PHYTOCHEMICAL SCREENING AND ANTICONVULSANT STUDIES OF ETHYL ACETATE FRACTION OF ETHANOLIC EXTRACT OF THE LEAVES OF *GLOBIMETULA BRAUNII* (LORANTHACEAE) PARASITING ON *PILIOSTIGMA THONINGII* (FABACEAE)

BY

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AHMADU BELLO UNIVERSITY,

ZARIA, NIGERIA

MARCH, 2013

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B.Sc Chem (BUK) 2008

M.Sc/Pharm.Sci/02072/2009-2010

A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES AHMADU BELLO UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF A MASTERS DEGREE IN PHARMACEUTICAL CHEMISTRY

DEPARTMENT OF PHARMACEUTICAL AND MEDICINAL CHEMISTRY,

FACULTY OF PHARMACEUTICAL SCIENCES,

AHMADU BELLO UNIVERSITY,

ZARIA, NIGERIA

MARCH, 2013

DECLARATION

I declare that the work in this thesis entitled: "**Phytochemical Screening and Anticonvulsant Studies of Ethyl acetate fraction of Ethanolic Extract of the Leaves of *Globimetula braunii* (Loranthaceae) Parasiting on *Piliostigma thoningii* (Fabaceae)"** has been performed by me in the Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, Zaria, under the supervision of Dr A. M. Musa, Dr M. I. Abdullahi and Dr M. I. Sule.

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 this or any other University.

_____ _____

Kamal Ja'afar Muhammad Date

CERTIFICATION

This thesis entitled: **''Phytochemical Screening and Anticonvulsant Studies of Ethyl acetate fraction of Ethanolic Extract of the Leaves of *Globimetula braunii* (Loranthaceae) Parasiting on *Piliostigma thoningii* (Fabaceae)''** by Kamal Ja'afar Muhammad meets the regulations governing the award of a Master of Science degree in Pharmaceutical and Medicinal Chemistry of Ahmadu Bello University, Zaria, and is approved for its contributions to knowledge and literary presentation.

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DEDICATION

To my beloved mother Hajiya Ummuhani Sulaiman Abdulkarim

List of Abbreviations

cm	centimeter
cm^{-1}	Wave number unit
g	gram
g/kg	gram per kilogram
Hz	Hertz
ml/kg	milliliter per kilogram
ml	milliliter
nm	nanometer
ppm	parts per million
UV	ultraviolet spectroscopy
IR	infrared spectroscopy
λ_{max}	maximum absorption wavelength
V_{max}	maximum absorption wavenumber
TLC	thin-layer chromatography
^{1}H-NMR	proton nuclear magnetic resonance spectroscophy
δ	chemical shift in ppm
s	singlet
d	doublet
dd	double doublet
m	multiplet
mg/kg	milligram per kilogram
MHz	mega hertz

%	percentage
EEG	electro encephalography
Hr	hour
R_f value	Retardation factor
β	beta
^{13}C-NMR	Carbon-13 nuclear magnetic resonance spectroscopy
DEPT	Distortionless Enhancement by Polarization Transfer
HMBC	heteronuclear multiple bond coherence
HSQC	heteronuclear single quantum coherence
COSY	correlation spectroscopy
LD_{50}	median lethal dose
EtOAc	ethyl acetate
$CHCl_3$	chloroform
MeOH	methanol

ABSTRACT

The ethyl acetate soluble fraction of ethanol extract of leaves of *Globimetula braunii,* a plant used in ethnomedicine for the treatment of epilepsy and diabetes among other ailments, was subjected to phytochemical and anticonvulsant screening. The phytochemical techniques employed were dry column vacuum chromatography and preparative thin layer chromatography. The anticonvulsant activity was studied using maximal electroshock test, pentylenetetrazole and 4-aminopyridine-induced seizures. The result of preliminary phytochemical screening carried out on the crude ethanol extract revealed the presence of saponins, carbohydrates, flavonoids, tannins, anthraquinones and steroids. Similarly, tannins, flavonoids and steroids/terpenes were found to be present in the ethyl acetate fraction. Extensive chromatographic separation of the ethyl acetate soluble fraction using silica gel, dry column vacuum chromatography, followed by crystallization led to the isolation of 3, 12-bis [2-(4-hydroxyphenyl) ethyl]-5, 7, 14, 16-tetrahydroxy-2, 11-dioxacyclooctadecane-1, 10-dione. The structure of this compound was established by spectral analysis including 1D and 2D NMR. In the pharmacological screening, 150 mg/kg of the fraction protected 83.33% of test animals against pentylenetetrazole-induced seizure in mice whereas sodium valproate a standard anti-epileptic drug offered 100% protection. In the 4-aminopyridine-induced seizure model, the fraction produced an increase in the mean onset of seizure in unprotected animals. The fraction did not exhibit a significant activity against maximal electroshock convulsion. The median lethal dose of the fraction was found to be 1261.91 mg/kg. These results suggest that the ethyl acetate fraction of *Globimetula braunii* Leaves extract possesses psychoactive compound that may be useful in the management of petit mal epilepsy.

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CHAPTER ONE

INTRODUCTION

1.0 General Introduction

Traditional medicine (also known as indigenous or folk medicine) comprises of knowledge that developed over generations within various societies before the era of modern medicine (WHO, 2008). It 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). By the late 1700's, William Withening's account of the Foxglove and its medicinal uses (1785) begun to appear in the United States before the advent of specific pharmaceuticals, traditional medicine was relied upon to treat many illnesses.

The World Health Organization (WHO) defines traditional medicine as; "the health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral-based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illness or maintain well being (WHO, 2009). WHO also notes, that "inappropriate use of traditional medicines or practices can have negative or dangerous effects" and that "further research is needed to ascertain the efficacy and safety of several of the practices and medicinal plants used by traditional medicine system (WHO, 2009).

The African continent has a long history of use of plants and up to 90% of the populations in some countries rely on medicinal plants as a source of drugs. (Hostettmann *et al*., 1996). The reason for relying on traditional medicine include 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).

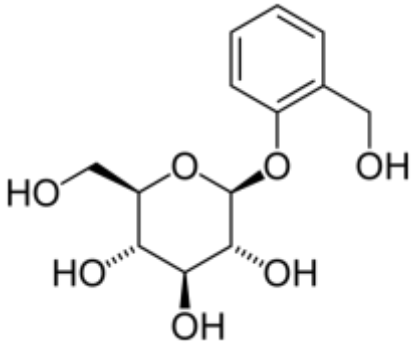
An estimated 25% of prescription drugs and 11% of drugs considered essential by the WHO are derived from plants and a large number of synthetic drugs are obtained from precursor compounds originating from plants (Rates, 2001). Of the top 150 proprietary drugs from the U.S. prescription, 57% contain at 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 structurally diverse potential bioactive organic molecule (Kinghorn, 1992), and higher plants as potential sources of new drugs is still highly unexploited. Only about 10% of the known species has been investigated phytochemically and less than that are investigated biologically (Hostettmann *et al*., 1996).

Development of drugs based on natural product has had a long history in United States, and in 1991 almost half of the best selling drugs were natural products or derivatives of natural

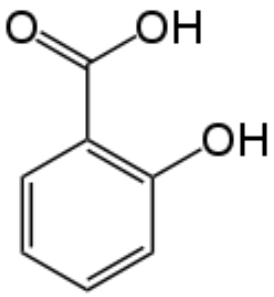
products. There has recently been a surge of interest in herbal remedies; and a Reuters/Zogby Poll in 2000 showed that about 40% of people in the U.S. had tried herbal remedies.

1.1 Brief History of Drug Discovery.

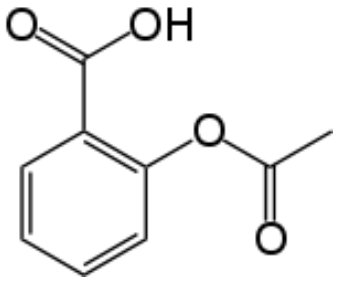
Therapeutic agents have played a major role in increasing average life expectancy worldwide in the last century. However, while many of the drugs in use in the last 50 years or more have been of synthetic origin, the Pharmacopoeias prior to that period were of natural origin (Pasteur, 1785). The treatment of disease with pure pharmaceutical agent is a relatively modern phenomenon. Among the earliest pure compounds discovered was salicin (I) isolated from the bark of the white willow*, Salix alba*, in 1825-26. It was subsequently converted to salicylic acid (II) via hydrolysis and oxidation. The use of salicylic acid, however often led to severe gastro intestinal toxicity. This was overcome when Felix Hoffmann of Bayer Company converted salicylic acid into acetylsalicylic acid (ASA) (III) via acetylation. Bayer then began marketing ASA under the trade name aspirin in 1899. Today, aspirin is still the most widely used analgesic and antipyretic drug in the world (Olaniyi, 2005).



I



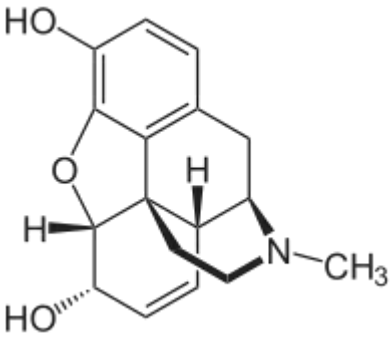
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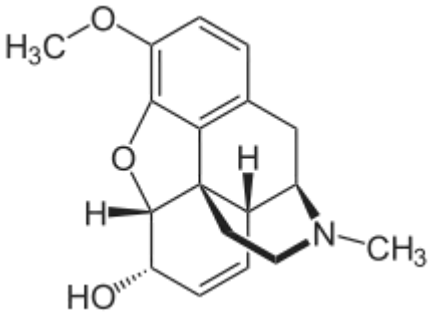
III

Plants-derived products have always been a rich source of lead compounds for potent pain killers such as morphine (IV) and codeine (V), isolated from the *Opium poppy* (Olaniyi, 2005).

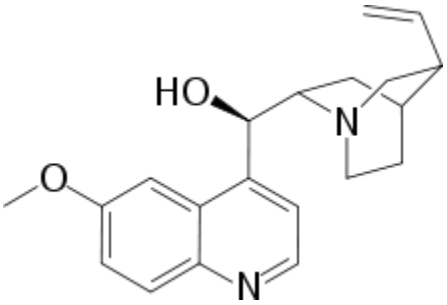
The antimalarial agent quinine (VI) was isolated from cinchona bark, and based on quinine structure, many classes of antimalarials were synthesized including the 4-aminoquinoline (chloroquine) (VII) and 8-aminoquinoline (primaquine) (VIII) (Olaniyi, 2005).



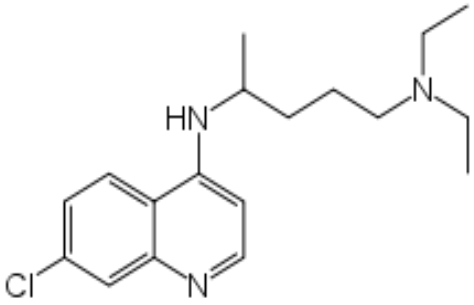
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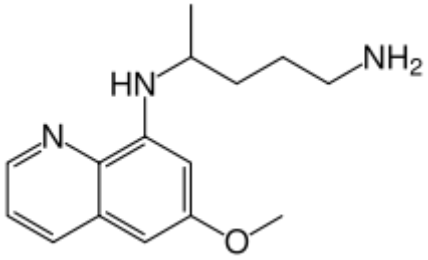
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VI

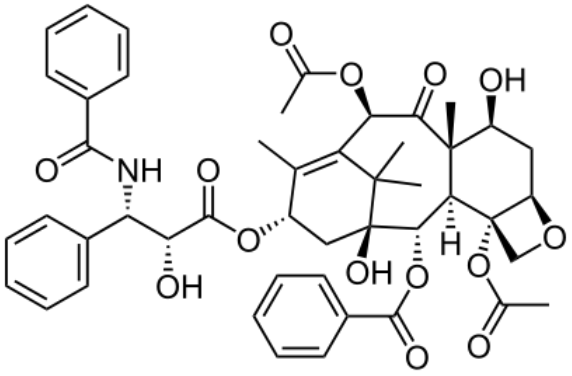


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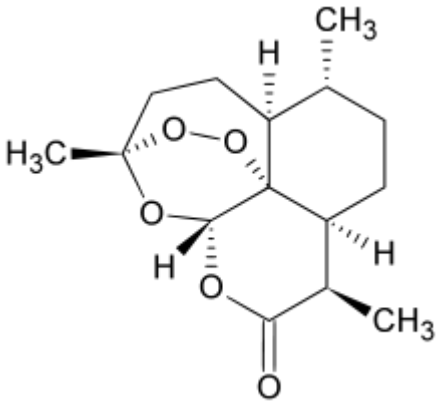


VIII

Clinically useful drugs which have been recently isolated from plants include the anticancer agent paclitaxel (Taxol) (IX), a natural product derived from the yew tree (Cutler and Cutler, 2000) and the antimalarial agent artemisinin (X) from *Artemisia annua*.

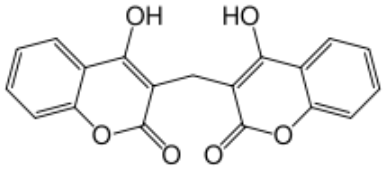


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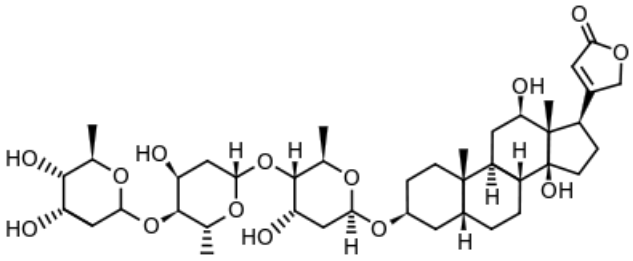


X

Natural products have been and still are an inexhaustible source of drug leads as well as drugs. Compounds isolated from natural products can be used directly as drugs e.g. anticoagulant dicoumarol (XI), isolated from the sweet clover hay *Melilotus officinalis*. Nature still supplies the best drug for the treatment of heart failure e.g. Digoxin (XII), as till date there is no synthetic drug capable of replacing it in heart failure (Olaniyi, 2005).

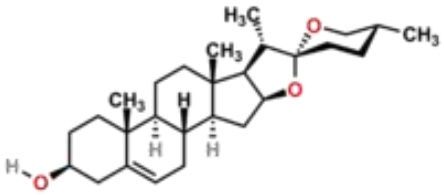


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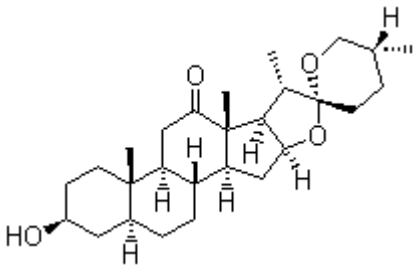


XII

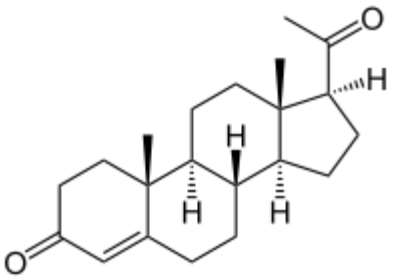
Drugs can also be semi synthesized from natural products leads e.g. the plants steroids diosgenin (XIII) and hecogenin (XIV) remain key precursors for the synthesis of many modern contraceptive drugs such as progesterone (XV) and its derivatives norethindrone (XVI).



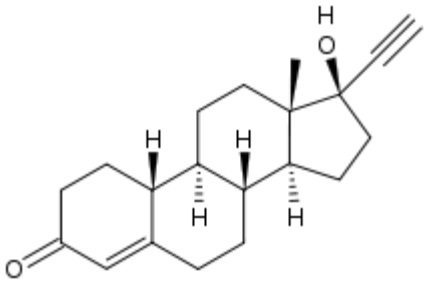
XIII



XIV



XV



XVI

1.2 Epilepsy.

Epilepsy is a common and diverse set of chronic neurological disorder characterized by seizures (Chang and Lowenstein, 2003). Some of the definitions of epilepsy require that seizures be recurrent and unprovoked, (Chang and Lowenstein, 2003; American Academy of Neurology, 2012; and WHO, 2009), but others require only a single seizure combined with brain alteration which increases the chances of future seizures. (Fisher *et al*., 2005).

The term seizures refers to a transient alteration of behaviour due to abnormal synchronized, and repetitive burst firing of neuronal populations in the central nervous system (CNS). Epilepsy is a syndrome of episodic brain dysfunction characterized by recurrent unpredictable spontaneous seizures. A partial seizure begins in a localized brain region, where as generalized seizures show wide spread involvement of both hemispheres from the outset. Epilepsy is usually controlled, but not cured with medication. However, over 30% of people with epilepsy do not have seizures control even with best available medications (Cascino, 1994; Engel, 1996). Seizures 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 (McNamara, 2006).

1.2.1 Mechanism of seizure

A seizure is a massive disruption of electrical communication between neurons in the brain, leading to the temporary release of excessive energy in a synchronized form neurons communicate with each other by firing electrical impulses. These impulses travel from the neuron along the axon, and then stimulate the release of neurotransmitters which flows across the synaptic cleff (Epilepsy Foundation of America, 2012).

If more excitatory than inhibitory transmitters are released the cell will fire, if more inhibitory neurotransmitters are released, the cell will not fire, since large numbers of cell are involved in even simple actions, the on/off action serves to control physical and mental functioning. However, if there is a consistently higher level of the excitatory neurotransmitters, or too few

inhibitory ones, the likelihood of seizures, an uncontrolled, continuing firing of neurons in the brain is increased. Some of the newer medications relate directly to this process and are designed to increase the level of inhibitory neurotransmitters, especially gamma-amino butyric acid (GABA), or to decrease the amount of the excitatory ones, such as glutamate (Epilepsy Foundation of America, 2012).

A sudden burst of neuronal firing may not be sufficient to cause an obvious seizure [although it might show up as a sudden spike on the (electro encephalography) EEG]. However, if the discharge of electrical energy has sufficient power and affects enough neurons, it will produce symptoms characteristic of the area in which the discharge took place. The result could be a sudden muscle jerk, an abrupt fall, a distorted vision. If the disturbance flashes across the whole brain at once, it could produce convulsive seizure, temporarily disrupting many of the functions of the brain. 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 [N-methyl-D-aspatate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) or Kainic acid], trigger seizures in experimental animals, *in vivo*. Conversely, pharmacological agents that enhance GABA medicated 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 pentylenetetrazole (PTZ) (McNamara, 2006).

