ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM THE AERIAL PARTS OF *Maesobotrya barteri* [HUTCH]

BY

CHRISTIANA ENE OGWUCHE

DEPARTMENT OF CHEMISTRY
FACULTY OF SCIENCES
AHMADU BELLO UNIVERSITY, ZARIA
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BY

Christiana Ene OGWUCHE, B.SC (HONS) CHEMISTRY (BSU)
M.Sc (ORGANIC CHEMISTRY, ABU)
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Declaration

I declare that the work in this thesis entitled " ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM THE AERIA PARTS OF *Maesobotrya barteri* [HUTCH**]**" has been performed by me, in the Department of Chemistry, under the supervision of Professors J.O. Amupitan, G.I. Ndukwe and Dr. (Mrs) R.G. Ayo. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

Christiana Ene OGWUCHE _____ _____

Signature Date

Certification

This thesis titled "ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM THE AERIAL PARTS OF *Maesobotrya barteri*" by Christiana Ene OGWUCHE, meets the regulation governing the award of the degree of Doctor of Philosophy (Ph.D) in Organic Chemistry of the Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

Professor J.O. Amupitan Chairman, Supervisory Committee	_____ Signature	_____ Date
Professor G.I. Ndukwe Member, Supervisory Committee	_____ Signature	_____ Date
Dr. (Mrs) R.G. Ayo Member, Supervisory Committee	_____ Signature	_____ Date
Professor V.O. Ajibola Head of Department	_____ Signature	_____ Date
Professor K. Bala Dean, School of Postgraduate Studies	_____ Signature	_____ Date

Dedication

This research work is dedicated to the glory of God for his infinite mercies, blessings, guidance and protection over me.

To my ever caring and goal-achieving Husband, Dr. Emmanuel Anawo Ogwuche, My Children (Emmanuel Ekondu and Tina-Treasure). Also my beloved parents, Mr Nicholas Samuel Ijigah and Mrs Justina Oyanu Ijigah, and to all my siblings.

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Abstract

The petroleum ether, chloroform, ethyl acetate and methanol extracts of the aerial plant parts of *Maesobotrya barteri* were screened for their antimicrobial activity and their chemical constituents. The preliminary phytochemical screening of the various extracts showed the presence of carbohydrates, saponnins, triterpenes, steroids, tannins, flavonoids, and cardiac glycosides. The result of the antimicrobial screening showed that all the extracts had significant activity against most of the microorganisms tested. The minimum inhibitory concentration (MIC), minimum bactericidal concentration and minimum fungicidal concentration (MBC/MFC) of the extracts showed that almost all the organisms tested were responsive to the extracts at a given concentration. It also showed that, ethyl acetate and chloroform crude extracts exhibited the highest activity against the bacterial *Salmonellae typhi* and *Shigella dysenteriae* and *Candida krusei* and *Candida stellatoidea* for fungi. The controls used were Sparlfloxacin 2mg/ml for the bacteria and Fluconazole 5mg/ml for the fungi. Chromatographic separation of the ethyl acetate and petroleum ether extracts of the aerial plant parts of *Maesobotrya barteri* afforded two compounds labelled as 1 and 2. These compounds (1 and 2) were characterised by 1D and 2D NMR spectroscopy and identified as β-amyrin and an isomer of betulinic and oleanolic acid. They were obtained from the ethyl acetate and petroleum ether fractions, a white crystalline and yellowish crystalline compound respectively. They showed significant antimicrobial activities. Compound (1) (β-Amyrin) from the ethyl acetate fraction was found to be active against most of the tested microbes and gave a zone of inhibition range between 26 - 32 mm. The MIC value for the most sensitive organisms were recorded at a concentration of 6.25 µg/ml and the other microbes were sensitive at 12.5 µg/ml with a corresponding MBC/MFC recorded at 25 µg/ml for some of the test microbes and 50 µg/ml for the others. Antimicrobial

result for compound (2), betulinic acid and oleanolic acid (as isomer) from petroleum ether fraction showed a zone of inhibition range between 24 - 30 mm. The MIC values for the most sensitive organisms were recorded to at a concentration of 6.25 µg/ml and the other microbes were sensitive at 12.5 µg/ml. The activity recorded by the crude and pure isolates on the organisms are in line with the ethnomedicinal claims of the plant based on their zones of inhibition as compared with those of standard drugs.

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ppm	Parts per million
uv	Ultraviolet
TLC	Thin layer chromatography
MIC	Minimum inhibitory concentration
MBC/MFC	Minimum bactericidal /fungicidal concentration
MHz	Megahertz
δ	Chemical shift in ppm
R_f	Retardation factor
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Correlation
COSY	Correlation Spectroscopy
DEPT	Distortionless Enhancement by Polarization Transfer

CHAPTER ONE

1.0 INTRODUCTION

1.1 Medicinal Plants

A medicinal plant is any plant which in one or more of its organs contains substances that can be utilized for therapeutic purposes or which are precursors for the synthesis of useful drugs (Sofowora, 2008). Plants have been used in traditional medicines for many years, but no sufficient scientific data exist to confirm their efficacy (Sofowora, 2008). In Africa and most developing countries of the world, there is an interest in the study of medicinal plants and their curative properties and almost all the ingredients used in the formulation of medicinal remedies in Africa come from plant sources (Harborne *et al.*, 1993). There is increasing scientific interest in the extraction and isolation of secondary metabolites from plants, as well as of biosynthetic, biochemical, pharmacological, chemotaxonomical, ecological, physiochemical and plant tissue culture studies as a result of modern isolation and pharmacological testing of purified substances rather than in the form of older galenical preparation (Silvia *et al.*,1998).

1.2 Background of the Use of Natural Product as Therapeutic Agent

Natural products are generally either of pre-biotic origin or originate from microbes, plants or animal sources (Nakanishi, 1999). As chemicals, natural products include such classes of compounds as terpenoids, polyketides, amino acids, peptides, proteins, carbohydrates, lipids, nucleic acids-bases, ribonucleic acids, deoxyribonucleic acids etc. Natural products are not just accidents or products of convenience of nature; they are a natural expression of the increase in complexity of organisms (Jarvis, 2000). Increase in

natural sources to provide treatments for pain, palliatives, or curatives for a variety of maladies or recreational use reaches back to the earliest point of history.

Nature has provided many things for humankind over the years, including the tools for the first attempt at therapeutic intervention (Nakanishi, 1999). Neanderthal remains have been found to contain the remnants of medicinal herbs (Holt and Chandra, 2002). The Nei Ching is one of the earliest health science anthologist ever produced and dates back to the thirtieth century BC (Nakanishi, 1999). Some of the first records on the use of natural product in medicine were written in cuneiform in Mesopotamia on clay tablets and date to approximately 2600 BC (Cragg and Newman, 2001[a]). Indeed many of these agents continue to exist in one form or another to this day as treatment for inflammation, influenza, cough and parasitic infestation. Chinese herbs guide document the use of herbaceous plants as far back in time as 2000 BC (Holt and Chandra, 2002). For a variety of reasons, the interest in natural products continues to this very day. The first commercial pure natural product introduced for therapeutic use is generally considered to be the narcotic morphine, marketed by Merck in 1826 (Newman *et al.*, 2000).

In recent years, a significant revival of interest in natural products as a potential source for new medicines has been observed among academia as well as pharmaceutical companies. Several modern drugs (~40% of the modern drugs in use) have been developed from natural products. More precisely, according to Cragg *et al.*, 1997, 39% of the 520 new approved drugs between 1983 and 1994 were natural products or their derivatives, and 60–80% of antibacterial and anticancer drugs were from natural origins. In the year 2000, approximately 60% of all drugs in clinical trials for the multiplicity of

cancers had natural origins. However, in most cases the term natural products refers to secondary metabolites, small molecules (mol wt <2000 amu) produced by an organism that are not strictly necessary for the survival of the organism. The study of botany in the area of medicine today is recognized as the most viable method of identifying new medicinal plants or refocusing on those earlier reports of bioactive constituents (Adjanohoun *et al*., 1991; Farnsworth, 1990). Natural products certainly play a crucial role in meeting this demand through the continued investigation of the world's biodiversity, much of which remains unexplored (Cragg and Newman, 2001a).

Sometimes, however, a straightforward natural product isolation route, irrespective of bioactivity, is also applied, which results in the isolation of a number of natural compounds suitable for undergoing any bioactivity screening (Sarker and Lutfun, 2007). The most extensively used plant components can be classified into alkaloids, tannins, steroids, phenolic compounds, terpenoids and carbohydrates (Hill, 1952).

Environmental samples from soil and marine habitats will offer access to an untapped reservoir of genetic and metabolic diversity (Cragg and Newman, 2001b). China has emerged as a major producer of a number of quality medicinal plant products including coumarins, eucalyptus, peppermint, spearmint, sassafras and valerian in addition to its established listings (Trease and Evans, 2009). This is also true for nucleic acids isolated from symbiotic and endophytic microbes associated with terrestrial and marine macro organisms.

1.3 The Medicinal Uses of Plants

Diverse people and cultures throughout the world have employed herbal medicine to treat diseases throughout the history of mankind. This situation still persists in most developing countries where two-third of the people have no access to modern medicine.

In the industrialized countries also, there is increase in the use of herbal drugs; in fact 25% of all prescribed drugs are substances derived from higher plants (Fransworth *et al*., 1985). Traditional medicine is now recognized by the World Health Organization (WHO) as a building block for primary health care (Akerele, 1998).

The medicinal use of herbs represents an important aspect of the history of medicine and has contributed immensely to the development of modern therapeutics (Akerele, 1998). Many modern day drugs are derived from plants; examples are reserpine, quinine, digoxine, morphine, codeine and host of others (Cannell, 2000).

1.4 Justification of the Research

The screening of plant extracts has had an impressive history of identifying active agents. The choice of the aerial part of *Maesobotrya barteri* as the plant parts of interest in this work was based on its vast medicinal importance among traditional medicine practitioners in Orokam, Benue state of Nigeria, other parts of the tropics, including West Africa. *Maesobotrya barteri* is used in these places to treat various ailments such as in the treatment of bruised or erupted skin, malaria, catarrh, vomiting, diarrhea, typhoid and eye pain (Dalziel, 1956). Therefore, there is reason for a scientific study to ascertain the medicinal potentials of this plant, isolate its active ingredient(s) and characterize them using usual organic analytical techniques.

1.5 Aim of the Research

The aim of this work is to:

investigate the ethnomedicinal claims of the aerial parts of *Maesobotrya barteri*, isolate and characterize some compounds(s) that may be responsible for the claimed ethno medicinal application.

1.6 Objectives of the Research

The objective of this research are:

i. Phytochemical screening using the crude extracts of the solvents above.

ii. Antibacterial screening of the extracts.

iii. Antifungal screening of the extracts.

iv. Analytical separations involving several consecutive steps of chromatographic and purification techniques.

v. Verification of the purity of the isolated compound(s) and testing for antibacterial antifungal activities.

vi. Structural elucidation and characterization of the isolated compounds using available spectral techniques.

CHAPTER TWO

2.0 LITERATURE REVIEW

Maesobotrya is a group of flowering plant belonging to the family Phyllanthaceae or Euphorbiaceae.

Kingdom: Plantae

Order: Malpighiales

Family: Phyllanthaceae or Euphorbiaceae.

Genus: *Maesobotrya*

The Euphorbiaceae is the 4th largest family of the angiosperms comprising over 300 genera and about 7,500 species distributed widely in tropical Africa (Gill, 2002). The euphorbiaceae plants are shrubs, trees, herbs or rarely lianas (Pandey, 2006). Many of these families are xerophytes and cactoid and most often with milky latex. The family provides food (Etukudo, 2003; Pandey, 2006) and varied medicinal properties used in ethnobotany (Agbovie *et al*., 2002; Gill, 2002; Kubmarawa *et al.*, 2007). However, some are also found to be toxic. For instance, ricin found in *Ricinus communis* is a well-known poisonous compound that elicits violent purgative action in man (Trease and Evans, 2002), while the leaves of *Euphorbia kamerunica* were found to be toxic to rats (Ajibesin *et al.*, 2002). The leaves of *Euphorbia kamerunica* is also a known irritant having *in vitro* cytotoxic activities (Abo and Evans, 1981).

2.1 Botanical Characteristics of *Maesobotrya* Species

Different communities have different names for the plant *Maesobotrya barteri*.

English: Bush Cherry

IDOMA: Enam-piepie

EDO*:* Orúrú

IGBO*:* Miri ọgu,

YORUBA*:* Mdun or Obomodu.

Statistics have shown that the Plant List includes 30 scientific plant names of species rank for the genus *Maesobotrya*. Of these 18 are accepted species names and 12 are a further scientific plant names of infraspecific rank for the genus *Maesobotrya*.

2.1.1 Description

Maesobotrya barteri

These dioecious trees or shrubs with a simple indumentum. Leaves alternate, often long-petiolate, stipulate, simple, entire or toothed, penninerved; The petioles are bipulvinate; stipules minute and deciduous (or foliaceous and persistent). Inflorescences axillary (or cauliflorous), solitary (or fasciculate), racemose (or subspicate); bracts usually 1-flowered; flowers shortly pedicellate. The male flowers, calyx (4)5-lobed, imbricate; petals absent; disc glands (4)5, alternating with the stamens, fleshy, contiguous; stamens (4)5(6), opposite the sepals; filaments free; anthers erect, dorsifixed, introrse, thecae parallel, longitudinally dehiscent; pistillode cylindric, not lobed. Female flowers : calyx as in the male; petals absent; disc hypogynous, cupular, entire; ovary (1)2(4)-locular, with 2 ovules per locule; styles short; stigmas bifid, recurved. Fruit subglobose or ellipsoid, subdrupaceous, tardily loculicidally dehiscent; pericarp thin; endocarp 1-locular by suppression. Seeds solitary by abortion, ellipsoid, ecarunculate; testa thin; albumen copious; cotyledons broad, flat, green. Species +/- 20, all in west tropical Africa, except one (1) in Uganda; southern tropical Africa. But most probably further species in northern Angola, Angola, Zambia (Emily, 2009).

Figure 1: The aerial parts of the plant *Maesobotrya barteri*

2.1.2 *Maesobotrya pynaertii*

This specie is not very typical of the genus. It grows into a taller tree than the other species, and has smaller leaves with shorter petioles. The bark is soft with deep vertical fissures running up the trunk. The slash has a pale brown layer on the outside, and a dull orange colour in the centre which oxidizes to a darker and brighter shade. The leaves have tiny tufts of hairs along the margin which require a lens to be seen properly. The domatia are scattered along the lamina close to the midrib rather than in the axils of the secondary veins. The species is dioecious with male and female flowers on different individuals. The flowers are produced in 5–10 cm long inflorescences on branches below the leaves. Its ecology is restricted to the seasonally flooded swamps of the centre of the Congo River Basin (Kawanabe, 1999).

2.1.3 *Maesobotrya floribunda*

Maesobotrya floribunda is dioecious shrub or small tree up to (-12)m tall, with drooping branches, bark grayish brown, longitudinally grooved, twigs angular, yellowish short-hairy. Leaves occur mainly in forest, often in open, seasonally inundated localities, from sea-level up to 1000 m altitude. It mainly grows on sandy loam, enriched with clay or organic material. Origin and geographic distribution *Maesobotrya floribunda* occurs from Cameroon and the Central African Republic south to DR Congo and Zambia. Uses in Congo include rubbing the leaves on the skin to treat prickly heat (Burkill, 1996).

