PHYTOCHEMICAL AND ANTIMICROBIAL SCREENING OF THE EXTRACTS OF THE WHOLE PLANT *CEROPEGIA DEIGHTONII* HUTCH AND DALZIEL (APOCYNACAE)

BY

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DEPARTMENT OF CHEMISTRY, FACULTY OF SCIENCE, AHMADU BELLO UNIVERSITY, ZARIA

JUNE, 2014

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JUNE, 2014

DECLARATION

I declare that the work in this thesis entitled: ***"Phytochemical and Antimicrobial Screening of the extracts of the whole plant Ceropegia deightonii Hutch and Dalziel (Apocynacae)"*** has been carried out by me in the Department of Chemistry of Ahmadu Bello University, Zaria under the supervision of Prof. G.I Ndukwe and Prof. J.O Amupitan. The information obtained 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 the award of another degree and/or diploma by me or anyone else at this or any other institution.

…… ……. ……..…….

Habibullah Awolowo Signature Date

CERTIFICATION

This thesis titled: ***"Phytochemical and Antimicrobial Screening of the extracts of the whole plant Ceropegia deightonii Hutch and Dalziel (Apocynacae)"*** by Habibullah Awolowo meets the regulations governing the award of a Master of Science degree in Organic chemistry of the Ahmadu Bello University Zaria, and is approved for its contribution to knowledge and literary presentation.

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Dean, SPGS

DEDICATION

This piece of work is dedicated to Almighty Allah (S.W.T), the Most Beneficent and Most Merciful.

ACKNOWLEDGEMENT

All praises and thanks are due to Allah (S.W.T) the Lord of the worlds, the Exalted from all that they ascribe to Him. May the peace and blessings of Allah (S.W.T) Who has taken neither a wife, nor a son (or offspring or children) be upon Prophet Muhammad; His servant and Messenger (S.A.W), his household, righteous companions and all those who follow them in righteous till the day of reckoning.

My profound gratitude goes to my supervisor, Prof. G.I. Ndukwe and Prof. J.O. Amupitan for their invaluable encouragements, instructions, advices and his unending, inestimable assistances during the course of this work. I say thank you sirs for your fatherly care! Obediently, my profound gratitude also is due to my parents for their love, care, prayers and support, and for showing me the right path in life, may Allah's (SWT) mercy and blessings continue to be upon them until they are admitted into the paradise! (Amin).

Concomitantly, my sincere gratitude also goes to all my lecturers in the Department for their dispositions towards me and in one way or the other contributed to the success of this piece of work and accomplishment of my studies. My sincere gratitude also goes to Dr A.M. Musa (Department of Pharmaceutical Chemistry), Mallam Y. M. Sani (Department of Pharmaceutical Chemistry), Dr J.D. Habila, Mallam Nasir Tajuddeen, Mallam Mubarak Dambatta, Mallam Muhammad Al-Amin and Ibrahim Abdulkadir for the role they played during the course of this research work. I say, thank you a lot, may Allah reward you abundantly! (Amin).

This acknowledgement would be incomplete without appreciating the efforts and support of my beloved darling, my sweetheart, the love of my life and the only sugar in my tea; Rahmatu Musa Danraka (Mrs. Habibullah)

Let me also seize this opportunity to express my thanks to my friends and colleagues, who have contributed in one way or the other towards the success of this project. I am particularly grateful to Mallam Ibrahim Yusuf of MTN NIG, Mrs. Kudirat Hassan of Access Bank Plc, Mr. Sadiq Hassan, Alh. Bashir Awolowo, Madinat Hassan and of my numerous friends and colleagues for their companionships, whom lack of space forbade their mention in this brief piece. May Allah (SWT) bless you all (Amin).

Abstract

Ceropegia deightoniii, a plant used in North Central Nigeria for the treatment of infectious diseases was subjected to phytochemical and antimicrobial analysis. The phytochemical screening was performed using standard procedures. The result of the phytochemical screening of the n-hexane, ethyl acetate and methanol extracts revealed the presence of cardiac glycosides, anthraquinones, saponins, terpenes, steroids, flavonoids and tannins. The antimicrobial activity against *S. aureus, S. feacalis, E.coli, S. Dysenteriae* and *C. Albicans* was tested using the agar diffusion method as outlined by the National Committee for Clinical Laboratory Standards (NCCLS). The results of the MIC and MBC showed that the plant extracts had remarkable activity against the tested microorganisms with inhibition zones ranging from 19mm to 26mm except against *S. typhi, P. aeruginosa and C. stellafoidea*. The MIC values ranged from 5.00 mg/ml to 20.00 mg/ml while the MBC/MFC values ranged from 20.00 mg/ml to 40mg/ml for all the plant extracts against both gram positive and gram negative microorganisms which indicate the broad spectrum of activity of the plant *Ceropegia deightonii*. Extensive chromatographic separation of the petroleum ether extract led to the isolation of lupeol, a pharmacologically active triterpenoid. The structure of the compound was established by spectral analysis including 1D and 2D NMR.The claims made by the local practitioners were therefore verified to be true to some extent.

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List of Abbreviations

cm	centimeter
cm^{-1}	per centimeter
Hz	Hertz
ml	milliliter
nm	nanometer
ppm	parts per million
UV	ultra violet
λ_{max}	wave length of maximum absorption
TLC	thin layer chromatography
δ	chemical shift in ppm
s	singlet
d	doublet
dd	double doublet
MHz	Mega hertz
%	percentage
R_f	retardation factor
HMBC	heteronuclear multiple bond correlation
HSQC	homo nuclear single quantum coherence
COSY	correlation spectroscopy
DEPT	distortionless enhancement by polarization transfer

CHAPTER ONE

1.1 INTRODUCTION

Ever since ancient times, in search for rescue for their disease, the people looked for drugs in nature. The beginnings of the medicinal plants' use were instinctive, as is the case with animals (Stojanoski, 1999). In view of the fact that at the time there was not sufficient information either concerning the reasons for the illnesses or concerning which plant and how it could be utilized as a cure, everything was based on experience. In time, the reasons for the usage of specific medicinal plants for treatment of certain diseases were being discovered; thus, the medicinal plants' usage gradually abandoned the empiric framework and became founded on explicatory facts. Until the advent of iatrochemistry in 16th century, plants had been the source of treatment and prophylaxis (Kelly, 2009). Nonetheless, the decreasing efficacy of synthetic drugs and the increasing contra-indications of their usage make the usage of natural drugs topical again.

Medicinal plants are of great importance to the health of individuals and communities. The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolic compounds (Hill, 1952). Many of these indigenous medicinal plants are used as spices and food plants. They are also sometimes added to foods meant for pregnant and nursing mothers for medicinal purposes (Okwu, 1999, 2001).

Natural products, such as plants extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drugdiscoveries because of the unmatched

availability of chemical diversity (Cosa *et al.*, 2006). According to the World Health Organization (WHO), a fact sheet on traditional medicine published by the body in 2008 states that more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. The use of herbal medicines in Asia represents a long history of human interactions with the environment. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases (Duraipandiyan *et al*., 2006). Due to the development of adverse effects and microbial resistance to the chemically synthesized drugs, men turned to ethnopharmacognosy. They found literally thousands of phytochemicals from plants as safe and broadly effective alternatives with less adverse effect. Many beneficial biological activity such as anticancer, antimicrobial, antioxidant, antidiarrheal, analgesic and wound healing activity were reported. In many cases the people claim the good benefit of certain natural or herbal products. However, clinical trials are necessary to demonstrate the effectiveness of a bioactive compound to verify this traditional claim. Clinical trials directed towards understanding the pharmacokinetics, bioavailability, efficacy, safety and drug interactions of newly developed bioactive compounds and their formulations (extracts) require a careful evaluation. Clinical trials are carefully planned to safeguard the health of the participants as well as answer specific research questions by evaluating for both immediate and long-term side effects and their outcomes are measured before the drug is widely applied to patients.

Medicinal plants are plants whose extracts can be used directly or indirectly for the treatment of different ailments. Therefore, the use of traditional medicine and medicinal plants in most developing countries, as a basis for the maintenance of good health, has been widely observed (Edward, 2001). Scientists throughout the world are trying to explore the precious

assets of medicinal plants to help the suffering humanity. Furthermore, in the world more than 30% of the pharmaceutical preparations are based on plants (Shinwari and Khan, 1998).

However, an increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants. The use of medicines from plants in the form of local medicine dates back to 4000-5000 B.C. While the medicinal values of these plants are due to the presence of small doses of active compounds which produces physiological actions in the human and animal body (Zaidi, 1998). Some of the important bioactive compounds found in medicinal plants are alkaloids, glycosides, resins, gums, mucilages etc. (Sack and Forehlich, 1982). It was observed that developed countries mostly imports raw materials of valuable medicinal plants from developing countries. Where they are screened, analyzed and used in drug preparations, and returned as high priced medicines to developing countries (Shinawie, 2002).

1.2 Bioactive compounds in medicinal plants

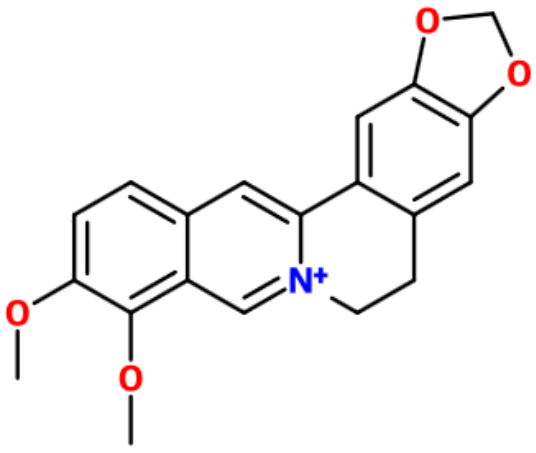
Bioactive compounds in plants are secondary plant metabolites eliciting pharmacological or toxicological effects in man and animals. Some of the important bioactive compounds found in medicinal plants are alkaloids, glycosides, resins, gums, mucilages, tannins, steroids, phenolic compounds etc. Some of which will be discussed in details.

1.2.1 Alkaloids

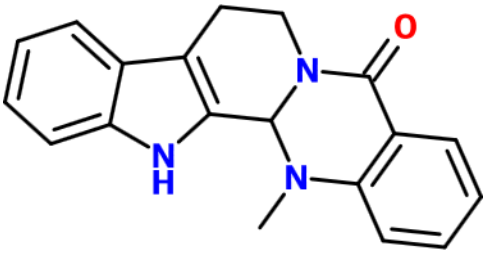
Alkaloids are important chemical compounds that serve as a rich reservoir for drug discovery. Several alkaloids isolated from natural herbs exhibit antiproliferation and antimetastasis effects on various types of cancers both in vitro and in vivo. Alkaloids, such as

camptothecin and vinblastine, have already been successfully developed into anticancer drugs (Jin-Jiang *et al.*, 2012).

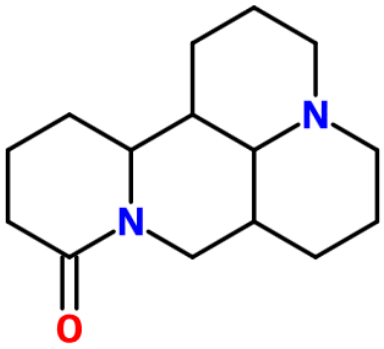
Alkaloids are a highly diverse group of compounds that contain a ring structure and a nitrogen atom. In most cases, the nitrogen atom is located inside the heterocyclic ring structure (Wang *et al.*, 2009). A classification based on biosynthetic pathways is mostly used to categorize different alkaloid (Wang *et al.*, 2009). Alkaloids have a wide distribution in the plant kingdom and mainly exist in higher plants, such as those belonging to *Ranunculaceae, Leguminosae, Papaveraceae, Menispermaceae, and Loganiaceae* (Wang *et al.*, 2009). Moreover, several alkaloids exhibit significant biological activities, such as the relieving action of ephedrine for asthma, the analgesic action of morphine, and the anticancer effects of vinblastine (Lee, 2011). In fact, alkaloids are among the most important active components in natural herbs, and some of these compounds have already been successfully developed into chemotherapeutic drugs, such as camptothecin (CPT), a famous topoisomerase I (TopI) inhibitor (Huang *et al.*, 2007) and vinblastine, which interacts with tubulin (Li *et al.*, 2007).



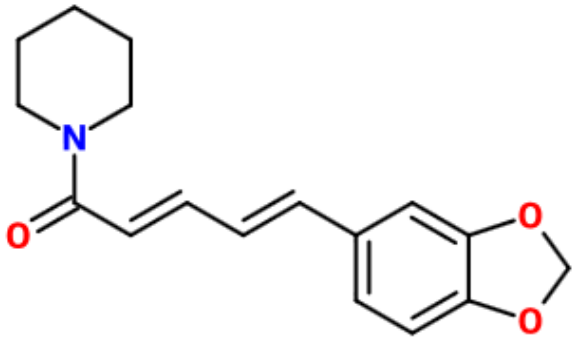
Berberin



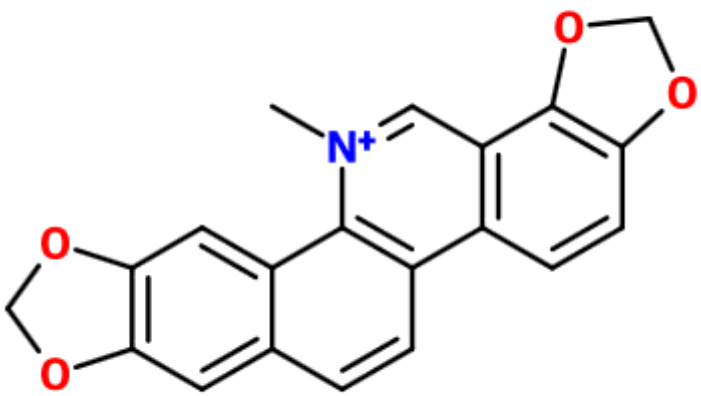
Evodiamine



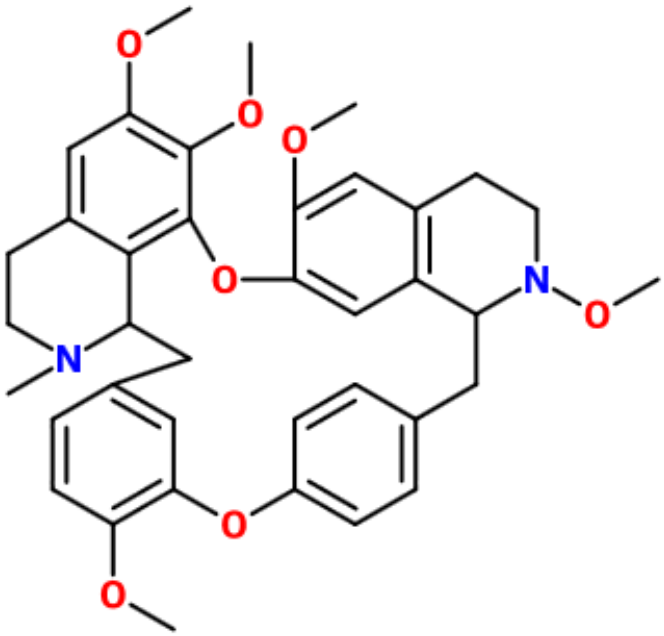
Maltrine



Piperine



Sanguimarine



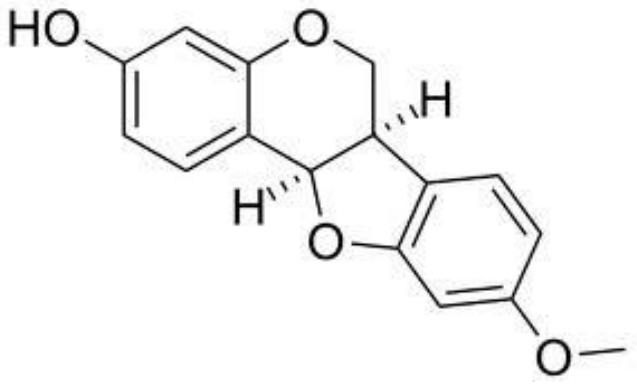
Tetrandrine

1.2.2 Phenolic compounds

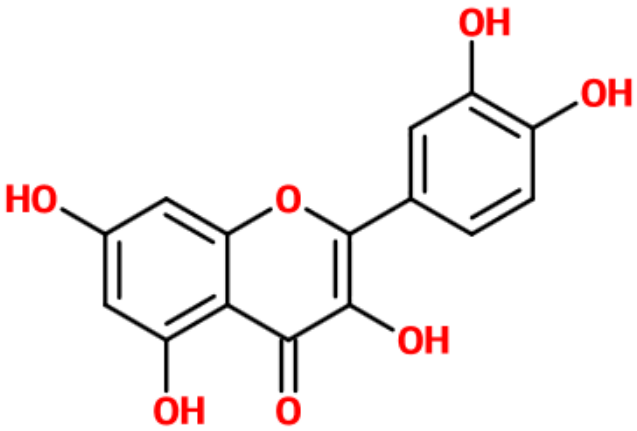
Phenolic compounds are widely distributed in plant kingdom and constitute one of the most important classes of natural and synthetic antioxidants. Phenolic compounds are well-known phytochemicals found in all plants. They consist of simple phenols, benzoic and cinnamic acid, coumarins, tannins, lignins, lignans and flavonoids (Khoddami *et al.,* 2013).