1.2.2 Status epilepticus and other convulsive emergencies (SE)

Status epilepticus refers to continuous seizures or discrete seizure with impaired consciousness in the interictal period. It is considered by neurologist as a neurological emergency (Lowenstein and Alldredge, 1998). 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).

The incidence of generalized convulsive status epilepticus GCSE among the general population ranges between 8 and 41 cases per 100,000. (Delorenze *et al*., 1996; Lothman, 1990), with a GCSE mortality rates in adults of 10% to 40%, the overall rates of SE was 6.2 per 100,000 with higher rates in children Younger than 5 years (7.5/100,000) and the elderly (22.3/100,000). In children GCSE is most common in the very Young, especially in those younger than 2 years. In this group more than 80% have either a febrile or acute symptomatic (Identifiable) etiology (Sillanpaa *et al.,* 2002).

1.3 Statement of Research Problem.

Epileptic Seizures result from abnormal excessive and hyper synchronous neuronal activity. About 50 million people worldwide have epilepsy and nearly 90% of epilepsy occurs in developing countries (WHO, 2009). Epilepsy is the second most common chronic neurological condition seen by neurologists (Onwuekwe *et al*., 2009). 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 50 million individuals estimated to have epilepsy in the 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 currently available anticonvulsant drugs 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 (Mc Namara, 2006).

Over 30% of people with epilepsy do not have seizures control even with best available medications (Cascino, 1994; Engel, 1996). Many plant extracts have been tested and found to have anticonvulsant activities. This makes it necessary to search for drugs that may be more potent. This will go a long way to proposing a template or lead that can be used for designing other drugs (Olagunju *et al*., 1999).

1.5 Justification for Plant Selection.

Globimetula braunii has been used in management of pains and diseases related to epilepsy. This has been validated scientifically by Olagunju *et al*., (1999). Literature search in scientific data base showed no phytochemical or pharmacological investigation aimed at isolating bio-active principles was ever undertaken.

1.6 Aim of the Study

The aim of this study is to isolate some of the bioactive compound present in the ethyl acetate soluble fraction of *Globimetula braunii* and characterized these compounds chemically and biologically.

1.7 Objective of the Study

The main objective of the present study is to identify and isolate some of the phytochemical constituents present in the plant using standard phytochemical methods. Anticonvulsant studies of the ethyl acetate leaves fraction of *Globimetula braunii* will also be investigated using standard laboratory models.

1.8 Statement of Research Hypothesis

The leaves of *Globimetula braunii* contain secondary metabolites with anticonvulsant activity.

CHAPTER TWO

2.1 Literature Review

Globimetula braunii (Engler) Van Tiegh is a member of the Loranthaceae family. The family is represented by 65 genera and 900 species found in the tropics and are mainly distributed in Africa, Asia, Australia, and South America, (Johnrii, 1987). It is a semi-parasitic shrub on trees with woody stem. The leaves are simple, leathery and evergreen. They are attached to the host by woody suckers often regarded as adventitious roots. The plants represent a family of plants commonly called mistletoe that possesses several local uses and are widely distributed in tropical countries like Malaysia and India. It is a bushy parasitic plant found in variety of host plants from Ghana to Nigeria and widely dispersed across central tropical Africa (Burkill, 1985). It is regarded as a threat to agricultural yield because of its parasitic characteristics (Dibong *et al*., 2008). Other members of the mistletoe family with reported biological activities include the European mistletoe (*Viscum album*) and *Psitta canthus calyculatus* (Gray and Flatt, 1999; Ohiri *et al*., 2003; Rodriguez-Cruz, e*t al.,* 2003).

2.2 Scientific Classification.

Kingdom: - Plantae.

Family: - Loranthaceae.

Genus: - Globimetula

Species: - Braunii

Sub species: - Braunii

Local names of *Globimetula braunii*

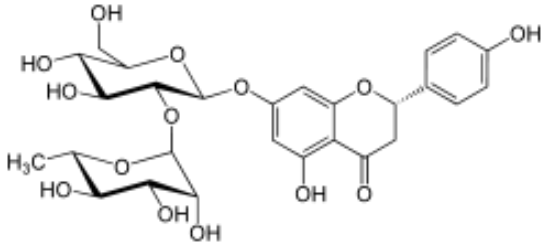
The local names include “mistletoe” in English, “Kauchii” in Hausa North Western Nigeria, and “Afomo onishano” in Yoruba, South Western Nigeria.

2.3 Ethnomedicinal uses of *Globimetula braunii*

The leaves of *Globimetula braunii* are used for the treatment of disease like cardiovascular diseases (Ouedraogo *et al*., 2004), hepatic illness (Phillipson and Wright, 1991), (Al-Ghaithi *et al*., 2004; Olagunju *et al*., 1999), malaria (Traore *et al*., 2000), and to aid delivery (Personal communication). It also has common application in treating rheumatism, epilepsy, infertility, stomach problems, and as a laxative. (Olagunju *et al*., 1999).

2.4 Phytochemistry of *Globimetula braunii* and other species in the family loranthaceae.

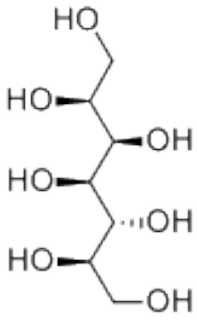
To the best of our search, there is no report on isolation of any compound from the leaves of *Globimetula braunii*; however, HPLC analysis of crude methanol and ethyl acetate fraction of the leaves revealed that extract of *Globimetula braunii* contained both naringin (XVII) and apigenin (XXIX) (Okpuzor., *et al* 2009).



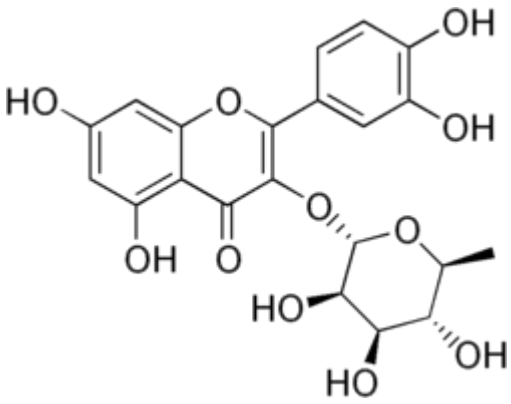
XVII

Quercitrin (XVIII) has been reported to be the taxonomic marker of Loranthaceae family, already isolated from three phyllogenetically linked genera of *Scurrula, taxillus, dendrophtoe and helixanthera*, but it was the first isolation of the 4”-O-acetyl quercitrin (XIX) in this family (Tilney and Lubke, 1974**).**

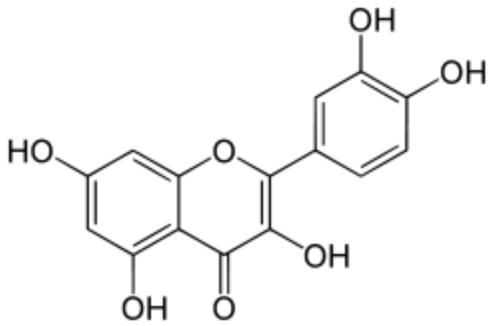
The isolation of perseitol (XX) (Ishizu *et al*., 2001), quercitrin (XVIII), a crystalline polyhydric alcohol 3-O-oxypyranoside quercetin (XXI) (Shibuya *et al*., 1999), were isolated from *Scurrula fusca*; and tannin (XXII) from *Scurrula liquidambaricolous* (Shen *et al*., 1993).



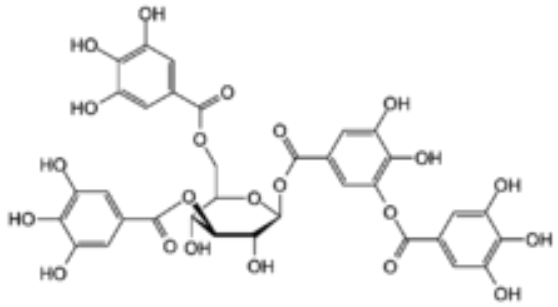
XX



XVIII

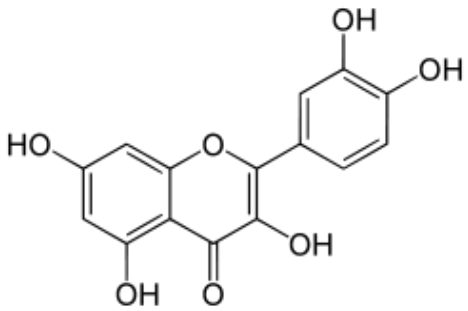


XXI

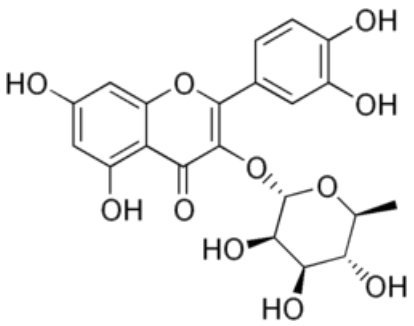


XXII

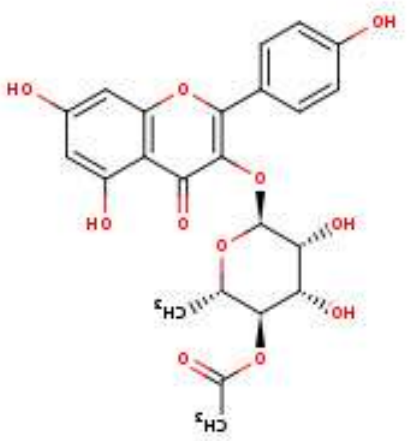
The isolation of 4˝-O-acetyl quercitrin (XIX), quercetin (XXI), and quercitrin (XVIII), have been reported from *Scurrula ferruginea Danser* (Loranthaceae) (Tilney and Lubke, 1974**)**



XXI



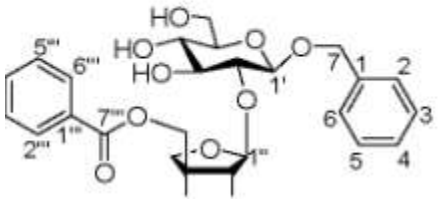
XVIII



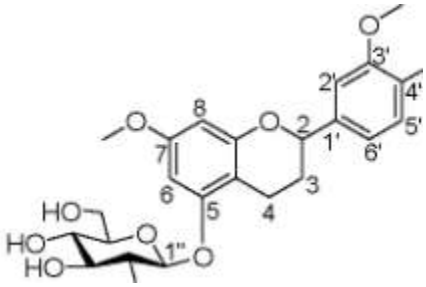
XIX

The isolation of two new phenolic glycosides, 1-O-benzyl-1-[5-O-benzoyl-β-D-apiofuranosyl (1→2)]-β-D glucopyranoside (XXIII), and 4′- hydroxy-7, 3′- dimethoxyflavan-5-O-β-D-

glucopyranoside (XXIV), have been reported from the dried whole plants of *Viscum articulatum* (Loranthaceae) (Leu *et al*., 2004).

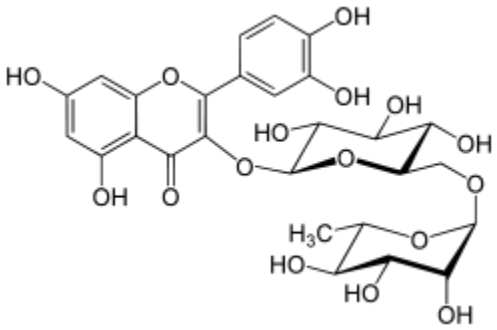


XXIII

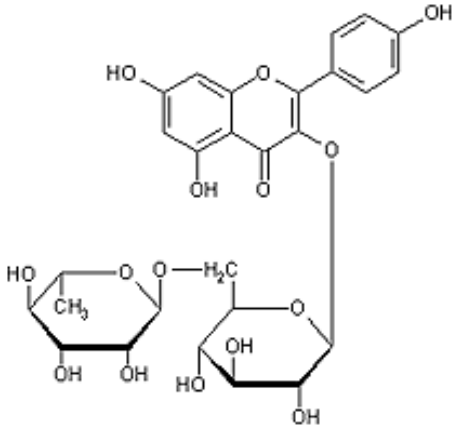


XXIV

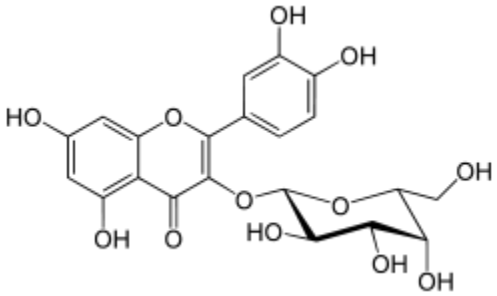
Rutin (XXV), nicotiflorin (XXVI) and hyperoside (XVII) have been reported to be isolated from the leaves of *Tripodanthus acutifolius* (Soberón, 2009).



XXV



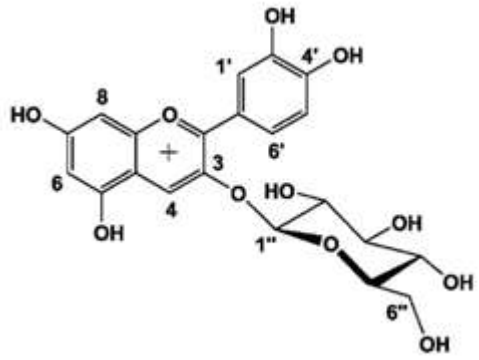
XXVI



XXVII

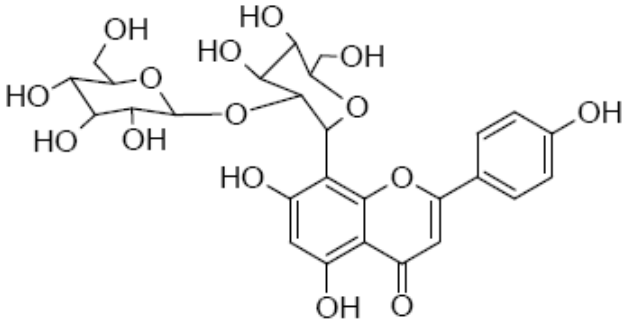
Cyanidin 3-O-β-glucopyranoside (XXVIII) was isolated from acidified methanolic extract of flowerers of the three bird-pollinated hemi parasitic plants. *Tapinanthus buvumae* (Rendle)

Danser, *Tapinanthus constrictiflorus* (Engl.) Danser and *Phragmanthera usuiensis* (Oliv.) M. Gilbert in the mistletoe family Loranthaceae (Fossen and Andersen, 1998).



XXVIII

Apigenin-8-O- β -D (2''-O- β -D-glucopyranosyl)-glucopyranoside (XXIX) have been reported to be isolated from a Bandaaka Vikshaadano, Vrkshruuhaa in the Indian Ayurverdic system of medicine *Dendrophthoe falcate* (Loranthaceae). This compound has been isolated for the first time in this genus (Gangwar and Saxena, 2010).



XXIX

2.5 Pharmacological Actions of *Globimetula braunii* and other Species in the Family Loranthaceae

The crude aqueous extract of *Globimetula braunii* was tested on isolated rat uteri. The determination of the oxytocic activity of the crude extract provides the starting point towards the determination of their potential use in humans to induce uterine contractions (Obuh and Nworgu, 2008).

Extract of *G. braunii* in different solvent systems were evaluated for lipid and blood pressure lowering activities. Cytotoxic activity of the isolated compound from *Scurrula ferruginea* Dancer were evaluated on four human cell lines, Quercetin XXI was the only biologically active

compound. Many studies have been conducted on quercetin which has been found to be one of the most active flavonol as anticancer agent with genistein (Agullo *et al*., 1996).

Globimetula braunii studies showed strong antioxidant and possesses anti-inflammatory activity. It has also been observed to be pre-oxidant that limits low density lipoprotein (LDL) oxidation (Okpuzor *et al.,* 2009).

CHAPTER THREE

3.0 METHODOLOGY

3.1 MATERIALS.

3.1.1 Solvents/Reagents.

Solvents 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 (15-40 µm) glass precoated plate (Park scientific Limited Northampton, U.K product). Sephadex LH-20 was obtained from sigma Co. USA.

3.1.2 Experimental animals.

Locally bred adult Swiss albino mice of either sex weighing (20±2 g) 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 period of study.

3.1.3 Collection, identification and preparation of plant materials.

The leaves of *Globimetula braunii* parasiting on *Piliostigma thonningii* were collected in October 2011, at the Botanical garden of medicinal plants of Sheda Science and Technology complex (SHESTCO), Abuja, Nigeria. This was confirmed and authenticated by U.S Gallah, of the herbarium section, Biological Sciences Department, Ahmadu Bello University, Zaria, Nigeria, , by comparing with existing specimens (No 9016 for *G. braunii* and No 7151 for *Piliostigma thnoningii*).

The leaves were washed with clean water; air dried under the shade for two weeks, size reduced with a laboratory blender and the powdered plant material stored for use.

3.2 Methods

3.2.1 Extraction and fractionation

The powdered plant material (2500 g) was extracted with ethanol using a soxhlet apparatus for 48 hours. The solvent was concentrated *in vacuo* to yield a solid residue (240 g) referred to as *Globimetula braunii* ethanolic leaves extract. The ethanol extract, (220 g) was suspended in distilled water, filtered and partitioned successively with n-hexane, chloroform, ethyl acetate and n-butanol to obtain n-hexane fraction 5.2 g (nHXF), chloroform fraction 15.4 g (CF), ethyl acetate fraction 53.6 g (EAF), n-butanol fraction 16.4 g (nBF), and the residual aqueous fraction 8.9 g (AF) respectively.

3.2.2 Preliminary phytochemical screening

Preliminary thin layer chromatography (TLC) was carried out on all the fractions. Of all the fractions, the TLC of EAF revealed the most promising spots and also enough quantity for laboratory study and was selected for further study.

Portions of the ethanol extract and that of ethyl acetate fraction were subjected to preliminary phytochemical screening using standard methods as summarized below.