2.2 Medicinal and Nutritive Applications *Maesobotrya* Species

In Nigeria, the specie *M. barteri* is under-exploited although the tree is of both medicinal and of nutritional importance (Ogbuagu *et al*., 2008). *Maesobotrya* species are used medicinally in different regions in Africa. It bears succulent black-purple

fruits that are edible and stain the tongue. The seeds are often with a conspicuous carbuncle, with the endosperm present or absent. The nutritive values of the fruit and seeds of *Maesobotyra floribunda* have been studied. Pierre *et al*., (2006), reported the isolation and structural determination via NMR of a Novel Sesquiterpenoid from the seed of *Maesobotyra floribunda* which also had antimicrobial activity. Okwu *et al*., (2013) reported that chewing sticks from *Maesobotrya barteri* and other medicinal plants were analyzed for major, minor and trace elements and was found that these chewing sticks when used without tooth paste to be very efficient, effective and reliable in cleaning the teeth of many people in Southern Nigeria. The teeth of the users of those chewing sticks are usually strong, clean, fresh and devoid of dental plaques. These results indicate the basis for the prevention and protection of the teeth against caries and plaques by the samples used. According to Fidele *et al*., 2013, plants of the Euphorbiaceae family (Alchornea sp., Croton sp., Discoglypremna sp., Drypetes sp., Fontainea sp., Macaranga sp., Maesobotrya sp., Neoboutonia sp., and Uapaca sp.) are shown to be rich in terpenoids (69.5%). According to the records of Fauna and Flora International of Liberia, *M. barteri* has bright red fruits ("bush cherry") that are delicious and are of welcome refreshment for thirsty travelers (Emily, 2009). In Congo a paste of pounded fruits of the Central Africa *Maesobotrya cordulata* is applied to treat psoriasis (Kawanabe, 1999). Pulverized leaves are applied to wounds to heal them and are applied to scarifications to treat oedema (Ramirez, 1988). In Congo a bark decoction of *Maesobotrya vermeulenii* is drunk and taken in bath to treat leprosy (Tane *et al.*, 1996). *Maesobotrya floribunda* leaves are ground and mixed with palm oil for external use (Burkill, 1996).

2.3 The Main Groups of Active Ingredients of Medicinal Plants

The medicinal action of some species of plant is determined by the constituents. Results have shown that plants contain some organic compounds, which have different functions to the plants and the animal kingdom. These constituents affect the conditions and functions of various organs in the human body, clear up residual symptoms or destroy the causes of diseases, in most cases, infections by microorganisms. The important active ingredients are alkaloids, saponins, tannins, glycosides, flavonoids, essential oils, fatty acids and essential minerals.

2.3.1 Flavonoids

They are polyphenolic substances present in plants and found mostly in seeds, fruits skin, bark or flowers. Flavonoids are divided into two main classes depending on the oxidative level of the central chiral ring which are flavonols and anthocyanidin; others include flavones, flavan-3-ol, cathechins and isoflavones (Finar, 1975).

The widespread distribution of flavonoids, their variety and relatively low toxicity compared to other active plant compounds (for instance alkaloids) mean that many animals, including humans, ingest significant quantities in their diet without adverse effect. Preliminary research indicates that flavonoids may modify allergens, viruses, and carcinogens and may be biological "response modifiers." *In vitro* studies show that flavonoids also have anti-allergic, anti-inflammatory, antimicrobial (Robert and Marlene, 2005), anti-cancer and anti-diarrhea activities (Leland, 2006).

Flavones are the most numerous class of naturally occurring oxygen heterocyclic compound and constitute an important group of yellow natural pigments. The whole group may be called flavonoids. Flavonoids are known to be antimicrobial metabolites (Ververdis *et al.*, 2007). Flavonoids are widely distributed in plants fulfilling many

functions including producing yellow or red/blue pigmentation in flowers and protection from attack by microbes and insects. They are most commonly known for their antioxidant activity. Examples of some flavonoids such as dihydrochalcone, flavan-3,4-diol, and epicatechin are represented as structures 1, 2 and 3 respectively (Jeffrey and Herbert, 1993).

2.3.2 Tannins

Tannins are colourless, non-crystalline polyphenolic substances rich in phenolic groups, capable of binding or precipitating water-soluble proteins (Hagerman and Butler, 1989). They are very complex aromatic compounds that are widely distributed in plants and known to be glycosides containing glucose combined with phenol or hydroxyl acid (Tedder *et al*., 1972). Tannins are extracted from various plant sources such as the fruits, leaves of brushes, the bark and heartwood of trees. Therapeutically, they have a stringent quality and can be used to stop bleeding externally and to dry mucus secretions in the treatment of digestive tract inflammation (Finar 1975).

The new technologies used to analyze molecules and structures have shown that a division into condensed and hydrolysable tannins is too simplistic (Muller and McAllan, 1992). They precipitate proteins from solution and they form a bluish-black colour with ferric salt, a property that is used in the manufacture of ink and in their identification in phytochemical analysis. Recent studies have demonstrated that products containing "Chestnut" tannins included at low dosage (0.5-0.2%) in the diet can be beneficial (Schisvone, *et al.*, 2008). It is believed that tannin isolated from the stem of *Myracrodruon urundenva* may have neuroprotective functions capable of reversing 6-hydroxydopamine induced toxicity. The plant has shown promise as a potential

therapeutic agent, which may be beneficial in patients with neurological diseases (Nobre and Helio, 2007). It has also been confirmed that chocolate liquor contains about 6% tannin (Robert and Marlene, 2005). An example of hydrolysable tannins which show anti-human-immunodeficiency-virus (HIV) activity by inhibiting reverse transcriptase is corilagin (Notka *et al.*, 2004). Tannins are represented as structures 4, 5 and 6 respectively (Jeffrey and Herbert, 1993).

2.3.3 Alkaloids

Alkaloids are basic substances, which contain one or more nitrogen atoms usually as an integral part of a cyclic system. They are toxic, crystalline, colourless and often optically active substances. Most alkaloids have bitter taste (Finar, 1975). Some individual alkaloids have been reported to be useful as growth regulators or as insect repellents (Harbone, 1973). Alkaloids are produced by a large variety of organisms, including bacteria, fungi, plants, animals and are part of natural products (also called secondary metabolites). The chemistry of alkaloids encompasses thousands of compounds of many structural types. It is believed that the first alkaloid that was isolated and studied is morphine which was discovered in 1805 (Leland, 2006). The word "alkaloid" originated from the Arabic, *al-qali* (an early form of soda ash) (Cordell, 1981). Many alkaloids have a bitter taste, and a large number of them exhibit potent physiological effects on mammals. For example, morphine shows narcotic effects; reserpine is an antihypertensive agent; atropine is a smooth muscle relaxant; cocaine is a local anaesthetic and a potent central nervous system stimulant; and strychnine is a nerve stimulant. Alkaloids act on a diversity of metabolic system in humans and other animals (Rhoades, 1979). Alkaloids are represented as structures 7, 8 and 9 respectively (Jeffrey and Herbert, 1993).

2.3.4 Phenols

They are members of a group of aromatic chemical compounds with weakly acidic properties and are characterized by hydroxyl group attached to the aromatic ring. The simplest of phenols derived from benzene is known as phenol. The structural classes of phenolic compounds include the polyphenolic (hydrolysable and condensed tannins) and monomers such as ferulic and catechol. Polyphenols have been reported to play a role in inactivating carcinogenic agent and inhibiting the expression of mutagens. (Harbone, 1993). Notable sources of natural phenols in human nutrition include berries, tea, beer, olive oil, chocolate or cocoa, coffee, etc; fruit based drinks (including cider, wine and vinegar) and vegetables. Herbs and spices, nuts (walnuts, peanut) and algae are also potentially significant for supplying certain natural phenols. Natural phenols can also be found in fatty matrices like olive oil (Gutfinger, 1981).

Phenolic compounds, when used in beverages, such as prune juice, have been shown to be helpful in the colour and sensory components, such as alleviating bitterness (Donovan *et al.,* 1998). Such foods containing natural phenols are generally considered as health food. The presence of natural phenols in food is one of the motivations for organic agriculture. Organic advocates claim that organically grown potatoes, oranges, and leafy vegetables have more phenolic compounds and these may provide antioxidant protection against heart disease and cancer (Asami, 2003). Some say that diets rich in whole and unrefined foods, like whole grains, dark green and yellow/orange-fleshed vegetables and fruits, legumes, nuts and seeds, contain high concentrations of antioxidant phenols, fibers and numerous other phytochemicals that may be protective against chronic diseases (Bruce *et al.,* 2000). An example of a Phenolic compound is represented as structures 10 and 11 respectively (Jeffrey and Herbert, 1993).

2.3.5 Saponins

Saponins have molecular structure similar to steroids. They are soap-like and hence have emulsifying properties. The formation of persistent foam during plant extraction or during the concentration of plant extracts is reliable evidence that saponins are present. Plants rich in saponins generally have anti-inflammatory properties (Finar, 1975).

In plants, most saponins which readily dissolve in water, are poisonous to fish (Robert, 2003). Many of the plants that contain saponins were historically used as soaps. These include soaproot (*Chlorogalum pomeridianum*), soapbark (*Quillaja saponaria*), soapberry (*Sapindus saponaria*), and soapnut (*Sapindus mukurossi*) (Hostettman and Marston, 1995). Saponins may be mono- or polydesmodic, depending on the number of attached sugar moieties. Saponins are a class of chemical compounds, one of the very many serves as anti-feedants and protects the plant against microbes and fungi (MetaCyc, 2008).

Another importance of the saponin class involves their complexation with cholesterol to form pores in cell membrane bilayers, example is in red cell (erythrocyte) membranes where complexation leads to red hemolysis on intravenous injection (Leland, 2006). In addition, the amphipathic nature of the class gives them activity as surfactants that can be used to enhance penetration of macromolecules such as proteins, they have also been used as adjuvant in vaccines (Rhoades, 1999). Examples of saponins are ∝-Amyrin and Solanine, as structures 12 and 13.

2.3.6 Steroids

Steroids form a group of structurally related tetracyclic triterpenoids whose structures are based on 1,2-cyclopentenophenanthrene skeleton. Many plant steroids occur as

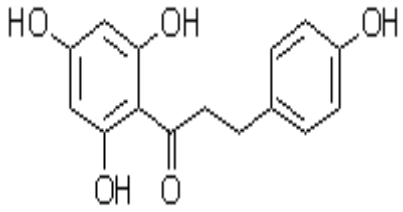
cardio-active glycosides and have the properties of stimulating heart muscle. The hydrolysis of such steroids yields one or more sugars and an aglycone.

Gonane is the simplest possible steroid and is composed of seventeen carbon atoms; bonded together to form four fused rings. The three cyclohexane ring (designated as ring A,B,C as shown in the structure of cholestane) form the skeleton of phenanthrene; ring D has a cyclopentane structure. Hundreds of distinct steroids are found in plants, animals and fungi or from cycloartenol. Phytosterols found in plants have many applications ranging from food additives, medicine to cosmetics (Sarker *et al*., 2007), represented as structrue 14 .

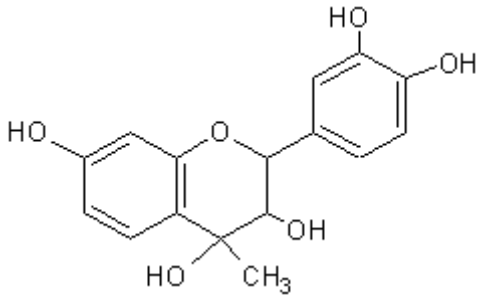
2.3.7 Glycosides

They are acetal or ketals of a sugar in their ring form. They consist of a sugar molecule joined to a non-sugar moiety, aglycone, via a glycosidic bond. Anthocyanins are glycosides that are naturally plant pigments and their non-sugar part are known as anthocyanidins. The water-soluble anthocyanins are responsible for the property of stimulating heart muscle and have been used as stimulants to increase heart muscle contraction in case of heart failure (Finar, 1975).

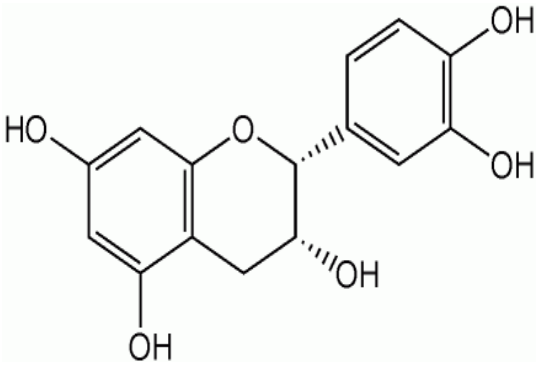
Many plants store important chemicals, as glycosides; if these chemicals are brought in contact with water and an enzyme and the sugar part is broken off, making the chemical available for use. Many of such plant glycosides are used as medications. In animals (including humans), poisons are often bound to sugar molecules in order to remove them from the body (Seawright, 1995). Glycosides play numerous important roles in living organisms.



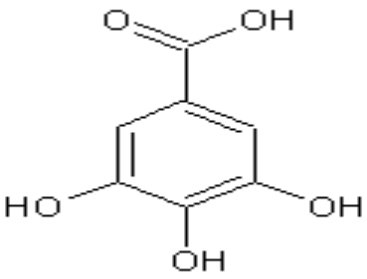
Dihydrochalcone (1)



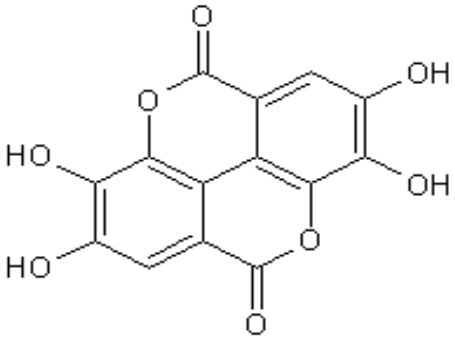
Flavan-3,4-diol (2)



Epicatechin (3)



Gallic acid (4)



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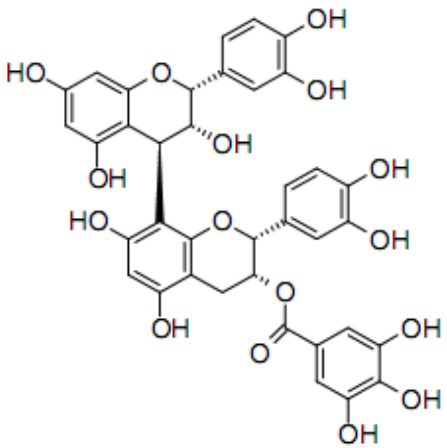
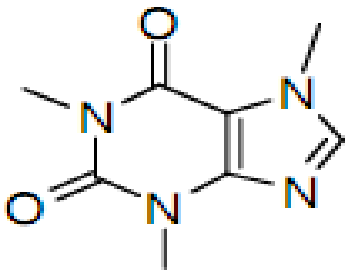
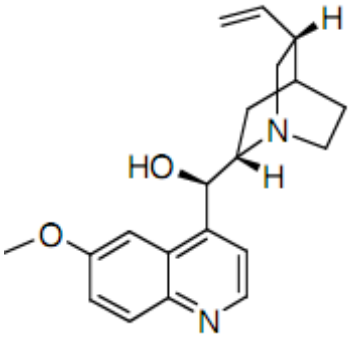


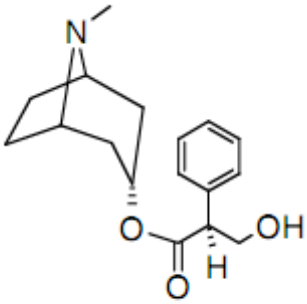
Hydrolysable tannin (6)



