ETHNOPHARMACOLOGY OF ANTIMALARIAL PLANTS USED AMONG THE LUHYA COMMUNITY IN KAKAMEGA EAST SUB-COUNTY

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A thesis submitted in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy (PhD) in Pharmacognosy of the University of Nairobi

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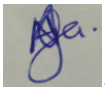
Nillian Ayuma Mukungu (BPharm, MSc.) ……  …… .. 3/11/2021 ….

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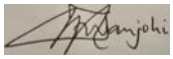
Prof. J.W. Mwangi, PhD  …… 3rd November 2021

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DEDICATION

This thesis is dedicated to my parents, Mr. Charles Mukungu and Mrs. Irene Mukungu; my husband Martin and my children; Yovela, Brighton and Deborah.

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
µl	Microlitre
ACT	Artemisinin-based Combination Therapy
AIDS	Acquired Immune Deficiency Syndrome
APAD	3-Acetyl Pyridine Adenine Dinucleotide
APADH	3-Acetyl Pyridine Adenine Dinucleotide Dehydrogenase
APHRC	African Population and Health Research Center
CARTA	Consortium for Advanced Research Training in Africa
CDC	Centers for Disease Control and Prevention
CQ	Chloroquine
CRA	Commission for Revenue Allocation
DDT	Dichlorodiphenyltrichloroethane
DELI	Double-site Enzyme-linked Lactate dehydrogenase Immunodetection
DMSO	Dimethyl Sulphoxide
ELISA	Enzyme-Linked Immunosorbent Assay
ERC	Ethics Review Committee
ESI	Electrospray Ionization
GLP	Good Laboratory Practice
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HIV	Human Immunodeficiency Virus
ICF	International Coach Federation
IC_{50}	Half Maximal Inhibitory Coefficient

IPTp	Intermittent Preventive Treatment in pregnancy
KEMRI	Kenya Medical Research Institute
KIPPRA	Kenya Institute for Public Policy Research and Analysis
KNBS	Kenya National Bureau of Statistics
KNH	Kenyatta National Hospital
LC-MS/MS	Liquid chromatography tandem-mass spectrometry
LDH	Lactate Dehydrogenase
mg	milligram
ml	milliliter
MS	Mass Spectroscopy
m/z	Mass-to-charge ratio
NACOSTI	National Commission for Science, Technology and Innovation
NBT	Nitro Blue Tetrazolium
NCAPD	National Coordinating Agency for Population and Development
NIAID	National Institute of Allergy and Infectious Diseases.
nm	nano meter
NMR	Nuclear Magnetic Resonance
NP-40	Nonidet P-40
OD	Optical Density
PIC	Prior Informed Consent
pLDH	Parasite Lactate Dehydrogenase
PVDF	Polyvinylidene difluoride
QE	Quercetin equivalent

RMPI	Roswell Park Memorial Institute
RTO	Retrospective Treatment Outcome
SP	Sulfadoxine/sulfalene-Pyrimethamine
UK	United Kingdom
UNICEF	United Nations Childrens' Fund
UoN	University of Nairobi
USAID	United States Agency for International Development
UV	Ultraviolet
v/v	Volume per volume
WHO	World Health Organization

OPERATIONAL DEFINITION OF TERMS

Ethnobotany: The study of how people relate with plants. It involves the observation of how people use plants for various purposes (Iwu, 2002).

Ethnomedicine: The healthcare system that includes beliefs and practices in relation to health and diseases within a given indigenous community (Bhasin, 2007).

Ethnopharmacology: The systematic study of traditional medicine products and evaluation of their biological activities (Katiyar *et al.*, 2012).

Phytomedicine: A medicinal product derived from plant material or a preparation with identified chemical substances derived from plants that contribute to its biological activity (Pandey *et al.*, 2011).

ABSTRACT

Malaria remains a major disease in Kenya responsible for high mortality especially in the children aged under five years and pregnant women. Many communities in Kenya still use plants for the management of malaria. An ethnobotanical survey was conducted among 21 herbal practitioners in Kakamega East sub-County in Western Kenya to identify antimalarial plants used by the Luhya community. Two plants, *Leucas calostachys* Oliv. and *Justicia betonica* L. were selected after the ethnobotanical survey for evaluation of their antimalarial activities and phytochemical constituents.

Most of the herbal practitioners interviewed were aged more than 50 years old and had a low level of formal education. A total of 42 plant species in 24 plant families were identified for the management of malaria. Two plants, *Rumex steudelii* Hochst.ex A. Rich and *Phyllanthus sepialis* Müll. Arg are reported for the first time for the management of malaria. The Lamiaceae family was the most frequently encountered for treatment for malaria in this community. *Melia azedarach* L, Aloe spp, *Ajuga integrifolia* Buch. Ham, *Vernonia amygdalina* Del., *Rotheca myricoides* (Hochst.) Steane and Mabb, *Fuerstia africana* T.C.E.Fr., *Zanthoxylum gilletii* (De Wild.) P.G. Waterman and *Leucas calostachys* Oliv. were the most commonly used plants for treatment of malaria. *Leucas calostachys* Oliv. and *Justicia betonica* L., were selected for antimalarial activity testing and phytochemical evaluation.

Extracts of *Leucas calostachys* and *Justicia betonica* were studied for activity against chloroquine-sensitive 3D7 *Plasmodium falciparum* and β-hematin inhibition. The

methanol extract and ethylacetate extract of *Leucas calostachys* and *Justicia betonica* respectively showed the best antiplasmodial activity and β-hematin inhibition activity. This is the first report of β-hematin inhibition activities of these plants.

Bio-assay guided fractionation led to the tentative identification of compounds from active fractions of *Leucas calostachys* and *Justicia betonica* using LCMS/MS. Eight flavonoidal compounds and 12 phenylethanoid were identified from the methanol fraction of *Leucas calostachys.* The flavonoids were: isorhamnetin, luteolin-7-O-glucoside, luteolin-4′-O-glucoside, luteolin diglucoside, apigenin-O-glucoside, genistein-O-glucoside, chrysoeriol-7-O-glucoside, and chrysoeriol-7-O-glucuronide. Seven phenylethanoid compounds were identified and included acteoside, isoactoeside, hydroxyacteoside, forthsoside B, samioside, alyssonoside and leucoseptoside A. Five other phenyethanoids could not be identified. This is the first report of any phytochemicals from *Leucas calostachys*.

Similarly, 13 compounds were identified from active fraction of *J. betonica* and these included five carotenoids and nine indoloquinolines. Four of the carotenoids were lutein/zeaxanthin, anhydrolutein I, anhydrolutein II and lutein 5, 6-epoxide. On the other hand, five of the indoloquinolines identified were 5H, 6H quinindolin-11-one, 10H indolo [3,2-b] quinoline, 6H indolo [2,3-b] quinoline, 11H indolo [3,2-c] quinoline and 7H indolo [2,3-c] quinoline. The rest of the compounds could not be identified using the LCMSMS. This is the first time carotenoids, 11H indolo [3, 2-c] quinoline and 7H indolo [2, 3-c] quinoline have been identified in this plant.

The results of this study supports the use of *Leucas calostachys* Oliv. and *Justicia betonica* L. in the treatment of malaria among the Luhya community in Kenya. It therefore justifies the continued search of antimalarial medicine from natural sources based on folkloric information.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Historical perspectives

Malaria is ancient disease which preceded human history. The oldest recorded malaria parasites, *Plasmodium dominicum,* which infected non-human primates, existed more than 30 million years ago (Poinar, 2005). This disease has affected human beings throughout history. The ancient writings of China describe symptoms associated with malaria such as fevers, enlarged spleen and epidemic episodes (Cox, 2010). Cuneiform scripts discovered in Mesopotamia describe malaria like symptoms as "deadly periodic fevers". Similarly, in Egypt, malaria antigens were discovered dating between 3200 and 1304 B.C (Institute of medicine (US), 2004). However, a more severe type of malaria arising from infection with *Plasmodium falciparum* causing fatality originated in Central and West Africa and spread to the rest of the world about 4000 years ago (CDC, 2012a). In India, malaria was known as "king of diseases" between 1500 and 800 B.C. The great philosopher, Aristotle (384-322 BC) referred to malaria fevers in his writings. The *Corpus Hippocratus,* by Hippocrates (450-370 BC), clearly describes malaria symptoms (Sherman, 1998).

Malaria rapidly spread in Europe in the first century in the crowded settlements and often affected the economic activities as many people fell ill during epidemics. It is believed

that malaria may have contributed the fall of Rome. The malaria epidemic of 79 AD led to migration of farmers from the fertile lands around Rome. This region remained unsettled until the 20th century when these marshy areas (Pontine Marshes) were drained and malaria eradicated in Europe (Dagen, 2020; Institute of medicine (US), 2004).

The greatest impact of malaria has been on Africa. This disease is still prevalent in the sub-Saharan Africa, although it is also found in other tropical and sub-tropical areas such as China, India and South and Central America (Olowe *et al.,* 2015). Malaria transmission is dependent on the climatic conditions which affect mosquito populations. High temperatures, increased humidity and rainfall in the sub-Saharan Africa lead to the proliferation of mosquitoes which in turn increase transmission of the malaria parasites. (Camponovo *et al.*, 2017).

For many years, the causative agent for malaria was unknown. It was believed to originate from the miasmas around swamps. In fact, malaria derives its name from Italian word "*mal'aria",* meaning "bad air". This belief was held on until 1880 when the French surgeon Alphonse Laveran, discovered that parasites were responsible for the transmission of malaria. Later, Ronald Ross, described the role of mosquito vector in its transmission (Cox, 2010).

1.2 Pathophysiology of malaria

Malaria is a protozoal disease caused by unicellular eukaryotic micro-organisms of the genus *Plasmodium*. This genus comprises of more than 100 species capable of infecting

several animal species including mammals, reptiles and birds (CDC, 2012b). Five of these species infect humans, namely: *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlensi. P. vivax* and *P. falciparum* are the most commonly encountered malaria parasites worldwide and responsible for the largest disease burden (Cox, 2010).

Malaria symptoms vary and can be classified as either mild or severe. This disease resembles flu-like symptoms and in most cases is accompanied by fever which usually sets in after two days from the time of infection. Before this, an individual may experience prodromal symptoms including backaches, anorexia, malaise, nausea and vomiting. At first, the fever may be irregular but regularizes after some days (Bartoloni & Zammarchi, 2012).

Mild malaria symptoms include fever often accompanied by chills, tremors, sweating and vomiting. If not managed, it progresses to the severe form which manifests as cerebral malaria, hypoglycemia, electrolyte disturbances, anemia and organ failure. Organs often affected include the kidney, liver and lungs. Severe malaria is often associated with fatality (Camponovo *et al.*, 2017). In malaria endemic regions, these symptoms are mainly seen in children under five years whereas most adults are asymptomatic because of the acquired partial immunity (Laishram *et al.*, 2012).

A typical malaria infection has three stages; cold, hot and sweating. During the cold stage, an individual feels sudden extreme coldness due to peripheral vasoconstriction. This results in a cold and goose pimpled skin, shivering and teeth chattering. This stage lasts about 30 minutes after which temperature rises leading to the hot stage. The patients

feels hot with a flushed skin and in most cases vomits. If not controlled, the temperature could rise further upto 40 °C with headache, extreme thirst and could alter consciousness. In young children, convulsions is common at this stage. This second stage lasts 2-6 hours after which the patient enters the sweating stage with generalized whole body sweats. This results in lowered body temperature and relief from the symptoms (Bartoloni & Zammarchi, 2012).

1.3 Life cycle of malaria parasites

Malaria are obligate parasites of insects and vertebrates. They are transmitted mainly by bites from infected female *Anopheles* species of mosquitoes which bite mainly at night and breed in water. Other rare transmission modes of malaria include blood transfusion, organ transplant and congenital disease (Filler *et al.*, 2003; Owusu-Ofori *et al.*, 2013).

The life cycle of malaria parasites involves several stages within the human as intermediate host and mosquito, the definitive host. First, the mosquito deposits numerous malaria sporozoites on biting humans. The sporozoites enter the liver through the blood stream. After 5 to 16 days, they divide into several merozoites (asexual replication) which enter the blood stream and invade the red blood cells (RBCs). However, some *P. vivax* and *P. ovale* parasites produce hypnozoites that become dormant within liver cells and are responsible for relapses weeks or months later after the initial infection. Once the merozoites invade the RBCs, they develop into “ring forms” which further mature into trophozoites. These replicate to form schizonts. Upon the rapture of the RBCs, the schizonts release numerous merozoites which continue propagating in the

RBCs (erythrocytic stage). The infection of RBCs is responsible for fever and other clinical symptoms associated with malaria. The time taken to complete the erythrocytic stage varies depending on the malaria parasite. For *P. falciparum, P. ovale* and *P. vivax,* this cycle lasts 48 hours (tertian malaria) whereas for *P. malariae* it takes 72 hours cycle (quartan malaria) thus the differences in periodic fevers associated with malaria. Some of the merozoites within RBCs can differentiate into gametocytes (sexual stage). When these gametocytes are ingested by the mosquitoes, they are fertilized and mature within the midgut to form infective ookinete. The ookinete migrates to the hemocele where it develops into an oocyst where sporozoites are formed. Upon maturation, the oocyst raptures and releases the sporozoites that re-infect humans through mosquito bites (CDC, 2012b). This life cycle is illustrated in Figure 1.1.

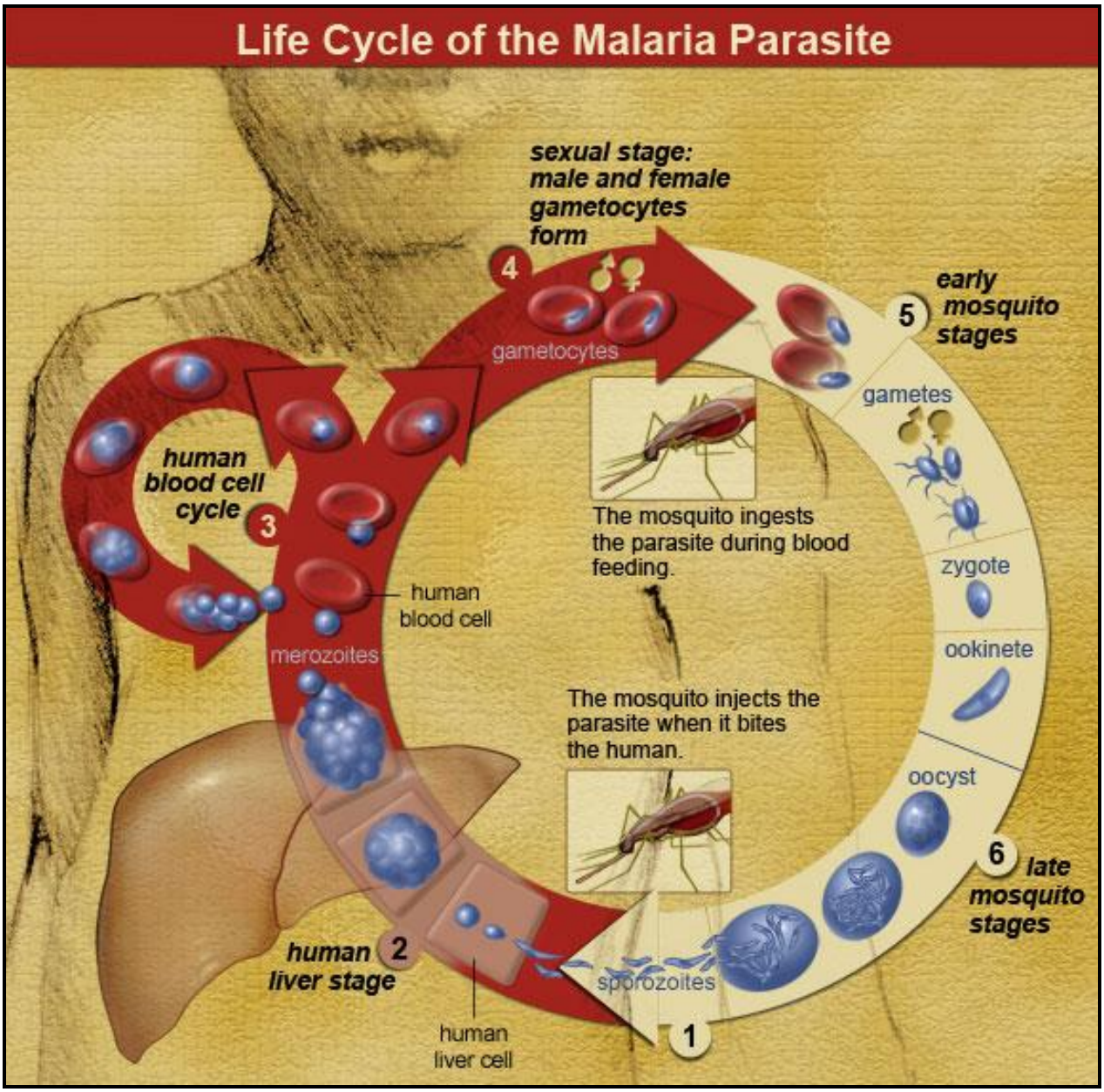


Figure 1. 1: Life cycle of malaria parasites.

Source: (National Institute of Allergy and Infectious Diseases (NIAID), 2012)

1.4 Epidemiology of malaria

Malaria remains a life threatening disease globally. The differences in the prevalence of different malaria parasites within different regions of the world gives rise to differences in malaria epidemicity observed globally.

1.4.1 Global malaria epidemiology

It is estimated that 3.2 billion people worldwide are at a risk of contracting malaria. Sub-Saharan Africa is highly affected by this disease. It is estimated that in 2018, there were about 228 million cases of malaria, majority of which were reported in Sub-Saharan Africa. In the same period, 405,000 deaths occurred due to malaria (WHO, 2018). Populations that are at high risk of malaria include children under the age of 5 years, pregnant women and people with low immunity such as HIV/AIDS patients. Most of the deaths from malaria occur among children under the age of five in Africa where it is estimated that a child dies every minute due to the disease (UNICEF, 2013; WHO, 2013). Immunity to malaria develops slowly and can only be protective from the adolescent period onwards (Doolan *et al.*, 2009). Lack of immunity in children may be a contributing factor to the high mortality rate in this age group.

The high disease burden in the Sub-Saharan Africa can be attributed to the fact that *P. falciparum*, responsible for most fatalities, is most prevalent in these region. This parasite is also associated with high epidemicity (Cox, 2010). Majority of people affected by malaria live in poor nations which lack adequate facilities thus many cases may go

unreported. Some individuals get treated with traditional medicines with such cases going unreported. It is therefore possible that malaria prevalence is higher than the estimated numbers (Cohee & Laufer, 2018).

There has been a concerted effort towards eradicating malaria worldwide. In the 1950s, the World Health Organization (WHO) set out to eradicate malaria. The effort involved landscaping measures, use of dichlorodiphenyltrichloroethane (DDT), a pesticide, to control the mosquitoes and use of the effective antimalarial drug, chloroquine. The war was soon lost because of the ban on use of DDT and the development of resistance to chloroquine (Schlitzer, 2007). Recently, the increase in global investment and action on malaria such as "Roll Back Malaria" has substantially improved malaria control since the year 2000 (UNICEF, 2013). This resulted in a 45 % decrease in global malaria mortality rate in the period 2000-12 and a decrease in malaria incidence rate from 72 to 59 per 1000 population at risk between the years 2010 and 2017 (WHO, 2019). This decline has been attributed to effective interventions including the use of insecticide treated nets (ITNs), indoor residual spray (IRS) and artemisinin-based combination therapies (ACT) (Zhou *et al.*, 2011). The interventions have been used on a large scale in many regions. This decline in morbidity and mortality in many countries has raised hope of elimination and eradication of malaria. Figure 1.2 shows the global malaria case incidences rate in the year 2018.

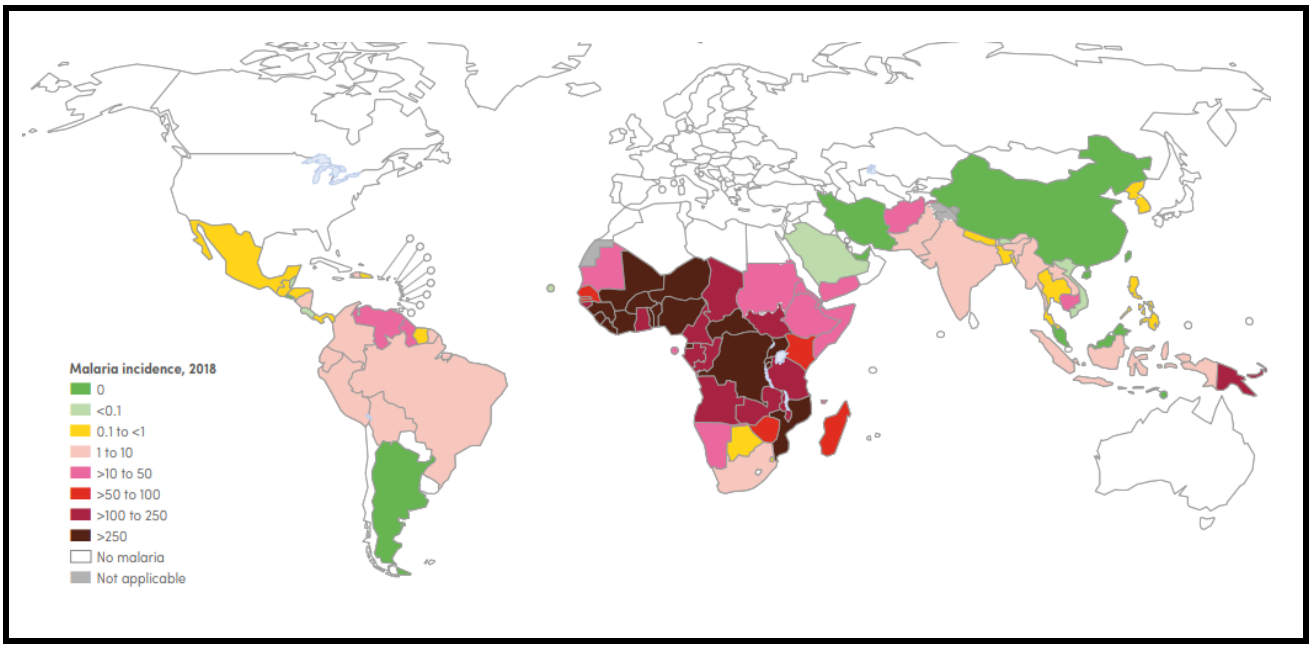


Figure 1. 2: World malaria case incidences rate (cases per 1000 people) in the year 2018.

Source : (WHO, 2019)

1.4.2 Epidemiology of malaria in Kenya

Kenya is ranked among the top 20 countries in the world with the highest burden of malaria (WHO, 2018). Majority of Kenyans (70 %) are at a risk of the disease. The disease burden is highest (> 40 % prevalence) in Western, a stable malaria transmission region (KNBS & ICF Macro, 2011; USAID, 2013). Central Kenya has the lowest prevalence of malaria in counties such as Laikipia, Nakuru, Nyeri and Muranga. The disease accounts for more than 15 % of outpatient consultations nationally and over 60 % in the endemic regions. Figure 1.3 shows the malaria prevalence in Kenya.

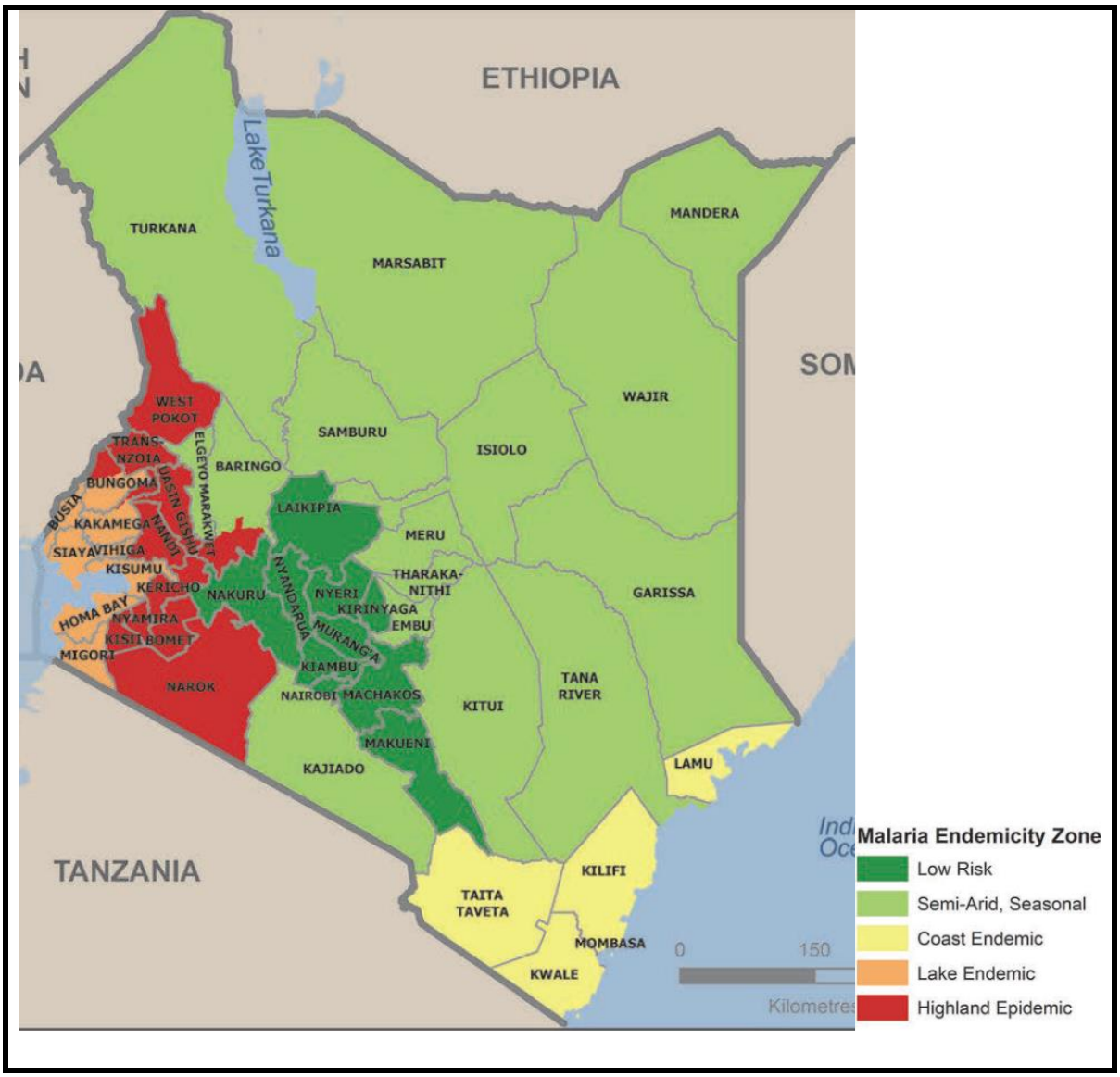


Figure 1. 3: Malaria Endemicity in Kenya.

Source: (NMCP, KNBS & ICF, 2016)

Kenya has put in place malaria control strategies in order to reduce its malaria burden. These includes; capacity building, prevention measures, case management, surveillance, monitoring and evaluation. This is contributed to the decline of malaria burden over the years. For, example, the malaria cases fell from 166.2 to 70.1 per 100,000 people

between 2004 and 2018 (WHO, 2018). However, some factors still hamper the successful elimination of the disease. For, instance, the development of resistance to pyrethrum derived insecticides decreased the efficacy of indoor residual spraying. Other insecticides such as organophosphates have been employed but they are not widely used due to the toxicity associated with them (Abong'o *et al.*, 2020; Bashir *et al.*, 2019).

Majority (96 %) of malaria transmission in Kenya is due to *Plasmodium falciparum* parasites. This is favored by the warm temperature experienced in most parts of the country. A small proportion (16 %) of infections are due to mixed infection with *P. ovale, P. malariae* or both. According to the Kenya Malaria Indicator Survey 2010, malaria prevalence in rural areas was almost three times that of urban areas. This could be attributed to several factors: most rural areas usually have lots of vegetation and stagnant water which favors the multiplication of mosquitoes that transmit malaria. Some rural areas also lack access to health facilities therefore infected patients lack proper management of malaria (Govoetchan *et al.*, 2014). Others still hold onto cultural beliefs that hinder their search for medical information even when they are sick (Mazigo *et al.*, 2010). Effective antimalarial drugs and proper prevention measures are necessary in lowering the malaria cases and deaths in Kenya.

1.5 Malaria chemotherapy and drug resistance

Drugs have been invaluable tool in the malaria management strategies over the decades. Antimalarial drugs are used either for prophylaxis or treatment of malaria infections.

Several classes of effective antimalarial drugs have been developed but their utility is hampered by development of resistance to these drugs by the parasites over time.

1.5.1 Antimalarial drugs

The treatment of malaria has a long history. Even before the discovery of malaria parasites in 1880s and 1890s, herbs were already use in the management of febrile illnesses. For example, Cinchona bark and qinghao *(Artemisia annua)* were used for management of fevers for over 5000 years ago (Meshnick & Dobson, 2001). Compounds derived from these plants (artemisinins and quinine) are currently the most effective drugs in the management of malaria.

Most antimalarial drugs target the parasites within the blood stream (erythrocytic schizonticides). During this stage of the infection, individuals usually experience clinical symptoms of malaria mainly characterized by episodes of fever. Treatment at this stage is critical for all Plasmodium parasites. However, in cases of *P. ovale* and *P. vivax* infection, it is necessary to use drugs acting on the exo-erythrocytic (tissue schizonticides) stages of the parasites to avoid relapse of the disease due to the presence of hypnozoites.

Drugs used for prophylaxis target liver stages of the malaria parasites and thus prevent initiation of the erythrocytic stage and manifestation of symptoms. Some antimalarial drugs target the sexual stage of the parasites (gametocides) and therefore prevent the

transmission of malaria from person to person. Chemical classes of antimalarial drugs and their therapeutic effects are shown in Table 1.1.

Table 1. 1: Antimalarial drugs and their therapeutic effects

Chemical class	Examples	Therapeutic effect
Aromatic-amino alcohols	Quinine, mefloquine, lumefantrine, halofantrine	
4-Amino quinolines	Amodiaquine, chloroquine	Erythrocytic schizonticidal
Sulphonamides	Sulphadoxine, sulfalene	
Sulphones	Dapsone	
Diaminopyrimidines	Pyrimethamine	
8-Aminoquinolines	Primaquine, tafenoquine, pamaquine	Tissue and erythrocytic schizonticidal; gametocidal
Hydroxynaphthoquinones	Atovaquone	
Biguanides	Proguanil	Tissue and erythrocytic schizonticidal
Tetracyclines	Doxycycline, minocycline	
Macrolides	Spiramycin, azithromycin.	
Sesquiterpenes	Artemisinin, artesunate, artemether, dihydroartemisinin	Erythrocytic schizonticidal and gametocidal

(Adopted with modification from Eholié *et al.,* 2009)

The 4-aminoquinolines have a similar range activity as aromatic amino alcohols in inhibiting only the blood stage of parasites. They are useful in treatment of malaria attacks caused by sensitive parasites. On the other hand, the 8-aminoquinolines, proguanil and atovaquone are effective in the tissue stages of parasites and are therefore useful in chemoprophylaxis (Crowth, 2011). The 8-aminoquinolines are active against both the dormant and active liver parasites whereas proguanil and atovaquone are only active against the liver parasites (Bassat, 2011). They are used together with other suppressive drugs in elimination of malaria due to *P. vivax.* The anti-folates (sulphonamides, sulphones and diaminopyrimidines) are known to suppress the erythroctic parasites but their efficacy is lower compared to the quinolines. They are often combined to give a synergistic antimalarial effect (Gregson & Plowe, 2005).

Antibiotics have also been used as adjuvants in the treatment of malaria. The tetracyclines, lincosamides and macrolides groups are effective and are often used in combination with other antimalarial drugs. New antibiotics are still being tested for their antimalarial potency. For example, a new antibiotic, tigecycline, exhibits good antimalarial activity (Sahu *et al.*, 2014). This group of drugs therefore have potential in malaria chemotherapy. Since the mode of action of antibiotics differ from other antimalarial drugs, there are lower chances of development of cross resistance between them and standard antimalarial drugs. Some of them also show synergistic effects when used with antimalarial drugs (Gaillard *et al.*, 2016).

The artemisinins are the main class of compounds in the malaria chemotherapy. Although the plant *Artemisia annua* had been used for many years in the management of febrile illnesses, it was only in the 1972 that the sesquiterpenes were isolated and demonstrated high antimalarial activity (Tu, 2011). The artemisinin and its derivatives are unique in that they attack the parasites in the early erythrocytic stages resulting in rapid clinical response (White, 2008). They are also effective at the sexual stage which prevents the transmission of parasites from person-to-person (van den Broek *et al.*, 2005; Bassat, 2011).

No single drug is effective against all the species of *Plasmodia* that infect humans. Treatment regimens differ regionally because different regions are affected by different species of parasites which with varying sensitivity to antimalarial drugs. The quinoline derivatives and the artemisinins are fast-acting erythrocytic schizonticides and are useful in acute malaria infections. Others such as proguanil, pyrimethamine and sulfonamides, although active at this stage, are slow acting therefore not preferred in acute malaria infections (Eholié *et al.*, 2009).

The artemisinins, although effective with rapid parasite clearance have a short half-life. To overcome this, they are administered in combination with other long-lasting antimalarial drugs such as lumefantrine (Artemisinin Combined Therapy-ACT) to prevent recrudescence of malaria infection. They form the current management regimens for uncomplicated *P. falciparum* malaria infections (van den Broek *et al.*, 2005).

Recently, WHO recommended the use of malaria vaccine in 3 African countries. The vaccine, (RTS, S) was adopted in the routine child immunization programs in in parts of Kenya, Malawi and Ghana since 2019 in a pilot study. The vaccine acts against *P. falciparum* and was shown to have a low efficacy of 39 % in preventing malaria in children 5-17 months old in the phase III clinical trials (WHO, 2020). However, the vaccine is only intended to supplement other malaria control strategies and not to replace them.

1.5.2 Antimalarial drugs resistance

The development of resistance to antimalarial drugs is a great threat to the effective fight against malaria. Various drugs have been developed to fight malaria parasites over the years but their effective life-spans have been cut short because of development of resistance. The successful development of synthetic antimalarials in the 1940s was a great milestone in malaria management. Chloroquine was preferred over quinine because of the low cost of production and fewer side effects. It was widely adopted as the drug of choice for malaria due to affordability and ease of access. However resistance to this drug developed very fast on the Thai-Cambodian border and spread throughout the world (Wongsrichanalai *et al.*, 2002).

Development of resistance to antimalarial drugs is not uniform but dependent on the geographical location as well as the malaria parasites involved. For example, although resistance to quinine was reported over 100 years ago in Brazil, this resistance is currently restricted to southeast Asia and western Oceania (Wongsrichanalai *et al.*, 2002).

Quinine is still effective in most of the other regions of the world as a drug of choice for management for severe malaria. On the other hand, chloroquine resistance is specific to *P. falciparum* parasites. Chloroquine is still useful in management of malaria caused by *P. ovale* and *P. malariae* parasites.

Resistance to other synthetic drugs employed in management of malaria after chloroquine, such as the sulfadoxine-pyrimethamine (SP), also developed over the years rendering them ineffective. For example, studies carried out in the Western Kenya show high resistance to SP, the only drug used in Intermittent Preventive Treatment in pregnancy (IPTp) (Iriemenam *et al.*, 2012).

Resistance to the artemisinins has been reported in South-East Asia, a historical source of emerging antimalarial drug resistance (Dondorp *et al*., 2009; Phyo *et al*., 2012). This poses a global threat as it may spread to neighboring countries and eventually to the rest of the world. There are already reports of high recrudescence rates of *P. falciparum* up to 10 % of patients after ACT treatment (LaCrue *et al.*, 2011; Cheng *et al.*, 2012). Recently, there are reports of resistance in Rwanda with delayed parasite clearance after treatment (Tacoli *et al.*, 2016). Therefore, there is urgency in development of newer antimalarial medicines before widespread resistance to the current mainstay treatment of malaria, the artemisinins. One strategy is to study the plants used in ethno medicine which can provide important leads in new drug development.

1.6 Use of plants as medicines

Plants have been employed in the management of human diseases from time immemorial. Herbal medicine is the oldest form of medical practice. Archeological evidence points to the practice of herbal medicine more than 60,000 years ago (Pan, *et al*., 2014). Before the advent of modern science where causes of diseases are well understood in most cases, humans instinctively chose locally available plants to treat illnesses (Petrovska, 2012). The practice of herbal medicine during this period was linked to cosmology and practiced within the limits of available tools and understanding. However, with experience, certain plants were preferred in management of certain illnesses. Traditional medicine knowledge was passed on from one generation to another orally. With the advent of human civilization, some of the traditional knowledge was recorded in documents or preserved as monuments. The earliest records of use of medicinal plants on clay tablets are attributed to the Sumerians of Mesopotamia which date back to more than 5,000 years ago (Pan, *et al*., 2014).

The practice of herbal medicine developed independently within different geographical regions. Some of the well-developed systems with a long history include Ayurveda (India) which dates back to more than 4000 years and Traditional Chinese Medicine (TCM) that is estimated to be over 5000 years old. The Greek physicians, Hippocrates and Dioscorides, greatly contributed to the development of traditional medicine in Europe. They described how to diagnose diseases as well as herbal medicines that could be used. In his book, "*De Materia Medica*", Dioscorides described the use 657 drugs of

plant origin in management of human diseases (Petrovska, 2012). This formed the basis of the modern conventional medicine. In Africa, this knowledge was mainly transmitted orally from one generation to another (Atemezing & Pavón, 2009). However, the practice of traditional forms of medicines declined as synthetic molecules were preferred to the traditional medicines (Abdullahi, 2011).

The 21st century witnessed a growing demand for the use of phytomedicines. This was necessitated by the emergence of drug resistant microbes as well as emergence of new diseases such as human immuno-deficiency virus (HIV) and other chronic illnesses that could not be adequately managed using the available drugs. Today, various forms of traditional medicine practices are still practiced in many parts of the world. These practices are no longer a preserve of certain regions but are now widespread in the form of complementary medicine (Bag, 2019). The World Health Organization (WHO) recognizes the important role traditional medicine plays in health care. Consequently, WHO has developed guidelines on the study of traditional medicines (Kasilo *et al.*, 2013).

1.7 Plants as sources of new antimalarial drugs

Communities living in malaria endemic areas have for many years used traditional preparations especially from plants for treatment of malaria (Ntie-Kang *et al.*, 2014). The diverse chemical structures of compounds with antimalarial activities from plants provides a rich library for discovery of new anti-malarial drugs. This is well illustrated by the discovery of quinine from *Cinchona* species and artemisinin from *Artemisinin annua*.

In South America, *Cinchona* species were used in treatment of febrile illnesses for many years. However, the first record of the use of this tree is attributed to the Jesuit missionaries. The record states that the Countess of Chinchon visited Peru in the 1630s with the Jesuits missionaries and she fell sick with fever. The natives of Peru treated her with the bark of a tree which was later named "The Cinchona tree". She later carried the bark, christened Jesuit bark, to Europe in 1638 where it was adopted for use in treating malaria-like fevers. Quinine, a quinoline alkaloid, was isolated from the plant by French pharmacists in 1820 (Renslo, 2013). This drug continues to be used in the management of severe malaria in most parts of the world (Toshihiro *et al.*, 2009). Chloroquine, an analogue of quinine, was synthesized in the 1940s but resistance to the drug by *P falciparum* developed very fast on the Thai-Cambodian border and spread throughout the world (Wongsrichanalai *et al.*, 2002). As a result, the compound is no longer used in the management of malaria.

In China, the sweet wormwood herb (*Artemisia annua*) was used for more than 2000 years in treating periodic fevers in form of herbal teas (Biesen, 2010; Renslo, 2013). Artemisinin, a sesquiterpene, was isolated in 1972 during a Chinese government project (Project 523) to identify antimalarial compounds from traditional Chinese medicines (Renslo, 2013).

The success stories about discovery of antimalarial drugs from plants has sparked the interest in search for more compounds from nature. Several plants continue to be used by local communities for management of malaria-like fevers. Scientific evaluation of such

plants is important in the discovery of new anti-malarial drugs. Several compounds isolated from plants have shown to possess varying *in vitro* and *in vivo* antimalarial activities (Ntie-Kang *et al.*, 2014).

1.8 Ethno-medical approach in drug discovery

Ethno-medicine is the study of indigenous medical systems which represents an important tool for successful discovery of drugs from natural products. It is important to understand the history of each community and their use of ethnic medicines (Cragg & Newman, 2002).

1.8.1 Health system in traditional communities

Most traditional societies have a holistic view of health. Disease conditions are usually viewed as a result of interrelated factors. Understanding the health system within societies is therefore important for ethno-medical studies. This entails the understanding of the society's view of disease, belief systems, attitudes towards health and illness, role of practitioners and choice of remedies.

Understanding of ethno medicine is well illustrated by Arthur Kleinman's (1978) "explanatory model" (Figure 1.4). The model proposes that a disease has more than one potential cause. Whenever an individual experiences ill-health, they usually try to review the circumstances leading to the disease and how it will eventually affect them (Kleinman, 1978). Interviews carried out during ethno-medical studies seek to understand the community's view of diseases and the remedies used.

