IDENTIFICATION OF ANTIMALARIAL AND LARVICIDAL COMPOUNDS FROM *Trichilia megalantha* HARMS AND *Trichilia welwitschii* C.D.C. (MELIACEAE)

BY

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ABSTRACT

Malaria is the second leading cause of death from infectious diseases in Africa. The global burden of malaria is increasing due to drug-resistant parasites and insecticide-resistant mosquitoes. Meliaceae has been known to possess antimalarial, larvicidal and other properties but many Nigerian *Trichilia s*pecies have not been investigated. Thus there is dearth of information on the antimalarial and larvicidal activities of *Trichilia megalantha* (TM) and *Trichilia welwitschii* (TW). Therefore, this study investigated the antimalarial and larvicidal activities of both plants.

Leaf, stem bark and root of TM (FHI 109556) and TW (FHI 109557) were extracted by maceration in methanol. The extracts (100-800 mg/kg) were subjected to *in vivo* mouse-model 4-day suppressive antimalarial evaluation using *Plasmodium berghei* ANKA strain. Toxicity was determined *in vivo* in mice and *in vitro* using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and brine shrimp lethality (BSL) assays. The larvicidal activities of extracts and compounds were evaluated on *Anopheles gambiae* larvae. Antioxidant activity was also investigated with 1, 1- diphenyl-2-picrylhydrazyl radical. Chloroquine, N, N-diethyl-3-methylbenzamide (DEET), gallic acid, doxorubicin and etopoxide were used as positive controls in the antimalarial, larvicidal, antioxidant, cytoxicity and BSL assays, respectively. Isolation of compounds was done using repeated column chromatography and high performance liquid chromatography. Structures of compounds were elucidated by spectroscopy (IR, UV, NMR and MS). Linear regression was used to determine 50% lethality concentration

(LC_{50}), 50% inhibitory concentration (IC_{50}) and 50% cytotoxic concentration (CC_{50}). Data were analysed using ANOVA and Student's t-test at $p = 0.05$.

The stem bark extract of TM had the highest chemo-suppression (100%) at 200 mg/kg in the antimalarial assay of the extracts tested against *P. berghei berghei*. The leaf of TW had 93.4% compared to chloroquine, which had 98.0%. In the larvicidal assay, the stem bark extract of TM was the most toxic, with an LC_{50} of 74.0 µg/mL, while DEET had 120.2 µg/mL. In the BSL assay, all the extracts were found to be non-toxic with LC_{50} > 1000 µg/mL as compared to etopoxide (7.46 µg/mL). The ethyl acetate fraction of TM stem bark showed antioxidant activity with IC_{50} of 25.37±1.46 µg/mL, while gallic acid had 23.44 ± 0.43 µg/mL. Both plant extracts did not produce any significant changes in haematological, biochemical, and histological parameters of animals used. Seven compounds were isolated from active extracts namely; Ursolic acid (1), lupeol (2), scopoletin (3), β- sitosterol (4), stigmasterol (5) and stigmastenone (6) from TM and 3,3′,4-tri-O- methyl ellagic acid (7) from TW. Ursolic acid, lupeol and scopoletin had chemo-suppression of 93.4%, 88.3% and 58.5%, respectively against *P. berghei,* while 3,3′,4-tri-O- methyl ellagic acid from TW had 75.8% chemosuppression. Lupeol from TM had the highest larvicidal activity on *An. gambiae* larvae with LC_{50} of 6.20 µg/mL.

Ursolic acid from *Trichilia megalantha* and 3,3′,4-tri-O-methyl ellagic acid from *Trichilia welwitschii* exhibited antimalarial activity. Lupeol with larvicidal activity was obtained from *Trichilia megalantha*. These plants could provide lead for antimalarial drug discovery and development.

Keywords: Malaria, Larvicidal compounds, *Trichilia megalantha*, *Trichilia welwitschii*.

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CERTIFICATION

I certify that this project was carried out by Fadare Dorcas Adenike in the Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan; under my supervision.

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DEDICATION

This thesis is dedicated to my beloved husband Dr. David Abimbola Fadare and to the memory of my mother Mrs. Bernice Monisola Oteyola

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ABBREVIATIONS

^{13}CNMR	13 Carbon Nuclear Magnetic Resonance
^{1}HNMR	Hydrogen Nuclear Magnetic Resonance
ACTs	Artemisinin-combination therapy
AIDS	Acquired Immune deficiency Syndrome
ANOVA	Analysis of variance
BSL	Brine shrimp lethality
CC	Column chromatography
CD_3OD	deuterated methanol
$CDCl_3$	deuterated chloroform
$CHCl_3$	Chloroform
CRIN	Cocoa Research Institute of Nigeria
CSP	Circumsporozoite protein
DCM	Dichloromethane
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethyl sulphoxide
DPPH	1,1-dipheyl-2-picry-hydrazyl
EGCG	Epigallocatechin gallate
ElMS	Electron Impact Mass Spectrometry
EtOAc	Ethyl acetate
GC	Gas chromatography
GC–MS	Gas chromatography Mass spectrometry

GDP	Gross Domestic Product
HIV	Human immunodeficiency virus
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Correlation
HPLC	High Performance Liquid Chromatography
Hz	Hertz
IAMRAT	Institute of Advance Medical Research and Training
IRS	Indoor Residual Spraying
ITNs	Insecticide-treated bed nets
LC	Lethal Concentration
m/z	Mass to.Charge ratio
MAP	Multiple antigen peptide
MeOH	Methanol
MHz	Megahertz
$^{\circ}$C	Degree centigrade
PCV	Packed cell volume
SERA	Serine rich protein
TLC	Thin layer chromatograph
WHO	World Health Organisation

CHAPTER ONE

INTRODUCTION

Malaria is one of the most common vector-borne diseases widespread in the tropical and subtropical regions which include much of sub-Saharan Africa, Asia, and Latin America. It is a life-threatening parasitic disease transmitted by female *Anopheles gambiae* mosquitoes and caused by the plasmodium parasite. The pathogenesis occurs during erythrocytic stages. A peculiarity of *Plasmodium falciparum* is its ability to adhere to vascular endothelium (cytoadherence) of erythrocytes infected with maturing parasites.

Many of the world's mosquito species, notably the most competent *Anopheles* vectors of human malaria, have highly selective host preferences and correspondingly adapted feeding behaviours (Lyimo and Ferguson, 2009; Takken and Verhulst, 2013). Most of the global malaria burden occurs in sub-Saharan Africa because of three endemic species of highly specialized mosquitoes that almost exclusively rely upon humans (*An*. *funestus* and *An*. *gambiae*), or upon humans and their cattle (*An*. *arabiensis*), for blood (Kiswewski *et al*., 2004). These exceptional vector species can mediate intense malaria transmission levels, more than four orders of magnitude in excess of that required to sustain stable endemic populations of the *P. falciparum* parasite (Smith *et al.,* 2007; Eckhoff, 2013).

The five species of *Plasmodium* which can infect and be transmitted by humans are: *Plasmodium vivax, P. falciparum*, *P. malariae, P. ovale* and *P. knowlesi* of which the first two are the most common. Infection by *P. falciparum* is the most deadly malaria infection (Caraballo and King, 2014). Despite considerable success of malaria control programs in the past, malaria still continues to be a major public health problem in several countries. Malaria is found throughout the tropical regions of the world and causes more than 100 million acute illnesses and at least half a million deaths, annually. Mortality and morbidity due to malaria are a matter of great concern throughout the world. The World Health Organization (WHO) estimated that in 2015, there were 207 million cases of malaria, with estimated death of about 473,000 people, many of whom were Africa children (WHO, 2014). Even though casualty in children below the age of 5 years is very high, the disease affects all age groups. Malaria also causes anaemia in children and pregnant women. It contributes to negative birth outcomes such as low birth weight, premature delivery, still birth and spontaneous abortion. Malaria is commonly associated with poverty and has a major negative effect on economic development (Gollin and Zimmermnn, 2007; Worrall *et al*., 2005). In Africa, it is estimated to result in losses of $12 billion USD per year due to increased healthcare costs, lost of ability to work and effects on tourism. (Greenwood *et al*., 2008).

Commercial antimalarial drugs such as chloroquine, when used as monotherapies, are rapidly losing their effectiveness (Talisuna *et al.,* 2004; Sowunmi *et al.,* 2005; Saar *et al.,* 2005, Koram *et al.,* 2005). Chloroquine, though effective as a blood schizontocidal, is ineffective or partially effective in resistant cases. The severe and complicated cerebral malaria due to *P. falciparum* is compounded by the chloroquine-resistant parasites

(Waters and Eidsten, 2012). Other drugs in use are sulfadoxine and pyrimethamine which also suffer resistance thus rendering it in effective in some parts of the world. Ouinine which is used in treatment of severe malaria is administered for a long period and associated with side effects. The most effective treatment for *P. falciparum* infection is the use of artemisinins in combination with other antimalarials (known as artemisinin-combination therapy, or ACTs), which decreases resistance to any single drug component (Wu, 2002). These additional antimalarials include; amodiaquine, mefloquine or sulfadoxine/pyrimethamine. Another recommended combination is dihydroartemisin and piperaquine (Keatin, 2012).

Drug resistance poses a growing problem in 21st-century malaria management. Resistance is now common against all classes of antimalarial drugs save the artemisinins (ACTs). The cost of artemisinins limits their use in the developing world (White, 2008). Malaria strains found on the Cambodia–Thailand border, Myanmar and Vietnam are resistant to combination therapies that include artemisinins, and may therefore be untreatable (Wongsrichanalai & Meshnick, 2008; WHO, 2013). There has been emerging resistance also in Laos (Briggs, 2014; Ashley *et al*., 2014). In the 2000s, malaria with partial resistance to artemisins emerged in Southeast Asia (O'Brien *et al*., 2011; Fairhurst *et al*., 2012). Exposure of the parasite population to artemisinin monotherapies in subtherapeutic doses for over 30 years and the availability of substandard artemisinins likely drove the selection of the resistant phenotype (Dondorp *et al*., 2009; DioÂ genes *et al*., 2010)

Despite the need, no effective vaccine exists, although efforts to develop one are ongoing. Spread of multidrug-resistant strains of *Plasmodium* and the adverse side effects of the existing anti-malarial drugs have necessitated the search for novel, well tolerated and more efficient antimalarial drugs (Perez *et al.,* 1994). In Africa, *P. falciparum* has developed widespread resistance to conventional over-the-counter drugs such as chloroquine, sulfadoxine-pyrimethamine, amodiaquine and other relatively inexpensive treatment options. In recent years, particularly for falciparum malaria, there has been an emphasis on the use of artemisinin-based medicines (van Agtmael *et al*., 1999a, 1999b). These are derived from the *Artemisia annua* shrub, although developing resistance to artemisinin monotherapies (confirmed in 2009) mean that use as single drug is not recommended. Instead, artemisinin-based combination therapies (ACTs, artemisinin taken together with other drugs) are advised (Capela *et al*., 2009). The use of ACTs can be extremely effective in treating malaria and has increased rapidly in recent years, but the use of this drug remains very low in some rural areas where the population prefers traditional, less expensive preparations. For example, range of African countries surveyed in 2007 and 2008, fell below the World Health Organisation target of 80% efficacy of the drug for the continent. (Fidock *et al*., 2004).

Development of new therapeutic approaches to malaria is imperative, since resistance of parasites to different anti-malarials is fast developing. The need for an alternative drug initiated intensive efforts for developing new anti-malarials from indigenous plants (Francis *et al*., 2007). Natural products are important sources of biologically active compounds and have potential for development of novel anti-malarial drugs (Zirihi *et al*.,

2005). Natural products are generally safer to mammals, including man (Okunade *et al*., 2001). Interest in plant as new anti-malarials has been stimulated by the isolation of artemisinin, a highly active compound against drug-resistant *P. falciparum* from *Artemisia annua*. The first anti-malarial developed was quinine, obtained from the stem bark of *Cinchona calisaya* and *Cinchona succirubra*. Synthetic anti-malarials were later developed based on the quinine template e.g chloroquine, primaquine, proguanil, pyrimethamine, mefloquine. Parasites have developed resistance to almost all of these anti-malarials. This necessitates the need for newer and effective anti-malarials. There are a series of new synthetic anti-malarials that have been developed and are undergoing different stages of drug trials.

The problem of resistance and side effects associated with antimalarial drugs urge for an increasing demand for active compounds with a new mode of action to replace the current ineffective drugs (Go, 2003).

1.1 Use of Larvicides for Control of Malaria

A larvicide is an insecticide that is specifically targeted against the larval stage of an insect. Their most common use is against mosquitoes. Larvicides may be contact poisons, stomach poisons, growth regulators, or (increasingly) biological control agents. In addition to the development of new antimalarial drugs, vector control has been found to be a good alternative to overcoming the burden of malaria. This approach largely relies on interruption of the disease transmission cycle by either targeting the mosquito larvae through spraying of stagnant water breeding sites or by killing the adult mosquitoes using

insecticides (Ghosh *et al*., 2012). Larviciding is a successful way of reducing mosquito densities in their breeding places before they emerge into adults. It is easier to control the insects at the larval stage when they are most accessible, concentrated within specific habitats and less mobile. Larviciding largely depends on the use of synthetic chemical insecticides—organophosphates (*e.g.* temephos, fenthion) and insect growth regulators (*e.g.* diflubenzuron, methoprene). The main limitations of the method are the resistance developed by mosquitoes to these insecticides because of their continuous use (Adams, 2006) and accumulation of chemicals which also affected the biological environment (Cova *et al*., 1995; Garry *et al.*, 1990). Hence, the use of economically feasible botanical and biodegradable extracts is considered to be an important alternative strategy for the control of malaria vector, *An gambiae*. Insecticidal effects of plant extracts vary not only according to plant species, mosquito species, geographical varities and parts used, but also due to extraction method adopted and the polarity of the solvents used during extraction. (Shaalan *et al*., 2005; Lyimo and Ferguson, 2009). Natural products are generally preferred because of their less harmful nature to non-target organisms and due to their innate biodegradability. Currently, mosquito control programme is focused more on the elimination of mosquitoes at larval stage with plant extracts. The advantage of targeting mosquito larvae is that they cannot escape from their breeding sites until the adult stage and also to reduce the overall pesticide use in control of adult mosquitoes by aerial application of adulticidal chemicals (Walker and Lynch, 2007).

1.2 Plants as source of antimalarial agents

For thousands of years, plants have formed the basis of sophisticated traditional medicine systems and more recently, natural products have been a good source of lead compounds, against diseases and infections. The most important lead compound against malaria is quinine, isolated from *Cinchona* bark, which was used as a template for chloroquine and mefloquine synthesis. More recently, artemisinin (Perez, *et al*., 1994), isolated from the Chinese plant *Artemisia annua*, has been used successfully against chloroquinr-resistant malaria.

Man has been dependent on plants from time immemorial. Plant materials are found to be present in or have provided the synthetic models for 50% orthodox drugs (Robbers *et al.*, 1996). The primary benefits of using plant-derived medicines are that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment. Over 90% of the drugs in hospitals today were introduced in the last 50-60 years; so it is easy to understand the important role that traditional medicine has played in the in drug discovery and development (Boye, 1985). The WHO estimates that up to 80% of the global population rely on plants for their primary health care, since; western pharmaceuticals are often expensive and are not easily accessible in developing countries. Thus, many communities turn to traditional medicine to treat malaria infection,, using preparations mainly based on medicinal plants claimed by traditional healers to be effective (Willcox and Bodeker 2004; Joshi and Joshi, 2000).

In China, for example, traditional medicine is largely based on some 5,000 plants and is used to treat 40% of urban patients and 90% of patients in rural areas. In 1991, more than

700,000 tones of plant material were used for medicine, 80% collected from the wild. Plants have contributed more than 7,000 different compounds in use today as heart drugs, laxatives, anti-cancer agents, hormones, contraceptives, diuretics, antibiotics, decongestants, analgesics, anesthetics, ulcer treatments and anti-parasitic compounds.

Africa is endowed with the tropical biome with a large expanse of the equatorial forest; plants which is often exploited as remedies for management of diseases and infections in various ethnomedicines (Sofowora, 1982). The Meliaceae plant family has been used for generations in Africa, India and tropical America to treat malaria (Muregi *et al*., 2003; 2004; 2007). In tropical America, *Cedrela odorata, Carapa quianensis* and *Swietenia mahagoni* have been used while in Africa and India the 'Neem' tree or *Azadirachta indica* is used. The extract of the bark and leaves of *Azadirachta indica* has been used in Thailand and Nigeria as anti-malarials from ancient times (Kraus, 1986; Eugene; 1992).

Several other plants used for the treatment of malaria in Nigeria include *Carica papaya*, F*icus thonningii*, *Trema occidentalis*, *Ocimum gratissimum*, *O. basilicum, Erythrina senegalaensis* (Ajaiyeoba *et al.*, 2006). Charaka in 2000 BC and Susruta in 1500 BC reported anti-malarial and antipyretic activity of neem. Ekanem (1978) reported schizontocidal activity of aqueous extract of neem leaves on *P. berghei* and Udeinya *et al*., (2008) also found anti-malarial activity in acetone/water extracts of neem leaves on chloroquine-sensitive *P. falciparum*. MacKinnon *et al.,* (1997) tested a series of sixty extracts of twenty-two Meliaceae for activity against *P. falciparum*, using both chloroquine-sensitive and chloroquine-resistant strains. The extracts showing the highest

activity against the chloroquine-sensitive strain were the leaves of *Azadirachta indica, Cedrela salvadorensis* and *Chukrasia tabularis*, the bark of *Trichilia glabra* and the wood of both *Cedrela odorata* and *Dysoxylum fraseranum*. The leaves of *A. indica, C. tabularis* and *C. salvadorensis* and the wood of *C. odorata* and *Guarea pyriformis* were the most active against the chloroquine-resistant strain. The common denominator in the Meliaceae is the presence of limonoids, in particular the limonoid gedunin (Deck *et al.*, 1998). In a study of *A. indica* wood extracts from different locations, the activity increased as the percentage of gedunin increased. MacKinnon *et al.,* 1997 prepared a series of nine derivatives of gedunin (Deck *et al*., 1998) in an attempt to establish some sort of structure–activity relationship. None of the derivatives was as active as gedunin but a number of important characteristics were identified. It was found that the presence of an α, β-unsaturated ketone in ring A was vital for activity and that the presence of a 7α-acetate group as well as the furan ring also contributed to the activity. In a survey of twenty-one compounds isolated from medicinal plants, Khalid *et al.,* (1986) found particular activity in gedunin isolated from *Melia azedarach*. This study found gedunin to be roughly as active as quinine. However, despite the promising *in vitro* activity of gedunin, Bray *et al.*, (1990) found that it did not inhibit *Plasmodium berghei* in mice. Work on the leaves of *Azadirachta indica* collected in India resulted in the isolation of four limonoids, of which meldenin (MacKinnon *et al*., 1997) was the most active against the chloroquine-resistant K1 strain of *P. falciparum* (Joshi *et al*., 1998). Further investigation on *A. indica* has been carried out by Jones *et al.,* (1994) and Dhar *et al.,* (1998). Jones and his co-workers studied azadirachtin and a series of seventeen semisynthetic derivatives and their affect *in vitro* on male gamete production from

malarial microgametocytes. Azadirachtin (Khalid *et al*., 1986) and three of the semisynthetic derivatives were found to inhibit the formation of mobile male gametes *in vitro*. The study indicated that the presence of a hemiacetal group at C-11 was vital to the activity. Dhar *et al.,* (1998) investigated the seeds of *A. indica*, and found that the extract was active against all the erythrocytic stages of *P. falciparum*. In addition to inhibiting the asexual stages of the parasite, the neem extracts also displayed a gametocytocidal effect. All stages of maturation of the gametocytes were affected, unlike artemisinin and primaquine that just affect the immature stages. Khalid *et al.,* (1998) isolated three limonoids of the mexicanolide type from *Khaya senegalensis*. One of the compounds, fissinolide (Bray *et al*., 1990), showed slight activity against chloroquine-resistant *P. falciparum*. A study on related species *Khaya grandifoliola* by Agbedahunsi *et al.,* (1998), indicated that the hexane extract of the stem bark was the most potent when tested against *P. falciparum in vitro* and *P. berghei* in mice. The results obtained were similar to those obtained with the reference drug, chloroquine diphosphate.

1.3 Rationale for this Study

i Malaria stands out as the most predominant disease of poverty. It contributes in a large extent to a viscious circle of disease-poverty-disease and reduces work capacity. In addition, malaria impairs physical and mental development in children, diminishes returns achieved through education and limits potential to contribute fully to social and economic growth of the country.

ii The ever increasing global population that prefers the use of natural products in treating and preventing medical problem with special reference to malaria.

iii The use of plants as a possible source of antimalarial agents has led to the discovery of potent drugs against malaria.

iv The predominance of drug-resistant parasites has necessitated the development of newer, safe, effective and inexpensive drugs.

v Development of resistance by malaria vectors to insecticides due to their continous use.

vi Negative effect of the accumulation of non-biodegradable chemicals on biology of the environment.

1.4 Objectives of the study

i. To extract authenticated *T. megalantha* and *T. welwitschii* plant parts into methanol.

ii. To determine the larvicidal properties of the extracts on *An. gambiae* mosquito.

iii. To screen *T. megalantha* and *T. welwitschii* plant extracts for antiplasmodial activity.

iv. To carry out antimalarial activity of plant extracts and fractions *in vivo* in mice.

v. To isolate and purify active compounds using chromatographic techniques.

vi. To elucidate structures of active compounds by spectroscopic methods.

vii. To evaluate toxicity properties of *T. megalantha* and *T. welwitschii* plant extracts.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria Parasites

Malaria is a parasitic infection caused by the species of *Plasmodium* parasites; *P. falciparum, P. ovale, P. vivax*, *P. knowlesi and P. malariae*. These 5 species differ in geographical distribution, microscopic appearance, clinical features and possibility of development of resistance to antimalarial drugs. *Plasmodium falciparum* accounts for the majority of instances of morbidity and mortality. The parasite is transmitted to human beings by infected female mosquitoes (Bloland, 2001; White, 2004). The parasite, vector and human transmission dynamics determine the burden of malaria in the different regions of the world. More than forty species of anopheline mosquitoes with different transmission potentials transmit human malaria. The most competent and efficient malaria vector, *Anopheles gambiae* is found exclusively in Africa due to the favourable condition (adequate rainfall, temperature and humidity) for breeding and survival (Kiszewski *et al*., 2004). Of the parasites that cause malaria, the most common are *P. falciparum* and *P. vivax*. *Plasmodium falciparum* is the most virulent. Transmission of disease through mosquito bites depends on factors such as rainfall patterns (mosquitoes breed in wet conditions), closeness of breeding sites to households, and the types of mosquito species in an area. Some regions have a fairly constant number of cases throughout the year ('malaria endemic' areas), while others have seasonal bouts of infection, usually coinciding with the rainy season. Malaria can assume many manifestations in an individual depending on parasite species and the pattern of

transmission. *Plasmodium malariae* and *P. ovale* infections cause little morbidity and almost no mortality while *P. vivax* infections are more severe and debilitating but are usually self-limiting in healthy individuals. *Plasmodium falciparum* infections are always life threatening in non-immune individuals.

Although malaria is a common disease it is both preventable and curable. Prevention focuses on reducing transmission through control of the malaria-bearing mosquito, primarily through the use of mosquito nets (treated with long-lasting insecticide) that offer overnight protection, and through indoor residual spraying of insecticides. In Africa, for example, more households now use nets than even a few years ago. In 2008, 31% of households had at least one net, compared to 17% in 2006 however; overall coverage for the continent is still low. The percentage of children under five years old using a net in Africa was 24% in 2008, well below the WHO target for the continent of 80%. The use of nets and indoor spraying as prevention interventions can be complemented by other vector control methods, such as the reduction of standing water where the mosquito breeds (Tiwary *et al*., 2007).

2.2 Malaria vector

Malaria parasites are exclusively transmitted by female adult mosquitoes of the genus *Anopheles* except a few cases of transplacental and blood transfusion-associated transmission (Collins and Paskewitz, 1995). Mosquitoes (Culicidae) are a family of about 3500 species within the order Diptera. They are divided into three sub-families: Toxorhynchitinae, Anophilinae and Culicidae (Clements, 1992). Mosquitoes are found

throughout the world except in regions that are permanently frozen (Antarctica). More than half of all mosquito species live in the humid tropics and sub-tropics, where the warm moist climate is favourable for rapid development and adult survival. The vectors of human malaria belong to the genus *Anopheles* and malaria is transmitted by different *Anopheles* species, depending on the region and the environment. Over 60 species of *Anopheles* are important vectors of human malaria with *An. gambiae*, *An. arabiensis* and *An. funestus* being the three most efficient vectors. These species are widely distributed in tropical Africa (Manson-Bhar & Bell, 1987). *Anopheles gambiae* is the principal vector of malaria parasite in West Africa and is probably the world's most efficient. It is an ubiquitous species that breeds in rainwater puddles, borrow pits, river pools or quiet backwaters (CDC, 2004).

Female mosquitoes take blood meals which are the link between the human and the mosquito hosts in the parasite life cycle to carry out egg production. The successful development of the malaria parasite in the mosquito (from the "gametocyte" stage to the "sporozoite" stage) depends on several factors. The most important is ambient temperature and humidity (higher temperatures accelerate the parasite growth in the mosquito). Like all mosquitoes, anophelines go through four stages in their life cycle: egg, larva, pupa, and adult. The first three stages are aquatic and last 5-14 days, depending on the species and the ambient temperature. The adult stage is when the female *Anopheles* mosquito acts as malaria vector. The adult females can live up to a month (or more in captivity) but most probably do not live more than 1-2 weeks in nature.

Adult females lay 50-200 eggs per oviposition singly and directly on water and hatch within 2-3 days. Mosquito larvae have a well-developed head with mouth brushes used for feeding. In contrast to other mosquitoes, *Anopheles* larvae lack a respiratory siphon and for this reason position themselves so that their body is parallel to the surface of the water. Larvae develop through 4 stages, or instars, after which they metamorphose into pupae. The larvae occur in a wide range of habitats but most species prefer clean, unpolluted water. All *Anopheles* larvae lack the respiratory siphons used as breathing tubes in most other mosquito genera, and therefore the larvae lie parallel to the water surface in order to breathe (Foster and Walker, 2009).The pupa is comma-shaped when viewed from the side. After a few days as a pupa, the dorsal surface of the cephalothorax splits and the adult mosquito emerges (Killeen, 2013; CDC, 2014).

2.3 Malaria Burden

The global burden of *P. falciparum* malaria increased through the 1990s due to drug-resistant parasites and insecticide-resistant mosquitoes; this is illustrated by re-emergence of the disease in areas that had been previously declared malaria-free (Griffien *et al*., 2010; Okell *et al*., 2011). The first decade of the 21st century has seen reduction. This can be attributed to improvement of socio-economic indices, deployment of artemisinin-combination drugs and insecticide-treated bednets. Chloroquine, the most effective anti-malarial ever developed, deployed since the 1930s, has witnessed non-effectiveness against *P. falciparum* and only marginally effective against *P. vivax*. Early evidence of resistance to artemisinins, the most important class of antimalarials, is now confirmed in the region of the Cambodia/Thailand border, Colombia, and Guinea.

The major obstacle to global malaria eradication remains the parasite's historical strongholds in Africa and the southern Pacific, where unusually efficient vectors saturate human populations with intense transmission that dramatically attenuates, and even negate, the impacts of drugs and vaccines (Killen, 2013; Huho *et al*., 2012; Shekalaghe *et al*., 2011). About 90% of all malaria deaths in the world today occur in Africa south of the Sahara. This is because the majority of infections in Africa are caused by *P. falciparum,* the most dangerous of the five human malaria parasites. It is also because the most effective malaria vector, the mosquito *An. gambiae* – is the most widespread in Africa and the most difficult to control. An estimated one million people in Africa die from malaria each year and most of these are children under 5 years old (WHO, 2015).

Malaria affects the lives of almost all people living in the area of Africa defined by the southern fringes of the Sahara desert in the north, and latitude of about 28° in the south. Most people at risk of the disease live in areas of relatively stable malaria transmission. Infection is common and occurs with sufficient frequency that some level of immunity invariably develops. A smaller proportion of people live in areas where risk of malaria is more seasonal and less predictable, because of either altitude or rainfall patterns. People living in the peripheral areas north or south of the main endemic area or bordering highland areas are vulnerable to highly seasonal transmission and to malaria epidemics (McGinn, 2002).

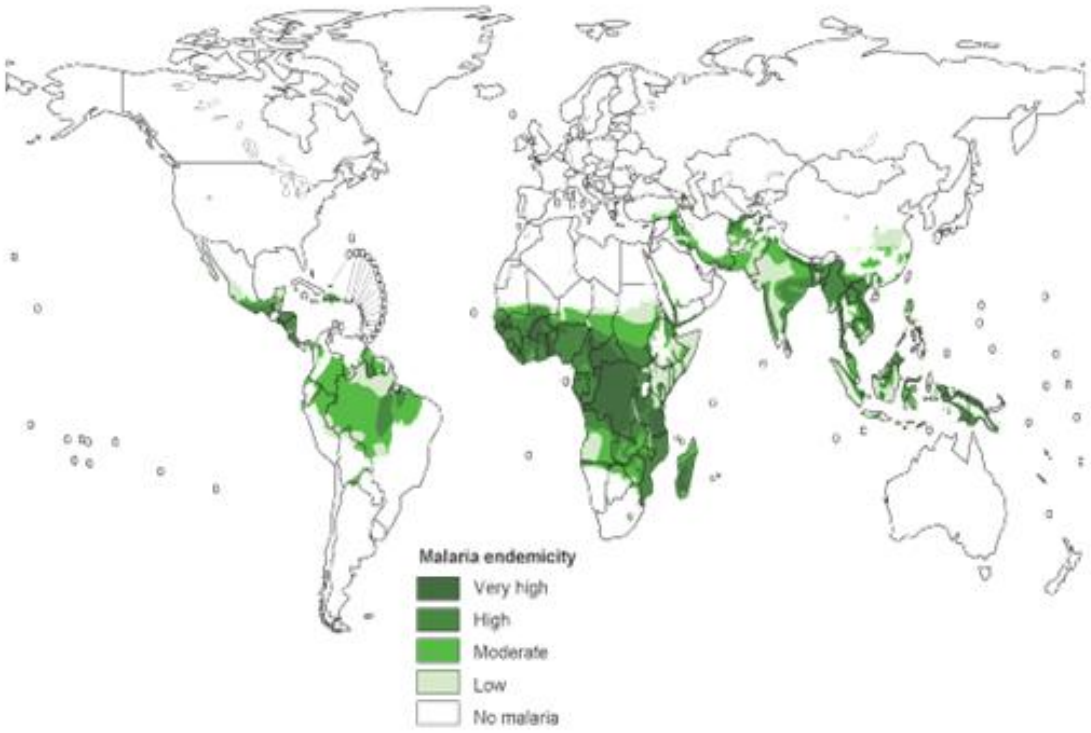


Fig. 2.1: Geographical Distribution of Malaria Endemicity

(Source: Hay *et al*., 2008)

In areas of stable malaria transmission, young children and pregnant women are the population groups at high risk of malaria morbidity and mortality. Most children experience their first malaria infections during the first year or two of life, when they have not yet acquired adequate clinical immunity, making these early years particularly dangerous. Ninety percent of all malaria deaths in Africa occur in young children. Adult women in areas of stable transmission have a high level of immunity, but this is impaired especially in the first and second pregnancies, with the result that risk of infection increases. Travelers or migrants coming from areas with little or no malaria transmission, who lack acquired immunity after exposure to several infective bites, are also vulnerable to attack (Collins and Paskewitz, 1995).

Malaria is endemic in some of the offshore islands to the west of mainland Africa – Sao Tome and Principe and São Tiago Island of Cape Verde. In the east, malaria is endemic in Madagascar, in the Comoro islands (both the Islamic Federal Republic of the Comoros and the French Territorial Collectivity of Mayotte). Malaria is a major public health problem and an important obstacle to economic development in most developing countries, particularly in Africa. Costs of treating the infection in terms of the burden on the health systems and loss of economic activity are enormous. In Africa, where malaria reaches a peak at harvest time and hits young adults, a single bout of the disease causes loss of 10 working days (WHO, 2000). It undermines investment in education since large amounts of development funds are channeled to disease prevention and treatment. In areas of high malaria endemicity, the learning capacity of about 60% of all school children is impaired. The disease is estimated to cost Africa between $3-12 billion

annually (McGinn, 2002). In Africa, the average cost for each nation to implement malaria control programmes is estimated at >$300,000 per year. Costs to endemic countries include control and lost of working days-estimated to be 1-5 % of GDP in Africa.

2.3.1 Global Distribution of Endemic Malaria

Although the geographical area affected by malaria has shrunk considerably over the past 50 years, control is becoming more difficult and gains are being eroded. This can be attributed to economic developments or agricultural activities that change land use such as road construction, creation of dams, irrigation schemes, mining, commercial tree cropping and deforestation.These result in an increase in breeding sites. Most malaria cases (around 85%) and deaths (~ 90%) are in the low-income nations of sub- Saharan Africa (the five main contributors to global deaths are the Democratic Republic of Congo, Ethiopia, Nigeria, Tanzania and Uganda), although Asia, Latin America, the Middle East and parts of Europe are also affected. Malaria is the fifth highest cause of death from infectious diseases globally and second in Africa, after HIV/AIDS. Malaria is present in 109 countries and territories, and in the future coverage may expand further as climate change allows mosquitoes and the parasite to colonize new areas (Fig. 2.1). In 2008, malaria was estimated to have caused nearly nine hundred thousand deaths globally, mostly among African children. It is estimated that one child dies from malaria every 30 seconds, and in Africa it is the leading cause of under-five mortality. In Africa 10% of mortality in children is directly attributable to malaria (Amadou *et al.,* 2001).

Pregnant women are also at high risk of malaria, with illness causing impaired foetal growth and high rates of miscarriage (Seal *et al*., 2010) and significant maternal deaths (up to 50% death rate in cases of severe disease), especially among HIV-infected women. Infection with HIV/AIDS reduces immunity to malaria and results in higher treatment failure. Malaria during pregnancy often contributes to maternal anaemia, premature delivery and low birth weight, leading to increased child mortality. The costs of malaria treatment are not only high for health maintenance, but the infection also results in significant economic losses. The annual Gross Domestic Product (GDP) is estimated to be reduced by as much as 1.3% in countries with high disease rates. In Africa, it is estimated that at least 12 billion USD per year is lost directly through illness, treatment and premature death. Aggregated losses over time have resulted in substantial differences in GDP between countries with and without malaria, particularly in Africa. In some countries with a heavy disease burden, malaria accounts for up to 40% of public health expenditure, up to 50% of in-patient hospital admissions, and to 60% of visits to out-patient health clinics. Management of malaria infection is therefore an essential part of global health improvement and economic development (Ntie-Kang *et al.,* 2014).

The infection is a major cause of illness, health care visits and hospitalizations in many areas of the tropics (Fischer and Bialek, 2002). Pregnant woman are also at increased risk of malaria infection in all areas where malaria is endemic (Diagne *et al.,* 1997).

2.3.2 Global Malaria

Malaria affects 3.3 billion people, or half of the world's population, in 106 countries and territories. World Health Organisation estimates 216 million cases of malaria occurred in 2010, 81% in the African region resulting in 655,000 malaria deaths in 2010, 91% in the African Region, and 86% were children under 5 years of age. Malaria is the third leading cause of death for children under five years worldwide, after pneumonia and diarrheal disease (Snow *et al*., 2005; Cibulskis *et al*., 2016).

2.3.3 Malaria in Africa

Thirty countries in Sub-Saharan Africa account for 90% of global malaria deaths. Nigeria, Democratic Republic of Congo (DRC), Ethiopia, and Uganda account for nearly 50% of the global malaria death. Malaria is the second leading cause of death from infectiou s diseases in Africa, after HIV/AIDS. Almost 1 out of 5 deaths of children under 5 in Africa is due to malaria (Good, 2001; Greenwood *et al*., 2008).

2.3.4 Malaria in Nigeria

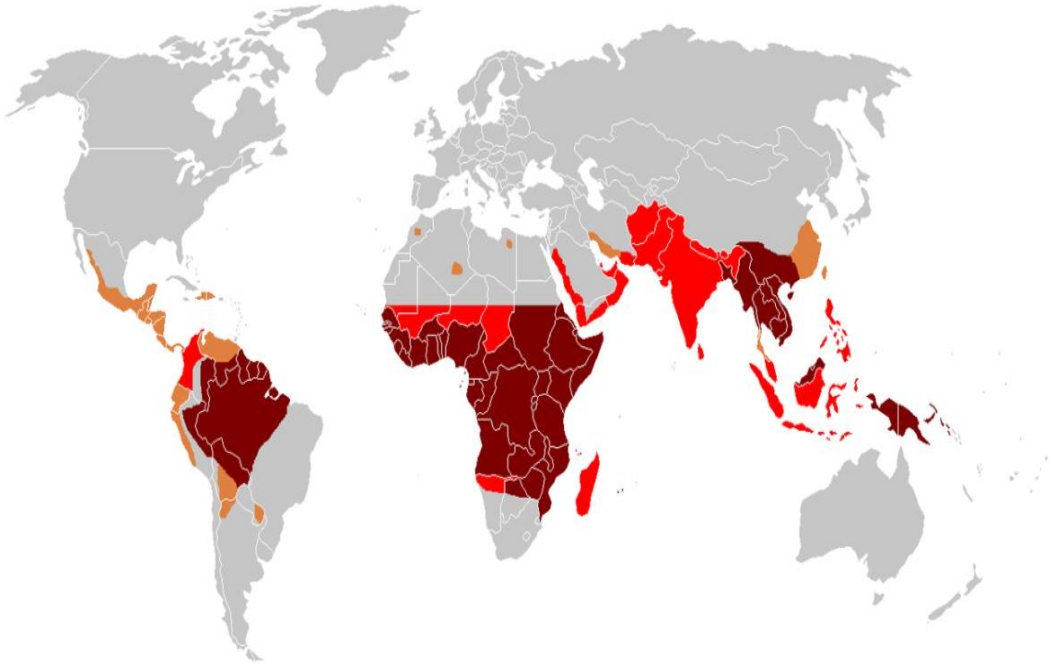
Malaria is a major public health problem in Nigeria where it accounts for more cases and deaths than any other country in the world. Malaria is a risk for 97% of Nigeria's population. The remaining 3% of the population live in the malaria free highlands. There are an estimated 100 million malaria cases with over 300,000 deaths per year in Nigeria which makes Nigeria the country with the highest number of malaria casualties

worldwide (Michael, 2008). This compares with 215,000 deaths per year in Nigeria from HIV/AIDS. Malaria contributes to an estimated 11% of maternal mortality. It accounts for 60% of outpatient visits and 30% of hospitalizations among children under five years of age (WHO, 2014). Malaria has the greatest prevalence, close to 50%, in children age 6-59 months in the South West, North Central, and North West regions while the least prevalence, 27.6%, in children age 6 to 59 months occur in the South East region (Fig. 2.3). Despite so many gains in malaria prevention and treatment, the widespread prevalence of counterfeit, substandard drugs is contributing to the alarming high number of malaria deaths and costs of health care in Nigeria.

2.3.5 Population at Risk of Malaria Infection

2.3.5.1 Children under the age of five

Malaria is among the most frequent cause of morbidity and mortality among children (Bloland 2001). *Plasmodium falciparum* is the principal cause of severe disease and death in children. Malaria burden is enomous in young children because clinical effects of malaria depend upon levels of immunity among other factors. Several exposure to malaria infection increases immunity in malaria endemic countries. Prior to acquiring immunity, they are protected initially by maternal factors like transplacental acquisition of maternal antibodies. The protective effect of these antibodies decrease during the first six months. Severe disease and death mostly occur between the ages of 1 and 5 (Roberts and Mathews, 2016).



	Elevated occurrence of chloroquine- or multi-resistant malaria
	Occurrence of chloroquine-resistant malaria
	No *Plasmodium falciparum* or chloroquine-resistance
	No malaria

Fig. 2.2: Distribution of Drug- Resistant *P. falciparum* Malaria

(Source: Gething *et al.,* 2011)

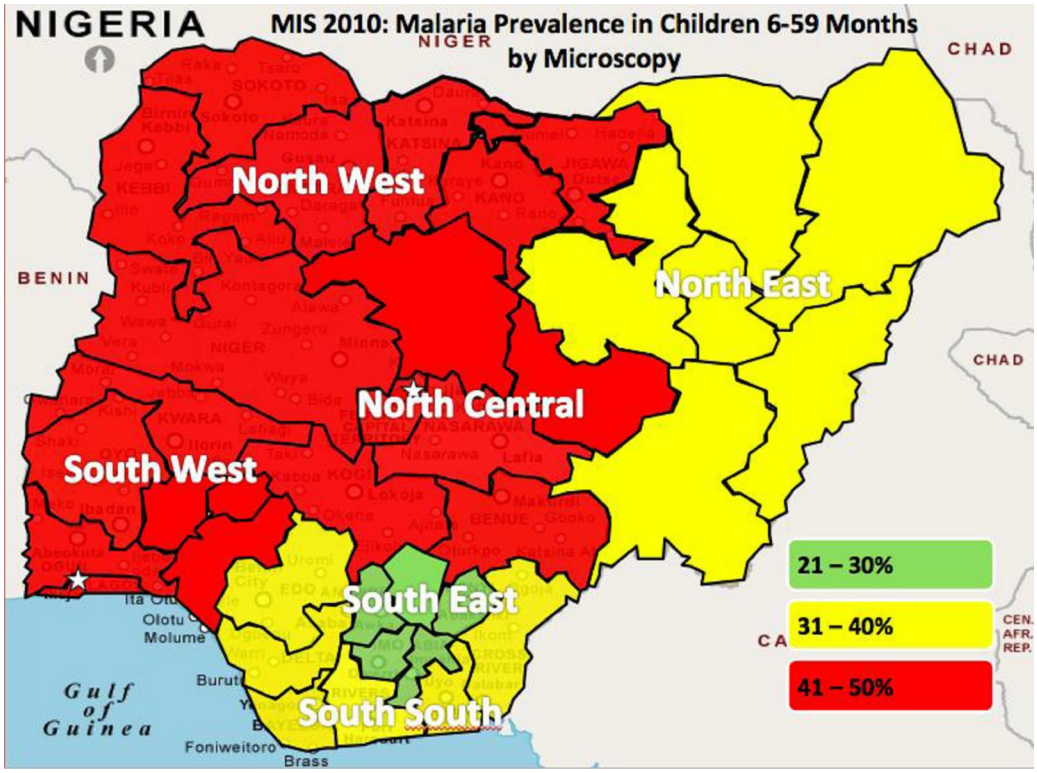


Fig. 2.3: Malaria Prevalence in Children 6-59 Months in Nigeria

(Source: US Embassy, 2011)

2.3.5.2 Pregnant women living in malaria endemic areas

Pregnant women have increased susceptibility to malaria infection than their non-pregnant counterpart. This is because during normal pregnancy the immune system is regulated to ensure that the foetus is not rejected as a foreign allograft leading to reduced immunity. Replication of parasite in the placenta alters transplacental nutrient transport, resulting in premature delivery, low birth weight and anaemia in the mother (Miller *et al*., 1994). In highly endemic areas malaria contributes to 2-15% of maternal anemia, 8-14% of low birth weight and 3-5% of infant deaths (Ofori *et al*., 2009).

2.3.5.3 Displaced Persons and Refugees in Endemic Areas

Wars in Africa and other parts of the world have led to problems of malaria transmission in displaced persons and refugees as a result of their living conditions. Collapse of health services due to social, political and civil unrest has led to resurgence of malaria in areas where it was formely eradicated (Pitt *et al*., 1998).

2.3.5.4 People Living with HIV/AIDS

Malaria causes anemia which may require blood transfusions, a procedure that increases the risk of HIV infection where universal blood screening is yet to be achieved. People living with HIV/AIDS are at an increased risk of clinical malaria, severe illness, hospitalization, and death. Malaria contributes to a temporary increase in viral load

among HIV-infected people which may worsen the clinical disease, increase mother-to-child transmission, and augment transmission in adults (Sanyaolu *et al*., 2013).

2.3.5.5 Non Immune Travellers to Endemic Areas

Over 20 million travellers from non-endemic countries visit malaria countries for employment and tourism. Malaria infection in this group is very severe because they lack immunity which can only be acquired after exposure to several malaria attacks. Complications in non-immune patients are cerebral malaria, severe anaemia, hypoglycemia, renal failure to mention a few (Miller *et al*., 1994).

2.4 Malaria Control Policies

Approriate malaria control strategies depend on local malaria endemicity (Griffin *et al*., 2010).

2.4.1 Vector Control

The aim of vector control is to decrease contact between humans and vectors of human disease. Control of the vector may prevent malaria and other mosquito borne diseases. Vector control should be selective, cost effective, sustainable, eco-friendly and applied in an integrated fashion utilizing local technologies and resources. As far as possible, community participation should be elicited and health education should be emphasized. These concepts should constitute the basic frame work in the planning for vector control (CDC, 2004).

2.4.1.1 Indoor Residual Spraying

Indoor residual spraying is the spraying of insecticides on the walls inside a home. After feeding, many mosquitoes rest on a nearby surface while digesting the blood meal, so if the walls of houses have been coated with insecticides, mosquitoes can be eliminated before transfer of the malaria parasite. The problem of physiological resistance to insecticides in the anopheline vector requires the introduction of new insecticides in the control of malaria, such as Indoor Residual Spraying (IRS) (Hill *et al*., 2006). Thus, IRS prevents transmission of infection. To be effective, IRS must be applied to over 70% of households in an area. Indoor Residual Spraying with DDT and dieldrin was the primary malaria control method used during the Global Malaria Eradication Campaign period (1955-1969). Though complete eradication was not achieved, it eliminated malaria from several areas and reduced the burden of malaria to a great extent. Due to the failure of the malaria eradication campaign and environmental concerns about residual insecticides, IRS is rarely employed today other than in a few focal areas (CDC, 2004).

2.4.1.2 Insecticide-Treated Bed Nets

Mosquito bed nets or curtains treated with the synthetic pyrethroids produces remarkable impact in lowering malaria transmission in areas where the vector is endophagic, biting rhythm coincides with the sleeping habits of the population and target vector species is sensitive to the insecticide.

Insecticide-treated bed nets (ITNs) are a form of personal protection that has repeatedly displayed reduction of several diseases and mortality due to malaria in endemic regions.

In community-wide trials in several African countries, ITNs have been shown to reduce all cases of mortality by about 20%. Insecticide-treated bed nets have been tried in the control of endemic malaria in India against *An. ninimus*, *An. fluviatilis* and *An. culicifacies* (Sharma and Dev, 2015).

In Indonesia, bed nets have been distributed in the transmigrant areas. Results have been highly encouraging in terms of malaria control and prevention from mosquito bites, but bed nets may not be suitable universally and requires the knowledge of the vector biting behaviuor, socio-cultural and sleeping habits of the target population and a strong health education support. (Rozendaal, 1989; Jana-Kara *et al*., 1995). Treated bed nets form a protective barrier around persons using them. However, mosquitoes can feed on people through the nets, and even a few smaller holes provide little or no protection (N'Guessan *et al*., 2007). The application of residual insecticide greatly enhances the protective efficacy of bed nets. The insecticides used for treatment kill mosquitoes and other insects, they also repel thus reducing the number of mosquitoes that enter the house and attempt to feed. Currently, only pyrethroid insecticides are approved for use on ITNs. They do not rapidly breakdown unless washed or exposed to sunlight. An important shortcoming of most insecticide treated bed nets is washing which significantly reduces their efficacy. To maintain the efficacy of ITNs, the nets must be retreated at intervals of 6-12 months, more frequently if the nets are washed (Luxemburger *et al.,* 1994).

2.4.1.3 Bioenvironmental Control of Mosquito Larvae

Bioenvironmental technology is cost effective, ecologically sound, and sustainable and can be linked with the income generating schemes .The larval habitats are destroyed by filling depressions that collect water, draining swaps or ditching marshy areas to remove standing water. People are educated to remove or cover standing water in cans, cups or rain barrels around houses to reduce available container-breeding mosquitoes sites. Mosquitoes that breed in irrigation water can be controlled through careful water management. In the application of this strategy, it is important to select areas amenable to bioenvironmental interventions as large marshy areas, low population density areas and irrigation tracts with extensive seepages and misuse of water may not respond to these methods (Sharma *et al*., 1993; Beier *et al*., 2008).

2.4.1.4 Biolarvicides

Biological control implies the use of predators, parasites or pathogens. The best known biological control agent is the top minnow or mosquito fish, *Gambusia affinis,* a native of southern USA, which has now been introduced into many tropical and sub-tropical countries to control mosquito larvae (Mittal, 2003). Another fish, the guppy *Poecilia reticulate* (Lebistes*)* is also used (Service, 1986). This can be adopted in large water bodies.

Biological control agents include toxins from the bacterium, *Bacillus thunringiensis var israelensis* (Bti). This is a biological or a naturally occurring bacterium found in soils. These products can be applied in the same way as chemical insecticides. They are very specific, affecting only mosquitoes, black flies and midges. It contains spores that produce toxins that specifically target and only affect the larvae of the mosquito, blackfly and the fungus gnat. Insects that are exposed to the *Bacillus* species have trouble digesting food they eat after the exposure and later die of starvation. There is no record of resistance to Bti till present day (Tetreau *et al.,* 2013).

Insect-growth regulators such as methropene are specific to mosquitoes and can be applied in the same way as chemical insecticides. Other potential biological control agents, such as fungi (e.g. *Laegenidium giganteum*) or mermithid nematodes (e.g. *Romanomermis culicivorax*) which are less efficient for mosquito control because they are not effective against all species of mosquito larvae are not widely used (Bedding, 1993; Kerwin, 2007). Source reduction is an ideal approach to mosquito control. Mosquito larvae are concentrated in defined areas, and source reduction eliminates mosquitoes before they reach the stage that is responsible for disease transmission.

The limitation of biological control methods is that they require a good understanding of the population dynamics of the vector. Besides, they are slow acting and therefore unsuitable in emergencies such as disease pandemics and epidemics, where insecticides are more appropriate intervention tools.

2.4.1.5 Release of genetically modified *Anopheles*

Genetic modification of malaria vectors aims to produce mosquitoes that are refractory to the parasite. This approach involves the use of genetically impaired insects to limit reproduction and survival of their own species in natural populations. Such insects are mass produced in the laboratory and released among wild populations in the field so that mating with normal insects will either not result in an offspring or lead to reduced fitness (sterility, failure to adapt properly to the environment) of the progeny. Genetic approaches include the sterile-insect release method, chromosomal translocations, hybrid sterility and cytoplasmic incompatibility. This approach is still several years from application in field settings (Scott *et al*., 2002).

