ISOLATION AND CHARACTERISATION OF ANTITUBERCULOSIS COMPOUNDS FROM THE LEAVES OF *Clerodendrum capitatum* (WILD), *Heeria insignis* (DEL) AND STEM BARK OF *Psorospermum senegalense* (SPACH)

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DECEMBER, 2015

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Ph.D/SCI/44752/12-13

A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES,

AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA,

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D) IN ORGANIC CHEMISTRY

DEPARTMENT OF CHEMISTRY

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AHMADU BELLO UNIVERSITY,

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DECEMBER, 2015

Declaration

I declare that the work in this thesis entitled "**Isolation and characterisation of antituberculosis compounds from the leaves of *Clerodendrum capitatum* (wild), *Heeria insignis* (del) and stem bark of *Psorospermum senegalense* (spach)***"* has been performed by me in the Department of Chemistry, Ahmadu Bello University, Zaria, Nigeria, under the supervision of Prof. R.G. Ayo, Prof. G.I. Ndukwe and Dr. J.D. Habila. The Information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma in any institution.

Hajara MOMOH

(Name of Student)	(Signature)	(Date)

Certification

This thesis entitled "ISOLATION AND CHARACTERISATION OF ANTITUBERCULOSIS COMPOUNDS FROM THE LEAVES OF *CLERODENDRUM CAPITATUM* (WILD), *HEERIA INSIGNIS* (DEL) AND STEM BARK OF *PSOROSPERMUM SENEGALENSE* (SPACH)" by Hajara MOMOH, meets the regulations governing the award of the degree of Doctor of Philosophy Degree in Organic Chemistry of the Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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Dedication

To Allah, the Lord and Sustainer of the universe.

Acknowledgement

All praises, adoration and thanks are due to Almighty Allah, the most beneficient, the most merciful who has taught man by the pen and made it possible for me to undertake and come to this level of this work. May his peace and blessings be showered on the nobel prophet, his household, companions and sincere believers till the end of time. I also thank Allah for blessing me with my ever dedicated, patient, encouraging and hard working supervisors in persons of Prof. (Mrs) R.G. Ayo, Prof. G. I. Ndukwe and Dr. J.D Habila whose unrelenting effort, useful suggestions, guidance, encouragement and support made it possible for me to come this far in this research work.

I thank the entire staff members of the Department of Chemistry, A.B.U, Zaria for their contributions in one way or the other to the success of this work.

Words cannot express my appreciation to my dear husband, Dr Ahmad Ismail for his patience, encouragement and tireless effort financially, morally and spiritually toward the success of this work, may Allah reward him abundantly. My profound gratitude goes to my parents, Alh S.A Momoh and Hajia Aminat Momoh for their care, moral, financial and spiritual support. May Allah reward them abundantly. I also appreciate the tolerance, moral support and prayers of my children and in-law, Mall M.J. Ismail, which were a great source of inspiration for me during the study.

I wish to acknowledge Dr Peters Oladosu of the National Institute of Pharmaceutical Research and Development (NIPRD) who helped in the antituberculosis screening of the crude and isolated compounds. I cannot forget to acknowledge my co-researchers (especially Mubarrak Dambatta) who were very supportive during the course of the experimental processes of this work. My thanks will be incomplete without specially thanking my in-law Mall. Jamiu Gaminana and his wonderful family for their warm hospitality and encouragement. May Allah reward you with goodness.

Finally, I must confess that all friends, brothers and sisters in relation, in islam and Christian friends alike have contributed to the success of this research, you are all acknowledged. Thank you all.

Abstract

Phytochemical studies, antimicrobial and antituberculosis screenings of extracts from the leafs of *Clerodendrum capitatum, Heeria insignis* and stem bark of *Psorospermum senegalense* were carried out. The phytochemical studies of the three plants revealed the presence of carbohydrates, cardiac glycosides, glycoside, saponins, streroids, triterpenes,

flavanoids and tannins. The antimicrobial screening of the hexane , dichloromethane, ethyl acetate and methanol extracts of the three plants showed that they were active against most of the test microorganisms namely *Shigella dysenteriae, Salmonella typhi, Corynebacterium ulcerans, Klebsiella pneumoniae, Staphylococcus aureus, Methicillin resistant Staphylococcus aureus, Proteus mirabilis, Streptococcus pneumoniae, Proteus vulgaris, Vancomycin resistant enterococci, Bacillus subtillis, Escherichia coli, Pseudomonas flourescense, Streptococcus pyogenes, Enterobacter specie, Streptococcus feacalis, Pseudomonas aeruginosa, Proteus rettgeris, Candida tropicalis, Candida pseudotropicalis, Candida krusei, Candida albicans and Candida stellatoid*. However, the ethyl acetate extract showed the highest activity of all the extracts. The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the extracts were determined. Antituberculosis screening of the hexane, dichloromethane, ethyl acetate and methanol extracts against *Mycobacteria bovis* showed that dichloromethane extracts of *C. capitatum and H. insignis* were most active while only the ethyl acetate extract of *P.senegalense* was active. *Heeria insignis* was the most active of the three plants. Chromatographic separation of the dichloromethane extract of *C. capitatum and H. insignis* and ethyl acetate extract of *P. senegalense* yielded six chemical substances which were characterized using 1-D and 2-D NMR spectra to be 3-hydroxylanost-7-en-29-carboxylic acid (C1), Betulin (C2 & H1), 3-hydroxy-7-lanostene (H3), a yet to be identified compound (H4) and α-amyrin (P2). Antimicrobial studies of the isolated compounds revealed that they were active against most of the test microorganisms. *S. dysenteriae* was the most sensitive to all the isolated compounds with MIC of 62.5 µg/mL and MBC of 125 µg/mL. Antituberculosis evaluation of the compounds showed that they were all active against *Mycobacteria bovis* with H3 being the most active with MIC of 125 µg/mL. Findings from this work clearly shows that these plants have potentials that can be explored in the search for anti-TB drugs from nature.

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List of Abbreviations and Acronyms

NMR: Nuclear Magnetic Resonance Spectroscopy

^{13}C NMR: C-13 Nuclear Magnetic Resonance Spectroscopy

^{1}H NMR: Proton Nuclear Magnetic Resonance Spectroscopy

1 D : One Dimensional

2 D : Two Dimensional

$CDCl_3$: Deuterated Chloroform

COSY : Correlation spectroscopy

d : Doublet

dd: Double Doublet

DEPT: Distortionless Enhancement by Polarization Transfer

HMBC: Heteronuclear Multiple Bond Correlation

HSQC: Heteronuclear Single Quantum Coherence

Hz : Hertz

m: Multiplet

ME: Methanol

EA Ethyl acetate

MIC: Minimum Inhibitory Concentration

MBC Minimum Bactericidal Concentration

MFC Minimum Fungicidal Concentration

BCG Baccille Calmette-Guérin

Mp: Melting Point

NOESY: Nuclear Overhauser Effect Spectroscopy

s : Singlet

t : Triplet

TLC : Thin Layer Chromatography

UV : Ultraviolet

EtOAc : Ethyl acetate

DMSO: Dimethyl Sulfoxide

MHB: Muller-Hinton Broth

SDA : Sabraud Dextrose Agar

DCM: Dichloromethane

MeOH: Methanol

HE Hexane

ZI Zone of Inhibition

CHAPTER ONE

1.0 Introduction

1.1 Medicinal Plants

Medicinal plants are natural sources of compounds that can be used against many diseases (Kubmarawa *et al.,* 2007). The medicinal values of these plants lie in bioactive compounds that produce definite physiological actions on the human body (Krishnaiah *et al.,* 2009). Medicinal plants have been shown by many studies as sources of diverse nutrients and non-nutrient compounds. Many of the medicinal plants display antioxidant and antimicrobial properties which can protect the human body against both cellular oxidation reactions and pathogens. Thus, it is important to characterize different types of medicinal plants for their antioxidant and antimicrobial potentials (Bajpai *et al.,* 2005; Mothana and Lindequist, 2005; Wojdylo *et al.,* 2007). In Africa and developing countries, it is estimated that 70 to 80% of people rely on traditional healers and herbal practitioners for their health needs (Agyare *et al.,* 2006) and medicinal plants are the main sources of remedies used in this therapy. Some of these medicinal plants have been used for the management of different disease conditions such as bacterial infections, parasitic infections, skin diseases, hypertension, pains and inflammation such as rheumatoid arthritis (Muthu *et al.,* 2006).

The World Health Organization (2000) defines traditional medicine as "the diverse health practices, approaches, knowledge and beliefs incorporating plant, animal and/or mineral based medicines, spiritual therapies, manual techniques and exercises applied singularly or in combination to maintain well-being, as well as to treat, diagnose or prevent illness". Traditional medicine utilizes biological resources and the indigenous knowledge of plant groups conveyed verbally through generations. This is closely linked to the conservation of biodiversity and the related intellectual property rights of indigenous people (Timmermans, 2003). It is however necessary to validate the information through an organized infrastructure

for it to be used as an effective therapeutic means, either in conjunction with existing therapies or as a tool in novel drug discovery. Although it is these traditional medicines that provide the link between medicine and natural products, it was not until the 19th century that active compounds were isolated and principles of medicinal plants identified (Phillipson, 2001). The isolation of morphine from opium by Serturner (1805) started the chemistry of natural products (Patwardhan *et al.,* 2004).

Despite the discovery of natural products from higher plants, the interest of chemists and pharmaceutical scientists shifted to production of synthetic compounds and modification of natural products to enhance biological activity, increase selectivity and to decrease toxicity and side effect. Aspirin is one such example and was the earliest of these modified natural products. In more recent years, industry has once again turned its interest to natural product research (Phillipson, 2001). This is as a result of drug-resistant microorganisms, side effect of modern drugs and emerging diseases for which no medicine is available (Phillipson, 2001).

Some classes of chemical compounds that have been isolated from natural products include; terpenoids, alkaloids, flavonoids and glycosides. These are the major classes of components of natural products (Kaisar *et al.,* 2011).

a) **Terpenoids:** These are modified terpenes, where methyl groups are moved or removed or oxygen atoms added. They represent the oldest group of small molecular products synthesized by plant and are probably the most widespread groups of natural products (Kaisar *et al.,* 2011).
b) **Alkaloids**: These are a group of naturally occurring chemical compounds that contain a basic nitrogen atom. They have a bitter taste and are generally white solids (Kaisar *et al.,* 2011).

c) **Flavonoids:** These are a class of plant secondary metabolites. They are polyphenolic compounds that are ubiquitous in nature and are categorized according to chemical structure into flavonols, flavones, and flavonones, isoflavones, catechins, anthocyanidins and chalcones. They have aroused considerable interest recently because of their potential beneficial effects on human health. They have been reported to have antiviral, anti-allergic, anti-platelet, anti-inflammatory, anti-tumor and antioxidant activities (Kaisar *et al.,* 2011).

d) **Glycosides:** These are molecules in which a sugar is bound to a non-carbohydrate moiety, usually as small organic molecule. Many plants store chemicals in the form of inactive glycosides (Kaisar *et al.,* 2011).

1.2 Natural products as leads in novel and active chemotypes

There is an urgent need to identify novel and active chemotypes as leads for effective drug development. Natural products including plants, animals and minerals have been the basis of treatment of human diseases. The current accepted modern medicine or allopathy has gradually developed over the years by scientific and observational efforts of scientists (Vickrant *et al.,* 2011). Natural products as crude materials with efficacy against various diseases have been selected by humans over many generations of practical experience. Such experiential evaluation is different from the scientific evaluation of orthodox medicine and is underestimated sometimes. In 2001, researchers identified 122 compounds used in modern medicine which were derived from ethnomedical sources, 80% of these have had an ethnomedical use identical or related to the current use of the active elements of the plant (Fabricant and Farnsworth, 2001). Most of the pharmaceuticals currently available to physicians such as aspirin, digitalis, quinine, opium, morphine, atropine, ephedrine, and digitoxin were developed from natural products (Vickrant *et al.,* 2011).

The substances that can inhibit pathogens and have little toxicity to host cells are considered candidates for developing new antimicrobial drugs. On the other hand, indiscriminate use of commercial antimicrobial drugs in the treatment of infectious diseases has resulted in multiple-drug resistance of many human pathogenic microorganisms. This situation has necessitated a more radical approach to the search for new antimicrobial substances from various sources which could be used as novel antimicrobial chemotherapeutic agents (Navarro *et al.*, 1996). The search for new antibiotics includes various sources such as the synthetic compounds, bioactive agents from aquatic microorganisms, and natural products including medicinal plants.

1.3 Statement of research problem

Infectious diseases are the number one cause of deaths world-wide and in tropical countries it accounts for approximately 50% of deaths (Iwu *et al.*, 1999).This is partly due to increasing incidence of multiple drug resistance. Bacterial resistance to almost all antibacterial agents have been reported (Ojiako, 2014) and this is largely due to indiscriminate use of antimicrobial drugs used in the treatment of infectious diseases. Apart from resistance, high costs, long periods of medication and undesirable side effects, some antibiotics have limited applications, hence there is serious need to develop safer and more active chemotherapeutic agents that will act over a short period of time.

1.4 Justification

*Heeria insignis (*DEL), *Psorospermum senegalense* (SPACH) and *Clerodendrum capitatum (*WILD*)* have been used in traditional medicine in many communities in northern Nigeria, for the treatment of various infectious diseases including tuberculosis. These plants are among plants claimed to treat tuberculosis and to the best of our knowledge, there is no reported

work on the antituberculosis activities of these, hence there is a need to justify scientifically the ethnomedicinal application of these plants.

1.5 Aim of the research

The aim of the research work is to validate the antituberculosis claim by traditional medicine practitioners associated with local uses of the stem bark of *Psorospermum senegalense* (Spach), leaves of *Heeria insignis* (Del) and *Clerodendrum capitatum* (Wild) and to isolate and characterise any possible antituberculosis compound(s) from these plants.

1.6 Objectives of the research

The aim stated above will be achieved through the following objectives:

i. collection and identification of the three plants parts,
ii. air drying, pulverising and extraction using known techniques,
iii. phytochemical screening of the crude plant materials,
iv. antimicrobial screening of the crude extracts of the plants,
v. antituberculosis screening of the crude extracts of the plant materials,
vi. isolation of phytochemicals present in the extracts and
vii. testing and characterisation of the isolated compounds for activities.

CHAPTER TWO

2.0 Literature Review

2.1 Tuberculosis

Tuberculosis, caused by *Mycobacterium tuberculosis* (MTB), or *Tubercle bacillus* (TB) is a widespread disease and in many cases fatal and infectious. The disease is caused by various strains of *Mycobacteria*. Tuberculosis typically infects the lungs, but can also infect other parts of the body. It is spread through the air when people who have an active TB infection cough, sneeze, or otherwise transmit respiratory fluids through the air **(**WHO, 2009). Most infections do not have symptoms, known as latent tuberculosis. About one out of ten latent infections eventually progresses to active disease which, if left untreated, kills more than 50% of those so infected **(**WHO, 2009).

2.1.1 Symptoms of tuberculosis

The classic symptoms of active TB infection are a chronic cough with blood-tinged sputum, fever, night sweats, unexplained weight loss, chest pain, or pain with breathing or coughing, fatigue, chills and loss of appetite. Infections of other organs cause a wide range of signs and symptoms varying according to the organs involved. For example, tuberculosis of the spine may give back pain, and tuberculosis of the kidneys might cause blood in urine (Dollin, 2010).

2.1.2 Treatment of TB

Effective TB treatment is difficult and requires administration of multiple antibiotics over a long period of time, due to the unusual structure and chemical composition of the *Mycobacterial* cell wall. The cell wall of *mycobacterium tuberculosis* contains an additional

layer beyond the peptidoglycan that is exceptionally rich in unusual lipids, glycolipids and polysaccharides such as mycolic acid, mycocerosic acid, phenolthiocerol, lipoarabinomannan, and arabinogalactan, which hinders the entry of drugs and makes many antibiotics ineffective (Reljic, 2007; Kolattukudy *et al*., 1997).

The standard treatment for TB is with the first line drugs isoniazid (along with pyridoxal phosphate to obviate peripheral neuropathy caused by isoniazid), rifampicin , pyrazinamide, and ethambutol for two months, then isoniazid and rifampicin alone for a further four months. The patient is considered to be free of living bacteria after six months (although there is still a relapse rate of up to 7%). For latent tuberculosis, the standard treatment is six to nine months of isoniazid alone, if the organism is known to be fully sensitive, then treatment is with isoniazid, rifampicin, and pyrazinamide for two months, followed by isoniazid and rifampicin for four months. Ethambutol need not be used. The second line drugs such as streptomycin, ciprofloxacin, moxifloxacin, ethionamide and cycloserinea are only used to treat disease that are resistant to first line therapy (i.e., for extensively drug-resistant tuberculosis (XDR-TB) or multidrug-resistant tuberculosis (MDR-TB) **(**WHO, 2009). Third-line drugs include drugs that may be useful, but have doubtful or unproven efficacy e.g. rifabutin, clarithromycin, linezolid, thioacetazone, arginine, vitamin D and bedaquiline **(**WHO, 2009).

2.1.3 Epidemiology

Tuberculosis is the second-most common cause of death from infectious disease after those due to HIV/AIDS (Cohn *et al.,* 1990). Roughly one-third of the world's population has been infected by *M. tuberculosis* (WHO, 2009) with new infections occurring in about 1% of the population each year (WHO 2010). However, most infections with *M. tuberculosis* do not cause TB disease and 90–95% of infections remain asymptomatic (Slutkin *et al*., 1988). Emerging mycobacterial drug resistance is further complicating the situation. After decades

of steady decline, the incidence of TB is also increasing in industralized countries, mainly as the result of outbreaks in particularly vulnerable groups (Von and Vuola, 2002).

The bacille Calmette-Guérin (BCG) vaccine has existed for 80 years and is one of the most widely used of all current vaccines. BCG vaccine has a documented protective effect against meningitis and disseminated TB in children. It does not prevent primary infection and, more importantly, does not prevent reactivation of latent pulmonary infection, the principal source of bacillary spread in the community. The impact of BCG vaccination on transmission of Mtb is therefore limited (Hoft, 2008).

2.2 Anti-Tubercular Plants

Many medicinal plants have been reported to exhibit antimycobacteria and anti-TB activities. Ibrahim *et al* (2012) reported the anti-TB activity of *Sterculia setigera* Del., leaf extracts. Nine medicinal plants including *Salvadora persica, Acacia senegale, Plumbago dawei, Loranthus acacia,* and *Acacia nilotica* used for the treatment of tuberculosis were studied in the work. The extracts were tested on four strains of *Mycobacteria* namely; *Mycobacterium tuberculosis* (Mtb), *Mycobacterium kansasii* (Mk), *Mycobacterium fortuitum* (Mf), and *Mycobacterium smegmatis* (Ms) using BACTEC MGIT 960 system. All the plants showed varying degrees of antibacterial activity on the four tuberculosis-causing strains (Richard *et al*., 2010). *Eurphobia scarlatina* was found to be the most active against both the slow (Mtb and Mk) and the fast (Mf and Ms) growers with Zero GUs at 0.5 mg/mL (Richard *et al*., 2010). Also Donfack *et al* (2014) screened 19 ethnobotanically selected plants including *Aframomum melegueta, Annickia chloranta, Anonidium mannii, Annona muricata* and *Chlorophytum macrophyllum* for their activity against *Mycobacterium smegmatis, Mycobacterium avium, Mycobacterium bovis. Mycobacterium tuberculosis and Mycobacterium ulcerans* using the Resazurin Microtiter Assay. Results showed that crude

extracts from all the plants mainly inhibited BCG, while interface fractions from *Annickia chlorantha* stem bark and stem displayed the strongest activity against *M. ulcerans*, with minimum inhibitory concentrations (MICs) of 1.95 and 7.81 µg/ml respectively. Similarly the antibacterial activity of the butanol extracts of *Alstonia scholaris* was carried out by Molly *et al* (2012). The Luciferase Reporter Phage (LRP) assay and an *in vitro* bioassay based on inactivation of viability by a procedure similar to neutralization test were used to study the inhibition of *Mycobacterium tuberculosis*. The LRP method showed that butanol extracts of flower and bark at concentrations of 100 and 500µg/ml respectively showed moderate bactericidal activity against clinical strains of sensitive and drug resistant *Mycobacterium tuberculosis* whereas the results of *in vitro* bioassay showed complete inhibition of the fast grower *Mycobacterium* species. The antibacterial activity of *Withania somnifera and Pueraria tuberosa* against *Mycobacterium tuberculosis* H37R was tested using minimum inhibitory concentration method (MIC) carried out after sensitivity testing. Aqueous extract of *Withania somnifera* (0.01-1.0 mg/mL) had significant effect against *M. tuberculosis* whereas *Pueraria tuberosa* in higher concentration showed no inhibition (Periyakaruppan *et al.*, 2012).

Heeria insignis, *Psorospermum senegalense* and *Clerodendrum capitatum* are used in northern Nigeria traditionally to treat various infections, including tuberculosis but this claims have not been validated by any scientific means hence, there is need to investigate the antituberculosis efficacy of these plants.

Table 2.1 Plant materials

Plants	**FAMILY**	**HAUSA NAME**	**USES**
Psorospermum senegalense (SPACH)	Hypericaceae	*Huda tukunya*	Kidney problems, Stomach ache, Tuberculosis, Skin infections, Protection against evil Leprosy and syphilis (Burkill, 1994).
Heeria insignis (DEL)	Anacardiaceae	*Hawaye Zaki*	Diarrhoea , venereal diseases, tuberculosis, tapeworm and hookworm, to increase lactation schistosomiasis, kidney trouble (Burkill, 1985).
Clerodendrum capitatum (WILD)	Verbenaceae	*Mashayi*	Tuberculosis, Diabetes mellitus, Obesity, Hypertention ,Erectile dysfunction, and Asthma (Neeta and Tejas, 2007).

2.3 The Verbenaceae Family

The Verbenaceae family commonly known as the *verbena* or *vervain* family comprises of 35 genera and 1,200 species.They are found mainly in the tropical and subtropical regions of the world (Heywood *et al*., 2008). This family is represented by herbs, shrubs and small trees known for heads, spikes or clusters of small flowers, of which many have aromatic odour. Recent phylogenetic studies have shown that numerous genera traditionally classified as Verbenaceae belong instead in Lamiaceae. The main difference between the two families is the ovary. Lamiaceae have a deeply four-lobed ovary with gynobasic style while the Verbenaceae have an unlobed ovary and a terminal style (Cronquist,1981; Cantino *et al.,* 1992).

2.3.1 The Genus *Clerodendrum*

Clerodendrum is a genus of flowering plants belonging to the family Verbenaceae. It is widely distributed in the tropical and warm temperate regions of the world, with most of the species occurring in tropical and northern Africa, Asia, Egypt and Madagascar. The genus represents herbs, shrubs and small trees usually growing up to 1–12 m tall, with opposite or whorled leaves and is well-known for its ornamental uses (Pallab *et al.*, 2014). Estimates of the number of species in *Clerodendrum* vary widely from about 150 to about 450, this is partly because about 30 species have been transferred to Rotheca, about 30 more to Volkameria, and one to Ovieda (Yao-Wu *et al*., 2010; Raymond *et al.*, 2004; Rosette and Bernard 2000).

The genus is widely used as medicines specifically in Indian, Chinese, Thai, Korean and Japanese systems of medicine for the treatment of various life-threatening diseases such as syphilis, typhoid, cancer, jaundice and hypertension (Neeta and Tejas, 2007). Few species of the genus are ornamentals and are cultivated for aesthetic purposes. The powder/paste form

and the various extracts of root, stem and leaves are reported to be used for the treatment of asthma, pyreticosis, cataract, malaria and diseases of blood, skin and lung (Neeta and Tejas, 2007).The major chemical components reported from the genus are phenolics, steroids, di- and triterpenes, flavonoids and volatile oils (Neeta and Tejas, 2007).

The genus *Clerodendrum* is reported to demonstrate versatile biological activities such as antitumurgenic (Liu *et al*., 2008), hypoglycemic, hypolipidemic (Devi and Sharma, 2004) hepatoprotective activity against CCl_4-induced liver injury in rats (Gopal and Sengottuvelu, 2008), anti-inflammatory (Choi *et al*., 2004), radical-scavenging activity (Vidya *et al*., 2007 and Chae *et al*., 2006), antidiarrhoeal (Rani *et al*., 1999), antinociceptive and antipyretic effects (Narayanan *et al*., 1999).

2.3.2 *Clerodendrum capitatum*



Plate 1

Clerodendrum capitatum (Willd) locally named "Gung" in Sudan and called *mashayi* in Hausa, is an indigenous tropical African plant, which grows fast, erect, well branched, having perennial under-shrub and grows up to 0.5-2 m high (Adeneye *et al*., 2008).

2.3.3 Medicinal Uses of *Clerodendrum capitatum*

In Sudan, the roots of this plant are used traditionally in the management of male erectile dysfunction (Mahmoud *et al*., 1995). In Nigeria, this plant is used to treat fever, diabetes mellitus, obesity, diarrhoea, asthma, pyreticosis, tuberculosis and hypertension (Siddig *et al*., 2012).

2.3.4 Pharmacological Investigation of members of *Clerodendrum* genus

(i) Antimalarial Activity

The antimalarial activity of some *Clerodendrum* species have been reported . Simonsen *et al* (2001) reported that the alcoholic extract of *Clerodendrum phlomidis* showed antimalarial activity against *Plasmodium falciparum* with an IC_{50} value of 48 µg/ml. In another report, *Clerodendrum inerme* inhibited the growth of larvae of *Aede saegypti, Culex quinquefasciatus and Culex pipiens* at 80 and 100 ppm concentration of petroleum ether extracts and it was found to have antimalarial activities (Kalyanasundaram and Das, 1985).

(ii) Antioxidant Activity

Organic and aqueous extracts of *Clerodendrum colebrookianum* were reported to show significant inhibition of lipid peroxidation *in vitro* and *in vivo* induced by $FeSO_4$ ascorbate in rats. Aqueous extracts showed stronger inhibitory activity over organic extracts (Rajlakshmi *et al.,* 2003). Isoacteoside, trichotomoside and jionoside D, three compounds isolated from *C. trichotomum*, when tested showed significant scavenging activity of intracellular reactive oxygen species produced by hydrogen peroxide suggesting their antioxidant properties (Chae *et al*., 2004).

(iii) Anti-Inflammatory Activity

Many species of the genus *Clerodendrum* have shown potent anti-inflammatory activity. *Clerodendrum phlomidis* was reported for significantly decreasing paw oedemas induced by carrageenan in rats at a dose of 1g/mL (Surendrakumar, 1988). Similarly *Clerodendrum petasites* was reported to show moderate anti-inflammatory activity in the acute phase of inflammation in rats (Panthong *et al*., 2003). Somasundram and Sadique (1986) reported that flavonoid glycosides of *Clerodendrum inerme* showed modulation in calcium transport in isolated inflamed rat liver and thereby showed reduction in inflammation. The alcohol extract of roots of *Clerodendrum serratum* showed a significant anti-inflammatory activity in carrageenan and also in the cotton pellet model in experimental mice, rats and rabbits (Narayanan *et al*., 1999).

(iv) Antimicrobial Activity

The hexane extract of *Clerodendrum colebrookianum* at concentrations of 1000 and 2000 ppm showed strong antibacterial activities against various Gram positive and Gram negative pathogens such as *S. aureus, Staphylococcus haemolyticus, E. coli, Pseudomonas aeruginosa* (Misra *et al*., 1995). Also two flavonoids from roots of *C. infortunatum*, cabruvin and quercetin, showed strong antifungal activity. The flavonoids showed activity against *Alternaria carthami and Helminthos porinoryzae,* the quercetin against *Alternaria alternate* and *Fusarium lini* at concentrations of 200, 500 and 1000 mg/ml (Roy *et al.*, 1996). Misaponin A, a triterpenoid saponin isolated from the roots of *Clerodendrum wildii*, showed potent antifungal activity against *Cladosporium cucumerinum* (Toyota *et al*., 1990).

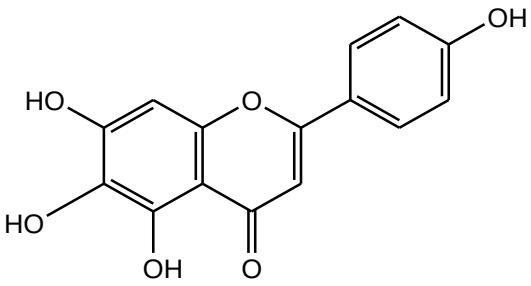
(v) Antiviral Activity

Mehdi *et al* **(**1997) studied the antiviral activity of *Clerodendrum inerme* against Hepatitis B virus. Result showed an ED_{50} value of 16 mg/mL. Also two phenyl propanoid glycosides

(acteoside and acteoside isomer) isolated from *Clerodendrum* trichotomum showed potent inhibition of HIV-1 integrase with IC_{50} values of 7.8 ± 3.6 and 13.7 ± 6.0 (Kim *et al.*, 2001).