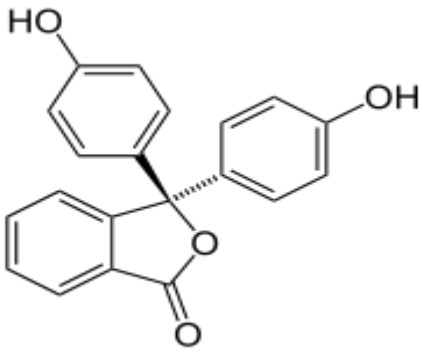
Caffeine (7)



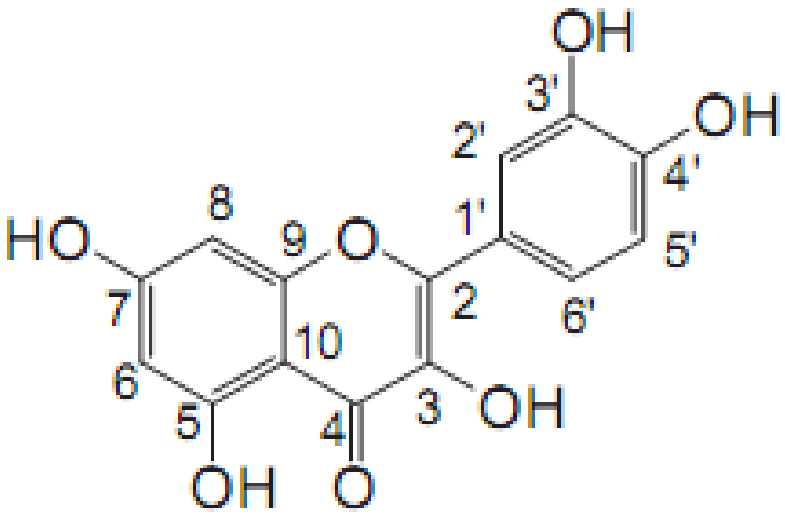
Quinine(8)



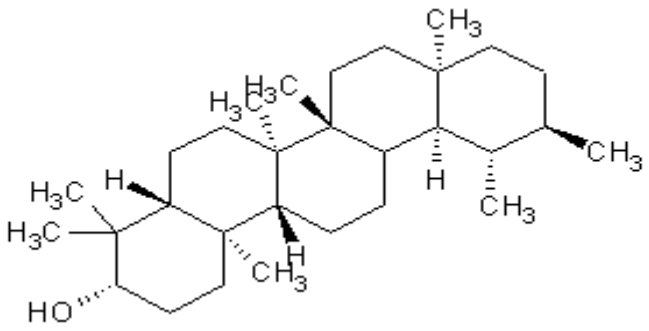
Atropine (9)



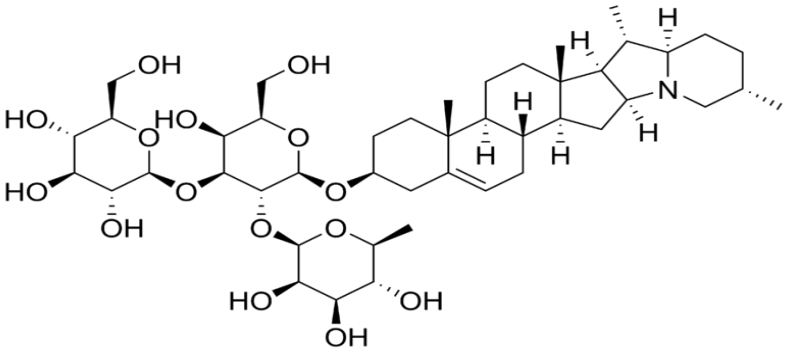
Phenolphthalein (10)



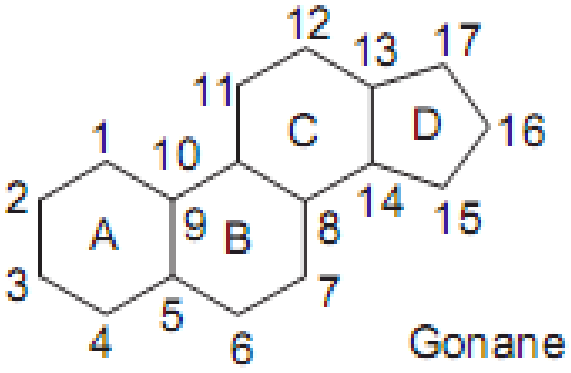
Quercetin (11)



∝-Amyrin (12)



Solanine (13)



(14)

2.4 Some Microorganisms and their Effects on the Human body

Most of the pathogenic antimicro-organisms used for the microbial test of the plant extracts include the following; *Staphylococcus aureus, Streptococcus pyogenes, Klebsiella pneumonia, Salmonella typhi, Escherichia coli, Candida albicans, Candida tropicalis, Candida krusei, Candida stellatoidea.*

2.4.1 Enterobacteriaceae

The enterobacteriaceae are a large, heterogeneous group of Gram-negative rods whose natural habitat is the intestinal tract of humans and animals. The family include many genera such as; Escherichia, Shigella, Salmonella, Enterobacter, Klebsiella, Serratia, Proteus, and others. Some enteric organisms e.g. *Escherichia coli*, are part of the normal flora and incidentally cause disease, while others, the *Salmonellae* and *Shigellae* are regularly pathogenic for humans. The enterobacteriaceae are facultative anaerobes or aerobes, ferment a wide range of carbohydrates, possess a complex antigen structure and produce a variety of toxins and other virulence factors.

(i). Klebsiella

Klebsiella pneumoniae is present in the respiratory tract and faeces of about 5% of normal individuals. It causes a small proportion (about 1%) of bacterial pneumonias. *K. pneumoniae* can produce extensive haemorrhagic necrotizing consolidation of the lungs. *Klebsiella* sp. rank among the top ten bacterial pathogens responsible for hospital-acquired infections (Podschun and Ullman, 1998). .

(ii). Escherichia coli

Escherichia coli is the most common cause of urinary tract infection and accounts for approximately 90% of first urinary tract infections in young women. It causes diarrhoea

and is common worldwide. These *E coli* are classified by the characteristics of their virulence properties and each group causes disease by a different mechanism (Atlas, 2004).

(iii). Salmonellae

Salmonellae are often pathogens for humans or animals when acquired by the oral route. They are transmitted from animals and animal products to humans, where they cause enteritis, systemic infections and enteric fever. Examples forms of salmonellae are as follows; *S. typhi, S. cholerasuis, S paratyphi A, S. paratyphi B. Salmonellae* produce diseases such as typhoid fever, headache, vomiting, nausea etc. *Salmonellae* vary in length and grow on simple media. The most important is *Salmonella typhi* which reaches the small intestine after ingestion of *Salmonellae*, from which they enter the lymphatic and then the blood stream. It can be treated with antibiotics (Podschun and Ullman, 1998). .

(iv). Staphylococci

Staphylococci are Gram negative spherical cells, usually arranged in grape like irregular clusters. They grow readily on many types of media and are active metabolically, fermenting carbohydrates and producing pigments that vary from white to deep yellow. The genus staphylococcus has at least 40 species. The three frequently encountered species of clinical importance are S*taphylococcus aureus*, *Staphylococcus epidermidis* and *Staphylococcus saprophyticus. S. aureus* is a major pathogen for humans. Almost every person will have some type of *S. aureus* infection during their life time, ranging from food poisoning or minor skin infections to severe life-threatening infections. *Staphylococci* are non-motile and do not form spores. Under the influence of drugs like penicillin, *Staphylococci* are lysed. *Staphylococci* are easily cultured on most

bacteriological media under aerobic or microaerophilic conditions. *Staphylococci* can produce disease both through their ability to multiply and spread widely in tissues and through their production of many extracellular substances. Some of these substances are enzymes. However, it is difficult to eradicate pathogen staphylococci from infected persons, because the organisms rapidly develop resistance to many antimicrobial drugs and the drugs cannot act in the central necrotic part of a suppurative lesion. It is difficult to eradicate the *S. aureus* carrier state (Bronner, 2004).

(v). Streptococci

The *Streptococci* are gram-positive spherical bacteria that characteristically form pairs or chains during growth. They are widely distributed in nature. Some are members of the normal human flora; others are associated with important human diseases attributable in part to infection by *Streptococci*, in part to sensitization to them. *Streptococci* elaborate a variety of extracellular substances and enzymes. The *Streptococci* are a large and heterogeneous group of bacteria and no one system suffices to classify them. Yet, understanding the classification is key to understanding their medical importance. Examples of *Streptococci* are *S. pyogenes, S. agalactiae, S. dysgalactiae, S. anginosus, S. pneumonia. S. pyogenes, S pneumococci* are susceptible to penicillin G and most are susceptible to erythromycin. Some are resistant to tetracycline. *S pneumococci* are sensitive to many antimicrobial drugs, early treatment usually result in rapid recovery and antibody response seems to play a much diminished role (Musher, 2010).

(vi). Pseudomonads

Pseudomonas aeruginosa belongs to the *Pseudomonads* group. *Pseudomonas aeruginosa* are Gram-negative, motile with aerobic rod and produce water-soluble

pigments. *Pseudomonas aeruginosa* is frequently present in small numbers in the normal intestinal flora and on skin of humans and it is a major pathogen of the group. *Pseudomonas aeruginosa* is notorious as an opportunist pathogen because of its innate resistance to many anti-microbial agent and disinfectants and therefore become dominant and important when more susceptible bacteria of the normal flora are suppressed (Prescott *et al.*, 1990).

Pseudomonas is now increasingly emerging as an opportunistic pathogen of clinical relevance. Several different epidemiological studies indicate antibiotic resistance in clinical isolates. Diseases caused by *Pseudomonas aeruginosa* include endocarditics, respiratory system infections, central nervous system infection, urinary tracts infection etc. The combination of gentamycin and carbencillins is frequently used to treat severe *Pseudomonas* infections.

(vii). *Bacillaceae*

The genus *Bacillus* is of the family bacillaceae and it is the largest in the order. The genus contains Gram-positive, endospore-forming and chemo-heterotrophic rod that are usually motile with peritrichous flagella. *Bacillus subtilis* is not considered as a human pathogen; it may contaminate food but rarely cause food poisoning (Ryan and Ray, 2004).

Bacillus subtilis can divide asymmetrically, producing an endospore that is resistant to environmental factors. *Bacillus subtilis* produces the proteolytic enzyme subtilisin and its spores can survive the extreme heating that is often used to cook food.

(viii). *Fungi*

Fungi constitute a large diverse group of heterotrophic organisms which exist as saprophytes, parasites or commensals. Most are found as saprophytes in the soil and on decaying plant material. About 180 of the 250,000 known fungi species are recognized as capable of causing diseases (mycosis) in man and animals (Rippo, 1988). Most of these are mould fungi but there are number of pathogenic yeast fungi and many are dimorphic. Dimorphic fungi usually assume the mould form when growing as saprophytes in nature and the yeast form when causing infection. In the laboratory, the tissue form can be induced by culturing at 37 ^{o}C on rich media such as blood agar, whereas the mould form develops when incubated at a lower temperature (22-27 ^{o}C) on a less rich medium such as Sabouraud's agar (Rippo, 1988).

Yeast infections affecting skin and nails and mucous membranes of the mouth and vagina, are usually caused by *Candida albicans* which are found as commensal of man. Yeast infections are generally endogenous in origin but can be transmitted sexually. There are relatively few therapeutically useful antifungal agents that are available. This is due mainly to the fact that fungi and man are both eukaryotes and most substances that kill or inhibit fungal pathogens are also toxic to the host. Most superficial Candida infections respond well to topical therapy with nystatin, amphotencin B or azoles.

Superficial Candida infections occasionally occur on the penis after intercourse with females with vaginal thrush. The yeast may also infect the outer ear (Roberts and Mackenzie, 1984). In oral candidosis, nystatin, ampholericin B and miconazole may be effective in lozenge or gel form. Most cases of vaginal candidosis can be treated successfully with a single application of an azole derivative with oral therapy with fluconazole or itraconazole (Zakirai, 2009).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents

i. HCl acid (concentrated).

ii. HNO_3 acid (concentrated).

iii. 2 M NaOH solution.

iv. Iron (III) chloride solution.

v. Mayer's reagent; potassium iodide (KI) solution.

vi. Dragendorff's reagent; Bismuth nitrate ($Bi(NO_2)_3$) and potassium iodide solution (KI)

vii. $CuSO_4$ solution

viii. Iodine: potassium (1:2) in distilled water

ix. KOH solution 2 M.

x. 2 % sodium nitroprusside.

xi. Magnesium powder (BDH)

xii. Acetic acid (glacial) (AR-BDH)

xiii. $NaHCO_3$ solution (saturated).

xiv Magnesium turning

xv. 0.5 M potassium iodide (KI) solution.

xvi. H_2SO_4 acid (concentrated).

xvii. Molisch reagent; 1 % α-naphtol in 8 % ethanol

xviii. Fehling's solution A (Copper (II) tetraoxosulphate (VI) solution) and B; (sodium potassium 2,3-dihydroxybutanedioate solution)

3.1.2 Solvents for extraction

The solvents used were: Petroleum ether 60-80 (Aldrich), Chloroform (Aldrich), Ethyl Acetate (JHD) and Methanol (JHD). All solvents used were redistilled by simple distillation.

3.1.3 Materials for chromatographic separations

Materials used for chromatographic separations were: Chromatographic Tank, Glass column, (20 x 20 cm) glass plates, Ultraviolet lamp (254 and 366 nm), Silica gel (Qualikens 60-120 mesh), 10 % H_2SO_4.

3.1.4 Materials for antimicrobial test

Microbiological media (nutrient broth): Muller Hinton agar.

Test Organisms: *Staphylococcus aureus, Streptococcus pneumonia, Salmonella typhi, Klebsiella pneumonia, Shigella dysenteriae, Escherichia coli, Candida stellatoidea, Candida krusei,* and *Candida tropicalis*

Petri Dishes, Sterile Pipette, 6 mm cork borer, Incubator, Autoclave, Dimethylsulphoxide (DMSO) 10%

3.2 Methodology

3.2.1 Collection of plant materials

The aerial parts of *Maesobotrya barteri* were collected from Orokam in Ogbadibo Local Government Area of Benue State in the month of July, 2013. They were properly identified at the Herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria-Nigeria; where a sample of it was deposited and assigned the voucher

specimen number 322. Thereafter, they were dried for three. The dried aerial parts were pounded using a wooden mortar and pestle into coarse powder.

3.2.2 Extraction of plant material

The dried powder plant material (980 g) was extracted using Soxhlet apparatus and a range of solvents starting with petroleum ether, chloroform, ethyl acetate and methanol. The extraction procedure were based on Standard techniques for obtaining organic constituents from dried plant tissue according to Harbone, (1984). Each extraction process was performed exhaustively, finally concentrated under reduced pressure, respectively weighed and kept in a desiccator.

3.3 Phytochemical Screening

The crude petroleum ether, chloroform, ethyl acetate and methanol extracts were subjected to phytochemical screening using standard techniques of plant secondary metabolites by Harborne (1984), Sofowora (1993) and Trease and Evans (2009), using the following procedures:

3.3.1 Test for carbohydrate

Molisch's Test

The sample (2 mL of each extract) was put into a test tube and a few drop of Molisch's reagent (10 % α-naphtol solution) added. Concentrated H_2SO_4 (1 mL) was allowed to flow down the side of the inclined tube so that the acid form a layer beneath the aqueous solution without mixing with it. the presence of a red ring observed at the inter-phase with a dull-violet precipitate indicates the presence of carbohydrate (Silva *et al*., 1998).

