Characterisation of antimalarial compounds from plants used in traditional health practices in Lake Victoria basin

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

I dedicate this project to God and to my parents **Mama Alice** and the late **Eldadi Kube**

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AOA	Antioxidant Activity
BCC	Behavioral and Chemical Change
COSY	COrrelation SpectroscopY
DC	Dead in Control
DDT	Dichlorodiphenyltrichloroethane
DEET	N,N-Diethyl-3-methylbenzamide
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethylsulphoxide
DPPH	1, 1-diphenyl-2-picrylhydrazyl
DT	Dead in Test
EIMS	Electron Ionization Mass Spectroscopy
FTIR	Fourier Transformer Infra Red
GLM	General Linear Model
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Correlation
IGR	Insect Growth Regulators
IR	Infra Red
LD_{50}	Dose required that kills 50% brine Shrimps
MeCN	Acetonitrile
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Over hauser Enhancement
NOESY	Nuclear Over hauser Effect Spectroscopy

ODS	Octadecylsilicane
SAS	Statistical Analysis System
SIT	Sterile Insect Technique
SPs	Synthetic Pyrethroids
TDR	Tropical Disease Research
TLC	Thin Layer Chromatography
TMS	Trimethyl Silane
UV	Ultra Violet
WHO	World Health Organization

ABSTRACT

Malaria, transmitted by bites of infected female anopheline mosquitoes, is an infectious disease caused by parasitic protozoa of the genus *Plasmodium*. The parasite infects human and insect hosts alternately. It remains a leading cause of morbidity and mortality in Kenya and is responsible for 2% of disease burden world wide with 90% of the cases in Africa. Prevention of malaria encompasses a variety of measures that may protect against infection or the development of the disease in infected individuals (vector control, protective clothing, and use of bed nets, vaccination, and chemotherapy or chemoprophylaxis). Parasite and vector resistance to drugs, and insecticides respectively coupled with ineffective repellants and absence of a vaccine have limited the control of the disease in most of the sub-Saharan Africa. With increasing cases of drug resistant parasites, expensive drugs and poor distribution of modern health facilities, there seems to be resurgence in use of herbal remedies to treat malaria and other infections before seeking conventional western remedies. Four plants: *Maytenus heterophylla* (Celastraceae); *Strychnos henningsii* and *S. usambarensis* (Loganiaceae); and *Periploca linearifolia* (Asclepediaceae), used for the treatment of malaria in the Lake Victoria basin were investigated for efficacy. Different plant parts were sequentially extracted with hexane, chloroform, ethyl acetate and methanol. The extracts were screened for *in vitro* anti-plasmodial activity against two *Plasmodium falciparum* isolates, D6 (chloroquine-sensitive) and W2 (chloroquine-resistant) strains and the activity (IC_{50}) determined. All the fractions were active against D6 and W2 strains (IC_{50} < 50 µg/ml). The activity of the plant extracts was slightly lower (IC_{50} 3.30 - 37.43 µg/ml) against W2 than D6 strain (IC_{50} 1.07- 25.78 µg/ml). The chloroform extracts of all the plants were the most active (IC_{50} 4.00 - 10.58 µg/ml. The methanol extract of *S. henningsii* had the highest activity (IC_{50} 1.07 ± 0.07 µg/ml) while the ethyl acetate fraction of *P. linearifolia* had the lowest activity

(IC_{50} 37.43 ± 0.96 µg/ml) against D6 strain. The extracts were also investigated for toxicity using brine shrimp larvae (*Artemia salina*). All the fractions and the aqueous extracts were not toxic (LC_{50} > 200 µg/ml). The fractions with low IC_{50} and high LC_{50} values may be used as a source of compounds for use in anti-malarial therapy or in combination with standard drugs. The methanol extracts were also tested for anti-oxidant activity using DPPH. The activity of the extracts was found to increase with concentration. The methanol extracts were found to have significant radical scavenging activity implying that the use of these plants as medicines may protect the human body against radicals which cause severe pathological conditions. The plants may therefore serve as a natural source of anti-oxidants. Isolation of three compounds (lupeol ester **(50)**, β-sitosterol **(54)** and β-amyrin **(55)**) from *P. linearifolia* fractions with high *in vitro* anti-plasmodial activity was achieved using chromatographic techniques (VLC, TLC and CC), and their characterization accomplished using spectroscopic techniques (1D and 2D NMR, IR and UV), chemical methods and MS spectrometry.

CHAPTER ONE

INTRODUCTION

1.1 Background

Malaria is an infectious disease caused by minute parasitic protozoa of the genus Plasmodium, which infect human and insect hosts alternately. There are four species of malaria that can infect humans: *Plasmodium falciparum, P. vivax, P. ovale and P. malariae* (Knell, 1991). Many other species of Plasmodium have been found in other animals. Of special interest for drug testing are organism such as *P. cathemerium* (avian malaria) and *P. berghei* (rodent malaria) (Markell *et al*., 1992). The malaria parasites are transmitted by bites of infected female anopheline mosquitoes. The vector may also transmit filariasis and arboviruses in addition to malaria. The mosquito injects infected saliva during blood feeding. While in humans the parasites multiply drastically, first in the liver then in the bloodstream. Female anopheline mosquitoes are infected if they suck blood from infected human hosts. The parasites multiply in the stomach-wall of the mosquito, and then migrate through her body to infect the salivary glands. When the infected mosquito feeds on human blood, it injects saliva containing the malaria parasites into the human host and the cycle starts again. Malaria may be acquired rarely from infected blood during transfusion or even from shared needles among drug addicts. Each malaria attack is severe, and may lead to the death of 70% of red blood cells, causing anaemia, coma and death (Knell, 1991).

1.2 Malaria as a killer disease and its control

Malaria is the leading killer disease in the tropics. More people are infected with malaria than any other disease, and the number affected or at risk is increasing remorselessly. Hopes that malaria might be eradicated have never been realised due to its complexity.

Control measures are becoming less effective, and the threat of epidemic malaria is increasing in many tropical areas. It is estimated that up to 500 million cases of malaria resulting in 2.5 million deaths are reported annually (WHO, 2005). However, malaria is a preventable and treatable disease but is becoming more difficult to control. Measures which guard against disease but not infection include vaccination, chemotherapy and chemoprophylaxis (WHO, 2001a). A three–fold approach has been taken in attempting to control malaria; vaccine development, chemotherapy and vector control.

1.2.1 Vaccine development

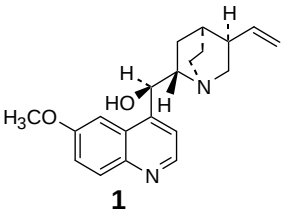
Vaccine development was first tried through induction of immunity by sporozites that had been inactivated by UV light or mechanical disruption in avian malaria (Kinoshita, 1998). Vaccination against the exoerythrocytic cycle development in mammalian malaria has been successfully achieved. It involved use of irradiated sporozoites obtained by dissection of infected mosquito glands or inoculated through the bite of infected irradiated mosquito (Kumar *et al.,* 2002). A pre-erythrocytic vaccine RTS, S/ASO2A has been tried in Mozambique resulting in 47% protective efficacy (Alonso *et al.*, 2000) SPf-66, pre-erythrocytic and asexual blood stage proteins of *P. falciparum*, is designed to block the parasite at its later merozite form, when it emerges from initial incubation in the liver. In Tanzania, the efficacy was found to be 31% in children below 5 years of age (Maire *et al.*, 2006).

However, the development of a suitable vaccine has been hindered by lack of suitable source of parasite cultures from which it can be prepared. The main problem is low immunogenecity of malaria parasites (Kwiatkowski and Marsh, 1997). Nevertheless, recent research in Mozambique has shown that children treated with RTS, S/ASO2A vaccine had a 30% lower risk of developing the malaria (D'Alessandro *et al*., 1995). This

shows that an effective vaccine could make a huge impact in the control of the disease. Unlike less complex organisms, parasites have developed ingenious ways of avoiding immune response in the hosts. For instance, the malaria parasite expresses different antigens at each stage of its life cycle, and is often able to change these antigens when the host mounts an immune response towards them. The difficulty in culturing malaria parasites in large quantities has also limited the development of vaccines (Graves and Gelband, 2003).

1.2.2 Chemotherapy

Vector control and vaccination emphasises prevention, while chemotherapy focuses on disease treatment. Quinine **(1)** and related alkaloids were the only anti-malarial drugs available prior to World War 1 (Knell, 1991). It was initially isolated from the stem bark of the South American cinchona tree, which is now grown more extensively in Java. Synthesis of the drug has not been commercially practical although many attempts have been made from 1920 (Knell, 1991). The discovery of quinine **(1)** inspired the search for synthetic analogues like chloroquine (CQ) **(2)**, amodiaquine (AQ) **(3)**, mefloquine (MQ) **(4)**, primaquine (PQ) **(5)** and tafenoquine **(6).** Other anti-malarial drugs include: diaminopyrimidines – pyrimethamine **(7)**; sulphonamides and sulphones – sulphadoxine **(8)**, sulphadizine **(9),** dapsone **(10)**; quinoline methanols – mefloquine **(4)**; antibiotics – tetracycline **(11)**, fluoroquinolones **(12)**; biguanides – proguanil **(13)**; phenathrene methanols – halofantrine **(14)**; naphthoquinoles –atovaquone **(15)**; iron-chelating agents – desferrioxamine; and sesquiterpene lactones - qinghaosu or arteminisin **(16)** and analogue [artemether **(17)** and artesunate **(18)**] (Bradley *et al*., 1996).



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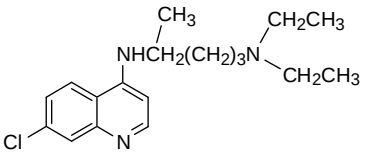

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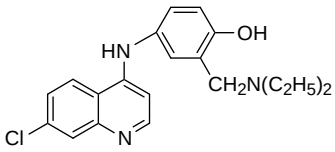

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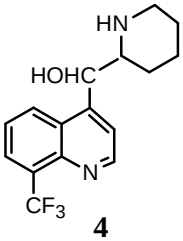

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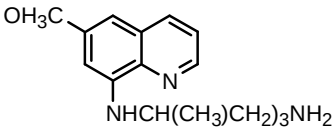

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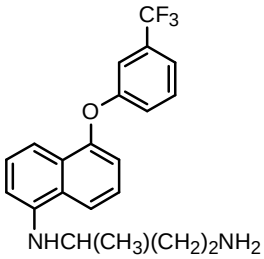

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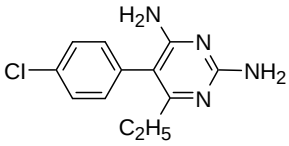

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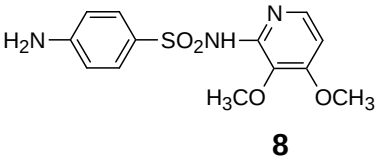

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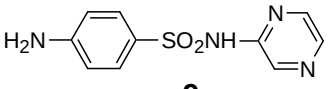

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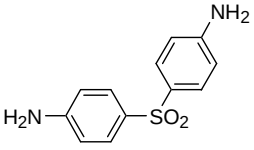

