BIO ACTIVITY-GUIDED ISOLATION OF THE ANTICONVULSANT PRINCIPLES OF METHANOLIC EXTRACT OF THE STEM-BARK OF *LONCHOCARPUS SER1CEUS* (POIR) FAMILY PAPILIONACEAE

BY

ABDULLAH1, SAKYNAH MUSA

DEPARTMENT OF PHARMACEUTICAL AND MEDICINAL CHEMISTRY

AHMADU BELLO UNIVERSITY, ZARIA-NIGERIA

SEPTEMBER, 2012

BIOACTIVITY-GUIDED ISOLATION OF THE ANTICONVULSANT PRINCIPLES OF METHANOLIC EXTRACT OF THE STEM-BARK OF *LONCHOCARPUS SERICEUS* (POIR) FAMILY PAPILIONACEAE

BY

ABDULLAHI, SAKYNAH MUSA, B. Pharm. (ABU, 2008)
M.Sc/Pharm.Sci/07505/2009-2010

A THESIS SUBMITTED TO THE
SCHOOL OF POSTGRADUATE STUDIES
AHMADU BELLO UNIVERSITY, ZARIA-NIGERIA

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF
MASTER OF SCIENCE DEGREE
IN
PHARMACEUTICAL AND MEDICINAL CHEMISTRY

DEPARTMENT OF PHARMACEUTICAL AND MEDICINAL CHEMISTRY
AHMADU BELLO UNIVERSITY, ZARIA-NIGERIA

SEPTEMBER, 2012

DECLARATION

I declare that the work in this thesis entitled: "**Bioactivity-Guided Isolation of the Anticonvulsant Principles of Methanolic Extract of the Stem-Bark of *Lonchocarpus sericeus* (poir) Family Papilionaceae**" has been performed by me in Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, Zaria, under the supervision of Dr M. I. Sule and Dr A. M. Musa.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at any university.

Abdullahi, Sakynah Musa

02/10/12

Date

CERTIFICATION

This thesis entitled: **BIOACTIVITY-GUIDED ISOLATION OF THE ANTICONVULSANT PRINCIPLES OF METHANOLIC EXTRACT OF THE STEM-BARK OF *LONCHOCARPUS SERICEUS* (POIR) FAMILY PAPILIONACEAE** by Abdullahi, Sakynah Musa meets the regulations governing the award of a Master of Science degree of Ahmadu Bello University, Zaria, and is approved for its contributions to knowledge and literary presentation.

Dept of Pharm & Clinical Pharmacy
Ahmadu Bello University
Zaria - NIGERIA

Dr M. I. Sule
Chairman, Supervisory Committee — 9/10/2012 Date

Dr A. M. Musa
Member, Supervisory Committee — 5/10/2012 Date

Dr U. U. Pateh
Head of Department — 09/10/12 Date

Prof. Osadebe O. Patience
External Examiner — 19/09/12 Date

Prof. A. A. Joshua
Dean, SPGS — Date

ACKNOWLEDGMENT

I wish to express my greatest indebtedness to my major supervisor Dr M. I. Sule not only for his effort to secure admission for me but also for his persistent support and encouragement during my course work. I also owe a debt of gratitude to my second supervisor Dr A. M. Musa not only for being with me in the laboratory throughout the course of this work, but, also for his wealth of skills and experience which he freely made available to me at the period of dire need. I am grateful to Dr Musa Ismail of Usmanu Danfodiyo University, Sokoto and Mr Dilip Jagjivan of School of Chemistry, University of KwaZulu Natal Durban for running the spectra. I also wish to express my gratitude and appreciation to Dr Abdullahi Yaro of Bayero University, Kano for assisting me with the pharmacological studies.

I want to thank the management of Ahmadu Bello University Zaria for waiving part of the tuition fees of my registration. I also want to acknowledge Ahmadu Bello University Mac Arthur Foundation for funding part of this research work.

I want to acknowledge the cooperation and assistance of the entire staff of the Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University Zaria, especially the Head Dr U. U. Pateh and our lecturers for the guidance and knowledge they imparted to us generously.

I would not forget to thank my senior colleagues, friends and course mates for the interactive sessions and the information we shared during the course work. I am also thankful to Dr Nafiu Abdu of the Department of Soil Sciences for his concern and guidance.

I remain thankful to my darling husband Kabir Abubakar (Asgar) who did not only take the pains of typing the manuscript of this thesis but considered the completion of the work within the stipulated time as "A TASK THAT MUST BE DONE". I am appreciative of your love and care.

Finally, I wish to express my sincere gratitude to my beloved parents Dr and Mrs. A. M. Abu-Abdissamad for the limitless love they showered upon me.

To my children Rufaydah and Suhayl, I have no access to English words that can express my love and gratitude to you; so, I only say "*Jazakumul'lahu khairan*".

DEDICATION

To my beloved parents Dr and Mrs. A. M. Abu-Abdissamad

ABSTRACT

The chloroform soluble fraction of methanol extract of stem-bark of *Lonchocarpus sericeus,* a plant used in ethnomedicine for the treatment of epilepsy and backache among other ailments, was subjected to phytochemical and anticonvulsant screening. The phytochemical techniques employed were column chromatography, gel filtration and preparative thin layer chromatography. The anticonvulsant activity was stud'.ed using maximal electroshock test, pentylenetetrazole and 4-aminopyridine induced seizures. Preliminary phytochemical screening of the crude extract revealed the presence of flavonoids, phenolic compounds, saponins, steroids and tritcrpenes while that of the chloroform soluble fraction re\ealed steroids, tritcrpenoids, phenolic compound and flavonoids. Extensive chromatographic separation of the chloroform soluble fraction using silica gel column chromatography, gel filtration and preparative thin layer chromatography led to the isolation of a tiiteipcnoid, lup-20(29)-ene-3(}-ol (Lupeol) and a chalcone, 2',4'-dihydroxy-3'-(4-hydroxy-4-methy[l] pertenyl)-cha!cone. Their structures were determined by spectral analysis including ID and 2D NMR. In the pharmacological screening, 150 mg/kg of the extract protected 50% of test animals against pentylenetetrazole induced seizure in mice whereas sodium valproate a standard anti-epileptic drug offered 100% protection. In 4-aminopyridine induced seizure model, the fraction at doses of 75 and 300 mg/kg protected 33.3% of the animals against mortality, but phenobarbitone (20 mg/kg) protected 100%. There was no activity against maximal electroshock convulsion. The mean l&hal dose was calculated as 1131.3 mg/kg. The results suggest that the chloroform fraction *of Lonchocarpus sericeus* stem-bark extract possess psychoactive compound that may be useful in the management of petit mal epilepsy.

TABLE OF CONTENT

Title Page.....

Declaration.....i

Certification.....ii

Acknowledgment.....iii

Dedication.....v

Abstract.....vi

Table of Content.....vii

List of Tables.....xiii

List of Figures.....xiv

List of Plates.....xvi

Chapter One: Introduction.....1

General Introduction.....1

Brief History of Drug Discovery.....3

Epilepsy.....11

Mechanisms of Seizure.....12

Status Epilepticus and Other Convulsive Emergencies.....12

Statement of Research Problem.....13

Justification of the Study.....13

Justification for Plant selection.....14

Objectives of the Study.....15

Research Hypothesis.....15

Chapter Two: Literature Review.....16

Plant Description.....16

Scientific Classification.....16

Ethnomedical Uses of *Lonchocarpus sericeus*.....17

Phytochemistry of *Lonchocarpus sericeus*.....17

Pharmacological Properties of *L. sericeus*.....18

Triterpenoids.....19

Chemical Tests for Triterpenoid.....21

Pharmacological Properties of Triterpenoids.....21

Spectral Properties of Triterpenoids.....22

UV and IR Spectra of Triterpenoids.....22

Proton NMR Spectra of Triterpenoids.....22

Flavonoids.....23

Chemical Test for Flavonoids.....25

Spectral Properties of Flavonoid.....25

UV and IR Spectra of Flavonoid.....25

Analysis of Flavonoid Structure by NMR.....26

NMR Spectroscopy.....26

^{13}C- NMR Spectroscopy.....26

Distortionless Enhancement by Polarisation Transfer (DEPT) Experiment.....26

Two Dimensional NMR Spectroscopy.....27

COSY Technique.....27

HSQC Technique.....27

HMBC Technique.....28

Chapter Three: Materials and Methods.....29

Materials.....29

Chemicals/Reagents.....29

Equipment.....29

Experimental Animals.....29

Plant Collection.....30

Methods.....30

Extraction and Fractionation.....30

Preliminary Phytochemical Screening.....31

Test for Steroids and Triterpenes.....31

Test for Flavonoids.....31

Test for Phenolic Compound.....32

Test for Saponins.....32

Column Chromatography.....32

Preparative Thin Layer Chromatography (PTLC).....33

Gel Filtration.....33

Anticonvulsant Studies.....34

Acute Toxicity Studies.....34

Pentylenetetrazole induced Seizure in Mice (PTZ).....35

4-Aminopyridine- Induced Seizure Test (4AP).....35

Maximal Electroshock Seizure Test in Chicks (MEST).....35

Chapter Four: Results.....37

Result of Column Chromatography.....39

Result of Phytochemical Screening.....40

Result of Preparative TLC.....41

TLC Profile of R_1.....42

Chemical Test on R_1.....42

UV NMR Spectral Analysis of R_1.....44

Proton NMR Spectral Analysis of R_1.....46

Carbon 13 NMR Spectral Analysis of R_1.....48

DEPT NMR Spectral Analysis of R_1.....50

HSQC Spectral Analysis of R_1.....52

COSY Spectral Analysis of R_1.....56

HMBC Spectral Analysis of R_1.....58

Comparison of Spectral data of R_1 with that obtained from the Literature.....61

TLC Profile of R_2.....63

Proton NMR Spectral Analysis of R_2.....65

Carbon 13 NMR Spectral Analysis of R_2.....69

DEPT NMR Spectral Analysis of R_2.....71

COSY Spectral Analysis of R_2.....74

HSQC Spectral Analysis of R_2.....77

HMBC Spectral Analysis of R_2.....82

Result of Acute Toxicity Studies.....87

Result of PTZ induced Seizure Test.....88

Result of 4-AP induced Mortality Test.....90

Result of MEST.....92

Chapter Five: Discussions.....94

Chapter Six: Summary Conclusion and Recommendations.....101

Summary.....101

Conclusion.....101

Recommendations.....101

Bibliography.....103

Dept of Pharmaceutical Chemistry
Faculty of Pharmaceutical Sciences
Ahmadu Bello University
ZARIA - NIGERIA

List of Tables

Table 4.1: Result of Preliminary Phytochemical Screening of CF.....38

Table 4.2 Result of Column Chromatography of CF.....40

Table 4.3 TLC Profile of R_1.....43

Table 4.4: Result of HSQC and DEPT Experiment of R_1.....55

Table 4.5: Spectral Data of R_1 Compared with that Obtained from the Literature.....62

Table 4.6: Result of HSQC and DEPT Experiment of R_2.....81

Table 4.7: Result of Major HMBC Correlations of R_2.....86

Table 4.8: Effect of CF against Pentylenetetrazol-Induced Seizure in Mice.....89

Table 4.9: Effect of CF against subcutaneous 4-Aminopyridine-induced seizure in mice.....91

Table 4.10: Effect of CF against Maximal Electroshock seizure in chicks.....93

Faculty of ...
Ahmadu Bello University
ZARIA - NIGERIA

List of Figures

Figure 4.1: UV spectrum of R_1.....45

Figure 4.2: Proton NMR spectrum of R_1.....47

Figure 4.3: Carbon-13 spectrum of R_1.....49

Figure 4.4: DEPT spectrum of R_1.....51

Figure 4.5: HSQC spectrum of R_1.....53

Figure 4.6: HSQC spectrum of R_1.....54

Figure 4.7: COSY spectrum of R_1.....57

Figure 4.8: HMBC spectrum of R_1.....59

Figure 4.9: HMBC spectrum of R_1 (Expansion).....60

Figure 4.10: Proton NMR of R_2.....66

Figure 4.11: Proton NMR of R_2 (Aliphatic region).....67

Figure 4.12: Proton NMR of R_2 (Aromatic region).....68

Figure 4.13: ^{13}C NMR spectrum of R_2.....70

Figure 4.14: DEPT experiment of R_2.....72

Figure 4.15: DEPT experiment of R_2 (Expansion).....73

Figure 4.16: COSY spectrum of R_2.....75

Figure 4.17: COSY spectrum of R_2 (Expansion).....76

Figure 4.18: HSQC spectrum of R_2.....78

Figure 4.19: HSQC spectrum of R_2 (Aliphatic region)..79

Figure 4.20: HSQC spectrum of R_2 (Aromatic region)...80

Figure 4.21: HMBC spectrum of R_2.....83

Figure 4.22: HMBC spectrum of R_2 (Aliphatic region).....84

Figure 4.23: HMBC spectrum of R_2 (Aromatic region).....85

List of plates

Plate 4.1: TLC profile of R_2.....64

CHAPTER O NE

INTRODUCT ION

1.0 General Introduction

The use of natural products with healing properties is as old as human civilization, and for a long time minerals, animals and plant products were the main sources of drugs (Rates, 2001). The World Health Organization (WHO) defines traditional medicine as practices, knowledge and belief systems which use minerals, plants and animal based remedies, spiritual therapies and exercise to prevent, treat and maintain well-being. The WHO estimated that 4 billion people (about 80% of the world population) depend on traditional medicine mostly herbal remedies for their primary health care needs (WHO, 2008). Herbal medicine is a major component of all indigenous peoples' traditional medicine and a common element in Ayurvedic, homeopathic, naturopathic, traditional oriental and Native American Indian medicine.

The African continent have a long history of the use of plants and in some African countries, up to 90% of the population rely on medicinal plants as a source of drugs (Hostettmann *et al.*, 1996).

The reason for relying on traditional medicine is due to economic reasons, accessibility and belief that natural products are more acceptable to the body and as such have fewer side effects than synthetic drugs (Sofowora, 1993; Kafura, 1999). Traditional medicine is still widely acceptable despite the fact that it is limited by lack of precise diagnosis, hygiene, standardization, and ethics in dosage and sometimes exaggerated claims which cannot be proven scientifically (Elujoba, 1999; Shok, 1999).

Recently, there is a growing interest in the use of natural products and folk medicine even in the developed countries. In the United States, 158 million adults use complementary and alternative medicine (CAM) and according to the United States of America Commission for CAM, Seventeen billion US dollars ($17b) was spent on traditional remedies in 2000 while in the United Kingdom, the annual expenditure in CAM is two hundred and thirty million dollars ($230 million). In Nigeria, Ghana, Mali and Zambia the first line treatment for 60% of children with fever, resulting from malaria is the use of herbal medicine at home (WHO, 2003). WHO notes that of 119 plants derived pharmaceuticals, about 74% are used in modern medicine in ways that correlated directly with their traditional uses as plant medicines by native cultures.

An estimated 25% of prescription drugs and 11% of drugs considered essential by the WHO are derived from plants and a large number of synthetic drugs are obtained from precursor compounds originating from plants (Rates, 2001). Of the top 150 proprietary drugs from the National prescription, 57% contain at least one major active compound of biological origin, and 17% are unmodified natural products from plants (Grifarn *et al.*, 1997). It is thus apparent that plants represent a treasure of structural diverse potential bioactive organic molecule (Kinghorn, 1992). Higher plant as potential sources of new drugs is still highly unexploited. Among the known species, only about 10% has been investigated phytochemically and the fraction subjected to biological or pharmacological screening is even smaller (Hostettmann, *et al.*, 1996).

Phytochemical examinations of plant constituents have been made possible by improved methods of extraction using supercritical carbon dioxide ($SC0_2$), separation and isolation of active constituents using modern chromatographic techniques, Computer Assisted Structure Elucidation (CASE), one dimensional proton Nuclear Magnetic Resonance (ID ^1H – NMR) technique, Carbon – 13 Nuclear Magnetic Resonance (ID ^{13}C NMR) technique, Distortionless

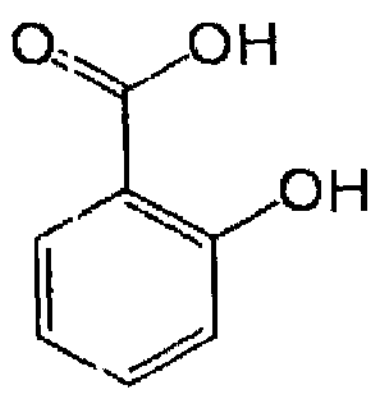
Enhancement by Polarisation Transfer (DEPT) and two dimensional proton and Carbon – 13 Nuclear Magnetic Resonance technique such as H –H COSY (proton – proton correlation spectroscopy). H-C COSY (proton - carbon correlation). HSMQC (Heteronuclear Single and Multiple Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Correlation) etc are used to determine chemical structures of the isolate (Venkat and Kathandaraman, 1998; Blinov *et al.*, 2003).

1.1 Brief History of Drug Discovery

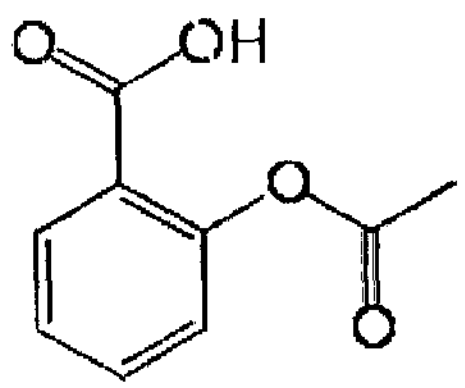
Therapeutic agents have played a major role in increasing average life expectancy worldwide in the last century. However, while many of the drugs in use in the last fifty years or more have been of synthetic origin, the pharmacopoeias prior to that period were of natural origin (Pasteur, 1785).

The medicinal value of plants had been recognized by almost every society on this planet. In the 19th and earlier centuries, natural product extracts, particularly those derived from botanical species, provided the main source of folk medicines. However, in the latter part of the 19th century, biologically active organic molecules began to be isolated in relatively pure form for medicinal use. For example, Salicylic acid (I), the precursor of Aspirin (II) was isolated in 1874 from willow bark. Various more potent pain killers, such as Morphine (III) and codeine (IV), were isolated from the Opium poppy (Olaniyi, 2005). The antimalarial agent quinine (V) was isolated from Cinchona bark. The leaves of the purple fox glove plant provided an excellent source of digitalis that was purified for use against heart disease. Although, synthesis of the first synthetic pharmaceutical drug, Aspirin (II) occurred in the latter half of the 19th century, it was not until the early 1900s that the recognition of aspirin as a universal pain reliever was realized and this discovery spawned the era of therapeutic agent (Olaniyi, 2005).

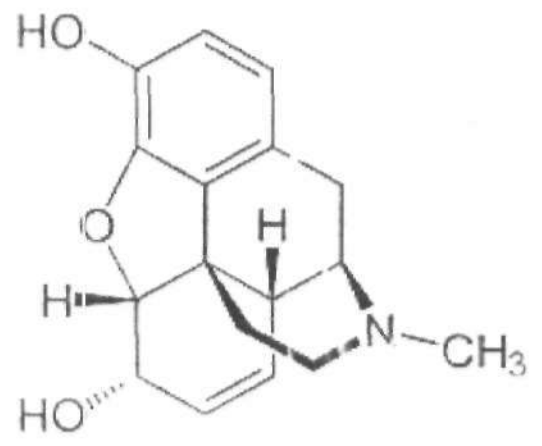
With time, more therapeutic agents were synthesized as analogues base on the structure of isolated natural product. For example, pethidine (VI) and pentazocine (VII) were synthesized based on morphine structure. Based on quinine structure, many classes of antimalarials were synthesized such as 4-aminoquinolines (chloroquine) (VIII) and 8-aminoquinolines (primaquine) (IX) (Olaniyi, 2005).



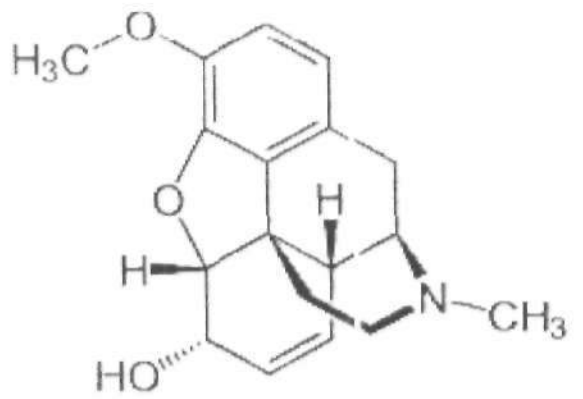
I



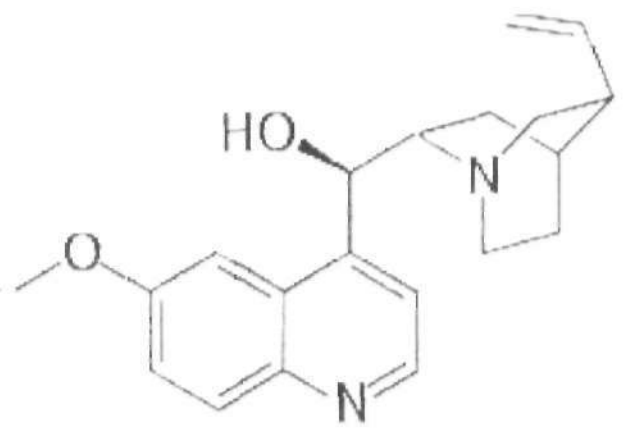
II



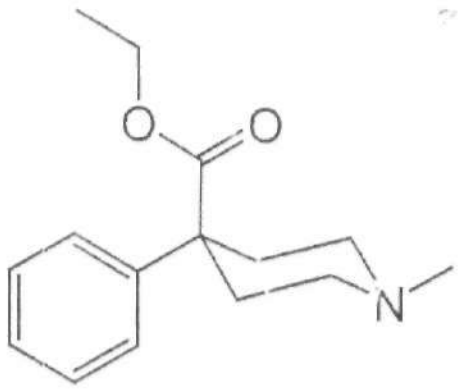
III



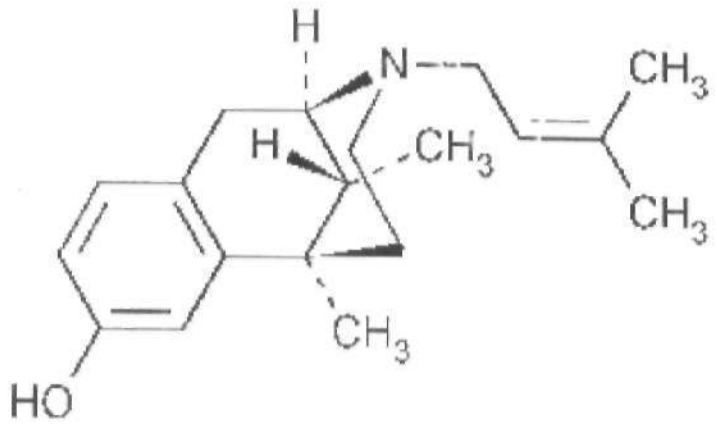
IV



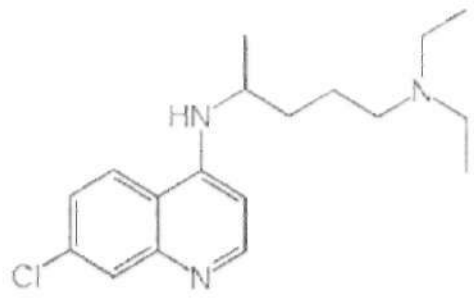
V



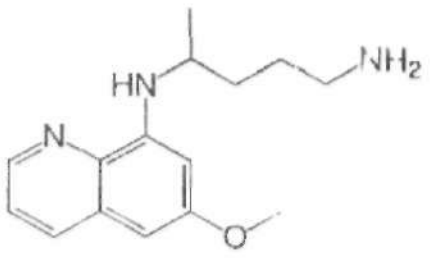
VI



VII

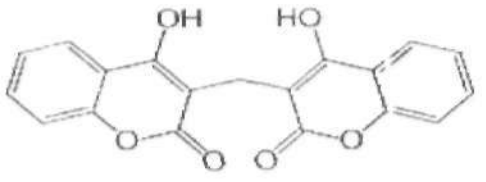


VIII

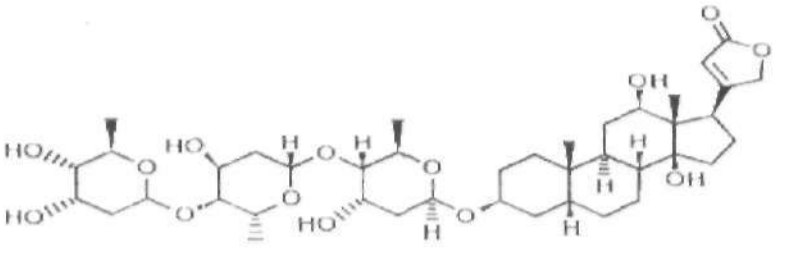


IX

Natural products have been and still are an inexhaustible source of drug leads as well as drugs. Lead compounds are still a *sine qua non* of drug design (Olaniyi, 2005). Lead compounds can be used directly as drug e.g development of anticoagulant dicoumarol (X) which acts by inhibiting the synthesis of prothrombin and other clotting factors such as vitamin k. This compound was isolated from the Sweet clover hay, *Melilotus officinalis* which had became moldy or spoiled. Nature still supplies the best drug for the treatment of heart disease. There is no synthetic drug capable of replacing digoxin (XI), a constituent of digitalis (Olaniyi, 2005).