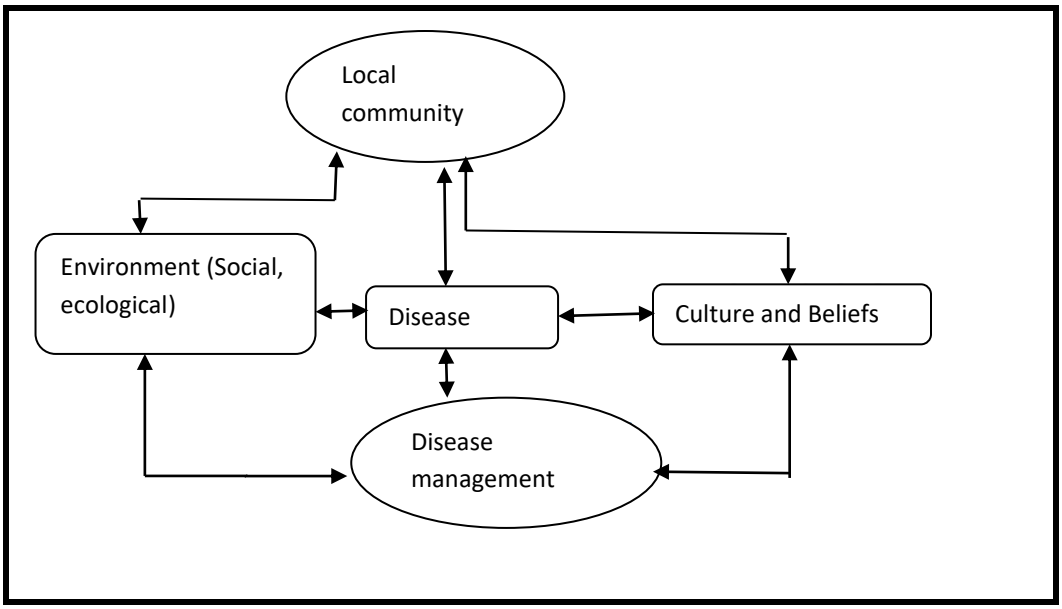


Figure 1. 4: Kleinman's Explanatory Model of Disease.

This model visualizes a disease as a result of culture, beliefs and environment. The management should also be focused not just on the disease itself but also the causative factors. It is a no wonder that the term malaria is derived from an Italian word, *"mal'aria",* meaning "bad air". The French people also referred to malaria as *"paludisme"* which means "rooted in the swamp" (CDC, 2012a). These communities related the disease to the environment where it was most prevalent. This relationship still remains relevant in the present modern world. It therefore follows that control of malaria involves not only the chemotherapeutic agents, but also the proper management of the environment to control the breeding of the vectors that transmit the disease.

Cultural beliefs and practices greatly impact on the health and healing practices within societies. These can guide the choice of remedies in management of diseases and care of the sick person. However, some of the beliefs held within indigenous communities can

impact negatively on disease management. For example some African communities believe that taking a sick child to hospital or administration of an injection could result in death (Maslove *et al.,* 2009). In such cases, a person with severe malaria requiring hospitalization may not access the required medical attention because of these cultural beliefs. Local communities especially in rural areas therefore need to be educated against such beliefs that hinder effective control and management of disease.

The understanding of the communities' view of health is also important to the scientist in understanding how to interpret the information given during the ethno-botanical research. Different approaches such as ethnobotany and retrospective treatment outcomes (RTO) are employed in successful ethno-botanical studies.

1.8.2 Methods used in ethno medicine

The success of drug discovery from plant depends on choosing the best candidate plant for further study. Scientists have over a long period of time devised various approaches of identifying the best candidate for this success. Several ethnomedical approaches, with varying degrees of success, have been described in ethno medicine.

Ethnobotanical studies are based on the relationship between plants and humans. There exists a complex interaction between people and plants and this varies from culture to culture. However, some general processes are common in many cultures (Ulysses, 2010). The relationship is evident through rituals and ceremonies performed in different communities. People use plants for various purposes such as food, medicine, building,

textiles, divination, cosmetics, music and social life. This indigenous knowledge is rapidly disappearing all over the world. Ethno-botanical studies are therefore needed to document most of this knowledge before it becomes extinct. Some regions such as Asia have a strong history of use of ethno-medicine as recorded in ancient books. However, most practices of ethno-medicine, especially in Africa, are undocumented. It is therefore necessary to have such information documented for future use (Mahomoodally, 2013; Mahwasane *et al.*, 2013).

Ethnobotany involves exploration of how plants are used within communities. In this approach, quantitative methods are employed in documenting plants used in disease management within various cultures (Heinrich, 2000). The method has been used successfully in the discovery of many drugs in clinical use (Fabricant & Farnsworth, 2001). However, the use of ethnobotany in drug discovery has some limitations. For instance, the clinical outcomes, which is a pointer to efficacy, of the plant are not considered important in ethnobotany. The method also does not clearly indicate why some plants are chosen over others (Graz *et al.*, 2005).

Plants can also be chosen based on climate and seasonality (Ulysses, 2010). Plants available in most seasons are chosen over those that occur occasionally. There is preference for use of native perennial resources among communities. This ensures consistency in the supply of specific plants employed in management of diseases throughout the year. However, this method leaves out potent plants that are available only at certain seasons.

Another method of drug discovery from ethno-medicine involves Retrospective Treatment Outcome (RTO) surveys which was proposed by Graz *et al.*, 2005. It involves asking individuals about the disease status they suffer from, what medicines they took and the outcomes of the treatment. Those plants with the best outcomes are selected for further investigation. This approach gives a high success rate in drug discovery. It also has an advantage that patients can describe any side effects related to the use of the medicines. For instance, RTO carried out in Mali by Graz *et al.*, (2005) resulted in selection of plants with higher efficacy compared to those selected through ethno-botany.

A more comprehensive method in ethno-medicine studies involves the integrated approach as postulated by Katiyar *et al.*, 2012. The authors highlighted the importance of using the knowledge of Ayurveda (traditional Indian medicine), documented and non-documented uses of plants and a proper literature review of chosen plants. All these areas can be analyzed to come up with the best candidate for the bio-assay guided fractionation.

The choice of any method will usually vary from place to place. Countries such as India and China have a very rich system of traditional medicines which are well documented (Katiyar *et al.*, 2012). However, in Africa most of the knowledge is passed from generation to generation through the word of mouth. Therefore, the integrated approach may not be fully utilized as described in literature. In such cases, working with the traditional practitioners and asking in-depth questions are necessary. Very few RTO

studies are available therefore this poses a challenge in the search of the best candidate plants for study. Ethnopharmacogical studies are important in evaluating the biological activities and phytochemical constituents of plants.

1.9 Ethnopharmacology in drug discovery

Ethnopharmacology facilitates intentional, focused and safe natural product drug discovery. The starting point in ethnopharmacology is usually the identification of the correct plant candidate(s) by use of ethnobotanical methods. Bioassay-guided fractionation is important in successful discovery of lead compound or formulation of standardizes extract. (Katiyar *et al.*, 2012).

1.9.1 Conceptual framework in ethnopharmacological studies

Since most traditional communities use plants in ethnomedicine, ethnopharmacology is a useful tool in the evaluation of the biological activities of such plants. These are based on reductionist approach which utilizes biological systems in drug discovery. The approach assumes that plants contain molecules that interact with specific target within the body to alleviate a disease condition (Mazzocchi, 2012). Figure 1.5 shows the development of modern drugs from ethnomedicine.

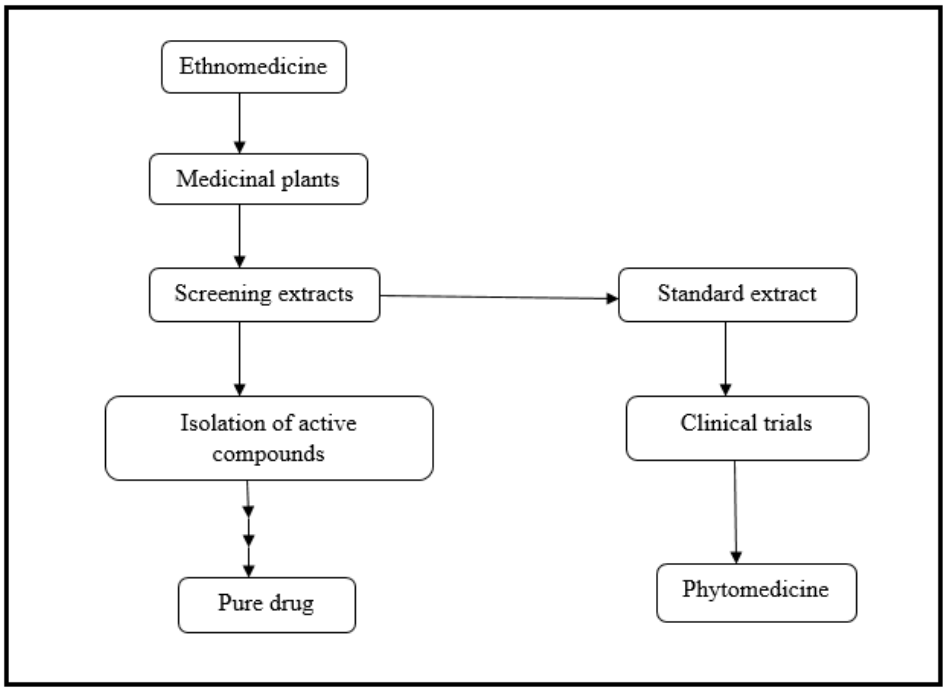


Figure 1. 5: Development of medicines from ethnopharmacology .

(Adapted from Ashok, 2007)

Ethnopharmacology has been successfully used in discovery of many drugs from natural sources. The current antimalarial drugs, quinine and artemisinins were discovered based on their traditional uses (Achan *et al.*, 2011; Tu, 2011).

Ethnopharmacological methods have for a long time been biased towards isolation of active compounds and evaluation of their chemical, biological and pharmacological activities (Etkin & Elisabetsky, 2005). However, more needs to be done by integrating social sciences in ethnopharmacological studies to validate traditional knowledge in the context of different cultures (Reyes-Garcia, 2010). When social sciences are involved, peoples' behaviors and practices can be studied. These could help in explaining the

reasons why communities prefer some remedies over others. For example, practices related to the use of the ecosystem affect the available natural resources. Involvement of anthropologists will define how culture affects the human behavior in terms of their health and wellbeing (Reyes-Garcia, 2010).

1.9.2 Ethnopharmacology in development of antimalarial drugs

Cinchona trees were used in management of fevers associated with malaria among the natives of South America. The cinchona alkaloids, quinine, quinidine, cinchonine and cinchonidine, were the first drugs used in management of malaria. Due to pressure on the South American cinchona trees, efforts were made to cultivate the trees elsewhere including Java and India (Karin & Toni, 2009). The Javan cinchona bark was preferred since it mainly contained quinine (Achan *et al.,* 2011). However, during the second World War, the supply of cinchona barks was cut off by the Japanese (Dewick, 2011). This necessitated production of synthetic drugs as alternatives to quinine. This led to the synthesis of 4-aminoquinoloines (chloroquine and amodiaquine) and the 8-aminoquinolines (primaquine, tafenoquine and pamaquine). Efforts were also made to synthesize quinine in 1944 by the American chemists, Woodward and Doering (Crowth, 2011). However, the laboratory synthesis is not economically viable as it involves many stages with only a small yield of the compound. For example, in 2001, Stork *et al.*, successfully carried out a stereo-controlled synthesis of quinine. The synthesis involved 20 steps and only yielded 7 % of quinine. For these reasons, Cinchona bark remains the main source of quinine.

1.9.3 Phytochemistry of medicinal plants

Phytochemistry is the study of plant chemicals which is essential in the discovery development of new drugs from natural products. Plants produce an array of secondary metabolites which have been found to be important in fighting and prevention of diseases in humans. These compounds are grouped based on their chemical structures in classes such as terpenoids, alkaloids, flavonoids, glycosides, phenylpropanoids and phenolics among others. Phytochemistry involves various techniques such as extraction, chromatography, isolation and structure elucidation.

Ethnopharmacological knowledge is critical in the phytochemistry procedures and subsequent success in identification of biologically active compounds. The way the plant is used in traditional medicine gives a pointer to the nature of compounds that elicit the biological effect. For example, plants whose vapors are inhaled may be a pointer to the volatile compounds as the biologically active entities. The importance of ethnomedical procedures is well illustrated in the discovery of artemisinins. During the screening of plants for antimalarials by Chinese scientists in project 523, artemisinin showed promising antimalarial activity but the activity was not reproducible in subsequent experiments. Literature search pointed to the preparation of the plant extract in cold water unlike in the experiment where heat was used. Extraction of the plant at low temperatures gave better results (Tu, 2011).

Successful extraction of secondary metabolites involves proper preparation and preservation of plant material to avoid degradation of target compounds. Suitable

extraction procedures for the target class of compounds are chosen based on standard protocols (Jones & Kinghorn, 2012). These compounds can then be evaluated for biological activity. Although often these secondary metabolites lack the required activity or only exhibit a fraction of the crude extract, their isolation is important as they can be important marker compounds in standardization of herbal products.

A review of compounds with anti-malarial activities from African plants revealed that over 300 phytochemicals have been isolated and tested for antiplasmodial activity (Batista *et al.*, 2009; Oliveira *et al.*, 2009; Onguén *et al.*, 2013a, 2013b) . Among the classes identified, alkaloids and terpenoids are the major groups of compounds with anti-malarial properties. Other compounds with antimalarial activity include: flavonoids, phenolics, lignans, polyacetylenes, xanthones, quinones, steroids and coumarins (Bero & Quetin-Leclercq, 2011; Ntie-Kang *et al.*, 2014; Onguén *et al.*, 2013a, 2013b). These studies were informed by traditional uses of these plants in African communities. Figure 1.6 shows selected compounds derived from plants with significant anti-plasmodial activity.

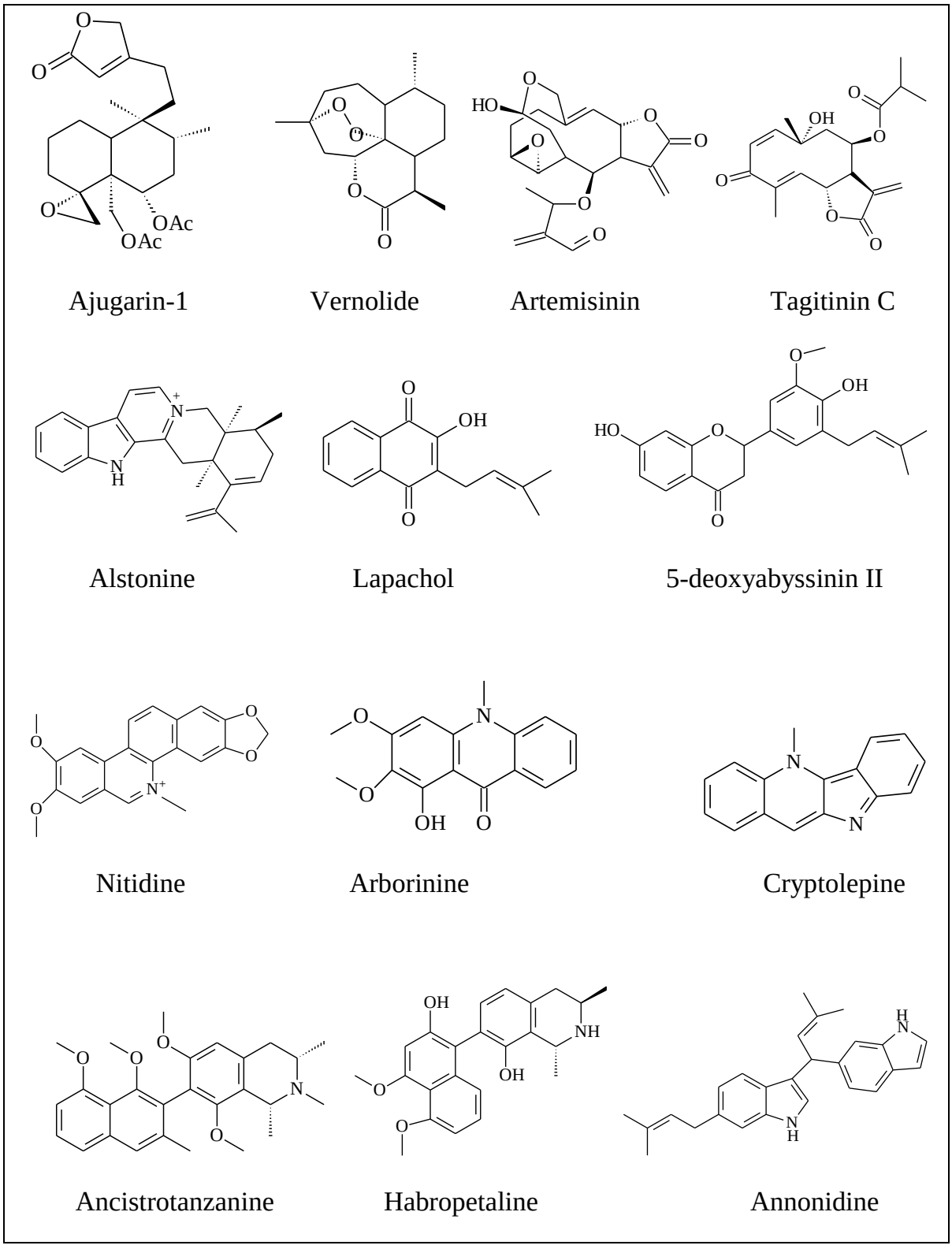


Figure 1. 6: Some antimalarial compounds isolated from African plants

The plant based antimalarials have a diverse chemical structures indicating that they act by different mechanisms from the known antimalarial drugs such as chloroquine in combating malarial parasites. The parasites may also not have developed resistance yet to such compounds making them useful as novel drugs in areas with multidrug resistance. For example, the artemisinins presented a different chemical class from classical antimalarial drugs such as aminoquinolines. There is therefore need to continue the search for antimalarial compounds with unique chemical structures for further development of new antimalarial drugs.

1.9.4 Biological assays for antimalarial activities

Biological assays are useful tools in verifying the pharmacological activities of plants. In testing for antimalarial activity, *in vivo* and *in vitro* methods are employed. *In vitro* methods allow for the evaluation of several drugs at the same time thus are useful tools in high throughput screening in drug discovery. Since the procedures are carried out on isolated parasites, they are not affected by individuals immune responses and reinfections within a person (WHO, 2001). However, the efficacy of a drug in *in vitro* studies does not guarantee effectiveness in clinical application. It is therefore necessary to carry out *in vivo* tests of components that show good *in vitro* activities. This is because other factors such as metabolism and absorption are necessary for a drug to attain *in- vivo* efficacy. Several in vitro and *in vivo* methods are available for study of antimalarial activity.

1.9.4.1 *In vitro* assays for antiplasmodial activity

Several *in vitro* assays have been developed over the years to evaluate the activity of antiplasmodial drugs. The first antiplasmodial assay, the macro technique was developed in 1968 and demonstrated both the *in vitro* growth of the parasites and the effects of antimalarial drugs on the growth (Sinha *et al.*, 2017). Several variations of the macro technique have led to many other *in vitro* tests. Currently, micro technique assays are utilized. These assays developed from the macro technique concept with variations in the culture of malaria parasites as described by Trager and Jensen, 1976. These methods include schizont maturation method, isotopic assay, parasite lactate dehydrogenase (pLDH) assay and histidine-rich protein II assay (HRP2 assay) (Noedl *et al.*, 2003; Krettli *et al.*, 2009).

The schizont maturation assay method was the first *in vitro* method developed for the assessment of effect of antiplasmodial drugs on malaria parasites. The method is based on the ability of drug to inhibit the maturation of parasites from the ring stage to the schizont stage. The schizonts are counted on thick films using microscopes. However, this method is limited to only synchronized malaria parasites and those with only one asexual form (Srinivas & Puri, 2002). The method is simple to carry out and economical thus used as an epidemiological tool for global baseline assessment and measuring the response of *P. falciparum* parasites to antimalarial drugs (WHO, 2001).

The second method utilizes the measurement of level of hypoxanthine, a known low molecular weight compound, required in the erythrocytic stage of malaria parasites in

serum-free culture medium (Asahi *et al.*, 1996; Desjardins *et al.*, 1979). In this method, the level of incorporation of hypoxanthine, labelled with radioactive tritium, into the parasites which is measured using spectrometric methods. Antiplasmodial activity is a measure of the inhibition of the uptake of the [^{3}H] hypoxanthine . This assay can evaluate several compounds at the same time and is highly reproducible. However, it has lost popularity due to the expensive equipment involved and the safety requirements for handling radioactive substances (Smilkstein *et al.*, 2004).

The third method is based on measuring activity of the enzyme**,** parasite lactate dehydrogenase developed by Makler *et al.*, (1993). This enzyme is important in the conversion of lactate to pyruvate. In *P. falciparum* the co-enzyme 3-acetyl pyridine adenine dinucleotide (APAD) is necessary in this reaction catalyzed by lactate dehydrogenase forming reduced APAD. The reduced APAD in turn reduces tetrazolium to form a blue product, formazan, which can be measured using a spectrophotometer (650 nm). The level increases with the increase in parasitemia (Makler *et al.*, 1993; Basco *et al.*, 1995). This fact that this method requires an initial high parasitemia of 1-2% and there is interference from pigments present in natural extracts limits its application (Kaddouri *et al.*, 2006).

Another assay method based on the lactate dehydrogenase enzyme activity was developed by Druilhe *et al.*, in 2001. Instead of using tetrazolium as in the earlier method, it utilizes the double-site enzyme-linked lactate dehydrogenase immunodetection assay (DELI), an immuno-enzymatic reaction. In this assay, there are 2 monoclonal

antibodies that recognize 2 different sites of LDH. One of the monoclonal antibodies develops a colorimetric reaction in the presence of peroxidase substrate, the level of which is determined using optical density (Brasseur *et al.*, 2001). DELI assay is more sensitive as it can be used even when parasitemia as low as 0.005% (Druilhe *et al.*, 2001). However, the reagents used in this assay are very expensive thus limiting its application.

A fourth method developed by Noedl *et al*. (2002) is based on the ability to quantify the water-soluble histidine-rich proteins II (HRP2) produced by *P. falciparum* parasites. Histidine rich proteins II are a group of histidine and alanine rich proteins produced during the active phase of multiplication of the parasites. The measurement of these proteins is done using enzyme-linked immunosorbent assay (ELISA) technique. The amount of proteins produced in presence of an antimalarial drug is inversely proportional to the growth inhibition ability of the drug. The method is relatively simple to perform, sensitive and highly reproducible and more sensitive than the isotopic methods (Noedl *et al.*, 2002; Sinha *et al.*, 2017).

Although *in vitro* assays involving the use of malaria parasites are commonly used in search for new compounds, these methods are not compatible with high throughput purposes. Recently other methods that do not require culturing of *Plasmodium* parasites have been developed as important tools in high throughput screening. For example, the use of *in vitro* β-hematin formation assay is becoming a more acceptable assay in high throughput screens. Malaria parasites digest hemoglobin releasing heme, a toxic compound. However, the malaria parasites have a mechanism for detoxifying the heme

by converting it into the malaria pigment, hemozoin. A synthetic analog of hemozoin, β-hematin, is composed of ferritoprotopophyrin [Fe (III) PPIX] which is crystalline in nature. In this assay, formation of β-hematin from heme is made possible by use of a catalyst. Porphyrins being highly colored absorb strongly in the visible region of electromagnetic spectrum, therefore basis for their analysis. The activity of an antimalarial drug is measured as a factor of inhibition of formation of β-hematin (Sinha *et al.*, 2017). The aminoquinoline class of antimalarials inhibit this pathway. The β-hematin inhibition method is summarized in Figure 1.7.

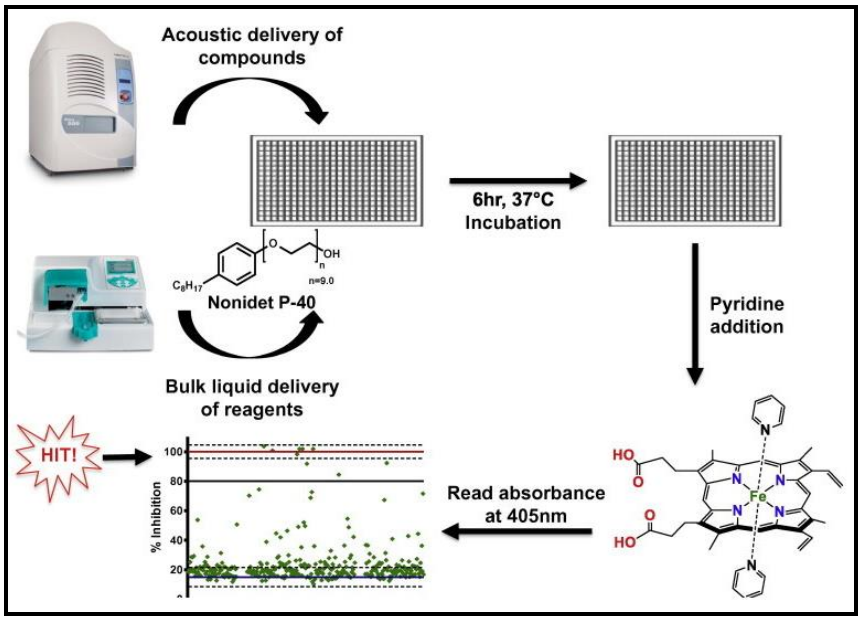


Figure 1. 7: Summary of the work flow in β-hematin inhibition assay.

(Sandlin *et al.*, 2014)

In this assay, pyridine is added after the incubation period. Pyridine forms a complex with hematin and not β-hematin to give a red color that is measured by UV/VIS spectroscopy at 405nm (Vargas *et al.*, 2011).

1.9.4.2 *In vivo* assays

In vivo antimalarial activity tests are the oldest forms of assays for assessing the effects of antimalarial drugs in mice infected with malaria parasites (Geary *et al.*, 1983). Donor mice are injected with the malaria parasites then sacrificed after attaining the required level of parasitemia. The blood obtained by cardiac puncture is diluted in physiological saline and injected intraperitonally into the test mice. Two tests are used: Peter 4-day suppressive test and the Rane's test.

Peter 4-day suppressive test method developed by Peter *et al* (1995) evaluates schizonticidal activity of the test extracts or compounds in mice infected with *P. berghei.* Treatment is started 3 hours after inoculation of the parasites and thereafter daily for 3 more days. After the fourth day, blood drawn from the tail is examined microscopically to determine the level of parasitemia.

A second method, Rane's test, is used as a follow up to the Peter 4-day suppressive test to tests the curative potential of extracts or compounds. Mice are inoculated with malaria parasites, and then the drug given after 72 hours. The doses are repeated daily for 5 days while checking the level of parasitemia daily. The mice are monitored over a period of 30 days to determine the mean survival time.

The *in vivo* methods are more accurate in determining the effectiveness of drugs since they take into account effects of host's immunity and pharmacokinetics of the drug within the body. However, these methods are limited to evaluation of only few drugs

because of the labour involved and the animal ethics requirements. To overcome this challenge, a novel *in vivo* method is proposed by Jimenez-Diaz *et al.*, (2013). In this assay, only 2 mice per group are used for compounds that show parasite growth inhibition and clear parasites within mice peripheral blood.

1.10 Study Problem

Plants are an important source of new drugs (Cragg & Newman, 2002). Communities living in malaria endemic areas have used many traditional preparations in the management of the disease. The systematic study of such plants can result in new drugs. Although many plants have been studied for antimalarial activity in Kenya, few studies have been carried out to document the plants used among the Luhya community, in Western Kenya, for the management of malaria (Dharani *et al.*, 2010; Nyunja *et al.*, 2009; Otieno & Analo, 2012). This knowledge may be lost if left undocumented. There is also need to validate the claimed antimalarial properties of these plants. This study therefore sought to evaluate the ethnopharmacology of plants used in Kakamega East sub-County for malaria management.

1.11 Study Justification

Malaria still remains a severe public health problem worldwide. In Kenya, malaria is the leading cause of morbidity and mortality (USAID & CDC, 2018). In the recent past, efforts to fight malaria have seen a dramatic decline in prevalence and death due to malaria worldwide. However, the development of antimalarial drug resistance threatens

the success gained over the years. There is therefore need to search for new antimalarial drugs. This research sought to evaluate antimalarial properties of plants used in management of malaria among the Luhya community. Similar work has been carried out in some communities in Kenya but no systematic studies have been carried out in the Luhya community.

Africa is endowed with enormous natural resources that remain unutilized. This research finding will provide information on the efficacy of the plants used in management of malaria in Kakamega East sub-County. This is important as evidence-based medicine is an important aspect of successful healthcare. It will form a basis for future research in antimalarial drug discovery. The research is important in the global search for new antimalarial agents. The isolated compounds could further be developed through semi synthesis to optimize activity and reduce toxicity. The pharmacophores can also be modified to yield better antimalarial agents.

1.12 Objectives

1.12.1 General objective

The aim of this study was to identify and document antimalarial plants used among the Luhya community of in Kakamega East sub-County and evaluate their antiplasmodial properties.

1.12.2 Specific objectives

The specific objectives of this study were:

1. To identify and document plants used in malaria management among the Luhya community in Kakamega East sub-County, Western Kenya.
2. To evaluate the *in vitro* antimalarial activities of selected plant extracts
3. To characterize antimalarial phytochemicals from selected plants.

CHAPTER TWO

ETHNOBOTANICAL SURVEY OF ANTIMALARIAL PLANTS USED AMONG THE LUHYA COMMUNITY, KAKAMEGA EAST SUB-COUNTY

2.1 Introduction

The Luhya community of Kenya is the second largest ethnic group in Kenya after the Kikuyu. The community consists of 16 sub-tribes with different linguistic dialects (Brenzinger, 1992). The Luhya community inhabits the defunct Western province of Kenya and parts of Rift Valley province. After the promulgation of the Kenyan constitution in 2010, the region was divided into 4 counties namely; Kakamega, Vihiga, Bungoma and Busia.

In the rural areas, the Luhya people engage mainly in peasant agriculture as the main source of livelihood (Rank, 2009). However, an increasing number of people have moved to urban centers in search of employment and education. The poverty level in this region is over 50 % (NCAPD, 2005; KIPPRA, 2013).

The Luhya community has a rich cultural heritage characterized by distinct practices such as circumcision, traditional dances and bull fighting among others. The practice of herbal medicine is core to this community (Ngetich, 2013).

Malaria remains a great burden among the Luhya community. Given that this is a rural community endowed with the flora rich, Kakamega forest, use of herbal medicine for

management of malaria is common (Nyunja *et al.*, 2009). Little is known about these plants used for malaria management in this community. Some ethnobotanical studies carried out among the Luhya community have revealed some plants which are used in management of malaria (Dharani *et al.*, 2010; Otieno & Analo, 2012). These plants include; *Melia azedarach, Trichilia emetica, Piper umbellatum, Cassia occidentalis*, *Harrisonia abyssinica*, and *Vernonia amygdalina*. However, no ethnopharmacological survey for antimalarial plants has been carried out in this community. This study identified the plants and formulations used in malaria management among the Luhya community residing in Kakamega East sub-County.

2.2 Methodology

2.2.1 Study design

An ethnobotanical survey was carried out in Kakamega East sub-County between the months of August and October 2015. This was a qualitative cross- sectional study to collect information on malaria diagnosis, plants used to treat malaria, their preparation and formulations and side effects associated with their use among the Luhya community.

2.2.2 Study area

Kakamega County is located in Western Kenya. The county lies within the longitudes 34° 20′ 35.29′′ E- 35° 09′ 27.04′′ E and latitudes 0° 05′ 19.12′′N- 0° 53′ 53.81′′ N. It boarders the counties of Bungoma to the North, Trans Nzoia to the North East, Uasin-Gishu and Nandi Counties to the East, Vihiga to the South, Siaya to the South West and Busia to the West. Administratively, it comprises 12 sub counties, 83 locations, 250 sub-

locations and 187 villages. According to the 2019 census, the county has a total population of 1, 867, 579 people within an area of 3,224.9 km^2 yielding a population density of 618 people per km^2. The poverty level is 57 %. The public health facilities in the county include: 1 county referral hospital, 12 sub county hospitals, 47 health centers, 123 dispensaries and 44 clinics. The most prevalent diseases in the county are malaria, diarrhea, skin diseases and respiratory tract infections (KNBS, 2019).

Kakamega East District is one of the 6 districts in Kakamega County. It has a population of 167,000 people in a total area of 445.5 km^2. The population density is estimated to be 358 people per km^2. The district is mainly a rural set-up with no major urban centers. The entire district is served with dispensaries and health centers. It is the home to the Kakamega forest, the only tropical rain forest in Kenya. Figure 2.1 shows the map of Kakamega county (NCAPD, 2005). This being a rural population, coupled with the proximity to Kakamega forest, there is a high prevalence of use of herbal medicine in management of malaria (Nyeko *et al.*, 2016; Wardle *et al.*, 2012).

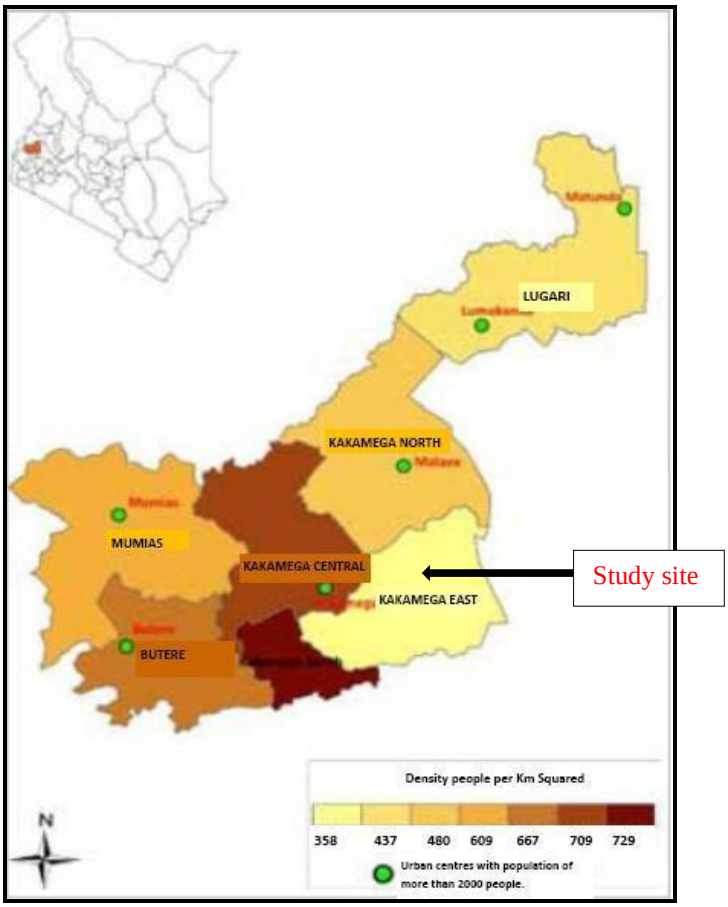


Figure 2. 1: Map of Kakamega County showing constituent sub-Counties

Source: (CRA, 2013)

2.2.3 Study population

The target population were traditional practitioners and other care givers who had resided in Kakamega East sub-County for at least years and use herbal medicines in management of malaria. The traditional medical practitioners were those well known by the community for treating different diseases whereas care givers were those who occasionally used plants to manage some disease especially among family members only.

Only those who agreed to sign the prior informed consent, resided in Kakamega East sub-County for at least 3 years prior to the study and had used herbal medicine in management of malaria were selected for interviews. Those who did not meet these criteria were excluded from the study.

2.2.4 Sample size, sampling and participant recruitment strategy

The principles of sampling for qualitative research were applied in this study (Guest *et al.*, 2006; Mason, 2010). For this method, a sample of at least 15 participants is acceptable. For this study, 21 traditional medicine practitioners were interviewed. Participants were recruited using the snowballing technique. In this method, community gate keepers were requested to identify the herbalists or homes where family members had a vast knowledge of use of herbal medicines. The participants also identified other practitioners of herbal medicine known to them.

2.2.5 Data collection

For the purposes of this study, malaria was defined as any febrile condition accompanied by any two of the following symptoms: sweating, chills, vomiting, fatigue, joint pains, headache or coughing. Information on use of plants for management of malaria was collected using semi-structured questionnaires (Appendix 2). The participants were asked to provide information on how they diagnosed malaria, plants used to manage malaria and how they prepared the medicines prior to administration. Participants were requested to identify some of the plants they used (if available within vicinity and through

ethnobotanical expeditions), describe and demonstrate how they prepared these medicines. Thereafter, the plants were collected with the help of a taxonomist and voucher specimens prepared and preserved at the university of Nairobi herbarium under the numbers NMA2015/1-42.

2.2.6 Quality assurance

A preliminary study was carried out to determine the suitability of the data collecting tool. The findings of this study were used to improve the tool. A research assistant was trained before induction into the study. Note taking for all interviews was carried out by two people. The notes were entered in the questionnaire within 24 hours after the interview to avoid any loss of data. All data entries were double checked against the original information.

2.2.7 Data management and analysis

The information was collected by taking notes and filling in questionnaires during the interviews. The information was transferred to Microsoft word 2013 and Excel 2013 sheets in computer for analysis. All the filled in questionnaires were filed and kept under lock and key. The soft copies on computer were backed up every 24 hours using a hard disk and flash disc. The mentioned or identified plants were collected with the help of a taxonomist and voucher specimens prepared and preserved at the University of Nairobi herbarium. Photos of the identified plants were be taken using a camera. Data was checked for any errors and corrected appropriately before analysis and storage. The data

was analyzed using Microsoft Excel 2013 and presented in form of frequency distribution tables, pie charts, graphs and percentages.

2.2.8 Ethical considerations

Ethical approval for this study was obtained from the Kenyatta National Hospital/University of Nairobi Ethics and Research Committee (P186/03/2015) and as attached in appendix 3. The community gate keepers, who included village elders and church leaders, approved the study to be conducted within the local villages. The participants in this study were provided with information on the nature of study, benefits and risks involved. The participants were also educated on the need for prior informed consent (PIC) before signing the PIC forms (Appendix 1).

Data collected from all participants was held in high confidence. No identifiers were used on any of the recorded material. The researchers and the participants signed the assurance of anonymity and confidentiality statement which was included in the PIC form. The data collected was used only for the purposes of this study.

The data gathered in this study belongs to the Luhya community in Kakamega East sub-County and University of Nairobi. Any utilization of this intellectual property in any technological advancement is guided by provisions of the United Nations Framework Convention on Biodiversity at the Earth Summit in Rio de Janeiro, Brazil in 1992 and the Nagoya Protocol on the access to genetic resources.

2.3 Results and discussion

2.3.1 Socio-economic characteristics of respondents

A total of 21 respondents who utilized antimalarial plants either for self-medication or for treating others, were interviewed. The majority (57 %) of these respondents were male. The age ranged between 21 and 85 years, with a mean age 49 years of and median age of 56 years (Figure 2.2). Majority (60 %) were aged above 50 years. Usually, the older members of the society have experience in the practice of traditional medicine and pass it on to the younger generation. However, the younger herbalists are not usually accepted by the community as they are considered inexperienced in traditional medicine (Lambert *et al.*, 2011).

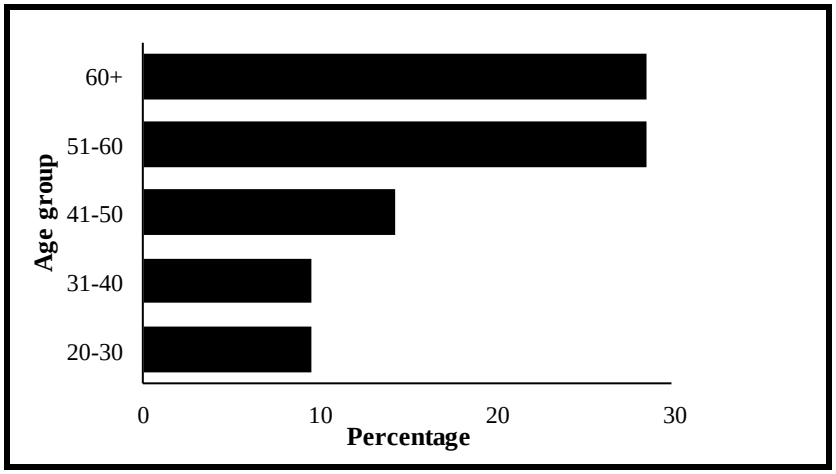


Figure 2. 2: Age groups of respondents

Most of the traditional medicine practitioners encountered in this study had a low level of education (Figure 2.3). The practice of traditional medicine has for a long time been restricted to the less educated since the more educated people view it as ancient and

outdated form of medicine that lacks scientific evidence. Most of the practitioners charged a small fee or no fee at all in managing the common diseases such as malaria since the plants are obtained locally. This was probably due to the fact that the patients came from the practitioner's close relatives or neighbors, therefore the treatment was considered a social responsibility. The practice of traditional medicine in this community was therefore not considered as a source of livelihood.

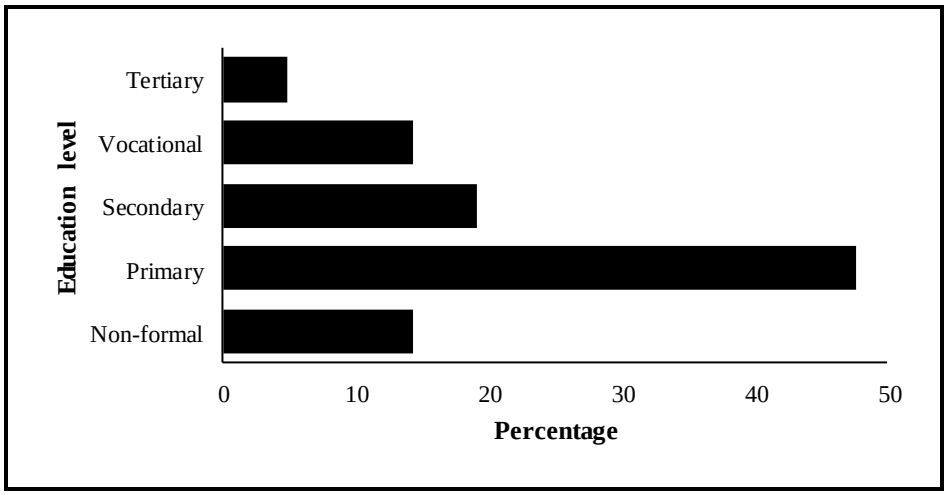


Figure 2. 3: Education level of respondents

2.3.2 Source of ethnomedical knowledge

Most (48 %) of the respondents had inherited the knowledge of traditional medicine through their family lineage as shown in Figure 2.4. However a relatively large proportion (43 %) had acquired the knowledge through other means such as interaction with known herbalists or by reading books about traditional medicine. Only one person had acquired the knowledge through training in a private traditional medicine facility within the County.