3.3.2 Test for flavonoids

Shinoda Test

A small quantity of magnesium powder and a few drop of conc. HCl were added to an alcoholic solution of each plant extract (2 mL). The appearance of an orange, or pink or red to purple colour indicates the presence of flavonoids (Sofowora, 1993).

Sodium Hydroxide Test: The extract (2 mL) was dissolve in 10 % aqueous sodium hydroxide solution and filtered to give yellow color, a change in color from yellow to colorless on addition of dilute HCl indicates the presence of flavonoids (Cannel, 2000).

3.3.3 Test for alkaloids

Mayer's reagent

Freshly prepared Mayer's' reagent (Potassium mercuric iodide solution) was added drop wise to each acidified solution of the extracts (1 mL). A white or creamy precipitate was observed indicating the presence of alkaloid (Silva *et al*., 1998).

Dragendorff's reagent test

An acidified solution of the extract was treated with Dragendorff reagent

(Bismuth nitrate and potassium iodide solution). An orange-brown precipitate was formed indicating the presence of alkaloid (Silva *et al*., 1998).

3.3.4 Test for tannins

A small quantity of each extract (0.5 g) was dissolved in distilled water (10 mL), boiled and filtered. To the filtrate was added few drops of 1% ferric chloride solution, a

formation of blue black or green precipitate was taken as evidence for the presence of tannins (Trease and Evans, 2009).

Lead Sub-acetate Test

To a small quantity of the extract (1 mL), three drops of lead sub-acetate was added. The solution was observed for the presence of green precipitate as evidence for the presence of tannins (Trease and Evans, 2009).

3.3.5 Test for saponins

Distilled water (5 mL) was added to 0.3g of each extract in a test tube and shaken vigorously for 30 seconds then allowed standing for 45 minutes. A honey-comb froth that persisted for 30 minutes was observed indicative of the presence of saponins (Silva *et al*., 1998)**.**

3.3.6 Test for glycoside ($FeCl_3$)

To about 0.5 g of the extract/fraction, 5 mL of conc. H_2SO_4 was added and boiled for 15 min. This was then cooled and neutralized with 20 % KOH. The solution was divided into two portions. three drops of ferric chloride solution was added to one of the portions and a green to black precipitate indicated phenolic aglycone as a result of hydrolysis of glycoside (Silva *et al*., 1998)**.**

3.3.7 Test for steroids

Salkowski test

The plant extracts were dissolved in 1ml chloroform respectively and 1mL conc. H_2SO_4 was added. The formation of a reddish-brown interphase was taken as an indication for the presence of was steroids (Silva *et al*., 1998)**.**

3.3.8 Lieberman-Buchard test

The extract was dissolved in chloroform and a few drops of acetic anhydride were added followed by concentrated sulfuric acid. The mixture was carefully mixed and the resulting solution was observed for blue, green, red or orange color that changes with time (Silva *et al*., 1998)**.**

3.4 Antimicrobial Screening

3.4.1 The test organisms

The microorganisms used for the test include *Streptococcus pyogenes, Staphylococus aureus, Salmonella typhi, Klebsiella pneumonia, Escherichia coli, Shigella dysenteriae, Candida stellatidea, Candida krusei,* and *Candida tropicalis*. The clinical strains were obtained from the Department of Medical Microbiology Ahmadu Bello University Teaching Hospital, Shika, Zaria. All the isolates were checked for purity and maintained in a slant of nutrient agar.

3.4.2 Culture media

The culture media used were Mueller Hinton agar (MHA) and Mueller Hinton broth (MHB). All the media were prepared according to manufacturer's specifications.

3.4.3 Preparation of inoculums of test organisms

The McFarland turbidity standard scale 1 was used to standardise the organisms. The scale was prepare y adding 9.9 mL of 1 % barium chloride ($BaCl_2$). Suspensions of the organisms

were made in sterile distilled water and compared with the McFarland turbidity standard, until the opacity match with the scale number 1, which correspobds to 1.5 x 10^6 CFU/mL.

3.4.4 Sensitivity test of the crude extracts

The agar well diffusion mehod was used as reported by Nostro, *et al*., (2000). The antimicrobial activity of the petroleum ether, chloroform, ethyl acetate and methanol extract of the aerial plant parts of *Maesobotrya barteri* were determined using stock concentration of 100 mg/mL. The standardised inocula of the isolates were uniformly streaked unto freshly prepared Mueller Hinton agar plates with the aid of a sterile swab stick. Using a sterile cork borer (8 mm in diameter), five appropriately labelled wells were bored into each agar plate. A 0.2 mL of the appropriate extract concentrate was placed in each well and then allowed to diffuse into the agar. An extra plate was streaked with the inocula isolate and ciprofloxacin standard (10 µg/disc) was place on it. The plate were incubation at 37^{o}C for 24 hours. While for the fungi, Sabouraud Dextrose Broth was used and the incubation period was 48 hours at 25 ^{o}C. The antimicrobial activities were expressed as diameter zone of inhibition produced by the plant extract.

3.4.5 Minimum inhibitory concentration

Minimum inhibitory concentration of the extract was carried out on the microorganisms that were sensitive to the extract and was done using broth dilution method as reported by Vollekova *et al*., (2001). Different concentration of the extract that exhibited antimicrobial activity against the test organisms were prepared in the test tube containing Mueller Hinton Broth (MHB). The organisms were inoculated into each tube containing the diluted extracts. The plate were incubation at 37 ^{o}C for 24 hours for

bacteria and 25 ^{o}C, for 48 hours for the fungi, The lowest concentrations of the extract which shows no turbidity was recorded as the minimum inhibitory concentrations.

3.4.6 Minimum bactericidal and fungicidal concentration

Minimum bactericidal and fungicidal concentrations of the extracts were carried out to check whether the test microbes were killed or only their growth was inhibited. Mueller Hunton agars were prepared according to the manufacturer's instruction, boiled to dissolve and were sterilized at 121 ^{o}C for 15 minutes, the media were cooled to 45 ^{o}C and the medium (20 ml) was poured in to sterile Petri dishes, the plates were covered and allowed to cool and solidify. The contents of the MIC in the serial dilution was inoculated on to the media, the media were incubated at 37 ^{o}C for 24 hrs for the bacteria and at 25 ^{o}C for 48 hrs for fungi, after which the plate were observed for colonies growth. The MBC/MFC was the plate with lowest concentrations of the extract without colony growth.

3.5 Methods of Isolation/Purification

Thin-Layer (TLC), column (CC) and Preparative Thin-Layer chromatographic techniques were adopted for the isolation and purification processes.

3.5.1 Thin Layer Chromatography (TLC)

Thin layer chromatography was carried out on aluminum TLC sheets precoated with silica gel 60 $PF_{254,}$ layer thickness of 0.2 mm.

Technique: One way ascending Spotting and development: Spots were applied manually using capillary tube; plates were dried using air blower and developed at room temperature using a Shandon chromatographic tank.

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

3.5.2 Column Chromatography (CC)

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

(a) Technique- Gradient elution.

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

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

(d) Column packing - Wet slurry method

(e) Sample loading- Ethyl acetate extract (15 g) was loaded by the dry loading method (Cannell, 2000); the sample was dissolved in minimum amount of suitable organic solvent, mixed with a small quantity of silica gel, dried, triturated and then loaded on top of the previously packed column.

Solvent System/Elution: Various solvent systems comprising 100 % Hexane, hexane/ethyl acetate mixtures, ethyl acetate 100 % and methanol % were used in eluting the column by gradient elution.

3.6 Column Chromatography of Ethyl acetate Extract

The ethyl acetate extract which was the most sensitive extract from the antimicrobial screening was subjected to column chromatography (CC) for fractionation. Fifteen grams (15 g) of the extract was chromatographed on silica gel column eluting with hexane 100 %, hexane/ethyl acetate mixtures (80:20, 70:30, 60:40, 30:70, 10:90), ethyl

acetate 100 % and methanol 100 % as solvent systems to give 102 fractions. the 102 fractions were pooled together based on similarity in their TLC profile to give 9 sub-fractions. After repeated column chromatography, further purification was achieved with preparative thin layer chromatography at the same solvent combination after which fraction EN_1 produced white crystalline solid (11.8 mg). Several trials using different solvent combinations showed a single spot which was invisible under the UV lamp but after spraying the TLC plate with 10 % H_2SO_4 and oven dried for 5 minutes at 60 ^oC still showed a single spot. The white crystalline substance was labeled compound 1which was insoluble in ethyl acetate but soluble in chloroform.

3.7 Column Chromatography of Petroleum Ether Extract

The petroleum ether extract (7.0 g) of the extract was chromatographed on silica gel column eluting with hexane 100 %, hexane/ethyl acetate mixtures (80:20, 60:40, 30:70), ethyl acetate 100 % and methanol 100 % as solvent systems to give 85 fractions. The 85 fractions were pooled together based on similarity in their TLC profile to give 6 sub-fractions. After repeated column chromatography, further purification was achieved with preparative thin layer chromatography at the same solvent combination after which fraction P_1 produced yellow crystalline solid (5.7 mg). Several trials using different solvent combinations showed a single spot which was invisible under the UV lamp but after spraying the TLC plate with 10 % H_2SO_4 and oven dried for 5 minutes at 60 ^oC still showed a single spot. The yellow crystalline substance was labeled compound 2 which was insoluble in ethyl acetate but soluble in chloroform.

3.8 Spectral Analysis

(a) 1D-NMR and 2D- NMR spectra were obtained at the school of chemistry and physic, University of Kwazulu-Natal Durban south Africa on a Bruker AVANCE spectrometer (600 MHz for ^{1}H and 400 MHz for ^{13}C), using the TMS peaks as standard. Chemical shift values were reported in parts per million (ppm) relative to TMS standard and coupling constants are give in Hz. The NMR solvent used for these measurement is deuterated chloroform.

CHAPTER FOUR

4.0 RESULTS

4.1 Result of Extraction of Plant Material

The extracts from the aerial parts of *Maesobotrya barteri* (980g) were weighed and the yield calculated. From the results shown in Table 4.1, the petroleum spirit extract had the highest yield followed by methanol extract, ethyl acetate extract, and lastly the chloroform extract.

TABLE 4.1: Percentage recovery of the various solvents extracts

Solvent	Yield of Extract (g)	Percentage Yield (%)
Petroleum spirit	12.60	1.29
Chloroform	8.43	0.86
Ethyl acetate	16.14	1.65
Methanol	13.44	1.37

4.2 Result of Phytochemical Screening of the Crude Extracts

The results of the phytochemical screening of the extracts of *Maesobotrya barteri* are shown in Table 4.2. The plant *M. barteri* contains carbohydrates, reducing sugars, cardiac glycosides, saponins, steroids, triterpenes, flavonoids, and tannins.

Table 4.2: Phytochemical constituents of the aerial parts of *Maesobotrya barteri*.

Constituents	Petroleum spirit extract	Chloroform extract	Ethylacetate extract	Methanol extract
Carbohydrate	-	+	+	+
Saponnins	-	-	-	+
Flavonoids	-	-	+	+
Tannins	-	-	+	+
Cardiac glycosides	-	-	+	+
Steroids	+	-	+	+
Triterpenes	+	-	+	+
Anthraquinone	-	-	-	-
Alkaloids	-	-	-	-

KEY: + = Present, - = Absent

4.3 Results of the Zones of Inhibition of the Crude Extracts

The antimicrobial test results (Table 4.3**)**, shows the zones of inhibition (mm) of the crude extracts of the aerial plant parts of *Maesobotrya barteri*. All the extracts had significant activity against the test microorganisms. The active controls used were ciprofloxacin 2 mg/ml for the bacteria and fluconazole 5 mg/ml for the fungi.

Table 4.3: Results of the zone of inhibition (mm) determination.

Organisms	P.E (mm)	E.E (mm)	C.E (mm)	M.E (mm)	CP (10µg/disc)	FZ (10µg/disc)
S. aureus	17	25	21	20	37	-
S. pyogenes	17	29	24	22	39	-
E. Coli	16	30	25	24	35	-
S. typhi	18	32	29	24	37	-
S. dysenteriae	17	30	27	23	42	-
K. pneumoniae	15	22	24	22	38	-
C. tropicalis	16	24	20	20	-	37
C. Krusei	15	25	22	21	-	35
C. stellatoidea	17	27	24	20	-	32

KEY: - = Not determined, P.E=Petroleum ether extract, E.E = Ethyl acetate extract, C.E = Chloroform extract, M.E= Methanol extract, CP= Ciprofloxacin , FZ= Fluconazole

4.4 Results of Minimum Inhibitory Concentration (MIC) of the Crude Extracts

The minimum inhibitory concentration (MIC) of the extracts (4.4) showed that almost all the organisms tested were responsive to the extracts at a given concentration.

Table 4.4: Result of minimum inhibitory concentration (MIC) determination.

Test organisms	P. E (mg/ml)	C.E (mg/ml)	E.E (mg/ml)	M.E (mg/ml)
S. aureus	15	7.50	7.50	7.50
S. pyogenes	15	7.50	3.25	7.50
E. coli	15	7.50	3.25	7.50
S. typhi	15	3.25	3.25	7.50
S. dysenteriae	15	3.25	3.25	7.50
K. pneumoniae	15	7.50	3.25	7.50
C. tropicalis	15	7.50	7.50	7.50
C. krusei	15	7.50	7.50	7.50
C. stellatoidea	15	7.50	7.50	7.50

Key: P.E=Petroleum ether extract, E.E = Ethyl acetate extract, C.E = Chloroform extract, M.E= Methanol extract,

4.5 Results of Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) of the Crude Extracts

The minimum bactericidal/fungicidal concentration (MBC/MFC) of the extracts (Table4.5) shows that almost all the organisms tested were responsive to the extracts at a given concentration.

Table 4.5: Result of minimum bactericidal/fungicidal concentration determination

Test organisms	P.E (mg/ml)	C.E (mg/ml)	E.E (mg/ml)	M.E (mg/ml)
S. aureus	30	30	15	30
S. pyogenes	30	15	7.5	15
E. coli	30	15	7.5	15
S. typhi	30	7.5	7.5	15
S. dysenteriae	30	7.5	15	15
K. pneumoniae	30	15	15	30
C. tropicalis	30	30	15	30
C. krusei	30	30	15	30
C. stellatoidea	30	30	15	30

Key: P.E=Petroleum ether extract, E.E = Ethyl acetate extract, C.E = Chloroform extract, M.E= Methanol extract,

4.6 Result of the Zones of Inhibition of the Pure Isolates

The antimicrobial test results (Table 4.6**)**, shows the zones of inhibition (mm) of the pure isolates from ethyl acetate and petroleum ether. All the pure isolates had significant activity against the test microorganisms. The active controls used were ciprofloxacin 2 mg/ml for the bacteria, fulcin and fluconazole (5 mg/ml) for the fungi.

Table 4.6: Result of zone of inhibition of the isolated compounds

Test Organism	Compd (1) ETA	Compd (2) PET	Sparfloxacin	Fluconazole
S. aureus	32	27	37	-
S. pyogenes	30	25	35	-
E. coli	32	30	38	-
K. pneumonia	32	32	40	-
S. typhi	30	30	42	-
S. dysenteriae	30	30	40	-
C. krusei	27	26	-	37
C. tropicalis	0	0	-	32
C. stellatoidea	25	25	-	37

Key: - = Not determined, 0 = No zone

4.7 Results of Minimum Inhibitory Concentration (MIC) of the Pure Isolates

The minimum inhibitory concentration (MIC), of the pure isolates shows that almost all the organisms tested were responsive to the extracts at a given concentration.

