ABSTRACT

Plants and their metabolic products have served as therapeutic weapons for treatment of various human and animal diseases. *Callichilia stenopetala* Stapf (family Apocynaceae) has been used by traditional practitioners in the South-eastern part of Nigeria as a remedy for the treatment of various ailments including different types of resistant malaria with their "re-current" fever. Malaria with emergence of its drug resistant parasites is a major health issue in the tropics and subtropical areas of the world. This study was aimed at evaluating *in vivo* antiplasmodial activities of methanolic extracts of some Nigerian medicinal plants. Antioxidant activity was included as a supporting factor, considering the relationship between oxidative stress and chronic diseases. Seven plants were initially evaluated for their antimalarial and antioxidant activities and the plant with the most promising activity was studied further. Phytochemical analysis and oral acute toxicity of methanolic extract of *C. stenopetala* was carried out using standard procedures. The methanolic extract of *C. stenopetala* was fractionated with water and organic solvents viz:- hexane, chloroform and ethyl acetate successively to obtain various fractions. Antiplasmodial activities of the crude extract and the obtained fractions of *C. stenopetala* were studied in detail against chloroquine sensitive *Plasmodium berghei berghei* NK 65 – infected mice using 4-day suppressive test procedure. The result was compared with some standard antimalarial agents, chloroquine phosphate and artesunate. An attempt was also made towards isolating and elucidating the structure(s) of constituent(s) thought to be responsible for the antimalarial activity of the plant. Bio-activity guided chromatographic isolation of two most active fractions – hexane and chloroform was done. The isolates obtained were subjected to spectral analysis for characterization using various spectrometric and chromatographic techniques: one-Dimensional (1D) and two-Dimensional (2D) NMR techniques, Attached Proton Test (APT), Correlation Spectroscopy (COSY), Nuclear Overhauser Effect Spectroscopy (NOESY), Heteronuclear Multiple Bond Correlation (HMBC), Heteronuclear Single Quantum Coherence (HSQC), Infra-red (IR), Gas Chromatography-Mass Spectroscopy (GC-MS) and X-ray crystallography. *In vitro* anti-oxidant activity of the crude extract, the obtained fractions and the pure isolates were also assessed using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and total phenolic content (TPC). The root bark of *C. stenopetala* was selected for the study based on its promising bioactivity. Results of its phytochemical analysis test showed presence of alkaloid, saponins, tannins, triterpenes and phenolic compounds. No mortality or evidence of adverse effects was observed in acute toxicity test. The crude extract, the hexane fraction and chloroform fraction demonstrated intrinsic antimalarial properties that were dose-dependent. A comparative analysis indicated that at dose 250 mg kg^{-1}, the crude extract, hexane and the chloroform fractions produced 69.3, 82.24 and 39.9% suppression of parasitaemia respectively against 82.1% for chloroquine (5 mg kg^{-1}) and 73.80% for artesunate (10 mg kg^{-1}). Three active compounds, structurally elucidated as vobtusine, α-amyrin acetate and lupeol acetate were isolated and they exhibited significant ($P< 0.05$) chemosuppression at 7 – 10 mg kg^{-1} (62.2, 67.61 and 68.65% respectively) compared with the standard drugs chloroquine and artesunate. The antioxidant activities of the crude extract and the fractions were dose-dependent, and the effect resides mainly in the aqueous and ethyl acetate fractions. Only vobtusine exhibited a significant free radical scavenging effect (68.7%) at 0.2 mg mL^{-1}. These results suggest that *C. stenopetala* root bark has antimalarial and antioxidant activities which may be due to the isolated compounds. This is the first report on antimalarial constituents of *C. stenopetala*, to the best of my knowledge.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Malaria has been recognized as one of the major killer diseases of the world, particularly in the tropics and sub-tropical regions. The high rate of mortality and morbidity has become of global concern, especially among children of under 5 years, pregnant women and adults visiting from non-endemic region to endemic region (Murray *et al.*, 2012).

In the era before year 2000, chloroquine was the drug of choice and mainstay of malaria chemotherapeutic control in Nigeria but this changed based on the recommendation of the World Health Organization (WHO) to artemisinin-based combination therapy (ACT) as the first line of drug of choice for the treatment of uncomplicated malaria. The re-emerging of malaria in many parts of the world and rapid increase of resistant strains of *P. falciparum* to most of the available anti-malarial drugs, as well as resistance of vectors to insecticides were the determining factors for the recommendation (Ridley 2002; Zirihi *et al.*, 2005). The recommendation for new malaria treatment regime became necessary because malaria-related morbidity and mortality was increasing in sub-Saharan Africa, primarily as a result of increased treatment failure of the common first line drugs—chloroquine (CQ) and sulphadoxine-pyrimethamine (SP). Traditionally, plants have played a great part as direct and indirect source for healthcare materials. They are instrumental to early pharmaceutical drug discovery and industry (Elujoba *et al*., 2005). The observed recent increase in the demand for alternative medicine may be due to cultural, economical, availability reasons or the need to try something else if the orthodox drugs are not giving desired results.

In malaria-endemic countries, several plants are utilized in traditional medicine for the treatment of malaria and/or fever. The use of these medicine plants have led to development of some drugs from them, such sourced/derived antimalarial drugs include quinine and artemisinin. Artemisinin is a direct natural product of the plant *Artemisia annua*, whereas chloroquine is a synthetic analogue of quinine, a natural product of *Cinchona* plant (Adesegun *et al.*, 2001; Batista *et al.*, 2009). However, there is a need to study and evaluate these plants known as medicinal plants and the active phytochemical compounds isolated from them for their efficacy, safety for human/animal consumption, dosage range and toxicity status.

The basic native methods of preparing and taking herbal drugs include aqueous or alcoholic extract of the whole plant or plant part taken orally, incisions, inhalation as vapour or topically etc. Modern technology has provided methods of extracting or isolating the active compounds to enhance activity, target desired effect and reduction in multiple adverse effects. The isolated active compounds could possibly serve as lead template for the creation of effective analogs.

The plant of choice, *Callichilia stenopetala* Stapf (family Apocynaceae) was selected after initially screening of nine plants obtained during ethnopharmacological survey. It has been used ethnomedicinally for the treatment of different diseases including malaria diseases. Its crude extract, fractions and phytoconstituent(s) were evaluated for antimalarial activity using *in vivo* assay against *Plasmodium berghei berghei*-infected mice as a good alternative in the treatment of malaria. The use of a 4-day suppressive model has been successively used in evaluation of antimalarial effect of many plants (Andrade-Neto *et al.*, 2003; Sudhanshu *et al.*, 2003). Adegboyega *et al.*, **(**2014) suggested that one way of attenuating resistance is by using new compounds that are not based on existing synthetic antimalarial agents. Preferably, the desired

new drugs should have novel modes of action or be chemically different from the drugs in current use.

1.2 STATEMENT OF THE PROBLEM

Malaria is a parasitic disease that affects more than 500 million people worldwide, killing between 60,000 (sixty thousand) and 2.7 million people annually, especially children and pregnant mothers (Percário *et al.*, 2012). It is caused by a single-celled parasite, *Plasmodium.* The parasite *P. falciparum* is usually transmitted to humans by a secondary vector, anopheles mosquitoes after a bite. Once in the body, *Plasmodium* has a complex life cycle and an inherent mechanism at evading the immune response. One of its key strategies is possession of high polymorphic natured proteins and the ability to undergo almost unlimited antigenetic variation by changing the antigens on the infected red blood cell surface. This inherent property results in significant challenges to vaccine design and rapid development of multi-drug resistant strains of *Plasmodium*. Drug resistant *Plasmodium*, particularly *P. falciparum* represents a major problem for both prophylaxis and clinical treatment of malaria infection (Phyo, 2012). There is a report of rapid spread of resistant malaria parasites to new areas and re-emergence of malaria in areas where the disease had been eradicated (McCollum, 2006). At present there are no drugs that can completely offer protection against malaria in all regions of the world.

Clinical resistance to the artemisinin and its combinations is currently being reported, suggesting that *P. falciparum* parasites have already developed the ability to grow in the presence of these antimalaria agents, leaving little or no alternative for malaria treatment. This strongly suggests the need for urgent and further research into new antimalarials (Noedl *et al.*, 2008).

1.3 AIM AND OBJECTIVES

The aim of this study was to determine the antimalarial activity of root bark of *Callichilia stenopetala* (Stapf.); to isolate, purify, characterize and elucidate the structures of compounds with possible antimalarial activities from root bark of *Callichilia stenopetala*.

1.4 SPECIFIC OBJECTIVES OF STUDY ARE

The specific objectives of this study are to:

- Collect some Nigerian medicinal plants used in treatment of malaria fever and screen the plants for antimalarial and antioxidant activities;
- Extract, fractionate, purify and isolate active compounds from active fraction(s) of selected plant for the study;
- Characterize and carry out structure elucidation of the isolated compounds;
- Carry out bioassay on the crude extract, fractions and isolated compounds for possible antimalaria and antioxidant activities.

1.5 SIGNIFICANCE OF STUDY

In Nigeria, malaria is a major health problem and the most widely diagnosed infectious disease in the country. It is the single most important cause of death especially among children under the age of 5 (23%); it is responsible for one out of ten deaths in pregnant women and causes the Federal Government of Nigeria over one billion Naira annually in treating malaria (Odugbemi, 2007). The disease is responsible for up to 60% of daily outpatient department (OPD) consultations in health facilities (FMOH, 2008). Chloroquine failure range of 50 – 95% has been reported for some parts of Nigeria (Falade *et al.*, 2005; FMOH, 2004). Report on the development of resistance to artemisinins and their associate drugs will cruelly limit the utility

of ACT in future, leaving little or no alternative treatment or malaria. Consequently to develop alternative therapy, research on anti-malaria is urgently vital.

About 75% of the population in Africa depends on traditional medicinal plants for malaria treatment due to inaccessibility of health facilities, financial status, cultural reasons or formerly experienced treatment failure (Olasehinde *et al.,* 2014). Lots of undocumented and unverified herbs are being used today as single and as poly-herbals in treatment of malaria fever. The diversity of chemical compounds found in these herbs could contribute greatly as an important source of molecular templates in the search for new and novel antimalarial drugs. *C. stenopetala* root bark, use in treatment of malaria fever was scientifically documented as a possibly safe, effective, affordable and active antimalarial agent. The possible active antimalarial compound(s) isolated from the plant may possibly be a source of new antimalarial agent or template.

1.6 DEFINITION OF OPERATIONAL TERMS

Ethnopharmacology:	The scientific study of plants used medicinally, especially as folk remedies, by different ethnic or cultural groups.
Traditional medicine:	This refers to the sum total of all knowledge and practical application, whether explicable or not used in diagnosis, prevention, and treatment of diseases conditions.
Free radicals:	Free radicals are atoms or groups of atoms with an odd (unpaired) number of electrons and can be formed when oxygen interacts with certain molecules.

Oxidative stress: Oxidative stress is essentially an imbalance between the production of free radicals and the ability of the body to counteract or detoxify their harmful effects through neutralization by antioxidants.

Antioxidants: Enzymes or other organic substances that is capable of counteracting the damaging effects of free radicals/oxidation in animal tissues.

Natural product: Natural products are the chemical compounds found in nature that usually has a pharmacological or biological activity for use in pharmaceutical drug discovery and drug design.

(Sofowora, 1993; Evans, 2009, Percário *et al.*, 2012)

1.7 LIST OF ABBREVIATIONS AND ACRONYMS

APT: Attached proton test

^{13}C: 13-Carbon NMR spectroscopy

CC: Column chromatography

$CDCl_3$: Deuterated chloroform

COSY: Correlation Spectroscopy

CQ: Chloroquine COSY: Correlation Spectroscopy

1D-NMR: One dimension Nuclear magnetic resonance spectroscopy

2D-NMR: Two dimension Nuclear magnetic resonance spectroscopy

DMSO: Dimethylsulfoxide

EtOAc: Ethyl acetate

GS-MS: Gas chromatography-Mass spectroscopy

^{1}H: Proton NMR spectroscopy

HMBC: Heteronuclear multiple bond correlation

HSQC: Heteronuclear single quantum coherence

J: Coupling constant

m/z: Mass to charge ratio

MeOH: Methanol

Mg: Milligrams

Min: Minutes

NMR: Nuclear magnetic resonance spectroscopy

NOESY: Nuclear overhauser effect spectroscopy

ppm: Part per million

ROESY: Rotating frame overhauser effect spectroscopy

TLC: Thin layer chromatography

UV: Ultra violet

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1. Medicinal plants and biodiversity

Plant is an integral part of human life and man has relied on it for ages for survival, as source of food (cereals, vegetables, fruits, beverages, drinks, spices, condiments, seasoning, etc.); for colouring or as dye-colours; ethno-medicine and ethno-veterinary medicine; pesticides and insecticides to protect the crops; wood for making implements (utensils, tools, musical instruments, boats, oars and other household goods); cordage; commercial plants; packaging material, and fuel (Shah, 2005). Medicinal plants have curative properties and have been part of man's civilization process, providing the starting material for the isolation or synthesis of conventional drugs. It has been reported that about 35 000 to 70 000 species of plants have been used at one time or another for medicinal purposes (Das-Prajapati *et al.*, 2003).

The complex traditional medicinal systems have been in existence for thousands of years, providing mankind with needed healthcare to date. Medicinal plant therapy is based on the empirical findings and experience of hundreds of thousands of years. Prescription drugs, over 121 in number sold worldwide are derived from several plant species of the rainforests (Shanley 2003). Many of today's drugs have been derived directly from plant sources or have provided the models for 50% western drugs and are worth about $6 billion a year (Balunas 2005, Chin *et al.*, 2006).

In 1977, the World Health Organization, described medicinal plant as "any plant which in its one or more of its organ contains substance(s) that can be used for the therapeutic purpose(s) or which are precursors for the synthesis of useful drugs" (Ugoh and Elibe, 2013). The substance could be found in one or more of the plant organ(s), which include, leaves, stem, barks or

roots. A plant is assumed to be a medicinal plant only when its biological activity has been ethnobotanically reported or scientifically established (Omotayo and Borokini, 2012). These plants are used by the traditional healers in treatment, prevention and health plan for various disease conditions like bleeding, malaria, pain, inflammation, boils, bronchitis, cold, cough, asthma, dysentery, ear complications, headache, leucoderma, pneumonia, renal complications, piles, scorpion bite, snake bite, skin diseases, child delivery complications, menstrual disorders, miscarriages, family planning etc. For instance, *Alternanthera pungens* (Family Amaranthaceae) is used in treatment of various disease conditions such as abdominal pains, gonorrhoea and haemorrhage, and to promote flow of milk, and abortion (Pei 2005); mustard seeds are used for bronchial and rheumatic complaints; Indian gooseberry (*Phyllanthus emblica*) used for coughs, asthma, jaundice and wounds; and neem (*Azadirachta indica* (L) as pesticide, dermatological and antibacterial activities. Neem has attracted dozens of patent applications, probably a highly celebrated medicinal tree globally (Kareru *et al.*, 2010).

The tropical forest is believed to be the source of a large proportion of the world's recognized medicinal plants. West Africa is blessed with a good biodiversity that they can boast of having plants with solution to wide range of health issues. These medicinal plants have been described by Sofowora (1993) as a reservoir of phytomedicines, the sleeping giants of drug development. The plant genetic resources of Nigeria are a veritable source of pharmaceuticals and therapeutics though a lot of documentations have to be done in relation to their safety and efficacy. Nigeria rainforest biodiversity resource is highly underutilized and the undiscovered plant species may possibly be the key to man's quest for total good health (Okigbo *et al.*, 2008).

The world's tropical rain forests are especially rich in biodiversity but there is rapid depletion of this natural resource worldwide due to rise in plant-based drugs and other human factors. In

Africa, Nigeria in particular, is under the pressures from degradation, unsustainable arable land use, urbanization, expansion in agriculture, over exploitation, construction, deforestation and indiscriminate exploitation of natural resources and industrialization are taking their toll (Obute and Osuji, 2002; Ayodele 2005), thereby causing gradual or fast extinction of valuable species. In view of the greatly increase in the demand for plants for medicinal purpose, a World Wide Fund for Nature (WWF) warned that the enormous market demand could have an irreversible impact on many species unless action is taken to regulate trade and urgent step taken to start domestic cultivation (Shan *et al.*, 2014). Some of these medicinal plant species that are under the threat of extinction have slow growth rates, low population densities, and narrow geographic distribution (Shan *et al.*, 2014), therefore they are more prone to fast extinction (Jablonski 2004). A good example is the sudden fame of the yew tree containing bioactive terpenoid taxol and its gradual steps towards extinct. Taxol, use in the treatment of ovarian and breast cancer, can be made semi-synthetically from one or more of the constituents of *Taxus baccata*, a yew tree that grows among pine forests at around 3000 m in the Himalayas. In a bid to meet with the global demand for this anticancer drug, Pharmaceutical companies have stripped forest areas of this species. In 1977 the plant was not considered important enough even to be included in a book on trees, but within 15 years it had become an endangered species due to the isolation of an active natural product. No plan for re-plant or replacement was made earlier due unconsidered arbitrary decimation of the yew tree population.

Every aspect and component of biodiversity, the plants, and ecosystem is under threat of extinction and therefore an urgent need for conservation of whatever that is remaining for current and future generations. Across the continent, the renewed interest in medicinal plants of Africa seems to be a little late, many of the medicinal plants have gone into extinction,

particularly the lesser-known medicinal plants before they are documented. Preserving the rich biodiversity ecosystem is a topic that cannot be over emphasized and at the long run also ensuring the availability of the needed medicinal plants (Shan *et al.,* 2014). The unpleasant conclusion is that the uncontrolled activities of the human race are the major cause in the vascular plant diversity reduction. With the disappearance of the flora, the eco-system balance is being lost too (Ticktin *et al.,* 2007; Wyatt and Silman 2010).

The need to study medicinal plants, aim at understanding, *inter alia,* widespread use of plants in traditional medicine, rescuing traditional medicinal plants and knowledge about them from imminent loss as well as the need for health for all.

2.2 ETHNOBOTANY AND TRADITIONAL MEDICINE

The term ethnobotany has been defined as the study of the reciprocal relationship between plants found in a locality or community and the traditional people that live within the same locality (Albuquerque *et al.,* 2005; Ritter *et al.,* 2014). Ethnobotany, in general, thus refers to the study about the utilization of plants for a wide variety of humans needs such as medicine, food, fodder, fibre/clothing, and goods required for their material culture and amenities.

Traditional medicine practice has existed in Africa and other cultures for centuries sustaining the cultural healthcare systems. In the far past it was the sole healthcare system available but suffered backset due to the re-emergence of a more scientific based modern medical practice. The recent renewed interest in the traditional medicine may be due to cultural reasons, a search for alternative healing system, financial and availability reasons. Traditional medicine is the sum total of all knowledge and practical application, whether explicable or not used in diagnosis, prevention, and elimination of physical, mental or social imbalance; and relying

exclusively on practice and experience, and observations handed down from generation to generation, whether verbally or in writing. Traditional medicine also refers to 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 illnesses or maintain well-being (Sofowora, 1993). A traditional healer can be defined as a person with competence to practice traditional medicine and at the same time uphold the total balance of the body systems (Togola *et al*., 2005).

There are many different systems of traditional medicine, and the philosophy and practices of each are influenced by the prevailing conditions, environment, and geographic area within which it first evolved, however, a common philosophy is a holistic approach to life, equilibrium of the mind, body, and the environment, and an emphasis on health rather than on disease. Generally, the focus is on the overall condition of the individual, rather than on the particular ailment or disease from which the patient is suffering, and the use of herbs is a core part of all systems of traditional medicine (Conboy *et al.,* 2007; Rishton 2008; Schmidt *et al.,* 2008). The use of herb or medicinal plant in reparation of medicine, known as the Herbal medicine, is a major component in traditional medicine systems such as in Siddha, Ayurvedic, Homeopathic, Naturopathic, Traditional Chinese medicine and Native America medicine (Pravin *et al.,* 2012). Their use is well established, widely acknowledged to have a high safety margin, effective and may be accepted by national authorities. Herbs include crude plant materials, such as leaves, flowers, fruit, seeds, stems, wood, bark, roots, rhizomes or other plant parts, which may be entire, fragmented or powdered.

The terms “complementary medicine”, “alternative medicine” and “non-conventional medicine” are used interchangeably with “traditional medicine” in some countries. Traditional medicine as

already stated involves the use of herbal, animal parts and minerals, but herbal medicine being the most widely used of the three (Orwa 2002; Pei 2005), showing the crucial part ethnobotany plays in the lives of people. Therefore the preservation of biodiversity cannot be over emphasized. Most traditional or ethnobotanical knowledge were obtained verbally thus when every specialist herbal healer that dies without an apprentice, the great medical knowledge base of his/her culture dies with him/her. It has also been observed that some traditional medicine practitioners tend to hide the identity of plants used for different ailments largely for fear of lack of patronage should the sufferer learn to cure himself. Another negative habit observed is that in order to mystify their trade, cultivation of the plants is not encouraged, thus all the collections are virtually from the wild. With the passing away of most of these practitioners with their wealth of knowledge, a huge loss is made in the body of knowledge dealing with plants that heal and ethnobotany. Some traditional healers have revealed important information to a few close relatives where any interest is shown in order to retain the trade or business within the family linage. This mode of information transfer is, however, grossly inadequate in that it lacks continuity. This accumulates to a frighten conclusion that the knowledge on the use of these species might disappear even faster than the plants due to modernization and urban drift.

World Health Organisation (WHO) estimates that up to 80% of the world's people rely on traditional medicine for their primary health care, based on the fact that western pharmaceuticals are often expensive, inaccessible or unsuitable in some "Africa diseases" (Hostettman and Marston 2002; WHO 2011). Most developing countries based on the WHO information and realizing the crucial roles traditional medicine plays, are urgently setting up research centers or funding for the study of medicinal plants available with the note that there was an abject neglect of this highly endangered but cheap alternative health care resource (Bhat

et al., 2013). Karou, in 2012 confirmed that approximately 80% of the population in Mali use traditional medicine as their only type of medicine. Available figures show that between 60 and 70% of Ghanaians rely on traditional medical systems for their health needs (Sarpong, 2000), while in Nigeria, a WHO survey estimated that up to 75% of the population patronize traditional medicine (Omoseyindemi, 2003). In Ghana, Mali, Nigeria and Zambia, the first line of treatment for 60% of children with high fever resulting from malaria is the use of herbal medicines at home. South Africa has an estimated 250 000 traditional healers providing healthcare to around 80% of the black population using knowledge that dates back as far as 1000 BC (Karou *et al.*, 2011).

In a number of industrialised countries many people regularly use some form of traditional complementary and alternative medicine (TCAM), including herbal supplements. Available statistics indicate that Germany (75%), Canada (70%)and England (47%) (Thomas *et al*., 2001) population makes use of herbal product(s).

The globalisation of traditional medicine has important implications for both the quality control of medicaments, the training, competence of practitioners, the safety and efficacy of both mass produced and individually compounded herbal products and documentation of any toxicity or adverse effect (Shia *et al.*, 2007; Urban and Separovic 2005).

2.3 PLANTS IN DRUG DEVELOPMENT

2.3.1 Ethnobotanical leads in natural product discovery

The search for new molecules, nowadays, has taken a slightly different route where the science of ethnobotany and ethnopharmacognosy are being used as guide to lead the chemist towards different sources and classes of compounds. The ethnobotanical approach is actually one of

several methods that can be applied in choosing plants for pharmacological studies. It is estimated that 265,000 flowering species grace the earth. Of these, less than half of 1 percent has been studies exhaustively for their chemical composition and medical value. In a world with limited financial resources, it is impossible to screen each of the remaining species for biological activity. Thus the strategy of collection of plants based on its local/indigenous medical usage seems to be logic, economical and can offer strong clue to the biological activities of the plants (Turschner and Efferth 2009).

Historically all herbal medicinal preparations were derived from plants, whether in the simple form of plant parts or in the more complex form of crude extracts, mixtures, etc. Today a substantial number of drugs are developed from plants which are active against a number of diseases. Natural products and their derivatives represent more than 50% of all the drugs in clinical use in the world. Higher plants contribute no less than 25% of the total. In the developed countries 25% of the medical drugs are based on plants and their derivatives (Principe, 2005) and the use of medicinal plants is well known among the indigenous people in rural areas of many developing countries.

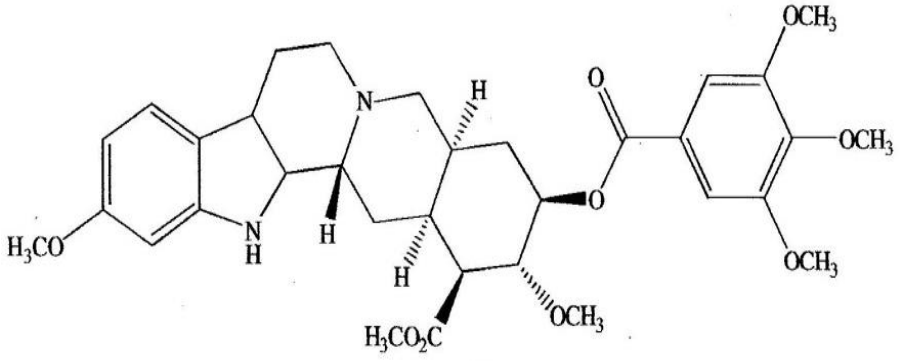
In the past, the healing powers of plants were discovered through trial and error, assumption that the appearance of plants may give clues to their medicinal properties (Doctrine of Signatures) etc. Some of the therapeutic properties attributed to plants through the above methods were proven to be erroneous, but still most medicinal plant therapy is based on the empirical findings of hundreds and thousands of years (Gurib-Fakim, 2006). Years of safe usage lays the foundation of ethnopharmacological research of medicinal plants.

In contrast to synthetic pharmaceuticals based upon single chemicals, many medicinal plants exert their beneficial effects through the additive or synergistic action of several

chemical compounds acting at single or multiple target sites associated with a physiological process (Okigbo *et al*., 2009). Thus, a single plant may, for example, contain bitter substances that stimulate digestion, anti-inflammatory compounds that reduce swellings and pain, phenolic compounds that can act as an antioxidant and venotonics, anti-bacterial and anti-fungal tannins that act as natural antibiotics, diuretic substances that enhance the elimination of waste products and toxins and alkaloids that enhance mood and give a sense of well-being.

In modern medicine, plants are used as sources of direct therapeutic agents, as leads for new synthetic compounds, and as a taxonomic marker for discovery of new compounds. They serve as raw material bases for the elaboration of more complex semi-synthetic chemical compounds. Modern medicine usually aims at isolation or synthesis of a patentable single compound or a magic bullet to treat specific health condition(s). The synthesis of plant sourced bioactive compounds is chemically difficult, because of their complex structure and high cost (Shimomura *et al*., 1997). The majority of these involve the isolation of the active chemical compound found in a particular medicinal plant and its subsequent chemical or laboratively modification towards getting a better moiety, such include; *Dioscorea* specie derived diosgenin from which all anovulatory contraceptive agents have been derived; reserpine **(1)** and other anti-hypertensive and tranquilizing alkaloids from *Rauwolfia* species; pilocarpine to treat glaucoma and dry mouth, derived from a group of South American trees (*Pilocarpus* specie) in the *Citrus* family; laxative agents from *Cassia* specie and as a cardiotonic agent to treat heart failure from digitalis species. Three of the major sources of anti-cancer drugs on the market or completing clinical trials were derived from North American plants used medicinally by the Native Americans: the Papaw (Asimina specie); the Western Yew tree (*Taxus brevifolia*),

effective against ovarian cancer and the May apple (*Podophyllum peltatum*) used to combat leukaemia, lymphoma lung and testicular cancer (Gurib-Fakim 2006).



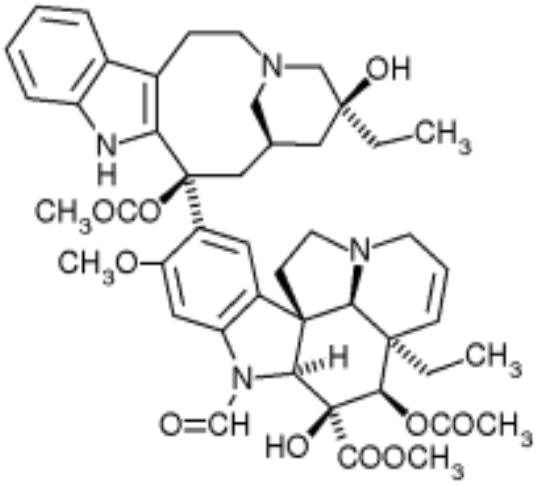
Reserpine (1)

The "Ebers papyrus", the Egyptian pharmaceutical record, indicates the use of willow leaves as an antipyretic agent. The ethnobotanical knowledge of this plant led to chemist interest in isolation of the bioactive compounds responsible for the observed desired activity. Salicin was isolated from the bark of the white willow, *Salix alba*, in 1825-26 (Vlachojannis *et al.*, 2011) as an active compound of the plant. The conversion to salicylic acid via hydrolysis and oxidation provided a potent antipyretic compound that was later manufactured and used worldwide. The severe gastrointestinal toxicity/adverse effect observed on the use of salicylic acid was solved by conversion of the acid to acetylsalicylic acid (ASA, Aspirin) via acetylation and marketed under the trade name aspirin in 1899. Aspirin is still the most widely used analgesic and antipyretic drug in the world.

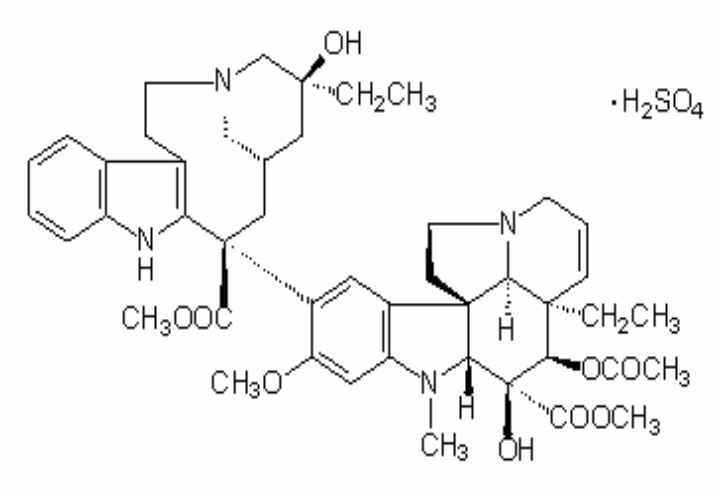
More recently, the vinca alkaloids, vinblastine **(3)** and vincristine (**2**) were isolated as antineoplastic agents from the Madagascan periwinkle, *Catharanthus roseus.* Through intensive structure–activity relationship (SAR) studies on **2** and **3**, semisynthetic congeners vindesine (deacetylvinblastine amide, VDS) and vinorelbine ($3^{/}$, $4^{/}$-dehydro-$7^{/}$ -nor-vinblastine) were

developed. The four compounds are now in clinical use for cancer treatment (Newman *et al.*, 2000).

More than 60% of cancer therapeutics on the market or in clinical testing is based on natural products (Brower, 2008). Early work on the American mandrake *(Podophyllum peltatum)* showed that its resinous constituents, which had been used as a drastic purgative, contained substances effective in the cure of venereal warts and podophyllin paint is still used for this and similar conditions. Further research work showed that some of the isolated compounds are cytotoxic and have antitumour action, though this does not seem to have led to clinical uses yet. More promising and exciting results have followed the investigation of *Vinca rosea*, a semi-



Vincristine (2)



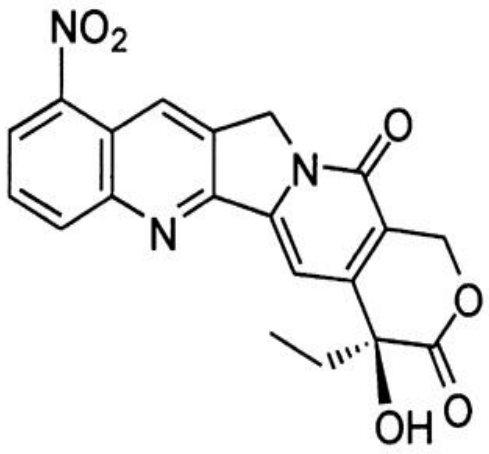
Vinblastine (3)

tropical periwinkle. This has been long used by many native societies as a cure for diabetes. Scientific investigation has not confirmed this claim, but workers noted that extracts of the plant lowered the resistance of mice to infection. This was found to be caused by diminished production of white blood cells and, since certain cancers (leukemias, Hodgkin's disease, etc.) are accompanied by an overproduction of white blood cells, *Vinca rosea* was screened for anti-cancer activity. About sixty alkaloids were isolated by Svoboda and co-workers and at least two of these, vincaleukoblastine (vinblastine) and eurocristine (vincristine), have been shown to be effective clinically in certain forms of cancer. These two compounds are the leading anticancer agents for the certain forms of cancer (Aniszewski 2007). This is an exciting development and is certainly encouraging the search for new cancer drugs from the plant kingdom. Cancer therapeutics from plants also includes paclitaxel, isolated from the Pacific yew tree (*Taxus brevifolia* Nutt, Taxaceae) and combretastatin, derived from the South African bush willow (Brower, 2008). Paclitaxel, a diterpenoid is used clinically in ovarian, breast, lung and prostate cancer effectively (Prakash *et al.*, 2013; Heinrich, 2003). Since its approval as an anticancer drug by the Food and Drug Administration in 1992, it has been used to treat several different

types of cancer including ovarian, breast, and lung (Cragg and Newman 2005; Prakash *et al.*, 2013).

Like paclitaxel, discodermolide targets tubulin by stabilizing microtubules, halting cell division, and thus triggering cell cycle arrest (Hung *et al*., 1996; Haar *et al*., 1996; Pryor *et al.*, 2002; Mukhtar *et al.*, 2014). It is a polyhydroxylated lactone isolated from the deep-sea sponge *Discodermia dissolute* (Porifera) of the Bahamas (Mukhtar *et al.*, 2014). It is an immunosuppresive and cytotoxic agent. In 1998, Novartis Pharma AG licensed this compound for development as a candidate agent for treatment of cancers.

Camptothecin **(4)**, is derived from the Chinese "happy tree" *Camptotheca acuminata* (Nyssaceae). Interest in camptothecin (**4**) grew as a result of its ability to inhibit topoisomerase I, which is the enzyme involved in many important cellular processes by interacting with DNA (Kroll *et al.*, 2007). With the structure of Camptothecin **(4)** acting as template, several products have been developed namely topotecan (hycamptamine) and irinotecan (CPT-11) (Friedman *et al*., 2003). Camptothecin **(4)** exhibited sever bladder toxicity during clinical trial while its derivative Irinotecan is less toxic. Irinotecan has much greater water solubility and is a prodrug, being metabolized *in vivo* by hydrolysis to give topoisomerase I inhibitor which is 1000 times more active than the parent compound. Irinotecan has been approved in the US against metastatic colorectal cancer and leukaemias. Topotecan has also been approved in the US for ovarian cancer and has been tested also among paediatric patients with resistant and recurrent solid tumours (Martinez *et al.*, 2003).



Camptothecin (4)

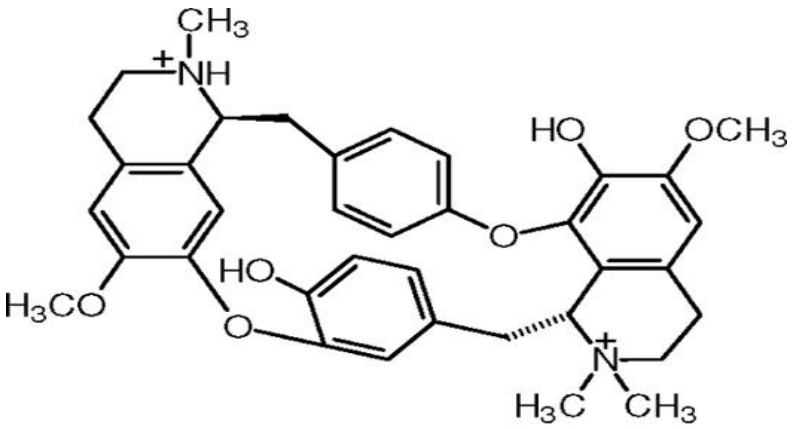
The isolation of the anti-malarial drug, quinine from the bark of *Cinchona* species (e.g. *C. officinalis*) was reported in 1820 by the French pharmacists, Caventou and Pelletier. In the early 1500s, Indian fever bark was one of the first medicinal plants to find appreciative consumers in Europe. The bark had long been used by indigenous groups in the Amazon region for the treatment of fevers, and was first introduced into Europe in the early 1600s for the treatment of malaria. Taken from the Cinchona tree (*Cinchona officinalis*), the bark was used as an infusion by native people of the Andes and Amazon highlands to treat fever. Jesuit missionaries brought the bark back to Europe. By the early sixteenth century, this medicine was known as 'Jesuit fever bark', quite a transformation.

Similarly, a potent antimalarial drug, a sesquiterpenoid endoperoxide, named artemisinin was isolated from *Artemisia annua* as a remedy against the multidrug resistant strains of *Plasmodium*, following the long use of this plant material as an antimalarial drug in the traditional Chinese medicine. Using the basic structure of artemisinin, semisynthetic compounds were synthesized with the aim of optimizing the pharmacology of the principal molecule

leading to the identification of artemether and dihydroartemisinin as potent antimalarial agents that are now in a widespread use around the world (Turconi *et al.*, 2014).

The name coca (*Erythroxylum coca*) comes from an Aymara word meaning 'tree'. In Andean cultures, the leaves of the coca tree have been primarily chewed to obtain perceived benefits. From ancient times, indigenous people have added alkaline materials such as crushed seashells or burnt plant ashes to the leaves in order to accentuate the pharmacologically active moiety of coca. In 1860, a German chemist Carl Koler isolated cocaine, the chemical responsible for the biological activity. He found that cocaine could act as a local anaesthetic in eye surgery. As the years passed, scientists observed that cocaine paralyzed nerve endings responsible for transmitting pain (Wielenga and Gilchrist, 2013). As a local anaesthetic, it revolutionized several surgical and dental procedures.

Pot curare arrowhead poison used in the East Amazon is predominately from the species *Strychnos guianensis*. Tube curare in the West Amazon is from *Chrondroden drontomentosum*; curare in modern medicine is made from this and named as tubocurarine **(5)**. The jaborandi tree (*Pilocarpus jaborandi*) secretes alkaloid- rich oil. Several substances are extracted from this aromatic oil, including the alkaloid pilocarpine, a weapon against the blinding disease, glaucoma. American Indians on the island of Guadeloupe used pineapple (*Ananas comosos*) poultices to reduce inflammation in wounds and other skin injuries, to aid digestion and to cure stomach ache.



Tubocurarine (5)

In 1891, an enzyme that broke down proteins (bromelain) was isolated from the fresh juice of pineapple and was found to break down blood clots. Other pharmaceuticals that have their origin in botanicals include atropine, hyoscine, digoxin, colchicine and emetine. Reserpine **(1)**, an anti-hypertensive alkaloid (*Rauwolfia serpentina*) became available as a result of work carried out by Ciba-Geigy in India. It is pertinent to note that most of these early discoveries were mainly based on traditional observation.

There are four basic ways in which plants that are used by traditional medicine are valuable for modern medicine or drug making company:

1. Plants from the tropics are sometimes used as sources of direct therapeutic agents (e.g. the alkaloid D-tubocurarine **(5)** is extracted from the South American jungle liana *Chondrodendron tomentosum* is widely used as a muscle relaxant in surgery. Another good example to illustrate this feature is Reserpine **(1)**, an important hypotensive agent extracted from *Rauwolfia*. The synthesis of such drugs is either impossible for the chemist or too expensive as compare to the isolation of the active compound from the plant.
2. Plants raw materials could serve as the starting materials for semi-synthetic compounds. An example of this includes saponin extracts that are chemically altered to produce

sapogenins necessary for the manufacture of steroidal drugs. Most steroids were obtained from extracts of neo-tropical yams of the genus *Dioscorea*.

3. New and unusual chemical substances found in plants could serve as blueprints for novel synthetic drug substances and example of this includes cocaine from *Coca* plants. *Erythroxylum coca*, has served as model for the synthesis of a number of local anaesthetics such as procaine.

4. Plants can also be used as taxanomic markers for the discovery of new compounds. The documented facts of a family can be used to investigate a closely related family in search of useful phytochemicals for human usage.

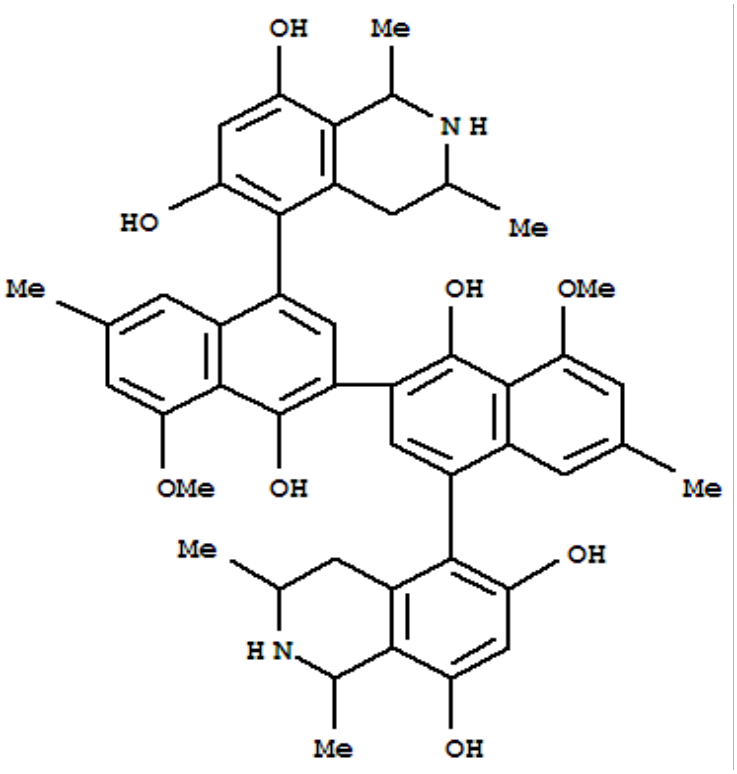
Table 1: Herbal drugs used in traditional medicine and which have given useful modern drugs

Botanical names	English names	Indigenous use	Origin	Uses in biomedicine	Biologically active compounds
Adhatoda vasica	–	Antispasmodic, antiseptic, insecticide, fish poison	India, Sri Lanka	Antispasmodic, oxytocic, cough suppressant	Vasicin (lead molecule for Bromhexin and Ambroxol)
Catharanthus roseus	Periwinkle	Diabetes, fever	Madagascar	Cancer chemotherapy	Vincristine, Vinblastine
Chondrodendron tomentosum	–	Arrow poison	Brazil, Peru	Muscular relaxation	D-Tubocurarine
Gingko biloba	Gingko	Asthma, anthelmintic (fruit)	Eastern China	Dementia, cerebral Deficiencies	Ginkgolides
Harpagophytum procumbens	Devil's claw	Fever, inflammatory conditions	Southern Africa	Pain, rheumatism	Harpagoside, Caffeic acid
Piper methysticum	Kava	Ritual stimulant, tonic	Polynesia	Anxiolytic, mild Stimulant	Kava pyrones
Podophyllum peltatum	May apple	Laxative, skin infections	North America	Cancer chemotherapy, Warts	Podophyllotoxin and lignans
Prunus africana	African plum	Laxative, Old man's disease	Tropical Africa	Prostate hyperplasia	Sitosterol
Ancistrocladus korupensis	–	Malaria, diabetes	Cameroon	HIV-1, HIV-2	Michellamine B **(6)**
Camptotheca acuminata	Happy tree, xi shu, tree of life	Wound	China, Tibet	Anti-cancer	Camptothecin
Cinchona spp	Peruvian bark, Jesuit bark, fever tree	Muscle relaxant to cease shivering due to low temperatures.	Andes (South America)	Antimalarial, antipyretic	Quinine

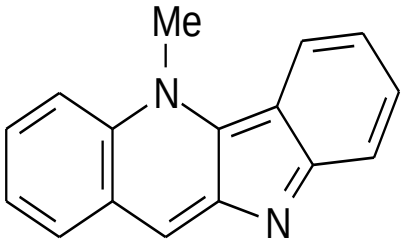
Voacanga Africana	–	Poison, stimulant, aphrodisiac, and ceremonial psychedelic, geriatric conditions, cardiac tonic	Africa and Asia	*Antiulcer, anti-addictive*	Ibogaine (Methylenedioxy-methamphetamine)
Taxus brevifolia	Pacific yew or western yew)	To make bows and paddles for canoes	Pacific Northwest of North America	Anticancer	Paclitaxel (Taxol)
Rauvolfia vomitoria	Devil's pepper, Indian snakeroot		Tropical Africa	Tranquilizer, antihypertensive	Reserpine yohimbine
Physostigma venenosium	Calabar bean	Ritual ordeal	Tropical Africa	Cholinesterase inhibitor	Physostigimine **(10)**
Cryptolepis sanguinolenta	Nibima, kadze, gangamau, Ghanaian quinine and yellow-dye root	Malaria and fever, Type II diabetes	West Africa	*Antimalarial, anticancer, antiinflammatory, antiviral*	Cryptolepine, isocryptolepine
Aspalathus linearis	Rooibos tea, red bush	Nervous tension, allergies and digestive problems	Southern Africa	Antitumor , Anti-ageing	Nothofagin **(11)**, Aspalathin **(12)**

(Sourced from Omotayo and Borokini 2012)

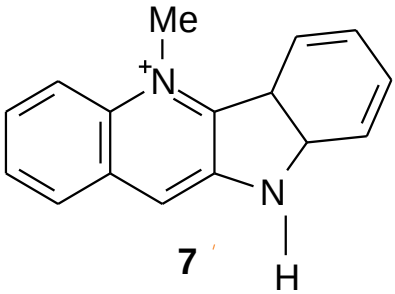
The structures of some drugs in Table 1



Michellamine B (6)

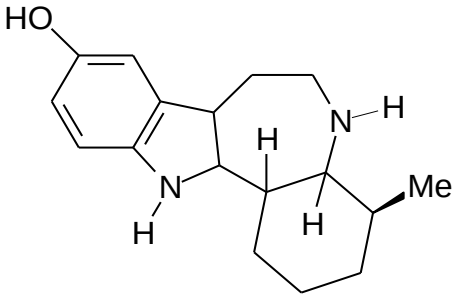


Cryptolepine (7)

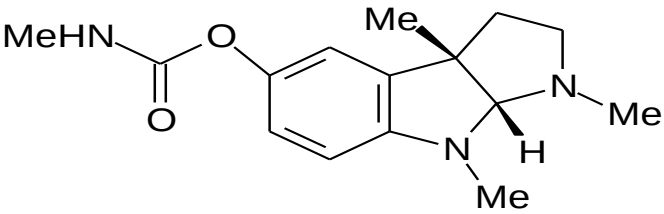


7

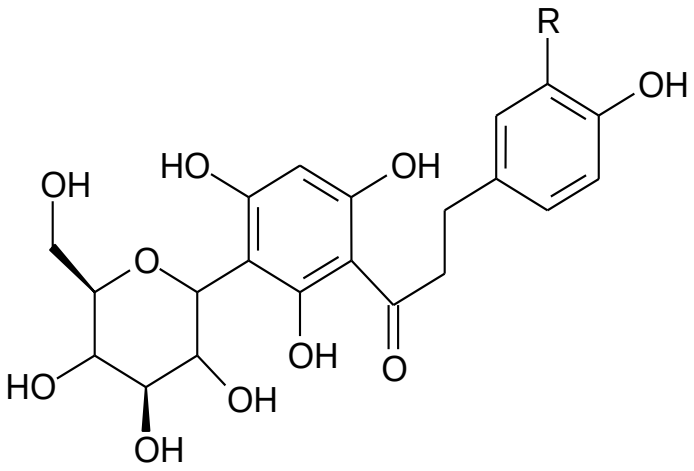
Isocryptolepine (8)



Ibogaine (9)



Physostigimine (10)



R_1=R_2=H= Nothofagin (11)

R_1=R_2=Glc=Aspalathin (12)

2.3.2 Some secondary plant metabolites in drug discovery

Secondary metabolites are small molecules and chemical compounds which are not essential for the growth and development of the producing organism but are formed during the plants' normal metabolically processes (Okigbo *et al*., 2009). These phytochemicals have importance because of their biological activities on other organisms and are normally produced as defense mechanism of the plant, enhance normal growth or facilitating reproduction process (Okigbo *et al*,, 2009).

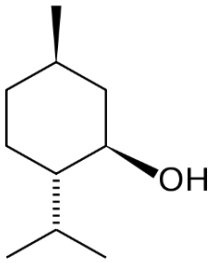
2.3.2.1 Terpenes

Terpenes are widespread throughout many, if not all plant families, and are characterized by a structure consisting of repeating isoprene units (Xu *et al*., 2004). The term may also refer to oxygen derivatives of these compounds that are known as the terpenoids. The theory that provided the first conceptual framework for a common structural relationship among the

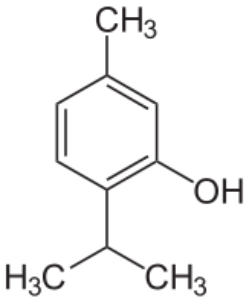
terpenes was first formulated by Wallach in 1887 after carrying out structural investigations of several terpenes. His theory stated that terpenes can be viewed as made up of one or more isoprene (2-methyl-1,3-diene) units joined together in a head to tail manner. Wallach's idea was further refined in the 1950 by Ruzicka's formulations of the biogenetic isoprene rule emphasizing mechanistic considerations of terpene synthesis in terms of electrophilic elongations, cyclizations and rearrangements (Maimone and Baran 2007).

Many plant terpenoids are toxins and feeding deterrents to herbivores or are attractants, and many possess pharmacological activity.

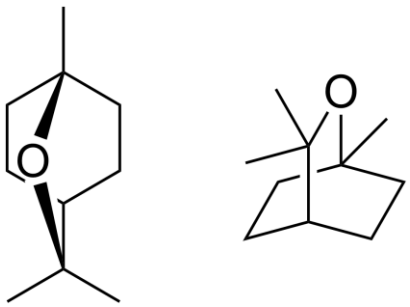
Terpenes are normally classified into groups based on the number of isoprene units from which they are biogenetically derived. These include the hemiterpene (one isoprene); Monoterpenes (two isoprene units), they are low molecular weight, and therefore volatile, hydrocarbons and are responsible for the fragrance of many plant species. Members of this subgroup include the fragrant and antimicrobial compounds, menthol **(13)**, thymol **(14)**, and 1,8- cineole **(15)** (Rasooli and Mirmostafa 2002; Gurib-Fakim 2006).



Menthol (13)



Thymol (14)



1,8- cineole (Eucalyptol) (15)

Sesquiterpenes (three isoprene units): Sesquiterpenes are also constituents of essential oils of many plants, e.g. bisabolol, humulene and caryophyllene. Sesquiterpene lactones are well known as bitter principles. They occur in families like the Asteraceae. These compounds possess a broad range of activities due to the a-methylene-clactone moiety and epoxides. Their pharmacological activities are anti-bacterial, anti-fungal, anthelmintic, anti-malarial (artemesinin **51)** and molluscicidal. Examples are Santonin, which is used as an anthelmintic and as an anti-malarial (Cantrell *et al*., 2001).

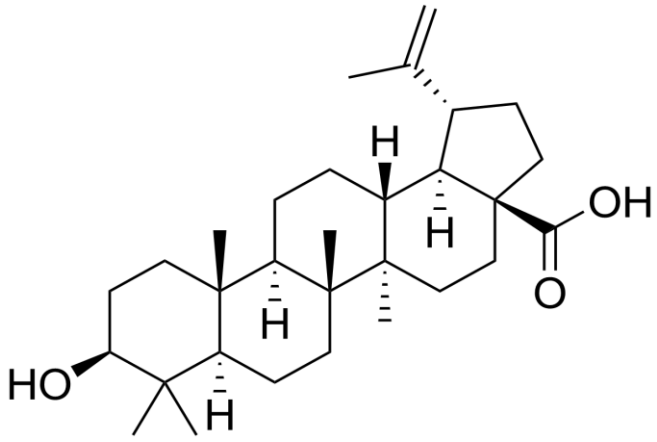
Diterpenes (four isoprene units): They are present in animals and plants. These compounds have some therapeutic applications. For example, Taxol and its derivatives are anti-cancer drugs. Other examples are Forskolin, which has anti-hypertensive activity. Zoapatanol is an abortifacient while Stevoside is a sweetening agent.

Triterpenes (six isoprene units) and tetraterpenes which are compounds based on eight isoprene units. Important among these are the C_{40} carotenoids etc. The higher terpenes are further subdivided into several subclasses based on the particular type of skeletons they possess.

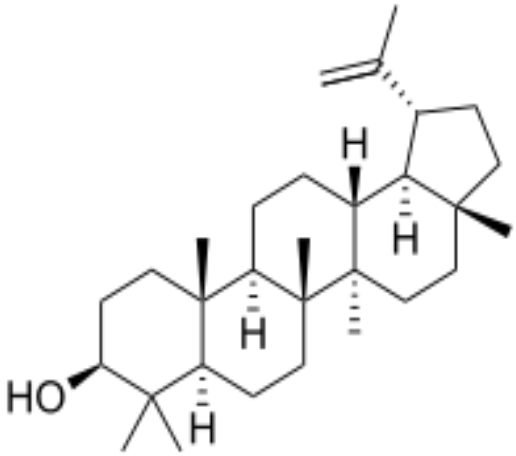
Terpenoids are variously said to have antiseptic activity, irritant properties, and sedative proper. Various studies have shown that they are active against bacteria, fungi, virus, protozoa (Karou *et al.,* 2011). There is a wide range of pharmacological activities studied in natural terpenoids. Based on these studies, betulinic acid **(16)**, a pentacyclic triterpene has been found to have

significant anti-tumor, anti-viral, anti-inflammatory, broncho-vasodilation, analgesic and anti-plasmodium activities.

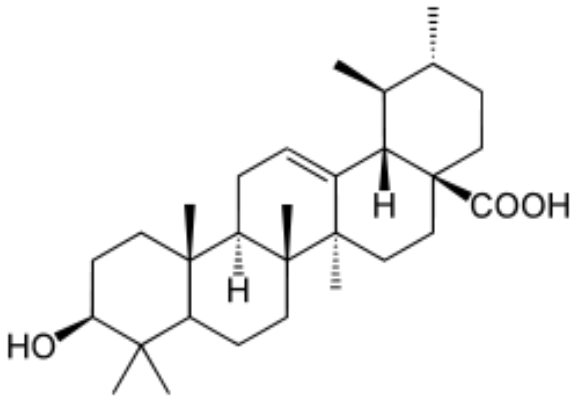
Other terpenoids like lupeol **(17)** and ursolic acid **(18)** have also been studied and found to have anti-cancer effect, anti-inflammation, hepatic and cardio protective effect (Dzubak *et al*., 2005; Tolstikova *et al*., 2006) and analgesic in ursolic acid **(18)**.



Betulinic acid (16)



Lupeol (17)



Ursolic acid (18)

Terpenoids are chemically lipid-soluble compounds and they can be extracted with petroleum ether generally. Sesquiterpene lactones, diterpenes, sterols and less polar triterpenoids extraction can be also performed by using benzene, ether and chloroform. Ethyl acetate and acetone extracts contain oxygenated diterpenoids, sterols and triterpenoids. Ethanol, methanol and water led to the extraction of highly oxygenated namely polar triterpenes as well as triterpenoid and sterol glycosides. Total extraction of the material carried out by any polar solvents such as acetone, aqueous methanol (80%) and aqueous ethanol and then re-extraction with hexane, chloroform and ethyl acetate is also leads to successive extraction of terpenoids and sterols (Harborne, 1998).

Gas-Liquid Chromatography (GLC) is known as the best method for analyses of terpenoids especially mono- and sesquiterpenoids. Thin layer chromatography (TLC) can be used as another rapid, useful method for terpenoids and sterols detection with concentrated H_2SO_4 and heating due to all terpenoids and steroids (except carotenoids) are colourless compounds. TLC is also allowing to the isolation of various classes of terpenoids on silica gel and silver nitrate impregnated silica gel coated plates (Harborne 1998).

Column chromatography is convenient method and simple isolation technique for various terpenoids especially sesqui-, di-, tri- and tetraterpenoids as well as sterols. As stationary phase silica gel, alumina, cellulose, sephadex, polyamid are used for the separation of different types of secondary metabolites but of this silica gel is the most extensively used adsorbent for particularly nonpolar and medium polar compounds including terpenoids and sterols. Silver nitrate impregnated silica gel also provide separation of terpenoids containing unsaturation (Sarker *et al*., 2006).

The general alicyclic nature of terpenoids frequently gives room to isomerism of the compounds. Different geometric conformations are possible in terpenoids due to the twisted cyclohexane ring, depending on the substitution around the ring. Therefore, stereochemistry is commonly found in terpenoids. These structural features may cause artifact formation during isolation procedure (Harborne, 1998).

2.3.2.2 Alkaloids

The term alkaloid has been defined as a cyclic organic compound containing nitrogen in a negative oxidation state, which has limited distribution in a limited number of plant species (Ober 2003). Families reported to be rich in alkaloids are: Liliaceae, Amaryllidaceae, Apocynaceae, Berberidaceae, Leguminosae, Papaveraceae, Ranunculaceae, Rubiaceae and Solanaceae. Based on their structures, alkaloids are divided into several sub-groups (Aniszewki 2007). Free alkaloids are soluble in organic solvents and react with acids to form water soluble salts. Berberine **(27)**, which is a quartenary ammonium alkaloid is an exception here. Most alkaloids are solids except for Nicotine, which is a liquid.

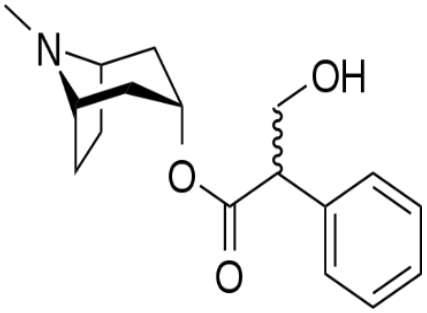
Alkaloids are usually classified according to their common structural motif, based on the metabolic pathway used to construct the molecule. When not much is not known of the biosynthesis of alkaloids, they were grouped under the names of known compounds, even some non-nitrogenous ones, for example the phenanthrenes, since this moiety appeared in the finished product or by the plants or animals from which they were isolated. When more is learned about a certain alkaloid, the grouping is changed to reflect the new knowledge, usually taking the name of a biologically-important amine that stands out in the synthesis process (Aniszewki 2007).