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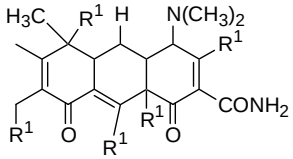

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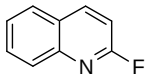

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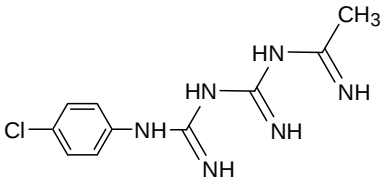

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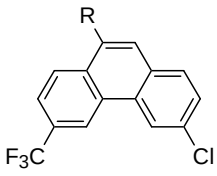

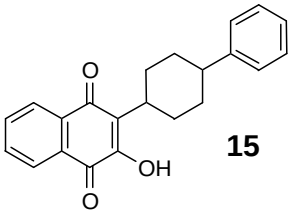

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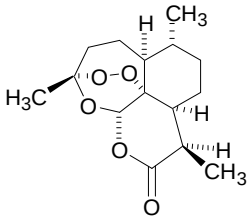
14 R = $CH(OH)CH_2CH_2N[(CH_2)_3CH_3]_2$



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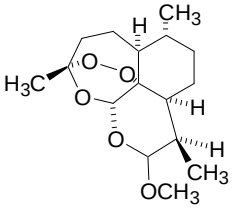

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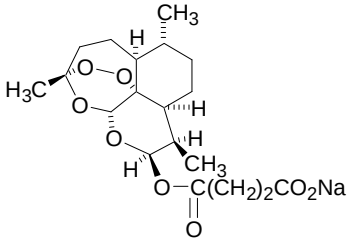

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CQ **(2)** was developed during World War II. Chloroquine-resistant *P. falciparum* strains were noted in South America and South East Asia in the late 1950s and subsequently in most areas of intense malaria transmission (Meyer, 1972). It has been noted that parasites that are resistant to chloroquine are often resistant to other synthetic quinoline based anti-malarial drugs (Sumawinata *et al.,* 2003). Quinine **(1)** has therefore emerged as the effective drug in such cases (Markell *et al.,* 1992). The increasing spread of CQ resistant *P. falciparum* strains has inspired numerous studies aimed at identifying new anti-malarial compounds. Research on identification of active plant constituents is important in the search for new anti-malarial compounds. Extracts of *Artemisia annua* have been used for centuries to treat malaria in China (Klayman, 1985). One of the recent achievements in treatment of malaria in China is the use of artemisinin **(16)** from *A. annua* (Klayman, 1985). Artemisinin is effective against both *P. falciparum* and *P. vivax*, and derivatives such as artemether **(17)** and sodium artesunate **(18)** are even more active, especially in patients with cerebral malaria (Sumawinata *et al.,* 2003).

Due to increasing cases of resistant strains of malaria parasites (Bradley and Bannister, 2001), expensive anti-malarial drugs coupled with poor distribution of modern health facilities in the developing countries, most people have turned to herbal remedies for the treatment of malaria and other infections (Akerele, 1990). Recognising the potential of herbal drugs in the expansion of health services, the World Health Organisation, in 1978 called for a comprehensive approach to medicinal plants (Akerele, 1990).

Most people living in rural areas of developing countries depend on traditional medicines (Kokwaro, 1993). In East and Central Africa, a lot of importance is attached to development of the medicinal plants industry essential for health care provision (Kokwaro, 1993). It is also capable of contributing to poverty alleviation, particularly among women in rural communities. However, it is necessary to use modern scientific methods to carry out systematic investigations to establish the efficacy and toxicity of medicinal plants commonly used by traditional medicine practitioners (TMPs) (WHO, 1996).

1.2.3 Chemoprophylaxis

Chemotherapy is the protection or prevention from development of a disease by the use of drugs. It is a method recommended for non- immune visitors to endemic areas and people living in such places. Due to increasing resistance of malaria parasite, it has become very hard to prescribe prophylactic drugs. CQ has been a drug of choice for prophylaxis against CQ-sensitive malaria. Its resistance is currently wide spread making it a less effective prophylactic drug. A combination of MQ and proguanil has been used for resistant malaria but its use has been limited to non endemic areas. Halofantrine which has commonly been used has poor bioavailability and may not be used in pregnant women. The use of prophylactic drugs has not been 100% effective in both travellers and people living in endemic areas.

1.2.4 Vector control

Vector control involves the use of methods targeted at controlling mosquito populations at larval or adult stages. Vector control involves the use of repellents, insect growth regulators (IGRs), adulticides, larvicides, biological control agents, Sterile Insect

Technique, genetic engineering and environmental management techniques (Srinivas *et al.*, 2000).

IGRs have been used to control mosquitoes (Mwangi and Rembold, 1988). They are slow acting, target specific and primarily active against the immature stages of mosquitoes, flies and other insects (Srinivas *et al.*, 2000). Some IGRs induces sterility while others induce morphological anomalies in the adult stages (Fradin and Day, 2002). Currently, the most commonly used IGR is Altosid®, which contains methoprene that is modelled on juvenile hormone as the active ingredient (Bradley *et al.*, 1996).

Biological control has been attempted using toxins produced by *Bacillus thuringiensis H-4* and *Bacillus sphaericus*) (Manasherob *et al.*, 1996). Fungi such as *Leptolegnia chapmani* (Zattau and McInnis, 1987) and *Toplocladium cylindrosporum* (Ravallec *et al.*, 1989) have shown some potential for mosquito control. The mermethid entomo-pathogenic nematode, *Romanomermis culcivorax*, has also been used against mosquito larvae (Walker and Lynch, 2007). Invertebrate predators of mosquito larvae have also been used ((Leon and Richard, 1990). Under natural conditions in rice fields, invertebrate predators (Coleptera, Dystiscidae, Hydrophylidae, Hemiptera, Belostomidae and Notonoctidae among others) can be responsible for drastic reductions in larval populations. Due to the use of vertebrate predators and agrochemicals coupled with natural population fluctuations, invertebrate predators can be eliminated or reduced (Leon and Richard, 1990). Vertebrate predators like larvivorous fish have also been used as a biological tool for mosquito control for nearly 100 years (Srinivas *et al.*, 2000). *Gambusa affinis* has been successfully used in controlling populations of *Culex tarsalis* and *Anopheles freeborni* in California rice fields and is by far the most commonly used fish for mosquito control (Hoy and Reed, 1970). However, it is expensive to rear and transport fish (Leon and Jonathan, 2007). The guppy fish, *Poecilia reticulate* Peters, and the Argentine Pearl

fish, *Cynolebias bellottii* Steindachner, are also useful for mosquito control (Walker and Lynch, 2007). *Tilapia sp* has been successfully used at Namanjalala in Kitale Kenya to control mosquito larvae. However, this campaign is facing serious shortage of skilled personnel (Wandera, 2001).

Synthetic larvicides have been used in mosquito control. Paris green ({Cu $(C_2H_3O_2)_2$.3Cu $(AsO_2)_2$}) has been used to control mosquito larvae, though it is expensive due to the high copper content and pollutes the environment (Rodgers and Rathburn, 1958). Inorganic larvicides are highly toxic to most aquatic organisms, burn foliage so easily due to large amounts of water-soluble arsenic in them (Rathburn, 1966).

Other chemical larvicides that have been used are the organic larvicides. The earliest used synthetic organic larvicides were the organochlorides, methoxychlor and benzene hexachloride (Gjullin and Richards, 1952). However, organochlorines are toxic to fish, other aquatic animals and birds. They also accumulate in the food chain, are persistent in the environment and harmful to the ozone layer. Due to development of resistance in mosquitoes and gnats, the organochlorine insecticides were replaced by organophosphates (OPs) such as malathion, parathion and fenithion among others. OPs are highly toxic to mammals and are therefore, not commonly used.

Another class of chemical larvicides used are botanical insecticides such as pyrethrin, rotenone and rynodin. Pyrethrin, an excito-repellant insecticide, is derived from the flowers *Chrysanthemum cinerariaefolium.* The insecticidal properties of the plant was first recognised around 1800 and originally used as powders or dusts from the dried flowers (Pohanish, 2002). The cost of extraction, volatility and the quick bio-degradability has limited their widespread use. Interestingly, no resistance has been reported for the natural pyrethrins. Structure-activity relationship studies of the insecticidal pyrethrins led to the development of synthetic pyrethroids (Elizabeth *et al.*,

2005). These include permethrin, cyahalomethrin, cypermethrin, fenvalerate and decamethrin. Decamethrin is a chloro analogue (FMC-45497) and has been found to be 50 -100 times more effective than the OPs. Synthetic pyrethroid resistance is emerging despite early optimism that its rapid toxicological action would not produce resistance (Malcom, 1988; Nkunya, 2002).

Repellents are substances that protect animals, plants or products from insect attack by making food or living conditions unattractive or offensive (Fradin and Day, 1998). Commercial repellents containing ingredients such as diethyl phthalate, diethyl carbamate, *N, N*-diethyl-3-methylbenzamide (DEET) and 2-ethyl-1, 3-hexanediol are in use (Elizabeth *et al.,* 2005). Essential oils from some plants have been reported to have repellents activity. The repellent plants include cedar, geranium, lavender, pine, cinnamon and rosemary among others. Studies have indicated that components in *Eucalyptus maculata citriodora* oil especially *p*-menthane-3, 8-diol (Schreck and Leonhardt, 1991) and eucamol (Satoh, 1995) are as effective as DEET (Watanabe and Shono, 1993). Synthetic repellents such as dimethyl phthalate and 2-ethyl-1, 3-hexanediol and essential oils have not provided a great impact in controlling the rate of inoculations and transmissions of malaria parasite since most of them are highly volatile and provided only transient protection against mosquitoes (Majori *et al.,* 1987).

Environmental management falls into three broad categories; environmental modification, environmental manipulation, and modification/manipulation of human habitation or behaviour. Environmental modification includes permanent physical transformations such as drainage, land filling and grading (Lawrence and Cynthia, 1990). Environmental manipulation includes recurrent measures that produce temporary conditions that are unfavourable to larval production, including regulation of water level, removal of vegetation, stream flushing, among others (Lawrence and Cynthia, 1990). The third

category involves activities that are designed to reduce contact with vectors and therefore pathogen transmission. These include location of human settlements away from the vector breeding sources, mosquito proofing of houses, use of insect-treated bed nets, curtains, clothes and other personal protection measures (WHO, 1999). Some of these procedures were effective in the past but were de-emphasized due to their high cost and labour-intensive nature.