2.4.1.6 Other Vector Control Methods

Fogging or area spraying is primarily reserved for emergency situations; halting epidemics or rapidly reducing adult mosquito populations when they have become severe pests. Fogging and area spraying must be properly timed to coincide with the time of peak adult activity, because resting mosquitoes are often found in areas that are difficult for insecticides to reach (e.g. under leaves, in small crevices). Personal protection measures involve the use of window screens, ITNs, repellants and wearing light-coloured clothes, long pants and long sleeve shirts (Esu et al., 2010).

2.4.1.6.1 Plant Based Insecticides

Plants have always been a rich source of chemicals and drugs for man (Arnason *et al*., 1989). In the 20th century, a few of these natural compounds like nicotine, rotenone and pyrethrins were used commercially as insecticides. However, plants produce thousands of other compounds that are insecticidal. Examples include compounds with actions on hormonal, neurological, nutritional or enzymatic properties with diverse modes (Armason 1989; Rosenthal, 2001).

2.4.2 Malaria Vaccine

Vaccines are the most cost effective tools for public health. They have contributed to a reduction in the spread and burden of infectious diseases and have played the major part in previous elimination campaigns for smallpox and the ongoing polio and measles initiatives. No effective vaccine for malaria has so far been developed. By their very nature, protozoa are more complex organisms than bacteria and viruses, with more complicated structures and life cycles. This creates a problem in vaccine development but also increases the number of potential targets for a vaccine. The complexity of the malaria parasite makes development of a malaria vaccine an up hill task. A completely effective vaccine is not yet available for malaria, although several vaccines are under development, there is none that targets parasitic infections. The epidemiology of malaria varies enormously across the globe hence it may be necessary to adopt very different vaccine development strategies to target the different populations (Mullin, 2014).

Theoretically, each developmental stage could have a vaccine developed specifically to target the parasite. Any vaccine produced would ideally have the ability to be of therapeutic value as well as preventing further transmission and is likely to consist of a combination of antigens from different phases of the parasite's development. The majority of research into malaria vaccines has focused on the *P. falciparum* strain due to the high mortality caused by the parasite and the ease of carrying out *in vitro/in vivo* studies.

(i) Type 1 vaccine is suggested for those exposed mostly to *P. falciparum* malaria in sub-Saharan Africa

(ii) Type 2 vaccine could be thought of as a 'travellers' vaccine', aiming to prevent all cases of clinical symptoms in individuals with no previous exposure. Problems with the current available pharmaceutical therapies include costs, availability, adverse effects and contraindications, inconvenience and compliance.

Malaria vaccine strategies can be categorized by their intended primary mode of protection and the stage of the parasite lifecycle which they target. The mode of action of the vaccine candidate will determine the type of trial that is used to evaluate its efficacy.

2.4.2.1 Pre-erythrocytic stage

Pre-erythrocytic vaccines are designed to prevent the establishment of the liver-stage of the malaria infection with the subsequent release of primary merozoites into the blood by targeting either the blood-borne sporozoites or the infected hepatocytes (Mullin, 2014).

Examples are circumsporozoite protein (CSP), multiple antigen peptide (MAP), De-oxyribonucleic acid vaccine (naked DNA) and RTS,S (Agnandji *et al.*, 2012; Bejon *et al.*, 2005; 2008; Plassmeyer *et al.*, 2009; Mahajan *et al*., 2010).

The RTS,S/AS01(RTS,S) vaccine is the most advanced candidate against the most deadly form of human falciparum malaria. It is a malaria vaccine developed through a partnership between GlaxoSmithKline Biologicals (GSK) and the PATH Malaria Vaccine Initiative (MVI), with support from the Bill & Melinda Gates Foundation and from a network of African research centres that performed the studies. The vaccine, RTS,S, is the first malaria vaccine to have undergone pivotal Phase 3 testing and obtained a positive scientific opinion by regulatory authority. It offers no protection against *P. vivax* malaria, which predominates in many countries outside Africa. The vaccine is being considered as a complementary malaria control tool in Africa that could potentially be added to and not replace the core package of proven malaria preventive, diagnostic and treatment interventions.

A Phase 3 trial of RTS,S/ASO1 began in May 2009 and completed enrolment in 2011. It included 15,460 children in seven countries in sub-Saharan Africa. The vaccine candidate is a pre-erythrocytic stage hybrid recombinant protein vaccine, based on the RTS,S recombinant antigen. The formulation comprises 25 μg of RTS,S with the AS01E adjuvant system. However there is still no generally accepted correlate of protection induced by RTS,S/AS01(White, *et al.*, 2015; WHO, 2016).

2.4.2.2 Asexual Blood-stage

This vaccine is expected to provide partially protection against malaria attacks. A perfect, anti-parasitic vaccine would completely prevent blood-stage infection, making the presence or absence of parasites an acceptable measure of vaccine performance. These include Merozoite surface Protein 1 and 2 (MSP-1, MSP-2), apical membrane protein (AMA-1), Serine rich protein (SERA) and ring infected erythrocyte surface antigen (RESA). Vaccine SPf66 was one of the earliest developed (Pattaroyo *et al*., 1987; 1988). It is a synthetic peptide vaccine containing antigens from the blood stages of malaria linked together with an antigen from the sporozoite stage, and is targeted mainly against the blood (asexual) stages. (Fairhurst and Wellems, 2006; Graves and Gelband, 2006a; 2006b; Raj *et al.*, 2014)

2.4.2.3 Sexual Stage

Malaria vaccines which target the sexual stage of the parasite, also called transmission-blocking vaccines, are being developed to interrupt the parasite life cycle. In this case, there would be no immediate health benefit to the vaccinated individual. Reduced transmission may lead to reduced morbidity and mortality in the community. Examples are *P. falciparum* transmission blocking vaccine candidate (Pfs-25) and a genetically engineered, attenuated vaccinia virus, multistage multicomponent *P. falciparum* vaccine known as NYV|C-pf7 (Kaslow, 1997; Blackman *et al.*, 1990).

2.4.2.4 Multi-stage Vaccine (pre-erythrocyte and asexual blood stages)

This is a multicomponent vaccine directed towards the pre-erythrocytic and asexual blood stages. It is expected to provide partial protection against malaria attacks. Example is cocktail peptide vaccine Spf66 which was the first synthetic vaccine to undergo extensive field trial in South America and Africa (Pattaroyo *et al*., 1987; 1988; Mahajan *et al*., 2010).

2.4.2.5 Challenges to Developing Malaria Vaccines

Even with the recent progress, accelerating development of malaria vaccines remains as complex as ever. Developers face myriad of challenges, including:

1. There are no known correlates of immunity for malaria vaccines; therefore, vaccine candidates can only be shown to work by going through clinical trials. The need for an empirical process makes developing malaria vaccines expensive and time consuming.
2. Owing to the above, the field would benefit from the availability of diverse target antigens and antigen delivery platforms capable of inducing a variety of immune responses. Few new antigens have been added to the malaria vaccine candidate arsenal over the past decade, and immune-enhancing adjuvants, which are few in number and largely controlled by for-profit entities, are not freely available.

3. The field needs additional, and more rigorously qualified, assays and models for assessing vaccine candidates to inform decision-making along the development pathway.
4. Various business models are needed for ensuring the availability of vaccines once developed.
5. Activities to ensure vaccine financing and use are crucial if a viable candidate is to advance through development and reach those in need.

2.5 Malaria: Poverty, health care and traditional medicines

Malaria disproportionately affects poor people who cannot afford treatment or who have limited access to health care facilities, and traps families and communities in a downward spiral of poor health and poverty. The cost and limited availability of many medicines mean that many populations in low-income nations may often rely on traditional herbal remedies as the first line of treatment for malaria, perhaps in half or more of cases in some poor countries in sub-Saharan Africa. Medicinal plants have clearly played an important role in malarial treatment for centuries. Since the 15th century, local people in South America and the Spanish (after their arrival) recognised the potency of the bark of the local *Cinchona ledgeriana* tree, which contains quinine, as an antimalarial. Various synthetic analogues of quinine have also since been developed for treatment. The use of artemisinin extracts from the *Artemisia annua* shrub – especially of artesunate, artemether and dihydroartemisinin – is another example of a traditional treatment (used originally in China) that has become increasingly important worldwide in recent years

(Sinclair *et al*., 2012). The interest in *Artemisia annua*, developing drug resistances, and the limited access of poor communities to modern drugs, have stimulated renewed interest in the current use and future potential of other plant products in treating malaria. Thiis may be as part of traditional health care practices and in developing new conventional medicines. Over a thousand plant species are identified by traditional healers as effective in the prevention and/or treatment of one or more of the recognized symptoms of malaria (Ajaiyeoba *et al.,* 2003; 2006; Ajibesin *et al*., 2008).

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2.6 Plants as Sources of Antimalarial Drugs

The use of natural products with theurapeutics properties dates back to ancient times. Plants have formed the basis of sophisticated traditional medicine systems and continue to provide mankind with new remedies (Samuelsson, 2004; Fournet & Muñoz, 2002; Garavito *et al*., 2006). High reliance on plants still remains the main stay of treatment in developing world with a high incidence of malaria and other protozoa; diseases (Munoz *et al.*, 2000; Mureji *et al*., 2003; Ajaiyeoba *et al.*, 2002; 2004; Hout *et al*., 2006; de Mesquita *et al*., 2007; Ibrahima et al., 2012). Antiplasmodial activity has been linked to several classes of secondary plant metabolites, including alkaloids, terpenoids, coumarins, flavonoids, chalcones, quinones and xanthones (Srivastava and Mishra, 1985; Sibandze and van Zyl, 2008; Muthaura *et al*., 2007). Of these, the antiplasmodial activity of the alkaloids is the most recognised (Caraballo *et al*., 2004, Saxena *et al*., 2003, Rukunga and Simons, 2006).

2.6.1 Alkaloids

One of the oldest and most important antimalarial drugs, quinine, is an alkaloid, a naturally occurring physiologically active nitrogenous base. Alkaloids are divided into a number of sub-groups and antiplasmodial activities have been reported for most of them (Basco *et al*., 1994; Federici *et al*., 2000; El Sayed *et al*., 2001; Wright *et al*., 2002; Ovenden *et al*., 2002; Rao *et al*., 2006)

2.6.1.1 Naphthylisoquinoline alkaloids

These alkaloids show remarkable activity against *P. falciparum*, both *in vivo* and *in vitro*. For example, dioncopeltines A, B and C isolated from *Triphophyllum peltatum* (Dioncophyllaceae) exhibit high antiplasmodial activity in *P.berghei* infected mice (Francois *et al*., 1997). Dioncophylline C cured infected mice completely after oral treatment with 50 mg kg^{-1} day^{-1} for 4 days without noticeable toxic effects. A novel hetrodimeric antiplasmodial napthylisoquinoline alkaloid, Korupensamine E and korundamine A, were isolated from another species of Dioncophyllaceae, *Ancistrocladus korupensis*. It is one of the most potent naturally occurring antiplasmodial napthylisoquinoline dimers with an IC_{50} value of 2.0 and 1.1μg/mL against *P. falciparum* respectively (Hallock *et al*., 1997; 1998).

Ancistrolikokines A, C and B and Korupensamine A isolated from *Ancistrocladus likoko* showed good to moderate antimalarial activities *in vitro* with IC_{50} values of 191, 6232, 538 and 24 ng/mL and 140, 924, 208 and 72 ng/mL against *P. falciparum* NF54 strain

and K1 strain, respectively. (Bringmann *et al.*, 2000a). The 5,8′-Coupled naphthylisoquinoline alkaloid, ancistroealaine-B from *Ancistrocladus ealaensis* exhibited activity against *P. falciparum* (IC_{50} = 0.52 µg/mL). Ancistroealaine-B represented pure 'ancistrocladaceae-type' alkaloid, *S*-configured at C-3 and equipped with an oxygen function at C-6 (Bringmann *et al*., 2000b). Bringmann *et al.* (2002) reported ancistrocongolines A–D from *Ancistrocladus congolensis* along with the known alkaloid Korupensamine A. All compounds exhibited antiplasmodial activities with ancistrocongoline B being the most active (IC_{50} = 0.15 µg/mL). The 7,3′-Coupled ancistrotectorine, ancistrocladidine and ancistrotanzanine C , possessing antimalarial activities against the K1 strain (IC_{50} = 0.1–0.7 µg/mL) have been reported from *Ancistrocladus tanzaniensis* (Bringmann *et al.,* 2004). Campbell *et al*., (1998) isolated four alkaloids, lycorine, 1,2-di-*O*-acetyllycorine, ambelline and crinine, from the bulbs of *Brunsvigia littoralis*. The alkaloids displayed very good activity (IC_{50} = 0.62 and 1.0 µg/mL, respectively), but were cytotoxic. Sener and co-workers isolated four groups of alkaloids, lycorine, crinine, tazettine, and galanthamine exhibiting antimalarial activity at different potencies, from amaryllidaceae plants namely *Pancratium maritimum*, *Leucojum aestivum*, and *Narcissus tazetta*, found in Turkey (Sener *et al.*, 2003). Haemanthamine and 6-hydroxyhaemanthamine were active with IC_{50} of 0.70 and 0.34 µg/mL, respectively, against *P. falciparum* (T9.96), while galanthamine IC_{50} = 4.38 µg/mL) and tazettine (IC_{50} = 5.42 µg/mL) had least activity against *P. falciparum* (K1).

2.6.1.2 Quinoline Alkaloids

Historically, quinine has been an important drug for the treatment of malaria, and remains so with the widespread occurrence of chloroquine-resistant strains of *P. falciparum* (Kayser *et al*., 2003). Using quinine as a lead structure, synthetic derivatives such as chloroquine and mefloquine with higher antimalarial activity were developed. Other natural quinoline derivatives, such as 2-*n*-propylquinoline, chimanine B and 2-*n*-pentylquinoline, have been shown to exhibit EC_{50} values of 25 to 50 µg/mL parasites causing cutaneous leishmaniasis activity (Cimanga *et al*., 1997).

2.6.1.3 Bisbenzylisoquinoline Alkaloids

The bisbenzylisoquinoline alkaloids can be divided into three categories: biscoclaurines, coclaurin-reticulines and bisreticulines, according to the nature, the number, and the attachment point of the bridges. A number of bisbenzylisoquinolines with antiprotozoal activity have been identified. Most have an IC_{50} value for *in vitro* antiplasmodial activity below 1.0 µg/mL (Hay *et al*., 2007). For instance, pycnamine from *Trichilia* sp. was found to have an IC_{50} value of 0.15 µg/mL (Dharani *et al*., 2010). The *in vitro* antiplasmodial activity of chrysopentamine, strychnopentamine and isostrychnopentamine isolated from the leaves of *Strychnos usambarensis* was determined against three *Plasmodium falciparum* cell lines in comparison to chloroquine and quinine, Chrysopentamine had the highest activity. It presented an IC_{50} around 500 nM against all tested *Plasmodium* lines (Frédérich *et al*., 2004).

The alkaloids exhibited antiplasmodial activity (IC_{50} 29–1500 nM) against D6 clone, IC_{50} 59–4030 nM against W2 clone of *P. falciparum*. (Angerhofer *et al*., 1999). The most elective alkaloids were (−)-cycleanine), (+)-cycleatjehine, (+)-cycleatjehenine , (+)-malekulatine , (−)-repandine and (+)-temuconine Mambu *et al*. reported strong antiplasmodial activity of (−)-curine and isochondodendrine isolated from the stem bark of *Isolonagh esquiereina,* IC_{50} = 353 and 892 nM, respectively (Mambu *et al*, 2000). Azaanthracene alkaloid a bis-benzylisoquinoline alkaloid (1*S*, 1'*R*)- rodiasine (IC_{50} = 1.14 μM) from *Pseudoxandra cuspidata* had (IC_{50} = 42.92 μM) (Roumy *et al*., 2006).

2.6.1.4 Protoberberine and Aporphine Alkaloids

Antimalarial activities of several protoberberine group containing alkaloids, which exhibited promising antiplasmodial activities were evaluated Wright *et al*. (2000). Dehydrodiscretine, IC_{50} = 0.64 μM) and berberine (IC_{50} = 0.96 μM) were the most active. In the aporphine group, norcorydine possessed the highest antiplasmodial activity (IC_{50} = 3.08 μM). Antimalarial activity of phenolic aporphine-benzylisoquinoline alkaloids isolated from the roots of *Thalictrum faberi* IC_{50} = 11.2- 24.2 μg/mL (Lin *et al.,* 1999). Hadranthine A obtained from *Duguetia hadranth* exhibited *in vitro* antimalarial activity against *P. falciparum* with IC_{50} = 120 μg/mL and selectivity index of >40 (Muhammad *et al.,* 2001).

2.6.1.5 Indole Alkaloids

The indole substructure is widely distributed in the plant kingdom. Some indoles are reported to possess antiplasmodial activity. For instance, cryptolepine and related indolequinolines isolated from *Cryptolepis sanguinolenta* were active *in vitro* against the W2, D6 and K1 strains of *P. falciparum*, with IC_{50} values ranging from 27 to 41 μg/mL (Kayser *et al*., 2003).

From the review of antiplasmodial indole alkaloids from natural sources by Frederich and co workers, 10′-Hydroxyusambarensine from the roots of *Strychnos usambarensis* displayed modest antimalarial activity (IC_{50} = 0.16 μg/mL, W2 strain). (Frederich *et al.*, 1999; Frederich *et al.*, 2008). Cryptoleptine *from Cryptolepis sanguinolenta* was the second most active after cryptolepine against K1 and T996 strains of *P. falciparum* with IC_{50} = 0.8 and 0.23 μM, respectively, (Paulo *et al*., 2000). Icajine, isoretuline and strychnobrasiline reversed CQ resistance at concentrations between 2.5 and 25.0 μg/mL, with an interaction factor (IF) of 12.82 for isoretuline on W2 strain. Icajine was found synergistic with mefloquine with an IF = 15.38 (Frederich *et al.*, 2001a).

Many dimeric or trimeric indolomonoterpenic alkaloids with antiplasmodial properties have been isolated from root-bark of *Strychnos icaja*, found in Central Africa (Barbaras *et al*., 2008; Frederich *et al.,* 2001b, 2002; Phillippe *et al.,* 2002; 2007). Antimalarial β-carboline alkaloids, canthin-6-one and 9-hydroxycanthin-6-one isolated from the roots of *Eurycoma longifolia* with IC_{50} of 2.2 and 2.3 μg/mL, respectively (Kuo *et al.,* 2003).

Chrysopentamine isolated from *S. usambarensis* displayed antiplasmodial activity against three *P. falciparum* cell lines FCA 20, FCB1-R and W2 with IC_{50} approximately 0.5 μM.(Frederich, 2004). Ellipticine and aspidocarpine isolated from *Aspidosperma vargasii* and *A. desmanthum*, respectively, exhibited significant *in vitro* inhibition of *P. falciparum* K1 strain, IC_{50} = 0.073 and 0,02 μM, respectively) (de Andrade-Neto *et al,*, 2007). Alstonine from *Picralima nitida* displayed *in vitro* antimalarial activity against *P. falciparum* comparable to quinine with IC_{50} value of 0.9 μg/mL (Okunji *et al.,* 2005). Naucleofficine A isolated from *Nauclea officinalis* was reported to have *in vitro* antimalarial activity (Sun *et al.,* 2008).

2.6.1.6 Phenanthridine and Benzophenanthridine Alkaloids

These alkaloids are mostly found within three plant families: the Papaveraceae, Fumariaceae and Rutaceae (Krane *et al*., 1984). Examples of antimalarial benzophenanthridine alkaloids obtained from plant sources are fagaronine and nitidine. The IC_{50} value of these alkaloids ranges from 0.09 to 0.11 μg/mL against *P. falciparum* (Gakunju *et al.,* 1995; Kassim *et al*., 2005).

2.6.2 Terpenoids

2.6.2.1 Monoterpenes

Monoterpenes constitute structurally simple antiprotozoal compounds. Piquerol A, isolated from *Oxandra espinata*, has been shown to exhibit low activity against *P. falciparum* strains, with an IC_{50} value of 100 μg/mL (Dharani *et al.,* 2010).

2.6.2.2 Sesquiterpenes

The discovery of artemisinin (qinghaosu), a sesquiterpene lactone endoperoxide, as an antimalarial constituent in the Chinese plant *Artemisia annua,* has prompted the investigation of other naturally occurring compounds with peroxide groups (O-O bonds) for their antiplasmodial activity (Robert *et al*., 2002). The 1,2,4 trioxane ring in artemisinin is essential for activity. After being opened in the *Plasmodium* cell it liberates singlet oxygen which is a strong cytotoxin. In addition to sesquiterpene endoperoxides, other sesquiterpenes with antiplasmodial activity have been reported. For example, activity has been documented for the germacranolide sesquiterpene lactones neurolenin A and B from *Neuroleaena lobata*, a medicinal plant used in Guatemala for the treatment of malaria infection (Francois *et al*., 1997). Tagitinin C, present in the leaves of *Tithonia diversifolia* was active against *Plasmodium falciparum* (Goffin *et al*., 2002)

2.6.2.3 Diterpenes

Diterpenes from many plant species are well known for their antiplasmodial properties (Kayser *et al*., 2003). However, most combine high antiparasitic activity with high cytotoxicity to mammalian cells (Oketch-Rabah *et al*., 1998). For example, the macrocyclic germacrane dilactone 16,17-dihydrobrachy-calyxolide from *Vernonia brachycalyx* shows antiplasmodial activity (IC_{50} = 17 μg/mL against *P. falciparum*) but also inhibits the proliferation of human lymphocytes at the same concentration (Oketch-Rabah *et al*., 1998). Other examples of antiplasmodial diterpenes are *E*-phytol and 6-*E*-geranylgeraniol-19-oic acid, isolated from *Microglossa pyrifolia* (Köhle *et al.,* 2002).

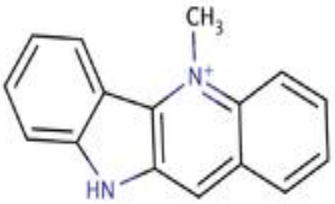
2.6.2.4 Triterpenes and Saponins

Triterpenes are known for their antiplasmodial activities, but exhibit some toxicity for humans and other mammals (Kayser *et al*., 2003). Betulinic acid was identified to be the antiplasmodial principle of *Triphyophyllum peltatum* and *Ancistrocladus heyneanus*. Bringmann *et al*. (1997), reported an IC_{50} value of 10 μg/mL for betulinic acid against *P. falciparum in vitro* and moderate cytotoxicity (IC_{50}> 20 μg/mL).

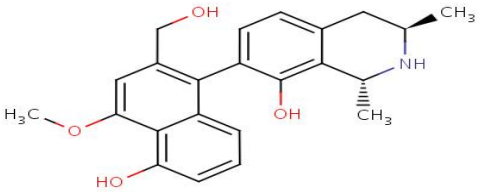
Saponins are also toxic to humans and their use as drugs is limited due to poor bioavailability, limited absorption in the gastrointestinal tract and haemolytic toxicity.
The plant *Asparagus africanus* has yielded a new steroidal saponin, muzanzagenin, with antiplasmodial activity (IC_{50} = 61 μM against the K39 isolate of *P. falciparum* (Oketch-Rabah *et al*., 1997).

2.6.3 Limonoids

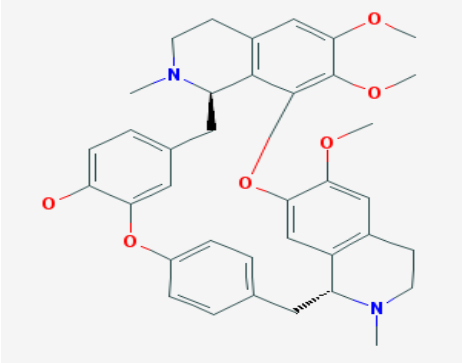
Limonoids are also known as bitter terpenoids (Kayser *et al.,* 2003). One well known plant family rich in limonoids is the Meliaceae, of which *Azadirachta indica*, the neem tree which is widely used as an antiplasmodial plant, is a representative (Karus *et al*., 1981. Nimbolide (IC_{50} = 0.0095 μg/mL, *P. falciparum* K1 strain) was the first agent to be identified as an active antiplasmodial principle in neem (Rochanakij *et al*., 1985). Subsequently, gedunin was also found to be active *in vitro* against *P. falciparum*, with IC_{50} values in the range of 0.7 to 1.7 μg/mL (MacKinnon *et al*., 1997) but did not show promising antimalarial activity *in vivo* mouse model. Limonoids from other Meliaceae



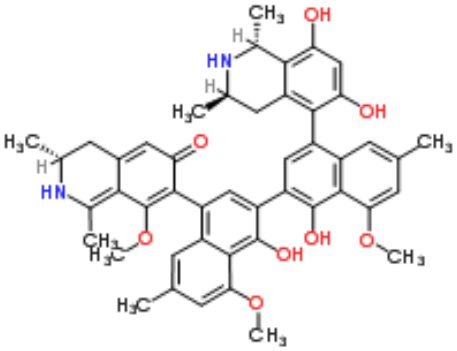
Crptolepin



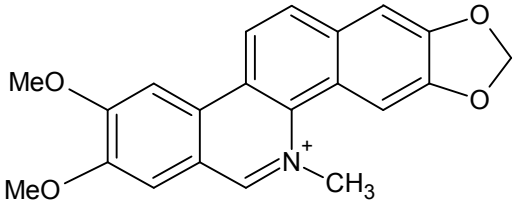
Dioncopentile A



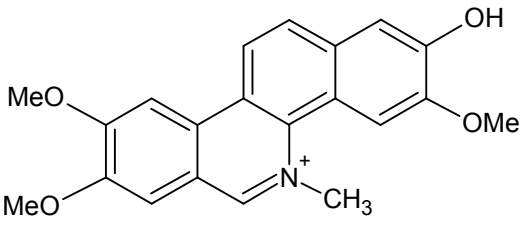
Pycnamine



Korundamine A

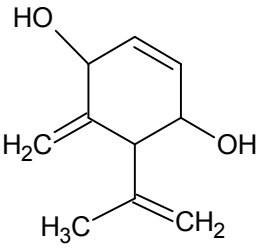


Nitidine

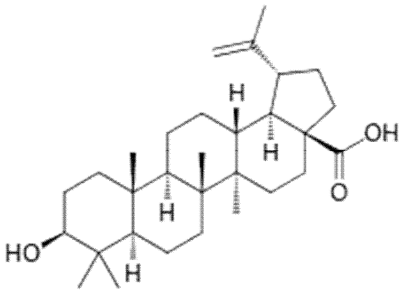


Fagaronine

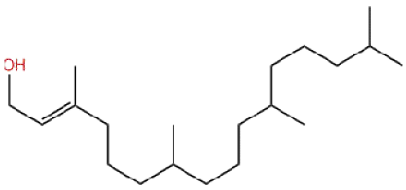
Fig. 2.4: Alkaloids with Antimalarial Activities



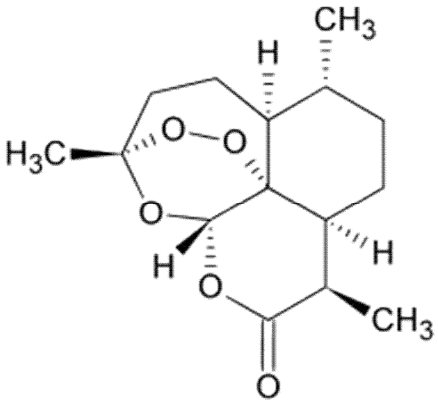
Piquerol A



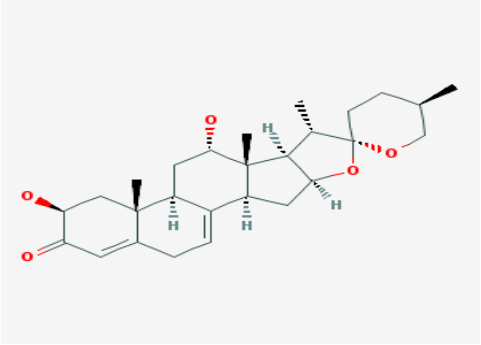
Betulinic acid



E- phytol



artemisinin



Muzazangenin

Fig. 2.5: Terpenoids and Saponins with Antimalarial Activity

plants were also found to possess antiplasmodial activity (Bickii *et al*., 2000; Agbedahunsi *et al*., 1998; Garcez *et al.*, 1997; Sengupta *et al*., 1960).

2.6.4 Quassinoids

They are bitter principles characterizing the Simaroubaceae plant family. Quassinoids are heavily oxygenated lactones, the majority with a C20 skeleton referred to as picrasane. However, C18, C19 and C25 quassinoids are also known. They are biosynthetically related to triterpenes, sharing the same metabolic precursors. *Brucea javanica* was one of the oldest Simaroubaceae plant screened for antiplasmodial activity. Brucein A, B, and C isolated from chloroform fraction of this plant were active against multi-drug resistant strain of *P. falciparum* comparable to mefloquine (Pavanand *et al*., 1988). Antimalarial activity has been reported for ailanthone (IC_{50} = 0.003 μg/mL) and 6α-tigloyloxychaparrinone (IC_{50} = 0.061 μg/mL) isolated from *Ailanthus altissima* (Okunade, 2003). Quassinoids like pasakbumin B and C, and eurycomanone possessing antimalarial activity were isolated from *E. longifolia* with IC_{50} = 22.6, 93.3 and 40.0 μg/mL, respectively, (Kuo *et al*., 2004; Chan *et al*., 2004). The most active compound in the group is reported to be simalikalactone D from *Simaba guianensis and S. orinocensis*, with an IC_{50} value of 0.017 – 0.03 μg/mL (Cabral *et al*., 1993, Muhammad *et al.,* 2004). Simalikalactone D was also identified to be responsible for the antimalarial activity of *Quassia amara* leaves (Bertani *et al.*, 2006). Antiplasmodial compounds, the samaderines B, E, X and Z obtained from *Quassia indica* were active against chloroquine resistant (K1) strain of *P. falciparum* with an IC_{50} of 0.14-0.21 μM (Kitagawa *et al*., 1996). Cedronin from *Simaba cendron* was active *in vitro* against both

chloroquine sensitive and resistant strains of *P. falciparum* and also *in vivo* against *P. vinckeri petteri* in mice (Moretti *et al*., 1998). Neosergeolide isolated from *Picrolemma sprucei*, has shown potent antimalarial activity, IC_{50} = 0.02 μM). (de Andrade-Neto *et al*., 2007). The activity of compounds in this group is due to the oxymethylene bridge. Other quassinoids such as brusatol, bruceantin and bruceins (A, B and C) and orinocinolide have been isolated and their antimalarial activity determined *(*Muhammad *et al.,* 2004). Although quassinoids are generally cytotoxic, few compounds such as glaucarubinone from *Simarrouba amara* were relatively selective against *P. falciparum in vitro* but were toxic *in vivo.*

2.6.5 Coumarins

The antiplasmodial activity of 2'-epicycloisobrachycoumarinone epoxide and its stereoisomer, isolated from *Vernonia brachycalyx*, have been reported. Both stereoisomers show similar *in vitro* activity against chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum,* IC_{50} = 54 μM against Dd2 strain (Oketch-Rabah *et al*., 1997). Clausarin and dentatin (IC_{50} = 0.1 and 8.5 μg/mL) from *Clausena harmandiana* (Yenjai *et. al*. 2000) were found active *in vitro*. A new coumarin derivative, 5,7-dimethoxy- 8-(3'-hydroxy-3'-methyl-1'-buteneyl)-coumarin, was isolated from *Toddalia asiatica* and was found to have IC_{50} values of 16.2 and 8.8 μg/mL against chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*, respectively (Oketch-Rabah *et al*., 1997a).

The stem bark of *Exostema mexicanum* is used as a quinine substitute for malaria treatment in Latin American folk medicine. Bioassay-guided fractionation of lipophilic and hydrophilic extracts from the stem bark yielded 4-phenylcoumarins. The most lipophilic compound, *O*-methylexostemin revealed the strongest antiplasmodial activity with IC_{50} = 3.60 µg/mL (Kohler *et al.*, 2001). The EtOAc extract of the stem bark of *Hintonia latiflora* showed total parasitemia suppression and chemo-suppression of schizont numbers in *P. berghei* infected mice. Antimalarial activity was associated with phenylcoumarins that suppressed the development of *P. berghei* schizonts *in vitro,* IC_{50} = 24.7 and 25.9 µM, respectively, (Argotte-Ramos, 2006).

Isoimperatorin isolated from methanol extract of the roots of *Zanthoxylum flavum* had IC_{50} values of 5.5 and 2.7 µM against D6 and W2, respectively (Ross *et al*., 2008). A new coumarinolignan, grewin obtained from *Grewia bilamellata* displayed antimalarial activity against D6 and W2 with IC_{50} 11.2 µM and 5.5 µM, respectively, without significant cytotoxicity (Ma *et al*., 2006). The compound 1-O-galloyl-6-O-luteoyl-a- D-glucose with an IC_{50} value of 2.21 mM (FCR3) was isolated from *Phyllanthus niruri* (Subeki *et al*., 2005).

2.6.6 Flavonoids

Flavonoids are widespread in the plant kingdom. Following the detection of antiplasmodial flavonoids in *Artemisia annua* there has been renewed interest in these compounds. Other *Artemisia* species have been screened. Exiguaflavanones A and B

isolated from *Artemisia indica* exhibited *in vitro* activity against *P. falciparum*, with EC_{50} values of 4.6 and 7.1 μg/mL, respectively (Chanphen *et al*., 1998).

Acetoxy-4′,5,7-trihydroxyflavanone isolated from *Siparuna andina,* showed high *in vitro* antimalarial activity (IC_{50} = 3.0 μg/mL) (Jenett-Siems *et al.*, 2000). 6-Hydroxyluteolin-7-*O*-(1″-α-rhamnoside) obtained from *Vriesea sanguinolenta* had IC_{50} = 2.13 and 3.32 μM against K1 and NF54 strains, respectively, (Bringmann *et al.,* 2000c). Acacetin, 7-methoxyacacetin, and genkwanin isolated from *A. afra*, possessed considerable antiplasmodial activity. Acacetin demonstrated highest activity (IC_{50} = 5.5 and 12.6 μg/mL against poW and Dd2 strain, respectively). *Andira inermis* yielded calycosin and genistein which were the first isoflavones to possess antiplasmodial activity with IC_{50} = 4.2 and 9.8 μg/mL and IC_{50} = 2.0 and 4.1 μg/mL against W2 and Dd2 strains, respectively, (Kraft *et al.*, 2000). Antiplasmodial activities of isoflavanquinones (abruquinone) from *Abrus precatorius* gave IC_{50} = 1.5 μg/mL (Limmatvapirat *et al.,* 2004). Antimalarial flavonol arabinofuranosides obtained from *Calycolpus warszewiczianus* had IC_{50} value of 14.5 μM (Torres-Mendoza *et al*., 2006). A flavone glycoside from *Phlomisbrun neogaleata*, (Kirmizibekmez *et al.*, 2004) and iridoid from *Scrophularia lepidota*, (Tasdemir *et al*., 2005) have been reported to inhibit FabI enzyme of *P. falciparum* (IC_{50} = 10.0 and 100.0 μg/mL, respectively). Other biflavones reported to possess moderate to good antimalarial activity include sikoanin B and C from *Wikstroemia indica* with IC_{50} = 0.54 and 0.56 μg/mL, respectively (Nunome *et al.*, 2004). Biflavanone isolated from the bark of *Ochna integerrima* ws found to be responsible for the antiplasmodial activity of the plant. The IC_{50} = 80.0 ng/mL (Ichino *et al.,* 2006a).

Green tea flavoniods catechins, which include (−) epigallocatechin gallate (EGCG), (−)-epicatechin gallate, (−)-epigallocatechin, and (−)-epicatechin inhibit PfENR reversibly with EGCG being the best ($K_i = 79 \pm 2.67$ nM) (Sharma *et al*., 2007). Heterophyllin, heteroflavanone C and artoindonesianin A-2 isolated from the stem bark of *Artocarpus champeden* were tested for their inhibitory activity against 3D7 strain of *P. falciparum*. All possessed interesting activity with inhibitory concentrations from 0.001 to 1.31 μM (Widyawaruyanti *et al*., 2007).

2.6.7 Chalcones

Phlorizidin, from *Micromelum tephrocarpum*, was one of the first chalcone glycosides reported to exhibit antiparasitic activity (Kayser *et al*., 2003). In traditional medicine, *M. tephrocarpum* is used to treat malaria because of its bitter taste, a property shared with quinine and other antimalarial herbs. Phlorizidin inhibits the induced permeability in *Plasmodium* infected erythrocytes to various substrates including glucose.

Licochalcone A isolated from *Glycyrrhiza inflata* and *G. glabra* has been identified as potent inhibitor of protease activities of *Plasmodium* (Chen *et al*., 1994,). (+)-Nyasol ($IC_{50} = 49$ μM) isolated from *Asparagus africanus* (Oketch-Rabah *et al.*, 1997b)*, (*and pinostrobin ($IC_{50} > 100$ μM) from *Cajanus cajan* (Ducker-Eshun *et al.,* 2004) possess weak antimalarial activity. Cajachalcone, 2′,6′-dihydroxy-4-methoxy chalcone was identified as the biologically active constituent from the leaf extract of *Cajanus cajan* (Ajaiyeoba *et al*., 2013). Cajachalcone had an IC_{50} value of 2.0 μg/mL (7.4 μM) against the multiresistant strain of *Plasmodium falciparum* (K1) in the parasite lactate

dehydrogenase assay. 5-Prenylbutein (IC_{50} = 10.3 μM) from *Erythrina abyssinica* (Yenesew *et al*., 2004) and prenylsubstituted dihydrochalcone (IC_{50} = 5.64 μM) from *Piper hostmannianum* - exhibiting antimalarial activity have been reported (Portet *et al*., 2007). Bartericin A, stipulin, 4-hydroxylonchocarpin from *Dorstenia barteri* var. *subtriangularis* and were active *in vitro* against *P. falciparum.* They had IC_{50} = 2.15, 5.13 and 3.36 μM, respectively, (Ngameni *et al.*, 2007).

2.6.8 Quinones

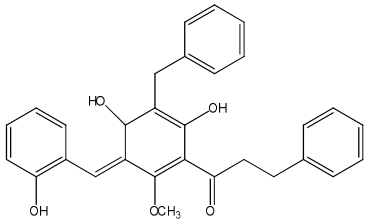
Quinone methides isolated from the roots of *Salacia kraussii* showed high antiplasmodial activity (IC_{50} = 94.0 and 27.6 ng/mL,) (Figueiredo *et al.*, 1998). Naphthoquinoid and isopinnatal possessing good antimalarial activity were reported from *Kigelia pinnata* with IC_{50} = 0.15 and 0.25 μM, respectively, (Weiss *et al.*, 2000). The mode of action of these furano- and hydroxy-naphthoquinones appears to be the inhibition of mitochondrial electron transport and respiratory chain by reduced oxygen consumption similar to that of atovaquone. Phenylanthraquinones and knipholone were isolated from *Bulbine frutescens* (Likhitwitayawuid *et. al*, 1998a; Abegaz *et al*., 2002). The glycoside displayed better activity. Newbouldiaquinone A from *Newbouldia laevis* moderately suppressed growth of *P. falciparum*, *in vitro* (Eyong *et al.*, 2006)

Benzoquinone metabolites from an endophytic fungus *Xylaria* sp (Tansuwan *et al.*, 2007) was reported to possess antimalarial activity (IC_{50} = 1.84 and 6.68 μM, respectively). Xestoquinone isolated from marine sponge, *Xesto spongia*, inhibited Pfnek-1 (IC_{50} = 1.1 μM), but was inactive towards PfPK7 and PfGSK-3 (Laurent *et al.*, 2006).

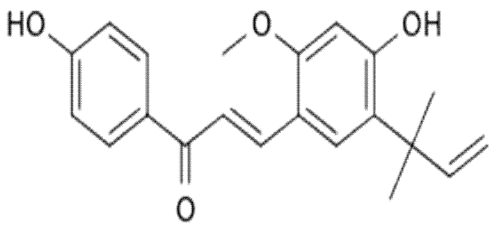
Plumbagin, a cytotoxic napthoquinone isolated from *Plumbago zeylanica*, has been found to exhibit antiplasmodial activity against chloroquine sensitive (D6) and resistant (W2) strains of *P. falciparum*, with IC_{50} values of 178 and 189 μg/mL, respectively (Jalalpure, 2011). Anthraquinones isolated from the tropical tree *Morinda lucida* have been tested for antiplasmodial activity *in vitro*: digitolutein, rubiadin-1-methyl ether and damnacanthal showed activity on chloroquine-resistant *P. falciparum* ($IC_{50} \approx 21$ to 83 μM) (Sittie *et al*., 1999). The anthraquinone benzoisoquinoline-5-10-dione has been isolated from *Psychotria camponutans* and tested against *P. falciparum.* It had IC_{50} = 0.84 μg/mL (Solis *et al*., 1995).

Cannabis sativa afforded 5-acetoxy-6-geranyl-3-npentyl- 1,4-benzoquinone, which displayed notable antimalarial activity against D6 and W2 clones with IC_{50} values of 7.5 and 7.0 mM, respectively. Among the metabolites isolated from the root extract of *Bauhinia purpurea* were bauhinoxepin I and bauhinoxepin J. The two compounds exhibited antimalarial activity against K1, with IC_{50} = 10.5 mM and IC_{50} = 5.8 mM (Boonphong *et al*., 2007). Bioactivity-guided fractionation of the ethanol extract of *Zhumeria majdae* led to the isolation of 12,16-dideoxy aegyptinone B. This compound exhibited antiplasmodial activity with IC_{50} values of 4.4 and 4.7 mM against D6 and W2 strains, respectively (Moein *et al*. 2008). From the roots of *Bulbine frutescens* the first sulfated phenylanthraquinones were isolated, together with their known sulfate-free analogues. Two of them, isoknipholone and sodium 40 -O-demethylknipholone 60-O-sulfate, presented promising activity against K1 with an IC_{50} of 0.28 mM for isoknipholone and an IC_{50} of 7.9 mM for the sulfated phenylanthraquinone (Mutanyatta

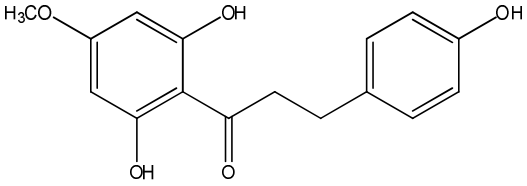
et al., 2005). The root of the African plant, *Bulbine frutescens* afforded two novel dimeric phenylanthraquinones, joziknipholones A and B were isolated. These two compounds exhibited strong activity against K1 with IC_{50} values of 164 and 270 nM, respectively (Bringmann *et al*., 2008). 10-(chrysophanol-70-yl)- 10-(x)-hydroxychrysophanol-9-anthrone and chryslandicin were isolated from the dichloromethane extract of the root of *Kniphofia foliosa* . They showed good activity against 3D7 with IC_{50} values of 0.5 and 1.0 mM, respectively (Wube *et al.*, 2005). Glaberianthrone, 3-geranyloxyemodin anthrone, 3-prenyloxyemodin anthrone, 2-geranylemodin and bianthrone 1a were isolated from the hexane extract of the stem bark of *Psorospermum glaberrimum*. Their IC_{50} values ranged between 1.68 -2.53 mM, against the W2 strain (Lenta *et al.*, 2008). From the root bark of *Harungana madagascariensis* was isolated new Bazouanthrone together with known compounds, feruginin A, harunganin , harunganol A and harunganol B . All the compounds were found to be moderately active against W2 with IC_{50} values from 1.8 – 5.0 mM (Lenta *et al*., 2007). Marcanine A was identified as the major active constituent *Polyalthia viridis*. It was found to show notable antimalarial activity with IC_{50} = 10.5 mM (Ichino *et al*., 2006b).



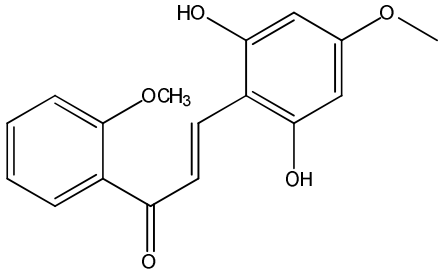
Uvaretin



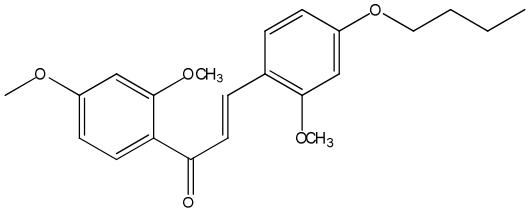
Licochalcone A



Asobogenin

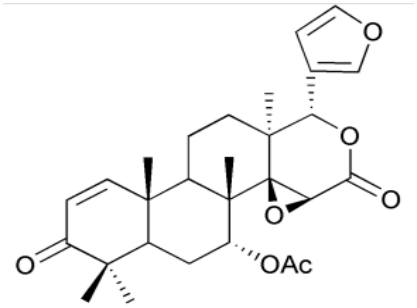


Cajachalcone

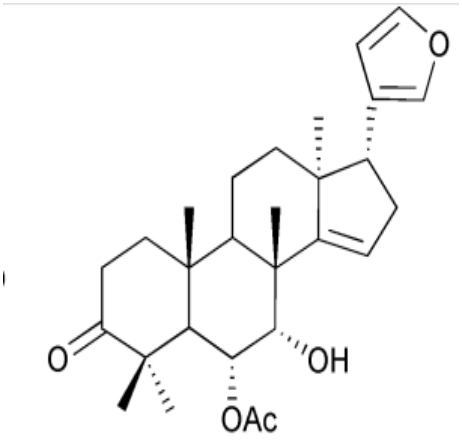


2,4-dimethoxy-4-butyloxychalcone

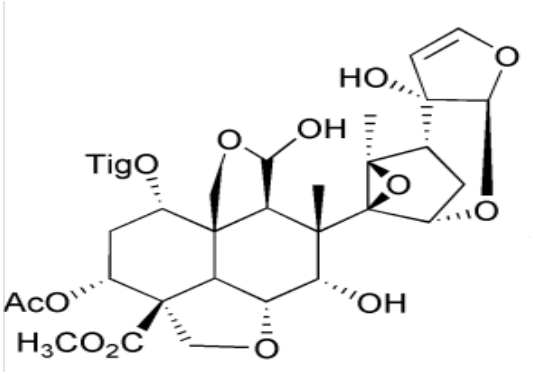
Fig. 2.10: Antimalarial Chalcones



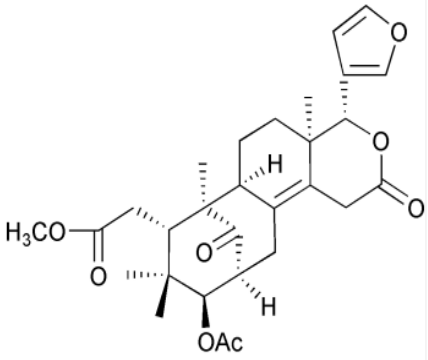
Gedunin



Meldenin

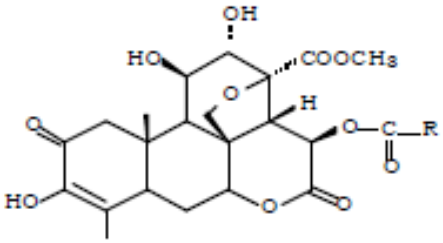


Fissinolide

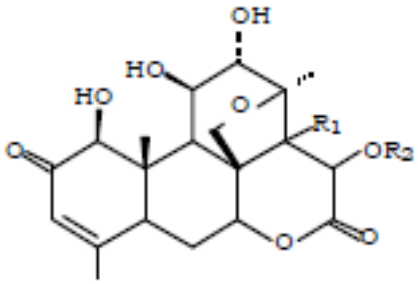


Azardirachtin

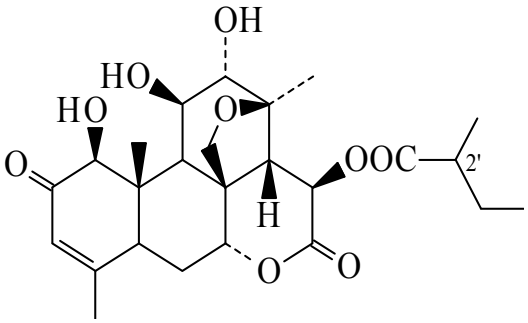
Fig. 2.6: Antimalarial Limonoids



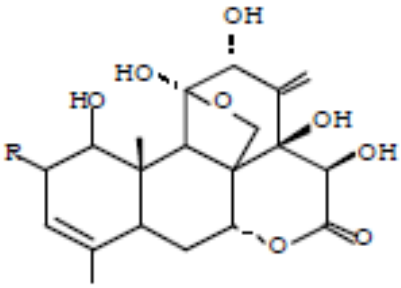
Brucein B, R=Me



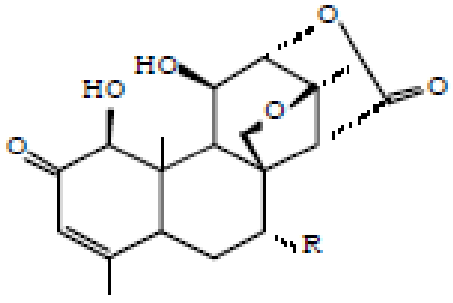
Brucein D, R1=OH; R2=H



Simalikalactone D

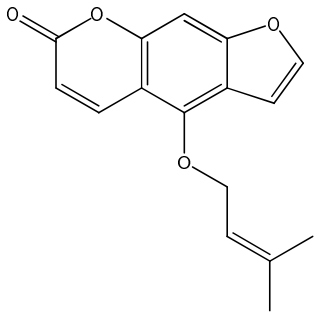


Eurycomanone, R=(O)

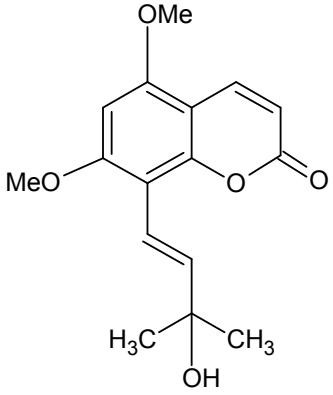


Cedronin, R=OH

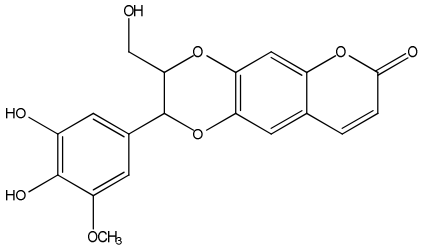
Fig. 2.7: Qaussinoids with Antimalarial Activity



Isoimperatorin

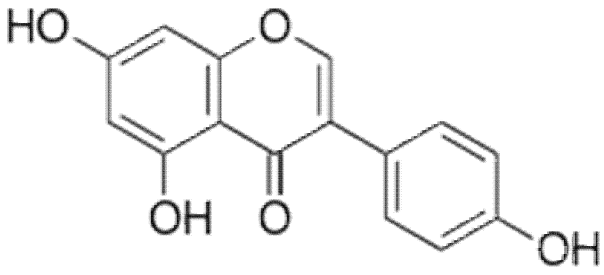


5,7-dimethoxy-8-(3′-hydroxy-3′-methyl-1′-buteneyl)-coumarin

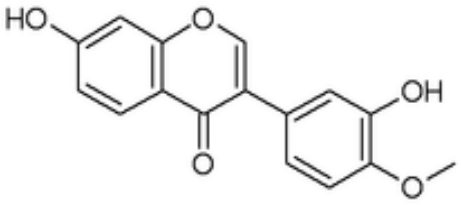


Grewin

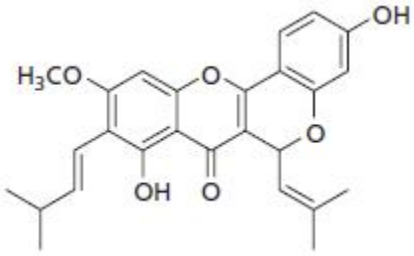
Fig. 2.8: Antimalarial Coumarins



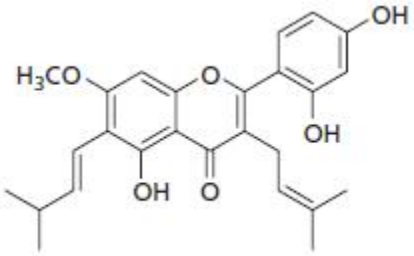
Genistein



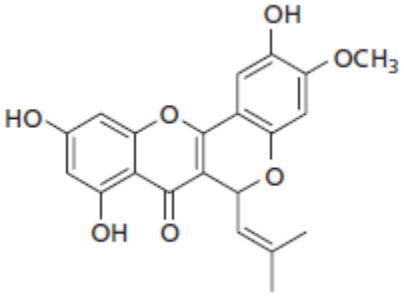
Calycosin



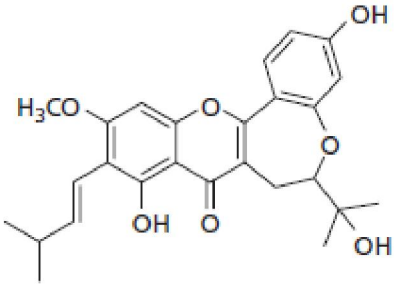
Cycloartocarpin



Artocarpin

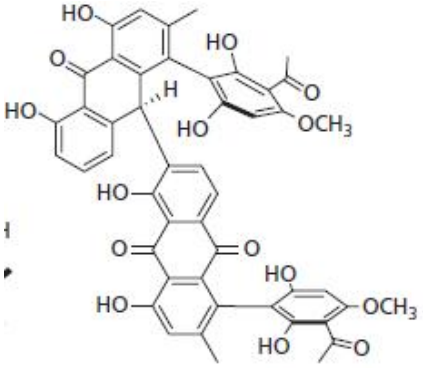


Artoindonesianin A-2

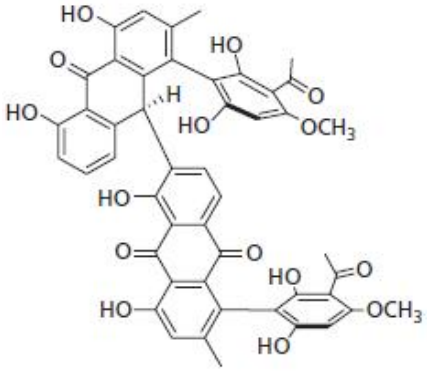


Chaplashin

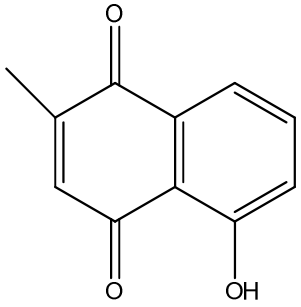
Fig. 2.9: Antimalarial Flavonoids



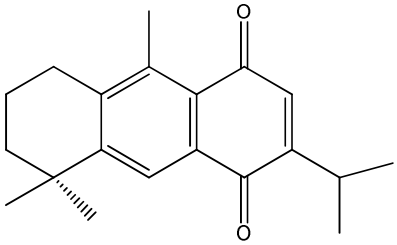
Joziknipholones A



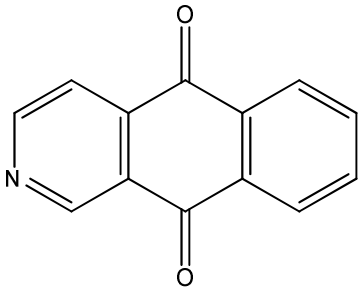
Joziknipholones B



Plumbagin



12,16-dideoxy aegyptinone B



Benzoisoquinoline-5-10-dione

Fig. 2.11: Antimalarial Quinones

2.6.9 Xanthones

Antiplasmodial xanthones have been isolated from *Garcinia cowa, Calophyllum caledonicum, Garcinia livingstonei* and *Garcinia mangostana.* Cowaxanthone had IC_{50} = 1.5 μg/mL, calothwaitesixanthone, IC_{50} = 2.7 μg/mL), and mangostin (IC_{50} = 17.0 μM, respectively (Mahabusarakam *et al.*, 2006; Hay *et al*., 2004; Likhitwitayawuid *et al*. 1998b). Garciniaxanthone, smeathxanthone A, smeathxanthone B and chefouxanthone were isolated from the roots of *Garcinia polyantha.* They exhibited antimalarial activity against NF54 with IC_{50} values ranging from 2.5 to 4.1 mM (Lannang *et al*., 2008).

