ANTIPLASMODIAL AND TOXICITY ACTIVITIES AND CHARACTERIZATION OF CHEMICAL COMPOUNDS EXTRACTED FROM SELECTED MEDICINAL PLANTS IN UGANDA

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A thesis submitted to the Graduate School in partial fulfillment for the requirements of the Doctor of Philosophy Degree in Chemistry of Egerton University

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DECLARATION AND RECOMMENDATION

DECLARATION

This thesis is my original work and has not been submitted for examination in any institution.

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RECOMMENDATION

We confirm that the work reported in this thesis was carried out by the candidate under our supervision.

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DEDICATION

This Thesis is dedicated to my mother Mrs. Wakumbaine Zeuliya, sons Kumbaine Jesse and Kodi Jayson for their moral and emotional support and encouragement they offered during the initial and final stages of the course.

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ABSTRACT

Malaria caused by *Plasmodium* parasite is at the moment the one of the highest killer disease in the tropics. In developing countries, where malaria is one of the most prevalent diseases, some people still rely on traditional medicine for the treatment of this disease. In the present study an ethnobotanical survey was conducted to document antimalarial medicinal plants. *In vitro* antiplasmodial and *in vivo* toxicity activities were carried out on crude root extracts and on the isolated pure compounds from *Oncoba spinosa*, *Acacia sieberiana* and *Euclea latideus*. Structure elucidation was also carried out on the isolated pure compounds. The ethnobotanical survey was conducted by use of semi-structured interviews and a guided questionnaire. The characterization of the isolated compounds was determined using NMR technique only. The antiplasmodial activity was performed using a fluorescence based SYBR Green 1 assay technique on 3D7 and Dd2 *Plasmodium* strains. Lorke's method was used to determine the *in vivo* acute toxicity of the extracts on mice. Thirty three plant species from 30 genera belonging to 23 families were documented, of these ten species (30.3 %) were recorded for the first time as antimalarial plants. Acute toxicity studies showed that all crude extracts of *E. latideus* and *A. sieberiana* had LD_{50} > 5000 mg/kg. The LD_{50} for hexane and CH_2Cl_2 extracts of *O. spinosa* were > 5000 mg/kg while the EtOAc and MeOH had 547.72 mg/kg. The EtOAc extract of *O. spinosa* had high activity of (IC_{50}) 3D7: 4.69 ± 0.01 µg/mL and Dd2: 3.52 ± 0.02 µg/mL. Extracts of *E. latideus* had high activity (IC_{50}) 3D7: (9.75-38.21) µg/mL and Dd2: (2.78-38.93) µg/mL. *A. sieberiana* extracts had the highest activity of (IC_{50}) 3D7: (4.45-27.32) µg/mL and Dd2: (3.38-21.87) µg/mL. Isolation resulted in the identification of eight known compounds which included; three triterpenoids Lupeol, betulin, 3β-(5-methoxyferuloyl)lup-20(30)-ene; two steroids β-sitosterol, stigmasitosterol; benzoic acid and an aliphatic acid chaulmoogric acid. Betulin and β-sitosterol had the highest activity (IC_{50}) 3D7: 3.71 and 5.51 µM, respectively. Antiplasmodial activities of the extracts (IC_{50}: 2.76- > 50) µg/mL, pure compounds (IC_{50}: 3.71- > 120.77) µM of the three plants and the controls (IC_{50}: 0.0056-0.0440) µg/mL showed significance among themselves at ($P < 0.05$). Extracts and compounds exerted a significant ($P < 0.05$) decrease in antiplasmodial activity compared to the standard controls. The findings show that the crude extracts and pure compounds have got high antiplasmodial activity and lack toxicity. Therefore the local communities can continue to use the three plants for the treatment of malaria and this justifies the ethanomedicinal use of the plants for the management of malaria.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
Ac	Acetate
ACTs	Artemisinin-based Combination Therapies
AL	Artemether-Lumefantrine
ACTs	Artemisinin-based Combination Therapies
AQ	Amodiaquine
AVMA	American Veterinary Medical Association
BEIR	Biodefense and Emerging Infectious Research
br	Broad Resonance
bs	Broad Singlet
δ	Chemical shift
^{13}C NMR	Carbon -13- Nuclear Magnetic Resonance
CC	Column Chromatography
CC_{50}	Cytotoxicity Concentration at 50%
CDC	Centerfor Disease Control
$CDCl_3$	Deuterated chloroform
CD_3OD	Deuterated methanol
$(CD_3)_2SO$	Deuterated dimethlysulfoxide
COSY	Correlation Spectroscopy
CQ	Chloroquine
d	Doublet
1-D	One dimension
2-D	Two dimension
dd	Doublet of doublets
ddd	Doublet of doublet of doublets
dt	Doublet of triplets
qt	Quartet of triplets
DDT	Dichlorodiphenyltrichloroethane
DCM	Dichloromethane
DEPT	Distortion less Enhancement by Polarization Transfer
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulphoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl

EC_{50}	Effective concentration at 50%
ED_{50}	Effective Dose at 50% concentration
ELISA	Enzyme-linked Immunosorbent Assay
ESI	Electron spray ionization
ESI-MS	Electron Spray Ionization - Mass Spectrometry
EtOAc	Ethyl acetate
FQ	Ferroquine
FT-IR	Fourier Transfer-Infrared
GC	Gas Chromatography
GC/MS	Gas Chromatography/Mass Spectrometry
GDP	Gross Domestic Product
GNP	Gross National Product
HEPES	(2- hydroxylethylpiperazine-N^1-2-ethane) Sulphoxide
HETCOR	Heteronuclear Correlation
HIV/AIDS	Human Immune Virus/Acquired Immune Deficiency Syndrome
HMBC	Heteronuclear Multiple Bond Coherence
HMQC	Heteronuclear Multiple Bond Quantum
^{1}H NMR	Proton Nuclear Magnetic Resonance
HPLC	High Performance Liquid Chromatography
HPLC-MS	High Performance Liquid Chromatography – Mass Spectrometry
HRMS	High Resolution Mass Spectroscopy
HSQC	Hetronuclear Single Quantum Coherence
Hz	Hertz
IC_{50}	Inhibition Concentration at 50%
IRS	Indoor residual spraying
ITN	Insecticide-treated mosquito net
J	Coupling constant
IR	Infrared
LC_{50}	Lethal concentration at 50%
LC-MS	Liquid Chromatography – Mass Spectrometry
LD_{50}	Lethal dose at 50%
m	Multiplet

MEME	Minimum Essential Medium with Earl's salts
MeOH	Methanol
mg/mL	Milligram per milliliter
µg/ML	Microgram per milliliter
MHz	Mega hertz
MBC	Minimum bacterial concentration
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
mM	Millimolar
µM	Micromolar
M.p	Melting point
MS	Mass Spectroscopy
NDA	National Drug Authority
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced)
ng/mL	nanogram per milliliter
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institute of Health
nM	nanomolar
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Over Hauser Effect
NOESY	Nuclear Over Hauser Enhancement Spectroscopy
OD	Optical density
pLDH	Parasite Lactate Dehydrogenase
PPK	Percentage of people who have knowledge about the use of a species
PR	Preference ranking
PRBC	Parasitized Red Blood Cells
ppm	Parts per million
q	Quartet
RBC	Red Blood Cells
RBM-WHO	Roll Back Malaria-World Health Organization
R_f	Retardation factor
RI	Resistance index
ROESY	Rotating frame Overhause Effect Spectroscopy

s	Singlet
SEM	Standard Error Mean
SI	Selectivity Index
sLOX	Soybean lipoxygenase
SP	Sulfadoxine-pyrimethamine
SPSS	Statistical Product and Service Solution
SRB	Sulforhodamine B
STDS	Sexual Transmitted Diseases
t	triplet
TB	Tuberculosis
Tdd	triplet of doublet of doublets
TFA	Trifluoroacetic acid
Tig	Tiglate
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
TOCSY	Totally Correlated Spectroscopy
UV	Ultraviolet
WHO	World Health Organization

CHAPTER ONE
INTRODUCTION

1.1 Background information

1.1.1 History of natural products

Dating back to prehistoric times man has used plants to alleviate and treat diseases. The use of medicinal plants has always been part of human culture and it began from the time of early civilization as evidenced by the earliest recorded uses found in Babylon (1770 BC) and in ancient Egypt (1550 BC) (Akintonwa *et al.*, 2009). The origin of pharmaceutical natural products research can be traced to 1805, when the German Pharmacist Sertürner isolated morphine from *Opium latex* and soon recognized the superior therapeutic properties of the pure compound. The isolation of morphine initiated the discovery of numerous important drug substances; such as emetine (1817), atropine (1819), quinine (1820), caffeine (1820), and digitoxin (1841) (Potterat and Hamburger, 2008).

The French pharmacists Pelletier and Caventou were particularly prolific in isolating numerous important alkaloids. Caventou established in the mid-1820s a factory for production of quinine which was to become the first commercial natural product and pure drug substance. Soon, factories in other European countries were established. For example, E. Merck Company in Germany (1827), and early pharmaceutical industry developed along with the discovery of an increasing number of plant alkaloids (Potterat and Hamburger, 2008).

In the 19th and well into the 20th Century, ethnopharmacology provided a number of compounds with unique pharmacological properties. This gave significant advances in pharmacotherapy, like quinine (1820) as antimalarial, cocaine (1860), the first local anesthetic. Other drugs include; tubocurarine (1935), a muscle relaxant enabling modern surgical procedures, and reserpine (1951) as a first effective antihypertensive. Towards the end of the 19th Century, rapidly growing understanding of organic synthesis and chemical structures led to first derivatives of natural products. Diacetylmorphine (1898) and acetylsalicylic acid (1899) were among the first compounds to be commercialized as pharmaceuticals (Soltan and Zaki, 2009). The contribution of plant derived natural products to modern pharmacotherapy is considerable. Out of 243 structures which Sneader identified as the starting point for the development of our modern drugs, some 60 compounds are of plant origin (Akintonwa *et al.,* 2009).

During the last decade, the uses of traditional medicine has expanded globally and gained popularity. The World Health Organization (WHO) estimated in 2001 that up to 80% of the world's population relies on traditional medicinal practices for some aspect of primary

health care even for some complicated and disturbing infections like tuberculosis (Akintonwa *et al.,* 2009; Soltan and Zaki, 2009). In the last few years there has been an exponential growth in the field of herbal medicine because of their natural origin, easy availability, efficacy, safety and less side effects. It has also been due to its efficient to cure age related disorders like memory loss, osteoporosis, immune disorders (Kamboj, 2000; Grover *et al.*, 2002).

1.1.2 Controling malaria using antimalarial medicinal plants

Historically, communities in tropical regions have used local plants as a means of preventing and treating malaria (Okigbo *et al*., 2009; Willcox *et al*., 2004). There over 1200 plant species have been used in treating malaria throughout the world (Willcox *et al.* 2004). Similarly, medicinal plants are widely used in Uganda to treat malaria (Katuura *et al.,* 2007; Ssegawa and Kasenene, 2007; Tabuti 2008). Some plants which are widely used as anti-malarials have been shown to be significantly active *in vitro* and *in vivo* against *Plasmodium* species (Willcox *et al*., 2011). For this reason, herbal medicine continues to play an essential role in covering the basic health needs in many developing countries, including Uganda (Kuglerova *et al*., 2011). Many existing antimalarial drugs have been produced from the active compounds of plants such as quinine from the *Cinchona* bark and artemisinin from *Artemisia annua* (Asteraceae) (Bloland *et al*., 2000; Willcox *et al*., 2004).

At present there very few drugs that can offer protection against malaria in all regions of the world. The need for novel chemotherapeutic agents against malaria is therefore acute. One approach to new chemotherapeutic agents is to identify drugs with novel action. Traditional medicinal plants used as antimalarial have the potential of providing novel antiplasmodial active compounds (Steele *et al*., 1999). The increasing prevalence of drug resistant strains of *P. falciparum* to standard antimalarial drugs necessitates a continuous effort to search for new antimalarial drugs with new modes of action, used alone or in combination (Kshirsagar *et al*., 2000). It is therefore of interest to screen traditional antimalarial medicinal plants for an evaluation of *in vitro* or *in vivo* antiplasmodial and toxicity (Muthaura *et al*., 2007).

In order to manage malaria, new knowledge and products are needed. Traditional herbal medicine has constituted a good basis for antimalarial lead discovery and drug development. The practice of plant traditional medicines in communities of Butebo County in Uganda is based on knowledge that has been passed orally from generation to generation. There are no written documents that are available on the antimalarial plants used to date.

The herbal preparations also lack standardization on quality, efficacy and safety. The herbal preparations are used in most cases in their crude forms.These are known to contain potent and toxic chemical substances which have long term effects when taken. The therapeutic claims on these herbal medicines have not yet been evaluated for efficacy and assessed for potential toxic effects. These claims must be validated in order to raise confidence among clients of traditional medicine. In Butebo County, water is the most solvent which is used to prepare the extracts and concoctions. Therefore in most cases the ingredients in traditional medicines may be in minute concentrations due their low solubility in water. This means large quantities of these medicines are needed for curative effects. The lack of suitable solvents implies that many useful plants may not show any curative properties despite some of them containing highly active compounds. This calls for a need to find better solvents with high solubility to use in the preparations of the herbal medicines (Kodi *et al*., 2017).

This research will form a basis for continued research into other medicinal plants of Uganda. This is to be done by setting standards as regards to efficacy assessment, toxicity tests, better formulations and presentations, as well as improved health care. The integration of the medicinal plants into the delivery of primary health care in order to meet the targets of National Health Policy (1999) and the National Drug Policy (1993) will be another important step. The isolated active components after being characterized may also act as models for the synthesis of compounds or their derivatives that might exhibit maximum biological activity with minimum toxicity.

1.2 Statement of the problem

Medicinal plants are used to treat malaria and fever in the rural communities in Uganda. Unfortunately, most of them lack documentation and have not been adequately investigated. Antiplasmodial screening has not been carried out using any scientific method to determine the efficacy of the active compounds either in crude form or as pure compounds. The crude extracts made may contain potent and toxic chemical substances that may have long term effects on the people. This calls for toxicity assessment to determine the safety of the herbal medicines. The identities of the active components are unknown, and therefore uncertainties exist regarding dosage preparations and effectiveness of the medicinal plants in the treatment of the disease. Bioassay guided isolation and structure characterization is needed to solve this problem. Therefore all traditional medicines must be subjected to safety, therapeutic efficacy and quality control in order to solve some of these problems. This needs

to be done so that the lives of people in the local communities who mostly use the medicinal plants for the treatment of malaria are not at risk.

1.3 Objectives

1.3.1 General objective

To determine antiplasmodial and toxicity activities and characterization of compounds isolated from selected medicinal plants from Uganda.

1.3.2 Specific objectives

(i) To document ethnobotanical information on traditional herbal medicines used to treat malaria in Butebo County, Uganda.

(ii) To determine the *in vitro* antiplasmodial activity of single crude plant extracts against *P. falciparum* organisms.

(iii) To determine the *in vivo* acute toxicity of crude plant extracts.

(iv) To determine the *in vitro* antiplasmodial activity of isolated pure compounds.

(v) To determine the chemical structures of the isolated pure compounds.

1.4 Justification

The human practice of antimalarial traditional medicines in rural communities is based on the knowledge that has been passed orally from generation to generation. Only a very few written documents are available that show tests carried out on antiplasmodial efficacy and toxicity assays of the locally available antimalarial plants. The results from this research can be used as a basis, for the identification and motivation for conservation of local species of antimalarial medicinal plants with high efficacy and with minimum toxicity. The findings can be used to identify plants with market potential that can help generate income for the local communities. This will provide alternative treatment for malaria that is cheap, accessible, safe and effective. The plants can be used to make drugs in pharmaceutical industries and medicine, in order to develop alternative drugs for the treatment of malaria. Structure characterization of the antiplasmodial active pure compounds may act as models for synthesis of derivatives that might exhibit maximum biological activity with minimum toxicity. Therefore, a research into the abundant medicinal plants as a way of providing alternative or complementary treatment is needed and is in line with Ministry of Health policy of integrating use of medicinal plants into delivery of improved medical services.

CHAPTER TWO
LITERATURE REVIEW

2.1 The global burden of malaria

Malaria is an ancient and one of the major fatal parasitic killer diseases of the world, having been recorded as early as 1500 B.C. (Roger and Richard, 2004). This parasitic disease remains a major public health problem and concern which affects hundreds of millions of people, particularly in tropical African developing countries (Njoroge and Bussmann, 2006). Globally, about 3.2 billion people live in areas prone to malaria transmission and 1.2 billion are at high risk. In 2016, malaria contributed to an estimated 216 million infections and about 445, 000 deaths where 91% of the deaths were in the African region and among those about 78% were children under the age of 5 years (WHO, 2017). It causes childhood death, anemia, low birth-weight, epilepsy, and neurological problems. All these are frequent consequences of malaria which compromise the health and development of millions of children throughout the tropical world (RBM-WHO, 2006a). Therefore, malaria is Africa's leading cause of under-five mortality (20 per cent) and constitutes 10 per cent of the continent's overall disease burden. It accounts for 40 per cent of public health expenditure, 30-50 per cent of inpatient admissions, and up to 50 per cent of outpatient visits in areas with high malaria transmission (RBM-WHO, 2006c). Cameroon figures are among the 18 countries bearing 90% of malaria deaths in Africa, with 71% of its population living in high transmission areas (WHO, 2008). In the western region of Cameroon, for example, malaria was shown to be the most important cause of infant mortality causing about 45% deaths and 54% hospitalizations for children under five (Zofou *et al.*, 2011).

Malaria is the single most important cause of ill health, death and poverty in Sub-Saharan Africa. The disease is believed to be a major obstruction to social and economic development in Africa. It causes enormous misery and suffering through the pain of fevers. The disease not only results in lost life and lost productivity because of illness and premature deaths, but it also hinders children in their schooling and social development. This is both through absence from school and permanent neurological or other damage associated with severe episodes of the disease (Sachs and Malaney, 2002; Kilama, 2005; United Nations, 2005).

The costs of malaria are not only high for peoples' health, but the disease also results in significant economic losses. Annual Gross Domestic Product (GDP) is estimated to be reduced by as much as 1.3% in countries with high disease rates. In Africa it is estimated that at least USD 12 billion per year is lost directly through illness, treatment and premature

death. Aggregated losses over time have resulted in substantial differences in GDP between countries with and without malaria, particularly in Africa. Malaria disease management is therefore an essential part of global health improvement and economic development (WHO, 2010).

Malaria is reported by the Ministry of Health (MOH) as the leading cause of morbidity and mortality in Uganda, accounting for approximately 8-13 million episodes per year, 30-50% of outpatient visits at health facilities, 35% of hospital admissions, 9-14% of hospital deaths (nearly half of those in children less than 5 years of age) and a great many deaths occurring outside of health-care settings (Uganda Ministry of Health, 2005). Available data include a Uganda Demographic and Health Survey (UDHS) in 2006 (Uganda Bureau of Statistics, 2007), a Uganda Malaria Indicator Survey (UMIS) in 2009 (Uganda Bureau of Statistics, 2010), and ongoing health facility-based data routinely collected through the Health Management Information System (HMIS).

2.2 The malaria epidemiology

Malaria is transmitted to humans by female *Anopheles* mosquitoes. If this insect bites a person with malaria it sucks some of the parasites along with blood cells. The protozoan then pass along with the blood into mosquito's stomach where they grow and reproduce, at this stage the parasites pass into the mosquito blood stream and eventually reach its salivary gland. When the mosquito bites a healthy person some parasites enter the human blood through the skin, where they travel to the liver and develop for about eight days. They then enter the blood stream and invade the red blood cells. The red blood cells provide oxygen rich environment and a food source for the parasites. In each blood cell, many parasites spore form and eventually they burst to release the spores which then enter other red cells. These cells later burst and a regular cycle of invasion and bursting develops. This bursting usually takes place every twenty four to forty eight hours. At the begining of each bursting cycle the host experiences chills, headache, high fever, heavy sweating, enlargement of the spleen and often fatal complications. These symptoms result from the parasites waste products being emptied into the blood. During the time between bursting of red cells, the host may feel well although weak. In some kinds of malaria, the cycle many continue for several weeks and then seem to disappear, but the symptoms may suddenly re-appear months later. In other forms of malaria, death may result within several hours after the onset of symptoms (Center for Disease Control, 2008; Cooper and Magwere, 2008) (**Figure 2**).

The type of malaria depends on the species of *Plasmodium* involved. Transmission of disease through mosquito bites depends on factors such as rainfall patterns (mosquitoes breed in wet conditions), how close breeding sites are to people, and the types of mosquito species in an area. Some regions have a fairly constant number of cases throughout the year ('malaria endemic' areas), while others have seasonal bouts of infection, usually coinciding with the rainy season. When malaria is contracted because prevention methods are not available or are not practiced effectively, the first symptoms of the disease; fever, headache, chills and vomiting usually appear 10 to 15 days after infection. Treatment then needs to be prompt to minimize serious health risks and allow the majority of deaths to be avoided. Several prescription drugs have been and still are used for treatment, although resistances to some commonly used medicines have developed rapidly (Avwioro, 2010) (**Figure 1**).

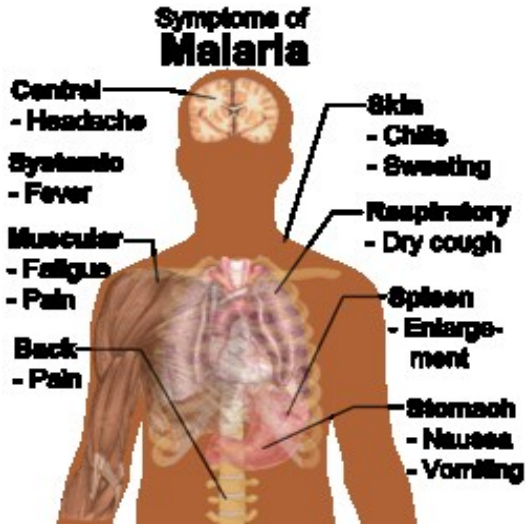


Figure 1: Symptoms of malaria

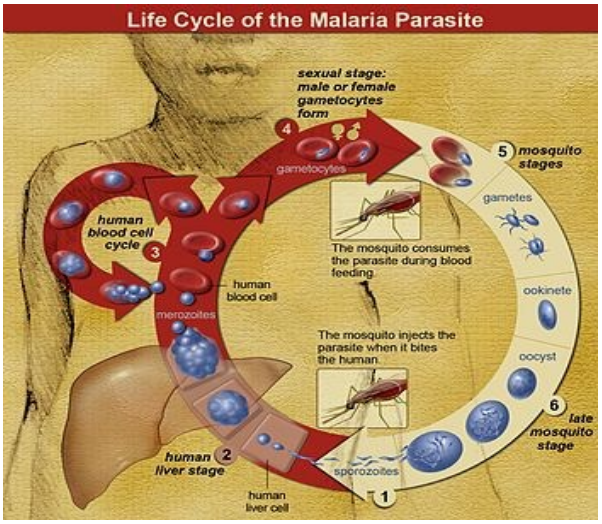


Figure 2: Life cycle of malaria parasite

file:///C:/users/HP/Documents/Malaria

To combat malaria, new drugs are desperately needed, but traditional mechanisms for drug development have provided few drugs to treat diseases of the developing world. In this challenging situation, there are some reasons for optimism. First, the determination of the genome sequence of *P. falciparum* offers a multitude of potential drug targets. Secondly, advances in malaria genetics offer improved means of characterizing potential targets. Thirdly, the recent increased participation of pharmaceutical companies in the antimalarial drug discovery and development process offers hope for the development of new, affordable drugs. However, there is lack of standardized systems for antimalarial drug efficacy screens (Center for Disease Control, 2012).

In the East African region the fight against malaria has been handled by using different methods in order to eradicate the disease. For example Kenya, Tanzania and Uganda have used almost the same strategies and drugs to fight the disease. In Uganda, malaria is responsible for more illness and death than any other single disease. While those also with low immunity pregnant women, children under five years and people living with HIV/AIDS are particularly most affected. However all people living in Uganda are at risk of being infected with malaria parasites and suffering from resulting illness. Malaria is the most common disease and kills the most people (Batega, 2004; Malaria Control Programme, 2005). It is the most frequent cause of attendance at health facilities accounting for 25-40% of out-patient attendances, 20% of in-patient admissions and 9-14% of in-patient deaths. More than 200 children die daily from the disease (Malaria Control Programme, 2005; Ministry of Health, 2006a).

In children it does not only lead to illness and death but also has long term consequences on their development through low birth weight, chronic anemia, reduced growth and in some cases severe mental retardation. In pregnancy, malaria may cause maternal anemia, premature births, low-weight babies (which is the principal contributor to infant mortality) and still births. The disease is responsible for nearly 60% of miscarriages and also contributes to poverty. First of all, many productive days are lost through sickness or tending to sick relatives. Secondly the cost of treatment or barriers to mosquito bites, such as insecticides or mosquito nets is high for the average Ugandan. The average cost of treating a single malaria episode is estimated at UGX 30,000 (1 USD = UGX 3,660). Indeed, a poor family can spend up to 25% of its income on malaria treatment and prevention (Ministry of Health Uganda, 2006b).

In most parts of Uganda, temperature and rainfall are sufficient to allow a stable, year round (perennial) malaria transmission at high levels with relatively little seasonal variability. Only in the high altitude areas in the Southwest, West and East is malaria transmission generally low. Tremendous progress has been made in the fight against malaria. This has been achieved through the improvement of health system performance and increased public knowledge about malaria (Ministry of Health Uganda, 2010).

Ninety percent of the population in Uganda lives in a high malaria-transmission area, and this creates a heavy burden on the health system (WHO, 2013). Malaria in Uganda has been ranked as a leading cause of morbidity and mortality. Uganda has some of the highest recorded malaria transmission rates in Africa, particularly in the areas around Lake Kyoga in Central Uganda (Kiwanuka *et al*., 2008). The Ministry of Health (MOH) officials in Uganda

estimated that each person in Apac District on average receives over 1500 malaria-infected bites every year. Previous estimates have also indicated that at least 12.3 million cases of malaria are registered across Uganda (Ministry of Health Uganda, 2010).

Some plants which are widely used as anti-malarials have been shown to be significantly active against *Plasmodium* species (Willcox *et al*., 2011). However, the importance of medicinal plants in the treatment of malaria seems to have been neglected by by many countries. Uganda. Uganda is yet to officially adopt such remedies in treating malaria at the national level. So far, the Uganda Malaria Control Strategic Plans (UMCSP) for 2006 and 2010 remain silent on the role of herbal medicines and medicinal plants in combating malaria (Ministry of Health Uganda, 2006b; 2010).

Malaria accounted for 40% of inpatient deaths in the financial year 2009/2010 and 31% in the financial year 2013/2014 according to the annual health sector performance report 2013. The disease consumes over 10% of the Ministry of Health budget and 25% of household incomes. Health Ministry statistics show that the country has been losing over (UGX) shillings 658 million annually in malaria related costs. The government of Uganda, through the Ministry of Health allocates approximately 100 billion Uganda shillings annually as recurrent budget to health according to Ministry Finance Planning and Economic Development Report (2002/3) and guidelines on management and utilization of government grants (2002). Approximately 25 billion of this is equivalent to 25% is budgetary expenditure spent on procurement of modern drugs. In spite of this investment, there is limited access to expensive conventional medicines by the majority of the rural population. This difficulty has been attributed `to the poor economic status of the population with an annual GNP per capita of US$ 320 and approximately 35% of the people living below the poverty (Ministry of Finance, Planning and Economic Development, Financial year District transfers 2002/2003).

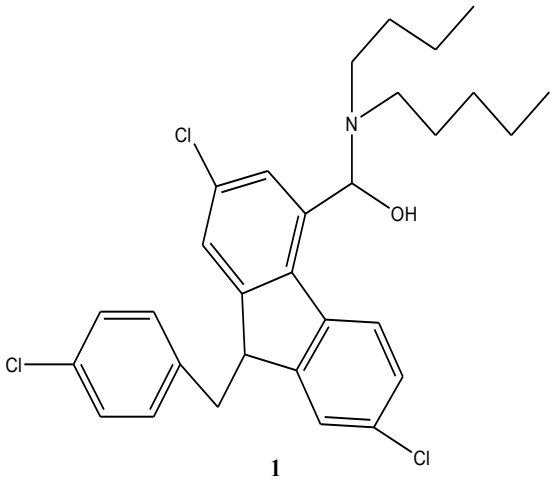
All the four human *Plasmodium* species occur in Uganda but *P. falciparum* is by far the most common contributing 90-98% of the parasite population. The second most common species is *P. malariae* with 1-3% as mono-infection but is more commonly found as a mixed infection with *P. falciparum* (up to 16% of childhood infections in highly endemic areas). Both *P. vivax* and *P. ovale* are rare and do not exceed 1-1.5% of malaria cases.

The most common vectors are *Anopheles gambiae s.l.* and *Anopheles funestus*, with *A. gambiae* being the dominant species in most places. It is only during the short dry seasons when permanent water bodies with breeding sites and in higher altitude areas is *A. funestus* found more frequently. Within the *A. gambiae* complex the predominantly anthropophilic *Anopheles gambiae s.s*. is by far the most common with *A. arabiensis* found in 1-10%. The

non-malaria vector, *A. quadriannulatus* is less than common. The *A. bwambae* sibling species of the *A. gambiae* complex is only found near the Semliki hot Springs in Bundibugyo District in Uganda. Species such as *A. pharaoensis* or *A. moucheti* although identified occasionally do not seem to play any significant part in malaria transmission (Onori, 1967a; Onori and Benthein, 1967b; Onori, 1969).

P. falciparum, the malaria parasite that is responsible for most attacks of malaria in Uganda, has developed resistance to the commonly used antimalarial drugs. As a result of widespread chloroquine and sulphadioxine-pyrimethamine resistance, 90% of Sub-Saharan African countries adopted policies of artemisinin based combination therapy (ACTs) for treatment of uncomplicated malaria by 2007. Chloroquine resistance was first documented in Uganda in 1988. The overall national treatment failure rate is 30% for chloroquine (CQ) and 10% for sulphadioxine-pyrmethamine (SP). These treatment failure levels are unacceptably high, leading to the need to change to ACTs. This increasing resistance further stresses the importance of effective preventive methods such as insecticide treated mosquito nets (ITN) (Kamya *et al*., 2002; Mbaisi *et al*., 2004).

Today, Uganda faces new challenges for malaria treatment because of increasing resistance of malaria parasites to chloroquine (CQ) and sulphadioxine-Pyrmethamine (SP). New and highly effective drugs artemether/lumefantrine (**1**) as the first line treatment for uncomplicated malaria, and artesunate/amodiaquine combination as the alternative are now available.



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Poverty is recognized as the main underlying cause of the poor health and malaria, as one of the principal contributions to poor health sanitation in the country. It is further

documented by health sub-district in Uganda concept paper (1999) that of the 37.58 million Ugandans, 49% live 5 km away from health facilities. In adition 86% live in rural areas with poor roads making accessibility to conventional treatment difficult. Secondly, Ministry of Finance Planning and Economic development Report (2002/2003) indicates inadequate provision and inequitable distribution of scarce resources. This is a general level of underdevelopment of service delivery and poor infrastructure. The existence of such situations mean that the majority of the population will still have to continue with the use of cheaper and more accessible herbal preparations in the management of many diseases. Therefore, the research into and improvement of new and cheaper alternative medicines is necessary whether in the form of crude herbal medicines or as components of conventional medicine.

2.3 Vector control techniques and treatment of malaria using compounds from medicinal plants

There are four types of human malaria pathogens: *Plasmodium vivax, P. falciparum, P. malariae and P. ovale*, of which the first two are the most common. *P. falciparum* is the most deadly malaria parasite of all; found throughout the tropical regions of the world that causes a severe, potentially life threatening infection. Other species of this genus infect animals such as *P. knowlesi* (monkeys), *P. berghei* (rodents) and *P. gallinaceum* (fowl) (Rukunga and Simons, 2006).

Various techniques have been employed to control the malaria vectors and many antimalarial are currently used to fight the disease. Malaria transmission can be reduced by preventing mosquito bites through distribution of inexpensive treated mosquito bed nets. This include nets that are conventionally treated (require regular retreatment) and long lasting insecticidal nets that retains the efficacy for at least three years (Malima *et al*., 2008). Insect repellants such as those that can be applied to skin or clothes or as repellant soaps or as mosquito coils can be used. Attractants can be used in case the mosquitoes need to be trapped (Zwiebel and Takken, 2004). Environmental modification and manipulation can also be done. This involves destruction of breeding sites through drainage of stagnant water and filling small ponds or water collecting depressions where mosquitoes lay their eggs. Water from the ponds can also be treated with old engine oil to prevent the mosquitoes from accessing the water in order to lay the eggs.

Larviciding is another method that can be used to control mosquito breeding sites. It involves the use of chemical insecticides in aquatic habitants such as edges of swamps, lakes, pools and river sides. Predatory fish that feed on mosquito larvae can be used to control vector diseases at larval stages. This method is effective than the chemical control one, because fish are harmless to both humans and wild life. The fish are both cheap to produce and exhibit minimal risks of mosquito resistance. One other method that has been used is that of insect pathogenic fungi. Adult *anopheles* mosquitoes have been controlled by a selected fungus (*Lagenidiam*, *Coelomomyces*, *and Culicinomyces*) and have seen promising results (Scholte *et al*., 2004). The fungus are applied to the surfaces inside houses where female mosquitoes rest after blood meals. Improvement on the hose can also contribute significantly to malaria transmission control. Plastering of walls and ceiling fills the crevices that serve as refuge for adult mosquitoes. Traditional mosquito bites can be prevented by burning leaves of fresh *Azadirachata indica* stems of plants such as *Ocimum basilium*, *Ocimum osuave* and *Lantana camara*.

The Ugandan government caused alarm among international donors when it recently announced it intended to use the insecticide DDT (dichlorodiphenyltrichloroethane) to fight malaria. Although the pesticide is banned under international convention except in the event of a "public health crisis," the health ministry in Uganda argued that the use of DDT is a cost effective, long term preventive measure that would reduce the economic burden placed on the country's health services by malaria. The indoor spraying of DDT has started in some parts of the country for example in the Northern and Eastern regions. Meanwhile, environmentalists insist DDT is a dangerous toxin, harmful to both the environment and human health (Ministry of Health Uganda, 2014).

Prophylaxis of the disease can be achieved by use of drugs like Artemisinin-based Combination Therapies-ACTs. This can be administered mainly to children, pregnant women and people with low immunity that are the most affected by the disease. However, there are some shortcomings with some of these methods used to reduce malaria transmission. In the first place most people living around water bodies use the mosquito nets for fishing and secondly the mosquitoes now bite people before going to their houses to sleep. This implies the use of mosquito nets and indoor spraying is rendered ineffective.

There are four major problems that are associated with the management of malaria. The most important problem is that the parasites which cause malaria are resistant to or are developing resistance to the most widely available, affordable and safest first line treatments such as chloroquine, amodiaquine, sulfadoxine (**2**)-pyrimethamine (**3**) and other virtually

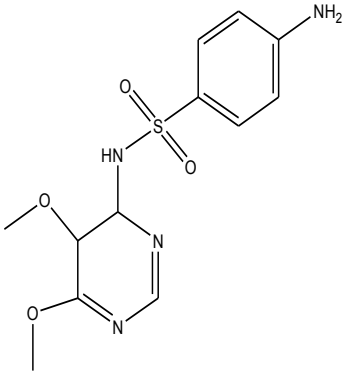
affordable drug treatment options (Kilama, 2005). The overall control of the mosquitoes which transmits malaria is made difficult also by their resistance to a wide range of insecticides. A new and rapidly developing problem is the widespread production of substandard and counterfeit antimalarial drugs. For example, in mainland South-East Asia 38 and 53% of "artesunate" blister packs sampled contained no active ingredient (Newton *et al*., 2006). In Cambodia, China, Indonesia, Thailand and Laos's counterfeit drugs have made the control of malaria difficult. Another example is in Kenya, where counterfeit drugs have been encountered in the market and some have been detected during laboratory testing. These included antimalarials, antibiotics, antiretrovirals and corticosteroid skin creams. Finally, many countries in Africa lack the necessary infrastructure and resources to manage and control malaria (WHO, 2004).

In Africa there is also a problem of most infections being treated on the basis of symptoms rather than a positive blood test (Biritwum *et al*., 2000). This leads to a huge amount of over diagnosis and consumption of unnecessary and costly drugs. Self-treatment with medicines purchased from a local store or pharmacy, which probably accounts for as much of 50% of drug use in Africa, may lead to the use of inappropriate drugs or under dosing. There is an urgent need to develop new drugs or vaccines for the treatment, management, prevention and control of malaria. This is due to the widespread suffering and death caused by malaria and the failure of the safest and most affordable antimalarials to treat the disease because of drug resistance (Kilama, 2005; Waako *et al*., 2005).

The World Health Organization estimates that about 80 per cent of the population in Sub-Saharan Africa turns to traditional medicinal remedies (herbal medicines) in their search for treatment (WHO, 2008; 2010). Many people living in developing countries do not have access to modern therapeutics, such as ACTs, to treat malaria. This is because of financial, socio-economical, geographical and cultural reasons. They use plants often in combination, for the health care management of malaria. Validated antimalarial phytomedicines formulated from traditional medicines have been reported in recent years. Some are government approved in different countries, for example, *Argemone mexicana* in Mali, whose antimalarial activity has been confirmed in clinical trials (Graz *et al*., 2010). In Burkina Faso, a combination of three plants have been used for several years as a curative antimalarial and recently studied for its benefits in the prophylaxis of malaria (Yerbanga *et al*., 2012).

Medicinal plants have been playing a vital role in the treatment of malaria for thousands of years. Over 1200 plants are reported to have antimalarial effects (Willcox *et al*., 2004). It is probable that some of them contain as yet undiscovered powerful antimalarial

compounds. Many well-known drugs listed in the modern pharmacopoeia have their origins in natural sources mainly from plants. For example, known potent antimalarial compound quinine was isolated from the bark of the *Cinchona* tree. Various other synthetic analogues of quinine have since been developed for malaria (Van Wyk *et al*., 1997; Rukunga and Simons, 2006). Over a past decade, the discovery of artemisinin from *Artemisia annua* has boosted research on plants in the search for new antimalarial compounds. A new group of antimalarials the artemisinin compounds, especially Artesunate (**4**), artemether (5) and dihydroartemisinin (**6**) have been deployed on an increasingly large scale (RBM-WHO, 2006b). These compounds produce a very rapid therapeutic response (reduction of the parasite biomass and resolution of symptoms) and are active against multidrug-resistant *P. falciparum* malaria. To date, no parasite resistance to these compounds has been detected. If used alone, the artemisinin's will cure *P. falciparum* malaria in seven days. However, studies in South East Asia have shown that combinations of artemisinin compounds with certain synthetic drugs (ACTs), produce high cure rates in just three days of treatments. As a response to increasing levels of antimalarials resistance, WHO recommends that all countries experiencing resistance to conventional monotherapies, such as chloroquine (**7**), amodiaquine (**8**), should use combination therapies preferably those containing artemisinin derivatives (ACTs) for *falciparum* malaria (RBM-WHO, 2006b).



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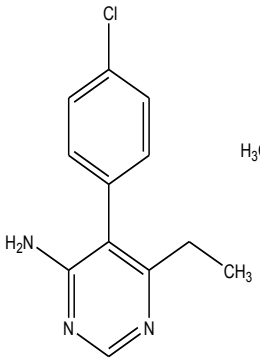

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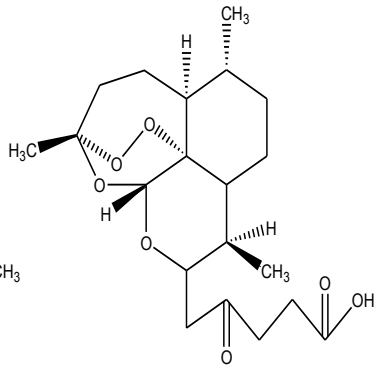

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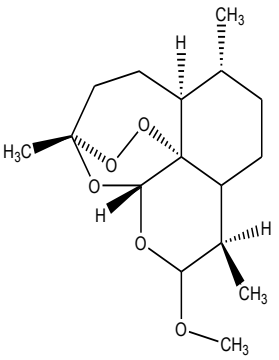

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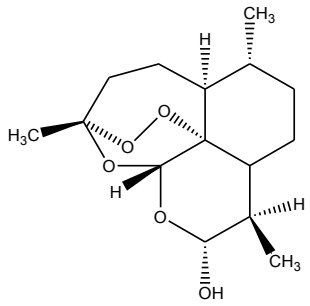

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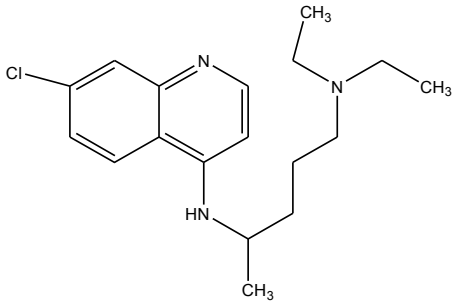

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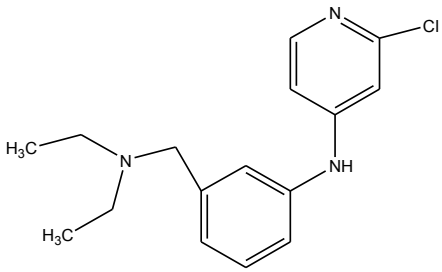

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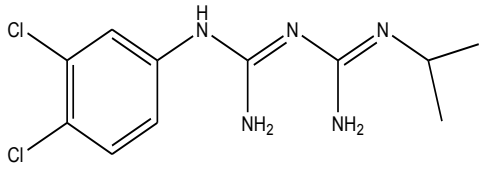
2.4 Resistance to antimalarial drugs

Resistance has been defined as the temporary or permanent ability of an organism and its progeny to survive or multiply despite the administration and absorption of a drug given in doses equal to or higher than those recommended but within tolerance of the subject. Drug resistance may lead to treatment failure, but treatment failure is not necessarily caused by drug resistance despite assisting with its development. A multitude of factors can be involved in the processes including problems with non-compliance and adherence, poor drug quality, interactions with other pharmaceuticals, poor absorption, misdiagnosis and incorrect doses given. The majority of these factors also contribute to the development of drug resistance (Cloete, 2003).

