

Title

Evaluation of biological activities of nine anti-inflammatory medicinal plants and characterization of antimicrobial compounds from *Pomaria sandersonii* and *Alepidea amatymbica*

EDDWINA MULEYA

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Promoter: Dr F. M. Mtunzi (Vaal University of Technology)

Co Promoters: Professor A.M. Sipamla (Vaal University of Technology)

Professor J.N Eloff (University of Pretoria, Phytomedicine Programme)

Dedicated to:
My Children
Innocent
Reneiloe Petunia & Russell
Kudakwashe
And also
Pearl Tinomukudzaishe

DECLARATION

I, the undersigned hereby declare that the research data contained in this study is my own original work carried out at Vaal University of Technology, Phytomedicine Programme, University of Pretoria, Department of Chemistry, and University of Botswana, Department of Chemistry. This thesis has not been submitted at any university before. I declare that all information sources used or quoted have been indicated and acknowledged by means of complete references.

Dr F.M Mtunzi

Professor A. M Sipamla

Professor J, N Eloff, Phytomedicine Programme, University of Pretoria

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ABSTRACT

Medicinal plants provide valuable alternative sources of drugs and drug discovery because many have been used in traditional practices for centuries to manage or treat various forms of ailments. The aim of this study was to evaluate the biological activities of nine medicinal plants used by Zulus in Mabandla village, KwaZulu-Natal province, South Africa to treat inflammation and to isolate selected active compounds against studied pathogens from *Alepidea amatymbica* and *Pomaria sandersonii*. The plants were selected on the basis of an ethnobotanical survey based on questionnaire response and verbal interviews that were conducted in Mabandla village with the local traditional healers and herbalists. The isolation of compounds from *Alepidea amatymbica* and *Pomaria sandersonii* was based on the bioassay based study which was carried out in this study.

Bioassay guided study involving in vitro anti-inflammatory measurement using soya bean derived 15 Lipoxigenase, free radical scavenging capacity against the ABTS^{••} radical cation and DPPH[•] radicals; antimicrobial and bioautography assays against *Staphylococcus aureus*, ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Escherichia coli*, ATCC25922, *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* were carried out using the plants extracts, fractions and pure compounds.

Isolation of compounds displaying biological activity was carried out by using open column chromatography and preparative thin layer chromatography (PTLC). The compounds were characterised by use of Nuclear Magnetic resonance, (NMR) and Mass Spectrometry (MS).

The DPPH sprayed TLC showed that all the nine plants contained antioxidants. Most of which were contained in polar fractions of acetone and methanol. Results of the assays displayed a range of biological activities comparable to the positive controls used for each assay. DPPH[•] scavenging displayed EC₅₀ values ranging between 1.008 and 467 µg/ml. The highest activity was observed with the methanol fraction of *Berkheya setifera* with an EC₅₀ value of 1.008 µg/ml followed by the crude extract of *Gunnera perpensa* with EC₅₀ value of 1.069 µg/ml. *Carissa bispinosa* hexane fraction had the lowest activity of 467.7 µg/ml.

The *Pomaria sandersonii* DCM extract had the highest ABTS^{••} radical scavenging activity by *Pomaria sandersonii* DCM extract, (1.273 µg/ml) for the ethyl acetate, (5.973 µg/ml) while the hexane fraction from *Eucomis autumnalis* had the lowest activity (929.4 µg/ml). The activity of *Pomaria sandersonii* extracts and fractions demonstrated that the plant contains antioxidants that react with both DPPH and ABTS radicals although higher activities were shown by ABTS as displayed by the lower EC₅₀ values. All the crude fractions and extracts had high to moderate antibacterial activities (20-625 µg/ml) and anti-fungal activities (20-2500 µg/ml).

Pomaria sandersonii crude and fractions had the highest antimicrobial activity compared to other plants. Some MIC values for *P. sandersonii* dichloromethane and ethyl acetate fractions (80 µg/ml in each case) compared well with gentamycin (4 µg/ml) since they showed same values against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*.

The dichloromethane, acetone and methanol fractions were also active (20 µg/ml) against both *Candida albicans* and *Aspergillus fumigatus*. Inhibition of pathogen growth demonstrated by the polar fractions of the studied plants

suggested that some of the active compounds would be soluble in water. A total of seven compounds were isolated from *Alepidea amatymbica* and *Pomaria sandersonii*. We propose three were new compounds after considering literature search involving closely related research to this investigation. These were two diterpenes from *Alepidea amatymbica*, namely, 14-acetoxo-12-oxokaur-16-en-19-oic acid labelled as 0657 and 16-hydroxy-kaur-6-en-19-oic acid given the label 06-2 in this study. The third suspected new compound is the chalcone dimer, which is referred to as EM86 in this study from *Pomaria sandersonii*. EM80-2 was obtained as a mixture of the cis and trans of 2', 4, 4',-trihydroxychalcone or 1-(2,4-dihydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one, from *Pomaria sandersonii*. The three diterpenes, 14-acetoxokaur-16-en-19-oic acid (0652), 13-hydroxy-16-kauren-19-oic acid (06B) and 14-oxokaur-16-en-19-oic acid (06431) were isolated from *Alepidea amatymbica* for the first time.

Isolated compounds were further tested as individual compounds and results showed that 16-hydroxy-kaur-6-en-19-oic acid (06-2) had weak activity against tested bacteria and fungi with the MIC: *Staphylococcus aureus* (320 µg/ml) and *Candida albicans*, (320 µg/ml). On the other hand 13-hydroxy-kaur-16-en-19-oic acid (06B) was more active against, *Staphylococcus aureus* (160 µg/ml) and *Aspergillus fumigatus* (40 µg/ml). The yellow compound that was isolated from *Pomaria sandersonii*, 1-(2, 4-dihydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one was antimicrobial with the following MICs: *Candida albicans*: 80 µg/ml; *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*: 160 µg/ml and *Aspergillus fumigatus*: 625 µg/ml.

There were two mixtures referred to as EM 49 and EM 77 from *Pomaria sandersonii* which were difficult to purify but had anti-microbial inhibitory activities worth reporting. EM49 had MIC against *Candida albicans* of: 160µg/ml; *Pseudomonas aeruginosa*: 320 µg/ml, *Escherichia coli*: 80µg/ml, *Enterococcus faecalis* 80µg/ml, and *Staphylococcus aureus*: 80µg/ml and *Aspergillus fumigatus*: 320µg/ml. EM 77 had MIC against *Escherichia coli*: 80 µg/ml and *Cryptococcus neoformans*: 80µg/ml. Further work on their purification need to be done since in this research we are just reporting on their high MIC activities.

The medicinal plants used to treat inflammation under different disease conditions in the Zulu community of Mabandla village, Kwa-Zulu Natal, South Africa have some relevant biological activities. The various antimicrobial, antioxidant and anti-inflammatory activities support the validity of their healing capacities that the traditional healers of the community claim to possess. Although there is evidence of good antimicrobial, antioxidant and anti-inflammatory activities by the crude extracts, the high levels of sucrose in *P. prunelloides* and glucose in *G. perpensa* should be borne in mind when using their decoctions in traditional medicine particularly by diabetic patients.

In vitro results for the antioxidant, antinflammatory and antimicrobial activities carried out in this investigation illustrate that the plants can be a source of treatment and management for inflammation related conditions. These therefore justify their use in Zulu traditional medicine. However, *in vivo* assays should be carried out in order to completely validate claims by the traditional healers that they treat inflammation related conditions.

GLOSSARY OF ABBREVIATIONS

¹³ CNMR	Carbon 13 nuclear magnetic resonance
13-HPODE	13-hydroperoxy-9,11-octadienoic acid;
¹ HNMR	Proton nuclear magnetic resonance
4-HNE	4-hydroxy-2-nonenal
9-HPODE	9-hydroperoxy-10, 12-octadecadienoic acid,
<i>A.f.</i>	<i>Aspergillus fumigatus</i>
ABTS	2, 2'-azinobis (3-ethylbenzothiazoline)-6-sulfonic acid
ABTS•+	2, 2'-azinobis (3-ethylbenzothiazoline)-6-sulfonic acid radical ion
AD	Alzheimer's disease
<i>C. a.</i>	<i>Candida albicans</i>
C4 LTB4	Dihydroxyeicosatetraenoate,
COSY	Correlated Spectroscopy
COXs	Cyclooxygenases(
DMSO	Dimethylsulphoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl),
<i>E.c.</i>	<i>Escherichia coli</i>
ET	Electron transfer
GLC	Gas liquid chromatography
HETE	Hydroxyeicosatetraenoic acid the enzymes:
HHE	4-hydroxy-2-hexenal;
HMBC	Heteronuclear multiple-bond coherence
HNE	4-hydroxy-2-nonenal,
HPETE	Hydroperoxyeicosatetraenoic acid;
HSQC	Heteronuclear single quantum coherence
LOXs	Lipoxygenases
LTC4	Cysteinyl leukotriene
LTS	Leukotriene synthase
MDA	malonyldialdehyde
MIC	Minimum inhibitory concentration
NDGA	Nordihydroguaiaretic acid
MRSA	Methicilin Resistant <i>Staphylococcus aureus</i>
NOESY	Nuclear Overhauser Effect Spectroscopy
NSAIDs	Non selective non- steroidal ANTI- INFLAMMATORY drugs
ORAC	Oxygen radical absorbance capacity

OXS	Oxygenases;
<i>P. a.</i>	<i>Pseudomonas aeruginosa</i>
PGs	Prostaglandins
POS	Peroxidases;
PTLC	Preparative Thin layer chromatography
PUFAs	Poly unsaturated fatty acids
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPTLC	Reverse phase thin layer chromatography
SODs	Superoxide dismutases
<i>S. a.</i>	<i>Staphylococcus aureus</i>

CHAPTER 1

1.1 INTRODUCTION

The use of medicinal plants in South African traditional medicine and other cultures in alleviating inflammatory conditions and other disease conditions is well documented (Van Staden 2008). Approximately 20,000 plant species have been recognized and documented for medicinal purposes by the World Health Organisation (WHO) (Gullece *et al.* 2006). Plants have been the major source of therapeutic agents to mankind and they still possess various phytochemical compounds that may be used in disease treatment. The therapeutic activities may be exerted through various mechanisms such as antimicrobial, anti-parasitic, antioxidant, anti-inflammatory, immune modulation (Adams & Bauer 2008). These plants may contain some novel bioactive compounds without the side effects associated with some conventional drugs. Therefore, it is necessary to investigate and determine the efficacy of medicinal plants scientifically in order to validate their ethno-pharmacological use. Also new therapeutic drugs with low toxicity compared to the conventional drugs may be identified and isolated from traditional medicinal plants. Proper documentation of medicinal plants is essential for recording and preservation of the traditional knowledge on ethno-botanical plants of medicinal importance in humans and veterinary medicine (Koné & Atindehou 2008).

Infections from bacteria, fungi, parasites and virus in humans and animals cause some serious diseases such as malaria, tuberculosis, diarrhoea and pneumonia (Green *et al.* 2010). The mechanism of action may involve direct cell damage, production of toxins and/or suppression of the immune system (Adams & Bauer 2008). The innate immune systems are usually recruited in an attempt to overcome any damages done by the infectious pathogens through the process of phagocytosis. Phagocytosis is activation of leukocyte cells' (neutrophils and macrophages) production, releasing respiratory burst such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) as microbicidal agents to kill the pathogens (Iwalewa *et al.* 2007).

Antibiotics are the primary drugs used in the treatment or elimination of these pathogens from the infested host (Shai *et al.* 2008). The development of resistant pathogens against the conventional drugs result in fresh challenges because infectious diseases previously under control are re-emerging with more virulence (Nkomo & Kambizi, 2009).

South African hospitals have reported on penicillin resistant and multi-resistant pneumococci (Eloff 1998). Most conventional drugs are also implicated to be toxic with some serious side effects (Maregesi *et al.* 2008).

Therefore, there is an urgent need for discovery and development of new drugs with high efficacy against the pathogens and minimal side effects to the host cells. Plants and plant preparations have been used in traditional practice to treat various kinds of diseases. Plant derived compounds could use a different mechanism of fighting pathogens from established antimicrobials and also possess a clinical value in treating diseases caused by resistant strains of pathogens (Eloff 1998). The plants under study are used to treat inflammation-related conditions by the Zulu traditional practitioners. Inflammation is a protective mechanism developed by the body as a way to fight or remove effects of negative stimuli such as microbial infections, allergy, injury, environmental hazard, inherited gene polymorphisms, or from dysfunctions of the immune system and stress (physical or oxidative) (Stables & Gilroy 2011; Westbrook *et al.* 2010). Oxidative burst derivatives such as superoxide, hydrogen peroxide, hydroxyl radical, and nitric oxide derived from phagocytosis initiate the production of inflammatory mediators like eicosanoids (leukotrienes (LTs), prostacyclin (PC) and prostaglandin (PG), and cytokines (interleukins and chemo attractants) (Westbrook *et al.* 2010; Iwalewa *et al.* 2007) during infection. Lipoxygenases (LOX) and cyclooxygenases (COX) are the rate-determining enzymes in the production of these mediators such as arachidonic acid. Inhibition of eicosanoids' biosynthesis is therefore a promising method for combating a number of diseases mediated by inflammation (Adams & Bauer 2008).

Non-steroidal anti-inflammatory drugs (NSAIDs) like aspirin are commonly used in treating pains and inflammation by non-selective inhibition of COX enzymes, COX 1 and COX 2. The inhibition of this enzymatic pathway causes gastrointestinal and renal tract damage, limiting their usage (Viji & Helen 2008). Oxidative burst that occurs under conditions of chronic inflammation can result in oxidative stress (Iwalewa *et al.* 2007). Oxidative stress defined as an imbalance between the production of reactive species and elimination in favour of the former is enhanced in disease conditions (infection and non-infection) (Aruoma 2007; Kris-Etherton *et al.* 2004). Host body immune system during reactive oxygen species (ROS) mediated inflammatory disorders produces ROS (superoxide, hydroxyl radical, hydrogen peroxide, hypochlorites) and RNS (nitrogen oxide, peroxynitrite) as agents for killing invading pathogens. The reactive

species can engage in reaction with phospholipids of the cell membrane to perpetuation of a chain reaction process referred to as lipid peroxidation. The products of lipid peroxidation are cytotoxic aldehyde molecules, including malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), 4-hydroxy-2-hexenal (4-HHE) and acrolein (Esterbauer *et al.* 1991) that cause cellular damage to DNA, proteins, structural carbohydrates, and lipids (Sies & Cadenas 1985), unless the radical that initiates and propagates the process is terminated by an antioxidant.

In this research work, (1) literature and ethno-botanical survey were carried out by verbal interviews with traditional practitioners on the use of some plants in treating inflammation in Mabandla village, Kwazulu-Natal Province in South Africa. (2) Phytochemical analysis of 9 plants (*Pentanisia prunelloides*, *Pomaria sandersonii*, *Alepidea amatymbica*, *Gunnera perpensa*, *Carissa bispinosa*, *Artemisia afra*, *Eucomis autumnalis*, *Ledebouria revoluta* and *Berkheya setifera*) identified for treating inflammation by the local of Mabandla village, Kwazulu-Natal Province in South Africa were also evaluated. (3) The biological activities of the extracts (antimicrobial, antioxidant and Lipooxygenase (LOX) inhibition) were carried out. (4) Isolation and characterization of bioactive compounds from *Pomaria sandersonii* and *Alepidea amatymbica* was also carried out. (5) Cytotoxicity tests were carried out on water extracts of *Pomaria sandersonii* (PS) and *Alepidea amatymbica*. (ALA). The results of these activities are hereby presented in this thesis.

1.2 AIM

The purpose of the study was to assess biological activities of secondary metabolites in medicinal plants used for treating inflammation and associated complications, and characterization of antimicrobial compounds from the plant extracts of *Pomaria sandersonii* and *Alepidea amatymbica*.

1.3 OBJECTIVES

- (i) To assess free radical scavaging activity of the plant extracts, fractions and isolated compounds.
- (ii) To assess the anti-inflammatory activities of the plant crude extracts
- (iii) To assess antimicrobial activities of plant crude extracts and fractions.

- (iv) To isolate and perform structural elucidation of biologically active compounds
- (v) To assess plant extract toxicity

1.4 THESIS OUTLINE

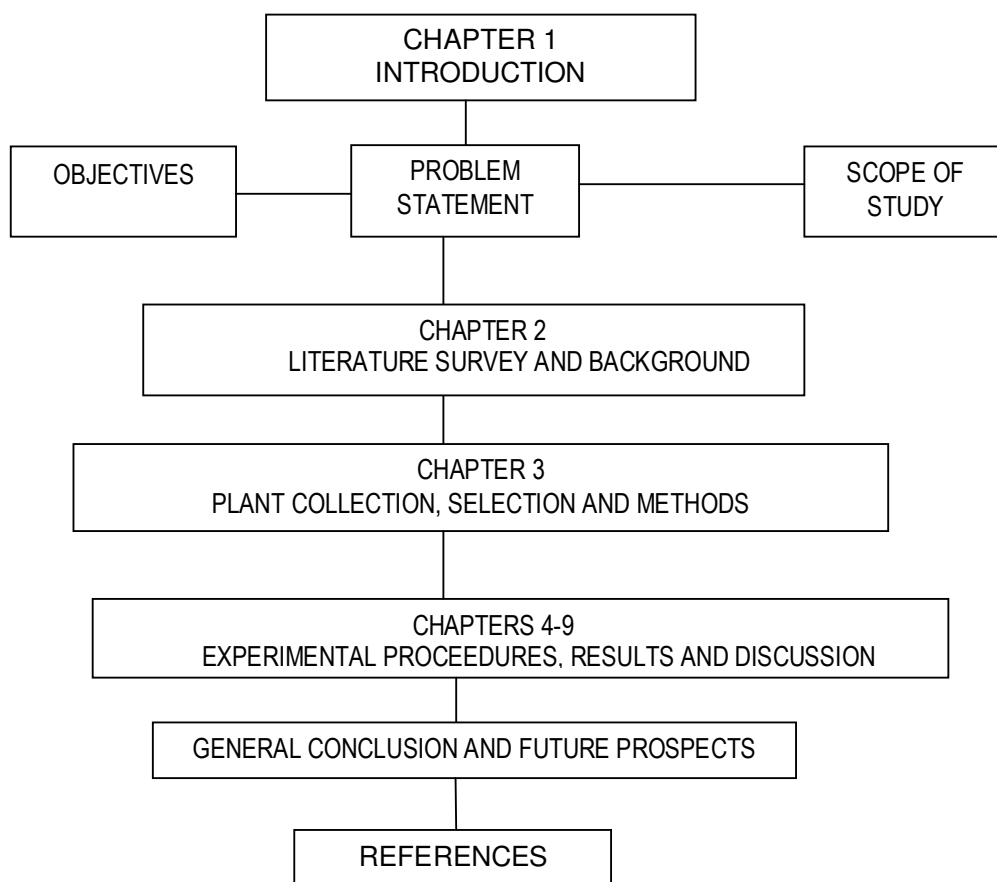


Figure 1: Thesis outline

CHAPTER 2

LITERATURE SEARCH

2.1 INTRODUCTION

In many African cultures, medicinal plants are valuable resources used to manage varieties of human and animal ailments especially in the rural areas where modern medicine is inaccessible and unaffordable. South Africa has one of the most diverse geographical floras in the world (Van Staden 2008) and has a cultural diversity with traditional healing being integral to each ethnic group (Van Vuuren & Viljoen 2008). Plant phytochemicals have also been a source of drugs or templates for drug development in modern medicine. Some of the drugs developed from medicinal plants include artemisinin, isolated from *Artemisia annua*, as anti-malarial drug (Hoareu & DaSilva 1999), kniphone isolated from *Kniphosa folisa* Hochst (Asphodelaceace) which is a selective inhibitor of lipoxygenases involved in biosynthesis of inflammatory mediators such as leukotrienes (Adams & Bauer 2008; Van Wyk 2008). Commercial herbal concoctions and decoctions are also readily available in the herbal shops that are widespread across South Africa. Examples of such concoctions include *umzimba omubi* (used to treat wounds, skin rashes, fungal infections and boils) (Ndhlala *et al.* 2009), *umuti wekukhwehlela ne zilonda* (used as cough mixture to treat chest infections and difficulty in breathing) (Ndhlala *et al.* 2009), *mvusa ukunzi* (energizer and increases man's sexual prowess) (Ndhlala *et al.* 2009), *imbiza ephuzwayo* (energy tonic, increases sexual prowess, relieves constipation, reduces stress, reduce high blood pressure, clears skin conditions, boosts vitality, helps to prevent arthritis, kidney problems and reduces general body pains) (Ndhlala *et al.* 2009), *vusa umzimba* (to treat wounds, rashes fungal infections, boils, chest infections and stops menstrual pains), (Ndhlala *et al.* 2009), *ingwe muthi mix*, *ibhubezi* (used to treat wounds, fungal infections, STDs, treatment of influenza, to reverse impotence, clean the body system and stimulate blood production) (Ndhlala *et al.* 2009) and *super new one hundred* (used for nervous disorders, skin conditions, stimulates blood production, boosts sexual performance, treats back pains, fights influenza and strengthens the body (Ndhlala *et al.* 2009). Herbal decoctions like *isihlambezo* are administered to women during child delivery to aid labour or reduce pains (Varga & Veale 1997). Some common fruit such as cranberry juice (*Vaccinium macrocarpon*) have been used to treat urinary tract infections (Heinrich *et al.* 2004) while lemon balm (*Melissa officinalis*), garlic (*Allium sativum*) and tee tree (*Melaleuca alternifolia*) are broad-spectrum antimicrobial agents (Heinrich *et al.* 2004). In addition to the need of producing more effective and safer drugs for disease control, the high cost of some conventional drugs and inaccessibility especially in developing countries where health delivery is still a major challenge is the driving force for the continued search for new drugs from natural source. There is also need for newer drugs against drug resistant microorganisms or new emerging infectious pathogens. The sections hereafter will discuss medicinal plants as antimicrobial, antioxidants, anti-inflammatory agents and their biologically active plant metabolites.

2.2 PLANT AND PLANT PREPARATIONS AS ANTIMICROBIAL AGENTS

The continued threat of infections and infectious diseases is still a major health problem worldwide despite the milestone achievements in understanding the mechanisms of disease pathogenesis. The emergence and spread of multidrug-resistant (MDR) microbial strains to many antibiotic drugs and increased cases of immune disorders have complicated the treatment of infectious diseases. Therefore more efficient anti-infectious agents that can also overcome the drug resistance mechanisms of microorganisms are desirable (Gibbons 2005). Medicinal plants and their preparations have been a source of many therapeutic drugs against infectious and non-infectious diseases (Watt & Breyer-Brandwijk 1962; Kokwaro 1976; Gelfand *et al.* 1985; Hutchings *et al.* 1996; Van Wyk *et al.* 1997). The medicinal importance of plant preparations is due to the presence of diverse phytochemicals acting synergistically or individually through various mechanisms to exert their activities. Natural products and their derivatives represent over 50% of all the drugs in clinical use in the world (Van Wyk *et al.* 2002). The form in which the dosage is prescribed varies from use as fresh or dried samples (chewing or stew), decoction (in water or milk) or infusion (in water), tinctures, poultice, snuff and fumes from the burning plant depending on the recommendation made by the traditional practitioner and on the type of disease. Many herbal traditional practitioners prescribe herbal mixtures from several medicinal plants for particular therapeutic regimes (Van Vuuren & Viljoen, 2008). Plant parts used for the treatment of a given condition may differ from one practitioner to another and species (Kokwaro 1976; Lemenih *et al.*, 2003; Steyn 2003). For example different parts of *Croton gratissimus* Burch var. *gratissimus* (Euphorbiaceae) such as, the root decoction is used for treating coughs, fever and sexually transmitted diseases like syphilis, while the bark is used to treat bleeding gums, abdominal disorders, skin inflammation, earache and chest complaints (Van Vuuren & Viljoen 2008). However, it was hypothesized that the same bioactive compounds may be present in all the parts of a particular medicinal plant, although the quantity may vary (Van Vuuren & Viljoen 2008).

Many medicinal plants have been listed as anti-infective agents in South African traditional medicine. Some of the plant extracts have been evaluated against various disease pathogens such as bacteria (Eloff *et al.* 2008), fungi (Moore *et al.* 2008), parasites (Eloff *et al.* 2008), and virus (Tshikalange *et al.* 2008). Some medicinal plants of South African origin are reported to have good antimicrobial activities and their bioactive components against bacteria; fungi and parasites with MICs of 200 µg/ml and below using two-fold serial dilution method are listed in Table 1.

Table 1: South African medicinal plants with MIC < 200 µg/ml against some pathogenic microorganisms

Name of organism	Traditional plant used for treatment	Antimicrobial compound isolated	Conventional treatment
<i>Staphylococcus aureus</i>	<i>Helichrysum aureonitens</i> , MIC range = 0.1-0.5 µg/ml; <i>Hermania saccifera</i> MIC 15.5 µg/ml, ; (Kambizi & Afolayan 2008; Essop <i>et al.</i> 2008; Hutchings <i>et al.</i> , 1996) <i>Gunnera perpensa</i> , MIC =39 µg/ml of isolate (Drewes <i>et al.</i> 2005); <i>Pentanisia prunelloides</i> , Ethanol extract, MIC = 200 µg/ml (Yff <i>et al.</i> 2002)	3,5,7-trihydroxyflavone; 17,19-diacetoxy-15-hydroxyabda-7,13-diene (Kambizi & Afolayan 2008) Palmitic acid from <i>Pentanisia prunelloides</i> (Yff <i>et al.</i> , 2002) , 2-methyl-6-(3-methyl-2-butenyl)benzo-1,4-quinone from <i>Gunnera perpensa</i> (Drewes <i>et al.</i> 2005)	Tetracycline; ciprofloxacin
<i>Staphylococcus epidermidis</i>	<i>Gunnera perpensa</i> MIC = 9.8 µg/ml and 187 µg/ml respectively of isolate (Drewes <i>et al.</i> 2005);	2-methyl-6-(3-methyl-2-butenyl)benzo-1,4-quinone and 6-hydroxy-8-methyl-2,2-dimethyl-2H-benzopyran (Drewes <i>et al.</i> 2005)	Tetracycline; ciprofloxacin
<i>Escherichia coli</i>	<i>Combretum erythrophyllum</i> , MIC range = 25-100 µg/ml of isolate; <i>Combretum woodii</i> , chloroform extract, MIC = 100 µg/ml; (Martini <i>et al.</i> 2004).	Ramnocitrin, Quercetin-5, 3-dimethylester, paramnocitrin (Martini <i>et al.</i> 2004).	Tetracycline, ciprofloxacin
<i>Enterococcus faecalis</i>	<i>Combretum erythrophyllum</i> MIC= 50 µg/ml; <i>Combretum woodii</i> MIC= 125 µg/ml (Martini <i>et al.</i> 2004, Van Vuuren 2008); <i>Gunnera perpensa</i> MIC=39 µg/ml of isolate (Drewes <i>et al.</i> 2005)	Rhamnazin (2',3',4-trihydroxyl-3,5,4'-trimethoxyl bibenzyl); combretastatin B5(Martini <i>et al.</i> 2004, Van Vuuren,2008, Eloff <i>et al.</i> 2005), 3-hydroxy-2-methyl-5-(3-methyl-2-butenyl)benzo-1,4-quinone from <i>Gunnera perpensa</i> (Drewes <i>et al.</i> , 2005).	Amphotericin ciprofloxacin
<i>Candida albicans</i>	<i>Helichrysum tenax</i> var <i>tenax</i> (MIC 62.5 µg/ml); (Kambizi & Afolayan, 2008; <i>Gunnera perpensa</i> . MIC 130 µg/ml and 37 µg/ml of isolates respectively (Drewes <i>et al.</i> 2005).	Ent-beyer-15-en-19-ol (Kambizi & Afolayan 2008); 2-methyl-6-(3-methyl-2-butenyl)benzo-1,4-quinone and 6-hydroxy-8-methyl-2,2-dimethyl-2H-benzopyran from <i>Gunnera perpensa</i> (Drewes <i>et al.</i> 2005).	amphotericin
<i>Pseudomonas aeruginosa</i>	<i>Salix carpensis</i> MIC = 62.5 µg/ml (Masika <i>et al.</i> 2006).	Catechol, 2-hydroxybenzyl alcohol (Masika <i>et al.</i> 2006)	Tetracycline
<i>Cryptococcus neoformans</i>	<i>Gunnera perpensa</i> , MIC range = 70 µg/ml and 75 µg/ml of isolate (Drewes <i>et al.</i> , 2005) <i>Terminalia prunioides</i> , hexane extract, (MIC = 80 µg/ml) (Masoko <i>et al.</i> 2005).	2-methyl-6-(3-methyl-2-butenyl)benzo-1,4-quinone and -hydroxy-8-methyl-2,2-dimethyl-2H-benzopyran from <i>Gunnera perpensa</i> (Drewes <i>et al.</i> 2005)	Amphotericin
<i>Bacillus cereus</i>	<i>Hermannia saccifera</i> (MIC = 19.5 µg/ml) (Van Vuuren <i>et al.</i> 2008); <i>Gunnera perpensa</i> MIC =18 µg/ml and 75 µg/ml (Drewes <i>et al.</i> 2005).	2-methyl-6-(3-methyl-2-butenyl)benzo-1,4-quinone (Drewes <i>et al.</i> 2005) and;6-hydroxy-8-methyl-2,2-dimethyl-2H-benzopyran (Drewes <i>et al.</i> 2005).	Tetracycline
<i>Babesia caballi</i>	<i>Elephantorrhiza elephantina</i> (MIC = 100 µg/ml) (Naidoo <i>et al.</i> (2005).		Imidocarb, diminazene
<i>Mycobacterium bovis</i> BCG	<i>Euclea natalensis</i> (MIC = 26 µg/ml) (McGaw <i>et al.</i> , 2008).	7-methyljuglone	Isoniazid and rifampicin
<i>M. kristinae</i>	<i>Helichrysum aureonitens</i> MIC range = 0.1-0.5 µg/ml (Van Vuuren,2008)	3,5,7-trihydroxyflavone (Van Vuuren 2008)	
<i>Klebsiella pneumoniae</i>	<i>Terminalia sericea</i> (MIC range = 3.8-31 µg/ml) (Eldeen <i>et al.</i> 2006); <i>Gunnera Perpensa</i> MIC= 187 µg/ml)	Anolignan B (Eldeen <i>et al.</i> 2006);6-hydroxy-8-methyl-2,2-dimethyl-2H-benzopyran (Drewes <i>et al.</i> 2005)	Tetracycline
<i>Bacillus subtilis</i>	<i>Aloe ferrox</i> (MIC = 62.5-125 µg/ml) (Kambizi <i>et al.</i> 2004).	Aloin (Kambizi <i>et al.</i> 2004)	Tetracycline
<i>Entamoeba histolytica</i>	<i>Elianthemum glomeratum</i> (Calzada & Alanis, (2007).	Tiliroside, kaempferol-3-O-(3",6"-di-O-E-p-coumaroyl)-β-D-glucopyranoside, astragalín, quercitrin and isoquercitrin (Calzada & Alanis 2007) .	Metronidazole, Emetine

<i>Caenorhabditis elegans</i> (Nematode), anti-helminthic	<i>Acorus calamus</i> L. (Araceae) (Fennel <i>et al.</i> 2004)	Not known	Levamisole , IC ₅₀ 4.7-6.9 µg/ml
<i>Entamoeba histolytica</i> (amoeba),	<i>Albizia adianthifolia</i> W. F. (Wight (Leguminosae) ; <i>Deinbollia oblongifolia</i> W. F. Radlk. (Anacardiaceae) and <i>Acorus calamus</i> L. (Araceae) (IC ₅₀ ranged between 0.313 to 5 mg/ml) (Fennel <i>et al.</i> 2004)	Not known	Metronidazole, IC ₅₀ = 0.2 µg/ml) (Fennel <i>et al.</i> 2004)
<i>Biomphalaria glabrata</i> (Mollusc)	<i>Zingiber officinale</i> L. (Zingiberaceae) (Fennel <i>et al.</i> 2004)	Not known	Gingerol, shogaol
<i>Schistosoma mansoni</i> (Schistosoma)	<i>Berkheya speciosa</i> O.Hoffm (Compositae) and <i>Trichilia emetica</i> Vahl (Meliaceae), were lethal at 6.25 mg/ml , <i>Euclea natalensis</i> A.DC. (Ebenaceae) (extracts, at a concentration of 3.13 mg/ml, killed 66% of the schistosomes) (Fennel <i>et al.</i> 2004)	Not known	5 mg/ml gingerol arrested the ability of miracidia to infect both snails and mice (Fennel <i>et al.</i> 2005)
<i>Plasmodium falciparum</i>	<i>Ajuga remota</i> Benth. (Lamiaceae) and <i>Caesalpinia volkensii</i> Harms (Fabaceae), IC ₅₀ values less than 2µg/ml (Fennel <i>et al.</i> 2004)	Not known	Chloroquine IC ₅₀ = 0.043µg/ml) (Fennel <i>et al.</i> 2004)
<i>Aspergillus fumigatus</i>	<i>Terminalia prunioides</i> acetone extract (MIC = 0.16 µg/ml) <i>Terminalia brachystemma</i> DCM extract, MIC 80 µg/ml (Masoko <i>et al.</i> 2005)		Amphotericin B
<i>Sporothrix schencki</i>	<i>Terminalia sericea</i> Methanol extract, MIC = 0.02 µg/ml) (Masoko <i>et al.</i> 2005)		Amphotericin B
<i>Microsporum canis</i>	<i>Terminalia mollis</i> Methanol extract, MIC = 0.02 µg/ml (Masoko <i>et al.</i> 2005)		Amphotericin B
<i>Mycobacterium tuberculosis</i>	<i>Euclea natalensis</i> .(MIC for pure compounds = 8, 10, 100, 0.5, 10 and 100 µg/ml, respectively) (Van Kooy <i>et al.</i> 2006) <i>Artemisia afra</i> isolate fraction from DCM extract MIC = 10 µg/ml (Ntutela <i>et al.</i> 2009)	Diospyrin; isodiospyrin; mamegakinone, 7-methyljuglone; neodiospyrin and shinanolene (Van Kooy <i>et al.</i> 2006) artemin and arsubin (Ntutela <i>et al.</i> 2009)	Rifampicin, isoniazid, ethambutol, streptomycin,
HIV/AIDs anti HIV= protease activity; Anti-HIV-1-Reverse transcriptase	<i>Geum japonicum</i> - ethyl acetate fraction compounds inhibited HIV-protease activity (100-42%); Crude extract of <i>Terminalia sericea</i> (IC ₅₀ = 43 µg/ml (Xu <i>et al.</i> 1996; Tshikalange, <i>et al.</i> 2008)	2α-19 α-dihydroxy-3-oxo-12-ursen-28-oic acid (74%); maslinic acid (100%);ursolic acid (85%) tormentic acid (49%); epipomolic acid (42%) in a concentration of µg/ml. For <i>Terminalia sericea</i> ,active compound not known (Xu <i>et al.</i> 1996; Tshikalange <i>et al.</i> 2008)	Adramycin (IC ₅₀ = 100 µg/ml (Tshikalange <i>et al.</i> 2008)
HIV-1replication in TR-cells(Appendino <i>et al.</i> 2007)	<i>Helichrysum decumbers</i> compound inhibit NF-κB IC ₅₀ = 5 µg/ml (Appendino <i>et al.</i> 2007)	aranol(Appendino, <i>et al.</i> 2007)	

2.3 ANTI-INFLAMMATORY MECHANISMS OF PLANT EXTRACTS

Inflammation is one of the first body's natural defence mechanisms to adverse conditions such as infection, stress and injury. Inflammatory response serves to facilitate the removal of any offensive stimuli or alleviate any damage caused by invading organisms (Iwalewa *et al.* 2007). The inflammatory mediators include mast cells, leukocytes (basophils, monocytes, neutrophils, eosinophils), tumour necrosis factor-alpha (TNF- α), interleukins (IL), interferons and colony stimulating factors (CSFs) (Kaplan *et al.* 2007), serotonin or 5-hydroxytryptamine (5-HT), histamine, cyclooxygenase (COX), lipoxygenase, reactive oxygen species (ROS) (Iwalewa *et al.* 2007). However, excessive elaboration of inflammatory mediators may be injurious to the host cells leading to various forms of disorders such as infectious diseases, arthritis, cancer and cardiovascular disease. Diseases associated with inflammation are also being treated using some medicinal plant preparations (Iwalewa *et al.* 2007). Inflammation mechanisms in the pathogenesis of several diseases and disorders are shown in Fig 2 (Iwalewa *et al.* 2007).

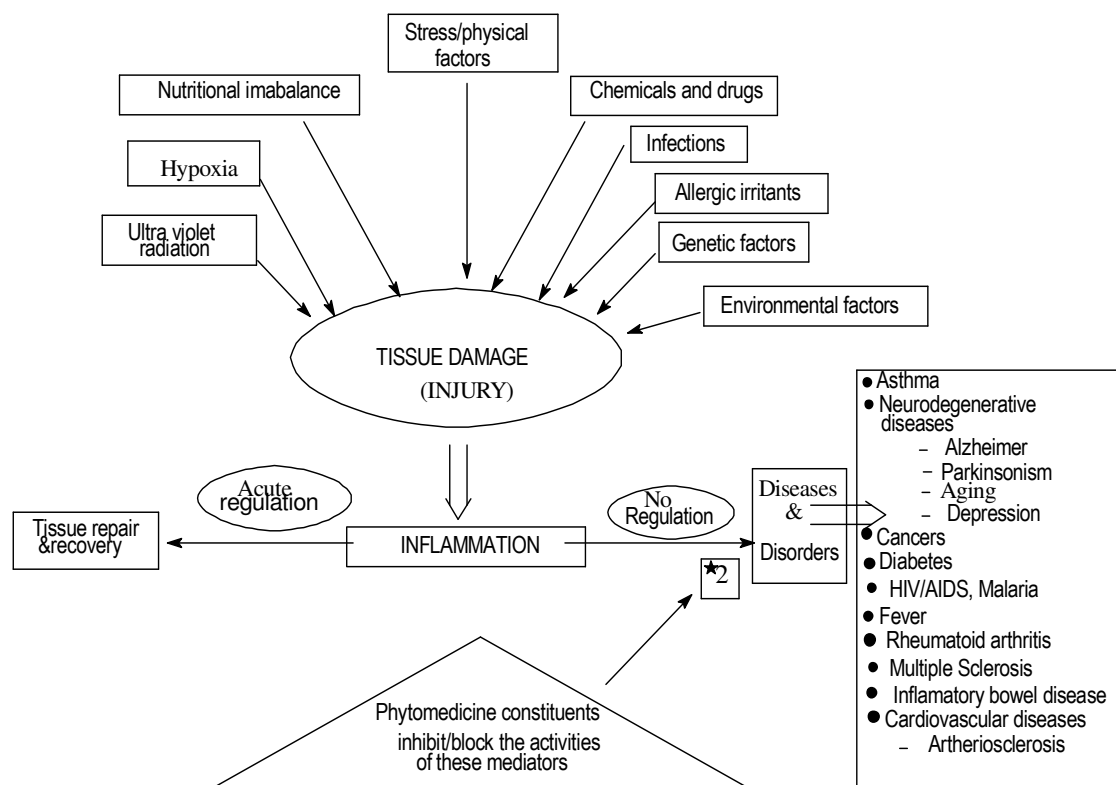


Figure 2: Schematic diagram of the tissue damage pathways and mechanisms of inflammation.

Site *2 of Fig 2.1, indicates the presence of platelets, lymphocytes (T cells, B cells, macrophages, nuclear factor kappa B, (NF κ B) and transcription factor (NF κ B) cells), that release more devastating pro-inflammatory cytokines like interleukin-1 β (IL-1 β), tumour necrosis factor alpha (TNF- α), cyclooxygenases (COXs), 5-lipoxygenase (5-LOX), nitric oxide, NO, reactive oxygen species, ROS in chronic inflammatory stage (Iwalewa 2007; Yang 2009).

Current treatment of inflammation is mainly by non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and indomethacin that exert anti-inflammatory actions by inhibiting cyclooxygenases (COXs). COXs exist as two isoforms: cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). Cyclooxygenase-1 (COX-1) is common in most cells under normal physiological conditions. COX-1 is responsible for maintaining the usual and normal intercellular defensive activities such as antiplatelet activity, vasodilation and cyto-protection (Iwalewa *et al.* 2007). COX-1 products from arachidonic acid are involved in the maintenance of the gastrointestinal tract (GIT) integrity through cyto-protective processes thereby reducing gastric acid secretion, increasing the thickness of the mucus layer and enhancing mucosal blood flow (Suleiman *et al.* 2007). Intestinal homeostasis depends on three processes namely interactions between the micro biota, intestinal epithelium and the host epithelium which are maintained by many regulatory mechanisms (Maloy & Powrie 2011). COX-2 is an inducible enzyme stimulated by agents such as tumour necrosis factor-alpha (TNF- α), lipopolysaccharide (LPS) and tumour-promoting factors to produce pro-inflammatory mediators (Viji & Helen 2008). High COX-2 levels are associated with cells involved with chronic inflammation in which large amounts of pro-inflammatory agents such as cytokines, prostaglandins, vasoactive amines (histamine and serotonin) and cytotoxic NO are present (Iwalewa *et al.* 2007). Modulation of the expression and activities of COX-2 is important in inflammation pharmacotherapy. However, classical NSAIDs are non-selective COX inhibitors with characteristic adverse effects such as GIT ulceration. COX-2 selective inhibitors associated with reduced adverse effect on gut intestinal tract and other tissues are the target of inflammatory therapy. Adverse renal effects such as a decrease in renal function have been reported in chronic applications (Iwalewa *et al.* 2007).

Another important enzyme involved in production of pro-inflammatory mediator from polyunsaturated fatty acids, (PUFAs) is lipooxygenase (LOX) such as 5-LOX, 12-LOX and 15-LOX. 5- lipooxygenase increases the production of cysteinyl leukotrienes such as leukotriene C₄, (LTC₄), leukotriene D₄, (LTD₄), and leukotriene E₄, LTE₄ (Viji & Helen 2008, Adams & Bauer 2008). Leukotrienes are chemical compounds involved in the pathogenesis of chronic inflammation-related diseases such as psoriasis, asthma, rheumatic disorders and other disorders (Adams & Bauer 2008). The COX and LOX enzymes share the same substrates (arachidonic acid or other PUFAs in the cell membranes) during the immune response reaction but differ in their products (Süleyman *et al.* 2007). Various South African medicinal plants have been identified as possessing anti-inflammatory activity. Some medicinal plants are used in traditional medicine to treat inflammatory and associated disorders are listed in Table 2 (Kaplan *et al.* 2007; Iwalewa *et al.* 2007).

Table 2: List of some of the plant species parts used to treat pain and inflammatory disorders.

Plant species	Parts used	Constituents	Ethno pharmacological usage	Mechanism of action
<i>Atractylodes lancea</i> THUMB DC (Asteraceae)	Rhizome	Atractylolone, (2-[(2'E)-3,7,-dimethyl-2,6-octadienyl]-4-methylphenol (Adams & Bauer 2008)	Rheumatic diseases, digestive disorders, mild diarrhoea and influenza (Adams & Bauer 2008))	5 LO inhibitory activity (IC ₅₀ = 2.9 µg/ml) (Adams & Bauer 2008))
<i>Kniphofia foliosa</i> Hochst (Asphodelaceae)	Roots	Anthraquinone knipholone (Adams & Bauer 2008)	Abdominal cramps and wound healing (Adams & Bauer 2008)	IC ₅₀ value for leukotriene biosynthesis inhibition=4.2 µM (Positive control: zileuton IC ₅₀ = 10.4 µM) (Adams & Bauer 2008)
<i>Siphonochilus aethiopicus</i> (Schweinf.) B.L. Burt (Zingiberaceae)	Rhizome	-	Pain and inflammation(McGaw <i>et al.</i> , 1997; Lindsey <i>et al.</i> 1999; Zschocke <i>et al.</i> 2000)	Prostaglandin synthesis inhibition. Ethanol extract rhizome 46%, leaves, 93% tubers, 83% stem 43% (Lindsey <i>et al.</i> 1999)
<i>Ocotea bullata</i> (Burch.) Baill. (Lauraceae)	Stem bark	Sibyllenone, ocobullenone (Jäger <i>et al.</i> 1996; Zschocke <i>et al.</i> 2000).	Headaches, urinary disorders, and stomach ailments (Jäger <i>et al.</i> 1996; Zschocke <i>et al.</i> 2000).	COX1 inhibitory activity. 97% activity from ethyl acetate fraction (Endomethacin, 0.5 µg, 66.5 %); Prostaglandin synthesis inhibition. 5-LOX inhibitor (Jäger <i>et al.</i> 1996; Zschocke <i>et al.</i> 2000).
<i>Eucomis autumnalis</i> (Mill.) Chitt. (Hyacinthaceae)	Bulb	Eucomin(for COX-1) Eucosterol (for COX-2) (Taylor & Van Staden, 2002)	Inflammation and headache (Taylor & Van Staden, 2002)	Eucosterol with Selective COX-2 inhibitory activity extract IC ₅₀ = 54 µM concentration of 250 µg/ml COX -1 inhibition IC ₅₀ = 46 µM (Taylor & Van Staden 2002)
<i>Evodia rutaecarpa</i> (Juss.) Benth (Rutaceae)	Fruit	1-methyl-2-nonyl-4-quinoline (Adams & Bauer, 2008)	Gastrointestinal problems, headache, menstrual complaints and mouth ulcers (Adams and Bauer, 2008)	5-LO inhibitory activity, IC ₅₀ = 12.2 µM (Positive control: zileuton IC ₅₀ = 10.4 µM) (Adams & Bauer 2008)
<i>Pentanisia prunelloides</i> Walp. (Rubiaceae)	Leaves and roots	Palmitic acid (Yff <i>et al.</i> 2002; Jager & Van Staden, 2005)	Treatment for swellings, sore joints and rheumatism inflammation(Yff <i>et al.</i> 2002; Jager and Van Staden, 2005)	COX-1 inhibitory activity, of leaf water extract = 74%, Root water extract =74%, Ethyl acetate extract leaf = 88%;Ethyl acetate root = 87%, Indomethacin,20 µM = 83% (Yff <i>et al.</i> 2002; Jager & Van Staden 2005)
<i>Bacopa monnieri</i> (L.) Wettest (Scrophulariaceae)	Whole plant	Bacoside A ₁ , A ₂ , A ₃ , bacosaponinins, brahime, herpestine, luteolin-7-glucoside (Viji & Helen 2008)	Treatment of asthma, epilepsy, insanity, inflammation, cardiovascular diseases (Viji and Helen 2008)	
<i>Combretum erythrophyllum</i> (Sond.) (Combretaceae)	Leaves		Back ache (McGaw <i>et al.</i> 2001)	Prostaglandin synthesis inhibition Ethanol extract= 90%(Lindsey <i>et al.</i> 1999)
<i>Cenchrus ciliaris</i> L. (Poaceae)	Underground runners		Dysmenorrhoea	Prostaglandin synthesis inhibition Ethanol extract= 99%(Lindsey <i>et al.</i> 1999)
<i>Solanum mauritianum</i> Scop.) (Solanaceae)	Leaves, bark		Dysmenorrhoea	Prostaglandin synthesis inhibition Ethanol extract= 97%(Lindsey <i>et al.</i> 1999))
<i>Terminalia sericea</i> Burch ex DC. (Combretaceae)	Fruit; Root	Anolignan B	Tuberculosis; stomach troubles, wounds, inflammation and sexually transmitted diseases	% prostaglandin synthesis by ethyl acetate root extract 85%; 37% for COX 1

Prostaglandin inhibition positive control: Indomethacin = 67% (Lindsey *et al.* 1999)

2.4 PLANT AND PLANT PREPARATIONS AS ANTIOXIDANTS

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are some of the molecular species produced in the body as a defensive measure when the body is exposed to chemical, mechanical and microbial stimuli (Kaplan *et al.* 2007). In normal physiological conditions, the ROS and RNS are an integral part of the body's immunological system as microbicidal agents and signalling molecules in some metabolic pathways. However in a pathological situation such as oxidative stress (referred to as imbalance between the generation and annihilation of ROS/RNS in favour of the former), reactive species are involved in aetio-pathology of many diseases (Sies & Cadenas 1985; Kaplan *et al.* 2007; Catalá 2009). These reactive species include superoxide anion, O_2^- , hydrogen peroxide, H_2O_2 , hydroxy radical, OH , organic hydroperoxide, $ROOH$, alkoxy, $RO\cdot$ and peroxy, ROO -radicals, hypochlorous acid, $HOCl$, and peroxynitrite, $ONOO^-$ (Gutteridge 1988). The mechanisms are toxic effects through production of peroxides and free radical intermediates that damage cell components including proteins, lipids and DNA (Sies & Cadenas 1985; Catalá, 2009). In degenerative disease conditions such as cardiovascular disorders, cancer, inflammation, arthritis, immune system decline, brain dysfunction, cataract, and Alzheimer's disease (AD), initiation and propagation result from oxidative damage to cellular components mucotaneous membrane by free radicals as presented in Figure 3 (Kaplan *et al.* 2007).

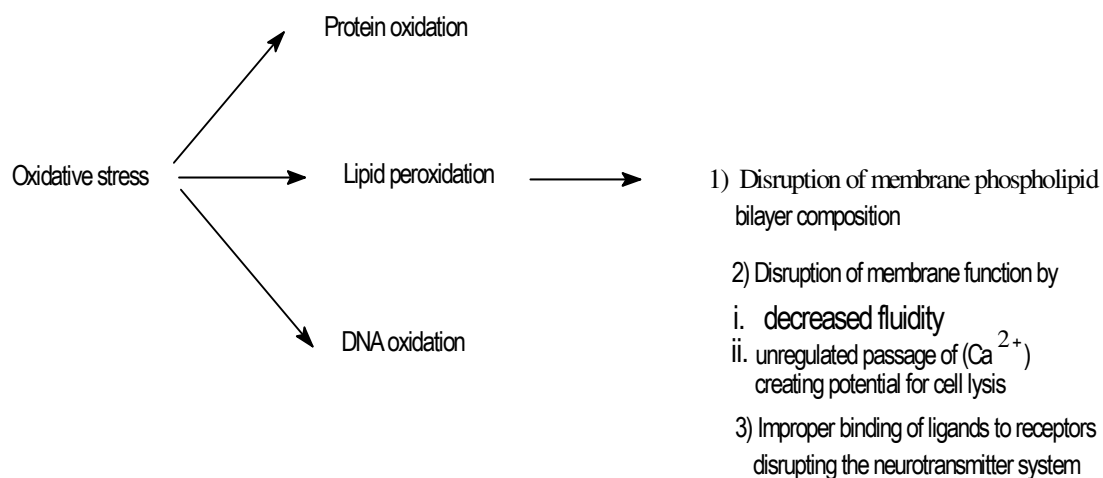


Figure 3: Mechanisms of oxidative stress leading to neuronal degeneration.

Lipid peroxidation is caused by free radicals attacking lipid molecules which are part of the cell membrane structure. The reaction occurs according to the free radical mechanism with the lipid molecules, which are the polyunsaturated fatty acids (PUFAs). Formation of more free radicals initiating even more chain reactions is the result. These reactions destroy the cell membrane structure and function (Spiteller 2001). Polyunsaturated fatty acids (PUFAs) and their metabolites have a number of physiological roles in the body including: energy provision, membrane structure,

cell signalling and regulation of gene expression (Catalá 2009). Lipid peroxidation free radical reaction mechanism occurs in three steps: initiation, propagation and termination (Figure 4) (Catalá 2009).

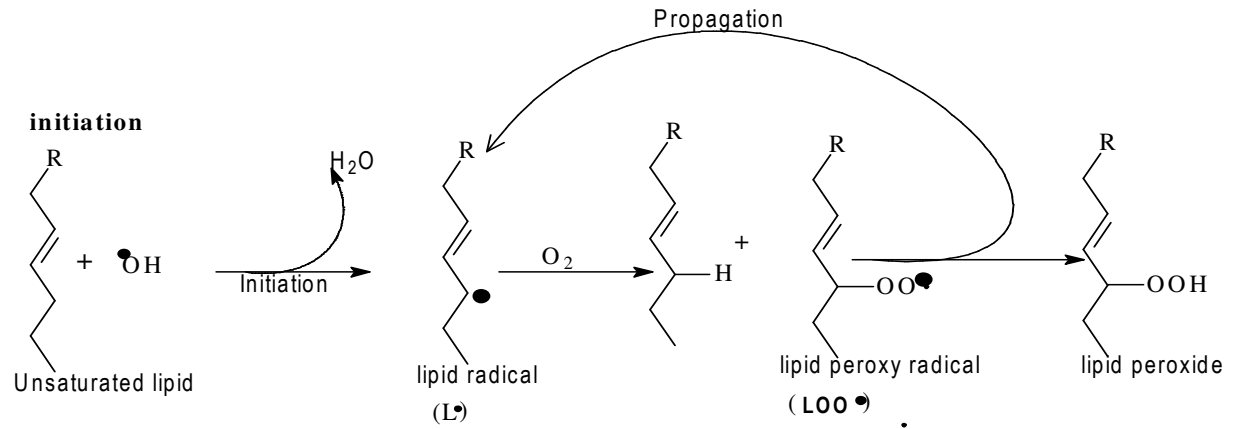
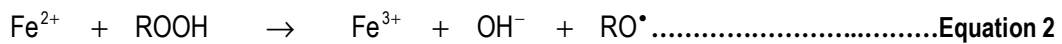
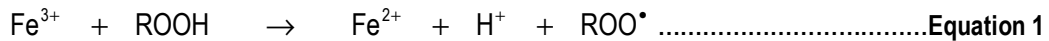
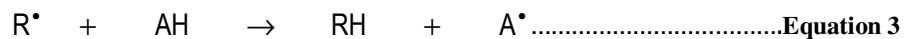


Figure 4: Lipid peroxidation reaction mechanism

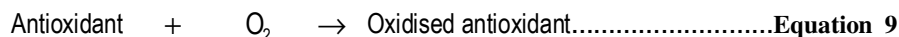
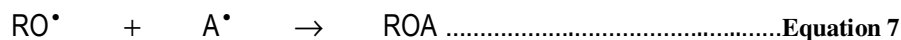
The LOO• (Figure 4) can abstract hydrogen from an adjacent fatty acid to produce a lipid hydroperoxide (LOOH) and a second lipid radical (Catala 2006). Free metal ions like Fe³⁺ can reduce LOOH forming peroxy and alkoxy radicals (equation 1 and 2) (Catala 2006).



This situation results in a condition of excess free radicals and destruction of cell membranes and other structures leading to local injury by disturbing the assembly of the membrane. Changes in fluidity, permeability, and inhibition of metabolic processes are the result and can cause eventual organ dysfunction. The LOOH can break down to reactive aldehyde products such as malondialdehyde (MDA), 4-hydroxy-2,2-nonenal (HNE), 4-hydroxy-2,2-hexenal (4-HNE) and acrolein which are more stable molecules than free radicals themselves. These products can diffuse from the original cells to attack targets far from the site of the original event causing more damage (Esterbauer *et al.* 1991). Antioxidants inhibit the formation of free alkyl radicals in the initiation step or interrupt the propagation of the free radical chain thereby delaying or slowing the chemical reaction rate of lipid oxidation (equation 3 to 9) (Havsteen 2002).

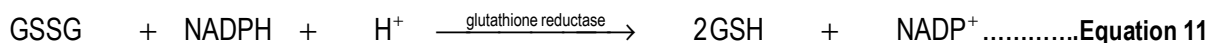
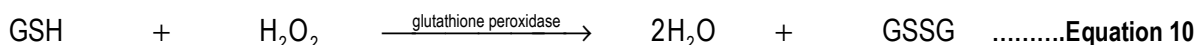


Termination reactions



The oxidized antioxidant (A•), which stabilizes the acquired reactive electron by resonance within its double bond conjugated structure, is not as reactive as the free radicals. The resultant species is not as destructive to its surrounding molecules as the free radical hence scavenging the free radical in the process (Havsteen 2002). The body has developed some endogenous defences to protect itself against free radicals like superoxide dismutases (SODs), catalases and glutathione peroxidase. These defences are not sufficient, resulting in increased levels of free radicals leading to tissue damage and disease.

Glutathione peroxidase enzyme also forms a redox cycle that is central for reduction of intracellular hydroperoxides. The enzyme reduces H₂O₂ to H₂O by oxidizing glutathione (GSH) (equation 10). The oxidized form glutathione (GSSG) is then catalysed by glutathione reductase (equation 11)



Superoxide dismutase (SOD) is another antioxidant enzyme that is endogenously produced and is present in every cell. SOD is reported to appear in three forms: (1) Cu-Zn SOD in the cytoplasm with two sub-units; (2) Mn-SOD in the mitochondrion and (3) Cu-SOD which is extracellular. The mode of action of the three antioxidant enzymes is that SOD eliminates the ROI by reducing superoxide to hydrogen peroxide. Catalase and selenium dependent glutathione peroxidase are responsible for reducing H₂O₂ to water. Trace metal cofactors copper, zinc or manganese for superoxide dismutase (SOD) and iron for catalase are therefore required. Superoxide dismutase's role is to dismutase O₂ to H₂O₂. If the nutritional supply of these minerals is inadequate, enzymatic defences against free radicals may be impaired. Plant extracts with good antioxidative activities of (Table 3) are valuable because they may be used as supportive therapy in various ailments. Active plant secondary metabolites (the natural antioxidants) are monohydroxy and polyhydroxy phenol compounds with various ring substitutions (Naseem *et al.* 2010).

Table 3: Some plants used to treat condition associated with oxidative stress

Plant species	Parts used	Bioactive constituents	Indications	Antioxidant activity Assays, EC ₅₀ (µg/ml)
<i>Agasthoma betulina</i> (Bergius) Pillans	Leaves	Limonene, menthone, diosphenol, l-pulegone and ψ-diosphenol (Moolla & Viljoen 2008)	Antispasmodic, antipyretic, cough remedy, diuretic, kidney and urinary tract infections, and other stomach ailments, rheumatism, gout and bruises (Moolla & Viljoen 2008)	DPPH, 100; ABTS, 37.75±0.54, anti-inflammatory 5 LO IC ₅₀ >100 (Moolla and Viljoen 2008)
<i>Agasthoma crenulata</i> (L) Pillans	Leaves	Limonene, methone, diosphenol, l-pulegone (Moolla & Viljoen 2008)	Antispasmodic, antipyretic, cough remedy, diuretic, kidney and urinary tract infections, cholera and other stomach ailments, rheumatism, gout and bruises (Moolla and Viljoen 2008)	DPPH, >100; ABTS, 33.32±0.33, anti-inflammatory 5 LO IC ₅₀ >100 (Moolla & Viljoen 2008)
<i>Aspalathus linearis</i> (Brum.f.) R. Dahlgr.	Leaves	(+)-Catechin, (-)-epicatechin, procyanindin, bi-fisetinidol-(4β,6: 4β,8)-catechin, aspalathin, dihydrochalcone glucoside, aspalalinin (Joubert & Schulz 2006)	Infantile colic, allergies, asthma, and dermatological problems (Joubert & Schulz 2006)	DPPH, 2.33, O ₂ scavenging=44.4, ABTS = 2.37, FRAP = 1.98 (Joubert & Schulz 2006)
<i>Salvia muiirii</i> L. Bol (Lamiaceae)	Leaves	Rosmaric acid (Kamatou <i>et al.</i> 2010)	Treatment of inflammation (Kamatou <i>et al.</i> 2010)	DPPH, 11.1±0.52 (Trolox 2.51 ± 0.41); ABTS, 11.9±1.52 (Trolox - 2.43 ± 0.07), 5-LOX anti-inflammatory assay, IC ₅₀ > 100 compared with 4.95 ± 0.07 NDGA (Kamatou <i>et al.</i> 2010) [for 5-LOX assay, NDGA, IC ₅₀ < 30: good activity; 30 < IC ₅₀ < 80 moderate activity; IC ₅₀ > 80: poor activity (Kamatou <i>et al.</i> 2010).
<i>Crossopteryx febrifuga</i> (Afzel) Benth. (Rubiaceae)	Root bark		Treatment of hookworm, syphilis ulcer (Maiga <i>et al.</i> 2008)	IC ₅₀ values (µg / mL) Methanol extract DPPH 35± 3, quercetin, 3.4±0.3; 15- LO inhibitory capacity, 35± 1 compared to quercetin , 11.5± 0.6 (Maiga <i>et al.</i> 2008)
<i>Diospyros abyssinica</i> (Hiern) F. White (Ebenaceae)	Leaves, fruit, and seed	Betulin, betulinic acid lupeol (Maiga <i>et al.</i> 2008)	Malaria, wound healing, dysentery (Maiga <i>et al.</i> 2008)	IC ₅₀ values (µg / mL) Methanol extract DPPH 16.6± 0.4, quercetin, 3.4±0.3; 15- LO inhibitory capacity 16±1.0, compared to quercetin , 11.5± 0.6 (Maiga <i>et al.</i> 2008)
<i>Lannea velutina</i> A. Rich (Anacardiaceae)			Treatment of diarrhoea and strained muscle (Maiga <i>et al.</i> 2008)	Methanol root extract DPPH 12 ± 2, quercetin, 3.4±0.3; 15-LO inhibitory capacity, 14± 1, compared to quercetin , 11.5± 0.6 (Maiga <i>et al.</i> 2008)
<i>Parathelypteris nipponica</i> (Franch. et Sav.) Ching	Rhizomes	Unknown	Inflammation, burn scald and acute liver damage (Fu <i>et al.</i> 2010)	Methanol extract: DPPH 2.00±0.02 mg/ml ;reductive ability 0.18±0.02 mg/ml ferric thiocyanate (FTC) assay 0.10±0.01 mg/ml superoxide anion 0.60±0.05 mg/ml, OH radicals 0.26±0.03 mg/ml, and hydrogen peroxide 0.45±0.03 mg/ml (Fu <i>et al.</i> 2010)
<i>Salvia schlechteri</i> Briq. (Lamiaceae)	Leaves	Rosmarinic acid, caffeic acid, carnosol and derivatives (carnosic acid (Kamatou <i>et al.</i> 2010)	Treatment of coughs, colds, bronchitis and female ailments (Kamatou <i>et al.</i> 2010)	DPPH, 1.61±0.03 (Trolox - 2.51 ± 0.41); ABTS, 17.5±2.05 (Trolox - 2.43 ± 0.07, 5-LOX anti-inflammatory assay, IC ₅₀ > 100 compared with 4.95 ± 0.07 NDGA [for 5-LOX assay, NDGA, IC ₅₀ < 30: good activity; 30 < IC ₅₀ < 80 moderate activity; IC ₅₀ > 80: poor activity] (Kamatou <i>et al.</i> 2010)
<i>Hermannia althaeifolia</i> L. (Malvaceae)	Flowers, stem, Leaves	Not known	Fever, cough, asthma, wounds, burns, eczema, stomach ache, as a purgative, heartburn, flatulence in pregnant women, colic, haemorrhoids (Essop <i>et al.</i> 2008)	DPPH, 14.73±0.79; ABTS, 11.86±0.64 5-LOX assay= 53.53±.0.23 with 100 µg/ml (Essop <i>et al.</i> 2008)

<i>Lythrum salicaria</i> L. (Lythraceae)	Whole plant	Tannins, flavone-C-glycosides and anthocyanins, Isoorientin, isovitexin and their derivatives (Tunalier <i>et al.</i> 2006)	Diarrhoea, Chronic intestinal catarrh, haemorrhoid, eczema, varicose veins, bleeding gums (Tunalier <i>et al.</i> , 2006)	Ethyl acetate fraction Iron(II) reduction 0.5± 0.00; DPPH IC ₅₀ , 2.7 ± 0.0 1; % inhibition Fe-thiocyanate 93.8 ± 0.9 (Tunalier <i>et al.</i> 2006).
<i>Combretum apiculatum</i> Sond. subsp <i>apiculatum</i> (Combretaceae)	Leaves	Quercetrin; kaempferol; (Eloff <i>et al.</i> 2008)	Treatment of coughs, abdominal disorders, bilharzia, back ache, colds, constipation, diarrhoea, fever, cancer (Eloff <i>et al.</i> 2008)	DPPH: 11.81±.85 µM; 47.36±0.3µM, respectively (Eloff <i>et al.</i> 2008)
<i>Combretum woodii</i> Dummer. (Combretaceae)	Leaves	Combretastatin B5	Treatment of coughs, abdominal disorders, bilharzia, back ache, colds, constipation, diarrhoea, fever, cancer (Ree <i>et al.</i> 1999; quoted in Eloff <i>et al.</i> 2008).	TEAC value of hexane extract = 2.3 (Ree <i>et al.</i> , 1999 quoted in Eloff <i>et al.</i> 2008).
<i>Gunnera perpensa</i> L. (Gunneraceae)	Rhizome		Treatment of rheumatic pains, cold, wound dressing, stomach ailments, menstrual pains, and treating female infertility and male impotence (Simelane <i>et al.</i> 2010).	50 mg/ml extract in methanol: DPPH IC ₅₀ =1.6, ABTS IC ₅₀ = 0.9, Superoxide scavenging=IC ₅₀ >5, Hydroxyl scavenging IC ₅₀ >5, NO scavenging IC ₅₀ =>5 (Simelane <i>et al.</i> 2010).
<i>Vitex obovata</i> ssp. <i>obovata</i> (Lamiaceae)	Aerial parts		Depression, venereal disease, malaria, asthma, allergy, wounds, skin diseases and snake bite. (Neuwinger 2000).	DPPH IC ₅₀ methanol extract= 33.06±1.68 µg/ml; Anti-malarial IC ₅₀ 14.35±.2.01 µg/ml; Toxicity = IC ₅₀ 7.19±.0.89 µg/ml (Nyiligira <i>et al.</i> 2008)
<i>Vitex obovata</i> ssp. <i>wilmsii</i> Gurke C. L. Bredenkamp & Botha (Lamiaceae)	Aerial parts		Depression, venereal disease, malaria, asthma, allergy, wounds, skin diseases and snake bite (Neuwinger 2000).	DPPH IC ₅₀ methanol extract =27.90 ±7.21 µg/ml; Anti-malarial IC ₅₀ 16.02± 3.07 µg/ml; Toxicity = IC ₅₀ 26.91±.3.77 µg/ml (Nyiligira <i>et al.</i> 2008).
<i>Vitex pooara</i> Corbishley. (Lamiaceae)	Aerial parts		Depression, venereal disease, malaria, asthma, allergy, wounds, skin diseases and snake bite.(Neuwinger 2000).	DPPH IC ₅₀ Methanol extract = 30,56±2.83 µg/ml; Anti-malarial IC ₅₀ 13.15± 2.05 µg/ml; Toxicity = IC ₅₀ 34,1±6.86µg/ml (Nyiligira <i>et al.</i> 2008).
<i>Vitex rehmanii</i> Gurke. (Lamiaceae)	Aerial parts	Cirsimaritin 12S, 16 S/R-dihydroxy- <i>ent</i> labda-7,13-diene-15,16-olide (Nyiligira <i>et al.</i> 2008).	Enema for stomach ache (Watt & Breyer Brandwijk 1962).	DPPH IC ₅₀ methanol extract 22.14±.1.74 µg/ml; Anti-malarial IC ₅₀ 9.16±.1.37 µg/ml; Toxicity = IC ₅₀ 7.67± 0.31 µg/ml (Nyiligira <i>et al.</i> 2008).
<i>Vitex zeyheri</i> Sond, ez Schauer.(Lamiaceae)	Aerial parts		Depression, venereal disease, malaria, asthma, allergy, wounds, skin diseases and snake bite (Neuwinger 2000).	DPPH IC ₅₀ methanol extract >100 µg/ml; Anti-malarial IC ₅₀ 12.42± 2.05µg/ml; Toxicity = IC ₅₀ 14.99 ± 4.64 µg/ml (Nyiligira <i>et al.</i> 2008)
Ascorbic acid				DPPH, 2.46±0.01 Essop <i>et al.</i> 2008).
Trolox				DPPH, 2.51±0.41; ABTS,2,43±0.07 (Kamatou <i>et al.</i> , 2010).

TEAC = trolox equivalent antioxidant capacity, NDGA = Nordihydroguaiaretic acid

2.5 CLASSES OF PHYTOCHEMICAL COMPOUNDS

Plant secondary metabolites with biological activity are important to drug discovery because they can be used for therapeutic purposes, modified to more potent derivatives or synthesized with improved activity and reduced toxicity (Kostova 2005). The phytochemical compounds are synthesized by the plant as a form of defence mechanism to fight disease conditions like fungal and bacterial attack or other disease causing conditions to the plants (Chong *et al.* 2009). The phytochemicals are broadly classified as follows: alkaloids, phenolics and terpenes. In the following section, a brief discussion will cover each of their pharmacological significance, chemical structures, biosynthesis and examples of plants where some of them have been reported to be sourced.

2.5.1 ALKALOIDS

Alkaloids are a group of plant secondary metabolites that contain heterocyclic ring systems with nitrogen. Alkaloids exhibit varied pharmacological activity, giving rise to their use as pharmaceuticals, stimulants, narcotics, and poisons (Facchini 2001). Anti-inflammatory quinoline alkaloids such as 1-methyl-2-nonyl-4-quinolone ($IC_{50} = 12.1 \mu M$), Evorcapine ($IC_{50} = 14.6 \mu M$), 1-methyl-2-(6'Z)-6'-undecenyl-4-quinolone ($IC_{50} = 10.0 \mu M$) and 1-methyl-2-(4'Z,7'Z)-4'7'-tridecenyl-4-quinolone ($IC_{50} = 10.1 \mu M$) exhibiting 5-LO inhibition in each case, (Figure 5) compared to zileuton ($IC_{50} = 12.3 \mu M$), a known 5-LO inhibitor, have been isolated from processed unripe fruits of *Evodia rutaecarpa* demonstrating strong leukotriene synthesis inhibitory capacity (Figure 6) (Adams & Bauer 2008). The plant is used traditionally to treat ailments such as GIT disorders, headache, menstrual complaints and mouth ulcers (Adams & Bauer 2008).

Other alkaloids that are used in clinical practice include the analgesics morphine and codeine, the anticancer compounds vinblastine and taxol, the gout suppressant colchicine, the muscle relaxant (C)-tubocurarine, the antiarrhythmic ajmaline, the antibiotic sanguinarine, and the sedative scopolamine. Caffeine is a central nervous system stimulant, cardiac and respiratory stimulant and is also used as an antidote to barbiturate and morphine poisoning (Aniszewski 2007). Emetine, an alkaloid in the root of *Cephaelis ipecacuanha*, is used in the treatment of amoebic dysentery and other infections.

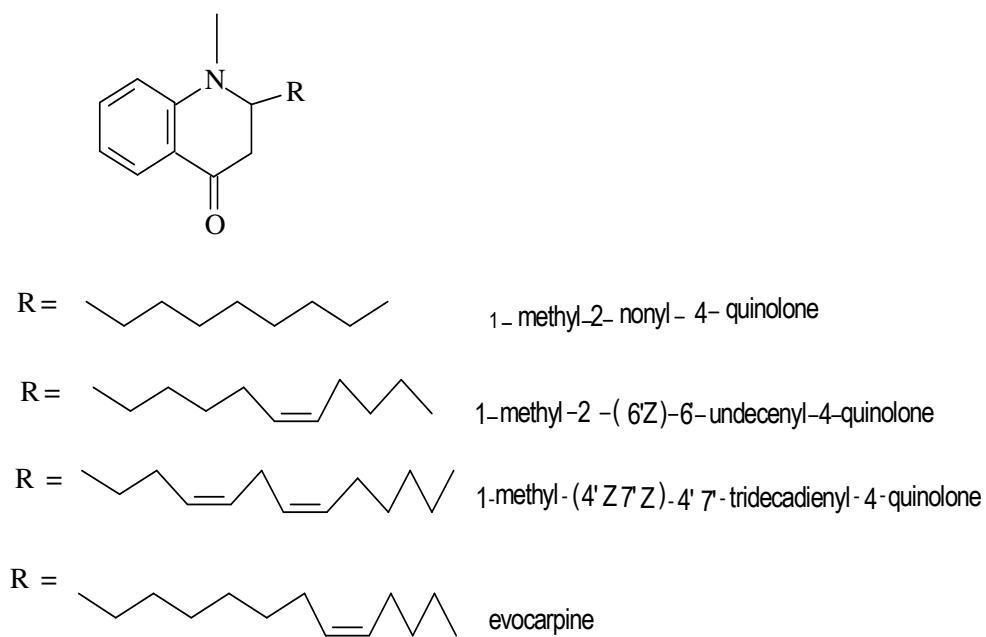


Figure 5: Anti-inflammatory quinolone alkaloids

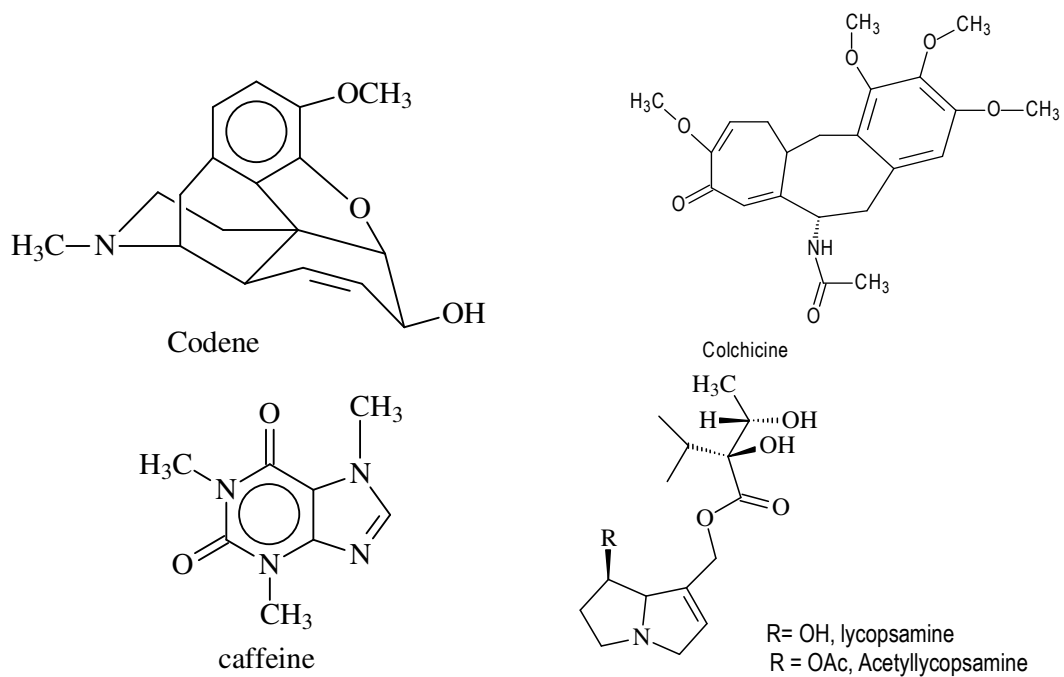


Figure 6: Plant derived alkaloids

Alkaloid biosynthesis

Biological precursors of most alkaloids are amino acids such as ornithine, lysine, phenylamine, tyrosine, tryptophan, histidine, aspartic acid and anthranilic acid (Plemenkov 2001). Pathways vary, depending on the type of alkaloid being synthesized. For example, tripenoid indole alkaloids (TIAs such as vindoline) come from tryptophan, benzyisoquinoline come from tyrosine and tropane (such as hyosyamine) alkaloids from ornithine (Figure 7) (Facchini 2001). Tropane alkaloids (reported to occur predominantly in Solanaceae) biosynthesis includes cholinergic drugs atropine, hyoscyamine, scopolamine and cocaine (Facchini 2001). Purine alkaloids include caffeine; theobromine and theacrine have a different biosynthetic pathway starting with xanthosine (Facchini 2001).