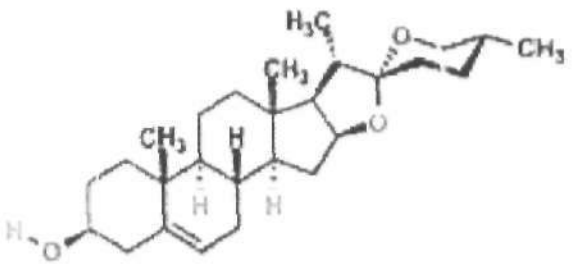


X

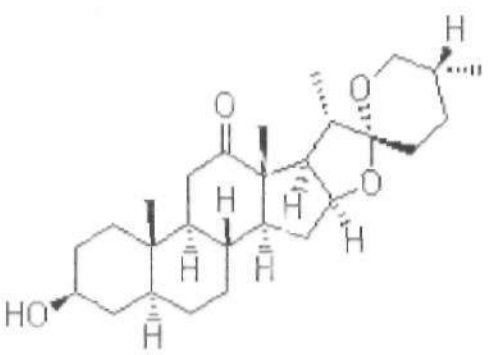


XI

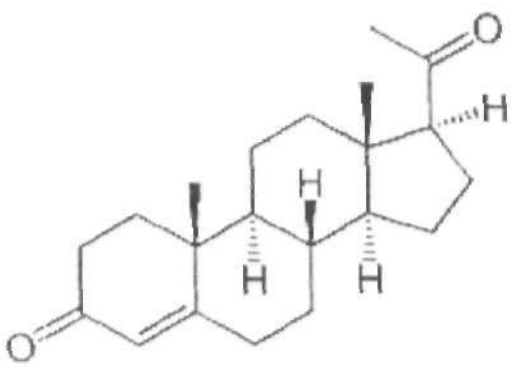
Drugs can also be semi synthesized from natural products used as leads e.g the plant steroids, diosgenin (XII) and hecogenin (XIII) remain key precursors for the synthesis of many modern contraceptive drugs such as progesterone (XIV) and its derivative norethindrone (XV) and other pharmaceuticals including Betamethasone (XVI) and beclomethasone (XVII) (Olaniyi, 2005).



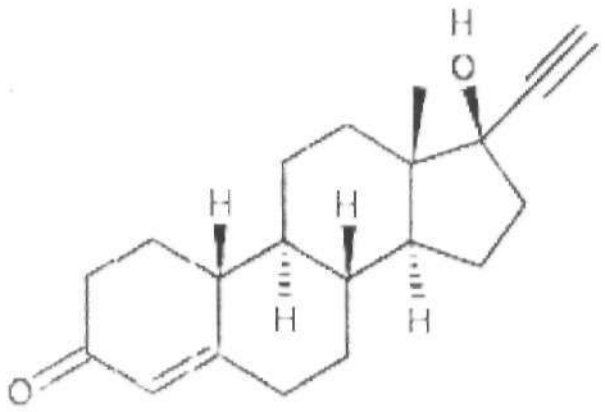
XII



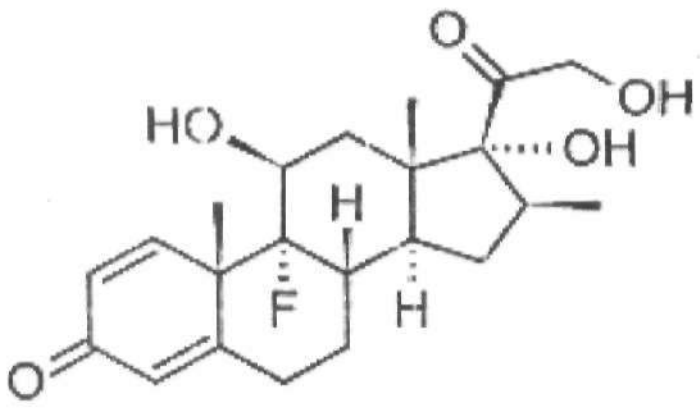
XIII



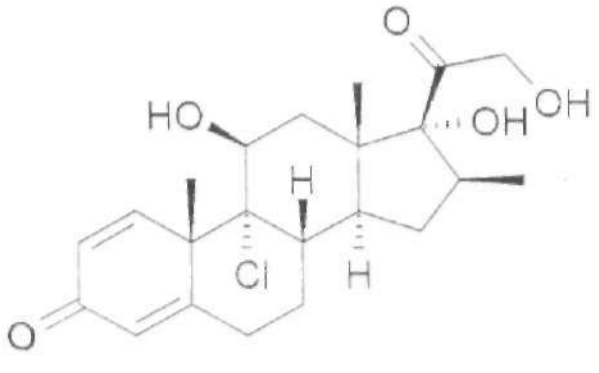
XIV



XV

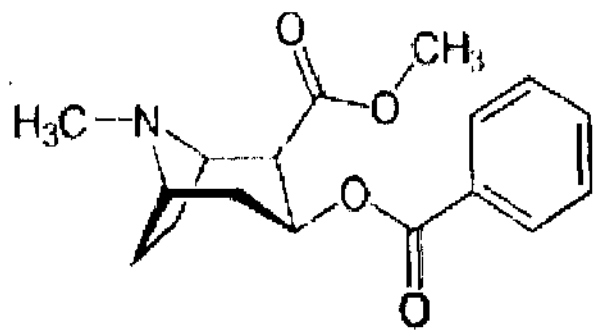


XVI

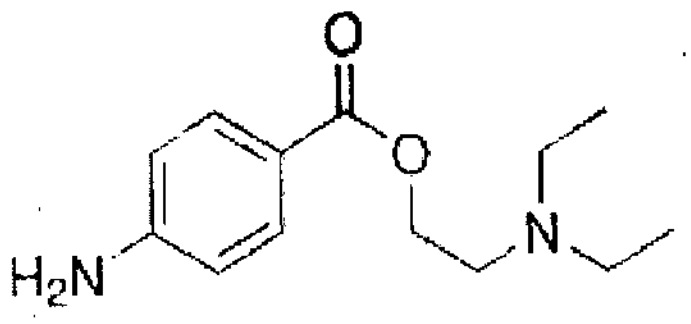


XVII

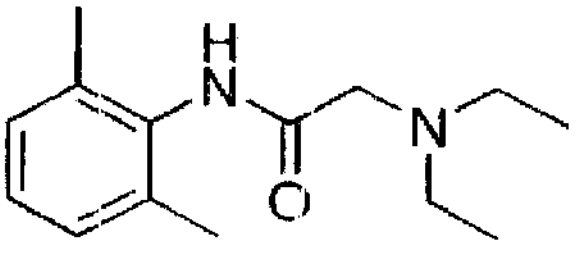
Natural products have also been utilized as chemical models or templates for the design and synthesis of many other important drugs (Exploitation of the lead) (Ganellin, 1993) e.g Cocaine (XVIII) from *Erythroxylon coca* leaves served as a lead for more potent and less toxic local anesthetics such as procaine (XIX) and Lidocaine (XX). These compounds are devoid of unwanted effects of cocaine such as addiction, tremor, urinary incontinence and agitation.



XVIII



XIX



XX

1.2 Epilepsy

The term epilepsy refers to a disorder of brain function characterized by the periodic and unpredictable occurrence of seizure. Seizure, on the other hand, refers to a transient alteration of behavior due to the disordered, synchronous and rhythmic firing of populations of brain neurons (McNamara, 2006).

Seizure can be non-epileptic when evoked in a normal brain by treatments such as electroshock or chemical convulsants or epileptic when occurring without evident provocation. Pharmacological agents in current use inhibit seizures, and thus are referred to as antiseizure drugs. Whether any of these agents prevent the development of epilepsy (epileptogenesis) is uncertain (McNamara, 2006).

The mechanisms of action of antiseizure drugs fall into three major categories. Drugs effective against the most common forms of epileptic seizures, partial and secondarily generalized tonic-clonic seizures, appear to work by one of two mechanisms. One to limit the sustained repetitive firing of neurons, an effect mediated by promoting the inactivated state of voltage activated Na^+ channels. A second mechanism appears to involve enhanced γ-aminobutyric acid (GABA) – mediated synaptic inhibition; an effect mediated either by pre synaptic or post synaptic action.

Drugs effective against absence seizure, a less common form of epileptic seizure, limit activation of a particular voltage-activated Ca^{2+} channel known as the T-current (McNamara, 2006).

1.2.1 Mechanisms of Seizure

The pivotal role of synapses in mediating communication among neurons in the mammalian brain suggested that defective synaptic function might lead to a seizure. That is a reduction of inhibitory synaptic activity or enhancement of excitatory synaptic activity might be expected to trigger a seizure; pharmacological studies of seizures supported this notion. The neurotransmitters mediating the bulk of synaptic transmission in the mammalian brain are amino acids, with GABA and glutamate being the principal inhibitory and excitatory neurotransmitters respectively (McNamara, 2006).

Pharmacological studies disclose that antagonists of the GABA receptor or agonists of different glutamate-receptor subtypes (NMDA, AMPA or Kainic acid) trigger seizures in experimental animals *in vivo*. Conversely, pharmacological agents that enhance GABA-mediated synaptic inhibition suppress seizures in diverse models. Glutamate-receptor antagonists also inhibit seizures in diverse models, including seizures evoked by electroshock and chemical convulsants such as pentylenetetrazol (PTZ) (McNamara, 2006).

1.2.2 Status Epilepticus and Other Convulsive Emergencies

Status epilepticus is a repeated attack of epilepsy occurring one after another. It is considered by neurologist as a neurological emergency. Mortality for adults approximates 20% (Lowenstein and Alldredge, 1998). The goal of treatment is rapid termination of behavioural and electrical seizure activity. The longer the episode of status epilepticus is untreated, the more difficult it is to control and the greater the risk of permanent brain damage (McNamara, 2006).

Two to four percent of children experience a convulsion associated with a febrile illness. From 25% to 33% of these children will have another febrile convulsion. A complicated febrile convulsion is one that the febrile seizure lasted more than 15 minutes and/or followed by a second seizure in the same day. A concern regarding an increased risk of developing epilepsy led many physicians to prescribe antiseizure drugs prophylactically after a febrile seizure (McNamara, 2006).

1.3 Statement of Research Problem

The epilepsies are common. Epilepsy is the second most common chronic neurological condition seen by neurologists. It is estimated that there are 5.5 million persons with epilepsy in India, two million in the USA and three million in UK. Although data on epilepsy in Nigeria are scanty, one quarter of the 40 million individuals estimated to have epilepsy in developing world live in Africa (Onwuekwe, *et al.*, 2009).

While medications and other treatments help many people of all ages who live with epilepsy, more than a million people continue to have seizures that can severely limit their school achievement, employment prospects and participation in all of life's experience (Epilepsy Foundation of America, 2010).

1.4 Justification of the Study

The entire currently available antiepileptics in clinical use are synthetic molecules and pharmacotherapy involving the use of these agents is symptomatic in that neither effective prophylaxis nor cure is available. Compliance with medication is a major problem because of the need for long term therapy together with unwanted effects that range in severity from minimal

impairment of the Central Nervous system (CNS) to death from Aplastic anaemia or hepatic failure (McNamara, 2006).

The active principles of many plant-derived drugs are secondary metabolites (Dobelis, 1993) thus basic phytochemical investigation of biologically active plant extract is very vital. This will go a long way to aid in discovery of lead that can be used for designing other drugs.

Nigeria, the fourth largest economy in Africa with an estimated $350 per capita annually, ranks near the bottom (158 out of 177 countries) in terms of per capita income with over half of the population living in poverty (PMNCH, 2007). This implies that very few people can afford orthodox medicine in treating their ailments. Thus, there is need to study plants with medicinal properties that can serve as alternative medicines for those that cannot afford orthodox medicine for treatment.

1.5 Justification for Plant Selection

Lonchocarpus sericeus is used in folk medicine for the treatment of several illnesses.

The ethnomedical use of the stem bark in the management of convulsion has been validated scientifically by Musa *et al.*, (2006).

Despite this, literature search in scientific data base revealed that no work has been reported yet on the isolation of any compound from the stem-bark of this plant. Therefore, this work is aimed at isolation of some bioactive constituents from the stem bark of the plant.

1.6 Objectives of the Study

The objective of this study is to carry out bioactivity-guided isolation of the anticonvulsant principles of methanolic extract of the stem-bark of *Lonchocarpus sericeus*.

1.7 Research Hypothesis

The stem bark of *Lonchocarpus sericeus* contains secondary metabolites with anticonvulsant activity.

CHAPTER TWO

LITERATURE REVIEW

2.1 Plant Description

Lonchocarpus sericeus is a shrub, or small tree that reaches up to 16m high. It is of dry deciduous, fringing and transition forest often near water. The plant flowers (with dense hanging racemes of purple flowers) mainly when leafless. It is frequently planted in villages as a shade tree and in Gardens and Cemeteries. The wood is a clear yellow, hard, very durable, taking a fine polish and resistant to water. The bark strips well and is a source of fibre. The flower has a marked smell of vanilla (Burkill, 1995).

2.1.1 Scientific Classification

Kingdom : Plantae

Tribe : Millettieae

Family : Papilionaceae

Genus : *Lonchocarpus*

Species : *sericeus*

Local Names

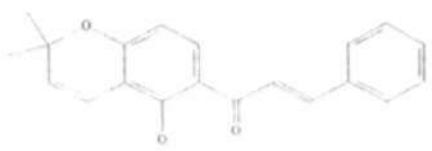
In English, the plant is known as Senegal Lilac or Cube root. In Hausa, it is called Farin Sansami, shunin biri, or furen 'yar sarki while in Igbo it is known as Njasi. In Yoruba it is popularly called Apapo.

2.1.2 Ethnomedical Uses of *Lonchocarpus sericeus*

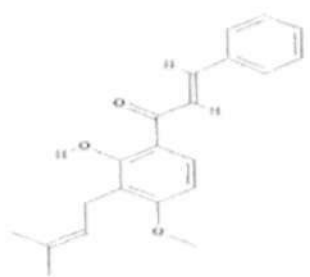
The main use of the bark is a stomachic and laxative for young children. It also has common application in treating convulsion and backache. It is often applied in lotion form on parasitic skin conditions and eruptions. It has been used against leprosy in Gambia. It is sometimes used locally and as a fish poison in South America. A root-decoction is used in Angola as a lotion for wounds and as an anti scorbutic. The leaves are used in Senegal to relieve headache. The seeds have insecticidal potential. (Burkill, 1995).

2.1.3 Phytochemistry of *Lonchocarpus sericeus* and other species in the genus

The isolation of two bioactive chalcones; Lonchocarpin (XXI) and Derricin (XXII) from the root have been reported (Fontenele, *et al.*, 2005).



XXI

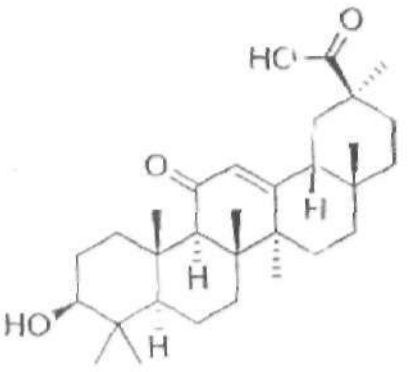


XXII

The occurrence of 2,5- dihydroxymethyl -3,4- dihydroxypyrrolidine in *L. sericeus* seed has been reported and has been shown to be responsible for glucosidase inhibition (Evans *et al.*, 1985).

The presence of Rotenone has also been reported (Burkill, 1995). The seed were also reported to have high proportions of Linolenic acid and relatively poor linoleic acid contents compared to soya beans (Ezeagu *et al.*, 1998).

Absence of alkaloids in the bark and root has also been reported. The presence of oleanane derivative, glycyrrhetinic acid (XXIII). a triterpenes of proven anti-arthritic activity was reported in the root and stem of *L. cyanescens*. (Burkil., 1995).



XXIII

To the best of our search, there is no report yet on isolation of any compound from the stem-bark of this plant but the result of preliminary phytochemical screening revealed the presence of important secondary metabolites (Musa *et al.*, 2006).

2.1.4 Pharmacological Properties of *L. sericeus*

The leave extract of *L. sericeus* has been reported to have hepatoprotective activity (Amegnona and Messanvi, 2009) while the methanol extract of stem-bark has been shown to have

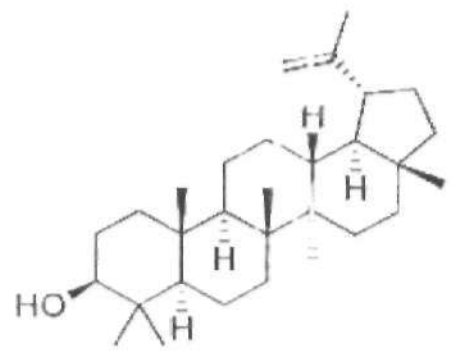
anticonvulsant activity (Musa *et al.*, 2006) and anti inflammatory activity (Musa *et al.*, 2007). Lonchocarpin and derricin isolated from the root have been shown to possess antiplatelet activity (Fontenele *et al.*, 2005).

The stem-bark has been tested for antimicrobial activity as a possible explanation for its use against skin infections in Nigerian Folk medicine. No action was found against either Gram +ve or Gram -ve organisms nor was there any fungistatic action (Burkill, 1995).

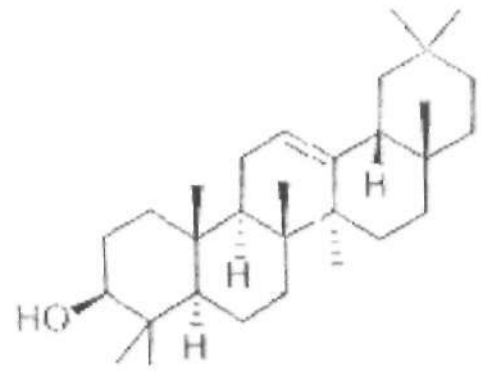
2.2 Triterpenoids

Triterpenoids are ubiquitous non-steroidal secondary metabolites of terrestrial and Marine Flora and fauna, occurring in the free form as well as in the form of ether, ester and glycoside. Although medicinal uses of this class of compounds have been limited, considerable recent work strongly indicates their great potential (Mahato and kundu, 1994). As the name implies, triterpenoids are isopentenoids composed of thirty carbon atoms and may possess acyclic, mono, di-, tri-, tetra- or pentacyclic carbon skeletons.

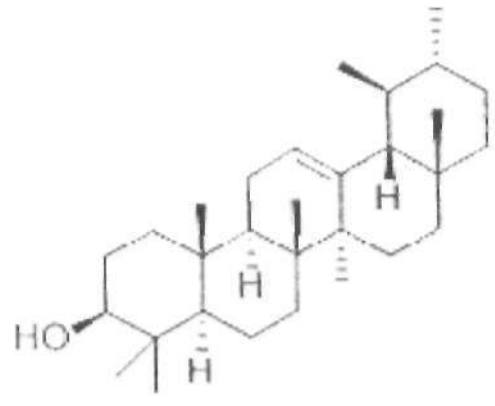
Pentacyclic triterpenoids are dominant constituents of this class and have been widely investigated. They are C-30 terpenoids derived from squalene and are comprised of various subgroups: Lupane group e.g lupeol (XXIV). Oleanane group e.g β-amyrin (XXV) and ursane group e.g α-amryin (XXVI).



XXIV



XXV



XXVI

2.2.1 Chemical Tests for Triterpenoid

1- Liebermann-Burchards' test: Triterpenoids undergo the Liebermann Burchards' reaction (acetic anhydride and concentrated sulphuric acid) to produce a brownish-red or violet ring at interphase with an upper green layer (Joan, 1994).

2- Salkwoskii test: Triterpenoids give a positive result to Salkwoskii test due to the presence of a steroidal nucleus (Sofowora, 1993).

2.2.2 Pharmacological Properties of Triterpenoids

Cytotoxic activity: isodiospyin and β-amyrin have been reported to have activity against HCT-8 colon tumor and P-388 lymphocytic leukemia with ED_{50} values of 4.9 and 0.59 μgml^{-1} respectively. (Yan *et al.*, 1989).

Anti inflammatory activity: Eleven (11) triterpene alcohols isolated from compositae flowers were evaluated with respect to their anti inflammatory activity against 12-0-tetradecanoylphorbol -13-acetate induced inflammation in mice. All of them showed marked inhibitory activity and their 50% inhibitory dose was 0.1-0.8 mg per ear (Toshihoro *et al.*, 1996). In addition, Lupeol, Betulin, Ursolic acid and Oleanolic acid have been found to have antimycobacterial action. Other bioactivities such as cytotoxic activity of lupeol and betulin, antihepatotoxic and antifertility activities of oleanolic acid as well as antileukemic and hepatoprotective activities of ursolic acid have been documented for triterpenoids (Toshihoro *et al.*, 1996).

2.2.3 Spectral Properties of Triterpenoids

Spectroscopic techniques are now routinely used for structure elucidation of natural products. Of all the physical methods, the NMR techniques have changed greatly during the last two decades especially with the growth of multiple pulse and 2D NMR. Other elementary methods include Ultraviolet (UV) and Infrared (IR) Spectroscopy.

2.2.3.1 UV and IR Spectra of Triterpenoids

UV is employed to detect isolated double bonds and conjugations. In simple dienes, the λ_{max} is 217-228 nm. If the diene is heteroannular, the λ_{max} is 230-240 nm; if the diene is homoannular, the λ_{max} is 256-265 nm and there is a weak band at 315-330 nm (Finar, 1975).

I.R is employed in terpenoid chemistry to detect the presence of OH (around 3400 cm^{-1}) an oxo group at 1750-1700 cm^{-1} for saturated and 1700-1600 cm^{-1} for the α, β- unsaturated carbonyl function. I.R spectroscopy is specifically useful for detecting the presence of isopropenyl group in terpenes usually between 895-885 cm^{-1} (Finar, 1975).

2.2.3.2 Proton NMR Spectra of Triterpenoids

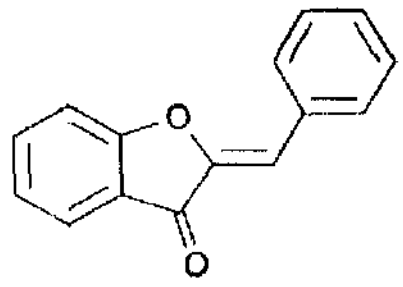
^1H NMR is used to detect and identify double bonds, determine the nature of end-groups present and the number of rings present e. g a multiplet signal at δ 3.25 for proton at C-6 is characteristic of α, β-unsaturated carbonyl compound while a mutiplet signal for C-8 methylene group has δ 4.78 which falls within the normal range for olefinic protons (Finar, 1975).

2.3 Flavonoids

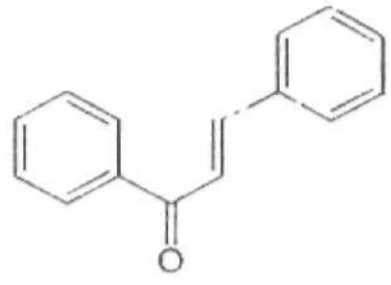
Flavonoids are polyphenolic compounds possessing 15 carbon atoms; two benzene rings joined by a linear three carbon chain. They constitute one of the most characteristic classes of compounds in higher plant. They are easily recognized as flower pigments although their occurrence is not restricted to flowers but include all parts of the plant. The chemical structure is based on a C_{15} skeleton with a chromane ring bearing a second aromatic ring in position 2, 3 or 4.