Table 2: Groups of alkaloid

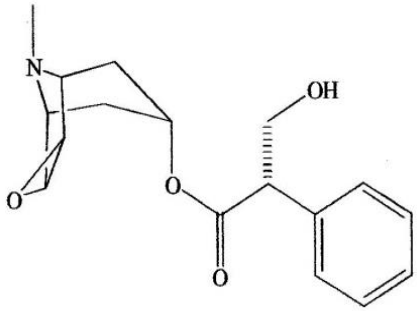
Group of Alkaloids	**Examples with Bioactivity**
Pyridine alkaloids	Piperine, coniine, trigonelline, nicotine
Pyrrolidine alkaloids	Hygrine, cuscohygrine
Tropane alkaloids	Atropine **19**, cocaine, ecgonine, scopolamine **20**
Quinoline alkaloids	Quinine **40**, quinidine **21**, strychnine **22**, brucine **23**
Isoquinoline alkaloids	Codeine **24**, thebaine , papaverine **25**, berberine **27**
Phenethylamine alkaloids	Mescaline **26**, ephedrine **30**, dopamine **29**, amphetamine **28**
Indole alkaloids	Ergine **32**, harmine **31**, yohimbine **33**, reserpine **1**
Purine alkaloids	Xanthine **35**, caffeine, theophylline **34**
Terpenoid alkaloids	Aconitine **36**, taxol
Vinca alkaloids	Vinblastine **3**, vincristine **2**
Miscellaneous alkaloids	Capsaicin

The nitrogenous characteristic of alkaloids enables them to act on the nervous system of vertebrates by interfering with essential receptors in the neural pathways. In this way, the compound can produce strong physiological responses from small doses, either as a therapeutic or as a poison (Aniszewki 2007). Alkaloids are believed to be waste products and of nitrogen

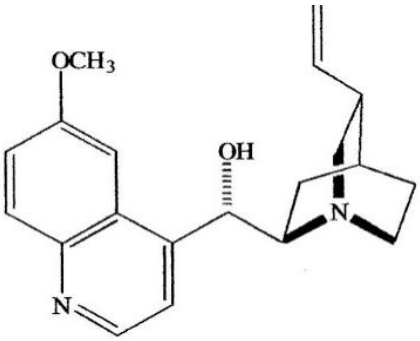
source. They are thought to play an important role in plant protection, germination and are plant growth stimulants (Ober 2003).



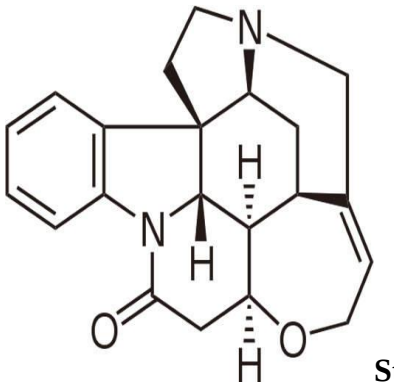
Atropine (19)



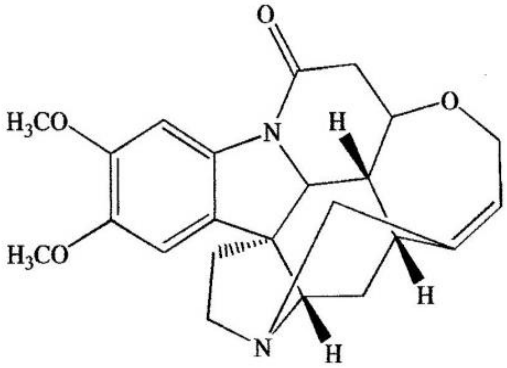
Scopolamine (20)



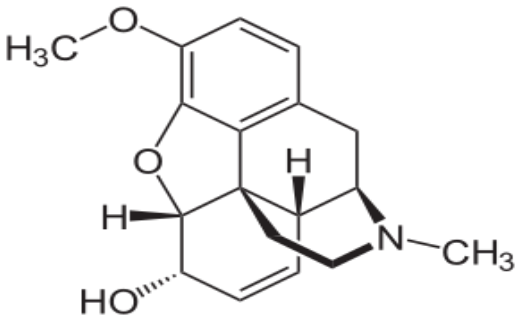
Quinidine (21)



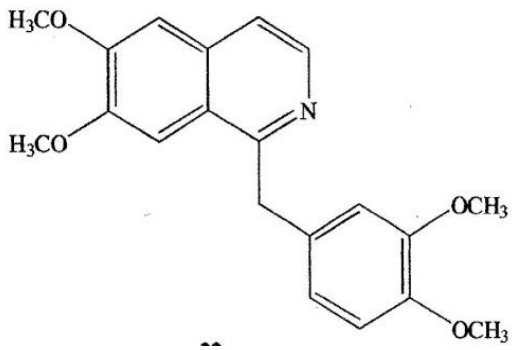
Strychnine (22)



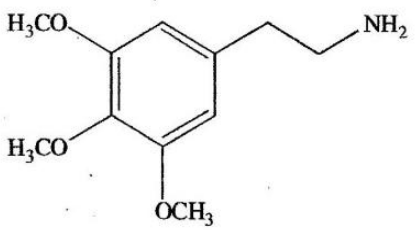
Brucine (23)



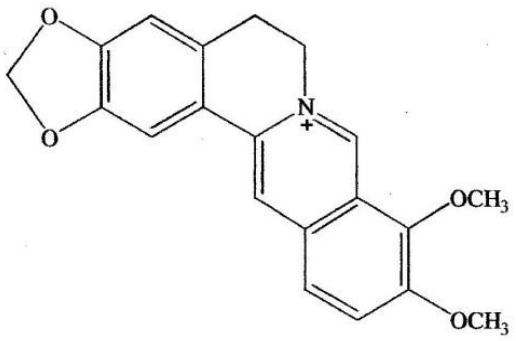
Codeine (24)



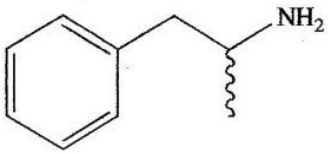
Papaverine (25)



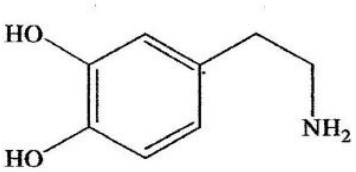
Mescaline (26)



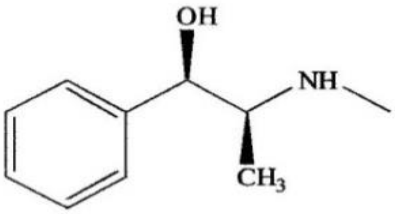
Berberine (27)



Amphetamine (28)



Dopamine (29)



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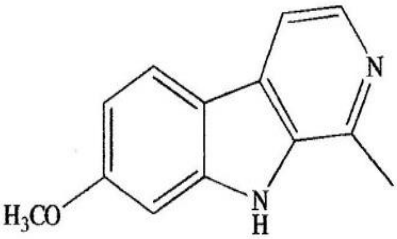
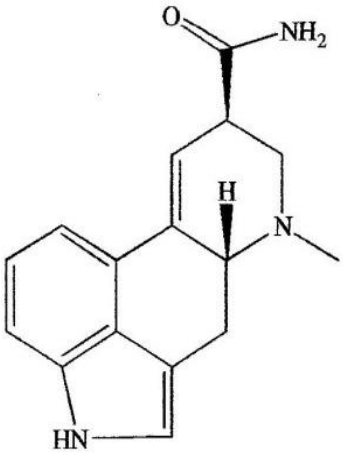
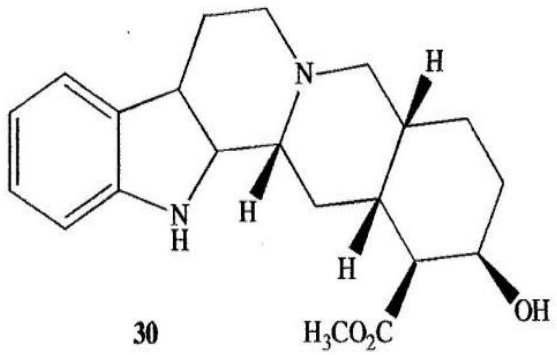


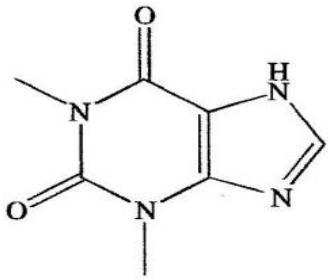
Harmine (31)



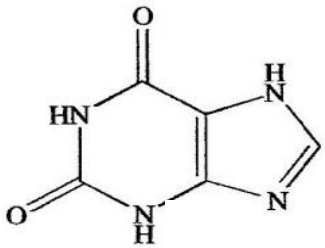
Ergine (32)

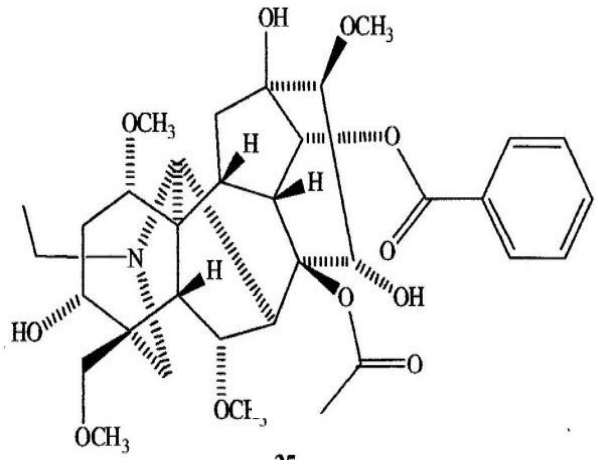


Yohimbine (33)



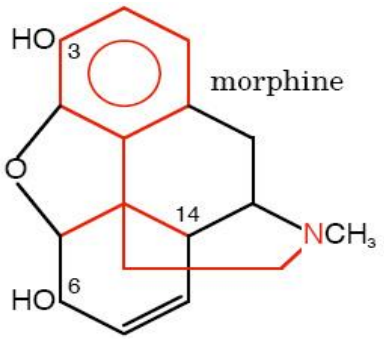
Theophylline (34)





Xanthine (35) **Aconitine (36)**

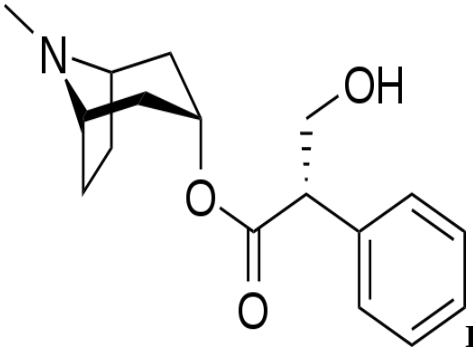
Scientists have observed that alkaloids are usually found localized in the part of the plant to be protected and be at sufficient concentration for adequate defense. Alkaloids are more likely to be found in inflorescences, young growing tips of plants, peripheral cell layers of stems and roots, these are parts of the plants that are easily attacked by herbivore. A good example is the concentration of morphine **(37)**, occurs about 0.3 to 1.5% in a whole capsule but up in crude opium, the air dried poppy latex is up to 25% or more. 0.8 to 2% of monoterpene alkaloids can be extracted from the *Rauwolfia* root but more than 5% in its root bark, suggesting peripheral location in the root bark parenchyma (Evans 2009; Cushniea *et al.*, 2014). Plants sequester alkaloids as passive defense mechanism by acting as feeding deterrents for predating insects. The bitter taste of many alkaloids may be importance as a feeding deterrent, quinine, strychnine **(22)**, brucine **(23)** (Patel *et al.*, 2012). The marine sponges in hostile marine ecosystem produce chemical defences in the form of alkaloids, such as ichthyotoxic and latruculins (Patel *et al.*, 2012).



Morphine (37)

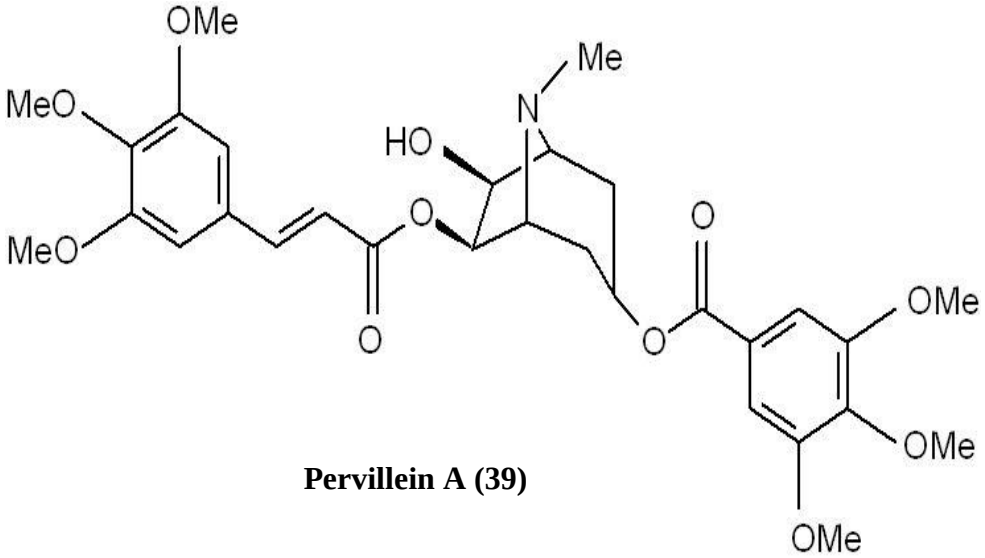
Common medicines derived from alkaloids include; atropine **(19)** (derived from the nightshade *Atropa belladonna*); galantamine; tropane; nicotinic acid - vitamin B3; vinblastine (**3**) (dimeric indole, anticancer); caffeine (psychostimulant from *Coffea* species); codeine **(24)**

(*Erythroxylum coca* Lam used as a local anesthetic and the treatment of cough); colchicine **(24)** in the treatment of gout; morphine **(37)** (*Papaver somniferum* used as indispensable analgesic); quinidine **(21)** (antiarrhythmic agent); quinine from the Cinchona tree and and L-hyoscyamine **(25)** (in the form of its racemic mixture known as atropine **(19)** as antispasmodic and for pupil dilation. Quinine, an antimalarial agent has been a lifesaving drug for human for many years and was the template from which some other antimalarial were developed from. Quinine is still the drug of choice in cerebral malaria and resistant malarial attack. Antibiotic and antiseptic activities are common biological activities of alkaloids (Cushniea *et al.,* 2014). Some are currently in use in medicine such as the berberine **(27)** in ophthalmics and sanguinarine in toothpaste.



Hyoscyamine (38)

Recently, from the extracts of *Erythroxylum pervillei* collected in Madagascar, nine tropane alkaloids were isolated, out of which seven are new compunds. Six of the new compounds (pervilleins A-F) were found to reverse multidrug resistance (MDR), using a KB-VI (vinblastine-resistant oral epidermoid carcinoma). These promising compounds show are novel inhibitors of the MDR phenotype (Kinghorn *et al*., 2003).



Pervillein A (39)

2.4 MALARIA

2.4.1 Introduction

Malaria is caused by a single-celled protozoan parasite of the genus *Plasmodium*. It is a major public health problem in warm climate and less economic empowered regions within the equator band in spite of the efforts of the World Health Organisation through various eradication programmes. It affects the productivity of individuals and the whole society, since it causes more energy loss, more debilitation and more loss of work capacity than any other human parasitic diseases (Sachs 2002). Malaria is preventable and curable at a cost, thus the density of malarial attack is commonly associated with degree of poverty and environmental cleanliness (Neil *et al*., 2013). It is most prevalent in poorer countries where prevention is difficult and prophylaxis is generally not an option.

Malaria occurs approximately in 130 countries and territories including the newly reported areas in Africa, Southeast Asia and South America (Snow *et al.,* 2005 and Korenromp 2005). Each year 300 to 500 million new cases are diagnosed and approximately 1.5 million people die of the disease; the majority of them being Africa children and pregnant women (Gwatkin and Gulliot, 2000; Barat *et al.,* 2004; Greenwood *et al.,* 2005). World Health Organization (WHO) in its World Malaria Report estimated that there were about 219 million cases of malaria in 2010 and an estimated 660,000 deaths (Neil *et al*., 2013). According to the latest WHO estimates, released in December 2014, there were about 198 million cases of malaria in 2013 (with an uncertainty range of 124 million to 283 million) and an estimated 584 000 deaths (with an uncertainty range of 367 000 to 755 000) WHO 2014. The re-emerging of malaria in many parts of the world is due to the rapid increase of resistance to most of the available anti-malarial drugs, as well as resistance of vectors to insecticides, weak health services, persistence of

poverty and population migration (Ridley 2002; Zirihi 2005; Wongsrichanalai and Meshnick, 2008; Souleymane *et al*., 2013).

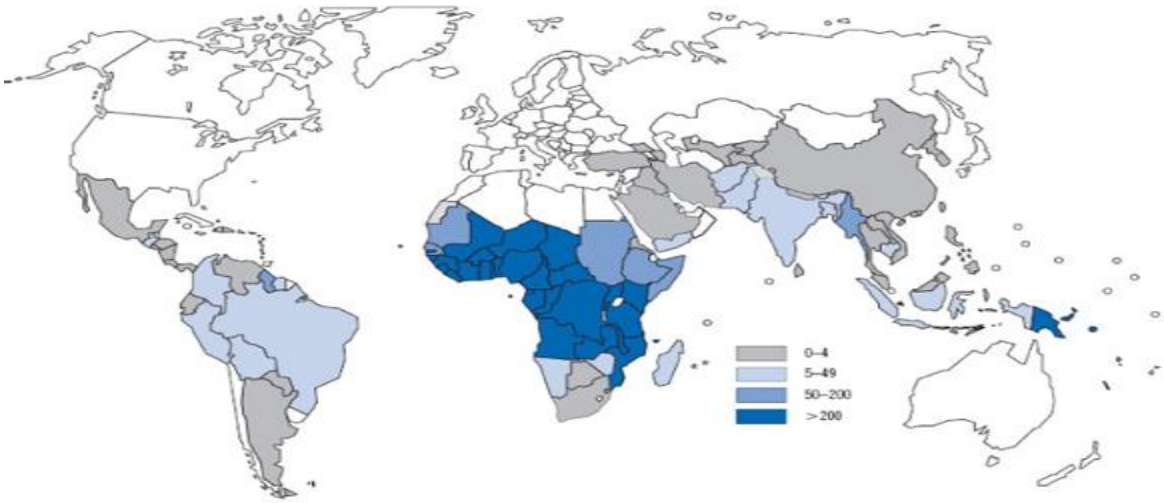


Figure 1: Estimated incidence of malaria per 1000 population in the year 2000 (WHO, 2008)

The malaria parasite, *Plasmodium*, is a very small, single-cell blood organism, or 'protozoan'. Over 40 species of the *Plasmodia* are in existence (Adesegun and Coker 2001). It lives as a parasite in other organisms, namely man and mosquito. The first scientist to isolate and identify the parasite in human blood was Laveran, in 1880 (Cox 2010). Human malaria protozoa are transmitted through bites of female *Anopheles* mosquitoes during blood meal. Ronald Ross, proved that the mosquito was the vector for malaria in humans by showing that certain mosquito species transmit malaria to birds. He proved the complete life-cycle of the malaria parasite in mosquitoes. He isolated malaria parasites from the salivary glands of mosquitoes that had fed on infected birds in 1897. In 1898 the Italian malariologists, Giovanni Battista Grassi, Amico Bignami, Giuseppe Bastianelli, Angelo Celli, Camillo Golgi and Ettore Marchiafava

demonstrated conclusively that human malaria was also transmitted by mosquitoes, in this case anophelines (Cox, 2010: Coker *et al*, 2001).

Four *Plasmodium* species are its aetiological agents. The 4 species that commonly infect man are:

- *P. falciparum*: It is responsible for 50% of all malaria cases worldwide and nearly responsible for all morbidity and mortality severe malaria. Found in the tropics and sub-tropics.
- *P. vivax*: The malaria parasite with the widest geographical distribution. Seen in tropical and sub-tropical areas but rare in Africa. It is responsible for 43% of all malaria cases in the world.
- *P. ovale*: This species is relatively rarely encountered. Primarily seen in tropical Africa, especially, the west coast, but has been reported in South America and Asia
- *P. malariae*: Responsible for only 7% of malaria cases. Occurs mainly in sub-tropical climates
- In recent years, some human cases of malaria have also occurred with *Plasmodium knowlesi* – a species that causes malaria among monkeys and occurs in certain forested areas of South-East Asia.

  (Ene *et al.*, 2009; Ngarivhume *et al.*, 2015)

2.4.2 Causative agent life cycle

This small single-cell organism, the *Plasmodium*, has three to four different forms. Each form is specialised in living in a certain place (Figure 1.6).

- The gametocyte is the form that infects the mosquito and reproduces itself, as if it were both sexes. When the mosquito has sucked blood containing gametocytes, these pass into the salivary glands of the mosquito, where they develop into a new form, the sporozoite.
- The sporozoite can be passed on to man when the mosquito bites, injecting its saliva into the tiny blood vessels. The sporozoite travels with the blood to the liver and enters the liver cells. In the liver some of the sporozoites divide (tachysporozoites) and become thousands of merozoites.

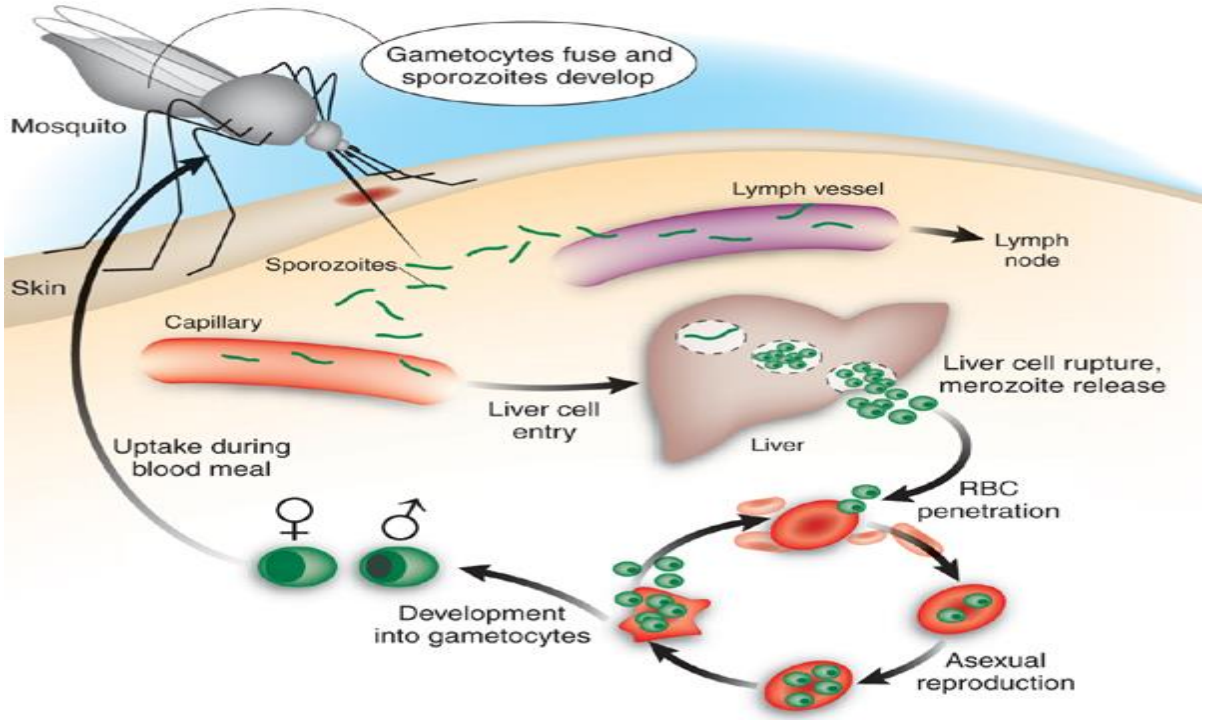


Figure 2: Causative agent life cycle (Ronan *et al.*, 2009)

- The merozoites are released from the liver to the blood where they are taken up by the red blood corpuscles. Some of these turn into ring-formed trophozoites that split again to form schizonts.
- Schizonts burst the red blood corpuscles at a certain moment, releasing the merozoites. This release coincides with the violent rises in temperature during the attacks seen in malaria.

The trophozoites that are left over during division can, in the course of the next day, develop into the sexual form, the gametocyte, which can be taken up by a blood-sucking mosquito and start another cycle.

The incubation period (time from infection to development of the disease) is usually about 10 to 15 days. This period can be much longer depending on whether any antimalarial medication has been taken.

P. ovale and *P. vivax* can produce a dormant form, hypnozoite, that can cause relapse of the disease months and even years after the original disease (relapsing malaria) because it's dormant in the liver cells. This is why it's important after infections, for patients to be treated with primaquine to kill the liver stages. Primaquine cannot be used by people with a condition called G6PD-deficiency.

In *P. vivax*, *P. ovale* and probably *P. malariae*, all stages of development subsequent to the liver cycle can be observed in the peripheral blood. However, in the case of *P. falciparum*, only the ring forms and gametocytes are usually present in the peripheral blood. Developing forms

appear to stick in the blood vessels of the large organs such as the brain and restrict the blood flow with serious consequences.

The four species have haemolytic component, where new brood of parasites break out of the red blood cells. This causes little or no harm, except with *P. falciparum* malaria where the parasites multiply very rapidly and may occupy 30% or more of the red blood cells causing a very significant level of haemolysis. One reason for this is that *P. falciparum* invades red blood cells of all ages whereas *P. vivax* and *P. ovale* prefer younger red cells, while *P. malariae* seeks mature red blood cells. The blood stage parasites are responsible for the clinical manifestations of the disease, and are the source of infection to mosquitoes.

2.4.3 Transmission

The intensity of transmission depends on factors related to the parasite, the vector, the human host, and the environment.

Vector: Transmission is more intense in places where the mosquito is relatively long-lived (so that the parasite has time to complete its development inside the mosquito) and stronger human-biting habit tends to have higher volume of malaria infection incidents. For example, the long lifespan and strong human-biting habit of the African vector species is the main reason why about 90% of the world's malaria deaths are in Africa (Karou *et al.*, 2011).

Human host: The human factor depends on the immunity level of the people living within a specified environment or when people with low immunity move into areas with intense malaria transmission, such as job seeking, or refugees. Partial immunity may be developed over years of exposure, and while it never gives complete protection, it does reduce the risk that malaria

infection will cause severe disease. (Doolan *et al.*, 2009; Behet *et al.*, 2014). For this reason, most malaria deaths in Africa occur in young children and pregnant women, whereas in areas with less transmission and low immunity, all age groups are at risk. Non-treated or inadequately treated individuals can be sources of infection to mosquitoes for a period of 2-3 years; the mosquitoes themselves remain infectious until death.

Environment: Transmission also depends on climatic conditions that may affect the number and survival of mosquitoes. Transmission is often seasonal, following the trend of the rainfall. The peak of infection if often from the start of the rainfall to just after the rainfall season, when stagnant water could readily be seen.

2.4.4 Clinical symptoms

The clinical course of *P. falciparum* following a bite by an infected mosquito, if infection does occur could be any of the following depending on the host and parasite factors;

- Asymptomatic parasitaemia (clinical immunity)
- Acute, uncomplicated malaria
- Severe malaria

In asymptomatic parasitaemia, the individuals have the malaria parasites in the periphera blood but no symptoms (act as reservoirs) or may develop anti-parasite immunity so that they do not develop parasitaemia following infection. It is normally found in adults and older children that have acquired natural immunity.

Acute or simple uncomplicated malaria is seen in individuals having some degree of immunity to malaria. There is manifestation of malaria but not life-threatening disease. Fever is the most constant symptom. Other symptoms include paroxysms of severe chills and rigors

(uncontrollable shivering), vomiting, diarrhoea (more commonly seen in young children), convulsions (commonly seen in young children) and pallor, resulting mainly from the lysis of red blood cells. Malaria also reduces the synthesis of red blood cells in the bone marrow. Other common clinical features are: jaundice (mainly due to haemolysis), anorexia, cough, headache, malaise, muscle aches, splenomegaly and tender hepatomegaly (Fidock *et al.*, 2004; Jones, 2006). These clinical features occur in "mild" malaria. However, the infection requires urgent diagnosis and management to prevent progression to severe disease.

Severe and complicated malaria, has a very high death statistics and mostly due to *P. falciparum*. Manifestations of sever malaria include; cerebral malaria, acute renal failure, severe malaria anaemia, pulmonary oedema, circulatory collapse, shock, hypoglycaemia, blackwater fever, and metabolic acidosis.

It is common for an individual patient to have more than one severe manifestation of malaria with a year.

2.4.5 Diagnosis

Blood is the most easily accessible diagnostic tissue. Variations in haematological parameters are influenced by any disease condition which affects the haemopoietic physiology. This is likely to happen with an endemic disease such as malaria that affects the host homeostasis. The target of malaria parasite is RBC (red blood cells) so that peripheral blood smear microscopical examination is the major diagnostic tool of the disease. However, this technique requires technical expertise and it is time consuming in repeated smear examinations (Rosenthal 2012).

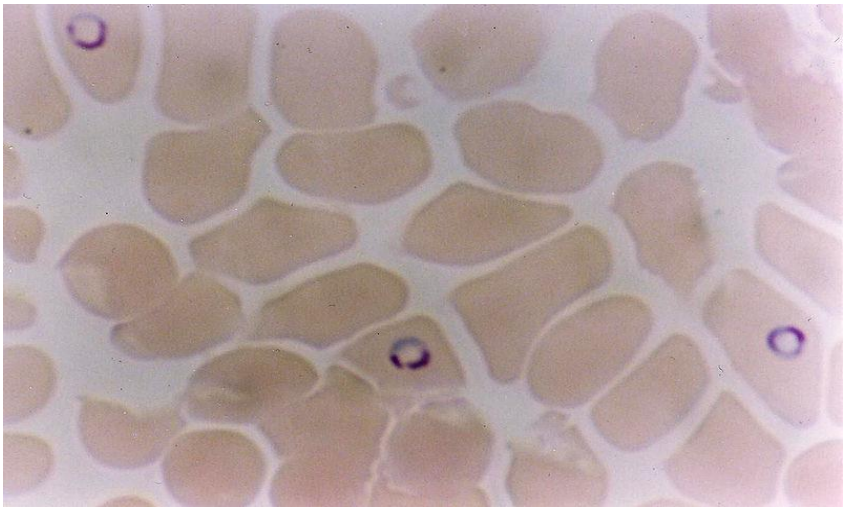


Figure 3a: Ring stage of *''Plasmodium falciparum''* in human red blood cells (Hempelmann, 2008)

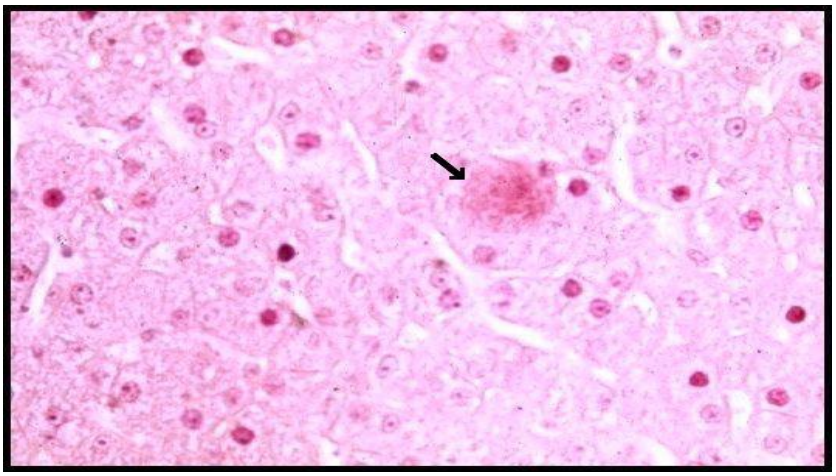


Figure 3b: Stained thin blood film showing *Plasmodium falciparum* infection

Section of liver showing a greatly enlarged parenchymal cell full of merozoites (see arrow) (Hempelmann, 2008)

2.4.6 Treatment/ Management of Malaria

Healthcare givers are advised to initiate antimalarial curative treatment on patients presenting fever (both for asymptomatic and symptomatic individuals) based on positive laboratory results (Hamainza *et al.,* 2014). The treatment cum control strategy is aimed at controlling human-to-mosquito transmission by providing chemotherapy to chronic parasite carriers, acting as reservoir. It may at the same time be complimentary to front-line interventions for preventing mosquito-to-human transmission, such as long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) of houses. Thus, accelerate declines in malaria burden in areas of low and seasonal transmission (Hamainza *et al.,* 2014).

The WHO recommended three main objectives of antimalarial treatment policy:

- Management of cases, to reduce morbidity and mortality by ensuring rapid, complete cure of the infection and thus preventing the progression of uncomplicated malaria to severe, potentially fatal disease, malaria-related anaemia and, during pregnancy, the negative impact of malaria on the fetus.
- To curtail the transmission of malaria by reducing the parasite reservoir of infection and infectivity.
- Use of intermittent preventive treatment (IPT) during pregnancy (FMOH, 2005; WHO, 2010; Bardají *et al.,* 2012)

Whichever treatment policy followed in the course of treatment the key factors to be watched out for in the drug, is the toxicity, safety, efficacy, readily availability, inexpensive and simple to use treatment regimen (Fernando *et al.,* 2011).

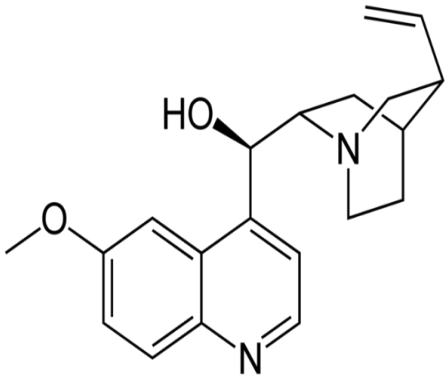
Antimalarial drugs can be termed as follows depending on the site of action:

- Schizontocide (blood Schizontocide) act on the asexual erythrocytic forms of all species of *Plasmodium falsiparium* and are of interest for use to achieve clinical or suppressive cure. Examples include pyrimethamine, sulphonamides and sulphones.
- Gametocytocides destroy sexual erythrocytic forms of plasmodium and thus prevent transimission of malaria to the mosquito. Examples include primaquine, chloroquine, mepacrine, quinine.
- Sporontocides, inhibit the formation of malarial oocysts and sporozoites in infected mosquitos. Example includes proguanil.

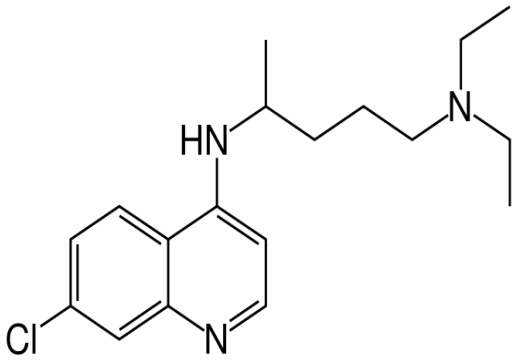
  (Enayati and Hemingway, 2010; Parija, 2011).

Pelletier and Caventou, in 1820 isolated Quinine **(40)** (Croteau *et al.*, 2000), an aminoquinoline alkaloid isolated from the bark of *Cinchona* species (Rubiaceae). It is one of the oldest and most important antimalarial drugs that has ever been used and is still relevant till date. For almost three centuries, this alkaloid was the sole active principle effective against *Plamodium falciparum* in tropical medicine. It is the lead responsible for the development of synthetic antimalarial drugs belonging to the classes of 4- and 8-aminoquinolines, such as chloroquine and primaquine, among others. Quinine **(40)** toxicity and short supply during the first and second world war spurred the research into the synthesis of analogues of quinine giving rise to the synthesis of chloroquine **(41)** in 1946. Early presumptive treatment of febrile illness with chloroquine **(41)** was the mainstay of malaria control in Nigeria until the immergence or indication of *P. falciparum* resistance to this drug. Not only was the drug very effect until resistance to it started, it was easily accessed drug and also at very affordable cost. Based on the evidence of unacceptable high level of reported resistance and upon the recommendation of the

WHO the first line of drug for the treatment of malaria was changed from chloroquine **(41)** to artemisinin-based combination therapy (ACT) officially (Saxena *et al.*, 2003).

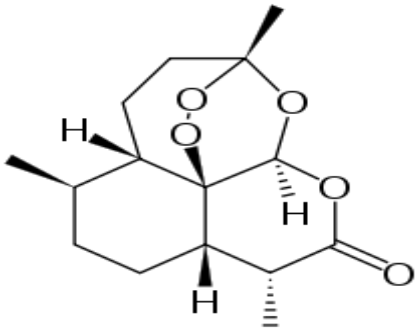


Quinine (40)

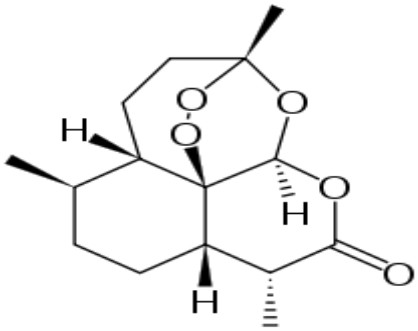


Chloroquine (41)

After several years of incursion in the synthetic drugs, another botanical derivative, artemisinin **(42)**, isolated from Chinese medicinal herb *Artemisia annua* was found to have a powerful antiplasmodial activity. Artemisinin **(42)** derivatives are now recommended by the World Health Organization worldwide (Adebajo *et al.*, 2014), in combination with other drugs, such as lumefantrine, amodiaquine, mefloquine, sulphadoxine-pyrimethamine (SP), as the first-line treatment of malaria.



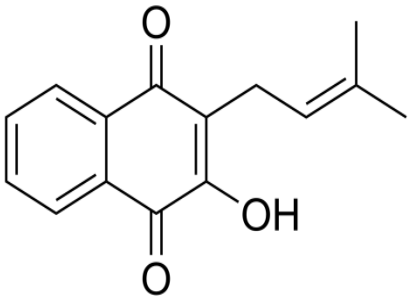
Artemisinin (42)



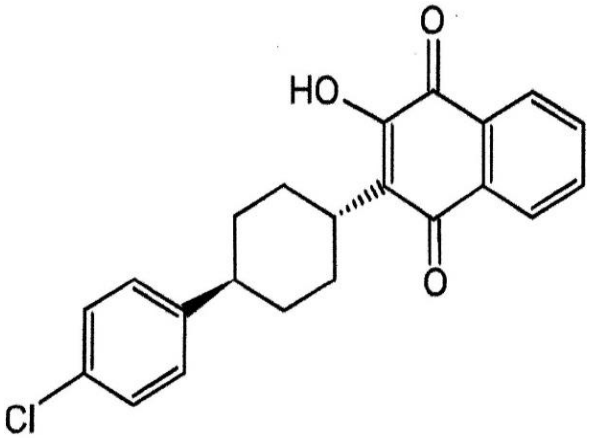
Artemether (43)

The new synthetic antimalarial drug atovaquone **(45)** (2-alkyl-3-hydroxynaphthoquinone compound) is an analogue of lapachol **(44)** (a prenylnaphthoquinone) from the *Tabebuia* species

(Bignoniaceae). The discovery of this drug provided a novel lead for antimalarials that resulted in the development of atovaquone **(45)**. When used in combination with proguanil provides an effective treatment for malaria (Looareesuwan *et al*., 1999; McKeage and Scott 2003)

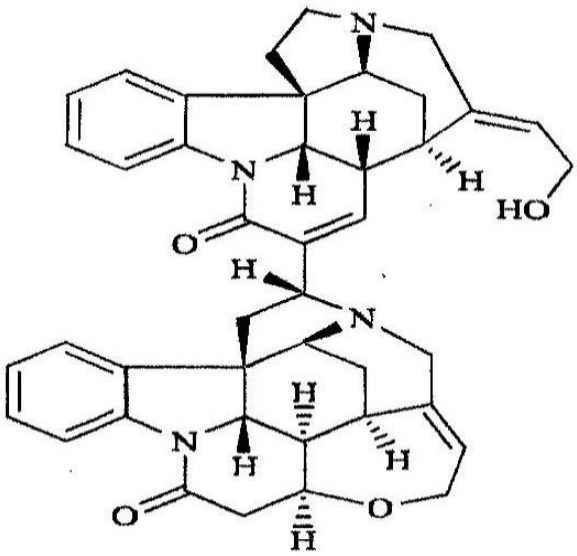


Lapachol (44)

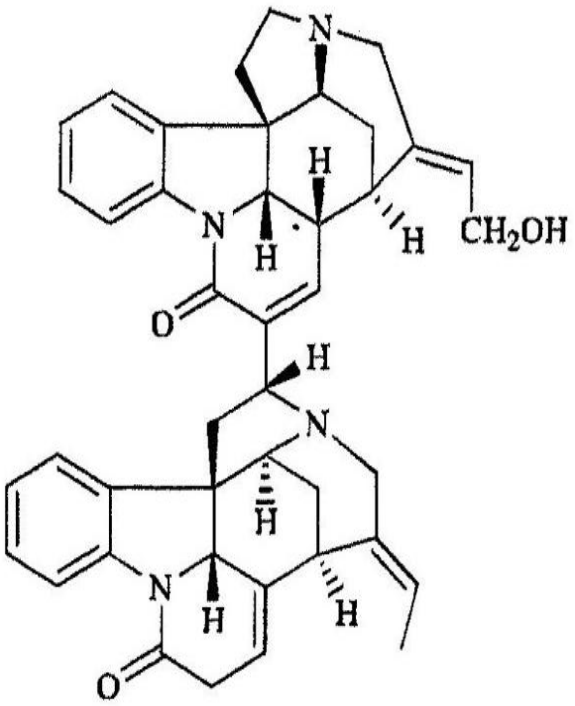


Atovaquone (45)

In alternative medicine, several plants are utilized in traditional medicine for the treatment of malaria and/or fever. Several studies have been done on some of these plants to evaluate their efficacy, dosage range, toxicity and renal clearance. In some studies a good number of pure compounds have been isolated having from very potent to weak antiplasmidial activities.



Strychnoguncine



18-Hydroxyisogucine

Structures of Indolomonoterpenoid alkaloids from *Strychnos spp* with selective antiplasmodial activity (Frederich *et al.,* 2002, Frederich *et al.,* 2003).

Several studies have been undertaken to evaluate not only the inhibitory effects of various plant extracts on *P. falciparum* (Wanyoike and Chhabra 2004) using *in vitro* culture, but also *in vivo* anti-malarial properties on *Plasmodium berghei*-infected mice (Andrade-Neto *et al.,* 2003; Sudhanshu-Saxena and Jain, 2003).

In malaria therapy, 11 drugs out of the antimalarials included in WHO therapeutic schemes for malaria treatment are natural products or their analogues or were design-based on the pharmacophores from natural products (Bourdy *et al*., 2008).

2.4.7 Mechanism of action

There is no one drug binding site with respect to malaria: different anti-malarial drugs have different modes of action, and not all are well described yet.

The exalt mechanism of action for most antimalarial agents are still heavily debated, however four mechanisms are regularly suggested:

- Disruption of parasite mitochondrial function
- Modulation of host immune function
- Interference with parasite transport proteins
- Haem detoxification

(Golenser *et al.,* 2006)

Haem detoxification: Intra-erythrocytic stages of malaria parasites consume and degrade huge quantities of hemoglobin in the food vacuole and release large quantities of redox active free heme as a by-product (Egan 2002). Free heme (ferriprotoporphyrin IX) is very toxic, and parasites detoxify free heme by forming hemozoin, mainly through the biocrystallization or biomineralization process. Molecules that inhibit parasite growth through binding to heme are potential antimalarials, and the inhibition of hemozoin formation is considered a valid target for developing new antimalarials. Also, the inhibition of hemozoin formation may develop oxidative stress due to the accumulation of free heme, which can generate highly reactive hydroxyl radical (·OH), and the malaria parasite is susceptible to oxidative stress (Kumar and Bandyopadhyay 2005; Kumar 2007). The parasite supplies antioxidant moieties to the host to avoid the formation of the oxidative stress and also possesses an efficient enzymatic antioxidant defense system including glutathione- and thioredoxin-dependent proteins. Mechanistic and structural work on these enzymes produces redox imbalance status and a basis for targeting the parasite (Wongtrakul *et al.,* 210). Therefore, the enhancement of oxidative stress to the parasite by any means is a promising strategy in developing new antimalarial agents.

The 4-aminoquinoline (chloroquine **(41)**, quinine **(40)**) appears to act by blocking the formation of hemozoin from heme molecules once they are liberated from hemoglobin (Egan, 2001). Antiparasitic effects are presumably engendered by the toxicity of free heme, possibly by disruption of membranes. It is unclear if other chemically related antimalarials act in a similar fashion. Antifolates inhibit the synthesis of folic acid by blocking the dihydrofolate reductase and dihydropteroate synthetase enzymes of the parasite (Golenser *et al*., 2006).

2.4.8 Resistance

Resistance to antimalarial medicines has been widely reported (Alker *et al.*, 2007; Ursing *et al*., 2007; Parija, 2011), particularly rapid spread of resistant malaria parasites to new areas and re-emergence of malaria in areas where the disease had been eradicated. Resistance of *P. falciparum* to previous generations of medicines, such as chloroquine and sulfadoxine-pyrimethamine (SP), became widespread in the 1970s and 1980s, undermining malaria control efforts and reversing gains in child survival. Drug resistant *Plasmodium*, particularly *P. falciparum* represents a major problem for both prophylaxis and clinical treatment of malaria infection (Phyo 2012).

Clinical resistance to the artemisinin **(42)** and its combinations is currently being reported, suggesting that *P. falciparum* parasites have already developed the ability to grow in the presence of these antimalaria agents, leaving little or no alternative for malaria treatment. This poses a potentially dangerous and severe scenario, if the resistance spreads to endemic areas in Africa (Porter-Kelley 2010; Aung 2012) since, no other effective antimalarial treatments are in sight. ACTs (Artemisinin Combinations Therapies) are World Health Organization identified most effective treatment (Wichmann *et al.*, 2004; Wells, 2010) for uncomplicated malaria infection.

To survive inside the human body, *P. falciparum* has developed several mechanisms through which it may escape the host immune system as well as antimalarial agents. While these survival strategies provide temporary parasitization, they often cause death to their host in the end. The most notorious survival mechanism of the malaria parasite is its ability to undergo almost unlimited antigenic variation through changing the antigens on the infected erythrocyte surface. Some of these erythrocyte antigens are specific receptor-adhesive ligands mediating adhesion of the infected erythrocyte in the intravascular microenvironments, especially the post-capillary regions. In addition, by sequestration, the parasite escapes nonspecific clearance in the spleen, which may improve its ability to reinvade and proliferate. This inherent property results in significant challenges to vaccine design and rapid development of multi-drug resistant strains of *Plasmodium*.

Vector control has been the buck of malaria control success story. This control is highly dependent on the use of pyrethroids (natural product), which are the only class of insecticides currently recommended for insecticide-treated net (ITNs) or long-lasting insecticidal nets (LLINs). Resistance to the pyrethroids and the other three classes of insecticides used for public health has been detected. The efficacy of transmission control by means of insecticide-treated nets and indoor residual spraying is thus challenged due to the increasing resistance to insecticides among the different species of mosquitoes, particularly in endemic areas such as Africa (Ranson *et al*., 2011). The development of new, alternative insecticides that are safe to human is a high priority, particularly for bed nets.

There is currently no effective vaccine against malaria. Some promising preliminary results have been seen, but no solution to this issue is expected over the next few years (Schwartz *et al*., 2012). Malaria control is becoming totally dependent on pharmacological treatments.

2.4.9 The health and economic burden of Malaria in Africa

The "direct" and "indirect" burden of malaria is based on its morbidity, mortality effect on human race and the economic effect on human development. Every year, about 300-500 new clinical attacks are reported. Of these, 2-3 million are severe (considering cerebral malaria and clinical death rate of over 1 million) which result in loss of school attendance, and lowered productivity at work. In some heavy-burden countries within the Africa continent, the disease accounts for:

- up to 40% of public health expenditures;
- 30% to 50% of inpatient hospital admissions;
- up to 60% of outpatient health clinic visits.
- Lose in man-hour to the nation and death

It has been reported to be a leading cause of disease and death among children less than five years, pregnant women and non-immune travellers/immigrants. Children under 5 in are the major at risk group in malarious regions of Africa. Malaria in pregnancy accounts for about 25% of cases of severe attack which result in 10 to 20% low birth weight in developing world. The Ministry of Health in Nigeria has reported in April 2004 that malaria is responsible for one out of ten deaths in pregnant women and has caused the Federal Government of Nigeria over one billion Naira annually in treating malaria (Odugbemi, 2007).

Africa contributes approximately 59% of the total global burden of clinical malaria and about 74% of the clinical *P. falciparum* burden (Korenromp 2005). In terms of mortality, Africa contributes an estimated 90% of the global mortality burden (WHO, 2014). Access to clinical care is difficult especially in the rural settings that commonly have very intense malaria transmission. Malaria is one of the health problems that increase the work load for health workers.

2.5 ANTIOXIDANTS

Free-radicals are generated in living organisms as part of the body's normal metabolic process. Increased radical activity and damage to the system can be as a result of chemical mobilization of fat stores under various conditions such as lactation, exercise, fever, infection and even fasting. Adrenalin and noradrenalin are stress hormones secreted by the adrenal glands under conditions of continuing and excessive emotional stress and are metabolized into simpler, albeit, free radical molecules.

These generated free radicals are unstable molecules that include the hydrogen atom, nitric oxide (NO) and molecular oxygen (O_2) and in an attempt to stabilize, they attack other molecules in the body potentially leading to cell damage and triggering the formation of another free radical resulting in a chain reaction. Considerable evidence have accumulated to implicate cellular damage arising from reactive oxygen species (ROS), at least in part, in human diseases such as neurodegenerative disorders (e.g. alzeimer disease, parkinson disease, multiple schlerosis, down's syndrome), inflammation, viral infections, autoimmune pathologies, and digestive system disorders such as gastrointestinal inflammation and ulcer (Repetto and Llesuy, 2002; Odukoya *et al.*, 2005).

Antioxidants are compounds that help to inhibit the many oxidation reactions caused by free radicals such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite thereby preventing or delaying damage to the cells and tissues (Odukoya *et al.*, 2005). The antioxidant capacity of compounds has been related to the prevention of several diseases including cancer, coronary heart diseases, inflammatory disorders, neurological degeneration, and aging (Pandey and Rizvi 2009) .

Reactive oxygen species (ROS), generated during malarial infection have been implicated in the induction of oxidative stress. Thus, as the severity of the malarial infection increases, more reactive oxygen species (ROS) are generated and infection induced oxidative stress also increases. This induces host tissue damage and at the same time kills the *Plasmodium* parasites. The body system in its compensatory manner tries to prevent the tissue damage, initiates activity to circumvent biological injury caused by the oxidative stress. It does this by conversion of unstable free radicals into H_2O_2 (hydrogen peroxide) by the antioxidant enzyme superoxide dismutase (SOD). The converted H_2O_2 are then removed by the catalase and the glutathione systems. Unlike the host, the parasite is not efficient in circumventing the oxidative stress. The level of parasitaemia in the body system determines the SOD inhibition rate because of the positive correlation between SOD and parasitaemia level (Asmah *et al.*, 2012).

Polyphenolic compounds are among the interesting antioxidant compounds isolated previously from natural product resources, including micro- and macroalgae. At least 8,000 different different bioactive compounds are considered to be polyphenols (Pandey and Rizvi, 2009).

In addition to polyphenolic compounds are other interesting antioxidants, such as carotenoids, and also found in natural product resources.

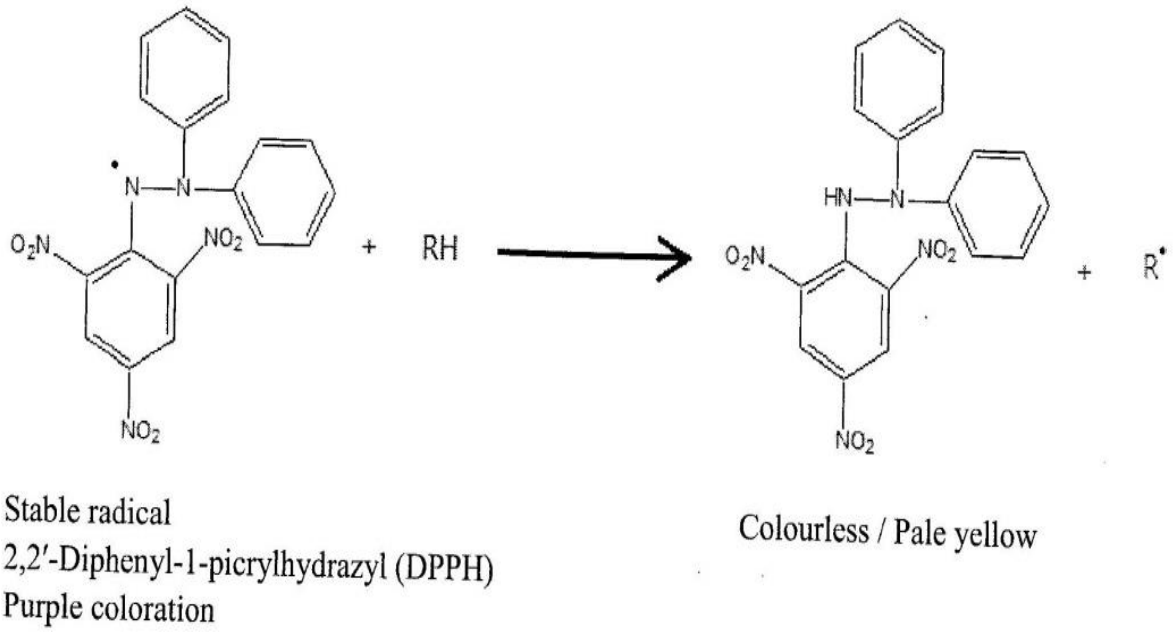
It is known that plant accumulate antioxidant chemicals as secondary metabolites through evolution as a natural means of surviving in a hostile environment (Manach *et al*., 2004).

2.5.1 Dipheny-1-picryl hydrazyl (DPPH) radical scavenging activity

Scavenging of the stable 2,2-dipheny-1-picryl hydrazyl (DPPH) radical by suspected antioxidants is a widely used method to estimate and compare antioxidant activities in a relatively short period of time compared to other more extensive methods. The ability of antioxidants to scavenge DPPH radical is thought to be related to their hydrogen donating abilities (Kosar *et al*., 2006).

DPPH can generate stable free radicals in ethanolic solution. It has a dark purple colour in methanol absorbing light at 517 nm. When a suspected antioxidant is added to this DPPH solution, the purple colour may decolourize. Since decrease in absorbance at 517 nm is directly proportional to the antioxidant potency of the compounds, absorbance can be measured by following the colour of the sample at this wave length (Kosar *et al*., 2006).

The scavenging ability of extracts/ compounds can be measured at various concentrations and compared with a known standard antioxidant. Based on the concentration of the extract/compounds needed to reduce the absorbance at 530 nm, an IC_{50} value can be estimated.



DPPH assay for the detection of radical scavenger.

2.6 PURIFICATION, ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM PLANT EXTRACTS

2.6.1 Purification and isolation of natural products

Natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity (Cos *et al.*, 2006).

The work or investigation in this area of science-natural products chemistry began with the work of Serturner, who first isolated morphine from opium. Extraction is the crucial first step in natural product isolation because it is necessary to extract/pull out the desired chemical components from the plant materials for further separation and characterization. The basic operation step of grinding is to obtain a homogenous sample and improving the kinetics of analytic extraction and also increasing the contact of sample surface with the solvent system. During extraction and isolation processes the potential active constituents are carefully protected to avoid or minimize lost, distorted or destruction during the preparation of the extract from plant samples or during isolation procedure The selection of solvent system largely depends on the specific nature of the bioactive compound being targeted. Different solvent systems are available to extract the bioactive compound from natural products. The extraction of hydrophilic compounds uses polar solvents, such as methanol, ethanol or ethyl-acetate. For extraction of more lipophilic compounds, dichloromethane or a mixture of dichloromethane/methanol in ratio of 1:1 is used.

The analysis of bioactive compounds present in plant extracts involves the applications of common phytochemical screening assays, chromatographic techniques such as HPLC and TLC as well as characterization and elucidation of the compound(s) structure(s). In recent years new

extraction techniques with significant advantages over conventional methods have been developed for extracting analytes from solid matrices. The advantages include, reduction in organic solvent consumption, reduction in sample degradation, reduction of extraction and clean-up time or even elimination of additional sample clean-up and concentration steps, improvement in extraction efficiency, selectivity, and/or kinetics, ease of automation, etc (Huie 2002; Kerem *et al*., 2005). These recent extraction techniques include supercritical fluid extraction (SFE), pressurised liquid extraction (PLE), microwave-assisted extraction (MAE), solid-phase microextraction (SPME), ultrasound- assisted extraction (UAE), superheated liquid extraction, and extraction with supercritical or subcritical water. Most of these methods have similar pros and cons with regard to solvent volume, extraction time and extraction efficiency.

Plant extracts usually occur as a combination of various types of bioactive compounds or phytochemicals with different polarities. Therefore their separation still possesses a big challenge for the process of isolation, identification and characterization of bioactive compounds. It is a common practice in isolation of these bioactive compounds that a number of different separation techniques such as TLC, column chromatography, flash chromatography, sephadex chromatography and HPLC, could be used to obtain pure compounds. The pure compounds are then used for the determination of structure and biological activity. These techniques are not only being restricted to plant sources but they are also being applied to microbial and even fungal sources of metabolites. Both commercial and non-commercial/academic sources and supply chains of any pure natural product compounds can be traced back ultimately to crude natural materials (extracts) that had gone through various purification steps.

2.6.2 Solvent- solvent partition/Fractionation

Some extraction techniques involve partition between two immiscible liquids. It involves the selective transfer of plant phytoconstituents/substance from one liquid phase to another. It is a first step towards isolation of plant components. This separation is governed by the principle of "partition coefficient" and it is independent of the concentration of the solute. In saponin separation from plant material, a single butanol-water partition step is often suffices to concentrate the saponin (s) in the butanol fraction, thereby providing a preliminary clean-up step (Hostettman and Marston 1995). The organic layer (butanol fraction) is then chromatographed for further clean-up exercised. This is the method used in the isolation of triterpene glycoside from *tetrapleura tetraptera*, a plant from Nigeria with molluscicidal activity (Hostettmann and Marston, 1995).

2.6.3 Chromatographic Methods

Chromatography is the method of choice in handling the problem of purification and isolation of compounds of interest from a complex natural mixture. The name chromatography was firstly used in 1906 by a botanist called Tswett; who worked to separate coloured plant pigments. Chromatographic methods are very flexible due to their separation principles.

Adsorption chromatography or liquid– solid chromatography: The sample components are adsorbed on the surface of the adsorbent and displace the initially loosely adsorbed solvent molecules. The stationary phase (the adsorbent) is often a polar solid (almost always silica or alumina), that is used in connection with a non- polar liquid. The (polar) functional groups of the sample components are then easily attracted by the adsorbent surface and displace the non - polar solute molecules.

Partition chromatography: This can be divided into liquid – liquid chromatography (LLC) and chromatography on chemically bonded phases . It involves two immiscible liquids, one as the mobile phase and the other being fixed on a solid support as the stationary phase.

Normal phase (NP) chromatography involves the use of polar stationary phase (usually on a silica or alumina support), the stationary phase is hydrophilic and therefore has a strong affinity for hydrophilic molecules in the mobile phase. Thus, the hydrophilic molecules in the mobile phase tend to bind (or "adsorb") to the column, while the hydrophobic molecules pass through the column and are eluted first. The term reversed-phase describes the chromatography mode that is just the opposite of normal phase, namely the use of a polar mobile phase and a non-polar (hydrophobic) stationary phase (Mehta, 2012).