CHAPTER TWO

LITERATURE REVIEW

2.1 Chemotherapy and chemoprophylaxis

Chloroquine **(2)** and other quinine analogs have been the most widely used drugs in most of the endemic regions. Since its development in the early 1940s, CQ became the drug of choice for malaria management. It is relatively cheap and therefore affordable in the poor countries, which are the worst affected. Other quinoline based synthetic anti-malarials include AQ **(3)**, MQ **(4)** and PQ **(5)** among others. However, the spread of chloroquine resistance has been reported in most areas in Africa, South America and South East Asia (WHO, 2001b). Although CQ-resistant *P. falciparum* has spread through almost all endemic areas, CQ remains the most widely used treatment for uncomplicated *P. falciparum* malaria. Varying degree of CQ resistance has been reported (WHO, 2000). Initially, there was overwhelming evidence which indicated that AQ **(3)** was significantly more effective than CQ **(2)** in Africa and should have presumably replaced it as one of the effective anti-malarial drug (Meyer, 1972). At first CQ- resistant *P. falciparum* strains retained some sensitivity to AQ **(3)**, later resistance to this drug soon followed (Draper, 1983). MQ **(4)** is also widely used for the treatment where multi-drug resistant acute falciparum malaria occurs (Kofi and Kue, 1985). However, resistance in non-immune individuals has been reported in Thailand (Mockenhaupt, 1995). PQ **(5)** has undergone preliminary trials in adults and children in South East Asia (Collins and Jeffery, 1996). It is a drug that works by inhibiting the electron transfer chain in the parasite. Reports have appeared calling attention to what has been termed PQ **(5)** resistance in *P. vivax* (Kulkarni *et al.*, 2000). Its use has adverse reactions that affect the gastro-intestinal system leading to reduced prescription of the drug. Gametocyte resistance of the drug has been

reported (Peters, 1990). Nevertheless, quinine **(1)** the natural template from which the aminoquinoline drugs were developed is still used as the last resort where resistance to other aminoquinolines has been observed. However, due to its negative side effects its use is restricted to life threatening malaria cases.

Pyremithamine **(6)**, proguanil **(13)** and sulpur-based anti-malarial drugs are examples of the most readily available antifoliates. Resistance of *P. falciparum* to pyremithamine **(6)** developed rapidly and was first observed in 1953 and is now widespread (Onori, 1982). Resistance of the asexual *P. falciparum* to proguanil **(13)** was also detected early and lack of prophylactic efficacy was confirmed in Thailand (Phillips, 1984).

Sulphur-based anti-malarial drugs such as sulfadoxine **(8)**, sulphadiazine **(9)** and dapsone **(10)** have also been used. However, their continued use can lead to kidney and liver poisoning (Berman *et al*., 1991). The resistance of sulphur-based compounds seems to be present in all stages of parasite metabolism (White, 1992).

Halofantrine® **(14)**, a phenanthrine-methanol, with activity against the erythrocytic stages of the malaria parasite has been used in areas with multiple-drug resistant *P. falciparum* (WHO, 1988). However, the drug can produce potentially fatal cardiac conduction abnormalities thus limiting its use (Boudreau, 1985). Resistance of halofantrine **(14)** has been reported in Thailand (Bradley and Bannister, 2001).

Atovaquone **(15)**, a hydroxynaphthoquinone, is currently being used mainly for the treatment of opportunistic infections in immuno-suppresed patients. It is effective against CQ-resistant *P. falciparum*, but resistance develops rapidly when used alone (Looareesuwan, 1985). It is therefore usually administered in combination with proguanil (Canfield *et al.,* 1995).

New anti-malarials consists of the sesquiterpene lactones like artemisinin **(16)** and derivatives such as artemether **(17)** and artesunate **(18)** (Bradley *et al.,* 1996). Artemisinin

(16) derivatives currently shows no cross resistance with the known anti-malarial drugs and as such are important for treating severe malaria in areas of multi-drug resistance (Bradley *et al*., 1996). However, resistance to artemisinin has been demonstrated *in vitro* (Bradley and Bannister, 2001). The drug must therefore be used sparingly to avoid resistance development.

Resistance has been reported for almost all anti-malarial drugs, necessitating research on combination therapy which involves the use of at least 2 anti-malarial drugs with different modes of action (WHO, 1997). The principle is based on potentiation of the drugs leading to reversal or delay of resistance (WHO, 1986). The first combination was Fansidar® (sulfadoxine + pyrimethamine) (SP) (WHO, 1986; Miller, 1986). Although SP is still reliable in most areas in Africa, resistance is increasing and could potentially develop to render the drug useless in the near future (WHO, 1996). A strategy that has received much recent attention is the combination of three anti-malarial drugs, such as mefloquine **(4)** and SP or chloroquine and SP (WHO, 2001a). In recent study done in Nigeria, the combination of chloroquine and SP (CSP) was found to be safe and had superior efficacy compared to chloroquine alone (WHO, 2001a).

Artemisinin combination therapy (ACT) is currently recommended for areas with chloroquine and SP resistance. Some African countries such as Zambia, S. Africa, Kenya and Zanzibar have adopted ACT as first line treatment for malaria after some drugs exceeded 15% resistance level. The most common ACT is amodiaquine (**3**) with an artemisinin derivative (WHO, 2001b).

Kenya has recently accepted Coartem® (a combination of artemether and lumefantrine) as a first line anti-malaria drug but it is too expensive for the poor. Currently, Coartem® is 10 times more expensive than SP drugs, going well beyond the reach of the majority of Kenyans (Gakuu, 2004). Other ACTs recommended in Kenya are amodiaquine plus

artesunate and mefloquine plus artesunate. Beside drug resistance, chemotherapy is limited by lack of effective case management, diagnosis and poverty levels in most endemic areas (Salako *et al.*, 1990). This is made worse by the existence of unofficial health care systems like unlicensed medicine vendors, traditional herbal healers, and spiritual healers which run parallel to the official system (Salako *et al.*, 1990).

The reduced efficacy of conventional anti–malarial drugs and their combinations, in all regions of the world due to resistance development, has enhanced the need for novel chemotherapeutic agents. However, few alternative drugs are under development, necessitating urgent efforts to identify new classes of anti-malarial compounds. Plants are a potential source of new anti-plasmodial compounds and therefore, the focus of much of the current anti-malarial drug research and development. The clinical utility of quinine and quinidine **(1)** isolated from cinchona bark, and the discovery of artemisinin from the Chinese herb, A*rtemisia annua*, has stimulated much interest in plants as potential sources of new anti–malarial drugs.

2.2 Plants used for malaria treatment

In the survey of traditional anti-malarial plants used in Lake Victoria basin, *Maytenus heterophylla, Strychnos usambarensis, Strychnos henningsii* and *Periploca linearifolia* were found to be commonly used to treat malaria. The investigation of the toxicity, anti-oxidant,

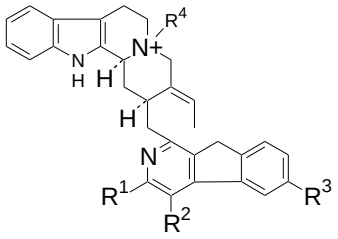
anti-plasmodial activity and isolation of pure compounds in these traditional anti-malarial plants was undertaken.

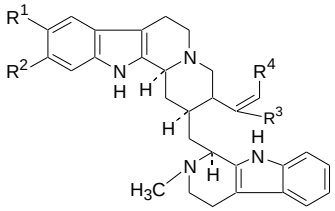
2.2.1 *Strychnos usambarensis (*Gilg)

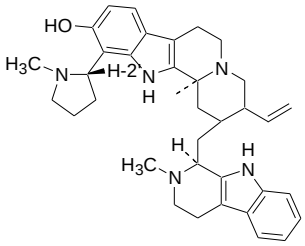
Strychnos usambarensis (Loganiaceae) grows into a shrub and has a grey bark which is sometimes smooth or granulated (Beentje, 1994). The local names: include *Akwalakwala* (Luo), *Gitarangui* (Kamba) and *Mutikani* (Kikuyu). The leaf extract is used as an anti-inflammatory (Chhabra *et al*., 1984) while the root extract is used to treat chest pains (Kokwaro, 1993).

Figure 1: *Strychnos usambarensis*

In previous phytochemical studies, several compounds including usambarensine **(19)**, dihydrousambarensine **(20)**, *N*-4-methylusambarensine **(21)**, 10'-hydoxyusambarensine **(22)**, usambarine **(23)**, dihydrousambarine **(24)**, 10-hydroxyusambarine **(25)**, 11-hydroxydihydrousambarine **(26)**, 10-hydroxydihydrousambarine **(27)**, 11-hydroxydihydrousambarine **(28)**, strychnopentamine **(29)** and isostrychnopentamine **(30)** have been isolated from *S. usambarensis* **(**Bisset *et al.*, 1973; Bonjean *et al.*, 1996; Caprasse *et al*., 1984).







19 R^1, R^2, R^3, R^4=H
20 R^1, R^2 =OH; R^3, R^4=H
21 R^1, R^2, R^3=H; R^4=OCH_3
22 R^1, R^2, R^4=H; R^3=OH

23 R^1, R^2, R^3, R^4 =H
24 R^1, R^2 =H; R^3, R^4=OH
25 R^1=OH; R^2, R^3, R^4=H
26 R^1, R^3, R^4=H; R^2=OH
27 R^1, R^3, R^4=OH; R^2=H
28 R^1=H; R^2, R^3, R^4=OH

29 α-H-2'
30 β-H-2'

The *in vitro* anti-plasmodial activity of the extracts and the isolated alkaloids against FCA and W2 strains of *P. falciparum* has been reported. High activity (IC_{50} < 1 µg/ml) was reported for the root and leaf extracts of *S. usambarensis*. The extract from the stem bark of *S. usambarensis* also had high activity (IC_{50} 1 µg/ml). Many alkaloids isolated from *S. usambarensis* were found to be active against the CQ–sensitive *P. falciparum* FCA 20 strain. Strychnopentamine **(29)** (IC_{50} 0.117 µM), isostrychnopentamine **(30)** (IC_{50} 0.120 µM), 10-hydroxyusambarensine **(22)** (IC_{50} 0.487 µM), dihydrousambarensine **(20)** (IC_{50} = 0.857 µM) were found to be the most active (Michel *et al.*, 1999). Seven other alkaloids: (usambarensine **(19)**, 10'-hydroxyusambarensine **(22)**, usambarine **(23)**, dihydryousambarine **(24)**, 11-dihydroxyusambarine **(28)** and tetradehydrolongicaudatine Y **(31)**, exhibited moderate anti-plasmodial activity (IC_{50} 1- 2 µM). The activity of the compounds against W2 strain, were of the same order as those of FCA strain. However, two compounds (usambaresine **(19)**, IC_{50} 1.3 µM and 10'-hydroxyusambarensine **(22)**, IC_{50}1.8 µM) were approximately twice less active against the resistant clone than the susceptible clone. The activity for dihydrousambarensine (IC_{50} 32 nM) was 30-fold higher than for the CQ-resistant strain (IC_{50} = 0.03 µM). However, its 90% inhibitory

concentration (IC_{90}) remained high (4.6 µM). The IC_{50} of strychnopentamine was the same (0.117 µM) for both strains, while its isomer, isostrychnopentamine, also exhibited the same activity but with a lower IC_{90} (0.6 µM) for W2 strain. On the other hand, some alkaloids, like strychnophylline **(32)** (IC_{50} = 5.0 µM) and the dihydroxyusambarine **(24)** (IC_{50} = 4.9 µM), were significantly less active against the W2 strain (Frederich *et al.*, 1999; Michel *et al.*, 1999).