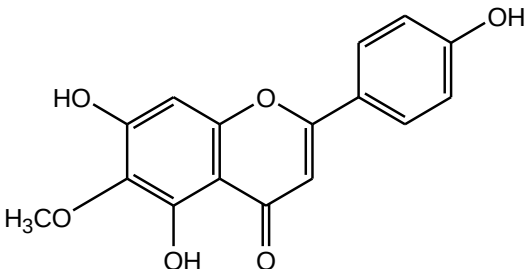
2.3.5 Some compounds isolated from *Clerodendrum* genus

A number of researchers have identified and isolated compounds from different species of *Clerodendrum*. The major compounds isolated from *Clerodendrum* are steroids, terpenoids and flavonoids are major among them (Praveen *et al.*,2012). Scutellarein (**1**) was isolated from the aerial parts of *C. indicum* (Jun and Handong, 1999), Apigenin (**2**) was isolated from the leaves and stem of *C. inerme* (Pandey *et al.*, 2006), Hispidulin (**3**), pectolinarigenin (**4**) and Luteolin (**5**) were isolated from the flowers of *C. phlomoides* (Bhattacharjee *et al.*, 2011). Similarly 7-hydroxy flavanone (**6**) and Cleroflavone (**7**) were reported from *C. inerme* (Shrivastava and Patel, 2007). Oleanolic acid (**8**) was also isolated from *C. inerme.*



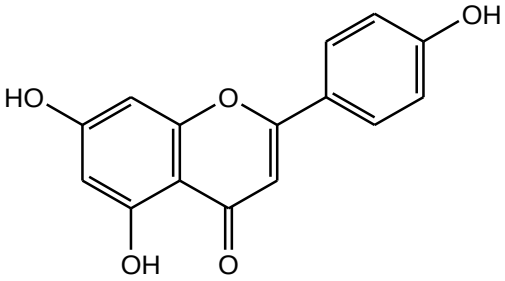
(**1**)

Scutellarein



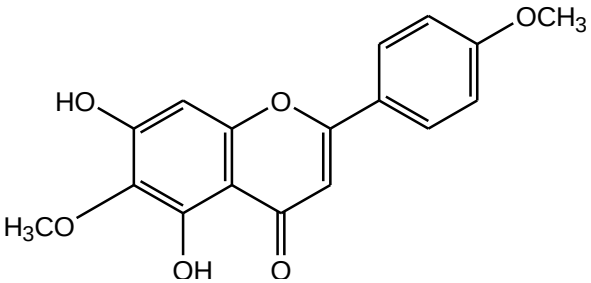
(**2**)

Apigenin



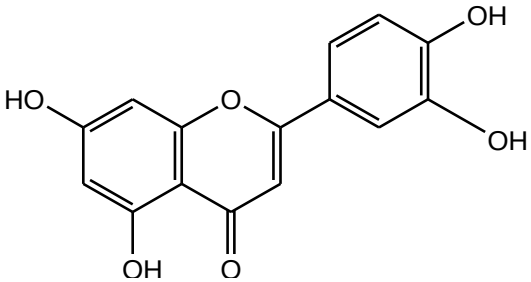
(**3**)

Hispidulin



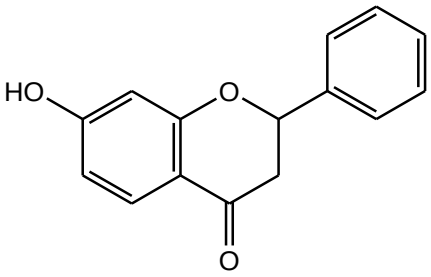
(**4**)

Pectolinarigenin



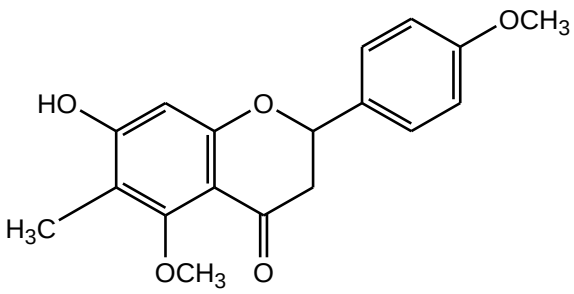
(**5**)

Luteolin



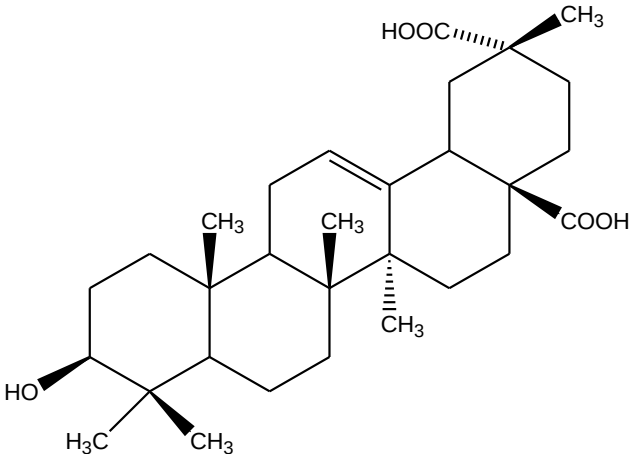
(**6**)

7-hydroxy flavanone



(7)

Cleroflavone

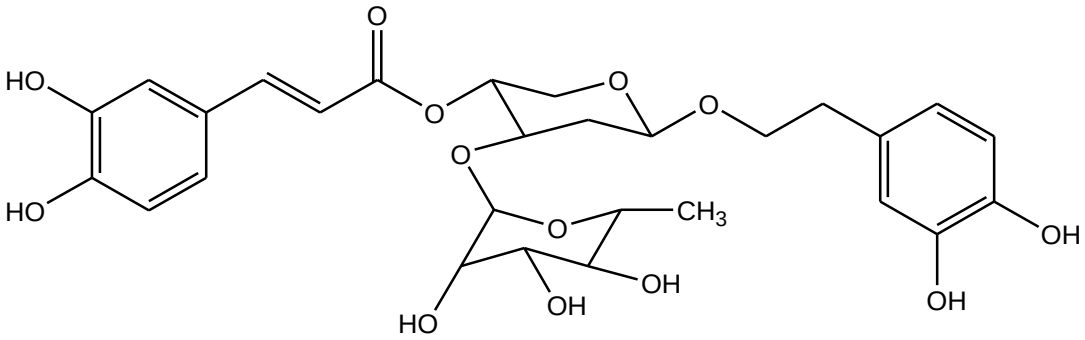


(**8**)

Oleanolic acid

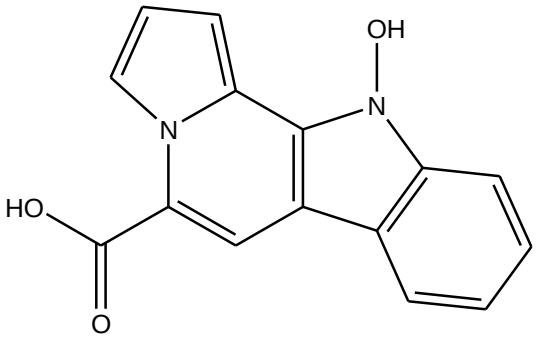
Some phenolic compounds have also been reported from *C. inerme*, they include acteoside (**9**), indolizino (**10**) and verbascoside (**11**) (Shrivastava and Patel, 2007).

Researchers have reported that the roots of *C. infortunatum* contain β-sitosterol (**12**), clerosterol (**13**), clerodolone (**14**), clerodone (**15**), which is identified as 5, 25-sigmastadien-3β-ol, lup_20 (30)-en-3β-diol-12-one and 3β-hydroxylupan-12-one, the roots of *C. phlomides* was reported to contain γ- sitosterol (**16**) **(**Gokani *et al.*, 2011).



(**9**)

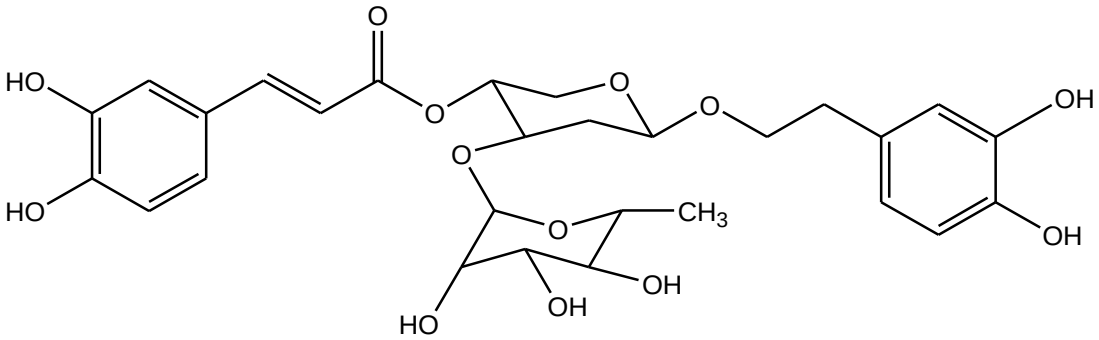
Acteoside



Indolizion [8,7-B] Indole
5-Carboxlic Acid

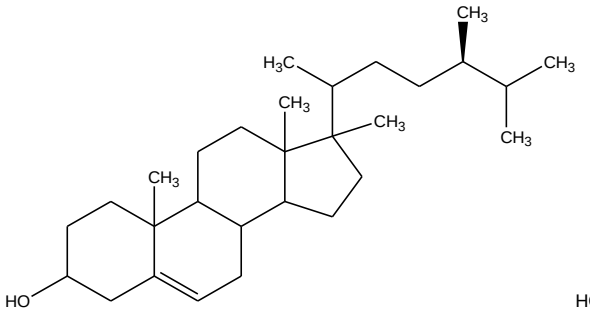
(**10**)

Indolizino



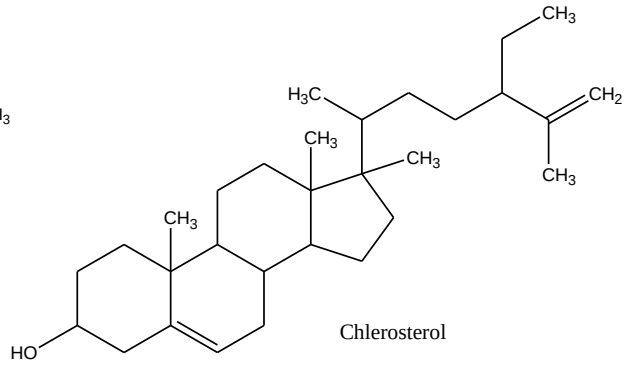
(**11**)

Verbascoside



(**12**)

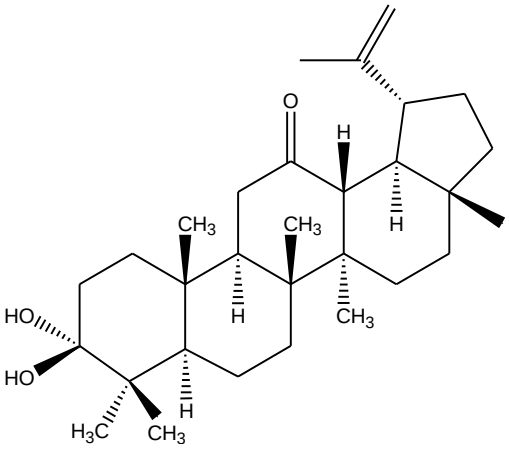
β-sitosterol



Chlerosterol

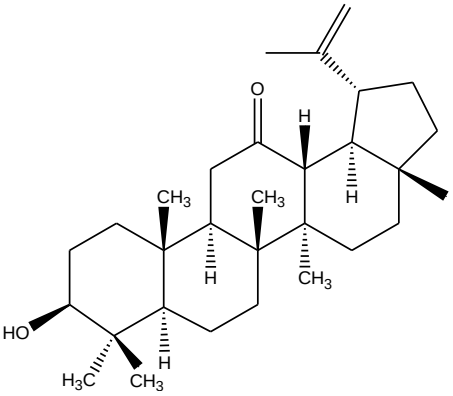
(**13**)

Clerosterol



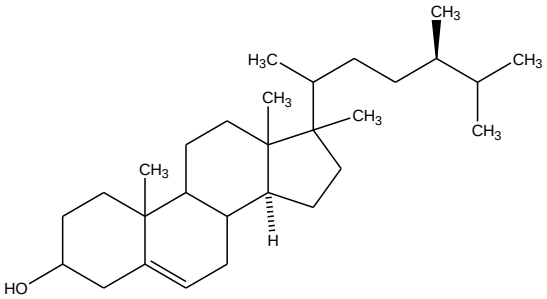
(**14**)

Clerodolone



(**15**)

Clerodone



(**16**)

γ- sitosterol

2.4 The Anacardiaceae family

The family Anacardiaceae which is also known as the cashew family includes approximately 800 species in 82 genera. They are found primarily in the tropics and subtropics but extending into the temperate zone. The Anacardiaceae includes primarily trees and shrubs (rarely lianas or subshrubs) with resin canals and clear to milky exudate. Members of the family are cultivated throughout the world for their edible fruits and seeds, medicinal compounds, valuable timber and landscape appeal. Some of the genus of Anacardiaceae, including mango (*Mangifera indica*), pistachio (*Pistacia vera*), cashew (*Anacardium occidentale*), and pink peppercorn (*Schinus terebinthifolia*), are enjoyed worldwide while other notables such as the pantropical Spondias fruits, the marula of Africa (*Sclerocarya birrea*) are restricted to localized cultivation and consumption and are not generally transported far distances to larger markets. (Mitchell and Mori, 1987).

2.4.1 The genus *Heeria*

The genus *Heeria* belongs to the family of Anacardiaceae in the major group Angiosperms (Flowering plants). The plant list includes 47 scientific plant names of species rank for the genus *Heeria*. The genus *Heeria* is monotypic, forming an evergreen tree that is found growing naturally in the south western Cape region of South Africa. The gum exudate from the bark has been used as an ointment to treat boils and abscesses (Von *et al*., 1996).

2.4.2 *Heeria insignis*



Plate 2

Heeria insignis (a synonym of O*zoroa insignis*) is locally named or called *hawayen zaki* or *kansheshe* in Hausa. It is a shrub or tree up to 6.5 m high, it is an indigenous African shrub found extensively in the southern savanna from Senegal to Niger and Nigeria (Dalziel, 1955).

2.4.3 Medicinal Uses of *Heeria insignis*

In northern Nigeria, this specie is used in treatment of tuberculosis, venereal diseases, tapeworm and hookworm, schistosomiasis and kidney trouble (Burkill, 1985) In Guinea-Bissau, the infusion of the roots is taken by women after childbirth to increase lactation (Abreu *et al.,* 1999).

2.4.4 Pharmacological Investigation of members of *Heeria* genus

(i) Antioxidant Activity

The aqueous and organic solvent extracts of the peeled stem of *Ozoroa insignis* exhibited very high antioxidant activity. It reduced 2,2- diphenylpicrylhydrazyl (DPPH) in the first minute by about 84.5% (Nyaberi *et al*., 2010).

(ii) Cytotoxic activity

The compound 6-pentadecylsalicylic acid isolated from the dichloromethane extract from twigs of *Ozoroa insignis* was reported to be active against marine crustaceans (Weidong *et al*., 2002). Also the crude methanol bark extract of *Ozoroa insignis*, showed *in-vitro* cytotoxic activity against Hep-G2 (human hepatocellular carcinoma), MDA-MB-231 (human mammary adenocarcinoma), and 5637 (human primary bladder carcinoma) (Rea *et al.,* 2003).

(iii) Anti-tumor Activity

In the antitumor screening of medicinal plants from Guinea-Bissau, (Abreu *et al*., 1999) observed that an ethanolic root extract of *O. insignis* showed moderate activity in KB, A 549 and MDA-MB cell lines, with IC_{50} values of 30.5, 22.0 and 15.5 µg mL^{-1}, respectively.

(iv) Antidiarrhoeal activity

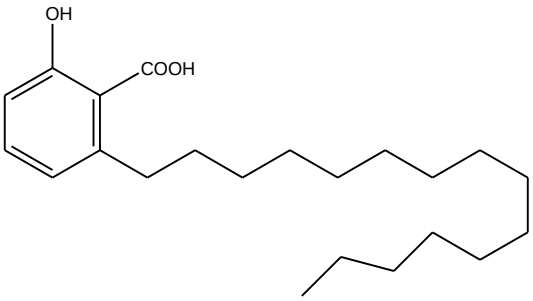
The antidairrhoeal activity of the methanol and dichloromethane extracts of the leaves of *Heeria insignis* using isolated rabbit jejunum and castor-oil induced diarrhoea in mice was investigated. On the isolated rabbit jejunum evaluation, both extracts produced concentration-dependent relation of isolated rabbit jejunum that was not blocked by phentolamine. In the castor oil-induced diarrheoeal test, each extract gave 80% protection at 200 mg/kg, which is comparable to loperamide 2 mg/kg with 80% protection (Agunu *et al*., 2011).

(v) Antimicrobial activity

The antibacterial activity of the methanol and dichloromethane extracts of the leaves of *Heeria insignis* was studied. It showed significant inhibitory effects against *Salmonella typhi*, *Pseudomous aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*. The methanol extract gave higher antibacterial activity than dichloromethane (Agunu *et al*., 2011). Also, Nyaberi *et al* (2010) determined the antimicrobial activity of the aqueous and organic extracts of the peeled stem charcoal of *Ozoroa insignis* using the cork and bore diffusion method against test organisms *Staphylococcus aureus* (ATCC 22923), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922) and *Candida albicans* (ATCC 90028). The chloroform and methanol extracts were significantly active against *P. aeruginosa, E. coli* and *S. aureus* compared to the aqueous extract that had no activity against any of the test organisms.

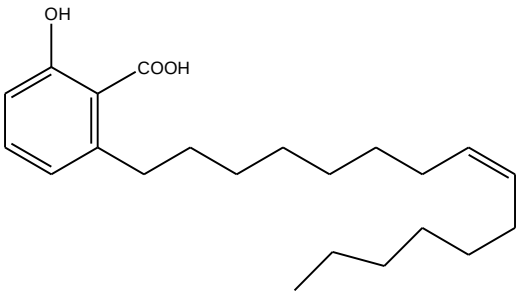
2.4.5 Some compounds isolated from *Heeria* genus

The isolation of anacardic acid (6-pentadecylsalicylic acid) (**17**) and ginkgoic acid (**18**) from the crude methanol bark extract of *Ozoroa insignis* has been reported by Rea *et al* (2003). The leaf and flower oils of *Heeria insignis* were found to contain myrcene (**19**), α-pinene (**20**) and β-pinene (**21**) (Ayedoun *et al*.,1998). A new orsellinic acid named ozoroalide (**22**) and anacardic acid methyl esters (**23**) were isolated from the roots of *Ozoroa insignis* and identified on the basis of spectroscopic methods (Pedro and Yonghong, 2007).



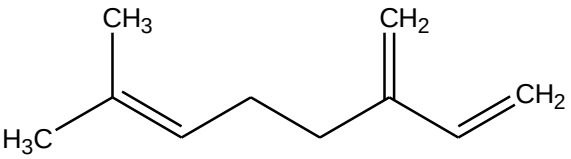
(**17**)

anacardic acid



(**18**)

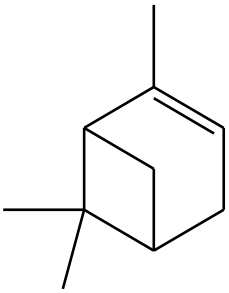
ginkgoic acid



(12)

(**19**)

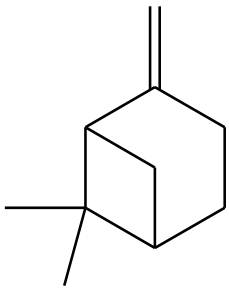
Myrcene



(13)

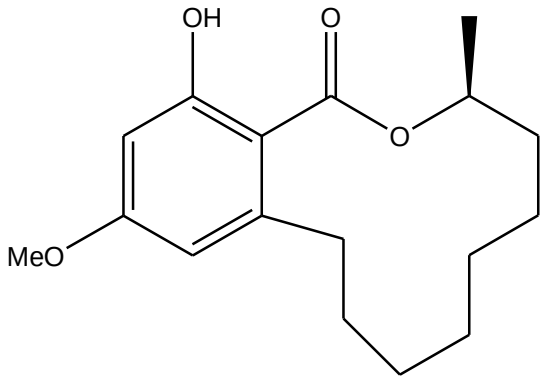
(**20**)

α-pinene



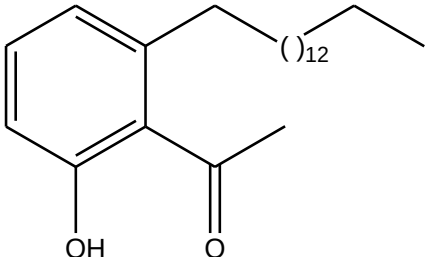
(**21**)

β-pinene



(**22**)

ozoroalide



(**23**)

Anacardic acid methyl ketone

2.5 The Guttiferae family

The family Guttiferae or Clusiaceae are a family of plants with about 37 genera and 1610 species (Gustafsson, 2002)**.** This family is represented by trees and shrubs often with milky sap, fruits and capsules for seeds. It is primarily tropical, it shows large variation in plant morphology (for example, three to ten, fused or unfused petals and many other traits). According to the APG III, this family belongs to the order Malpighiales. The APG III system reduced the circumscription of this family to just 14 genera and about 595 species. Previous circumscriptions have often included the family Hypericaceae as a subfamily within Guttiferae (Gustafsson, 2002).

2.5.1 The genus *Psorospermum*

The genus *Psorospermum* comprises 55 species mostly shrubs or small trees growing in tropical regions of South America, Africa and Madasgascar. Most of the species of the genus have been used for centuries in the ethnomedical traditions of indigenous African populations as febrifugal, antidote against poisons, purgative, stomachic and as a remedy for the treatment of leprosy, skin diseases (like dermatitis, scabies and eczemas) and subcutaneous wounds. (Francesco *et al*., 2013)**.**

2.5.2 *Psorospermum senegalense*



Plate 3

The plant *Psorospermum senegalense* is a plant that belongs to the family of *Hypericaceae*. Its local name in Hausa language is *Huda Tukunya*. It is found in the bush and wooded savanna of the Sandanian zone, recorded from Senegal to Sierra Leone. The plant is also found in other places like Dakar and Guniea.They are shrubs, the branches and young leaves clothed with a pale rusty-brown tomentum, deciduous on the old wood and partially on the upper surface of the leaves. Flowers densely tomentose, tomentose pedicels equalling or twice as long as the calyx. Stamens 5–8 in each phalange, ovules solitary, ascending. (Burkil, 1994).

2.5.3 Medicinal uses of *Psorospermum senegalense*

The plants are attached to millet by the Tenda to discourage bees from nesting on it. The plant enters into a Fulani magical treatment to confer protection against evil (Burkill, 1994). The plant has a general usage in Senegal for all skin infections. A bark decoction of the root is used in washes and bathes for common dermal troubles and for herpes, eczema, leprous and syphilitic conditions. In Nigeria decoction of the leaves is used in the treatment of tuberculosis. In the Ferlo region, a decoction of bark from the main stem and of the roots is added to baths and is made up into draughts for pains in the joints in attacks of fever (Lawal and Abu, 2013). A filtrate from a prolonged boiling of the leaves is deemed in Senegal to alleviate respiratory trouble and is taken to treat leprosy. An oil film comes to the surface of this preparation which can be separated off on cooling. This is used externally for skin troubles (Burkill, 1994).

2.5.4 Pharmacological Investigation of members of *Psorospermum genus*

(i) Antimalarial Activity

Bioassay-guided fractionation of the n-hexane extract of the stem bark of *Psorospermum glaberrimum* showed that this crude extract exerted a good anti-plasmodial activity against *P.*

falciparum W2 strain, with IC_{50} of 0.87 l g/mL, among the compounds, resulting from the purification of the extract, 3-geranyloxy emodinanthrone and acetylvismione D were seen to exert a good level of anti-plasmodial activity providing IC_{50} values of 1.68 and 0.12 l M respectively (Ndjakou *et al*., 2008).

The extracts of different polarity from the leaves of *P.senegalense* was studied as an anti plasmodial remedy against *Plasmodium falciparum*. It was found that the dichloromethane extract exerted a valuable activity (IC50 = 10.03 g/mL) (Jansen *et al.*, 2010).

(ii) Antimicrobial activity

A bianthrone named Adamabianthrone isolated from the bark extract of *Psorospermum adamauense* was tested against five Gram-positive bacteria (*Citrobacter freundii, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Salmonella typhi),* three Gram negative bacteria *(Bacillus cereus, Staphylococcus aureus, and Staphylococcus faecalis),* and two fungi *(Candida albicans and Microsporum audounii).* The lowest MIC value of (27.34 mg/mL) was recorded for *S. faecalis*, while other values ranged from 54.68 to 218.74 mg/mL. adamabianthrone did not show activity against *C. albicans* (Tsaffack *et al*., 2009). The antimicrobial activity of the root extract of *Psorospermum corymbiferum* was tested and it was found to be active against a wide panel of microorganisms, including bacteria and fungi like *B. subtilis, S. typhi, S. aureus, P. aeruginosa, Proteus mirabilis, Bacillus cereus,* and *C. albicans* (Zubair *et al*., 2009a, b).

(iii) Anti cancer activity

Kupchan *et al* (1980) provided the first evidence of the anti-leukemic properties of *Psorospermin* isolated from the root of *P. febrifugum* on P-388 cell line, also 3-geranyloxyemodin anthrone isolated from the same plant was proven to have anti-leukemic effect on P-388 cell line, although with much less efficacy when compared to *Psorospermin*

(Amonkar *et al*. 1981). Leet *et al* (2008) performed a preliminary screening of the *in vitro* anti-cancer activity of the dichloromethane and methanol extracts from the wood stems and roots of *Psorospermum molluscum* Hochr and found that both exerted a potent effects in a panel of mammalian cancer cells with IC_{50} values which ranged between 0.2 to 4.0 g/ mL.

(iv) Anti-leishmanial activity

Extracts of the leaves and root bark of *P. guineense* obtained after maceration with dichloromethane, methanol and water were tested as anti-leishmanial agents. The result from this study indicated that only the dichloromethane extract of root bark was active against both the extracellular and the intracellular form of leishmania major with percentages of survival of 3.5 and 14.0 % at a dose of 35 g/mL respectively (Ahua *et al*., 2007).

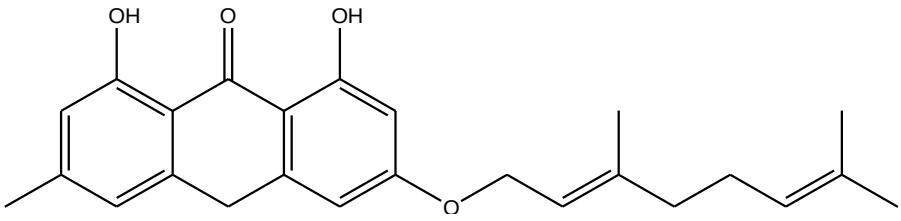
(v) Anti-inflammatory activity

An essential oil obtained by stem distillation from the leaves and roots of *P. tenuifolium* was assayed for its anti-inflammatory activity using the tetradecanoylphorbol-13-acetate induced ear oedema in mice. At concentration values of 5.0 and 2.5 mg/mL, a significant anti-inflammatory effect with percentages of oedema reduction of 92.3 and 76.9 % respectively was recorded (Zubair *et al*., 2009a, b).

2.5.5 Some compounds isolated from *Psorospermum* genus

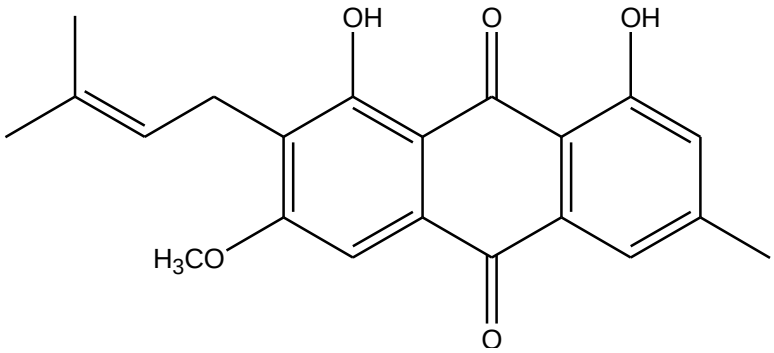
The secondary metabolites isolated from *Psorospermum* genus include alkaloids, anthraquinones, anthrones and bianthrones, vismiones, flavonoids, long chain alcohols, steroids, tannins, terpenes, and xanthones (Francesco *et al*., 2013). Tsaffack *et al* (2009) investigated the chemical composition of the bark extract of *Psorospermum adamauense* obtained after maceration with a 1:1 mixture of methanol/methylene chloride and revealed the presence of 3-geranyloxyemodin (**24**). Also Vismiaquinone (**25**), a- and b-amyrin (**26**) and (**27**) were isolated from the methanol extract of the leaves of *Psorospermum*

androsaemifolium (Poumale *et al*., 2008, 2011). Kouam *et al.*, (2010) reported the isolation and characterization of friedelan-3-one (**28**), friedelan-3-ol (**29**), and finally betulinic acid (**30**) have been reported from *Psorospermum aurantiacum* (Kouam *et al*., 2010).



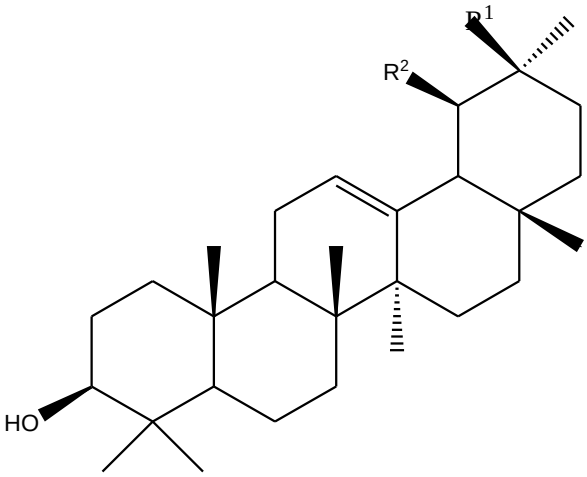
(**24**)

3-geranyloxyemodin anthrone



(**25**)

Vismiaquinone

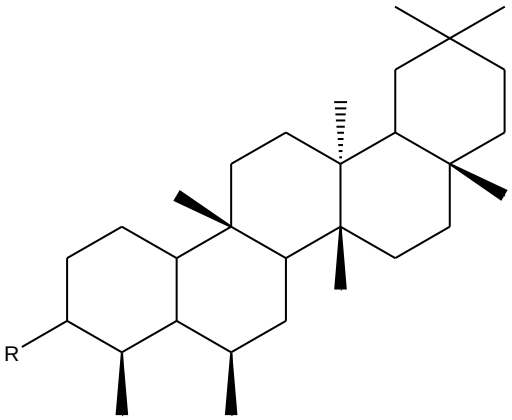


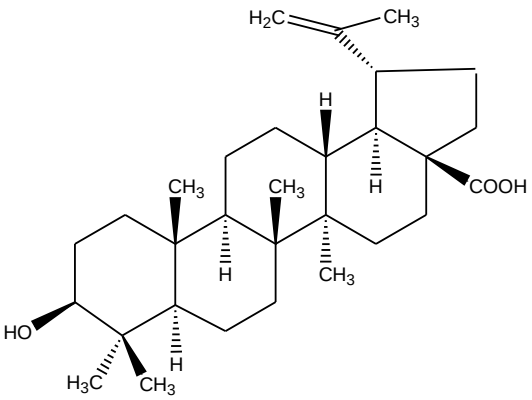
(**26**) R^1 = H, R^2 = CH_3

a-amyrine

(**27**) $R^1 = CH_3$, $R^2 = H$,

b-amyrine





(**28**) R = =O

Friedelan-3-one

(**30**)

Betulinic acid

(**29**) R = β-OH

Friedelan-3-ol

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1.0 Materials

3.1.1 Solvents/Reagents

The solvents used were methanol, ethyl acetate, dichloromethane and n-hexane. They were sourced from JHD Guanghua Chemical Factory Co., Ltd. Guandong, China. They were of general purpose grade and were distilled before use.

3.1.2 Equipment

The melting points of the isolated compounds were determined using the Gallenkamp melting point apparatus at the Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University Zaria.

^{1}H NMR and ^{13}C NMR were run on a 600 MHz Bruker AVANCE spectrometer obtained at the School of Chemistry and Physics, University of Kwazulu-Natal Durban, South Africa.

3.1.3 Plant Material

The plant materials were collected fresh from Zaria, Nigeria in September, 2013. The plants were authenticated at the Department of the Biological Sciences, Ahmadu Bello University, Zaria, Nigeria. A voucher specimen number 900688 for *Clerodendrum capitatum,* 900206 for *Psorospermum senegalense* and 014 for *Heeria insignis* were deposited there. The fresh leaves of *Clerodendrum capitatum, Heeria insignis* and stem bark of *Psorospermum senegalense* were air-dried, pulverized using wooden motar and pestle and stored till needed

3.2 Extraction of Plant Materials.

To the sample (500 g) in a 2 litre conical flask was added methanol (1.5 L), stopped and kept for one week. The mixture was filtered and the filterate was concentrated *in- vacuo* at 40^{o}C using a *rota vapor*. The respective concentrated methanol extracts were partitioned with hexane, dichloromethane and ethyl acetate. The resulting extracts were concentrated *in vacuo* at 40˚C using rotary evaporator.

