PHYTOCHEMICAL AND ANTIMICROBIAL STUDIES OF *MORINDA LUCIDA* (BENTH) LEAF AND *UAPACA TOGOENSIS* (PAX) STEM BARK EXTRACTS

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NIGERIA

MARCH, 2015

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DEPARTMENT OF CHEMISTRY,
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NIGERIA

MARCH, 2015

DECLARATION

I declare that the work in this dissertion entitled **"Phytochemical and antimicrobial studies of *Morinda lucida* (Benth) leaf and *Uapaca togoensis* (Pax) stem bark extracts"** was carried out by me in the Department of Chemistry, Facaulty of Science, Ahmadu Bello University, Zaria, Nigeria under the supervision of Prof. G. I. Ndukwe, Dr. M. S. Sallau and Dr. (Mrs) R. G. Ayo. The Information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this work dissertation was previously presented for another degree or diploma at this or any other Institution.

_____ _____

Andrew Atai OMACHI Signature Date
Ph.D./SCIE/07491/2008-09

`

CERTIFICATION

This dissertation entitled "PHYTOCHEMICAL AND ANTIMICROBIAL STUDIES OF *MORINDA LUCIDA* (BENTH) LEAF AND *UAPACA TOGOENSIS* (PAX) STEM BARK EXTRACTS", by Andrew Atai OMACHI, meets the regulations governing the award of the degree of Doctor of Philosophy (Ph.D) in Chemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This dissertation is dedicated to my Lord and Saviour, Jesus Christ, who made it possible for me to undertake the programme successfully.

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LIST OF ABBREVIATIONS

δ	=	chemical shift
COSY	=	COrrelation SpectroscopY
DEPT	=	Distortionless Enhancement by Polarization Transfer
DMSO	=	Dimethylsulfoxide
NMR	=	Nuclear Magnetic Resonance
1D-NMR	=	One Dimensional Nuclear Magnetic Resonance
2D-NMR	=	Two Dimensional Nuclear Magntic Resonance
LD_{50}	=	oral mean lethal dose
^{13}C	=	Carbon-13
^{1}H	=	Proton
MHZ	=	Mega hertz
MS	=	Mass spectrometry
HMBC	=	Heteronuclear Multiple Bond Correlation
HSQC	=	Heteronuclear Single Qantum Coherence
NOESY	=	Nuclear Overhauser Enhancement Spectroscopy
MIC	=	Minimum Inhibitory Concentration
HIV	=	Human Immunodeficiecy Virus
HSV1	=	Herpes Simplex Virus type1
MBC	=	Minimum Bactericidal Concentration
MFC	=	Minimum Fungicidal Concentration
TLC	=	Thin Layer Chromatography
CC	=	Open column chromatography

VLC	=	Vacuum Liquid Chromatography
PTLC	=	Prepartive Thin Layer Chromatography
PET	=	Petroleum ether
CF	=	Chloform
ETAC	=	Ethyl acetate
MET	=	Methanol
IC_{50}	=	50 % inhibitory concentration
Fig	=	Figure
ppm	=	Parts per million
amu	=	Atomic mass unit

ABSTRACT

The aims of the study were phytochemical and antimicrobial studies of *Morinda lucida* Benth (Rubiaceae) leaf and *Uapaca togoensis* Pax (Phyllanthaceae, a segregate of Euphorbiaceae *Sensu lato*) stem bark extracts. Air-dried leaves of *Morinda lucida* were powdered and extracted with the four selective solvents viz petroleum ether (60-80^{0}C), chloroform, ethyl acetate and methanol in order of increasing polarity. Similarly, air – dried stem bark of *Uapaca togoensis* were powdered and extracted with the three selective solvents viz n-hexane, ethyl acetate, and methanol. Phytochemical screening was carried out using standard methods. The results showed that the *Morinda lucida* leaf extracts were made up of carbohydrates, glycosides, flavonoids, cardiac glycosides steroids and triterpenoids and *Uapaca togoensis* stem bark extract was composed of carbohydrates, glycosides, tannins, flavonoids, saponins, cardiac glycosides, steroids and triterpenes. The extracts were screened for activity against Methicilin-resistant/multidrug-resistant *Staphylococcus aureus* (MRSA), Vancomycin-resistant Enterococci (VRE), *Staphylococcus aureus, Corynebacterium ulcerans, Streptococcus pyogenes, Escherichia coli, Salmonella typhi, Shigella dysenteriae, Pseudomonas aeruginosa, Proteus mirabilis,* and *Neisseria gonorrhea, Candida albicans, Candida stellatoidia, Candida krusei,* and *Candida tropicalis*. Antimicrobial study revealed that the *Morinda lucida* leaf extract is both antibacterial and antifungal. All the extracts were active against the same set of bacterial and fungal pathogens. The *Morinda lucida* were active against MRSA, *S. aureus, S. pyogenes, E. coli, S. typhi, S. dysenteriae, P. mirabilis, N. gonorrhea, C. albicans* and *C. tropicalis* but were not active against VRE, *C. ulcerans, P. aeruginosa, C. stellatiodia* and *C. krusei*. Also, antimicrobial activity tests showed that *Uapaca togoensis* stem bark extract is both antibacterial and antifungal. All the extracts have activity against the same set of bacterial and

fungal pathogens. The extracts of *Uapaca togoensis* were active against MRSA, *S. aureus, C. ulcerans, E. coli, S. typhi, S. dysenteriae, P. mirabilis, N. gonorrhea, C. albicans* and *C. tropicalis* but have no activity against VRE, *S. pyogenes, P. aeruginosa* and *C. stellatoidea*. The antimicrobial activities of the *Morinda lucida* and *Uapaca togoensis* extracts justify the use of these plants in folkloric medicine to treat some infectious diseases. By chromatographic techniques taraxeryl hexadecanoate was isolated from the chloroform extract of *Morinda lucida* and betulinic acid was isolated from ethyl acetate extract of *Uapaca togoensis*. These compounds showed similar pattern of antimicrobial activities as the parent extract.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Medicinal Plants

Many studies have shown that medicinal plants exhibit diverse biological activities such as antioxidant, antimicrobial, anticancer, anti-inflammatory, etc. The biological activities of medicinal plants are due to their combination of bioactive compounds which are secondary metabolites. The major classes of plant secondary metabolites are alkaloids, phenolic compounds, glycosides, anthraquinones and terpenoids. Secondary metabolites have restricted distribution in the plant kingdom; the composition of plant's secondary metabolites varies among species, genus, or family due to genetic and environmental factors. Also, the secondary metabolites are structurally diverse and this confers on each of them unique biological properties.

Bioactive compounds have been found to exhibit biological activities which are related to those of the parent plant. Thus, the biological activities of medicinal plants signify the kind of bioactive compounds which are present in its extract. The information on the constituent secondary metabolites of the plants clarifies their uses in ethnomedicine.

The ethnomedicinal plants have been the major sources of drugs and lead compounds for drug synthesis (Adzu *et al*., 2011; Halilu *et al.*, 2013). However, despite the importance of bioactive plant compounds to pharmaceuticals, only a few medicinal plants have been investigated for their phytochemicals and biological activities.

Phytochemical screening is the means of identifying new sources of therapeutically and industrially important bioactive compounds from plants (Aman *et al*., 2012). The phytochemical screening involves botanical identification, extraction with suitable solvents, purification and characterization of the bioactive constituents of medicinal plants (Bandaranayake, 2006). Antimicrobial activity has formed the basis of many applications of bioactive compounds in

pharmaceuticals, food preservation, alternative and natural therapies (Kemal *et al.,* 2013). The evaluation of the antimicrobial activity of natural products has become critical due to the increased prevalence of microbial antibiotic resistance to the most common antibiotics (antimicrobial agents). Therefore, in this study the phytochemical and antimicrobial studies of the two medicnal plants, *Uapaca togoensis* Pax (Phyllanthaceae, a segregate of Euphorbiaceae *sensu lato*) and *Morinda lucida* Benth (Rubiaceae), which are used in ethnomedicine to treat many diseases including microbial infections such as respiratory diseases, fever, gonorrhea, vomitting, desentry, etc (Kemabonta and Okogbue, 2000; Soladoye, 2005; Koné *et al.*, 2007; Odutuga *et al.,* 2010) were investigated to discover the phytochemical constituents responsible for their antimicrobial activities . In this study, *M. lucida* and *U. togoensis* were screened for their phytochemical constituents and antimicrobial activities against both resistant and standard bacterial and fungal pathogens to isolate the antibacterial and antifungal constituents and characterize them using the usual organic analytical techniques.

1.2. Statement of the Problem

Though *U. togoensis and M.lucida* are traditional medicinal plants in use in West Africa and Central Africa to treat various diseases including microbial infections, no phytochemical and antimicrobial studies on these plants has been carried out to discover secondary metabolites resposible for its antimicrobial activities.

1.3. The Aims of the Study

The aims of the study were:

i. Phytochemical and antimicrobial studies of *Uapaca togoensis* (Pax) stem bark extracts.

ii. Phytochemical and antimicrobial studies of *Morinda lucida* (Benth) leaf extracts.

1.4. The Objectives of the Study

The objectives of the study were:

i. Collection and botanical identification of the plant materials.

ii. Air drying, pulverizing and solvent extraction of the pulverized plant materials.

iii. Phytochemical screening of the crude extracts, bioassay-guided fractionation of the crude extracts, and isolation of some phytochemicals of *M. lucida* and *U. togoensis* and their characterization.

iv. Determine the susceptibility of some antibiotic resistant microbial strains and standard strains of some bacteria and fungi to *M. lucida* leaf extract and *U. togoensis* stem bark extract.

v. Determine the susceptibility of some resistant microbial strains and standard strains to isolated compounds from *M. lucida* and *U. togoensis.*

1.5. Justification of the Study

Most of the traditional knowledge of medicinal plants is in the form of oral knowledge and the active ingredients of herbal remedies are unknown to the traditional healers. Herbal remedies generally lack standardization. This constitutes hinderance to acceptibility by the general public and the integration into the official national health care system of many countries. The correct identification of herbal material and the active ingredients is essential to quality control, safety and efficacy, acceptibility by the general public and possible integration into the national health care system. The phytochmical and antimicrobial studies of *U. togoensis* and *M.lucida* would provide information on of the therapeutic potentials of these medicinal plants, quality parameters and data about these plants.

1.6. The Scope of the Study

The research work covered collection and identification of the plant materials, air-drying and pulverizing of the dried plant material, solvent extraction of pulverized plant material, preliminary phytochemical screening of the extracts to determine the major phytochemicals present, isolation of compounds and their structural characterisation, the antibacterial and antifungal assays of the extracts and compounds isolated from them. The antimicrobial activity parameters determined were zone of inhibition (ZI), minimum inhibitiory concentration (MIC) and minimum bactericidal/ fungicidal concentration (MBC/MFC).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Phytochemical Screening and Antimicrobial Activities of *Morinda lucida* Benth Leaf Extract

2.1.1 Scientific classification of *Morinda lucida* Benth

Family: Rubiaceae

Subfamily: *Rubioideae*

Tribe: *Morindeae*

Genus: *Morinda*

Species: *Morinda lucida* Benth

2.1.2 Vernacular names

English: *Brimstone trees*.

Igala: *Ọgẹlẹ*

Yoruba: *Oruwo*

Ibo: *Nfia*

Ebira: *Ugigo*

2.1.3 The family, Rubiaceae

Rubiaceae is a family of flowering plants, variously called the madder family, bedstraw family or coffee family. The family takes its name from the madder genus *Rubia*. The plants included in the family are *Cofea arabica*, *Morinda lucida*, *Cinchona* (whose bark contains quinine). The Rubiaceae is one of the five largest plant families, including Orchidaceae, Compositae, Leguminosae, and Gramineae. The family includes trees, shrubs, climbers, herbaceous plants and ant plants. Rubiaceae is subdivided into four subfamilies, namely Rubioideae, Cinchonoideae, Ixoroideae and Antirheoideae with about 50 tribes. According to the

world Rubiaceae checklist, there are 611 genera and 13,143 species (Davis *et al*., 2009). Psychotria, with 1834 species is the largest genus in the family. With many large and poorly defined genera, the family was not extensively researched as others. According to Karou *et al.* (2011). Rubiaceae are used as ornamental trees and in African folk medicine to treat several diseases including malaria, hepatitis, cough, hypertension, diabetes and sexual weakness. Following leads supplied by traditional healers, biological screening have shown many of the plants to possess antimicrobial, antidiabetic, antihypertension, antioxidant and anti-inflammatory activities. The family consists of many genera that serve as valuable medicinal plants. According to Karou *et al*. (2011), great attention has been paid to species such as *Nauclea latifolia, Morinda lucida, Mitragyna inerms* and *Crossopteryx febrifuga* but these plants have not been systematically studied and so, several compounds should be waiting to be discovered.

2.1.4 The genus, *Morinda*

The genus, *Morinda*, is among the genera of Rubiaceae that serve as valuable medicinal plants. It comprises about 80 species and occurs throughout the tropics. In Africa 5 species are found and they are: *Morinda lucida* Benth, *Morinda citrifolia L., Morinda geminate DC, Morinda Longiflora* and *Morinda Morindoides* (Bak). The distinctive characteristics of *Morinda lucida* is the comparatively small flowering and fruiting heads on long slender peduncles. Morinda species yield yellow and red dyes and are used to dye textiles in Nigeria, Ghana, Congo DR and Cote d'Ivoire.The *Morinda* species are bitter tasting and the bitter-tasting roots are used as flavouring for food and alcoholic beverages. The wood is excellent for making charcoal, and for construction, mining props, furniture, canoes, poles and fuel wood. Many species, including those from Africa, are important medicinal plants, widely applied against various kinds of fevers and infections (Zimudzi and Cardon, 2005).

2.1.5 Origin and geographic distribution of *M. lucida*

In Africa, *M. lucida* occurs mainly in West Africa and Central Africa. In West Africa it occurs in the coastal countries including Nigeria (Zimudi and Cardon, 2005). Fig 2.1 shows the distribution of *M. lucida* in Africa.

2.1.6 Description of *M. lucida*

Morinda lucida (Benth) is an evergreen shrub of up to 18m height (Plate III). The bark (Plate IV) is grey in color and the leaves (Plate I and II) are about 7-15 cm long by 3.5-7.5 cm broad. Flowers (Plate I) are bi-sexual, regular, heterostylous, and fragrant. Calyx is cup-shaped, about 2 mm long, persistent. Corolla is salver-shaped, about 1.5 cm long, white or greenish yellow. Lobes are ovate-lanceolate; up to 5 mm x 2.5 mm. Ovary is inferior, 2-celled. Style is 8-11mm long with stigma lobes 4-7 mm long. Stamens are 5, inserted in the Corolla throat, with short filaments. Fruits are a drupe; several together arranged into an almost globose succulent syncarp 1-2.5 cm in diameter, soft and black when mature (Zimudzi and Cardon, 2005).

2.1.6 Phytochemistry of *M. lucida*

Phytochemical screening of *M. lucida* leaf extract was found to contain saponins, tannins, anthroquinones, alkaloids and unsaturated steroids and triperenes (Ogunrade and Onifade, 2009). However, the preliminary phytochemical screening of stem bark, root bark and leaf extracts of *M. lucida*, showed that saponins were expressed in all parts of the plant but anthraquinones were present in the stem bark and root bark of the plant (Adomi and Umukoro, 2010). According to Adomi and Umukoro (2010), the maximum accumulation of chemical constituents occurs at the time of flowering and then declines at the beginning of fruiting stage.



Fig. 2.1: Distribution map of *Morinda lucida* Benth(Rubiaceae) in Africa.

Source: *Morinda lucida*; Prota Database. www. Protabase. Org. Retrived 12/07/2012.

Key:

■ Countries where *M*. l*ucida* is present.

□ Countries where *M*. l*ucida* is absent.



Plate I: Leaves and flowers of *Morinda lucida*

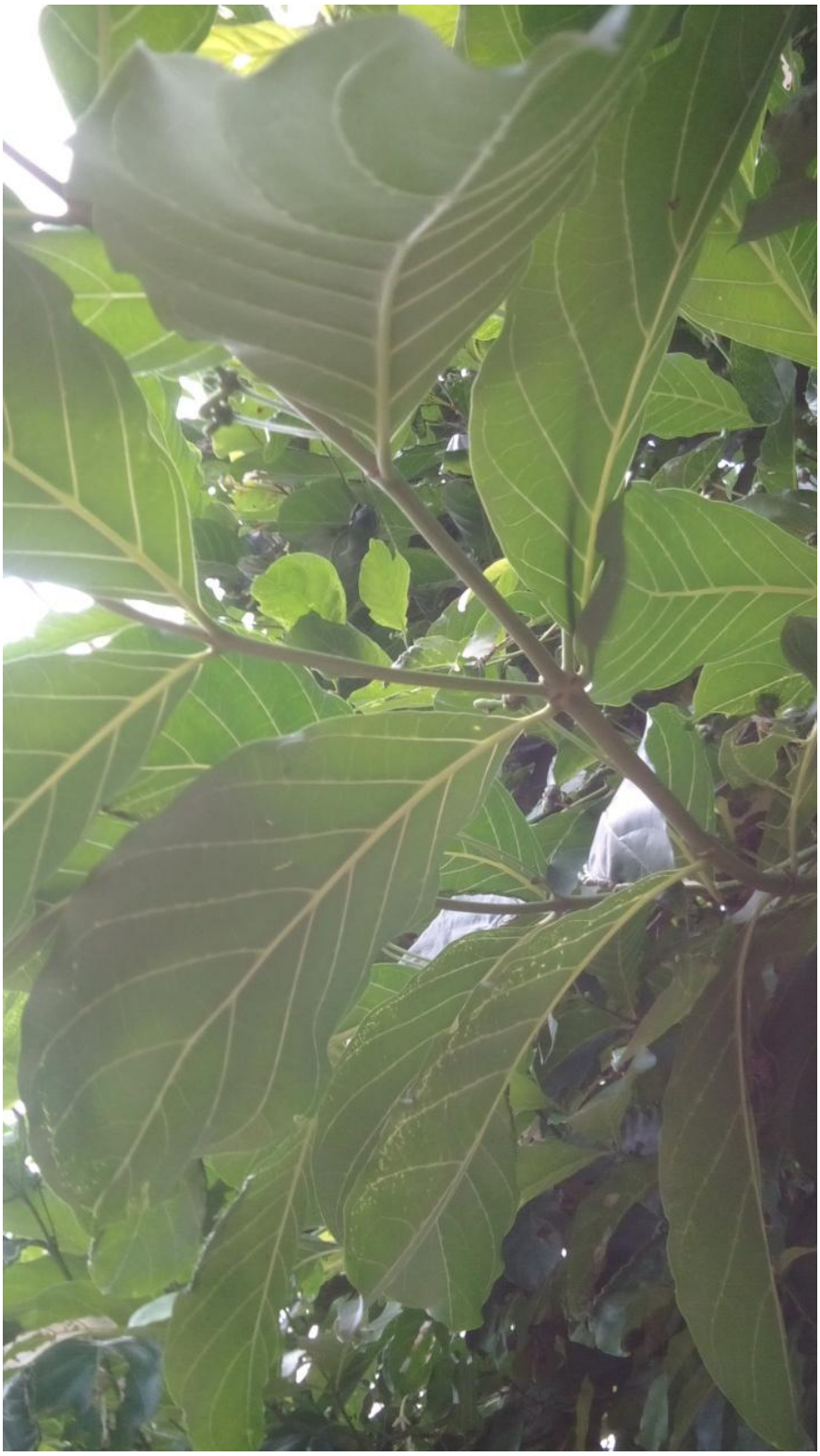


Plate II: Underneath surface of *Morinda lucida* leaves



Plate III: *Morinda lucida tree*



Plate IV: *Morinda lucida* stem

In a similar phytochemical analysis of aqueous extract of *M. lucida*, Omale *et al.,* (2011) found that the aqueous extract showed prominence of alkaloids and flavonoids (12.00 and 9.40 mg/g respectively). Ogunlana *et al.,* (2008) have found *M. lucida* stem bark extract to achieve a maximum antioxidant activity of 6 % within 40 minutes. Phytochemical screening of methanol extract of *M. lucida* by Nweze (2012) indicated the presence of long chain alkanol, fatty acid, β-sitosterol/stigmasterol, aromatic/terpenoid compound. Ayinla and Oseni (2013) reported that the phytochemical screening of the methanolic leaf extract showed presence of anthraquinones, alkaloids, saponins, tannins and cardiac glycosides. Phytochemical screening of the crude methanol extract of *M. lucida* root by Umar *et al.* (2012) showed the presence of saponins, flavonoids, alkaloids and tannins. Prelimnary phytochemical screening by Addy *et al.* (2013) on the *M. lucida* leaf extract indicated the presence of saponins, anthraquinones, cardenolides, alkaloids, sterols and tannins. Ayoade *et al.* (2014) showed that the phytochemical constituents of *M. lucida* extract are carbohydrate, proteins, glycosides, flavonoids, steroids, terpenoids, saponins, tannins, alkaloids, fats and oils. The phytochemical screening of the aerial part of *Morinda lucida* revealed that it contains alkaloids, saponins, tannins, flavonoids, and terpenoids and steroids (Kikakedimau *et al.,* 2012).