Resistance to all known antimalarial drugs, with the exception of the artemisinin derivatives, have developed to various degrees in several countries (Bloland and WHO, 2001). The urgency generated by drug resistant strains of malaria has accelerated antimalarial drug research over the last two decades. While synthetic pharmaceutical agents continue to dominate research, attention increasingly has been directed to natural product. The success of artemisinin, isolated from *Artemisia annua*, and its derivatives for the treatment of resistant malaria has focused attention on the plants as a source of antimalarial drugs (Etkin, 2003).

New antimalarial drugs must meet the requirements of rapid efficacy, minimal toxicity and low cost. Immediate prospects for drugs to replace chloroquine and Sulphadoxine-pyrimethamine (SP) include amodiaquine (a CQ-like quinoline) and chlorproguanil (**9**)-dapsone (**10**). These replacements will probably provide a few years of efficacy, particularly in Africa. However, they already suffer from some cross resistance with CQ and SP. This increases the likelihood that full blown resistance to these drugs will emerge rapidly (Winstanley, 2001; Lang and Greenwood, 2003). High on the list of mid-term replacements are artemisinin derivatives. However, these drugs have very short half-lives, which necessitate their use in combination with a longer acting drug. Therefore, new drugs

are needed in order to avoid an ever increasing toll of malaria on tropical areas. It is imperative to rapidly put into action strategic plans for the discovery and development of novel antimalarial compounds that are not affected by pre-existing mechanisms of drug resistance. Ideally, new drugs for uncomplicated *P. falciparum* malaria should be efficacious against drug resistant strains. They should provide cure within a reasonable time (ideally three days or less) to ensure good compliance, be safe, suitable for small children and pregnant women. In addition they should have appropriate formulations for oral use and, above all, be affordable (Ridley, 2002; Nwaka and Ridley, 2003).



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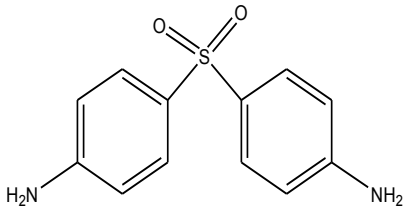

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2.4.1 Spreading and prevention of resistance

There is no single factor that explains the greatest degree of influence on the spread of resistance. However a number of causes associated with an increase have been acknowledged. These include aspects of economics, human behaviors, pharmokinectics and the biology of vectors and parasites. Preventing malaria infections developing has a major effect on the potential rate of development of resistance by directly reducing the number of cases of malaria which decreases the requirement for antimalarial therapy. Preventing the transmission of resistant parasites limits the risk of resistant malarial infections becoming endemic. This can be controlled by a variety of non-medical methods including insecticide treated bed nets, indoor residual spraying, environmental controls (such as swamps draining) and personal protective methods such as mosquito repellents. Chemoprophylaxis is also important in the transmission of malaria and resistance in defined populations (for example travelers) (Center for Disease Control, 2012).

A hope for future of antimalarial therapy is the development of an effective malaria vaccine. This could have enormous public benefits, providing a cost effective and easily applicable approach to preventing onset of malaria. It also prevents the transmissions of gametocytes, which reduces the risk of resistance developing. Antimalarial therapy could be also be diversified by combining a potentially effective vaccine with current chemotherapy, which will be reducing the chance of vaccine resistance developing (WHO, 2010).

2.5 Ethnobotanical survey done on some antimalarial medicinal plants

Ethnobotany is the study of how communities of a particular region use indigenous plants for food, clothing, medicine and other activities (Aiyeloja and Bello, 2006). The documentation is crucial for the conservation and utilization of biological resources (Muthu *et al*., 2006).

Ethno-medicinal study is today recognized as the most viable method of identifying new medicinal plants or refocusing on those earlier reported for bioactive constituents (Adjanahoun *et al*., 1991). Ethnobotanical survey is an important step in the identification, selection and development of the therapeutic agents from medicinal plants. In Ethnobotany and natural products chemistry, the mode of preparation and administration of herbal preparations are often crucial variables in determining efficacy in pharmacological evaluation. Use of phytomedicines for treatment of fevers and malaria is a known practice in many rural communities in Africa. However, these plants are taken orally by the people without any consideration of possible toxic effect of components in such plants. The surveys have shown that these traditional medicines have been found to be effective especially in the treatment of malaria which is of great concern to any African nation (WHO, 2002).

The constant evolution of the malaria parasite has rendered the cheapest and most widely available antimalarial treatments ineffective. This is because of the recent reports on increasing resistance of *P. falciparum* to artemisinin based compounds (Cui *et al*., 2012). Accordingly, there is deep concern that this parasite will soon develop total resistance to such orthodox treatments. Therefore, the search for newer more effective malaria cures is a major force of global research to day. This calls for an urgent need to explore and utilize the naturally endowed rich biodiversity of indigenous communities through research that could translate to benefits for mankind. Such investigations on medicinal and beneficial plants could provide useful leads for the synthesis of important active compounds. Various studies have been documented with over 1200 plant species from 160 families used in the treatment of malaria or fever (Willcox and Bodeker, 2004). Similar investigations have been carried out in many African nations like Ethiopia (Bekalo *et al*., 2009), Kenya (Njoroge and Bussmann, 2006; Muthaura *et al*., 2007), Ghana (Asase *et al*., 2005), Cameroon (Titanji *et al*., 2008), Nigeria (Idowu *et al*., 2010; Dike *et al*., 2012) and Zimbabwe (Ngarivhume*et al*., 2015)

Many communities in Africa have much elaborated plant knowledge (Barrow, 1996). Most knowledge on medicinal plants is transferred orally in these communities (Fratkin, 1996). There is therefore the danger of losing this precious cultural heritage. In view of the rapid loss of natural habitats, traditional community life, cultural diversity and knowledge of

medicinal plants, an increasing number of ethnobotanical inventories need to be established (Van Wyk and Gericke, 2000). Traditional medicines are widely used in many parts of the world and their importance is on the rise. To many communities, they are perceived to provide holistic treatment for physical as well as psycho-spiritual illnesses therefore preferred to orthodox medicine. However, knowledge of traditional practices and materials is being lost with time because of non-documentation. The problem is aggravated by extermination of indigenous resources with environmental degradation and deforestation (Tabuti *et al*., 2003).

Forty-one species belonging to 17 families were encountered during a study in Ghana. Of the 17 families studied, Leguminosae and Anacardiaceae predominated in terms of number of species used to treat malaria. Eight plant species which had not been previously documented for the treatment of malaria in Ghana were identified (Asase *et al*., 2005). *Azadirachta indica* (Meliaceae), *Morinda lucida* (Rubiaceae) and *Nauclea latifolia* (Rubiaceae) which were noted to have been utilized in the treatment of malaria in Ghana were also identified to be used in the South-Western regions of Nigeria (Mshana *et al*., 2001).

Dike *et al.* (2012) carried out an ethnobotanical survey for potential antimalarial plants in South-western Nigeria. Information in this survey was collected by interviewing indigenous people, using a semi-structured questionnaire. Twenty two plant species used in the treatment of malaria belonging to 18 families were identified and documented. Information such as common vernacular names, plant parts used methods of preparation and previous scientific reports were documented. Of the plants identified during the survey, *Azadirachta indica* (12.9%), *Alstonia congensis* (11.9%) and *Cymbopogon citratus* (11.3%) showed the highest incidence of encounter, whereas *Nauclea latifolia* recorded the lowest incidence of encounter (0.2%). The traditional usage of *Persea americana* and *Ludwigia peruviana* in the treatment of malaria was reported for the first time. They concluded that although a large number of traditionally used plants for the treatment of malaria were identified, scientific validation of the traditional claims of antimalarial properties was needed.

Idowu *et al*. (2010) carried out an ethnobotanical survey of herbal medicine used for treatment of malaria fever in 17 communities in Ogun State, South-West Nigeria. According to their results, 38 plant species belonging to 24 families were used in herbal antimalarial recipes. Among the plants mentioned, the most frequently used were *Morinda lucida* (7.87%), *Lawsonia inermis* (7.41%), *Citrus medica* (6.94%), *Sarcocephalus latifolius* (6.48%) and *Morinda morindiodes* (6.48%). Results showed that irrespective of plant and part (leave, fruit, stem bark or root bark) or combinations of the plant parts, water and aqueous extract from fermented maize were the main medium of herbal antimalarial

preparations. Modes adminstration included drinking, bathing and steam inhalation of the aqueous herbal preparations for 4-10 days or until symptoms of malaria disappear. About 65% of all the plants mentioned in the survey have been documented to have toxic effect on the liver and kidney of experimental mice. Continuous consumption of these plants could therefore have pathological effects on the consumers. Therefore, this shows the need for more research in order to identify lead compounds in indigenous antimalarial plants with less or no toxicity.

Ngarivhume *et al*. (2015) carried out an ethnobotanical survey to document how malaria is conceptualized and diagnosed by traditional healers in Zimbabwe. Twenty six species were identified which comprised of trees (38.5%), shrubs (30.8%), climbers (23.0%) and herbs (7.7%), belonging to 16 families. Most plant species belong to the Apocynaceae (15.4%), Compositae (11.5%) and Leguminosae (11.5%), followed by the Aristolochiaceae, Rubiaceae and Cucurbitaceae (7.7%). The other ten families contributed one species each (3.8%). Most of the plant parts used to treat malaria are stored as dried powders in closed bottles. The powders are always soaked in hot or cold water and the infusion is taken as the active medicine. Only 25% of the healers referred the malaria patients that do not respond to their treatment to hospital. Their survey underscores the need to preserve and document traditional healing for managing malaria. This is for purpose for more future scientific research on the plants to determine their efficacy and their safety.

Muthaura *et al.* (2007) in their study found out that in Kenya, most people especially in rural areas use traditional medicine and medicinal plants to treat many diseases including malaria. They documented medicinal plants traditionally used to treat malaria by the Digo community of Kwale district. Traditional health practitioners were interviewed with standardized questionnaires in order to obtain information on medicinal plants traditional used for management of malaria. Twenty five species in 21 genera and 16 families were encountered during the study. Celestraceae, Leguminosae and Rubiaceae families represented the species, most commonly used while three plant species were documented for the first time for the treatment of malaria.

According to literature there is no record of indigenous antimalarial herbs commonly used in Butebo County in Eastern Uganda. However, a number of surveys have been carried out in Uganda to document the use of antimalarial herbal medicines (Namukobe *et al*., 2011; Anywar *et al*., 2016; Tugume *et al*; 2016). It is assumed that these studies can help in documenting data that will assist policy makers. But few studies have been undertaken to

investigate the antiplasmodial efficacy and safety of plants claimed to have antimalarial therapeutic value (Willcox, 1999).

Stangeland *et al*. (2011) performed an ethnobotanical study of plants used to treat malaria in Nyakayojo Subcounty in South Western Uganda. Thirty two (67%) of the species used by the respondents are documented for antimalarial use in other studies, and nearly half (44%) had documented antiplasmodial activity. Fifty six species, distributed among 47 genera and 23 families, were reported to be used. Review of ethnomedical use relevant to malaria, biological activity and chemical studies of the 48 scientifically identified species was done. *Vernonia amygdalina* was by far the most commonly used plant (86% of respondents), and mainly used were the leaves. Other commonly used plants included *Aloe species*, *Justicia betonica*, *Vernonia adoensis* and *Tithonia diversifolia* by 68, 39, 29 and 21% of the respondents. It was common to use several plants together in a recipe (43% of recipes). The most common mode of administration was oral, but bathing and steam baths were used as well. Leaves were by far the most commonly used plant part (in 85 % of recipes). The literature survey may indicate a possible explanation for the use of several plants.

A study on ethanomedicinal use, preference for species, and ecological viability of plants used for treating malaria was carried out among the communities living around the Sango Bay Forest Reserve in Southern Uganda. In this study semi-structured interviews and informal discussions were used to collect ethnobotanical information. Sixteen species representing 11 families and 14 genera were reportedly used to treat malaria, including four new reports. The three species were found to be highly valued in the treatment of malaria: *Hallea rubrostipulata* (Rubiaceae), *Warburgia ugandensis* (Canellaceae) and *Syzygium guineense* (Myrtaceae) and similarly used by the local people as determined by the clustering procedure. The recognition of the use of traditional medicine by the local communities as an integral and essential part of their health care system is vital in the conservation and sustainable utilization of these plants (Ssegawa and Kasenene, 2007).

An ethnobotanical survey on plants used for traditional treatment of malaria in Mbarara District, Western Uganda was also investigated. From this study 20 species belonging to 18 genera and 12 families were identified. These include; *Vernonia amygdalina, Aloe sp., Pseudarthria hookeri, Lantana trifolia, Mangifera indica, Venonia lasipus, Cassia occidentalis, Conyza sp., Nicotiana tabaccum, Maesa lanceolata* and many others. Most of the plants were from the family Asteraceae. Other families included Verbenaceae, Anacardiaceae, Agavaceae, Fabaceae, Cucurbitaceae, Solanaceae, Labiatae, Rutaceae, Flacourtiaceae, Ceasalpinoidea and Myrsinaceae. Plant parts were used either singly or in

combinations for the treatment of malaria. The medicines are prepared by adding water to freshly pounded materials then is squeezed to obtain a liquid that is taken orally (decoctions). Also dried or fresh materials are boiled in water and the liquid taken orally (concoctions). The most commonly used plant parts were the leaves. Water was the most common solvent and the oral route was the most commonly used method of administration. From their study they concluded that a number of plants in Mbarara district can be used as sources of herbal remedies for malaria. A number of these plants can be included in the national primary healthcare package after scientific studies on safety and efficacy are done (Katuura *et al*., 2007).

Although there is widespread use of traditional herbal remedies in the management of malaria, scientific understanding of the plants is however, largely unexplored (WHO, 2002). Ethnobotanical information about antimalarial plants used in traditional herbal medicine, is essential for further evaluation of the efficacy and safety of plant antimalarial remedies. Efforts are now being directed towards discovery and development of new chemically diverse antimalarial agents (Clarkson *et al*., 2004). The reputed efficacies of these plants have been experienced and passed on from one generation to the other. Promising herbal medicines identified in this way can then be subjected to pharmacological screening, toxicological screening, and phytochemical analysis. Clinical trials to confirm their efficacy, safety and determining the administration doses should be investigated (WHO, 2000).

2.6 Classes of phytochemical compounds from antimalarial medicinal plants

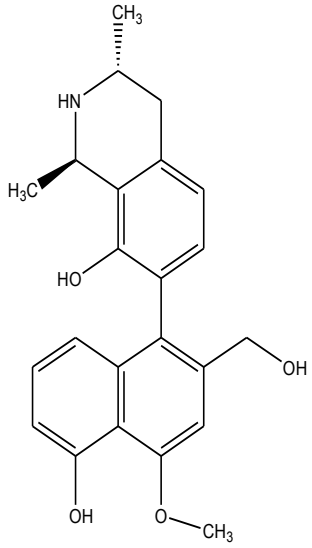
The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body (Zhang and Bjorn, 2009). The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, phenolic compounds and others (Edeoga *et al.,* 2005). The antiplasmodial activity has been linked to a range of several classes of the secondary plant metabolites including alkaloids, sesquiterpenes, triterpenes, flavanoids, limonoids, quassinoids, xanthones, quinones and phenolic compounds. Among these, alkaloids have been the most important and have shown very interesting antiplasmodial activities (Saxena *et al*., 2003; Rukunga and Simons, 2006).

2.6.1 Alkaloids

Alkaloids are naturally occurring amines that have heterocyclic structures and occur in approximately 20% of all plant species (Rao *et al.,* 2009; Zhang and Bjorn, 2009). One of the oldest and most important antimalarial drugs, quinine, belongs to this class of compounds and is still relevant. A few of naturally occurring alkaloids belonging to different groups are described below.

Naphthylisoquinoline alkaloids have shown remarkable activity against *P. falciparum in vivo* and *in vitro* models. Extracts from the species of *Triphophyllum peltatum* (Dioncophyllaceae) and the isolated compounds; dioncopeltine A (**11**), dioncophylline B (**12**) and C (**13**) exhibited high antiplasmodial activity. Dioncopeltine A was shown to suppress *Parasitaemia* almost totally while dioncophylline C cured infected mice completely after oral treatment with 50 mg/kg daily for 4 days without noticeable toxic effects (Francois *et al*., 1997). The dimeric antiplasmodial napthylisoquinoline alkaloid heterodimer, korundamine A (**14**), has been isolated from another species, *Ancistrocladus korupensis* in the same family. It is one of the most potent naturally occurring antiplasmodial naphthylisoquinoline dimmers yet identified by *in vitro* screening with an EC_{50} of 1.1 μg/mL against *P. falciparum* (Hallock *et al*., 1998).

A number of different *bis*benzylisoquinolines alkaloids with antiprotozoal activity have been identified. *In vitro* antiplasmodial activity (IC_{50}) of most bisbenzylisoquinolines is below 1.0 μg/mL. For instance, pycnamine (**15**) from *Trichilia* spp. was found to have IC_{50} value of 0.15 μg/mL. However, monomeric benzylisoquinolines do not have potential antiplasmodial activity (Kayser *et al*., 1998).



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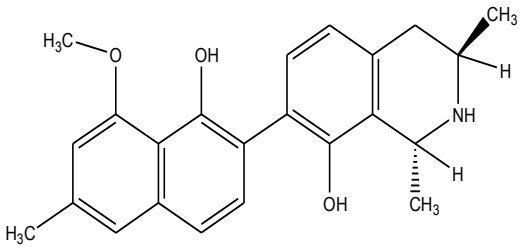

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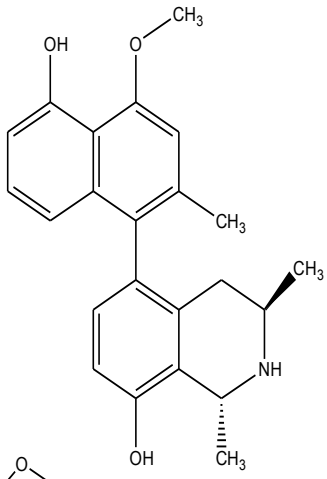

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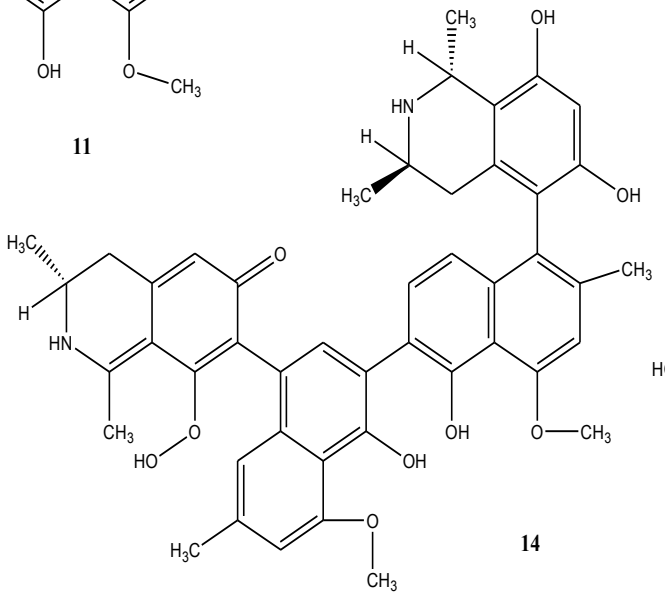

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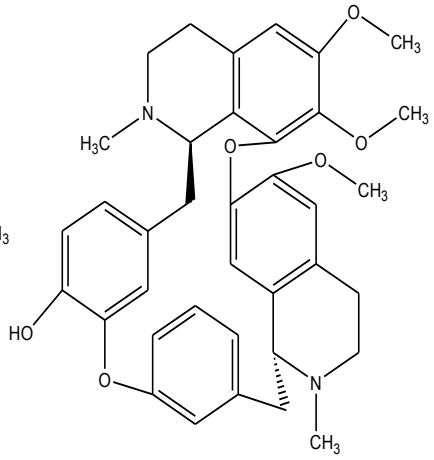

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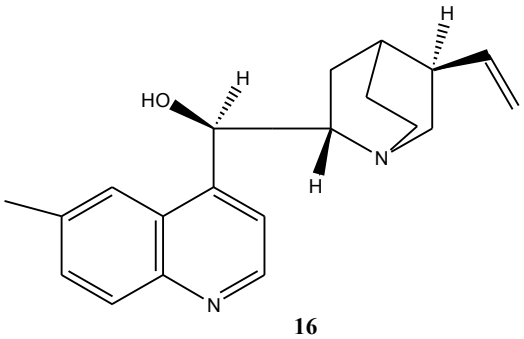
Quinoline alkaloids the group to which quinine belongs has been used up to the middle of this century for the treatment of malaria. Following the widespread development of chloroquine-resistant strains of *P. falciparum* it has become important again (Kayser *et al*., 1998). Quinine (**16**) was the lead structure in the discovery of synthetic derivatives (like chloroquine and mefloquine that have higher anti-malarial activity.

Indoles comprise of a group of alkaloids with varied biological activity in which the indole sub-structure is widely distributed in the plant kingdom. Some indoles are reported to possess antiprotozoal activity for instance; Cryptolepine (**17**) and related indole-quinolines isolated from *Cryptolepis sanguinolenta* were active *in vitro* against *P. falciparum* strains W2, D6 and K1 with IC_{50} ranging from 27-41 µg/mL (Kayser *et al*., 1998).



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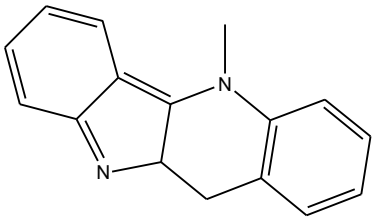

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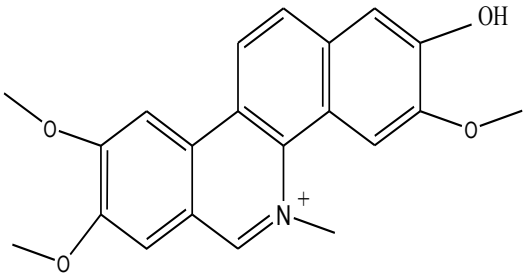
These alkaloids are mostly found within three plant families only Papaveraceae, Fumariaceae and Rutaceae (Krane *et al*., 1984). Some examples of benzophenanthridine alkaloids obtained from plant sources include fagaronine (**18**) and nitidine (**19**). Antimalarial activities of these alkaloids ranged from IC_{50} 9 to 108 µg/mL against *P. falciparum* (Gakunju *et al*., 1995).



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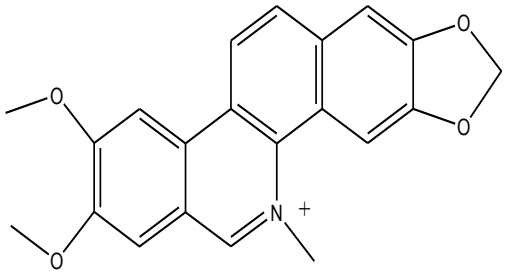

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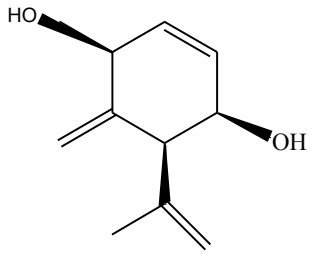
2.6.2 Terpenoids

In plants, terpenoids represent a chemical defense against environmental stress and provide a repair mechanism for wounds and injuries. Interestingly, effective ingredients in several plant derived medicinal extracts are also terpenoid compounds of monoterpenoid, sesquiterpenoid, diterpenoid, triterpenoid and carotenoid groups (Salminen *et al.,* 2008; Gordien *et al.,* 2009).

Monoterpenes are examples of simple antiprotozoal drugs. Piquerol A (**20**) isolated from *Oxandra espinata* (Annonaceae) was shown to exhibit an IC_{50} value of 100 µg/mL against *P. falciparum* (Kayser *et al*., 1998). This was relatively very low activity compared to many other compounds.

The discovery of artemisinin (**21**), a novel sesquiterpene lactone endoperoxide, an antimalarial constituent from the Chinese plant *Artemisia annua*, prompted the investigation

of some other naturally occurring peroxides for their antiplasmodial activity. Klayman (1985) investigated this plant that has been used for many centuries in Chinese traditional medicine for the treatment of fever and malaria. The sesquiterpene lactone with a peroxide grouping is responsible for its reputable antiplasmodial activity (IC_{50} = 3.4 µg/mL).



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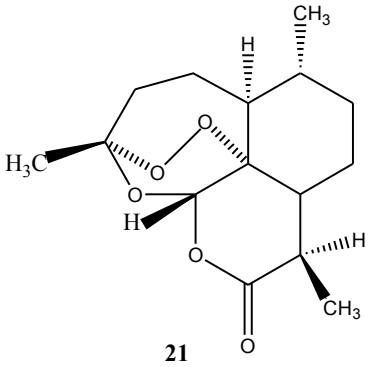

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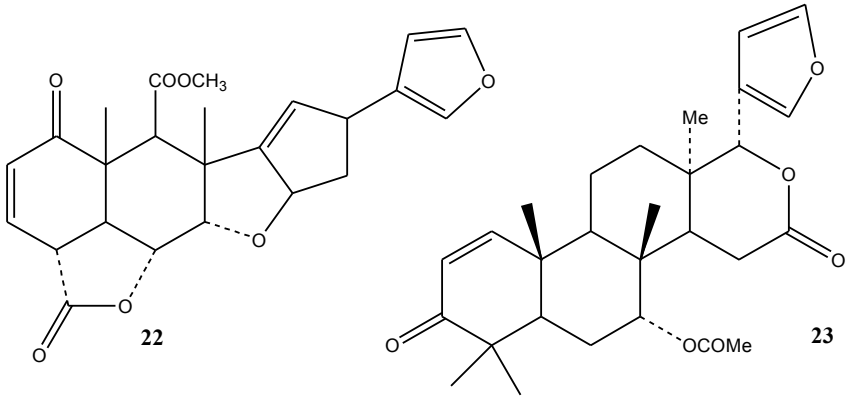
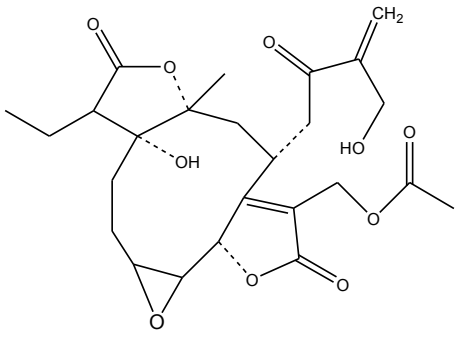
Limonoids are also known as bitter terpenoids (Kayser *et al*., 1998). One well known plant family rich in these is Meliaceae. *Azadirachta indica*, the neem tree, widely used as an antiplasmodial plant in Asia belongs to this family. Nimbolide (**22**) (IC_{50} = 0.95 µg/mL, *P. falciparum* K1) was the first to be identified as the active antiplasmodial principle of the neem tree. Subsequently, gedunin (**23**) was also found to be active *in vitro* against *P. falciparum* parasites with IC_{50} values in the range of 0.72-1.74 µg/mL (Khalid *et al*., 1989; MacKinnon *et al*., 1997).

A study on the seed extracts of neem, for its inhibition of the growth and development of asexual and sexual stages was investigated. The extracts were tested on drug sensitive and resistant strains of human malaria parasite *P. falciparum*. The antiplasmodial effect of the neem components was observed on parasites previously shown to be resistant to other antimalarial drugs like chloroquine and pyrimethamine. The activity was due to limonoids of which gedunin (**23**) was the most active and showed activity, IC_{50} = 0.72 µg/mL (Bray *et al*., 1990; Dhar *et al*., 1998).



Diterpenoids from many plant species are well known for their biological activity and are among the most widely distributed terpenoids in the plant kingdom (Kayser *et al*., 1998). However, most of them contain high anti-parasitic activity with high cytotoxicity to mammalian cells. The macrocyclic germacrane dilactone-16,17-dihydrobrachy-calyxolide (**44**), from *Vernonia brachycalyx* (Asteraceae) showed antiplasmodial activity (IC_{50} = 17 µg/mL on *P. falciparum*). However, it also inhibited the proliferation of human lymphocytes at the same concentration indicating general toxicity (Oketch-Rabah *et al*., 1998). Other antiplasmodial diterpenoids are phytol (**45**) and 6-*E*-geranylgeraniol-19-oic acid (**46**) isolated from *Microglossa pyrifolia* (Asteraceae). These compounds have been found to have high antiplasmodial activity, IC_{50} 8.5 µg/mL (PoW), 11.5 µg/mL (Dd2) and IC_{50} 12.9 µg/mL (PoW), 15.6 µg/mL (Dd2), respectively (Köhler *et al*., 2002).



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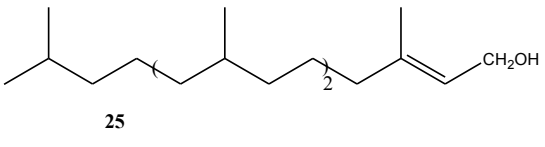

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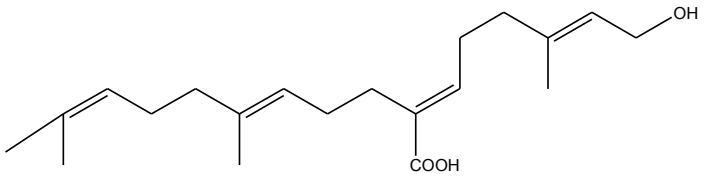

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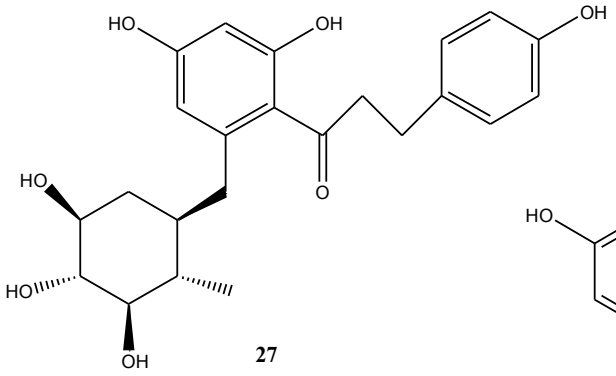
2.6.3 Chalcones

Phlorizidin (**27**), from *Micromelum tephrocarpum* (Rutaceae), was one of the first chalcone glycoside reported to exhibit anti-parasitic activity (Kayser *et al*., 1998). In ethno-medicine *M. tephrocarpum* is used to treat malaria because of the bitter taste, a property shared with quinine (**16**) and other antimalarial herbs. The most promising compound in this class of natural products is licochalcone A (**28**). It was first isolated from *Glycyrrhiza glabra* (Liquorice) (Fabaceae) and was the subject of intensive preclinical studies (Chen *et al*., 1994).



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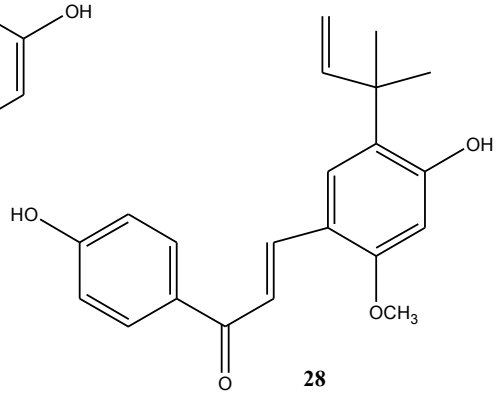

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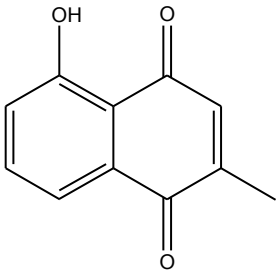
2.6.4 Naphthoquinones

Plumbagin (**29**), a cytotoxic napthoquinone has been isolated from *Plumbago zeylanica* and found to exhibit antiplasmodial activity (IC_{50} 178.12 and 188.8 µg/mL) against chloroquine-sensitive (D6) and resistant (W2) isolates, respectively (Lin *et al*., 2003).



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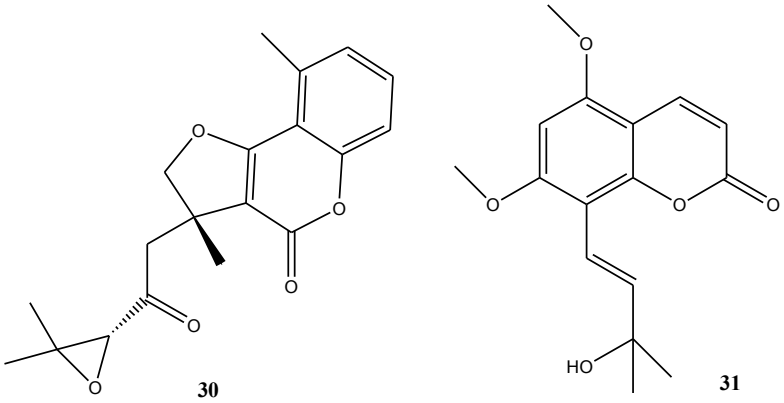
2.6.5 Coumarins

The antiplasmodial activity of 2'-*epi*cycloisobrachycoumarinone (**30**) epoxide and its stereoisomer isolated from *Vernonia brachycalyx*, has been reported. Both stereoisomers show similar *in vitro* activity against chloroquine-sensitive and chloroquine-resistant strains

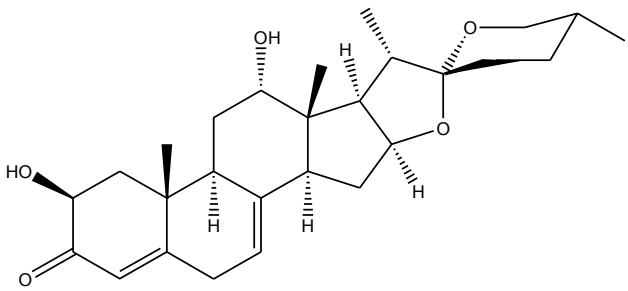
of *P. falciparum* with IC_{50} values of 0.11 and 0.15 µg/mL, respectively (Oketch-Rabah *et al*., 1997b). A new coumarin derivative, 5,7-dimethoxy-8-(3'-hydroxy-3'-methyl-1'-buteneyl)-coumarin (**31**), was isolated from *Toddalia asiatica.* This compound was found to have IC_{50} values of 16.2 and 8.8 µg/mL against CQ-sensitive and CQ-resistant strains of *P. falciparum*, respectively (Oketch-Rabah *et al*., 2000).



30 **31**

2.6.6 Saponins

Saponins from plant sources are known for their biological activity, but exhibit some toxicity to humans and other mammals. The use of saponins as drugs is limited to due to the poor bio-availability, reduced absorption in the gastrointestinal tract and haemolytic toxicity when given orally. The plant *Asparagus africanus* (Liliaceae) yielded a new steroidal saponin, muzanzagenin (**32**) that exhibited antiplasmodial activity of EC_{50} = 61 µM against K39 isolate of *P. falciparum,* was isolated (Oketch-Rabah *et al*., 1997a).



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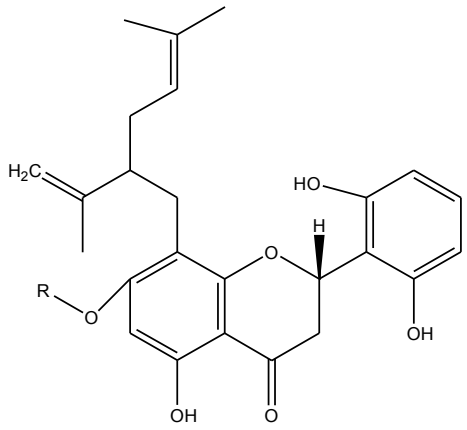
2.6.7 Flavonoids

Flavonoids are widespread in fruits, vegetables, teas and other medicinal plants. They have received great attention and have been studied extensively. This is because they are a group of biologically active plant compounds (Yuan *et al*., 2009). They are perhaps among

the thirty most important single groups of phenolics comprising of a group of over 4,000 aromatic plant compounds. These include anthocyanins, proanthocyanidins, flavonols and catechins (Carvalho *et al*., 2010). Following the detection of antiplasmodial flavonoids from *Artemisia annua* (Asteraceae), this class of compounds has attracted renewed interest. As part of a multi-disciplinary research programme on antiplasmodial drugs, additional *Artemisia* species have been screened in Thailand. The exiguaflavanone A (**51**) and B (**52)** isolated from *Artemisia indica* (Asteraceae) exhibited *in vitro* activity against *P. falciparum* with EC_{50}values of 4.6 and 7.1 µg/mL, respectively (Chanphen *et al*., 1998).

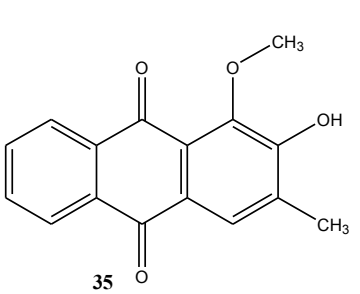


33, R = H
34, R = CH_3

2.6.8 Simple aromatic compounds

Anthraquinones and xanthones belong to a group related to naphthoquinones in structure and biological activity. The main chemical difference between the groups is the tricyclic aromatic system with a *para*-quinoid substitution. Anthraquinones isolated from the tropical tree *Morinda lucida* (Rubiaceae) were tested for antiplasmodial activity *in vitro*. Digitolutein (**35**), rubiadin-1-methyl ether (**36**) and damnacanthal (**37**) showed activity on Chloroquine-resistant *P. falciparum* isolate (EC_{50} = 21.4-82.9 µM) (Sittie *et al*., 1999). Other rare anthraquinones have been identified as potential antiplasmodial drugs. From *Psychotria camponutans* (Rubiaceae), the benzoisoquinoline-5-10-dione (**38**) has been isolated and tested against *P. falciparum* (EC_{50} = 0.84 µg/mL) (Solis *et al*., 1995). Antiplasmodial xanthnones have been isolated from *Garcinia cowa* (Guttiferae). Preliminary screening of five prenylated xanthones demonstrated significant activity against *P. falciparum in vitro* with IC_{50} ranging between 1.5 and 3.0 µg/mL. Cowaxanthone (**39**) displayed high

antiplasmodial potential (EC_{50} = 1.5 µg/mL) compared to that of Pyrmethamine (IC_{50} = 2.8 µg/mL) (Likhitwitayawuid *et al*., 1998).



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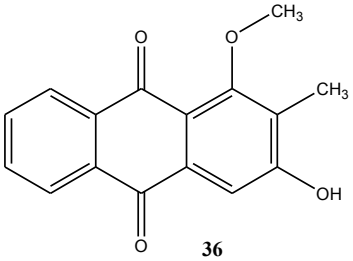

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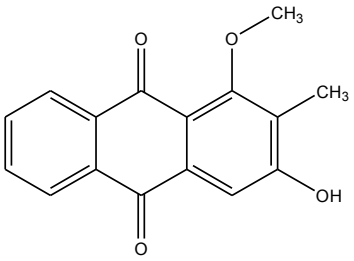

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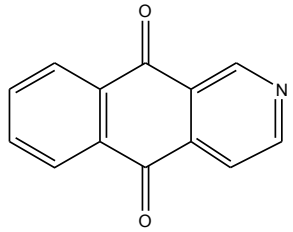

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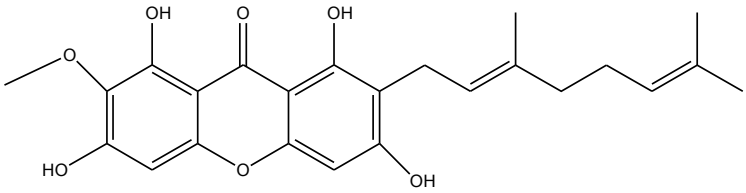

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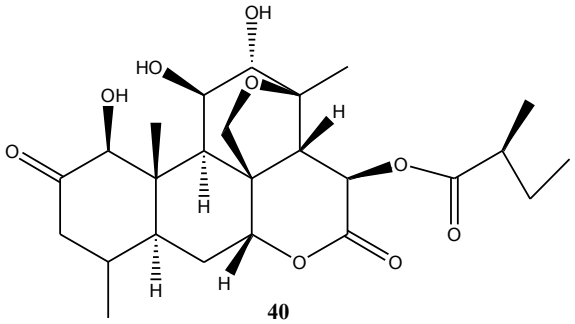
2.6.9 Quassinoids

The quassinoids are heavily oxygenated lactones with majority of C_{20} basic skeleton named as picrasane. However, C_{18}, C_{19} and C_{25} quassinoids are also known. They have varying numbers of different oxygen-containing groups. A wide spectrum of biological properties has been reported from this class of compounds. They are biosynthetically related to triterpenoids and share the same metabolic precursors. The most active compound in this group is simalikalactone D (**40**) from *Simaba guianensis* (Simaroubaceae) with IC_{50} < 1.7 µg/mL. Activity of the compounds in this group is due to the methylene-oxygen bridge (Cabral *et al*., 1993).



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2.7 Biosynthesis of some secondary metabolites

2.7.1 The mevalonate pathway to steroid synthesis

Triterpenes are a group of structurally diverse C_{30} molecules, consisting of six isoprene units. They are all derived from the basic biosynthetic precursor of all terpenoids. The sterol skeletons possess 5α, 8β, 10β, 13β, 14α, 17α, and 20α configurations, consistent with their stereospecific formation from the protosteroid cation (**Figure 3**). The dimethylallyl diphosphate (DMAPP) combines with isopentenyl diphosphate (IPP) *via* the enzyme prenyl transferase to produce geranyl diphosphate (GPP). The addition of a C5 unit to GPP yields farnesyl diphosphate (FPP) (Mercer, 1984).