The pharmacological and medicinal properties of flavonoids have been extensively investigated several activities have been reported especially as antioxidants, antiproliferative, anti-inflammatory, analgesic and cytotoxic (Maria, *et al.*, 2012).

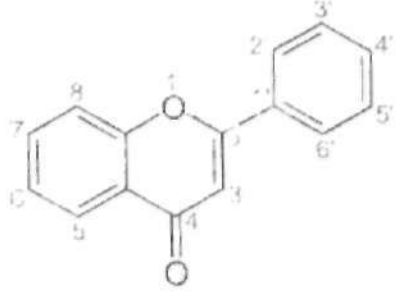
The Genus *Loncohocarpus* is known as a prolific source of flavonoids including aurones (XXVII), chalcones (XXVIII), flavones (XXVIX), flavonols (XXX), flavans (XXXI), flavanones (XXXII) and stilbenes (XXXIII) (Lima *et al.*, 2009).



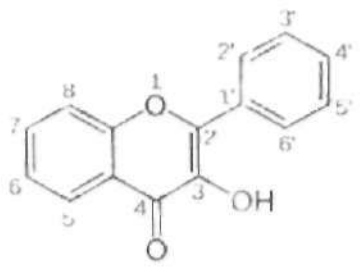
XXVII



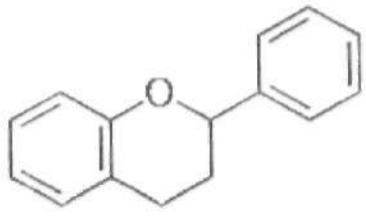
XXVIII



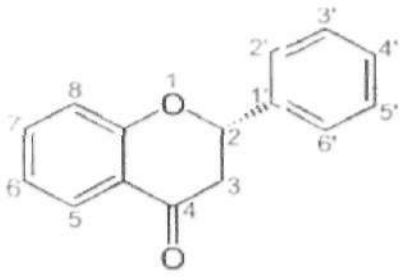
XXVIX



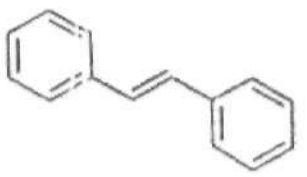
XXX



XXXI



XXXII



XXXIII

2.3.1 Chemical Test for Flavonoids

1- Ferric Chloride test: Polyphenols are able to reduce Fe^{3+} to Fe^{2+}. The production of Fe^{2+} can be monitored by the formation of Prussian blue Fe_4 [Fe $(CN)_6]_3$ (Cannell, 2000).

2- Shinoda's test: Flavonoids also produce pink or red colour with concentrated hydrochloric acid in the presence of Magnesium chip (Silva *et al.*, 1998).

2.3.2 *Spectral Properties of Flavonoids*

2.3.2.1 UV and IR Spectra of Flavonoids

UV spectroscopy is applied in flavonoids chemistry because of conjugation of both ring A and B with the carbonyl group. Two major absorption peaks are observed and are referred to as band I (300-380 nm) and band II (240-280 nm). They correspond to Cinnamoyl system and benzoyl system respectively (Mabry, 1969).

I.R spectroscopy is employed to detect the presence of free hydroxyl group in the region of 3300 cm^{-1}, carbonyl group around, 1685 cm^{-1} and aromatic double bonds which absorbs between 1500 cm^{-1} and 1610 cm^{-1} (Mabry, 1969).

2.3.2.2 Analysis of Flavonoids Structure by NMR

Proton signals obtained in NMR spectra of trimethylated flavonoids generally occur in the range of 0-8 ppm on a δ scale. The aromatic region gives a series of doublet in the range of 6-8 ppm. C-13 resonances occur predominantly in the range of 90-185 ppm. Chemical shift values are influenced by electron density such as carbonyl and oxygenated aromatic carbons which resonate at low field (130-185 ppm) whereas hydrocarbon and other aliphatic carbons are generally represented by signal in the range 0-110 ppm (Mabry *et al*., 1969).

2.4 NMR Spectroscopy

2.4.1 ^{13}C- NMR Spectroscopy

The assignment of ^{13}C NMR signals begins with an inspection of the proton noise (broad band) decoupling spectra (taking into consideration the chemical shift values) and usually backed up by ^{13}C multiplicity data, e. g DEPT, APT, INEPT etc (Mahato and Kundu, 1994).

2.4.2 Distortionless Enhancement by Polarisation Transfer (DEPT) Experiment

The DEPT experiment is the most commonly used method to determine the multiplicity of ^{13}C signals. The DEPT spectrum contains only signals arising from protonated carbons. The signals arising from carbons in CH_3 and CH groups (i.e those with an odd number of attached protons) appear oppositely phased from those in CH_2 groups (i.e those with an even number of attached protons) e. g signals from CH_3 and CH groups point upwards while signals from CH_2 groups point downwards (Field *et al*., 1995).

2.4.3 Two Dimensional NMR Spectroscopy

Since the advent of pulsed NMR Spectroscopy, a number of advanced two-dimensional techniques have been devised. These methods yield valuable information for the solution of complex structural problems. A few of these techniques include Correlation Spectroscopy (COSY). Nuclear Overhauser Enhancement Spectroscopy (NOESY). Heteronuclear Single Quantum Coherence (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC) (Mahato and Kundu, 1994).

2.4.3.1 COSY Experiment

The COSY spectrum shows which pair of nuclei in a molecule are coupled. The COSY spectrum is a symmetrical spectrum which has the ^{1}H NMR spectrum of the substance as both of the two chemical shift axes.

The COSY spectrum has a diagonal set of peaks (open circles) as well as peaks which are off the diagonal (closed circles). The off diagonal peaks are the important signals since they occur at positions where there is coupling between the two protons on both axes. In a single COSY spectrum, all of the spin-spin coupling pathways in a molecule can be identified (Field *et al.*, 1995).

2.4.3.2 HSQC Experiment

The HSQC spectrum is a heteronuclear analogue of the COSY spectrum. The HSQC spectrum has the ^{1}H NMR spectrum of the substance on one axis and the ^{13}C spectrum on the second axis. Unlike COSY and NOESY, the HSQC does not have diagonal peaks. The peaks occur at positions where protons are coupled to carbons and therefore, the correlation between the protons

(in the ^{1}H NMR spectrum) and the carbon nuclei (in the ^{13}C spectrum) can be obtained (Field *et al.*, 1995).

Using a HSQC spectrum, it is usually possible to easily assign all of the resonances in the ^{1}H NMR spectrum (i.e establish which proton in a molecule gives rise to each signal in the spectrum) using spin-spin coupling information. The ^{13}C spectrum can be assigned by correlation to the proton resonance (Field *et al.*, 1995).

2.4.3.3 HMBC Experiment

The HMBC experiment detects long range coupling between proton and carbon (two or three bonds away) with great sensitivity. The experiment can be adjusted to detect relatively large coupling constants (4-10 Hz) (Mahato and Kundu, 1994).

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals/Reagents

The chemicals used were of high quality and the reagents used were of analytical grade. Silica gel for column chromatography was of mesh size 60-120 µm (Qualikems product) while that for thin layer chromatography (TLC) was 60 GF_{254} glass precoated plate. Sephadex LH_{20} was obtained from Sigma Co. USA.

3.1.2 Equipment

Helios zeta thermo scientific Ultraviolet (UV) spectrophotometer UVZ 164617, 1002E, England available at Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, Zaria, Nigeria.

Bruker AVANCE -300 Japan (100 MHz and 400 MHz) Nuclear Magnetic Resonance Spectrometer available at School of Chemistry, University of KwaZulu Natal, Durban, South Africa.

3.1.3 Experimental Animals

Locally bred adult Swiss albino mice of either sex weighing 20 ± 2g were obtained from Animal House Facility of the Department of Pharmacology and Therapeutics, Ahmadu Bello University Zaria-Nigeria. Day old Ranger cockerels (30 ± 5 g) were obtained from Zarm farms Kwara State, Nigeria. The mice, maintained on standard rodent feed and water *ad libitum*, were housed in

polypropylene cages at room temperature throughout the study. All experimental protocols were in accordance with the guidelines for the care and use of experimental animals as accepted internationally and in compliance with Ahmadu Bello University, Zaria-Nigeria Research Policy. The experiments were conducted in a quiet laboratory between the hours of 900 h to 1600 h.

3.1.4 Plant Collection

The tree growing wild in Kongo Campus of Ahmadu Bello University Zaria was identified on the field in June, 2010 using descriptions in the literature (Burkill, 1995). The stem-bark was stripped off and its identity was confirmed and authenticated by Messrs U. S. Gallah and Musa Mohammad of the Herbarium Section, Department of Biological Sciences, Ahmadu Bello University Zaria by comparing with a previous Herbarium specimen of voucher number 1085. The stem-bark was washed, air dried under the shade for several weeks, size reduced using mortar and pestle and referred to as the plant material.

3.2 METHODS

3.2.1 Extraction and Fractionation

The plant material was subjected to extraction with methanol in a Soxhlet apparatus for 72 hours. The extract was concentrated *in vacuo* to yield a solid residue referred to as the methanolic extract of *L. sericeus* (MESL) (Brain and Tuner, 1975). The colour and weight was recorded. MELS was suspended in distilled water and partitioned successively with n-Hexane, Chloroform and Ethyl acetate to obtain n-Hexane fraction (nHXF), chloroform fraction (CF) and ethyl acetate fraction (EAF) respectively.

Preliminary TLC was carried out on all the fractions. Of all the fractions, the TLC of CF revealed the most prominent spots and therefore, was selected for further study.

3.2.2 Preliminary Phytochemical Screening

CF was subjected to preliminary phytochemical screening using standard methods as follows;

3.2.2.1 Test for Steroids and Triterpenes

a) Liebermann-Burchards Test: A 0.2 g of the extract was dissolved in 2ml of chloroform. 2ml of acetic anhydride was added to the solution and shaken. 2 drops of concentrated sulphuric acid was added carefully down the side of the tube to form a lower layer. A brownish-red or violet ring at the zone of contact of the two liquids and the upper layer turning green denotes the presence of steroids and triterpenes (Silva *et al.*, 1998).

b) Salkwoski test: A 0.2 g of the extract was dissolved in chloroform and 2ml of concentrated sulphuric acid was carefully added down the side of the test tube to form a lower layer. A reddish brown colour at the interphase indicates the presence of steroidal nucleus (Sofowora, 1993).

3.2.2.2 Test for Flavonoids

a) Ferric chloride test: One gram of the extract dissolved in methanol was boiled with water and filtered. To 2ml of the filterate, 2ml of freshly prepared ferric chloride solution was added; green, blue or violet colouration indicates the presence of phenolic hydroxyl group (Cannell, 2000).

b) Shinoda' test: A 0.2 g of the extract was dissolved in methanol. Some pieces of magnesium chip were added followed by five drops of concentrated hydrochloric acid. A pink or red colour indicates the presence of flavonoids (Silva *et al.*, 1998).

3.2.2.3 Test for Phenolic Compounds

A 0.2 g of the extract dissolved in methanol was boiled with water and filtered. Two drops of ferric chloride was added to the filtrate. Formation of a blue-black or green precipitate indicates the presence of Tannins (Trease and Evans, 1996).

3.2.2.4 Test for Saponins

A 0.5 g of the extract was shaken with water in a test tube. A froth which persists for 15 minutes is indicative of saponins (Silva *et al.*, 1998).

3.2.3 Column Chromatography

The following column conditions were employed:

a) Technique: Gradient elution.

b) Column: Glass column (750 cm x 3.5 cm) with sintered disc at the bottom.

c) Stationary phase: Silica gel, 60-120 mesh size.

d) Column packing: Wet slurry method.

e) Sample loading: Dry load method (Cannell, 2000)

CF (5 g) was adsorbed into silica gel and chromatographed over silica gel packed column. The column was eluted continuously with Hexane, then Hexane: chloroform mixture, chloroform, chloroform: ethylacetate mixture and finally methanol.

3.2.4 Preparative Thin Layer Chromatography (PTLC)

The following conditions were employed:

a) Technique: one way ascending

b) Spotting and development: Spots were applied manually with the aid of a capillary tube; plates were dried using air blower and developed at room temperature using a Shandon Chromatank.

c) Spraying reagent: 10% sulphuric acid

d) TLC plate: Fluka Silica gel precoated glass plate 20 cm x20 cm with layer thickness of 0.25 mm.

A thin line about 2 cm from the bottom of the plate was drawn with a pencil. The sample to be separated was dissolved in minimum amount of Chloroform to give an approximate concentration of 20 mg/ml. it was then applied uniformly along the thin line using capillary tube. The plate was allowed to dry after which it was developed using Hexane: ethylacetate (9:1) as solvent system. The developed plate was air dried in a fume cupboard and the position of the band of interest was marked with pencil and scrapped off the backing of the plate on to a foil. The scrapped sorbent was size reduced using pestle and Mortar, transferred to sintered glass funnel and washed repeatedly with chloroform. The solution obtained was evaporated to give the compound which was coded as R_1 (Gibbons and Gray, 1998).

3.2.5 Gel Filtration

Fraction 4 was subjected to repeated gel filtration using sephadex LH_{20} and was eluted with absolute methanol to obtain compound R_2.

Both compounds R_1 and R_2 were subjected to spectral analysis.

UV was recorded on a Helios zeta thermo scientific Ultraviolet spectrophotometer in methanol. ^{13}C and ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy was carried out on a Bruker AVANCE -300 Japan (100 MHz and 400 MHz) spectrometer in deuterated Chloroform ($CDC1_3$) using Tetra methyl silane (TMS) as standard.

3.2.5 Anticonvulsant Studies

CF was made into an emulsion using acacia. The emulsion was screened for anticonvulsant activity using standard procedures viz:

3.2.5.1 Acute Toxicity Studies

The method described by Lorke (1983) was adopted. All mice were injected intraperitoneally (i.p). The study was divided into two phases. In the first phase, nine mice of either sex were divided into three groups of three mice each. Group I received 10 $mgkg^{-1}$ extract while groups II and III received 100 $mgkg^{-1}$ and 1000 $mgkg^{-1}$ respectively. The animals were observed for signs and symptoms of toxicity including mortality for twenty-four hours after treatment. In the second phase, four mice of either sex were divided into four groups of one mouse each. The first mouse received extract at a dose of 200 $mgkg^{-1}$ while the second, third and fourth received the extract at doses of 400 $mgkg^{-1}$, 800 $mgkg^{-1}$ and 1600 $mgkg^{-1}$ respectively. The mice were also observed for twenty four hours. The final LD_{50} was calculated as the square root of the product of the lowest lethal dose and the highest non-lethal dose *i.e* the geometric mean of consecutive doses for which 0 and 100% survival rates were recorded.

3.2.5.2 Pentylenetetrazole Induced Seizure Test in Mice (PTZ)

The method previously described by Swinyard (1989) was employed. Thirty mice were randomly divided into five groups of six mice each. Group one (negative control group) was given 10 $mlkg^{-1}$ i.p of normal saline, group two, three and four were given 75 $mgkg^{-1}$, 150$mgkg^{-1}$ and 300 $mgkg^{-1}$ i.p of CF respectively. Group five (positive control) was given 200 $mgkg^{-1}$i.p valproic acid. Thirty minutes post-treatment, 100 $mgkg^{-1}$ of freshly prepared solution of s.c PTZ (CD_{97}) was administered to each mouse. The mice were observed for thirty minutes for presence or absence of threshold seizures (i.e an episode of clonic spasm of at least five seconds duration).

3.2.5.3 4-Aminopyridine- Induced Mortality Test in Mice (4-AP)

The method previously described by Yamaguchi and Rogawski (1992) was adopted. Thirty albino mice were randomly divided into five groups of six mice each. Group one (negative control) was given 10 $mlkg^{-1}$ of normal saline, groups two, three and four were given 75 $mgkg^{-1}$, 150 $mgkg^{-1}$ and 300 $mgkg^{-1}$ i.p of CF respectively and group five was administered 20 $mgkg^{-1}$ phenobarbitone to serve as positive control. After thirty minutes, 15 $mgkg^{-1}$ of freshly prepared 4-AP was administered subcutaneously to each mouse. Ability of the extract to protect the mice from lethality within a thirty minute observation period was considered an indication of anticonvulsant activity.

3.2.5.4 Maximal Electroshock Seizure Test in Chicks (MEST)

The method of Swinyard and Kupferberg (1985) was employed. Day old ranger cockerels (fifty in number) were randomly divided into five groups of ten chicks each. The first group received normal saline (10 $mlkg^{-1}$) i.p, second, third and fourth groups were administered 75 $mgkg^{-1}$, 150 $mgkg^{-1}$ and 300 $mgkg^{-1}$ i.p of CF respectively and the fifth group was administered 20 $mgkg^{-1}$ of phenytoin. Thirty minutes later, maximal electroshock was administered to induce seizure in the

chicks using Ugobasile electroconvulsive machine (Model 7801) with corneal electrodes placed on the upper eyelids of the chicks. The shock duration, frequency and pulse width were set and maintained at 0.8 s, 100 pulses/sec and 0.6 ms respectively. A current of 90 mA which produced tonic seizures in 80% of the control chicks, was used throughout the study. An episode of tonic extension of the hind limbs was regarded as full convulsion, while lack of tonic extension of hind limbs was regarded as protection. In unprotected animals, the recovery time was recorded.

CHAPTER FOUR

RESULTS

4.1 Result of Phytochemical Screening

Preliminary phytochemical screening of CF revealed the presence of flavonoids, phenolic compounds, steroids and triterpenoids (Table 4.1).

Table 4.1: Table showing the result of preliminary phytochemical screening of CF

Constituent	Test	Observation	Inference
1. Steroids and triterpenes	a. Liebermann burchards test	Brownish-red ring	+ ve
	b. Salkwoskii test	Reddish brown colour at interphase	+ ve
2. Flavonoids	a. Shinodas test	Red colouration	+ ve
	b. Ferric chloride test	Green colouration	+ ve
3. Saponins	Froth test	No froth	- ve
4. Phenolic compound	a. Lead subacetate test	Yellow colouration	+ve
	b. Ferric chloride test	Greenish black colouration	+ve

Key

+ ve present

- ve absent

4.2 Result of Column Chromatography

A total of one hundred and four (104) fractions, 25ml each were collected. The fractions were pooled together based on their TL C profile to give nine (9) major fractions, and the column was finally washed with methanol to give the tenth fraction (Table 4.2).

Table 4.2: Table showing the result of column chrom. tography of CF

Fraction	Collection	Number of Spots
1	1-36	No clear spot
2	37-49	3
3	50-55	3
4	**56-65**	**4**
5	**66-69**	**3**
6	70-73	5
7	74-80	4
8	81-90	4
9	91-104	5
10	105	>6

4.3 Result of Preparative TLC

Fraction 5, when subjected to PTLC gave three bands of R_f values 0.28, 0.44 and 0.69.

4.4 TLC profile of R_1

The second band with R_f value 0.44 was scrapped off the plate and washed with chloroform to obtain 6mg of white needles which was coded as R_1. R_1 gave a single homogenous spot with two solvent systems (Table 4.3).

4,5 Chemical Test on R_1

It gave a positive test with Salkowskii and Liebermann Burchard's reagent.

Table 4.3: Table showing the TLC profile of R_1

Solvent system	Number of Spot	Colour of spot after heating	R_f Value
Hex: EtOAc (9:1)	1	Maroon	0.44
Hex: EtOAc (5:1)	1	Maroon	0.65

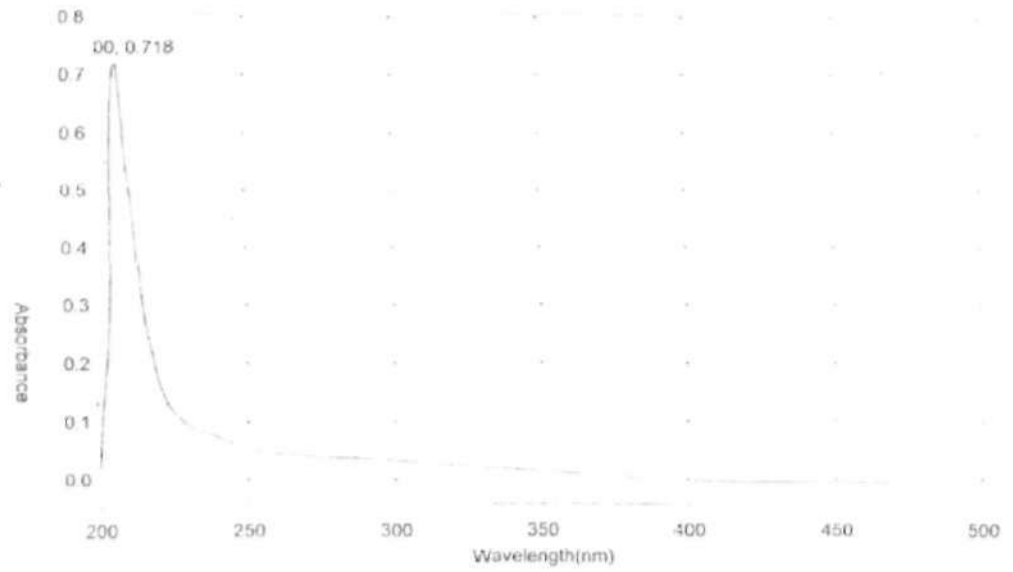
4.6 UV Spectral Analysis of R_1

The UV spectrum of R_1 showed wavelength of maximum absorbance at 207nm (Figure 4.1).

THERMO ELECTRON – VISIONpro SOFTWARE V3.00

Operator Name	I.M SALISU	Date of Report	5/25/2011
Department	PHARM CHEMISTRY	Time of Report	12:20:46 PM
Organisation	ABU		
Information	Pharm Chem		

Scan Graph



Results Table - scan001,Sakina,Cycle01

nm	A
207.00	0.718

Peak Pick Method
Find 8 Peaks Above -3.0000 A
Start Wavelength 200.00 nm
Stop Wavelength 500.00 nm
Sort By Wavelength

Sensitivity Auto

Page 1. Scan Graph

Figure 4.1: UV spectrum of R_1

4.7 Proton NMR Spectral Analysis of R_1

The ^1H NMR spectrum revealed the presence of seven tertiary methyl protons at δ 0.77, 0.79, 0.85, 0.94, 0.97, 1.05 and 1.65 (3H-each). a sextet of one proton at δ 2.37, a multiplet at δ 3.2 and a pair of broad singlet at δ 4.55 and δ 4.70 (1 H each) (Figure 4.2).