3.3. Preliminary Phytochemical Screening

The extracts were subjected to various phytochemical tests to identify the constituent secondary metabolites using standard methods (Harborne, 1988; Sofowora, 1993). The metabolites tested for included: carbohydrates, tannins, saponnis, flavonoids, anthraquinones, cardiac glycosides, steroids, terpenes and alkaloids.

3.3.1. Test for carbohydrates (Molischs test)

To 1 g of the sample in 20 ml test tube was added 5 ml of distilled water and heated on a water bath for 5 mins. The mixture were filtered. To each filtrate, four drops of Molisch's reagent was added and stirred. Concentrated sulphuric acid (3 mL) was carefully added to the mixture from the side of the test tube to form a lower layer. Appearance of a purple colour at the interface of the extracts indicate the presence of carbohydrate (Trease and Evans, 1996).

3.3.2. Test for tannins (ferric chloride test)

To 1 g of the respective samples in 20 ml test tube was added 5 ml of distilled water and boiled. The mixture were filtered and subjected to the following test independently and respectively. Two drops of ferric chloride was added to the filtrate, formation of green precipitate was observed (Trease and Evans, 1996).

3.3.3. Test for flavonoids (Shinoda test)

To 1 g sample in 20 mL test tube was added methanol (5 mL). The samples were subjected to the following test independently and respectively. Three pieces of magnesium chips were added followed by a few drops of concentrated hydrochloric acid. A purple colour was observed which indicated the presence of flavonoids (Sofowora, 1993).

Sodium Hydroxide test

To 1 g sample in 100 mL beaker was added 10 % aqueous sodium hydroxide (5 mL) and filtered. The filterate was yellow and few drops of HCl was added then it turned colourless which indicated the presence of flavonoids (Cannell, 2000).

3.3.4. Test for Anthraquinones (Free Anthraquinones)

To 1 g sample in 20 mL test tube was added benzene (10 mL), shaken and filtered. The filterate were subjected to the following test independently and respectively. 10% ammonia solution (5 mL) was added to the filtrate, the mixture was shaken. No colour change was observed in the ammoniacal layer (Lower phase) that indicated the absence of free anthraquinone. (Cannell, 2000).

Combined anthraquinones

To 1 g sample in 20 mL test tube was added aqueous sulphuric acid (10 mL). The mixture was boiled and filtered hot and subjected to the following test independently and respectively. The filtrate was shaken with benzene (5 mL), the benzene layer was separated and to half its own volume, 10 % NH_4OH was added. A pink colouration was observed in the ammonia phase (lower phase) which indicates the presence of combined anthraquinone or anthraquinone derivatives (Cannell, 2000).

3.3.5. Test for saponins (Frothing test)

To each sample (0.5 g) in 20 mL test tube was added distilled water (5 mL) and shaken. Frothing which persisted for 15 minutes indicated the presence of saponins.

3.3.6. Test for glycoside ($FeCl_3$ test)

To each sample (0.5 g) in 20 mL test tube was subjected to the following test independently and respectively. Conc. H_2SO_4 (5 mL) was added and boiled for 15 min. This was then cooled and neutralized with 20% KOH. The solution was divided into two portions. Three drops of ferric chloride solution was added to one of the portions, and a green to black precipitate was observed (Silva *et al.*, 1998).

3.3.7. Test for cardiac glycoside (Kella-Killani test)

To each sample (0.5 g) in 20 mL test tube was added glacial acetic acid (5 mL) containing traces of ferric chloride. The test tube was held at an angle of 45˚ and concentrated sulphuric acid (1 mL) was added carefully down the side. A purple ring colour at the interface was observed.

3.3.8. Test for Steroids/Terpenes (Liebermann-Buchard test)

To each sample (0.5 g) in 20 mL test tube was added chloroform (1 mL) and a few drops of acetic anhydride were added followed by concentrated sulfuric acid. The mixture was carefully mixed and a blue colour that changed with time was observed in the resulting solution (Silva *et al.*, 1998).

Salkowski test

To each sample (0.5 g) in 20 mL test tube was added chloroform (1 mL) and to it concentrated sulfuric acid (1 mL) was added down the test tube to form two phases. Formation of yellow colouration was observed and taken as an indication for the presence of sterols/triterpenes (Silva *et al.,* 1998).

3.3.9. Test for Alkaloids

The each sample (0.5 g) in 20 mL test tube was added 1% aqueous hydrochloric acid (5 mL) and stirred on a water bath and filtered. The filterate (3 mL) was divided into three. To the first portion, three drops of freshly prepared Dragendoff's reagent was added and an orange to brownish precipitate was observed. To the second portion 1 drop of Mayer's reagent was added and yellowish colour precipitate was observed. To the third portion 1 drop of Wagner's reagent was added which give a reddish- brown precipitate. (Silva *et al.,* 1998).

3.4 Antimicrobial Activity Studies on Extracts and Isolates

The antimicrobial activities of all the extracts, isolated compounds and standard drugs (Ciprofloxacin, Sparfloxacin and Fluoconazole) were determined using pathogenic organisms *in vitro*.

3.4.1 Test Organisms

The organisms used were clinical isolates obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital (ABUTH), Zaria, Nigeria. The test microorganisms used were *Shigella dysenteriae, Salmonella typhi, Corynebacterium ulcerans, Klebsiella pneumoniae, Staphylococcus aureus, Methicillin resistant Staphylococcus aureus, Proteus mirabilis, Streptococcus pneumoniae, Proteus vulgaris, Vancomycin resistant enterococci, Bacillus subtillis, Escherichia coli, Pseudomonas flourescense, Streptococcus pyogenes, Enterobacter specie, Streptococcus feacalis,*

Pseudomonas aeruginosa, Proteus rettgeris, Candida tropicalis, Candida pseudotropicalis, Candida krusei, Candida albicans and Candida stellatoide were grown on a nutrient agar slant in an incubator at 37^{o} C for 24 h for the bacteria and at 25˚C for 24 hours for the fungi.

3.4.2. Preparation of the Plants Extracts

Stock solution of the respective plant extract were prepared by dissolving 0.3g of the *Psorospermum senegalense* and *Clerodendrum capitatum* extracts and 0.4g of *Heeria insignis* crude extracts in 10 ml of dimethyl sulphoxide (DMSO) to obtain a concentration of 30 mg/ml and 40mg/ml respectively. From the stock solution, two fold serial dilution of the extract of concentrations of 30 mg/ml, 15 mg/ml, 7.5 mg/ml, 3.25 mg/ml and 1.02 mg/ml were obtained for the *P. senegalense* and *C. capitatum* extracts and concentrations of 40 mg/ml, 20 mg/ml, 10 mg/ml, 5 mg/ml and 2.5 mg/ml were obtained for *H. insignis* extract. The well diffusion method of Nostro *et al.,* 2000 were used in the antimicrobial screening.

3.4.3. Preparation of culture media

The culture media used were Mueller Hinton agar (MHA) and Mueller Hinton broth (MHB).They were prepared according to the manufacturer's instruction and were sterilized at 121˚C for 15 minutes. The media were cooled to 45˚C and 29 ml of the sterilized media were poured asceptically into sterilized petri dishes and allowed to cool and solidify.

3.4.4. Antimicrobial sensitivity testing

The sterilized Mueller Hinton agar were inoculated with 0.1 ml standard inoculums of the test organisms. The inoculums were spread evenly over the surface of the medium by the use of a sterile inoculating loop. Standard cork borer of 6 mm diameter was used to bore a well at the centre of each inoculated medium and 0.1 ml of the solution of the extracts was introduced into each well. The inoculated plates were then incubated at 37˚C for 24 hours for the bacteria and at 25˚C for 24 hours for the fungi after which each plate was observed for zone of inhibition of growth. Cyprofloxacin, sparfloxacin and fluconazole were used as standard drugs for comparison.

3.4.5 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations (MIC) were determined for the extracts using nutrient broth dilution method in accordance with (Vollekova *et al.,* 2001). Nutrient broth was prepared according to the manufacturer's instructions. Mc-farlands turbidity scale number 0.5 was prepared to give turbid solution. Normal saline was prepared and was dispensed into test tubes and the microorganisms were then inoculated and incubated at 37 ˚C for 6 hours. Dilution of the test microorganisms in the normal saline was performed until the turbidity marched that of the Mac farlands scale by visual comparison. At this point the microorganisms had a concentration of about 1.5 x 10^8 cfu/ml. Two fold serial dilutions of the extracts in the broth were performed to obtain the concentration of 30 mg/ml, 15 mg/ml, 7.5 mg/ml, 3.25 mg/ml and 1.02 mg/ml for the *P. senegalense* and *C. capitatum* extracts and concentrations of 40 mg/ml, 20 mg/ml, 10 mg/ml, 5 mg/ml and 2.5 mg/ml were obtained for *H. insignis* extracts respectively. Having obtained the different concentrations of the extract in the broth, 0.1 ml of the standard inoculum of the test microorganism in the normal saline was then inoculated in to the different concentrations. Incubation was made at 37 °C for 24 hrs after which each broth was observed for turbidity. The lowest concentration of the extract in the broth which showed no turbidity was recorded as the minimum inhibitory concentration (MIC).

3.4.6 Minimum Bactericidal Concentration & fungicidal concentration (MBC/MFC)

The minimum bactericidal concentration/minimum fungicidal concentrations (MBC/MFC) were determined according to Bauer *et al.,* 1977. The content of the MIC in the serial dilution was sub-cultured onto the prepared medium and incubation was done at 37 ˚C for 24 hrs for the bacteria and at 25˚C for 24 hours for the fungi Thereafter each plate of the medium was observed for colony growth. The value obtained in the plate with lowest concentration of the extracts without colony growth was recorded as the MBC/MFC

3.5 Antibacterial assay

The anti tuberculosis activity of the extracts was carried out using the minimum inhibitory concentration determination by the broth microdilution method as described by Oladosu *et al.*, 2013.

3.5.1. Preparation of Extract

To 100 mg sample in 50 mL sterile bottle was added 0.5 mL dimethyl sulphoxide (DMSO) and 0.5 mL distill water. The mixture were further diluted (1:10) in 7H9 Middlebrook broth to give 10 mg/mL concentration.

3.5.2. Preparation of *Mycobacterium bovis* (BCG)

Five hundred micro litre (500 µL) of test organism *Mycobacterium bovis* (BCG) freshly prepared stock was inoculated into 50 ml of sterile Middlebrook 7H9/ADC broth medium and incubated at 30^0C for 5-7 days. The optical density of resulting culture was measured using a UV spectrophotometer. The optical density (OD) of the resulting culture was determined at 650 nm.

3.5.3. Antituberculosis screening

The microbroth dilution in sterile 96 microwell plate method was employed for the determination of anti-Tb activity of the compounds. Into each well of 96 microwell plate was transferred 50 µl of sterile 7H9 broth starting from well 2 to 12. To each of the first well was added 100 µl of 10% DMSO, 100 µl of 25 µg/ml solution of rifampicin (control drug, prepared by dissolving 250 mg of rifampicin powder in 10 ml DMSO and diluted to 1: 1000 by dispensing 25 µl of rifampicin in 25 ml 7H9 Middlebrook broth) and 100 µl of each plant fraction. Using a multichannel pipettor, 50 µl was carefully removed from well 1 to 2, mixed thoroughly and the process continued to well 11 from which 50 µl was withdrawn and discarded. The wells were inoculated with 50 µl of diluted BCG culture and incubated at 30^0C for a period of seven days. The results were confirmed by staining the wells with

tetrazolium dye after the incubating period. The reduction of tetrazolium salt from colourless to brightly coloured derivative in the wells is an indication of sample inactivity but if the dye remained colourless, that confirmed the activity of the sample. The last well where there was no growth is regarded as the minimum inhibitory concentration (MIC) of the sample (Oladosu *et al.,* 2013).

3.6 Chromatographic Procedure

3.6.1 Thin Layer Chromatography (TLC)

Thin layer chromatography was carried out on TLC aluminum sheet of silica gel 60 PF_{254} pre coated with layer thickness of 0.2 mm using various solvent systems comprising hexane/ethyl acetate mixtures (95, 90, 85, 80, 85,70, 60, %).

Spotting and Development: Spots were applied manually using capillary tube; plates were dried using air blower and developed at room temperature using a Shandon chromato tank.

Detection of Spots: Spots on TLC plates were visualized under UV light (254 and 366 nm) and spraying with 10% sulphuric acid, followed by heating at 110˚C for 5-10 min.

3.6.2 Column Chromatography

The following column conditions were employed in running the column chromatography.

(a) Technique - Gradient elution.

(b) Column - A glass column of dimensions 75 by 3.5 cm was used.

(c) Stationary phase - Silica gel of 60 – 120 mesh size.

(d) Column packing – This was done by the wet slurry method.

(e) Sample loading – The sample was loaded by the dry loading method (Cannell, 1998). The sample was dissolved in minimum amount of suitable organic solvent, mixed with small

quantity of silica gel, dried, triturated and then loaded on top of the previously packed column.

Solvents System of Elution: Various solvent systems comprising 100 % hexane, hexane/ethyl acetate mixtures (90, 80, 70, 60, 50, 40, 30, 20, 10 %), ethyl acetate 100 % and methanol 100 % were used in eluting the column by gradient elution.

3.7 CHROMATOGRAPHIC SEPARATION

3.7.1 Column Chromatography of dichloromethane extract of *H. insignis*

The dichloromethane extract (6 g) of *H. insignis* was chromatographed over silica gel packed column of dimension 75 by 3.5 cm, the column was eluted continuously using n-hexane and followed by mixtures of n-hexane : ethyl acetate. Fifty fractions of 100 mL each were collected. The 50 fractions were pooled together based on similarity in their TLC profile to give 9 sub-fractions. Repeated column chromatography of sub fractions 4 gave 71 sub-fractions. Sub-fractions number 17– 47 showed 4 spots and were pooled together and labelled as $HF4_{17\text{-}47.}$

3.7.2 Preparative thin layer chromatography of fraction $HF4_{17\text{-}47}$

Fraction $HF4_{17\text{-}47}$ which showed four spots was subjected to preparative TLC. Three out of the four spots were isolated as pure compounds and labelled as compound H1 (6.4 mg), H3 (7.1 mg) and H4 (5.7 mg). These were subjected to NMR spectroscopic analysis and the resulting spectra used to elucidate their chemical structures.

3.7.3 Column Chromatography of dichloromethane extract of *C. capitatum*

The dichloromethane extract (5 g) of *C. capitatum* was chromatographed on a silica gel packed column of dimension 75 by 3.5 cm. The column was eluted continuously using n-Hexane and then n-Hexane : Ethyl acetate mixtures and fifty nine fractions of 100 ml each

were collected. The 59 fractions were pooled together based on similarities in their TLC profile to obtain 9 sub-fractions. Repeated column chromatography of sub fraction 3 gave 33 sub-fractions. Sub-fractions 8 – 15 consisted of three spots and were pooled together and labelled as $CF3_{8\text{-}15.}$ Repeated column chromatography of sub-fractions 4 also gave 29 sub-fractions. The sub-fractions 16 – 18 consisted of two spots and were pooled together and labelled as $CF4_{16\text{-}18.}$

3.7.4 Preparative thin layer chromatography of Fraction $CF3_{8\text{-}15}$

Fraction $CF3_{8\text{-}15}$ which showed three spots was subjected to preparative TLC. One out of the three spots was isolated as a pure compound and labelled compound C1 (5.4 mg) and this was also subjected to spectroscopic analyses to elucidate its chemical structures.

3.7.5 Preparative thin layer chromatography of Fraction $CF4_{16\text{-}18.}$

Fraction $CF4_{16\text{-}18}$ which showed two spots was subjected to preparative TLC. One out of the two spots was removed as pure compound and labelled compound C2 (5.8 mg) and was subjected to spectroscopic analysis to elucidate its chemical structures.

3.7.6 Column Chromatography of dichloromethane fraction of *P. senegalense*

The ethyl acetate extract (5 g) of *P. senegalense* was chromatographed on silica gel packed column of dimension 75 by 3.5 cm the column was eluted continuously using n-Hexane and then n-Hexane : Ethyl acetate mixtures and sixty one fractions of 100ml each were collected. The sixty one fractions were pooled together based on similarities in their TLC profile to give 10 sub-fractions. Sub-fraction 6 gave two spots and was labelled PF6.

3.7.7 Preparative thin layer chromatography of Fraction PF6

Fraction PF6 which showed two spots was subjected to preparative TLC. One out of the two spots was removed as pure compound and labelled compound P2 (7.4 mg) and this component was subjected to spectroscopic analysis to elucidate its chemical structures.

3.8 Melting Point Determination

The melting points of the isolated compounds were determined using the Gallenkamp melting point apparatus at the Department of Pharmaceutical and Medicinal Chemistry, A.B.U, Zaria.

3.9 Spectral Analyses

The compounds were subjected to NMR analysis measured on a Bruker Advance (600 MHZ) spectrophotometer.The chemical shift (δ) were recorded in parts per million (ppm) relative to TMS and coupling constants are given in Hz. The solvent used for these measurements is deuterated chloroform.

CHAPTER FOUR

4.0 RESULTS

Table 4.1 shows the result of the percentage recovery yield after extraction of the three plants. In all three cases methanol extract had the highest percentage recovery yield.

Table 4.2 shows the results of the phytochemical screening of the extracts of the leaves of *Heeria insignis* (Del), *Clerodendrum capitatum* (Willd) and stem bark of *Psorospermum senegalense.* The results revealed the presence of carbohydrate, glycosides, cardiac glycoside, saponins, steroids and triterpenes, flavonoids, tannins and alkaloids, while anthraquinones were present only in the methanol extract of *Psorospermum senegalense* but absent in all other extracts of the other plants.

Table 4.3 - 4.3.2 shows the results of the antimicrobial susceptibility tests, expressed in terms of diameter of zones of inhibition, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts against the test organisms.

The zone of inhibition (ZI) of *C. capitatum* extracts on the microorganism ranged from 17-26 mm (hexane); 23-26 mm (dichloromethane); 26-29 mm (ethyl acetate) and 20-23 mm (methanol) against the entire test organisms except *Corynebacterium ulcerans*. The results of the minimum inhibitory concentration (MIC) showed that ethyl acetate fraction inhibited the growth of all test organisms at a low concentration of 3.25 mg/mL except *S. aureus* and *C. albicans* which were inhibited at 7.5 mg/mL. Higher MIC values were observed for hexane (7.5-15 mg/mL), dichloromethane and methanol fraction all showed MIC at 7.5 mg/mL. The microorganisms were completely killed at a higher concentration; ethyl acetate (MBC/MFC: 7.5-15 mg/mL); dichloromethane (MBC/MFC: 15 mg/mL); methanol (MBC/MFC: 15-30 mg/mL) and hexane (MBC/MFC: 30 mg/mL).

The result of the zone of inhibition (ZI) of *H. insignis* extracts on the microorganism ranged from 20 to 34 mm against all test organism with the exception of *P.aeruginosa*, *S. feacalis* and *C. albicans*. Minimum inhibitory concentration (MIC) determination revealed that a low concentration of 5 mg/mL of ethyl acetate and dichloromethane inhibited the growth of all test organisms with the exception of *VRE* which was inhibited at 10 mg/mL. These extracts also showed bactericidal and fungicidal effect at 10 mg/mL, while the rest of the extracts showed MIC, minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) ranging from 10 to 40 mg/mL. The hexane, dichloromethane, ethyl acetate and methanol extracts of *Psorospermum senegalense* displayed potential antimicrobial activity against seven out of the twelve tested bacteria. The ethyl acetate extract exhibited the highest zone of inhibition (28 mm), MIC (3.25mg/ml) and MBC/MFC(15 mg/ml) against *Streptococcus pyogenes.*

Table 4.4 shows the result of the anti-tuberculosis activity of the crude extracts of the three plants. The anti-tuberculosis activity of the crude extracts of the three plants revealed that the dichloromethane fraction of *Heeria insignis* and *Clerodendrum capitatum* both showed the highest activity with MIC of 0.675 mg/mL, followed by ethyl acetate extract (1.25 mg/ml) of *H.insignis*, hexane and methanol extracts were inactive. The ethyl acetate and methanol extracts of *C. capitatum* inhibited the growth of *Mycobacterium bovis* at MIC of 2.5 mg/mL, no activity was recorded for the hexane fraction. *Psorospermum senegalense* showed the least activity out of the three plants with MIC of 2.5 mg/ml only in the ethyl acetate extract.

Table 4.5 shows the result of TLC of dichloromethane extracts of *C. capitatum* using hexane: ethyl acetate (7:3).

Table 4.6 shows the Fraction obtained from the column chromatography of dichloromethane extract.

Table 4.7 shows the result of TLC of dichloromethane extracts using hexane: ethyl acetate (7:3).

Table 4.8 shows Fractions obtained from column chromatography of dichloromethane extract of *H. insignis.*

Table 4.9 shows TLC profiles of ethyl acetate extract using hexane: ethyl acetate (1:1).

Table 4.10 shows Fractions obtained from column chromatography of ethyl acetate extract.

Table 4.11 shows the TLC profiles of the isolated compounds.

Table 4.12 shows the ^{13}C NMR ($CDCl_3$) assignment of C1.

Table 4.13 shows the ^{13}C NMR ($CDCl_3$) assignment of C2 and H1.

Table 4.14 shows the ^{13}C NMR ($CDCl_3$) assignment of H3.

Table 4.15 shows the ^{13}C NMR ($CDCl_3$) assignment of P2.

Table 4.16 shows result of zones of inhibition of the isolated compounds The zone of inhibition (ZI) of the isolated compounds on the microorganism ranged from 20-21 mm (C1); 23-29 mm (C2); 22-32 mm (H1); 22-31 mm (H3); 20-30 mm(H4) and 23-28 mm (P2) against the entire test organisms except *S. feacalis, Enterobacter SP, and C. stellatoidea* which were not sensitive to C1 and C2. *VRE, P.aeruginosa, P. rettgeris C. pseudotropicalis* were insensitive to H, H3 and H4 while *MRSA, P.aeruginosa, C. stellatoidea, C. pseudotropicalis* were not sensitive to P2.

Table 4.17 shows result of minimum inhibitory concentration (µg/ml) of the isolated compounds. The results of the minimum inhibitory concentration (MIC) showed that C1, C2, H, H3 and H4 inhibited the growth of all test organisms at concentration ranging between 62.5 -125 µg/mL with the exception of P2 which had MIC of 125 µg/mL.

Table 4.18 shows result of minimum bactericidal /fungicidal concentration (µg /ml) of the isolated compounds which ranged between 125-500 µg/mL.

Table 4.19: Shows result of minimum inhibitory concentration (µg/ml) of the isolates against *Mycobacterium bovis* which revealed that H3 had the highest activity against *Mycobacteriunm bovis* with MIC of 125 µg/ml, all the other compounds isolated also showed activity at MIC 250 µg/ml.

4.1 Result of extraction of the Leaf of *Clerodendrum capitatum*, *Heeria insignis* and Stem Bark of *Psorospermum senegalense*

Table 4.1: Result of extraction

SN	SAMPLES	WEIGHT USED	SOLVENTS USED	WEIGHT OF CRUDE EXTRACT(g)	% RECOVERY YIELD
1	*Clerodendrum capitatum* /Leaves	500 g	Hexane	10.46	2.092
			DCM	8.34	1.668
			EtOAc	7.44	1.488
			MeOH	80.12	16.024
2	*Heeria insignis*/ Leaves	400 g	Hexane	7.40	1.85
			DCM	8.50	2.125
			EtOAc	6.25	1.56
			MeOH	69.38	17.345
3	*Psorospermum senegalense*/Bark	650 g	Hexane	4.75	0.731
			DCM	21.09	3.244
			EtOAc	8.63	1.327
			MeOH	108.04	16.615

DCM = dichloromethane extract, EtOAc = Ethyl acetate extract, MeOH = Methanol extract

4.2. Result of Phytochemical Screening

Table 4.2: Results of phytochemical screening of extracts from the plants

	C. capitatum				*P.senegalense*				*H. Insignis*			
Metabolites	HE	DCM	EA	ME	HE	DCM	EA	ME	HE	DCM	EA	ME
Carbohydrate	-		+	+	-		+	+	-		+	+
Cardiac glycoside	+		+	+	+	+	+	+	+		+	+
Tannins	-	-	+	+	-	-	-	-	-		+	+
Saponins	-	-	-	+	-	-	+	+	-	+	-	+
Flavonoids	-	-	+	+	-	+	+	+	-	+	+	+
Anthraquinones	-	-	-	+	-	-	-	+	-	-	-	-
Steroids	+	+	+	+	+	+	+	+	+	+	-	+
Triterpenes	+	+	+	+	+	+	+	+	+	+	-	+
Glycosides	+	+	+	+	+	+	+	+	+	+	+	+
Alkaloids	-	+	+	+	-	+	+	+	-	+	+	+

Key: + = present, - = absent, HE = hexane extract, DCM = dichloromethane extract, EA = Ethyl acetate extract, ME = Methanol extract

4.3 Result of antimicrobial activity of the plant extracts

4.3.1 Result of Zones of Inhibition

Table 4.3: Zones of Inhibition (mm) of the extracts and standard drugs

Pathogens	*C.capitatum*				*P.senegalense*				*H.insignis*				CPX	SFX	FC Z
	HE	DCM	EA	ME	HE	DCM	EA	ME	HE	DCM	EA	ME			
S. aureus	18	24	26	22	20	25	27	24	20	29	32	24	37	41	01
S.pnoumoniae	17	25	28	22	18	22	27	20	-	-	-	-	38	37	0
C.ulcerans	0	0	0	0	-	-	-	-	-	-	-	-	0	0	30
Mrsa	20	23	27	23	-	-	-	-	22	27	31	25	35	35	0
E. coli	18	23	27	20	19	25	27	22	-	-	-	-	32	35	0
K.pneumonia	0	0	0	0	0	0	0	0	-	-	-	-	40	37	0
P. mirabilis	19	24	28	20	-	-	-	-	-	-	-	-	0	36	0
S.dysenteriae	19	26	29	23	-	-	-	-	22	30	32	26	39	40	0
S.typhi	18	24	29	21	-	-	-	-	-	-	-	-	41	32	0
P.retgeris	-	-	-	-	-	-	-	-	0	0	0	0	35	37	0
P. aeruginosa	-	-	-	-	-	-	-	-	0	0	0	0	0	32	0
Enterobactersp	-	-	-	-	-	-	-	-	21	27	33	24	34	35	0
Vre	-	-	-	-	0	0	0	0	20	26	30	24	0	35	0
S. pyogenes	-	-	-	-	20	24	28	22	-	-	-	-	35	37	0
B. subtillis	-	-	-	-	22	- 27	31	25	21	31	34	26	31	34	-26
P. vulgaris	-	-	-	-	0	0	0	0	-	-	-	-	0	32	0
P. flourescense	-	-	-	-	0	0	0	0	-	-	-	-	0	30	0
S.Feacalis	-	-	-	-	-	-	-	-	0	0	0	0	34	37	0
C. pseudotropicalis	-	-	-	-	18	23	27	20	21	29	33	25	0	0	32
C.albicans	18	23	26	21	-	-	-	-	0	0	0	0	0	0	36
C.tropicalis	0	0	0	0	18	24	26	21	-	-	-	-	0	0	35
C.krusei	18	24	28	21	0	0	0	0	-	-	-	-	0	0	34
C. stellatoidea	-	-	-	-	-	-	-	-	20	28	32	24	0	0	37

Key: 0 = no activity, - = not determined , HE = hexane extract, DCM = dichloromethane extracts, EA = Ethyl acetate extract, ME = Methanol extract, CPX=ciprofloxacin, SFX = Sparfloxacin, FCZ = fluconazole

4.3.2 Result of Minimum Inhibitory Concentration (MIC):

Table 4.3.1: Minimum inhibitory concentration (MIC) of the extracts in mg/mL

Pathogens	*C.capitatum*				*P.senegalense*				*H.insignis*			
	HE	DCM	EA	ME	HE	DCM	EA	ME	HE	DCM	EA	ME
S. aureus	15	7.5	7.5	7.5	7.25	3.25	7.5	7.5	5	5	10	10
S.pnoumoniae	15	7.5	3.25	7.5	15	7.5	3.25	7.5	-	-	-	-
MRSA	7.5	7.5	3.25	7.5	-	-	-	-	10	5	5	10
E. coli	15	7.5	3.25	7.5	15	7.5	3.25	7.5	-	-	-	-
K.pneumonia	15	7.5	3.25	7.5	0	0	0	0	-	-	-	-
P.mirabilis	15	7.5	3.25	7.5	-	-	-	-	-	-	-	-
S.dysenteriae	15	7.5	3.25	7.5	-	-	-	-	10	5	5	10
S.typhi	15	7.5	3.25	7.5	-	-	-	-	-	-	-	-
P.retgeris	-	-	-	-	-	-	-	-	0	0	0	0
P. aeruginosa	-	-	-	-	-	-	-	-	0	0	0	0
Enterobactersp	-	-	-	-	-	-	-	-	10	5	5	10
VRE	-	-	-	-	0	0	0	0	10	10	5	10
S. pyogenes	-	-	-	-	7.5	3.25	7.5	7.5	-	-	-	-
B. subtillis	-	-	-	-	-7.5	3.25	3.25	7.5 -	10	5	5	10 -
P. vulgaris	-	-	-	-	0	0	0	0	-	-	-	-
P. flourescense	-	-	-	-	0	0	0	0	-	-	-	-
S.feacalis	-	-	-	-	-	-	-	-	0	0	0	0
C. pseudotropicalis	-	-	-	-	15	7.5	3.25	7.5	10	5	5	10
C.albicans	15	7.5	7.5	7.5	-	-	-	-	0	0	0	0
C.tropicalis	0	0	0	0	15	7.5	7.5	7.5	-	-	-	-
C.krusei	15	7.5	3.25	7.5	0	0	0	0	-	-	-	-
C.stellatoidea	-	-	-	-	-	-	-	-	10	5	5	10

Key: 0 = no activity, - = not determined, HE = hexane extract, DCM = dichloromethane extracts, EA = Ethyl acetate extract, ME = Methanol extract

4.3.3 Result of Minimum Bactericidal/Fungicidal Concentration (MBC)/(MFC)

Table 4.3.2: Minimum bactericidal/fungicidal concentration (MBC/MFC) of the extracts in (mg/mL)

Pathogens	*C.capitatum*				*P.senegalense*				*H.insignis*			
	HE	DCM	EA	ME	HE	DCM	EA	ME	HE	DCM	EA	ME
S. aureus	30	15	15	30	30	15	15	15	20	10	10	20
S.Pneumoniae	30	15	15	30	30	30	15	30	-	-	-	-
MRSA	30	15	15	30	-	-	-	-	20	20	10	20
E. coli	30	15	15	30	30	15	15	30	-	-	-	-
K.pneumonia	30	15	15	30	0	0	0	0	-	-	-	-
P.mirabilis	30	15	15	30	-	-	-	-	-	-	-	-
S.dysenteriae	30	15	7.5	15	-	-	-	-	20	10	10	20
S.typhi	30	15	15	30	-	-	-	-	-	-	-	-
P.retgeris	-	-	-	-	-	-	-	-	0	0	0	0
P. aeruginosa	-	-	-	-	-	-	-	-	0	0	0	0
Enterobacter sp	-	-	-	-	-	-	-	-	20	20	10	20
VRE	-	-	-	-	0	0	0	0	40	20	10	20
S. pyogenes	-	-	-	-	30	15	15	30	-	-	-	-
B. subtillis	-	-	-	-	30	15	7.5	15	20	10	10	20
P. vulgaris	-	-	-	-	0	0	0	0	-	-	-	-
P. flourescense	-	-	-	-	0	0	0	0	-	-	-	-
S.feacalis	-	-	-	-	-	-	-	-	0	0	0	0
C. pseudotropicalis	-	-	-	-	30	15	15	30	20	10	10	20
C.albicans	30	15	15	30	-	-	-	-	0	0	0	0
C.tropicalis	0	0	0	0	30	15	15	30	-	-	-	-
C.krusei	30	15	15	30	0	0	0	0	-	-	-	-
C.stellatoidea	-	-	-	-	-	-	-	-	40	10	10	20