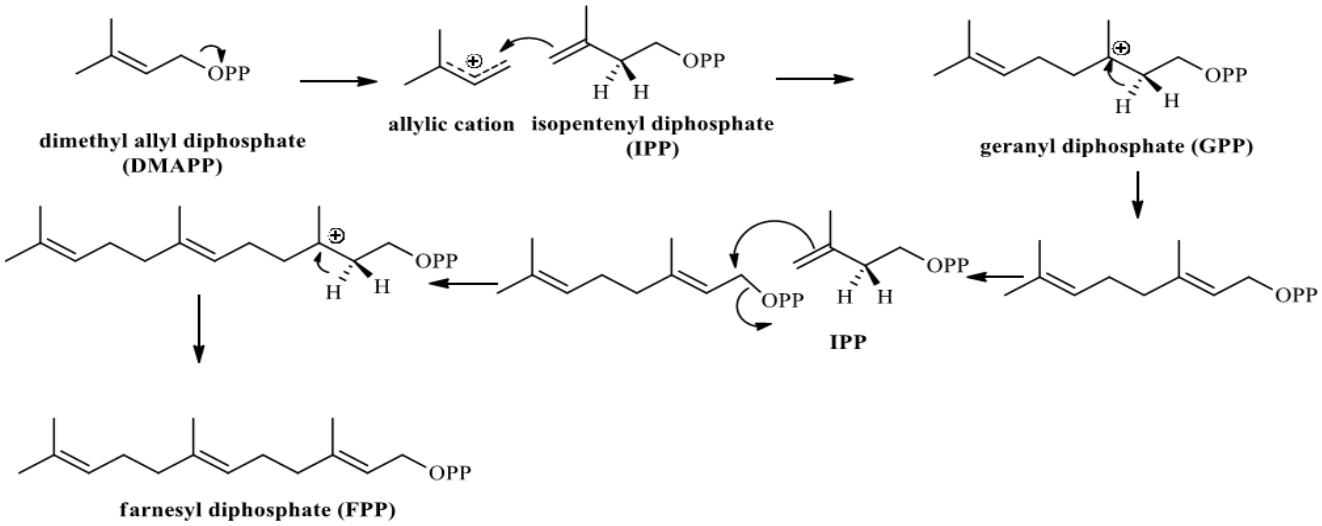


Figure 3: Formation of farnesyl diphosphate (FPP) (Dewick, 2002

Triterpenes (C_{30}) are formed by two molecules of FPP joining togethertail to tail to yield C_{30}acyclic polyene squalene (**Figure 4**) *via* the enzyme squalene transferase. The symmetric olefin undergoes oxidation to form *S*-oxidosqualene *via* reaction of NADPH and O_2 catalyzed by squalene epoxidase. The introduction of a proton to the epoxide group allows the opening of the ring and the generation of a tertiary carbocation. This allows electrophilic addition to the double bond, formation of a six-membered ring and the production of a new tertiary cation. This process continues producing a tertiary carbocation after each ring formation. The cyclization of squalene-2,3-oxide folded in a chair–boat– chair–boat– conformation generate a 17β-side chain (17α-hydrogen) at C_{20}. After the cyclization of 2,3-oxidosqualene to form lanosterol, several sequential transformations occur to form cholesterol in mammals and ergosterol in fungi. Loss of the C-4 methyl and C-14 methyl, reduction of C-24 double bond and a series of double bond shifts occur in the formation of

ergosterol. However, the synthesis of ergosterol has three additional steps, 15 resulting in two additional double bonds at C-7 and C-22 and a methyl group at C-24 of the ergosterol side chain. Ergosterol has an hydroxy group at C-3 of the sterol ring and a double bond at C-5. These structural differences make ergosterol remarkably suited for fulfilling both the cellular and the membrane requirements of the organism in which they are the most abundant sterol (Benveniste, 1986).

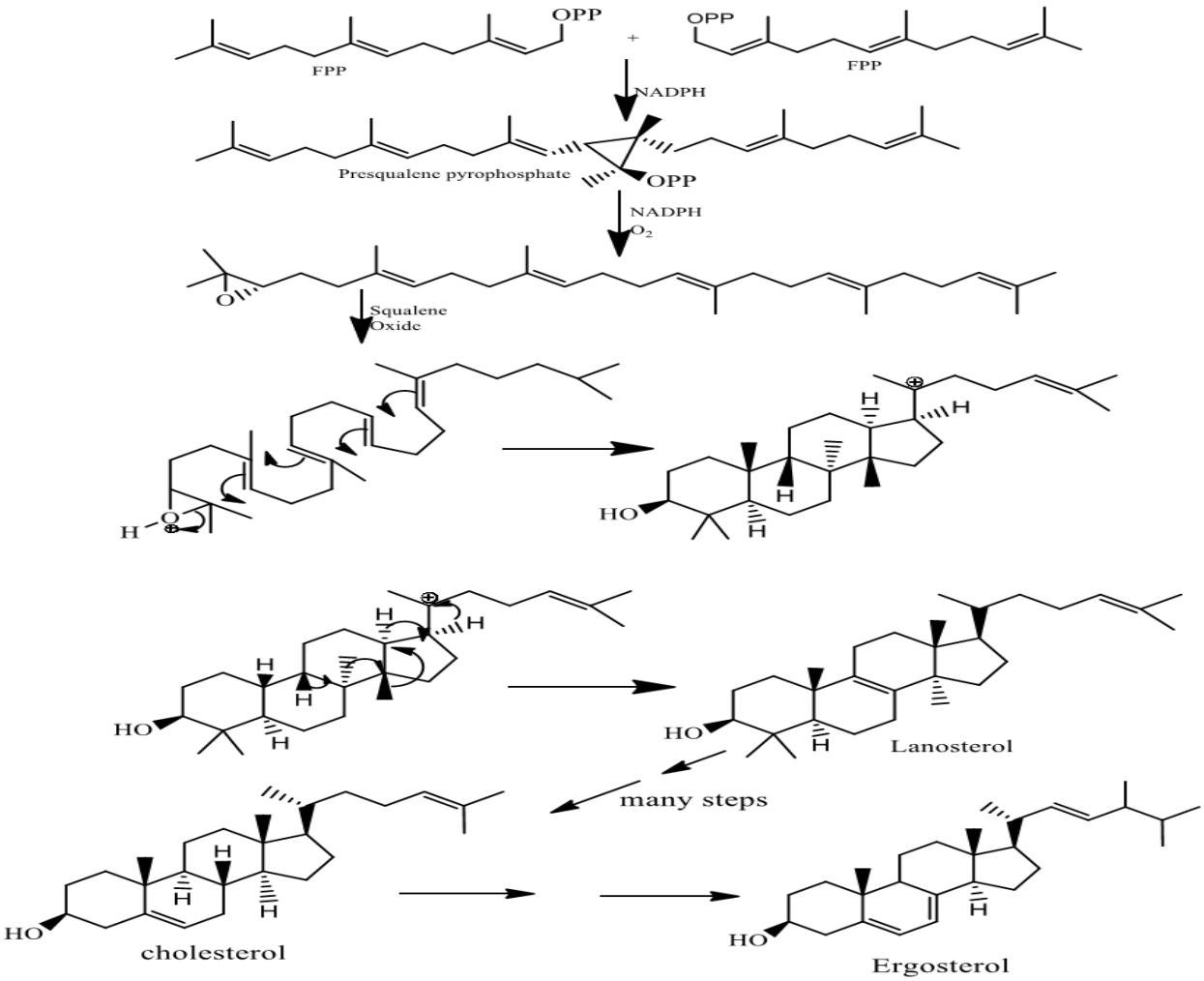


Figure 4: Biosynthetic pathway of triterpenoids from Farnesyl pyrophosphate (FPP) (Dewick, 2002)

2.8 Local use of the antimalarial medicinal plants and classification of the plants investigated

Nine plant species: *Croton macrostachyus*, *Oncoba spinosa*, *Steganotaenia araliacea, Acacia sieberiana, Ormocarpum trachycarpum, Acacia hockii, Euclea latideus, Cassia hirsute* and *Chamaecrista nigricans* were collected from different parts of the Butebo County. These plants were selected from the thirty three antimalarial plants documented

during an ethnobotanical survey after literature search using different data bases (Kodi *et al*., 2017). The plants are reportedly used for the treatment of malaria, fever and other diseases. However, the study investigated only three plants namely; *Oncoba spinosa, Euclea latideus* and *Acacia sieberiana* in the study. These plants had not been investigated with no documented antiplasmodial activity; toxicity assays carried out by other researchers. A summary of the local uses of the three plants is shown below (**Table 1**).

Table 1: Summary of plant species that were studied, their local names and uses.

Botanical name	Local name	Family name	Local uses
O. spinosa	Ekalepulepu	Flacourtiaceae	Malaria, epilepsy, syphilis, wounds skin disease, headaches, sexual impotence, stomach ache
E. latideus	Emusi	Ebenaceae	Malaria, swelling of legs, ringworms
A. sieberiana	Etiriri	Mimosaceae	Malaria, cough, epilepsy, Dysentery

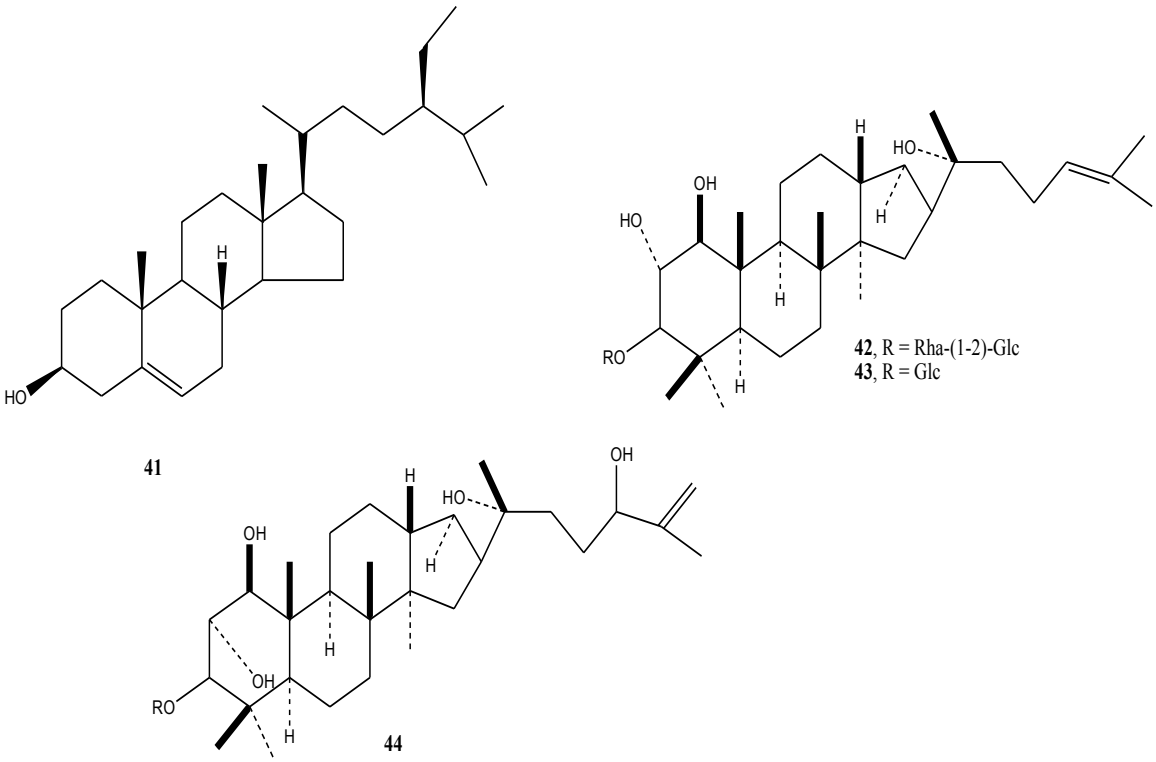
2.9 A review of the chemical investigations of the medicinal plants under study

In this section the phytochemical studies, biological activities and traditional uses of the three plants in the study are discussed. The investigations carried out on the phytochemical compounds isolated from the three plant species and those in the same genus are explained.

2.9.1 A review of the chemical investigations of the genus *Oncoba* (Flacourtiaceae)

Some species in the family of Flacourtiaceae are used as pain relievers by natives in the Amazon. This is not surprising, considering that the bark of temperate species of Salicaceae the same family, was the original source of aspirin. Several phytochemical constitutes have been reported which include: Phenolic glycosides, lignan and sterol like β-sitosterol (4**1**), polysaccharide, flavonoids and condensed tannins, alkaloids, clerodane diterpenes, terpenoids and sugars. Coumarins such as scoparone and aesculetin have also been reported. Other compounds include flacourtin, pyrocatechol, homaloside D, poliothrysoside, β-D-glucopyranoside, hesperitin, vicenin, lapachol, caprionic acid, casearia clerodane I to VI, casearvestrin A to C, hesperitin, ramantoside and butyrolactone lignan disaccharides (Balogun *et al*., 2013).

Previous phytochemical studies on the genus *Oncoba* afforded three tetracyclic triterpenoid from the species *O. mannii*. which included 1β,2α,3β,20(*R*)-tetrahydroxydammar-24-ene-3-*O*-β-*D*-glucopyranoside (4**2**), 1β,2α,3β,20(*R*)-24-pentahydroxydammar-25-ene-3-*O*-β-*D*-glucopyranoside (chilianoside H) (4**3**) and 1β,2α,3β,20(*R*)-tetrahydroxydammar-24-ene-3-*O*-α-*L*-rhamnopyranosyl-(1 → 2)-β-*D* glucopyranoside (4**4**) (Garo *et al*., 2009). Compounds (4**2**) and (4**3**) completely inhibited growth of two strains of *Staphylococcus aureus* including MRSA (strain ATCC BAA-40), at 16 µg/mL, while compound (4**4**) showed weak activity at the same concentration.



The use of *O. spinosa* (**Plate 1**) against *P. falciparum* parasites is not documented very well although it has been reported by some communities to treat malaria (Kodi *et al*., 2017). Different parts of the tree have been reported to have medicinal and non medicinal values. *O. spinosa* belongs to the family Flacourtiaceae (Salicaceae). It is a small tree of about 13 m high which grows under conditions of higher rainfall, deciduous, secondary and fringing forest from Senegal to West Cameroon. It is widely distributed in tropical Africa and Arabia (Hutchinson and Dalziel, 1954). The leaves of the plant are traditionally used in South-West of Nigeria for the treatment of diabetes and cancer, while the seed oil is drunk as

a fever remedy (Balogun *et al*., 2013). In Ivory Coast, the plant has a good is used as an aphrodisiac. In Tanzania the leaf sap is drunk as a remedy for malaria cure. Secondary metabolites consisting of tannins, steroids and triterpenoids have been found in the leaves, bark and roots, while traces of flavones in the leaves. The plant has given negative results in anti-biotic tests. A decoction made from small chips of the wood is drunk for a week as a treatment for stomach-ache and loss of appetite. The fruit, combined with karite butter (*Vitellaria paradoxa*), is also used in the treatment of stomach-ache and loss of appetite. Oil obtained from the seeds is taken orally for treating fever and when applied externally it has been used in the treatment of leprosy. However, the oil contains no chaulmoogric acid (**45**) and is considered useless against the leprosy *bacillus* although it has also been used in the treatment of other skin-complaints. A decoction of leafy twigs is used as a wash on sores while that the leaf-sap and roots, combined with *Schrebera trichoclada*, is used in the treatment of vertigo. The root is used as antidysenteric and a strengthening tonic (Balogun *et al*., 2013).

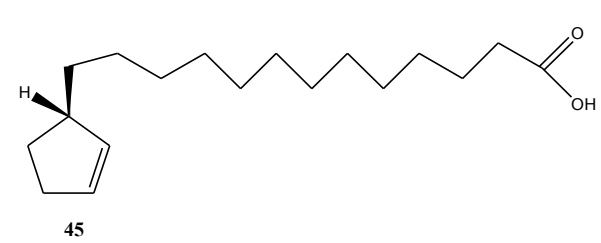
The α-glucosidase inhibitory, radical scavenging and cytotoxicity activities of the aqueous and chloroform extracts of the leaves of *O. spinosa* have been reported (Balogun *et al*., 2013). Their study showed that the crude and aqueous extracts showed better activities than the standard antidiabetic drug (acarbose) in a concentration dependent manner.

The aqueous and chloroform extracts had antioxidant activities that compares to ascorbic acid at concentrations 125 and 250 µg/mL. The brine shrimp lethal dose assay indicated the cytotoxicity of the chloroform and hexane extracts, this was further established using cervical (HeLa) and Lung (A549) cancer cell lines. The results support the folkloric use of the plant as antihyperglycemic and antineoplastic agent (Balogun *et al*., 2013).

The methanol extract of the fruits of this plant collected in Yemen demonstrated antimicrobial, anticancer and antioxidant activities. Phytochemical screening of *O. spinosa* leaves revealed the presence of anthraquinones, alkaloids, phenols, sterols, tannins, carbohydrates and flavonoids (Mothana *et al*., 2011).

Djouossi *et al.* (2015) in their effort to search for novel antimicrobial/antioxidant agents from Cameroonian medicinal plants used traditionally to treat human microbial infections and oxidative related diseases carried research on the plant. They investigated phytochemically and biologically the methanol extracts of the leaves of *O. spinosa* and isolated five known compounds. They included kaempferol (4**6**), quercetin (4**7**), apigenin-7-O-β-D-glucuronopyranoside (4**8**), quercetin-3-O-β-D-galactopyranoside (4**9**) and quercetin-3-O-α-L-rhamnopyranosyl (1→6) β-D-glucopyranoside (**50**). The *in vitro* antibacterial,

antifungal and antioxidant activities of the MeOH extracts and compounds (4**6-50**) were evaluated.



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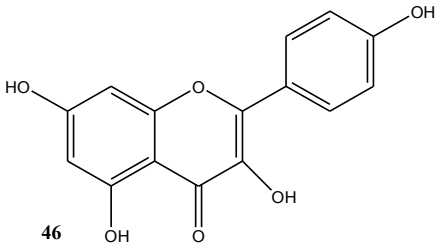

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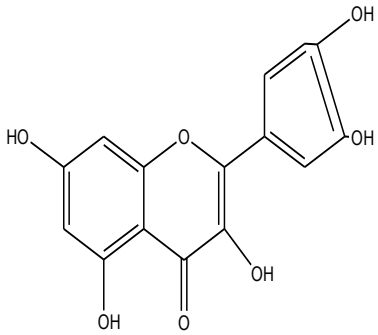

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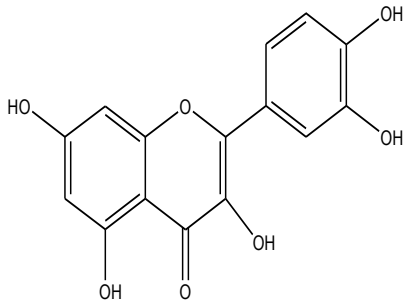

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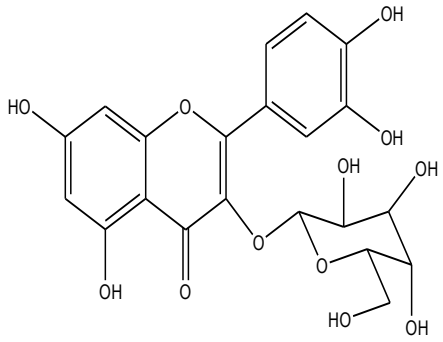

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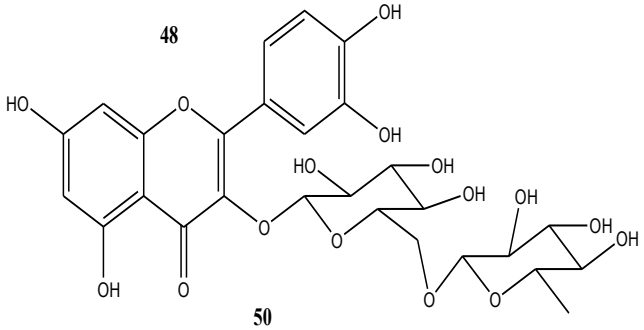

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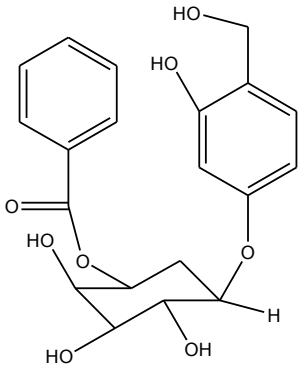
Another study was investigated on methanol crude extract of *O. spinosa* leaves. In this study isolation and determination of cytotoxic activities of the extracts and the isolated compound was conducted. Flacourtin, 3-hydroxy-4-hydroxymethylphenyl-6-*O*-benzoyl-β-D-glucopyranoside (**51**) was isolated and characterized. The LD_{50} values obtained for cytotoxic activities were less than 1000 µg/mL for flacourtin and the plant extracts. The preliminary toxicity assay indicated that flacourtin and the plant extracts were not cytotoxic therefore the traditional uses of the plant may pose no danger (Balogun *et al*., 2016).



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Addae-Kyereme *et al.* (2001) assessed the *in vitro* antiplasmodial activities of some Ghanaian plants traditionally used for fever/malaria treatment. In their investigation the methanol crude extract from the seeds of *O. spinosa* were tested against *P. falciparum.* Their results indicated that the methanol crude extract of the seeds had IC_{50} > 100 µg/mL that was considered not active. The literature on *Oncoba* genus shows that they treat a number of diseases and contain various active compounds. Therefore there was need to test the crude extracts for antiplasmodial, toxicity activities and determine the compounds present in *Oncoba spinosa* to justify its use by the local communities.

2.9.2 A review of the chemical investigations of the genus *Acacia* (Mimosaceae)

The family mimosaceae has 56 genera and 2800 species which are found in tropical and subtropical region. The Mimosaceae plants are climber (*Acacia caesia, Acacia pennata*), tree (*Acacia catechu, Albizia lebbeck, Prosopis* spp.), shrub (*Acacia farnesiana*), deciduous tree (*Acacia leucophalia*), tree (babul), low erect tree (*Acacia senegal*).

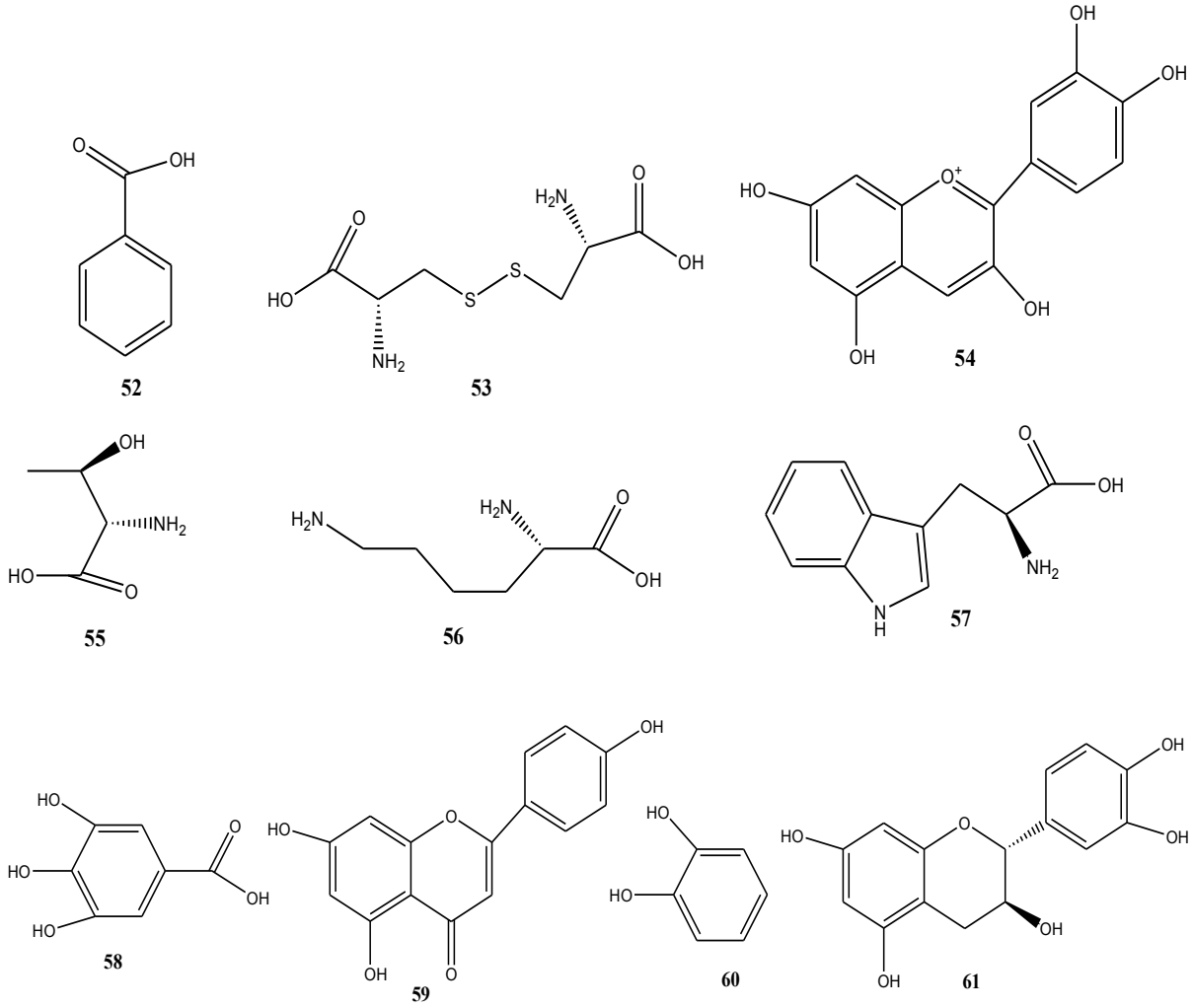
A. lebbeck is widely distributed in India and is also found in South Africa and Australia. It is a host of lac insect and is reported to have antiseptic, antidysentric and anti-tubercular activities. *A. caesia* is an important plant and its roots are used in pleurisy, measles, smallpox and bronchitis. It has cleansing property and protects the skin against microorganism. *A. catechu* is used in cancerous sores, bronchitis, pain in chest, strangulation of intestine and facilitating child birth. It's alcoholic (50% v/v) extract of stem has antiviral, antifungal and plasmolytic activity. *A. catechu* extract has the following chemical substances: flavonoids, β-galactose, λ-arabinose, δ-rhamnose, λ- glucuronic acid and aldobiuronic acid.

A. farnesiana is pharmagnostically used in treatment of convulsions (root), sores (powder), eye disease (leaf), epilepsy (stem bark), rabies (bark smeared and drunk), cholera

(as a prophylactic, with other plants), and snake bite. Its bark is used as astringent, demulcent and antifertility. The extract of this plant has the following chemical substances: benzaldehyde, anisaldehyde, decylaldehyde, cuminaldehyde, ketone, cresol, benzyl alcohol, linalool, terpineol, nerolidol, farnesol, ethylphenols, coumarin, butyric acid, palmitic acid, benzoic acid (**52**), salicylic acid, eicosane (Joshi, 1982). *Acacia modesta* is used as restorative and it has the following chemical components: oetacosanol, α-amyrin, betulin, ψ-sitosterol, γ-sitosterol and pinotol, octacosane. *Acacia nilotica*, is locally known as babul is used in syphilis, cholera, dysentery and leprosy.

A. *nilotica* in pharmacy is used in preparing emulsions, tablets, pills while the decoction of its bark is used in gurgle. It has following chemical components: galactose, l-arabinose, 4-oldobiuronic acids and arabinose, polyphenols, tannin, octacosanol, betulin, β-amyrin, β-sitosterol (4**1**), gallic acid and condensed tannins. *A. Senegal* traditionally is used externally and applied to nodules in leprosy. It is applied externally to cover sore nipples, burns and other inflamed areas. It has following chemical components: Ceryl alcohol, betulin, sitosterol, uvaol, octacosanol and β-amyrin. *Acacia sinuate* is used as a cathartic (leaf), an emetic, expectorant and apparent (Ali *et al*., 2012)

A number of plants from the genus of *Acacia* have been studied for antiplasmodial activity. Malviya *et al*. (2011) carried out a comprehensive review on ethnopharmacological claims of *A. nilotica*. The plant is richer source of cysteine (**53**), cyanidin (**54**), threonine (**55**), lysine (**56)**, tryptophan (**57**), potassium, phosphorus, magnesium, iron and manganese. Seeds contain high percentage of phenolic constituents consisting of *m*-digallic acid, gallic acid (**58**), protocatechuic and ellagic acids, leucocyanidin, and 3,4,5,7-tetrahydroxyflavan-3-ol and (-) epicatechol. The leaf contains apigenin (**59**), 6-8-bis-D-glucoside, rutin, 8% digestive protein (12.4% crude protein). The bark contains tannin (12-20%), terpenoids, saponins and glycosides, phlobetannin, gallic acid, protocatechuic acid, pyrocatechol (**60**), (+) – catechin (**61**), (-) epigallocatechin-5,7-digallate. The root contains octaconsanol, betulin, β-amyrin, Kaempferol and β-sitosterol. The gum is composed of galactoaraban which gives on hydrolysis L-arabinose, D-galactose, L-rhamnose, D-glucuronic acid and 4-O-methyl- D-glucuronic (Chaubal *et al*., 2006).



Antiplasmodial activity studies have been conducted on *A. nilotica*. One of the investigations was carried against *P. falciparum* 3D7 (CQ sensitive) and Dd2 (CQ resistant) strain. The ethyl acetate extract possessed the highest activity (IC_{50} = 1.5 µg/mL). The methanol extract of *A. nilotica* seed exerted high activity on both 3D7 and Dd2 strains with IC_{50} of 0.9 and 4.1 mg/mL, respectively (El-Tahir *et al*., 1999).

In another study aqueous root extract of *A. nilotica w*as analyzed for antiplasmodial activity in mice and acute toxicity. All doses of the extract administered produced significant, dose dependent, chemo suppressive activity against CQ-sensitive *P. berghei* NK 65 infected micein the suppressive, curative and prophylactic tests. This was comparable to the group treated with chloroquine. The oral median lethal dose (LD_{50}) of the extract in mice was 5000 mg/kg body weight. The results of this study showed that the aqueous root extract of *A. nilotica* is safe and has antiplasmodial activity (Alli *et al*., 2011).

Acacia sieberiana (**Plate 2**) with the English name "White thorn" is a member of the family Fabaceae- Mimosoideae. This tree grows up to 15 m high with light-coloured bark and often with a flat crown. The leaves, 10-15 cm long have straight white thorns at their base. The branches and often the leaves are covered with yellow hairs. The flower heads are cream-coloured and spherical. The seeds are contained in straight pods, 8-12 cm long and 2-3 cm broad. The tips of young shoots are intensively browsed particularly toward the end of the dry season. *A. sieberiana* grows in the savannah and woodland. It occurs with various botanical characteristics in the entire Sahel and other semi-arid regions in Africa. The tree possessed some ethnobotanical history; decoction of the root is taken as remedy for stomach-ache. The bark, leaves and gums are used to treat tapeworm, bilharzia, haemorrhage, colds, diarrhoea, gonorrhoea, coughs, kidney problems, syphilis, ophthalmia, rheumatism, fever, infectious diseases, inflammation, pain in the back and disorders of the circulatory system. The pods serve as an emollient, and the roots for stomach-ache, acne, tapeworms, urethral problems, oedema and dropsy (Orwa *et al.*, 2009).

Acacia sieberiana is a legume and it hosts rhizobium bacteria in its roots. The bacteria fix nitrogen gas from the air and without requiring nitrogen fertilizer or soil nitrates. They convert it into nitrogen compounds necessary for plant nutrition. Ultimately, surrounding plants also benefit from the increase in available nitrogen, which means that plants such as *A. sieberiana* species are of particular ecological importance (Orwa *et al.*, 2009). The use of *A. sieberiana* against *P. falciparum* is not documented, although it has been reported by some communities to treat malaria (Kodi *et al*., 2017). However, various studies have been conducted on the plant. An investigation on the anti-inflammatory activity of the alcoholic extract of leaves of *A. sieberiana* was carried out. The methanol extract of leaves of *A. sieberiana* resulted in isolation of certain polyphenolic compounds (Ndamistso *et al*., 2013). From their findings it was found out that ethanol extract of *A. sieberiana* showed a good anti-inflammatory activity against acute inflammation. This revealed one of the pharmacological bases for the ethanomedicinal use of *A. sieberiana*.

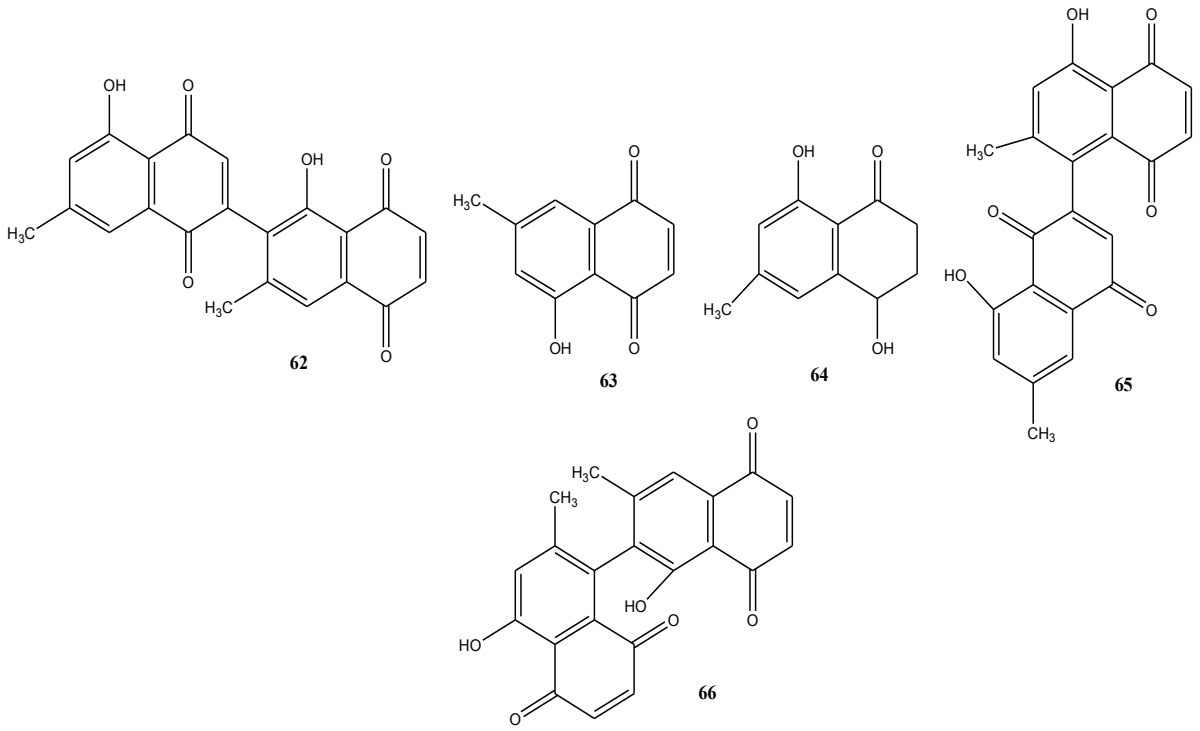
In another study *A. sieberiana* was one of plants used for anthelmintic activity of plant species traditionally used in the treatment of intestinal parasites and their symptoms. This activity was assessed using a standard motility assay against a levamisole resistant strain of the nematode *Caenorhabditis elegans*. According to the findings anthelmintic activity was confirmed in 12 plant species. The activity demonstrated the presence of molecules in these plants of having a broad spectrum. The results provide support for further study of these plants and their compounds as possible treatments for parasitic worm infections (Carrie *et al*.,

2010). Literature shows that the *Acacia* plants are used as medicinal remedies for various diseases including *A. sieberiana.* However no antiplasmodial toxicity screening and charactization has determined on this plant.

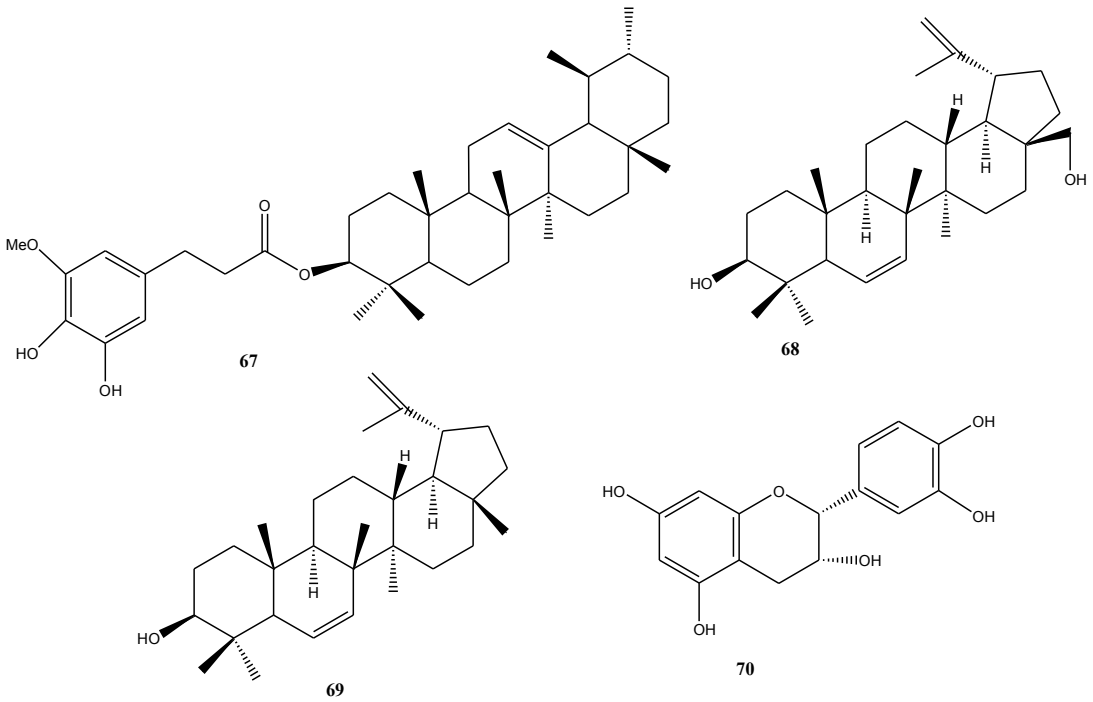
2.9.3 A review of the chemical investigations of the genus *Euclea* (Ebenaceae)

Ebenaceae family to which *Euclea latideus* (**Plate 3**) belongs occurs mainly in the lowlands of the tropical and, to a lesser extent, in subtropical regions of the old and new Worlds. The small genus *Euclea* is restricted to Africa and Southern Arabia whereas the large genus *Diospyros* is pantropical. Many of its species are usualy small to medium-sized trees in the forest understory, with an often remarkably low population density. Only few species penetrate the mountains and extratropical warm temperate regions. Ebenaceae are the source of several economically important products, the most valuable being their fruits and timber (ebony). Naphthoquinones, terpenoids (especially lupanes, ursanes, oleananes, and taraxeranes), benzopyrones, polyphenols, and tannins are all widely distributed and very characteristic for Ebenaceae. Other compounds are steroids, naphtalene-based aromatics, hydrocarbons, lipids, amino acids, carotenoids, and sugars (Mallavadhani *et al*., 1998). Naphthoquinones occur in several parts of the plants, especially in the bark, and are active against fungi, bacteria, mollusks, insects, worms and termites. Their derivatives and oxidative decomposition products are responsible for the dark brown to black colored tissues of the bark, heartwood, fruits, and leaves. Idioblasts containing tannins occur in various parts, including fruits (Neuwinger, 1998; Yonemori *et al*., 2000).

Euclea natalensis is a plant species that belongs to the family Ebenaceae common in tropical and subtropical regions of Africa, specifically on the East coast of Southern Africa. Many *Euclea* species are widely gathered by indigenous people because of their medicinal properties. Roots of these plant species are frequently used to treat respiratory complications such as chest pains, bronchitis, pleurisy and asthma. Ground root powder is topically applied in cases of leprosy and is used by some ethnic groups to treat toothache and headache. The bioactivity encountered is attributable to naphthoquinones, which are common phenolic compounds in the Ebenaceae family. According to the literature, the following naphthoquinones have been isolated from *E. natalensis:* diospyrin (**62**), 7-methyljuglone (**63**), shinanolone (**64**), neodiospyrin (**65**) (Prozesky *et al.*, 2001) and isodiospyrin (**66**) (Khan, 1985). The naphthoquinones present in *E. natalensis* have been found to have inhibitory activity against *Mycobacterium tuberculosis* (Lall and Meyer, 2001).



Deutschländer *et al*. (2011) investigated antidiabetic activities on crude and isolated pure compounds from *E. undulata.* Alpha-amylase results indicated that *E. undulata* inhibited alpha-amylase with IC_{50} = 2.80 µg/mL. Results obtained indicated that the plant extract had the ability to lower blood glucose levels to some extent and in different manners. This confirms the ethanomedicinal use of these four species in the treatment of diabetes. Phytochemical studies of a crude acetone extract of the root bark of *E. undulata* produced a new α-amyrin-3O-ß-(5-methoxy)ferulic ester (**67**), and three known compounds; betulin (**68**), lupeol (**69**) and epicatechin (**70**). *In vitro* assays on C2C12 myocytes revealed that **68** (21.4%) and **69** (166.3%) were active in lowering blood glucose levels. Compounds **27** (IC_{50} 4.79 µg/mL) and to a lesser extent **70** (IC_{50} 5.86 µg/mL) and **69** (IC_{50} 6.27µg/mL) inhibited alpha-glucosidase. These results indicated that the acetone crude extract does contain compounds that display hypoglycemic activity.



From the available literature not much research has been carried out on *E. latideus*. There is no documented information on antiplasmodial screening done on the plant and other species in this family. However, the plant is reported to be used as an antimalarial by the local communities in Butebo County (Kodi *et al*., 2017).

E. latideus, *A. sieberiana*, *O. spinosa* are used as antimalarial plants and little scientific literature data exist to validate antimalarial properties of the above three medicinal plants. It was important that their claimed antimalarial properties are investigated. This was done in order to establish their efficacy and determine their potential as sources of new antimalarial drugs. In the present work, evaluation of the *in vitro* antiplasmodial activity and *in vivo* acute toxicity tests of three plants used in traditional medicine against malaria and fever was under taken.