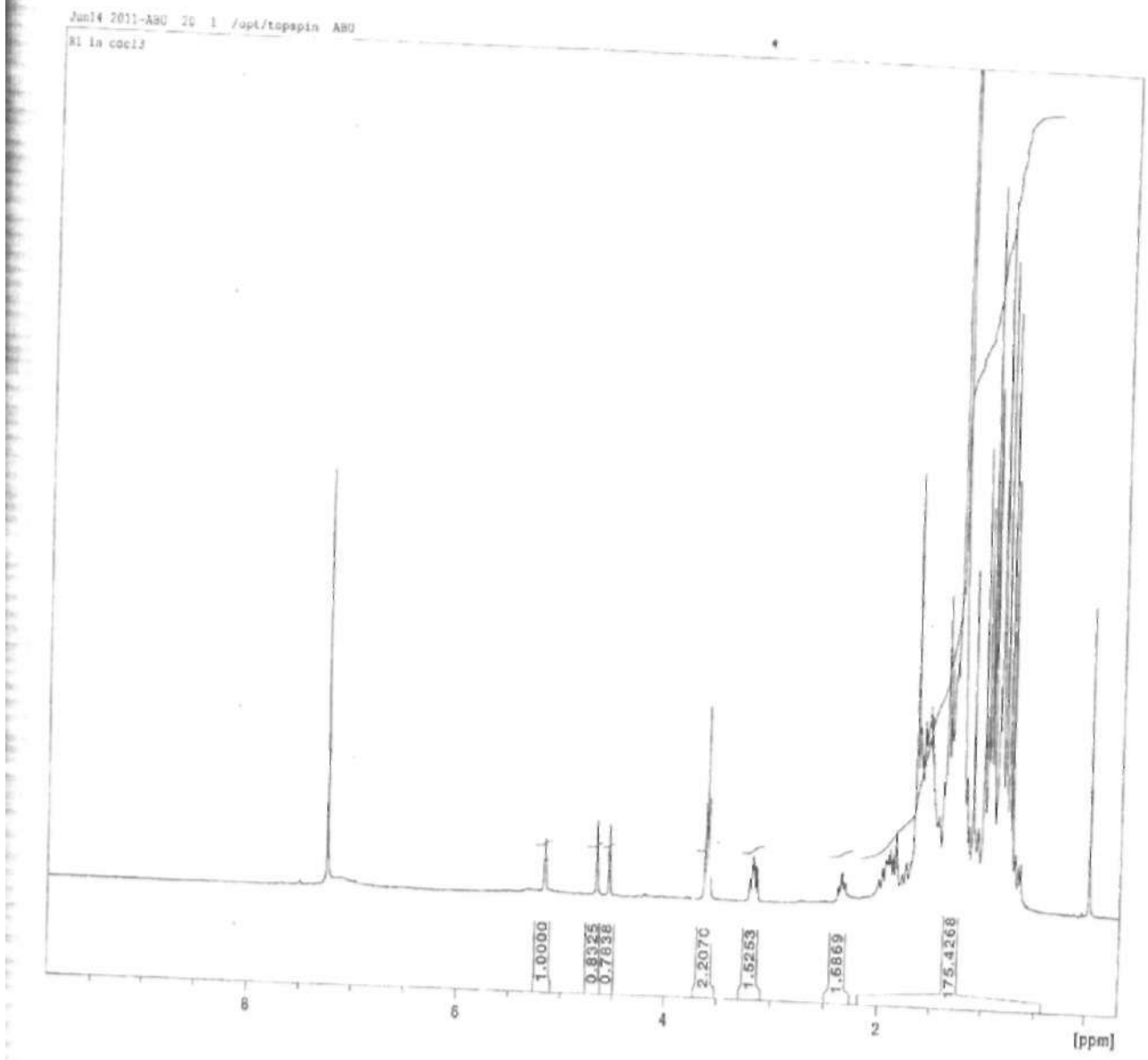


Figure 4.2: Proton NMR spectrum of R_1

4.8 Carbon 13 NMR Spectral Analysis of R_1

The result of ^{13}C NMR showed seven methyl groups at [δc: 28.0 (C-23). 18.0 (C-28). 16.1 (C-25). 16.0 (C-26). 15.5 (C-24). 14.8 (C-27) and 19.5 (C-30)], an exomethylene group at [δc: 109.3 (C-29) and 151.0 (C-20)] and a secondary hydroxyl bearing carbon at [δc: 79.0 (C-3)] (Figure 4.3).

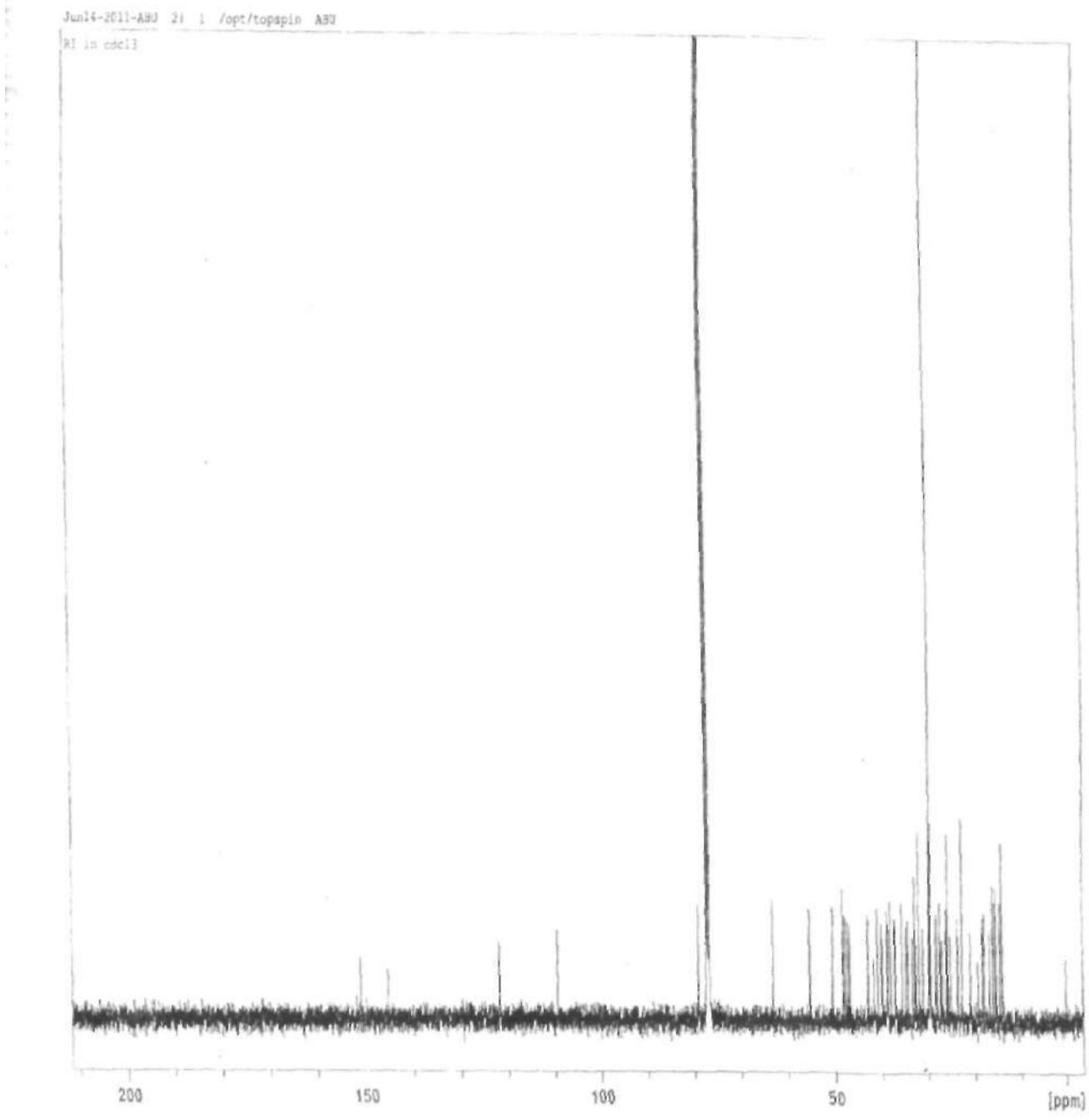


Figure 4.3: Carbon-13 spectrum of R_1

4.9 DEPT NMR Spectral Analysis of R_1

The results of DEPT experiment revealed the presence of seven methyl carbons at C-23, C-24, C-25, C-26, C-27, C-28 and C-30; eleven methylene carbons at C-1, C-2, C-6, C-7, C-11, C-12, C-15, C-16, C-21, C-22 and C-29; six methine carbons at C-3, C-5, C-9, C-13, C-18 and C-19 and six quaternary carbons at C-4, C-8, C-10, C-14, C-17 and C-20 (Figure 4.4).

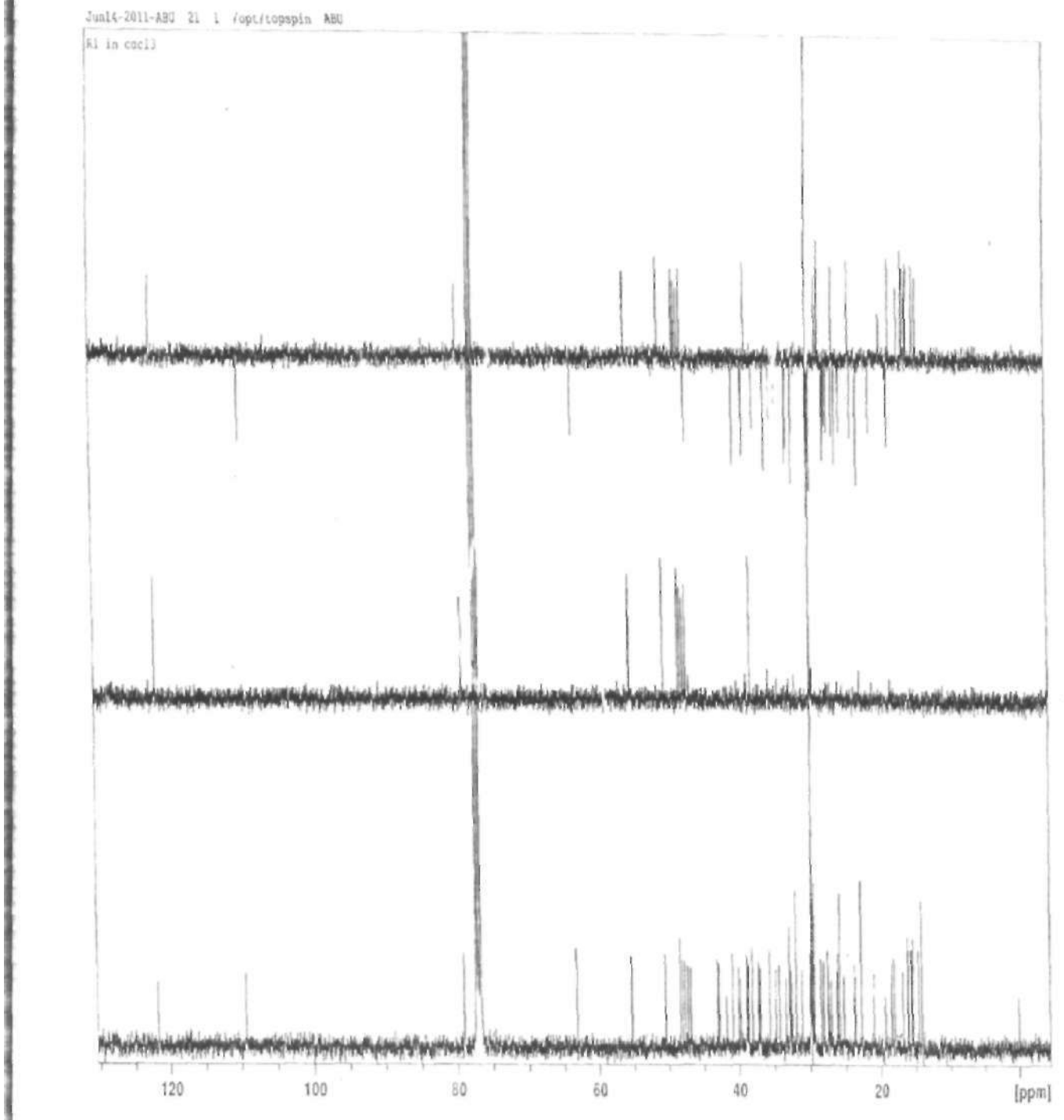


Figure 4.4: DEPT spectrum of R_1

4.10 HSQC Spectral Analysis of R_1

The HSQC of R_1 is as shown in Figures 4.5 and 4.6 and was used to assign protons on their respective carbons (Table 4.4).

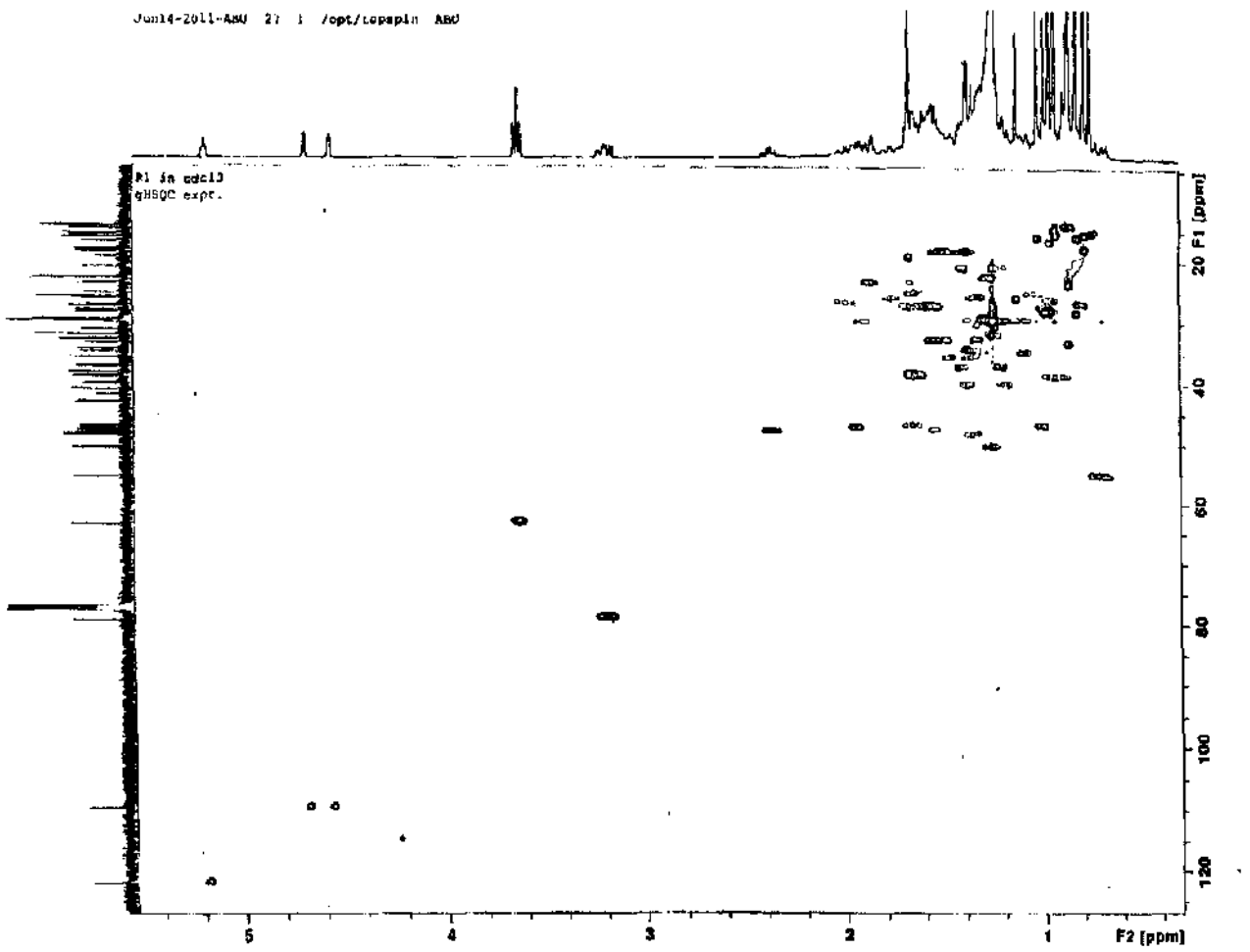


Figure 4.5: HSQC spectrum of R_1

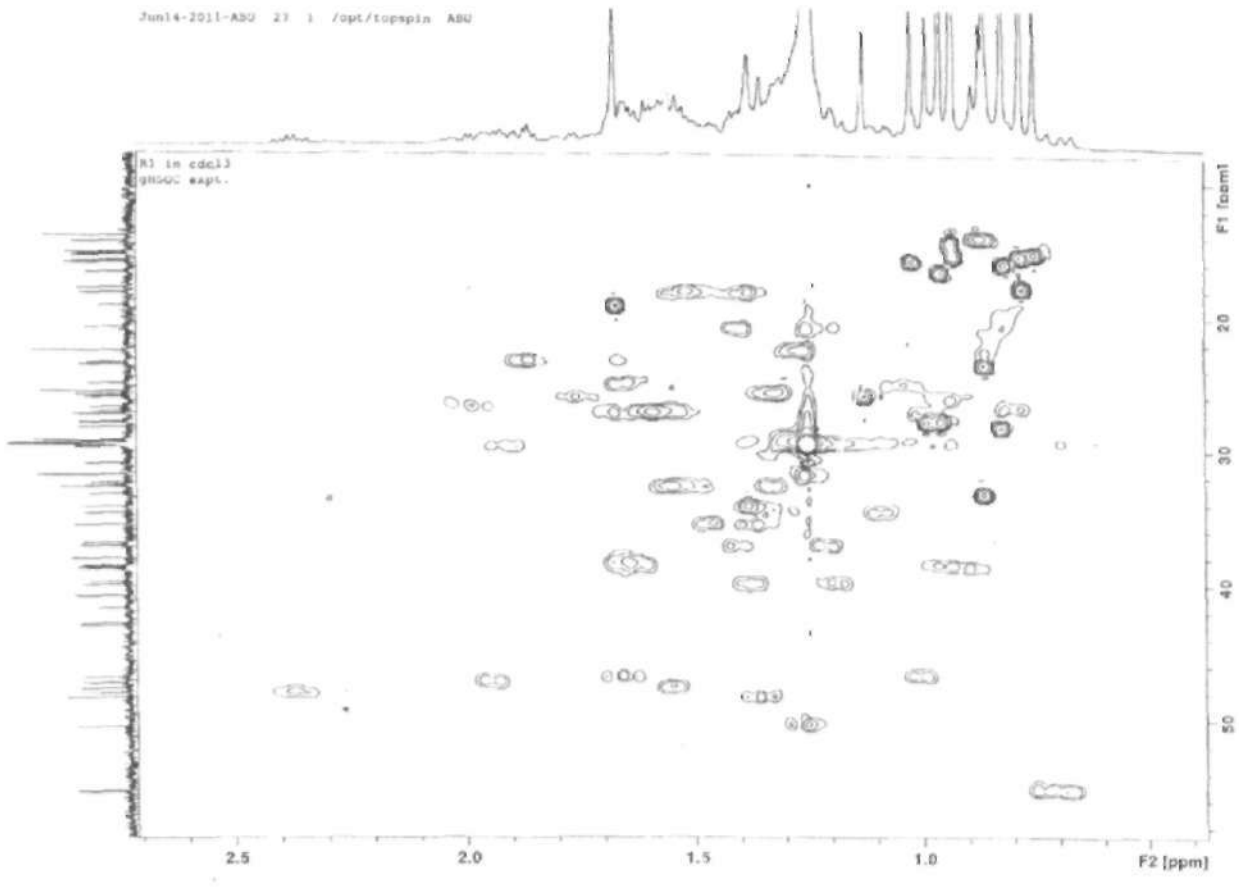


Figure 4.6: HSQC spectrum of R_1

Table 4.4 Table showing the result of HSQC and DEPT experiment of R_1

S/No	^{1}H	^{13}C	DEPT
1	1.38	38.7	CH_2
2	1.60	27.4	CH_2
3	3.2	79.0	CH
4		38.9	C
5	0.6	55.5	CH
6	1.5	18.5	CH_2
7	1.36	34.2	CH_2
8		40.9	C
9	1.3	50.5	CH
10		37.2	C
11	1.4	21.0	CH_2
12	1.65	25.2	CH_2
13	1.60	38.1	CH
14		42.9	C
15	1.55	27.1	CH_2
16	1.38	35.5	CH_2
17		43.0	C
18	1.92	48.3	CH
19	2.37	48.0	CH
20		151.0	C
21	0.6	29.9	CH_2
22		40.0	CH_2
23	0.97	28.0	CH_3
24	0.77	15.5	CH_3
25	0.79	16.1	CH_3
26	0.85	16.0	CH_3
27	0.94	14.8	CH_3
28	1.05	18.0	CH_3
29	4.55, 4.7	109.0	CH_2
30	1.65	19.5	CH_3

4.11 COSY Spectral Analysis of R_1

The COSY spectrum of R_1 showed some cross peaks such as between δ_H 2.37, H-19 and one Sp^3 methylene proton signal (δ_H 1.37, H-21) and another Sp^3 methine proton signal (δ_H 1.89, H-18); and between oxygenated methine proton signal (δ_H 3.2, H-3) and Sp^3 methylene signal (δ_H 1.60, H-2) (Figure 4.7).

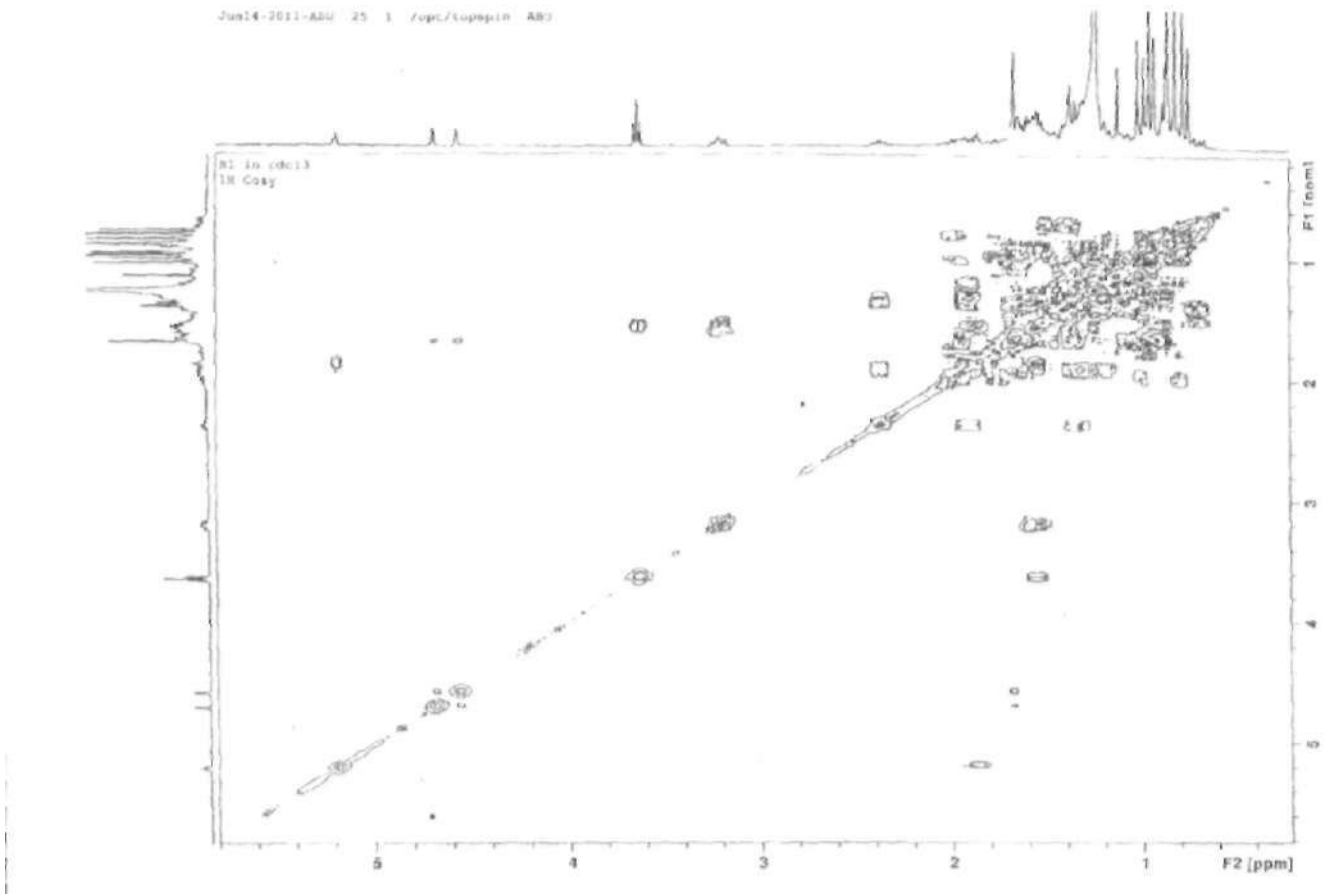


Figure 4.7: COSY spectrum of R_1

4.12 HMBC Spectral Analysis of R_1

In the HMBC spectrum, the methine proton signal at (δ_H 3.2, H-3) showed cross peaks with a methyl carbon signal at (δc 28.0, C-23) and (δc 18.5, C-6). The sextet methyl signal at δ_H 2.37 (H-19) showed cross peaks with two methylene carbon signals at (δc 29.9, C-21) and (δc 109.0, C-29). a methine carbon signal [δ_c 48.3 (C- 18). a methyl carbon signal [δ_c 19.5 (C-30)] and a quaternary carbon signal [δ_c 151.0 (C-20)]. The pair of broad singlet of olefinic proton at δ_H 4.55 and 4.70 showed cross peaks with a methylene carbon signal [δ_c 48.0 (C-19) and δ_c 19.5 (C-30)]. (Figures 4.8 and 4.9).