In a review, Lawal *et al.* (2012) claimed that the major constituents of *M. lucida* are alkaloids, triterpenoids, anthraquinones and anthraquinols (Fig.2.2). Most of these compounds were isolated from the wood bark, stem and leaves of *M. lucida*. Adesogan (1973) isolated ten anthraquinones and anthraquinols from light petroleum extract of the stem of *M. lucida*. The anthraquinones isolated were damnacanthal (**1**), alizarin-l-methyl ether (**2**), rubiadin-l-methyl ether (**3**), soranjidol (**4**), oruwal (9,10-dimethoxyanthracene-2-aldehyde) (**5**), oruwalol (**6**) 1-hydroxy-2-methyl-anthraquinone (**7**), 1-methoxy-2-methylanthraquinones (**8**), rubiadin (**9**),

nordamnacanthal (**10**), and munjistin (**11**). Adesogan (1973) proposed that oruwalol ($C_{17}H_{14}O_4$) is either 5-hydroxyoruwal (**6a**) or 8-hydroxyoruwal (**6b**). Other compounds isolated from *M. lucida* include digitolutein (**12**), ursolic acid (**13**), oleanolic acid (**14**), lucidin (**15**), oruwacin (**16**), an iridiod ferulate (Adesogan, 1979; Stephens *et al.*, 2008), morindone (1, 5, 6-trihyhdroxy-2-methylanthraquinone) (**17**). Adesogan (1979) isolated pseudopurpurin (**18**). Demagos *et al.*, (1981) isolated from the stem heartwood of *M. lucida* four anthraquinones, namely 3-hydroxyanthraquinone-2-carbaldehyde (**19),** morindone-5-methyl ether (**20**), munjistin methyl ester (**21**), damnacanthol-ω-methyl ether (**22**). In addition to the four anthraquinones, Demagos *et al.* (1981) isolated two glycosides namely soranjidiol 6-o-β-primveroside (**23**) and lucidin 3-o-β-primveroside (**24**). Dossch *et al.* (1981) isolated methyl 1, 3-dihydroxyanthraquinone-2-carboxylate (**25)** from the heartwood of *M.lucida*. Rath *et al.* (1995) isolated 2-hydroxy-3-hydroxymethylanthraquinone (**26**) from *M. lucida*. Gonza'lez *et al.* (1997) also isolated 3-hydroxy-1-methoxy-2-(methoxymethyl) anthraquinone (**27**).

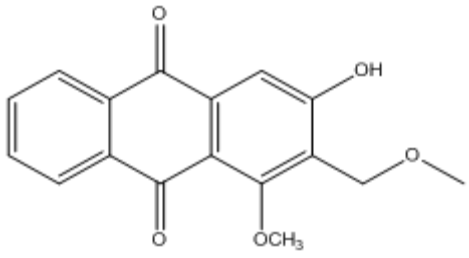
2.1.8 Ethnomedicinal uses

The leaf, stem bark, root bark and fruit-seed of *M. licida* are widely used in rural areas in West and Central Africa for the treatment of many diseases (Odutuga *et al.,* 2010). Decoctions and infusions are used in the treatment of infectious diseases such as malaria, trypanosomiasis, leprosy, dysentery and gonorrhea (Kemabonta and Okogbue, 2000; Soladoye, 2005). Among the Yorubas, the fresh leaves of the plant are often macerated in palm wine and the tincture which is bitter taken orally for the treatment of diabetic patients (Odutuga *et al*., 2010). The bark of *M. lucida* is widely used in Cameroon as a decoction

Fig. 2.2. Phytochemicals from *Morinda lucida* (Benth)

Fig 2.2. Phytochemicals from *Morinda lucida* (Benth) (contd.)

Fig. 2.2. Phytochemicals from *Morinda lucida* (Benth) (contd.)



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Fig. 2.2. Phytochemicals from *Morinda lucida* (Benth) (contd.)

for treating Diabetes mellitus (Agbor *et al*., 2012). Oliver-Bever (1986) reported the use of a weak decoction of the stem bark to treat severe jaundice. According to Burkill (1997) the stem bark, roots and leaves which are bitter and astringent are used in the treatment of fever, malaria, jaundice and dysentery.

2.1.9 Toxicity

Medicinal plants are often assumed to be efficacious and safe; however, there are some reports on poisonings consecutive to plant-based-medicine administration (Cowan, 1999; Fennel *et al*., 2004). Therefore, renewed interest is accorded to toxic effect of plant extracts. According to Afolayan and Yakubu (2009) and Oyedemi *et al.* (2010), toxicity studies in animals are commonly used to assess potential health risk in humans, caused by intrinsic adverse effects of chemical compounds or plant extracts. Many assays have been used to evaluate the toxicity of plant extracts. The main assays use animal models to better understand the *in vivo* manifestations of the plant. The results are expressed in LD_{50} (Oral mean lethal dose), the drug concentration killing 50 % of the animals.

Agbor *et al*. (2012) conducted acute and sub-acute toxicity study on mice and found that *Morinda lucida* stem bark aqueous extract is well tolerated at low dose administration but may be toxic at dose level of 5 g Kg^{-1} at sub-acute administration. James *et al.* (2011) in a study to investigate the *in vivo* antiplasmodial effect of *M. lucida* aqueous leaf extract in mice calculated body weight the oral median lethal dose (LD_{50}) to be 6400 mg Kg^{-1} (6.4 gKg^{-1}). Toxicological evaluation of ethanolic root extract of *Morinda lucida* Benth in male Wistar rats by Olunu *et al.*, (2012) revealed that the extract is unlikely to be haematotoxic, hepatotoxic and nephrotoxic if consumed repeatedly at 50, 100, 200 and 300 mg/kg body weight. The leaf extract of *M. lucida* has also been found to have reversible antispermatogenic properties (Raji, *et al.*, 2005). Another

commonly used assay to evaluate the toxicity of plants is based on the reduction of cell amount in cell culture and the result is expressed as IC_{50}, the drug concentration killing 50 % of the cells. Ajaiyeoba *et al.* (2006) screened some Nigerian antimalarial plants for *in vitro* cytotoxicity test using brine shrimp IC_{50} of 2.6, 389.9 and 9368 μgml^{-1} for *Morinda lucida* bark, *Morinda lucida* leaves and *Nauclea latifolia* bark respectively, versus 449.1 $\mu g\ ml^{-1}$ for chloroquine phosphate and found that *M. lucida* was less toxic than chloroquine phosphate and *N. latifolia*. Akintowa *et al*. (2009) using Allium cepa test demonstrated that *M. lucida* at high concentrations exhibited mitostatic effect. According to Karou *et al.* (2011) the results of Ames test showed alteration of at least three biochemical characteristics of the normal organisms, thus demonstrating mutagenicity.

2.1.10 Antimicrobial activities of *M. lucida* (Benth)

The extracts of *M. lucida* were tested for antibacterial and antifungal activity using the agar diffusion assay or the broth microdilution assay, or using both the agar diffusion assay and broth microdilution assay. In agar diffusion assay, the antimicrobial activity is expressed as inhibition zone diameter around the disk or the well. In microdilution assay, the antimicrobial activity is expressed in terms of drug concentration killing microorganism. The lowest concentration showing no visible growth in the broth medium is recorded as the Minimum Inhibitiory Concentration (MIC) and the lowest concentration killing 99.9 % of the microbial inocular is taken as the Minimum bacteridal concentration (MBC) if the microbe is bacteria or minimum fungucidal concentration (MFC) if the microbe is a fungus. Using the agar diffusion assay, Adomi (2008) screened the aqueous extract of *Morinda lucida* with 100 mg/ml extract solution and obtained zone diameter ranging from 14 to 25 mm with gram positive and gram negative bacteria. Ogundare and Onifade (2009) by *in vitro* experiment using the agar well

diffusion method investigated the inhibitory activity of the leaf extract of *M. lucida* on *Escherichia coli* and found that 25 mg/ml of the extract inhibited *E. coli* growth with zone of inhibition measuring 5 mm. In *in vivo* experiment conducted by Ogundare and Onifade (2009) by feeding albino rats with the leaf extract of *M. lucida* infected with *E. coli*, they found that the extract is able to prevent the establishment of *E. coli* infection in the rats. According Ogundare and Onifade the *in vivo* results imply that the extract can be used in the treatment of the symptoms of infections caused by enteropathogenic *E. coli.* Umeh *et al.* (2005) investigated the antibacterial activities of the plants *Mitrogyna inermis, M. lucida, Khaya senegalensis* and *Nauclea latifolia* using an indicator-based microdilution assay and found that antibacterial activity varied according to the type of plant, part of plant used, extraction solvent and the test organism. According to Umeh *et al.* (2005) the extracts of these plants in water, methanol, chloroform and petroleum ether inhibited the growth of bacteria in broth cultures but that petroleum ether and chloroform extracts of the plants have more inhibitory effect than methanolic and aqueous extracts. By *in vitro* experiment using agar well diffusion and disc diffusion methods, Fakoya *et al.* (2014) investigated possible antimicrobial activity of both aqueous and methanol extracts of the root stem bark, and leaf of *M. lucida* on Gram-negative enterobacteria *E. coli, Salmonella typhi, Salmonella paratyphi,* and *Salmonella typhorium*. They found that *M. lucida* extracts were more active against all the tested bacteria than the standard antibiotics chloramphenicol and ciprofloxacin. Fakoya *et al.* (2014) concluded that the extracts of *M. lucida* has the potentials of inhibiting the growth of *E.coli* and *Salmonella* species, thereby suggesting the potency of *M. lucida* extracts in the treatment of infections in which *E. coli* and *Salmonella* species are implicated. *M. lucida* has also been shown to possess anti-trypanosomal and antiplasmodial activities.

The protozoan genus *Trypanosoma* is the cause of the protozoan disease trypanosomiasis, a potentially fatal disease of humans and animals in tropical Africa and South America (Atawodi, 2005; Alli *et al*., 2011; Ene *et al*., 2014). Human African typanosomiasis (HAT), also known as "sleeping sickness", is caused by two protozoan subspecies of Trypanosoma; *Trypanosoma brucei gambiense* in West and Central Africa, and *Trypanosoma brucei rhodisiense* in South and East Africa. A third protozoan subspecies of *Trypanosoma, Trypanosoma brucei brucei*, is the causative agent of African Animal Trypanosomiasis (AAT). The infection is transmitted by the bite of infected tsetse fly of the genus "glossina" (Alli *et al*., 2011) Asuzu and Chineme (1990) investigated the effect of *Morinda lucida* leaf extract on *Trypanosoma brucei brucei* infection in mice and found that the extract possess trypanocidal activity. Also, Alli *et al.* (2011) investigated the antitrypanosomal activity of aqueous leaf extract of *M. lucida* using albino rats infected with *Trypanosoma brucei brucei* and found that the aqueous leaf extract of *M. lucida* possess trypanocidal activity and are of the view that *M. lucida* leaf extract could be useful as a source of new trypanocidal agent from medicinal plant. Nweze (2012) investigated anti-trypanosomal activity of methanol extract of *M. lucida* using *in vitro* cultures of *Trypanosoma brucei brucei* and found that the extract showed anti-trypanosomal activity against *Trypanosoma brucei brucei* with an MIC value of 25 µg/ml.

The protozoan disease, malaria, is caused by *Plasmodium* species, *Plasmodium falciparum, Plasmodium malariae*, *Plasmodium vivax* and *Plasmodium ovale* (Dikasso *et al.*, 2006). Malaria is transmitted to humans by the bite of *Anopheles* mosquitoes and is the single most important cause of ill-health, death and poverty in the sub-saharan Africa (Omale *et al.*, 2011; Umar *et al.*, 2012; Ayinla and Oseni, 2013; Bello *et al*., 2009; Ayoade *et al*., 2014). The works of Omale *et al*. (2011), Umar *et al*., (2012), Ayinla and Oseni (2013), Bello *et al.* (2009)

and Ayoade et *al*. (2014) confirms antimalarial activity of *M. lucida* extracts. Bello *et al.* (2009) confirmed antimalarial activity of *M. lucida* leaf extract to be MIC of 0.6 mg/ml.

2.2 The Phytochemical Screening and Antimicrobial Activities of *Uapaca togoensis* Pax

2.2.1 Scientific classification

Kingdom: Plantae

Sub-kingdom: Angiosperms

Order: Malpighiales

Family: Phyllanthaceae

Sub-family: *Antidesmatoideae*

Tribe: *Uapaceae*

Genus: *Uapaca*

Species: *Uapaca togoensis* Pax (synonyms: *Uapaca guignardii* A. Chev. ex Beille, *Uapaca Somon* Aubrév & Leandi)

2.2.2 Vernacular names

English: *Charcoal, Somon*

Igala: *Apọ*

Hausa: *Kafaffago*

Ibo: *Obia*

Yoruba: *Ajegbé*

2.2.3 The family, *Phyllanthaceae* Martynov (Malpighiales; Euphorbiaceae *sensu lato*)

Phyllanthaceae is a family of flowering plants in the eudicot order Malpighiales. It is most closely related to the family picrodendraceae. Phyllanthaceae is most notable for having two ovules in each locule of the ovary. This trait distinguishes it from Euphorbiaceae. In

addition, unlike many of the Euphorbiaceae, none of Phyllanthaceae have latex, and only a very few produce a resinous exudates. The phyllanthaceae are mostly trees, shrubs or herbs with a few climbers, or succulents, and one species, *Phyllanthus fluitans*, aquatic. The phyllanthaceae is divided into two subfamilies- antidesmatoideae and phyllanthoideae which are divided into six tribes and four tribes respectively. The six tribes of antidesmatoideae are Bischofieae, Uapaceae, Spondiathus, Scepeae, Jablonskieae and Antidemsmateae while the four tribes of the subfamily phyllanthoideae are Bridelieae, phyllantheae, Wielandieae and Poranthereae. Phyllanthaceae comprises 2000 species spread among the subfamilies, tribes, subtribes and genera (Hoffman *et al.*, 2006).

2.2.4 The genus, *Uapaca*

Uapaca is the sole genus in the tribe Uapaceae and subtribe Uapacinae of the family phyllanthaceae, a segregate of the Euphorbiaceae *Sensu lato* (Kadiri, *et al*., 2013). According to Kadiri, *et al.* (2013), the genus *Uapaca* comprises about 60 species worldwide, of which 49 are confined to African continent. Out of the 49 species in Africa, only nine are represented in West Africa. The species are *U. heudelotii* Baill, *U. togoensis* Pax, *U.staudtii* Pax, *U. paludosa* Aubrév & Léandri and *U. vanhouttei* Pax. *U. mole*, *U. acuminata*, *U. guineensis* and *U. pynaertii.* The two which are commonly used in folkloric medicine in West African subregions are *U. heudelotii* Baill and *U. togoensis* Pax (Kadiri *et al.*, 2013).

In folkloric medicine, *U. heudelotii* Baill is used for treating skin infections, female sterility, pile, rheumatism, as an emetic, mouthwash and gargle for tooth-troubles. Apart from its medicinal use, *U. heudelotii* Baill is a source of highly priced hard timber. *U. togoensis* Pax is used for treating female infertility, and as a restorative wash against fatigue. According to Koné *et al.* (2007), decoctions of leaves or stem bark of *U. togoensis* is used in the treatment of

respiratory diseases such as cough and pneumonia, fever, rheumatism, vomiting and epilepsy in Northern Cote'd'Ivoire. Besides its medicinal use, *U. togoensis* is used for making charcoal.

The genus *Uapaca* is easily recognized in damp localities by the numerous stilt roots on which the base of the tree rests but usually without stilt roots on dry sites. Though other species such as *Santri trimera* also have stilt roots, they are flattened but round in cross section in *Uapaca*. According to Kadiri *et al.* (2013), "generic constant features in *Uapaca* are the hypostomatic leaf, polygonal cell shape, straight anticlinal wall, anomocytic and paracytic stomata. Cell wall thickness varies from 1.0-3.0 µm and stomatal index ranges from 22.4 % to 34 %". According to Kadiri *et al.* (2013), "special stomatal types can be used to distinguish the species: pericytic stomatal type only in *U. heudelotii* and *U. paludosa*, tetracyclic stomata in *U. vanhouttei* and anisocytic stomata is absent in *U. staudtii* only. Conical non-glandular trichomes are present in *U. togoensis* on both surfaces but are restricted to the abaxial surface in *U. vanhouttei*. Conical glandular trichomes were recorded only in *U. togoensis* on both surfaces in *U. vanhouttei* on the abaxial surface".

2.2.5 Origin and geographic distribution

U. togoensis Pax is found in West Africa and Central Africa (Kadiri *et al.*, 2013). They occur mainly in the rainforest zones.

2.2.6 Description of *U. togoensis*

U. togoensis Pax is an evergreen tree (Plate VIII) that usually grows up to 20 metres tall, though those of 30 metres tall occur (Breteler, 2013). Delvaux *et al.* (2009) said that the heights range is from 10 to 15 m in Central Benin Republic. According to Breteler (2013) the stem (Plate IX) is around 20 cm in diameter usually with stilt roots, the stilt roots are usually absent when the tree grows in Savannah. A study of leaf (Plates V, VI, VII) epidermal morphology of the five

West African species of *Uapaca* genus has revealed that the generic constant features are polygonal cell shape, straight anticlinal wall pattern, hypostomatic leaf, paracytic and anomocytic stomata types (Kadiri *et al*., 2013). According to Kadiri *et al* the distinguishing characteristics are absence of anisocytic stomata in *U. staudtii*, presence of pericytic stomata in *U. heudelotii* and *U. paludosa* and presence of tetracyclic stomata in *U. vanhouttei*, presence of conical non-glandular trichomes in *U. togoensis* on both surfaces but which are restricted to the abaxial surface in *U. Vanhouttei*. Also, both *U. togoensis* and *U. Staudtii* contains calcium oxalate.

2.2.7 Phytochemistry of *U. togoensis*

There is paucity of data on phytochemical constituent of *U. togoensis*.

2.2.8 Ethnobotany of *U. togoensis*

This is concerned with the use of plants in the society. *U. togoensis* Pax is used both in traditional medicine and for making charcoal (Koné *et al*., 2007; Kadiri *et al*., 2013). In folkloric medicine, *U. togoensis* is used in the treatment of infectious diseases such as respiratory diseases (a disease caused by *Streptococcus pneumonia* and *Staphylococcus aureus*), malaria fever, epilepsy, vomiting and rheumatism (Koné *et al*., 2007).