From this study, it was evident that more people with no family history of practice of traditional medicine were interested in the practice. This could be due to the high demand and increasing acceptability of traditional medicine as is the trend globally (Abdullahi, 2011). This may be the reason for the higher number (52 %) of people in this study practice herbalism even though they did not inherit the practice.

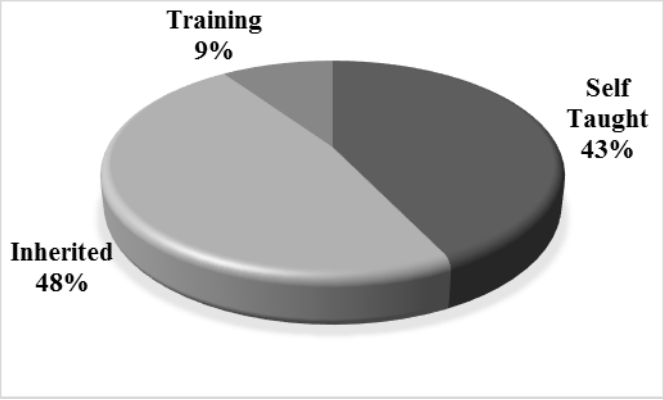


Figure 2. 4: The source of knowledge on use of traditional medicines

2.3.3 Plants reported for management of malaria in Kakamega East sub-County

The collected information on plants used in management of malaria in the Luhya community is summarized in Table 2.1.

Table 2. 1: Plants used in the management of malaria among the Luhya community, Kakamega East sub-County

Voucher number	Local name	Plant name	Family	Growth form	Plant Part Used	Mentions	Mode of preparation
NMA2015/01	Not available	*Justicia betonica*	Acanthaceae	Herb	Aerial	3	Pound, add cold water/Boil
NMA2015/02	*Busangula*	*Rhus natalensis*	Anacardiaceae	Herb	Leaves, Stem Bark	1	Boil in water
NMA2015/03	*Shikata/Achoka*	*Carissa edulis*	Apocynaceae	Shrub	Root Bark	1	Boil in water/Inhale steam
NMA2015/04	*Shituti*	*Acmella caulirhiza/ Spilanthes acmella*	Asteraceae	Herb	Aerial Part	2	Pound, add cold water
NMA2015/05	*Ing'oi*	*Microglossa pyrifolia*	Asteraceae	Shrub	Root, Leaves	2	Boil in water
NMA2015/06	*Masambu malulu/libinzo*	*Tithonia diversifolia*	Asteraceae	Shrub	Leaves	2	Pound, add cold water
NMA2015/07	*Musulilitsa*	*Vernonia amygdalina*	Asteraceae	Shrub	Leaves	4	Pound in cold water/ Boil
NMA2015/08	*Lusiola*	*Markhamia lutea*	Bignoniaceae	Tree	Stem Bark	2	Boil in water
NMA2015/09	*Mutsulio*	*Spathodea campanulata*	Bignoniaceae	Tree	Stem Bark	3	Boil without crushing
NMA2015/10	*Apachi*	*Warbugia ugandensis*	Canellaceae	Tree	Leaves, Stem Bark	3	Boil in water
NMA2015/11	Not available	*Cucumis aculeatus*	Curcurbitaceae	Climber	Leaves	1	Pound, add cold water
NMA2015/12	*Musutsu*	*Croton macrostachys*	Euphorbiaceae	Tree	Stem Bark	1	Boil in water
NMA2015/13	*Musenzeli*	*Albizia gummifera*	Fabaceae	Tree	Stem Bark	2	Boil without crushing
NMA2015/14	*Murembe*	*Erythrina abyssinica*	Fabaceae	Tree	Stem Bark	1	Boil in water
NMA2015/15	*Lubinu*	*Senna didmobotrya*	Fabaceae	Shrub	Leaves	1	Boil in water
NMA2015/16	*Imbindi*	*Senna occidentalis*	Fabaceae	Shrub	Root	1	Pound, add cold water
NMA2015/17	*Musila*	*Hanungana madagascariensis*	Hypericaceae	Tree	Stem Bark	1	Boil in water
NMA2015/18	*Imbuli yu mtakha*	*Ajuga integrifolia*	Lamiaceae	Herb	Aerial	5	Pound, add cold water
NMA2015/19	*Shiteng'oteng'o*	*Clerodendrum johnstonii*	Lamiaceae	Shrub	Leaves	1	Pounded in cold water/Boil
NMA2015/20	*Shisilangokho*	*Clerodendrum myricoides*	Lamiaceae	Shrub	Rootbark, Leaves	4	Boil in water/Roast
NMA2015/21	*Muvesemu*	*Fuerstia africana*	Lamiaceae	Herb	Aerial	4	Boiled or roasted
NMA2015/22	*Lumetsani*	*Leucas calostachys*	Lamiaceae	Herb	Aerial	4	Pound, add cold water/Boil/Steam
NMA2015/23	*M'monyi*	*Ocimum kilimandscharicum*	Lamiaceae	Herb	Aerial	1	Inhale steam
NMA2015/24	*Shilokha*	*Prectranthus barbatus*	Lamiaceae	Shrub	Leaves	2	Chew bud/boil in water

Voucher number	Local name	Plant name	Family	Growth form	Plant Part Used	Mentions	Mode of preparation
NMA2015/25	*Muarubaini*	*Melia azedarach*	Meliaceae	Tree	Leaves, Stem Bark	10	Boil in water
NMA2015/26	*Munyama*	*Trichelia emetica*	Meliaceae	Tree	Stem Bark	1	Boil in water
NMA2015/27	*Mukoye*	*Cissampelos mucronata*	Menispermaceae	Climber	Root	1	Chewing
NMA2015/28	*Mutoto*	*Ficus thonningii*	Moraceae	Tree	Stem Bark Root	2	Boil in water
NMA2015/29	*Mushevesheve*	*Maesa lanceolata*	Myrsinaceae	Tree	Bark, Stem Bark	1	Boil in water
NMA2015/30	Not available	*Flueggea virosa*	Phyllanthaceae	Shrub	Aerial	1	Boil in water
NMA2015/31	Not available	*Phyllanthus sepialis*	Phyllanthaceae	Shrub	Leaves	1	Boil in water
NMA2015/32	*M'monyo/Mkungune*	*Pittosporum manii syn P. viridiflorum*	Pittosporaceae	Shrub	Leaves, Stem Bark	1	Boil in water
NMA2015/33	*Shikachi*	*Rumex abyssinicus*	Polygonaceae	Herb	Leaves	2	Pound, add cold water
NMA2015/34	*Alukhava*	*Rumex steudelii*	Polygonaceae	Herb	Root	2	Pound, add cold water
NMA2015/35	*Butunduli*	*Rubus pinnatus*	Rosaceae	Shrub	Leaves, Fruits	1	Pounded in cold water/chew
NMA2015/36	*Shihunya bukundu*	*Clausena anisata*	Rutaceae	Shrub	Leaves	1	Boil in water
NMA2015/37	*Shikuma*	*Zanthoxylum gilletti*	Rutaceae	Tree	Stem Bark	4	Boil in water
NMA2015/38	*Mayengo*	*Physalis peruviana*	Solanaceae	Shrub	Leaves	1	Inhale steam
NMA2015/39	*Indalandalu*	*Solanum incanum*	Solanaceae	Shrub	Root	1	Pound, add cold water
NMA2015/40	*Shimenenwa*	*Lantana trifolia*	Verbenaceae	Shrub	Leaves	2	Boil in water/steam
NMA2015/41	*Muhoko*	*Rhoicissus tridentata*	Vitaceae	Climber	Root	1	Boil in water
NMA2015/42	*Shikakha*	*Aloe vera*	Xanthorrhoeaceae	Herb	Leaves	9	Boil in water

A total of 42 plant species belonging to 40 genera within 24 families were identified. A large proportion of these plants were from the Lamiaceae (18 %), Fabaceae (9 %), and Astaraceae (9 %) families as illustrated in Figure 2.5.

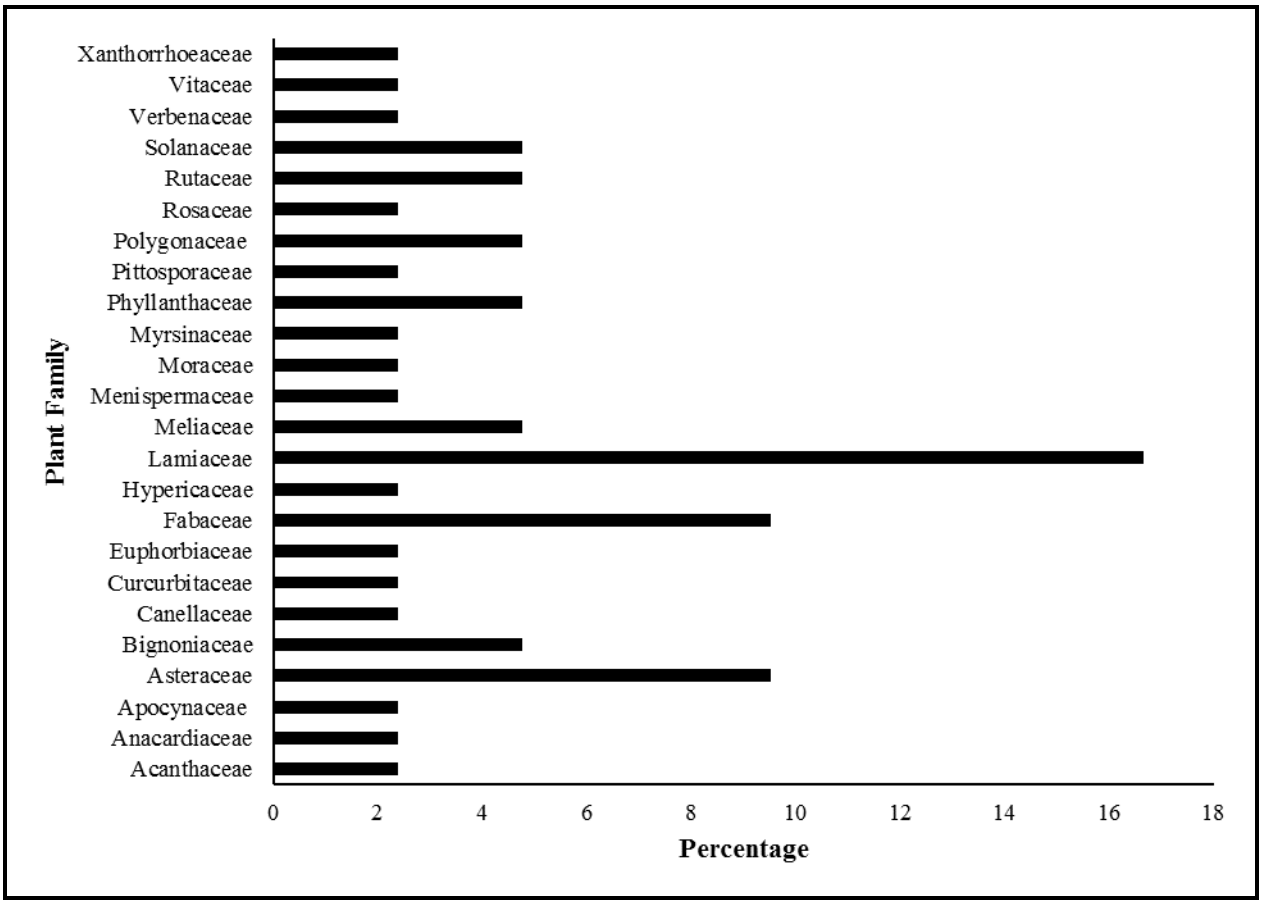


Figure 2. 5: Frequency of use of plant families in management of malaria in Kakamega East sub-County.

The Lamiaceae (Labiatae) family was the most common family used in the management of malaria in Kakamega East sub-County. The family is distributed throughout the world and known for its aromatic and medicinal properties. The family was also the most utilized among the Sidama people of Boricha District in Ethiopia in a similar study (Asnake *et al.*, 2016) and ranked second among plant families used in the Lesser Himalayas communities in Pakistan (Shah *et al.*, 2014). This is a popular family in the treatment of malaria in traditional medicine. Past phytochemical studies into this family

reveal that it is rich in terpenoids, which contributes to its aromatic properties. Some of these terpenoids have demonstrated antiplasmodial activities such the ajugarins (diterpene), hosloppones (diterpene) and ferruginol (monoterpene) from *Ajuga integrifolia*, *Hoslundia opposita* and *Fuerstia africana* respectively (Ntie-Kang *et al.*, 2014; Shadrack *et al.*, 2016).

Most of the identified plants were shrubs (42%) and trees (27 %) while the rest were climbers (24%) and herbs (7 %). Shrubs and herbs are usually the most utilized forms of traditional medicine due to ease of availability. The eight commonly cited plants in this study are shown in Figure 2.6.

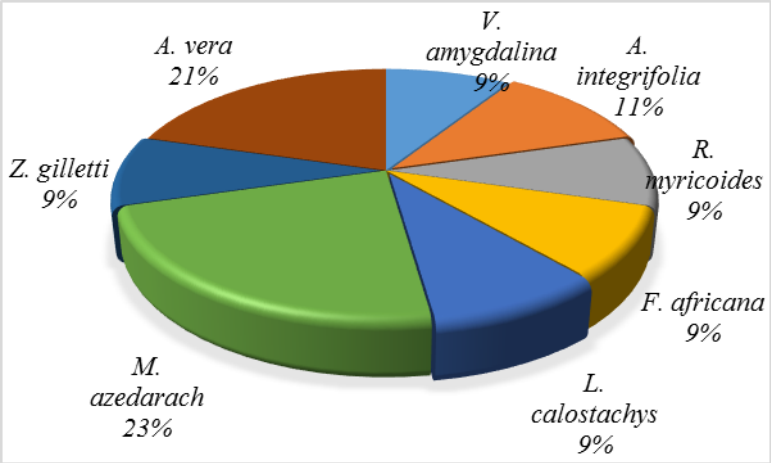


Figure 2. 6: Most commonly used antimalarial plants for management of malaria in Kakamega East sub-County

The commonly utilized plants for the management of malaria in Kakamega East sub-County are well known antimalarial plants (Gitua *et al.*, 2012; Hayelom *et al.*, 2012; Qureshi *et al.*, 2016). In fact, some of them have been extensively studied and exhibit

good antiplasmodial activities with identified active phytochemicals. For example, nitidine, a compound isolated from *Z. gilletti* is a potential antimalarial lead compound (Bouquet *et al.*, 2012; Muganga *et al.*, 2014). This shows that the traditional medical practitioners in this community have a good understanding of antimalarial plants.

Most of the identified plants had a local Luhya name *(Isukha* or *Kabras* dialects) among the local communities in Kakamega East sub-County. However four of the plants could not be identified by their local names. These plants may have been introduced into the region. For example, *Justicia betonica* L. was referred to as the dark "*Imbuli yu mutakha*" which also refers to *Ajuga integrifolia*. Use of similar names for *Justicia betonica* L. and *Ajuga integrifolia* Buch-Ham. could be due to the similar use of both plants for malaria management or due to their bitter taste. However, the 2 plants have very different morphological characteristics. On the other hand *Cucumis aculeatus* was referred to as *"Khaseveve"* due to its leaves having morphological similarity to a pumpkin plant's leaves which is used as a vegetable in this community. Pumpkin leaves are known as *"Liseveve"* in the *Isukha* dialects of Luhya language.

The lack of names for some of these plants may be explained by theory of ecological succession. In this theory, changes in the living organisms within a given ecology are likely to occur over a period of time. This results in emergence of new plants and disappearance of some plants due to the changes in ecology (Chang & Turner, 2019). The introduction of new plants may affects the naming of such plants by communities.

The naming of the new plants within the community may be based on the properties or morphological properties of known plants.

2.3.4 Plant parts used for management of malaria

The most commonly used plant parts were the leaves (36 %) and stem barks (25 %) while fruits were the least used plant parts (2%) used as shown in Figure 2.7.

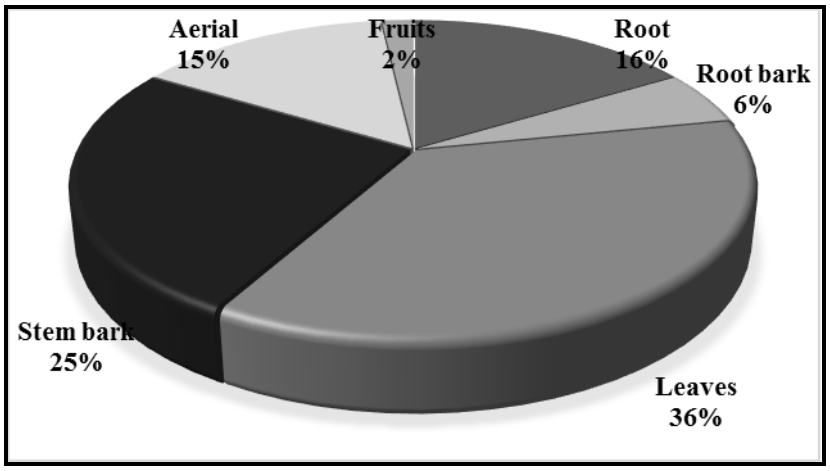


Figure 2. 7: Plant parts used for management of malaria in Kakamega East sub-County

The use of leaves, flowers and fruits is the least destructive approach and therefore support the conservation of medicinal plants. However, the use of roots, and whole plants are the most destructive leading to unsustainable harvesting of medicinal plants (Chen, S.-L. *et al.*, 2016). Similarly, the use of stem barks can lead to destruction and eventual depletion of medicinal plants. Some strategies have been employed in the preservation of medicinal plants. For example, proper procedures for bark harvesting can be utilizes to

preserve the plants. Another strategy employed in conservation of plant resources includes the use of alternative renewable plant parts such as leaves instead of roots. Indeed, some studies have shown that the use of alternative parts have yielded comparable chemical and biological activities (Jena *et al.*, 2017; Zschocke *et al.*, 2000).

2.3.5 Herbal medicines preparations

Various methods of preparing herbal medicines were encountered in the study. The most common preparations were decoctions (55 %) which were made by boiling plant material before use. Other methods included cold maceration with water (24 %), steaming (9 %), roasting (6 %) to obtain ash or chewing (6 %) as shown in Figure 2.8. In most of the cases, the plant material was harvested and freshly prepared just before use. However, in cases where the plants are not easily accessible, the plant material was preserved by drying under shade and kept for future use.

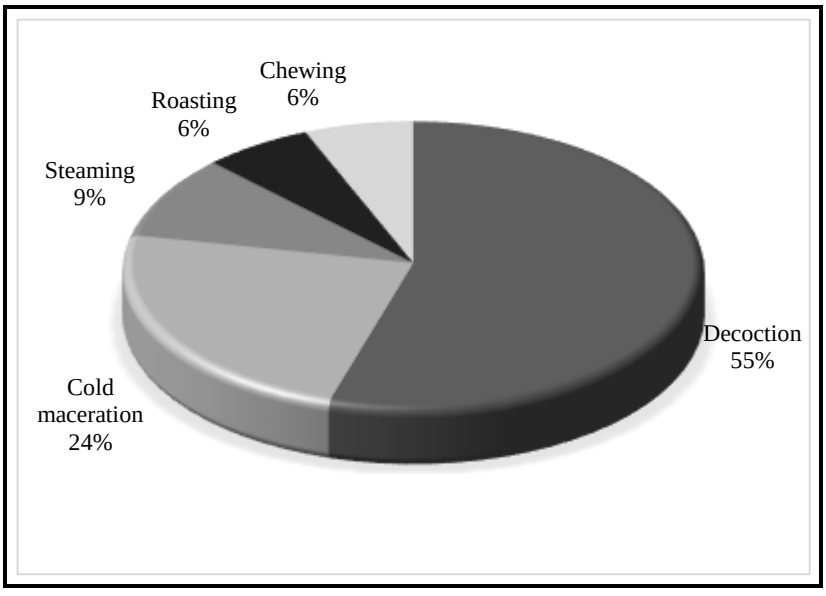


Figure 2. 8: Preparation methods for antimalarial herbal medicines in Kakamega East sub-County

These findings are similar to other ethnobotanical studies which show that decoction is the commonest method of preparation of herbal medicines (Abdul *et al.*, 2018; Tugume & Nyakoojo, 2019). Decoctions are preferred for hard plant materials such as roots and barks. Boiling enhances the extraction of the active phytochemicals from the plant material thereby increasing the potency of the preparation compared to cold macerations. However, in some instances, boiling may destroy the thermolabile components such as alkaloids. In some instances where boiling is unavoidable, controlled conditions such as use of Soxhlet extraction in the laboratory is utilized to avoid destruction of such compounds. Boiling herbal medicines may also help kill some of the micro-organisms that contaminate most herbal medicines (de Sousa Lima *et al.*, 2020; Van Vuuren *et al.*, 2014). Decoction preparations are not stable when stored over a long period of time unless a preservative is added (Kumadoh & Kwakye, 2017).

2.3.6 Sources of plant material

The herbal medicines used for malaria among the study population was mainly obtained from the wild (77 %) while only a small fraction was cultivated (23 %). Previous studies carried out in Kenya show that most of the herbal products are exclusively obtained from the wild which is unsustainable (McMullin *et al.*, 2012). The availability of wild herbal medicines is threatened by deforestation worldwide. It is estimated that at least one plant species is lost every 2 years worldwide (Chen, S.-L. *et al.*, 2016). The increasing demand of herbal medicines for commercialization has further increased pressure on the

dwindling wild sources (Hilonga *et al.*, 2019). Cultivation of medicinal plants is a viable alternative if the practice of traditional medicine is to be sustained for future generations.

2.4 Literature review on identified plants

The identified plants in this study have been used in many communities for the management of various ailments. Most of them are commonly used for the management of malaria and other febrile illnesses. A total of 38 out of the 42 reported plants have been tested for *in vivo* and/or *in vitro* antiplasmodial activities as summarized in Table 2.2.

Table 2. 2: Literature review of the plants used for management of malaria among the Luhya community of Kakamega East sub-County

Plant name	Traditional uses	*In vitro* and *in vivo* antimalarial activities	Identified antimalarial compounds
Justicia betonica L.	Lower cholesterol, paralysis, earaches, headaches, bruises diarrhea, vomiting, constipation, pain and inflammation and Malaria (Gangabhavani & Ravishankar, 2013)	Ether aerial parts extract had IC_{50} of 13.36 µg/ml (Bbosa *et al.*, 2013) MeOH aerial parts 69.6 µg/ml (Muregi *et al.*, 2003)	No reference
Searsia natalensis Bernh.ex C. Krauss	Malaria (Gathirwa *et al.*, 2011), diarrhea, HIV (Mugisha *et al.*, 2014)	$CHCl_3$ leaf extract had IC_{50} of 1.8 µg/ml	No reference
Carissa edulis L.	Sickle cell anemia, fever, epilepsy, pain (Ya'u *et al.*, 2013), malaria (Orwa *et al.*, 2007)	DCM, stems extract had IC_{50} of 33 µg/ml (Clarkson *et al.*, 2004)	No reference
Spathodea campanulata P. Beauv.	Malaria, herpes, fever, diabetes, dysentery, ulcers, HIV (Lim, 2013)	Ethanolic leaf extract had IC_{50} >68 µg/ml (Rangasamy *et al.*, 2008)	Lapachol (Ntie-Kang *et al.*, 2014)
Markhamia lutea (Benth.) K.Schum.	Malaria (Lacroix *et al.*, 2009)Anemia, diarrhea, microbial and parasitic infections (Ali *et al.*, 2015)	EtOAc, Leaf extract exhibited 70 % parasite suppression (Lacroix *et al.*, 2011)	Musambins A-C & Musambiosides A-C(Lacroix *et al.*, 2009)

Plant name	**Traditional uses**	***In vitro* and *in vivo* antimalarial activities**	**Identified antimalarial compounds**
Warbugia ugandensis Sprague	Worms, fever, gonorrhea, syphilis (Lacroix *et al.*, 2011) (Were *et al.*, 2010)	DCM, stem bark extract of IC_{50} of 8 µg/ml (Wube *et al.*, 2008) with 69% parasite inhibition (Were *et al.*, 2010)	Coloratane sesquiterpenes.(Onguén *et al.*, 2013b) (Onguén *et al.*, 2013a)
Vernonia amygdalina Del.	Febrifuge, vermifuge, laxative, malaria, wounds and as appetizer (Ifeoma & Chukwunonso, 2011)	Ethanolic leaf extract IC_{50} of 9.83µg/ml (Omoregie *et al.*, 2011) *In vivo* parasite suppression of between 57.2–72.7 % in combination with CQ (Challand & Willcox, 2009)	Vernolide, Vernodalin, Vernodalol & Hydroxyvernolide (Onguén *et al.*, 2013b)
Tithonia diversifolia (Hemsl.) A. Gray	Diabetes mellitus, sore throat, menstrual pain, malaria, wounds (Owoyele *et al.*, 2004)	ether extract of aerial parts had IC_{50} of 0.75 µg/ ml(Goffin *et al.*, 2002) whereas the methanolic extract had 74% parasitemia suppression (Oyewole *et al.*, 2008)	Tagitinin C (Onguén *et al.*, 2013b)
Acmella caulirhiza Del.	Toothache, throat and gum infections, dysentery, rheumatism, immune booster and malaria (Grubben & Denton, 2004)	DCM extract had IC_{50} < 10 µg/ml (Owuor *et al.*, 2012)	No reference
Microglossa pyrifolia (Lam.) Kuntze	Malaria (Jeruto *et al.*, 2011)Headache, cough, flu, cleansing airway (Moshi *et al.*, 2012)	DCM, Leaf extract had IC_{50} of <15 µg/ml (Muganga *et al.*, 2014)	Diterpeness (Kohler *et al.*, 2002)
Cucumis aculeatus Cogn	Diarrhea, leprosy, migraines, wounds, gonorrhea (Engels *et al.*, 1991), malaria (Njoroge & Bussmann, 2006)	Aqueous fruit extract had IC_{50} of >30 µg/ml (Gakunju *et al.*, 1995)	No reference
Croton macrostachuys Hochst. ex Del.	Diabetes, dysentery, wounds, malaria, purgative, stomachache (Gelaw *et al.*, 2012)	DCM, leaf extract IC_{50} of 2µg/ml (Owuor *et al.*, 2012)	No reference
Harungana madagascariensis Lam. ex Poir.	Anemia, malaria (Iwalewa *et al.*, 2008), fever, nephrosis, jaundice, gastrointestinal disorders (Okoli *et al.*, 2002)	Ethanolic stem bark extract had IC_{50} of <0.5 µg/ml and showed between 28.6–44.8% Parasite suppression (Iwalewa *et al.*, 2008)	Bazouanthrone, Feruginin A, Harunganin, Harunganol A &B (Ntie-Kang *et al.*, 2014)
Rotheca myricoides (Hochst.) Steane & Mabb.	Measles, malaria, asthma, wounds, gonorrhea, rabies and eye disease (Hayelom *et al.*, 2012)	Methanolic leaf extract, IC_{50} of 9.51-10.56 µg/ml and 82 % parasite suppression at 600mg/kg (Deressa *et al.*, 2010)	No reference

Plant name	Traditional uses	*In vitro* and *in vivo* antimalarial activities	Identified antimalarial compounds
Leucas calostachys Oliv.	Colds, headache (Okello, S. V. *et al.*, 2010) malaria (Nyambati *et al.*, 2013)	Aqueous whole plant extract had IC_{50} of 0.8µg/ml with parasite inhibition of 3.5-5.2 % (Nyambati *et al.*, 2013)	No reference
Ocimum kilimandscharicum Gürke	Colds, cough, analgesic, sedative, diarrhea, measles (Soni *et al.*, 2012), malaria (Owuor *et al.*, 2012)	DCM, Leaves and twigs had IC_{50} of < 1.5 (Owuor *et al.*, 2012)	No reference
Fuerstia africana T.C.E.Fr.	Eye ailments, toothache (Kipkore *et al.*, 2014) malaria (Muganga *et al.*, 2010)	Pet-ether extract of aerial parts had IC_{50} of 1.5 µg/ml (Kigondu, Matu, *et al.*, 2011) Methanolic leaf extract had IC_{50} <15 µg/ml with > 70 % parasite suppression (Muganga *et al.*, 2014)	Ferruginol (Onguén *et al.*, 2013b)
Clerodendrum johnstonii Oliv.	Abscess, hernia (Quattrocchi, 2012), malaria (Jeruto *et al.*, 2011; Pascaline *et al.*, 2011)	No reference	No reference
Plectranthus barbatus Andrews	Gastritis, respiratory disorders, cough, analgesic, hypertension, stomachache, epilepsy (Fernandes *et al.*, 2012) malaria, break fevers (Al-Musayeib *et al.*, 2012)	Methanolic extract had IC_{50} of 6.5 µg/ml, (Al-Musayeib *et al.*, 2012)	No reference
Ajuga integrifolia Buch.-Ham	Vermifuge, toothache, hypertension, stomachache fever (Hailu & Engidawork, 2014), malaria (Gitua *et al.*, 2012)	Aqueous leaf extract exhibited 90% parasite suppression (Gitua *et al.*, 2012)	Ajugarin-1(Onguén *et al.*, 2013b) ergosterol-5,8-endoperoxide (Ntie-Kang *et al.*, 2014)
Albizia gummifera (J.F.Gmel.) C.A.Sm.	Malaria, bacterial infections, skin diseases, stomachache (Kokila *et al.*, 2013)	The alkaloidal fraction had IC_{50} of 0.06 µg/ml while spermine alkaloid exhibited parasite suppression of 43 to 72% (Rukunga *et al.*, 2007)	Spermine alkaloids *(*Rukunga *et al.*, 2007)
Senna occidentalis (L.) Link	Malaria , vermifuge, analgesic, laxative, hepatoprotective, diuretic and febrifuge (Silva *et al.*, 2011)	EtOH root bark extracts had IC_{50}<3 µg/ml whereas 200 mg/kg of EtOH & DCM extracts exhibited >60% parasitaemia suppression.(Tona *et al.*, 2001)	Quinones (Kayembe *et al.*, 2010)
Senna didmobotrya (Fresen.) H.S.Irwin & Barneby	Intestinal worms, skin diseases, jaundice, veneral diseases, malaria, fever (Nagappan, 2012)	DCM/MeOH. Twigs extract had IC_{50} of 9.5 µg/ml (Clarkson *et al.*, 2004)	No reference

Plant name	**Traditional uses**	***In vitro* and *in vivo* antimalarial activities**	**Identified antimalarial compounds**
Erythrina abyssinica DC.	Abortion , cough, malaria (Lacroix *et al.*, 2011)	EtOAc, Bark extract showed 83 % parasite suppression (Lacroix *et al.*, 2011)	5- deoxyabyssinin II and homobutein (Ntie-Kang *et al.*, 2014)
Trichilia emetica Vahl	Diabetes, hypertension (Konaté *et al.*, 2014), malaria (Diarra *et al.*, 2015)	DCM/MeOH (1:1), leaves & twigs extract had of IC_{50} of 3.5 µg/ml (Clarkson *et al.*, 2004)	Kurubasch aldehyde (Bero *et al.*, 2009)
Melia azedarach L.	Hepatoprotective, malaria, skin diseases, ulcers, fever, vermifuge, asthma (Qureshi *et al.*, 2016)	DCM, Leaf extract had IC_{50} of 28 µg/ml (Lusakibanza *et al.*, 2010)	No reference
Cissampelos mucronata A.Rich	Antisnake venom, veneral diseases, malaria, menstrual disorders, wounds, febrifuge (Nondo *et al.*, 2011)	EtOAc root extract had IC_{50} <3.91 with active compound, curine IC_{50} of 0.24 (Omole, 2012)	Curine (Ndiege, 2011)
Ficus thonningii Blume	Malaria (Falade *et al.*, 2014), Diabetes, diarrhea, mental illness, gonorrhea, urinary tract infections (Dangarembizi *et al.*, 2013)	Hexane, leaf extract IC_{50} of 10.4 µg/ml (Falade *et al.*, 2014)	No reference
Flueggea virosa (Roxb. ex Willd.) Royle	Fever, stomachache, rheumatism, pneumonia, epilepsy, body pains (Magaji *et al.*, 2007) malaria (Al-Rehaily *et al.*, 2015)	MeOH/H_2O leaves extract had IC_{50} of 7.8 (Willcox *et al.*, 2011a) Alkaloids: securinine and viroallosecurinine had IC_{50} values of 2.7 and 2.9 µg/mL respectively (Al-Rehaily *et al.*, 2015)	Securinine and Viroallosecurinine (Al-Rehaily *et al.*, 2015)
Phyllanthus sepialis Müll.Arg.	Tonic in pregnancy (PROTA, 2008) dental hygiene (Bussmann *et al.*, 2006)	No reference	No reference
Pittosporum viridiflorum Sims	Chest complains, purgative, male impotence, asthma, coughs (Dold & Cocks, 2012) malaria (Nyongbela *et al.*, 2013)	DCM whole plant extract had IC_{50} of 3 µg/ml (Clarkson *et al.*, 2004)	Triterpenoid estersaponin, (Nyongbela *et al.*, 2013) Pittoviridoside (saponins) (Seo *et al.*, 2002)
Rumex abyssinicus Jacq.	Wounds, liver diseases, malaria, gonorrhea (Mulisa *et al.*, 2015)	DCM, root extract had IC_{50} <15 µg/ml, (Muganga *et al.*, 2014)	No reference
Rumex steudelii Hochst.ex A. Rich	Antifertility, tonsillitis, wounds, eczema, hemorrhoids, leprosy (Solomon *et al.*, 2010) (Gebrie *et al.*, 2005)	No reference	No reference

Plant name	Traditional uses	*In vitro* and *in vivo* antimalarial activities	Identified antimalarial compounds
Maesa lanceolata Forssk	Malaria	$CHCl_3$ leaf extract IC_{50} of 1.6 µg/ml (Katuura *et al.*, 2007) DCM/MeOH (1:1) twigs extract IC_{50} of 5.9 µg/ml (Clarkson *et al.*, 2004)	No reference
Rubus pinnatus Wild	Bleeding gums, expectorant, demulcent, diarrhea (Quattrocchi, 2012), malaria (Lacroix *et al.*, 2011)	EtOAc, leaf extract exhibited 20 % parasite suppression (Lacroix *et al.*, 2011)	No reference
Zanthoxylum gilletii (De Wild.) P.G.Waterman	Stomachache, gonorrhea, back pain, urinogenital infections (Gaya *et al.*, 2013).malaria (Masinde, 2014)	DCM/MeOH (1:1) stem bark extract had IC_{50} of 2.52, 1.48 and 1.43 µg/ml against W2, D6 and 3D7 strains. (Masinde, 2014)	Nitidine (Muganga *et al.*, 2014) Seasamine ; 8-acetonyldihydrochelerythrine (Masinde, 2014)
Clausena anisata (Willd.) Hook.f. ex Benth.	Vermifuge, febrifuge, measles, hypertension, malaria, analgesic, rheumatism (Okokon *et al.*, 2012)	DCM, root extract had IC_{50} of 10 µg/ml (Clarkson *et al.*, 2004)	No reference
Physalis peruviana L.	Malaria, rheumatism, hepatitis, dermatitis, diuretic (Ramadan *et al.*, 2015)	No reference	No reference
Solanum incanum L.	Pneumonia, liver pain, headache, toothache, stomachache, sore throat (PROTA, 2008), malaria (Nguta *et al.*, 2010)	$CHCl_3$/MeOH, leaf extract showed 31 % parasite suppression (Murithi *et al.*, 2014)	No reference
Lantana trifolia L.	Common cold, asthma, epilepsy, madness, childhood cerebral malaria, sickle cell anemia.(Nalubega *et al.*, 2013)	Pet-ether aerial parts extract had IC_{50} of 13.2 µg/ml	No reference
Rhoicissus tridentata (L.f.) Wild & R.B.Drumm.	Dysmenorrhea, uterotonic, indigestion, pregnancy &childbirth. (Mukundi *et al.*, 2015), malaria (Gakunju *et al.*, 1995)	Aqueous root extract had IC_{50} > 40 µg/ml (Gakunju *et al.*, 1995)	No reference
Aloe species	Colds, malaria, stomachache, anemia (PROTA, 2008)	Ether leaf extract *of A.dawei*, IC_{50} of 7.9 µg/ml (Bbosa *et al.*, 2013)	Aloin A/B, microdontin A/B (Hintsa *et al.*, 2019)

$CHCl_3$= Chloroform, DCM =Dichloromethane, EtOAc= Ethyl Acetate, MeOH= methanol, Pet-ether= Petroleum ether

In vitro antiplasmodial activity is classified as high (IC50 <5 μg/ml), promising (5<IC50 <15 μg/ml), moderate (15<IC50 <50 μg/ml) and inactive (IC50 >50 μg/ml) (Lekana-Douki *et al*., 2011). Of the identified plants, 14 have high antiplasmodial activity with *Albizia gummifera*, *Leucas calostachys*, *Tithonia diversifolia* and *Hanungana madagascariensis* having the significant antiplasmodial activity of <1 μg/ml.

The *in-vitro* antiplasmodial activities of these plants was compared to the relative frequency of citation (RFC) of various plants as antimalarial remedies. The RFC is used to determine the level of agreement between different practitioners on use of herbal medicine for a given disease. It indicates the importance placed on certain plants for use in given disease states within the community. RFC is often useful in selection of plants for further pharmacological and phytochemical studies. The RFC was calculated using standard methods (Khan *et al.*, 2014) as;

$$\mathrm{RFC} = \frac{FC}{N} \; ; \; 0 < RFC < 1$$

Where FC is frequency of citation of a given plant species and N is total number of informants in the study.

In this study, RFC value of less than 0.09 was considered low whereas RFC value above 0.09 was considered high. All the plants with reported *in-vitro* antiplasmodial activity were further classified according to their level of antiplasmodial activity and their relative frequency of citation based on the ethnobotanical survey as shown in Figure 2.9.

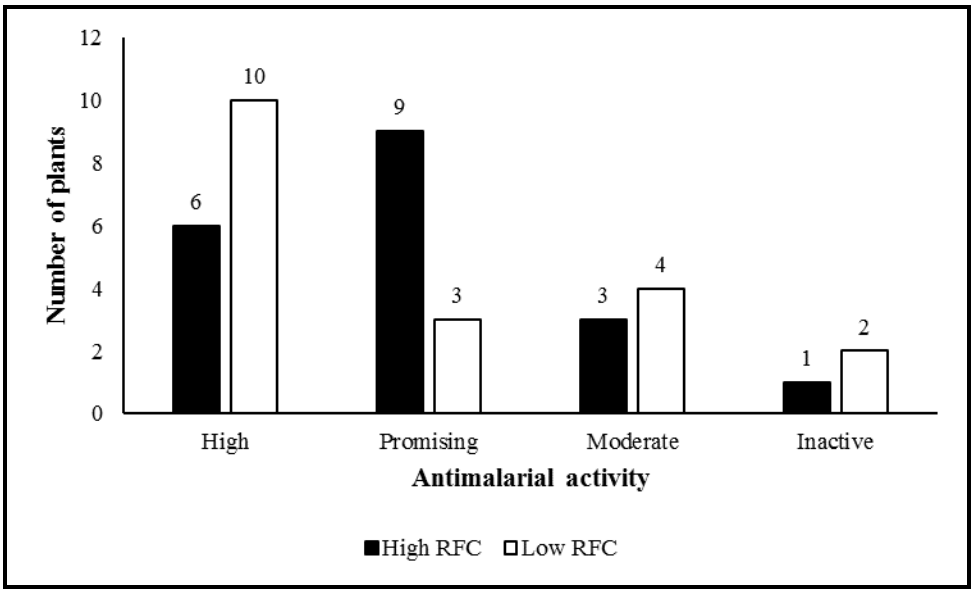


Figure 2. 9: Comparison of reported antimalarial activity and frequency of use of antimalarial plants in Kakamega East sub-County

From this analysis, most of the plants with low RFC had a higher *in-vitro* antiplasmodial activity compared to those highly cited in the high antimalarial category. However, most of the plants with promising *in-vitro* antiplasmodial activity also had high RFC values. This indicates that most herbal practitioners use plants with known antimalarial activities but not necessarily the most potent ones. Therefore, that frequency of citation is not necessarily a pointer to the potency of the plant against diseases.

However, it should be noted that although some plants demonstrate *in vitro* antiplasmodial activity, this may not necessarily translate to *in vivo* activity. Other factors such as bioavailability and toxicity have to be considered. For example, although *Tithonia diversifolia* has promising *in-vitro* antiplasmodial activity; however, it was found to be toxic to the liver and kidney, therefore limiting its widespread use in the

management of malaria (Elufioye et al., 2009). However, in most cases, the selectivity index (SI) of most antimalarial plants is high therefore they are utilized with minimal toxicities (Kigondu, Rukunga, *et al.*, 2011).

2.5 Conclusion

This study led to the documentation of antimalarial plants used among the Luhya community in Kakamega East sub-County. Most of the identified plants have demonstrated *in vitro* antiplasmodial properties in previous studies. This indicates the herbal practitioners in this community have a good knowledge of antimalarial plants used in traditional medicine. These plants should be studied in the search for newer antimalarial drugs. More ethnobotanical studies should also be conducted among the Luhya community to document plants used in management of other prevalent diseases.

The work in this chapter is published in the Journal of Ethnopharmacology ((Mukungu *et al.*, 2016) and part of the paper is attached as Appendix 5.

CHAPTER THREE

ANTIPLASMODIAL ACTIVITIES OF SELECTED ANTIMALARIAL PLANTS.