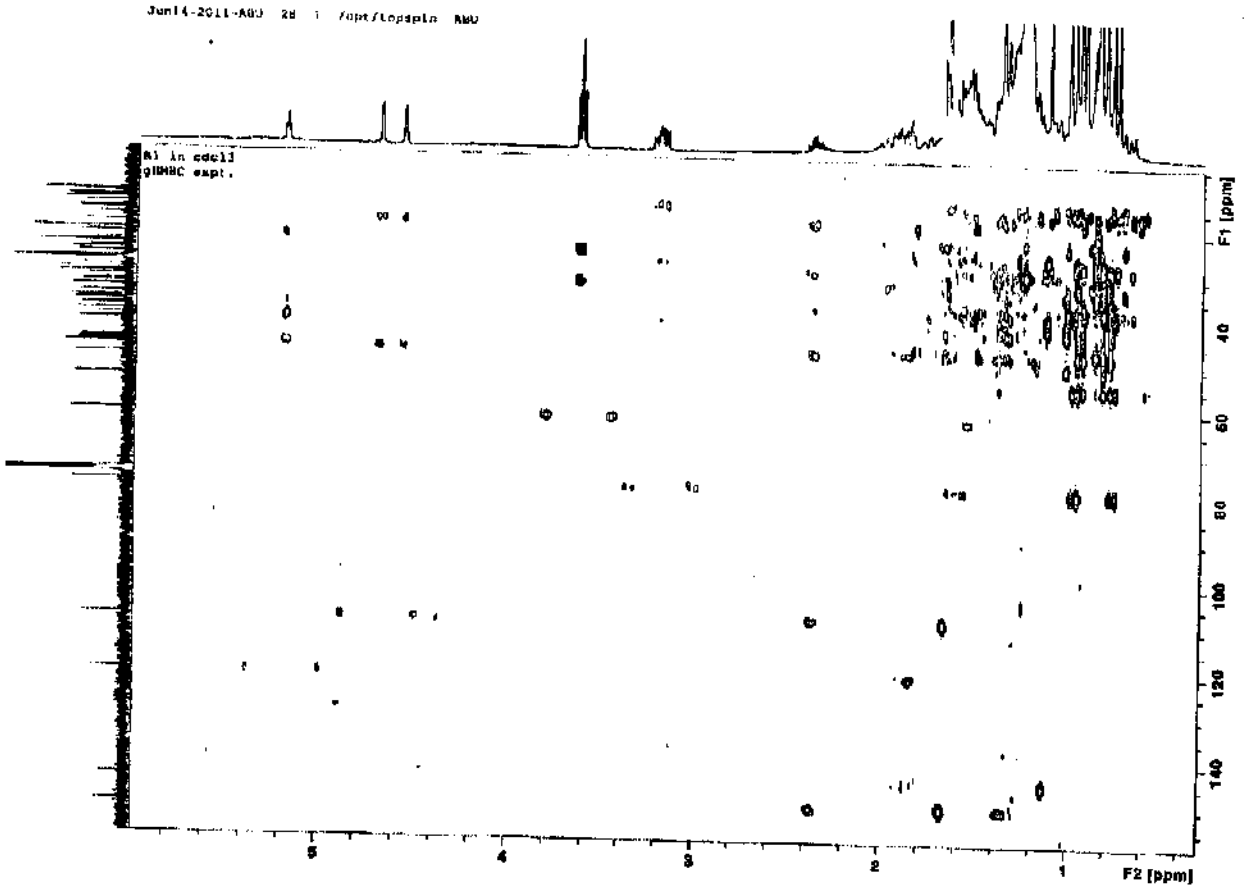


Figure 4.8: HMBC spectrum of R_1

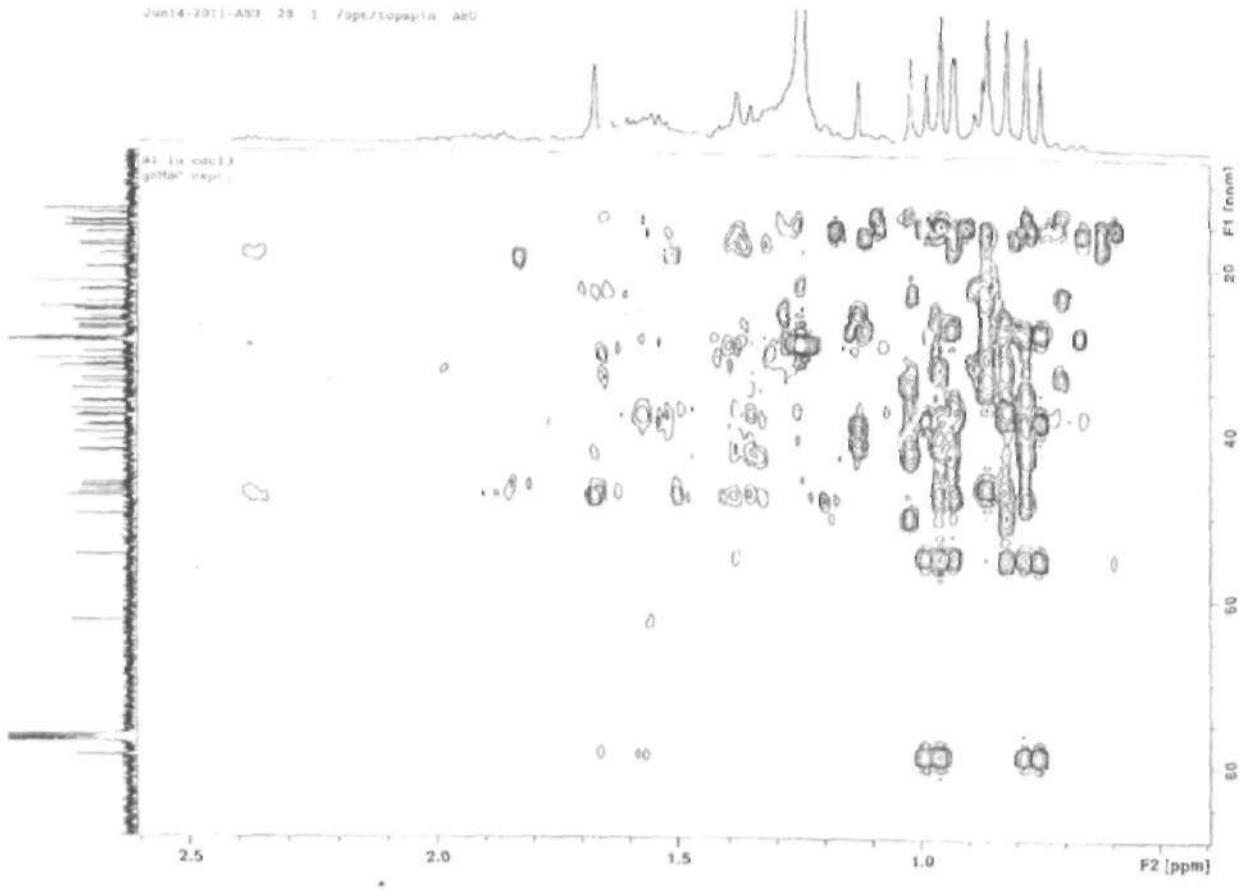


Figure 4.9: HMBC spectrum of R_1

4.13 Comparison of the Spectral data of R_1 with that obtained from the Literature

The spectral data of R_1 was compared with Lupeol isolated by Jain and Bari (2010) as shown on table 4.5.

Table 4.5: Table comparing the spectral data of R_1 with that obtained from the literature

Position	δ_c (Reference)	δ_c (R_1)	δ_H (Reference)	δ_H (R)
1	38.7	38.7	1.68	1.38
2	27.4	27.4	1.61	1.60
3	79.0	79.0	3.18	3.20
4	38.9	38.9		
5	55.3	55.5	0.69	0.60
6	18.3	18.5	1.54	1.50
7	34.3	34.2	1.41	1.36
8	40.8	40.9		
9	50.4	50.5	1.28	1.30
10	37.2	37.2		
11	20.9	21.0	1.42	1.40
12	25.1	25.2		1.65
13	38.0	38.1		1.60
14	42.8	42.9		
15	27.4	27.1		1.55
16	35.6	35.5		1.38
17	43.0	43.0		
18	48.3	48.3		1.92
19	48.0	48.0	2.39	2.37
20	151.0	151.0		
21	30.0	29.9		0.60
22	40.0	40.0		
23	28.0	28.0	0.98	0.97
24	15.4	15.5	0.77	0.77
25	16.1	16.1	0.84	0.79
26	16.0	16.0	1.04	0.85
27	14.5	14.8	0.97	0.94
28	18.0	18.0	0.79	1.05
29	109.3	109.0	4.56, 4.69	4.55, 4.70
30	19.3	19.5	1.69	1.65

4.14 TLC Profile of R_2

Repeated gel filtration of fraction 4 afforded 5.2 mg of a yellow amorphous compound coded R_2.

R_2 gave a single homogenous spot with solvent system of Hexane:Ethyl acetate (9:1) (Plate 4.1).

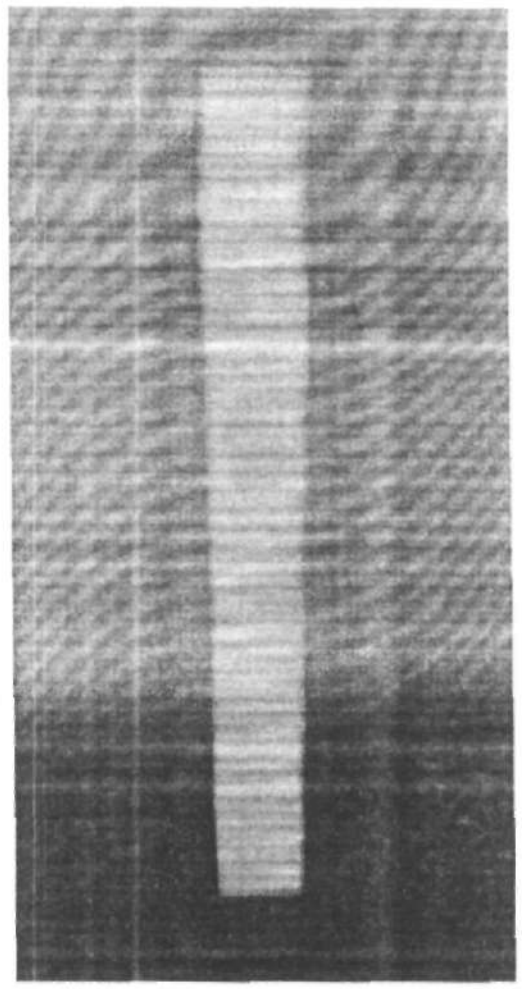


Plate 4.1: TLC profile of R_2

4.15 Proton NMR Spectral Analysis of R_2

The ^1H NMR spectrum of R_2 disclosed signals for two tertiary methyl protons at δ_H 0.87 and δ_H 1.50, characteristic signals for aromatic methine protons, a methylene proton and a singlet around δ_H 13.7 for free hydroxyl group (Figure 4.10).

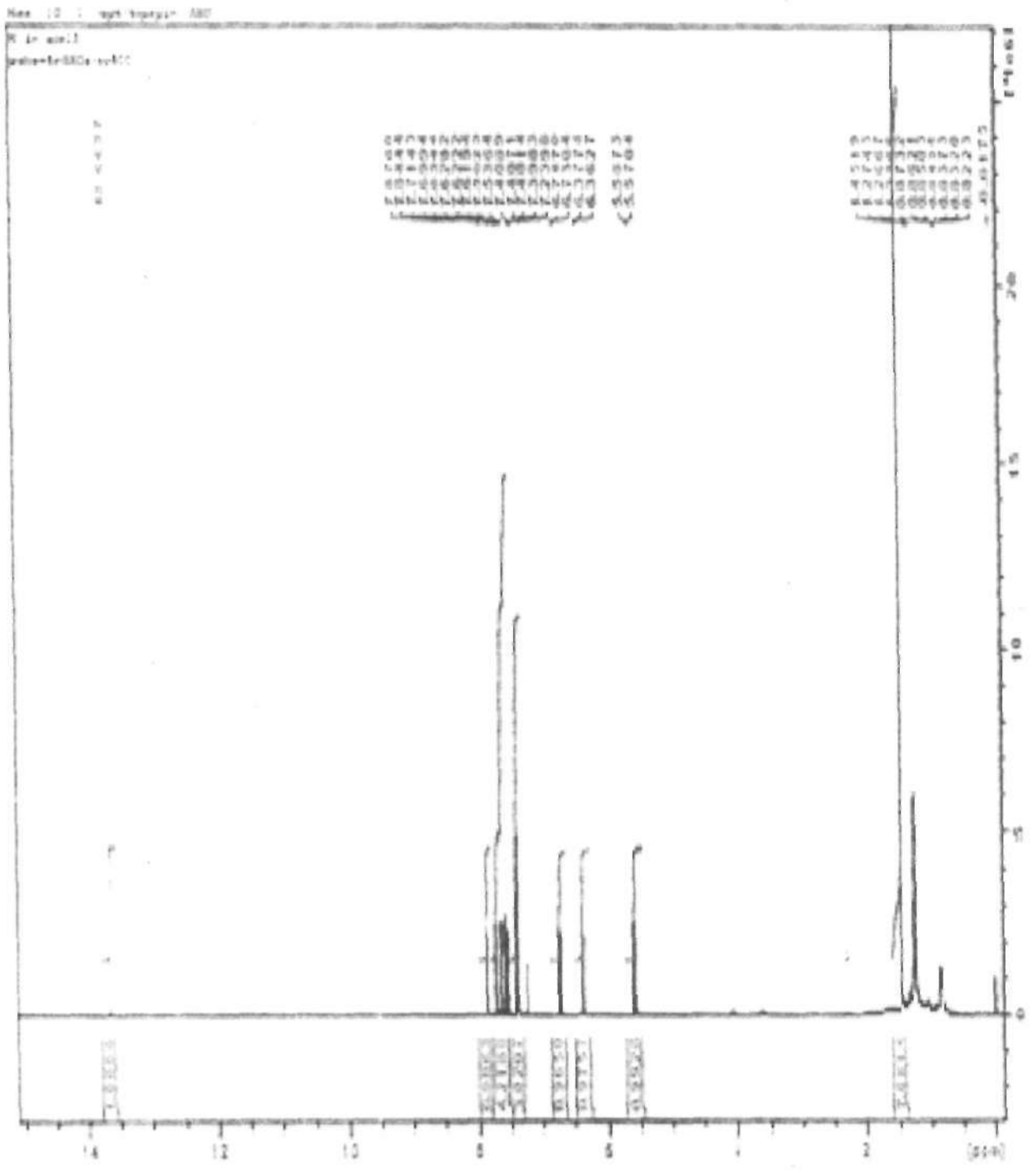


Figure 4.10: Proton NMR of R_2

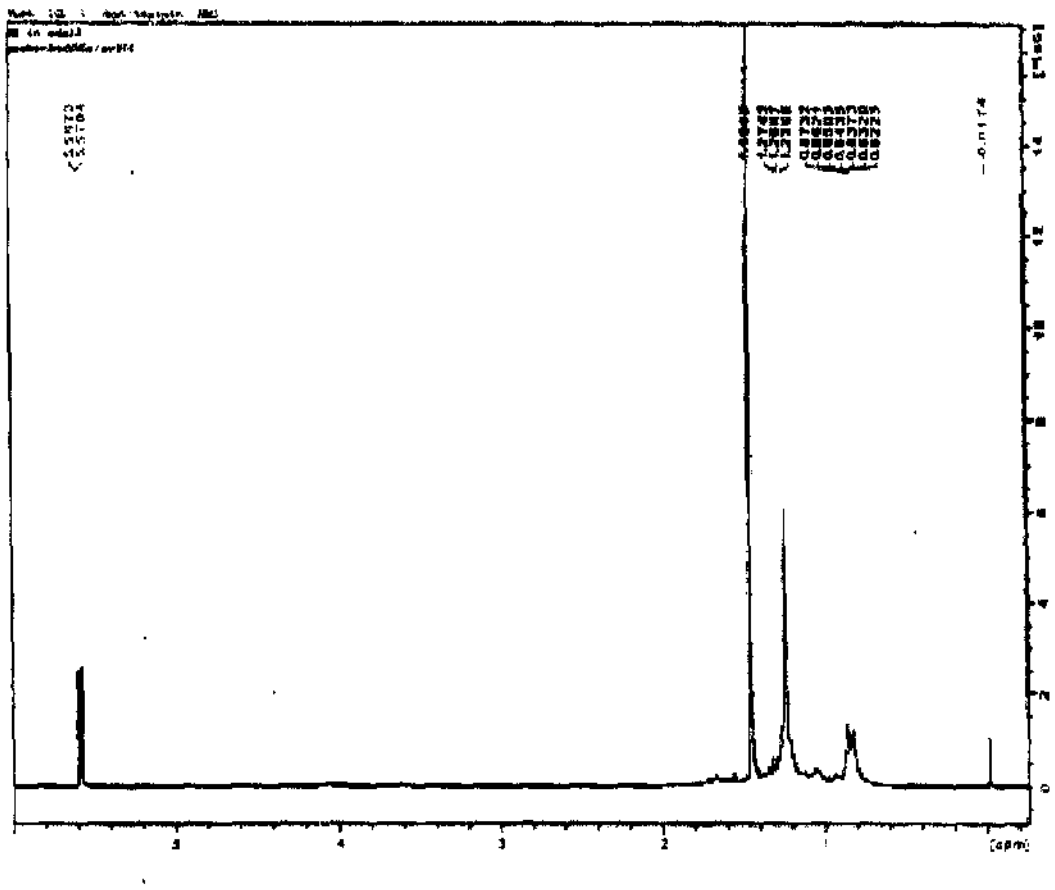


Figure 4.11: Proton NMR of R_2 (Aliphatic region)

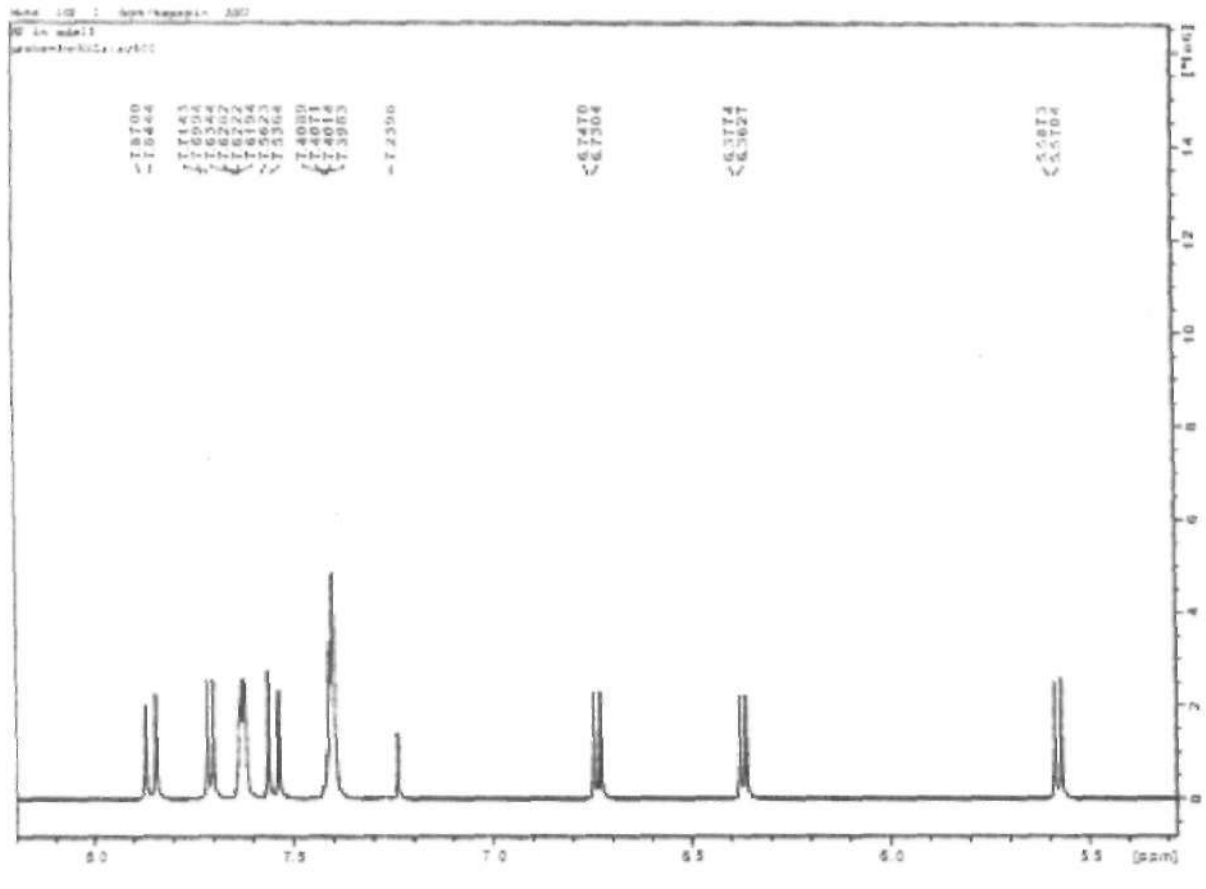


Figure 4.12: Proton NMR of R_2 (Aromatic region).

4.16 Carbon 13 NMR Spectral Analysis of R_2

The ^{13}C spectrum revealed a downfield signal at δ_C 191.9, characteristic signals for aromatic ring, a hydroxyl bearing carbon signal at δ_C 78.0 and two methyl carbon signals at δ_C 14.2 and 28.4 (Figure 4.13).

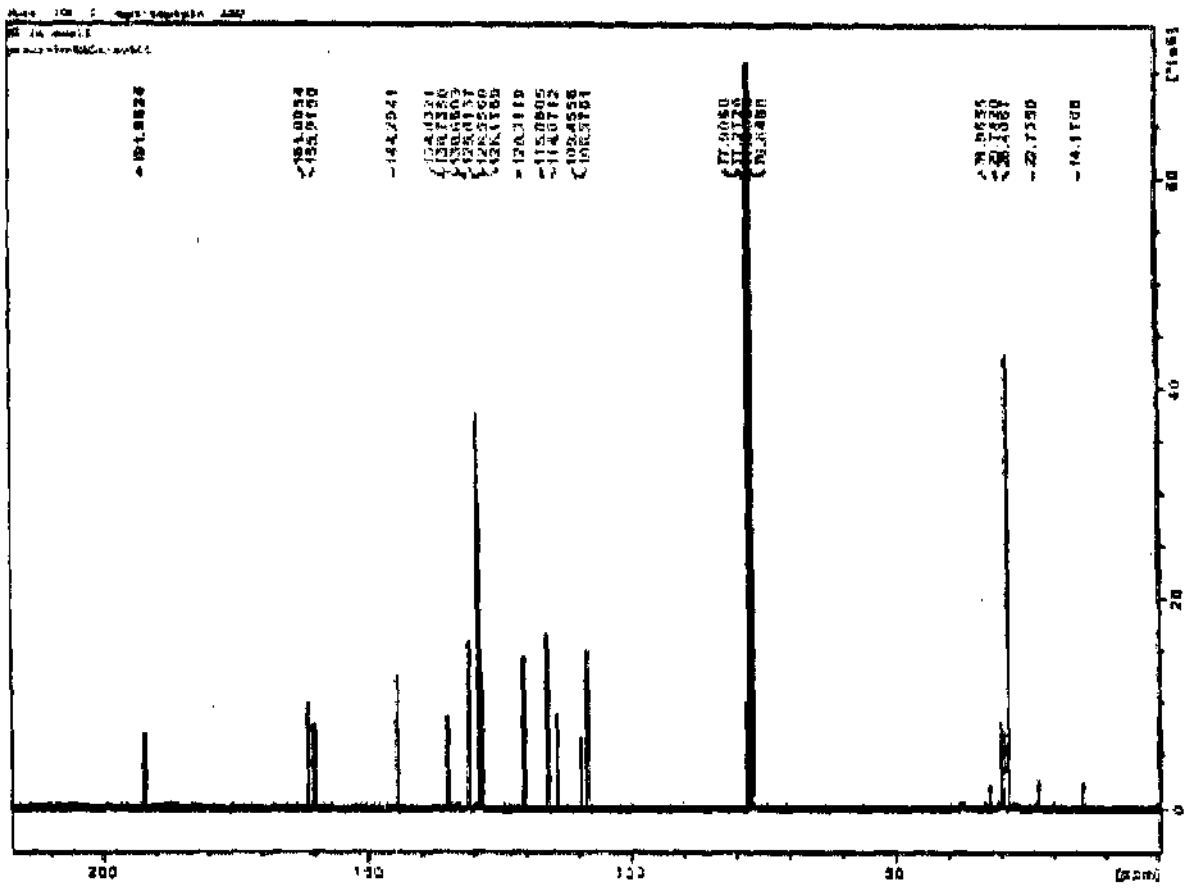


Figure 4.13: ^{13}C NMR spectrum of R_2

4.17 DEPT NMR Spectral Analysis of R_2

The DEPT experiment disclosed two carbon resonances for methyl groups, a methylene carbon at δ_C 29.7, nine aromatic methine carbons at δ_C 144.2, 130.8, 130.7, 129.0 128.6, 128.2, 120.3, 115.9 and 78.0 and seven quaternary carbons at δ_C 191.9, 161, 159.9, 134.8, 114.1, 109.5 and 78.0 (Figures 4.14 and 4.15).