2.2.9 Pharmacological/Biological investigations

The antimicrobial activities of the ethanol extracts from 16 plant species including *U.* togoensis used in Brazilian folk medicine was determined against *Staphylococcus aureus, Micrococcus flavus, Bacillus cereus, B. subtilis, Salmonella enteretidis, Escherichia coli, Pseudomonas aeruginosa,Proteus mirabilis, Serratia marcescens,Mycobacterium phlei, M. smegmatis and M. fortuitum*, and the yeast *Candida albicans* and *C. krusei.* It was found that *U. togoensis* was among the bioactive component (Sant'Ana *et al.,* 2006). According to Koné, *et al.*

(2007), 90 % ethanol extract of *U. togoensis* leaves/stem bark is potent against *S. pneumonia* and *S. aureus*. Ethanol extracts from 50 plants species including *U. togoensis* used in North Côte d'Ivoire as traditional remedies for bacterial diseases were screened for *in vitro* activity against Gram negative bacteria (*E. coli* and *P. aeruginosa*) and Gram positive bacteria (*S. aureus, E. faecalis, S. pyogenes* and *B.subtilis*. *U. togoensis* was among the 10 plants with promising level of activity against the bacteria and the antibiotic resistant strains. The fruit extract from *U. togoensis* were screened for their antiproliferative activities on both sensitive and resistant cancer lines. The fruit extract of *U. togoensis* demonstrated a strong cytotoxicity on many drug-resistant and sensitive cancer cell lines (Keute and Efferth, 2015).



Plate V: Leaves and fruits of *Uapaca togoensis*



Plate VI: Underneath surface of *Uapaca togoensis* leaf



Plate VII: Upper surface of *Uapaca togoensis* leaves



Plate VIII: *Uapaca togoensis* tree



Plate IX: *Uapaca togoensis* stem

CHAPTER THREE

3.0 EXPERIMENTAL

3.1 Materials

Dry *M. lucida* leaves, *U. togoensis* stem bark, petroleum ether (60-80 ^{0}C), chloroform, ethyl acetate and methanol. Soxhlet extractor, heating mantle, Gallentkamp Thermo chiller, Rotary Vacuum evaporator, Vacuum pump, 100 ml beakers (36), Sinter funnel (porosity 3), capillary tubes, chromatographic tanks, TLC Silica gel 60 F_{254} Aluminium sheets (20x20 cm). TLC Silica gel 60H, PTLC spreader and applicator and automatic micropipette, and chromatographic coloumns. GC/MS: Agilent Gas chromatogram (6890N model) coupled to 5973N Mass Selective Detector. NMR spectrometer: Bruker Avance 400 MHz tunned to 400 MHz for ^{1}HNMR and 100 MHz for ^{13}C NMR.

All the solvents were products of Sigma Aldrich and were distilled before use. TLC precoated plates and powders were Merck Products.

3.2 Collection and Identification of Plant Material

Samples of the plants were collected from the wild at Inye, Ankpa Local Government Area of Kogi State, Nigeria, in October, 2010. The fresh leaf samples of the plants were taken to Department of Biological Sciences, Kogi State University Anyigba, Kogi State, Nigeria and were identified and later confirmed at the Herberium section, Department of Biological Sciences, Faculty of Science, Ahmadu Bello University Zaria. Specimen voucher number of *M. lucida* Benth is 1862 and that of *U. togoensis* is 965. The plant materials were collected following their traditional medicinal use. In the area, decoction of *M. lucida* shoot is drunk to treat fever, and meningitis while decoction of *U. togoensis* shoot is drunk to treat measles, fever, and chickenpox

3.3 Drying and Grinding

The plant materials were air-dried on a frame about 3 meters above the ground. After drying, the plant materials were pulverized in a clean mortar and the resulting powder was packed into a dry, clean plastic container with screw cap for subsequent works.

3.4 Extraction

Plant material (1Kg) was weighed into clean cotton bag and extracted in Soxhlet apparatus with the four selective solvents in the order petroleum ether (60-80 ^{0}C)/n-hexane, chloroform, ethyl acetate and finally methanol. Extraction with each of the solvents was continued until the solvent was no longer extracting as evidenced by change of colour to colourless. The extract obtained by each solvent was concentrated on vacuum rotary evaporator and the concentrate was air-dried by evaporation in sample dish to a constant weight. The dried extracts were subsequently screened for class of phytochemical compounds (preliminary phytochemical screening) and antimicrobial activities and the bioactive extract was fractionated by either Vacuum liquid chromatography (VLC) or Column chromatography (CC).

3.5 Phytochemical Screening

The method of Trease and Evans (1996) was used for the preliminary phytochemical screening of the extract. Each of the extracts obtained by the four selective solvents was tested as follows:

3.5.1 Test for carbohydrate and/or glycosides

a. Molisch's test**:** 2-3 drops of Molisch's reagent (10 % ethanolic α-naphthol solution) was added to a little quantity of extract in a test tube and about 2 ml of concentrated sulphuric acid (H_2SO_4) was run down the side of the test tube to form a layer. A purple to violet color at the interface indicated presence of carbohydrates.

b. Fehlings test**:** i. To the extract (2 ml), 5 ml of Fehling's solution (1:1 solution of Fehling solutions A and B = $CuSO_4$/tartarate) was added and boiled for a few minutes. A brick-red precipitate indicated the presence of free reducing sugar.

c. Glycosides. To a small portion of the extract, 5 ml of dilute sulphuric acid was added and boiled waterbath for 10 minutes. This was cooled and neutralized with 20 % potassium hydroxide solution and the solution was divided into two portions:

i. To the first portion, Fehling solution (5 ml) was added and boiled. A brick red precipitate indicated presence of sugar from hydrolysis of glycoside.
ii. To the second portion, about 3 ml of ferric chloride solution was added. A green to blue indicated the presence of phenolic aglycone from hydrolysis of glycosides.

3.5.2 Test for tannins

a. Ferric chloride ($FeCl_3$) test: The extract (0.1 g) was dissolved in water and filtered. To the filtrate few drops of ferric chloride was added. Formation of a blue-black precipitate indicated hydrolysable tannin and green precipitate indicated the presence of condensed tannin (non-hydrolysable tannin).

b. Lead sub-acetate test: Lead sub-acetate [$(CH_3CO^-_2)_2Pb^+_2.2Pb(OH)_2$] solution (3 drops) was added to the solution of the extract. A coloured precipitate indicated tannins.

c. Bromine water test: To a small portion of the extract few drops of bromine water were added. A buff color indicated condensed tannins and a no color change indicated hydrolysable tannins.

3.5.3 Test for flavonoids: a. Shinoda test: The extract (0.5 g) was dissolved in about 2 ml of 50 % methanol and boiled. Metallic magnesium and 5 drops of conc. HCl were added; a red or orange colour indicated the presence of flavonoid aglycone.

b. Sodium hydroxide test: Few drops of aqueous sodium hydroxide solution were added to 5 ml of extract. A yellow colouration showed the presence of flavonoid.

c. Ferric chloride test: To a small portion of the extract, few drops of ferric chloride solution were added. A greenish precipitate indicated presence of phenolic nucleus.

3.5.4 Test for Saponins

i. Frothing test: The extract (0.1 g) was dissolved in 10 ml of distilled water. This was shaken vigorously for 30 seconds and allowed to stand for 30 minutes. A honey comb was formed for more than 30 minutes and this indicated presence of saponin.

ii. .Haemolysis test: The extract (0.1 g) was dissolved in 5 ml distilled water and filtered. 2 ml of 1.8 % aqueous sodium chloride solution was filled into a test tube and 2 ml of distilled water was filled into the second test tube. To each of these test tubes with its content was added 2 ml of the extract filtrate. In addition, animal blood (5 drops) was added to each test tube with the aid of a stringe. The content of test tube was mixed by inverting the test tube. Then, the test tube was observed for 15 minutes for haemolysis (settling down of red blood cells) in the tube containing the extract filtrate and its absence in the tube containing distilled water. Haemolysis in the test tube containing filtrate and its absence in the test tube containing distilled water indicates presence of saponins.

3.5.5 Test for cardiac glycoside

a. Salkowsky test: The extract (0.5 g) was dissolved in 2 ml of chloroform and 5 drops of concentrated H_2SO_4 were added to form a lower layer. A reddish-brown colour at interface indicated the presence of steroidal ring.

b. Kella-Killiani Test: Extract was dissolved in glacial acetic acid containing traces of ferric chloride. With the test tube held at an angle of 45^0, 1 ml of concentrated sulphuric acid (H_2SO_4) was added down the side of the test tube to form a lower layer at the bottom. Purple ring at the interface indicated presence of desoxy sugars and a plane green in the upper acetic acid layer indicated the presence of cardiac glycosides.

c. Kedde's test: To the extract (0.1 g) was added 1ml of 3,5-dinitrobenzoic acid in 95 % ethanol solution. The solution was made alkaline with 5 % sodium hydroxide solution. Appearance of purple-blue color indicates the presence of cardenolides.

3.5.6 Test for anthraquinones

a. Borntrager's test: The extract (0.1 g) was shaken with 10 ml of chloroform and filtered. 5 ml of 10 % ammonia solution was added to the filterate and stirred. The production of a pink red or violet colour indicated the presence of free anthraquinones.

b. Modified Borntrager's test: The extract (0.1 g) in a test tube was boiled with 10 % hydrochloric acid (5 ml) for 3 minutes. (This would hydrolyse the glycosides to yield aglycones, which are soluble in hot water). This was filtered while hot and the filtrate was cooled and extracted with 5 ml of benzene. The benzene layer was collected and shaken gently in a test tube with half its volume of 10 % ammonia solution. A rose pink to cherry red of the lower ammonia layer indicated presence of anthraquinone derivatives.

3.5.7 Test for steroids and triterpenes (Lieberman Burchards Test)

Equal volume of acetic anhydride was added to the extract in a test tube. To this 1 ml of concentrated sulphuric acid was added down the side of the test tube. The colour change was observed immediately and later. Red, pink or purple colour change indicated the presence of triterpenes while blue or blue green indicated the presence of steroids.

3.5.8 Test for alkaloids

a. Dragendoff's test (solution of potassium bismuth iodine). The Dragendoff's reagent (3 drops) was added to the extract (0.1 g). Formation of red precipitate indicated the presence of alkaloids.

b. Wagner's test (Iodine in potassium iodide): The Wagner's reagent (3 drops) were added to small amount of the extract. Formation of whitish precipitate indicates alkaloids.

c. Mayer's test (potassium mercuric iodide): Mayer's reagent (3 drops) was added to a sample of the extract (0.1 g) in a test tube. Formation of cream precipitate indicated alkaloid.

3.6 Antimicrobial activities screening

The antimicrobial activities tests were carried out using Kirbybauer method (Cheesbrough, 2006) with *M. lucida* leaf and *U.togoensis* stem bark extracts in petroleum ether (60-80 ^{0}C)/n-hexane, chloroform, ethyl acetate and methanol against some pathogenic microorganisms and some resistant strains. The microbes were obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria. All the isolates were checked for purity and were maintained in slants of nutrients agar for the bacteria and slants of sabouraud dextrose agar for the fungi. Well diffusion method was used to determine the antimicrobial activities of the extracts from the plants. 0.1 g of each extracts was weighed and dissolved in 10 ml of the solvent of extraction to obtain a concentration of 10 mg/ml of each extract. This was the intial concentration used to check the antimicrobial activities of the extracts.

3.6.1 Determination of the zone of inhibition (ZI) of microbial growth

Blood and Sabouraud dextrose agars were the media used as the growth medium for the test organisms and each media was prepared according to the manufacturer's instruction; dissolved and were sterilized by autoclaving at 121 ^{0}C for 15 mins. The media were allowed to cool to 45 ^{0}C and 20 ml of the sterile media was then poured into sterile petri dishes and the

media were covered and allowed to cool and solidify. The blood agar media was then seeded with 0.1 ml standard inoculums of the bacteria; the inoculum was spread evenly over the surface of the media by the use of sterile swab. Also, the sabouraud dextrose agar was inoculated with 0.1 ml of the fungi and spread evenly with the use of sterile swabs. The plates were dried at 37 ^{0}C for 30 mins. By the use of a standard cork borer of 6 mm in diameter a well was cut at the centre of each inoculated plate and 0.1 ml of the solution of the extract was filled into each well on the inoculated media. The inoculated plates were then incubated at 37 ^{0}C for 24 hours for the bacteria and at 26- 28 ^{0}C for 48 hours for the fungi after which each plate was observed for the zones of inhibition of growth. The zones were measured with a transparent ruler and the result recorded in millimeters (mm).

3.6.2 Determination of minimum inhibition concentration (MIC)

The MIC of each extract was determined against the test organisms using broth dilution method. Nutrient and sabouraud dextrose broth was prepared according to the manufacturer's instruction. 10 ml of each of the broth was dispensed into test tubes and sterilized at 121 ^{0}C for 15 mins and then allowed to cool. Normal saline was prepared and dispensed into test tubes and the test microorganisms were then inoculated into the normal saline. Incubation was done at 37 ^{0}C for 6 hours for bacteria and 26-28 ^{0}C for fungi. Dilution of the test microorganisms in the normal saline was performed until the turbidity matched that of Mc-Farland's scale by visual comparison at the point the test microorganisms has a concentration of about $1.5x10^8$ cfu/ml obtained using Mc-Farland standard number 0.5. Two-fold serial dilution of each extract in the broth was performed to obtain the concentrations of 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml and 0.625 mg/ml. The initial concentration was obtained by dissolving 0.1 g of the extracts in 10 ml of the sterile nutrient broth. Having obtained the different concentrations of the extract in the

broth, 0.1 ml of the standard inoculums of the test microorganisms in the normal saline was then inoculated into the different concentrations of the extract in the broth. The broth was then incubated at 37 ^{0}C for 24 hours for bacteria and 26-28 ^{0}C for fungi after which the broths in test tubes were checked for turbidity (growth). The lowest concentration of the extract in the broth which showed no turbidity was recorded as the minimum inhibition concentration (MIC).

3.6.3 Determination of minimum bactericidal/fungicidal concentration (MBC/MFC) of the extracts

The MBC/MFC of the extract was carried out to determine whether the test microorganisms were killed or only their growth was inhibited. For this purpose, blood agar and sabouraud dextrose agar were prepared according to manufacturer's instruction; they were boiled and sterilized to 121 ^{0}C for 15 minutes, cooled to 45 ^{0}C and was poured into sterile petri dishes. The plates were covered and the media were allowed to cool and solidify. The contents of the MIC in the serial dilution were then sub-cultured onto the prepared media and the media were then incubated at 37 ^{0}C for 24 hours for bacteria and 26-28 ^{0}C for 48 hours for the fungi, after which the media was observed for colony growth. The MBC/MFC was the lowest concentration of the extracts without colony growth.

3.7 Fractionation of Extracts

3.7.1 Vacuum liquid chromatography (VLC) of extracts

The extract was pre-adsorbed onto celite. To do this, the extract was dissolved in the volume of the extractant just enough to dissolve it and then celite was added to saturate it. The mixture was thoroughly mixed with a stirrer. The celite preadsorbed with extract was air dried. Packing of column: the sintered funnel (250 ml, porosity 3) was used as the column. The sintered funnel was set on a Buchner flask with circular rubber at the junction to make it air leak free. Then the Buchner flask was connected to vacuum pump and vacuum was applied. While the

vacuum is applied, the sintered funnel was dry-packed with silica gel 60G (Merck) to a height of 5 cm. The silica gel was pressed at the top with a rubber bung to ensure uniform tight packing into a hard layer. A circular piece cut from a filter paper was placed on top of the silica gel to cover it. The vacuum was put off and the pure, less polar component (petroleum ether 60-80 ^{0}C) of the solvent system was poured on the filter paper and vacuum was reapplied. While the vacuum is applied, the solvent was sucked through the layer of silica gel with the solvent front moving uniformly until the silica gel was sucked dry. Then, the celite preadsorbed with extract was added to the top of the silica gel in the column. After loading the column with preadsorbed celite, another circular piece of paper cut from filter paper was placed on the preadsorbed celite to cover it and the elutant was poured directly on the filer paper disc. The elution was done by step gradient (developed once in the nonpolar component of the solvent mixture and subsequently increase the solvent polarity after each elution by adding the more polar solvent of the mixture in increasing proportion) using a solvent system comprising petroleum ether/n-hexane and ethyl acetate. 50 ml portion of the solvent system was used to develop and collect fractions. After the initial elution with pure petroleum ether, the solvent system (mixture of petroleum ether and ethyl acetate) was composed by increasing the more polar component (ethyl acetate) in steps of 1 %, 2 %, 3 etc. up to 10 % and subsequently in steps of 15 %, 20 %, 25 %, etc, up to 100 % of the volume of the solvent system used for each elution.As the solvent system was poured on top of the packing, under vacuum, it moved through the extract pre-adsorbed celite and then through the silica gel into the Buchner flask. Each fraction collected was transferred into a 100 ml beaker and the solvent left to evaporate. The Buchner flask was changed for a clean one after each fraction collection. After allowing the volume of the fractions to decrease by evaporation to about 1/50 of the initial volume, the TLC of the fractions was done

and fractions with identical chromatogram were combined to get a new set of fraction, out of which the fraction of interest was selected for the isolation of the compound of interest. Visualisation of the chromatogram was done first under UV light and then by spraying with 10 % aqueous sulphuric acid solution.

3.7.2 Thin layer chromatography (TLC)

Thin layer chromatography (TLC) was done with the commercial TLC plate, TLC silica gel $60F_{254}$ aluminium sheets. The TLC plates prior to use were activated in the oven at 150 ^{0}C. For the purpose of monitoring a 20x20 cm TLC plates were divided into small ones; 4x5 cm, 2x5 cm, 5x5 cm etc. At about 1 cm from the breadth of the TLC plate, the origin line was drawn with pencil. Samples, was (were) spotted on the origin with the aid of capillary tube(s). Where the samples to be spotted are many, they were spotted on the origin at intervals of 1cm from each other. After the spot had dried, the edge of the plate with the spot was diped into the solvent system in a closed chamber or chromatographic tank saturated with the vapour of the solvent system for development of the chromatogram. The development was stopped when the solvent front was about 1 cm from the opposite edge of the plate. Immediately after the removal of the plate from the closed chamber, the solvent front was marked with pencil. The migration of spot, R_f, was calculated by the formula.

$$R_f = \frac{Spot\ distance\ from\ the\ origin}{Solvent\ front\ distance\ from\ the\ origin}$$

The chromatographic fractions 1 to 10 from VLC were monitored with TLC using petroleum ether; ethyl acetate of ratio 9:1 for the development, fractions 11 to 18 was developed with petroleum ether; ethyl acetate 7:3 and fractions 19-29 was developed with petroleum ether: ethyl acetate 1:1. Homogeneity of a spot was checked in two different solvent systems. Developed plates were visualized by UV light (254 nm) and spraying with 10 % H_2SO_4 solution.

3.7.3 Preparative thin layer chromatography (PTLC)

PTLC require TLC grade sorbent; and Kieselgel 60G (merck) was used. Other materials are a spreader, applicator and six of 20x20 cm glass plates. The plates were washed, cleaned and dried in the oven. Six of the plates were mounted on the spreader and the spreader locked. 45 g of the sorbent, in this case Kieselgel 60G was dissolved in 90 ml of distilled water in a conical flask (500 ml) and shaken vigorously for 2 minutes. The applicator was set at the end of the spreader and the slurry was poured across the length of the applicator. The applicator was pulled by hand steadily over the six plates and the sorbent was left to air dry for about 30 minutes. After this, the plates were carefully packed into a plate holder and activated in an oven at 115 ^{0}C for 4 hours after which oven was put off and the plates allowed to cool down gradually in the oven prior to use. The sample to be separated was spotted as a line about 1 cm from an edge of the plate with the aid of capillary tubes. 50 ml of the solvent system (petroleum ether/ethyl acetate) was measured into the chromatographic tank and shaken to allow the vapour saturate the closed chamber quickly. When the sample on the plate has dried, the edge of the plate was dipped into the solvent system in the chromatographic tank and closed. Care was taken so that the sample line on the plate does not dip into or make contact with the solvent system. After developing the plate, the plate was removed from the closed chamber when the solvent front is about 1cm from the top edge of the plate in the closed chamber. After bringing out the developed plate from the closed chamber, it was allowed to air dry for 5 minutes and then viewed under the UV light (254 nm or 366 nm) where the boundaries of the band was marked out with a spatula. Then, the marked out band was scraped into a beaker and desorbed with chloroform/methanol (8:2) for 30 minutes. This was filtered and the filtrate allowed to air dry in 100 ml beaker. The homogeneity

of the spot of isolated compound was checked in different solvent systems and spray detection with 10 % H_2SO_4 solution.