3.1 Introduction

Based on the ethnobotanical survey and literature review, two plants; *Leucas calostachys* and *Justicia betonica* were selected for further studies. The two plants demonstrated *in vitro* antiplasmodial activities in previous studies (Bbosa *et al.*, 2013; Nyambati *et al.*, 2013). However, no phytochemicals with antimalarial properties have been isolated from these plants. The description, use in traditional medicine and reported biological activities for *Leucas calostachys* Oliv. and *Justicia betonica* L. is reported hereafter.

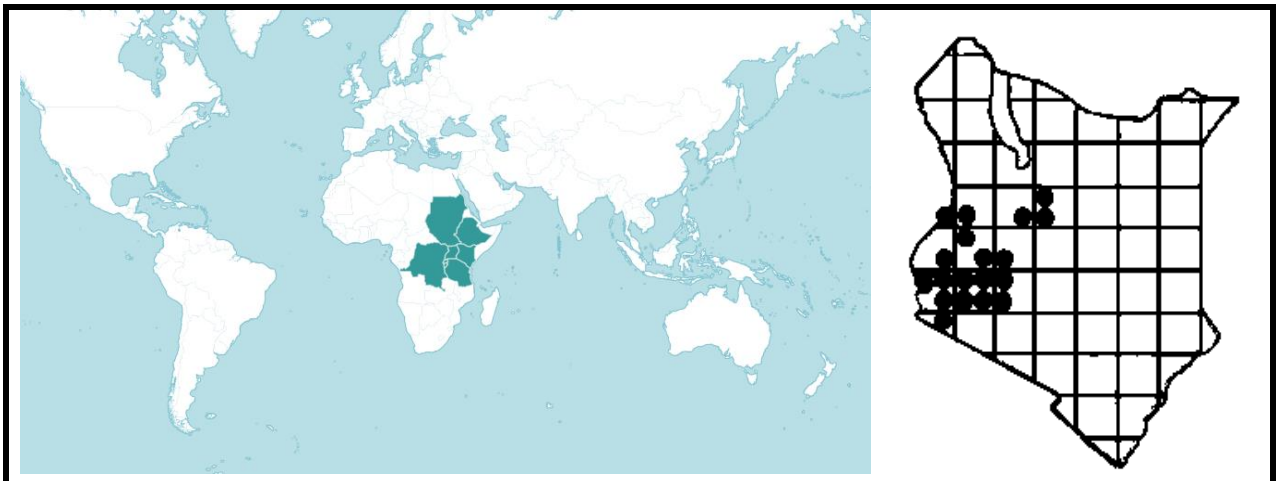
3.2 *Leucas calostachys*

3.2.1 Taxonomy, description and distribution of *Leucas calostachys* Oliv. (Lamiaceae)

Leucas calostachys Oliv. belongs to the flowering plant family Lamiaceae (Labiatae or mint family). The Lamiaceae family consists of over 200 genera with over 7000 plant species distributed throughout the world. The plants in the family, often herbs or shrubs, are characterised by square stalks, simple opposite leaves and aromatic smell. Many plants in the family such as mint, peppermint, thyme, lavender, basil, and rosemary are used for culinary purposes due to their spicy qualities (Ramasubramania, 2012).

In Kenya, this family is represented by several genera such as *Ocimum, Ajuga, Mentha, Thymus, Leucas*, and *Precranthus* (Agnew & Agnew, 1994). Many of these are utilized in traditional medicines for management of various diseases such as colds, fever, infections and malaria (Githinji & Kokwaro, 1993; Okach *et al.*, 2013). During the current ethnobotanical study among the Luhya community, plants in the family were the most commonly used in the management of malaria. A total of seven (7) plants from this family were reported for use in malaria management. Most of them have demonstrated *in vitro* antiplasmodial activities in previous studies (Mukungu *et al.*, 2016). Among them, compounds with antimalarial activity have been isolated from *Ajuga integrifolia* and *Fuerstia africana* (Kuria *et al.*, 2001; Ntie-Kang *et al.*, 2014). Although *Leucas calostachys* was among the commonly used plants in malaria management, at the time of this study, no phytochemicals had been isolated.

The genus *Leucas* comprises of at least 130 plant species distributed mainly in Africa and Eastern Asia. *Leucas calostachys* is endemic to Eastern Africa within the countries of Burundi, Ethiopia, Kenya, Rwanda, Sudan, Tanzania, Uganda and Zaire. It is found in the bushy or wooded grassland. In Kenya, the plant is distributed in the western region and towards the central part of Kenya as shown in Figure 3.1. Some of its local names in Kenya include; *Lumetsani* (Luhya), *Bware* (Luo), *Muhuithia mweru* (Kikuyu) *Ngechepchat* (Kipsigis) and *Engejenii* (Maasai) (Beentje *et al.*, 1994).



(a) (b)

Figure 3. 1: Distribution of *Leucas calostachys* worldwide (a) and in Kenya (b)

(Beentje *et al.*, 1994)

Leucas calostachys is a small erect shrub with almost square pubescent stalks. It has obovate, opposite petiolate leaves with many hairs on the upper surface. The leaf margins are crenated and the surface has prominent venation (Paton *et al.*, 2009) as shown in Figure 3.2.

Figure 3. 2: *Leucas calostachys* Plant

(Photo by NAM, October 2017 at Ileho, Kakamega East, sub-County)

3.2.2 Uses of *Leucas calostachys* in traditional medicine

The leaves, roots or entire aerial parts of *Leucas calostachys* are used in traditional medicine for management various diseases. The plant is used in management of gastro-intestinal disorders such as amoebiasis, diarrhea, stomachache, constipation, heartburn and peptic ulcers. It is also used in the treatment of respiratory disorders such as pneumonia, coughs, chest pain, flu and colds. Other disease conditions include wounds, kidney disorders, malaria, muscle pull, skin disorders, cancer and arthritis. It is also be used as a food substitute and in management of animal diseases such East Coast fever in cattle and poultry diseases (Agnew & Agnew, 1994; Chouhan & Singh, 2011; Jeruto *et*

al., 2011; Kigen *et al.*, 2014). It is the most commonly used plant in ethnomedicine among the Keiyo community of Elgeyo Marakwet County in Kenya (Kigen *et al.*, 2014).

3.2.3 Previous biological studies on *Leucas calostachys* Oliv.

Leucas calostachys has been tested for various pharmacological and biological activities including antiplasmodial, antimicrobial and analgesic potential.

Nyambati *et al.*, (2013) tested the *in vitro* antiplasmodial activity of methanolic and aqueous extracts of *L. calostachys* whole plant against *Plasmodium knowlesi* parasites. These extracts showed good antiplasmodial activity with inhibition concentrations (IC_{50}) of 3.45 µg/ml and 0.79 µg/ml respectively. Another study by Jeruto *et al.* (2015) showed poor activity within the the chloroform and methanol extracts, with IC_{50} values of 40.2 and 88.4 µg/ml respectively against chloroquine sensitive (D6) *Plasmodium falciparum* strains. Muregi *et al.* (2004) also tested various extracts of *L. calostachys* leaves on K39 chloroquine-sensitive *P.falciparum* parasites. The chloroform, hexane and methanol extracts showed moderate to poor activity of IC_{50} values of 36, 66 and 87 µg/ml respectively. The aqueous and ethylacetate extracts lacked activity.

Based on its use in management of various infections both in man and animals, *L calostachys* has been tested for antimicrobial activity. The methanol and acetone extracts of the leaves had moderate activity against methicillin resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacteria. The extracts lacked activity against *Bacillus*

cereus, E.coli and the fungus, *Candida albicans*. The methanol, acetone and aqueous leaf extracts were non- toxic against brine shrimp (Cherotich, 2015).

3.3 *Justicia betonica* L. (Acanthaceae)

3.3.1 Taxonomy, description and distribution

Justicia betonica, commonly referred to as White Shrimp plant or Squirrel's tail, is a member of the Acanthaceae family. This is one of the largest plant families found in the subtropical and tropical regions of the world with over 4300 species within over 430 genera. The genus *Justicia* is the largest in the family with over 600 species. It is native to Africa, Asia, New Caledonia, Southern and Northern America. *Justicia betonica* is found in most of the countries in Sub-Saharan Africa and Indian sub-continent as shown in the map in Figure 2.8. The plant grows well in damp places especially along waterways and it is known to be an invasive weed.

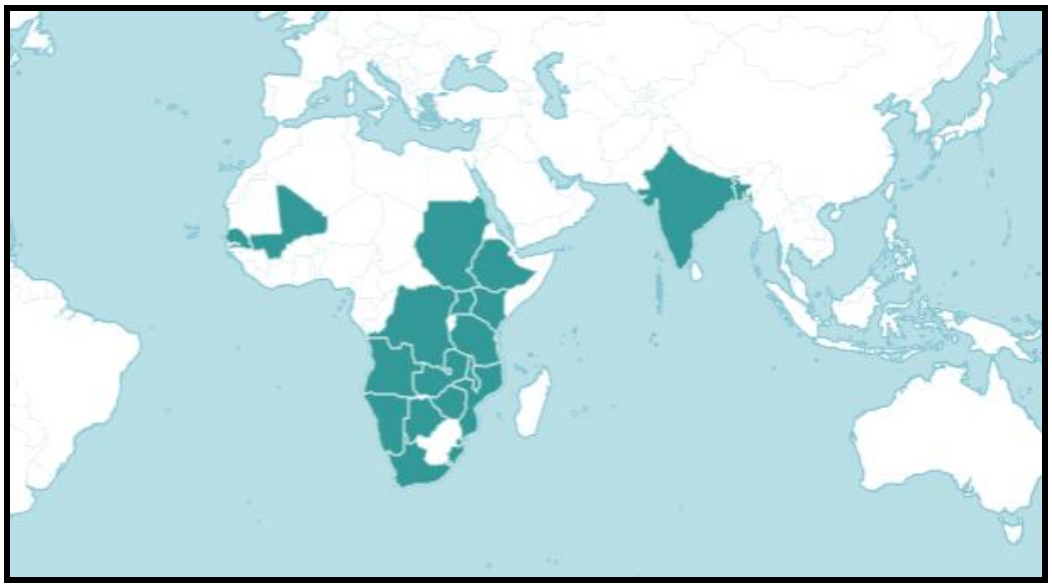


Figure 3. 3: World distribution of *Justicia betonica*
(Beentje *et al.*, 1994)

Justicia betonica is a shrub that that grows upto 2 m with several stems either erect or decumbent arising from the rhizome or taproots. They form a dense ground cover and the stems easily develop roots at the nodes when they come in contact with soil. It has glabrous leaves which are simple and oval to lanceolate in shape with wavy margins. They measure upto 14 cm long and 11 cm wide. It is dark green in color on the upper whereas the lower one is paler. They have a terminal inflorescences with characteristic lavender or purple flowers within variegated bracts (International Plant Names Index, 2018). The photo in Figure 3.4 shows the plant in its natural habitat.

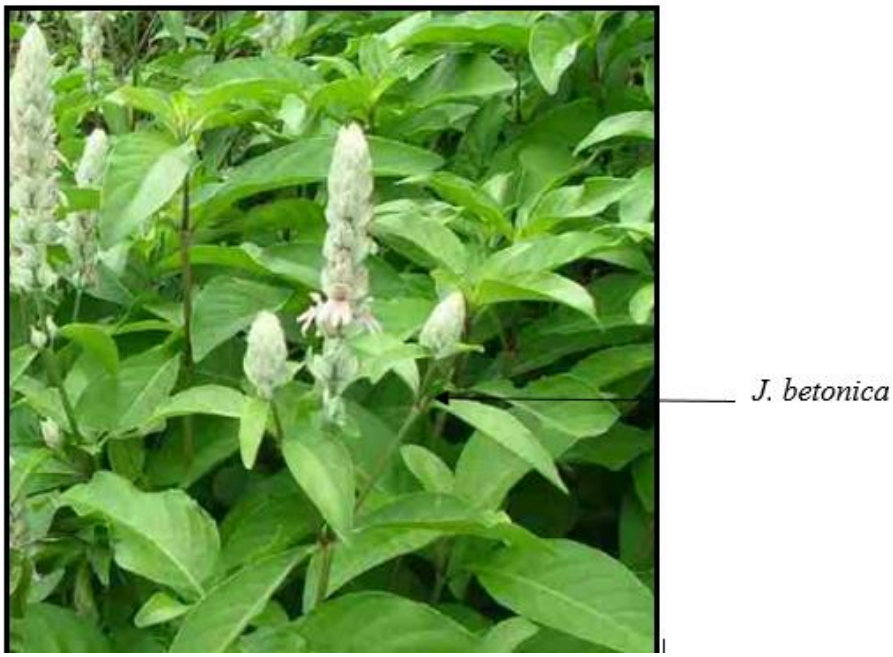


Figure 3. 4: *Justicia betonica* plant

(Photo: keyserver.lucidcentral.org)

3.3.2 Uses of *Justicia betonica* in traditional medicine

Justicia betonica is used as an ornamental plant, as food or in traditional medicine in Africa and the Indian subcontinent. In traditional medicine, it is used in management of snake bites (Bekalo *et al.*, 2009), malaria (Bbosa *et al.*, 2013), pain and inflammation, gastro-intestinal, (Gangabhavani & Ravishankar, 2013) genito-urinary and respiratory infections, and cancers (Jeruto *et al.*, 2011). It is also used as a dye for clothes in Uganda (Wanyama *et al.*, 2011).

3.3.3 Previous biological studies on *Justicia betonica*

Bbosa *et al.*, 2013 investigated the antiplasmodial activity of the ether extract of *J. betonica*. In the study, the ether extract was active with an IC_{50} value of 13.36 μg/ml.

Muregi *et al.*, 2004 tested the methanol extract of *Justicia betonica* and it demonstratedd a mild antiplasmodial activity with an IC_{50} value of 69.6 μg/ml.

The anecdotal use of *J. betonica* in many infections including wounds and skin infections prompted the study of its antimicrobial activity by some researchers. The non-polar extracts demonstrated moderate activity against *E. coli, Salmonella, streptococcus* and *Staphylococcus* species (Sasikumar *et al.*, 2007).

Many of the *Justicia* species are used in management of cancers in traditional medicine (Jeruto *et al.*, 2007; Ngbolua, 2019). *In vitro* studies of *Justicia betonica* demonstrated that the methanol and ethanol extracts are cytotoxic against human cervical carcinoma and hepatoma cells. The plant also inhibits platelet aggregation and has antioxidant, analgesic and anti-inflammatory effects (Corrêa & Alcântara, 2012).

3.4 Experimental

3.4.1 Plant material collection, authentication and preparation

The aerial parts of the *Leucas calostachys* and *Justicia betonica* were harvested from their natural habitat in Ileho, Kakamega East sub-County during the month of August 2017. They were identified by Mr. Patrick Mutiso, University of Nairobi botanist, and voucher specimens deposited at the University of Nairobi Herbarium, Chiromo under the codes *Leucas calostachys* (NMA2015/22) and *Justicia betonica* (NMA2015/01). The materials were air dried under ambient conditions. The dried plant materials were coarsely powdered and stored in air tight plastic containers until needed for the study.

3.4.2 *In vitro* antiplasmodial assay using microscopy

The preparation and extraction of plant materials for antiplasmodial activity was carried out at the School of Pharmacy, University of Nairobi. The *in-vitro* antiplasmodial activity testing was carried out at the department of tropical and infectious diseases, Institute of Primates Research (IPR), Nairobi, Kenya.

3.4.2.1 Materials and reagents

General purpose grade methanol, ethanol, dichloromethane and ethylacetate were obtained from Kobian Ltd, Kenya. These solvents were distilled in the laboratory before use. Dimethyl Sulfoxide (DMSO) was from BDH chemicals, UK.

Roswell Park Memorial Institute (RPMI) 1640 and Albumax II were obtained from InVitrogen Life Technologies, New Zealand. Phenazine ethosulphate (PES), glucose, sodium bicarbonate ($NaHCO_2$), D-sorbitol and N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) were obtained from Sigma-Aldrich, USA. Chloroquine phosphate standard was from Kampala Pharmaceutical Industries Ltd, Uganda. Gentamycin was obtained from Sigma-Aldrich, Dorset, UK. Giemsa stain was purchased from TCS Biosciences, UK.

Non-infected O-positive whole blood was donated by National Blood Transfusion Centre, Nairobi, Kenya and collected in heparinized tubes (BD Vacutainer®, Franklin Lakes, NJ, USA). Chloroquine-sensitive 3D7 *Plasmodium falciparum* parasites were donated by the KEMRI laboratory (Kilifi Kenya).

3.4.2.2 Instrumentation

An electric hammer mill (Muharata, Kenya) was used to pulverize the plant materials. A rotary evaporator (Heidolph Electro GmbH & Co. KG, Kelheim, Germany) consisting of a VV2000® Heidolph rotary vacuum evaporator and a WB2000® water bath connected to a Laborota4000 chiller (Polyscience, Niles, USA) and a diaphragm vacuum pump (KNF Neuberger GmbH, Freiburg, Germany) was used in the concentration of plant extracts. An Indesit refrigerator (Indesit, Italy) was used to store crude plant extracts and blood products at temperatures of 4-6 °C. High Speed Micro Centrifuge – D3024® (DragonLab LLC, USA) was used to centrifuge blood sample. A light microscope (Partec-Sysmex, Japan) was used to visualize the malaria parasites. A Freez I® incubator (Analis, Suarlee,

Belgium) was used in the incubation of the malaria parasites. Centrifuge tubes, culture flasks and 96-well plates and culture were obtained from Corning, UK. A Bioflow® laminar flow cabinet (Vermeulin L. J BVBA, Westmalle, Belgium) was used in handling micro-organisms and blood components. Ultrasonic water bath (E/MC Corp. Rai Research, USA) was used in dissolution plant extracts and cultures.

3.4.2.3 Preparation of plant test samples

For this test, 100 g each of dried and powdered *Leucas calostachys* and *Justicia betonica* were separately extracted with dichloromethane, ethylacetate and methanol respectively by cold maceration over 24 hours. Each extract was concentrated in vacuo at 40 °C and kept in amber colored glass bottles in a refrigerator until use. Stock solutions of 1 mg/ml were prepared by dissolving the extracts in aliquots of DMSO until dissolution followed by ethanol with the aid of ultrasonic water bath set at 40°C for half an hour.

3.4.2.4 Preparation stock solutions and culture medium

Human serum was prepared from whole blood by leaving it overnight at 4 °C to separate. In a laminar flow, the serum was collected in sterile 50 ml tubes and placed in a water bath at 56 °C for 1 hour to inactivate immunoproteins. Upon cooling, the inactivated serum was stored at -20 °C until ready for use.

Incomplete culture media (ICM) was prepared by dissolving RPMI-1640 supplemented with 2 mM L-glutamine, 7 mM D-glucose, 25 μg/ml gentamicin sulphate, 37.5 mM HEPES and 6 mM NaOH in a liter of millipore water. The pH was adjusted to 7.2.

Complete media (CM) was made by addition of 10 % human serum to the ICM. Bicarbonate was added to both ICM and CM before use.

Red blood cells stock suspension was prepared by adding whole blood to an equal volume of incomplete media. This was centrifuged at 5000 rpm for 4 minutes and the supernatant and buffy upper layer discarded. The procedure was repeated three times. The pellet, consisting of red blood cells was re-suspended in complete media and stored at 4°C.

Chloroquine stock solution of 620 μg/ml was prepared by dissolving 1 mg of chloroquine phosphate standard in water.

3.4.2.5 Parasite culture

Chloroquine sensitive *Plasmodium falciparum,* 3D7 strain, which had been cryopreserved in liquid nitrogen were thawed to room temperature before their culture. These parasites were cultured according to the method described by Trager & Jensen, (1976) with slight modifications. An amount of 13 ml of complete media buffered with $NaHCO_3$ was measured into T-25 cm^3 culture flask. To this, 800 μl of 50 % red blood cells (3 % hematocrit) was added followed by 1 ml of parasites and incubated at 37 °C under microaerophilic conditions (90% nitrogen, 5 % carbon dioxide, and 5 % oxygen). After 24 hours of incubation, the culture media which formed the supernatant was aspirated off and new culture media added. The parasitemia levels were monitored by making a thick smear from the culture sediment. This was observed under a light

microscope with giemsa staining. The parasitemia levels were maintained between 2-5 %. A homogenous parasite stage was obtained by addition of D-sorbitol to synchronize the parasite culture as described by Lambros and Vanderberg, 1979.

3.4.2.6 Determination of antiplasmodial activity of the plant extracts

The antiplasmodial activity of *Justicia betonica* and *Leucas calostachys* was determined by culturing the *P. falciparum* parasites in the presence of plant extracts. This was a screening method to determine the active fractions of the plant extracts. Microscopy was used to determine the activity of the plant extracts.

From the various plant extracts stock solutions of 1 mg/ml, series dilutions were made ranging between the concentrations of 500 μg/ml to 1 μg/ml in DMSO/ethanol and complete culture medium to a maximum concentration of 0.5 % ethanol in 96 well plates. The final well volume was 250 μl composed of 3 % hematocrit and 1 % parasitemia. The tests were done in duplicates. The positive control consisted of chloroquine and was used in place of the plant extracts. The negative control wells were prepared with neither plant extracts nor chloroquine. The culture plates were incubated at 37 °C with 5 % CO_2 for 48 hours. After the 48 hours, the culture plates were removed from the incubator and the supernatant removed using a micropipette. The pellet was mixed and used to make a smear which was fixed with methanol and air dried. It was then stained with giemsa stain for 10 minutes, rinsed with distilled water and air dried. This was examined under a light microscope with magnification of ×100 for the presence of parasites. In this method the presence of parasites in the blood film indicated low or no activity in the plant extract

whereas lack of parasites shows that the plant extract had a high activity against the *Plasmodium falciparum* parasites. The activity of the plant extracts was measured using minimum inhibitory concentration (MIC). The extracts with highest antiplasmodial activity were selected for β-hematin inhibition assay.

3.4.3 β-hematin inhibition assay

Malaria parasites depend on the host's hemoglobin during the intra-erythrocytic stage of malaria infection. They enzymatically metabolize the hemoglobin within their acidic food vacuoles releasing toxic heme and amino acids. However, the parasites are able to detoxify the free heme by converting it into hemozoin (β-hematin) through polymerization as shown in Figure 3.5. The hemozoin is stored in neutral lipid droplets.

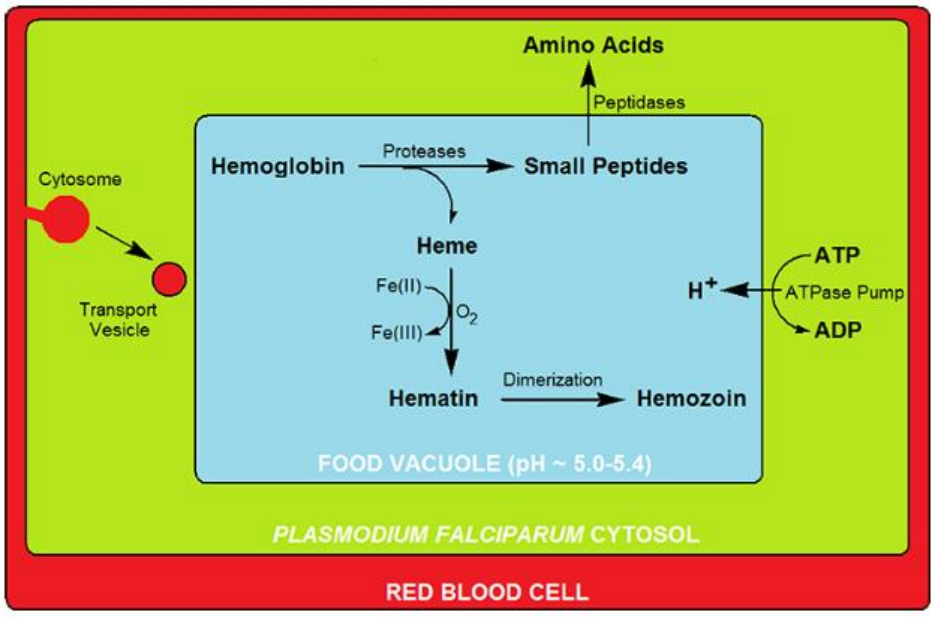


Figure 3. 5: Formation of β-hematin in *Plasmodium falciparum*
(Régis *et al.*, 2016)

Hemozoin formation is essential for the survival of malaria parasites. Therefore, agents that inhibit this process are potential antimalarial agents. In this study, bovine hemin, a commercial product was used in the formation of β-hematin in presence of acetate, temperature of 60 °C and pH 7.4. By the use NP-40, the hemin was converted into β-hematin. The non-aggregated heme was solubilized by addition of pyridine to form a colored compound that is assayed using calorimetry. This assay method developed by Ncokazi and Egan (2005) and modified by Sandlin *et al*. (2014). This study was carried out at the Center for Exploratory Research, Hitachi, Japan.

3.4.3.1 Materials and reagents

General purpose grade solvents were from Kobian Ltd., Nairobi Kenya. These solvents were distilled before use. Analytical grade hexane, dichloromethane, chloroform, acetone, ethyl acetate, and methanol and HPLC grade methanol, acetonitrile were from Wako Pure Chemical Industries, (Tokyo, Japan). The HPLC solvents were degassed before use.

Nonidet P-40 **(**NP-40) and chloroquine diphosphate were from Nacalai Tesque Inc. (Kyoto, Japan). Pyridine, dimethysulfoxide (DMSO) and hemin chloride were from Tokyo Chemical Industry Co. Ltd (Tokyo, Japan). HEPES buffer solution was from Dojindo (Tokyo, Japan). Sodium acetate, glacial acetic acid and acetone was from Wako Pure Chemical Industries Ltd (Tokyo, Japan).

3.4.3.2 Instrumentation

An SH 8000Lab microplate reader from Corona Electric Company (Tokyo, Japan). Millipore Filter unit (0.22µm) MS® PVDF syringe filter was from Membrane Solutions (Tokyo, Japan). Savant SPD1010 SpeedVac concentrator was from Thermo Fisher Scientific (New York, USA). A refrigerator (Sanyo Labcool, MPR-1410) was from Sanyo Electric Co. Ltd (Osaka, Japan). Rotavapor RII connected to a Buchi V-700 vacuum pump and Vacuum Controller V -850 was obtained from BÜCHI Labortechnik AG (Meierseggstrasse 40, Switzerland). Flat bottom 96-well plates were obtained from Tokyo Chemical Industry Co. Ltd, (Tokyo, Japan).

3.4.3.3 Preparation of plant extracts and fractions

The powdered plant materials of *Justicia betonica* and *Leucas calostachys* were separately extracted with dichloromethane (DCM)/methanol (MeOH) (1:1) using distilled general grade solvents. Each extract was reduced in vacuo to give dark green colored extracts that were stored under refrigerator until use. The plant extracts under this study were prepared by chromatography.

Based on the *in-vitro* antiplasmodial assay, the methanol extract of *L. calostachys* had the highest activity. In the β-Hematin inhibition assay, this fraction was obtained through subjecting the DCM/MeOH extract to column chromatography by gradient elution using the hexane, ethylacetate and methanol to give 5 fractions. The methanol fraction was

further fractionated using preparative HPLC to yield 15 fractions (F1-F15) which were tested in the β-Hematin assay.

On the other hand, the DCM/MeOH extract of *Justicia betonica* was subjected to gradient elution column chromatography to give 5 fractions (DCM, 50 % EtOAc, EtOAc, 50 % MeOH and MeOH). Each of these fractions were tested in the β-hematin assay. The EtOAc fraction was further subjected to column chromatography with gradient elution using hexane and EtOAc to give 4 fractions (JB F1-JB F4). Out of these, JB F4 which were tested for its dose-dependent β-hematin inhibition activity.

3.4.3.4 Preparation of stock solutions for β-hematin inhibition assay

Hemin stock solution (100 ml, 25 mM) was prepared by dissolving 1.64 g of hemin chloride in DMSO. This was sonicated for 1 minute and then filtered through a 0.22 μm PVDF membrane filter. From this stock solution, 177.6 μl aliquot was measured and suspended in 20 ml of 2 M acetate buffer (pH 4.9) to make the hematin suspension for use in the assay. This suspension was prepared within an hour of use. The stock solution (384 μM) of NP-40 was prepared in water. Pyridine solution was prepared adding 50 % pyridine, 20 % acetone, water and 200 mM HEPES buffer solution at pH 7.4.

Chloroquine diphosphate was dissolved in water to make a 100 mM stock solution. From this, a serial dilution (0-100 mM) was made in DMSO for determination of IC_{50} of chloroquine diphosphate. Stock solutions (40 mg/ml) of *Leucas calostachys* fractions were made by dissolving each fraction in water. Conversely, 2 stock solutions for *Justicia*

betonica (10 mg/ml and 5 mg/ml) were prepared in DMSO. Quantitative determination of *Justicia betonica* fraction 4 (JB F4) was done by making serial dilutions ranging from 0-20 mg/ml stock solution in DMSO. All solutions were shaken in a water bath to aid dissolution. The negative control consisted of DMSO.

3.4.3.4 The β-hematin assay

The procedure described by Sandlin *et al*., 2014 was adopted in this study. In this test, solutions were added to 96 U shaped microtiter plates in the order of 1 µl of test compound/standards, 19 µl of water, NP-40 stock solution (5 µl), acetone (7 µl) and finally the hemin suspension (25 µl). These plates were incubated in a water bath under agitation at 45 rpm and temperature of 37 °C for 6 hours. The analysis was done using pyridine-ferrochrome assay. This involved the addition of 15 µl of acetone and 8 µl of pyridine solution to each well and the incubation under water bath repeated for a period of 30 minutes. The absorption was measured at 405 nm using the microplate reader.

3.4.3.5.1 Data analysis

The β-hematin inhibition activity for each of the test samples was tested relative to the positive and negative controls. For each of the tests carried out on the extract or drug ($A_{Analysis}$), there were 2 controls; control analysis ($A_{Analysis;\ Blank}$) whose absorption was read without allowing the 6 hour period for the reaction time of pyridine, and a blank control ($A_{CLTBlank;\ Blank}$) carried out with DMSO only. The absorption arising from unreacted hematin was calculated as;

$$Change\ in\ absorption\ (\Delta A_{Analysis}) = A_{Analysis} - A_{Analysis;Blank}$$

The residual absorbance ($\Delta A_{CLT;\ Blank}$) of the plant/drug (not as a result of β-hematin) was calculated as follows;

$$(\Delta A_{CLT;Blank}) = A_{CLT;Blank} - A_{CLTBlank;Blank}$$

B-hematin synthesis inhibition by the plant/drug was calculated as;

$$I_{Analysis} = \Delta A_{Analysis} - (\Delta A_{CLT;Blank})$$

A positive $I_{Analysis}$ was indicative of active extract/drug whereas a negative $I_{Analysis}$ indicated inactive extract/drug.

The percent β-inhibition activity of the test samples was calculated as;

$$\frac{I_{Analysis}\ of\ test\ sample}{I_{Analysis}\ of\ standard} \times 100\ \%$$

3.5 Results and Discussion

3.5.1 *In vitro* antiplasmodial activity testing using microscopy

In the test for antiplasmodial activity of the *Justicia betonica* and *Leucas calostachys* plant extracts, microscopy was used to determine the activity of each extract. Minimum inhibitory concentration (MIC) was used to compare the activity of various extracts. The results for the *in vitro* antiplasmodial activity are illustrated in Table 3.1.

Table 3. 1: Antiplasmodial activity of *Justicia betonica* and *Leucas calostachys* extracts against chloroquine sensitive *P. falciparum* 3D7

Extract		Concentration (µg/ml)					
		500	**250**	**125**	**62.5**	**31.5**	**MIC**
Justicia betonica	MeOH	+	+	+	+	+	>500
	EtOAc	-	-	-	+	+	125
	DCM	+	+	+	+	+	>500
Leucas calostachys	MeOH	-	-	-	-	+	62.5
	EtOAc	-	-	-	-	+	62.5
	DCM	-	-	-	+	+	125

Key: + parasites observed, - no parasites observed
MeOH-Methanol; EtOAc –Ethylacetate; DCM-dichloromethane

The ethylacetate extract of *Justicia betonica* and the methanol and ethylacetate extracts of *Leucas calostachys* showed the highest activity against the chloroquine sensitive *P. falciparum* parasite with minimum inhibitory concentration (MIC) of 125 µg/ml and 62.5

μg/ml respectively. This was a preliminary screening test to identify active fractions which would be further studied for β-hematin inhibition activity and phytochemistry.

The *in-vitro* antiplasmodial activity results obtained in this study are in agreement with previous studies on these plants. For example, the methanolic and aqueous extracts of *L. calostachys* were active against *P. knowlensi* with an IC_{50} of 3.45 μg/ml and 0.79 μg/ml respectively (Nyambati *et al.*, 2013). On the other hand, the ether extract of *J. betonica* demonstrated good antiplasmodial activity with EC_{50} of 13.36 μg/ml whereas the methanol extract only exhibited a mild activity with EC_{50} of 69.6 μg/ml (Bbosa *et al.*, 2013; Muregi *et al.*, 2004). These studies are comparable to the present study where the methanol extract of *Leucas calostachys* and ethylacetate extracts of *Justicia betonica* were the most active against the chloroquine sensitive *P. falciparum* parasites. These active fractions were selected for β-hematin inhibition assay.

Although demonstration of *in vitro* antiplasmodial activity is important in ascertaining the antimalarial properties of plants, some plants used in traditional medicine for management of malaria have also been shown to alleviate other symptoms associated with malaria infection. For instance, some antimalarial plants lower the fever or pain which are common symptoms of malaria. In fact, *J. betonica* is used in traditional medicine in the management of headache, vomiting, pain and inflammation which are symptoms accompanying malaria. The ethanolic extract of this plant demonstrated significant analgesic (Eddy's hot plate method in rats and acetic acid induced writhing in mice) and anti-inflammatory (carrageenan-induced paw edema in rats and human red

blood cells membrane stabilization assay) effects (Gangabhavani & Ravishankar, 2013). Although the more polar fractions of *J. betonica* had insignificant anti-plasmodial activity, these other antimalarial properties may be the reason why the plant is commonly used in management of malaria.

Leucas calostachys belongs to Lamiaceae (mint) family which is used for management of pain and inflammation (Uritu *et al.*, 2018). The family is a rich source of anti-oxidants and anti-inflammatory agents making it important in management of malaria (Carović-Stanko *et al.*, 2016; Chouhan & Singh, 2011). It is believed that anti-oxidants modulate plasmodial infection (Arrey *et al.*, 2013; Percário *et al.*, 2012). A review of the genus *Leucas* by Chouhan & Singh, (2011) showed that the genus is rich in phenolic compounds which serve as anti-oxidants. Most phenolic compounds such as flavonoids and tannins are soluble in polar solvents. The antiplasmodial activity of the methanol extract of *L. calostachys* in this study may be attributed to presence of phenolic compounds.

Many plants from the Lamiaceae family are used in folklore as antimalarial and most have demonstrated antiplasmodial activities in their methanol or ethanol extracts. They include; *Plecranthus* species, *Fuerstia africana, Ajuga integrifolia, Phlomis brunneogaleata,* and *Hyptis suaveolens.* Few plants in this family have their antiplasmodial activity within their non-polar fractions which are rich in terpenes. Such plants include *Hoslundia opposita, Ocimum sanctum, Ocimum kilimandscharicum* and

Salvia radula, (Onguén *et al.*, 2013b; Tariq *et al.*, 2016; Titanji *et al.*, 2008). Lamiaceae family is therefore a potential source of newer antiplasmodial agents.

3.5.2 β-Hematin assay

β-hematin inhibition assay is a simple, quick, robust and cost effective method used in identification of antimalarial compounds. This method has been utilized by many researchers in evaluating many plant extracts and compounds for antimalarial properties (Akkawi, 2014; Attieh *et al.*, 2015; Batista *et al.*, 2009; Sandlin *et al.*, 2014)

3.5.2.1 Determination of IC_{50} of chloroquine diphosphate

Chloroquine diphosphate, a known inhibitor of β-hematin formation was used to as a positive standard in this assay. A series of dilutions ranging from 8 mM to 80 uM in in DMSO. A sigmoidal dose-response curve (Figure 3.6) was generated to determine the IC_{50} of the chloroquine diphosphate standard using the ED50plus v1.0 software.

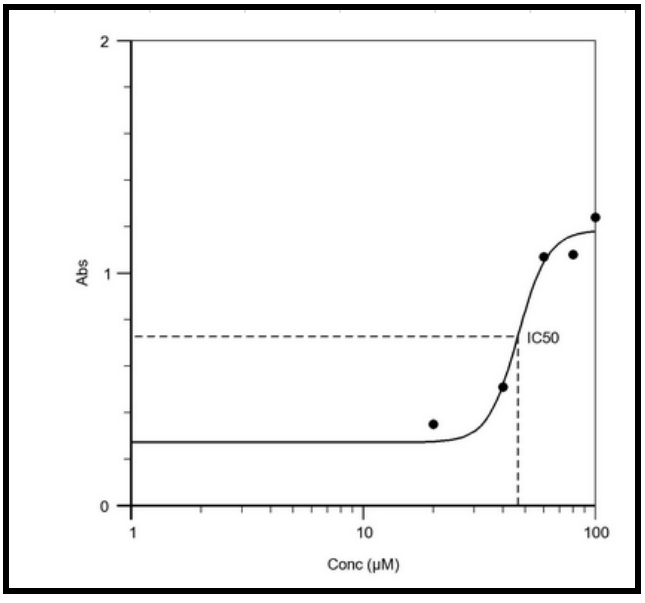


Figure 3. 6: Dose response assay for chloroquine diphosphate

The IC_{50} of chloroquine diphosphate was determined as 46.3µM. This was comparable to similar studies where the value was determined as 53.0 µM (Sandlin *et al.*, 2014).

3.5.2.2 *Leucas calostachys* activity

The $I_{Analysis}$ for each of the test fractions, each at a fixed concentration of 0.7 mg/ml, was calculated. Only 5 fractions were active with positive $I_{Analysis}$ values. The activity of these active fractions was compared to chloroquine diphosphate to give the percent inhibition as shown in Figure 3.7.

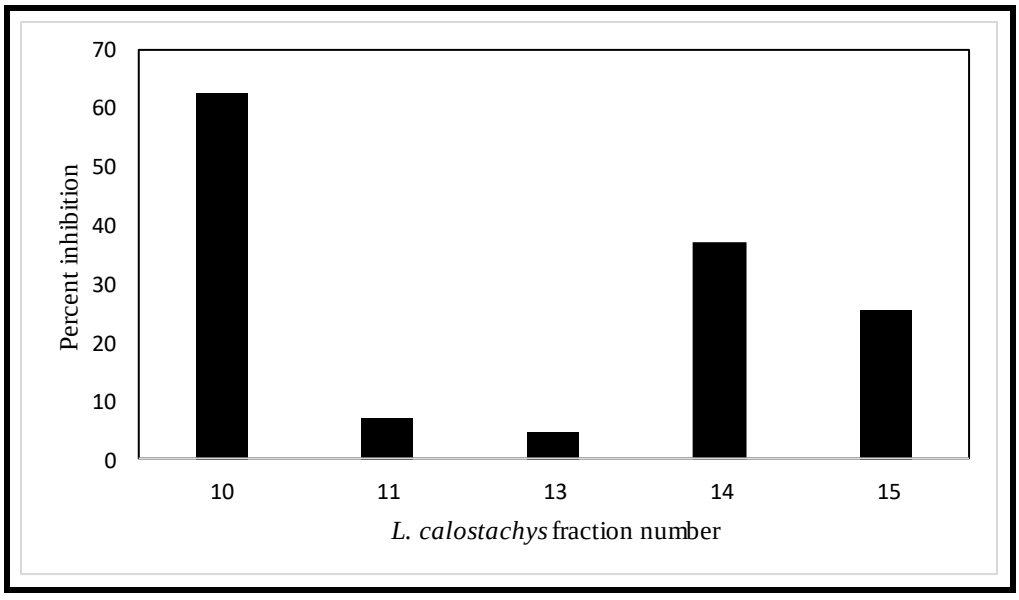


Figure 3. 7: β-hematin inhibition assay of *Leucas calostachys* fractions.

The *Leucas calostachys* fraction 10 had the highest activity followed by fractions 14 and 15 respectively. Fraction 11 and 13 had the least activity with β-hematin inhibition of less

than 10 %. The fraction 10 was therefore selected for identification of potential antimalarial compounds.

3.5.2.3 *Justicia betonica* L. activity

Following the column chromatography fractionation of DCM/MeOH extract of *Justicia betonica* L., 4 fractions (DCM, EtOAc, 50 % MeOH in EtOAc and MeOH) were obtained. Each of these fractions was tested for β-hematin inhibition activity at 2 dose levels; 0.088 mg/ml and 0.175 mg/ml. The $I_{Analysis}$ at each concentration was calculated and the percent inhibition obtained as shown in Figure 3.8.