Primarily phenolic compounds are of great importance as cellular support material because they form the integral part of cell wall structure by polymeric phenolics (Gupta *et al.,* 2010), bioactive polyphenols have attracted special attention because they can protect the human body from the oxidative stress which may cause many diseases, including cancer, cardiovascular problems and ageing (Robards *et al.,* 1999). The phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites. A number of studies have focused on the biological properties such as antiapoptosis, anti-ageing, anticarcinogen, anti-inflammation, anti-artherosclerosis, cardiovascular protection and improvement of the endothelial function, as well as inhibition of angiogenesis and cell proliferation activity (Han *et al.*,2007). Phenolic compounds have been extensively used in disinfections and remain the standards with which other bacteriocides are compared (Okwu, 2001).

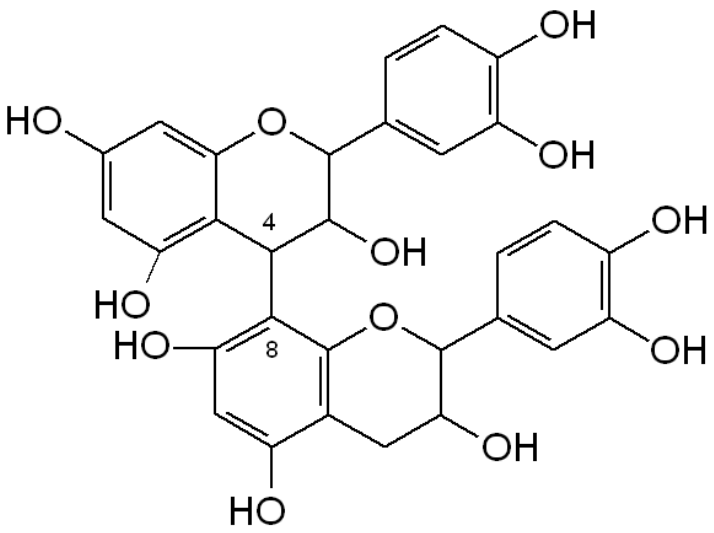
Quercetin is a flavonoid that possesses various degrees of antioxidant or free radical scavenging properties (Korkina and Afana, 1997). Below are examples of common phenolic compounds of plant origin.



Medicarpin



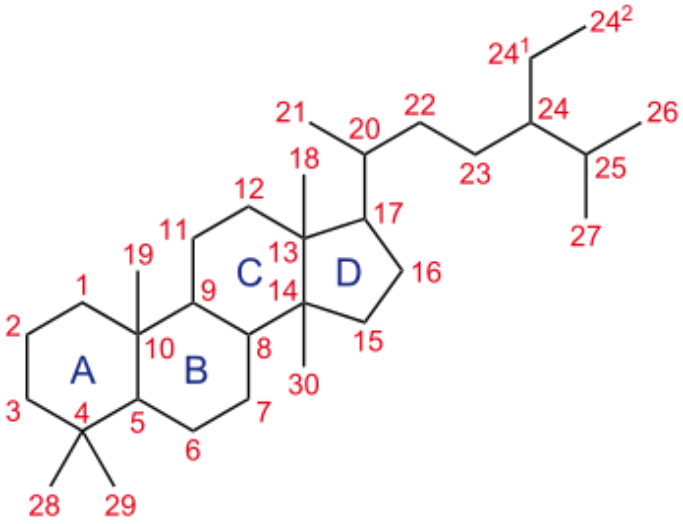
Quercetin



Procyanidin

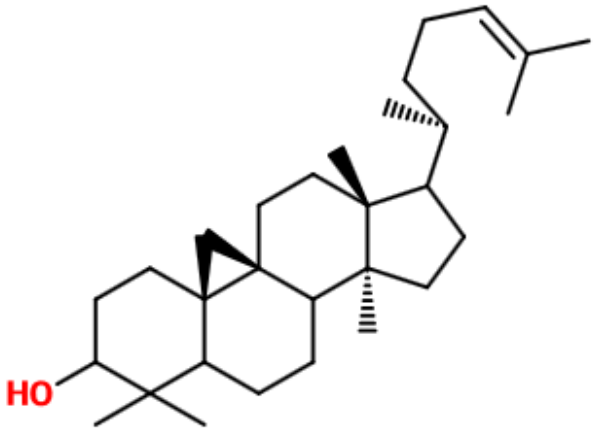
1.2.3 Steroids

A steroid is a type of organic compound that contains a characteristic arrangement of four cycloalkane rings that are joined to each other. Examples of steroids include the dietary fat cholesterol, the sex hormones estradiol and testosterone and the anti-inflammatory drug dexamethasone. The core of steroids is composed of seventeen carbon atoms bonded together that take the form of four fused rings: three cyclohexane rings (designated as rings A, B and C in the figure below) and one cyclopentane ring (the D ring).

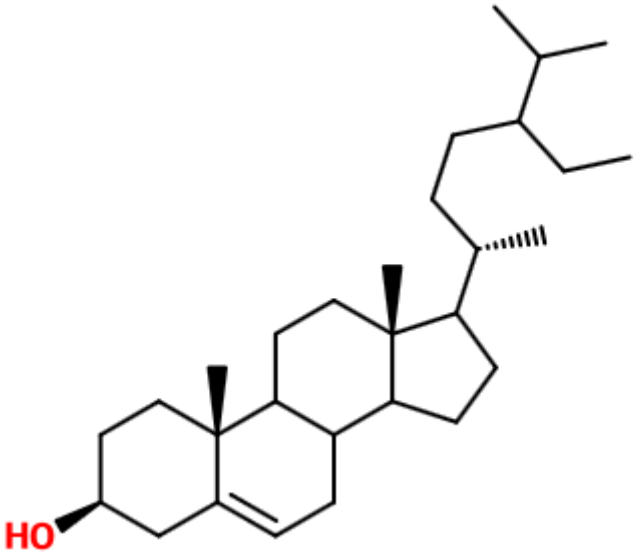


Structure of steroids

The steroids vary by the functional groups attached to this four-ring core and by the oxidation state of the rings. Sterols are special forms of steroids, with a hydroxyl group at position-3 and a skeleton derived from cholestane (Moss, 1989). Hundreds of distinct steroids are found in plants, animals and fungi. All steroids are made in cells either from the sterols lanosterol (animals and fungi) or from cycloartenol (plants). Both lanosterol and cycloartenol are derived from the cyclization of the triterpene squalene.



Cycloartenol



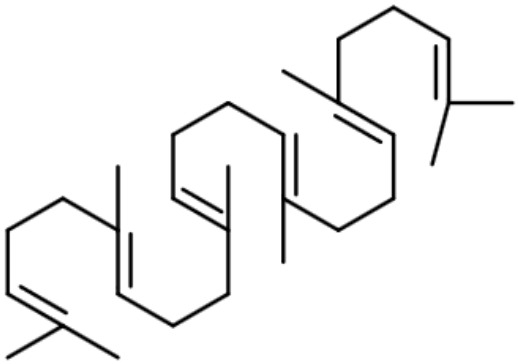
Sitosterol

1.2.4 Terpenes and Terpenoids

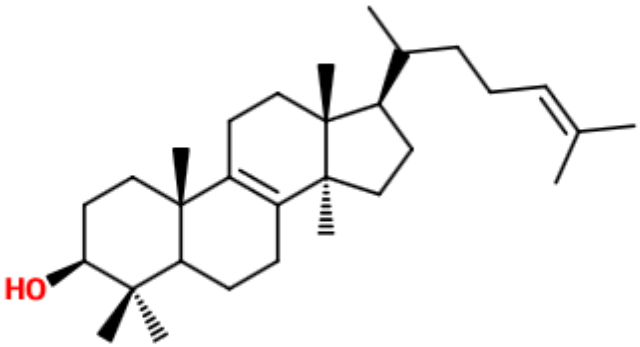
Terpenes are specialized plant metabolites that act as attractants to pollinators and as defensive compounds against pathogens and herbivores. Terpenes and terpenoids are the primary constituents of the essential oils of many types of plants and flowers. These compounds belong to the isoprenoid group. Even though isoprene itself has not been found in nature, its polymers, terpenic hydrocarbons and their oxygen derivatives are very often present in large quantities in different plant species. The resin produced by most plants is a viscous liquid, composed mainly of volatile fluid terpene (Finar, 1964). The single isoprene unit, therefore, represents the most basic class of terpenes and the hemiterpenes. An isoprene unit bound to a second isoprene is the defining characteristic of terpene, which is also a monoterpene with ten carbon atom skeleton; (C10). Sesquiterpenes contain three isoprene units (C15), while diterpenes (C20) and triterpenes (C30) contain two and three terpene units respectively. Tetraterpenes consist of four terpene units, polyterpenes more than four such units. In nature, terpenes occur predominantly in the form of hydrocarbons, alcohols and their glycosides, ethers, aldehydes, ketones, carboxylic acids and esters.

Apart from broad structural diversity, terpenes also exhibit a wide array of biological actions. Essential oils have antimicrobial, antiparasitic, insecticidal and antioxidant/prooxidant activities that often represent the combined bioactivity of multiple components (Stepnowski, 2012). Many plant terpenoids are cytotoxic towards tumour cells and are applied as chemotherapeutic or chemo-preventive compounds (Bifulco, 2005). Terpenes play an important role as signal compounds and growth regulators (phytohormones) of plants (Stepnowski, 2012). Many insects metabolize the terpenes which they may have obtained from their plant food for synthesizing growth hormones and pheromones (Stepnowski, 2012). Many plant and insect

resins like Lac resin also have economically beneficial properties, useful for varnishes and adhesives (Ghorai, 1996). Examples of some common terpenes are squalene, geraniol, and lanosterol.



Squalene



Lanosterol

1.2.5 Tannins

Tannins has astringent properties, hastens the healing of wounds and inflamed mucous membrane. Tannins contribute property of astringency i.e. fasten the healing of wounds and inflamed mucous membrane and have received considerable attention in the fields of nutrition, health and medicine, largely due to their physiological activity, such as antioxidant, antimicrobial and anti-inflammatory properties. Tannins are complex moieties produced by majority of plants as protective substances, they have wide pharmacological activities. They have been used since past as tanning agents and they possess astringent, anti-inflammatory, antidiarrheal, antioxidant and antimicrobial activities (Killedar and More, 2010).

1.3 AIM AND OBJECTIVES OF THE RESEARCH

1.3.1 Aim of the research

The aim of this project was to authenticate or otherwise, the traditional medicinal uses of the plant *Ceropegia deightonii*..

1.3.2 Objectives of the research

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

1. Collection of the plant sample.
2. Proper botanical identification of the plant.
3. Air drying and pulverization of the plant.
4. Extraction of the pulverized plant.

5. Phytochemical screening of the crude extracts.
6. Antimicrobial screening of the crude extracts.
7. Chromatographic separation and purification of the various components of the crude extracts.
8. Structural elucidation and characterization of the isolated compounds using various spectroscopic techniques

1.4 RESEARCH JUSTIFICATION

Numerous medicines in use today are extracted from plants. About 50 to 60% of pharmaceutical drugs are either of natural origin or obtained through use of natural products as starting points in their synthesis (Balandrin *et al*., 1993). Plants have been a rich source of medicines because they produce a host of bioactive molecules, most of which probably evolved as chemical defences against predation or infection (Cox and Balick, 1994).

It is against this background that thequest to investigate the active ingredients in *Ceropegia deightonii* that are responsible for the cure of the various ailments as claimed by the traditional medicine practitioners started.

1.5 SCOPE AND LIMITATION OF THIS RESEARCH

1.5.1 Scope of this research

This research work is expected to cover solvent extraction of the plant, phytochemical screening of the plant extracts, antimicrobial screening of the crude extracts and isolated

compound(s), isolation of active compound(s) if any and characterization and structural elucidation of the isolated compound(s).

1.5.2 Limitations of this research

However, due to unavailability of some modern analytical equipments and the high cost of running the analysis overseas, a good number of the active ingredients in the plant may not be fully profiled.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The family Asclepiadaceae

According to Angiosperm Phylogeny Group (APG II), the *Asclepiadaceae* is a former plant family now treated as a subfamily (subfamily *Asclepiadoideae*) in the *Apocynaceae* family (Bruyns and Endress, 2000). They form a group of perennial herbs, twining shrubs, lianas or rarely trees but notably also contain a significant number of leafless stem succulents. The name comes from the type genus *Asclepias* (milkweeds). There are 348 genera, with about 2,900 species. They are mainly located in the tropics to subtropics, especially in Africa and South America.

Apocynaceae is a family of flowering plants that includes trees, shrubs, herbs, stem succulents, and vines, commonly called the dogbane family. Members of the family are native to European, Asian, African, Australian and American tropics or subtropics, with some temperate members (Bruyns and Endress, 2000). Many species are tall trees found in tropical rainforests, but some grow in tropical dry (xeric) environments. There are also perennial herbs from temperate zones. Many of these plants have milkylatex, and many species are poisonous if ingested. Some genera of *Apocynaceae*, such as *Adenium*, have milky latex apart from their sap, and others, such as *Pachypodium*, have clear sap and no latex.