3.2.2.1 *Test for Steroids/Tterpenes*

Liebermann-Buchard test: A small portion of the extract was dissolved in chloroform. Equal volume of acetic anhydride was added, and concentrated sulphuric acid was added down the test tube. The solution was observed for the presence of a brown ring at interphase (Silva *et al.,* 1998).

Salkowski test: A small quantity of the extract was dissolved in 1ml chloroform and to it 1ml of concentrated sulphuric acid was added down the test tube. Formation of red or yellow colouration was taken as an indication for the presence of sterols (Sofowora, 1993).

3.2.2.2. *Test for Flavonoids.*

Shinoda Test: A small quantity of the extract was dissolved in methanol. Some pieces of magnesium chip were added followed by five drops of concentrated hydrochloric acid. It was observed for the appearance of pink, orange or red to purple colour indicates the presence of Flavonoids (Silva *et al*., 1998).

Sodium hydroxide test: 2 ml of the extract was dissolved in 10% aqueous sodium hydroxide solution. The solution was observed for the presence of a yellow colour, a change in colour from yellow to colourless on addition of dilute hydrochloric acid indicates the presence of flavonoids (Silva *et al*., 1998).

Ferric chloride test: A drop of ferric chloride was added to a small quantity of the extract. The solution was observed for the formation of a green precipitate (Trease and Evans, 1996).

3.2.2.3 *Test for Tannins*

Ferric chloride test: A small quantity of the extract was boiled with water and filtered. Two drops of ferric chloride was added to the filtrate, formation of a blue-black, or green precipitate was taken as evidence for the presence of tannins (Trease and Evans, 1996).

Lead sub-acetate test: To a small quantity of the extract, three drops of lead sub-acetate was added. The solution was observed for the presence of green precipitate (Trease and Evans, 1996).

3.2.2.4 *Test for Saponins*

A small quantity 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).

3.2.2.5 *Test for Alkaloids.*

0.5 g of the extract was stirred with 5 ml of 1% aqueous hydrochloric acid on a water bath and filtered. 3 ml of the filtrate was divided into three. To the first 1 ml few drops of freshly prepared Dragendoff reagent was added and observed for formation of orange to brownish precipitate. To the second, one drop of Meyer reagent was added observed for formation of white to yellowish or cream colour precipitate. To the third, 1 ml of wagner reagent was added to give a brown or reddish or reddish-brown precipitate (Trease and Evans, 1996).

3.2.2.6 *Test for Anthraquinones.*

Free anthraquinone: The extract was shaken with 10 ml of benzene, the content was filtered, and 5 ml of 10% ammonia solution was added to the filtrate, the mixture was shaken. Presence of a pink, red, or violet colour in the ammoniacal layer (lower phase) indicates the presence of free anthraquinone (Trease and Evans, 1996).

Combined anthraquinone: The extract was boiled with 10 ml of aqueous sulphuric acid and filtered hot. The filtrate was shaken with 5 ml benzene, the benzene layer was separated and half its own volume. 10% ammonium hydroxide was added. A pink, red or violet colouration in the ammonia phase (lower phase) indicates the presence of combined anthraquinone or anthraquinone derivatives (Trease and Evans, 1996).

3.2.2.7 *Test for Carbohydrates.*

Fehling's Test: 1 ml of extract was added to 2 ml Fehling's solution and boiled for 5 minutes. A red precipitate indicates the presence of reducing sugar (Silva *et al*., 1998).

Molisch's Test: 1 ml of the extract was added to 1 ml molish's reagent and 1ml Concentrated sulphuric acid was carefully added. A reddish ring indicates the presence of carbohydrates (Silva *et al*., 1998).

3.3 Chromatographic Procedure.

3.3.1 Thin layer chromatography (TLC).

Thin layer chromatography was carried out using TLC precoated plates.

Technique: One way ascending.

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

Solvent system: various solvent systems were used, they include:

a. Hexane: Ethyl acetate (2:1)
b. Chloroform: Ethyl acetate (1:1)
c. Chloroform :Ethyl acetate (1:2)
d. Chloroform :Ethyl acetate (9:1)
e. Chloroform (100%)
f. Chloroform: Ethyl acetate (7:3)
g. Ethyl acetate: Chloroform (9:1)
h. Ethyl acetate: Methanol (1:1)
i. Chloroform: Ethyl acetate (1:2)
j. Chloroform: Ethyl acetate (5:1)
k. Ethyl acetate :Ethanol (9:1)
l. Ethanol (100%)
m. Ethyl acetate: Chloroform: Methanol: Water (15:8:4:1)
n. n-butanol: acetic acid: water (4:1:5).

Detection: Spots on TLC plates were visualized under normal day light and by spraying with 10% sulphuric acid, followed by heating at 110^0C for 5-10 min.

3.3.2 Gel filtration chromatography

The following conditions were employed in running the gel filtration chromatography.

a) Gel filter: Sephadex LH-20

b) Packing: The Sephadex was suspended in the eluent and left standing to swell for 24 hours prior to use. It was then poured into the column and allowed to set tightly

c) Sample loading:- The sample was dissolved in a small volume of the eluent and applied on top of the column

d) Solvent (eluting): - The column was eluted continuously with Methanol 100%

3.3.3 Dry column vacuum chromatography.

The following conditions were employed in running dry column vacuum chromatography (DCVC)

a) Column packing: The sinter glass funnel was filled with up to 7 cm silica gel for thin layer chromatography and tapped to give a level surface. Vacuum was applied and the surface was pressed firmly to give a completely leveled, well compacted bed of 4.5 cm high.

b) Sample Application: The compound mixture was pre-adsorbed on Merck silica gel 60 (15-40 μm).

c) Solvent system: The column was developed by gradient elution using chloroform, Chloroform: ethyl acetate mixture, ethyl acetate, and finally methanol solvent system.

d) Solvent (eluting): - Vacuum was applied to the column.

3.3.4 Spectral analysis.

a) UV Analysis

b) FTIR Analysis

c) Proton and ^{13}Carbon NMR Spectra.

NMR spectra were obtained on a Bruker AVANCE–AIII Germany (600 MH_Z for ^{1}H and 125 MH_Z for ^{13}C) spectrometer in deutrated dimethyl sulfoxide (DMSO) using Tetra methyl silane (TMS) as standard.

3.4 Chromatographic Separation.

3.4.1 Thin layer chromatography of the ethanol extract of *Globimetula braunii.*

The crude ethanol extract of *Globimetula braunii* was subjected to thin layer chromatography using pre-coated glass plates. The solvents system used were hexane, ethyl acetate: n-hexane (1:10), ethyl acetate: n-hexane (3:10), ethyl acetate: n-hexane (1:2). Ethyl acetate: chloroform (2:3) ethyl acetate: chloroform (4:5) and ethyl acetate (100%).

3.4.2 Thin layer chromatography of the n-hexane and Chloroform fractions of *Globimetula braunii.*

The n-hexane and chloroform fractions of *Globimetula braunii* were subjected to thin layer chromatography using pre-coated TLC glass plate. The solvent system used were hexane, n-hexane: ethyl acetate (2:1).

3.4.3 Thin layer chromatography of the ethyl acetate fraction of *Globimetula braunii*.

The ethyl acetate fraction of *G. braunii* was subjected to thin layer chromatography using precoated TLC glass plate. The solvents system used were chloroform: ethyl acetate (1:1), chloroform: ethyl acetate (1:2), ethyl acetate (100%).

3.4.4 Thin layer chromatography of the n-butanol fraction of *Globimetula braunii.*

The n-butanol fraction of *Globimetula braunii* was subjected to thin layer chromatography using precoated TLC glass plate. Solvents system used were n-butanol, acid anhydride and water (4:1:5) and chloroform, ethyl acetate, methanol, and water (15:8:4:1).

3.5 Pharmacological Studies

3.5.1 Acute toxicity studies

The method previously described by Lorke (1983) was adopted. All mice were injected intra peritoneally (i.p). The study was divided into two phases. In the first phase, nine mice of either sex were divided into groups of three mice each. Group one received 10 mg/kg extract while groups II and III received 100 mg/kg and 1000 mg/kg respectively. The animals were observed for signs and symptoms of toxicity including mortality for fourty eight 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 600 mg/kg while the second, third and fourth received the extract at doses of 1000 mg/kg, 1600 mg/kg and 2,900 mg/kg respectively. The mice were also observed for fourty eight hours. The median lethal dose was calculated as the square root of the products of the lowest lethal dose and the highest non – lethal dose i.e. geometric mean of consecutive doses for which 0 and 100% survival rates was recorded.

3.5.2 Pentylenetetrazole induced seizure 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 ml/kg i.p of normal saline, group two, three and four were given 75 mg/kg, 150 mg/kg and 300 mg/kg i.p of EAF respectively. Group five (positive control) was given 200 mg/kg i.p sodium valproate. Thirty minutes post-treatment, 90 mg/kg of freshly prepared solution of *SC*. 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).

3.5.3 4- Aminopyridine induced seizure test (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 ml/kg of normal saline, groups two, three and four were given 75 mg/kg. 150 mg/kg and 300 mg/kg i.p of EAF respectively and group five was administered 20 mg/kg i.p phenobarbitone to serve as positive control. After thirty minutes, 13.5 mg/kg of freshly prepared 4-AP was administered subcutaneously to each mouse. Ability of the extract to protect the mice from lethality within a thirty minutes observation period was considered an indication of anticonvulsant activity.

3.5.4 Maximal electroshock seizures test in chicks (MEST).

The method of Swinyard and Kupferberg (1985) was employed. Fifty chicks were randomly divided into five groups of ten chicks each. The first group received normal saline (10 ml/kg) i.p, second, third and fourth groups were treated with 75 mg/kg, 150 mg/kg and 300 mg/kg i.p of EAF respectively, and the fifth group was administered 20 mg/kg of phenytoin, thirty minutes

later, maximal electroshock was administered to induce seizures 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 0.8 m/s respectively. A current of 70 mA which produced tonic seizures in 80% of the control chicks, were used throughout the study. An episode of tonic extension of the hind limbs was regarded as full convulsion, while lack of tonic extension of the hind limbs was regarded as protection. In unprotected animals, the recovery time was recorded.

3.6 Statistical Analysis

Data were expressed as mean **±** SEM and the mean values of the control groups were compared with the mean values of the treated groups using one way ANOVA followed by posthoc Dunnet's t-test for multiple comparison. Results were considered statistically significant at ($P < 0.05$). The lowest confidence level is usually indicated $P < 0.05$ (95% C.I).

CHAPTER FOUR

4.1 Results of Phytochemical Screening

The results of preliminary phytochemical screening of the crude ethanol extract of G*lobimetula braunii* revealed the presence of saponins, tannins, carbohydrates, flavonoids, anthraquinones, and steroids/terpenes (Table 4.1). Similarly, tannins, flavonoids and steroids/terpenes were found to be present in the ethyl acetate fraction (Table 4.2).

Table 4.1 Results of phytochemical analysis of ethanol extract of *Globimetula braunii* leaves

Constituents	Test	Observation	Inferences
Steroids/Terpenes	a. Lieberman burchard	Greenish upper and brown ring at interphases was observed	+
	b. Salkwoski	Red ring with at the interphases	

Flavonoids	a. Sodium hydroxide	Yellow colouration was observed	+
	b. Shinoda test	Red colouration was observed	
	c. Ferric chloride test	Green precipitate was observed	
Tannins	a. Ferric chloride test	Green precipitate was observed	+
	b. Strong lead sub-acetate test	Green precipitate was observed	
Saponins	frothing	Froth which persisted for up to 15 minutes was observed	+
Alkaloids	a. Wagner's test	No change was observed	_
	b. Meyer's test	No change was observed	
	c. Dragendroff's test	No change was observed	
Anthraquinones	a. Bontrager's test	Bright pink colour was observed in the upper layers	+
Carbohydrates	a. Molisch's test	A reddish coloured ring was observed at interphases	+
	b. Fehling's test	A brick red precipitate was observed	

Keys + = present – = not present

Table 4.2 Results of phytochemical analysis of ethyl acetate fraction of *Globimetula braunii* leaves

Constituents	Test	Observation	Inferences
Steroids/Terpenes	a. Lieberman burchard	Greenish upper and brown ring at interphases was observed	+
	b. Salkwoski	Red ring with at the interphases	
Flavonoids	d. Sodium hydroxide	Yellow colouration was observed	+
	e. Shinoda test	Red colouration was observed	
	f. Ferric chloride test	Green precipitate was observed	
Tannins	a. Ferric chloride test	Green precipitate was observed	+
	b. Strong lead sub-acetate test	Green precipitate was observed	
Saponins	Frothing	Froth which persisted for up to 15 minutes was observed	_
Alkaloids	d. Wagner's test	No change was observed	_
	e. Meyer's test	No change was observed	
	f. Dragendroff's test	No change was observed	
Anthraquinones	b. Bontrager's test	Bright pink colour was observed in the upper layers	_
Carbohydrates	a. Molisch's test	A reddish coloured ring was observed at interphases	_

	b. Fehling's test	A brick red precipitate was observed

Keys + = present − = not present

4.2 Result of Thin Layer Chromatography

4.2.1 Result of Thin Layer Chromatography of crude ethanol extract

Thin layer chromatographic analysis of the crude ethanol extract using ethyl acetate: hexane (3:10) as solvent system yielded 4 spots (Table 4.3)

Table 4.3 TLC of the crude ethanol extract using ethyl acetate: n-hexane (3:10)

Spot	R_f value	Colour in 10% H_2SO_4 acid
1	0.31	Pale green
2	0.42	Pale green
3	0.47	Yellow
4	0.53	Brown

Thin layer chromatographic analysis of the crude ethanol extract using ethyl acetate: hexane (5:10) as solvent system yielded 7 spots (Table 4.4).

Table 4.4 TLC of crude ethanol extract using ethyl acetate: n-hexane (1:2)

Spot	R_f value	Colour in 10% H_2SO_4 acid
1	0.33	Pale green
2	0.39	Pale green

3	0.42	Yellow
4	0.48	Brown
6	0.55	Brown
7	0.6	Violet

4.2.2 Result of Thin Layer Chromatography ethyl acetate fraction

Thin layer chromatographic analysis of the ethyl acetate fraction using chloroform: ethyl acetate (1:2) as solvent system yielded 5 spots (Table 4.5).

Table 4.5 TLC of ethyl acetate fraction using chloroforms: ethyl acetate (1:2)

Spot	R_f value	Colour in 10% H_2SO_4 acid
1	0.11	Brown
2	0.19	Brown
3	0.25	Pale brown
4	0.58	Light green
5	0.90	Green

Thin layer chromatographic analysis of the ethyl acetate fraction using chloroform: ethyl acetate (1:1) as solvent system yielded 4 spots (Table 4.6).

Table 4.6 TLC of ethyl acetate fraction using chloroform: ethyl acetate (1:1)

Spot	R_f value	Colour in 10% H_2SO_4 acid
1	0.13	Dark brown
2	0.23	Green
3	0.48	Brown

4	0.83	Green

4.3 Result of Dry Coloum Vacuum Chromatography

Ethyl acetate fraction was chromatographed over loose Merck silica gel 60 (15-40 µm) packed columns. The column was eluted continuously using chloroform, chloroform: ethyl acetate mixtures and ethyl acetate (Table 4.7). Twenty one fractions were collected (100 – 650 ml).

The fractions were pooled together based on their TLC profile to give eight major fractions and the column was finally washed with methanol to give the ninth fraction.

Table 4.7 Showing the Result of Dry Column Vacuum Chromatography of EAF

Fraction	Eluting solvent	Solvent system	Number of spots
1	Chloroform 100%	chloroform : Ethyl acetate1:1	1

2	Chloroform :ethyl acetate 5:95%	chloroform : Ethyl acetate1:1	4
3	Chloroform :ethyl acetate 10:90%	chloroform : Ethyl acetate1:1	5
4	Chloroform :ethyl acetate 15:85%	chloroform : Ethyl acetate1:1	5
5	Chloroform :ethyl acetate 20:80%	chloroform : Ethyl acetate1:1	5
6	Chloroform :ethyl acetate 25:75%	chloroform : Ethyl acetate1:1	6
7	Chloroform :ethyl acetate 30:70%	chloroform : Ethyl acetate1:1	7
8	Chloroform :ethyl acetate 35:65%	chloroform : Ethyl acetate1:1	7
9	Chloroform :ethyl acetate 40:60%	chloroform : Ethyl acetate1:1	7
10	Chloroform :ethyl acetate 45:55%	chloroform : Ethyl acetate1:1	6
11	Chloroform :ethyl acetate 50:50%	chloroform : Ethyl acetate1:1	6
12	Chloroform :ethyl acetate 55:45%	chloroform : Ethyl acetate1:1	4
13	Chloroform :ethyl acetate 60:40%	chloroform : Ethyl acetate1:1	5
14	Chloroform :ethyl acetate 65:35%	chloroform : Ethyl acetate1:1	5
15	Chloroform :ethyl acetate 70:30%	chloroform : Ethyl acetate1:1	5
16	Chloroform :ethyl acetate 75:25%	chloroform : Ethyl acetate1:1	3
17	Chloroform :ethyl acetate 80:20%	chloroform : Ethyl acetate1:1	3
18	Chloroform :ethyl acetate 85:15%	chloroform : Ethyl acetate1:1	3
19	Chloroform :ethyl acetate 90:10%	chloroform : Ethyl acetate1:1	2
20	Chloroform :ethyl acetate 95:5%	chloroform : Ethyl acetate1:1	2
21	Ethyl acetate 100%	chloroform : Ethyl acetate1:1	2

4.4 Isolation of Compound K1

Fraction one consisting of one major spot with little impurities when subjected to crystallization in chloroform and ethyl acetate gave single homogeneous spot coded K1 (22.4 mg). K1 was subjected to chemical and spectroscopic analysis to elucidate its chemical structure.

4.4.1 Thin Layer Chromatographic Analysis of Compound K1

TLC analysis of the isolated compound K1 using chloroform: ethyl acetate (1:1) and (2:1), then sprayed with 10% sulphuric acid revealed single homogeneous spot with R_f value 0.68 (Plate 1) and 0.57 (Plate 2) respectively (Table 4.8).