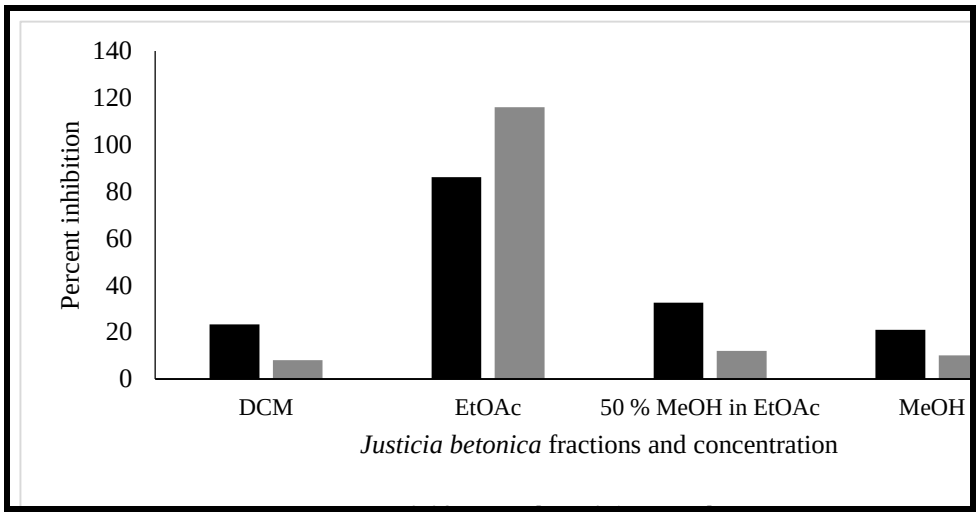


Figure 3. 8: β-hematin inhibition activity of *Justicia betonica* fractions

The EtOAc fraction exhibited the highest inhibition activity against β-hematin of over 50 % at both dose levels. The rest of the fractions had a percent inhibition of less than 50 % at both dose levels. Further fractionation of the ethylacetate fraction yielded 4 fractions

(F1-F4), of which fraction 4 (50 % hexane in EtOAc) was tested for β-hematin inhibition activity and results shown in Figure 3.9.

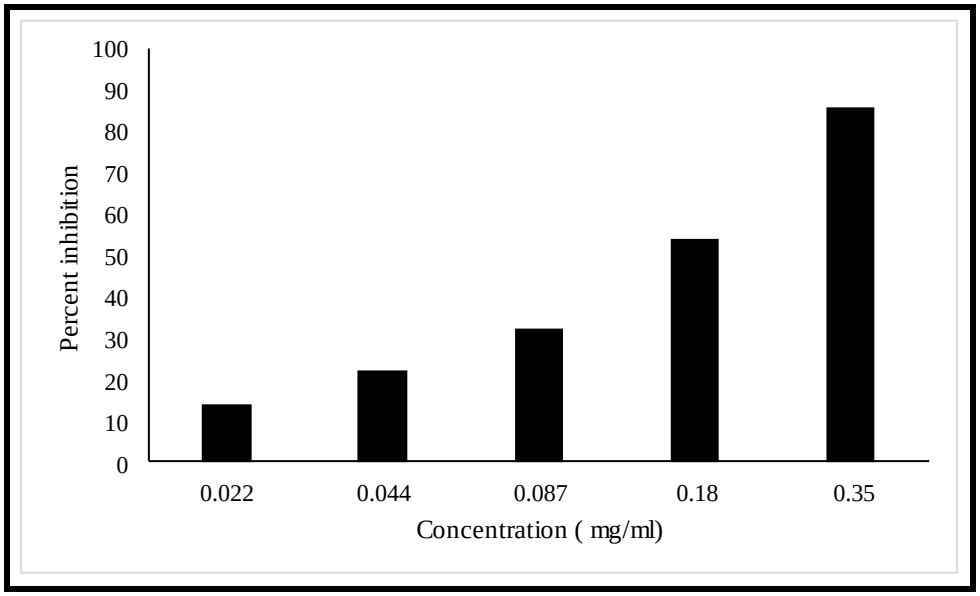


Figure 3. 9: β-hematin inhibition activity of *Justicia betonica* fraction 4

The *Justicia betonica* F4 (JB F4) which was derived from the active fraction (EtOAc fraction) exhibited dose dependent activity against formation of β-hematin. Concentration of 0.18 mg/ml and above exhibited inhibition activity of more than 50 %.

Although the antiplasmodial activity of *Leucas calostachys* Oliv.and *Justicia betonica* L. has been reported, assessment of their β-hematin inhibition activity has never been determined. In this study both plants showed activity against formation of β-hematin.

The use of β- hematin inhibition assay has been widely utilized in the study of purified antimalarial compounds as well as plants extracts (Abiodun, 2018; Heshmati *et al.*, 2018; Vargas *et al.,* 2011). The quinoline class of antimalarial is known to inhibit the formation of β-hematin by binding on heme monomers preventing its polymerization. Apart from the quinolines, other scaffolds known to inhibit this pathway include; benzamides, benzimidazoles, and triacylimidazoles (Fong *et al.*, 2015). Other studies based on plant extracts reveal that many other compounds possess β-hematin inhibition activity. Hashimati *et al.* (2018) screened the genus Scrophularia using the β- hematin formation assay and found out that the active fractions contained methoxylated flavonoids, methoxylated coumarins and diterpenoids. Another study by Riscoe *et al* (2005) indicated that xanthones inhibit the β-hematin pathway. It is hypothesized that there are several mechanisms by which these phytochemicals inhibit the formation of β-hematin which may be different from that of quinolines. Some of these compounds have multiple targets against the malaria parasites. For example, the triaryl imidazoles poses both gametocidal and schizonticidal effects. This multiple activity gives them advantage over the quinolines where malaria parasites have developed resistance (Fong *et al.*, 2015; Sandlin *et al.*, 2014).

β-hematin formation assay method can easily be adapted for high throughput screening of antimalarial compounds. Several studies carried out using this method concluded that there is a high correlation (72 % for pure compounds and 50 % for extracts) between β-hematin formation assay and parasite inhibition assay (Fong *et al.,* 2015; Vargas *et al.,*

2011). In this study, there was a correlation between the antiplasmodial activity and the β-hematin inhibition assay in both plants. The active fractions in the antiplasmodial active were similarly active in the β-hematin formation assay.

3.6 Conclusion

The methanol fraction of *Leucas calostachys* aerial parts, and the ethylacetate fraction of *Justicia betonica* aerial parts demonstrated both *in vitro* antiplasmodial growth inhibition and β-hematin inhibition activities. These results verify the use of these plants in management of malaria within the Luhya community in Western Kenya. This study is the first to demonsatrate β-hematin inhibition activity of these plants. The active fractions, methanol fraction of *L. calostachys* and ethylacetate fraction of *J. betonica*, were selected for purification and identification of possible antimalarial compounds.

CHAPTER FOUR

STRUCTURE ELUCIDATION OF COMPOUNDS WITH ANTIMALARIAL ACTION IDENTIFIED FROM *LEUCAS CALOSTACHYS* OLIV. AND *JUSTICIA BETONICA* L.

4.1 Introduction

Plant derived compounds have played a crucial role in the discovery of antimalarial drugs throughout human history. These compounds, including quinine and artemisinins, are still the mainstay in current management of malaria. Due to rapid emergence of resistance to antimalarial drugs by malaria parasites, there still need for search of new antimalarial agents from nature. An analysis of antimalarial compounds derived from plants which exhibit antimalarial activity shows that they belong to various chemical classes including alkaloids, quinones, terpenoids, flavonoids, xanthones, phenolics, lignans among many others (Onguén *et al.*, 2013a). Therefore, antimalarial phytochemicals are diverse in their chemical nature.

The various phytochemicals studied for their antimalarial activities exhibit different mechanisms of action against antimalarial parasites. For example quinine, a quinoline alkaloid, acts by inhibiting the formation of β-hematin leading to accumulation of toxic heme within the parasites and eventual parasite death. On the other hand, the artemisinins act by producing free radicles that cause protein damage and hindering parasites'

proteasome function leading to parasite death (Bridgford *et al.*, 2018). The mode of action of several other phytochemicals is still not well understood. Some studies indicate that some flavonoids may act in similar way to quinine by inhibiting formation of β-hematin. Some phytochemicals demonstrate activity against malaria via more than one mechanism of action (Sandlin *et al.*, 2014). The structural diversity and multiple modes of action of phytochemicals make them desirable candidates against multi drug resistant malaria parasites.

Several studies have been conducted to identify phytochemical constituents of *Leucas calostachys* and *Justicia betonica* (Bbosa *et al.*, 2013; Hassan *et al.*, 2019; Subbaraju *et al.*, 2004). However, no compounds with antimalarial activity have been identified from these plants. This section describes the various classes of phytochemicals identified from these plants.

4.1.1 Reported phytoconstituents of *Leucas calostachys*.

Preliminary screening of *Leucas calostachys* reveals that it contains alkaloids, saponins, glycosides, phenolics (flavonoids, tannins, lignans, coumarins), terpenoids, phytosteroids, aliphatic long chain compounds (Okach *et al.*, 2013). Although no specific compounds have been identified from this plant, many other plants in the genus such as *Leucas aspera* and *Leucas alluaudii* among others have been widely studied. Among the compounds isolated from the genus include organic acids, phenolics, alkaloids, steroids, terpenoidal compounds and glycosides (Chouhan & Singh, 2011; Das *et al.*, 2012). The compounds identified from genus *Leucas* are summarized in the Table 4.1.

Table 4. 1: Compounds isolated from other *Leucas* species

Class	Examples
Organic acids	Benzoic acid derivatives Peroxyacid (urticic acid)
Phenolic compounds	Long chain alkylphenols Lignans Flavonoids Coumarins
Alkaloids	Nicotine
Steroids	Ubiquitous phytosterols Leucisterol
Terpenes (including saponins)	Essential oils Diterpenes Triterpenes (oleanane, prostane and lactone types)
Glycosides	Flavonoidal glucosides Phenylethanoid glycosides Diterpenoidal glucosides
Others	Proteins, carotenoids, long chain compounds, Fatty acids,

(Chouhan & Singh, 2011; Hassan *et al.*, 2019)

Many of the *Leucas* species have a rich aroma due to monoterpenes and sesquiterpenes such as menthol and thymol (Chouhan & Singh, 2011). A unique group of diterpeness found in this genus include leucosperones, leucosperols and leucasdins. Prostane type of triterpenes known as leucastrins have also been isolated from the genus (Miyaichi *et al.*, 2006; Sadhu *et al.*, 2006).

4.1.2 **Reported phytoconstituents of *Justicia betonica*.**

Preliminary screening of *Justicia betonica* revealed the presence of steroids; triterpenoids, alkaloids, glycosides, saponins (Bbosa *et al.*, 2013). The plant contains high levels of flavonoids and other phenolic compounds. It is estimated that the leaves contain 2.86 mg Quercetin Equivalent (QE) of flavonoids (Biju *et al.*, 2013).

Subbaraju *et al.*, (2004) isolated an indoloquinoline class of alkaloids with anti-tumor activities from the methanolic extract of the whole plant of *J. betonica*. They include; quindoline class (jusbetonin and 10 H quindoline) and the quinindoline class (6 H quinindoline and 5 H, 6 H quinindolin-11-one). Jusbetonin, a glycoside, was isolated from the methanol fraction of methanolic extract whereas the other 3 compounds were isolated from the EtOAc fraction of the methanolic extract. Their chemical structures of these alkaloids are illustrated in Figure 4.1.

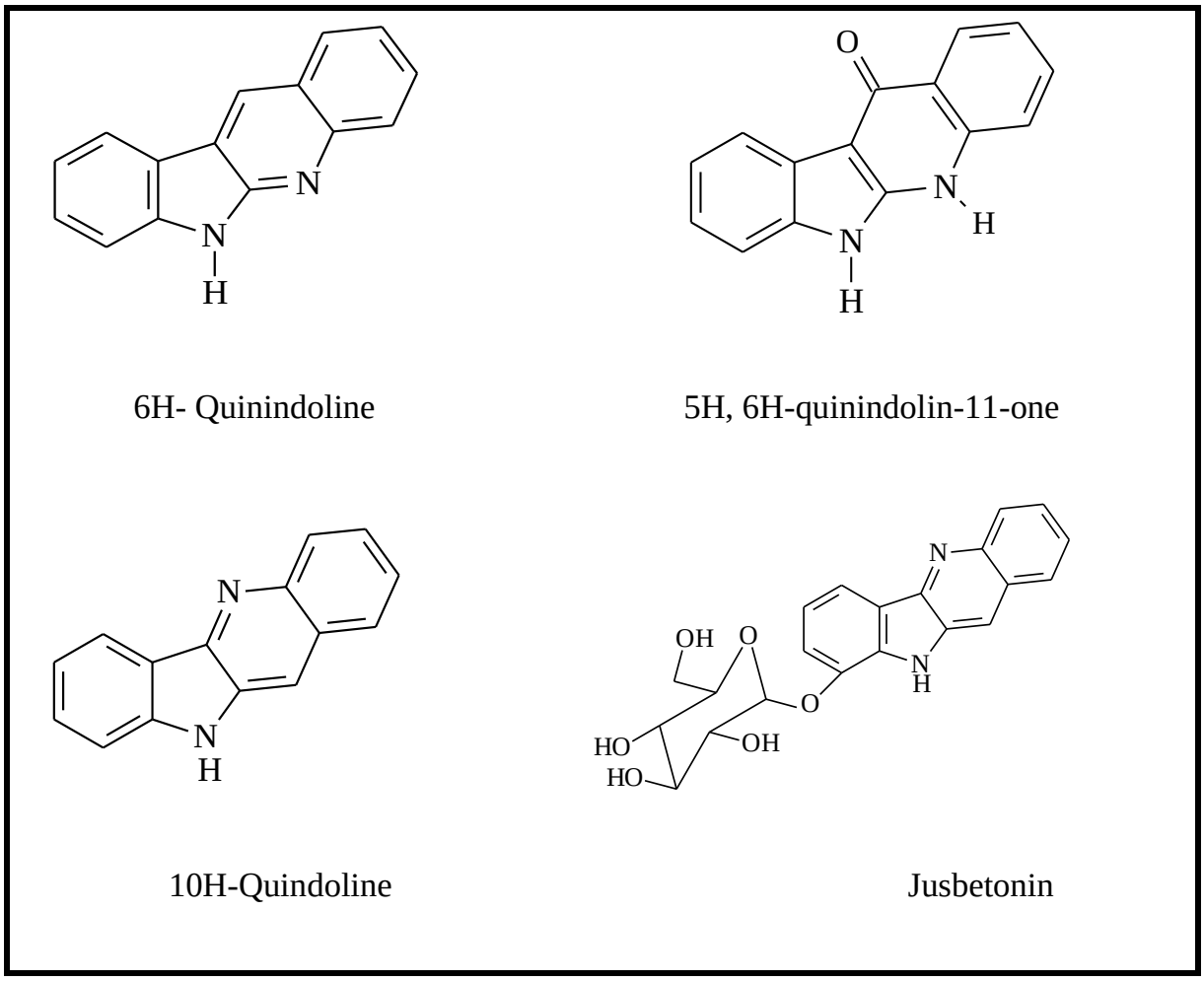
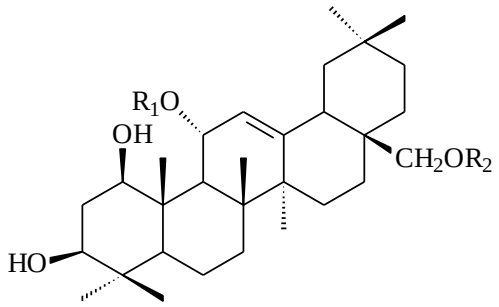
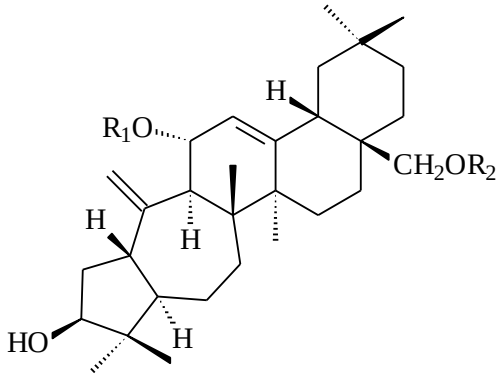


Figure 4. 1: Chemical structures of alkaloids isolated from *J. Betonica*

Two different types of triterpenoidal glycosides were also isolated from the aqueous portion of ethylacetate extract of *J. betonica.* The first class is made of an olean-12-ene-1 β,3β,11α,28-tetraol (Kanchanapoom *et al.*, 2004) whereas the second class is composed of A-nor-B-homo-oleanan-10,12-diene-3β,11α,28-triol nucleus (Kanchanapoom *et al.*, 2005). Their chemical structures are illustrated in Table 4.2.

Table 4. 2: Chemical structures of Triterpenoid glycosides isolated from *J. betonica*

Structure	Name	Substituents	
		R_1	R_2
	Justicioside A	H	β-D-Glc- β-D-Glc
	Justicioside B	H	β-D-Glc- β-D-Glc- β-D-Glc
	Justicioside C	Me	β-D-Glc- β-D-Glc
	Justicioside D	Me	β-D-Glc- β-D-Glc- β-D-Glc
olean-12-ene-1 β,3β,11α,28-tetraol nucleus			
	Justicioside E	H	β-D-Glc- β-D-Glc
	Justicioside F	H	β-D-Glc- β-D-Glc- β-D-Glc
	Justicioside G	Me	β-D-Glc- β-D-Glc- β-D-Glc
A-nor-B-homo-oleanan-10,12-diene-3β,11α,28-triol nucleus			

4.2 Experimental

The *in vitro* antiplasmodial activity and β-hematin inhibition assay, showed highest activity with the methanol fraction and EtOAc fractions for *Leucas calostachys* and *Justicia betonica* respectively. These extracts were fractionated using column chromatography and high perfomance liquid chromatography (HPLC) for the isolation of possible antimalarial compounds. The compounds were tentatively identified using electrospray ionisation tandem mass spectroscopy (ESI-MS/MS) and reports from literature.

4.2.1 Solvents, reagents and equipment

4.2.1.1 Solvents and reagents

General grade methanol, ethanol, dichloromethane and ethylacetate were from Kobian, Ltd, (Nairobi, Kenya). These solvents were distilled in the laboratory before use. Analytical grade hexane, dichloromethane, chloroform, acetone, ethylacetate, and methanol and HPLC grade methanol and acetonitrile used in column chromatography were all from Wako Pure chemical Industries (Tokyo, Japan). The HPLC solvents were degassed before use.

4.2.1.2 Column chromatography

Open column chromatography was performed using glass column measuring 2 cm internal diameter and 50 cm long equipped with a glass wool filter. The columns were

fitted with solvent reservoir all from Nacalai Tesque, Inc. (Kyoto, Japan). Normal phase silica gel of porosity 60Å and particle size 63-200 μm was from Sigma–Aldrich GmbH (Seelze, Germany).

4.2.1.3 Thin layer chromatography

Thin layer chromatography was performed on pre-coated aluminium plates with 0.2 mm thick layer of normal phase silica gel 60 GF_{254} (Supelco Analytical™) with a fluorescent indicator was from Merck KGaA (Darmstadt, Germany). Ultraviolet light lamp (Min UVIS®) from Desaga GmbH (Heidelberg, Germany) was used to visualize the thin layer chromatograms.

4.2.1.4 Analytical HPLC

The analytical HPLC system consisted of an L-2000 Hitachi LaCrom Elite from Hitachi (Tokyo Japan), equipped with an organiser, L2130 pump, 7125i Rheodyne Model manual injector with a 20 μl loop and L-2400 single wavelength UV detector. Chromatographic separation of the compounds was achieved with analytical columns: YMC Triart C18 (150×3.0 mm) and YMC Triart C8 (150×3.0 mm) both from YMC, Co. Ltd. (Kyoto, Japan). The samples were filtered through a 0.45 μm syringe filter (As One cooperation, China) before injection into the HPLC system. UV detection of eluents was performed at a wavelength of 254 nm. The data acquisition system was supported by OpenLab control (Agilent technologies, USA).

4.2.1.5 Semi-preparative HPLC

Semi-preparative HPLC was performed on a Hitachi LaCrom elite L-2000 series equipped with an organizer, pump (L2130), Reodyne Model manual injector with a 50 µl loop and UV detector. The chromatographic separation of the compounds was achieved with semi-preparative columns: YMC Triart C18 (250x5.0mm) and YMC Triart C8 (250x5.0mm) both from YMC, Co. Ltd. Kyoto, Japan. The fractions eluting from the column were collected using Gilson FC 203 B fraction collector from Gilson Inc. (Middleton, USA). UV detection of samples was performed at a wavelength of 254 nm. OpenLab control from Agilent technologies (Santa Clara, USA) was used to acquire the data.

4.2.1.6 Liquid chromatography- Mass spectrometry

Thermo Scientific Q Exactive Focus benchtop LC-MS/MS System (ThermoFisher Scientific Inc. Bremen, Germany) consisting of HPLC and mass spectrometer components were used to obtain the mass spectrum data. The HPLC consisted of a Thermo Scientific™ Dionex™ Ultimate™ RSLC 3000 system with a rapid separation quaternary pump, a diode-array detector (DAD), a thermostated rapid separation column compartment and an auto sampler was used to fractionate the samples before analysis by the mass spectrometer.

The Mass spectrometer (Q Exactive Focus, Thermo Scientific™) consisted a quadrupole ion filter and Orbitrap mass analyzer. Thermo Scientific™ Xcalibur™ instrument

control and data processing software was used to acquire the data on a computer with Intel® microprocessor. The comuter was also fitted with Microsoft® Windows® 7 operating system and Microsoft Office software package (Microsoft, Redmond, Washington, USA).

4.2.1.7 Other equipment

Rotavapor RII connected to a Buchi V-700 vacuum pump and Vacuum Controller V -850 was from BÜCHI Labortechnik AG, (Meierseggstrasse 40, Switzerland) was used to concentrate large volumes of extracts before use in chromatography. Savant SPD1010 SpeedVac concentrator from Thermoscientific, (New York, USA) was used to concentrate smaller volumes of extracts. A Sanyo Labcool, MPR-1410 refrigerator from Sanyo Electric Co. Ltd, (Osaka, Japan) was used to store all extracts and fractions at 4-8 °C until use.

4.2.2 Preparation of plant extracts and fractions

For this purpose, 1 kg *Leucas calostachys* Oliv. and 500 g of *Justicia betonica* L. plant powders were used. The plant powders were extracted three times by cold maceration using general grade dichloromethane (DCM) and methanol (MeOH) mixture (1:1) over a period of 24 hours. The extracts were filtered, combined and reduced to dryness in vacuo on a rotary evaporator at 40 °C. The extracts were stored in airtight containers under refrigeration (4-8 °C) until use.

4.2.3 Fractionation of *Leucas calostachys* extracts

4.2.3.1 Chromatographic separation of Leucas *calostachys*

Leucas calostachys DCM/MeOH (50:50) extract (20 g) was re-dissolved in MeOH/DCM (50:50) and adsorbed onto 17 g of silica gel. The solvent was evaporated in vacuo using a rotary evaporator at 40 °C and loaded onto a column containing 50 g of silica gel. It was gradient eluted with hexane, EtOAc-hexane (50:50), EtOAc, MeOH-EtOAc (50-50), and MeOH. The collected fractions were monitored using thin layer chromatography and similar fractions combined to give 5 fractions. The MeOH fraction was subjected to semi-preparative HPLC.

Semi-preparative HPLC trial runs were performed using different mobile phases of MeOH and acetonitrile (ACN) with the following buffers: 0.1 % acetic, 0.01 % formic and 10 mM ammonium formate. The optimized conditions was achieved using mobile phase consisting of ACN and MeOH and 0.01% formic acid buffer with a gradient elution of increasing volume of ACN (60-100 %) on YMC Triart C18 column. The elution time was 30 minutes at a flow rate of 3.0 ml/min. A wavelength of 254 nm was chosen for the detection of compounds.

In this analysis volume of 50 µl of the MeOH fraction, derived from the column chromatography, was injected during each cycle. With the help of a fraction collector, each fraction was collected over 2 min interval giving total of 15 fractions. A total of 10 cycles were run and the respective fractions combined and concentrated using a

SpeedVac® concentrator. The schematic presentation of the *Leucas calostachys* fractionation is illustrated in Figure 4.2.

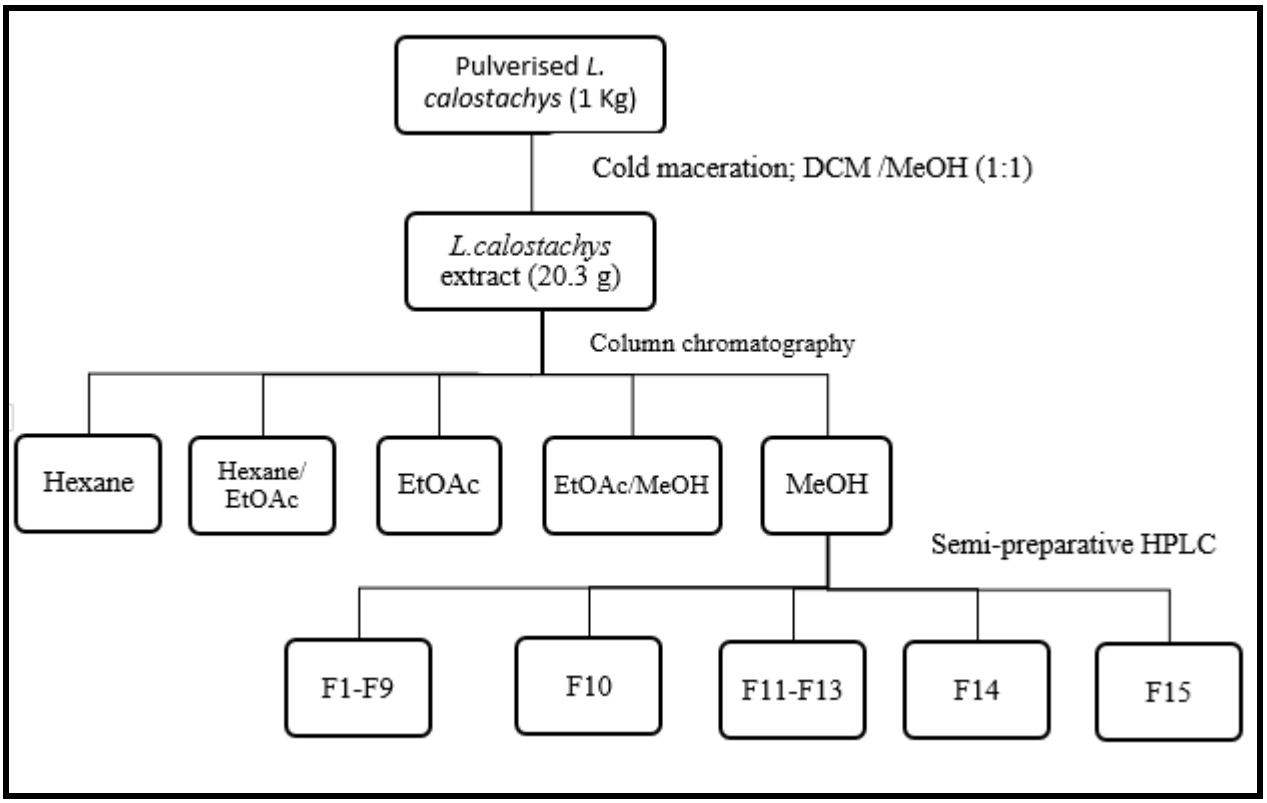


Figure 4. 2: Extraction and fractionation of *Leucas calostachys*

4.2.3.2 HPLC-ESI-Mass spectroscopy for *Leucas calostachys*

The *Leucas calostachys* fraction 10 (F10) obtained from the semi-preparative HPLC exhibited the highest β-hematin inhibition activity. This fraction was subjected to liquid chromatography tandem mass spectroscopy to identify possible antimalarial compounds. The fraction 10 was separated on YMC Triart C18 (150 X 3.0mm) column using linear gradient of 60 %-100 % ACN in MeOH as the mobile phase. A volume of 2.0 µl was

injected at flow rate of 0.5 ml/min over a period of 30 minutes. The pump pressure was kept at 146 bars and column temperature at 10 °C.

4.2.4 Fractionation and Isolation *Justicia betonica*

4.2.4.1 Chromatographic separation of *Justicia betonica*

The *Justicia betonica* DCM/MeOH extract (10 g) was re-dissolved in DCM/MeOH (50:50). This was adsorbed onto silica gel and evaporated to dryness and then loaded onto a column packed with silica gel in DCM. Gradient elution was performed using DCM, EtOAc-DCM (50:50), EtOAc, MeOH-EtOAc (50:50) and MeOH respectively. These fractions were monitored using TLC and similar fractions combined giving a total of 5 fractions. Each fraction was evaporated to dryness and a portion tested for β-hematin inhibition activity. The ethylacetate fraction, which showed the highest activity was further fractionated using column chromatography with gradient elution with hexane and EtOAc from 20-50 % EtOAc to give a total of 4×200 ml fractions. The fractionation of *Justicia betonica* is illustrated in Figure 4.3.

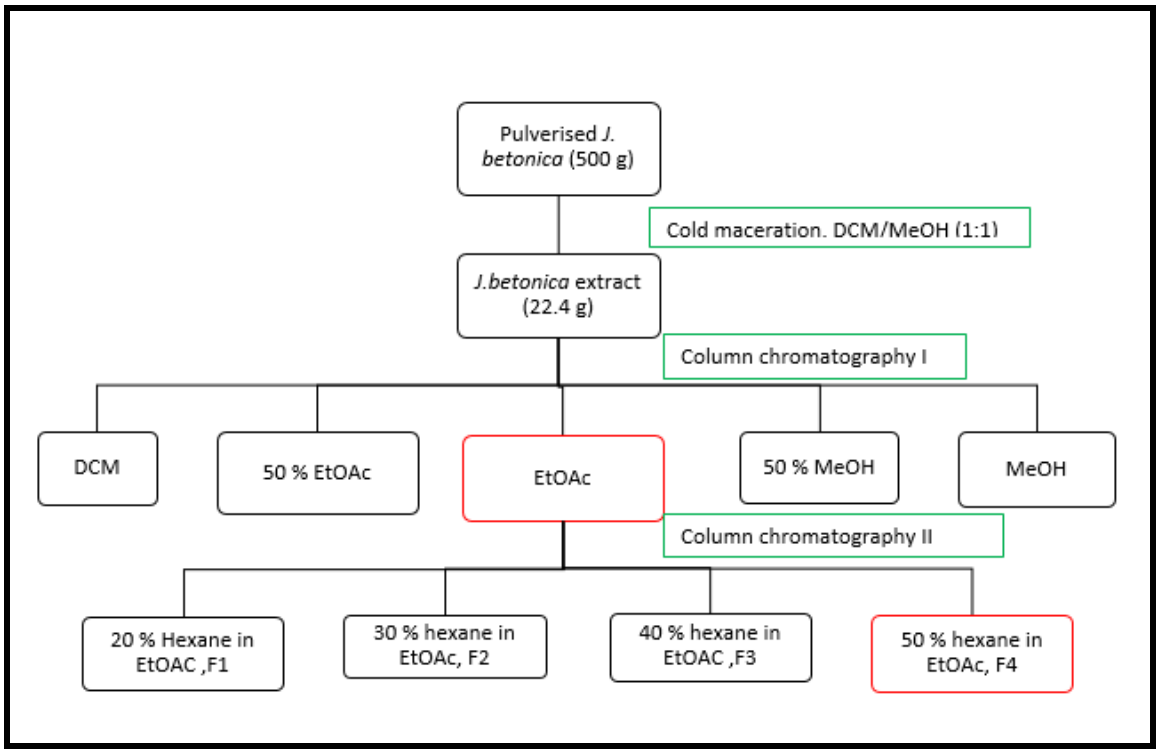


Figure 4. 3: Extraction and fractionation of *Justicia betonica*

Fraction 4 (hexane-EtOAc; 50:50) was an orange colored eluent (Figure 4.4) and exhibited a dose-dependent β-hematin inhibition activity.

Figure 4. 4: *Justicia betonica* active Fraction 4

Fraction 4 eluent was reduced in vacuo and purified by crystalisation using acetone to give orange to purple colored crystals whose purity was monitored by analytical HPLC.

The chromatogram of purified *Justicia betonica* L. fraction 4 (JB 4) is shown in Figure 4.5. These crystals were subjected to ESI-MS/MS for the identification of possible antimalarial compounds.

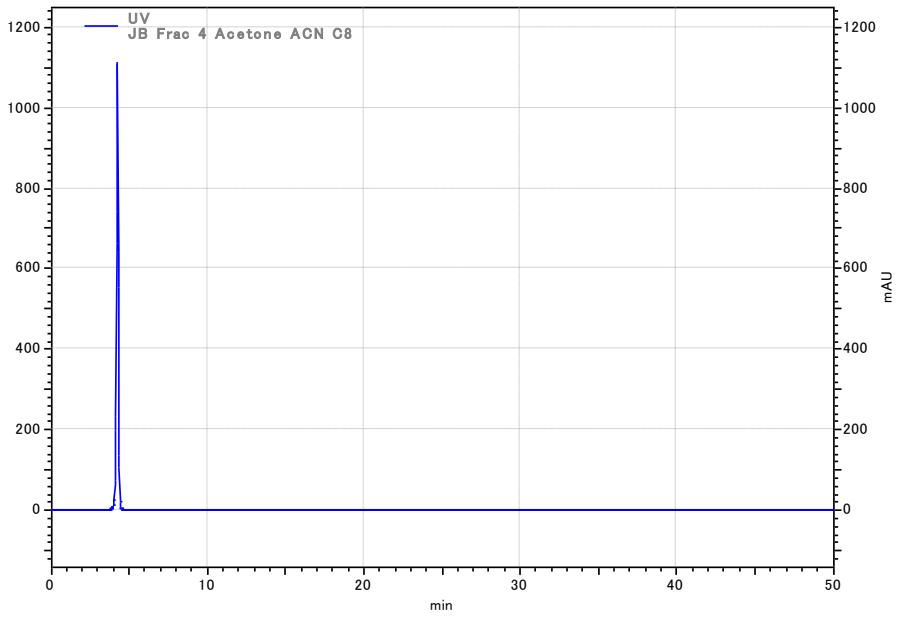


Figure 4. 5: HPLC chromatogram of purified *Justicia betonica* fraction 4

4.2.4.2 HPLC-Mass spectroscopy for *Justicia betonica*

The *Justicia betonica* Fraction 4 obtained was separated on YMC Triart C8 (150 X 3.0 mm) column using linear gradient of 60 %-100 % ACN in MeOH as the mobile phase. A volume of 2.0 µl was injected at flow rate of 0.5 ml/min over a period of 30 minutes. The pump pressure was kept at 146 bars and column temperature at 10 °C.

4.3 Results

4.3.1 Extraction yields for *Leucas calostachys* Oliv. and *Justicia betonica* L.

The powdered plants were extracted using DCM/MeOH (50:50) and resulted in a percentage yield of 2.0 % and 4.5 % for *Leucas calostachys* and *Justicia betonica* respectively.

4.3.2 Identification of compounds from *Leucas calostachys*

Leucas calostchys Oliv. was extracted DCM/MeOH (50:50) and resulted in a percentage yield of 2.0 % of a dark green crude extract. The bio-assay fractionation of this extract resulted in F10 with the highest β-hematin inhibition activity. The F10 was subjected to LC-MS/MS for identification of constituent compounds.

ESI-MS full scans modes were performed to identify both protonated and deprotonated molecular ions. This was followed by ESI-MS/MS analysis in both negative and positive modes to identify product ions arising from the precursor ion so as to deduce the fragmentation pattern of these compounds**.** The chromatogram of this fraction is shown in Figure 4.6.

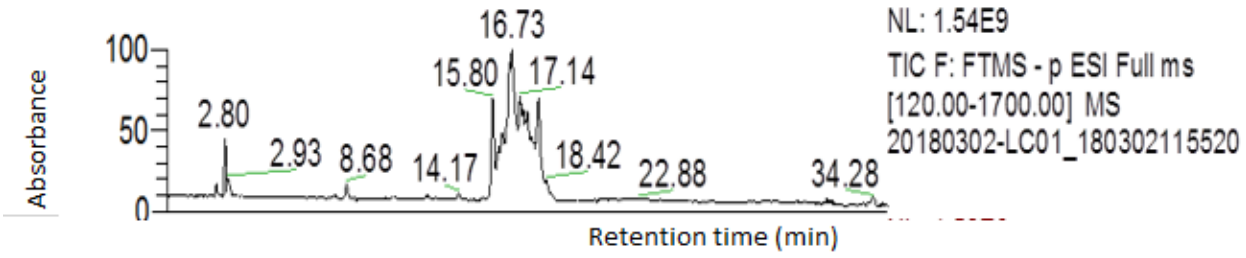


Figure 4. 6: HPLC chromatogram of *Leucas calostachys* methanolic fraction.

Most of the compounds eluted between 14th and 18th minutes of the chromatogram. The identification of the compounds was successfully achieved based on accurate mass and fragmentation patterns from previous published literature data.

Fragmentation patterns of various polyphenolic compounds have been extensively studied and their data were compared with mass spectroscopy data obtained in this study (Ablajan et al., 2006; Ibrahim *et al.*, 2015; Li *et al.*, 2016). From this analysis, the identified compounds were polyphenolics broadly classified into two main groups: flavonoidal glycosides and phenylethanoid glycosides.

4.3.2.1 Flavonoidal compounds

There were 8 flavonoidal compounds identified from *Leucas calostachys.* Domon and Costello, (1988) proposed the fragmentation pattern of flavonoidal glycosides as illustrated in Figure 4.7. This pattern was utilized in characterization of the flavonoidal compounds based on the ms^2 data.

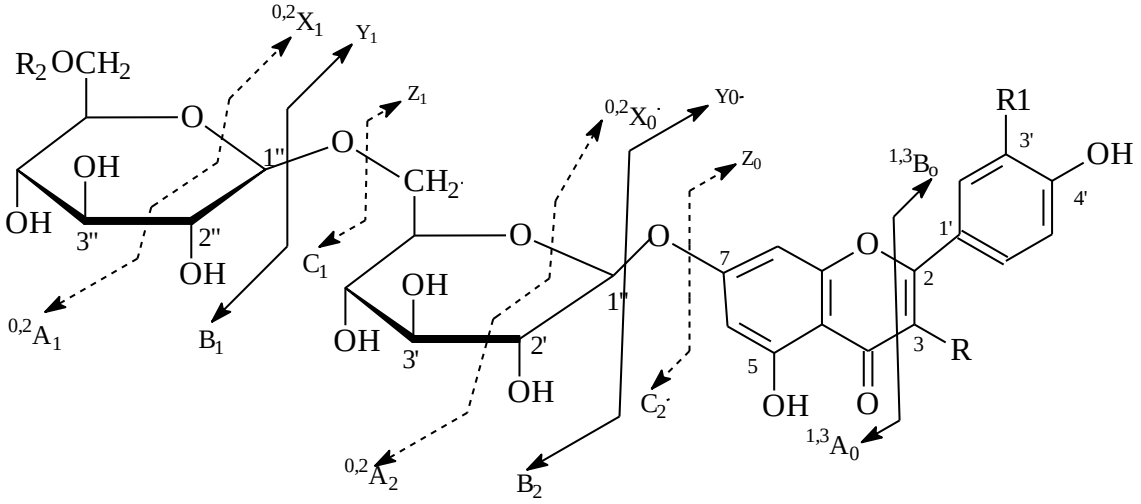
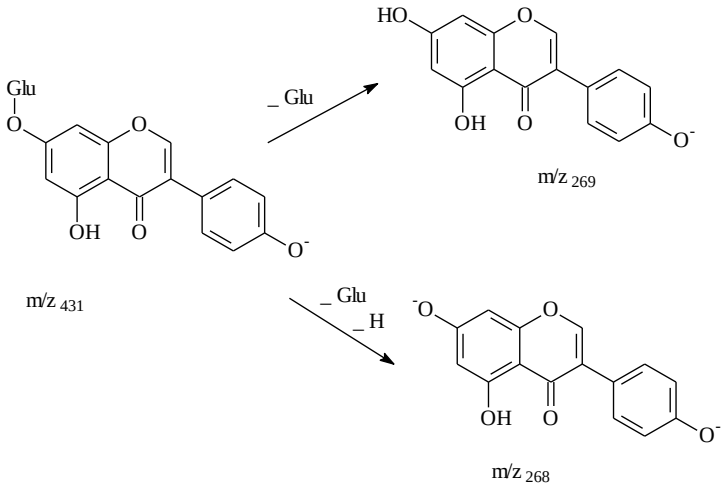


Figure 4. 7: Fragmentation pattern of flavonoidal glycosides

The deprotonated molecular ions $[M-H]^-$ for compounds **1** (tR =11.87) and **4** (tR=15.30) with m/z of 447.09 and compound **8** (tR= 17.37) with m/z of 609.13 showed a similar product ion with m/z of 285.04 which indicated a deprotonated aglycone fragment of either luteolin or kaempferol. These compounds were tentatively identified as having originated from luteolin since only $[Y_0-H]^-$ fragment was observed unlike in kaempferol where both $[Y_0-H]^-$ and $[Y_0-2H]^-$ product ions should be present in the ion spectrum of $[M-H]^-$ (Ablajan *et al*., 2006). Although compound **1** was isomeric to compund **4** with a deprotonated ion $[M-H]^-$ of m/z 447.09, the ms^2 spectrum showed a fragment $[Y0-2H]^-$ of m/z 284 which was absent in compound **4**. The main fragment of m/z 285.04 is due to the loss of one O-hexose sugar as seen in compound **4**. The higher intensity of fragment of m/z 285 $[M-H-162]^-$ than the fragment of m/z 284 $[M-2H-162]^-$ is indicative of 7-OH glycosylation (Li *et al.*, 2016). Compound **1** was therefore identified as luteolin -7-O-glucoside. The product ion of m/z 285.05 was due to the loss of two glucose residues in compund **8** $[M-H-162-162]^-$. The 2 glucose residues in compound **8** were O-glucosides likely linked to one another since m/z values of 161.02 and 323.07 were observed resulting from $[B_1-H]^-$ and $[B_2-H]^-$ fragments respectively. The fragmentation profile of these compounds are similar to luteolin glucosides found in literature (Ibrahim *et al.*, 2015; Li *et al.*, 2016). Compounds **1, 4** and **8** were identified as luteolin -7-O- glucoside, luteolin -4′-O-glucoside and luteolin diglucoside respectively.