Antiplasmodial and cytotoxicity activities of allanxanthone C, norcowanin, mangostin and Tovophyllin A isolated from the methanol extract of the stem bark of *Allanblackia monticola* showed that they were found to be active against the two Plasmodium strains tested, with IC_{50} = 1.3 - 4.1 mM on (FcM29) and IC_{50} on = IC_{50} = 6.3- 7.8 mM (F32) and also showed weak cytotoxicity against human melanoma A375 cells (Azebaze *et al*., 2006; 2007). The dimeric xanthone garcilivin A obtained from the root bark of *Garcinia livingstonei* (Mbwambo *et al*., 2006) showed a higher and nonselective antiparasitic activity and cytotoxicity (IC_{50} 2.0 μM against MRC-5 cells) than its diastereoisomer garcilivin C (IC_{50} 52.3 μM). A new prenylated xanthone, 5-O-methylcelebixanthone and cochinchinone C were isolated from roots of *Cratoxylum cochinchinense*. The two compounds exhibited antimalarial activity against K1 with IC_{50} values of 8.9 and 6.3 mM, respectively (Laphookhieo *et al*., 2006). A xanthone derivative, gaboxanthone, was isolated from the seed shells of *Symphonia globulifera* and evaluated against W2. It had an IC_{50} value of 3.5 in the antiplasmodial assay (Ngouela *et al*., 2006).

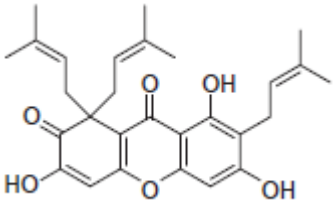
The whole plant of *Swertia alata* was investigated and three xanthones, swertiaperennine, swertianin and decussatin, were isolated and tested for antimalarial activity. The results indicated that all xanthones had IC_{50} values < 50 mM (Karan *et al*., 2003). I*n vivo* antimalarial study of swertiaperennine in the *P. berghei* test model reduced parasitemia by 17.60% at a dose of 10 mg/kg.

2.6.10 Stilbenes

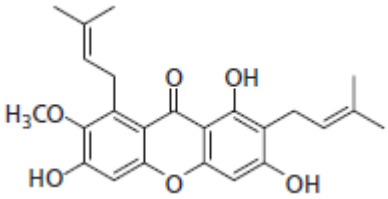
In vitro antiplasmodial activity of piceid-(1-6)-β-D-glucopyranoside isolated from the MeOH extract of the leaves of *Parthenocissus tricuspidata* against D10 strain of *P. falciparum* had IC_{50} value of 5.3 mM (Son *et al*., 2007). It showed *in vivo* activity against *P. berghei* in mice intraperitoneally and exhibited significant blood schizontocidal activity in 4-day early infection, in preventive and curative treatment, with chemosuppression of 59 and 44% at 5 mg/kg per day, respectively, and an LD_{50} > 500 mg/kg (Park *et al*., 2008). E-Resveratrol-3-O-a-L-rhamnopyranosyl- (1-2)-b-D-xylopyranoside, a stilbene glycoside was isolated from an n-butanol-soluble fraction of the root of *Pleuropterus ciliinervis*. It showed moderate cytotoxicity and antimalarial activity against D10 with an IC_{50} of 3.9 mM (Lee *et al*., 2008). It also demonstrated moderate antimalarial activity *in vivo* when tested against *P. berghei* in mice intraperitonially (Moon *et al*., 2008).

2.6.11 Essential oils

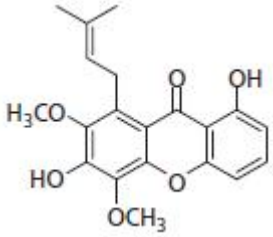
The essential oil from the leaves and stem of *Tetradenia riparia* exhibited moderate antimalarial activity against strains of *P. falciparum* (Campbell *et al*., 1997). Essential oils obtained from *Artemisia vulgaris*, *Eucalyptus globulus*, *Myrtus communis*, *Juniperus communis*, *Lavandula angustifolia, Origanum vulgare*, *Rosmaricus officinalis* and *Salvia officinalis* were active against two strains of *P. falciparum*; FcB1-Columbia and a Nigerian chloroquine resistant strain. *M. communis* and *R. officinalis* oils were most active inhibiting *P. falciparum* at a concentration ranging from 150 to 270 mg/mL (Milhan *et al*., 1997). The essential oil of *Salvia repens* exhibited an IC_{50} of 1.7 mg/mL with β-phellandrene, β-caryophyllene, limonene and camphor as major components (Kamatou *et al*., 2005).



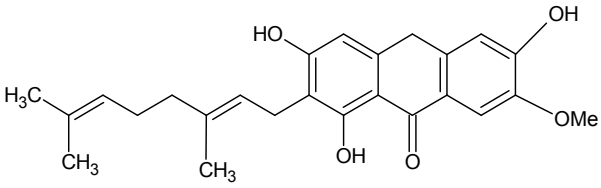
Allanxanthone C



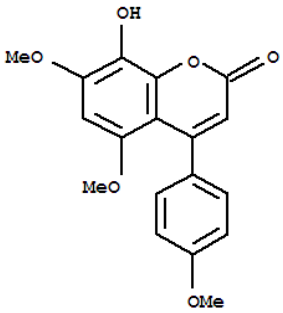
Mangostin



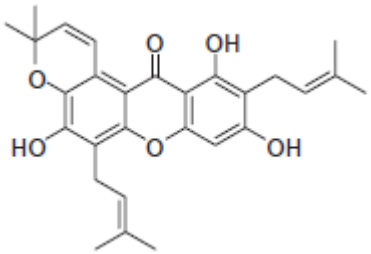
5-O-methylcelebixanthone



Cowaxanthone

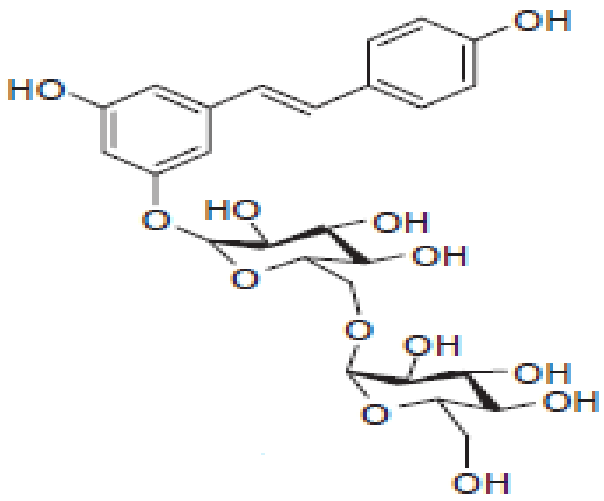


Exostemin

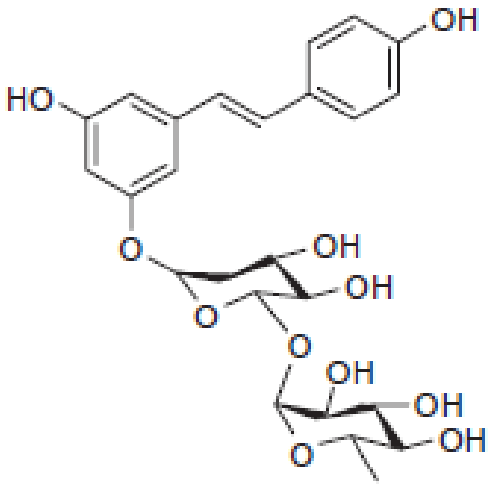


Tovophyllin A

Fig. 2.12: Antimalarial Xanthones



piceid-(1-6)-β-D-glucopyranoside



(E)-resveratrol-3-O-a-L-rhamnopyranosyl- (1-2)-b-D-xylopyranoside

Fig 2.13:Antimalarial Stilbenes

2.7 Review of Plant Extracts and Compounds Exhibiting Larvicidal Properties

After several problems encountered from indiscreet and over application of synthetic insecticides in nature, re-focus on phytochemicals that are easily biodegradable and have no ill-effects on non-target organisms was valued. Thus, the search for new bioactive compounds from the plant kingdom and an effort to determine its structure and commercial production has been initiated. At present phytochemicals make up to 1% of world's pesticide market (Isman, 1997).

Earliest reports of the use of plants against mosquito larvae is credited to Campbell *et al.* (1933) who found that plant alkaloids like nicotine, anabasine , methylanabasine and lupinine extracted from the Russian weed, *Anabasis aphylla,* killed larvae of *Culex pipiens, Cx. territans,* and *Cx. quinquefasciatus.* Several groups of phytochemicals such as alkaloids, steroids, terpenoids, essential oils and phenolics from different plants have been reported previously for their insecticidal activities. Plant families Asteraceae, Rubiaceae, Ranunclaedae, Euphobiaceae and Meliaceae rank high among the families that are frequently screened for larvicidal, adulticidal and repellent activities (Shaalan *et al.*, 2005; Ranaweera, 1996).

Steam distilled extract of *Callitris glancophylla* was toxic to *Aedes aegypti* larvae with an IC_{50} value of 0.69 mg/mL while lowest LC_{50} recorded for a botanical compound (pipercide) from *Piper nigrum* fruits was 0.004 mg/mL against *Cx. pipiens pallens* larvae (Park *et al.*, 2002). Larvicidal and adult emergence inhibition activity of ethanol extract of *Centdella asiatica* against *Cx. quinquefasciatus* at different temperatures showed a

varied activity with LC_{50} ranging between 6.84 at 19^{o}C to 1.12 ppm at 31^{o}C (Rajkumar and Jebanesan, 2005).

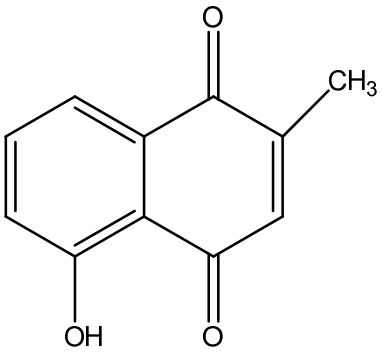
Larvicidal properties of the leaf and rhizome essential oils of the Nigerian *Curcuma longa* showed that both leaf and oils were toxic to the *An. gambiae* larvae with LC_{50} values of 0.029 and 0.017 µg/mL, respectively (Ajaiyeoba *et al*., 2008a). The hexane soluble fraction of *Quassia africana* afforded simalikalactone D which was found to be responsible for the larvicidal property of the plant. It had IC_{50} value of 1.25 µg/mL against *An. gambiae* larvae (Sama *et al*., 2014). Essential oils from *Ocimum spp* and *Cymbopogon citratus* have been found to show both larvicidal and repellancy activities against different mosquito species (Adebayo *et al*., 1999; Gbolade *et al*., 2000; Oyedele *et al*., 2002). Furthermore, the essential oils of *Ocimum americanum* and *O. gratissimun* were shown to be as potent as *L. sidoides* and *Cymbopogon citratus* in the larvicidal activity against *Ae. aegypti* and caused 100% mortality at a concentration of 100 ppm (Ranawwera, 1996; Cavalcanti *et al*., 2004).

The leaf and tuber of *Curcuma raktakanda* were investigated for larvicidal activities against the early fourth instar larvae of four mosquito species, viz *Cx. sitiens, Ae. aegypti, Cx. quinquefasciatus,* and *An. stephensi.* The petroleum ether extract of the leaf and tuber exhibited toxicity towards all the test species with tuber showing more activity than leaf (Latha and Ammini, 2000). Several natural and modified triterpenoids were found active against *Ae. aegypti* larvae (da Silva *et al*., 2016).

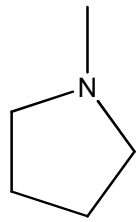
The larvicidal activity of methanol and ethanol extracts of five aromatic plant species against *Ae. albopictus* and *Cx. quinquefasciatus* larvae varied according to plant species . Methanol extract of *Aristolochia saccata* roots was found to be the most effective against *Ae. albopictus* larvae followed by ethanol extracts of *A. saccata, Annona squamosa* leaf and methanol extract of *A. squamosa* leaf respectively. The LC_{90} values of methanol extract of fruit/pericarp of *Gymnopetelum cochinchinensis*, bark of *Caesalpinea* species and ethanol extract of stem of *Piper* species were obtained at <200 ppm but methanol extract of seeds of *G. cochinchinensis* and stem of *Piper* species gave at <358 ppm against *Ae. albopictus* larvae. Ethanol extract of leaf of *A. squamosa* was found to have the most promising larvicidal activity against *Cx. quinquefasciatus* larvae. Methanol and ethanol extracts of *A. saccata* (root), methanol extract of *A.squamosa* (leaf) showed LC_{90} values at <100 ppm while methanol extract of *G. cochinchinensis* (fruit/ pericarp), methanol and ethanol extract of *Piper* species showed at <200 ppm and methanol extract of *G. cochinchinensis* (seed) showed at >302 ppm against *Cx. quinquefasciatus* larvae. The extracts of *Cymbopogon citratus* and *Abrus precatorius* were found most effective with LC_{50} value of 24 and 30 mg/L, respectively. (Nazar *et al*., 2009). Larvicidal amides have been isolated from the plant *Achillea millefolium*. The larvicidal activity of the extract was linked to the presence of N-2-methylpropyl- *(E,* E)-2,4-decadieneamide. Other long chain amides with larvicidal activity are e *N-isobutyl 2E,* 4E-octadieneamide from *Fagara macrophylla* (Rutaceae) which exhibited activity against *Cx. pipiens* larvae and *N-isobutyl-2£,* 4£, *8E,* 10Z-dodeca-2,4,8,10-tetraeneamide from *Spilanthes mauritiana* (Compositae) which showed activity against *Ae. aegypti* larvae.

The essential oil of *Tetradium glabrifolium* fruits exhibited larvicidal activity against the fourth-instar larvae of *Aedes albopictus*, with an LC_{50} value of 8.20 μgm/L. The two isolated constituents, 2-tridecanone (LC_{50} =2.86 μg/mL) and 2-undecanone (LC_{50} =9.95 μg/mL) possessed stronger larvicidal activity than d-limonene (LC_{50} =41.75 μgmL−1) against the early fourth-instar larvae of *Ae. albopictus* (Liu *et al*., 2015).

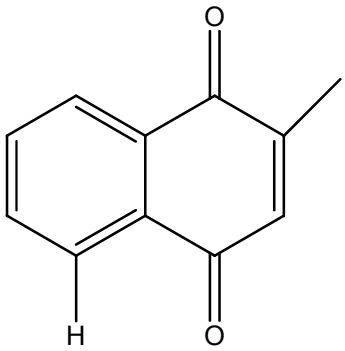
Screening of the root bark extracts of five Meliaceae species (*Turraea abyssinica*, *Turraea wakefeldii*, *Turraea mombassana* , *Trichilia roka* and *Melia volkensii* and fractions for toxicity and long-term effects on *Anopheles gambiae* indicated that larvicidal effects of the extracts appeared to be largely associated with limonoids of medium polarity (Ndungu *et al*., 2004). Azadirachtin (limonoid) isolated from *Azadirachta indica* A. Juss has been reported to have larvicidal activity against various mosquito species (Zebitz, 1984; 1986). It also disrupts the growth and metamorphosis of insects by interfering with the production of ecdysone and juvenile hormones (Chamagne *et al.,* 1989).



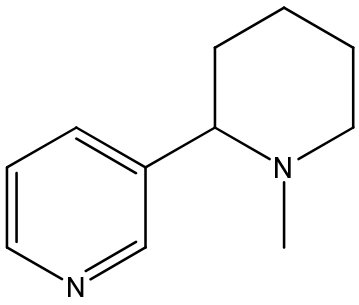
Plumbagin (*Plumbago zeylanica*)



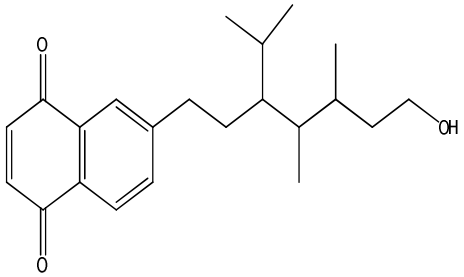
N-methyl pyrollidine (*Celastraceae spp*)



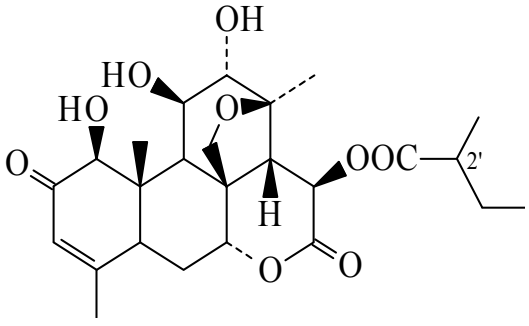
Juglone (*Juglans regia*)



Methyl anabasine (*Canabis ruderalis*)



Cordiaquinone A (*Cordia curassavica)*



Simalikalactone D (*Quassia africana*)

Fig 2:14: Some Larvicidal Compounds from Plants

2.8 Plant Family Meliaceae

The Meliaceae, or the Mahogany family, is a flowering plant family of mostly trees and shrubs (and a few herbaceous plants, mangroves) in the order Sapindales. They are characterised by alternate, usually pinnate leaves without stipules, and by syncarpous, (Pennington and Styles, 1975) apparently bisexual (but actually mostly cryptically unisexual) flowers borne in panicles, cymes, spikes, or clusters. Most species are evergreen, but some are deciduous, either in the dry season or in winter. The family includes about 50 genera and 600 species, with a pantropical distribution. Species of the Meliaceae family show with relative frequency the presence of limonoids, which are secondary metabolites derived from the metabolic pathway of terpenoids (Gualtieri *et al*., 2012). The metabolites present in this family are mainly limonoids, which are triterpenes modified with high oxygenation. These compounds are also known as meliacins because of its bitter taste. These request special attention because they are considered the major chemosystematics markers of the Meliaceae family (Taylor, 1981; 1984; Mulholland and Taylor, 1980; Murata *et al*., 2008).

Traditionally in certain parts of Africa, some Meliaceae species are used for treatment of febrile illnesses and malaria. In West Africa, the *Meliaceae* species *A. indica* is used for treatment of malaria. In tropical America, members of the Meliaceae family, *Cedrela odorata, Carapa guianensis* and *Swietenia mahagoni* have been used in traditional medicine for the treatment of fevers, a characteristic symptom of malaria (MacKinnon *et al*. 1997).

2.9 The Genus *Trichilia*

Trichilia (Meliaceae) is a genus of trees, rarely shrubs. It has about 90 species, distributed mostly in lowland areas of tropical America, with 18 species in Africa, 6 in Magadascar and two in the Indo-Malay peninsula. It also occurs in the forests of the lower Amazon (Reitz, 1984). The *Trichilia* species are found as trees (20 to 30 m in height) or groves (3 to 10 m height) with pinnate leaves and young shoots, or trifoliate. Flowers are normally unisexual (dioic plant) with four to five petals. The seeds are fleshy, partially or completely surrounded by a thin rim or Chubby. The *Trichilia* name is derived from the Greek "*Tricho*", which refers to the three locules of the ovarian and three valves in the fruit (Patricio and Cervi, 2005; Komane *et al.*, 2011).

Surveys of the Meliaceae family have identified the genus *Trichilia* as a potential source of substances with insecticidal action (Matos *et al*., 2009; Lima *et al*., 2011), similar to *Azadirachta indica*, the best known species within the family in this respect. This genus is known as a rich source of secondary metabolites, including limonoids, triterpenoids and steroids (Dal Piaz *et al*., 2012; Ramirez *et al*., 2000; Vieira *et al*., 2013; Wang *et al*., 2008; Zhang *et al*., 2011). Trichilia species are traditionally used for the treatment of many diseases such as asthma, gastric affections, hepatitis, cirrhosis and dysmenorrhea (Sanogo, 2011).

Genus *Trichilia* is important because of medicinally important tetranortriterpenoids or limonoids (Kirtikar and Basu, 1993). Limonoids show a wide range of biological

activities including anti-feedant and growth regulatory properties in insects and antifungal, bactericidal and antiviral activities in laboratory animals and humans (Koul and Isman, 1992; Nakatani *et al*., 1981; 1998).

2.9.1 Reported Biological Investigations of *Trichilia* species

Trichilia heudelotti leaf extract showed both antibacterial and antifungal activities against all the strains of bacteria tested. Its hexane and chloroform fractions inhibited 6 and 14% of the fifty multidrug resistant bacteria isolates from clinical infections, respectively. *Trichilia heudelotti* extracts gave EC_{50} of 4.00 µg/mL using the TLC staining and 1,1-diphenyl-2-picry-hydrazyl (DPPH) free radical scavenging assay. Therefore, the plant possesses strong antioxidant activities (Aladesanmi *et al*., 2007).

Wood and leaf methanol extracts of *T. gabla* and *T. americana* had insecticidal activity, inhibiting the growth of *Spodoptera litura* (Wheeler *et al*., 2001). Insecticidal activity of *T. claussenii in vitro* observed by Matos *et al*., (2009) obtained 100% larval mortality on *Spodoptera frugiperda* at 1000 mg/kg when delivered in an artificial diet. Besides mortality, the extract inhibited or delayed the larval development by 1-3 days. *Melia azedarach* and *Trichilia claussenii* had *in vitro* anthelmintic effect against sheep gastrointestinal nematodes (Cala *et al*., 2012). Crude aqueous extract of *T. monadelpha* was tested for *in vivo* mice antimalarial activity against Chloroquine sensitive NK65 *Plasmodium berghei*. The result showed that the plant demonstrated significant chemosuppresive activity on day 4 (Olorunniyi, 2013). The dichloromethane extract of leaf of *Trichilia emetica* had prominent antiplasmodial activity (IC_{50}: 12 µg/mL [95% CI:

12–14]), and also exhibited a good binding activity to the $GABA_A$–benzodiazepine receptor (Bah *et al*., 2007).

Antimicrobial activity of *T. quadrijuga, T. dregeana* and *T. cassareti* showed that extracts of the plants inhibited the growth of *Staphlococcus aureus* and *S. epidermidis* while *T. silvatica n*-butanol leaf extracts inhibited growth of *Streptococcus salivarius* and *S. mutans*. *Trichilia ramalhoi* was found to possess trypanocidal activity (Eldeen *et al*., 2007; Vieira *et al*., 2014). *Trichilia glabra* leaf possesses antiviral activity (Cella *et al*., 2004). *Trichilia connaroides* dichloromethane and chloroform extract of the seeds had activity against *Plasmodium falciparum* (Kumar *et al.,* 2011). Different morphological parts of *T. emetica* have been reported to possess antibiotic, antiplasmodial, anti-inflammatory, anti cancer and hepatoprotective activities (Germano *et al*., 2005, 2006; Traore *et al*., 2007, Komane *et al*., 2011). The chloroform extract of dried leaves of *Trichilia connaroides*, was screened for analgesic and antiinflammatory activity, using chemical, thermal and formalin-induced inflammation in Swiss mice and Wistar rats. Chloroform extract showed significant and dose-dependent analgesic, and antiinflammatory activity (Ashok *et al*., 2006).

2.10 Description of *Trichilia megalantha* Harms

Medium-sized tree of 30 m high and above found in the moist semi-deciduous forest in Ivory Coast and Nigeria, and recently recorded from Liberia (Nimba). The base is with low and blunt buttresses. The bark is grey or reddish, rough and scaly; when slashed pale pink or yellowish white, rapidly turning (reddish) brown on exposure, fibrous, faintly

scented, sometimes exuding some cream-collared, tacky latex from near the cambium. Fruit 3-chambered, stalked, slightly 3-lobed, 1.5 - 2.5 cm across, densely covered with a pale brown indumentums. Seeds 1 or 2 in each chamber, black, partly covered with an orange-red aril. (Burkill, 1985).

2.11 Description of *Trichilia welwitchii* C.D.C.

Synonyms *Trichilia caloneura* Pierre ex Pellegrin

Trichilia kisoko De Wild.

Trichilia oddoni De Wild.

Trichilia pynaertii De Wild.

Trichilia zenkeri Harms

In the past *T. welwitchii* has been much confused with *T. monadelpha* but it differs in its two celled ovary and fruit. A small to medium-sized tree, ± 10–20 m high, bole cylindrical 15–30 cm diameter bearing a dense crown of ascending branches; of rain-forest, deciduous forest and secondary jungle; in the SE corner of Nigeria, and common over the Congo basin. It is also found in Gabon and Angola. It usually fruits in May. The wood is probably used for similar purposes as *T. monadelpha* (Burkill, 1985).

This species has rather dull fruits which, when ripe, split open to reveal two to three shiny black seeds with bright red arils seeds with bright red arils. The seeds hang suspended from the fruit by a fragile thread that moves gently in the slightest breeze.Two important characters that separate it from*Trichilia rubescens,* which is similar, are the presence of a little white latex in the bark (absent in *T. rubescens*) and the roundish petiole base

(winged in *T.rubescens*). When both species are placed side by side the tertiary venation is tighter on the underside of the leaflets in *T. welwitschii* (Klopper *et al*., 2006).

2.11.1 Ethnobotanical uses of *Trichilia welwitschii*

The bark is used medicinally as treatment for dropsy, swellings, oedema and gout. In DR Congo a bark decoction is administered as enema to treat haemorrhoids and other abdominal disorders and as an abortifacient whereas pounded young leaves are applied to syphilitic sores. It is also a 3rd class timber used for carpentary and related applications (Louppe *et al.*, 2008).

2.11.2 Chemical constituents of *Trichilia welwitschii*

The seeds of *T. welwitschii* C.D.C. (Meliaceae) yielded three limonoids, dregeanin DM4, rohituka 3 and trichilia lactone D5. The bark yielded 28,29-dinorcycloart-24-ene-3,4,6-triol , sitosterol-3-O-β-D-glucoside, 4-hydroxy-N-methyl-L-proline, stigmasterol and sitosterol. (Tsamo *et. al*, 2013).

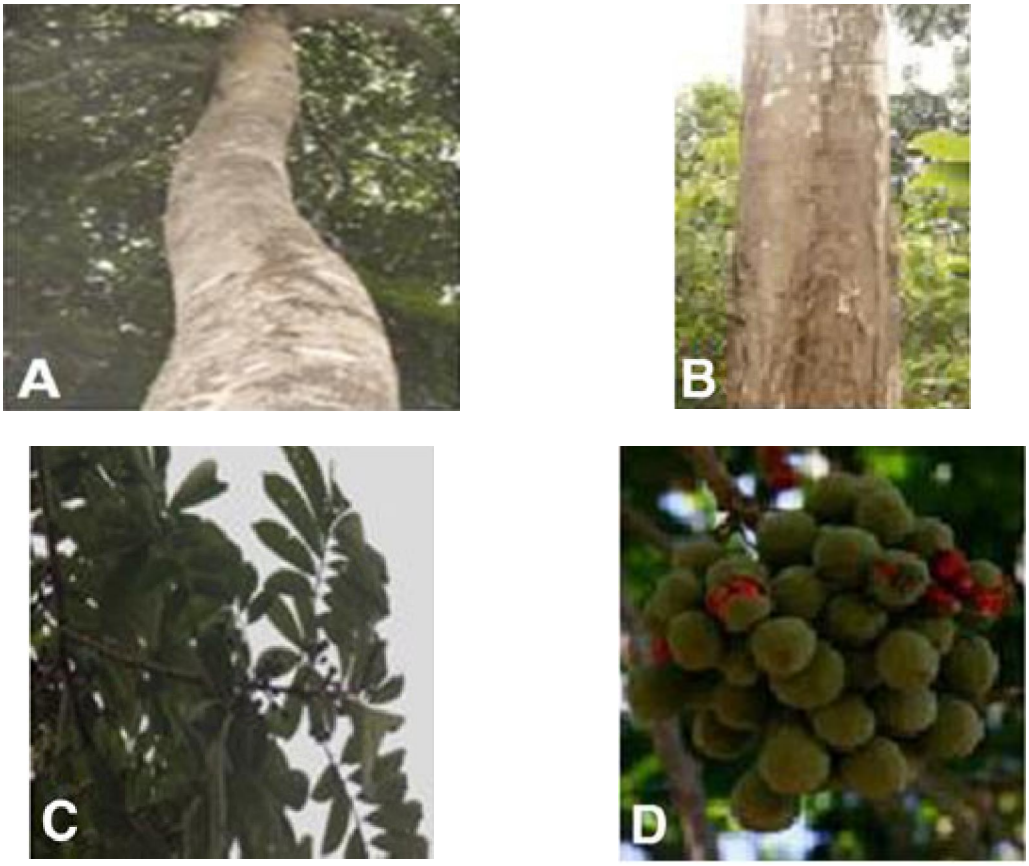


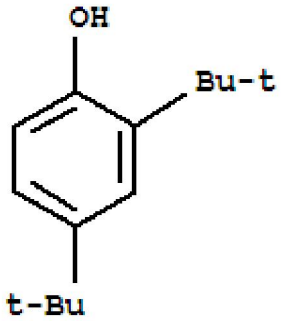
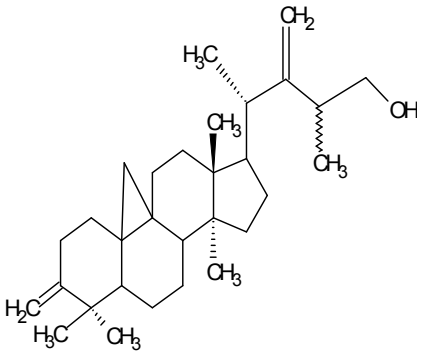
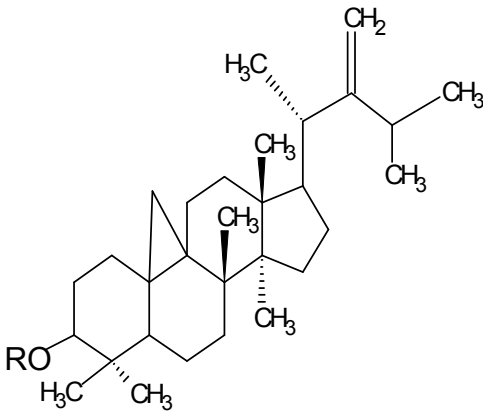
Fig. 2.15: ***Trichilia megalantha***

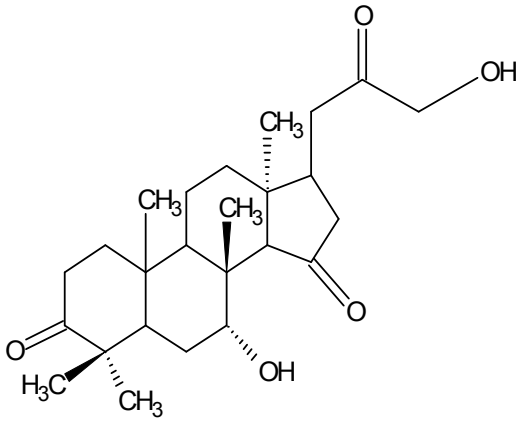
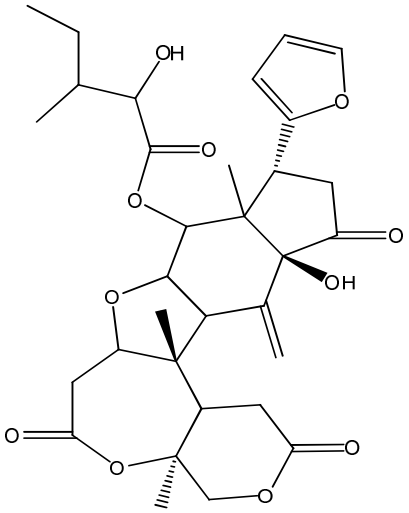
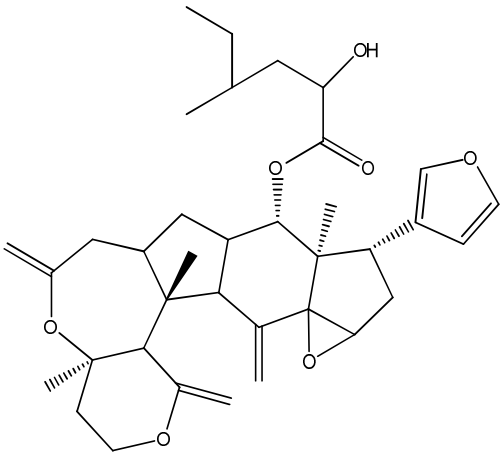
A tree, B stem, C leaves, D fruits

Fig. 2.16: ***Trichilia welwitschii***

A Tree. B fruits, C Leaves

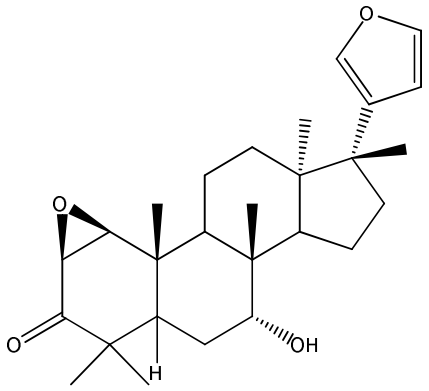
Table 2.1 : Previously Isolated Compounds From *Trichilia* Species

Name	Structure	Reference
T. connaroides Phenol,2,4-bis(1,1-dimethylethyl)		Senthilkumar *et.al,* (2012)
T. claussenii 24-methylene-26-hydroxycycloartan-3-one		Pupo *et. al,* 1997
T. claussenii 24-methylen-cicloartanol etherified R= $CH_3(CH_2)_nCO$		Pupo *et. al,* 2002

T. estipulate 21,24,25,26,27-pentanor-15,22-oxo-7a,23-dihydroxy-apotirucalla(eupha)-1-en-3-one.		Cortez *et. al*, 2000; FaÂtima das *et al*,. 2000
T. welwitschii Rohituka 3		Tsamo *et al.*, 2013
T. welwitschii Dregeanin DM4		Tsamo *et al.*, 2013

T. havanensis

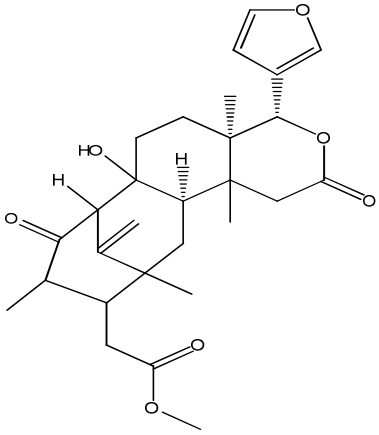
(1*α*,1*α*;21,23-Diepoxy-7r-hydroxy-24,25,-26,27-tetranor-apotirucalla-14,20,22-trien-3-one



Rodrı´guez *et al,* 2003

T. connaroids

Trichiconicin A

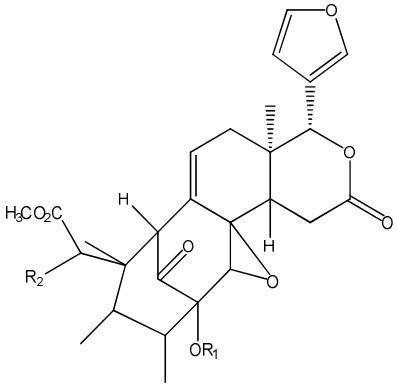


Liu *et al.*, 2014

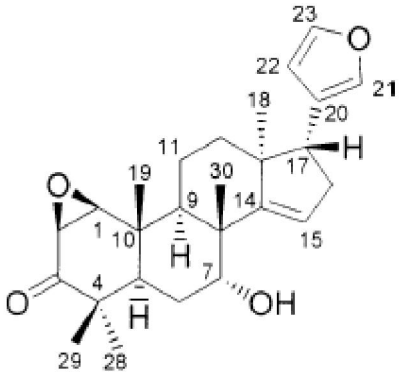
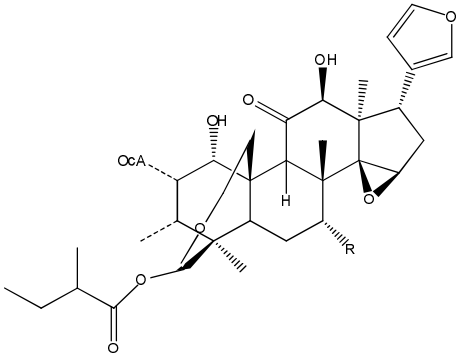
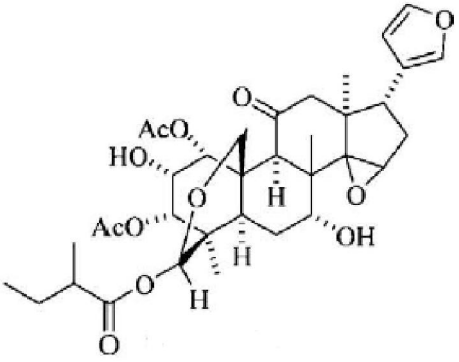
T. sinensis

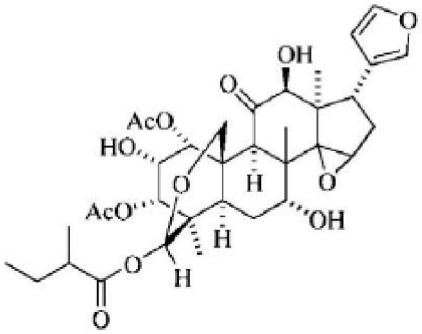
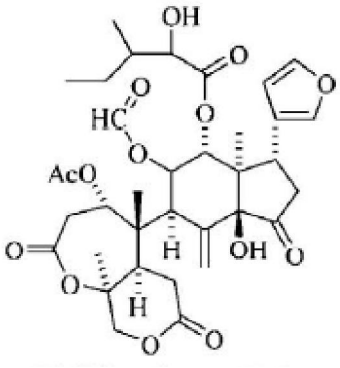
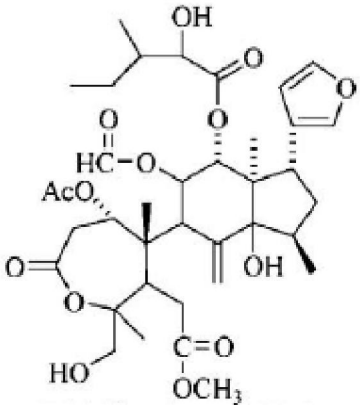
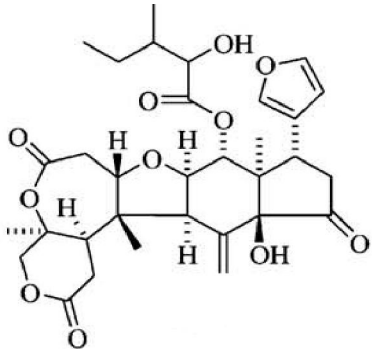
Trichinenlide B-E

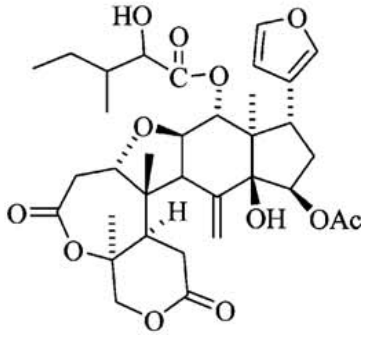
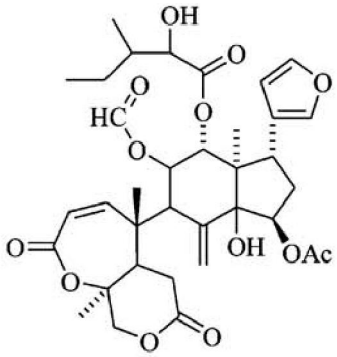
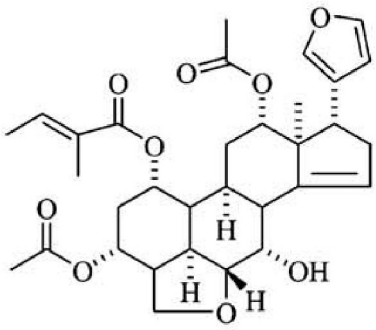
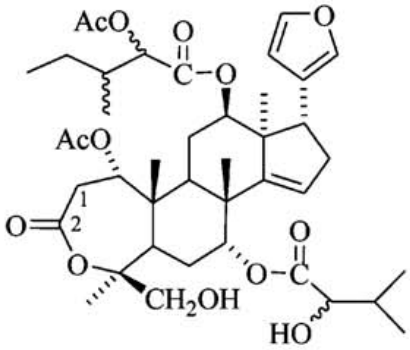
	R1	R2
B	A1	H
C	AI	OH
D	T1g	H
E	T1g	OH



Jin-Biao Xu *et al.,* 2013

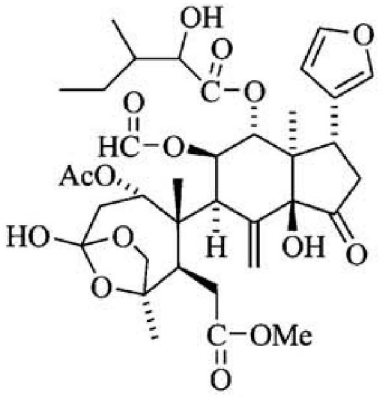
T. havanensis 1*â*,2*â*;21,23-diepoxy-7R-hydroxy-24,25,26,27-tetranor-apotirucalla-14,20,22-trien-3-one		Rodrı´guez *et al.*, 2003
T. emetica Trichilin A and B A, R=H B, R=OH		Komane *et al.*, 2011
T. connaroids Trichilin C		Mulholland *et al.*, 2000
T. roka Trichilin D		Nakatani *et al.*, 1985

T. roka Trichilin F		Nakatani *et al.*, 1993
T. roka Trichilia substance Tr-B		Nakatani *et al.,*1984
T. roka Trichilia substance Tr-C		Nakatani *et al.,*1984
T. emetica Rohituka 3		Komane et al., 2011

Rohituka-5		Guanatilaka *et al*., 1998
Rohituka-7		Guanatilaka *et al*., 1998
Trichilinin		Geng *et al*., 2010
Dregeana 4		Tsamo *et al*., 2013

T. rubra

Nymania 1

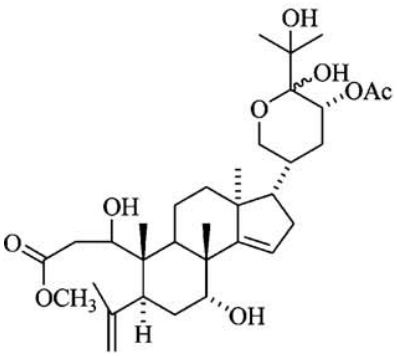


Musza *et al*., 1994

T. emetica

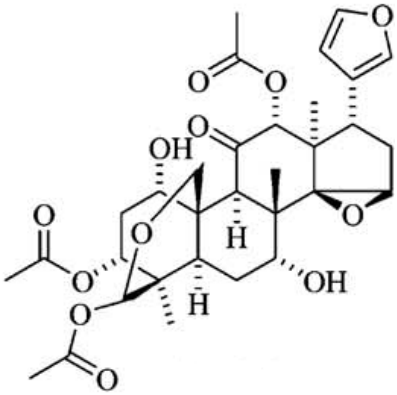
Seco-A-protolimonoid

methyl-1(*S*),23(*R*)-diacetoxy-7(*R*),24,25-trihydroxy-20(*S*)-21,24-epoxy-3,4-*seco*-apotirucall-4(28),14(15)-dien-3-oate



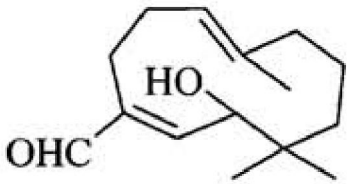
Guanatilaka *et al*., 1998

Sendanin



Kubo and Klocke, 1982

Kurubasch aldehyde



Maminata *et al*., 2007

T. catigua

cinchonains

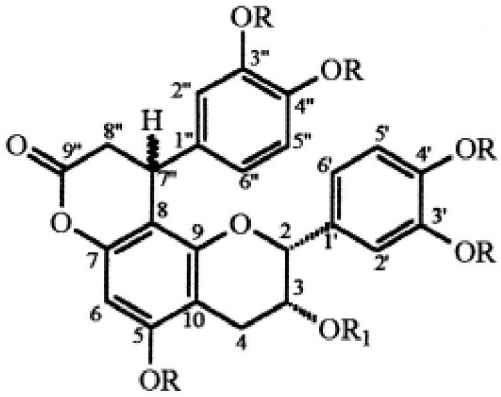
1 and 2

1. H-7β, R=R_1=H

1a H-7β, R=Me, R_1=H

2. H-7α, R=R_1=H

1a H-7α, R=Me, R_1=H



Pizzolatti *et al.*, 2002

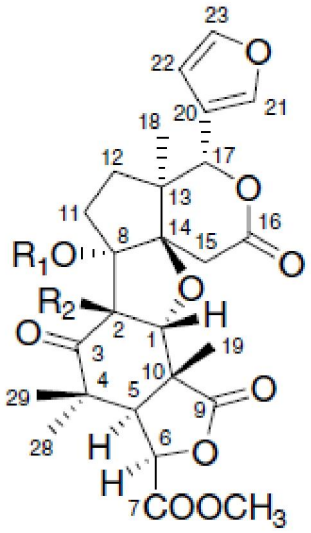
T. connaroids

Trijugins A-C

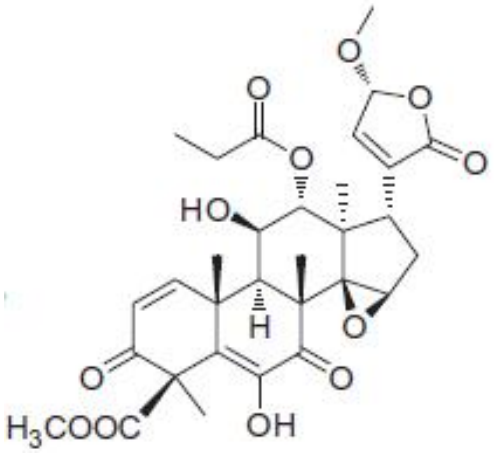
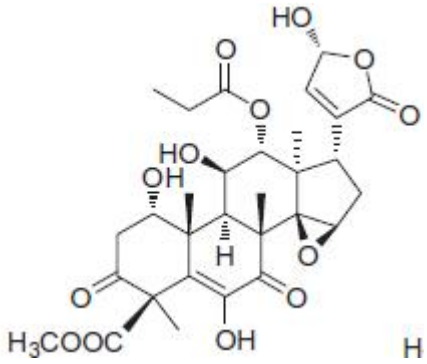
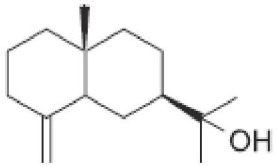
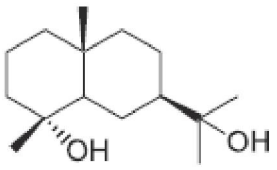
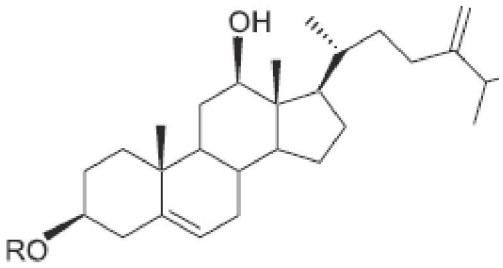
A R_1=Ac, R_2= H

B R_1=Ac, R_2= OH

C R_1=H, R_2= H



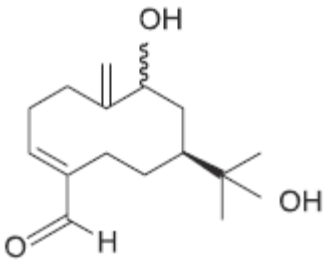
Fang *et al.*, 2010; Zhang *et al.*, 2003

T. americana americanolide C		Kai- long *et al.*, 2015
T. americana Americanolide D		Kai- long *et al.*, 2015
T. claussenii β-eudesmol		Pupo *et al.*, 2002
T. claussenii Cryptomeridiol		Pupo *et al.*, 2002
T. lepidota 22,25-Dihydroxy-9β,19-cyclolanost-23-en-3-one		Pupo *et al.*, 2002

Pupo *et al.,* 2002

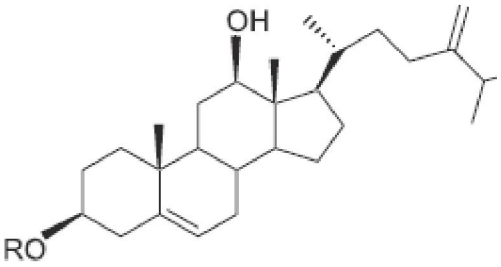
T. lepidota

germacra-3,10(14)-dien-9,11-diol-4-carbaldehyde



Pupo *et al,*, 2002

T. lepidota



1. R=$CO(CH2)_{14}CH_3$

   24-methylene-12β-hydroxycholest-4-en-3-palmitate

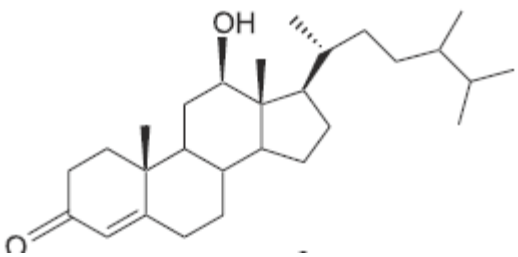
2. R=H.