Key: 0 = no activity, - = not determined, HE = hexane extract, DCM = dichloromethane extract, EA = Ethyl acetate extract, ME = Methanol extract

4.4 Result of Anti-tuberculosis activity

Table 4.4: Antituberculosis activity of the extracts

	C. capitatum				*P.senegalense*				*H. insignis*				Rf
Extract Concentration (mg/ml)	HE	DCM	EA	ME	HE	DCM	EA	ME	HE	DCM	EA	ME	
5	-	+	+	+	-	-	+	-	-	+	+	-	+
2.5	-	+	+	+	-	-	-	-	-	+	+	-	+
1.25	-	+	-	-	-	-	-	-	-	+	+	-	+
0.625	-	+	-	-	-	-	-	-	-	+	-	-	+
0.3125	-	-	-	-	-	-	-	-	-	-	-	-	+

Key: - = No activity , + = Activity, HE = hexane extract, DCM = dichloromethane extract, EA = Ethyl acetate extract, ME = Methanol extract, Rf = Rifampicin

4.5 RESULT OF CHROMATOGRAPHIC SEPARATION

4.5.1 Thin layer chromatography of the dichloromethane Extract of *C. capitatum*

Table 4.5: TLC of dichloromethane extracts of *C. capitatum* using hexane: ethyl acetate (7:3)

Spot	R_f Value	Colour in 10% H_2SO_4
1	0.80	Orange
2	0.70	Dark green
3	0.55	Purple

4.5.2 Column Chromatography of dichloromethane extract of *C. capitatum*

Table 4.6: Fractions from column chromatography of dichloromethane extract of *C. capitatum*

Fraction	Eluting solvent	Number of major spots
1	Hexane: ethyl acetate(05:95%)	3
2	Hexane: ethyl acetate (10:90%)	3
3	Hexane: ethyl acetate (20:80%)	2
4	Hexane: ethyl acetate (30:70%)	1
5	Methanol	3

4.5.3 Thin layer chromatography of the dichloromethane extract of *H. insignis*

Table 4.7: TLC of dichloromethane extract of *H. insignis* using hexane: ethyl acetate (7:3)

Spot	R_f Value	Colour in 10% H_2SO_4
1	0.82	Orange
2	0.68	Dark green
3	0.55	Purple

4.5.4 Column Chromatography of dichloromethane extract of *H. insignis*

Table 4.8: Fractions from column chromatography of dichloromethane extract of *H. insignis*

Fraction	Eluting solvent	Number of major spots
1	Hexane: ethyl acetate (05:95%)	2
2	Hexane: ethyl acetate (10:90%)	3
3	Hexane: ethyl acetate (20:80%)	3
4	Hexane: ethyl acetate (30:70%)	1
5	Methanol	2

4.5.5 Thin layer chromatography of the ethyl acetate extract of *P. senegalenses*

Table 4.9: TLC profiles of ethyl acetate extract of *P. senegalenses* using hexane: ethyl acetate (1:1)

Spot	R_f Value	Color in 10% H_2SO_4
1	0.66	Yellow
2	0.55	yellow
3	0.40	Orange
4	0.22	Purple

4.5.6 Column Chromatography of ethyl acetate extract of *P. senegalenses*

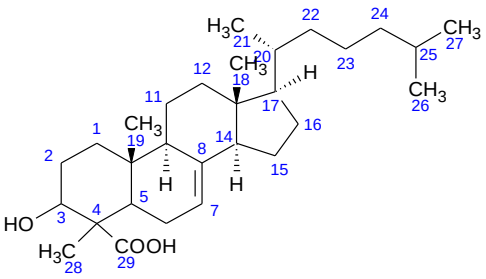
Table 4.10: Fractions from column chromatography of ethyl acetate extract of *P. senegalenses*

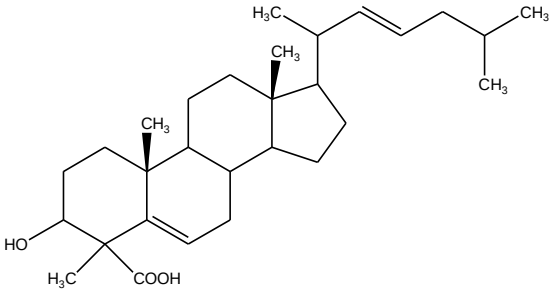
Fraction	Eluting solvent	Number of major spots
1	Hexane: ethyl acetate (05:95%)	3
2	Hexane: ethyl acetate (10:90%)	3
3	Hexane: ethyl acetate (20:80%)	2
4	Hexane: ethyl acetate (30:70%)	1
5	Methanol	1

4.6 TLC analysis of isolated compounds

Table 4.11: TLC profiles of the isolated compounds

Compound code	Solvent system	Number of spot(s)	Colour of spot on heating	R_f value
C1	Chloroform: Ethyl acetate (9.5:0.5)	1	Dark green	0.46
C2	Hexane: Ethyl acetate (8:2)	1	Orange	0.58
H1	Hexane: Ethyl acetate (8:2)	1	Orange	0.58
H3	Chloroform: Ethyl acetate (8.5:1.5)	1	Dark green	0.59
H4	Chloroform: Ethyl acetate (9:1)	1	Dark	0.41
P2	Chloroform: Ethyl acetate (8.5:1.5)	1	Purple	0.52



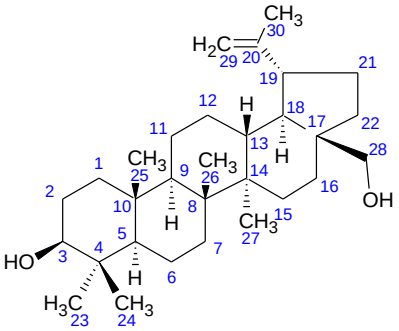


C1: 3-hydroxylanost-7-en-29-carboxylic acid zymosterol

Table 4.12: ^{13}C NMR ($CDCl_3$) assignment of C1

Carbon position	^{13}C δ (ppm) C1	^{13}C δ (ppm) Lit.	CHn
1	37.3	37.2	CH_2
2	36.1	31.5	CH_2
3	71.8	71.1	CH
4	45.9	43.3	C
5	42.9	139.6*	CH
6	24.3	117.5*	CH_2
7	121.7	31.8	CH
8	140.8	31.9	C
9	50.2	51.3	CH
10	39.8	34.3	C
11	25.4	21.1	CH_2
12	36.5	39.5	CH_2
13	42.3	43.3	C
14	56.1	55.9	CH
15	24.3	24.7	CH_2
16	28.2	28.5	CH_2
17	56.8	55.2	CH
18	12.2	12.1	CH_3
19	18.8	19.0	CH_3
20	37.3	40.3	CH
21	19.0	21.4	CH_3
22	36.5	138.2*	CH_2
23	24.7	129.5*	CH_2
24	40.5	39.5	CH_2
25	29.1	31.9	CH
26	22.6	22.7	CH_3
27	23.1	23.0	CH_3
28	21.1	14.1	CH_3
29	178.1	177.4	COOH

***= position of double bonds on the closest literature found (zymosterol)** Ezuruike *et al.*, 2015



C2, H1: Betulin

Table 4.13: ^{13}C NMR ($CDCl_3$) assignment of C2 and H1

Carbon position	^{13}C δ (ppm) C2	^{13}C δ (ppm) H1	^{13}C δ (ppm) Lit.	CHn
1	38.9	38.9	38.9	CH_2
2	27.5	27.5	27.5	CH_2
3	79.1	79.1	79.2	CH
4	38.9	38.9	38.8	C
5	55.3	55.3	55.4	CH
6	18.4	18.4	18.4	CH_2
7	34.4	34.3	34.3	CH_2
8	41.6	41.5	41.0	C
9	50.5	50.5	50.5	CH
10	37.4	37.2	37.4	C
11	30.0	29.0	20.9	CH_2
12	25.5	25.2	25.3	CH_2
13	37.2	37.2	37.2	CH
14	42.9	42.9	42.8	C
15	27.3	27.3	27.1	CH_2
16	29.2	29.4	29.2	CH_2
17	47.8	47.8	47.9	C
18	48.0	48.0	47.9	CH
19	48.4	48.4	48.8	CH
20	150.9	150.9	150.6	C
21	29.8	29.9	29.8	CH_2
22	34.3	34.3	34.1	CH_2
23	28.1	28.1	28.1	CH_3
24	18.4	15.7	15.4	CH_3
25	19.3	16.1	16.2	CH_3
26	20.9	16.0	16.1	CH_3
27	18.3	15.6	14.8	CH_3
28	60.1	59.1	60.6	CH_2
29	109.3	109.3	109.8	CH_2
30	21.1	20.9	19.2	CH_3

Tijjani *et al.*, 2012

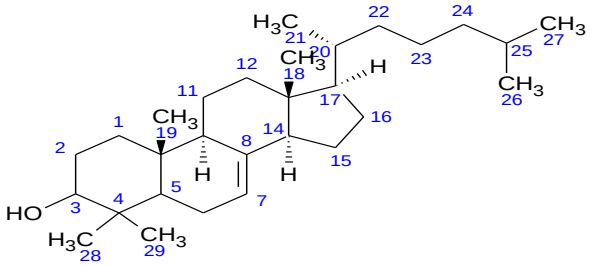


Table 4.14: ^{13}C NMR ($CDCl_3$) assignment of 3-hydroxy-4-dimethyl -7-lanostene (H3)

Carbon position	^{13}C δ (ppm) H3	^{13}C δ (ppm) Lit.	CHn
1	36.1	37.11	CH_2
2	29.2	31.42	CH_2
3	71.8	71.05	CH
4	42.3	37.92	C
5	42.3	40.20	CH
6	24.3	29.62	CH_2
7	121.7	117.39	CH
8	140.8	139.60	C
9	50.2	49.41	CH
10	36.5	34.17	C
11	23.1	21.52	CH_2
12	37.3	39.53	CH_2
13	45.9	43.35	C
14	56.1	55.01	CH
15	21.1	22.93	CH_2
16	29.2	27.93	CH_2
17	56.8	56.11	CH
18	11.9	11.83	CH_3
19	18.8	13.03	CH_3
20	39.8	36.18	CH
21	19.8	18.82	CH_3
22	36.2	36.10	CH_2
23	24.3	23.88	CH_2
24	42.3	39.47	CH_2
25	28.2	27.99	CH
26	23.1	22.54	CH_3
27	23.1	22.81	CH_3
28	28.2	*	CH_3
29	26.2	*	CH_3

*** The closest literature had 27 carbons Wilson *et al.*, 1996**

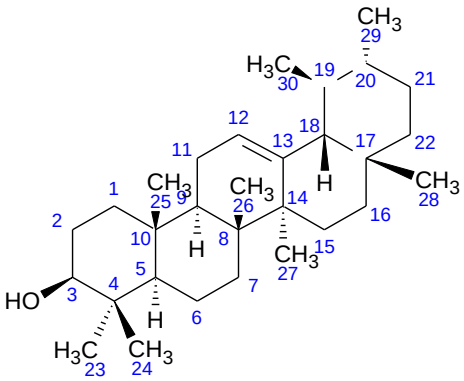


Table 4.15: ^{13}C NMR ($CDCl_3$) assignment of P2 (α-amyrin)

Carbon position	^{13}C δ (ppm) P2	^{13}C δ (ppm) Lit.	CHn
1	38.6	38.5	CH_2
2	24.1	23.4	CH_2
3	79.1	80.9	CH
4	38.8	37.7	C
5	55.2	55.3	CH
6	18.3	18.6	CH_2
7	32.9	32.9	CH_2
8	39.5	39.7	C
9	47.6	47.7	CH
10	39.5	36.8	C
11	24.2	22.8	CH_2
12	125.8	124.2	CH
13	138.0	139.5	C
14	42.0	42.1	C
15	28.1	28.2	CH_2
16	27.2	26.7	CH_2
17	36.7	33.8	C
18	52.8	59	CH
19	39.1	39.7	CH
20	37.1	39.7	CH
21	30.6	31.3	CH_2
22	41.0	41.6	CH_2
23	29.6	28.1	CH_3
24	15.5	15.8	CH_3
25	15.5	14.2	CH_3
26	16.9	16.8	CH_3
27	17.1	17.6	CH_3
28	28.0	28.8	CH_3
29	23.6	23.3	CH_3
30	23.3	21.4	CH_3

Rajnish *et al.*, 2015

Table 4.16: Determination of Zones of Inhibition of the Isolated Compounds

Test organisms	C1	C2	H1	H3	H4	P2
MRSA	27	29	24	22	20	-
VRE	24	27	-	-	-	23
S. aureus	26	26	27	29	27	24
S.feacalis	-	-	24	27	26	24
B. subtilis	27	25	30	31	29	26
P.aeruginosa	24	27	-	-	-	-
Enterobacter sp	-	-	25	24	24	24
P. retgeris	21	24	-	-	-	25
S. dysenteriae	29	28	32	29	30	28
C. stellatoidea	-	-	24	25	23	-
C. pseudotropicalis	23	25	-	-	-	-
C.albicans	20	23	22	24	23	25

Key: **- =** not active; P2 = α-Amyrin, H4= yet to be identified compound

C1 = 3-hydroxylanost-7-en-29-carboxylic acid
C2, H1= Betulin; H3 = 3-hydroxy-7-lanostene

Table 4.17: Minimum Inhibitory Concentration (µg/ml) of the Isolated Compounds

Test Organisms	C1	C2	H1	H3	H4	P2
MRSA	62.5	62.5	125	125	125	-
VRE	125	62.5	-	-	-	125
S. aureus	62.5	125	62.5	62.5	62.5	125
S.feacalis	-	-	125	62.5	125	125
B. subtilis	62.5	125	62.5	62.5	62.5	125
P. aeruginosa	125	62.5	-	-	-	-
Enterobacter sp	-	-	125	125	125	125
P. retgeris	125	125	-	-	-	125
S. dysenteriae	62.5	62.5	62.5	62.5	62.5	62.5
C. stellatoidea	-	-	125	125	125	-
C. pseudotropicalis	125	125	-	-	-	-
C.albicans	125	125	125	125	125	125

Key: **- =** not active; P2 = α-Amyrin, H4= yet to be identified compound

C1 = 3-hydroxylanost-7-en-29-carboxylic acid
C2, H1= Betulin; H3 = 3-hydroxy-7-lanostene

-

Table 4. 18: Minimum Bactericidal /Fungicidal Concentration (µg /ml) of the Isolated Compounds

Test Organisms	C1	C2	H1	H3	H4	P2
MRSA	250	125	250	500	500	-
VRE	250	250	-	-	-	250
S. aureus	250	250	250	125	250	250
S. feacalis	-	-	250	250	250	250
B. subtilis	250	250	125	125	125	250
P. aeruginosa	250	250	-	-	-	-
Enterobacter sp	-	-	250	250	250	250
P.retgeris	500	250	-	-	-	250
S. dysenteriae	125	125	125	125	125	125
C. stellatoidea	-	-	500	250	500	-
C. pseudotropicalis	500	250	-	-	-	-
C. albicans	500	500	500	250	500	250

Key: - = not active; P2 = α-Amyrin , H4= yet to be identified compound

C1 = 3-hydroxylanost-7-en-29-carboxylic acid
C2, H1= Betulin; H3 = 3-hydroxy-7-lanostene

Table 4.19: Minimum Inhibitory Concentration (µg/ml) of the Isolates against *Mycobacterium bovis*

Concentration of isolates (µg/ml)	C1	C2	H1	H3	H4	P2
500	+	-	+	+	+	+
250	+	-	+	+	+	+
125	-	-	-	+	-	-
62.5	-	-	-	-	-	-

Key: **- =** not active; P2 = α-Amyrin , H4= yet to be identified compound

C1 = 3-hydroxylanost-7-en-29-carboxylic acid
C2, H1= Betulin; H3 = 3-hydroxy-7-lanostene

4.7 Chemical Tests on the Isolated Compounds

The compounds gave orange colour in the Liebermann-Buchard test for steroids/triterpenes.

4.7.1. Melting Point Determination of the compounds

The melting points of C2 and H1 were found to be 255 - 256°C. That of P2 was found to be 185 -187°C.

4.8 Result of spectral analysis

The spectra results are shown in Figures 4.01 to 4.32.