Compounds **2** and **3** (tR = 14.13min and tR=15.10 min respectively) were isomers with m/z of 431.09 $[M-H]^-$. The ms^2 specta for both compounds suggest the loss of one

hexose sugar residue to give aglycone molecule of either apigenin or genistein (Kachlicki *et al.*, 2016). Compound **2** had a prominent peak of m/z 268 $[M-2H-162]^-$ due to the loss of sugar residue characteristic of apigenin glucoside. Therefore compound **2** was identified as apigenin 7-O-glucoside. On the other hand compound **3** gave two intensive fragments of m/z 269 $[M-H-162]^-$ and 268 $[M-2H-162]^-$ due to the loss of hexose sugar as illustrated in Figure 4.8. The spectrum is similar to the reported pattern for genistein 7-O-glucoside (Soukup et al., 2012).



Key: - Glu= loss of Glucose molecule; -H = loss of hydrogen molecule

Figure 4. 8: Fragmentation pattern of Genistein 7-O-glucoside.

Compound **5** (tR= 15.61 min) with m/z of 461.10 $[M-H]^-$ showed a fragments of m/z 299.05 [M-H-162] indicating a loss of hexose sugar residue and m/z of 283 [M-H-162-CH_3]. This fragmentation pattern is similar to that of chrysoeriol-7-O-glucopyranoside

(Hassan *et al.*, 2019; May *et al.*, 2017). The proposed fragmentation pattern is illustrated in Figure 4.9.

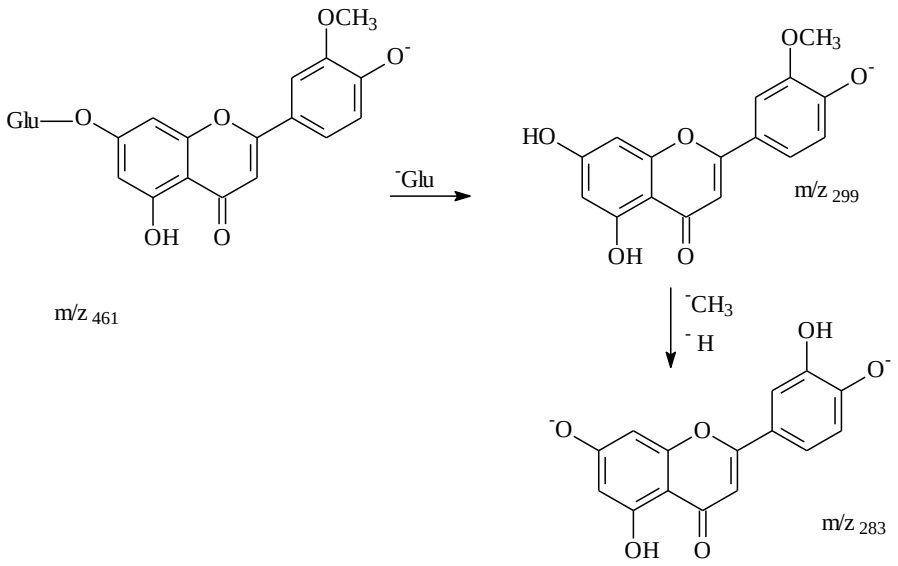


Figure 4. 9: Fragmentation pattern of compound 5.

Compound **6** (tR=16.30min) with m/z of 475. 08 $[M-H]^-$ showed a product ions with m/z of 299 [M-H-176] and 285 [M-H-176-14] due to successive loss of glucuronyl and methyl groups. The pattern is characteristic of chrysoeriol 7-O-glucuronide (Hassan *et al.*, 2019).

Compund **7** (tR=16.80 min) with a molecular ion $[M-H]^-$ with m/z of 315.05 showed an ms^2 spectrum with main fragment of m/z 300.02 due to the loss of methyl group. This fragmentation pattern is characteristic of isorhamnetin and is illustrated in Figure 4.10.

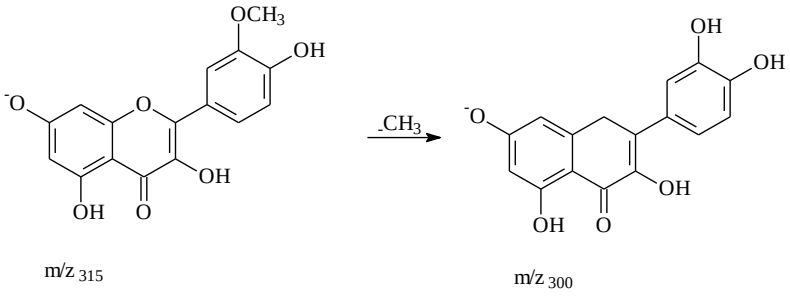


Figure 4.10: Fragmentation pattern of compound 4 (isorhamnetin).

Their mass spectra information and proposed identifications of the flavonoidal compounds are summarized in Table 4.3. Their respective LC-MS/MS spectra are shown in appendix 5.

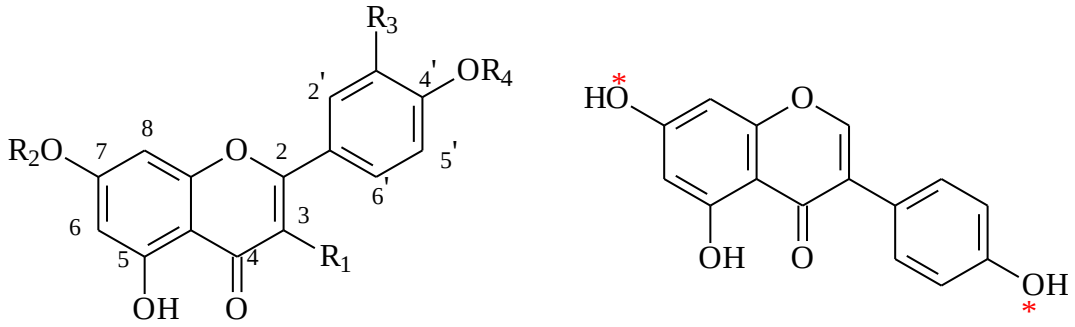
Table 4. 3: Mass spectrum data of identified flavonoids from *Leucas calostachys*.

No.	t_R	m/z[M-H]$^-$	Formula	HPLC-ESI-ms^2	PROPOSED ID/Ref
1	11.87	447.0939	$C_{21}H_{20}O_{11}$	[447], 310 (10),286 (15), **285** (100), 284 (40), 174 (10)	Luteolin -7-O-glucoside (cynaroside) (Li *et al.*, 2016; Marston & Hostettmann, 2006)
2	14.13	431.0986	$C_{21}H_{20}O_{10}$	[431], 311(3), 269 (40), **268** (100)	Apigenin-O- glucoside
3	15.10	431.0989	$C_{21}H_{20}O_{10}$	[431], 311 (3), **269** (100), 268 (90)	Genistein-O-glucoside
4	15.30	447.0915	$C_{21}H_{20}O_{11}$	[447], **285** (100), 286 (15)	Luteolin 4′- O-glucoside (Pikulski & Brodbelt, 2003; Zhao *et al.*, 2014)
5	15.61	461.1094	$C_{22}H_{22}O_{11}$	[461], **299** (100), 284 (38), 283 (40), 269 (10)	Chrysoeriol 7-O-glucoside
6	16.30	475.0887	$C_{22}H_{20}O_{12}$	[475], 299 (60), **285** (100), 284 (90)	Chrysoeriol 7-O-glucuronide(Quispe *et al.*, 2018)
7	16.80	315.0515	$C_{16}H_{12}O_7$	[315], **300** (100), 228 (7), 201 (10), 165 (5), 136 (20), 132 (7), 65 20)	Isorhamnetin?(Marston & Hostettmann, 2006)

8	17.37	609.1259	$C_{27}H_{30}O_{16}$	[609], 447 (3), 323 (5), **285** (100), 161 (10)	Luteolin diglucoside (Chen, H.-J. *et al.*, 2012)

Key: tR=retention time, [ ] = molecular ion, ( ) = percentage

All the flavonoids occurred as glycosides apart from isorhamnetin. These flavonoids belong to the flavone sub-class apart from genistein which is an isoflavanoid and isohamnetin which falls under the flavanol sub-class. The chemical structures of these flavonoids are shown in Figure 4.11.



Flavone/flavanol Isoflavone

Figure 4. 11: Chemical structures of flavonoids present in *L. calostachys*

Flavonoid	**R1**	**R2**	**R3**	**R4**
Isorhamnetin	OH	H	OCH_3	H
Apigenin-7-O-glucoside	H	Glucosyl	H	H
Luteolin 7-O-glucoside	H	Glucosyl	OH	H
Luteolin 4′- O-glucoside	H	H	OH	Glucosyl
Luteolin diglucoside	H	H/2 GluOH	H/2 Glu	
		(2 Glucosyl either at R_2 or R_4)		
Chrysoeriol-7-O-glucoside	H	Glucosyl	OCH_3	H
Chrysoeriol-7-O-glucuronide	H	Glucuronyl	OCH_3	H
Genistein-O-glucoside	Glucosyl at either of the * positions			

4.3.2.2 Phenylethanoid compounds.

Another class of polyphenolic compounds identified from methanolic fraction 10 of *Leucas calostachys* were the phenylethanoid glycosides. These are glycosides whose aglycone moieties are substituted with aromatic acids such as caffeic acid, cinnamic acid, ferulic acid, coumaric acid and their related isomers. They form glycosides through either glycosidic or ester bonding with various sugars such as apiose, glucose, arabinose, rhamnose, xylose, lyxose and allose. These compounds easily produce $[M-H]^-$ in the negative ion ESI-MS spectra. They also produce characteristic ions originating from both the aromatic acid and sugar moieties in the ms/ms spectra (Gong *et al.*, 2016).

Compound **9** of m/z 639.1939 $[M-H]^-$ (tR=6.10 min) had the chemical formulae deduced as $C_{29}H_{36}O_{16}$ (calculated $C_{29}H_{35}O_{16}$, 639.1923). A product ion peak of m/z 621.1835 was due to the loss of water molecule $[M-H-H_2O]^-$ indicating the presence of β-OH group. The product ion of m/z 459.1517 was due to the further loss of caffeoyl moiety $[M-H-H_2O\text{-caffeoyl}]^-$. The characteristic peaks for caffeoyl moiety were also present of m/z 161.0235 and m/z 179.0342. This fragmentation pattern is similar to that reported in literature for β-hydroxyacteoside (Cardinali *et al.*, 2012; Gong *et al.*, 2016).

Compounds **10** and **16** (tR, 7.92 min and 10.38 min respectively) were isomeric compounds with deprotonated molecular ions [M-H]- of m/z 651.1934 and 651.1937 respectively. They both had a product ion peak observed of m/z 487.14 resulting from the loss of 164 amu [M-H-164amu]. However, compound **16** showed other prominent product ion peaks of m/z 323.0780, 251.0562 and 233.0458 which were lacking in

compound's **10** ms^2 spectrum. The product ion peak of m/z 323.0780 in compound **16** could have resulted from the loss second 164 amu, $[M-H-164amu-164amu]^-$. On the other hand, compound **10** exhibited a ion of m/z 325.0930 which could be due to loss of the caffeoyl group $[M-H-164\ amu\text{-}caffeoyl]^-$. The characteristic caffeoyl moiety peaks were present of m/z 179.03 and 161.02 in both compounds **10** and **16** spectra. However no literature reports were found of similar compounds. These could be novel compounds whose identities were not determined and further work is required to identify them.

Compounds **11** and **17** (tR, 8.07 min and 10.60 min respectively) of m/z 755.24 $[M-H]^-$ were isomers whose molecular formular was determined as $C_{34}H_{44}O_{19}$ (calculated, $C_{34}H_{43}O_{19}$, 755.2398). The ms^2 spectra showed product ion of m/z 593.2093 due to the loss of 162 amu. This was likely the loss of a caffeoyl moiety from the molecular ion $[M-H\text{-}caffeoyl]^-$. Another product ion was observed of m/z 461.1674 which reprents further loss of 132 amu. This was indicative of a further loss of pentose sugar moiety, $[M-H\text{-}caffeoyl\text{-}pentosyl]^-$. The product ion of m/z 461.1674 represent the core skeleton, $[rhamnosyl\text{-}glucosyl\text{-}phenyethanoyl]^-$, present in most phenyethanoid molecules. The characteristic ion peaks of m/z 161.02 $[caffeoyl\text{-}H\text{-}H_20]^-$. and m/z 179.03 $[caffeoyl\text{-}H]^-$ also indicated presence of caffeoyl moiety. These compounds were tentatively identified as fortythoside B and samioside having apiose as the pentose sugar. The fragmentation pattern was similar to those reported in literature (Agar & Tath, 2020; Cardinali *et al.*, 2012; Kyriakopoulou *et al.*, 2001). The two compounds differ in their attachment of the pentose sugar. The apiose is attached at C-6 of glucose molecule in fortythoside B and

C-2 of rhamnose moiety in samioside respectively. However, this study could was not able to ascertain the exact compounds allocated to the elution times.

Compounds **12** and **19** were isomers with molecular ion of m/z 623.1990 and 623.1987 eluted at 8.60 min and 11.01 min respectively. The chemical formulae was determined as $C_{29}H_{36}O_{15}$ (calculated, $C_{29}H_{35}O_{15,}$ 623.1976). These compounds showed ion peaks of m/z 461.16 as a result of loss of 162 amu. This corresponds to loss of caffeoyl moiety [M-H-caffeoyl]$^-$. Another ion peak was observed of m/z 315.10 due to another loss of 146 amu. This represents loss of rhamnosyl moiety in addition to the loss of caffeoyl moiety [M-H-caffeoyl-rhamnosyl]$^-$. The characteristic peaks due to caffeoyl moiety were also observed of m/z 161.02 [caffeic acid-H-H_2O]$^-$ ion and 179.03 [caffeic acid-H]$^-$. These data is superimposable with reported literature mass spectra values for verbascoside (acteoside) and isoverbascoside (isoacteoside) (Attia *et al.*, 2018) (Gong *et al.*, 2016). However, this study could not ascertain which compound eluted earlier than the other therefore, could not allocate the compounds to the specific run times. Their fragmentation pattern of verbascoside is illustrated in Figure 4.12.

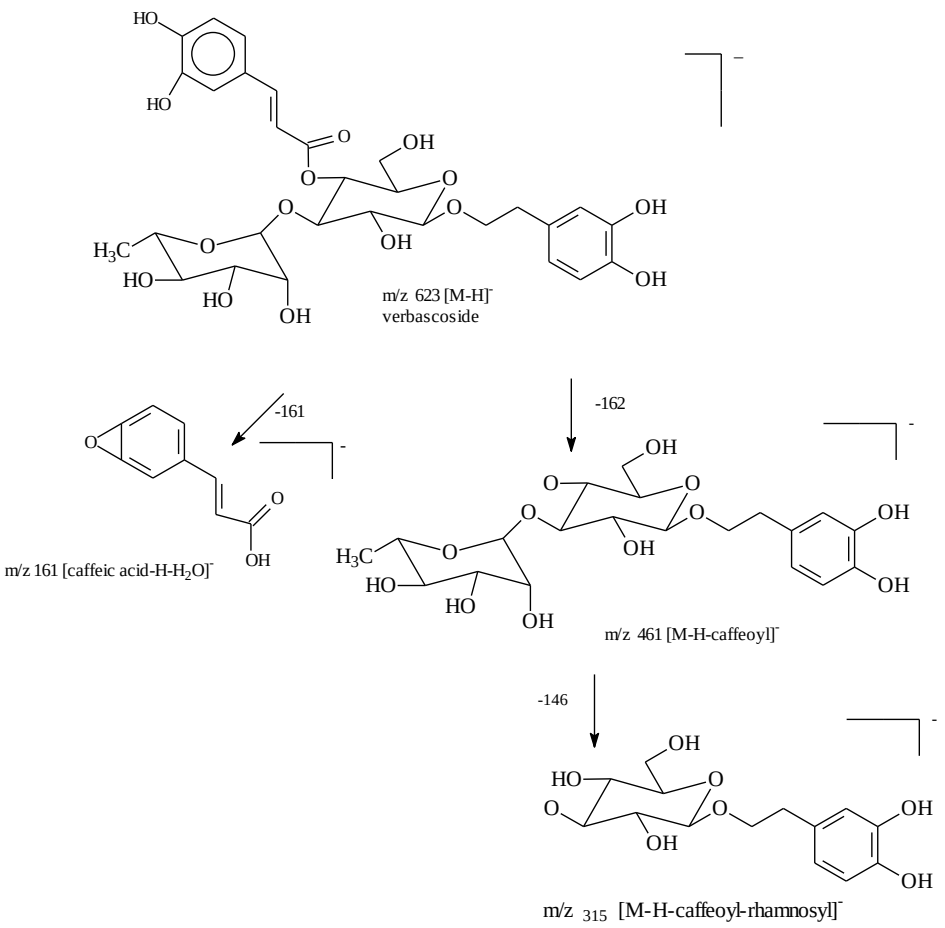


Figure 4. 12: Fragmentation pattern for verbascoside.

Compound **13** (tR=9.08 min), of m/z 637. 1779 $[M-H]^-$ showed ion peak of m/z 487.1463 and 325.0929 representing $[M-H-150amu]^-$ and $[M-H-150amu-caffeoyl]^-$ respectively. The 150 amu could be due to the loss of vanilloyl moiety. The product ion peaks observed of m/z 179.0342 and m/z 161 are characteristic caffeoyl product ions. The ms^2 spectrum of this compound was similar to that of compound **10** except for the molecular ion peaks. There was an extra 14 amu for compound **10** which is probably due to a CH_2 group. Compound **13** is likely the demethylated form of compound **10**. The

identity of this compound was also not determined and further spectroscopic work should be carried out on it.

Compound **14** (tR, 9.74 min), of m/z 735.2210 [M-H]- showed a main product peak of m/z 623.1988 due to loss of 112 amu. This study was unable to determine the lost molecule represented by the 112 amu. The rest of the fragmentation pattern is similar to that of verbascoside/isoverbascoside which had m/z of 623.19. It was therefore concluded that the compounds is a substituted verbascoide/isoverbascoside whose identity could not be determined in this study.

Compound **15** (tR=10.18 min), of m/z 769.2573 $[M-H]^-$ had a molecular formular of $C_{35}H_{46}O_{19}$ (calculated $C_{35}H_{45}O_{19,}$ 769.2555). There was a product ion peak of m/z 593.2096 due to loss of 176 amu indicating a possible loss of feruloyl moiety $[M-H\text{-feruloyl}]^-$. A further loss of a pentose sugar moiety with 132 amu gave rise to product ion of m/z 461.1666 [M-H-feruloyl-pentose]$^-$. The characterist peaks for feruloyl moiety were also observed of m/z 193 [feruloyl-H], 175.0394 [feruloyl-H-H2O] and 161.0233 $[\text{feruloyl-}CH_3]^-$. Based on these fragmentation patterns and literature values, the compound was identified as alyssonoside (Agar & Tath, 2020; Petreska *et al.*, 2011).

Compound **18** (tR=10.82) of m/z 637.2147 $[M-H]^-$ had a molecular formula $C_{30}H_{38}O_{15}$ (calculated $C_{30}H_{37}O_{15}$, 637.2132). This compound follows a similar fragmentation pattern to other phenyethanoids. There is loss of 176 amu likely due to loss of feruloyl moiety to give fragment of m/z 461.1670 $[M-H\text{-feruloyl}]^-$, a common structure in most phenylethanoids. There is a characteristic feruloyl moiety ion peaks observed of m/z

175.0392 [feruloyl-H-H2O]$^{-}$, 193.0500 [feruloyl-H]$^{-}$ and 161.0235 [feruloyl-CH_3]$^{-}$.This compound was therefore identified as lucoseptoside A in line with previous studies (Grzegorczyk-Karolak & Kiss, 2018; Petreska *et al.*, 2011).

Compound **20** (tR, 14.22) with molecular ion [M-H]$^{-}$ of m/z 803. 2424 showed a small product ion of m/z 641.2121 likely due to the loss of caffeoyl molecule [M-H-caffeoyl]$^{-}$. However, there was a higher peak of m/z 623.1990 which was probably due to the further loss of water molecule [M-H-H_2O-caffeoyl]$^{-}$. Further loss of 162 amu, most likely attributed to another caffeoyl moiety, gave rise to [rhamnosyl-glucosyl-phenyethanoyl] moeity as seen in compounds **11,12 17** and **19**. No previous reports of such compound was found in literature. A similar phenyethanoids compound with m/z of 803 isolated by Grzegorczyk-Karolak & Kiss, 2018 remains unidentified. However, in their report, the peak of m/z 641 was the most abundant whereas in this study, the peak of m/z 623 was the most abundant.

The mass spectroscopic data and the identity of all the phenylethanoid compounds found in *Leucas calostachys* are summarized in Table 4.4.

Table 4. 4: Phenylethanoid compounds identified from *Leucas calostachys*.

No.	tR	$[M-H]^-$	Mol. Formulae	ms^2	Proposed Identity/Ref.
9	6.10	639.1939	$C_{29}H_{36}O_{16}$	[639.19], 621.18(30), 459.15(10), 179.03(55), **161.02(100)**	β-OH-acteoside (Gong *et al.*, 2016)
10	7.92	651.1934		[651.19], 487.14(10), **179.03(100),** 161.02(50)	Unidentified phenylethanoid
11	8.07	755.2414	$C_{34}H_{44}O_{19}$	[755.24], 593.20(40), 461.16(10), 623.20(5), 179.03(5), 161.02(50)	Fortythoside B or Samioside (Cardinali *et al.*, 2012)
12	8.60	623.1990	$C_{29}H_{36}O_{15}$	[623.19], 461.16(20), 179.03(5), **161.02(100)**	Verbascoside (Acteoside)(Gong et al., 2016; Grzegorczyk-Karolak & Kiss, 2018)
13	9.08	637.1779		[637.17], 487.14(10), 325.09(5), **179.03(100)**	Unidentified phenyethanoid
14	9.74	735.2210		[735.22], **623.19(100),** 461.16(20) 179.03(5), 161.02(90)	Unidentified phenylethanoid
15	10.18	769.2573	$C_{35}H_{46}O_{19}$	[769.25], 593.20(40), 637.21(5), 461.16(10), 193.05(2), 175.03(30) $161.02(10)^-$	Alyssonoside
16	10.38	651.1934		[651.19], 623.19(20), 487.14(15), 323.07(25), **179.03(100),** 161.02(90)	Unidentified phenylethanoid
17	10.60	755.2414	$C_{34}H_{44}O_{19}$	[755.24], 593.20(30), 461.16(5), 179.03(5), **161.02(30)**	Fortythoside B or Samioside (Gong *et al.*, 2016)
18	10.82	637.2147	$C_{30}H_{38}O_{15}$	[637.21], 461.16(40), 193.05(20), **175.03(100),** 161.02(40)	Leucoseptoside A (Grzegorczyk-Karolak & Kiss, 2018)
19	11.01	623.1987	$C_{29}H_{36}O_{15}$	[623.19], 461.16(30), 179.03(5), **161.02(100)**	Isoverbascoside (Isoacteoside)(Gong et al., 2016)
20	14.22	803.2414		[803.24], **623.19(100),** 461.16(20), 175.03(2), 161.02(30)	Unidentified phenylethanoid

Key: [ ] - molecular ion, ( ) – product ion percent peak; **bold** –base peak

The chemical structure of the 8 identified phenylethanoids from *L. calostachys* are shown in Figure 4.13.

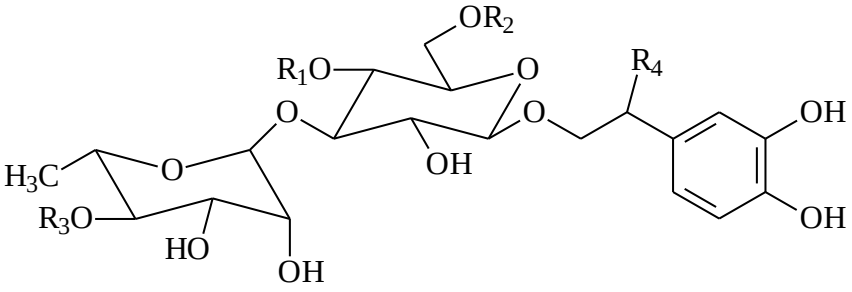


Figure 4. 13: Chemical structures of phenylethanoids identified from *L. calostachys*

Phenylethanoid	**R_1**	**R_2**	**R_3**	**R_4**
β-hydroxyverbascoside (β-hydroxyacteoside)	caffeoyl	H	H	OH
Fortythoside B	caffeoyl	β-D-Apiose	H	H
Verbascoside (acteoside)	caffeoyl	H	H	H
Alyssonoside	feruloyl	β-D-Apiose	H	H
Samioside	caffeoyl	H	β-D-Apiose	H
Leucosceptoside A	feruloyl	H	H	H
Isoverbascoside (isoacteoside)	H	caffeoyl	H	H

Most of the phenyethanoids identified in this study were related pointing to a similar biosynthetic pathway. Figure 4.14 summarized fragmentation patterns of these compounds.

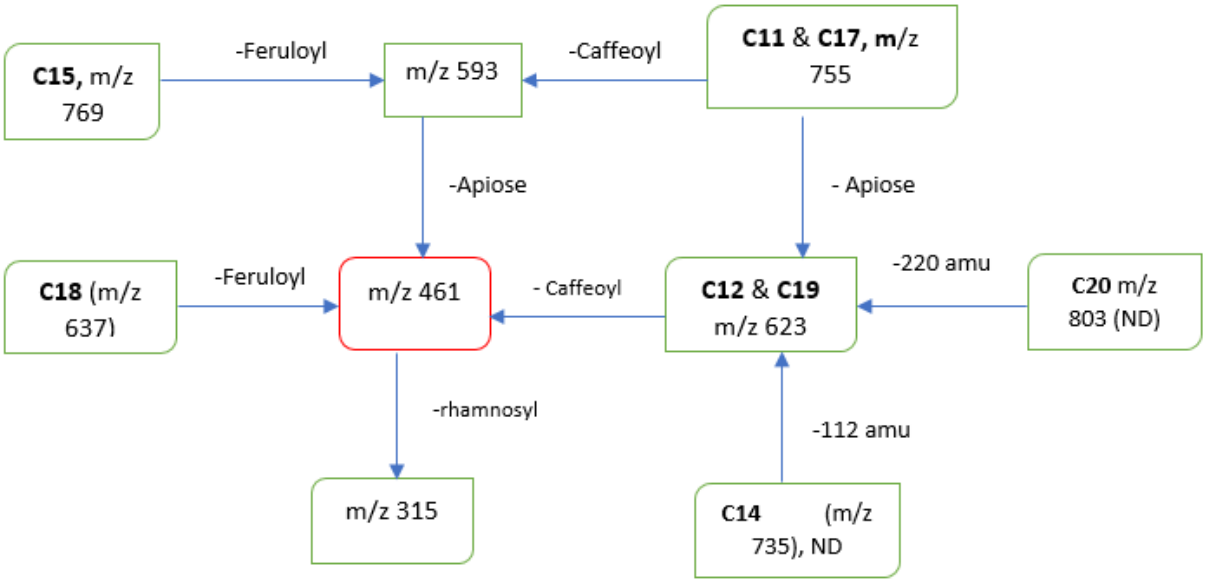


Figure 4. 14: Summary of fragmentation patterns of related phenyethanoids form *Leucas calostachys*

Key: C=compound; ND =structure not determined

4.3.3 Identification of Compounds from *Justicia betonica*

The *Justicia betonica* active fraction 4 (JB F4) was subjected to LC-MS/MS for identification of compounds. The ESI-MS full scans and ESI-MS/MS analysis were performed performed to identify both protonated and deprotonated ions. The HPLCchromatogram of this fraction is shown in Figure 4.15.

Figure 4. 15: HPLC chromatogram of *Justicia betonica* ethylacetate fraction (JB F4).

The identification of the compounds was successfully achieved based on the ESI-MS/MS data and fragmentation patterns from previous published studies. The identified compounds were carotenoids and indoquinoline alkaloids.

4.3.3.1 Carotenoids

A total of 5 carotenoids were identified from JB F4. Their mass spectra is shown in appendix 3b. The general fragmentation pattern of carotenoids is well described in literature and briefly illustrated in Figure 4.16 (van Breemen *et al.*, 2012).

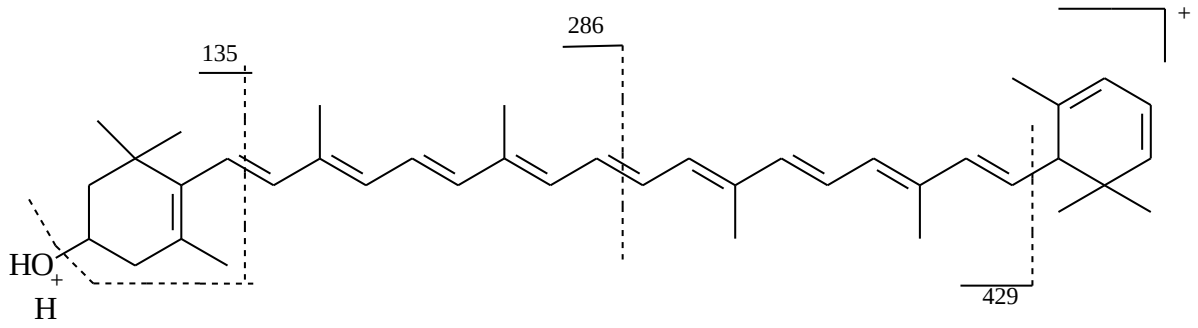


Figure 4. 16: Fragmentation pattern of carotenoids

The fragmentation pattern usually shows peaks indicating abundant fragmentation of the polyene chain. These peaks include those of m/z 175, 145 and 119. These are the most abundant peaks in all the spectra of carotenoids.

Compound **A** (tR, 5.69 min) has a molecular ion peak $[M+H]^+$ of 602.43. There were product ion peaks of m/z 584.42 and 566.41 likely due to loss of H_2O molecules, $[M+H-H_2O]$ and $[M+H- H_2O-H_2O]$ respectively. It has characteristic peaks to epoxy carotenoids. This compound has similarity in spectrum to compound D which was also an epoxy carotenoid. However, no literature report similar to this compound was found. The compound was therefore not fully identified.

Compound **B** (tR, 9.91 min) with molecular ion, $[M+H]^+$ of m/z 569.43 and $[M+NH4]^+$ of m/z 584.42 had molecular formula determined as $C_{40}H_{56}O_2$ (calculated for $C_{40}H_{57}O_2$, 569.4361). The compound was tentatively identified as either lutein or zeaxanthin. The ms/ms was not obtained for this compound therefore the fragmentation pattern could not be determined. The structures are shown in Figure 4.17.

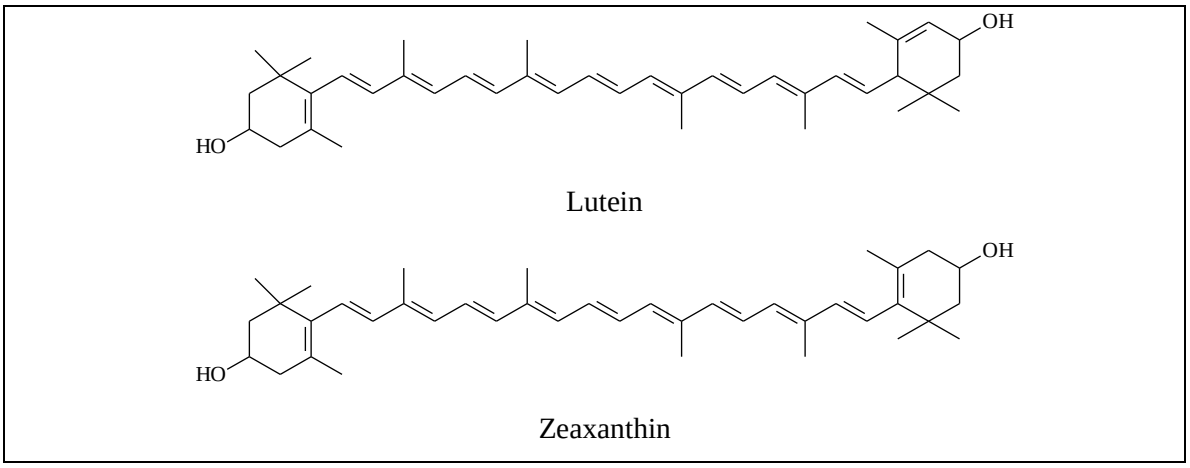


Figure 4. 17: Possible Structures of Compound A (Lutein or Zeaxanthin)

Compounds **C** and **E** (tR, 9.96 min and 12.12 min) of m/z 551.42 were isomers of molecular formula $C_{40}H_{54}O$ (calculated for $C_{40}H_{55}O$, 551.4256). The chromatogram of compounds B and D showed 2 main peaks at retention times 9.89 and 12.09 minutes respectively, which indicated that there were isomers as shown in Figure 4.18.

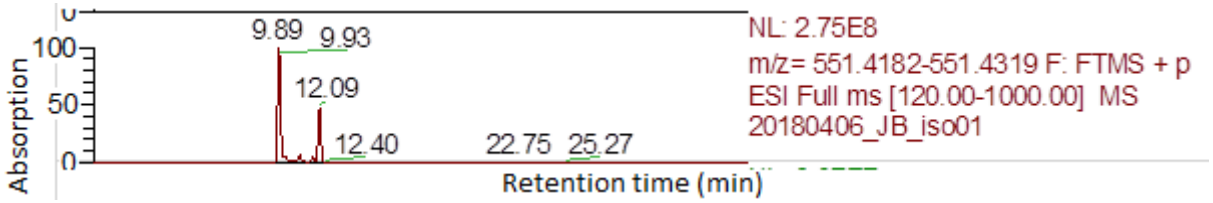


Figure 4. 18: HPLC chromatogram of compounds with m/z of 551.42

The fragmentation pattern is indicative of monohydroxy carotenoids which showed loss of water molecule $[M+H-H_2O]^+$ of m/z 533.42. The ratio of $[M+H]^+/[M+H-H_2O]^+$ was much higher than 1 indication the presence of an non-allylic OH on the ring (Mercadante *et al.*, 1997). These are characteristic fragmentation patterns of anhydrolutein II and III (Figure 4.19) as reported in literature (Maoka et al., 2005). Anhydroluteins rarely occur

in plants. They were first identified in a dietary source as artefacts or as products of lutein metabolism by Waldron *et al* 1993. They are often isolated from human plasma as dehydration products of lutein (Krinsky *et al.*, 2004).

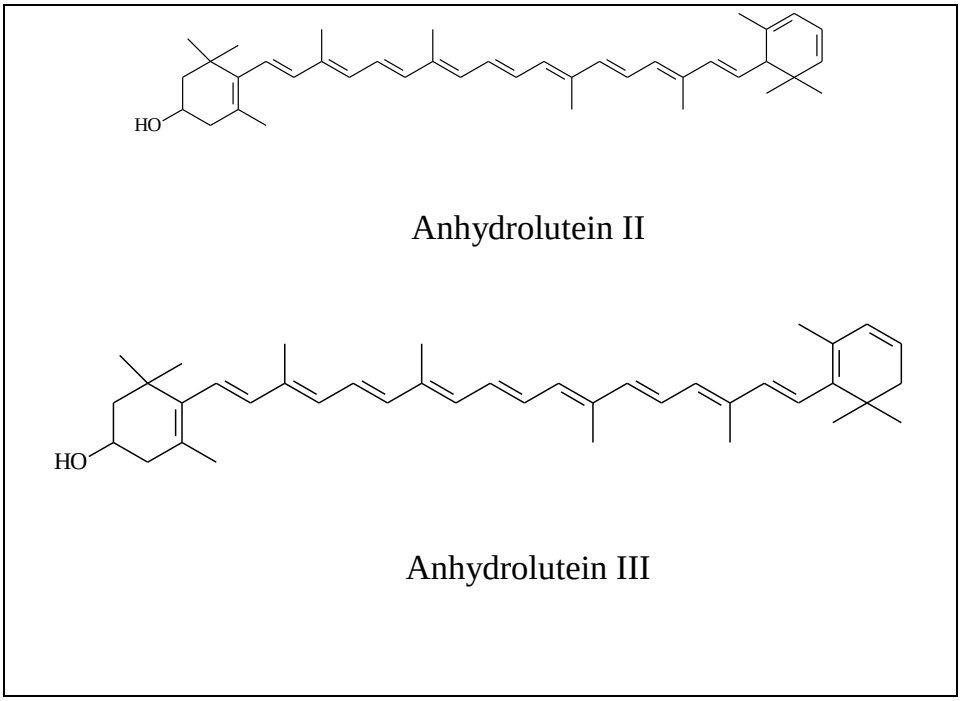


Figure 4. 19: Chemical structures of Anhydrolutein II and Anhydrolutein III

Compound **D** (tR, 10.00 min) with pseudo molecular ion $[M+H]^+$ of m/z 585.43 with molecular formula $C_{40}H_{56}O_3$ (calculated for $C_{40}H_{57}O_3$, 585.431) had peaks of m/z 352, 221 and 181 which are characteristic of epoxy carotenoids. The compound was tentatively identified Lutein 5, 6 epoxide (Figure 4.20) based on similar mass spectrum reported previously (Koizumi *et al.*, 2018). The presence of $[M+H-18]^+$ and $[M+H-18-18]^+$ of m/z 567.42 and 549.35 respectively differentiates this compound from its isomer antheraxanthin, which usually lacks these peaks (Meléndez-Martínez *et al.*, 2005).

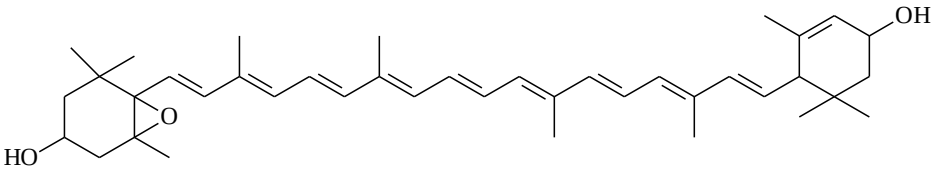


Figure 4. 20: Chemical Structure of lutein 5, 6-epoxide

The summary on data of carotenoids found in *Justicia betonica* is illustrated in Table 4.5.

Table 4. 5: Identified carotenoids from *Justicia betonica*

No.	tR (min)	$[M+H]^+$	Mol. Formulae	ms^2	Proposed Identity
A	5.69	602.43		[602.43], 584.42 (30), 566.41 (10), 464.33 (10), 351.27 (20), 175.15 (50), **145.10 (100),** 119.09 (80).	Not identified
B	9.91	569.43	$C_{40}H_{56}O_2$	-	Lutein/Zeaxanthin
C	9.96	551.42	$C_{40}H_{54}O$	[551.4241], 533.4155 (2), 175.1481 (70), 145.1012 (70) **119.0857 (100)**	Anhydrolutein I/ II
D	10.00	585.43	$C_{40}H_{56}O_3$	[585.4229], 567.4232 (20), 549.4106 (10), 492.3541 (10), 157.1011 (60), 145.1012 (90), **119.0857 (100)**	Lutein 5, 6-epoxide
E	12.12	551.42	$C_{40}H_{54}O$	[551.4242], 533.4143 (2), 175.1481 (70), 145.1012 (70), **119.0858 (100)**	Anhydrolutein I/ II

Key: [ ] - molecular ion; ( )- percent peak; bold –base peak

4.3.3.2 Indoloquinolines

The second class of compounds identified from *Justicia betonica* were indole quinoline alkaloids.

Compound **F**, (tR, 4.59 min) with a molecular ion peak $[M+H]^+$ at 235.0865 had molecular formula, $C_{15}H_{10}N_2O$ (calculated for $C_{15}H_{11}N_2O$, 235.0872). The ms^2 data was not available for this data. However, based on previously isolated compounds from *Justicia betonica*, this compound was tentatively identified as 5H, 6H Quinindolin-11-one whose chemical structure is shown in Figure 4.21 (Subbaraju *et al.*, 2004).

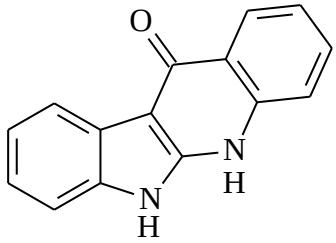


Figure 4. 21: Chemical structure of 5H, 6H Quinindolin-11-one

There were 8 isomeric compounds (compounds **G-N**) with m/z of 219.09 which eluted between 3.6 minute and 4.8 minutes as seen in Figure 4.22.

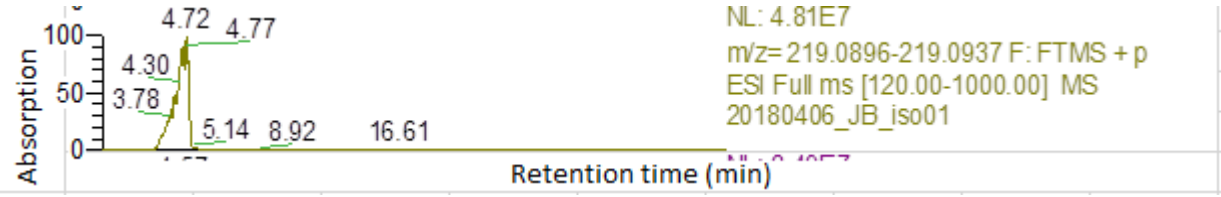


Figure 4. 22: HPLC chromatogram of compounds with m/z of 219.09

These m/z values of 219.09 with molecular formula $C_{15}H_{10}N_2$ (calculated for $C_{15}H_{11}N_2$, 219.0923) corresponds to unsubstituted indoloquinoline molecular ion $[M+H]^+$. There are four possible isomers of indoloquinoline molecule namely, indolo[3,2-b]quinoline, indolo[2,3-b] quinoline, Indolo[3,2-c]quinoline and indolo [2,3-c]quinoline reported in literature. So far, only indolo [2,3-c]quinoline, has not been isolated from natural sources (Aksenov *et al.*, 2017). Their chemical structures are shown in Figure 4.23.