   24-methylene-12β-hydroxycholesterol

T. lepidota

Pupo *et al.*, 2002

24-methyl-12β-hydroxycholest-4-en-3-one



CHAPTER THREE

MATERIALS AND METHODS

3.1 General Experimental Procedures

3.1.1 Materials and Reagents

Separating funnel, Hexane, Chloroform, Ethyl Acetate, Methanol, distilled water, Silica gel G (70-230 mesh), Iodine tank, Ultraviolet lamp, Spray reagents, weighing balance (Mettler PC 400 & H80, England), microscope, glass slides, glycerol, Giemsa stain, 250 mL disposable cups, Larvae of *Anopheles gambiae* mosquito, malaria parasite (CQ resistant *Plasmodium berghei* ANKA strain), precoated fluorescent (F_{254}) aluminum plate

3.1.2 Column chromatography (CC)

Glass columns of different lengths and widths were used for column chromatography. Except otherwise stated, Silica gel (Kieselgel 60, 70-230 mesh ASTM) was used as adsorbent. Mobile phases of varying organic solvents were used. In most cases, gradient elution was used.

3.1.3 Thin Layer Chromatography (TLC)

Column fractions were analyzed by thin layer chromatography (TLC) and pre-coated plates (Whatman® KC 18F silica gel 60A) were used. The plates were activated at 100^{o}C

for 1 hour prior to use. Several solvent systems were used for the development of the TLC plates.

3.1.4 Plant Collection and Authentication

Trichilia megalantha plant materials were collected from Cocoa Research Institute of Nigeria (CRIN), Ibadan while *T. welwitschii* was obtained along Ajibode Road, University of Ibadan Campus, Nigeria. The plants were chosen based on phytochemical targeting which involves the collection of members of plant family (Meliaceae) known to be rich in bioactive compounds and were known to possess both antimalarial and larvicidal properties. The plant specimens were identified and authenticated by botanist at Forestry Research Institute of Nigeria, Ibadan. A voucher specimen of plant samples were deposited in the herbarium with FHI numbers 109556 and 109557 for *Trichilia megalantha* and *T. welwitschii* respectively, for identification.

3.2 Sample Preparation

The plants parts were dried under the shade between 28-32^{o}C, ground and were stored in cellophane bags at room temperature. Powdered plant materials were extracted for 72 hours in methanol by maceration. The macerated samples were filtered and replaced with fresh methanol. The filtrate collected was pooled and concentrated. The extracts were stored in a refrigerator prior to use.

3.3 Partitioning of crude extracts of *T. megalantha* stem

Methanol extract (650 g) was dissolved in a solvent mixture containing water and methanol. The mixture was introduced into a separating funnel and made up to 500 mL. This was partitioned into hexane six times using 1000 mL each time. The hexane fractions were combined and concentrated to dryness. The same procedure was carried out with dichloromethane, ethyl acetate and butanol (Figure 3.1).

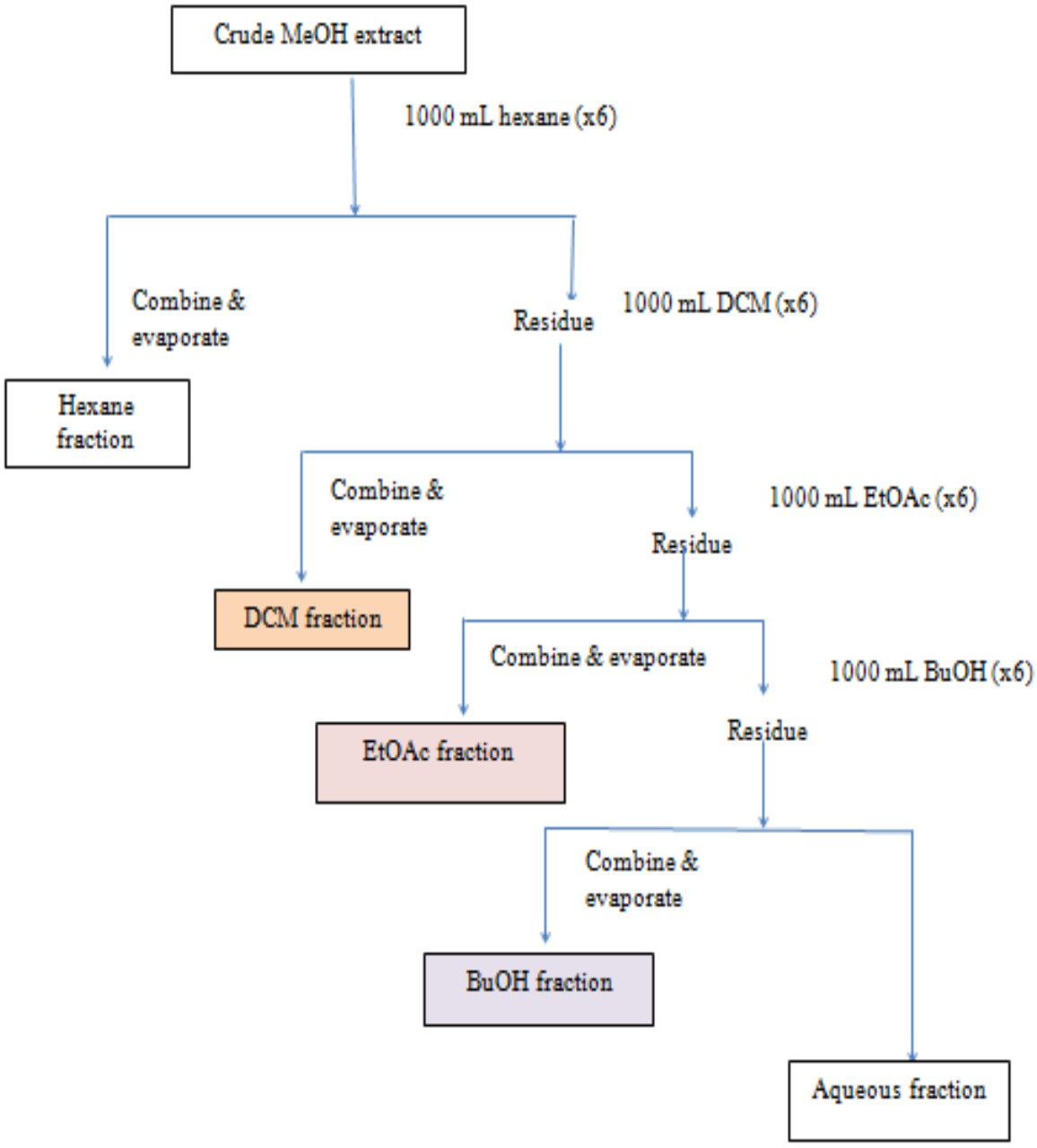


Fig. 3.1: Extraction and Liquid-liquid Partitioning of *Trichilia megaantha* Stem Bark

3.4 Spectroscopic measurements

One dimensional (1D) NMR (^{1}H NMR, ^{13}C NMR, DEPT 135 and DEPT 90), and 2D NMR (HMQC and HMBC) spectra, were recorded in deuterated solvents ($CDCl_3$ or in CD_3OD or pyridine) on Bruker AM-400 or 600 MHz spectrometers. Chemical shifts were measured in ppm (δ) and coupling constants (*J*) are given in Hz.

Mass spectra (Electron Impact), (EIMS) was recorded on Varian MAT 312 double focusing spectrometer or on a Finnigan MAT 311 with MASS PEC data system. Peak matching and field desorption (FD) experiments were performed on Finnigan MAT 312X mass spectrometer.

3.5 *In vitro* antiplasmodial assay

3.5.1 Parasite

The culture of asexual stages obtained from chloroquine sensitive (D6) and chloroquine resistant (W2) strains of *P. falciparum* strain was used.

3.5.2 Parasite cultivation

The *in vitro* culture experiments were performed using the D6 clone of the NF54 strain (chloroquine sensitive) and the W2 (Chloroquine resistant) *P. falciparum* strain. Parasites

were cultured using the Trager and Jensen method (1987). The parasites were cultured at 37°C under a low-oxygen atmosphere (5% oxygen, 5% carbon dioxide, and the remainder nitrogen) in human A^+ type erythrocytes at a hematocrit of 3 to 5%. Synchronized ring-phase cultures were obtained by two consecutive treatments at intervals of 48 h with a 5% (wt/vol) solution of D-sorbitol (Sigma-Aldrich) as described by Lambros and Vanderberg, 1979. The development and growth of parasites were analyzed on thin blood smears of cultures stained with 10% Giemsa stain thrice a week. Whem parastaemia was >5% and predominantly late trophozoites, preschizonts or schizonts, parasites were diluted with uninfected red blood cells at 5% hematocrit to lower parasitaemia and allow continuous growth.

3.5.3 Assessment of *in vitro* Antimalarial Assay

The antimalarial activity of *T. megalantha* and *T. welwitschii* was determined against chloroquine sensitive (D6) and chloroquine resistant (W2) strains of *P. falciparum* by measuring plasmodial LDH activity according to the procedure of Makler et al., (1993). Suspension of red blood cells infected with D6 or W2 strain of *P. falciparum* (200 µL, with 2% parasitemia and 2% hematocrit in RPMI 1640 medium supplemented with 10% human serum and 60 µg/mL Amikacin) is added to the wells of a 96- well plate containing 10 µL of serially diluted samples (plant extracts and fractions). The plate is incubated at 37^OC, for 72 h in a modular incubation chamber with 90% N_2, 5% O_2, and 5% CO_2. Parasitic LDH activity is determined by mixing 20 µL of the incubation mixture with 100 µL of the Malstat™ reagent (Flow Inc., Portland, OR) and incubating at room temperature for 30 min. Twenty microliters of a 1:1 mixture of

NBT/PES (Sigma, St. Louis, MO) is then added and the plate is further incubated in the dark for 1 h. The reaction was then stopped by adding 100 µL of a 5% acetic acid solution and the absorbance read at 650 nm. Artemisinin and chloroquine were included as the drug controls. IC_{50} values are computed from the dose response curves of growth inhibition using XLfit 4.2.

3.6 *In vivo* Antimalarial Assay in Mice

3.6.1 Experimental Animals

Swiss albino mice of both sexes weighing between 20-25 g housed in the animal house at Institute of Advanced Medical and Postgraduate Research Training (IAMRAT), College of Medicine, Ibadan, were used. The animals were fed on standard feed pellets and water *ad libitum.*The test animals were put randomly into six groups each containing five mice.

3.6.2 Parasites

Chloroquine resistant ANKA strain of *Plasmodium berghei* obtained from Dr D. kyle of the Division for experimental therapeutics, Walter Reed Army Institute for Research, Washington DC, United States of America and maintained by serial passage in the Malaria Research Laboratory, IMRAT, University of Ibadan was used.

3.6.3 Preparation of parasite suspension for inoculation

The parasitized red blood cells used for inoculation were obtained by cardiac puncture from an infected donor mouse with between 18-20 %. The blood was diluted to desired

parasite density in 0.9% NaCl solution (Kendall McGraw, Laboratories, Inc, U.S.A.). Each mouse was inoculated intravenously with $1x10^6$ parasitized red blood cell suspension in normal saline (0.2 mL). The day of inoculation was defined as day zero (D0) and subsequent days D1, D2… (Tona *et al*., 2001)

3.6.4 Assessment of the antimalarial effects of extracts/drug (compounds)

The Peter's 4-day suppressive test was done. Briefly, *Plasmodium berghei* infected healthy Swiss albino mice (male 6 -8 weeks old, approx. (20–25 g). Thirty-six infected mice were randomly divided into six groups. The first four groups were treated orally with the *T. megalantha* extracts in different doses (100, 200, 400 and 800 mg dried plant product/kg body weight). The fifth group of mice (positive control) was given only water which served as negative control. The 6^{th} group of mice received chloroquine, 10 mg/kg body weight, as standard anti-malarial. The extracts/water was administered to the animals once daily for four days from D0 post infection till D3 post infection, while chloroquine was administered daily till D2 post infection. (Peters and Robinson, 1992; Tona *et al*., 2001).

On day-4 post inoculation, thin blood films were prepared from the tail vein of all the animals to monitor the parasitaemia. Each thin film was air dried, fixed with methanol and stained with Giemsa stain. Giemsa stained blood films were examined under a high power objective (x100) to quantify the parasite density. Parasitaemia was determined by counting the number of parasitized erythrocytes among at least 1000 red blood cells.

The suppression of parasitaemia in relation to the control was assessed using the formula by Fidock *et al.*, 2004.

Average (Av)% suppression

$$= \frac{100.\ X\ Av\%\ parasitaemia\ in\ control - Av\%\ parasitaemia\ in\ test}{[Av\%\ parasitaemia\ in\ control}$$

3.6.5 Statistical analysis

The mean percentage parasitaemia at different doses was compared with the control group using Student's *t* test. P-values < 0.05 were considered to be significant.

3.7 Brine shrimp lethality assay

The cytotoxicity of the plants extracts was evaluated using the nauplii larvae of brine shrimp, *Artemia salina,* in the brine shrimp lethality assay (McLaughlin & Rogers, 1991). Brine shrimp eggs were hatched in natural seawater obtained from the Bar Beach, Ikoyi, Lagos, Nigeria, and incubated for 48 h in 3.8 g/L seawater. After hatching, the nauplii were collected and treated with selected concentrations (five dilutions, 10–5000 mg/mL) of plant extracts and etoposide was included as positive control.

3.8 Evaluation of Larvicidal activity of *T. megalantha* and *T. welwitschii*

3.8.1 Preparation of stock solution

Stock solutions of each extract were prepared at 2000 µg/mL with ethanol by dissolving 4 mg of extract in 2 mL of EtOH. The stock solution was serially diluted with water to prepare the working solutions of 15.65, 31.25, 62.5,125, 250, 500 and 1000 µg/mL.

3.8.2 Determination of larvicidal activity

The 4th instar larvae of *An. gambiae* were collected from several mosquito breeding sites in Ibadan, Nigeria. The larvae were washed in clean water before assay. Standard methods for assaying larvicidal activity as recommended by the World Health Organisation were followed in all experiments (WHO, 2005). Preliminary bioassays evaluation of larvicidal activity of *T. megalantha* and *T. welwitschii* parts were performed with early 4th instar (5-6 mm) larvae of *An. gambiae.* The extracts and compounds were in triplicate using 20 larvae for each assay. The larvae were placed into 250 mL disposable plastic cups containing 100 mL of graded concentrations of the test solution and incubated at 28-32^{o}C. Larvae were considered dead when they were unable to reach the surface of the solution when pricked with needle. The number of dead larvae was determined at the start of the experiment (0 h) and 24 and 48 h thereafter. An aqueous solution of ethanol (1%) was employed as the negative control while N, N-diethyl-3-methylbenzamide (DEET) was the positive control.

3.8.3 Data Analysis

The data obtained were statistically analysed. Mean of three replicates and standard error of the mean (M ± SEM) were determined. Statistical significance was determined by graphpad[4] prism software. One way ANOVA was used to compare parameters within groups. All the data were analyzed at a 95% confidence interval ($P<0.05$). Concentration of the extract required to kill 50% (LC_{50}) of the larvae present was determined after 48 h using anon-linear regression in a Graphpad prism[4] software.

3.9 *In vitro* Antioxidant DPPH Assay

Free radical scavenging activity of *T. megalantha* extracts and fractions were evaluated using 1,1-diphenyl-2- picrylhydrazyl (Sharma and Bhat, 2009). Graded concentrations (6.25 – 400 µg/mL) of test solution in 0.1 mL were added to 0.9 mL of 0.1 µm solution of DPPH in methanol. Methanol only (0.1 mL) was used as experimental control. After 30 min of incubation at room temperature, the reduction in the number of free radical was measured by reading the absorbance at 517 nm. Gallic acid and N-acetyl cysteine were used as reference standard. The scavenging activity of the extracts corresponded to the intensity of quenching DPPH. The percentage inhibition was calculated from the following equation:

$$\% \text{ inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of test sample})}{\text{Absorbance control}} \times 100$$

3.10 *In vitro* Cytotoxicity Assay

Cell lines (PC-3 and Vero) was cultured in Dulbecco's modified Eagle medium (containing 10% fetal bovine serum) in flasks, and kept in 5% CO_2 incubator at 37 ^{o}C. Upon confluency, cells were harvested and plated in 96-well plate (seeding density 8,000 cells/well for PC-3/Vero) in 100 µL medium. After 24 hours, test sample (50 µg/mL) was added in triplicate and incubated at 37^{o}C for 48 hours. After incubation, 200 µL MTT at 0.5 mg/mL was added to each well and incubated at 37^{o}C for 3 hours. Thereafter, 100 µL DMSO was added and absorbance was taken at 570 nm using micro-plate reader (van Meerloo *et al*., 2011). Doxorubicin (50 µM) was used as positive control and the negative control contains none of the test samples. The percentage inhibition or decrease in viable cells was calculated by following formula:

$$\% \text{ Inhibition} = \frac{100 - (\text{mean OD (test sample)} - \text{mean OD (negative control)} \times 100)}{\text{(mean OD (positive control)} - \text{mean OD(negative control)}}$$

3.11 *In vivo* Toxicity Test of the Crude Plant Extracts

3.11.1 Acute toxicity

The crude methanol extracts of stem bark of *T. megalantha* and *T. welwitchii* were evaluated for their toxicity in healthy Swiss albino mice aged 6-8 weeks and weighing 27-32 g. For each extract tested, 24 mice were used by randomly distributing them into four groups of six mice per cage using the Organization for Economic Cooperation and Development (OECD) guidelines. Any mortality within 24 h of drug administration was considered as toxicity of the drug. (Lorke, (1983)

3.11.2 Subacute toxicity

Forty-two animals were divided into seven groups (n=6). The sub-acute oral toxicity of the crude methanol extracts of both the *Trichilia* species were evaluated in mice using the procedure described by the Organization for Economic Cooperation and Development (OECD, 2008).

Subacute toxicity was determined using weight, blood chemistry (liver and kidney function tests), haematological and histological parameters before and after treatment. The mice in group one, two and three were given orally 250, 500, and 1000 mg/kg doses of *T. megalantha* extract. The extracts in each case was administered orally over a period of twenty eight days using oral cannula. Mice were observed continuously for one hour after the treatment; intermittently for 4 h, and thereafter over a period of 24 h. The mice were also observed for gross behavioral changes such as feeding, movement and other signs and symptoms of toxicity manifestations for 24 h and observed daily for mortality during the 28 days period (Pillai *et al*., 2011).

Briefly, Groups I-III animals were administered orally with graded doses of methanol extract of *T. megalantha* stem bark (250, 500, 1000 mg/kg), groups IV-VI received *T. welwitschii* stem bark extract while group VII (untreated) served as control. The animals received daily doses of extract till D28.

Food intake was measured daily while body weights of animals were taken weekly. On the 29 day of the experiment, blood was collected through the optical sinus. Vital organs such as the heart, liver, kidney, lung and spleen were harvested, blotted with tissue paper and weighed. Samples of the lung, heart, liver, kidney, and lung were fixed in 10% neutral buffered formaldehyde for histopathological examination.

3.11.3 Determination of Packed Cell Volume (PCV) of mice treated with methanol extracts of *T. megalantha* and *T. welwitschii* stem bark

Packed cell volume (PCV) or Heamatocrit level (HCT) was determine every week through the 28 day experiment (Alexander and Griffiths, 1993). Blood collected in sealed heparinized capillary tubes was centrifuged at 3,800 rev/min for 5 min. The values were read using Hawkley microheamatocrit reader.

3.11.4 Biochemical Estimations of serum of mice treated with methanol extracts of *T. megalantha* and *T. welwitschii* stem bark

Blood collected in heparinized tubes were centrifuged at 3000 rpm for 10 min. The plasma was separated and analyzed for various parameters such as Glutamic oxaloacetic Transaminase (AST), Glutamic pyruvic Transaminase (ALP), Alkaline phosphatase (ALP), to evaluate renal and hepatic function using the procedure outlined in a commercial kit (Randox kit RX MONZA AP 542). AST; catalyses the transfer of an amino group from L-aspartate to 2-oxoglutarate to form oxaloacetate and L-glutamate.

Oxaloacetate spontaneously decarboxylates to form pyruvate under the strongly acidic conditions. ALT; catalyses the transfer of an amino group from L-alanine to 2-oxoglutarate to form pyruvate and L-glutamate. An increase in pyruvate concentration corresponds with the levels of AST and ALT activities. The pyruvate concentration is determined spectrophotometrically in the form of hydrazone, which is produced by reaction with 2,4-dinitrophenylhydrazine in an alkaline medium. Increased AST and ALT activity is indicative of liver damage or disease.

3.11.4.1 Aspartate Aminotransferase (AST) Determination in serum of mice treated with methanol extracts of *T. megalantha* and *T. welwitschii* stem bark

Blank: About 0.1 mL of distilled water and 0.5 mL of reagent 1(Phosphate buffer, L-aspartate, 2-oxoglutarate and Sodium azide) were measured into 10 mL sample bottle. These were thoroughly mixed and incubated for 30 min at 37ºC. Then 0.5 mL of reagent 2 (2,4-dinitrophenyl hydrazine) was added and the mixture was allowed to stay for 20 min at RT (28-32 ºC) after which 5 mL of 0.4 mol/L NaOH was added.

Sample: mixture of 0.1 mL of the samples and 0.5 mL of reagent 1 was incubated for 30 min at 37ºC. After which 0.5 mL of reagent 2 was added and the mixture was allowed to stay for 20 minutes at RT (28-32 ºC) then 5 mL of 0.4 mol/L NaOH was added. After 5 min the absorbance were read at 546 nm wavelength using a spectrophotometer. The activities of AST in the plasma were obtained by extrapolation from the standard curve (Huang *et al*., 2006).

3.11.4.2 Alanine Aminotransferase Determination (ALT) in serum of mice treated with methanol extracts of *T. megalantha* and *T. welwitschii* stem bark

Blank: Distilled water (0.05 mL) wad added to 0.25 mL reagent R1 (Phosphate buffer, DL-α-alanine, 2-oxoglutarate and Sodium azide) and incubated at 37ºC for 30 min. Thereafter 0.25 mL reagent R2 was added and allowed to stand at RT for 20 min, which was then followed by the addition of 2.5 mL NaOH (0.4 mol/L).

Sample: The sample (0.05 mL) was added to 0.25 mL reagent R1 and incubated at 37ºC for 30 min. Then 0.25 mL reagent R2 was added and allowed to stand at RT for 20 min, which was then followed by the addition of 2.5 mL NaOH (0.4 mol/L). Absorbance of sample was read against blank at 546 nm after 5 min (Huand *et al*., 2006).

3.11.4.3 Alkaline Phosphatase Determination (ALP) in serum of mice treated with methanol extracts of *T. megalantha* and *T. welwitschii* stem bark

This was carried out using the semi micro method as below. 10 mL of reagent R1a (diethanolamine buffer (1 mol/L, pH 9.8) and $MgCl_2$ (0.5 mmol/L) was added to vial of reagent R1b (p-nitrophenylphosphate ;10 mmol/L). 1 mL of the resultant reagent was added to 0.02 mL of sample and the absorbance read immediately at intervals of 0 s, 60 s, 120 s and 180 s (Galind, 2010). ALP activities were calculated using the formula:

$U.I = 2760 \times \Delta A$

Where U.I = activity in international units

ΔA = change in absorbance

3.11.5 Histological examination

Following blood collection, vital organs such as lungs, heart, liver, kidney and spleen were removed and weighed. The collected organs were fixed in 10% buffered formalin and embedded in paraffin. Histology sections (4-5 μm thick) were stained with hematoxylin and eosin for evaluating histological alterations (Krause, 2001). Photomicrographs of relevant stained sections were taken.

3.11.6 Statistical analysis

The values were expressed as mean ± SEM. Statistical analysis was determined by one way analysis of variance (ANOVA), P values ≤ 0.05 were considered as significant.

3.12 Isolation of Compounds

3.12.1 Isolation of compound TMH 27

The hexane soluble fraction of *T. megalantha* (30 g) was chromatographed on silica gel (230–400 mesh; 120 g) in a glass column (60×6.5 cm). Elution was with Hex: EtOAc mixtures of increasing polarity in stepwise gradient while collecting 200 mL portion. Collected fractions (480) were monitored by TLC and pooled into nine subfractions (A-I). Fraction A was oil and was subjected to GC-MS analysis.

Fraction C (94-120) was fractionated in a column chromatography over silica gel and eluted successively using gradient mixtures of n-hexane, hexane-EtOAc, EtOAc, EtOAc–

MeOH and MeOH to give five fractions C1 (2.5 g), C2(7.0 g), C3 (8.5 g), C4 (3.0 g) and C5 (4.0 g).

Fraction C4 (3.0 g) was further subjected to column chromatography over silica gel. Sixty-four fractions were collected. Fractions 24-28 afforded TMH 27.

3.12.2 Isolation of compound DAF-1

Fraction D (121-195; 4.675g) from CC of Hexane fraction was chromatographed over silica gel (230–400 mesh) and eluted successively using gradient mixtures of n-hexane, hexane-EtOAc, EtOAc, EtOAc–MeOH and MeOH. A total of 105 fractions (100 mL) were collected. Fractions 69 and 70 afforded DAF 1 (12 mg).

3.12.3 Isolation of compound TMH1

Column chromatography of fractions F (230-236) on silica gel and eluted with mixtures of n-hexane, hexane-EtOAc, EtOAc, EtOAc–MeOH and MeOH gave 60 fractions of 100 mL each. Based on similar TLC characteristics subfractions 24-28 were pooled (silica gel G, Hex: EtOAc (70:30). Compound TMH 1 precipitated from this subfraction.

3.12.4 Isolation of compounds from *T. megalantha* DCM fraction

Column chromatography of DCM fraction (20 g) over silica gel (230–400 mesh) using a gradient of n-hexane, n-hexane-EtOAc (1:1), EtOAc, EtOAc-MeOH (1:1) and MeOH. Three hundred and fifty four (354) fractions of 250mL eachwere collected to yield 6 subfractions, A1 (3 g), A2 (336 mg), A3 (5.0 g), A4 (3.3 g) A5 (4.5 g) and A6 (3.8 g)

3.12.5 Isolation of compound DAF-H 27

Fraction A1 was rechromatographed on silica gel G and eluted with Hex: EtOAc and $CHCl_3$: CH_3OH mixtures. A total of 82 fractions (100 mL portion) were collected and monitored with TLC. Fractions 19-21 (silica gel G, Hex: EtOAc, 9:1), were pooled and chromatographed on silica. Thirty five (35) fractions were collected of 20 mL each. Compound DAF- H27 crystallized from fractions 24 and 25.

3.12.6 Isolation of compound TMH 47

Fraction A1 from *T. megalantha* DCM fraction (subfractions 24-39) eluted with 80-85% Hex in EtOAc were pooled and further purified on a smaller glass column (50 ×1.3 cm). It was eluted with hexane then 75-95% Hex in EtOAc mixtures of increasing polarity. (silica gel G, Hex : EtOAc (50:50) while collecting 50 mL each. Collected subfractions (61) were monitored by TLC. Compound TMH 47 precipitated from subfractions 31-34.

3.12.7 Isolation of compound DAF- 2

Fractions A2 (111-138; 336 mg) of DCM soluble fraction of *T. megalantha* was chromatographed on Sephadex gel using DCM: MeOH mixtures (1:1) affording 34 fractions. Fractions 24-29 precipitated a yellow crystalline solid DAF-2 (12 mg).

3.12.8 Isolation of compound TMH-70B

Fraction A3 (5.0 g) of the DCM soluble fraction of *T. megalantha* was chromatographed on silica in a glass column (44×5 cm). Elution was with Hex, Hex: EtOAc, EtOAc and EtOAc : MeOH mixtures of increasing polarity in stepwise gradient while collecting 100 mL portion. Collected subfractions (206) were monitored by TLC. Subfractions 92-108 eluted with Hex: EtOAc (85:15) were pooled based on similar TLC characteristics (silica gel G, Hex:EtOAc (60:40). Compound **TMH 70B** (18 mg) crystallized out from this pooled subfraction.

3.13 Analysis of oils from *T. megalantha* Hexane Fraction A

Gas chromatographic (GC) analysis of the oil was performed on a Shimadzu GC 17A, using a fused silica capillary column (30 m × 0.25 mm i.d.), coated with 5% diphenyl dimethyl siloxane (DB-5), equipped with Flame Ionization Detector. Helium was used as carrier gas at a flow rate of 1.2 mL/min. Oven temperature was programmed from 60 to 200^{o}C at 2^{o}C/min and then held isothermal at 200^{o}C for 20 min; injector temperature, 250^{o}C; detector temperature, 250^{o}C; 0.2 μL of sample injected in a split ration of 50%. Gas chromatography-Mass-spectrometry (GC–MS) data were obtained on a Shimadzu QP–500, fitted with the same column and under similar temperature programme as mentioned above for GC analysis.

3.13.1 Identification of compounds

Chemical structures of compounds were elucidated using one dimensional (1D) NMR (^{1}HNMR, ^{13}CNMR, DEPT 135, DEPT 90) and 2D NMR recorded in deuterated solvents (CDCl3, CD3OD) on Bruker AM- 500 or 600 MHz spectrometers. Chemical shifts (δ) and coupling constant (J) were measured in ppm and Hz, respectively.

From the GC-MS analysis, compounds were identified by comparing the retention indices of peaks on DB-5 column with literature values19,20, computer matching against the library spectra (NIST-1, NIST-2, Wiley and Adams Library).

CHAPTER FOUR

RESULTS

4.1 Yield of extracts and fractions

Leaf extracts of the both plants had the highest yield while the root bark had least extractive value. The butanol fraction (34.7%) had highest percentage yield while the DCM (8.7%) fraction was the least (Table 4.1).

4.2 *In vitro* Antiplasmodial Activities of Methanol Extract of *T. megalantha* and *T. welwitschii* on *Plasmodium falciparum*

The leaf extract of *T. welwitschii* (9.0 5 µg/mL) showed the highest antiplasmodial followed by *T. megalantha* (12.27 µg/mL) stem bark. The most sensitive strain to the extracts was the chloroquine sensitive *P. falciparum* D6 (Table 4.2).

4.3 *In vivo* Antimalarial Activities of Methanol Extract and Fractions of *T. megalantha* and *T. welwitschii* on *Plasmodium berghei* in Mice

Trichilia megalantha stem bark was the most active extract (100% chemosuppression). Least activity was observed for the leaf extract (Table 4.3). Highest antimalarial activity was demonstrated by the leaf extract of *T. welwitschii* (93.4% chemosuppression) while the root bark had the least activity (Table 4.4). The stem bark chloroform fraction of *T. megalantha* was the most active fraction followed by the ethyl acetate fraction, hexane soluble fraction and methanol fraction (Table 4.5).

The effect of graded doses of extracts of *T. megalantha* and *T. welwitschii* on the period of survival of infected animals as shown in Tables 4.3 and 4.4 indicated that treated animals survived longer than the untreated animals. Survival of animals treated with 100-800 mg/kg *T. megalantha* stem bark ranged from 16-25 days while those treated with CQ (10 mg/kg) was 23 days.

4.4 Larvicidal Activities of Methanol Extract and Fractions of *Trichilia megalantha* and *Trichilia welwitschii.*

All tested extracts showed larvae mortality. Of the crude extracts screened, *T. megalantha* stem bark showed highest activity of 100% mortality at 1000 µg/mLwhile the leaf showed the least activity (38%). The root bark of *T. welwitschii* was more toxic to the larvae (100%). The next in activity was the stem while the leaf showed the least larvae toxicity of 43% mortality. Hexane fraction of *T. megalantha* demonstrated the highest larval mortality. Fraction C from *T. megalantha* stem hexane fraction showed highest percentage mortality (100%) at the highest concentration tested (Figures 4.1-4.5).

4.5 Results of toxicity studies of *T. megalantha and T. welwitschii* extracts in Mice

The results of acute toxicity of stem bark of *T. megalantha and T. welwitschii* indicated that LD_{50} of the extracts are higher than 5000 mg/kg and no changes were observed in any behavioral parameters in mice. Body weight (Fig.4.6-4.7) of mice increased along with the time in subacute model ranging between 22.7-26.2 g.

The biochemical (alkaline phosphatase, acid phosphatase) (Table 4.10), hematological parameters (PCV) and histological studies of liver, heart, lung, spleen and kidney indicated either no or less alteration in the treated group. No deleterious morphological changes were observed in the histological analysis of the major vital organs (Fig 4.8-4.14).

4.6 GC-MS Analysis of *T. megalantha* Hexane Fraction A.

Fraction A contained mainly fatty acids. The major constituents were Myristic acid, Linoleic acid and eicosatrienoic acid (Table 4.14).

Table 4.1: Percentage yields of extracts and fractions of *T. megalantha* and *T. welwitschii*

Plant extract/Fraction	Weight (g)	% Yield
T. megalantha		
Leaf	60.0	16.9
Stem bark	650.0	12.5
Root	35.0	6.9
T. megalantha stem		
Hexane	2.2	7.3
Chloroform	33.7	11.2
Ethyl acetate	23.5	8.8
Butanol	22.5	34.7
Aqueous	250.0	74.7
T .welwitschii		
Leaf	11.8	6.2
Stem bark	8.3	2..7
Root	6.0	4.0

Table 4.2: *In vitro* Antiplasmodial Activity of *T. megalantha* and *T. welwitschii* extracts on *Plasmodium falciparum* (D6 and W2)

Extracts	IC_{50} µg/mL (Mean ±SD)	
	D6	W2
T. megalantha		
Leaf	> 50.0	> 50.0
Stem bark	12.27±0.13	25.65 ±7.01
Root	>50	>50
T. welwitschii		
Leaf	9. 05±3.41	10.55±0.15
Stem bark	>50	>50
Root	>50	>50
Chloroquine	0.163	-
Artemisinin	0.026	-

Table 4.3: Response of Swiss Albino Mice Infected with *P. berghei* ANKA Strain to *Trichilia megalantha* Methanol Extracts

Dose (mg/kg)	Parasitaemia ± SEM (%)	Parasite Suppression (%)	Survival time ± SEM (%) (days)
T. megalantha			
Leaf			
100	7.55 ± 1.3	Nil	8.75 ± 0.6
200	3.60 ± 0.4	50.4	12.2 ± 0.7
400	1.64 ± 0.5	77.4*	18.1 ± 1.1*
800	0.00 ± 0.0	100.0*	21.0 ± 1.2*
Stem bark			
100	1.32 ± 1.2	82.0*	16.8 ± 0.6*
200	0.00 ± 0.0	100.0*	18.2 ± 0.7*
400	0.00 ± 0.0	100.0*	22.8 ± 1.1*
800	0.00 ± 0.0	100.0*	25.0 ± 1.2*
Root bark			
100	2.01 ± 1.2	72.3*	13.8 ± 0.5
200	1.81± 0.3	75.0*	14.2 ± 0.7
400	0.36 ± 0.2	95.0*	20.1 ± 1.1*
800	0.09 ± 0.2	98.8*	26.0 ± 1.2*
CQ[a]	0.30 ± 0.2	96.2	23.5 ± 1.2
Untreated	7.25		9.5 ± 0.5

Values are mean ±SEM, n=5 in each group;
* Significantly active when compared with the control; P< 0.05
[b] CQ Chloroquine (10mg/kg).

Table 4.4: Response of Treatment in Swiss Albino Mice Infected with *P. berghei* to *Trichilia welwitschii* Methanol Extracts

Dose	Parasitaemia ± SEM (%)	Parasite Suppression ± SEM (%)	Survival time (days)
Leaf			
100	2.45 ± 0.25	61.4*	14.8 ± 0.6*
200	0.85 ± 0.10	88.3*	19.2 ± 0.7*
400	0.65 ± 0.12	91.0*	20.4 ± 1.0*
800	0.48 ± 0.05	93.4*	24.0 ± 1.2*
Stem bark			
100	7.01 ± 1.36	3.5	8.3 ± 0.4
200	6.05 ± 0.47	16.6	11.2 ± 0.5
400	2.58 ± 0.51	64.3*	18.5 ± 0.1*
800	0.50 ± 0.04	90.7*	22.0 ± 1.2*
Root bark			
100	3.01 ± 1. 25	58.5*	14.5 ± 0.6
200	2.66 ± 0.36	63.3*	14.2 ± 0.7
400	2.01 ± 0.21	72.3*	18.1 ± 1.1*
800	1.81 ± 0.22	75.8*	18.4 ± 1.5*
CQ[a]	0.30 ± 0.20	96.2	23.5 ± 1.2
Untreated	7.25 ± 0.25		9.5 ± 0.5

Values are mean ±SEM, n=5 in each group;
* Significantly active when compared with the control; $P < 0.05$
[a] CQ Chloroquine (10 mg/kg).

Table 4.5: Response of Swiss Albino Mice Infected with *P. berghei* to *Trichilia megalantha* Stem Bark Fractions

Dose (mg/kg)	Parasiteamia± SEM (%)	Parasite Suppression ± SEM (%)	Survival time (days)
TMSH			
100	4.94 ± 1.4	40.5	10.8 ± 0.6
200	4.27 ± 0.5	48.5	10.2 ± 0.7
400	4.06 ± 0.5	54.8*	12.0 ±1.2
800	1.96 ± 0.4	66.0*	12.3 ± 0.6
TMSC			
100	1.08 ± 0.3	79.0*	16.8 ± 0.6*
200	0.75 ± 0.5	85.7*	17.2 ± 0.7*
400	0.56 ± 0.47	89.1*	22.3 ±1.2*
800	0.25 ± 0.4	95.0*	25.0 ± 0.7*
TMSE			
100	1.35 ± 1.4	74.0*	16.3 ± 0.6*
200	1.30 ± 0.5	74.7*	18.1 ± 0.7*
400	1.21 ± 0.5	76.4*	19.0 ± 1.2*
800	0.20 ± 0.4	96.1*	22.3 ± 0.7*
TMSM			
100	4.94 ± 0.3	3.1	8.7 ± 0.6
200	4.29 ± 0.3	16.3	10.2 ± 0.7
400	4.08 ± 0.5	20.5	11.5 ±1.5
800	1.97 ± 0.4	61.6*	12.2 ± 0.4
CQ[a]	0.10 ± 0.1	98.2	22.4 ± 0.2
Untreated	5.12 ± 0.3	0	10.1 ± 1.0

TMSH: *T. megalantha* hexane fraction, TMSC: *T. megalantha* DCM fraction, TMSE: *T. megalantha* EtOAc, TMSM: *T. megalantha* MeOH fraction
Values are mean ±SEM, n=5 in each group;
* Significantly active when compared with the control; $P < 0.05$
[a] CQ Chloroquine (10 mg/kg).

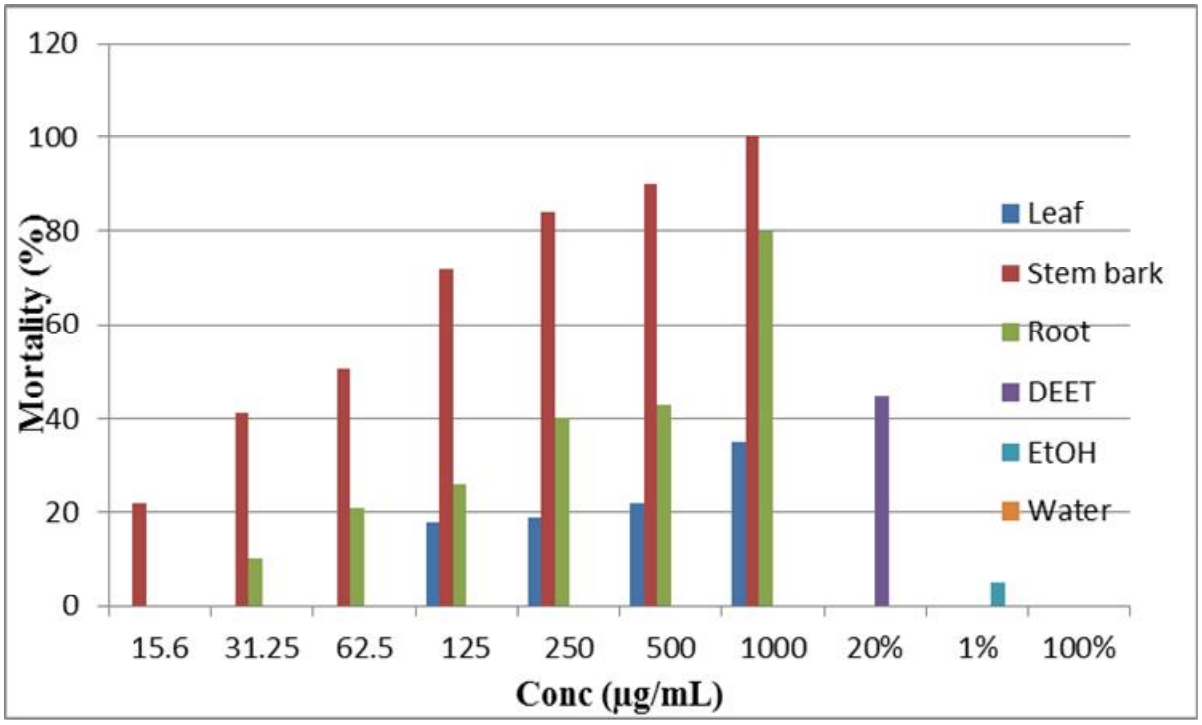


Fig. 4.1: Larvicidal activities of *T. megalantha* extracts against *An. gambiae*

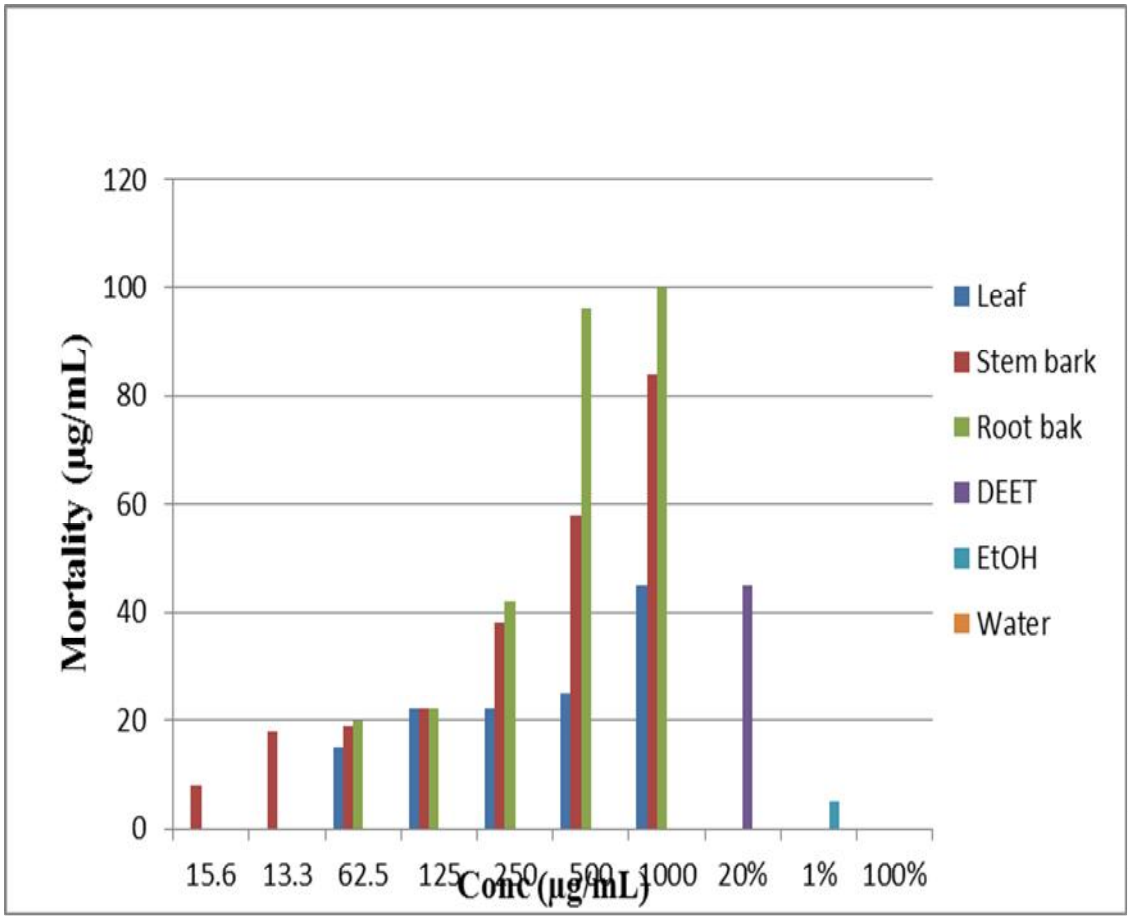


Fig. 4.2: Larvicidal activities of *T. welwitschii* extracts against *An. gambiae*

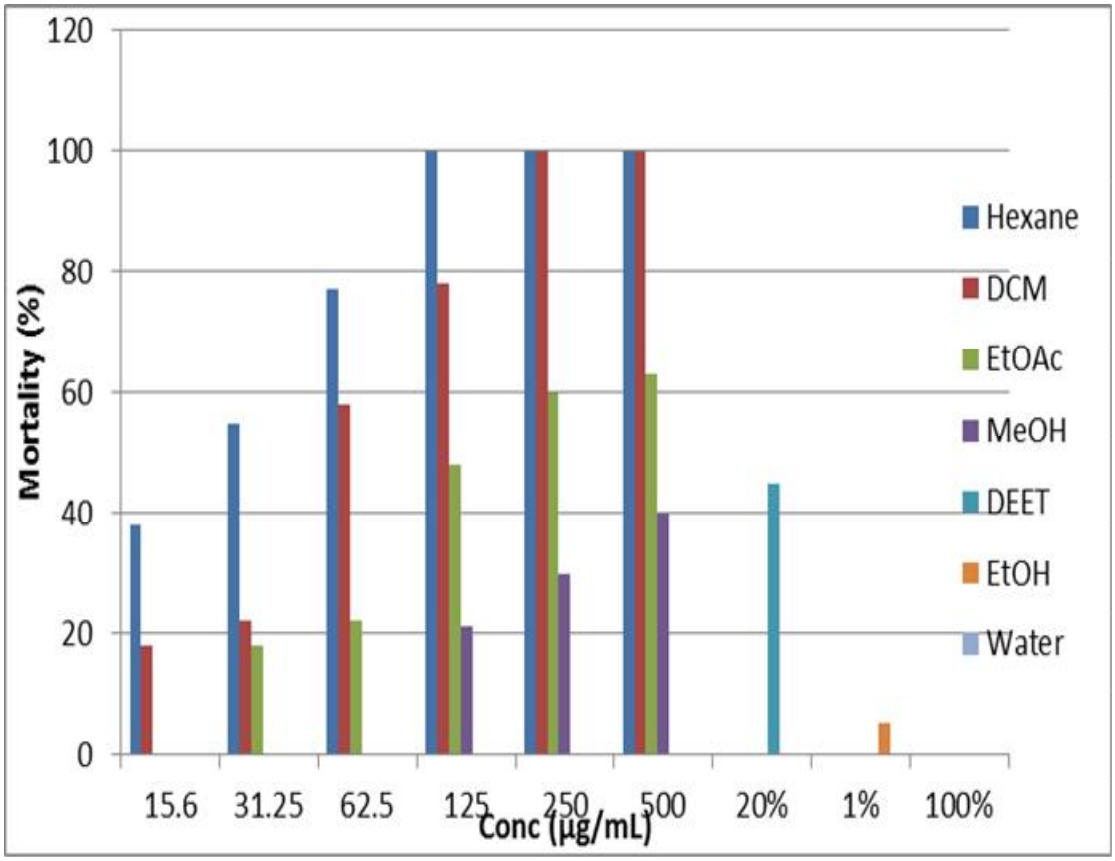


Fig. 4.3: Larvicidal activity of fractions of *T megalantha* stem bark against *An. gambiae*

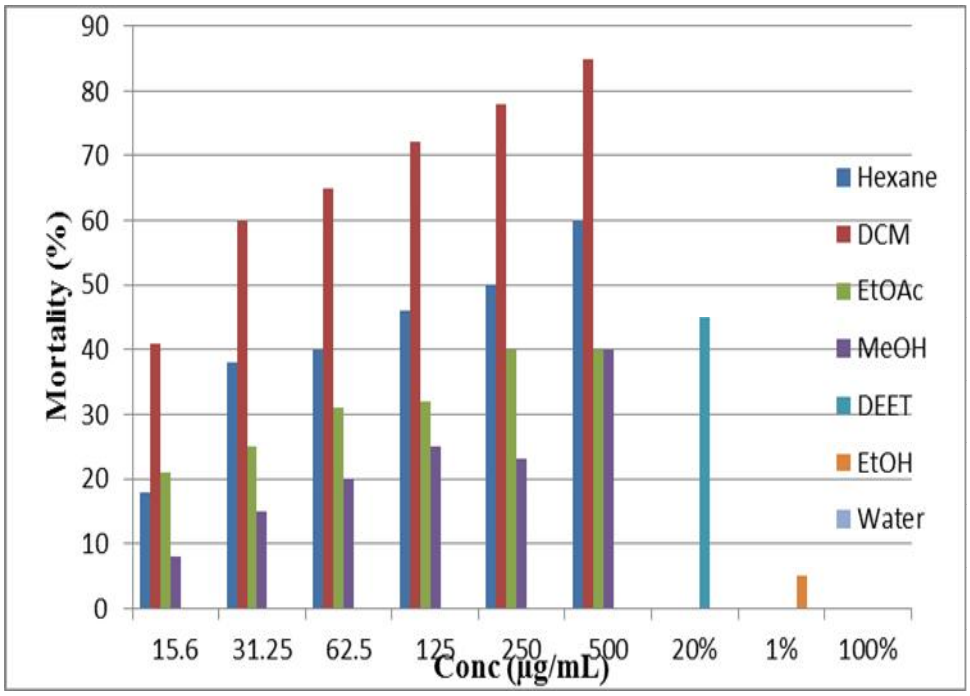


Fig. 4.4: Larvicidal activity of fractions of *T. welwitschii* root against *An. gambiae* larvae.