Table 4.7: Result of Minimum Inhibition Concentration of isolated compounds (□g/ml)

Test organisms	Compound 1 (□g/ml)	Compound 2 (□g/ml)
S. aureus	6.25	6.25
S. pyogenes	12.5	12.5
E. coli	6.25	12.5
S. typhi	6.25	6.25
S. dysenteriae	6.25	6.25
K. pneumonia	6.25	6.25
C. Krusei	12.5	12.5
C. tropicalis	12.5	12.5
C.stellatoidea	12.5	12.5

4.8 Results of Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) of the Isolate

The minimum bactericidal/fungicidal concentration (MBC/MFC) of the pure isolate showed that almost all the organisms tested were responsive to the extracts at a given concentration. Minimum bactericidal concentration and minimum fungicidal concentration (MBC/MFC)**.**

Table 4.8 Result of Minimum Bactericidal concentration and minimum Fungicidal Concentration of the isolated compounds (1 and 2)

Test organisms	Compound 1	Compound 2
S. aureus	25	25
S. pyogenes	25	25
E. coli	25	25
S. typhi	25	25
S. dysenteriae	25	25
K. pneumonia	25	25
C. Krusei	25	25
C. tropicalis	50	25
C. stellatoidea	50	50

4.9 Result of 1D and 2D NMR of Compound 1

4.9.1 ^{1}H NMR of compound 1

The proton NMR spectrum of compound 1 revealed the presence of the deshielded protons between δ 7.2 and 7.6 ppm and that of the secondary hydroxyl group at δ 5.3 ppm. It also revealed the protons of the various methyl and methylene groups.

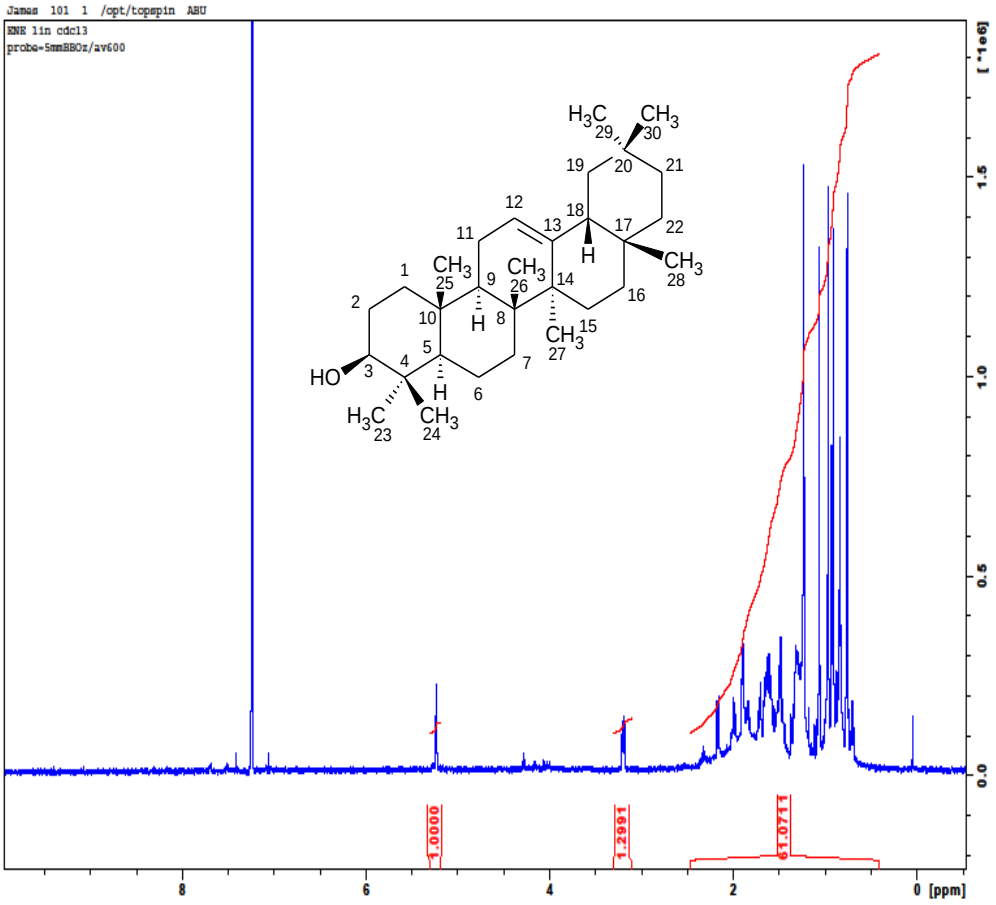


Figure 2: ^{1}H-NMR spectrum of compound (1)

4.9.2 ^{13}C NMR of compound 1

The **^{13}C** NMR spectrum of compound 1 revealed a total of 30 carbon signals.

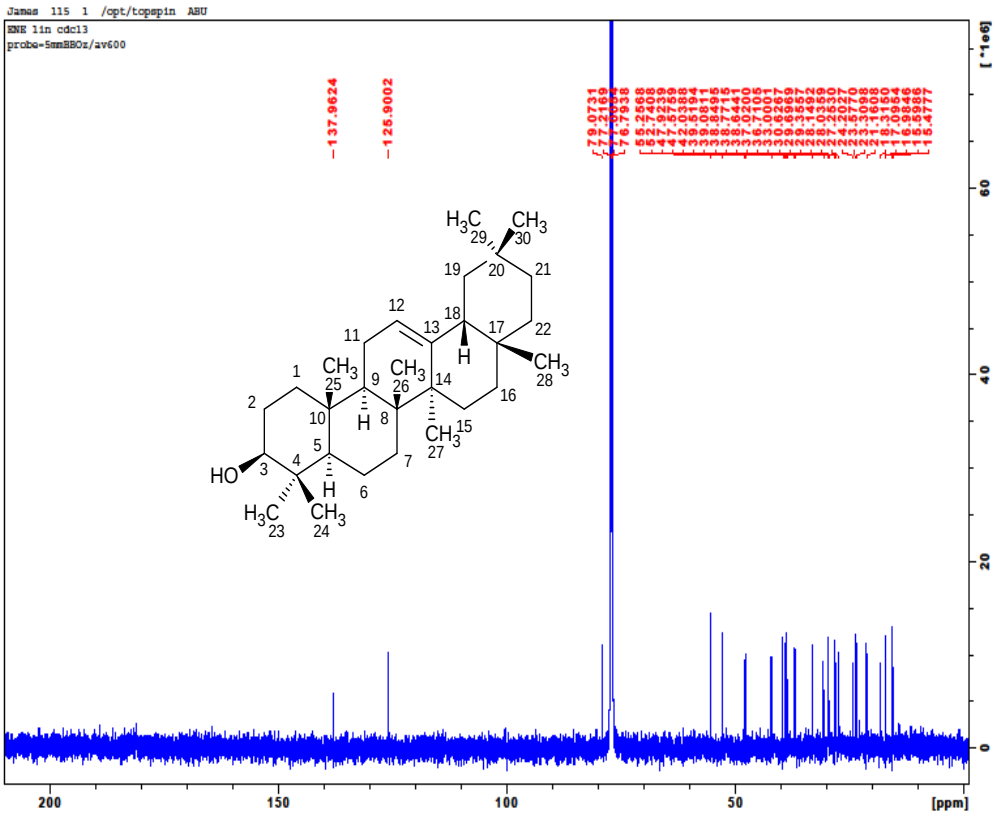


Figure 3: ^{13}C-NMR spectrum of compound (1)

4.9.3 DEPT experiment of compound 1

The DEPT spectrum of compound 1 revealed the presence of eight methyl group signals, ten (10) methylene group signals and six quaternary carbons signals.

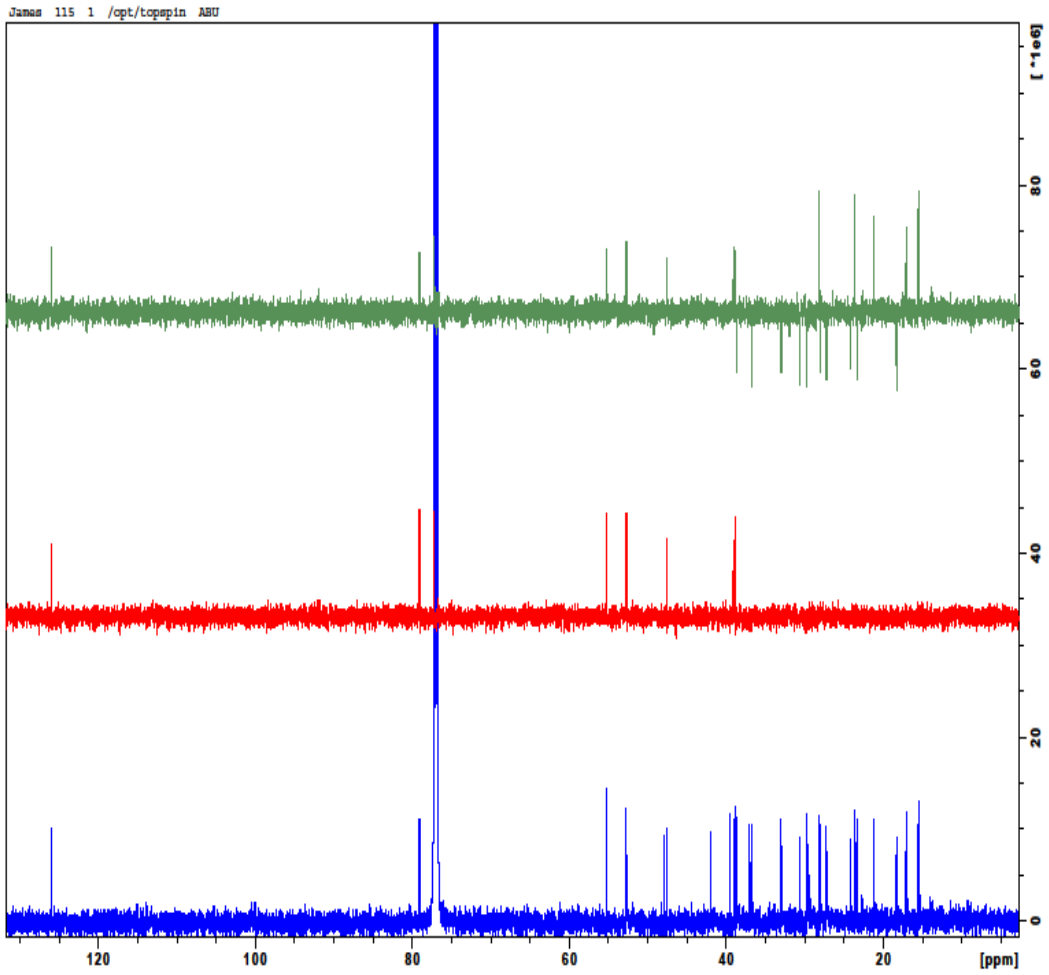


Figure 4: DEPT experiment spectrum of compound (1)

4.9.4 The 2D ^{1}H-^{1}H COSY correlation of compound 1

The COSY spectrum of compound 1 showed the correlation between the protons at the chemical shift 3.2 ppm and 3.1 ppm, 5.3 ppm and 2.4 ppm and also between 1.5 ppm and 3.2 ppm.

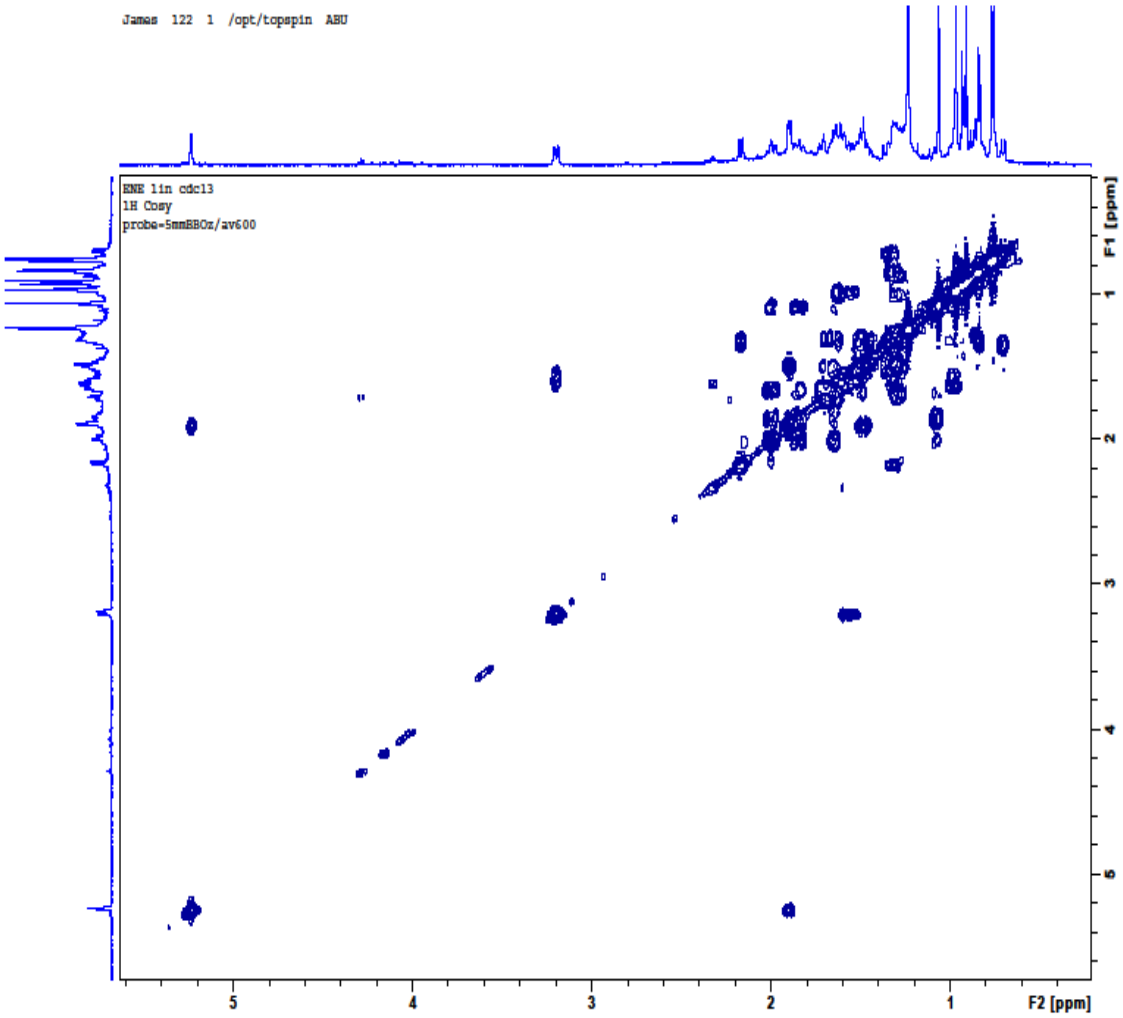


Figure 5: COSY experiment spectrum of compound (1)

4.9.5 NOESY experiment of compound 1

The NOESY spectrum of compound 1 shows the correlation between protons.