Table 4.8 Table showing the TLC profile in K1

Solvent system	Number of spot	Colour of spot After heating	Rf value
chloroform: ethylacetate 1:1	1	Violet	0.68
chloroform: ethylacetate 2:1	1	Violet	0.57

4.5 TLC Profile of K1

chloroform: ethylacetate1:1 chloroform: ethyl acetate 2:1





Plate 1 Plate 2

Plate 1 and Plate 2: TLC profile of K1 in chloroform: ethylacetate1:1 and chloroform: ethyl acetate 2:1 respectively

4.6 Chemical Test on Compound K_1

Green colour precipitate was observed on addition of drop of 10% aqueous iron (III) chloride to the volume of the compound.

4.7 Spectroscopy

4.7.1 Results of UV spectrum of Compound K1

Compound K1 displayed λmax (MeOH) nm at 279 and 272 (Figure 1).

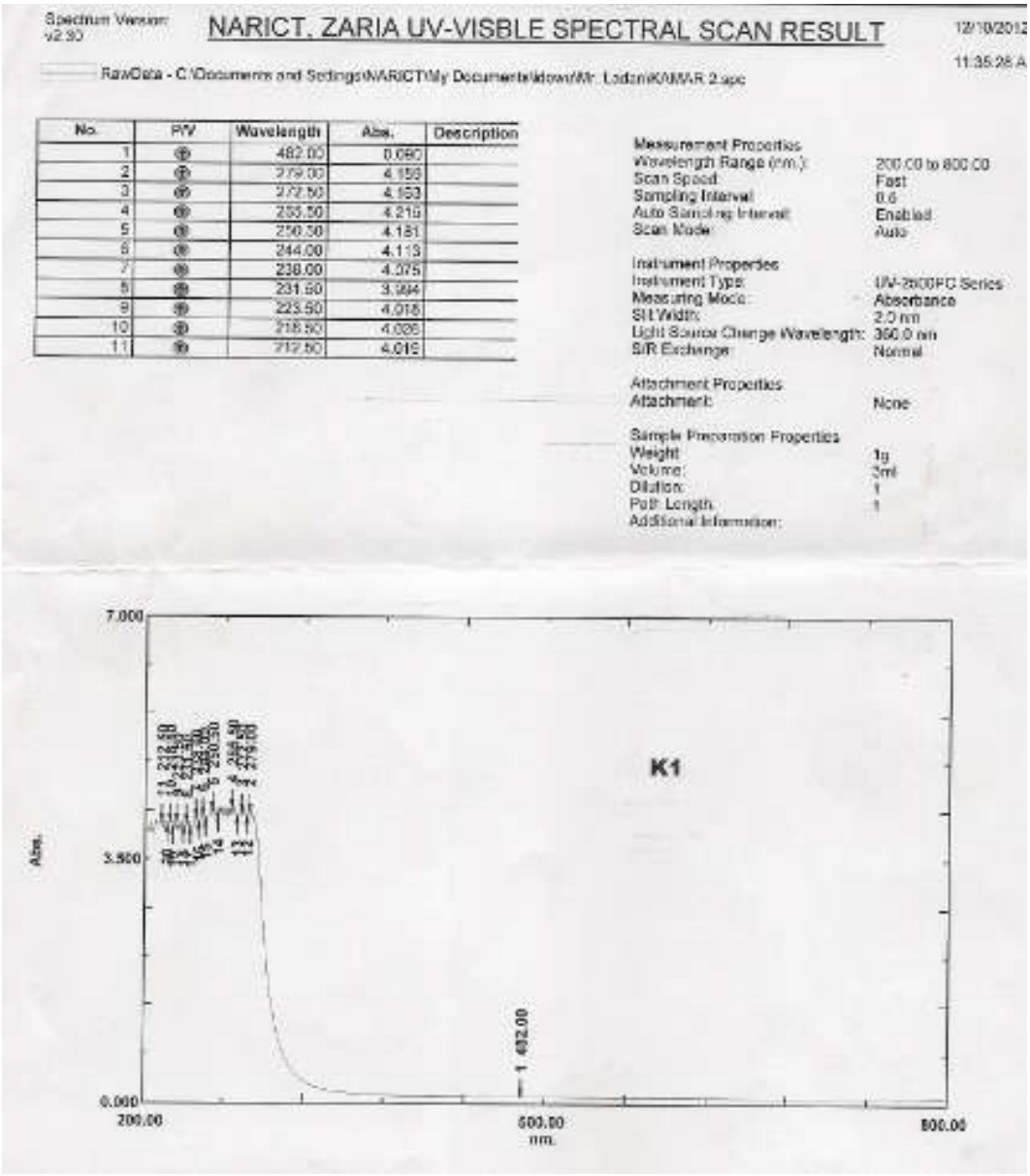
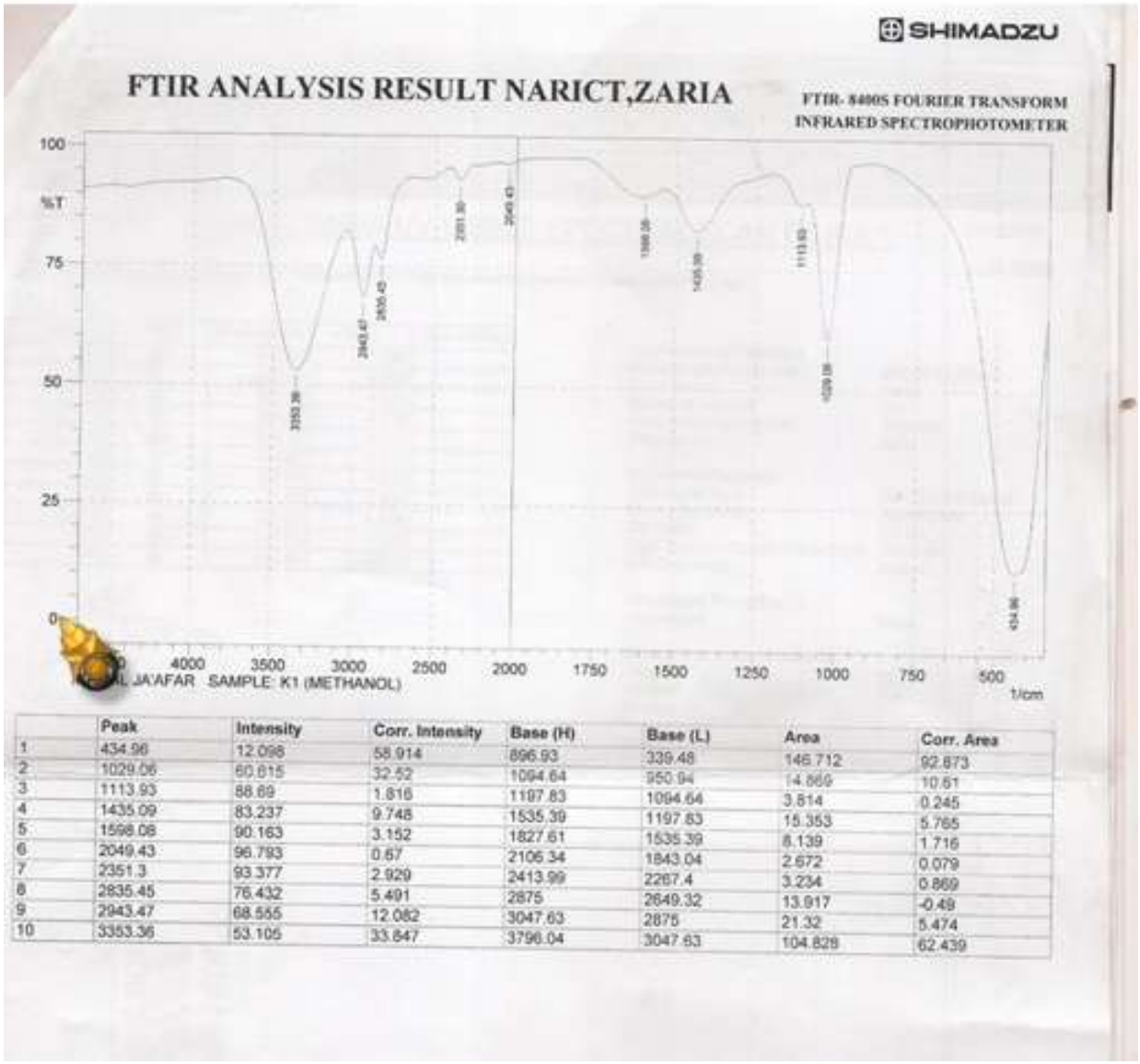


Figure 1: UV spectrum of Compound K1

4.7.2 Result of FTIR spectrum of Compound K1

The IR spectrum shows a very intensely broad band at 1029, 2943, and 3353 cm^{-1} (Figure 2).



	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	434.96	12.098	58.914	896.93	339.48	146.712	92.873
2	1029.06	60.615	32.52	1094.64	950.94	14.869	10.61
3	1113.93	88.69	1.816	1197.83	1094.64	3.814	0.245
4	1435.09	83.237	9.748	1535.39	1197.83	15.353	5.765
5	1598.08	90.163	3.152	1827.61	1535.39	8.139	1.716
6	2049.43	96.793	0.67	2106.34	1843.04	2.672	0.079
7	2351.3	93.377	2.929	2413.99	2267.4	3.234	0.869
8	2835.45	76.432	5.491	2875	2649.32	13.917	-0.49
9	2943.47	68.555	12.082	3047.63	2875	21.32	5.474
10	3353.36	53.105	33.847	3796.04	3047.63	104.828	62.439

Figure 2: FTIR spectrum of Compound K1

4.7.3 Results of NMR

^{1}H NMR (δ ppm, 600 MHz, DMSO-6).

9.15 (s, OH), 6.97 (d, 2H), 6.66 (d, 2H), 4.82 (m, H), 4.26 (m, H) 3.52 (m, H2.91, (d, H), 2.89(d, 2H) 2.56 (m, 2H), 2.53 (m, 2H) 2.48 (m, 2H), 1. 92(m, 2H) 1.87, (m, 2H), 1.85 (m, 2H), 1. 67 (m, 2H), 1. 63 (m, 2H), 1.56 (t, 2H)

The ^{1}H NMR chemical shift values of compound K1 (Figures 3 and 4) revealed proton resonances in the aromatic region δ_H 6.66 and 6.97. The downfield resonance at δ_H 9.15 was due to phenolic OH absorption. The two protons in the aromatic region integrated for 2, each. They exhibited doublet (ortho coupled) splitting pattern and their calculated *J* value (8.34)

The chemical shifts observed in the aliphatic region at δ_H 4.82, 4.26, and δ_H 3.52 are due to oxymethine protons, where as the other resonances observed at δ_H 2.91,, 2.89, 2.56, 2.53 2.48, 1.92, , 1.87, 1.85 and 1.56 are due to methylene protons.

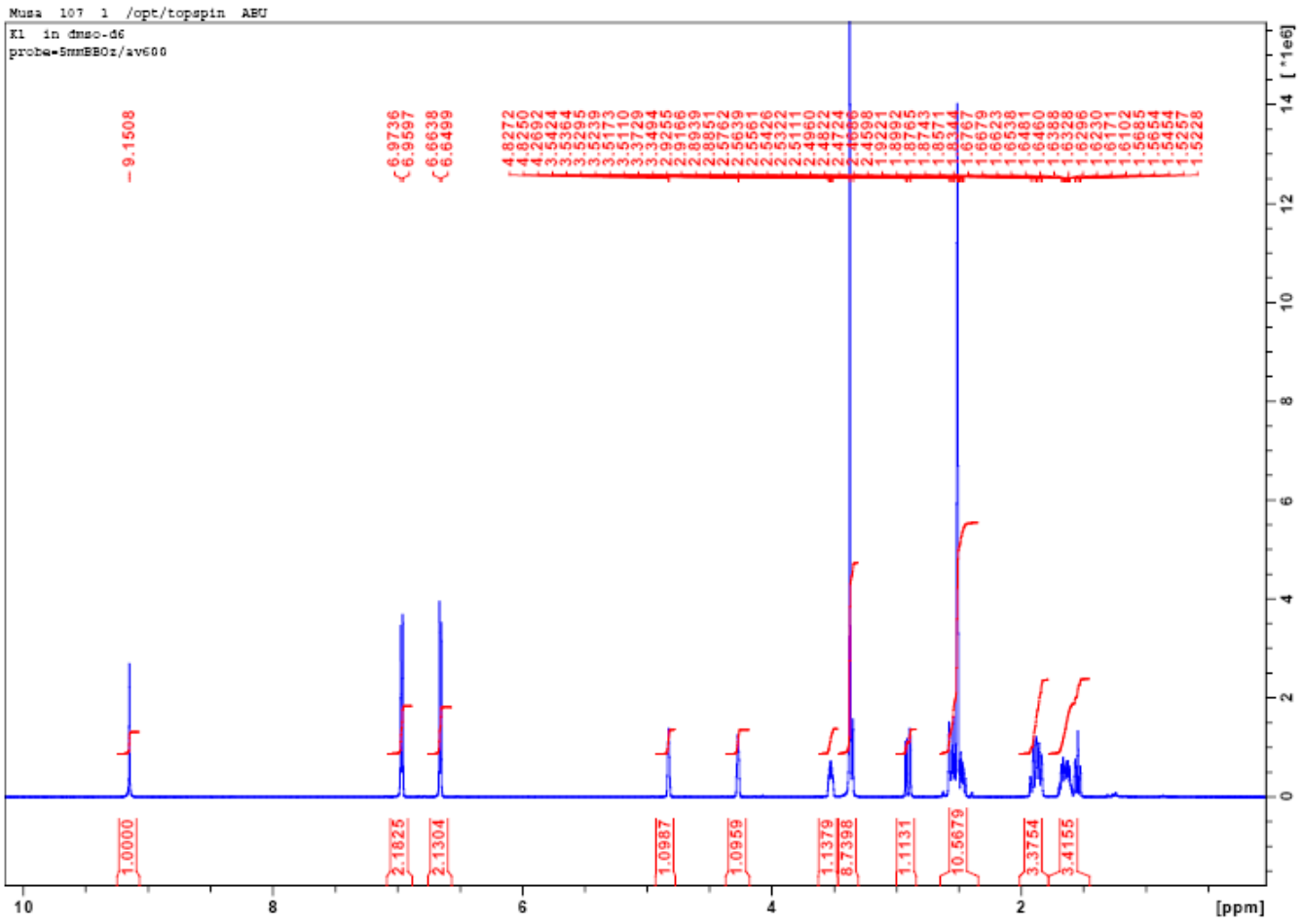


Figure 3: Proton NMR spectrum of K1

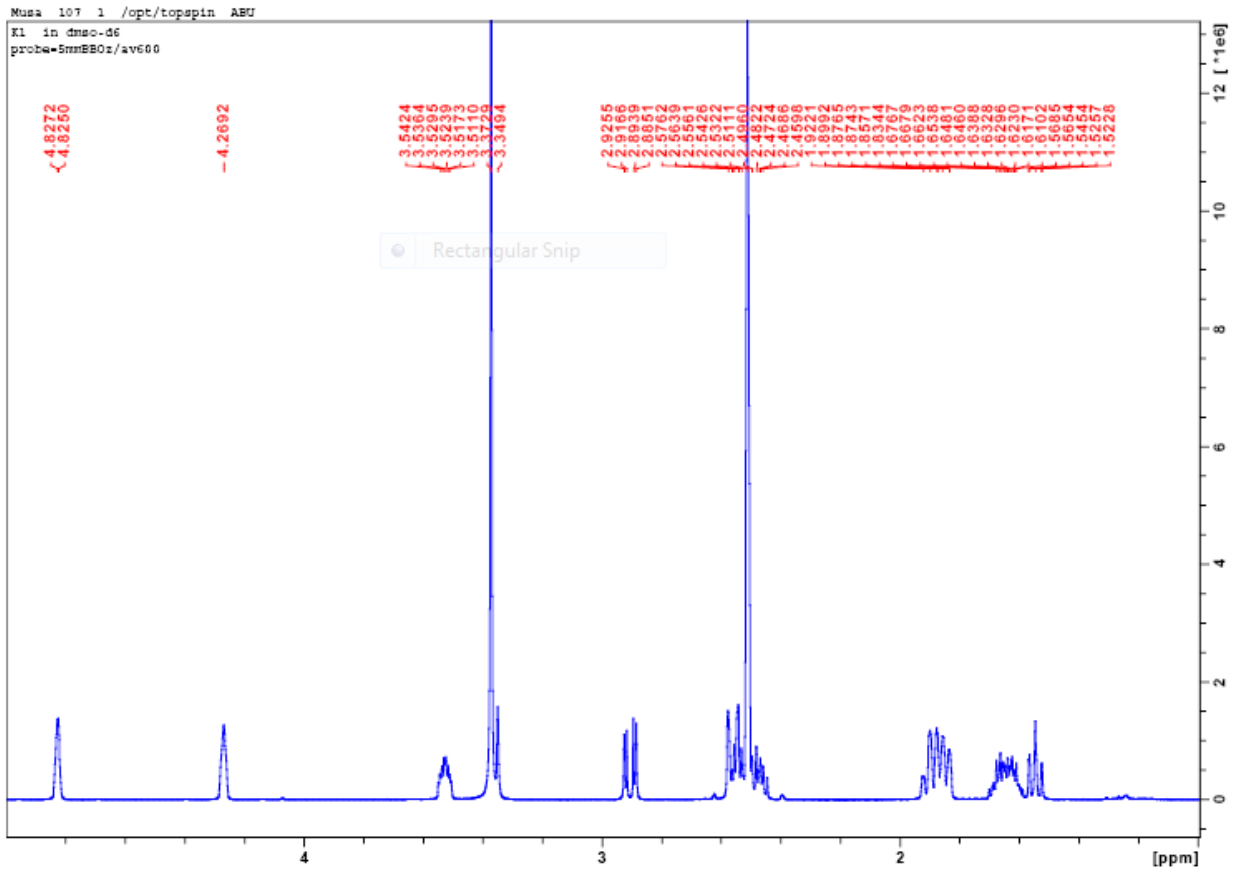


Figure 4: Expanded Proton NMR Spectrum of K1

^{13}C NMR (δ ppm, 125 MHz, DMSO) & DEPT experiments

169.97 (q), 155.75 (q), 132.13 (q), 129.59 (CH), 115.50 (CH), 73.11 (CH), 65.76 (CH), 64.50 (CH), 37.92 (CH_2), 36.93 (CH_2), 36.49 (CH_2), 30.38 (CH_2) and 29.43 (CH_2)

The ^{13}C-NMR spectrum of compound K1 (Figure 5) and the DEPT experiment (Figure 6, 7, 8 and 9) indicate K1 contains (three quartenery, five methine and five methylene groups). A down field signal at δ_C 169.97 is a characteristic signal for carbonyl esters. The oxygenated carbon signal at δ_C 155.50 is due to phenolic carbon. The methine resonances at δ_C 129.59 and 115.50 are due to aromatic ring. The spectrum also showed resonances at δ_C 73.11, 65.77 and 64.50

assignable to oxymethine carbons. The DEPT experiments also revealed the presence of 5 overlapping methylene groups at δ_C 37.92, 36.93, 36.49, 30.38 and 29.48.