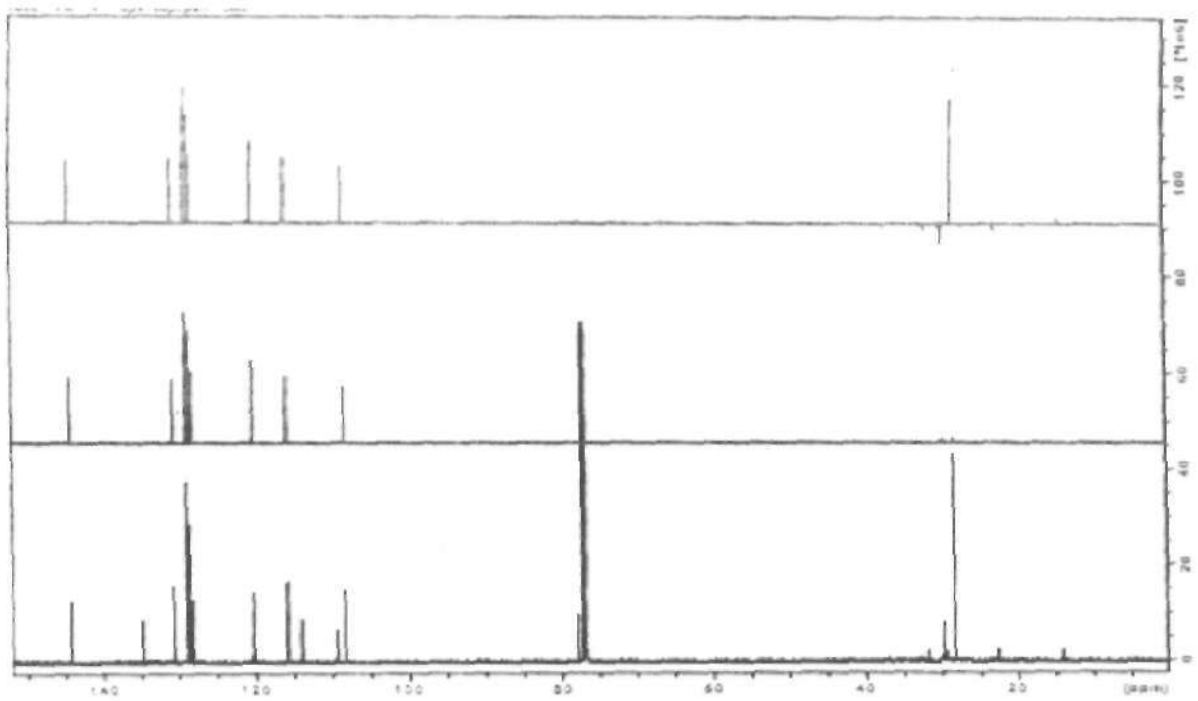


Figure 4.14: DEPT experiment of R_2

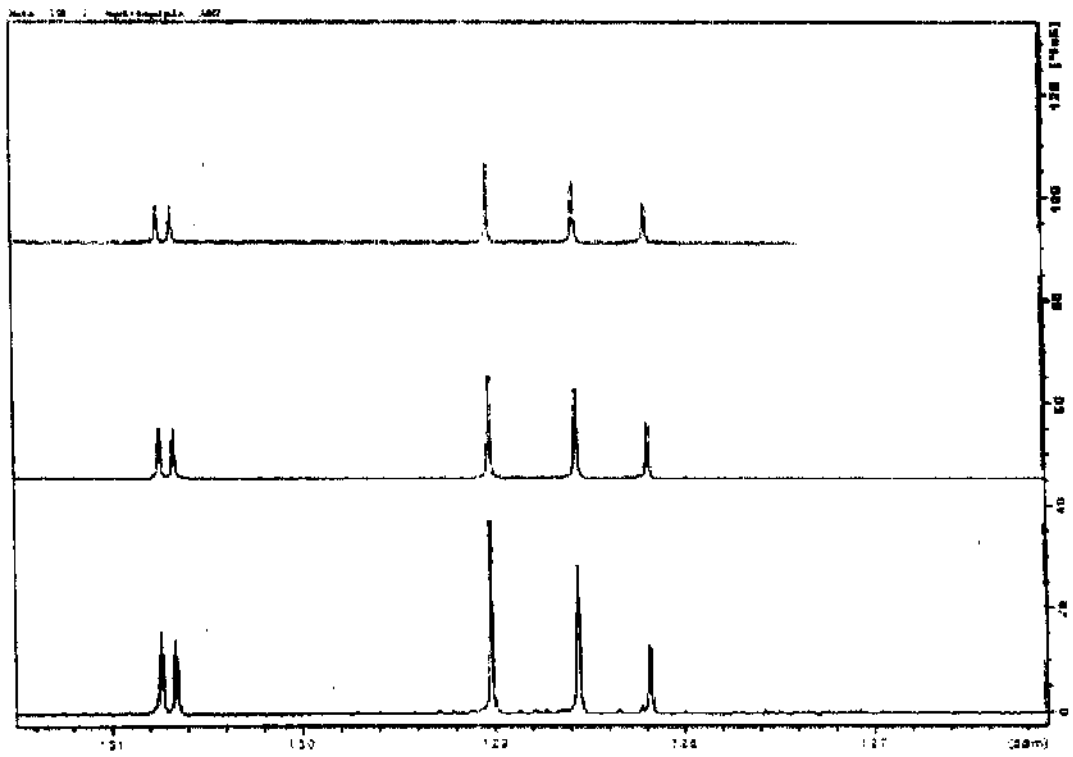


Figure 4.15: DEPT experiment of R_2

4.18 COSY Spectral Analysis of R_2

The COSY spectrum of R_2 showed four important cross peaks between δ_H 7.55 (H-α) and δ_H 7.86 (H-β); 5.58(H-2'') and δ_H 6.74(H-1''); δ_H 6.37 (H-5') and δ_H 7.71 (H-6') and δ_H 7.40 (H-2, 4, 6) and δ_H 7.63 (3, 5) indicating protons that are attached on adjacent carbons (Figures 4.16 and 4.17).

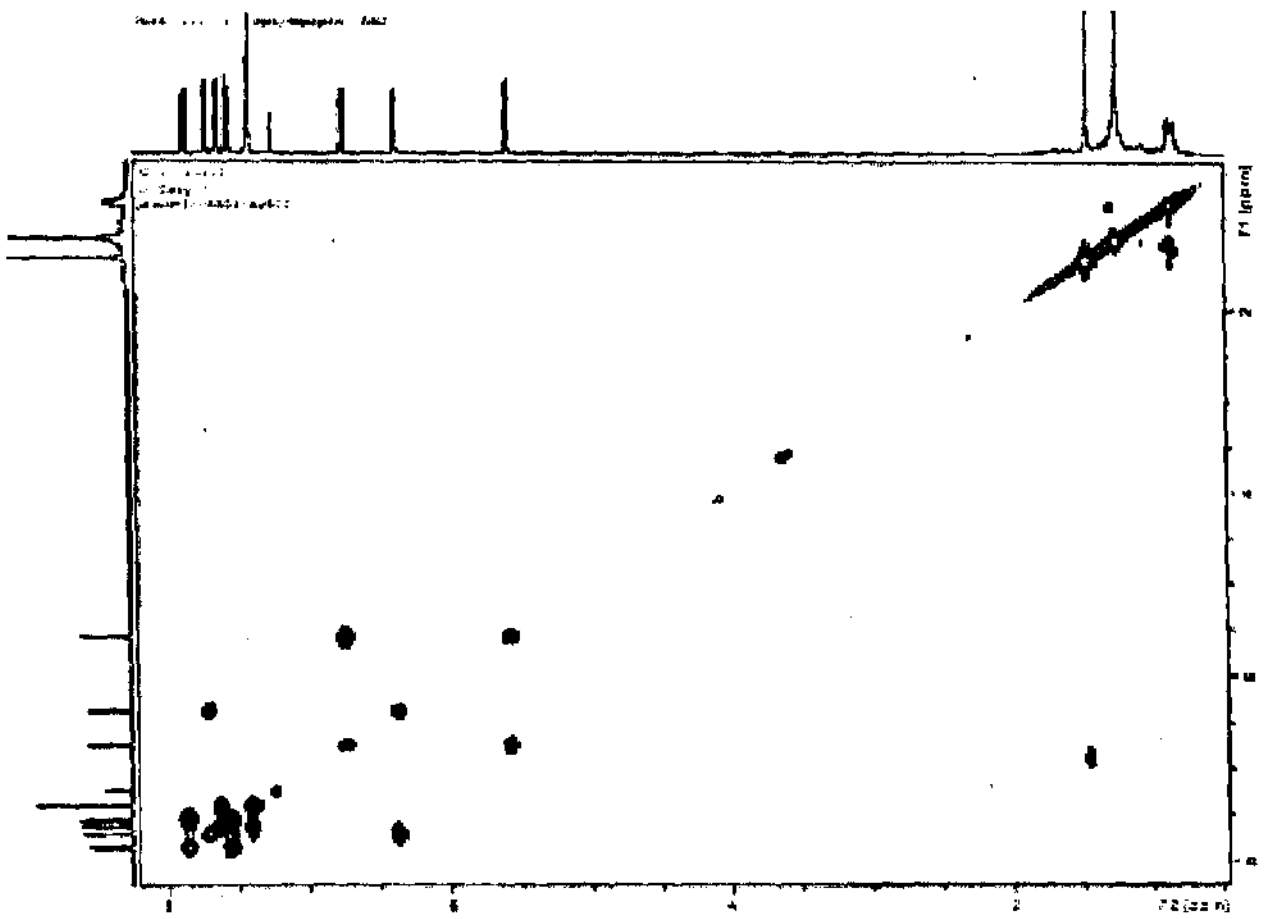


Figure 4.16: COSY spectrum of R_2

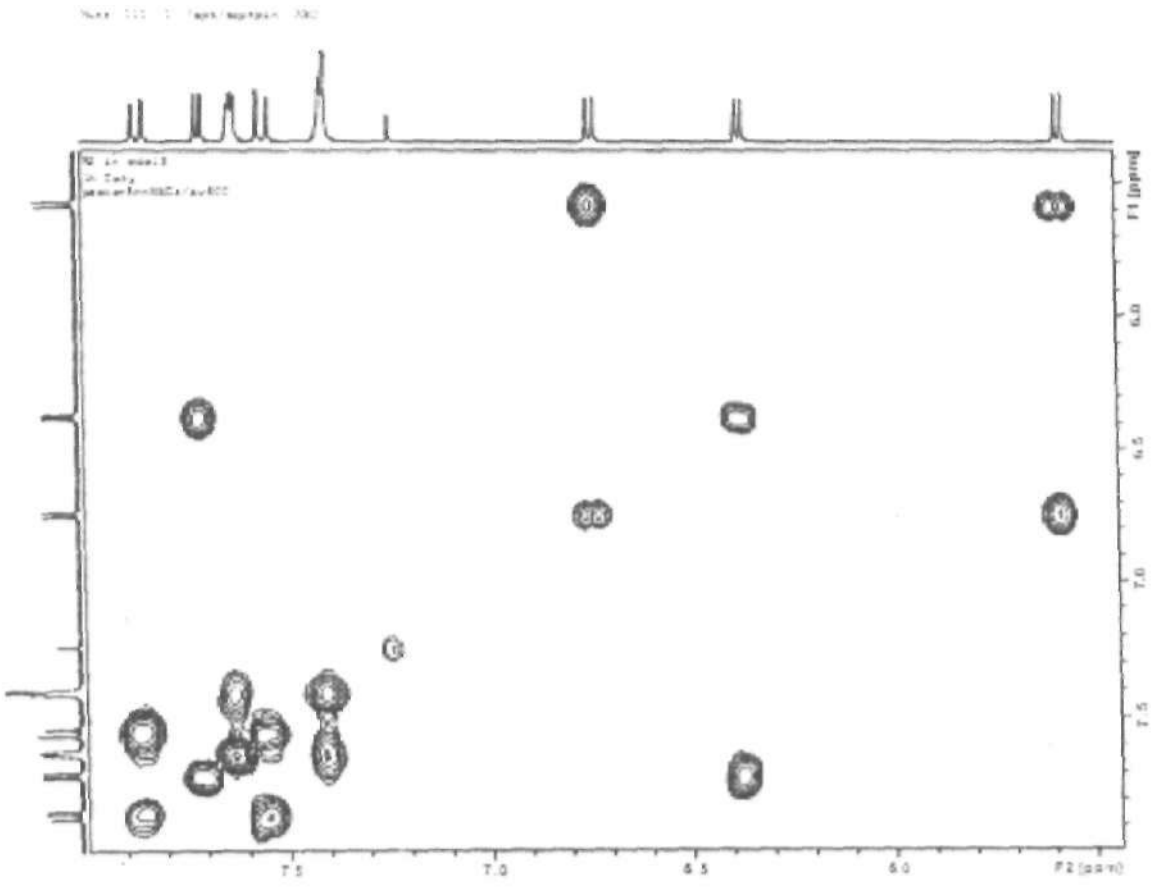


Figure 4.17: COSY spectrum of R_2 (Aromatic region)

4.19 HSQC Spectral Analysis of R_2

The HSQC spectrum of R_2 is as shown in figures 4.18, 4.19 and 4.20 and was used to assign protons on their respective carbons (Table 4.6)

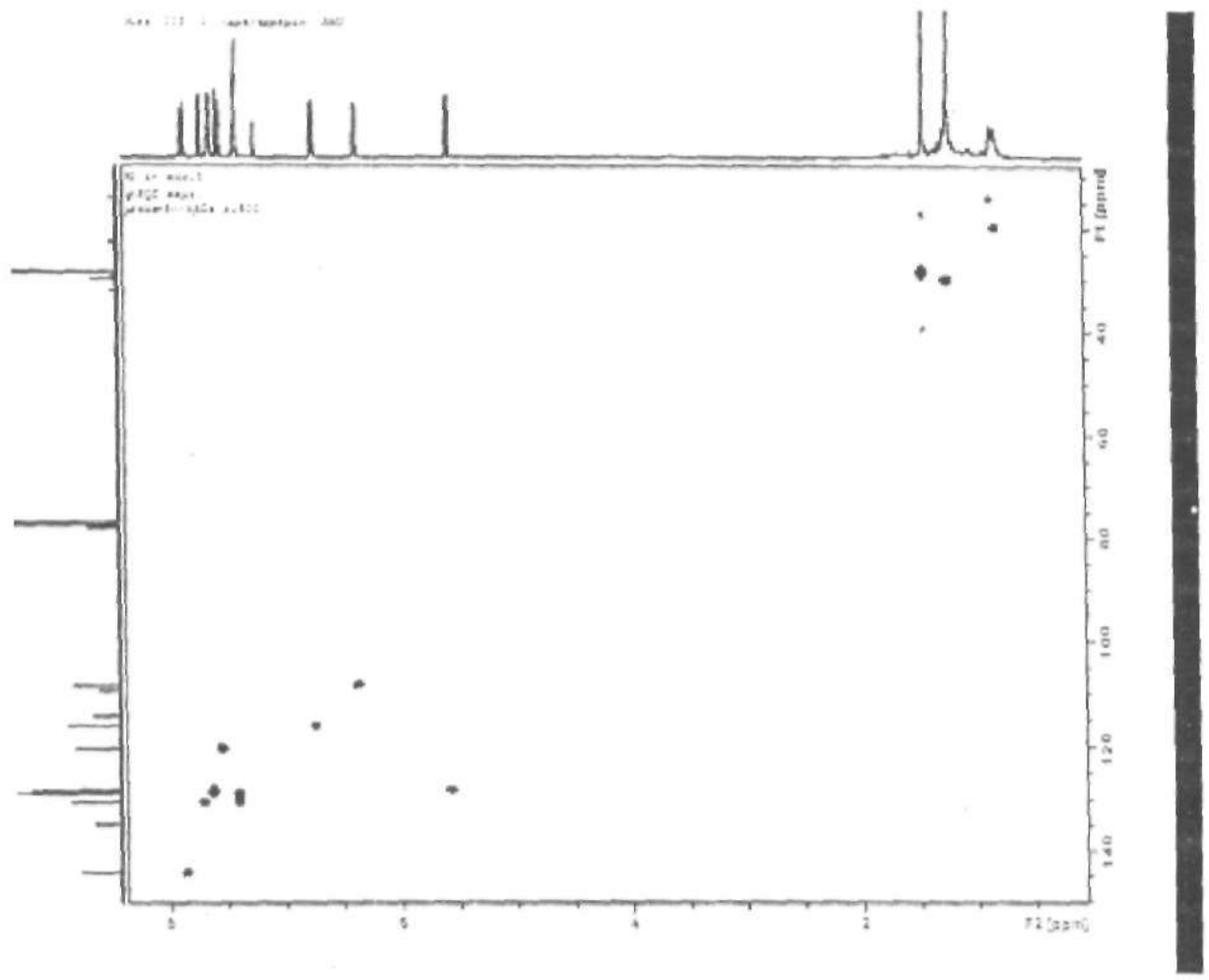


Figure 4.18: HSQC spectrum of R_2

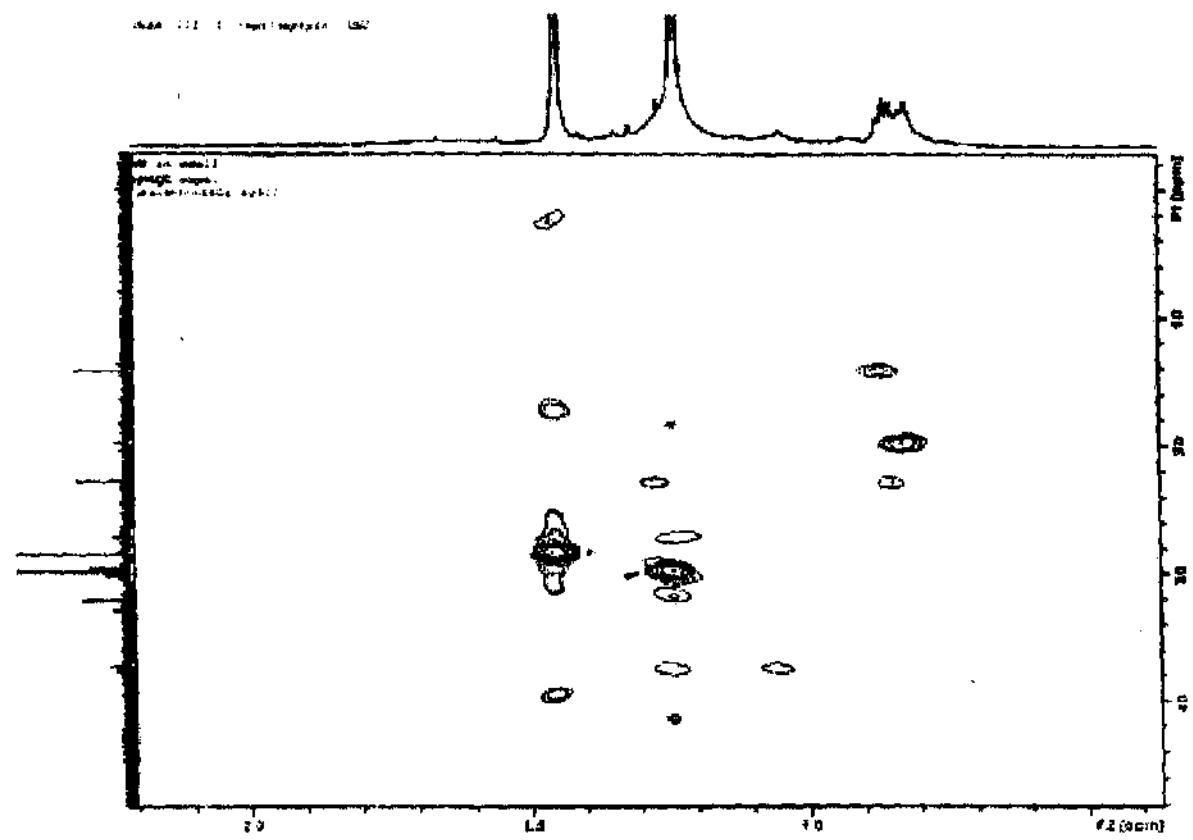


Figure 4.19: HSQC spectrum of R_2 (Aliphatic region)

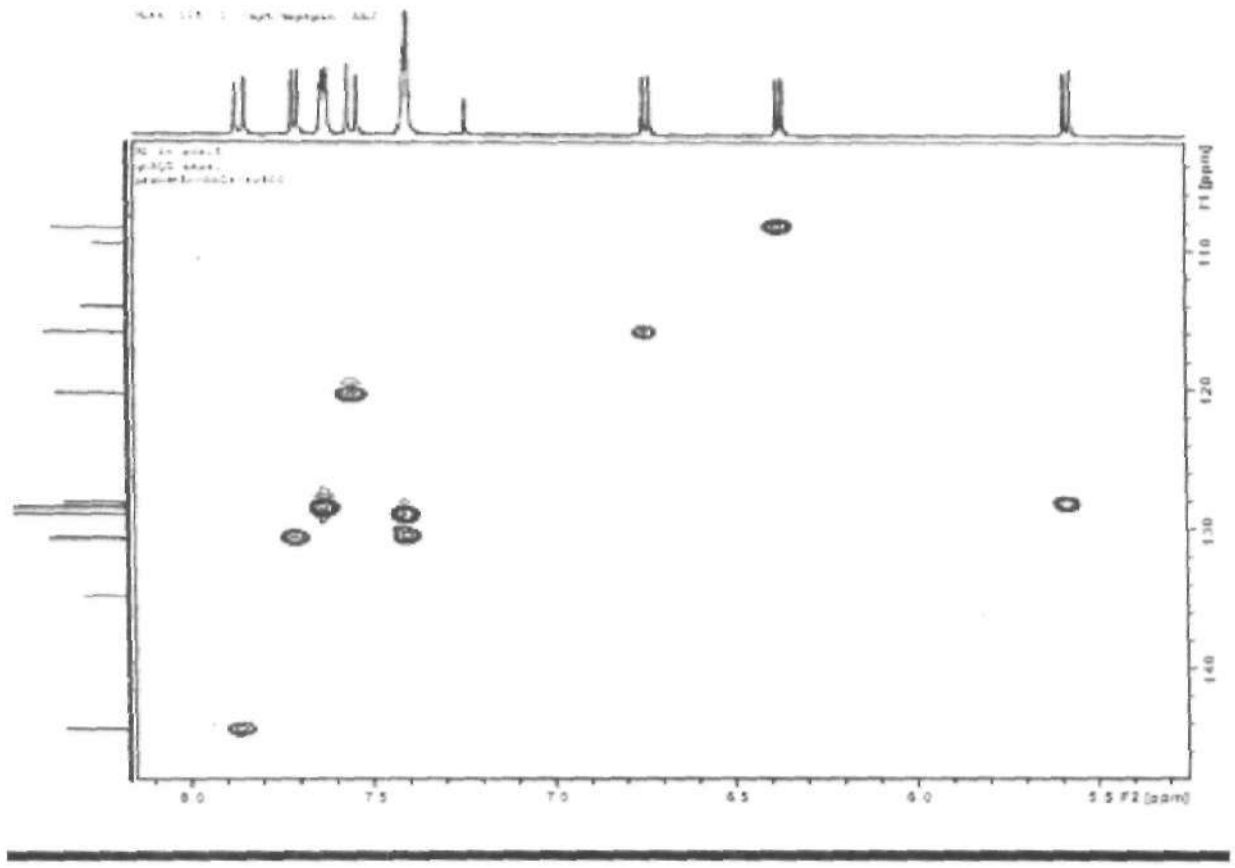


Figure 4.20: HSQC spectrum of R_2 (Aromatic region)

Table 4.6: Table showing the result of HSQC and DEPT experiment of R_2

Position	δ_C (ppm)	δ_H (J) Hz	DEPT
1	134.8		C
2, 4	128.6	7.40 (1.5, 6.4)	CH
3, 5	128.2	7.63 (1.7, 9.0)	CH
6	130.7	7.40 (1.5, 6.4)	CH
1'	109.5		C
2'	161.0		C
3'	114.1		C
4'	159.9		C
5'	108.4	6.37 (8.8)	CH
6'	130.8	7.71 (8.9)	CH
C=O	191.9		C
1''	115.9	6.74 (10.0)	CH
2''	129.0	5.58 (10.1)	CH
3''	29.7	1.26 (4.6)	CH_2
4''	78		C
5''	14.2	0.87 (6.7)	CH_3
6''	28.4	1.50	CH_3
α	120.3	7.55 (15.5)	CH
β	144.2	7.86 (15.4)	CH

4.20 HMBC Spectral Analysis of R_2

The HMBC spectrum was used to establish the connectivity between the different parts of the molecule (Figures 4.21, 4.22 and 4.23) (Table 4.7).