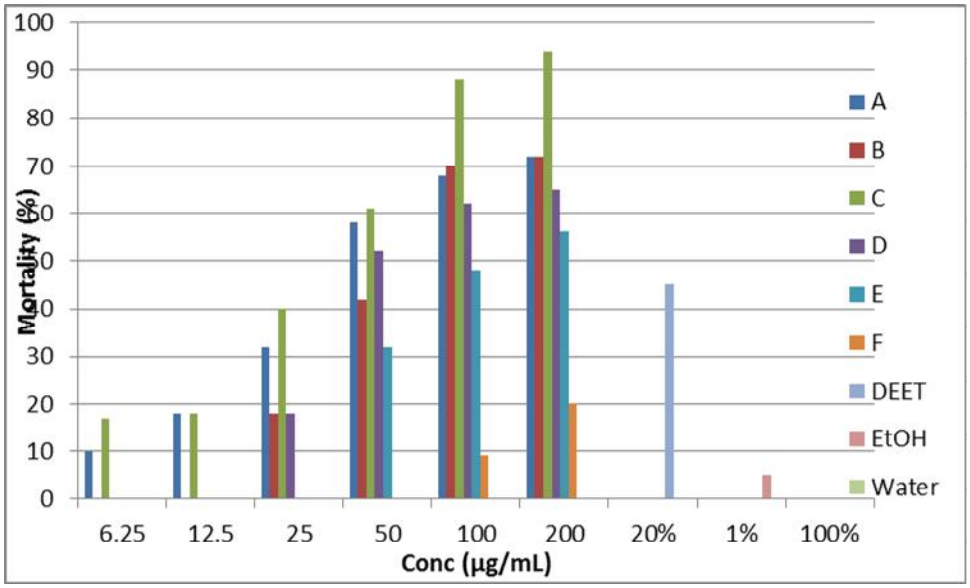


Fig. 4.5: Larvicidal activity of column fractions of *T. megalantha* stem bark Hexane fraction against *An. gambiae*

Table 4.6: Antioxidant Activities of *Trichilia megalantha* Extracts and Fractions

Extract/fraction	IC_{50} ± SEM mg/mL
T. megalantha	
Leaf	204.12 ± 1.60
Stem bark	160.73 ±1.21
Root	inactive
T. megalantha Stem	
Hexane	302.41 ±1.82
DCM	244.42±1.58
EtOAc	25.37±1.46
BuOH	12.80 ±0.58
Aqueous	inactive
Gallic acid (μM)	23.43± 0.43
N-acetyl cysteine (μM)	11. 44 ± 0.7

Table 4.7: Brine Shrimp Lethality Assay of extracts and fractions of *T. megalantha*

Extract/fraction	LC_{50} ± SEM (µg/mL)
T. megalantha	
Leaf	1233. 15
Stem bark	1499.13
Root bark	1421.06
T. megalantha Stem bark	
Hexane Fraction	10.22
DCM Fraction	19.34
EtOAc Fraction	107.58
Butanol Fraction	113.96
Aqueous Fraction	1034.21
Etoposide	7.46

Table 4.8: *In vitro* Cytotoxicity (MTT) activities of *Trichilia megalantha* Extracts and Fractions on PC-3 Cancer Cell line

Extract/fraction	% Inhibition at (50 µg/mL)	Interpretation
Leaf	14.6	Inactive
Stem bark	14.6	,,
Root bark	39.2	,,
Stem bark		
Hexane	12.8	,,
DCM	16.70	,,
EtOAc	36.08	,,
Butanol	26.04	,,
Aqueous	4.35	,,
Doxorubicin (50 µM)*	99.12	Active

*IC_{50} 0. 31 ± 0.036

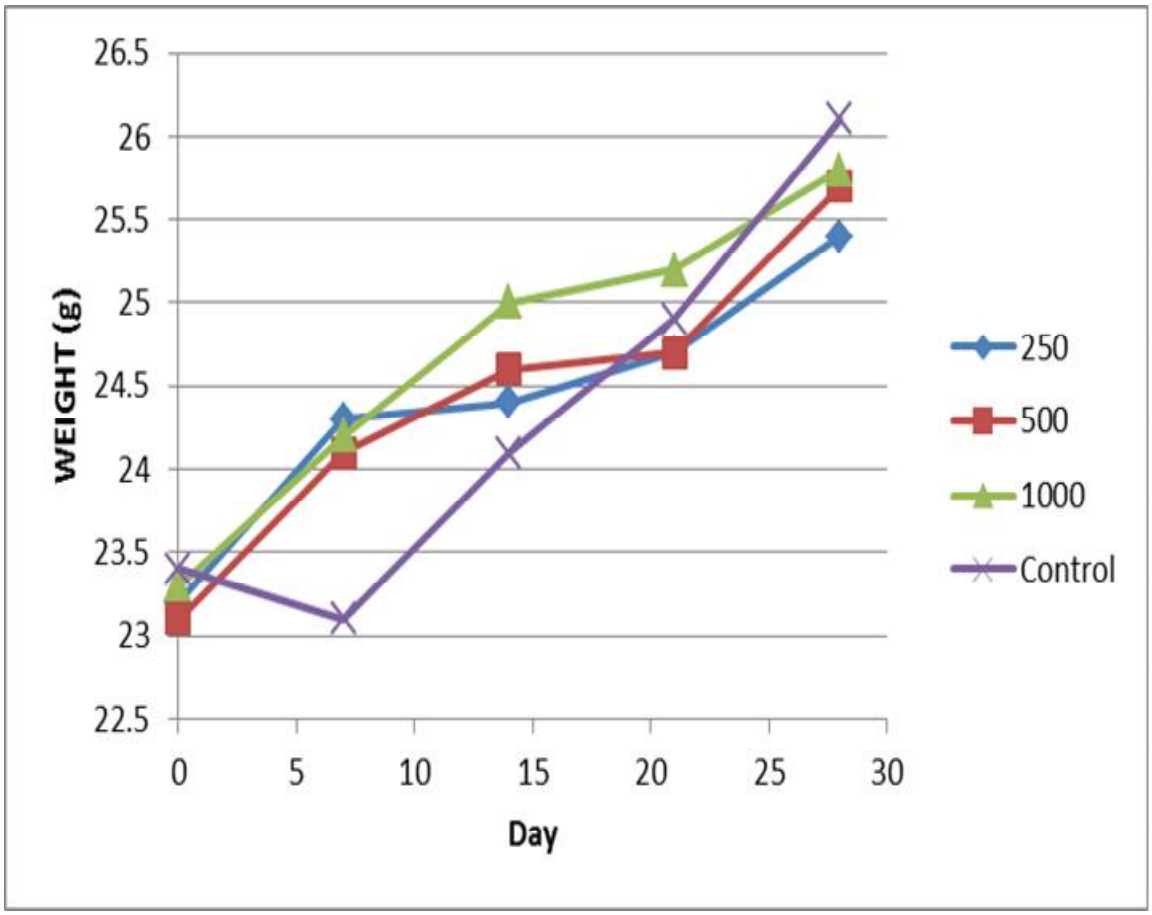


Fig. 4.6: Effect of *Trichilia megalantha* stem bark extract on the body weight of mice

(mean ±SEM, n=6)

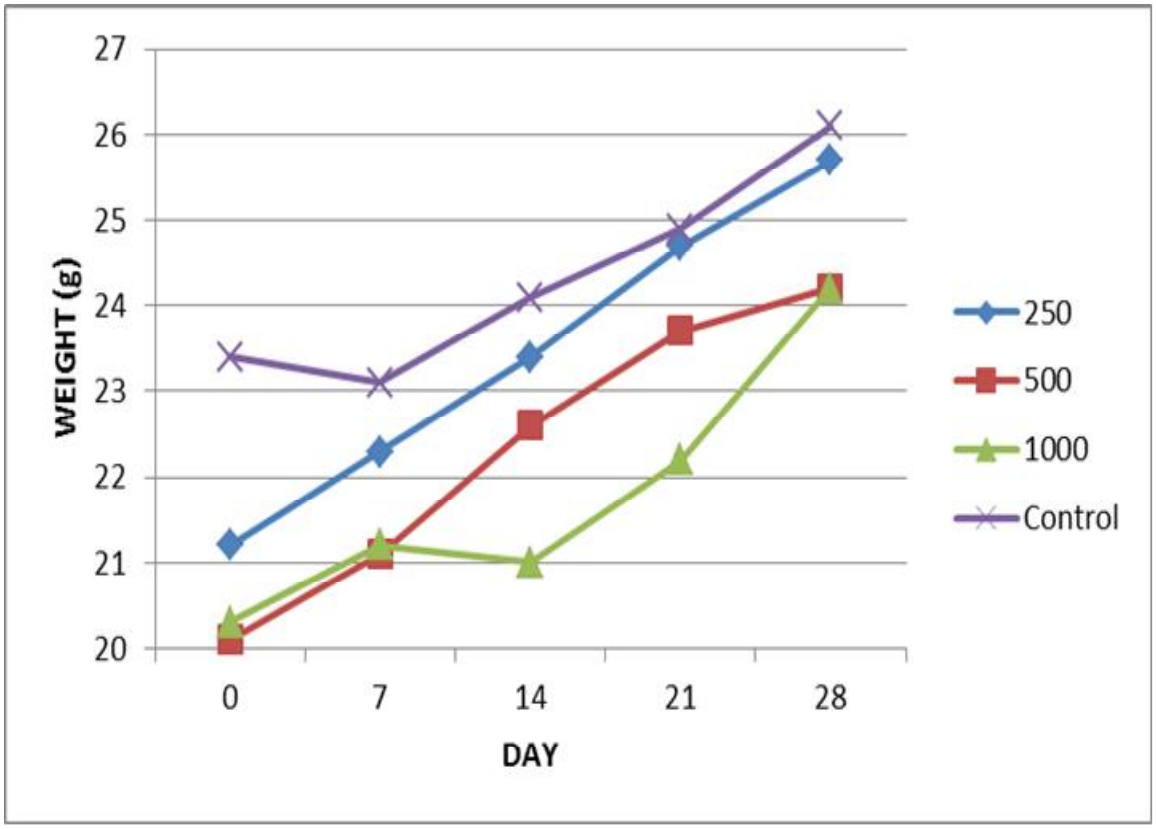


Fig. 4.7: Effect of *Trichilia welwitschii* stem bark extract on the body weight of mice

(mean ±SEM, n=6)

Table 4.9: Effect of methanol extract of Stem of *T. megalantha* and *T. welwitschii* on PCV of Mice

	PCV (%)				
Extract/con mg/kg	Day 0	Day 7	Day 14	Day 21	Day 28
T. megalantha					
250	51.74 ±2.53	48.23±4.79	43.00 ±3.07	48.32±3.22	43.22±1.22
500	51.74±2.47	43.66 ±5.27	40.51± 2.68	46.98±2.53	43.67±2.53
1000	54.00±2.31	47.66 ±2.53	48.25 ±2.53	48.87±2.53	49.69±4.89
T. welwitschii					
250	51.22±2.24	50.23±4.79	48.00±3.05	48.72±3.02	49.22±1.21
500	50.71±2.47	43.67±5.22	40.51±2.31	46.98±2.46	46.67±2.13
1000	52.01 2.31	47.64±2.50	48.25±2.11	48.65±2.51	50.69± .19
Untreated	49.93±5.60	50.84±2.90	49.34±2.53	48.89±2.53	49.69±2.53

(Mean ± SEM, N=6)

Table 4.10: Effect of *T. megalantha* and *T. welwitschii* S tem bark Extracts on the Weight of Vital Organs in Mice

	Weight (g) of Vital organs				
Extract/conc mg/kg	Heart	Liver	Kidney	Lung	Spleen
T. megalantha					
250	0.14±0.01	1.74±0.06	0.57±0.02	0.25±0.02	0.20±0.02
500	0.13±0.00	1.56±0.19	0.38±0.01	0.27±0.05	0.31±0.07
1000	0.14±0.01	1.78±0.24	0.39± 0.02	0.29±0.01	0.20±0.02
T. welwitschii					
250	0.12± 0.01	1.64±0.06	0.57±0.02	0.26 ±0.02	0.20±0.02
500	0.13±0.00	1.58±0.19	0.48±0.01	0.27±0.05	0.29±0.07
1000	0.14±0.01	1.77±0.24	0.40± 0.02	0.28±0.01	0.21±0.02
Untreated	0.12±0.01	1.74±0.06	0.38±0.05	0.28±0.02	0.28±0.02

(Mean ± SEM, N=6)

Table 4.11: Effect of Stem bark of *T. megalantha* and *T. welwitschii* on Blood Chemistry Values in Mice

Extract/(con mg/kg)	AST (IU/L)	ALT(IU/L)	ALP(IU/L)
T. megalantha			
250	30.90±7.95	215.70±39.04	29.90±8.92*
500	41.20±2.80	233.60± 24.82	44.40±7.56*
1000	36.60 ±6.84	214.70±31.09	30.70±10.7*
T. welwitschii			
250	30.94±7.98	218.70±32.04	32.90±5.92*
500	42.20±1.80	230.60±24.22	41.20±7.54*
1000	34.60±6.70	218.70 ±21.09	30.55± 9.7*
Untreated	40.20±3.07	235.50±21.16	14.70±1.65

*Statistically significant when compared with control animals $p \leq 0.05$
(Mean ±SEM, n=6)

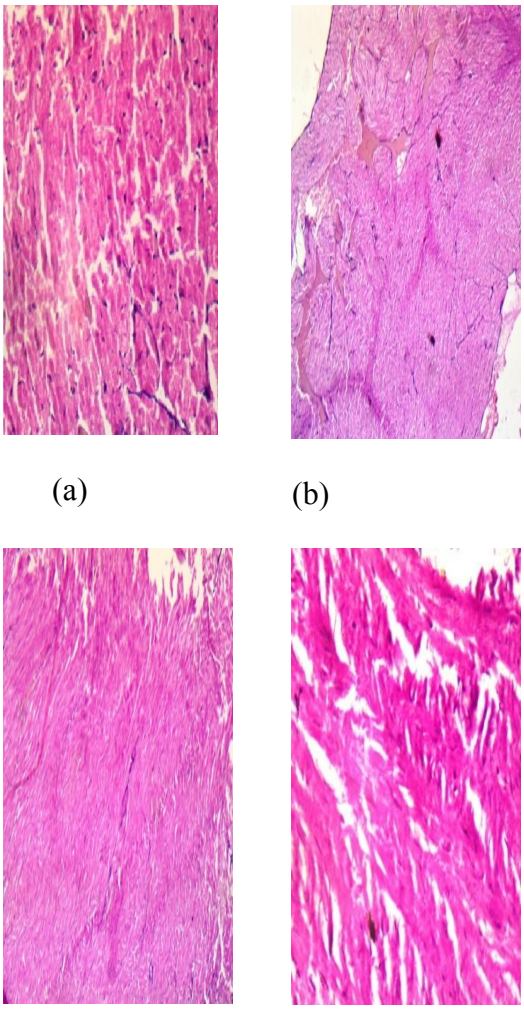


Fig. 4.8: Histology of the heart of mice after 28-day administration of methanol extract of *T. welwitschii* stem bark

(a) 250 mg/kg (b) 500 mg/kg, (c) 1000 mg/kg (d) distilled water

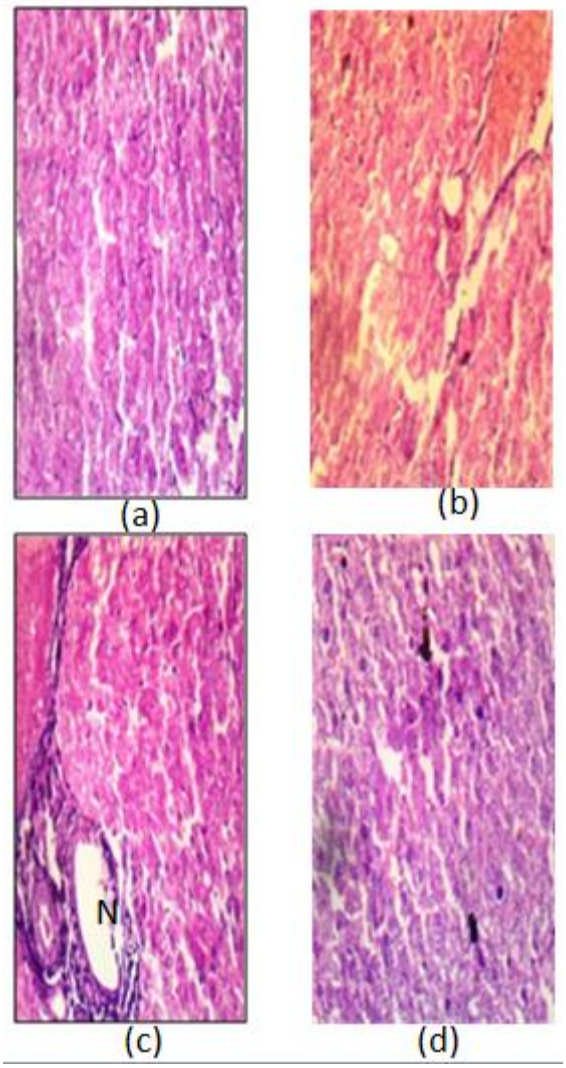
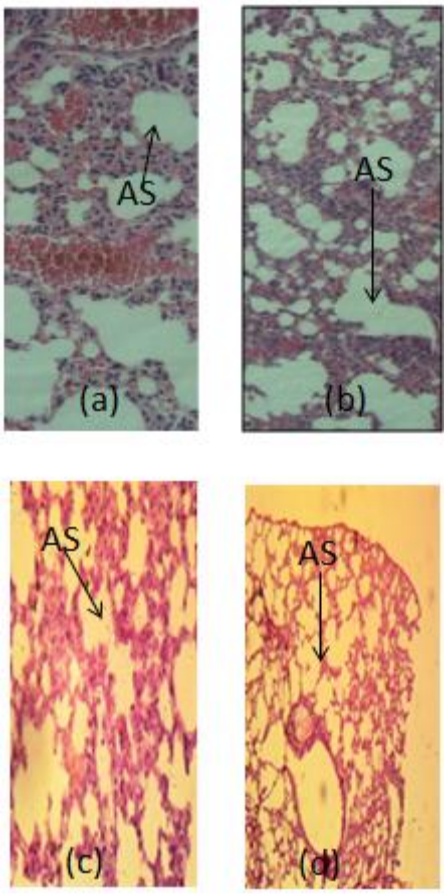


Fig. 4.9: Histopathology of the liver of mice after 28-day administration of methanol extract of *T. welwitschii* stem bark

(a) 250 mg/kg,(b) 500 mg/kg,and (c) 1000 mg/kg (d) distilled water

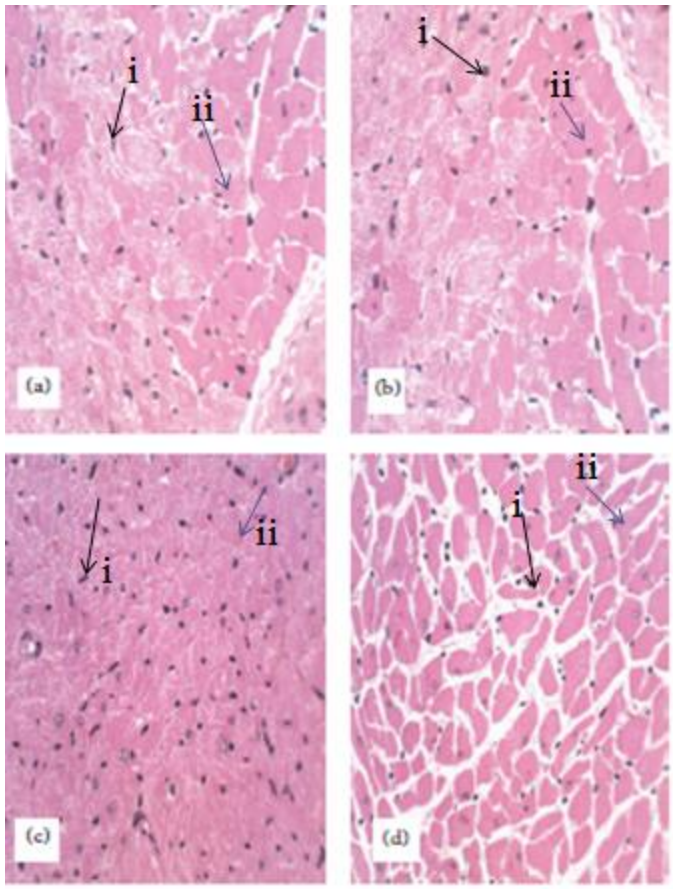
No visible lesions except few hepatocytes with very large nuclei (N) which have prominent multiple nucleoli in the liver of the group that received 1000 mg/kg



AS = Alveoli sac

Fig. 4.10: Histology of lung of mice after 28-day administration of methanol extract of *T. welwitschii* stem bark

(a) 250 mg/kg, (b) 500 mg/kg, (c) 1000 mg/kg, (d) distilled water



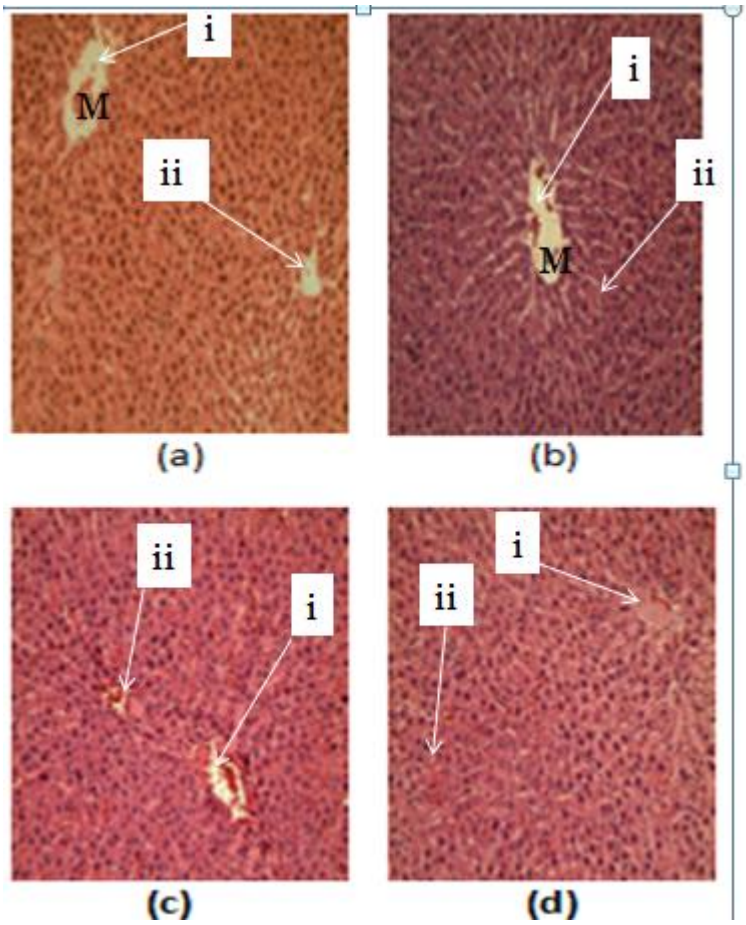
i= Nucleus of mycocyte

ii= Blood vessel

Fig 4.11: Histology of heart of mice after 28-day administration of methanol extract of *T. megalantha* stem bark

(a) 250 mg/kg, (b) 500 mg/kg, (c) 1000 mg/kg, (d) distilled water

There is architectural difference in the appearance of the heart of animals treated with 1000 mg/kg dose but no visible lesions were seen in the heart of mice that received lower doses (250 mg/kg) of *T. megalantha* stem bark extracts.



i= Portal triad

ii= Central vein

Fig. 4.12: Histology of liver of mice after 28-day administration of methanol extract of *T. megalantha* stem bark

(a) 250 mg/kg, (b) 500 mg/kg, (c) 1000 mg/kg *T.* (d) distilled water

There is mild to moderate vacuolar change (M) of the hepatocytes in the liver of animal treated with 250, 500 and 1000 mg/kg.

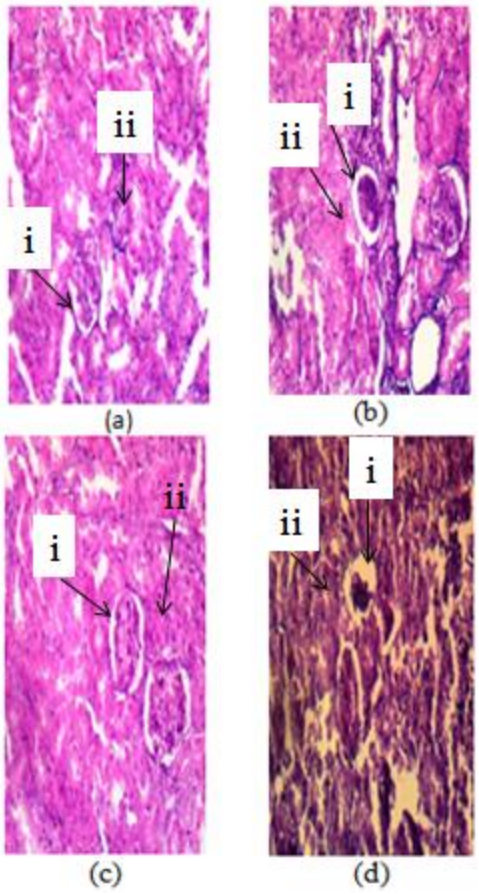
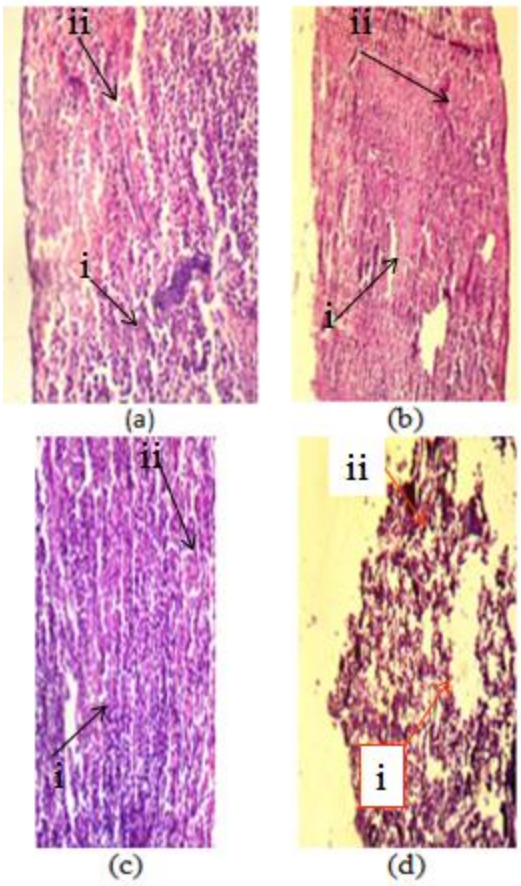


Fig. 4.13: Histology of kidney of mice after 28-day administration of methanol extract of *T. megalantha* stem bark

(a) 250 mg/kg, (b) 500 mg/kg, (c) 1000 mg/kg, (d) distilled water



i= White pulp

ii= Red pulp

Fig. 4.14: Histology of spleen of mice after 28-day administration of methanol extract of *T. megalantha* stem bark

(a) 250 mg/kg, (b) 500 mg/kg, (c) 1000 mg/kg, (d) distilled water

Table 4.12: Larvicidal Activity of Isolated Compounds from *T. megalantha* Stem and *T. welwitschii* Leaf

Plant	Compound	LC_{50} (µg/mL)
T. megalantha	TMH1 (Lupeol)	6.2
	TMH 70B (Ursolic acid)	15.6
T. welwitschii	FD 84 3,3′,4-tri-O-methylEllagic Acid	48.1
	DEET	120.0
	Ethanol	-

Table 4.13: Response to Treatment in Swiss Albino Mice Infected with *P. berghei* to Compounds Isolated from *T. megalantha* Stem and *T. welwitschii* Leaf

Dose(mg/kg)	Parasitaemia ± SEM (%)	Parasite Suppression ± SEM (%)
TMH 1 (Lupeol)		
50	2.45 ± 0.25	61.4*
100	0.85 ± 0.10	88.3*
TMH70B (Ursolic acid)		
50	2.58 ± 0.51	64.3*
100	0.48 ± 0.05	93.4*
FD84 (3,3,4-tri-o-methyl ellagic acid)		
50	3.01 ± 1. 25	58.5*
100	1.81 ± 0.22	75.8*
DAF 2 (Scopoletin)		
50	Not active	Not active
100	4.06 ± 0.51	54.8*
CQ[a]	0.38± 0.20	94.6
Untreated	7.25± 0.25	

*Statiscally significant when compared to untreated animals $p \leq 0.05$
[a] Chloroquine (10 mg/kg)
(Mean ±SEM, n=3)

4.7 Spectra data of isolated compounds

Bioassay guided fractionation and isolation from hexane and DCM fractions of *T. megalantha,* led to the identification of six compounds and oils while *T. welwitschii* afforded one compound.

4.7.1 Isolation and Spectral Data of compound TMH 1 (Lupeol)

TMH-1, R_f = 0.62 (silica gel, Hex: EtOAc, (9.5:0.5), off white amorphous solid (402 mg), was isolated by repeated column chromatography of the hexane soluble fraction of *T. megalantha* stem bark.

EIMS *m/z* (rel. int.): 426 [M^+] (2), 411 [M^+ - CH_3] (3), 408 [M^+ -H_2O] (3), 218 (5), 207 (6), 189 (58), 163 (80), 135 (57), 107 (68), 105 (55), 79 (54), 41 (100);

^{1}H NMR: δH: 0.75, 0.78, 0.81, 0.92, 0.94, 1.02 (Me-28, Me-23, Me-24, Me-25, Me-26, Me-27), 1.67 (3H, br d, *J*=0.5 Hz, Me-30), 3.18 (1H, dd, *J*=9.6, 6.2 Hz, *H*α-3), 4.56 (1H, d, *J*=0.4 Hz, *Ha*-29), 4.67 (1H, dq, *J*=0.4, 0.5 Hz, *Hb*-29)

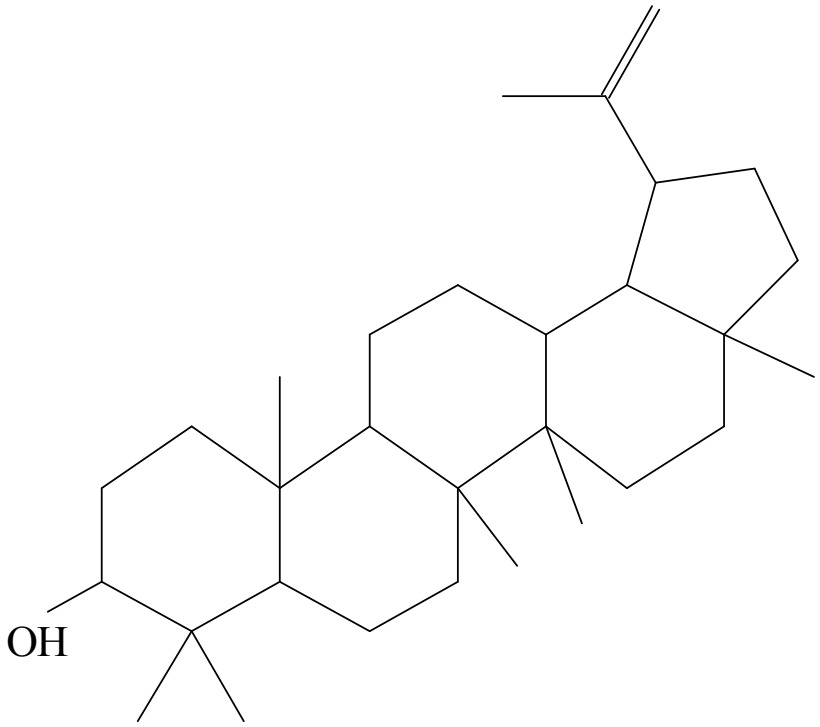


Fig. 4.15: Structure of TMH 1- Lupeol

4.7.2 Isolation and Spectral Data of compound TMH 70B (Ursolic acid)

Compound TMH 70B was obtained as white powder (45 mg) from hexane fraction of *T. megalantha.* Melting point = 283-285°C.

^{1}H NMR: δH; 2.52 (1H, d, J = 11.0 Hz), 1.24 (s), 1.02 (s), 0.93 (s), 1.05 (s), 1.22 (s), 0.97 (s), 0.99 (d, 6.1).

EIMS *m/z* (rel. int.): 464.2 (5%), 396.2 (100%), 382 (50%), 268 (28%), 255.2 (30%), 147.0(35%).

^{13}C NMR data is presented in Table 5.2.

The spectra data compares to those described in the literature and indicated that compound **TMH 70B** is Ursolic acid **(**Fig 4.16).

For information on spectra data, see Appendix 4A-4F

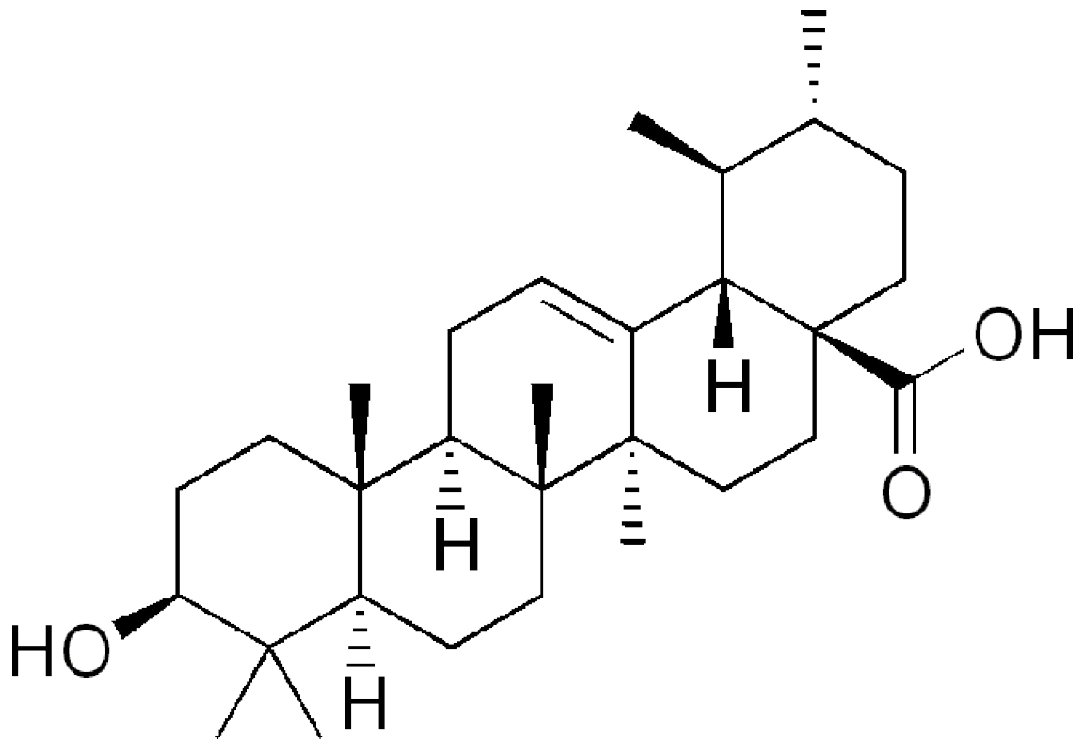


Fig. 4.16: The structure of TMH 70B (Ursolic acid)

Synonyms: 3-beta-3-hydroxy-urs-12-ene-28-oic-acid

4.7.3 Isolation Spectral Data of compound DAF- H27 (β- Sitosterol)

Compound **DAF-H27** (50.2 mg) precipitated from fractions 69-70 from TM hexane fraction as a white compound. R_f = 0.73, (Hexane: EtOAc 7:3).

Melting point = 136-137 ºC.

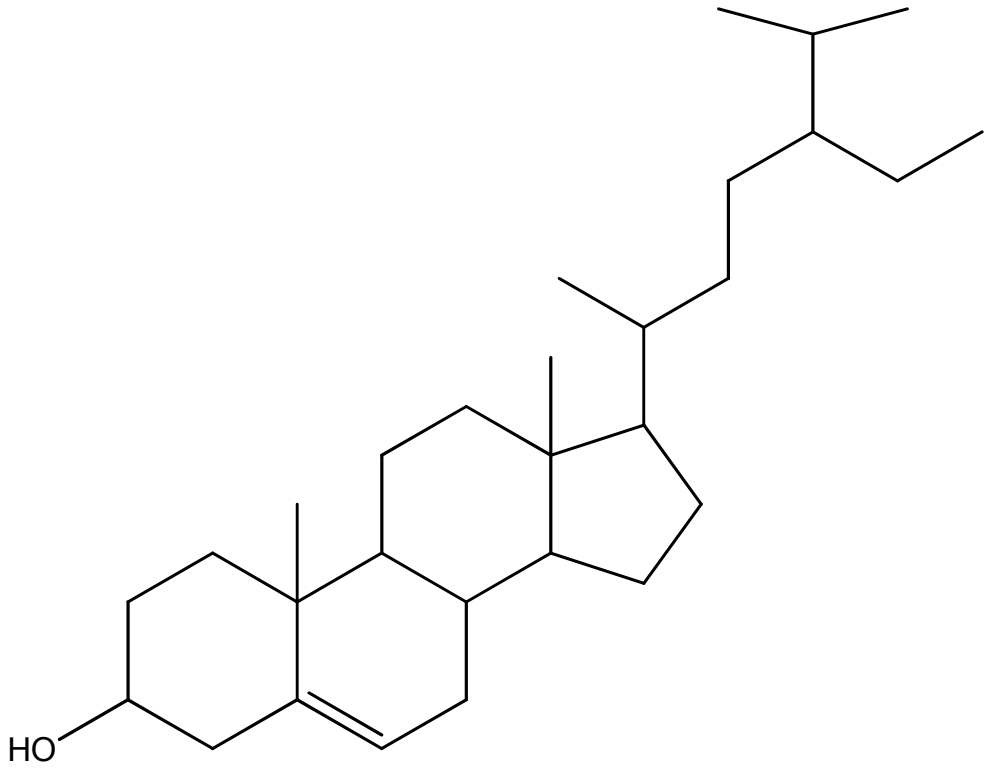


Fig. 4.17: The structure of β- Sitosterol

4.7.4 Isolation and Spectral Data of compound TMH 47 (Stigmasterol)

Compound **TMH 47** (42 mg) was obtained from fractions 14-18 (silica gel G, Hex: EtOAc (95:5). It precipitated out of the solution as a white powder.

Melting point was 162-164 ˚C.

The EI-MS showed a molecular ion at m/z 412.5 calculated for $C_{29}H_{48}O$.

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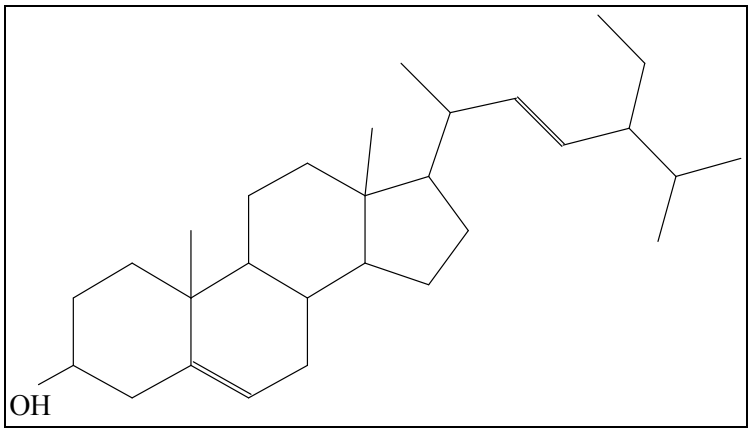

Fig. 4.18: The structure of Stigmasterol

4.7.5 Isolation and Spectral Data of compound DAF 1 (Stigmaste-4-en-3-one)

Compound **DAF 1** $R_{f=}$ 0.7, silica gel G, Hexane: EtOAc (80:20), a white powder (8 mg), was isolated by repeated column chromatography of the hexane fractions of *T. megalantha.*

Visible as a purple spot when sprayed with cerric sulphate,

EI-MS *m/z* (rel. int. %) z 412.4; 370; 229; 192; 124(100%); 95; 54

The EI-MS of compound **DAF 1** showed a molecular ion at m/z 412.4 calculated for $C_{29}H_{48}O$. ^{13}C NMR data is shown in Table 5.3

For information on spectra data, see Appendix 3A-3F

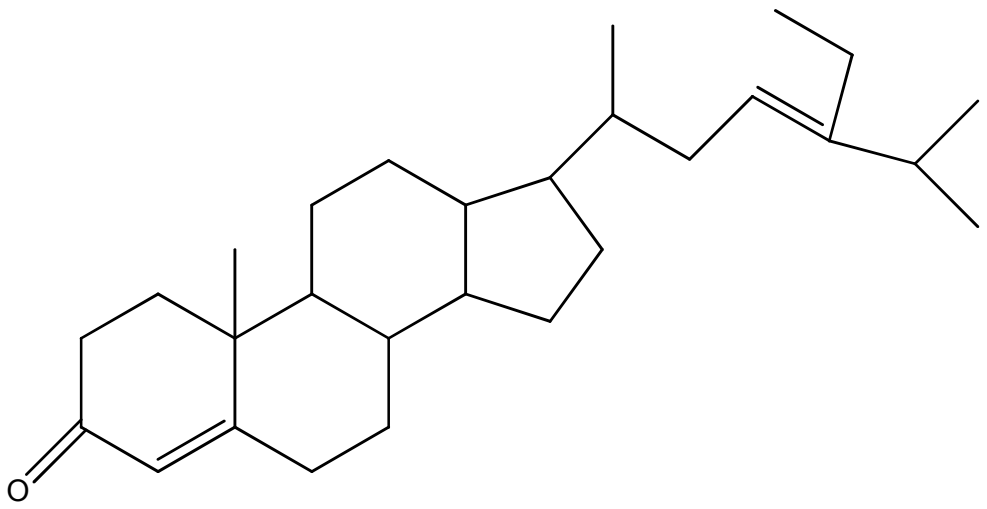

Fig. 4.19: Structure of Stigmastenone

4.7.6 Isolation and Spectral Data of compound FD 84 (3,3,4-tri-o-methylellagic acid)

Compound **FD 84** was obtained as a pale yellow amorphous powder.

EIMS m/z 345 $[M+H]^+$), 344(100%), 329(31%); 301(28%), 285.9(30%), 55.0(30%).

For information on spectra data, see Appendix 6A-6F

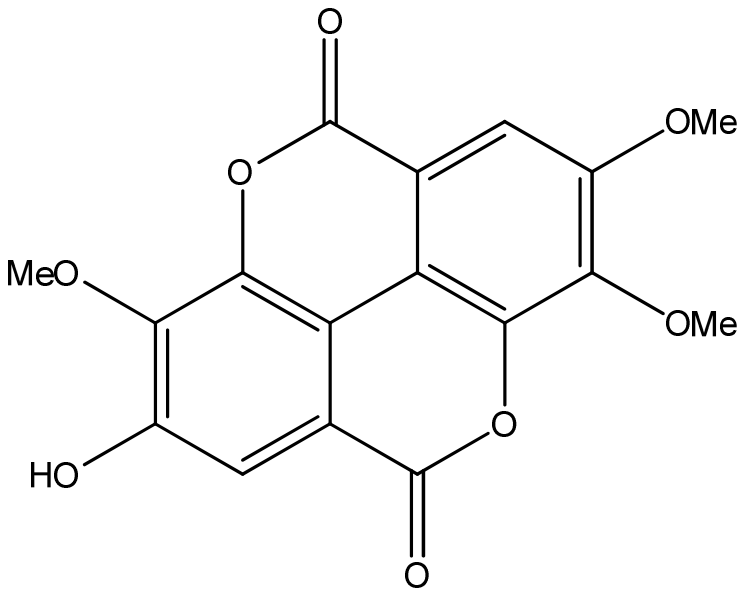

Fig. 4.20: Structure of 3,3′,4-tri-o-methyl ellagic acid

4.7.7 Isolation and Spectral Data of Compound DAF 2 (Scopoletin)

This compound was isolated as a yellow crystalline solid (14.2 mg, R_f, 0.81 silica gel, 3:7 ethyl acetate- hexane). Melting point = 202-204 ^oC.

^{13}C-NMR δ (ppm): 161.4 (C-2); 144.0 (C-6); 149.7 (C-7); 150.0 (C-9); 111.5 (C-10); 113.4 (CH-3); 143.3 (CH-4); 107.5 (CH-5); 103.2 (CH-8); 56.4 (OCH_3).

^{1}H-NMR δ (ppm): (6.25; *d*; J = 9.4 Hz; H-3); (7.59; *d*; J = 9.4 Hz; H-4); (6.82; *s*; H-5); (6.89; *s*; H-8); (3.93; *s*; OCH_3).

LRMS *m/z* (rel. int.): 192 (100%); 177 (63%); 164 (30%); 149 (59%); 121 (30%).

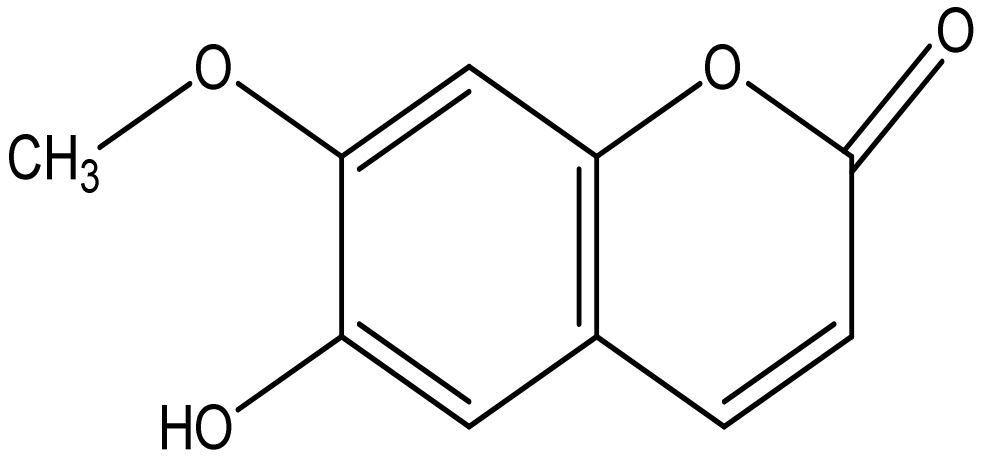

Fig. 4.21: Structure of Scopoletin

Table 4.14: GC-MS Analysis of Contituents of fixed oil from Hexane Fraction A of *T. megalantha* stem bark

S/N	Retention time (min)	Composition	Chemical formula
1	19.115	5,9-Undecadien-2-one, 6,10-dimethyl	$C_{13}H_{22}O$
2	21.326	trans-Z-α-Bisabolene epoxide	$C_{15}H_{24}O$
3	21.721	1,3-Heptadiene, 3-ethyl-2-methyl-	$C_{10}H_{18}$
4	21.945	Cubenol	$C_{15}H_{26}O$
5	22.015	8,11,14-Eicosatrienoic acid, (Z,Z,Z)-	$C_{20}H_{34}O_2$
6	22.166	1-Naphthalenol	$C_{15}H_{26}O$
7	22.163	4-Isopropyl-1,6-dimethyl-1,2,3,4,4a,7,8,8a-oc	$C_{15}H_{26}O$
8	23.121	Methyl tetradecanoate	$C_{15}H_{30}O_2$
9	33.515	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$
10	34.048	Methyl 13-octadecenoate	$C_{19}H_{36}O_2$
11	50.444	cis-13,16-Docasadienoic acid, methyl ester	$C_{23}H_{42}O_2$
12	51.225	6,9,12,15-Docosatetraenoic acid, methyl ester	$C_{23}H_{38}O_2$
13	53.221	9,12-Octadecadienoyl chloride, (Z,Z)-	$C_{18}H_{31}ClO$
14	58.577	Tetracosa-2,6,10,14,18-pentaen-22-ol, 2,6,10,15,19,23-hexamethyl-23-methoxy-, alltrans	$C_{31}H_{54}O_2$

CHAPTER FIVE

DISCUSSION

The dearth of chemotherapeutic agents against malaria infection coupled with the challenge of drug resistant infections necessitate the need to direct concerted effort towards the development of new potent, safe, cheap and affordable antimalarials (Kapoor and Kumar, 2005). Although some vaccines are still undergoing clinical trials, this may constitute the best long term control option. The highly adaptive nature of malaria parasites however, accentuates the difficulty in obtaining an effective one (Alonso *et al*., 2005; Aponte *et al*., 2007; Bejon *et al*., 2008; Guinovart *et al*., 2009). Nature remains an ever evolving source for compounds of medicinal importance due to their potential usefulness in preventing and treating a wide array of diseases. Since ancient times, the use of medicinal plants for the treatment of parasitic diseases like malaria is well known and documented. The plant kingdom has provided two of the most important drugs for the treatment of malaria infection, quinine from *Cinchona calisaya* Wedd. and *Cinchona succirubra* Pav. Ex Klozsht (Bruce-Chatt, 1988), and artemisinin from *Artemisia annua* (Klayman, 1985). Several compounds isolated from nature also form a rich source of diverse structures for optimization to obtain improved therapeutics. The Meliaceae plant family has emerged as a potent source of insecticides because of their use as growth regulators against many insect pests (Jacobson, 1987; Gajmer *et al*., 2002; Banchio *et al*., 2003; Wandscheer *et al*., 2004).