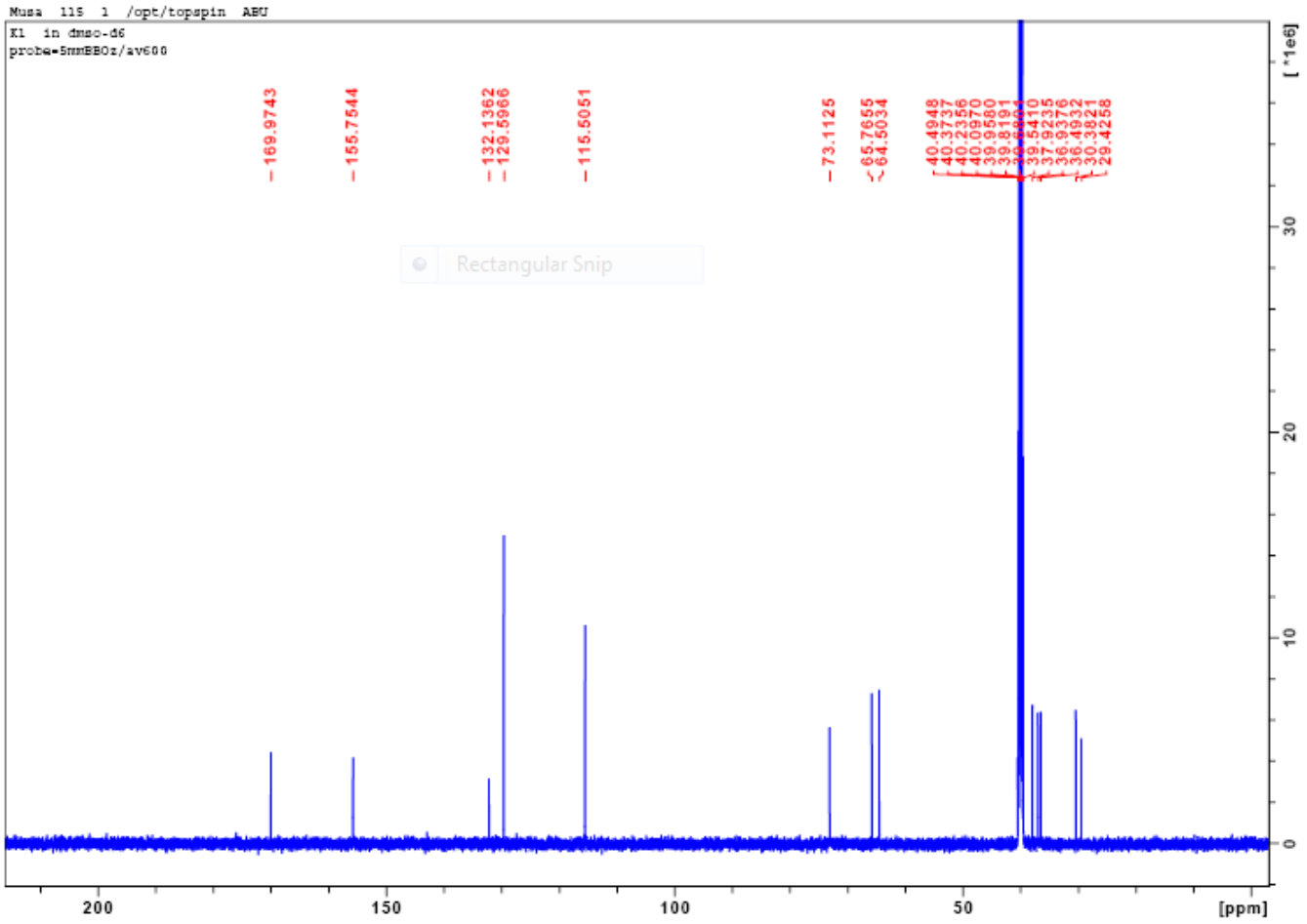


Figure 5: ^{13} Carbon NMR Spectrum of K1

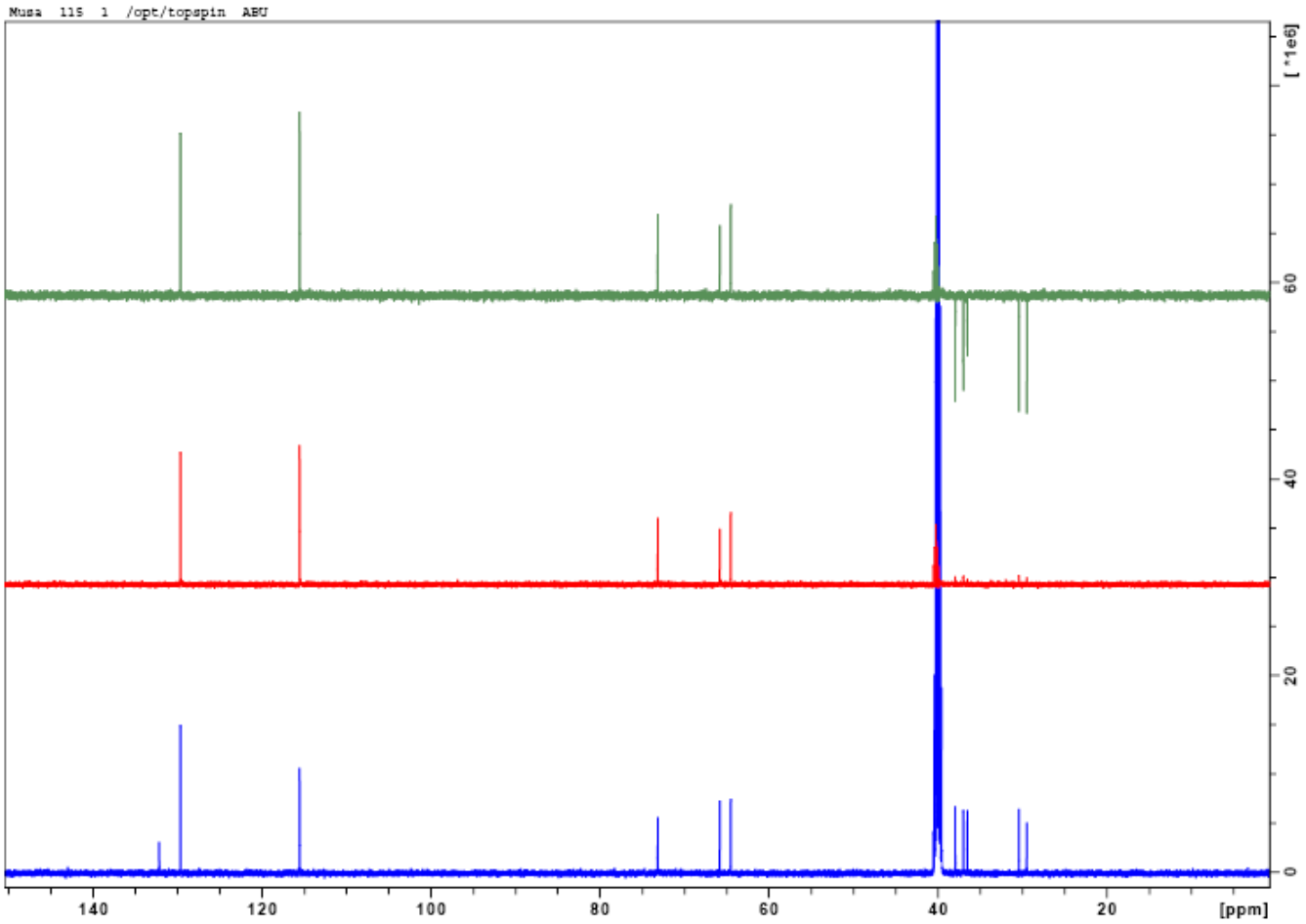


Figure 6: DEPT Spectrum of K1

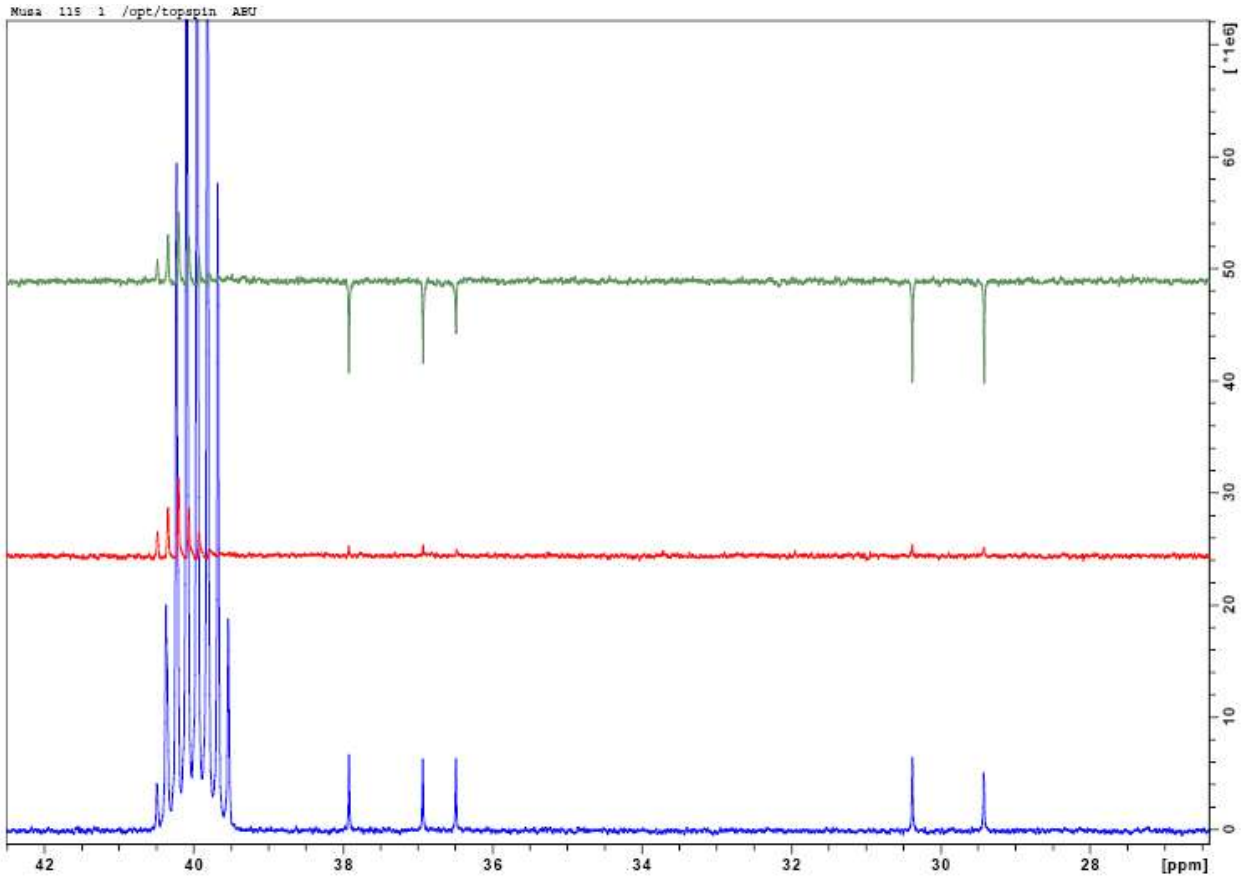


Figure 7: Expanded DEPT Spectrum of K1 (1)

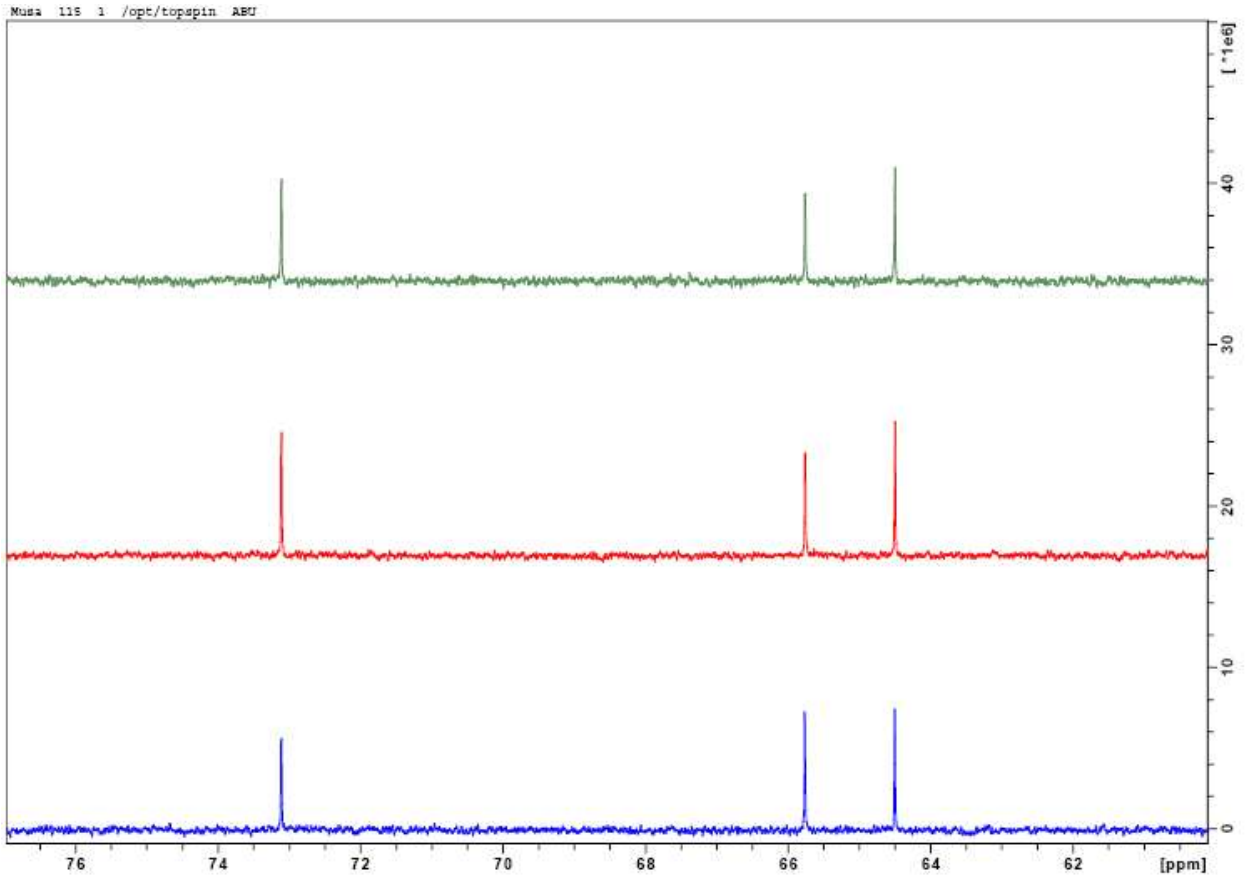


Figure 8: Expanded DEPT Spectrum of K1 (2)

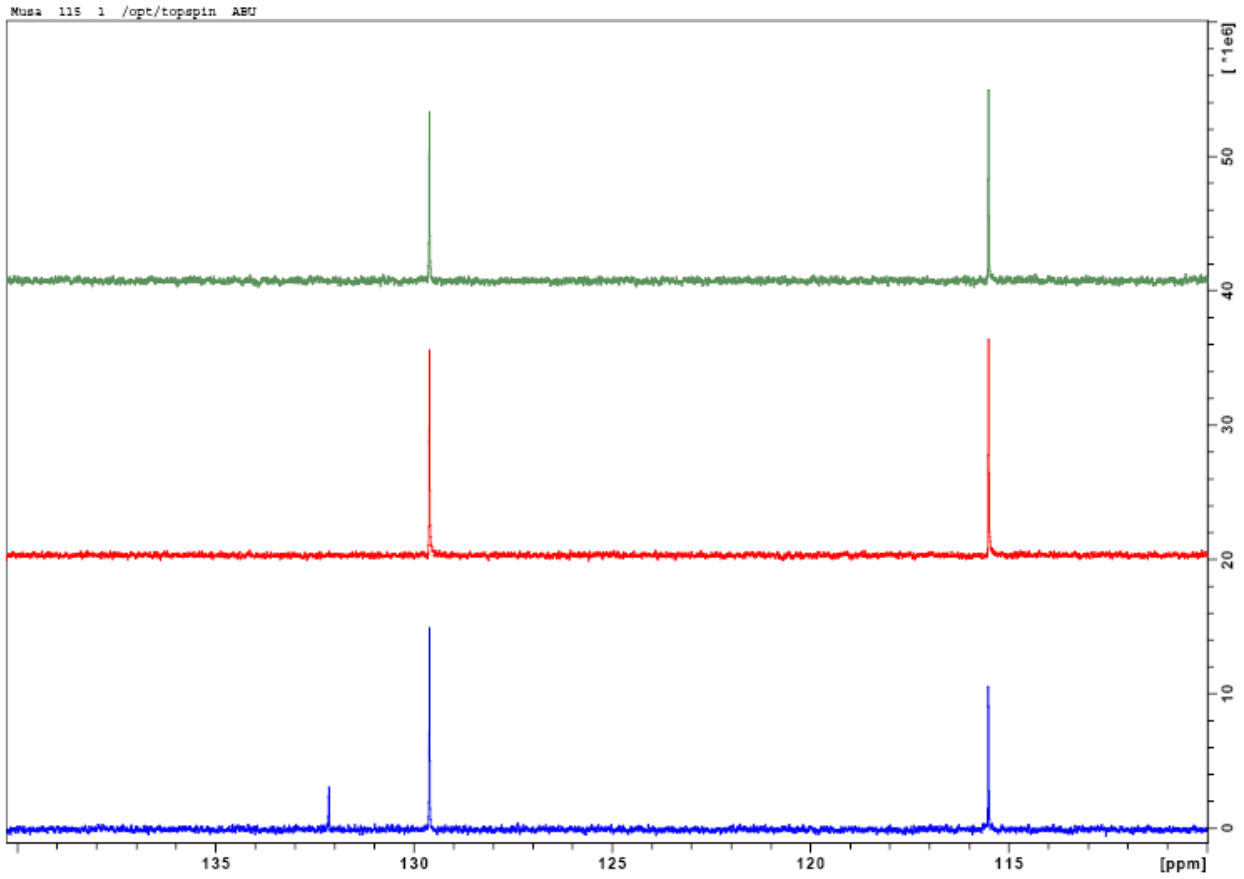


Figure 9: Expanded DEPT Spectrum of K1 (3)