3.7.4 Open column chromatography

The sorbent used was 60-200 mesh silica gel and the solvent system was n-hexane/ethyl acetate. Two types of column were used; the bigger one was 1m long with internal diameter of 5cm and smaller on was a 50 ml burrette. A small piece of cotton wool was inserted into the column to surport the sorbent and subsequently filled to the middle with n-hexane. Slurry of the silica gel in n-hexane was poured into the column containing n-hexane and allowed to settle. After the silica gel had settled, another piece of cotton wool was inserted to cover the column of silica gel. The crude extract was preadsorbed to the silica gel and loaded on the column of silica gel. Step gradient elution was used to collect the fractions. For each solvent polarity, 300-500 ml of the solvent system was used to collect fractions of 100 ml each. The smaller column was used for the purification of isolated compounds by the same procedure used in the fractionationation of the crude. In this case 100 ml of solvent system was used for elution to collect fractions of 20 ml each.

CHAPTER FOUR

4.0 RESULTS

4.1 Phytochemical Screening and Antimicrobial Activities of *M. lucida* Leaf Extract.

4.1.1 Extraction of *M. lucida* leaves

The extract yields from the extraction of *M. lucida* leaves are presented in Table 4.1. The petroleum ether (PET) extract was 60.5 g, chloroform (CF) extract was 25.0 g, ethyl acetate (ETAC) extract was 8.2 g and methanol extract was 27.8 g. The extraction solvents have different polarity and the extraction of the plant metabolite is based on the principle of "like dissolves like". Thus, the extraction with solvents of different polarity is based on their respective selectivity for substances of similar polarity. Hence, nonpolar solvents (e.g. n-hexane or petroleum ether) are used to solubilize and extract mostly lipohilic compounds such as alkane, fatty acids, pigments, waxes, sterols, some terpenoids, alkaloids, and coumarins (Cowan, 1999; Seidel, 2006). The medium-polarity solvents (e.g. chloroform, ethyl acetate) are used to extract compounds of intermediate polarity such as some terpenoids, alkaloids, and phenolics, while the more polar solvents (e.g. methanol, ethanol, acetone) are used to extract the more polar compounds such as flavonoid glycosides, tannins and some alkaloids (Cowan, 1999; Seidel, 2006). A selective extraction is usually performed sequentially using selective solvents in order of increasing polarity. This allows a preliminary separation, and hence fractionation, of the metabolites present in the plant material within distinct extracts and this simplifies the complex mixture of the plant metabolites for the isolation of the constituent compounds (Seidel, 2006). The polarity of the four selective solvents used is in the order: petroleum ether < chloroform < ethyl acetate < methanol. Petroleum ether which was used first is nonpolar and extracts lipophilic compounds. Petroleum ether was followed by chloroform which was follwed by ethyl acetate.

Both chloroform and ethyl acetate are of medium polarity and they both extract metabolites of medium polarity. That these two solvents extracts essentially the same thing. Methanol was used last and extracts polar metabolites. As extraction is based on the selectivity of the solvents for the metabolites, the distribution of the extract recovery yields of the four solvents must be a reflection of the polarity distribution of the plant metabolites. The extract yield of petroleum ether is approximately 50 % of the entire extracts and that of methanol is 22.9 %. The extract yield from both chloroform and ethyl acetate solvent constitute approximately 27.1 % of the entire extract. This implies that about one-half of the entire extract is composed of lipophilic compounds. In otherwords, the *M. lucida* metabolites are composed mainly of lipophilic compounds. Though both chloroform and ethyl acetae are of medium polarity, chloroform is of lower polarity than ethyl acetate and it was used to extract before ethyl acetate. As both solvents extract essentially the same thing, the chloroform that was used first extracted almost all that ethyl acetate could have extracted leaving the ethyl acetate to extract only those metabolites whose polarities are beyond that which can be extracted by chloroform. The extract yield of either chloroform or ethyl actate depends on the polarity distribution of the metabolite molecules in the medium polarity range. Where most of the molecules have polarities within the range that can be extracted by chloroform, the chloroform extract yield would be larger than that of ethyl acetate but where most of the molecules have polarities outside the range that can be extracted by chloroform the ethyl acetate extract yield would be greater than that of chloroform. In this case where the chloroform extract is more than that of ethyl acetate most of the metabolite molecules have polarities within the range that chloroform can extract. This is what is observed.

Table 4.1: Recovery yields from extraction of *M. lucida* leaves using different solvents.

Solvent	**Mass of extract (g)**	**% recovery yield**
Petroleum ether (60-80^{0}C)	60.5	6.05
Chloroform	25.0	2.50
Ethylacetate	8.2	0.82
Methanol	27.8	2.78

4.1.2 Preliminary phytochemical screening of *M. lucida* leaf extracts

The results are presented in Table 4.2.The phytoconstituents of both petroleum ether and chloroform extracts were steroids and triterpenes, of ethyl acetate extract were carbohydrates, flavonoids, cardiac glycosides, steroids and the methanol extract were carbohydrates, flavonoids, saponins, cardiac glycosides, and steroids. This means that the *M. lucida* secondary metabolites are steroids, triterpenes, flavonoids, saponins, and cardiac glycosides. The secondary metabolites viz tannins, anthraquinones and alkaloids were absent.

The biological functions of flavonoids include protection against allergies, inflammatory disorders, platelet stickiness and hence platelet aggregation, diarrhea, microbes, ulcers, hepatotoxins, viruses, and tumors (Okwu and Iroabuchi, 2009; Kumar *et al.*, 2010). Flavonoids have antioxidant, antipyretic (fever-reducing), analgesic (pain-relieving), and spasmolytic (spasm-inhibiting) activities (Krishnaiah *et al.,* 2009). Flavonoids are used in treatment of diarrhea. It has been shown that the flavonoids epicatechin, quercetin and luteolin inhibit the development of fluids that results in diarrhea (Krishnaiah *et al.,* 2009). Flavonoids are polyphenolic compounds possessing 15 carbon atoms that form a carbon skeleton of two rings joined by a chain of three carbon atoms. Plants used to accelerate labour in Southern Nigeria were found to contain high level of flavonoids and phenolic compounds. The ability of the plants to accelerate labor may be due to their high content of flavonoids and phenolic compounds (Okwu *et al.,* 2009). Phenolic compounds have the ability to block specific enzymes that causes inflammatory disorders. They also modify the prostaglandin pathways, and thereby protect platelets from clumping (Okwu *et al.,* 2009). Prostaglandins can act to regulate menstruation, prevent conception, and induce child birth or abortion, lower blood clothing and possibly act as decongestants (Okwu *et al.,* 2009). The presence of phenolics in plants indicates they could act

as anti-clothing agents, antioxidants, immune enhancers and hormone modulators (Okwu *et al.*, 2009). Phenolic compounds acts as stimulating agents (Kenner and Requena, 2009). For these reason herbs rich in phenlics are used as aphrodisiac. For example, *Emblica officinalis* used in Ayurveda medicine (Krishnaiah *et al.,* 2009).

Triterpenes/triterpenoids have wide spectrum of biological activities. These include antibacterial, antifungal, antiviral, cytotoxic, analgesic, and anticancer. For example, the pentacyclic triterpenoid betulinic acid has been shown to inhibit HIV (Cowan, 1999). Clinical studies have revealed that terpenoids strengthen the skin, increase the concentration of antioxidants in wounds, and restore inflamed tissues by increasing blood supply (Krishnaiah *et al.,* 2009). Consequently, terpenoids have been widely used in herbal medicine for burns, psoriasis, prevention of scar formation following surgery, recovery from an epistomy following vaginal delivery of a newborn, and treatment of external fistulas (Krishnaiah *et al.,* 2009). Also, the presence of terpenoids is the reason why plants rich in terpenoids are used as a rejuvenating agents and terpenoids has been found to be a very useful for anti-aging and overall beauty enhancement (Krishnaiah *et al.,* 2009). Terpenoids reduces insulin requirement in diabetics by between 30 to 50 % (Divit, 1986; Krishnaiah *et al.,* 2009). Also, terpenoids reduces complications associated with diabetes and lower sugar level in blood (Krishnaiah *et al.,* 2009). Terpenoid is a heart-frendly phytochemical and plants rich in terpenoids are very popular among patients with high blood pressure and diabetes. Terpenoids improve lung functions. This is the reason plants rich in terpenoids are used for respiratory treatment (Krishnaiah *et al.,* 2009). For example, the plant *Hibiscus rosa-sinensis* is used to soothe irritated tissues and the mucous membranes that line the respiratory tract, which eases hacking coughs and other respiratory ailments (Krishnaiah *et al.,* 2009).

Saponins show anti-inflammatory activity and plants rich in saponins are used in the treatment of inflammations (Kenner and Requena, 1996). Some saponins have been found to possess antimicrobial activity (Osborn, 2003). Saponin is used in medicine as gentle blood cleanser (Kenner and Requena, 1996). Saponins bind to cholesterol to form insoluble complexes excreted via the bile. This prevents cholesterol reabsorption and results in low serum cholesterol. Thus, saponins have been found to be potentially useful for the treatment of hypercholesterolemia (Malinow *et al.*, 1977; Olaleye, 2007). Saponin reduces the cholesterol in blood and blood pressure. Thus, saponins reduce the risk of cardiovascular diseases such as hypertension and are used in the treatment of hypertension (Malinow *et al.*, 1977; Krishnaial *et al*, 2009). Also, saponin is used to treat hypoglycaemia (a disorder usually associated with diabetes).

Cardiac glycosides, also referred to as cardenolides, are cardioactive. Examples of cardiac glycosides are digitoxin and digoxin from foxglove (*Digitalis purpurea*, family Scrophulariaceae) which is used from 18th cencury for the treatment of heart disease. Both saponins and cardiac glycosides are cardioactve glycosides. The active component of cardioactive glycosides (cardiac glycosides and saponins) is the steroidal aglycone. Cardioactive glycosides have their effect on the contraction of heart muscle and, in proper doses; some can be used to treat various forms of heart failure. Cardioactive glycosides can have medicinal or toxic effects. The difference between the medicinal and toxic effect of substance is a matter of dosage. Thus, foxglove and lily-of-the-valley (*Convallaria majalis*) which contain moderate level of cardioactive glycosides have beneficial medicinal effect whereas milkweed (*Asclepias*) and oleander (*Nerium oleander*) which contain toxic level of cardioactive glycosides are toxic.

Some steroids have cardiotonic activity and some are sex hormones. For example, the steroid hormones, progestogens (e.g, progesterone) and the estrogens (e.g, estradiol) are female hormones responsible for female sexual characteristics, for the maintenance of pregnancy and for the control of menstral cycle. Modified estrogens and progestogens are used in oral contraceptives. Some breast cancers are estrogen dependent. Androgens (e.g, testosterones) are male sexual hormones. Among other properties is that they have a stimulating effect on the development of muscle (anabolic effect). Anabolic steroids are used in stimulating the growth of beef cattle. The cortical steroids (e.g, cortisone) are produced by the adrenal cortex and the two main functions are regulation of mineral balance and conversion of protein to carbohydrate and its storage as glycogens. Cortical steroids have immunosuppressive activity and reduce inflammation. They are used in the treatment of rheumatoid arthritis, asthma, and in creams for reducing inflammation. The cardenolides and bufadienolides groups of steroids are important for their cardiotonic activity and they possess insecticidal and antimicrobial properties. Plants rich in steroids (e.g, *Cheoma rutidosperma*) are used as vegetable for expectant mothers or breast feeding mothers to ensure their hormonal balance, since steroidal structure could serve as starting material for the synthesis of steroid hormones (Edeoga *et al*., 2005).

Each secondary metabolite in *M. lucida* performs many biological functions. Biologically active compounds have been found to exhibit biological activities which are related to those of the parent plant. Thus, the extract of *M. lucida* can perform any biological function known for its constituent secondary metabolite.

Table 4.2: Phytochemical Constituents of *M. lucida* leaf extract

Test	PET	CF	ETAC	MET
1. Carbohydrate/glycoside				
a. Molisch test	-	+	+	+
b. Fehlings test	-	+	+	+
2. Tannins				
a. Ferric chloride test	-	-	-	-
b. Lead sub-acetate test	-	-	-	-
3. Flavonoids				
a. Shinoda test	-	-	+	+
b. NaOH test	-	-	+	+
4. Saponins	-	-	-	+
a. Frothing test				
5. Cardiac glycoside				
a. Salkowsk test	-	-	+	+
b. Kella-Killiani test	-	-	+	+
6. Anthraquinone	-	-	-	-
7. Steroid & triterpene	+	+	+ (steroid)	+ (steroid)
8. Alkaloids				
a. Dragendoff's test	-	-	-	-
b. Wagner's test	-	-	-	-
c. Meyer's test	-	-	-	-

Key: PET = Petroleum ether ETAC = Ethyl acetate + = Present
CF = Chloroform MET = Methanol - = Absent

4.1.3 Antimicrobial activities of *M. lucida* leaf extracts

The parameters determined were the zone of inhibition (ZI), minimum inhibitiory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC).

4.1.3.1 . Zone of Inhibition (ZI) of M.lucida extracts

The results are presented in Table 4.3. A zero vaue of ZI indicates that the nmicroorganism is resistant to the extract while nonzero value of ZI indicates that the microorganism is sensitive. The extracts were found to be active against MRSA, *S. aureus, S. pyogenes, E. coli, S. typhi, S. dysenteriae, p. mirabilis, N. gonorrhea, C. albicans C. tropicalis.* The ZI of petroleum extract ranged from 22 to 25 mm for the bacteria and is 24 mm for the fungi; the ZI of chloroform extract 24 to 27 mm for bacteria and is 24 to 25 mm for fungi; the ZI of ethyl acetate 22 to 26 mm for bacteria and is 21 to 22 mm for fungi; and the ZI of the methanol extract varied from 22 to 26 mm for bacteria and varied from 22 to 23 mm for fungi. The ZI of erythromycin (antibacterial) varied from 27 to 40 mm and the ZI of fluconazole (antifungal) varied from 35 to 38 mm. The ZI range for all the extracts are about the same and are lower than those for erythromycin and fluconazole. This means the extracts have equal activity and are not as active as the positive controls. However, their activities are of the same pattern as the positive controls implying that they can serve substitutes of the positive controls where they are not available.

4.1.3.2. Minimum inhibitiory concentration (MIC) and minimum bactericidal/ fungicidal concentration (MBC/MFC) of M. lucida leaf extracts

. The MIC and MBC/MFC are presented in Table 4.4. The MIC of the extracts was 10 mg/ml against sensitive bacteria and fungi except for the bacteria, *E. coli* and *S. dysenteriae* which the MIC was 5 mg/ml. The MBC/MFC of the extracts was 20 mg/ml against the sensitive bacteria and fungi except for the bacteria, *P. mirabilis*, which MBC/MFC was 40 mg/ml or the fungi, *C. albicans* and *C. tropicalis*, which the MBC/MFC was 40 mg/ml.

4.1.3.3 The Zone of inhibition of isolated compounds

The zone of inhibition was determined for the compound (AOC1) isolated from the chloroform extract and the result obtained is presented in Table 4.5. The isolated compound was active against MRSA, *S. aureus, S. pyogenes, E. coli, S. typhi, S. dysenteriae, P. mirabilis, N. gonorrhea, C. albicans* and *C. tropicalis.* The ZI varied from 20 to 24 mm for the bacteria and from 20 to 21 mm for the fungi.

4.1.3.4 The MIC and MBC/MFC of compound AOC1 isolated from chloroform extract

The MIC and MBC/MFC of the compound AOC1 isolated from chloroform extract is presented in table 4.6. The MIC was 25 mg/ml for the bacteria except *P. mirabili* for which it was 50 mg/ml and the MIC was 50 mg/ml for the fungi. The MBC/MFC of the extract was 100 mg/ml for both the bacteria and fungi.

Table 4.3: Zones of inhibition of *M. lucida* leaf extracts, erythromycin, and fluconazole, and the extractants (mm)

Test organism	PET Extract (mm)	CF extract (mm)	ETAC extract (mm)	MET extract (mm)	Erythromycin (mm)	Fluconazole (mm)	PET (mm)	CF (mm)	ETAC (mm)	MET (mm)
Methicilin Resistant Staph aureaus (MRSA)	25	26	25	26	35	0	0	0	0	0
Vancomycin Resistant Enterococci (VRE)	0	0	0	0	0	0	0	0	0	0
Staphylococcus aureus	22	26	24	22	40	0	0	0	0	0
Corynebacterium alcerans	0	0	0	0	0	0	0	0	0	0
Streptococcus pyogenes	24	24	24	22	34	0	0	0	0	0
Escherichia coli	25	27	23	24	30	0	0	0	0	0
Salmonella typhi	24	25	24	24	27	0	0	0	0	0
Shigella dysenteriae	25	27	26	23	32	0	0	0	0	0
Pseudomonas aeruginosa	0	0	0	0	0	0	0	0	0	0
Proteus mirabilis	22	24	22	22	35	0	0	0	0	0
Neisseria gonorrhea	25	27	26	24	37	0	0	0	0	0
Candida albicans	24	25	21	23	0	37	0	0	0	0
Candida stellatoidea	0	0	0	0	0	35	0	0	0	0
Candida krusei	0	0	0	0	0	38	0	0	0	0
Candida tropicalis	24	24	22	22	0	35	0	0	0	0

Key: PET = petroleum ether (60-80^{0}C), CF = chloroform, ETAC = ethyl acetate, MET = methanol.

Table 4.4: The MIC and MBC/MFC of *M. lucida* leaf extracts on different bacterial/fungal species

Test organism	Antimicrobial parameter (mg/ml)	PET Extract (mg/ml)	CF extract (mm/ml)	ETAC extract (mm/ml)	MET extrac (mg/ml)
Methicilin resistant staph aureus (MRSA)	MIC	10	10	10	10
	MBC	20	20	20	20
Vancomycin resistant enterococci (VRE)	MIC	-	-	-	-
	MBC	-	-	-	-
Staphylococcus aureus	MIC	10	10	10	10
	MBC	20	20	20	40
Corynebacterium ulcerans	MIC	-	-	-	-
	MBC	-	-	-	-
Streptococcus pyogenes	MIC	10	10	10	10
	MBC	20	20	20	40
Escherichia coli	MIC	10	05	10	10
	MBC	20	20	20	20
Salmonella typhi	MIC	10	10	10	10
	MBC	20	20	20	20
Shigella dysenteriae	MIC	05	05	05	10
	MBC	20	20	20	20
Pseudomonas aeruginosa	MIC	-	-	-	-
	MBC	-	-	-	-
Proteus mirabilis	MIC	10	10	10	10
	MBC	20	20	40	40
Neisseria gonorrhea	MIC	05	05	10	10
	MBC	20	20	20	20
Candida albicans	MIC	10	10	20	10
	MFC	20	20	40	20
Candida stellatoidea	MIC	-	-	-	-
	MFC	-	-	-	-
Candid krusei	MIC	-	-	-	-
	MFC	-	-	-	-
Candida tropicalis	MIC	10	10	10	10
	MFC	20	20	40	40

Key: PET= Petroleum ether, CF = Chloroform, ETAC = Ethy acetate, MET = Methanol, MIC = Minimum inhibitiory concentration, MBC= Minimum bactericidal concentration, MFC = Minimum fungicidal concentration.