2.2.2 *Strychnos henningsii (*Gilg)

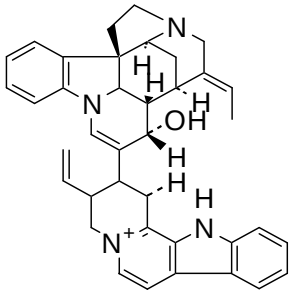
It is a shrub or a tree of about 2.5 - 12 m belonging to the Loganiaceaea family and Strychnos genus. Local names include: *Akwalakwala lyech* (Luo), *Kara*, *Karrah* (Borana), *Muteta* (Kamba, Kikuyu), *Mase, Legutuet* (Kipsigis), *Olduyesi* (Maasai), *Muchambe* (Meru), *Chibulukwa* (Samburu), *Hadesa* (Somali), *Turubupwa*, *Turkukwa* (Tugen) and *Yopoliss* (Turkana). It has a pale grey bark that is rough. The leaves are glossy, leathery and elliptic (Beentje, 1994). The flowers are white cream or yellow.

Figure 2: *Strychnos henningsii*

Strychnos henningsii root extract is drunk as a treatment of chest pains and internal injuries (Kokwaro, 1993) while fresh root is chewed for snake bite treatment. The

leaf extract is used for treatment of rheumatism and gastrointestinal complains (Chabbra *et al.,* 1984).

Strychnopentamine **(29)** and isostrychnopentamine **(30)** have been isolated from *S. henningsii* root bark (Bosly, 1951). The anti-plasmodial alkaloids isostrychnofoline **(33)** (IC_{50} 1.2 µM) and holstiine **(34)** (IC_{50} 1 µM), have also been isolated from *S. henningsii* root bark (Frederich *et al.,* 2002).



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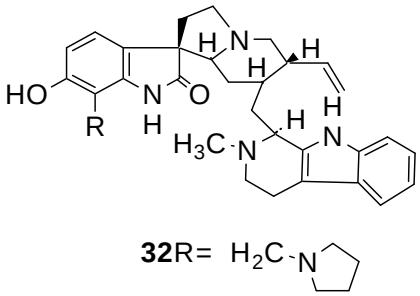
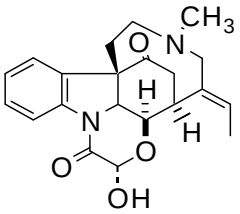


32 R= H_2C-N

33 R=H



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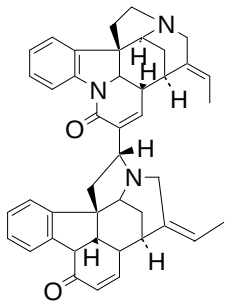
Other *Strychnos* species that exhibit moderate anti-plasmodial activity (IC_{50} 10 - 30 µg/ml) (Frederich *et al.,* 1998; 1999; Michel *et al.,* 1999) include *S. gossweileri* (Quetin-Leclercq *et al.,* 1991), *S. guianensis* (Wright *et al.,* 1990), *S. variabilis, S. angolensis* and *S. memecyloides*. Sungucine **(35)**, from *Strychnos icaja* roots, exhibited good anti plasmodial activity, (IC_{50} 3 µM) (Bisset *et al.,* 1973; Kambu *et al.,* 1979).



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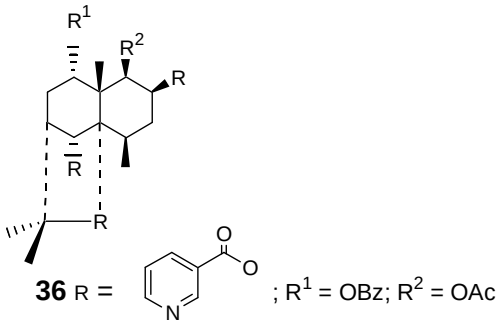
2.2.3 *Maytenus heterophylla* (Eckl. and Zeyh)

It is a shrub or a small tree of about 1.5 – 6 m from the family Celastraceae. The local names include: *Anguong'o* (Luo), *Mdunga Mdeewe* (Swahili), *Mokalakala* (Malakote), *Muthuthi* (Kikuyu), *Kigorwet* (Kipsigis, Ndorobo), *Kumwayakhafu* (Luhya), *Teerkolwa* (Marakwet) and *Olaimorongai* (Maasai) (Beentje, 1994; Kokwaro, 1993). The leaves are often, but not always fasciculated on short shoots. It produces white, cream or yellow flowers.

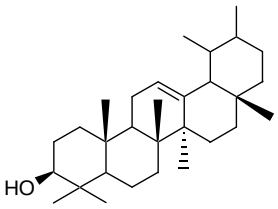
Figure 3: *Maytenus heteropylla*

Its roots are boiled and the liquid drunk as an anti-helminthic and also for the treatment of hernia. Roots are also used as a cure for syphilis (Kokwaro, 1993).

Most plants in the family contain a class of sesquiterpene nicotinoyl alkaloids (Schaneberg *et al.,* 2001) with insecticidal activity (Sekar *et al.,* 1996). The alkaloids are characterized by a dihydroagarofuran sesquiterpene bonded as a diester to a substituted nicotinic acid. 1β-Acetoxy-9α-benzoyloxy-2β, 6α-dinicotinoyloxy-β-dihydroagarofuran **(36)** and β-amyrin **(37)** have been isolated from *M. heterophylla* (Orabi *et al.,* 2001).



36 R = ; R^1 = OBz; R^2 = OAc



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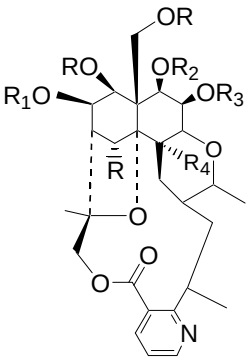
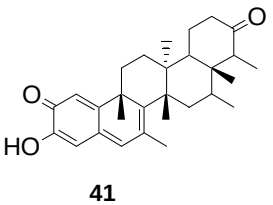
Other Maytenus species include: *M. puttericoides, M. buchananii, M. krukovii* and *M. arbutifolia*. Maytein **(38)** was isolated from *M. krukovii* (Sekar *et al.,* 1995). Putterine A **(39)** and putterine B **(40)** were isolated from *M. putterlickoides* (Schaneberg *et al.,* 2001). Leaves of *M. diversifolia* contain maytenin **(41)** (Melo *et al.,* 1974) which has been used to treat cancer (Nozaki *et al.,* 1990).



38 R, R_1, R_2 = Ac; R_3 = Bz; R_4= OH

39 R_1= ; R, R_2, R_3 = Ac; R_4 = OH

40 R_1 = ; R, R_2 ,= Ac; R_3 = $(CH_3)_2CH_2CO$-



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Hot water extracts of *M. heterophylla, M. senegalensis* and *M. acuminate* from Kenya have been screened for *in vivo* anti-malarial activity in mice against a CQ resistant *P.*

berghei NK65 alone and in combination with CQ. The root bark extracts of all the three plants had moderate activity (33 - 49 % parasitaemia suppression). In combination with CQ, the root bark extract of *M. heterophylla* had improved parasitaemia suppression (38 - 66%) indicating a synergistic interaction (Muregi *et al.*, 2006).

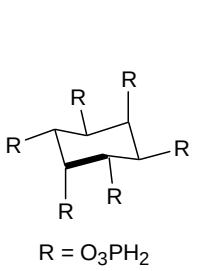
2.2.4 *Periploca linearifolia* (Dill and A.Rich).

It is a climbing herb or a real liana upto 10 m long belonging to the Asclepiadaceae family. Local names include: *Yathi.Rateng* (Luo), *Mwembaiguru* (Kikuyu), *Sinendet* (Kipsigis, Okieikpokot), *Muhalia* (Luhya), *Osinendei, Osinande* (Maasai), *Esinindet* (Marakwet) and *Chokilewa* (Turkana). The leaves are linear or narrowly elliptic. It produces cream or greenish-yellow flowers (Beentje, 1994). The plant is mostly woody and much branched. The young stems are densely pubescent (Beentje, 1994).

Figure 4: *Periploca linearifolia*

The bitter leaf extract is used as a tonic alterative and as an anti-helminthic. It is also used for treatment of eye infections, bronchitis and ulcers (Jain and Sharma, 1967). In India, the plant is used to treat diabetes (Dixit and Panday, 1984).

The leaf extract contains phytin **(42)**, anthraquinone **(43)**, gemnamagenin **(44)**, gymnemic acid II **(45)** (Nagaraju and Rao, 1990; Hiji, 1993), betaine **(46)**, cholin **(47)** and lupeol **(48)** (Hichri *et al.*, 2002).



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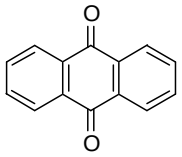

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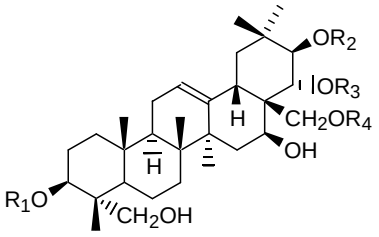
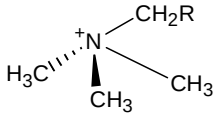
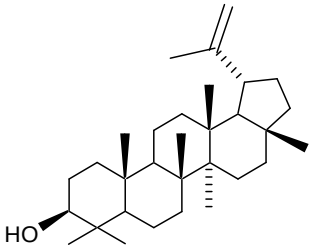


44 R_1,R_2,R_3,&R_4=H

45 R_2,R_3,R_4= methylbutanoate;R_1=gluc



46 R = CO_2H
47 R = CH_2OH



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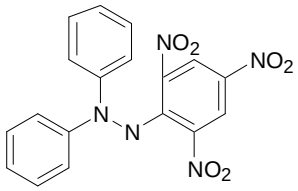
2.3 DPPH radical scavenging

Radicals are a group of atoms joined together in a particular structure and take part in most chemical reactions (Brand-Williams *et al.*, 1995). Some free radicals are presumed to have deleterious effects in the human body. Anti-oxidants scavenge for the free radicals and protect the human body against radicals which may cause severe pathological conditions such as ischemia, anaemia, asthma, arthritis, inflammation and neuro-degeneration (Nakayoma and Yamada, 1995). There is a preference for anti-oxidants from natural rather than from synthetic sources (Abdalla and Roozen, 1999). There is therefore a parallel increase in the use of methods for estimating the efficiency of such substances as anti-oxidants in natural products (Sa′nchez-Moreno, 2002; Schwarz *et al.*, 2001). Thearapeutic activity of some drugs has been attributed to the ability to interfere with free radical processes (Kourotinakis *et al.*, 2002). For example, chloroquine (CQ) **(2)**

which is commonly used in treating malaria acts directly or indirectly and alters anti-oxidant status that makes certain organs more susceptible to stress. Several studies have shown that CQ causes increased lipid peroxidation and decreased enzymic and non-enzymic anti-oxidants and results into side effects such as cinchonism, hypoglycemia and hypotension. In this case different concentrations of methanol extracts of selected medicinal plants were assessed for their anti-oxidant activity using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) (**49)**.