Plate 1: **Photo of *O. spinosa***

Plate 2: **Photo of *A. sieberiana***

Plate 1: **Photo of *Euclea latideus***

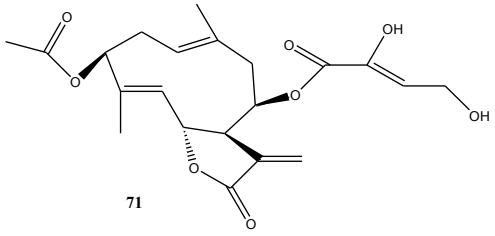
2.10 Some reported antiplasmodial activities of compounds and crude extracts from medicinal plants

Ayoola *et al*. (2008) in a study of phytochemical screening determined the antioxidant activities of four medicinal plants traditionally used in the treatment of malaria in South Western Nigeria. The ethanolic extracts of the leaves of *Carica papaya* (Caricaceae), stem bark of *Mangifera indica* (Anacardiaceae), leaves of *Psidium guajava* (Myrtaceae) and the leaves of *Vernonia amygdalina* (Compositae) were used in the study. The plant parts commonly used in the locality in malaria therapy were employed in this study. All the plants showed potent inhibition of DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging activity, *P. guajava* being the most potent. The free radical scavenging (antioxidant) activities of these plants probably contribute to the effectiveness of the above plants in malaria therapy.

Muthaura et al. (2007) analyzed the methanolic and water extracts of five medicinal plant species used for the treatment of malaria in traditional/culture health systems of Kwale people in Kenya. These extracts were tested for antiplasmodial activity against *P. falciparum* and *P. berghei*, respectively and for their cytotoxic effects. The most active extracts (IC_{50} < 10 μg/mL) screened against CQ sensitive (D6) and resistant (W2) *P. falciparum* clones, were the water and methanol extracts of *Maytenus undata* (Celasteraceae), methanol extracts of *Flueggea virosa* (Euphorbiaceae), *Maytenus putterlickioides and Warburgia stuhlmannii* (Canellaceae). These extracts showed various cytotoxic levels on vero E6 cells with the water extract of *M. undata* exhibiting least cytotoxicity. These results indicate that there is potential for isolation of a lead compound from the extracts of the five plants.

Muthaura *et al.* (2007b) in another investigation evaluated ten plant extracts commonly used by the Meru community of Kenya for the *in vitro* and *in vivo* antiplasmodial, cytotoxicity and animal toxicity activities. The water and methanol extracts of *Ludwigia erecta* (Onagraceae) and the methanol extracts of *Fuerstia Africana* (Lamiaceae) and *Schkuhria pinnata* (Asteraceae) were screened. The extracts exhibited high antiplasmodial activity (IC_{50} < 5 μg/mL) against CQ sensitive (D6) and resistant (W2) *P. falciparum* clones. The cytotoxicity of these highly active extracts on vero E6 cells were in the range 161.5-4650.0 μg/mL with a selectivity index (SI) of 124.2-3530.7. These results suggest that there is potential to isolate active nontoxic antimalarial principles from these plants. *S. pinnata* is widely used and believed to be an efficacious antimalarial medicinal plant. Preliminary information from literature suggests it is safe and bioactive, with low toxicity values (LC_{50}) above 1000 μg/mL (Muñoz *et al*., 2000; Bussmann *et al*., 2011).

In South Africa other studies have been done on *S. pinnata* DCM/MeOH (1:1) crude extracts of the whole plant against *P. falciparum* NF parasite to give IC_{50} of 2.19 μg/mL (Makoka *et al*., 2013). In the same investigation they isolated two pure componds Schkuhrin I (**71**) and Schkuhrin II (**72**) which were screened on *P. falciparum* NF-54 and NF strains to give IC_{50} of 2.05 and 1.67 μg/mL. These results show that both crude extrcts and pure componuds are effective antimalarial remedies. The antiplasmodial results also give the justification for the use of these plants in traditional medicine by the local community.



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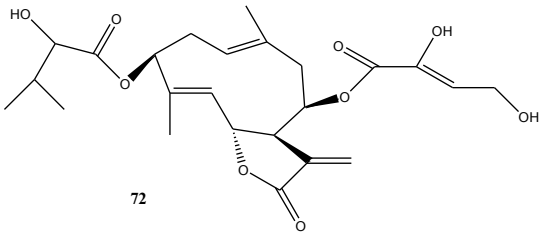
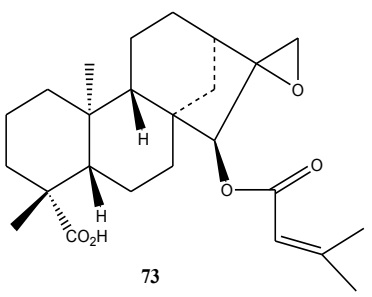


72

Mohammed *et al*. (2014) carried out evaluation of antimalarial activity of aqueous and methanol extracts of the leaves of *Acokanthera schimperi* (Apocynaceae) and *Croton macrostachyus* (Euphorbiaceae) from Ethopia against mice infected with CQ sensitive strain of *P. berghei*. To assess the effect of extracts of the plants on the parasite, a 4-day suppressive standard test was performed using *P. berghei* (ANKA strain). Extracts of the leaves of both plants significantly ($P < 0.05$) suppressed *Parasitemia* in dose dependent manner at all dose levels. The findings from their study may support the traditional use of the plants to treat malaria. However, further pharmacological, toxicological and phytochemical studies are required to evaluate the potential of the plants towards the devb elopment of new antimalarial agent.

Sebisubi *et al.* (2010) studied the antimalarial activity of *Aspilia pruliseta*, a medicinal plant from Uganda. *A. pruliseta* (Asteraceae) is a medicinal plant indigenous to Uganda and the neighboring countries of East Africa. It has been used extensively by the rural population for the treatment of fevers and malaria. During the antimalarial evaluation of this plant, four non-toxic diterpenes were isolated that possessed moderate activity against CQ sensitive (D6) and CQ resistant (W2) clones of *P. falciparum*, with IC_{50} values ranging from 14 to 23 μM. These moderately active compounds included the previously undescribed diterpene, *ent*-15β-senecioyloxy-16,17-epoxy-kauran-18-oic acid (**90**) that demonstrated an IC_{50} value of 23.4 μM against clone D6, but was devoid of activity against clone W2. A known diterpene 16,17-epoxy-15-[(2-methyl-1-oxo-2-butenyl)oxy]-4α,15β(E)-kauran-18-oic acid (**91**) was also isolated during the bioactivity-guided fractionation of the total chloroform extract of *A. pruliseta*. The moderate activities of selected diterpenes of *A. pruliseta* could account collectively for the historical and enduring use of this plant in traditional African medicine.



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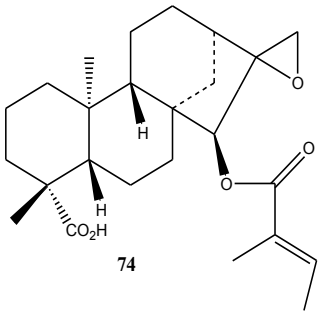

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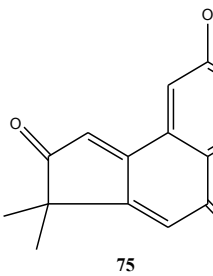
Namukobe *et al*. (2015) in their study of antiplasmodial compounds from the stem bark of *Neoboutonia macrocalyx* isolated five compounds from the EtOAc and aqueous crude extracts. The isolated compounds were assessed for antiplasmodial activity against the CQ sensitive Sierra Leone I (D6) and CQ-resistant Indochina I (W2) strains of *P. falciparum*. Chemical investigation of the ethyl acetate extract of *Neoboutonia macrocalyx* bark resulted in the identification of one not previously described diterpenoid; neoboutomacroin (**75**) in addition to the four known compounds which included, a phenanthrene; 3,6-dihyroxy-1,7-dimethyl-9-methoxyphenanthrene (**76**), a sterol; 3-O-Acetyloleuritolic acid (**77**) and two diterpenoids; simplexin (**78**) and montanin (**79**). Compounds **75** and **79** displayed high antiplasmodial activity of IC_{50} values less than 10 µg/mL against both strains. However, all the compounds tested displayed high cytotoxic activity against MRC5 cell line with IC_{50} less than 10 µM. Their study provided the evidence for use of this plant as an antimalarial in traditional medicine. Their study provided the evidence for use of this plant as an antimalarial in traditional medicine.



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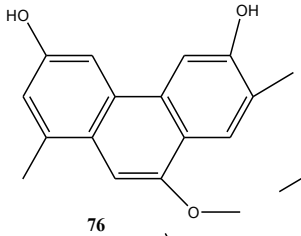

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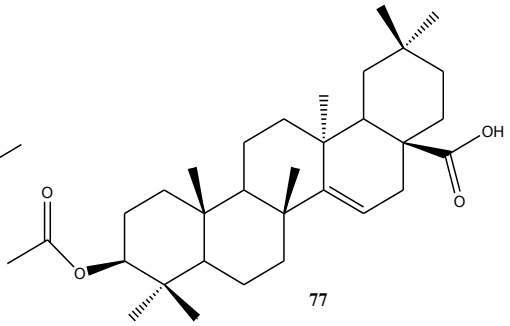

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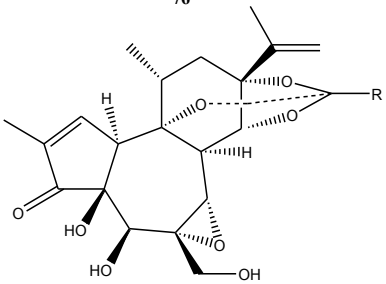


78, R = $CH_2(CH_2)_7CH_3$
79, R = $CH_2(CH_2)_9CH_3$

A vast number of rural communities rely on traditional medicine for treating common and specific health problems. In Sub-Saharan Africa where malaria is endemic and in other parts of the world, plants are extensively used for treating periodic fevers and malaria. Medicines based on traditional knowledge of wild plants serve as some of the most common treatments for malaria in Uganda (Katuura *et al*., 2007).

Katuura *et al*. (2007) investigated the antiplasmodial activity of ten medicinal plants used to treat malaria in South Western Uganda. The study plants were *Bothlioclines longpipes* (Asteraceae), *Toddalia asiatica* (Rutaceae), *Maesa lanceolate* (Myrsinaceae), *Indigofera emerginella* (Papilionaceae), *Lantana trifolia* (Verbanaceae), *Vernonia lasiopus* (Asteraceae), *Trimmeria bakeri* (Flacourtiaceae), *Rhus natalensis* (Anacardiaceae), *Erythrophleum pyrifolia* (Ceasalpinaceae) and *Conyza sp* (Asteraceae). Extracts were subjected to *in vitro* antiplasmodial screening against wild strains of *P. falciparum* using the nitro-tetrazolium blue-based lactate dehydrogenase assay. The antiplasmodial activity of the crude extracts of plants ranged from high to moderate (EC_{50}: 1.60-50.00 µg/mL). However, some of the extracts had no antiplasmodial activity with inhibitory concentrations above 50 µg/mL. Their study showed that extracts of several medicinal plants used in traditional treatment of against malaria and fever in Western Uganda, had antiplasmodial activity against the blood stage of *P. falciparum*. Lack of *in vitro* antiplasmodial activity in some plants may have been due to the methods of extraction used, which were not the same as those used in traditional practice. Furthermore, the specific traditional preparation techniques often employ mixtures of plants (Tabuti *et al*., 2003). Their study relied on an *in vitro* system, it is known, however, that some compounds that show *in vitro* activity may not possess *in vivo* activity due to pharmacokinetic and immunological factors (Waako *et al*., 2005). This calls for further studies to investigate the efficacy and safety of these plants in an *in vivo* system.

Another study of three Ugandan medicinal plants was under taken to investigate their antioxidant and antiplasmodial activity (Stangeland *et al*., 2010). The plants included: *Hallea rubrostipulata* (Rubiaceae), *Vernonia adoensis* (Asteraceae) and *Zanthoxylum chalybeum (Rutaceae).* The plants were tested for antioxidant activity using three assays (DPPH), ferric reducing ability of plasma (FRAP) and total phenol content. In addition the antiplasmodial activity using an Enzyme-Linked Immunosorbent Assay on *P. falciparum* CQ sensitive strain MRA-285 line was also done. The strongest antiplasmodial activity was found in the water extract of *H. rubrostipulata* (IC_{50} = 1.95 µg/mL) which is regarded as very high activity. They found that all extracts from *V. adoensis* had very high antiplasmodial activity ranging

from 2.14 to 2.83 µg/mL. Four of the *Z. chalybeum* extracts demonstrated high activity as well (IC_{50} ranging from 2.72-3.94 µg/mL). It was only the methanol extract that had low activity (IC_{50} = 10.92 µg/mL).

This work assessed the *in vivo, in vitro* antiplasmodial and toxicity screening of some families of species of antimalarial plants. Emphasis was put on the families and species of antimalarial medicinal plants that were documented during the ethnobotanical survey (Kodi *et al*., 2017).

According to literature research the antiplasmodial efficacy, toxicity and characterization of active compounds from the antimalarial medicinal plants of *Oncoba spinosa, Acacia sieberiana* and *Euclea latideus* has not been investigated. The plants are used in their crude form without any knowledge of their antiplasmodial activity and safety. The local communities do not know the effectives the preparations they use in the treatment of malaria. The solvents used are not able to extract the active compounds from the plant material leading to doses with low concentrations of active compounds. Therefore, it is viable to carry out scientific investigations to validate the use of the three medicinal plants in the treatment of malaria in the rural communities of Butebo County found in Eastern Uganda (Kodi *et al*., 2017).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Summary of activities carried out in the study

This section describes the steps involved in the analysis and the instruments that were used in carrying out the experiments. The flow chart below gives a brief summary of the activities that were carried out to achieve the objectives of the research. These included an ethnobotanical survey, collection of plant materials, extraction of crude samples, bioassay activity tests, isolation, purification and structure elucidation (**Figure 5**).

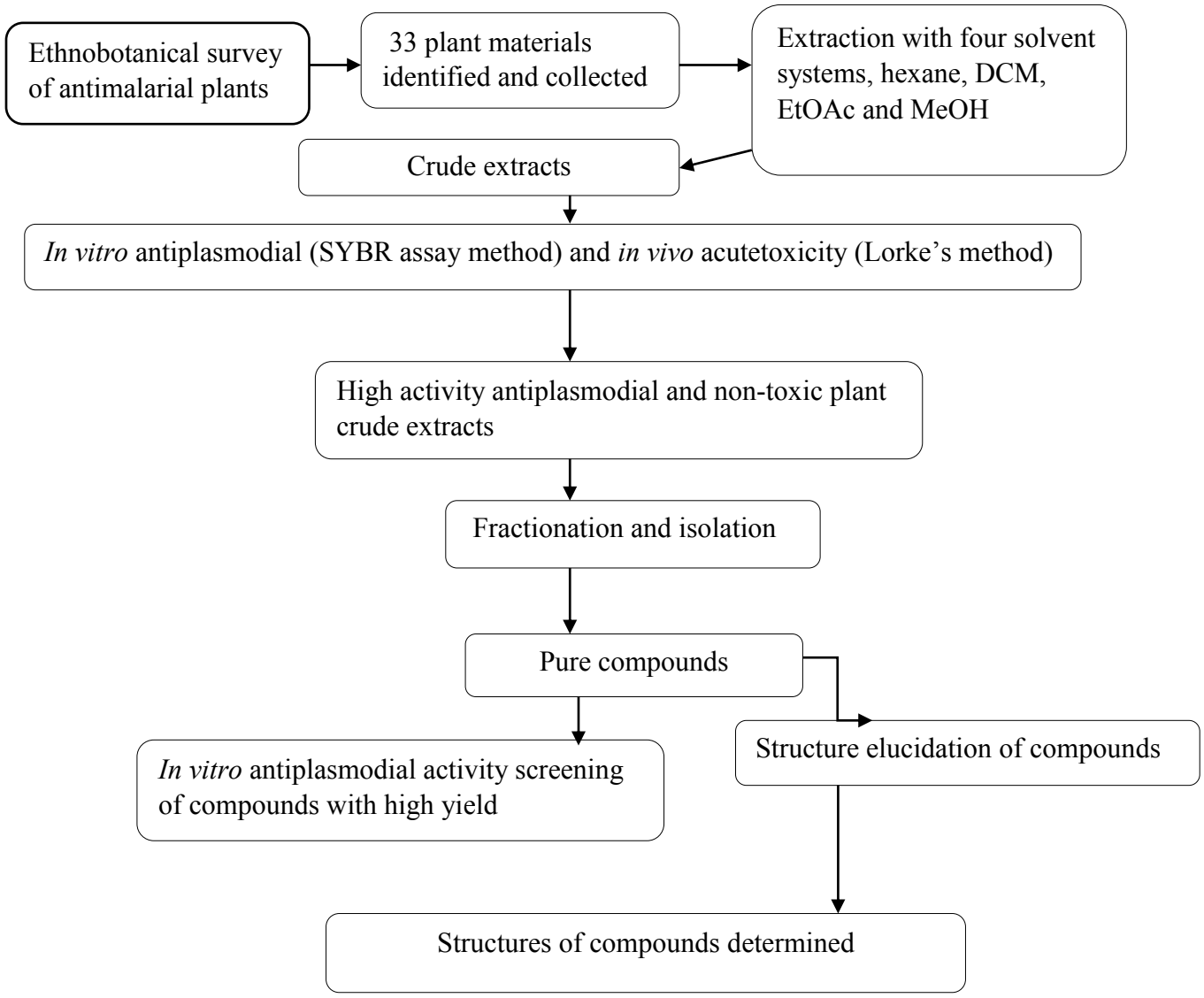


Figure 5: Flow scheme showing activities carried out in the study

3.2 Study site

An ethnobotanical study was carried out in Butebo County composed of five sub counties namely; Kakoro, Kabwangasi, Petete, Butebo and Kibale positioned between 33° 45′- 34° 13′ E and 1° 12′ - 1° 18′ N in Pallisa District, located in Eastern Uganda (**Figure 6**). The study was aimed at documenting medicinal plant species used in treating malaria and fever among the local people. The study was conducted during dry and rain seasons in December - January and April-August, respectively in 2014 (Kodi *et al*., 2017). This was aimed at targeting plants that grow during dry and wet seasons.

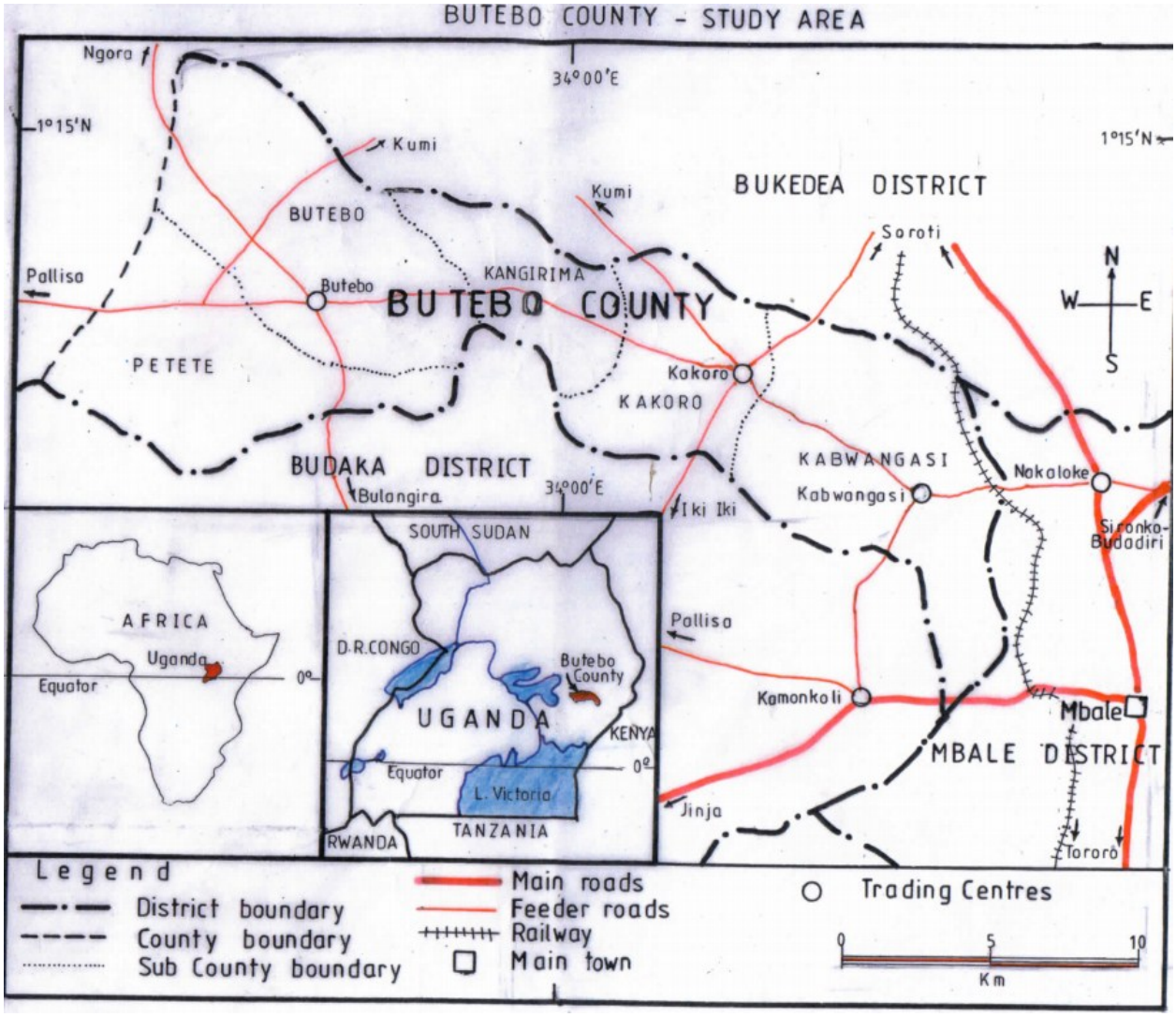


Figure 6: **Map of Butebo County showing the five Sub-counties (Source: Makerere University, Geography Depatment)**

3.3 Medicinal plants selection criteria

After the identification of the plant species by a taxonomist, Mr. Protase Rwaburindore they were screened using different data bases and electronic library data to check for documented antimalarial use and antiplasmodial activity in other studies reported in literature. The screening was used as a basis for the selection of plants in the study. Preference ranking given by the respondents was also considered to select the plant species to be investigated. Plants were ranked according to their level of effectiveness in the treatment of malaria by the local people. From the thirty three plant species identified, ten were considered for potential plants for this study. The ten plants were screened using the dictionary of natural products on CDROM by Chapman and Hall in order to get the ones not previously investigated. The three plants were Oncoba spinosa, Euclea latideus and Acacia sieberiana.

3.4 Collection of Ethnobotanical information and plant identification

The plants collected and information documented was obtained by interviewing respondents using semi-structured, open and closed ended questionnaires. They were administered to 50 respondents that included traditional healers and herb sellers using Snowball sampling method. Ten respondents from the five Sub-Counties were selected for the interview. The questions in the questionnaire were translated in the two local languages (*Ateso* and *Lugwere*) for better understanding in order to give the correct responses. Information collected included knowledge on the use of medicinal plant species, the local names of important plant species, plant parts used, dosage, methods of herbal preparation, mode of administration, duration of treatment, traditional uses and other questions as indicated in the questionnaire **[Appendix 2].** Field surveys were done on farm land, in forest reserves to identify the plants and also to find out the habitats in which they are found. Medicinal plants were photographed, collected, dried and taken for identification by a taxonomist, Mr. Protase Rwaburindore. The voucher specimens were deposited at the Department of Botany Herbarium, Makerere University Kampala, Uganda for future reference.

3.5 Preparation of test compounds

3.5.1 Chemicals and test reagents

All the chemicals used for the extraction, fractionation and chromatography were of analytical grade and purchased from Sigma-Aldrich representative in Kenya (Kobian Kenya Limited).

3.5.2 Ethical considerations

The experimental protocol was approved by the Departmental Committee for Research and Ethics Department of Pharmacology Makerere University. Each animal was used only once. For ethical reason, all animals were sacrificed at the end of the study (Leary *et al*., 2013). Experimental protocol was followed according to Guidelines for Care and Use of Laboratory Animals in Biomedical Research (Council, 2010). All rules were followed as well as specific national laws where applicable.

3.5.3 General preparation of crude extracts

Plant materials were washed to remove the soil, cut into small parts and then air dried at room temperature in a shade for 21 days (Sofowora, 1982). The dried plant material was pounded using a clean mortar and pestle and then blended into fine powder with electric blender (Thomas-Wiley Mill Model 4). The powdered sample was bagged in black plastic bags and stored in an air-tight container ready for extraction. Crude plant extracts were prepared by grinding of 800-1000 g of air dried powdered plant material for each plant species. This was done in sequential cold extractions with 1200-2000 mL of *n*-hexane, dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH) at room temperature for 72 hours (Newton *et al*., 2002) in Winchester bottles of 2.5 L with intermittent agitations. This was aimed at extracting compounds from low to high polarity (**Figure 7**). The process was repeated twice for each solvent and after the third extraction, the same crude plant powder was air dried and further treated three times with the next solvent. In all the three stages, the extracts were filtered through cotton wool, then Whatman filter paper (Whatman® No. 1). Finally after filtration the crude extracts were concentrated, under reduced pressure in a water bath at 40-45 ºC, by using a rotatory evaporator machine (BUCHI-R 205). They were then transferred to weighed containers and put in the oven to dry completely at 40°C to produce solid materials. Their mass yields were calculated based on dry weight and expressed as percentage yield of the powered material using the equation shown below. Samples of the

solid residues obtained were transferred into sterile containers and kept in a refrigerator at 4°C for antiplasmodial and toxicity assays.

Extract yield (%) = $\frac{W1}{W2} \times 100$, where, W1 = net weight of crude extracts (grams), W2 = total weight of medicinal plant powder (grams).

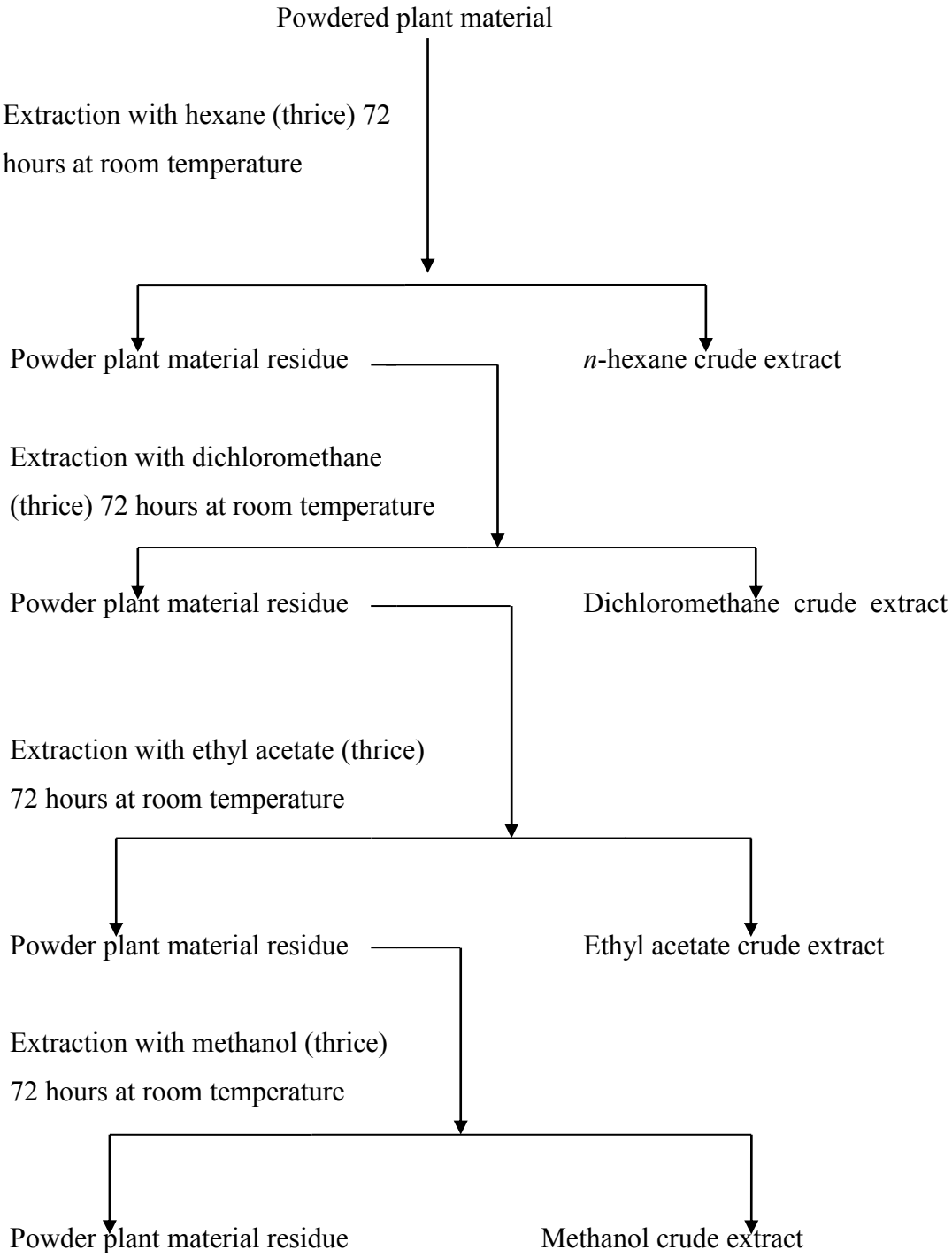


Figure 7: **Schematic representation of the extraction procedure of crude extracts from the medicinal plants**

3.6 Purification and analysis of compounds

3.6.1 General chromatography

For the three plants under the study only two crudes extracts were fractionated, these included that of dichloromethane and EtOAc. Methanol crude extracts had promising activity and were considered for future isolation and characterization. The *n*-hexane crude extracts were considered to have fats. Gravity Column chromatography was used for fractionation and isolation of pure compounds that was run on Merck silica gel 60 (70-230 mesh). Merck 9385 silica gel was used as the adsorbent and elution was allowed to proceed by gravity. Different sized columns were used in the isolation procedure ranging from 1-8 cm in diameter depending on the amount of sample available. Solvents used in the column chromatographic process included: n-hexane, dichloromethane, ethyl acetate, diethyl ether and methanol. Before addition of the crude extracts and fractions obtained, some silica gel was added followed by the solvent used for extraction then dried in a fume cupboard or at temperatures below 45 ºC. The resulting product was then introduced in powder form into a column packed using n-hexane. Thin layer chromatography was used to monitor the chromatographic process. Analytical TLC was performed using silica gel (0.2 mm thick) on aluminum backed plates (Merck Art. 5554) which contained a fluorescent indicator (GF254). The detection was accomplished by the plates first being visualized with a UV lamp at 254 and 365 nm wave lengths. This was followed by development with anisaldehyde spray reagent consisting of anisaldehyde, conc. H_2SO_4 and methanol in a ratio of 1:2:97 followed by heating in an oven at a temperature of 100° C. The purity of all isolated compounds was confirmed by TLC analysis using dichloromethane-diethyl ether solvent systems, packed in bottles and stored in a refrigerator awaiting spectroscopic analysis.

3.6.2 Spectroscopic analysis of pure compounds

Identification of the pure compounds was achieved by ^{1}H and ^{13}C NMR spectroscopy. The pure compounds were analyzed using a Bruker avance ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) to get the spectral data. The purified compounds were analysed using one and two dimensional (1-D and 2-D) NMR experiments, respectively. However, the two dimensional NMR analysis was only carried out on purified samples whose mass had reasonable quantity. The various spectroscopic techniques used to elucidate the structures included: 1-D (^{1}H, ^{13}C, DEPT) and 2-D (COSY, NOESY, ROESY, HMBC and HSQC). COSY, NOESY, ROESY, HSQC and HMBC experiments were recorded with gradient enhancements using sine shape gradient pulses. The structures were simulated using ACD

NMR manager program and TOPSPIN 3.1 software to obtain the chemical shifts of proton and carbon. The chemical shifts (δ) were expressed in parts per million (ppm) relative to tetramethylsilane (TMS δ = 0) as internal standard and the coupling constants (*J*) given in Hz. The pure samples were either dissolved in deuterated chloroform ($CDCl_3$) with solvent signals (δ_H 7.26 ppm and δ_C 77.23 ppm) as reference or deuterated methanol (CD_3OD) with solvent signals at (δ_H 3.31 ppm, δ_H 4.87 ppm and δ_C 49. 1 ppm). The pure samples were dissolved in 5 mL of deuterated solvent in a 5 mm NMR tube and the data processed using TOPSPIN 3.1 software. Using the NMR spectroscopic experiments structures were proposed based on the interpretation of the spectra and compared with known compounds reported in literature.

3.7 Fractionation and purification of the crude extracts of *Oncoba spinosa*

The dichloromethane crude extract (2.6 g) yellow in colour was eluted with hexane: ethyl acetate mixtures of increasing polarity (0-100% ethyl acetate) in the ratios of 100:0 to 0:100 then washed with 100% methanol. A total of 12 broad fractions (S1-S12) were collected and combined on the basis of their TLC profiles (**Figure 8**). Pooled fraction (S2 and S3) were eluted with a gradient of EtOAc-hexane (2:3, 1:1), respectively then washed with 100% EtOAc, to give 29 fractions labelled A1-A29. TLC analysis pooled combined fractions of A2 -A10 and A11 and A12. Combined fraction A2-A10, was eluted with 100% hexane then EtOAc: hexane (1:19, 1:4) respectively to give a white powder that was a pure compound **OS2** (293.4 mg, 1.41%). Pooled fractions A11 and A12 were eluted with EtOAc-hexane, 1:9 then 100% ethyl acetate to give 40 fractions labelled C1-C40. TLC analysis afforded fraction C27 and C28), that was cleaned using EtOAc: hexane, 1:4 and then 100% EtOAc to give white crystals of a pure compound **OS4** (2.6 mg, 0.10%). Fractions (S6 and S7) were eluted with solvent system of increasing polarity of EtOAc-hexane (2:3, 1:1) respectively followed by 100% EtOAc. This gave 28 fractions labelled G1-G28. TLC profile analysis gave pooled combined fraction, G21 and G22 which was eluted with EtOAc-hexane, 1:1 followed by 100% EtOAc to give a pure compound **OS1** (29.8 mg, 1.15%), of white crystals.

The brown EtOAc crude (6.84 g), was eluted with hexane-EtOAc mixture of increasing polarity of (0-100%), finally washed with 100% MeOH. This resulted in 13 broad fractions identified as OE1-OE13. The fractions were combined according to their TLC profiles to give fraction OE3-OE12 that was eluted with EtOAc-hexane (3:17, 1:4) respectively then followed by 100% EtOAc. This yielded 38 fractions labelled AE1-AE38.

This pooled fraction AE1-AE24 was eluted with diethyl ether-dichloromethane (DEE-DCM) (4:21, 13:21), respectively followed by 100% EtOAc to give compound **OS3** (40.9 mg, 0.60%) with white crystals. Four pure compounds were isolated from this plant, three from the dichloromethane and one from the ethyl acetate crude extracts.

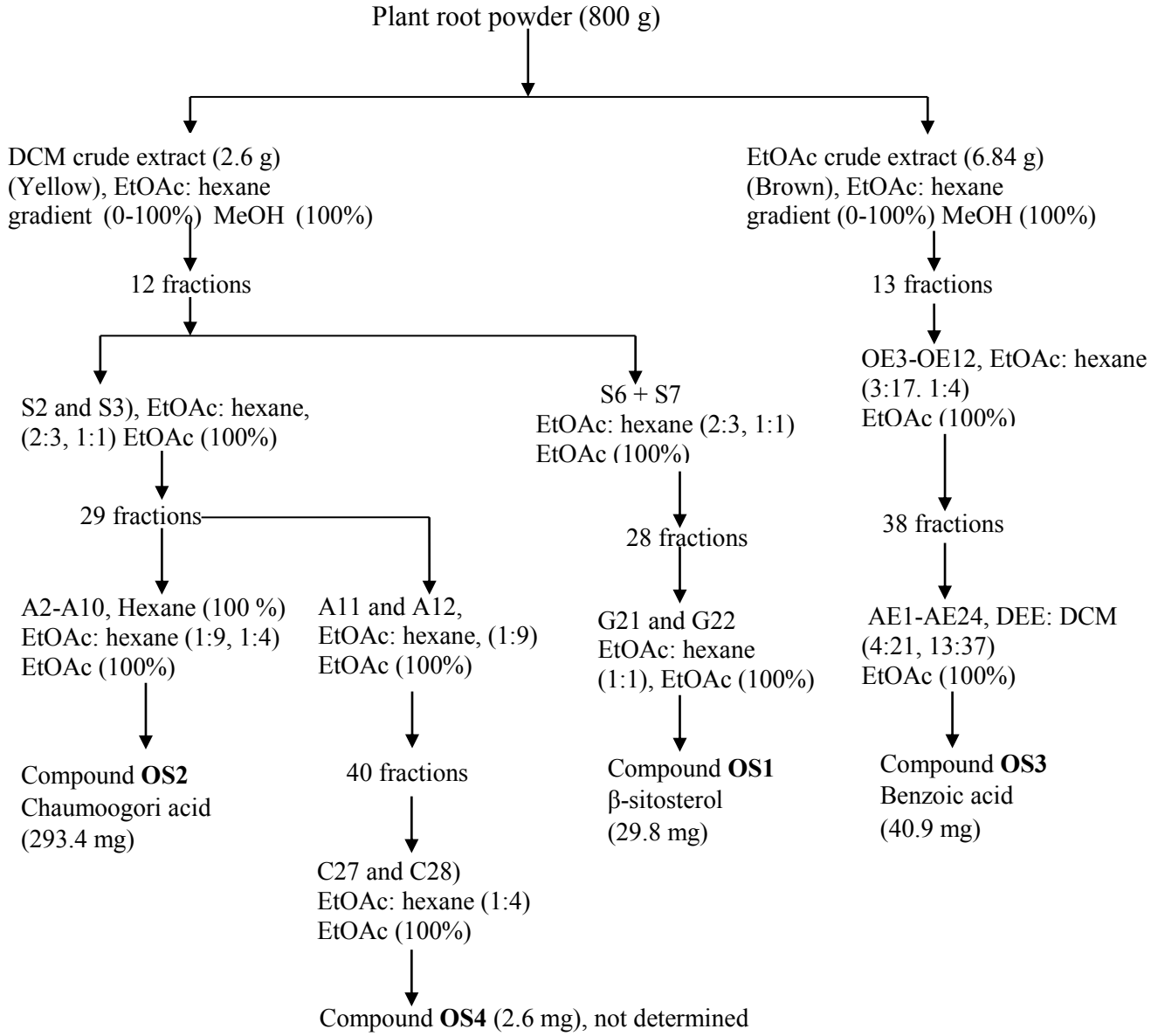


Figure 8: Flow chart showing procedure for isolation of pure compounds from *O. spinosa*

3.8 Fractionation and purification of the crude extracts *Euclea latideus*

The brown dichloromethane crude extract (7.24 g) was eluted with increasing polarity of EtOAc: hexane (0-100%), followed by 100% MeOH. Eleven broad fractions M1-M11) were collected which pooled fraction M3-M10 (**Figure 9**). This combined fraction was eluted with EtOAc: hexane (1:4, 3:7) respectively then washed with 100% EtOAc to give 45 fractions B1-B45. These fractions pooled fraction B15-B23 that was eluted with EtOAc:

hexane, 1:4, 1:3, 1:4 to give 61 fractions (D1-D61). Repeated column chromatography with elution of EtOAc: hexane (1:3, 1:4), respectively then followed by diethyl ether-DCM (1:24) yielded black crystals of a pure compound **EL3** (172.3 mg, 2.38%).

The light green crude extract of EtOAc (5.18 g) was eluted with increasing polarity of EtOAc : hexane (0 -10%) followed by MeOH 100 % to yield 14 broad fractions J1-J14). This gave pooled fraction J4 and J5 which was eluted with increasing polarity of EtOAc: hexane (1:3, 3:7), respectively followed by 100 % EtOAc that resulted in 33 fractions EA1-EA33. The pooled fraction EA 14-EA33 was eluted with diethyl ether: dichloromethane (DEE: DCM), 2:23 followed by 100% EtOAc to afford two pure compounds **EL1** (26.0 mg, 0.50%) UV active and **EL2** (189.6 mg, 3.66%), respectively which were all white powders. Three pure compounds were isolated from *E. latideus*, two were from the EtOAc crude extract and one from the dichloromethane.