Table 4.5: The zone of inhibition (ZI) of compound AOC1 (mm)

Test organisms	mm
Methicilin resistant Staph aureus (MRSA)	22
Vancomycin resistant enterococci (VRE)	0
Staphylococcus aureus	22
Corynebacterium ulcerans	0
Streptococcus pyogenes	20
Escherichia coli	22
Salmonella typhi	21
Shigella dysenteriae	23
Pseudomonas aeruginosa	0
Proteus mirabilis	20
Neisseria gonorrhea	24
Candida albicans	21
Candida stellatoidea	0
Candid krusei	0
Candida tropicalis	20

Key: ZI = Zone of inhibition.

Table 4.6. The MIC and MBC/MFC of AOC1 on different bacterial/fungal species

Test organism	Antimicrobial parameter	µg/ml
Methicilin resistant staph aureus (MRSA)	MIC	25
	MBC	100
Vancomycin resistant enterococci (VRE)	MIC	-
	MBC	-
Staphylococcus aureus	MIC	25
	MBC	100
Corynebacterium ulcerans	MIC	-
	MBC	-
Streptococcus pyogenes	MIC	25
	MBC	100
Escherichia coli	MIC	25
	MBC	50
Salmonella typhi	MIC	25
	MBC	100
Shigella dysenteriae	MIC	25
	MBC	100
Pseudomonas aeruginosa	MIC	-
	MBC	-
Proteus mirabilis	MIC	50
	MBC	100
Neisseria gonorrhea	MIC	25
	MBC	50
Candida albicans	MIC	50
	MFC	100
Candida stellatoidea	MIC	-
	MFC	-
Candida krusei	MIC	-
	MFC	-
Candida tropicalis	MIC	50
	MFC	100

Key: ZI = Zone of inhibition, MIC = Minimum inhibitory concentration, MBC = Minimum bactericidal concentration, MFC = Minimum fungicidal concentration.

4.1.4 Spectral data of phytochemicals isolated from *M.lucida* leaf extract

4.1.4.1 *Isolation and characterisation of AOC1 (taraxeryl hexadecanoate)*

AOC1 was isolated from the VLC fractions 10 and 11 of chloroform extract by preparative TLC as orange solid using 7:3 mixture of petroleum ether (60-80^{o}C): ethyl acetate. The structure elucidation was by MS and 1D-NMR (^{1}H & ^{13}C). The EIMS spectrum (Fig. 4.1) showed peaks as followed: M/Z (rel. int.,%) 683.4(M^{+}), 658.3, 623.2, 593.3, 563.2, 533.2, 503.2, 459.9, 429.1, 385.1, 355.2, 312.6, 270.3(12.8), 241(3.8), 240(77), 227.3(15.4), 185.2(7.7), 143.2(20.5), 111.2(3.8), 74.1(100), 59(9.0), 57(16.7), 55(25.6).

The ^{1}H NMR spectrum (Fig. 4.2) showed peak values 0.6698, 0.7076, 0.7399, 0.8302, 0.8477, 09060, 0.9325, 0.9465, 0.9682, 0.9829, 1.0196, 1.0444, 1.1157, 1.1327, 1.3644, 1.3981, 1.4456, 1.4648, 1.4794, 1.4959, 1.5124, 1.5293, 1.5736, 1.6503, 1.6803, 1.7250, 1.8111, 1.8402, 2.0135, 2.1045, 2.1346, 2.2183, 2.2369, 2.2551, 2.1721, 2.3536, 2.3720, 2.3894, 2.7422, 2.7626, 2.7792, 3.6631, 3.6806, 3.6980, 3.7153, 4.0679, 4.0854, 4.1031, 4.1202, 4.5506, 4.5683, 4.6489, 4.6666, 5.0975, 5.3169, 5.3318, 7.2402, 8.0391.

The ^{13}C NMR spectrum (Fig. 4.3) showed peak values 13.0559, 13.1009, 13.2354, 15.3498, 17.3953, 18.0221, 18.3022, 18.5609, 18.6934, 18.7278, 18.8006, 20.0188, 20.4199, 21.5632, 21.6074, 21.6803, 21.6993, 22.4133, 23.4499, 23.7816, 24.7043, 25.3846, 26.1865, 26.8044, 26.9608, 27.2325, 28.0960, 28.1408, 28.2544, 28.3136, 28.3519, 28.4515, 28.5167, 28.5823, 28.6906, 30.9164, 31.1887, 31.6527, 31.7763, 33.3838, 33.8442, 35.4798, 35.5689, 35.6148, 35.9979, 36.2164, 36.2763, 36.3465, 36.4112, 37.1502, 38.3584, 38.7160, 38.7988, 38.8407, 41.2977, 57.4082, 59.1214, 59.7687, 75.6978, 76.0159, 76.2176, 76.3329, 116.3803, 124.0139, 129.1796, 134.1730, 142.6682, 160.0726, 172.9174.

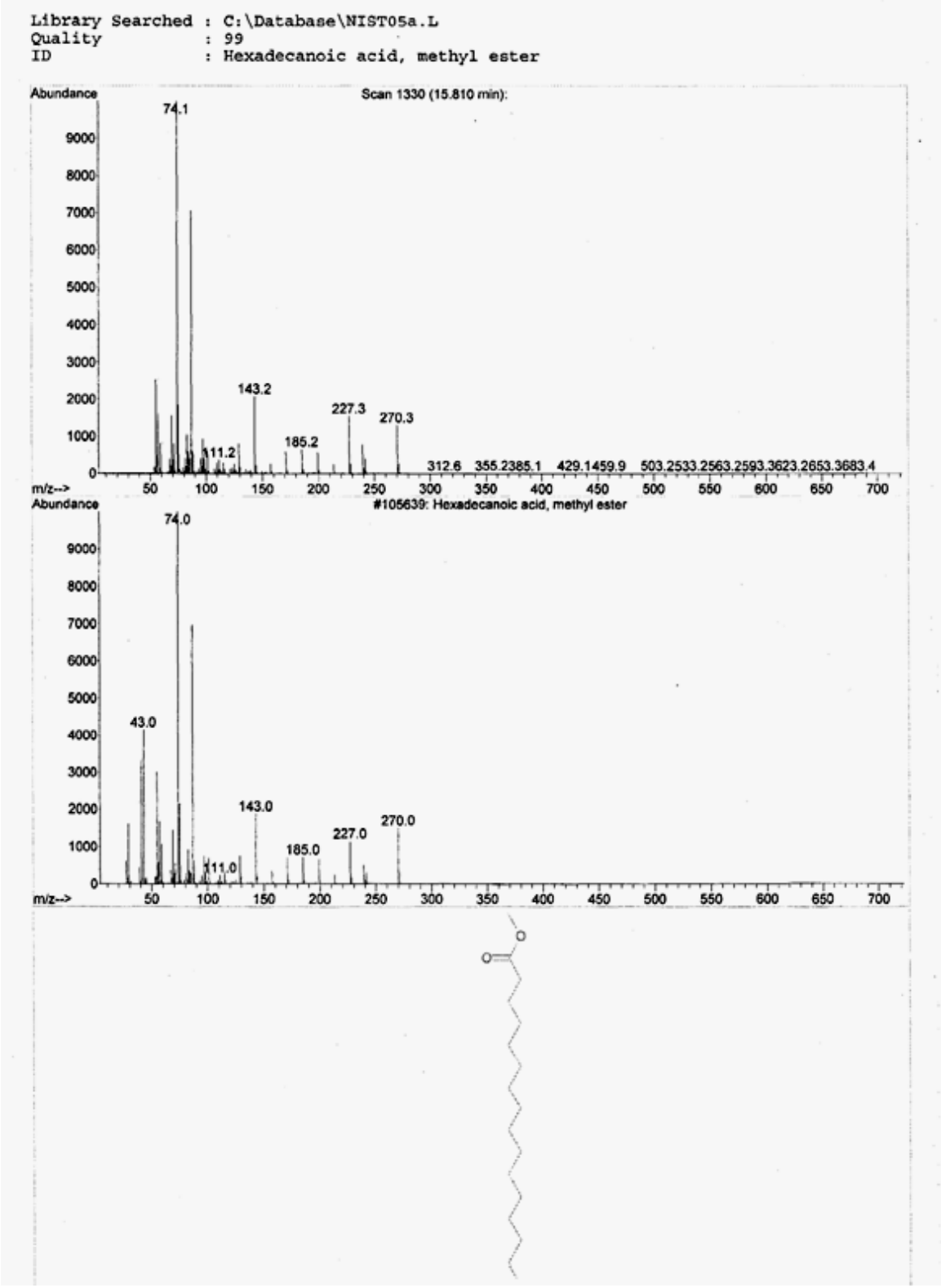


Fig. 4.1 Mass spectrum of AOC1 (taraxeryl hexadecanoate)



4.2 Phytochemical and Antimicrobial Screening of *U. togoensis* Pax Stem Bark Extracts

4.2.1 Extraction of the *U. togoensis* stem bark

The recovery yield of extracts from the extraction of *U. togoensis* stem bark is presented in Table 4.7. The dried crude extract obtained from n-hexane was 4.4076 g, ethyl acetate was 18.5046 g and methanol was 176.36 g. These extraction solvents are of different polarities. The n-hexane is nonpolar and therefore lipophilic, the ethyl acetate is medium polarity and the methanol is polar. Therefore, extraction by these solvents is based on the principle of "like dissolves like" and hence the selectivity of each solvent. On the basis of their selectivity, n-hexane extracts lipohilic compounds, methanol extracts polar compounds, and ethyl acetate extracts compounds of intermediate polarity. The methanol extract is 88.5 % of the entire extract and 17.64 % of the plant material. This means the *U. togoensis* stem is composed mainly of polar compounds.

4.2.2 Preliminary phytochemical screening of *U. togoensis*

The results of the preliminary phytochemical screening are shown in Table 4.8. The n-hexane extract contain mainly steriods and terpenes; the ethyl acetate extract contains mainly glycosides, tannins, cardiac glycosides, steriods and terpenes; the methanol extract contains carbohydrates, glycosides, tannins, flavonoids, saponins, cardiac glycosides, alkaloids, steroids and triterpenes. None of the extracts contain anthraquinone. This means the stem bark extract of *U. togoensis* contains glycosides, tannins, cardiac glycosides, flavonoids, saponins, alkaloids, steroids and triterpenes.

Tannins and flavonoids are phenolic compounds. The biological activities of flavonoids have already been enumerated. Tannins are astringent and possess antihaemorrhagic, antimicrobial and antidiarrheac properties (Krishnaiah *et al.,* 2009). Tannins aid wound-healing. According to Okwu *et al.* (2009), the abundant presence of tannins and saponins may be

responsible for the haemostatic activity of plants containing them where they arrest bleeding from damaged or injured vessels by precitating proteins to form vascular plugs.

Some alkaloids present in plants function as spasmolytic (spasm-inhibiting), anesthetic, anti-cholinergic, antipyretic, analgesic, and antimicrobial agents. For example, berberine is active against trypanosomes and plasmodia Cowan, 1999).

The phytochemical analysis has shown that *U. togoensis* stem bark extract is rich in secondary metabolites. As the biological activities of plants secondary metabolites are related to those of the parent plant, the *U. togoensis* stem bark extract must possess all the biological activities of the secondary metabolites found in it.

Table 4.7. Recovered extract yields from extraction of *U. togoensis* stem bark (1kg) using different solvents

Solvent	Mass of extract (g)	Recovery yield (%)
n – hexane	4.4076	0.44
Ethylacetate	18.5046	1.85
Methanol	176.3600	17.64

Table 4.8: Phytochemical constituents of *U. togoensis* stem bark

Test	Methanol extract	Ethyl acetate extract	n-hexane extract
1. Carbohydrate			
a. Molisch test	+	+	-
b. Fehlings test	+	-	-
2. Glycosides			
a. Fehlings test	+	+	-
b. Ferric chloride test	+	+	-
3. Tannins			
a. Ferric chloride test	+	+	-
b. Bromine water test	-	-	-
c. Lead sub-acetate test	+	+	+
4. Flavonoids			
a. Shinoda test	+	-	-
b. Sodium hydroxide test	+	-	-
c. Ferric chloride test	+	+	-
5.Saponins			
a. Frothing test	+	-	-
b. Haemolysis test	+	-	-
6. Cardiac glycoside			
a. Keller-killiani test	+	+	+
b. Kedde's test	+	+	-
7. Anthraquinones			
a. Free anthracene derivatives	-	-	-
b. Combined anthracene	-	-	-
8. Steroids and Triterpenes			
a. For steroids	+	+	+
b. For triterpenes	+	+	+
9. Alkaloids			
a. Dragendoff's test	+	-	-
b. Wagners test	+	-	-
c. Meyers test	+	-	-

Key: + = Present, - = Absent

4.2.3 Antimicrobial activities of *U. togoensis* stem bark extract

4.2.3.1 *Zone of Inhibition (*ZI*) of U. togoensis stem bark extracts*

The ZI results are presented in Table 4.9. The zero ZI value indicates the microbe is resistant while the nonzero indicates the microbe is sensitive. The MRSA, *S. aureus, C. ulcerans, E. coli, S. typhi, S. dysenteriae, P. mirabilis, N. gonorrhea, C. albicans, C. krusei and C.tropicalis* are sensitive to the extracts but VRE, *S. pyogenes, P. aeruginosa and C. stellatoidea* are resistant.

4.2.3.2 *Minimum Inhibition Concentration (MIC) and Minimum bactericidal/fungal concentration (*MBC/MFC*) of U. togoensis stem bark extracts*

The MIC and MBC/MFC of the stem bark extracts of *U. togoensis* are presented in Table 4.10. The MIC of n-hexane extract varied between 5 mg/ml and 10 mg/ml but the MBC/MFC is 20 mg/ml. In the case of ethyl acetate extract, the MIC varied between 2.5 mg/ml and the MBC/MFC varied between 5 mg/ml and 10 mg/ml. In the case of methanol extract the MIC is 5 mg/ml and the MBC/MFC varied between 10 mg/ml and 20 mg/ml.

4.2.3.3 Antimicrobial activities of compounds isolated from U. togoensis (Pax)

The zone of inhibition of compound AOUE1 isolated from U. togoensis stem bark ethyl acetate extract:

The zone of inhibition of compound AOUE1 isolated from *U. togoensis* stem bark ethyl acetate extract is presented in Table 4.11. As revealed in Table 4.11, the compound AOUE1 was found to be active against MRSA, *S. aureus, C.ulcerans, S. pyogenes, E. coli, S. dysenteriae, P. aeruginosa, N. gonorrhea, C. albicans, C. stellatoidia* and *C. tropicalis* but not active against VRE, *S. typhi, P. mirabilis,* and *C. krusei.* The ZI of AOUE1 ranged from 26 to 35 mm for bacteria and from 25 to 30 mm for fungi. The ZI of ciprofloxacin ranged from 35 to 41 mm and the ZI of the fluconazole ranged from 31 to 37 mm.

The Minimum Inhibitiory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) of AOUE1:

The MIC and MBC/MFC of AOUE1 are shown in Table 4.12. As shown in Table 4.12, the MIC of AOUE1 is 6.25 µg/ml and the MBC/MFC is 12.5 µg/ml.

Table 4.9: Zone of inhibition of *U. togoensis* stem bark extracts and the positive controls

Test organism	n-Hexane extract (mm)	ETAC extract (mm)	MET extract (mm)	Erythromycin (mm)	Fluconazole (mm)
Methicillin resistant *Staphylococcus aureaus* (MRSA)	20	27	23	35	0
Vancomycin resistant *enterococci* (VRE)	0	0	0	0	0
Staphylococcus aureaus	21	32	24	37	0
Corynebacterium ulcerans	18	25	22	40	0
Streptococcus pyogenes	0	0	0	38	0
Escherichia coli	18	24	22	39	0
Salmonella typhi	21	27	24	37	0
Shigella dysenteriae	22	30	25	40	0
Pseudomonas aeruginosa	0	0	0	0	0
Proteus mirabilis	18	23	20	35	0
Neisseria gonorrhoae	18	24	21	37	0
Candida albicans	17	22	20	0	35
Candida stellatoidea	0	0	0	0	37
Candida krusei	20	27	23	0	38
Candida tropicalis	18	24	21	0	35

Key: ETAC = Ethyl acetate. MET = Methanol

Table 4.10: The MIC and MBC/MFC of *U. togoensis* stem bark crude extracts

Test organism	Antimicrobial Parameter	n-Hexane ether extract (mg/ml)	ETAC extract (mg/ml)	MET extract (mg/ml)
Methicillin resistant staphylococcus aureaus (MRSA)	MIC	5	2.5	5
	MBC	20	10	20
Vancomycin resistant enterococci (VRE)	MIC	-	-	-
	MBC	-	-	-
Staphylococcus aureaus	MIC	5	2.5	5
	MBC	20	5	10
Corynebacterium ulcerans	MIC	10	5	5
	MBC	20	10	10
Streptococcus pyogenes	MIC	-	-	-
	MBC	-	-	-
Escherichia coli	MIC	10	5	5
	MBC	20	10	20
Salmonella typhi	MIC	5	2.5	5
	MBC	20	5	10
Shigella dysenteriae	MIC	5	2.5	5
	MBC	20	5	10
Pseudomonas aeruginosa	MIC	-	-	-
	MBC	-	-	-
Proteus mirabilis	MIC	10	5	5
	MBC	20	20	20
Neisseria gonorrhoae	MIC	10	5	5
	MBC	20	10	20
Candida albicans	MIC	10	5	5
	MFC	20	10	20
Candida stellatodea	MIC	-	-	-
	MFC	-	-	-
Candida krusei	MIC	5	2.5	5
	MFC	20	10	10
Candida tropicalis	MIC	10	5	5
	MFC	20	10	20

Key: ETAC = Ethyl acetate. MET = Methanol, MBC = Minimum bactericidal concentration, MFC = Minimum fungicidal concentration

Table 4.11: The zone of inhibition (ZI) of the compound AOUE1 isolated from ethyl acetae extract of *U. togoensis* stem bark

Test organism	AOUE1 (mm)	Ciprofloxacin (mm)	Fluconazole (mm)
Methicillin resistant *Staphylococcus aureaus* (MRSA)	29	35	0
Vancomycin resistant *Enterococci* (VRE)	0	0	0
Staphylococcus aureaus	34	37	0
Corynebacterium ulcerans	29	34	0
Streptococcus pyogenes	35	40	0
Escherichia coli	30	41	0
Salmonella typhi	0	35	0
Shigella dysenteriae	26	0	0
Pseudomonas aeruginosa	27	39	0
Proteus mirabilis	0	35	0
Neisseria gonorrhoae	27	0	0
Candida albicans	25	0	35
Candida stellatodia	30	0	34
Candida krusei	0	0	37
Candida tropicalis	26	0	31

Table 4.12: The MIC and MBC/MFC of AOUE1 on different bacterial/fungal species

Test organism	Antimicrobial parameter	AOUE1 (μg/ml)
*Methicilin resistant staph aureus (*MRSA*)*	MIC	6.25
	MBC	12.5
*Vancomycin resistant enterococci (*VRE*)*	MIC	-
	MBC	-
Staphylococcus aureus	MIC	6.25
	MBC	12.5
Corynebacterium ulcerans	MIC	6.25
	MBC	12.5
Streptococcus pyogenes	MIC	6.25
	MBC	12.5
Escherichia coli	MIC	6.25
	MBC	12.5
Salmonella typhi	MIC	-
	MBC	-
Shigella dysenteriae	MIC	6.25
	MBC	12.5
Pseudomonas aeruginosa	MIC	6.25
	MBC	12.5
Proteus mirabilis	MIC	-
	MBC	-
Neisseria gonorrhea	MIC	6.25
	MBC	12.5
Candida albicans	MIC	6.25
	MFC	12.5
Candida stellatoidea	MIC	6.25
	MFC	12.5
Candida krusei	MIC	-
	MFC	-
Candida tropicalis	MIC	6.25
	MFC	12.5

Key: MIC = Minimum inhibitory concentration, MBC = Minimum bactericidal concentration, MFC = Minimum fungicidal concentration.