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The DPPH radical ($C_{18}H_{12}N_5O_6$) is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalization also gives rise to the deep purple color, characterized by an absorption band in methanol solution centered at about 520 nm. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of the purple color. Representing the DPPH radical by Z• and the donor molecule by AH, the primary reaction can be represented by the chemical equation 1:

Equation 1: Z •+ AH = ZH + A•

2.4 *In vitro* anti-plasmodial assay

In vitro assays are performed during rational drug discovery, in order to determine the activity of target molecules before the toxicity in the real organism is determined. Hypoxanthine is the most important parasite growth promoting component. *P. falciparum*

requires hypoxanthine formed from ATP catabolism in red blood cells (RBC) for nucleic acid synthesis and energy metabolism. In *in vitro* anti-plasmodial assay, the test drug and radioactive-labeled hypoxanthine are added to the cultures and the uptake of hypoxanthine by the parasite monitored. The ability of the test drug to inhibit uptake of the hypoxanthine is a measure of its capability to inhibit parasite growth. It is usually determined by a liquid scintillation counter. Generally, anti-plasmodial activity of IC_{50} >50 µg/ml is considered to be inactive (Sixsmith *et al.*, 1984).

2.5 Brine shrimp toxicity assay

Brine shrimp [*Artemia Salina* (Leach)] assay is used as a basic indicator of toxicity. Their eggs are readily available and hatch easily within 48 hours in seawater to provide a large number of larvae (nauplii) for experimental use (McLaughlin *et al.*, 1991). Brine shrimp toxicity bioassay is inexpensive and easily utilizes large number of organisms for statistical validations. The method requires relatively small amount of samples (2 -20 mg or less). The assay involves killing of the brine shrimps. Their use in experimental work has so far not been objected by animal rights advocates (McLaughlin *et al.*, 1991). An extract with a lethal concentration (LC_{50}) upto 200 µg/ml is considered lethal or toxic. In the current study, they were used as basic indicators of the toxicity of traditional anti-malarial herbs.

2.6 Rationale and Justification

Many rural indigenous communities have a long history of the use of plant-derived substances in traditional medicine and protection against pests and vectors. However, these products contain complex mixtures of chemical constituents of which several remain unknown. Chemical and biological investigations of medicinal or pesticidal plant

products have often led to the isolation of structurally interesting biologically useful compounds. The search for new, effective, less toxic and environmentally safe compounds as opposed to the more toxic ecologically harmful, non-biodegradable synthetic products has recently been intensified. Consequently, the project aims at enhancing awareness on the value of traditional anti-malarial plants and conservation of wetland biodiversity. The results obtained from these studies will contribute immensely to the WHO goal of reduction of the malaria burden in Africa to half by the year 2010.

2.7 Statement of the problem

Herbal anti-malarial drugs are common, accessible and acceptable to local indigenous communities which have no access to modern health facilities and drugs. The anti-plasmodial efficacy and toxicity of the herbal extracts and the bioactive chemical principles from some plants that are used in Lake Victoria basin have not been investigated.

2.7.1 Hypothesis

Plants in the Lake Victoria basin that are used in traditional malaria therapy may contain principles that inhibit the growth of *P. falciparum* and can be developed into safe herbal drugs or combinations for the treatment of malaria.

2.7.2 Objectives

2.7.2.1 General objective

To investigate the anti-plasmodial activity and the active principles of selected plants which are commonly used by traditional medical practitioners in Lake Victoria basin for treatment of malaria.

2.7.2.2 Specific objectives

i. To extract and screen *Strychnos usambarensis, S. henningsii, Maytenus heterophylla* and *Periploca linearifolia* the extracts for anti-plasmodial activity.

ii. To extract and screen *Strychnos usambarensis, S. henningsii, Maytenus heterophylla* and *Periploca linearifolia* the extracts for toxicity using brine shrimp assay.

iii. To carry out anti-oxidant activity of the methanol extracts

iv. To undertake in vitro activity guided fractionation and isolation of anti-plasmodial compounds from *Strychnos usambarensis, S. henningsii, Maytenus heterophylla* and *Periploca linearifolia* extracts with high activity.

v. To characterize the anti-plasmodial compounds from *Strychnos usambarensis, S. henningsii, Maytenus heterophylla* and *Periploca linearifolia* using chemical, physical and spectroscopic methods.

CHAPTER THREE

MATERIALS AND METHODS

3.1 General procedures

All solvents including Methanol, *n*-hexane, chloroform and ethyl acetate were purchased from Kobian Kenya Ltd., Nairobi and were of laboratory grade. They were distilled before use. Dimethylsulphoxide (DMSO) and sulphuric acid were of analar grade.

The glassware that was used were soaked in chromic acid overnight, washed and rinsed with tap water followed by acetone. The clean glassware was then dried in an oven at 110 ^{o}C for 1 hr.

Sulphuric acid (25%), which was used as a general purpose TLC locating agent, was made by pouring concentrated acid (25 ml) slowly into distilled water (75 ml). Vanillin-sulphuric acid spray, used for detection of terpenoids, was prepared by dissolving vanillin (0.5 g) into absolute ethanol (25 ml) followed by slow addition of concentrated sulphuric acid (100 ml).

3.1.1 Plant material

Plant materials were collected from the Lake Victoria basin on the Kenyan side.

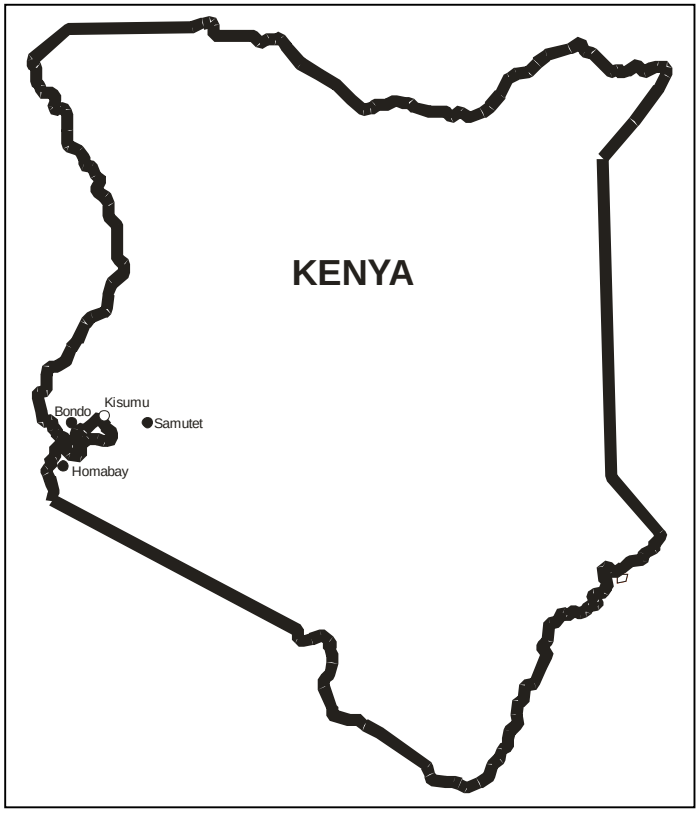
(i). *Maytenus heterophylla* was sampled from Rongo in Homa Bay.

(ii). *Periploca linearifolia* was sampled at Samutet on Kisumu – Kericho road.

(iii). *Strychnos usambarensis* was sampled from Got Ramogi Hill in Bondo District.

(iv). *Strychnos henningsii* was sampled from Got Ramogi Hill in Bondo District.

Figure 5: Map of Keya showing where plant materials were collected

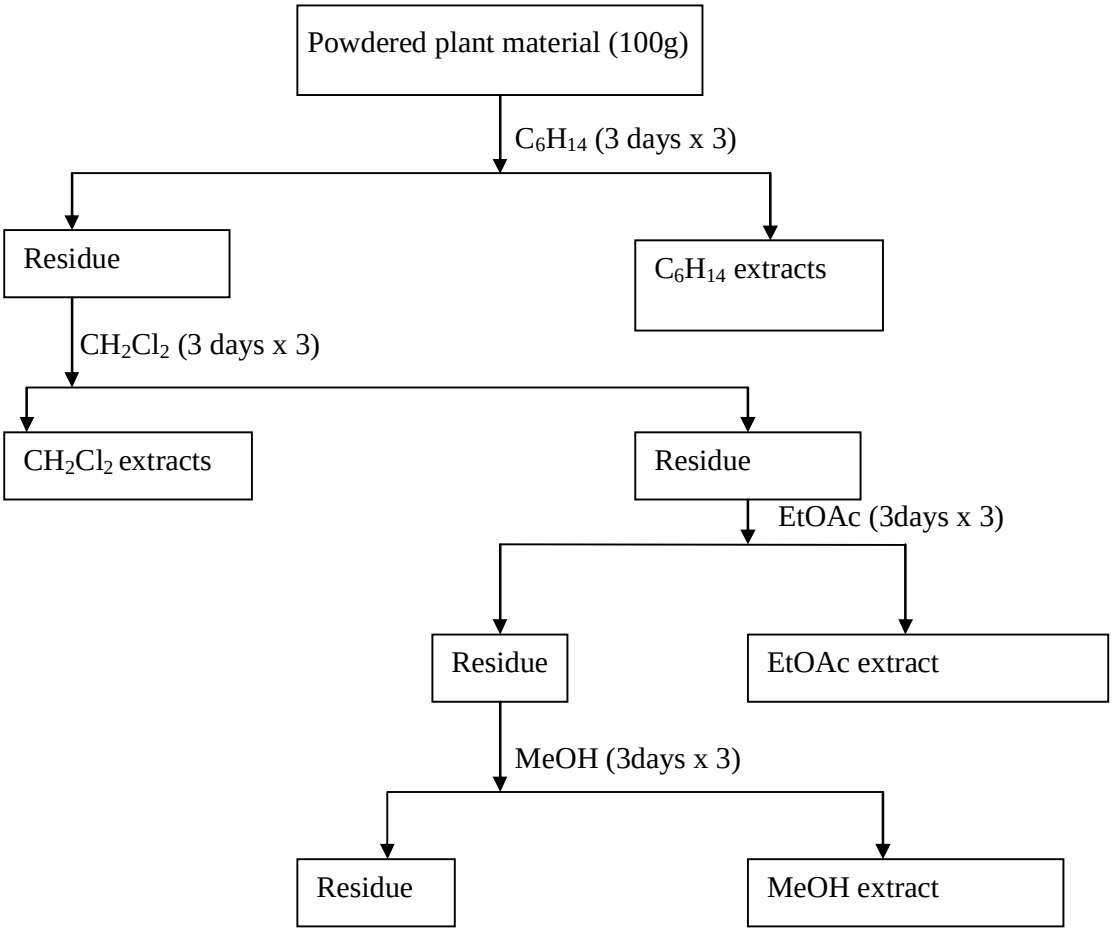


The plants were then identified by a taxonomist at the Department of Botany Herbarium at the University of Nairobi. Voucher specimens were deposited at the Herbarium and the numbers assigned as follows: *M. heterophylla* (2004/19), *P. linearifolia* (2004/24), *S. usambarensis* (2004/25), and *S. henningsii* (2004/26). The plants were air-dried under shade for 14 days and ground into powder using a laboratory mill.

3.1.2 Extraction

For each plant part, 100 g of the powder was extracted sequentially using hexane, chloroform, ethyl acetate and methanol (Scheme 1). The extracts were then decanted and filtered through Whatman filter paper and the crude extracts concentrated under reduced pressure. The dry samples were then stored in a freezer at -4^{0} C.

Scheme **1**: Procedure for extraction of plant material.



3.2 Isolation, purification and structural elucidation

The different plant extracts with high anti-plasmodial activity were subjected to chromatographic separation methods. Combinations of chromatographic techniques (VLC, MPLC, CC and TLC) were used to fractionate and purify the extracts.