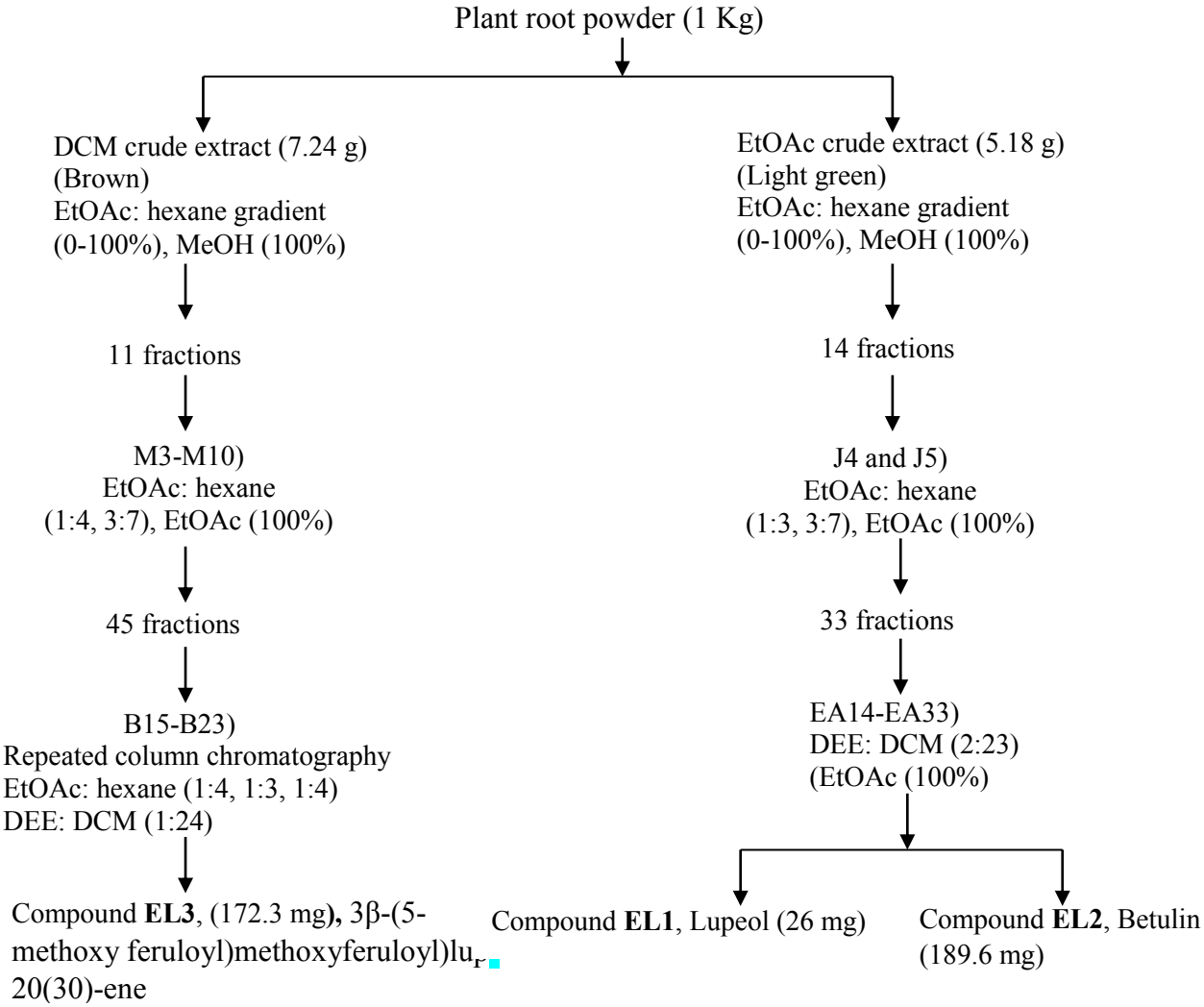


Figure 9: Flow chart showing procedure for isolation of pure compound from *E. latideus*

3.9 Fractionation and purification of the crude extracts of *Acacia sieberiana*

The brown EtOAc crude extract (5.86 g) was eluted with EtOAc: hexane (0-100%) followed by 100% MeOH. Five broad fractions were collected M1-M5 based on their R_f values asthey were eluted from the column (**Figure 10**). TLC profile analysis gave pooled fraction M5 and M6 that was eluted with EtOAc: hexane (1:4, 3:7), respectively followed by 100% EtOAc to yield a yellow gel which was a pure compound **AS1** (195.9 mg, 3.34%).

The brown dichloromethane crude extract (4.96 g) was eluted with EtOAc: hexane gradient (0-100%), then 100% MeOH. The elution afforded 12 broad fractions DN1-DN12) that pooled fraction DN5-DN7. Fraction DN5-DN7 was eluted with increasing polarity of EtOAc: hexane (3:7, 7:13 and 2:3), respectively followed by 100% EtOAc. In this elution 32 fractions DA1-DA32 were collected that pooled fraction DA6-DA18). Repeated column chromatography of fraction DA6-DA18 with elution of EtOAc: hexane 3:7, then 100% EtOAc afforded a pure solid compound **AS2** (16.8 mg, 0.34%) with white crystals.

Only two pure compounds were isolated from this plant species. One from dichloromethane and the other from EtOAc crude extract as described above. Other pure compounds were isolated as fatty acids that were not considered for 2-D NMR spectroscopy.

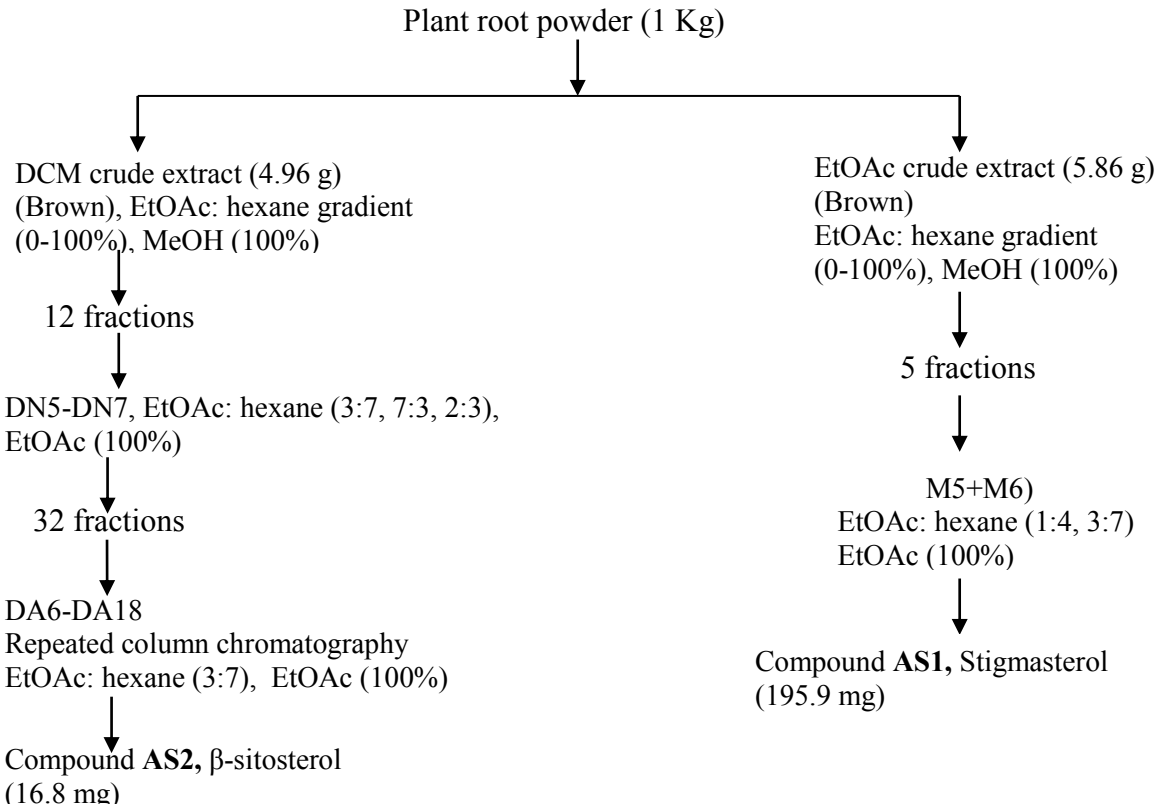


Figure 10: Flow chart showing procedure for isolation of compounds from *A. sieberiana*

3.10 Biological assay activity tests

The biological activities were tested on both crude and pure compounds. These included the *in vitro* antiplasmodial activities that were determined on pure and crude extracts. *In vivo* acute toxicity was only measured on crude extraxts in mice.

3.10.1 Preliminary bioassay screening of crude extracts

The crude extracts were subjected to preliminary activity screening tests against *P. falciparum* (Chloroquine sensitive and Chloroquine resistant) parasites, cultivated using established methods (Trager and Jensen, 1976), as describe in section, **3.10.2**. Only active medicinal plants were extracted using other solvents and considered for *in vitro* antiplasmodial test.

3.10.2 *In vitro* antiplasmodial tests

The extracts and compounds were assayed using a non-radioactive Malaria SYBR Green I assay technique (Smilkstein *et al*., 2004) with modifications (Yenesew *et al*., 2012) to determine a concentration that inhibits growth of 50% of parasites in culture (IC_{50}). Two different *P. falciparum* strains, CQ sensitive (3D7) and CQ resistant (Dd2) were used. The following reagent was obtained through Biodefense and Emerging Infections Research Resources Repository (BEI Resources), National Institute of Allergy and Infectious Diseases (NIAID), National Institute of Health (NIH): *P. falciparum*, Strain Dd2, MRA-150, contributed by David Walliker and 3D7, MRA-102, contributed by Daniel J. Carucci. These isolate were grown as described (Trager and Jensen, 1976); with minor modifications (Johnson *et al*., 2007).

Drugs, extracts and pure compounds were dissolved in 99.5% dimethylsulfoxide (DMSO) (Sigma-Aldrich). This was followed by dilution in complete Roswell Park Memorial Institute 1640 series of Cell Culture Media (RPMI 1640) enriched with human serum. The RPMI 1640 medium was prepared accordingly as described by Akala *et al*. (2011). In summary the basic culture medium was prepared from RPMI 1640 powder (10.4 g; augmented with 2 g glucose and 5.95 g of HEPES, dissolved to homogeneity in 1 L of de-ionized water and sterilized with a 0.2 µM filter). Complete RPMI 1640 media, used for all parasite culture and drug dilutions, consisted of basic RPMI 1640 media with 10% (v/v) human ABO pooled plasma, 3.2% (v/v) sodium bicarbonate and 4 mg/mL hypoxanthine. At the same time two-fold serial dilutions of drugs were prepared to generate 10 dilutions of

each drug for testing that consisted of chloroquine (1.953-1000 ng/mL), mefloquine (0.488-250 ng/mL) and test samples (97.7-50,000 ng/mL). These were prepared on a 96-well plate, such that the final proportion of DMSO was 0.025- 0.05%. Chloroquine and mefloquine were used as standard controls while 0.4% DMSO was used as the negative control. The culture adapted *P. falciparum* at 2% hematocrit and 1% *Parasitemia*, were then added on to the plate containing dose range of drugs and incubated in a gas mixture (5% CO_2, 5% O_2, and 90% N_2) at 37 °C. The assay was terminated 72 hours later by freezing at -80 °C for 24 hours. After thawing 100 µL of SYBR Green I solution which contained (0.2 µL of 10,000 x SYBR Green I (Invitrogen)/mL) in lysis buffer {Tris (20mM; pH 7.5), EDTA (5 mM), saponin (0.008%, w/v), and Triton X-100 (0.08%, v/v)} was added to each well. This was followed by gently mixing using the Beckman Coulter Biomek 2000 automated laboratory work station. The plates were incubated for 5-15 minutes at room temperature in the dark. Parasite growth inhibition was quantified by measuring the per-well relative fluorescence units (RFU) of SYBR Green 1 dye using the Tecan Genios Plus (Tecan US, Inc., Durham, NC). This was done with excitation and emission wavelengths of 485 and 535 nm, respectively, and with the gain set at 60. The reference anti-malarial drugs, chloroquine and mefloquine were tested along the test compounds and a minimum of three separate determinations was carried out for each sample. Differential counts of relative fluorescence units (RFUs) were used in calculating 50% inhibition concentration (IC_{50}) for each drug. The IC_{50} values were given as mean of two or three independent experiments and the results were presented as mean $\pm$ SD (standard Deviation).

The resistance index (RI) for each crude extract and isolated compounds tested was determined to assess the activity of the *Plasmodium* on the CQ resistant strain. It was calculated as the ratio between IC_{50} of the resistant value of the strain to the sensitive value of the strain calculated. RI = IC_{50} of resistant strain (Dd2)/ IC_{50} of sensitive strain (3D7). The RI value determines whether the test samples have activity against the resistant strain of *P. falciparum.*

3.10.3 *In vivo* acute toxicity (LD_{50}) test

The estimated lethal dose (LD_{50}) of the crude extracts in mice was performed using the method described (Lorke, 1983). A total of 230 mice weighing (13.0-30.0) g obtained from Department of Pharmacology Makerere University were used to carry out the *in vivo* acute toxicity experiments. The mice were kept in cages in a ventilated room and fed with a pelletized grower mash. They were also provided with clean drinking water. The weight of

each mouse was measured and the dose calculated for all the dose levels. The tests were done in two phases. In the first phase, nine (9) mice were divided into 3 groups of 3 mice per group. After overnight fast (24 hours) the animals in the first phase received doses of 500, 1000 and 2000 mg/kg weight body. The surviving animals were sacrificed under chloroform anesthesia. When no death was observed in the first phase, then higher doses were administered in the second phase. In the second phase, also 9 mice, 3 per group were treated with doses of 3000, 4000 and 5000 mg/kg weight body. One mouse was used as control and received an equivalent volume of distilled water. When death occurred in the first phase then four groups of four animals each was used. These group of animals received doses of 600, 700, 800 and 900 mg/kg body weight. The stock solution was prepared by dissolving 0.2 g of the crude extract in 2 mL of distilled water to give a concentration of 100 mg/mL. The crude extracts were then administered using a cannula attached to a graduated syringe. The animals were given food and water four hours post drug administration. Toxicity signs such, writhing, decreased motor activity, decreased body/limb tone, decreased respiration, loss of appetite, feeling sleep, depression, gasping for air, palpitation and mortality (death) that occurred within 24 hours was recorded. After which the LD_{50} was determined.

For each dose level, the volume administered was calculated using (Tedong *et al*., 2007) equation as follows:

$$V = \frac{D \times P}{C}$$

D = dose used (mg/kg body weight), P = body weight,

C = concentration (mg/mL) and V = volume.

3.11 Data analysis

3.11.1 Ethnobotanical information

In order to analyze ethnobotanical information, Microsoft Excel package 2013 was used where descriptive statistical methods were employed. The information obtained through the ethnobotanical interviews was analyzed and expressed as percentages. Pie charts and bar graphs were used to express the following parameters: Taxonomic diversity, growth forms, parts of the plant used to treat malaria. The percentage of people who have knowledge about the use of a species in the treatment of malaria was evaluated using the formula:

$$PPK = \frac{NPICP}{TNPI} \times 100,$$ Where: PPK = percentage of people who have knowledge about the use of a species in the treatment of malaria

NPICP = number of people interviewed citing species

TNPI = Total number of people interviewed

Preference ranking (PR) method was also employed, where plants are ranked according to their level of effectiveness in the treatment of malaria by the local people. Each rank was given an integer (1, 2 or 3) with the most effective plants assigned a value of 3 (Asase *et al*., 2005). Availability of literature of previous studies on the plants identifiedthat included, used as antimalarial plants, antiplasmodial and toxicity activity tests, clinical trials, extraction solvents utilized was also used. Phytochemical compounds isolated from the antimalarial plants were used determine the previous research investigated.

3.11.2 Antiplasmodial bioassay activity tests

In vitro antiplasmodial test results were given as the inhibition concentration (IC_{50}), this represents the drug concentration capable of inhibiting 50% of the *P. falciparum*. Differential counts of relative fluorescence units (RFUs) were used in calculating 50% inhibition concentration (IC_{50}) for each drug. This was done by an equation generating a sigmoidal concentration- response curve (variable slope), with log transformed drug concentrations on the *X*-axis and relative fluorescent units (RFUs) on the *Y*-axis (Graphpad Prism for Windows, version 4.0; Graphpad Software, Inc., San Diego, CA) (Bacon *et al*., 2007; Johnson *et al*., 2007).

The IC_{50} values above 100 µg/mL for crude extracts were considered inactive (O'Neil *et al*., 1985). This is in line with WHO guidelines (2011) and basic criteria for antiparasitic drug discovery. In describing *in vitro* antiplasmodial activities of natural products, pure compounds were classified in five groups (Batista *et al*., 2009) ansd also crude extracts (WHO, 2011) (**Table 2**).

Table 2: Antiplasmodial activity classification of crude extracts and pure compounds

Sample	IC_{50} (µg/mL, µM)	Classification
Crude extract	< 5	High
	5-15	Promising
	15-50	Moderate
	50-100	Weak
	> 100	Inactive
Pure compound	< 1	Potent/Excellet
	1-20	High
	20-100	Moderate
	100-200	Low
	> 200	Inactive

3.11.3 Toxicity bioassay activity tests

The LD_{50} values were calculated as the geometric mean of the highest non-lethal dose (with no deaths) preceding the lowest lethal dose (where deaths occurred).

$$LD_{50} = \sqrt{(D_o \times D_{100})}$$

LD_{50} = median lethal dose

D_o = highest dose that gave no mortality

D_{100} = lowest dose that produced mortality

The general toxicity activity was considered: $\leq$ 1 mg/kg (extremely toxic), 1-50 mg/kg (highly toxic), 50-500 mg/kg (moderately toxic), 500-5000 mg/kg (slightly toxic), 5000-15000 mg/kg (practically non-toxic), and $\geq$ 15000 mg/kg (harmless) (WHO, 2011).

3.11.4 Statistical analysis

Data on *Parasitemia* was analyzed using windows SPSS version 16. Statistical significance was determined with the Biostat 1.0 software package using one way ANOVA and student's *t-test*. These were transformed in *P*-values to compare results at 95% confidence level (α = 0.05). This was used to compare results between doses, among treatment and control dose levels. The differences between means was considered significant when $P < 0.05$ (Kirkwood *et al*., 2003).

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 Ethnobotanical survey

4.1.1 Plant information and taxonomic diversity

Thirty three plant species from 30 genera belonging to 23 families were documented. Twenty three plants have been documented in literature as antimalarial medicinal plants in Uganda and other Countries (**Table 3**).

All the medicinal plants were reported in the two local languages used which included *Ateso a*nd *Lugwere*. The most frequently used medicinal plants were from the families; Rutaceae and Lamiaceae (13.0% each) that had three plant species each. This was followed by Euphorbiaceae, Mimosaceae, Celastraceae, Meliaceae, Asteraceae and Papillionaceae (8.7% each) with two plant species each. The remaining fifteen families Polygalaceae, Flacourtiaceae, Umbelliferae, Sapotaceae, Apocynaceae, Simaroubaceae, Ebenaceae, Aristolochiaceae, Anacardiaceae, Caricaceae, Lauranceae, Myrtaceae, Verbenaceae, Poaceae and Celastraceae with 4.3% had one plant species each (**Figure 11**) (Kodi *et al*., 2017).

Ten species (30.3%) were identified and documented for the first time in Uganda to treat malaria these included: *Oncoba spinosa, Steganotania araliacea, Acacia sieberiana, Ormocarpium trachycarpum, Acacia hockii, Euclea latideus, Cassia hirsuta*, *Chamaecrista nigricans*, *Aristolochia tomentosa* and *Buttyrospermuum paradoxum.* Information on the correct identification of these medicinal plants that include the species name, families, local names, plant parts used and other diseases treated including malaria is summarized in (**Table 3**). Among the plants identified: *C. nigricans* (90%), *Z. chalybeum* (84%)*, S. pinnata* (80%), *O. basilicum* (78%), *E. latideus* (74%), *E. abyssinica* (72%), *A. indica. and O. spinosa* (70 %) had the highest PPK values with corresponding PR values of 3, 3, 3, 2, 3, 2, 3 and 3, respectively. *A. hockii* (28%), *C. papaya* (26%), *C. reticulate, M. indica and C. macrostachyus* (24%) each *and A. tomentosa* (8%) had the lowest PPK values with PR values of 2, 2, 2, 1, 2, 2, respectively (**Table 5**). The high PR values are in agreement with the plants that had high PPK values used in treating malaria (**Table 5**). An ethnobotanical survey in Ghana and in Nigeria identified that *Azadirachta indica* had the highest PPK values of 29.3%, 12.8% and PR values of 2, 3, respectively. *Cymbopogon citratus* was documented as one of the plants with the highest value of PPK (percentage of people who have knowledge about the use of a species in the treatment of malaria (PPK) and preference ranking (PR) of 11.3 and 3 respectively second to *A. indica* (Asase *et al*., 2005; Dike *et al*., 2012). The information on frequently used antimalarial plant species is also an important lead to the

species that can be targeted for antiplasmodial tests, toxicological tests, and phytochemical analysis. This was also noted by (Hassan-Abadallah *et al*., 2013) that plant species with high fidelity level values are considered potential candidates for further pharmacological investigations and deserve priority investigations.

The antimalarial plants that have been documented in Uganda and other Countries and cited in this study are: *F. virosa, S. longipedunculata, E. abyssinica, M. azedarach, C. edulis, H. abyssinica, Z. chalybeum, P. guajava, C. sinensis, S. pinnata, L. camara, C. papaya, M. indica, A. indica, P. americana, B. pilosa, C. citratus, P. barbatus, M. senegalensis, C. reticulate, O. gratssiumum, O. basilicum and C. macrostachyus*. The families of Lamiaceae and Rutaceae had the highest number of species documented; this information is in agreement with results from other researchers (Nguta *et al*., 2011) which showed the same trend.The 23 antimalarial documented plant species in this study are high compared to other similar ethnobotanical surveys done in Uganda. For example only 20 were documented in Cegere Sub-county (Anwyar *et al*., 2016), 20 medicinal plants in Kibale (Namukobe *et al*., 2011), 20 species in Mbarara District (Katura *et al*., 2007). However, the number was lower compared to surveys in other regions in Uganda that include: 48 in Nyakayojo, Mbarara District in Western Uganda (Stangeland *et al*., 2011) and 86 species in Mpigi District, Central Uganda (Adia *et al*., 2014).

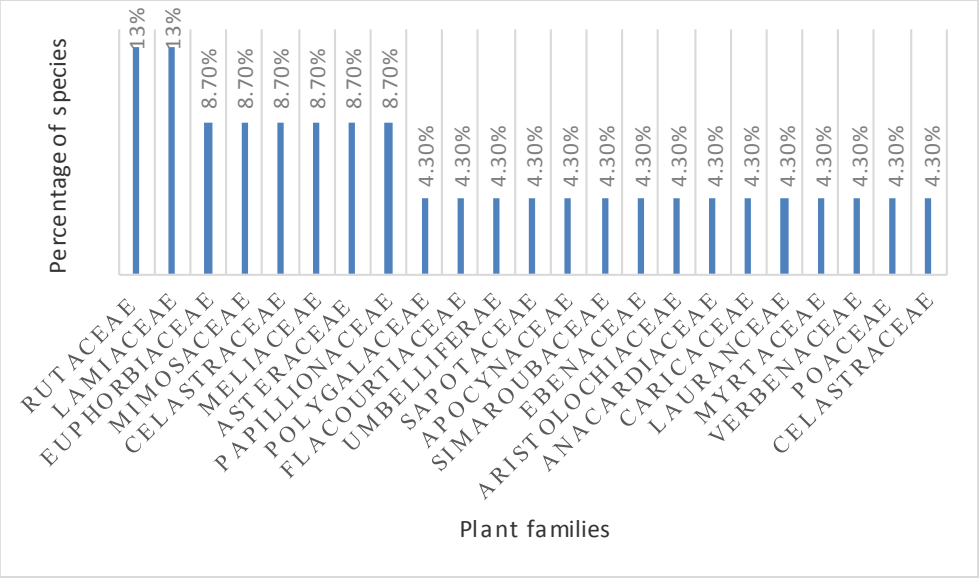


Figure 11: Plant families used in treating malaria in Butebo County (Kodi *et al*., 2017)

Table 3: Taxonomic diversity, growth forms, plant parts, local names, habitats of medicinal plants and diseases

	Botanical name	Family name	Local name (s)	Parts used	Growth habit	Habitat	Related and other iseases treated
1	*Croton macrostachyus*	Euphorbiaceae	Ookota	R & S	T	GL	Stomach ache, TB, Coughs, fever asthma, skin diseases
2	*Flueggea virosa*	Euphorbiaceae	Alakasi	R & L	SH	WG	Chest pains, miscarriage,
3	*Ocimum basilicum*	Lamiaceae	Emopim	L	H	G	Fever, Eye cataract, headache
4	*Securidaca longipedunculata*	Polygalaceae	Eliloi	R	SH	OG	Measles, Body pains, cough Hernia, Snake bite, ticks control, Skin disease, Infertility Diarrhoea, tooth ache
5	*Oncoba spinosa*	Flacourtiaceae	Ekalepulepu	R	SH	GL	Epilepsy, syphilis, wounds Skin disease, Headaches Sexual impotence, Stomach ache
6	*Steganotania araliacea*	Umbelliferae	Ematule	R & L	T	OG	Swollen body, measles

7	*Erythrina abyssinica*	Papilionaceae	Engosorot	R	T	GL	TB, Stomach ache, tooth ache Deafness, sterility, Uterine fibroids Fever, leprosy, arthritis, burns, Body swellings, Syphilis
8	*Acacai sieberiana*	Mimosaceae	Etiriri	R	T	WG	Cough, Epilepsy, Dysentery
9	*Ormocarpum trachycarpum*	Papillionaceae	Ederut	R	SH	GL	Snake bite, Pneumonia
10	*Melia azedarach*	Meliaceae	Elira	L	T	HS	Parasitic worms, Fever, Skin disease & itching Wounds, Body pains
11	*Butyrospermuum paradoxum*	Sapotaceae	Ekunguri	R	T	WG	Labour pains, headaches
12	*Carrisa edulis*	Apocynaceae	Ekamuriei	R	SH	WG	Cough, Epilepsy, Syphilis, Diarrhoea, Snake bite, measles Dysentery, fever, Chest pain, Headaches, TB, Polio

13	*Harrisonia abyssinica*	Simaroubaceae	Ekeroi	R & L	SH	WG	Syphilis, Snake bite, Fever, Wounds, Hania, abdominal pains (menstruation)
14	*Acacia hockii*	Mimosaceae	Ekisim		T	OG	Diarrhoea, Dysentery, Syphilis
15	*Euclea latideus*	Ebenaceae	Emusi	R	SH	GL	Swelling of legs, Ringworms
16	*Cassia hirsuta*	Caesalpinioideae	Kasagalansansi	R	H	RS	Stomach ache
17	*Zanthoxylum chalybeum*	Rutaceae	Eusuk	R & L	T	WG	Fever, Coughs, Colds, Chest pain, TB, Asthma, Sterility Body swellings, Stomach aches
18	*Chamaecrista nigricans*	Caesalpiniaceae	Epeduru lo didi	L	H	G	Hypertension, Labour promotion, Retained placenta
19	*Schkuhria pinnata*	Asteraceae	Apunait	L	H	G	Chest pains, treatment of ears Wounds, skin diseases, diabetes Cough
20	*Aristolochia tomentosa*	Aristolochiaceae		S	C	GE	Wounds, snakebites, skin diseases

21	*Melia indica.*	Anacardiaceae	Omuyembe	L	T	HS	Venereal diseases, pain killers Diarrhoea, dysentery, cough fever, Cough, syphilis
22	*Carica papaya*	Caricaceae	Papali	L	T	HS	Cough, Cancer, snake bite sterility, pain killers, antidotes promotes labour, lactation stimulant, venereal disease
23	*Persea Americana*	Lauranceae	Ovakedo	L	T	HS	Antibacterial, antifungal, Worms parasites, high blood pressure, wounds, cough
24	*Azadirachta indica*	Meliaceae	Neem	L, R, SD &RB	T	HS	Cough, Syphilis, skin disease chicken pox, vomiting, fever, lice, diabetes, obesity, Nausea
25	*Psidium guajava*	Myrtaceae	Mapera	L & RB	T	HS	Cough, wounds, typhoid, Measles, diarrhoea, dysentery, fever, diabetes, small pox
26	*Lantana camara*	Verbenaceae	Kanpanga	L	SH	RS	TB, pneumonia, chest pain, snake bite, wounds, measles, ringworms

27	*Citrus sinensis*	Rutaceae	Omucungwa	R	T	HS	Cough, reduce vomiting, diabetes
28	*Citrus citrates*	Poaceae	Akisube	L	H	C	Cough, cancer, fever, indigestion
29	*Bidens pilosa*	Asteraceae	Kalala	L, WP & R	H	G	Wounds, skin diseases, diabetes
30	*Plectranthus barbatus*	Lamiaceae	Ebiriri omutano	L, S, R & WP	H	G	Snakebites, fever, heart diseases, respiratory problems
31	*Maytenus senegalensis*	Celastraceae	Echomai	R	T	F	Toothaches, wound healing, skin diseases, respiratory treatment chest pain, fever
32	*Citrus reticulata.*	Rutaceae	Omuqugwa	R	T	C	weight reduction, cancer skin diseases
33	*Ocimum gratissimum*	Lamiaceae	Omujaja	L	H	G	Chest pain, treatment of ears

S- Stem, R- root, L- Leaves, RB- root bark, SD- seeds, WP- whole plant, T- tree, SH-shrub, H- herb, C-climber, GL- grassland, WG-Wooded grassland, G- grassland, OG- open grassland, HS-homestead, RS- road side, GE- garden edge, G- garden

4.1.2 Respondent details

A total of 50 respondents interviewed comprising of females (34 %) and males (66 %) with age groups of 20 or less (0%) , 21-30 (8%), 31-40 (14%), 41-50 (16%), 51-60 (24%), and above 60 (38%) with the highest number of people interviewed. They consisted of mainly Bateso (68%) and Bagwere (32%) whose main occupation is farming. The educational background consisted of 34% with no formal education, 20% had reached primary level, 44% had secondary education and those with tertiary education were 2% (**Table 4**). The females (34%) had the least number while males (66%) the highest. This was in agreement with other ethnobotanical studies carried out (Anywar *et al*., 2016) who had the highest value (53.3%) of the respondents as males while the rest were females. In an ethnobotanical survey conducted in Oyam district, Nothern Uganda, the females and males were 41.8% and 58.2% respectively (Kamatenesi *et al*., 2011).

In the African culture the belief is that traditional healers should be male (Okello and Ssegawa, 2007). In society older people aged between 51-80 years for example in this study (62%) had more knowledge on medicinal plants and their uses. This is due to long direct contact with plant resources than young people. On the other hand, younger people have little interest in traditional medicine in general and there appears to be a risk of knowledge loss if nothing is done to motivate them. Younger people are exposed to modern education and therefore not interested in learning and practicing ethanomedicinal wisdom that would give them indigenous knowledge. The respondents were dominated by the Ateso (68%) who had a strong background in knowledge on medicinal plants than the Bagwere (32%). The highest level of education attained was that of tertiary institution. Most of the respondents were educated up to the level of Secondary education (98%). Differences in medicinal plants knowledge among age groups was also reported in other studies investigated in Ethiopia (Chekole *et al*., 2015).

Table 4: **Demographic data of the respondents**

	Sex		Tribe		Age (years)						Educational status			
	M	F	Bat	Bag	0 - 20	21 - 30	31 - 40	41 - 50	51 - 60	61 - 90	No formal education	PE	SE	TE
N	33	17	34	16	0	4	7	8	12	19	17	10	22	1
%	66	34	68	32	0	8	14	16	24	38	34	20	44	2

KEY: M- male, F- Female, Bat- Bateso, Bag- Bagwere, N- number of respodents,
PE-Primary education, SE-Secondary education, TE-Tertiary education

4.1.3 Indigenous knowledge on the antimalarial medicinal plants

The local communities in Butebo County use the antimalarial medicinal plants for the treatment of other different diseases. These include: gynecological issues, digestive disorders, skin infections, respiratory tract infections**,** arthritis and inflammation, neurological and nervous system disorders, erectile dysfunction and impotence, poisonous animal bites, hypertension, painful body parts, body odour, headaches and fatigues, diabetes, STDs and venereal diseases among others. The use of one plant to treat several ailments is probably attributed to presence of many metabolites in one particular plant this is because the same molecule can be active against different pathogens.

Most of the medicinal plant species collected and identified in the study area were also used in other regions of Uganda and other parts of Africa to treat the same or different ailments. The use of the same plant species for similar or different ethanomedicinal uses in different countries is a reliable indication of the bioactivity potential of the documented plant species (Maroyi, 2013). Using the same species in different cultures over a long period suggests strongly that these species may be effective in the treatment of malaria. It is however, important to validate all claims of therapeutic efficacy and safety. This should be done by undertaking pharmacological, toxicological, and controlled clinical studies. Validation of traditional medicinal practices is important because it may generate higher confidence and therefore wider use of such species. Wider acceptance of traditional herbal remedies can yield significant benefits for primary health care. They can also extend the market and create value addition for the herbal medicines (Van Wyk *et al*., 2004).

All the respondents reported that the patients received included both men and women of different age groups ranging from children to elderly people. The number of patients received on daily basis ranged between averages of 6-10. These were diagnosed using symptoms of the disease and interviews. The patient's preferred decoctions and infusions

(**Table 5**), which were prepared from either boiling water or hot water. Some diseases were reported to have been treated successfully after failing to be treated in clinics or hospitals.

The knowledge about the medicinal plants was received from parents, grandparents, in laws and through dreams. This information included the dose of the medicinal plants and part of the plant used to treat the diseases. The information was passed on to next chosen person in the clan especially those who were still energetic. This explains the reason why the plants were collected and prepared by only the family members so that the information could not be passed on to other families. Most of the respondents reported that they were not willing to share the information about the medicinal plants. This was because the medicinal plants generate income to specific families.

Poverty, availability, being cheap and effective, lack of medicine in hospitals and distant medical facilities were cited by respondents as reasons for preferring medicinal plants to modern synthetic medicine. The same reasons were also noted (Anywar *et al*., 2016) in their study of antimalarial plants in Cegere Sub-County, Northern Uganda in an ethnobotanical survey that they conducted. Some healers cited that some plants were effective but not used because of being poisonous. There were side effects caused by some medicines that were handled by reducing the dose or stopping the medication. The expiry date was determined by the formation of moulds, acquiring bad smell, change of colour and fermentation of the medicinal preparations. The old and expired medicines were either thrown away or destroyed by burning (Kodi *et al*., 2017).

In order to promote the herbal medicines in future the respondents reported that there should be registration of all the people involved in herbal medicinal practice for recognition. They also recommended the legislation of a policy to stream line the herbal medicine treatment. This was to assist them know each other in to order to form associations that would assist them to look for funding and training in standard practices to improve on the quality of the medicines. They also cited lack of scientific testing (efficacy and safety), deforestation and storage as major challenges in the practice of the herbal medicine. They could not determine the correct dose of the preparations and it was becoming difficult to find the raw materials (plants) for the medicines (Kodi *et al*., 2017).

4.1.4 Growth forms of plants, habitats and plant parts used

The most commonly used plant part was the roots (44.68%), followed by leaves (38.30%), stem (6.38%), root bark and whole plant (4.30% each), the least was the seeds with 2.13% (**Figure 12**). The growth habits included; trees (48.48%) with the highest value, followed by shrubs and herbs (24.24% each) and the climbers with the least percentage of (3.03%) (**Figure 13**). The largest habitat of the medicinal plants was found in the homesteads (21.10%), followed by wooded grassland and garden with (18.20% each), grasslands (15.20%), open grassland (9.10%), cultivated and roadside (6.10%), garden edge and forest had the lowest value of 3.0% each (**Figure 14**). Information on growth forms, habitats and plant parts used is summarized in **Table 3**. Other ethnobotanical surveys also showed that roots had the highest percentage of the plant parts used followed by leaves (Katura *et al*., 2007; Muthaura *et al*., 2007; Kamatenesi *et al*., 2011). The results got from the growth habits above had the same findings in an ethnobotanical survey of antimalarial plants investigated in Ghana (Asase *et al*., 2005).The homesteads that occupied the largest habitat was also cited by (Anywar *et al*., 2016) in their survey of antimalarial medicinal plants in Uganda.

Collection of the bark and root is damaging and makes species vulnerable to over exploitation. Harvesting the bark in large quantities can destroy the plant because the protective role of the bark to the plant will be affected. On the other hand uprooting plants especially in case of herbs and shrubs causes total destruction of the plant. Debarking and uprooting of medicinal plant species negatively affects the sustainability of the species in use. Harvesting of roots on the other hand is more destructive as it often involves uprooting whole plants which consequently affects regeneration for sustainable use (Cunningham, 1996).

Flueggea virosa, Steganotia araliacea, Harrisonia abyssinica, Zanthoxylum chalybeum, Psidium guajava, Bidens pilosa, Plectranthus barbatus, Azadirachta indica and Croton macrostachyus, have more than one plant part that is used (**Table 3**). Preserving of these plants may be achieved if the harvesting of bark and root is avoided and harvesting of leaves which is less destructive is promoted. The use of leaves is less destructive if small quantities are collected but not so if large quantities are harvested. Most studies have shown that leaves of different plants possess bioactive ingredients against different diseases and pathogen (Ogbonna *et al*., 2013; Searels *et al*., 2013). Since harvesting of leaves is less destructive than harvesting roots or barks, it is necessary to test leaves for efficacy against different diseases. This should be for those plants where roots and barks are mostly harvested to minimize dangers of over exploitation. Leaves of *A. indica* (El Tahir *et al*., 1999), *S. longipedunculata* (Bah *et al*., 2007), *L. camara* (Ranpariya *et al*., 2016), *C. papaya* (Melariri

et al., 2012)*, and P. guajava* (Melariri *et al*., 2012) have been found to be effective against malaria therefore the harvesting roots of this plants can be avoided. Therefore in order to preserve the medicinal plants it better to harvest plant parts that do not destroy the whole plant or the harvesting should not be done continuously and in large quantities (Katema *et al*., 2013).

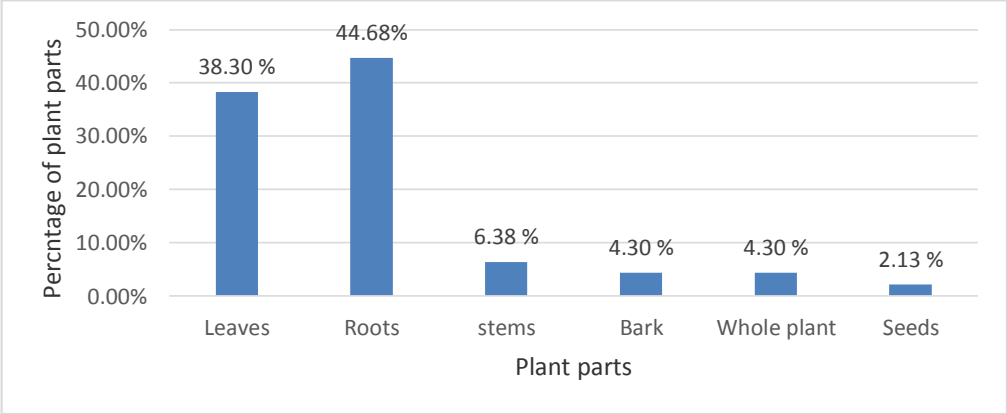


Figure 12: **Medicinal plant parts used**

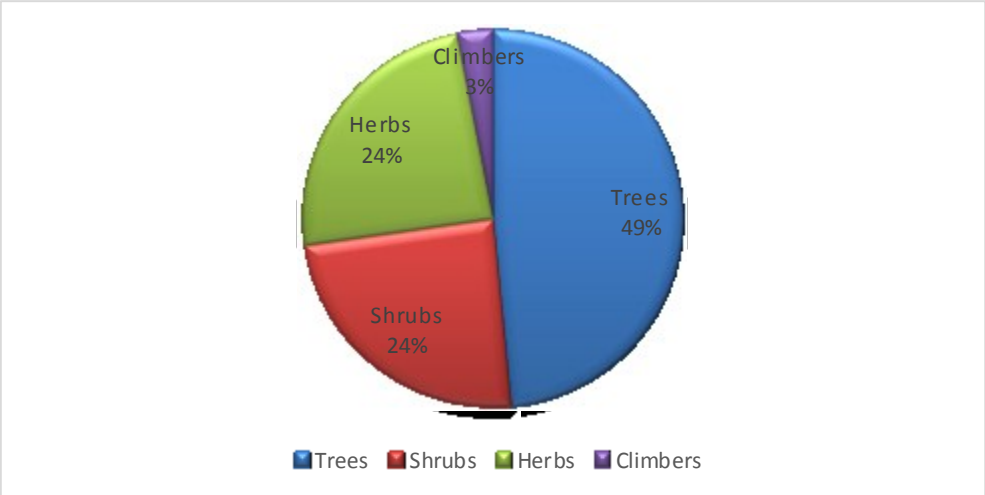


Figure 13: Growth habits of medicinal plants

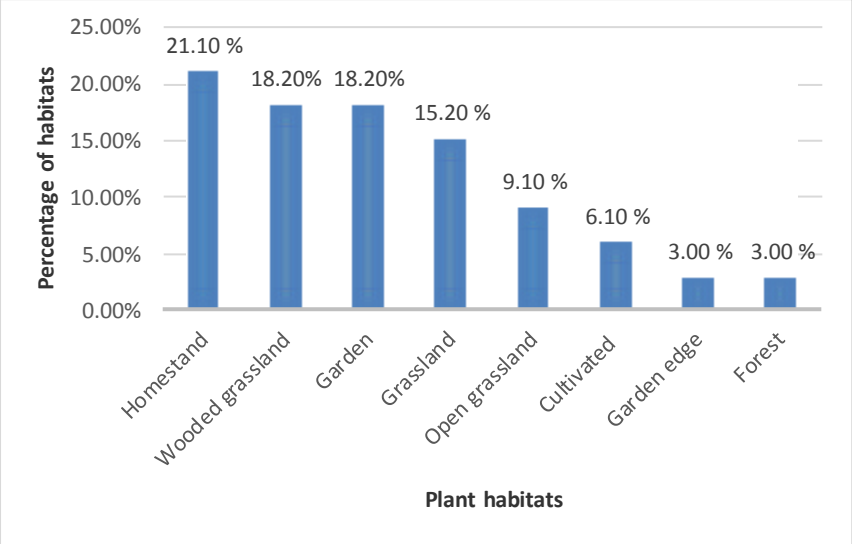


Figure 14: Habitats occupied by the plants

4.1.5 Herbal preparation and administration

Most of the plant materials were dried in the shade, pounded into a powder and taken orally as water decoctions (76.50%) with the highest percentage and infusions (23.50%). The decoctions and infusions were taken as single monotherapy preparations. However, the some respondents reported mixing more than one plant in their preparations in the treatment of other diseases. A summary of the species name, mode of preparation, method of administration, percentage of people who have knowledge about the use of a species in the treatment of malaria and preference ranking values are shown in (**Table 5**). The preparations were made by boiling the medicinal plant powder in water that was made by adding 2-5 spoons of the powder to a half a liter of hot or cold water. This was taken twice daily for about a week to complete a full dose. The dose taken depended on the age of patient, but some respondents reported that sometimes weight was considered to determine the dose. This was also cited in other antimalarial ethnobotanical surveys (Tabuti, 2008; Anywar *et al*., 2016). That is why some medicines have to be taken for a longer period of time and also it may lead to under dose or over dose. Therefore the dose of these herbal medicines has to be determined for effective treatment of malaria in the communities.