2.6.3.1 Thin Layer Chromatography (TLC)

TLC is a simple, quick, and inexpensive procedure that gives the researcher a quick answer as to how many possible components/compounds are in a mixture and the degree of purity of the compounds. Very little quantity of the test sample is required. TLC is also used to support the identity of a compound in a mixture when the R_f of a compound is compared with the R_f of a known compound. Separations in TLC involve distributing a mixture of two or more substances between a stationary phase and a mobile phase (Gibbons 2006). The stationary phase is a thin layer of adsorbent (usually silica gel or alumina) coated on a plate. The mobile phase is a developing liquid which travels up the stationary phase, carrying the samples with it. Components of the samples will separate on the stationary phase according to how much they adsorb on the stationary phase versus how much they dissolve in the mobile phase.

The advantage of TLC is that the samples do not have to undergo the extensive clean-up steps, and the ability to detect a wide range of compounds, using reactive spray reagents. Non-

destructive detection (fluorescent indicators in the plates, examination under a UV lamp) also makes it possible for purified samples to be scraped off the plate and be analyzed by other techniques (Sarker *et al.,* 2006; Pyka 2014). However, there is always the possibility of extraction of some impurities from the TLC plates such as the binders, the fluorescent indicators etc which are not shown in UV. So it is highly recommended to do a final purification step by gel filtration using sephadex LH-20. Different functional groups can be detected by different reagents (Jeon *et al.,* 2015) such as;

Alkaloids: Dragendorff's reagent

Cardiac glycosides: Antimony trichloride

Sugar: Aniline phthalate

Amino acids: Ninhydrin

TLC allows also the quantitative determinations, either by the measurement of the density of spot obtained with a suitable spray reagent using densitometer or by scraping the relevant band of TLC separation off the plates. The isolated compound can then be eluted off the scraped band using appropriate solvent and measuring the UV absorbance. The quantity of ginsenoside in *Panax ginseng* spray dried extract was performed at 530 nm after spraying the TLC plates with vanillin-sulphuric acid and confirmatory determination was done using HPLC (Kim *et al.,* 2013).

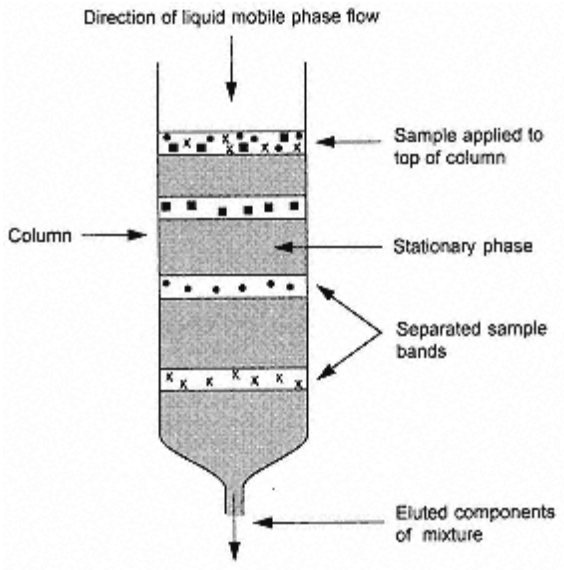
2.6.3.2 Column Chromatography (CC)

CC consists of a column of particulate material such as silica or alumina that has a solvent passed through it at atmospheric, medium or low pressure. The separation can be liquid/solid (adsorption) or liquid/liquid (partition). The columns are usually glass or plastic with sinter frits to hold the packing. Most systems rely on gravity to push the solvent through, but medium

pressure pumps are commonly used in flash CC. The sample is dissolved in solvent and applied to the front of the column (wet packing), or alternatively adsorbed on a coarse silica gel (dry packing/slurry). The solvent elutes the sample through the column, allowing the components to separate. The solvent is usually changed stepwise, and fractions are collected according to the separation required, with the eluting products usually monitored by TLC (Guiochon, 2001; Jeon *et al.*, 2015).

Open column chromatography is often the first fractionation step for most crude extract isolation process. In general it has a poor resolution result, materials are lost due to irreversible adsorption on the silica gel and the long length of time needed to perform the separation. Chloroform-methanol-water as eluent is commonly practiced here and allows the application of gradient elution technique. Bidesmosidic saponin was obtained by silica gel column chromatography with the solvent system acetone-n-propanol-water (35: 35: 5) (Borel *et al.*, 1987). Initial fractionation of methanol extract of *Tetrapleura tetraptera* (Leguminosae) fruits was by this method with application of a gradient elution of chloroform-methanol-water (85: 11: 1 -> 70: 30: 5 -> 50: 50: 0) (Maillard *et al.*, 1989). A complex mixture of triterpene glycosides, 2,9,16-trihydoxypalmitic acid glycosides of polygalacic acid from *Crocosmia crocosmiiflora* (Iridaceae) were isolated by strategy which involved open-column chromatography on silica gel, eluting with n-butanol-ethanol-water (5: 1: 4, upper layer) and chloroform-methanol-water (60: 29: 6). Final purification was by HPLC (Asada *et al.*, 1989a). In the separation process of dammarane glycosides actinostemmosides A-D from *Actinostemma lobatum* (Cucurbitaceae) open column chromatography was employed at various stages (Iwamoto *et al.*, 1987).

The use of reverse phase (RP) sorbent and sephadex are currently being employed in open-column chromatograph but mostly after an initial silica gel separation step or other separation technique such as drop counter current chromatography (DCCC).



Open column chromatography showing the stationary phase (solid packing material) and the mobile phase (liquid)

The limitations of column chromatography such as slow separation and irreversible adsorption of sample to solute had led to better improved column techniques like the flash chromatograph and vacuum liquid chromatography.

2.6.3.3 Vacuum liquid chromatography (VLC)

The technique used here is simply reduction of pressure to increase the flow rate of the mobile phase through a short bed of adsorbent. As opposed to flash chromatography, the column is allowed to run dry after each fraction is collected. This is similar to preparative TLC where plates can be dried after a run and then re-eluted. Different chromatographic supports have been employed in VLC: silica gel (both normal and reversed-phase), Al_2O_3 (aluminium oxide), CN

(acetonitrile), DIOL (polyols) and polyamide. The most popular eluent is hexane with increasing proportions of ethyl acetate. VLC is mainly used for the fractionation of natural products prior to other separation steps such as RPC (reverse phase chromatography), and HPLC (high pressure liquid chromatography) (Hostettmann *et al*., 1998; Reid and Sarker 2006; Otto 2008).

Some saponin active constituents of *Schefflera impressa* (Araliaceae) were isolated through the application of this method of isolation (Srivastava and Jain, 1989). Three active constituents of *Collinsolnia canadensis* root (Lamiacea) were isolated by the process which involved VLC on silica gel as the only chromatographic tool (Joshi *et al.*, 1992).

2.6.3.4 High Pressure Liquid Chromatography (HPLC)

The principle of HPLC, a chromatographic technique, in simplified words the application of pressure at the inlet of a liquid chromatographic column to improve on its resolution performance. HPLC is employed in the separation of a mixture of compounds. It is used in phytochemical and analytical chemistry to identify, quantify and purify the individual components of the mixture (Piana *et al.*, 2013). Currently, due to the improved efficiency of the columns, this technique is gaining popularity among various analytical techniques as the main choice for fingerprinting study for the identification and quality control of herbal plants (Fan *et al.*, 2006).

A long column is not needed because separation in HPLC is very efficient. Columns are usually 10 –30 cm long, with an internal diameter of 4 mm, having large surface area due to fine packing that can be used. Separation is by different components of the sample being carried forward at different rates by the moving liquid phase, due to their differing interactions with the stationary and mobile phases. Flow rate and column dimensions can be adjusted to minimize

band broadening. The required pressures are supplied by pumps that could withstand the involved chemicals. In addition to the normal phase columns, (non-polar solvent and polar surface such as silica), there are reverse phase (RP) columns as well. The latter, normally, involves the use of a polar solvent (water, methanol, acetonitrile etc.) and a non-polar surface. RP HPLC is the method of choice for larger non-volatile molecules. Reversed-phase chromatography is the most commonly used separation technique in HPLC due to its broad application range. The reasons for this include the simplicity, versatility, and scope of the reversed-phase method as it is able to handle compounds of a diverse polarity and molecular mass (Prathap *et al.,* 2013). Evaporative light-scattering detector is mostly used here as a universal detector (Guiochon, 2001). This separation technique lingers on speed and high resolution, but over loading of the column or over speeding of the elution affect the degree of resolution (Hostellmann *et al.,* 1998).

The Detector: The HPLC is usually linked to a spectrometer (e.g. ultra violet or mass spectrometry) that acts as the detector. The amounts passing through the column are small, so solutes are analyzed as they leave the column. The length of time it takes for a compound to reach the detector allows the component to be identified. A chromatogram is obtained for the sample, which aid in characterization of the isolated components.

Thus preparative HPLC can be useful for obtaining ultrapure standard compounds, labeled compounds, trace impurities, decomposition products of pharmaceuticals, metabolites and higher molecular weight bio-materials (example, peptides).

The quantitative determination of the ginsenosides by HPLC is a currently preferred method, which gives better results than those obtained by colourimetric, GC and TLC-fluorimetric techniques. Analytical HPLC is sometimes capable of resolving mixtures of closely related

compounds. Miyahara *et al* (1983) reported a successful separation of glycosides of rhodeassapogenin, its 25R-epimer (isorhodeasapogenin) and the 25(27)-dehydro-derivative (convallamarogenin) by HPLC, using a TSK-GEL LS-410 C-18 column at low temperature (0°C), with 90% methanol at 0.5 ml/min and a Radial Pak C-18 column with 80% methanol at 1 ml/min (Hostettmann and Marston, 1995).

Semi-preparative RP-HPLC was used to fractionate and isolate the extract of *Radix imperatoriae* (the root of masterwort), the obtained fractions were analyzed by LC-UV-ESI-MS and 600 MHz microcoil 1 H NMR spectroscopy, to obtain oxypeucedanin hydrate, an antimicrobial active compounds (Gökay *et al.*, 2010).

The ambient extraction and HPLC analysis method were applied to six lots of goldenseal root powder from three different vendors to determine their alkaloid content. The HPLC analysis was done with a Zorbax Rapid Resolution Eclipse XDB-C18 column, which provides high resolution and excellent peak shape of six alkaloids in 15 minutes. This analysis provides reliable quantitative results of the alkaloids in goldenseal, including berberine and hydrastine.

2.6.3.5 Gel permeation chromatography (Size Exclusion Chromatography)

This is a clean-up chromatographic technique that is becoming increasingly popular. Gel Chromatography or sometimes referred to as Size Exclusion Chromatography (SEC). It is a non-destructive method for the recovery of a high quantity compound or fraction, or extract. It is generally a low-resolution chromatography technique and thus it is often reserved for the final, "polishing" step of purification. This technique employs cross-linked dextran which upon contact with a suitable solvent swells up to form a gel matrix and the ability of molecules to move through a column of gel that has pores of clearly defined sizes. The larger molecules

cannot enter the pores, and therefore, they move faster through the column and elute first. Slightly smaller molecules can enter some pores, and so take longer to elute, while small molecules can be delayed further.

The advantage of the technique is simplicity, isocratic, and large molecules rapidly elute. However, the columns are expensive and sensitive to contamination; consequently they are mainly used in applications where alternative separation techniques are not available, and samples are fairly clean. The commonly used gel in natural products lab is sephadex LH-20. It is a method or choice for large molecules such as proteins, polypeptides carbohydrates and to separate chlorophyll from compounds of interest, where usually chlorophyll elutes first (Guiochon 2001; Reid and Sarker 2006; Bucar 2013).

2.6.3.6 Gas Chromatography (GC)

GC is the use of a carrier gas to convey the sample in a vapour state through a narrow column made from usually fused silica tubes (0.1 to 0.3mm ID) that have refined stationary phase films (0.1 to 5 μm) bound to the surface and cross linked to increase thermal stability. The column is installed in an oven that has temperature control, and the column can be slowly heated up to 350-450 °C starting from ambient temperature to provide separation of a wide range of compounds. The carrier gas is usually hydrogen or helium under pressure, and the eluting compounds can be detected several ways. Once GC has separated a mixture, the components can be identified using known retention times. For unknown compounds the solutes are collected individually and analysed using methods such as, e.g. flames (flame ionization detector), by changes in properties of the carrier (thermal conductivity detector), or by mass spectrometry. The availability of "universal" detectors such as the FID (flame ionisation

detection) and MS (mass spectrometer), makes GC the appropriate tool in the investigation of essential oils.

For each compound in a mixture one peak is observed on the chromatogram. In the particular set of operating conditions relating to the column, the retention time will increase with the size and polarity of the compound. To find the concentration of a particular compound, the peak height should be measured. GC is used to analyse blood samples for the presence of alcohol. The availability of modified cyclodextrins as stationary phases made it possible to separate enatiomers, the determination of enatiomeric ratios and absolute configurations. However, GC is restricted to molecules (or derivatives) that are sufficiently stable and volatile to pass through the GC system intact at the operating temperatures (Niessen, 2001; König, and Hochmuth, 2004).

The alkaloid rich *Epipremnum aureum* (Linden and Andre) Bunting, family, Araceae, possesses many pharmacological activities such as antibacterial, antifungal, calming effect and relaxation (Srivastava *et al.*, 2011; Meshram and Srivastava, 2014). Recent advances in the use of GC coupled to MS have allowed a chemically guided isolation and characterization of twenty six bioactive alkaloids from *Epipremnum aureum*. GC-MS data were recorded in a GCMS-2010 Shimadzu instrument operating in EI mode at 70ev. A Restek-5MS column (30m x 0.25mm x 0.25µm) was used. The oven temperature program was 1000 to 2500 C at 50 C min-1 and held for 5 min at 2500 C and from 2500 C to 2800 C at 100 C min-1 and held for 10 min at 2800 C. The identification of the alkaloids was confirmed by comparing of their fragmentation pattern, m/z value, and retention time with those of authentic compounds.

2.6.3.7 Counter-current chromatography

Counter-current chromatography (CCC) is a type of liquid-liquid chromatography, where both the stationary and mobile phases are liquids. The operating principle of CCC equipment requires a column consisting of an open tube coiled around a bobbin. The bobbin is rotated in a double-axis gyratory motion (a cardioid), which causes a variable gravity (G) field to act on (an example of HPCCC system) the column during each rotation. This motion causes the column to see one partitioning step per revolution and components of the sample separate in the column due to their partitioning coefficient between the two immiscible liquid phases used. There are many types of CCC available today. These include HSCCC (High Speed CCC), droplet counter-current chromatography (DCCC), rotation locular counter-current chromatography (RLCCC) and HPCCC (High Performance CCC). HPCCC is the latest and best performing version of the instrumentation available currently (Reid and Sarker, 2006; Otto 2008).

2.6.4 Fractional Crystallization

Crystallization is an old but an important method for the purification of compounds from the mixture. This purification method is based on differences in solubility of the components of a mixture in a particular solvent. Crystallization mostly depends upon the inherent character of the compound which forms the crystals at the point of super- saturation in solvent in which it is soluble.

Compounds such as; sugars, glycosides, steroids, triterpenoids, flavonoids etc exhibit crystalline nature with certain exceptions (Florence *et al.*, 2006). This is a very valuable method for resolution of often otherwise intractable mixtures.

The most commonly used procedure for the purification of a solid material by recrystallisation from a solution involves the following steps:

- The impure material is dissolved in a suitable solvent, by shaking or vigorous stirring to form a near-saturated solution.
- The solution is then set aside to cool to room temperature or left in the refrigerator so that the dissolved substance slowly crystallises out.
- The crystals are separated from the mother liquor, either by centrifuging or by filtering, under suction, through a sintered glass, a Hirsch or a Buchner funnel. Usually, centrifuging is much preferred because of the much greater ease and efficiency of separating crystals and mother liquor, and also because of the saving of time and effort, particularly when very small crystals are formed or when there is entrainment of solvent.
- The crystals are washed free from mother liquor with a little fresh cold solvent and then dried.

A greater degree of purity is also to be expected if the crystallization and recrystallization process is repeated several times, especially if different solvents are used. The advantage of several crystallizations from different solvents lies in the fact that the material sought, and its impurities are unlikely to have similar solubility as solvents and temperatures are varied.

After crystallization the crystals can be removed from the mother liquor by filtering with sintered glass at the pump and perhaps air-drying by suction. Another means of drying the crystals is by heating in an oven above the boiling point of the solvent (but below their melting

point), followed by cooling in a desiccator. Where this treatment is in-advisable, it is still often possible to heat to a lower temperature under reduced pressure, for example in an Abderhalden

pistol. In cases where heating above room temperature cannot be used, drying must be carried out in a vacuum desiccator containing suitable absorbants (Florence *et al.*, 2006; Reid and Sarker 2006).

Where water is the solvent to be removed, the safest way is to dry in a vacuum desiccator over concentrated sulphuric acid, phosphorus pentoxide, silica gel, calcium chloride, or some other desiccant. Where substances are stable in air and melt above l00^{o}C drying in an air oven may be adequate. In other cases, use of an Abderhalden pistol may be satisfactory (Florence *et al.*, 2006).

2.6.5 Other separation methods

Other separation methods described by Hostettmann1 and Marston 1995, are centrifugal thin-layer chromatography (CTLC), overpressure layer chromatography (OPLC), flash chromatography (FC), liquid chromatography (low-pressure liquid chromatography (LPLC), medium-pressure liquid chromatography (MPLC), high-pressure liquid chromatography (HPLC), and centrifugal partition chromatography (CPC).

Evaluation of recent literature shows that CTLC, OPLC, RLCC, and DCCC have been rarely used since 2000. FC is still used often but mainly as part of a multi-step isolation procedure. The main separation technologies used in recent years are methods of liquid chromatography such as MPLC and semi-preparative HPLC, as well as CPC, mainly as high-speed counter-

current chromatography (HSCCC) or high-performance centrifugal partition chromatography (HPCPC). Multi-step chromatographic operations have mostly been used, e.g. a combination of FC for pre-purification and semi-preparative HPLC for final purification.

In spite of the high resolution attainable on these techniques, a one (1) step separation step is not always possible. A pre-fractionation step may be needed and also a clean-up step may also be needed.

2.7 CHARACTERIZATION AND STRUCTURE ELUCIDATION OF BIOACTIVE COMPOUNDS

2.7.1 Spectroscopic Techniques

Structure elucidation depends on classical spectroscopic techniques such as: Nuclear Magnetic Resonance (NMR) 1-D and 2-D Proton NMR as well as C-13 NMR, Infra-Red (IR), Mass Spectrometry (MS) and X-Ray analysis.

2.7.1.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

Spectroscopy is the study of the interaction of electromagnetic radiation (EMR) with matter. NMR spectroscopy is the study of interaction of radio frequency (R_f) of the EMR with unpaired nuclear spins in an external magnetic field to extract structural information about a given sample. NMR spectroscopy is routinely used by chemists to study chemical structure of molecules using one dimensional technique (1D-NMR) only or in combination with two-dimensional techniques (2D-NMR) for more complicated molecular structures (Hostettmann and Wolfender, 2001). The 1D and 2D NMR involve protons (^{1}H) and carbons (^{13}C).

Figure 4: Equipment for measuring Nuclear Magnetic Resonance (NMR)

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2.7.1.2 One Dimensional NMR

1D-Proton NMR (1H-NMR) and 1D-Carbon NMR (13C-NMR)

Proton NMR is a plot of signals arising from absorption of RF during an NMR experiment by the different protons in a compound under study as a function of frequency (chemical shift). The area under the plots provides information about the number of protons present in the molecule, the position of the signals (the chemical shift) reveals information regarding the chemical and electronic environment of the protons, and the splitting pattern provides information about the number of neighboring (vicinal or geminal) protons.

Carbon NMR is a plot of signals arising from the different carbons as a function of chemical shift. The signals in ^{13}C NMR experiments normally appear as singlets because of the decoupling of the attached protons. Different techniques of recording of the 1D carbon NMR has been developed so that it is possible to differentiate between the various types of carbons such as the primary, secondary, tertiary and quaternary from the 1D ^{13}C NMR plot. The range of the chemical shift values differs between the 1H (normally 0-10) and 13C NMR (normally 0-230) that arises from the two nuclei having different numbers of electrons around their corresponding nuclei as well as different electronic configurations (Kwan and Huang, 2008; Breton and Reynolds, 2013**)**.

2.7.1.3 Two dimensional NMR (2D-NMR)

The 2D-NMR experiments are concerned with structural elucidation of natural products and include the homonuclear ^{1}H, (COSY, NOESY) and the heteronuclear ^{1}H, (HMQC, HMBC).

COSY and NOESY

COSY is a plot that shows coupling among neighbouring protons. It provides information on the connectivity of the different groups within the molecule (Kwan and Huang, 2008; Breton and Reynolds, 2013**)**.

Drawing a straight line from any of the dark spots to each axis, one can see which protons couple with one another and which are therefore attached to neighboring carbons.

2D NOESY is a homonuclear correlation via dipolar coupling; which correlate nuclei through space (distance smaller than 5Å) and enables the assignment of relative configuration of substituents at chiral centers(Kwan and Huang 2008; Breton and Reynolds 2013**)**.

Similar to the COSY, it is possible to see which protons are nearer to each other in space by drawing a straight line from any of the dark spots to each axis of the plot (Hostettmann and Wolfender, 2001).

HMQC and HMBC

The HMQC experiment provides correlation between protons and their attached heteronuclei through the heteronuclear scalar coupling. The spots in the spectrum indicate which protons signal is attached to which carbon. The HMBC experiment detects long range coupling between proton and carbon (two or three bonds away) with great sensitivity. The experiment can be adjusted to detect relatively large coupling constants (4-10 Hz) or smaller. This experiment in conjugation with ^{1}H, ^{1}H-COSY enables the elucidation of the skeleton of the compound under study (Hostettmann and Wolfender, 2001).

2.7.2 Other Spectroscopic Methods

These include the infrared (IR) spectroscopy which offers information relating to the functional groups, and the ultraviolet (UV) spectroscopy which reveals information relating to the presence of sites of unsaturation in the structure. These two methods are becoming less important in structure elucidation of natural products due to the superiority of information obtained from the NMR experiments with much less sample amounts.

2.7.2.1 Gas Chromatography/Mass Spectrometry (GC/MS)

GC equipment can be directly interfaced with rapid scan mass spectrometer of various types. GC and GC/MS are unanimously accepted methods for the analysis of volatile constituents of herbal medicines, due to their sensitivity, stability and high efficiency. Gas chromatography coupled with mass spectrometry (GC/MS) thus, is a powerful technique for the analysis of complex botanical extracts. It provides abundant information for structural elucidation of the compounds when tandem mass spectrometry (MS) is applied. The GC/MS data is used to identify individual fragments to obtain structural information.

In GC/MS a mixture of compounds to be analyzed is initially injected into the GC where the mixture is vaporized in a heated chamber (injector). The gas mixture travels through a GC column carried by a carrier gas, where the compounds become separated as they interact with the stationary phase of the column. The separated compounds then immediately enter the mass spectrometer that generates the mass spectrum of the individual compounds. (Hostettmann and Wolfender, 2001; Guo *et al.,* 2006; Teo *et al.,* 2008).

2.7.2.2 Mass Spectrometry (MS)

MS is an analytical technique that involves generating charged particles (ions) from molecules of the analyte. The generated ions are analyzed to provide information about the molecular weight of the compound and its chemical structure.

2.7.2.3 X-ray Crystallography

X-ray Crystallography is a scientific method used to determine the arrangement of atoms of a crystalline solid in three dimensional space. This technique takes advantage of the interatomic spacing of most crystalline solids by employing them as a diffraction gradient for x-ray light, which has wavelengths on the order of 1 angstrom (10^{-8} cm).

The discovery of X-rays in 1895 enabled scientists to probe crystalline structure at the atomic level. X-ray diffraction has been in use in two main areas, for the fingerprint characterization of crystalline materials and the determination of their structure. Each crystalline solid has its unique characteristic X-ray powder pattern which may be used as a "fingerprint" for its identification. Once the material has been identified, X-ray crystallography may be used to determine its structure, how the atoms pack together in the crystalline state and what the interatomic distance and angle are etc. X-ray diffraction is one of the most important characterization tools used in solid state chemistry and materials science. The size and shape of the unit cell for any compound can easily be determined using X-ray diffraction.

2.8 NIGERIAN MEDICINAL PLANTS USED IN TREATMENT OF MALARIA

Nigeria is enriched with medicinal plants used for various disease states. A lot of such plants are being used for the treatment of malaria. The use of some plant species for malaria treatment runs across all ethnic and cultural groups in the country, for example, *Alstonia boonei* (Apocynaceae) and *Azadirachta indica* (Meliaceae).

Alstonia boonei, a high tree of up to 33 m, belongs to a highly priced medicinal plant species, especially in situations where affordable antimalarial drugs are found ineffective, due to drug-resistant malaria parasites. The plant stem bark or leaves are administered as decoction or "teas" and sometimes as an ingredient in malaria "steam therapy" (Majekodunmi *et al.*, 2008).

Plants of the Meliaceae family are commonly used for malaria treatment in Nigeria, which include *Azadirachta indica*, *Khaya senegalensis* and *Khaya grandifoliola*. *Azadirachta indica* (neem tree) is a commonly used decoction against fever and/or malaria in all the ethnical groups in Nigeria and Africa as a whole. It is an evergreen, fast growing tree, up to 25 m in height and is used in traditional medical practice in form of an aqueous decoction of the leaves, stem bark and root (Obih and Makinde, 1985). It is also used as "steam bath" during the course of malaria treatment. The plant has been exhibited to have high prophylactic, moderate suppressive and a very minimal curative schizonticidal effect in mouse model of malaria (Isah *et al.*, 2003).

Khaya plants are widely used for medicinal purposes in Nigeria. It is gradually becoming uncommon to find an intact *Khaya* tree near homes. The decoctions of the stem barks of *Khaya senegalensis* and *Khaya grandifoliola* are extensively used as antimalarial remedies (Adebayo *et al.*, 2003; Bumah *et al.*, 2005).

Morinda lucida (Rubiaceae) is a moderately coarse wood (9–18 m in height) bearing a dense crown of slender crooked branches. The aerial parts, stem bark or root bark of *Morinda lucida* are widely used in Nigeria for treatment of malaria and other tropical diseases (Avwioro *et al.*, 2005). A seasonal variation in its antimalarial activity has been reported (Adebayo and Krettli, 2011).

The stem bark of *Enantia chlorantha* (Annonaceae), an ornamental tree is used against fever/malaria by traditional medicine practitioners in the South-eastern -forest regions of Nigeria. Other plants used for malaria treatment in Nigeria, include, *Quassia amara* and *Quassia undulata* (Simaroubaceae). Both plants are known to have been used for curative and preventive purposes in malarial control (Adebayo and Krettli, 2011). Aqueous decoction of *Nauclea latifolia* (Rubiaceae) root bark is used locally in treatment of malaria. *Carica papaya* (Caricaceae), commonly referred to as paw paw, is widely grown in the tropics for its edible fruit and also used as a weak decoction of its leaves against malaria. The aqueous extract of the root of *Fagara zanthoxyloides* (Rutaceae) is used for malaria treatment in Nigeria (Odebiyi and Sofowora, 1979).

The antimalarial plants are used in form of monotherapy or are taken together in combined therapies, poly herbal, known as 'Agbo-Iba' in South-west of Nigeria and "Ogwu-Iba" in South-east part of Nigeria. An example of such multi-herbal extract is "Agbo-Iba" made up of *Cajanus cajan* (pigeon pea) leaf, *Euphorbia lateriflora* leaf, *Mangifera indica* leaf and bark, *Cassa alata* leaf, *Cymbopogon giganteus* leaf, *Nauclea latifolia* leaf, and *Uvaria chamae* bark (Nwabuisi, 2002). So many of such poly herbal exist depending on the herbal practitioner recipe.

Some of the medicinal plants used as folkloric medicine in the treatment and control of malaria and malarial fever have been investigated using both *in-vitro* and *in-vivo* models. Examples of the plants that have been reported to exhibit activities *in-vitro* and *in-vivo* include *Khaya grandifoliola* (Agbedahunsi *et al.,* 1998; Bickii *et al.,* 2000), *Azadirachta indica* (Obih and Makinde, 1985; MacKinnon *et al.,* 1997; Isah *et al.,* 2003), *Vernonia amygdalina* (Masaba, 2000; Abosi and Raseroka, 2003), *Momordica balsamina* (Benoit-Vical *et al.,* 2006) and *Picralima nitida* (Okokon *et al.,* 2007a), *Picralima nitida* (Okokon *et al.,* 2007a), *Quassia amara* (Ajaiyeoba *et al.,* 1999; Bertani *et al.,* 2005).

2.8.1 Isolated Compounds Characterized as Antimalarials From Nigerian Medicinal Plants

The active antimalarial principles of some medicinal plant species used in Nigerian folk medicine for malaria treatment have been isolated, of which majority are alkaloids, followed by limonoids. Some of the alkaloids isolated are:

(a) Fagaronine, a benzophenanthridine alkaloid derived from the root extract of *Fagara zanthoxyloides*. It inhibited *Plasmodium falciparum* growth *in vitro* at low IC_{50} (0.018 g/ml) (Kassim *et al.,* 2005).

(b) Palmatine and jatrorrhizine, protoberberine, are alkaloids isolated from *Enantia chlorantha* and are bioactive against malarial parasites (Adebayo and Krettli, 2011)

(c) *Picralima nitida*: indole and dihydroindole alkaloids isolated from the plant include, akuammiline, akuammidine, akuammine, akuammigine, akuammicine, picraline, and alstonine (Ansa-Asamoah *et al.,* 1990). All the alkaloids exhibited different levels of

activities against *Plasmodium falciparum* comparable to chloroquine and quinine (Okunji *et al.*, 2005), and alstonine was the most active.

(d) Five limonoids were isolated from the wood extracts of *Azadirachta indica* plants of which gedunin, and meldenin were the most active against *Plasmodium falciparum* (Joshi *et al.*, 1998). Limonoids active against malaria parasites were isolated from *Khaya grandifoliola* (methylangolensate, 7-deacetylkhivorin, 1-deacetylkhivorin, and 6-acetylswietenolide and gedunin) (Bickii *et al.*, 2000).

(e) Azadirachtin (a tetranortriterpenoid), which occurs in 4–6 g/kg amounts in neem seed kernels inhibits the formation of mobile microgametes in vitro, an activity linked to the presence of a hemiacetal group at C-11 (Jones *et al.*, 1994).

(f) Ursolic acid isolated from the stem bark of *Spathodea campanulata*, suppressed infection and prolonged the survival of mice infected with *Plasmodium berghei* (Amusan *et al.*, 1996).

(g) Anthraquinones from *Morinda lucida*, the most active being damnacanthal; structure–activity studies showed that an aldehyde group at C-2 and a phenolic hydroxy group at C-3 enhanced activity against *Plasmodium falciparum* (Koumaglo *et al.*, 1992; Sittie *et al.*, 1999).

(h) Sesquiterpene lactone, Tagitinin C, present in the leaves of *Tithonia diversifolia* was active against *Plasmodium falciparum* (Goffin *et al.*, 2002).

(i) Ajoene, a metabolite of *Allium sativum*, was active against *Plasmodium berghei* in mice (Perez *et al.*, 1994).

(j) Simalikalactone D was identified to be responsible for the antimalarial activity of *Quassia amara* leaves (Bertani *et al.,* 2006).

Other phytochemicals with antiplasmodial activities in Nigerian folk medicinal plants are:

A novel friedelane triterpenoid named endodesmiadiol, and known compounds friedelin, canophyllol, canophyllal, cerin, morelloflavone, volkensiflavone, 8-deoxygartanin, 3β-acetoxyoleanolic acid and 1,8-dihydroxy-3-isoprenyloxy-6-methylxanthone were isolated from the ethyl acetate extract of *Endodesmia calophylloides* (Guttiferae), a tree found in Nigeria, Cameroon, Gabon and Angola. All the isolated compounds were found to be antimalarial active with IC_{50} values ranging from 7.2 to 23.6 μM and could be interesting sources for new potential antimalarial leads (Ngouamegne, *et al.,* 2008).

A bioassay-guided fractionation of the crude methanol stem bark extract from *Cassia siamea* L. (Leguminosae), using the parasite lactate dehydrogenase assay and multi-resistant strain of *P. falciparum* (K1) for assessing the *in vitro* antimalarial activity has led to the isolation of two bioactive compounds- Emodin and lupeol **(17)**. They were isolated from the ethyl acetate fraction by a combination of chromatographic techniques. Both compounds were found to be the active principles responsible for the antiplasmodial property of *C. siamea*, (Ajaiyeoba *et al.,* 2005).

From the stem bark of *Pentadesma butyracea* Sabine (Clusiaceae), a multipurpose rain forest species commonly called “butter tree” and widely distributed in tropical West Africa including Cameroon and Nigeria, four new xanthones named butyraxanthones A-D, together with six known xanthones and a triterpenoid (lupeol), were isolated and assayed *in vitro* for antiplasmodial activity against the *P. falciparum* chloroquine-resistant FcB1 strain. All of these

xanthones exhibited good antiplasmodial activity with IC_{50} values ranging from 4.4 to 8.0 μM (Zelefack *et al.*, 2009).

Alstonia species (Apocynaceae) are traditionally used in Africa and South-East Asia for the treatment of malaria. The investigation of several *Alstonia* species, including *A. angustifolia, A. scholaris, A. macrophylla,* and *A. glaucescens,* resulted in the discovery of a series of antiplasmodial bis-indole alkaloids, of which villalstonine and macrocarpamine were the most active against the CQR K1 strain *in vitro* (IC_{50} 270 and 360 nM, respectively) (Wright *et al.*, 1993, Keawpradub *et al.*, 1999).

2.9 NIGERIAN MEDICINAL PLANTS HAVING ANTIOXIDANT ACTIVITIES

Africa is blessed with enormous biodiversity resources. It is known that plant accumulate antioxidant chemicals as secondary metabolites through evolution as a natural means of surviving in a hostile environment (Manach *et al.*, 2004). Antioxidant potential of some Nigeria medicinal plant has been understudied and found to play important role in the chemoprevention of diseases that has their etiology and pathophyiology in reactive oxygen species.

The *in vivo* antioxidant potential of *Sacoglottis gabonensis* stem bark on 2,4 dinitrophenylhydrazine-induced membrane peroxidation has been reported (Maduka and Okoye, 2002). *Sacoglottis gabonensis* is used as beverage additive in Nigeria.

Aqueous and ethanolic extracts of a Nigerian popular spice and food additive, leaves harvested from eastern has been demonstrated to significantly increased the activity of superoxide dismutase and the level of reduced glutathione peroxidase as well as the levels of glutathione peroxidase and glucose-6-phosphate, while decreasing lipid peroxidation (Atawodi, 2005).

Spices and herbs are recognized as sources of natural antioxidants that can protect from oxidative stress (Repetto and Llesuy, 2002).

Phytochemical and antioxidant screening of some Nigerian plants of Apocynaceae, of *Strophantus hispidus* (stem and root), *Voacanga africana* (stem and leaf) and *Thevetia neriifolia* (stem and leaf) were investigated and were reported as being active as against free radicals (Ayoola *et al.*, 2008). *E.angolense*, *K.senegalensis*, *A. leiocarpus*, *Psidium guajava* (guava) Myrtaceae and *Aloe vera* Liliaceae were found to be good radical scavengers with the degree of inhibition compared to the standard ascorbic acid used (Olajide *et al* ., 2011). Research study has suggested a possible synergistic antioxidant interaction, which could be used to enhance the medicinal applications of the plants involved. Such report has been made for the combination of *Psidium guajava* (guava) and *Aloe vera* (Olajide *et al* ., 2011).

Vernonia amygdalina, a vegetable and herb has been used in various part of Nigeria for the treatment of several ailments ranging from diabetes, malaria, cancer and for general wellbeing. This local treatment has been backed up in recent times scientifically. The antioxidant activities of different extracts of *Vernonia amygdalina (* aqueous, methanol, hexane, ethyl acetate and butanol) using three methods: scavenging effect on 2,2-diphenyl-1-picryhydrazyl radical (DPPH), hydroxyl radical and peroxide oxidation by ferric thiocynate method by Oloyede and Ayila (2012). All fractions showed significant antioxidant activity, when compared with antioxidant standards (butylated hydroxyl anisole BHA, ascorbic acid and α-tocopherol). *Telfairia occidentalis* Hook f. commonly called fluted pumpkin, another leafy vegetable that is consumed in different parts of the country because of the numerous nutritional and medicinal attributes ascribed to it has been found to exhibit antioxidant activity. It is reported to suppress or prevent the production of free radical and scavenge already produced free radical, lower lipid

peroxidation status and elevates antioxidant enzymes (such as superoxide dismutase and Catalase) both *in vitro* and *in vivo* (Oboh *et al* ., 2006).

The antioxidant properties of mushroom have been reported. They are regarded as organisms which possess naturally occurring antioxidants. This is correlated with their phenolic and polysaccharide compounds (Mau *et al* ., 2005). Three species of Pleurotus florida, P. pulmonarius and P. citrinopileatus and others reacted positively to antioxidant test procedures (Mau *et al* ., 2005).

2.10 TAXONOMY AND BOTANICAL DESCRIPTION OF APOCYNACEAE

The scientific classification for this family is Eukaryotes > Archaeoplastida > Chloroplastida > Charophyta > Streptophytina > Plantae (Land plants) > Tracheophyta (vascular plants) > Euphyllophyta > Lignophyta (woody plants) > Spermatophyta (seed plants) > Angiospermae (Flowering plants) > Eudicotyledons > Core Eudicots > Asterids > Euasterid I > Order: Gentianales > Apocynaceae

The Apocynaceae was initially recognized as "Apocynum" by Adanson, in 1763, and later established as "Apocineae" by Jussieu (1789), in a work that marked the beginning of valid supra-specific names for spermatophytes (McNeill *et al.* 2006). Subsequently, the group was divided by Brown (1810) into two families of similar sizes and easily distinguished by the presence (Asclepiadaceae *s.l.*) or absence (Apocynaceae *s.str.*) of pollinaria.

It was a widely circumscribed family based on presence of laticifers, abundant endosperm (the term "Perispermo", as used by Jussieu 1789, was later understood as endosperm), an often contorted corolla limb, and mainly bifollicular fruits (Sennblad and Bremer 1996)

Apocynaceae is a family of flowering plants that includes trees, shrubs, herbs, stem succulents, and vines (Wiart 2006). A common term for the family is dogbane family, after the American plant known as dogbane, *Apocynum cannabinum*. The Apocynaceae comprise approximately 5,000 species and are widely distributed (about 250 genera and 2000 species according to Ng FSP. 2006). The family show variable habit, from magna trees to climbing vines, yet, they can be easily recognized by the presence of latex and a style-head derived from the fusion of two carpels at the apex of the styles. Characteristically many of these plants have milky latex, 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. The leaves are simple, usually opposite and decussate, or whorled; lacking stipules. Flowers are usually showy, actinomorphic, aggregated in cymose or racemose inflorescences (rarely fasciculate or solitary). They are perfect (bisexual), with a synsepalous, five-lobed calyx united into a tube at the base. Inflorescences are terminal or axillary. Five petals are united into a tube with four or five epipetalous stamens. The style is expanded at the apex into a massive clavuncle just below the stigma. The ovary is usually superior, bicarpellary, and apocarpous, with a common fused style and stigma. The fruit is a drupe, a berry, a capsule or a follicle. Members of the family are native to European, Asian, African, Australian and American tropics or subtropics, with some temperate members. Many species are tall trees found in tropical rainforest, but some grow in tropical dry (Xeric) environments. There are also perennial herbs from temperate zones (Rapini, 2004).

Included in the Apocynaceae family are five subfamilies: Apocynoideae, Asclepiadoideae, Periplocoideae, Rauvolfioideae, and Secamonoideae. Apocynaceae has actually come around as

a joining of two families, the Apocynaceae sensu Stricto and the Asclepiadaceae (Endress and Bruyns 2000; Endress *et al.* 2007: Simões *et al.*, 2007; Ibrahim *et al.*, 2014).

The family Asclepiadaceae has been relegated to the subfamily Asclepiadoideae where they are treated separately because they form a distinctive group, with distinct features (Livshultz *et al.* 2007; Simões *et al.*, 2007; Livshultz 2010). *Callichilia*, having seven species and *Voacanga* having 12 species are examples of genera of Apocynaceae that are native to Africa.

2.11 MEDICAL/ECONOMIC USES OF APOCYNACEAE

In traditional medicine, Apocynaceae species are generally used to treat gastrointestinal ailments, fever, malaria, pain and diabetes (Wiart 2006). The roots, leaves and latex have been reported to be used in treatment of skin, liver diseases, leprosy, dysentery, worms, ulcers, tumours and ear aches (Rajakaruna *et al.*, 2002). The roots and leaves of *Calotropis gigantea* (L.) Aiton have been studied and its folkloric use in the treatment of skin and liver diseases, leprosy, dysentery, worms, ulcers, tumours and ear aches has been confirmed (Rajakaruna *et al.*, 2002). The wound healing properties of its latex has also been proofed scientifically (Nalwaya *et al.*, 2009). *Kopsia fruticosa* (Ker.) A. DC. is used to treat sore and syphilis, and has cholinergic effects. Leaves and barks of *Dyera costulata* Hook have been used for treating fever, inflammation and pain (Subhadhirasakul *et al.*, 2003). Stems, leaves and latex of *Alstonia angustiloba* Miq. have been used for gynaecological problems and skin sores in Indonesia (Mulyoutami *et al.*, 2009) and its leaves are externally applied to treat headache in Malaysia (Lin 2005).

Among many plants used as antibacterials, the members belonging to Apocynaceae have been reported to have better effect against pathogens and these plants are being locally available (Hadi and Bremner, 200; Hussain and Gorsi, 2004; Suresh *et al.*, 2008).

A traditional treatment for pleurisy comes from *Asclepias tuberosum* (milkweed) (Shariff and Sudarshana, 2006). Cardiac glycosides with positive effect on failing heart has been sourced from this family and such include the Acokanthera, Apocynum, Cerbera, Nerium, Thevetia and Strophantus. The alkaloids reserpine **(1)** and rescinnamine, use in the treatment of high blood pressure and some forms of psychosis are phytochemicals from *Rauvolfia serpentine* (Indian snakeroot) (Shariff and Sudarshana, 2006).

The anticancer and antimalarial properties of Apocynaceae (Wiart 2006; Patel *et al.*, 2010) was investigated in Ng FSP 2006 study on leaf extracts of five selected species of Apocynaceae used in traditional medicine (*Alstonia angustiloba*, *Calotropis gigantea*, *Dyera costulata*, *Kopsia fruticosa* and *Vallaris glabra*). The study showed that *V. glabra* leaf extracts has wide spectrum of activities as antiproliferative and antiplasmodial agent. Some species that have been reported as having cytotoxic activity also include; *Allamanda* (Schmidt *et al.*, 2006), *Alstonia* (Keawpradub *et al.*, 1999; Jagetia and Baliga 2006), *Cerbera* (Chang *et al.*, 2000; Nurhanan *et al.*, 2008), *Nerium* (Siddiqui *et al.*, 1995; Pathak *et al.*, 2000), *Plumeria* (Kardono *et al.*, 1990) and *Tabernaemontana*. *Alstonia*, are also known to have antimalarial properties (Schwikkard and Heerden 2002). *Catharanthus roseus* yields alkaloids, vinblastine **(3)** and vincristine **(2)** used in clinical treatment of cancer.

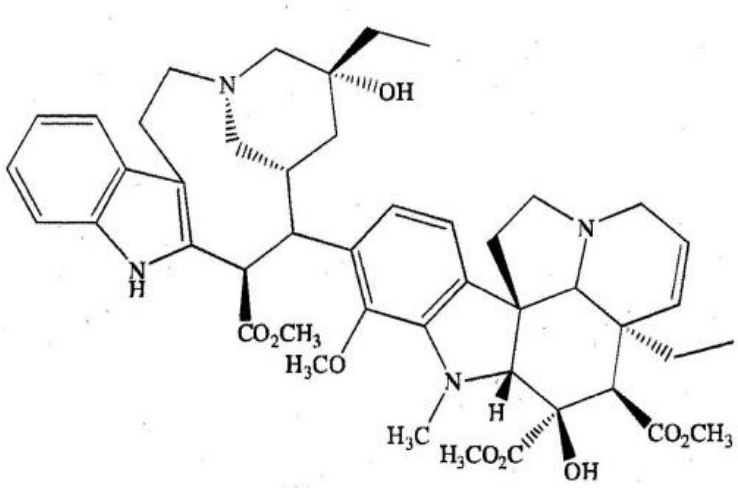
The sap from the genera *Carpodinus*, *Landolphia*, *Hancornia*, *Funtumia*, and *Mascarenhasia* were used as a commercial source of inferior rubber. The juice of *Acokanthera* species such as

A. venenata and the milky juice of the Namibian *Pachypodium* have been used as venom for arrow tips by the Bushmen tribe (Patel *et al.*, 2010). Several genera are grown as ornamental plants due to their flower, this include Amsonia (bluestar), Mandevilla (Savannah flower), *Adenium* (desert-rose) *Nerium* (oleander), *Vinca* (periwinkle) and *Carissa* (Natal plum, an edible fruit) (Patel *et al.*, 2010).

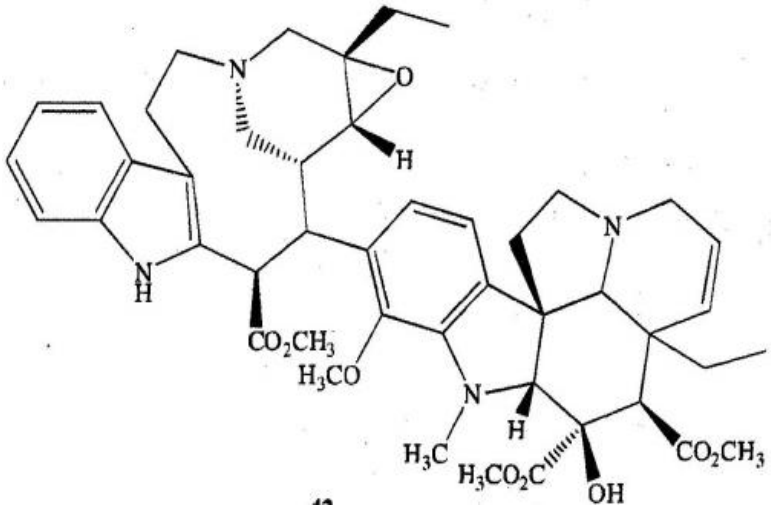
2.12 PHYTOCHEMISTRY: ALKALOIDS OF APOCYNACEAE FAMILY (DOGBANE)

All alkaloids from the alkaloid rich dogbane family (Apocynaceae) have strong biological and medicinal effect (Tadeusz 2007). A large number of alkaloids have been isolated from this family. Many of them are currently use in cancer chemotherapy. Alkaloids are especially abundant in the following genera: Devil's-pepper (*Rauvolfia* L.), Periwinkle (*Catharanthus* G. Don), Milkwood (*Tabernaemontana* L.), *Strophanthus* (*Strophanthus* DC.), *Voacanga* (*Voacanga* U.) and *Alstonia* (*Alstonia* R. Br.).

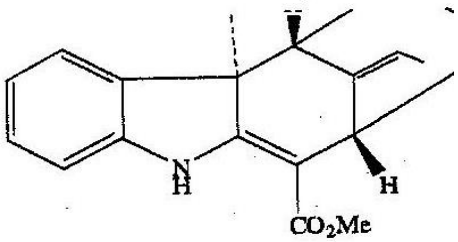
Indole alkaloids have been isolated as a major component. Besides indole alkaloids, there are also steroidal alkaloids, monoterpenoid piperidine alkaloids (skytanthine type), spermidine alkaloids and dimeric piperidine derivatives. The indole alkaloids comprise a large and complex group of naturally occurring organic compounds possessing the indole or dihydroindole (indoline) nucleus. Examples of indole alkaloids from the family Apocyanaceae include: vinblastine **(3)**, vincristine **(2)**, vinrosidine (**46)**, vincoside**,** vallesiachotamine**,** strychnine **(22),** vincamine (**49),** kopsine **(51),** catharanthine **(50)** and vinleurosine **(47)**. These alkaloids form some of the most potent drugs available to man for the treatment of a variety of cancers.



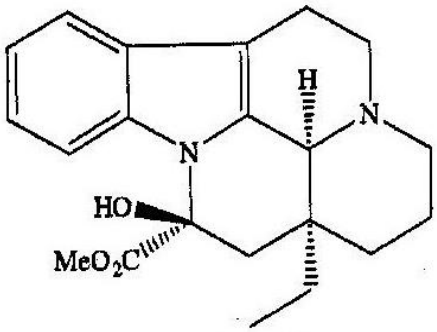
Vinrosidine (46)



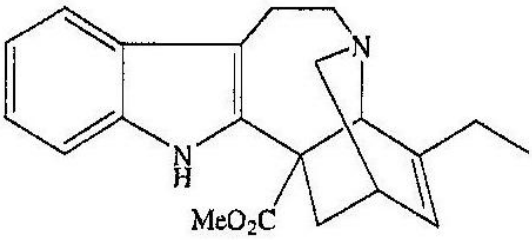
Vinleurosine (47)



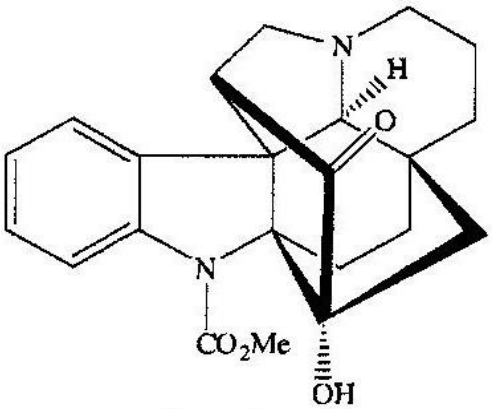
Condylocarpine (48)



Vincamine (49)

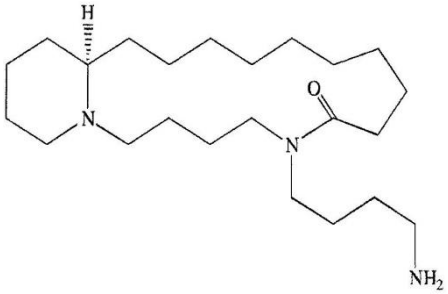


Catharanthine (50)

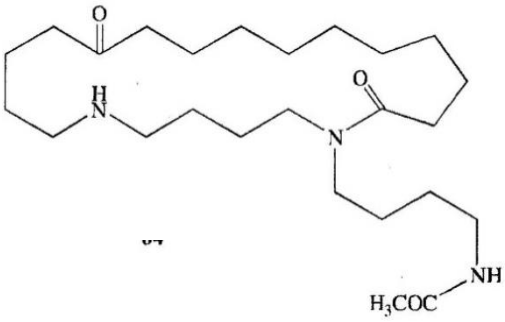


Kopsine (51)

Only a few examples of spermidine alkaloids of the Apocynaceae have been isolated, example being oncinotine (**52)** and *N*-acetyloncinotine (**53)** from *Oninotis nitida* (Cordell, 1981).



Oncinotine (52)



***N*-acetyloncinotine (53)**

Deserpine is a bioactive alkaloid isolated from the roots of *Rauwolfia canescens* (Varchi *et al*., 2005). Its chemical structure differs from reserpine **(1)** only by absence of a metoxy group but shows an interesting profile of biological activity. It has been employed in clinical practice for the treatment of hypertension and as a tranquilizer and also in clinical control of other cardiac disorders. Deserpine is a compound with limited availability from natural sources (Varchi *et al*., 2005).

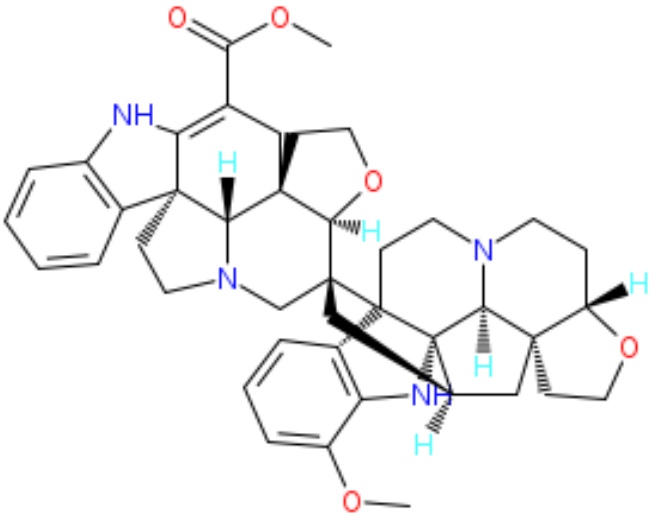
The alkaloids isolated from heynana milkwood (*Tabernaemontana heyneana* Wall.) have shown both bioimpact and uterotrophic activity during *in vivo* test on animal (Srivastava *et al*., 2001).

More than 180 biologically active alkaloids have been isolated from the genus *Alstonia*, making this genus one of the most important in terms of potential alkaloid use. One of the newly isolated alkaloids of *Alstonia* plants is menilamine. Investigations into the possible medical use of menilamine as distinguished it as a prospective new anti-malarial alkaloid (Macabeo *et al.*, 2005).

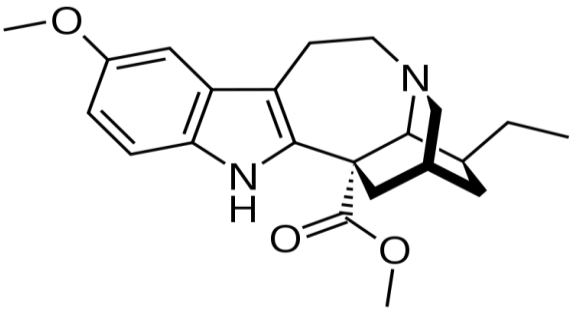
Alkaloids from *Alstonia macrophylla* Wall. Ex G. Don growing in Thailand, during bioassay have display strong bioactivities and are considered to be of potential use in medicine. The family continues to be an interesting source of novel drugs.

Table 3: Some physiological important Alkaloids of Apocynaceae family

Genera	Specie	Alkaloids Isolated	Reference
Devil's-pepper (*Rauvolfia* L.)	*Rauvolfia serpentina*	Reserpine and rescinnamine	Varchi *et al*., 2005
	Rauvolfia serpentina	Five new indole alkaloids (N_b-methylajmaline, N_b-methylisoajmaline, 3-hydroxysarpagine, yohimbic acid and isorauhimbic acid)	Itoh *et al.*, 2005
	Rauwolfia capra(quinine tree)	Quinine	Varchi *et al*., 2005
	Rauwolfia canescens	Deserpine	Varchi *et al*., 2005
Periwinkle (*Catharanthus* G. Don)	*Catharanthus roseus and Vinca* spp	Ajmalicine, catharanthine, leurosine, vindoline, vindolinine, vinblastine, vincristine, vindesine and alioline, iboga alkaloids	Varchi *et al*., 2005
	Vinca difformis Pourr	Vincamajine, vincamedine, vincadifformine, akuammidine, vellosimine, vincadiffine, difforlemenine, difforine and normacusine. iboga alkaloids	Garnier and Mahuteau, 1986
Milkwood (*Tabernaemontana* L.)	Iboga	Iboganine, other iboga alkaloids	
	Heynana (*T. heyneana* Wall.)	Ervatine, tabersonine, coronaridine, heyneanine, voacristine, voacristine hydroxyindolenine, hydroxyibogamine and coronaridine hydroxyindolenine	Srivastava *et al*., 2001
	T. elegans	Apparicine, 16-S-hydroxy-16, 22-dihydroapparicine, tubotaiwine, vobasine, vobasinol, tabernaemontaninol, tabernaemontanine, isovoacangine, dregamine, dregaminol, dregaminol-methylether, 3-R/S-hydroxytabernaelegantine B, 3-methoxy-tabernaelegantine C, 3-R/Shydroxy- conodurine, tabernaelegantine A, B, C, and D	Heijden *et al*., 1986
Strophanthus (*Strophanthus* DC.)			
Voacanga (*Voacanga* U.)	*V. Africana; V. grandifolia*	Voacangine, Vobtusine and Voacamine (predominating types), Dregamine, Tabersonine, Voacamine, Coronaridine, iboga alkaloids	
Alstonia (*Alstonia* R. Br.)	*Alstonia macrophylla* Wall. Ex G. Don	Talcarpine, pleiocarpamine, alstoumerine, 20-Epiantirhine, alstonerine, alstophylline, macralstonine, villalstonine, alstomacroline and macrocarpamine	Keawpradub *et al*., 1999



Callichiline (54)



Voacangine (55)

2.13 GENUS: *Callichilia*

The genus is restricted to Africa. In 1902 Stapf described the genus C*allichilia* based on 5 species, some of which he removed from *Tabernaemontana.*

Markgraf (1923) reduced its size by separarting Ephippiocarpa, based on *C. orientalis*. Pichon 1948, reduced Callichilia by placing *C. barteri* in the monotypic genus *Hedranthera.*

Recent authors, prefers the original genus concept as described by Stapf.

Alkaloids of *C. barteri* and *C. subsessilis* have been analyzed, and some structures were found, peculiar to these species. Of these, only alkaloid vobtusine was found in both species.

Erect glabrous shrubs or lianas, with white latex in every part of the plant, except in the corolla-lobes. Branches are unarmed, terete and lenticellate.

Most *Callichilia* species occur in the rain forest belt of Central Africa from Nigeria to Zaire, where *C. bequaertii* is the most widely distributed specie, while *C. monopodialis* is endemic in mountainous region in Cameroun. *C. subsessilis* occurs in the rain forests West Africa. The six species from Centra and West Africa are either understory shrubs or lianas and are easily recognized by their large conspicuous flowers.

Callichilia belongs to the tribe of the *Tabernaemontacea*, which was discussed by Pichon (1948) and Leeuwenberg (1976).