Chromatography and purification was done using column chromatography (CC) on Kieseigel silica gel 60 (0.63-0.2 mm/70-230 Mesh ASTM, Merck and Aldrich, Germany) and eluted with a slow gradient of different solvent systems. During packing of both columns the slurry method was used. The plant extract was dissolved in minimum possible solvent, mixed with equal amount of silica gel, and ground into fine powder and

the solvent removed. The dry powder was introduced at the top of the column and covered with a small amount of silica and cotton wool to minimize disturbances during the addition of the eluting solvent. Different solvent systems were used and the presence of any pure fractions monitored by TLC. Analytical pre-coated plastic (Polygram ® sil G/UV $_{254}$) and aluminium (Alugram® sil G/UV $_{254}$, Machery-Nagel GmbH and Co., Germany) TLC plates were used. TLC analysis of extracts and fractions assisted in establishing the best solvent system for separation, complexity of extracts and purity of the isolates. Visualisation of the spots developed on a TLC plate was done using long and short wavelengths (365 and 254 nm) on an ENF-240 C/F UV lamp (Spectronics Co., Westbury). The TLC plate was then sprayed using vanillin-sulphuric acid or 25% sulphuric acid followed by heating for 10 minutes at 110 ºC in an oven. Fractions that showed presence of similar compounds were combined and concentrated to give pure or semi- purified portions. The purity of the compounds was checked by spotting them on a TLC plate and developing the plate using different solvent systems (Hayashi *et al.,* 1990).

Characterization of the purified compounds was done by physical, chemical methods and spectroscopic: UV, IR, MS, 1D (^{1}H, ^{13}C and DEPT) and 2D NMR techniques. The 2D NMR techniques used included COSY, NOESY and HETCOR (Duddeck and Dietrich, 1998).

3.2.1 Spectroscopic analysis

3.2.1.1 Melting point

Melting points of pure samples in open capillary tubes were recorded using Gallenkamp melting point apparatus (Sanyo, West Sussex-UK) and were uncorrected.

3.2.1.2 Infra red spectroscopy (IR)

IR spectra of pure samples were recorded on a Perkin-Elmer 593 spectrophotometer FTIR series (Tokyo, Japan) measured as KBr pellets.

3.2.1.3 Ultra violet spectroscopy (UV)

UV spectra of pure compounds were recorded on a Shimadzu UV-160A UV-Vis spectrophotometer (Tokyo, Japan).

3.2.1.4 Nuclear magnetic resonance (NMR)

^{1}H, ^{13}C, COSY, DEPT, NMR spectra were recorded on a Bruker 300 machine using $CDCl_3$ as solvent and TMS as internal standard. Chemical shifts were recorded in δ units (ppm) and coupling constants (J) in Hz. The ^{13}C NMR multiplicity was determined by the DEPT experiments.

3.2.1.5 Mass spectrometry (MS)

MS analyses were carried out in Finigan Mat SSQ700 single quadruple instrument and Autospec time of flight (TOF) spectrometer EI-MS and ES-MS;.

3.3 Bioassay methods

3.3.1 Brine shrimp toxicity test

The toxicity of the extracts was monitored by the brine shrimp lethality test (Meyer *et al.*, 1982). Brine shrimp, *Artemia salina* Leach eggs (Interpet Ltd., Dorking, England) were hatched in artificial seawater prepared by dissolving 38 g of sea salt (Sigma Chemicals Co., UK) in one litre of distilled water. After 48 h of incubation at room temperature (22-26 ^{o}C), the larvae (nauplii) were attracted to one side of the vessel with a light source and collected with a Pasteur pipette. Nauplii were separated from eggs by aliquoting them three times in small beakers containing sea water (Alkofahi *et al.,* 1989). Plant extracts were dissolved in 5 µl dimethylsulphoxide (DMSO), diluted with artificial sea salt water so that the final concentration of DMSO did not exceed 0.05%. The plant extract (50 µl, 4000 ppm) was placed in row one and a two-fold dilution carried out down the column. The last row had sea salt water and DMSO only and served as the drug-free control. To each well 100 µl suspension containing 10 naplii was added and the micro-titre plate incubated for 24 h (Solis *et al.,* 1995). The plates were then examined under a microscope (x12.5) and the number of the dead nauplii in each well counted. Each experiment was replicated three times and average values determined. The activity of each extract was determined from the 50% lethality concentration fifties (LC_{50}) using Finney Probit analysis (McLaughlin *et al.,* 1991).

3.4 Preparation of drugs for *in vitro* anti-plasmodial assay

Stock solution of crude extracts (10 ml, 250µg/ml) were made with sterile deionized water and filtered through 0.45 and 0.22 µm micro-filters under a laminar flow hood. The water insoluble extracts were first dissolved in dimethylsulphoxide (DMSO) (solvent concentration < 0.02%) (Elueze *et al.,* 1996) and sonicated to a homogenous solution.

Stock solutions of CQ, MQ and quinine (1 µg/ml) were similarly prepared in sterile water. All the drug solutions were stored at -20 ^{o}C and retrieved only during use.

3.4.1 *In vitro* anti-plasmodial assays

The *in vitro* anti-plasmodial assays were carried out in collaboration with Kenya Medical Research Institute (KEMRI). The *in vitro* semi-automated micro-dilution assay technique that measures the ability of extracts to inhibit the incorporation of [G-^{3}H]-hypoxanthine into the malaria parasite was used (Desjardins *et al.*, 1979; Muregi *et al.*, 2003). Laboratory-adapted *P. falciparum* cultures of W2 and D6 were used in this study.

The parasite culture medium was that described by Trager and Jensen (1976). Human type O+ erythrocytes (< 28 days old) were used as host cells and the cultures incubated at 37 ^{o}C in a mixture of 5% O_2, 5% CO_2 and 90% N_2. Aliquots (25 µl) of culture medium were added to all the wells of a 96-well flat-bottomed micro-culture plate (Costar Glass Works, Cambridge, UK). The test solutions (25 µl) were added, in duplicate; to the first wells and a Titertek motorized hand diluter (Flow Laboratories, Uxbridge, UK) used to make two-fold serial dilutions of each sample over a 64-fold concentration range. A suspension (200 µl, 1.5% v/v) of parasitized erythrocytes (0.4% parasitaemia) in culture medium and growth rate (>3-fold per 48 h) were added to all test wells. Non-parasitized erythrocytes were used in control experiments. The plates were incubated at 37 ^{o}C in a mixture of 5% O_2, 5% CO_2 and 90% N_2. After 48 h, each well was pulsed with 25 µl of culture medium containing 0.5 µCi, of [G-^{3}H]-hypoxanthine and plates incubated for 18 h. The contents of each well were harvested onto glass fibre filters, washed thoroughly with distilled water, dried and the radioactivity measured by liquid scintillation counter. Computation of the drug concentration causing 50% inhibition of [G-^{3}H]-hypoxanthine

uptake (IC_{50}) was done by interpolation of logarithmic transformation of concentration and counts per minute (cpm) value using:

$$IC_{50} = \text{antilog}\left[\log X_1 + \frac{[(\log Y_{50} - \log Y_1)(\log X_2 - \log X_1)]}{(\log Y_2 - \log Y_1)}\right]$$

Where Y_{50} is the cpm value midway between parasitized and non-parasitized control cultures and X_1, Y_1, X_2 and Y_2 are the concentrations and cpm values for the data points above and below the cpm midpoints respectively (Sixsmith *et al.*, 1984).

3.5 Preparation of samples for DPPH radical scavenging

The dried methanol extracts of the four plants were made to different concentrations (1500, 1000, 500, 250 ppm). Caffeic acid (3, 4-dihydroxycinnamic acid, Riedel de Haen, Seezle, Germany) was used as a control. The radical scavenging activity of the tested fraction was expressed as the % decrease in concentration compared with caffeic acid (% activity of caffeic acid).

3.5.1 DPPH radical scavenging test

A solution of DPPH (10^{-4} M) was prepared by dissolving 0.0039 g of the radical DPPH in 100 ml MeOH. The solution attained a deep purple colour and was left in the refrigerator at -4 ^{o}C for 2 h in order for the absorbance to stabilize. A calibration curve of concentration versus absorbance of DPPH was constructed as follows: the 10^{-4} M solution of DPPH was diluted with MeOH, the solutions vortexed, left in the dark and their absorbance measured in a UV-Vis spectrophotometer (Shimadzu UV-160A) at λ_{max} 520 nm. The calibration curve of absorbance (y) versus concentration (x) of DPPH was expressed by the following equation:

$y = 11050x$; $R=0.9998$

For the radical scavenging test, methanol was used as the blank. DPPH (10^{-4} M, 2.9 ml) solution was placed in a cuvette and the absorbance measured at t = 0 (A_0). Subsequently, 0.1 ml solution of each of the tested extract was added to the above volume of DPPH and the absorbance measured at regular time intervals until its value reached a plateau (steady state, A_T). The concentration of the mixture of DPPH with each extract was estimated using the calibration curve (y= 11050x; R=0.9998). For each of the extracts tested, the following parameters : % decrease in DPPH absorbance, % decrease in DPPH absorbance as compared to that of cafffeic acid, were estimated in order to evaluate the radical scavenging activity of the samples. All experiments were done in duplicate and the percent scavenging effect (ΔC) was determined by comparing the absorbance of solution containing the test sample to that of control solution without the test sample taking the corresponding blanks using the equation:

$$\Delta C = C_O\text{-}C_T/C_O \text{ x100 \%}$$

Where C_O is the concentration of DPPH before the addition of each sample, and C_T the final concentration of the mixture (DPPH + each sample)

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Extraction

The powdered root bark from each plant was extracted sequentially using hexane, chloroform, ethyl acetate and methanol. Table 1 summarizes the percentage yields of crude extracts obtained from extraction with various solvents.

Table 1: The percentage yield of plant extracts

Plant	% yield C_6H_{14}	$CHCl_3$	EtOAc	MeOH
S. usambarensis	0.18	0.25	0.37	0.55
S. henningsii	0.13	0.18	0.37	0.53
P. linearifolia	0.68	0.62	1.38	1.95
M. heterophylla	0.15	0.39	0.20	1.00

Root bark used

Periploca linearifolia methanol extract gave the highest yield (1.95 %) followed by ethyl acetate (1.38 %), while *Strychnos henningsii* hexane extract gave the lowest yield (0.13 %). Generally, *P. linearifolia* and *S. henningsii* extracts gave the highest and the lowest yields respectively, for all solvents except the ethyl acetate fraction of *M. heterophylla*. Methanol was used as an extraction solvent instead of water that is normally used in herbal drug preparations since it has comparable polarity.

4.2 *In vitro* anti-plasmodial assay

The chloroform, ethyl acetate, and methanol extracts of *S. usambarensis*, *S. henningsii*, *M. heterophylla* and *P. linearifolia* were screened for *in vitro* anti-plasmodial activity against CQ-sensitive (D6) and resistant (W2) *P. falciparum* strains (Table 2).