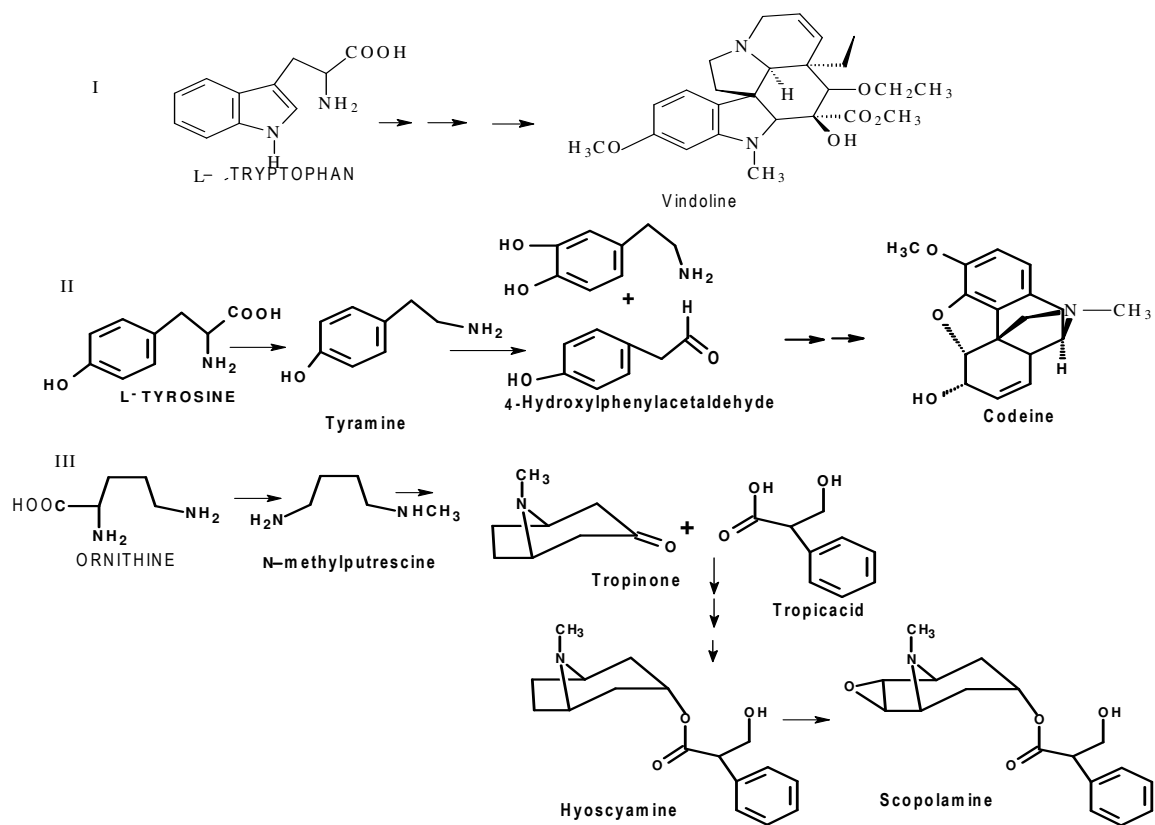


Figure 7: Biosynthesis of alkaloids, from tryptophan (TIAs) tyrosine alkaloids and ornithine

2.5.2 PHENOLIC COMPOUNDS OF PHARMACOLOGICALLY VALUED MEDICINAL PLANTS

Phenolic secondary metabolites have diverse chemical structures classified as flavonoids, tannins, stilbenes, chalcones, and coumarins. Each of these groups demonstrates a variety of biochemical and pharmacological properties (table 4) such as antioxidant, antimicrobial, anti-inflammatory, anti-tumour promotion, anti-HIV, antileishmanial (Eloff *et al.* 2008). Plant families such as Asteraceae, Combretaceae, Verbenaceae, Hyacinthaceae, and many others which are rich in phenolics secondary metabolites are used in South African traditional medicines for treating a variety of disease conditions. Phenolic compounds are further sub-divided into simple phenolic, flavonoids, stilbenoid, coumarins, hydrolysable tannins and condensed tannins. The representative structures and some pharmacological activities of these groups of compound are given in Figure 8 and Table 4 respectively.

Table 4: Some polyphenols with pharmacological properties

Name of active polyphenol and plant source	Class of polyphenol	Biological activity
Combretastatin B5 , 1, from <i>Combretum woodii</i> ,1 (Eloff <i>et al.</i> 2005).	Stilbenoid (Eloff <i>et al.</i> 2005).	<i>S. aureus</i> , MIC = 16 µg / ml; <i>P. aeruginosa</i> and <i>E faecalis</i> MIC for both = 125 µg/ml (Eloff <i>et al.</i> 2005) Combretastatin B5 also demonstrated antioxidant capacity about eight times of vitamin E with a TEAC value of 7.9 (Zishiri 2005).
Bartericin A1, 2, from <i>Dorstenia barteri var subtriangularis</i> (Moraceae) (Ngameni <i>et al.</i> 2008)	Dipyrrenylated chalcone (Ngameni <i>et al.</i> 2008)	Anti-malarial, activity against <i>Plasmodium falciparum</i> IC ₅₀ values of 2.15 µM (Ngameni <i>et al.</i> 2008).
Nepetin from <i>Eupatorium arnotianum</i> Griseb (Clavin <i>et al.</i> 2007).	Flavonoid (Clavin <i>et al.</i> 2007).	Anti-inflammatory activity on TPA(12-O-tetradecanoylporbol-13-acetae) induced mouse ear edema inhibition =46.9% compared to positive control indomethacin =60%.(Clavin <i>et al.</i> 2007).
3'4'-dihydroxyflavanone 7-O-β-D-glucoside 3 from <i>Bidens parviflora</i> Willd (Wang <i>et al.</i> 2007).	Flavanone glucoside (Wang <i>et al.</i> 2007).	Anti-allergic activity in histamine release from rat mast cells stimulated by an antigen-antibody reaction, IC ₅₀ =57.7 µg / ml compared to positive control, indomethacin 52.6 µg/ml (Wang <i>et al.</i> 2007).
3',4'-dihydroxy aurone 6-O-β-D-glucoside, from <i>Bidens parviflora</i> Willd (Wang <i>et al.</i> 2007).	Aurone (Wang <i>et al.</i> 2007).	Anti-allergic activity in histamine release from rat mast cells stimulated by an antigen-antibody reaction, IC ₅₀ =83.3 µg/ml compared to positive control, indomethacin 52.6 µg / ml (Wang <i>et al.</i> 2007).
Vatdiospyridol, 4 from <i>Vatica diospyroides</i> Sym. (Dipterocarpaceae) (Kinghorn <i>et al.</i> 1999).	Oligostilbenoid, a resveratrol tetramer (Kinghorn <i>et al.</i> 1999).	Cytotoxic against colon cancer, breast cancer (Kinghorn <i>et al.</i> 1999).
<i>Trans-resveratol</i> 5 from <i>Vitis amurensis</i> (Vitaceae)	Stilbene	COX- 2 inhibition IC ₅₀ = 0.535 µM and COX 2 = 0.996 µM (Roupe <i>et al.</i> 2006).
(-)-epigallocatechin(2β-O-7',4β-8')-epicatechin-3'O-gallate, 6 from <i>Camallia sinensis</i> (L.) O. Kuntze var <i>sinensis</i> (cv., Yabutika)(Mukai <i>et al.</i> 2008).	Tannin (Mukai <i>et al.</i> 2008).	Anthelmintic against egg bearing adults of <i>Caenorhabditis elegans</i> LC ₅₀ =49 µmol L ⁻¹ compared to anthelmintic mebendazole LC ₅₀ =49 µmol L ⁻¹ used as a positive control (Mukai <i>et al.</i> 2008).
Rhapontigenin,7 (3,3', 5 -trihydroxy-4'-methoxystilbene) from <i>Rhei undulatum</i> (Roupe <i>et al.</i> 2006).	Stilbene (Roupe <i>et al.</i> 2006).	Inhibits histamine release by 90% at concentrations of 78 µM (Roupe <i>et al.</i> 2006). Inhibits NO production IC ₅₀ = 48 µM (Roupe <i>et al.</i> 2006).
Galangin (3,5,7-trihydroflavone) 8 from <i>Helichrysum aureonitens</i> Sch. Bip (Meyer & Afolayan 2005).	Flavonoid (Meyer & Afolayan 2005).	Inhibited the growth of <i>Bacillus cereus</i> <i>Enterobacter cloacae</i> and <i>Micrococcus kristinae</i> at 100 µg / ml (Laurens <i>et al.</i> 2008).
<i>trans</i> -petrostilbene (trans-3,5-dimethoxy-4'-hydroxystilbene) 9 from <i>Pterocarpus marsupium</i> , (Roupe <i>et al.</i> 2006)	Stilbene (Roupe <i>et al.</i> 2006).	Scavenge DPPH radicals with an EC ₅₀ = 30 µM (Roupe <i>et al.</i> 2006) Anti-cancer activity of B 16 melanoma F10 cells by 40% at concentration of 40 µM (Roupe <i>et al.</i> 2006)
3-O-methylquercetin from <i>Helichrysum odoratissimum</i> (L.) Sweet (Van Puyelde, <i>et al.</i> 1989).	Flavonoid (van Puyelde, <i>et al.</i> 1989).	Antibacterial activity against <i>Salmonella typhimurium</i> MIC = 50 µg/ml; <i>Stahylococcus aureus</i> MIC = 6.25 µg/ml; <i>Candida albicans</i> MIC = 12.5 µg/ml; (Van Puyelde <i>et al.</i> 1989).
1,3,6,8-tetrahydroxy-2,5- dimethoxyxanthone 10 from <i>Securidaca longepedunculata</i> Frese. (Polygalaceae) (Meyer <i>et al.</i> 2008).	Xanthone (Meyer <i>et al.</i> 2008).	% relaxation of pre-contracted rabbit corpus carvenosal muscle, = 97% as compared to Viagra, 100 % (Meyer <i>et al.</i> 2008).
piceatannol (trans-3, 4, 3', 5'-tetrahydroxystilbene) from <i>Euphorbia lagascae</i> (Roupe <i>et al.</i> 2006).	Stilbene (Roupe <i>et al.</i> 2006).	COX- 2 inhibition IC ₅₀ = 4.13 µM and COX 2 = 0.0113 µM (Roupe <i>et al.</i> 2006).

NF_KB = nuclear factor K B which is a pro-inflammatory factor

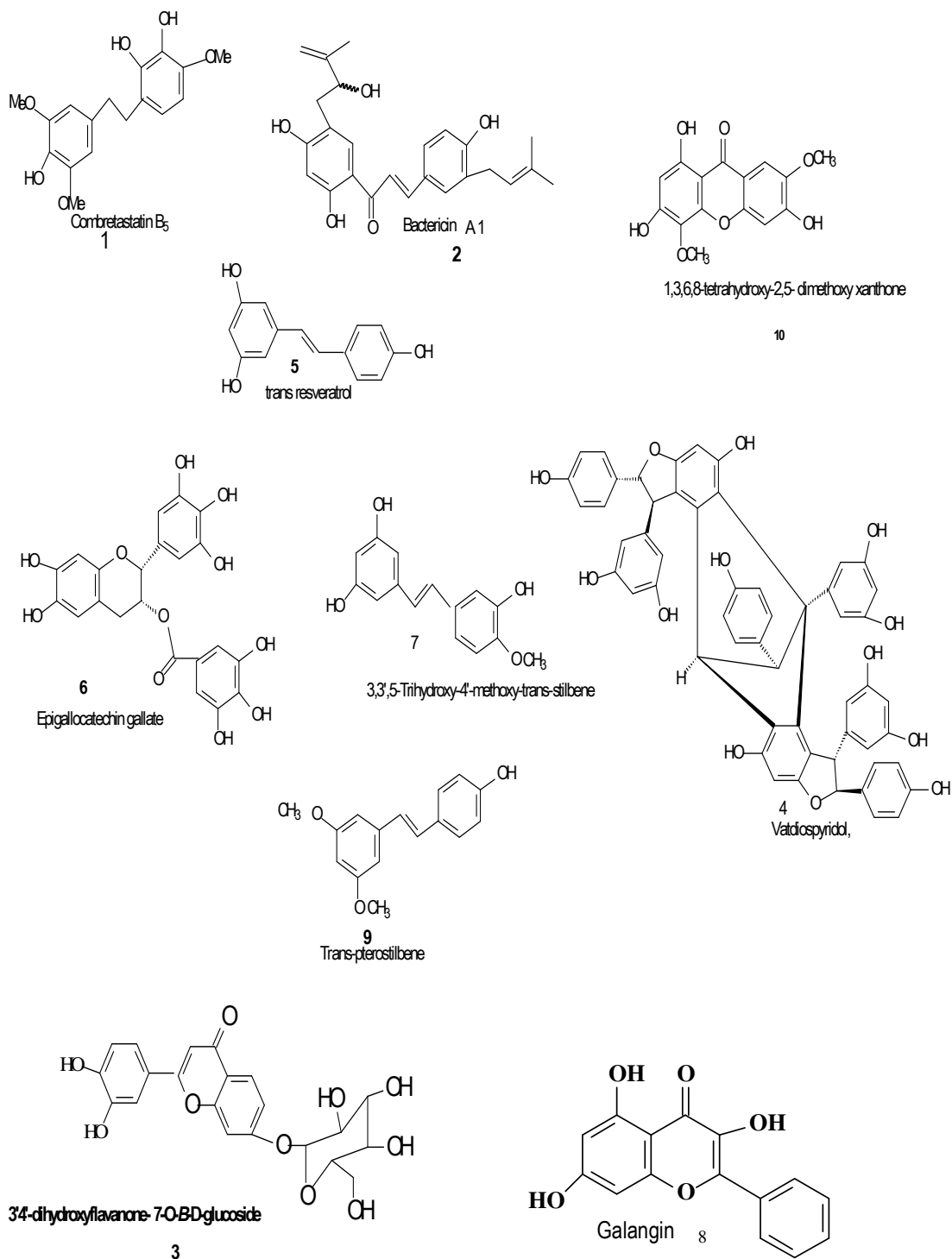


Figure 8: Structures of some polyphenols with pharmacological properties

2.5.3 POLYPHENOL BIOSYNTHESIS

Polyphenols are a family of aromatic molecules that are derived from phenylamine and malonyl-coenzyme A (CoA); via the phenylpropanoid pathway Figure 9 (Winkel-Shirley 2001).

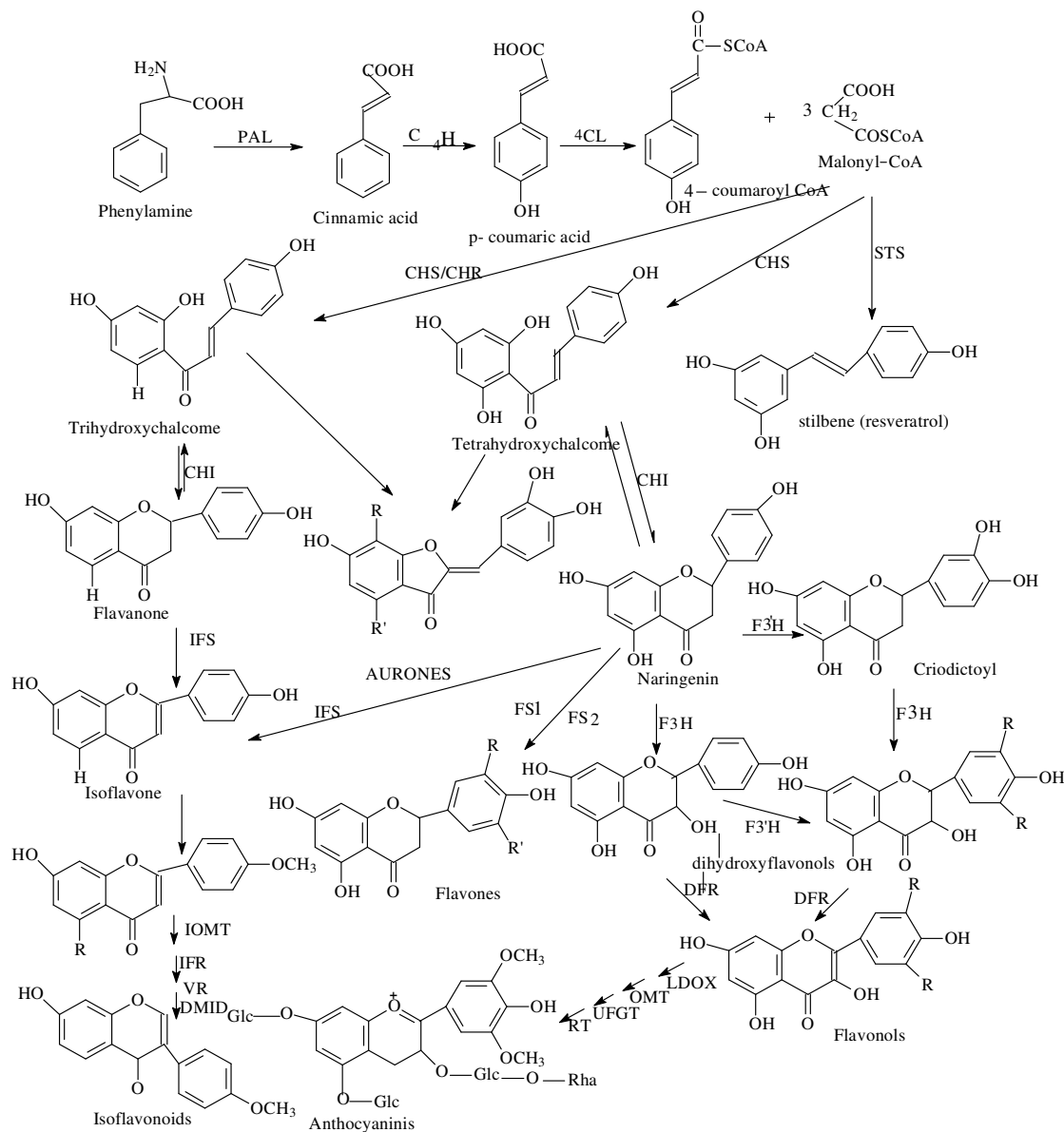


Figure 9: The phenylpropanoid pathway polyphenol biosynthesis.

Enzyme (Figure 9) names are abbreviated as follows: cinnamate-4-hydroxylase (C₄H), chalcone isomerase (CHI), chalcone reductase (CHR), chalcone synthase (CHS), 4-coumaroyl:CoA-ligase (4CL), dihydroflavonol 4-reductase (DFR), 7,2'-dihydroxy, 4'-methoxyisoflavanol dehydratase (DMID), flavanone 3-hydroxylase (F₃H), flavone synthase (FSI and FSII), flavonoid 3' hydroxylase (F₃'H) or flavonoid 3'5' hydroxylase (F₃'5'H), isoflavone O-methyltransferase (IOMT), isoflavone eductase (IFR), isoflavone 2'-hydroxylase (I₂'H), isoflavone synthase (IFS), leucoanthocyanidin dioxygenase (LDOX), leucoanthocyanidin reductase (LCR), O-methyltransferase (OMT), Phenammonia-lyase (PAL), rhamnosyl transferase (RT), stilbene synthase (STS), UDPG-flavonoid glucosyl transferase (UFGT), and vestitone reductase (VR) (Winkel-Shirley 2001).

The garlic acid (IC_{50} DPPH, =8.4 μ M) (Yokozawa *et al.* 1998) is esterified to a core polyol and the galloyl groups may be further esterified or oxidatively cross linked to yield more complex hydrolysable tannins. Tannins are secondary plant metabolites, whose anti-oxidative and anti- inflammatory capacity and anthelmintic activity have been demonstrated (Table 4) on different nematodes of sheep (Athanasiadou *et al.* 2001).

2.5.4 TRITERPENOIDS AND SAPONINS

Terpenes are compounds made up of 5 carbon units, referred to as isoprene units put together in a regular pattern. The number of isoprene units in the terpene determines its complexity and size (Table 5). Carotenoids, steroids, and gibberellins are examples of polymeric isoprene derivatives, which consist of at least thousands of different phytochemicals found in a wide variety of plant species (Volkman 2005).

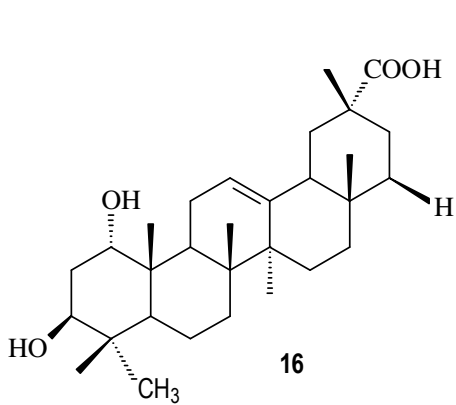
Table 5: Class, numbers of isoprene units, example and sources of terpenes.

Classes of terpenes	Number of isoprene units	Length of Carbon chain	Example	Source of example
Monoterpenes	2	C ₁₀	(-)-Menthol Neptalactone Geraniol	Peppermint oil Catnip oil Rose oil
Sesquiterpenes	3	C ₁₅	Zingiberene Farnesol	Ginger oil Lily of the valley oil
Diterpenes	4	C ₂₀	Retinol(Vitamin A)	Cod liver oil
Triterpenes	6	C ₃₀	Squalene	Shark liver oil
Tetraterpenes	8	C ₄₀	Lycopene	Tomato pigment

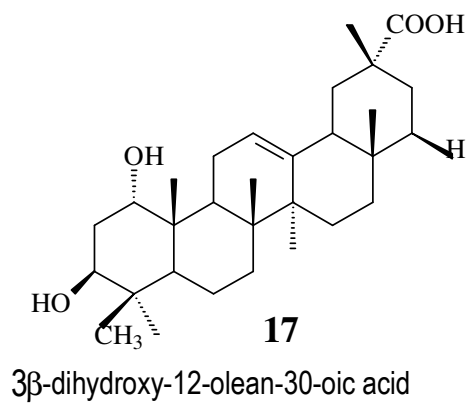
Steroids are terpene derived, but carbons are rearranged or even removed during biosynthesis. Sesquiterpenes and diterpenes serve as pheromones, defensive agents, visual pigments, antitumor drugs and signal transduction networks while monoterpenes are common fragrances and flavours (Volkman 2005). Terpene derivatives like cholesterol are important membrane constituents, precursors of steroid hormones and bile acids in humans and animals. Higher oligoterpenes function as photoreceptive agents, cofactor side chains and constitute natural polymers (Wendt & Schilz 1998). A number of terpenoids with good biological activity (Figure10) have been isolated from many medicinal plants that is used traditionally in treating ailments such as infectious diseases, inflammation, wound, hypertension (Anger *et al.*

2006). Saponins are plant secondary metabolites, consisting of a triterpenoid, steroid or steroidal glycoalkaloid molecule bearing one or more sugar chains (Osbourn 1998). Saponins dissolve in water to form colloidal solutions that foam upon shaking and their surface-active properties distinguish these compounds from other glycosides (Tyler *et al.* 1981). Examples of some saponins (structures 16-21) are in Figure 10.

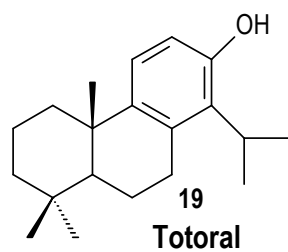
- 1 α , 3 β -dihydroxy-12-oleanen-29-oic acid, **16**. MIC in $\mu\text{g/ml}$ towards *Staphylococcus aureus* (ATCC 29213), 125; *Pseudomonas aeruginosa* (ATCC 27853), >250; *Escherichia coli* (ATCC 25922), 16; *Enterococcus faecalis* (ATCC 29212), 125 (Anger *et al.* 2006)) and strong inhibition of 3 α -hydroxysteroid dehydrogenase with an IC_{50} of 0.3 $\mu\text{g/ml}$, (Katerere *et al.* 2003; Rogers and Subramony 1988)
- 1-hydroxy-12-olean-30-oic acid **17**, demonstrated MIC in $\mu\text{g/ml}$ towards *Staphylococcus aureus* (ATCC 29213), 94; *Pseudomonas aeruginosa* (ATCC 27853), >250; *Escherichia coli* (ATCC 25922), >250; *Enterococcus faecalis* (ATCC 29212), 24 (Anger *et al.* 2006; Mukherjee *et al.* 1994).
- 3, 3 α -dihydroxy-12-oleanen-22-one, **18**. MIC in $\mu\text{g/ml}$ towards pathogens *Staphylococcus aureus* (ATCC 29213), 125; *Pseudomonas aeruginosa* (ATCC 27853), >250; *Escherichia coli* (ATCC 25922), 16; *Enterococcus faecalis* (ATCC 29212), 125 (Anger *et al.* 2006; De Sousa *et al.* 1990).
- Totoral, **19**, a terpene with MIC = 2- $\mu\text{g/ml}$, increases the activity of methicillin against MRSA (Gibbons 2006).
- 12S,16S/R-dihydroxy-*ent* labda-7,13-diene-15,16-olide **20**, from methanol extract exhibited anti-malarial activity, IC_{50} 0.09 \pm 0.02 $\mu\text{g/ml}$; compared to a positive control, chloroquine IC_{50} 0.12 \pm 0.04 $\mu\text{g/ml}$ and toxicity IC_{50} 1.27 \pm 0.21 $\mu\text{g/ml}$ compared to chloroquine Toxicity IC_{50} 519.9 $\mu\text{g/ml}$ MIC in $\mu\text{g/ml}$ towards *Staphylococcus aureus* 4.0 $\times 10^{-3}$; *Bacillus cereus*, 1.00 $\times 10^{-3}$; *Escherichia coli*, 3 $\times 10^{-3}$; *Enterococcus faecalis* 6 $\times 10^{-3}$; *Salmonella typhimurium*, 4 $\times 10^{-4}$; *Cryptococcus neoformans* 3 $\times 10^{-4}$ (Nyiligira *et al.* 2008)



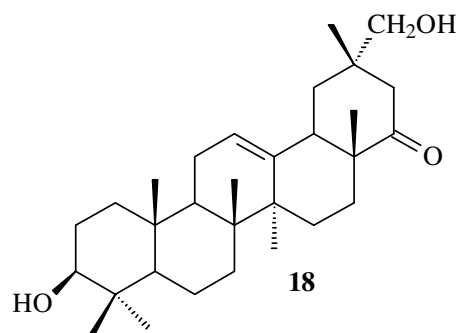
1 α ,3 β -dihydroxy-12-oleanen-29-oic acid



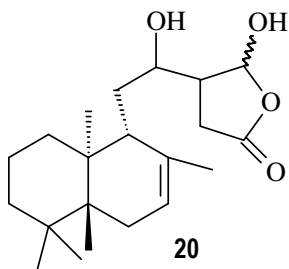
3 β -dihydroxy-12-olean-30-oic acid



Totoral



3,30-dihydroxyl-12-oleanen-22-one



12S,16S/R-dihydroxy-ent labda-7,13-diene-15,16-olide

Figure 10: Structures of biologically active terpenes

Terpene biosynthesis

Synthesis of terpenes occurs by reactions involving formation of the building blocks such as 3, 3 dimethylallyl pyrophosphate (Figure 11). Various terpenes are then formed such as terpenol, limoene, followed by more complex ones depending on the organism and enzymes involved.

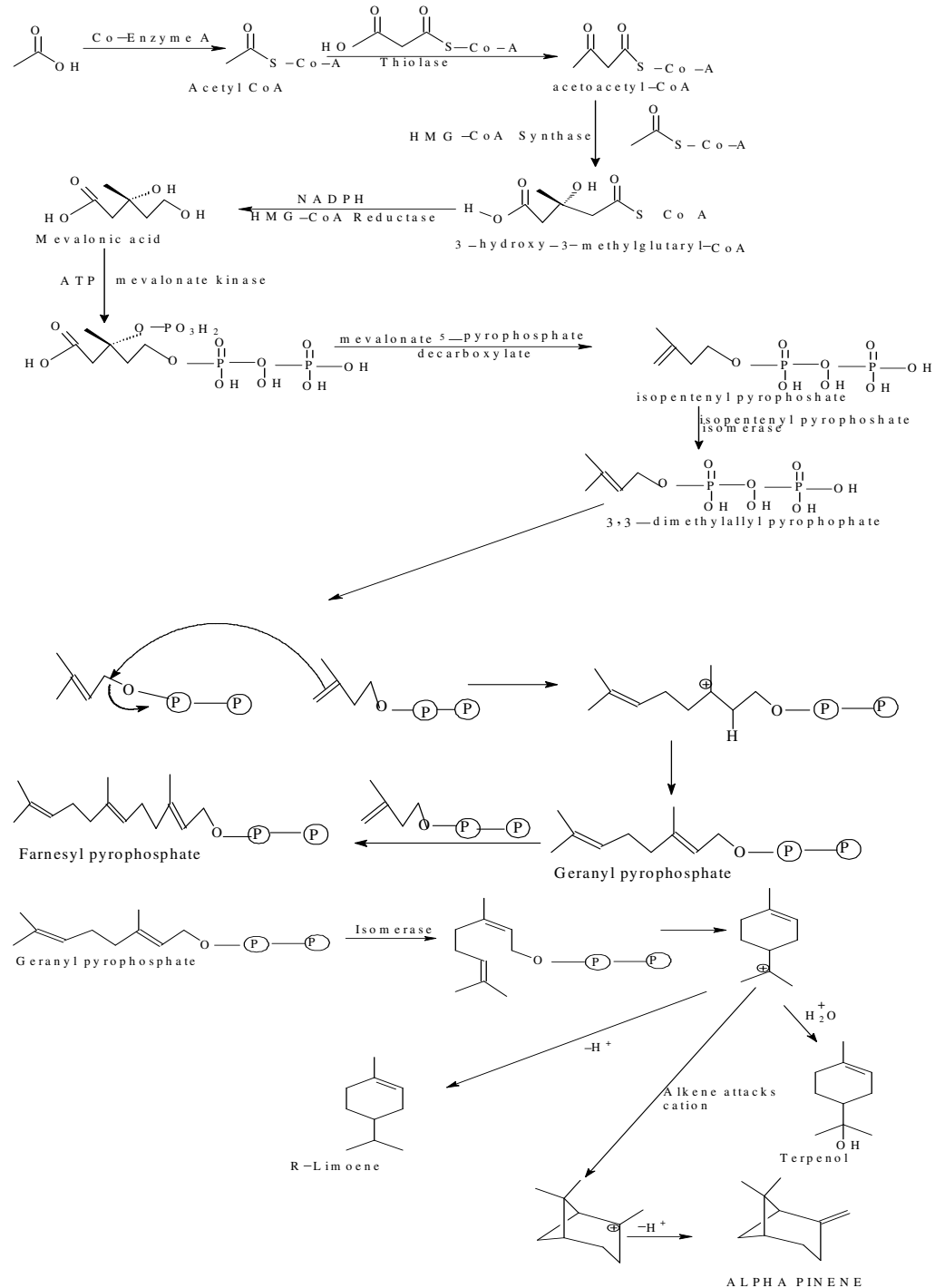


Figure 11: Biosynthesis of terpenes

Saponins are involved in a number of bodily functions and processes including membrane-permeabilising, stimulating the immune system, hypo-cholesterolaemic and anti-tumour acting and they also affect growth, feed intake and reproduction in animals (Francis *et al.* 2002). Saponins are antifungal, antiviral agents, protozoacidal, molluscidal, and antioxidants. They assist in the correction of impaired digestion of protein and the uptake of vitamins and minerals in the gut (Francis *et al.* 2002). Saponins are used in the pharmaceutical industry as raw materials for synthesis of steroidal drugs (Estrada *et al.* 2000). They are the main constituents of many plant drugs and are responsible for many pharmacological properties. Two triterpenoid saponins lotoidoside D and lotoidoside E (figure 12) which were isolated from *Glinus lotoides* exhibit anti-tumour properties (Yan *et al.* 2006). Jujubogenin, [3-O- α -L-arabinofuranosyl-(1 \rightarrow 2)- β -D-glucopyranosyl-(1 \rightarrow 3)]- β -L-arabinopyranoside, MIC of 50- μ g/ml against *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* was isolated from *Colubrina retusa* L.(Rhamnaceae) (Sparg *et al.* 2004).

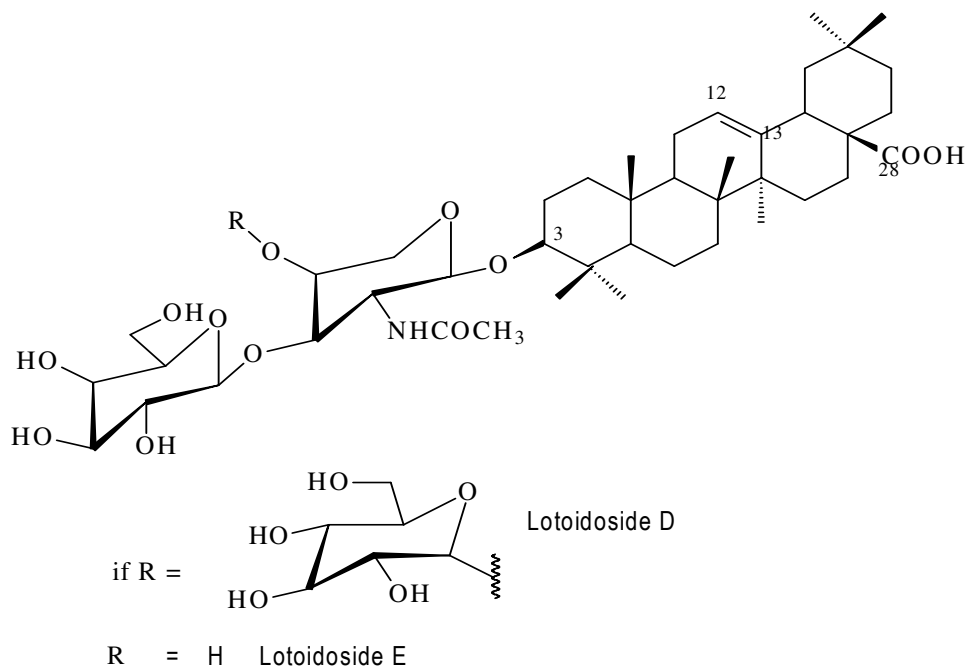


Figure 12: Natural triterpenoid saponins bearing N-acetylglucosamine with anti-tumor activity .

Hederacolchiside A₁, a saponin isolated from *Hedera helix* L. (Araliaceae) demonstrated antileishmanial activity on the parasite *Leishmania infantum* against both promastigote (IC₅₀ of 1.2 ± 0.1 μ M) and amastigote (IC₅₀ of 0.053 ± 0.002- μ M) forms of the parasite (Delmas *et al.*, 2000).

2.6 CONCLUSION

The chemotherapeutic properties of medicinal plants are demonstrated by varied biological activities of different types of plant extracts and pure compounds. This presents the possibility that new and more effective drugs can be obtained from medicinal plants either as the active phytochemicals themselves or as pathogen resistance modulators (Gibbons 2005). Although some plant extracts or pure compounds may be cytotoxic, organic synthesis could be used to come up with less poisonous but effective drugs based on the isolates. Indigenous peoples in South Africa have been using plants of medicinal value for centuries, however, most of them have not yet been scientifically validated (Van Staden 2008). This presents a challenge in ensuring that all medicinal plants used in South Africa are identified, characterized and documented for efficacy and safety (Street *et al.* 2008).

Biological activities of plant extracts may be due to synergism (additive) effects of individuals among the complex components acting through different mechanisms. As a result, development of resistance to plant extracts with anti-infectious activities may be minimal compared to a single compound. Some medicinal plants are being over-harvested and have been on the red list of extinction, therefore investigations of their pharmacological properties are essential for pharmacopeia. Many traditional healers harvest the stem and root from the wild for their healing concoctions and this threatens the plants biodiversity and population stability (Street *et al.* 2008). It is important for research to check if the biologically active compounds in the stem and root parts are also present in the leaves. This would encourage use of leaves instead of destroying the whole plant by removing its roots and stem.

CHAPTER 3

EXPERIMENTAL PROCEDURES

PHYTOCHEMICAL ANALYSIS

3.1 INTRODUCTION

Plant material exists as a complex mixture of carbohydrate, protein, lipid, lignin and a variety of plant secondary metabolites like phenolics, terpenoids and alkaloids. Plant secondary metabolites are the target of investigation for biological activities because of their diverse chemical structure and proven therapeutic potential in traditional medicine (Süleyman *et al.* 2007). Many factors such as biotic (plant age, species genus and plant part) and abiotic (season, ecological, geographical location and climate) conditions influence plant capacity to synthesized phytochemicals (Joubert *et al.* 2008). Therefore, collection of plant materials for biological activities evaluation must be performed at optimal times of the year, plant age and in a known ecological environment (Street *et al.* 2008). The season of the year is important during plant collection because some plants have demonstrated seasonal variation in the composition of some of their secondary metabolites (Van Heerden 2008). Geographical origin also affects the composition of some medicinal plants (Van Wyk 2008).

In medicinal plant research, plants are sourced from various locations include wild collection, organized markets, National Botanical Gardens and in some cases, herbarium specimens (Eloff 1999). The advantage of collecting plants from National Botanic gardens and herbariums lies with ease of plant identification, preservation and future reference. However, many plants used by traditional practitioners in the rural areas and herbal outlets are sourced from the wild with no proper identification or documentation (Makunga *et al.* 2008). For proper documentation, medicinal plants for scientific evaluation need to be clearly identified, voucher specimen prepared and deposited at an established herbarium. Medicinal plant materials are pre-treated prior to investigation. Some of the treatments include washing, drying, grinding, storage, and extraction process.

3.1.1 Washing

Plant materials are washed with water as a pre-treatment method to remove environmental contaminants such as dust from leaves, soil from roots and any other extraneous materials that may adhered to the plant samples.

3.1.2 Drying

The cleaned plant materials are dried at optimal conditions to preserve to essential ingredient and increase the shelf life of the material. Some of the drying methods include air dried at room temperature under shade (Parekh &

Chanda 2007), oven drying at a relatively low temperature (preferably below 50°C) (Buwa & Van Staden 2006), sun drying (Liu *et al.* 2009) and freeze drying (Eloff 1998).

3.1.3 Grinding

Plant materials are usually ground as a means of reducing the particle sizes and also to increase the surface area necessary to maximize the yield of the phytochemicals that are bound within the plant matrix.

3.1.4 Storage

Plant materials need to be stored and preserved under dry and dark conditions. Damp or humid conditions could lead to hydrolysis of different compounds and fungal growth could alter the powder composition. Heavy fungal infections can affect biological activity of some plant extracts (Kotse & Eloff, 2002).

3.2 METHODS OF SAMPLE PREPARATION

3.2.1 Extraction

Extraction of phytochemicals from the plant matrix is carried out using suitable solvents (solid-liquid) as a means of eliminating unwanted solid plant material and increasing the concentration of targeted components. A wide range of solvents such as hexane, cyclohexane, petroleum ether, diethyl ether (non-polar), chloroform, ethyl acetate (medium polar), and alcohols, acetone or water (polar) are usually employed in solid-liquid extraction processes. Depending on the compound(s) of interest, solvent polarity choice is important although water or ethanol is commonly used as extractants in the form of decoctions, infusions or tinctures by traditional healers. Organic solvents extracts or fractions usually exhibit better biological activities than water extracts (Parekh & Chanda 2007b.) and therefore solvent choice in scientific evaluation of medicinal plant extracts is important.

Various extraction methods such as soxhlet extraction (Eloff 1998), steam distillation (Singh *et al.* 2010), maceration in a solvent (solid-liquid extraction at room temperature with shaking) (Eloff, 1998; Steenkamp *et al.* 2004) are used in medicinal plant research. For biologically active volatile components, fresh plant sample is used in solid-liquid extraction protocols at low temperature to avoid loss of the volatile components (Liu *et al.* 2009). These protocols include hydro-distillation (HD), microwave assisted extractions (ME) and liquid supercritical CO₂ extractions (Liu *et al.* 2009). Solid-liquid maceration has the advantage over other methods in that it is a fast, cheap and effective method of obtaining crude extracts from plant material. Extraction methods can affect the yield of some components because of the difference

in solubility in the solvents or instability to the conditions employed during extraction (Liu *et al.* 2009). Ultra sound assisted extraction, UAE, hydro distillation, HD, microwave assisted extraction, ME, and liquid supercritical CO₂ extractions, (Liu *et al.* 2009) require specialized equipment but may be ideal if equipment is available, for the isolation of some components. Choice of solvent is determined by the chemical properties of the components of interest to be extracted (Eloff 1998; Kotze & Eloff 2002). Acetone was reported to be the best extractant to produce crude extracts because it dissolves many hydrophilic and lipophilic components is miscible in almost all other extractants, volatile and less toxic to test organisms compared to other solvents (Eloff 1998). In this study, acetone was used for crude extractions.

3.2.2 Liquid-liquid fractionation

Fractionation (liquid-liquid partition) involved the use of two immiscible solvents of different polarities to separate extract components of similar polarity or solubility into the same group. This is one of the first stages in the isolation of active components from plant tissues and also a means of potentiating the activity of an extract. The first step in the process is involved in dissolving the extracts in water followed by fractionating with solvents of increasing polarity in the order of hexane, dichloromethane, ethyl acetate, butanol and finally residual water fraction (Eloff *et al.* 2005). In bioactivity guided isolation, biological assays such as bioautography, minimum inhibitory concentration and MIC, determination are performed to ensure that antimicrobial compounds are targeted for isolation.

3.3 PHYTOCHEMICAL ANALYSIS

3.3.1 Chromatographic techniques in the analysis of medicinal plant extracts

Chromatography is a method of separating components of mixture by principle of differential solubility, size or molecular weight in the eluting solvent (mobile phase) and adsorption on the adsorbent (stationary phase). Chromatographic techniques including planar (paper chromatography (PC), thin layer chromatography (TLC), preparative TLC (PTLC), reverse phase thin layer chromatography (RPTLC)) and column (open column chromatography (OCC), gas chromatography (GC), and high performance liquid chromatography (HPLC)) are used as a single technique or in combination to isolate compounds (Harborne, 1998).

Planar chromatography techniques are similar in their qualitative methods with storable results and images which can be kept as the extract fingerprints for future reference. TLC is suitable for separating lipid soluble compounds such as lipids, steroids, quinones and chlorophylls while PC is mostly applicable for highly polar compounds such as carbohydrates, amino acids, nucleic acid bases, organic acids and phenolic compounds. TLC has advantages over PC in that a number of different adsorbents may be used on a glass, aluminium or other inert support for the stationary phase

(Harborne, 1998). Examples of adsorbents commonly used are silica gel, aluminium oxide, celite, calcium hydroxide, ion exchange resin, magnesium sulphate, polyamide, Sephadex, polyvinylpyrrolidone and cellulose (Harborne 1998). This makes TLC more versatile as compared to PC. The more compact nature of adsorbent in TLC increases its speed compared to PC and is an advantage when using labile compounds. TLC can separate less than μg amounts and is therefore more sensitive than PC (Harborne 1998). However, planar chromatography has general disadvantage of not being quantitative in its chromatograms.

Preparative TLC (PTLC), which is a form of thin layer chromatography, but with a thicker layer of adsorbent, is used to clean a contaminated compound with few impurities after fractions are eluted from open column chromatography (Hostettmann *et al.* 1998). The mixture is separated into bands by elution on preparative TLC plates, then the bands of interest scraped off followed by extracting the compound of interest from the adsorbent by use of a suitable solvent (Hostettmann *et al.* 1998). The major advantages of PTLC when compared to other analytical quantitative techniques are that it is a quick, easy, cost effective method of separating between milligrams to a gram of pure compounds (Hostettmann *et al.* 1998). It has disadvantages over automated methods due to poor detection limit, control of elution and long time for method development and restricted stationary phase such as silica, alumina, cellulose and reverse phase (Cannel 1998). PTLC also contains binders, fluorescent indicators in unknown compositions and may contaminate the sample being separated (Hostettmann *et al.* 1998)

Two dimensional TLC, in which the sample is spotted on the TLC in the usual way, followed by developing and drying, followed by the same plate being rotated through 90° and developed the second time (Hostettmann *et al.* 1998). During the second elution, compounds are separated further, enhancing resolution (Hostettmann *et al.* 1998). The resulting chromatogram may then be observed under UV or stained, detecting if contaminants are present (Hostettmann *et al.* 1998).

Gas liquid chromatography, GLC is suitable with separation of volatile compounds. GLC provides both quantitative and qualitative data since the area under the peaks on chromatograms are directly related to the concentrations of the components under study. GLC can also be hyphenated to a mass spectrometer (MS) to determine the chemical structure of the compounds (Harborne 1998). The disadvantage of GLC is derivatisation of higher boiling compounds to a volatile compound such as conversion into esters (Harborne, 1998).

High performance liquid chromatography, HPLC, like the GC provides both quantitative and qualitative data on non-volatile compounds (Harborne, 1998). The pre-packed column in HPLC can be with silica compounds (for non-polar compounds) or reverse phase C_{18} bonded phase column (RP-HPLC for polar compounds). Some chromatographic methods such as planar, HPLC, and GC are used to produce chromatograms or chemical fingerprints to assess the chemical constituents of medicinal (Stafford *et al.* 2005) or fractions (Olivier *et al.* 2008), in phylogenetic analysis to determine if certain secondary metabolites are common to plants of the same family or even different families

(Koorbanally *et al.* 2006), check purity (Olivier *et al.* 2008), and stability of compounds (Stafford *et al.* 2005). Column chromatography and PTLC are used for purifying fractions.

3.4 MATERIALS AND METHODS

3.4.1 Basis for selection of plants for studies

Selection of plants was based on the ethno-botanical survey conducted in Mabandla village with six traditional healers who service the community. The healers identified plants which they commonly used for treatment of inflammation-related diseases (Table 3.1). Some other common diseases treated with the same medicinal plants include headache, stomach ache, diarrhoea, vomiting, back ache, chest pains, kidney condition, insomnia, anxiety, cough, flu and fever (Questionnaire 2009).

3.4.2 Ethno-botanical survey of plant used in inflammatory at Mabandla Village, Kwa-Zulu Natal

An ethno-botanical survey was conducted with the chief, Mr Langford S.I. Dlamini Sibakhulu, together with six traditional healers (4 males and 2 females) of Mabandla village to establish the common methods of medicinal plant usage in the village. Questionnaires were administered to the chief and traditional healers.

3.4.3 Plant selection and collection

Selections of plants were based on the ethno-botanical survey as stated above and literature documentations of anti-inflammatory medicinal plants of Zulu medicinal plants (Table 6) were collected from Mabandla village of UMzimkhulu, Local Municipality, KwaZulu-Natal, South Africa with the aid of traditional healers of the village (Mr Sanoyi Paulos Dlamini). Identification of plants was done at the South Africa National Biodiversity Institute Pretoria and where the voucher specimens of the plants were kept (Table 6)

3.4.4 Plant treatment

Plant root and bulb materials were washed, air dried at room temperature for two to three weeks and subsequently ground to powder. Dried plant pulverised material was stored in an airtight container at room temperature. Crude extracts were made by shaking plant powder in a ratio of 1 g to 10-ml solvent for 2hr (Eloff 1998). Excess solvent

was recovered on a rotary vapour until the extract was concentrated. The concentrate was dried at room temperature in the fume hood. The crude plant extract was stored in the fridge at 4–7°C for future use.

Table 6: A list of anti-inflammatory medicinal plants collected for the study

Scientific name	Zulu Name	Voucher specimen number	Ethno-botanical use	Part of plant used
<i>Pentanisia prunelloides</i> , (<i>P. p.</i>) Klotzsch ex Eckl & Zeyh.) Walp. (Rubiaceae)	Isicimamilo	1200-1	Aches and pains	Roots
<i>Gunnera perpensa</i> (<i>G.p.</i>) L. (Gunneraceae)	Ugobho	2537-1	Painful body after birth	Roots
<i>Eucomis autumnalis</i> (<i>E.a.</i>) Mill Chitt. (Hyacinthaceae)	Umathunga	3790-4001	Waist pains	Roots
<i>Pomaria sandersonii</i> (<i>P.s.</i>) (Harv) B. B. Simpson & G.P. Lewis, comb.nov (Leguminosae)	Isitholwane	14806-0	Build strength after child birth	Roots
<i>Alepidea amatymbica</i> <i>Al.a.</i>) Eckl. & Zeyh (Apiaceae)	Ikhatazo	2116-0	Cough	Roots
<i>Ledebouria revoluta</i> (<i>L.r.</i>) L.f. Jessop (Hyacinthaceae)	Icibudwane	758-3	Sores and skin eruptions	Bulbs
<i>Artemisia afra</i> (<i>At.a</i>) Jacq.ex Wild (Asteraceae)	Mhlonyane	3166-4001	Coughs, colds, headaches and fever	Leaves
<i>Carissa bispinosa</i> (<i>C.b.</i>) (L.) Desf. Ex Brenan	Umvhusankunzi	984-2	Male impotency	Roots
<i>Berkheya setifera</i> (<i>B.s.</i>) D.C. (Asteraceae)	Ulwimi lwenkomo	1342-0	Treats children, ailments like coughs and diarrhoea	Roots and leaves

3.4.5 Phytochemical analysis

3.4.5.1 Preliminary phytochemical analysis

Solvents:

The following solvents in Table 7 were used in various experiments of this work:

Table 7: List of solvents used in the study

Solvent	Grade	Supplier
Hexane (HEX)	Analytical reagent	Merck
Dichloromethane (DCM)	Analytical reagent	Merck
Chloroform (CHL)	Analytical reagent	Merck
Ethyl Acetate (ETAC)	Analytical reagent	Merck
Acetone (ACE)	Analytical reagent	Merck
Methanol (MET)	Analytical reagent	Merck
Formic acid (FA)	Analytical reagent	Merck

The nine medicinal plants crude extracts qualitative phytochemical analysis was determined as follows:

Alkaloids: (200 mg plant powder in 200 ml acetone followed by filtering. 2 ml filtrate + 1% HCl + 6 drops of Dragendorff reagent, orange precipitate would indicate presence of alkaloid).

Saponins: (200 mg plant powder in 200-ml acetone followed by filtering Frothing test: 0.5 ml filtrate + 5 ml distilled water; frothing persistence indicated presence of saponins). **Cardiac glycosides** (200 mg plant powder in 200-ml acetone followed by filtering. Keller Killani test: (2 ml filtrate + 1 ml glacial acetic acid + FeCl₃ + conc. H₂SO₄); green-blue colour would indicate presence of cardiac glycosides).

Terpenoids: (Liebermann-Buchard reaction: 200 mg plant material in 20 ml chloroform, filtered; 2 ml filtrate + 2 ml acetic anhydride indicated presence of terpenoids). **Flavonoids:** (200 mg plant material in 20 ml ethanol, filtered; a 2 ml filtrate + conc. HCl + magnesium ribbon, pink tomato red colour would indicate presence of flavonoids).

Tannins: (200 mg plant powder in 20 ml distilled water, filtered, 2-ml filtrate + 2 ml FeCl₃; blue black precipitate would indicate presence of tannins).

3.4.5.2 Solvent extraction

The acetone crude extract was obtained by shaking plant powder with solvent in a ratio of 1:10 for two hours followed by filtering. The crude extracts were concentrated by rotary vacuum evaporation and then further air-dried at room temperature under a fan.

3.4.5.3 Liquid-liquid extraction (Fractionation)

Crude extracts were then dissolved in 70% acetone in a separating funnel and were shaken first with Hexane, then DCM, Ethyl acetate, acetone and methanol successively, to produce fractions of different polarities. Fractions were concentrated on a rotary evaporator and then air dried at room temperature under a fan. The fractionation protocol is shown on Figure 13.

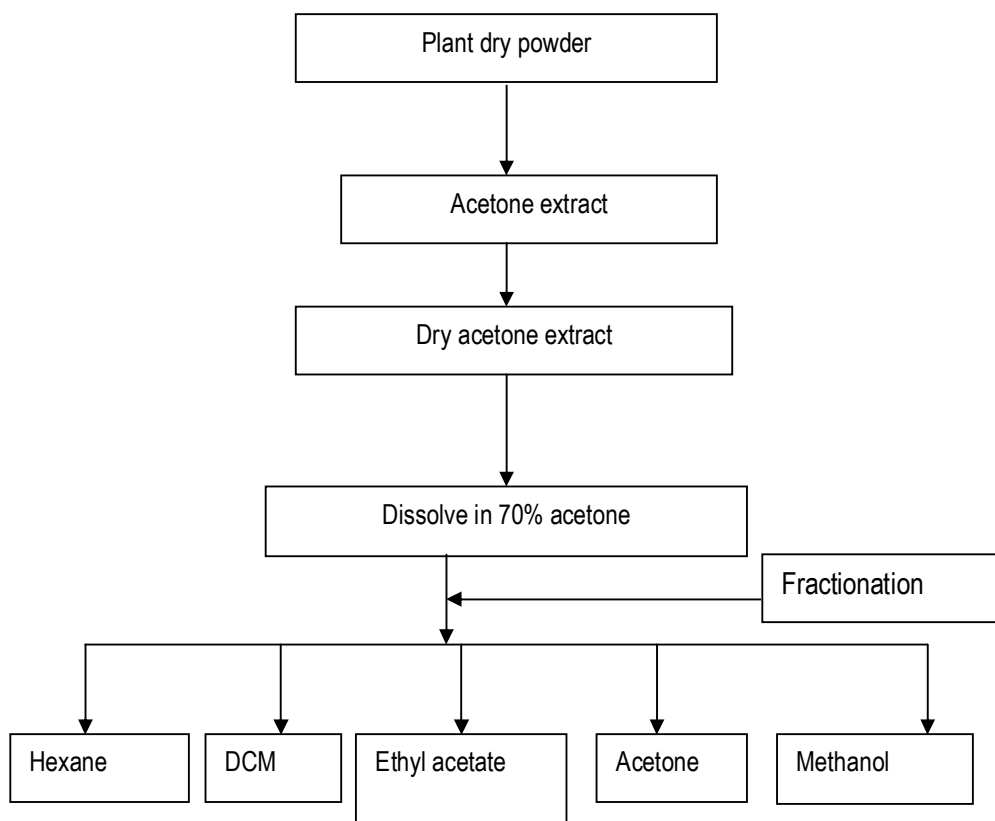


Figure 13: Fractionation protocol

3.4.5.4 Phytochemical profiling

To determine the chemical composition of the extract/fraction, 10 µL of each extract was spotted on TLC, dried and eluted with solvent combination of varied polarities on TLC silica gel 60 F₂₅₄ to obtain a fingerprint chromatogram. The mobile phases used include ethyl acetate: methanol: water (EMW) ratio of 10:1.35:1, Dichloromethane: Ethyl acetate: formic acid (DCM: ET: FA) ratio of 40:10:1. Separated components were visualized under visible and ultraviolet light (254 and 360 nm, Camac Universal lamp TL-600), sprayed with vanillin spray (1 g vanillin dissolved in 28 ml methanol and 1 ml conc. sulphuric acid) and heated at 105 °C in an oven.

3.5 RESULTS

Results for the ethno-botanical survey, yields of crude extracts and fractions, phytochemical profiling and qualitative analysis are listed in the following Tables 9; 10 and Figures 14 to 19.

3.5.1 Ethno-botanical survey

A brief survey was conducted to identify medicinal plants used for treatment of inflammation related conditions. Two different questionnaires were used for the village chief and traditional practitioners. Their responses are summarised in Table 8.

Table 8: A summary of ethno-botanical survey carried out with traditional practitioners and village chief at Mabandla village, KwaZulu-Natal, South Africa.

Item	Response
Names of traditional healers	Mr Sanoyi Paulos Dlamini (Male, 60 yrs), Mr Philimon Cira (Male, 53 yrs), Mr Alias Moabi (male, 71), Mr Mfundo Somaxhana (male, 46), Ms Jabu Base (female, 50) and Ms Noluthando Ndzimande (Female,35)
Nature of training obtained prior to practise as traditional healer	Inherited skills from dead fathers or grandfathers or other close relatives who were traditional healers. No formal training.
Diseases or conditions treated	Headache, stomach ache, diarrhoea, vomiting, back ache, chest pains, kidney condition, insomnia, anxiety, cough, flue and fever
Plant processing and storage	Ground plant powder is boiled with water, cooled and stored in a bottle. About half a cup is taken orally three times a day.
Source of plants for medicines	Plants are collected from the nearby mountains, forests and grasslands.
HIV AIDS awareness	Yes and treat patients who show symptoms of cough, diarrhoea, loss of weight and appetite.
Collaboration with hospitals and clinics nearest to them	Most of their patients cannot afford doctors and conventional medication.

3.5.2 Yields of extracts and fractions

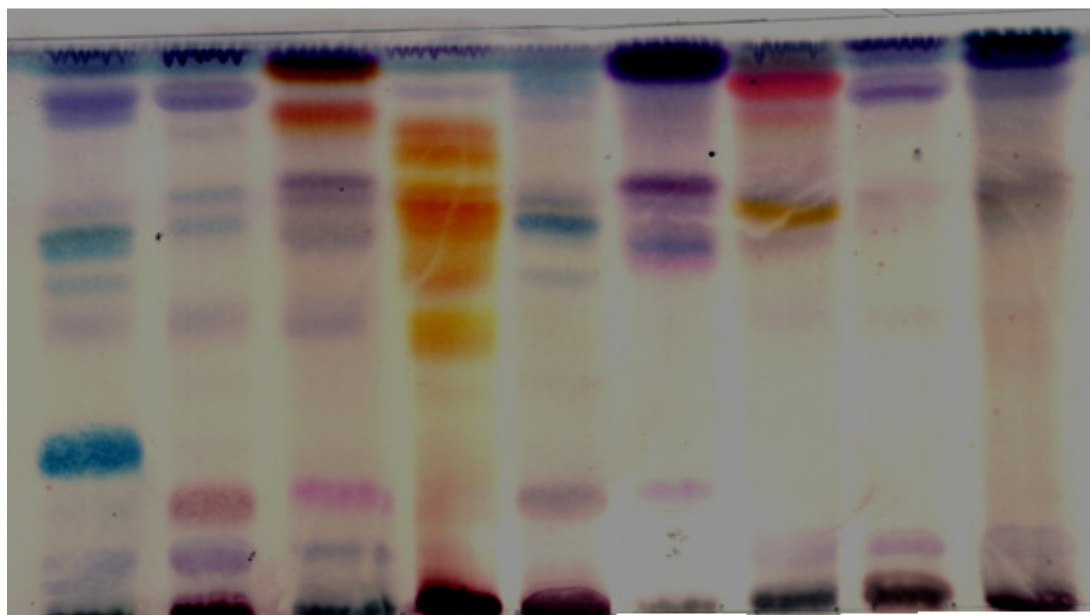
Different plants have different yields of extracts and fractions (Table 9). The highest extract yield was obtained *Pomaria sandersonii* followed by *Alepidea amatymbica*. A significant amount (35%) of *Alepidea amatymbica* extract was hexane and DCM soluble indicating that more than 50% of components are non-polar. Only *Pentanisia prunelloides* and *Gunnera perpensa* had water soluble fractions.

Table 9: The yield in grams of the crude extracts and fractions of the extracts from 200g plant powder

Plant species	Crude extract	HEX	DCM	ETAC	ACE	METH	Water
<i>P. sandersonii</i>	15.61	0.10	1.26	3.55	5.71	4.93	
<i>A. amatymbica</i>	15.30	5.33	6.32	0.14	0.13	3.63	
<i>P. prunelloides</i>	6.1	0.07	0.05	-	0.60	0.43	4.93
<i>C. bispinosa</i>	5.60	0.10	0.50	0.61	1.32	2.03	
<i>L. revoluta</i>	5.55	0.51	0.07	0.3	0.4	0.5	
<i>B. setifera</i>	4.50	0.23	0.90	0.04	0.05	3.16	
<i>A. afra</i>	5.76	1.03	0.66	0.06	3.15	0.06	
<i>G. perpensa</i>	12.90	0.22	0.05	0.06	0.52	5.19	4.67
<i>E. autumnalis</i>	5.31	0.01	0.25	0.03	0.03	2.98	

3.5.3 Phytochemical profiling

The preliminary phytochemical investigation was carried out and the results displaying the fingerprinting of plant extracts are presented in Figure 14. Results of preliminary qualitative phytochemical screening are also shown in Table 10. The finger print results in Figure 14 indicate from the difference in R_f values that acetone crude extracts contain a variety of compounds which form different colours with vanillin/sulphuric acid spray. *Pomaria sandersonii*, *Eucomis autumnalis*, *Gunnera perpensa*, *Artemisia afra* and *Pentanisia prunelloides* displayed a high variety of bands reflecting the presence of different phytochemicals that they contain



G.p *P.p* *E.a* *P.s* *A.ta* *Al.a* *L.r* *C.b* *B.s*

Figure 14: Phytochemical profile of the acetone crude extracts.

The chromatogram was eluted by DCM: Ethyl acetate: Formic acid (40:10:1) and derivatized with vanillin-sulphuric acid indicating the variety of plant crude acetone extracts. *G. p.* = *Gunnera perpensa*, *P. p.* = *Pentania prunelloides*, *E. a.* *Eucomis autumnalis*, *Ata* = *Artemisia afra*, *Ala*, *Alepidea amatymbica*, *L. r.* = *Ledebouria revoluta*, *B. s.* *Berkheya setifera*

Hexane fraction phytochemical screening

Hexane contains some non-polar components of fractions in figure 15. *Artemisia afra* followed by *Alepidea amatymbica* displayed the highest band density of all the hexane soluble fractions.

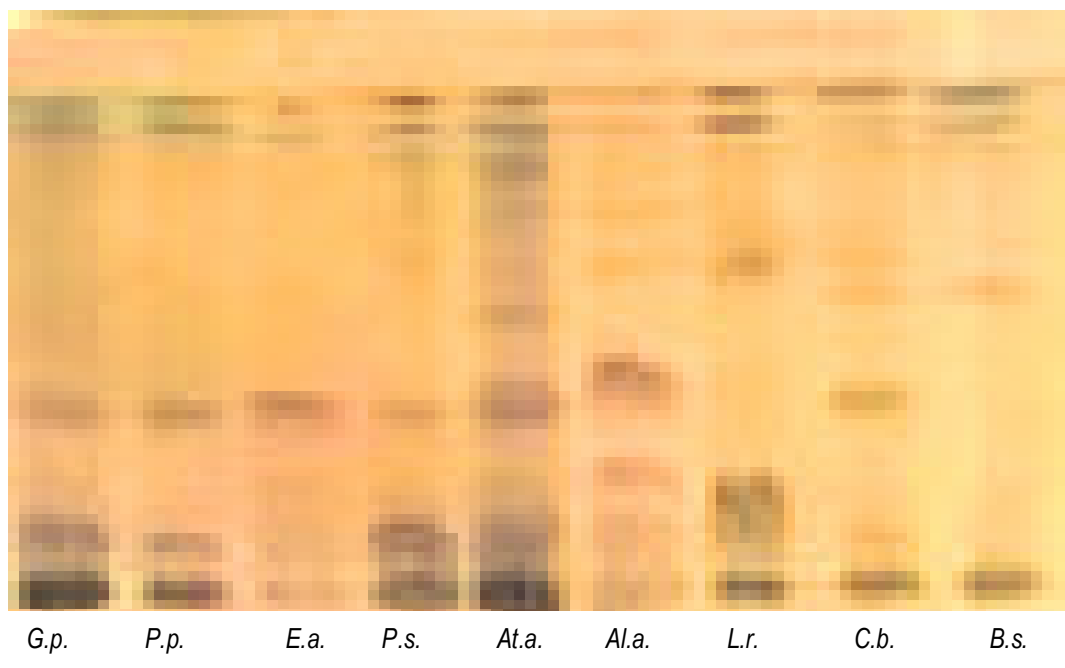


Figure 15: Hexane fractions: The TLC eluted with DCM: Ethyl acetate: formic acid (40:10:1)

This was followed by spraying with vanillinic sulphuric acid. *G. p.* = *Gunnera perpersa*, *P. p.* = *Pentania prunelloides*, *E. a.* *Eucomis autumnalis*, *Ata* = *Artemisia afra*, *Ala*, *Alepidea amatymbica*, *L. r.* = *Ledebouria revoluta*, *B. s.* *Berkheya setifera*.

Dichloromethane fraction phytochemical screening

There are more compounds extracted by DCM for *E. autumnalis*, *P. sandersonii*, *A. afra* and *A. amatymbica* as is displayed in the vanillin sprayed TLC on figure 16. The various coloured bands illustrate the different functional groups present in the DCM fraction. *A. amatymbica* DCM fraction contains large amounts of the secondary metabolite that forms a purple complex (R_f value of 0.78) with vanillinic sulphuric acid as displayed in Figure 16. Most terpenes have purple or ink coloured complexes with vanillinic sulphuric acid.

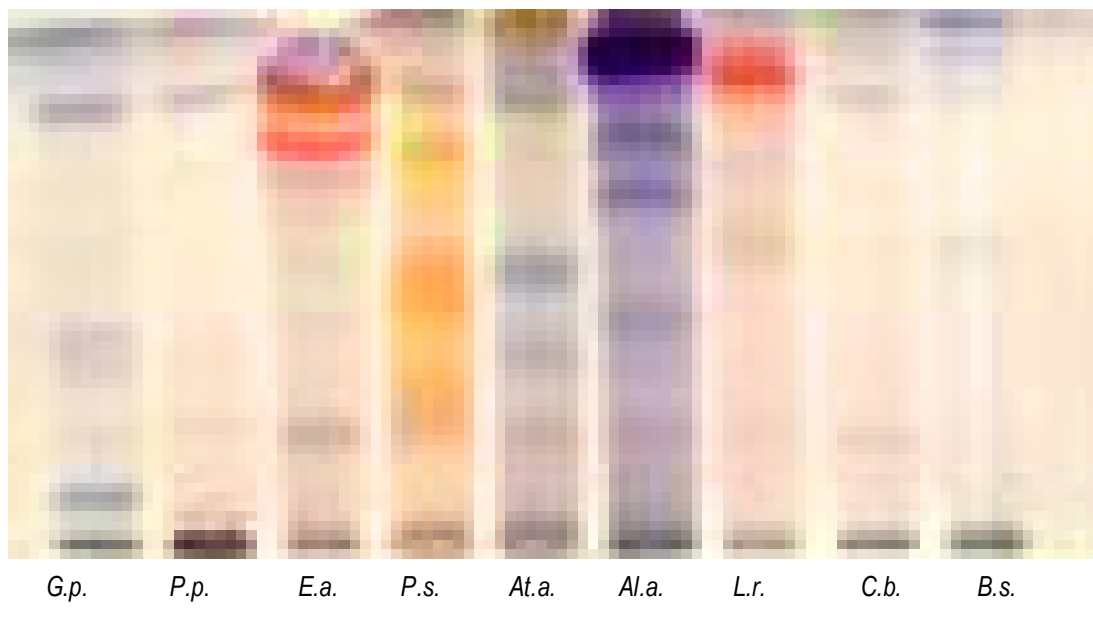


Figure 16: DCM fractions The TLC eluted with DCM Ethyl acetate: formic acid (40:10:1)

This was followed by spraying with vanillinic sulphuric acid. *G. p.* = *Gunnera perpensa*, *P. p.* = *Pentania prunelloides*, *E. a.* = *Eucomis autumnalis*, *Ata* = *Artemisia afra*, *Ala*, *Alepidea amatymbica*, *L. r.* = *Ledebouria revoluta*, *B. s.* = *Berkheya setifera*

Ethyl acetate fraction phytochemical screening

Ethyl acetate did not extract anything for *P. prunelloides*, and was a poor extractant for *E. autumnalis*, *C. bispinosa* and *B. setifera*. *Pomaria sandersonii*, *Alepidea amatymbica*, *Artemisia afra* and *Ledebouria revoluta* ethyl acetate fraction indicated presence of some plant metabolites as displayed in Figure 17.

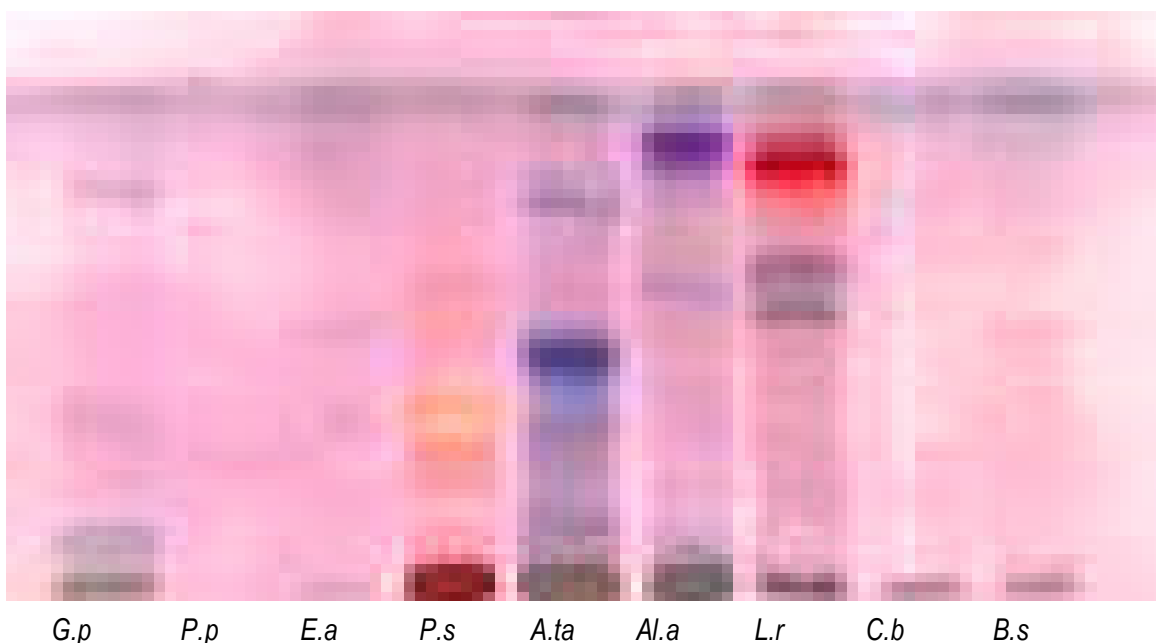


Figure 17: Ethyl acetate fractions The TLC eluted with DCM: Ethyl acetate: formic acid (40:10:1)

This was followed by spraying with vanillinic sulphuric acid. *G. p.* = *Gunnera perpensa*, *P. p.* = *Pentanisia prunelloides*, *E. a.* = *Eucomis autumnalis*, *A.ta* = *Artemisia afra*, *Al.a*, = *Alepidea amatymbica*, *L. r.* = *Ledebouria revoluta*, *B. s.* = *Berkheya setifera*

More compounds are in the acetone fractions in *P. sandersonii*, *A. afra* and *A. amatymbica* than there are in the rest of the plants used in this study as displayed in Figure 18.

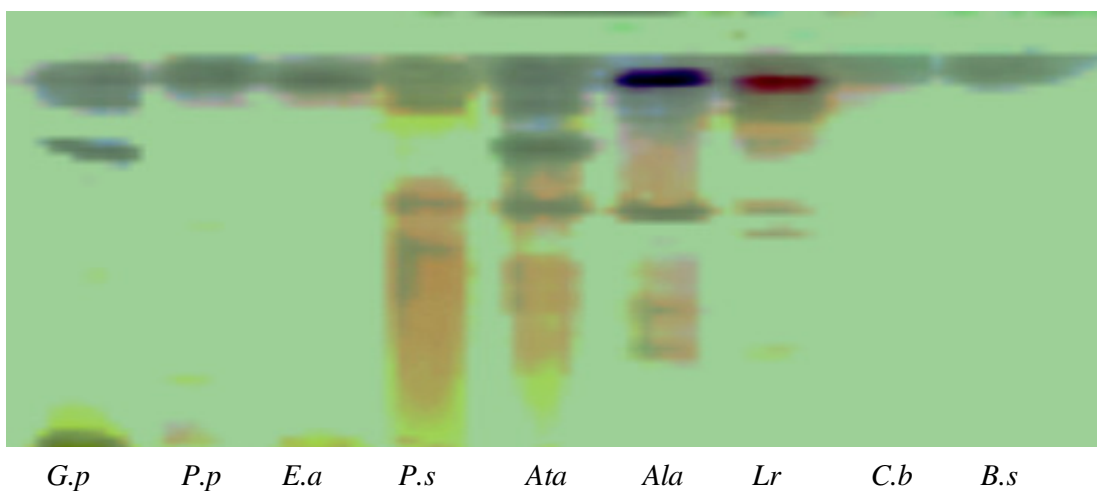


Figure 18: Acetone fractions. The TLC eluted with DCM: Ethyl acetate: formic acid (40:10:1)

This was followed by spraying with vanillinic sulphuric acid. *G. p.* = *Gunnera perpensa*, *P. p.* = *Pentanisia prunelloides*, *E. a.* = *Eucomis autumnalis*, *Ata* = *Artemisia afra*, *Ala*, = *Alepidea amatymbica*, *L. r.* = *Ledebouria revoluta*, *B. s.* = *Berkheya setifera*

Pomaria sandersonii contains the highest density of compounds that make coloured complexes with vanillin as displayed in Figure 19. *B. setifera* methanol fraction did not show compounds which complex with vanillin, indicating that most of its polar compounds are soluble in acetone and ethyl acetate.

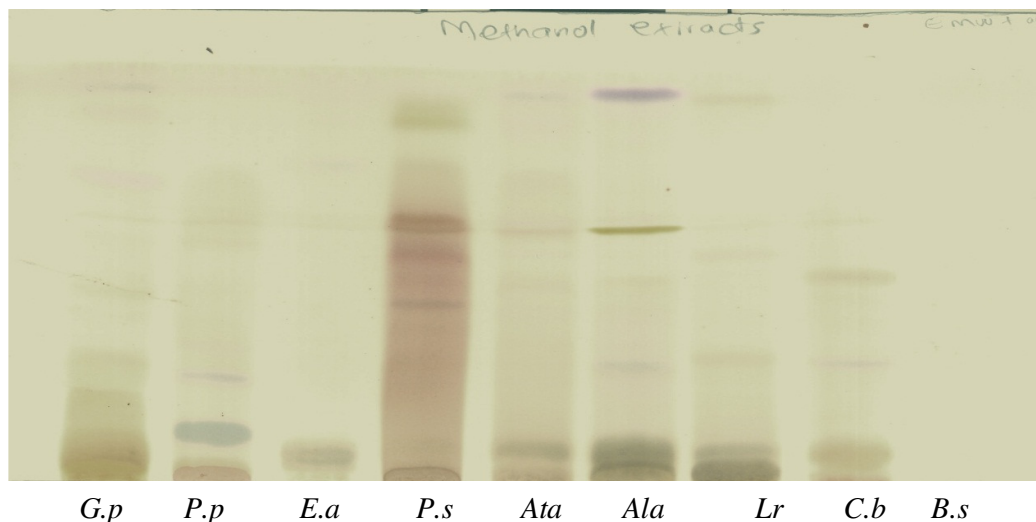


Figure 19: Methanol fractions. TLC eluted with EMW.