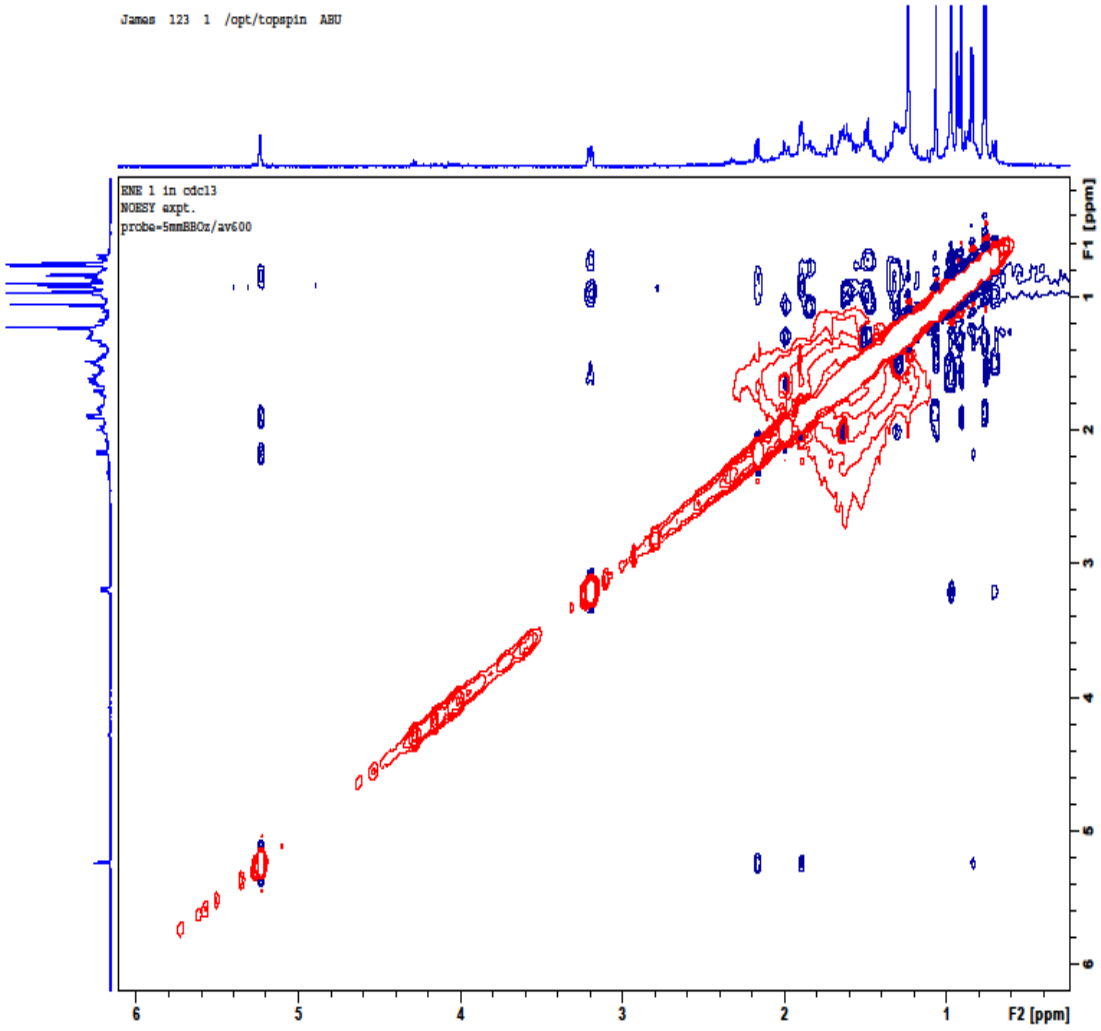


Figure 6: NOESY experiment spectrum of compound (1)

4.9.6 HSQC experiment of compound 1

The HSQC spectrum of compound 1 showed the correlation between the protons and corresponding carbons.

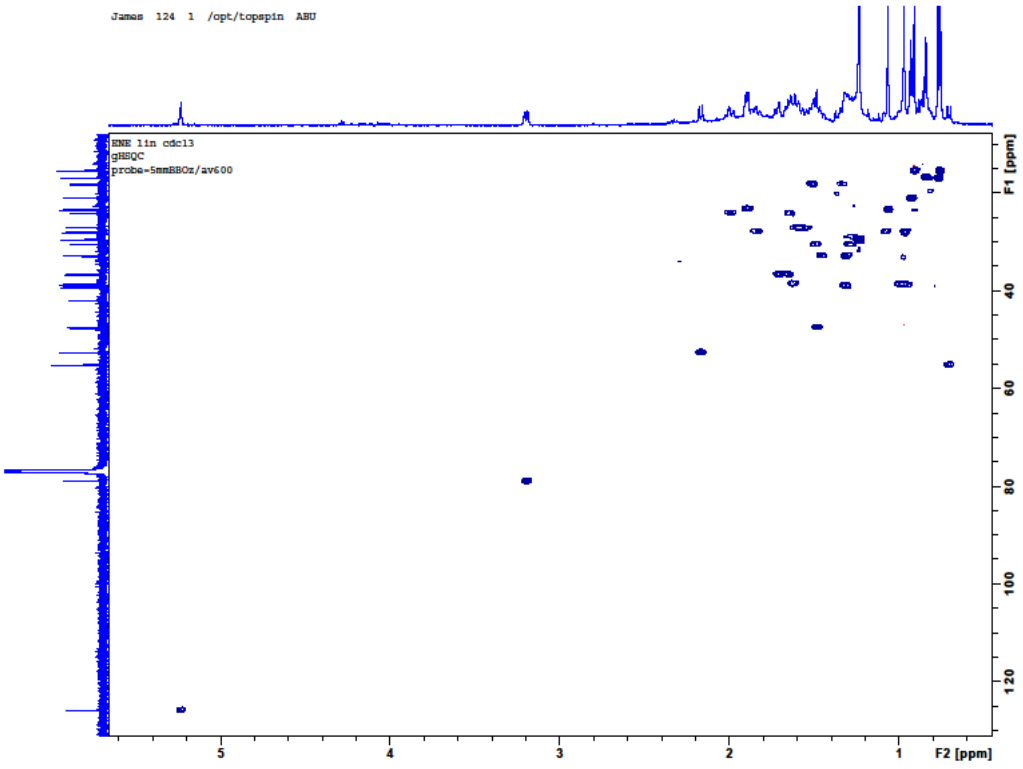


Figure 7: HSQC experiment spectrum of compound (1)

4.9.7 HMBC experiment of compound 1

The HMBC spectrum of compound 1 showed the correlation between proton and carbons 2,3,4 or 5- bonds away.

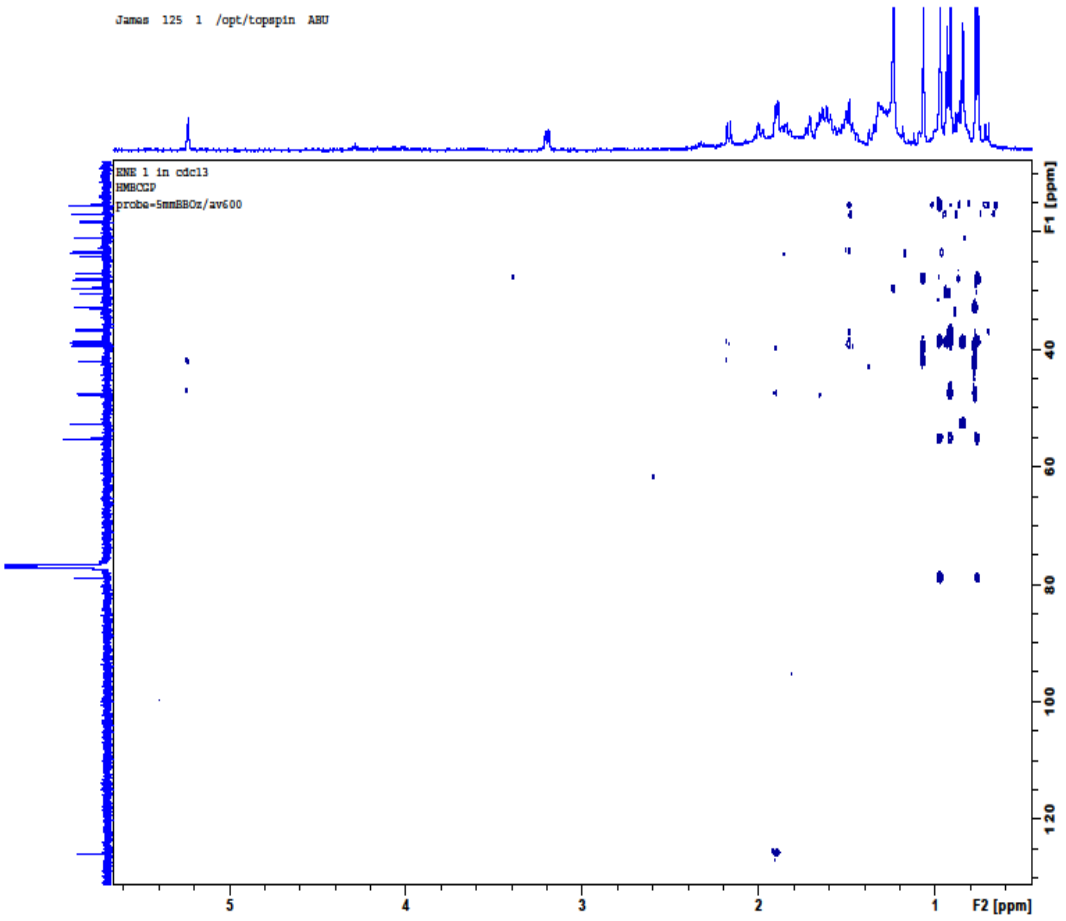
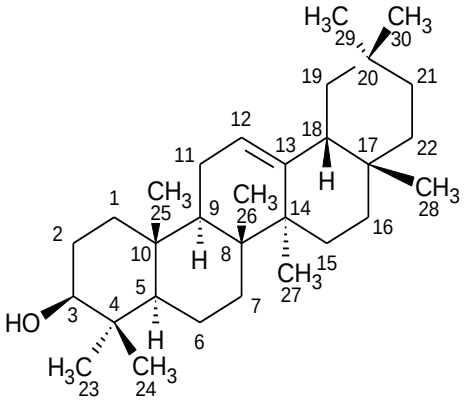


Figure 8: HMBC experiment spectrum of compound (1)

Table 4.9: Comparison of ^{13}C NMR spectral data of β-amyrin obtained from the aerial parts of *M. barteri* with literature, Hernández *et al.*, 2012 and Manal *et al.*, 2009

Carbon Position	^{13}C-NM (ppm) Experimental	^{13}C-NMR (ppm) Literature	^{13}C-NMR (ppm) Literature
1	**38.8**	38.7	37.3
2	**27.2**	27.2	28.2
3	**79.0**	79.3	71.8
4	**38.7**	38.5	37.3
5	**55.2**	55.1	36.8
6	**18.3**	18.6	21.1
7	**38.6**	32.4	42.3
8	**39.5**	39.8	45.9
9	**47.9**	47.6	50.1
10	**36.7**	36.9	36.5
11	**23.5**	23.6	24.3
12	**125.9**	121.7	121.7
13	**137.4**	145.2	140.8
14	**42.0**	41.7	44.3
15	**29.3**	26.2	26.1
16	**21.1**	26.1	23.1
17	**29.6**	32.6	48.4
18	**47.5**	47.2	45.8
19	**39.0**	46.8	39.8
20	**30.6**	31.0	36.4
21	**33.0**	34.7	34.0
22	**37.0**	37.1	31.7
23	**28.0**	28.0	29.3
24	**15.5**	15.4	19.8
25	**15.4**	15.4	19.1
26	**16.9**	16.8	18.9
27	**24.2**	25. 9	18.3
28	**17.0**	28.4	19.2
29	**28.1**	33.8	36.1
30	**23.3**	23.7	19.4



Compound 1 (β-Amyrin or β-Amyrenol)

4.10 Result of 1D and 2D NMR of Compound 2

4.10.1 ^{1}H NMR of compound 2

The proton NMR spectrum of compound 2 (an isomer of Betulinic and oleanolic acid) revealed the presence of the deshielded protons between the chemical shift 7.7 and 4.6 ppm for betulinic acid and the chemical shift δ5.3 and 7.5 for oleanolic acid. It also revealed the protons of the various methyl and methylene groups.

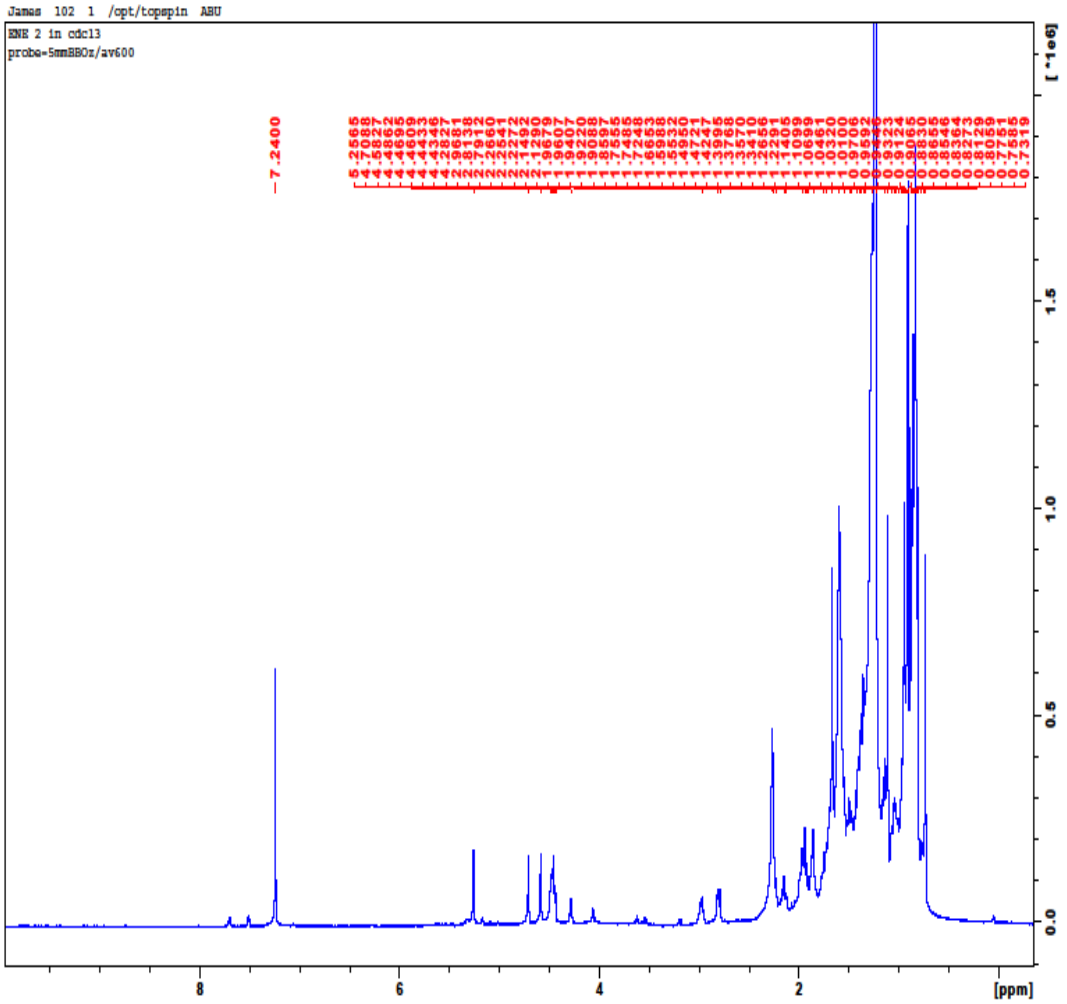


Figure 9: ^{1}H-NMR spectrum of compound (2)

4.10.2 ^{13}C NMR of compound 2

The ^{13}C NMR spectrum of compound 2 reveals signals that correspond to two isomeric triterpenes (oleanolic and betulinic acid).