The genus *Trichilia* (Meliaceae) comprises about 419 species that are known to possess biological and pharmacological activities. Several studies have shown that plant crude extracts can be highly toxic to *An. gambiae* and other mosquito species (Banchio *et al*., 2003; Ndung'u *et al*., 2004a; Ndung'u *et al*., 2004b; Pavela, 2009; Kamaraj *et al*., 2008).

The scantiness of chemotheraupitic arsenal to combact malaria infection and the problem of drug resistance infections coupled with emergence and rapid multiplication of insecticide resistant vectors necessitate the need to direct concerted effort towards the search for new potent antimalarial and larvicidal agents. Historically, majority of antimalarial drugs have been derived from medicinal plants or from structures modeled on plant derived compounds. These include the quinoline-based antimalarials as well as artemisinin and its derivatives. In endemic countries like Nigeria, accessible treatments against malaria are mainly based on the use of traditional herbal remedies. Medicinal plants are commonly used in the management of malaria. The importance of diverse medicinal plants lies not only in their chemotherapeutic value in traditional healthcare but also in their potential as sources of new chemical entities for drug discovery.

Results of the *in vitro* antimalarial activity of *T. megalantha* and *T. welwitschii* extracts (Table 4.2) showed that methanol extract of *T. welwitschii* was the most active with IC_{50} values of 9.0 and 10.5 µg/mL against D6 and W2 falciparum strains used respectively. A low activity was observed with *T. megalantha* stem bark (IC_{50}, 12.2 and 26.65 µg/mL). Other plants parts were not active in the assay.

In this study, crude extracts of *T. megalantha* and *T. welwitschii* plant parts were assessed for antimalarial activities and were found to show various abilities to suppress parasite growth in the four day suppressive test. *T. megalantha* showed intrinsic antimalarial activity judging by its percentage chemosuppression in comparison with that of chloroquine. Of all the morphological parts screened, the stem bark gave the highest suppression of parasite growth. It completely cleared the parasite, *P. berghei*, in mice at dose as low as 200 mg/kg. It was followed by the root bark while the leaf demonstrated lowest chemosuppresssion. Parasitaemia on day 4 ranged from 0.00 to 6.21 in animals treated with selected doses (100-800 mg/kg) of extract while for CQ and negative control was 0.10 and 5.52 respectively (Table 4.3) The percentage suppression of parasite growth ranged from 49.04 to 100% in animals infected and treated with extracts of the leaf, stem bark and root in a dose dependent manner. Treatment of infected animals with standard dose of chloroquine (10 mg/kg) resulted in 96% suppression of parasite growth.The multiple comparison tests indicated that all the mice treated with the three extracts resulted in reduced parasite load as compared to their respective negative control groups.

Fractions from the stem bark of *T. megalantha* showed statistically significant ($P<0.05$) chemosuppression against *P. berghei* at all dose levels tested compared to the mice in the untreated group on day 4 (Table 4.5). The mice treated with chloroquine had parasites suppression of 98% on day 4, while treatment with the highest dose of the DCM fraction exerted 96.1% chemosuppression on the same day.

The antimalarial activity of crude methanol extract of *T. welwitschii* is shown in Table 4.4. The parasitaemia of animals infected with *P. berghei* after four day treatment with 100-800 mg/kg extract ranged from 0.48% - 7.01%. Parasitaemia in animals treated with Chloroquine, the standard drug, was 0.30% while infected untreated animals had 7.25%. The highest antimalarial activity was displayed by the leaf extract with 93.4% chemosuppression followed by the stem bark (90.7%) while the root had the least activity (75.8%). These activities are comparable to that of Chloroquine (96.2%).

Moreover, mice treated with the leaf, stem bark and root bark extracts of *T. megalantha* survived longer than mice in the corresponding negative control groups. The group treated with 800 mg/kg of the root bark of *T. megalantha* had the longest time of 26 days. Group treated with chloroquine survived till day 23. Survival time increases as the dose increases. The mean survival time of the animals treated with varying doses of extract was statistically significant ($p< 0.05$), (except the lowest dose) when compared to the negative control group mice. Similarly the mice treated with the fractions survived significantly longer than mice in the negative control.

In all cases, remarkable chemosuppression of parasitaemia by extracts translated into a longer mouse survival. Survival rate of experimental animals is an important factor in the assessment of the efficacy of the treatment regimen of drugs. The mean survival time of mice treated with varying doses of extract ranged from 10.2 to 26.0 days. Survival time for animals that received chloroquine was 24 days while the untreated group survived till day 10. In animals treated with fractions, the survival time ranged from 8.7 to 25 days.

Relative to the mice in untreated control group, mice treated with doses of *T. megalantha* survived up to a further two weeks. Similarly the mice treated with the fractions survived significantly longer than mice in the negative control. The mean survival time of the mice treated with the fractions increased as the dose increase. Animals treated with 400 mg/kg of chloroform fraction of *T. megalantha* had the same survival time with group that received chloroquine (10 mg/kg) while group that received 800 mg/kg had a longer survival time of 28 days. This showed the efficacy of extracts of *T. megalantha* and *T. welwitschii* in the management of malaria. The multiple comparison tests indicated that all the mice treated with the three extracts resulted in reduced parasite load as compared to their respective negative control groups. Fractions from the stem bark of *T. megalantha* showed statistically significant ($P< 0.05$) chemosuppression against *P. berghei* at all dose levels tested mice compared to the mice in the untreated group on D4.

The efficacy of phytochemicals against mosquito larvae can vary significantly depending on plant species, plant parts used, age of plant parts (young, mature or senescent), solvent used during extraction as well as upon the available vector species (Van Wyk *et al*., 2000). Assessment of the larvicidal effect of extracts of the leaves, stem bark and root bark (15.63 –1000.00 µg/mL) of *T. megalantha* and *T. welwitschii* against early 4th instar larvae of *Anopheles gambiae* showed various larval toxicities. All extracts were significantly toxic to the larvae (Figure 4.1-4.2). Activity was observed to be concentration- dependent in the experiments. Results showed that all crude extracts tested were toxic to the larvae but the larvae were more susceptible to *T. megalantha* stem bark extract. At the highest concentration tested (1000 µg/mL), it killed the entire larvae.

Among fractions tested, the hexane soluble fraction exhibited the highest toxicity (100% mortality at 250 µg/mL) while the methanol soluble fraction was the least toxic (40%) (Figure 4.3 & Figure 4.4). For the plants tested, larvicidal activity was observed to reside mainly in the non-polar extracts. In the case of *T. welwitschii* the crude methanol root extract was the most active, exhibiting 100% larval mortality at 1000 µg/mL, followed by the stem bark while the leaf displayed lowest activity.

Comparing the extracts of the two species, *T. megalantha* extract showed better larvicidal activity than *T. welwitschii*. Analysis of results using GraphPad Prism, showed that the stem bark extract of *T. megalantha* was the most toxic, LC_{50} of 94 µg/mL followed by the root (LC_{50} of 525 µg/mL). Larval mortality was considerably reduced in leaf extracts of both plants when compared to the root and stem bark extracts.

The results of larvicidal assay of fractions obtained by partitioning the crude methanol extracts into hexane, chloroform and ethyl acetate showed that the non-polar fractions were the most active. The hexane fraction of the stem bark of *T. megalantha* was the most active exhibiting 100% mortality at 250 µg/mL, followed by the chloroform fraction while the methanol fraction had the least activity. The DCM fraction of *T. welwitschii* root demonstrated the highest larval toxicity (LC_{50} 22.5 µg/mL) followed by the hexane fraction (LC_{50} of 250.7 µg/mL). Methanol fraction had the least toxicity to the larvae (LC_{50} of >500 µg/mL). The LC_{50} values of sub-fractions obtained by subjecting the hexane fraction of *T. megalantha* stem to column chromatography are given in Figure

4.5. In each case, the toxicity of the fraction was found to increase with concentration. At 200 µg/mL percentage mortality of Fractions A, B and C were 72% and 94% respectively. The LC_{50} of fraction A was 43.48 µg/mL. Fraction B was moderately toxic to the larvae exerting an LC_{50} of 65.65 µg/mL Fraction C was the most potent fraction from *T. megalantha* stem hexane fraction with an LC_{50} of 42.98 µg/mL. Fractions D and E were moderately toxic. They produced LC_{50}s of 45.13 and 123.1 µg/mL, respectively. Fraction F appears not to be toxic against the larvae. It was observed that the moderately polar fractions were more potent that the polar fractions.

Several studies have demonstrated that moderately polar extracts are more toxic to mosquitoes than the polar extracts. Study carried out by Latha and Ammini, (2000) demonstrated that the petroleum ether extract of the leaves and tuber of *Curcuma raktakanda* exhibited high toxicity towards larvae of *Culex quinquefasciatus, Culex sitiens*, *Aedes aegypti* and *Anopheles stephensi.*

Furthermore, the result of DPPH antioxidant activity (Table 4.6) of leaf, stem and root bark of *T. megalantha* extracts and stem bark fractions showed that radical scavenging ability was pronounced in the butanol fraction when compared to other fractions. It had IC_{50} of 12.80 ± 0.58 mg/mL as compared to gallic acid and N- acetyl cysteine of 23.44 ± 0.43 mg/mL and 11.44 ± 0.7 mg/mL, respectively. The EtOAc fraction was the next in activity with IC_{50} value 25.37 ± 1.46 mg/mL. Hexane and DCM fraction had moderate DPPH radical scavenging activity. The root extract was not active in the antioxidant assay.

Selectivity of plants/drugs is a relevant characteristic for defining lead candidates. Results from cytotoxicity studies differentiate between general toxicity and specific toxicity. A potential chemotherauptic agent must be toxic to the parasite but har.mless to the host. The brine shrimp lethality assay also revealed that the methanol extracts of *T. megalantha* stem bark was not toxic with CC_{50} greater than 1000 µg/mL as compared to etopoxide with CC_{50} of 7.46 µg/mL (Table 4.7). The *in vitro* cytotoxicity assay results showed that all the plant parts tested were not cytotoxic against PC3 cell lines used in this study (Table 4.8). This showed that the extracts of *T. megalantha* and *T. welwitschii*

In recent times, herbal medicines have received greater attention as an alternative to clinical therapy and the demand for these remedies has currently increased. The need to ascertain the safety and efficacy of traditional and herbal products is imperative. Investigation of acute toxicity is the first step in the toxicological analysis of herbal drugs. Toxicity studies in animal models have been found to have a good predictability for human toxicities close to 70-80% (Olson *et al*, 2000; Kola & Landis, 2004), In acute toxicity study, methanol extract of the stem bark of *T. megalantha* and *T welwitschii* did not show any mortality or toxic effect up to the dose of 5000 mg/kg during the observational period of 24 hours. For all doses there were no deaths reported. Both extracts did not produce any significant changes in behaviour, breathing, cutaneous effects, sensory nervous system responses. They gained weight with no adverse clinical signs of toxicity at any dose. Throughout the 14-day observation period, there were no significant changes in behaviour in any of the mice, nor did they produce any variations

in the general appearance.These results showed that in single dose, It appears there are no adverse effects of methanol extract of the plants indicating that the medium lethal dose (LD_{50}) is higher than 5000 mg/kg in mice. According to Kennedy & Sherman (1986) and Schorderet (1992) substances that present LD_{50} higher than 5000 mg/kg of body weight by oral route may be considered practically non-toxic. Thus the methanol extract of stem bark of *T. megalantha* and *T. welwitschii* can be classified in the category of substances with low toxicity.

The result of the 28 days sub-acute toxicity study showed that administration of extracts of both plants did not show any significant changes in behaviour or locomotor activity, no ataxia and no sign of intoxication were observed during the 28 day period. No difference in growth was noticed between the control group and treated groups (Fig. 4.6 - 4.7). No change in fur coating, eyes and respiratory function. There were some apparent macroscopic or microscopic changes observed in the organs analyzed but there was absence of any gross pathological lesion in organs. All animals gained weight, without a statistical difference between the animals in either sex (Fig. 4.6). A 11% increase in body weight was observed in the untreated animals. This same trend was observed in animals treated with different doses of extracts.The packed cell volume (PCV), of the animals in all the groups was not statistically different as shown in Table 4.9. Hematological changes such as anemia are often accompanied with bone marrow toxicity (Rhiouani *et al*., 2008; Koshy *et al*., 2011). According to (Onyeyilli *et al*., 1998) anemia that results after administration of agent can be a result of lysis of blood cells. However no such anemia is observed after chronic treatment with the extracts suggesting that there is no

lysis of blood cells. This showed that the drug is non-toxic in nature. Table 4.10 shows the effects of extracts of *T. megalantha* and *T. welwitschii* on the weights of principal organs. There was no significant difference ($P > 0.05$) between the organs weights of the extract treated groups compared to the control.

Alanine aminotransferase (ALT) belongs to a group of enzymes called aminotransferases. The enzyme ALT been found to be in highest concentrations in the liver, with decreasing concentrations found in the kidneys, heart, skeletal muscle, pancreas, spleen and lung tissue respectively. ALT measurements are used in the diagnosis and treatment of certain liver diseases (e.g. viral hepatitis and cirrhosis) and heart diseases. It is often tested in combination with Aspartate aminotransferace (AST) as part of a liver panel with ALT levels being higher in most types of liver disease. Elevated ALT and AST serum levels, combined with histopathological evidence, are used to identify acute hepatocellular injury, which is essential for investigating and recognizing chemical-induced liver toxicity (Ramaiah, 2007).

Results of histological studies provided supportive evidence for biochemical analysis. Organ weight changes are markers of toxicology and risk assessment of drugs, chemicals and food additives (Michael *et al*., 2007). The primary organs which are affected by toxicants are heart, liver, lung, kidney and spleen due to metabolic changes (Lazaro *et al*., 2002). No abnormalities were detected in histopathology of organs of heart, liver, spleen, kidney and lung of control group (Figure 4.5- 4.15). The changes in body weight have been used as an indicator of adverse effects of chemicals. In the subacute toxicity study,

mice treated with various doses of methanolic extract of *T. megalantha* had a progressive increase in body weight. This could be an indication that the drug does not affect the feed utilisation ratio of the animals. The increase in weight was not significantly different from that of the control. The result of the haematological status after 28 days of oral administration of methanolic extract of both plants showed an insignificant reduction in PCV of the treated animals compared to that of the control (Table 4.9). This was confirmed by the histopathological result of the spleen of animals that received the extract which was evident in the lack of expansion of the red pulp of the spleen. All the other parameters in all treated group remained normal without any significant difference. Changes in the weight of vital organs (Table 4.10) were not statistically significant.

Transaminases (GOT and GPT) and ALPs are good indices of liver and kidney damage respectively. Results of serum biochemistry are displayed in Table 4 11. There were no deleterious changes found in the level of the liver enzymes and ALPs in serum of treated groups with control animals which is indicative of no hepatocellular damage. Liver AST and ALT activities were found to be similar in all the experimental groups although a marked significant ($p > 0.05$) increase in the liver ALP activities were observed in the animals treated with the extracts of *T. megalantha* and *T. welwitschii*. However enzymatic activities of the vital organs were within the normal limits with no statistically significant elevation in any of the treated groups.

Further more, gross examination of internal organs like liver, lung, heart, spleen and kidney were also found to be normal. No tangible changes were detected in the

architecture of the cells in the various organs of treated animals when compared to the control. There were numerous hepatocytes with large nuclei (N) with prominent multiple nucleoli in the liver of mice that received 1000 mg/kg of *T. welwitschii* indicative of low grade toxicity at high doses. In the spleen of animals that received 250 mg/kg of *T. welwitschii*, no visible lesion was observed except few fairly large distinct follicles (right) separated from the splenic sinuses (left) beneath the capsule. There is mild to moderate vacuolar change of the hepatocytes in the liver of animal treated with 250 mg/kg and 500 mg/kg of *T. megalantha* while the kidney of animals that received 1000 mg/kg revealed few foci of individual cell necrosis in the renal tubular epithelium; glomeruli tufts appear to contain polymorph nuclear cells (suggestive of a glomerulitis). These results demonstrate that methanol extract of *T. megalantha* and *T. welwitschii* did not produce any significant change in haematological, biochemical, and histopathological parameters of animals used in this study at lower doses. It can therefore be concluded that the administration of this extract at the dosages studied (250–500 mg/kg body weight) appears to be safe.

Chromatograhic separation of hexane and dichloromethane fractions of *T. megalantha* led to the isolation of six compounds: TMH 1(lupeol), DAF 27 (β-sitosterol), DAF 1 (Stigmastenone), TMH 70B (Ursolic acid), TMH 47 (stigmasterol) and DAF 2 (scopoletin) while *T. welwitschii* afforded one compound FD 84(3,3,4′-tri-o-methylellagic acid).

The compound TMH 1 was obtained as a precipitate (402 mg) from the hexane fraction of *T. megalantha*. The EIMS of the compound had the [M+] at m/z 426.3 consistent with the formula $C_{30}H_{50}O$. The spectrum also presents other fragment ions at m / z 218 and 207 from the Retro Diels-Alder fragments. The 1H NMR spectrum ($CDCl_3$, 400 MHz) shows six signals of three protons each at δH 0.74, 0.77, 0.81, 0.92, 0.94, 1.01 corresponding to protons of six methyl bound to quaternary carbons. The singlet of three protons δH 1.66 attributable to the vinylic methyl (C-30). - two doublets of one proton each at δH 4.66 and 4.54 (2H, d, J = 1.9 Hz) attributable to ethylydene group were also observe: - one multiplet at δH 3.16 corresponding to the hydroxyl proton, comparing all

Table 5.1: ^{13}CNMR Spectral Data of compound TMH 1

Position	^{13}C-NMR (δ) *,	^{13}C-NMR (δ) ($CDCl_3$, 400 MHz)	Multiplicity
1	38.7	40.79	C
2	27.4	27.91	C
3	38.13	38.18	CH
4	48.23	48.25	CH
5	42.9	43.00	C
6	38.8	38.53	CH_2
7	34.2	34.4	CH_2
8	40.8	40.7	CH
9	50.4	50.2	C
10	37.1	37.8	CH
11	20.9	20.9	C
12	25.1	25.1	C
13	38.0	38.5	CH_2
14	42.8	42.8	CH_2
15	27.4	27.4	CH_2
16	35.5	35.6	CH_2
17	43.0	43.8	CH_2
18	48.2	48.3	CH_2
19	47.83	47.96	CH
20	150.9	151.0	CH_2
21	29.83	29.84	CH_2
22	39.93	39.93	CH_3
23	28.0	28.0	CH_3
24	15.4	18.02	CH_3
25	16.1	15.79	CH_3
26	15.9	15.7	CH_3
27	14.5	14.7	CH_3
29	18.0	18.0	C
30	109.2	109.39	CH_2
31	19.3	19.31	CH_3

*Sholichin *et al*., 1980

these spectral data to those obtain from the literature led to the identification of the TMH 1 structure as lupeol Fig. 5.1 (Sholichin *et al*., 1980; Ghulam *et al*., 2000; Jamal *et al*., 2008).

Compound TMH 70B (45 mg) a white powder was isolated from hexane fraction of *T. megalantha*. It is an ursane triterpene. 1H NMR, a doublet at 5.35 is attributed to the oléfinic proton at H-12; a triplet at 4.60 attributed to hydroxyl proton at H-3; the single proton at 3.64 is H-18. but the methyl zone (0.99 to 065) showing seven methyl groups or generally for this type of triterpene; the reason is because of Me-28 who is replaced by carbonyl group at 179. The mass of ursolic acid is 456 but the mass spectra is given with adduct ion (M+X) X m/z 464; X= Li. The ^{13}C-NMR spectrum of TMH 70B (Table 5.2) shows 30 signals, consisting of seven quaternary carbons, seven methines, nine methylenes and seven methyls deduced from the DEPT experiments. The most downfield signal resonated at δ179 is attributed to the carboxylic acid (C-28). The appearance of signals at δ122.6 and 139.0 indicated the presence of a double bond in urs-12-ene triterpenoid.The combined spectra data analysis using 1H-**,** 13C-NMR, DEPT**,** COSY and HSQC shows that TMH 70B is a pentacyclic triterpene (Gnoatto *et al*., 2008; Moghaddam *et al*., 2007). Comparing the data with literature identifies TMH 70B as ursolic acid.

Table 5.2: ^{13}CNMR Spectral Data of TMH 70B (Ursolic acid)

Carbon Position	δ 13C (ppm)	δ 13C (ppm)*	DEPT	δ 1 H (ppm)
1	38.1	38.4	CH2	
2	28.2	28.1	CH2	
3	77.2	78.1	CH	3.43 (1H, br *s*)
4	38.1	38.4	C	
5	55.9	55.8	CH	
6	19.8	18.8	CH2	
7	33.9	33.6	CH2	
8	39.7	40.0	C	
9	49.9	48.3	CH	
10	37.4	37.4	C	
11	23.6	23.6	CH2	
12	122.6	125.6	CH	5.50 (1H, br *s*)
13	139.7	139.7	C	
14	42.5	42.5	C	
15	28.7	28.7	CH2	
16	24.9	24.9	CH2	
17	48.0	48.0	C	
18	53.5	53.5	CH	2.52 (1H, *d*, *J* = 11.0 Hz)
19	39.7	39.5	CH	
20	38.1	39.1	CH	
21	31.8	31.1	CH2	
22	37.0	37.3	CH2	
23	28.2	28.5	CH3	1.24 (*s*)
24	14.1	15.7	CH3	1.02 (*s*)
25	18.7	16.6	CH3	0.93 (*s*)
26	19.0	17.4	CH3	1.05 (*s*)
27	23.0	23.8	CH3	1.22 (*s*)
28	173.4	180.0	C	
29	17.5	17.5	CH3	0.97 (s)
30	21.4	21.4	CH3	0.99 (*d*, 6.1)

*Babalola and Shode, 2013).

Compound DAF H27 (110.2 mg) precipitated from *T. megalantha* hexane fraction. A Co-TLC and the EI-MS of compound DAF H27 which showed a molecular ion at m/z 414 and direct TLC comparison with the reference commercial β- sitosterol with gave an identical R_f 7.4; (Hex: EtOAc 4:1) values confirms the compound to be β- sitosterol.

Compound TMH 47 (42 mg) was obtained as a white powder. From the ^{1}H-NMR spectrum, H-3 proton appeared as a triplet of a double doublet (tdd) at δ 3.25 (J = 4.5 and 1.1 MHZ) and H-6 olefinic proton showed a multiplet at δ 5.14. Two olefenic protons appeared downfield at δ 4.14 (m) and δ 4.61 (m) which were identical with the chemical shift of H-22 and H-23, respectively of Stigmasterol (Li *et al*., 2006). Six methyl protons also appeared at δ 1.07, δ 1.26, δ 0.91, δ 1.01, δ 1.00 and δ 0.97. The data compared to the structure of stigmasterol from literature (Habib *et al*., 2007; Jamal *et al*., 2009; Moghaddam *et al*., 2007). By comparing spectral with literature (Shukla *et al*., 2002), the structure of this compound was elucidated and identified as stigmasterol. ($R_{f=}$ 0.64, violet color in UV, visible as a dark blue spot when sprayed with cerric sulphate reagent), TMH 47 was also identified by co-TLC with an authentic sample of stigmasterol.

Compound DAF 1 was obtained as a white amorphous solid. The molecular formula of compound DAF 1 was found to be $C_{29}H_{48}O$ by LRMS m/z 412.4 ;370; 229; 192; 124(100%); 95; 54.The ^{1}H NMR spectrum shows the presence of six methyl groups at 1.18, 0.92, 0.85, 0.84, 0.82, 0.71 ppm and one olefinic proton at 5.72 ppm. The ^{13}C NMR spectrum of it shows 29 carbon signals, including two olefinic carbons at 123.7 and 171.7 ppm, one conjugated ketone at 199.7 ppm (Table 5.3). Interpretation of its 2D NMR

Table 5.3: ^{13}CNMR Spectral Data of DAF 1 (Stigmastenone)

Position	^{13}C-NMR(δ), $CDCl_3$, 500 MHz)	^{13}C-NMR (δ)*	Multiplicity
1	35.66	35.7	CH2
2	33.97	34.0	CH2
3	199.68	199.7	C
4	123.72	123.8	CH
5	171.73	171.7	CH
6	33.8	33.9	CH2
7	32.9	33.0	CH2
8	35.59	35.7	CH2
9	53.78	53.9	CH2
10	38.58	38.6	CH2
11	21.14	21.1	CH2
12	39.59	39.7	CH
13	42.36	42.4	CH
14	55.84	55.9	CH2
15	24.16	24.2	CH2
16	28.18	28.2	CH
17	55.9	56.1	CH
18	12.12	12.0	CH3
19	17.37	17.4	CH3
20	36.1	36.1	CH2
21	18.68	18.7	CH3
22	32.02	32.1	CH2
23	26.01	26.1	CH2
24	45.79	45.9	CH
25	29.1	29.2	CH
26	19,81	19.8	CH3
27	19.00	19.1	CH3
28	23.0	23.1	CH2
29	12.2	12.0	CH3

* Hoa *et al*., 2014

(HSQC and HMBC) suggests that compound DAF 1 is a sterol which has identical NMR spectral data with those of stigmast-4-en-3-one (Hoa *et al;* 2014).

Compound FD 84 (3,3,4′-tri-O-methylellagic acid) was obtained as a pale yellow amorphous solid from *T. welwitschii*. The EI-MS spectrum displayed a molecular ion peak at *m/z* 345 [M+H]+ consistent with a molecular formula of $C_{17}H_{12}O_8$, with 12 degrees of unsaturation The ^{1}H-NMR spectrum (C_5D_5N) showed the presence of two aromatic protons at δ 7.82 (s, 1H) and δ 7.56 (s, 1H), three methoxyl signals at δ 4.19 (s, 3H), δ 4.14 (s, 3H) and δ 3.85 (s, 3H). The 13C-NMR spectrum (C_5D_5N) showed two asymmetric benzene rings, three methoxyl signals at δ 61.34, δ 61.34 and δ 56.63 (Table 5.4). By comparing spectral with literature the structure of this compound was elucidated and identified as 3,3,4-tri-O-methylellagic acid (Adigun *et al*., 2000)

Table 5.4: ^{1}HNMR and ^{13}CNMR Spectral Data of FD84 (3,3,4-tri-O-Methyl ellagic acid)

Position	^{1}H-NMR (δ), ()	^{13}C-NMR (δ) $CDCl_3$, 500MHz	^{13}C-NMR (δ)*	^{13}C-NMR (δ)*
1		111.21		112.42
2		140.96		141.60
3		140.20		141.38
4		152.63		152.71
5	7.56 (1H s H-5)	111.66	7.75(1Hs,H-5)	113.44
6		112.53		112.43
7		158.33		158.48
1′		111.96		112.68
2′		141.49		142.05
3′		140.84		140.10
4′		153.81		147.34
5′	7.83(1H s H-5′)	107.47	7.30(1H s H-5′)	101.75
6′		113.38		114.02
7′		158.82		161.05
OCH3	4.20	56.73	4.10	61.68
OCH3	4.10	61.31	4.02	62.20
OCH3	3.86	61.02	3.82	62.14

*Adigun *et al.,* 2000

Compound DAF 2 was a yellow crystalline solid. Molecular weight data analysis result by ES-MS (m/z 193.1, $[M+H]^+$), which was consistent with $C_{10}H_8O_4$ and combined with 1DNMR (1H- and 13C-NMR spectral data used to characterized the chemical structure of DAF 2 (Table 5.5). The 1H-NMR spectrum showed four aromatic protons (δH 6.25, 6.82, 6.89, and 7.58 ppm), and one methoxy group (δH 3.93 ppm), combined with ten carbons from 13C-NMR. Detailed analysis of the 1D and 2D NMR and 13-CNMR spectra and comparison with literature allowed the establishment of the structure of compound DAF 1 as scopoletin.

From the results of larvicidal activity of the isolated compounds against the larvae of *An. gambiae* mosquito (Table 4.13), the most toxic compound was **TMH 1** (lupeol) which had IC_{50} value of 6.2 µg/mL. **TMH 70B** (Ursolic acid) and **FD 84** (3,3,4-tri-O- methyl ellagic acid) were 15.6 µg/mL and 48.1 µg/mL, respectively. Evaluation of larvicidal activity of Ursolic acid and its derivatives against larvae of *Ae. aegypti* showed varying degree of toxicity (da Silva *et al*., (2016).

The results of the 4-day suppressive test of the isolated compounds against ANKA strain *P. berghei* showed that the compounds possess varying degree of chemosuppressive ability. **TMH 70B** was the most active compound, showing highest chemosuppression of parasite growth. At 100 mg/kg, it had a chemosuppression of 93.5%. This was followed by **TMH 1** (88.3%). **DAF 2** was the least active with chemosuppression of 58.5%.

Table 5.5: ^{13}CNMR Spectral Data of DAF 2 (Scopoletin)

Position	^{13}C-NMR (δ), $CDCl_3$, 500 MHz)	^{13}C-NMR (δ)*	Multiplicity
1	161.5	160.8	C
2	113.4	113.3	CH
3	144.0	144.7	CH
4	111.2	112.1	C
5	107.5	109.9	CH
6	143.3	146.0	C
7	150.2	151.9	C
8	103.2	102.7	CH
9	149.7	149.6	C
10	56.4	56.1	OCH_3

*Zhang *et al*., 2011

Lupeol has been reported to inhibit chloroquine sensitive 3D7 strain of *P. falciparum* (Ziegler *et al*., 2002). Lupeol isolated from *Vernonia brasiliana* was shown to display antiplasmodial activity (Alves *et al.,* 1997). Lupeol from stem bark of *Cassia siamea* was reported to be responsible for its antimalarial activity (Ajaiyeoba *et al*., 2005, 2008b). Lupeol isolated from the hexane fraction of *T. megalantha* stem bark was active in the four day suppressive test. Ursolic acid is widely found in the peels of fruits, as well as in herbs and spices like rosemary and thyme. Ursolic acid isolated from *T. megalantha* stem bark was active in the *in vivo* antimalarial study. It had 93.5% chemosuppression. Ursolic acid isolated from extracts of *Morinda lucida*. Benth. leaf exhibited an *in vitro* antiplasmodial activity against a chloroquine-sensitive *P. falciparum* strain had IC_{50} values 3.1 ± 1.3 µg/mL. *In vivo* study, at a daily dose of 200 mg/kg body weight produced 97.7% chemosuppression (Cimanga *et al*., 2006).

Methanol extract of the leaf of *T. welwitschii* afforded **FD 84 (**3,3′,4-tri-O-methyl-Ellagic acid). This compound was first identified by Adigun *et al*, 2000. Ellagic acid is a polyphenol found in numerous fruits and vegetables, and this molecule seems to be a primary component of several tannin-bearing antimalarial plants found in the African flora (Vattem *et al*., 2005). Ellagic acid from *Psidium friedrichsthalianum* possesses antioxidant and anti-inflammatory activities (Flores *et al*., 2013). Several reports have shown the antimalarial efficacy of ellagic acid (Verotta *et al*., 2001; Banzouzi *et al*; 2002; Reddy *et al*., 2007; Soh *et al*., 2009). Ellagic acid from *Alchonea cordifolia* demonstrated antiplasmodial activity against McF29 with IC_{50} of 0.029 ug/mL (Banzouzi *et al*., 2002).

Antimalarial activity of 3,3′,4-tri-O-methyl-ellagic acid from *T. welwitschii* (Table 4.13) showed significant chemosuppression of parasite growth against *P. berghei* ANKA strain more effectively than ellagic acid. The methoxy group may probably increase the activity. This is the first report of antimalarial activity of the compound.

Scopoletin, a coumarin was isolated from the DCM fraction of *T. megalantha* as a yellow crystalline solid in the present study. It had been isolated earlier from other *Trichilia* species e.g *T. Cassareti, T. elegans, T. estipulate and T. lepidota.* Scopoletin isolated from *Macaranga gigantifolia* exhibited anticancer properties. It has strong cytotoxic activity against P-388 murine leukemia cells with IC_{50} value 17.42 µg/mL (Darmawan *et al*., 2012).

Several coumarins have been found to show antiplasmodial activities *in vitro* and *in vivo* in animal models. Anti-malarial coumarins have been identified by Cubukcu *et al*., 1990 and Noster *et al*. 1990 from *Artemisia arbrotanum* (Asteraceae) and *Toddalia asiatica* (Rutaceae), respectively. The results of the antiplasmodial assays against the chloroquine-susceptible 3D7 and chloroquine- resistant Dd2 strains of *P. falciparum*, showed that the compound from *Vernonia brachycalyx* was weakly active, with IC_{50} values of 160 µM and 54 µM, while for compound from *Toddalia asiatica*, the IC_{50} values were 111 µM and 54 µM, respectively. In addition, Oketch-Rabah *et al*. isolated a new anti-malarial coumarin, 5,7-dimethoxy-8-(30-hydroxy-30-methyl- 10-butene) coumarin from the roots of *Toddalia asiatica*. This compound showed moderate activity against the chloroquine-sensitive K39 and chloroquine-resistant V1/Strains of *P. falciparum* strains, with IC_{50} values of 16.2 µg mL−1 and 8.8 µg mL−1, respectively (Oketch-Rabah *et al*., 1997). The

anti-malarial coumarin 7-hydroxy 6-methoxycoumarin or scopoletin isolated from the dichloromethane leaf extract of *Schefflera umbellifera* (Araliaceae), demonstrated moderate activity *in vitro* against both the chloroquine-susceptible (D10) and chloroquine resistant (K-1) strains of *P. falciparum* (Mthembu *et al.,* 1989)

In vivo antimalarial study of scopoletin isolated from *T. megalantha* showed a moderate activity against chloroquine resistant ANKA strain *P. berghei* infected mice with percentage chemosuppression of parasite growth of 58.5% on D4 at 100 mg/kg. This is a confirmation of earlier report on the *in vitro* antimalarial activity of scopoletin.

CHAPTER SIX

CONCLUSIONS

Nature remains an ever evolving source for compounds of medicinal importance. The use of medicinal plants for the treatment of various ailments and as insecticides is well known and documentaion spanning ages. A glowing example includes the use of *Cinchona sp* for the treatment of malaria infection has been known for centuries. Several compounds isolated from nature also form a rich source of diverse structures for lead optimization to obtain improved therapeutics.

Medicinal plants consist of many components and their biological activities are not usually attributable to a single moiety. The demonstrated high larvicidal activities of extracts and fractions and oils of *T. megalantha* and *T. welwitschii* indicate that could be studied further as sources of environmentally friendly vector control agents for malaria.

The study showed that

- Meliaceae plant family provides potentially valuable sources of antimalarial and larvicidal agents that can be further developed
- The extracts of *T. megalantha* and *T. welwitschii* possess promising antimalarial activity with minimal or low cytotoxicity.
- Plant-based compounds may be effective alternatives to conventional synthetic insecticides for the control of *An. gambaie.*They are specific target insects, less expensive, easily biodegradable to non-toxic products, and potentially suitable for use in mosquito control programme.

- There are no adverse effects of methanol extract of the plants in mice indicating that the medium lethal dose (LD_{50}) is higher than 5000 mg/kg in mice.
- No abnormalities were detected in histopathology of organs of heart, liver, spleen, kidney and lung of animals treated with lower doses of *T. megalantha* and *T. welwitschii* compared to the control group.

Many members of the Meliaceae plant family have been evaluated for antimalarial and larvicidal activities. There is no previous report on antimalarial, larvicidal and toxicity evaluation of *T. megalantha* and *T. welwitschii* plants and parts. Six compounds were isolated and characterized from *T. megalantha* for the first time. The result of this study *T. megalantha* and *T. welwitschii* plant and parts have demonstrated significant antimalarial activity.

In conclusion this study provides evidence of non-toxic effect of extracts of *Trichilia megalantha* and *Trichilia welwitschii* in mice and as such gives credence to the safety of both plants at lower doses as herbal remedies in management of malaria and larvicides against *Anopheles gambiae*. However, detailed toxicological studies of the lead compounds is required.

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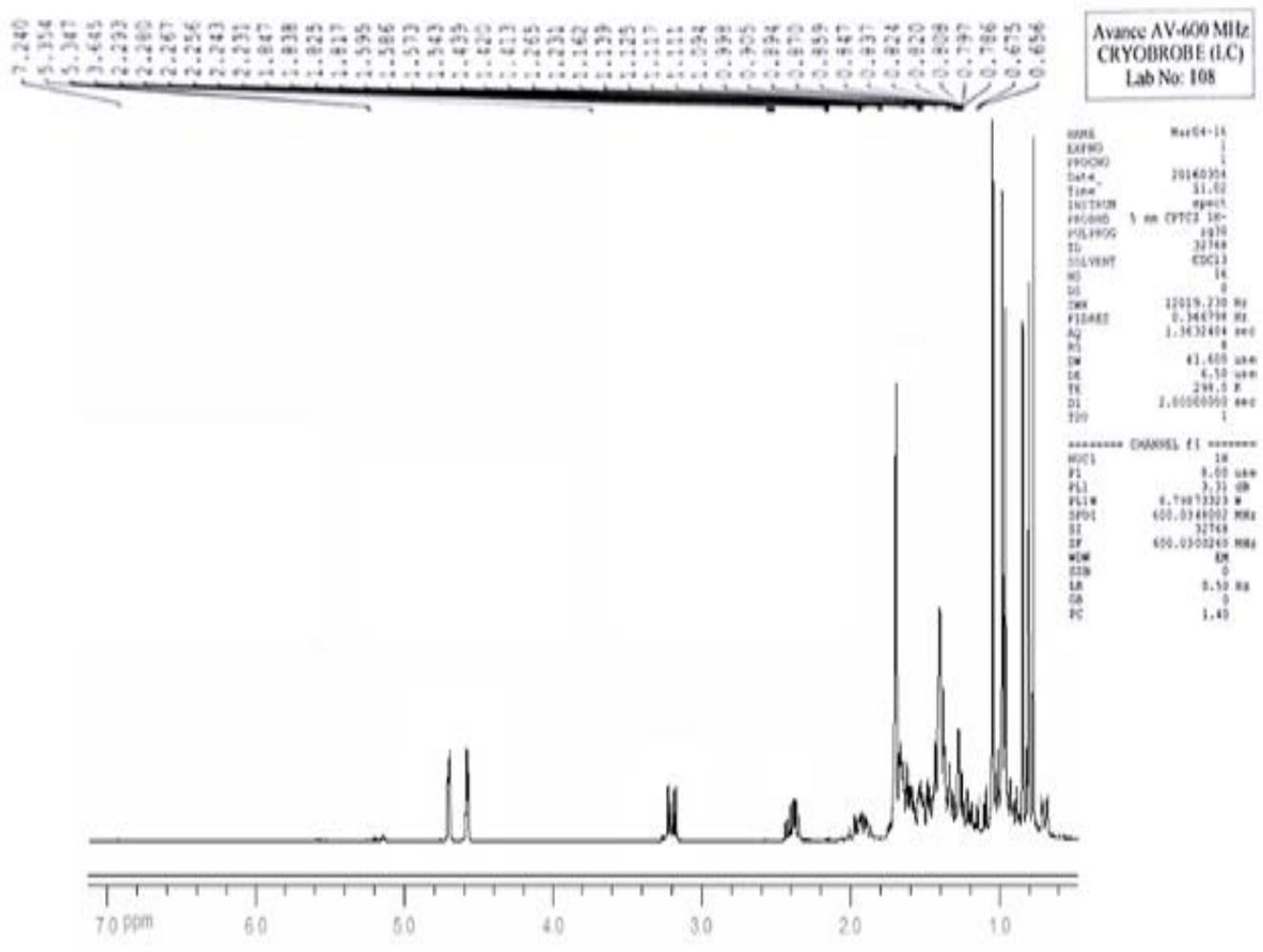
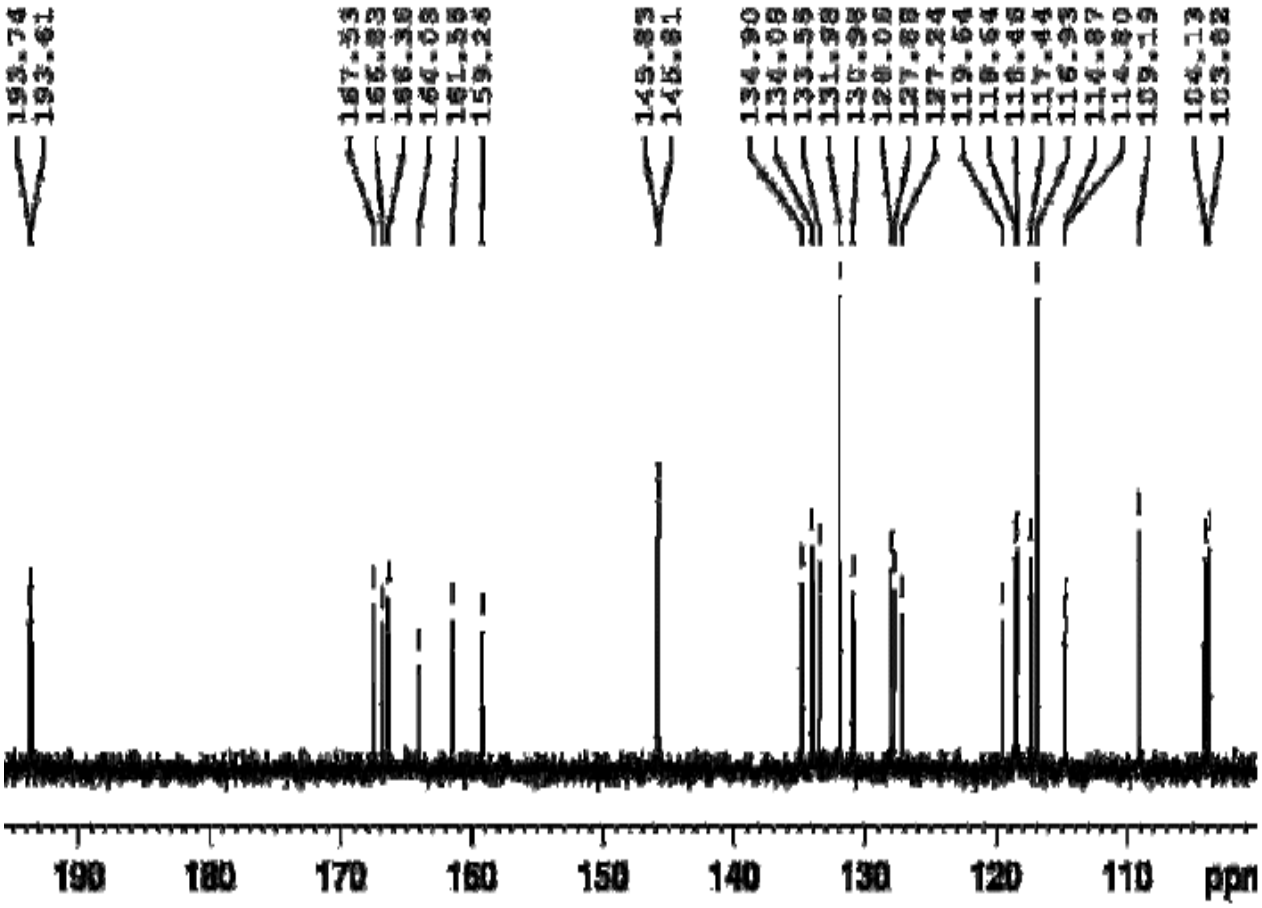
APPENDIX 1A: ^{1}HNMR Spectrum of TMH 1 (Lupeol)



APPENDIX IB: ^{13}CNMR Spectrum of TMH 1 (Lupeol)

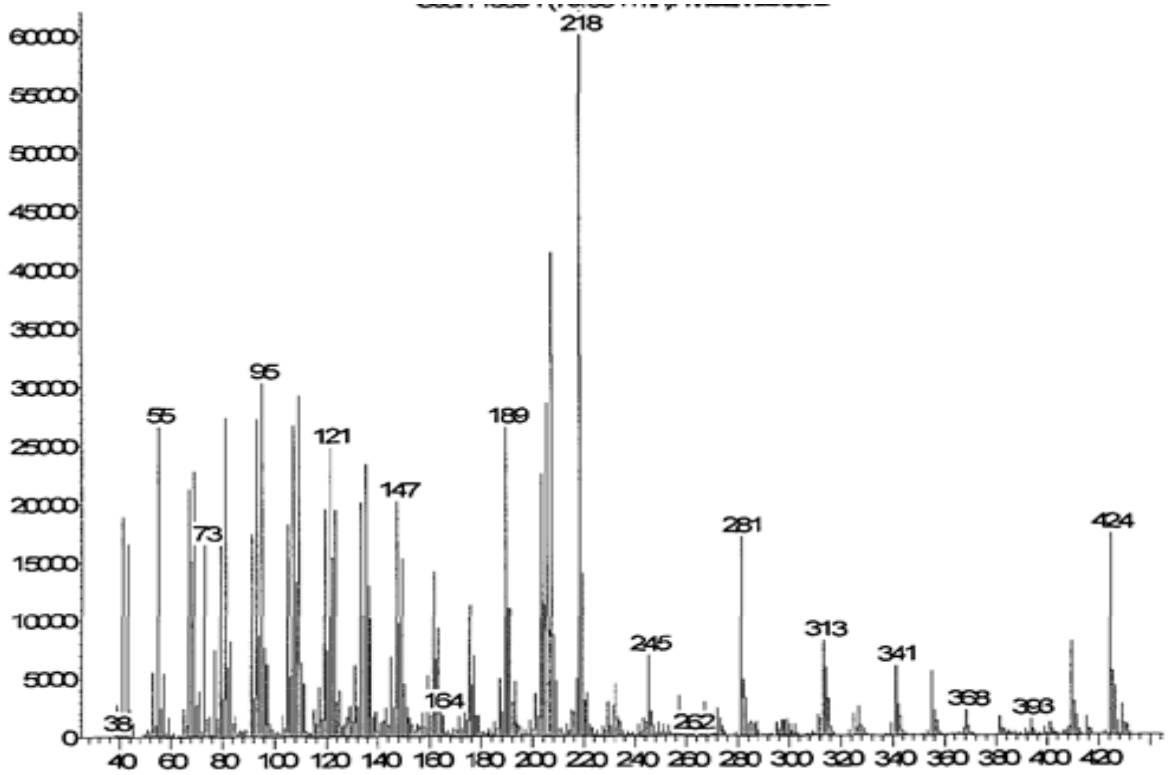


APPENDIX 1C: EI-MS of TMH 1 (Lupeol)

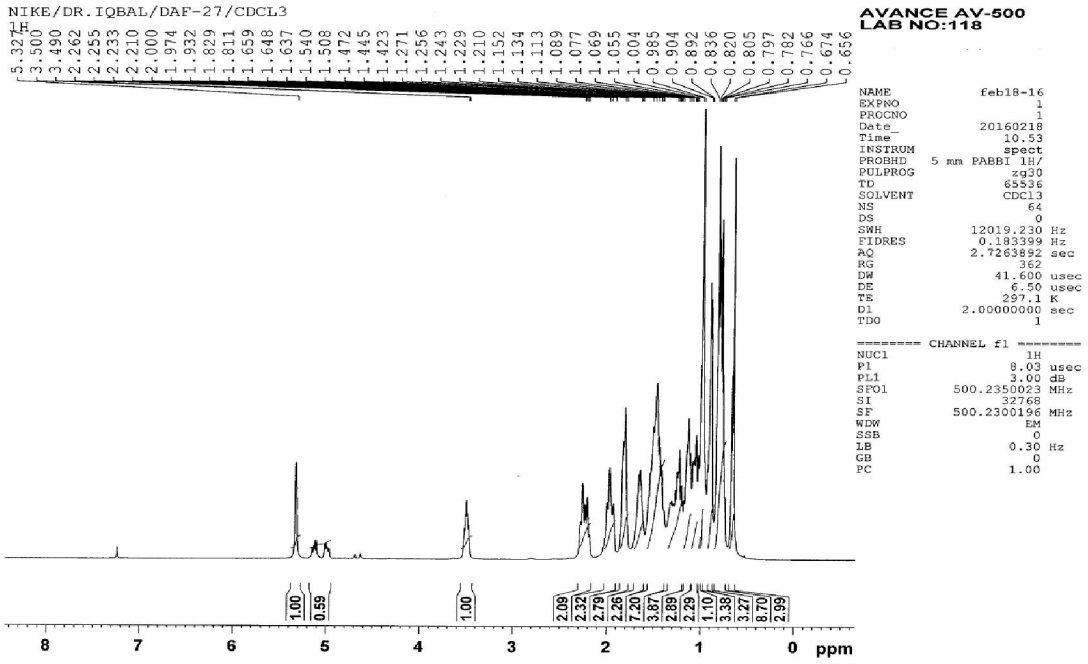
File: TMH 1
Sample: Nike/DR.IQBAL
Instrument: JEOL MS 600H 1