This was followed by spraying with vanillic sulphuric acid. *G. p.* = *Gunnera perpensa*, *P. p.* = *Pentanisia prunelloides*, *E. a.* = *Eucomis autumnalis*, *Ata* = *Artemisia afra*, *Ala.* = *Alepidea amatymbica*, *L. r.* = *Ledebouria revoluta*, *B. s.* = *Berkheya setifera*

3.5.4 Qualitative phytochemical analysis of the extracts

The qualitative analysis of extracts indicated that there is a variety of secondary metabolites in all the nine plants. The test for steroids was positive in the *C. bispinosa* and *B. setifera* water extracts. Anthraquinones were detected in *P. sandersonii*, *L. revoluta*, and *C. bispinosa*. Table 10 is a summary of the tests that were performed on the plant water extracts. The observations in the qualitative analysis reflect the phytochemical profiling by vanillin TLC shown in Figure 14. Phenols, alkaloids, saponins and glycosides reducing sugars and terpenes are present in all plants used in this study. However, fractionation yielded water soluble fractions which are likely to be sugars only from *P. prunelloides* and *G. perpensa*.

Table 10: Qualitative phytochemical analysis of the plant extracts

Plant species	P	A	S	G	St	AL	T	R
<i>G. perpensa</i>	+	-	+	+	-	+	+	+
<i>P. prunelloides</i>	+	-	+	+	-	+	+	+
<i>E. autumnalis</i>	+	-	+	+	-	+	+	+
<i>P. sandersonii</i>	+	+	+	+	-	+	+	+
<i>A. afra</i>	+	-	+	+	-	+	+	+
<i>A. amatymbica</i>	+	-	+	+	-	+	+	+
<i>L. revoluta</i>	+	+	+	+	+	+	+	+
<i>C. bispinosa</i>	+	+	+	+	+	+	+	+
<i>B. setifera</i>	+	-	+	+	-	+	+	+

P=phenolics A= Anthraquinones, S= saponins, G=glycosides St=steroids, Al =alkaloids, T=triterpenoids, R=reducing sugars

3.6 DISCUSSION

The qualitative analysis of crude extracts and thin layer chromatograms of the crude extracts and fractions displayed the presence of different types of plant secondary metabolites. The plants were sampled by the traditional practitioners who provided the information of ethno botanical use listed in Table 6.

3.6.1 Ethnobotanical survey

The ethno-botanical survey indicated that the rural community of Mabandla village is dependent on the health delivery system of their local traditional healers' knowledge of plants in their immediate environment and prescriptions during disease management. The major reasons for this dependence appear to be the prohibitive cost and availability of conventional medicines from the survey. Traditional practitioners also claim that medicinal plant use is effective in managing some disease conditions especially if patients consult in the early stages of the disease. Documented scientific research on the plants is therefore necessary because there is no formal training associated with the practise of prescribing medication in this community. With the increase of urbanisation, this indigenous knowledge faces the possibility of being shunned by the younger generation and may go extinct. Furthermore, because the healers claim that the plants offer cures for some disease conditions, it is possible that they could present newer and more potent drugs to diseases like tuberculosis and malaria, whose pathogens are becoming more and more virulent to known drugs and HIV/AIDS, whose cure is still to be discovered.

3.6.2 Phytochemical screening

The vanillin-TLC in figure 14 (different R_f values) and the preliminary phytochemical screening in table 10 displayed that a variety of secondary metabolites are present in all the plants. The difference in chemical composition was expected because the plants are used to treat different disease conditions. However complete characterisation is necessary in order to scientifically distinguish the mechanism of action of each plant. Reports on some chemical composition of extracts of *Artemisia afra*, (Liu *et al.* 2009) *Eucomis autumnalis*, (Koorbanally *et al.* 2006) *Gunnera perpensa*, (Drewes *et al.* 2005) *Pentanisia prunelloides* (Yff *et al.* 2002) *Alepidea amatymbica* (Somova *et al.* 2001) *Ledebouria revoluta* (Moodley *et al.* 2006) have been made but they are not exhaustive.

Different solvents (Table 7) were employed to isolate components from crude extract into fractions of different polarity from the hexane soluble, which is non-polar to methanol and water soluble (most polar). The TLCs in figures 14 to 19 of the different fractions also displayed different densities of bands illustrating the difference in the polarity that a given solvent can extract. Methanol demonstrated the highest amounts of different compounds. Phytochemicals have different polarity, for example, terpenes are relatively non-polar when compared to the alkaloids phenolics and saponins. Fractionation is the first step in the isolation of chemical compounds from crude plant extracts. *Artemisia afra* hexane fraction has the highest density of bands displayed in figure 3.2. More than 50 volatile secondary metabolites have been isolated from *Artemisia afra* (Liu *et al.* 2009) and could explain the presence of the high density of bands in the non-polar solvent. The DCM fraction TLC *Alepidea amatymbica* (figure 16) displayed large non-polar bands with R_f value of about 0.8. This is because of the presence of DCM soluble kaurene derivatives that have been isolated (Somova *et al.* 2001).

3.7 CONCLUSION

Interesting phytochemical composition has been exhibited by the crude extracts and fractions of the plants under study. Further investigation into the biological activities of the fractions is necessary in order to target compounds of interest for isolation. Compounds with antimicrobial and antioxidant activities will be the target of isolation in the following sections of this study.

CHAPTER 4

ANTIOXIDANT ACTIVITIES OF NINE MEDICINAL PLANTS USED IN TREATING INFLAMMATORY AILMENTS

4.1 INTRODUCTION

Oxygen and nitrogen are both essential in many metabolic processes and some molecules are reduced to reactive oxygen species (ROS) and reactive nitrogen species (RNS) during normal and pathological conditions due to incomplete transfer of electron (Gac *et al.* 2010). The activated macrophages and neutrophils of the immune system is defence mechanisms generate pro-inflammatory molecules including ROS/RNS as anti-infectious agents to fight microbial infection. These reactive species are also generated by some normal body metabolites such as respiratory process (Iwalewa *et al.* 2007). The ROS/RNS involved in the process of phagocytosis function as antimicrobial agents and are important in fighting against infectious pathogens. ROS/RNS may function during cell defense as signalling molecules, stimulating cell proliferation or modulate enzyme activities (Kaplan *et al.* 2007). These reactive species include superoxide anion, O_2^- , hydrogen peroxide, H_2O_2 , hydroxy radical, $\cdot OH$, organic hydroperoxide, ROOH, alkoxy, RO and peroxy, ROO radicals, hypochlorous acid, HOCl, and peroxynitrite, $ONOO^-$ (Gutteridge 1995). However, overproduction of ROS/RNS without an efficient process of balancing the process results in oxidative stress. This condition is defined as an imbalance between the production of ROS/RNS and the biological system's ability to readily detoxify the reactive intermediates (Lykkesfeldt & Svendsen 2007).

Oxidative damage to cellular components such as the cell membrane by free radicals is associated with the development of degenerative diseases including cardiovascular disorders, cancer, inflammation, arthritis, immunosuppression or immuno-stimulation, brain dysfunction, Alzheimer's disease, AD and cataract (Kaplan *et al.* 2007). The reactive species interact with biological molecules such as DNA causing mutagenicity and genotoxicity. Oxidative attack on protein in cell membranes could result in the malfunctioning of some enzymes. Interactions with phospholipids of the cell membrane could also initiate lipid peroxidation causing injury and tissues damage (Govindarajan *et al.* 2005). The cytotoxic by products of lipid peroxidation include malonyldialdehyde (MDA) and 4-hydroxy-2, 3-nonenal (4-HNE) (Stables & Gilroy 2011).

The body is endowed with an elaborate endogenous antioxidant system for balancing the generation and annihilation of the reactive species to its optimal level. These include superoxide dismutases (SODs, catalases and glutathione peroxidases) (Kaplan *et al.* 2007). However, the endogenous antioxidant mechanisms are sometimes overwhelmed, leading to ROS/RNS overproduction and the consequently oxidative stress with the accompanied health disorders.

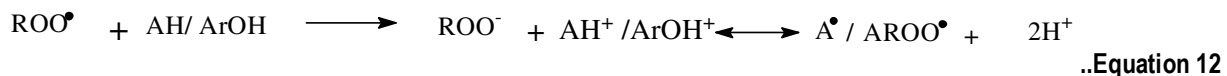
4.1.1 Antioxidant capacity in plant extracts.

Bioavailable medicinal plants and dietary antioxidants (vitamins C, carotenoids, alkaloids and phenolics) react with the ROS and RNS to form a stable non-reactive molecule thereby providing protection against oxidative stress (Kamat *et al.* 2000). Many medicinal plants have been screened and their efficacies against some biologically relevant oxidative mechanisms are validated (Table 2.3) (Van Staden 2008; Sies & Cadenas 1985; Kaplan *et al.* 2007; Catalá 2009).

4.1.2 Methods of assessing antioxidant assay

The measurement of free radical scavenging activity is achieved by comparing the reaction of the plant sample reaction with artificial radicals such as DPPH and ABTS. Other assays also involve measurement of scavenging capacity of biologically relevant oxidants such as superoxide, peroxynitrite, hydroxyl radical and others which are artificially liberated and then the course of the reaction is followed spectrophotometrically (Huang *et al.* 2005).

Methods of antioxidant capacity assays may be broadly classified as electron transfer (ET) and hydrogen transfer (HAT) based assays (Huang *et al.* 2005). The HAT mechanisms of antioxidant action can be summarized by equation 4. In this equation, the aryloxy radical (ARO•) formed from the antioxidant phenol (ArOH) and A• are stabilized by resonance. An example of the HAT-based assay is the oxygen radical absorbance capacity (ORAC), which applies a competitive reaction scheme in which antioxidant and substrate kinetically compete for thermally generated peroxy radicals through the decomposition of azo compounds such as ABAP (2,2'-azobis(2-aminopropane) dihydrochloride (Huang *et al.* 2005). The Electron transfer (ET) mechanism action is based on equation 12.



The antioxidants react with a coloured oxidizing agent instead of peroxy radical and the reaction is followed by colour change using a UV spectrophotometer and absorbance is correlated to the concentration of antioxidants in the sample (Huang *et al.* 2005) The ET- based assays include 2,2'-azinobis (3-ethylbenzothiazoline)-6-sulfonic acid ABTS /Trolox-equivalent antioxidant capacity (TEAC), 1, 1-diphenyl-2-picrylhydrazyl (DPPH), Folin- Ciocalteu-(RCR) , ferric reducing ability (FRAP) and cupric ion reducing antioxidant capacity assay (CUPRAC) using different chromogenic redox reagents with different standard potentials (Huang *et al.* 2005). The ABTS^{•+} and DPPH[•] radical assays will be discussed in more detail in the account to follow because they will form the basis of assessing free radical scavenging capacity of the plant extracts and fractions in this study.

4.1.3 The 2, 2'-azinobis (3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) assay

In the ABTS^{•+} assay, antioxidants (ArOH) donate electrons or protons to ABTS^{•+} radical (figure 20) to form a stable compound. The pre-formed radical monocation blue/green chromophore of ABTS^{•+} is generated at room temperature for 12-16 hours prior to the start of the reaction. The neutral ABTS molecule is oxidized to an unstable ABTS^{•+} radical with potassium persulfate (equation 13).



The ABTS^{•+} chromophore (blue/green) has absorption maxima at wavelengths 415 nm, 645 nm, 734 nm and 815 nm (Huang *et al.* 2005). The extent of decolourization determined as percentage inhibition of the ABTS^{•+} radical cation as a function of concentration and time are explained relative to the reactivity of trolox used as a standard, under the same conditions.

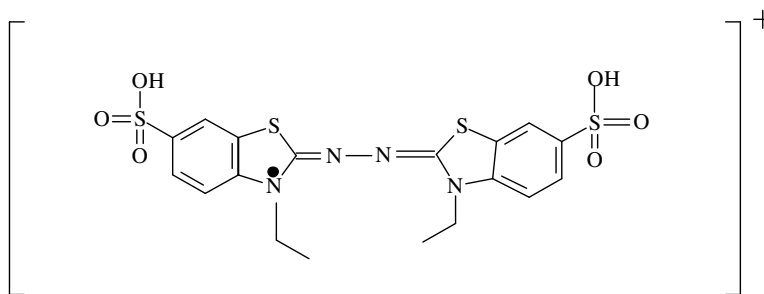


Figure 20: The 2, 2'-azinobis (3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) radical

4.1.4 The DPPH assay

The assay is based on equation 15 in which the DPPH• (figure 4.3) which is the [2, 2-di (4-*tert*-octylphenyl)-1-picrylhydrazyl] stable radical with $\lambda_{\max} = 515$ nm which is decolourized from purple to yellow during the reaction (Huang *et al.* 2005). The stability of the free radical arises as a result of the delocalization of the spare electron over the whole molecule (Figure 21).

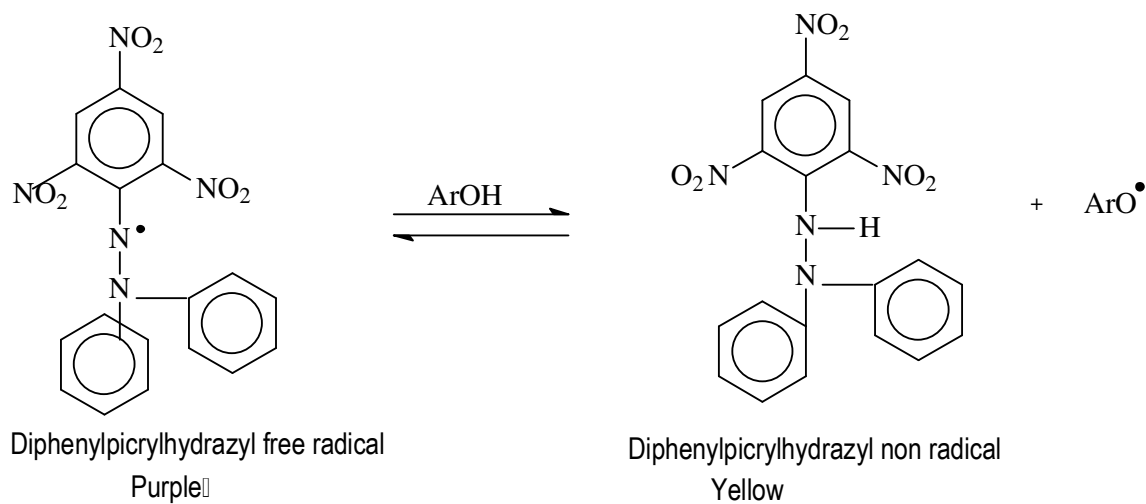


Figure 21: The DPPH redox reaction

When the DPPH radical is mixed with a substrate acting as a hydrogen atom donor, a stable non-radical form of DPPH is obtained with a simultaneous violet colour change to pale yellow.

4.2 MATERIALS AND METHODS

4.2.1 Plant extracts preparation:

Plant root and bulb materials were treated as described in section 3.4.4 to produce acetone crude extracts. Fractions were prepared using the protocol as summarised in Figure 13

4.2.2 DPPH Antioxidant bioautography

Crude plant extracts and fractions TLC chromatograms were prepared as described in section 3.4.5.4. The chromatograms were then developed by spraying with 0.2% DPPH in methanol solution.

4.2.3 DPPH antioxidant assay

For DPPH antioxidant assay, using 96 well plates and a VERSAmax™ tunable micro plate reader (Labotech), 40µL of 0.5 mg/ml plant extract and fraction was determined by a twofold serial dilution in 160 µL of 0.0025% of DPPH (total volume 200-µL) or 40-µL of methanol (negative control) or trolox (positive control). After 30 min the absorbance was measured at 516 nm wavelength. The free radical DPPH· scavenging (reduction) was calculated from the equation: Activity [% of DPPH reduction] = $(A-A_x)/A \times 100\%$, where A is absorbance of DPPH solution with methanol, A_x is absorbance of a DPPH solution with a tested fraction solution or trolox (positive control).

4.2.4 ABTS^{•+} Scavenging assay

The ABTS^{•+} radical cation was prepared by mixing 7mM ABTS stock solution and incubating for 12-16h in the dark at room temperature until a steady absorbance was obtained to indicating that the reaction was complete. 40µl of 0.5 mg/ml plant extract and fraction was diluted using deep well plates and a VERSAmax™ tunable micro plate reader (Labotech), by a twofold serial dilution in 160 µL of the ABTS^{•+} radical cation (total volume 200µL). 40 µL of methanol was the negative control and trolox the positive control. Absorbance was measured at 734 nm after about six minutes.

4.3 RESULTS

The results summarise the findings of two types of antiradical evaluation, namely, the DPPH• bioautography and free radical scavenging capacity against DPPH• and ABTS^{•+} radicals. These are discussed in the sections that follow.

4.3.1 DPPH bioautography

The DPPH• sprayed TLC displayed that all the nine plants contain antioxidants, most of which are polar, contained in polar fractions of methanol and acetone. The test samples exhibit moderate to good antiradical activity. DPPH• bioautography results are represented as chromatograms displayed in Figures 22-27.

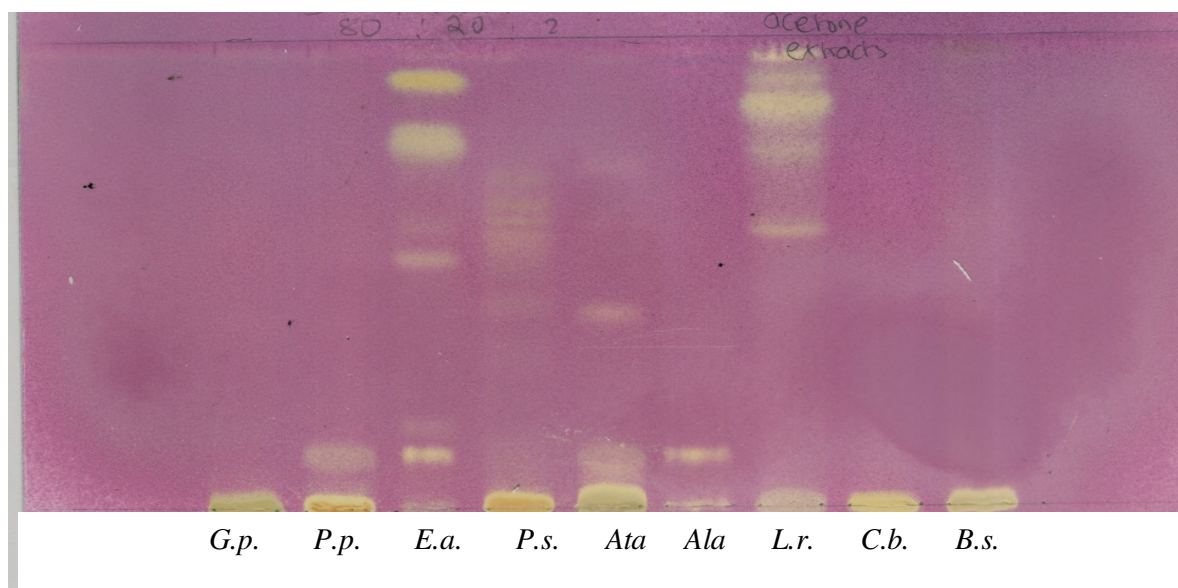


Figure 22: Antioxidant activities of acetone crude extracts.

The chromatogram was eluted with DCM: Ethyl acetate: Formic acid (40:10:1) and sprayed with DPPH indicating the variety of plant crude acetone extracts. *G. p.* = *Gunnera perpensa*, *P. p.* = *Pentanisia prunelloides*, *E. a.* = *Eucomis autumnalis*, *Ata* = *Artemisia afra*, *Ala*, = *Alepidea amatymbica*, *L. r.* = *Ledebouria revoluta*, *B. s.* = *Berkheya setifera*

Hexane fraction contains some non-polar components of the fractions most of which do not react with DPPH• free radical except for *G. perpensa*, *P. prunelloides*, *P. sandersonii* and *A. afra*.

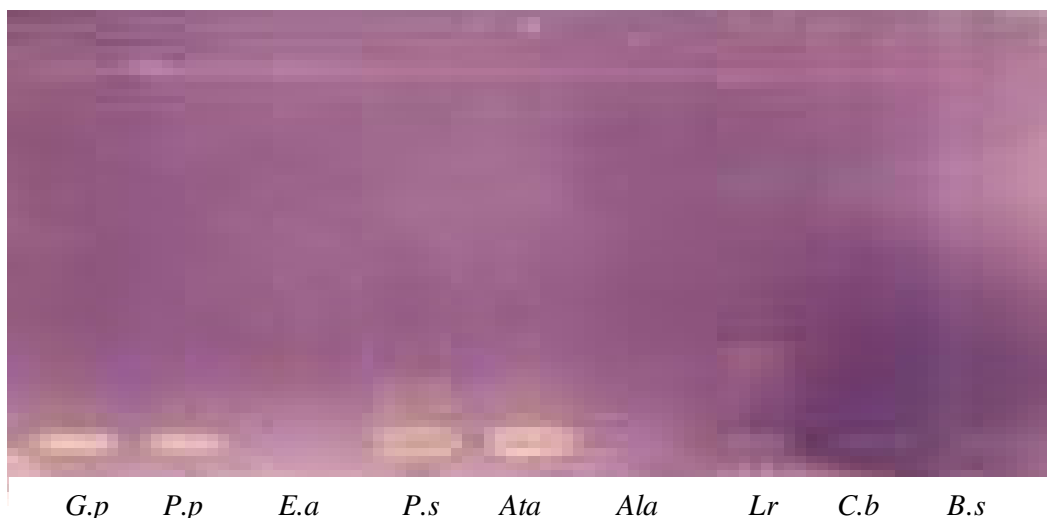


Figure 23: Antioxidant activities of hexane fractions.

The TLC eluted with DCM, Ethyl acetate: formic acid (40:10:1) and sprayed with DPPH• showing the compounds with free radical activity. *G. p.* = *Gunnera perpensa*, *P. p.* = *Pentania prunelloides*, *E. a.* *Eucomis autumnalis*, *Ata* = *Artemisia afra*, *Ala*, = *Alepidea amatymbica*, *L. r.* = *Ledebouria revoluta*, *B. s.* *Berkheya setifera*

More compounds displaying antioxidant activity are in *E. autumnalis* as is displayed in figure 24 on the DPPH• sprayed TLC.

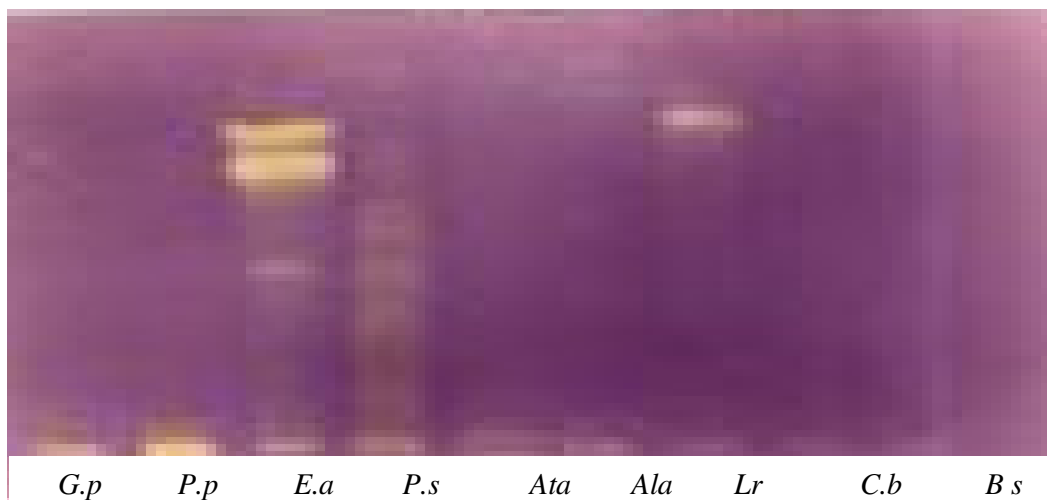


Figure 24: Antioxidant activities of DCM fractions.

The TLC developed with DCM: Ethyl acetate: formic acid (40:10:1) and sprayed with DPPH• showing the compounds with free radical activity. *G. p.* = *Gunnera perpensa*, *P. p.* = *Pentania prunelloides*, *E. a.* *Eucomis autumnalis*, *Ata* = *Artemisia afra*, *Ala*, = *Alepidea amatymbica*, *L. r.* = *Ledebouria revoluta*, *B. s.* *Berkheya setifera*

Ethyl acetate extracted some DPPH• free radical scavenger in *L. revoluta*, *P. sandersonii*, *A. amatymbica* and *A. afra*.

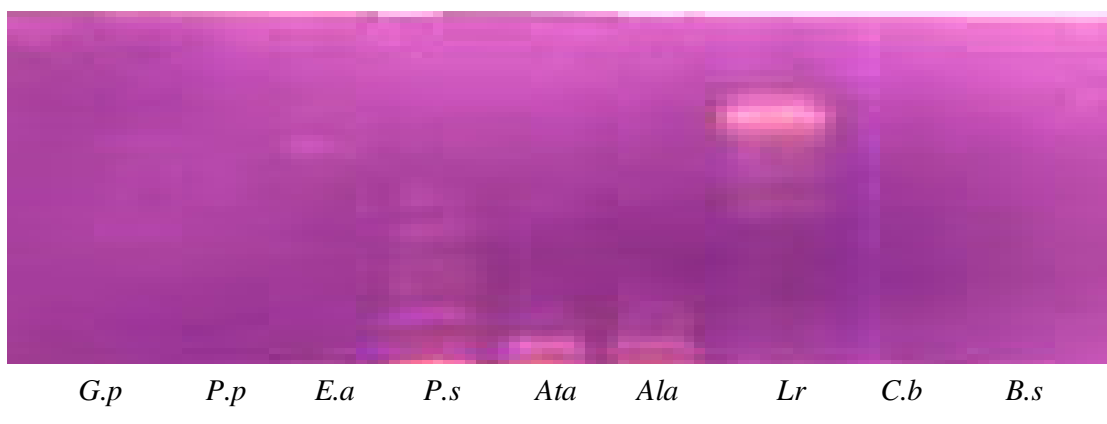


Figure 25: Antioxidant activities of ethyl acetate fractions.

The TLC eluted with DCM: Ethyl acetate: formic acid (40:10:1) and sprayed with DPPH showing the compounds with free radical activity. *G. p.* = *Gunnera perpensa*, *P. p.* = *Pentania prunelloides*, *E. a.* = *Eucomis autumnalis*, *Ata* = *Artemisia afra*, *Ala*, = *Alepidea amatymbica*, *L. r.* = *Ledebouria revoluta*, *B. s.* = *Berkheya setifera*

Acetone fractions of *P. prunelloides*, *E. autumnalis*, *C. Bispinosa*, *B. setifera* and *L. revoluta* have few bands from the DPPH sprays.

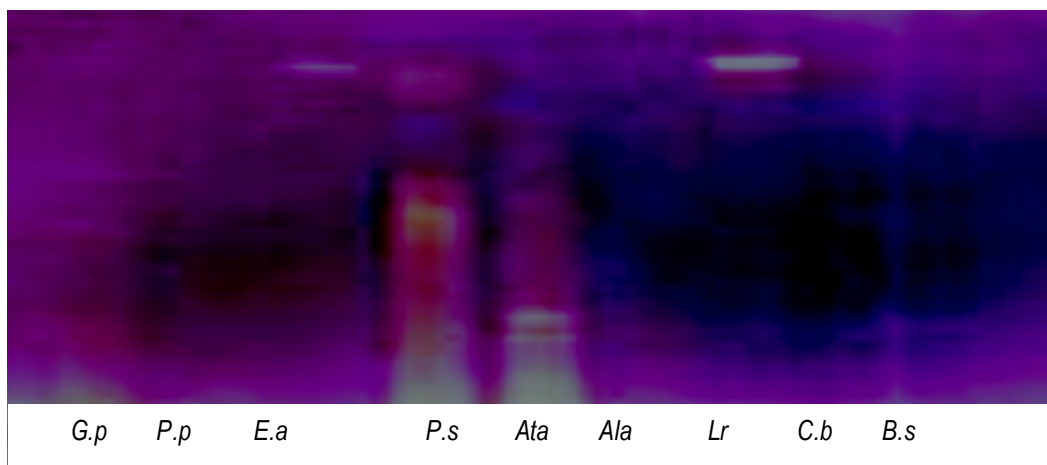


Figure 26: Antioxidant activities of acetone fractions.

The TLC developed with DCM Ethyl acetate: formic acid (40:10:1) and sprayed with DPPH showing the compounds with free radical activity. *G. p.* = *Gunnera perpensa*, *P. p.* = *Pentania prunelloides*, *E. a.* = *Eucomis autumnalis*, *Ata* = *Artemisia afra*, *Ala*, = *Alepidea amatymbica*, *L. r.* = *Ledebouria revoluta*, *B. s.* = *Berkheya setifera*

Methanol fraction contains the highest amount of *antioxidants* as demonstrated by the many clear bands on the DPPH sprayed TLC in figure 27 for *G. perpensa*, *P. sandersonii*, *A. afra*, *A. amatymbica*, *L. revoluta* and *C. bispinosa* plant fractions. There is an absence of DPPH free radical activity in *E. autumnalis* and *B. setifera* methanol fractions.

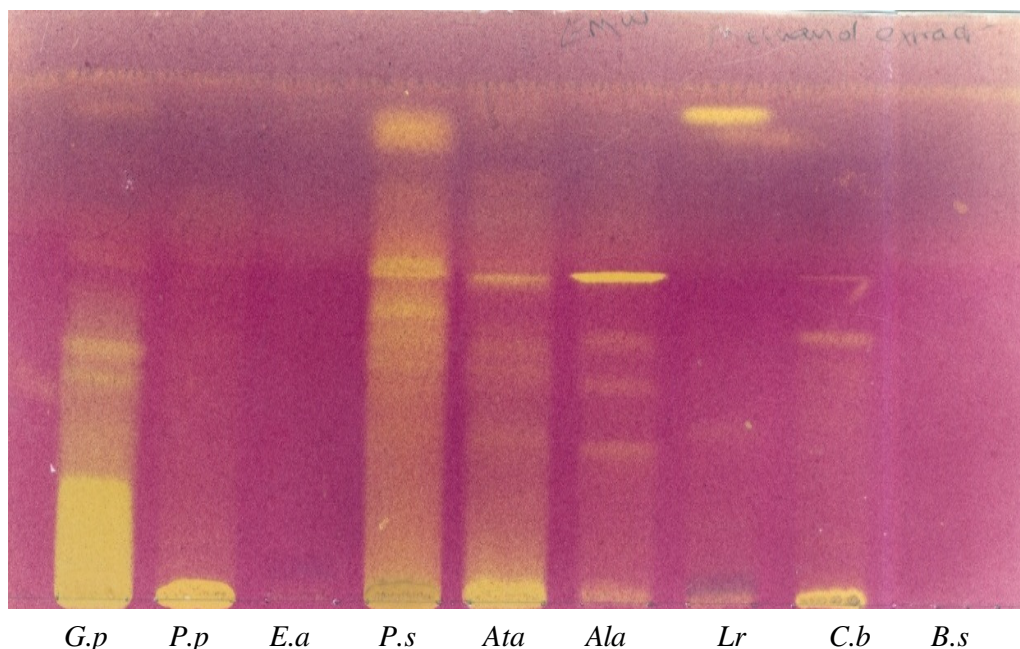


Figure 27: Antioxidant activities of methanol fractions

The TLC developed with EMW and sprayed with DPPH showing the compounds with free radical activity. *G. p.* = *Gunnera perpersa*, *P. p.* = *Pentania prunelloides*, *E. a.* = *Eucomis autumnalis*, *Ata* = *Artemisia afra*, *Ala*, = *Alepidea amatymbica*, *L. r.* = *Ledebouria revoluta*, *B. s.* = *Berkheya setifera*

4.3.2 Free radical scavenging ability of crude extracts and fractions

In order to assess antioxidant scavenging capacity of plant extracts and fractions, DPPH• and ABTS• free radical scavenging assays were carried out and expressed as EC₅₀. Results are presented in Tables 11 to 21 and Figures 28 to 36.

4.3.3 DPPH free radical scavenging

DPPH• free radical scavenging activities of the extract and fractions determined as EC₅₀ values ranged between 1.008 and 467 µg/ml. The highest activity is obtained from methanol fraction of *Berkheya setifera* with EC₅₀ value of 1.008 µg/ml followed by crude fraction of *Gunnera perpersa* with EC₅₀ value of 1.069 µg/ml. *Carissa bispinosa* hexane fraction displayed the lowest activity of 467.7 µg/ml. In general, the more polar fractions and crude extracts are the most active antioxidants for both ABTS•• and DPPH• radical assays (Tables 11-21)

4.3.4 ABTS^{•+} free radical scavenging

Highest ABTS^{•+} radical scavenging was demonstrated by *Pomaria sandersonii* DCM, (1.273 µg/ml) for the Ethyl acetate, (5.973 µg/ml). Figures 28-36 and the corresponding Tables 11 – 21 display a comparison of activities (DPPH[•] and ABTS^{•+}) of the crude extracts and fractions of the nine plants. The lowest activity was displayed by hexane fraction from *Eucomis autumnalis* (929.4 µg/ml).

Pomaria sandersonii

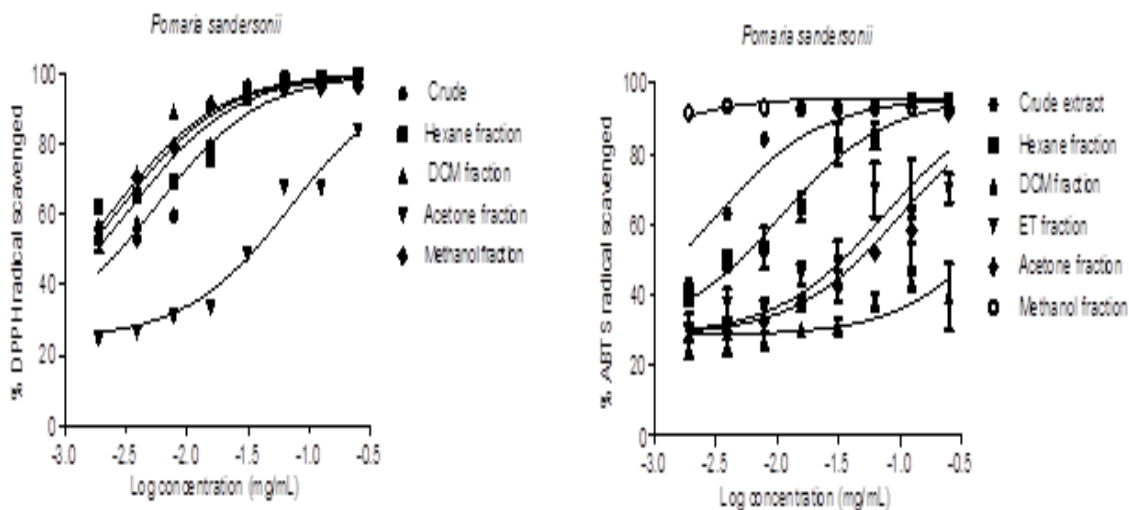


Figure 28: Plots of % free radical scavenging against log concentration in mg/ml

All samples display some anti-radiative activity although the crude extract DPPH[•] and ABTS^{•+} for *Pomaria sandersonii* was the highest compared to all of the plant's samples. DCM fraction was more reactive with ABTS^{•+} radical than with DPPH[•] as reflected by their curve in Figure 28 and the respective EC₅₀ value in Table 11 while the hexane fraction is more active with DPPH[•] than with ABTS^{•+} radical.

Table 11: EC₅₀ values for free radical scavenging activity of the extracts and fractions for *Pomaria sandersonii*

	DPPH			ABTS ^{•+}		
	EC ₅₀	95%CL	R ²	EC ₅₀	95%CL	R ²
C	7.691	5.681 to 10.41	0.8636	1.274	0.9439 to 1.719	0.9381
H	19.73	14.55 to 26.77	0.9261	150	114.8 to 196.0	0.8084
DCM	36.23	26.66 to 49.24	0.9219	1.662	1.262 to 2.188	0.7836
ET	13.51	9.974 to 18.30	0.967	5.973	4.722 to 7.555	0.9569
AC	4.165	3.058 to 5.674	0.9401	1.274	0.9439 to 1.719	0.9381
MET	31.36	23.08 to 42.59	0.7374	150	114.8 to 196.0	0.8084

C= crude extract, H= hexane fraction, DCM= dichloromethane fraction, ET = ethyl acetate fraction, AC = acetone fraction and MET= methanol fraction CI= confidence limit

Alepidea amatymbica

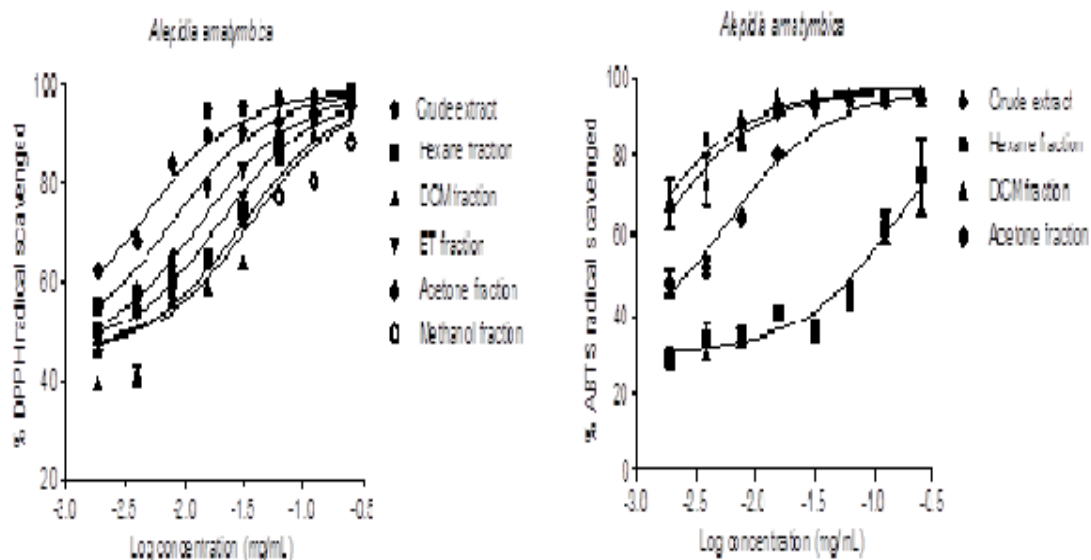


Figure 29: Plots of % free radical scavenging against log concentration in mg/ml (DPPH on right and ABTS on left side)

Alepidea amatymbica DCM fractions also displayed difference in reactivity towards the two radicals. EC₅₀ values for the crude and hexane samples displayed higher activity towards DPPH• than ABTS^{•+} while the rest of the fractions displayed the reverse.

Table 12: EC₅₀ values for free radical scavenging activity of the extracts and fractions at *Alepidea amatymbica*

	DPPH• activity			ABTS•+		
	EC ₅₀	95%CL	R ²	EC ₅₀	95%CL	R ²
C	74.88	56.75 to 98.80	0.9013	217	156.6 to 300.7	0.819
H	124.7	93.68 to 166.1	0.4685	2433	652.5 to 9072	0.1159
DCM	106.3	80.17 to 141.0	0.8608	28.14	21.34 to 37.10	0.883
ET	13.95	10.59 to 18.38	0.9772	1.109	0.7414 to 1.658	0.7298
AC	5.863	4.422 to 7.772	0.9122	0.0834	0.005391 to 1.290	-11.14
MET	4.51	3.384 to 6.010	0.907	2.614	1.918 to 3.563	0.6107

C= crude extract, H= hexane fraction, DCM= dichloromethane fraction, ET = ethyl acetate fraction, AC = acetone fraction and MET= methanol fraction

Pentanisia prunelloides

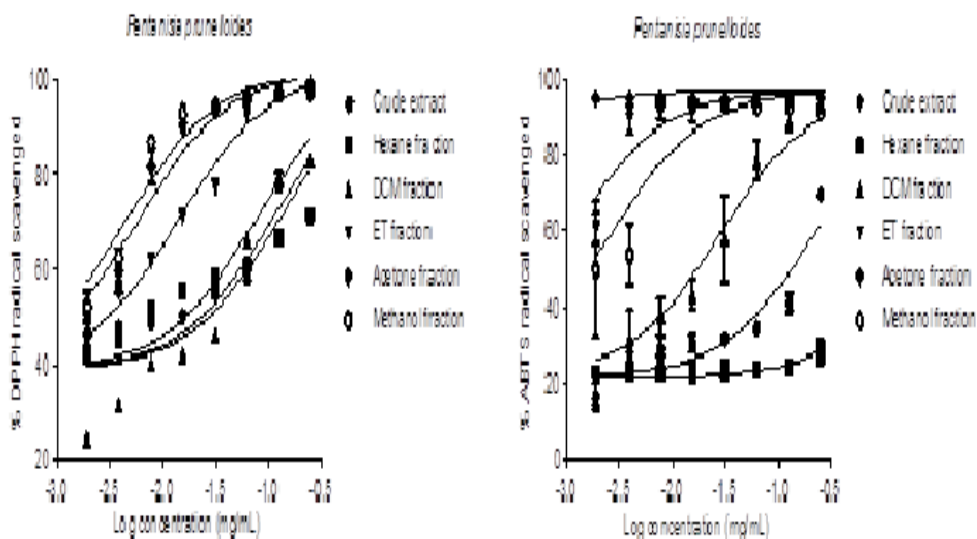


Figure 30: Plots of % free radical scavenging against log concentration in mg/ml (DPPH on right and ABTS on left side)

The EC₅₀ values for *Pentanisia prunelloides* hexane, DCM and acetone fractions indicate that they were more reactive with DPPH• radical while there was a comparable activity with both radicals with the methanol fractions.

Table 13: EC₅₀ values for free radical scavenging activity of the extracts and fractions *Pentanisia prunelloides*

	DPPH•			ABTS••		
	EC ₅₀	95%CL	R ²	EC ₅₀	95%CL	R ²
C	5.609	4.519 to 6.961	0.9101	3.163	2.265 to 4.415	0.8895
H	3.573	2.870 to 4.448	0.8379	10.68	7.854 to 145.3	0.9141
DCM	2.935	2.349 to 3.667	0.9117	797.9	429.0 to 1484.	0.3895
ET	-	-	-	-	-	-
AC	70.61	55.92 to 89.14	0.9617	99.55	72.47 to 136.7	0.8674
MET	2.619	2.090 to 3.282	0.9787	0.1647	0.03148 to 0.8613	-3.704

C= crude extract, H= hexane fraction, DCM= dichloromethane fraction, ET = ethyl acetate fraction, AC = acetone fraction and MET= methanol fraction

Ledebouria revoluta

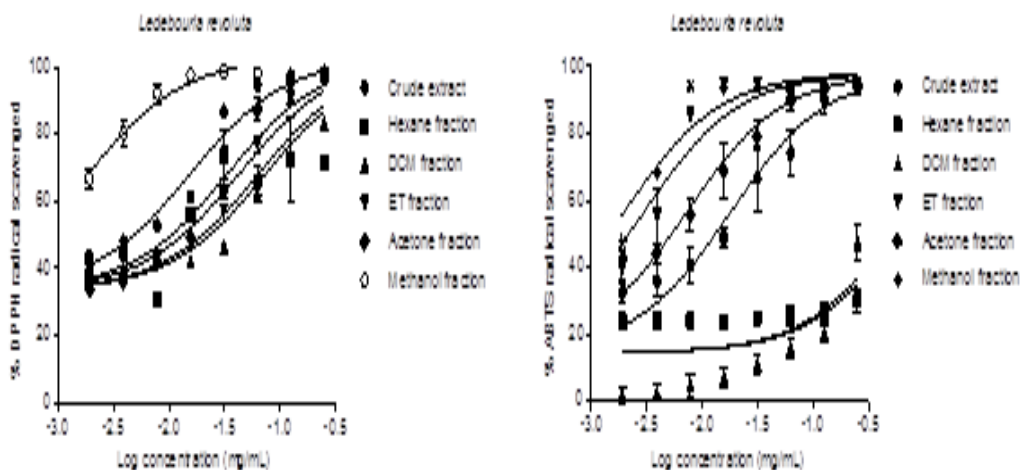


Figure 31: Plots of % free radical scavenging against log concentration in mg/ml (DPPH on right and ABTS on left side)

Ledebouria revoluta methanol fractions displayed high activity EC₅₀ of about 2 µg/ml for both DPPH• and ABTS•• radicals. The ethyl acetate fraction was active against the ABTS•• radical (EC₅₀ of 2.937 µg/ml) but was less active against the DPPH• radical (EC₅₀ of 42.58 µg/ml).

Table 14: EC₅₀ values for free radical scavenging activity of the extracts and fractions at for *Ledebouria revoluta*

	DPPH•			ABTS••		
	EC ₅₀	95%CL	R ²	EC ₅₀	95%CL	R ²
C	15.46	11.23 to 21.28	0.9542	17.79	13.43 to 23.57	0.8541
H	63.93	46.43 to 88.03	0.4444	759.2	436.4 to 1321	-2.948
DCM	74.21	53.82 to 102.3	0.8916	686.8	407.6 to 1157	0.4636
ET	42.58	30.97 to 58.55	0.9635	2.937	2.153 to 4.006	0.8513
AC	33.4	24.29 to 45.92	0.9411	7.152	5.374 to 9.518	0.8881
MET	1.867	1.271 to 2.743	0.8778	1.957	1.395 to 2.745	0.8601

C= crude extract, H= hexane fraction, DCM= dichloromethane fraction, ET = ethyl acetate fraction, AC = acetone fraction and MET= methanol fraction

Carissa bispinosa

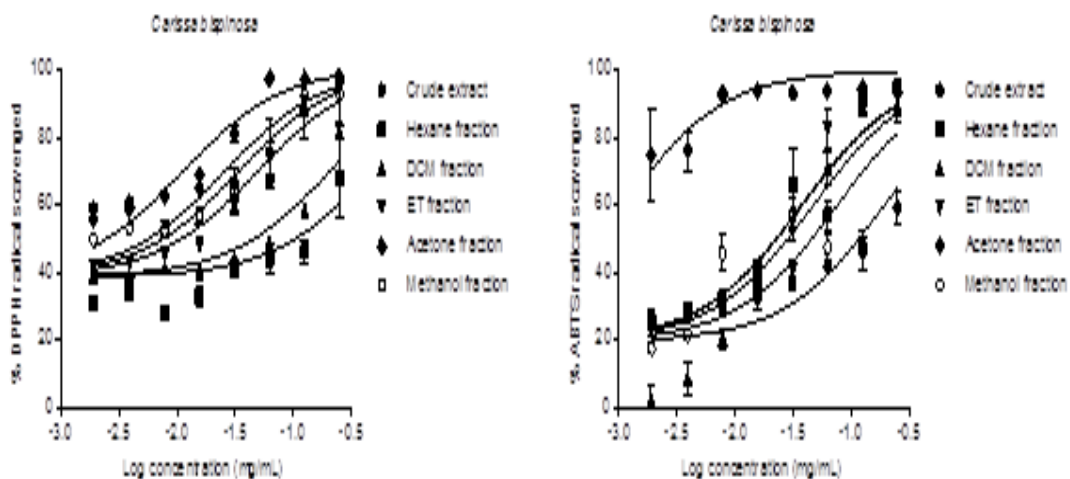


Figure 32: Plots of % free radical scavenging against log concentration in mg/ml

Carissa bispinosa displayed low activity with the exception of the acetone fraction DPPH• (EC₅₀=11.18 µg/ml) and ABTS•• (EC₅₀=1.108 µg/ml).

Table 15: EC₅₀ values for free radical scavenging activity of the extracts and fractions at for *Carissa bispinosa*

	DPPH •			ABTS••		
	EC ₅₀	95%CL	R ²	EC ₅₀	95%CL	R ²
C	25.45	16.68 to 38.83	0.3931	190.6	123.1 to 295.1	0.5893
H	467.7	264.0 to 828.5	0.4972	36.62	24.57 to 54.57	0.8654
DCM	205.5	131.4 to 321.3	0.905	46.56	31.24 to 69.37	0.8499
ET	48.96	32.28 to 74.27	0.9394	37.49	25.15 to 55.87	0.8916
AC	11.18	7.267 to 17.20	0.9131	1.108	0.6115 to 2.007	0.3412
MET	33.67	22.13 to 51.24	0.6624	75	50.22 to 112.0	0.5984

C= crude extract, H= hexane fraction, DCM= dichloromethane fraction, ET = ethyl acetate fraction, AC = acetone fraction and MET= methanol fraction

Berkheya setifera

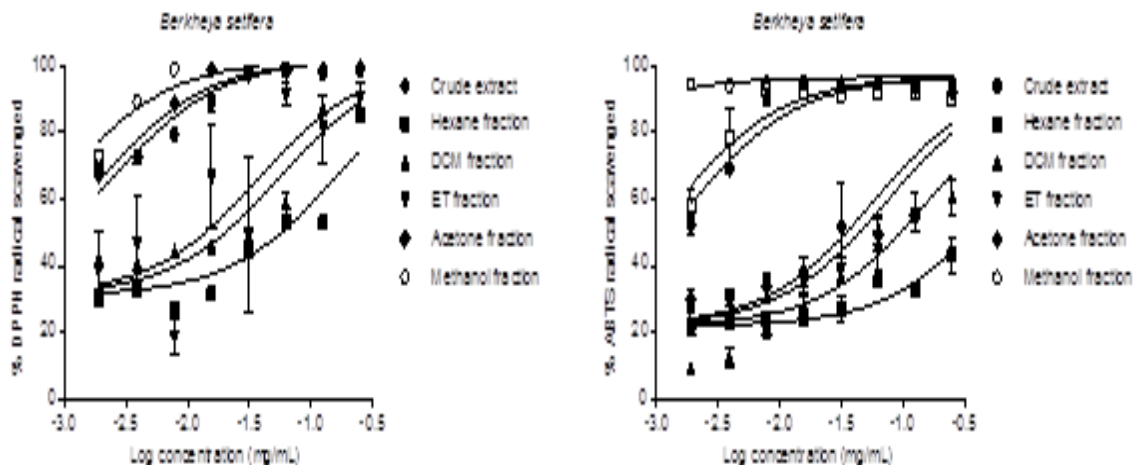


Figure 33: Plots of % free radical scavenging against log concentration in mg/ml (DPPH on right and ABTS on left side)

Berkheya setifera crude and acetone fraction had comparable activity against both radicals of about 2 µg/ml in each case. However the highest activity for the *Berkheya setifera* samples was displayed by methanol fractions for both radicals.

Table 16: EC₅₀ values for free radical scavenging activity of the extracts and fractions at for *Berkheya setifera*

	DPPH•			ABTS•+		
	EC ₅₀	95%CL	R ²	EC ₅₀	95%CL	R ²
C	2.471	1.541 to 3.963	0.887	1.967	1.377 to 2.811	0.8966
H	157.8	98.39 to 253.0	0.8773	577.1	348.7 to 955.0	0.6111
DCM	55.3	35.89 to 85.21	0.8615	158.1	114.1 to 219.0	0.7746
ET	38.11	24.82 to 58.50	0.4636	57.15	42.37 to 77.09	0.8006
AC	2.016	1.231 to 3.304	0.9341	73.03	53.99 to 98.79	0.6948
MET	1.008	0.5376 to 1.890	0.8343	1.463	0.9902 to 2.163	0.6945

C= crude extract, H= hexane fraction, DCM= dichloromethane fraction, ET = ethyl acetate fraction, AC = acetone fraction and MET= methanol fraction

Gunnera perpensa

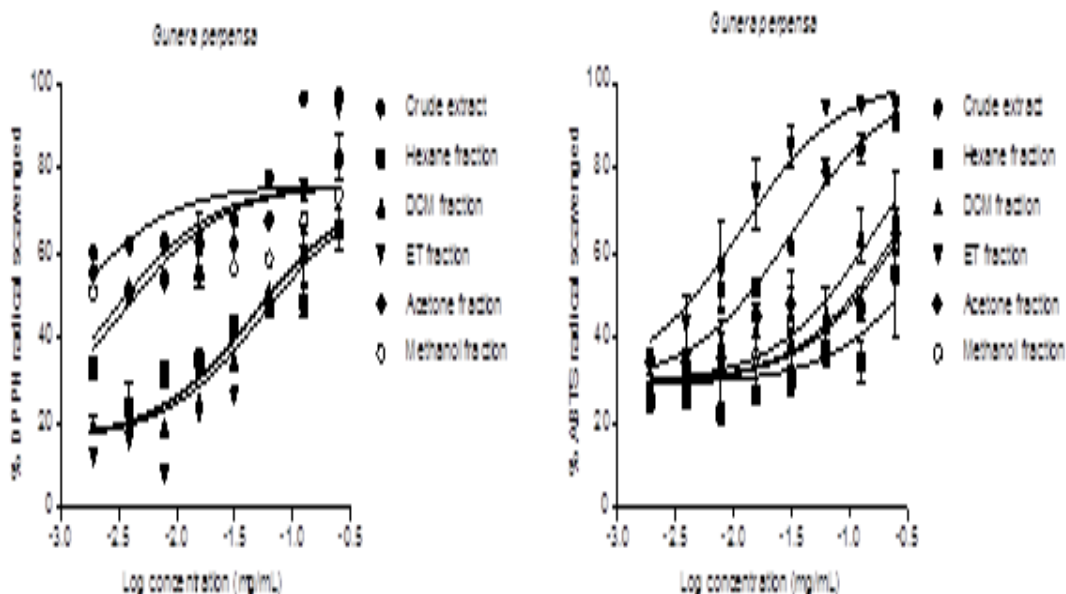


Figure 34: Plots of % free radical scavenging against log concentration in mg/ml (DPPH on right and ABTS on left side)

Gunnera perpensa crude and fractions were more active with DPPH• (57.67 µg/ml) than ABTS•+ (11.39 µg/ml) except for the ethyl acetate fraction.

Table 17: EC₅₀ values for free radical scavenging activity of the extracts and fractions for *Gunnera perpersa*

	DPPH•			ABTS••		
	EC ₅₀	95%CL	R ²	EC ₅₀	95%CL	R ²
C	1.069	0.5222 to 2.188	0.346	32.49	21.55 to 48.96	0.9302
H	46.88	28.06 to 78.30	0.5588	646.5	349.0 to 1198	0.4257
DCM	48.71	29.15 to 81.41	0.8813	164.9	109.5 to 248.1	0.6992
ET	57.67	34.42 to 96.63	0.7889	11.39	7.437 to 17.45	0.8678
AC	2.795	1.622 to 4.815	0.2938	261.5	168.5 to 405.8	0.4348
MET	2.795	2.005 to 5.783	0.2938	292.9	186.5 to 460.2	0.4577

C= crude extract, H= hexane fraction, DCM= dichloromethane fraction, ET = ethyl acetate fraction, AC = acetone fraction and MET= methanol fraction

Eucomis autumnalis

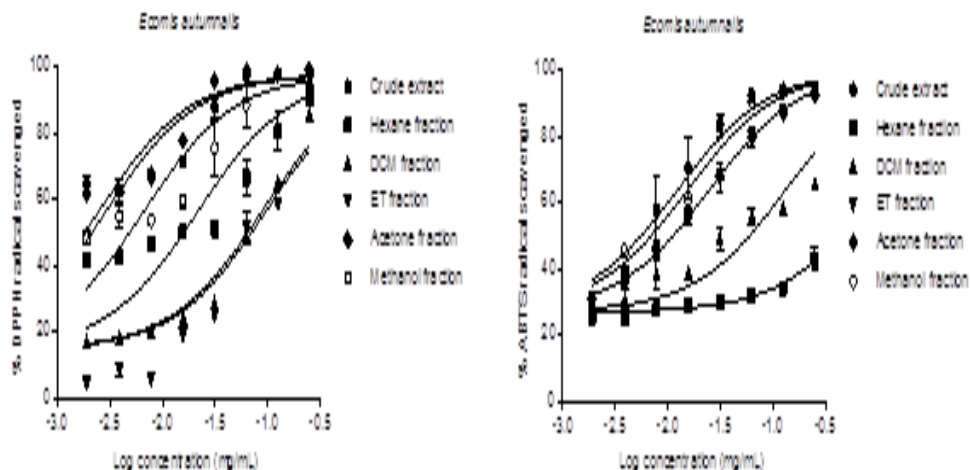


Figure 35: Plots of % free radical scavenging against log concentration in mg/ml (DPPH on right and ABTS on left side)

Eucomis autumnalis crude and acetone fraction displayed high DPPH• free radical scavenging activity of EC₅₀ of 2.891 µg/ml and 2.41 µg/ml respectively. ABTS•• free radical activity for the plant sample was generally lower for all samples except for the ethyl acetate moderate activity of 2444 µg/ml.

Table 18: EC₅₀ values for free radical scavenging activity of the extracts and fractions for *Eucomis autumnalis*

	DPPH•			ABTS••		
	EC ₅₀	95%CL	R ²	EC ₅₀	95%CL	R ²
C	2.891	1.980 to 4.221	0.7544	12.18	9.461 to 15.68	0.8771
H	21.67	15.25 to 30.79	0.5432	929.4	557.5 to 1550	0.6797
DCM	87.71	61.00 to 126.1	0.9615	130	100.7 to 167.9	0.7260
ET	95.32	66.15 to 137.3	0.8442	24.44	19.03 to 31.37	0.9709
AC	2.461	1.668 to 3.631	0.7564	24.44	19.03 to 31.37	0.9709
MET	6.621	4.650 to 9.428	0.7286	15.17	11.80 to 19.52	0.9446

C= crude extract, H= hexane fraction, DCM= dichloromethane fraction, ET = ethyl acetate fraction, AC = acetone fraction and MET= methanol fraction

Artemisia afra

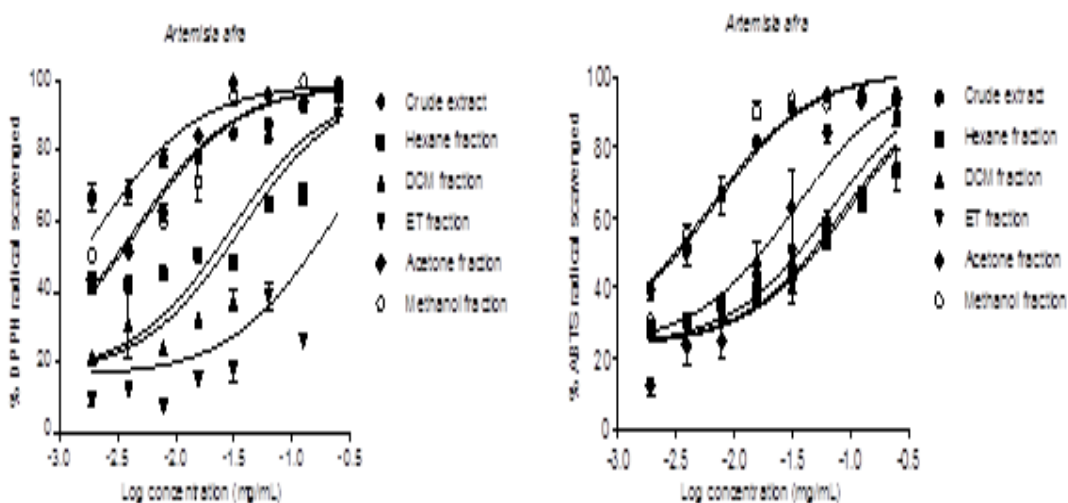


Figure 36: Plots of % free radical scavenging against log concentration in mg/ml (DPPH on right and ABTS on left side)

Artemisia afra crude extract and methanol fraction DPPH• and ABTS•• free radical scavenging activity as displayed by the curves on figure 36 and the EC₅₀ values at 95% confidence limit. High activity is also displayed by the acetone fraction DPPH• anti-radical activity.

Table 19: EC₅₀ values for free radical scavenging activity of the extracts and fractions for *Artemisia afra*

	DPPH•			ABTS••		
	EC ₅₀	95%CL	R ²	EC ₅₀	95%CL	R ²
C	2.113	1.362 to 3.277	0.5043	6.447	4.843 to 8.581	0.9697
H	29.5	20.08 to 43.35	0.4448	96.66	72.85 to 128.3	0.8541
DCM	36.03	24.50 to 52.99	0.8452	89.08	67.21 to 118.1	0.9166
ET	199.3	128.1 to 309.9	0.6795	68.57	51.86 to 90.67	0.9308
AC	4.393	2.961 to 6.517	0.9288	32.95	24.94 to 43.53	0.8637
MET	4.715	3.184 to 6.980	0.8696	6.028	4.525 to 8.032	0.8994

C= crude extract, H= hexane fraction, DCM= dichloromethane fraction, ET = ethyl acetate fraction, AC = acetone fraction and MET= methanol fraction

4.4 DISCUSSION

4.4.1 TLC DPPH scavenging activity

Methanol fractions (Tables 11-21) exhibited the highest numbers of compounds possessing DPPH free radical scavenging activities (most concentrated in *Pomaria sandersonii* and *Gunnera perperensa*) as depicted on the chromatogram. The exception is the DCM extract from *Eucomis autumnalis* which shows three bands of free radical scavenging activities. Non-polar fractions of hexane on the chromatograms have few antioxidant bands on their TLCs.

4.4.2 ABTS and DPPH scavenging assay results

The nine plant fractions and crude extracts free radical scavenging activities displayed in figures 28 to 36 demonstrated the possibility of capability to be used in the treatment of oxidative stress conditions. Oxidative stress, which is caused by an oxidative burst, is triggered by chronic inflammation which also causes pain (Iwalewa *et al.* 2007). EC₅₀ (Effective Concentration) values express the value of plant extract/fraction in µg/ml to decrease the absorbance of DPPH• or ABTS•• radicals by 50% (Antolovic *et al.* 2002).

Alepidea amatymbica polar fractions (methanol for DPPH• radical scavenging and Ethyl acetate, acetone and methanol fractions for ABTS•• radical cation) displayed high activity of 0.08 to 2.6 µg/ml and low activity from crude extracts, DCM and ethyl acetate (Tables 13 and 14). *Alepidea amatymbica* aqueous extracts are used traditionally to treat inflammation.

Artemisia afra forms part of southern African indigenous medicines with a wide range of applications including treatment of conditions associated with chronic inflammation such as rheumatism, fever, diabetes, asthma, malaria and wounds (Liu *et al.* 2009; Van Wyk 2008). *A. afra* volatile oils have been reported to demonstrate antioxidant activity when sprayed by DPPH• during a TLC screening method of evaluation and during non-enzymatic lipid peroxidation in liposomes (Liu *et al.* 2009). In this investigation, high free radical scavenging activities have been demonstrated during this study (Table 11 - 19). Simelane *et al.* (2010) have reported DPPH and ABTS scavenging activity IC₅₀ of 1.6 µg/ml for DPPH and 0.9 µg/ml for ABTS values which are comparable (EC₅₀ of 1-2.8 µg/ml) to those obtained in this study.

Several reports on the biological activities of *Artemisia afra* have been made including the isolation and characterisation of volatile and non-volatile metabolites (Steenkamp 2003 & Liu *et al.* 2009). The fact that *Artemisia afra* crude fraction and polar fractions of acetone and methanol displayed high activity (EC₅₀ for DPPH• radical, 2.113 µg/ml with crude 4.393 µg/ml with acetone fraction, 4.715 µg/ml, with methanol fraction and EC₅₀ with ABTS•• radical cation, 6.447 µg/ml and 6.208 µg/ml from crude and methanol fraction respectively) demonstrates why the aqueous extracts are used in traditional medicine. *Eucomis autumnalis* crude fraction had EC₅₀ with ABTS•• radical cation, 12.89 µg/ml and 6.21 µg/ml against DPPH. *Eucomis autumnalis* is used to treat various inflammation related diseases ranging from post-operative recovery, healing fractures to treating syphilis (Koorbanally *et al.* 2006).

Pentanisia prunelloides fractions had activity of 2.2- 5.6 µg/ml against the DPPH• radical and could explain why the root extracts are used for treatment of inflammation related conditions (Steenkamp 2003; Lindsey *et al.* 1999). *Ledebouria revoluta* is used in traditional medicine for wound dressing, sores, coughs, backaches, gastroenteritis and during pregnancy (Hutchings 1996 & Watt Breyer-Brandwijk 1962). The methanol fraction of *Ledebouria revoluta* had comparable DPPH free radical scavenging capability of 1.867 µg/ml to that of *Berkheya setifera* methanol fraction with EC₅₀ value of 1.008 µg/ml and *Gunnera perpensa* crude acetone extract EC₅₀ value of 1.069 µg/ml. This illustrates the reasons why *Gunnera perpensa* is used for treating inflammation related diseases.

Crude acetone extract, DCM and Ethyl acetate fractions of *Pomaria sandersonii* displayed higher ABTS•• scavenging capability was higher than corresponding DPPH scavenging ability. *P. sandersonii* samples had the highest number of fractions with ABTS•• scavenging capability of 1.274 µg/ml to 5.973 µg/ml (for the crude, DCM, ethyl acetate, acetone and methanol) compared to all the plants studied although its hexane fraction activity of 150 µg/ml was low. *Carissa bispinosa* DPPH scavenging ability of crude extract and fractions are the lowest active samples of all the nine plants. *Gunnera perpensa* acetone, methanol fraction and crude extract had activity of DPPH free radical scavenging at 2.795 µg/ml and 2.795 µg/ml respectively. In earlier literature, *Gunnera perpensa* methanol and aqueous extracts had

analgesic and anti-inflammatory activity (Nkomo *et al.* 2010). *Gunnera perpensa* methanol extracts had ABTS•• (78.45%) and DPPH• (78%) scavenging at a concentration of 50 µg/ml (Simelane *et al.* 2010).

4.5 CONCLUSION

The most active antioxidants were contained in *Pentania prunelloides*, *Pomaria sandersonii*, *Gunnera perpensa* and *Eucomis autumnalis* methanol fractions because they exhibited free radical activity against both ABTS•• and DPPH• free radicals. These plants are used for treating the various disorders related to inflammation by the traditional Zulu practitioners. The activities validate claims by the traditional healers that they use the plants for curing the various inflammation related conditions. The activity of *Pomaria sandersonii* extracts and fractions demonstrated that the plant contains antioxidants that react with both DPPH and ABTS radicals although higher activities were shown by ABTS as displayed by the lower EC₅₀ values. These results were produced from *in vitro* assays using artificial radicals ABTS and DPPH therefore the plant extracts and fractions activity *in vivo* still need to be carried in order to fully validate their activities as antioxidants in the body.

CHAPTER 5

Antibacterial and antifungal activities of nine medicinal plants used for treating inflammatory ailments

5.1 INTRODUCTION

Infectious diseases are still a serious challenge in health management despite the discovery and use of antibiotics. The situation is complicated by the emergence of more virulent and resistant pathogens to existing drugs and the increasing cases of immuno-compromised conditions especially HIV infection, diabetes and old age. These necessitate continuous efforts in the discovery of new classes of safer and more potent anti-infectious agents that can overcome resistance mechanisms (Gibbons 2005). In South Africa and other communities world-wide people use plants as sources of traditional remedies to treat infectious diseases and general ailments. Natural products and their derivatives represent over 50% of all the drugs in clinical use in the world (Van Wyk *et al.* 2002). Many therapeutic regimes used in traditional medicines are usually herbal mixtures from several medicinal plants (Van Vuuren & Viljoen 2008).

The use of plants as medicine forms part of traditional medicine therapy to manage various ailments and diseases. It is estimated that 80% of Zulu patients who use orthodox medicine also consult traditional healers (Jager *et al.* 1996; Liu *et al.* 2008). Infectious diseases in association with inflammation complications is one of the major health challenges faced by Mabandla community. The pathogenic bacteria implicated in some diseases of the community and South Africa in general are *Escherichia coli* (causes gastroenteritis); *Enterococcus faecalis* (causes endocarditis), *Pseudomonas aeruginosa* which causes inflammation and sepsis in the lungs, urinary tract and kidneys), *Staphylococcus aureus* causes tonsillitis, scarlet fever, minor skin infections, impetigo, boils, abscesses and scalded skin syndrome (Hamer 2007). Some of the fungal pathogens also associated diseases in the community include *Candida albicans* which is one of the important opportunistic yeast involved in oropharyngeal and genital candidosis (Rex *et al.* 1995). *Aspergillus fumigatus* is implicated in causing a range of diseases called aspergillosis whose symptoms include cough, fever, wheezing, skin sores and vision problems (Walsh *et al.* 2008).

Many of the plants secondary metabolites are constitutive, existing in healthy plants in their biologically active forms, but others occur as inactive precursors and are activated in response to tissue damage or pathogen attack (Osbourne 1996). A diverse range of bioactive molecules such as alkaloids, polyphenols and terpenoids are responsible for some of the therapeutic potential of medicinal plant extracts (Yff *et al.* 2002). The aim of this study is to determine the antimicrobial activities of the selected plants.

5.2 BIOLOGICAL ASSAYS

5.2.1 Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration is a quantitative measure of anti-infective properties of pure drugs or mixtures as in the case of medicinal plant extracts. It represents the lowest concentration of an antimicrobial agent that inhibits the visible growth of a microorganism after incubation for a given length of time of the test sample and the organism. The efficacy of antimicrobials from biological extracts, fractions and isolates are carried out alongside known drugs such as amphotericin B, neomycin and others to ensure that the organism being investigated is a susceptible strain (Ncube *et al.* 2008). Minimum bactericidal concentrations (MBCs) are the lowest concentration of antimicrobials that will prevent the growth of an organism after subculture on to antibiotic-free media (Andrew 2001).

Two methods commonly used in evaluating antimicrobial activities of plant extracts include the diffusion (include agar well diffusion, agar disk diffusion) and dilution (agar dilution and broth micro/macro-dilution) (Ncube *et al.* 2008). Agar diffusion methods have some limitations because some antimicrobial components of an extract especially the non-polar may be insoluble in the agar and therefore would not exhibit the activities accurately (Eloff 1998). The diffusion techniques are therefore only useful in identification of leads but not quantification of bioactivity (Ncube *et al.* 2008). In agar disk diffusion assay, 6-mm sterile paper disks saturated with plant extract/fraction/isolate are placed onto a suitable agar medium which is pre-inoculated by a test organism, incubated at 37 °C for 24hr for bacteria and 48 hr for fungi, and zones of inhibition are measured (Ncube *et al.* 2008). In agar well diffusion, wells of between 6 and 8 mm are punched on the agar into which a culture of the test organism has been spread evenly. Fixed volumes of the plant extract/fraction or isolate of known concentrations are then introduced into the wells, incubated and followed by measuring the zones of inhibition (Ncube *et al.* 2008).

A broth micro-dilution technique in which 96-well plates and tetrazolium salts indicate bacterial growth has been found to be quick, worked with many different plants, gave reproducible results and required only 10-25 µl extract (Eloff 1998). The method can distinguish between microbicidal and micro-biostatic effects. Permanent records are produced with more sensitivity than the agar diffusion methods (Eloff, 1998). In the agar dilution assay, a stock solution of the test solution is incorporated into molten agar at different concentrations and the test organism is introduced by streaking onto the agar plates, which are incubated and the minimum concentration determined as the concentration of the test solution inhibiting visible growth of test organism on the agar plate (Andrews 2001).

5.2.2 Bioautography

Bioautography is a qualitative method of determining biological activities of crude extracts, and fractions. The biological activity could be antifungal (Masoko & Eloff 2006), antibacterial (Martini & Eloff 1998), tyrosinase inhibition (Momtaz *et al.* 2008), and antioxidant activity (Matthew & Abraham 2006). The two methods commonly used for the determination of antimicrobial activity are agar diffusion and direct TLC (Jacob & Walker 2005). In agar diffusion, a suitable TLC solvent system is chosen for optimum separation of constituents in an active extract or fraction. After obtaining the fraction or extract TLC chromatogram, and it is dried free of solvent, the TLC is placed face down in a plate on the surface of agar containing a strain of pathogen of interest for a period of about 30 minutes and then removed (Jacob & Walker 2005). The plates are incubated at 35 °C for 24 hours and zones of inhibition are compared with the TLC for active components (Jacob & Walker 2005). However it has a disadvantage that the active components, which may be lipophilic, may not diffuse into the agar in order to exhibit the biological activity (Jacob & Walker 2005). In direct TLC bioautography a suspension of a microorganism in a suitable broth is applied uniformly on a developed TLC plate by a spray gun (Begue & Klein 1972). The sprayed TLC is incubated in a humid atmosphere at 35 °C for 24hr (Masoko & Eloff 2006). Zones of microorganism inhibition are visualized by spraying a dehydrogenase-activity-detecting reagent such as tetrazolium salt; metabolically active organisms convert the tetrazolium salt into a correspondingly intensely coloured formazin. Antimicrobial compounds appear as clear spots against a coloured background (Masoko & Eloff 2006). The dye *p*-iodonitrotetrazolium violet (INT) is usually used for the assay. The amount of formazin, which is the red dye produced by the enzymatic reaction increases over time (about 4hr) in the humid incubator. Results are scanned for record (Masoko & Eloff 2006).

Bioautography has advantages of providing a rapid and simple method of identifying the number of biologically active constituents present in an extract and requires very small amounts of sample (less than one milligram) (Jacob & Walker 2005). The original activity in the crude extract may be different from the isolated constituent on the TLC because of absence of synergy which may otherwise be present in the crude extracts (Jacob & Walker 2005). In addition, there is an assumption that the constituent does not decompose during TLC, which could happen. The activity once identified, should be confirmed by a micro-plate serial dilution assay (Jacob & Walker 2005).

5.3 MATERIALS AND METHODS

5.3.1 Plant selection and collection

Plants under study listed in Table 3.4.4 were used for the study. The chief traditional healer, Mr S. Dlamini organised the collection of the different plants by digging the plant roots from the mountain areas of Mabandla village in Kwazulu-Natal. The plant roots were packed in labelled bags and were brought to the Chemistry laboratory for processing.

5.3.2 Bioautography

For bioautography, TLC was developed as described in section 3.3.4.2 and air dried for 72hr to ensure all the solvents are evaporated from the plate. The chromatograms were sprayed with concentrated broth of actively growing *S. aureus* and *E. coli* cells and incubating at 37°C in a chamber of 100% relative humidity for 16hr. Plates were sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (Sigma) and re-incubated for another 30 min to 2 hr. Clear white zones against the purple background on the chromatogram indicate inhibition (Begue & Kline 1972).

5.3.3 Minimum inhibitory concentrations (MIC)

Minimum inhibitory concentrations (MIC) of *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC25922, *Candida albicans* and *Aspergillus fumigatus* were determined by twofold serial dilution on 96-well micro plate (Eloff 1998). To each well 100 µl of distilled water were added. And 100 µl of extract (at 10 mg/ml in acetone) was added to the first well of the micro titre plate followed by serial dilution up to well eight. The solvent blank and antibiotic (such as amphotericin B) were included as negative and positive control respectively. To each well, 100 µl of an overnight culture of microbes, diluted with Mueller Hinton broth (bacteria) or Sabouraud broth (fungi) was added and followed by incubation overnight at 37 °C. The effective concentration of the test sample in well one was 2.5 mg/ml and well eight was 0.19 mg/ml. 40 µl 0.2 mg/ml INT (iodonitrotetrazolium violet, Sigma) solution was added. Colour change was noted after 30, 60, and 120 min to obtain the lowest concentration where growth was inhibited (MIC).