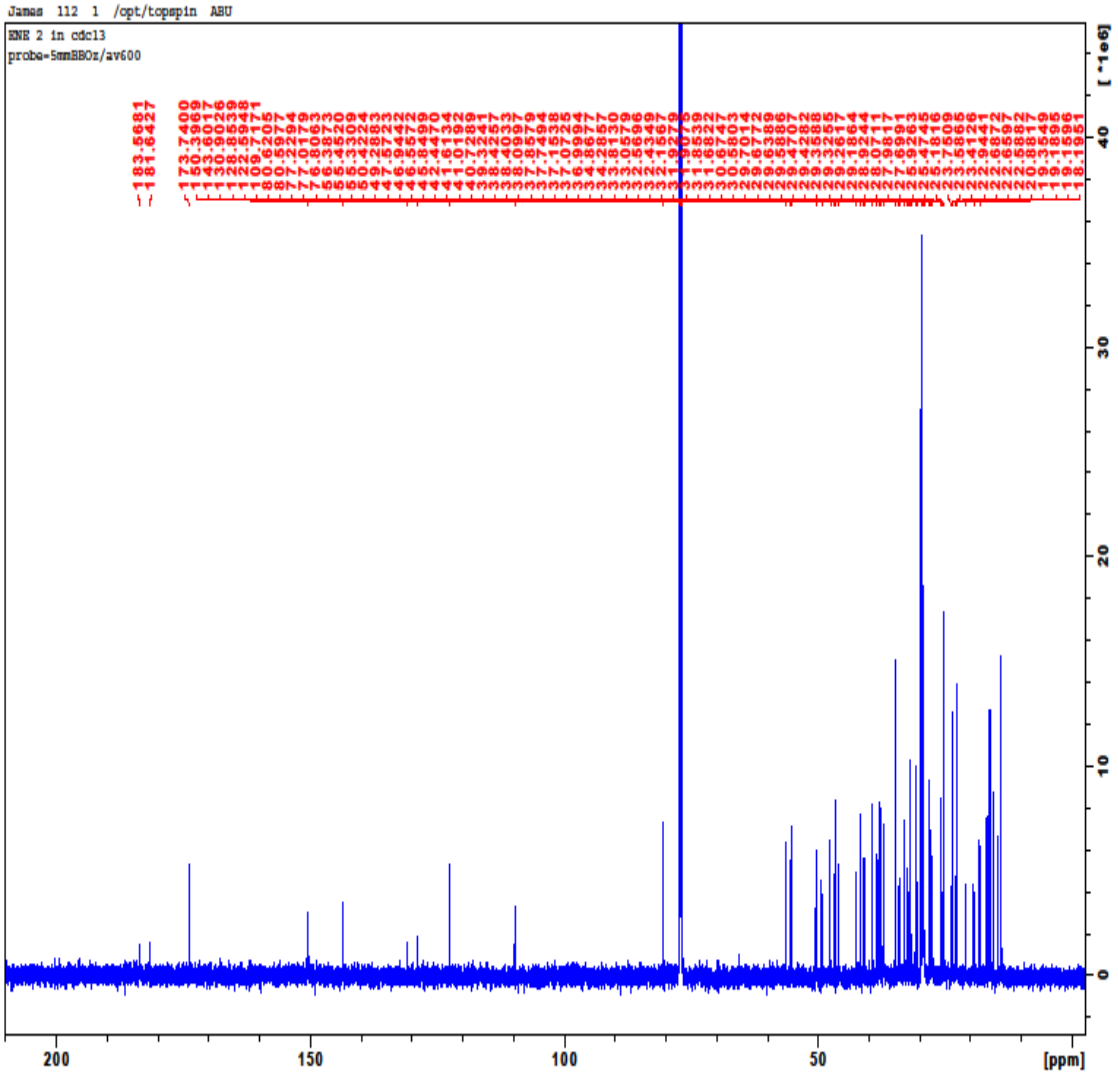


Figure 10: ^{13}C-NMR spectrum of compound (2)

4.10.3 DEPT experiment of compound 2

The DEPT spectrum of compound 2 revealed the presence of six methyl group signals, twelve (12) methylene group signals, six methine signals and six quaternary carbons signals.

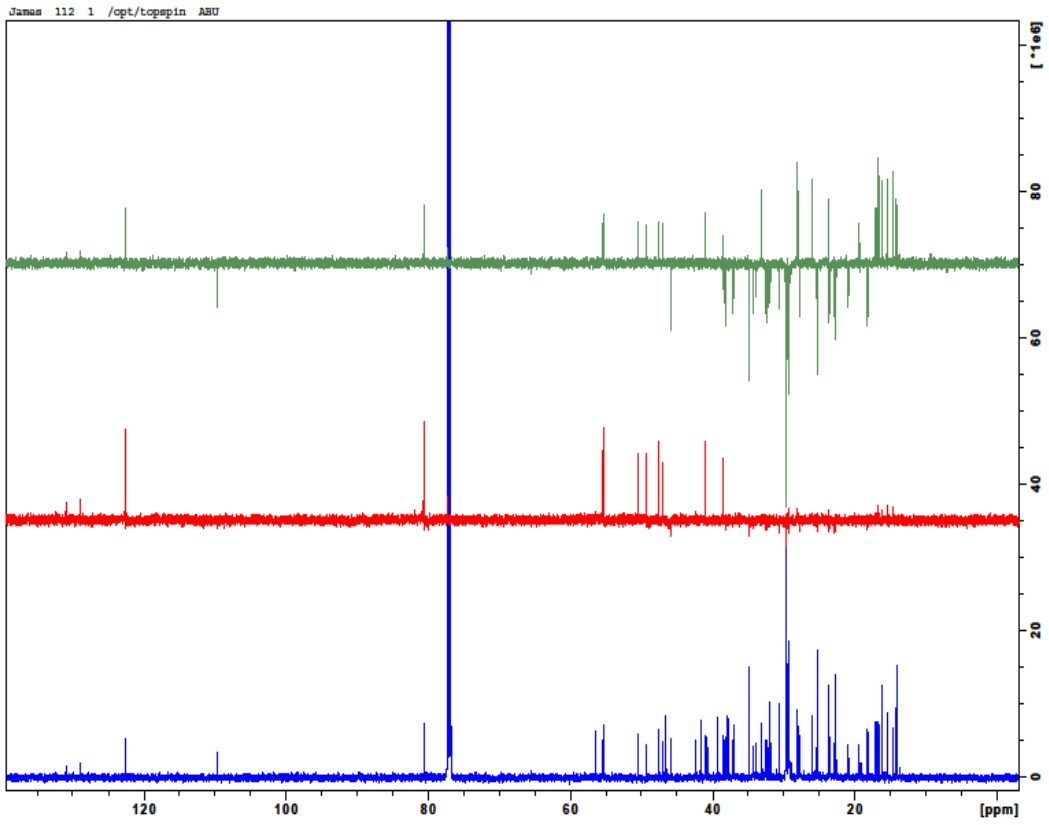


Figure 11: DEPT experiment spectrum of compound (2)

4.10.4 The 2D ^{1}H-^{1}H COSY correlation of compound 2

The COSY spectrum of compound 2 showed the correlation between the protons at the chemical shift 5.2 ppm and 2.1 ppm, 3.5 ppm and 5.2 ppm and also between 1.8 ppm and 5.2 ppm.

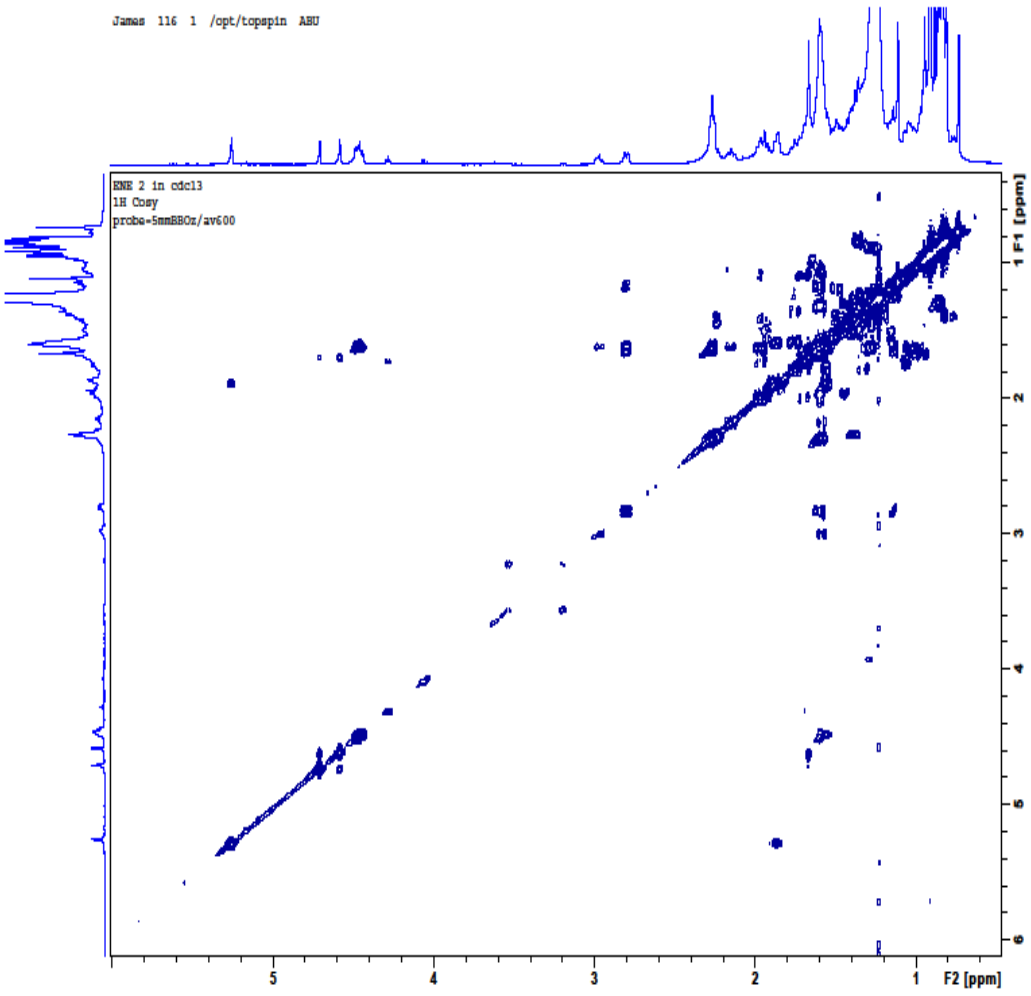


Figure 12: COSY experiment spectrum of compound (2)

4.10.5 NOESY experiment of compound 2

The NOESY spectrum of compound 2 showed the correlation between the protons.

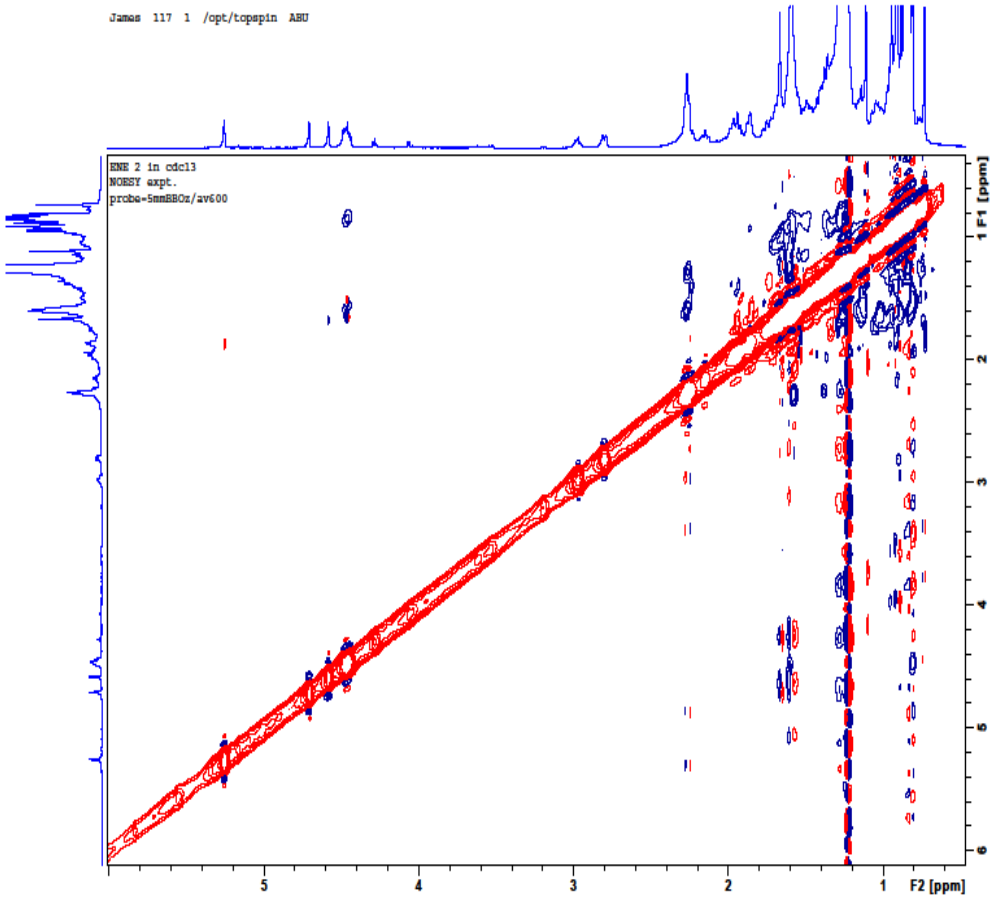


Figure 13: NOESY experiment spectrum of compound (2)

4.10.5 HSQC experiment of compound 2

The HSQC spectrum of compound 1 showed the correlation between the protons and corresponding carbons.