The 2D ^{1}H-^{1}H COSY (Figure 10, 11, and 12), correlation spectra of compound K1 indicated the protons that are situated in the same environment and showed the nature of both the aromatic substitution and aliphatic regions of K1. It confirmed the ortho coupled protons (δ_H 6.97 and 6.66 due to aromatic ring) and virtually showed all the correlated protons in the molecule, thus: 4.26//2.89; 4.26//2.91; 4.26//1.87;

2.91//2.54; 2.89//2.53;

4.82//1.87; 4.82//1.9; 4.82//1.85;

3.52//1.85; 3.52//1.67; 3.52//1.63; 3.52//1.56;

2.56//1.67; 2.48//1.63;

The 2D correlations (^{1}H-^{1}H) and the HSQC results facilitated the sub structures consisting of two aliphatic chains and phenolic rings;

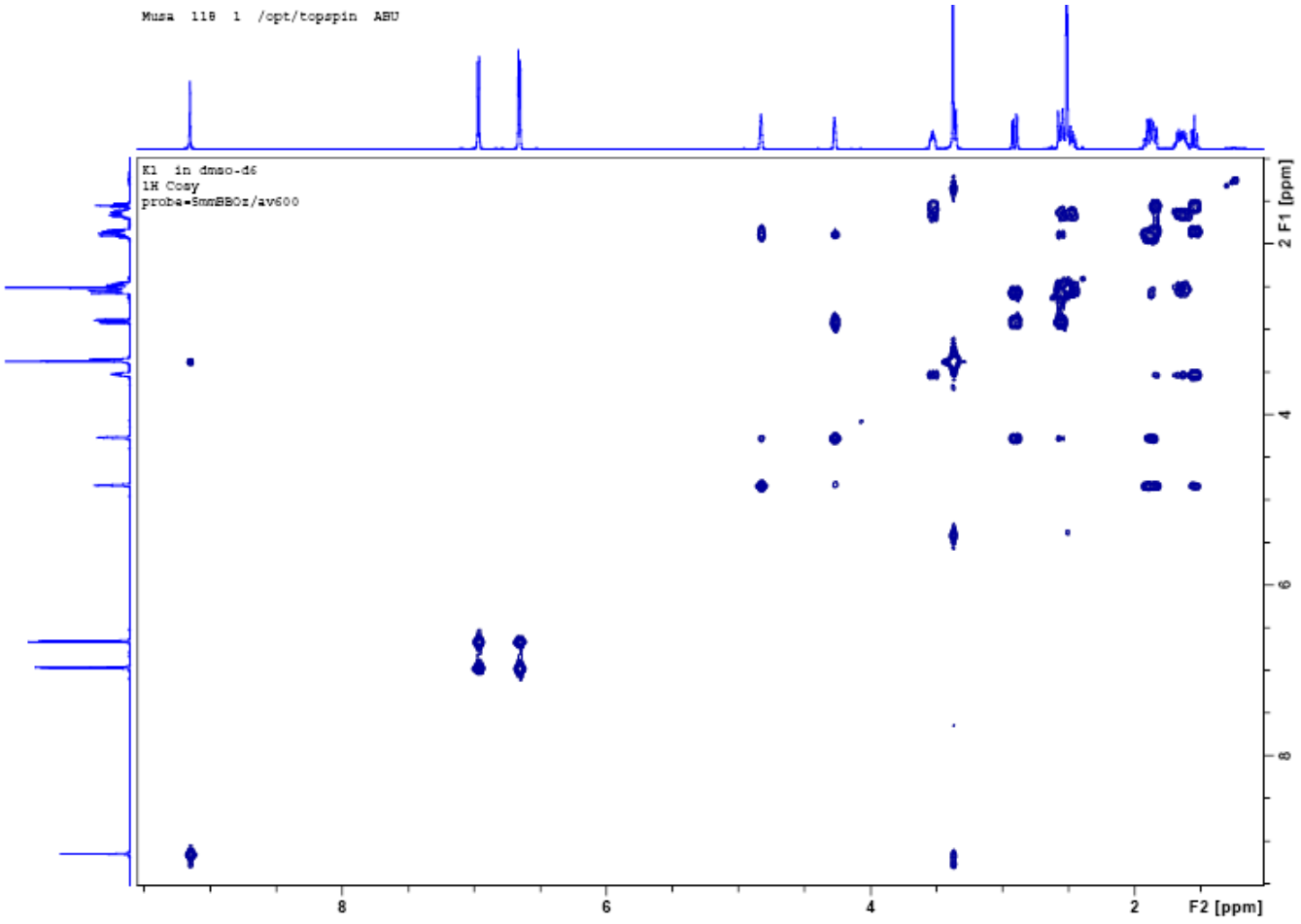


Figure 10: COSY spectrum of K1

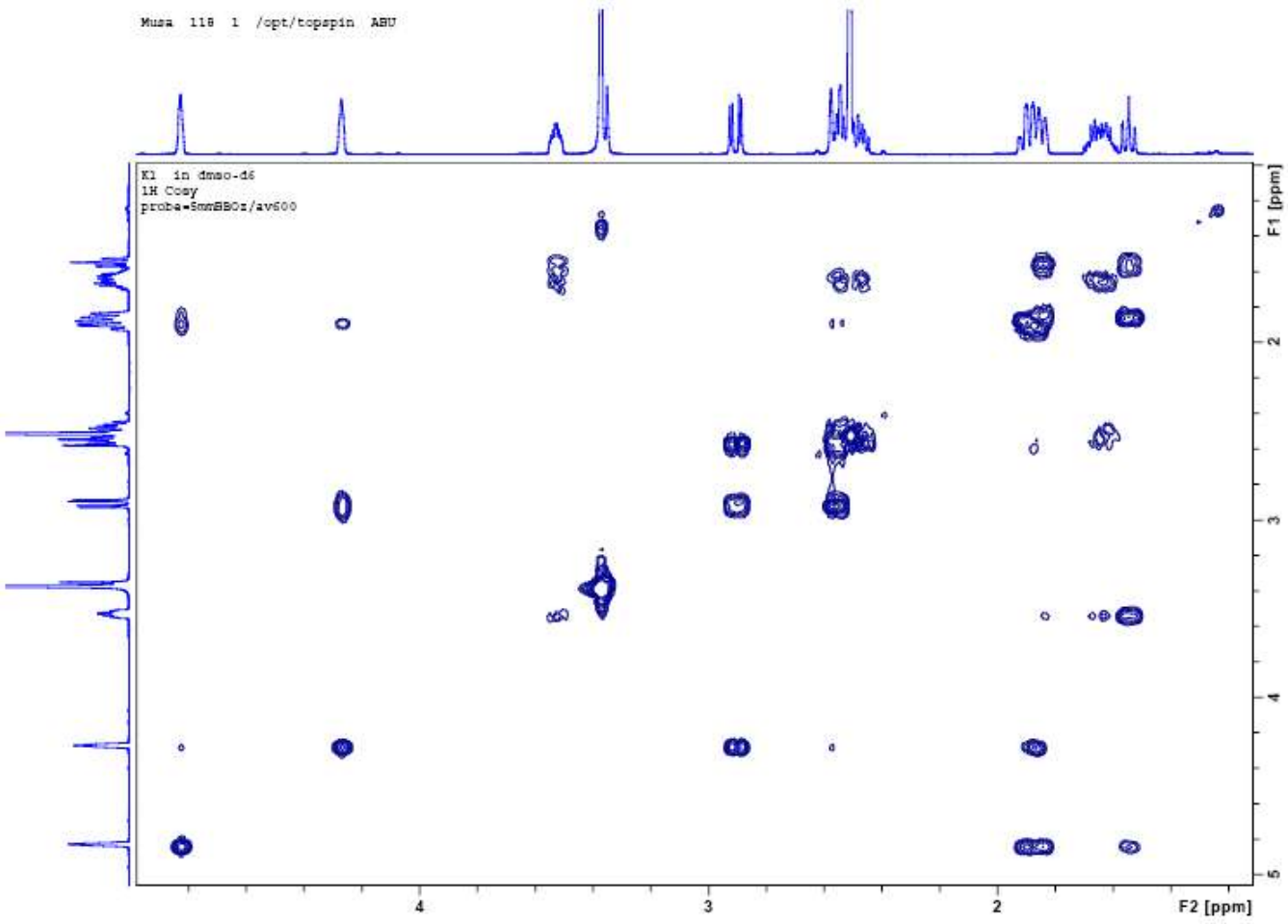


Figure 11: Expanded COSY spectrum of K1 (1)

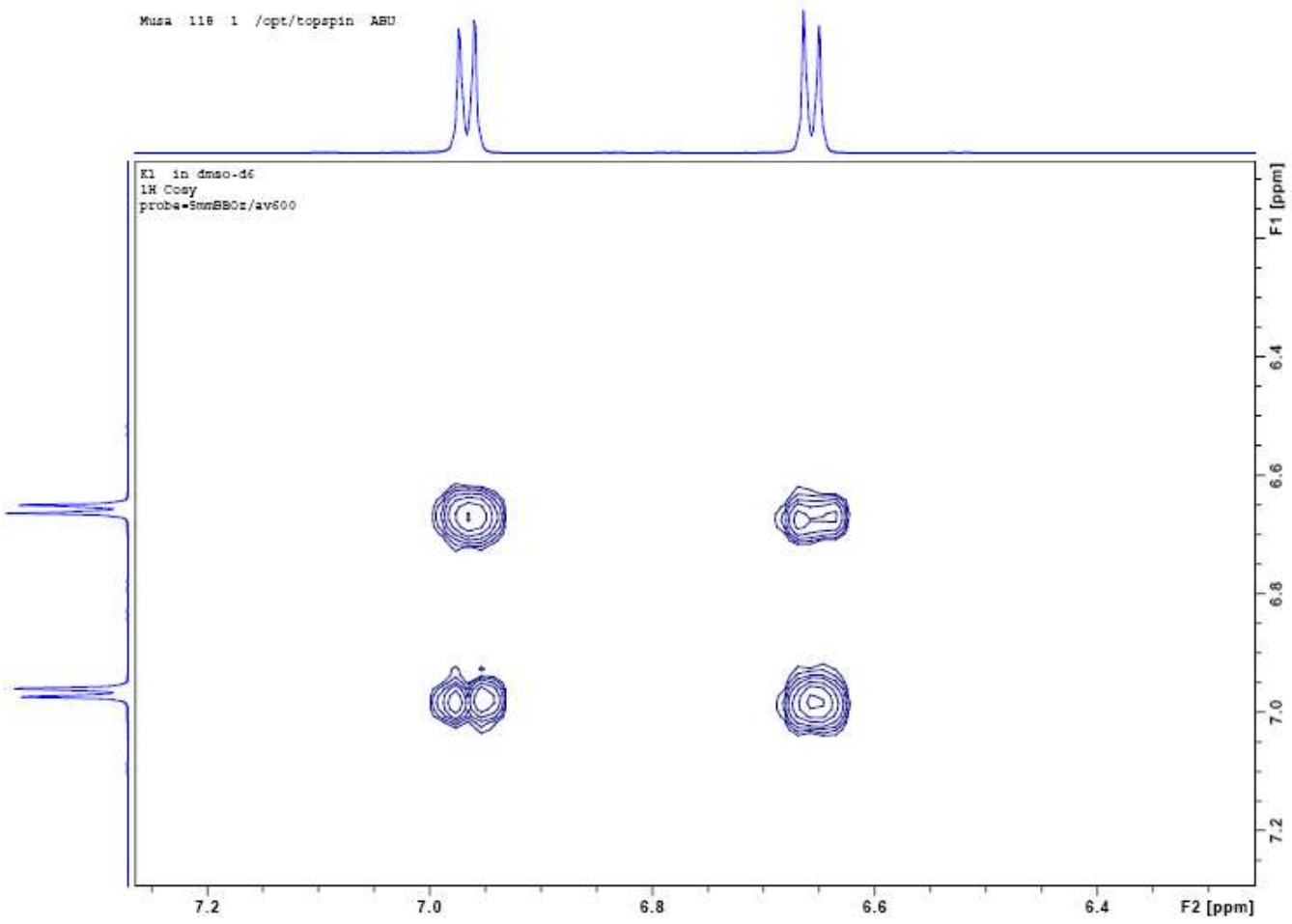


Figure 12: Expanded COSY spectrum of K1 (2)

The HSQC (Figure 13, 14, and 15) spectrum showed attachment of protons to their respective carbon atoms. The spectra extensively overlapped suggestive of a dimer.

C-129.59/H-6.97; C-115.5/H-6.66; CH due to aromatic ring

C-73.11/H-4.82; C-65.76/H-4.26; C-64.50/H-3.52; aliphatic CH, due to oxymethines and at C-36.49/H-2.91; C-36.49/H-2.89; C-36.93/H-1.85; C-36.93/H-1.56; C-37.92/H-1.67; C-37.92/H-1.63; C-36.49/H-2.54; 36.49/H-2.53; C-30.38/H-2.56; C-30.38/H-2.48 due to methylene groups