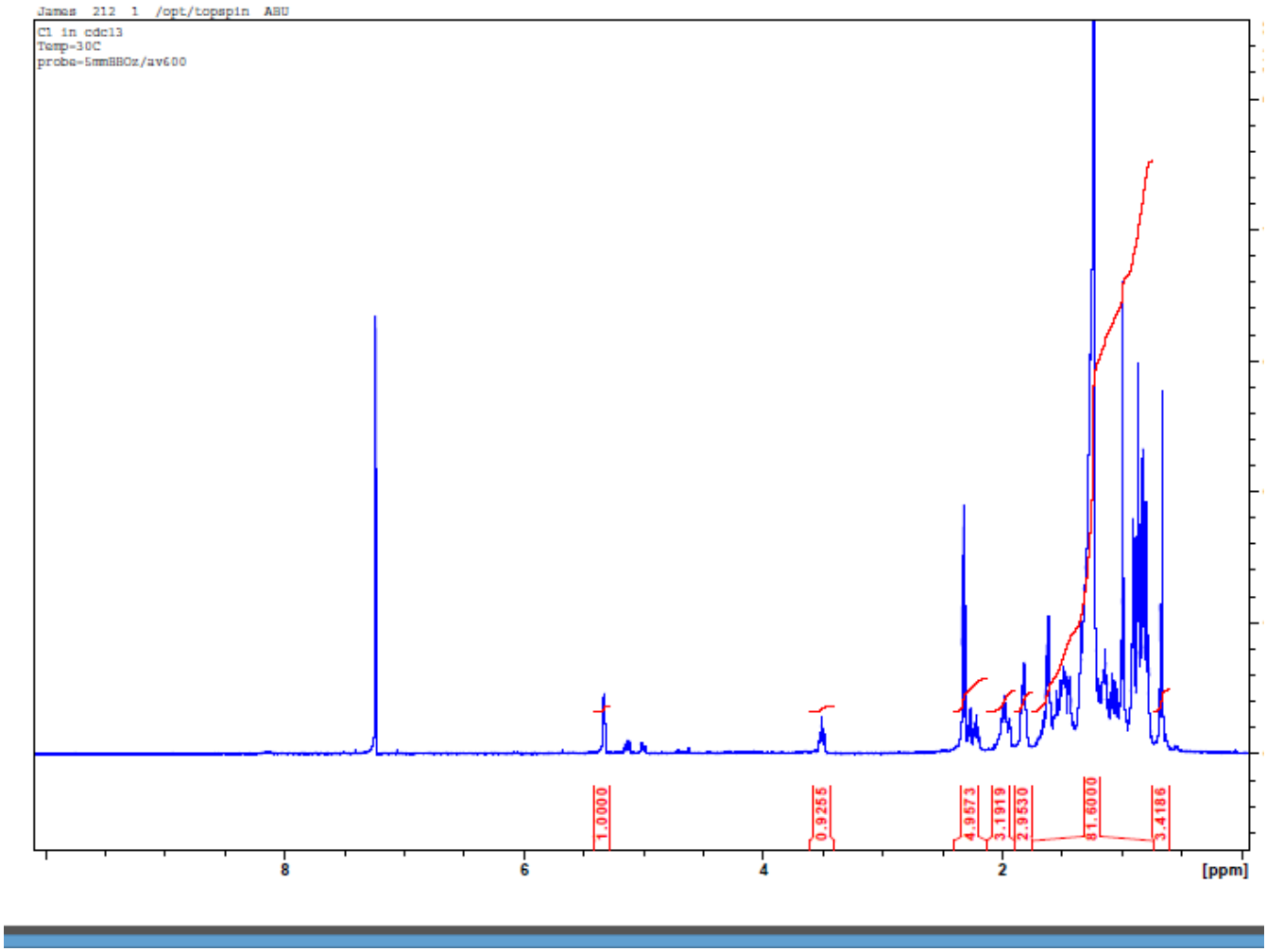


Figure 4.01; ^{1}H NMR spectrum of C1

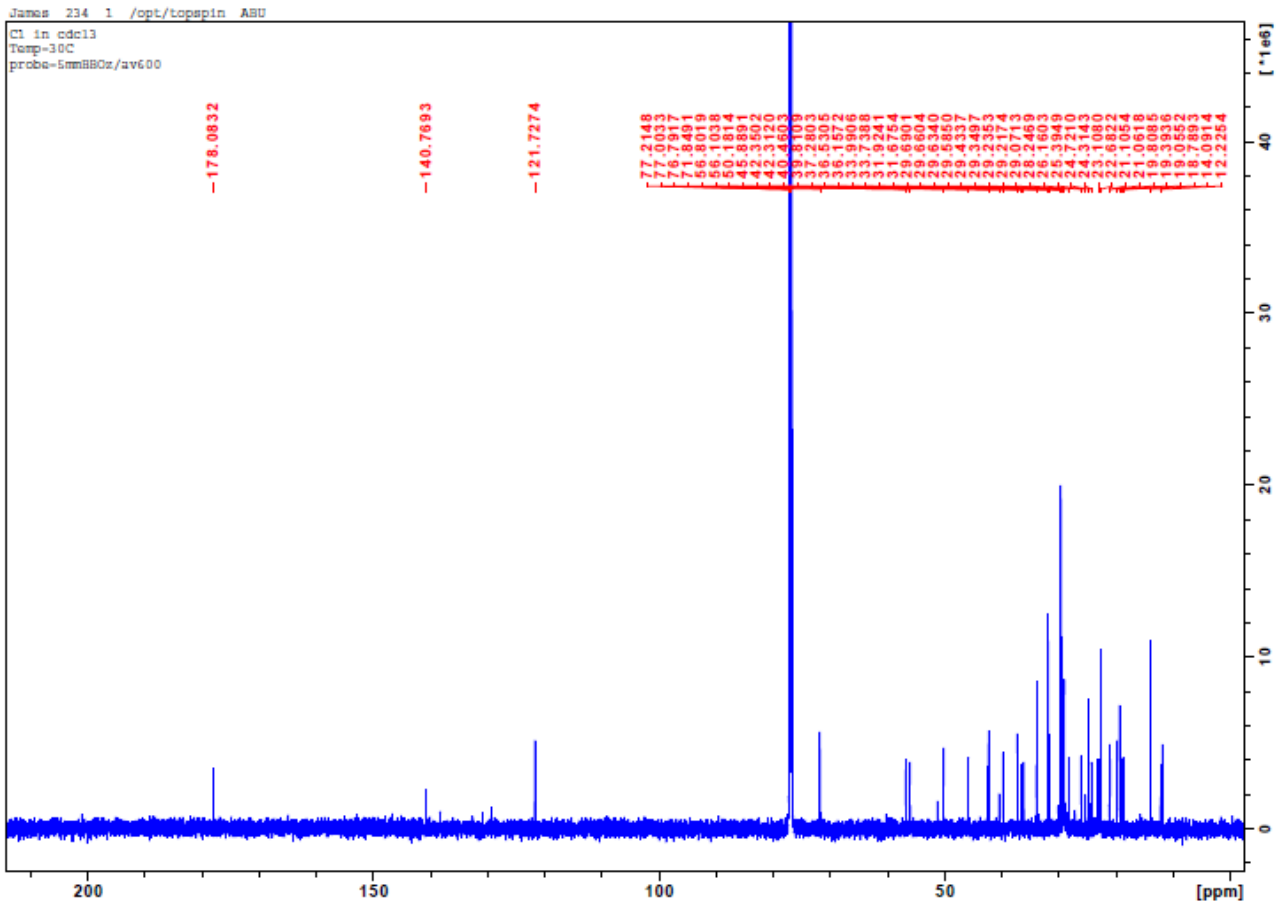


Figure 4.02: ^{13}C NMR spectrum of C1

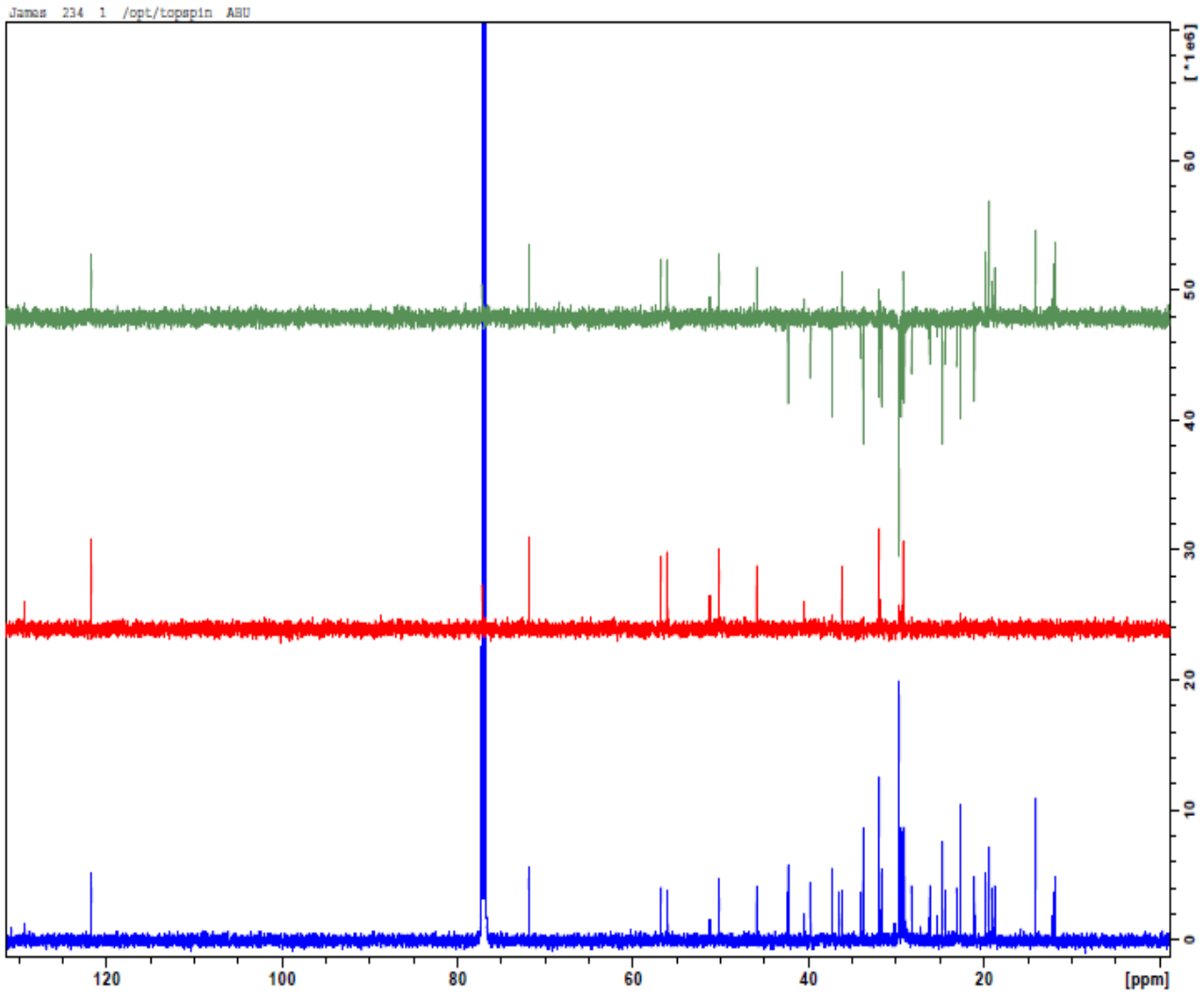


Figure 4.03: DEPT spectrum of C1

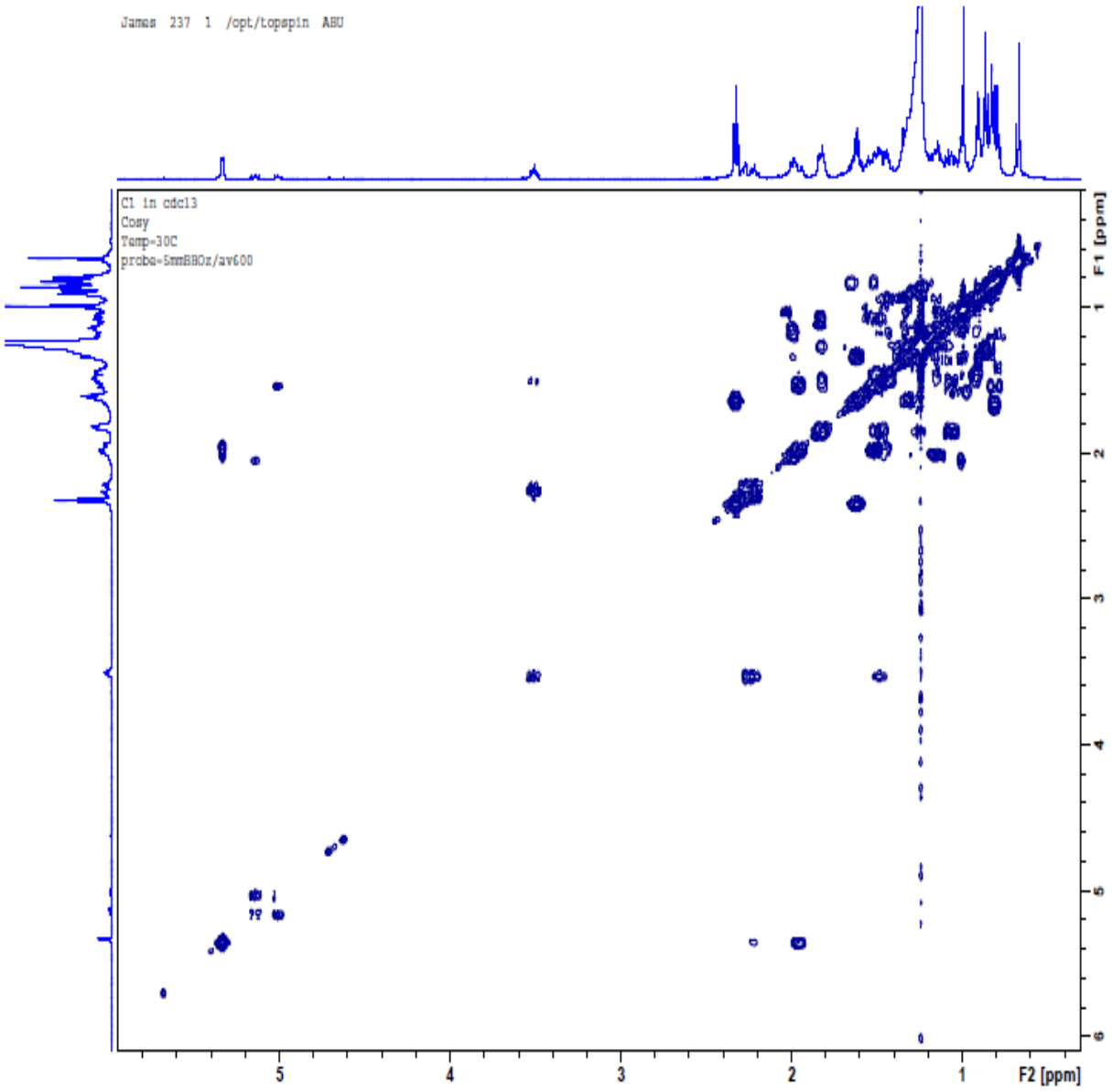


Figure 4.04 COSY spectrum of C1

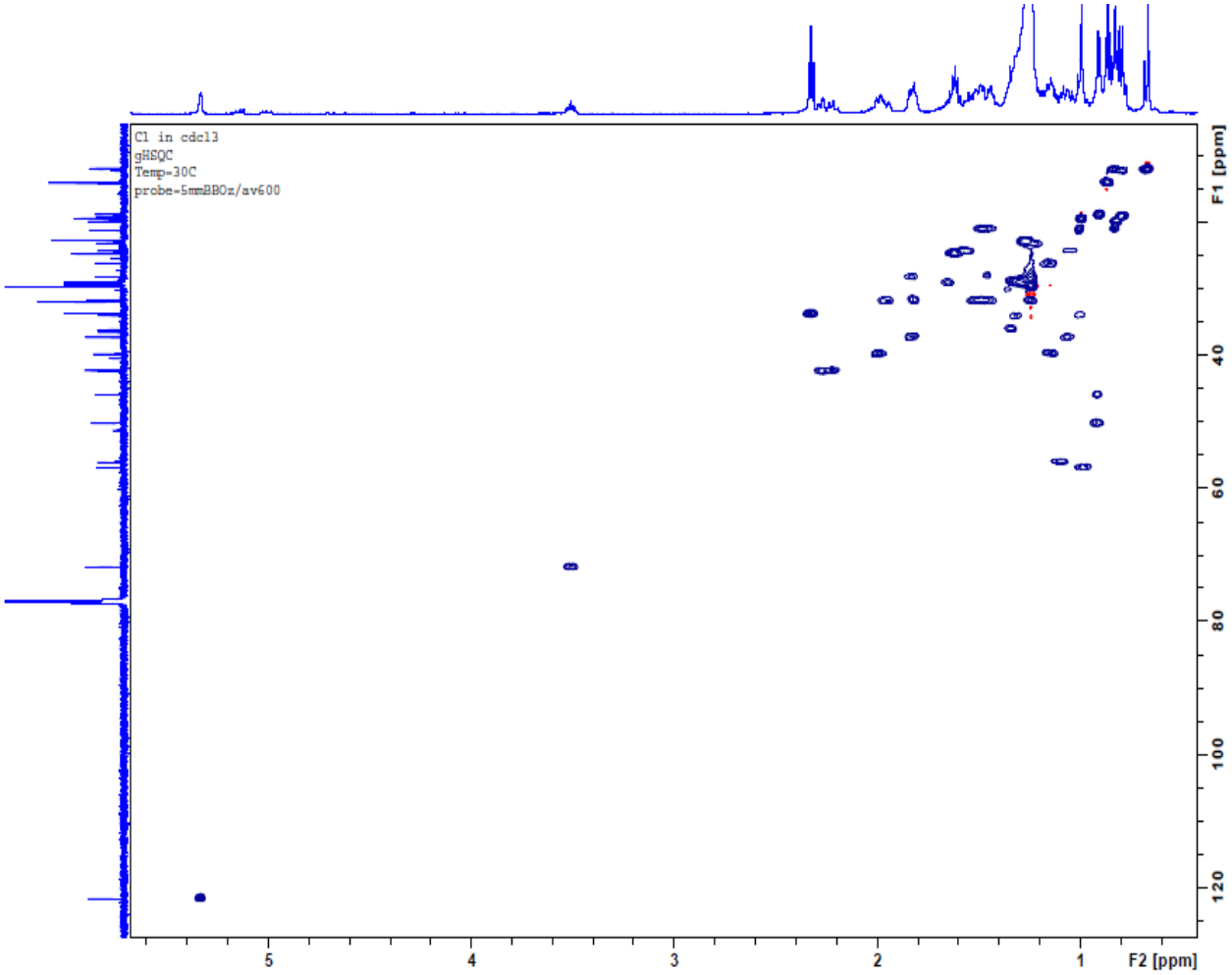


Figure 4.05: HSQC spectrum OF C1

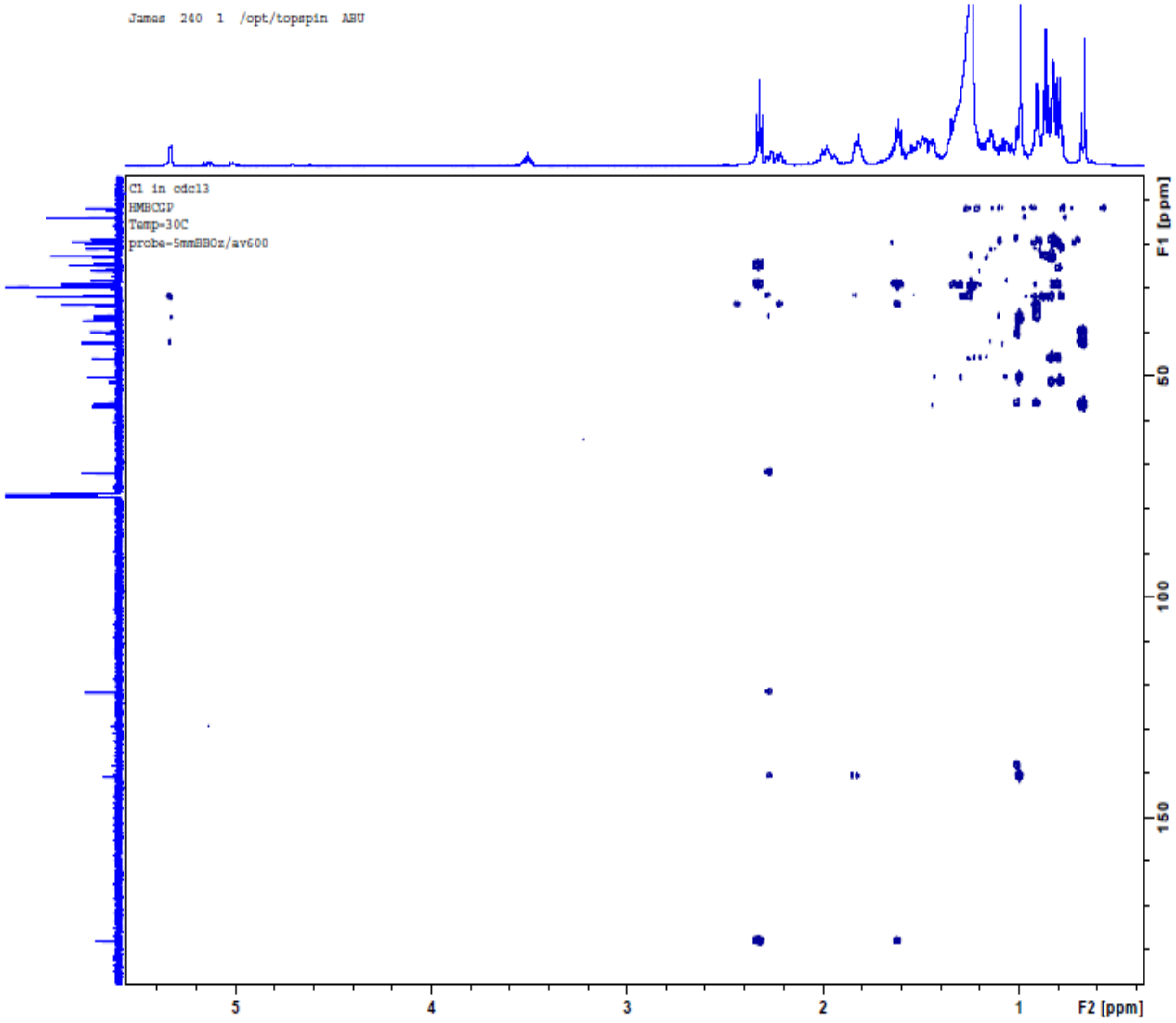


Figure 4.06: HMBC correlation spectrum of C1

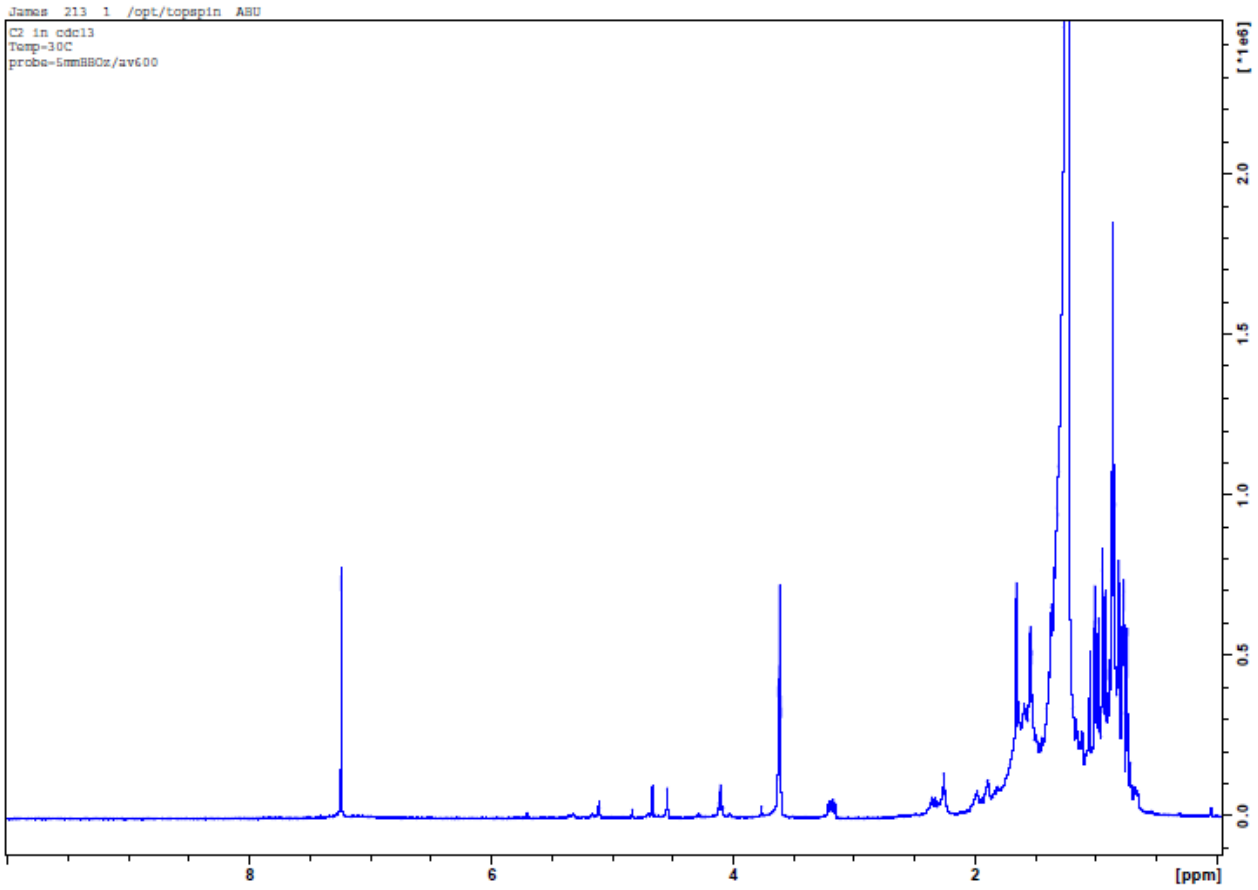


Figure 4.07: ^{1}H NMR spectrum of C2

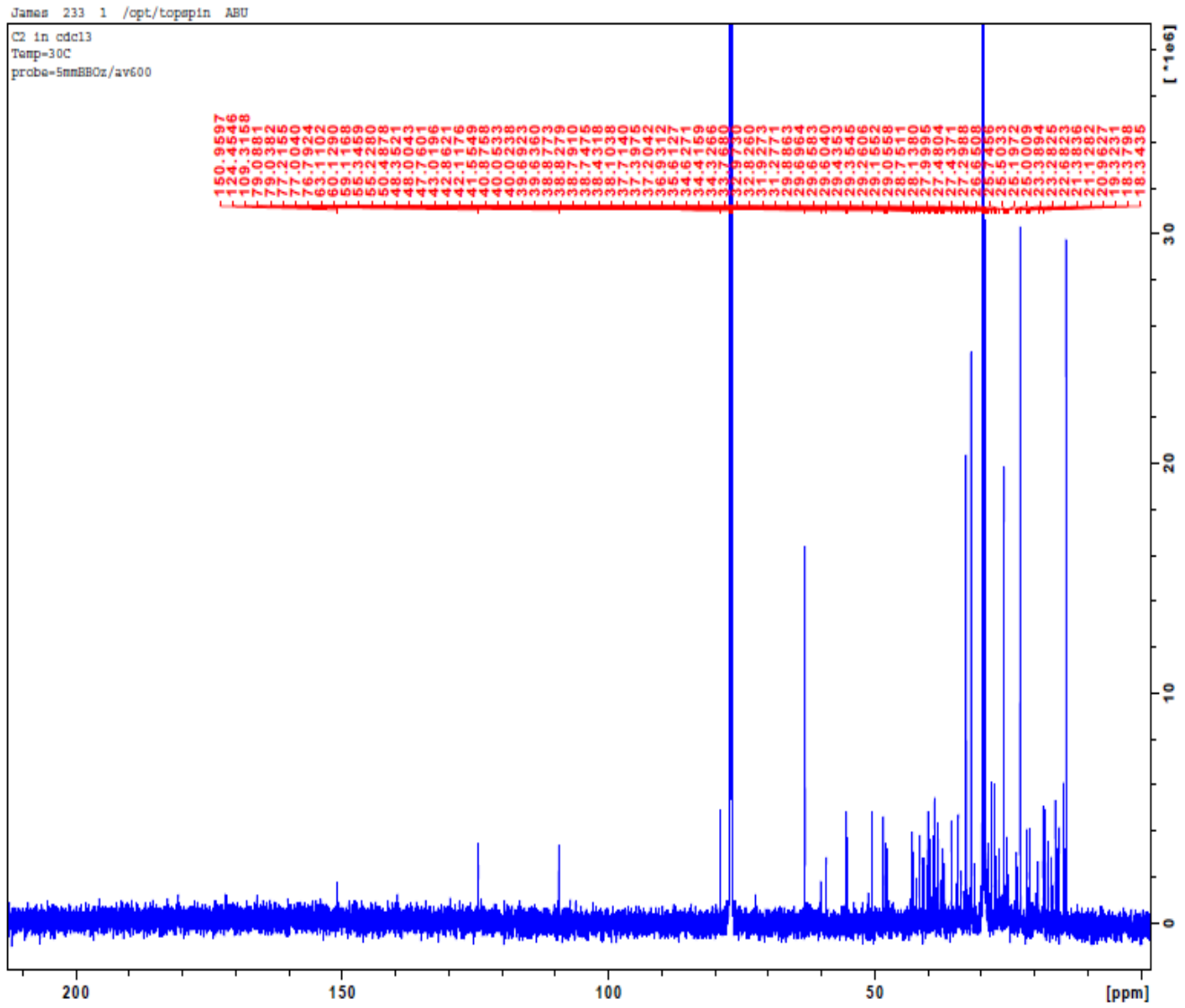


Figure 4.08: ^{13}C NMR spectrum of C2

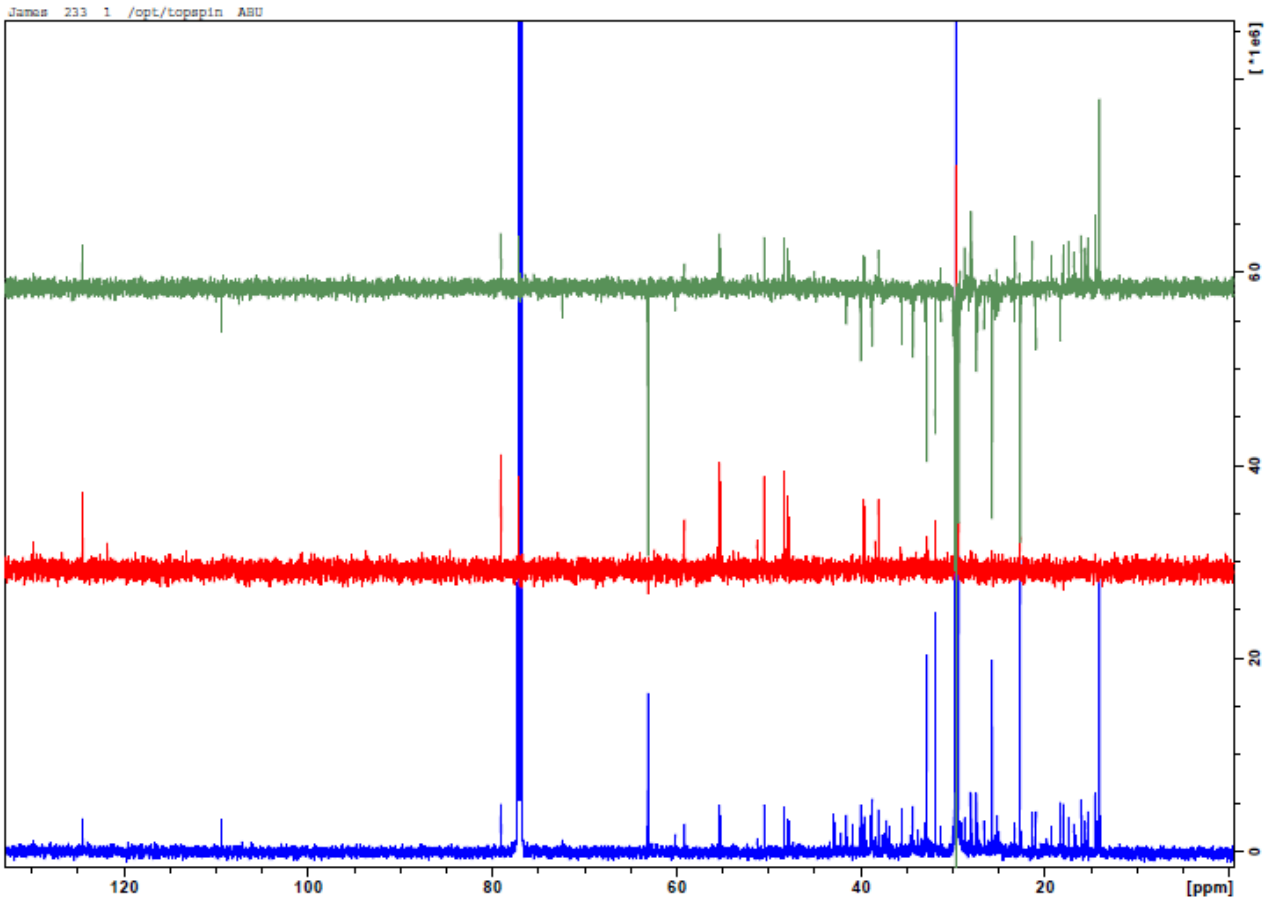


Figure 4.09: DEPT experiment of C2.

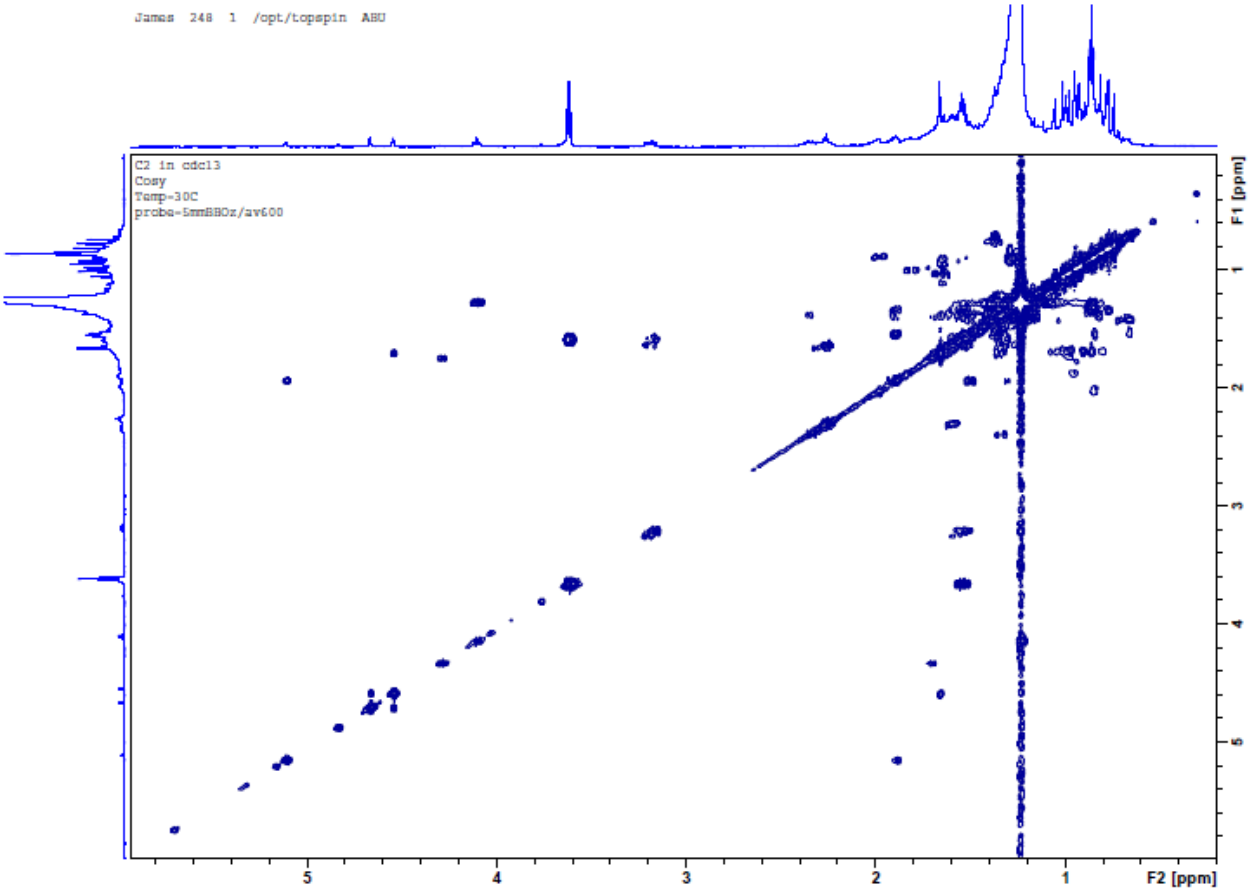


Figure 4.10: COSY correlation spectrum of C2.