2.1.1 The genus *Ceropegia*

Ceropegia is a genus of plants within the family *Apocynaceae*. It was named by Carl Linnaeus, who first described this genus in volume 1 of his Species plantarum, which appeared in 1753. Linnaeus thought that the flowers looked like a fountain of wax. From this the scientific name was derived: '*keros*' meaning wax and '*pege*' meaning fountain (Pooley, 1998). They have many common names including lantern flower, parasol flower, parachute flower, bushman's pipe, string of hearts, snake creeper, wine-glass vine, rosary vine, and necklace vine. *Ceropegia* species have attracted much attention from botanists, horticulturalists, gardeners and succulent plant enthusiasts.

The genus *Ceropegia* belongs to the *Asclepiadoideae* (Milkweed) sub-family within the family *Apocynaceae*. *Ceropegia* comprises more than 180 species, all restricted to the Old World. The plants are found in tropical and subtropical habitats from Canary Islands and West-Africa as far as Australia, with main distribution areas in East-Africa, India, Madagascar and China (Meve and Liede-Schumann, 2007). The table below shows the list of some *Ceropegia* species (Pooley, 2008) (Dyer, 1983).

Table 2.0: List of some *Ceropegia* species

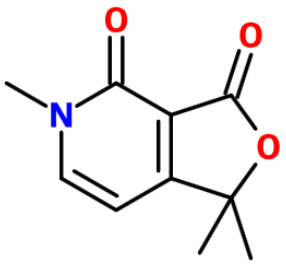
S/No	Specie	S/No	Specie
1	*Ceropegia Africana*	31	*Ceropegia fusca*
2	*Ceropegia ampliata*	32	*Ceropegia galeata*
3	*Ceropegia antennifera*	33	*Ceropegia intermedia*
4	*Ceropegia Arabica*	34	*Ceropegia gemmifera*
5	*Ceropegia arenaria*	35	*Ceropegia haygarthii*
6	*Ceropegia aridicola*	36	*Ceropegia hians*
7	*Ceropegia aristolochoides*	37	*Ceropegia juncea*
8	*Ceropegia armandii*	38	*Ceropegia krainzii*
9	*Ceropegia ballyana*	39	*Ceropegia leroyi*
10	*Ceropegia barbarta*	40	*Ceropegia linearis*
11	*Ceropegia barkleyi*	41	*Ceropegia lugardae*
12	*Ceropegia bonafouxii*	42	*Ceropegia multiflora*
13	*Ceropegia bosseri*	43	*Ceropegia nilotica*
14	*Ceropegia cancellata*	44	*Ceropegia pachystelma*
15	*Ceropegia carnosa*	45	*Ceropegia petignatii*
16	*Ceropegia ceratophora*	46	*Ceropegia racemosa*
17	*Ceropegia chrysantha*	47	*Ceropegia radicans*
18	*Ceropegia cimiciodora*	48	*Ceropegia rendallii*
19	*Ceropegia crassifolia*	49	*Ceropegia robynsiana*
20	*Ceropegia debilis*	50	*Ceropegia rupicola*
21	*Ceropegia decidua*	51	*Ceropegia sandersonii*
22	*Ceropegia denticulate*	52	*Ceropegia senegalensis*
23	*Ceropegia devecchii*	53	*Ceropegia seticorona*
24	*Ceropegia dichotoma*	54	*Ceropegia somaliensis*
25	*Ceropegia dimorpha*	55	*Ceropegia stapeliiformis*
26	*Ceropegia distincta*	56	*Ceropegia stentii*
27	*Ceropegia elegans*	57	*Ceropegia gemmifera*
28	*Ceropegia filiformis*	58	*Ceropegia haygarthii*
29	*Ceropegia fimbriata*	59	*Ceropegia hians*
30	*Ceropegia verrucosa*	60	*Ceropegia juncea*

2.1.2 Chemical compositions of *Ceropegia* species

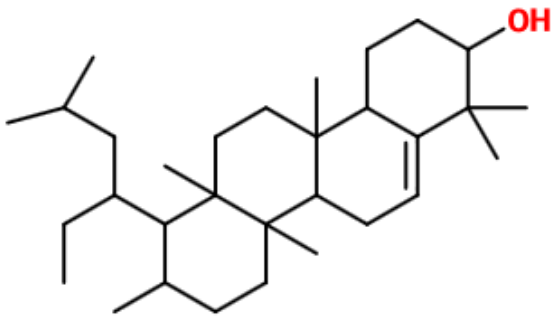
The investigation for the phytochemistry of genus *Ceropegia* possibly began in 1990 (Sivakumar *et al.,* 1990). So far, a variety of interesting but limited compounds have been isolated and identified from the plant of this genus which are alkaloids. Cerpegin has been isolated and identified as a new pyridine type alkaloid from *Ceropegia juncea* together with lupeol, which are relatively rare in nature. Based on spectroscopic methods the structure of this alkaloid has been elucidated as 3,4-dioxo-1,1,5-trimethyl-1,3,4,5-tetrahydrofuro-[3,4-*c*]-pyridine. The total alkaloid fraction exhibited promising hepatoprotective, antipyretic, analgesic, local anesthetic, anti-ulcer, and mast-cell stabilizing, tranquilsing and hypotensive activities and was devoid of side effects as noted out by the sub-acute toxicity studies (Adibatti *et al.*, 1991). Cerpegin, a novel pyridine alkaloid isolated from *Ceropegia juncea* has shown an analgesic effect (not involving the opioid pathway) against acetic acid induced writhing in mice. The chloroform extract of *Ceropegia juncea* was found to be toxic at doses above 400 mg/kg and the mice showed excitation, irritability, convulsions and respiratory paralysis (Sukumar *et al.*, 1995), and (Sekar and Francis, 1998) reported it was used as alternative source for renewable energy due to presence of polyphenol, oil and hydrocarbon.

Alkaloids, triterpenes, phenolics, flavonoids, tannins, saponins, and carbohydrates were identified from *Ceropegia*. Cerpegin is a rare naturally occurring pyridinone alkaloid isolated from *Ceropegia juncea* and it is used in traditional Indian medicine as tranquillizer, anti-inflammatory, analgesic and antiulcer (Adibatti, 1985). Triterpenes seems to be rare in *Ceropegia*. Only two triterpenes were identified, 2-Guimarenol and lup-18-en-3-beta-ol from *Ceropegia dichotoma (*Gonzalez *et al.*, 1973).

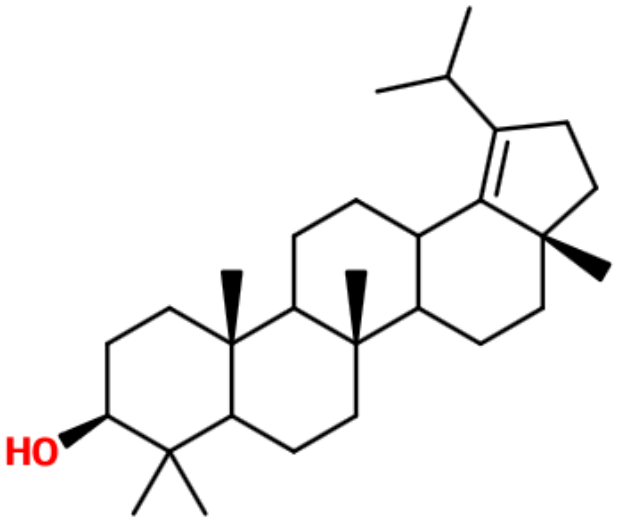
Glycosides also seem to be rare in *Ceropegia*. Steroidal glycosides and their derivatives were identified from aerial parts of *Ceropegia fusca* (Garcı´a, 2011). *Ceropegia* Species has revealed the presence of volatile oil and terpenes, in *Ceropegia woodii*, 41 peaks were isolated, of which 24 compounds were identified. Of the total volatile matter, 70.73% was terpenes, 5.82% was taxanes, and 1.52% was ketones (Meng *et al.*, 2010). Á-pinene, camphene, ocimene and eucalyptole (43.91% of total volatile matter) in *Ceropegia woodii*, (Meng *et al.*, 2010)



Cerpegin



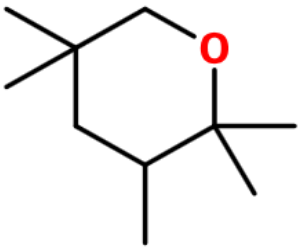
2-Guimarenol



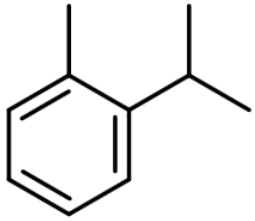
Lupenol



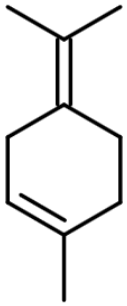
Sabinene



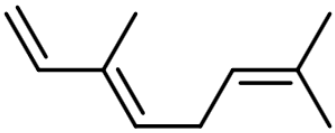
Eucalyptol



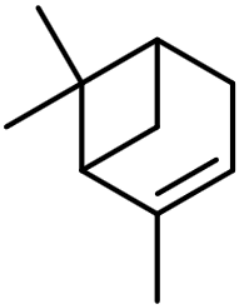
O-cymene



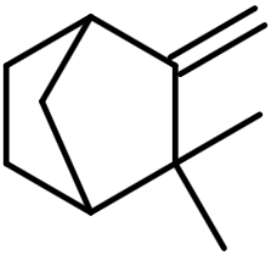
Terpinolene



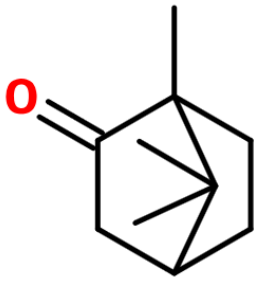
Ocimene



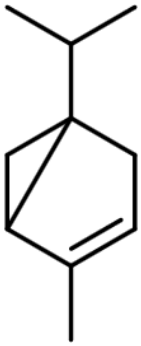
Alpha pinene



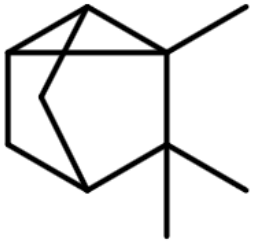
Camphene



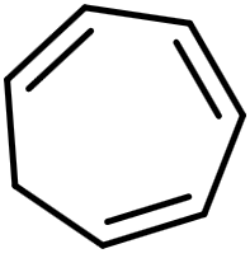
L-camphor



Thujene



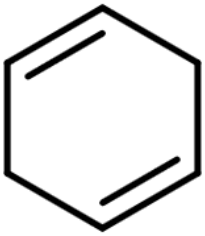
Tricycline



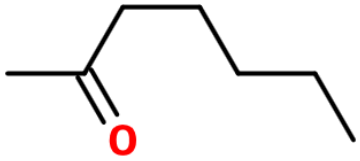
1,3,5-cycloheptatriene



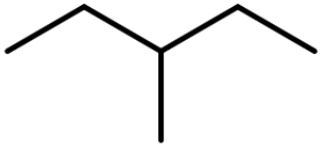
Undecane



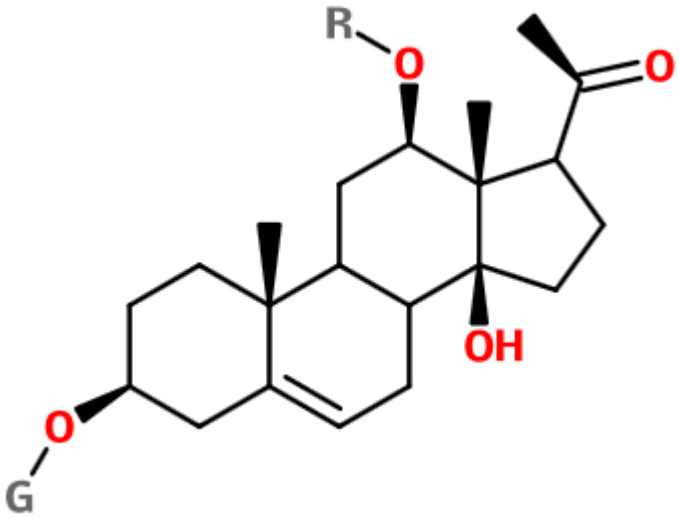
Cyclohexa-1,4-diene

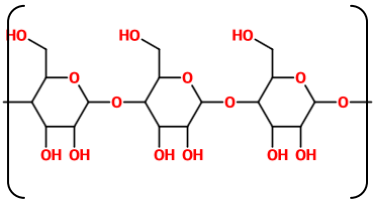


2-heptanone

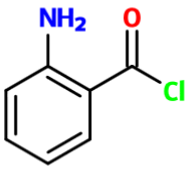


3-methyl pentane



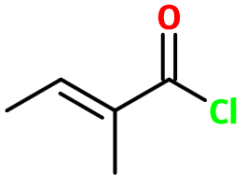


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2.1.3 Phytochemical studies on *Ceropegia* species

A research carried out on the preliminary phytochemical screening of the methanolic extract of the leaf and tuber of *Ceropegia bulbosa* and *Ceropegia attenauta* for the presence of steroids, reducing sugars, alkaloids, saponins, tannins, flavonoids, terpenoids, glycosides and vitamins C /ascorbic acid (Riddhu and Payal, 2014). The results are presented in Table 2.1 below.

Table 2.1: Preliminary phytochemical screening/characterization of leaf and tuber extract of *Ceropegia bulbosa*

S/no.	Sample compound	*Ceropegia bulbosa*	
		Leaves	Tuber
1	Alkaloids	+	+
2	Tannins	+	+
3	Glycosides	+	+
4	Vitamin C	-	-
5	Steroids	+	+
6	Flavonoids	+	+
7	Starch	+	+
8	Terpenoids	+	+
9	Volatile oil	+	+
10	Potassium salts	+	+

The preliminary phytochemical screening of *Ceropegia pusilla* tuber was carried out, it was found that the qualitative phytochemical analysis of this plant confirm the presence of various secondary metabolites like alkaloids, glycosides, tannins, saponin, flavonoids, steroid, triterpenes and phenol as presented in Table 2.2 (Kalimuthu and Prabakaran, 2013). The results

suggest that *Ceropegia pusilla* possess the phytochemical properties for curing various ailments and also potential anti-inflammatory, antimicrobial and antioxidant and leads to the isolation of new and novel compounds.

Table 2.2: Phytochemical analysis of methanolic extract of *Ceropegia pusilla.*

S/No.	Secondary metabolites	Ethanol extract of *C. pusilla*
1	Alkaloids	+
2	Glycosides	+
3	Tannins	+
4	Saponins	+
5	Flavonoids	+
6	Steroid	+
7	Terpenoids	+
8	Phenol	+

Gas chromatogram of the methanolic extract of the leaves of *Ceropegia pusilla* showed 53 peaks indicating the presence of fifty three compounds (Kalimuthu and Prabakaran, 2013). The active principles of some of the compounds with their retention time (RT), molecular formula, % area and compound name are presented in Table 2.3. The results pertaining to GC-MS analysis led to the identification of number of compounds from the GC fractions of the methanolic extract of *Ceropegia pusilla* tuber. These compounds were identified through mass spectrometry attached with GC. In addition to this, the results of the GC-MS profile can be used as pharmacognostical tool for the identification of the plant. The presence of various bioactive

compounds confirms the application of *Ceropegia pusilla* for various ailments by traditional practitioners (Kalimuthu and Prabakaran, 2013).