4.2.4. Spectral data of phytochemicals isolated from *U. togoensis* stem bark

4.2.4.1 *Isolation and characterization of AOUE1*

Compound AOUE1 was isolated from the combined open column chromatographic fractions 25 to 38 by step gradient elution of 8.0 g of the ethyl acetate extract with 1:9 n-hexane/ethyl acetate solvent system. The isolated compound AOUE1 was further purified by washing with methanol and subsequently dried to a constant mass of 4.7727 g. The dried AOUE1 was white powder with melting point at 285-288 ^{0}C. Its structure characterization was done by 1D-NMR (^{1}H, ^{13}C and DEPT) and 2D- NMR (COSY, HMBC, HSQC, and NOESY) experiments. The ^{1}H NMR and ^{13}C NMR spectrum of the solution of AOUE1 in DMSO-d_6 was obtained at 30 ^{0}C with probe 55mmBBOz/av600.

The ^{1}H NMR spectrum (Fig.4.4) showed singlets at chemical shifts (δ) 4.64, 4.53, 4.19, 2.50, 1.61, 0.89, 0.86, 0.73, 0.60 and multiplets at 2.90, 2.30, 2.10, 1.75, 1.65, 1.40, 1.20, 1.0 ppm.

The ^{13}C NMR spectrum (Fig.4.5) showed peak values at 14.8658, 16.2277, 16.2582, 16.4104, 18.4460, 19.4325, 20.9479, 25.5844, 27.6363, 28.5760, 29.6858, 30.6097, 32.2147, 34.4179, 36.8342, 37.2161, 38.0924, 38.7570, 38.9747, 39.6085, 39.7475, 39.8867, 40.0257, 40.1649, 40.3041, 40.4433, 40.5622, 40.7525, 42.4932, 47.0984, 49.0593, 50.4310, 55.3901, 55.9079, 77.2876, 110.0679, 150.8167, 177.7000.

The DEPT spectrum (Fig.4.6) consists of six CH signals at δ 38.7570, 47.0984, 49.0593, 50.4310, 55.3901, 77.2876; eleven CH_2 signals at ô 18.4460, 20.9479, 25.5844, 27.6363, 29.6858, 30.6097, 32.2147, 34.4179, 37.2161, 38.9747, 110.0679; six CH_3 signals at δ 14.8658, 16.2277, 16.2582, 16.4104, 19.4325, 28.5760; seven C signals at ô 38.0924, 39.6085, 40.7525 42.4932, 150.8167, 177.7000.

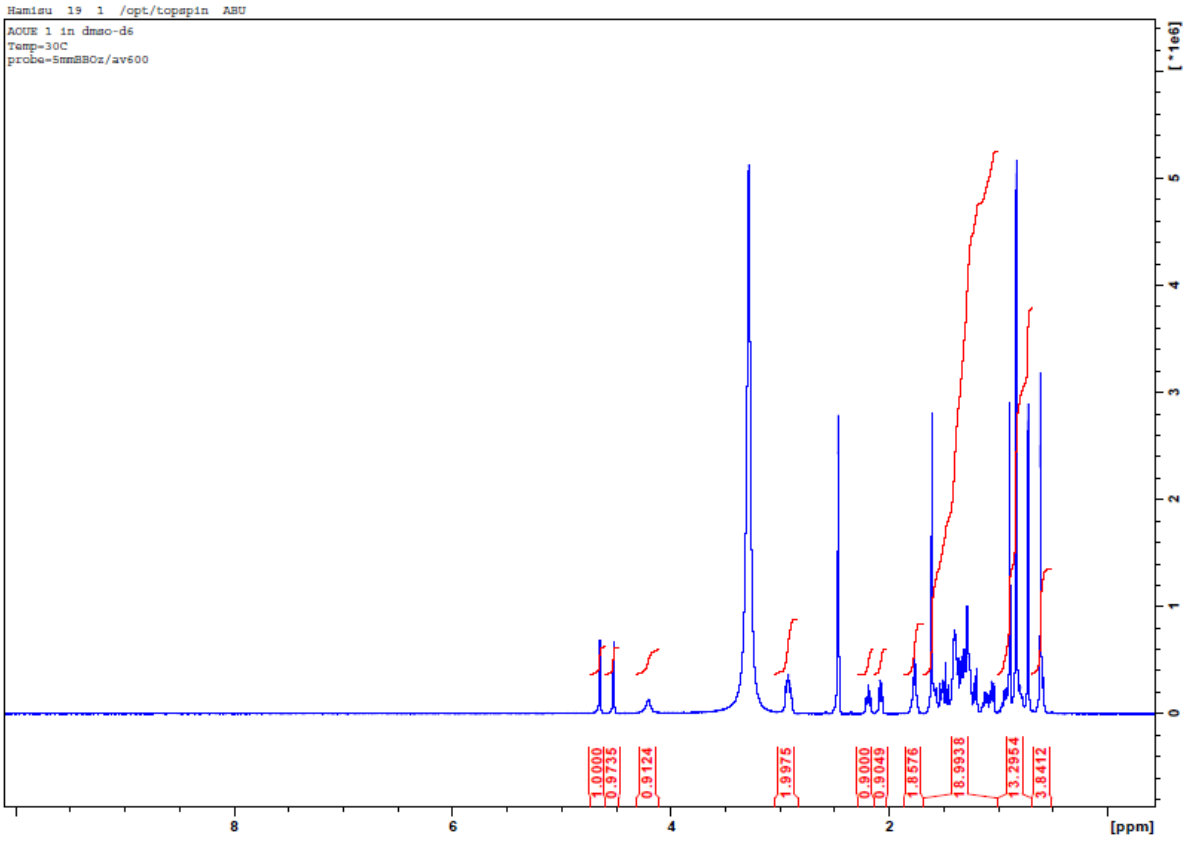


Fig. 4.4. ^{1}H NMR spectrum of AOUE1 (Betulinic acid)

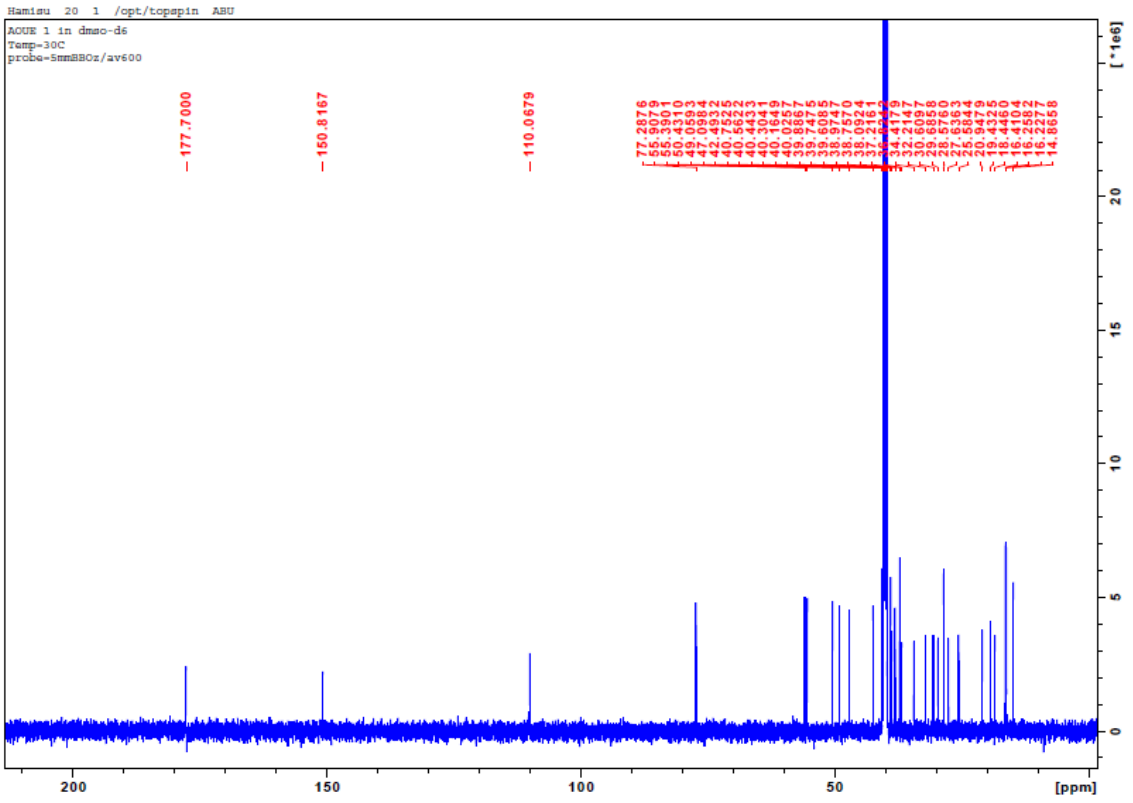


Fig.4.5 ^{13}C NMR spectrum of AOUE1 (Betulinic acid)

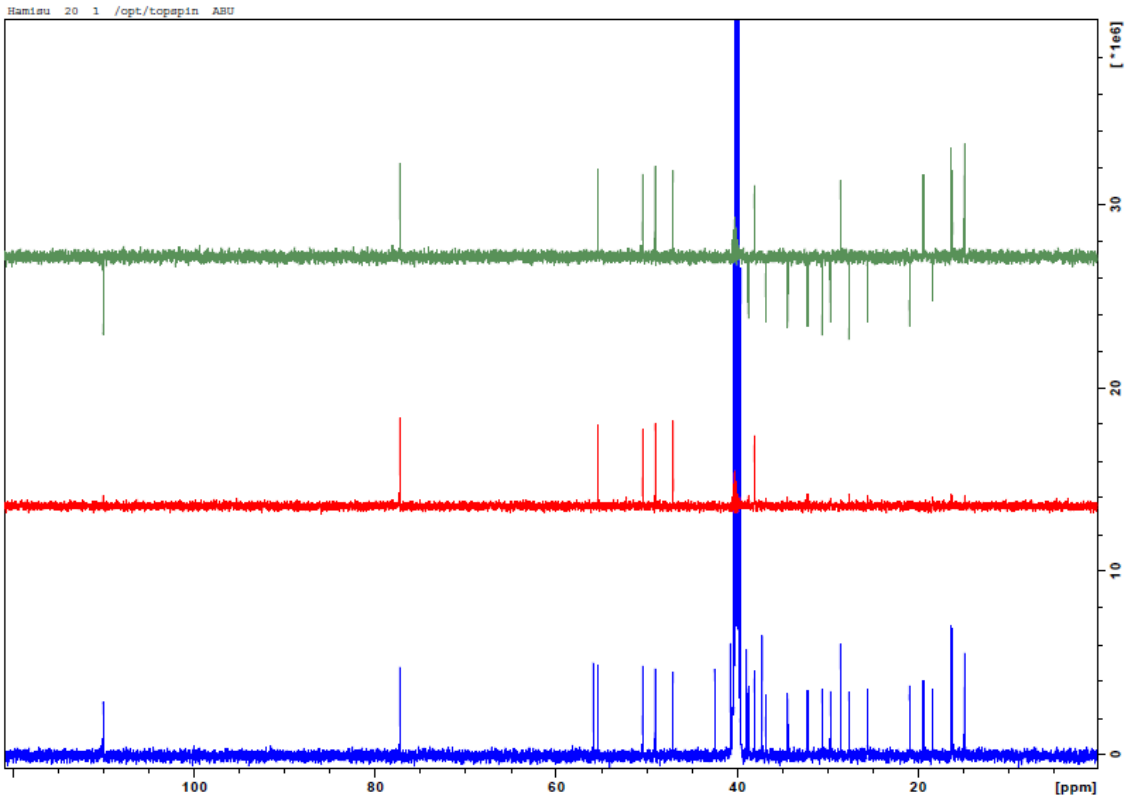


Fig. 4.6 DEPT spectra of AOUE1 (Betulinic acid)

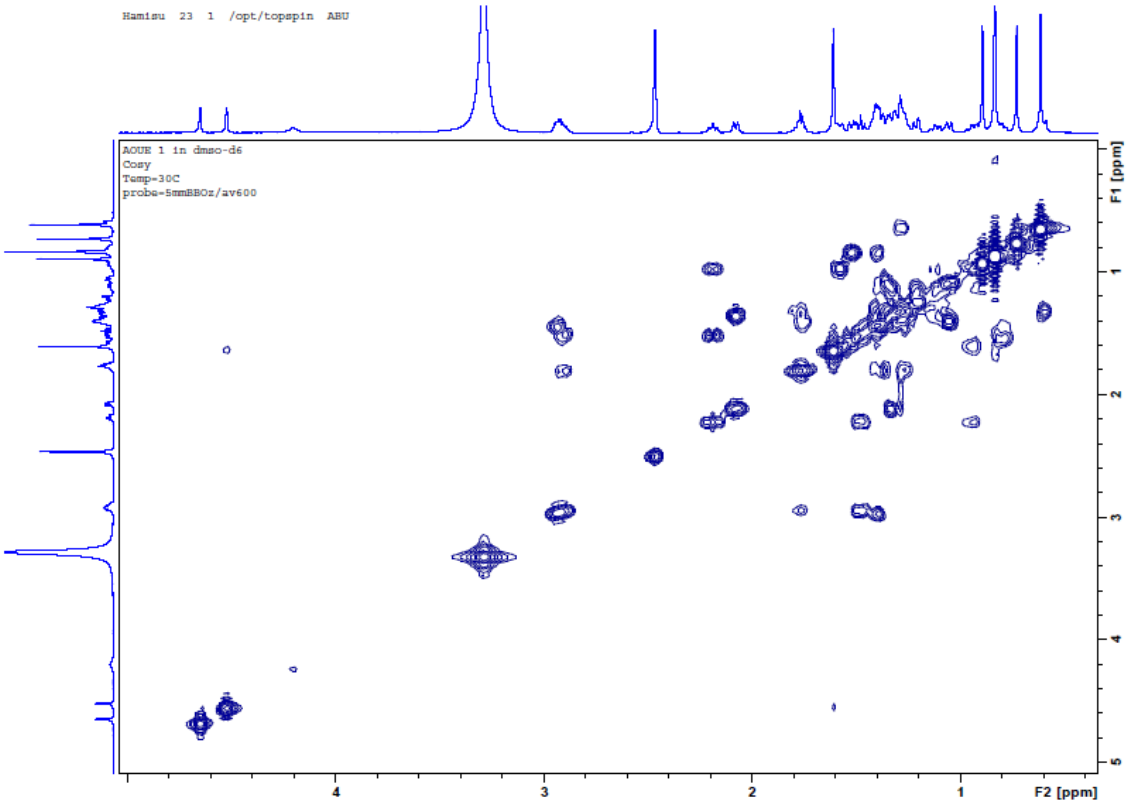


Fig. 4.7. COSY spectrum of AOUE1 (Betulinic acid)

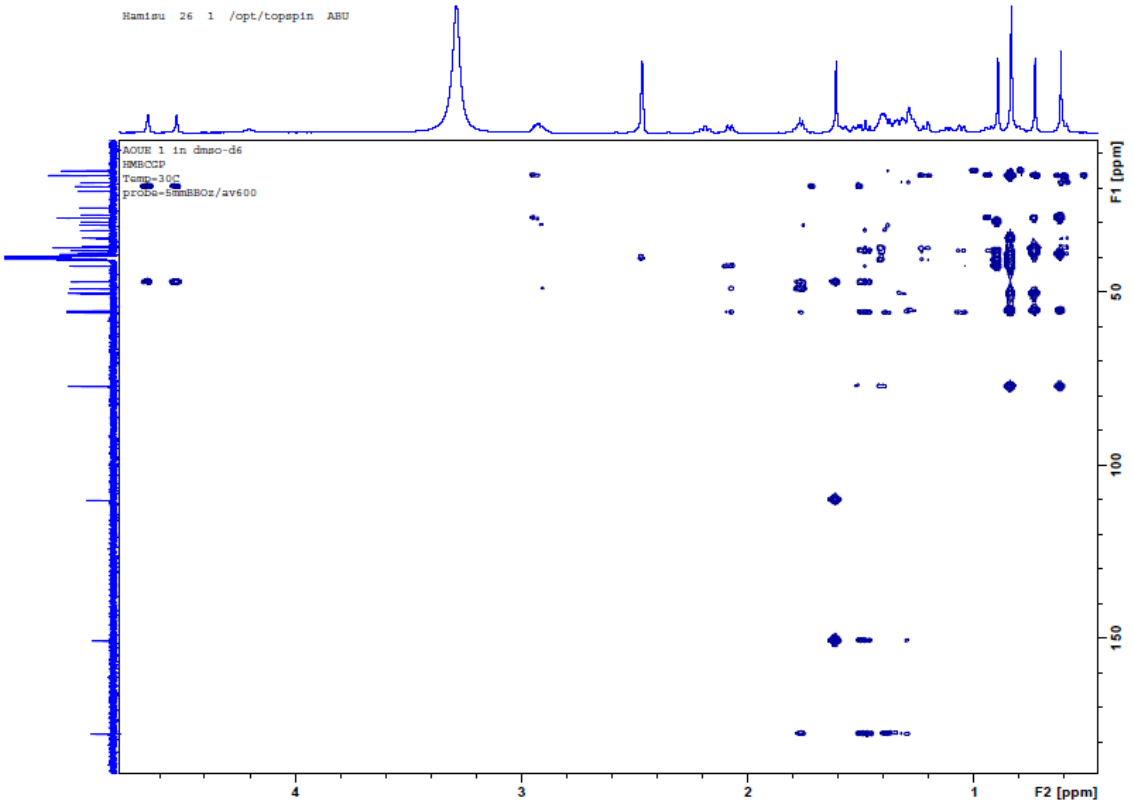


Fig. 4.8. HMBC spectrum of AOUE1 (Betulinic acid)

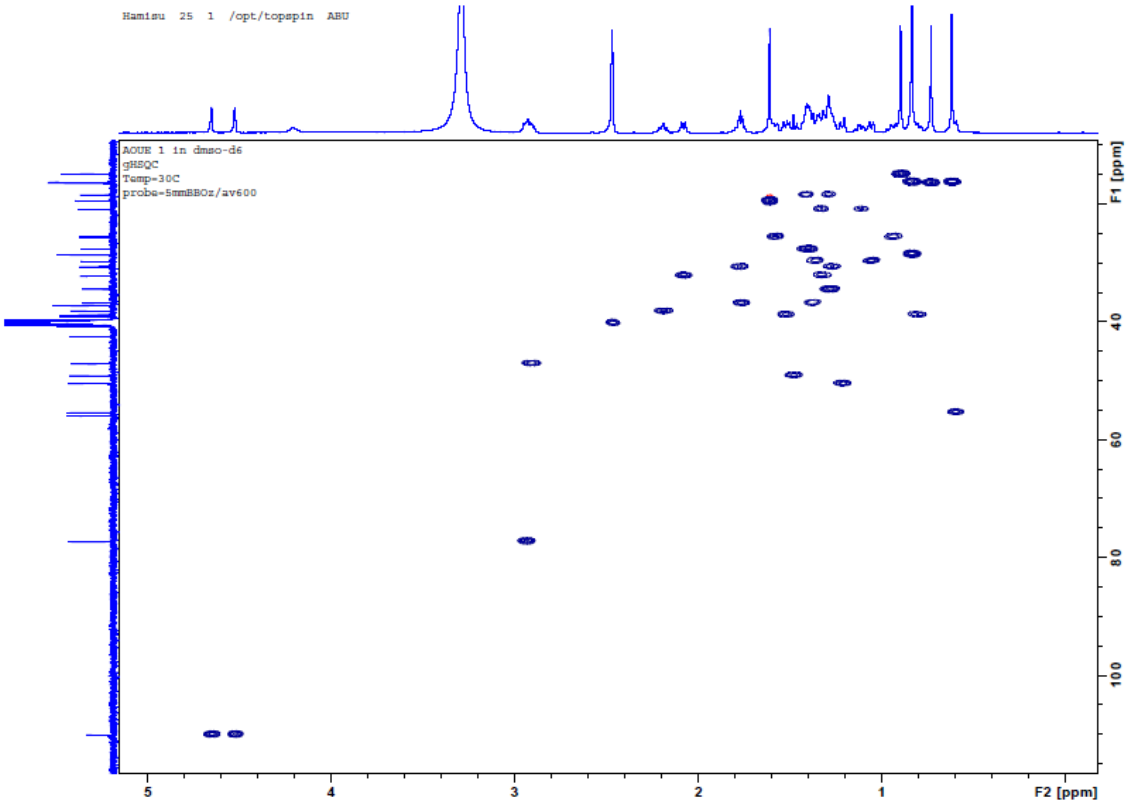


Fig. 4.9. HSQC spectrum of AOUE1 (Betulinic acid)