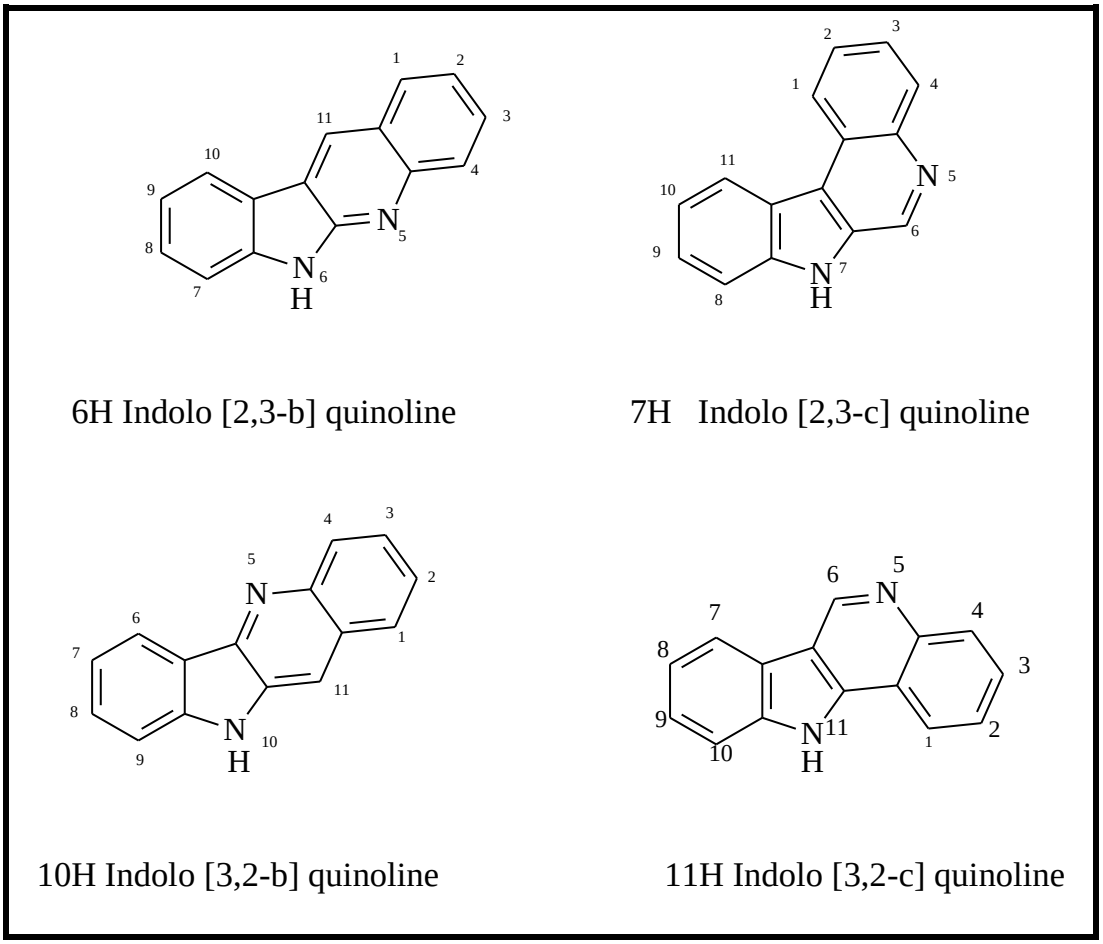


Figure 4. 23: Chemical structures of indoloquinoline isomers.

From this study, we propose that four (4) of the identified isomeric compounds include all these possible isomers shown in Figure 4.19. Indolo [3,2-b] quinoline (Quindoline) and Indolo [2,3-b] quinoline (quinindoline) were isolated from *Justicia betonica* (Subbaraju *et al.*, 2004). Indolo [3, 2-c] quinoline was isolated from the West African plant, *Cryptoleria sanguinolata* together with other related indoloquinolines. The indolo [2, 3-c] quinoline has so far not been isolated from natural sources (Afonso *et al.*, 2016; Fanny & Klaus, 2013; Prakash & Perunninakulath, 2016). The other 4 compounds could not be identified based on literature and therefore are possible new compounds.

Table 4.6 shows the summarized data and identity of indoloquinoline alkaloids from *Justicia betonica*

Table 4. 6: Indoloquinoline alkaloids identified from *Justicia betonica*

No.	tR (min)	$[M+H]^+$	Mol. Formulae	ms^2	Proposed Identity
F	4.59	235.0865	$C_{15}H_{10}N_2O$	-	5H, 6H Quinindolin-11-one
G-N	3.61 3.78 3.96 4.17 4.35 4.53 4.71 4.88	219.09	$C_{15}H_{10}N_2$	[219.09], 218.08(20), 165.07(10), 106.07(5)	10H Indolo [3,2-b] quinoline (Quindoline) 6H Indolo [2,3-b] quinoline (Quinindoline) 11H Indolo [3,2-c] quinoline 7H Indolo [2,3-c] quinoline 4 other new compounds

Key: [ ] - molecular ion; ( ) - percent peak; mol - molecular

4.4 Discussion

This study reports the identification of 20 compounds from *Leucas calostachys* for the first time. However, several other flavonoids have been isolated from the genus *Leucas*. They include baicalein, cirsimaritin, acacetin, chrysoeriol, apigenin, 5-hydroxy-7,4′-dimethoxyflavone, tricin, pillion and gonzalitosin I among others (Chouhan & Singh, 2011). Flavonoids are widely distributed plant polyphenolic compounds which differ in structures based on the degree of hydroxylation, polymerization and conjugation. Based on the variation in chemical structure, flavonoids are classified into 6 sub-classes; flavones, flavonols, flavan-3-ols, flavanonol, flavanones and isoflavones. They occur in plants as aglycones, their methyl derivatives or as glycosides.

The biological activities of flavonoids are structure dependent (Kumar & Pandey, 2013). They possess various pharmacological activities including; anti-oxidants, antiviral, antibacterial, antifungal and antiplasmodial properties. The hydroxyl groups in flavonoids are responsible for the anti-oxidant activity through free radical scavenging and metal chelation. Flavonoids also offer protection against various diseases by inducing protective enzymes within the body (Kumar *et al.*, 2013).

Flavonoid containing plants are important in the management of malaria. Malaria pathogenesis is associated with inflammation and oxidative stress (Ty *et al.*, 2019). The anti-inflammatory and anti-oxidant activity of flavonoids, coupled with the induction of

protective enzymes are important in preventing/slowing the progression of malaria infection. In addition, flavonoids are known to possess antiplasmodial effects. They inhibit the growth of malaria parasites as well as potentiating the effects of other antimalarial drugs such as artemisinins (Bilia *et al.,* 2008; Czechowski *et al.,* 2019). They are believed to act by inhibiting the biosynthesis of fatty acid within the malaria parasites (Ntie-Kang *et al.,* 2014). The active extracts from Kenyan antimalarial plants such as *Erythrina abyssinica*, *Milletia usaramensis* and *Derris trifoliate* are rich in flavonoids (Yenesew *et al.,* 2003; Yenesew *et al.,* 2004).

Several isolated flavonoids have shown *in vitro* antiplasmodial activities (Bero *et al.,* 2009; Vargas *et al.,* 2011) while some demonstrated β-hematin inhibition activity. In the study by Vargas *et al.,* 2011, apigenin and luteolin exhibited both *in vitro* antiplasmodial and β-hematin inhibition activities. However, apigenin-7-glucoside, isorhamnetin and quercetin only exhibited *in vitro* antiplasmodial activity. Moreover, some flavonoids such as catechine, epicatechine and morin lacked *in vitro* antiplasmodial activity but showed β-hematin inhibition activities. Among the identified flavonoids in this study, isorhamnetin, luteolin 7-O-glucoside and chrysoeriol 7-O-glucoside have showed moderate *in vitro* antiplasmodial activities against K1 *P. falciparum* in previous studies (Pan, W.-H. *et al.,* 2018).

Phenylethanoids are widely distributed in medicinal plants where they play an important role both in prevention and treatment of various diseases such as infections, inflammation

and tumors. They are also potent anti-oxidants (Xue & Yang, 2016). Several plants used in traditional medicine in management of malaria have phenylethanoids. *Stachytarpheta cayennensis* (Verbenaceae) commonly used in Latin America for treatment of malaria is rich in phenyethanoids such as acteoside, isoacteoside, leucosceptoside A, martynoside and jionoside. *Duranta erecta*, a herb used in China and India for management of malaria is also rich in phenylethanoid, acteoside, in addition to flavonoids and iridoid compounds (Srivastava *et al.*, 2019). Phenyethanoids also contribute to the antimalarial properties of plants such as *Ajuga laxmannii*, *Stachys lavandulifolia*, and *Grevillea "Poorinda Queen*" (Atay *et al.*, 2016; Delazar *et al.*, 2011; Ovenden *et al.*, 2011).

Justicia betonica is a well-known plant in folklore for treatment of malaria in Eastern Africa and Indian sub-continent (Corrêa & Alcântara, 2012; Okello, D. & Kang, 2019). Although the antiplasmodial activity of this plant is also well established, no phytochemicals were attributed to this effect. In this study, the active fraction was found to contain carotenoids and indoloquinoline alkaloids.

Indole quinoline alkaloids are a rare group of alkaloids that were first isolated from the West African plant, *Cryptolepis triangularis*. Cryptolepine, the major alkaloid in the plant exhibits strong activity against multidrug resistant strain of *P. falciparum*, K1 with activity against plasmodium gametocyte (Forkuo *et al.*, 2017). However, it is too toxic for use as antimalarial agent as it causes liver necrosis and damage to DNA (Ademir *et al.*, 2008; Rocha e Silva *et al.*, 2012).

Indoloquinolines alkaloids have also been isolated from the Amazonian *Aspidosperma* species which are used in traditional medicine for treatment of malaria by indigenous people of Brazil (Kffuri *et al.*, 2015). *Aspidosperma* contains alkaloids, ellipticine and olivacine, which have excellent antimalarial activity just like the cryptolepines. However they are less toxic compared to cryptolepine (Rocha e Silva *et al.*, 2012). The plant *Sida acuta* Burm. (Malvaceae) is commonly used in Ivory Coast by traditional healers in malaria treatment (Banzouzi *et al.*, 2004). An investigation into the antimalarial phytochemicals from this plant revealed it contains the indoloquinoline alkaloids; cryptolepine, quindoline, quindolinone and cryptolepinone among others (Aravind, 2017; Karou *et al.*, 2008). Indoloquinolines are therefore potential compounds in development of newer antimalarial drugs. They also act synergistically with artemisinins in *in vivo* and *in vivo* tests against malaria parasites (Forkuo *et al.*, 2016).

The antiplasmodial activities of some natural indoloquinolines are shown in Table 4.7 while their chemical structures are shown in Figure 4.24.

Table 4. 7: Antiplasmodial activities of natural indoloquinolines

Compound	Natural source	Antiplasmodial activity (IC_{50})		Reference
		CQ resistant	**CQ sensitive**	
Ellipticine	*Aspidosperma vargasii*	0.35-0.81μM	0.35μM	(Rocha e Silva *et al.*, 2012)
Olivacine	*Aspidosperma ulei*	1.2-1.4 μM	1.2 μM	(Rocha e Silva *et al.*, 2012)
Cryptolepine	*Cryptolepis sanguinolenta*, *Sida acuta*	0.20-0.91 μM	0.9 μM	(Grellier *et al.*, 1996; Rocha e Silva *et al.*, 2012)
Isocryptolepine	*Cryptolepis sanguinolenta*	0.8 μM	0.56 μM	(Grellier *et al.*, 1996)
Neocryptolepine	*Cryptolepis sanguinolenta*	1.5 μM	-	(Montalvo-Acosta & Ibarra, 2015)
Quindoline	*Cryptolepis sanguinolenta*, *Justicia betonica*, *Sida acuta*	>229 μM	>229 μM	(Paulo *et al.*, 2000)

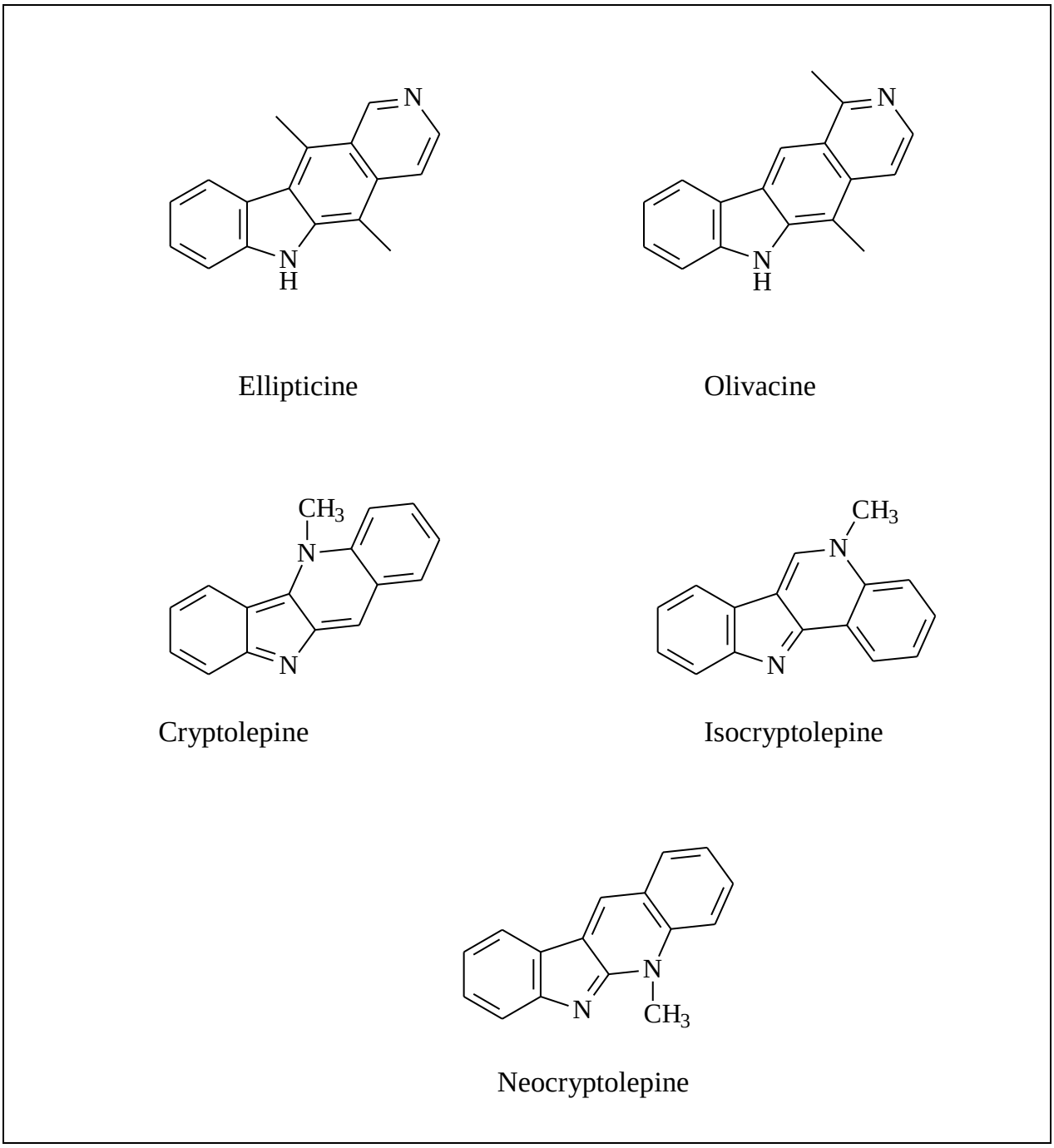


Figure 4. 24: Chemical structure of natural indoloquinolines with antimalarial activity

Indoloquinolines exert various biological activities through intercalation into DNA therefore making them cytotoxic (Lisgarten *et al.*, 2002). However, the antimalarial activity does not depend solely on the intercalation within DNA but partly to the inhibition of formation of hemozoin, similar to that of 4-aminoquinolines such chloroquine. (Onyeibor *et al.*, 2005; Wright *et al.*, 2001). Fanny and Klaus (2013) established that N-5 methylation is responsible for the DNA intercalation and thus cytotoxic effect of indoloquinolines. However, the indoloquinoline compounds present in *J. betonica* lack the N5 substitution and therefore less likely to have the cytotoxicity effect.

Justicia betonica also contains carotenoids which have also demonstrated *in vitro* activity against *P. falciparum*. Lycopene, a non-pro-vitamin A carotenoid induces parasite cytotoxicity by production of reactive oxygen species within the parasite (Agarwal *et al.*, 2014). Carotenoids are also known for their anti-oxidant and immune boosting properties (Milani *et al.*, 2017). All these effects are important in combating malaria infection. Past studies show that administration of carotenoids increases the potency of antimalarial drugs. For example, administration of lycopene increased the potency of chloroquine in Swiss albino mice infected with *P. berghei* parasites (Iswari *et al.*, 2016). Moreover, the provitamin A carotenoids provide Vitamin A, an important compound in prevention of malaria. Children in malaria endemic areas usually have a low levels of vitamin A. In a double-blinded placebo-controlled trial in Papua New Guinea, supplementation of Vitamin A decreased malaria infections by 30 % in school children (Shankar, 2000). It is

unclear how supplementation with vitamin A decreases malaria infection (SanJoaquin & Molyneux, 2009).

4.5 Conclusion

The study identified antiplasmodial compounds from *Leucas calostachys* Oliv.and *Justicia betonica* L. using LC-MS/MS. Flavonoids and phenylethanoids in *Leucas calostachys* Oliv. whereas carotenoids and indoloquinoline alkaloids in *Justicia betonica* L. are responsible for the antimalarial activity of these plants.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Ethnopharmacological approach plays an important role in identifying potential sources of new drugs. This is an interdisciplinary field involving traditional medicine practitioners, botanists, chemists and pharmacologists. In the current study of antimalarial plants used among the Luhya community of Western Kenya, 21 traditional herbal practitioners were involved. Most of the traditional medicine practitioners in this community had low level of education and were male. The traditional medicine knowledge in the community was acquired mainly through inheritance.

The plants used in Luhya community of Kakamega East sub-County are potential sources of antimalarial medicines. In this study, 41 plants were identified for management of malaria. Most of these plants belonged to the Lamiaceae family. Apart from *Rumex steudelii* Hochst.ex A. Rich and *Phyllanthus sepialis* Müll. Arg, all the other plants identified in this study have been reported elsewhere for treatment of malaria.

The traditional medicine practitioners obtain their plants from the wild which possess danger to the future sustainability of herbal medicine. Majority of these plants were shrubs whereas leaves were the commonly utilized parts. Decoction was the main form of herbal medicine preparation in managing malaria.

Two of the encountered plants, *Leucas calostachys* Oliv. and *Justicia betonica* L. were investigated for their antimalarial properties using both *in-vitro* antiplasmodial test and β-hematin inhibition assay. The methanol extract of *Leucas calostachys* Oliv. and the ethylacetate fraction of *Justicia betonica* L. exhibited high antimalarial properties.

The structure elucidation of antimalarial compounds in this study was achieved using liquid chromatography tandem mass spectroscopy (LC-MS/MS). This is a powerful analytical technique which is highly sensitive and selective and allows the characterization of molecules on a large scale. In this study, the spectroscopic data obtained was compared with data available in literature.

The bio-assay guided fractionation of *Leucas calostachys* Oliv. methanol fraction and the LC-MS/MS led to identification of 8 flavonoids and 12 phenylethanoids as the potential antimalarial compounds. The flavonoids included; isorhamnetin, luteolin-7-O-glucoside, luteolin-4′-O-glucoside, luteolin diglucoside, apigenin-O-glucoside, genistein-O-glucoside, chrysoeriol-7-O-glucoside and chrysoeriol-7-O-glucuronide while the phenylethanoids included; acteoside, isoactoeside, hydroxyacteoside, forthsoside B, samioside, alyssonoside and leucoseptoside A. Previous studies reveal the antimalarial properties of flavonoids and phenylethanoids. This is the first time antimalarial compounds have been identified from *Leucas calostachys* Oliv.

Analysis of antimalarial compounds from *Justicia betonica* L. led to identification of 13 compounds; 4 carotenoids (lutein/zeaxanthin, anhydrolutein I, anhydrolutein II and Lutein 5,6-epoxide) and 9 indoloquinoline alkaloids of which 5 were identified as 5H, 6H

quinindolin-11-one, 10H indolo [3,2-b] quinoline, 6H indolo [2,3-b] quinoline, 11H Indolo [3,2-c] quinoline and 7H Indolo [2,3-c] quinoline. However, 4 other indoloquinoline isomers could not be identified based on the LC-MS/MS and available literature. This is the first time of identifying antimalarial compounds from *J. betonica* L.

5.2 Recommendations

This study identified 41 antimalarial plants used among the Luhya community of Kakamega East sub-county. More ethnobotanical studies should be carried out to identify antimalarial plants in the entire Kakamega East sub-County.

Among the identified antimalarial plants in this study, 4 of them; *Rumex steudelii* Hochst.ex A. Rich, *Phyllanthus sepialis* Müll. Arg, *Physalis peruviana* L. and *Clerodenderon johnstonii* Oliv. have not been studied for any antimalarial properties. More research is needed to study their antimalarial properties. In addition, some of the plants have only been studied for their *in-vitro* antiplasmodial activities. Further work should be carried out to identify the antimalarial compounds from those plants exhibiting promising antimalarial activity such as *Croton macrostachuys* Hochst. ex Del., *Searsia natalensis* Bernh.ex C. Krauss, *Maesa lanceolata* Forssk and *Ocimum kilimandscharicum* Gürke.

Although antimalarial compounds were identified from the active fractions of *Leucas calostachys* and *Justicia betonica* in this study, further work is required to test their

individual antimalarial properties. Validation and safety studies should also be carried out on the active compounds. Although the active fractions and pure compounds should be standardized and commercialized.

Although LC-MS/MS is a quick and important technique in identifying compounds, some compounds could not be identified using available databases. Further work should be carried out using other techniques such as NMR to identify the new compounds. There will also be need of structure modification and study of structure activity relationship on the active isolated compounds.

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APPENDICES

Appendix 1: Volunteer Information and Prior Informed Consent (PIC) Form

UNIVERSITY OF NAIROBI

COLLEGE OF HEALTH SCIENCES

SCHOOL OF PHARMACY

DEPARTMENT OF PHARMACOLOGY AND PHARMACOGNOSY. P. O. Box 19676, 00202 NAIROBI, TEL. 0202 725099

Introduction

The practice of traditional/complementary medicine is common worldwide in management of various diseases. In Kenya, plants are often used in management of malaria. These plants can be developed further as a source of new antimalarial drugs. This study seeks to identify the plants used in management if malaria among the Luhya community.

Purpose for the interview

This interview seeks to determine the understanding of malaria among the Luhya community and identify plants commonly used in its management. The study will also look at their preparations, dosages and administration of these herbal drugs.

Rights of participants

Your participation in this study is voluntary and you may choose to withdraw from it at any time. You may be asked to identify and demonstrate how you prepare some of your medicine. All information you give us will be held in high confidentiality.

Benefits

All the information you give to us will be respected, preserved and maintained as intellectual property of the community. The community will be informed of the efficacy and toxicity of these plants in treatment of malaria. This is to encourage their proper use and conservation for the future generations. Any future benefits that may be accrued from this study in form of commercialization of products and patents will be provided for in accordance with prior mutual agreement with the community with the commercializing entity.

Assurance of anonymity and confidentiality

All the information that we shall obtain from you will be kept confidential and will not be shared with any other parties apart from the researchers. The information will only be used for the purpose of this study. Your name will not be used anywhere in the course of this study.

Ethics and Research Committee

The ethics committee in this study ensures that the research will be conducted in ethical manner and the rights of the participants are protected. In case your rights are violated, contact:

The Secretary, Prof. Chindia

The KNH/UoN Ethics and Research Committee

P.O. Box 19676-00202 Nairobi

Tel 020-2726300 Ext 44355

In case you require any further information, you may contact me or any of my supervisors whose contacts are indicated below:

Nillian Ayuma Mukungu

Department of Pharmacology and Pharmacognosy

University of Nairobi, Kenya

Tel : +254721291660

E-mail: nillyanne2004@yahoo.com

Supervisors:

1. Professor J.W. Mwangi

   Department of Pharmacology and Pharmacognosy

   University of Nairobi, Kenya

   Tel: +254 722825966

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2. Dr. F. A. Okalebo

   Department of Pharmacology and Pharmacognosy

   University of Nairobi, Kenya

   Tel: +254737434204

   E-mail: okalebof@yahoo.com

3. Dr. K.O. Abuga

   Department of Pharmaceutical Chemistry

   University of Nairobi, Kenya

   Tel: +254722984195

Assurance of anonymity and confidentiality statement

I do hereby assure you that ALL the information you have provided will be kept strictly anonymous and confidential and will be made available ONLY to the researchers named above and used only for research purposes. Any other use of the traditional knowledge and associated materials including but not limited to commercial development, may only proceed after concluding a co-operative research development agreement. Results of the work will be communicated to the community.

Any unauthorized commercial use of the above without the said agreement shall be subject to an automatic 20 – 50% royalty rate of gross revenue from such sales together with the damages.

Signature/Fingerprint: _____ Date: _____

(Participant)

Signature: _____ Date: _____

(Researcher)

Statement of Consent

I..... (name of participant) hereby give my full consent and conscious to participate in this study and declare that to the best of my knowledge the information that I have provided are true, accurate and complete.

Date..... (Signature/fingerprint of participant)…….

Witnessed by

…… Signature…….

Name

Appendix 2: Data Collecting Tool

TITLE OF THE STUDY

A. Eligibility Checklist

a) Has lived in Kakamega East sub-County for one year? Yes/No

b) Do you agree to take part in this study? Yes/No

c) Have you used plants in management of malaria? Yes/No

B. Demographic information

Sex.....

Age.....

Religion……

Village/Area of residence.....

Level of Education (Tick)

No formal education	
Primary	
Secondary	
Tertiary	
Technical/Vocational	

Type of practitioner

Herbalist	

practitioner	
Care giver in household	

C. Views on use of herbal medicines

i. Why would you prefer herbal products over conventional drugs?

ii. What are the shortcomings of herbal medicine?

D. Knowledge of malaria

a) What is malaria? *(Ask about the vernacular name and the causes of malaria*).

b) Have you managed someone suffering from malaria using herbs? Yes/No

c) If yes, how did the patients present? *(Probe about the symptoms of malaria known to the practitioner)*

E. Plants used in management of malaria

a) Mention the plants you have used in malaria management

i. ----------

ii. ----------

iii. ----------

iv. ----------

v. ----------

vi. ----------

vii. ----------

viii. ----------

ix. ----------

x. ----------

Among the mentioned plants, which ones are most effective?

Are there any plants used for prevention of malaria?

The form below will be filled for each plant mentioned above.

Data about medicinal plant and its use:

Plant (Local name).....

Habit (Tree/ Herb/ Shrub/Climber/…...)

Identification features…….

……..

……..

…….

Plant part used.....

Cultivated/ Wild.....

How is the availability of the plant in the plant in this area?

Easily available	
Sometimes	
Rarely	
Not found	

Method of plant preparation

Mode of administration.....

By mouth	
inhalation	
Taking bath	
Application on the skin	
Others (specify)	

Dosage (amount) Adults.....

Children…….

Frequency of administration

…….

Duration of usage ……

Can this plant be used by pregnant women?

When was the last time you used this plant?

Less than 30 days ago	
More than a month	
Last year	

Any other plant(s) used together with the plant

If mixed, how do you mix the various components?

What is the role of each of the components?

Other uses (if any)…….....…….....

Side effects associated with use of the plant.

Any special considerations necessary for the plant

(E.g. time of harvesting, geographical location, maturity, season to harvest).

Could you please tell me what influences your choice of plants to use for malaria management?

F. Is the key informant willing to show the plants they use? If yes, conduct a field visit and collect samples.

Remarks:

Plant identified as …… (Botanical name and family)

Signature of Researcher

Appendix 3: Ethics Approval Letter

UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
P O BOX 19676 Code 00202
Telegrams: varsity
(254-020) 2726300 Ext 44355

KNH/UON-ERC
Email: uonknh_erc@uonbi.ac.ke
Website: http://erc.uonbi.ac.ke
Facebook: https://www.facebook.com/uonknh.erc
Twitter: @UONKNH_ERC https://twitter.com/UONKNH_ERC

KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP, Nairobi

13 JUL 2015

Ref: KNH-ERC/A/312

13th July 2015

Nillian Ayuma Mukungu
U803/98508/2015
Dept.of Pharmacology and Pharmacognosy
School of Pharmacy
University of Nairobi

Dear Nillian

RESEARCH PROPOSAL – ETHNOPHARMACOLOGY OF ANTIMALARIA PLANTS USED AMONG THE LUHYA COMMUNITIES IN KAKAMEGA COUNTY (P186/03/2015)

This is to inform you that the KNH/UoN-Ethics & Research Committee (KNH/UoN-ERC) has reviewed and **approved** your above proposal. The approval periods are 13th July 2015 12th July 2016.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH/UoN ERC before implementation.
c) Death and life threatening problems and serious adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH/UoN ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH/UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Clearance for export of biological specimens must be obtained from KNH/UoN-Ethics & Research Committee for each batch of shipment.
g) Submission of an *executive summary* report within 90 days upon completion of the study This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/or plagiarism.

For more details consult the KNH/UoN ERC website **www.erc.uonbi.ac.ke**

Yours sincerely,

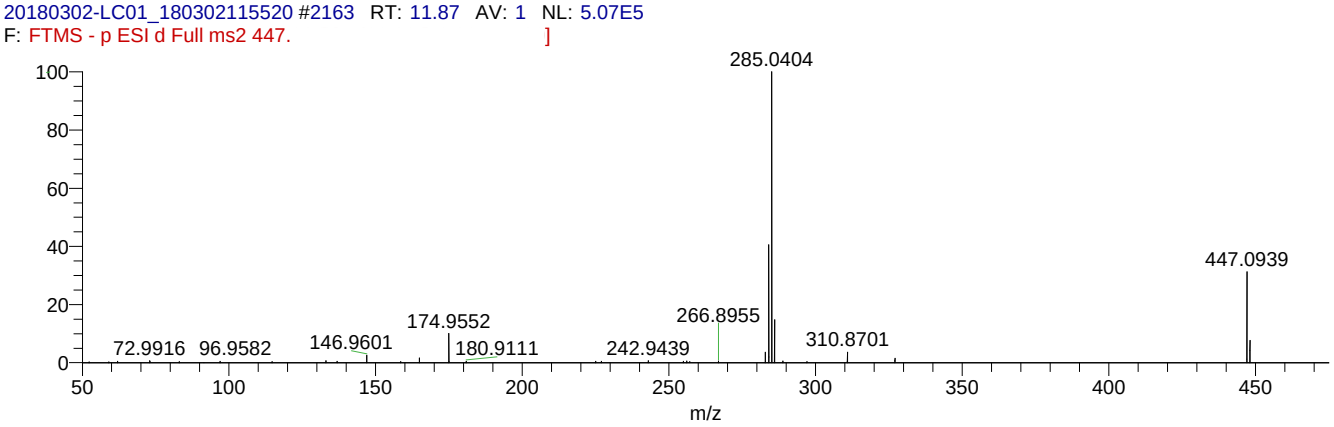
PROF. M. L. CHINDIA
SECRETARY, KNH/UON-ERC

Appendix 4: Mass spectrum of identified Compounds

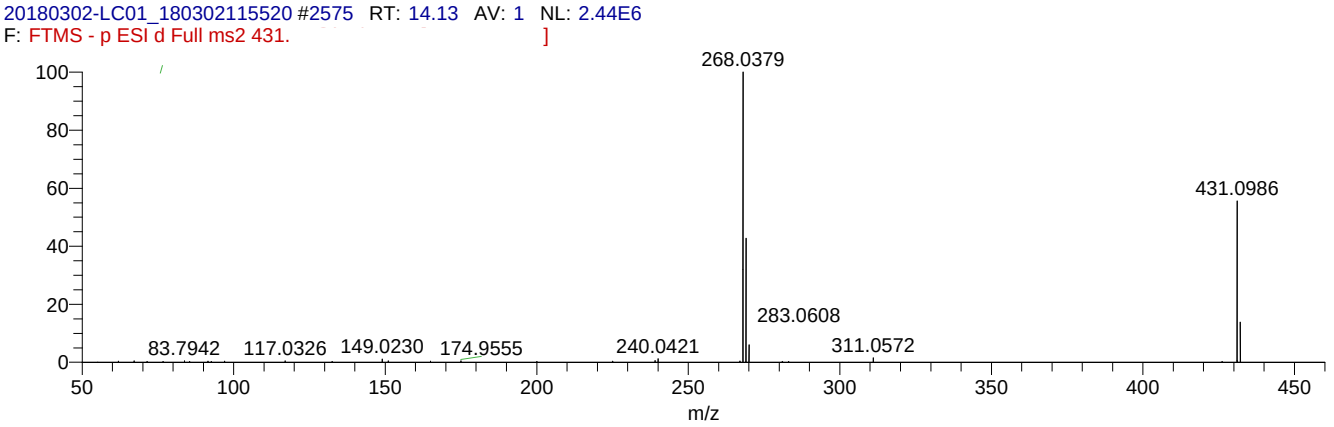
Appendix 4 a: *Leucas calostachys*

I Flavonoids (Compounds 1-8)

Compound **1**

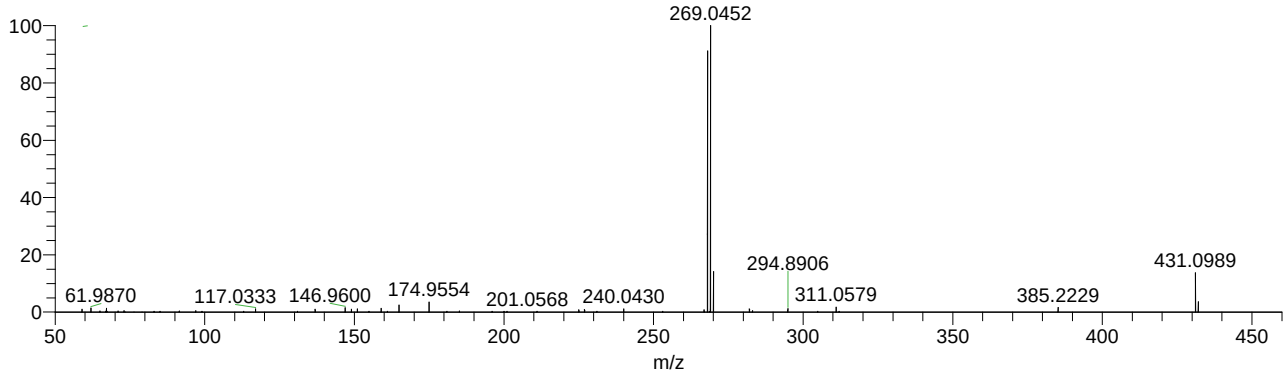


Compound **2**



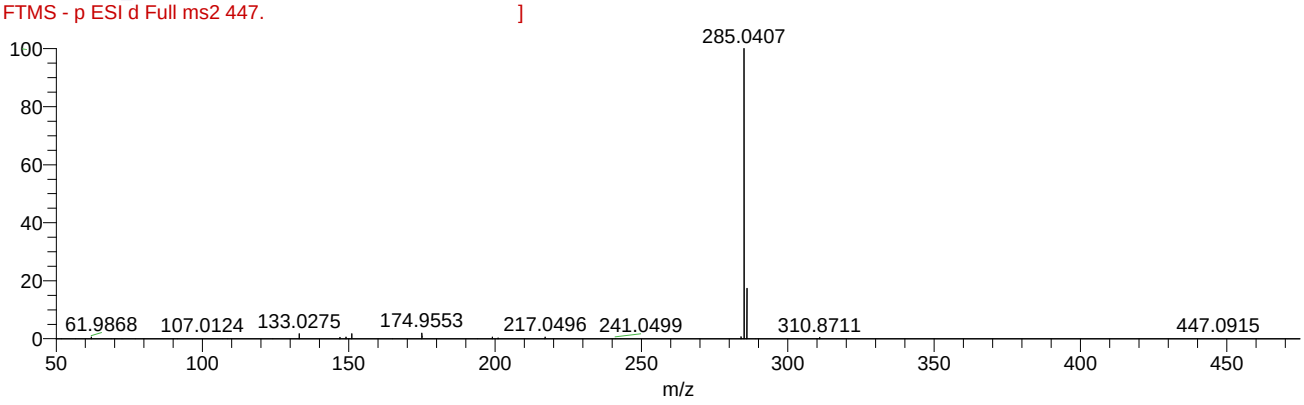
Compound **3**

20180302-LC01_180302115520 #2751 RT: 15.10 AV: 1 NL: 5.12E5
F: FTMS - p ESI d Full ms2 431.]



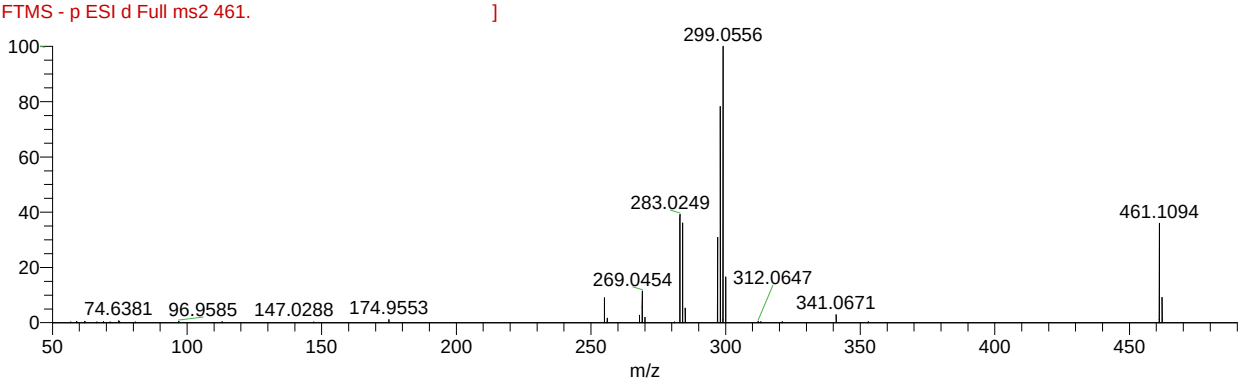
Compound **4**

20180302-LC01_180302115520 #2787 RT: 15.30 AV: 1 NL: 3.56E6
F: FTMS - p ESI d Full ms2 447.]

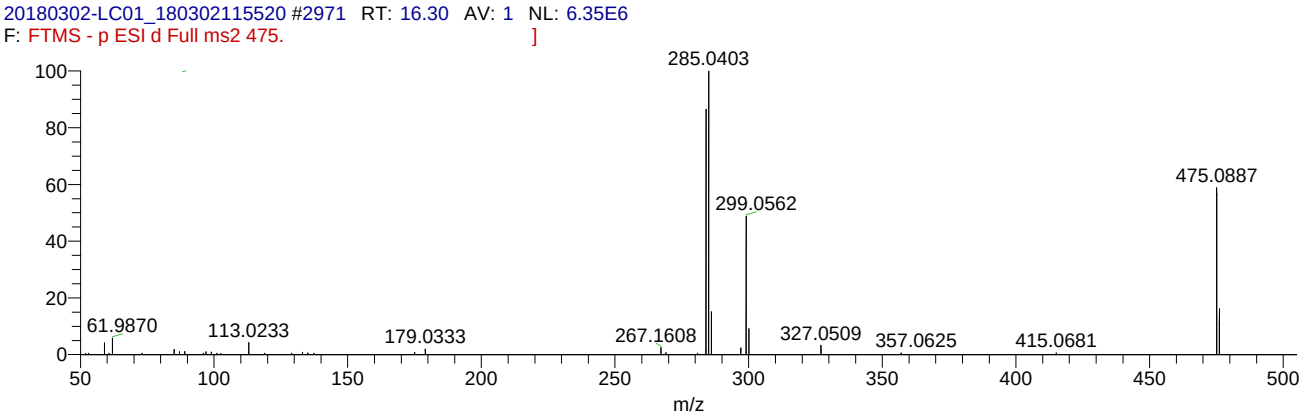


Compound **5**

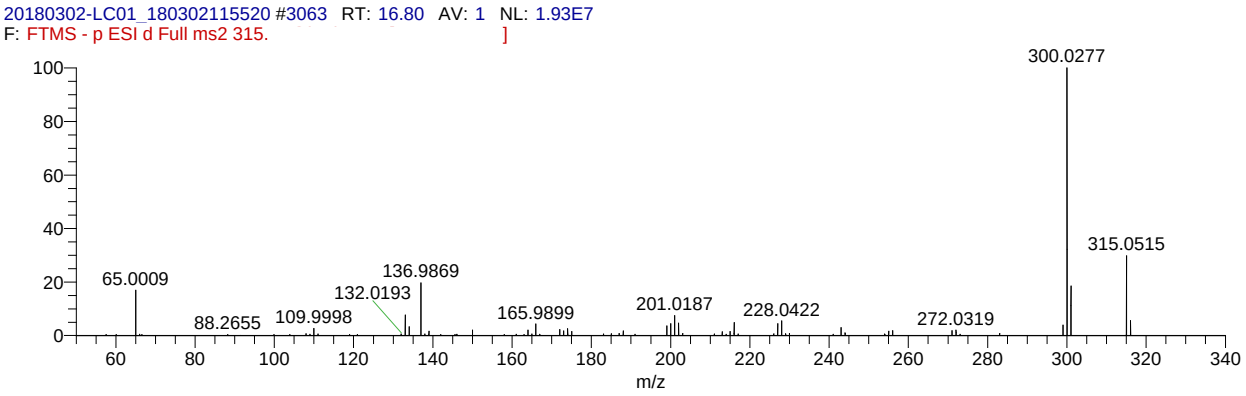
20180302-LC01_180302115520 #2843 RT: 15.61 AV: 1 NL: 1.82E6
F: FTMS - p ESI d Full ms2 461.]