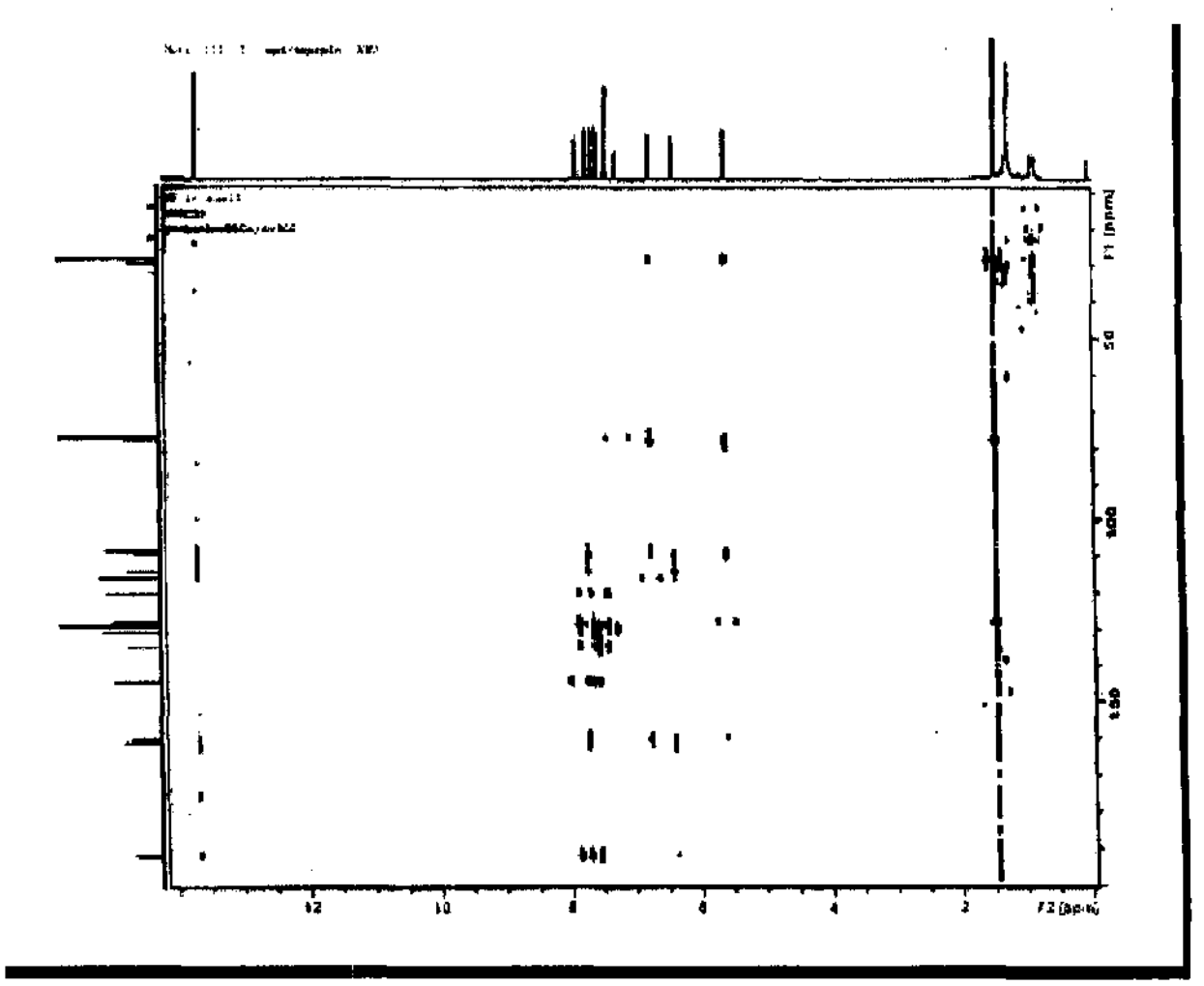


Figure 4.21: HMBC spectrum of R_2

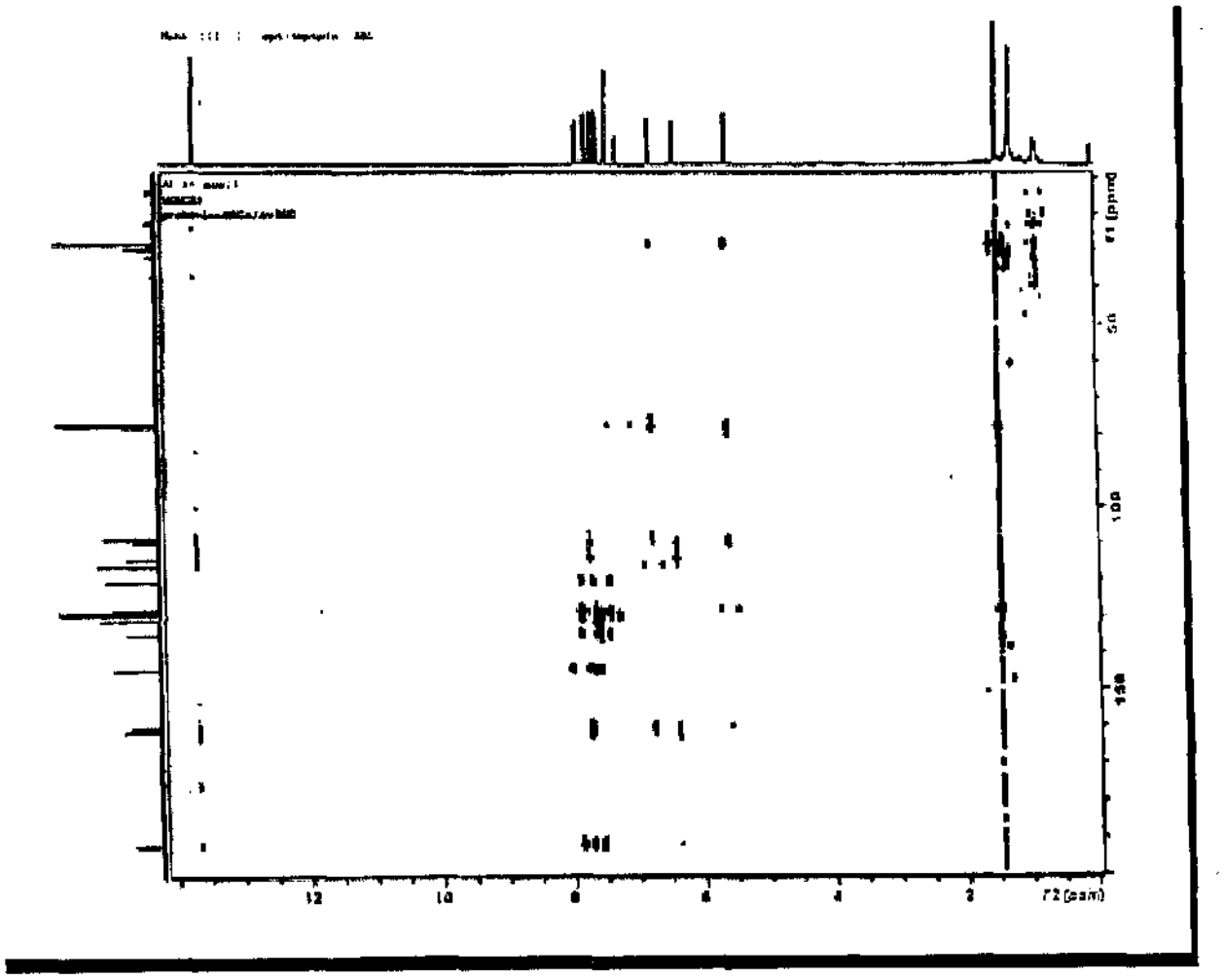


Figure 4.21: HMBC spectrum of R_2

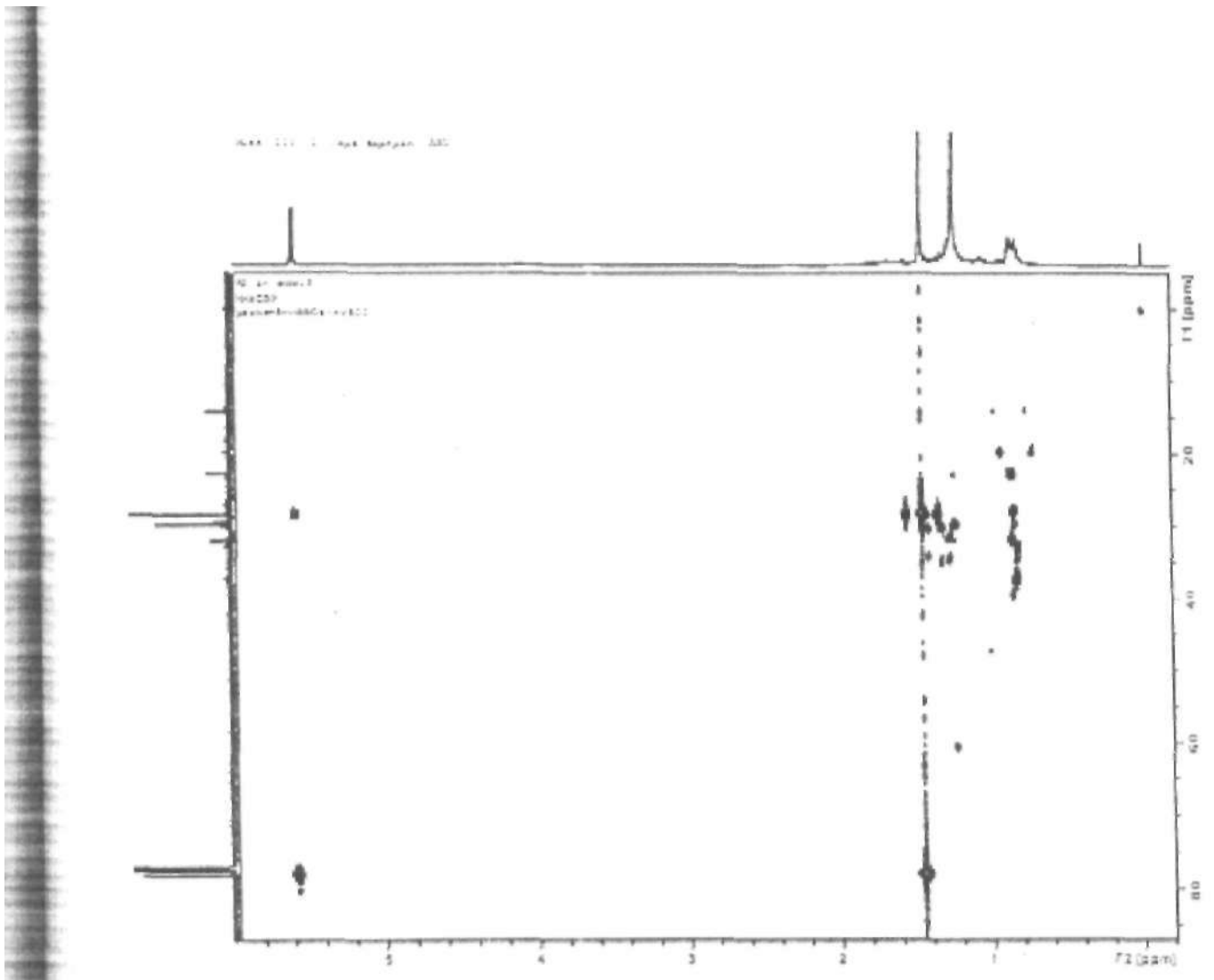


Figure 4.22: HMBC spectrum of R_2 (Aliphatic region)

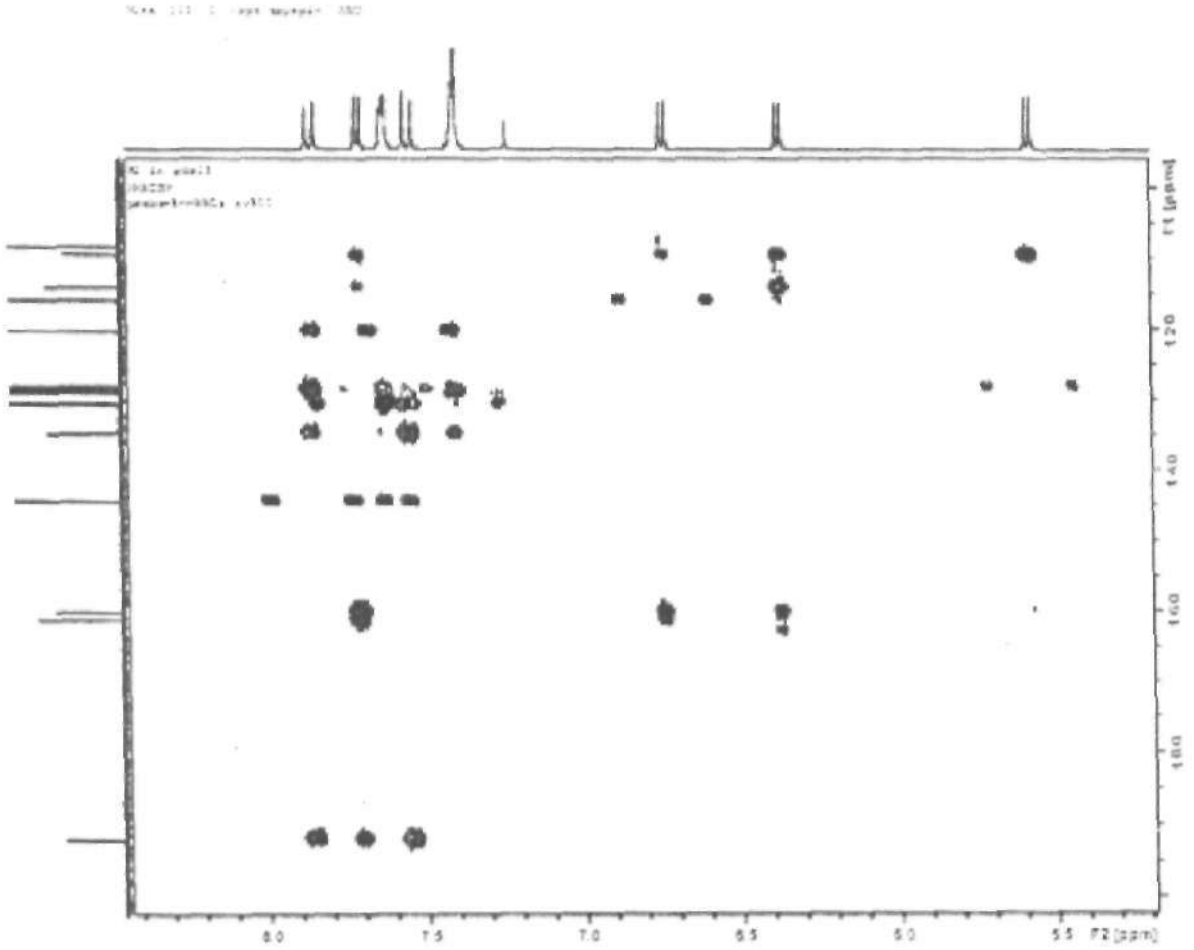


Figure 4.23: HMBC spectrum of R_2 (Aromatic region)

Table 4.7: Table showing the result of major HMBC correlations of R_2

δ_H	δ_C
7.86 (H-β)	C=O, C-1, C-6, C-2, C-α
7.71(H-6')	C=O, C-2', C-4', C-1'
7.63 (H-3,H-5)	C-1, C-3, C-5, C-2, C-4
7.55 (H-α)	C=O, C-β, C-1
7.40(H-2,H-4)	C-1, C-2, C-4, C-3, C-5, C-α
6.74(H-1")	C-2', C-4', C-1"
6.37(H-5')	C-4', C-3', C-1', C-5'
5.58(H2")	C-2", C-1", C-4", C-3"

4.21 Result of Acute Toxicity Studies

In the first phase, death was observed only in the group that received 1000 mg/kg. In the second phase, the highest dose for which the animal survived was 800 mg/kg while 1600 mg/kg was the lowest lethal dose. Therefore, the LD_{50} was calculated as 1131.3 mg/kg.

4.22 Result of PTZ induced Seizure Test

All the control animals exhibited threshold seizures and loss of righting reflex with tonic forelimbs extension. The extract exhibited anticonvulsant effect on seizure induced by subcutaneous PTZ. It offered maximum protection against threshold seizure with 50% protection at a dose of 150mg/kg (Table 4.8)

Table 4.8: Effect of CF against Pentylenetetrazol-induced seizure in mice

Treatment ($mgkg^{-1}$)	Percentage protection	Mean Onset of Seizure in Unprotected animals (min)	Percentage protection against Mortality
Normal saline	-	6.33 ± 1.2	13.33
CF 75	16.7	5.6 ± 1.75	50.00
CF 150	50.00	8 ± 0.58	83.33
CF 300	33.33	5.8 ± 1.39	67.67
VPA (200)	100.00	-	100.00

Protection against seizure and mortality expressed as percentages; Mean onset of seizure expressed as mean ± SEM; CF: Chloroform soluble fraction; VPA: Valproic acid; n=6

4.23 Result of 4-AP induced Mortality Test

The extract did not produce a significant protection against 4-AP induced mortality compared to the control. However, the extract produced an increase in the mean onset of seizure in unprotected animals (Table 4.9)

Table 4.9: Effect of CF against subcutaneous 4-Aminopyridine-induced Mortality in mice

Treatment ($mgkg^{-1}$)	Percentage protection	Mean Onset of Seizure in Unprotected animals (min)	Percentage protection against Mortality
Normal saline	13.33	15.8 ± 1.2	33.33
CF 75	23.33	22.5 ± 2.9	33.33
CF 150	13.33	17.4 ± 1.44	13.33
CF 300	26.67	19.75 ± 3.66	33.33
PBT (20)	100	-	100

Protection against seizure and mortality expressed as percentages; Mean onset of seizure expressed as mean ± SEM; CF: Chloroform soluble fraction; PBT: Phenobarbitone; n=6

4.24 Result of MEST

All the control animals exhibited seizures after the delivery of electroshock. The extract did not protect the animals against tonic hind limbs extension at all the tested doses (Table 4.10)

Table 4.10: Effect of CF against Maximal Electroshock seizure in chicks

Treatment ($mgkg^{-1}$)	Percentage protection	Mean Recovery time in Unprotected animals (min)
Normal saline	0/10	4.0 ± 0.68
CF 75	0/10	5.2 ± 0.57
CF 150	0/10	7.2 ± 0.65
CF 300	0/10	6.1 ± 0.78
Phenytoin (20)	7/10	5.67 ± 1.86

Protection against seizure expressed as percentages; Mean recovery time expressed as mean ± SEM; CF: Chloroform soluble fraction; n=10

CHAPTER FIVE

DISCUSSIONS

The preliminary phytochemical screening of the chloroform fraction revealed the presence of phenolic compounds, flavonoids, steroids and triterpenes. The triterpenoid isolated in this work could be responsible for the anti-inflammatory activity of the extract reported by Musa *et al.*, (2007). Silica gel column chromatography of the chloroform fraction using Hexane: chloroform for gradient elution followed by preparative TLC led to the isolation of R_1 which was soluble in chloroform.

The structure of R_1 was elucidated by spectroscopic analysis as well as by comparison of its spectral data with previously reported values.

The UV spectrum of R_1 showed wavelength of maximum absorbance at 207nm. This indicates the absence of conjugation in the system.

The ^{1}H NMR spectrum revealed the presence of seven tertiary methyl protons at δ 0.77, 0.79, 0.85, 0.94, 0.97, 1.05 and 1.65 (3H-each). A sextet of one proton at δ 2.37 was ascribed to 19β – H. The H-3 proton showed a multiplet at δ 3.2 while a pair of broad singlet at δ 4.55 and δ 4.70 (1 H each) is indicative of exomethylene double bond at H-29. These resonances are typical of lupane type triterpenoid (Jain and Bari, 2010).

The result of C-13 and DEPT NMR showed seven methyl groups at [δc: 28.0 (C-23). 18.0 (C-28). 16.1 (C-25). 16.0 (C-26). 15.5 (C-24). 14.8 (C-27) and 19.5 (C-30)], an exomethylene group at [δc: 109.3 (C-29) and 151.0 (C-20)] and a secondary hydroxyl bearing carbon at [δc: 79.0 (C-3)], in addition to ten methylene, five methine and five quaternary carbons. The deshielded

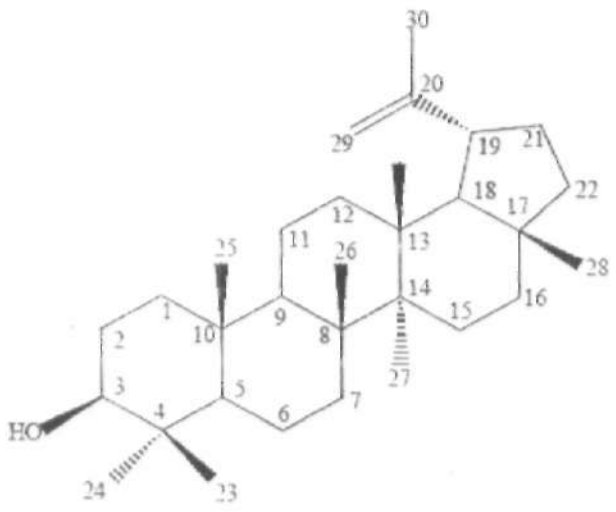
signal at δc 79.0 was due to C-3 with a hydroxyl group attached to it. The deshielding of C-23 methyl could be due to the influence of adjacent C-3 hydroxyl group. The ^{13}C resonances confirmed the presence of lupane type triterpenoid (Jain and Bari, 2010).

Confirmation of the final structure of R_1 was accomplished by 2D NMR experiments (COSY and HMBC).

The COSY spectrum of R_1 showed some cross peaks such as between δ_H 2.37, H-19 and one Sp^3 methylene proton signal (δ_H 1.37, H-21) and another Sp^3 methine proton signal (δ_H 1.89, H-18); and between oxygenated methine proton signal (δ_H 3.2, H-3) and Sp^3 methylene signal (δ_H 1.60, H-2).

In the HMBC spectrum, the methine proton signal at δ_H 3.2 (H-3) showed cross peaks with a methyl carbon signal (δc 28.0, C-23) by J_3 correlation. The sextet methyl signal at δ_H 2.37 (H-19) showed cross peaks with two methylene carbon signals [δc 29.9 (C-21) and δc 109.0 (C-29)], a methine carbon signal [δ_c 48.3 (C- 18). a methyl carbon signal [δ_c 19.5 (C-30)] and a quaternary carbon signal [δ_c 151.0 (C-20)]. The pair of broad singlet of olefinic proton at δ_H 4.55 and 4.70 showed cross peaks with a methylene carbon signal [δ_c 48.0 (C-19) and δ_c 19.5 (C-30)] by J_3 correlation.

Based on the result of 1D and 2D NMR and comparing the data obtained with that in the literature, R_1 was suggested to contain lupeol with the following structure:



Lup-20(29)-ene-3β-ol

In addition to resonances due to lupeol, there are proton shift at 5.2 due to olefinic proton and at 3.7 due to proton adjacent to OH. The C-13 and DEPT NMR also revealed two olefinic carbons at 121.0 and 145.0 and methylene carbon bearing hydroxyl group at 63.0. All these together with additional methyls, methylene and other quaternary carbons suggest the presence of likely another triterpene. The structure could not be deduced due to so many overlaps.