Run Date 02-13-2016 (Time Run 14:53: 06)

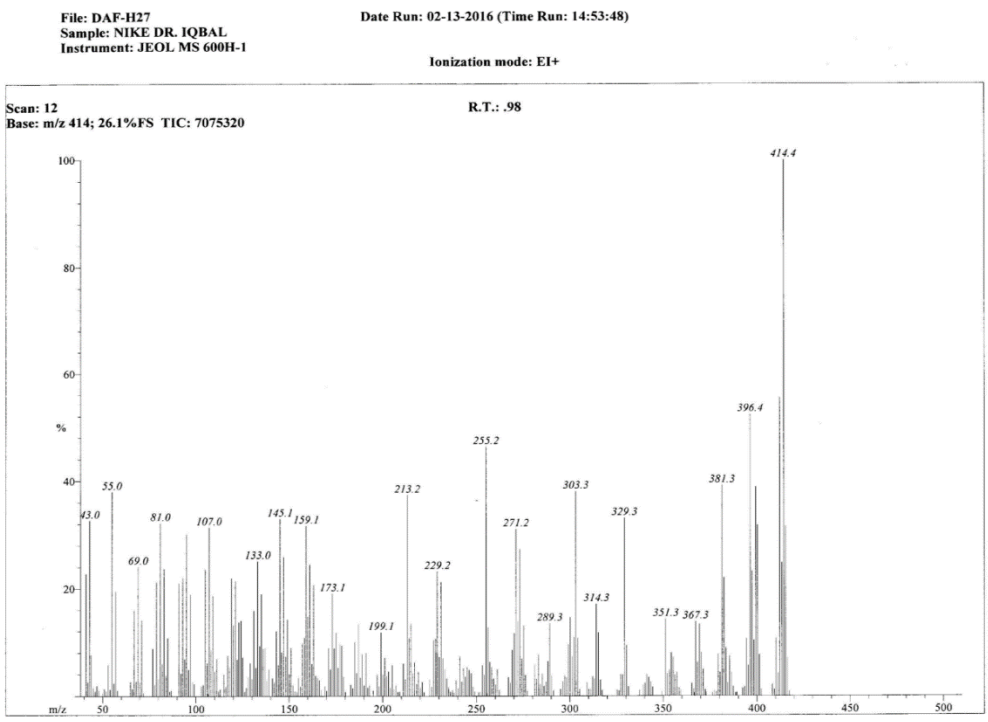
Ionization mode: EI^+



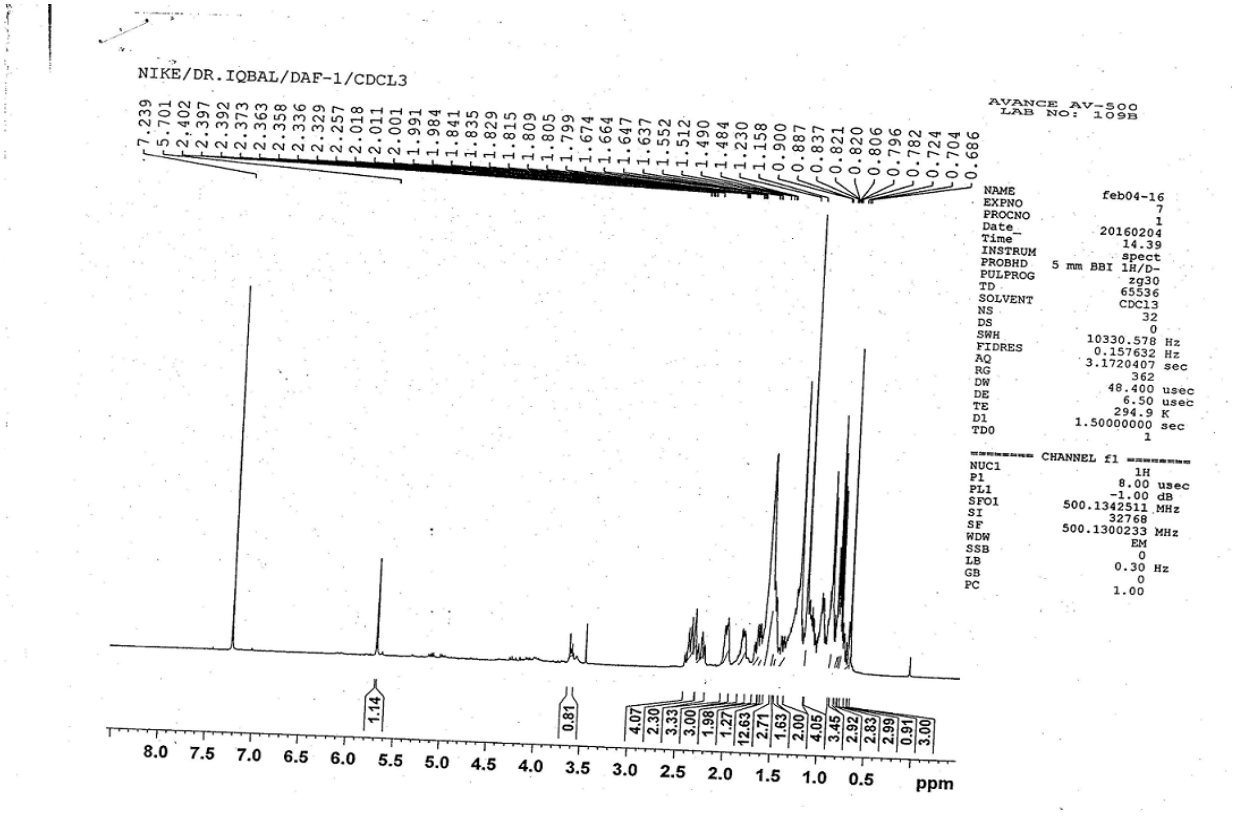
APPENDIX 2A: ^{1}HNMR Spectrum of DAF H27 (β-sitosterol)



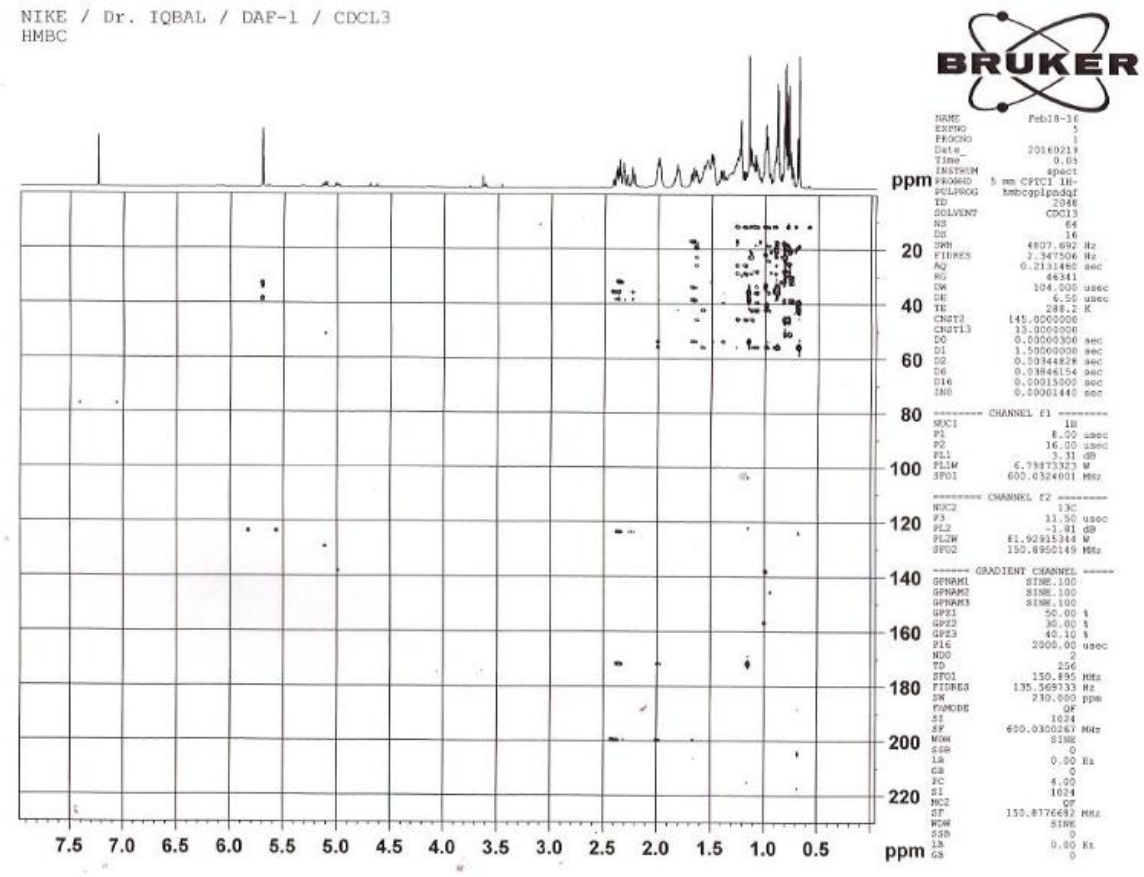
APPENDIX 2B: EI-MS of DAF H27 (β-sitosterol)



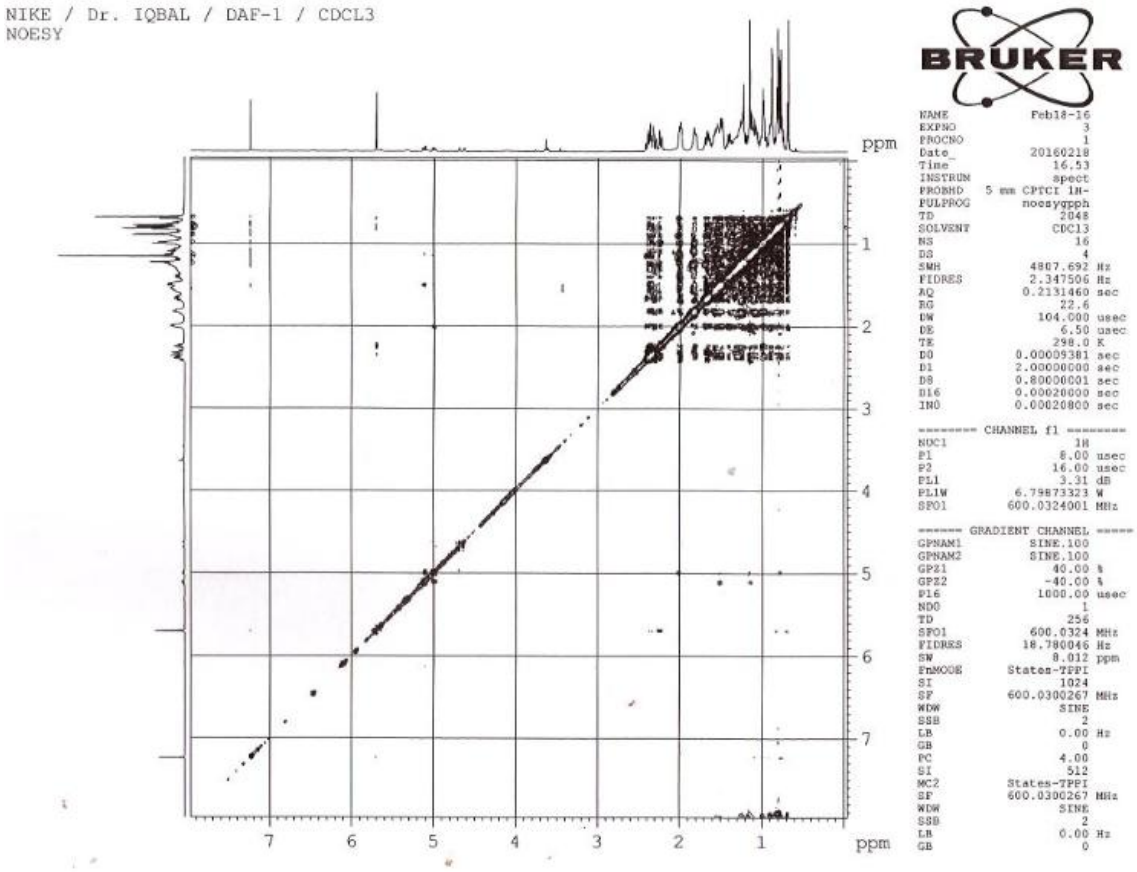
APPENDIX 3A: ^{1}HNMR Spectrum of DAF 1 (Stigmastenone



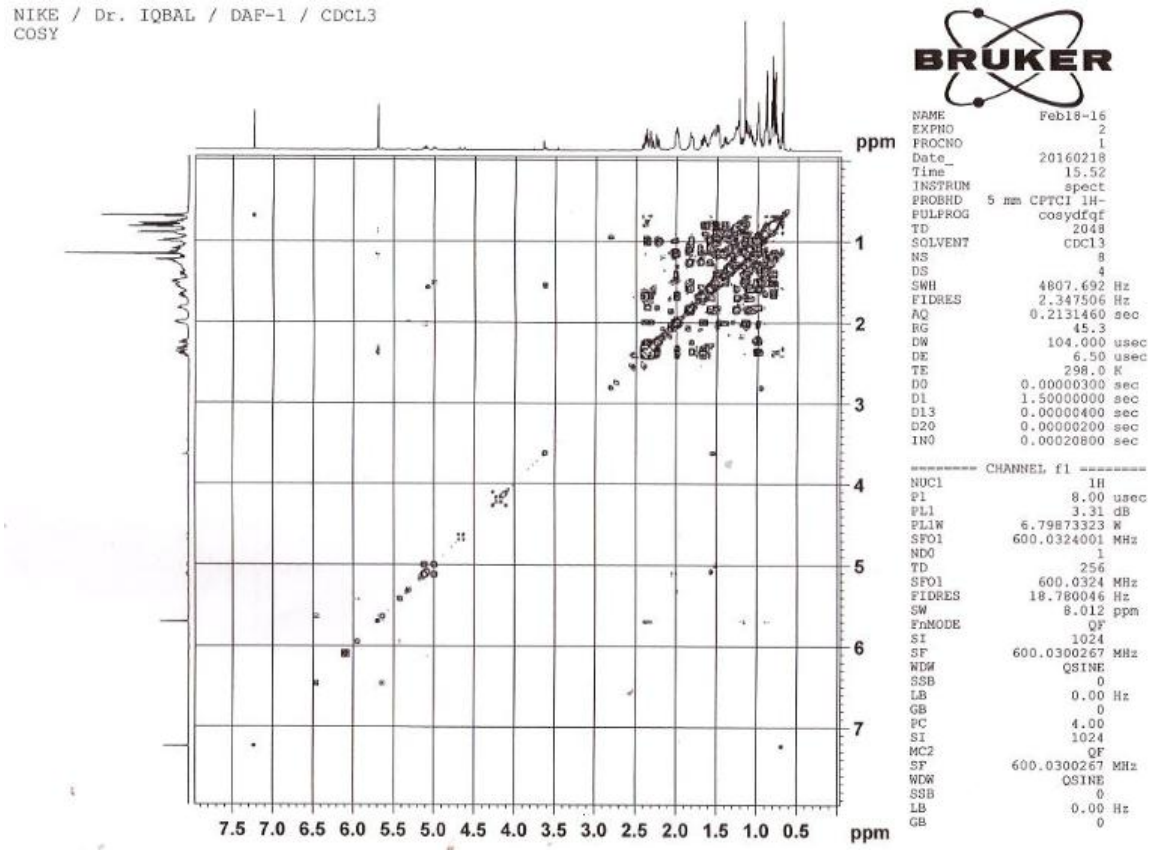
APPENDIX 3B:2D NMR Spectrum (HMBC) of DAF 1 (Stigmastenone)



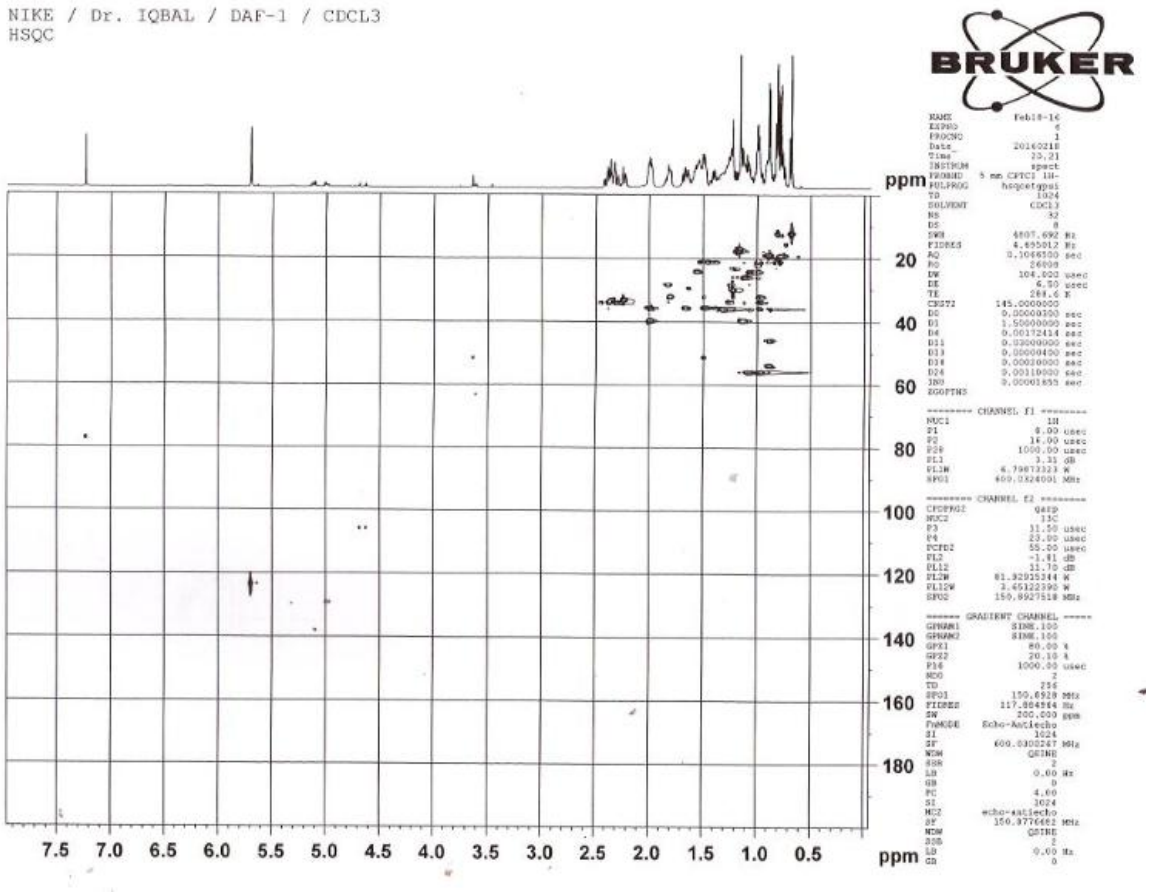
APPENDIX 3C: 2D NMR spectrum (NOESY) of DAF 1 (Stigmastenone)



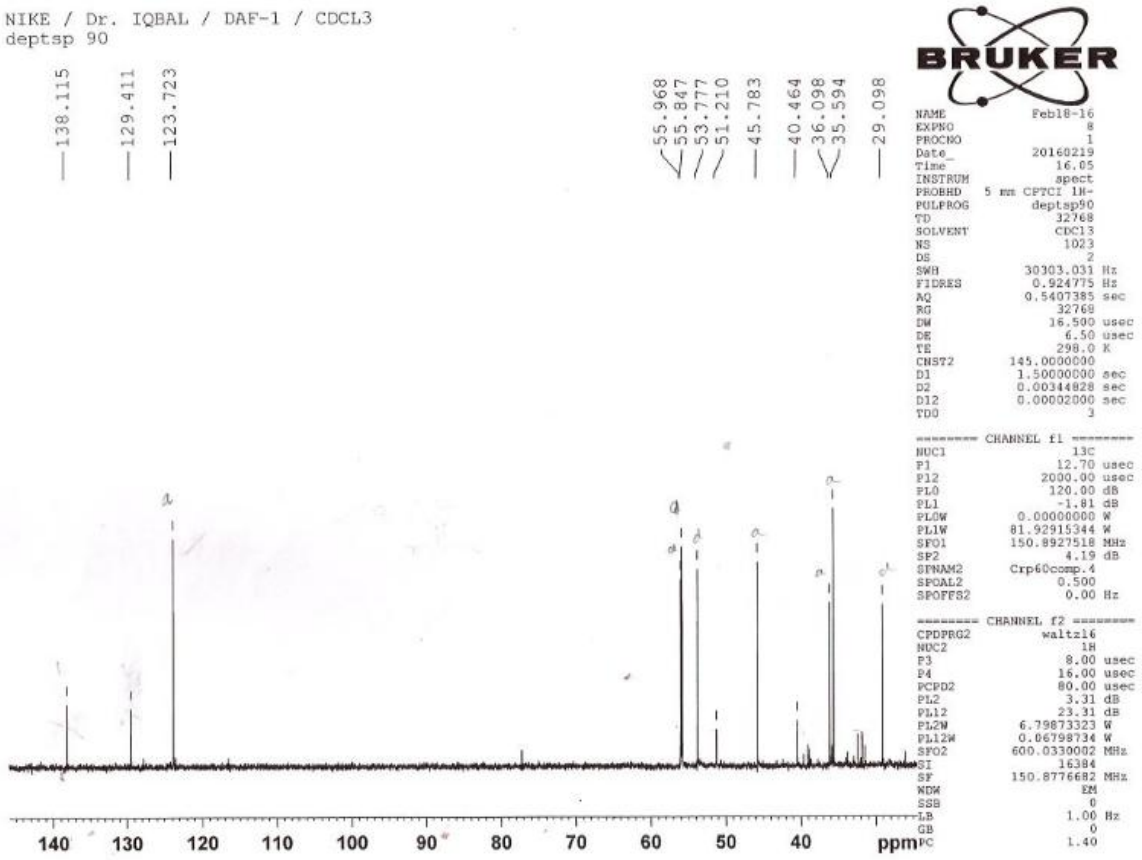
APPENDIX 3D: : 2D NMR spectrum (COSY) of DAF 1 (Stigmastenone)



APPENDIX 3E: 2D NMR (HSQC) spectrum of DAF 1 (Stigmastenone)



APPENDIX 3F:13C Dept 90 of DAF 1 (Stigmastenone)



APPENDIX 3G: EI-MS of DAF 1 (Stigmastenone)

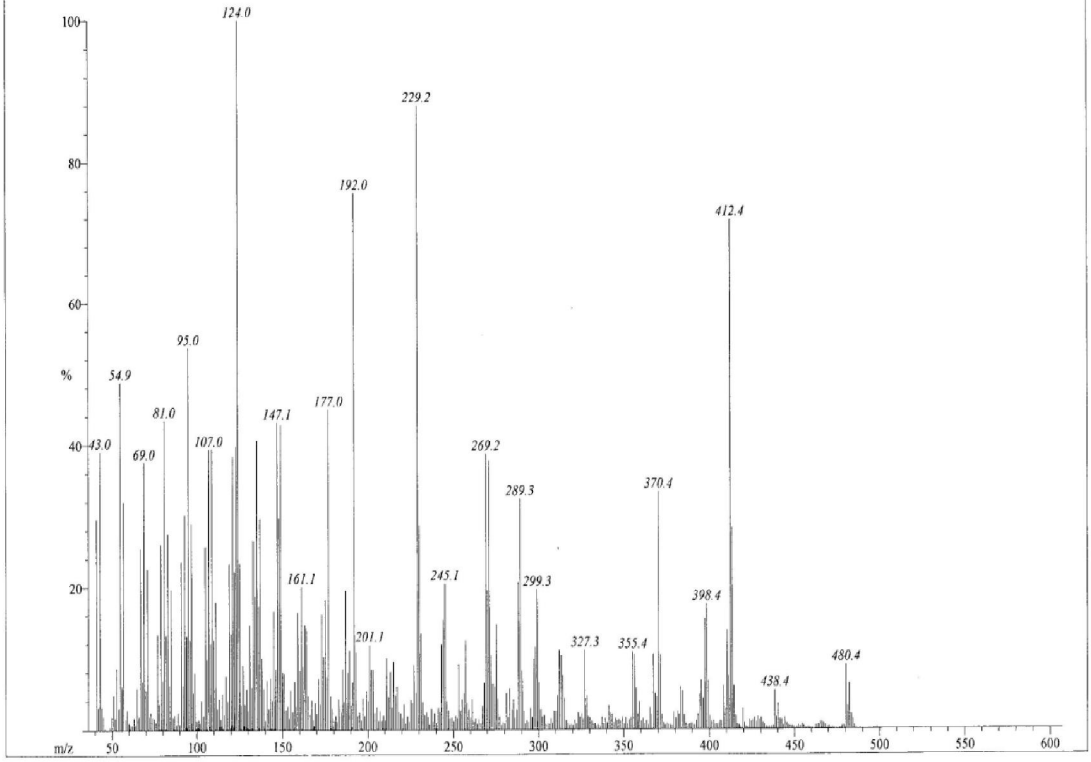
File: DAF-1
Sample: NIKE/ DR. IQBAL
Instrument: JEOL MS 600H-1

Date Run: 02-13-2016 (Time Run: 15:18:13)

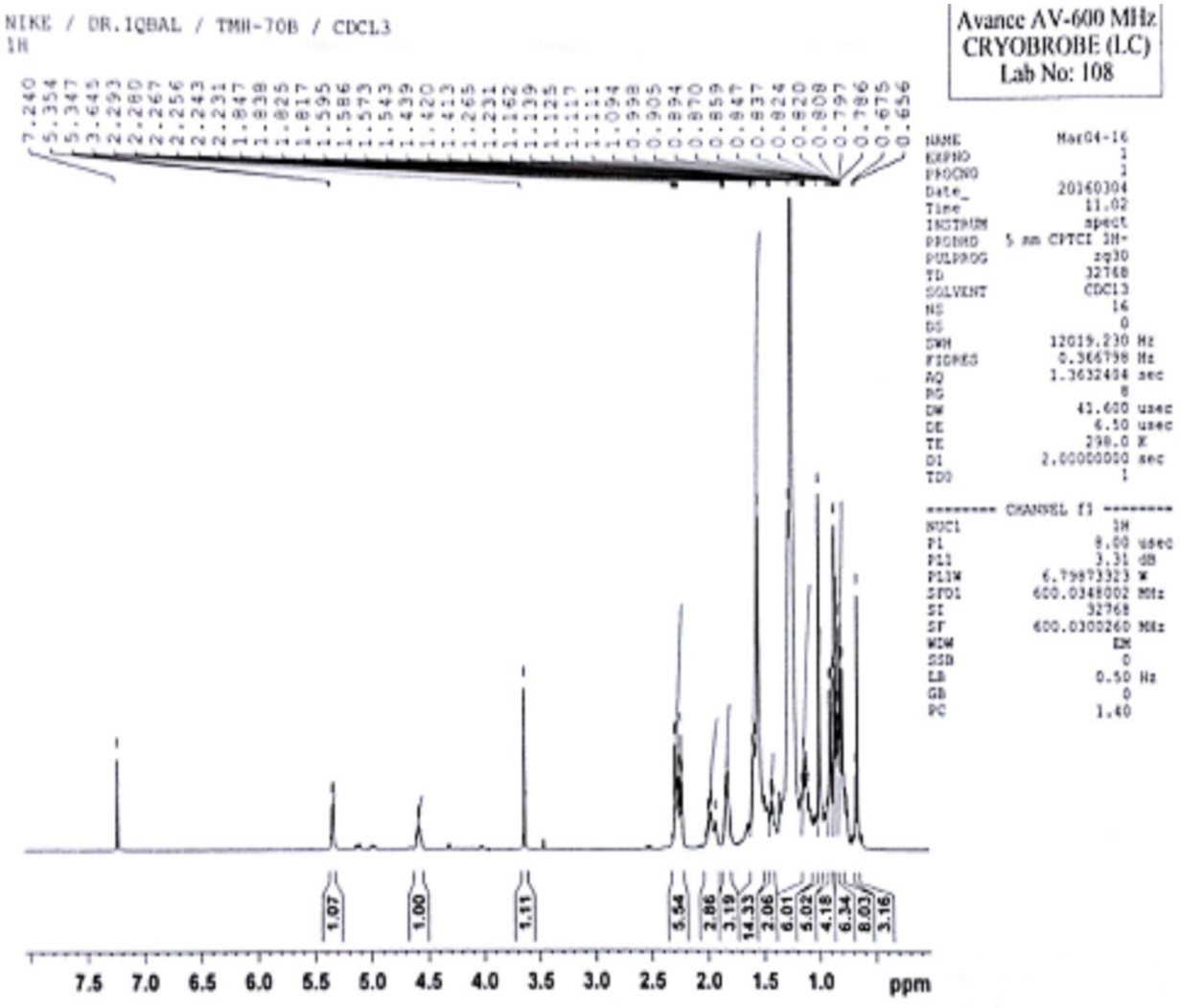
Ionization mode: EI+

Scan: 12
Base: m/z 124; 99.5%FS TIC: 33058662

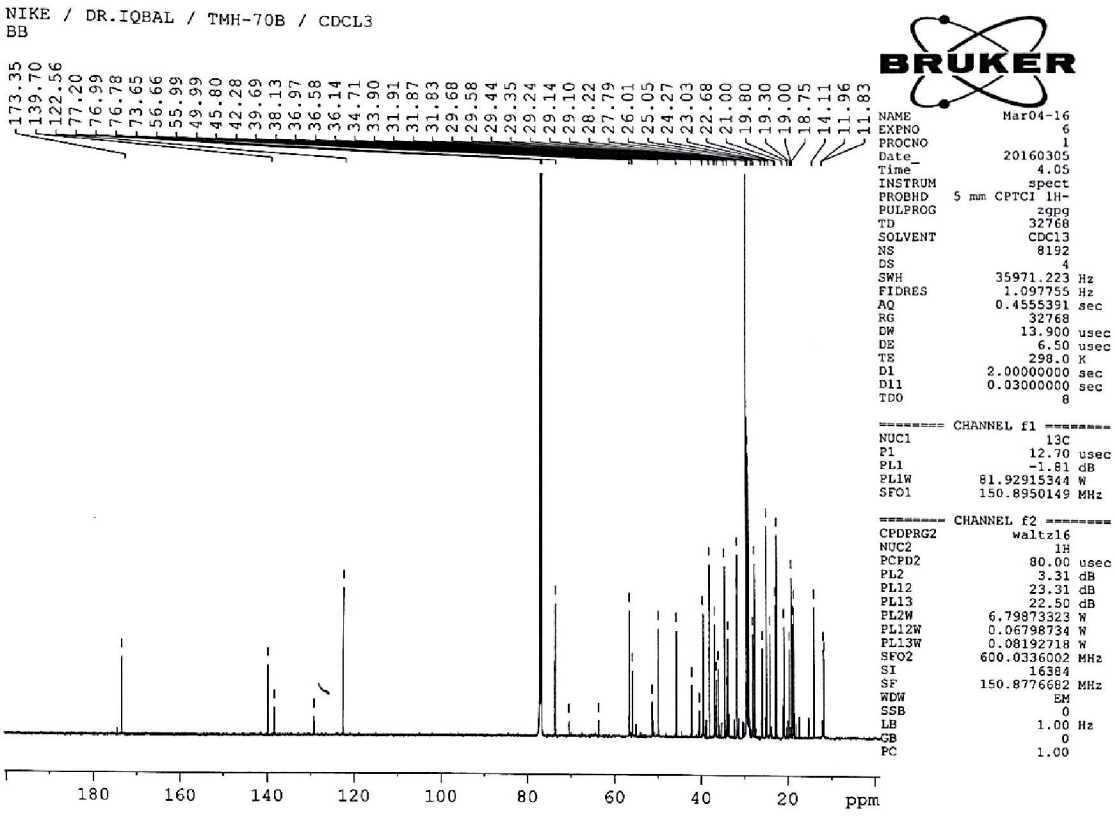
R.T.: .98



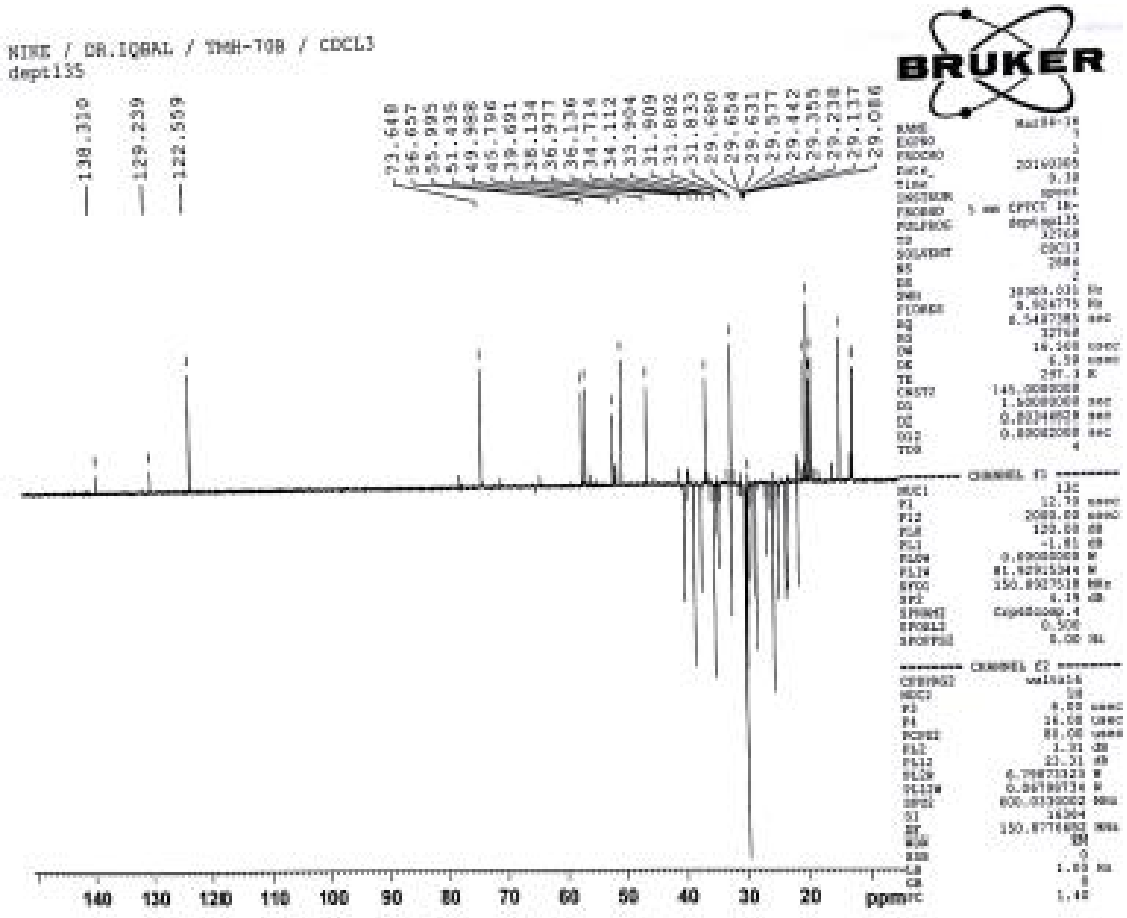
APPENDIX 4A: ^{1}HNMR spectrum of TMH 70B (Ursolic acid)



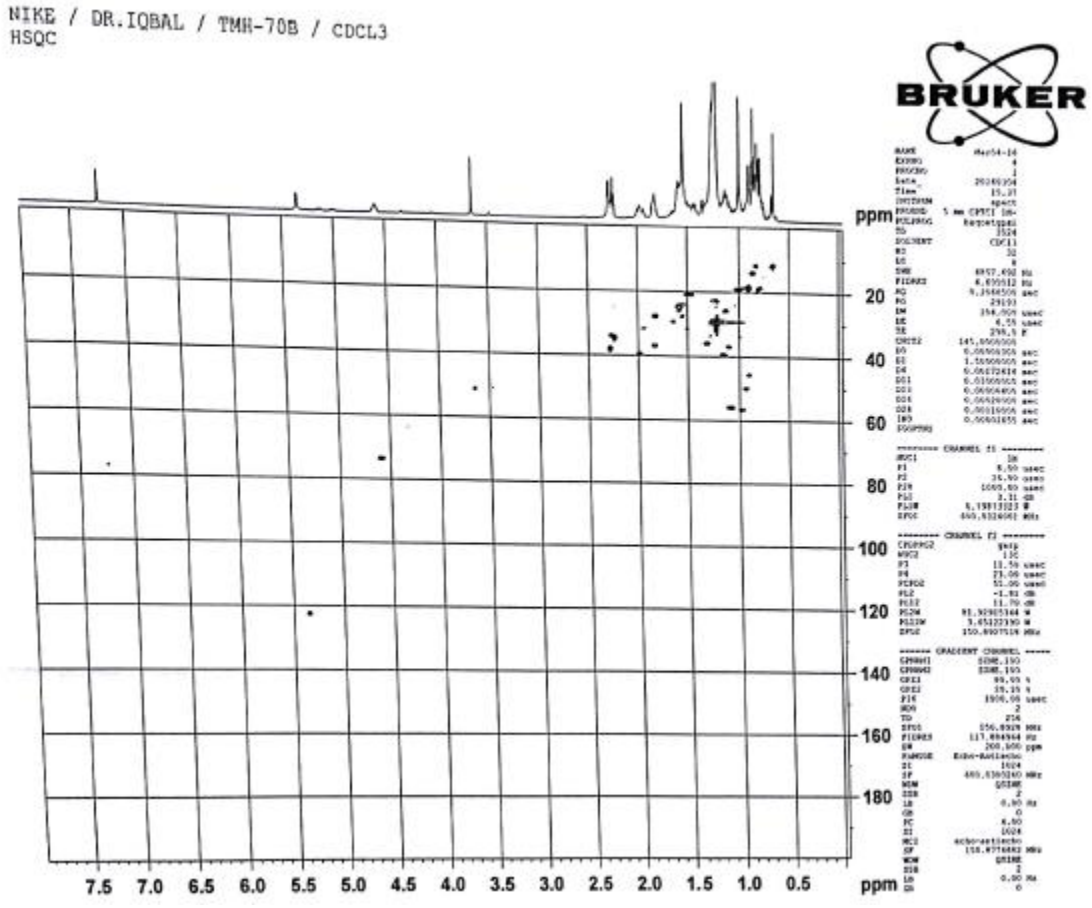
APPENDIX 4B: ^{13}CNMR Spectrum of TMH 70B (Ursolic acid)



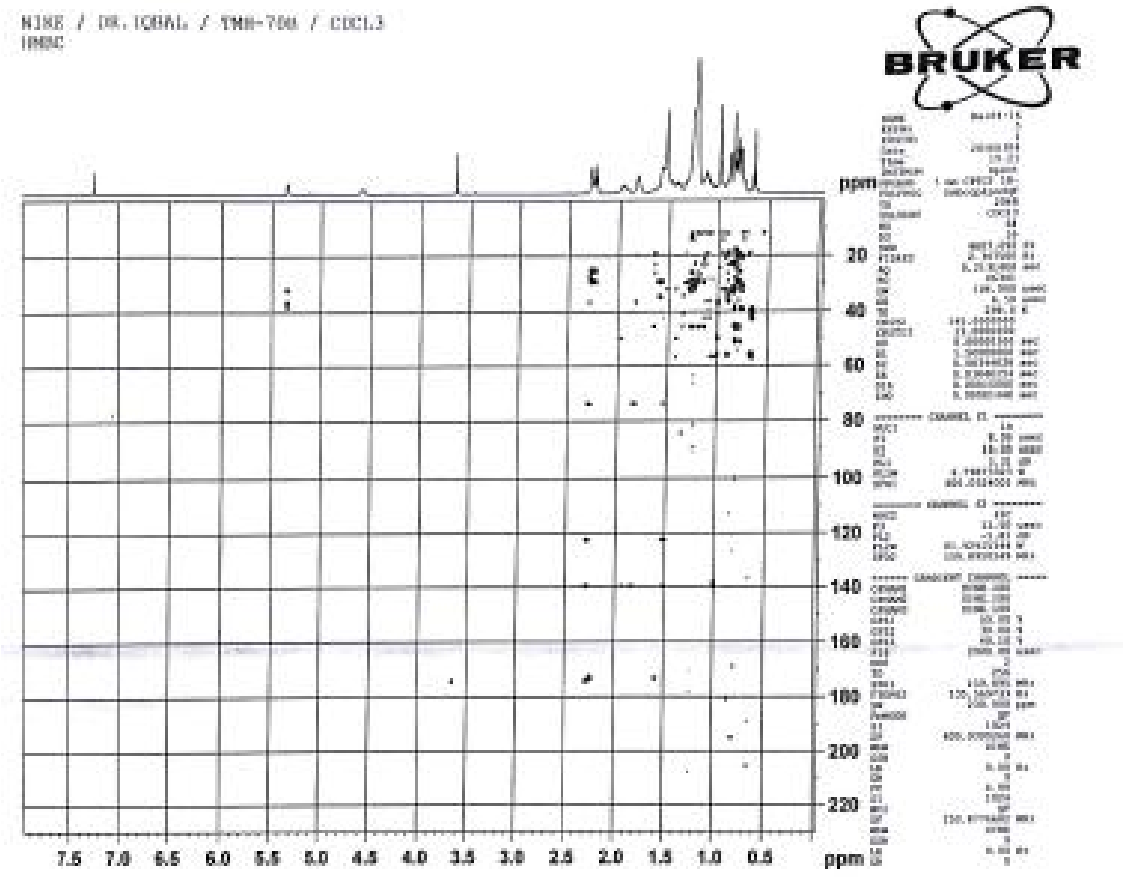
APPENDIX 4C: 13CNMR DEPT 135 Spectrum of TMH 70B (Ursolic acid)



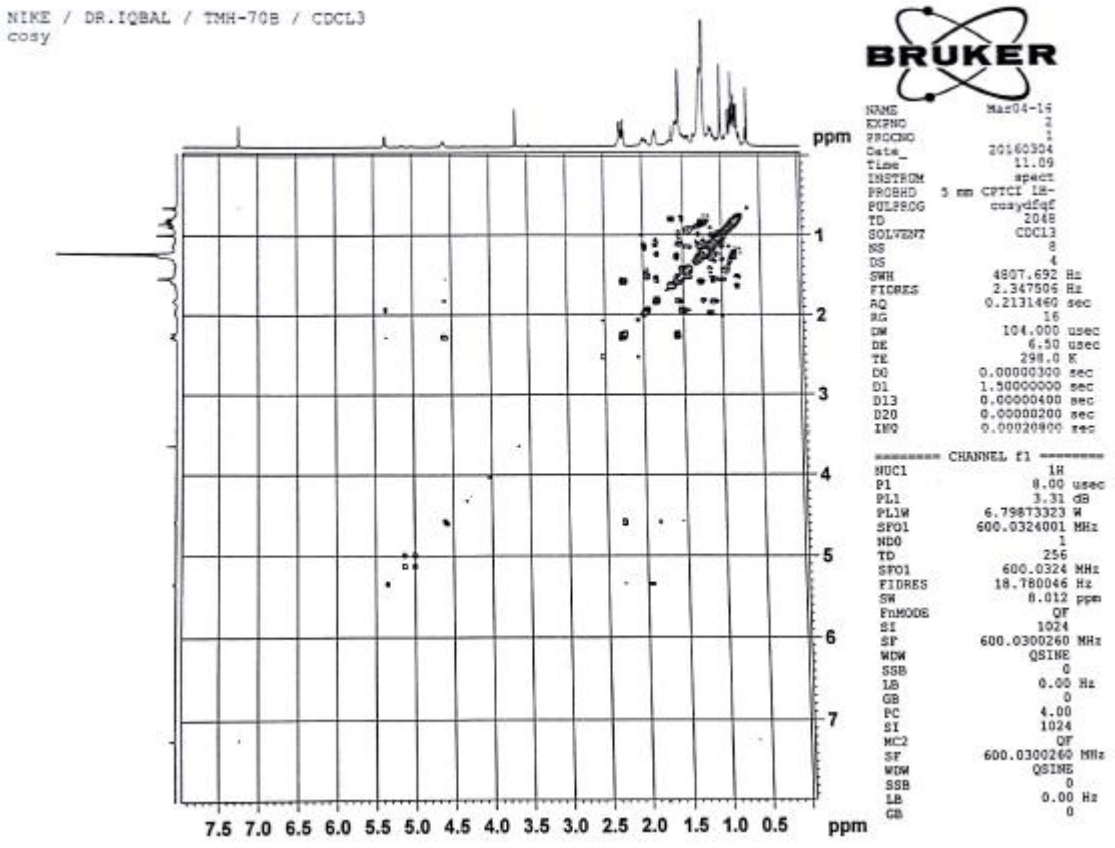
APPENDIX 4D: 2D NMR (HSQC) Spectrum of TH 70B (Ursolic acid)



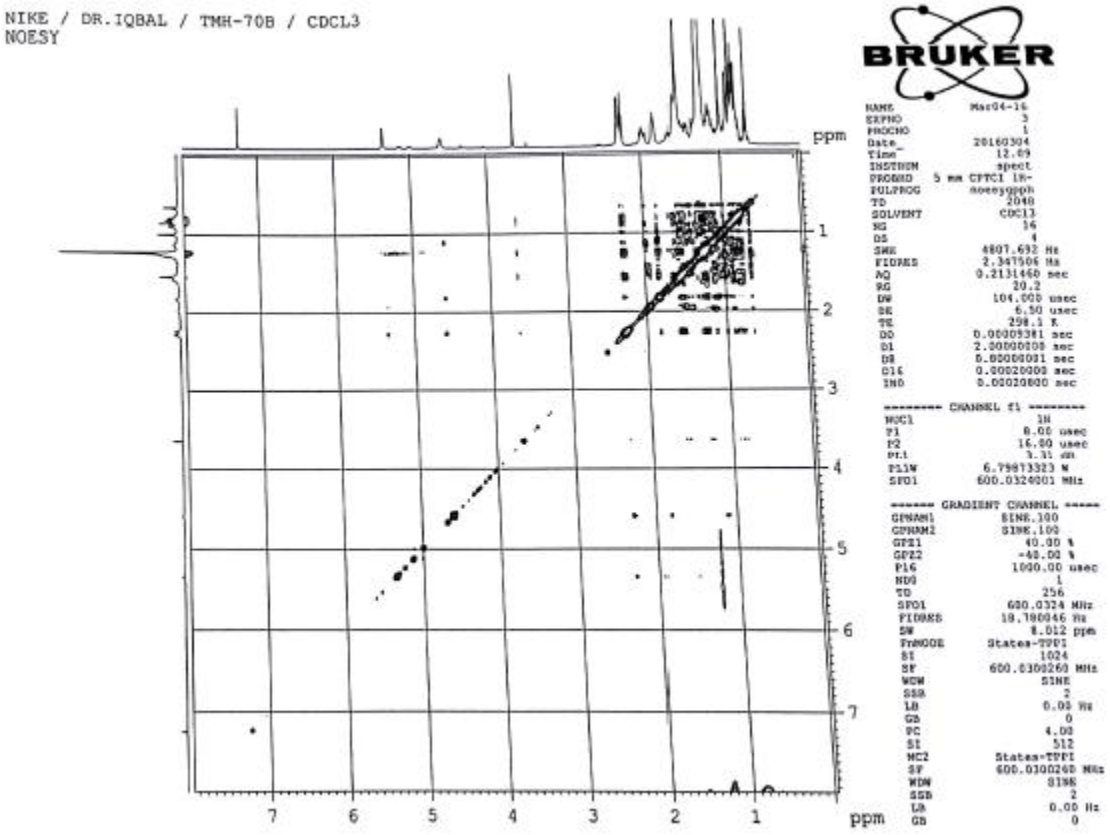
APPENDIX 4E: 2D NMR (HMBC) Spectrum of TMH 70B (Ursolic acid)



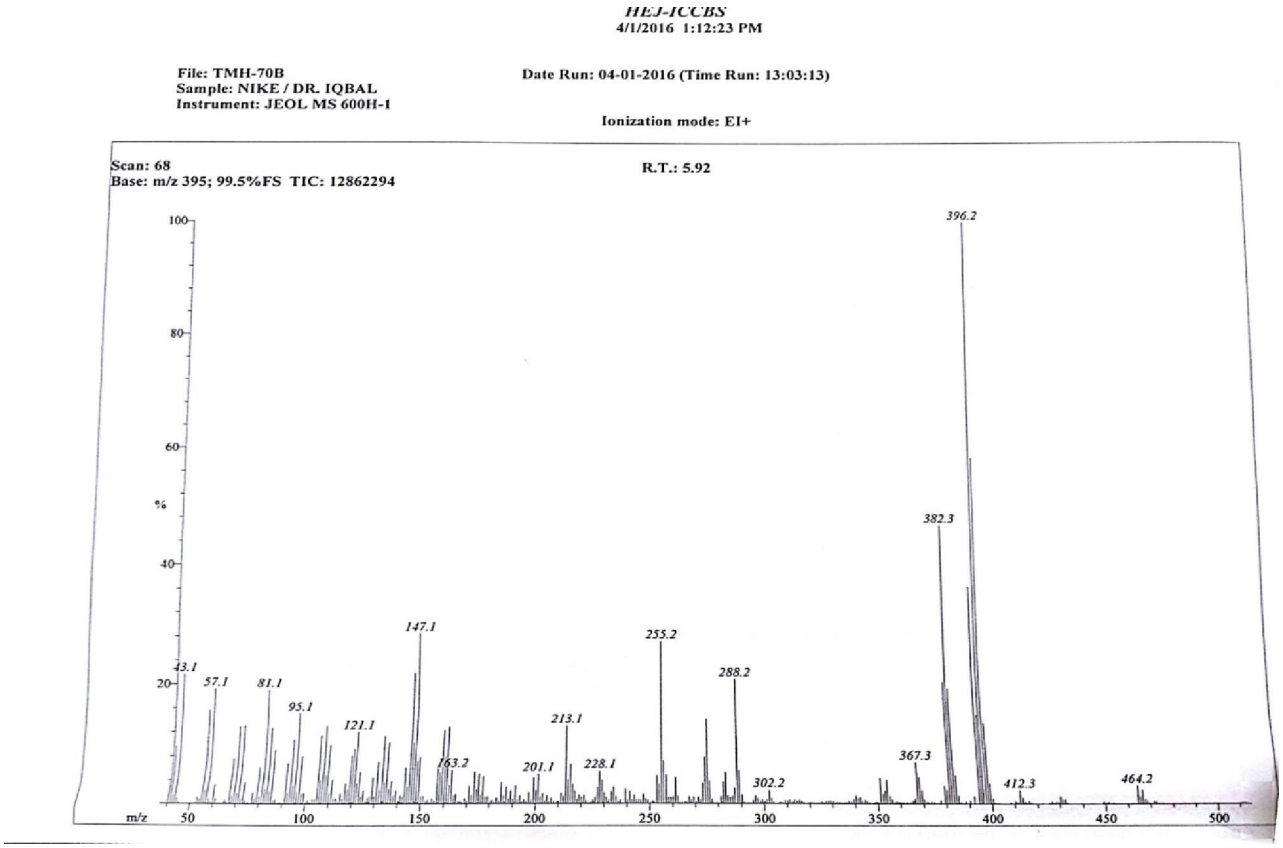
APPENDIX 4F: 2D NMR (COSY) Spectrum of TH 70B (Ursolic acid)