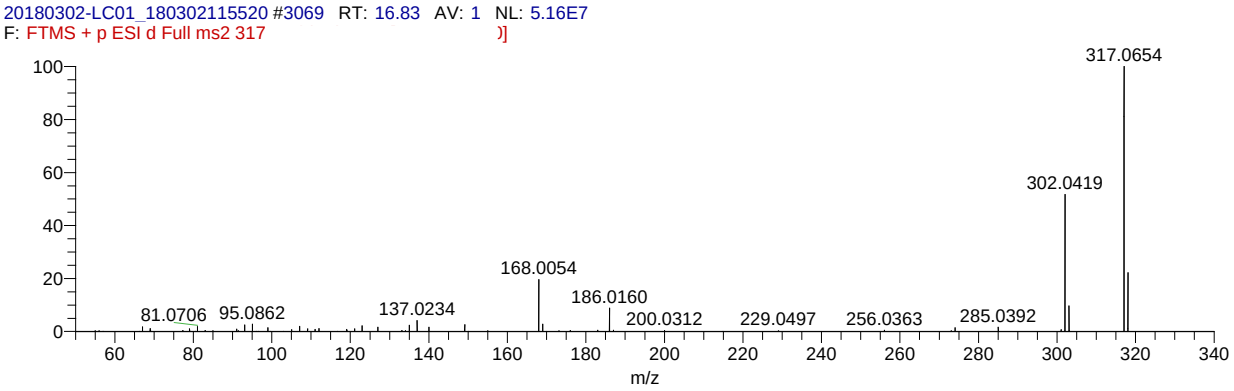
Compound **6**



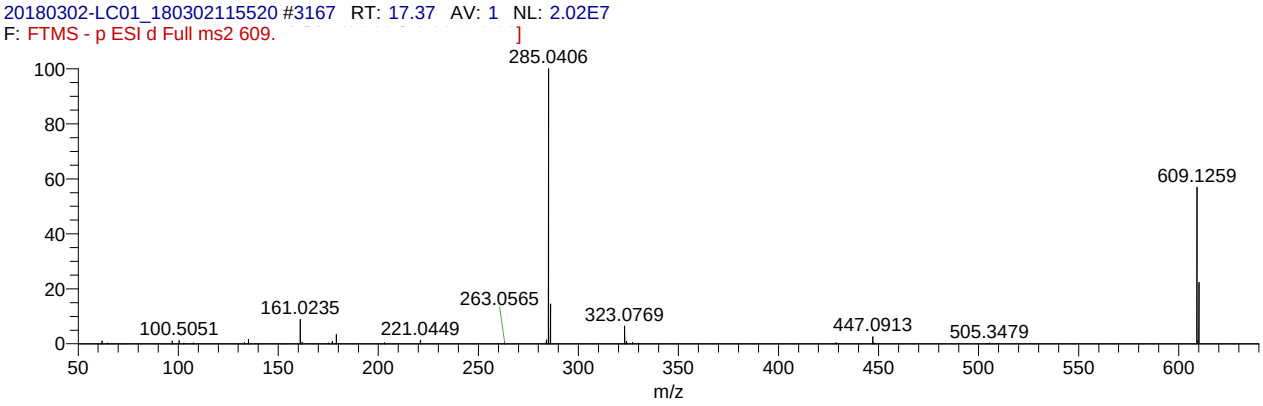
Compound **7** (deprotonated)



Compound **7** (protonated)

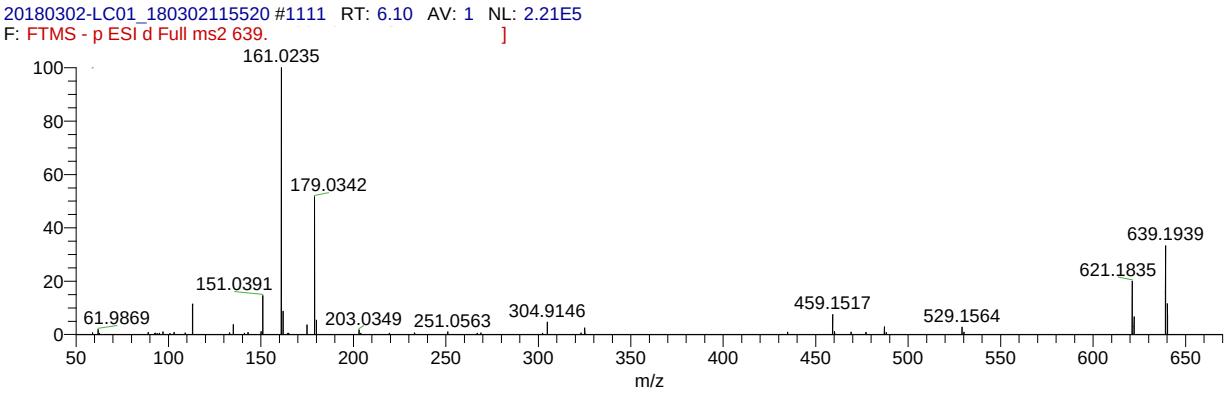


Compound **8**

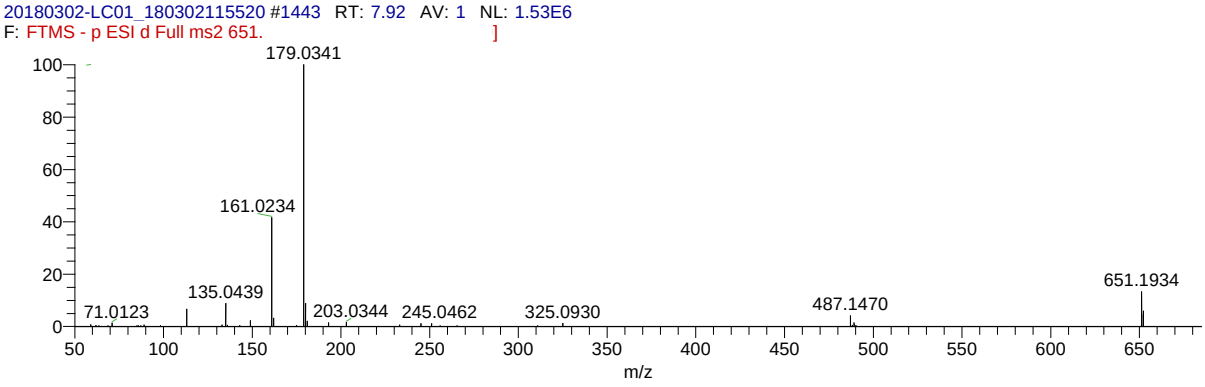


II Phenylethanoids (Compounds 9-20)

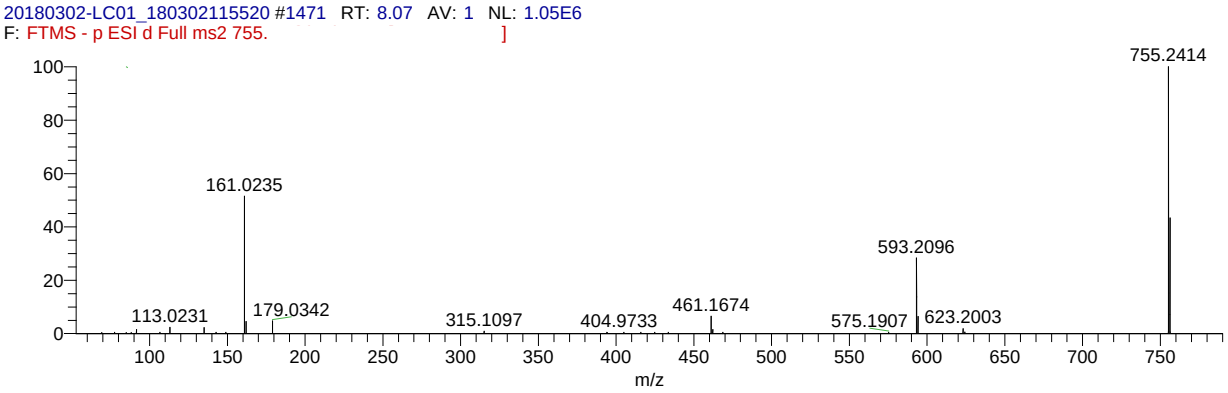
Compound **9**



Compound **10**



Compound **11**



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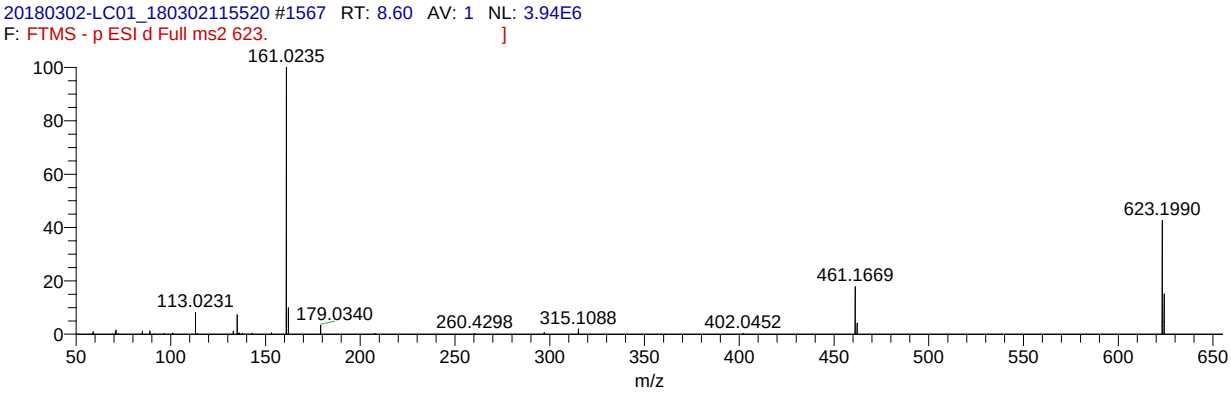

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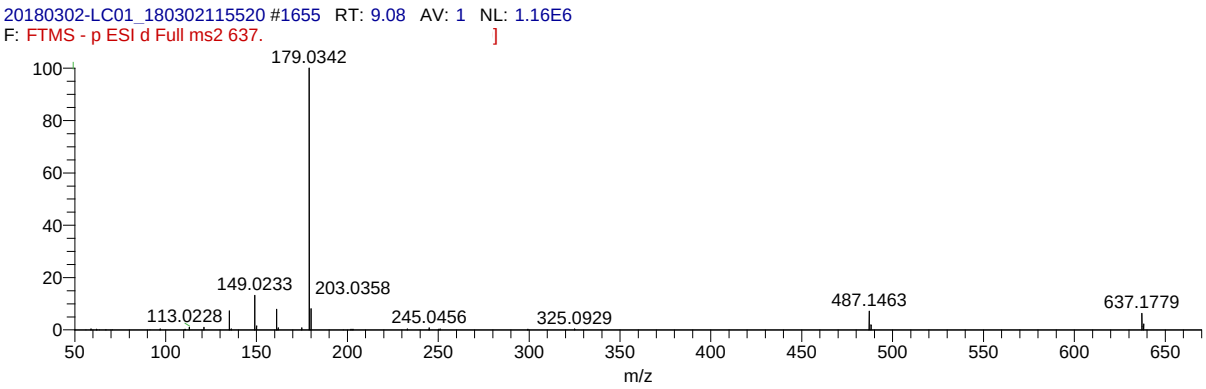

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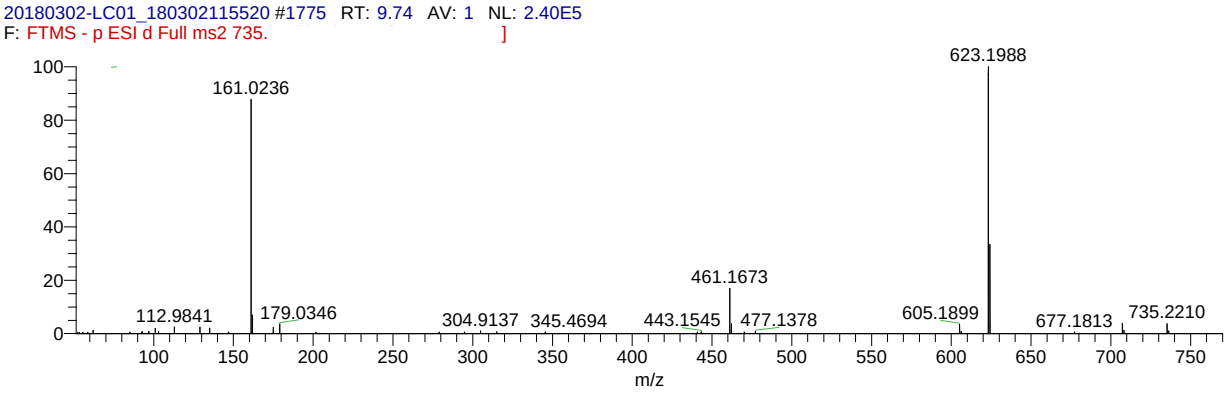

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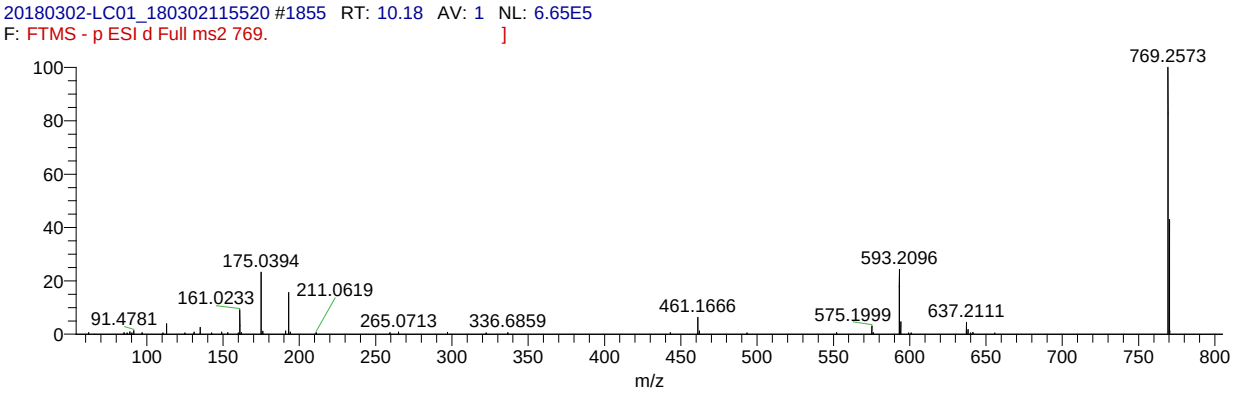

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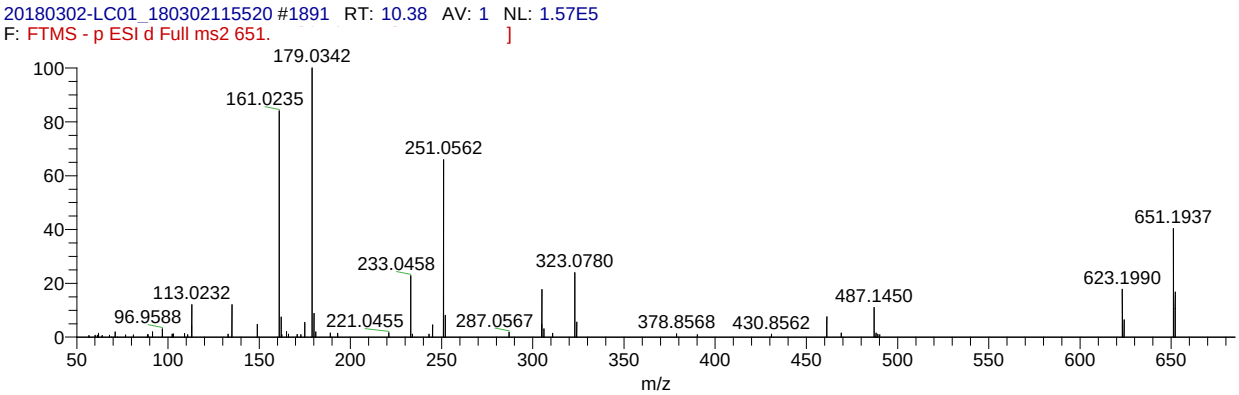

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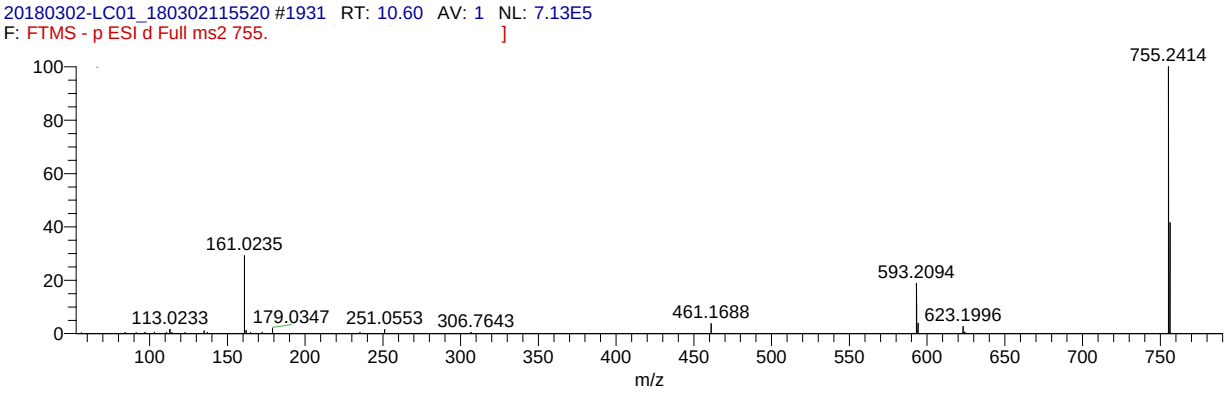

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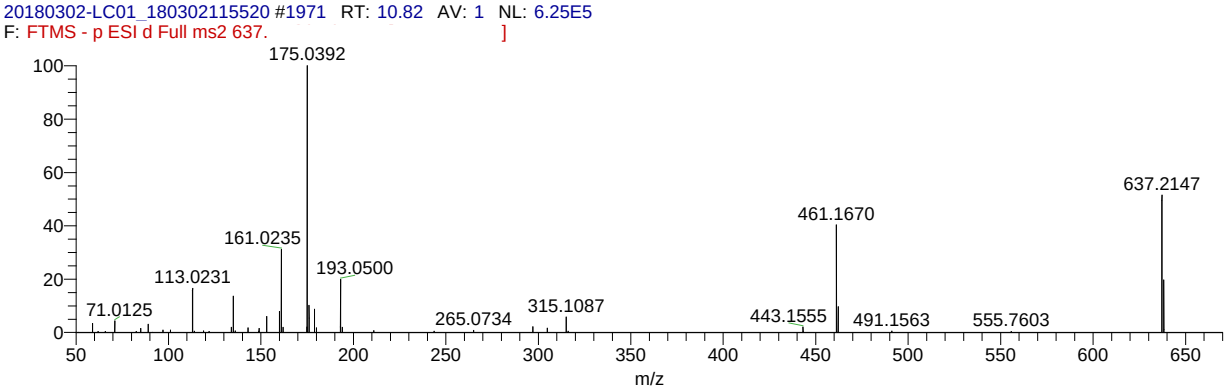

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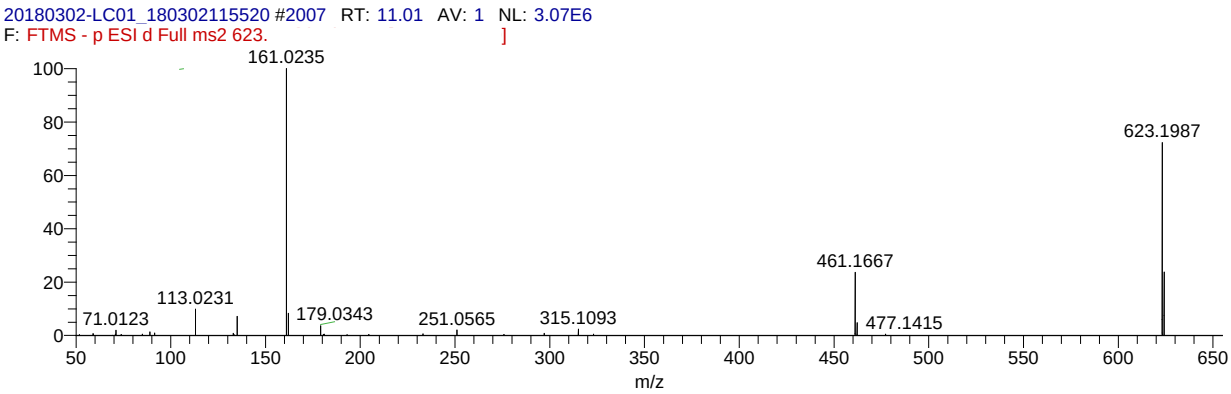

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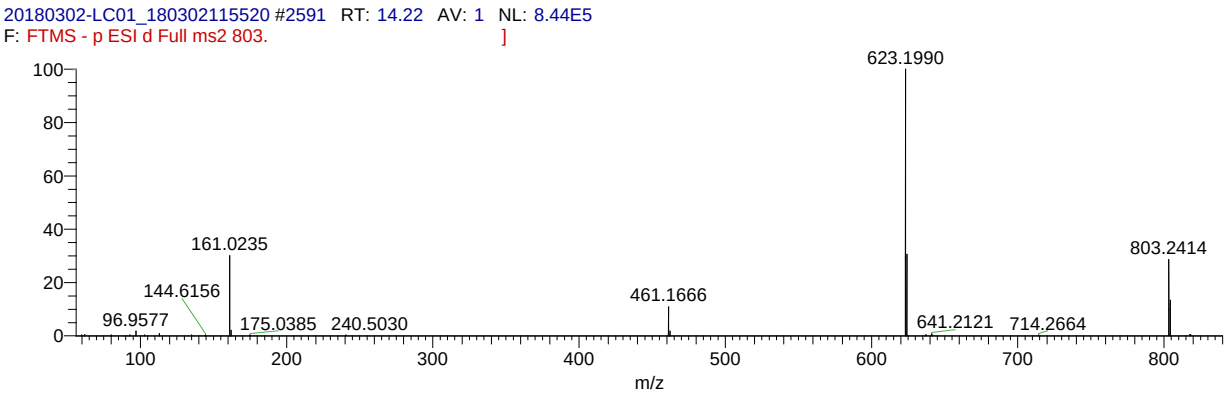
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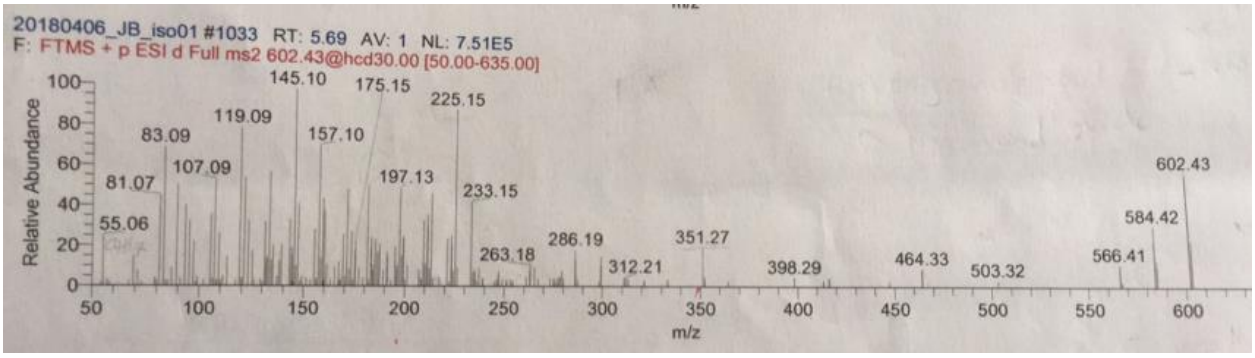
F: FTMS - p ESI d Full ms2 803.]
623.1990
161.0235
803.2414
144.6156
461.1666
96.9577
175.0385
240.5030
641.2121
714.2664
100
80
60
40
20
0
100
200
300
400
500
600
700
800
m/z

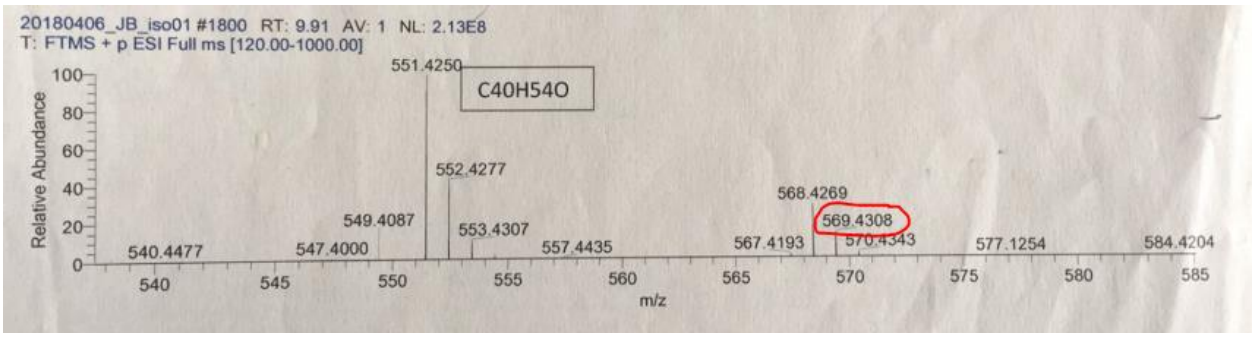
Appendix 4 b: *Justicia betonica*

I Carotenoids

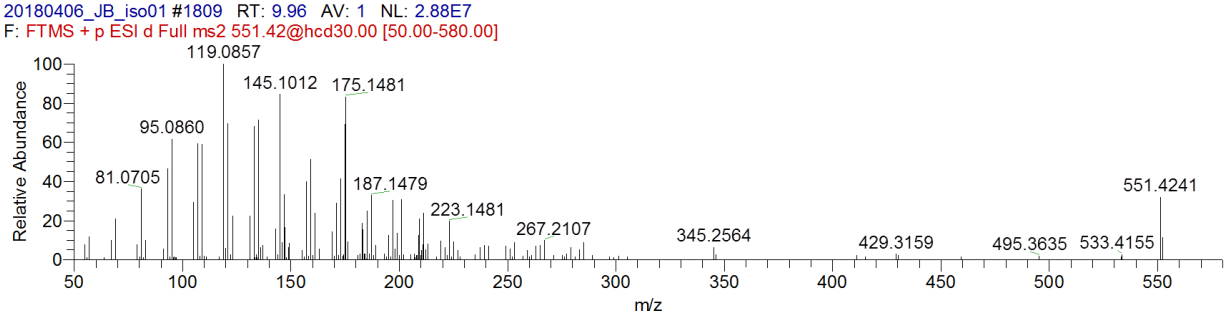
Compound **A**



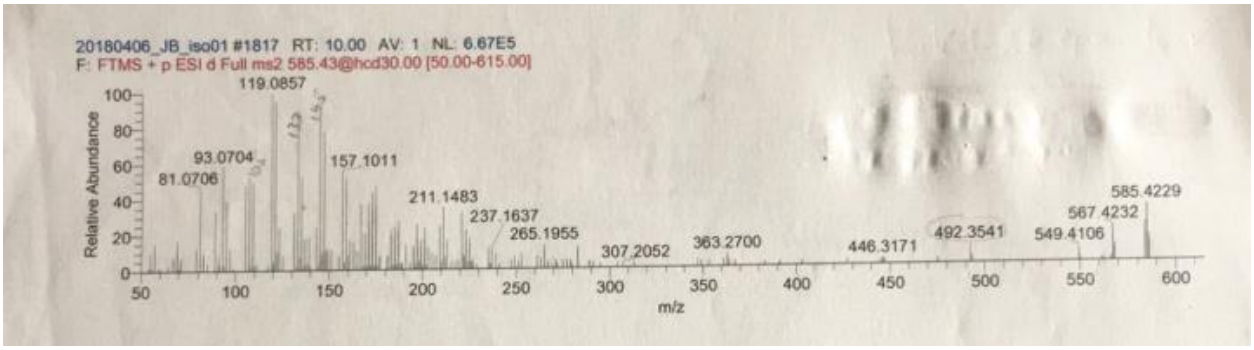
Compound **B**



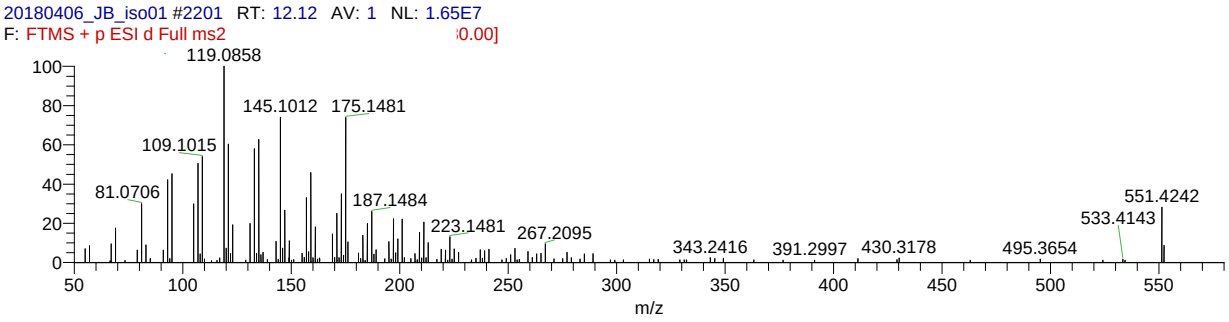
Compound **C**



Compound **D**

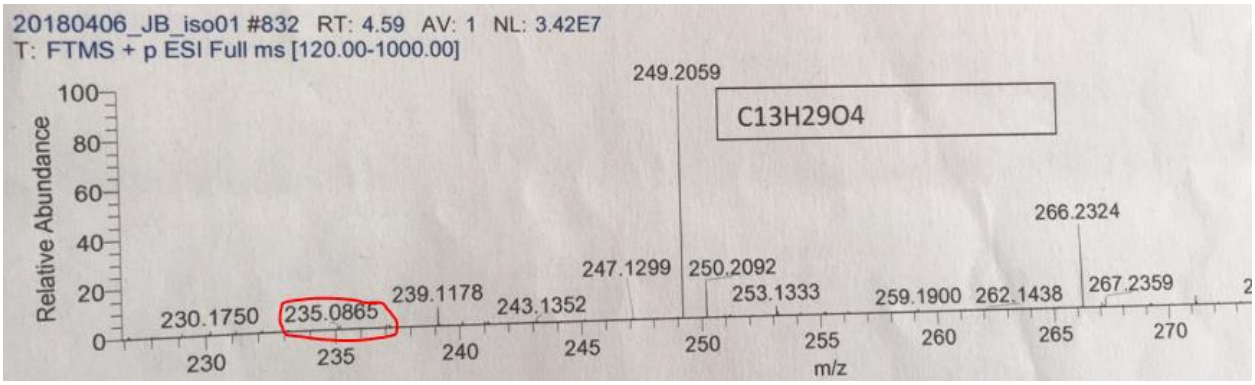


Compound **E**

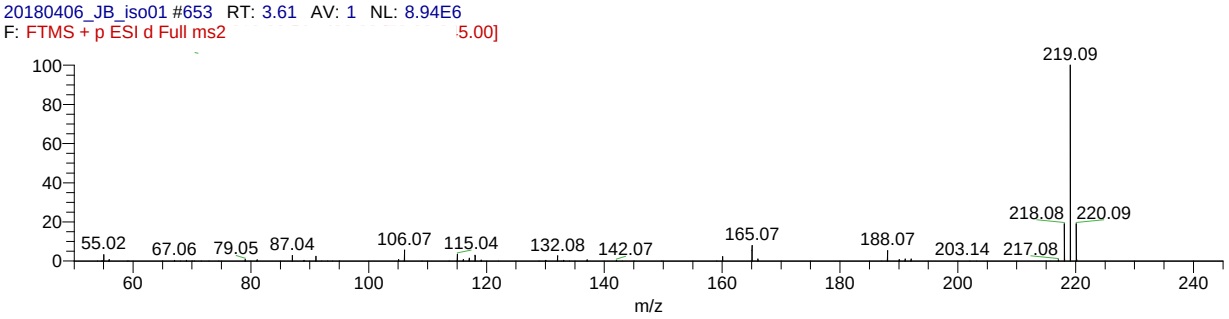


II Indoloquinolines alkaloids

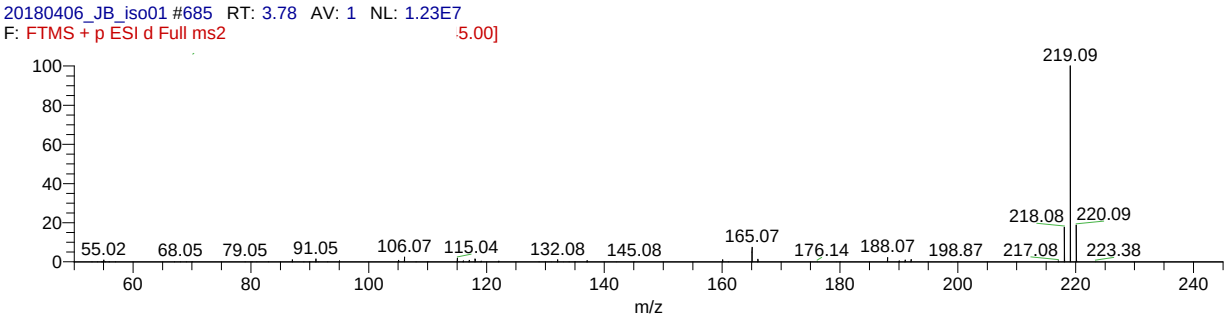
Compound **F**



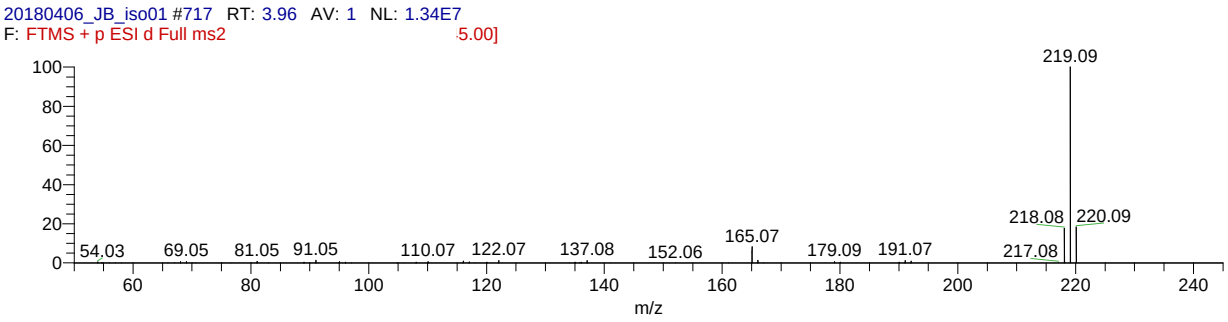
Compounds **G**



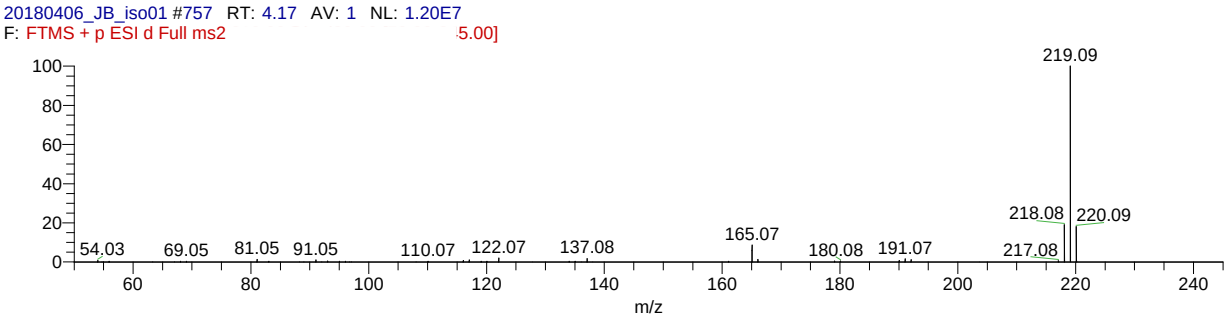
Compounds **H**



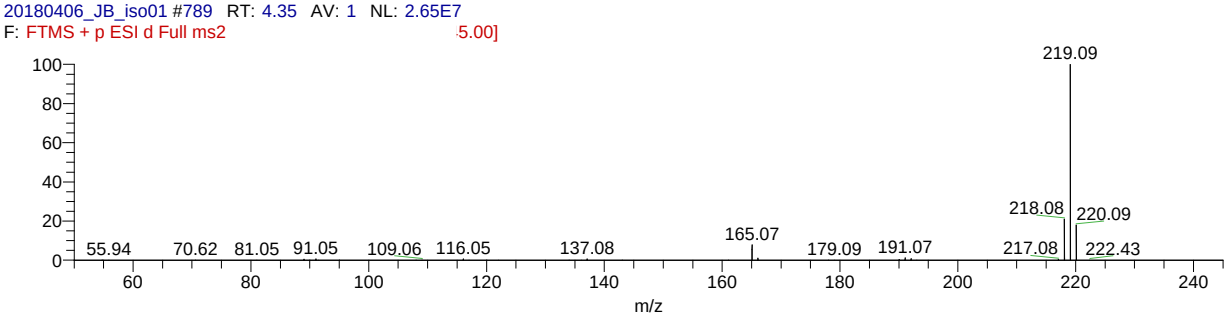
Compounds **I**



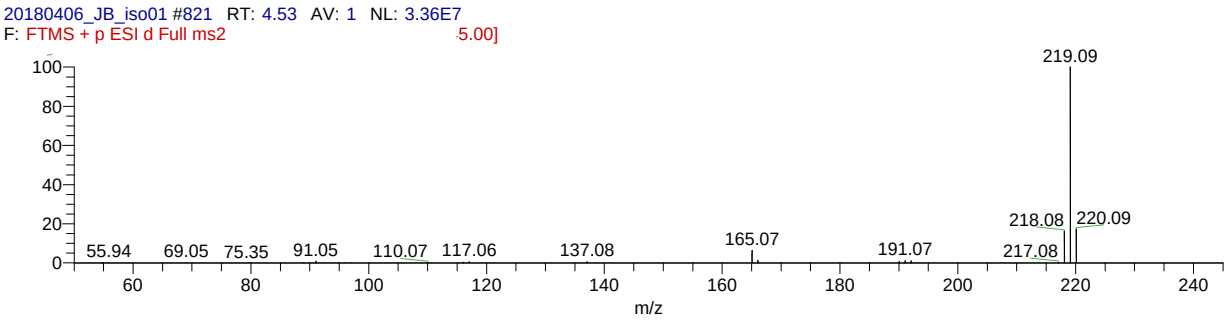
Compounds **J**



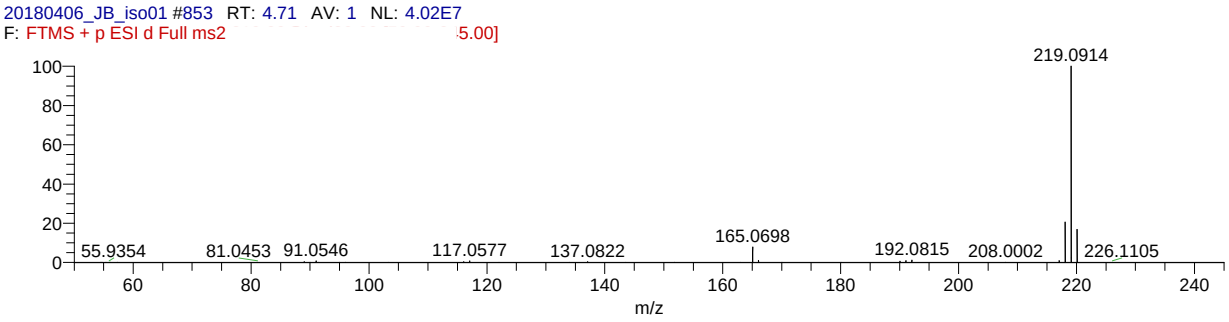
Compounds **K**



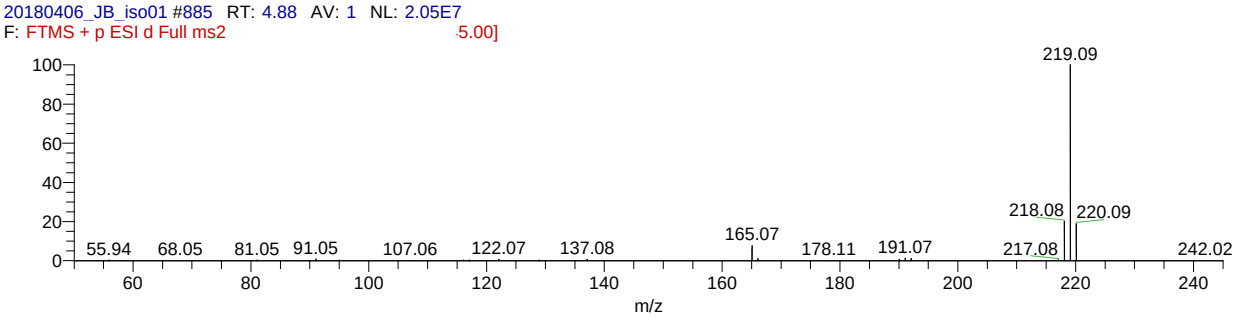
Compounds **L**



Compounds **M**



Compounds **N**



Appendix 5: Published work

Part of this thesis work has been published in refereed journals as part of the requirements for PhD studies at the University of Nairobi. The full published papers are hereafter annexed for reference.

Citation:

Mukungu N.A., Abuga K.O., Okalebo F.O., Ingwela R.T. and Mwangi J.W. (2016). Medicinal plants used for management of malaria among the Luhya community of Kakamega East sub-County, Kenya. *J. Ethnopharmacol.* 194, 98–107