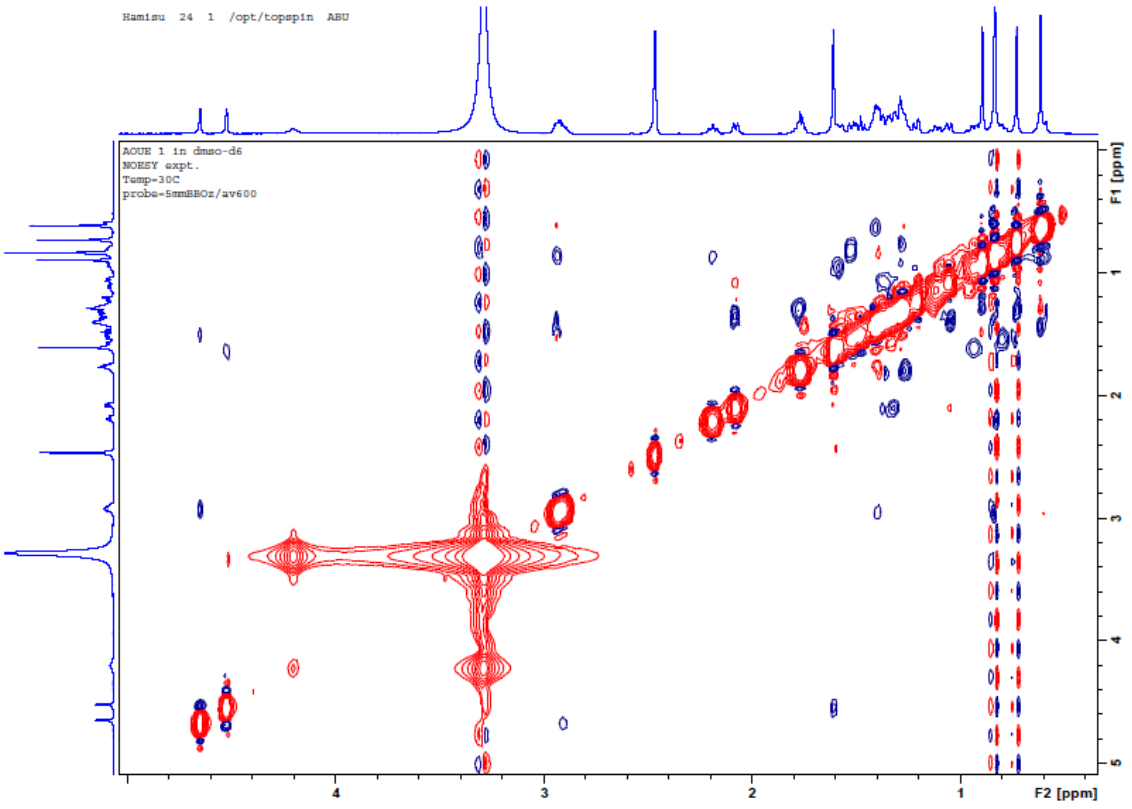


Fig. 4.10 NOESY spectrum of AOUE1 (Betulinic acid)

CHAPTER FIVE

5.0 DISCUSSION OF RESULTS

5.1 Phytochemical Screening and Antimicrobial Activities of *M. lucida* Leaf Extracts

5.1.1 Extraction of *M. lucida* leaf

The leaves collected were air-dried immediately after harvest. The drying was to prevent decay. Then the dried leaves were subsequently pulverized into powder with pestle and mortar. The shredding into power was meant to improve the subsequent extraction by rendering the leaves more homogenous and increasing the surface area for solvent extraction. 1 Kg of the powder leaves was extracted successively by four selective solvents in order of their increasing polarities. After extraction with each solvent, the extract solution was first concentrated *in vacuo* with rotary evaporator and subsequently air-dried to a constant weight. The dried crude extracts were 60.5 g by petroleum ether, 25.0 g by chloroform, 8.2 g by ethyl acetate and 27.8 g by methanol (Table 4.1). Petroleum ether is nonpolar, methanol is polar and both chloroform and ethyl acetate are of medium polarity (Seidel, 2006). Petroleum ether extract essentially nonpolar metabolites (e.g. fatty acids, sterols, and some terpenoids) while methanol extracts polar substances such as flavonoid glycosides tannins, and some alkaloids, and the medium-polarity solvent (chloroform and ethyl acetate) extracts compounds of intermediate polarity such as flavonoids and some alkaloids (Seidel, 2006). The amount of extract obtained by petroleum ether was about the same as the amount of extract obtained by the other three solvents. This means that half of *M. lucida* leaf extract is essentially lipophilic compounds (fatty substances). As chloroform and ethyl acetate are about the same polarity they are able to extract compounds of about the same polarity. Consequently, as chloroform was used before ethyl acetate it extracted almost all that ethyl acetate could have extracted. Hence, chloroform extract is more than ethyl

acetate extract. The amount of methanol extract is about a quarter of the whole extract. This means the polar component of *M. lucida* leaf extract is far smaller than the fatty component. Therefore, we conclude that *M. lucida* leaf extracts comprises mainly fatty substances.

5.1.2 Phytochemical components of *M. lucida* leaf extracts

From Table 4.2 the petroleum ether and chloroform extracts comprises steroids and triterpenes, the ethyl acetate extract comprises some carbohydrates/glycosides, flavonoids, cardiac glycosides, and steroids, and the methanol extract comprises some carbohydrates/glycosides, some flavonoids, saponins, cardiac glycosides and some steroids. Therefore, it can be concluded that the *M. lucida* leaf, extract contain triterpenes, glycosides, flavonoids, saponins, cardiac glycosides and steroids. This finding shows absence of anthraquinones and alkaloids in *M. lucida* leaf extract. The absence of anthraquinones from the leaves of *M. lucida* is in agreement with the report of Adomi and Umukoro (2010) who said that anthraquinones are present only in the stem bark and root bark. Similarly, the reports of Nweze (2012), Umar *et al.* (2012), Kikakedimau *et al.* (2012) and Ayodele *et al.* (2014) did not indicate presence of anthraquinone in the *M. lucida* extract. However, the reports of Ogunrade and Onifade (2009), Ayinla and Oseni (2013) and Addy *et al* (2013) on the phytochemical screening of the *M. lucida* leaf extract showed presence of anthraquinone. Also, many researchers have isolated anthraquinones from the heartwood and leaves (Adesogan, 1973; Adesogan, 1979; Demagos *et al.,* 1981; Dossch *et al.,* 1981; Rath *et al.,* 1995; Gonza'lez *et al.,* 1997; Stephen *et al.,* 2008). Adomi and Umukoro (2010) attributed the inconsistence in the result of the phytochemical screening of *M. lucida* extract to seasonal variation which can affect the chemical composition of the plant and its biological activities. According to Adomi and Umukoro (2010),

maximum accumulation of chemical constituents occurs at the time of flowering and then declines at the beginning of fruiting stage.

M. lucida extracts are used in ethnomedicine to treat microbial infections including malaria, trypanomiasis, leprosy, dysentery, gonorrhea, diabetes, jaundice and fever (Kembonta and Okogbue, 2000; Soladoye, 2005; Odotuga *et al.,* 2010; Agbor *et al.,* 2012). The presence of flavonoids and saponins which have antidiabetic, anti-inflammatory and antimicrobial activities justify the use of *M. lucida* extract in ethnomedicine to treat dysentery, fever, and diabetes. Terpenoids are active against bacteria, viruses, fungi and protozoa (Cowan, 1999). This justifies the use of *M. lucida* extract to treat the protozoan diseases malaria and trypanosomiasis.

5.1.3 Antimicrobial activities of *M. lucida* leaf extracts

5.1.3.1 *The zone of inhibition (ZI) of M. lucida leaf extracts*

Chemical substances which have antimicrobial activities produce zone of inhibition (ZI) of microbial growth in agar diffusion assay. The chemical substances which have no antimicrobial activities do not produce ZI in agar diffusion assay. The pathogen against which a chemical substance produce zone of inhibition is said to be sensitive or susceptible to the chemical substance; the pathogen against which a chemical produce no zone of inhibition is said to be resistant to the chemical substance. From Table 4.3, it is seen that the *M. lucida* leaf extracts produce no ZI against VRE, *Psueodomonas aeruginosa*, *corynebacterium alcerans candida stellatoida*, and *candida krusei*. This means that, VRE, *P. aeruginosa, C. alcerans, C. stellatoida,* and *C. krusei* are resistant to *M. lucida* leaf extracts. Hence, microbial infections of these microbes cannot be treated with *M. lucida* leaf extracts. Also, from Table 4.3 it is seen that MRSA, *S. aureus, S. pyogenes, E. coli, S. typhi, S. dysenteriae, P. mirabilis, N. gonorrhea, C. albicans, and C. tropicalis* are sensitive to *M. lucida* leaf extracts as the leaf extract produce ZI

against these microbes. As MRSA is sensitive to *M. lucida* extract it means that the leaf extract can be used to treat MRSA infections, an antibiotic resistant strain of *S. aureus*.

Though the *M. lucida* leaf extract is not active against *P. aeruginosa*, a gram negative bacterium but is active against the other gram negative bacteria *S. dysenteriae, S. typhi* and *E. coli*. Also, the leaf extract is active against the gram positive bacteria *S. aureus* and *S. pyogenes*. This means that antimicrobial activities of *M. lucida* leaf extract does not depend on whether the bacteria is gram positive or gram negative. *S. pyogenes* and *E. coli* are known to cause septicemia, a systemic disease caused by the spread of microorganisms and their toxin via the circulating blood (also called 'blood poisoning'). This means the herbal medicine of *M. lucida* can be used to treat septicemia. Infection of *S. aureus, S. typhi, E. coli and C. albicans* are known to cause nausea, vomiting, and diarrhea. As *M. lucida* leaf extractive is active against *S. aureus, S. typhi, E. coli,* and *C. albicans* it means the herbal medicine can be used to treat nausea, vomiting and diarrhea. *S. aureus* is known to cause abdominal pain. As the *M. lucida* leaf extract is active against *S. aureus* it means the herbal medicine can be used to treat abdominal pain. *E. coli* and *S. dynsenteriae* are known to cause gastrointestinal tract infection. As the *M. lucida* leaf extract is active against *E. coli,* and *S. dysenteriae* it means that the extracts of the leaf of *M. lucida* can be used to treat gastrointestinal tract infection. *S. aureus* is known to cause endocarditis, an inflammation of the innermost tunic of the heart. *S. pyogenes* is known to cause strep throat. As the *M. lucida* leaf extract is active against *S. aureus* and *S. pyogenes* it means that the extracts of the leaf of *M. lucida* can be used to treat endocarditis and strep throat.

From these findings, it can be concluded that the herbal medicine of *M. lucida* leaf extract can be used to treat septicemia, typhod fever, nausea, vomiting, diarrhea, abdominal pain, gastrointestinal tract infection, endocarditis and step throat. This finding justifies the traditional

use of the herbal medicine of *M. lucida* leaf extract to treat some microbial infections. Also, *M. lucida* leaf extract have activity against both bacterial and fungal pathogens with a few exceptions.

5.1.3.2. *The Minimum Inhibitiory Concentration (MIC) and Minimum bactericidal/fungicidal Concentration (MBC/MFC) of M. lucida leaf extracts*

In Table 4.4, it is seen that the MIC of the extracts is either 5 mg/ml or 10 mg/ml. The MIC of the extracts is 10 mg/ml against all the sensitive pathogens (MRSA, *S. aureus, S. pyogenes, E. coli, S. typhi, S. dysenteriae, C. tropicalis,* and *C. albicans*) except the petroleum ether extract which has MIC of 5 mg/ml against *N.gonorrhea,* chloroform which has MIC of 5 mg/ml against *E. coli, S. dysenteriae,*and *N. gonorrhea,* ethyl acetate extract which has an MIC of 5 mg/ml against *S. dysenteriae* and 20 mg/ml against *C. albicans*. As the MIC of the extracts for majority of the sensitive test microbial pathogens is 10mg/ml, it can be concluded that *M. lucida* leaf extract has an MIC of 10 mg/ml against sensitive microbial pathogens. Generally, the MBC/MFC of *M. lucida* extract is either double or quadruple of the MIC. The MBC/MFC of *M. lucida* leaf extracts is 20 mg/ml against most of the sensitive microbial pathogens exception of ethyl acetate extract which has MBC of 40 mg/ml against one bacterium viz *P, mirabilis* and the two fungi viz *C. albican* and *C. tropicalis,* and methanol extract which has MBC of 40 mg/ml one bacterium, *S. pyogenes.* As the *M. lucida* leaf extracts have both MIC and MBC/MFC for each of the sensitive microbial pathogen which is not equal it can be concluded that the *M. lucida* leaf extract could be either microbistatic or microbicidal depending on the concentration administered to an infected person.

5.1.4 Isolation and structure characterization of AOC1 (taraxeryl hexadecanoate)

The characterization here is based on structural informations from Electron impact mass spectrum (EIMS) and NMR (^{1}H, ^{13}C) of AOC1 and comparison of these data with published

data. The MS spectrum of AOC1 shows the largest mass to 683.4. The observable peaks in the spectrum are those from m/z = 55(25.6 %) to m/z = 270.3(12.8 %). The base peak appears at m/z = 74.1. The molecular ion is not known. The MS library search suggests methyl hexadecanoate, a fatty ester. Methyl esters of fatty acids have characteristic spectra. The molecular ion at m/z = 270 is clearly seen with an ion at m/z = 239 (i.e. [M-31]) representing loss of a methoxy group. In this case, the spectra does not contain an ion peak at m/z =239. Another characteristic of MS of methyl ester of fatty acids is an ion peak at m/z=227 (i.e $[M-43]^+$) which represents loss of a C_3 unit involving carbons 2 to 4 and an ion peak at m/z=74 which is the McLafferty rearrangement ion, CH_3OC $(=OH^+)$ CH_2.

The McLafferty rearrangement is a site specific rearrangement in which a hydrogen atom from carbon 4 of the aliphatic chain migrates to the carbonyl group, presumably through a six-membered ring structure transition state. According to Williams (2013), if one of the hydrogen atoms on carbon 4 is substituted, the McLafferty ion will be appreciably lower in intensity than expected. And, if both hydrogens on carbon 4 are substituted, the McLafferty ion cannot form. Also, if there is a substituent on carbon 2, the M/Z value of the McLafferty ion will be increased according to the size of the substituent. For example, substituting the two hydrogens on carbon 2 increases the mass of McLafferty rearrangement ion by 2. Furthermore, a long homologous series of related ions (14 amu apart) at m/z=87, 101,115,129, 143,159,199 etc of general formula $[CH_3OCO(CH_2)n]+$ is evidence that there it is unlikely the fatty acid chain contain other functional group. Though we do not have this long homologus series of related ions (14 amu apart) in the spectrum, the presence of ion peaks at m/z=227 and at m/z=74 suggest that there could be a long chain of fatty acid group in the compound.

In the ^{1}H NMR, there is no evidence for the presence of the –OH of an acid. Mehta *et al* ., (2006) assigned the resonance signal at δ 2.35 to the methylenes protons at α to the ester group and δ 4.18 for $–CH_2$-O-CO-. This signal appears at δ 2.3536 for α methylene group and at δ 4.11202 for $–CH_2$-O-CO- group.

The CH_3O signal should appear as singlet at δ 3.65 but what we have is a multiplet from 3.6631-3.7153 suggesting absence of CH_3O group. According to Mehta *et al.,* (2006), the rest of the methylenes resonate at δ 1.30. In this case a strong signal at δ 1.3644 is assigned to methylene signals. The β-methylene protons to the ester group appear as multiplet at δ 1.59-1.63 ppm. Thus, the multiplet at δ 1.5736-1.6803 was assigned to β-methylene protons to the ester group. The terminal CH_3 protons appear as multiplet at δ 0.85-0.88 ppm. In our case, this occurs at δ 0.8302-0.8477 ppm.

In ^{13}C NMR the methoxy carbon when present resonates at 51.29 ppm (Mehta *et al.,* 2006). This signal is absent in our ^{13}C NMR spectrum of AOC1. Therefore, the fatty acid ester is not a methyl ester of fatty acid. The methylene and methyl carbons of fatty acid moiety appear in the range from δ 13.92 to 29.7 ppm. This can be found from 13.0559-38.8407 ppm. The terminal methyl carbons resonate at δ 14.2 and this occurs at δ 13.1009 ppm. According to Mehta *et al.,* (2006), the methylene carbons α- and β- to ester group resonates at δ 34.5 and 32.00 respectively. This is found at δ 33.8442 and δ 31.7763 ppm for the α- and β-carbons respectively. The carbon of the ester group resonated at δ 172.917 ppm, the signal at δ 160.0726 ppm is due to olefinic C-14 and δ 116.3803 ppm to the second olefinic C-15 of taraxerol (Koay *et al.,* 2013). Therefore in comparison with the published data (Mehta *et al.,* 2006; Koay *et al.,* 2013) the compound AOC1 was identified as hexadecanoic acid ester (B) of taraxerol (A), that is, taraxeryl hexadecanoate ($C_{48}H_{82}O_2$).

5.1.5 Antimicrobial activities of AOC1 (taraxeryl hexadecanoate)

The parameters determined were ZI, MIC and MBC/MFC. The ZI is shown in Table 4.5 and the MIC and MBC/MFC is shown in Table 4.6.

From Table 4.5, the ZI of AOC1 ranged from 20 to 24 mm for the sensitive organisms. The compound AOC1 is not active against VRE, *P. aeruginosa, C. stellatoidia* and *C. krusei* but is active against MRSA, *S. aureus, S. pyogenes, E. coli, S. typhi, S. dysenteriae, P. mirabilis, N. gonorrhea, C. albicans* and *C. tropicalis*. The compound is active against all the microbial strains for which the extracts have activity. AOC1 has MIC of 25 mg/ml against the sensitive bacteria MRSA, *S. aureus, S. pyogenes, E. coli, S. typhi, S. dysenteriae, N. gonorrhea* and MIC of 50 μg/ml against the sensitive fungi; *C. albicans* and *C. tropicalis*. The MBC/MFC for both bacteria and fungi is 100 mg/ml. This means the compounds AOC1 have both bacteristatic/fungistatic and bactericidal/fugicidal activity. As the compound AOC1 has activity against both bacteria and fungi, it can be concluded that AOC1 is both antibacterial and antifungal.