Table 2.3: Activity of phytocomponents identified in methanolic extract of *Ceropegia pusilla* by GC-MS

S/No.	RT	Compound name	Molecular formula	Molecular weight	Peak area
1	2.08	3-Phenylnitropropane	$C_9H_{11}NO_2$	165	4.61
2	2.65	1,2-Diphenyl-1-butanol	$C_{16}H_{18}O$	226	8.04
3	5.03	(+,-)-16,N-bis(methoxycarbonyl)-N-norsalutaridine	$C_{22}H_{23}NO_8$	429	1.75
4	6.13	3-{[(t-Butyl)dimethylsilyl]oxy}-21-hydroxypregna-5,16-dien-21-one - Acetate	$C_{29}H_{46}O_4Si$	486	3.09
5	8.86	Trifluoromethylcyclopentadiene	$C_6H_5F_3$	134	2.24
6	11.65	Ethyl Azacyclohexane-2-ylideneacetate	$C_9H_{15}NO_2$	169	1.87
7	14.34	3-tert-Butoxycarbonylaminophenyl isonitrile	$C_{12}H_{14}N_2O_2$	218	6.25
8	15.89	(E)-1,2-Bis(2-bromo-4,5-dimethoxyphenyl)-N,N-dimethylethenylamine	$C_{20}H_{23}Br_2NO_4$	499	6.48
9	19.56	1-(m-Nitrostyryl)naphthalene	$C_{18}H_{13}NO_2$	275	2.08
10	20.51	2,4,6-Tribromobenzaldehyde	$C_7H_3Br_3O$	340	3.72
11	21.29	2-Homophthalimidobenzoic acid	$C_{16}H_{11}NO_4$	281	2.34
12	21.06	Fac-(Tricarbonyl) (dimethylphosphinoethane)(1,3-dimethylimidazolidin-2-ylidene)chromium	$C_{16}H_{30}CrN_2O_3P_2$	412	2.68

S/No.	RT	Compound name	Molecular formula	Molecular weight	Peak area
13	21.80	6-(4-Fluorophenyl)-3-cyano-4-[N-[(phenyl)(4-chlorophenyl)methyl]piperazino]-2H-pyran-2-one	$C_{29}H_{23}ClFN_3O_2$	499	2.76
14	24.14	9-((1-methylpiperidin-3-yl)methyl)-10-thiaanthracene	$C_{20}H_{23}NS$	309	1.59
15	25.32	Oleanolic acid	$C_{30}H_{48}O_3$	456	5.63
16	25.81	1-Acetyl-4-(N,N-dimethylcarbamoylmethyl)-2-methoxyindoline	$C_{15}H_{20}N_2O_3$	276	1.98
17	27.83	4-[(tert-Butyldimethylsilyl)oxy]-2,4-diphenyl-3-butenal Dimethylhydrazone	$C_{24}H_{34}N_2OSi$	394	3.48
18	28.30	[2'-Amino-1'-(4''-chlorophenyl)-5'-benzoyl-1',4',5',6'-tetrahydro-6'-thioxo]-spiro[cyclohexane-1,4'-pyridine]-3'-carbonitrile	$C_{24}H_{22}ClN_3OS$	435	4.54
19	30.46	3-Cyano-2-ethoxy-6-oxo-6,7-dihydro-4-phenyl-7-(4-nitrophenyl)-1,7,10-anthyridine	$C_{26}H_{17}N_5O_4$	463	1.80
20	33.51	2,3,7,8-Tetraethyl-5-phenyl-[18]porphyrin	$C_{34}H_{34}N_4$	498	1.56
21	34.37	24-Methylene-5à-cholest-24(28)-en-3á,6á,15à,16á,26-pentol	$C_{28}H_{48}O_5$	464	1.74
22	35.08	Fenproporex	$C_{12}H_{16}N$	188	2.25
23	35.61	Tricarbonyl[(1-4-ü)-5-(2-amino-3-methoxy-5-methylphenyl)-1,3-cyclohexadiene]iron	$C_{17}H_{17}FeNO_4$	355	3.96

S/No.	**RT**	**Compound name**	**Molecular formula**	**Molecular weight**	**Peak area**
24	36.02	Benzyl(5-iodo-2-trifluoromethylsulfonyloxy) benzoate	$C_{15}H_{10}F_3IO_5S$	486	4.37
25	39.58	2-Butoxy-3-fluoro-4-quinolinecarboxylic acid	$C_{14}H_{14}FNO_3$	263	2.31
26	41.42	(Z)-1,2,3,4,5,6,7,8-Octadeoxy-10,11:12,13-di-O-isopropylidene-D-manno-tridec-6-enitol	$C_{19}H_{34}O_5$	342	1.70
27	42.23	24-methylcholesta-5,7,24(28)-trien-3á-ol	$C_{28}H_{44}O$	396	4.54
28	42.56	7,7a-Dihydro-3-(methylimino)-cis-1H,3H,5H-thiazolo[3,4-c]thiazole	$C_6H_{10}N_2S_2$	174	2.34
29	42.95	Endo-9-anti-11-Dichloro-syn-11-fluorotricyclo[5.3.1.0]undecane	$C_{11}H_{15}C_{l2}F$	236	6.29
30	43.76	1,1'-(1,5-Pentylidene)-2,2'-bis (trichloroacetyl)bis(pyrrole)	$C_{17}H_{15}C_{l6}N_2O_2$	489	2.01

2.1.4 Antimicrobial studies on *Ceropegia* species

A research was carried out on the antimicrobial activity of methanol extract of the whole plant of *Ceropegia pusilla* which was assayed against five species of bacteria including *Shigella sonnei*, *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus* sp., and four species of fungi including *Aspergillus* sp., *Mucor*sp., *Penicillium* sp., and *Candida albicans* by agar well diffusion method. The Results of antimicrobial activity assays are presented in Tables 2.4 and 2.5. (Kalimuthu *et al.*, 2013).

Table 2.4: Antimicrobial activity of methanol extract of *Ceropegia pusilla* whole plant.

Methanol extract of	*Escherichia coli*	*Klebsiella sp.*	*Shegilla sp.*	*Staphylococcus sp.*	*Bacillus sp*
In vitro plant	R	S	R	R	R
Invivo plant	R	S	R	I	R

Table 2.5: Antifungal activity of methanol extract of *Ceropegia pusilla* whole plant

Methanol extract of	*Aspergillus sp.*	*Mucor sp.*	*Penicillium sp.*	*Candida albicans*
In vitro plant	R	R	I	I
Invivo plant	R	S	S	S

Key note: R-Highly Resistant, I-Intermediate (Moderately sensitive) S-Sensitive

2.2 Biological activities of *Ceropegia* species

The sweet-sour leaves of *Ceropegia pusilla* are edible and are considered to be tonic and digestive (Kirtikar and Basu 1935). It is used as an Antidote for snake bite (Duraisamy and subramaniam 2010**)**. The tubers are edible (Mabberly, 1987) and contain starch, sugars, gum, albuminoids, carbohydrates, fats, crude fibre, and the medicinally important alkaloid Ceropegin (Kirtikar and Basu, 1935) (Nadkarni, 1976) (Jain and Defillips, 1991). The root tubers also contain starch, sugars, gum, albuminoids, fats, crude fiber and valuable constituents in many traditional Indian Ayurvedic drug preparations, The Ceropegin was an analgesic drug, tranquilizer and known to use against ulcers, inflammation that are active against ulcers, inflammation etc., (Adibatti *et al.,* 1991). The boiled or roasted tubers are edible and a rich source of carbohydrates (Nikam and Savanth, 2007).

These species are of economic importance (Jagtap and Singh, 1999) due to their starchy edible tubers with medicinal value. The fresh tubers of these species are usually boiled before they are eaten to remove the bitterness. The active compound of tuberous roots is the alkaloid Ceropegin which is active against diarrhea and dysentery inflammation of gums and delirious fevers of parturition (Nadkarni, 1976). The tubers then again are a kind of energy source, among other things they are used to suppress fatigue. Furthermore the sticks with which the Toda stamp milk to butter (http://www.biodiversitylibrary.org). The alkaloid Ceropegin from the tubers of *Ceropegia bulbosa* was used in Bihar to cure cold, sneezing and eye diseases (Kirtikar and Basu, 1935). The crude extracts of *Ceropegia tuberose* were active against different bacterial strains (Vijayakumar *et al*., 2013)

2.3 Review of *Ceropegia deightonii*

2.3.1 *Taxonomic classification*

Kingdom: Plantae

Subkingdom: Viridaeplantae

Division: Tracheophyta

Subdivision: Spermatophytina

Class: Magnoliopsida

Subclass: Asteridae

Order: Gentianales

Family: Asclepiadaceae

Genus: *Ceropegia*

Specie: *Ceropegia deightonii* Hutch and Dalziel

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

The chemicals used were of high quality and the reagents used were of analytical grade. The solvents were distilled before being used. Silica gel 60G for thin-layer chromatography was used throughout. The plant *Ceropegia deightonii* was collected in fresh condition at Makurdi, in Benue state, Nigeria, on 21 February, 2013, identified and authenticated at the Herbarium in Biological Sciences Department, Ahmadu Bello University, Zaria. The plant material was dried indoors in a well-ventilated room for 3 weeks and then size reduced in a wooden mortar. The pulverised plant material was packaged and labelled appropriately with specimen number 1740. NMR spectra were obtained on a Bruker AVANCE spectrometer (400 MHz for ^{1}H and 125 MHz for ^{13}C), using the TMS peaks as standard. Chemical shift values (δ) were reported in parts per million (ppm) relative to TMS standard.

3.2 Methods

3.2.1 Extraction of the plant *Ceropegia deightonii*

About 800g of the dried and pulverized plant material was extracted with redistilled methanol using soxhlet extractor. The extract was then concentrated using a rotary evaporator at about 40°C. Percentage recovery of the crude extracts was calculated as percentage crude extract of plant material used.

3.2.2 Preparation of stock solution for antimicrobial studies

Exactly 1.0 g of the methanol extract was weighed into Eppendorf tubes and dissolved in 10ml Dextrose saline to produce extract solutions of 100 mg/ml stock solution. Three other extract concentrations (20mg/ml, 40mg/ml and 80mg/ml) were prepared from the 'stock' extract solution by appropriate dilution with Dextrose saline. Extract solutions were prepared just before use (Mann *et al.,* 2009). These concentrations were used for the in vitro studies.

3.2.3 Phytochemical Screening

Chemical tests were carried out on the plant extract to identify the secondary metabolites such as alkaloids, flavonoids, saponins, tannins, anthraquinones, cardiac glycosides and steroids/terpenes using standard procedures as described by (Sofowora, 1982, 1993) (Edeoga *et al.*, 2005).

3.2.3.1 Test for Carbohydrates

Molisch's test (General test for Carbohydrates): The aqueous extract (2ml) in a test tube was added three drops of Molischts reagent followed by three drops of concentrated sulphuric acid down the side of the test tube which was allowed to form a lower layer. A purple to violet coloured interface indicate the presence of carbohydrates. The mixture, when shaken gently and allowed to stand for two minutes, followed by dilution with water (5ml) would produce a dull violet precipitate-another indication of carbohydrates (Trease and Evans, 1972, 1989, 2002).

Fehling's test for free reducing sugars: 5m1 of a mixture (1:1) of Fehling's solution A and Fehling's solution B was added to the extract (2ml) dissolved in water and the mixture

boiled on water bath for 5 minutes. A brick-red precipitate indicates the presence of reducing sugars.

3.2.3.2 Test for Anthraquinone Glycosides

To the extract (0.2 g) was added benzene (10 ml) and shaken for 5 minutes. 10% ammonia (5 m1) solution was added and shaken. The presence of a pink, red or violet colour in the ammoniacal (lower layer) phase indicates the presence of free anthraquinones. For the anthraquinone glycosides, the extract (0.2 g) was boiled with dilute sulphuric acid (10 ml) and filtered while hot. The filtrate was shaken with benzene (5 ml), the benzene layer was separated and to it, 10% ammonia solution (3 ml) was added and mixture shaken. The layers were allowed to separate. A pink, red or violet colouration in the lower layer shows the presence of anthraquinone glycosides (Sofowora, 1993).

3.2.3.3 Test for Saponin Glycosides

Frothing test: the extract (0.2g) was shaken with water (5m1) in a test tube for 30 seconds. A persistent froth for 15 minutes or when warmed on water bath indicates the presence of saponins (Sofowora, 1993).

3.2.3.4Test for Steroids and Terpenoids

(a) Salkowski's test for steroids: the extract (0.5g) was dissolved in chloroform and concentrated sulphuric acid (2ml) was carefully added down the side of the test tube to form a lower layer. A reddish brown colour at the interface indicates the presence of steroidal ring (Sofowora, 1993).

(b) Liebermann -Burchard's test: the extract (0.5g) was dissolved in chloroform (2ml) and filtered into a clean, dry test tube. Acetic anhydride (2ml) was added to the filtrate and shaken. Few drops of concentrated sulphuric acid were 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 sterols and terpenes.

3.2.3.5 Test for Tannins

(a) Ferric chloride test: the extract (0.2g) was stirred with water (5ml) and filtered. To the filtrate (2ml) in a test tube, two drops of ferric chloride solution were added. A green or greenish black precipitate indicate the presence of condensed tannins, while a blue or bluish black precipitate shows the presence of hydrolysable tannins (Trease and Evans, 1989).

(b) Lead subacetate test: To the aqueous extract (1ml), three drops of lead subacetate solution was added. A coloured precipitate indicates the presence of tannins (Trease and Evans, 2002).

3.2.3.6 Test for Alkaloids

General test: About (0.2 g) of the extract was stirred with 1% hydrochloric acid (5 ml) on a water bath. To the filtrate (1 ml) was added, 2-3 drops of the following reagents were added:

- Wagner reagent solution of iodine in potassium iodide
- Mayer's reagent (Potassium mercuric iodide solution)
- Dragendroffs reagent (Potassium bismuth iodide solution)

The presence of precipitate in most or all of the above reagents indicates the presence of alkaloids (Trease and Evans, 1989), that is;

- Light brown to brownish precipitate- Wagner's reagent
- White or creamy white precipitate - Mayer's reagent
- Orange or orange-yellow precipitate –Drangendroff's reagent

3.2.3.7 Test for Cardiac Glycosides

The extract (1.0 g) was dissolved in water. Strong lead subacetate solution (1 ml) was added and filtered. The filtrate was divided into two for the following tests: **Keller-killiani Test (for Steroidal nucleus)**: To the first portion of the filtrate was added chloroform, the chloroform layer (lower) was separated and evaporated to dryness on water bath. The residue was dissolved in 3.5% ferric chloride (3 ml) solution in glacial acetic acid. The mixture was allowed to stand for a minute before it was transferred to a test tube. Concentrated sulphuric acid (1.5 ml) was added down the side of the test tube to form a lower layer on standing, the presence of a brown colour at the junction of the two liquids and a pale green colour in the upper layer indicates the presence of cardiac glycosides (Brain and Turner, 1975).