Some preparations were always prepared instantly to increase their effectiveness while those in powder form were stored. The stored medicines were kept in closed containers and in the dark to avoid contamination and degradation by sunlight, respectively. The storage containers consisted of; plastic papers, plastic bottles and glass bottles that were labelled by names and part of the plant used. Most of the respondents reported that the stored medicines

were used within a period of not more than 6 months to avoid changing into poisonous substances. This was done to avoid the decrease in the effectiveness and fermentation which is caused due long periods of storage (Kodi *et al*., 2017).

Decoctions and infusions have been cited else where as the most common method of preparation of herbal remedies. Other ethnobotanical surveys have also cited that most herbal preparations are taken orally as decoctions and infusions (Katura *et al*., 2007; Ssegawa and Kasenene, 2007)**.** The choice of oral administration may be related to the use of some solvents such as water that are commonly believed to serve as a vehicle to transport the medicine. Boiling is effective in extracting plant materials and at the same time preserves the herbal remedies for a longer period compared to cold extraction. However, both decoctions and cold extracts do not offer long shelf life for the preparations. This implies the herbalist continuously harvest medicinal plants which put them under a lot of pressure that may lead to over exploitation.

Table 5: Species names, modes of preparation, administration, PPK and PR values

	Botanical name	Mode of preparation	PPK	PR
1	*Crotn macrostachyus*	Decoction	24	2
2	*Flueggea virosa*	Decoction	54	2
3	*Ocimum basilicum*	Infusion	78	2
4	*Securindadaca longipedunculata*	Decoction	34	3
5	*Oncoba spinosa*	Decoction	70	3
6	*Steganonia araliacea*	Decoction	58	2
7	*Erythrina abyssinica*	Decoction	72	2
8	*Acacia sieberiana*	Decoction	66	3
9	*Ormocarpum trachycarpum*	Decoction	48	2
10	*Melia azedarach*	Decoction	56	1
11	*Butyrospermuum paradoxum*	Decoction	52	2
12	*Carrisa edulis*	Decoction	68	2
13	*Harrisonia abyssinica*	Decoction	42	3
14	*Acacia hockii*	Decoction	28	2
15	*Euclea latideus*	Decoction	74	3
16	*Cassia hirsuta*	Infusion	38	2
17	*Citrus citrates*	Decoction	56	3
18	*Zanthoxylum chalybeum*	Decoction	84	3
19	*Chamaecrita nigricans*	Infusion	90	3
20	*Schkuhria pinnata*	Infusion	80	3
21	*Citrus sinensis*	Decoction	34	1

22	*Aristolochia tomentosa*	Infusion	8	2
23	*Mangifera indica*	Decoction	24	1
24	*Carica papaya*	Decoction	26	2
25	*Lantana camara*	Decoction	40	2
26	*Persea Americana*	Decoction	44	2
27	*Azadirachta. indica*	Decoction and infusion	70	3
28	*Psidium guajava*	Decoction	52	2
29	*Bidens pilosa*	Infusion	36	2
30	*Plectranthus barbatus*	Infusion	30	2
31	*Maytenus senegalensis*	Decoction	60	3
32	*Citrus reticulate*	Decoction	24	2
33	*Ocimum gratissimum*	Decoction	50	2

The twenty three species (69.70%) identified in the ethnobotanical survey have been documented as antimalarial medicinal in Uganda and other parts of the World mainly East Africa and other regions of Africa. The crude extracts and phytochemical compounds that have been isolated have demonstrated high antiplasmodial activity. The antiplasmodial activies of the crude extracts and phyto-constituents are in agreement with the claimed therapeutic uses given by the respondents. However, the ten plant species that were documented for first time as antimalarial remedies need to be screened for antiplasmodial activity and toxicity by *in vitro* and *in vivo* standard tests. This should be done in order to justify their local usage (Kodi *et al*., 2017).

4.2 Compounds from *Oncoba spinosa* (Flacourtiaceae)

This plant yielded four pure compounds, three of which had been isolated earlier from other plant species. From the dichloromethane crude extract three pure compounds were isolated, which included a steroid β-sitosterol (**OS1**) (29.8 mg), chaulmoogric acid (**OS2**) (293.40 mg) and compound **OS4** (2.60 mg) whose structure is not yet elucidated. The ethyl acetate crude extract gave only benzoic acid (**OS3**) (40.90 mg) (**Figure 15)**.

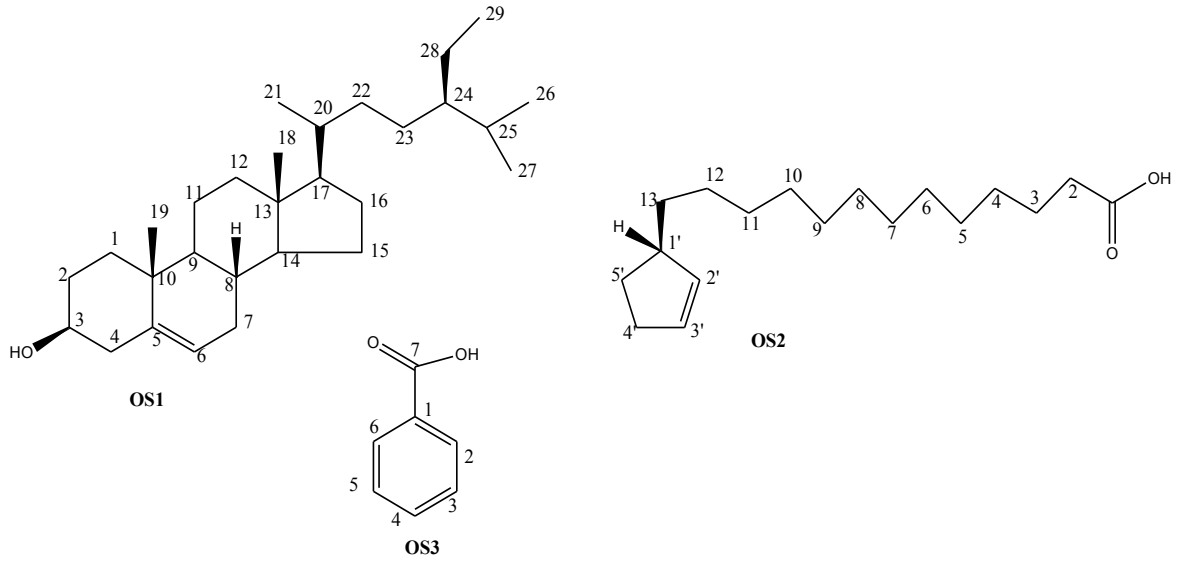
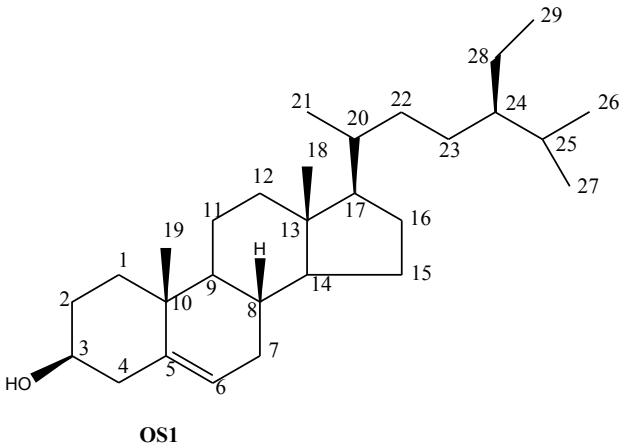


Figure 15: **Structures of compounds isolated from *O. spinosa***

4.2.1 Structure elucidation of β-sitosterol (OS1)



Compound **OS1** was isolated from the dichloromethane crude extract as white crystals (29.8 mg). Its structure was determined based on 1-D and 2-D NMR experiments (**Table 6)** and identified as a steroid. The spectra used in the identification of this compound is shown in spectra [**Appendix 1: K1 andK2].**

The ^{1}H NMR spectrum established six methyl protons at δ_H 0.68 (3H, s, H-18), 0.81 (3H, s, d, *J* = 6.78 Hz, H-26), 0.82 (3H, t, *J* = 6.9 Hz, H-29), 0.84 (3H, s, H-19), 0.92 (3H, d, *J* = 6.55 Hz, H-21) and 1.01 (3H, s, H-27). There was a triplet of doublet of doublets observed at δ_H 3.52 (1H, tdd, *J* = 4.42, 11.11 Hz, H-3) due to a proton attached a hydroxyl group. This is an indication of a hydroxymethine proton in steroids or triterpene nucleus. A broad doublet of one proton was seen at δ_H 5.35 (1H, br, d, *J* = 5.2 Hz, H-6), which

corresponded with the ^{13}C NMR resonance at δ_C 121.9 in the HSQC spectrum. Multiplets of peaks were observed at δ_H 2.25 (H-4), while the remaining proton signals were seen as multiplets between δ_H (0.91- 2.30) (**Table 6).** There are proton signals seen between δ_H (0.7-1.8) which are a result of overlapping of methyl and methylene protons. This phenomenon is seen by the frame work of steroids (Yun-song *et al*., 2007).

Some of the major correlations in the HSQC spectrum are that of the olefinic proton resonating at δ_H 5.35 that correlated with the carbon at δ_C 121.9 (C-6). The hydroxymethine proton at δ_H 3.52 correlated with the carbon at δ_C 72.0 (C-3). The six methyl protons showed correlations with their respective carbons at δ_H 0.68 with δ_C 12.1 (C-18), δ_H 0.81 with δ_C 20.0 (C-26), δ_H 0.82 with δ_C 12.2 (C-29), δ_H 0.84 with δ_C 19.6 (C-19), δ_H 0.92 with δ_C 19.0 (C-21) and δ_H 1.01 with δ_C19.3 (C-27). The carbon atoms absorbing at δ_C 50.0 (C-4) and 46.1 (C-24) are seen to correlate with the methine protons resonating at δ_H 0.92 and 0.93, respectively. The methylene protons that resonated at δ_H 2.24 and 2.29 correlated with carbon atoms at δ_C 42.5 (C-4). The remaining protons resonated with their respective carbons with the chemical shifts (**Table 6).**

Table 6: **^1H and ^{13}C NMR spectroscopic data for β-sitosterol (OS1) in $CDCl_3$ compared against literature values. Literature (Rashed *et al*., 2016) [^1H NMR 400 MHz, ^{13}C NMR 100 MHz, $CDCl_3$, *J* in Hz]**

Position of carbon	^{13}C NMR (125) MHz)	^{13}C NMR (100 MHz) literature	^1H NMR (500 MHz)	^1H NMR (400 MHz) literature
1α	37.5 (CH_2)	37.6	1.82 m	-
1β			1.85 m	-
2α	31.9 (CH_2)	31.5	1.95 m	-
2β			1.99 m	-
3	72.0 (CH)	71.6	3.52 (1H, tdd , *J* = 4.42, 11.11 Hz)	3.52 m
4α	42.5 (CH_2)	42.8	2.24 m	-
4β			2.29 m	-
5	141.0 (C)	140.5	-	-
6	121.9(CH)	121.5	5.35 (1H, br, d , *J* = 5.20 Hz)	5.37 m
7α	34.2 (CH_2)	33.9	1.00 m	-
7β			1.51 m	-
8	32.1 (CH)	31.8	1.51 m	-
9	50.4 (CH)	50.4	0.92 m	-
10	36.7 (C)	36.7	-	-

11α	21.3 (CH_2)	21.1	1.46 m	-
11β			1.50 m	-
12α	40.0 (CH_2)	39.9	1.99 m	-
12β			2.02 m	-
13	42.5 (C)	42.8	-	-
14	57.0 (CH)	56.5	1.00 m	-
15α	24.5 (CH_2)	24.5	1.06 m	-
15β			1.58 m	-
16α	28.5 (CH_2)	28.5	1.25 m	-
16β			1.84 m	-
17	56.3 (CH)	57.3	1.11 m	-
18	12.1 (CH_3)	12.0	0.68 (3H, s)	0.75 (3H, s)
19	19.6 (CH_3)	19.6	0.84 (3H, s)	1.09 (3H, s)
20	36.4 (CH)	35.9	1.36 m	-
21	19.0 (CH_3)	18.7	0.92 (3H, d, *J* = 6.55 Hz)	0.98 (3H, d, *J* = 6.50 Hz)
22α	35.9 (CH_2)	34.2	0.91 m	-
22β			1.35 m	-
23α	26.3 (CH_2)	26.3	1.15 m	-
23β			1.83 m	-
24	46.1 (CH)	46.4	0.93 m	-
25	29.4 (CH)	29.2	1.66 m	-
26	20.0 (CH_3)	19.8	0.81 (3H, d, *J* = 6.78 Hz)	0.85 (3H, d, *J* = 6.70 Hz)
27	19.3 (CH_3)	19.2	1.01 (3H, s,)	0.81 (3H, d, *J* = 6.70 Hz)
28α	23.3 (CH_2)	23.5	1.22 m	-
28β			1.25 m	-
29	12.2 (CH_3)	11.8	0.82 (3H, t, *J* = 6.90 Hz)	0.92 (3H, t, *J* = 7.40 Hz)

The data obtained from the ^{13}C NMR and DEPT spectra showed that compound **OS1** had 29 carbon peaks. These included six methyl groups observed at δ_C 12.1 (C-18), 12.2 (C-29), 19.0 (C-21), 19.3 (C-27), 19.6 (C-19) and 20.0 (C-26). A double bond showing the olefinic carbons was seen at δ_C 141.5 (C-5) and 121.9 (C-6), while a carbon atom attached to a secondary hydroxyl group was observed at δ_C 72.0 (C-3). The remaining carbon signals were attributed to the eleven methylene, nine methine and three quaternary carbon atoms. The ^{13}C NMR signals seen were also in agreement with that reported in literature (Pateh *et al*., 2009; Rashed *et al*., 2016).

In order to assign the remaining part of compound **OS1** 2-D NMR spectra were interpreted, which included COSY, HMBC and NOESY. In the COSY spectrum a number of cross peak correlations were seen. There were cross peak correlations between the methine

proton at δ_H 1.66 (H-25) with two methyl protons at δ_H 0.81 (H-26), δ_H 1.01 (H-27) and a methine proton at δ_H 0.93 (H-24). Another correlation was observed between the hydroxymethine proton of δ_H 3.52 (H-3) with two methylene protons at δ_H 2.25 (H-4) and δ_H 1.97 (H-2). The doublet of the olefinic proton at δ_H 5.35 (H-6) showed cross peak correlation with a methylene proton at δ_H 1.51 (H-7). Some methyl proton correlations were observed between δ_H 0.81 (H-26) with a methine proton at δ_H 1.66 (H-25), while δ_H 1.01 (H-27) also showed cross peak correlation with the methine proton at δ_H 1.66 (H-25). The methyl proton δ_H 0.92 (H-21) correlated with another methine proton at δH 1.36 (H-20).

In the HMBC spectrum the olefinic proton at δ_H 5.35 (H-6) showed correlation with methylene carbon peaks at δ_C: 45.5 (C-4), 34.2 (C-7) and long range cross peak correlation with a methine carbon at δ_C 32.1 (C-8) and a quaternary carbon at δ_C 36.7 (C-10). Some of the correlations from the NOESY experiments included that between the hydroxymethine proton at δ_H 3.52 (H-3) with two methylene proton signals at δ_H 2.24, 2.29 (2H-4) and δ_H 1.85, 1.82 (2H-1). Furthermore, there were some cross peak correlations shown between the methine proton at δ_H 5.35 (H-6) with the methylene carbon protons at δ_H 2.24, 2.29 (2H-4), δ_H 1.95, 1.99 (2H-2) and a methine carbon peak at δ_H 1.51 (H-8). Other cross peak correlations for NOESY and HMBC are summarized in (**Table 6**)**.**

Therefore, on the basis of 1-D and 2-D NMR experiments above and comparison with data reported from literature (Odiba *et al*., 2014; Rashed *et al.,* 2016), the structure of compound **OS1** was proposed to be that of β-sitosterol also known as stigmast-5-en-3β-ol. This investigation reports for the first time the isolation of β-sitosterol from *O. spinosa*. However, β-sitosterol is reported to have islolated before from other plant species that include: The MeOH/DCM extract of *Odontonema strictum* (Acanthaceae) (Luhata *et al*., 2015), petroleum-ether extract of *Dimocarpus longan lour* (Khaled and Gerda, 2013), ethanol extract of honey bee propolis (*Apis mellifera*) (Odiba *et al*., 2014) and many others.

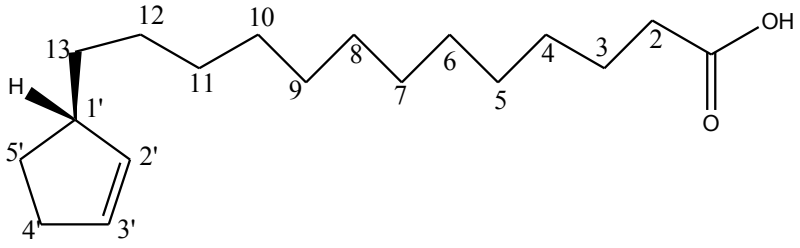
Some bioassay activities have been conducted on β-sitosterol which includes antimicrobial activity (Odiba *et al*., 2014). Their results gave zones of inhibition ranging from (27-34 mm) against the tested microorganisms. The minimum inhibitory (MIC) and minimum bacterial concentration (MBC) values were as low as 12.5-25 and 25-100μg/Ml, respectively against test bacteria's while the minimum fungicidal concentration (MFC) value was 50 μg/mL against *Candida krusei*.

Anti-cancer activity was investigated by Afrianti *et al*. (2015) against MCF7 (breast cancer cell line) and T47D (breast cancer stem cell line). The β-sitosterol in their study was isolated from the EtOAc extract of snake fruits (*Salacca edulis*). Cytotoxic assay revealed

that it inhibited the proliferation and viability of MCF7 at (IC_{50} = 45.414 µg/mL) and T47D at (IC_{50} = 1.1942 µg/mL). This shows that the plants from which β-sitosterol have biological activity therefore justifies their use in ethnomedicine.

On the same basis this study screened this compound for antiplasmodial activity to verify the antimalarial activity of *O. spinosa* (Kodi *et al*., 2017). According to this study β-sitosterol showed high antiplasmodial activity of (IC_{50}) value of (chlorqiune sensitive 3D7: 5.51 µM), which was among the highest from the five isolated pure compounds tested. However, it was inactive against the second strain of parasite with IC_{50} value of (chloroquine resitant Dd2: > 120.77 µM).

4.2.2 Structure elucidation of chaulmoogric acid (OS2)



OS2

Compound **OS2** was isolated from the dichloromethane crude extract as white crystals (293.4 mg). The structure of this compound was determined using 1-D and 2-D NMR experiments (**Table 7)** and the spectra used to interpret the data are shown **[Appendix 1: K3 and K4]**.

In the ^{1}H NMR two triplets of doublet of doublets for two coupled olefinic protons at δ_H 5.68 (1H, tdd, *J* = 2.05, 4.02 Hz, H-2′) and 5.68 (1H, tdd, *J* = 2.05, 3.94 Hz, H-3′) were established. A broad singlet was observed at δ_H 2.61 (1H, br, s, H-1′) due to the methine proton, while a triplet was seen at δ_H 2.35 (2H, t, *J* = 7.49 Hz, H-2) accounting for the methylene protons. Other multiplets of peaks were observed for methylene protons at δ_H 2.24 (2H, m, H-4′) and 2.02 (2H, m, H-5′). There was a resonance of multiplets (quintet) observed due to methylene protons δ_H 1.63 (2H, qn, *J* = 7.49 Hz, H-3). All the remaining methylene protons showed broad singlets at δ_H 1.26 (26H, br s, H-4-H-13).

The ^{13}C NMR and DEPT spectra established the presence of 18 carbon atoms. This was composed of 14 methylene, three methine and one quaternary carbon atom. Unsaturation was shown at carbon atoms δ_C: 135.5 (C-2′), 130.0 (C-3′) and that for the carbonyl group δ_C 178.8 (C-1). The DEPT spectrum confirmed the presence of one the quaternary carbon atom

at δ_C 178.8(C-1) by showing the absence of its peak. The HSQC spectrum showed carbons and protons directly attached to them. The olefinic protons 5.68 (H-2′, H-3′) showed correlation with carbon atoms at δ_C 135.5 and 130.0, respectively. The methine proton δ_H 2.61 showed cross peak correlation with the carbon δ_C 45.6. The methylene protons that resonated at δ_H 2.35, 2.24, 2.02 and 1.63 correlated with carbons δ_C: 33.9 (C-2), 32.0 (C-4′), 29.88 (C-5′) and 24.7 (C-3), respectively.

Table 7: **^{1}H and ^{13}C NMR spectroscopic data for Chaulmoogric acid (OS2) in $CDCl_3$ compared against literature values. Literature (Sivaraman *et al*., 2017) [^{1}H NMR 400 MHz, ^{13}C NMR 100 MHz, $CDCl_3$, *J* in Hz.]**

Position of carbon	^{13}C NMR (125 MHz)	^{13}C NMR (100MHz) Literature	^{1}H NMR (500 MHz)	^{1}H NMR (400 MHz) Literature
1	178.8 (C)	172.4	-	-
2α	33.9 (CH_2)	34.1	2.35 (2H, t, *J* = 7.49 Hz)	2.30 (2H, t, *J* = 7.20 Hz)
2β			2.36	-
3α	24.7 (CH_2)	24.9	1.63 (2H, qn, *J* = 7.49 Hz)	1.60 (2H, s)
3β			1.70 m	-
4	29.2 (CH_2)	29.1	1.26 (br, s)	1.26 (br, s)
5	29.4 (CH_2)	29.4	1.26 (br, s)	1.26 (br, s)
6	29.6 (CH_2)	29.6	1.26 (br, s)	1.26 (br, s)
7	29.7 (CH_2)	29.6	1.26 (br, s)	1.26 (br, s)
8	29.6 (CH_2)	29.6	1.26 (br, s)	1.26 (br, s)
9	29.9 (CH_2)	29.8	1.26 (br, s)	1.26 (br, s)
10	29.7 (CH_2)	29.8	1.26 (br, s)	1.26 (br, s)
11	28.0 (CH_2)	28.0	1.26 (br, s)	1.26 (br, s)
12α	29.1 (CH_2)	29.2	1.26 (br, s)	1.26 (br, s)
12β			1.37 m	-
13α	36.2 (CH_2)	36.2	1.26 (br, s)	1.26 (br, s)
13β			1.35 m	1.35
1'	45.6 (CH)	45.5	2.61 (1H, br, s)	2.60 (1H. br, s)
2'	135.5 (CH)	135.1	5.68 (1H, tdd, *J* = 2.05, 4.02 Hz)	5.66 m
3'	130.0 (CH)	129.7	5.68 (1H, tdd, *J* = 2.05, 3.94 Hz)	5.66 m
4'α	32.0 (CH_2)	32.0	2.24 m	2.21 m
4'β			2.36 m	-
5'α	29.9 (CH_2)	29.9	1.26 (br, s)	1.26 (br, s)
5'β			2.02 m	2.01 m

The assignments of protons to carbons were also accomplished by the COSY, HMBC and NOESY experiments. In the COSY spectrum there were cross peak correlations between the olefinic protons δ_H 5.68 (H-2′, H-3′) with a methine proton δ_H 2.61 (H-1′) and methylene

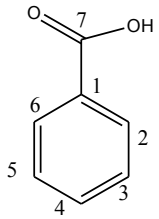
protons δ_H 2.01 (H-5′) and 2.36 (H-4′). Correlations were also observed between the methine proton δ_H 2.61 (H-1′) and the methylene protons δ_H (2.01_β, 1.26_α, H-5′) and (1.26_α, 1.35_β, H-13). Other cross peak correlations were seen between δ_H 2.35 (H-2) with δ_H 1.63 (H-3), δ_H 2.24 (H-4′) with δ_H 2.01 (H-5′), δ_H 1.26 (H-4) with δ_H 1.63 (H-3).

In the HMBC spectrum protons resonating at δ_H 5.68 (H-2′, H-3′) showed correlation with carbons δ_C 45.6 (C-1′), 29.88 (C-5′) and 32.0 (C-4′). More cross peak correlations were observed between the methylene protons δ_H (2.01_β, 1.26_α, 2H-5′) and carbon atoms δ_C: 135.5 (C-2′), 130.0 (C-3′), 32.0 (C-4′), 36.2 (C-13), 45.6 (C-1′). There existed coupling in the NOESY between the methine proton δ_H 2.61 (H-1′) with the methylene protons δ_H 1.26_α, 1.35_β, 2H-13), (2.01_β, 1.26_α, H-5′) and δ_H 1.63 (H-3). The methylene protons resonating at δ_H (2.35_β, 2.26_α, 2H-2) had space correlations with other methylene protons δ_H 1.63 (H-3) and 1.26 (H-4).

The spectroscopic analysis results of ^1H and ^{13}C NMR assignments were in correct agreement with that reported in literature (Blaise *et al*., 1997; Xu *et al*., 2007; Sivaraman *et al*., 2017). Therefore, the structure of compound **OS2** was proposed to be of chaulmoogric acid also known as 13-(2-cyclopentenyl)tridecanoic acid. This is the first report on the isolation of this compound from *O. spinosa.* However literature reports show the isolation and characterization of chaulmoogric acid from plant species such as: *Hydnocarpus anthelminthica* seed (Flacourtiaceae) (Wang *et al*., 2010), hexane extract of *Hydnocarpus pentandra* seeds (Flacourtiaceae) (Sivaraman *et al*., 2017) and *Xylosma controversum* (Xu *et al*., 2007).

Studies on some biological activities of this compound has been investigated which include anticancer, anti-inflammatory, hypolipidaemic activities (Gupta *et al*., 2005). Antifeedant, larvicidal, pupa mortality and adult malformation activities have been investigated by Sivaraman *et al*. (2017). Anti-tuberculosis activity of this compound was also investigated where it significantly inhibited Mtb H37Rv growth with MIC value 9.82 µM (Wang *et al*., 2010). Antileprotic activity is also reported to have been assessed on this compound (Barbosa-Filho *et al*., 2007). However, reports from literature show that no antiplasmodial activity screening studies have been conducted on chaulmoogric acid. Therefore, this study carried out the *in vitro* antiplasmodial activity of chaulmoogric acid for the first time. Chaulmoogric acid from this investigation displayed moderate activity of IC_{50} value (chloroquine sensitive Dd2: 68.67 µM) while it was found inactive against the second strain of parasite with IC_{50} (chloroquine resitant 3D7: > 178.57 µM).

4.2.3 Structure elucidation of benzoic acid (OS3)



OS3

Compound **OS3** was isolated from the EtOAc crude extract as a white crystalline solid (40.9 mg). Its structure was elucidated using the 1-D and 2-D experiments (**Table 8)** of which the some spectra are shown in [**Appendix 1: K5 and 6].**

The ^{1}H NMR spectrum showed five aromatic protons that consisted of four doublet of doublets. These protons resonated at δ_H 8.08 (1H, dd, J = 7.89, 1.70 Hz, H-6, H-2), 7.46 (1H, dd, J = 7.15, 1.13 Hz, H-5, H-3) and a triplet at δ_H 7.47 (1H, t, J = 7.15 Hz, H-4). The chemical shifts of these protons confirmed the presence of a benzene ring. The HSQC spectrum established the direct attachment of these protons to their respective carbons.

The ^{13}C NMR and DEPT spectra established the presence of seven carbon atoms that consisted of five methine and two quaternary carbon atoms. The methine carbons absorbed at δ_C: 134.0 (C-4), 130.3 (C-2, C-6) and 128.4 (C-3, C-5) while the quaternary carbons resonated at 169.6 (C-7) and 130.6 (C-1). The absence of the carbon signals in the DEPT spectrum for the quaternary carbon atoms confirmed their presence in the structure of the molecule.

In the COSY spectrum proton to proton correlations existed between aromatic methine protons, δ_H 7.47 (H-4) with 7.46 (H-5, H-3), 8.08 (H-6) with 7.46 (H-5), 7.46 (H-5) with 7.47 (H-4) and 8.08 (H-6), 7.46 (H-3) with 8.08 (H-2) and 7.47 (H-4). Carbon to proton correlations were established using the HMBC spectrum, some of them included: δ_H 7.47 (H-4) with carbons δ_C 130.3 (C-2, C-6), 8.08 (H-6) with 130.3 (C-2), 134.0 (C-4) and 169.6 (C-7), 8.08 (H-2) with 128.4 (C-3), 134.0 (C-4), 8.08 (H-6) and 169.6 (C-7). The methine proton 7.46 (H-5) correlated with 128.4 (C-3), 134.0 (C-4) and 130.3 (C-6). Proton to proton space correlations were observed in the NOESY spectrum. The methine proton δ_H 7.47 (H-4) showed space cross peak correlations with 7.46 (H-3), 8.08 (H-2, 6) and 7.46 (H-5). Other space couplings were observed between 8.08 (H-6) with 8.08 (H-2), 7.47 (H-4) and 7.46 (H-5), 8.08 (H-2) with 7.46 (H-3) and 7.47 (H-4), 7.46 (H-5) with 7.46 (H-3), 7.47 (H-4) and 8.08 (H-6), 7.46 (H-3) with 8.08 (H-2), 7.47 (H-4) and 7.46 (H-5).

Comparison of ^{1}H and ^{13}C NMR spectral data with that reported in literature (Xu *et al*., 2007; Abdullah *et al*., 2016) the structure of compound **OS3** was proposed to that of a phenolic compound known as benzoic acid. This study reports the isolation of benzoic acid from *O. spinosa* for first time. Xu *et al*. (2007) isolated benzoic acid form *Xylosma controversum*.

Antibacterial activity of benzoic acid was investigated by Goutam *et al*. (2016). Their results gave maximum antibacterial activity with zones of inhibitions of 22 ± 0.8 mm and 15 ± 0.8 mm against gram positive *Staphylococcus aureus* and gram negative *Aeromonas hydrophila*, respectively. Derivatives of benzoic have showed a wide range activity. Among the compounds, 3,4-dihydroxy benzoic acid showed significant activity *in vitro* on soybean lipoxygenase (sLOX) assay. The sLOX inhibitory activity was found to be 74.04±2.6% (Sudha and Srinivasan, 2014).

Antiplasmodial activity screening of benzoic acid is not reported in literature. This study could not determine antiplasmodial activity of benzoic acid because the entire sample was sent for spectroscopic analysis.

Table 8: ^{1}H and ^{13}C NMR spectroscopic data for benzoic acid (OS3) in $CDCl_3$ compared against literature values. Literature (Abdullah *et al*., 2016) [^{1}H NMR 600 MHz, ^{13}C NMR 150 MHz, MeOD, *J* in Hz.

Position of carbon	^{13}C NMR (125 MHz)	^{13}C NMR (150 MHz) Literature	^{1}H NMR (500 MHz)	^{1}H NMR literature (600 MHz)
1	130.6 (C)	129.4	-	-
2	130.3 (CH)	130.3	8.08 (1H, dd, *J* = 7.89, 1.70, H-6)	8.12 (1H, dd, *J* = 7.68, 1.68, H-6)
3	129.2 (CH)	128.4	7.46 (1H, dd, *J* = 7.15, 1.13, H-5)	7.45 (1H, dd, *J* = 7.20, 1.08, H-5)
4	134.0 (CH)	133.8	7.57 (1H, t, *J* = 7.15)	7.62 (1H, t)
5	129.2 (CH)	128.4	7.46	7.45
6	130.3 (CH)	130.3	8.08	8.12
7	169.6 (C)	172.8	-	-

4.2.4 Structure elucidation of compound OS4

The structure of compound OS4 has not yet been elucidated and its spectra are shown in appendix 1 [K7 and K8].

4.3 Compounds from *Euclea latideus* (Ebenaceae)

This plant species gave three pure compounds which included that from the dichloromethane crude extract which yielded one pure compound, a pentacyclic triterpene 3β-(5-methoxyferuloyl)lup-20(30)-ene (EL3) (172.3 mg). The other two pure compounds were isolated from the EtOAc extract, including the triterpenoid Lupeol (EL1) (26.0 mg) and betulin (EL2) (189.6 mg) (**Figure 16**).

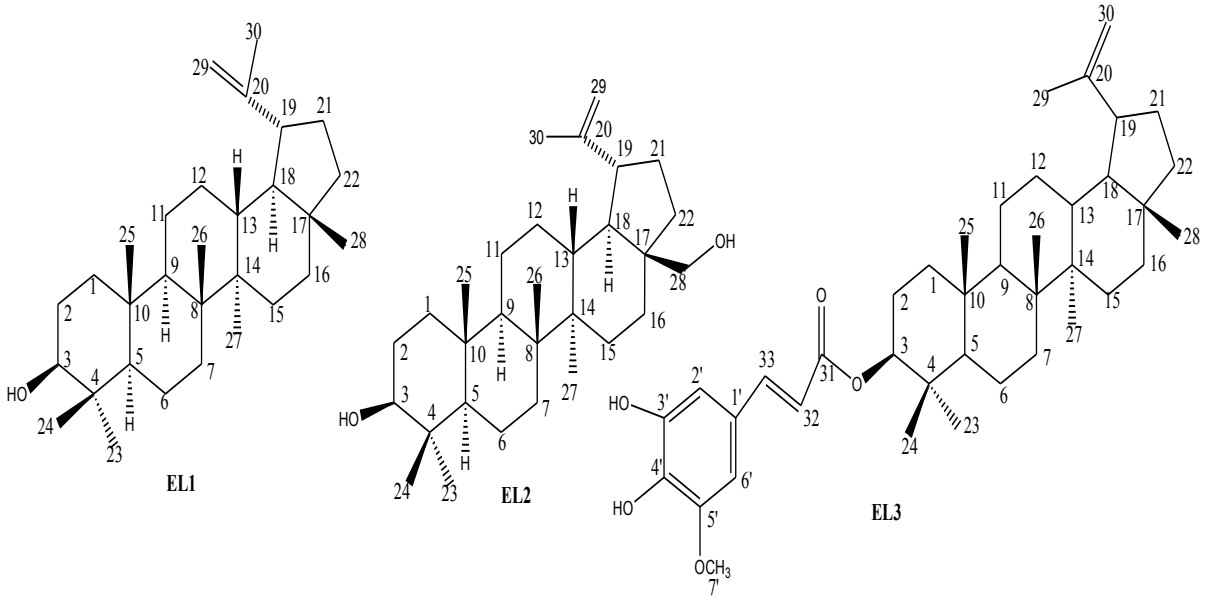
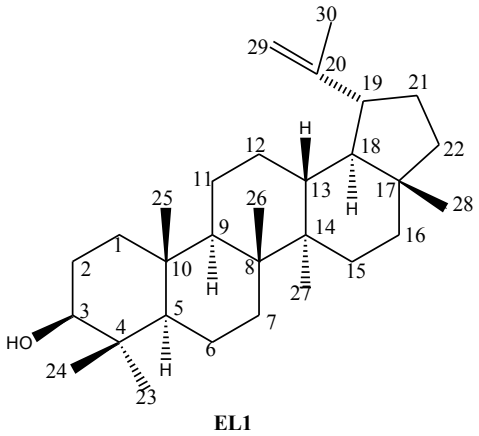


Figure 16: Structures of compounds isolated from *E. latideus*

4.3.1 Structural elucidation of lupeol (EL1)



Compound **EL1** was isolated as a white powder (26.0 mg). Its structure was determined basing on the ^{1}H NMR, ^{13}C NMR, DEPT and 2-D NMR experiments (**Table 9**) and identified as a pentacyclic triterpenoid. The spectra used in elucidation of the structure are shown in [**Appendix** 1: **K9 and K10].**

The ^{1}H NMR spectrum showed the presence of seven tertiary methyl protons at δ_H 0.78 (H-24), 0.81 (H-28), 0.85 (H-25), 0.97 (H-27), 0.99 (H-23), 1.05 (H-26), 1.71 (H-30) (each 3H, s). It also showed two olefinic protons that appeared as broad singlets at δ_H 4.72 (1H, br, s, H-29b) and 4.59 (1H, br, s, H-29a) representing two non-equivalent germinal exomethylene protons of a terminal bond. A doublet of doublets was seen at δ_H 3.22 (1H, dd, J = 4.95, 11.22 Hz, H-3). This represented an axial oxymethine proton (H-3α) attached to a secondary carbon atom that is bonded to an equatorial hydroxyl group (3β-OH) (Mulholand and Nair, 1994). A doublet was observed at 0.70 (1H, d, J = 9.14 Hz, H-5) and a doublet of doublet of doublets at δ_H 2.38 (1H, ddd, J = 5.84, 11.01 HZ, H-19). At δ_H 1.71 (3H, br s, H-30) a broad 3H- proton singlet was seen indicating an isopropenyl group. The remaining protons were seen between δ_H 1.10 and 1.94 as complex multiplets. This showed lupane type of structure.

Table 9: **^{1}H NMR and ^{13}C NMR spectroscopic data for Lupeol (EL1) compared against literature values. Literature (Rashed *et al*., 2016) [^{1}H NMR 400 MHz, ^{13}C NMR 100 MHz $CDCl_3$, J in Hz]**

Position of Carbon	^{13}C NMR (125 MHz)	^{13}C NMR (400 MHz) Literature	^{1}H NMR (500 MHz)	^{1}H NMR (100 MHz) Literature
1α	38.7 (CH_2)	38.6	0.89 m	-
1β			1.68 m	-
2α	27.5 (CH_2)	27.7	1.68 m	-
2β			1.58 m	-
3	79.0 (CH)	78.6	3.22 (1H, dd, J = 4.95, 11.22 Hz)	3.25 (1H, dd, J = 5.6, 10.80 Hz)
4	38.9 (C)	38.8	-	-
5	55.3 (CH)	55.8	0.70 (1H, d, J = 9.14 Hz)	(1H, d, J = 9.10 Hz)
6α	18.3 (CH_2)	18.7	1.38 m	-
6β			1.50 m	-
7α	34.3 (CH_2)	34.6	1.30 m	-
7β			1.41 m	-
8	40.9 (C)	40.8	-	-
9	50.5 (CH)	50.8	1.27 m	-
10	37.2 (C)	37.7	-	-

11α	20.9 (CH_2)	21.4	1.28 m	-
11β			1.43 m	-
12α	25.2 (CH_2)	25.5	1.10 m	-
12β			1.67 m	-
13	38.0 (CH)	38.7	1.68 m	-
14	42.9 (C)	43.3	-	-
15α	27.4 (CH_2)	27.7	1.05 m	-
15β			1.62 m	-
16α	35.6 (CH_2)	35.9	1.48 m	-
16β			1.52 m	-
17	43.0 (C)	43.3	-	-
18	48.3 (CH)	48.7	1.38 m	-
19	48.0 (CH)	48.4	2.38 (1H, ddd, J = 5.84, 11.01 Hz)	2.35 (1H, dt, J = 5.5, 10.90 Hz)
20	151.0 (C)	151.5	-	-
21α	29.9 (CH_2)	29.4	1.34 m	-
21β			1.94 m	-
22α	40.0 (CH_2)	39.8	1.22 m	-
22β			1.40 m	-
23	28.0 (CH_3)	28.5	0.99 (3H, s)	0.98 (3H, s)
24	15.4 (CH_3)	15.8	0.78 (3H, s)	0.75 (3H, s)
25	16.3 (CH_3)	16.6	0.85 (3H, s)	0.85 (3H,s)
26	16.0 (CH_3)	16.3	1.05 (3H,s)	1.08 (3H, s)
27	14.6 (CH_3)	15.3	0.97 (3H,s)	0.96 (3H, s)
28	18.0 (CH_3)	18.4	0.81 (3H, s)	0.80 (3H, s)
29α	109.3(CH_2)	108.8	4.59 (1H, br, s,)	4.58 (1H, s)
29β			4.72 (1H, br, s)	4.68 (1H, s)
30	19.3 (CH_3)	19.5	1.71 (3H, s)	1.75 (3H, s)

The ^{13}C NMR spectrum of compound **EL1** showed thirty carbon atoms (signals), which is typical of triterpenoid skeleton. The DEPT spectrum showed seven methyl groups at δ_C: 14.5 (C-27), 15.4 (C-24) 16.0 (C-26), 16.1 (C-25), 18.0 (C-28), 19.3 (C-30) and 28.0 (C-23). The peaks of two olefinic carbons showing an exomethylene group at 151.0 (C-20) and 109.3 (C-29) were seen indicating the presence of a double bond. Using the DEPT spectrum, eleven methylene, six methine and six quaternary carbon atoms were determined. The quaternary carbon atoms at δ_C: 151.0 (C-20), 43.0 (C-17), 42.9 (C-14), 40.9 (C-8), 38.9 (C-4) and 37.2 were confirmed by their absence in the DEPT spectrum. The spectrum also showed a deshielded signal at δ_C 79.0 (C-3) indicating a carbinol carbon.

The HSQC spectrum showed resonances of two olefinic protons at δ_H 4.59 and 4.72 that correlated with methylene carbon at δ_C 109.3 (C-29). The methine proton that resonated

at δ_H 3.22 correlated with methine carbon δ_C 79.0 (C-3). The methine protons resonating at δ_H 2.38 and 0.70 showed cross peak correlations with carbon atoms δc 48.0 (C-19) and 55.3 (C-5) respectively. The seven methyl protons resonating at different signals had cross peak correlations with their respective carbons: δ_H 0.99 with δc 28.5 (C-23), δ_H 1.71 with δc 19.3 (C-30), δ_H 0.81with δc 18.0 (C-28), δ_H 0.85 with 16.1 (C-25), δ_H 1.05 with δ_C 16.0 (C-26), δ_H 0.78 with δc 15.4 (C-24) and δ_H 0.97 with δ_C 14.6 (C-27). Correlations of other proton resonances with their respective carbons are shown in (**Table 9)**.

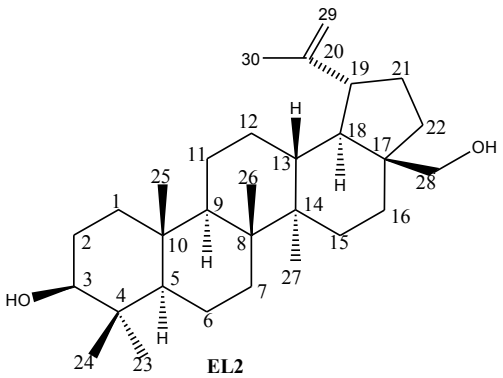
The proposed structure of compound **EL1** confirmed using 2-D NMR experiments that included COSY and HMBC. In the COSY spectrum cross peak correlations were seen between a methine proton at δ_H 2.38 (H-19) with a methylene proton peak (δ_H 1.34, H-21) and a methine proton signal (δ_H 1.38, H-18). Another cross peak correlation was seen between oxygenated methine proton signal (δ_H 3.22, H-3) and sp^3 methylene peak (δ_H 1.38, H-2). There was a correlation between sp^3 methine proton (δ_H 1.38, H-18) with (δ_H 2.38, H-19). Another cross peak correlation existed between the methylene proton (δ_H 1.40, H-22) with (δ_H 1.34, H-21).