2.13.1 Differentiating the *Callichilia* species:

Callichilia subsessilis, Stapf. can easily be distinguished from *C. stenopetala* by the very narrow sepals of *C. stenopetala*. Other diagnostic intra-generic presentation of some epidermal features of this species as reported by Green in his Ph.D research findings on Apocynaceae include:

- The genus *Callichilia* generally present 4 – 5 sided cell geometry.
- *C. monopodialis* and *C. stenopetala* have 5 – 6 sided cells outline with that of *C. stenopetala* being more curved at the edges.
- Cells of C. subsessilis are variable, ranging between 4 and 6.
- *C. barteri* is 5 – 6 sided in cell geometry.
- Acuminate trichomes were observed in C. subsessilis.
- *Callichilia* genus basically present paracytic type of stomata, with slight variation as presented in *C. subsessilis* and *C. stenopetala* possessing brachyparacytic and hemiparacytic subtypes repectively.
- Two types of stomata are found in *C. edulis*, the paracytic and the anisocytic. (Figure 5a and 5b)

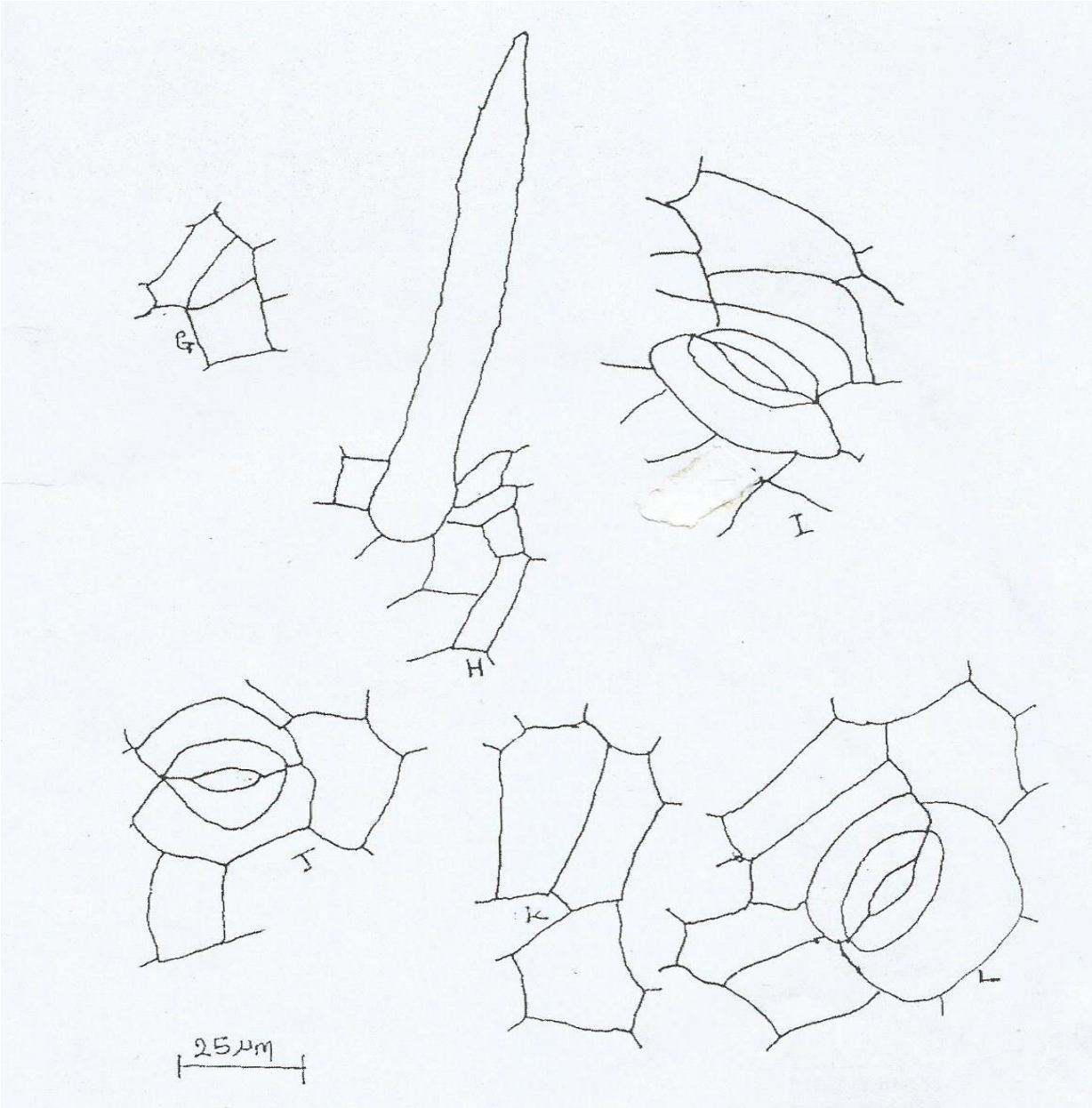


Figure 5a: ***Callichilia*** **spp microscopy (a)**

Callichilia subsessilis **(G) 4-6 sided cells, (H) acuminate trichome, (I) brachyparacytic stoma, (J) parasitic stoma;**

C. barteri **(K) 6 sided cell, (L) paracytic stoma**

Source: Green, 1995

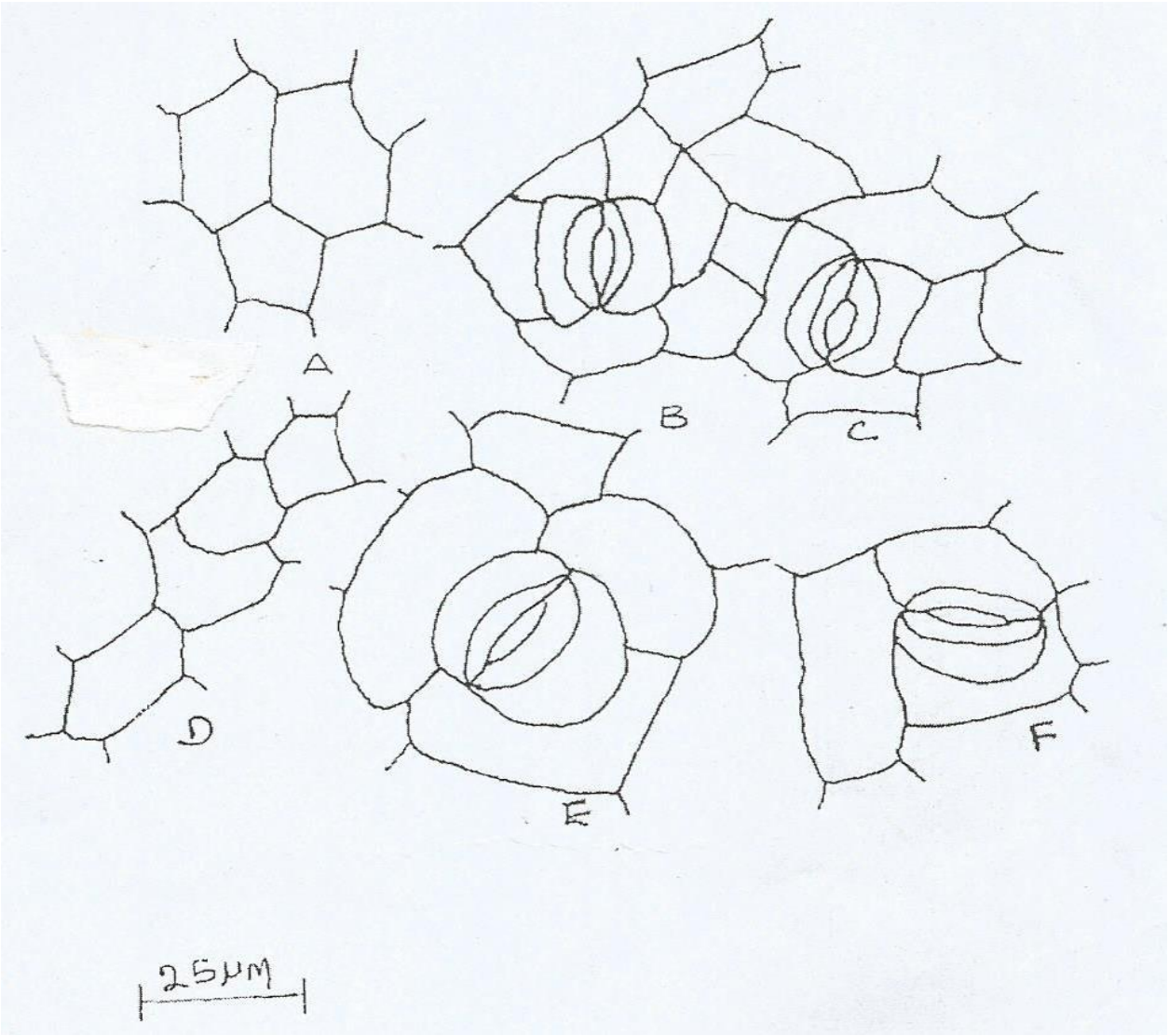


Figure 5b: ***Callichilia*** **spp microscopy (b)**

C. monopodiatis **(A) 5-6 sided cells, (B) paracytic stoma (C) hemi-paracytic stomata**

C. stenopetala **(D) 5-6 sided cells, (E) paracytic stomata, (F) hemi-paracytic stomata**

Source: Green, 1995

Table 4: Macromorphological Comparison of *Callichilia Spp*

TAXONOMIC PARAMETER	C. barteri	C. microcalyx	C. mannii	C. monopodialis	C. stenosepala	C. subsessilis
Ecology	Rain forest	Rain forest	Rain forest	Rain forest	Rain forest	Rain forest
Habitat	Secondary forest	Closed forest	Closed forest	Understorey forest	Secondary forest	Secondary forest
Habit	Erect shrub	Liane	Liane	Erect shrub	Erect shrub	Erect shrub
Stem size	1m high	3m	6m	2m	1.5m	2.5m
Leaf shape	Obovate to oblong	Oblong to ovate	Oblong to elliptic	Oblong to elliptic	Oblong to elliptic	Oblong to elliptic
Leave length	8-12cm	12-25cm	8-18cm	15-23cm	8-17cm	10-18cm
No. of lateral nerves	6-8 pairs	6-10 pairs	5-9 pairs	5-10 pairs	8-12 pairs	8-12 pairs
Leave texture	Coriaceous	Coriaceous	Coriaceous	Coriaceous	Coriaceous	Coriaceous
Inflorescence	Terminal and at branching point, cyme	Terminal cyme	Terminal cyme	Terminal cyme	Terminal cyme	Terminal cyme
Calyx	Green persistent	Green	Medium green	Green	Pale green	Green
Corolla	White	White	White	White	White	White
Fruit	A pair of yellow berry when ripe	Pair of berry, yellow when ripe	A pair of berry, yellow when ripe	A pair of big follicles 35cm long 5.5cm wide with blunt ends	A pair of berry yellow	P air of berry, yellow with red speckless

Source: Green, 1995

2.13.2 *Callichilia stenopetala* Stapf

Kingdom: Plantae

Sub-kingdom: Tracheobionta

Division: Magnoliophyta

Class: Magnoliopsida

Sub-class: Asteridae

Order: Gentianales

Family: Apocynaceae

Genus: Callichilia

Local name (Igbo): Utu nkita

Callichilia stenopetala Stapf, Flora of Tropical Africa (1904): 4(1): 602; Johnson Liberia 2: 626 (1906); Hutchinson and Dalziel, Fl. W. Trop. Afr. 2: 39 (1939)

Heterotypic synonym: *Callichilia subsessilis* (Benth.) Stapf, Flora of Tropical Africa 4(1): 132 (1902).

Callichilia stenopetala was described by Stapf (1904) in the Flora of Tropical Africa under addenda and therefore it was not included in the key to the species. *Callichilia stenopetala* was distinguished from *C. subsessilis* Stapf by having very narrow sepals, that is, the width of sepals of *C. stenopetala* is smaller. The other two diagnostic characters that he gave were higher insertion of the stamens and the shortness of the corolla lobes (Beentje 1978).

A glabrous shrub with slender branches. The leaves are lanceolate to elliptic –lanceolate, acutely acuminate (acumen slender), gradually attenuated or acuminate below and contracted ate the base 3 to 7 inches long. The leaves are broad membranous and has very short petioles.

Inflorescences are umbelliform at the early stage but gradually lengthen out and racemiform with 2 to 3 flowers out at a time. The sepal is narrow, lanceolate and acuminate while the corolla is white, tube slender, constricted at the middle and about 1.5 inches long (Beentje, 1978; Green, 1995; Otto, 2008).

The roots are narrow and quite long. The root is used in the treatment of recurrent and persistent fever, for treatment of tooth ache and dental problems, oral hygiene, in managing convulsion and insect bites. For dental problem the patient is asked to use as a chewing stick, while retaining the juice within the gum area (oral interview).

Figure 6: ***Callichilia stenopetala*** **Plant showing leaf arrangement**

Uruagu, Nnewi in Anambra state, Nigeria

Figure 7: *Callichilia stenopetala* plant showing its fruits

Uruagu, Nnewi in Anambra state, Nigeria

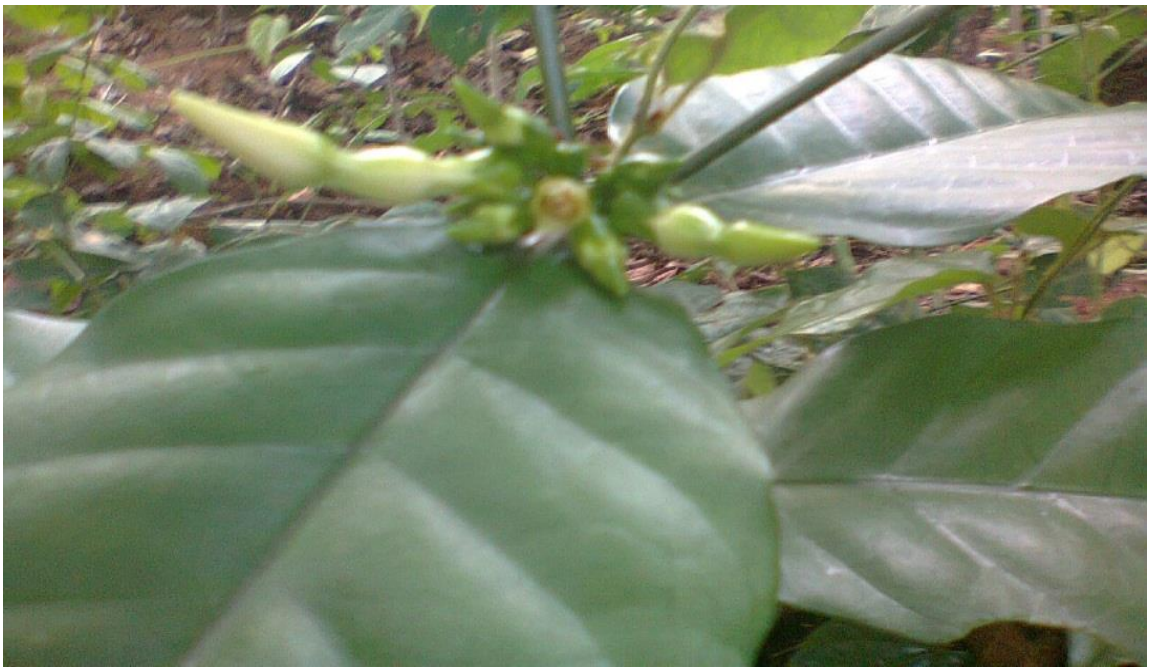


Figure 8: ***Callichilia stenopetala*** **showing its flower**

Uruagu, Nnewi in Anambra state, Nigeria

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1. SOLVENTS, REAGENTS AND EQUIPMENT

3.1.1 Solvents

Petroleum ether, Hexane, Chloroform, Ethyl acetate, Methanol, Butanol, Deuterated chloroform ($CDCl_3$, Sigma). Solvents used where both analar grades (for isolation purpose) and analytical grades (for extraction purpose).

3.1.2 Chemicals

TLC silica gel (Merck), Tween 80 (Sigma-Aldrich Co.), Chloroquine (Evans, Nigeria), Gallic acid, Ascorbic acid (Pharmaceitical Chemistery department, University of Lagos), Artesunate (Greenfield Pharmaceutical, China)

3.1. 3 Equipment

Soxhlet extractor, Separating funnels, Weighing balance, Rotary evaporator (Buchi), Analytical Thin Layer Chromatographic plates (TLC Silicagel 60 F_{254}, Merck), Chromatographic columns, Ultraviolet lamp (254 and 366 nm), Sintered glass funnel, Frosted slides, Fisher- Johns melting point apparatus, Fischer Scientific Pittsburg Pa, Vacuum pump/vacuum trap, Varian 680-IR spectrophotometer, Shimadazu GCMS-QP2010 (GC-MS), Bruker-Advance 600MHz FT-NMR Spectrometer, Perkin Elmier Spectrum 100 FT-IR Spectrometer, UV spectrophotometer (T90 Spectrometer, Leicestershire, England), Bruker Kappa Apex Duo (crystallography)

3.1.4 Reagents

- DPPH (1, 1-diphenyl-2- picrylhydrazyl) (Sigma-Aldrich USA)
- **Fehling Solution:** Solution A was prepared by dissolving 7.0 g of copper sulphate and 0.1 mL of sulphuric acid with sufficient distilled water and made up to 100 mL mark. Solution B was prepared by dissolving 35.2 g of sodium potassium tartrate and 15.4 g of sodium hydroxide in sufficient distilled water and made up to 100 mL mark. Equal volume of solutions A and B were mixed immediately before using.
- **Barfoeds Reagent:** It was by dissolving 13.3 g of crystallized neutral copper acetate in 200 mL of 1% acetic acid solution.
- **Iodine Solution:** It was prepared by dissolving a mixture of 2.5 g iodine crystals and 5.0 g of potassium iodide (KI) in 25 mL of distilled water.
- **Wagner's Reagent:** This was prepared by dissolving a mixture of 2.0 g of iodine and 3.0 g of potassium iodide in distilled water. The solution was made up to the 100 mL mark by further addition of distilled water.
- **Dragendorff's Reagent:** 0.85 g of bismuth nitrate was dissolved in 10 mL of acetic acid and 40 mL distilled water was added to give a stock solution A. 8.0 g of potassium iodide was equally dissolved in 20 mL of distilled water to give of stock solution B. 5 mL of each of the stock solutions A and B were mixed with 20 mL 33% acetic acid followed by addition of 100 mL distilled water to give the Dragendorff's reagent.
- **Molisch's Reagent:** (10% solution of $\propto$ - naphthol in alcohol).
- **Ammoniacal Silver Nitrate Solution:** It was prepared by dissolving 3.0 g of silver nitrate in 50 mL of distilled water followed by drop wise addition of ammonia solution

until the initial precipitate of silver oxide dissolved. The resulting solution was made up to 100 mL with distilled water in a volumetric flask.

- **Meyers Reagent:** 1.36 g of mercuric chloride was dissolved in 60 mL of distilled water to give solution A. 5.0 g of potassium iodide was dissolved in 20 mL of distilled water to give solution B. both solutions A and B were mixed together and made up to 100 mL mark in a volumetric flask.
- 1% Picric acid solution: It was prepared by dissolving 1 g of anhydrous trinitrophenol in 100 mL of hot water, cooled and filtered. The container was labeled as such.
- **20% Sodium hydroxide solution:** It was prepared by dissolving 80 g of NaOH in approximately 200 mL distilled water and made up to volume (1 L). The prepared solution in the flask was kept in the oven at 20 0C and maintained for 1 hour.
- **10% conc. Sulphuric acid in ethanol:** It was prepared by dissolving 10 mL of conc. Sulphuric acid in 90 mL of ethanol.
- **1% vanillin in sulphuric acid:** It was prepared by dissolving 1.00 g of vanillin powder in 100 mL of concentrated sulphuric acid.

3.1.5 Parasite and Animals

Plasmodium

The rodent parasite *Plasmodium berghei berghei* chloroquine sensitive strain (NK 65 strain) was obtained from National Institute of Medical Research (NIMR), Yaba, Lagos and identified by Dr. Aina of NIMR, Yaba, Lagos and Mr. Ota, technologist in the Department of Physiology, College of Medicine, University of Lagos. The *Plasmodium* was maintained in University Animal House Laboratory by serial passage of the parasite into uninfected mice.

Animals

The animals used in this study were Swiss albino mice of both sexes, weighing between 18 g and 22 g. They were sourced from the animal house of University of Lagos, Idi-Araba. They were kept in groups of five in separate plastic cages, and given standard laboratory diet and water *ad libitum* and maintained under laboratory conditions of room temperature - 12 h light and 12 h dark cycle. The animals were allowed to acclimatize for 7 days preceding the experiments. All experimental protocols were in compliance with internationally accepted principles for laboratory animal use and care.

Animal management and handling

The animal experiments were conducted in compliance with International Guiding Principles for research involving animals as recommended by the Declaration of Helsinki and Guiding Principles in the Care and Use of Animals (World Medical Association and American Physiological Society, 2002). The minimum number of mice was used, which depended on the bio-activity guided isolation procedure results. Thirty (30) mice were used for the oral acute toxicity.

The living conditions of the experimental animals were appropriate for their species and contributed to their health and comfort. The animals were accommodated in clean, well ventilated but grouped caging. Suitable nutritional, uncontaminated food and water were provided at all times. Feeding and drinking devices used were those that enable easy food/water intake and care was taken to ensure that their feedings/water were not contaminated with feaces and urine. Frequent replacement of bedding was observed. Appropriate restraint was employed to minimize risk of injury to the animal and personnel during blood collection from the tail for

blood smear. Record was kept for proper documentation, aid animal care, data collection and reliability of animal experiments.

All animals used for research were euthanized in a humane manner which minimized distress and maximized smooth and event-free euthanasia. Euthanasia procedures were carried by skilled personnel. To avoid potentially stressful transfer of mice from one cage to another before the procedure, the transparent home cage with tight fitting lids was used. The animals were made comfortable in their home cage before initiating the flow of $CHCl_3$ (chloroform) gas, the euthanasia gas used. After assurance of death, the carcasses were bagged and labeled appropriately for proper disposal. The cage used as chamber was sanitized after each use, to ensure good sanitation.

3.2 SEARCH AND COLLECTION OF PLANTS

Survey of some Nigerian medicinal plants used in treatment of recurrent-fever and malaria fever in the South-eastern part of Nigeria was done. The survey took the form of random oral interview of traditional practitioners, herb sellers, elderly people and mothers in the chosen locality. The plants mentioned frequently were noted, collected, identified and subjected to antimalarial and antioxidant bioassay. The selection of the plant for the study was based on the plant with the most effective antimalarial activity. Antioxidant activity was included in the bio-screening as a supporting factor, considering the relationship that exist between free radical generation and malarial infection. Nine (9) medicinal plants were collected from various locations within South- east region of Nigeria and subjected to bio-screening (antimalarial and antioxidant) and from the result obtained a plant, *Callichilia stenopetala* root bark was selected as the plant to be studied futher.

3.2.1 Collection and Taxonomical Identification of Study Plant

The plant for the study (fresh roots of *Callichilia stenopetala* Stapf, Apocynaceae) was collected at Uruagu village of Nnewi town in Anambra state, South- East region of Nigeria. The plant was noted to grow along parts where rain water/flood runs, that is along water course. The collection took place between January 2011 and March 2012. Bulk of the plant was collected between April -July 2011 and the same period 2012. It was authenticated by taxonomist, Mr. Ozoko of University of Nigeria Nsukka, Enugu state (preliminary identification) and further confirmed by Mr. Daramola, a taxonomist and collector, formerly of the University Herbarium, Department of Botany, Faculty of Science, University of Lagos Akoka. The herbarium specimen was prepared and deposited with voucher number LUH 3622.

3.2.2 Pre-extraction Preparation of *C. stenopetala*

The roots collected were washed in water, dried at room temperature after which they were grinded into powder, using a Warring mechanical blender and kept in a dry container until needed.

3.3 EXTRACTION AND FRACTIONATION OF C. STENOPETALA ROOT BARK

About 1.4 kg of pulverized plant material (root bark of *C. stenopetala*) was successively extracted with 90% methanol by continuous Soxhlet extractor. The cooled aqueous-methanol (MeOH) extract was filtered, pooled and dried using a rotary evaporator (Büchi Labortechnik GmbH). The dried extract obtained was 134.00 g. Extract (118 g) was suspended in 700 mL distilled water and successfully fractionated with *n*-hexane (3 × 700 mL), chloroform (3 × 700 mL) and ethyl acetate (3 × 700 mL) to yield *n*- hexane (17.46 g), chloroform (22 g), ethyl acetate (7.66 g), and aqueous residue (68 g) fractions, respectively (Figure 11). Some extract

was lost as emulsion. The extract and fractions were kept in airtight sample bottles and were stored at 4°C for use in anti-plasmodial bioassay, free radical inhibitory activity and toxicity.

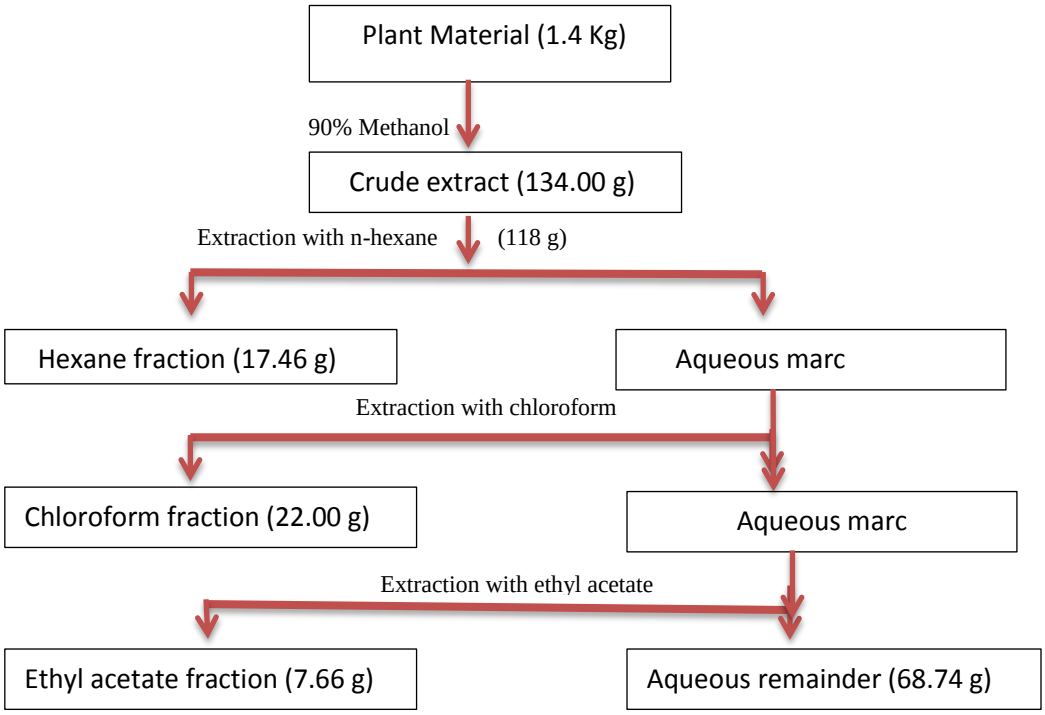


Figure 9: Schematic representation of extraction and partitioning of crude extract

3.4 PHYTOCHEMICAL SCREENING OF C. STENOPETALA

The dried plant was tested for the presence of secondary metabolites using standard methods in phytochemical screening. The metabolites tested include; tannins, saponins, flavonoids, anthraquinone derivatives, cardiac glycosides, steroids, triterpenes and alkaloids (Sofowora 1993; Onawumi *et al.*, 2012). The fractions, hexane, chloroform, ethyl acetate and aqueous remainder from the methanolic extract of the study plant were specifically tested for alkaloids.

Table 5: Phytochemical screening

S/N	Metabolite	Method
1	Tannins and phenols	2% solution of $FeCl_3$
2	Saponins	Frothing test
3	Flavonoids	Ammonia test
4	Anthraquinones	Brontrager's test
5	Triterpenes and steroids	Leiberman's and Salkowski's test
6	Alkaloids	Dragendorff's test

3.4.1 Test for Anthraquinones

Brontrager's test

The crude extract (0.1g) of *C. stenopetala* was dissolved in 10 mL of chloroform and shook properly and filtered. 5 mL of 10% of ammonia solution was added to the filtrate and stirred. There was no colouration observed in any of the extracts.

3.4.2 Test for Phenols and Tannins

Crude extract (20 mg) was mixed with 2 mL of 2% solution of $FeCl_3$. A blue-green or black coloration or precipitation indicated the presence of phenols and tannins.

3.4.3 Test for Saponins

Frothing test

The persistent frothing test for saponin described by (Yadav and Agarwala 2011) was used. To 20 mg of the extract, 5 mL of tap water was added. The mixture was vigorously shaken and heated to boil. Frothing that persisted for 30 min shows the presence of saponin.

3.4.4 Test for Alkaloids

Dragendorff's Test:

Crude extract 5 mg was mixed with 10 mL of 1% HCl and heated gently. Few drops of the following reagents, Dragendorff's, Mayer's and Wagner's were added separately and colour and precipitate formation was observed.

3.4.5 Test for Flavonoids

Ammonia test

The crude extract (20 mg) dissolved in 5 mL of distilled water and filtered. 5 mL of diluted ammonia solution was added to a portion of the aqueous filtrate of the plant extract, followed by addition of concentrated sulphuric acid. A yellow colouration was observed in each extract, indicating the presence of flavonoids.

Alternative test for flavonoids

1 g of the powdered plant (*C. stenopetala*) sample was heated with 10 mL of ethyl acetate over a steam bath for 5 min. The mixture was filtered and 5 mL of the filtrate was shaken with 1 mL

of dilute ammonia solution. A yellow colouration indicated the presence of flavonoids.

3.4.6 Test for Steroids and Triterpenes

Leiberman's test

Crude extract was mixed with each of 2 mL of chloroform and 2 mL of acetic acid. The mixture was cooled in ice. Carefully concentrated H_2SO_4 was added. A colour change from violet to blue to green indicated the presence of steroidal nucleus, glycine portion of glycoside.

Salkowski's Test

Crude extract was mixed with 2 mL of chloroform. Then 2 mL of concentrated H_2SO_4 was added carefully and shaken gently. A reddish brown colour indicated the presence of steroidal ring, glycone portion of the glycoside.

3.5 PHARMACOLOGICAL SCREENING

The extract and/or fractions and isolated compounds were screened for various pharmacological activities:

I. *In-vivo* activities:

- Acute toxicity study
- Anti-malarial activity

II. *In-vitro* activities:

- Antioxidant activity
- Total phenolic content

3.5.1 Acute Toxicity test in Mice

The toxic effect of *C. stenopetala* extract was determined on albino mice, using increasing high doses. Extract dose range of 500 – 8,000 mg kg^{-1} were administered to groups of mice orally. A total of 30 mice were used for the test. The mice were grouped, each group containing five (5) animals weighing between (18 - 22 g). The mice were subjected to 24 h fasting before administering the extracts dissolve in 5 % tween 80. Animals in the negative control group where given 0.2 mL of 5 % tween 80 orally. All animals were kept at room temperature in cross ventilated rooms without illumination at night. The mice were observed for toxicity, distress, abnormal behaviour and fatalities over 48 h and up to 7 days afterwards.

3.5.2 *In vivo* Anti-Plasmodial Assay of Crude Extract, Fractions and the Isolates

The anti-plasmodial activities of the *C. stenopetala* root bark extract and the fractions were assessed by the classic 4-day suppressive test (Peters, 1967). Mice, weight 18 - 22 g were inoculated intraperitoneally (i.p.) with blood containing 1 x 10^7 parasitized (CQ-sensitive *P. berghei berghei* NK 65 strain) erythrocytes contained in 0.2 mL inoculum on Day zero (D_0). Plant extract was solubilized in 5% tween 80 and administrated within 1 h post-inoculation of mice with the parasite, at different concentrations (250, 500 and 1000 mg kg^{-1}/day) in a dose volume of 0.2 ml. Groups of five mice (five mice in each group), were dosed orally using cannula for 4 consecutive days (D_0 – D_3). On D_4 (day 4 post treatment), tail blood smears were taken, fixed and stained with 10% Giemsa in phosphate buffer, pH 7.2 for 30 min and examined under microscope at 100 x. The % suppression of parasitaemia was calculated by comparing the parasitaemia present in infected controls with those of tested mice. Chloroquine (5 mg kg^{-1}) and artesunate (10 mg kg^{-1}) were used as positive control while 5% tween 80 was the negative control.

$$\text{Average Parasitaemia (\%)} = \frac{\text{Number of parasitized RBC}}{\text{Total number of RBC}} \times 100$$

$$\text{\% suppression} = \frac{\text{APU} - \text{APTg}}{\text{APU}} \times 100$$

Where APU is average parasitaemia in negative control and APTg is average parasitaemia in test group. The above procedure was done for the crude extract, the obtained fractions and the isolates to assess their anti-plasmodial activity.

3.5.3 Evaluation of Antioxidant Activity of Crude Extract, Fractions and the Isolates

The determination of the free radical scavenging activity of crude extract was carried using the DPPH (1, 1-diphenyl-2- picrylhydrazyl) assay as described by (Mensor *et al*. 2001) with a slight modification. This is an indicator of free radical scavenging capacity. DPPH radical is reduced from a stable free radical, which is purple in colour to diphenylpicryl hydrazine, which is yellow. Various concentrations of 0.01, 0.02, 0.05, 0.2, 0.5 and 1 gm mL^{-1} of sample in methanol were prepared. To 1 mL of each solution, 3 mL of methanol was added and then 1 mL of a 1 mM DPPH in methanol was added to make up to 5 mL. The mixture was shaken and allowed to stand at room temperature in a dark chamber for 30 min. The change in colour from deep violet to light yellow was then measured at 517 nm on a spectrophotometer.

The experiment was repeated with ascorbic acid (0.005, 0.01, 0.02, 0.05, 0.1, 0.2 mg mL^{-1}) which served as positive control. All determinations were carried out in triplicates. The same procedure was repeated using blank solution DPPH without the extract.

The decrease in absorbance was then converted to percentage scavenging activity (% SA) using the formula:

DPPH radical scavenging activities percentage (%SA) = $[(A_b-A_s)/A_b] / x100$

Where:

A_b= Absorbance of the blank solution

A_s= Absorbance of the test (extract) solution or the standard (ascorbic acid)

The scavenging reaction between (DPPH) and an antioxidant (HA) can be written as:

(DPPH) + (H-A) → DPPH-H + (A) Purple Yellow

3.5.4 Determination of the Total Phenolic Content of the Crude Extract (TPC)

The total phenolic content of the crude extract was determined with the modified Folin-Ciocalteu reagent (McDonald *et al*., 2001). For the preparation of the gallic acid calibration curve, 1 mL aliquots of 0.005, 0.01, 0.02, 0.05, 0.1 and 0.2 mg mL^{-1} ethanolic gallic acid solutions were mixed with 5 mL Folin-Ciocalteu reagent (diluted tenfold) and 4 mL (75 g L^{-1}) sodium carbonate. The absorbance was read after 1 h at 610 nm and the calibration curve drawn. Ethanolic extract of crude extract (1 mL) was mixed with the same reagent as described above and after 1 h, the absorbance was measured for the determination of the total plant phenolic content. All determinations were performed in triplicates (n = 3). The blank solution was made up of 5 mL Folin-Ciocalteu reagent, 4 mL sodium carbonate solution and 1 mL ethanol. The total phenolic content was expressed as milligrams of gallic acid equivalent (GAE) per gram of the methanolic extract calculated thus;

$T = CV/M$

Where

T = total phenolic content, mg/ml of extract, in GAE

C = the concentration of gallic acid in the plant extract established from the calibration curve, mg/ml

V = the volume of extract, mL

M = the weight of methanolic crude extract, g

3.6 PURIFICATION, ISOLATION AND SPECTROSCOPIC CHARACTERIZATION OF COMPOUNDS

3.6.1 Chromatographic Materials

Silica gel 60 F_{254} pre-coated aluminum sheets 20 x 20 cm wide and 0.25 mm thick (Merck, Germany), for analytical purpose were used for TLC analysis in this isolation of natural products research.

Silica gel 60 (0.063 - 0.200 mm mesh and 0.040 - 0.063 mm) (Merck, Germany) and Sephadex LH-20 (Sigma-Aldrich, St. Louis, MO, USA) were used as stationary phases for column chromatography. Various detecting agents used were: UV (254 and 366 nm), spraying with 1% Vanillin/H_2SO_4 and Dragendorff for detecting alkaloids.

3.6.1.1 Thin Layer Chromatography (TLC) Plates

Silica gel 60 F_{254} pre-coated aluminum sheets 20 x 20 cm wide and 0.2 mm thick (Merck, Germany) were used for the TLC analysis. With a pencil, the starting line (2 cm from the bottom of the plate) and finishing line (about 4 - 5 cm from top of the plate, solvent front) were marked. Samples were applied by using capillary tube on the starting line.

3.6.1.2 Preparation of the TLC Development Chamber

Enough of the appropriate developing solvent system was added into a development chamber/jar so that it is 0.5 to 1 cm deep in the bottom of the jar and covered with a tight fitting lid. The jar was closed tightly, and left to stand for about 30 min so that the atmosphere in the jar becomes saturated with the solvent.

Different solvent systems were used for the various TLC done.

3.6.1.3 Developing the Plates

After preparing the development chamber and spotting the samples on the TLC plates, the plates were ready for development. The plates were placed gently inside the development chamber and the mobile phase allowed running till almost at the top end of the plates (solvent front). The plates were removed from the chamber and the solvent front traced with a pencil.

The developed plates were first observed under the UV (254 and 366 nm) for locating chromophoric compounds and pencil marked seen spots. Further detection was achieved by spraying with 1% Vanillin/H_2SO_4. The sprayed plates were heated at 100^oC for about 2 to 3 min. Spots of different colours developed according to the nature of the compounds. 1% Vanillin/H_2SO_4 spray is generally used to visualize hydrocarbon compounds. This was prepared by dissolving 1g of vanillin powder in 100 mL of H_2SO_4. Other detecting reagents used were Dragendorff for detecting alkaloids and DPPH.

3.6.1.4 Preparation of Open Column Chromatography (CC)

Silica gel 60 (0.063 - 0.200 mm and 0.040 - 0.063 mm) (Merck, Germany) and Sephadex LH-20 (Sigma-Aldrich, St. Louis, MO, USA) were used as stationary phases for column chromatography (Hostettmann *et al.* 1998,). Various solvent systems were used for the packing and for elution.

The sephadex LH-20 was soaked over 24 hours with the solvent prior to packing and elution. The elution was usually 1 mL/min but slower when better resolution was required for gel filtration.

The columns were wet packed manually with gentle tapping to ensure that no air bubble was formed, thus the entrapment of air was avoided and care was taken not to dry the column

throughout the running of any of the fractionations. The samples were either introduced to the columns as slurry (dry loading) or solution (wet loading).

3.6.2 Separation of Chemical Constituents of Hexane Fraction of *C. stenopetala* (Hex)

Silica gel: 0.040 – 0.063 mm (Merck)

The Hexane fraction (4.5 g) was subjected to silica gel CC (7 by 75 cm). Step-wise gradient elution with solvent system dichloromethane: methanol (DCM: MeOH) was used, starting with a non-polar solvent (DCM, 100%) and gradiently increasing the polarity with MeOH. Two hundred and eighty (282) successive fractions were collected at 25 mL/20 min. Similar fractions were combined affording 22 fractions (C_1 – C_{22}) based on TLC analysis. The TLC solvent systems used were 100% DCM and Hex: EtOAc (1: 1). Fraction C_2 (750 mg) was subjected to further purification (rechromatography CC), eluting gradiently with solvent system Hexane: DCM. Eighty (80) fractions were collected at 15 mL/8 min. Similar fractions were combined based on TLC analysis to give rise to 10 fractions ($C_{2;1}$ - $C_{2;10}$). TLC mobile phase solvent system used was Hex: DCM (5: 6). Fraction $C_{2;8}$ (18-19) precipitated glassy cube like crystals that settled at the bottom of the glassware collector (120 mg). This was further purified by re-crystallization by dissolving in 6 drops of hexane and 20 mL of ethyl acetate added to the super saturated solution. This was covered with aluminum foil and left untouched/undisturbed. Glassy and cubed crystals were formed by the second week. The crystals where removed from the mother liquor by filtering with sintered glass and dried by pump suction and further air dried, affording 84 mg of compound **1**.

Fraction $C_{2;10}$ (27-31) precipitated as fine network like crystals (like cobwebs) that settled at the top of the glassware collector (211 mg). This was also further purified by re-crystallization by

forming super saturated solution in EtOAc and 20 mL of hexane affording 26 mg of compound **2**.

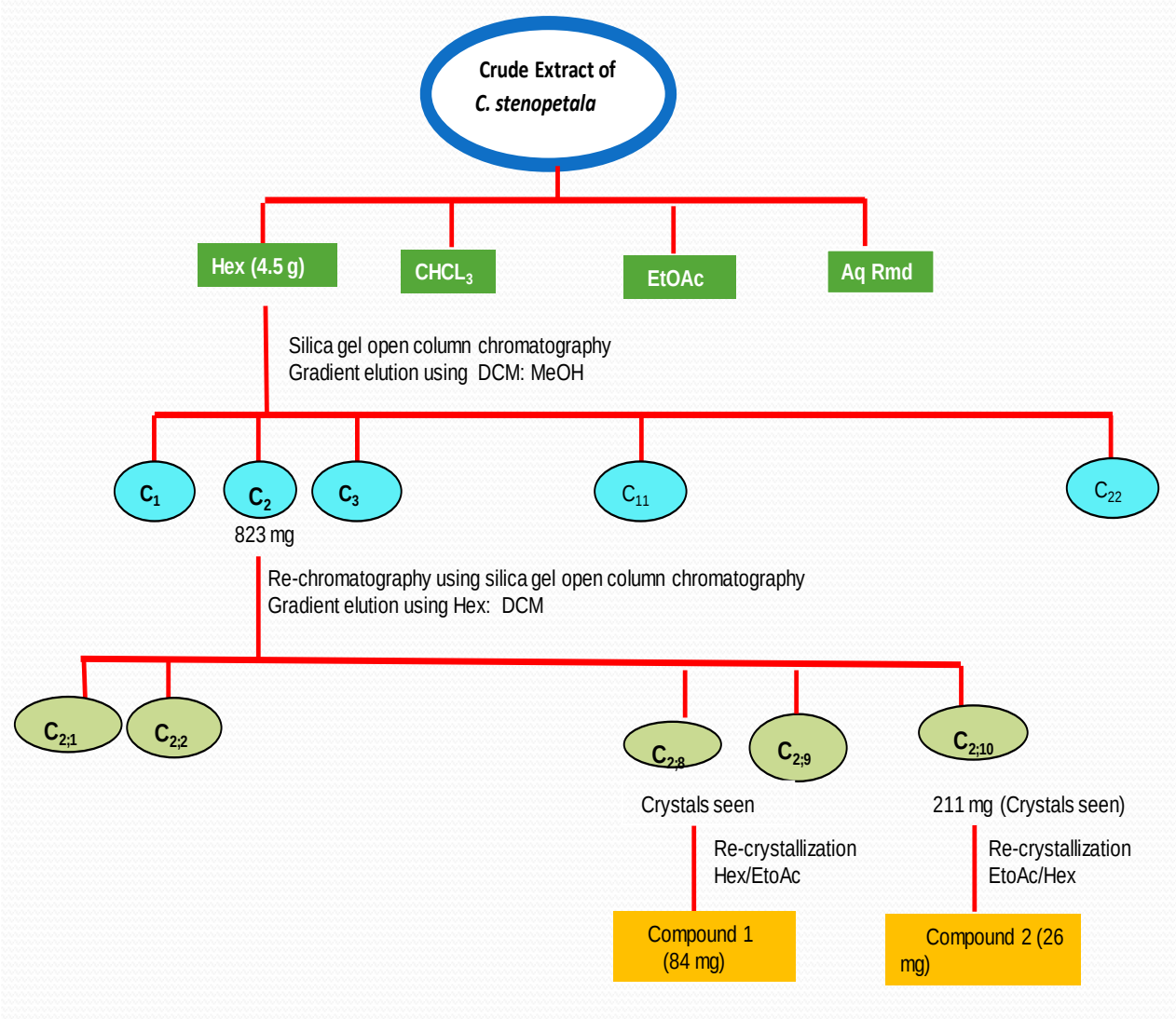


Figure 10: Isolation scheme of compounds 1 and 2

3.6.3 Separation of Chemical Constituents of Chloroform Fraction of *C. stenopetala* ($CHCl_3$)

The $CHCl_3$ fraction (10 g) was subjected to open CC (7 by 75 cm). The column was eluted with a solvent system of EtOAc: MeOH: H_2O (100: 13.5: 10). One hundred and sixty three (163) fractions were collected at 20 mL/30 min. Similar fractions were combined affording 8 fractions (V_1 – V_8) based on TLC analysis on silica gel. The TLC solvent system was $CHCl_3$: MeOH: H_2O (65: 35: 5) for the initial fractions and EtOAc: MeOH: H_2O (100: 13.5: 10) for the later fractions. Fraction V_3 (32 – 40) had crystals that were round shaped that settled at the bottom and stuck to the walls of the glassware collector. This was further purified by re-crystallizing using $CHCl_3$: MeOH (1:10) yielding whitish-yellow compound **3** (704 mg).

3.6.3.1 Recrystallisation

The crystal collected above (fraction 3, test tubes 32 – 40, weight 754 mg - weigth before final re-crystallisation) was dissolved in 10 mL of $CHCl_3$ (very readily soluble in $CHCl_3$ at room temperature) to form a super saturated solution formed. 100 mL of methanol was added to the super saturated solution and covered very closely with aluminum foil and left untouched/undisturbed. Small slight yellowish white round crystals were seen by the third day.

The crystals were filtered out by the 7th day using sinter glass from the mother liquor under suction pump. The sinter glass funnel containing the dried crystal was covered with perforated aluminum foil to allow further air drying. 704 mg of compound **3** was gotten, percentage yield was 7.00%.

3.6.4 Isolation of Chemical Constituents of EtOAc Fraction

The above process for $CHCl_3$ fraction was repeated for the EtOAc fraction (4 g) to afford the same compound **3**. The same eluting solvent system and column packing system were used. Crystal deposition was observed in the fraction (34 – 41). A yield of 489 mg of compound **3** was gotten after recrystallization. Co-TLC of compound obtained (from both fractions) showed them to have the same R_f value. Both have the same solubility property, same colour on TLC and ^1H NMR spectra.

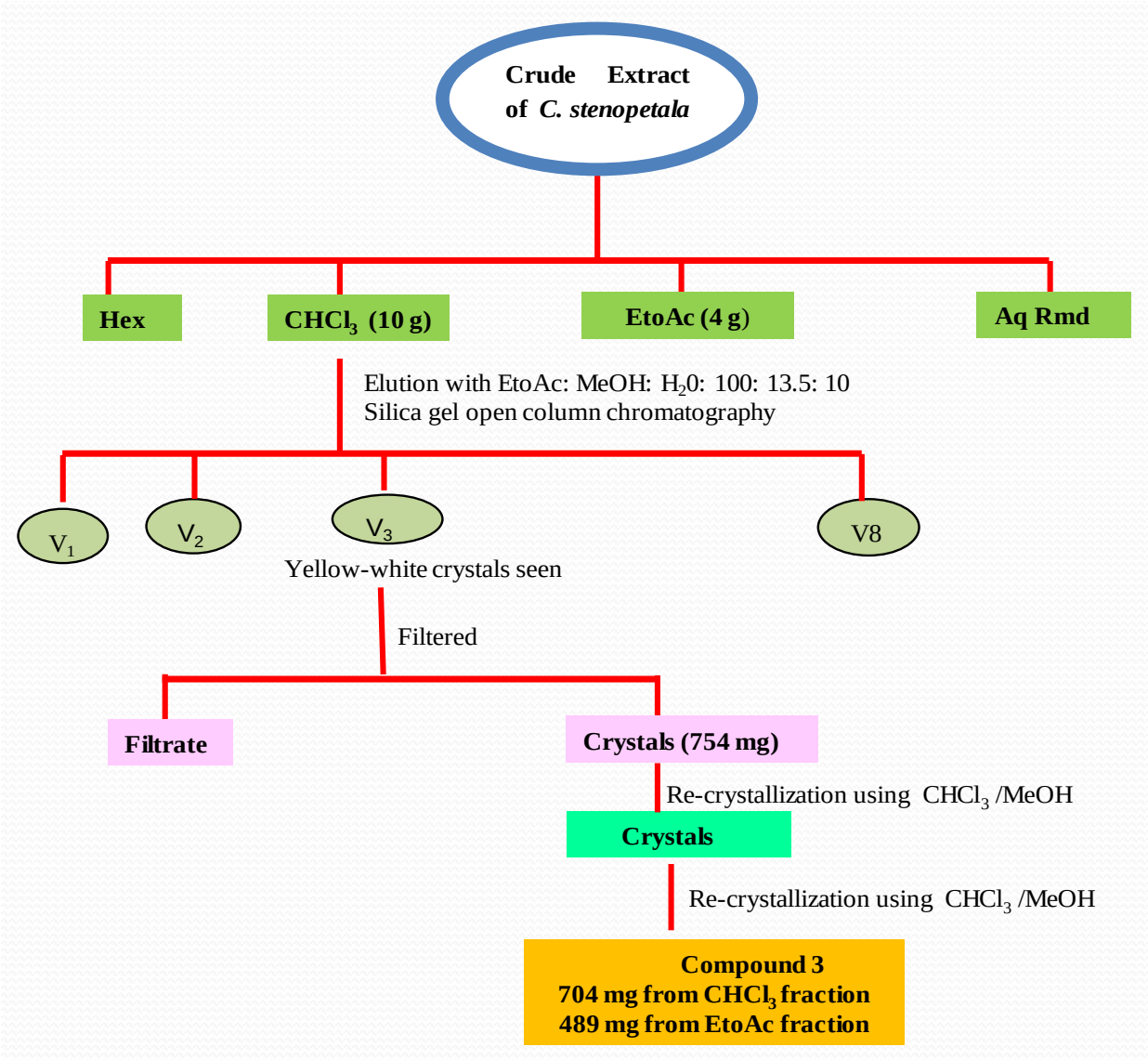


Figure 11: Isolation scheme of compound 3

3.6.5 GC-MS Studies

Compounds 1, 2, 3 were subjected to GC-MS, as part of a process towards determination of their configurations. The instrument Shimadazu GCMS-QP2010 was used for the experimental analysis. The operating temperatures include; column (AOC-20i) oven temperature: 40.0 ^{o}C; injection temperature 120 ^{o}C; ion source temperature 220.0 ^{o}C and interface temperature 300.0 ^{o}C. Carrier gas Helium was used at a flow rate of 0.76 mL/min and average velocity of 31.4 cm/sec. The temperature was programmed to rise by 10°C/min and to be held for 2 min from an initial temperature of 90 ^{o}C to a final temperature of 200 ^{o}C.

3.6.6 Spectroscopic Analysis

This technique is an important method in structure elucidation of constituents from of natural products and can be done in many different ways. All the isolated compounds were subjected spectroscopic evaluation.

3.6.6.1 Nuclear Magnetic Resonance Spectra (NMR)

Nuclear magnetic resonance spectrometry (NMR) was used in the bid to unraveling the structures of the isolated compounds. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance II 600 spectrometer (600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR). Deuterated chloroform was used as solvent for the various samples and the operating temperature was set at 25°C. The shifts were indicated in ppm (part per million) relative to tetramethylsilane (TMS) which served as an internal standard for ^{1}H spectra, and the deuterated solvent shift as reference for ^{13}C spectra. In order to observe homo- and heteronuclear correlations between proton and carbon atoms of the analytes, complementary two dimensional (2D) experiments were performed. For advance and 2D spectra including APT, COSY, NOESY, ROESY, HSQC and HMBC, the standard pulse sequence and processing macros were employed as provided in the

original software.

3.6.6.2 Infrared Spectroscopy (IR)

Varian 680-IR spectrophotometer was used. A thin microfilm of the isolated compound was prepared, placed in the optical path of the instrument and scanned over 450-4000 cm^{-1} frequency region at 1 cm^{-1} interval. The absorption bands were obtained and recorded as frequency (cm^{-1}). The spectrum was interpreted to deduce the functional groups present in the compounds.

3.6.6.3 X-ray Diffraction (Crystallography)

Bruker Kappa Apex Duo was used.

3.6.7 Melting Point Determination

The melting points of the solid isolates were determined using the Fisher- Johns apparatus, Fischer Scientific Pittsburg Pa. A small sample of the compound was placed on the microscopic field and a gradual increase in temperature was constantly applied until melting of the compound was observed.

Statistics

The results were analyzed statistically using one-way ANOVA/two-tailed student t-tail to identify the difference between the treated and control groups. $P < 0.05$ is considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 PLANT SEARCH AND DETERMINATION OF PLANT FOR STUDY

The search for medicinal plants used locally within the South-eastern part of Nigeria in treatment of malaria produced seven (7) different medicinal plants. These 7 medicinal plants produced 9 crude extracts because 2 of the collected medicinal plants (*Callichilia stenopetala* Stapf. Apocynaceae and *Petersianthus macrocarpus* P Beauv. Liben) have two methods of application, utilizing two different plant parts (Table 6). Thus, nine (9) different medicinal plant crude extracts/agents were bio-screened. Noted in the plant search is that both *C. stenopetala* Stapf. root bark and leaf are two different treatment preparations in the malaria fever management. These 2 parts of this plant were evaluated for their antiplasmodial and free radical scavenging activities (Tables 7 – 9). The result indicated that the *C. stenopetala* Stapf. root bark has better bio-activities as analysised than *C. stenopetala* Stapf. leaf (Table 9).

Noted also, is the use of either the stem bark or leaf of *P. macrocarpus* P Beauv. Liben in preparation of herbal medicine against malaria fever (Table 6). These 2 parts of this medicinal plant were evaluated for their antiplasmodial and free radical scavenging activities (Tables 7 – 9). *Petersianthus macrocarpus* stem bark was recorded to have better activities in parasitaemia chemosuppression and free radical inhibition than the leaf crude extract preparation (Table 7 – 9).

The remaining five (5) plants, (*Asystasia gangentica*, *Oldenlandia affinis*, *Oldenlandia corymbosa*, *Borreria ocymoides* and *Picralima nitida*) subjected to bio-screening showed

varying antiplasmodial and antioxidant activities as indicated by the antimalarial and DPPH free radical scavenging activity studies (Tables 7 – 9).

The bioassay result summary (Table 9) indicated that 5 of crude extracts/agents were very potent as antiplasmodial and as antioxidant agents; 2 crude extracts/agents exhibited moderate activity as antiplasmodial and very good antioxidant activity, while 2 crude extracts /agents exhibited moderate antiplasmodial and poor antioxidant activities (Table 9).

Four (4) different plant families were represented namely; Acanthaceae, Apocynaceae, Rubiaceace and Lecythidaceae. Apocynaceae and Rubiaceace have the highest number of plants in the list, which may be indicating the high rate of occurrence of antimalarial activity in these families. The part of the plants used for the antimalarial herbal preparations were noted, which include the root bark, the leaf, stem bark etc. The route of administration of the medicinal plants were basically via oral and preparation mainly decoction.

Table 6: List of some medicinal plants used in treatment of malaria fever

Plants	Family	Part used
Asystasia gangentica T. Anderson	Acanthaceae	Aerial
Callichilia stenopetala Stapf	Apocynaceae	Root bark
Callichilia stenopetala Stapf	Apocynaceae	Leaf
Oldenlandia affinis (R & S) DC	Rubiaceace	Aerial
Oldenlandia corymbosa Linn	Rubiaceace	Whole plant
Petersianthus macrocarpus (P Beauv.) Liben	Lecythidaceae	Stem bark
Petersianthus macrocarpus (P Beauv.) Liben	Lecythidaceae	Leaf
Borreria ocymoides	Rubiaceace	Aerial
Picralima nitida Durand and Hook	Apocynaceae	Leaf

The search for medicinal plants used locally within the South-eastern part of Nigeria in treatment of malaria fever and/or re-current fever produced seven (7) different medicinal plants. Two (2) of the collected medicinal plants (*Callichilia stenopetala* Stapf. Apocynaceae and *Petersianthus macrocarpus* P Beauv. Liben) have two methods of application, root bark and leaf; stem bark and leaf respectively (Table 6). Thus, making it nine (9) different medicinal plant crude extracts/agents bio-screened.

Table 7: Suppressive activity of some antimalarial medicinal plants crude extracts at 500 mg kg^{-1}

Plants	Dose mg kg^{-1}	Average Parasitaemia	% Parasitaemia Suppression
Asystasia gangentica		1.70	45.68
Callichilia stenopetala (Root bark)		0.60	80.30
Callichilia stenopetala (Leaf)		2.20	29.70
Oldenlandia affinis		2.14	31.63
Oldenlandia corymbosa		1.38	55.91
Petersianthus macrocarpus (stem bark)		0.66	78.90
Petersianthus macrocarpus (Leaf)		2.19	30.03
Borreria ocymoides		0.87	72.20
Picralima nitida		0.52	83.39
Neg control (5% Tween 80)	0.2 ml	3.13	
Positive control (Chloroquine)	5	0.56	82.11

n = 5

The nine (9) different crude extracts obtained from the seven (7) medicinal plants exhibited varing degrees of parasitaemia chemosuppression. The crude extracts exhibiting 30 to 40% *in vivo* parasitaemia suppression in mice malaria models were considered to be moderately active (Muregi *et al.*, 2006; Pereira *et al.*, 2014).

Table 8: DPPH free radical scavenging activity of some antimalarial medicinal plants crude extracts

Plants	Absorbance at 517 nm 1 mg mL^{-1}	% Inhibition (DPPH)
Asystasia gangentica (Aerial)	1.477 ± 0.03	23.59
Callichilia stenopetala (Root bark)	0.30 ± 0.50	84.50
Callichilia stenopetala (Leaf)	0.44 ± 0.01	76.92
Oldenlandia affinis (Aerial)	1.494 ± 0.41	22.71
Oldenlandia corymbosa (Whole plant)	0.356 ± 0.02	81.58
Petersianthus macrocarpus (Stem bark)	0.16 ± 0.03	91.72
Petersianthus macrocarpus (Leaf)	0.24 ± 0.28	87.58
Borreria ocymoides (Aerial)	0.605 ± 0.11	68.72
Picralima nitida (leaf)	0.535 ± 0.01	72.30
Blank	1.933 ± 0.02	

Values are expressed as mean ± SEM; n = 3

The free radical scavenging activity of the medicinal plant crude extracts using DPPH test procedure indicated that only 2 of the crude extracts (aerial part of *Asystasia gangentica* and aerial part of *Oldenlandia affinis*) exhibited poor activity.

Table 9: Bio-activities summary of some antimalarial medicinal plants crude extracts

Plants	Family	Part used	% Parasitaemia Suppression (500 mg kg^{-1})	% Inhibition (DPPH) 1 mg mL^{-1}
Asystasia gangentica T. Anderson	Acanthaceae	Aerial	45.68	23.59
Callichilia stenopetala Stapf	Apocynaceae	Root bark	80.30	84.50
Callichilia stenopetala Stapf	Apocynaceae	Leaf	29.70	76.92
Oldenlandia affinis (R & S) DC	Rubiaceace	Aerial	31.63	22.71
Oldenlandia corymbosa Linn	Rubiaceace	Whole plant	55.91	81.58
Petersianthus macrocarpus (P Beauv.) Liben	Lecythidaceae	Stem bark	78.90	91.72
Petersianthus macrocarpus (P Beauv.) Liben	Lecythidaceae	Leaf	30.03	87.58
Borreria ocymoides	Rubiaceace	Aerial	72.20	68.72
Picralima nitida Durand and Hook	Apocynaceae	Leaf	83.39	72.30

The different crude extracts exhibited varying parasitaemia suppression and DPPH percentage inhibition but no relationship/correlation is observed between the two bio-activities in these crude extracts.

4.2 Extraction of *C. stenopetala* root bark

Methanol (90%) was chosen as the solvent of extraction because of its close resemblance to medium used in soaking the herb locally before administration and preliminary studies also showed methanol extract to be the most potent. The yields of the extraction and fractionation are shown in Table 10.

Percentage yield of *C. stenopetala*

Weight of plant material = 1.4 kg
Weight of extract obtained = 134.00 g

$$\% \text{ yield} = \frac{\text{weight of extract}}{\text{weight of plant material}} \times 100$$

$$\frac{134}{1400} \times 100 = 9.57\%$$

4.2.1 Fractionation of *C. stenopetala* root bark

Table 10: Liquid – Liquid fractionation of crude extract of *C. stenopetala* root bark

Fractions	Qty (g) obtained (118.00 g)	Percentage yield (%)
Hexane	17.46	14.79
Chloroform	22	18.64
Ethyl acetate	7.66	6.49
Aqueous residue	68	57.63

Key: Qty: Quatity

More of the mid- polar to polar constituents were extracted due to the extraction technique using methanol.