3.2.3.8 Test for Flavonoids

Shinoda's test: the extract (0.2 g) was diluted with ethanol. Some pieces of magnesium filing were added followed by five drops of concentrated hydrochloric acid. A pink or red colour indicates the presence of flavonoids (Silva *et al.*, 1998).

Sodium hydroxide test: the extract (0.2 g) was dissolved in water (2 ml) and filtered. 10% aqueous sodium hydroxide (2 ml) solution was added. A Yellow solution indicates the presence of flavonoids. On addition of dilute hydrochloric acid, the solution becomes colourless (Brain and Turner, 1975).

Ferric Chloride Test: The extract (0.1 g) was boiled with water and filtered. To the filtrate (2ml), 2 drops of 10 % ferric chloride solution were added. A green or blue colour indicates the presence of phenolic nucleus.

3.3. Antimicrobial Screening

3.3.1 Test Organisms

The bacteria for this research viz. *Staphylococcus aureus; Streptococcus feacalis; Escherichia coli; Salmonella typhi; Shigella dysenteriae; Pseudomonas aeruginosa; Candida albicans and Candida stellafoidea* were gotten from Ahmadu Bello University Zaria Teaching Hospital. All the micro-organisms were checked for purity and maintained in slants of agar.

3.3.2 Cultivation and Standardization of test Organisms

A loop full of each of the test organisms was taken from the agar slant and sub cultured into test tubes containing sterile nutrient agar and sabouraud dextrose medium. The test tubes were then incubated for 24 hours at 37 °C for two days. The broth culture was standardized using sterile normal saline to obtain a density of 106cfu/ml for bacteria.

3.3.3 Preparation of Culture Media

The prescribed quantities of the dehydrated bacteriological culture media was weighed and hydrated with distilled water according to the manufacturer's specification. Where necessary, gentle heat was applied to aid dissolution and the resultant suspensions were dispensed into clean bottles and sterilized at 121 °C for 15 minutes in an Adelphi bench autoclave.

3.3.4 Antimicrobial Profile (susceptibility test)

The antibacterial screening was carried out using the agar diffusion method as described by (Lino and Deogracios, 2006) with slight modifications. Overnight cultures of the various bacteria in blood agar were sterilized to produce inoculums size of 10^6cfu/ml. The medium was seeded with standard inoculums (0.1 ml) of the micro-organism (mc-forland 0.5). The inoculums were sprayed evenly by the use of sterile swab over the surface of the medium; the seeded plants were allowed to dry at 37 °C for 30 mins inside incubator. A standard cork borer (6 mm) was used to cut a well at the center of each seeded medium used and the solution of the extracts (10mg/ml of each) was then introduced into each hole on the surface of the medium. In one medium was introduced sterile water (0.1 ml) to serve as negative control. The medium was incubated at 37 °C and 27 °C for 24 hours and 2-7 days for the bacteria and fungi respectively after which the plates were observed for zones of inhibition. The zones of inhibition were measured with a transparent rule and the results recorded.

3.3.5 Determination of Minimum Inhibitory Concentration (MIC)

This was done using broth dilution method as described by (Vollekova *et al.,* 2001) modified by (Usman *et al.,* 2007). In this method, 10ml nutrient broth (prepared using manufacturer's specifications) was dispensed into test tubes and sterilized at 121 °C for 10 minutes and allowed to cool. Mc-farland's turbidity standard scale number 0.5 was prepared to give a turbid solution. Normal saline was inoculated with each of the test micro-organism and incubated at 37 °C for 6 hours to make a turbid suspension of the micro-organisms. After incubations, dilution of the micro-organism in normal saline was done until the turbidity (l.5 x l06cfu/ml) matched that of the Mc-Forland scale by visual comparison. From the suspension of

the micro-organism in normal saline, 0.1ml was inoculated into the different concentrations of the extract in the nutrient broth. The broths were incubated at 37 °C for 24hours. The results were recorded after 24hours.

3.3.6 Determination of Minimum Bactericidal Concentration (MBC)

Blood agar was prepared according to manufacturer's instruction, sterilized at 121 °C for 15 minutes. It was poured into sterile petri-dishes. The plates were allowed to cool and solidify. The contents of the MIC test tubes in the serial dilution were then sub-cultured on to the prepared plates and the plates were then incubated at 37 °C for 24 hours. The results were recorded after 24 hours. (Vollekova *et al*., 2001) (Usrnan *et al.,* 2007).

3.4 Column Chromatographic separation of extracts

A glass column of dimensions (75 by 3.5cm) was used. The Column was packed using wet slurry method and the sample was loaded by the dry loading method (Cannell, 1998); the sample was dissolved in minimum amount of suitable organic solvent, mixed with a small quantity of silica gel, dried, triturated and then loaded on top of the previously packed column. Various solvent systems comprising petroleum ether/ethyl acetate mixtures were used in eluting the column by gradient elution with Silica gel (60 – 120 mesh size) as the *Stationary phase*. The eluates were collected in fractions of 100ml each in a beaker. The Thin layer chromatography (TLC) of the collected fractions were carried out and fractions with very similar TLC profiles were pooled together to give major fractions. One of the major fractions with very few prominent spot(s) on its TLC profile was washed with petroleum ether several times and the

prominent spots were separated using a preparative TLC. A white powdery solid was obtained from the preparative TLC and was named ALFA.

3.4.1 Isolation of ALFA

45 fractions, 100 ml each were collected from the column. The fractions were pooled together based on their TLC profile to give two major fractions and the column was finally washed with methanol to give the third fraction. Fraction 2 consisting of one major spot and two minor spots was washed with petroleum ether several times and separated using preparative TLC to give 5.5 mg of ALFA consisting of just one spot. ALFA was subjected to spectroscopic analysis to elucidate its chemical structure.

3.4.2 TLC analysis of ALFA

TLC analysis of ALFA using two different solvent systems, Petroleum ether:Ethyl acetate (8:2, R_f0.6) and (9:1, R_f0.4) gave a single homogenous spot. Spraying with 10% H_2SO_4 and heating gave a purple colored spot.

3.5 Spectroscopic analyses of isolate(s)

^{1}H and ^{13}C NMR spectra: NMR spectra were obtained on a Bruker AVANCE spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C) with $CDCl_3$ solvent, using the TMS peaks as standard. Chemical shift values (δ) were reported in parts per million (ppm) relative to TMS standard.

CHAPTER FOUR

4.0 RESULTS

4.1 Phytochemical screening of the plant extracts

The result of the phytochemical screening of the plant *Ceropegia deightonii* is shown in Table 4.1 below.

Table 4.1: The result of the phytochemical screening of the plant *Ceropegia deightonii*

Phytochemical	Petroleum ether	Ethyl acetate	Methanol
Anthraquinones	-	-	-
Cardiac Glycosides	+	+	+
Saponins	-	+	+
Steroids and tri-terpenes	+	-	-
Flavonoids	-	+	+
Tannins	-	+	+
Alkaloids	-	-	-

Key: + = Present, - = Absent.

4.2 Antimicrobial screening of the plant extracts

The results of the antimicrobial susceptibility tests, expressed in terms of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and diameter of zones of inhibition of the test organism are shown in Tables 4.2, 4.3 and 4.4 respectively.

Table 4.2: Sensitivity test of the microorganisms to varying concentrations of the extracts

Test Organisms	Mean Zone of inhibition (mm)				
	EA extract	**ME extract**	**Pet-E extract**	**Ciprofloxacin**	**Fluconazole**
S. aureus	26	23	20	37	0
S. feacalis	25	21	20	39	0
E. coli	26	23	19	30	0
S. typhi	0	0	0	32	0
S. dysenteriae	27	23	20	42	0
P. aeruginosa	0	0	0	30	0
C. albicans	24	21	19	0	47
C. stellatoidea	0	0	0	0	41

ME = Methanol, EA = Ethyl acetate, Pet-E = Petroleum ether

Drug concentrations = Ciprofloxacin (5 µg/ml), Fluconazole (5 µg/ml).

Table 4.3: Determination of the Minimum Inhibitory Concentration (MIC)

Test Organisms	Ethyl acetate extract (mg/ml)	Methanol extract (mg/ml)	Petroleum ether extract (mg/ml)
S. aureus	10	10	20
S. feacalis	10	10	10
E. coli	10	10	20
S. typhi	-	-	-
S. dysenteriae	5	10	20
P. aeruginosa	-	-	-
C. albicans	10	10	20
C. stellatoidea	-	-	-

Table 4.4: Determination of the Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) of the extracts against the microbes.

Test Organisms	Ethyl acetate extract (mg/ml)	Methanol extract (mg/ml)	Petroleum ether extract (mg/ml)
S. aureus	20	20	40
S. feacalis	20	40	40
E. coli	20	20	40
S. typhi	-	-	-
S. dysenteriae	20	20	40
P. aeruginosa	-	-	-
C. albicans	20	40	40
C. stellatoidea	-	-	-

4.3 Chromatographic separation of the extract of the plant

The petroleum ether extract of *Ceropegia deightonii* was subjected to thin layer chromatography using pre-coated silica aluminium TLC plate and Petroleum ether:Ethyl acetate (9:1) as solvent system to determine the profile of this extract. Two prominent spots were observed after visualization.

Table 4.5: Colour of observed spots and their R_f values

Spot	R_f Value	Color in 10% H_2SO_4
1	0.25	Pink
2	0.18	Purple

Table 4.6: TLC analysis of ALFA using two different solvent systems.

Solvent system	Number of spot	Color of spot	R_f value
Petroleum ether:Ethyl acetate (8:2)	1	Purple	0.6
Petroleum ether:Ethyl acetate (9.1)	1	Purple	0.4

4.4 Spectral analyses of ALFA

The isolate ALFA was subjected to spectroscopic techniques and reported as follows;