5.2 Phytochemical Screening and Antimicrobial Activities of *U. togoensis* Stem Bark Extract

5.2.1 Extraction of *U. togoensis* Pax stem bark.

The recovery yield of the extracts by each solvent indicates the solubility and polarity particulars of the metabolites in the plant. As shown in Table 4.7, the n-hexane extracted 0.44%, the ethyl acetate extracted 1.85%, and the methanol extrcted 17.64% of the plant material. This indicates that *U. togoensis* stem bark contain a little non-polar substances but contain majorly polar substances.

5.2.2 Phytochemical components of *U. togoensis* stem bark extracts

From Table 4.8, it is seen that the n-hexane extract contains steroid and triterpenes; the ethyl acetate extract contains glycosides, cardiac glycosides, tannins, steroids and triterpenes; the methanol extracts comprises, carbohydrate, glycosides, saponins, cardiac glycosides, tannin, flavonoids, alkaloids, steroids and triterpenes. Therefore, it can be concluded that *U. togoensis* stem bark contains carbohydrates, glycosides, saponins, cardiac glycosides, tannins, flavonoids, alkaloids, steroids and triterpenes.

U. togoensis extract is ised in ethnomedicine to treat respiratory diseases, malaria, fever, epilepsy, vomiting and rheumatism (Koné *et al.*, 2007). The flavonoids, saponins and terpenoids which have anti-inflammtory, antimicrobial, and antiprotozoan activities justifies their use in ethnomedicine to treat respiratory diseases, malaria, fever, vomiting.

5.2.3 Antimicrobial activities of *U. togoensis* stem bark extracts

5.2.3.1 *The Zone of Inhibitions (ZI) of the U. togoensis extracts*

From Table 4.9, it is seen that the ZI of the extracts is zero for VRE, *S. pyogenes, P. aeruginosa* and *C. stellatoidea*. This means that these microbial pathogens are resistant to *U. togoensis* stem bark extracts. Thus, the *U. togoensis* stem bark extract cannot treat microbial

infections in which VRE, *S. pyogenes, P. aeruginosa* and *C. stellatoidea* have been implicated. The ZI have nonzero values for MRSA, *S. aureus, C. ulcerans, E. coli, S. typhi, S. dysenteriae, P. mirabilis, N. gonorrhea, C. albicans, C. krusei and C. tropicalis.* This means that these microbial pathogen are sensitive to *U. togoensis* stem bark extract. Thus, *U. togoensis* stem bark extract can be used to treat microbial infections in which these pathogens have been implicated. This justifies the ethnomedical use to treat gonorrhea and other microbial infections. As the extract is active against both bacteria and fungal pathogens, it can be concluded that *U. togoensis* stem bark extract is both antibacterial and antifungal. From Table 4.9, it is observed that the ZI of n-hexane extract ranges from 17 to 22 mm, that of ethyl acetate extract ranges from 22 to 32 mm, and that of methanol extract ranges from 20 to 25 mm. In general, the ZI of *U. togoensis* stem bark extracts ranges from 17 to 32 mm but those of the controls; erythromycin ranges 35 to 40 mm and that of fluconazole rnages from 35 to 38 mm. The concentration of the extracts used for the determination of ZI was 0.02 mg/ml ($2x10^{-2}$ g/ml) while those of the controls were 5 µg/ml ($5x10^{-6}$ g/ml or 0.0005 x 10^{-2} g/ml), a ratio of 4000:1 for extract: erythromycin or fluconazole. Despite that the concentration of the extracts is 4000 times as that of either erythromycin or fluconazole, none of the extracts produced a ZI as large as those of erythromycin or fluconazole. This means that *U. togoensis* stem bark extract is not as active as erythromycin and fluconazole. However, the extracts are active against those microbial pathogens which erythromycin or fluconazole have activity.

5.2.3.2 The minimumiInhibitiory concentration (MIC) and minimum bactericidal concentration (MMC) of the U. togoensis stem bark extracts

It is seen from Table 4.10 that the MIC of n-hexane extract is 5mg/ml for the sensitive pathogenic microorganisms MRSA, *S. aureaus, S. typhi, S. dysenteriae* and *C. krusei* but 10 mg/ml for *C. ulcerans, E. coli, P. mirabilis, N. gonorrhea, C. albicans* and *C. tropicalis*. Thus,

the MIC of n-hexane extract is either 5 mg/ml or 10mg/ml with the larger MIC, 10 mg/ml, being the one active against most of the microbes. However, the MBC/MFC of the n-hexane extract is 20 mg/ml for all the microbes.

The MIC of ethyl acetate extract varies between 2.5 μg/ml and 5 mg/ml. However, the MBC/MFC of ethyl acetate extract is not a constant but fluctuates between 10 mg/ml and 20 mg/ml. The MIC of 5 mg/ml has static effect against all the pathogenic microbes. Thus, the microbistatic concentration of ethyl acetate extract is 5 mg/ml. The MBC/MFC of ethyl acetate extract is higher than the MIC; the MBC/MFC being twice or quadruple of the MIC. As the MIC and MBC/MFC are not equal it means the ethyl acetate extract at the concentration of 5 mg/ml is not microbicidal but is microbistatic. The MIC of methanol extract is 5 mg/ml for all the sensitive microbes. With respect to MBC/MFC, the MBC/MFC of methanol extract is either 10 mg/ml, or 20 mg/ml. None of the MBC/MFC is equal to the MIC. The MBC's are larger than the MICs. This means that the methanol extract is microbistatic at MIC of 5 mg/ml.

In general, all the extracts have activity against the same set of microbes. As all the extracts have activity against the same set of microbes, it is likely that the active ingredients is present in all the extracts but to a different extent.

5.2.4 Isolation and structure characterization of AOUE1 (betulinic acid)

5.2.4.1. *The isolation of AOUE1*

4.7727 g of AOUE1 was isolated from 8.0 g of the ethyl acetate crude extract by open column chromatography. The entire ethyl acetate extract was 18.5046 g. Therefore, 18.5046 g of ethyl acetate extract would have yielded 11.0396 g representing 1.10 % of the plant material. This indicates that *U. togoensis* is rich in AOUE1.

5.2.4.2 Structure characterisation of AOUE1

The structure characterization of AOUE1 was done by ID-NMR (^{1}H, ^{13}C and DEPT) and 2D-NMR (HMBC, COSY, HSQC, NOESY) and by comparison of these NMR data with published data (Kwon *et al.*, 2003; Uddin, *et al.*, 2011; Adzu *et al.*, 2011; Prakash and Prakash, 2012). It was found that the NMR data of AOUE1 corroborates those of betulinic acid.

The ^{1}H NMR spectrum of AOUE1 showed six tertiary methyl singlets at δ 0. 60, 0.73, 0.86, 0.89, 1.61, and 2.50 ppm; two signals due to the olefinic protons of the exocyclic double bond at δ 4.53 and 4.64 ppm; one 3-H signal at δ 4.19 ppm. Careful examination of ^{13}C NMR and the DEPT spectra reveals 30 signals made up of signals due to six CH, eleven CH_2, six CH_3 and seven C. The chemical shifts(δ) of the carbons are as follows: C-1 (38.9747; 39.1*), C-2 (27.6363; 28.1) C-3 (77.2876; 78.2), C-4 (39.6085; 39.6), C-5 (55.3901; 56.2), C-6 (18.4460; 18.8), C-7(34.4179; 34.9), C-8 (40.7525; 41.2), C-9(50.4310; 51.0), C-10 (38.0924; 37.9), C-11 (20.9479; 21.3), C-12 (25.5844; 26.2), C-13 (38.7570; 38.7), C-14(42.4932; 42.9), C-15(30.6097; 31.3), C-16 (32.2147; 32.6), C-17(55.9079; 56.4), C-18(47.0984; 47.8), C-19(49.0593; 49.8), C-20(150.8167; 152.20), C-21(29.6858; 30.6), C-22(37.2161, 37.4), C-23(28.5760; 28.5), C-24(16.2277; 16.2), C-25(16.4104; 16.8), C-26(16.2582; 16.4), C-27(14.8658; 15.0), C-28 (177.7000; 178.4), C-29(110.0679; 109.4), C-30(19.4325; 19.3).

The asterisked value (*), that is, the value on the right is literature value (Prakash and Prakash, 2012). The presence of acid group in the structure was identified at C-28 by the HMBC correlations shown by structure C. Based on the ID and 2D NMR data, the structure of AOUE1 was identified as betulinic acid, that is, 3β-hydroxy-lup-20(29)-en-28-oic acid ($C_{30}H_{48}O_3$) (structure D).

5.2.5.3 Antimicrobial activities of AOUE1 (betulinic acid)

The ZI of AOUE1 is shown in Table 4.11 and the MICs and MBCs/MFCs are shown in Table 4.12. The ZI shows that the compound AOUE1 is active against MRSA, *S. aureus, C. ulcerans, S. Pyogenes, E. coli, S. dysenteriae, P. aeruginosa, N. gonorrhea, C. albicans, C. stellatoidia* and *C. tropicalis.* This means these microbial strains including the resistant strain, MRSA, are sensitive to AOUE1. Those which are not sensitive to AOUE1 are VRE*, S. typhi, P. mirabilis* and *C. krusei*. The compound AOUE1 has activity against both bacteria and fungi with a few exceptions. Thus, AOUE1 is both antibacterial and antifungal. Whereas the ZI of the crude extracts of *U. togoensis* ranges from 20 to 25 mm, the ZI of AOUE1 ranges from 26 to 35 mm indicating that AOUE1 is more active than the crude extract of *U. togoensis*. The ZI of ciprofloxacin, an antibacterial antibiotic, ranges from 35 to 41 mm and the ZI of fluconazole, an antifungal antibiotic, ranges from 31 to 37 mm. This means that AOUE1 is not as potent as ciprofloxacin and fluconazole. However, as ciprofloxacin is not active against *S. dysenteriae* and *N. gonorrhea* whereas the crude extract and AOUE1 are active, the crude extract or AOUE1 may be substituted for ciprofloxacin in an infection where these pathogens have been implicated.

C. Key HMBC Correlations of Betulinic acid

D. Betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid)

CHAPTER SIX

6.0 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

In this study, phytochemical studies and antimicrobial activities of *M. lucida* and *U. togoensis* were done. Both plants are used in traditional medicine to cure a variety of human ailments. *M. lucida* is used in the traditional medicine to treat diabetes, hypertension, malaria, gonorrhea, dysentery, trypanosomiasis and leprosy. *U. togoensis* is used in folkloric medicine to treat respiratory diseases, malaria, epilepsy, vomiting and rheumatism. The selection of these plants for study was based on their folkloric use.

6.1.1 *M. lucida* phytochemical constituents and the antimicrobial activities of the leaf extracts

Phytochemical screening and antimicrobial studies of *M. lucida* has been carried out against many pathogenic microbes but not against resistant strains of microbes. Therefore, in this study antimicrobial activities of *M. lucida* against two resistant pathogenic microbial strains namely methicilin resistant *Staphylococcus aureus*/multidrug-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE) were carried out. In addition, some pathogenic microbes including those which the antimicrobial activities of *M. lucida* has not been studied were studied. Out of the fifteen pathogenic microbes used for the study only four bacteria and one fungi has been studied with *M. lucida* extract. The bacteria already studied are *S. aureus, E. coli, P. auruginosa* and *S. typhi* and the fungi already studied is *candida albicans.* In addition to these five pathogenic microbes, antimicrobial activities of *M. lucida* extracts against *C. ulcerans, S. pyogenes, P. mirabilis, N. gonorrhea, C. stellatoidea, C. krusei, and C. tropicalis* were studied. The leaf extracts of *M. lucida* were used to study the antimicrobial activities of *M.*

lucida. Four extracts of the *M. lucida* leaves were obtained using four selective solvents by Soxhlet extraction. The solvents were used for the extraction in order of their increasing polarity starting with the one of lowest polarity, petroleum ether (60-80^{0}C), and then chloroform, ethyl acetate, and methanol. Each of these solvents extracts the plant material according to its polarity. Thus, the four solvents used in the extraction yielded four extracts. Phytochemical screening of the extracts was carried out by the method of Trease and Evans (1996). The phytochemical screening showed that *M. lucida* leaf extracts contain carbohydrates, glycosides, flavonoids, cardiac glycosides, and steroids. The petroleum extract constitute 49.79% of the entire extract indicating that *M. lucida* leaf extracts is composed mainly of fatty substances. Antimicrobial activities screening of the crude extracts shows that VRE, *C. alcerans, P. aeruginosa, C. stellatoidea* and *C. krusei* are resistant to the extracts while MRSA, *S. aureus, S. pyogenes, E. coli, S. typhi, S. dysenteriae, P. mirabilis, N. gonorrhea, C. albicans,* and *C. tropicalis* are sensitive to the extracts. As the extracts are active against MRSA but not active against VRE both of which are resistant strains, we conclude that *M. lucida* extract could treat some antibiotic resistant strains including MRSA. Also, *M. lucida* extract is not selective and is both antibacterial and antifungal. The drug, erythromycin, shows activity against only bacteria and the drug fluconazole shows activity against fungi only meaning that the drugs have selective activities. Erythromycin is antibacterial and fluconazole is antifungal. The antibacterial activity of erythromycin is of the same pattern as that of *M. lucida* extract: the drug is active against the same set of bacterial strains which *M. lucida* extract have activity and is inactive against the same set of bacteria against which *M. lucida* extract is inactive. Similarly, the antifungal activity of fluconazole is of the same pattern as those of *M. lucida* extracts. The MIC of *M. lucida* against the sensitive pathogens is 10 mg/ml and the MMC is 20 mg/ml. As the *M. lucida* extracts were

active against bacterial and fungal pathogens, it can be concluded that the use of *M. lucida* extracts to treat infectious diseases by traditional healers is justified. A compound AOC1 was isolated from the chloroform extract and was identified by comparison of ^{1}H NMR and ^{13}C NMR data with published data as taraxerol hexadecanoate. The antibacterial and antifungal activities of this compound follow the same pattern of *M. lucida* extract.

6.1.2 *U. togoensis* phytochemical constituents and the antimicrobial activities of the stem bark extracts

The phytochemical and antimicrobial activities studies of *U. togoensis* were carried out against fifteen pathogenic microbial strains including two antibiotic resistant strains. There appears to be no such studies reported in literature to date. The pathogenic microbial strains whose sensitivity to *U. togoensis* extracts were determined were MRSA, VRE, *S. aurens, C. alcerans, S. pyogenes, E. coli, S. typhi, S. dysenteriae, P. aeruginosa, P. mirabilis, N. gonorrhea, C. albicans, C. stellatoidea, C. krusei and C. tropicalis.* The stem bark extracts of *U. togeoensis* was used for the study. The extracts were obtained by Soxhlet extraction using the three selective solvents n-hexane, ethyl acetate and methanol. 1 kg of the pulverized stem bark was extracted sequentially in order of increasing polarity to yield 4.4076 g crude extracted by n-hexane, 18.5056 g crude extract by ethyle acetate and 176.360 g crude extract by methanol. The methanol extract constitute 88.5% of the entire extract indicating that *U. togoensis* stem bark extract is largely made up of polar substances. Phytochemical screening of the extracts by the method of Trease and Evans (1996) shows that *U. togoensis* stem bark extract contain carbohydrates, glycosides, tannins, flavonoids, saponins, cardiac glycosides, alkaloids, steroids and terpenes. Antimicrobial activities studies against the pathogenic microbial strains shows that *U. togoensis* stem bark is active against both bacterial and fungal strains. Thus, we conclude that *U. togoensis* stem bark extract have both antibacterial and antifungal activities. All the extracts

have activity against the same set of pathogens. The extracts are active against MRSA, *S. aureus, C. ulcerans, E. coli, S. typhi, S. dysenteriae, P. mirabilis, N. gonorrhea, C. albicans* and *C. tropicalis* but inactive against VRE, *S. pyogenes, P. aeruginosa,* and *C. stellatoidea*. As the extract is active against MRSA but inactive against VRE, we conclude that *U. togoensis* may treat infections of some antibiotic resistant microbial strains including MRSA but excluding VRE. The selective drug, erythromycin is active against the same set of bacteria which the extract have activity meaning that the *U. togoensis* stem bark extract can serve as a substitute for erythromycin. Whereas the extract is active against *C. albican, C. krusei* and *C. tropicalis*, the extract is inactive against *C. stellatoidea.* On the other hand, the fungal selective drug, fluconazole, is active against all the four fungi but *U. togoensis* extract is not. The *U. togoensis* is active against all the fungi except *C. stellatoidea*. Thus, the *U. togoensis* extract cannot serve as a substitute for fluconazole in the treatment of infection caused by *C. stellatoidea*. That *U. togoensis* extract is both antibacterial and antifungal justifies the folkloric use of *U. togoensis* in treating some microbial infections. The compound AOUE1 was isolated from the ethyl acetate crude extract and it constitutes 5.54% of the entire extract. By means of 1D and 2D NMR, the compound was structurally characterized as betulinic acid. That the ethyl acetate extract of *U. togoensis* constitutes 5.54% of the entire extracts indicates that *U. togoensis* is a rich source of betulinic acid. Whereas the crude extract is inactive against *S. pyogenes, P. aeruginosa* and *C. stelltoidea,* AOUE1 is active against these pathogens. Also, whereas the crude extract is active against *S. typhi, P. mirabilis* and *C. krusei,* the isolated compound AOUE1 is inactive against these pathogens. These means there are other compounds in *U. togoensis* besides AOUE1 that possess antimicrobial activities against those pathogens which AOUE1 have no activity.

6.2 Conclusions

i. Phytochemical screening of *M. lucida* leaf extracts shows that the extract contains carbohydrates, glycosides, flavonoids, saponins, cardiac glycosides, steroids and triterpenes.

ii. Antimicrobial assays revealed that MRSA, *S. aureus, S. pyogenes, E. coli, S. typhi, S. dysenteriae, P. mirabilis, N. gonorrhea, C. albicans, and C. tropicalis* are sensitive to *M. lucida* leaf extracts but VRE, *C. ulcerans, P. aeruginosa, C. stellatoidea,*and *C. krusei* are not.

iii. The *M. lucida* extracts is active against certain bacteria (Gram positive and Gram negative) and fungi and so, *M. lucida* leaf extracts is both antibacterial and antifungal.

iv. Though *M.lucida* is active against bacteria, it is not as potent as the antibiotic, erythromycin and though the extract is active against fungi it is not as potent as the antifungal, fluconazole.

v. Phytochemical screening of *U. togoensis* stem bark revealed that the extract contains carbohydrates, glycosides, tannins, flavonoids, saponins, cardiac glycosides, alkaloids, steroids and triterpenes.

vi. Antimicrobial assays of the *U. togoensis* stem bark extracts revealed that the extracts have activity against MRSA, *S. aureus, C.ulcerans, E. coli, S. typhi, S. dysenteriae, P. mirabilis, N. gonorrhea, C. albicans, C. kursei* and *C. tropicalis* but are active against VRE, *S. pyogenes, P. aeruginosa* and *C. stellatoidea.*

vii. The *U. togoensis* stem bark extracts is active against some bacteria (Gram positive and Gram negative) and fungi and is both antibacterial and antifungal.

viii. *U. togoensis* extract is rich in betulinic acid which is anti-HIV, anti-HSV1, anticancer, etc.

6.3 Recommendations for Further Works

The following recommendations are suggested for further studies on *M. lucida* (Benth) and *U. togoensis* (Pax).

i. In this work, only two antibiotic resistant strains were screened for their sensitivity to the extracts of *M. lucida* and *U. togoensis.* Therefore, it is recommended that more resistant microbial strains be screened for sensitivity to the extracts of these plants to ascertain the spectrum of activity of then extracts against antibiotic resistant microbial strains.

ii. Further isolation of the phytochemicals in n-hexane and methanol extracts is necessary to fully understand the metabolite profile of the plant.

iii. Toxicological assessment of *U. togoensis* should be carried out to assess the safety of the herbal remedy.

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