5.4 RESULTS

5.4.1 Bioautography

From the microbial bioautograms the crude extract of *P. prunelloides*, *P. sandersonii*, and *A. amatymbica* have some zone of growth inhibition against *S. aureus* while *P. prunelloides*, *P. sandersonii*, *A. amatymbica* and *E. autumnalis* have zones of inhibition against *Escherichia coli*. Methanol extract were compared with acetone extracts in the bioautograms as displayed in Figures 37 and 38. It is worth noting that the methanol *Alepidea amatymbica* extract did not display any activity against both *Staphylococcus aureus* and *Escherichia coli*. There are a number of bands of activity against the same organisms with both *Alepidea amatymbica* and *Pomaria sandersonii* acetone extracts as displayed by both bioautograms. The high inhibition of pathogen growth displayed by polar fractions (on the base of chromatograms) indicates that most of the active compounds would be soluble in water, justifying the plants use in traditional practise.

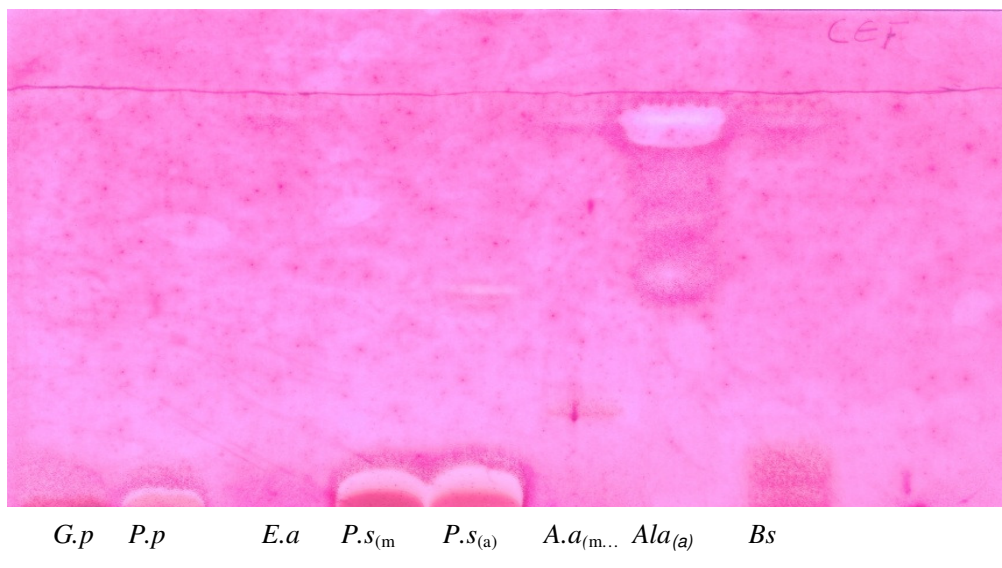


Figure 37: Bioautography to illustrate crude extracts inhibitory activity against *Staphylococcus aureus*

TLC Clear spots show antibacterial activity. The red area shows bacterial activity. Solvent was EMW. *G. p.* = *Gunnera perpensa*, *P. p.* = *Pentania prunelloides*, *E. a.* = *Eucomis autumnalis*, *A. a.* = *Alepidea amatymbica*, *B. s.* = *Berkheya setifera* m=methanol extract, a= acetone extract

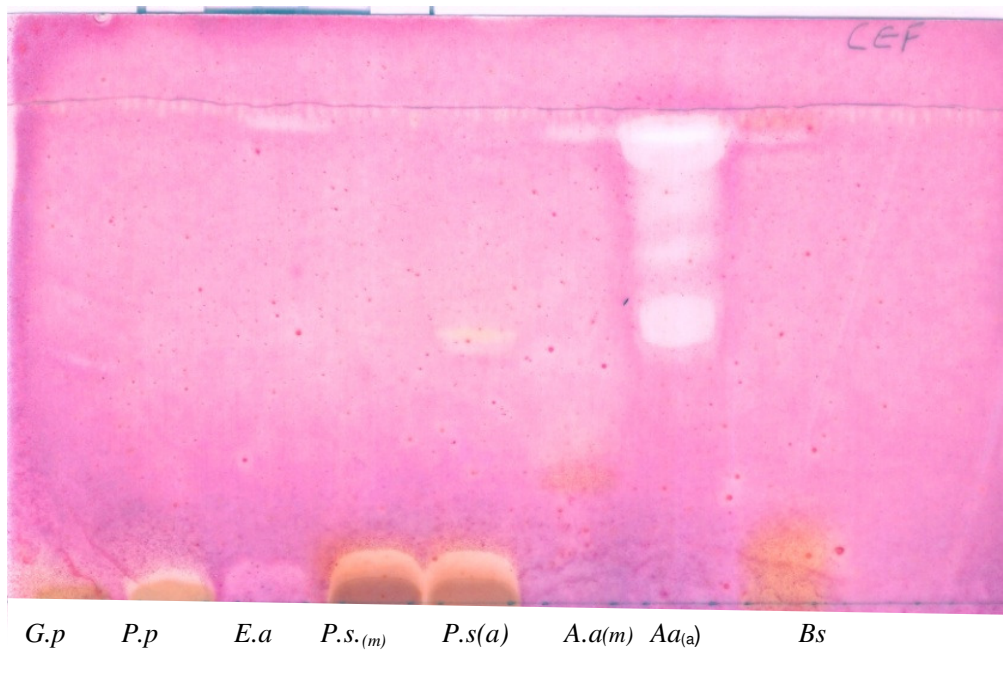


Figure 38: Bioautography to illustrate crude extracts inhibitory activity against *Escherichia coli* by crude extracts

Clear spots show antibacterial activity. The red area shows bacterial activity because the *p*-iodonitrotetrazolium chloride dye (INT) changes from colorless to red when bacteria are present. *G. p.* = *Gunnera perpensa*, *P. p.* = *Pentanisia prunelloides*, *E. a.* = *Eucomis autumnalis*, *A. a.* = *Alepidea amatymbica*, *B. s.* = *Berkheya setifera* *m*=methanol extract, *a*= acetone extract

The acetone extracts displayed highest number of bands with *Escherichia coli* inhibitory activity compared to the rest of the crude extracts. Acetone extractant was therefore the better than methanol for extracting antibacterial compounds from *Alepidea amatymbica*.

5.4.2 Minimum Inhibitory Concentrations, MIC

The results of the MICs are presented in Tables 20 (antibacterial) and 21 (antifungal). All of the crude extracts and fractions had antibacterial activity (MIC: 20-625 µg/ml) and anti-fungal activity (MIC: 20-2500 µg/ml). *P. sandersonii* crude and fractions demonstrated higher activity compared to other plants. Some MIC values of *P. sandersonii* DCM and ethyl acetate (80 µg/ml in each case) compared well with gentamycin (20 µg/ml) same value against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*. Anti-fungal activities of the DCM, acetone and methanol fractions were also active (20 µg/ml) for both *Candida albicans* and *Aspergillus fumigatus*. The high inhibition of pathogen growth displayed by acetone and methanol fractions from all the plants would also indicate that most of the active compounds would be soluble in water. It is possible that some of the active DCM soluble components with activities of 20-160 µg/ml as displayed on Table 21 may not be accessible to the traditional hears who mostly use water as their extractant.

Artemisia afra crude and fractions were also active against the pathogens tested. The acetone and methanol fraction inhibition (20 µg/ml) against *Escherichia coli* was of pharmacological importance. The activity by extracts and fractions against the fungi *Candida albicans* and *Aspergillus fumigatus* was in the range of 160–625 µg/ml except for the polar fractions of acetone and methanol where there was lower inhibition.

Carissa bispinosa was the most active crude extract (MIC: 20 µg/ml) against *Escherichia coli* compared to the rest of the crude extracts tested. The hexane extract had an activity of 20 µg/ml against *Escherichia coli*. The rest of the extracts of *C. bispinosa* had activity of 160-320 µg/ml against all the bacteria tested. Inhibition against the fungi of 160–625 µg/ml was worth considering except for the acetone fraction which was lower against *C. albicans* (2500 µg/ml). Inhibition against the tested bacteria by *Alepidea amatymbica* was high to moderate against the bacterial strains used in this study (160-320 µg/ml) for all fractions and crude extract. There was significant activity against the fungi tested with MIC of 160–625 µg/ml.

Pentanisia prunelloides extract and fractions' activity against bacteria had MIC range of 160-320 µg/ml for all fractions and crude extract and anti-fungal activity was 625 µg/ml for all the extracts and fractions. *Berkheya setifera* crude extract and fractions activity against bacteria and fungi was (160–320 µg/ml). The hexane fraction has good activity against *Staphylococcus aureus* (20 µg/ml) and (625 µg/ml) against *E. faecalis*. *Gunnera perpensa* crude extracts and fraction also had activity with MIC of 160-320 µg/ml. The methanol extract showed high activity of 80 µg/ml towards *Pseudomonas aeruginosa*. Anti-fungal inhibition is high to low with MIC range of 160–625 µg/ml although the acetone fraction had little inhibition (2500 µg/ml) towards *Aspergillus fumigatus*.

Ledebouria revoluta had MIC range of 20-320 µg/ml antibacterial activity. The inhibitory activity of 20 µg/ml by the polar fractions of acetone and methanol against *E. coli* and *C. albicans* were pharmacologically significant. The methanol fractions had the least inhibitory activity of 1250 µg/ml against *C. albicans*. According to the Zulu traditional healers of Mabandla village, these plants are used widely in their community for treatment of inflammation. The various activities demonstrated by each of them validated their uses although it is important to investigate their cytotoxicity.

Table 20: Antibacterial Activities of the crude extracts and fractions expressed as MIC ($\mu\text{g/ml}$)

Organisms tested	<i>Pomaria sandersonii</i>				<i>Alepidea amatymbica</i>				<i>Pentanisia prunelloides.</i>			
	<i>S.a.</i>	<i>E.f.</i>	<i>E.c.</i>	<i>P.a.</i>	<i>S.a.</i>	<i>E.f.</i>	<i>E.c.</i>	<i>P.a.</i>	<i>S.a.</i>	<i>E.f.</i>	<i>E.c.</i>	<i>P.a.</i>
Crude	160	40	80	80	320	320	320	160	160	320	320	320
Hexane	160	160	160	320	160	160	160	160	320	320	320	160
DCM	80	40	80	80	160	320	160	320	320	320	320	160
ETAc	80	40	80	80	320	320	320	320	320	160	160	320
Acetone	160	160	60	160	160	160	160	160	160	630	320	320
Methanol	160	160	160	160	160	320	630	320	160	320	320	160
Gentamycin	4	4	4	4	4	4	4	4	4	4	4	4

Table 20 continued

Organisms tested	<i>Carissa bispinosa</i>				<i>Berkheya setifera</i>				<i>Ledebouria revoluta</i>			
	<i>S. a.</i>	<i>S. a.</i>	<i>E. f.</i>	<i>E. c.</i>	<i>P. a.</i>	<i>E. f.</i>	<i>E. c.</i>	<i>P. a.</i>	<i>S. a.</i>	<i>E. f.</i>	<i>E. c.</i>	<i>P. a.</i>
Crude	320	320	160	160	160	320	20<	320	320	160	320	160
hexane	160	160	320	160	360	320	20<	160	80	160	320	80
DCM	160	320	160	160	320	320	320	160	160	160	320	160
ETOAc	160	320	160	160	320	160	160	320	160	630	160	160
Acetone	160	160	320	20<	320	160	320	160	320	320	320	320
Methanol	320	160	160	20<	320	320	320	160	320	160	160	320
Gentamycin	4	4	4	4	4	4	4	4	4	4	4	4

Table 20 continued

Organisms tested	<i>Gunnera perpensa</i>				<i>Eucomis autumnalis</i>				<i>Artemisia afra</i>			
	<i>S. a.</i>	<i>E. f.</i>	<i>E. c.</i>	<i>P. a.</i>	<i>S. a.</i>	<i>E. f.</i>	<i>E. c.</i>	<i>P. a.</i>	<i>S. a.</i>	<i>E. f.</i>	<i>E. c.</i>	<i>P. a.</i>
crude	320	160	320	160	160	160	630	320	320	630	160	320
hexane	160	320	320	160	160	320	60	160	160	320	630	20
DCM	320	320	320	160	160	160	160	160	160	320	320	160
Ethyl acetate	630	630	320	160	320	320	160	160	320	630	160	160
Acetone	630	160	160	160	320	320	160	320	160	320	20<	160
Methanol	320	160	160	80	160	320	160	320	160	320	20<	160
Gentamycin	4	4	4	4	4	4	4	4	4	4	4	4

Table 21: Antifungal activities of the extracts and various fractions expressed as MIC ($\mu\text{g/ml}$)

	<i>P. sandersonii</i>		<i>A. amatymbica</i>		<i>P. prunelloides</i>		<i>C. bispinosa</i>		<i>B. setifera</i>		<i>G. perpensa</i>		<i>E. autumnalis</i>		<i>A. afra</i>		<i>L. revoluta</i>	
	<i>C. a</i>	<i>A. f</i>	<i>C. a</i>	<i>A. f</i>	<i>C. a</i>	<i>A. f</i>	<i>C. a</i>	<i>A. f</i>	<i>C. a</i>	<i>A. f</i>	<i>C. a</i>	<i>A. f</i>	<i>C. a</i>	<i>A. f</i>	<i>C. a</i>	<i>A. f</i>	<i>C. a</i>	<i>A. f</i>
Crude	625	320	625	160	625	625	320	625	160	625	320	625	160	625	625	160	625	320
Hex	625	160	625	160	625	625	160	625	160	625	160	625	160	160	625	160	625	320
DCM	20<	320	625	160	625	625	160	625	160	625	160	625	160	320	625	160	625	320
ETAc	1250	320	625	160	625	625	160	625	160	625	160	625	160	160	625	160	625	320
ACE	20<	320	625	160	625	625	160	2500	320	625	160	2500	320	320	1250	320	20	320
MET	20<	320	625	160	625	625	160	160	320	625	160	160	320	625	1250	320	1250	320
AMP	13	0.6	13	0.6	13	0.6	13	0.6	13	0.6	13	0.6	13	0.6	13	0.6	13	0.6

Hex= Hexane, DCM= dichloromethane, ETAc = ethyl acetate, ACE = acetone, Met = methanol, AMP= Amphotericin B

5.5 DISCUSSION

Results for antibacterial and antifungal screening are displayed in Tables 20 and 21. A total of 54 plant crude extracts and fractions from nine plant species were investigated. The medicinal plants used to treat inflammation under different disease conditions in the Zulu community of Mabandla village, Kwazulu-Natal, South Africa have displayed antimicrobial activities. The various antimicrobial activities demonstrate the validity of the healing capacities that the traditional healers of the community claim to possess. Invasion of the body by organisms such as *Escherichia coli*, *Candida albicans*, *Pseudomonas aeruginosa* and other such pathogens can result in chronic inflammation (Iwalewa *et al.* 2007). Plant extracts that contain antimicrobial activity against microbial pathogens can offer an alternative therapy of managing disease with adequate consideration for safety in their administration. Ethno-medicine is one method of discovering new drugs which may offer a solution to cure diseases caused by pathogens which have resistance to known drugs (Ncube *et al.* 2007).

In this investigation, *Gunnera perpensa* extracts and fractions have good broad based antimicrobial activities against the organisms tested using the micro plate dilution method. The most active fraction was the methanol fraction (80 µg/ml) against *Pseudomonas aeruginosa*. *Gunnera perpensa* fractions (hexane, DCM, ethyl acetate, acetone and methanol) have good activity of 160 µg/ml against *Candida albicans*. The antimicrobial activities of water, ethyl acetate and ethanol fractions of the root extract of *Gunnera perpensa* against some bacteria and fungi has been reported before (*Bacillus subtilis*, 12.5 mg/ml) (Buwa & Van Staden 2006). Crude water extract of *Gunnera perpensa* have antimicrobial activity against *Escherichia coli* (0.78 mg/ml); *Klebsiella pneumoniae* (0.78 mg/ml); and *Candida albicans* (25 mg/ml) while the corresponding ethyl acetate and ethanol fractions were less active (Buwa & Van Staden 2006). However, the reported values are not considered to be significantly high by the phytomedicine program. This explains the use of the plant by South African Traditional healers as treatment against venereal diseases (Buwa & Van Staden 2006). Drewes *et al.* (2005) isolated 1,4-benzoquinone derivatives, which were identified as 2, methyl-6-(3-methyl-2-butenyl) benzo-1,4-quinone (MIC of 18 µg/ml against *Bacillus cereus*) and 3-hydroxy-2-5-(3-methyl-2-butenyl)benzo-1,4-quinone (MIC of 37 µg/ml against *Candida albicans* and 75 µg/ml and against *Cryptococcus neoformans*) and considered as the active antimicrobial components against the bacteria and fungi. Although this characterisation is not exhaustive, it explains why *Gunnera perpensa* is so active against the organisms tested (Tables 20 and 21). South African traditional healers use

Gunnera perpensa aqueous decoction to induce labour, facilitating the expulsion of the placenta and relief of dysmenorrhoea (Simelane *et al.* 2010). *Gunnera perpensa* also displayed antinociceptive and anti-inflammatory activity (Nkomo *et al.* 2010).

Artemisia afra acetone and methanol fractions had MIC of 20 µg/ml against *Escherichia coli* and 160-320 µg/ml for the crude extract and all fractions against the organisms tested (Table 20 and 21) against *Aspergillus fumigatus* and *Staphylococcus aureus*. *Artemisia afra* is used in traditional medicine in the treatment of a variety of diseases ranging from respiratory infections to dysmenorrhoea, diabetes and malaria (Suliman *et al.* 2010; Liu *et al.* 2009). The plant is rich in terpenes such as artemisia alcohol, camphene (Chagonda *et al.* 1999), camphor and artemisia ketone (Liu *et al.* 2009) and displayed a variety of biological activities (using disc diffusion method) against bacteria such as *Staphylococcus aureus* (MIC 2.0 mg/ml), *Mycobacterium smegmatis* (MIC 1.9 mg/ml), fungi such as *Candida albicans* (% minimum inhibitory percentage of 0.25) and protozoa such as *P falciparum* (IC₅₀ of 4.4 µg/ml) tested in their investigations (Liu *et al.* 2009). *A. afra* is used to treat various inflammatory related diseases as displayed in Table 6.

Eucomis autumnalis had MIC of 160-320 µg/ml from the crude extract and fractions against the organisms tested except for the crude extract against *E coli* with MIC of 630 µg/ml, which was lower than the plant fractions activities. *Eucomis autumnalis* also inhibited totally prostaglandin synthesis and displayed preferential COX-2 inhibitory activity (Fennel *et al.* 2004) and these pharmacological properties make the plant suitable for treating inflammation (Stafford *et al.* 2005). Activities reported on the activities of fractions and crude extracts against the organisms in this study are reported for the first time.

Alepidea amatymbica had MIC of 160-320 µg/ml by the crude extract and fractions against the organisms tested except for the methanol fraction against *Escherichia coli* in which the activity of 630 µg/ml, which was low compared to the inhibitory activities of the other plants in the study. *Alepidea amatymbica* crude and fractions also had anti-fungal activity (MIC: 160 µg/ml against *Aspergillus fumigatus*). Earlier literature reported much lower antibacterial and anti-fungal activities of *Alepidea amatymbica* with water, DCM, ethanol and petroleum ether extracts using the same method of Eloff, 1998 (Mulaudzi *et al.* 2009). The reason for the difference could be due the fact that in this study, fractions from liquid-liquid fractionation were used and not crude extracts where the different solvents were used to extract directly from plant

powder. *Alepidea amatymbica* petroleum ether, DCM and ethanol extracts also displayed above 90% COX-2 activity (Mulaudzi *et al.* 2009). *Alepidea amatymbica* is used in the treatment of various conditions of inflammation including asthma, influenza and diarrhoea (Somova *et al.* 2001)

Pentanisia prunelloides, which is used traditionally for the treatment of dysmenorrhoea, had good to moderate (MIC: 160-320 µg/ml) antibacterial activity from the crude extract and fractions against the organisms tested except for the acetone fraction against *Enterococcus faecalis* in which the activity of MIC 630 µg/ml, which was low. Yff *et al.* (2002) reported antibacterial activity by water, ethanol and ethyl acetate extracts by the same method of Eloff *et al.* (1998). Activities are lower (MIC: 0.39-2.5 mg/ml) than those reported here presumably because fractions as opposed to crude extracts, were used for assays in this study. *Ledebouria revoluta* had good activities (MIC: 20 µg/ml) for both acetone and methanol fractions against *E. coli* and good-moderate activity (MIC: 160-320 µg/ml) against the rest of the bacterial strains used. Anti-fungal activity for *Ledebouria revoluta* methanol fraction (MIC: 20 µg/ml) is high against *Candida albicans*. The bulb infusions of *Ledebouria revoluta* are used for diarrhoea treatment in goats and leaf decoctions for gall sickness Dold & Cocks (2001) quoted by McGaw *et al.* (2008).

Pomaria sandersonii, *Carissa bispinosa* and *Berkheya setifera* have shown good to moderate antimicrobial activity. *P. sandersonii* DCM and ethyl acetate fractions have higher MIC value (80 µg/ml respectively) which compared well with gentamycin (4 µg/ml) same value against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa* thereby justifying *P. sandersonii* extract use by traditional practitioners as part of post-natal care to women.

5.6 CONCLUSION

Plant fractions from *P. sandersonii*, *Carissa bispinosa*, *Eucomis autumnalis*, and *Gunnera perpensa* have good antimicrobial activity as exhibited by their MIC values. The nine plants in this investigation have also exhibited good antimicrobial activity.

CHAPTER 6

Anti-inflammatory activities of nine medicinal plants used in treating inflammatory ailments in Zulu traditional medicine of South Africa

6.1 INTRODUCTION

Inflammation plays significant roles in the progression of many diseases such as microbial and parasitic infections, cardiovascular, gastrointestinal, neurodegenerative, and respiratory ailments with or without recognizable bacterial or viral aetiology (Kaplan *et al.* 2007). Treatment of inflammation-associated diseases is a major challenge in Mabandla village of KwaZulu-Natal. Traditional healers use some medicinal plants available in the local environment to manage the various diseases (Table 6).

6.1 Diseases' mechanism of inflammation

Inflammatory response to stimuli involves increased blood flow, increased vascular permeability, destruction of tissues via the activation and migration of leucocytes (Iwalewa *et al.* 2007). The release of reactive oxygen derivatives (oxidative burst) (Westbrook *et al.* 2010), and local inflammatory mediators, such as prostaglandins (PGs) (Iwalewa *et al.* 2007), leukotrienes (Adams & Bauer 2008), and platelet-activating factors (Iwalewa *et al.* 2007) induced by phospholipase A₂, cyclooxygenases (COXs), and lipoxygenases (LOXs) characterise progression of inflammation (Iwalewa *et al.* 2007). Arachidonic acid is a key biological intermediate that is converted into a large number of bioactive eicosanoids (Adams & Bauer 2008). The two rate determining pathways of arachidonic acid metabolism are the COX-1 and 2 which result in the formation of both PGs and the lipoxygenase, LOX pathway, which is responsible for the formation of leukotrienes from mast cells and other enzymes and products (figure 39). The LOXs initiate oxidative peroxidation of polyunsaturated fatty acids (PUFAs), such as arachidonic acid forming short chain aldehydes, diketones and alkanes, thereby inflicting cellular damage in the process (Kühn & Donnell 2006).

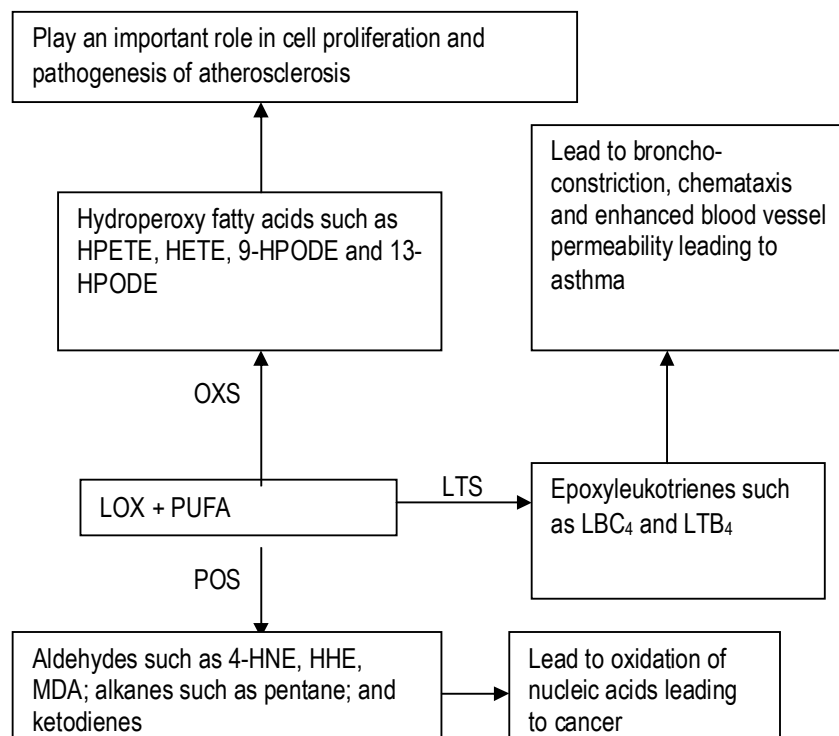


Figure 39: Summary of some LOXs activities illustrating their action on unsaturated fatty acids and products.

9-HPODE, 9-hydroperoxy-10, 12-octadecadienoic acid, 13-HPODE, 13-hydroperoxy-9,11-octadienoic acid; HNE, 4-hydroxy-2-nonenal, HHE, 4-hydroxy-2-hexenal; MDA, malondialdehyde, LTC₄ Cysteinyl leukotriene C₄ LTB₄, dihydroxyeicosatetraenoate, HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid the enzymes: POS, peroxidases; OXS, oxygenases; LTS, Leukotriene synthase.

Products of LOXs catalysed oxygenation during lipid peroxidation LPO are involved in the development of many chronic diseases (Figure 39), (Spiteller 1998). The LOXs are classified with respect to their position specificity of arachidonic acid oxygenation (5-LOX, 9-LOX 12-LOX and 15-LOX) (Rackova *et al.* 2007). 15-LOX catalyses the insertion of molecular oxygen on the arachidonic acid skeleton at carbon 15 position to produce hydroperoxyeicosatetraenoic acid (15(S)-HPETE) or on linoleic acid at position 13 to produce 13 hydroperoxylinoleic acid (13(S)-HPODE) (Ye *et al.* 2005). Oddly, inflammation is an indispensable early form of protective mechanisms developed by the body to fight against microbial infection or tissue injury. However, prolonged and excessive inflammatory response is detrimental to body cells involved. The COX pathway (COX-1 and COX-2) of arachidonic acid metabolism produces the prostaglandins PGG₂ and PGH₂, which are subsequently converted into further prostaglandins, prostacyclin and thromboxanes (TXs) (Ye *et al.* 2005).

6.1.1 Therapeutic treatment

Non-selective non-steroidal anti-inflammatory drugs (NSAIDs) such as acetylsalicylic acid or aspirin have been used as the major therapeutics in the treatment of inflammation and associated complications (Viji & Helen 2008). The mechanisms of action of NSAIDs include inhibiting the activity of COX enzymes, thereby reducing the production of pro-inflammatory prostaglandins with the resultant effects of reducing pain (analgesic), fever (anti-pyretic), and inflammation (Viji & Helen 2008). The uses of NSAIDs are associated with serious deleterious side effect, despite their therapeutic efficacy (Viji & Helen 2008). These include damage to the kidneys, worsening asthma and, most importantly, damage to the upper GI tract. The effects of NSAIDs on the Gastro Intestinal (GI) tract range from dyspepsia to ulceration, bleeding and death (Westbrook *et al.* 2010). Selective inhibition of COX 2 inhibition is associated with adverse side effects such as decreased renal function (Helen 2007). This justified the need for a continuous search for safer and more effective anti-inflammatory drugs. Many medicinal plants are used in various traditional medicines to treat inflammations and any resultant complication. Therefore, medicinal plants used in traditional medicine still represent a reservoir as starting point in search of new anti-inflammatory therapy (Iwalewa *et al.* 2007). In this study, validation of the ethno-pharmacological use of these plants was conducted by evaluating the lipoxygenase enzyme inhibitory activities of the extracts and their fractions of various polarities.

6.1.2 Theory of the LOX assay

The lipid peroxidation process which involves three steps of introduction of atmospheric oxygen into the hydrocarbon chain of the PUFA such as linoleic acid shown in figure 40 can be studied by following the UV absorbance of the oxidation products (Spiteller 1998). In vitro measurement of LOX activity can be effected by a reaction medium containing 15-LOX, linoleic acid in buffer at pH 9 for 30 to 90 seconds after adding plant extract. Increase of absorbance at 234 nm indicates the formation of hydroperoxylinoleic acid (Rackova *et al.* 2007).

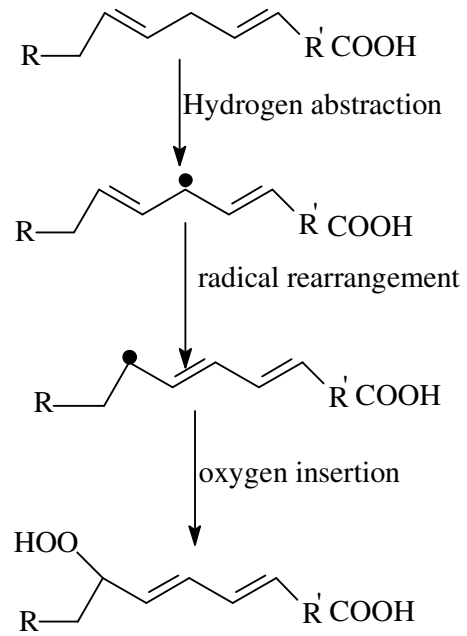


Figure 40: Formation of the conjugated double bond during LOX oxygenation of a PUFA

LOX catalysis is bimolecular which is dependent on the concentration of the lipid substrate and atmospheric oxygen (Kühn 2006).

6.2 MATERIALS AND METHODS

6.2.1 Plant extracts preparation:

Plants were treated as in section 3.4.4. The acetone extracts were made to 10 µg/ml and were further used for the anti-inflammatory assay.

6.2.2 Lipoxygenase inhibition

Inhibitory activity of the plant extracts against 15-soybean lipoxygenase (15-LOX) enzyme was evaluated as described by Malterud & Rydland (2000) in borate buffer (0.2-M, pH 9.00). Increase in absorbance at 234 nm was read 5 min at interval of 30s after addition of 15-LOX, using linoleic acid (134-µM) as substrate. The final enzyme concentration was 167 µg/ml. Test substances were added as DMSO solutions (final DMSO concentration of 1.6%) while DMSO alone was added in control experiments. The enzyme solution was kept on ice, and controls were measured at intervals throughout the experimental periods to ensure that the enzyme activity was constant. All measurements were carried out three times.

6.3 RESULTS

Results for the 15-LOX anti-inflammatory assay are displayed in Figure 41 and Table 22. *Pomaria sandersonii* sample had the highest percentage 15-LOX inhibition, EC_{50} of 7.22 $\mu\text{g/ml}$ followed by *Pentanisia prunelloides* that demonstrated a 15-LOX inhibitory activity (80%) with EC_{50} value of 15.98 $\mu\text{g/ml}$. *Eucomis autumnalis* EC_{50} = 42.76 $\mu\text{g/ml}$ displayed moderate LOX activity. *Ledebouria revoluta* was the least active EC_{50} = 1030 $\mu\text{g/ml}$. *Alepedia amatymbica* EC_{50} = 115 $\mu\text{g/ml}$, and *Gunnera perpensa* EC_{50} = 81.18 $\mu\text{g/ml}$ exhibited low activity.

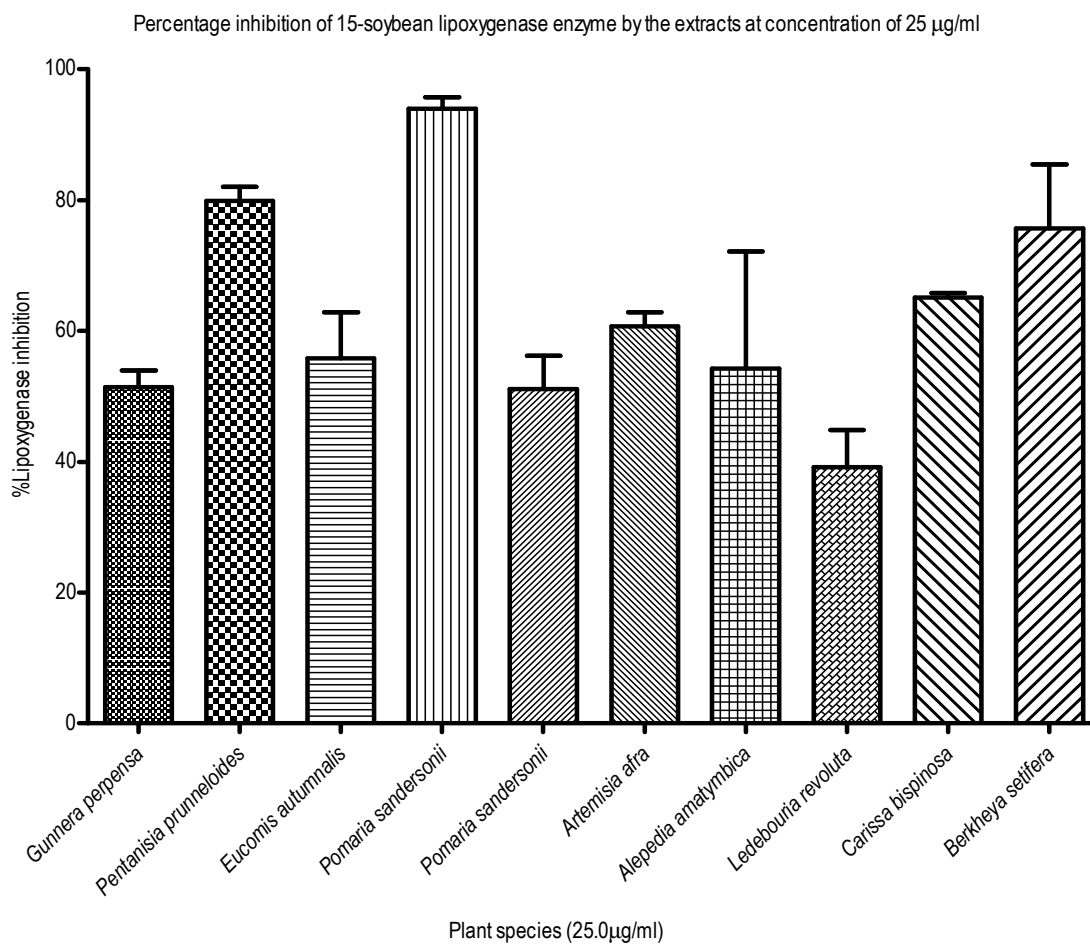


Figure 41: % soyabean derived 15-LOX- Inhibition by the various plant crude extracts

Table 22: Anti-inflammatory activity expressed as EC₅₀ values for the crude and fractions in µg/ml.

plant species	EC ₅₀	95%CL
<i>G. perpensa</i>	81.18	29.26 to 225.2
<i>P. prunelloides</i>	15.98	7.506 to 34.02
<i>E. autumnalis</i>	42.76	19.10 to 95.70
<i>P. sandersonii</i>	7.225	3.150 to 16.57
<i>P. sandersonii</i>	85.74	30.02 to 244.9
<i>A. afra</i>	21.84	10.34 to 46.12
<i>A. amatymbica</i>	113.4	33.13 to 387.9
<i>L. revoluta</i>	1030	0.4237 to 2.504
<i>C. bispinosa</i>	12.24	5.647 to 26.54
<i>B. setifera</i>	11.91	5.482 to 25.89

6.4 DISCUSSION.

The increase in absorption at 234 nm is a sign of the formation of the hydroperoxylinoleic acid and hence a measure of the activity of the 15-LOX. The rhizome of *A. amatymbica* LOX inhibition was about 50% but previous reports with petroleum ether and dichloromethane extracts of *A. amatymbica* rhizome has been reported to demonstrate COX-1 and COX-2 inhibition of above 90% (Mulaudzi *et al.* 2009; Stafford *et al.* 2005).

Eucomis autumnalis bulbs had a 58% LOX inhibition activity during this study. Previous reports indicated that *E. autumnalis* bulbs inhibited prostaglandin synthesis 90% relative to indomethacin (0.5 µg/ml) at 65% and also displayed selective COX-2 activity (Taylor & Van Staden 2002; Koorbanally *et al.* 2006). *Eucomis autumnalis* extracts had moderate 15-LOX activity with EC₅₀ value of 42.76 µg/ml. *Pentanisia prunelloides* had high 15-LOX inhibitory activity (80%) with EC₅₀ value of 15.98 µg/ml. *Pentanisia prunelloides* water ethanol and ethyl acetate extracts in earlier reports displayed cyclooxygenase inhibition of 65-87% (Yff *et al.* 2002). This is consistent with earlier reports of COX-1 inhibitory activity, of leaf water extract = 74%, root water extract = 74%, ethyl acetate extract leaf = 88%; ethyl acetate root = 87% when compared to the positive control of Indomethacin, 20 µM = 83% (Yff *et al.* 2002; Jager & Van Staden 2005).

Phospholipids such as arachidonic or linoleic acid are common substrates to both the COXs and LOXs producing prostaglandins and leukotrienes respectively (Schneider *et al.* 2006). However further study need to be carried out in order to establish if the extracts if the plant extracts are capable of dual LOX and LOX inhibition.

6.5 CONCLUSION

The plants displayed some lipoxygenase activity and are therefore anti-inflammatory. 15-LOX from soybean was used to assess the *in vitro* inhibitory activity. These findings should therefore be cautiously applied to the anti-inflammatory activity in humans because the mechanism of human derived 15 LOX may be different from that which is soybean derived. Cyclooxygenases (COX1 and COX-2), lipoxygenases (LOXs) and cytochrome P450 monooxygenases are three classes of enzymes which catalyse the formation of eicosanoids such as prostaglandins, thromboxanes, leukotrienes and other oxygenated derivatives of the lipid mediators (such as arachidonic and linoleic acid). In this study only 15 LOX inhibitory activity was studied. The crude plant extracts' other anti- inflammatory activities still need to be investigated in order to determine the other *in vivo* biological activities.

CHAPTER 7

Isolation and identification of antimicrobial compounds from *Pomaria sandersonii* and *Alepidea amatymbica*.

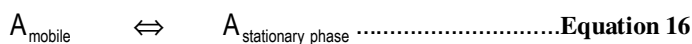
7.1 INTRODUCTION

In this Chapter symbols were used to represent different pure compounds obtained after isolation and purification. EM80-2 and EM86 represent two chalcones isolated from *Pomaria sandersonii* ethyl acetate roots fraction respectively. On the other hand, 06B, 06-2, 0652, 0657 and 06431 represent five diterpenoids isolated from the hexane extract of *Alepidea amatymbica* roots. Furthermore, the three diterpenes 0652, 0657, 06431 and EM86 biological activities were not determined in this study due to limited resources and time.

Isolation and identification of biologically active compounds from roots of *Pomaria sandersonii* and *Alepidea amatymbica* is a necessary step in establishing and documenting the active components of the two medicinal plants. In this research, several steps were conducted in an attempt to isolate pure compounds and their characterization, namely, liquid-liquid fractionation column chromatography by bioassay-guided protocol and characterization was achieved by using selected methods.

7.1.1 Column Chromatography

Column chromatography was used to separate and isolate different fractions-into their pure form. In column chromatography, the stationary phase, a solid adsorbent, is placed in a vertical glass (usually) column and the mobile phase, a liquid, is added to the top and flows down through the column (by either gravity or external pressure). The mixture to be analysed by column chromatography is applied on top of the column. Different components in the mixture have different interactions with the stationary and mobile phases; they are carried along with the mobile phase to varying degrees to attain separation. Analyte A is distributed between the mobile and stationary phases according to the equilibrium illustrated in equation 16.



The equilibrium constant, K, of equation 7.1 is termed the partition constant and it shows is the ratio between the molar concentrations of the analyte in stationary phase to the molar concentration in the mobile phase. (Chandrul & Srivastava 2010)

7.1.2 Reverse phase and normal phase chromatography

In reversed phase chromatography the column is non-polar and the analyte molecule binds to an immobilized hydrophobic molecule on the stationary phase as a more polar solvent is used as the eluent. This partitioning occurs as a result of the analyte having hydrophobic patches at its surface for binding to the matrix. Normal-phase employs polar stationary phase and non-polar-solvents and is used for separating analytes that are too hydrophobic or hydrophilic for separation using reversed-phase. Silica gel (SiO_2) and alumina (Al_2O_3) are two adsorbents commonly used for normal phase chromatography. These adsorbents are in different mesh sizes (Glowniak *et al.* 1996).

7.1.3 Nuclear Magnetic resonance

Nuclear magnetic resonance (NMR) spectroscopy is a method used in the structural elucidation of organic molecules. The method produces information about the spin under applied magnetic field of atomic nuclei of some elements possessing odd atomic number or mass or both such as ^1H , ^2H , ^{13}C , ^{14}N , ^{17}O , and ^{19}F which may be in the molecule to be elucidated. The nucleus, being charged has a magnetic field which generates a magnetic moment, μ , which interacts with applied magnetic field and precess about its axis (Kumirska *et al.* 2010). The frequency at which the nuclei precess is proportional to the applied magnetic field. The precession generates an oscillating electric field of the same frequency. If radio-frequency waves of same frequency are supplied (by a magnetic field) energy can be absorbed and effect a spin change on the atomic nucleus, resulting in resonance characteristic to the particular nuclei. The energy transfer is measured and processed to yield an NMR spectrum for the nucleus concerned. The resonant frequency of the energy transition is dependent on the effective magnetic field at the nucleus. The field is affected by electron shielding which in turn is dependent on the chemical environment for example, the more electronegative the element is, the higher the resonant frequency. The ratio of nuclear shielding and applied magnetic field for a particular nucleus is its chemical shift. Tetramethylsilane ($\text{Si}(\text{CH}_3)_4$, TMS) resonant frequency is the reference frequency during ^1H NMR measurements. Groups of protons can interact (J-coupling) with each other giving spaces between chemical shift values or peaks on the spectra resulting in the multiplicity of splitting pattern for the molecule. Coupling is measured in Hertz and is given a symbol J. The multiplicity of a multiplet is given by the number of equivalent protons in neighbouring atoms plus one (n+1 rule). Equivalent nuclei do not interact with each other and coupling constant, J, is not dependent on applied magnetic field (Pavia *et al.* 2009).

7.1.3.1 Different NMR techniques

There are several NMR techniques employed in structural elucidation of a molecule including carbon-13, ^{13}C NMR, proton NMR, ^1H NMR, two dimensional NMR including heteronuclear multiple-bond coherence, HMBC, heteronuclear multiple-quantum coherence, HMQC, and heteronuclear single quantum coherence, HQSC, Nuclear Overhauser Effect Spectroscopy, NOESY, Correlated Spectroscopy, COSY (Kumirska *et al.* 2010). In two dimensional NMR, data is

collected after two different time domains elapse, firstly acquisition of free induction domain FID, (t_2) and secondly successively incremented delay, (t_1). During this time domains, the nuclei are made to interact with each other in different ways depending on pulse sequences applied (Kumar *et al.* 2011). The theory and application of the different NMR techniques is summarised in Table 25.

Table 23: A summary of some NMR techniques used and their applications.

Name of technique	Theory and J coupling	Application
Carbon-13, ^{13}C NMR	Gives the coupling between adjacent carbon nuclei	Indicates the number of carbons in the molecule in their different chemical environments
Proton NMR, ^1H NMR	Chemical shift is a result of hydrogen nucleus in a vicinal position	The spectra indicates the number of adjacent protons
Heteronuclear multiple-bond coherence, HMBC	Coupling between ^1H ^{13}C or ^1H - ^{15}N which are 2-3 bonds away. (J_{CH} -coupling.)	Reduce the overcrowding and overlaps of peaks that are found in one dimensional spectra
Correlated Spectroscopy, COSY	Gives information on spin-spin coupling of neighbouring protons up to four bonds. (J_{H} -coupling of protons.)	This assists in giving information when there are overlapping multiplets obtained from a one dimensional spectrum.
Nuclear Overhauser Effect Spectroscopy, NOESY	Distinguishes protons that are close to each other in space even if they are not bonded. (J_{H} -coupling in space.)	The technique is also used as a measure of the distance between the protons in the molecule. A NOESY spectrum yields through space correlations <i>via</i> spin-lattice relaxation
Heteronuclear single quantum coherence, HQSC	Gives information on spin-spin coupling of ^1H to ^{13}C (J_{CH} -coupling.)	This is also used to determine the C-H bonds in the molecule

7.1.3.4 Mass spectra

Mass spectrometry is based on motion measurements recorded as mass: charge ratio (m/z) of sample derived charged particles as they move in an electric or magnetic field. Information that is obtained from the mass spectrum is the

molecular mass of a compound. The mass spectrometer is made up of three parts, an ion source, an analyser (such as quadrupoles, ion trap and time of flight) and an ion detector which converts the sample ions energy to an electrical signal that is read by a computer (Kumirska *et al.*, 2010). Sample is introduced into the ion source either by direct inlet such as syringe, septum inlet or probe or by indirect inlet such as gas chromatograph, HPLC, liquid electrophoresis column. Ionisation of sample occurs by a variety of methods such as electron impact (EI), chemical ionization, CI, electrospray ionisation, ESI, and matrix assisted laser desorption ionisation (MALDI) (Kumirska *et al.* 2010).

7.2 MATERIALS AND METHODS.

Fractions were prepared as reported in section 3.4.4. The ethyl acetate and hexane fractions were further purified by column chromatography as represented in Figures 42 and 43.

7.2.1 Isolation of compounds from *Pomaria sandersonii*

A mass of 4.89 g of the ethyl acetate fraction was dissolved in a minimum amount of acetone and silica gel to produce slurry with the solution and dried under a fan at room temperature. A column was set up for chromatography with 196 g of silica gel 60. After adding the ethyl acetate fraction impregnated on silica, elution was commenced with hexane: ethyl acetate in a ratio 90:10. The solvent system was changed with polarity ratio progression of elution as illustrated on Table 24.

Table 24: Solvent ratio used for elution of *Pomaria sandersonii* ethyl acetate fraction

Chloroform	Ethyl acetate
90	10
85	15
80	20
75	25
70	30
65	35

Isolated compounds obtained were obtained as mixtures. The 10th, 11th and 12th fractions were prepared for further purification by smaller column purification because they contained the antibacterial compounds.

7.2.1.1 Smaller Column Purification

Fractions 10 (110 mg), 11 (380 mg) and 12 (870 mg) were subjected to further column chromatography in a smaller column using 50-g of silica gel 60. For the 10th fraction, 85 test tubes from the column were collected and fractions 70-71 yielded a pure compound EM 80-2. For the 11th fraction, 53 test tubes were collected from the column and test tubes 17 to 40 yielded 20 mg of pure compound EM 86.

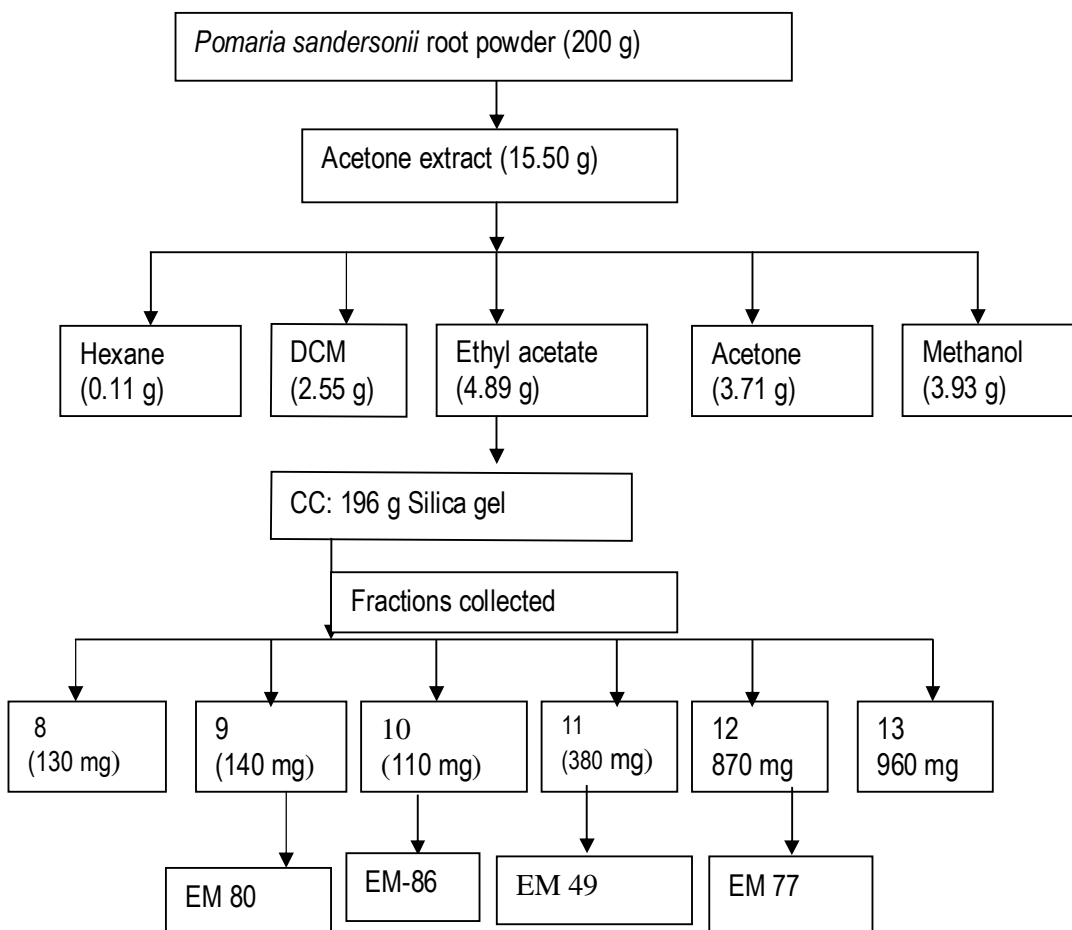


Figure 42: Isolation protocol used to isolate pure compounds from *Pomaria sandersonii*

7.2.2 Isolation of antimicrobial compounds from *Alepidea amatymbica*

6.32 g of the hexane fraction was dissolved in minimum amount of acetone and silica gel to produce slurry which was then dried under fan at room temperature. 190 g of Silica gel 60 was used to set up a column for chromatography. Hexane: ethyl acetate was used as eluent and polarity was varied as illustrated in Table 25. A total of 36 fractions were collected from the column. Compound 06B was eluted as the 8th, 0652 was the 52nd fraction while 06-2 came out in the 65-67th fractions during column elution. 0657 and 06431 were obtained by preparative TLC of the 43rd and 57th fractions from the column.

Table 25: Solvent ratio used for elution of *Alepidea amatymbica* hexane fraction

Hexane	Ethyl acetate
100	0
100	1
100	2
100	3
100	4
100	5
100	6
100	8
100	10

7.2.3 Microdilution assay

The minimum inhibitory concentrations of the isolated compounds were determined as described previously in section 5.3.3 (Eloff 1998).

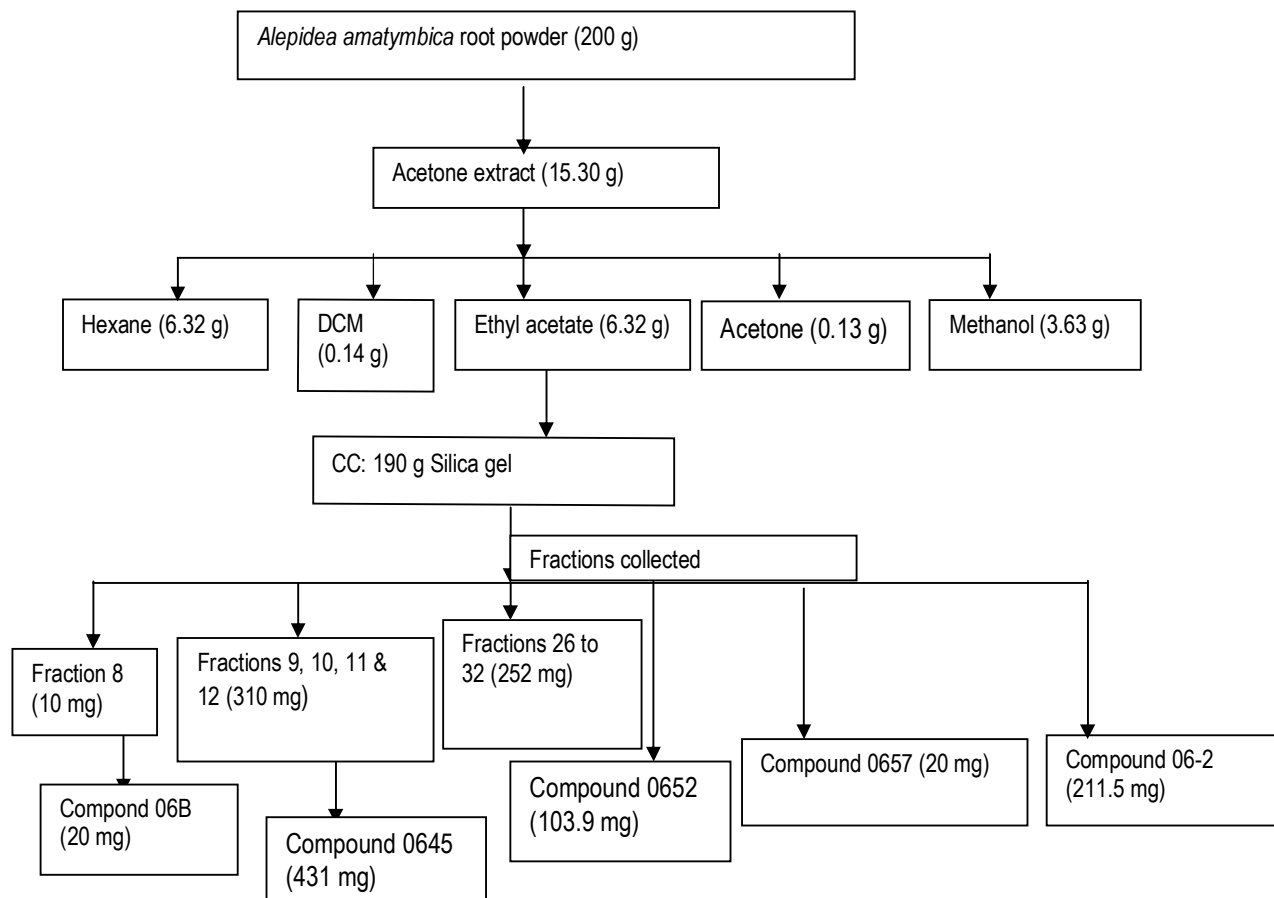


Figure 43: Isolation protocol used to isolate pure compounds from *Alepidea amatymbica*

Bioautography assays with *Staphylococcus aureus*, ATCC 29213 and *Escherichia coli*, ATCC25922 were carried out to identify the R_f values of the active fractions and pure compounds. Minimum inhibitory concentration against *Staphylococcus aureus*, ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Escherichia coli*, ATCC25922 was also carried out as described in section 5.3.3.

7.3 RESULTS

Seven compounds were isolated from the two medicinal plants *Alepidea amatymbica* and *Pomaria sandersonii*. For positive identification and structural elucidation of the isolated active compounds, NMR and MS experiments were carried out. The NMR and MS spectra are in appendices (Figures 51 -108). The mixture EM 49 and EM 77 displayed anti-microbial inhibitory activities. Results of all the experiments are summarised in Tables 28, 27 and 29 for three of the compounds, 06B, 06-2, EM80-2 and mixtures from *Pomaria sandersonii*. The antimicrobial activities of the pure compounds for *Alepidea amatymbica* and *Pomaria sandersonii* expressed as MIC ($\mu\text{g/ml}$) are displayed in figure Table 31.

7.3.1 Structure elucidation of pure compounds.

7.3.1.1 General characteristics of isolated chalconoids from *Pomaria sandersonii*

Two chalcones were isolated from *Pomaria sandersonii* ethyl acetate fraction. The NMR spectra of EM 80-2 and EM 86 were similar basic 15 carbon skeleton. They show difference due to absence of an olefinic group on αC and βC double bond in EM 86 and we have suggested that it is a dimer while EM 80-2 is a mixture of cis-trans isomers. Absence of $-\text{CH}_2$ signal on DEPT 135 for both compound spectra is also typical of chalcone spectra. Typical fragmentation pattern has been suggested for both chalcones in Figure 44. The spectra of all compounds are in the Appendix section.

7.3.1.2 Characterisation of EM 80-2

EM80-2 was isolated as a yellow amorphous powder with a melting point range of 200- 204 $^{\circ}\text{C}$. EM 80-2 was eluted from column with ethyl acetate: hexane (3:100) solvent. The NMR and MS spectra are in Appendix 1. TOF MSMS showed the molecular ion, $\text{M}+1$, at m/z 257 implying the molecular formula to be $\text{C}_{15}\text{H}_{12}\text{O}_3$ with the based peak at m/z 256 (100%). Other prominent peaks are at m/z 130 [$\text{M}-\text{C}_9\text{H}_7\text{O}^+$], 116, [$\text{M}-\text{C}_8\text{H}_4\text{O}^+$], 102, [$\text{M}-\text{C}_8\text{H}_6^+$] and 88 (100%), [$\text{M}-\text{C}_7\text{H}_4$] which are typical of chalcone fragmentation as displayed in Figure 44. The fragmentation pattern is indicative of a chalcone with three hydroxyl groups (Katerere 2000; Portet *et al.* 2008; Itagaki *et al.* 1966). ^1H NMR displayed typical phenolic resonance ranging δ 6.38-8.20. Protons attached following carbons δ 166.2, 144.6, 133.8, 132.8, 130.1, 125.6, 117.5, 116.9 and 103.5, have their resonating frequency which was the same as those of the Trans isomer for similar carbons. The coupling constant, $^3\text{J}_{\text{H,H}} = 9$ Hz is typical of olefin protons that are *trans* to each other on the double bond between C- α δ 132.8 and C- β δ 144.3. The $^3\text{J}_{\text{H,H}}$ *cis* coupling signal in the range of 12-18 Hz) is hidden behind the *trans* and was not apparent on the ^1H NMR spectrum. The two doublets at δ_{H} at 7.90 *d* 1H (2.10 Hz), 1H and 7.86, *d* ($J=2.10\text{Hz}$) 1H are

diagnostic of 3'/5' protons on the chalcone ring. The ^1H NMR (300-MHz), ^{13}C NMR, $^2\text{J}/^3\text{J}$ from HMBC and COSY for EM80-2 spectral data is shown in Table 26. NMR displayed resonances of δ 132.8 and 144.3 that is typical of α and β carbons on double bond between the two rings in a chalcone and also the keto carbon (β' -C) (Katerere 2000, Adesanwo 2009).

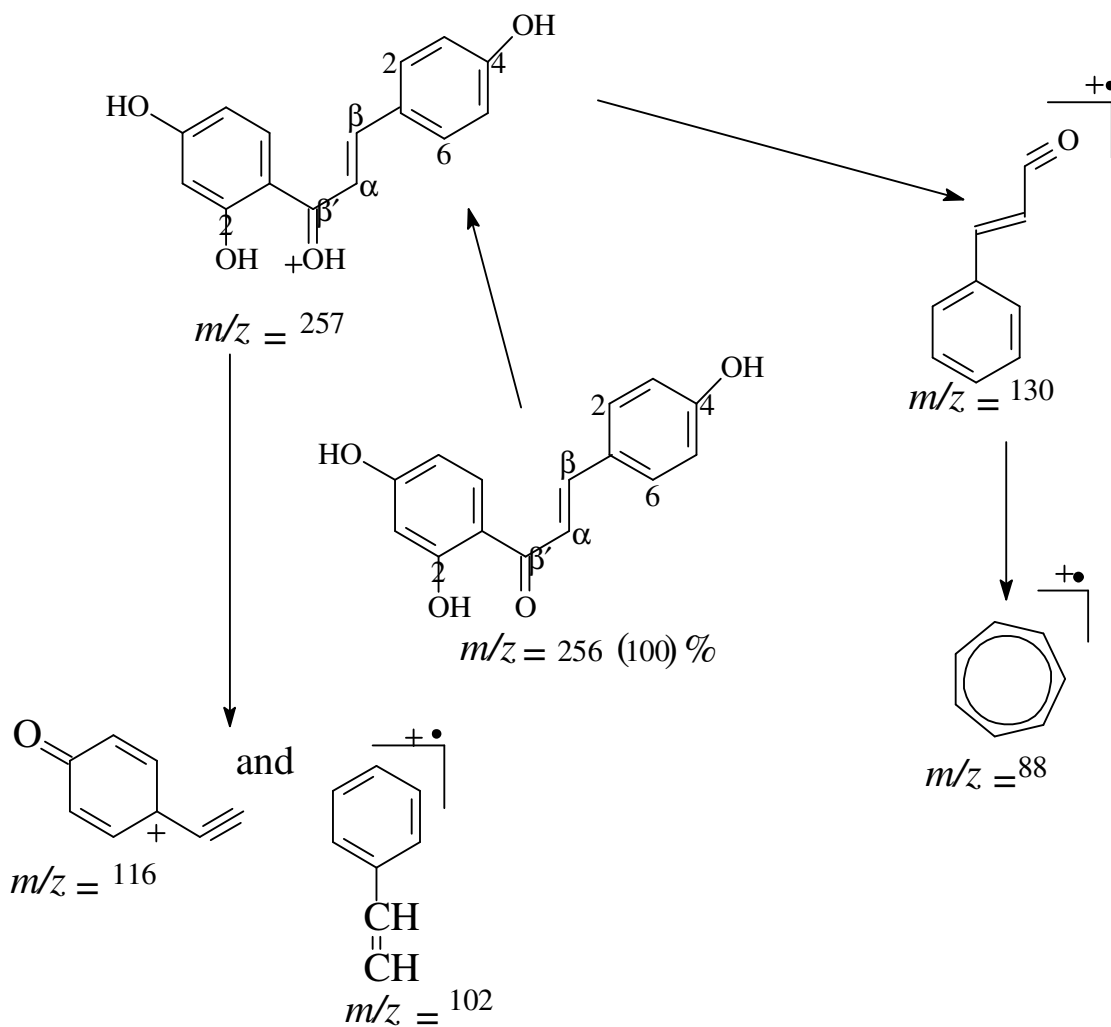


Figure 44: Typical fragmentation patterns in chalcones as exemplified by EM 80-2

Table 26: ¹³C NMR (300 MHz) and ¹H NMR (300 MHz) data δC (ppm) δH(ppm) and ²J/³J from HMBC and COSY for EM80-2 and EM86 in Acetone

EM 80-2				EM 86		
C	δ/ppm	¹ H, ¹ J/Hz	HMBC 2J/ 3J	δ/ppm	¹ H, ¹ J/Hz	HMBC 2J/ 3J
β	144.2	7.90 <i>d</i> (J=2.10), 1H	192.0 ,	43.1	3.18, <i>dd</i> , (J=3.5, 2.0)1H	128.7, 168.5
α	132.8	7.86, <i>d</i> (J=2.10) 1H		41.8	4.55, <i>t</i> ,	43.1, 118.7, 128.7, 151.6, 168.5
β'	192.0	-		168.5	-	
1'	113.7	-		128.7	7.26, <i>d</i> , (J=8.7), 2H	41.8, 128.7, 157.0
2'	166.7	-		157.8	-	
3'	102.9	6.38, <i>d</i> , (J= 2.10), 1H	113.7	117.7	6.57, <i>m</i> , 1H	118.7, 151.6, 157.8
4'	164.8	-		157.0		
5'	107.8	6.45, <i>dd</i> (J=8.9,2.3) 1H		103.4	6.57, <i>m</i> , 1H	103.4, 111.7, 118.7, 151.6, 157.8
6'	132.5	8.17 <i>d</i> (J=9.0) 1H		151.6	-	
1	126.7	-		129.8	-	
2/6	131.0 x 2	7.75 <i>d</i> (J= 8.70) 1H		128.6	7.13, <i>d</i> (J=8.1), 1H	41.8, 129.7, 157.8
3/5	115.9 x2	6.90 <i>d</i> (J= 8.70) 1H	126.7	115.9	6.81, <i>d</i> , (J= 8.7)2H	115.9, 129.8, 157.0
4	160.1	-			-	

7.3.1.3 Characterisation of EM 86

The pale yellow flaky crystals with a melting point range of 272-278 °C were eluted from the ethyl acetate fraction of *Pomaria sandersonii*. The M⁺ of 282.28 (100%) appeared on the TOF MS-MS ESI spectrum. The NMR and Ms spectra are in Appendix 2. The fragment of ¹³C NMR displayed 15 carbons. DEPT 135 did not detect any CH₂ carbons. The carbon resonance at δ 168.5 ppm is an indication of a keto carbon (β'-C) (Katerere 2000; Adesanwo 2009). The other quaternary carbons with δ 157.8 C-2'; 157.0 C-4'; 151.6 C-6' and 129.8 C-1 because they are not detected by the DEPT 135 as displayed in figure 61 (in the appendix) although the same are on the ¹³C NMR spectra. HMQC correlations have a ¹J coupling are in table 28. HMBC correlations display that the α-carbon resonating at 41.8ppm ¹J coupling with the proton δ 4.55, *t* and protons δ 3.18, *dd*, (J -3.5, 2.0)H couple with carbon at 128.7 ppm. The resonance of the α-C and β-H is unusual for a simple chalcone and implies that there is no double bond between the chalcone rings. The ¹J correlation of α-H 3.18, *dd*, (J -3.5, 2.0) proton with the keto carbon (β'-C) and the β proton 4.55, *t* with the α-C 43.1 as displayed by the HMBC spectrum implies that EM 86 is a dimer. This also confirmed by a MSMS ESI fragment at *m/z* 522 (35%). Table 28 gives a summary of all the ¹³C NMR and ¹H NMR data δC(ppm) δH(ppm) and ²J/³J from HMBC and COSY for EM 80-2 and EM 86.

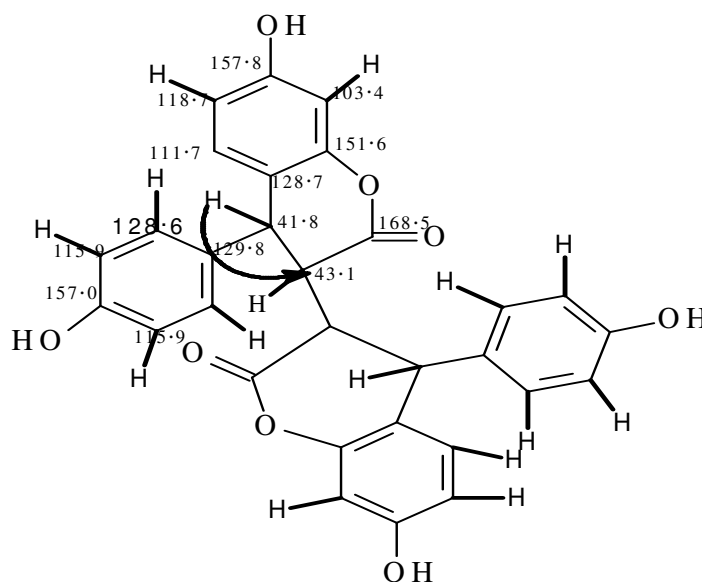


Figure 45: Significant HMBC correlations for EM 86

7.3.1.4 Compounds from *Alepidea amatymbica*

Five diterpenoids were isolated in this investigation from a hexane fraction of the *Alepidea amatymbica* root. A comparison to known diterpenes as proposed by *ent*- 2 β - acetohydroxykaur-16-en-19-oic acid (Langat *et al.* 2012), kaurenoic acid and its derivatives (Ronan *et al.* 2007), *ent*- 15 α -hydroxy-*ent*-kaur-16-en-19-oic acid (Somova *et al.* 2001), 12 α -hydroxy-*ent*-16-en-19-oic acid (Ortega *et al.* 1985) and *ent*-15 β -acetoxo(-)-kaur-16-en-19-oic acid (Cannon *et al.* 1965) in combination with NMR and mass spectra of the five compounds enabled structure elucidation. The NMR and MS spectra of the diterpenes are in Appendix 3-7. The ¹H NMR spectra of each of the five diterpenes isolated are diagnostic with δ_{H} 1.12 to 1.28 ppm for C-18 and δ_{H} 1.55 to 0.88 for the C- 20 methyl singlets. The ¹³C NMR showed 20 carbon resonance including exocyclic alkene C-16 resonances δ 152.0 to 155.8 with the exception of 06-2 which had an oxymethine carbon resonance at δ 82.5 s (C-16) and a methyl C-17 δ 28.5. For the rest of the four diterpenes, C-17 for 06-B, 06-52, 06431 and 0657 resonated between δ 102.5 and 107.0. These resonances for the four terpenes are characteristic of the exocyclic double bonds in a diterpenes on C16 and C-17 (Langat *et al.* 2012). The ¹³C NMR displayed C- 19 carboxylic δ 178.2 to 180.4 which is typical of kaurenoic acids (Ronan *et al.* 2007).

7.3.1.5 Characterisation of 06B

06B is colourless crystalline solid displaying melting point of 215-216 °C. M⁺ *m/z* = 318 which is consistent with molecular formula of C₂₀H₃₁O₃. Table 27 summarises NMR spectra structural elucidation for 06B. The NMR and MS spectra are in Appendix 3. The low resolution ESI MS displayed *m/z* fragments consistent with those proposed in Figure 46. The ¹³C NMR showed 20 carbon resonance including exocyclic alkene carbon resonances at δ 155.8 (C-16) and at 102.5 (C-17) (Vieira *et al.* 2002) a carbonyl resonance at 178.6 ppm (Pavia *et al.* 2009), an oxymethine carbon resonance at δ 73.4 (C-13) which is typical of a kaurenoic acid (Langat *et al.* 2012; Ortega *et al.* 1985). The ¹H NMR showed presence of two methyl groups with proton resonance at δ 1.20 ppm s, 3H-20 and δ 1.13 ppm 3H-18. Placement of the OH group on C-13 was as a result of the HMBC correlation by the C-11 protons (δ 1.31 *m*) and C-12 protons (δ 1.92 *m*) with the quaternary carbon C-13 (δ 76.4), as displayed by the HMBC spectrum, as a result of ²J/³J coupling summarised in table 7.16. The COSY spectrum indicates that proton δ 4.72, s(C-17) coupled with the protons δ 1.64-1.66, *m*, 2H (C-12). The two singlet protons δ 2.95 s, 2.89 ppm, s 2H (C-14) also exhibited coupling with δ 1.64-1.66, *m*, 2H (C-12) and also with δ 2.24, *d* (*J* = 2.7) 2H (C-15) on the COSY spectrum. The COSY spectrum also displays correlation between δ 2.61 ppm, *m*, 2H (C-9) with δ 1.20 s (CH₃) on C-20. The protons δ 2.24, *d* (*J* = 2.7) 2H (C-15). 06B should therefore be 13-hydroxy-kaur-16-en-19-oic acid. The stereochemistry of the compound was not determined.