In the HMBC spectrum the methine proton at (δ_H 3.22, H-3) showed correlation with the methyl carbon signals of (δ_C 28.0, C-23), (δ_C 15.8, C-24) and methylene carbon signal δ_C 18.3, C-6). The sextet signal at (δ_H 2.38, H-19) showed cross peak correlations with two methylene carbon signals (δ_C 29.9, C-21) and δ_C 109.3 C-29), a methine carbon signal δ_C 48.3, C-18), a methyl carbon signal δ_C19.3, C-30) and with a quaternary carbon signal δ_C 151.0, C-20). The pair of broad singlets of olefinic protons at (δ_C 4.59 and 4.72, H-29) showed cross peak correlation with a methine carbon signal at δ_C 48.0, C-19) and a methyl peak (δ_C 19.3, C-30). Other HMBC cross peak correlations were observed between a number of protons and carbon atoms. Therefore from the above spectroscopic data the structure of compound **EL1** was suggested to be that of Lupeol also known as lup-20-(29)-en-3β-ol. This was after comparison of its ^1H and ^{13}C NMR spectral data with that from reported literature (Abdullahi *et al*., 2013; Khaled *et al*., 2014; Rashed *et al*., 2016). There is no reported literature on the isolation of Lupeol from *E. latideus* therefore this is the first time that lupeol is isolated from this plant species. Lupeol has been isolated before from a number of plant species that include: *Pistacia chinesis* (Anacardiaceae) (Rashed *et al*., 2016), *Lonchocarpus sericeus* (Papilionaceae**)** (Abdulahi *et al*., 2013), *Acacia ataxacantha* (Fabaceae) (Abdou *et al*., 2016), *Liquidanbar styraciflua* (Hamamelidaceae) (Khaled *et al*., 2014), *Diospyros rubra* (Ebenaceae) (Prachayasittkul *et al*., 2010) and many more.

Lupeol has been investigated for antimicrobial and antioxidant activities. In one study it showed moderate antimicrobial activity with MIC > 50 µg/mL and moderate antioxidant activity with an IC_{50} value of 16.77 µg/mL (Abdou *et al*., 2016). Determination of α-glucosidase enzyme inhibition assay was investigated at concentration of 0.006 mM. The findings of this investigation gave 64.4% inhibition with IC_{50} value of 0.002 ± 0.004 mM (Mohamed *et al*., 2009). Literature also reports it to exhibit antitumor, anti-inflammatory, antiprotozoal and chemo preventive properties (Gallo and Sarachine, 2009). In other studies it has exhibited anti-inflammatory, anti-angiogenic activities and acetylchloinesterase activity (Khaled *et al*., 2014).

Antiplasmodial activity of lupeol is documented in literature. Dominique *et al*. (2015) carried out activity against lupeol isolated from *Ampelozizyphus amazonicus* that gave IC_{50} (chloroquine sensitive 3D7: 8.30 and chloroquine resistant 2Dd: 54.22 µM). The current study the activity of lupeol that displayed moderate antiplasmodial activity against both strains of parasite with (IC_{50}) values of (chloroquine sensitive 3D7: 56.13 µM, and chloroquine resistant Dd2: 59.01 µM).

4.3.2 Structural elucidation of betulin (EL2)



EL2

Compound **EL2** was isolated from EtOAc crude extract as a white powder (189.6 mg). The structure of this compound was proposed basing on ^{1}H NMR, ^{13}C NMR and complete assignment using 2-D NMR spectral data (**Table 10**). All the spectra used to interpret the data are shown **[Appendix I: K11 and K12].**

The ^{1}H NMR spectrum of compound **EL1** displayed singlet signals of six tertiary methyl groups at δ_H: 1.68 (H-30), 1.02 (H-26), 0.95 (H-27), 0.94 (H-23) 0.83 (H-25) and 0.75 (H-24). The observation of a signal at 1.68 indicated one methyl group attached to a double

bond. In addition it showed a ^{1}H doublet of doublet (1H, dd, J = 4.81, 11.43 Hz, H-3) due to a hydroxylmethine proton at δ_H 3.18. Its chemical shifts and coupling constant confirmed its assignment to the hydroxymethine at C-3, with the proton having an axial (α) orientation when an equatorial (β) hydroxyl is present (Mulholand and Nair, 1994). Other doublets of two coupled protons had resonances at δ_H 3.79 and 3.32 (1H, d, J = 10.80 Hz), showing the presence of two an equivalent protons of a hydroxymethylene group (-CH_2OH, C-28). This was confirmed by proton resonances in the HSQC spectrum of the methylene carbon that had a signal at δ_C 60.7. The protons signals at δ_H 4.62 and 4.52 (1H, br, s, H-29) indicated the presence of two non equivalent germinal exomethylene protons of terminal methylene group. This corresponded well with ^{13}C NMR signal at 109.9 in the HSQC spectrum. There were other major signals of doublet of doublet of doublets at δ_H 2.38 (1H, ddd, J = 5.84, 10.96 Hz, H-19) and a doublet signal at δ_H 0.68 (1H, d, J = 10.64 Hz, H-5). The remaining protons were assigned as multiplets as summarized in (**Table 10**).

Table 10: **^{1}H and ^{13}C NMR spectroscopic data for betulin (EL2) in $CDCl_3$ compared against literature values. Literature (Zahid *et al*., 2013) [^{1}H NMR 300 MHz, ^{13}C NMR 75 MHz, $CDCl_3$, J in Hz]**

Position of carbon	^{13}C NMR (125 MHz)	^{13}C NMR (75 MHz) literature	^{1}H NMR (500 MHz)	^{1}H NMR (300 MHz) literature
1α	38.7 (CH_2)	38.7	0.90 m	-
1β			1.62 m	1.63 m
2α	27.0 (CH_2)	27.1	1.53 m	-
2β			1.57 m	1.59 m
3	79.2 (CH_2)	78.9	3.18 (1H, dd, J = 4.81, 11.43 Hz)	3.17 (1H,dd, J = 4.90, 10.80 Hz)
4	38.9 (C)	38.9	-	-
5	55.5 (CH)	55.3	0.68 (1H, d, J = 10.64 Hz)	0.65 (1H, d, J = 9.40 Hz)
6α	18.3 (CH_2)	18.3	1.32 m	1.40 m
6β			1.53 m	-
7α	34.2 (CH_2)	34.3	1.32 m	-
7β			1.00 m	1.05 m
8	41.1 (C)	41.0	-	-
9	50.6 (CH)	50.4	1.19 m	1.23 m
10	37.2 (C)	37.2	-	-
11α	20.8 (CH_2)	20.1	1.17 m	1.15 m
11β			1.38 m	-
12α	25.2 (CH_2)	25.2	1.64 m	1.51 m

12β			1.71 m	-
13	37.3 (CH)	37.3	1.61 m	1.51m
14	42.9 (C)	42.7	-	-
15α	27.4 (CH_2)	27.4	0.95 m	-
15β			1.54 m	1.56 m
16α	29.2 (CH_2)	29.2	1.19 m	-
16β			1.93 m	1.93 m
17	28.0 (C)	27.9	-	-
18	48.9 (CH)	48.8	1.61 m	1.51 m
19	48.0 (CH)	47.8	2.38 (1H, ddd, *J* = 5.85, 10.96 Hz)	2.37 (1H, ddd, *J* = 4.0, 8.0 Hz)
20	150.7 (C)	150.0	-	-
21α	29.7 (CH_2)	29.8	1.32 m	1.35 m
21β			1.98 m	-
22α	34.0 (CH_2)	34.0	1.33 m	-
22β			1.85 m	1.83 m
23	21.1 (CH_3)	27.9	0.94 (3H, s)	0.95 (3H, s)
24	15.4 (CH_3)	15.3	0.75 (3H, s)	0.74 (3H, s)
25	16.1 (CH_3)	16.1	0.83 (3H, s)	0.80 (3H, s)
26	16.0 (CH_3)	16.0	1.02 (3H, s)	1.00 (3H, s)
27	14.8 (CH_3)	14.8	0.95 (3H, s)	0.96 (3H, s)
28α	60.7 (CH_2)	60.6	3.26 (1H, d, *J* = 10.80 Hz)	3.31 (1H, d,*J* = 12.0 Hz)
28β			3.73 (1H, d, *J* = 10.80 Hz)	3.78 (1H, d, *J* = 12.0 Hz)
29α	109.9 (CH_2)	110.0	4.52 (1H, br, s)	4.56 (1H, s)
29β			4.62 (1H, br, s)	4.66 (1H, s)
30	19.1 (CH_3)	19.1	1.68 (3H, s)	1.66 (3H, s)

quaternary carbon δ_C 150.7 (C-20) and one methylene carbon δ_C 109.9 (C-29) confirmed the presence of a terminal double bond.

The HSQC spectrum of compound **EL2** showed olefinic protons resonating at δ_H 4.52 and 4.62 that correlated with δ_C 109.9 (C-29). The hydroxymethine proton resonating at δ_H 3.18 (H-3) correlated with the methine carbon at δ_C 79.2 (C-3), while the protons of the hydroxymethylene group that resonated at δ_H 3.32 and 3.79 showed correlation with the methylene carbon atom at δc 60.7 (C-28). The methine proton resonating at δ_H 2.38 was observed to correlate with the methine carbon at δ_C 48.0 (C-19). The methine proton that resonated at δ_H 0.69 showed cross peak correlation with the carbon δ_C 55.5 (C-5). The spectrum also showed methyl protons that resonated with their respective carbon atoms.

The ^{13}C NMR spectrum showed thirty carbon signals that were clarified by DEPT experiments that indicated that compound **EL2** was a triterpenoid. It showed the presence of six methyl groups at δ_C 19.09 (C-30), 16.1 (C-25), 16.0 (C-26), 15.4 (C-24), 14.8 (C-27) and 21.1 (C-23). It also revealed the presence of twelve methylene, six methine and six quaternary carbon atoms that were confirmed by their absence in the DEPT spectrum at δ_C: 150.7 (C-20), 42.7 (C-14), 41.0 (C-8), 38.9 (C-4), 37.2 (C-10), 28.0 (C-17) in the molecule. The These included: δ_H 0.94 with δ_C 21.1 (C-23), δ_H 1.68 with δ_C 19.1 (C-30), δ_H 0.83 with δ_C 16.1 (C-25), δ_H 1.02 with δ_C 16.0 (C-26), δH 0.75 with δ_C 15.4 (C-24), δH 0.95 with δc 14.8 (C-27). Attachment of protons to other carbons in the HSQC spectrum is shown in **Table 10**.

Complete assignment of protons of the proposed structure was established using 2-D NMR experiments that included COSY, HMBC and ROESY spectrum. In the COSY cross peak correlations existed between olefinic protons δ_H 4.52 (H_α-29) and δ_H 4.62 (H_β-29) with the methyl proton signal δ_H 1.68 (H-30). Cross peak correlation were established between the two hydroxymethylene groups δ_H 3.79 (H_α-28) and δ_H 3.32 (H_β-28). There were other cross peak correlation between the methine proton δ_H 1.68 (H-5) with a methylene proton δ_H 1.53 (H_α- 6) .In the HMBC spectrum there was correlation between protons and carbons. The olefinic protons δ_H 4.52 (H_α-29) and 4.62 (H_β-29) correlated carbons δ_C 48.0 (C-19) and methyl carbon δ_C 19.1 (C-30). The hydroxymethine proton δ_H 3.18 (H-3) showed cross peak correlation with carbons δC 15.4 (C-24), 27.0 (C-2), 38.7 (C-1) and 38.9 (C-4). Correlations existed of 2 and 3 bonds between the methylene protons δ_H 1.38 (H_β-11) and δ_H 1.17$_\alpha$-11 with carbons at δ_C: 55.5 (C-5), 42.9 (C-14), 41.0 (C-8), 34.2 (C-7). The methine proton that resonated at δ_H 2.38 (H-19) showed cross peak correlations with carbons at δ_C: 19.1 (C-30), 29.7 (C-21), 37.3 (C-13), 48.9 (C-18) and 109.9 (C-29).Correlations were observed in the ROESY spectrum included that between the olefinic protons δ_H 4.52 (H_α-29) and 4.62 (H_β-

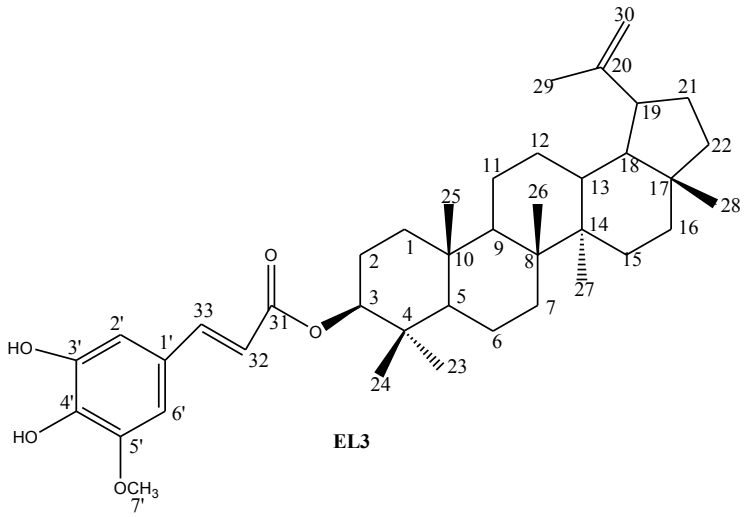
29) with the methine protons at δ_H 1.61 (H-18) and 2.38 (H-19). Space cross peak correlations were seen between the methine proton δ_H 2.38 (H-19) with methylene protons δ_H 3.79 (H_α-28), 4.62 (H_β-29), 1.85 (H_β-22), 1.61 (Hα-12) and a methine proton 1.61 (H-13). Space coupling correlations were seen between the methyl protons δ_H 0.95 (3H-27) with the methylene protons δ_H: 4.62 (H_β-29), 1.61 (H_α-12), 1.54 (H-15) and the methine protons of δ_H 1.61 (H-13), 1.61 (H-18). Proton cross peak correlations existed between the carbinol proton δ_H 3.18 (H-3) with methylene protons δ_H: 1.53 (H_β-6), 1.32 (H_α-6), 1.53 (H_α-2), 0.90 (H_α-1), 1.62 (H_β-1), methine proton 0.68 (H-5) and methyl protons 0.83 (3H-25).

After comparing the ^{1}H NMR and ^{13}C NMR spectral data with that from literature the structure of compound was proposed to be a pentacyclic triterpene, betulin also known as lup-20(29)-ene-3β,28-diol (PraChayAsittikul *et al*., 2010; Zahid *et al*., 2013). Although the isolation and biological activities of betulin is well published, no literature is available concerning its isolation from *E. latideus.* Therefore, this study reports the isolation of betulin for the first time from *E. latideus.*

Betulin has been isolated from various plant species among many that include: Dichloromethane extract of *Scheffleria umbellifera* (Araliaceae) (Mthembu *et al*., 2010), methanol crude extract of *Viburum nervosum* (Zahid *et al*., 2013), dichloromethane extract of *Diospyros rubra* (Ebenaceae) *(Parachayasittikul et al*., 2010), ethyl acetate stem bark extract of *Diospyros mespiliformis* (Ebenaceae) (Mohamed *et al*., 2009). A number of biological activities have been conducted on betulin that include: Antimicrobial screening against *Streptococcus pyogenes* gave a minimum inhibitory concentration (MIC) value of 85 µg/mL in an investigation by Parachayasittikul *et al*. (2010). The α-glucoside enzyme inhibitory potential was also investigated by Mohamed *et al*. (2009). At a concentration of 0.003 mM, their study gave percentage inhibition value of 75 with IC_{50} 0.46 ± 0.002 mM. Assessment of the anti-mycobacterial activity of betulin from the methanol bark extract of *Alnus incana* (Betulaceae) was conducted by Li *et al*. (2015). Betulin in their study displayed a MIC value of 12.5 µg/mL and an IC_{50} of 2.4 µg/mL against *M. tuberculosis* (H37Ra). Its derivatives betulinic acid and betulone in the same study showed lower anti-mycobacterial activities with IC_{50} values of 84 and 57 µg/mL, respectively against *M. tuberculosis* (H37Ra).

The antiplasmodial activity of betulin is documented in literature however the current study assessed the activity of betulin isolated from *E. latideu*. In the current study betulin exihibited high antiplasmodial activity against both strains that had IC_{50} values of (chloroquine sensitive 3D7: 3.71 µM, chloroquine resistant Dd2: 17.40 µM).

4.3.3 Structural elucidation of 3β-(5-methoxyferuloyl)lup-20 (30)-ene (EL3)



EL3

Compound **EL3** was isolated as a black crystalline solid (172.3 mg) from the dichloromethane crude extract. The proposed structure of compound **EL3** was based on the interpretation done on ^{1}H NMR, ^{13}C NMR, COSY, HMBC, HSQC, DEPT and NOESY experiments. The spectra of these experiments are shown **[Appendix I: K13 and K14].**

The ^{1}H NMR spectrum showed seven methyl groups all being singlets. They consisted of six tertiary methyl groups located on saturated carbons. These included: δ_H: 0.95 (3H, s, C-27), 1.04 (3H, s, H-26), 0.88 (3H, s, H-25), 0.92 (3H, s, H-24), 0.79 (3H, s, H-28), 0.89 (3H, s, H-23). There is one isopropylidene methyl group (MeC=CH_2) present at δ_H1.69 (3H, s, H-29) and a methoxyl (-OCH_3) singlet at 3.91 (3H, s, H-7′). The HSQC spectrum confirmed its location on this carbon at δ_H56.3. Proton signals were observed at δ_H 4.69 and 4.57 due to two coupled nonequivalent germinal olefinic protons. Both of these protons were doublets 4.69 (1H, d, *J* = 2.47 Hz, C-30) and 4.57 (1H, d, *J* = 2.47 Hz, H-30). A proton signal was observed at δ_H 4.59 (1H, m, H-3) due to a deshielded methine proton on a carbon that has an oxygen atom attached to an ester. The HSQC spectrum established its placement at δ_C 80.9. Two doublets of 5-methoxyferulic ester signals due to coupled *trans*-substituted protons on the ArCH=CH- were observed at δ_H 7.53 (1H, d, *J* = 15.86 Hz, H-32$_\alpha$,) and 6.27 (1H, d, *J* = 15.86 Hz, H-33$_\beta$). Two doublet signals due to two aromatic methine protons appeared at δ_H 6.82 (1H, d, *J* = 1.56 Hz, H-2′) and 6.65 (1H, *J* = 1.74 Hz, H-6′). These two protons were placed on their respective carbons by the HSQC spectrum at 109.3 (H-2′) and 102.9 (H-6′). A doublet of doublets was observed at δ_H 2.37 (1H, dd, *J* = 5.77 Hz, 11.0 Hz, H-19) (**Table 11**).

Table 11: **^{1}H and ^{13}C NMR spectroscopic data for 3β-(-5-methoxyferuloyl) lupen-20(30)-ene (EL3) in $CDCl_3$ compared against literature values. Literature (Mebe *et al*., 1998) [^{1}H NMR 400 MHz, ^{13}C NMR 100 MHz, $CDCl_3$, *J* in Hz].**

Position of Carbon	^{13}C NMR (125 MHz)	^{13}C NMR (100MHz) literature	^{1}H NMR (500 MHz)	^{1}H NMR (400 MHz) literature
1α	38.4 (CH_2)	38.4	1.02 m	1.13 m
1β			1.69 m	1.67 m
2α	23.8 CH_2)	23.8	1.69 m	1.60 m
2β			1.91 m	-
3	80.9 (CH)	80.9	4.59 m	4.50-4.60 m
4	38.7 (C)	39.0	-	-
5	55.4 (CH)	55.4	1.40 m	-
6α	18.2 (CH_2)	18.2	1.40 m	1.41 m
6β			1.50 m	1.52 m
7α	34.2 (CH_2)	34.2	1.02 m	-
7β			1.38 m	-
8	40.9 (C)	40.8	-	-
9	50.4 (CH)	50.3	1.26 m	1.55 m
10	37.1 (C)	37.1	-	-
11α	21.0 (CH_2)	20.9	1.21 m	-
11β			1.40 m	-
12α	25.1 (CH_2)	25.1	1.07 m	-
12β			1.69 m	-
13	38.1 (CH)	38.0	1.69 m	-
14	42.9 (C)	42.8	-	-
15α	27.4 (CH_2)	27.4	1.01 m	0.96 m
15β			1.69 m	1.83 m
16α	35.6 (CH_2)	35.6	1.37 m	-
16β			1.46 m	-
17	43.0 (C)	43.0	-	-
18	48.0 (CH)	48.0	1.37 m	1.31 m
19	48.3 (CH)	48.3	2.37 (1H, dd, *J* = 5.77, 11.0 Hz)	2.30 m
20	151.0 (C)	150.9	-	-
21α	29.8 (CH_2)	29.8	1.26 m	-
21β			1.91 m	-
22α	40.0 (CH_2)	40.0	1.20 m	-
22β			1.40 m	-
23	28.0 (CH_3)	28.0	0.89 (3H, s)	0.87 (3H , s)
24	16.7 (CH_3)	16.7	0.92 (3H, s)	0.89 (3H, s)
25	16.2 (CH_3)	16.2	0.88 (3H, s)	0.86 (3H, s)
26	16.0 (CH_3)	16.0	1.04 (3H, s)	1.02 (3H, s)

27	14.5 (CH_3)	14.5	0.95 (3H, s)	0.93 (3H, s)
28	18.0 (CH_3)	18.0	0.79 (3H, s)	0.76 (3H, s)
29	19.3 (CH_3)	19.3	1.69 (3H, s)	1.66 (3H, s)
30α	109.4 (CH_2)	109.3	4.57 (1H, d, *J* = 2.47 Hz)	4,58 (1H, br d, *J* = 2.40 Hz)
30β			4.69 (1H, d, *J* = 3.47 Hz)	4.70 (1H, br d, *J* = 2.40 Hz)
31	167.1 (C)	167.2	-	
32	144.5 (CH)	144.4	7.53 (1H, d, *J* = 15.86 Hz)	7.54 (1H, d, *J* = 15.80 Hz)
33	116.9 (CH)	116.8	6.27 (1H, d, *J* = 15.86 Hz)	6.29 (1H, d, *J* = 15.80 Hz)
1'	126.7 (C)	126.6	-	
2'	109.3 (CH)	109.2	6.82 (1H,d, *J* = 1.56 Hz)	6.79 (1H, br, d, *J* = 1.80 Hz)
3'	144.0 (C)	143.9	-	-
4'	134.6 (C)	134.5	-	-
5'	147.0 (C)	146.9	-	-
6'	102.9 (CH)	102.9	6.65 (1H, d, *J* = 1.74 Hz)	6.63 (1H, br, d, *J* = 1.80 Hz)
7'	56.3 (CH_3)	56.2	3.91 (3H, s)	3.88 (3H, s)

The ^{13}C NMR and DEPT experiments established the composition of the structure to have forty carbon atoms. It showed distinct carbon signals for lupene moiety. The outstanding difference was that the C-3 signal was deshielded to δ_C 80.9 compared to a free alcohol at δ_C 78.8. This confirmed the linkage between the lupene and ferulosyl moieties at C-3. Eight methyl signals were observed at δ_C: 14.5 (C-27), 16.0 (C-26), 16.2 (C-25), 16.7 (C-24), 18.0 (C-28), 19.3 (C-29), 28.0 (C-23) and 56.3 (C-7′). The methyl group at δ_C 56.3 confirmed its attachment to a methoxy group. The ^{13}C NMR further established six double bonds, three belonging to the aromatic ring located between δ_C: 126.7 (C-1′) and 102.9 (C-6′), 144.0 (C-3′) and 109.3 (C-2′), 134.6 (C-4′) and 147.0 (C-5′). Two double bonds are located between δ_C: 144.5 (C-32_α) and 116.9 (C-33_β), 151.0 (C-20) and 109.4 (C-30), while one is due to carbonyl group at 167.1 (C-31). The methoxy carbon atom was seen to absorb at δ_C 56.3 (C-7′). The ^{13}C NMR and DEPT experiments further determined the presence of eleven methylene and ten methine groups. The DEPT spectrum confirmed the presence of eleven quaternary carbon atoms by their absence in its spectrum, these include δ_C: 167.1 (C-31), 151.0 (C-20), 147.0 (C-5′), 144.0 (C-3′), 134.6 (C-4′), 126.7 (C-1′), 38.7 (C-4), 43.0 (C-17) 42.9 (C-14), 40.9 (C-8) and 37.1 (C-10).

The HSQC spectrum of compound **EL3** showed correlations of carbon atoms and protons directly attached to them. There were correlations between the two germinal olefinic

protons δ_H 4.57 and 4.69 with the carbon at δ_C 109.4 (C-30). The *trans*-substituted protons that resonated at δ_H 7.53 and 6.27 were observed to correlate with carbons δ_C 144.5 (C-32) and 116.9 (C-33) respectively. The two aromatic methine protons resonating δ_H 6.83 and 6.65 were seen to correlate with carbon atoms at δ_C 109.3 (C-2′) and 102.9 (C-6′) respectively. The methine proton resonating at δ_H 4.59 was observed to correlate with carbon atom δ_C 80.90 (C-3), while the methoxy carbon absorbing at δ_C 56.3 (C-7′) correlated with protons resonating at δ_H 3.91. The methine proton that resonated at δ_H 2.37 correlated with carbon atom δ_C 48.3 (C-19). All other methyl protons resonating at different chemical shifts were observed to correlate with their respective carbons as follows: δ_H 1.69 with δ_C 19.3 (C-29), δ_H 0.79 with δ_C 18.0 (C-28), δ_H 0.92 with δ_C16.7 (C-24), δ_H 0.88 with δ_C 16.2 (C-25), δ_H 1.04 with δ_C 16.0 (C-26) and δ_H 0.95 with δ_C 14.5 (C-28). The remaining protons resonated at different signals that correlated with their respective carbons (**Table 11**).

The ^{1}H and ^{13}C NMR, HSQC assignments were confirmed with COSY, HMBC and NOESY experiments. In the COSY spectrum the methine protonδ_H 7.53 (H-32) showed correlation with another methine proton δ_H 6.27 (H-33). The olefinic protons at 4.57_α and 4.69_β (2H-30) showed cross peak correlation with the methyl protons δ_H 1.69 (3H-29). The methine proton δ_H 2.37 (H-19) showed correlation with another methine proton δ_H 1.37 (H18) and the methylene proton δ_H 1.91_β (H-19). The methylene protons δ_H 1.40α and 2.05_β, (2H-11) showed cross peak correlation with a methine proton δ_H 1.26 and the methylene protons δ_H 1.69_β and 1.07_α (2H-12).

In the HMBC spectrum the olefinic methine proton δ_H 7.53 (H-32α) correlated with carbons δ_C: 167.1 (C-31), 126.7 (C-1′), 116.9 (C-33), 109.3 (C-2′) and 102.9 (C-6′). The olefinic methylene protons (δ_H 4.57_α, 4.69_β, 2H-30) were observed to correlate with carbons δ_C 48.3 (C-19), 19.3 (C-29). The hydroxymethine proton δ_H 3.91 (H-3) showed cross peak correlation with carbons δ_c 167.1 (C-31) and 16.7 (C-24). The correlation between the hydroxymethine proton and with carbon δ_c 167.1 (C-31) proved the linkage between the lupene and ferulosyl moieties. Furthermore the methine proton δ_H 2.37 (H-19) showed cross peak correlation with carbons δ_C 151.0 (C-20), 109.4 (C-30), 48.0 (C-18), 38.1 (C-13), 29.8 (C-21) and 19.3 (C-29). The methyl protons δ_H 0.79 (3H-28) correlated with carbons 48.0 (C-18), 48.3 (C-19), 35.6 (C-16) and 43.0 (C-17).

In the NOESY spectrum correlations were seen between olefinic proton δ_H 7.53 (H-32α) with protons δ_H 6.82 (H-2′), 6.65 (H-6′) and 6.27 (H-33_β). The olefinic proton δ_H 6.27 (H-33_β) was observed to correlate with protons δ_H 7.53 (H-32α), 6.82 (H-2′) and 6.65 (H-6′). The olefinic protons δ_H 4.57_α and 4.69_β (2H-30) showed cross peaks with protons δ_H 2.37 (H-

19), 1.37 (H-18) and 1.69 (H-29). The methyl protons δ_H 1.69 (3H-29) correlated with olefinic protons δ_H (4.57$_\alpha$, 4.69$_\beta$, H-30) and the methine proton 2.37 (H-19).

Comparison of the above spectral data with that reported in literature, the structure of the compound was proposed to be 3β-(-5-methoxyferuloyl) lupen-20(30)-ene (Mebe *et al*., 1998; Ohsaki *et al*., 2004; Rudiyansyah *et al*., 2006). The isolation of this compound from *E. latideus* is being reported for the first time. However, it is reported to have been isolated from dichloromethane crude extract of roots of *E. divinorum* (Mebe *et al*., 1998).

Cytotoxic activity of this compound has been investigated by Mebe *et al*. (1998) against a number of cell lines (Effective at 50%, ED_{50} < 20 µg/mL). It only showed activity against two cell lines P-388 (murine lymphocytic leukemia) and ZR-75-1 (human breast cancer) at ED_{50}, 2.1 and 4.2 µg/mL, respectively. However, antiplasmodial activity screening of this compound is not reported in literature. This study could not determine the *in vitro* antiplasmodial activity because the compound was insoluble in dimethlysulfoxide, because other solvents active against P. falciparum parasites.

4.4 Compounds from Acacia sieberiana (Mimosaceae)

Two known steroids were isolated from this plant, one from the dichloromethane extract identified as β-sitosterol (**AS2**) (16.8 mg). The β-sitosterol isolated from this plant was the same as that from the dichloromethane crude extract of *O. spinosa* in section **4.2.1**. The second steroid was from the ethyl acetate crude extract identified as stigmasterol (**AS1**) (**Figure 17**).

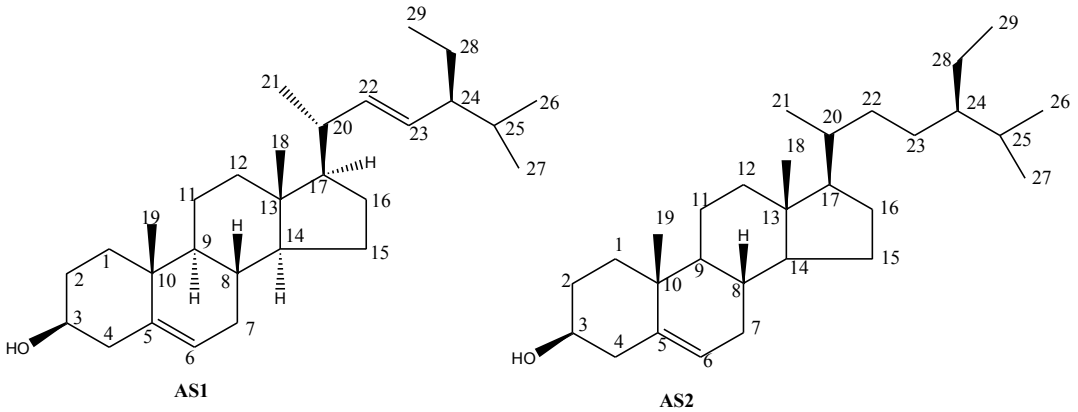
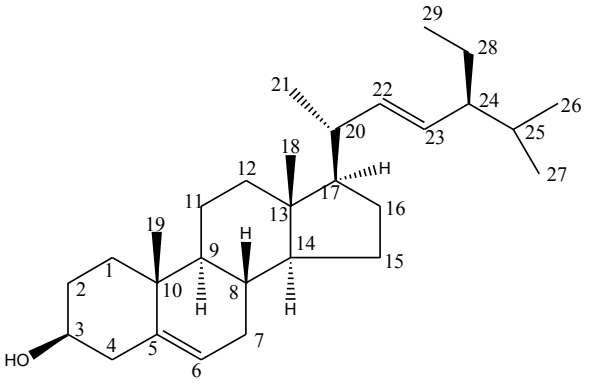


Figure 17: **Structures of compounds isolated from *A. sieberiana***

4.4.1 Structure elucidation of stigmasterol (AS1)



AS1

Compound **AS1** was isolated as a white crystalline solid (195.9 mg) from the ethyl acetate crude extract. The proposed structure of compound **AS1** was based on interpretation of spectra from ^{1} H NMR, ^{13}C NMR and 2-D experiments (**Table 12**) of which some spectra are shown **[Appendix I: K15 and K16].**

Table 12: **^{1}H and ^{13}C NMR spectroscopic data for stigmasterol (AS1) in $CDCl_3$ compared against literature values. Literature (Yusuf *et al*., 2015) [^{1}H NMR 400 MHz, ^{13}C NMR 100 MHz, $CDCl_3$, *J* in Hz].**

Position of carbon	^{13}C NMR (125 MHz)	^{13}C NMR (100 MHz) literature	^{1}H NMR (500 MHz)	^{1}H NMR (400 MHz) literature
1	37.5 (CH_2)	37.3	1.83 m	1.85 m
2	31.9 (CH_2)	31.7	1.97 m	1.46 m
3	72.0 (CH)	71.8	3.52 (1H,tdd, *J* = 4.57, 11.03 Hz)	3.52 m
4	42.5 (CH_2)	42.3	2.26 (2H, tdd, *J* = 4.88, 13.08 Hz)	2.27 m
5	141.0 (C)	140.8	-	-
6	121.9 (CH)	121.7	5.33 (1H, br,d, *J* = 5.04 Hz)	5.35 (br, s)
7	34.2 (CH_2)	32.0	1.51 m	1.96 m
8	32.1 (CH)	31.9	1.51 m	1.48 m
9	50.4 (CH)	50.2	0.92 m	0.93 m
10	36.7 (C)	36.5	-	-

11	21.4 (CH_2)	21.2	1.46	1.49 m
12	40.7 (CH_2)	39.7	2.00 m	1.16 m
13	42.4 (C)	42.2	-	-
14	57.1 (CH)	56.9	1.00 m	1.05 m
15	24.5 (CH_2)	24.3	1.06 m	1.56 m
16	28.5 (CH_2)	28.9	1.84 m	1.70 m
17	56.3 (CH)	56.0	1.11 m	1.13 m
18	12.1 (CH_3)	12.1	0.68 (3H, s)	0.69 (3H, s)
19	19.6 (CH_3)	21.0	1.01 (3H, s)	1.01 (3H, s)
20	40.0 (CH)	40.5	2.00 m	2.02 m
21	23.3 (CH_3)	23.1	0.90 (3H, d, J = 6.46 Hz)	1.02 (5H, d, J = 7.50 Hz)
22	138.5 (CH)	138.3	5.13 (1H, dd, J = 8.67, 15.13 Hz)	5.10 m
23	129.5 (CH)	129.3	5.00	5.03 m
24	51.5 (CH)	51.2	1.54 m	1.53 m
25	29.4 (CH)	29.2	1.66 m	1.65 m
26	20.0 (CH_3)	19.0	0.91 (3H, d, J = 6.78 Hz)	0.82 (3H, d, J = 6.50 Hz)
27	19.3 (CH_3)	19.4	0.98 (3H, s)	0.78 (3H, s)
28	25.6 (CH_2)	25.4	1.25 m	1.15 m
29	12.4 (CH_3)	12.3	0.82 (3H, t, J = 6.78 Hz)	0.80 (3H, t, J = 7.50 Hz)

In the ^{1}H NMR there was a presence of six high intensity signals showing the six methyl groups of three singlets δ_H: 0.68 (3H, s, H-18), 1.01 (3H, s, C-19), 0.98 (3H, s, H-27), two doublets 0.90 (3H, d, J = 6.46 Hz, H-21), 0.91 (3H, d, J = 1.73 Hz, H-26) and a triplet 0.82 (3H, t, J = 6.78 Hz, H-29). Three olefinic protons were observed that included a doublet δ_H 5.33 (1H, d, J = 5.04 Hz, H-6), doublet of doublets δ_H 5.13 (1H, dd, J = 8.67, 15.13 Hz, H-22) and another doublet of doublets δ_H 5.00 (1H, dd, 8.67, J = 15.14 Hz, H-23). The two doublet of doublets at δ_H 5.13 and 5.00 are coupled as shown by their coupling constants also indicates that they are cis protons. A triplet of doublet of doublets was seen at δ_H 3.52 (1H, tdd, J = 4.57, 11.03 Hz, H-3), indicating that the proton was attached to an oxygenated carbon

atom. Another triplet of doublet of doublets was observed to resonance at δ_H 2.26 (2H, tdd, *J* = 4.88, 13.03 Hz, H-4), while signals at δ_H 2.00 (H-20) showed multiplets of peaks. The presence of a cluster of signals between δ_H 0.65-2.26 suggests a steroidal nucleus (Pretsch *et al*., 2000). Other protons of this compound showed signals as shown in **Table 12**.

The spectra of ^{13}C NMR and DEPT experiments showed that compound **AS1** is composed of unsaturated aliphatic molecule with 29 carbon atoms. This consisted of six methyl carbons at δ_C: 12.4 (C-29), 19.6 (C-19), 12.1 (C-18), 19.3 (C-27), 20.0 (C-26), 23.3 (C-21), nine methylene groups, eleven methine carbons and three quaternary carbon atoms. The quaternary carbons were confirmed in the DEPT spectrum by their absence at δ_C: 141.0 (C-5), 36.7 (C-10) and 42.4 (C-13). This indicated the steroidal nature of compound **AS1** (Pateh *et al*., 2009). The resonances appearing on the downfield of the spectra δ_C: 141.0 (C-5), 121.9 (C-6), 138.5 (C-22) and 129.5 (C-23) showed the unsaturation of double bonds. The angular carbon atoms appeared at peaks δ_C: 19.6 (C-19) and 12.1 (C-18) (Habib *et al*., 2007; Pateh *et al*., 2009). The signal appearing at δ_C 72.0 (C-3) accounts for carbon atom 3 of β-hydroxyl group which confirms the compound as stigmaterol instead of β-sitosterol (Li *et al*., 2006; Jain and Bari, 2010). Compound **AS1** differs from β-sitosterol by the presence of a double between C-22 and C-23, instead of a single bond.

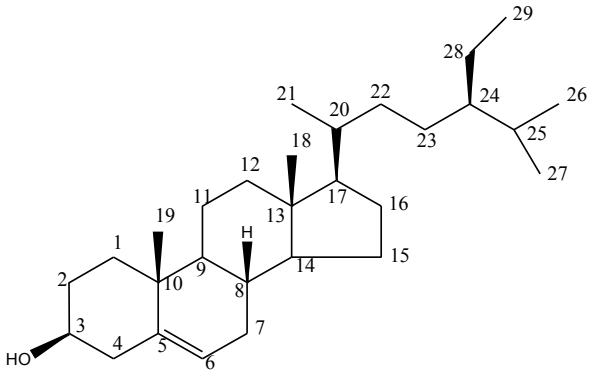
The complete assignment of protons was achieved by the HSQC, HMBC COSY and NOESY spectroscopic data. After comparison of 1-D and 2-D NMR spectral data with that from reported literature, the structure of compound **AS1** was proposed to be that of stigmasterol also known as (24*S*)-5,22-stigmastadien-3β-ol (Pateh *et al*., 2009; Luhata *et al*., 2015; Yusuf *et al*., 2015). This is the first report of isolation of stigmasterol from *A. sieberiana*. However, isolation of stigmasterol has been reported from a number of plant species. Some of the plant species from which stigmasterol has been isolated include: Dichloromethane twigs of *Keetia leucantha* (Rubiaceae) (Bero *et al*., 2013), DCM/MeOH extract of *Odontanema strictum* (Acanthaceae) (Luhata and Munkombwe, 2015), acetone stem bark of *Vitex schiliebenii* (Verbenaceae) (Nyamoita *et al*., 2013) and many more.

Stigmasterol has been screened for bioassay activities in a number of investigations. Some of these studies include: From the acetone stem bark of *Vitex schiliebenii* (Verbenaceae) (Nyamoita *et al*., 2013) isolated stigmasterol and carried out its larvicidal activity against *Anopheles gambiae* larvae. The larvicidal activity from their findings gave an IC_{50} of 8.145 ppm against late and early fourth instar larvae after 72 hours. Stigmasterol from *Mesua beccariana* (Clusiaceae) stem bark displayed strong inhibition on the Raji cell proliferation with IC_{50} values less than 5 µg/mL and also the proliferation of SK-MEL-28

and HeLa cancer cells were strongly inhibited by pure stigmasterol (Soek *et al.*, 2013). Antimycobacterial activity of stigmasterol from the ethanol aerial parts of *Knowltonia vesicatoria* (Ranunculaceae) against drug-sensitive *Mycobacterium tuberculosis* was determined and found to be active with an MIC of 50.00 μg/mL (Labuschangne *et al*., 2012).

This study screened the isolated stigmasterol for antiplasmodial activity to give moderate activity against the two strains of parasite with IC_{50} values of (chloroquine resistant Dd2: 68.67 µM, chloroquine sensitive 3D7: 29.64 µM).

4.4.2 Structure elucidation of β-sitosterol (**AS2**)



AS2

Structure identification of this compound was determined in section **4.2.1** under *O. spinosa* where it was also isolated from the dichloromethane crude extract. The antiplasmodial activity of β-sitosterol from *A. seberiana* could not be determined due to the low yield that was obtained during isolation.

4.5 Biological assay experiments

4.5.1 Test samples for bioassay activity screening

O. spinosa gave yields of (0.51-3.01) % for the crude extract while for the pure compounds the yields ranged between (0.10-11.28) percent. The yields for *E. latideus* were in the range: (0.36-2.36) % for the crude extracts and (0.45-3.66) % for the pure compounds. *A. sieberiana* yielded (1.09-2.64) % for the crude extracts and (0.34-3.34) % for the pure compounds. Among the crude extracts the MeOH extract of *O. spinosa* gave the highest yield (3.01%), while the hexane extract of *E. latideus* had the lowest (0.36%). Betulin (**EL2**) gave the highest yield (3.66%) and **OS4** (0.1%) had the lowest for the pure compounds (**Table 13**).