Table 2: *In vitro* anti-plasmodial activity (IC_{50} ±SD μgml^{-1}) of extracts of selected medicinal Plants

		IC_{50} µg/ml ±SD	
Plant	Extract	D6	W2
S. usambarensis	$CHCl_3$	10.58±2.90	24.37±0.40
	EtOAc	15.65±4.29	24.00±2.20
	MeOH	23.82±1.36	27.92±0.70
S. henningsii	$CHCl_3$	5.35±1.32	16.20±2.38
	EtOAc	25.07±0.96	37.43±0.96
	MeOH	1.07±0.07	26.25±0.04
M. heterophylla	$CHCl_3$	4.00±0.44	5.56±0.78
	EtOAc	18.95±0.10	29.04±0.85
	MeOH	13.07±1.60	19.53±4.40
P. linearifolia	$CHCl_3$	8.65±1.73	24.69±0.73
	EtOAc	25.78±1.57	36.13±1.87
	MeOH	1.60±0.01	3.30±0.01
Chloroquine	H_2O	0.024±0.001	0.003±0.001
Mefloquine	H_2O	0.010±0.001	0.020±0.001
Quinine	H_2O	0.120±0.001	0.190±0.001

All the extracts had IC_{50} < 50 µg/ml. Generally, the activity of all the fractions against D6 (sensitive strain) was higher compared to that of W2 strain. The anti-plasmodial activity (IC_{50}) of the extracts was in the range of 1.07 - 25.78 and 3.30 - 37.43 µg/ml for D6 and W2 strains, respectively.

P. linearifolia methanol extract was the most active extract against W2 strain (IC_{50} 3.30 ± 0.01 µg/ml), followed by *M. heterophylla* chloroform extract (IC_{50} 5.56 ± 0.78 µg/ml). All the other extracts had IC_{50} > 15 µg/ml.

S. henningsii methanol extract (IC_{50} 1.07 ± 0.07 µg/ml) showed the highest activity against the D6 strain followed by *P. linearifolia* methanol extract (IC_{50} 1.60 ± 0.01 µg/ml). All

the methanol extracts showed high activity (IC_{50} 1.07 - 13.07µg/ml) against the D6 strain apart from *S. usambarensis* (IC_{50} 23.82 ± 1.36 µg/ml)

The ethyl acetate extracts showed the lowest activity (IC_{50} 15 - 26 µg/ml and 24 - 37 µg/ml for D6 and W2 strains, respectively) for all the four medicinal plants. The chloroform extracts showed moderate activity (IC_{50} 4 - 11 µg/ml) against D6 strain for all the four plants.

Despite *S. henningsii* and *S. usambarensis* being in the same genus they showed different anti-plasmodial activity levels. For the chloroform and methanol extracts, *S. henningsii* was more active than *S. usambarensis* while for ethyl acetate extracts the reverse was observed. From the chemical literature information, more alkaloids have been found in *S. henningsii* than in *S. usambarensis*. These results are in agreement with those reported on previous anti-plasmodial assay of other *Strychnos* species (Frederick *et al.,* 2002; Kiriria *et al.,* 2006).

The observed anti-plasmodial activity of the extracts may explain the widespread use of these plants in treatment of malaria in the Lake Victoria basin. However, the activity of the plant extracts were much less than for the standard drugs (IC_{50} 0.01- 0.03 µg/ml) used in malaria treatment.

4.3 Brine shrimp toxicity test

All the fractions were screened against brine shrimp nauplii and the results summarized in Table 3.

Table 3: Brine shrimp toxicity assay of selected anti-plasmodial plant extracts

Plant	Extract	LC_{50}±S.D
S. usambarensis	C_6H_{14}	327.69±5.24
	$CHCl_3$	479.42±0.47
	EtOAc	707.65±6.60
	MeOH	422.42±4.95
	H_2O	962.11±3.20
S. henningsii	C_6H_{14}	457.18±3.17
	$CHCl_3$	554.72±6.48
	EtOAc	591.29±7.84
	MeOH	344.76±4.66
	H_2O	282.16±2.80
M. heterophylla	C_6H_{14}	806.72±5.17
	$CHCl_3$	493.12±2.32
	EtOAc	335.79±3.44
	MeOH	549.47±2.70
	H_2O	528.32±2.42
P. linearifolia	C_6H_{14}	622.55±3.97
	$CHCl_3$	353.91±2.97
	EtOAc	313.79±2.90
	MeOH	370.85±2.76
	H_2O	322.16±3.80
Mefloquine	H_2O	342.15±2.43
Chloroquine	H_2O	280.12±1.67
Quinine	H_2O	312.65±1.84

All the extracts showed low toxicity (LC_{50} >200 µg/ml) to brine shrimps. *Periploca linearifolia* EtOAc extract (LC_{50} 313.79 ± 2.90) was the most toxic while *S. usambarensis* methanol extract (LC_{50} 979.42 ± 4.95) was the least toxic.

Strychnos usambarensis (LC_{50} 327.69 ± 5.24) was the most toxic and *M. heterophylla* (LC_{50} 806.72 ± 5.17) the least toxic hexane extracts. Chloroform extracts had a narrow toxicity range (LC_{50} 353.91 ± 2.91- 554.72 ± 6.48 µg/ml) with *P. linearifolia* and *S. henningsii* being the most and least toxic respectively. The ethylacetate extract of *P linearifolia* was the most toxic (LC_{50} 313.79 ± 2.90 µg/ml) while that of *S. usambarensis* was the least toxic (LC_{50} 707.65 ± 6.60 µg/ml). *Strychnos henningsii* (LC_{50} 344.76 ± 4.66 µg/ml) was the most toxic while *S. usambarensis* (LC_{50} 979.42 ± 4.95 µg/ml) the least toxic methanol extracts. *Periploca linearifolia* aqueous extract (LC_{50} 322.16 ± 3.80 µg/ml) was the most toxic while *S. usambarensis* (LC_{50} 528.32 ± 2.42 µg/ml) the least

toxic. The standard drugs showed toxicity in the range of (LC_{50} 280.12±1.67- 542.15± 2.43 µg/ml). Chloroquine was the most toxic and mefloquine the least toxic.

In almost all cases the brine shrimp toxicity showed varied results compared to the anti-plasmodial activity. From the results it is confirmed that brine shrimp toxicity does not necessarily imply anti-plasmodial activity (Muregi *et al.*, 2003). The low toxicity of aqueous extract confirms that the plants are useful in traditional malaria therapy.

4.4 Radical scavenging activity

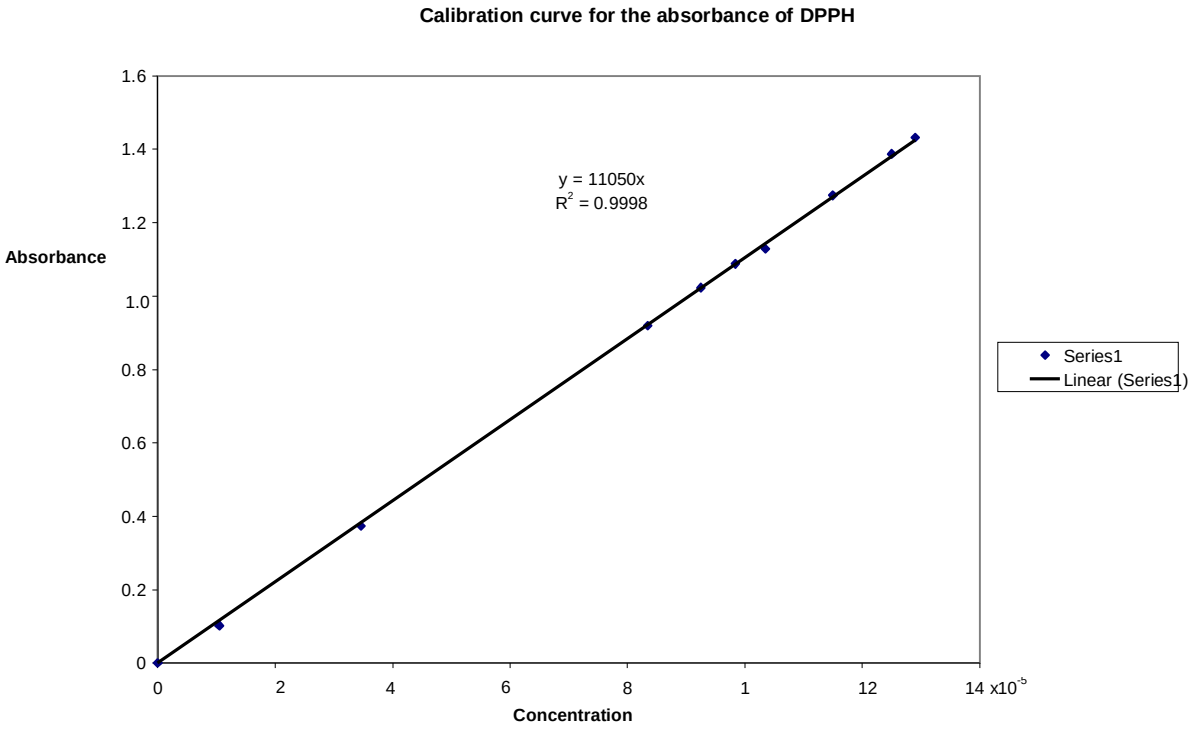
UV absorbance data (Table 4) of different concentrations of DPPH were used to obtain a calibration curve (Fig. 1).

Table 4: UV absorbance data of standard DPPH solutions

Concentration	Absorbance 1st reading	2nd reading	Average
1.05×10^{-5}	1.02×10^{-1}	1.02×10^{-1}	1.02×10^{-1}
3.46×10^{-5}	3.73×10^{-1}	3.73×10^{-1}	3.73×10^{-1}
8.34×10^{-5}	9.19×10^{-1}	9.19×10^{-1}	9.19×10^{-1}
9.25×10^{-5}	1.02	1.02	1.022
9.84×10^{-5}	1.09	1.09	1.09
1.00×10^{-4}	1.11	1.11	1.11
1.15×10^{-4}	1.13	1.13	1.13
1.20×10^{-4}	1.32	1.32	1.32
1.25×10^{-4}	1.33	1.33	1.33
1.29×10^{-4}	1.43	1.43	1.43

Solution made in methanol

Figure 6: Calibration curve for free radical scavenging activity of DPPH



Mean values used; SD < 5% in all experiments; equation for the line: y = 11050x

All experiments were carried out at different concentrations of the samples in order to investigate the effect of concentration on the anti-oxidant activity. The results (Table 5) were expressed as the % activity of each sample related to caffeic acid which is a strong DPPH radical scavenger.