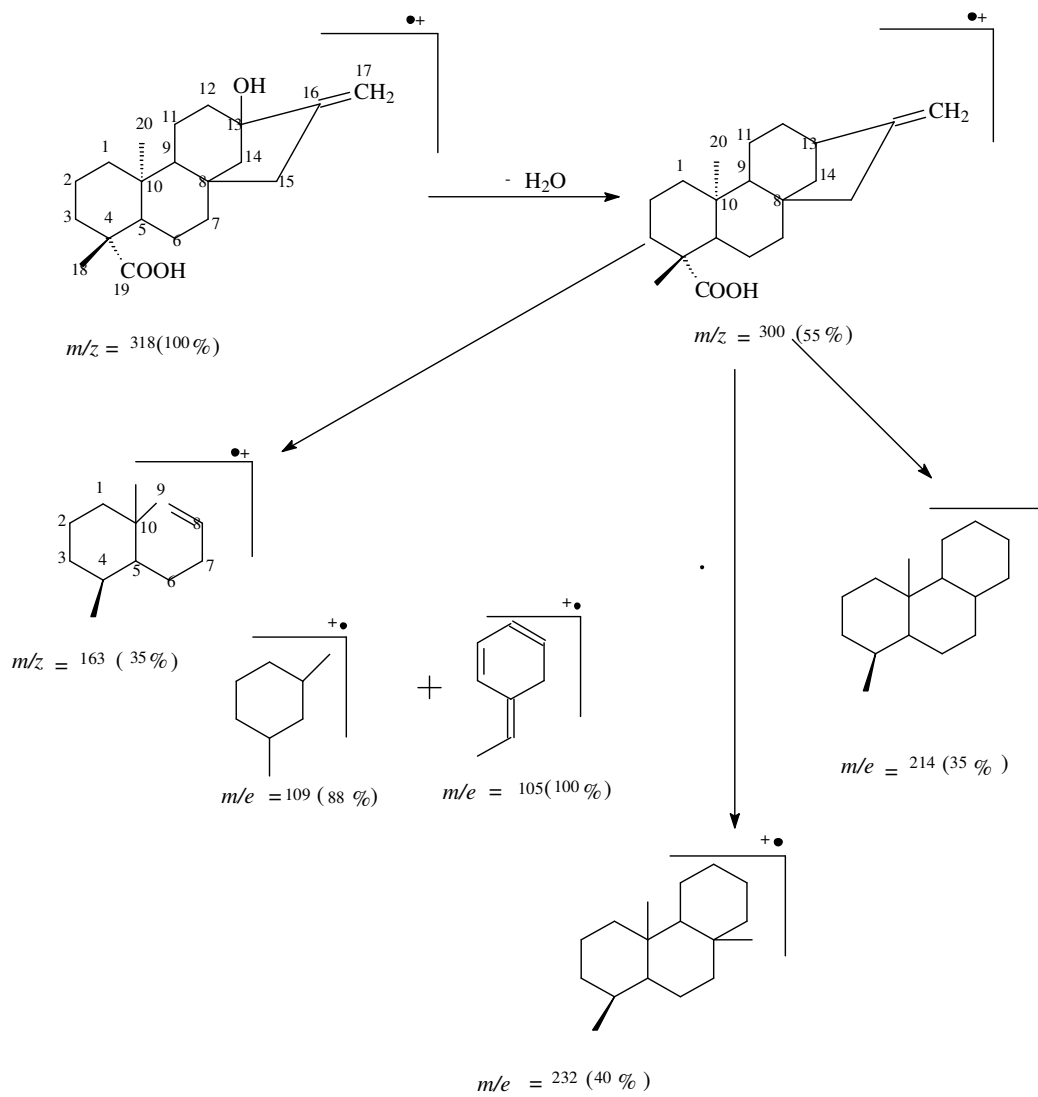


Figure 46: Suggested fragmentation pattern for 06B

Table 27: ¹³C NMR (300MHz) and ¹H NMR (300MHz) data δC (ppm) δH (ppm) and ²J/³J from HMBC and COSY for 06-2 and 06B in CDCl₃

C	06-2		06B			
	δH (ppm)	² J/ ³ J	δH(ppm)	² J/ ³ J		
1	41.3	1.79, (m), 2H	55.5, 82.5	40.4	2.24, d (J= 2.7) 2H	
2	19.0	1.39, (m), 2H	61.3, 43.3	19.1	1.20, m, 2H	49.4, 43.3, 37.8
3	39.8	1.87, (m), 2H		37.8	1.75, m, 2H	
4	43.3	-	-	49.4	-	-
5	55.5	1.15, (s), 1H	178.3, 61.3, 38.6, 43.3,	49.3	1.75, m, 1H	17.3, 28.6, 34.3, 32.2
6	126.5	5.55, (dd), (J = 2.7Hz), 1H	61.3, 49.9, 43.3	32.2	1.92, m, 2H	76.4, 42.5
7	133	5.94, (t), (J = 7.2 Hz), 1H	61.3, 49.9, 37.9	34.3	1.20-1.75, m, 2H	76.4, 43.9, 42.5
8	39.4	-	-	43.9	-	-
9	61.3	1.44, (m), 1H		42.5	2.61, m, 2H	34.3,
10	38.6	-	-	43.3	-	-
11	34.0	1.75, (m), 2H		21.9	1.87, m, 2H	
12	21.9	1.80, (d) (J= 1.5Hz), 2H	28.5q	28.9	1.64-1.66, m 2H	
13	49.9	2.19, (d), (J=2.4Hz),	133.0, 126.5, 82.5, 61.3, 41.3	76.4	-	-
14	37.9	2.16, (s), 2H	133.0, 126.5, 82.5, 58.6, 41.3	36.1	1.31, m, 2H	76.4, 43.9, 28.9, 17.3
15	58.6	1.38-1.53, (m), 2H	133.0, 82.5, 61.3, 34.0	43.8	2.95 s, 2.89, s 2H	
16	82.5	-	-	155.8	-	-
17	28.5	1.25, (s), (CH3)	82.5, 58.6, 49.9	102.5	4.78, s, 4.72, s 2H	
18	25.3	1.20, (s), (CH3)	178.3, 55.5, 39.8, 43.3, 34.0	28.6	1.20, s(CH3)	49.3, 43.9, 37.8, 178.6, 36.1
19	178.3	-	-	178.6	-	-
20	15.3	0.88, (s), (CH3)	55.5, 41.3, 38.6	17.3	1.13, s(CH3)	32.2, 43.9, 49.3, 76.4

7.3.1.6 Characterisation of 06-2

06-2 was a white crystalline acetone soluble compound. A mass of 211.5 mg of 06-2 was eluted with 100:6 hexane ethyl acetate solvent polarities. Its melting point range is 230-232 °C. Table 27 summarises NMR spectra structural elucidation for 06-2. The NMR and MS spectra are in Appendix 4. The MS data M+1 peak was found to be 318 but M+ 1 peak at 301 was also observed because under EI conditions alcohols dehydrate to give a peak at 300 as proposed in figure 46. The ¹³C NMR showed 20 carbon resonance including alkene carbon resonances at δ 126.4 (C-6) and at 133.0 (C-7), a carbonyl resonance at 178.3-ppm, an oxymethine carbon resonance at δ 82.5 (C-16) which are the characteristics of a kaurenoic acid (Langat *et al.* 2012). The ¹H NMR showed the presence of three methyl groups with proton resonances at δ 0.88(s, 3H-20), δ1.25 (s 3H-18), and δ1.93 (s 3H-17). DEPT 135 displayed seven methylene carbons, methyl carbons and five methine carbons. The proton on C6 δ 5.55, (*J* = 2.7Hz), 1H and C7 δ 5.94, (*t*), (*J* = 7.2 Hz), 1H are deshielded by the π bond between the two carbons. Placement of the OH group on C-16 was as a result of coupling displayed by the HMBC spectrum with the methyl protons on C17 (δ1.93 s 3H-17). HMBC spectrum also showed coupling of proton (δ 0.88 Hz H-18) ³*J* correlation with the tertiary carbon C19 δ 178.3. The double bond placement on C-6 is justified by the COSY spectrum that displayed a ³*J* proton correlation between the C 7 proton δ 5.94, (*t*), (*J* = 7.2 Hz), 1H C-7) with C 14 protons δ 2.16, (*s*), 2H. Another COSY ³*J* correlation with protons on C-7 is with proton on-C-5 δ 1.44, (*m*), 1H and ²*J* correlation with δ 5.55, (*dd*), (*J* = 2.7Hz), 1H-(C-6) hence the double bond is between C-6 (δ 126.5) and C 7 (δ 133.0). Similar kaurenoic acids have been reported on before because Somova *et al.* (2001) synthesised 15α-hydroxy-*ent*- kaur-16-en-19oic acid from xylopic acid and Ortega *et al.* (1985) isolated *ent*-12β-hydroxykaur-16-en-19-oic acid from *Stevia eupatoria*. The compound 06-2 is 16- hydroxy-kaur-6- en-19-oic-acid.

7.3.1.7 Characterisation of 0652

EM0652 is white crystalline solid soluble in chloroform with a melting point range of 219-220 °C. A mass of 103.9 mg was eluted from the column with solvent 100: 2 Hexane: ethyl acetate. . Table 28 summarises NMR spectra structural elucidation for 0652. The NMR and MS spectra are in Appendix 5. The ¹³C NMR displayed 22 carbon resonance including exocyclic alkene carbon resonances at δ 155.1, *d* (C-16) and at 103.2 *d* (C-17), a carbonyl resonance at 170.1, an oxymethine carbon resonance at δ 69.4 ppm *s* (C-14). The ¹H NMR displayed presence of three methyl groups with proton resonance at δ 1.23 *s*, 3H-18 and δ 0.94 3H-18 and the relatively deshielded δ 1.98 3H-22 because of the presence of the carbonyl group at C-21. Placement of the O-COCH₃ group on C-14 was as a result of its proton ³*J* coupling with the C-17 (δ 103.2) and also the C-13 proton (δ 2.68, *d*) displaying a ¹*J* coupling with the C-17 (δ 103.2), as confirmed by the HMBC spectrum. The COSY spectrum indicates that proton δ 4.73, *s* (C-17) is coupled with the protons δ 1.98 *s*, 3H (C-22). The M⁺ of 359 confirms the assignment of the molecule. In comparison to Somova *et al.* (2001) who isolated 11α- acetoxo *ent*- kaur-16-en-19-oic acid, 0652 is its isomer since they only differ by the position of the acetoxo group. The name of 0652 is

14 α - acetoxy *ent*- kaur-16-en-19-oic acid or xylopic acid (Somova *et al.* 2001). Xylopic acid was isolated from *Xylopia aethiopica* (Somova *et al.* 2001).

Table 28: ¹³C NMR (300MHz) and ¹H NMR (300MHz) data δC (ppm) δH(ppm) and ²J/³J from HMBC and COSY for 06431, 0657 and 0652 in CDCl₃

C	06431			0657			0652		
		δ H-(ppm)	λJ/λJ		δH (ppm)	λJ/λJ		δ H-(ppm)	λJ/λJ
1	43.5	1.57-1.75 (m), 2 H	55.6, 36.7, 36.5	41.9	2.37, m, 2H	152.0, 42.2, 33.5,32.7	39.2	1.88, m, 2H	56.9, 47.8, 37.7,
2	19.6	1.61, (m) 2H		33.5	1.71, m, 2H	32.7, 21.9, 41.9	19.0	1.98,m 2H	183.7
3	36.7	1.40-1.70 (m), 2H		32.7	1.51, m, 2H		41.1	1.63, m 2H	
4	48.3	-		30.9	-		38.6	-	-
5	55.6	1.66 (m), 2H		48.6	2.19-2.52, s1H	213.2	56.9	1.15, t, 1H	60.9,15.4.,39.2, 19.0
6	35.9	2.15-2.35 (m),2H	55.6, 48.3,	33.7	1.83, m, (J=2.7Hz) 2H		21.7	1.90, m, 2H	155.1
7	41.8	1.40-1.60 (m), 2H		33.0	1.48-2.19, m, 2H	21.9, 41.9	37.7	2.18, m, 2H	21.7,
8	56.9	-		57.0	-		43.8	-	-
9	84.9	-		85.5	-		60.9	1.33, t, 1H	69.4, 56.9, 47.8 43.8, 38.6, 15.4
10	22.0	-		52.5	-		43.1	-	-
11	36.5	1.50 (m) 2H	36.7	36.0	2.13-2.52, m, 2H	170.2	40.2	1.01, m, 2H	15.4
12	32.8	2.20-2.40, (m) 2H	152.5	213.2	-		47.8	2.56, m, 2H	43.8
13	42.1	2.77, (s) 1H		42.2	2.89, s 1H,		42.2	2.68, d 1H	60.9, 103.2
14	212.9	-		73.0	4.92, d,(J =3.0Hz)1H	15.5	69.4	5.09,t, (J= 2.7 Hz) ,1H	103.2
15	33.6	1.75(d) (J= 3.6 Hz) 2H	56.9	43.6	1.83, d, (J= 2.7Hz) 2H		39.5	1.24,m,2H	15.4,
16	152.0	-		152.0	-		155.1	-	-
17	106.9	4.90, (s);5.0 (s) 2H	33.6, 41.8, 42.1,	107.0	5.08 s, 4.99, s 2H	42.2,	103.2	4.73, d 2H	42.2
18	22.0	1.12 3H (s)	33.6, 33.7, 48.3, 55.6, 180.4	21.9	1.22, s, (CH3)	178.2, 73.0, 52.5, 48.6	29.0	1.28, s, (CH3)	15.4, 69.4, 56.9, 42.3, 37.7
19	180.4	-		178.2	-		183.7	-	-
20	18.0	1.39 3H (s)	55.6, 43.5, 33.6, 33.7, 84.9	15.5	1.55, s, (CH3)	32.7, 48.6, 85.5	15.4	0.94, s (CH3)	56.9, 39.2,60.9
21				170.2	-		170.1	-	-
22				21.2	2.19, s, (CH3)	213.2	21.6	1.98, s, (CH3)	

7.3.1.8 Characterisation of 06431

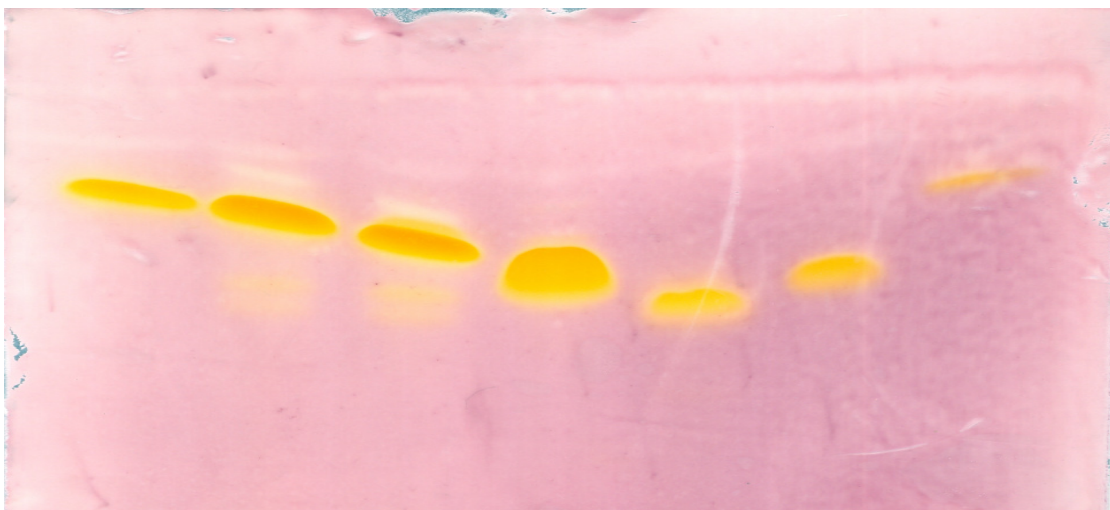
06431 is a brown waxy liquid. The M+1 peak from the MS-MS of m/z 317 implied the molecular formula of $C_{20}H_{28}O_3$. Table 30 summarises NMR spectra structural elucidation for 06431. The NMR and MS spectra are in Appendix 6. The ^{13}C NMR showed 20 carbon resonance including alkene carbon resonances at δ 106.9 (C-17) and at 152.0, a carbonyl resonance at 180.4, resonance typical of an oxymethine carbon at δ 84.9 (C-9) (Langat *et al.*, 2012). The 1H NMR displayed presence of two methyl groups with proton resonance at δ 1.39 (s, 3H-20) and δ 1.20 (s, 3H-18) and exocyclic proton resonances (4.90, s, 5.0, s, 2H-17). HMBC spectrum also displayed correlation of C-18 proton (δ 1.12) coupling with C-2, (δ 36.5) and C-3 (δ 36.7) placing the C-20 methyl group on quaternary C-10. The position of the keto group on C 14 was based on the COSY spectrum which exhibited a correlation between C-17 protons (δ 4.90, (s) and 5.0 (s) coupling with C-12 protons (δ 2.20-2.40, *m*) and the C13 proton (δ 2.77) with C-15 proton (δ 1.75, *d*) ($J = 3.6$ Hz). The placement of the OH group on C-9, δ 84.9 was based on its correlation with protons on C-20 (δ 1.39, s) as displayed by the HMBC spectrum. A summary of all the 1J , 2J and 3J coupling is summarised in Table 28. An isomeric kaurenoic acid 15 β -Acetoxy-(-)-kaur-16-en-19-oic acid has been synthesised before by acetylation of 15 β -Hydroxy-(-)-kaur-16-en-19-oic acid (Cannon *et al.* 1965). Somova *et al.* (2001) also isolated another isomer *ent* -15-oxkaur-16-en-19-oic acid from *Xylopiia aethiopa*. Although the stereochemistry was not determined 06431 is 14-oxokaur-16-en-19-oic acid. This is the first time the acetate of kaurenoic acid has been isolated from *Alepidea amatymbica*. Langat *et al.* (2012) synthesised *ent*-14-oxokaur-16-en-19-oic acid by Jones oxidation of *ent*-14S*-hydroxykaur-16-en-19-oic acid.

7.3.1.9 Characterisation of 0657

0657 is a brown waxy solid, and acetone soluble. A mass of 64.2 mg was isolated from the column chromatography with solvent hexane: ethyl acetate ratio 1000:30 polarity. The M+ 1 peak of m/z 358 confirms the mass of the compound to be $C_{22}H_{30}O_4$. Table 28 summarises NMR spectra structural elucidation for 0657. The NMR and MS spectra are in Appendix 7. The ^{13}C NMR showed 22 carbon resonance including exocyclic alkene carbon resonances at δ 152.0 ppm (C-16) and at 107.0 ppm (C-17), two carbonyl resonances at δ 178.2 and δ 170.2, typical of an oxymethine carbon resonance at δ 85.5 (C-9). The 1H NMR showed presence of three methyl groups with proton resonances at δ 2.19 s, 3H-22; δ 1.22 ppm 3H-20 and 1.55, s, 3H-18. According to Somova *et al.* (2001); Cannon *et al.* (1965); Langat *et al.* (2012) and other publications on kaurenoid isolation, the diterpenoid 0657 is a new compound because it contains both the acetate group on C14 and a keto group on C12. The name proposed for 0657 is *ent*-14 acetoxo12-oxokaur-16-en-19-oic acid.

7.4 BIOAUTOGRAPHY

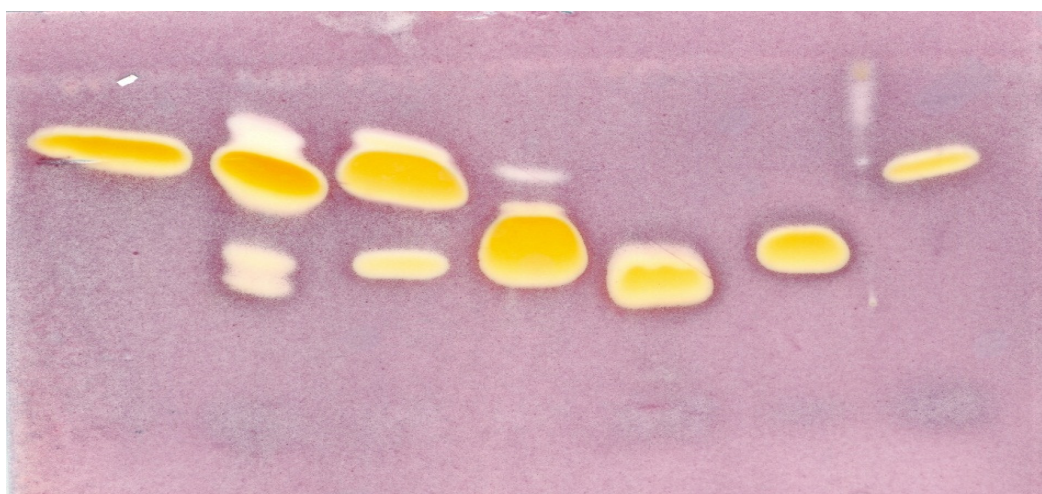
Bioautography results indicated that the *Pomaria sandersonii* fractions and pure compounds were active against *E. coli* and *S. aureus*, Figure 47.



EM 80-2

EM49 EM77

Figure 47: Bioautography to illustrate activity against *Escherichia coli* by EM 80-2 and mixtures from *Pomaria sandersonii*



EM80-2

EM49 EM77

Figure 48: Bioautography to illustrate activity against *Staphylococcus aureus* by EM 80-2 and mixtures from *Pomaria sandersonii*

7.4.1 Minimum inhibitory concentration for pure compounds and mixtures

Minimum inhibitory concentration against *Staphylococcus aureus*, ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Escherichia coli*, ATCC25922 *Candida albicans*, (*C. a.*), *Cryptococcus neoformans* (*C. n.*) and *Pseudomonas aeruginosa* (*P. a.*) results are displayed in Table 29. *Pomaria sandersonii* EM80-2 had the highest total activity among the pure compounds (1665 µg/ml) against all the organisms tested. It is worth noting that EM80-2 displayed high activity with 160 µg/ml against *Staphylococcus aureus*, *Enterococcus faecalis*, *Cryptococcus neoformans* respectively and 80 µg/ml against *Candida albicans*. However, it was the mixture EM49 which displayed highest activity against all organisms (1040 µg/ml) in this study. The least active was compound 0645, which was a mixture of diterpenes from *Alepidea amatymbica* with total activity of 3020 µg/ml against all organisms tested.

Compound 06B had the highest antifungal activity against *Aspergillus fumigatus* 40 µg/ml. The mixture EM77 demonstrated highest activity against *Cryptococcus neoformans* of 80 µg/ml. Compounds separated from *Pomaria sandersonii* demonstrated high anti-microbial activity (80-620 µg/ml), making it a more active plant than *Alepidea amatymbica*.

Table 29: Antimicrobial activities of the pure compounds and mixtures from *Alepidea amatymbica* and *Pomaria sandersonii* expressed as MIC (µg/ml)

Sample	<i>E. c.</i>	<i>S. a.</i>	<i>E. .f.</i>	<i>P. a</i>	<i>C. a.</i>	<i>C. n.</i>	<i>A. f.</i>	<i>T. activity</i>
06-2	620	320	125	620	320	620	250	2875
0645	250	250	625	125	625	500	625	3020
06-B	125	160	320	500	125	500	40	1770
EM80-2	160	160	160	160	80	320	625	1665
EM49	320	80	80	320	160	80	320	1365
EM77	80	320	160	625	625	80	320	2130
+ve cntrl	0.4	0.4	0.4	500	125	250	0.6	
-ve cntrl	500	500	500	500	500	500	500	

+ve control, (cntrl) for bacteria gentamycin and amphotericin B for fungi activity assay; -ve control, (cntrl) was acetone. T. activity = Total activity

7.5 DISCUSSION

Alepidea amatymbica has shown that its root hexane fraction is rich in diterpenes because five pure compounds from the diterpenoids family were isolated. Although diterpene kaurene derivatives were isolated before from the same plant (Somova *et. al.* 2001), this study also isolated closely related compounds. Compounds, 13-hydroxy-kaur-16-en-19-

oic acid (06B), which is also isomeric to the known kaurenoic acid, 12 α -hydroxy-ent-kaur-16-en-19-oic acid, whose common name is steviol (Ortega *et al.* 1985).and 13-hydroxy-kaur-16-en-19-oic acid (06B) have been isolated for the first time from *Alepidea amatymbica* in this study. Steviol was reported first by Bridal & Lavieille (1931) isolated from *Stevia rebaudiana* (Bertoni) and from *Stevia eupatoria* (Ortega *et al.* 1985).

The microdilution assay demonstrated high antimicrobial activity by 13-hydroxy-kaur-16-en-19-oic acid (06B), C₂₀H₃₁O₃ 40 μ g/ml against *Aspergillus fumigatus* to low activity of 500 μ g/ml against *Cryptococcus neoformans* and *Pseudomonas aeruginosa* respectively. 16-hydroxy-kaur-6-en-19-oic acid (06-2) was active against tested bacteria and fungi with the MIC: *Staphylococcus aureus* (320 μ g/ml) and *Candida albicans*, (320 μ g/ml). Naturally occurring kaurene derivatives also occur abundantly in other plants such as *Wedelia paludosa* D.C and *Xylopi frutscens* AUE (Garcia *et al.* 2007). Kaurenoic acid, which has a similar basic kaurenoid structure to 06B and 06-2 are reported to have biological activities, including antimicrobial, cytotoxic anti-inflammatory and antiprotozoal properties (Vieira *et al.* 2002).

Other investigations carried out with closely related kaurenoic diterpenes from *Alepidea amatymbica* and *Xylopi aethiopica* were found to also display significant systemic hypotensive and coronary vasodilatory effect accompanied with bradycardia (heart rate under 60 beats per minute in adult human) (Somova *et al.* 2001). The other three diterpenes 0652, 06431 and 06431 biological activities were not determined in this study due to lack of resources by the time that they were isolated.

EM80-2 is 1-(2, 4-dihydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one also known as isoliquiritigenin is a chalcone isolated from *Pomaria sandersonii*. Isoliquiritigenin has been isolated before from licorice (*Glycyrrhiza uralensis*), shallot, *Sinofranchetia chinensis*, *Dalbergia odorifera*, and soybean (Cuendet *et al.* 2010). During this investigation, EM80-2 also exhibited high antibacterial activity in the range of 80-160 μ g/ml against *Staphylococcus aureus*, ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, and *Escherichia coli*, ATCC25922. Isoliquiritigenin has been reported before to exhibit potent antioxidant, anti-inflammatory, phytoestrogenic, tyrosinase inhibitory and exhibited significant inhibitory effects of carcinogenesis in various tumour models (Cuendet *et al.* 2010). Isoliquiritigenin has also been reported before to exhibit antibacterial activity against *Staphylococcus aureus*, *Streptococcus mutans*, and *Actinomyces naeslundii* with inhibitory activity ranging 15.6-62.5 μ g/mL (Oldoni *et al.* 2011). This is the first time that isoliquiritigenin was isolated from *Pomaria sandersonii*.

EM86 appears to be a dimer of EM80-2. Its biological activity was not determined because of the lack of resources at the time of isolation. There were two mixtures referred to as EM49 and EM77 from *Pomaria sandersonii* which were difficult to purify but displayed high anti-microbial inhibitory activities worth reporting. EM 49 displayed MIC of *Candida albicans*: 160 μ g/ml; *Pseudomonas aeruginosa*: 320 μ g/ml, *Escherichia coli*: 80 μ g/ml, *Enterococcus faecalis* 80 μ g/ml, and *Staphylococcus aureus*: 80 μ g/ml and *Aspergillus fumigators*: 320 μ g/ml. EM77 displayed MIC of *Escherichia coli*: 80 μ g/ml and *Cryptococcus neoformans* 80 μ g/ml. Further work on their purification need to be done since in this research we are just reporting on their high MIC activities

7.6 CONCLUSION

Total of seven compounds were isolated from *Alepidea amatymbica* and *Pomaria sandersonii*. These were two diterpenes from *Alepidea amatymbica*, namely, 14-acetoxo-12-oxokaur-16-en-19-oic acid labelled as 0657 and 16-hydroxy-kaur-6-en-19-oic acid (06-2) in this study. The third suspected new compound is the chalcone dimer, which is referred to as EM86 in this study from *Pomaria sandersonii*. EM80-2 was obtained as a mixture of the cis and trans of 2', 4, 4',-trihydroxychalcone or 1-(2,4-dihydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one, from *Pomaria sandersonii*. The name of 0652 is xylopic acid, which is also known as 14-acetoxokaur-16-en-19-oic acid, 06B is 13-hydroxy-16-kauren-19-oic acid and 06431 is 14-oxokaur-16-en-19-oic acid which were isolated from *Alepidea amatymbica* for the first time in this study. 0652 is not a new compound because Langat *et al.* (2012) synthesised ent-14-oxokaur-16-en-19-oic acid from ent-14-hydroxykaur-16-en-19-oic acid by Jones oxidation. There is no report that has been made of the kaurenoic acid being isolated from a plant before.

13-hydroxy-kaur-16-en-19-oic acid, C₂₀ H₃₁ O₃ which is isomeric to the known 12 α -hydroxy-ent-kaur-16-en-19-oic acid, steviol has been isolated from *Alepidea amatymbica* for the first time. Somova *et al.* (2001), synthesised 15 α -hydroxy-ent-kaur-16-en-19-oic acid from xylopic acid. The plant root powders of *Alepidea amatymbica* and *Pomaria sandersonii* contain compounds with antimicrobial activities. They can be used as a source for pure compounds that are being used as lead compounds in drug discovery.

CHAPTER 8

Cytotoxicity determination of crude acetone extracts of *Alepidea Amatymbica* and *Pomaria sandersonii*

8.1 INTRODUCTION

The use of medicinal plants to control various disease conditions creates the necessity to assess their cytotoxicity levels. In vitro assessments are normally performed in the initial assessments to ensure informed plant use. There are various methods used for in vitro assessments such as MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazolium salt), XTT, (sodium 3-[1(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitrobenzene sulfonic acid hydrate), Trypan blue (TB), SRB, (Sulforhodamine,B) and the clonogenic assay (Mosmann 1983; Berridge *et al.* 1996).

The MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) reduction assay is one of the most frequently used methods for measuring cell proliferation and cytotoxicity (Mosmann 1983). The reaction for monitoring cytotoxicity levels is based on the fact that tetrazolium salts such as MTT which is yellow, are metabolically reduced to highly coloured formazans by enzymes present in living cells called microsomal NADPH-cytochrome P450 reductase (CPR) (Berridge *et al.* 1996).

The reaction is summarised by the scheme in Figure 49. The intensity of colour (measured spectrophotometrically) of the MTT formazan produced by living, metabolically active cells is proportional to the number of live cells present (Mosmann 1983). In the Phytomedicine Laboratory, University of Pretoria, viable cell growth after incubation with the test compound is determined using the tetrazolium-based colorimetric assay (MTT assay) described by Mosmann (1983).

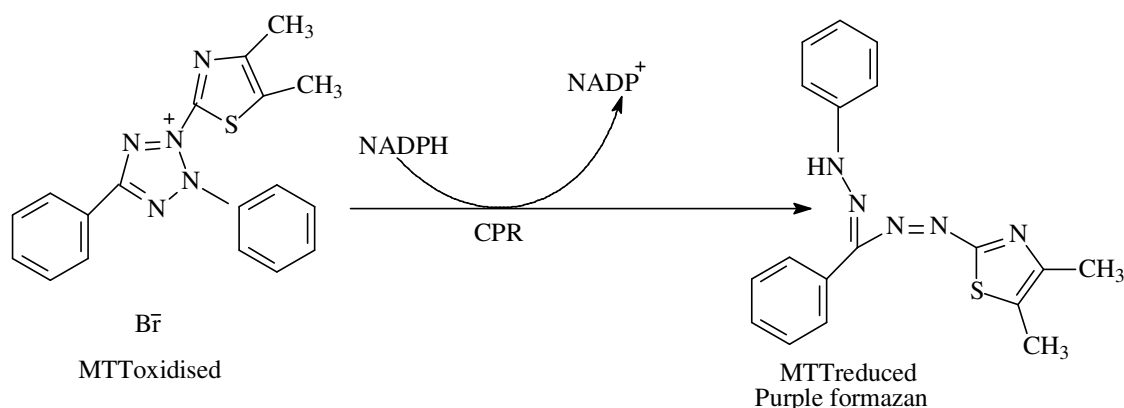


Figure 49: Reduction of yellow tetrazolium salt MTT% to purple formazan

8.2 MATERIALS AND METHODS

8.2.1 Plant Material

The roots of *Alepidea amatymbica* and *Pomaria sandersonii* were collected from Mabandla village in Kwazulu-Natal as described in section 3.4.3.

8.2.2 Preparation of crude extracts

The crude acetone extracts preparation was carried out as described in section 3.4.4.

8.2.3 CYTOTOXICITY ASSAY

Cells of a sub-confluent culture were harvested and centrifuged at 200 rpm for 5 min, and re-suspended in growth medium to 5×10^4 cells/ml. The growth medium used was Minimal Essential Medium (MEM, Sigma) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Sigma). A total of 200 μ l of the cell suspension were pipetted into each well of columns 2 to 11 of a sterile 96-well micro titre plate. Growth medium (200 μ l) was added to wells of columns 1 and 12 to minimize the “edge effect” and maintain humidity. The plates were incubated for 24hr at 37 °C in a 5% CO₂ incubator, until the cells are in the exponential phase of growth. The MEM was aspirated from the cells, and replaced with 200 μ l of test compound at differing concentrations (serial dilution prepared in growth medium). The cells were disturbed as little as possible during the aspiration of medium and addition of test compound. Each dilution was tested in quadruplicate. The micro-titre plates were incubated at 37 °C in a 5% CO₂ incubator for a defined contact period of two days with 10 mg/ml acetone extract. Untreated cells and positive control (doxorubicin, Pfizer Laboratories) were included.

After incubation, 30 μ l MTT (Sigma, stock solution of 5 mg/ml in PBS) was added to each well and the plates were incubated for a further 4hr at 37 °C. After incubation with MTT the medium in each well was carefully removed, without disturbing the MTT crystals in the wells. The MTT formazan crystals were dissolved by adding 50 μ l DMSO to each well. The plates were shaken gently until the MTT solution was dissolved. The amount of MTT reduction was measured immediately by detecting absorbance in a micro -plate reader (Versamax, Molecular Devices) at a wavelength of 570 nm and a reference wavelength of 630 nm. The wells in column 1, containing medium and MTT but no cells were used to blank the plate reader. The LC₅₀ values were calculated as the concentration of test compound resulting in a 50% reduction of absorbance compared to untreated cells.

8.3 RESULTS

8.3.1 CYTOTOXICITY ASSAY

The results of the cytotoxicity assays carried out on bovine dermis and monkey vero cells are displayed in Table 30. Results demonstrate that although they crude extracts are relatively not cytotoxic, *Alepidea amatymbica* (42.086 µg/ml) is relatively more cytotoxic to bovine dermis cells than *Pomaria sandersonii* (51.794 µg/ml) and a reverse trend was displayed when monkey vero cells were used (Table 30)

Table 30: Two day MTT assay 50 000 cells/ml on acetone crude extracts

Bovine dermis cells	Assay 1 EC₅₀ (µg/ml)	Assay 2 EC₅₀ (µg/ml)	Average EC₅₀ (µg/ml)	SD
<i>Alepidea amatymbica</i>	39.144	45.029	42.086	4.161
<i>Pomaria sandersonii</i>	47.111	56.476	51.794	6.622
Doxorubicin (+ control)	8.617	9.770	9.193	0.815
Vero cells				
<i>Alepidea amatymbica</i>	84.289	71.792	78.041	8.836
<i>Pomaria sandersonii</i>	39.197	37.974	38.586	0.865
Doxorubicin (+ control)	17.083	16.947	17.015	0.096

8.4 DISCUSSION

Crude extract with EC₅₀ less than 20 µg /ml are considered cytotoxic by the American National Cancer Institute (NCI) (Joshi *et al.* 2011). Both crude acetone extracts have much lower toxicity than 20 µg/ml. Both bovine dermis and monkey vero cell cytotoxicity assays demonstrated that crude acetone extracts of *Pomaria sandersonii* and *Alepidea amatymbica* displayed much lower toxicity levels when compared to that of doxorubicin. Doxorubicin is antibiotic used in the treatment of various types of cancers (Tokarska-Schlattner *et al.* 2005). The drug kills both normal and cancer cells which characterises its undesirable side effect during the treatment of cancer patients (Tokarska-Schlattner *et al.* 2005).

8.5 CONCLUSION

Acetone crude extracts demonstrated that they can be used as drugs because they are both antimicrobial and not cytotoxic when compared to some drugs used to treat cancer such as doxorubicin.

CHAPTER 9

Isolation and identification of sugars from *Pentanisia prunelloides* and *Gunnera perpensa*.

9.1 INTRODUCTION

P. prunelloides and *G. perpensa* in combination with a number of different plants such as *Agapanthus africanus* (L.) Hoffmg., *Asclepias frutcosa* L., *Callilepis laureola* L., *Clivia minita* (Lindl) Regel, *Combretum erythrophyllum*, *Crinum* sp., *Rhoicissus tridentata* (L. f.) Wild and Drum, *Scadoxus puniceus* (L.) Fris and Nordal, *Typha capensis* (Rorb) N.E. Br and *Vernonia neocorymbosa* Hilliard are used as ingredients to produce a decoction called *Isihlambezo* by Zulu traditional practitioners (Varga & Veal 1997). *Isihlambezo* is prescribed by traditional healers in South Africa as herbal remedy during pregnancy to initiate contractions, facilitating expulsion of the placenta and cleaning of the womb after birth and prevent post-partum haemorrhage (Varga & Veal 1997). *G. perpensa* is also prescribed to alleviate indigestion during pregnancy while *P. prunelloides* is used in the treatment of pregnancy-related infection (Varga & Veal 1997). Leaf and root extracts of *P. prunelloides* and *G. perpensa* are also used to relieve inflammation, bacterial and viral infections including (Yff *et al.* 2002). Active compounds in *P. prunelloides* responsible for the antibacterial activity are listed in Table1. *G. perpensa* has exhibited both antibacterial and antifungal activity as displayed in Table1. Phytochemical screening of *G. perpensa* displayed presence of alkaloids, flavonoids, steroids, saponins, tannins and glycosides (Simelane *et al.* 2010). Varga & Veal also reported that the toxicity of *Isihlambezo* has not been reported although some traditional healers ascribe a number of foetal and maternal healths to the decoction. Root extracts of the two plants are also some of the herbal treatments employed in Zulu traditional medicine to treat dysmenorrhoea (Steenkamp 2003). Roots extracts of *P. prunelloides* root and leaf have displayed COX- 1 inhibition (Yff *et al.* 2002). *G. perpensa* extracts have demonstrated DPPH and ABTS radical scavenging (78% and 78.45% respectively) (Simelane *et al.* 2010). The antioxidant and anti-inflammatory capability contributes to *G. perpensa*'s ability to treat pain related conditions.

9.2. MATERIALS AND METHODS.

9.2.1. Isolation of crude extract.

A mass of 200 g of plant extract was extracted with 2-litres methanol by soxhlet for four hours. The extract solution was then concentrated by rotary vapour under vacuum. The extract was then air dried under a fan at room temperature. Fractions were prepared as reported in section 3.3.4.3. For *Pentanisia prunelloides* and *Gunnera perpensa*, sucrose and glucose were precipitated out by adding excess acetone to the aqueous fraction.

9.3 RESULTS

A mass of 200 g of *Pentanisia prunelloides* and *Gunnera perpensa* dry powder produced 4.93 g sucrose and 4.63 g glucose respectively. TLC for the sugar samples is displayed in Figure 50.

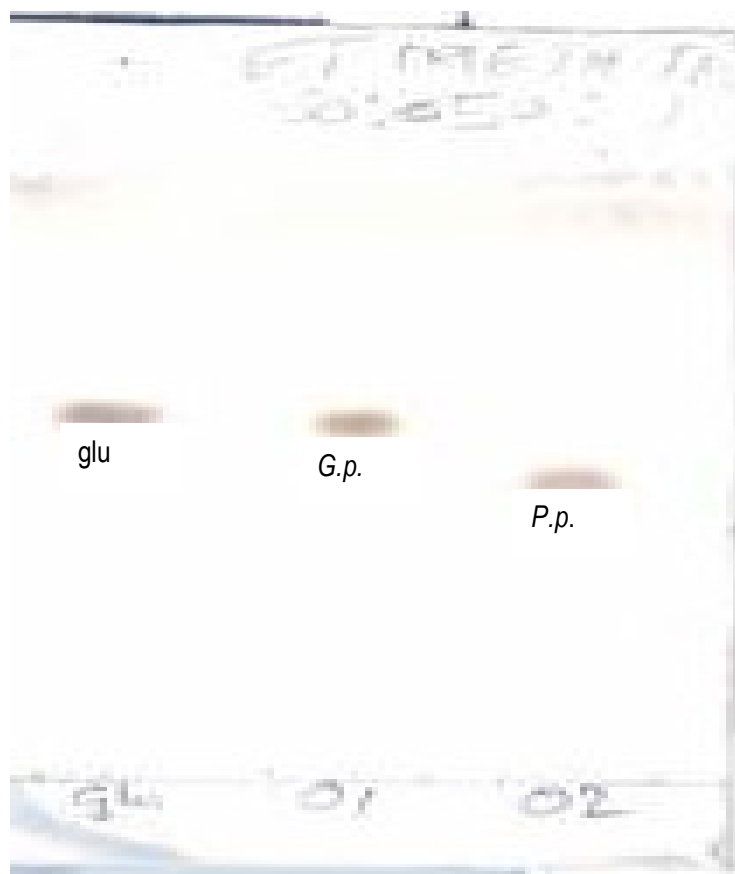


Figure 50: TLC of sugar samples. Glu = glucose, G. p and P. p. = samples from G.p and P.p respectively

R_f values of 0.48 for the *Gunnera perpensa* sample and 0.31 for the sample from *Pentanisia prunelloides*. The solvent used for elution was Ethyl acetate: methanol 50:50. The R_f value of the *Gunnera perpensa* sample displayed the same R_f value of glucose as illustrated in the chromatogram in figure 50. The sugars were identified by NMR spectroscopy as glucose and sucrose and the spectra are in the appendix section (Figure 109-114).

9.3.1 Structural Elucidation.

Assignment of peaks from ^{13}C NMR and ^1H NMR spectra obtained in this study (appendix section) was compared to values obtained from literature (Bagno *et al.* 2007; Tyrell & Prestegard 1986). Tables 31 and 32 are a summary of the structural elucidation for the sucrose and glucose isolated in this study.

Table 31: ^{13}C NMR (300 MHz) and ^1H NMR (300 MHz) data δ C (ppm) and δ H (ppm) for sucrose

C	δ /ppm	^1H , $^1\text{J}/\text{Hz}$
1	82.5	5.19
2	71.6	5.51
3	72.8	4.20
4	62.0	2.50
5	71.6	3.16
6	62.0	3.19
1'	60.1	3.30
2'	91.7	5.05
3'	74.2	4.42
4'	72.8	4.38
5'	76.9	3.17
6'	60.4	3.18

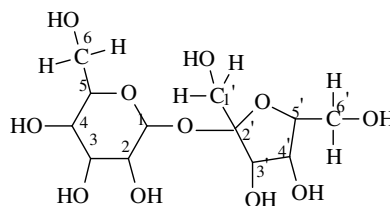
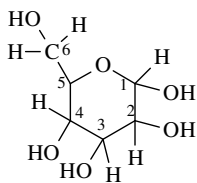


Table 32: ^{13}C NMR (300 MHz) and ^1H NMR (300 MHz) data δ C (ppm) and δ H (ppm) for Glucose

C	δ /ppm	^1H , $^1\text{J}/\text{Hz}$
1	92.2	6.3
2	73.1	3.2
3	72.4	3.7
4	72.0	4.3
5	70.6	3.5
6	61.2	4.3



9.4 DISCUSSION

P. prunelloides, crude methanol extract produced 60% sucrose component and for *G. perpensa*, 36% was glucose. While decoctions from both plants can be used as energy drink because of their high sugar content, in patients suffering from diabetes mellitus, extra care needs to be taken when managing other disease conditions. Both plants should be used with caution on diabetes patients because in traditional practice, water is used to make the decoctions instead of methanol and is likely to dissolve both sugars more.

In the condition of diabetes mellitus, inadequate insulin and its malfunction results in the malfunction of blood glucose regulation with the result of an increase in blood sugar levels (Singh 2010). Symptoms of diabetes include increased thirst, increased urinary output and ketonaemia and ketouria, that is, presence of ketone bodies in the blood and urine (Singh 2010). Diabetes mellitus is a group of syndromes characterized by polydipsia (drinking large amount of water), polyuria (excretion of large amount of dilute urine) and glycosuria (excretion of glucose in urine) (Singh 2010). Medications to manage other conditions such as pregnancy and child delivery should not contain sucrose and glucose especially when treating patients suffering from diabetes as this would introduce other complications associated with raised blood glucose levels.

9.5 CONCLUSION

Although there is evidence of good antimicrobial, anti-oxidative and anti-inflammatory activities by the crude extracts, the high levels of sucrose in *P. prunelloides* and glucose in *G. perpersa* should be borne in mind when using their decoctions in traditional medicine.

CHAPTER 10

General conclusion and future prospects

10.1 INTRODUCTION

The purpose of the study in this research was to assess biological activities of secondary metabolites in selected medicinal plants used for treating inflammation and associated complications and characterization of antimicrobial compounds from the plant extracts of *Pomaria sandersonii* and *Alepidea amatymbica* roots. The specific objectives were to assess free radical scavenging activity of the plant extracts, different fractions obtained by solvent-solvent extraction and isolation of biologically active compounds. Furthermore, antimicrobial and anti-inflammatory activities of the plant crude extracts and different fractions were tested. Biologically active compounds were isolated, characterized and their structures elucidated using NMR and MS.

Antibacterial, anti-inflammatory, antioxidant and cytotoxicity assays carried out demonstrated that the nine plants under study used in Zulu traditional medicine to treat and manage inflammation possessed some biological activities against tested microbes and showed potential as antioxidants and anti-inflammatory agents. The chemotherapeutic properties of the studied medicinal plants were demonstrated by their varied biological activities as observed in the results obtained for different plant extracts; fractions, pure compounds and as displayed in ABTS^{•+} and DPPH scavenging assays in Figures 27-36, Tables 28-36; antimicrobial activities Tables 22 and 23; soya bean derived LOX inhibitory activity in Figure 41 and Table 24, and MIC for pure compounds and mixtures, Table 31.

Infectious disease is an illness resulting from the invasion of the host species by a pathogenic microbial agent and the outcome of the disease depends on the degree of success of the invading pathogen and immune system of the host (National Institute of Health US 2007). About 70% of the global HIV/AIDS epidemic is reported to be in Sub-Saharan Africa and the impact of cancer in the same region has not been fully researched (Mbulaeteye *et al.* 2011). Infectious diseases are considered a major threat to human health, because of the unavailability of vaccines or limited chemotherapy even in the developed parts of the world, although developing countries are carrying the major part of the burden. Infectious diseases pose considerable treatment challenges, especially given the recent appearance of several highly virulent pathogens as well as the rising number of immuno-compromised patients worldwide with particular effect in Africa (Spellberg *et al.* 2008; Carlet *et al.* 2012). Since inflammation can play a role in the initiation and development of diseases such as cancer (Srivastava *et al.* 2009), plants that display anti-inflammatory activities are important in the control and management of disease.

The chemotherapeutic properties of medicinal plants were demonstrated by varied biological activities of different types of plant extracts and pure compounds in this study. This presents the possibility that new and more effective drugs can be obtained from medicinal plants either as the active phytochemicals themselves or as pathogen resistance modulators (Gibbons 2005). Although some plant extracts or pure compounds may be cytotoxic, organic synthesis could be used to come up with less poisonous but effective drugs based on the isolates. Indigenous peoples in South Africa have been using plants of medicinal value for centuries, however, most of them have not yet been scientifically validated (Van Staden 2008). This presents a challenge in ensuring that all medicinal plants used in South Africa are identified, characterized and documented for efficacy and safety (Street *et al.* 2008). Biological activities of plant extracts may be due to synergism (additive) effects of individuals among the complex components acting through different mechanisms. As a result, development of resistance to plant extracts with anti-infectious activities may be minimal compared to single compounds.

Some medicinal plants are being over-harvested and have been on the red list of extinction; therefore investigations of their pharmacological properties are essential for pharmacopeia. Many traditional healers harvest the stem and root from the wild for their healing concoctions and this threatens the plants biodiversity and population stability (Street *et al.* 2008). It is important for research to check if the biologically active compounds in the stem and root parts are also present in the leaves. This would encourage use of leaves instead of destroying the whole plant by removing its roots and stem.

10.2 PRELIMINARY INVESTIGATIONS

The qualitative analysis of crude extracts and thin layer chromatograms of the plant crude extracts and fractions of the studied plants in this research displayed the presence of different types of plant secondary metabolites (figures 14-19 and table 10). The plants were sampled by the traditional practitioners who provided the information on their ethnobotanical use. The TLCs of the different fractions also displayed different densities of bands illustrating the difference in the polarity that a given solvent can extract. Methanol fractions displayed the highest number and amounts of different compounds. Phytochemicals have different polarity, for example, terpenes are relatively non-polar when compared to alkaloids, phenolics and saponins.

10.3 ANTIMICROBIAL ASSAYS

Alepidea amatymbica had antibacterial activity ranging 160-320µg/ml from the crude extract and fractions against the organisms tested except for the methanol fraction against *Escherichia coli* in which the activity of 630 µg/ml, which was relatively lower. *Alepidea amatymbica* crude and fractions also had anti-fungal activity (160-µg/ml) against *Aspergillus fumigatus*. Lower antibacterial and antifungal activities of *Alepidea amatymbica* water, DCM, ethanol and petroleum ether extracts have been reported before using the same method of Eloff (1998).

Pentanisia prunelloides, which is used traditionally for the treatment of dysmenorrhoea, had good to moderate (160-320 µg/ml) antibacterial activity from the crude extract and fractions against the organisms tested except for the acetone fraction against *Enterococcus faecalis* in which the activity of 630 µg/ml, which was low. Yff *et al.* (2002) reported antibacterial activity by water, ethanol and ethyl acetate crude extracts by the same method of Eloff (1998). Activities are higher (0.39-12.5 µg/ml) than those reported here presumably because there is more synergism in crude extracts since fractions were used for assays in this study.

P. sandersonii DCM and ethyl acetate fractions displayed had MIC value (80 µg/ml in each case), which compared well with gentamycin's (20 µg/ml) same value against *S. aureus*, *E. faecalis*, *E. Coli* and *P. aeruginosa* thereby justifying *P. sandersonii* extract usage by traditional practitioners as part of post-natal care to women (Mulaudzi *et al.* 2009). *Gunnera perpensa* extracts and fractions had good broad based antimicrobial activities against the organisms tested using the 96 micro-plate dilution method. The most active fraction was the methanol fraction (80 µg/ml) against *Pseudomonas aeruginosa*.

Gunnera perpensa fractions (hexane, DCM, ethyl acetate, acetone and methanol) displayed good activity of 160-µg/ml against *Candida albicans*. The antimicrobial activities of water, ethyl acetate and ethanol fractions of the root extract of *Gunnera perpensa* against some bacteria and fungi have been reported before (*Bacillus subtilis*, 12.5 mg/ml active)(- Buwa & Van Staden 2006). Crude water extracts of *Gunnera perpensa* showed antimicrobial activity against *Escherichia coli*-(0.78 mg/ml); *Klebsiella pneumonia* (0.78 mg/ml); and *Candida albicans* (25 mg/ml) while the corresponding ethyl acetate and ethanol fractions were less active (Buwa & Van Staden 2006). However, the reported values are not considered to be significantly high by the phytomedicine program. This explains the use of the plant by South African Traditional healers as treatment against venereal diseases (Buwa & Van Staden 2006).

Artemisia afra acetone and methanol fractions had activities (20 µg/ml) against *E. coli* and good to moderate activity (160-320 µg/ml) for the crude extract and all fractions against the organisms tested (tables-5.1 and 5.2) against *Aspergillus fumigatus* and *Staphylococcus aureus*. *Eucomis autumnalis* had activity (160-320 µg/ml) from the crude extract and fractions against the organisms tested except for the crude extract against *E coli* in which the activity of 630 µg/ml was observed and considered

Ledebouria revoluta hexane fraction had activities of 80 µg/m) against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The acetone fraction of *Ledebouria revoluta* had activity of 20 µg/ml against *Candida albicans* and activity of (160-320 µg/ml) against the rest of the bacterial strains tested. The bulb infusion of *Ledebouria revoluta* is used to treat diarrhoea in goats and leaf decoction for gall sickness (Dold & Cocks 2001 quoted by McGaw *et al.* 2008).

Pomaria sandersonii, *Carissa bispinosa* and *Berkheya setifera* displayed good to moderate antimicrobial activity. *P. sandersonii* DCM and ethyl acetate fractions had activity with MIC value of 80 µg/ml against *S aureus*, *E. faecalis*, *E Coli* and *P aeruginosa* respectively comparing well with gentamycin's (4 µg/ml) thereby justifying *P. sandersonii* extract usage by traditional practitioners use as post-natal care to women.

The micro-dilution assay had antimicrobial activity by 06B, 13-hydroxy-16-kauren-19-oic acid, C₂₀ H₃₁ O₃ 40 µg/ml against *Aspergillus fumigatus* illustrating the relevance of the plants usage in traditional practise. 06-2, ent-16-hydroxy-16-kauren-19-acid is a white crystalline acetone soluble compound which was also isolated from *Alepidea amatymbica*. This compound was also found to have antimicrobial activity ranging between 320-1250 µg/ml. EM80-2 is 1-(2, 4-dihydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one also known as isoliquiritigenin is a chalcone isolated from *Pomaria sandersonii*. It was the most active antimicrobial compound isolated in this study with activities ranging between 80 to 620 µg/ml).

10.4 ANTIOXIDANT ASSAYS

Alepidea amatymbica polar fractions (methanol for DPPH• radical scavenging and ethyl acetate, acetone and methanol fractions for ABTS•• radical cation) had activity of 0.08 to 2.6 µg/ml and low activity for crude extracts, DCM and ethyl acetate fractions.

Crude acetone extract, DCM and ethyl acetate fractions of *Pomaria sandersonii* displayed higher ABTS•• scavenging capability than corresponding DPPH scavenging ability. *P. sandersonii* samples displayed the highest number of fractions with ABTS•• scavenging capability of 1.274 µg/ml to 5.973 µg/ml (for the crude, DCM, ethyl acetate, acetone and methanol) compared to all the plants studied although its hexane fraction activity was low. *Gunnera perpensa* acetone, methanol fraction and crude extract had activity of DPPH free radical scavenging and 2.795 µg/ml respectively.

The fact that *Artemisia afra* crude fraction and polar fractions of acetone and methanol had high activity (EC₅₀ for DPPH• radical, 2.113 µg/ml; crude 4.393 µg/ml; acetone fraction, 4.715 µg/ml; methanol fraction and EC₅₀ with ABTS•• radical cation, 6.447 µg/ml and 6.208 µg/ml from crude and methanol fraction respectively) demonstrate why the aqueous extracts are used in traditional medicine in the treatment of a variety of diseases ranging from respiratory infections to dysmenorrhoea, diabetes and malaria (Suliman *et al.* 2010; Liu *et al* 2009). The methanol fraction of *Ledebouria revoluta* had comparable DPPH free radical scavenging capability of 1.867 µg/ml to that of *Berkheya setifera* methanol fraction with EC₅₀ value of 1.008 mg/ t and *Gunnera perpensa* crude acetone extract EC₅₀ value of 1.069 µg/ml

10.5 ANTI-INFLAMMATORY ASSAYS

The studied plant crude extracts displayed some lipoxygenase inhibitory activity. Soybean derived 15-LOX was used to assess the *in vitro* anti-inflammatory activity. These findings should therefore be cautiously applied to the anti-inflammatory activity in humans because the mechanism of human-derived 15-LOX may be different from that which is soybean derived. Cyclooxygenases (COX-1 and COX-2), lipoxygenases (LOXs) and cytochrome P450 monooxygenases

are three classes of enzymes which catalyse the formation of eicosanoids such as prostaglandins, thromboxanes, leukotrienes and other oxygenated derivatives of the lipid mediators (such as arachidonic and linoleic acid) (Iwalewa *et al.* 2007). In this study only soya bean based 15-LOX inhibitory activity was studied. The plant extracts' inhibitory anti-inflammatory activities still need to be carried out to investigate the other possible biological activities.

10.6 CYTOTOXICITY

Crude extracts with EC₅₀ values less than 20- μ g/ml are considered cytotoxic by the American National Cancer Institute (NCI) (Joshi *et al.* 2011). Both crude acetone extracts have much lower toxicity than 20 μ g/ml because all values were higher. Both bovine dermis and monkey vero cell cytotoxicity assays demonstrated that crude acetone extracts of *Pomaria sandersonii* and *Alepidea amatymbica* displayed much lower toxicity levels when compared to that of doxorubicin. Doxorubicin is an antibiotic used in the treatment of various types of cancers (Tokarska-Schlattner *et al.* 2005). The drug kills both normal and cancer cells which characterises its undesirable side effect during the treatment of cancer patients (Tokarska-Schlattner *et al.* 2005).

10.7 ISOLATION AND IDENTIFICATION OF PURE COMPOUNDS

Alepidea amatymbica has shown that its root hexane fraction is rich in diterpenes because five pure compounds from the diterpenoids family were isolated. The name of 0652 is 14 acetoxo-12-oxokaur-16-en-19-oic acid or xylopic acid (Somova *et al.* 2001). Xylopic acid was also isolated from *Xylopiya aethiopia* (Somova, *et al.* 2001). 06431 is 14-oxokaur-16-en-19-oic acid. This is the first time the acetate of kaurenoic acid has been isolated from *Alepidea amatymbica*. Langat *et al.* (2012) synthesised the same kaurenoic acid, *ent*-14-oxokaur-16-en-19-oic acid by Jones oxidation of *ent*-14-hydroxykaur-16-en-19-oic acid. According to Ortega *et al.* 1985; Somova *et al.* 2001; Cannon *et al.* 1965; Langat *et al.* 2012 and Buckingham & Bradley 1994, the diterpenoid 0657 is a new compound because it contains both the acetate group on C14 and a keto group on C12. The name proposed for 0657 is 14 acetoxo-12-oxokaur-16-en-19-oic acid.

EM80-2 is 1-(2, 4-dihydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one also known as isoliquiritigenin and a chalcone dimer referred to as EM 86 were isolated from *Pomaria sandersonii*. Isoliquiritigenin has been isolated before from licorice (*Glycyrrhiza uralensis*), shallot, *Sinofranchetia chinensis*, *Dalbergia odorifera*, and soybean (Cuendet *et al.* 2010). During this investigation, EM80-2 also had high antibacterial activity in the range of 80-160 μ g/ml against *Staphylococcus aureus*, ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, and *Escherichia coli*, ATCC25922. Isoliquiritigenin has been reported before to exhibit potent antioxidant, anti-inflammatory, phytoestrogenic, tyrosinase inhibitory and exhibited significant inhibitory effects of carcinogenesis in various tumour models (Cuendet *et al.* 2010). Isoliquiritigenin has also been reported before to exhibit antibacterial activity against *Staphylococcus aureus*, *Streptococcus mutans*, and *Actinomyces naeslundii* with inhibitory activity ranging 15.6 to 62.5 μ g/ml (Oldoni *et al.* 2011). This is the first time for isoliquiritigenin be isolated from *Pomaria sandersonii*. The naming of the chalcone dimer EM86 is still to be confirmed because it appears to be new chalcone dimer (Buckingham and Bradley,

1994). EM86 appears to be a dimer of 1-(2, 4-dihydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one. The biological activity of EM86 was not determined because of the lack of resources at the time of isolation

10.8 CONCLUSION

Total of seven compounds were isolated during this study. We propose three were new compounds after considering literature search involving closely related research to this investigation. These include two diterpenes, namely, 0657, which are 14 acetoxo-12-oxokaur-16-en-19-oic acid, 06-2, 16-hydroxy-kaur-6-en-19-oic-acid and the chalcone dimer, which is referred to as EM86 in this study from *Pomaria sandersonii*

The most active antioxidants were contained in *Pentanisia prunelloides*, *Pomaria sandersonii*, *Gunnera perpensa* and *Eucomis autumnalis* methanol fractions because they exhibited free radical activity against both ABTS⁺ and DPPH free radicals. The nine plants in this investigation have also exhibited good anti-microbial activity. Although there is evidence of good antimicrobial, anti-oxidative and anti-inflammatory activities by the crude extracts, the high levels of sucrose in *P. prunelloides* and glucose in *G. perpensa* indicate that precautionary measures should be taken when using their decoctions in traditional medicine because of the high sugar content in the roots. The presence of the sugars is based on the isolation of glucose and sucrose from the two plants as shown in Chapter 8. Based on the results from sugar levels in *P. prunelloides* *G. perpensa* decoctions can be used as energy drinks.

In vitro results for the antioxidant activities of the plant crude extracts and fractions carried out in this investigation illustrated that the plants can be used for inflammation-related conditions which justify their usage in Zulu traditional medicine. However, *in vivo* assays should be carried out in order to completely validate claims by the traditional healers that they cure inflammation-related conditions. The root was used for the study as was recommended by the healers of Mabandla village. This study has confirmed that *Alepidea amatymbica* is rich in diterpenes because Somova *et al.* (2001) also isolated four different kaurenoic acids from the same plant.

However comparative studies could be carried out with the leaves so that if comparable activities exist, use of the leaves can be recommended so as to save the plant. EM80-2, which is 1-(2, 4-dihydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one and 06B, which is ent-13-hydroxy-16-kaur-19-oic acid, C₂₀ H₃₁ O₃ were reported to be lead compounds in cancer treatment in HIV treatment research respectively (Cuendet *et al.* 2010; Louvel *et al.* 2013). Therefore the two plants can be used as source of lead compounds in drug discovery for cancer (Cuendet *et al.* 2010), HIV treatment (Louvel *et al.* 2013) and natural sweeteners (Sharma *et al.* 2008) because research is already under way using steviol as a lead compound for newer drugs.

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APPENDIX 1

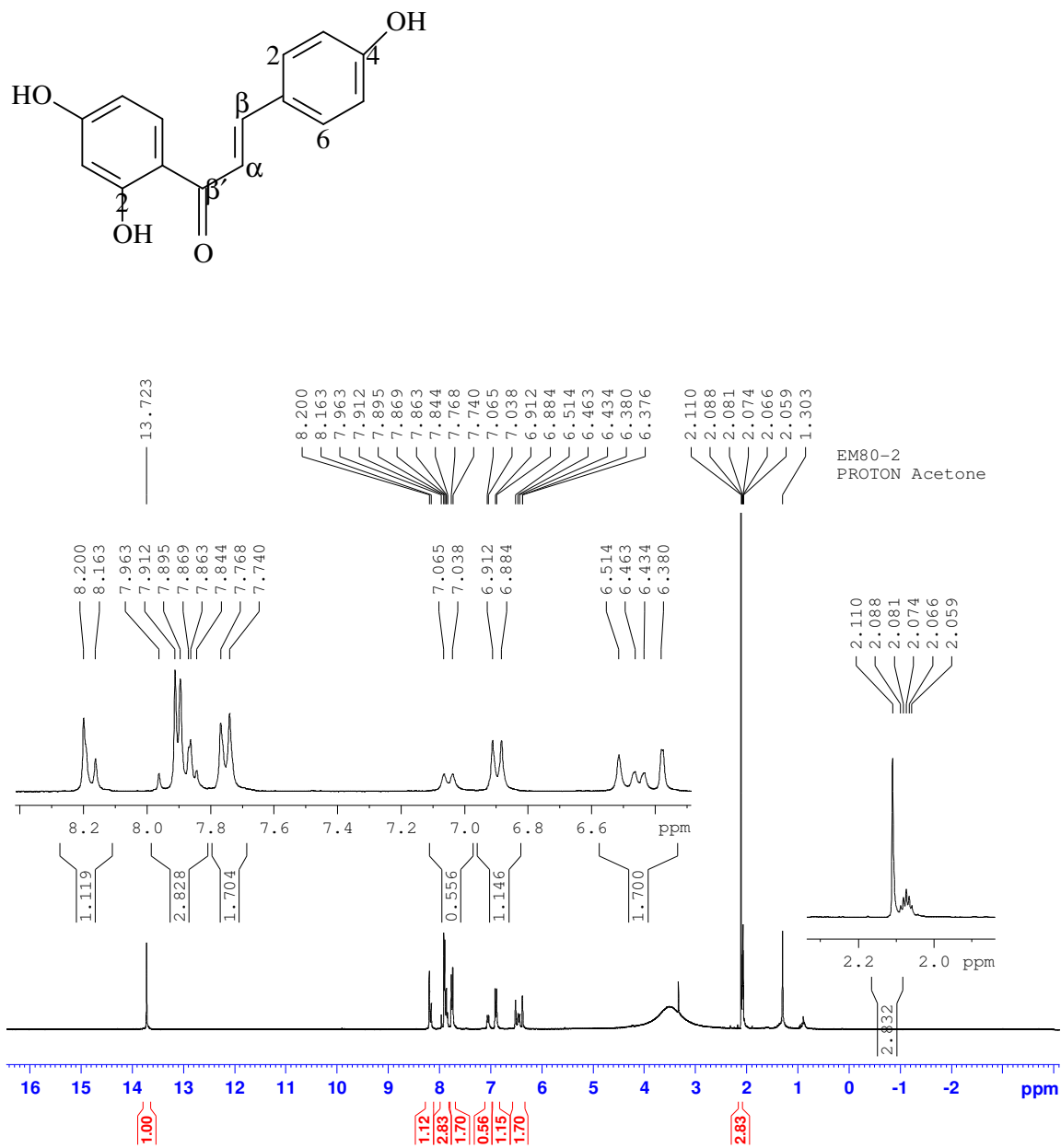


Figure 51: ¹H NMR spectrum (300 MHz) for EM80-2

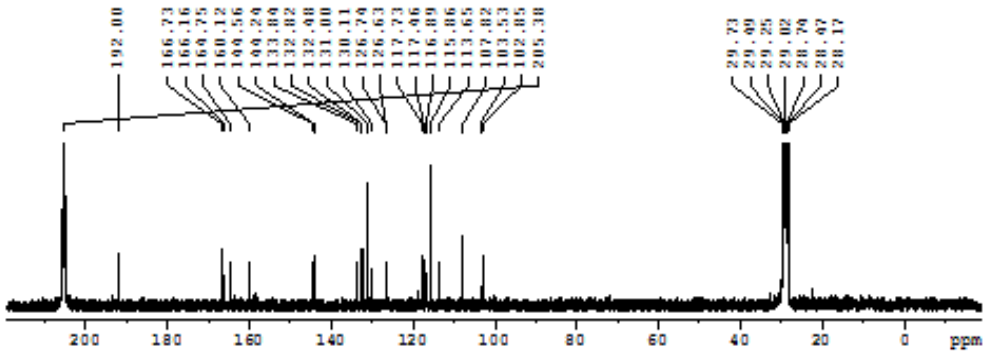
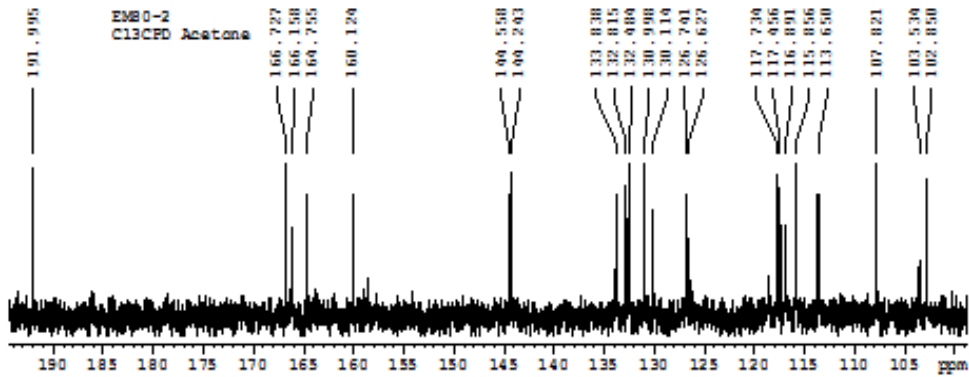
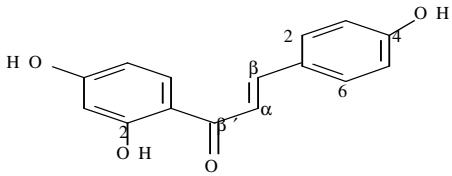
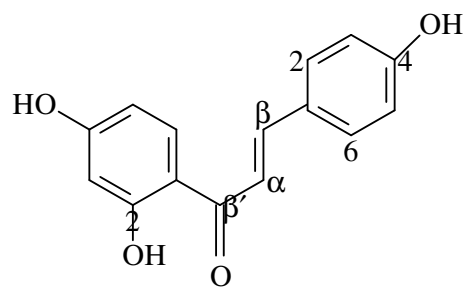