Table 13: Percentage yields of crude extracts and pure compounds

Species name	Weight of Plant material (g)	Extraction solvent	Crude extract		Pure compounds		
			Weight (g)	% yield (w/w)	Name of compound	Weight (mg)	% yield (w/w)
Oncoba spinosa	800	Hex	9.50	1.19			
		DCM	4.11	0.51	β-sitosterol (**OS1**)	29.80	1.15
					Chaulmoogric acid (**OS2**)	293.40	11.28
					Compound **OS4**	2.60	0.10
		EtOAc	10.46	1. 33	Benzoic acid (**OS3**)	40.90	0.60
		MeOH	24.11	3.01			
Euclea latideus	1000	Hex	3.60	0.36			
		DCM	10.22	1.02	3β-(5-methoxyferulo-yl)lup-20(30)-ene (**EL3**)	172.30	2.38
		EtOAc	8.21	0.82	Lupeol (**EL1**)	26.00	0.50
					Betulin (**EL2**)	189.60	3.66
		MeOH	23.60	2.36			
Acacia sieberiana	1000	Hex	26.40	2.64			
		DCM	10.90	1.09	β-sistosterol (**AS2**)	16.80	0.34
		EtOAc	12.50	1.25	Stigmasterol (**AS1**)	195.90	3.34
		MeOH	11.60	1.16			

4.5.2 In *vitro* antiplasmodial activity screening of crude extracts and pure compounds

The bioassay for *in vitro* antiplasmodial activity was determined using two strains of parasite for both crude extracts and pure compounds. These included the chloroquine drug sensitive (3D7) and chloroquine resistant (Dd2) strains of *P. falciparum*. Two positive controls were used that included chloroquine diphosphate (CQ) and mefloquine hydrochloride (MQ).

In describing *in vitro* antiplasmodial activities, pure compounds were considered to be inactive when they had IC_{50} > 200 µM, whereas those with an IC_{50} of 100-200 µM were considered to have low activity; IC_{50} of 20-100 µM, moderate activity; IC_{50} of 1-20 µM, high activity; and IC_{50} < 1 µM excellent/ potent antiplasmodial activity (Batista *et al*., 2009). Similarly activities of crude extracts were classified into five classes according to their IC_{50} values: high activity (IC_{50} < 5 µg/mL); promising activity (5 µg/mL < IC_{50} < 15 µg/mL); moderate activity (15 µg/mL < IC_{50} < 50 µg/mL); weak activity (50 µg/mL < IC_{50}< 100 µg/mL), inactive IC_{50} > 100 µg/mL (WHO, 2011).

According to this classification of *in vitro* antiplasmodial activity the root extracts of hexane, dichloromethane and methanol of *O. spinosa* with IC_{50} > 50 µg/mL against both 3D7 and Dd2 strains were considered inactive. The root EtOAc crude extract had high activity of 4.69 ± 0.01 µg/mL and 3.52 ± 0.02 µg/mL against 3D7 and Dd2 strains, respectively. The EtOAc had the highest antiplasmodial activity out of the four crude extracts tested for this plant against both strains of *Plasmodium*. The root crude extracts of *E. latideus* exhibited activities of: (IC_{50}) 3D7: 38.21 ± 0.49 µg/mL (hexane extract); (28.07 ± 1.65 µg/mL) (dichloromethane extract) and (IC_{50}) Dd2: 38.93 ± 0.54 µg/mL (hexane extract) all displayed moderate activity. Promising activity was shown by crude extracts (IC_{50}) 3D7: (12.86 ± 1.86 µg/mL) (EtOAc extract); (9.75 ± 1.47 µg/mL) (MeOH extract) and Dd2: (13.97 ± 2.59 µg/mL) (MeOH extract). High activity from this plant was demonstrated by the crude extracts: (IC_{50}) Dd2: 2.78 ± 0.02 µg/mL (dichloromethane extract) and (4.37 ± 0.97 µg/mL) (EtOAc extract) (**Table 14**).

Acacia sieberiana crude extracts exhibited better activities of which the methanol extract had high activity (IC_{50}) 3D7: (4.45 ± 0.19) µg/mL and Dd2: 3.38 ± 0.30 µg/mL. Promising activity was exhibited by the crude extracts (IC_{50}) 3D7: 11.83 ± 0.05 µg/mL (hexane extract), 6.61 ± 0.01 µg/mL (EtOAc extract) and Dd2: 9.17 ± 0.77 µg/mL (EtOAc extract). More activities on the two strains included: moderate activity of (IC_{50}) Dd2: 15.24 ± 0.36 µg/mL (hexane extract), 21.87 ± 2.78 µg/mL (DCM) and 3D7: 27.32 ± 0.30 µg/mL (DCM).

Table 14: ***In vitro*** **antiplasmodial activities of crude extracts and isolatedcompounds against 3D7 and Dd2 strains of** ***P. falciparum***

Name	**Test samples**	**Antiplasmodial activity IC_{50} (µg/mL)**	
		3D7 strain (CQ Sensitive)	**Dd2 strain (CQ Resistant)**
Oncoba spinosa	Hex	$> 50^{a}$	$> 50^{b}$ (> 1)
	DCM	> 50	> 50 (> 1)
	EtOAc	4.69 ± 0.01^{a}	3.52 ± 0.02^{b} (0.75)
	MeOH	> 50	> 50 (> 1)
Euclea latideus	Hex	38.21 ± 0.49^{a}	38.93 ± 0.54^{b} (1.02)
	DCM	28.07 ± 1.65^{a}	2.78 ± 0.02^{b} (0.10)
	EtOAc	12.86 ± 1.86^{a}	4.37 ± 0.97^{b} (0.34)
	MeOH	9.75 ± 1.47^{a}	13.97 ± 2.59^{b} (1.43)
Acacia sieberiana	Hex	11.83 ± 0.05^{a}	15.24 ± 0.36^{b} (1.29)
	DCM	27.32 ± 0.30	$21.87 \pm 2.7\ 8^{b}$ (0.80)
	EtOAc	6.61 ± 0.01^{a}	9.17 ± 0.77^{b} (1.39)
	MeOH	4.45 ± 0.19^{a}	3.38 ± 0.30^{b} (0.76)
Pure compounds	β-sitosterol (**OS1**)	2.28 ± 0.01 [5.51]^{*a}	> 50 [120.77]^{*b} (> 21.93)
	Chaulmoogric acid (**OS2**)	> 50 [> 178.57]*	18.76 ± 3.23 [67.00]^{*b} (< 0.38)
	Lupeol (**EL1**)	23.91 ± 0.05 [56.13]^{*a}	25.14 ± 0.01 [59.01]^{*b} (1.05)
	Betulin (**EL2**)	1.64 ± 0.02 [3.71]^{*a}	7.69 ± 1.21 [17.40]^{*b} (4.69)
	Stigmasterol (**AS1**)	12.21 ± 4.57 [29.64]^{*a}	28.29 ± 1.34 [68.67]^{*b} (2.32)
	3β-(5-methoxyferuloyl)lup-20(30)-ene (**EL3**)	NT	NT
Reference Standards	Chloroquine diphosphate (CQ)	0.0093 ± 0.0099^{a}	0.0440 ± 0.0102^{b} (4.73)
	Mefloquine hydrochloride (MQ)	0.0056 ± 0.0011^{a}	0.0161 ± 0.0132^{b} (2.88)

Values are expressed as mean ± SD (n = 3).

Values with the same superscript(a or b) in the same column are significantly different ($P < 0.05$)

Values in *IC_{50} are expressed in µM (Micromolar)

Values enclosed in parenthesis represent resistance index ratio (RI) of IC_{50} CQ-resistant strain (Dd2 /IC_{50} CQ-sensitive strain 3D7).

NT– Not tested (Insoluble in dimethylsulfoxide)

The antiplasmodial activities displayed by the crude extracts of all the three plants were not due to the toxicity of the plants but the real activity of the compounds. This is because all the crude extracts of *E. latideus* and *A. sieberiana* were practically non-toxic while the *O. spinosa* extracts were both non-toxic and slightly toxic (**Table 15**).

The root ethyl acetate extract of *O. spinosa* was found to be active against both CQ sensitive 3D7 and CQ resistant Dd2 strains with resistance index of 0.75 than the hexane, dichloromethane and methanol extract which showed resistance indices (IC_{50} of resistant strain Dd2 / IC_{50} of sensitive strain 3D7) of > 1. Extracts of *E. latideus* had high activity on both strains that gave better resistance indices of 0.10 and 0.34 for dichloromethane and ethyl acetate crude extracts, respectively. The hexane and methanol crude extracts had lower activity that displayed higher indices of 1.02 and 1.43, respectively against the two strains of parasite. *A. sieberiana* crude extracts had high activity against CQ resistant Dd2 strain with indices: 1.29 (hexane extract), 0.76 (MeOH extract), 1.39 (EtOAc extract) and 0.80 (DCM extract) (**Table 14**). The resistance indices of all the crude extracts of the three plants were better than the reference standards which had 2.88 and 4.73 for mefloquine and chloroquine, respectively (**Table 14**). The resistance indices (0.10-1.43) exhibited by the crude extracts of the three plants suggest that some of these extracts have promising high activity against CQ resistant Dd2 strain of *P. falciparum*. These results indicate the possible explanation for the traditional use of these medicinal plants against malaria. The investigation showed for the first time that compounds present in extracts of *E. latideus*, *A. sieberiana* and *O. spinosa* displayed antiplasmodial activity that ranged from high activity to being inactive. This study reports for the first time on antiplasmodial activity of crude extracts from *O. spinosa*, *E. latideus* and *A. sieberiana*.

The isolated pure compounds were active against the 3D7 (CQ sensitive) and Dd2 (CQ resistant) strains of *P. falciparum* (**Table 14**). They exhibited high and moderate activity against the two strains of *Plasmodium* compounds. The pure compounds with (IC_{50}) 3D7: 5.51 µM (β-sitosterol (**OS1)**) and 3D7: 3.71 µM, Dd2: 17.40 µM (betulin (**EL2)**) showed good activity. Those that displayed moderate activity included compounds with (IC_{50}) 3D7: 56.13 µM, Dd2: 59.01 µM (Lupeol (**EL1)**); 67.00 µM (chaulmoogric acid (**OS2)** and Dd2: 68.67 µM, 3D7: 29.64 µM (stigmasitosterol (**AS1)**). Two pure compounds were found to be inactive on both the two strains with (IC_{50}) 3D7: > 178.57 µM (chaulmoogric acid) and Dd2: > 120.77 µM (β-sitosterol (**OS1**)). Betulin (**EL2**) (IC_{50}; 3D7: 3.71 µM; Dd2: 7.40 µM) and β-sitosterol (**OS1**) (IC_{50}; 3D7: 5.51 µM) registered the highest activity. Betulin still showed the highest activity against both strains of IC_{50} 3D7: 3.71 and Dd2: 17.40 µM. The

antiplasmodial activities of the isolated compounds correlate well with the activities of the crude extracts from which they were isolated. Therefore, they were responsible for the various activities demonstrated by the crude extracts. Three isolated compounds were not very active against the CQ resistant strain as shown by their resistance indices: β-sitosterol (**OS1)** (> 21.93), stigmasitosterol (**AS1)** (2.32), betulin (**EL2**) **(**4.69) and Lupeol (**EL1**) (1.05). Chaulmoogric acid showed the highest cross resistance against both strains with resistance index of < 0.38 (**Table 14**).

Antiplasmodial activities of crude extracts of the three plants (Hex, DCM, EtOAc, MeOH) (IC_{50}: 2.78- > 50) µg/mL and pure compounds (IC_{50}: 3.71- > 178.57) µM showed significant difference with the reference standards (IC_{50}: 0.0056-0.0440) µg/mL (CQ and MQ) for both the two strains of parasite CQ sensitive 3D7 and CQ resistant Dd2 at (P < 0.05). This was due to the decrease in activity of both the crude and pure compounds compared to the reference standards. There was also significant difference observed between the antiplasmodial activities of the crude extracts of different extraction solvents (Hex, DCM, EtOAc, and MeOH). All the crude extracts and pure compounds exerted a significant (P < 0.05) decrease in antiplasmodial activity for the two strains of *Plasmodium* compared to the two standard controls (CQ and MQ) (**Table 14**).

Similar studies on antiplasmodial activity of crude extracts have been investigated on other species from the Flacourtiaceae family. Evaluation of antiplasmodial activity on the aerial parts of *Scolopia zeyheri* (Flacourtiaceae) was studied by Sylvain *et al*. (2013). The hexane, dichloromethane and methanol extracts were tested against CQ resistant FcBI and CQ sensitive F32 strains of parasite. Their findings gave (IC_{50}) FcBI: 24.5 ± 2.12 µg/mL (hexane extract), 29.3 ± 6.7 µg/mL (dichloromethane extract), > 50 µg/mL (methanol). The CQ resistant strain gave (IC_{50}) F32: > 50 µg/mL (hexane and dichloromethane extracts), 7.5 ± 2.1 µg/mL (methanol extract). Another *in vitro* antiplasmodial investigation was conducted in South Africa on root dichloromethane, dichloromethane-methanol and water crude extracts of *Flacourtia indica* (Flacourtiaceae) against CQ sensitive D10 *P. falciparum* strain using the parasite lactate dehydrogenase (pLDH) assay (Clarkson *et al*., 2004). Their findings gave IC_{50}: 86.5 µg/mL (DCM), 78 µg/mL (DCM/MeOH), 78 µg/mL (water) which showed that the extracts were inactive. A study from the same Flacourtiaceae family was assessed on *Trimeria grandifolia* dichloromethane/methanol leaf extracts against CQ sensitive 3D7 strain (Muganga *et al*., 2010). Their results also gave IC_{50} > 50 µg/mL, which was regarded inactive. These results are also in agreement with those reported by Addae-Kyereme *et al*. (2001) in which methanol crude extracts of the seeds of *O. spinosa* had IC_{50} > 100 µg/mL and

also regarded inactive. The results are in the same range to those got for *O. spinosa* in the current study where the ethyl acetate crude extracts of *O. spinosa* had IC_{50} values of 3.5 µg/mL and 4.69 for 3D7 and Dd2 strains, respectively. The remaining three extracts of methanol, dichloromethane and hexane which were inactive had $IC_{50} > 50$ µg/mL (**Table 14**).

Euclea latideus root crude extracts of hexane, dichloromethane, ethyl acetate and methanol had good activity (IC_{50}) 3D7: (9.75 ± 1.47-38.21 ± 0.49) µg/mL and Dd2: (2.78 ± 0.02-38.93 ± 0.54) µg/mL. The activities of the four extracts were classified as having high and moderate activity. Some antiplasmodial activity studies have been investigated on some *Euclea* species. In a study of South African medicinal plants against chloroquine-sensitive strain (D10) of *P. falciparum* reported antiplasmodial activity of 134 species (Clarkson *et al*., 2004). The roots, leaves and twigs of *Euclea natalensis* and *Euclea undulata* were among the plants tested. All the water crude extracts were inactive with $IC_{50} > 100$ µg/mL while the activities of the DCM/MeOH extracts had IC_{50} between 4.6-11.0 µg/mL. Another investigation was carried out on *Euclea divinorum* (Ng'ang'a, 2011). In this investigation the crude extracts were screened against chloroquine (CQ susceptible and resistant strains of *P. falciparum* (D6 and W2, respectively). The dichloromethane and EtOAc crude extracts obtained from the leaves showed high *in vitro* antiplasmodial activity in the range of IC_{50} = 6.12 ± 0.45-17.29 ± 1.44 µg/mL and 8.42 ± 1.06-12.09 ± 0.67 µg/mL against CQ-susceptible strain, respectively. The activities of these previous investigations are within the range exhibited by the crude extracts of *E. latideus*.

The crude extracts of *Acacia sieberiana* had highest activity of (IC_{50}) 3D7: (4.45 ± 0.19-27.32 ± 0.30) µg/mL and Dd2: (3.38 ± 0.30-21.87 ± 2.78) µg/mL. In a study (Clarkson *et al*., 2004) of species from the Fabaceae family to which *Acacia sieberiana* belongs were screened for antiplasmodial activity. The crude extracts of the twigs, roots and whole plants of DCM/MeOH (1:1) and water of *Acacia nilotica*, *Acacia tortilis* and *Elecphtorrhiza elephantine* were tested against D10 CQ sensitive. Their results showed IC_{50} values of 4.8-32.0 µg/mL and 32- > 100 µg/mL for DCM/MeOH and water extracts, respectively. In another study on the DCM, MeOH and aqueous crude extracts of the leaf/twig of *Schrankia leptocarpa* (Mimosaceae) was conducted against CQ 3D7 strain (Bero *et al*., 2009). Their findings gave IC_{50} 34.3 µg/mL (DCM extract), > 100 µg/mL (MeOH extract) and > 100 µg/mL (aqueous extract). Lenta *et al*. (2007) investigated the MeOH crude extract of the stem bark of *Acacia zygia* (Mimosaceae) from selected Cameroonian medicinal plants, against *P. falciparum* K1 CQ-resistant strain. The IC_{50} value that was registered for the methanolic stem bark extract was 1.0 µg/mL. A number of antiplasmodial activity studies have been

conducted on *A. nilotica* (Mimosaceae). One of the investigations was carried against *P. falciparum* 3D7 (CQ sensitive) and Dd2 CQ resistant strain (El-Tahir *et al*., 1999). The ethyl acetate extract possessed the highest activity (IC_{50} = 1.5 µg/mL). The methanol extract of *A. nilotica* seed exerted high activity on both 3D7and Dd2 strains with IC_{50} of 0.9 mg/mL and 4.1 mg/mL, respectively. Another study assessed the acetone stem bark of *Acacia zanthoploea* (Mimosaceae) against the South African isolate (*P. falciparum* UP1, CQ resistant strain) (Prozesky *et al*., 2001). The stem bark extract exhibited an activity of IC_{50}; 10.10 µg/mL which was regarded to have highactivity. The antiplasmodial activities investigated in previous studies above were in agreement with those obtained for *A. sieberiana* in the current study because they are within the same range.

The resistance index (RI) for ethyl acetate crude extract of *O. spinosa* was 0.75 while that for hexane, dichloromethane and methanol were > 1. *E. latideus* displayed resistance indices between 1-1.43. Similar investigations carried out on extracts of ethyl acetate and methanol of *Phyllanthus emblica*, *Syzygium aromaticum*, and *Abrus precatorius* were found to be as active against CQ-resistant Dd2 and INDO as against CQ-sensitive 3D7 giving resistance indices of 1-1.5 (Bagavan *et al*., 2011). In another study on antiplasmodial activity of ethyl acetate crude extracts of five plant species; the resistance indices of the plants ranged from 0.78 to 1.28 (Kaushik *et al*., 2013). Antiplasmodial properties and bioassay-guided fractionation of ethyl acetate extracts from *Carica papaya* leaves was carried out on the CQ-sensitive D10 and CQ-resistant Dd2 strains (Melariri *et al*., 2011). In their investigation they registered (IC_{50}) D10: 2.96 ± 0.14 µg/mL, Dd2: 3.98, which gave RI value of 1.34. These values of resistance indices are in the same range as the crude extracts for *A. sieberiana, O. spinosa* and *E. latideus.* This suggests that crude extracts from previous studies also had cross resistance against CQ resistant strains.

The antiplasmodial activity of all the crude extracts of; hexane, dichloromethane, EtOAc, MeOH (*O. spinosa*); hexane (*E. latideus* and *A. sieberiana*) and the pure compound (lupeol) against 3D7 and Dd2 strains did not differ significantly ($P > 0.05$) (**Table 14**). Therefore, these extracts and the pure compound may lack cross-resistance with CQ resistant strain. This phenomenon can be attributed to differences in the mode of actions of the different bioactive compounds in the extracts and that of the pure compound (Muthaura *et al*., 2007).

In the present study eight compounds were isolated and their structures elucidated, however only five compounds were assessed for *in vitro* antiplasmodial activities. These included lupeol (**EL1**), Betulin (**EL2**), β-sitosterol (**OS1**), stigmasitosterol (**AS1**) and

chaulmoogric acid (**OS2**). The activity of 3β-(5-methoxyferuloyl)lup-20(30)-ene (**EL3**) could not be determined because it was not soluble in DMSO. Benzoic acid (**OS3**) and **OS4** had poor yields which only enabled the determination of spectroscopic data.

The *in vitro* antiplasmodial activity of compounds from *O. spinosa* was assessed on the two strains of *Plasmodium* (CQ sensitive 3D7 and CQ resistant Dd2). In the current investigation β-sitosterol isolated from this plant had (IC_{50}) 3D7: 5.51 μM, Dd2: > 120.77 μM. Both the DCM crude extract from which it was isolated and the β-sitosterol had resistance indices in the same range > 1 and > 21.93 respectively. This indicates that both the crude and isolated compound were not very active against the resistant Dd2 strain. Mwangi *et al*. (2010) carried out *in vitro* antiplasmodial on β-sitosterol isolated from methanol crude extract of leaves of *Teclea trichocarpa* against CQ resistant *P. falciparum* KI strain. Their investigation gave IC_{50} of 8.20 μg/mL (19.81 μM), which correlates with the IC_{50} value got from this study. However, the dichloromethane extract from which it was isolated was inactive against both strains of parasite with IC_{50} > 50 μg/mL. This shows that there was antagonic interaction of the compounds in its crude form, which explains the increased activity of pure compound alone.

Increased activity of compounds than their crude extracts was also cited by Mokoka *et al*. (2013). In their investigation on *Schkuhria pinnata* DCM/MeOH (1:1) whole plant crude extracts against CQ NF54 sensitive strain gave IC_{50} of 2.19 μg/mL. This IC_{50} value was less than those of the isolated compounds schkuhrin I and schkuhrin II with (IC_{50}) NF54: 2.05 and 1.67 μg/mL respectively. Jullian *et al.* (2005) also carried out a bioassay-guided fractionation of the trunk bark extract of *Laetia procera* (Flacourtiaceae) that led to the isolation of six clerodane diterpenoids: casearlucin A, casamembrol A and four laetiaprocerines. The diterpenoids exhibited antiplasmodial activity with IC_{50} values of 0.57-6.04 μM on F32 strain and 0.54-27.5 μM on FCb1 strain. In another study on hexane and dichloromethane extracts of the bark of *Casearia grewiifolia* (Flacourtiaceae) four new clerodane diterpenes (caseargrewiins) and two known clerodane diterpenes were isolated (Kanokmedhakul *et al*., 2005). All compounds exhibited antimalarial activity against *P. falciparum* K1 with IC_{50}values of 3.6-7.9 μM, but they were also cytotoxicity. This shows that the Flacourtiaceae family has some species that exhibit high antiplasmodial activity.

Chaulmoogric acid isolated from *O. spinosa* was among the pure compounds tested fro antiplasmodial activity. This compound exhibited activity of IC_{50} 3D7: > 178.57 μM and Dd2: 67.00 μM which was different from the DCM crude extract from which it was isolated.

The DCM crude extract was completely inactive against both strains while this compound showed increased activity in its pure form on Dd2 strain. This result is similar to a study by Muganga *et al*. (2010) in which the isolated compound showed increased activity than the crude extract from which it was isolated. The isolated compounds methyl canadine, nitidine and chelerythine (IC_{50}) 3D7: 2.01, 0.17 and 1.35 µg/mL, respectively from the ethanol root bark of *Z. chalybeum* had increased activity than its crude extract (IC_{50} 42.5 and 41.5) µg/mL of MeOH and DCM, respectively.

Isolation and purification of the ethyl acetate crude extracts of *E. latideus* afforded triterpenoids lupeol (**EL1**) and betulin (**EL2**) that were tested for antiplasmodial activity. Lupeol had activity of IC_{50} 3D7: 23.91 ± 0.05 µg/mL (56.13 µM) and Dd2: 25.14 ± 0.01 µg/mL (59.01 µM). This activity was low as compared to that of its ethyl acetate crude extract from which it was isolated that was highly active on both strains of parasite (IC_{50} 3D7: 12.86 ± 1.86 µg/mL, Dd2: 4.37 ± 0.97 µg/mL). The crude also had a resistance index of 0.34 which low as compared to that of the pure compound that had 1.05. This suggests that there was enhancement of activity by other chemical constituents present in the extract which may have acted synergistically. The crude extract was also more active against the CQ resistance strain than the pure compound as shown by their indices.

Similar findings in which the pure compound had low activity compared to the crude extract from which it was isolated was also registered by Sebisubi *et al*. (2010). In their study the isolated four diterpenes had (IC_{50}) D6: 14.3-24.4 µg/mL, W2: 17.5-18.4 µg/mL which were less active compared to the chloroform crude extract that displayed (IC_{50}) D6: 7.89 µg/mL, W2: 8.74 µg/mL. Antiplasmodial activity screening of lupeol (**EL1**) is documented in literature. Some of the studies were investigated on 3D7 and Dd2 parasitic strains (Dominique *et al*., 2015). Their investigation gave IC_{50} values of 80.30 and 54.22 µM on the two strains of parasites, respectively on lupeol isolated from *Ampelozizyphus amazonicus* from Nigeria. Another study on lupeol isolated from the ethyl acetate leaf extract of *Cassia siamea* (Ajaiyeoba *et al*., 2008) gave high activity of IC_{50} 5.0 µg/mL (11.73 µM) against *P. falciparum* KI strain of parasites. Antiplasmodial screening of lupeol isolated from the DCM/MeOH crude extract of the bark of *A. zygia* was assessed against *P. falciparum* KI strain to give $IC_{50} > 0.078$ µg/mL (Abdalla and Laatsh, 2012). Ziegler *et al*. (2002) have also reported the *in vitro* inhibitory activity of lupeol against CQ-sensitive 3D7strain of *P. falciparum*, with an IC_{50} value of 11.8 mg/mL (27.70 µM) which was shown to cause a human erythrocyte shape towards stomatocytes. There is difference in the activity of Lupeol isolated from *E. latideus* and that from other plant species registered from previous studies.

However, all plant species used recorded activity that ranged from high to moderate activity. This suggests that regardless of the species, the lupeol isolated demonstrated good antiplasmodial activity.

The second compound betulin (**EL2**) isolated from *E. latideus* displayed antiplasmodial activity of (IC_{50}) 3D7: 1.64 ± 0.02 µg/mL (3.71 µM), Dd2: 7.21± 1.21 µg/mL (17.40 µM). It exhibited high activity on 3D7 strain and low activity on Dd2 strain compared to the ethyl acetate crude extract from which it was isolated (**Table 14**). It also had a resistance index of 4.69 showing that it was less reactive to the CQ Dd2 resistance strain compared to the ethyl acetate crude extract which had a value of 0.34. These results are in agreement to those in a study in which the activity of the pure compounds was higher than the crude extracts (Namukobe *et al*., 2015). They evaluated the ethyl acetate and aqueous extracts of *Neoboutonia macrocalyx* against CQ sensitive (D6) and CQ resistant (W2) strains of *P. falciparum*. The isolated pure compounds montanin and neoboutomacroin had IC_{50} 2.3-3.9µg/mL and 3.6-4.9 µg/mL on CQ D6 and CQ W2 strains, respectively. The ethyl acetate crude extract had IC_{50}: 12.7-5.7 µg/mL while the aqueous extract was inactive on both the two strains of parasites.

Antiplasmodial activity screening investigated on betulin has been reported in literature. Mthembu *et al*. (2010) conducted an *in vitro* antiplasmodial activity against the *P. falciparum* CQ-susceptible strain (D10). The study was investigated on betulin isolated from the dichloromethane leaf crude extract of *Scheffleria umbellifera* which gave high activity of IC_{50} of 3.2 µg/mL (7.24 µM). However the activity of betulin in their study compared well with that of the dichloromethane extract (IC_{50} D10: 3.7 µg/mL) form which it was isolated. According to (Ziegler *et al*., 2004) betulin isolated from several plant families such as *Rhamnaceae* (*Ziziphus vulgaris*) and Labiatae (*Zataria multiflora*) showed moderate activity (IC_{50} < 27.12 µM and < 27 µM, respectively). Another investigation was conducted on betulin isolated from *Ampelozizyphus amazonicus* (Dominique *et al*., 2015). Their findings gave IC_{50} values of 17.08 and 14.22 µM against 3D7 CQ sensitive and Dd2 CQ resistant parasites, respectively which were regarded as good activity. An evaluation was also conducted on betulin isolated from *Uapaca nitida* that was inactive with IC_{50} of 500 µg/mL (Chaudhuri *et al*., 2002; Hernry *et al*., 2006). However, betulin isolated from *Croton argrophylloides* was reported to inhibit *P. falciparum* strains with moderate activity of IC_{50}; 27.15 µM (Monte *et al*., 1988). The activities of betulin from *E. latideus* were in agreement with some values documented in previous studies above.

A. sieberiana yielded two pure compounds β-sistosterol (**AS1**) and stigmasterol (**AS2**). Stigmasterol from this study registered moderate activity of IC_{50} 3D7: 29.64 µM and Dd2: 66.67 µM. The ethyl acetate crude extract from which it was isolated exhibited IC_{50} 3D7: 6.61 ± 0.02 µg/mL and 9.17 ± 0.77 µg/mL. The pure compound had an index of 2.32 compared to that of the crude extract (1.39). These results show that there was decrease in activity of stigmasterol in comparison to crude extract. This shows that there was synergistic interaction of the constituents in the crude extract which accounted for its increased activity. Furthermore the crude extract was more active against the Dd2 resistant parasite as shown by the low value of resistance index. Melariri *et al*. (2011) recorded similar finding from the ethyl acetate crude extract and isolated pure compounds against CQ-3D7 sensitive and CQ-Dd2 resistant strains on *C. papaya*. The isolated compounds, linoleic and linolenic acids showed IC_{50} of 6.88 and 3.58 µg/mL, respectively. The study demonstrated high antiplasmodial activity of the ethyl acetate crude extract of *C. papaya* leaves with an IC_{50} of 2.96 µg/mL when compared to the activity of the fractions and isolated compounds. According to literature antiplasmodial activity of stigmasterol has been investigated. Bero *et al*. (2013) isolated stigmasterol from dichloromethane crude extract of *Keetia leucantha*. They screened it against chloroquine sensitive 3D7 *P. falciprum* parasites using the *in vitro* model. Their findings gave IC_{50} value of > 100 µg/mL which was considered inactive. However, the DCM crude extract from which it was isolated had (IC_{50}) 3D7: 11.3 ± 3.8 µg/mL. These results agree well with those for stigmasterol above in which the crude extract had high activity compared to the pure compound. The current investigation reports the antiplasmodial activity of compounds isolated from *O. spinosa*, *A. sieberiana* and *E. latideus* for first time.

4.5.3 *In vivo* acute toxicity tests of crude extracts

The present work was approved by the Ethical Committee for using animals at Makerere University, Department of Pharmacology (number 1250). According to the LD_{50} values of acute toxicity calculated, the crude extracts where classified into two groups. The first group included extracts in which the LD_{50} > 5000 mg/kg and considered to be practically nontoxic. These extracts included that of hexane, dichloromethane, ethyl acetate, methanol of *E. latideus* and *A. sieberiana*. *O. spinosa* had only hexane and dichloromethane extracts with LD_{50} > 5000 mg/kg. The second group was categorized as slightly toxic with LD_{50} of 547.72 mg/kg, this consisted of ethyl acetate and methanol crude extracts of *O. spinosa* (**Table 15**).

During the first phase of administration for *E. latideus* extracts at doses 500, 1000 and 2000 mg/kg, for the hexane extract animals looked normal after 6 hours of dosing. When the dose was increased to 3000 and 4000 mg/kg the animals rested for about 15 minutes and at 5000 mg/kg their movements in the cage slowed down in first 30 minutes after dosing. The dichloromethane extract had the same observations at 500 and 1000 mg/kg as that for the hexane extract, however, at 2000, 3000, 4000 mg/kg there was a small reduction in the movement of the animals. Finally at dose of 5000 mg/kg the animals were observed not to move freely as before. For the ethyl acetate crude extract at all doses 500-5000 mg/kg there was no observable effect in the behavior of the animals. Also no signs of toxicity were seen for the methanol crude extract at doses 500-200 mg/kg, however, at 3000 and 4000 mg/kg the movement of the animals slowed down in the first hour after dosing. The movement of the animals reduced for about an hour and then they rested in the corners of the cage.

There was no change in behavior in animals for all doses 500-5000 mg/kg for all the crude extracts of *A. sieberiana*. This was the same behavior seen for hexane and dichloromethane crude extracts for *O. spinosa* at all doses 500-5000 mg/kg. The ethyl acetate extract recorded no observable change in behavior at a dose of 500 mg/kg. At doses of 1000 and 2000 mg/Kg, there was retarded movement, restless in breathing and animals became less active and all the tested animals died. The same trend of results was recorded for the methanol crude extract. When doses were changed to 600, 700, 800 and 900 mg/kg the ethyl acetate and methanol extracts recorded mortality death (**Table 15).** The stock solution for all the doses administered was prepared by dissolving 0.2 g of the crude extract in 2 mL of distilled water to give a concentration of 100 mg/mL.

Determination of acute toxicity is the first step in the toxicological analyses of herbal drugs. In the present study Lorke's procedure (Lorke, 1983) was used because it offers the advantage that when doses are correctly chosen adequate information is obtained using only few animals, irrespective of the material tested and the route of administration. In the current study the acute toxicity of most of the crude extracts of the three plants had LD_{50} > 5000 mg/kg and these were considered non-toxic. The LD_{50} was 547.72 mg/kg in only two crude extracts of *O. spinosa* which were regarded as slightly toxic (**Table 15**). The methanol crude extract of the seeds of this plant was investigated by Addae-Kyereme *et al*. (2001). Their results showed that the seed crude extract was non-toxic to larvae of *brine shrimps* at IC_{50} of 250 µg/mL. However, the antiplasmodial activity of the methanol seed extract was found to

be inactive (IC_{50} >100 µg/mL). The reported acute toxicity values from literature agree with those obtained in the current study for the three plants.

Table 15: ***In vivo*** **acute toxicity activities of crude extracts against mice**

Scientific name	**Extraction solvent**	**Mortality**							
		Phase 1 dose (mg/Kg)			**Phase 2 dose (mg/Kg)**				**LD_{50} (mg/Kg)**
		500	1000	2000	3000	4000	5000		>5000
E. latideus	Hex	0	0	0	0	0	0		>5000
	DCM	0	0	0	0	0	0		>5000
	EtOAc	0	0	0	0	0	0		>5000
	MeOH	0	0	0	0	0	0		>5000
A. sieberiana	Hex	0	0	0	0	0	0		>5000
	DCM	0	0	0	0	0	0		>5000
	EtOAc	0	0	0	0	0	0		>5000
	MeOH	0	0	0	0	0	0		>5000
O. spinosa	Hex	0	0	0	0	0	0		>5000
	DCM	0	0	0	0	0	0		>5000
					600	700	800	900	
	EtOAc	0	3	3	1	2	3	3	547.72
	MeOH	0	3	3	1	2	3	4	547.72

Doses 500-5000 mg/Kg, number of mice used was 3

Doses 600-900 mg/Kg, number of mice used was 4

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The study shows that Butebo County has a wide diversity of plant species used as remedies for malaria. The ethnobotanical survey showed that many of the identified medicinal plants had documented antiplasmodial activity and are used as antimalarial remedies**.** Based on this, the claimed therapeutic treatment of these plants is highly justified. This gives a measure of credibility to the plants that were documented for the first time awaiting studies to determine their efficacy and safety. Therefore this confirms the continuous use of the plants by the local communities as antimalarial medicines.

The results show that the crude extracts of *O. spinosa, A. sieberiana* and *E. latideus* have high antiplasmodial activity. This antiplasmodial activity exhibited by these plants is attributed to the corresponding high activities that were shown by the pure compounds. In addition the resistance indices of the crude extracts were low showing that they were equally active against the chloroquine resistant Dd2 parasite strain. The crude extracts of the three plants showed significant difference in antiplasmodial activity with the reference standards for both the two strains of parasite CQ sensitive 3D7 and CQ resistant Dd2 at ($P < 0.05$). There was a significant decrease in antiplasmodial activity at ($P < 0.05$) for the two strains *Plasmodium* compared to the two stardard controls. This shows that the plants have promising antiplasmodial activity against CQ resistant Dd2 strain of *P. falciparum*. Therefore, the plants can be taken in their crude form to offer prophylactic cure after clinical trials. The findings of study support the continuous and persistent use of the medicinal plants in traditional treatment of malaria by the local communities in Butebo County.

The plants were classified as slightly toxic for *O. spinosa* (LD_{50} = 547.72 mg/Kg) and practically non-toxic (*E.latideus* and *A. sieberiana*) with LD_{50} > 5000 mg/Kg. This implies that the good antiplasmodial activity of the crude extracts is not due to their toxicity and they have a large safety margin. The apparent lack of high toxicity of the crude extracts of the plants possibly explains why they are taken without any side effects. Therefore the medicinal plants can be considered to be safe for consumption in their crude form if incorporated into an oral formulation for the treatment of malaria.

The analyses revealed for the first time that compounds present in the crude extracts of *O. spinosa*, *E. latideus* and *A. sieberiana* have high antiplasmodial activity. However, the pure compounds were not very active against the CQ resistant parasite because of the high resistance indices that were displayed. Antiplasmodial activities of the pure compounds

showed significant difference with the reference standards for both the two strains of parasite CQ sensitive 3D7 and CQ resistant Dd2 at ($P < 0.05$). All the pure compounds exerted a significant ($P < 0.05$) decrease in antiplasmodial activity for the two strains of *Plasmodium* compared to the two stardard controls. The antiplasmodial activities of the isolated pure compounds observed make these plants a source which could serve to isolate new lead structures for drug development. This accounts for collectively the enduring use of the plants against malaria by the local communities.

Nine pure compounds were isolated from the three medicinal plants in the study. Lupeol (**EL1**), betulin (**EL2**) and 3β-(5-methoxyferuloyl)lup-20(30)-ene (**EL3**) from *E. latideus*. *O. spinosa* yielded; β-sitosterol (**OS1**), chaulmoogric acid (**OS2**), benzoic acid (**OS3**) and compound **OS4**. From *A.sieberiana* stigmasitosterol (**AS1**) and β-sitosterol (**AS2**) were isolated. All these compounds are known and have been isolated from other species of plants apart from compound **OS4** that has its structure not yet elucidated. Antiplasmodial screening was only done on five of the isolated compounds.

According to findings from this study the antiplasmodial activity of crude extracts and isolated pure compounds of *Acacia sieberiana*, *Euclea latideus* and *Oncoba spinosa* was found to be promising. The toxicity results also show that the plants are non-toxic suggesting that the plants are safe to be used in their forms. Therefore this confirms the ethanomedicinal use of the three plants by the local communites for the treatment of malaria.

5.2 Recommendations

The following recommendations are suggested for the future work for this study for effective management of malaria;

(i) Local communities be sensitized on the sustainable use and conservation of the medicinal plant species documented. This is because most of the plants had documented antimalarial activity and are used as antimalarial remedies in other regions. Plant species documented for the first time need to be subjected to modern scientific studies to establish their efficacy and safety. This will help to preserve and document these plants which may be lost due to erosion of old age.

(ii) The crude extracts should be subjeced to *in vivo* antiplasmodial activity screening in animal models so as to compare the *in vitro* and *in vivo* activities of the extracts. Studies on combined crude extracts that had good *in vitro* antiplasmodial activity should be assessed to determine the *in vitro* and *in vivo* synergistic and antagonic interactions. Crude extracts of the leaves, flowers, seeds and stem bark of the three plants should aslo be tested for both *in vitro*

and *in vivo* antiplasmodial activity. This would help control harvesting plant parts that result in destroying the medicinal plants. The plants should be further investigated towards development into an antimalarial phytomedicines and recommended as antimalarials in known dosages. However, this should be done after detailed *in vivo* antimalarial evaluation and toxicological studies. This recommendation should be given especially to people in local communities where conventional drugs are unaffordable, unavailable and the health facilities are inaccessible.

(iii) Histological analysis needs to be determined on vital organs such as the liver, kidney and pancreas during acute toxicity tests. This should be done with aim of finding out if the crude extracts are responsible for any histological changes. Sub-acute and chronic toxicity tests be carried out to determine the effects of repeated doses of the extracts on the major systems for example the heart of the animals which are caused by long term exposure of the drugs. This can be used to determine the effect of the medicine on the vital organs of people in the local communities.

(iv) The isolated compounds be subjected to *in vivo* antiplasmodial activity screening so as to compare the *in vitro* and *in vivo* activities of the extracts. Pure compounds that had high *in vitro* antiplasmodial activity should be screened to determine the *in vitro* and *in vivo* synergistic and antagonic interactions. The highly active compounds are potential candidate molecules which need further investigation in combination with standard antimalarials in view of developing new antimalarials for effective treatment of clinical malaria.

(v) The roots were the only part of the plant that was used for the isolation and characterization of the compounds from the three medicinal plants in the study. Therefore, studies need to be carried out on different parts of the same plants and compare the activities of the isolated compounds. This should involve isolation and elucidation of the structures of the bioactive compounds from the leaves, seeds flowers; stem bark and other plant parts. Isolation and characterization be carried out on hexane and methanol crude extracts of the three plants because of the promising antiplasmodial activity that was exhibited by them.

Since the three plants studied and the isolated compounds have been found to have antiplasmodial activity and are safe, the local communities in Butebo County should continue to use the plants for malaria therapy. The plants can also be used in the treatment of other ailements apart from malaria due to lack of toxicity. Solvents like ethanol should be used instead of water to get high concentrations of the active componds in the preparations made. The 23 plants that were found to have antiplasmodial activity need to be planted on a large scale to provide income after the findings from this study have been given to them.

There is need for community awareness and education concerning the values of medicinal plant species of the area especially among the young people. Government should develop policy to integrate use of medicinal plant species in health care at national level that have high antiplasmodial activity and lack of toxicity. Deforestation of areas containing medicinal plants can be restricted for the medicinal plants to be easily available to the local communities when needed.

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APPENDICES