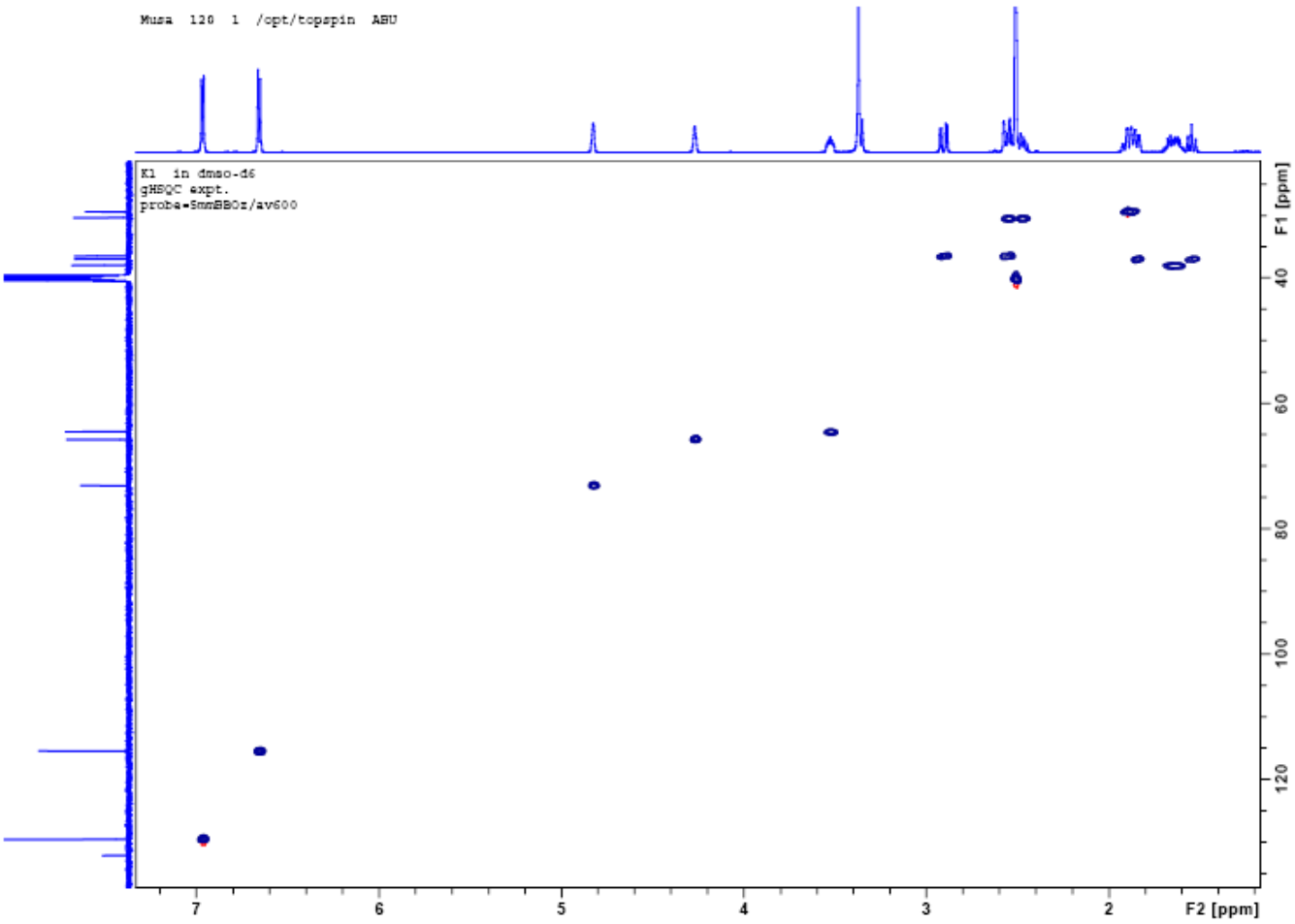


Figure 13: HSQC spectrum of K1

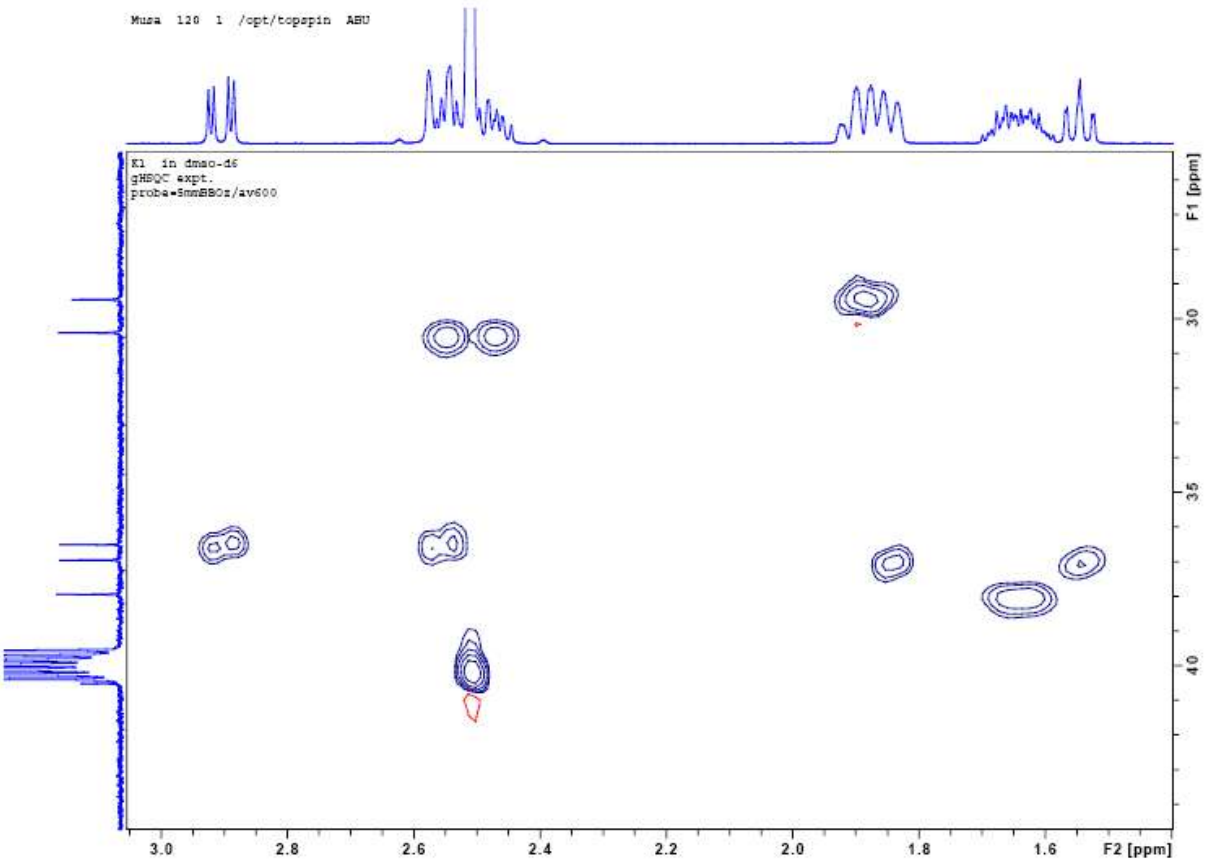


Figure 14: Expanded HSQC spectrum of K1 (1)

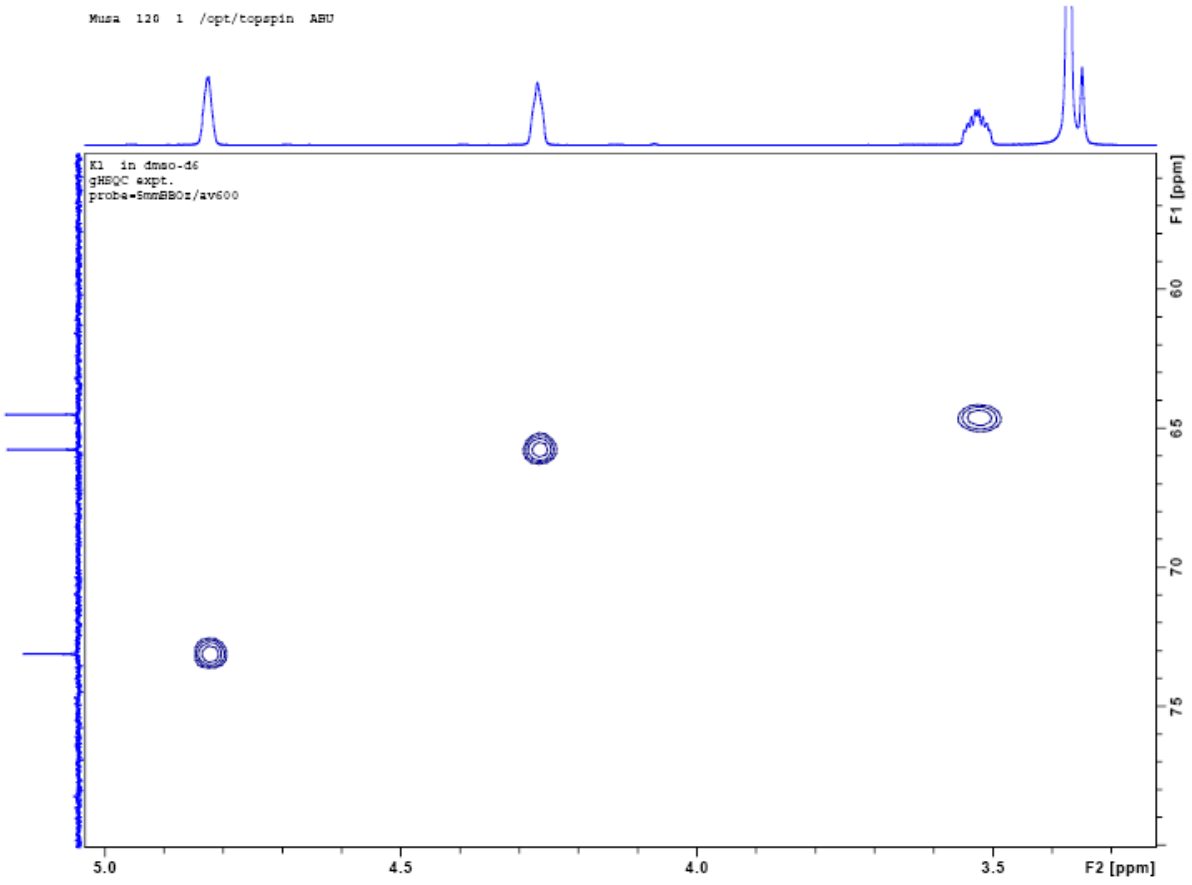


Figure 15: Expanded HSQC spectrum of K1 (2)

The Hetero multiple bond correlations (HMBC) between protons and carbons in the molecule (up to 3 bonds) facilitated correct assignment and established the structure. This connectivity facilitated the assignments of the ^{1}H and ^{13}C NMR signals, as well as the location of each substituent on the phenolic ester molecule. As shown on HMBC (Figure 16) and the expanded HMBC for aliphatic and aromatic regions (Figure 17 and 18) respectively, in the HMBC spectrum, the hydroxyl proton signal at (δ_H 9.15) showed correlation with carbon signals at δ_C 115.5 and 155.75. The aromatic proton at δ_H 6.97 showed cross peaks with carbon signals at δ_C 30.38, 115.50, 129.59 and 155.75 while the second aromatic proton δ_H 6.66 exhibited correlations with carbons at δ_C115.5, 132.13 and 155.75.

The proton at δ_H 3.52 showed correlations with the carbons at δ_C 30.34 and 37.92, this together with the ^1H-^1H COSY confirmed the sub structure.

The protons at δ_H 2.91 and 2.89 both showed cross peaks with carbon signals at δ_C 36.4, 65.76 and the carbonyl carbon 169.97. The proton at δ_H 2.54 and 2.53 correlates with the carbonyl carbon and 65.76; the protons at δ_H 2.56 and 2.48 each correlate through J_1 with δ_C 30.38, 37.92 (J_2), and 132.12 and through J_3 with carbons at δ_C 64.50 and 129.59. The protons at δ_H 1.92 and 1.87 correlate with δ_C 36.49, 65.77 and 73.11; the protons at δ_H 1.67 and 1.63 correlate with carbons at δ_C 30.34, 36.93, 64.50 and 132.12 while the protons at δ_H 1.56 correlate with δ_C 37.92 and 64.50.

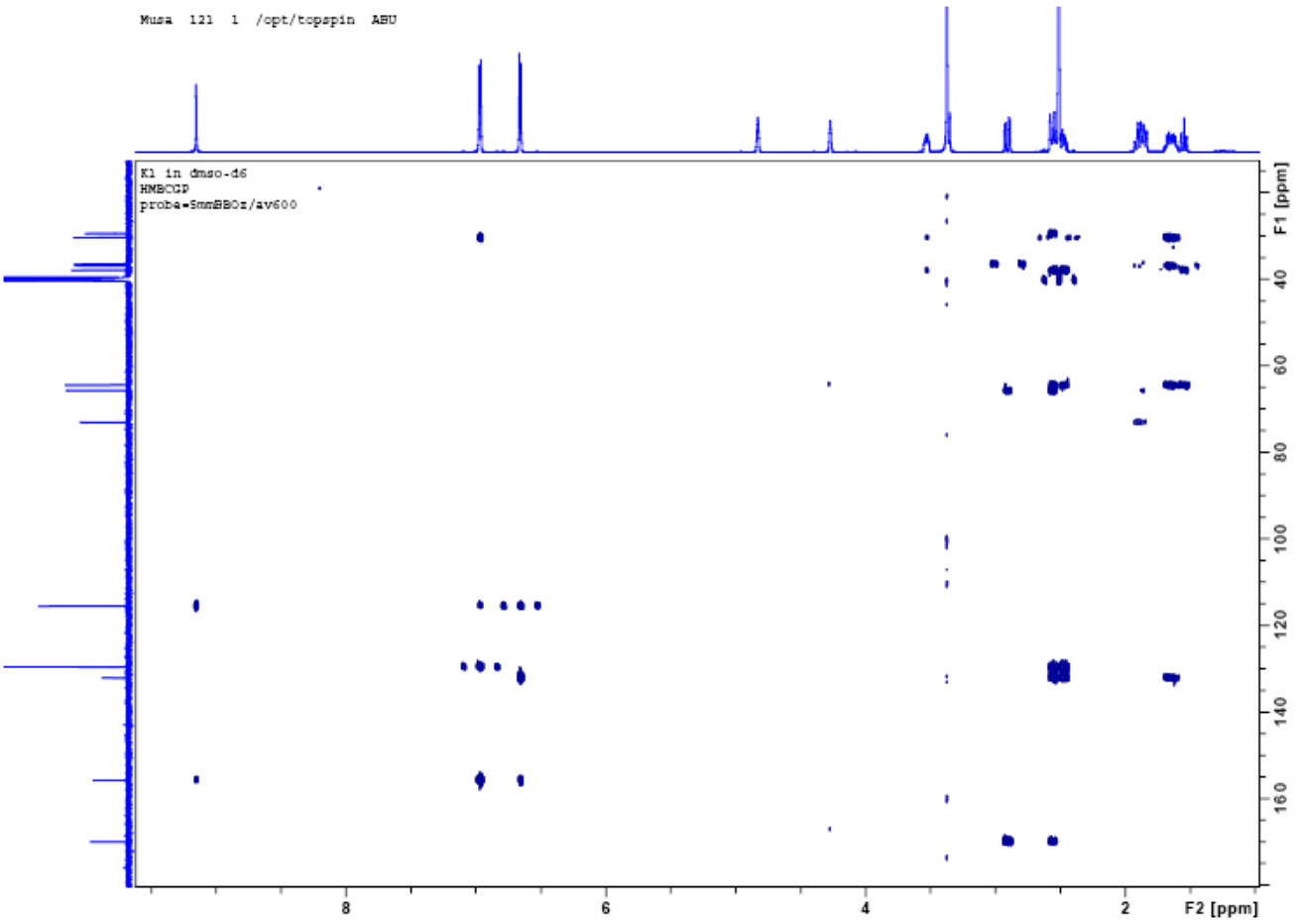


Figure 16: HMBC spectrum of K1

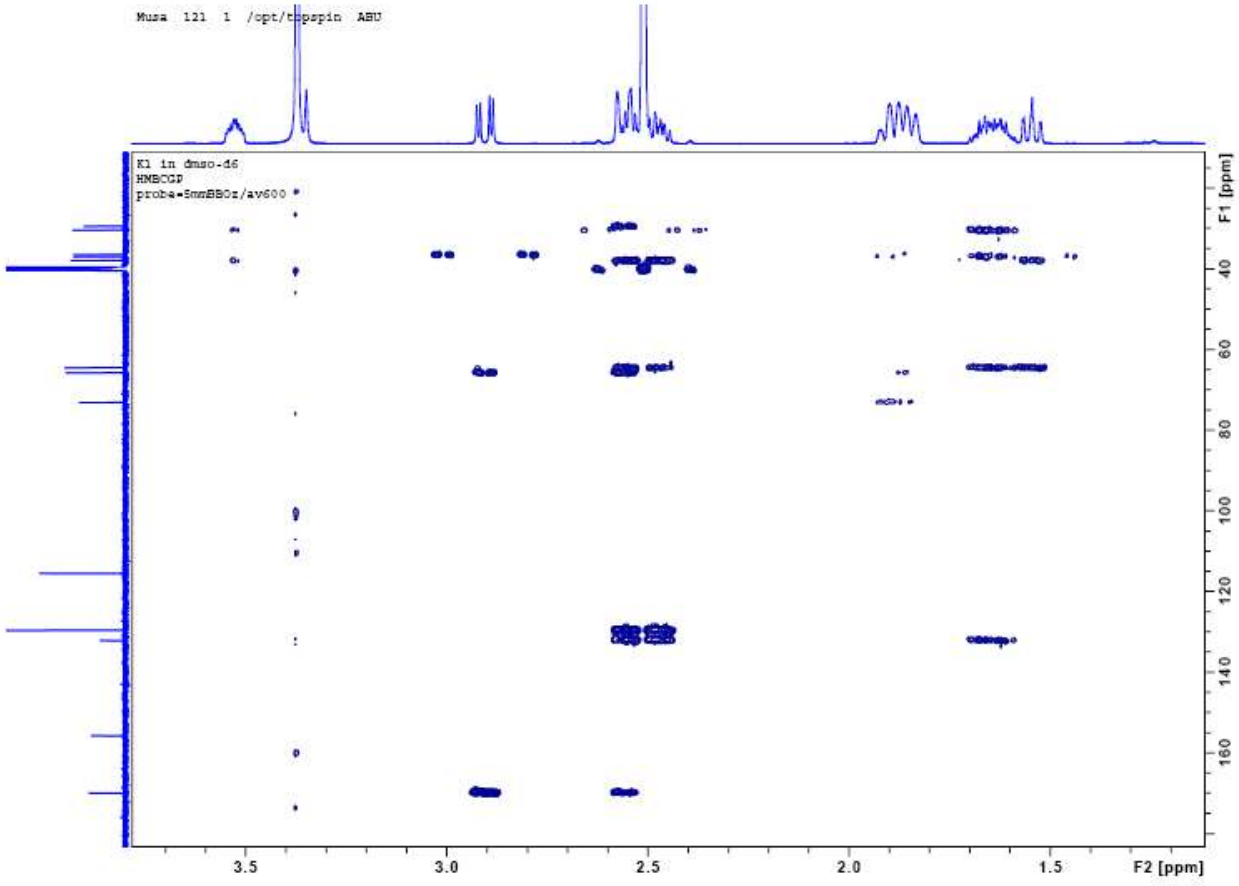


Figure 17: Expanded HMBC spectrum for aliphatic region of K1

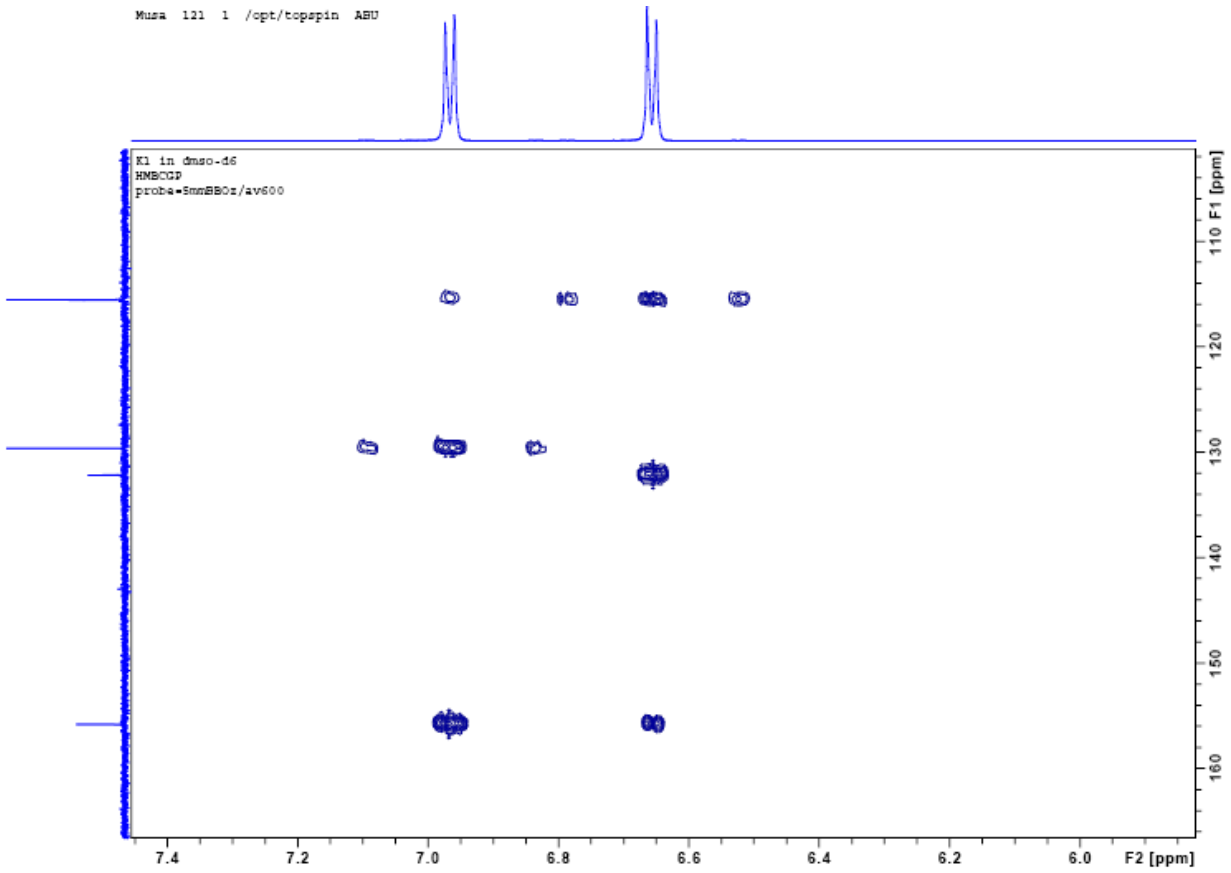


Figure 18: Expanded HMBC spectrum for aromatic region of K1

Table 4.9 Summary of ^{1}H NMR, ^{13}C NMR, DEPT, HSQC, and HMBC of Compound K1

Position	^{1}H	^{13}C	DEPT	HMBC
1	---	169.97	C	----
2	----	----	----	----
3	3.52	64.5	CH	C- 1a, 2a, 4
4	1.85	36.93	CH_2	C- 4, 5, 7
5	4.82	73.11	CH	
6	1.91	29.48	CH_2	C- 4, 5
7	4.26	65.77	CH	
8	2.91	36.49	CH_2	C- 1, 7, 8,
9	2.54	36.49	CH_2	C- 1, 7, 8, 9,