4.3 PRELIMINARY PHYTOCHEMICAL ANALYSIS OF C. STENOPETALA ROOT BARK

Upon preliminary phytochemical screenings, the crude extract tested positive for alkaloids, flavonoids, saponins, terpenoids, sterols, phenols and tannin (Table 11). The extract of *C. stenopetala* tested positive to the presence of saponins which are known to possess both antimicrobial and anti-inflammatory activities (Mathias *et al*., 2007). Blue-black precipitate exhibited on testing with 1% ferric chloride, which indicated the presence of tannins. Tannins have been reported to possess antimicrobial properties, and as a result, it is a very useful plant metabolite that could lead to drug production.

The fractions, chloroform and ethyl acetate showed high percentage of alkaloids, while the hexane and the aqueous fractions showed very low percentage of alkaloidal content (Table 12; Figure 12).

Table 11: Phytochemical screening of the crude extract of *C. stenopetala* root bark

Test	*C. stenopetala* Root bark
Alkaloid	+++
Alkaloid (after purification)	+++
Saponin	++
Phenol	+ +
Flavonoid	++
Terpenoid	+
Steroid	++
Anthraquinone	-
Tannin	+

KEY: -

- - Absence
- + Presence

The result of the phytochemical test presence in the crude extracts of *C. stenopetala*.

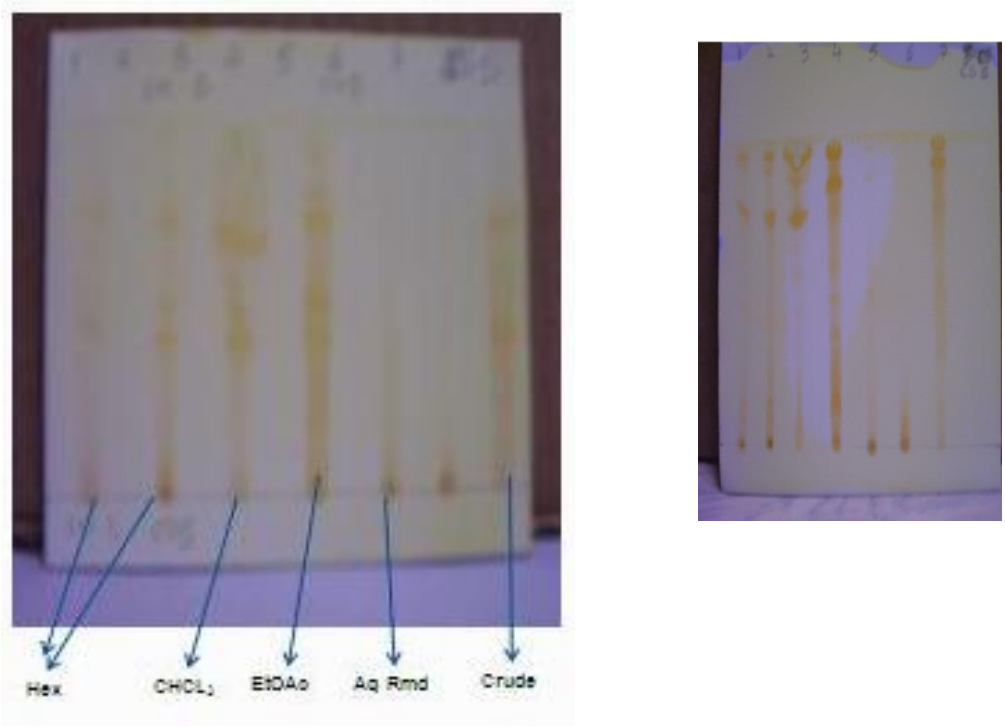


Figure 12a and 12b: TLC chromatograms showing the alkaloidal content of various fractions of *C. stenopetala*

Detecting agent : Dragendorff

Solvent sys: EtOAc: MeOH; H_2O (100: 13.5: 10)

Stationary phase: Silica gel

TLC chromatography of the various fractions of *C. stenopetala*, confirmed the presence of alkaloid in the various fractions of *C. stenopetala.* The TLC plates showed that there were less of alkaloidal compounds in the hexane and aqueous fraction. Increase in alkaloidal content was observed within the mid-polar fractions, chloroform and ethyl acetate.

Table 12: Alkaloidal content of the fractions

Fractions	Qty (+)
Hexane	+
Chloroform	+++
Ethyl acetate	+++
Aq residue	+
Rauwolfia (Positive control)	+++

KEY: -

- - Absence
- + Presence

Table showing the alkaloidal content of the individual fraction of *C. stenopetala*. Chloroform and ethyl acetate fractions, both have very high content of alkaloids compared with the positive drug- *Rauwolfia.*

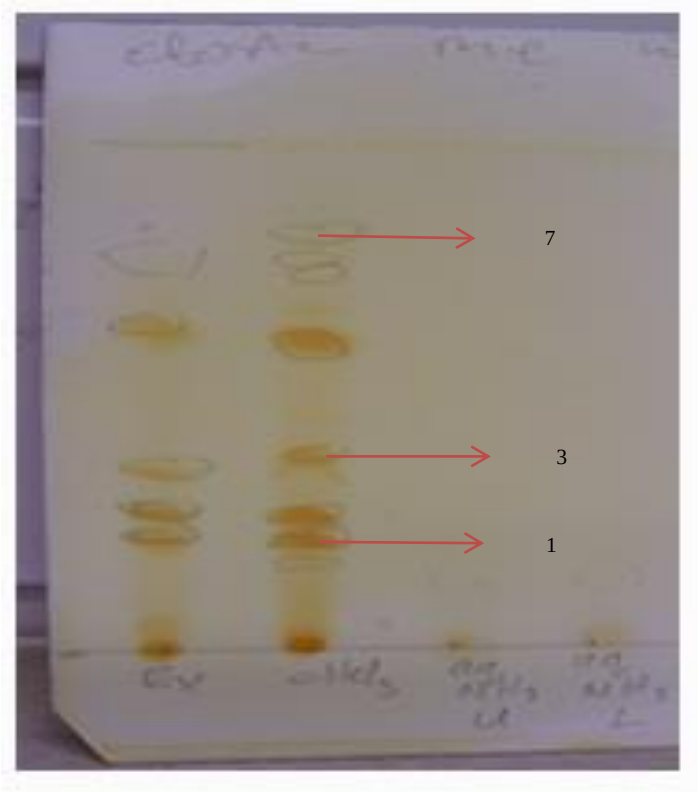


Figure 13: TLC chromatograms of the $CHCl_3$ fraction of *C. stenopetala* showing alkaloidal content

Detecting agents: UV (pencil mark) and Dragendorff (Brown-yellow colour)

Mobile phase: EtOAc: MeOH: H_2O (100: 13.5: 10)

Stationary phase: Silica gel F_{254}

Table 13: TLC result of alkaloidal content of chloroform ($CHCl_3$) fraction

Spot	UV (254 nm)	R_f	Dragendorff reagent	R_f
1	Blue	0.26	Brown-yellow	0.26
2	Blue	0.32	Brown-yellow	0.32
3	+ve	0.45	Brown-yellow	0.45
4	+ve	0.51	-ve	-
5	Blue	0.69	Brown-yellow	0.69
6	+ve	0.78	-ve	-
7	+ve	0.89	-ve	-

Table showing the calculated R_f values of different separated spots detected using UV and Dragendorf as detecting agents.

4.4 PHARMACOLOGICAL ACTIVITY

4.4.1 Acute Toxicity

Oral administration given in graded doses (500 to 8,000 mg kg^{-1}) produced no noticeable deleterious effect 24 h after dosing and up to 7 days afterwards. No physical or behavioural changes were observed in the experimental mice, thus no visible sign(s) of distress or overt toxicity like loss of appetite, hair erection, sleep, respiratory distress, stretching of the entire body, salivation, diarrhoea and the like was observed. This suggests that LD_{50} of the methanolic crude extract of *C. stenopetala* was greater than 8000 mg kg^{-1}.

4.4.2 Antimalarial activity

4.4.2.1 Antimalarial Activity of Crude Extract of *C. stenopetala*

The four-day suppressive Peters method used for this test revealed that the root bark crude extract of *C. stenopetala* has significant activity ($P< 0.05$) that is dose dependent compared with the reference standard chloroquine 5 mg kg^{-1} and artesunate 10 mg kg^{-1} against chloroquine sensitive strain of *Plasmodium berghei berghei,* NK 65 strain. The 500 and 1000 mg kg^{-1} of the crude extract exhibited parasite suppression (80.31 and 89.46% respectively) compared to the positive controls, chloroquine and artesunate (82.11 and 73.80%) respectively (Table 14).

Table 14: Suppressive activity of crude extract of *C. stenopetala* root bark on parasitaemia in mice

Treatment	Dose (mg kg^{-1})	Average Parasitaemia	% Chemosuppression
5% tween 80	0.2 ml	3.13 ± 0.11	
Chloroquine	5	0.56 ± 0.05	82.11
Artesunate	10	0.83 ± 0.06	73.80
C. stenopetala root bark extract	250	0.96 ± 0.19	69.32
	500	0.60 ± 0.08	80.31
	1000	0.33 ± 0.06	89.46

Values are expressed as mean ± SEM; n = 5

The root bark crude extract of *C. stenopetala* has significant activity ($P < 0.05$) that is dose dependent compared with the reference standard chloroquine and artesunate against chloroquine sensitive strain of *Plasmodium berghei berghei*, NK 65 strain..

4.4.2.2 Anti-malarial Activity of the Fractions

The hexane, chloroform and ethyl acetate fractions, dose-dependently reduced parasitaemia level compared to the negative group. The hexane fraction at 250 and 1000 mg kg^{-1} has chemosuppression of 82.24 and 95.43% respectively, reduction which is comparable to that of chloroquine (Table 15). The chloroform fraction caused a chemosuppression of 39.99 and 83.72% respectively at the dose levels of 250 and 1000 mg kg^{-1} respectively. Ethyl acetate and aqueous fractions dose-dependently reduced parasitaemia level at a much less extend compared with the two standard control drugs. The rank order of chemosuppression of the solvent fractions at 500 mg kg^{-1} was hexane (91.88%) > chloroform (71.42%) > ethyl acetate (32.81%) > aqueous (11.15%) (Table 15).

The antimalarial activity resides mainly in the non-polar fractions particularly the hexane fraction which dose dependently suppressed the growth of the *Plasmodium* in the 4-day suppressive test (D_0 - D_4) Table 15.

Table 15: Anti-malarial activity of fractions *C. stenopetala* on parasitaemia in mice

Treatment	Dose mg kg^{-1}	Average Parasitaemia	% Parasite Chemosuppression
5% tween 80 in distilled water		45.46 ± 4.33	
Chloroquine	5	8.1 ± 4.12	82.22
Artesunate	10	11.90 ± 1.21	73.80
Hexane	250	8.10 ± 2.50	82.24
	500	3.70 ± 0.62	91.88
	1000	2.08 ± 0.17	95.43
Chloroform	250	27.34 ± 5.79	39.99
	500	13.01 ± 3.62	71.42
	1000	7.40 ± 6.79	83.72
Ethyl acetate	250	37.28 ± 12.54	18.17
	500	30.61 ± 3.33	32.81
	1000	26.82 ± 3.51	41.13
Aqueous Residue	250	42.57 ± 9.39	6.56
	500	40.48 ± 3.40	11.15
	1000	33.64 ± 9.04	26.16

NB: extracts were dissolved in 5% tween 80
Values are expressed as mean ± SEM; n = 5

The hexane, chloroform and ethyl acetate fractions significantly ($P < 0.05$) and dose-dependently reduced parasitaemia level compared to the negative group.

4.4.3 Anti-oxidant Activity

4.4.3.1 Anti-oxidant Activity of the Crude Extract and the Fractions

The crude extract of the root bark of *C. stenopetala* exhibited free radical scavenging activity that is dose-dependent (Figure 14) and activity resides more in the polar fractions than the non-polar fractions as compared with a known standard, ascorbic acid (vitamin C). The DPPH scavenging activity of hexane fraction is the least potent while ethyl acetate was the most potent fraction (Figure 15).

The total phenolic content of the crude extract was 70 mg g^{-1} gallic acid equivalent.

Table 16: **DPPH Free radical scavenging activity of the crude extract and Vit C**

ABSORBANCE AT 517 NM		
Conc mg ml^{-1}	**% Inhibition C**	**% Inhibition VC**
0	0	0
0.005	0	9.93 ± 0.107
0.01	0	17.24 ± 0.010
0.02	0	25.92 ± 0.001
0.05	4.094 ± 0.001	91.05 ± 0.000
0.1	10.45 ± 0.037	93.19 ± 0.002
0.2	25.26 ± 0.013	93.8 ± 0.001
0.5	47.61 ± 0.012	-
1	84.54 ± 0.013	-
2	94.41 ± 0.011	-
5	95.12 ± 0.003	-

Table showing the DPPH radical scavenging activity of the crude extract of *C. stenopetal* root bark and Vitamin C (used as positive control) at 517 nm.

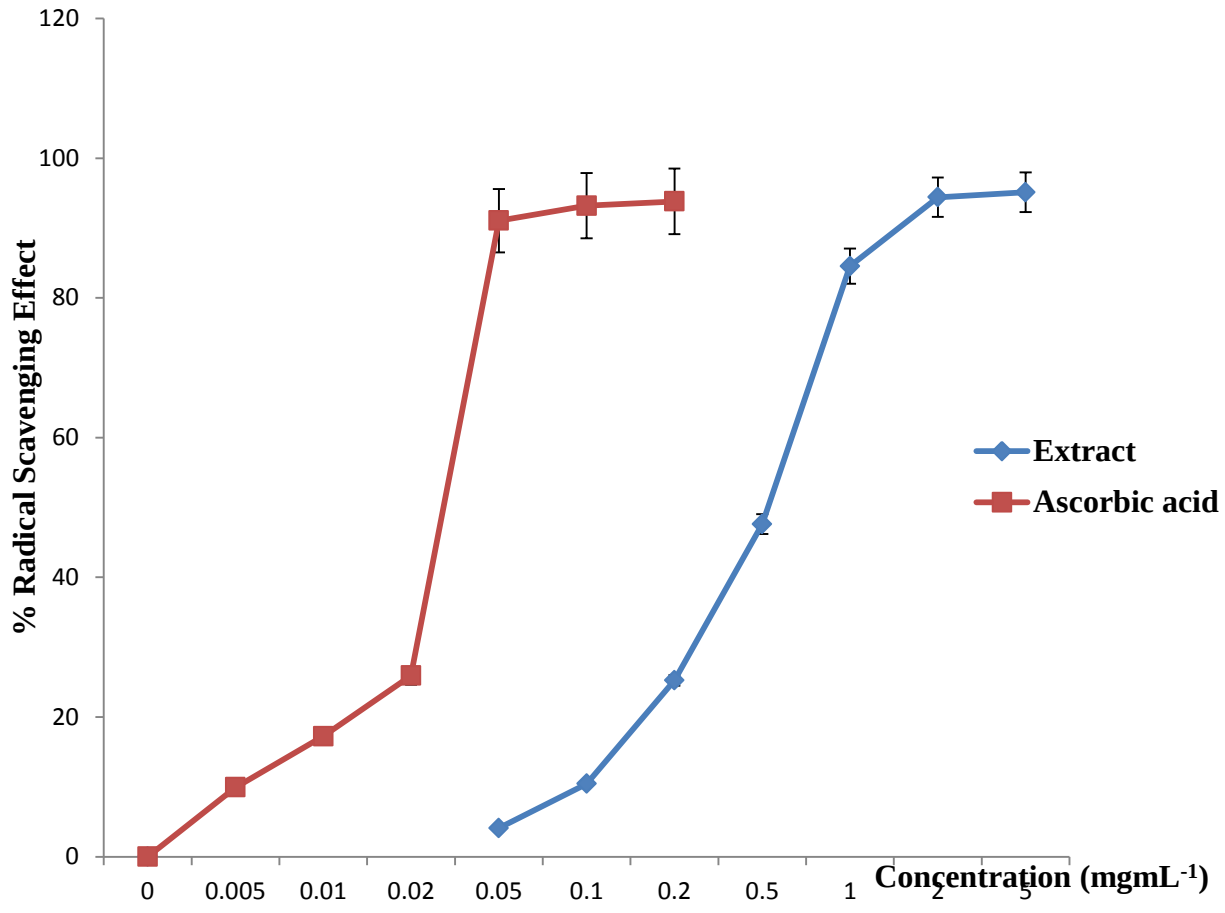


Figure 14: DPPH Free radical scavenging activity of crude extract and Vit C

Graph showing that DPPH radical scavenging activity of the crude extract of *C. stenopetal* root bark and Vitamin C (used as positive control) at 517 nm. Observed activities for both drugs are dose-dependent.

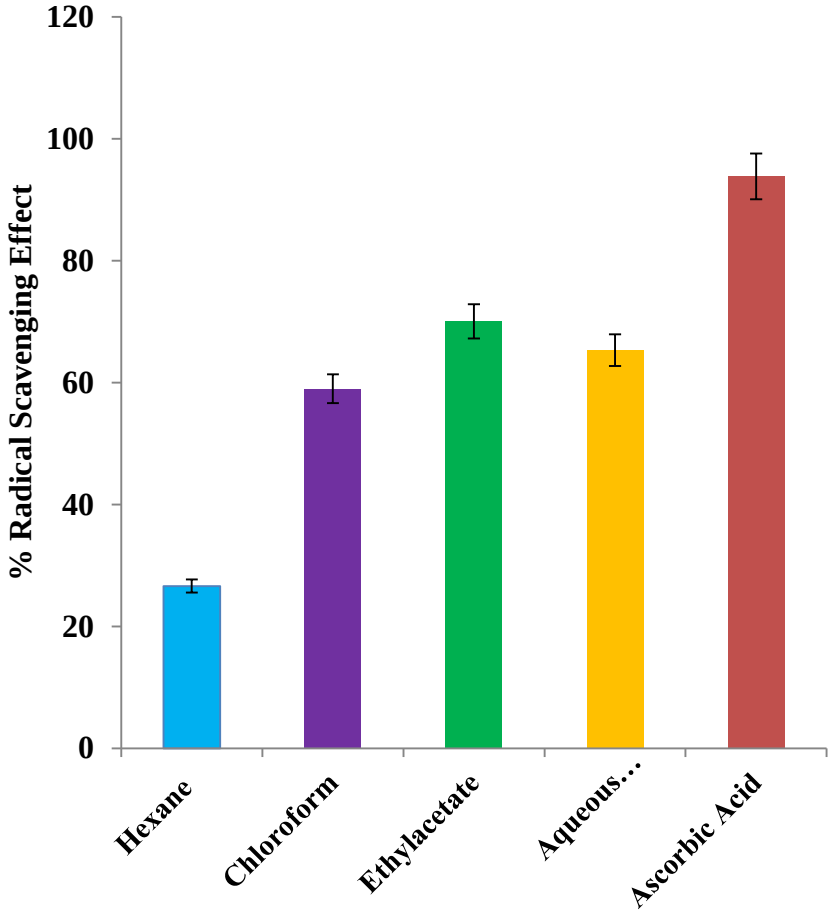


Figure 15: DPPH Free radical scavenging activity of fractions of *C. stenopetala* root bark

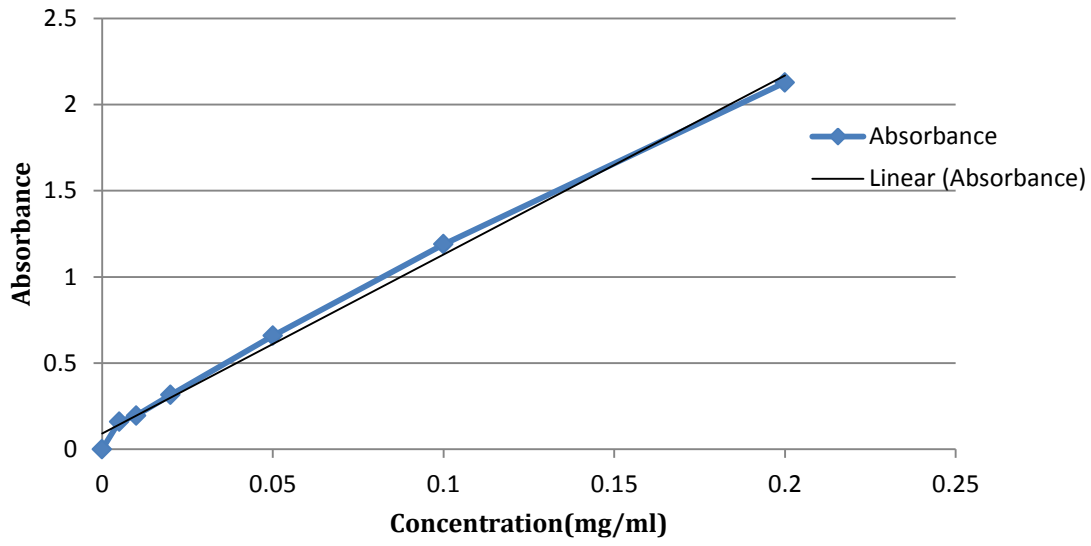
The result of this study reveals that DPPH radical scavenging activity resides more in the polar fractions than the non-polar fractions as compared with a known standard, ascorbic acid (vitamin C).

4.4.4 Determination of total phenolic content (TPC)

Table 17: Calibration data for gallic acid

S/N	Conc mg mL^{-1}	Absorbance at 765 nm
1	0.2	2.128 ± 0.176
2	0.1	1.187 ± 0.016
3	0.05	0.659 ± 0.007
4	0.02	0.315 ± 0.009
5	0.01	0.195 ± 0.005
6	0.005	0.160 ± 0.002

Absorbance value of gallic acid (765 nm) was used to plot the standard gallic acid curve.



Y= 12.24x; R^2= 0.995

Figure 16: Calibration curve for Gallic acid

The total phenolic content of the unknown (*C. stenopetala*) was determined by extrapolating the determined value of *C. stenopetala* on the calibrated Gallic acid curve.

Absorbance of extract at 610 nm = 0.689

Extrapolated conc. in terms of gallic acid = 0.07

Weight of ethanolic plant extract = 0.001 g

Vol of extract solution introduced = 1 mL

TPC = 1mL x 0.07/0.001 g

= 70.00 mg g^{-1}

4.4.5 Antimalarial and Antioxidant Activities of the Isolates

The *in vivo* antimalarial screening of the isolated compounds 1, 2, 3 showed that they were significantly active ($p \leq 0.05$) against chloroquine sensitive strain of *Plasmodium berghei* at various tested doses. Only vobtusine among the isolated compounds was able to exhibit significant scavenging of radicals at 0.2 mg mL^{-1} (68.7%) compared with the reference standard, Vitamin C (Table 18, 19 and Figure 17)

Table 18: Antimalarial activity of the isolates on parasitaemia in rats

Compounds	Dose mg kg^{-1}	Average Parasitaemia	% Parasite Chemosuppression
Compound 1 (α-Amyrin acetate)	10	7.44 ± 1.35	67.61
Compound 2	10	7.20 ± 0.51	68.65
Compound 3 (Vobtusine)	7	8.68 ± 0.46	62.20
Chloroquine	5	1.36 ± 0.142	94.08
7% DMSO in distilled water	0.2 mL	22.97	

Note: Albino rats (Wistar strain, 80-100 g) were used for the antimalarial activity

The *in vivo* antimalarial screening of the isolated compounds 1, 2, 3 showed that they were significantly active ($p \leq 0.05$) against chloroquine sensitive strain of *Plasmodium berghei.*

Table 19: Summary of bioactivity profile of the isolates

Compounds	Dose mg kg^{-1}	% Parasite Chemosuppression	DPPH Free radical Scavenging activity (0.2 mg mL^{-1}) % Inhibition
Compound 1 (α-Amyrin acetate)	10	68.65	14.00
Compound 2	10	67.61	5.80
Compound 3 (Vobtusine)	7	62.20	68.70
Control +Ve (CQ)	5	93.38	-
Vit C			93.80
7% DMSO in distilled water	0.2 mL	-	-

Note: Rats (80-100 g) were used for the antimalarial activity of the isolates.

The *in vivo* antimalarial screening of the isolated compounds 1, 2, 3 showed that they were significantly active ($p \leq 0.05$) against chloroquine sensitive strain of *Plasmodium berghei.* But only vobtusine among the isolated compounds exhibited free radical scavenging activity.

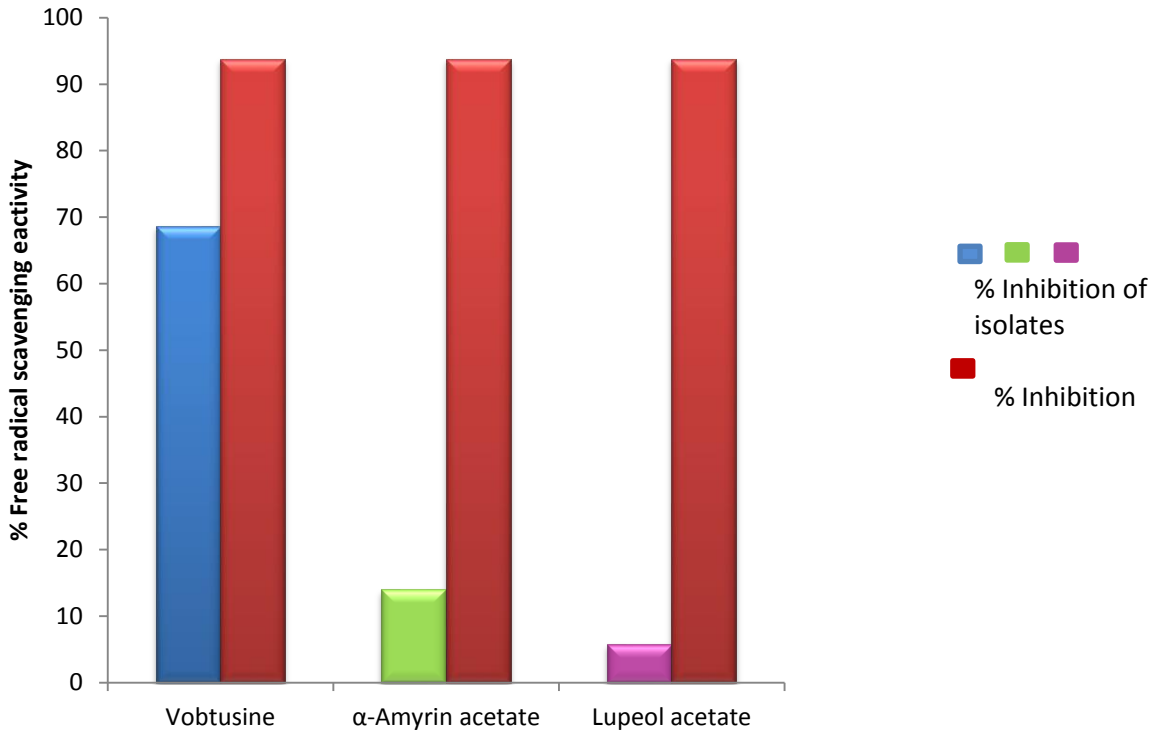


Figure 17: DPPH free radical scavenging activity of the isolates (0.2 mg mL^{-1})

Graph showing the DPPH radical scavenging activities of the isolated compounds and only vobtusine among the isolated compounds exhibited free radical scavenging activity

4.5 PURIFICATION, ISOLATION AND CHARACTERIZATION OF ANTIMALARIAL COMPOUNDS FROM ACTIVE FRACTIONS OF THE CRUDE EXTRACT

4.5.1: Hexane fraction: Isolation and spectroscopic results for characterization of compounds 1 and 2

The hexane fraction (the most active fraction) was fractionated using silica gel on open column chromatography and this resulted in isolation of two compounds (compound **1** and compound **2**).

The isolated compounds were further purified or cleaned up by recrystallization. The structure of compound **1** was confirmed using x-ray diffraction (Figure 31 – 33).

4.5.1.1: Isolation of compound 1

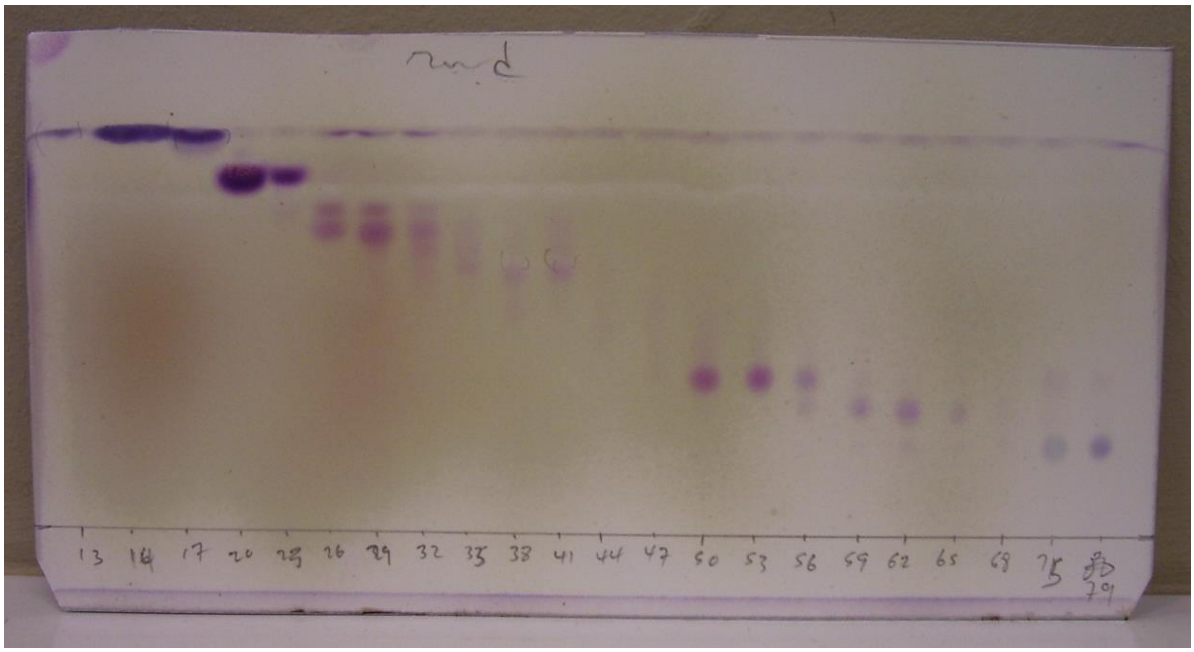
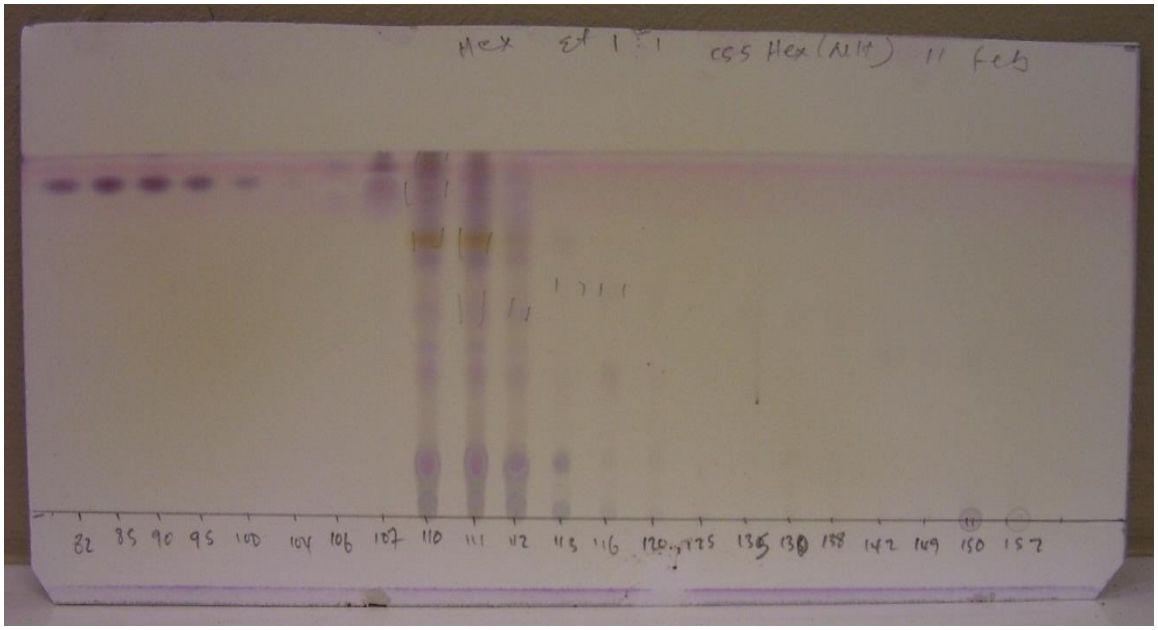


Figure 18: TLC Chromatogram of hexane fraction (1)

Solvent system Hex: DCM (5: 6)

Stationary phase: Silica gel F_{254}



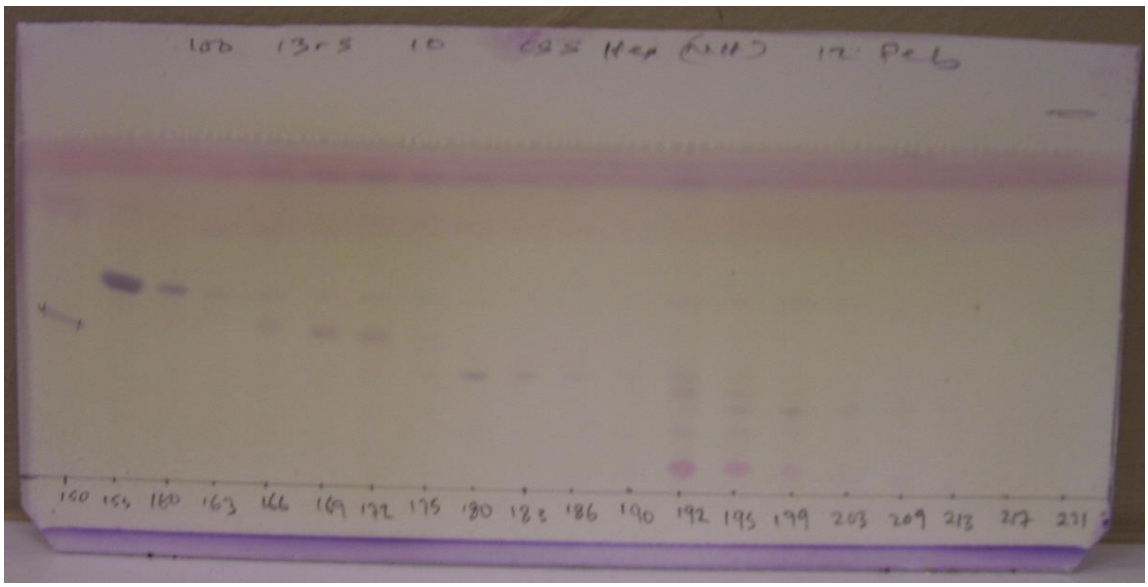
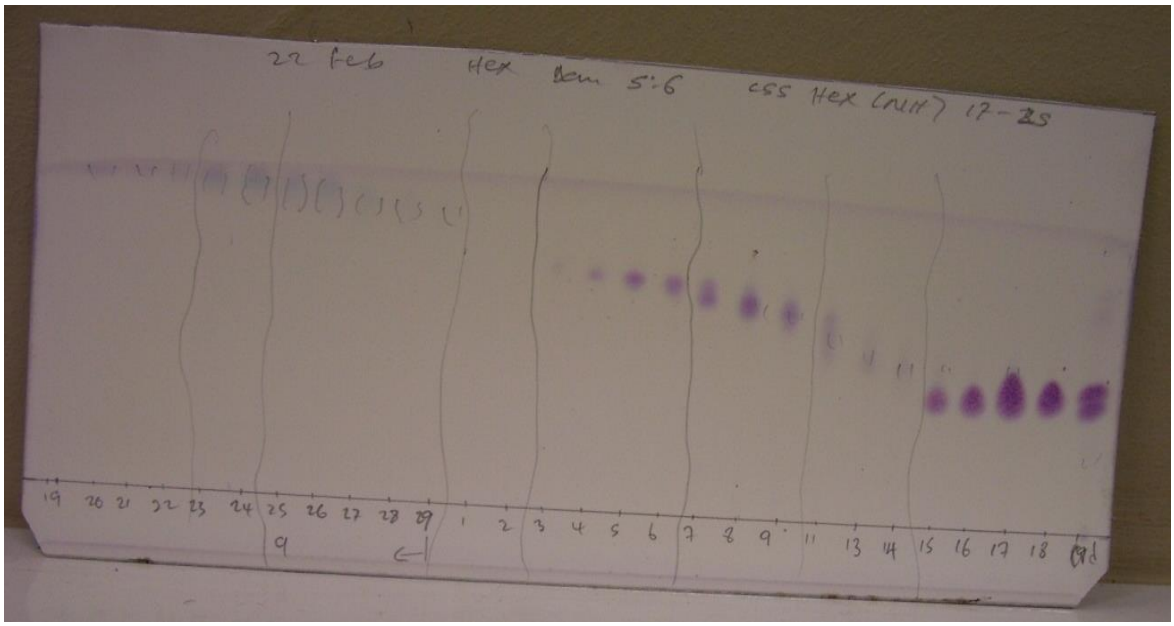


Figure 19: TLC Chromatogram of hexane fraction (2)

Solvent system Hex: DCM (5: 6)

Stationary phase: Silica gel F_{254}

Re-chromatograph of fraction C_2



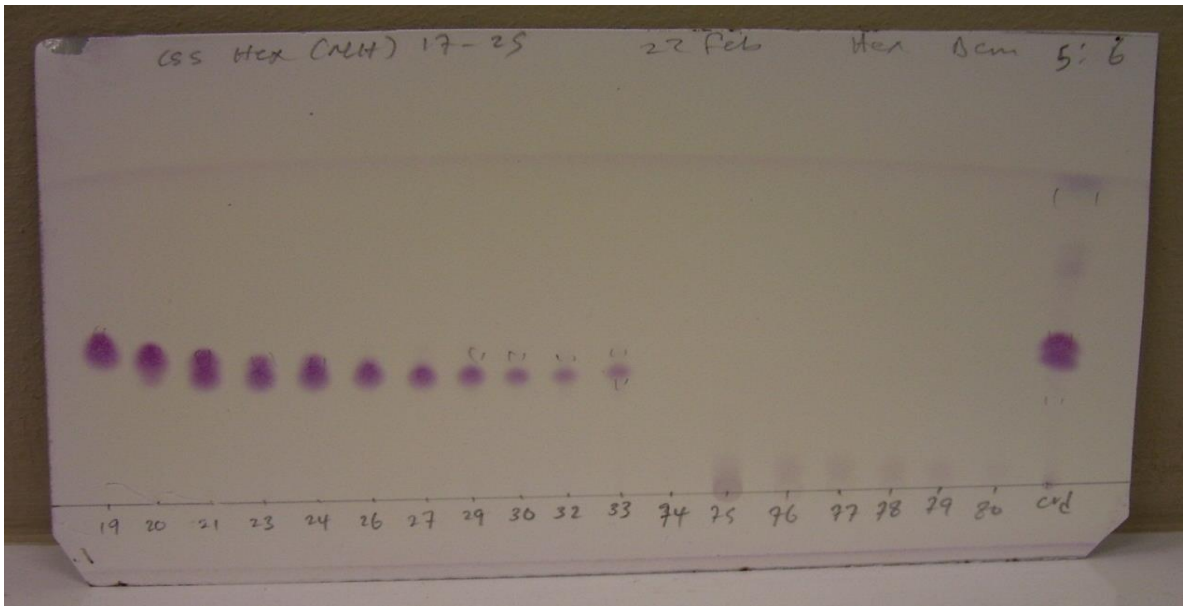


Figure 20: TLC of re-chromatogram of C_2 of hexane fraction

Mobile phase: Hex: DCM (5: 6)

Stationary phase: Silica gel F_{254}

Figure 21: TL Chromatogram, Illustration of the level of purity of compound 1 after recrystallization

Mobile phase: Hex: DCM (5: 6)

Stationary phase: Silica gel F_{254}

R_f value: 0.6 (Hex: DCM, 5: 6)

Melting point: 242°C - 243 ^{o}C

Spot detector: Day light: No colour

UV: Inactive

1% vanillin/H_2SO_4: Violet

Dragendorff: No colour reaction

4.5.1.2: Spectroscopic results for characterization and elucidation of compound 1

The compound **1** was isolated as colourless cube like crystals from hexane fraction of methanolic extract of *C. stenopetala* root bark.

The GC-MS of compound **1** (Figure 25) showed molecular ion peak at m/z 468.40 $(M)^+$ which is in agreement with the molecular formular $C_{32}H_{52}O_2$ (calculated m/z 468). The molecular ion in the GC-MS showed characteristic fragmentation pattern with m/z 453 $(M\text{-}CH_3)^+$ and 408 $(M\text{-}CH_3\ COOH)^+$.

The IR spectrum (Figure 26) displayed absorptions at 1730 and 1695 cm^{-1} due to the carboxyl and olefinic functions respectively (Figure 26). While 1060 and 2976 cm^{-1} were for (-C-O-) and (-C-H-) respectively.

The study of ^1H NMR spectrum ($CDCl_3$, 600 MHz) of the compound **1** showed signals for methyls, methylenes and methine protons (Figure 22). In the up-field region of the spectrum, singlets each of three protons resonating at δ 0.88, 0.98, 1.28, 1.05, 1.15, 0.80, 0.81, 0.91 were assigned to tertiary methyls. A double doublet of one proton at δ 4.53 was assigned to H-3 proton geminal to acetate group. While a singlet of three protons resonating at δ 2.05 was assigned to methyl of acetate group attached at C-3.

The ^{13}C NMR (APT) of compound **1** (Figure 23 and 24; Table 20) showed thirty two signals including nine methyls, nine methylenes, six methines and six quaternary carbons.

The ^1H – ^{13}C correlations were determined by HSQC spectrum (Figure 30). In the ^{13}C NMR spectrum of compound **1**, the up-field resonance at δ 28.1, 15.8, 14.2, 16.9, 23.2, 28.8, 17.5 and 21.4 were due to the tertiary methyls while the signals at δ 21.5 was assigned to carbon of methyl of acetate group. Also, in the down field region, the resonance at δ 171.1 and 124.3 were

assigned to the carbonyl carbon and olefinic C-12 while the signal at δ 139.6 was due to the quaternary C-13 carbon.

The point of attachment of acetate group was identified by HMBC correlation of H-3 δ 4.53 to carbonyl carbon δ 171.1 (Figure 29).

There is also NOESY correlation of H-12 δ 5.15 to H-18 δ 1.34 as well as H-11 δ 1.95 and H-29 δ 0.81 (Figure 27).

The structure was finally established from the X-ray diffraction studies (Figure 31-33). The chemical shift of the signals and other physical data were found identical to α-amyrin acetate (Figure 34) (Sisay and Abeba 2005; Shan, *et al.*, 2014)