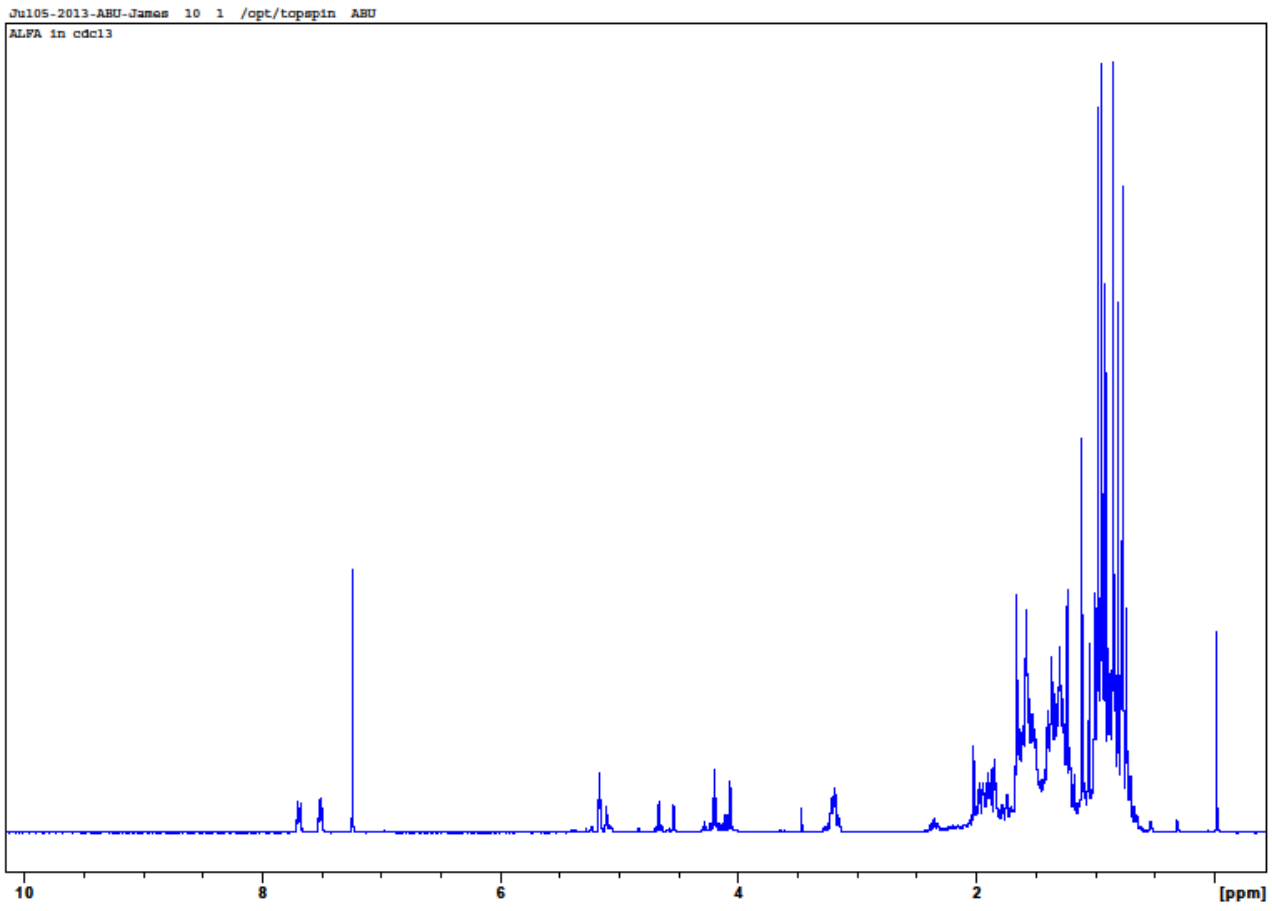


Figure 4.1: Proton NMR spectrum of ALFA

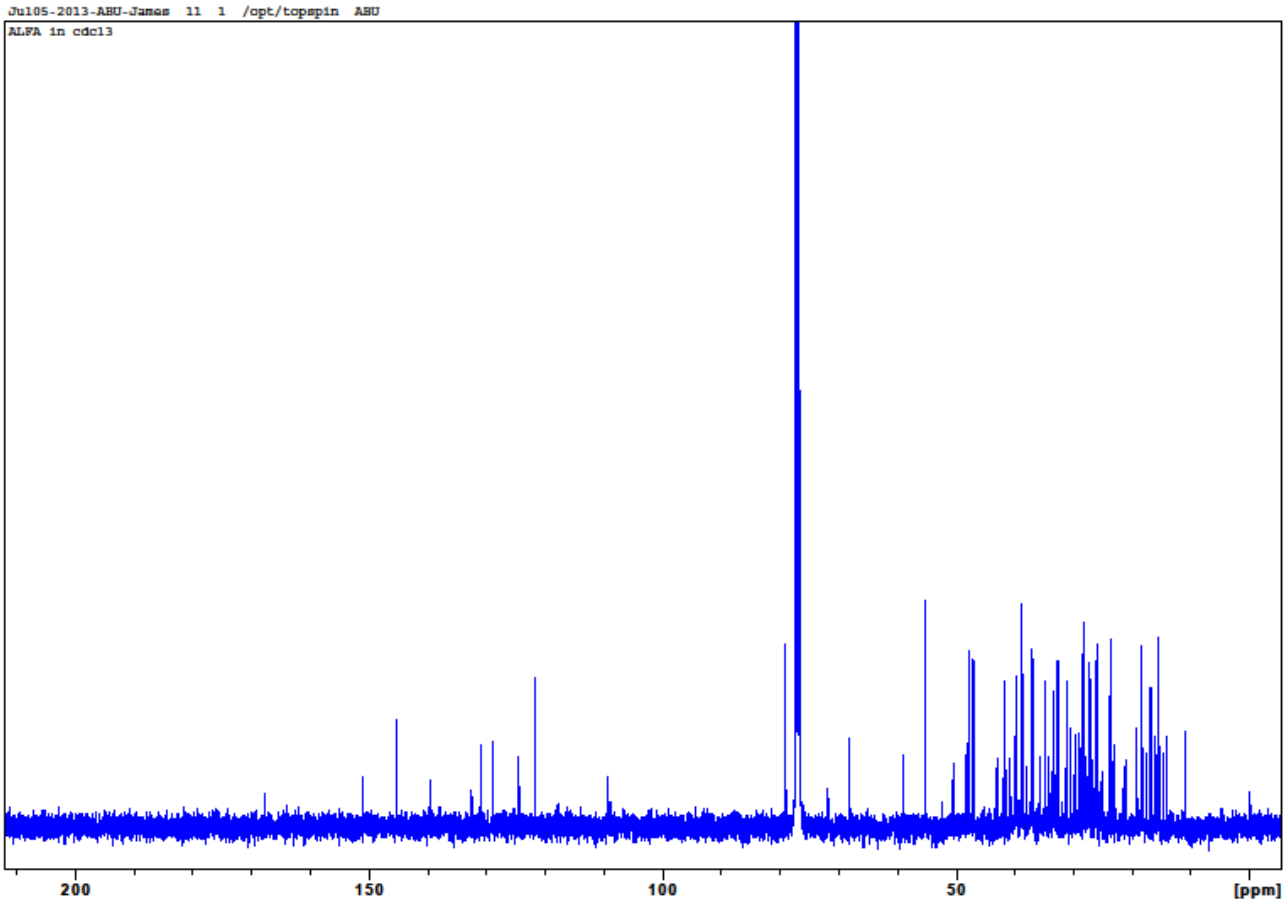


Figure 4.2: ^{13}**C NMR spectrum of ALFA**

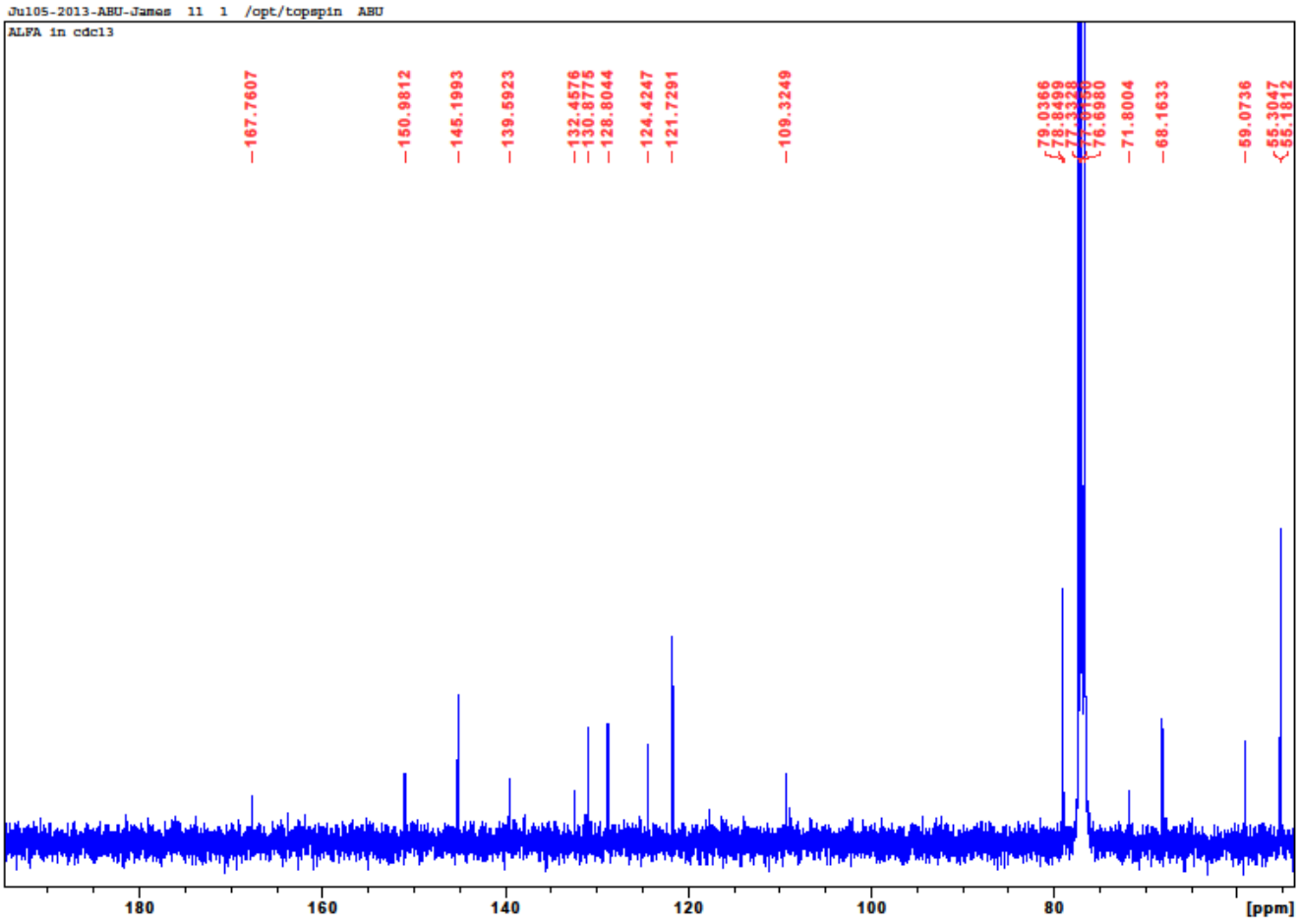


Figure 4.3: Expanded (1) ^{13}C spectrum of ALFA

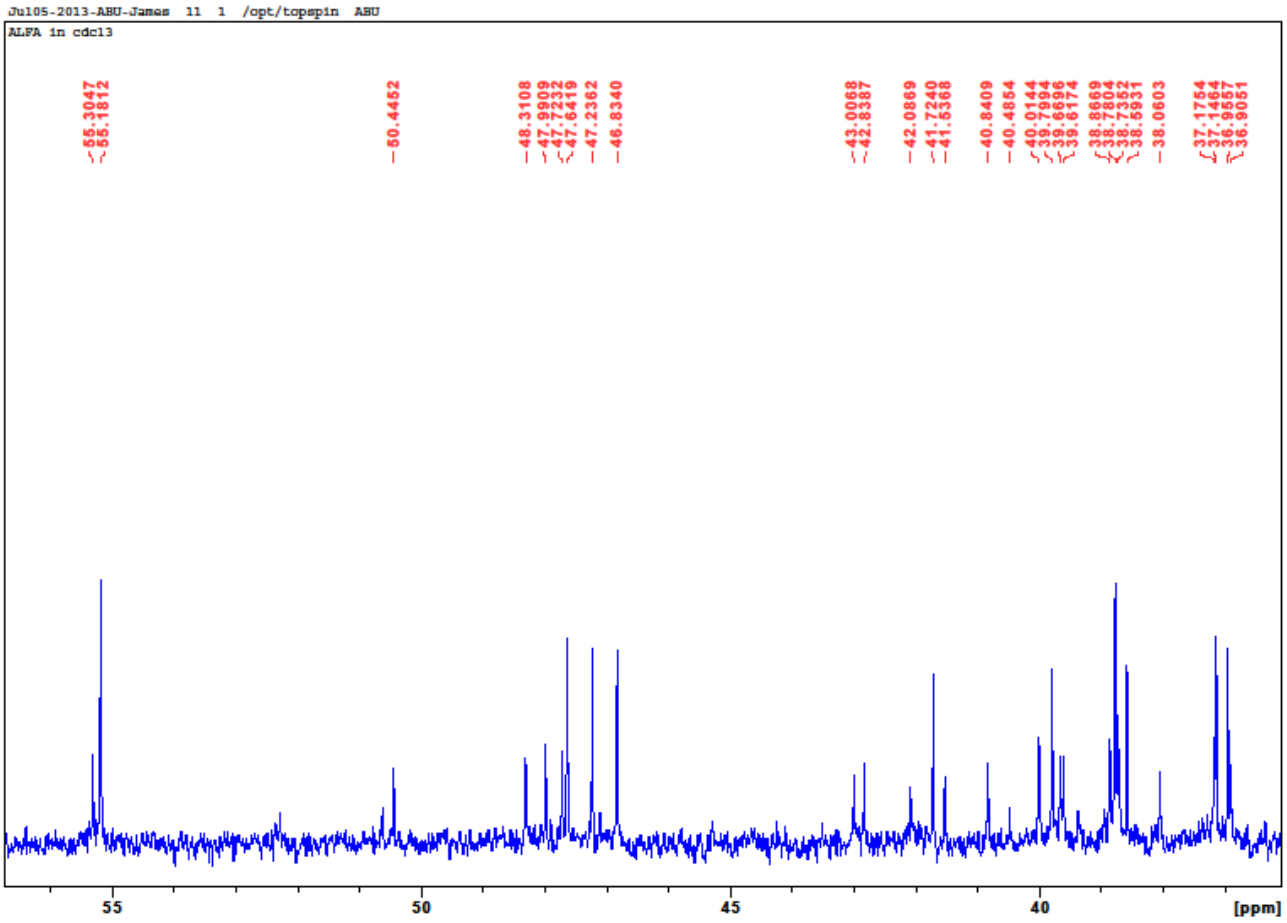


Figure 4.4: Expanded (2) ^{13}C spectrum of ALFA

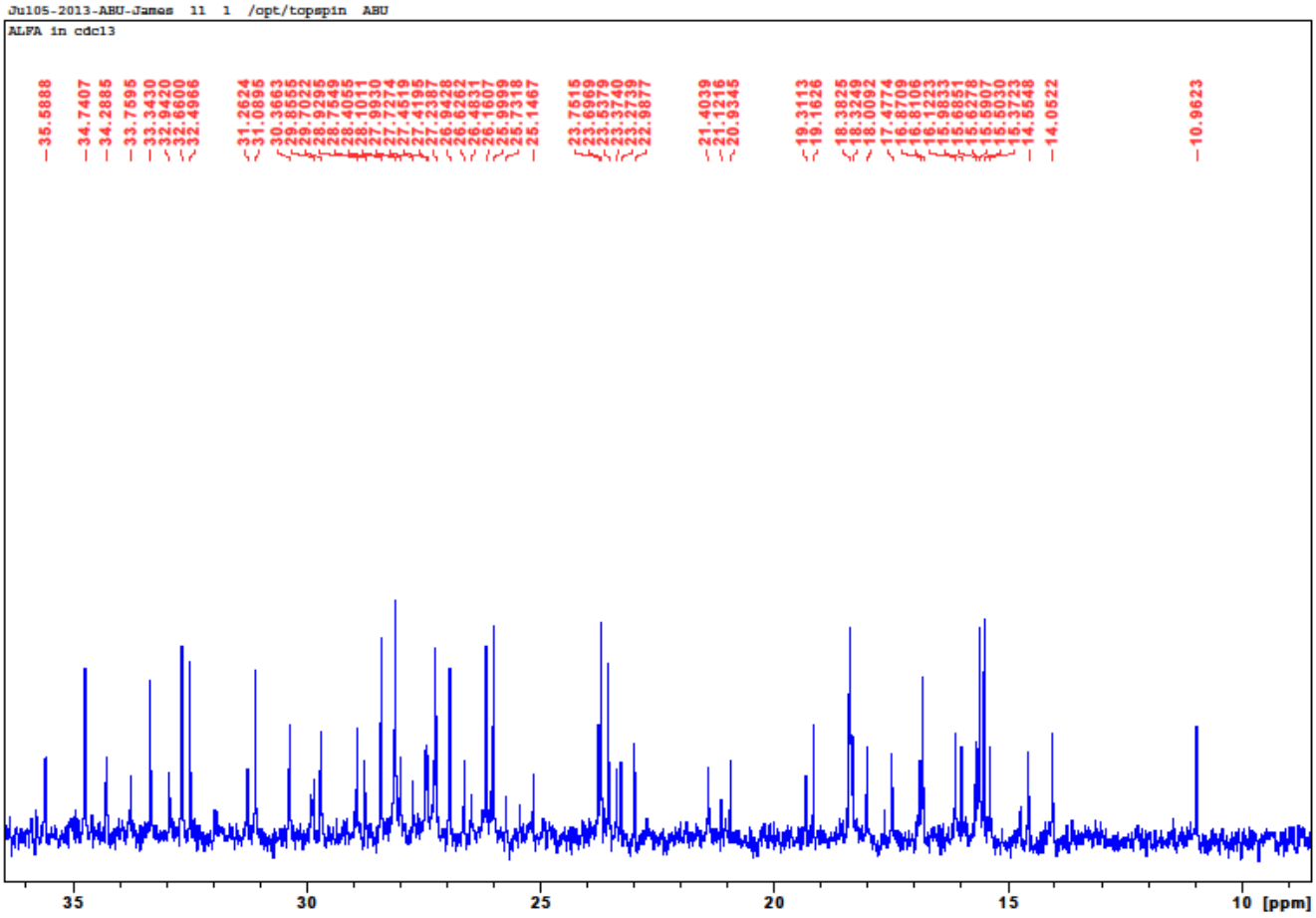


Figure 4.5: Expanded (3) ^{13}C spectrum of ALFA

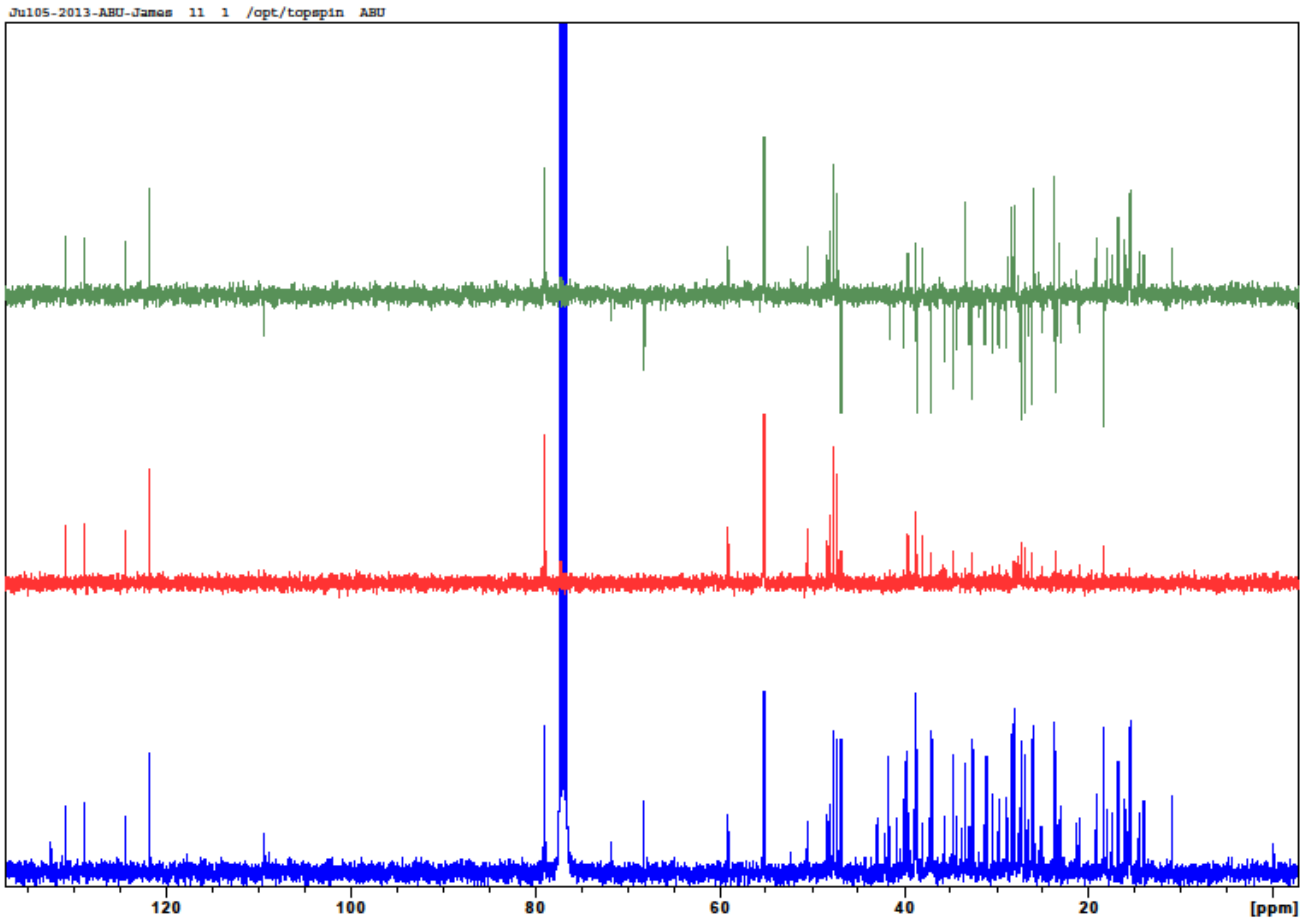


Figure 4.6: DEPT experiment of ALFA

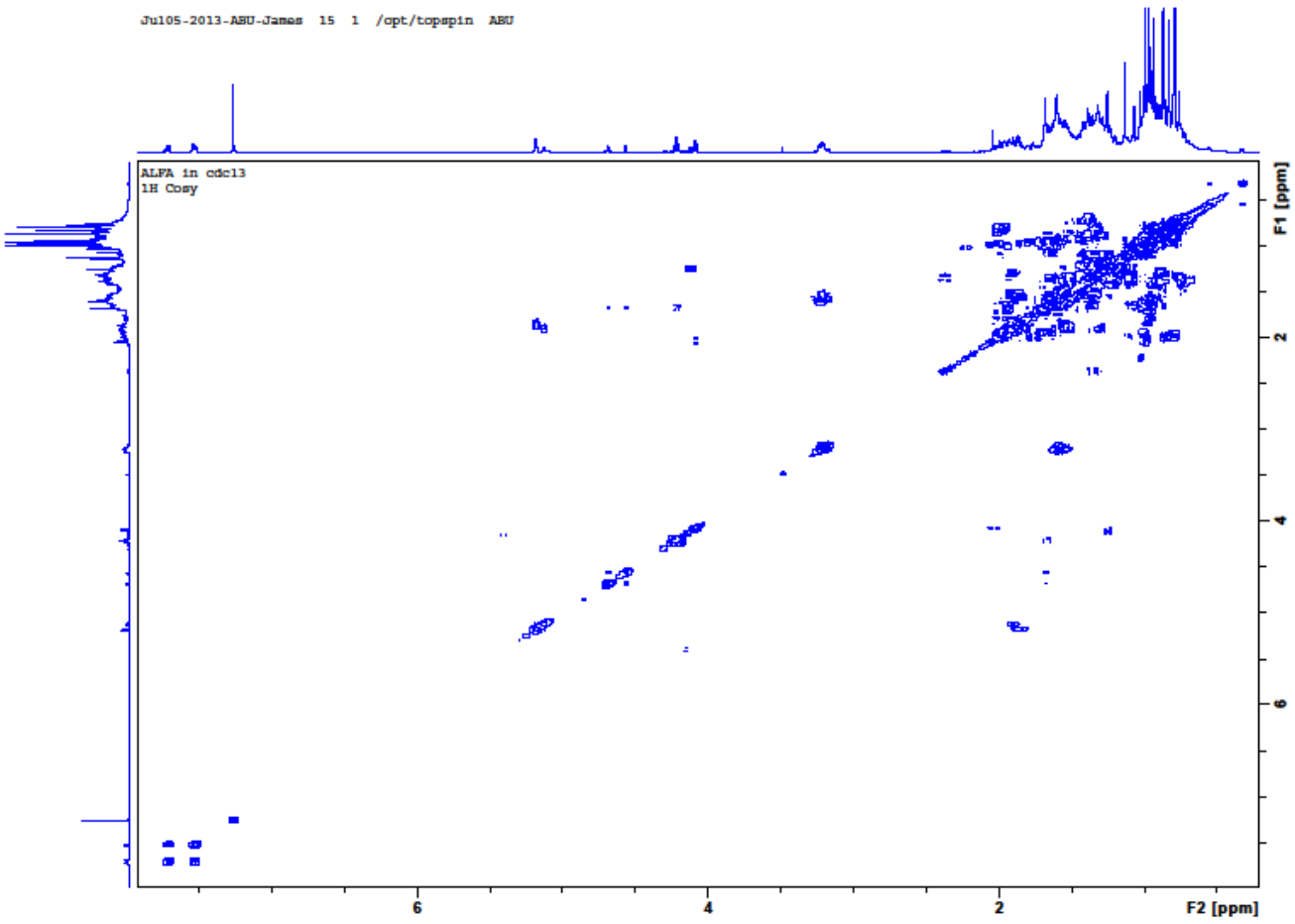


Figure 4.7: COSY correlation spectrum of ALFA

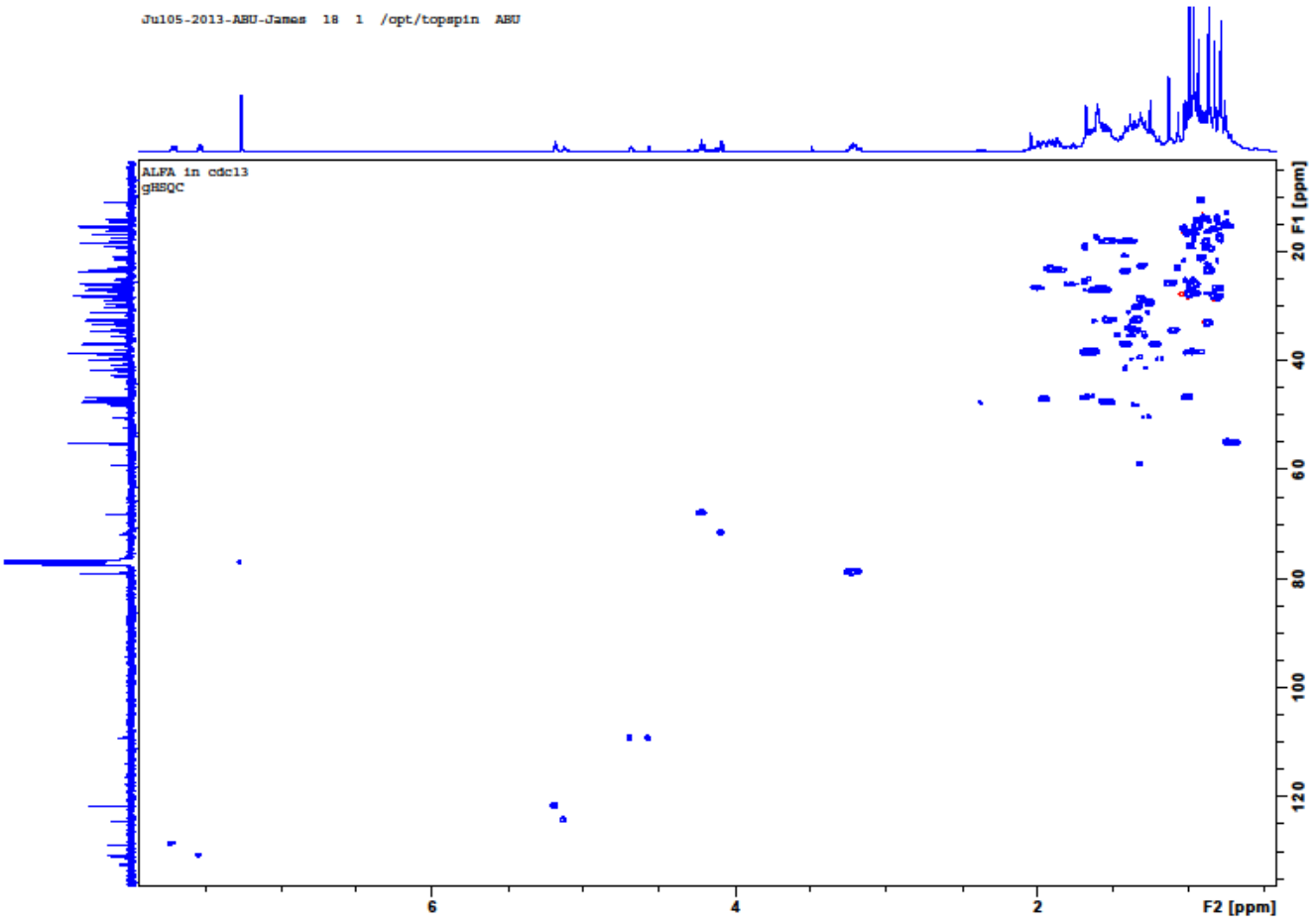


Figure 4.8: HSQC spectrum of ALFA

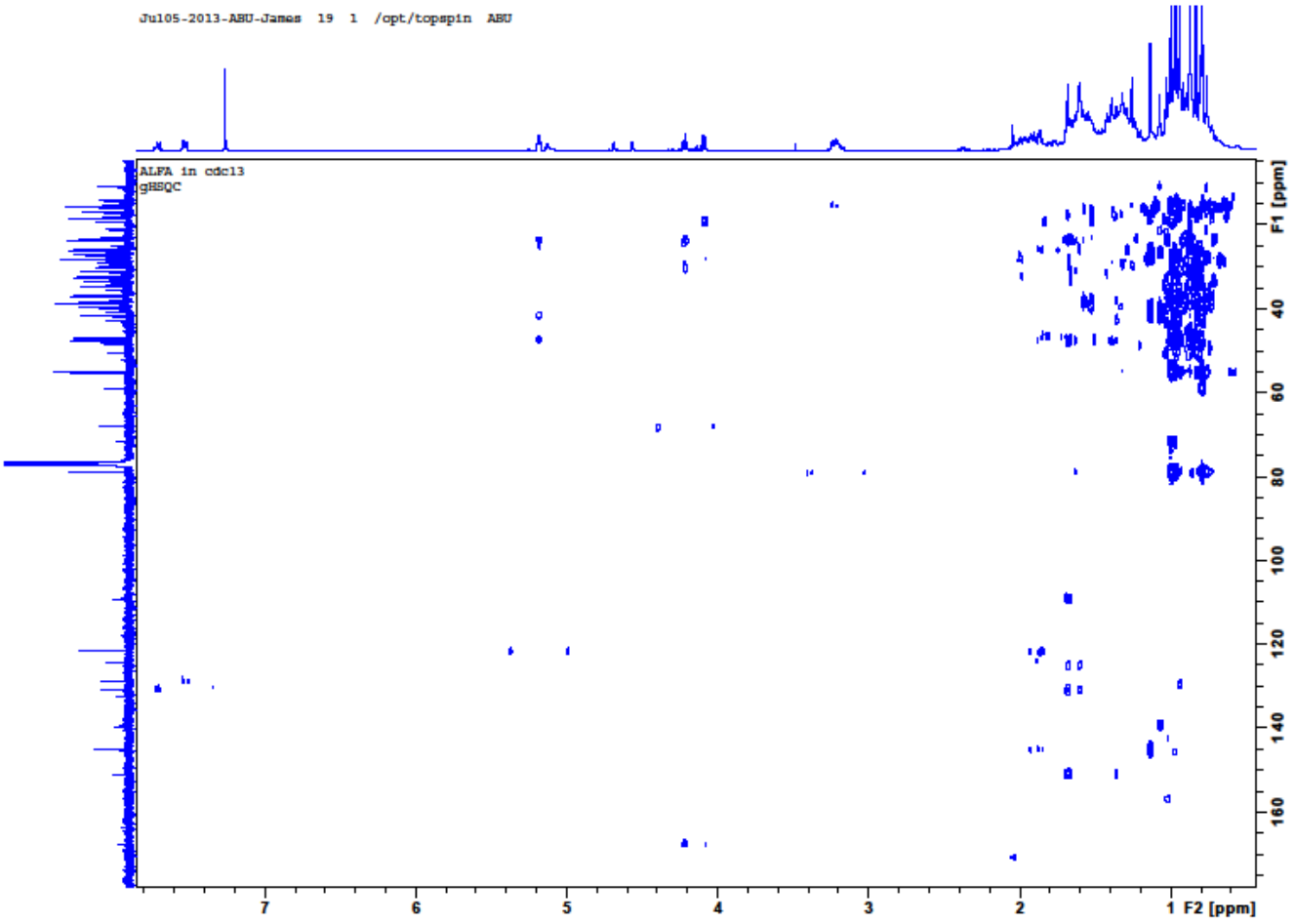


Figure 4.9: HMBC spectrum of ALFA

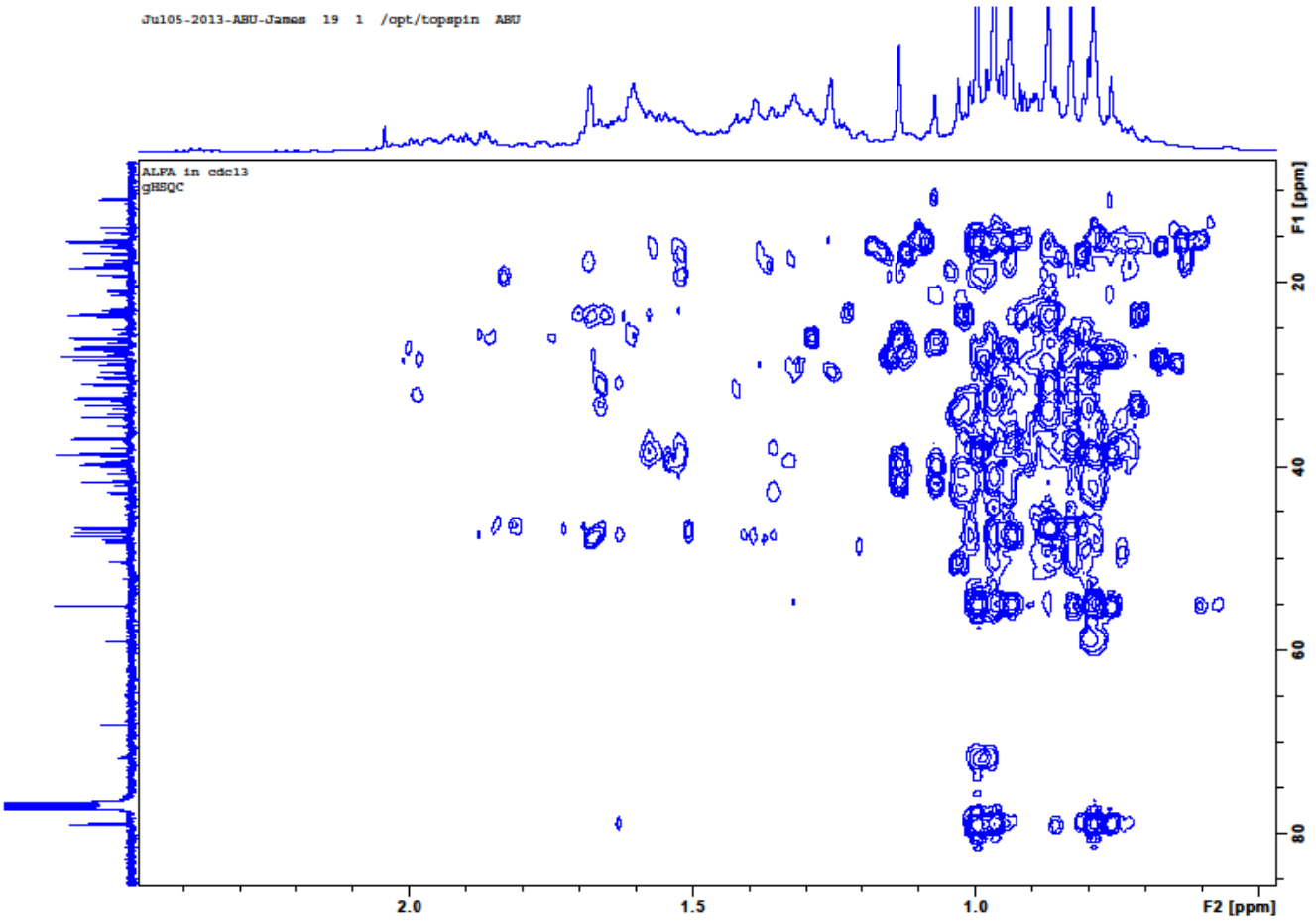


Figure 4.10: Expanded HMBC spectrum of ALFA

Table 4.7: Comparison of ^{1}H NMR, ^{13}C NMR obtained experimentally with those in literature.

Position	^{1}H experimental	^{1}H literature	^{13}C experimental	^{13}C literature	CH_n
1	1.33	1.36	38.0	38.2	CH_2
2	1.57	1.59	25.1	25.3	CH_2
3	3.20	3.16	79.0	79.1	CH
4	1.16	1.11	38.8	38.9	C
5	1.42	1.39	55.3	55.5	CH
6	1.20	1.16	18.4	18.5	CH_2
7	1.37	1.39	34.2	34.5	CH_2
8	1.15	1.16	40.8	41.0	C
9	1.38	1.36	50.4	50.6	CH
10	1.42	1.40	37.1	37.3	C
11	1.42	1.40	21.4	21.1	CH_2
12	1.42	1.40	27.4	27.5	CH_2
13	1.42	1.40	38.8	39.0	CH
14	1.39	1.36	42.8	43.0	C
15	1.39	1.36	27.7	27.6	CH_2
16	1.39	1.36	35.5	35.8	CH_2
17	1.18	1.16	43.0	43.2	C
18	1.46	1.43	48.3	48.5	CH
19	2.20	2.18	47.9	48.1	CH
20	1.73	1.71	150.9	151.1	C
21	1.55	1.51	30.3	30.0	CH_2
22	1.43	1.43	40.4	40.2	CH_2
23	1.15	1.11	28.1	28.2	CH_3
24	1.14	1.11	15.6	15.6	CH_3
25	1.18	1.16	16.1	16.3	CH_3
26	1.18	1.16	15.9	16.2	CH_3
27	1.34	1.36	14.5	14.7	CH_3
28	1.18	1.16	18.3	18.2	CH_3
29	4.65, 4.52	4.68, 4.56	109.3	109.5	CH_2
30	4.73	4.75	19.3	19.5	CH_3

CHAPTER FIVE

5.0 DISCUSSION

Soxhlet extraction of the plant material using solvents of varying polarities i.e. petroleum ether, ethyl acetate and methanol followed by concentration using a rotary evaporator gave three different extracts for the solvents; Petroleum ether, methanol and ethyl acetate extracts.

Phytochemical analysis of the Petroleum ether, methanol and ethyl acetate extracts indicate the presence of saponins, steroids, flavonoids, tannins, alkaloids and terpenes. Cardiac glycosides were found to be present in all the extracts. Saponins, flavonoids and tannins were found to be present in the ethyl acetate and methanol extracts. Steroids/tri-terpenes were found to be present in petroleum ether extract only as shown in Table 4.1.

Methanolic and ethyl acetate extracts indicated the presence of tannins. Tannins have been shown and reported to possess antimicrobial properties, and as a result, it is very useful plant metabolite in drug synthesis and production. The plant extracts indicated good number of vital secondary metabolites that are pre-cursor for drug synthesis due to their activities, especially flavonoids which possess various degrees of antioxidant and free radical scavenging properties (Korkina and Afana, 1997).

The antimicrobial activity against *S. aureus, S. feacalis, E. coli, S. Dysenteriae* and *C. Albicans* was tested using the tube dilution method as outlined by the NCCLS. The crude extracts were tested against eight pathogens; *Staphylococus aureus, Streptococus feacalis, Escherichia coli, Salmonella typhi, Shigella dysenteriae, Pseudomonas aeruginosa, Candida*