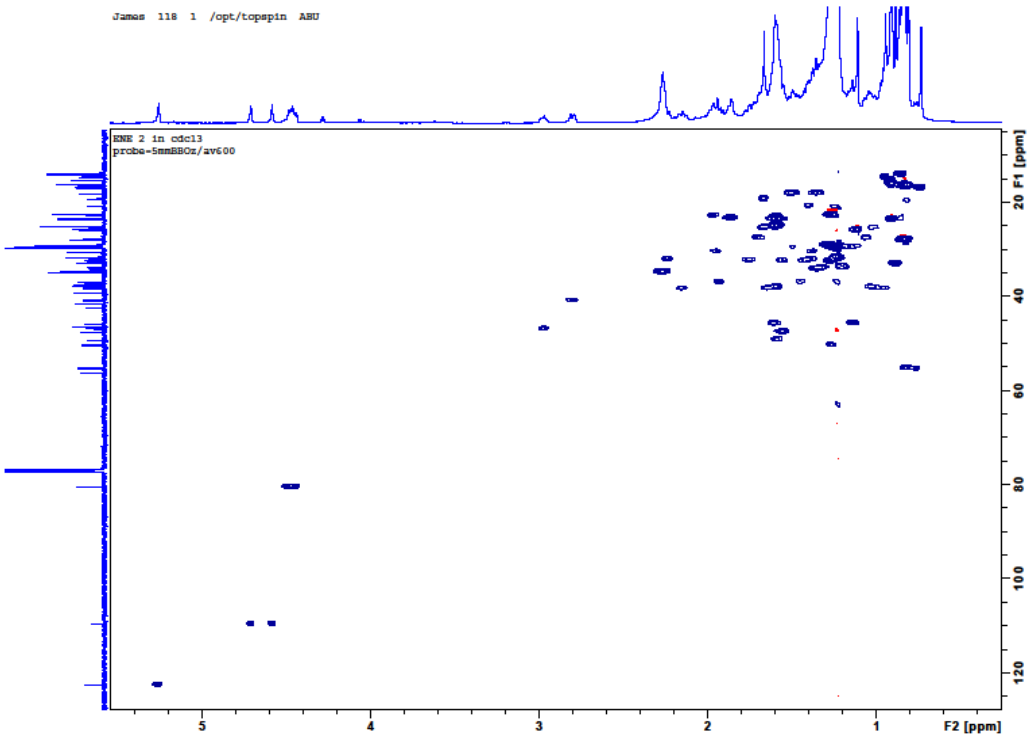


Figure 14: HSQC experiment spectrum of compound (2)

4.10.6 HMBC experiment of compound 2

The HMBC spectrum of compound 1 showed the correlation between proton and carbons 2,3,4 or 5- bonds away.

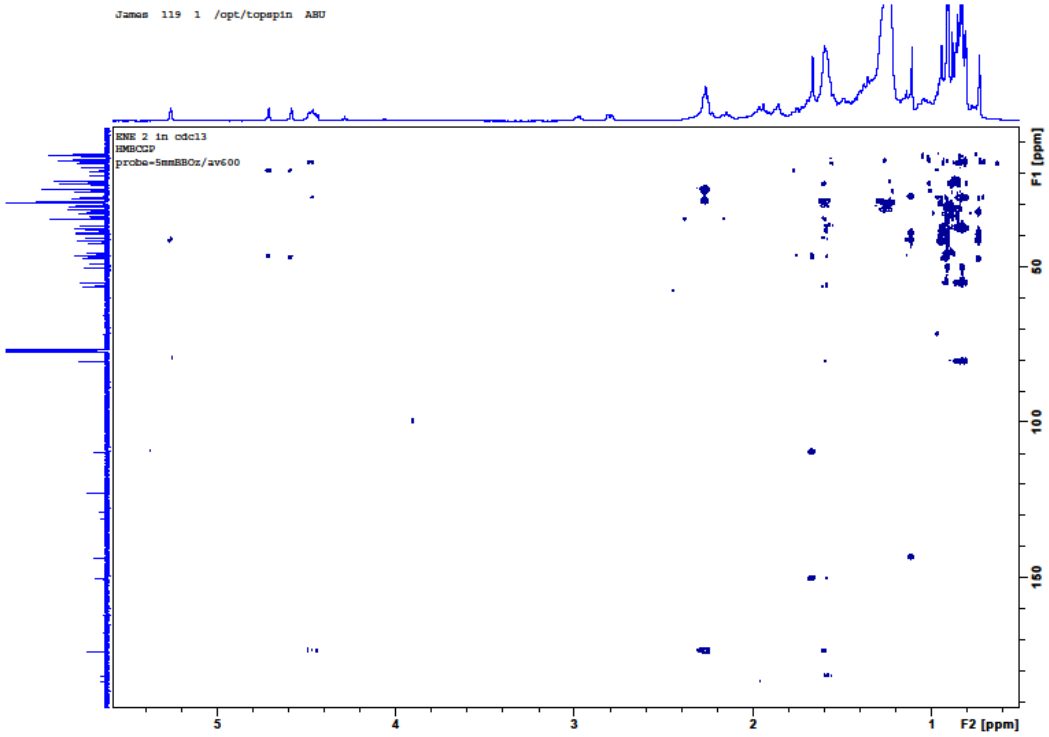


Figure 15: HMBC experiment spectrum of compound (1)

Table 4.10: ^{13}C NMR spectral data of compound (2) in comparison with literature, Onoja *et al*, 2012 and Okwute *et al*., 2014

C Position	^{13} C δ (ppm) Oleanolic acid Experimental	^{13} C δ (ppm) Literature	^{13} C δ (ppm) Betulinic acid Experimental	^{13} C δ (ppm) Literature
1	**37.7**	38.4	**38.4**	39.1
2	**27.9**	27.2	**29.2**	28.1
3	**77.2**	79.0	**77.0**	78.4
4	**38.4**	38.4	**38.0**	39.4
5	**55.4**	55.2	**55.3**	55.7
6	**19.1**	18.3	**18.1**	18.6
7	**34.8**	32.6	**34.2**	34.7
8	**39.3**	39.2	**40.7**	40.9
9	**47.5**	47.6	**50.4**	50.8
10	**37.0**	37.0	**37.1**	37.3
11	**27.6**	22.9	**25.1**	21.1
12	**122.5**	122.6	**25.4**	25.9
13	**143.6**	143.6	**37.8**	38.4
14	**41.6**	41.6	**42.4**	42,4
15	**28.9**	27.6	**30.5**	31.1
16	**22.6**	23.4	**32.1**	32.7
17	**46.9**	46.5	**56.3**	56.3
18	**41.0**	41.0	**46.5**	47.6
19	**45.8**	45.8	**49.2**	49.5
20	**36.9**	30.6	**150.3**	150.7
21	**33.8**	33.8	**29.6**	30.1

22	**32.4**	32.4	**32.5**	37.5
23	**28.0**	28.1	**29.3**	28.5
24	**23.7**	15.5	**22.9**	16.3
25	**22.6**	15.3	**23.4**	16.2
26	**19.1**	17.1	**22.5**	16.2
27	**25.9**	25.9	**20.8**	14.8
28	**181.6**	182.3	**183.5**	178.7
29	**33.0**	33.0	**109.7**	110.3
30	**23.5**	23.5	**19.3**	19.4

Oleanolic acid

Betulinic acid

CHAPTER FIVE

5.0 DISCUSSION

The result of the percentage yield of the extract reported in Table 4.1 shows that the petroleum spirit extract had the highest yield followed by methanol extract, ethyl acetate extract, and lastly the chloroform extract.

The result of the phytochemical analysis of the ethyl acetate and methanol revealed the presence of carbohydrate, flavonoid, saponins, tannins, steroids/triterpenes, cardiac glycoside while the petroleum ether and chloroform extract had steroids/triterpenes as the major constituents (Table 4.2). Majority of these plant constituents have been reported to have antimicrobial properties (Bruce *et al.*, 2000; Ververdis *et al.*, 2007).

The zones of inhibition (Table 4.3), the minimum inhibitory concentration (MIC) (Table 4.4) and minimum bactericidal/fungicidal concentration (MBC/MFC) (Table 4.5) of petroleum ether, chloroform, ethyl acetate and methanol extract of the aerial plant parts of *Maesobotrya barteri* showed remarkable activities on nines organisms tested against. All the extracts had significant activity against the test microorganisms and this is in line with reports from other *Maesobotrya* species according to Pierre *et al*., 2006. The MIC studies of the crude petroleum ether extract from the aerial parts of *Maesobotrya barteri* inhibited the growth of *Staphylococcus aureus, Streptococcus pyogenes, Klebsiella pneumoniae, Escherichia coli, Shigella dysenteriae, Salmonellae typhi, Candida stellatoidea, Candida krusei* and *Candida tropicalis* at the concentration 15 mg/ml with a corresponding MBC at 30 mg/ml for the microorganisms.

The MIC studies of the ethyl acetate crude extract of the aerial parts of *Maesobotrya barteri* (Table 4.4) inhibited the growth of *Staphylococcus aureus, Candida krusei* and *Candida tropicalis* at a concentration of 7.5 mg/ml, with corresponding MBC at 15 mg/ml while *Streptococcus pneumonia, Salmonella typhi, Klebsiella pneumoniae, Escherichia coli, Shigella dysenteriae,* and *Candida tropicalis* had their MIC at 3.25 mg/ml with a corresponding MBC at 7.5mg/ml.

The MIC studies of the crude chloroform extract from the aerial parts of *Maesobotrya barteri* (Table 4.4) which inhibited the growth of *Staphylococcus aureus, Streptococcus*

pyogenes, Escherichia coli, Klebsiella pneumoniae, Candida stellatoidea, Candida krusei and *Candida tropicalis* at a concentration 7.5mg/ml with a corresponding MBC at 30mg/ml while *Shigella dysenteriae* and *Salmonellae typhi* were at 3.25mg/ml with a corresponding MBC at 15mg/ml.

The MIC studies of the crude methanol extract from the aerial parts of *Maesobotrya barteri* (Table 4.4) inhibited the growth of *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumoniae, Shigella dysenteriae, Salmonellae typhi Candida stellatoidea, Candida krusei* and *Candida tropicalis* at a concentration 7.5mg/ml with a corresponding MBC at 30mg/ml and 15mg/ml for the microorganisms *Streptococcus pyogenes, Escherichia coli, Shigella dysenteriae* and *Salmonellae typhi*.

The zones of inhibition (Table 4.6), the minimum inhibitory concentration (MIC) (Table 4.7) and minimum bactericidal/fungicidal concentration (MBC/MFC) (Table 4.7) of compounds (1) and (2) from the ethyl acetate and petroleum ether fraction showed remarkable activities on nines organisms tested against. The zone of inhibition of compound (1) and (2) were compared with those of standard drugs ciprofloxacin 2 mg/ml for the bacteria, fulcin and fluconazole (5 mg/ml), this are in line with microbial studies reported by Nostro *et al.*, (2000).

Compound (1) was proposed to be β-Amyrin ($C_{30}H_{50}O$, 426.7 g/mol) using 1D-NMR and 2D-NMR spectroscopic analyses. Antimicrobial screening reported from other natural products has confirmed the bioactive properties of β-Amyrin. Jianhong-ching *et al.*, (2009) reported that β-amyrin isolated from *Ardisia elliptica,* a medicinal plant used for alleviating chest pain, fever, liver poisoning and parturition complications was found to be six times as active as aspirin in inhibiting platelets aggregation. According

to Neveen *et al.*, (2013), β-amyrin isolate from *Laurencia microcladia,* a marine algae was also found to have antibacterial activity.

Compound (2) was proposed to be an isomer of betulinic acid and oleanolic acid ($C_{30}H_{48}O_3$, 453 g/mol) using 1D-NMR and 2D-NMR spectroscopic analyses. Antimicrobial screening reported from other natural products has confirmed the bioactive properties of the isomers (betulinic acid and oleanolic acid). Betulinic acid is a naturally occurring pentacyclic triterpenoid which has been reported to have potentials such as an immunomodulatory, anti-malarial, anti-inflammatory, and anti-cancer agent, (Fujioka *et al*., 1994; Kim *et al*., 1998; Tan *et al*., 2003; Mansour *et al*., 2012).

Also, oleanolic acid isolated from the roots of *L. camara* was reported to have antiurolithiatic activity, (Narendra and Ameuta, 2013). Oleanolic acid is one of the main active components in the fruit of *Ligustrum lucidum*, reported to possesses anticancer, antimutagenic, anti-inflammatory, antioxidative and antiprotozoal activities (Mahato *et al*., 1988; En-Qin *et al*., 2011).

Antimicrobial screening reported from other natural products has confirmed the microbial properties of betulinic acid and oleanolic acid. However, this is the first time it will be isolated from the aerial parts of *Maesobotrya barteri*.

The structure of compound 1 and compound 2 was elucidated using Nuclear Magnetic Resonance Spectroscopy (NMR) (1-DNMR and 2-DNMR) and also by comparing the obtained data with already existing literature.

The ^{13}C NMR spectrum (Figure 3) of compound 1 showed thirty (30) major recognizable carbon signals, eight methyl groups at δ37.0200 (C-22), 28.0359 (C-23), 15.5986 (C-24), 15.4777 (C-25), 16.9846 (C-26), 24.2027 (C-27), 17.0954 (C-28), 28.1492 (C-29), 23.3098 (C-30) and a secondary hydroxyl bearing carbon 79.0731 at

(C-3). It also showed some recognizable signals at δ 125.9002 and 137.9624 ppm which is assignable to the double bond at C-12 and C-13. In addition, ten methylene groups, six quaternary carbons atoms were observed from DEPT experiment. The chemical shift at δ137.962, 125.9002 which are C-12 and C-13 and the consistent flow of methyl groups from C-23 to C-30 were characteristic peaks for a β- Amyrin type of skeleton (Manal *et al.*, 2009; Hernández *et al.*, 2012).

The structure of compound 2 was elucidated using Nuclear Magnetic Resonance Spectroscopy (NMR) (1-DNMR and 2-DNMR) and also by comparing the obtained data with already existing literature, the structure was proposed.

The isomers were obtained as a yellowish crystalline substance with the molecular formula is $C_{30}H_{48}O_3$. The ^{1}H NMR and ^{13}C NMR spectral data assigned values reported for 3β-dihydroxylup-20(29)-ene-28-oic acid (betulinic acid) and 3β - hydroxyolean-12-en-28-oic acid (oleanolic acid). The ^{13}C NMR indicated thirty carbon signals in the broad band decoupled spectrum; six methyl, twelve methylene, six methine and six quaternary carbon atoms from DEPT experiments. The chemical shifts at 183.56, 150.39 and 109.71 were the characteristic peaks for betulinic type of skeleton, assigned to C-28, C-20 and C-29 respectively (Ahmad, 1994 and Mei-Ing *et al* 1999). While The chemical shifts at 181.64, 122.59 and 143.60 were the characteristic peaks for oleanolic type of skeleton, assigned to C-28, C-12 and C-13 respectively. The oxygen deshielding chemical shift at 77.2 was assigned to C-3 (Seebacher *et al.*, 2003).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

This work describes the phytochemical screening, isolation, characterization and antimicrobial activities of various fractions and isolated compounds obtained from *Maesobotrya barteri* (Hutch), which is a plant used in Ethno medicinal practices in Nigeria.

Preliminary phytochemical screening of the ethyl acetate, methanol, chloroform and petroleum ether extracts of the aerial plant parts *Maesobotrya barteri* revealed the presence of steroids , triterpenes, flavonoid, saponins, cardiac glycoside and tannins.

Column chromatography of the ethyl acetate and petroleum ether extract followed by preparative thin layer chromatography led to the isolation of β-amyrin (compound 1) and an isomer of betulinic acid and oleanolic acid (compound 2).

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

6.2 Conclusion

The screening of plant extracts has had an impressive history of identifying active agents. The choice of the aerial parts of *Maesobotrya barteri* as the plant parts of interest in this work was based on its vast medicinal importance among traditional medicine practitioners in Orokam, Benue state region of Nigeria. Two compounds were isolated from this plant namely β-Amyrin and the isomers, betulinic and oleanolic acids. However based on literature and research findings, these two compounds are all been reported from the aerial plant parts of *Maesobotrya barteri* for the first time.

From the antimicrobial activities of the isolated compounds the use of these plants in the various diseases related ailment by the natives has been justified.

6.3 Recommendations

The various crude extracts from aerial plant parts of *Maesobotrya barteri* contain other bioactive compounds or phytochemicals. These should be isolated and characterized. *In vivo* studies should be carried so as to determine their mode of action and toxicity if any.

Other parts of the plant *Maesobotrya barteri* like the root should be subjected to phytochemical and biological study in order to fully exploit the chemical constituents present in this plant.

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