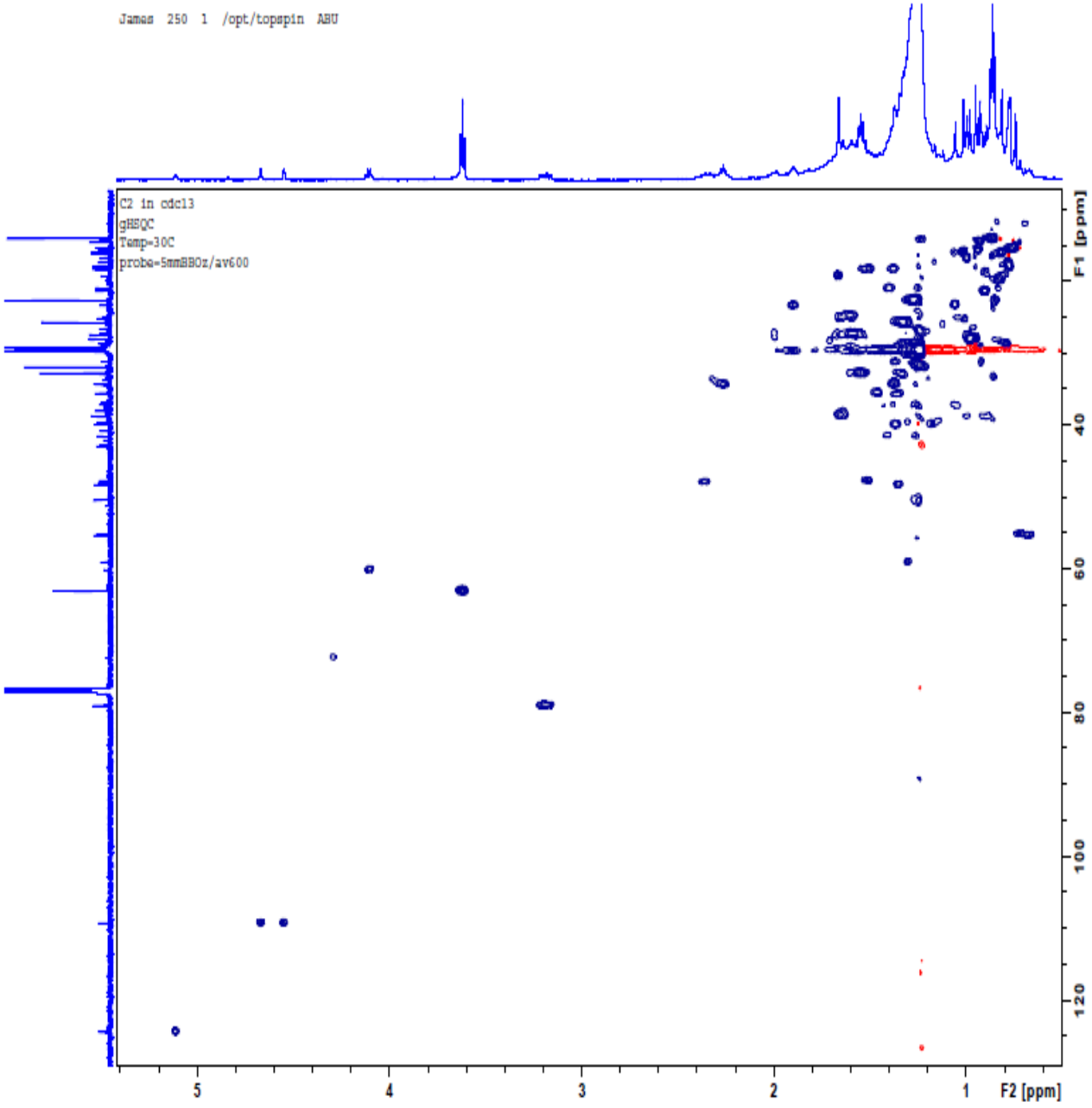


Figure 4.11: HSQC spectrum of C2

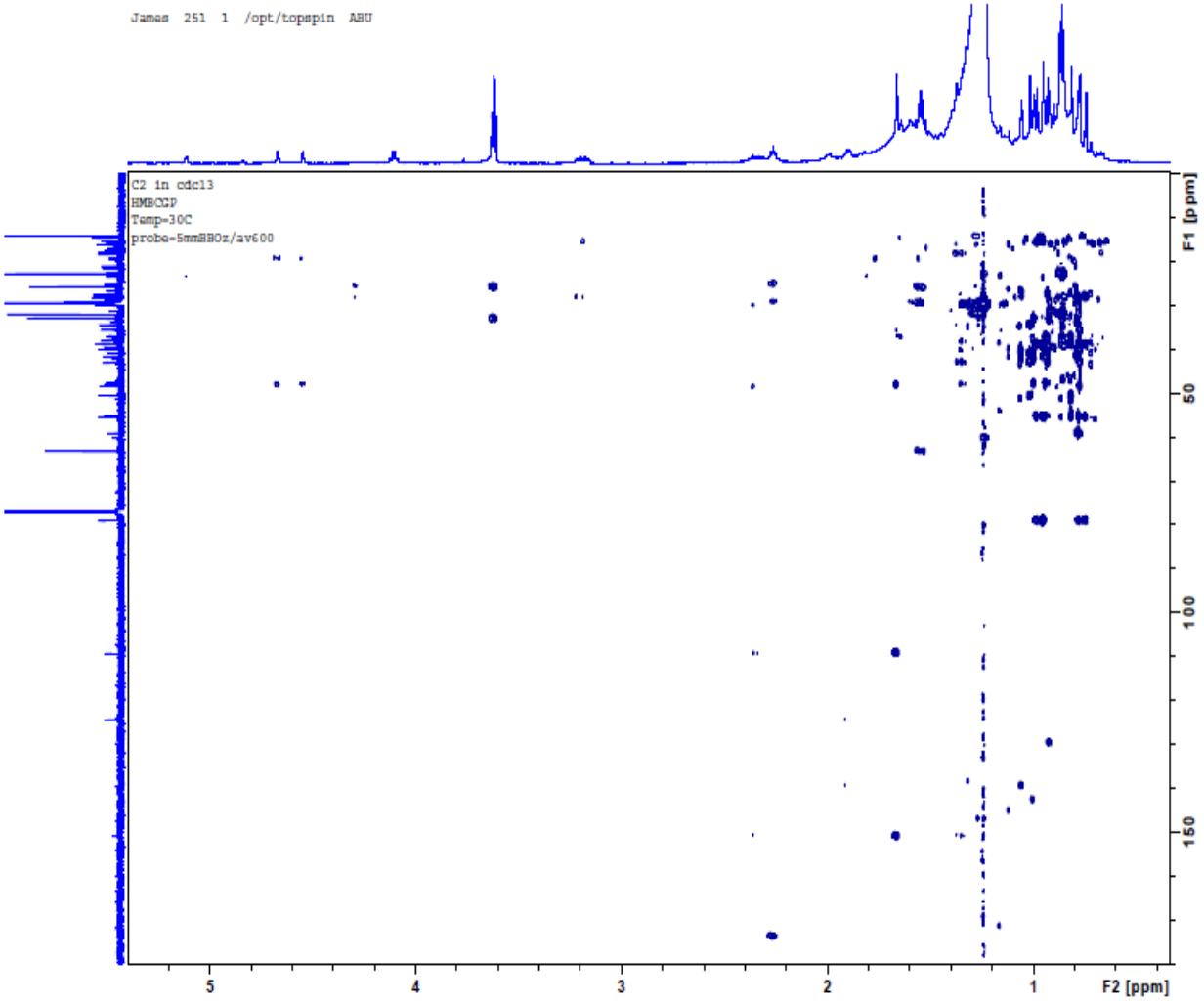


Figure 4.12: HMBC correlation spectrum of C2

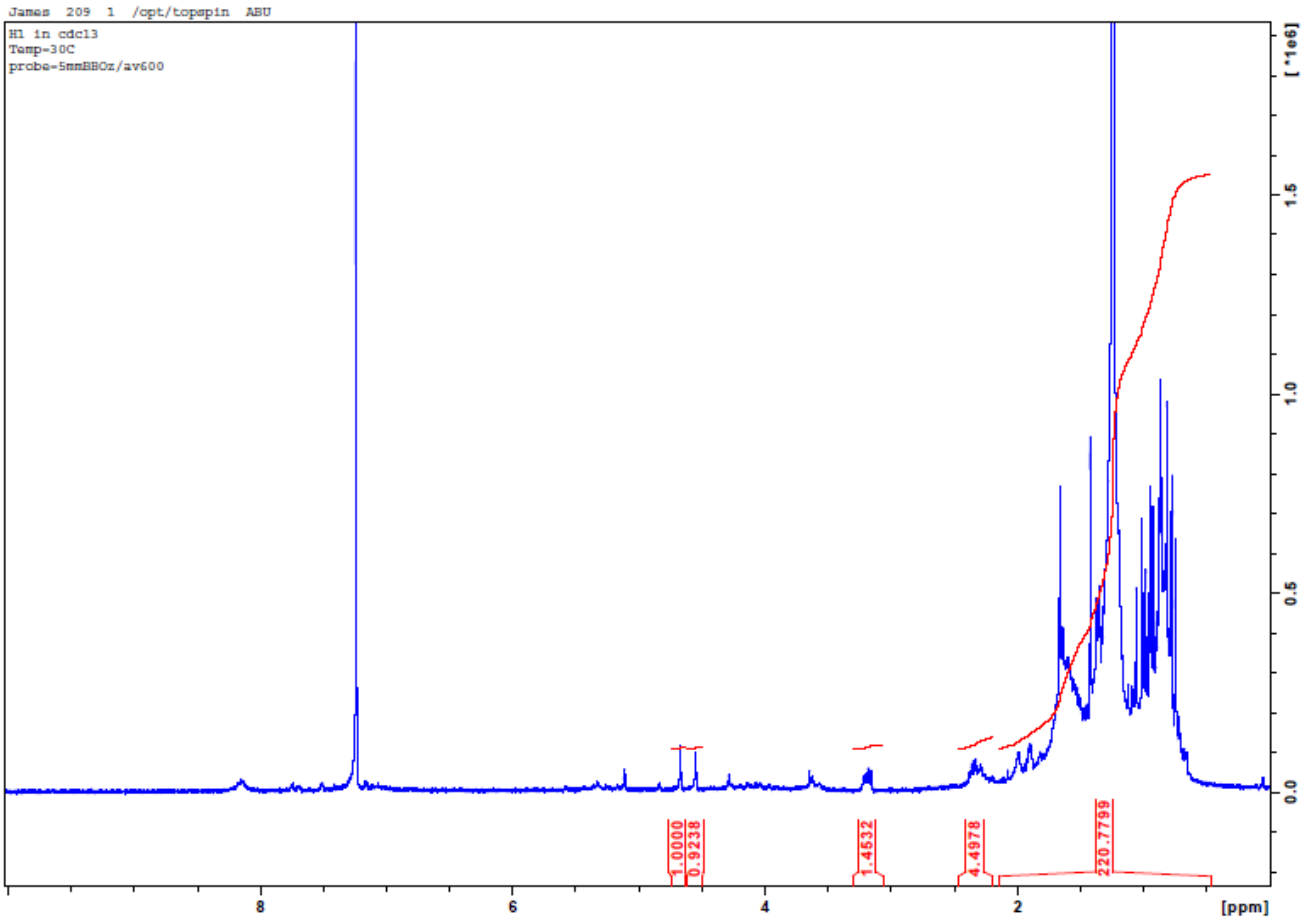


Figure 4.13: ^{1}H NMR spectrum of H1

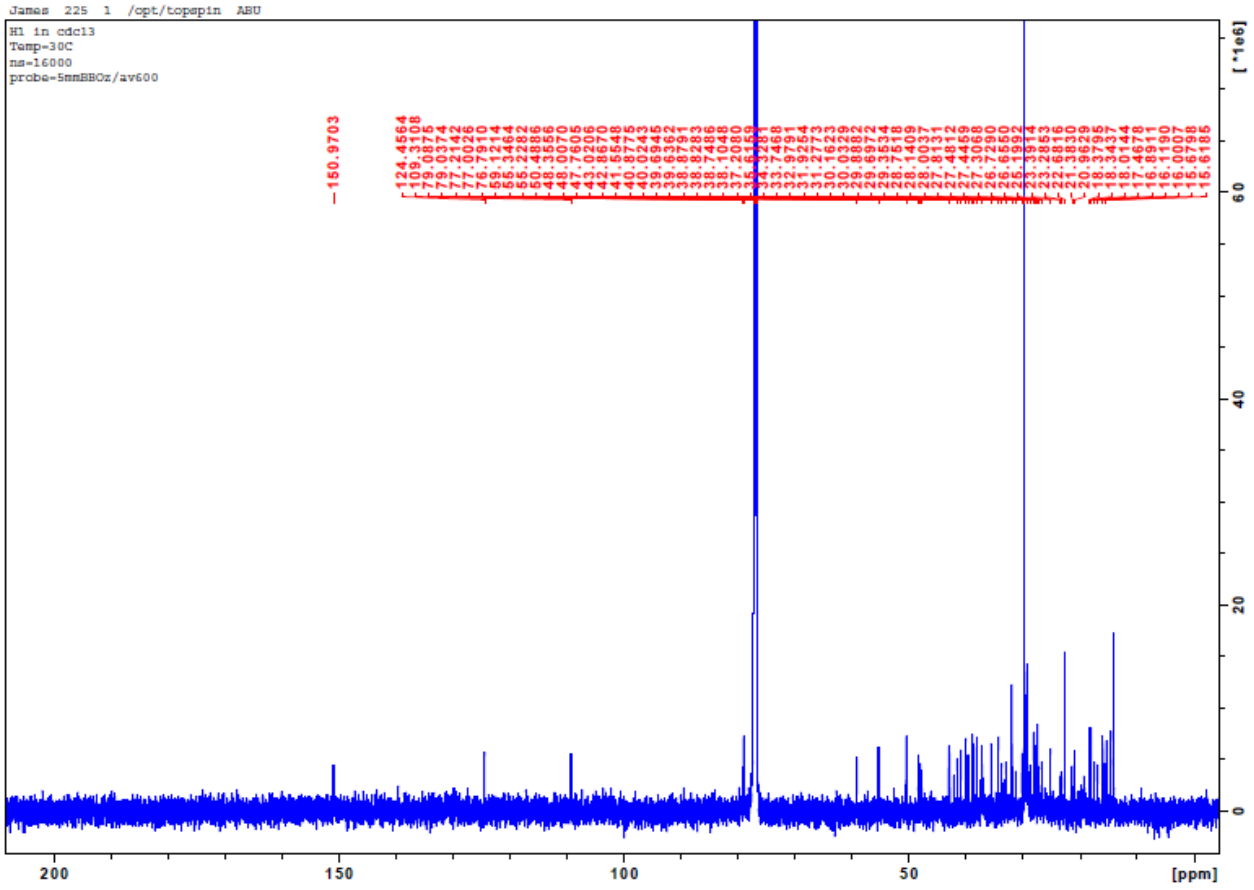


Figure 4.14: ^{13}C NMR spectrum of H1

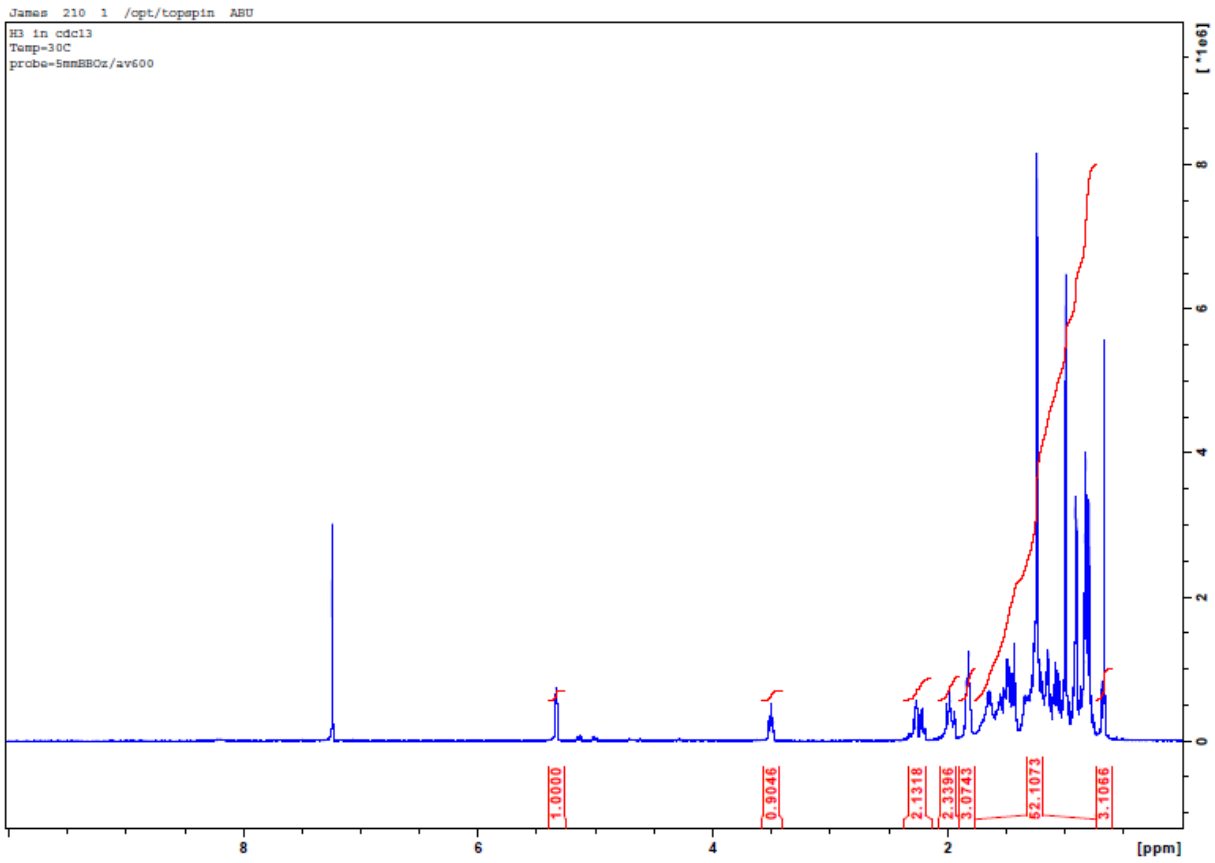


Figure 4. 15: ^{1}H NMR spectrum of H3

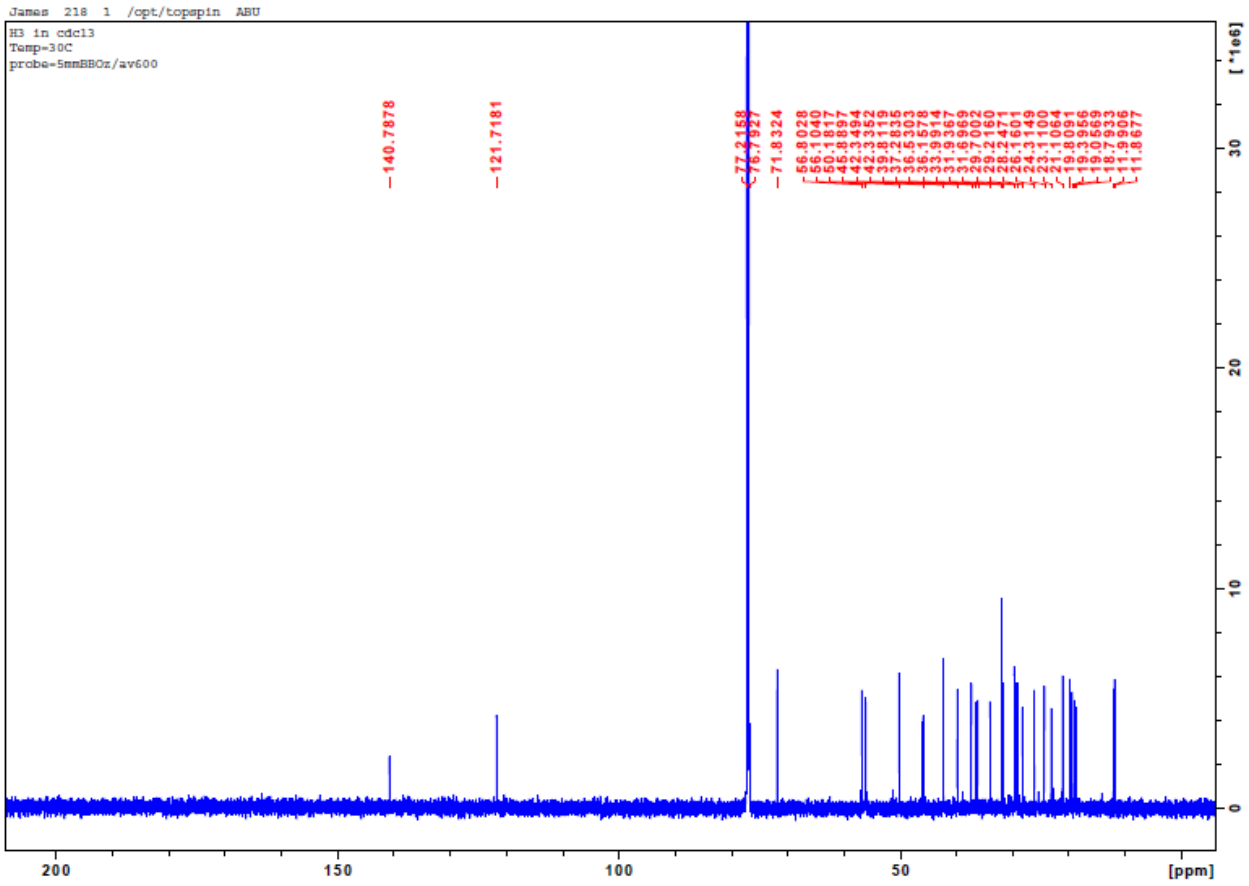


Figure 4.16: ^{13}C NMR spectrum of H3

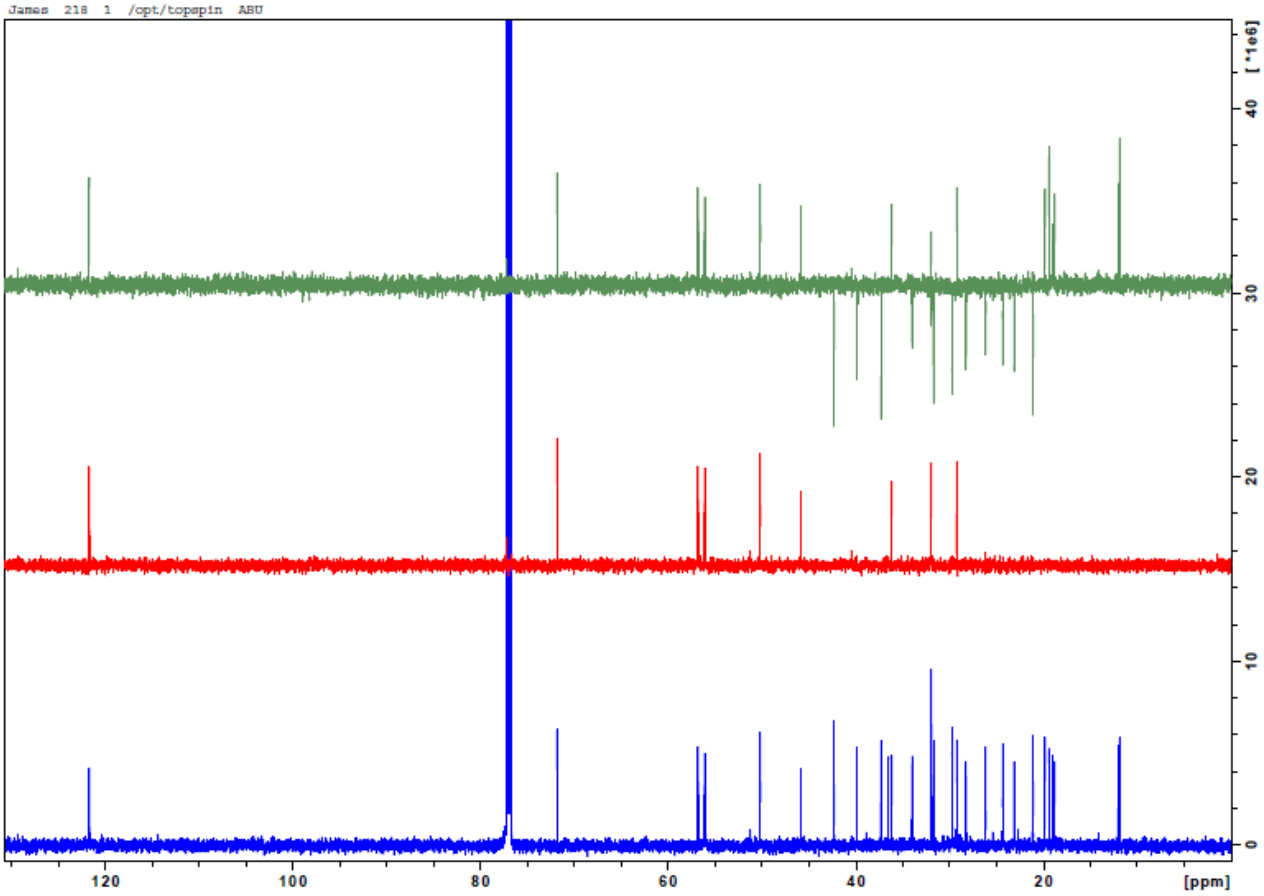


Figure 4.17: DEPT experiment of H3

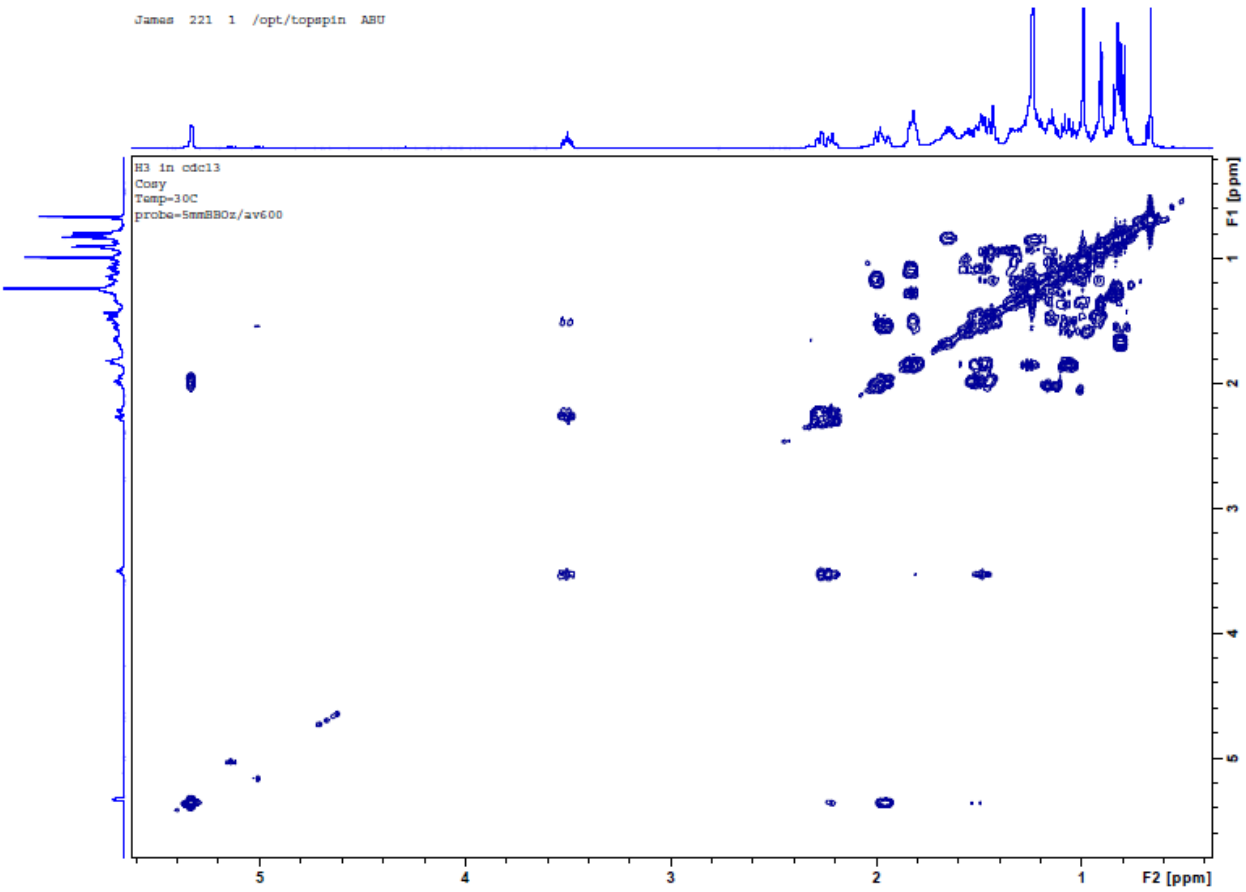


Figure 4.18: COSY correlation spectrum of H3.