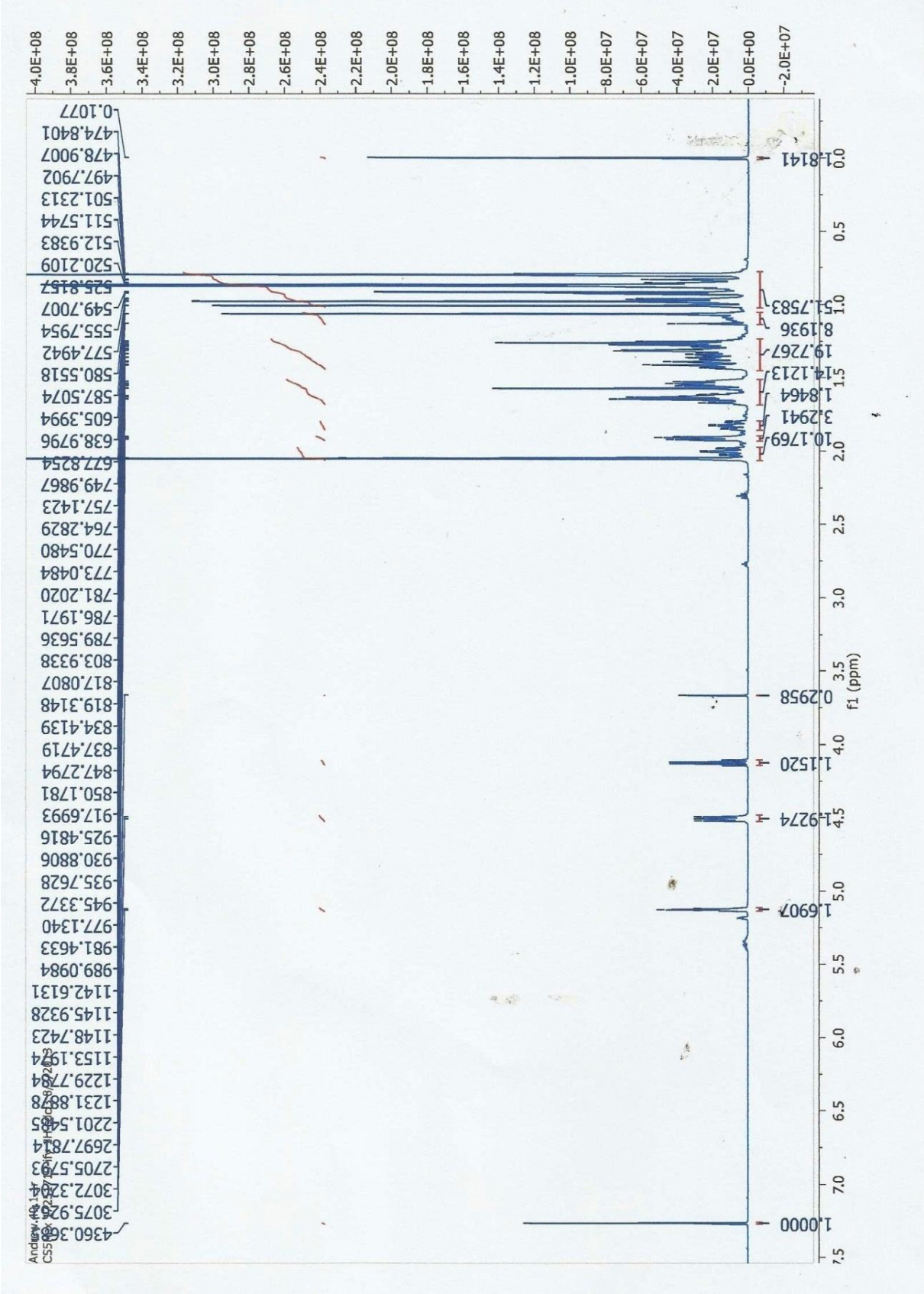


Figure 22: ^{1}H NMR spectrum of compound 1

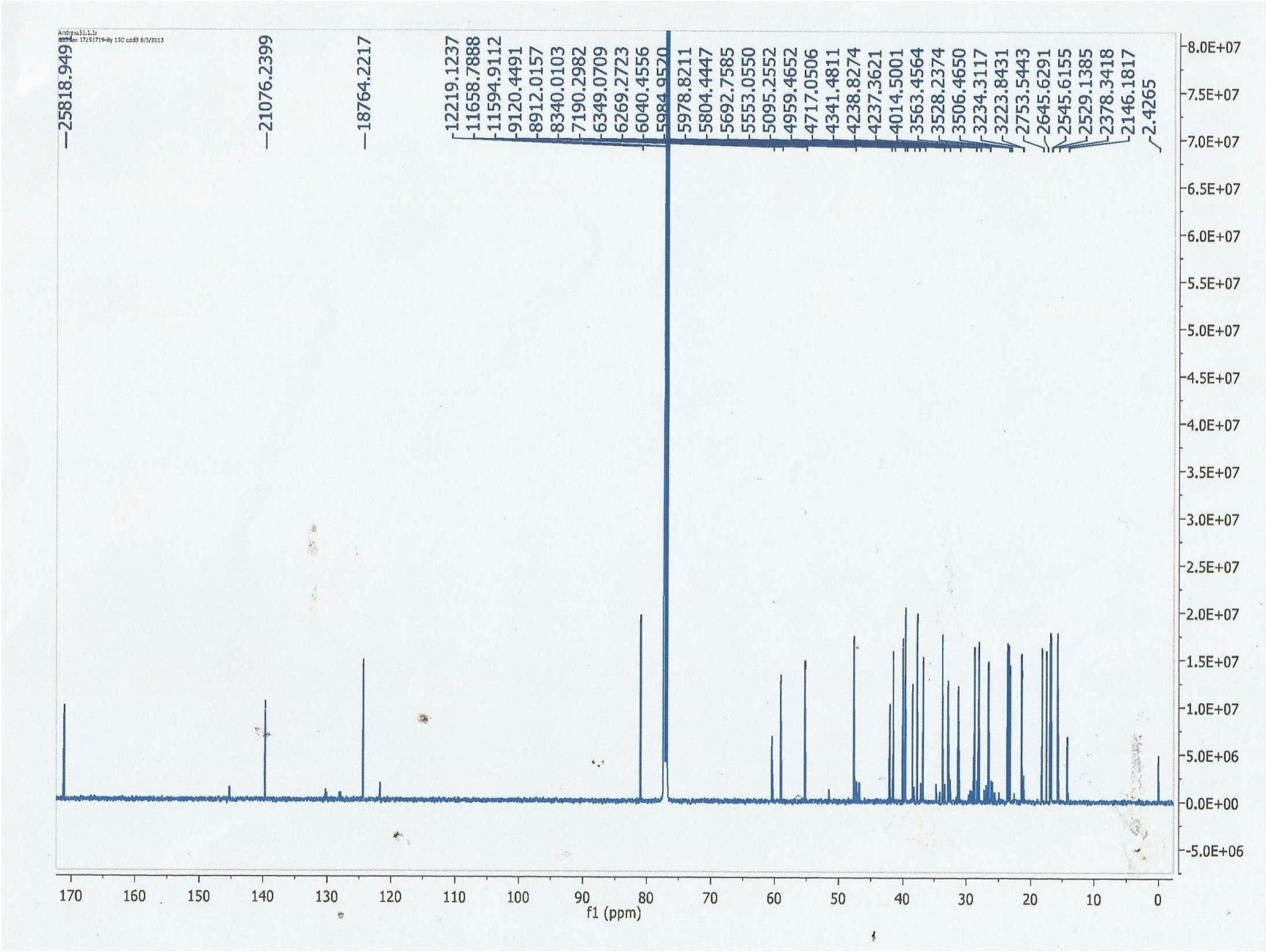


Figure 23: ^{13}C NMR spectrum of compound 1

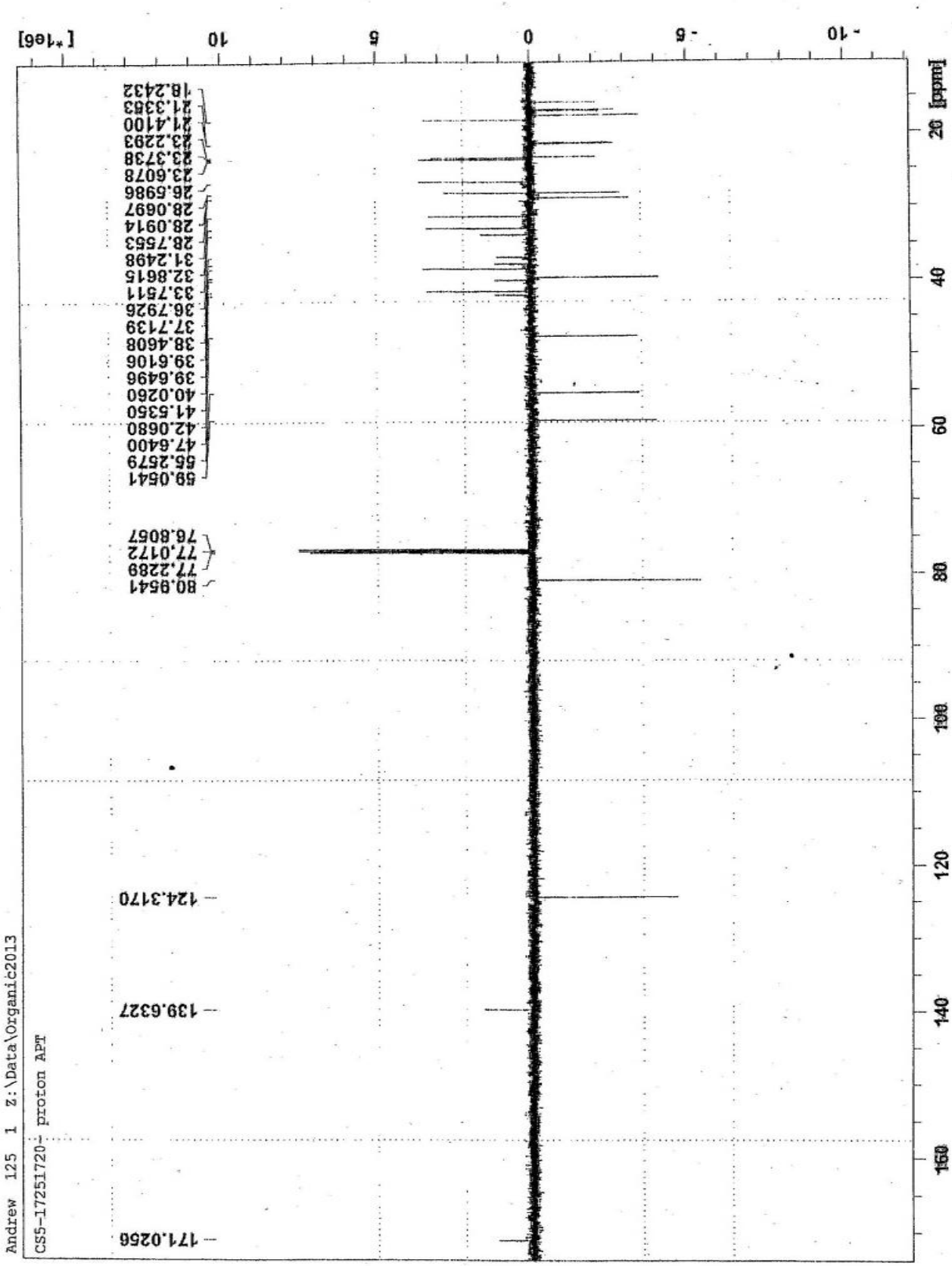


Figure 24: ATP spectrum of compound 1

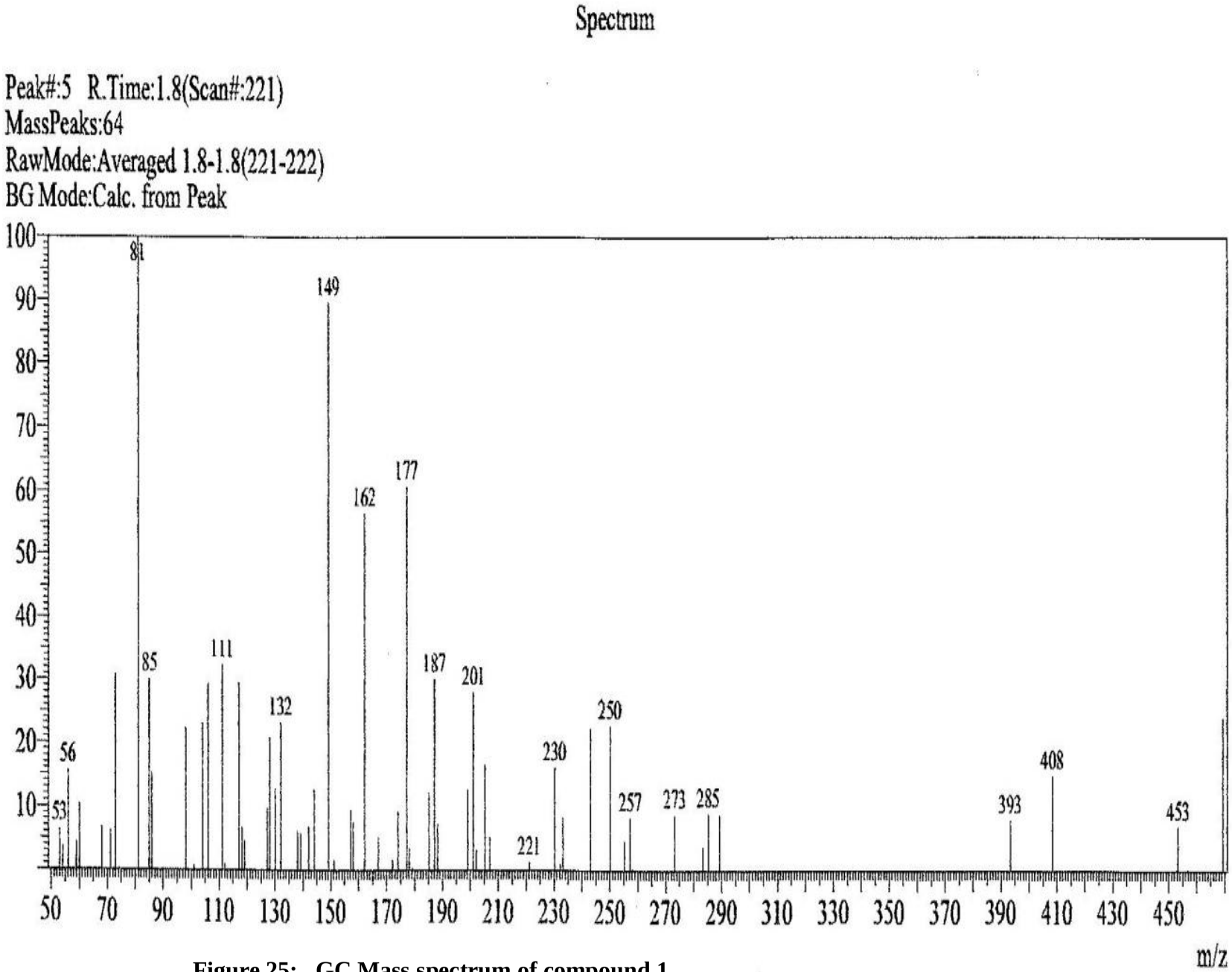


Figure 25: GC Mass spectrum of compound 1

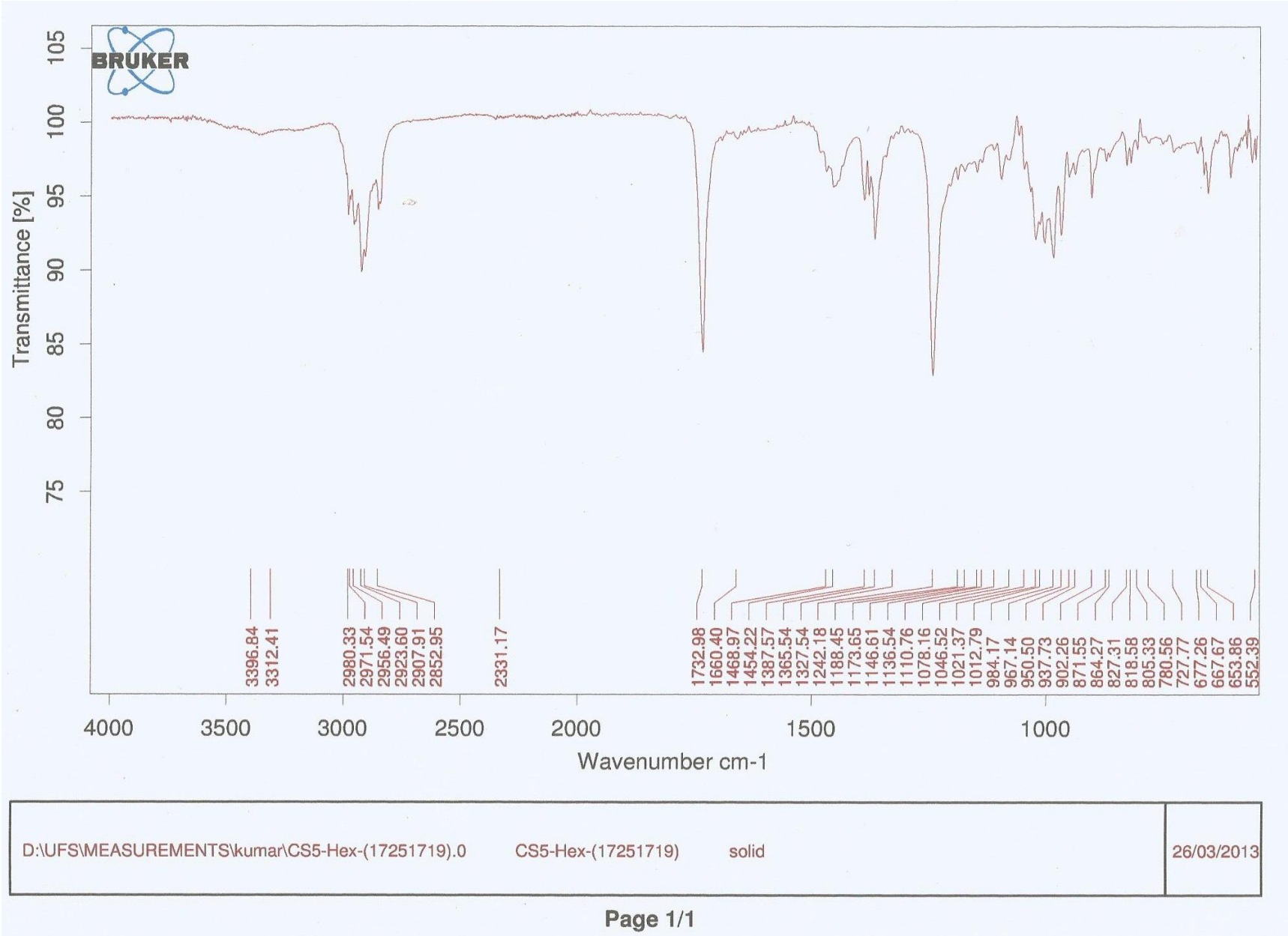


Figure 26: IR spectrum of compound 1

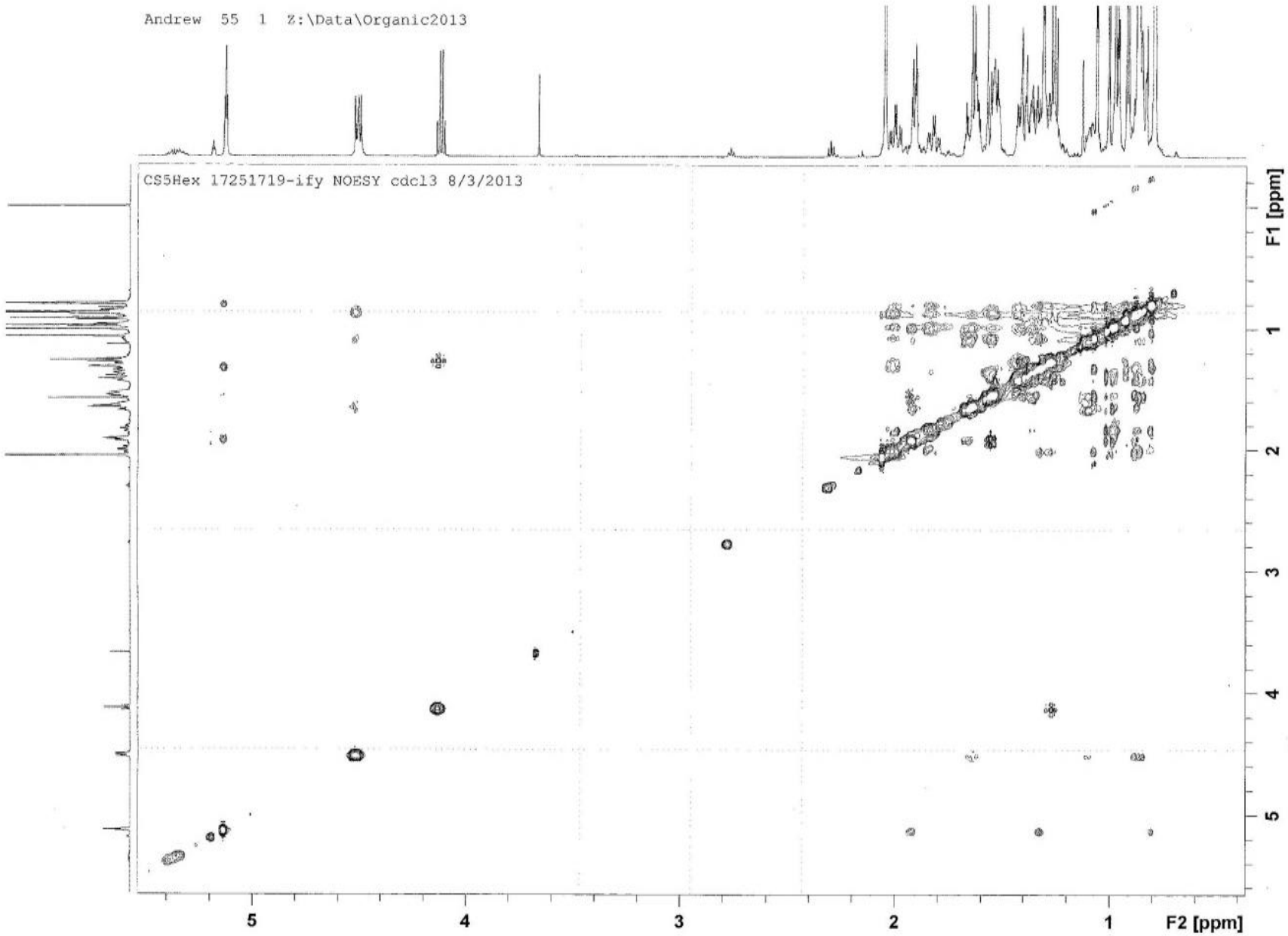


Figure 27: NOESY spectrum of compound 1

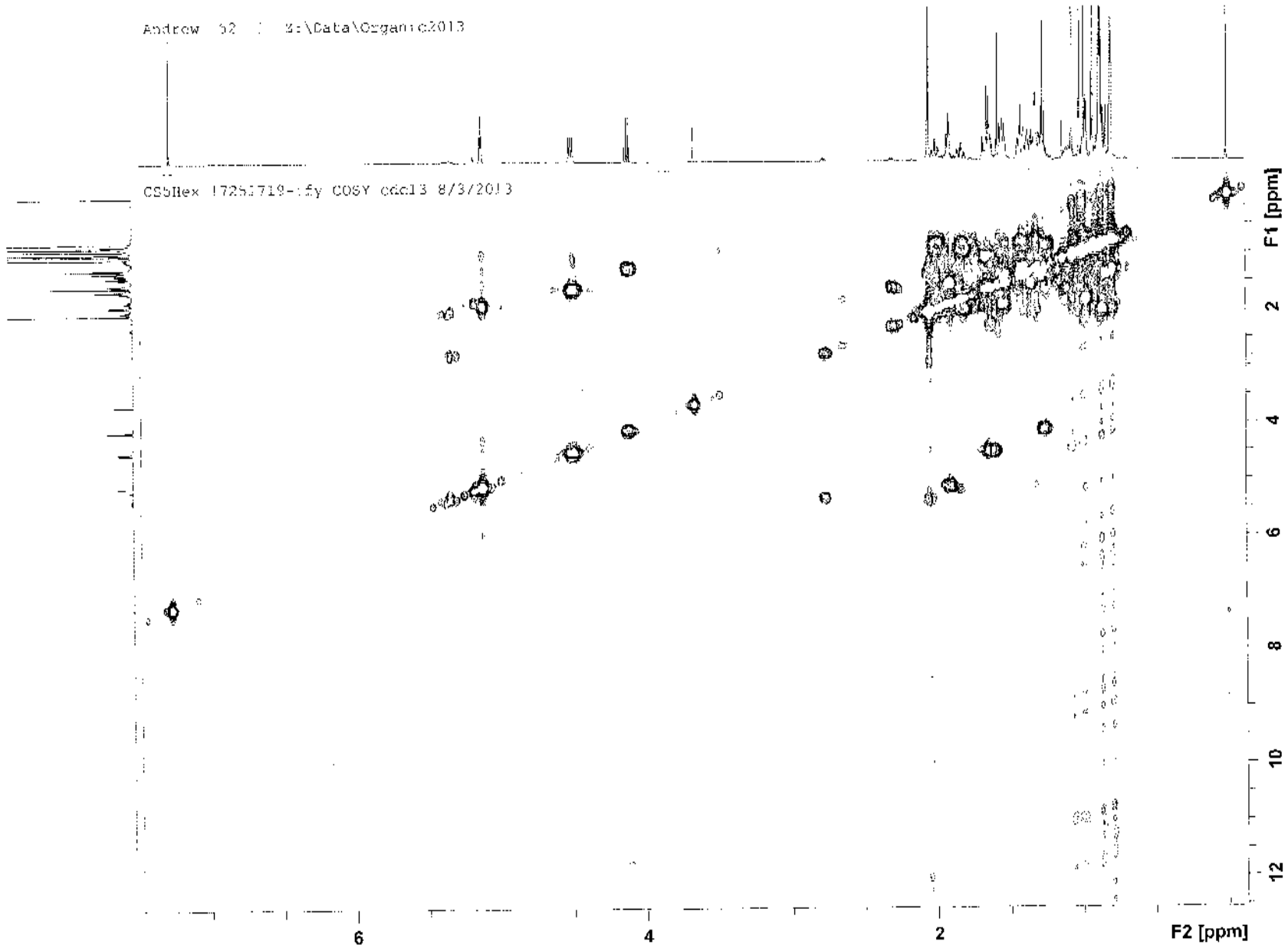


Figure 28: COSY spectrum of compound 1

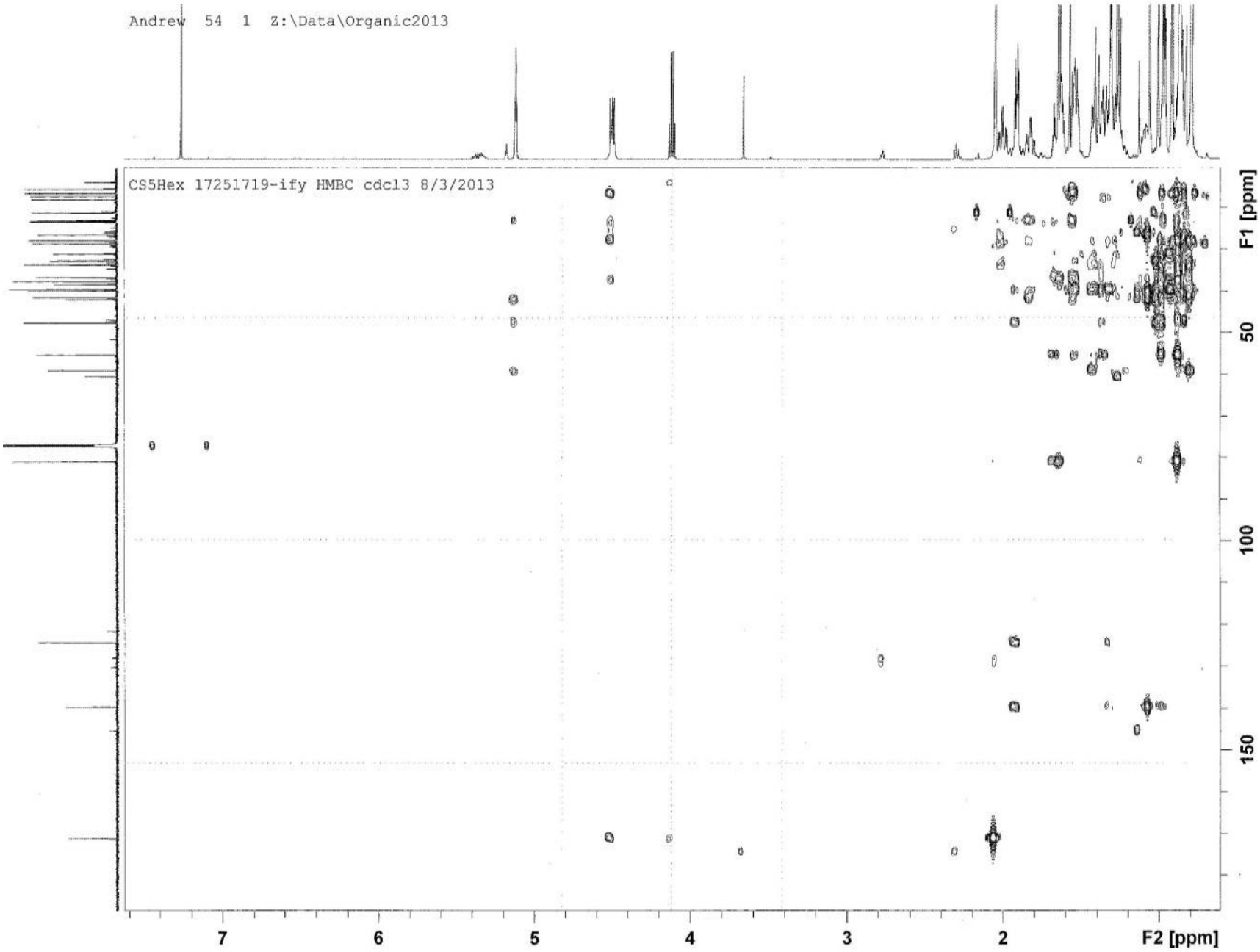


Figure 29: HMBC spectrum of compound 1

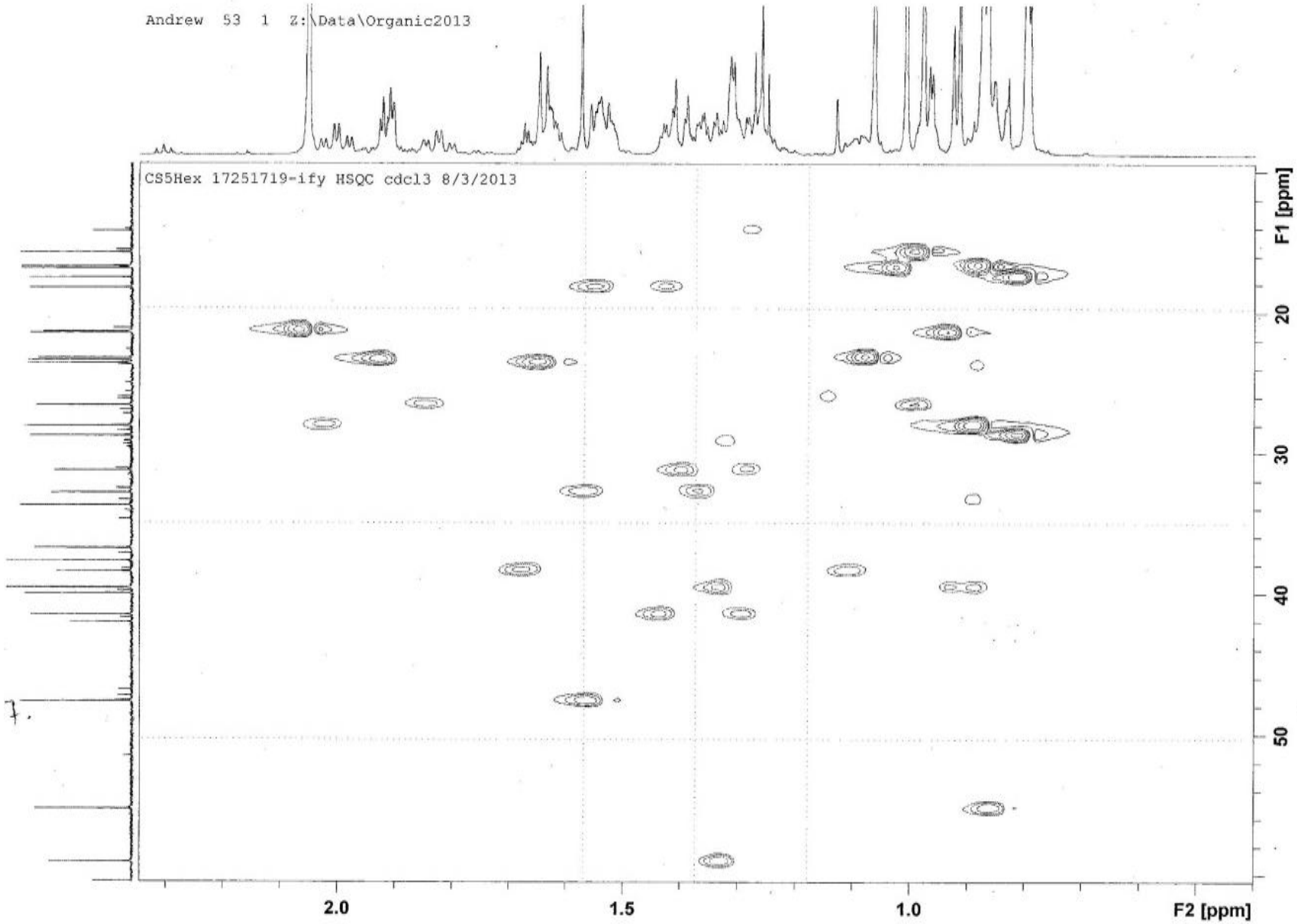


Figure 30: HSQC spectrum of compound 1

X-ray picture of compound 1 at various angles performed in Prof John Davies laboratory, University of Cambridge, USA

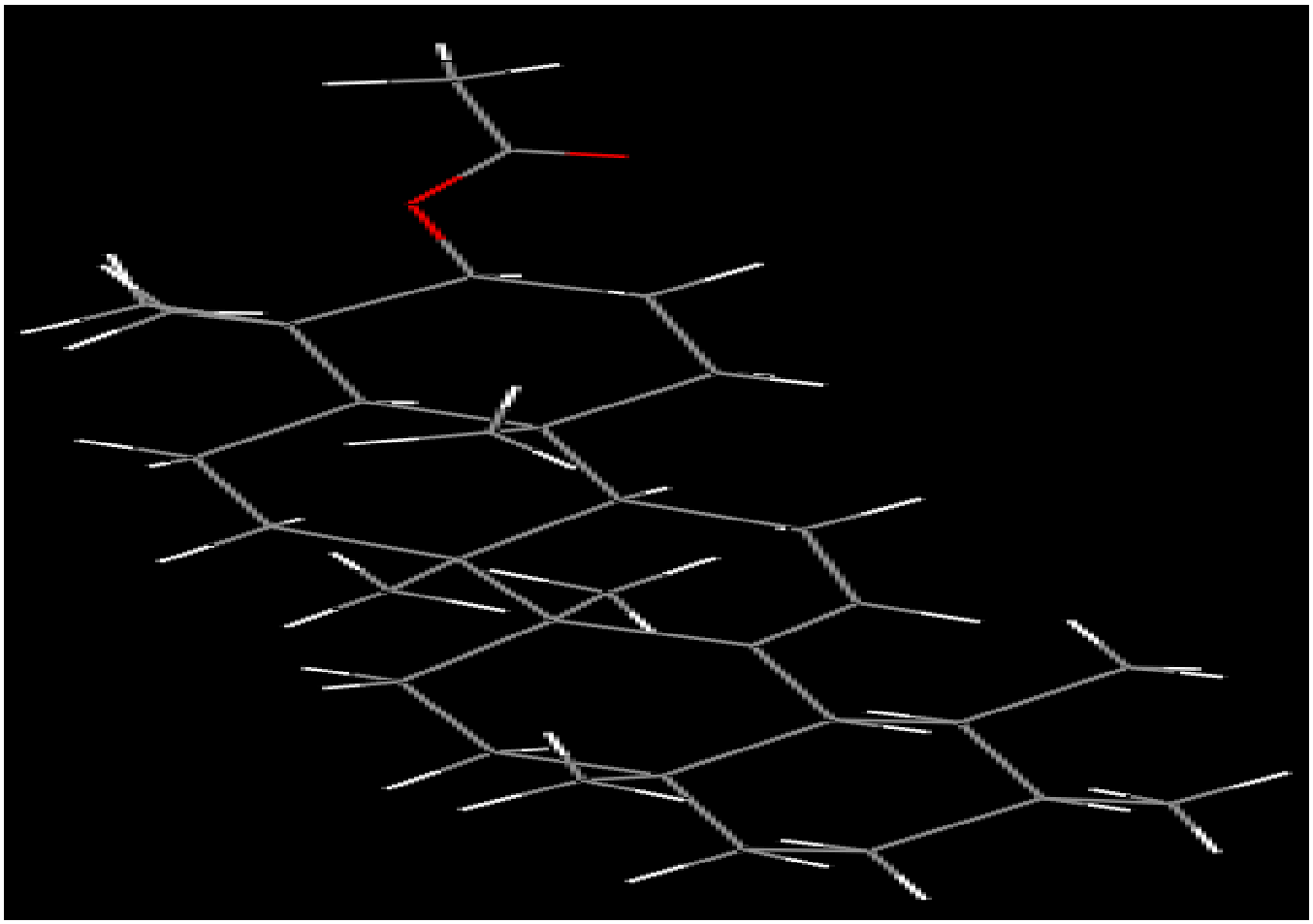


Figure 31: 3 Dimensional Ball and Stick minimized energy mode for compound 1 (1)

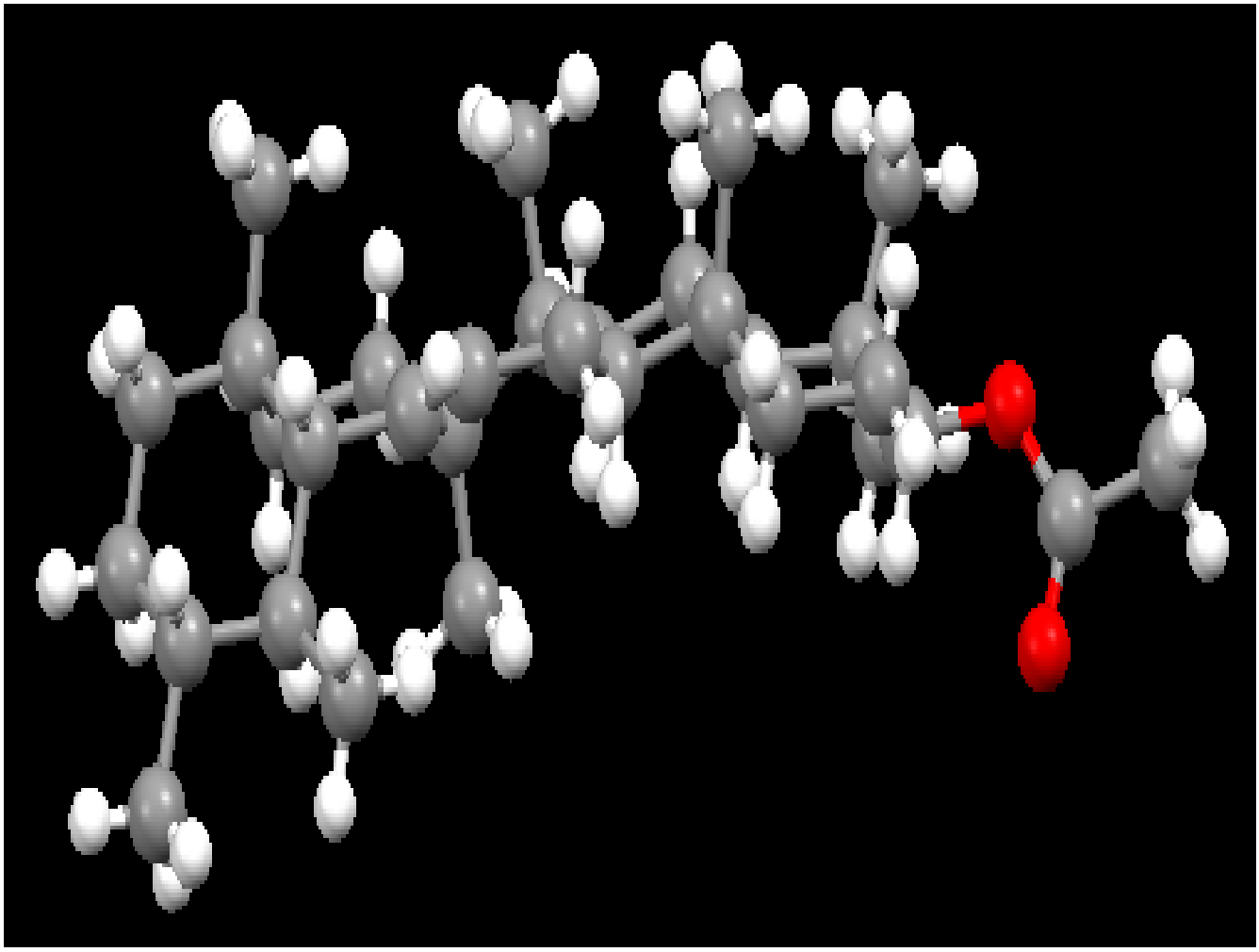


Figure 32: 3 Dimensional Ball and Stick minimized energy mode for compound 1 (2)

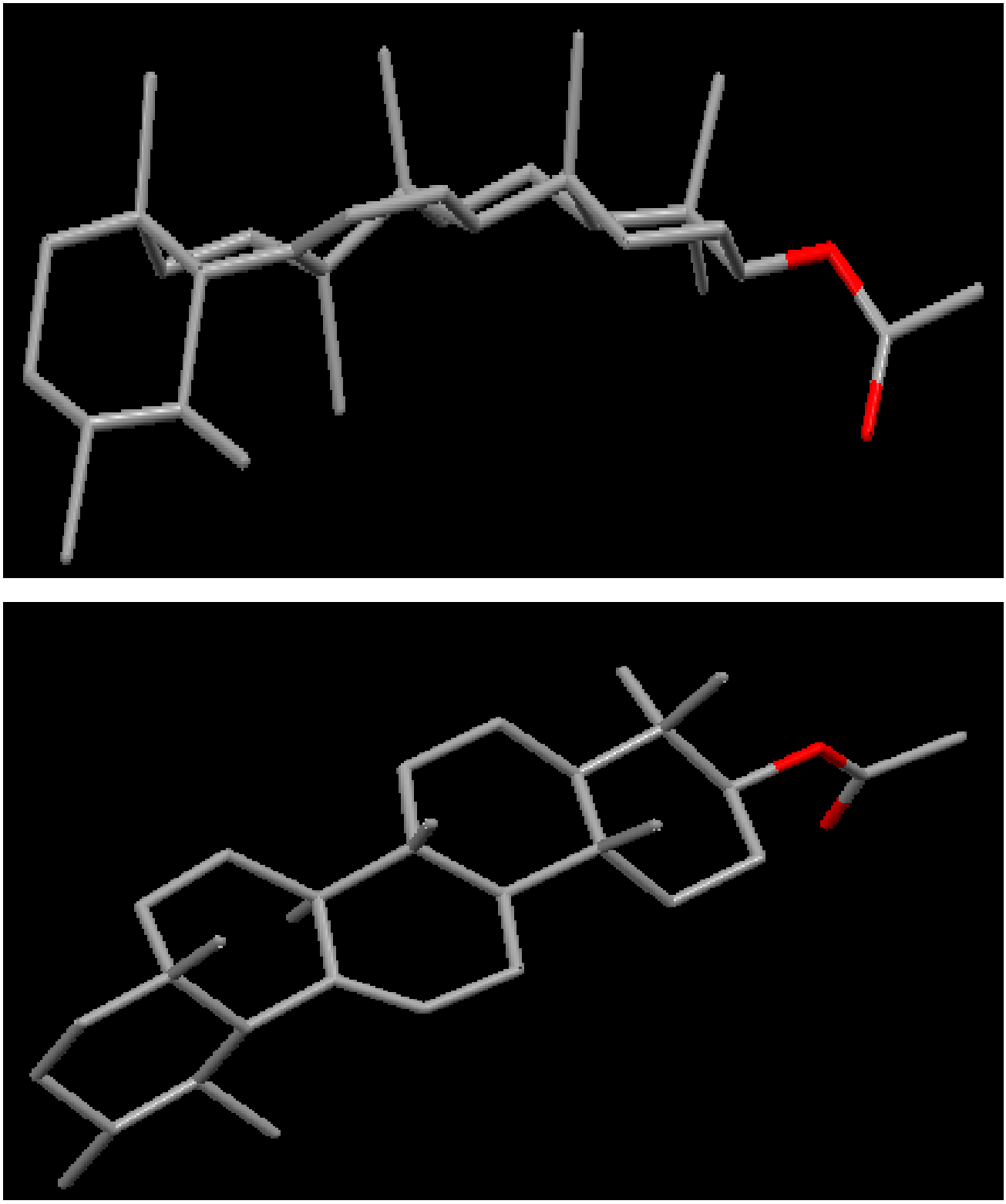


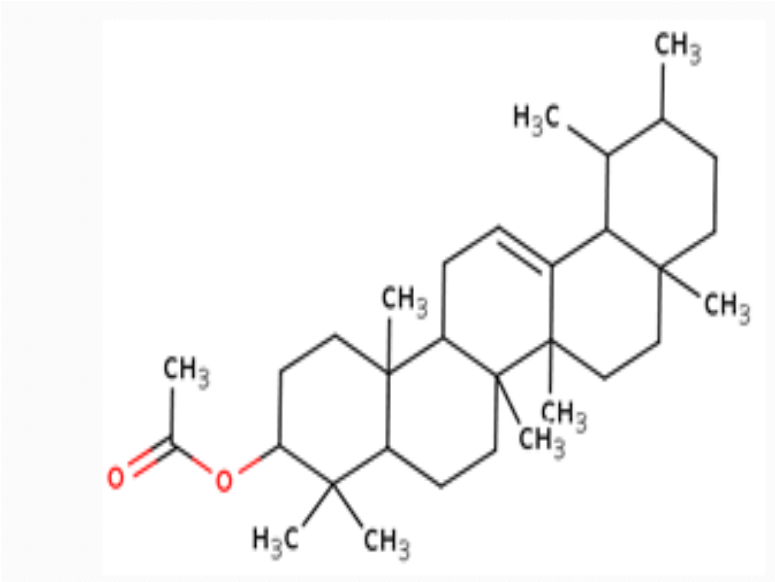
Figure 33: 3 Dimensional ball and stick minimized energy mode for compound 1 (3)

Table 20: ^{13}C NMR and ^{1}H-NMR main signals for compound 1*

Position	δ_C	δ_H (multiplicity)	Multip
1	38.5	1.69 (*t*) 1.10 (*t*)	CH_2
2	23.4	1.62 (*q*) 0.91 (*q*)	CH_2
3	81.0	4.50 (1H, *dd*)	CH
4	37.7	-	C
5	55.3	0.87 (*t*)	CH
6	18.2	1.55 (*q*) 1.45 (*q*)	CH_2
7	32.9	1.57 (*t*) 1.34 (*t*)	CH_2
8	40.6	-	C
9	47.6	1.57 (*t*)	CH
10	36.8	-	C
11	23.4	1.95 (*d*) 1.60 (*d*)	CH_2
12	124.3	5.12 (*t*)	CH
13	139.6	-	C
14	42.1	-	C
15	28.1	2.12 (*t*) 0.9 (*t*)	CH_2
16	26.6	1.85 (*t*) 0.99 (*t*)	CH_2
17	33.8	-	C

Position	δ_C	δ_H (multiplicity)	Multiplicity
18	59.0	1.34 (*d*)	CH_2
19	39.7	0.93 (*t*)	CH_2
20	39.6	1.34 (*q*)	CH_3
21	31.3	1.4 (*q*) 12.9 (*q*)	CH_3
22	41.5	1.44 (*t*) 1.29 (*t*)	CH_3
23	28.1	0.88 (*s*)	CH_3
24	15.8	0.98 (*s*)	CH_3
25	14.2	1.28 (*t*)	CH_3
26	16.9	1.05 (*s*)	CH_3
27	23.2	1.15 (*s*)	CH_3
28	28.8	0.80 (*s*)	CH_3
29	17.5	0.81 (*s*)	CH_3
30	21.4	0.91 (*s*)	CH_3
31; (-CO)	171.1		C
32; (-COMe)	21.5	2.05 (3s)	CH_3

*Assignments were based on 1D and 2D NMR experiments



α-Amyrin acetate

Figure 34: Structure of compound 1

- Molecular formula: $C_{32}H_{52}O_2$
- Systematic name: (3β)-Olean-12-en-3-yl acetate
- $M^+ = 468.40$ (experimental)
- Melting point: 242-243 ^oC (experimental)

4.5.1.3: Isolation of compound 2

Compound **2** was isolated as white crystals. The NMR data (Table 21) suggested a lupane skeleton**.**

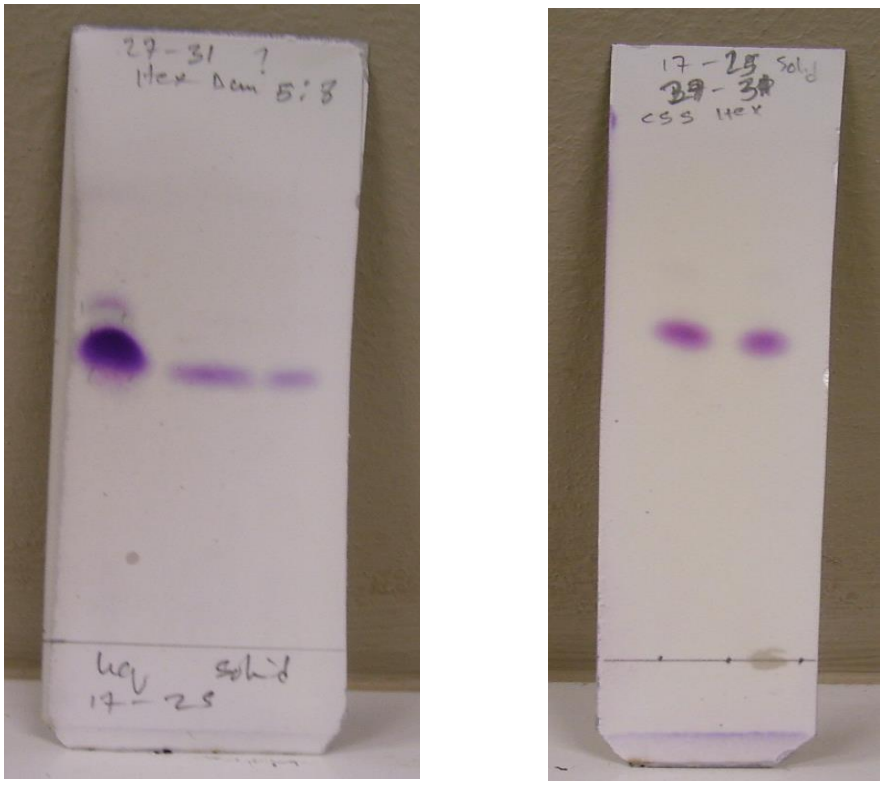


Figure 35: Illustration of the level of purity of compound 2 after recrystallization

TLC solvent system Hex: DCM (5: 6)

RF value: 0.56 (Hex: DCM; 5: 8)

Stationary phase: Silica gel

Melting point: 210°C - 212 ^{o}C

Spot detectors: Day light: No colour

UV: Inactive

1% vanillin/H_2SO_4: Violet- pink

Dragendorff: No colour reaction

4.5.1.4: Spectroscopic results for characterization and elucidation of compound 2

Compound **2** was isolated as white crystals from hexane fraction of the crude extract of *C. stenopetala* root bark.

The GC-MS of the compound (Figure 39) showed molecular ion peak at m/z 468.40 (M^+) which is in agreement with the molecular formular $C_{32}H_{52}O_2$.

The proton NMR spectrum (δ ppm, $CDCl_3$) showed sharp singlets at 0.79, 0.84, 0.85, 0.86, 0.94 and 0.96, indicated the presence of methyl groups at C-25, C-27, C-23, C-26, C-24 and C-28 positions respectively (Figure 37). The three protons of methyl group at C-30 position were confirmed by the presence of a sharp singlet at 1.68. The presence of three protons of acetyl group at 2.05 as a sharp singlet confirmed its position at C-3. The proton present at carbon atom C-3 was observed at 4.45 as a double doublet. A pair of broad singlet at 4.56 and 4.68 was assigned to the vinylic protons attached at C-29.

The ^{13}C NMR (APT) of the compound **2** (Table 21; Figure 38) showed thirty two (32) signals including eight methyls, eleven methylenes, six methines and seven quaternary carbons.

The ^{1}H - ^{13}C correlations were determined by the HSQC spectrum (Figure 44). In the ^{13}C NMR spectrum of compound **2** (Figure 36 and 38) the up-field resonance at δ 27.94 (C-3), 14.49 (C-24), 17.99 (C-25), 16.59 (C-26), 15.96 (C-29) and 19.28 (C-28) were due to tertiary methyls while the signals at δ 21.33 was assigned to carbon of methyl of acetate group. The signals observed at 109.35 and 150.99 were assigned to carbon-carbon double bond at C-29 and C-30 carbon atoms respectively. The absorption of methyl group at C-30, which is attached to olefinic carbon atoms appeared at 20.93. The presence of absorption at 80.97 showed the

presence of an acetoxy group attached at C-3 position. The absorptions appearing at 171.03 and 21.33 clearly indicated the presence of acetoxyl (-$OCOCH_3$) group.

The carbonyl signal at δ 206.99 and methyl at 30.9 were signals due to acetone used to wash the NMR tube.

On the basis of above spectral analysis compound **2** was identified as lupeol acetate. The identity was further confirmed by comparing the observed values with reported data (Jamal *et al.,* 2008).