APPENDIX 4G: 2D NMR (NOESY) Spectrum of Ursolic acid

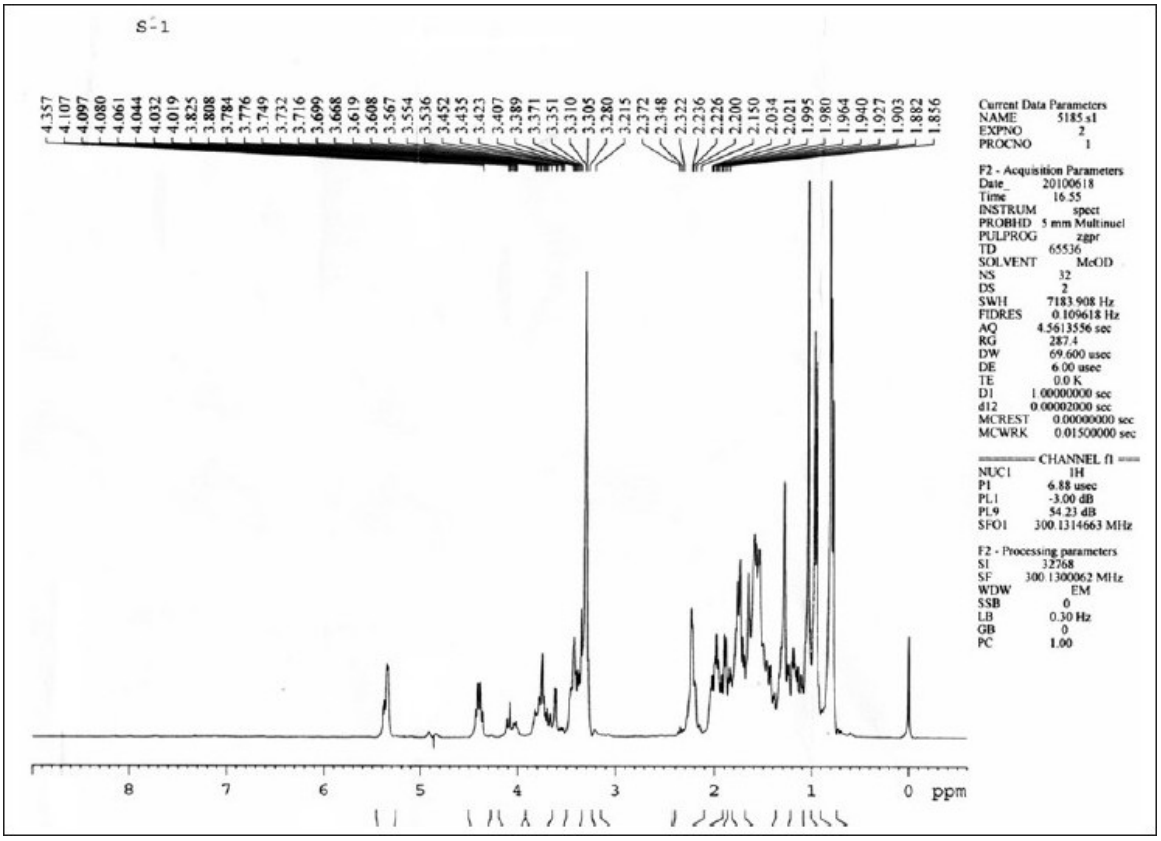


APPENDIX 4H: EI-MS of Ursolic acid

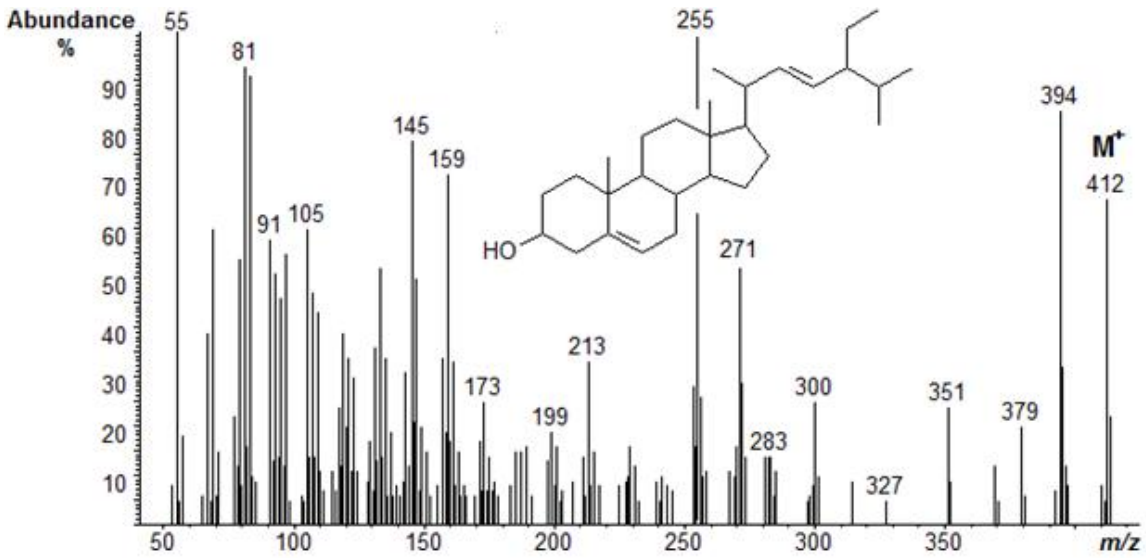


APPENDIX 5A: ^{1}HNMR Spectrum of TMH 47 (Stigmasterol)

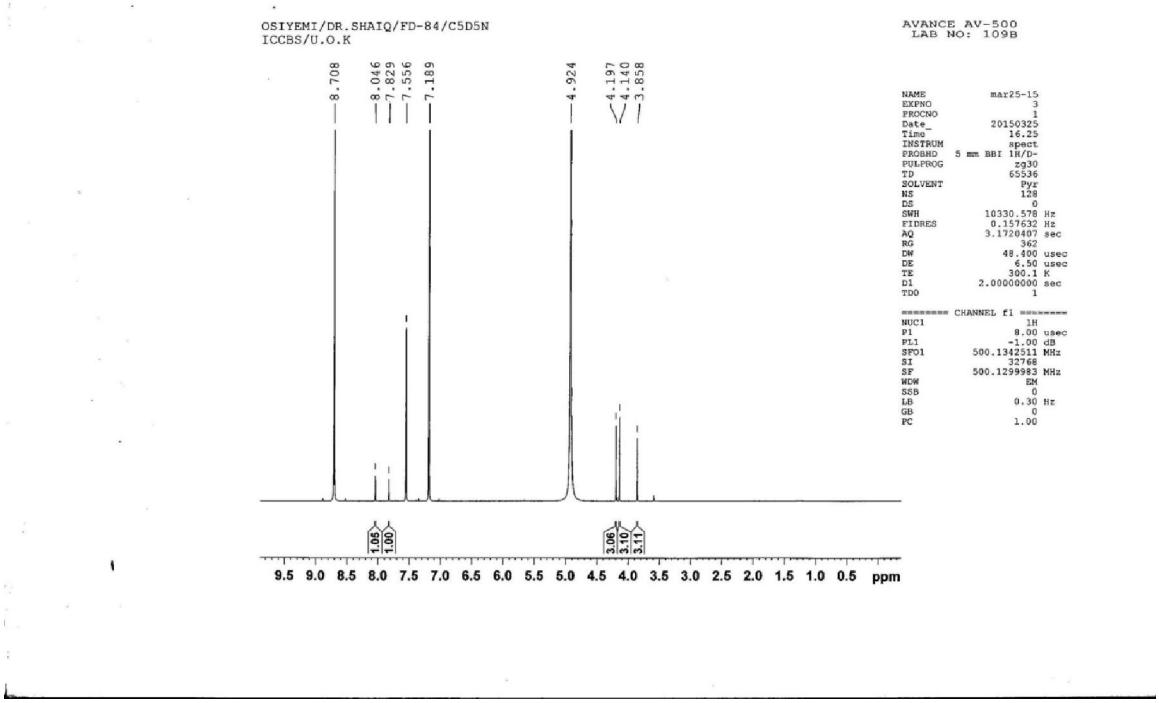
Nike/DR IQBAL/TMH 47/CDCl3



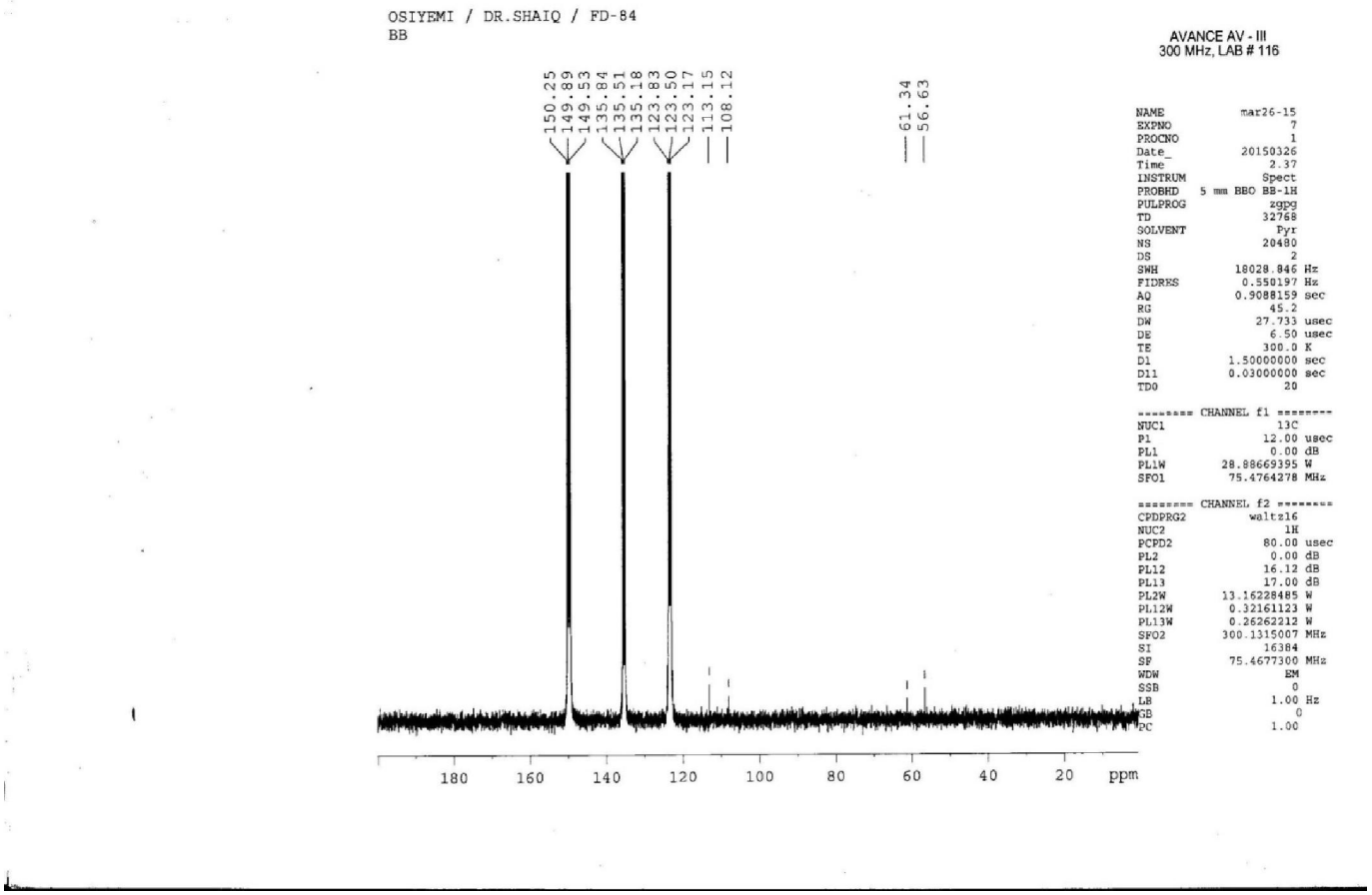
APPENDIX 5B: EI-MS of TMH 47 (Stigmasterol)



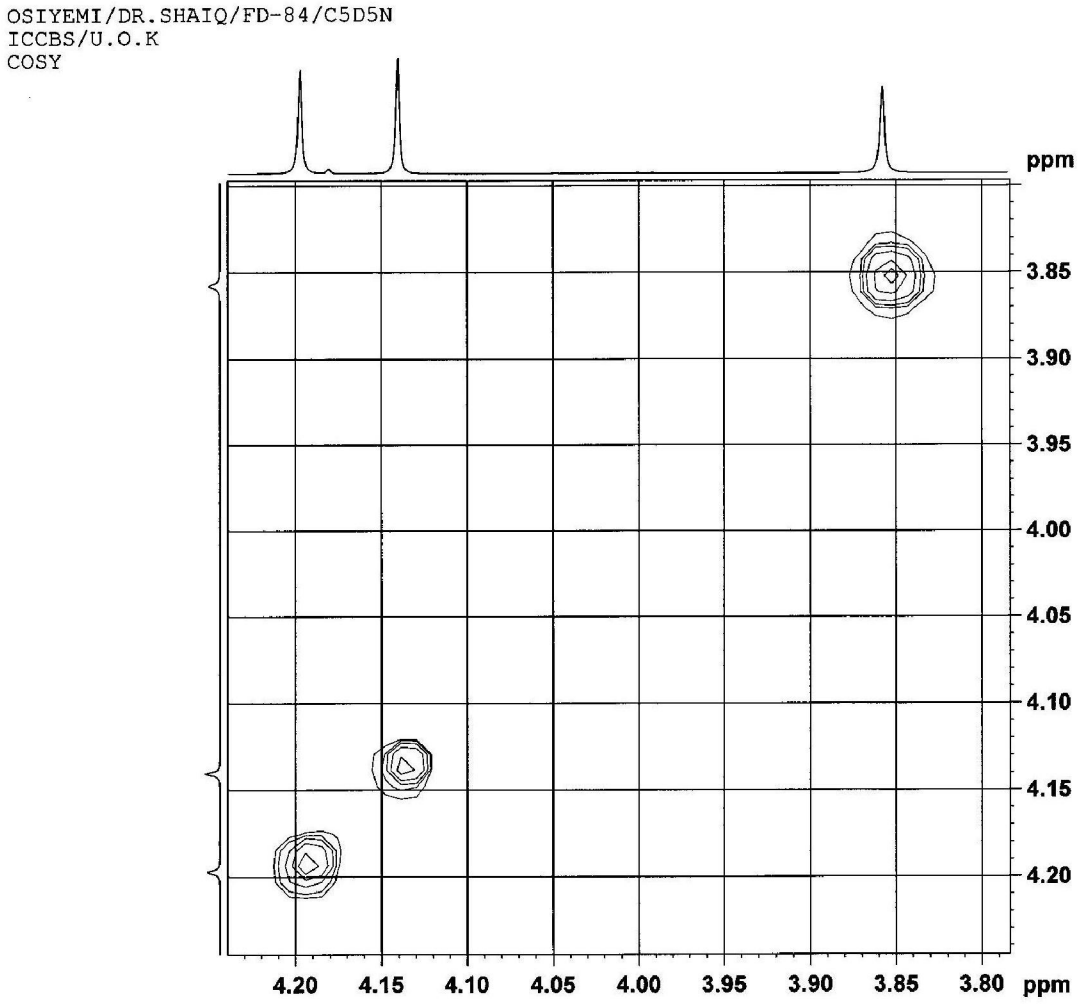
APPENDIX 6A: ^{1}HNMR Spectrum of FD 84 (3,3′,4-tri-O-methylellagic)



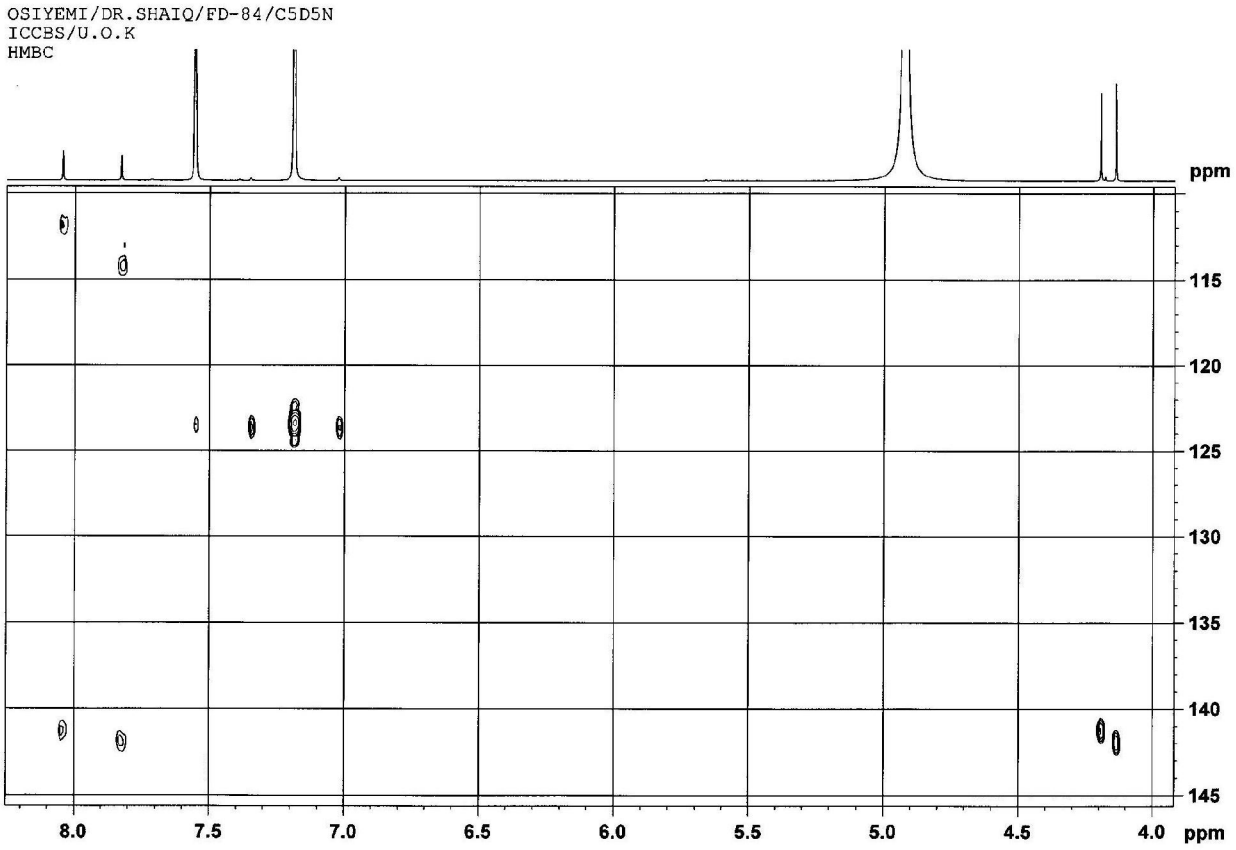
APPENDIX 6B: ^{13}CNMR Spectrum of FD 84 (3,3′,4-tri-O-methylellagic)



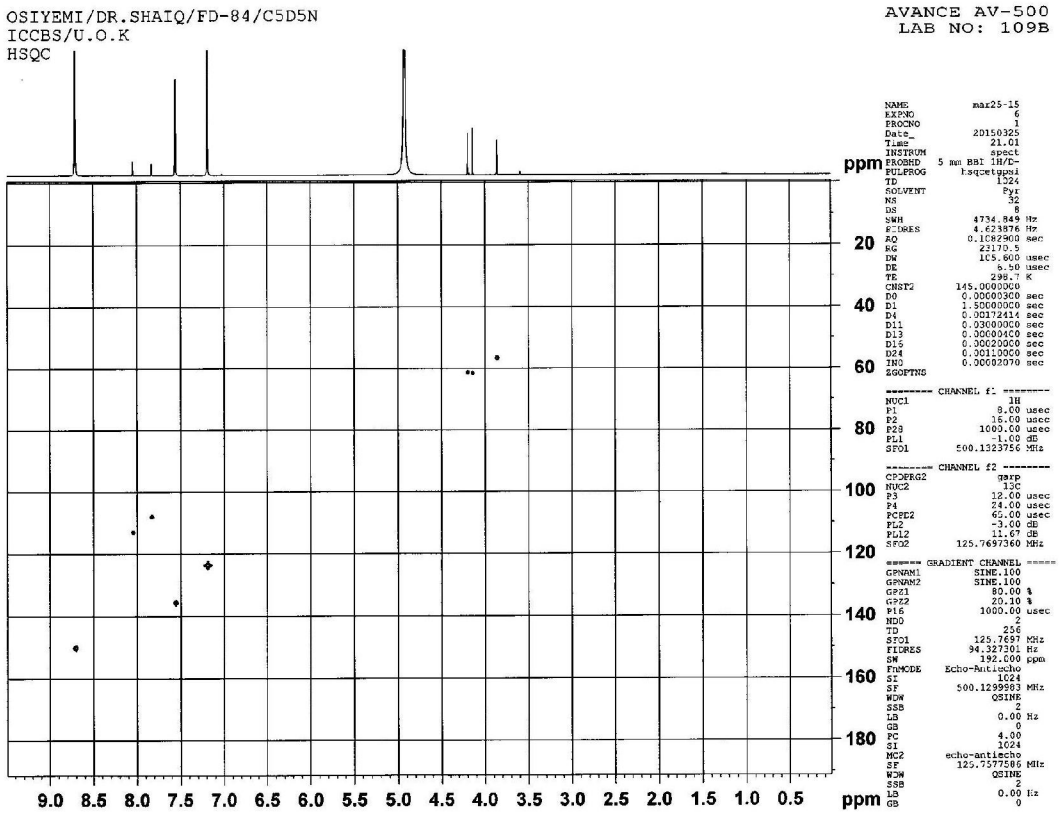
APPENDIX 6C: 2D NMR (COSY) Spectrum of FD 84 (3,3′,4-tri-O-methylellagic)



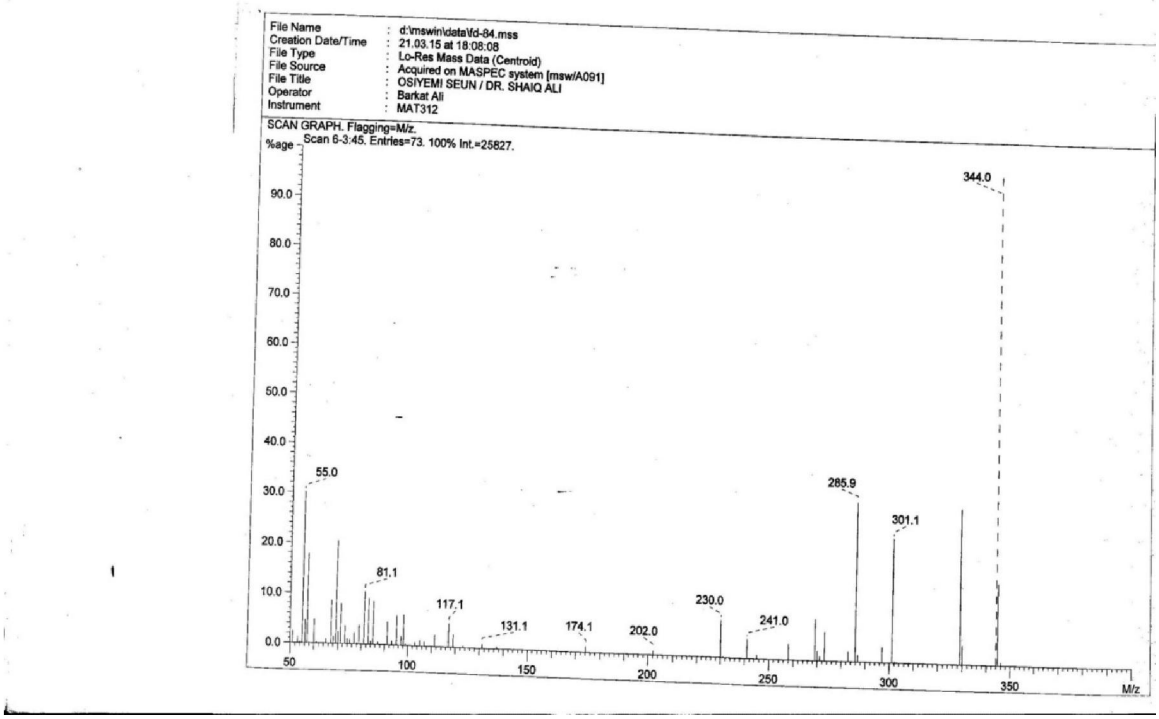
APPENDIX 6D: 2D NMR (HMBC) Spectrum of FD 84 (3,3′,4-tri-O-methylellagic)



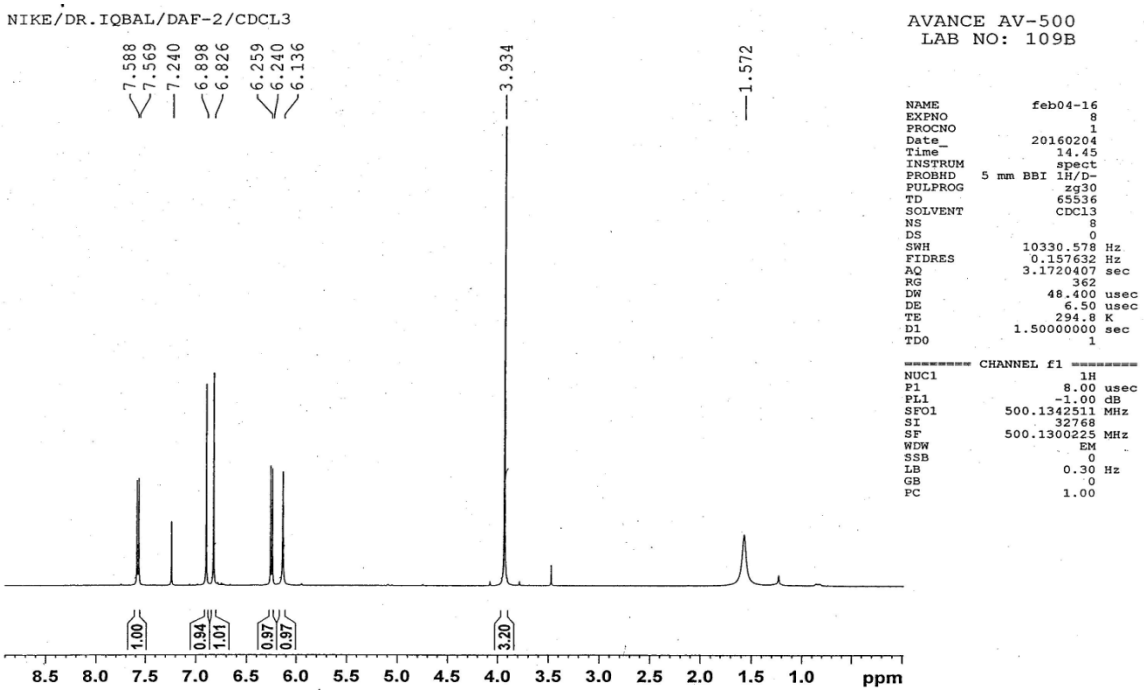
APPENDIX 6E: 2D NMR (HSQC) Spectrum of FD 84 (3,3′,4-tri-O-methylellagic)



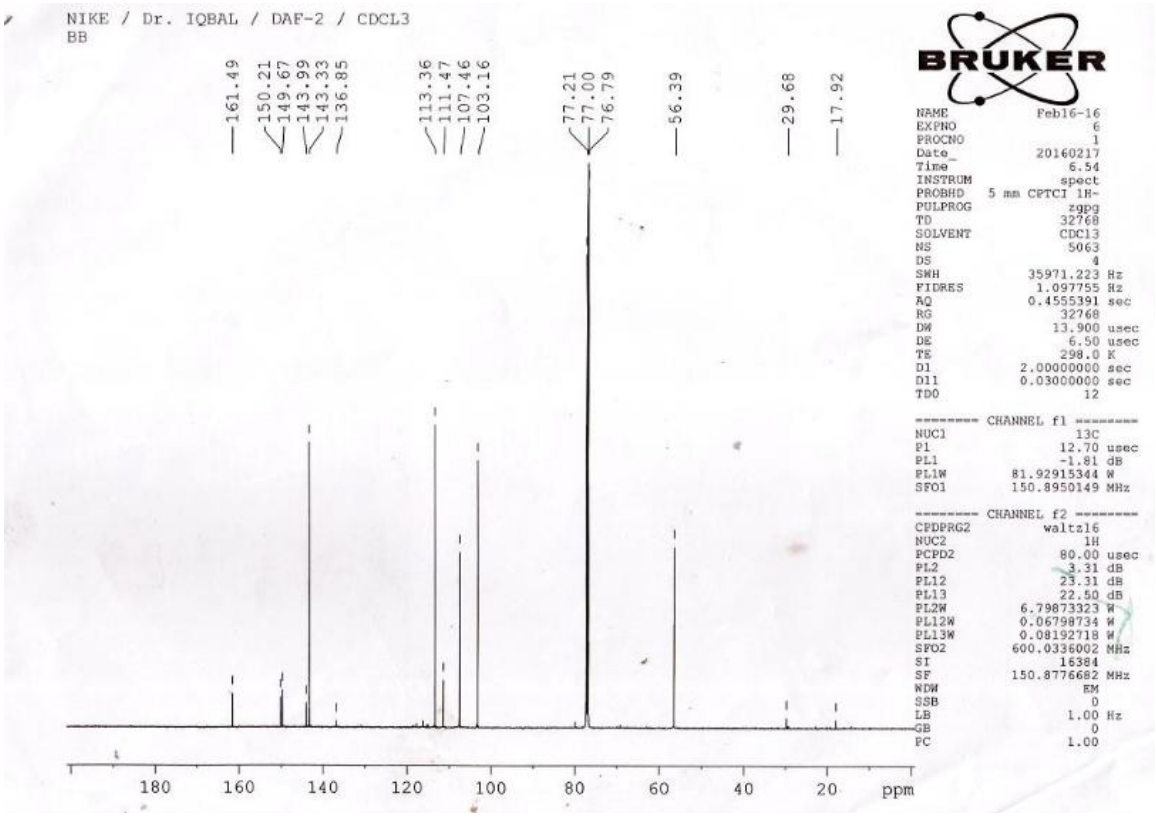
APPENDIX 6F: EI-MS of FD 84 (3,3′,4-tri-O-methylellagic)



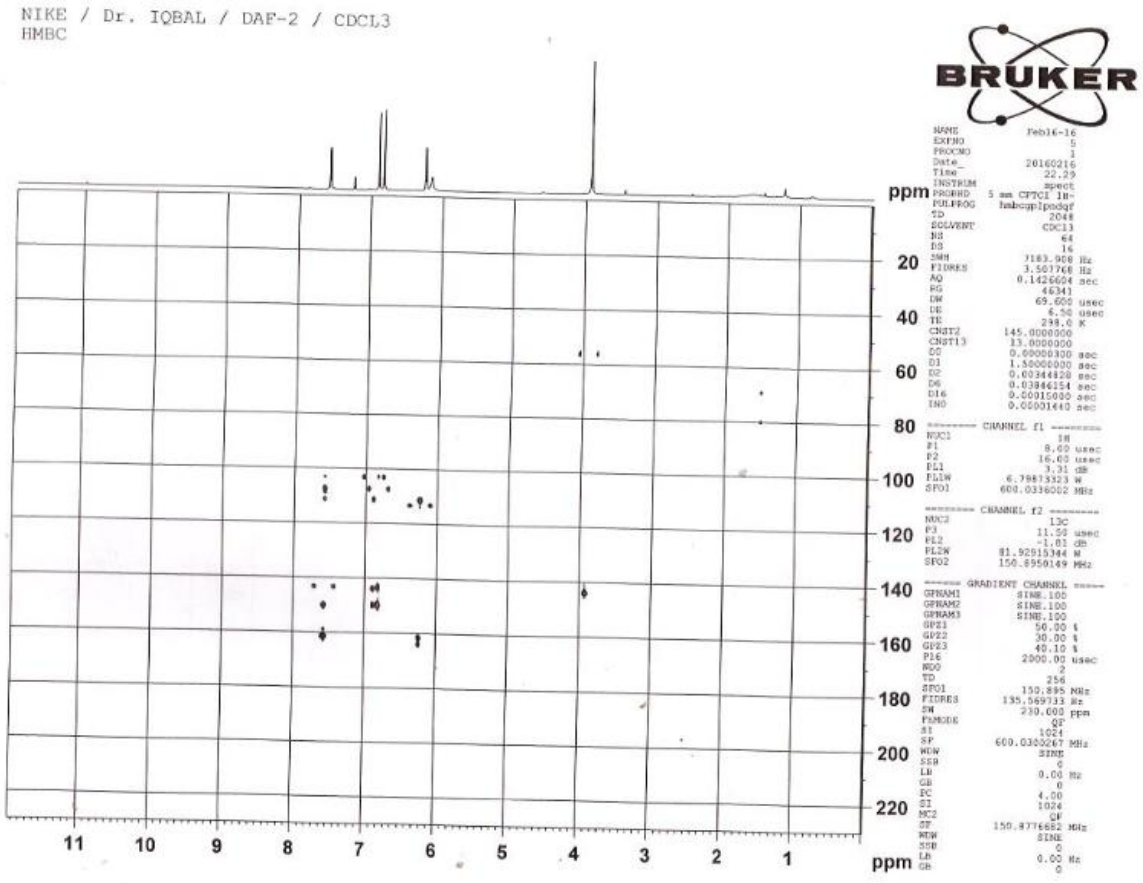
APPENDIX 7A: ^{1}HNMR Spectrum of DAF 2 (Scopoletin)



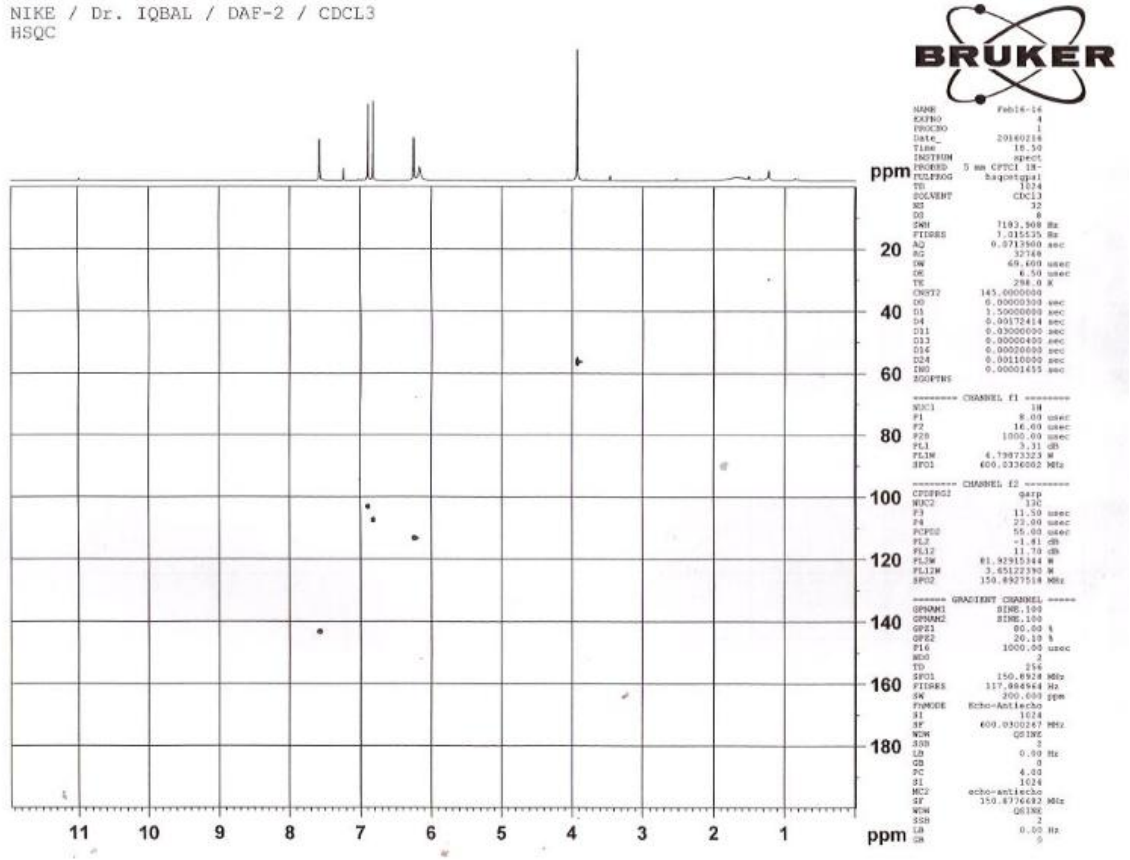
APPENDIX 7B: ^{13}CNMR Spectrum of DAF 2 (Scopoletin)



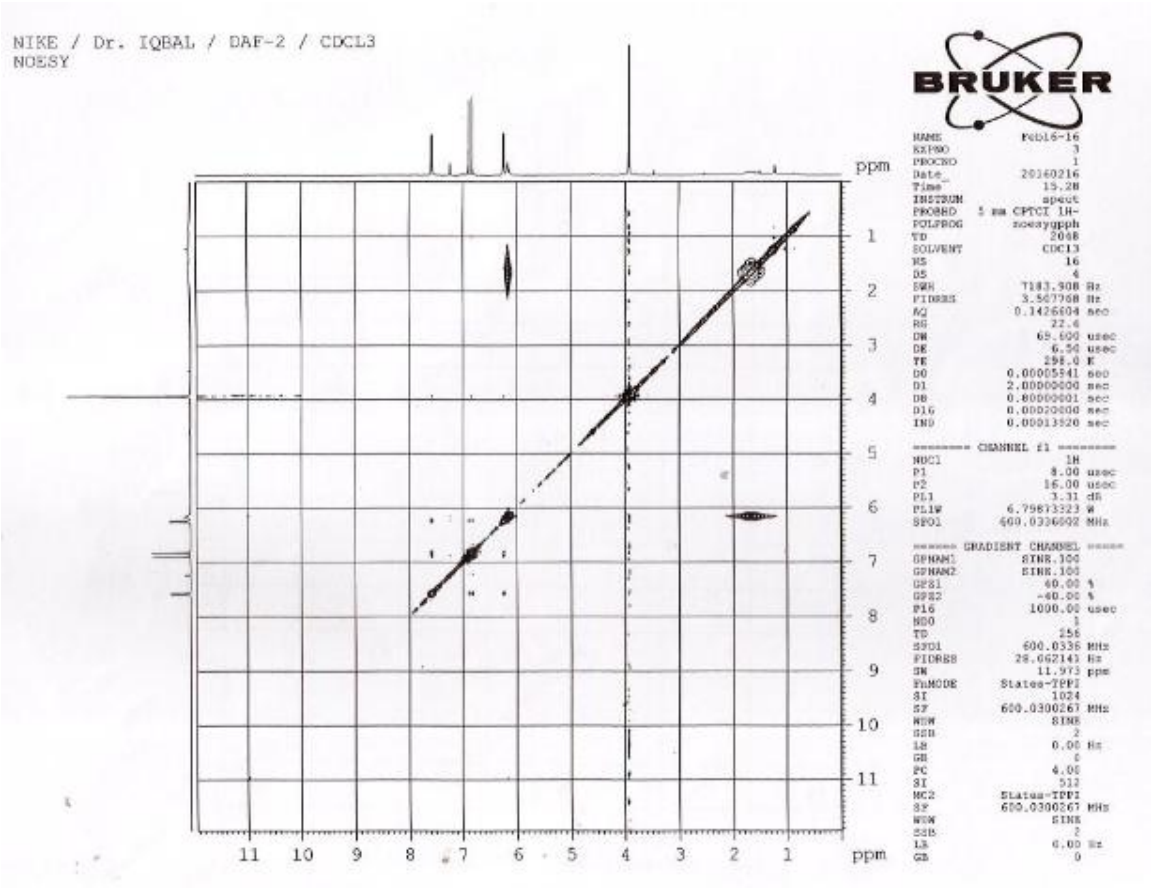
APPENDIX 7C:2D NMR(HMBC) of Spectrum of DAF 2 (Scopoletin)



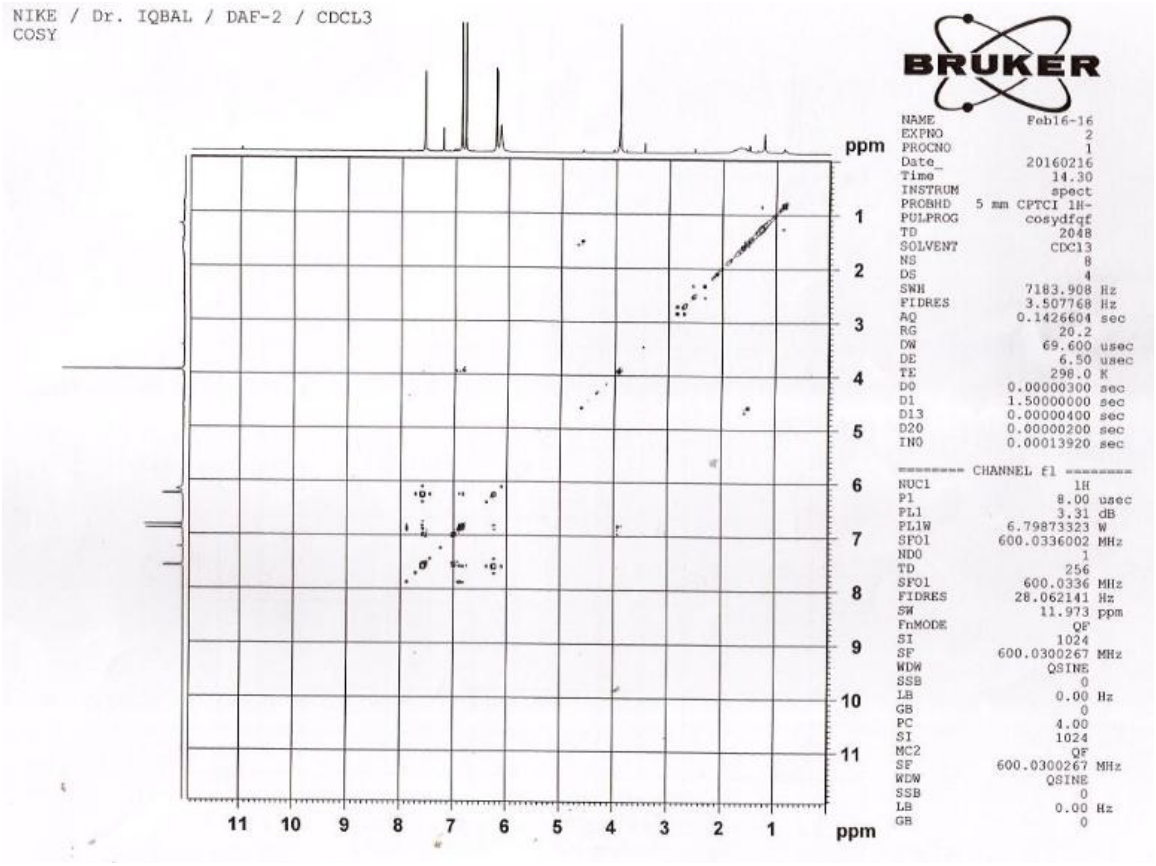
APPENDIX 7D: 2D NMR (HSQC) Spectrum of DAF 2 (Scopoletin)



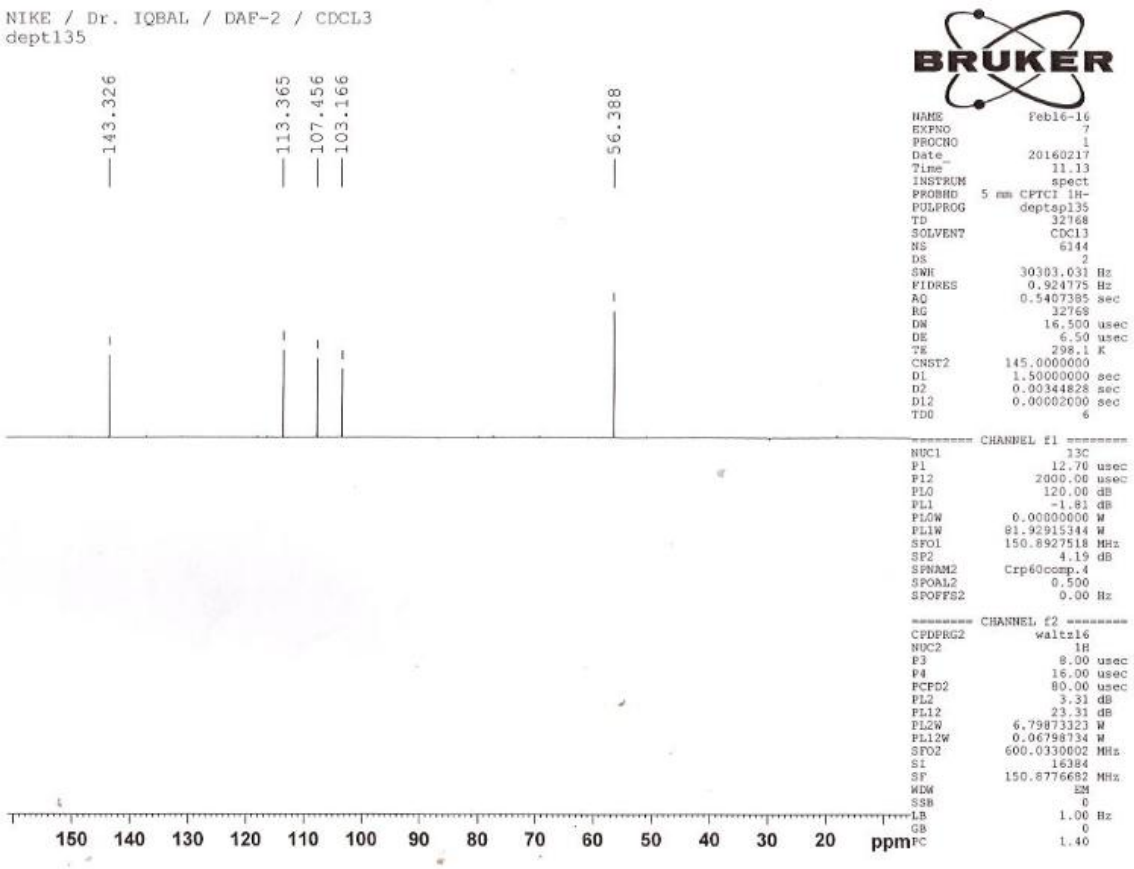
APPENDIX 7E:2D NMR (NOESY) Spectrum of Scopoletin



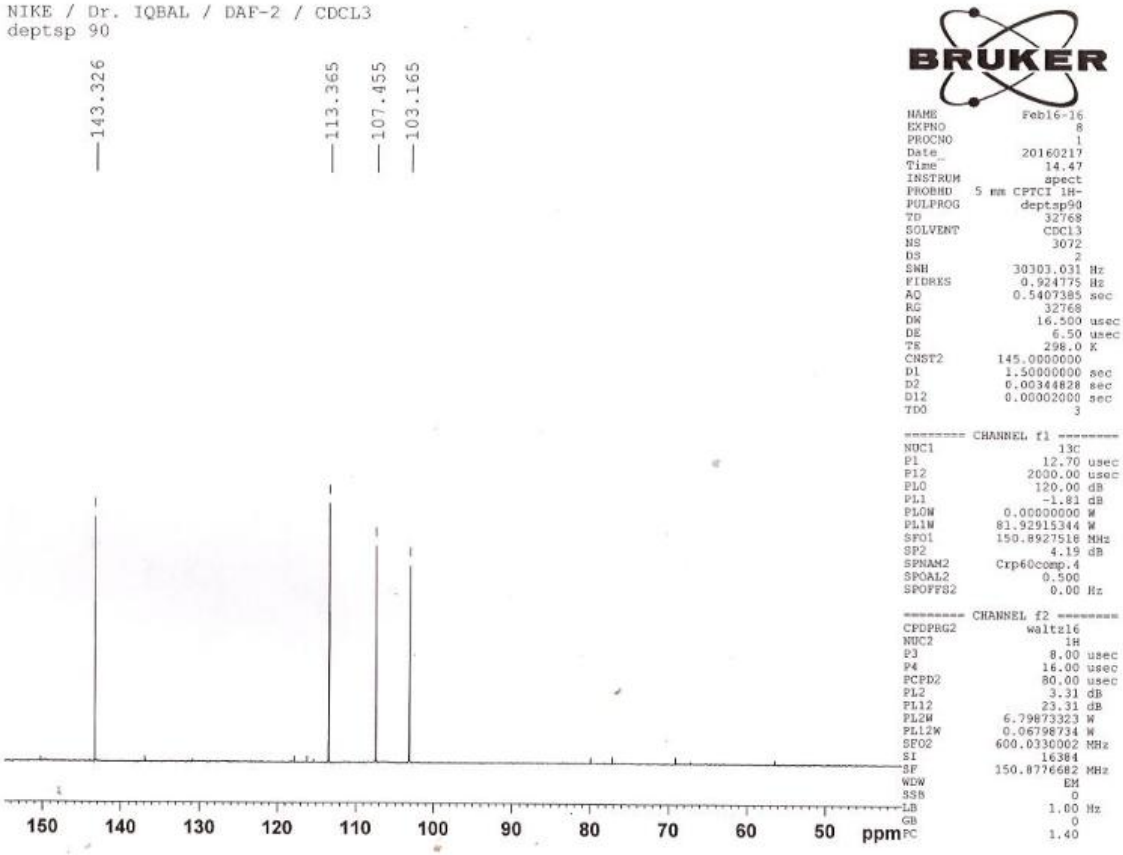
APPENDIX 7F: 2D NMR (COSY) Spectrum of Scopoletin



APPENDIX 7G:13C dept 135 Spectrum of DAF 2 (Scopoletin)



APPENDIX 7E: 13C DEPT 90 Spectrum of DAF 2 (Scopoletin)



APPENDIX 7F: EI-MS Spectrum of DAF 2 (Scopoletin)