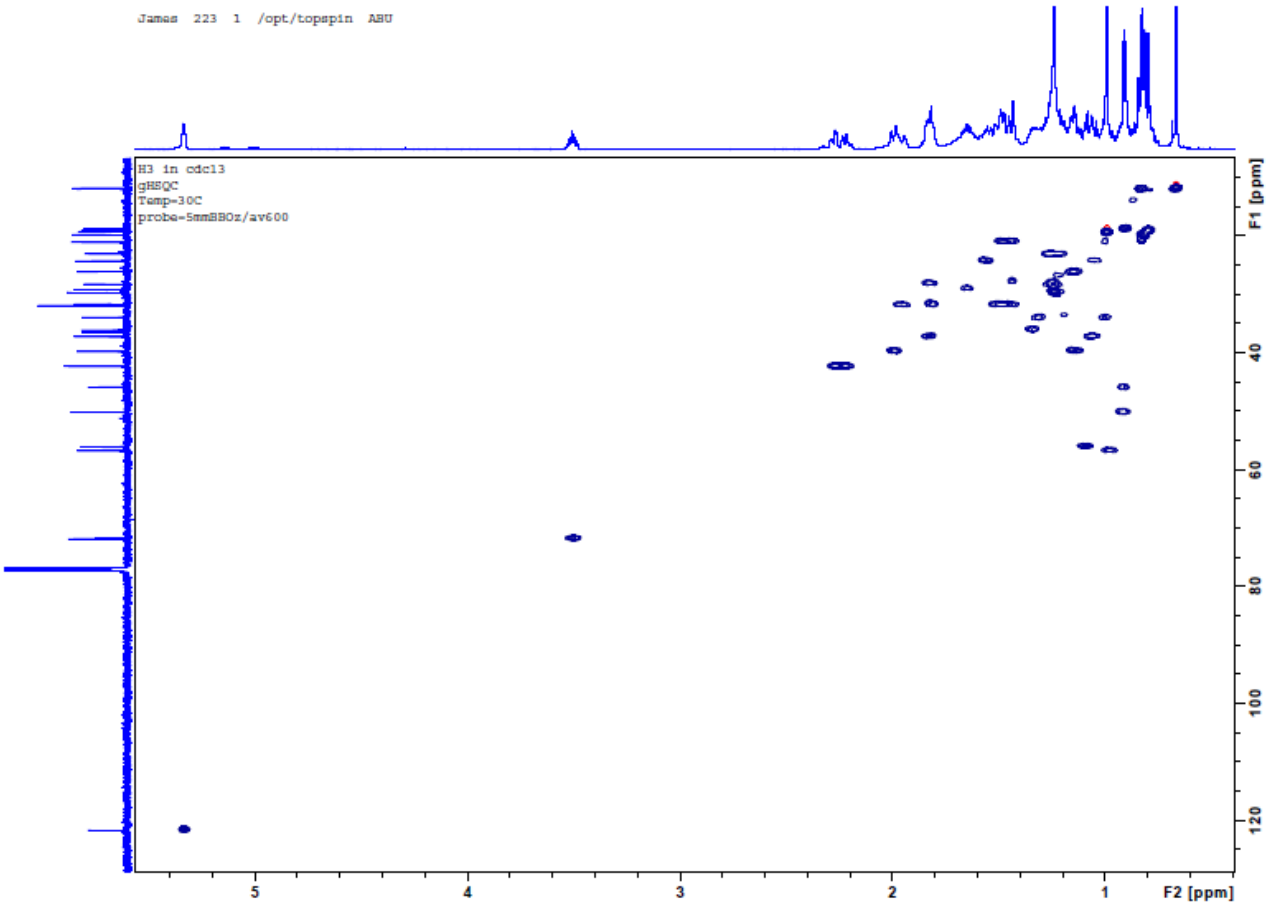


Figure 4.19: HSQC spectrum of H3

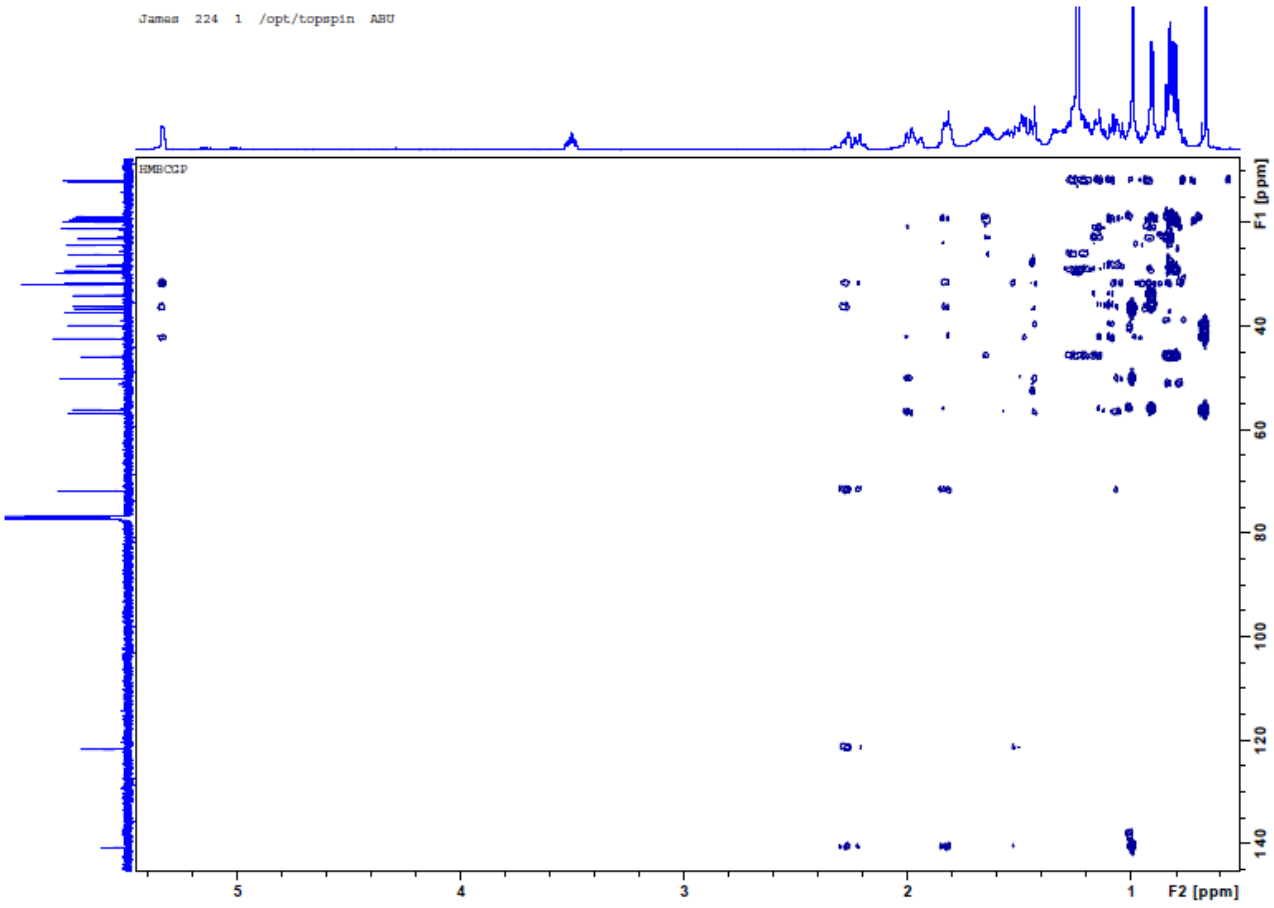


Figure 4.20: HMBC correlation spectrum of H3

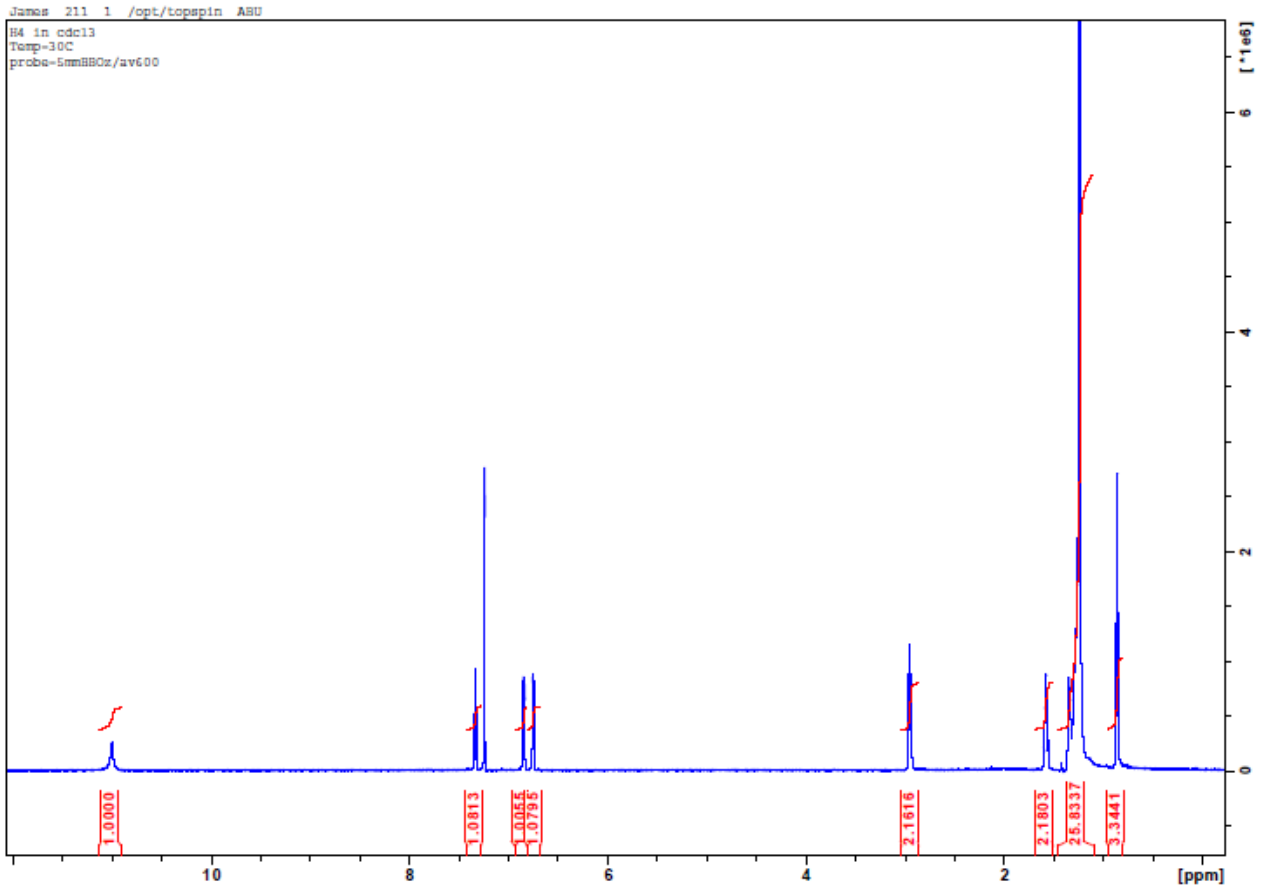


Figure 4.21: ^{1}H NMR spectrum of H4

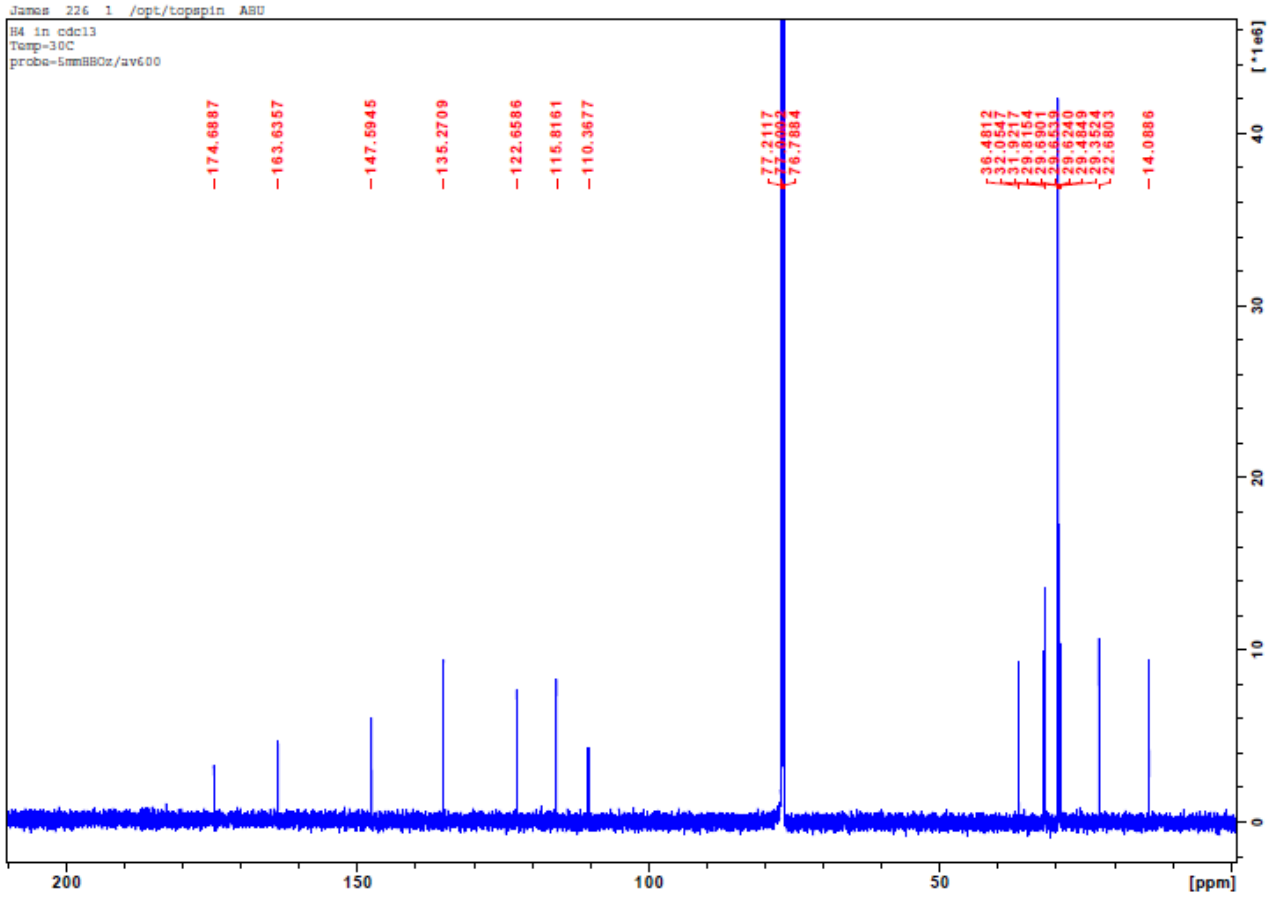


Figure 4. 22: ^{13}C NMR spectrum of H4

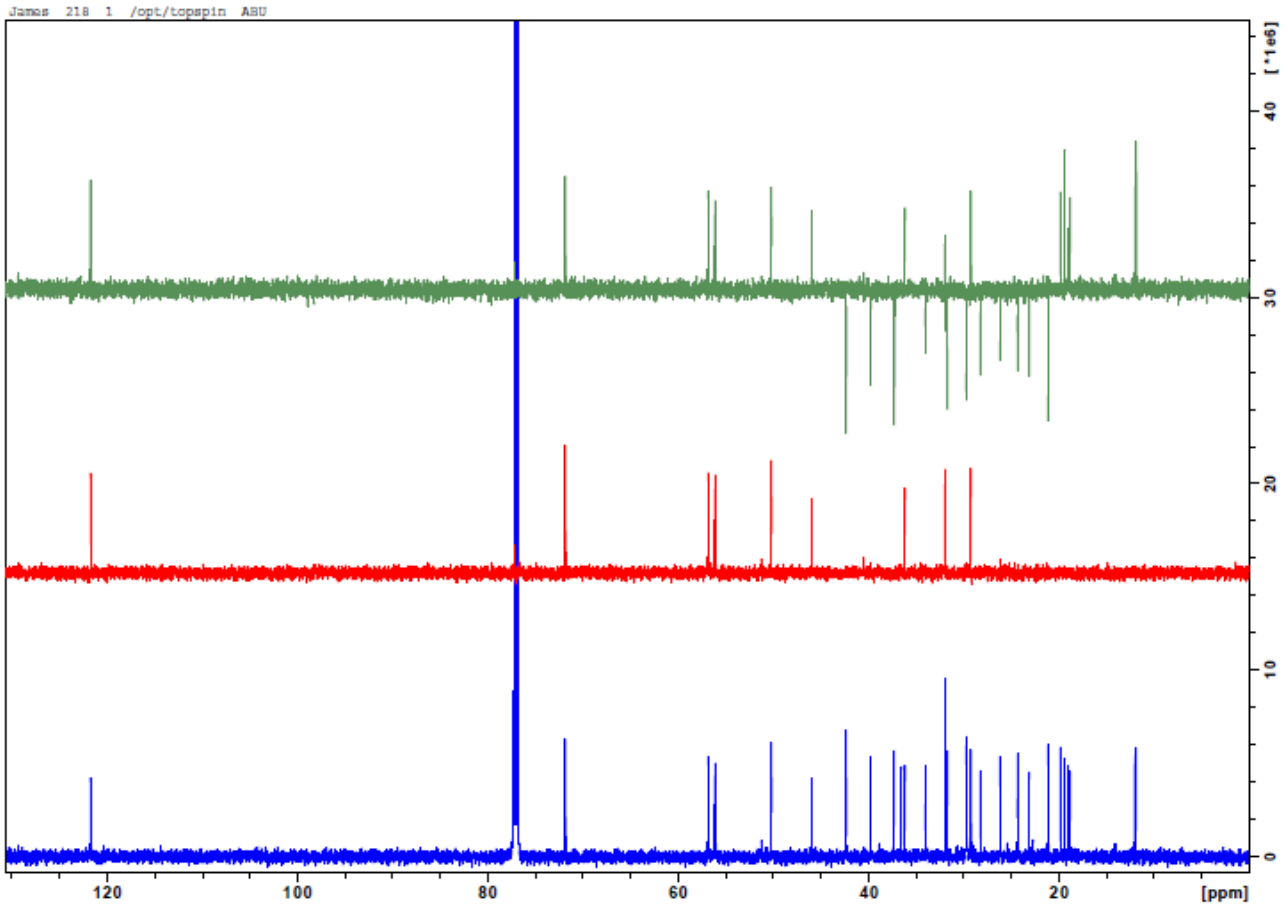


Figure 4. 23: DEPT experiment of H4.

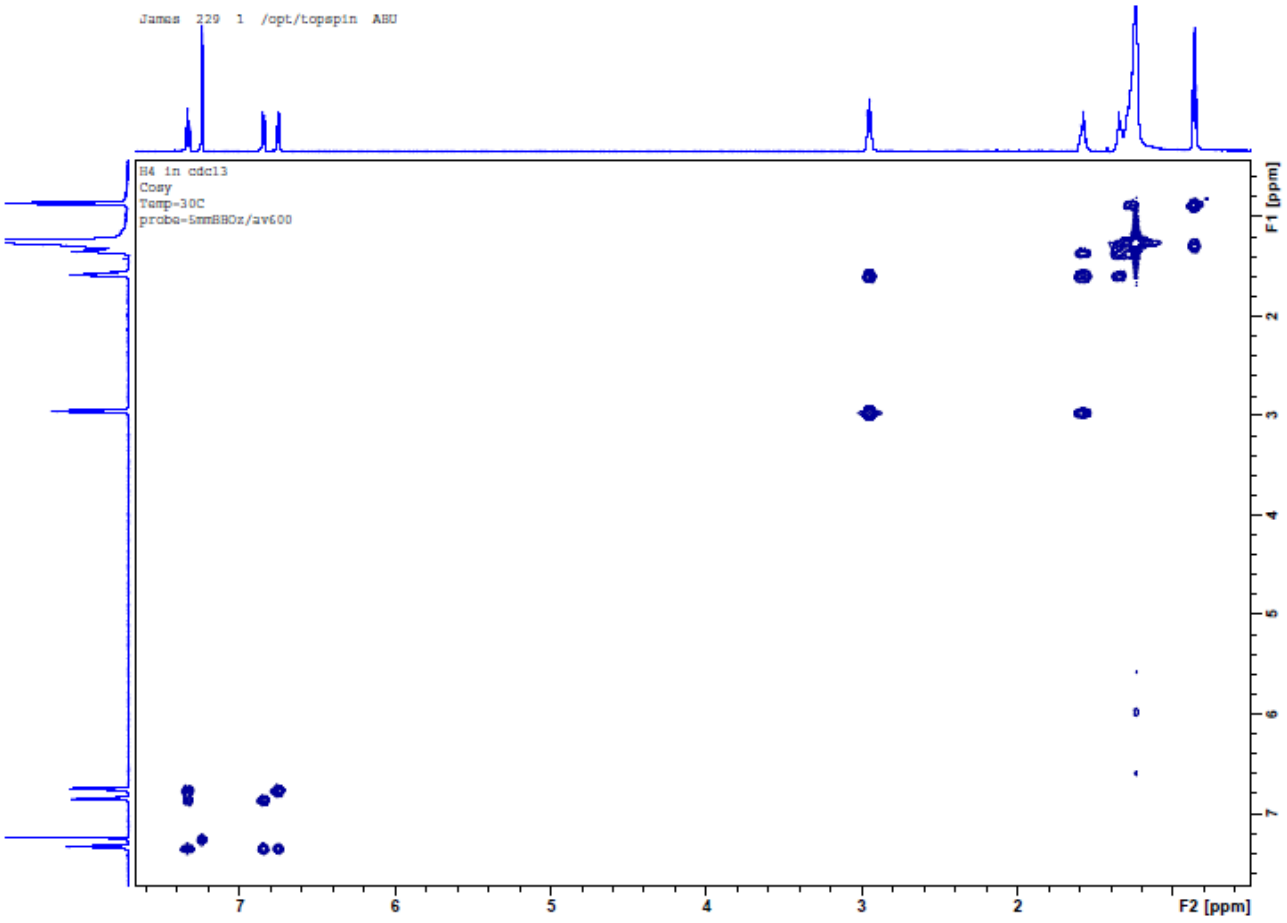


Figure 4.24: COSY correlation spectrum of H4.

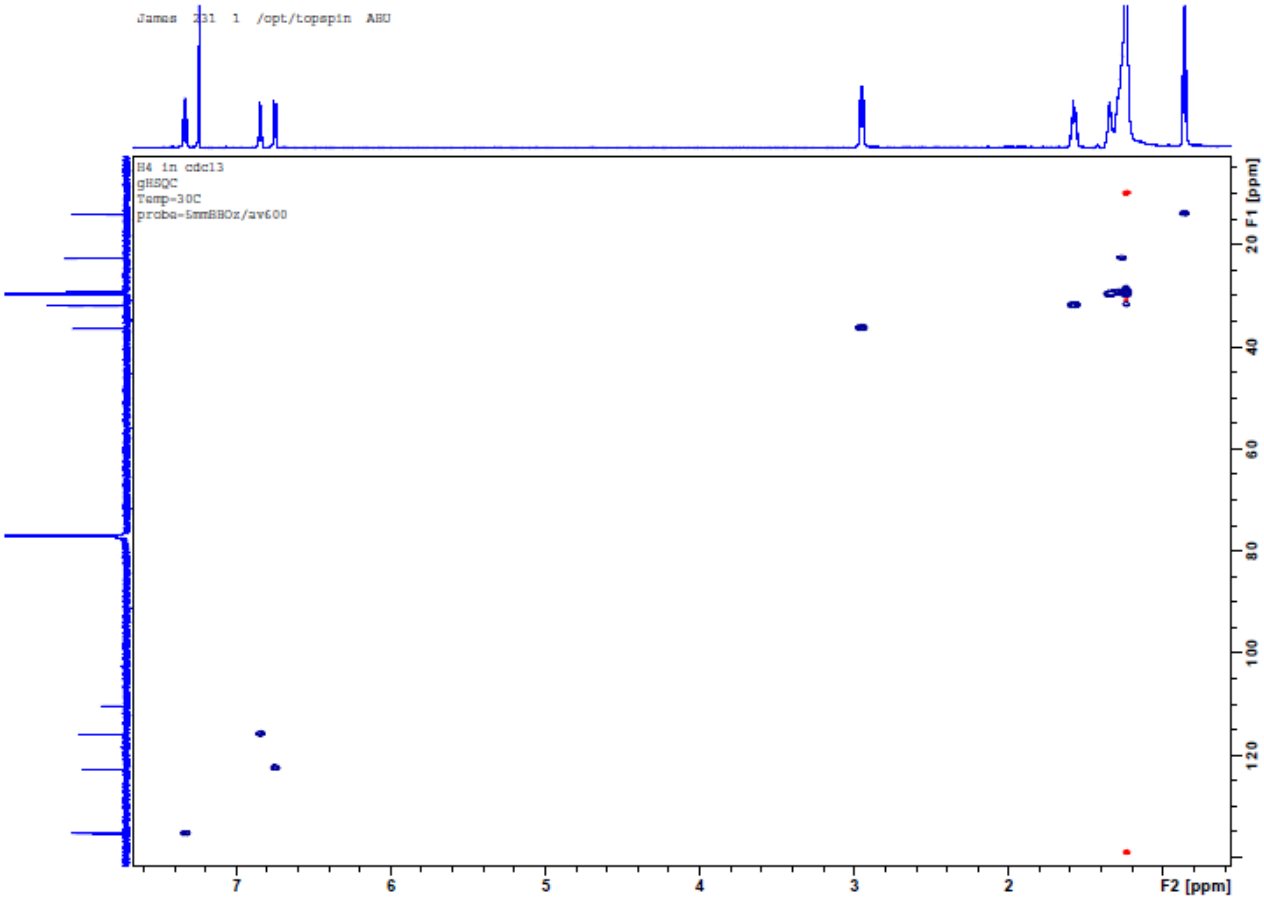


Figure 4.25: HSQC spectrum of H4

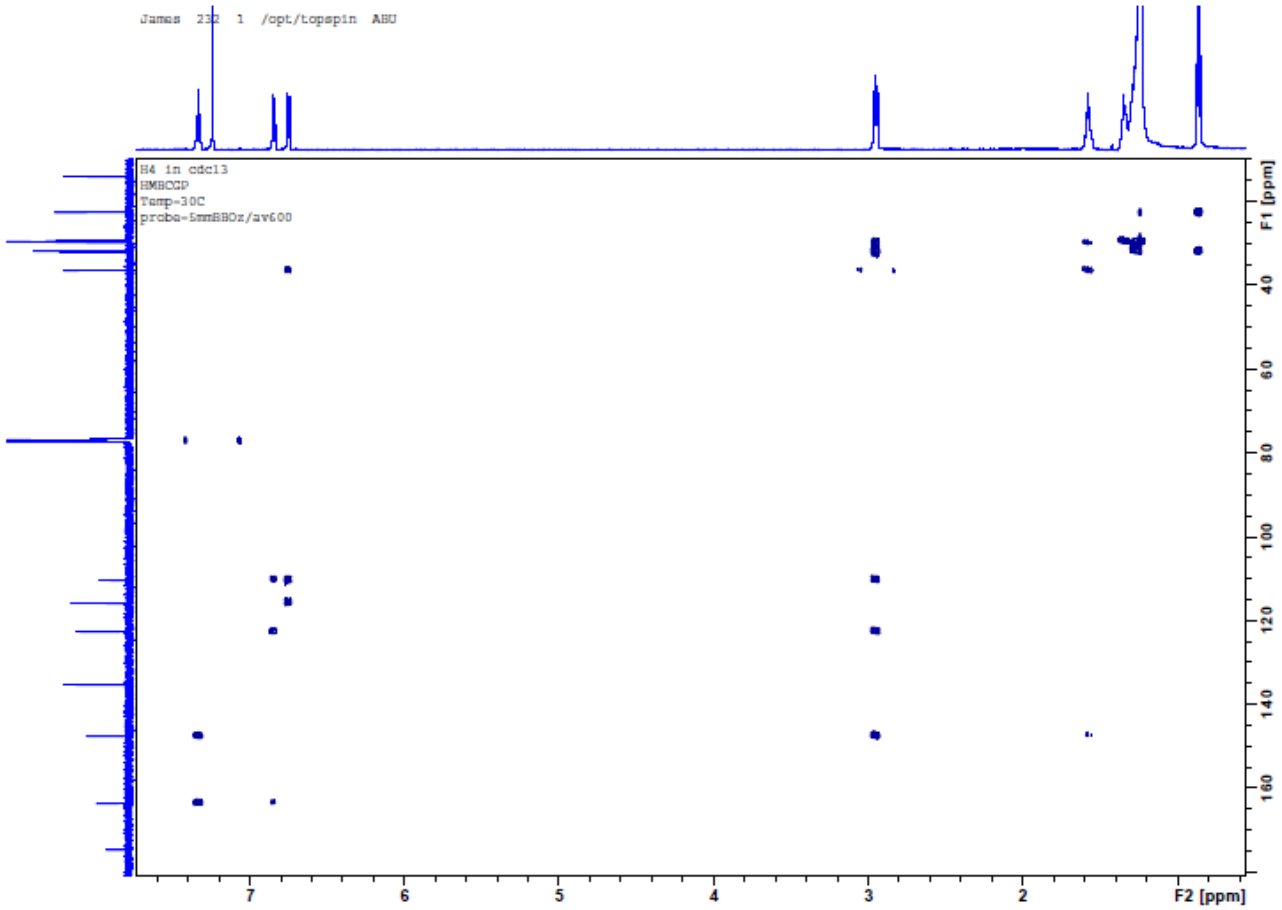


Figure 4.26: HMBC correlation spectrum of H4

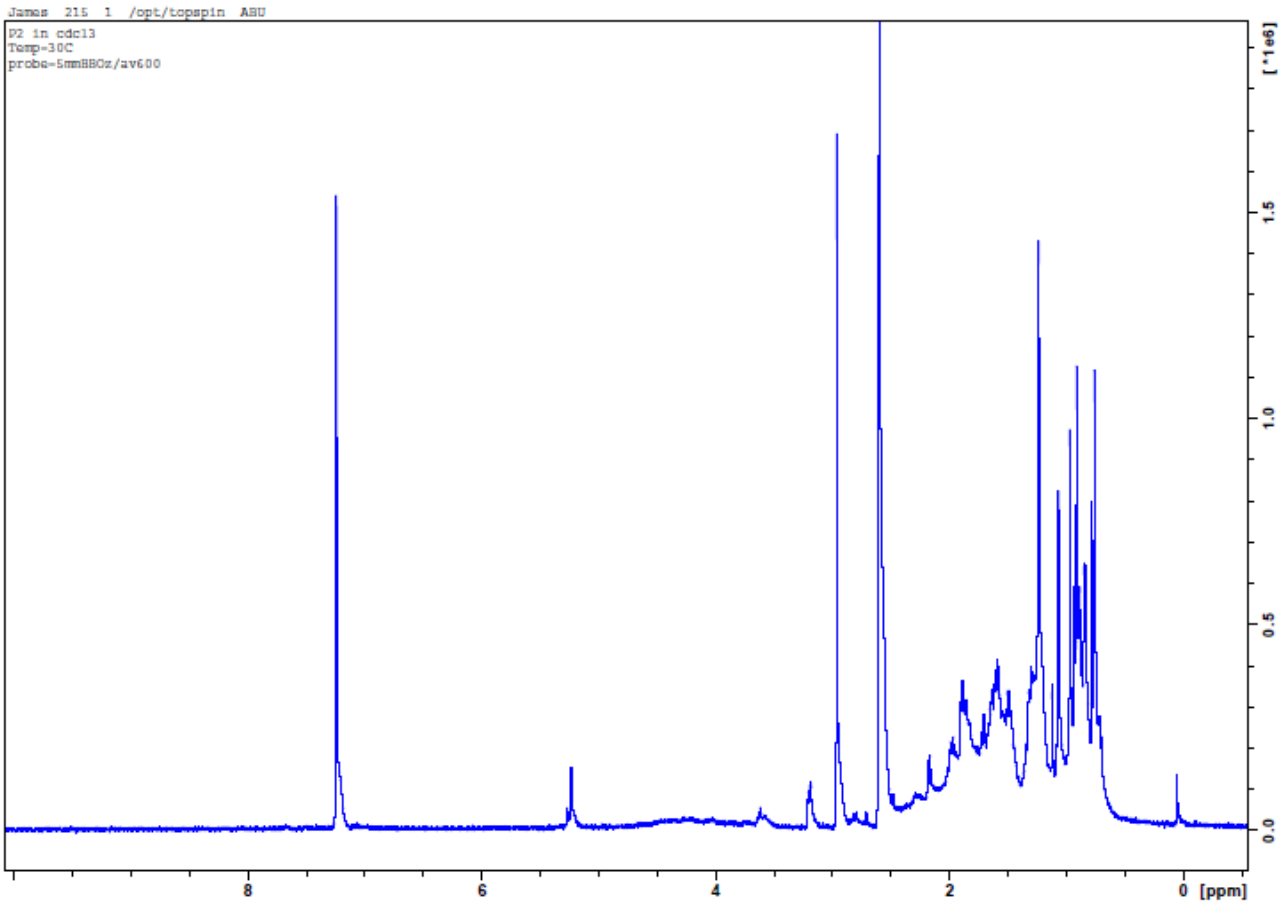


Figure 4.27: ^{1}H NMR spectrum of P2

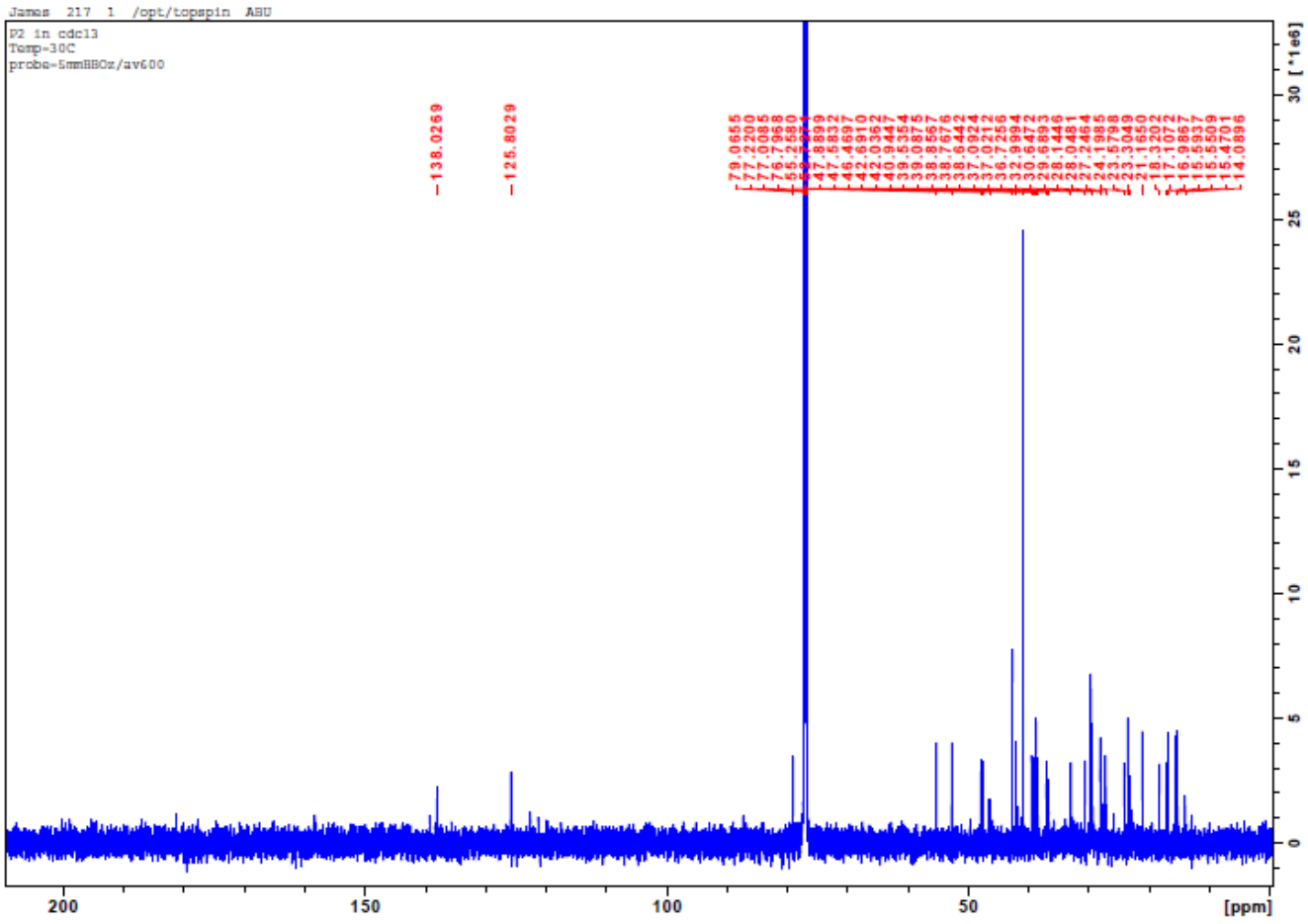


Figure 4.28: ^{13}C NMR spectrum of P2

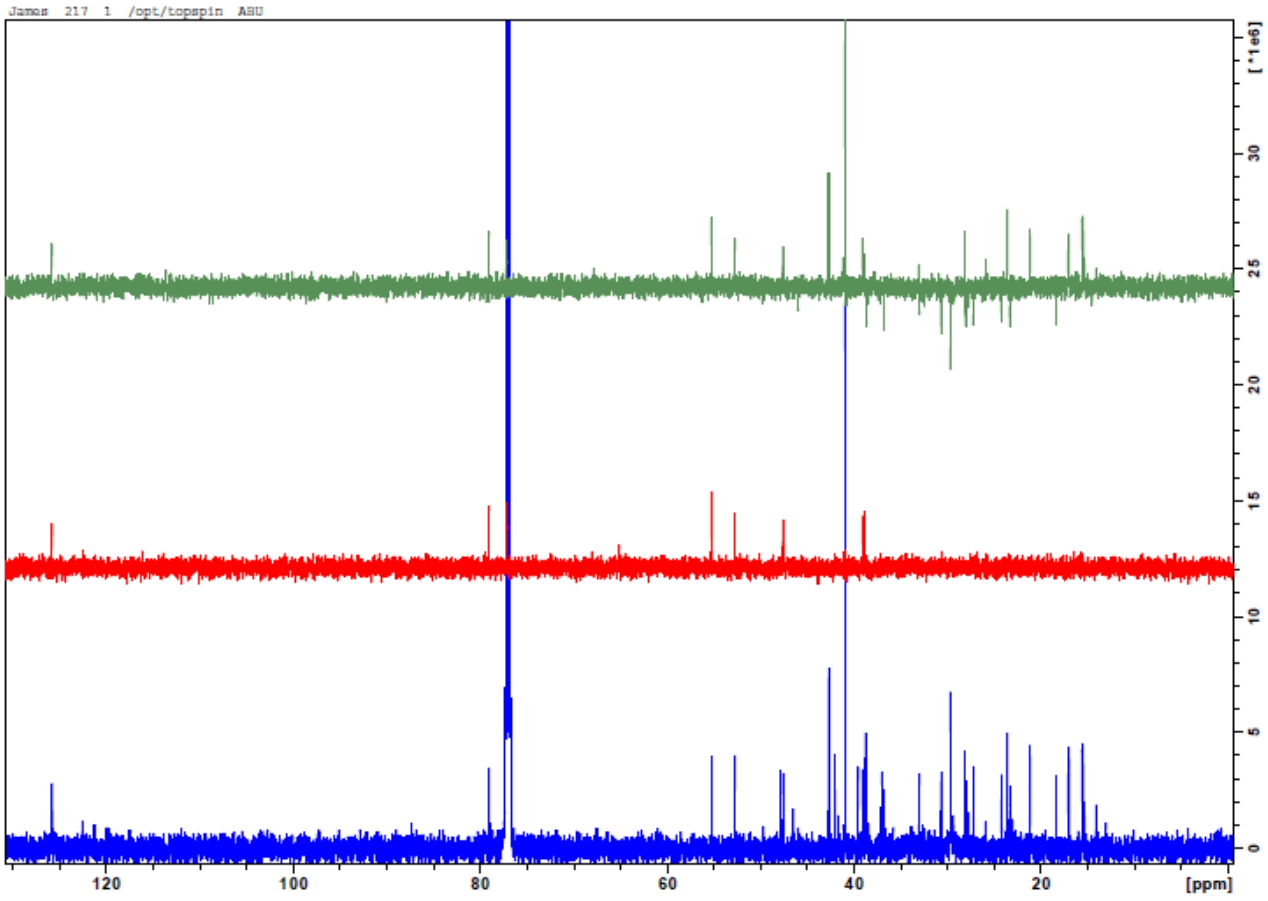


Figure 4.29: DEPT experiment of P2.