albicans and Candida stellafoidea. The results obtained are shown in table 4.2. All the three (3) extracts showed activity against all the tested microorganisms except against *Salmonella typhi, Pseudomonas aeruginosa* and *Candida stellafoidea*. The ethyl acetate have shown to be the most active of all, followed by the methanol extract, then pet ether extract.

Table 4.2 showed the zone of inhibition of the extracts against the test organisms with inhibition zones ranging from 19 to 26mm. The ethyl acetate being the most active of all shows most toxicity on the test organism, followed by methanol, while the pet ether extract shows the least. All the extracts were dissolved in DMSO, which has zero activity against all tested pathogens

The result of the Minimum Inhibitory Concentration (MIC) of petroleum ether extract was shown in Table 4.3. It revealed that the three (3) extracts could inhibit the growth of five (5) out of the eight (8) tested microorganisms. The MIC values ranged from 5.00 mg/ml to 20.00 mg/ml.

The result of the Minimum Bactericidal Concentration (MBC) of the petroleum ether, ethyl acetate and methanolic extract was shown in Table 4.4. The MBC for petroleum ether extract was 40mg/ml. for all the test organisms, the MBC for ethyl acetate shows that for all the test organisms was 20 mg/ml. The MBC of methanolic extract was 20mg/ml for *Staphylococus aureus, Escherichia coli* and *Shigella dysenteriae*. MBC values ranged from 20.00 mg/ml to 40mg/ml for all the plant extracts against both gram positive and gram negative microorganisms which indicate the broad spectrum of activity of the plant *Ceropegia deightonii.*

Result of TLC of the petroleum ether extract was shown in table 4.5. Petroleum ether and ethyl acetate at ratio of 9:1 gave the best profile with two prominent spots. After developing the plate, the colours and R_f of the spots were; 0.18 for the purple spot and 0.25 for the pink spot.

Column chromatographic separation followed by preparative TLC of the petroleum ether extract led to the isolation of a white solid compound coded ALFA. ALFA was a white powdery solid with melting point range of 214-217°C, which showed positive to steroids and triterpenes test of Salkowski and Liebermann-Buchard and was obtained from the chromatographic separation of petroleum ether extract.

The structure of ALFA was determined using ^{1}HNMR (Fig 4.1), ^{13}CNMR (Fig 4.2), Distortionless Enhancement polarization Transfer (DEPT) (Fig 4.6), Correlation spectroscopy (COSY) (Fig 4.7), Heteronuclear Multiple Bond correlation (HMBC) (Fig 4.9) and Heteronuclear single Quantum Correlation (HSQC) (Fig 4.8).

The proton NMR spectrum of ALFA revealed signals for overlapping methine, methelyne and methyl protons between δ_H: 0.75 and 1.25ppm, the downfield signals at 4.52 and 4.65 are characteristic of the olefinic protons of Lupeol (Jamal *et al.,* 2008). The signal at 3.20 was assigned to the position 3 of lupeol (Jamal *et al.*, 2008).