Figure 52: ¹³C NMR spectrum (300 MHz) for EM80-2



EM80-2
 C13DEPT135 Acetone

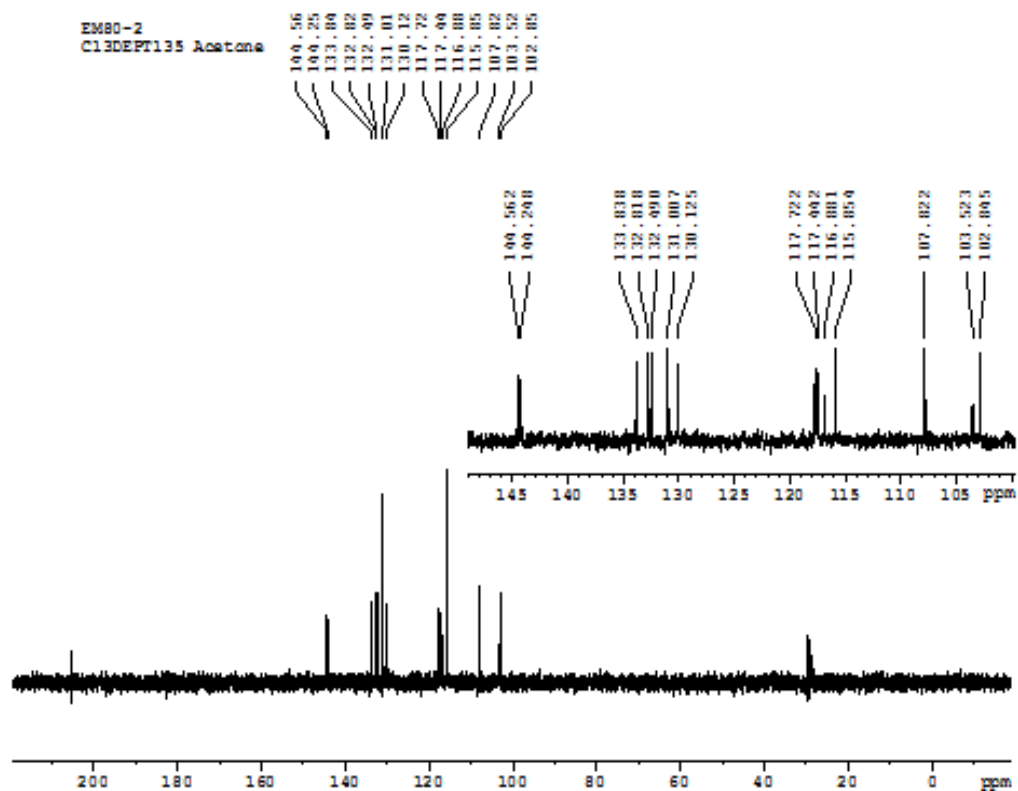


Figure 53: DEPT 135 spectrum (300 MHz) for EM80-2

EM80-2
HSQCGP Acetone

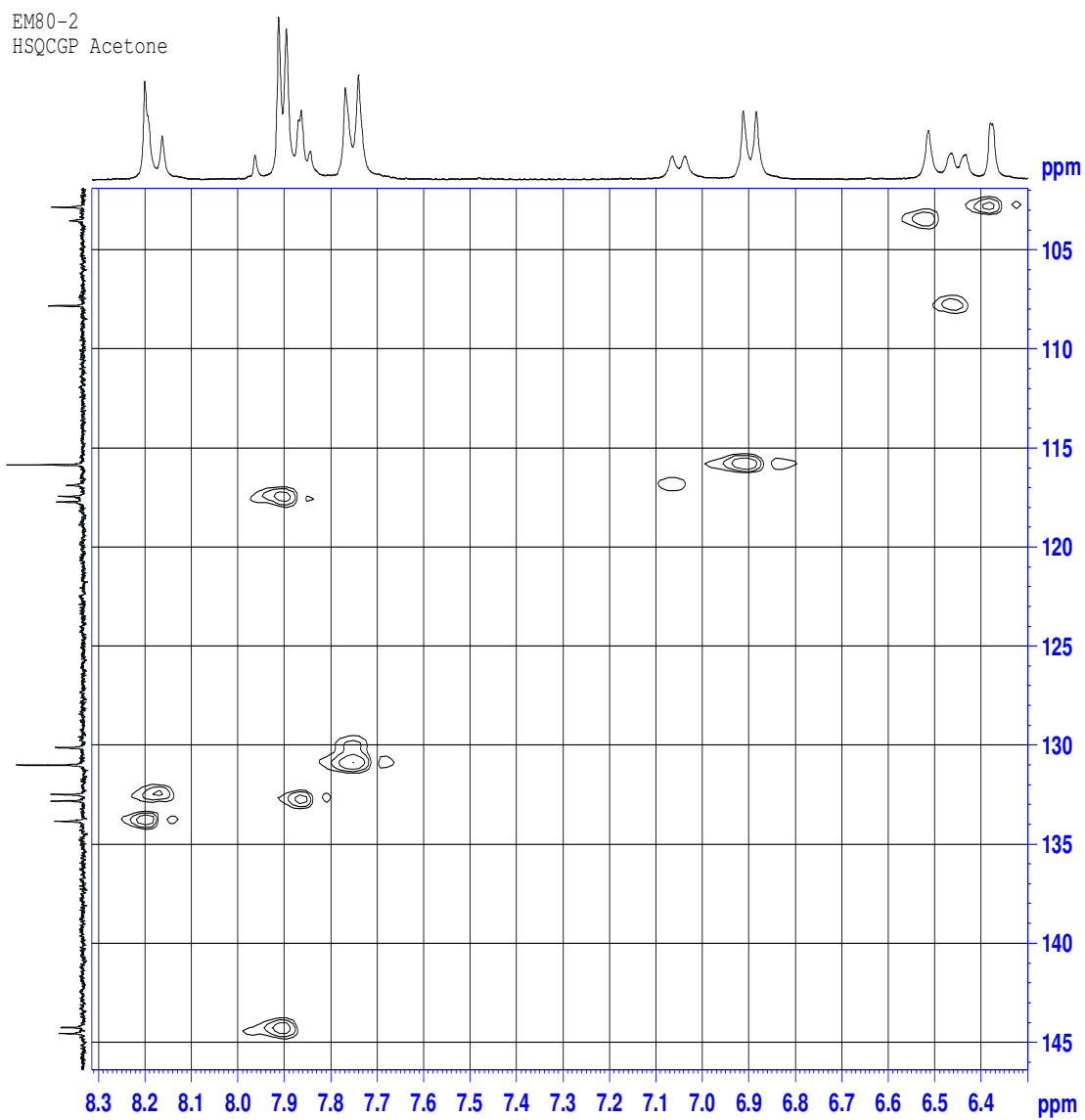


Figure 54: HSQC spectrum (300 MHz) for EM80-2

EM80-2
HMBCGPND Acetone

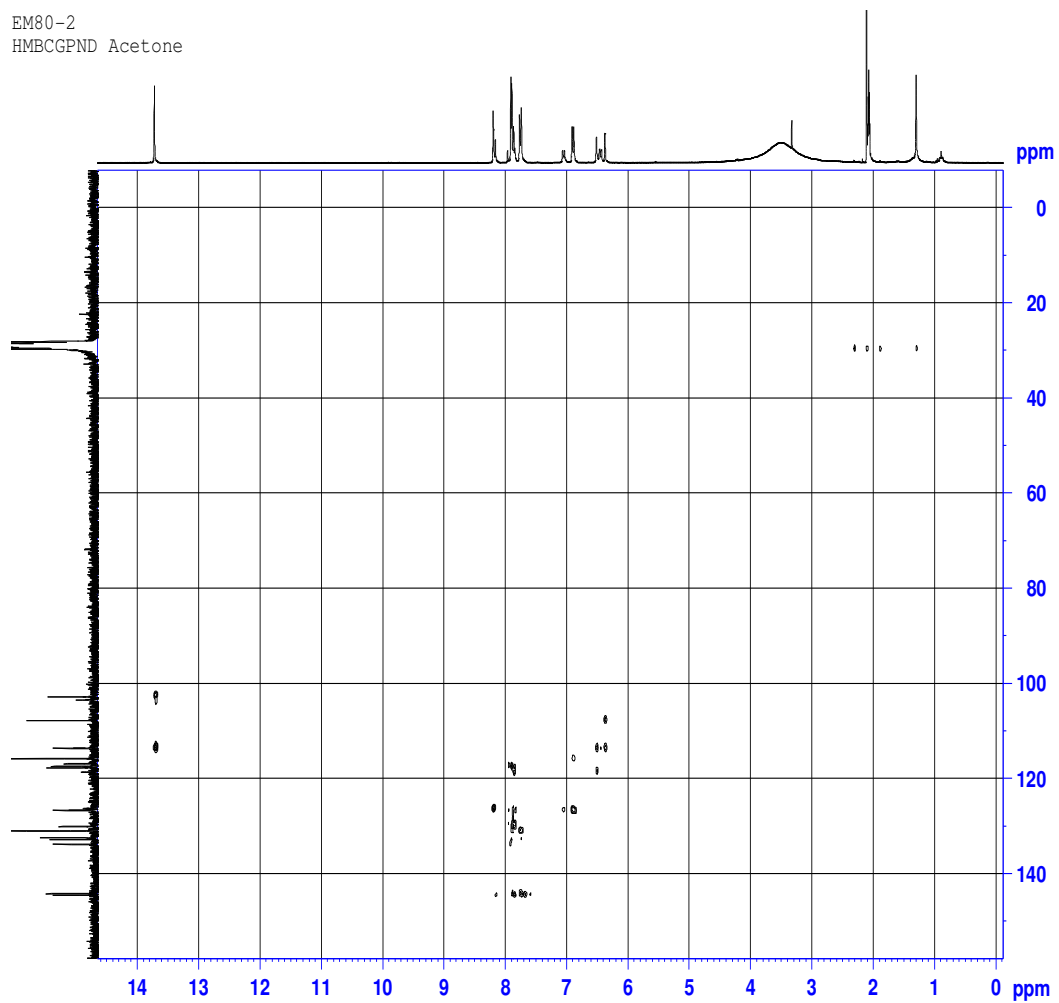


Figure 55: HMBC spectrum (300 MHz) for EM80-2

EM80-2
HMBCGPNDAcetone

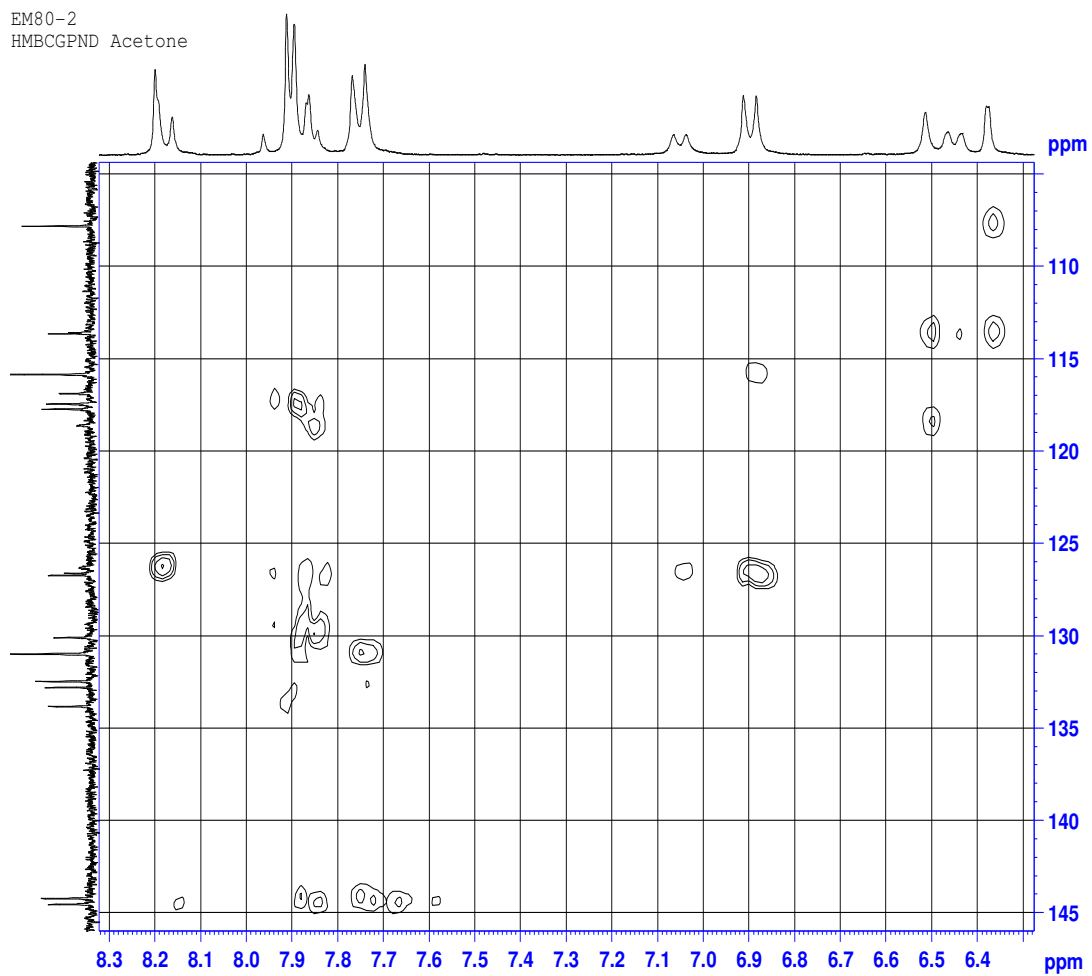


Figure 56: Expanded HMBC spectrum (300 MHz) for EM80-2

EM80-2
COSYCPBW Acetone

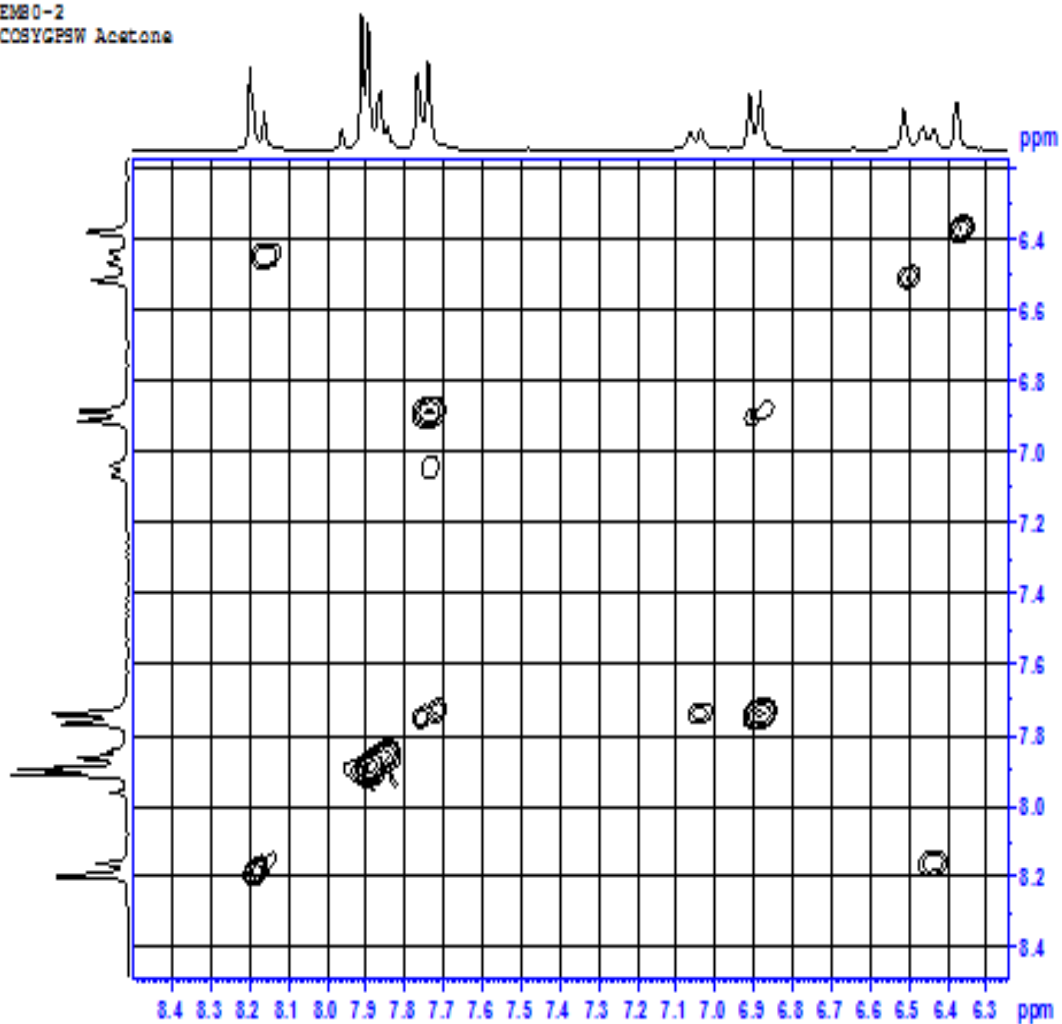


Figure 57: COSY spectrum (300 MHz) for EM80-2

15-Nov-2012
EM802-1

1: TOF MSMS ES+
BPI
1.12e4

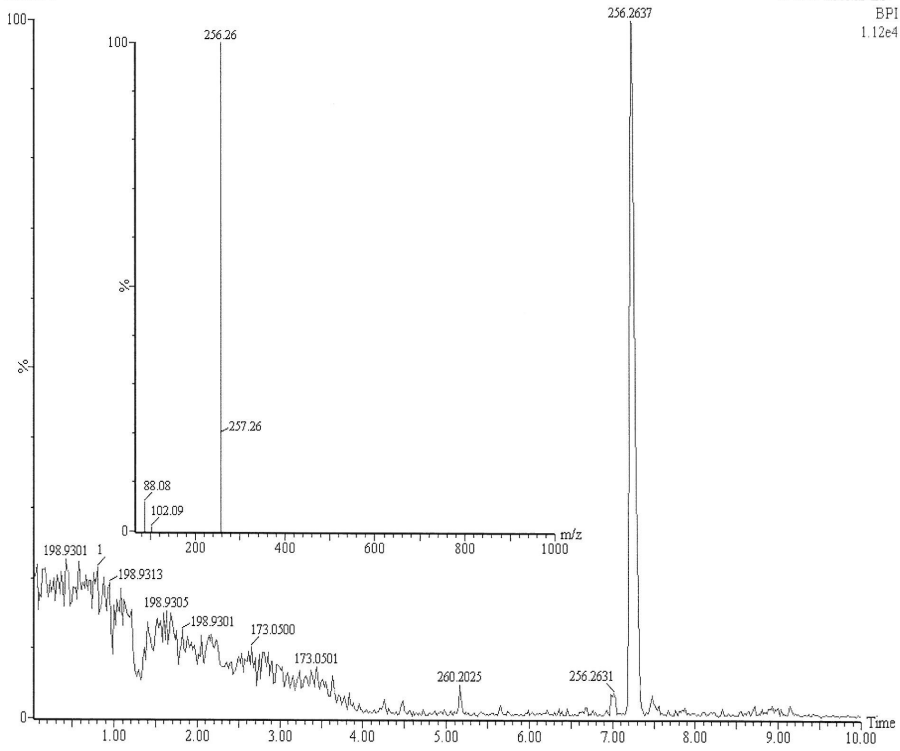


Figure 58: TOF MS-MS spectrum for EM80-2

APPENDIX 2

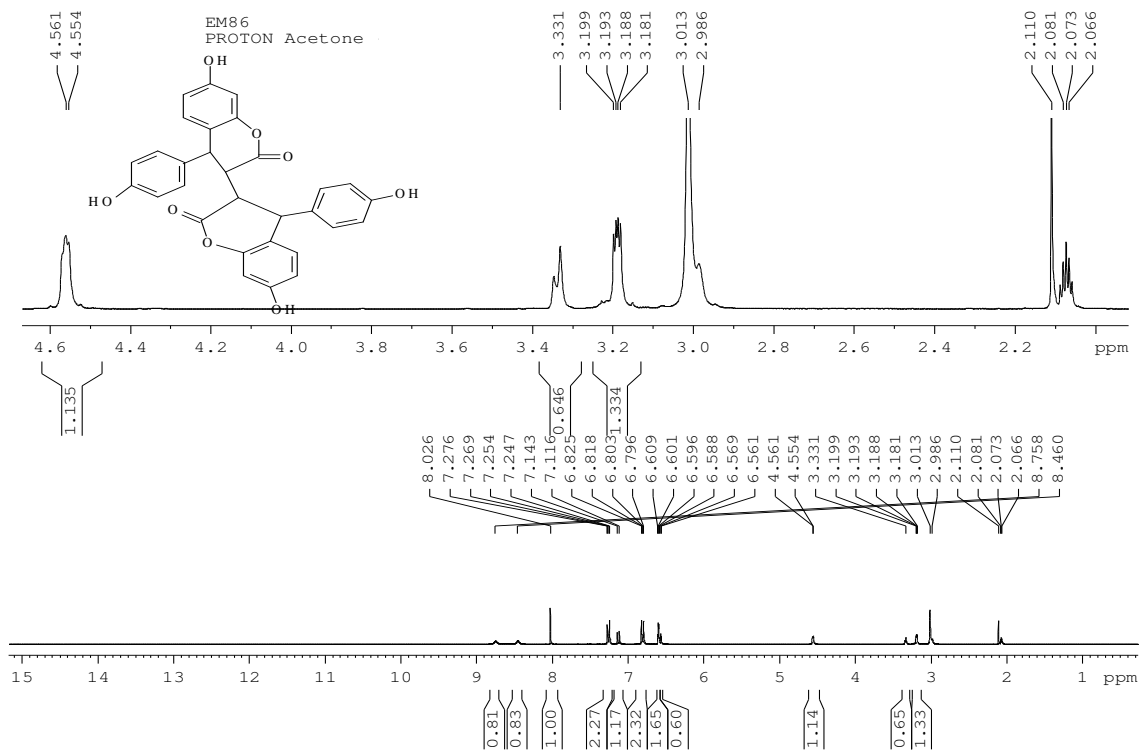


Figure 59: ^1H NMR spectrum (300 MHz) for EM86

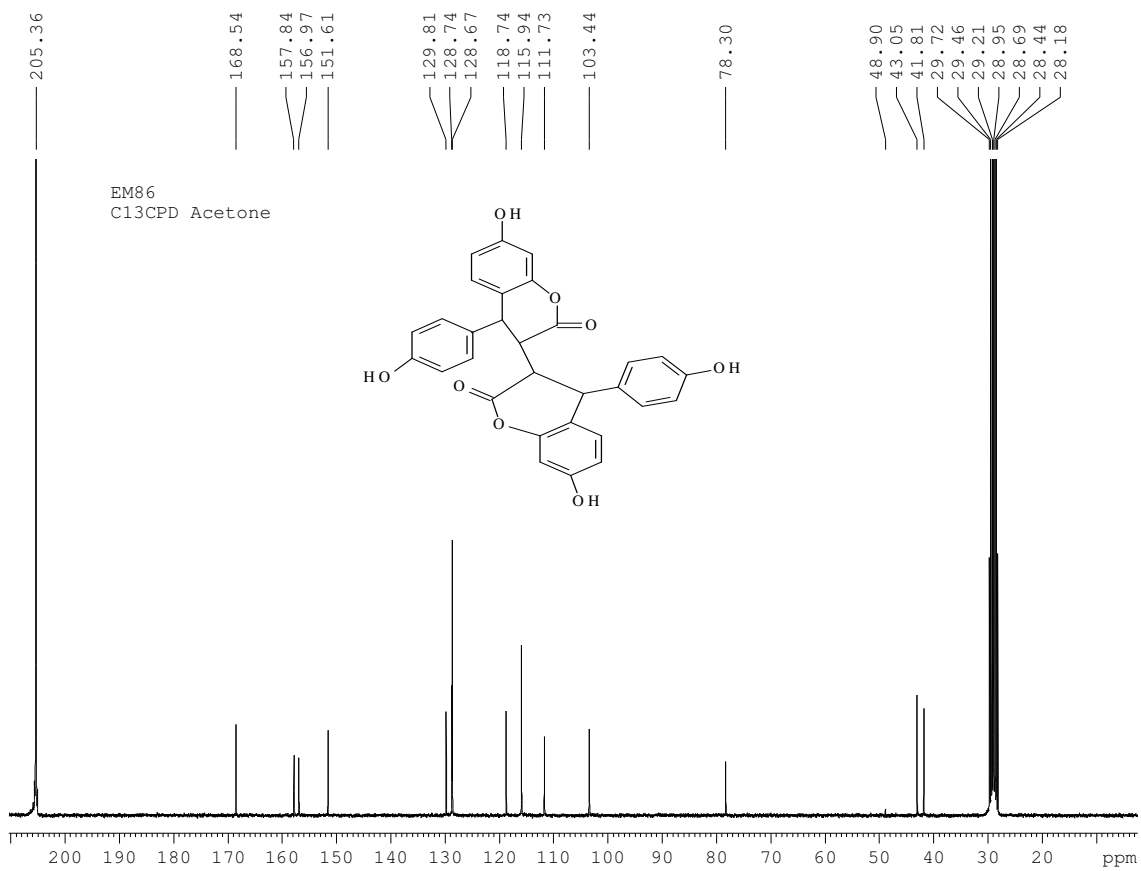


Figure 60: ^{13}C NMR spectrum (300 MHz) for EM86

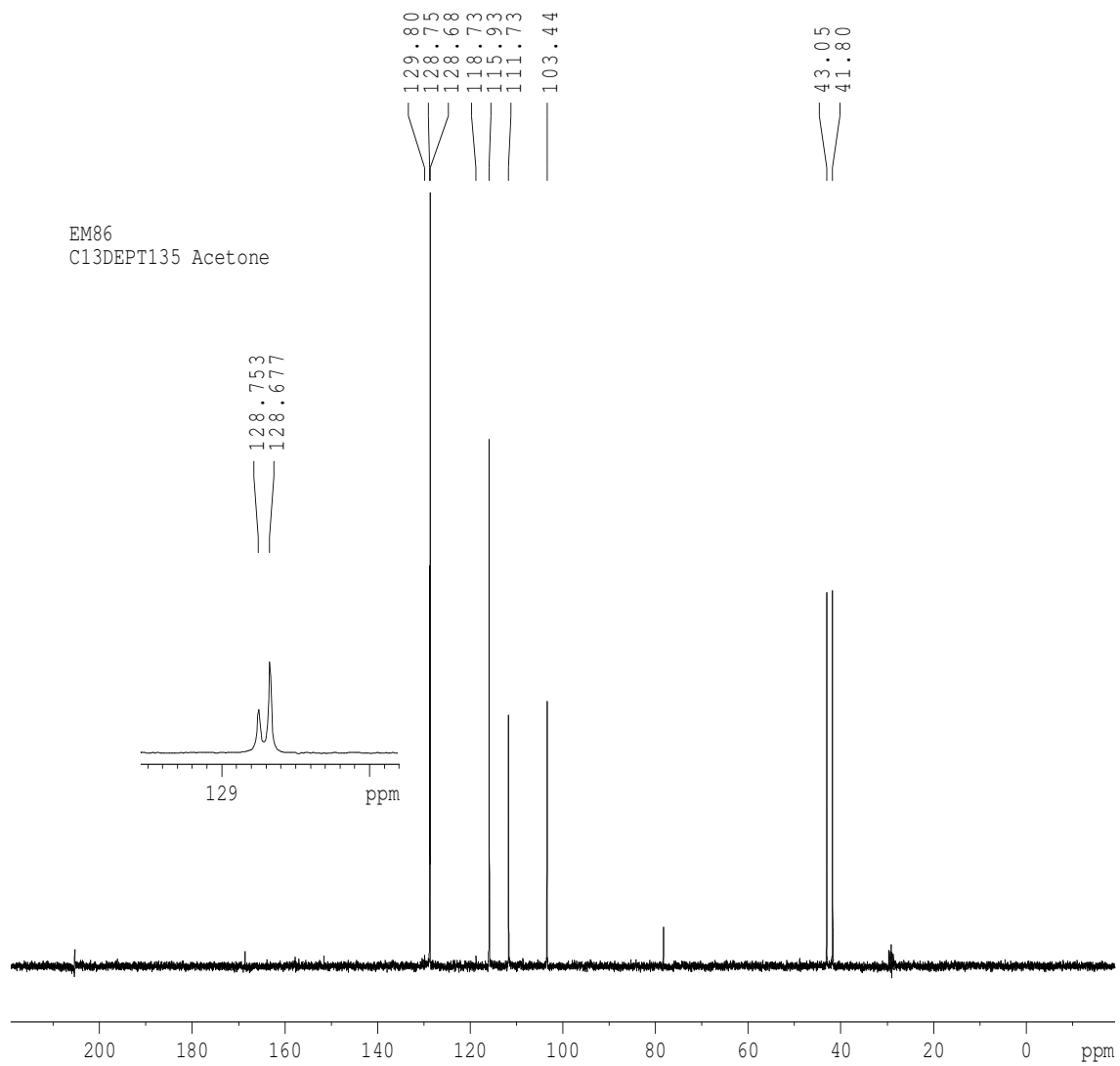


Figure 61: DEPT135 spectrum (300 MHz) for EM86

EM86
HMQCGP Acetone

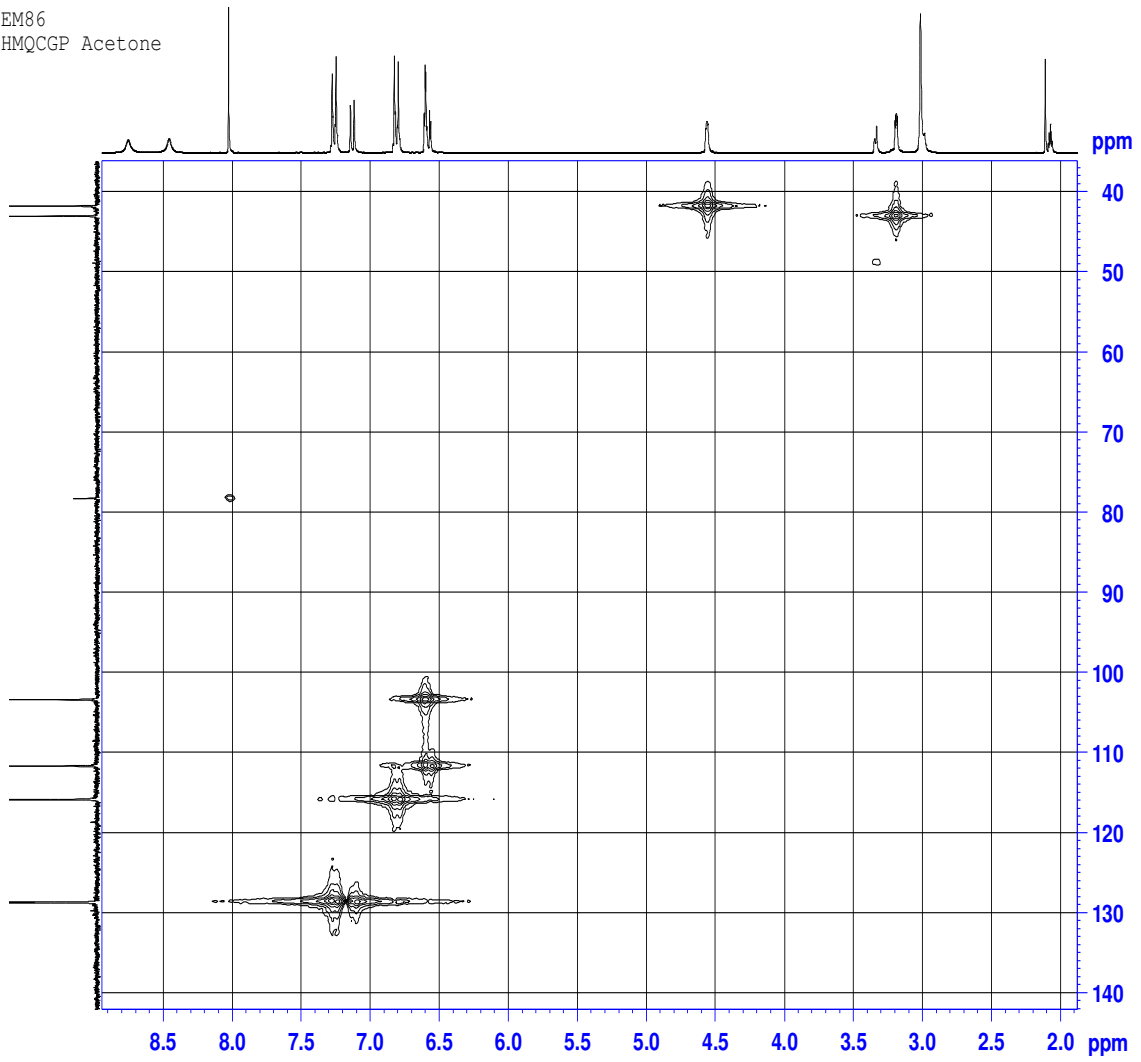


Figure 62: HMQSC (300 MHz) for EM86

EM86
HMBCGPND Acetone

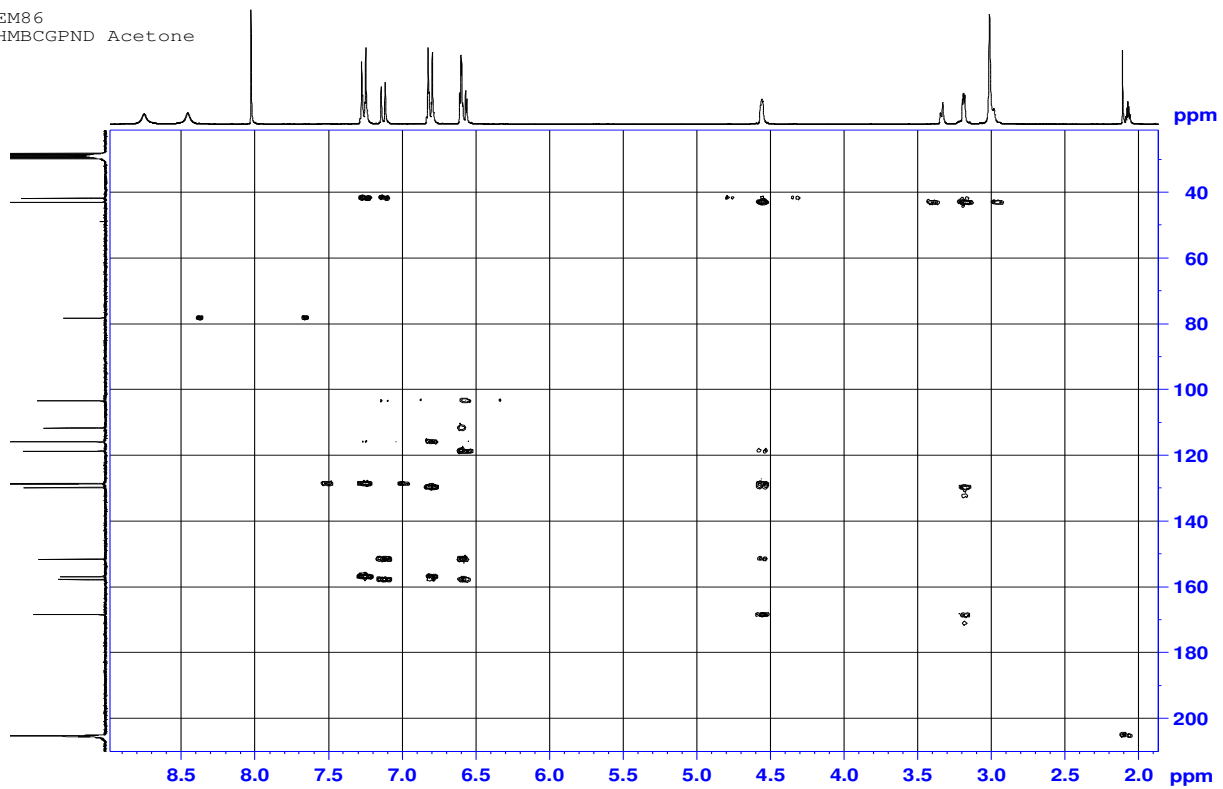


Figure 63:HMBC spectrum (300 MHz) for EM86

15-Nov-2012
EM86-4

1: TOF MSMS ES+
BFI
4.61e4

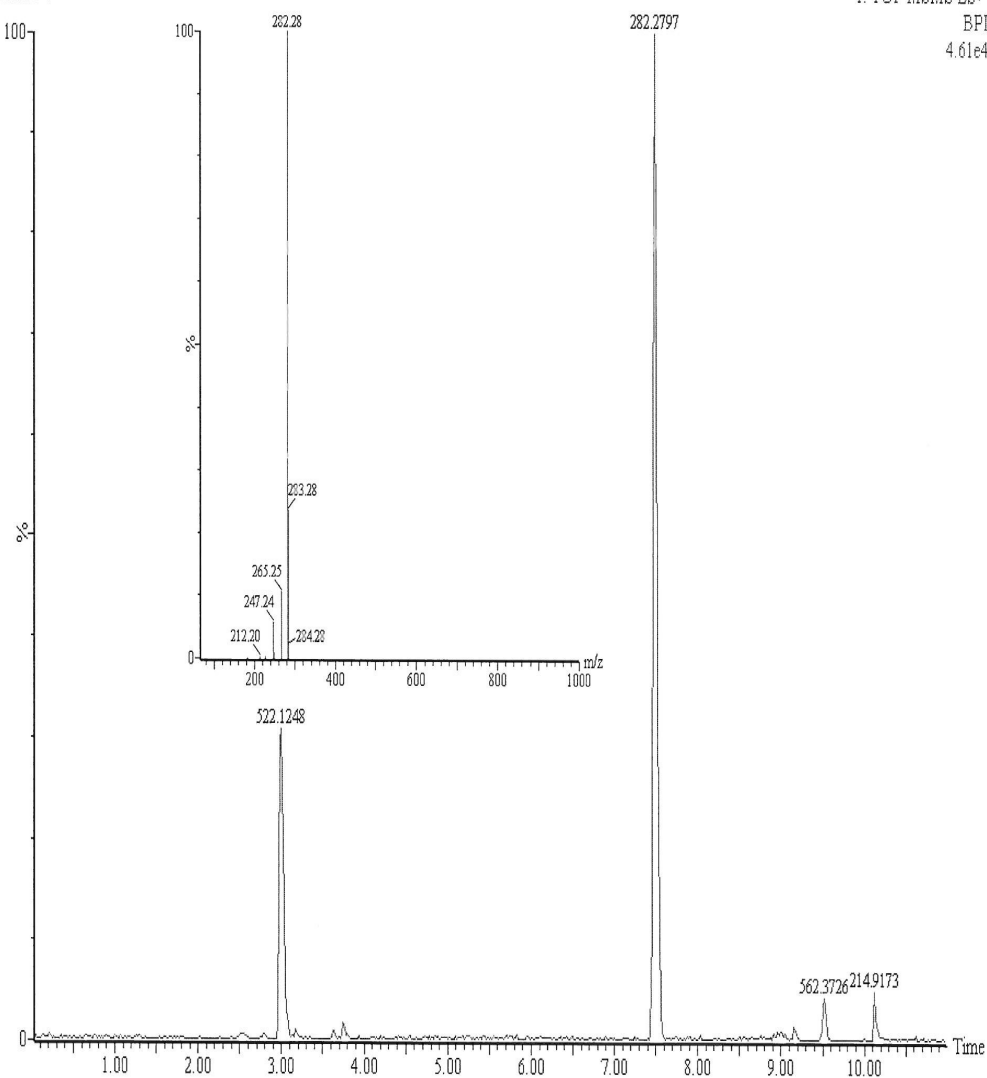


Figure 64: TOF MS-MS spectrum for EM86

APPENDIX 3

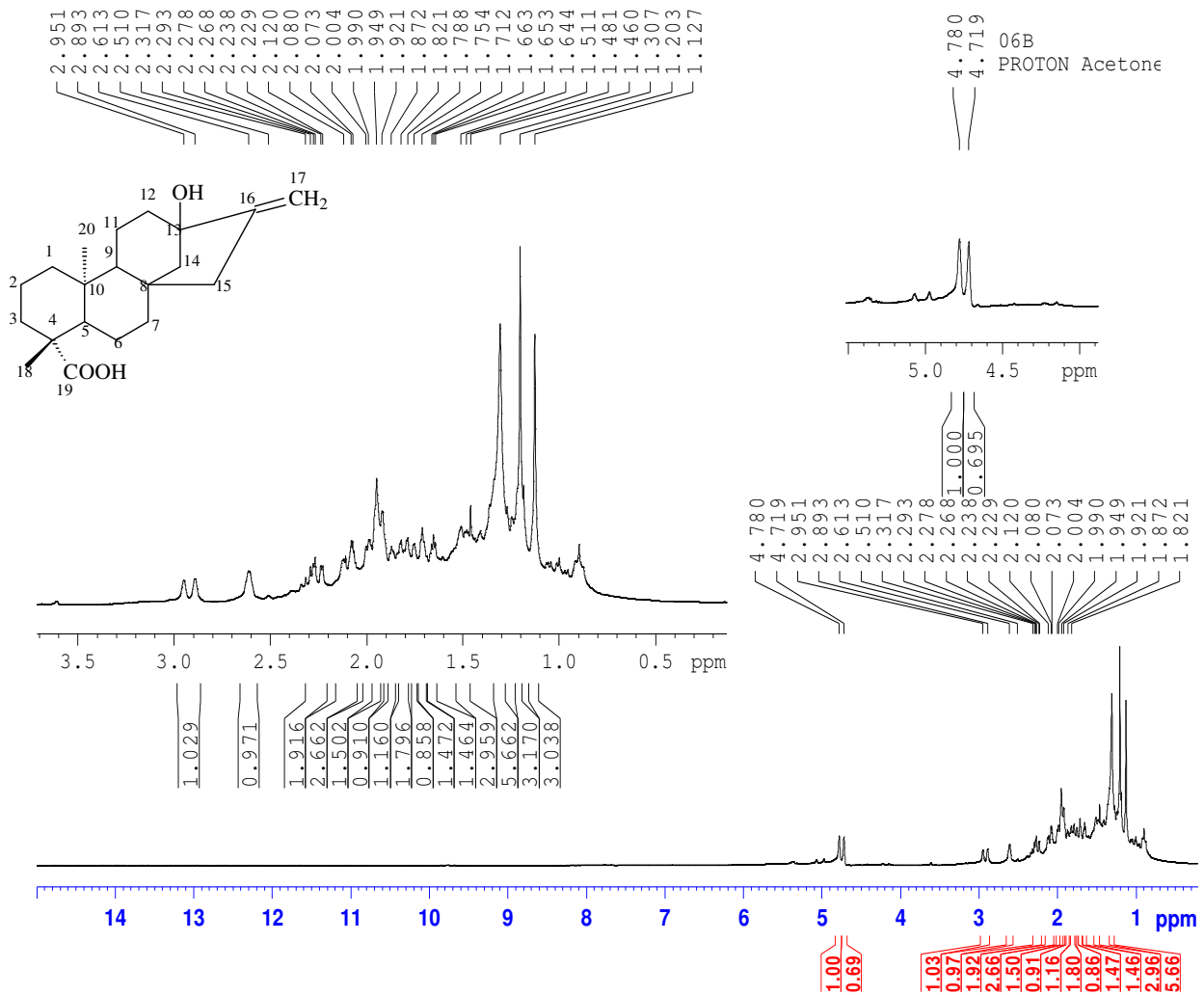


Figure 65: ¹H NMR spectrum (300 MHz) for 06B

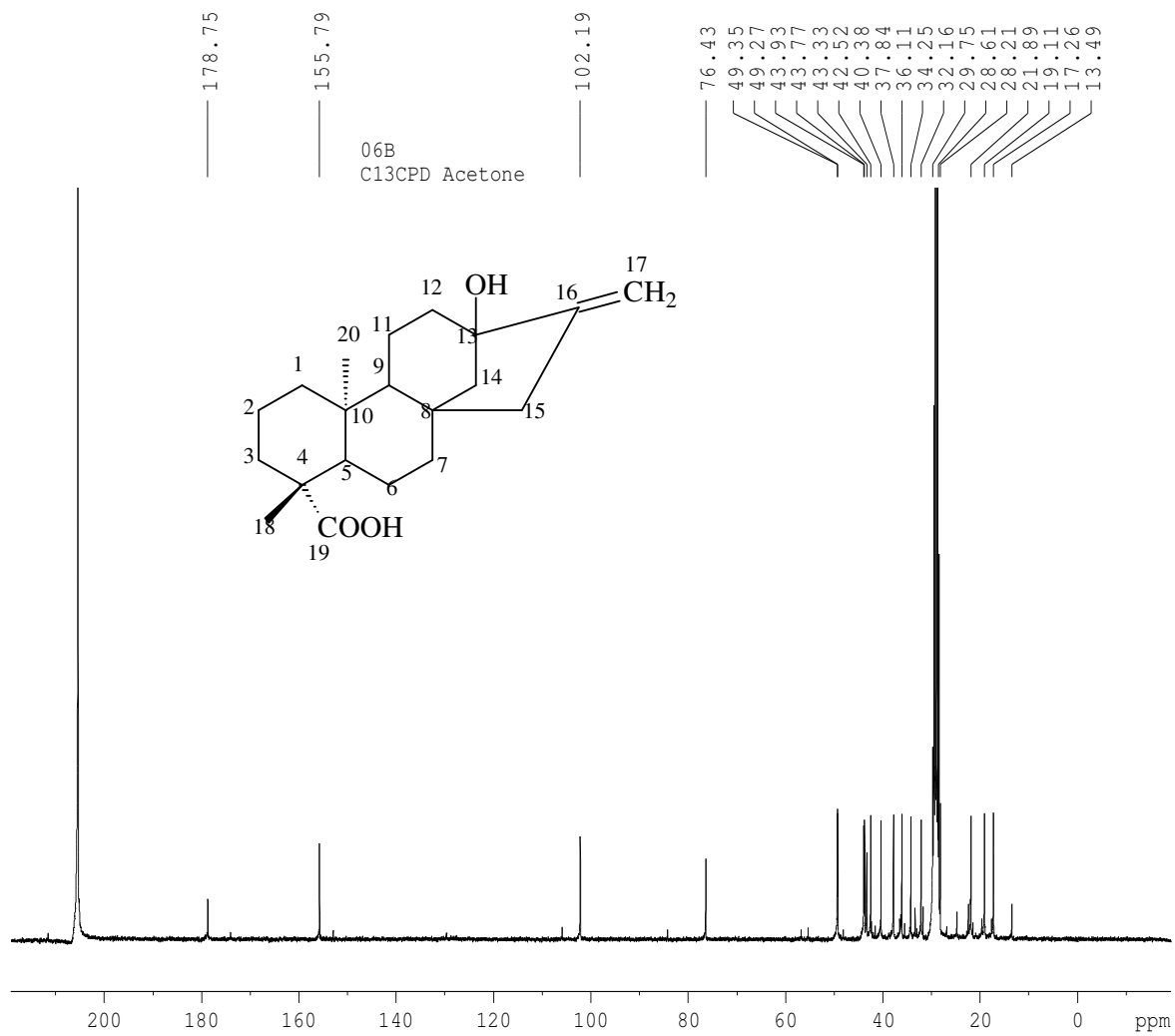


Figure 66: ¹³C NMR spectrum (300 MHz) for 06B

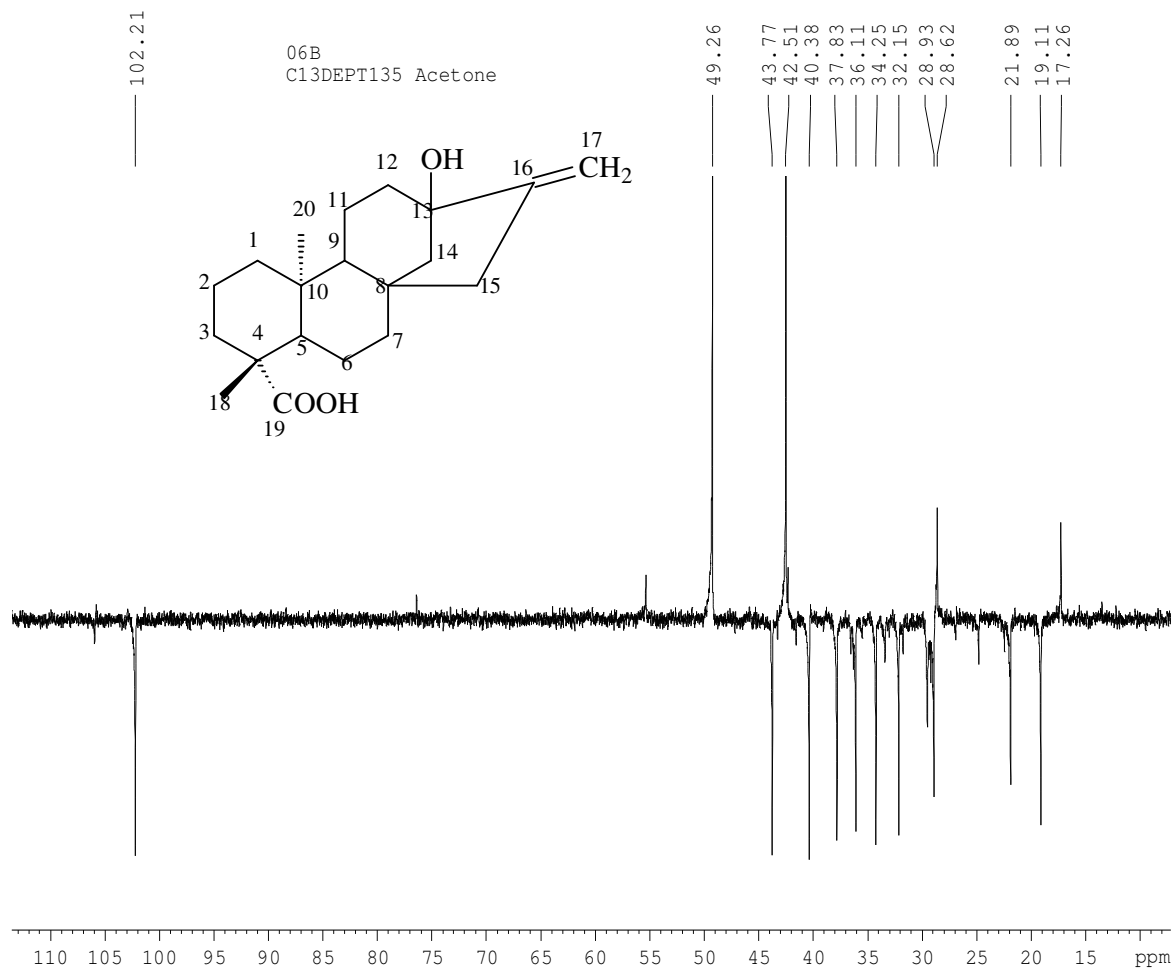


Figure 67: DEPT 135 spectrum (300 MHz) for 06B

06B
HMBCGPNDAcetone {C:\Stumbo} stumbo 4

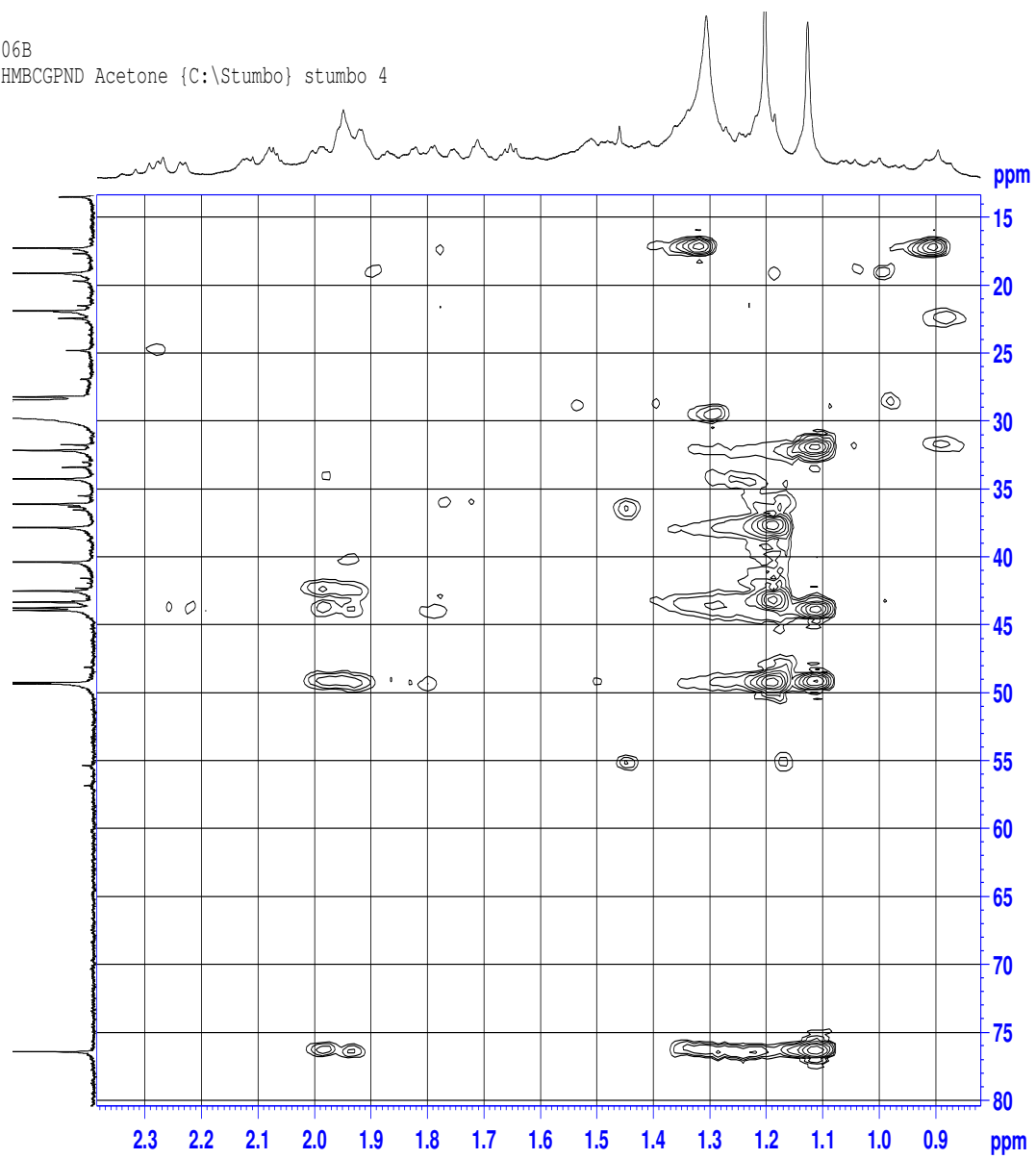


Figure 68: HMBC spectrum (300 MHz) for 06B

06B
COSYGPSW Acetone

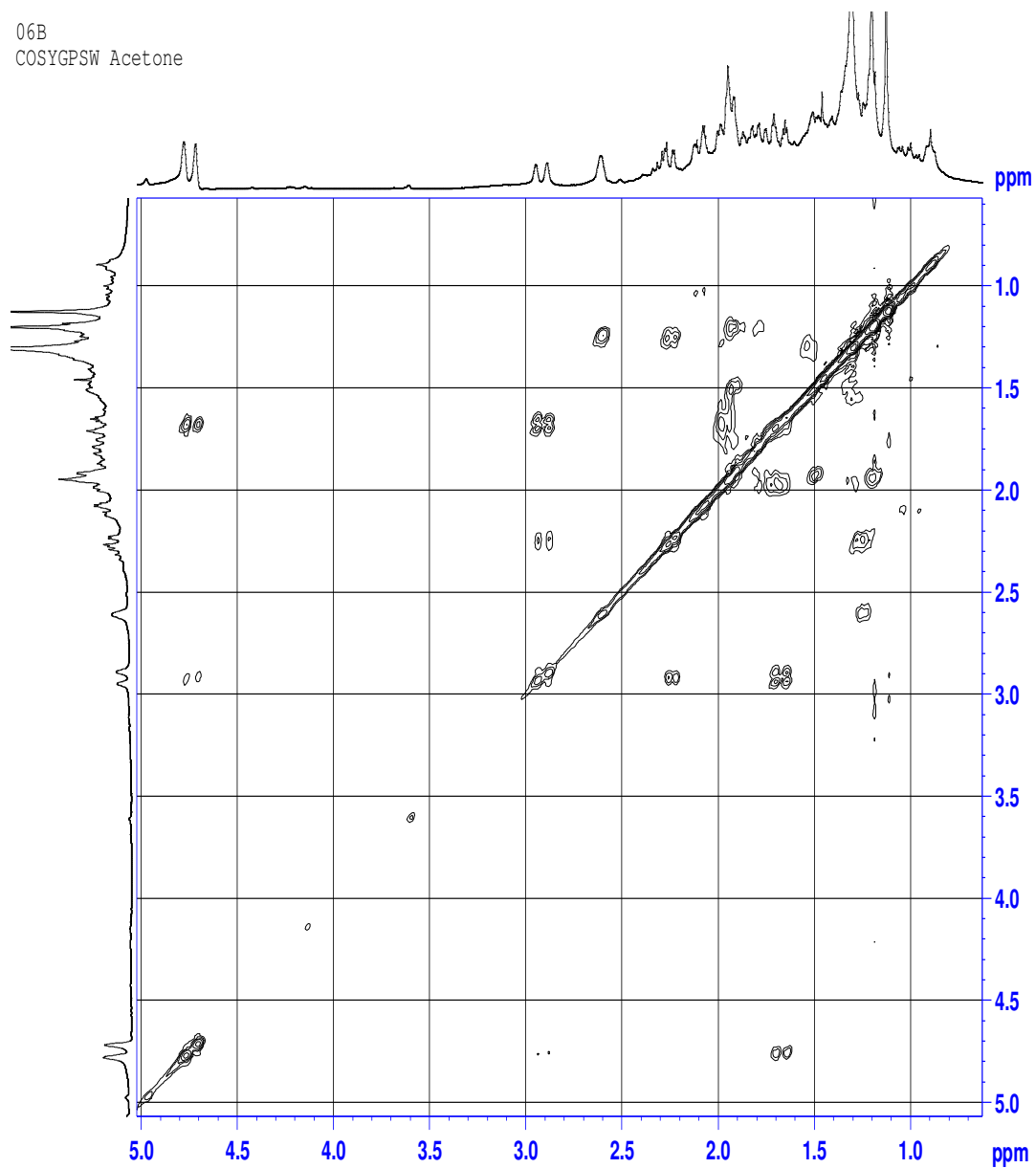


Figure 69: COSY spectrum (300 MHz) for 06B

06B
COSYGPSW Acetone

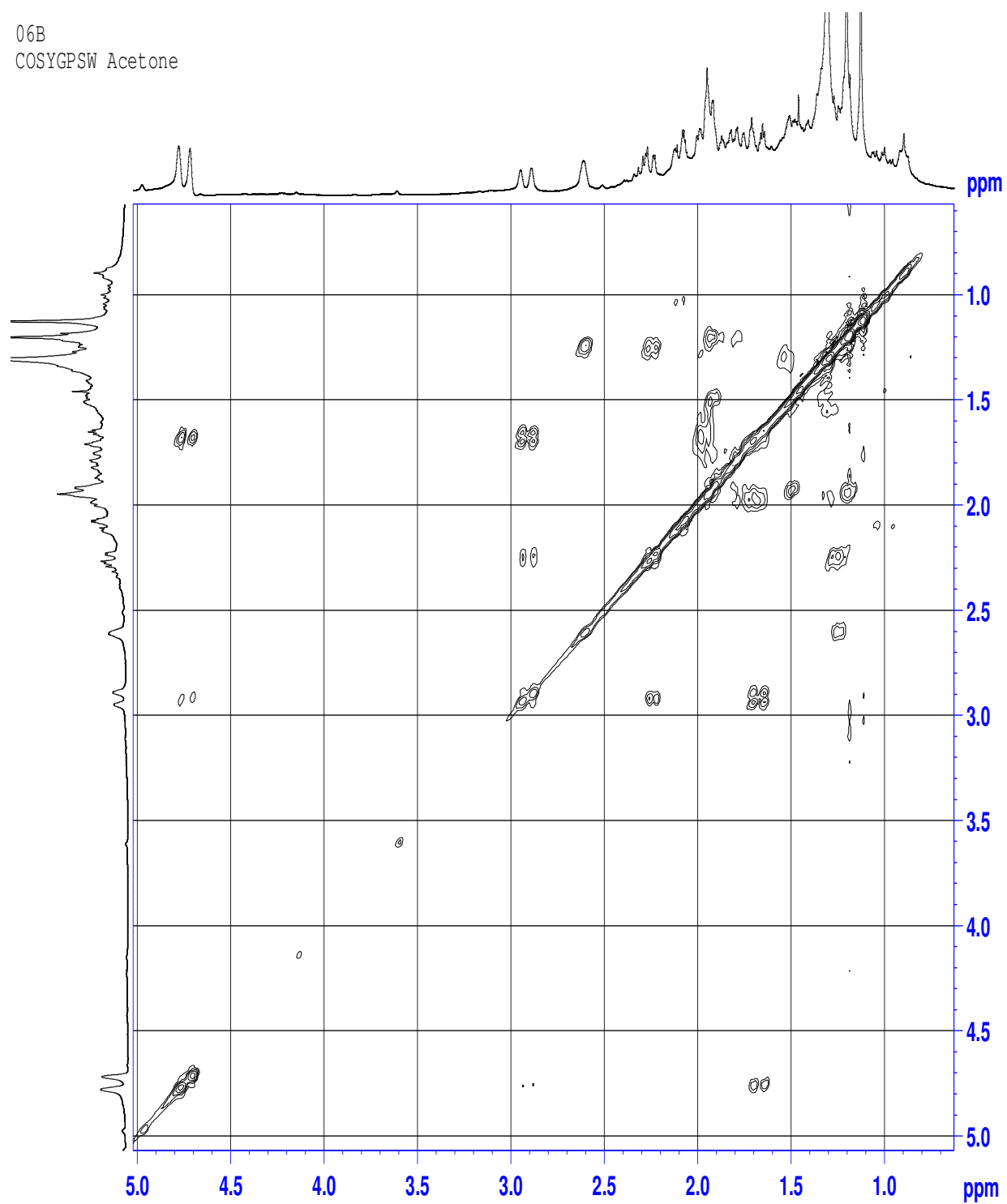


Figure 70: Expanded COSY spectrum (300 MHz) for 06B

EM06b #97 RT: 2.83 AV: 1 NL: 5.92E5
T: + c EI Full ms [49.50-500.50]

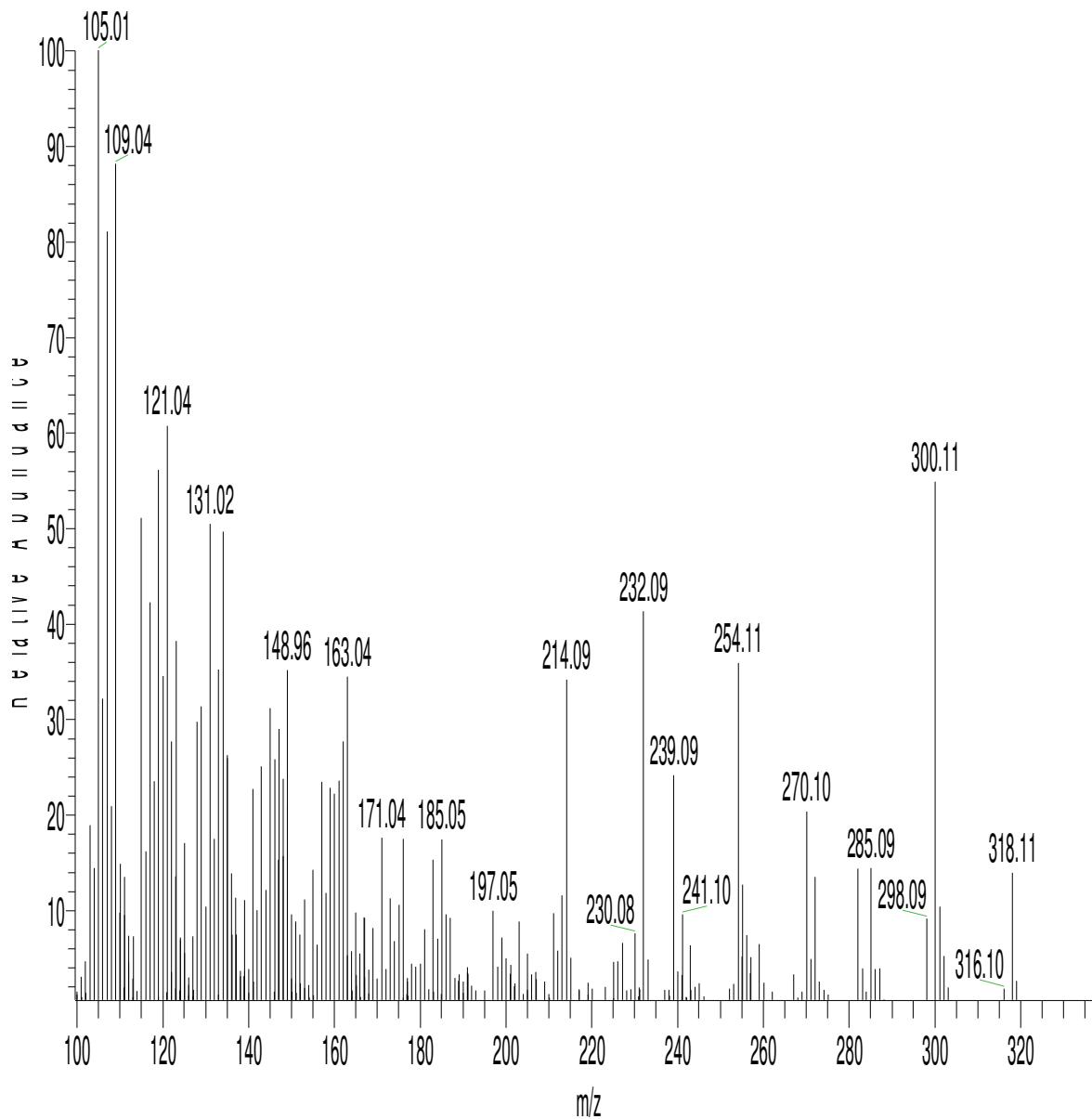


Figure 71: Low Resolution ESI MS Spectrum for O06B

EM06B_HREI-c1 #33 RT: 0.96 AV: 1 NL: 4.39E5
T: +c EI Full ms [303.50-333.50]

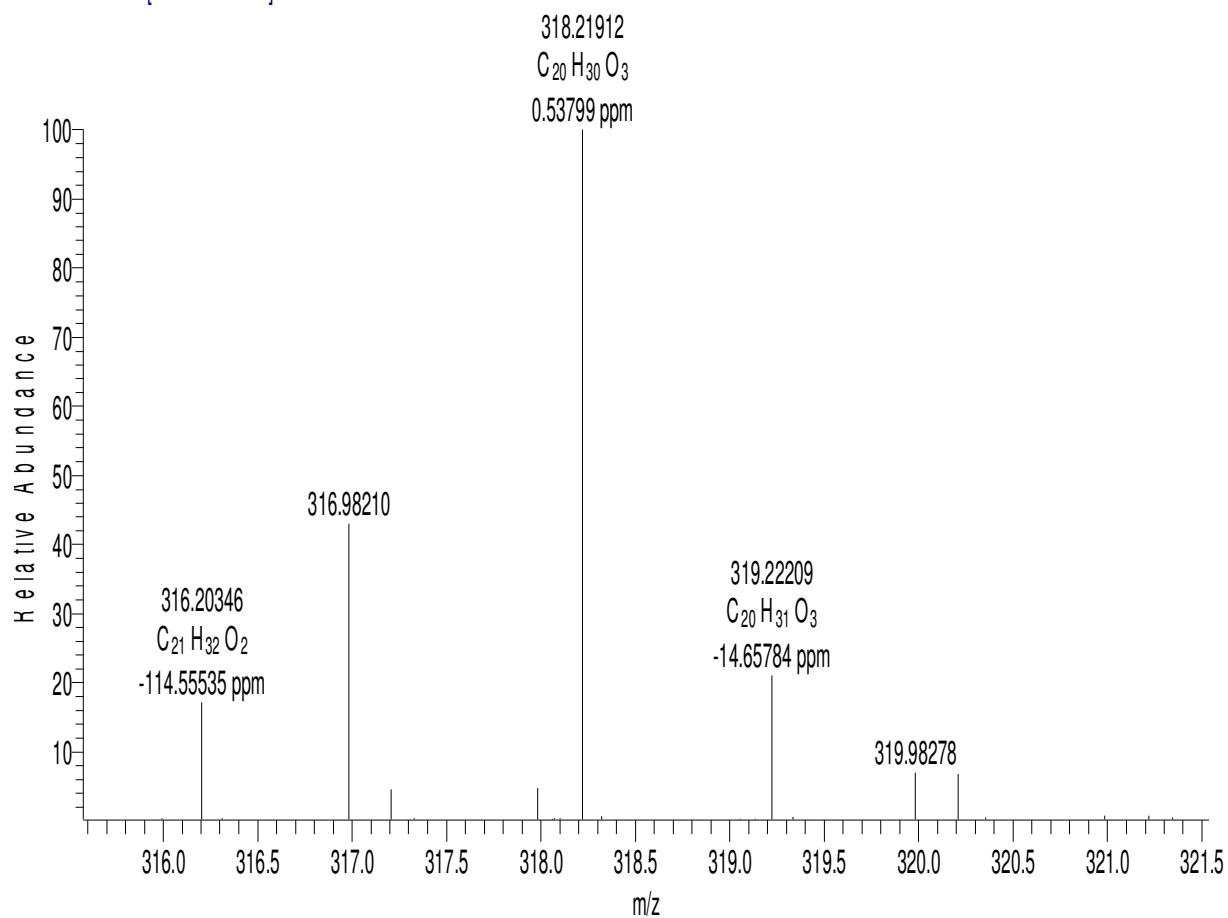


Figure 72: Accurate mass spectra scan ESI MS Spectrum for O06B

APPENDIX 4

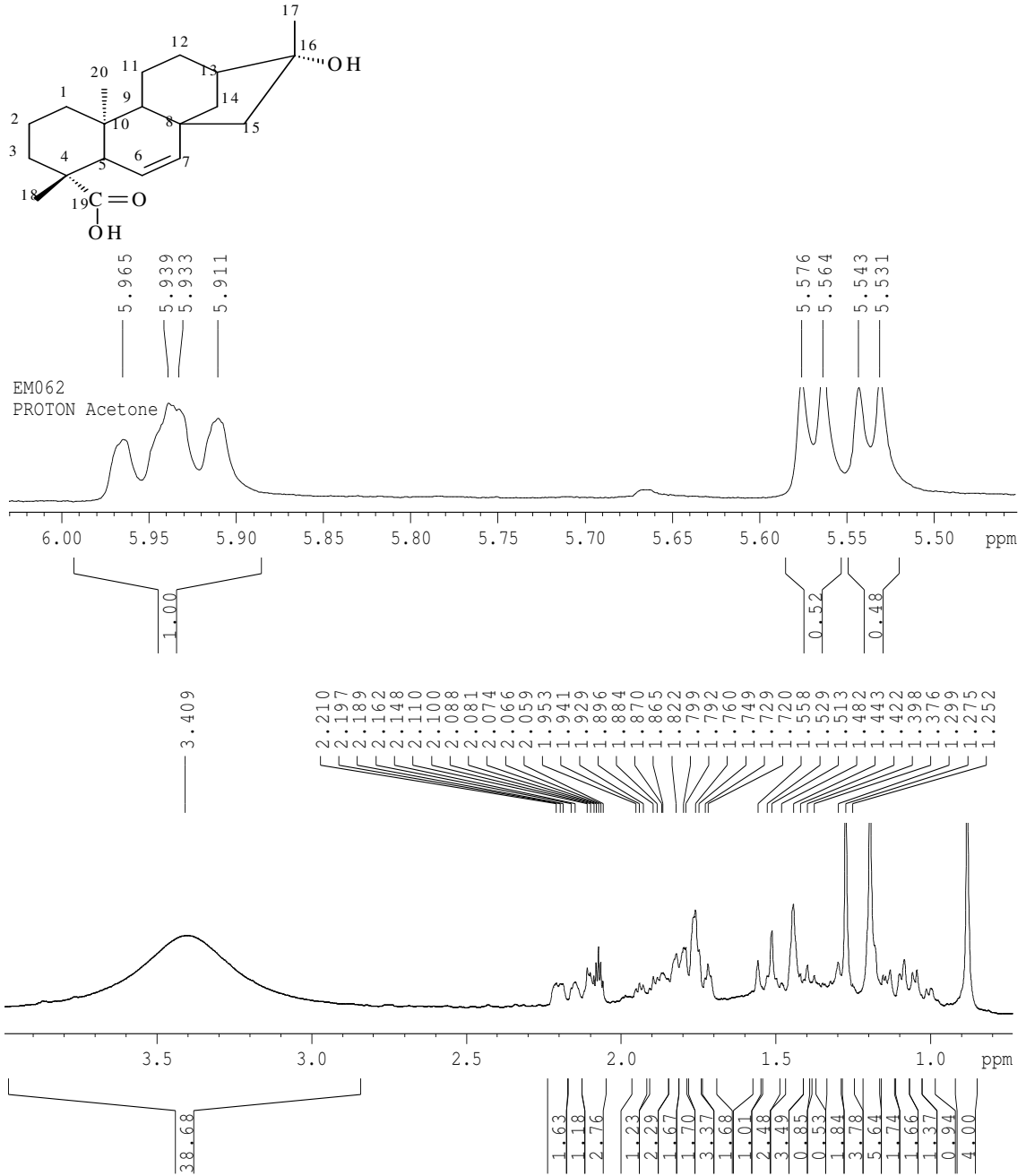


Figure 73: ¹H NMR spectrum (300 MHz) for 06-2

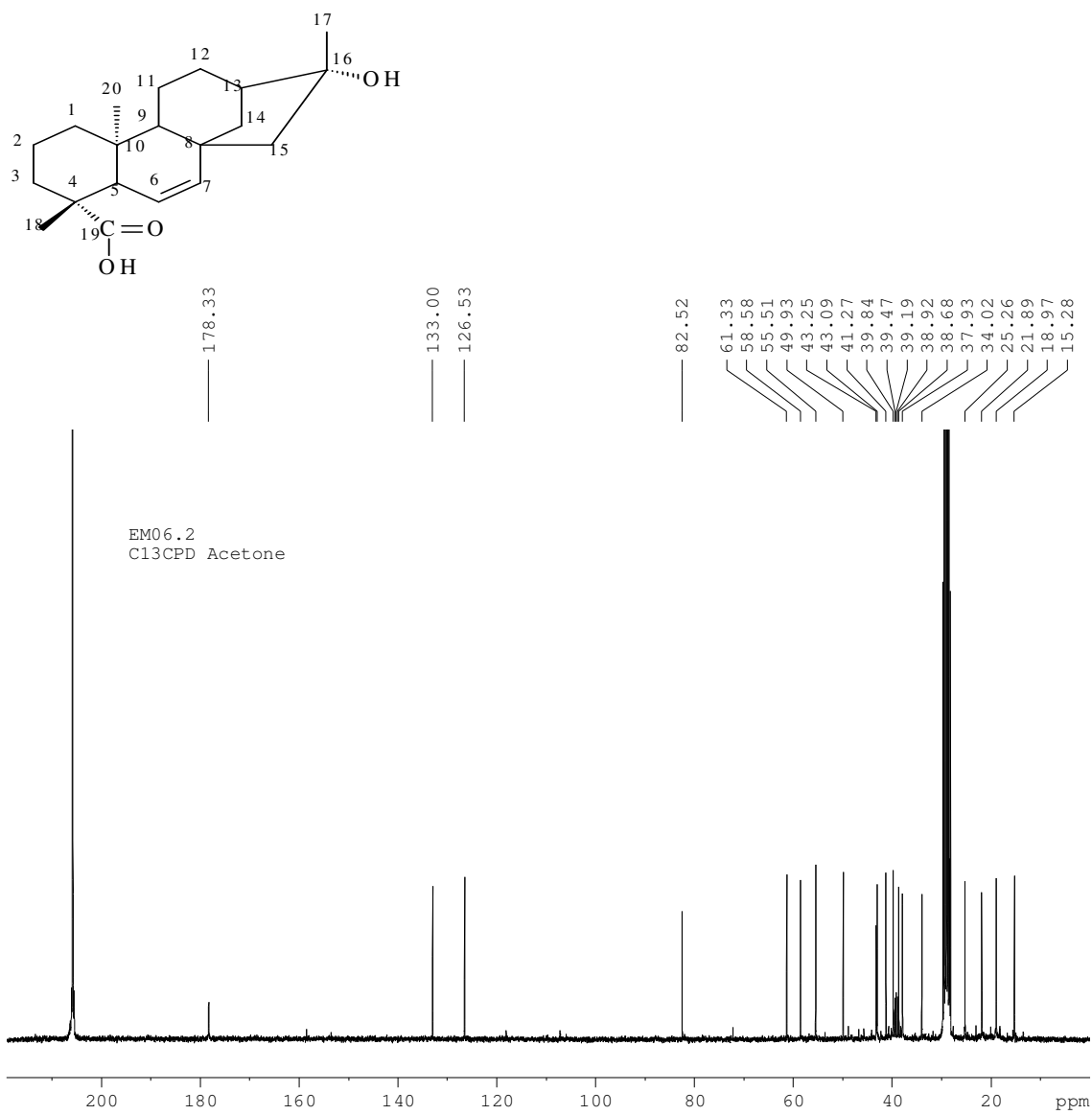


Figure 74: ^{13}C NMR spectrum (300 MHz) for 06-2

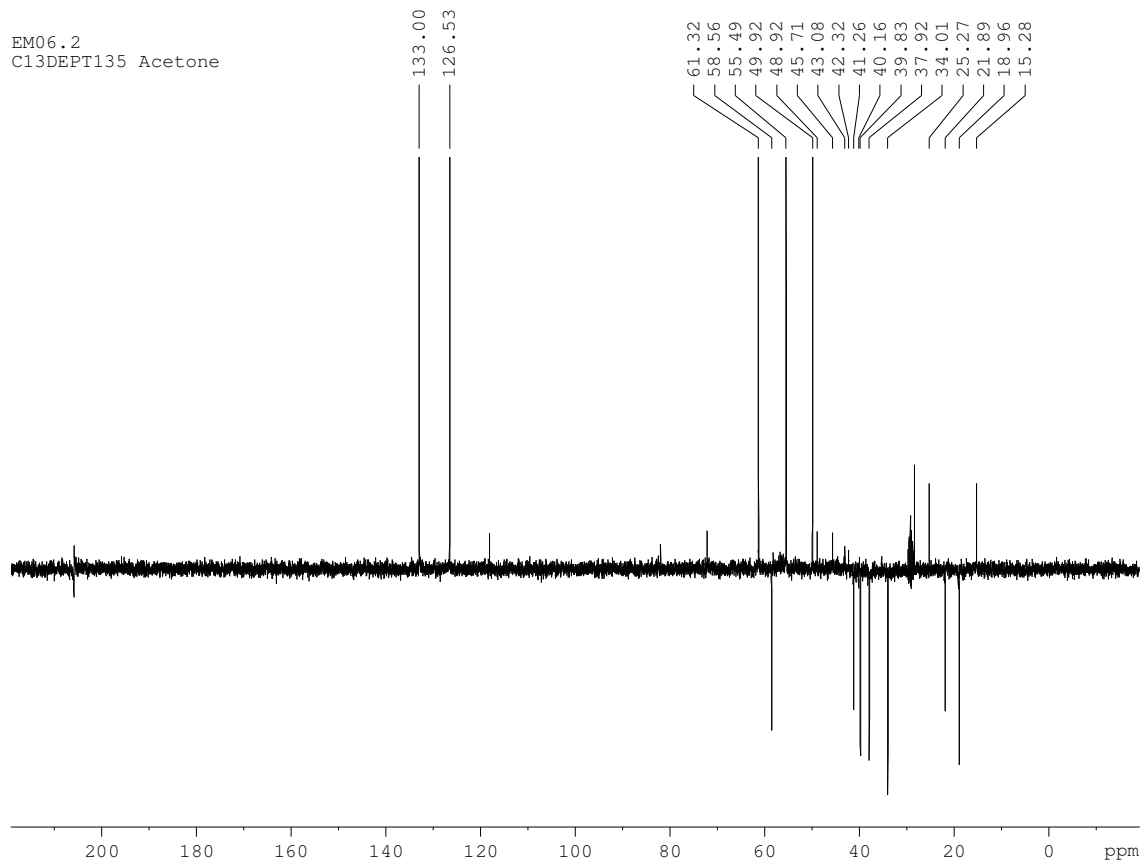


Figure 75: DEPT135 spectrum (300 MHz) for 06-2

EM06.2
HMOCGP Acetone

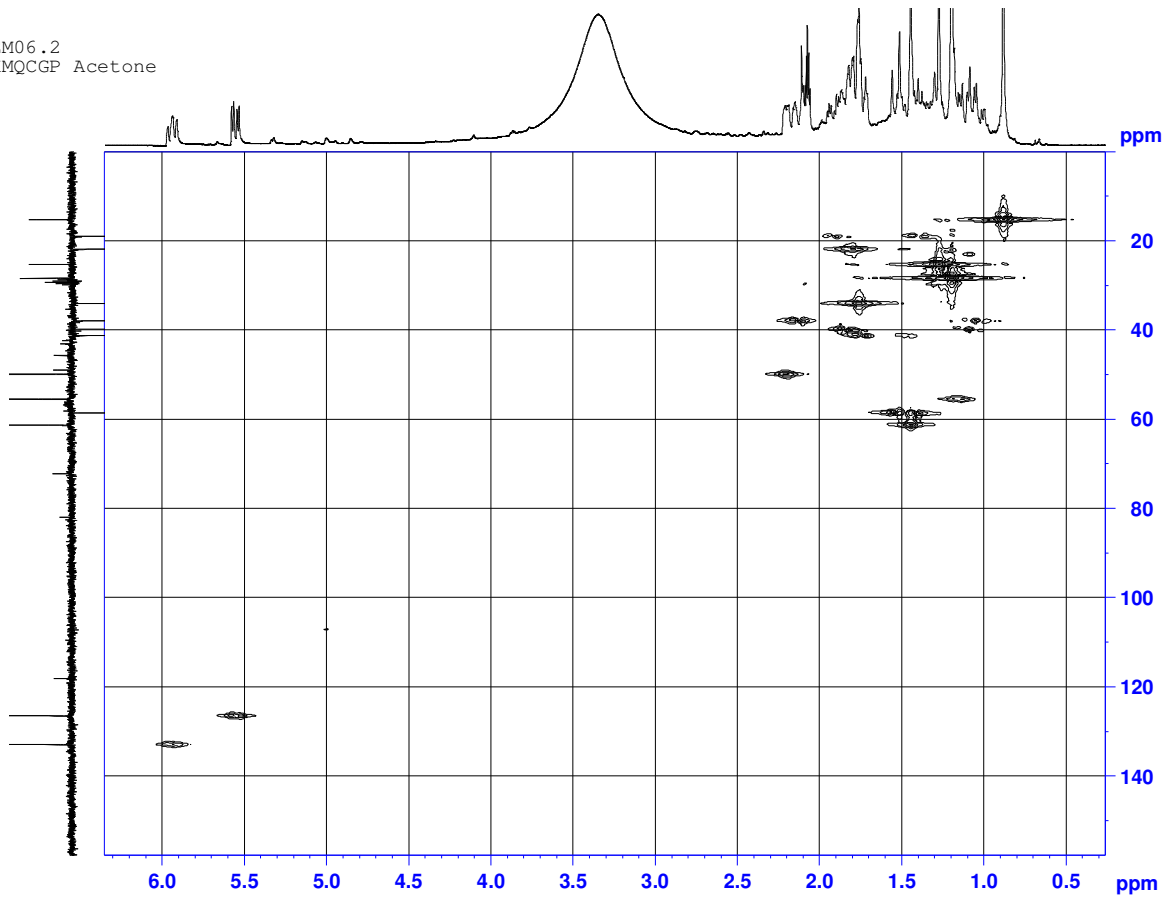


Figure 76: HMQC spectrum (300 MHz) for 06-2

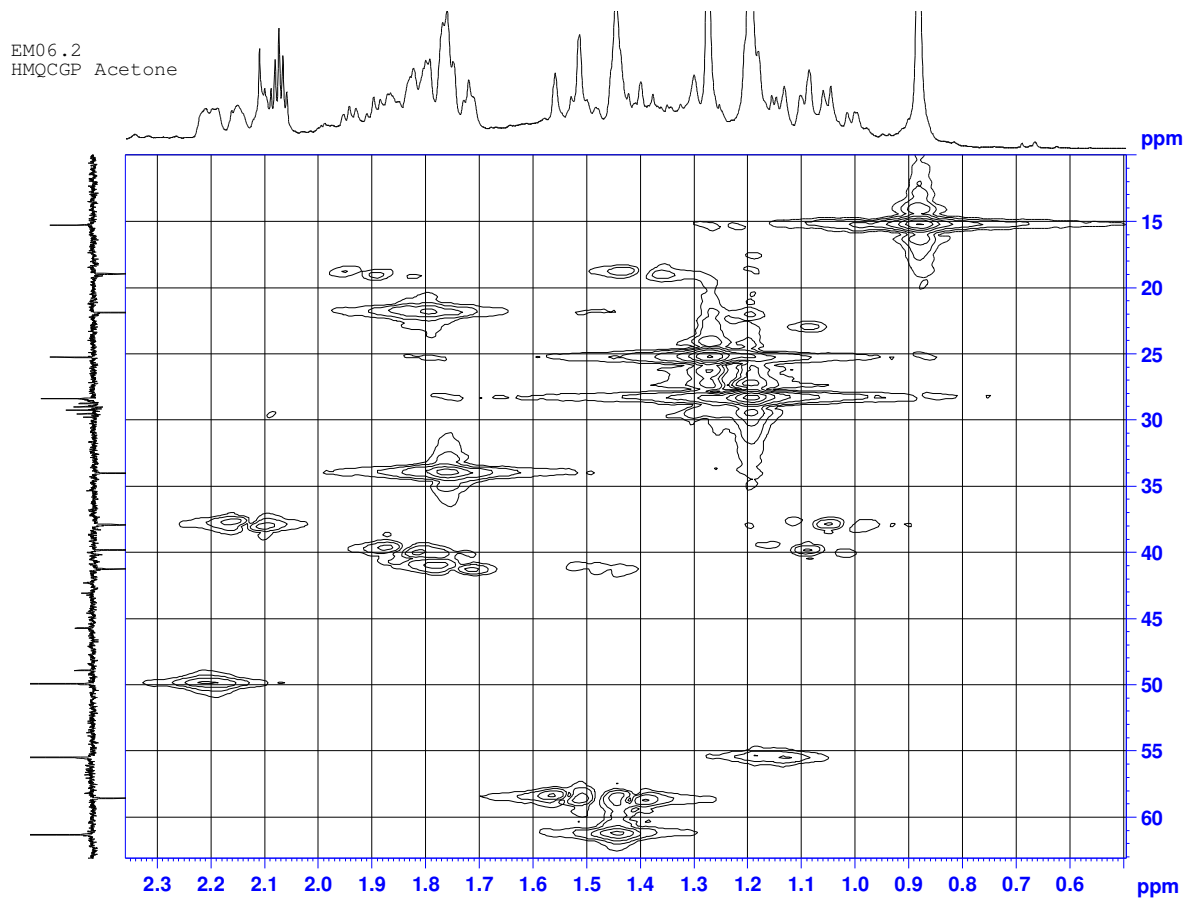


Figure 77: Expanded HMQC spectrum (300 MHz) for 06-2

EM06.2
HMBCGPND Acetone

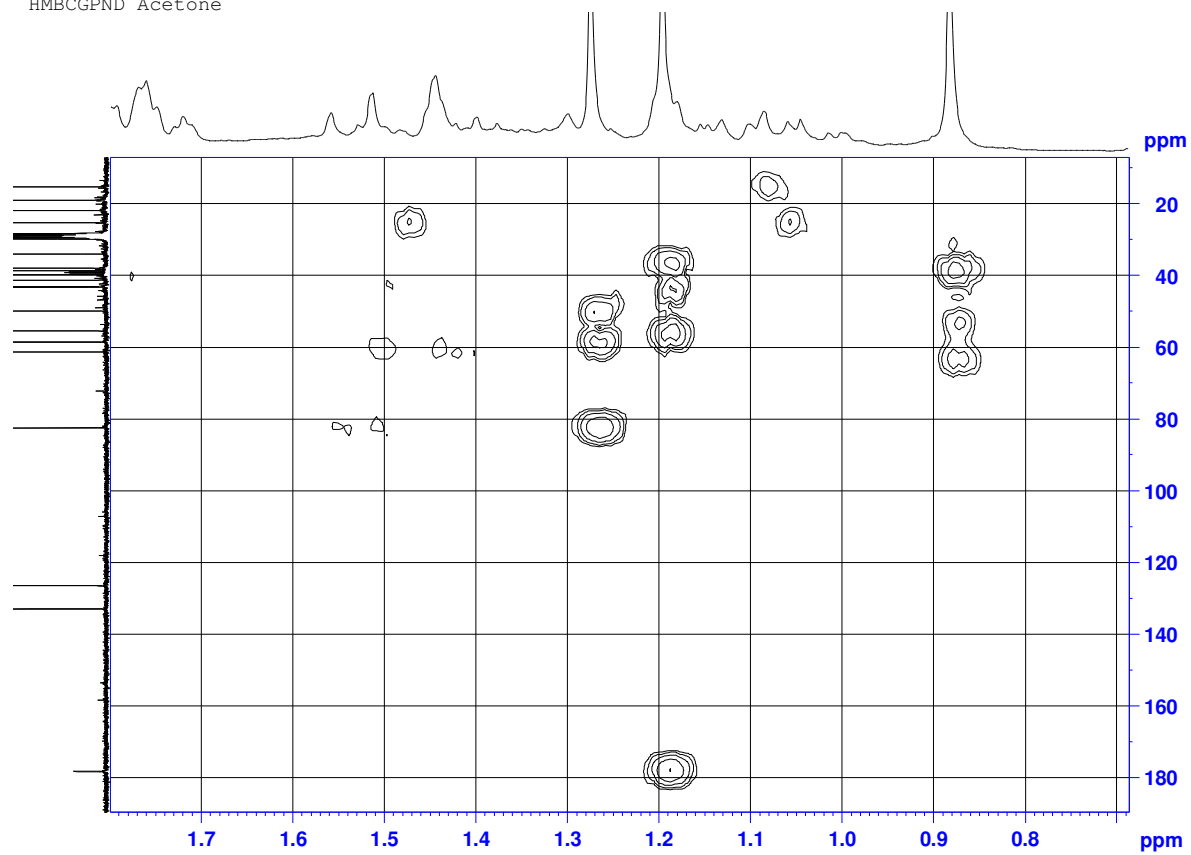


Figure 78: HMBC spectrum (300 MHz) for 06-2

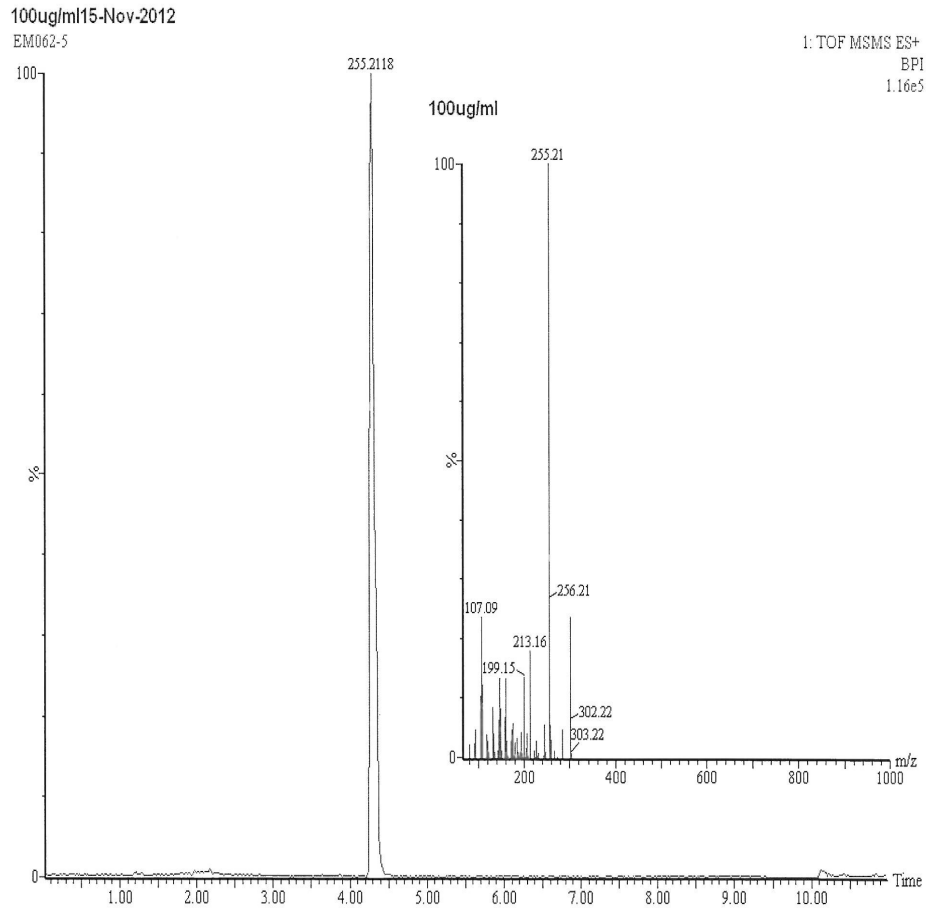
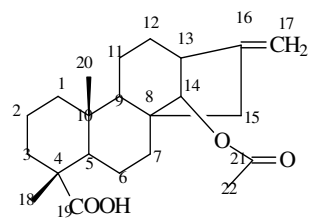