10	----	169.97	C	
11	----	----	----	----
12	3.52	64.5	CH	C- 1b, 2b, 13
13	1.56	36.93	CH_2	C- 1b, 12,
14	4.82	73.11	CH	
15	1.87	29.42	CH_2	C- 13, 14, 16
16	4.26	65.77	CH	
17	2.89	36.49	CH_2	C- 10, 16, 17
18	2.53	36.49	CH_2	C- 10, 16, 18
1a	1.67	37.92	CH_2	C- 1a, 2a, 3, 1’
2a	2.56	30.38	CH_2	C- 1a, 2a, 1,’2’
1b	1.63	37.92	CH_2	C-1b, 2b, 12, 1’’
2b	2.48	30.38	CH_2	C-1b, 2b, 1’’2’’
1’	----	132.13	C	
2’	6.97	129.59	CH	C- 2a, 1’, 2’, 4’, 6’
3’	6.66	115.5	CH	C-1’, 3’, 4’, 5’
4’	-----	155.75	C	
5’	6.66	115.5	CH	C-1’, 3’, 4’, 5’
6’	6.97	129.59	CH	C- 2a, 1’, 2’, 4’, 6’
1’’	----	132.13	C	
2’’	6.97	129.59	CH	C-2b, 1’’, 2’’, 4’’, 6’’
3’’	6.66	115.5	CH	C-1’’, 3’’, 4’’, 5’’
4’’	-----	155.75	C	
5’’	6.66	115.5	CH	C-1’’, 3’’, 4’’, 5’’
6’’	6.97	129.59	CH	C-2b, 1’’, 2’’, 4’’, 6’’

4.8 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 high test dose for which the animal survived was 1000 mg/kg while 1600 mg/kg was the lowest lethal dose. Therefore, LD_{50} was calculated as 1261.91 mg/kg. The animals displayed hypo locomotion and respiration depression before death.

4.9 Result of Pharmacological Studies

4.9.1 Effect of EAF on *SC* PTZ induced Seizure

All animals in the control group exhibited myoclonic jerk and some exhibited threshold seizures and loss of righting reflex with tonic hind limbs extension. The extract exhibited anticonvulsant effect on seizure induced by subcutaneous PTZ. It offered maximum protection against threshold seizure by protecting 83.33% at a dose of 150 mg/kg (Table 4.10).

Table 4.10 Effect of ethyl acetate fraction against pentylenetrazole induced seizure

S/N	Treatments mg/kg	Mean onset of jerking	Quantal protection of jerking	Mean onset of spasm,	Quantal protection against seizure	Mean time of death, mins	Quantal protection against death	Percentage protection against seizure	Mortality %
1	Normal saline 10 ml/kg	3.67± 0.55	0/6	4.80± 0.58	1/6	13.60 ± 2.54	1/6	16.67	83.33
3	75	4.17± 0.48	0/6	7.40± 1.91	1/6	9.5± 1.66	2/6	16.67	66.67
4	150	3.60± 0.24	1/6	8.00± 0.00	5/6	8.0± 0.00	5/6	83.33	16.67
5	300	5.30± 0.93*	0/6	10.33± 2.33	3/6	12.00 ± 4.00	4/6	50.00	33.33
6	Sodium	7.00±	5/6	0	6/6	0	6/6	0.00	0.00

Valproate (200)	0.70

Protection against seizure and mortality expressed as percentage; mean on set of lower Jerking, seizure and death expressed as mean ± SEM. *P < 0.05 (compared with normal saline treated control). n=6.

4.9.2 Effect of EAF on SC 4- aminopyridine induced Seizure

The fraction produced a reasonable protection against 4-AP induced seizure compared to the control. However, the fraction produced an increase in the mean onset of seizure in unprotected animals (Table 4.11).

Table 4.11 Effect of ethyl acetate fraction against subcutaneous 4- Amino pyridine induced seizure.

S/N	Treatment mg/kg	Mean onset of Jerking, Mins	Quantal Protection against Jerking	Mean onset of seizure/Death, mins	Quantal protection against seizure/death	Percentage protection	Percentage protection against Mortality
1	Normal saline 10 ml/kg	14.33 ± 1.70	0/6	15.00 ± 2.66	0/6	0.00	0.00
3	75	14.50 ± 1.01	0/6	14.67 ± 1.52	0.6	0.00	0.00
4	150	15.33 ± 0.61	0/6	15.33 ± 2.04	0/6	0.00	0.00
5	300	19.67 ± 2.4	0/6	19.67 ± 1.26	0/6	0.00	0.00
6	Phenobar	24.6 ± 0.45	0/6	24.60 ± 2.25	1/6	0.00	16.67

bitone

Protection against seizure and mortality expressed as percentages; Mean onset of seizure expressed as mean ± SEM. n=6.

4.9.3 Effect of EAF on Maximal Electroshock induced Seizure

All the control animals exhibited seizures after delivery of electroshock. The fraction did not protect the animals against tonic hind limbs extension at all the tested doses (Table 4.12).

Table 4.12 Effect of Ethyl acetate fraction against Maximal Electroshock induced seizure in Chicks.

S/N	Treatments mg/kg	Mean time of recovery Mins	Quantal Protection	Mortality %
1	Normal saline 10 ml/kg	6.2 ± 0.32	0/10	0.00
2	75	8.3 ± 0.87	0/10	0.00
3	150	6.20 ± 0.49	0/10	0.00
4	300	12.3 ± 3.12	0/10	0.00
5	Phenytoin 20	5.0 ± 2.0	8/10	0.00

CHAPTER FIVE

DISCUSSION

The result of preliminary phytochemical screening carried out on the crude ethanol extract revealed the presence of saponins, carbohydrates, flavonoids, cardiac glycoside, anthraquinones and steroids, while that of the ethyl acetate fraction revealed the presence of tannins, flavonoids and steroids/terpenes. These phytochemical constituents have been reported to be associated with different pharmacological activities of plants (Das *et al*., 1989, and Cowan, 1999). 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). Flavonoids and triterpenes are classified as active antimicrobial compounds, while tannins have astringent properties, hasten healing of wounds and inflame mucous membrane and good antibacterial activity (Rojas *et al*., 1992)

Dry column vacuum chromatography of the ethyl acetate fraction of ethanol extract using chloroform for gradient elution followed by crystallization led to the isolation of compound K1. The single spot displayed by the compound when subjected to TLC using two different solvent systems suggested that the compound is single and pure.

The positive test observed with iron (III) chloride indicated the presence for phenolic hydroxyl group.

The UV spectrum of compound K1 (Figure 1) showed wavelength of maximum absorbance at 279 nm, and 272 nm. This indicates the presence of conjugation in the system (Wade, 2006).

The IR spectrum of compound K1 (Figure 2) shows a very intensely broad band at 3353 cm^{-1} that is the characteristic of 0-H Stretching vibrations for hydroxyl group in phenol. The absorption band at 1598 cm^{-1} was typical C - C stretching double bond in aromatic ring. The C-H stretching band was shown at 2943 cm^{-1}. The absorption at 1113 cm^{-1} is a characteristic of C-O stretching for esters (Wade, 2006).

The ^{1}H NMR chemical shift values of compound K1 revealed the presence of a downfield resonance at δ_H 9.15 assignable to a phenolic OH absorption; it also exhibited an ortho coupled absorption in the aromatic region at δ_H 6.97 and 6.67, (*J,* 8.34), each of the two protons integrated for 2, suggesting a 1, 4-disubsituted ring system.

The chemical shifts observed in the aliphatic region at δ_H 4.82, 4.26, and 3.52 are due to oxymethine protons, while the other resonances observed at δ_H 2.91, 2.89, 2.56, 2.54 ,2.53 2.48, 1.91, , 1.87, 1.85 and 1.56 are due to methylene protons.

The integration and expansion of the intensely overlapping aromatic and aliphatic regions of the spectra disclosed proton at δ_H 2.91 was not one but two (a methylene), as further revealed in the DEPT experiments. This suggests the structure of KI as a dimer.

The ^{13}C-NMR spectrum and the DEPT experiments indicated K_I contains three quaternary, five methine and five methylene groups. The down field signal at δ_C 169.97 is characteristic for carbonyl esters (Silverstein *et al*., 2005). The oxygenated (quaternary) carbon signal at δ_C 155.50 is due to a phenolic carbon. The resonances at δ_C 129.59 and 115.50 represent aromatic methine groups while the chemical shift values at δ_C 73.11, 65.77 and 64.50 are assignable to oxymethine (aliphatic) carbons. The DEPT experiments also disclosed five overlapping methylene (CH_2) groups situated on carbons at δ_C 37.92, 36.93, 36.49, 30.38 and 29.48; the intensity of the resonances as well as the 2D (HSQC, ^{1}H-^{1}H and the HMBC) correlation spectra also showed multiple /overlapping absorptions further indicating the molecule to be a dimer.

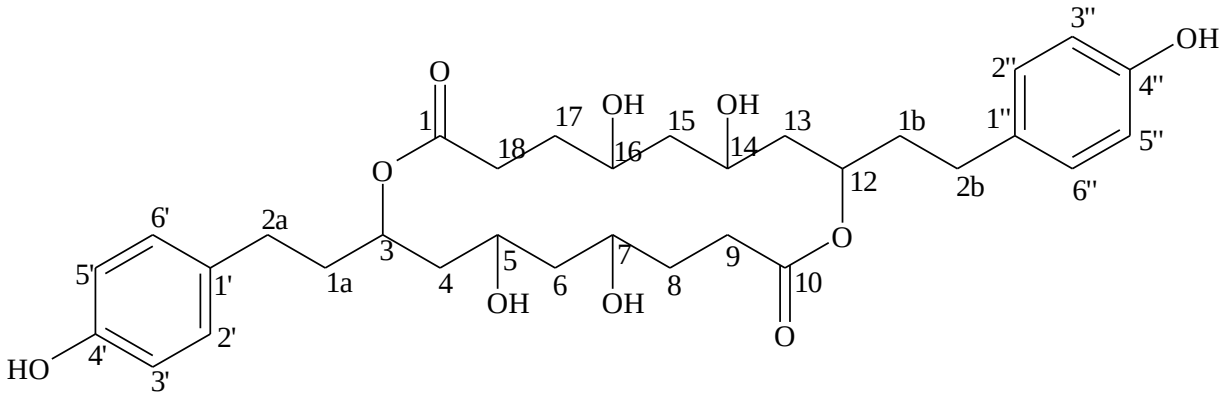
The 2D ^{1}H-^{1}H COSY correlation spectra of compound K1 indicated the protons that are situated in the same environment and revealed the nature of the aromatic and aliphatic regions. It confirmed the presence of aromatic ortho coupled protons, the aliphatic methine (CH) and the methylene groups thereby facilitating the elucidation of K1. Together with the HSQC, pair of phenolic ring, aliphatic chain, ethinyl unit and carbonyl ester group were obtained as sub structures.

The Heteromultiple Bond Correlations (HMBC) between protons and carbons in the molecule (up to 3 bonds) facilitated correct assignment and established the structure. This connectivity allowed assignments of the ^{1}H and ^{13}C NMR signals, as well as the location of each substituent on the phenolic ester molecule. The hydroxyl proton signal at (δ_H 9.15) showed correlation with

carbon signals at δ_C 115.50 and 155.75. The aromatic proton at δ_H 6.97showed cross peaks with carbon signals at δ_C 30.38, 115.50, 129.59 and 155.75 while the second aromatic proton δ_H 6.66 exhibited correlations with carbons at δ_C 115.50, 132.12 and 155.75. This established the phenolic ring with linkage to aliphatic system via carbon at δ_C 30.38

The HMBC confirmed the linkage of ethinyl sub-structure (C-1 a/b & C-2 a/b) with both the phenolic ring and the aliphatic chain; furthermore, the proton at δ_H 3.52 showed cross peaks with the carbons at δ_C 30.34, 37.92 and 36.93 further establishing the linkage between the phenolic rings and the aliphatic chain.

The HMBC correlations reinforced the dimeric nature of the molecule; the protons at δ_H 2.91 and 2.89 both showed cross peaks with carbon signals at δ_C 36.49, 65.76 and the carbonyl carbon 169.97. The protons at δ_H 2.54 and 2.53 correlate with the carbonyl carbon and δ_C 65.76; the protons at δ_H 2.56 and 2.48 correlate through J_1 with δ_C 30.38, (J_2) 37.9, and 132.12 and through J_3 with carbons at δ_C 64.50 and 129.59. The protons at δ_H 1.91 and 1.87 correlate with δ_C 36.4, 65.7 and 73.1; the protons at δ_H 1.67 and 1.63 correlate with carbons at δ_C 30.34, 36.9, 64.5 and 132.12 while the protons at δ_H 1.85 and 1.56 correlate with δ_C 37.92 and 64.50. The results of the 1D and 2D spectral data are summarized on Table (4.9) and allowed us to propose the structure of K1 as 3, 12-bis [2-(4-hydroxyphenyl) ethyl]-5, 7, 14, 16-tetrahydroxy-2, 11-dioxacyclooctadecane-1, 10-dione, Figure below.



3, 12-bis [2-(4-hydroxyphenyl) ethyl]-5, 7, 14, 16-tetrahydroxy-2, 11-dioxacyclooctadecane-1, 10-dione.

The data presented in this study provide scientific evidence that EAF obtained from the crude ethanol extract of *G. Braunii* leaves may contain psychoactive principles that are relevant to the management of convulsive disorder. The intraperitoneal median lethal dose of ethyl acetate fraction found to be 1261.91 mg/kg in mice suggests that it is relatively toxic (Matsumura, 1985). However, is it relatively safe at the dose employed in the studies.

EAF protected mice against pentylenetetrazole and significantly delayed the onset of myoclonus jerks and tonic seizures. It is widely accepted that PTZ causes seizures by blocking the major GABAergic inhibitory pathways (Desarro *et al.*, 1999). Standard antiepileptic drugs such as diazepam and phenobarbitone are thought to produce their effects by enhancing GABA mediated inhibition in the brain (MacDonald *et al*., 1995). Seizures induced by PTZ can also be blocked by drugs such as ethosuximide by reducing T-Type Ca^{2+} currents (Meldrum *et al*., 1996).

Activation of the N-methyl-D aspirate (NMDA) receptor system is also involved in the initiation and propagation of PTZ- induced seizures (Velisek *et al*., 1999 and Yudkoff *et al*., 2006). In this regard, drugs such as felbamate that block glutamatergic excitation mediated by NMDA receptor have demonstrated anticonvulsant activity against PTZ induced seizures (Macdonald *et al*., 1995). It is therefore possible that the anticonvulsant effect shown in this study by EAF against seizures produced by PTZ might be due to enhancement of GABAergic neurotransmission, inhibition of T-type Ca^{2+} current or blockade of glutamatergic neuro transmission mediated by NMDA receptor, which are not tested in this study.

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). Agents which protect animal against MEST have been shown to be beneficial in the management of generalized tonic clonic seizure. Therefore, the absence of anticonvulsant ability in MEST suggests that EAF 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). EAF failed to abolish the spontaneous discharges induced by 4- aminopyridine (4-AP) in mice. 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.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Preliminary phytochemical screening of the methanol extract of *G. braunii* revealed the presence of saponins, tannins, carbohydrates, flavonoids, anthraquinones and steroids. Similarly, tannins, flavonoids and steroids/terpenes were found to be present in the ethyl acetate fraction

Dry Column Vacuum Chromatography, followed by crystallization led to the isolation of 3, 12-bis [2-(4-hydroxyphenyl) ethyl]-5, 7, 14, 16-tetrahydroxy-2, 11-dioxacyclooctadecane-1, 10-dione

The MEST and PTZ tests are assumed to identify anticonvulsant drugs effective against generalized tonic-clonic seizure and petitmal seizure respectively (Loscher *et al*., 1988 and DeDyn *et al*., 1992). EAF failed to protect mice against maximal electro shock and 4-AP at the dose tested. Pharmacological studies on the ethyl acetate soluble fraction showed that 150 mg/kg protected 83.33% of test animals against pentylenetetrazol induced seizure in mice; as such may be useful in the management of petitmal epilepsy.

6.2 Conclusion

The research has achieved the stated objectives. To the best of our search, this is the first time this isolation is being reported. The new structure will add to the global natural product data base and may serve as a model for the synthesis of new anticonvulsant compound.

Based on the findings in this work, it can be concluded that the use of *G. braunii* in the management of convulsion has scientific basis.

6.3 Recommendations

In order to fully validate the ethno medicinal use of the leaves, it is recommended that

1. The isolated compounds should be subjected to pharmacological screening to determine their biological activity and toxicological profile.
2. Other compounds should be isolated and subjected to biological/pharmacological activity based on the folkloric claims of the plant.
3. Anticonvulsant activity should also be carried out on other fractions of the ethanol extract to investigate their anticonvulsant potential.
4. Detailed anticonvulsant studies exploiting other seizure models should be carried out on EAF to ascertain the mechanism of its anticonvulsant activity.

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