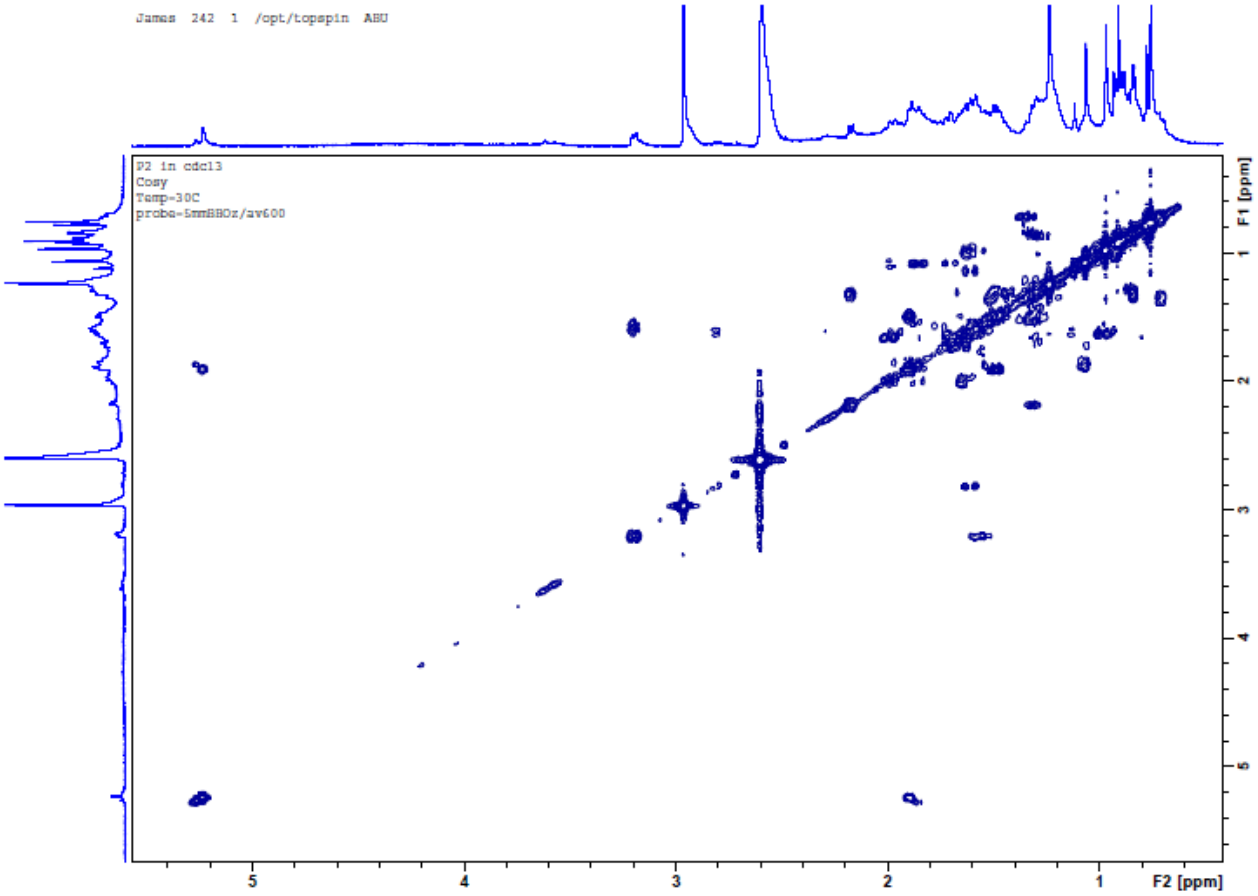


Figure 4.30: COSY correlation spectrum of P2.

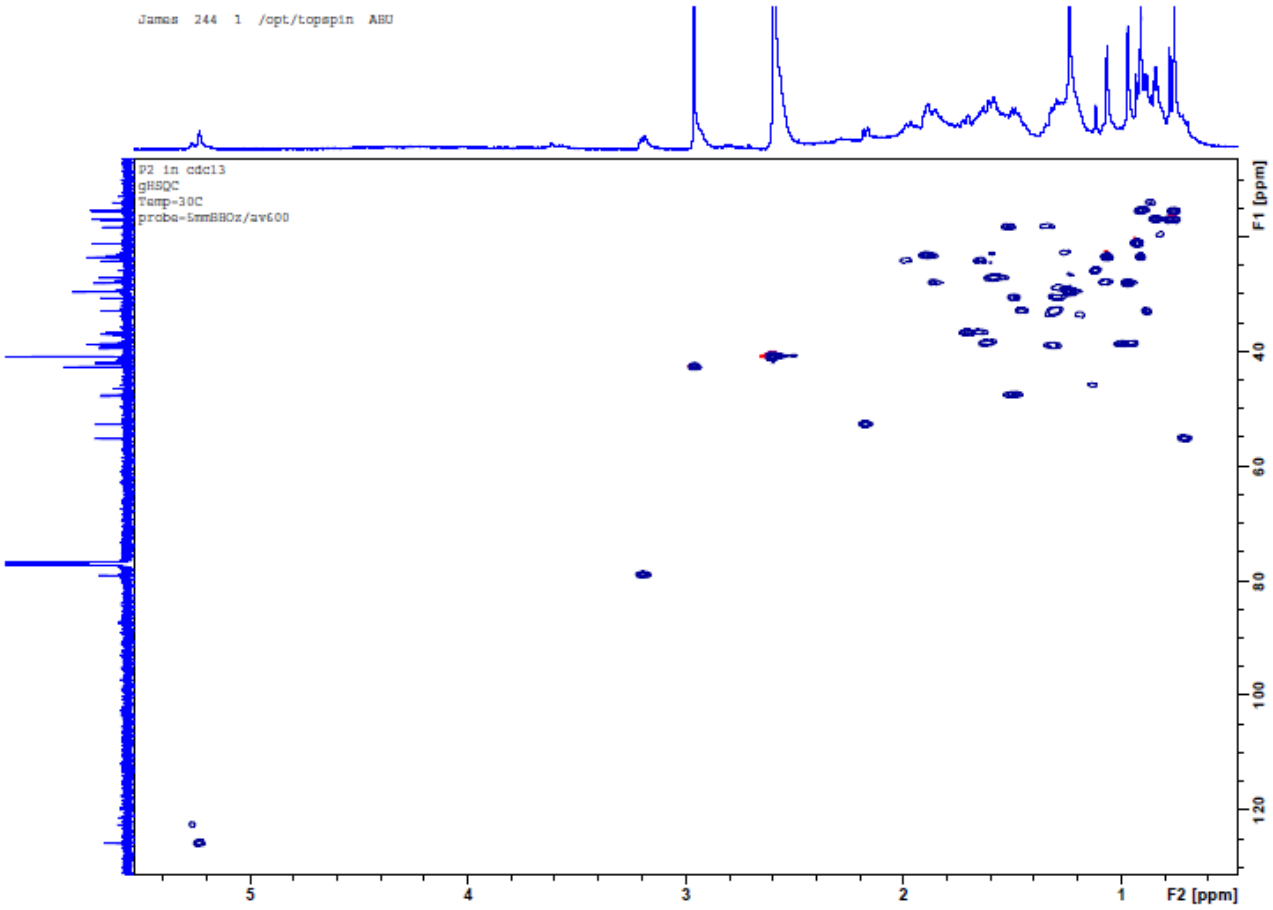


Figure 4.31: HSQC spectrum of P2

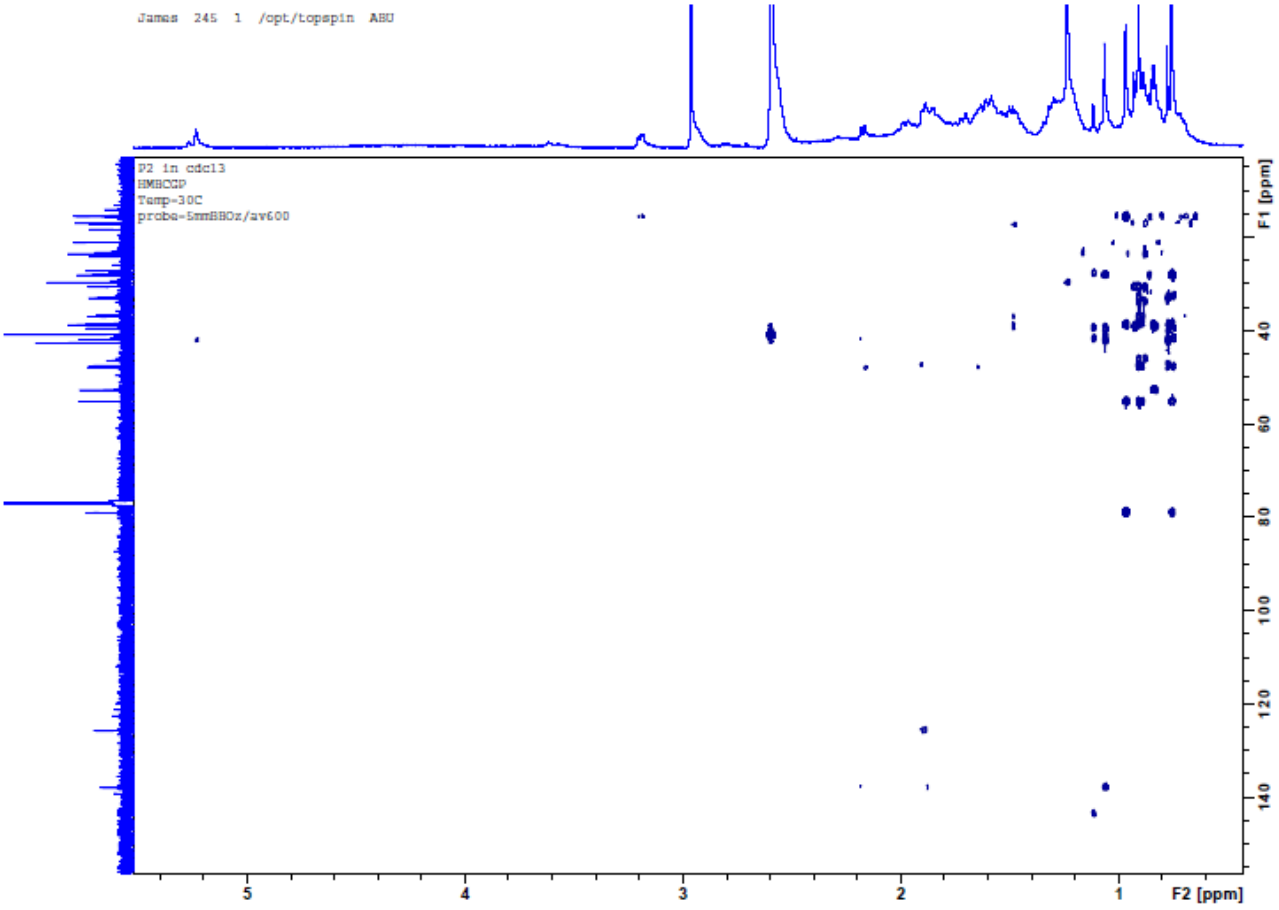


Figure 4.32: HMBC correlation spectrum of P2

CHAPTER FIVE

5.0 DISCUSSION

Tuberculosis, also called TB, is currently a major health hazard due to multidrug-resistant forms of Bacilli (Ramachandran and Balasubramanian, 2014). Global efforts are being made to eradicate TB using new drugs with new modes of action, higher activity, and fewer side effects in combination with vaccines. For this reason, unexplored new sources need to be examined for development of drugs. In an effort to expand the spectrum of anti TB and antibacterial agents from natural resources, *Heeria insignis*, *Psorospermum senegalense* and *Clerodendrum capitatum* were investigated. These plants have been widely used in traditional medicinal treatment of tuberculosis and other infectious diseases.

5.1 Extraction of the Leaves of *H. insignis, C. capitatum* and Stem Bark of *P. senegalense*

The results of the extraction generally indicated that the more polar solvents had the highest percentage recovery yield indicating that the plants contains polar secondary metabolites as shown in Table 4.1. Thus phytocompounds such as alkaloids, flavonoids, quinones, steroids and triterpenoids with polar moieties, polyhydroxylated phenolics, glycoside such as saponins might be present in the plants. These polar groups, if present, have been reported to have anticancer, antioxidant, antiinflamatory and antimicrobial properties (Singh *et al.*, 2002; Stapleton *et al.*, 2004; 2007; Shah *et al*., 2008).

5.2 Phytochemical Screening of the Leaves of *H. insignis, C. capitatum* and Stem Bark of *P. senegalense*

The results of the phytochemical analysis of the ethyl acetate and methanol extracts of the leafs of *Heeria insignis*, *Clerodendrum capitatum* and stem bark of *Psorospermum senegalense* revealed the presence of saponins, alkaloids, flavonoids, glycosides, cardiac

glycosides and tannins, while the hexane and dichloromethane extract had steroids/triterpenes as the major constituents. Tannins were completely absent in all the extracts of *Psorospermum senegalense* but were present in the ethyl acetate and methanol extracts of *Clerodendrum capitatum* and in the ethyl acetate and methanol extracts of *Heeria insignis*. Anthraquinones were present only in the methanol extract of *Psorospermum senegalense* but absent in all other extracts of the other plants as shown in Table 4. 2. The presence of steroids, triterpenoids, flavonoids, tannins, saponins and anthraquinones in these plants could be the linked to the observed antituberculosis and antimicrobial properties in the plants.

This is because these secondary metabolites have been scientifically proven to act as antioxidants, antiinflamatory, anticancer and antimicrobial agents (Singh *et al.*, 2002; Stapleton *et al.*, 2004; 2007; Shah *et al*., 2008).

5.3 Antimicrobial Screening of the Leaves of *C. capitatum, H. insignis,* and Stem bark of *P. senegalense*

5.3.1 Antimicrobial Screening of the the Leaves of *C. capitatum*

Antimicrobial screening showed that all the extracts of *C. capitatum* exhibited moderate to good antibacterial and antifungal activities. Zone of inhibition (ZI) determination showed inhibition which ranged from 17-26 mm (hexane), 23-26 mm (dichloromethane), 26-29 mm(ethyl acetate) and 20-23 mm (methanol) against the entire test organism except *Corynebacterium ulcerans*, as compared to the standard drugs used as positive control (ciprofloxacin, sparfloxacin; 32 - 42 mm, Fluconazole; 34 – 36 mm). The ethyl acetate was found to be the most active with ZI of 29 mm against *S. dysentriae* and *S. typhi*, ZI of 28 mm was recorded against *C. krusei*. The results of the minimum inhibitory concentration showed that ethyl acetate fraction inhibited the growth of all test organisms at a low concentration of 3.25 mg/ml except *S. aureus* and *C. albicans* which were inhibited at 7.5 mg/ml. Higher MIC values were observed for hexane (7.5-15 mg/ml), dichloromethane and methanol fractions

showed MIC at 7.5 mg/ml. The microorganisms were not only inhibited but completely killed at concentration: ethyl acetate (MBC/MFC; 7.5-15 mg/ml), dichloromethane (MBC/MFC; 15 mg/ml), methanol (MBC/MFC; 15-30 mg/ml) and hexane (MBC/MFC; 30 mg/ml).

5.3.2 Antimicrobial Screening of the Leaves of *H. insignis*

The extracts of *H. insignis* were found to be active against *B. subtilis, Enterobacter sp, S. aureus, S. dysenteriae, MRSA, C. pseudotropicalis and C. stellatoidea,* but not on *VRE, S. feacalis, P.aeruginosa, P.rettgeris and C.albicans*. The result of the zone of inhibition (ZI) of the hexane extract showed ZI which ranged from 20 to 22 mm, the dichloromethane (26-31 mm), methanol (20 and 26 mm) while ethyl acetate showed ZI ranging from 20 to 34 mm against all test organism with the exception of *P. aeruginosa*, S. *feacalis* and *C. albicans*. Minimum inhibitory concentration (MIC) result revealed that a concentration of 10 mg/mL of hexane and methanol inhibited the growth of all test organisms. Also, a concentration of 5 mg/mL of ethyl acetate and dichloromethane inhibited the growth of all test organisms with the exception of *VRE*. The dichloromethane and ethyl acetate extracts also showed a bactericidal and fungicidal effect at 10 mg/mL, while the rest of the extract showed MIC, MBC and MFC ranging from 10 to 40 mg/mL. The ethyl acetate extract exhibited the highest activity against *B. subtilis* with ZI of 34 mm, MIC of 5mg/mL, MBC/MFCof 10mg/mL. This activity against *B. subtilis* was comparable with the standard antibiotic Sparfloxacine (34 mm) and even more active than Ciprofloxacine (31 mm) as shown in Table 4.3.

5.3.3 Antimicrobial Screening of Stem bark of *P. senegalense*

The antimicrobial sensitivity test of the stem bark extracts of *Psorospermum senegalense* showed moderate to good activity. The Determination of zone of inhibition (ZI) showed inhibition ranging from 18-22 mm (hexane), 20-27 mm (dichloromethane), 25-31 mm (ethyl acetate) and 20-24 mm (methanol) against the entire test organisms. The ethyl acetate extract

had the highest zone of inhibition of 31 mm against *Bacillus subtilis*. The results of the minimum inhibitory concentration (MIC) showed that ethyl acetate fraction inhibited the growth of all test organisms at a low concentration of 7.5 mg/mL except *Pseudomonas aeruginosa* and *Candida krusei* which had MIC of 15 mg/mL. Higher MIC values were observed for dichloromethane (15 mg/mL), hexane and methanol fraction all showed MIC at 15 to 30 mg/mL. The microorganisms were bactericidal at a higher concentration; ethyl acetate (MBC/MFC; 15-30 mg/mL), dichloromethane (MBC/MFC; 30-60 mg/mL), methanol and hexane (MBC/MFC; 60 mg/mL). The high activity demonstrated by the ethyl acetate extract over the other extracts could be attributed to the presence of more secondary like flavonoids, alkaloids and glycosides metabolites present in the ethyl acetate extract.

5.4 Antituberculosis Screening of the Leaves of *H. insignis*, *C. capitatum* and stem bark of *P. senegalense*

The antituberculosis activity of the crude extracts of the three plants parts revealed that the plants had varying degree of activity against *mycobacterium bovis.* The dichloromethane fraction of *Heeria insignis* and *Clerodendrum capitatum* both showed the highest activity with MIC of 0.675 mg/Ml, followed by ethyl acetate extract (1.25mg/ml) of *H.insignis*, hexane and methanol extracts were not active. The ethyl acetate and methanol extracts of *C. capitatum* inhibited the growth of *Mycobacterium bovis* at MIC 2.5 mg/mL, no activity was recorded for the hexane fraction. Extracts from *Psorospermum senegalense* showed the least activity out of the three plants with MIC of 2.5 mg/ml only in the ethyl acetate extract. Results are presented in Table 4.6.

5.5 Isolation, Purification and Characterization of Isolates

Silica gel column separation of the most active extracts followed by preparative thin layer chromatography led to the isolation of 6 compounds coded C1 and C2 from dichloromethane

extract of *C. capitatum*, H1, H3 and H4 from dichloromethane extract of *H. insignis* and P2 from the ethyl acetate extract of *P. senegalense.*

5.5.1 Isolation, Purification and Characterisation of Isolates from *C. capitatum*

5.5.2 Isolation and Characterisation of C1

Compound C1 was isolated as white crystal. It gave a positive result in the test for steroids (Silva *et al.,* 1998). Its ^{1}H-NMR spectrum revealed three regions typical of the steroidal nucleus at 0.5 ppm – 2.5 ppm representing overlapping methyl, methylene and methine protons, an oxymethine proton at 3.5ppm assignable to the position 3 of steroidal nucleus and a single unsaturated proton signal at 5.22 ppm. The ^{13}C NMR spectrum of compound C1 revealed a total of 29 carbon signals. The signal between 11.8 ppm and 56.7 ppm are typical of the region of overlapping methyl, methylene and methine carbon atoms, an oxymethine carbon signal at 71.8 typical of position 3 of 3-hydroxylanost-7-en-29-carboxylic acid, and finally the unsaturated carbon signals at 121.7 and 140.8 ppm were assignable to a two carbon olefinic system. The signal at δ_H 178.1 ppm indicated the presence of a carboxyl group in the compound. The DEPT spectrum revealed a total of 6 methyl, 10 methylene, 8 methine and from these 5 quartenary carbons where established from the decoupled ^{13}C spectrum.These NMR data are very similar to the data reported in the literature for 3-hydroxylanost-7-en-29-carboxylic acid (Ezuruike *et al*., 2015).Therefore, C1 was assigned to be 3-hydroxylanost-7-en-29-carboxylic acid.

5.5.3 Isolation and Characterisation of C2

Compound (C2) was isolated as a white solid with melting point of 255 - 256°C. The ^{13}C NMR spectrum indicated a total of 30 carbon atoms, suggesting a triterpenoidal nucleus. The ^{1}H NMR spectrum showed six methyl signals at δ_H 1.65, 0.99, 0.97, 0.96, 0.80 and 0.75 ppm. A doublet of doublets at δ_H 3.18 ppm characteristic of an α-oriented proton at C-3.Doublets

for geminal protons at δ_H 4.70 and 4.59 ppm, along with the methyl signal at δ_H 1.65 ppm, suggested that compound C2 was a lupane-type triterpenoid. A pair of oxymethylene doublets at δ_H 3.75 and 3.23 ppm, instead of a seventh methyl singlet around δ 0.8 ppm, indicated the presence of a second hydroxyl proton in the molecule. The ^{13}C NMR spectrum further suggested compound C2 as a lupane-type triterpene derivative. A total of a total of 29 carbon signals was observed from the spectrum. The characteristic pair of sp^2 hybridized carbon atoms comprising the double bond of lupeol was observed at δ 150.9 and 109.3 ppm. Oxygenated carbon shifts were observed at δ 79.1 and 60.1 ppm, respectively. The DEPT Spectra revealed a total of 6 methyl, 12 methylene and 6 methine. Spectra data revealed a lupane-type triterpenoidal nucleus with two hydroxyl groups at C-3 and C-28. Consequently, after comparing these NMR data with data in the literature (Himanshu *et al.*, 2013, Tjjani *et al*. 2012), the compound was assigned to be a known structure 20(29)-lupene-3,28-diol, more commonly known as betulin.

5.5.4 Isolation, and Characterisation of Isolates from *H. insignis*

5.5.5 Isolation and Characterisation of H1

Compound (H1) was isolated as a white amorphous solid with melting point of 255 - 256°C.. ^{13}C NMR spectrum indicated a total number of thirty carbon atoms, which indicates a possible triterpenoidal nucleus The ^{1}H NMR spectrum showed signal at δ 1.65, 0.99, 0.97, 0.96, 0.80 and 0.75 ppm which are similar to the methyl groups attached to a triterpenoidal nucleus. A doublet of doublets was observed at δ 3.18 ppm, characteristic of an α-oriented hydrogen at C-3 of a 3 β-hydroxy triterpene. Doublets for geminal protons at δ 4.60 and 4.55 ppm along with the methyl group at δ 1.65 ppm suggest that **H1** was likely a lupeol-type triterpene derivative. Another pair of doublets at δ 3.70 and 3.20 ppm, instead of a seventh methyl singlet around δ 0.8 ppm indicated the presence of a second hydroxyl proton in the molecule. The ^{13}C NMR spectrum further suggested **H1** as a lupeol-type triterpene derivative.

A total of 29 carbon signals were observed in the spectrum. The characteristic pair of sp^2 hybridized carbons atoms typical of the double bond of lupeol was observed at δ 150.9 and 109.3 ppm . Oxygenated carbon shifts were observed at δ 79.1 and 59.1 ppm, respectively. The DEPT spectrum revealed a total of 6 methyl, 12 methylene, 6 methine and 6 quartenary carbons atoms. Consequently, the compound was proposed to be the known structure, lup 20 (29)-en-3, diol, commonly known as betulin after comparing with available spectral literature

5.5.6 Isolation and Characterisation of H3

Compound **H3** was isolated as a white crystalline substance. It gave a positive result for the test for steroids (Silva *et al.,* 1998). Its ^{1}H-NMR spectrum revealed three regions typical of the steroidal nucleus at 0.5 ppm – 2.5 ppm representing overlapping methyl, methylene and methine protons; an oxymethine proton at 3.5 ppm assignable to the position 3 of steroidal nucleus and a single unsaturated proton signal at 5.4 ppm. The ^{13}C NMR spectrum of H3 revealed a total of 29 carbon signals, 7 of which were methyl signals, 10 were methylene carbon signals, 8 were methine and there were 4 quaternary signals, these data is typical of 3-hydroxy-7-lanostene (Wilson *et al.,* 1996). The signals between 11.8 ppm and 56.7 ppm represent a region of overlapping methyl, methylene and methine carbon atoms, an oxymethine signal at 71.8 typical of position 3 of 3-hydroxy-7-lanostene, and finally the unsaturated carbon signals at 121.7 and 140.8 ppm were assigned to a two carbon olefinic system. These NMR data are very similar to the data for 3-hydroxy-7-lanostene and a comparison with data reported for 3-hydroxy-7-lanostene showed good agreement (Wilson *et al*., 1996). Therefore, the structure of H3 was proposed to be 3-hydroxy-7-lanostene.

5.5.7 Isolation of H4

Compound **H4** was isolated as a white powdery substance, **H4** is yet to be identified, however phenolic fatty ester is suspected.

5.5.8 Isolation, and Characterisation of Isolates from *P. senegalense*

5.5.9 Isolation and Characterisation of P2

Compound **P2** was isolated as a white amorphous solid with melting point of 185 -187°C.It gave positive result to the Salkowski's test for steroid/triterpenes. Its ^{1}H-NMR spectrum revealed three regions typical of the triterpenoidal nucleus at 0.5 ppm to 2.5 ppm representing overlapping methyl, methylene and methine protons, an oxymethine proton at 3.2 ppm and a single unsaturated proton signal at 5.22 ppm were observed. The ^{13}C NMR spectrum of P2 revealed a total of 30 carbon signals, 8 of which were methyl signals, 9 were methylene carbon signals, 7 were methine and there were 6 quaternary signals. These data are typical of α-amyrin (Joyce *et al.,* 2013, Rajnish *et al.,* 2015, Niaz, 2013). The signal between 10.9 ppm and 55.6 ppm represent a region of overlapping methyl, methylene and methine carbon atoms, an oxymethine carbon signal at 79.0 typical of position C-3 of α-amyrin and finally the unsaturated carbon signals at δ125.8 and δ138.0 ppm were assignable to two carbon olefinic system. This NMR data are very similar to the data for α-amyrin and a comparison with data reported for α-amyrin showed good agreement. Therefore, the structure of P2 was considered to be α-amyrin.

The compound isolated from the plants are majorly steroid and triterpenes and these classes of compounds have been reported to demonstrate antibacterial properties (Collins and Charles, 1987). Triterpenes are a class of chemical compounds composed of three terpenes or six isoprene units with the molecular formula $C_{30}H_{48}$ with the most important example being squalene as it forms the basis of almost all steroids. Triterpenes are widely distributed in edible and medicinal plants and are an integral part of the human diet. They have been

evaluated for use in drugs, cosmetics and healthcare products. Screening plant material has identified plants as promising and highly available sources of triterpenes (Szakiel *et al*., 2012).

Steroids are organic compounds that contain characteristic arrangement of four cycloalkane rings joined to one another. Examples of steroids include the dietary lipid cholesterol, the sex hormones, estradiol and testosterone (Desmond and Gribaldo, 2009). The steroids are among the most widely used class of drugs and their role in the therapy of pulmonary, inflammatory, dermatological and oncological diseases have been well documented (Grover *et al.,* 2007). Isolated steroids have been reported to possess pharmacological activities such as antifungal, antibacterial and antioxidant activity (Govindappa *et al.,* 2011). All the extracts of all the plants showed the presence of steroids and triterpenes in the phytochemical screening and inhibited the growth of organisms like *S. aureus, B.subtillis*, and *S. typhii*, this observed activity might be attributed to the presence of steroids and triterpenes in these extracts.

H3 had the highest activity against *Mycobacteriunm bovis* with MIC of 125 µg/ml, all the other compounds isolated also showed activity at MIC 250 µg/ml. The sensitivity of the isolated compounds against *Mycobacterium bovis* and the other microorganisms indicates that the chemical compounds can further be developed for the fight against these microorganisms and the use of the plant in the treatment of tuberculosis and other infectious diseases is justified. The sensitivity of *Mycobacterium bovis* to the isolated compound implies that the compounds are potential sources of antituberculosis drugs.

CHAPTER SIX

6.0 Summary, Conclusion and Recommendation

6.1 Summary

The fresh leaves of *Clerodendrum capitatum, Heeria insignis* and stem bark of *Psorospermum senegalense* were collected from Zaria, Kaduna State, Nigeria in the year 2012 during rainy season, pulverized to powder using wooden mortar and pestle.

Cold extraction maceration using hexane, dichloromethane, ethyl acetate and methanol were used to extract the phytocompounds in the plants. Extracts obtained were concentrated under reduced pressure and dried at room temperature. The yield of extracts were highest with more polar solvents (ethyl acetate and methanol) indicating higher polar metabolites' quantity.

Preliminary phytochemical screening of the hexane, dichloromethane, ethyl acetate and methanol extracts of the leaves of *Heeria insignis* and *Clerodendrum capitatum* and stem bark of *Psorospermum senegalense* revealed the presence of carbohydrate, glycosides, cardiac glycoside, saponins, steroids and triterpenes, flavonoids, and alkaloids, tannins were only present in the extracts of *Psorospermum senegalense* while anthraquinones were present only in the methanol extract of *Psorospermum senegalense.*

Antimicrobial studies of the crude extracts and isolated compounds showed broad spectrum antimicrobial activity against tested micro organisms.

Silica gel column purification of the dichloromethane extracts of *H. insignis, C.capitatum* and ethyl acetate extracts of *P.senegalense* followed by preparative thin layer chromatography led to the isolation of six compounds which were separately considered to be 3-hydroxylanost-7-en-29-carboxylic acid and Betulin from dichloromethane fraction of *C. capitatum;* Betulin, and 3-hydroxy-7-lanostene and a yet to be identified compound (H4) from dichloromethane

fraction of *H. insignis* and α-Amyrin from the ethyl acetate fraction of *P. senegalense.* The isolated compounds also demonstrated good activity against *Mycobacterium bovis* (one of the bacteria that causes tuberculosis).

6.2 Conclusion

The results from this studies indicate that the plant extracts offer significant potential for the development of novel anti tuberculosis therapies and treatment of several diseases caused by tested microorganisms. *Heeria insignis* was found to be the most active of the three plants on the antimicrobial and antituberculosis activity. Also H3 isolated from *Heeria insignis* was found to be the most active on *Mycobacterium bovis.* Findings from this research validates the claim of using *Heeria insignis (*DEL), *Psorospermum senegalense* (SPACH) and *Clerodendrum capitatum* (WILD) for the treatment of tuberculosis and other infectious diseases. To the best of our knowledge there is no reported work on the anti-tuberculosis activity of these plants prior to this work. Although the compounds have been isolated from different plants previously and the antimicrobial and antituberculosis activity of betulin have been reported, this is the first report of their isolation from *Heeria insignis (*DEL), *Psorospermum senegalense* (SPACH) and *Clerodendrum capitatum.* Therfore, this work will add to the global database of natural products.

6.3 Recommendations

This study encompasses only the leaves of *H. insignis* and *C.capitatum* and stem bark of *P. senegalense* since one of the aims is to validate the use of the plant for the treatment of tuberculosis. The following are therefore recommended:

1. other parts of the plants could be screened for their anituberculosis activities so as to also justify the general use of the plant as antitubercular plant,

2. other pharmacological studies could be conducted on the crude and various fractions from the leaves and stem bark to explore the efficacy of the plant as medicinal plant.
3. structural modification of the isolated compounds for enhanced anti tuberculosis activity could be carried out.

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