Figure 79: TOF MSMS spectrum (300 MHz) for 06-2

APPENDIX 5



EM06-52

C13CPD CDC13

183.69

170.11

155.13

103.17

69.37
60.88
59.86
47.82
43.77
43.06
42.25
41.12
40.20
39.53
39.20
38.56
37.70
28.99
21.71
18.95
15.45

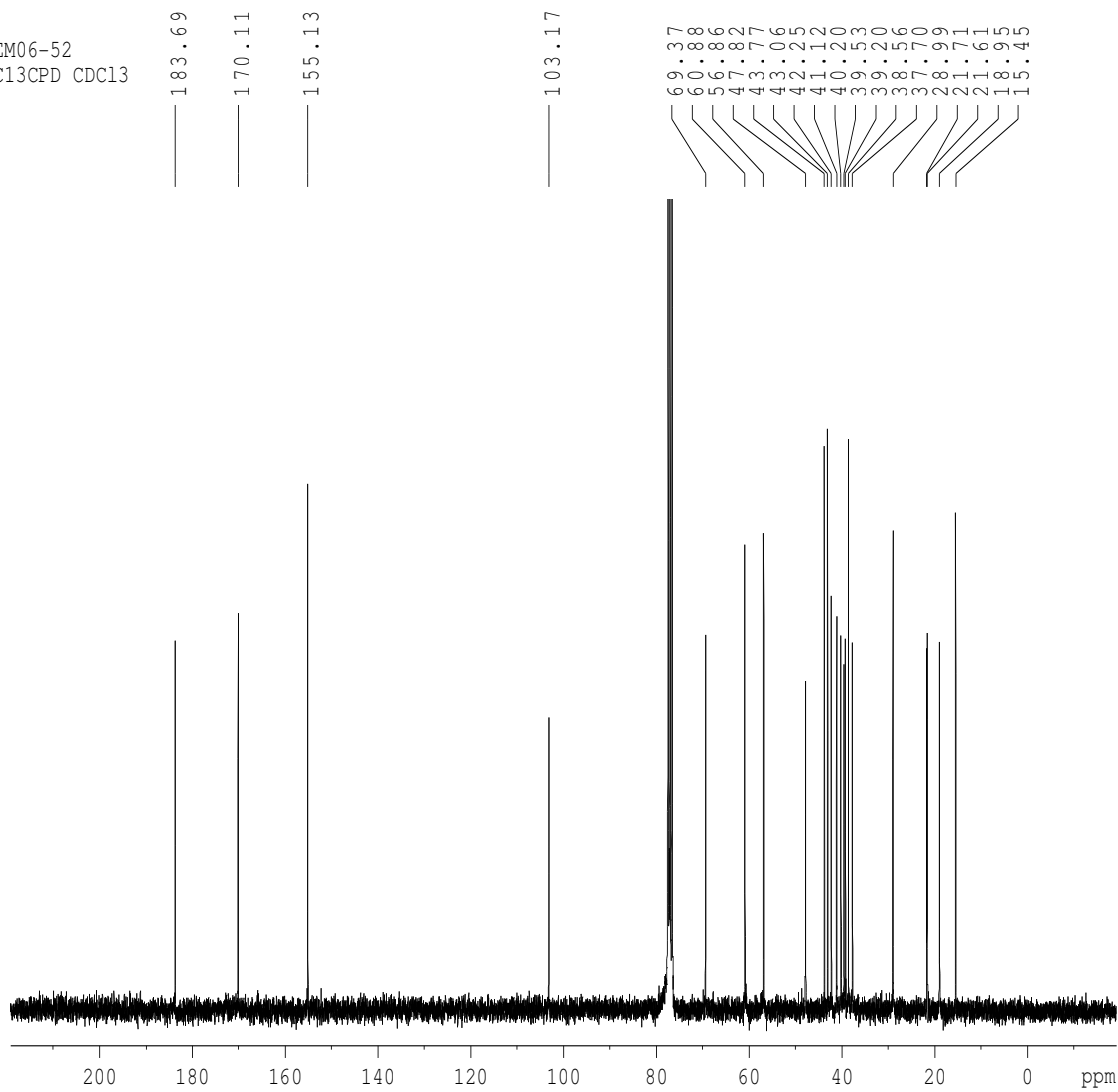


Figure 80: ¹³C NMR spectrum (300 MHz) for 0652

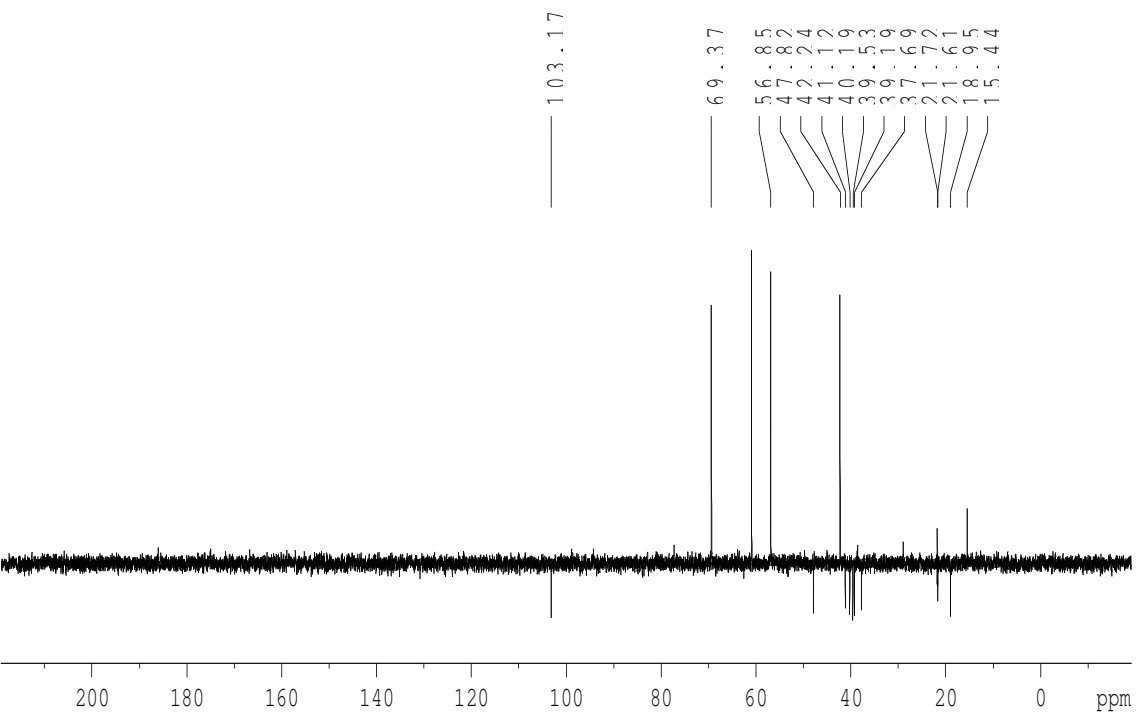
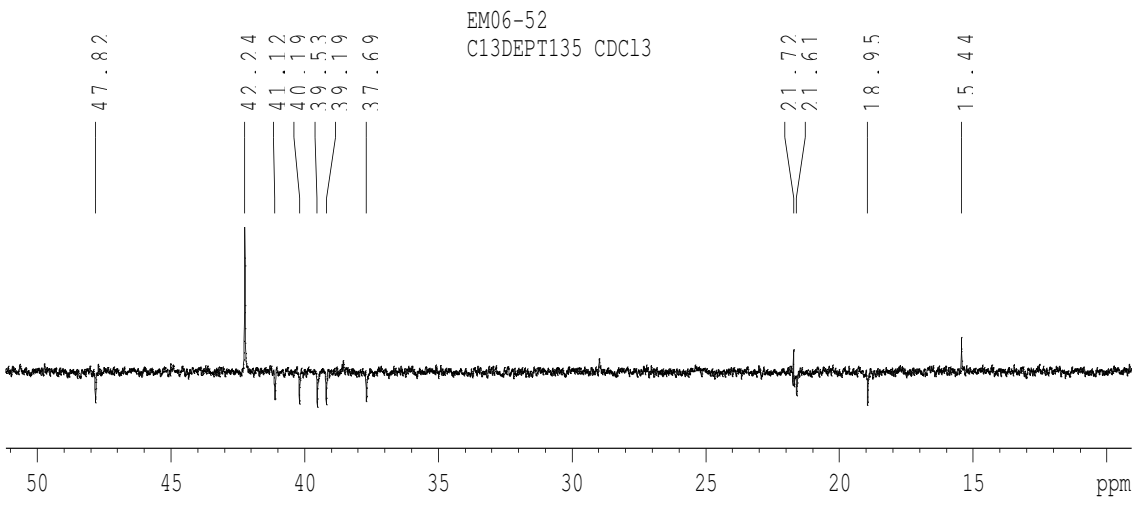
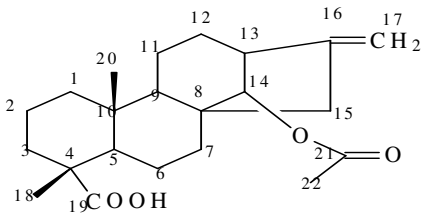


Figure 81: DEPT135 spectrum (300 MHz) for 0652

EM06-52
HSQCGP CDC13

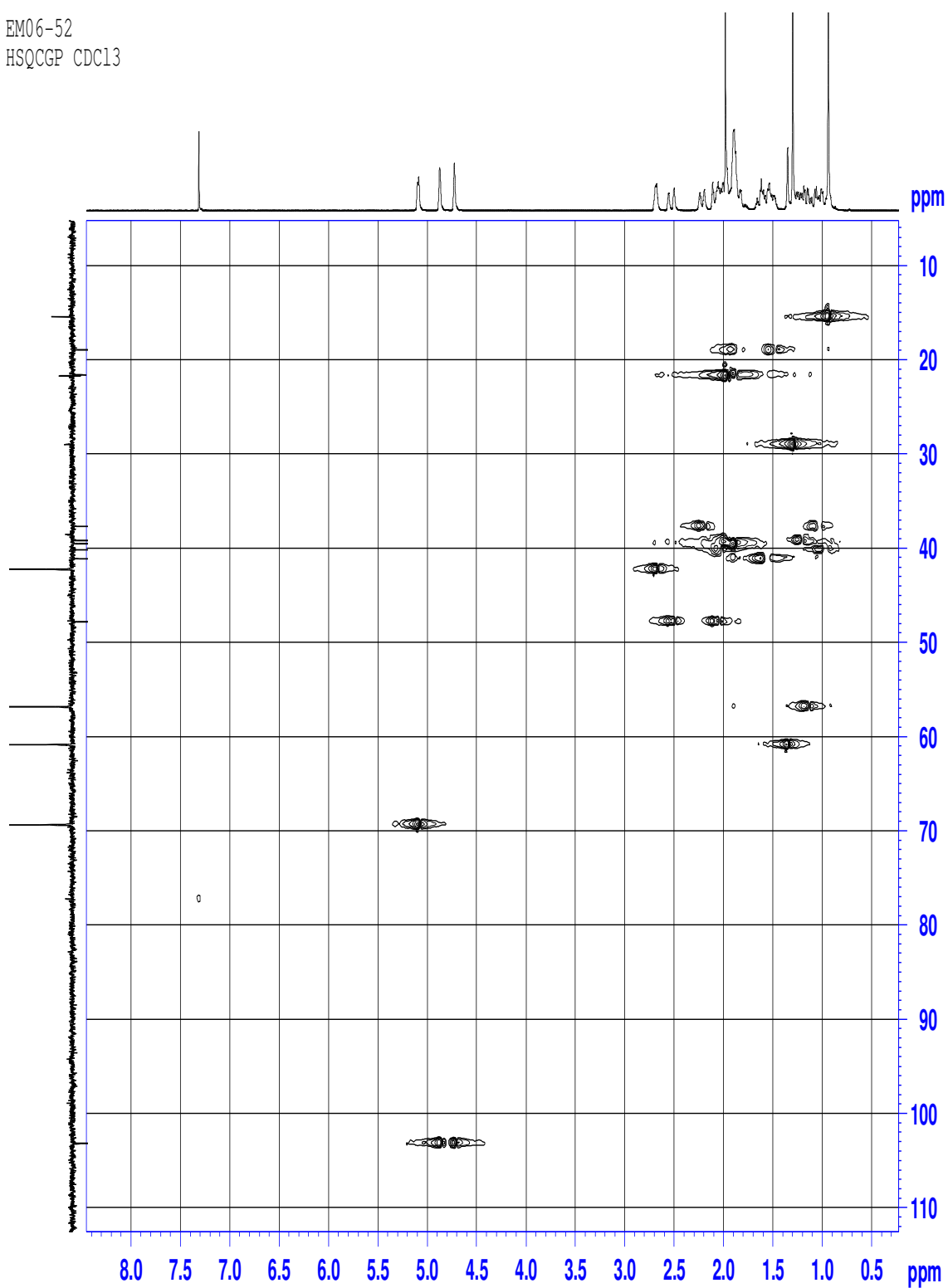


Figure 82: HSQC spectrum (300 MHz) for 0652

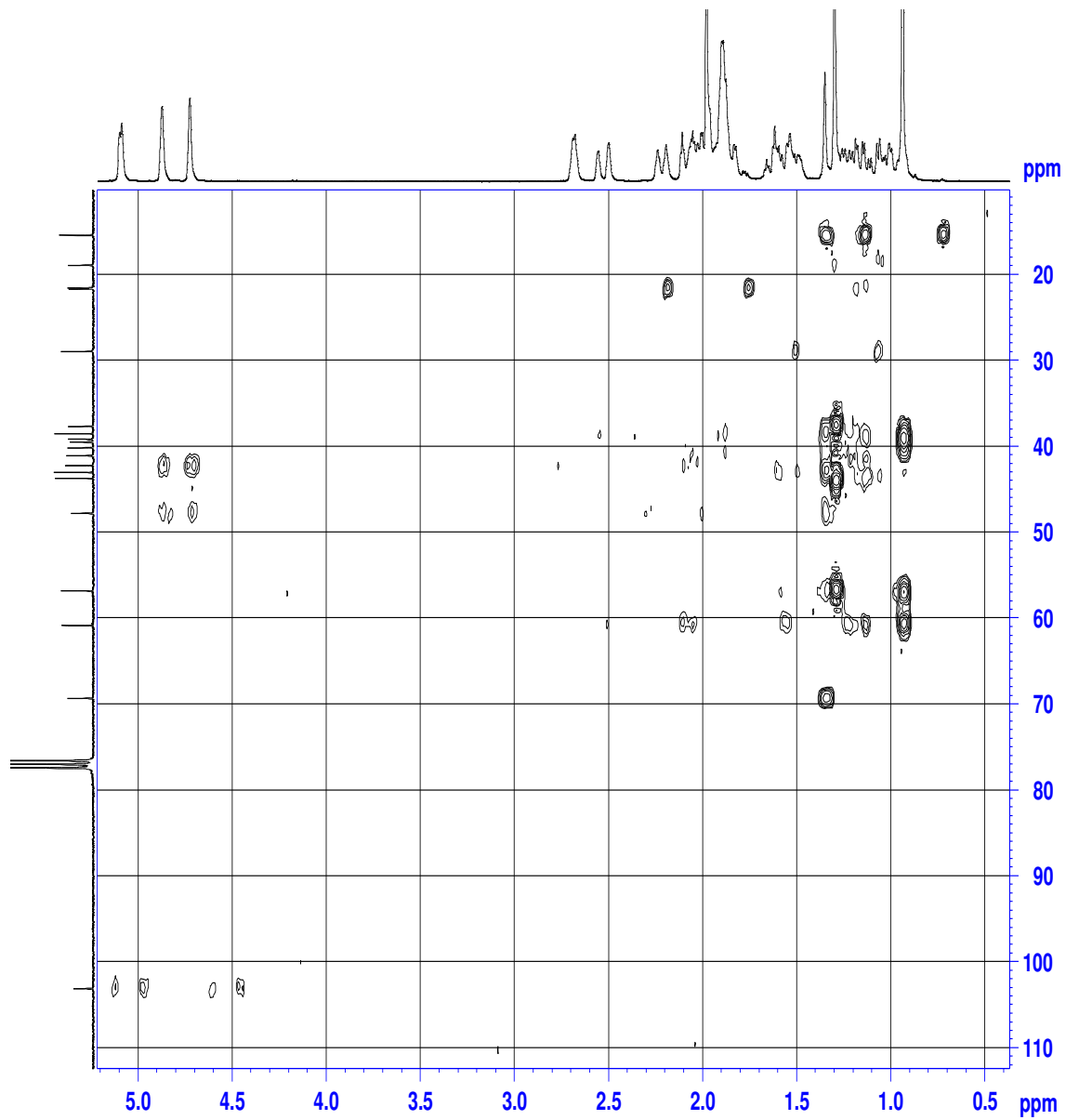


Figure 83: HMBC spectrum (300 MHz) for 0652

EM06-52
COSYGPSW CDCl₃

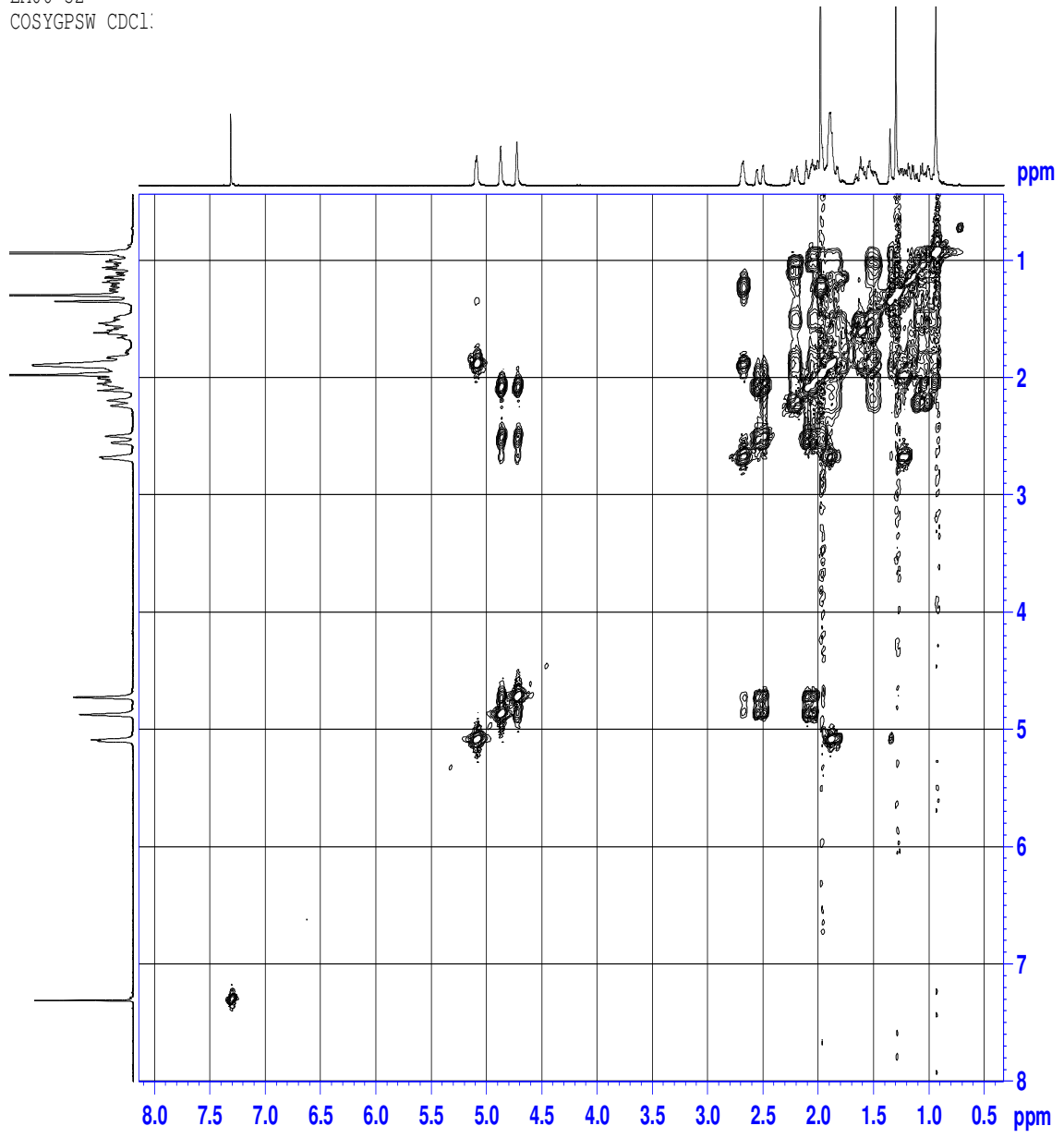


Figure 84: COSY spectrum (300 MHz) for 0652

EM06-52
COSYGPSW CDCl₃

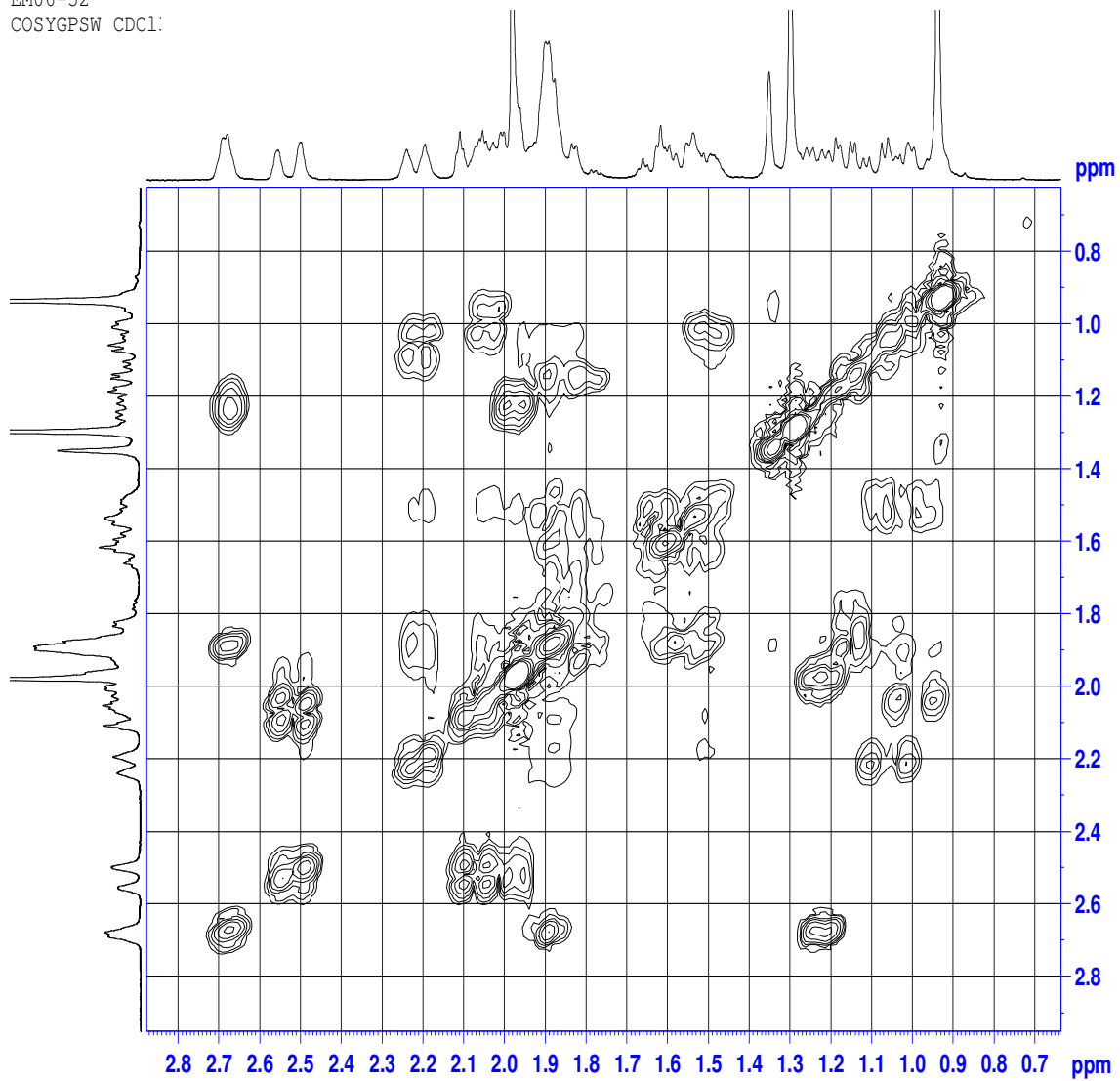


Figure 85: Expanded COSY spectrum (300 MHz) for 0652

EM 0652

DK_TUT_130211_6.1192 (0.295) Cm (1192:1201-1224:1236)

1: TOF MS ES-
6.04e5

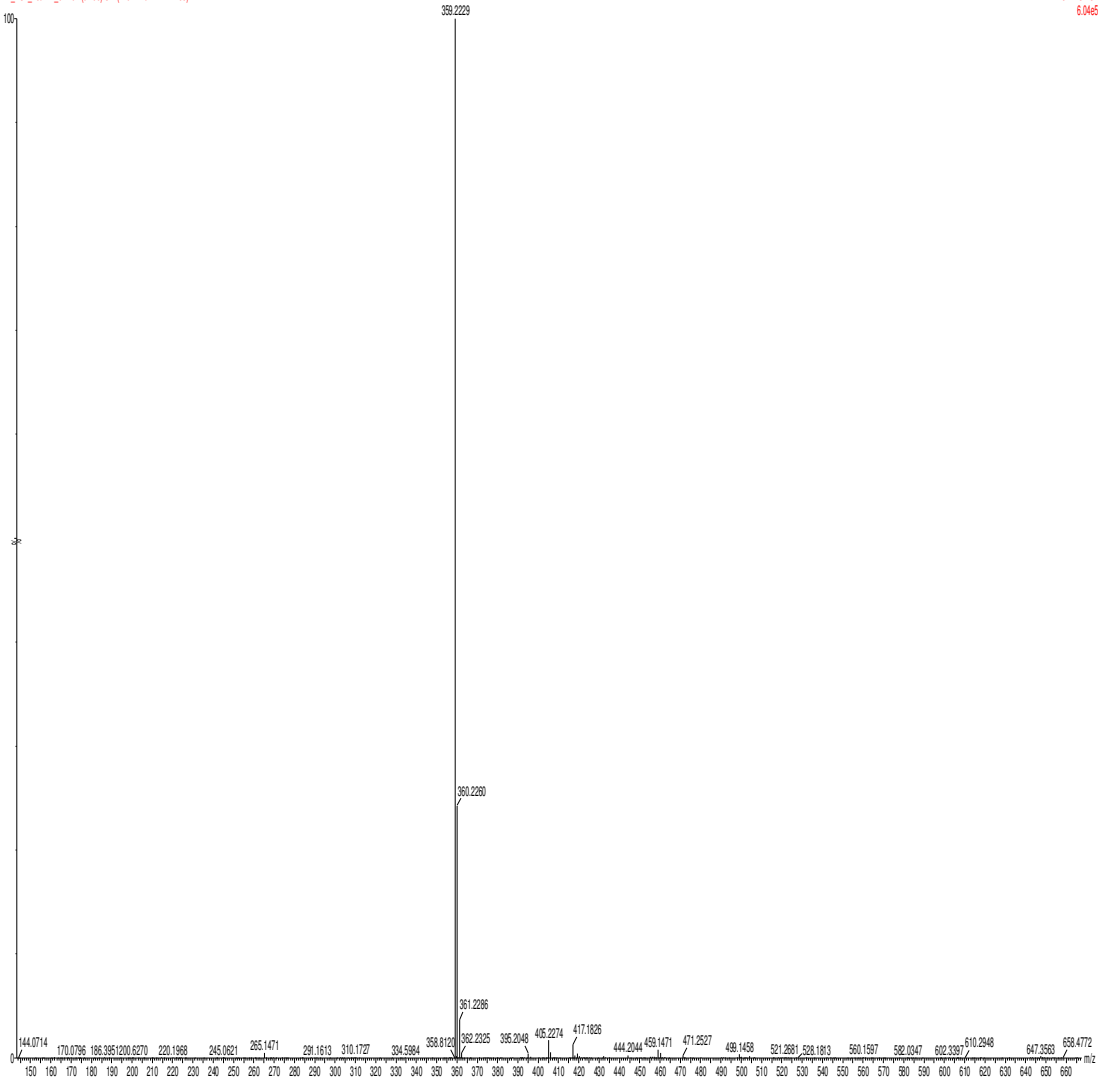


Figure 86: TOF MS-MS spectrum for 0652

APPENDIX 6

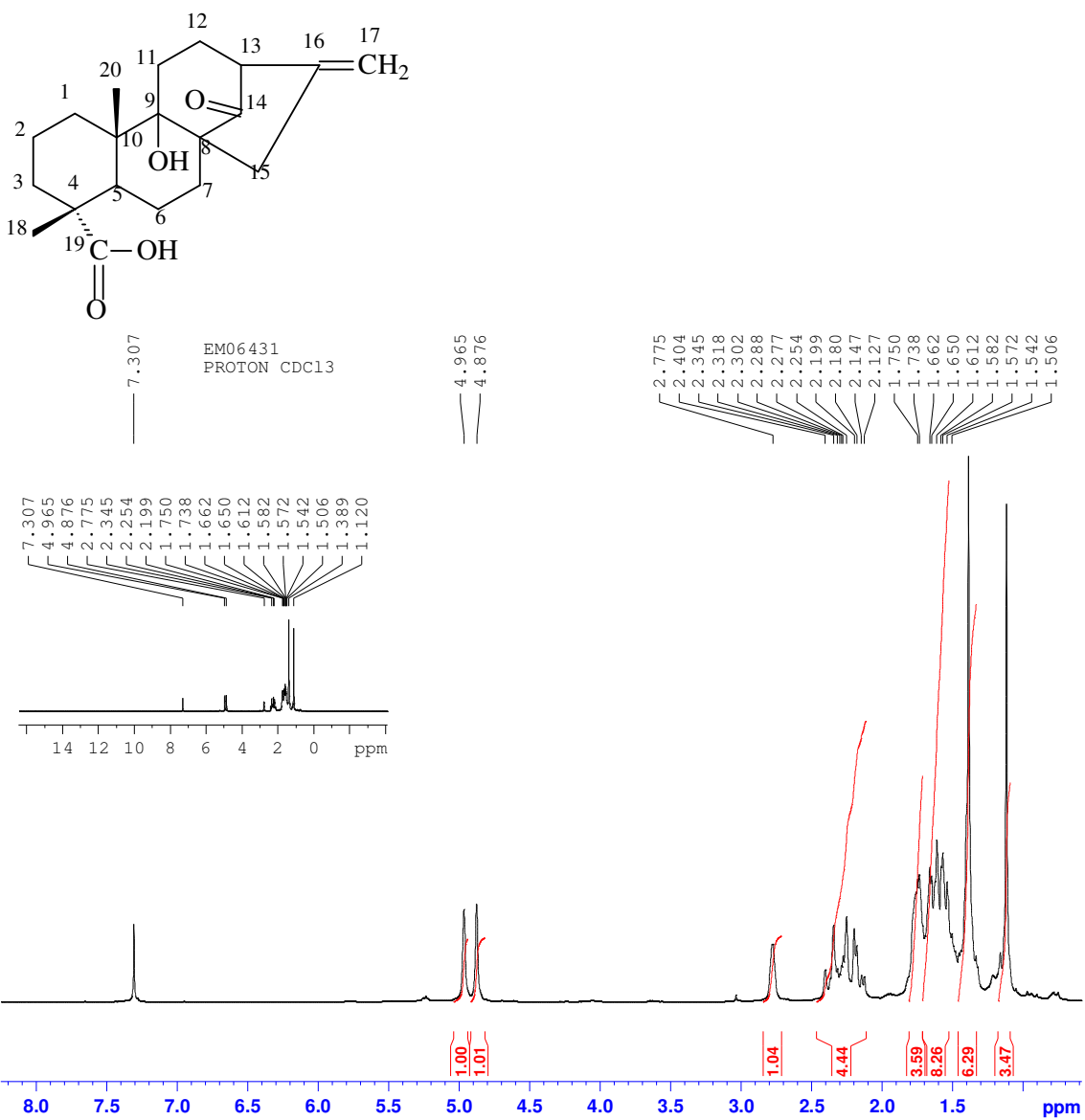
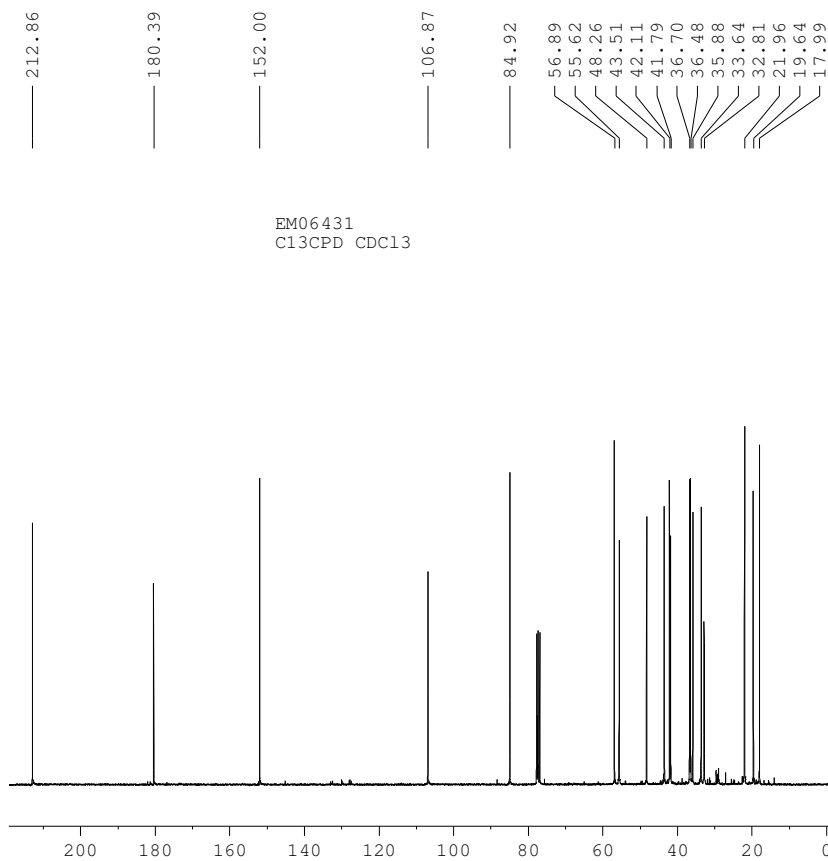
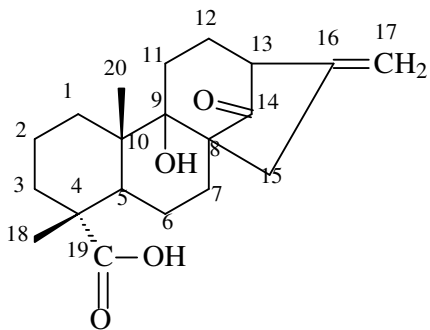


Figure 87: ¹H NMR spectrum (300 MHz) for 06431



```

Current Data Parameters
NAME          EM06431
EXPNO        11
PROCNO       1

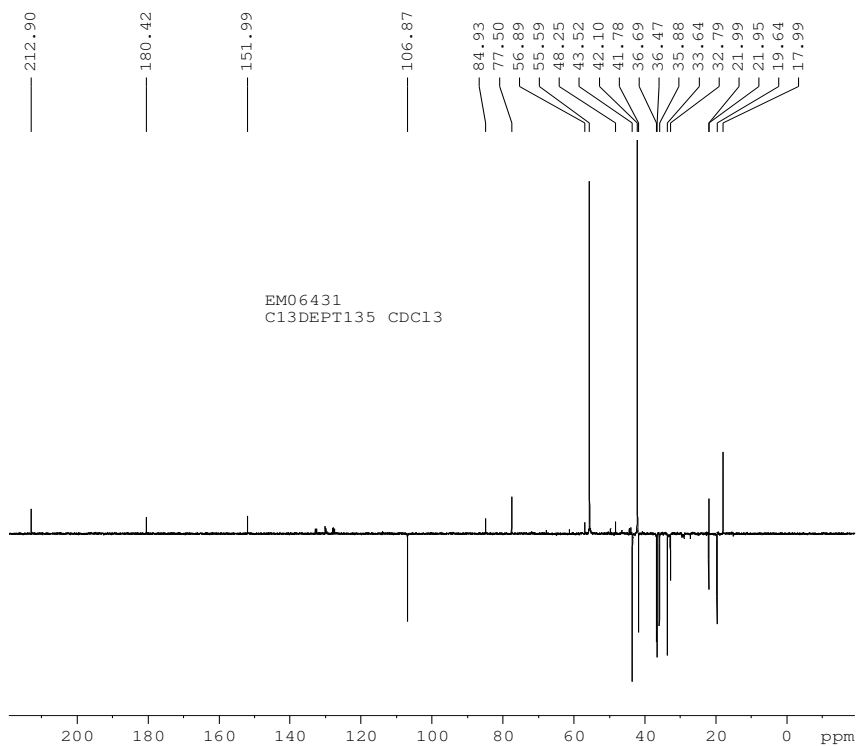
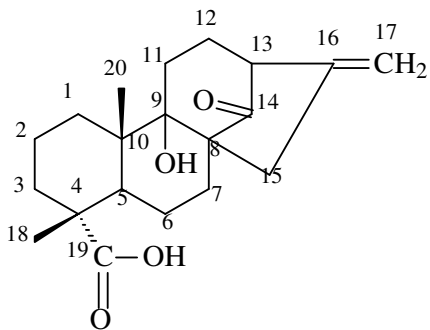
F2 - Acquisition Parameters
Date_        20120807
Time         1.04
INSTRUM      spect
PROBHD       5 mm QNP 1H/15
PULPROG      zgpg30
TD           65536
SOLVENT      CDCl3
NS           2048
DS           4
SWH          17985.611 Hz
FIDRES       0.274439 Hz
AQ           1.8219508 sec
RG           13004
DW           27.800 usec
DE           6.00 usec
TE           296.2 K
D1           2.00000000 sec
d11          0.03000000 sec
DELTA        1.89999998 sec
TD0          1

===== CHANNEL f1 =====
NUC1          13C
P1            8.50 usec
PL1           -2.00 dB
SFO1          75.4752953 MHz

===== CHANNEL f2 =====
CPDPRG2       waltz16
NUC2          1H
PCPD2         80.00 usec
PL2           0.00 dB
PL12          18.06 dB
PL13          18.00 dB
SFO2          300.1312005 MHz

F2 - Processing parameters
SI            32768
SF            75.4677490 MHz
WDW           EM
SSB           0
LB            1.00 Hz
GB            0
PC            1.40
  
```

Figure 88: ¹³C NMR spectrum (300 MHz) for 06431



```

Current Data Parameters
NAME          EM06431
EXPNO         12
PROCNO        1

F2 - Acquisition Parameters
Date_         20120807
Time          2.44
INSTRUM       spect
PROBHD        5 mm QNP 1H/15
PULPROG       dept135
TD            65536
SOLVENT       CDC13
NS            1536
DS            4
SWH           17985.611 Hz
FIDRES        0.274439 Hz
AQ            1.8219508 sec
RG            16384
DW            27.800 usec
DE            6.00 usec
TE            295.2 K
CNST2         145.000000
D1            2.00000000 sec
d2            0.00344828 sec
d12           0.00002000 sec
DELTA         0.00001082 sec
TDO           1

----- CHANNEL f1 -----
NUC1          13C
P1            8.50 usec
P2            17.00 usec
PL1           -2.00 dB
SFO1          75.4752953 MHz

----- CHANNEL f2 -----
CPDPRG2       waltz16
NUC2          1H
P3            10.00 usec
P4            20.00 usec
PCPD2         80.00 usec
PL2           0.00 dB
PL12          18.05 dB
SFO2          300.1312005 MHz

F2 - Processing parameters
SI            32768
SF            75.4677490 MHz
WDW           EM
SSB           0
LB            1.00 Hz
GB            0
PC            1.40

```

Figure 89: DEPT 135 spectrum (300 MHz) for 06431

EM06431
HSQCGP CDC13

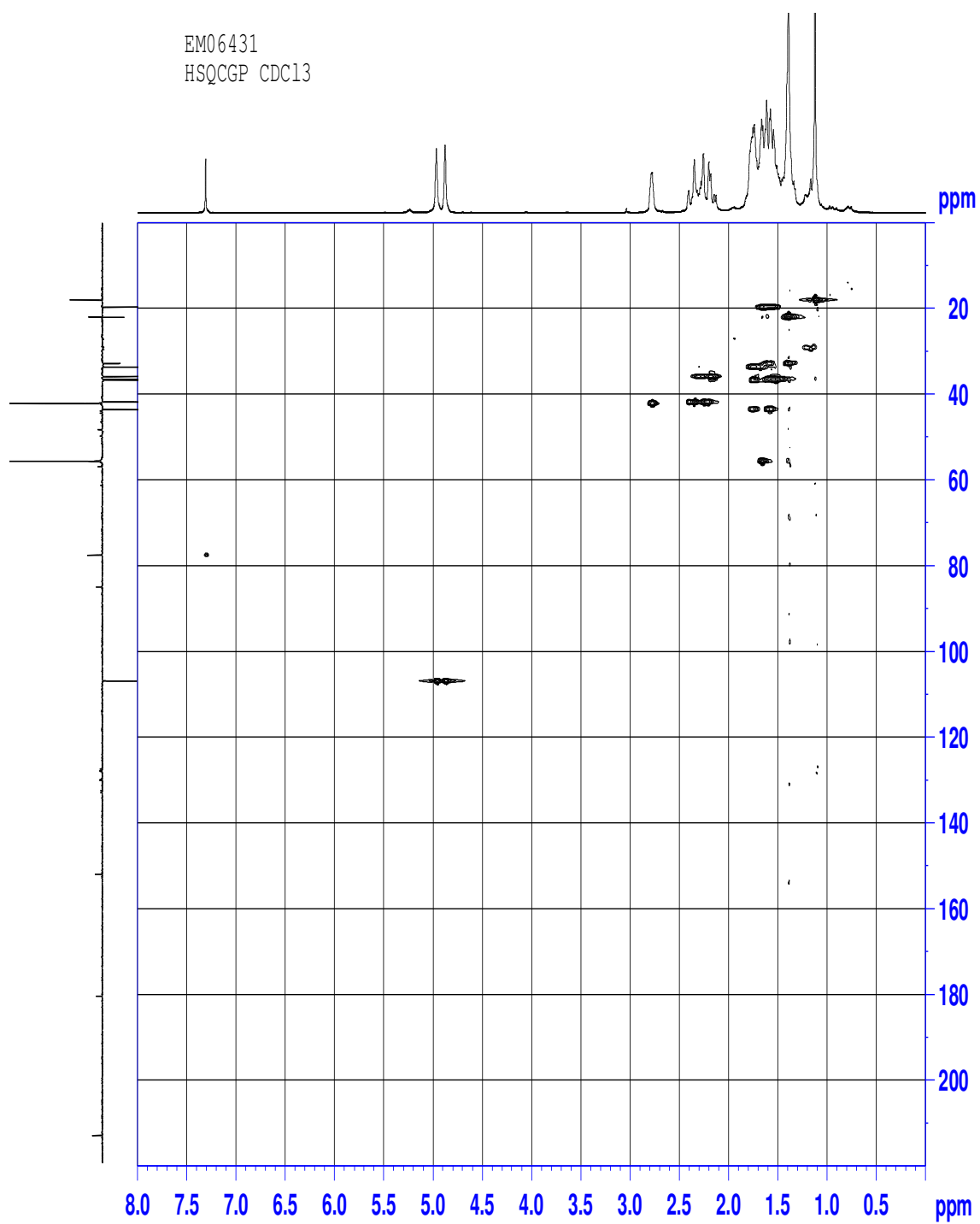


Figure 90: HSQC spectrum (300 MHz) for 06431

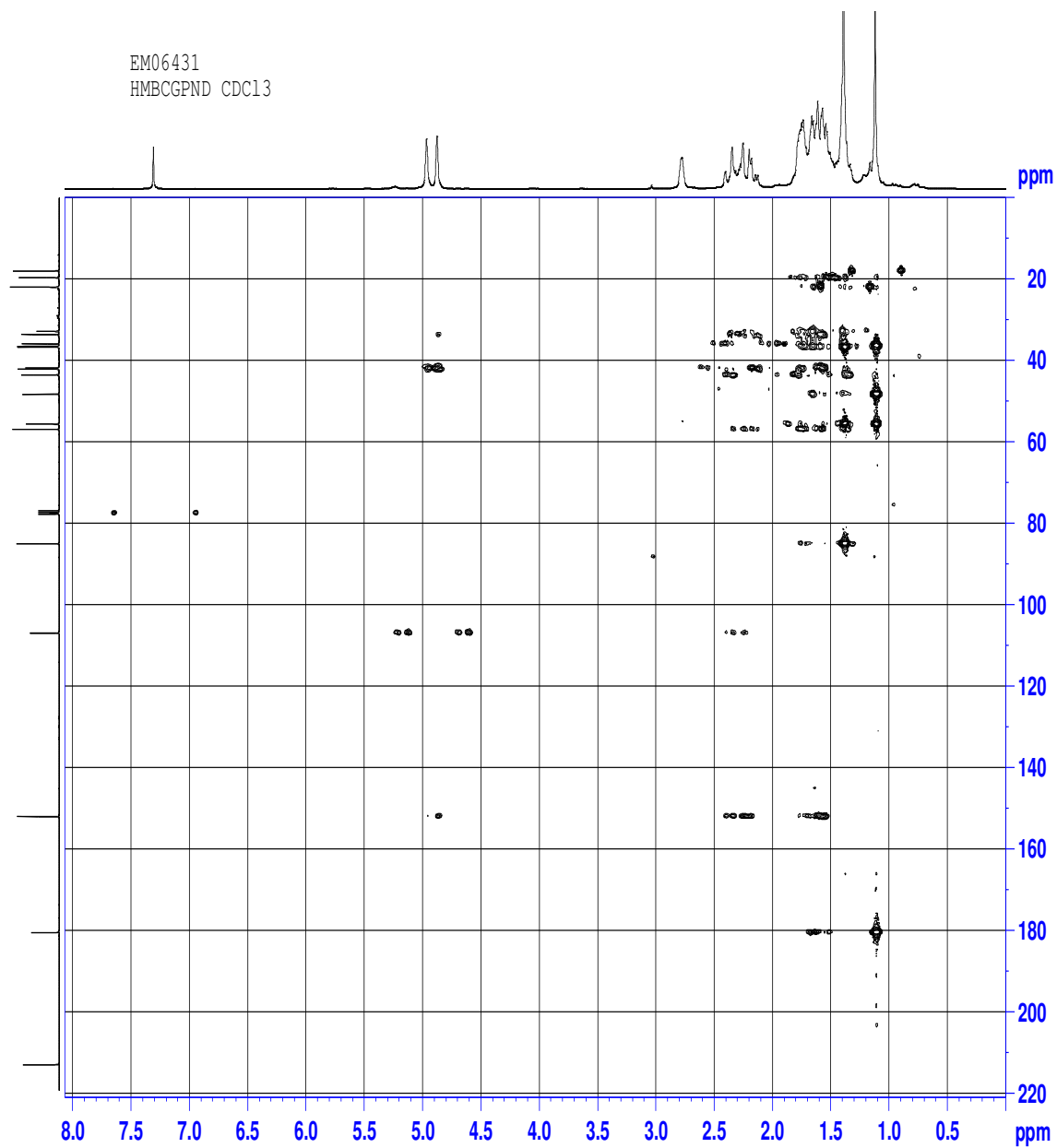


Figure 91: HSQC spectrum (300 MHz) for 06431

EM06431
HSQC GP CDC13

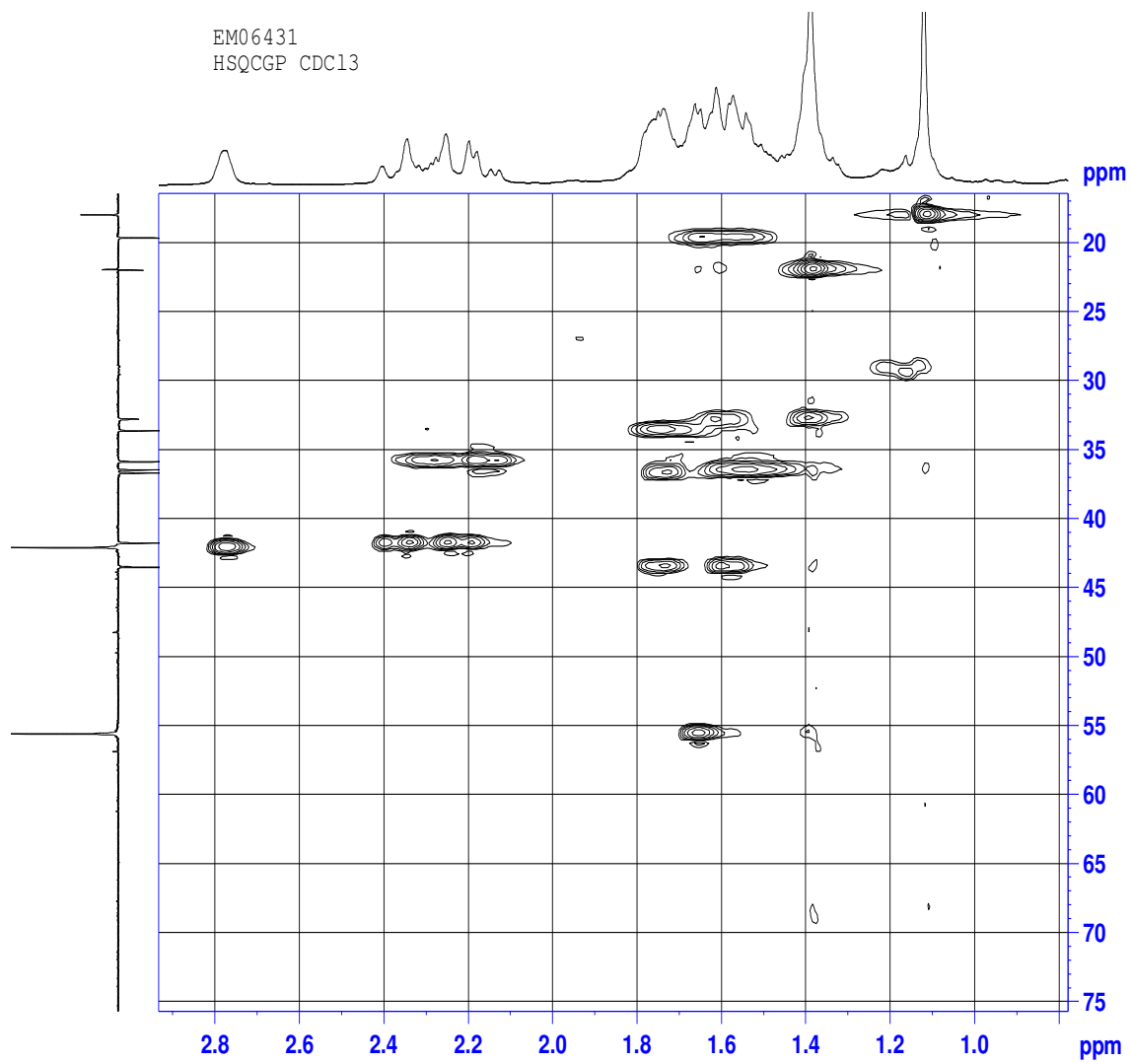


Figure 92: Expanded HMBC spectrum (300 MHz) for 06431

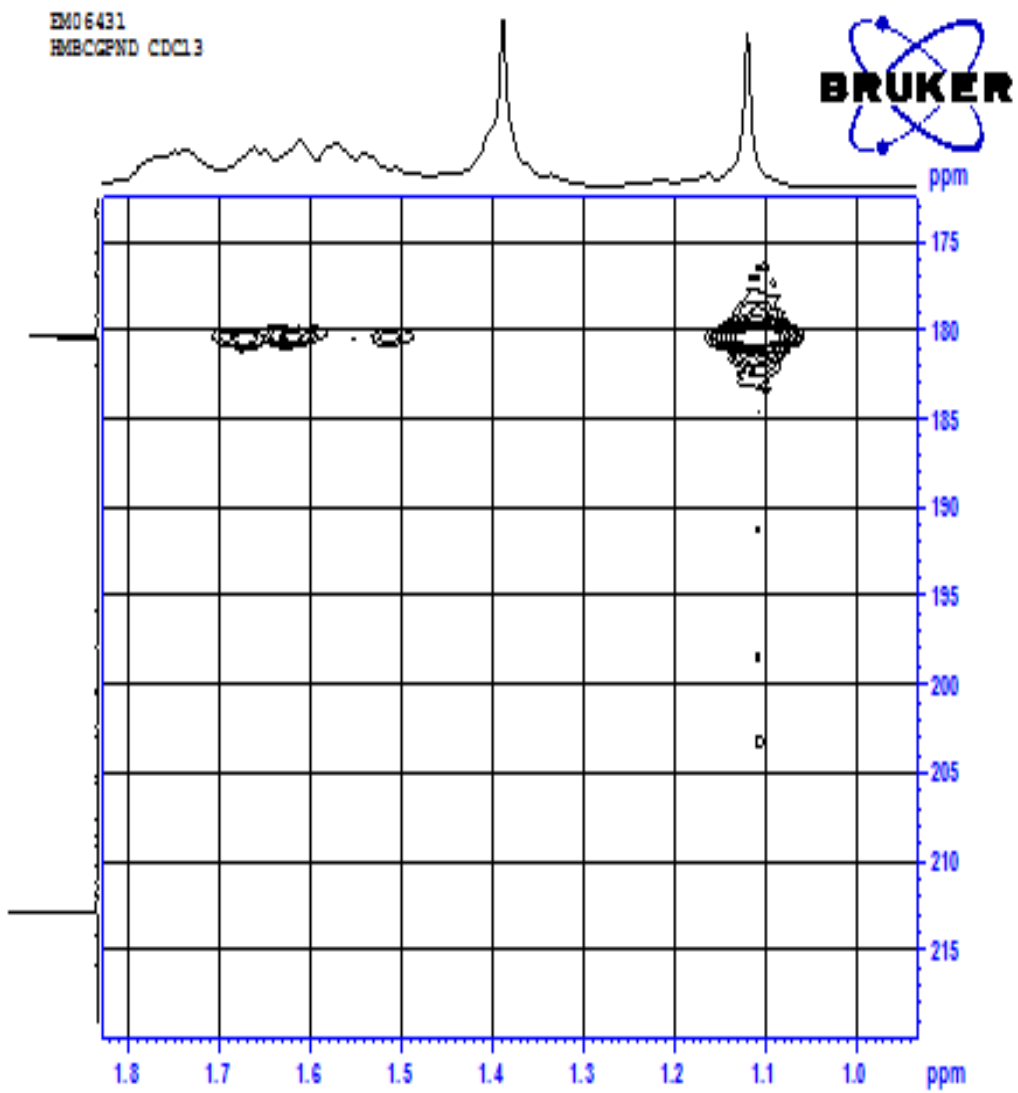


Figure 93: Expanded HMBC spectrum (300 MHz) for 06431

EM06431
COSYGPSW CDC13

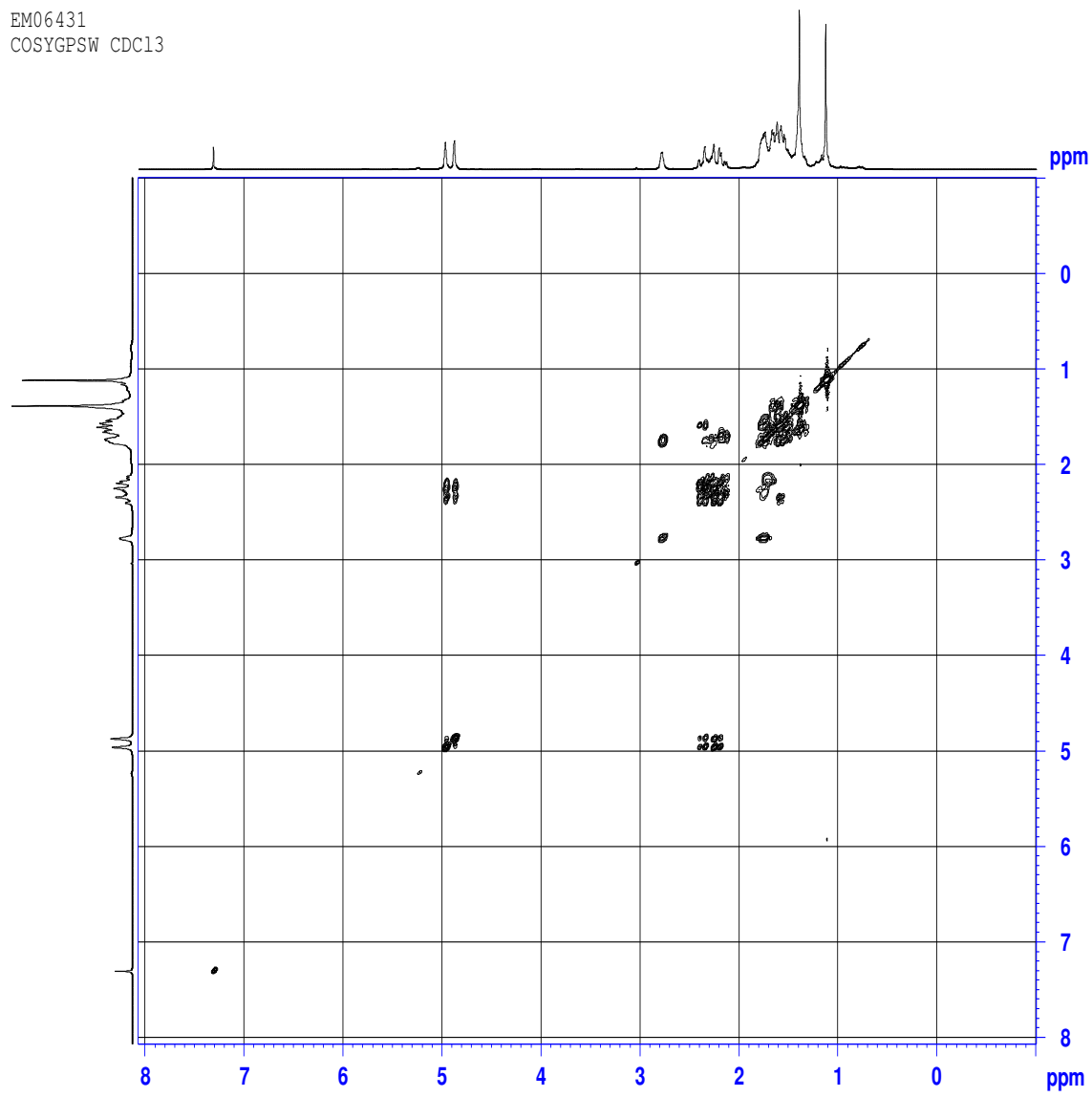


Figure 94: COSY spectrum (300 MHz) for 06431

EM06431
HMBCGPNDCDC13

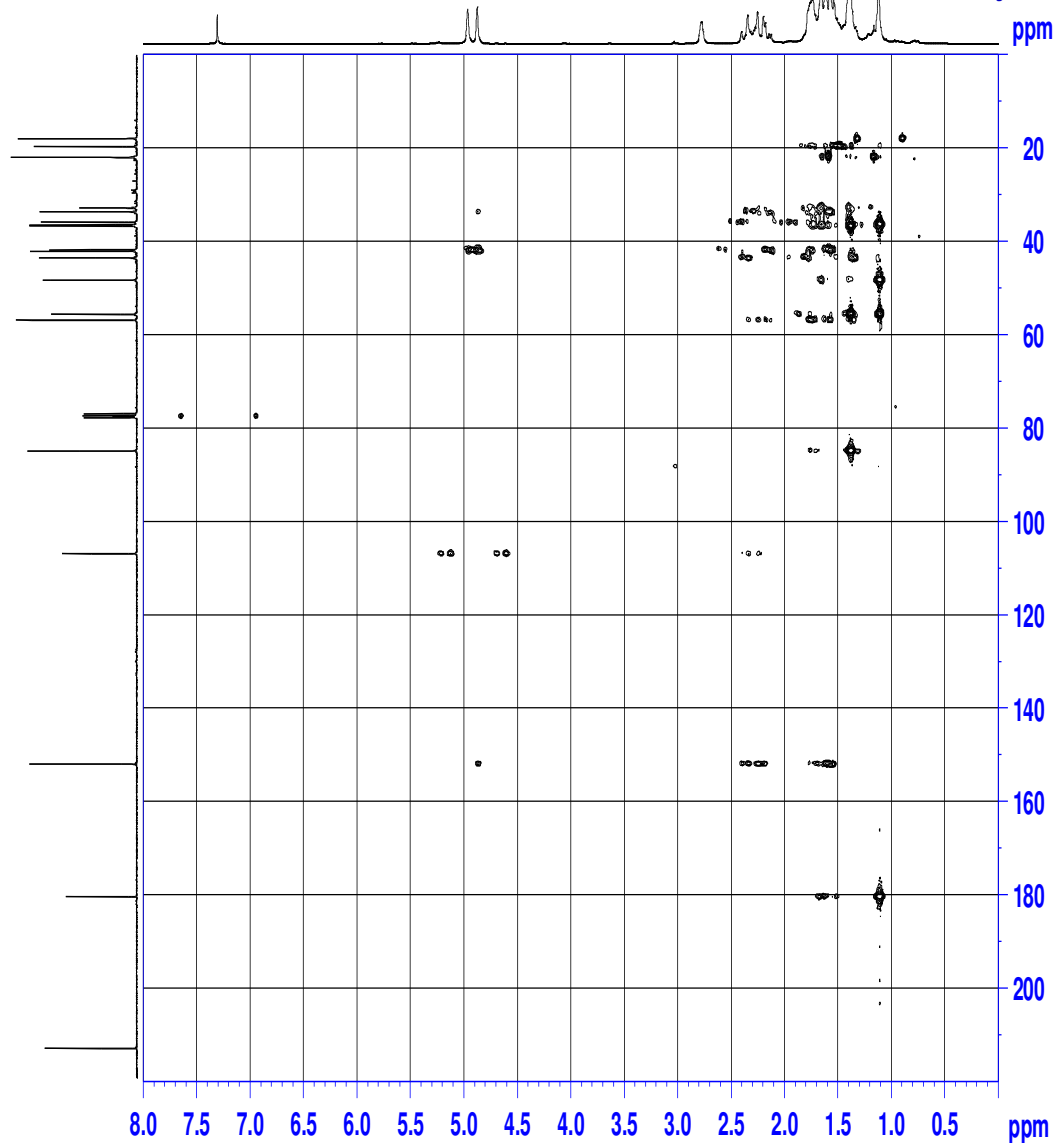
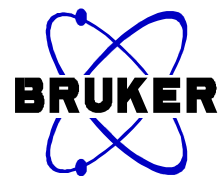