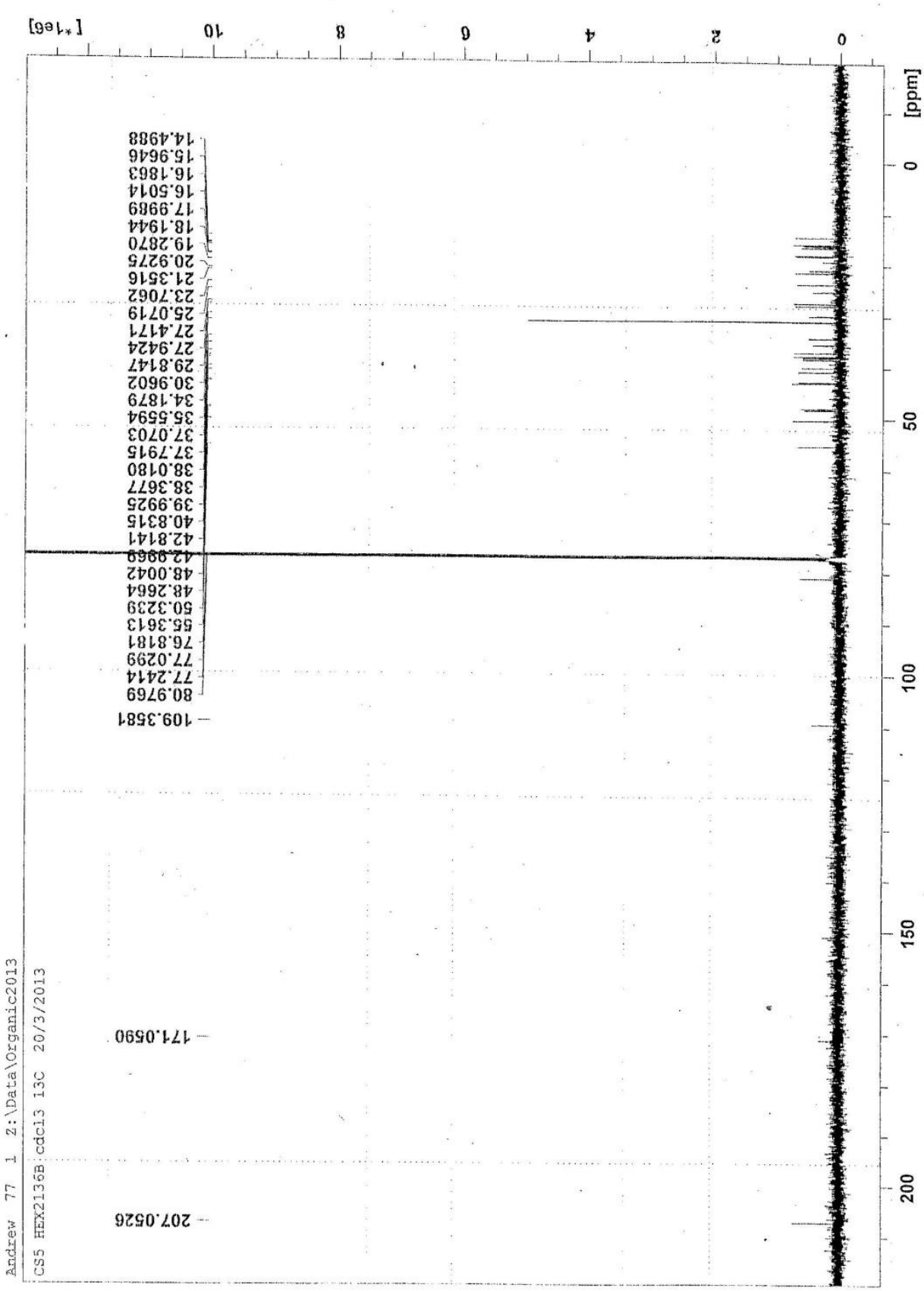


Figure 36: ^{13}C NMR spectrum of the compound 2

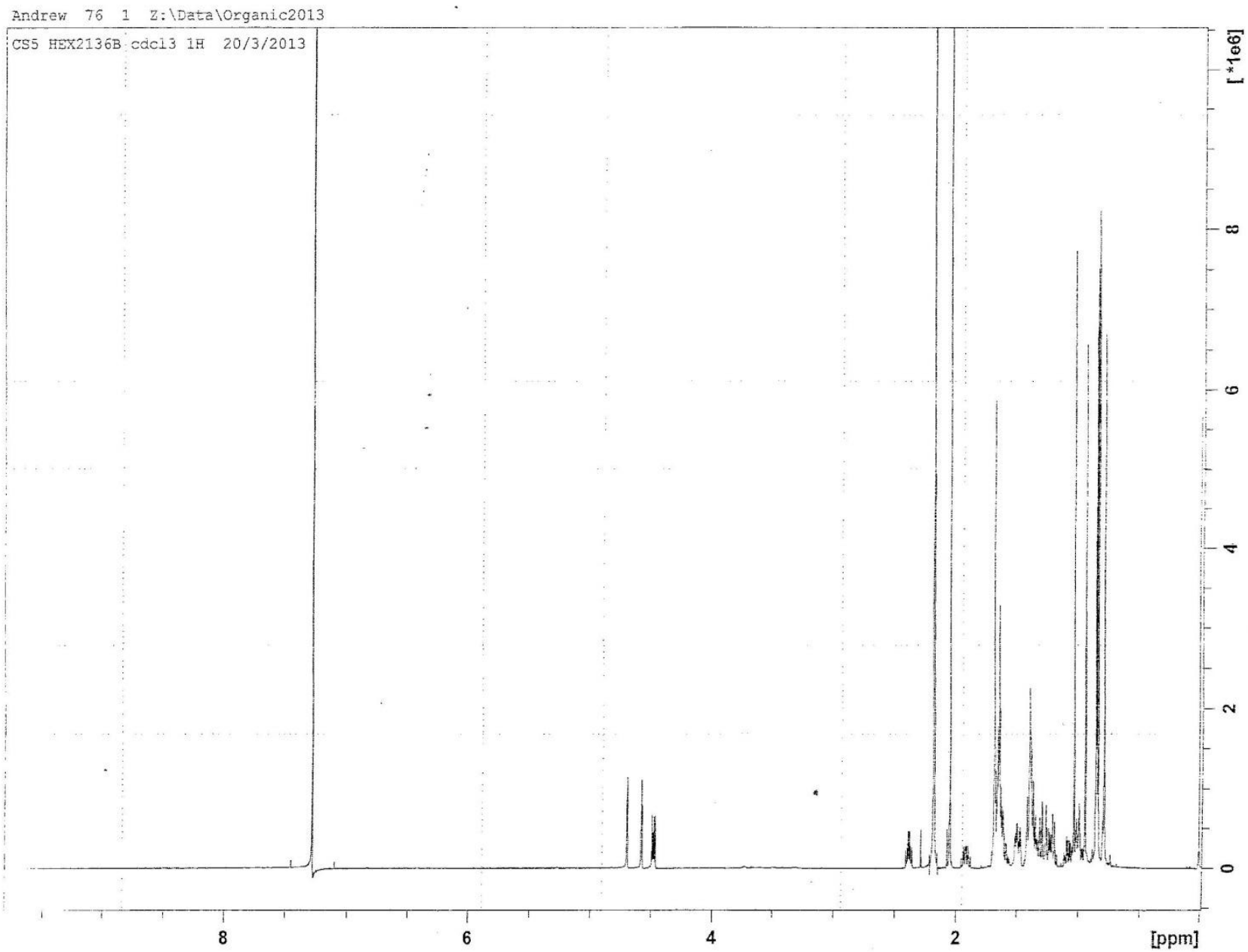


Figure 37: ^{1}H NMR spectrum of compound 2

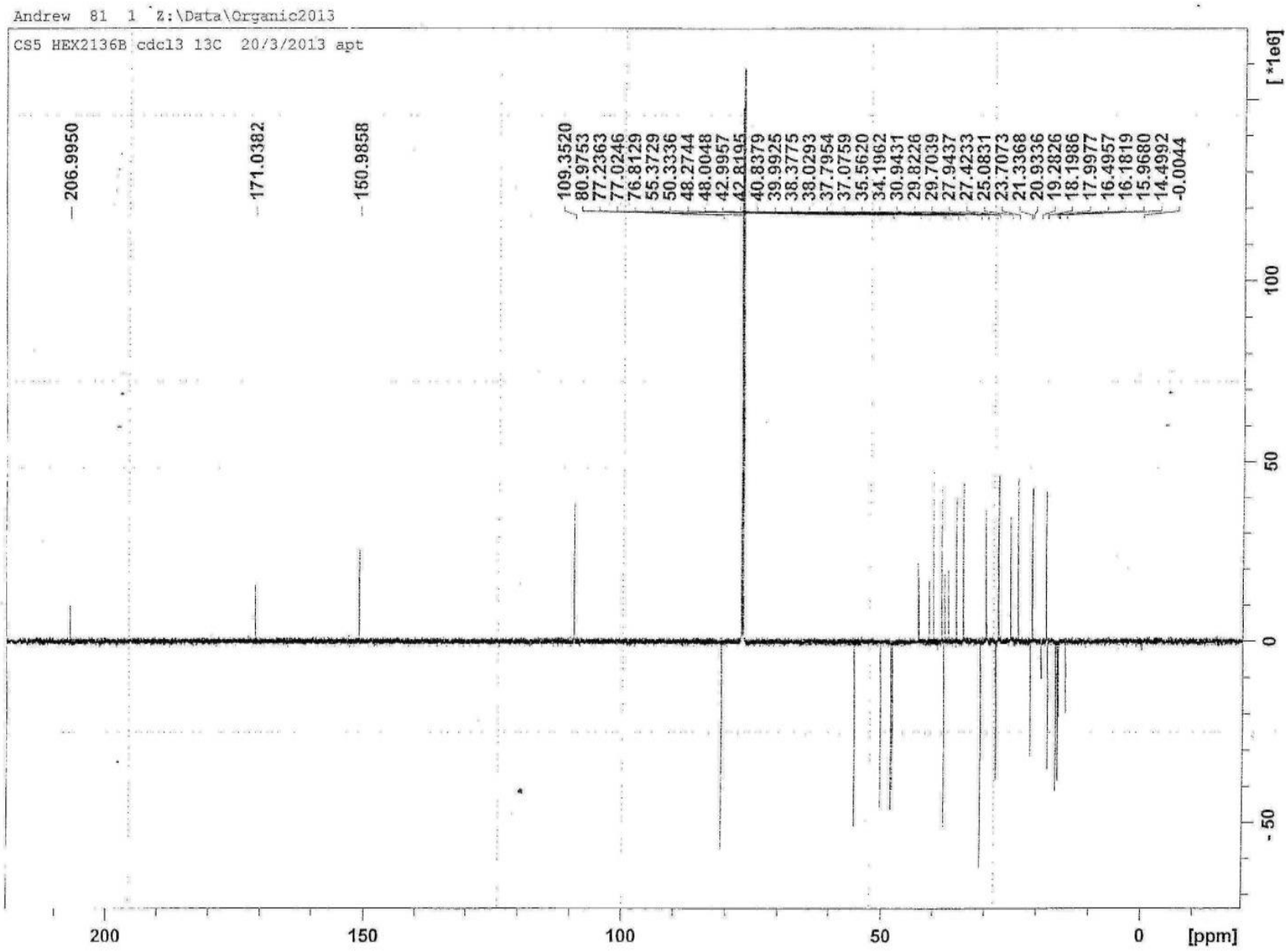


Figure 38: APT spectrum of compound 2

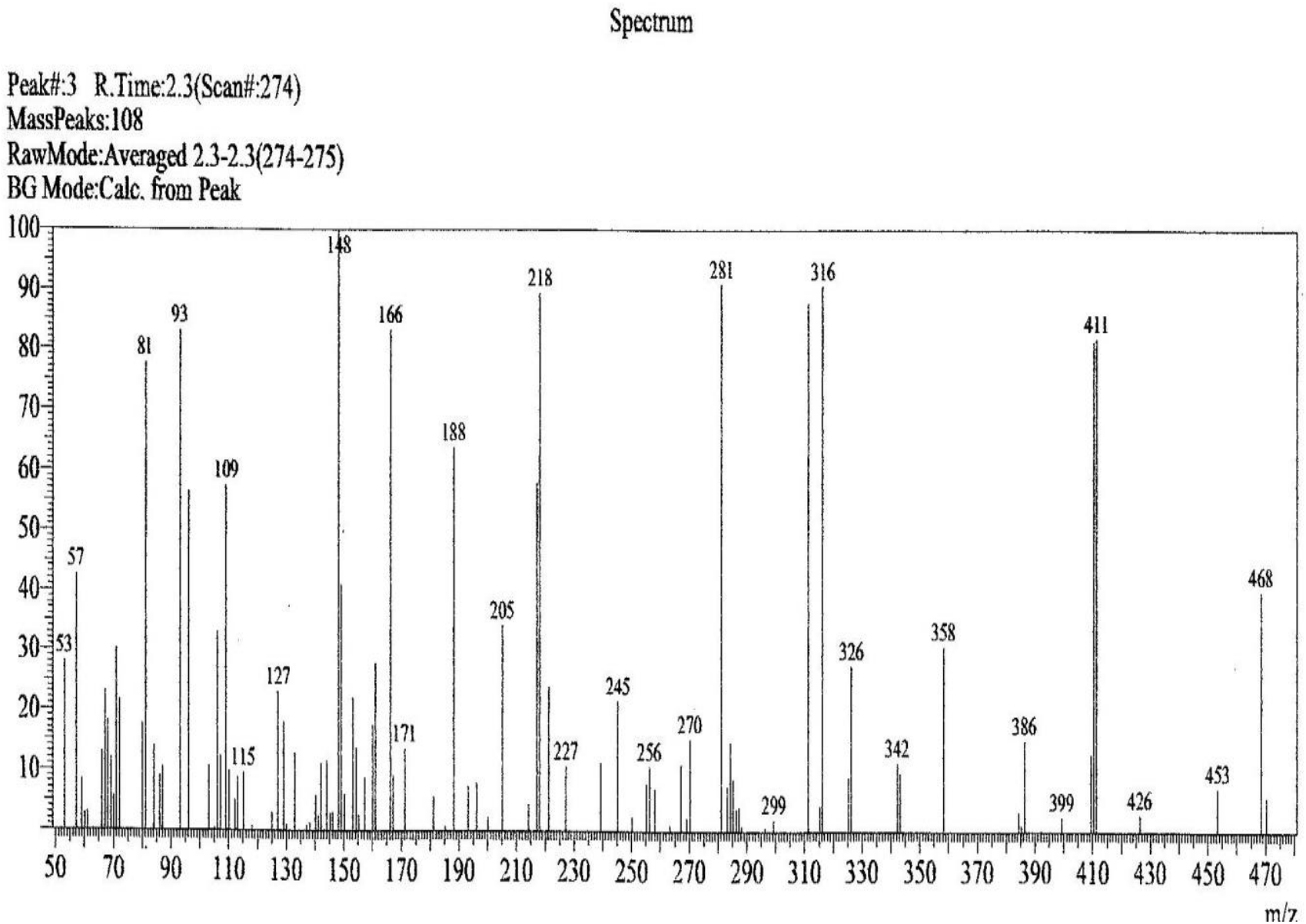


Figure 39: GC Mass spectrum of compound 2

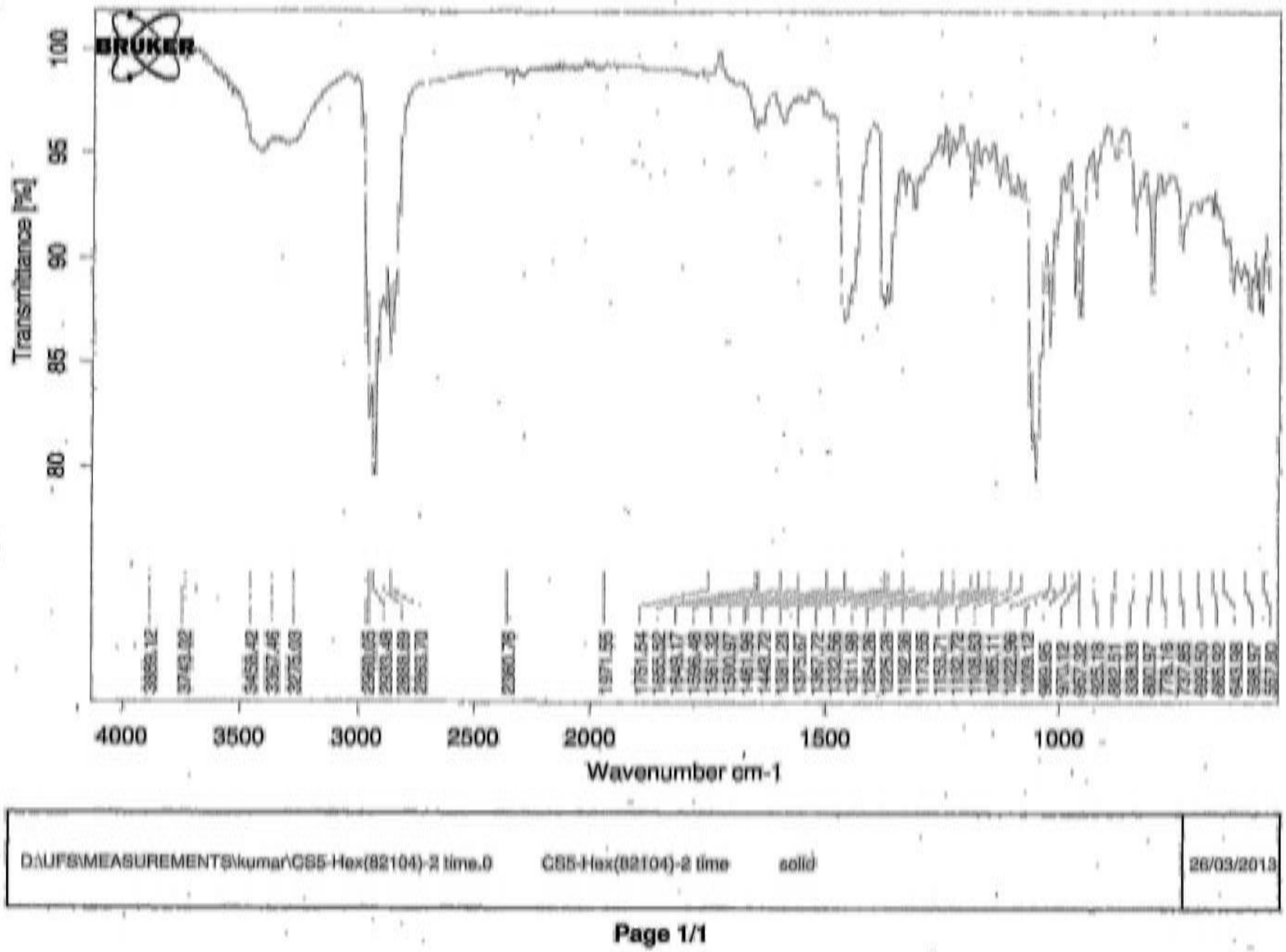


Figure 40: IR spectrum of compound 2

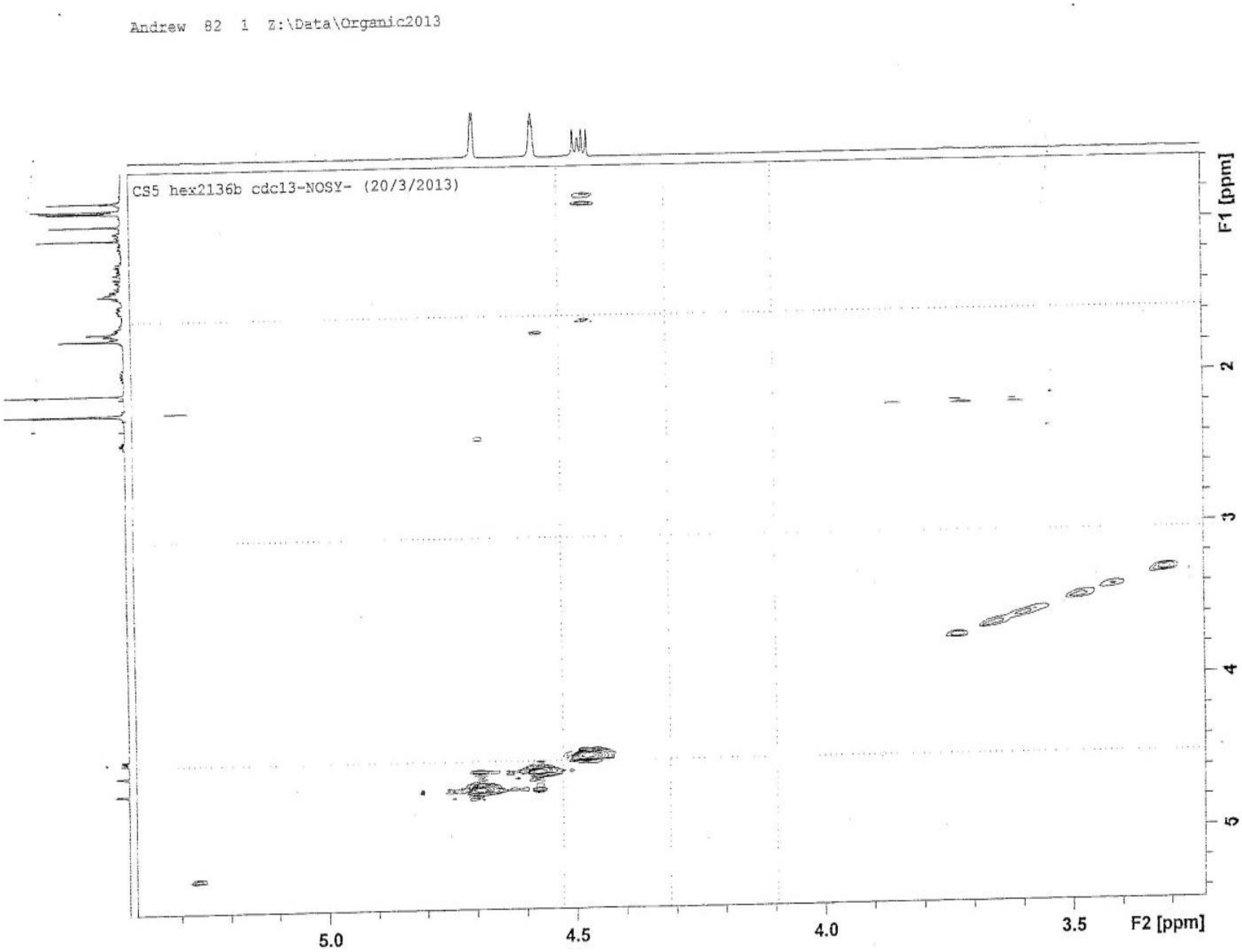


Figure 41: NOESY spectrum of compound 2

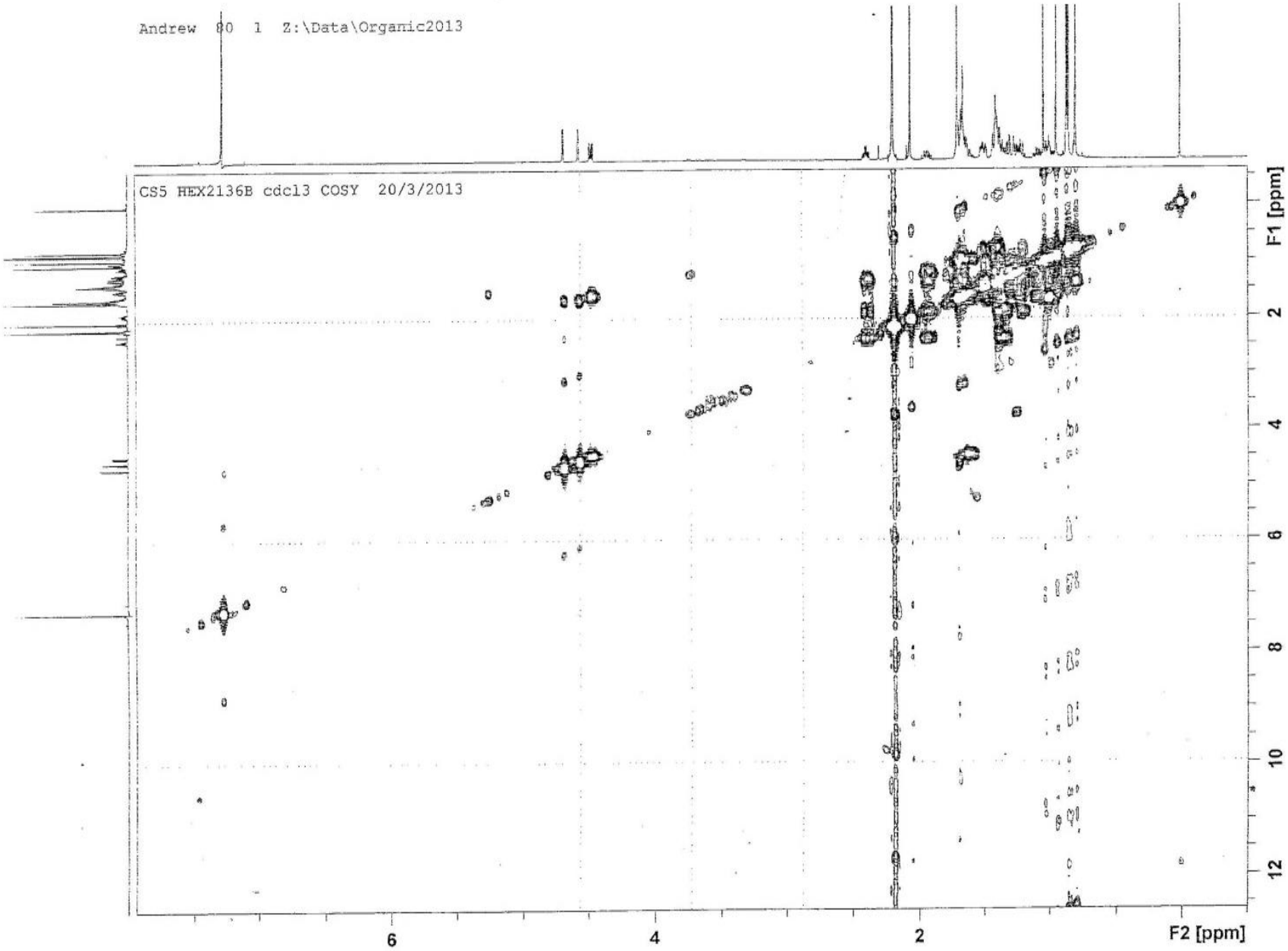


Figure 42: COSY spectrum of compound 2

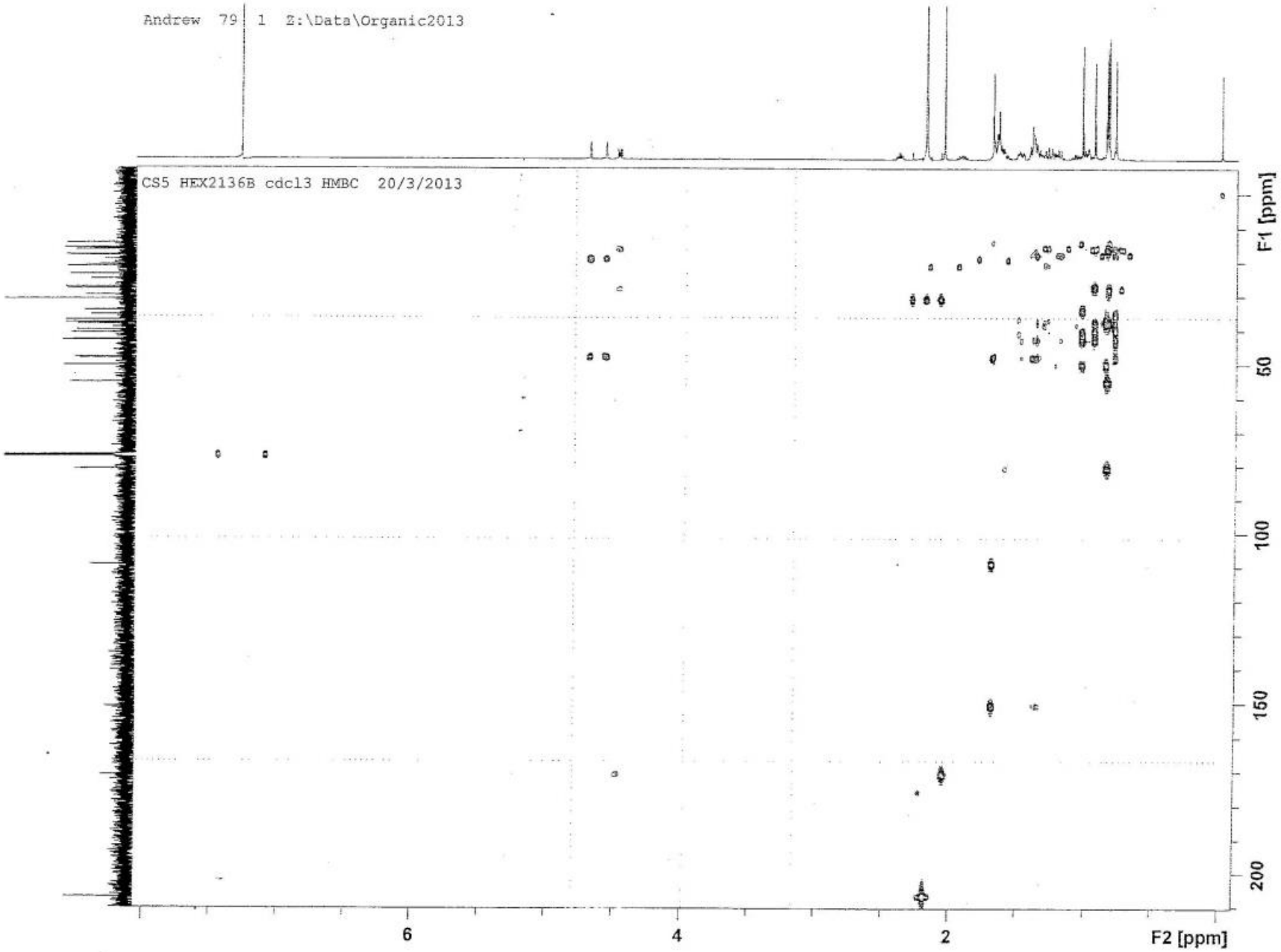


Figure 43: HMBC spectrum of compound 2

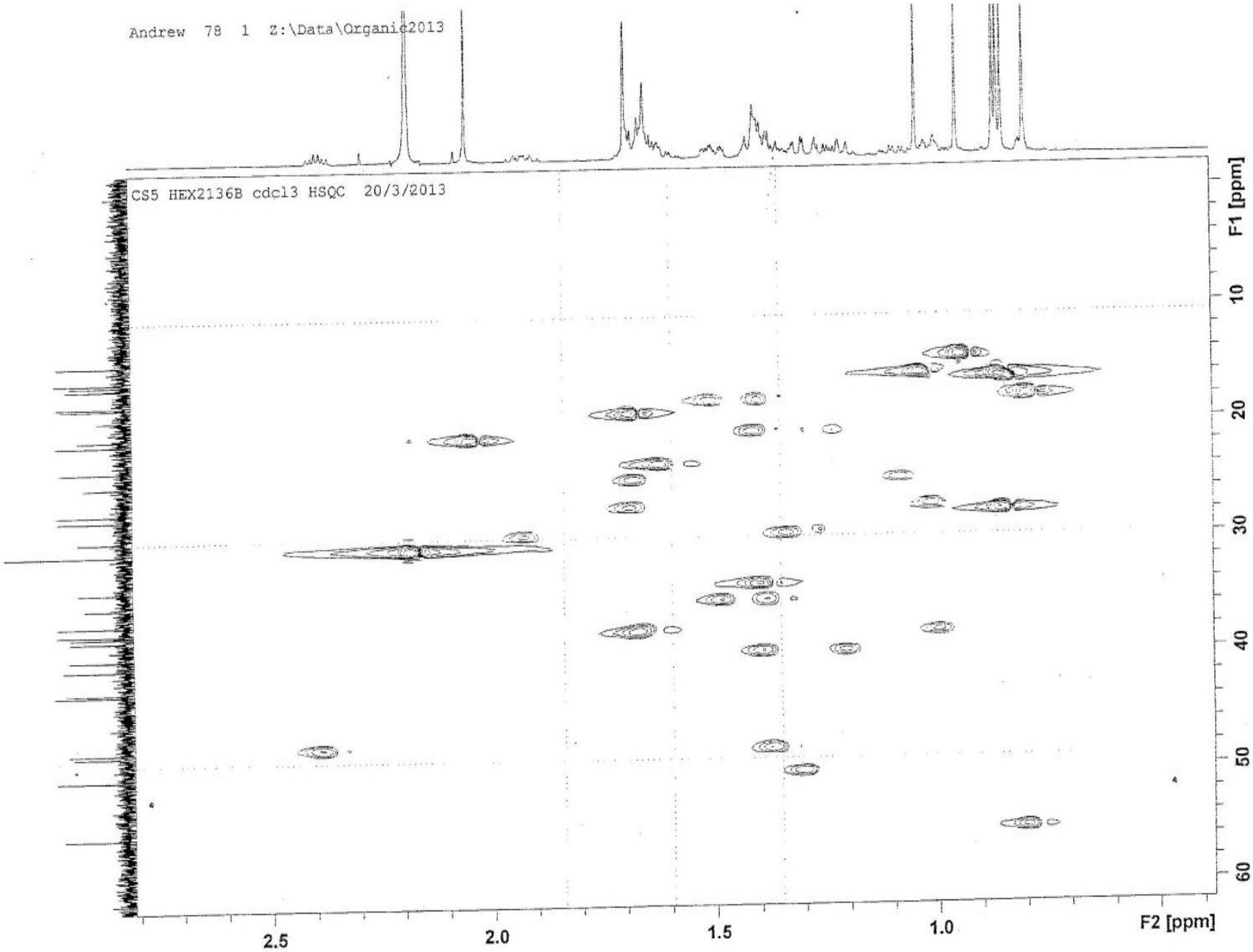
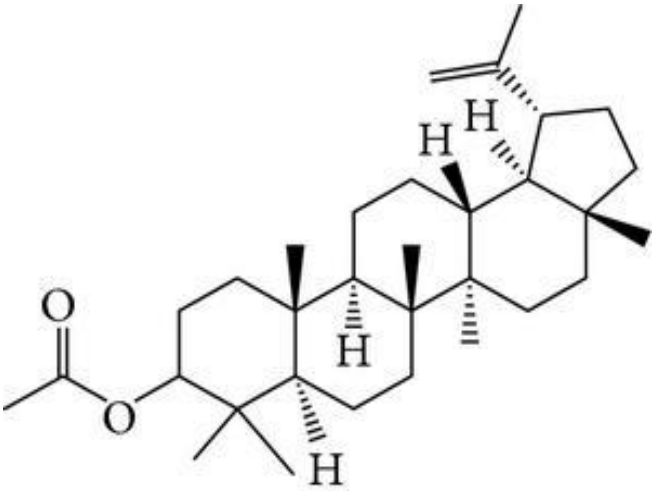


Figure 44: HSQC spectrum of compound 2

Table 21: ^{13}C NMR and ^{1}H-NMR main signals for compound 2

Position	δ_C	δ_H	APT (Multiplicity)
1	38.38		CH_2
2	27.42		CH_2
3	80.97	4.442 (*dd*)	CH
4	37.79		C
5	55.37	0.78 (*m*)	CH
6	18.19		CH_2
7	34.19		CH_2
8	39.99		C
9	50.33	1.32 (*m*)	CH
10	37.07	1.37 (*m*)	C
11	23.70		CH_2
12	25.08		CH_2
13	38.02	1.0 (*m*)	CH
14	42.99		C
15	29.70		CH_2
16	35.56		CH_2
17	42.81		C
18	48.27	1.37	CH
19	48.00		CH
20	150.99		C
21	29.82		CH_2
22	40.83	1.39 (*m*); 1.21 (*m*)	CH_2
23	27.94	0.85 (*s*)	CH_3
24	14.49	0.94 (*s*)	CH_3
25	17.99	0.79 (*s*)	CH_3
26	16.49	0.86 (*s*)	CH_3
27	15.96	0.84 (*s*)	CH_3
28	19.28	0.96	CH_3
29	109.35	4.68; 4.57	CH_2
30	20.93	1.68 (*s*)	CH_3
$OCOCH_3$	171.03		C
$OCOCH_3$	21.33	2.05 (*s*)	CH_3



Lupeol acetate

Figure 45: Structure of compound 2

- Molecular formula: $C_{32}H_{52}O_2$
- Synonyms: 3-Acetyllupeol; Lupeyl acetate; Lupenyl acetate; 3-O-Acetyllupeol;Lupeol 3-acetate;
- $M^+ = 468.40$ (experimental)
- Melting point: 210-212°C (experimental)

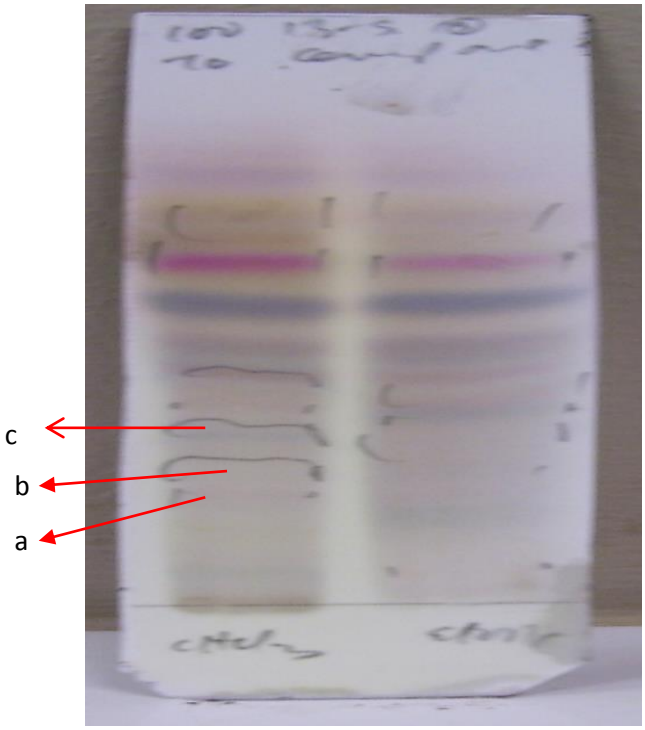
4.5.2 Chloroform Fraction: Isolation and Spectroscopic Results for Characterization of Compound 3

Compound **3** was isolated from chloroform and ethyl acetate fractions as amorphous solid and recrystallized as small, round, whitish-yellow crystals using chloroform and methanol.

Yield: 704 mg from $CHCl_3$ fraction and 489 mg from EtOAc fraction

- IR (KBr), V_{max}: 3330 cm^{-1} (N-H) (Figure 54)
- The results of ^{1}H NMR and ^{13}C NMR are presented in (Table 22; Figures 50 – 53)
- GC-MS: 718 $[M]^{+}$, 700 $(M-H_2O)^{+}$, 644 $(M- C_2H_2O_3^{+})$, 504 (M-214) (Figure 55)
- X-ray crystallography: Figures 60 – 62

4.5.2.1: Isolation of compound 3



Detecting agent: 1% vanillin in H_2SO_4 reagent; UV active (Pencil marked)

Mobile phase: EtOAc: MeOH: H_2O (100: 13.5: 10)

Stationary phase: Silica gel

Figure 46: TLC chromatogram of Chloroform fraction

Table 22: TLC results of Chloroform fraction

Spots	R_f
a	0.26
b	0.32
c	0.43
Blue Band	0.65
Purple/violet Band	0.79
Yellow Band	0.89

This table descripts the R_f of the various prominent and selected spots separated during the TLC (Figure 46). The spots that separated first (lower in the TLC plates) are the more polar

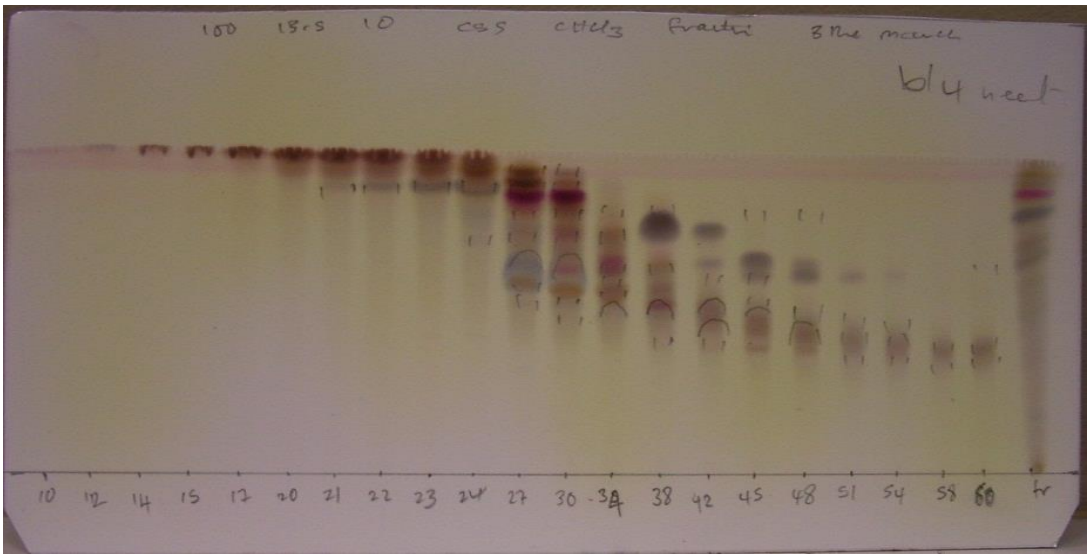


Figure 47: TLC Chromatogram of open column chromatography of $CHCl_3$ fraction

Compound **3** observed as crystals in test tubes 32 - 40

Mobile phase: $CHCl_3$: MeOH: H_2O (65: 35: 5)

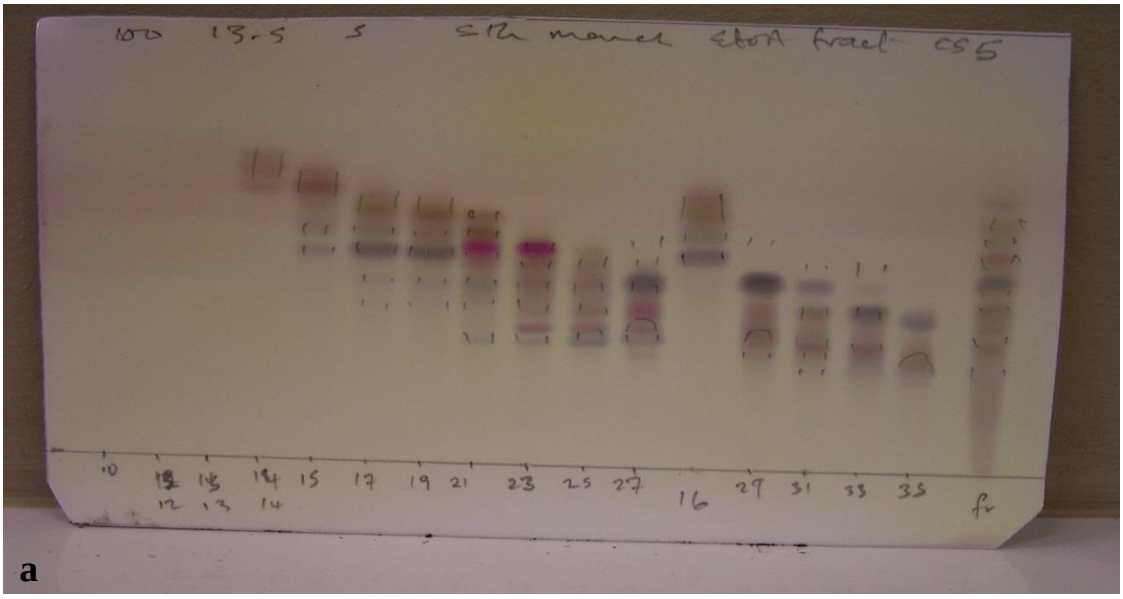
Stationary phase: Silica gel

Table 23: TLC results of test tube/fraction 34 from chloroform fraction (Figure 47)

Spots	R_f
1	0.05
2	0.56
3	0.59
4	0.67
5	0.74
6	0.82

Table 23 shows the R_f values of separated spots/compounds in CC collected fraction 34 (Figure 47). Spot 1 is the lowest visible separation on the TLC plate (in terms of position), while spot 6 is the highest spot on the TLC plate.

Ethyl acetate fraction



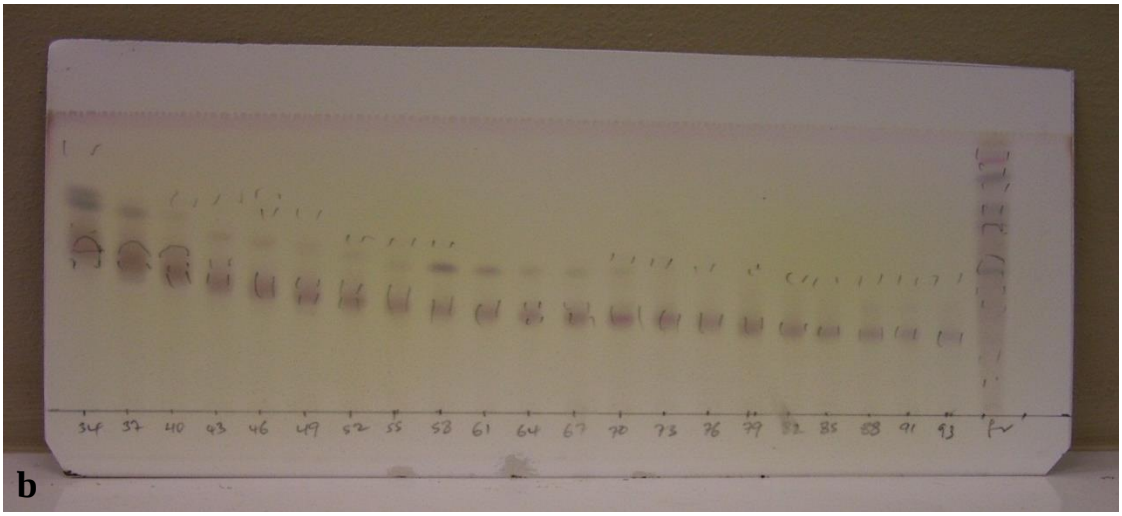


Figure 48a and b: TLC Chromatogram of open column chromatography of EtOAc fraction

Crystals were observed in test tubes 34 to 41

Mobile phase: $CHCl_3$: MeOH: H_2O (65: 35: 5)

Stationary phase: Silica gel

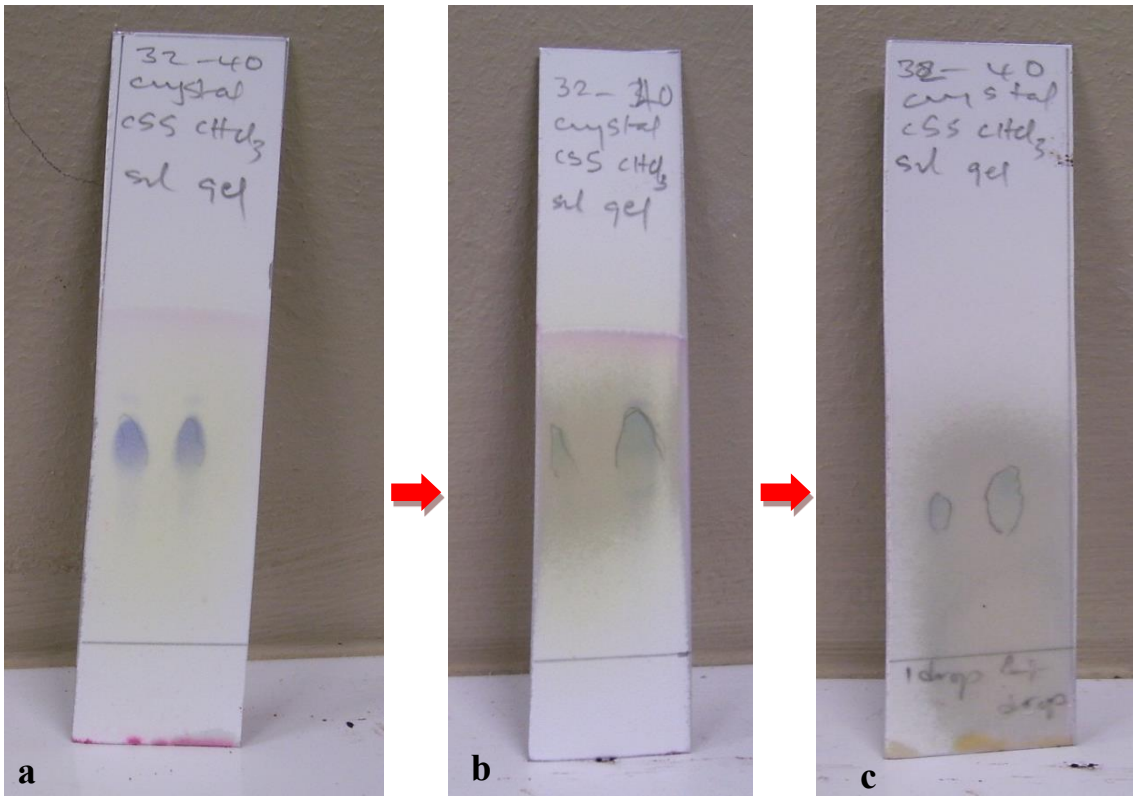


Figure 49: TLC Chromatogram of compound 3

a - Before recrystallization (purification), b and c - after recrystallization (purification).

Mobile phase: $CHCl_3$: MeOH: H_2O (65: 35: 5)

Stationary phase: Silica gel F_{254}

- R_f value: 0.67
- Melting: point: 305 ^oC
- Yield: 704 mg from $CHCl_3$ fraction and 489 mg from EtOAc fraction
- Detecting agents: Colourless with day light

  Active under UV 254 nm

  Blue colour with 1% vanillin/H_2SO_4

  Brownish-yellow colour for Dragendorff

4.5.2.2: Spectroscopic results for characterization and elucidation of compound 3

The following spectra was recorded for compound **3**. Compound **3** was isolated as whitish-yellow crystals from chloroform and ethyl acetate fractions of methanol extract of root bark of *C. stenopetala*.

The GC-MS of compound **3** (Figure 55) showed the molecular ion peak at m/z 718.40

The ^{13}C-NMR spectrum (APT) of compound 3 (Table 24; Figure 51 - 53) showed forty three signals including 2 methyls, 15 methylenes, 13 methines and 13 quaternary carbons.

The ^{1}H – ^{13}C correlations were determined by HSQC spectrum (Figure 59). In the down field region, the resonance at δ 134.6, 114.8, 118.4, 111.0, 145.3, 137.5, 137.9, 121.6, 120.7, 127.8, 109.4, and 143.2 and were assigned to aromatic carbons (Figure 51). The signal at δ 168.8 was assigned to carbonyl whilst 167.1 and 94.5 for olefinic C-2 and C-16.

The study of ^{1}H-NMR spectrum (Figure 50) showed signal down the field due to -NH- proton at δ 8.90 singlets with seven aromatic protons at 6.64, 6.68, 6.80, 6.85, 7.12 and 7.17. The presence of 2 methyl group as $-CO_2CH_3$ and $-Ar-OCH_3$ was proven by the NMR resonance at δ_C 168.8 and δ_H 3.80 (*s*) as well as δ_C 145.3 and δ_H 3.72 (*s*) in the HMBC experiment (Figure 58).

There was COSY correlations from H-23$^{/}$ to H-22$^{/}$ and ROESY correlation from H-16$^{/}$ to H-22$^{/}$ and H- of the $-OCH_3$ to H-23$^{/}$. The lack of (M^{+}-28) peak suggests that the methylenes 23$^{/}$ and 22$^{/}$ bridge in the dimer is bound to part A.

The molecular ion in the GC-MS showed characteristic fragmentation pattern of aspidosperma type skeleton; m/z 718.4 ($C_{43}H_{50}O_6$ N_4 = M), 700.45 (M – H_2O), 562.30 ($C_{35}H_{36}O_4$ N_3 = M –

156), 504.30 (M – 214). The fragmentation process and signal observed are typical characteristic of α-methylene-indoline and β-anilino-acryl ester grouping of aspidospermine. This suggested that the compound is a bis-indole alkaloid composed of two aspidospermedine type units linked by a spirocyclic system involving C-14 (two bonds) of one unit with C $22^{/}$ of another unit together with an additional carbon attached to N-1.

The IR spectrum showed peak at 3330 cm^{-1} (N-H) (Figure 54). Study of the IR spectrum shows an NH band and signals (1280 and 1250 cm^{-1}, -C-N- stretches) characteristics of the β -anilino-acryl ester group (Figure 54).

The compound **3** was elucidated as Vobtusine (Figure 63).