The ^{13}C NMR spectrum of ALFA revealed the presence of olefinic carbon atoms at 150.9 and 109.3; these values are in good agreement with those reported in literature for lupeol (Blair *et al.,* 1970) (Jamal *et al.,* 2008). The signal at 79.0 is due to an oxymethine carbon and was assigned to position 3 of lupeol. Other signals observed in the ^{13}C NMR spectrum of ALFA between 14.5 and 55.3 ppm were due to overlapping methine, methelyne and methyl carbon atoms.

The DEPT experiment also revealed the presence of C atoms to be thirty (30) in the compound just like the ^{13}CNMR, but this experiment distinguishes the carbon atom into C, CH, CH_2 and CH_3. From the DEPT it can be seen that we have six quaternary carbons at 38.8 for C_4, 40.8 for C_8, 37.1 for $C_{10,}$ 42.8 for $C_{14,}$ 43.0 for C_{17} and 150.9 for C_{20} respectively. Six (6) methine carbon atoms (-CH) were observed at 79.0 for C_3, 55.3 for C_5, 50.4 for C_9, 38.8 for C_{13}, 48.3 for C_{18}, and 47.9 for C_{19}. Eleven (11) carbon atoms identified to be methylene carbons ($-CH_2$) were observed at 38.0 for C_1, 25.1 for C_2, 18.4 for C_6, 34.2 for C_7, 21.4 for C_{11}, and 27.4 for C_{12}, 27.7 for C_{15}, 35.5 for C_{16}, 30.3 for C_{21}, 40.4 for C_{22}, and 109.3 for C_{29}While the remaining seven carbon atoms were all methyl carbons ($-CH_3$) at 28.1 for C_{23}, 15.6 for C_{24}, 16.1 for C_{25}, 15.9 for C_{26}, 14.5 for C_{27}, 18.3 for C_{28} and 19.3 for C_{30} respectively. Therefore, the DEPT experiment revealed the presence of seven CH_3, eleven CH_2, six CH and six C.

After comparing these data with the data in literature (Blair *et al.*, 1970) (Jamal *et al.*, 2008), the structure of ALFA was determined to be lupeol with molecular formular $C_{30}H_{50}O$. (Figure 5.1).

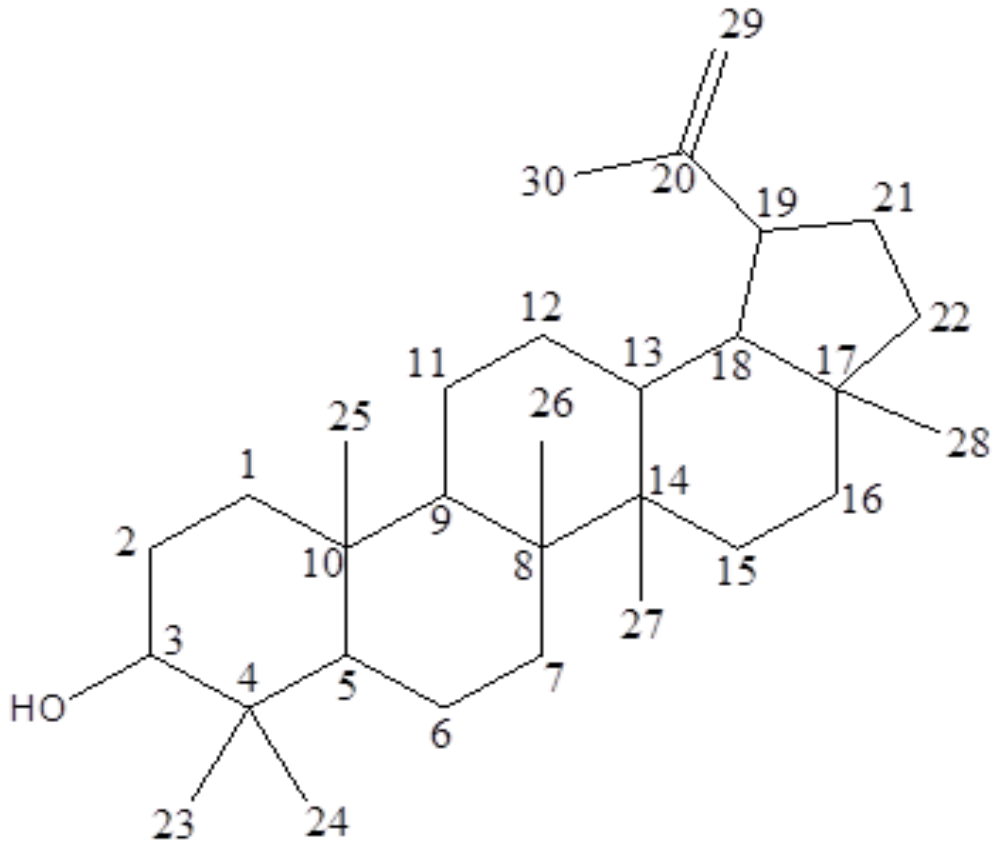


Figure 5.1: The proposed structure of ALFA (Lupeol)

CHAPTER SIX

6.0 Summary, Conclusion and Recommendation

6.1 Summary

The whole plant of *Ceropegia deightonii* collected from Makurdi, Benue state having been properly identified at the Herberium unit of Biological science department of A.B.U Zaria, the plant was subjected to air drying, pulverization and extraction. The crude extracts obtained were further subjected to preliminary phytochemical screening and then antimicrobial activity test. Petroleum ether fraction was subjected to chromatographic separations where a compound named ALFA was obtained, which after undergoing some spectroscopic analysis revealed to be a steroid called Lupeol.

6.2 Conclusion

Ceropegia deightonii a plant used in traditional medicine for the treatment of several diseases such as Mumps, Skin diseases, Typhoid, Swollen wounds, Pile, Diarrhea etc. Preliminary phytochemical screening revealed the presence of some secondary metabolites which are responsible for the observed antimicrobial activity seen against the tested microorganisms. So, the claim made by the local practitioners was verified to be true to some extent.

6.3 Recommendations

Base on the work carried out on the plant *Ceropegia deightonii*, the following recommendations were made; More compounds should be isolated from all the plant's parts using solvents of different polarity with the intention of revealing all the possible compounds with antimicrobial activity and other biological activity or pharmacological activity test should be carried out on the plant to explore the epicacy of the plant as medicinal plant.

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