Figure 95: Complete HMBC spectrum (300 MHz) for 06431

15-Nov-2012
EM06431-5

1: TOF MSMS ES+
TIC
3.96e6

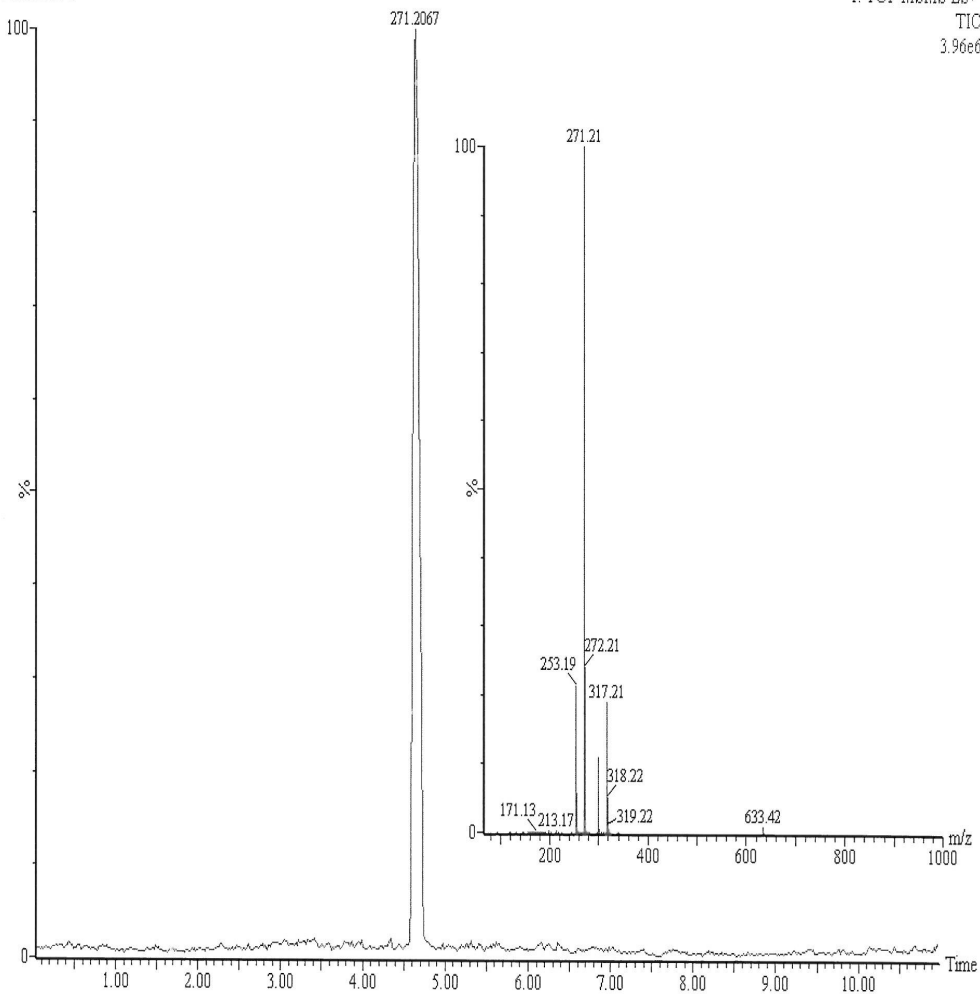


Figure 96: TOFMS-MS spectrum for 06431

APPENDIX 7

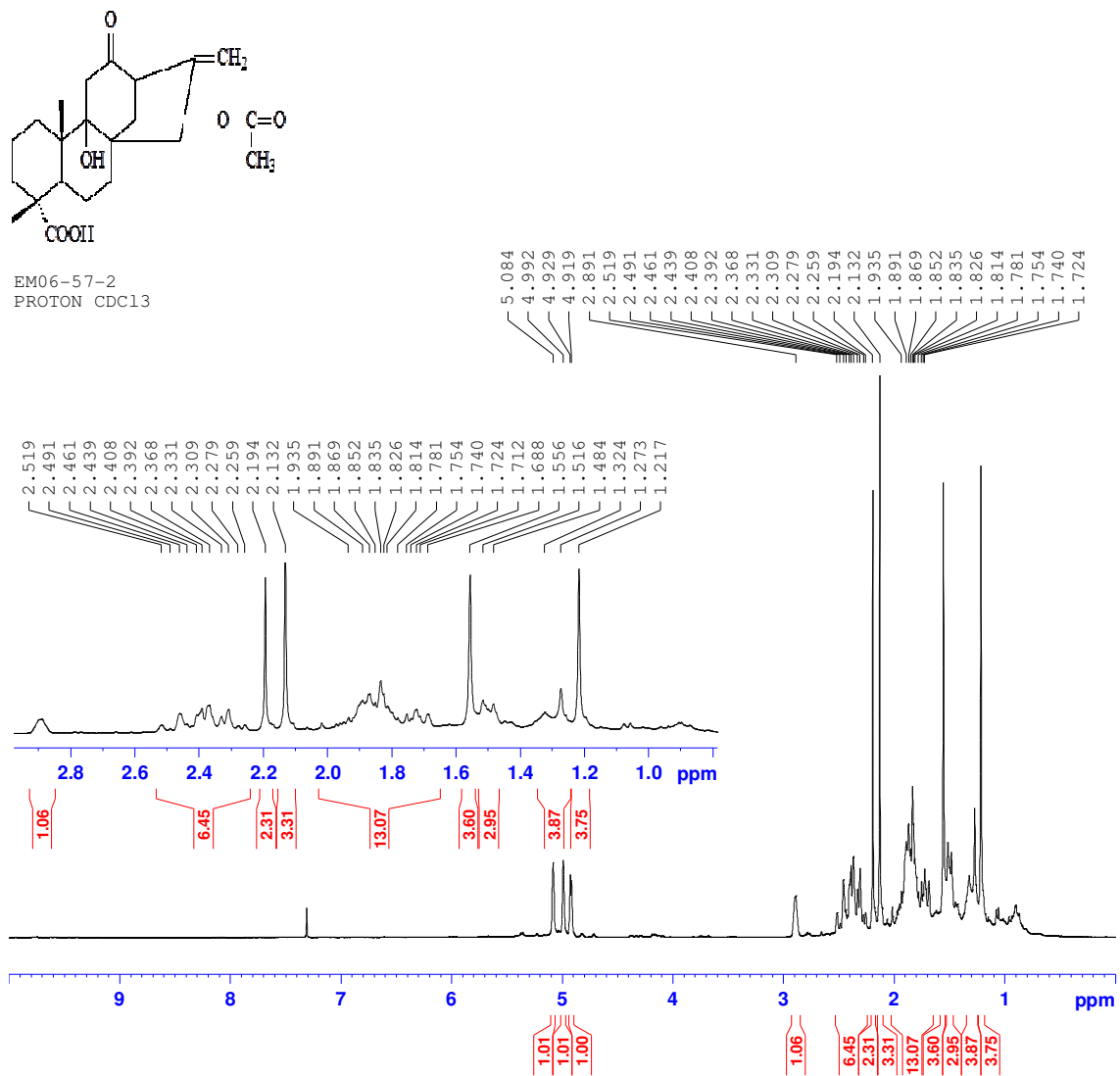
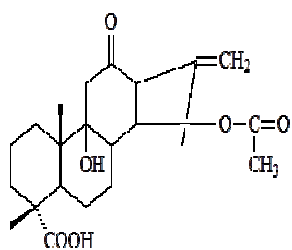


Figure 97: ¹H NMR spectrum (300 MHz) for 0657



EM06-57
C13CPD CDC13

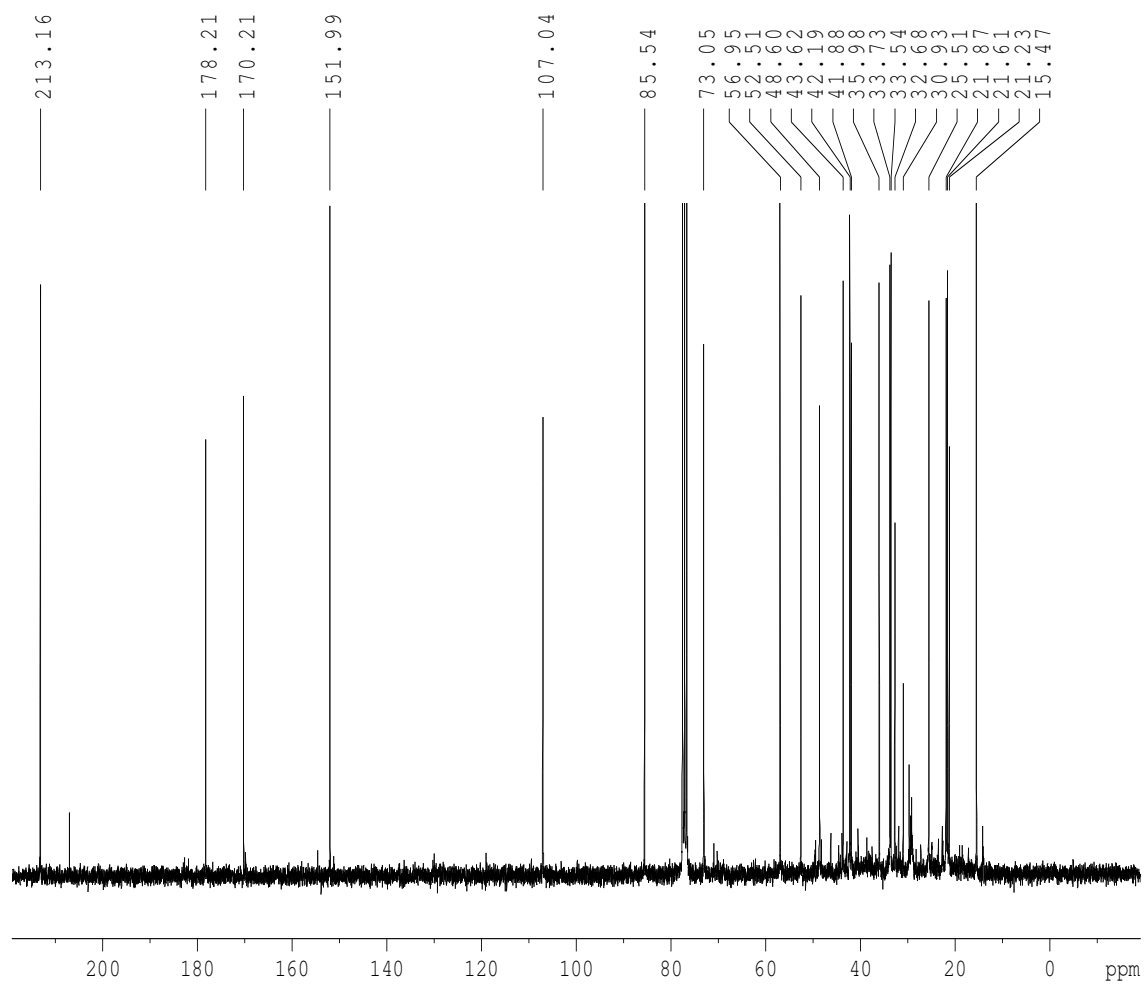
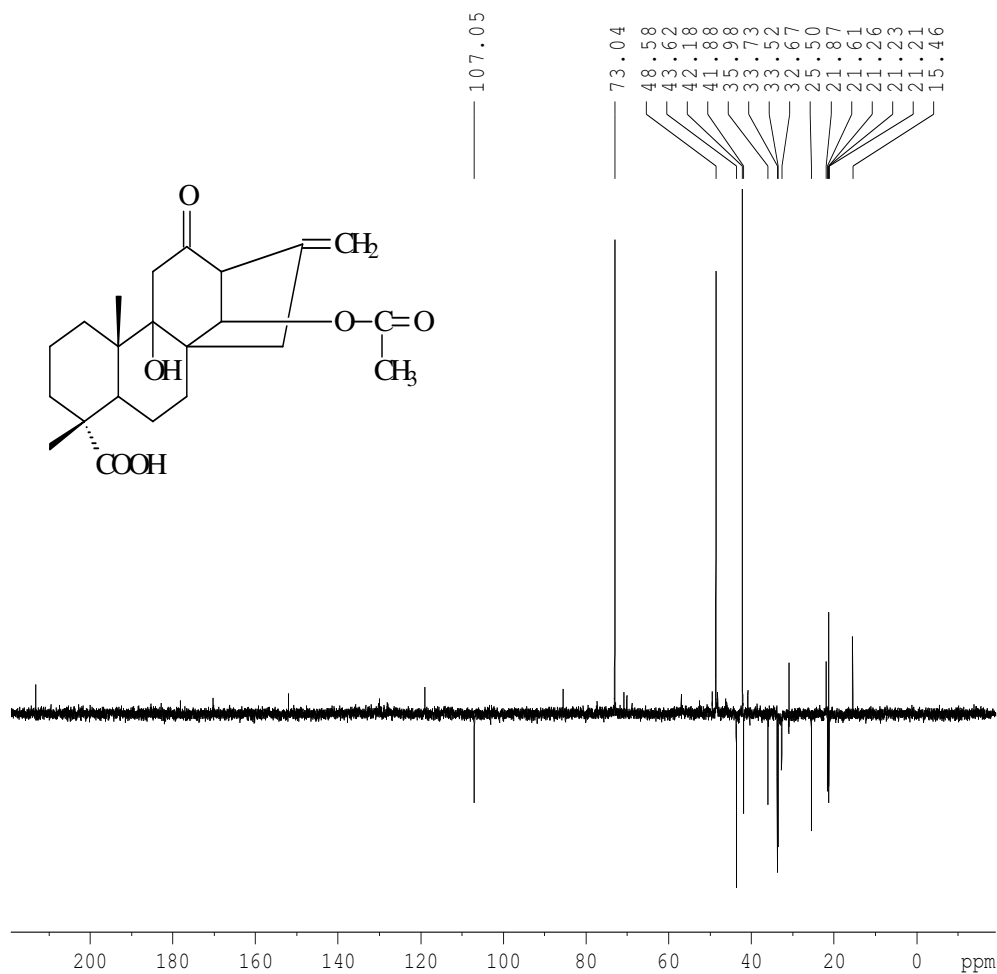


Figure 98: ¹³C NMR spectrum (300 MHz) for 0657



```

Current Data Parameters
NAME      EM-06-57
EXPNO    2
PROCNO   1

F2 - Acquisition Parameters
Date_    20120806
Time     12.47
INSTRUM  spect
PROBHD   5 mm QNP 1H/1
PULPROG  zgpg30
TD        65536
SOLVENT  CDCl3
NS        1536
DS        4
SWH       17985.611 Hz
FIDRES    0.274439 Hz
AQ        1.6219508 sec
RG        1024
DW        27.800 usec
DE        6.00 usec
TE        296.2 K
CONST2    145.000000
d1        2.0000000 sec
d2        0.00344828 sec
d12       0.00002000 sec
DELTA     0.00001082 sec
TD0       1

===== CHANNEL f1 =====
NUC1      13C
P1        8.50 usec
P2        17.00 usec
PL1       -2.00 dB
SFO1      75.4752953 MHz

===== CHANNEL f2 =====
CPDPRG2   waltz16
NUC2      1H
P3        10.00 usec
P4        20.00 usec
PCPD2     80.00 usec
PL2       0.00 dB
PL12      18.00 dB
SFO2      300.1312005 MHz

F2 - Processing parameters
SI        32768
SF        75.4677490 MHz
WDW       EM
SSB       0
LB        1.00 Hz
GB        0
PC        1.40
    
```

Figure 99: DEPT 135 spectrum (300 MHz) for 0657

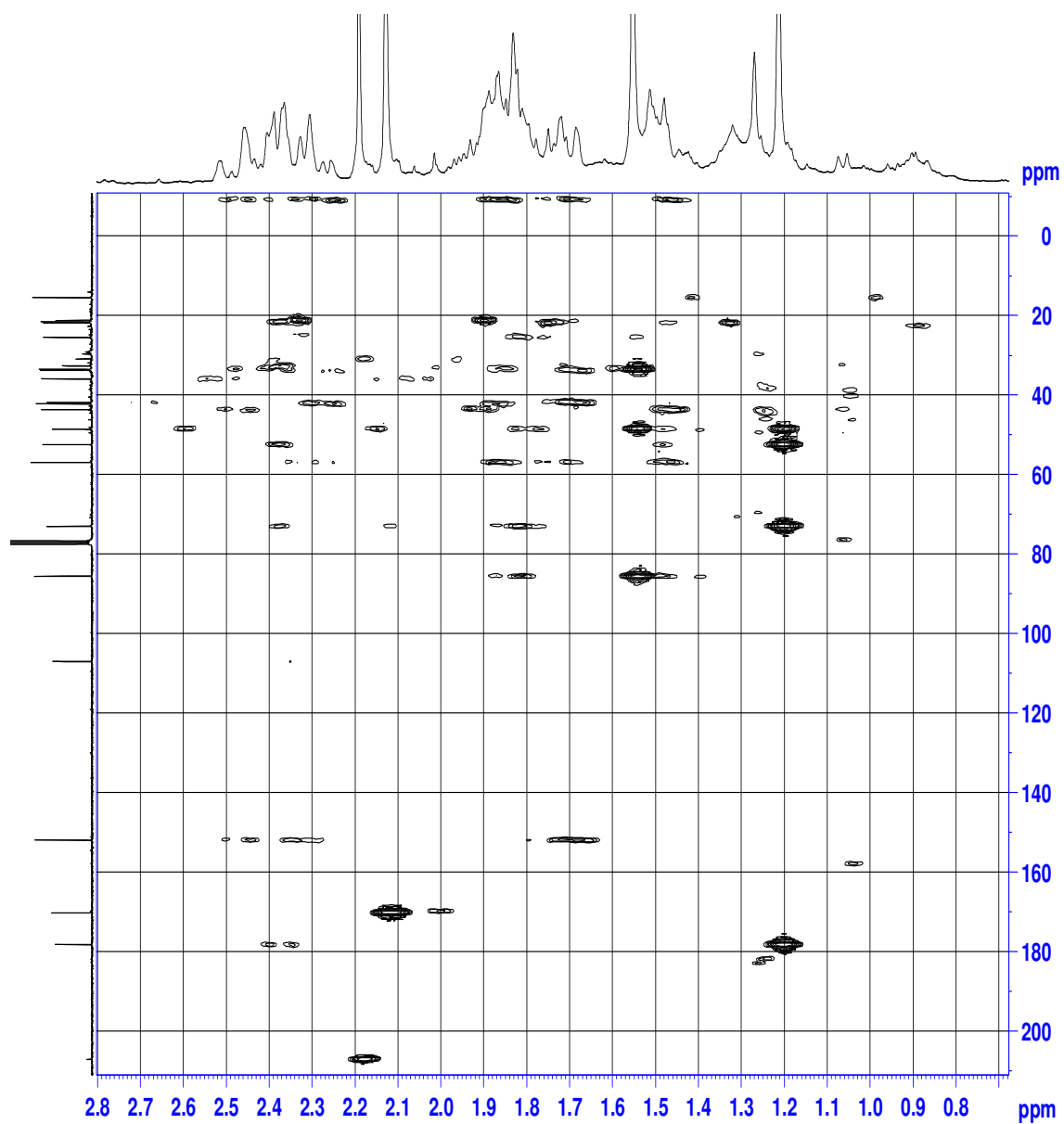


Figure 100: HMBC spectrum (300 MHz) for 0657

EM06-57
HSQCGP CDC13

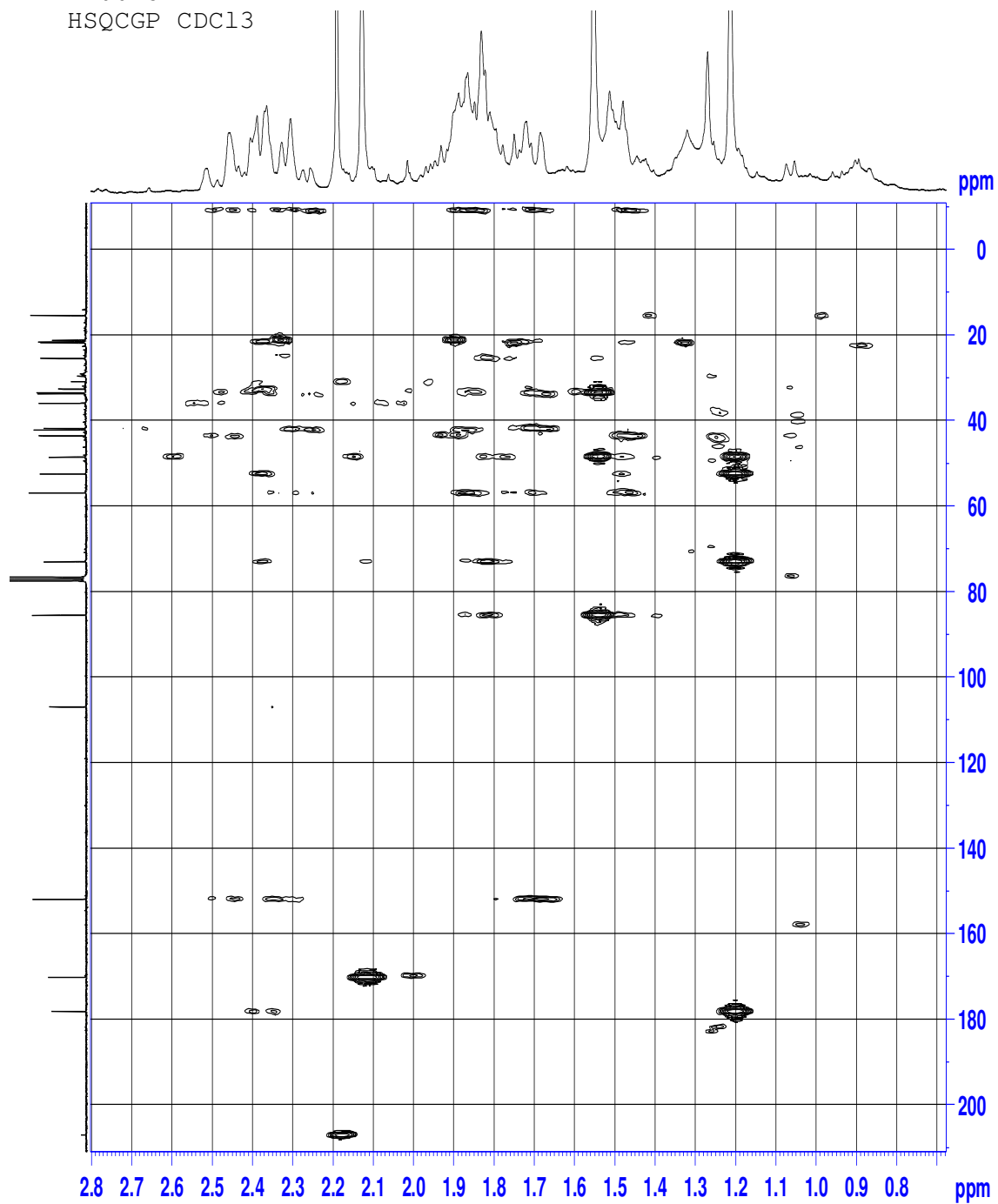


Figure 101: HSQC spectrum (300 MHz) for 0657

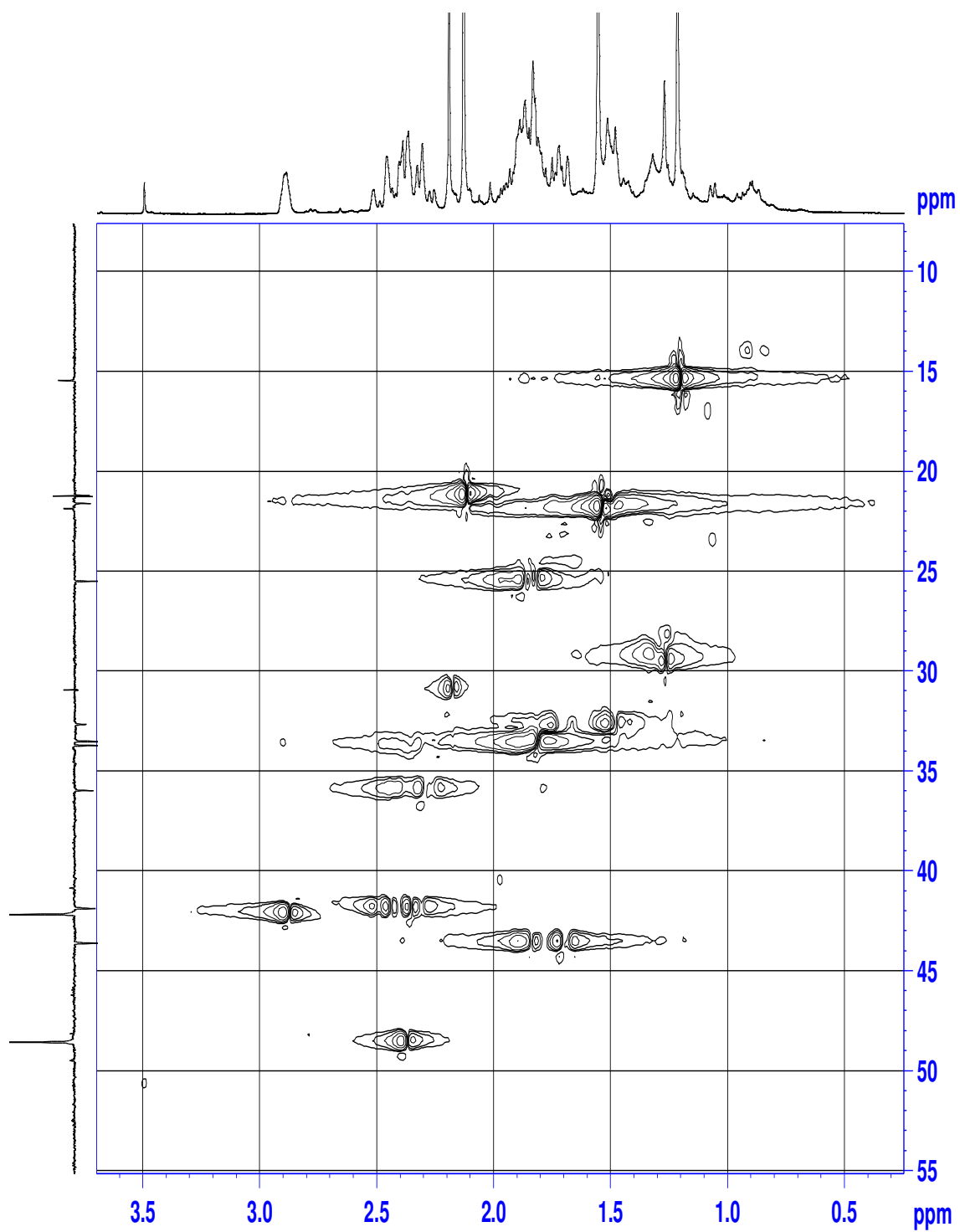


Figure 102: Expanded HSQC spectrum (300 MHz) for 0657

HMBCPND CDCI3

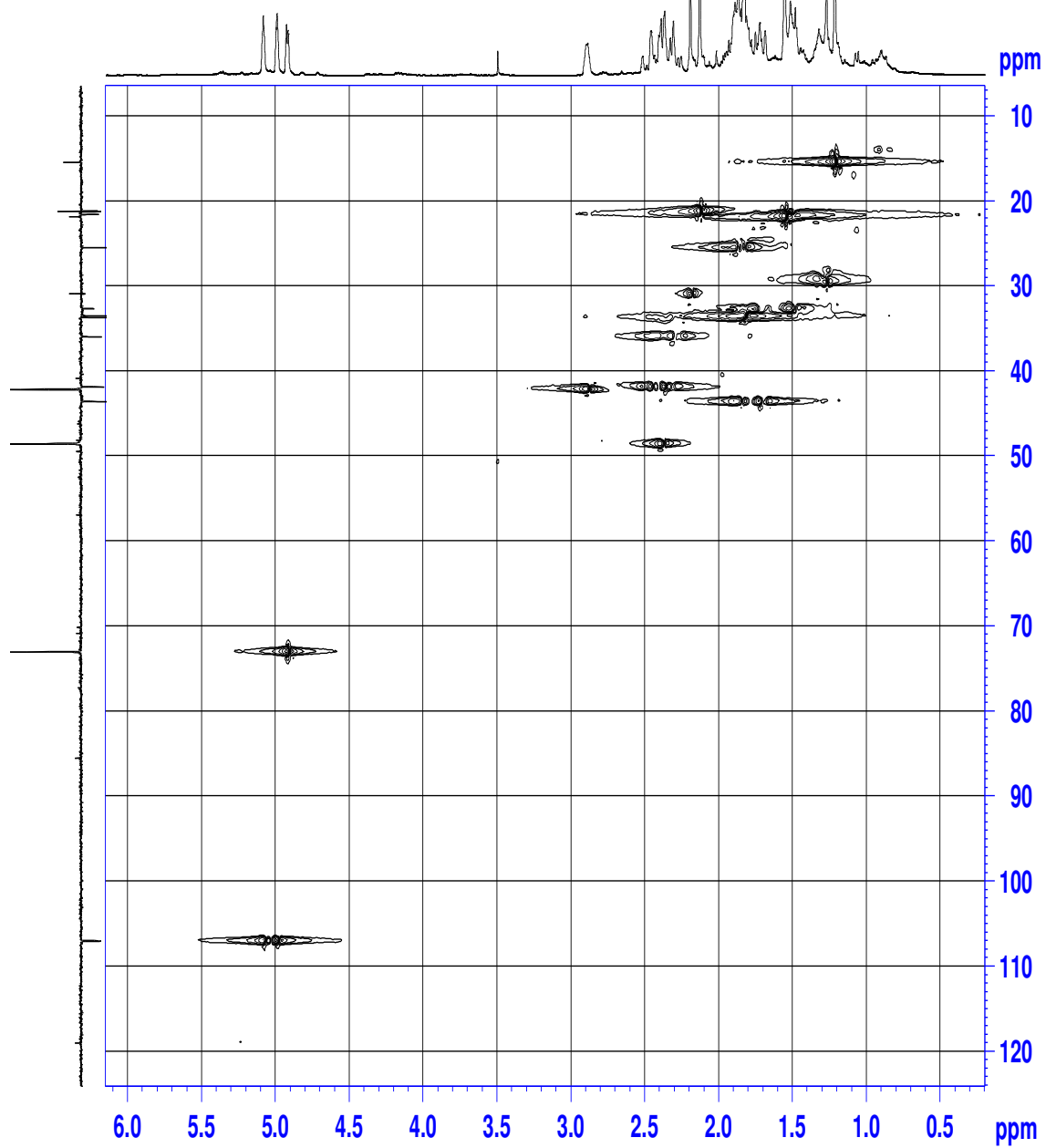
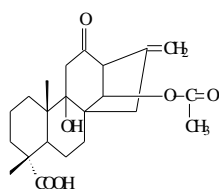


Figure 103: Expanded HMBC spectrum (300 MHz) for 0657

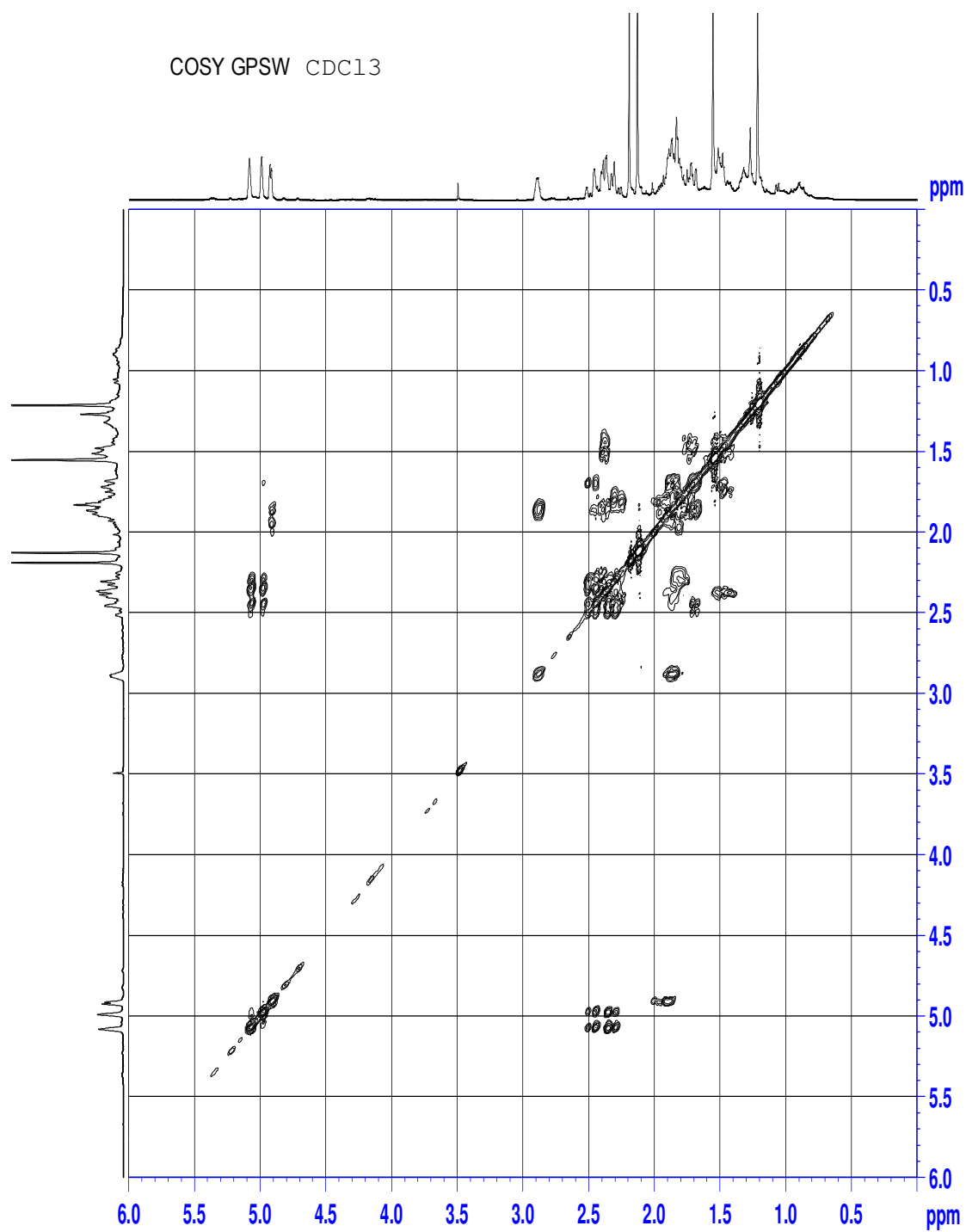


Figure 104: COSY spectrum (300 MHz) for 0657

15-Nov-2012
EM0657-1

1: TOF MSMS ES+
BPI
6.37e5

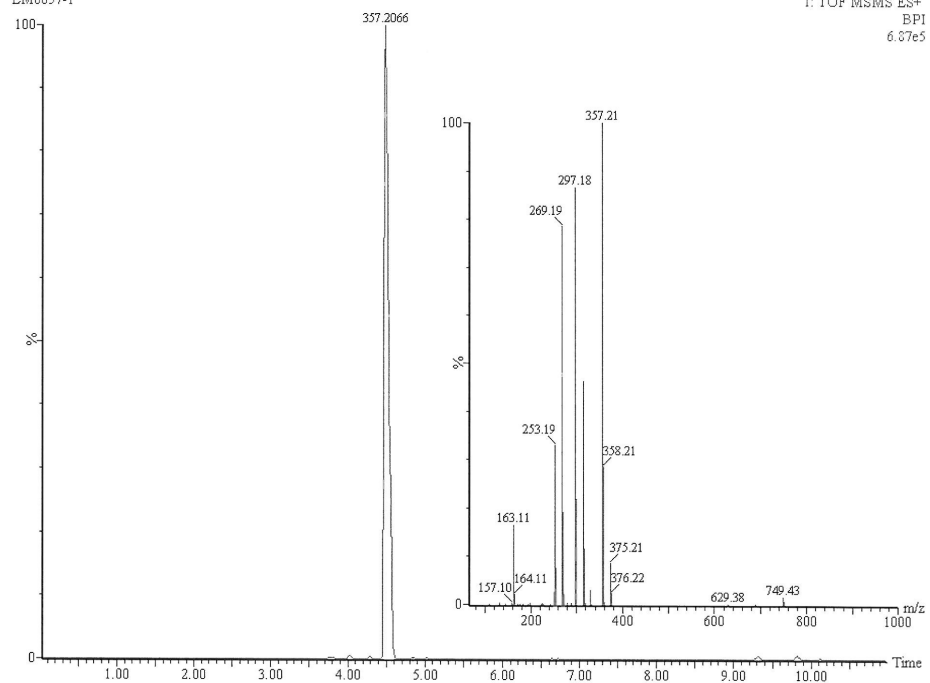


Figure 105: TOF MS-MS spectrum for 0657

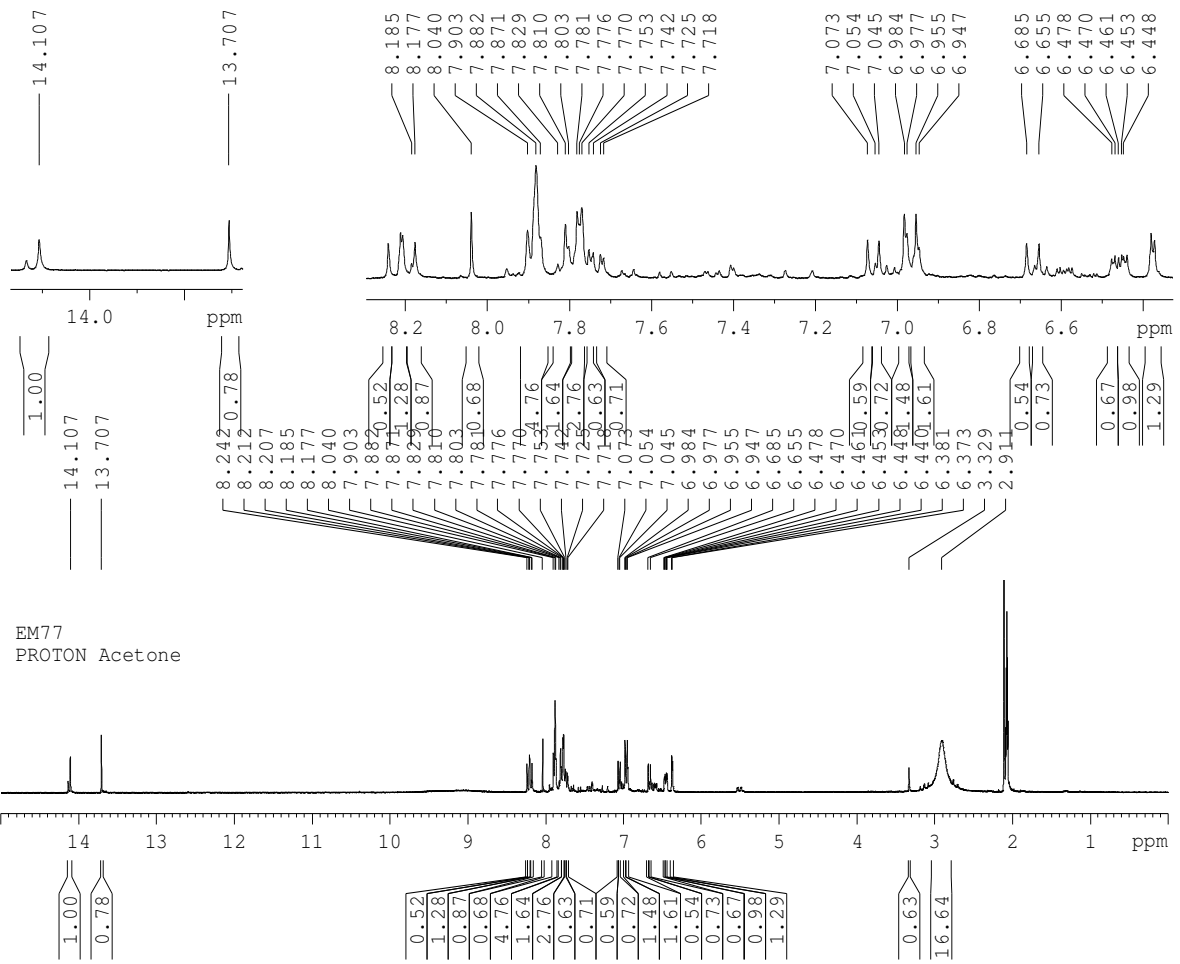


Figure 106: ¹H NMR spectrum (300 MHz) for EM77

APPENDIX 8

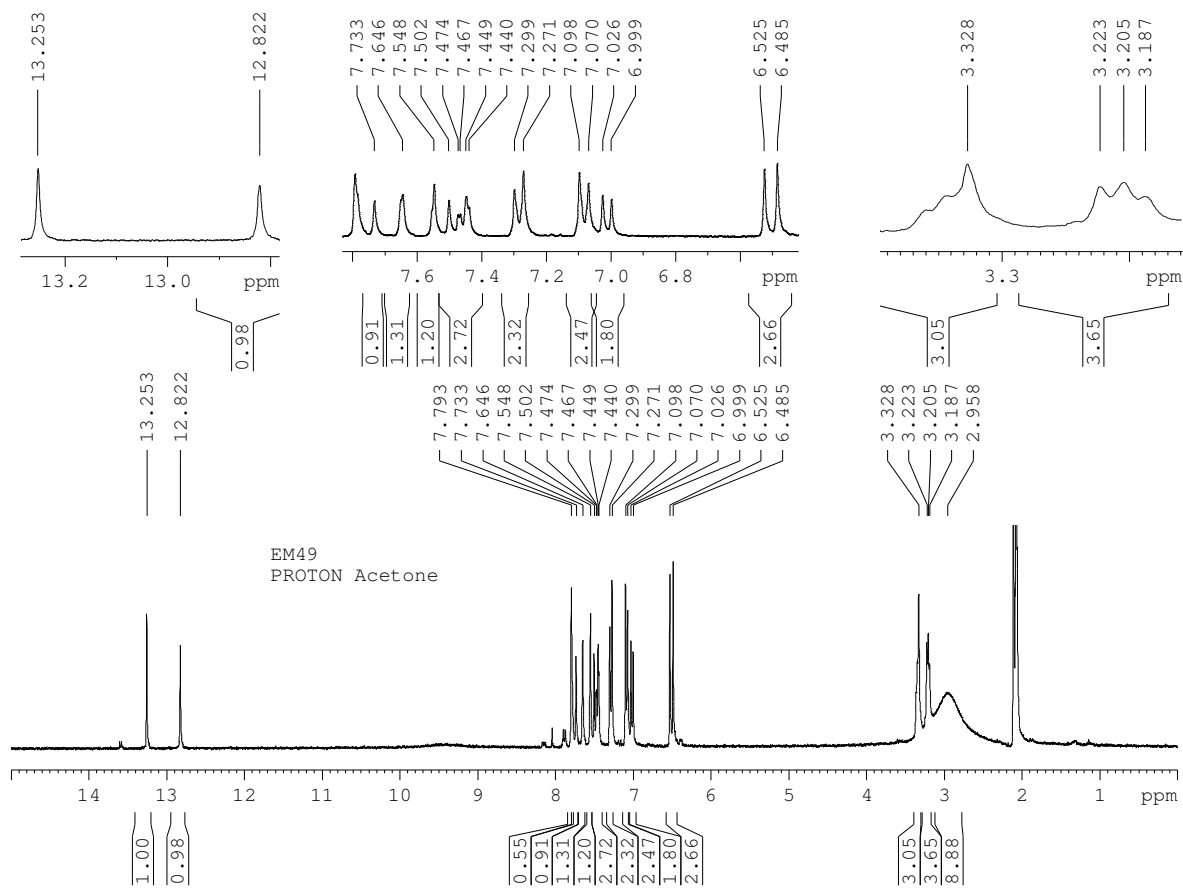
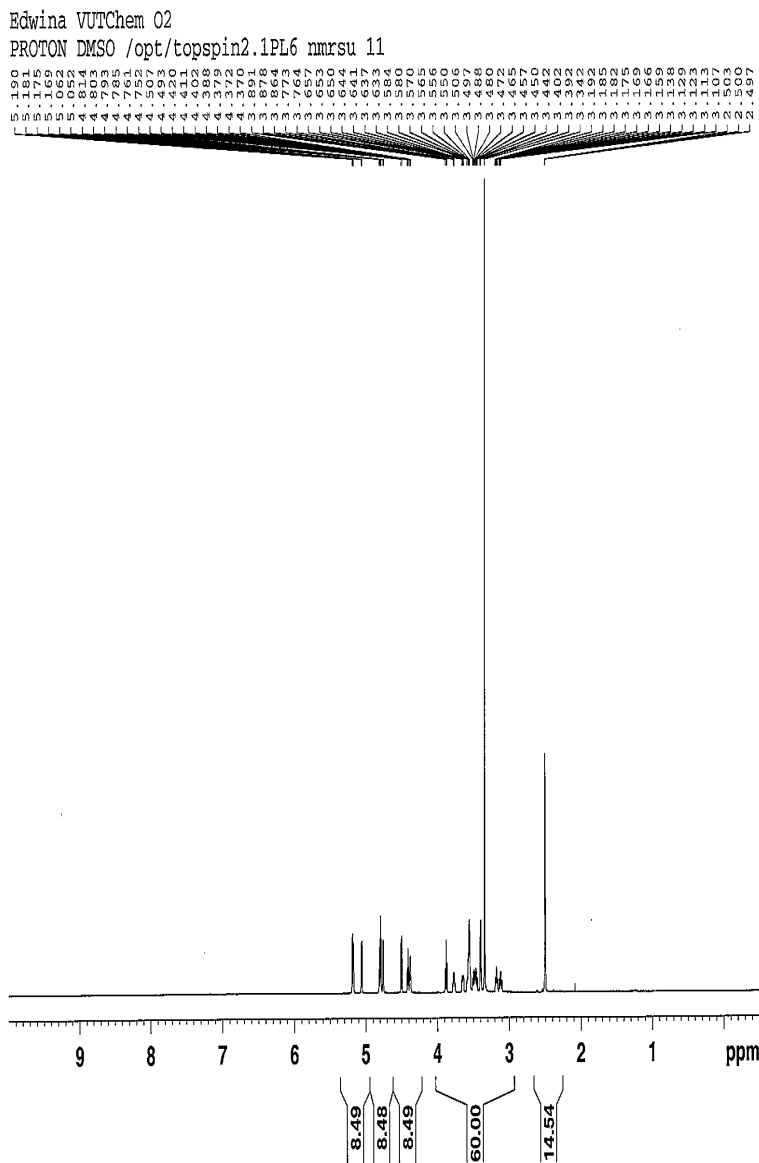


Figure 107: ¹H NMR spectrum (300 MHz) for EM49

APPENDIX 9



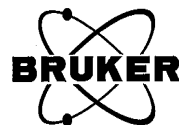
NAME Jun14-2011-nmrsu
 EXPNO 50
 PROCNO 1
 Date_ 20110614
 Time 16.13
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zg30
 TD 65536
 SOLVENT DMSO
 NS 16
 DS 2
 SWH 12335.526 Hz
 FIDRES 0.188225 Hz
 AQ 2.6564426 sec
 RG 128
 DW 40.533 usec
 DE 6.50 usec
 TE 294.5 K
 D1 1.00000000 sec
 TDO 1

==== CHANNEL f1 =====
 NUC1 1H
 P1 13.30 usec
 PL1 -1.00 dB
 PL1W 23.90681839 W
 SFO1 600.1737063 MHz
 SI 32768
 SF 600.1700021 MHz
 WDW EM
 SSB 0
 LB 0.30 Hz
 GB 0
 PC 1.00

Figure 108: ¹H NMR spectrum (600MHz) for sucrose isolated from *Pentania prunelloides*

Edwina VUT Chemistry O2
 C13DEPT90 DMSO /opt/topspin2.1PL6 nmrsu 11

91.70
 82.52
 74.21
 72.81
 71.58
 69.77
 39.99
 39.85
 39.54
 39.44



```

NAME      Jun15-2011-nmrsu
EXPNO     22
PROCNO    1
Date_     20110615
Time      19.57
INSTRUM   spect
PROBHD    5 mm PABBO BB-
PULPROG   dept90
TD         65536
SOLVENT   DMSO
NS         128
DS         4
SWH        36057.691 Hz
FIDRES     0.550197 Hz
AQ         0.9088159 sec
RG         2050
DW         13.867 usec
DE         6.50 usec
TE         295.1 K
CNST2     145.0000000
D1         2.0000000 sec
D2         0.00344828 sec
D12        0.00002000 sec
TDO        1
  
```

```

===== CHANNEL f1 =====
NUC1      13C
P1         10.00 usec
P2         20.00 usec
PL1        3.00 dB
PL1W      48.09095001 W
SFO1      150.9279578 MHz
  
```

```

===== CHANNEL f2 =====
CPDPRG2   waltz16
NUC2       1H
P3         13.30 usec
P4         26.60 usec
PCPD2     95.00 usec
PL2        -1.00 dB
PL12       16.08 dB
PL2W      23.90681839 W
PL12W     0.46829745 W
SFO2      600.1724007 MHz
SI         32768
SF         150.9129425 MHz
WDW        EM
SSB        0
LB         1.00 Hz
GB         0
PC         1.40
  
```

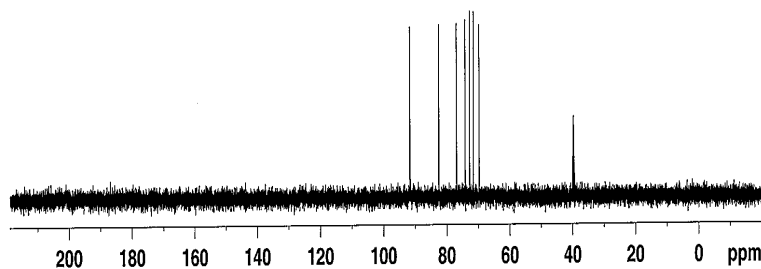


Figure 109: ¹³C NMR spectrum (600MHz) for sucrose isolated from *Pentanisia prunelloides*

Edwina VUT Chemistry O2
 C13DEPT135 DMSO /opt/topspin2.1PL6 nmrsu 11

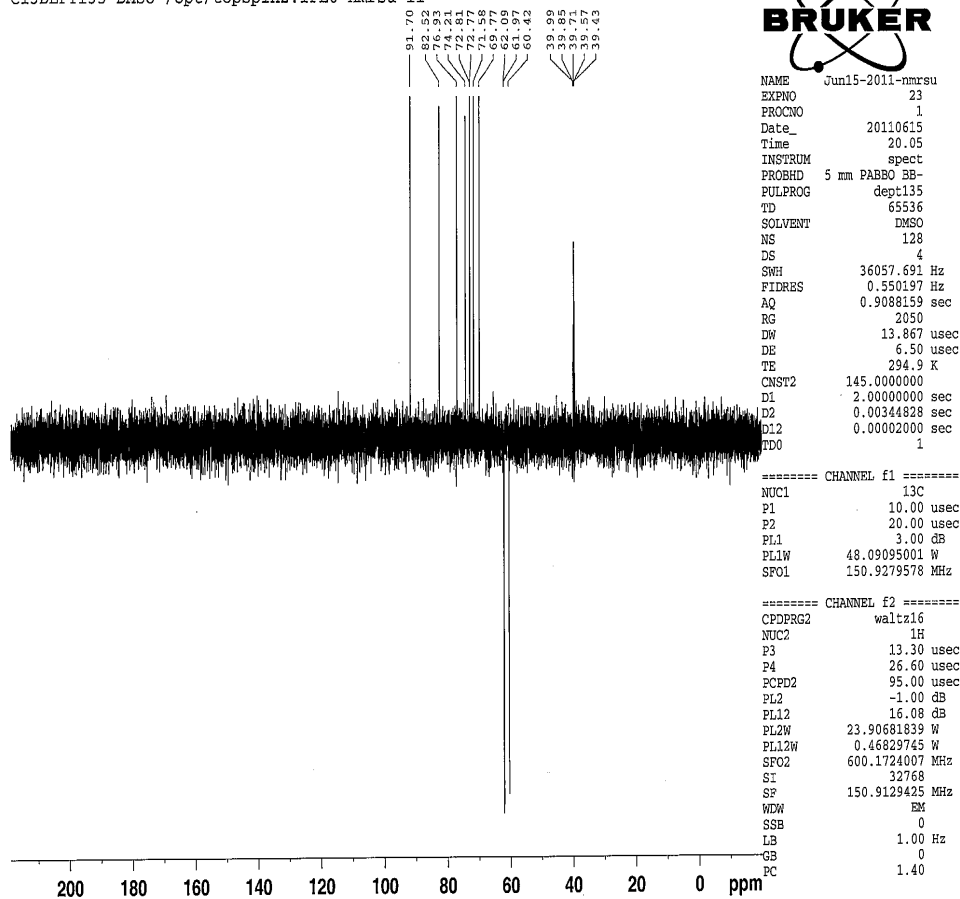


Figure 110: DEPT 135 spectrum (600MHz) for sucrose isolated from *Pentania prunelloides*

Edwina VUT Chemistry O2
COSYGPSW DMSO /opt/topspin2.1PL6 nmrsu 11

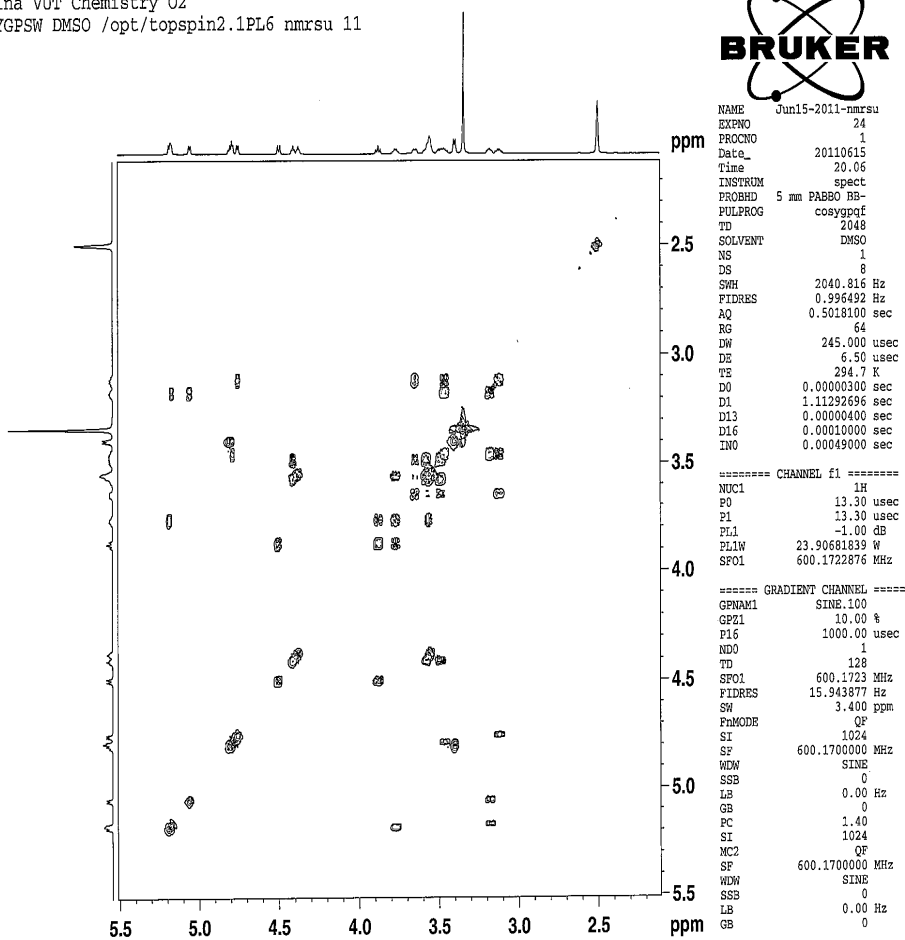


Figure 111: COSY spectrum (600MHz) for sucrose isolated from *Pentania prunelloides*

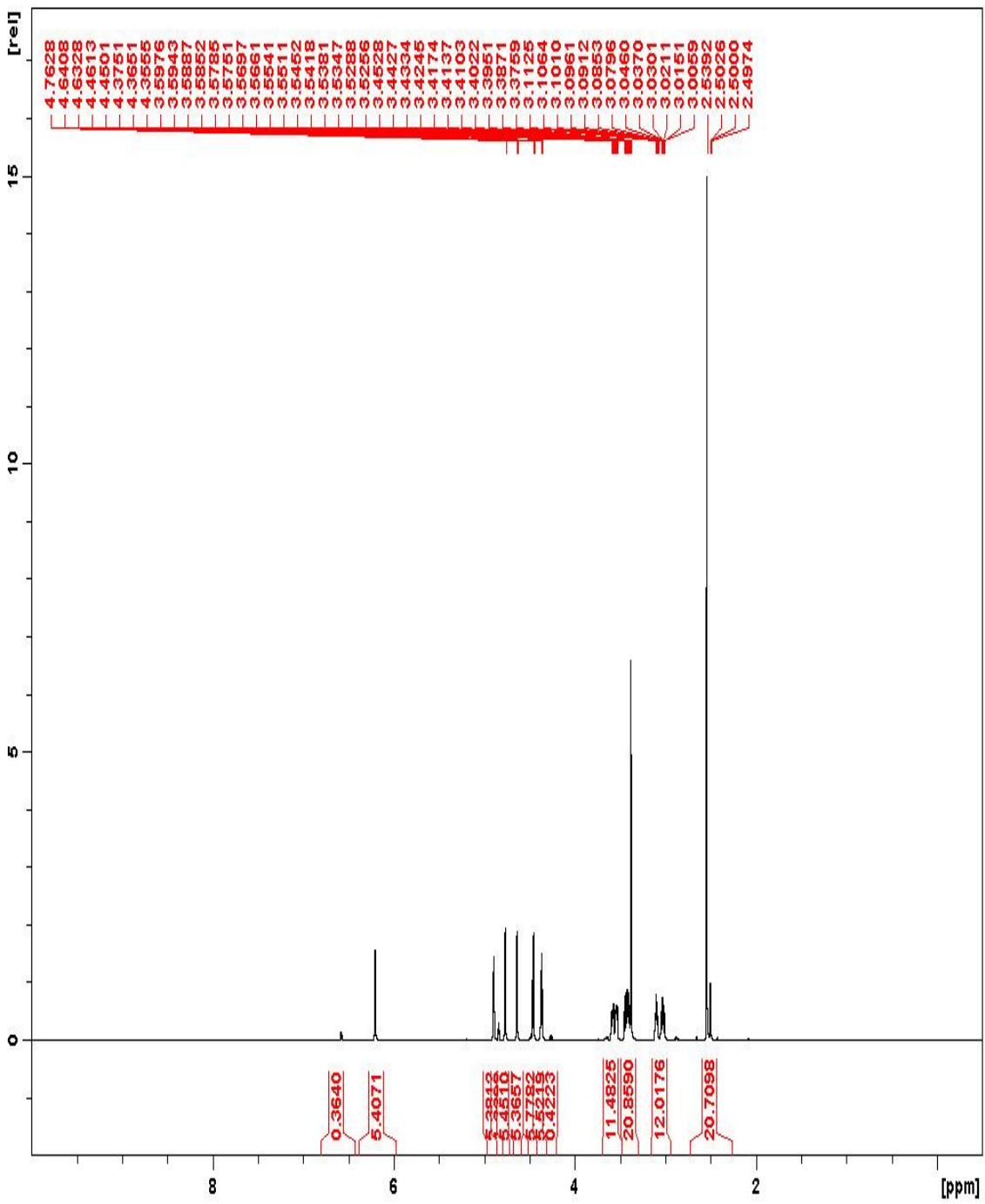


Figure 112: ¹H NMR spectrum (600MHz) for glucose isolated from *Gunnera perpensa*

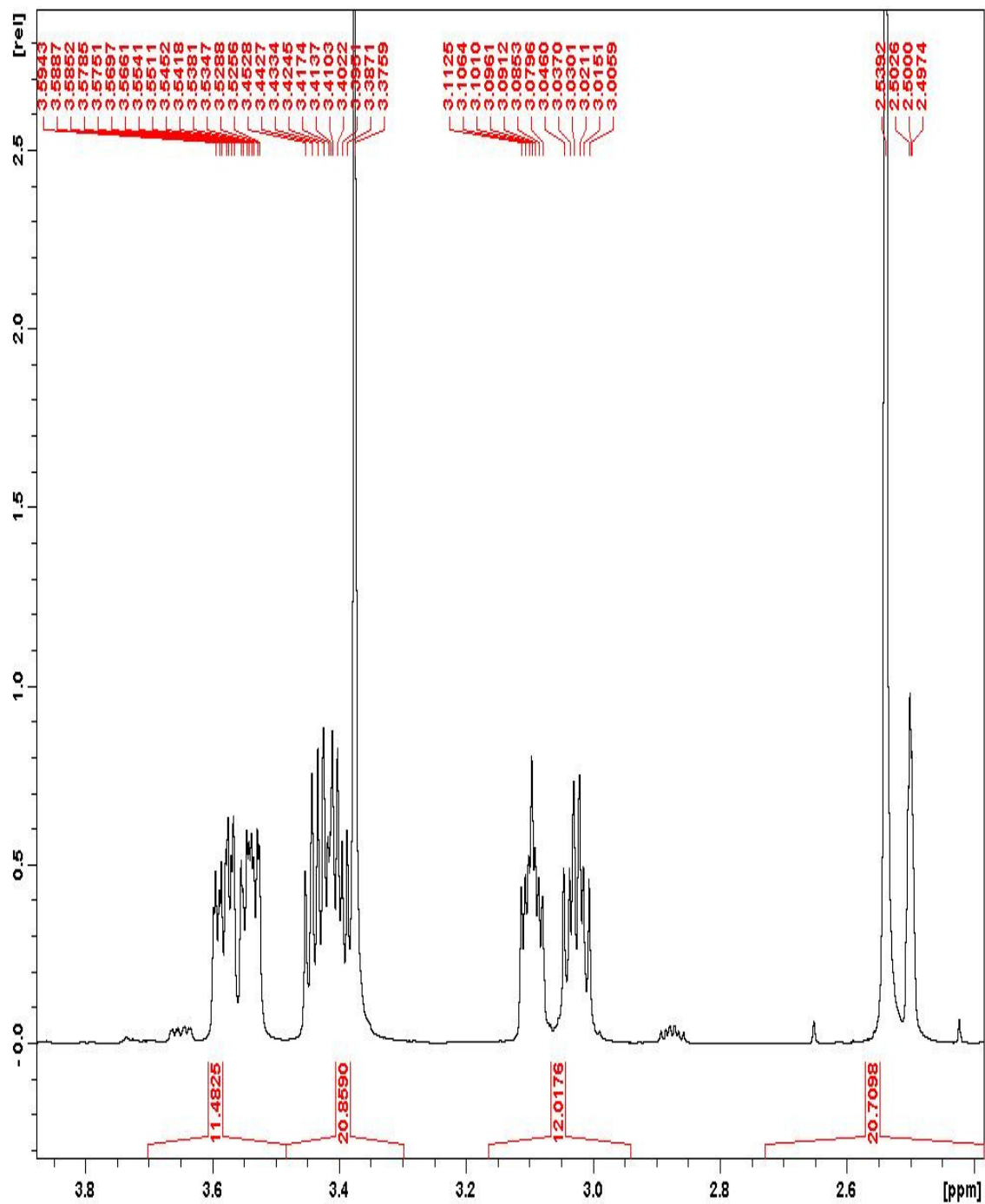


Figure 113: Expanded ¹H NMR spectrum (600MHz) for glucose isolated from *Gunnera perpensa*

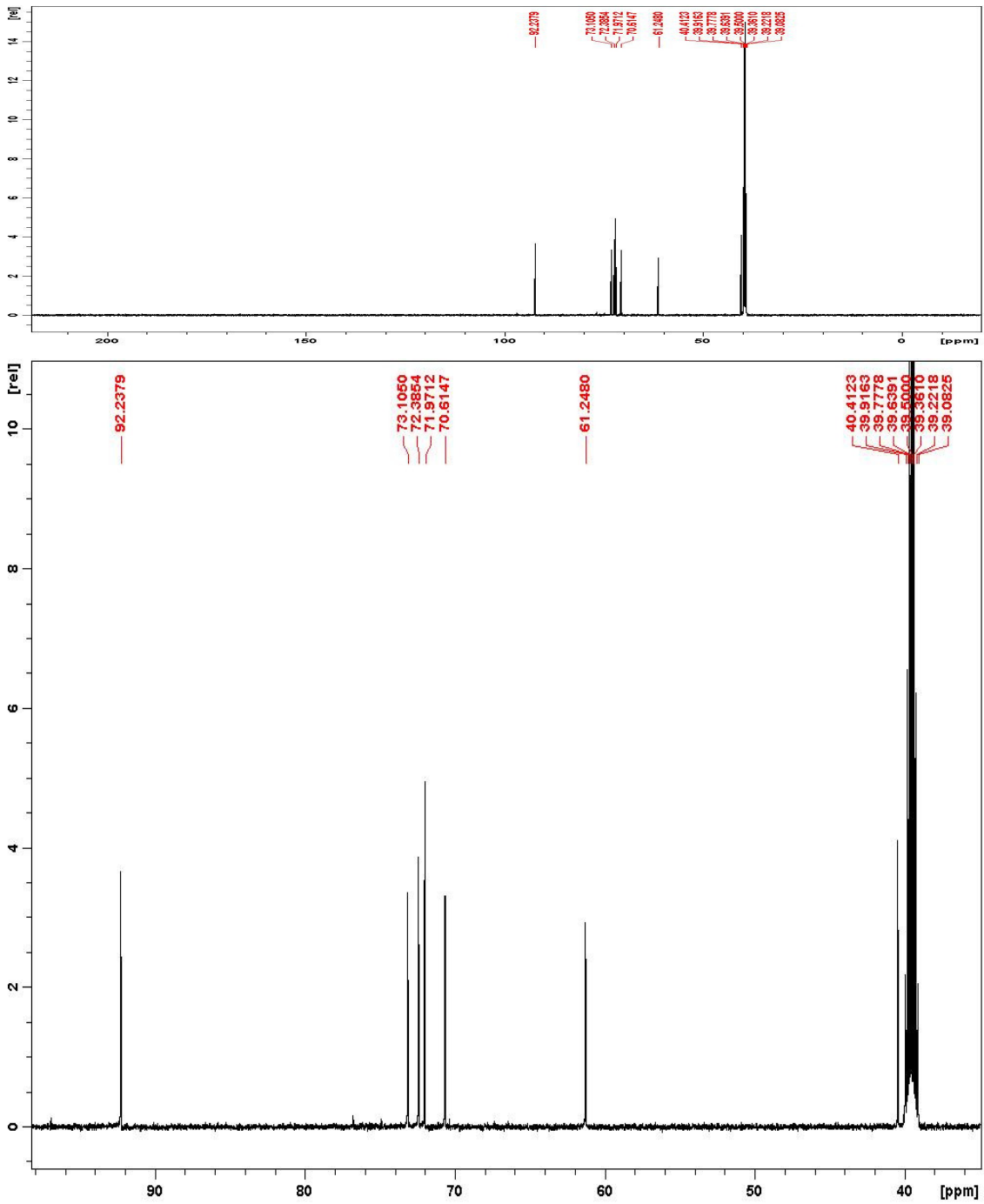


Figure 114: ^{13}C NMR spectrum (600MHz) for glucose isolated from *Gunnera perperna*

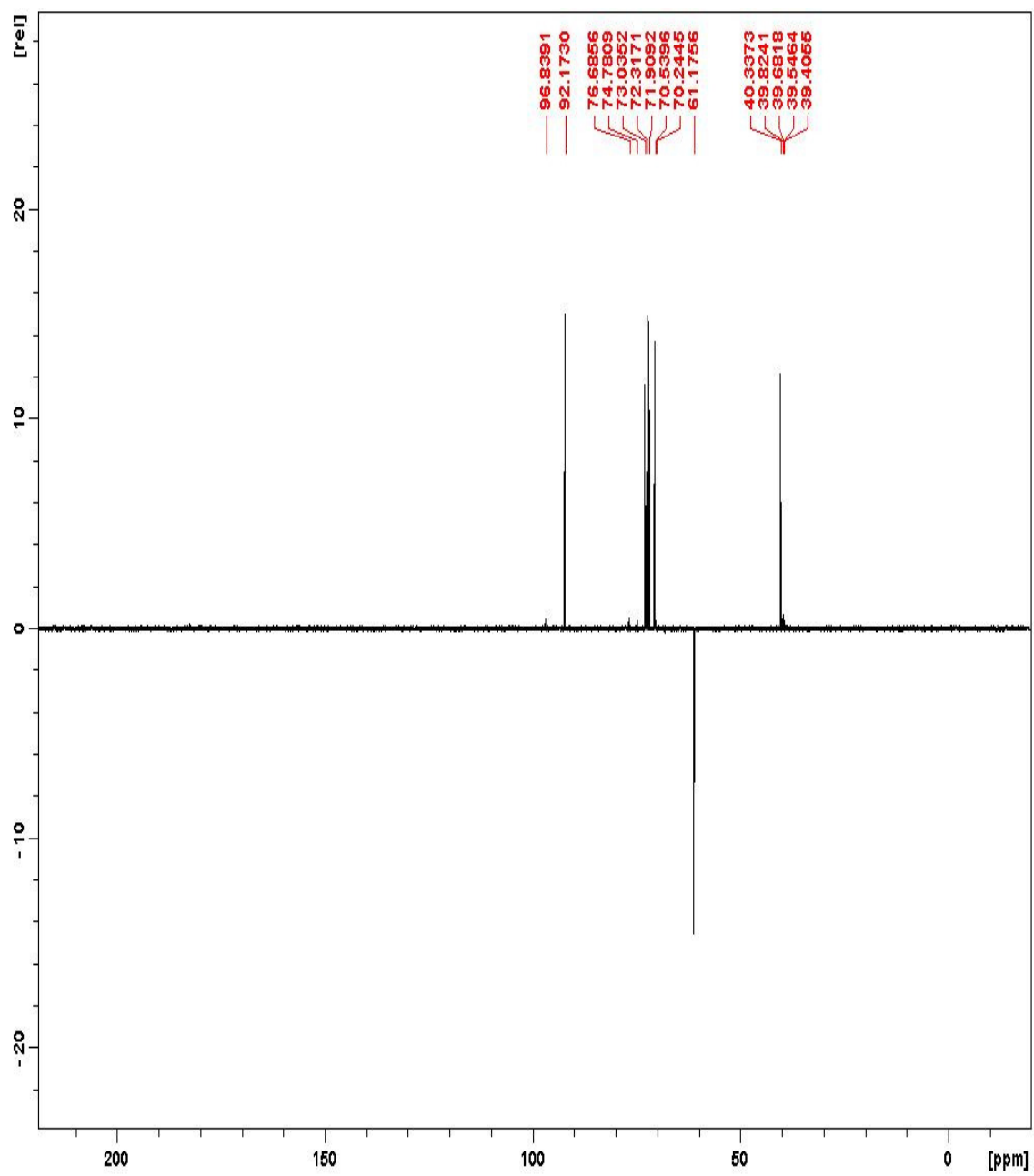


Figure 115: DEPT 135 spectrum (600MHz) for glucose isolated from *Gunnera perpersa*

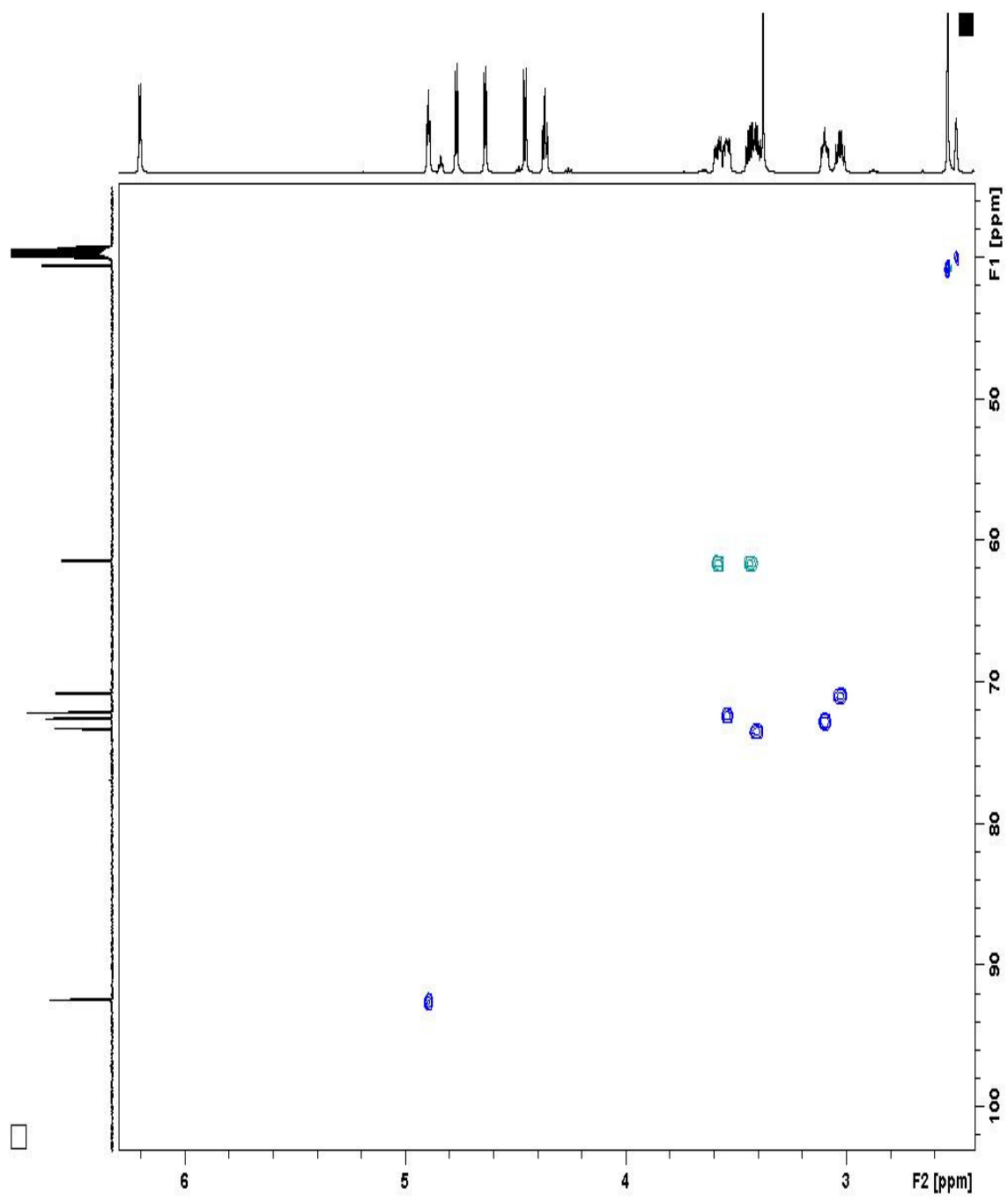


Figure 116: HMPC spectrum (600MHz) for glucose

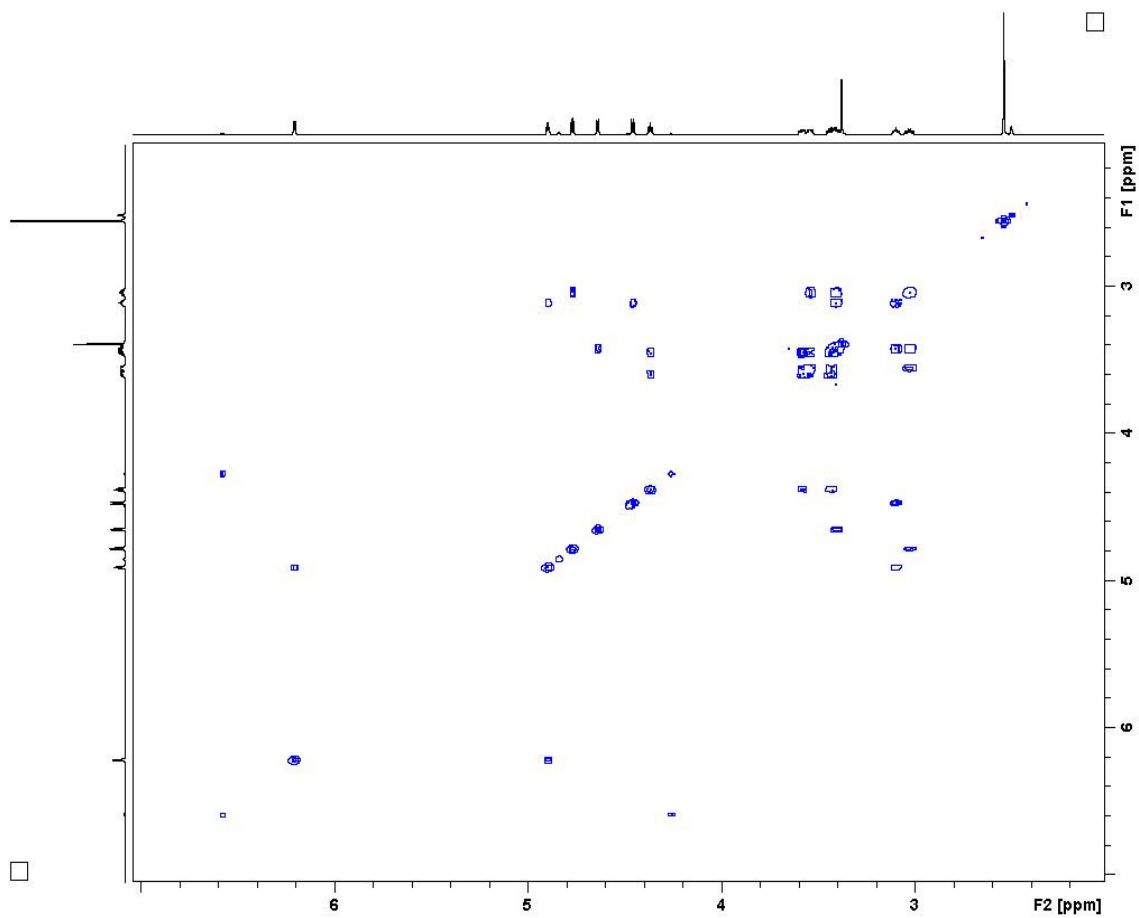


Figure 117: COSY spectrum (600MHz) for glucose