Triterpenes have rare reports in the fungal and animal kingdom (Lutta *et al.*, 2008) but its known to have vast occurrence in diverse plant families (Connolly and Hill, 2008) and is even found in propolis (Pereira *et al.*, 2002). Lupeol has been studied for more than a century. Recently, researches regarding biotransformation, chemoprevention, mechanisms of action, derivative synthesis and methods of detection have been carried out with lupeol.

Lupeol is produced by several organisms from squalene oxide. Dammarane and Baccharane skeletons are formed as intermediates. The reaction is catalyzed by lupeol synthase.

Lupeol has a complex pharmacology in humans, displaying antiprotozoal, antimicrobial, anti-inflammatory, cytotoxic and chemo preventive properties (Geetha and Varalakshmi, 2001).

The ^{1}H NMR spectrum of R_2 exhibited signals for ortho coupled hydrogens at δ_H 6.37 (d, 1H, J=8.8 Hz, H-5') and δH 7.71 (d, 1H, J=8.9 Hz, H-6') due to 1,2,3,4 tetra substituted aromatic ring. It also revealed the presence of two hydrogens at δH 7.55 (d, 1H, J=15.5 Hz, H-α) and δH 7.86 (d, 1H, J=15.4 Hz, H-β); this is typical of olefinic hydrogen in trans arrangement. It also showed two multiples at δH 7.40 (3H) and δH 7.63 (2H) corresponding to the five protons of a second monosubstituted aromatic ring. The downfield signal at δH 13.7 (s) indicates the presence of a free hydroxyl group. Other resonances at δ_H 5.58 (d, 1H, J=10.1 Hz, H-2'') and δ_H 6.74 (d, 1H, J=10 Hz, H-1'') typical of olefinic protons in cis-arrangement, δ_H 1.26 (d, J=4.6Hz, H-3'') due to methylene group integrating for two protons and 0.87(m) integrating for two methyl proton are indicative of the presence of a prenyl moiety as a side chain of the molecule.

The ^{13}C spectrum of R_2 showed 21 signals due to two benzene rings, a prenyl group at δ_C 115.9, a carbonyl carbon at δ_C 191.9 and the α and β carbons appeared at δ_C 120.3 and δ_C 144.2 respectively.

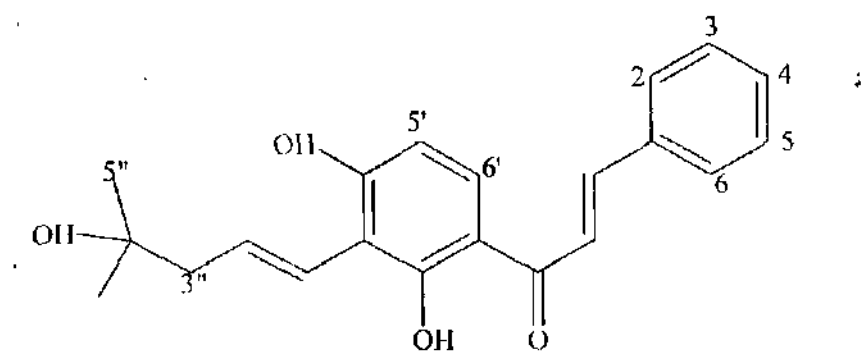
The DEPT experiment disclosed two carbon resonances for methyl groups, a methylene carbon at δ_C 29.7 and nine methine carbons at δ_C 144.2, 130.8, 130.7, 129.0, 128.6, 128.2, 120.3, 115.9 and 78.0.

The assignment of the carbons and the placement of the prenyl side chain were achieved using 2D NMR experiments.

The COSY spectrum of R_2 showed four important cross peaks between δ_H 7.55 (H-α) and δ_H 7.86 (H-β); 5.58(H-2'') and δ_H 6.74(H-1''); δ_H 6.37 (H-5') and δ_H 7.71 (H-6') and δ_H 7.40 (H-2, 4, 6) and δ_H 7.63 (3, 5) indicating protons that are attached on adjacent carbons.

In the HMBC spectrum, a common J_3 correlation between δ_H 7.63 (H-3'5') and δ_H 7.55 (H-α) and a J_2 correlation between and δ_H 7.86 (H-β) and δ_H 7.40 (H-6, 2) to a quaternary carbon at δ_C (134.8) confirm its assignment as C-1 also a common J_3 correlation between δ_H 7.86 (H-β) and δ_H 7.71 (H-6) to a quaternary carbonyl carbon at δ_H 191.9 confirm that C=O is linked to C-1' of ring A .

In the prenyl side chain, the methine proton signal at δ_H 5.58 (H-2'') showed cross peak with a quaternary carbon signal at δ_C 78.0 by J_3 correlation and a methylene carbon signal at δ_C 29.7 by J_2 correlation. The methyl hydrogens showed J_3 correlation to the methyl carbons of their respective partners and to the methylene carbon at δ_C 29.7. They also showed J_2 correlation to the quaternary carbon bearing the hydroxyl group at δ_C 78.0 on which they are attached. A J_3 correlation between δ_H 5.58 an a J_2 correlation between δ_H 6.64 to a quarternary carbon at δ_C 114.1 (C-3') indicates that the prenyl moiety is joined to the molecule through C-3' carbon. Thus the structure below is proposed for R_2



2',4'-dihydroxy-3'-(4-hydroxy-4-methyl pentenyl)-chalcone

To the best of our search, this is the first report of the isolation of this compound. However, the isolation of many similar prenylated chalcones such as dihydrocordoin (Alejandro and Luis, 2009) was reported from the same genus.

Chalcones (trans-1,3-diaryl prop-2-ene-1-one) are α,β unsaturated ketones consisting of two aromatic rings having diverse array of substituents. They are precursors of open chain flavonoids which are abundant in edible plants. They are key precursors in the synthesis of many biologically important heterocyclic compounds such as benzothiazepine, pyrazolines, 1,4 diketones and flavones (Rahman, 2011). Chalcones and its derivatives have attracted increasing attention due to numerous pharmacological applications. They have displayed a broad spectrum of pharmacological activities among which are antimalarial (Lim, *et al.*, 2007). anticancer (Achanta, *et al.*, 2006). anticonvulsant (Kaushik, *et al.*, 2010). anti-inflammatory (Zhang, *et al.*, 2010) and antioxidant (Sivakumar, *et al*, 2010).

The intraperitoneal median lethal dose of CF found to be 1131.3 mg/kg in mice suggests that it is relatively toxic (Matsumura, 1985). However, it is relatively safe at the doses employed in the studies.

Anticonvulsant activity in subcutaneous PTZ test identifies compounds that can raise the seizure threshold in the brain (White *et al.*, 1998). One of the mechanisms by which such drugs act is the suppression of T-type calcium currents in thalamic neurons (Rho and Sanker, 1999). Similarly, agents such as the barbiturates and benzodiazepines, which enhance GABAergic transmission, are known to be protective against PTZ-induced seizure. The ability of CF to protect the animals against PTZ-induced seizure suggest that CF contain compounds useful in petit mal epilepsy since all the antiepileptic drugs that are effective in the treatment of petit mal epilepsy can

suppress various seizure patterns induced by pentylenetetrazol (Loscher *et al.*, 1991). The biphasic nature of the anticonvulsant activity of the extract may be due to the presence of other constituents in the extract which in high doses may be pro-convulsants.

Maximal electroshock seizure can be prevented by Sodium channel blockers such as phenytoin, valproate, felbamate and lamotrigine (Sayyah *et al.*, 2004) or agents that block glutamatergic neurotransmission mediated by N-methyl-D-Aspartate (NMDA) receptors (Subramaniam *et al.*, 1995). The absence of anticonvulsant activity in MEST suggests that CF may not be useful in the treatment of generalized tonic clonic and partial seizures.

K^+ channels play a significant role in controlling all aspect of neuronal excitability (Wickenden, 2002). Sodium channel blockers, such as phenytoin which prevent seizure spread effectively antagonize seizures induced by K^+ channel blocker such as 4-Aminopyridine while those with specific actions on other cellular targets may be weak or inactive, presumably because they are unable to attenuate the spread of intense (non-NMDA receptor mediated) excitation evoked by 4-Aminopyridine (Yamaguchi and Rogawski, 1992). The inability of the fractions to produce significant activities against 4-AP induced seizure suggests that they may likely not be interacting with k^+ channel in producing their anticonvulsant activities. Triterpenes and steroids, among other phytochemicals have been reported to possess anticonvulsant activity in some experimental seizure models such as PTZ (Katsure *et al.*, 2002; Chauhan *et al.*, 1988). These constituents may be responsible, either singly or in combination for the anticonvulsant property of the chloroform fraction of *Lonchocarpus sericeus*.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Preliminary phytochemical screening of the methanol extract of *L. sericeus* revealed the presence of triterpenes, steroids, phenolic compounds, saponins and flavonoids.

Extensive phytochemical investigation of the chloroform soluble fraction led to the isolation of lup-20(29)-ene-3β-ol and 2',4'-dihydroxy-3'-(4-hydroxy-4-methyl pentenyl)-chalcone.

Pharmacological studies on the chloroform soluble fraction showed that 150mg/kg protected 50% of test animals against pentylenetetrazol induced seizure in mice; as such may be useful in the management of petit mal epilepsy.

6.2 Conclusion

Based on the findings in this work, it can be concluded that the use of *L. sericeus* in the management of convulsion has scientific basis. The chalcone isolated from the chloroform soluble portion of the extract may be partly responsible for the anticonvulsant activity. The isolation of lupeol reported in this work may also be partly responsible for the previously reported anti-inflammatory activity of the plant (Musa *et al.*, 2007)

6.3 Recommendations

In order to fully validate the ethnomedicinal uses of the stem bark, it is recommended that

1. Detailed anticonvulsant studies exploiting other seizure models should be carried out on CF to ascertain the mechanism of its anticonvulsant activity.

2. Anticonvulsant activity should also be carried out on other fractions of the methanol extract to investigate their anticonvulsant potential.
3. The isolated compounds should be subjected to pharmacological screening to determine their medicinal value and safety margin.
4. More compounds should be isolated and subjected to biological/pharmacological activity based on the folkloric claims of the plant.

BIBLIOGRAPHIES

Achanta, G., Modzelewska, A., Feng, L., Khan, S. R. and Huang, P. (2006). A Boronic Chalcone Derivative Exhibits Potent Anticancer Activity through Inhibition of the Proteasome, *Molecular Pharmacology*, 70:426-433

Alejandro, Y. and Luis, M. P., (2009). Isocordoin Derivatives from the Root Extract of *Lonchocarpus xuul*, *Journal of Mexican Chemical Society*, 53(1):12-14

Amegnona, A. and Messanvi, G., (2009). Hepatoprotective Effect of *Lonchocarpus sericeus* Leaves in CCl_4-Induced Liver Damage, *Journal of Herbs, Spices and Medicinal Plants*, 15(2):216-226, Retrieved on May 10, 2012, from http://www.informaworld.com

Blinov, R. A., Carison D., Flyasber, M. E., Martin G. E., Martirosian, E. R., Molodotsiv, S. and Williams A. J., (2003). Computer Assisted Structure Elucidation of Natural Products with Limited 2D NMR Data; Application of Structure System, *Magnetic Resonance Chemistry 41*: 359-372

Brain, K. R.and Turner, T. D., (1975). *The Practical Evaluation of Phytopharmaceutical*, Wright Scientechnica, Bristol, pp 11-35

Burkill, H. M., (1995). *The Useful Plants of West Tropical Africa*, Second Edition, the Royal Botanic Gardens, Kew, pp 388-392

Cannell, R..J. P., (2000). *Methods in Biotechnology Natural Products Isolation*, Humana Press, New Jersey, pp 1-52

Chauhan, A. K., Dobhal, M. P. and Joshuo, B. H., (1988). A review of Medicinal Plant Showing Anticonvulsant Activity, *Journal of Ethnopharmacology*, 22: 11-23

Connolly, J. H. and Hill, R. A., (2008). Triterpenoids. *Natural Product Reports* 25, 794-830

Dobelis, I. M (1993). *Magic and Medicine of Plants*, The Readers Digest Association Inc. Pleasant, New York, pp 8-48

Elujoba, A. A., (1999). *Pharmacognosy for Health and Culture, the PHC Jungle Connection*, Inaugural Lecture Series 134, Obafemi Awolowo University Press Limited, Ile-Ife

Epilepsy Foundation of America (2010). Retrieved from http://www.epilepsyfoundation.com

Evans, S. V., Fellows L. E., Shing T. K. M. and Fleet G. W. J., (1985). Glucosidase Inhibition by Plant Alkaloids which are Structural Analogues of Monosaccharide, *Phytochemistry*, 24(9):1953-1955

Ezeagu, I. E., Petzke, K. J., Lange, E., Metges, C. A., (1998). 'Fat Content and Fatty Acid Composition of Olis Extracted from Selected Wild-gathered Tropical Plant Seeds from Nigeria', *Journal of the American Oil Chemists Society*, 75 (8):1031-1035

Field, L. D., Sternhell, S. and Kalman, J. R., (1995). *Organic Structures from Spectra*, Second Edition, John Wiley and Sons,, No city of publication mentioned, pp 57-70

Finar, I. L., (1975). *Organic Chemistry*, Vol. 2, Stereo-Chemistry and the Chemistry of Natural Products, Fifth Edition, Longman, London, pp 789-799

Fontenele, J. B., Leal, L. K. A. M., Ferreira, M. A. D., Silvera, E. R. and Viana, G. S. B., (2005). Antiplatelet Effect of Lonchocarpin and Dericin Isolated from *Lonchocarpus sericeus*, 43(8):726-731. Retrieved May 12, 2012, from www.infomaheathcare.com,

Ganellin, C. R., (1993). General Approach to Discovering New Drugs: A Historical Perspective, in Ganellin C. R., and Robert, S. M., (Eds.). Medicinal Chemistry: The Role of Organic Chemistry, *Drug Research*, Second Edition, pp 121-122

Geetha, T. and Varalakshmi, P., (2001). Anti-inflammatory Activity of Lupeol Linoleate in rats, *Journal of Ethnopharmacology*, 76(1): 77-80

Gibbons, S. and Gray, A. I., (1998). 'Isolation by Planar Chromatography' in Cannell, R. J. P., (Eds.). *Natural Products Isolation*, Humana Press, New Jersey, pp 209-246

Grifarn, F., Newman, D., Fairfield, A. S., Bharttacharya, B., Grupenho J. T., (1997). 'The Origin of Prescription Drugs' in Grifarn, F., and Rosenthal, J., (Eds.). *Biodiversity and Human Health*, Island Press Washington DC, pp 1-75

Hostettmann, K., Wolfinder J. L., Rodriguez, S. and Marston, A., (1996). 'Strategy in the Search for Bioactive Plant Constituents', in Hostettman K., Chiyanganya, F., Maillard, M., and Wolfinder, J. L., (Eds.). *Chemistry, Biological and Pharmacological Properties of Afriical Medicinal Plants*, Proceedings of the Symposium by International Organization for Chemical Sciences in Development, (IOCD). University of Zimbabwe Publications Limited, pp 29-34

Jain, P. S. and Bari, S. B., (2010). Isolation of Lupeol Stigmasterol and Campesterol from Petroleum Ether Extract of Woody Stem-Bark of *Wightia tinctoria*, *Asian Journal of Plant Sciences*, 9(3):163- 167

Joan, C., (1994). *Methodology for Analysis of Vegetable Drugs*, no city of publication mentioned pp 19-21

Kafura, I., (1999). Immense Help from Nature Workshop, Elikaf Health Service Limited, no city of publication mentioned, pp 12-13

Katsure, V. S., Katsure,S. B and Chopde, C. T., (2002). Anticonvulsive Activity of *Butea Monosperma* Flowers in Laboratory Animals, *Pharmacol Biochem Behar*, 72: 965-972

Kaushik, S., Kumar, N. and Drabu, S., (2010). Synthesis and Anticonvulsant Activities of Phenoxychalcones, *The Pharmaceutical Research*, 3:257-262

Kinghorn, A.(1992). 'Plant as Source of Medicinally and Pharmaceutically Important Compounds', in Nigg, H. N., and Seigler D., (Eds.). *Phytochemical Resources for Medicine and Agriculture*, Plenum Press, New York, pp 45-60

Lim, S. S., Kim, H. S. and Lee, D. U., (2007). Invitro Antimalarial Activity of Flavonoids and Chalcones, *Bulletin of the Korean Chemical Society*, 28:2495-2497

Lima, A. F., Mileo, P. G., Androde Neto, M. Brazfilho, R., Silveira, E. R. and Pessoa O. D., (2009). *Magnetic Resonance Chemistry*, 47:165

Lorke, D., (1983). A New Approach to Practical Acute Toxicity Testing, *Archives of Toxicity*, 54: 275-289

Loscher, W., Fassbender, C. P. and Nolting, B., (1991). The role of Technical, Biological and Pharmacological Factors in the Laboratory Evaluation of Anticonvulsant Drugs III Pentylenetetrazole Seizure Model, *Epilepsy Research*, 8: 171-189.

Lowestein, D.H and Alldredge, B. K., (1998). Status Epilepticus *National England Journal of Medicine*, 38: 970-976

Lutta, K.P, Bil, C., Akenga, A. and Cornelius, W., (2008). Antimicrobial Marine Natural Product from the sponge *Axinella infundibuliformis*. *Records of Natural Products* 2:116-127

Mabry, T. J., Markham, K. R. and Thomas, M. B. (1969). *The Systematic Identification of Flavonoids*, Springer- Verlag, Berlin, chapter 5:8

Mahato, S. B. and Kundu, A. P., (1994). ^{13}C NMR Spectra of Pentacyclic Trierpenoids, A Compilation and Some Salient Features, *Review Article*, 98: 1517

Mahato, S. B., Nandy, A. K. and Roy, G., (1993). Triterpenoids, *Phytochemistry*, 31(7): 2199

Maria, G. B. C., Rochanne, M. S., Paulo N. B., Helcio, S. D., Otilia D. L. P., Raimundo B. and Maria R. J. A., (2012). Furanoflavones and other Chemical Constituents of *Lonchocarpus obtusos*, *Journal of Brazilian Chemical Society*, 0(0):1-5

Matsumura, F., (1985). *Toxicology of Insecticides*, Second Edition, Plenum Press, New York, pp. 588

McNamara J. O., (2006). 'Pharmacotheraphy of the Epilepsies' in Brunton L. L., Lazo, J. S., and Parker, K. L., (Eds.). *Goodman and Gilman's the Pharmacological Basis of Therapeutics*, Eleventh Edition, McGraw Hill, New York, pp 8-48

Musa, A. M., Yaro, A. H. and Abubakar, M. S., (2006). Anticonvulsant Activity of Methanol Extract of the Stem Bark of *Lonchocarpus sericeus*, Poir (Papilionaceae), *Journal of Tropical Biosciences*, 6: 17-20

Musa, A.M., Yaro, A.H. and Anuka J.A. (2007). Phytochemical Screening and Evaluation of Analgesic and Anti-Inflammatory Activities of *Lonchocarpus sericeus* Papilionaceae, *Nigerian Journal of Pharmceutical Research*, 6, (1): 1-5

Olaniyi, A. A (2005). *Essential medicinal chemistry*, Third Edition, Hope Publication, Ibadan, pp 1-70

Onwuekwe, I. O., Onodugo, O.D., Ezeala-Adikaibe, B., Aguwa E. N., Ejim, E. C., Ndukuba, K., Abadom, T. R., Illo, C. K. and Onyejizu C., (2009). Presentation and Pattern of Epilepsy in Nigerian Africans: A Study of Trends in the South East, *Transaction of the Royal Society of Tropical Medicine and Hygiene*, 3(8):785-789. Retrieved May 20, 2012, from http://www.tropicalmedandhygienejrnl.net

Partnership for Maternal, New Born and Child Health (PMNCH) Summary Report September, 16-22, 2007

Pasteur L., (1785). *Historical Background of Drug Discovery,* Retrieved May 20, 2012, from www.uga-cdd.org/background.php. Available on line 2006

Pereira, A., Nascimento, E. and Aquinoneto, F., (2002).Lupeol Alkanoates in Brazilian Propolis *Zeitschrift fur Naturforschung* 57c: 721-726

Rahman, M. A., (2011). Chalcones: A Valuable Insight into the Recent Advances and Potential Pharmacological Activities, *Chemical Science Journal*, 2:29, Retrieved online from: http://astonjournals.com/csj, on 31st August, 2012, pp 1-6

Rates, S. M., (2001). Plants as a Source of Drugs *Toxican*, 39: 603-619

Rho, J. M. and Sanker, R., (1999). The Pharmacological Basis of Antiepileptic Drug Actions, *Epilepsia*, 40: 1471-1483

Sayyah, M., Nadjafnia L. and M. Kamalinejad, (2004). Anticonvulsant activity and chemical composition of *Artemisia dracunculus* L. essential oil, *Journal of Ethnopharmacology*, 94: 283-287

Shok, M., (1999). *Allopathic (Orthodox) and Alternative (Homeopathic, Traditional, Herbal etc) Medicines; The Need for Productive Co-operation*, An Inaugural Lecture, Ahmadu Bello University Press Limited, pp 9-10

Silva G. L., Lee I., and Douglas K. A. (1998). *'Special problems with extraction of plants'*. In Cannell J. P. R (eds). Natural Products Isolation. Humana Press Publishers, New Jersey (USA) pp 356-358

Sivakumar, P. M., Prabhakar, P. K. and Double, M., (2010). Synthesis, Antioxidant Evaluation and Quantitative Structure Activity Relationship Studies of Chalcones, *Medicinal Chemistry Research*, 19:1-17

Sofowora, A.,(1993). *Medicinal Plants and Traditional Medicine in Africa*, Second Edition, Spectrum Books Limited, Ibadan, pp 134-156

Subramaniam,S, Rho, J.M, Penix L. Donevan S D, Fielding, R. P and M. A. Rogawski, (1995). Felbamate Block of the N-methyl-D-aspartate Receptor, *Journal of Pharmacology*, 273(2): 878-886

Swinyard, E. A. and Kupferberg H. J. (1985). Antiepileptic Drugs; Detection, Quantification and Evaluation, *Federation Proceedings*, 44: 39-43

Swinyard, E. A., (1989). Laboratory Evaluation of Antiepileptic Drugs, Review of Laboratory Methods, *Epilepsia*, 10: 107-119

Toshihoro, A., Ken. Y., Hirotoshi, O., Yoshimasa, K., Sakae, Y., Michio, T., Kunio, K. and Toshtake, T., (1996). Triterpene-alchohols from the Flowers of Compositae and their Anti-inflammatory Effects, *Phytochemistry*, 43(6):1255-1260

Trease, K. and Evans, W. C., (1996). *Textbook of Pharmacognosy*, Fourteenth Edition, Balliere, Tindall, London, pp 251-293

Venkat, E., and Kathandaraman, S., (1998). 'Super Critical Fluid Methods', in Cannal, R. J., (Eds.). *Methods in Biotechnology Natural Products Isolation*, Humana Press, New Jersey, pp 91-110

White, H. S., Wolf, H. H., Woodhead, J. H. and Kupferberg, H. J., (1998). 'The National Institute of Health Anticonvulsant Drug Development Program Screening for Efficacy', in French, J., Leppik, I. E. and Ditcher. M. A., (Eds.). *Antiepileptic Drug Development: Advances in Neurology*, Luppincott-raven Publishers, Philadelphia, Vol. 76 pp 29-39

WHO, (2003). Traditional medicine in developing countries, in http://www.WHO.Traditional medicine.mht.

WHO, (2008). WHO Global Burden of Disease: 2004 Update, Retrieved May 25, 2012 from www.who.int/healthinfo/globalburden-disease/2004reportupdate/en/index.html.

Wickenden, A.D., (2002). Potassium Channels as Antiepileptic Drug Target, *Journal of Neuropharmacology*, 43:1055-1060

Yamaguchi, S. I. and Rogawski, M. A., (1992). Effects of 4-aminopyridire-induced Seizure in Mice, *Epilepsy Research*, 11: 9-16

Yan X. Z, Kuo Y, Lee T, Shih T, Chen C. H., Donald, R. M., Andrew, T. M., Lee K., (1989). Cytotoxic Components of *Diospyros morrisiana*, *Phytochemistry*, 28 (5): 1541-1543

Zhang, X. W., Zhad, D. H., Quan, Y. C., Sun, L. P., Yin, X. Y. and Guan, L. P., (2010). Synthesis and Evaluation of Anti-inflammatory Activity of Substituted Chalcone