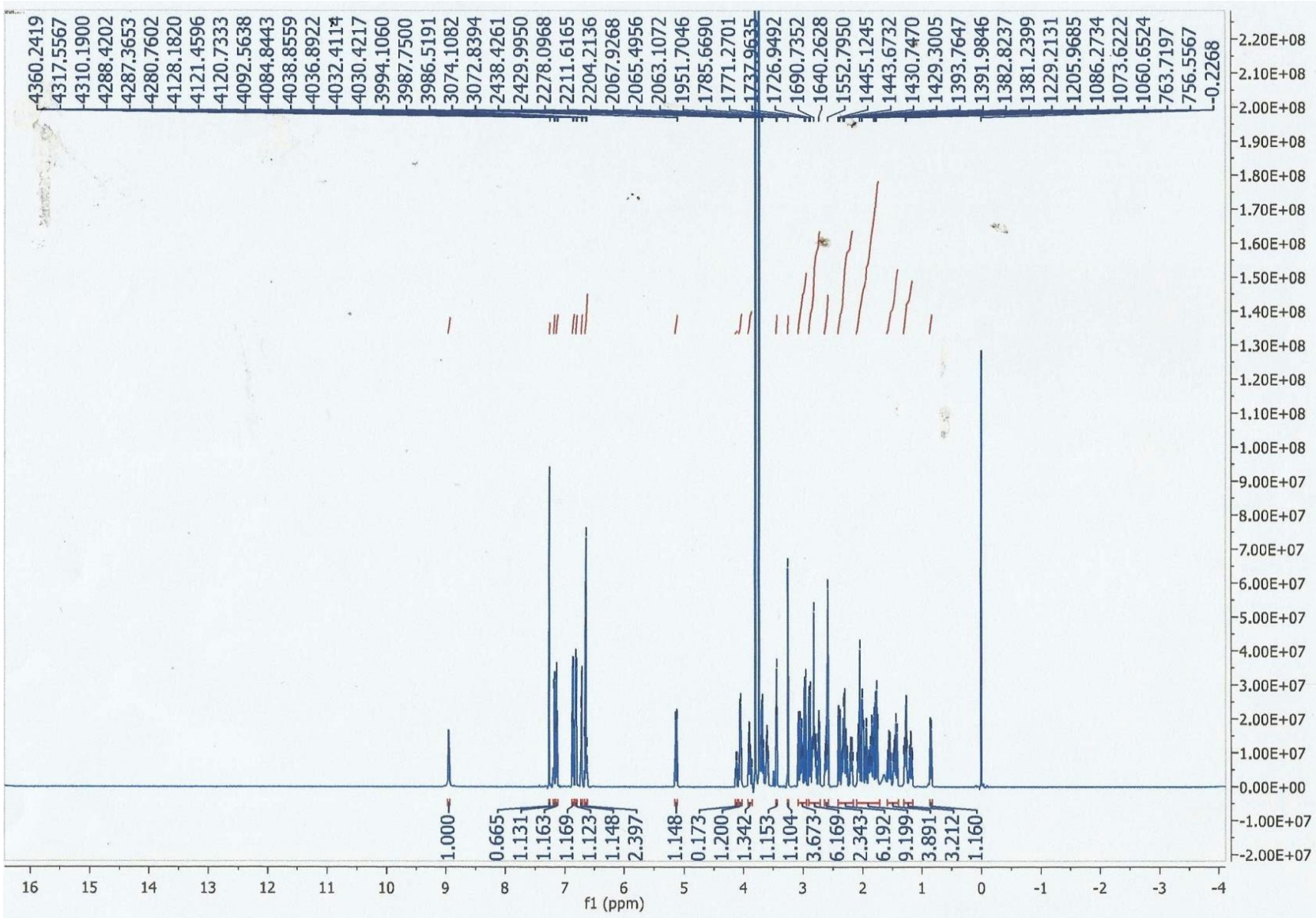


Figure 50: ^{1}H NMR spectrum of compound 3

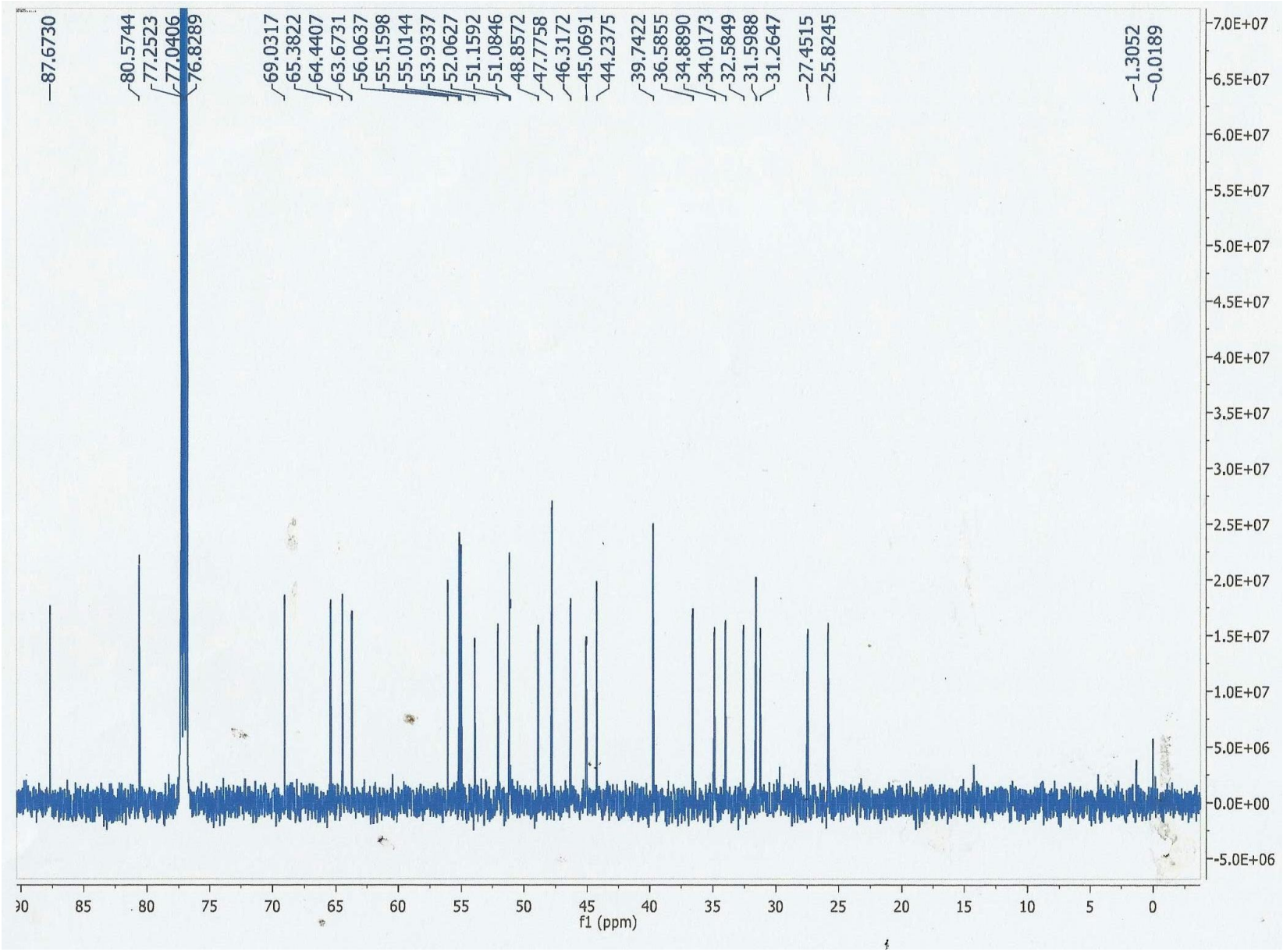


Figure 51: ^{13}C NMR spectrum of compound 3

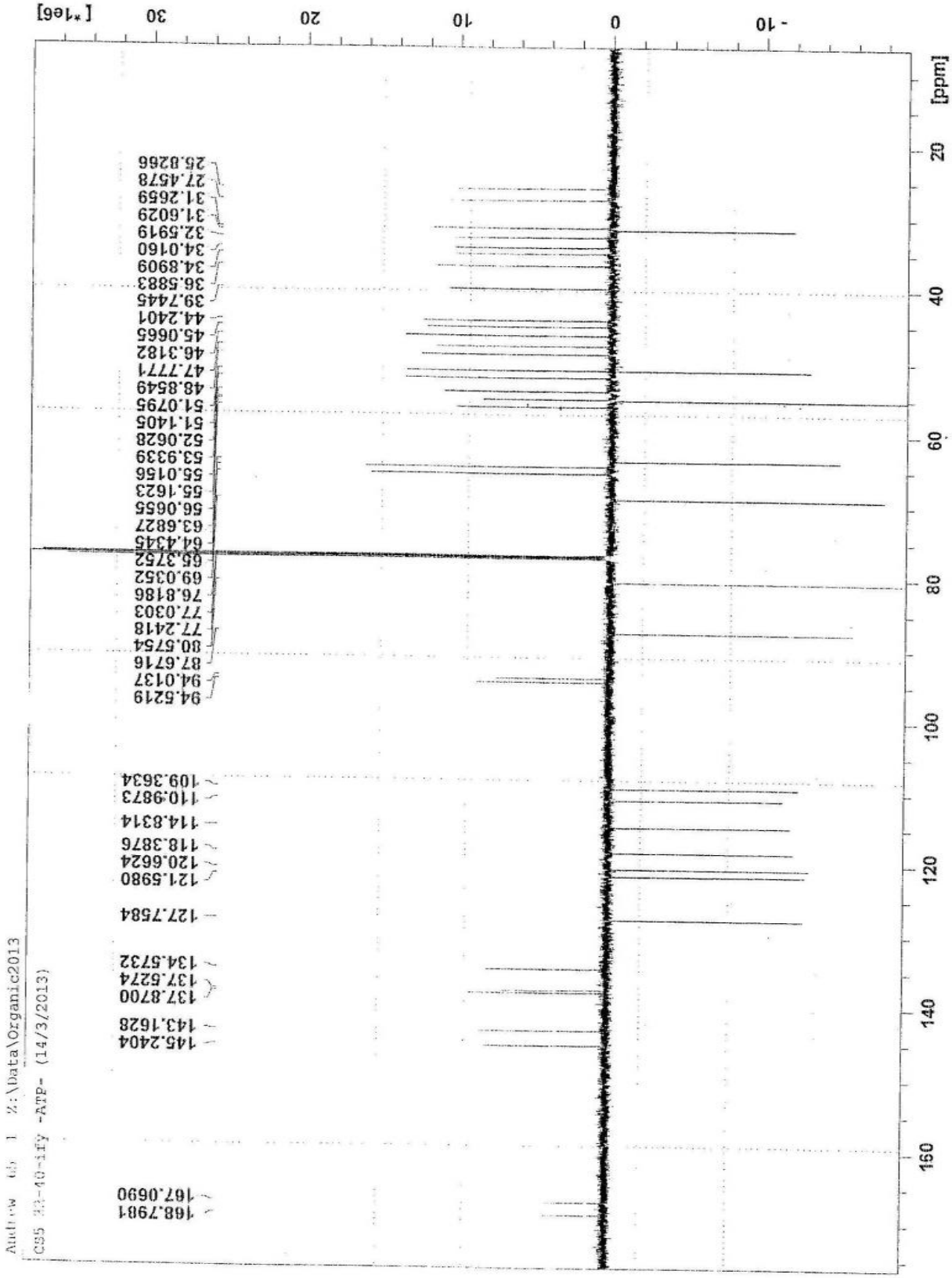


Figure 52: APT spectrum of compound 3

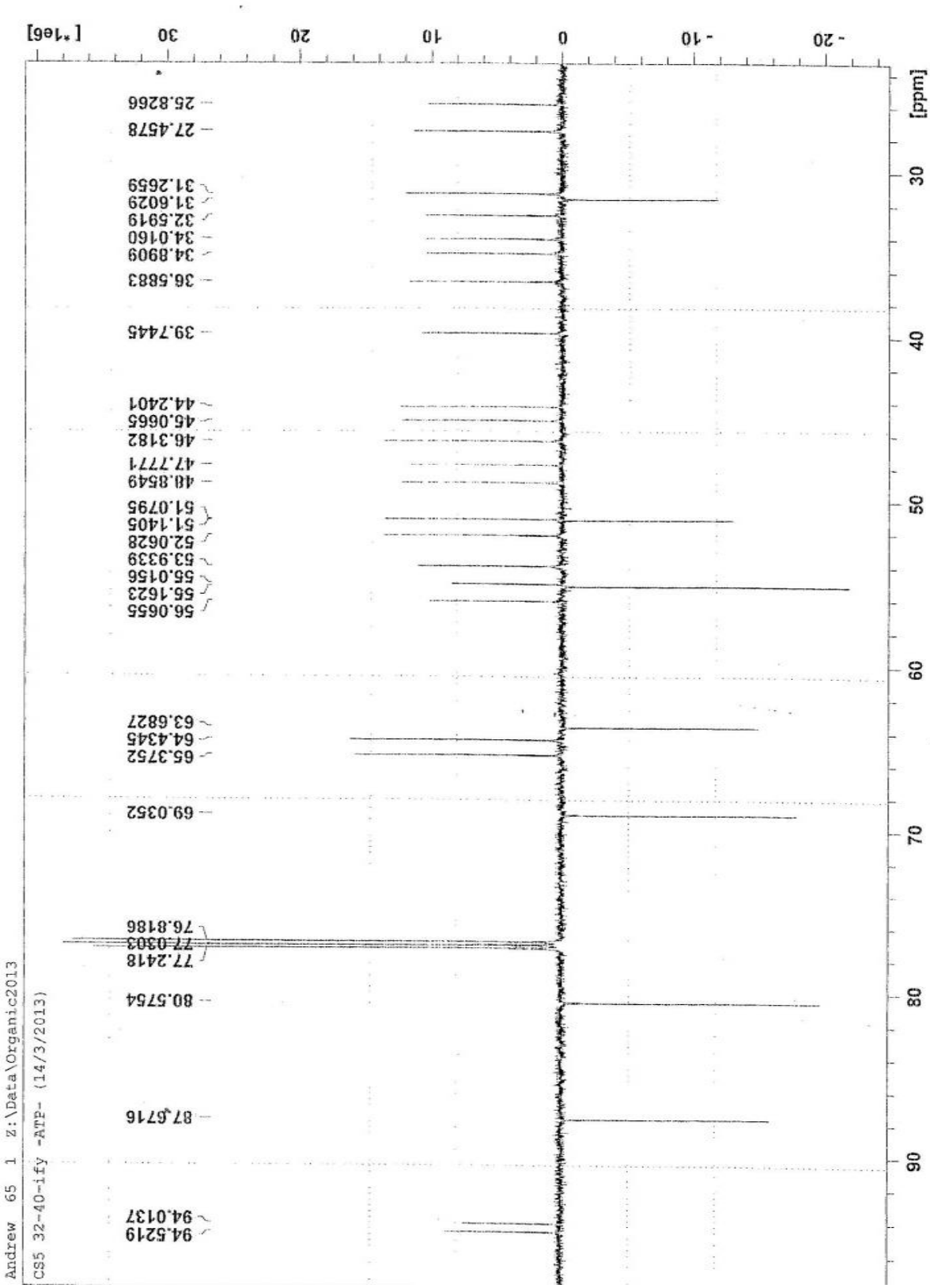


Figure 53: Expanded APT spectrum of compound 3

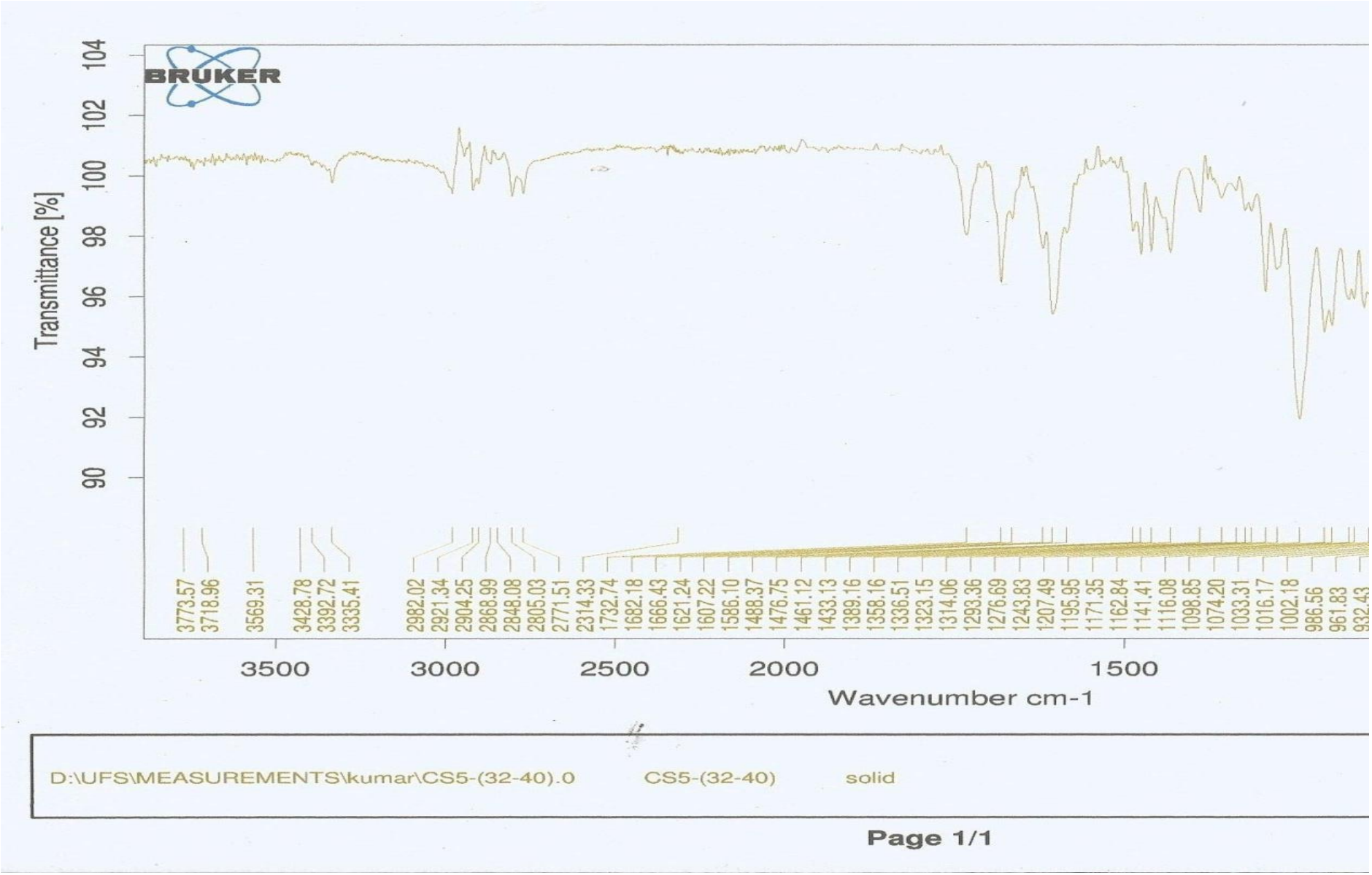


Figure 54: IR spectrum of compound 3

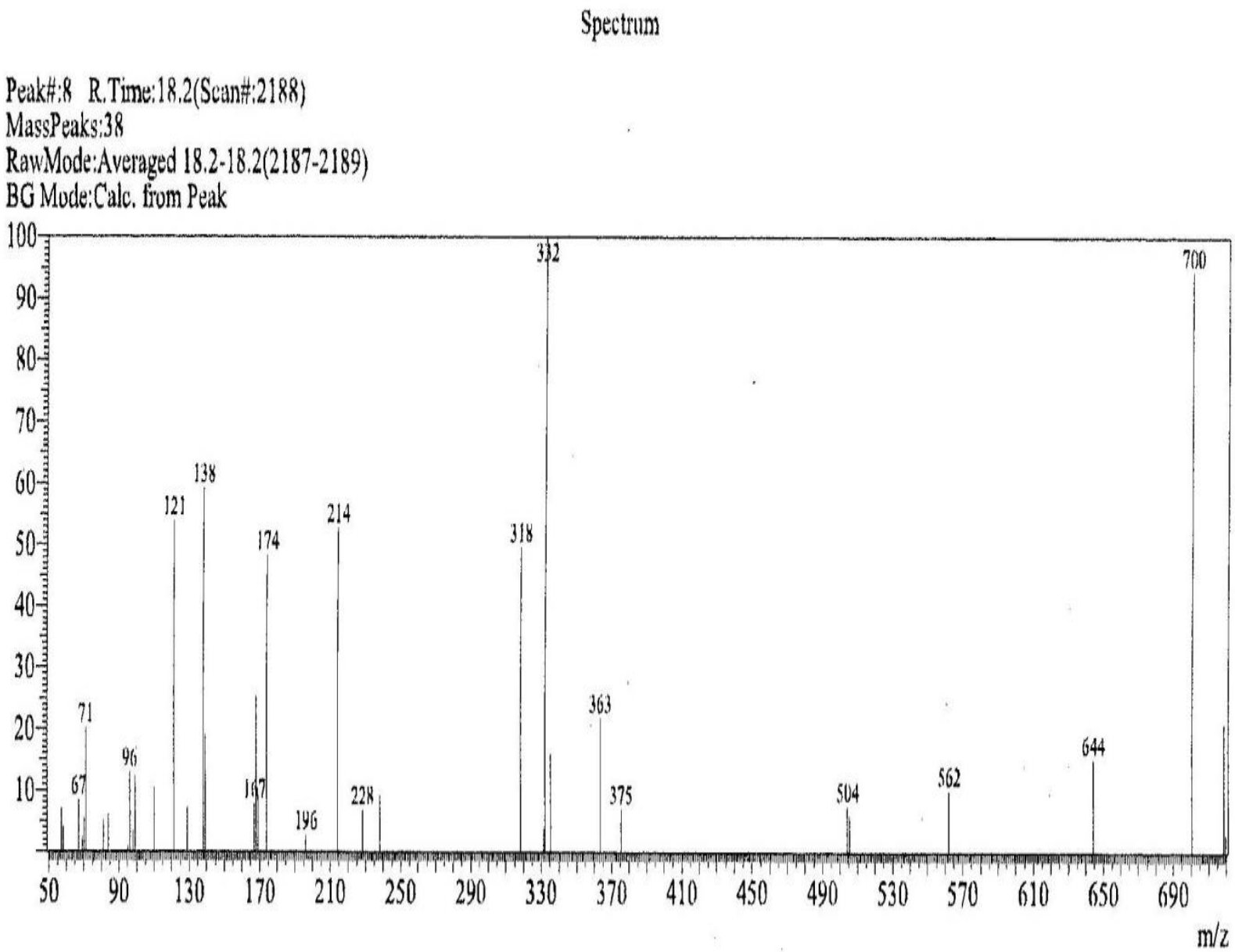


Figure 55: GC-MS spectrum of compound 3

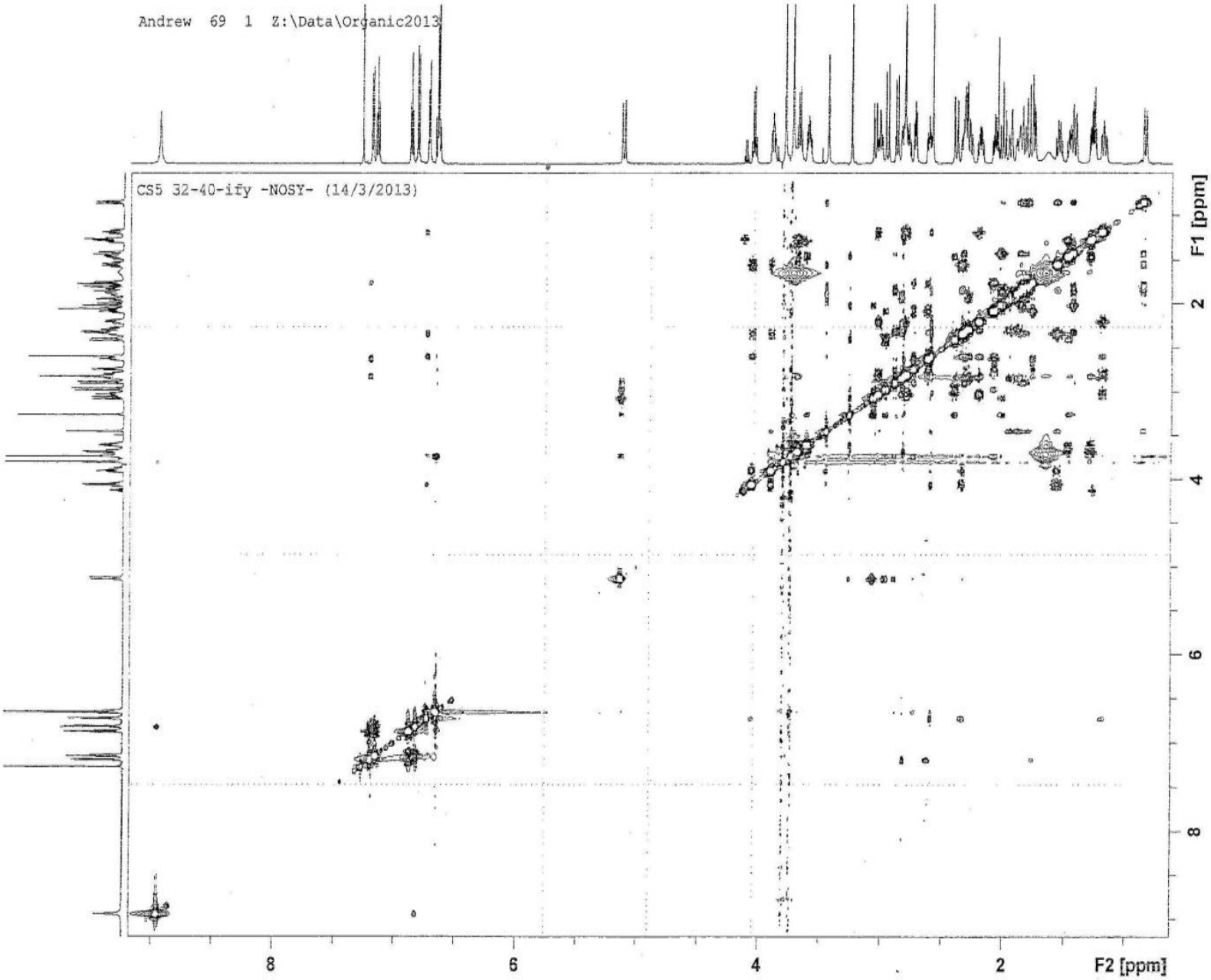


Figure 56: NOESY spectrum of compound 3

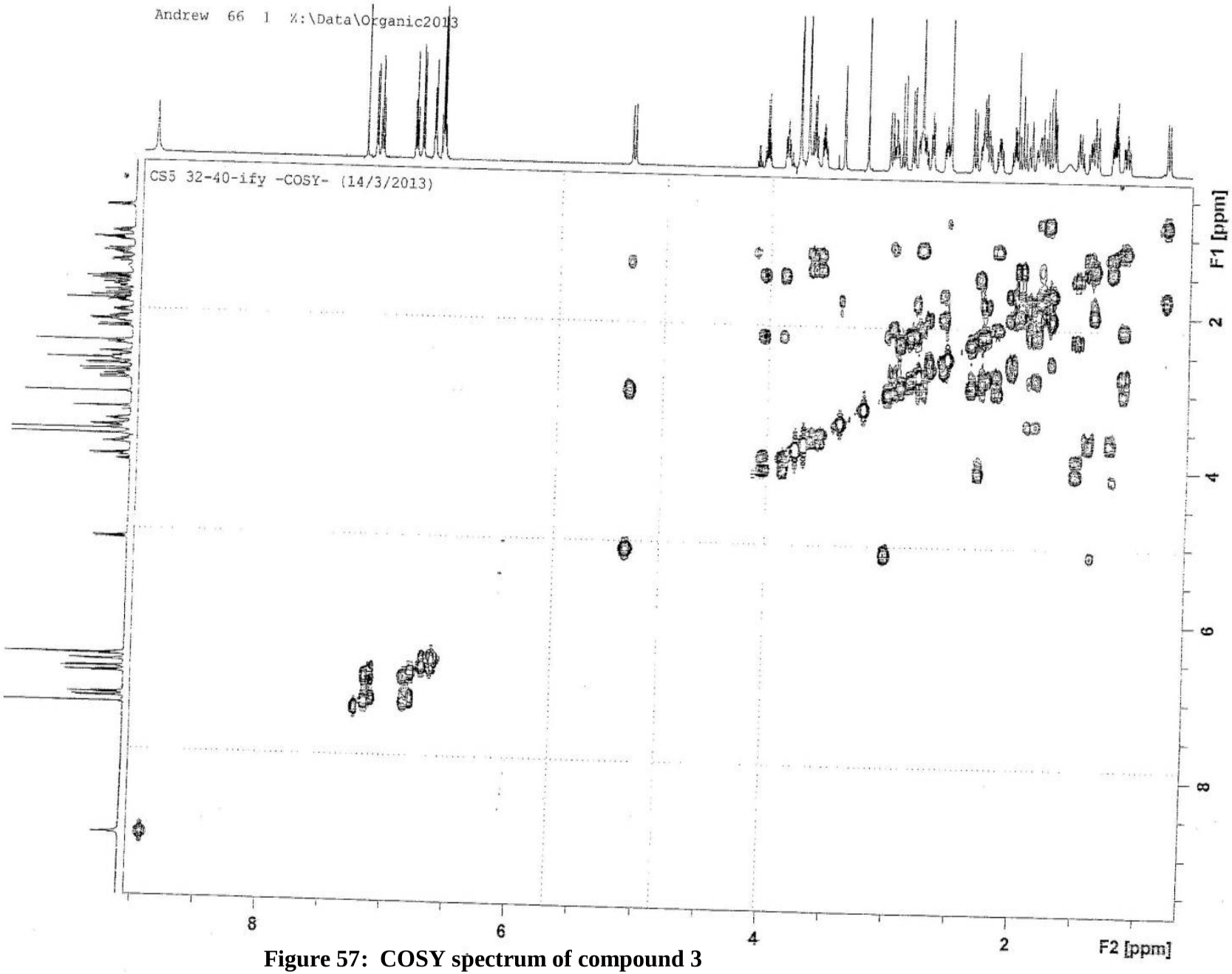


Figure 57: COSY spectrum of compound 3

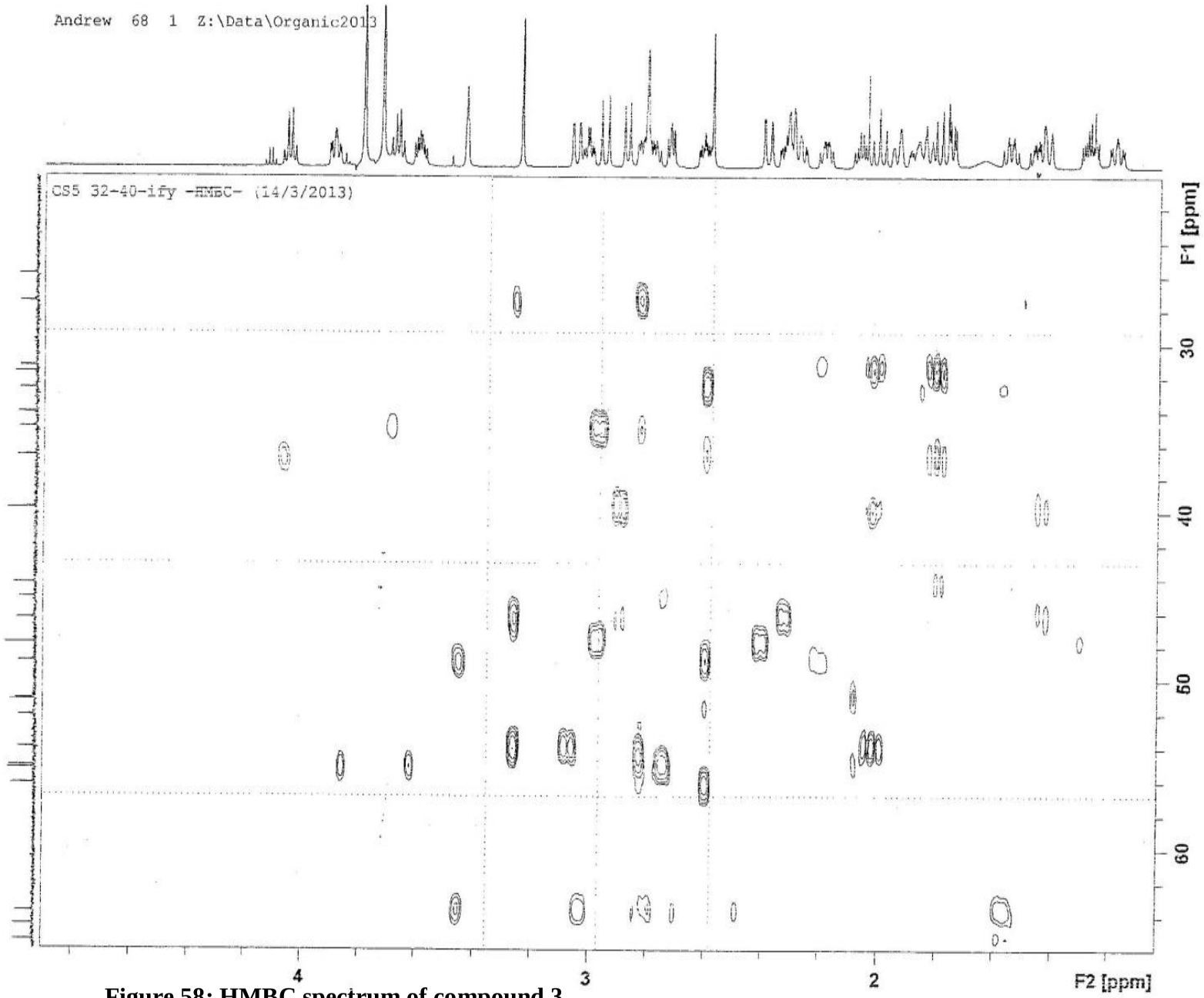


Figure 58: HMBC spectrum of compound 3

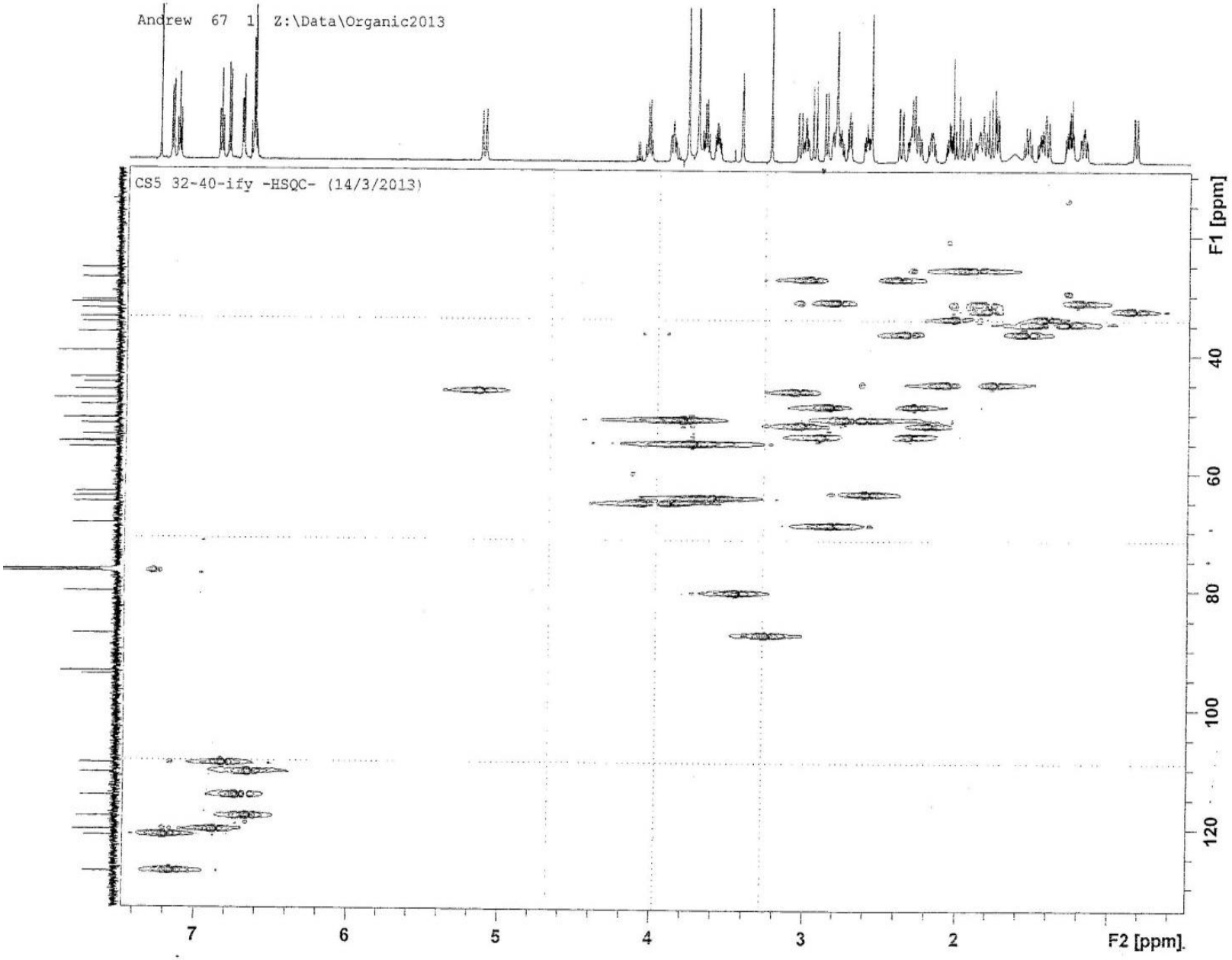


Figure 59: HSQC spectrum of compound 3

X-ray picture of compound 3 at various angles performed in Prof John Davies laboratory, Cambridge University

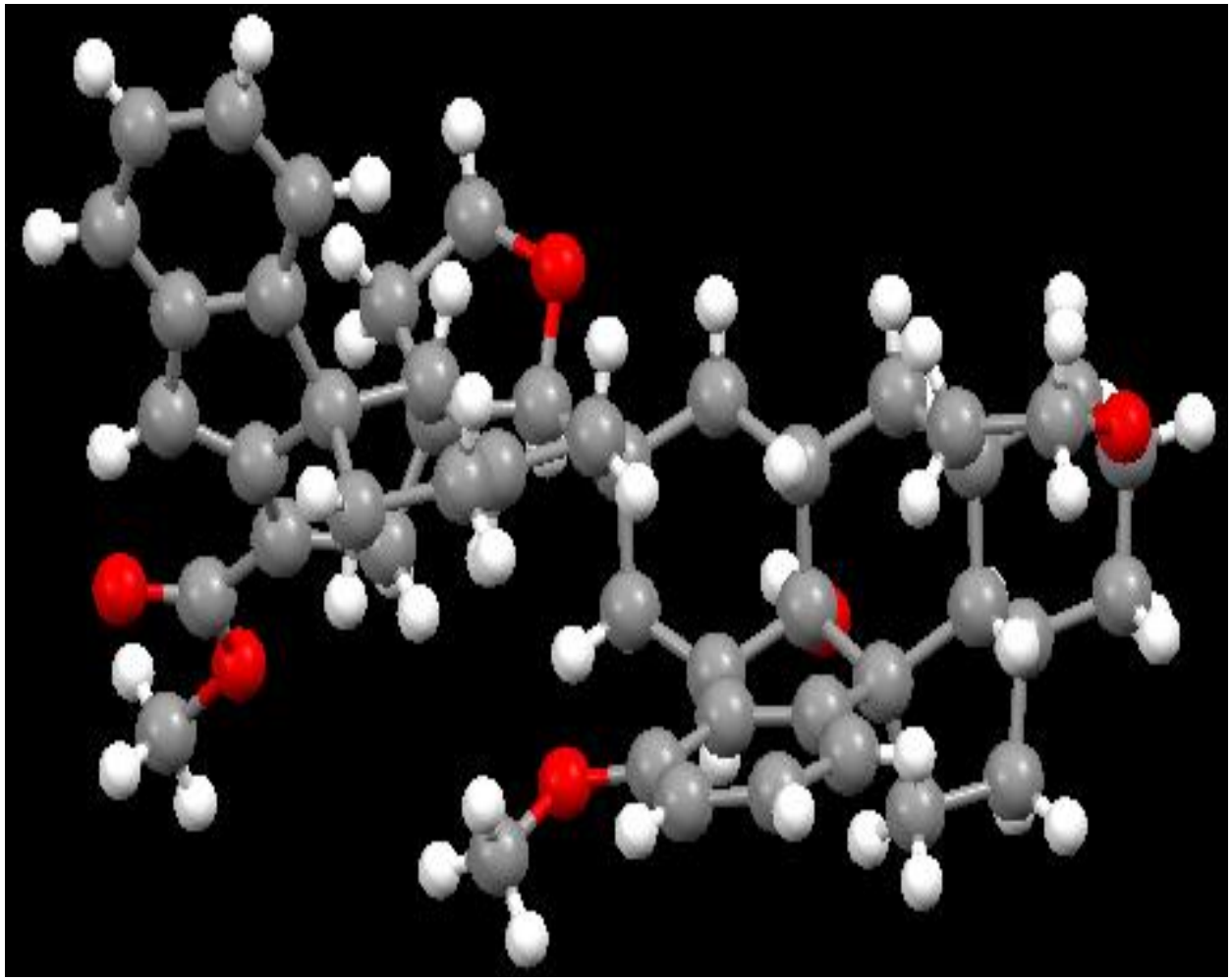


Figure 60: 3 Dimensional Ball and Stick minimized energy mode for compound 3

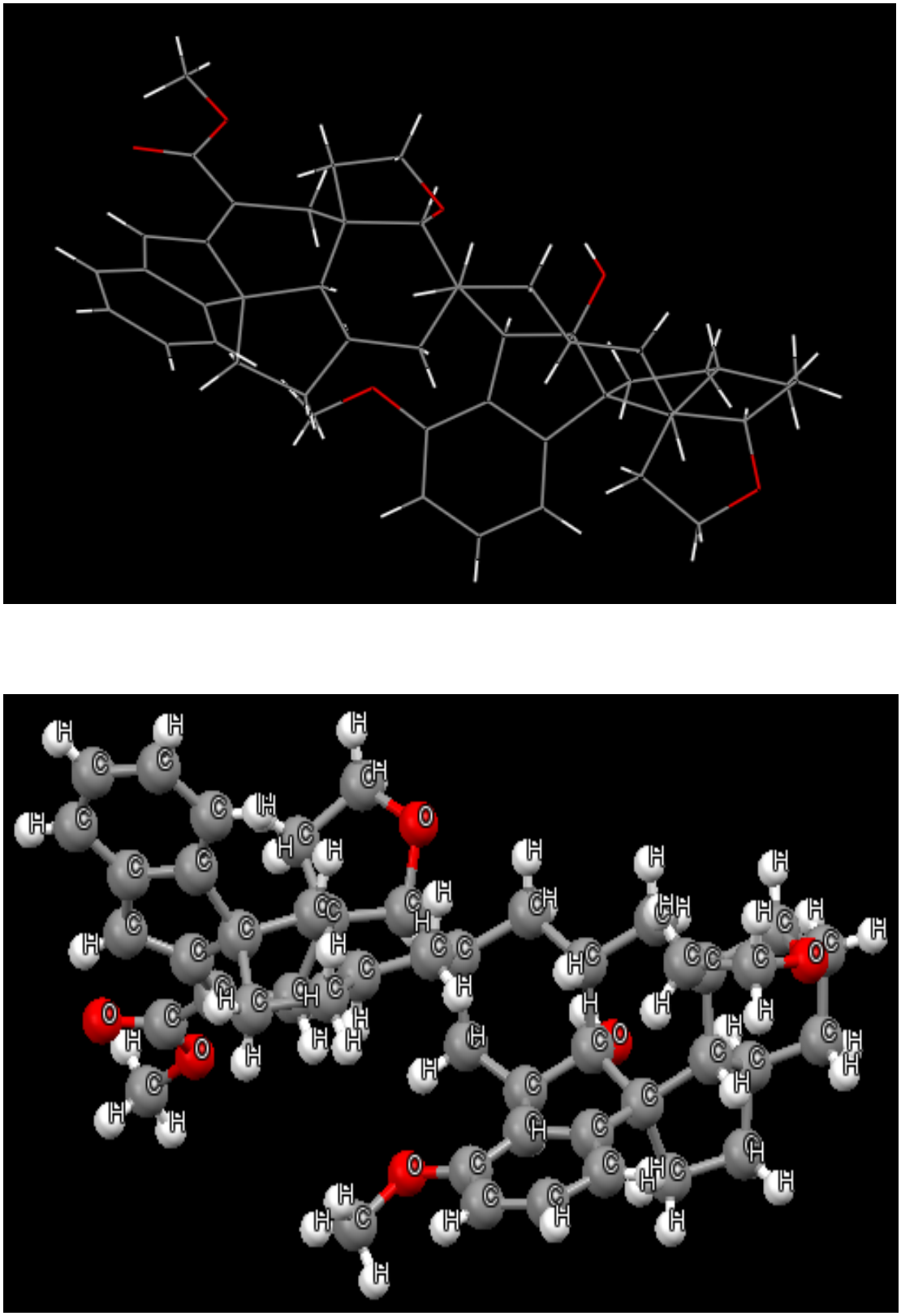


Figure 61: 3 Dimensional Ball and Stick minimized energy mode for compound 3

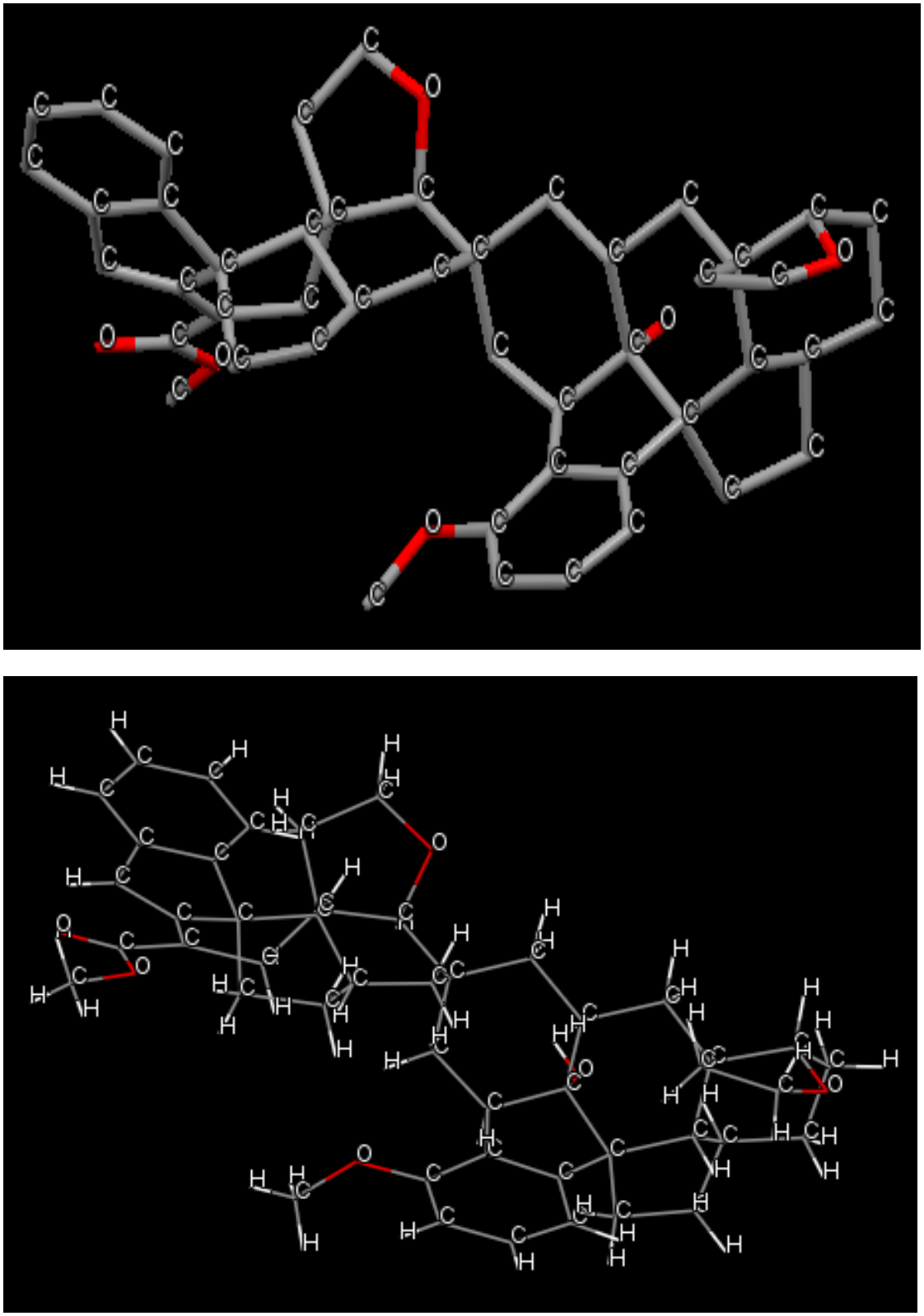


Figure 62: 3 Dimensional Ball and Stick minimized energy mode for compound 3

Table 24: ^{13}C NMR and ^{1}H-NMR main signals for compound 3

Position	δ_C	δ_H (multiplicity)	APT multiplicity
2	167.1	-	-
3	53.9	2.85(*d*) 2.40(*d*)	CH_2
5	51.1	2.70(*t*) 2.55(*m*)	CH_2
6	45.0	2.10(*m*) 1.78(*m*)	CH_2
7	55.0	-	C
8	137.9	-	C
9	121.6	7.17 (*d*)	CH
10	120.7	6.85 (*t*)	CH
11	127.8	7.12 (*t*)	CH
12	109.4	6.80 (*d*)	CH
13	143.2	-	C
14	39.7	-	C
15	87.7	3.25(*s*)	CH
16	94.5	-	C
17	27.4	2.41 (*s*) *2.95* (*s*)	CH_2
18	64.4	3.61(*m*) 3.75 (*m*)	CH_2
19	34.9	1.15 (*m*) 1.25 (*m*)	CH_2
20	47.8	-	C
21	69.0	2.81(*s*)	CH
	168.8 (C=O)		C
	51.2 (OMe)	3.80 (*s*)	CH_3

2′	94.0	-	
3′	48.9	2.80(*m*) 2.30(*m*)	CH_2
5′	52.1	2.99(*m*) 2.22(*m*)	CH_2
6′	31.3	1.15(*t*) 2.75(*m*)	CH_2
7′	56.1	-	C
8′	134.6	-	C
9′	114.8	6.72 (*d*)	CH
10′	118.4	6.68 (*t*)	CH
11′	111.0	6.64 (*t*)	CH
12′	145.3	-	C
13′	137.5	-	C
14′	25.8	1.88 (*m*) 1.99(*m*)	CH
15′	80.6	3.45(*s*)	CH
16′	31.6	1.81 (*m*)	CH
17′	32.6	0.89 (*d*) 1.85 (*d*)	CH_2
18′	65.4	3.89 (*m*) 3.95 (*m*)	CH_2
19′	36.6	1.55 (*t*) 2.35 (*m*)	CH_2
20′	44.2	-	C
21′	63.7	2.55(*s*)	CH
22′	34.0	0.7(*d*) 1.75(*m*)	CH_2
23′	46.3	3.10(*d*) 5.09(*d*)	CH_2
	55.2 (Ome)	3.72 (*s*)	CH_2

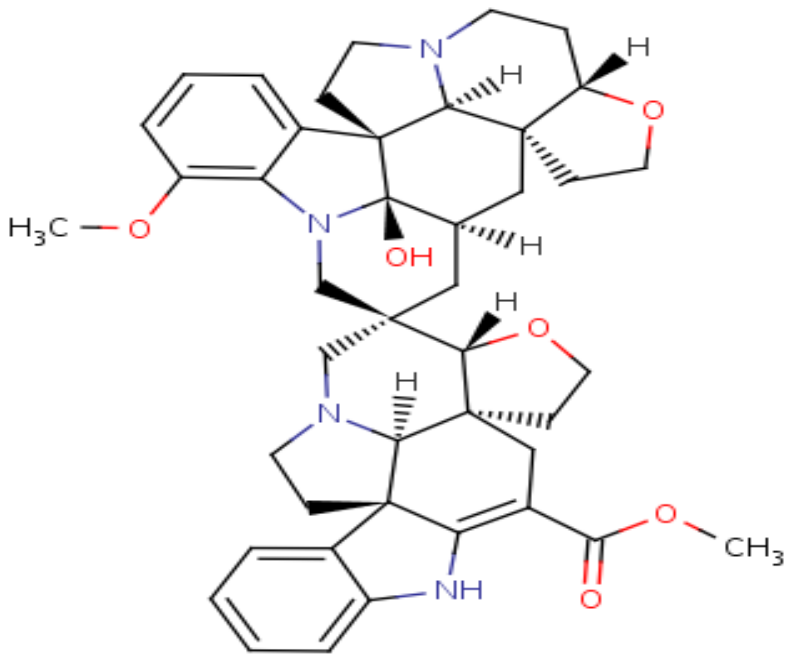


Figure 63: The structure of Vobtusine (compound 3)

Molecular formula: $C_{43}H_{50}N_4O_6$

M^+ = 718.40 (experimental)

Melting point: 305 ^oC (experimental)

Physical appearance: White-yellow small crystals

CHAPTER FIVE

5.0 DISCUSSION

Plants have served as a source of new pharmaceutical products and inexpensive starting materials for the synthesis or semi-synthesis of some known drugs. Plant and animal tissues consist of different classes of compounds with markedly different structures and bioactivities, which can act as "leads" in synthesis of other functional drugs. Quinine **(40)** and artemisinin **(42)** are natural products which are the "lead templates" in the synthesis of some important drugs currently available in clinical treatment of malaria. Chemical modifications of quinine and artemisinin "lead" led to the synthesis of series of highly potent and more therapeutically useful drugs. The two plants from which the "lead" compounds were isolated from have been used for centuries by the local population as traditional medicine against fever in South America and in Asia respectively. Parasite resistance to the drugs and their derivatives is threating treatment-result profile world-wide (Phyo *et al.,* 2012). This triggered the search for new antimalarial "lead" of natural origin using *in vitro* and *in vivo* studies.

The study involved the use of the traditional and low-cost 4-day suppressive assay for testing antimalarial activity. It involves a microscopic evaluation and comparing parasitized and uninfected erythrocytes of test animals. The methodology is accurate and has the advantage of permitting observation of the effect of test drugs on different intra-erythrocytic stages of the parasite (Fidock *et al.,* 2004, Krettli *et al.,* 2009). The *Plasmodium berghei* NK 65 (chloroquine sensitive) was used in predicting treatment outcomes of the antimalarial study plant due to its high sensitivity to chloroquine.

The most important initiating step in the analysis or isolation of the bioactive constituents present in medicinal plants or herbal preparations is good extraction to obtain crude extract. For

easy of isolation procedure, the obtained extract may be suspended in water, and re-extracted with lipophilic solvents in successive increase of solvent polarity, from hexane to polar solvent to obtain fractions. Afterward, each fraction is subjected to separation or purification by other clean-up methods such as chromatography. In this study, bio-activity guided isolation technique was employed for the isolation and purification of active compounds, which were also evaluated for bio-activity.

A plant search was employed in the quest for antimalarial biological active compounds from plants. The medicinal plants selected for the preliminary screening was based on their reputed and long use in a specified local population in treatment or management of malaria fever or re-current fever. Selecting plant for study or research based on its informed ethnobotanical relevance is a better and cost saving approach towards new drug development (Cox and Balick, 1994; Fennella *et al.*, 2004). The information gotten from indigenous medicine formed the rational for the selection of the medicinal plants subjected to phytochemical and pharmacological investigations in this study. Seven (7) antimalarial medicinal plants were collected and subjected to preliminary bio-screening to aid determine the most effective which was selected for further study. These 7 plants were collected for the preliminary bioassay due to their popular use in the course of malaria treatment in the South-eastern part of Nigeria. Noted during the plant indigenous search, was that different parts of 2 of the selected plants (root bark of *Callichilia stenopetala*; leaf of *C. stenopetala* Stapf. Apocynaceae and stem bark of *Petersianthus macrocarpus*; and leaf of *P. macrocarpus* P Beauv. Liben) are used differently/separately in malaria fever management in this locality. Each of these different parts of the 2 plants (*C. stenopetala* and *P. macrocarpus*) were regarded and treated as different treatment regime on their own. Thus, making it nine (9) different medicinal plant crude

extracts/agents bio-screened (Table 6). Comparing the antimalarial activities of *P. macrocarpus* stem bark and leaf indicated that the stem bark possesses more potent antimalarial activity than the leaf (Table 7 and 9). This observation justified the documented report of Dibua *et al.*, 2013, on the continuous folkloric use of stem bark of *P. macrocarpus* as antimalarial remedies due to its efficacy.

The popularity of a medicinal plant in treatment of an ailment may be directly related to its possible efficacy, low toxicity effect and availability in the stated locality. The frequency at which the plants of the family Apocynaceae and Rubiaceae were mentioned as antimalarial agents was more than the other families (Table 6), which may indicate the possibility of members of such families likely to possess antimalarial activity (Wong *et al.*, 2011). The 9 medicinal plant crude extracts (Table 6), *Asystasia gangentica, Callichilia stenopetala* leaf, *Callichilia stenopetala* root bark, *Oldenlandia affinis, Oldenlandia corymbosa, Petersianthus macrocarpus* leaf, *Petersianthus macrocarpus* stem bark, *Borreria ocymoides* and *Picralima nitida* all exhibited different levels of antimalarial and antioxidant activities. The antimalarial potentials of the plants and their corresponding free radical scavenging activities are shown in Tables 7, 8, 9. There was no specific correlation or relationship observed in the relationship between the antimalarial and antioxidant activities of these plant crude extracts. Five (5) plants were very potent as antiplasmodial and as antioxidant agents; 2 plants (*Callichilia stenopetala* leaf and *Petersianthus macrocarpus* leaf) exhibited moderate activity as antiplasmodial and good antioxidant activity; 2 plants have moderate antiplasmodial and poor antioxidant activities (*Oldenlandia affinis* and *Asystasia gangentica*) (Table 9). Most of these plants are used in the form of mono-therapy and occasionally were mentioned as part of a combined therapy, polyherbal (oral interview during the plant search survey). Whether used in combination as

multi-herbal preparation or given singly as mono-therapy, it is referred as "ogwu-iba" in the South-Eastern locality of Nigeria. Inference from the information gotten during the plant search indicated that combination therapy, when applied, has no fixed rule but depends on patient presentation and traditional health giver discretion. The common route of administration of these medicinal plants is oral. They are prepared as decoction or infusion of the part(s) of the plant of interest (oral interview during the plant search survey) for use in malaria treatment. Recent research reports or documentation validated some of the ethnobotanical use of these medicinal plants in treatment of malaria fever or re-current fever and malaria symptoms.

The standard 4-day suppressive test commonly used for evaluation of antimalarial activity of plants involves the determination of level of such activity by calculating the percent inhibition of parasitaemia (Madara *et al.,* 2012). Substances exhibiting 30 to 40% *in vivo* parasitaemia suppression in mice malaria models are considered to be moderately active (Muregi *et al.,* 2006, Pereira *et al.,* 2014). Crude extracts of *Picralima nitida* leaf, *Borreria ocymoides* aerial part, *C. stenopetala* root bark and *Petersianthus macrocarpus* stem bark exhibited high activities of both biological effects (Table 9). *Picralima nitida* leaf extract has both very high antimalarial and free radical scavenging activity (83.39% and 72.30%) respectively more than that recorded for *C. stenopetala* root extract (80.30 and 84.50% respectively). This observation is in line with past reports of some herbs and poly-herbs exhibiting both antimalarial and antioxidant effects (Reddy *et al.,* 2007). However, extensive work/research has been done on *P. nitida* thus the selection of *C. stenopetala* as the plant for further study in the current research work (Iwu and Klayman, 2002; Okokon *et al.,* 2007a; Osayemwenre *et al.,* 2014).

Thus all the 9 tested plants extracts exhibited moderate to good activity as antiplasmodial but the root bark of *C. stenopetala* (which was very potent) was selected for further study on its

antimalarial activity. *Callichilia stenopetala* is a seasonal plant and found mostly during the raining season, when malaria attack/infection is at its peak in the South-eastern part of Nigeria. It may be the nature's way of providing solution to environmental challenges. It has folkloric use in management of recurrent fever, oral hygiene oriented problems, treatment of infections, excessive menses, bleeding gums and mouth sores (oral interview).

Plants are very complex in their composition and their therapeutic activity depends on their chemical constituents which can be extracted using various solvents (Marston, 2002). The polarity of the solvent used for extraction determines the type of constituents extractable (Marston, 2002). Methanol used as the solvent system for the extraction of active constituents of *C. stenopetala* was based on the traditional method of soaking the plant in water (aqueous decoction) or alcohol before use. Both methanol and water are polar solvents. The choice of the solvent was also influenced by the ability of this solvent to extract a large spectrum of both polar and non-polar compounds compared with other solvents. The yields of the extraction and fractionation are shown in Table 10. Following fractionation, a simple process of pooling similar compounds of similar polarity together as a fraction and thereby making isolation of compounds easier (Marston, 2002). Thus hexane fraction contains non-polar constituents, chloroform and ethyl acetate fractions containing moderately polar constituents, while aqueous residue contains the polar constituents.

Preliminary phytochemical analysis revealed that the study plant (*C. stenopetala*) has a good number of phytochemical constituents which include alkaloids, flavonoids, saponins, terpenoids, sterols, phenols and tannins (Table 11). The curative properties of medicinal plants have been stipulated to be due to the presence of various secondary metabolites they contain

(Sofowora 1993). Antimalarial activity exhibited by the *C. stenopetala* may be attributed to its high alkaloid, phenol and terpenoid constituents (Hoet *et al.,* 2004: Tarkang *et al.,* 2013). The oldest antimalarial drug, quinine, belongs to alkaloid class of compounds and is still much relevant in malaria chemotherapy. The antimalarial activities of terpenoids such as artemisinin have been established in copious literature (Okokon and Nwafor 2007). The presence of phenols and tannins in the extract may be responsible for the free radical scavenging activity observed in the crude extract and polar fractions. The high reactivity of the hydroxyl group contained in phenols, flavonoids and tannins aid in formation of stable reaction products thereby are regarded as good scavengers of free radicals. Phenolic compounds have been reported to have physiological activities due to their ability to prevent or control oxidative stress related disorders (Odukoya *et al.,* 2005; Percário *et al.,* 2012). The compounds isolated in this study may very well serve as template for the synthesis of more potent compounds in future.

At dose range of 500 – 8,000 mg kg^{-1}, no significant changes were observed in the *C. stenopetala* crude extract treated mice. The acute toxicity result of this study suggested that the oral medial lethal dose (LD_{50}) of the extract should be above 8,000 mg kg^{-1}. The wellness parameters evaluated were, skin fur, behavioural pattern, tremors and GIT upset etc. Many of the herbal preparation ingredients currently available to practitioners have long history of use as herbal remedies and longtime usage of these herbal remedies is assumed to reflect their level or degree of safety. WHO, 2004 guidelines recommended that no specific restrictive regulatory action should be undertaken for such long time used herbs unless there is new evidence which will demand a revise of the risk-benefit assessment.

The *in vivo* model was used for this study based on its ability to account for the possible pro-drug effect and possible involvement of the immune system in eradication of the parasites (Madara *et al.,* 2012). The murine model of 4-day suppressive test evaluates the antimalarial activity of test drugs on early infections. The antiplasmodial activity of *C. stenopetala* root bark extract and fractions were established based on this test. The crude extract assay results show a reduction in the percentage parasitaemia in dose-dependent manner (Table 14). The activity profile of the fractions (250 mg kg^{-1}) decreased with polarity; hexane>chloroform>ethyl acetate > aqueous fraction (Table 15). The hexane and chloroform fractions were found to possess higher blood schizontocidal activity than the other fractions, indicating the possible localization of the active ingredients in these two fractions. Some active compounds may be found in the ethyl acetate fraction, which could explain its lower antimalarial activity. Antimalarial bio-active alkaloids, phenols and terpenoids compounds have been isolated and reported in some medicinal plants (Bantie *et al.,* 2014). The crude extract of *C. stenopetala* contains these metabolites (Table 11). These phytochemical constituents contained in the plant may have an individual bio-activity or synergistic effect to exert their bio-activity (Bantie *et al.,* 2014).

Antioxidant activity of *C. stenopetala* root bark extract and fractions were evaluated *in vitro* using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and total phenolic content (TPC). The antioxidant activities of compounds are usually associated with their ability to inhibit oxidative damage by scavenging of free radicals (Tarkang *et al.,* 2013). Total phenolic content assay correlates with the antioxidant activity of extract contents. The TPC of *C. stenopetala* was 70 mg g^{-1} gallic acid equivalent. Though the hexane fraction showed a remarkable level of free radical scavenging property, the polar fractions were better in mopping up of the free radicals.

This observed trend is probably due to the presence of the OH groups associated with polar compounds (Figure 15).

Free radicals induce oxidative stress *in vivo* that may lead to oxidative modification or damage of some biological structures, such as proteins, lipids, DNA, giving rise to generative diseases (Okeola *et al.,* 2011). The role of oxidative stress during malaria is still unclear. However recent studies suggest that malarial infection has the ability to activate the immune system which causes the release of reactive oxygen species (ROS) with the potency of inducing oxidative damage and cell destruction (Onyesom *et al.,* 2010; Onyesom *et al.,* 2012). The generation of reactive oxygen and nitrogen species (ROS and RNS) associated with oxidative stress during malaria infection thereby has been demonstrated to play a very important role in the development of systemic complications in the host and apoptosis to the malaria parasites (Percário *et al.,* 2012; Tarkang *et al.,* 2013). Oxidative stress result from increased production of free radicals during malarial infection, a fact suggested by increased oxidative stress marker in infected erythrocyte cells compared to uninfected cells (Percário *et al.,* 2012; Tarkang *et al.,* 2013). The deleterious consequences of increased cell rigidity and increased lipid peroxidation reported during oxidative stress in human malaria have been linked to increased mortality of adults and children with malaria (due to increased anemia) (Percário *et al.,* 2012). *Callichilia stenopetala* root bark crude extract and its fractions exhibited free radical mopping ability (Figure 14 and 15). Thus use of *C. stenopetala* root bark crude extract and its fractions in management of malaria infection may ameliorate symptoms of malaria, reverse or minimize the oxidative damage to hosts caused by the malarial parasites, reduce mortality and generally improve the malaria treatment routine. The suppressive and DPPH radical scavenging activities of the crude extract of *C. stenopetala* at 500 mg kg^{-1} are 80.31 and 84.50% respectively (Table 9

ad 14). Chloroquine, an antimalarial drug kills malaria parasites by generating of free radicals which at the long run induces biological injury to the host due to oxidative stress. Herbal antioxidants isolated from *Curcuma longa* have been reported to have prevented hepatotoxicity in rats treated with chloroquine (Percário *et al.,* 2012) in a laboratory investigation, indicating the bio-ability of antioxidants when present in medicinal plants.

The most potent antimalarial effective fraction(s) are the non-polar ones while the free radical scavenging activity is observed in the polar fractions, suggesting that taking the crude extract during malaria infection may be more beneficial than a purified fraction (Tables 9, 14, 15 and 17). The caution/fear on the crude extract being more toxic than the fraction(s) due to the many possible constituents, may not really arise because the oral acute toxicity test at a value far higher than the given dose resulted in no death of any of the test animals.

TLC is used to support the identification of a compound and involves the spraying of phytochemical screening reagents, which cause colour changes according to the phytochemicals existing in a plant extract; or by viewing the plate under the UV light. This was used throughout the course of the purification and isolation to monitor, identify and confirm purity of isolated compounds. Three known compounds were isolated from root bark extract of *C. stenopetala* by employing bioassay-guided fractionation, as shown in Figures 10, 21, and 34 for compound **1**; Figures 10, 35 and 45 for compound **2**; Figures 11, 49 and 63 for compound **3**.

The compound **1** was isolated from hexane fraction as colourless cube like crystals with R_f value 0.6 in C_6H_{14}: DCM (5: 6) from hexane fraction of methanolic extract of *C. stenopetala* root bark. The GC-MS of compound **1** (Figure 25) showed molecular ion peak at m/z 468.40 $(M)^+$ which is in agreement with the molecular formular $C_{32}H_{52}O_2$ (calculated m/z 468). From

the information on the spectra and its analysis (Figure 22 – 30), compound 1 was elucidated to be α-amyrin acetate, a pentacyclic triterpene. The final confirmation of the structure was established from the X-ray diffraction studies (Figure 31-33). The chemical shift of the signals and other physical data were found identical to literature reported α-amyrin acetate (Figure 34) (Sisay and Abeba 2005; Shan, *et al.*, 2014)

Compound **2** was isolated as white crystals, with R_f value 0.56 in Hex: DCM (5: 8) from hexane fraction of the plant, *C. stenopetala*. The GC-MS of the compound (Figure 39) showed molecular ion peak at m/z 468.40 (M^+) which is in agreement with the molecular formular $C_{32}H_{52}O_2$. Compound **2** was identified as lupeol acetate based on the available spectral analysis, ^{13}C NMR, ^{1}H NMR, APT, HSQC, HMBC, NOESY correlation, GC-MS, IR etc. (Figure 36 – 44). The identity was further confirmed by comparing the observed values with reported data (Jamal *et al.*, 2008). Lupeol acetate is a pentacyclic triterpene. It has been reported to be pharmacologically active triterpenoid. It has several potential medicinal properties (Fotie *et al.*, 2006).

From the results of this study, the two compounds (**1** and **2**), all pentacyclic triterpenes have good antimalarial activity (67.61 and 68.65%; Table 18) but presented significantly poor activity as antioxidants (14.0 and 5.8% at 10 mg kg^{-1}; Figure 17, Table 19). The antimalarial activities of these pure compounds were observed to increase here with decrease in the ability of the compounds to mop up free radicals. This is in opposite direction as against the bio-activities (Plasmodial chemosuppression and free radical scavenging) of the 9 tested medicinal plant crude extracts (Table 9) were no correlation was observed. The isolate, α-amyrin acetate at 67.61% chemosuppression has 14% ability to mop up free radicals. The triterpene, lupeol acetate (compound 2) has 68.65% chemosuppressive activity and exhibited a decreased ability

to mop up free radicals (5.8%). In line with the past reported action of sesquiterpene, artemisinin, observed antimalarial and antioxidant activities of compounds 1 and 2 is due to these isolated compounds initiating the production of free radicals on contact with iron, a common metal in the body, within erythrocytes (Zhang *et al.,* 2010). This induces oxidative stress, which is a very effective mechanism in the destruction process of malaria parasites in the host. Several studies have demonstrated the involvement and effectiveness of oxidative stress in the mechanism of action of some antimalarial drugs (Hartwig *et al.,* 2009; Klonis *et al.,* 2011). In other words, lowering the antioxidant activity of the body system improves the antimalarial activity of such drugs. Documented studies have demonstrated the involvement of oxidative stress in the mechanism of action of artemisinin and some other antimalarial drugs, thus discouraging the con-current administration of antioxidants with such drugs (Klonis *et al.,* 2011; Percário *et al.,* 2012).

Malaria parasites are highly vulnerable to oxidative stress during the part of their life cycle when they inhabit erythrocytes. The parasites live in a pro-oxidant rich environment containing oxygen and iron, which produce a large amount of reactive oxygen species. Management of oxidative stress in malaria parasite is tightly regulated through active redox and antioxidant defense systems. The elevation of oxidative stress as a result of inhibition of any component of this defense system leads to redox imbalance and ultimately parasite death. Identification of key molecules, which has the ability to disrupt parasite redox balance by altering key redox reactions and promote oxidative stress in parasites, is an effective approach to develop novel antimalarial drugs. A number of currently used drugs, especially the endoperoxide antimalarial, appear to act by increasing oxidant stress, and compounds 1 and 2 which exhibit this trend may be a source of new antimalarial drug.

Several triterpenes and their derivatives have been reported possessing promising antimalarial activity *in vitro* and *in vivo* against various strains of *P. falciparum* (Suksamrarn *et al.,* 2006). Compounds 1 and 2 have proved in this study to be one of such triterpenes. Lupeol ester derivatives have been reported to have antimalarial activities (Fotie *et al*., 2006; Kumar *et al*., 2008).

The compound **3** (Figure 63) was isolated as whitish-yellow round crystals from chloroform and ethyl acetate fractions of methanol crude extract of root bark of *C. stenopetala*. The GC-MS of compound **3** (Figure 55) showed the molecular ion peak at m/z 718.40 which was in agreement with the molecular formula $C_{43}H_{50}N_4O_6$ (calculated m/z 718.880). The ^{13}C-NMR spectrum (APT) of compound **3** (Table 24; Figure 51 - 53) showed forty three carbon signals confirming the above formular. The ^{1}H – ^{13}C correlations were determined by the D-NMR spectra Figure 56 - 59. The molecular ion in the GC-MS showed characteristic fragmentation pattern of aspidosperma type skeleton; m/z 718.4, (α-methylene-indoline and β-anilino-acryl ester grouping of aspidospermine). Thus confirming compound **3** to be a bis-indole alkaloid composed of two aspidospermedine type units. The peak at 3330 cm^{-1} (N-H) (Figure 54) is characteristic of the β -anilino-acryl ester group . The chemical shifts and other physical data obtained when compared with literature data, suggested the compound to be identical to vobtusine (Figure 63; Mei *et al.,* 2012). The structure was eventually established from X-ray diffraction study (Figure 60 - 62).

This is the first reported case of the compound as antimalarial and antioxidant bio-active agent, to the best of my knowledge. The compound was last isolated from *C. stenopetala* and evaluated then as possible cardiovascular agent in the mid- late 1960's. In this report vobtusine has moderate *in vivo* plasmodial chemosuppression activity at 7 mg kg^{-1} (62.20%) against the

chloroquine sensitive strain of *Plasmodium berghei berghei* (NK 65) in the 4-day suppressive test. It also exhibited a moderate antioxidant activity at 0.02 mg mL^{-1} (68.7%) compared with the standard vitamin C (93.80%). Vobtusine thus exhibited both antioxidant and antiplasmodial activities. These results suggest that the reduction of reactive oxygen species by vobtusine may have no direct role in its antiplasmodial action and other mechanism of activity may be implicated. The presence of ferriprotoporphyrin IX (iron) from the heamoglobin digestion inside the malaria parasite could change the properties of vobtusine since these metal ions could significantly change primary antioxidant activities to pro-oxidants (Labieniec and Gabryelalc, 2006). In line with the above statement, this may be one of vobtusine mechanism of activity.

Recent investigations have also shown that another possible mechanism in which vobtusine could be acting is interfering with the mechanisms of the disease by modulating the cellular signaling pathway and not by directly inducing the parasites to death. This approach has shown very promising results, with high rates of schizonticide and antiparasitic activity, but with minor changes in the host redox balance (Al-Adhroey *et al.,* 2011; Akanbi *et al.,* 2012; Percário *et al.,* 2012). Some established antioxidant plants which have been tested for this purpose: high rate of schizonticidal and antiparasitic activities include *Piper betle* L. leaves (Al-Adhroey *et al.,* 2011), *Anogeissus leiocarpus* (Akanbi *et al.,* 2012), *Nigella sativa* seeds (Okeola *et al.,* 2011) and flavonoids from *Artemisia annua* L. (Ferreira *et al.,* 2010). They all exhibited high antimalarial activity, when tested in mice infected with *P. berghei*. The administration of glutathione (an antioxidant) promoted reduced parasitaemia and increased survival of mice infected with *P. berghei* (Ghashgaeinia *et al.,* 2010). Furthermore, the use of antioxidant supplements during malaria infection can reverse or minimize the oxidative damage to hosts caused by the use of antimalarial drugs (Percário *et al.,* 2012).

Vobtusine (compound **3**) belongs to a group of complex alkaloids (bisindole alkaloids) which consists of a wide variety of structures, depending on the identity of the monomeric alkaloid components. Bisindole alkaloids are clinically important group, in which a cleavamine-type unit is attached via C-16 to the aromatic ring of an aspidosperma unit. Vindoline; vinblastine **(3)** and vincristine are the best known examples.

The family Apocynaceae has proved to be rich source of indole alkaloids which are active against *Plasmodium in vitro* under micro-range, *in vivo* and with good selectivity index (Philippe *et al.,* 2007). The monoindole alkaloids isolated from *Strychonos icaja* were devoid of any significant antiplasmodial activity while several of its oxygenated bisindole alkaloids (sungucine derivatives) were all highly active compounds (Frederich *et al.,* 2000; Philippe *et al.,* 2007). Keawpradub *et al.,* 1999, noted that *Alstonia* species (Apocynaceae) are traditionally used in Africa for their antimalarial properties and their antimalarial properties have been attributed to bisindole alkaloidal content (villalstonine, macrocarpamine). The antimalarial activity of *Tabernaemontana fuchsiaefolia* was attributed to bisindole alkaloids, the principal one being voacamine (Frederich., *et al.,* 2008). The observed antimalarial property of vobtusine is in line with past reported cases of bis-indole alkaloids being antimalarial active.

This study shows that vobtusine has dual activity as antimalaria and as antioxidant defense. Its antioxidant ability could ameliorate the progress of malarial infection and probably prevent its sequelae development of systemic complications caused by malaria infection (Percário *et al.,* 2012).

Synergistic and potentiative effect of the multi-phytoconstituents contained in the studied crude extract may have favoured enhanced efficacy of the crude extract considering its

chemosuppression of 87% at the dose of 250 mg kg^{-1}. Synergistic and potentiative drug combinations have been shown to achieve one or more favourable results: enhanced efficacy of action (both desired and undesired), decreased dosage at equal or increased level of target inhibition, reduced or delayed development of drug resistance, targeting multiple points of action and simultaneous reduction of toxic effects (Ma *et al.*, 2009; Ferreira *et al.*, 2010). Potentiation can exist between different phytochemicals within a single plant extract or with different plants taken concurrently. Experimental studies have shown that there is synergy between the various cinchona alkaloids (quinidine, cinchonine and cinchonidine). The poly-phytoconstituents may also likely reduce or delay development of drug resistance and simultaneous reduction of toxic effects.

SUMMARY OF FINDINGS

Findings from the results obtained in this study are as listed below

1. The plant search produced seven (7) medicinal plants with history of long use and acclaimed efficacy from which nine (9) crude extracts were obtained.
2. Nine (9) medicinal plants crude extracts namely, *Asystasia gangentica* (aerial),, *Callichilia stenopetala* (root bark), *Callichilia stenopetala* (leaf), *Oldenlandia affinis* (aerial), *Oldenlandia corymbosa* (whole plant), *Petersianthus macrocarpus* (leaf), *Petersianthus macrocarpus* (stem bark), *Borreria ocymoides* (aerial), *Picralima nitida* (leaf) use in treatment of re-current fever or malaria in the South-Eastern part of Nigeria were screened for antimalarial and antioxidant activities. The following results were obtained:
    I. Five (5) plant crude extracts (*Picralima nitida* leaf, *Petersianthus macrocarpus* stem bark, *Borreria ocymoides* aerial part, *C. stenopetala* root bark and *Petersianthus macrocarpus* stem bark) have potent antimalaria and antioxidant activities.
    II. Two (2) plant crude extracts (*Callichilia stenopetala* leaf and *Petersianthus macrocarpus* leaf) have moderate antimalarial and good antioxidant activities.
    III. Two (2) plant crude extracts (*Oldenlandia affinis* and *Asystasia gangentica*) have moderate antimalarial and poor antioxidant activities.
    IV. There was no observed correlation between antimalaria and antioxidant activities of these plant crude extracts.
2. Extraction, fractionation, purification and isolation of the methanolic crude extract:

I. Fractionation gave 4 different fractions (semi purification).

II. 3 bioactive compounds were purified and isolated using chromatographic techniques.

3. Characterization and structure elucidation of isolated compounds resulted to 3 named structures, vobtusine, α-amyrin acetate and lupeol acetate using modern spectroscopic equipment.

4. Bioassay of crude extract, fractions and isolated compounds established their possible bioactivity as:

   I. Bioactive secondary metabolites present in *C. stenopetala* root bark were established.

   II. The crude extract of *C. stenopetala* root bark has dose dependent plasmodia chemosuppression.

   III. Antimalarial activity resides in the non-polar fractions, particularly the hexane fraction.

   IV. The isolated compounds (vobtusine, α-amyrin acetate and lupeol acetate) were all active in chemosuppression of *Plasmodium*

   V. Only compound 3 (vobtusine) showed significant activity as free radical scavenging agent.

CONCLUSION

This study has shown that root bark of *C. stenopetala* has antimalarial and antioxidant activities and consists of more than one antimalarial active phytoconstituents. Chromatographic analysis of *C. stenopetala* yielded the bisindole alkaloid, vobtusine and two triterpenes which were elucidated as α-amyrin acetate and lupeol acetate. The isolated compounds showned potent activity against chloroquine sensitive strain of *Plasmodium.*

CONTRIBUTIONS TO KNOWLEDGE

1) This study established the antimalarial potential of *C. stenopetala* root bark for the first time, to the best of my knowledge.

2) Three compounds were isolated, elucidated structurally and confirmed to be α-amyrin acetate, lupeol acetate and vobtusine. α-Amyrin acetate was isolated from the plant for the first time and its possible use as antimalarial chemotherapeutic agent established.

3) Vobtusine antimalarial potential and free radical scavenging activity were established here for the first time, to the best of my knowledge.

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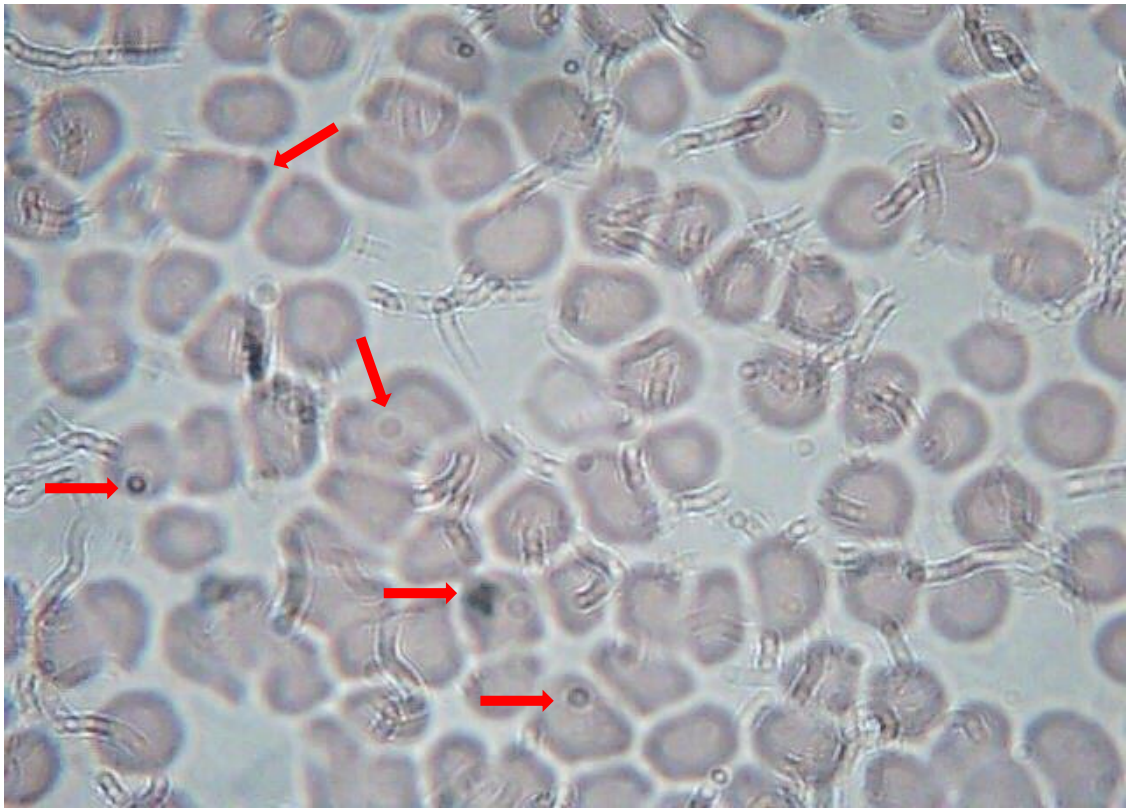

C. stenopetala partially cleared *Plasmodium berghei* blood stage form in Geimsa stained smears from mice

COLLEGE OF MEDICINE
UNIVERSITY OF LAGOS
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THE RESEARCH GRANTS AND EXPERIMENTATION ETHICS COMMITTEE (RGEEC) OF THE COLLEGE OF MEDICINE OF THE UNIVERSITY OF LAGOS, IDI-ARABA

RESEARCH PROPOSAL REVIEW STATUS SHEET

Title of the Proposal	**Isolation and Characterization of Antimalarial Compounds from Root Bark of Callichilia Stenopetala Stapf. (Apocynaceae)**
Protocol ID	**RGEEC /22/2015**
Version	**2 (Revised)**
Principal Investigator (s)	**Mrs. I. C. Orabueze**
Investigator's Affiliation	**Department of Pharmacognosy, Faculty of Pharmacy, University of Lagos, Idi-Araba, Lagos, Nigeria**
Date of Ethics Committee's discussion of the protocol	**Monday, May 11, 2015**
Ethics Committee's Recommendation	✓ 1. **Full Approval** 2. Conditional Approval 3. Not Approved 4. Rejected (*See attached explanatory notes to decision of the Committee*)
Ethics Committee's Comments	
Special Recommendations	
Name and Signature of Ethics Committee's Chair	**A. O. Okanlawon**
Date:	**June 18, 2015**

IMPORTANT

1. *Any changes to the proposal or to the attachments (informed consent/study instruments etc.) should be approved by RGEEC before being implemented.*
2. *The approval for this proposal is valid for a period of* ***one year only****.*
3. *Please resubmit this proposal for a Continuing Review at least 2 months before the next re-approval period.*

Explanatory notes to the decisions of the Research Grants and Experimentation Ethics Committee.

[X] The proposal is ***Approved as submitted***. No modifications are required.

[ ] The proposal is ***Conditionally Approved; requires amendments and/or clarifications.*** Final approval is contingent upon an adequate response by the Principal Investigator, to the satisfaction of the reviewers or the Chair on behalf of the RGEEC.

[ ] The proposal is ***Not approved; requires additional information and/or rewriting***. A revised version of the proposal should be re-submitted.

[ ] The proposal is ***Rejected.*** The proposal is ethically unacceptable, for the reasons stated as attached. The Principal Investigator may submit a new proposal that takes into consideration the ethical issues raised by the Committee. If you do not agree with the Committee's assessment, please feel free to submit an appeal to the Chair of the RGEEC, through the Secretariat.

Mr. Ekene, the plant collector

Uruagu, Nnewi in Anambra state, Nigeria