Table 5: Radical scavenging activity of methanol extracts of selected anti-plasmodial plants

Extract	Conc. (ppm)	Co	C_T	ΔC	ΔC_S
Caffeic Acid	500	1.07×10^{-4}	3.70×10^{-6}	96.54	100
P.linearifolia	250	1.02×10^{-4}	4.68×10^{-5}	54.11	56.10
	500	9.25×10^{-5}	3.45×10^{-5}	62.70	64.90
	1000	8.33×10^{-5}	2.97×10^{-5}	64.34	66.90
	1500	8.35×10^{-5}	2.68×10^{-5}	68.70	69.01
M. heterophylla	250	9.37×10^{-5}	5.99×10^{-5}	36.07	37.37
	500	8.77×10^{-5}	4.89×10^{-5}	44.24	45.83
	1000	8.75×10^{-5}	3.57×10^{-5}	59.20	61.32
	1500	8.83×10^{-5}	3.99×10^{-5}	54.81	56.78
S. usambarensis	250	9.19×10^{-5}	8.06×10^{-5}	12.10	12.74
	500	9.76×10^{-5}	8.18×10^{-5}	16.18	16.77
	1000	9.83×10^{-5}	8.15×10^{-5}	17.09	17.70
	1500	9.75×10^{-5}	7.83×10^{-5}	19.69	20.40
S. henningsi	250	9.26×10^{-5}	8.23×10^{-5}	11.12	11.52
	500	9.83×10^{-5}	8.14×10^{-5}	17.20	17.81
	1000	9.87×10^{-5}	7.78×10^{-5}	21.18	21.93
	1500	9.37×10^{-5}	5.99×10^{-5}	36.07	33.36

Mean values 2 readings; C_O - initial DPPH concentration before addition of sample; C_T - final DPPH concentration + sample ΔC - decrease in DPPH Conc (%); ΔC_S - decrease in DPPH conc (%) compared to caffeic acid

All the methanol extracts showed a concentration-dependent anti-oxidant activity, with the highest radical scavenging activity at 1500 ppm. Unlike other extracts, the free radical

scavenging activity of *P. linearifolia* at 250 ppm still showed high activity. Caffeic acid reduced the concentration of DPPH by 96.5% and was equated to 100% radical scavenging activity. The free radical scavenging effect of the plant extracts and the positive control (caffeic acid) in a DPPH free radical system showed that the anti-oxidant directly reacted with DPPH free radical. *Maytenus heterophylla* showed a concentration-dependent activity with a slight variation. The methanol extracts of *P. linearifolia* and *M. heterophylla* showed high anti-oxidant activity while *S. usambarensis* and *S. henningsii* showed low activity. Since the methanol extracts of these plants also showed a high anti-plasmodial activity, their anti-oxidant activity may protect the body against severe pathological conditions.

4.5 Identification of isolated compounds.

4.5.1 Purification of *P. linearifolia* extract

P. linearifolia methanol extract (210 g) was fractionated using VLC to give 12 fractions (Table 6).

Table 6: VLC fractionation of *Periploca linearifolia* methanol extract

Solvent	Fraction	Mass (mg)
Hexane	F_1	23.0
Hexane: CH_2Cl_2 (70:30)	F_2	773.0
Hexane: CH_2Cl_2 (50:50)	F_3	88.6
Hexane: CH_2Cl_2 (25:75)	F_4	27.0
CH_2Cl_2	F_5	43.3
Hexane: EtOAc (80:20)	F_6	108.0
Hexane:EtOAc (50:50)	F_7	251.1
Hexane: EtOAc (10:90)	F_8	33.5
EtOAc	F_9	14.7
EtOAc: MeOH (70:30)	F_{10}	19.5
EtOAc: MeOH (50:50)	F_{11}	57.4
MeOH	F_{12}	28.7

4.5.2 Lupeol ester (50)

The fraction eluting with *n*-hexane-CH_2Cl_2 70:30 (F_2) and *n*-hexane-EtoAc 80:20 (F_6) were chromatograhed on Sephadex LH-20 reverse phase column (CC) using MeCN:H_2O 1:1 to give a white amorphous solid **(50) (**10.8 mg; R_f 0.55 SiO_2, CH_2Cl_2-MeOH 20:1, mp 78-81 ºC). TLC analysis of **50** and spraying with vanillin-sulphuric acid gave purple spots, indicating a terpenoid skeleton. UV spectrum revealed absorption at λ_{max} ($CHCl_3$) 229 and 283 nm. The IR spectrum (Appedix 1g) indicated the presence of OH absorptions at 3489 and a C=C at 1643 cm^{-1}. A carbonyl group was suggested by the presence of the band at 1729 cm^{-1}. ^{1}H NMR spectrum (Appendix1a and 1b) revealed 32 signals.

^{1}H NMR revealed peaks at δ 0.81 (s, 3H), 0.86 (s, 3H), 0.88 (s, 3H), 0.90 (s, 3H), 0.96 (s, 3H), 1.05 (s, 3H), 1.71 (s, 3H) corresponding to H-23, 24, 25, 26, 27, 28 and 30, respectively (Table 7).

Table 7: ^{1}H NMR (125 MHz, $CDCl_3$) data for lupeol ester **(50)**

Position	$\delta_{(lit)}$	$\delta_{(obs)}$
1	1.38 (m, 2H)	1.42 (m, 2H)
2	1.68 (m, 2H)	1.67 (m, 2H)
3	4.85 (1H, dd, J=4.9, 11.6 Hz)	4.55 (1H, dd, J=4.6, 11.0 Hz)*
4	-	-
5	1.39 (m, 2H)	1.42 (m, 2H)
6	1.40 (m, 2H)	1.42 (m, 2H)
7	1.36 (m, 2H)	1.28 (m, 2H)*
8	-	-
9	1.39 (m, 1H)	1.42 (m, 1H)
10	-	-
11	1.40 (m, 2H)	1.42 (m, 2H)
12	1.68 (m, 2H)	1.68 (m, 2H)
13	1.68 (m, 1H)	1.68 (m, 1H)
14	-	-
15	1.36 (m, 2H)	1.28 (m, 2H)*
16	1.36 (m, 2H)	1.28 (m, 2H)*
17	-	-
18	1.68 (m, 1H)	1.68 (m,1H)
19	2.38 (dd, J = 5.1, 10 Hz)	2.55 (dd, J = 5.4, 11.0 Hz)*
20	-	-
21	1.51(1H, ddd, 12.0, 8.5, 15 Hz)	1.51(1H, ddd, 12.0, 8.5, 15 Hz)
22	1.43 (m, 2H)	1.42 (m, 2H)
23	0.83 (s, 3H)	0.81 (s, 3H)
24	0.84 (s, 3H)	0.86 (s, 3H)
25	0.89 (s, 3H)	0.88 (s, 3H)
26	0.95 (s, 3H)	0.90 (s, 3H)
27	0.97 (s, 3H)	0.96 (s, 3H)
28	1.03 (s, 3H)	1.05 (s, 3H)
29	4.75 and 4.89 (br, s)	4.59 and 4.70 (br, s)*
30	1.75 (s, 3H)	1.71 (s, 3H)
1’	-	-
2’	2.83 and 2.85 (1H, dd, J=4.9, 14.7 Hz)	2.55 and 2.56 (1H, dd, J=6.7, 14.7 Hz)*
3’	4.54 (1H, brs)	4.53(1H, brs)
4’	1.43 (m, 2H)	1.42(m, 2H)
5’-(n’-3)[10]	1.29 (m, 2H)	1.28 (m, 2H) x 11
(n’-2)	1.28 (m, 2H)	1.28 (m, 2H)
(n’-1)	1.28 (m, 2H)	1.28 (m, 2H)
n’=18	0.88 (t, 3H)	0.81 (t, 3H)

* Large difference as a result of use of pyridine-d_5 solvent

The peak at δ 2.55 (m, 1H) was attributed to H-19. Olefinic protons were observed at δ 4.59 (1H, H-29b) and 4.70 (1H, brs H-29a). The signal at δ 4.55 (dd, J = 11.2, 5.4 Hz, 1H)

and 2.55 (t, J =7.3 Hz, 2H) were assigned to H-3 of acylated lupeol and to H-2' in the fatty acid chain, respectively.

The ^{13}C NMR spectrum (Appendix 1c and 1d) revealed 44 signals and the data is summarized in Table 8.

Table 8: Comparison of ^{13}C NMR (125 MHz, $CDCl_3$) data for lupeol **(48)** and lupeol-3-hydroxyoctadecanoate **(50)**

	δ_C ppm	
Carbon	lupeol (**48**)	lupeol-3-hydroxyoctadecanoate **(50)**
1	38.7 (t)	38.4 (t)
2	27.4 (t)	27.5 (t)
3	78.9 (d)	81.4 (d)
4	38.8 (s)	38.4 (s)
5	55.3 (d)	55.7 (d)
6	18.3 (t)	18.2 (t)
7	34.2 (t)	34.2 (t)
8	40.8 (s)	41.6 (s)
9	50.4 (d)	50.4 (d)
10	37.1 (s)	37.1 (s)
11	20.9 (t)	21.0 (t)
12	25.1 (t)	25.4 (t)
13	38.0 (d)	38.1 (d)
14	42.8 (s)	43.0 (s)
15	29.0 (t)	29.3 (t)
16	35.5 (t)	35.6 (t)
17	43.0 (s)	43.0 (s)
18	48.2 (d)	48.3 (d)
19	47.9 (d)	48.0 (d)
20	150.9 (s)	150.9 (s)
21	29.8 (t)	29.9 (t)
22	40.0 (t)	40.1 (t)
23	28.0 (q)	28.0 (q)
24	15.4 (q)	16.0 (q)
25	16.1 (q)	16.2 (q)
26	16.5 (q)	16.6 (q)
27	14.5 (q)	14.5 (q)
28	18.0 (q)	18.2 (q)
29	109.3 (t)	109.3 (t)
30	19.3 (q)	19.3 (q)
1’		172.8 (s)
2’		41.7 (t)
3’		68.3 (d)
4’		38.4 (t)
5’		24.1 (t)
6’		29.4 (t)
7’		29.5 (t)
8’		29.6 (t)
9’		29.6 (t)
10’		29.7 (t)
11’		29.7 (t)
12’		29.7 (t)
13’		29.7 (t)
14’		29.7(t)
15’		29.7 (t)
16’		31.9 (t)
17’		22.7 (t)
18’		14.1 (q)

From the ^{13}C NMR (Appendix 1d) and DEPT (Appendix 1e) analysis two olefinic carbon signals were located at δ 109.3 and 150.9. 8 Methyls at δ 14.1 (C-18'), 14.5 (C-27), 14.5 (C-24), 16.2 (C-25), 16.6 (C-26), 18.2 (C-28), 19.3 (C-30), and 28.0 (C-23); 10 methylenes at δ 38.4 (C-1), 27.5 (C-2), 18.2 (C-6), 34.2 (C-7), 22.7 (C-11), 25.4 (C-12), 29.3 (C-15), 35.6 (C-16), 29.9 (C-21), 40.9 (C-22), 41.0 (C-2'), 29.3 (C-4'); 6 methines at δ 81.4 (C-3), 55.7 (C-5) ,50.4 (C-9), 38.1 (C-13), 48.3 (C-18), 48.0 (C-19) and 5 quaternary centers at δ 38.0 (C-10), 38.4 (C-4), 41.6 (C-8), 43.0 (C-14), 43.0 (C-17) were identified. From the ^{13}C NMR (Appendix 1c and 1d) and DEPT (appendix 1e) data the presence of a terminal-alkene is indicated by the presence of the signals at δ 150.9 (s) and 109.3 (t) which are in agreement with literature value for lupeol (Shashi and Asish, 1994). The presence of an ester group was suggested by the signal at δ 172.8. This led to the conclusion that the isolated compound is a lupeol fatty acid ester. The signal at δ 68.3 is characteristic of a secondary alcohol (Kalsi, 1995). The compound therefore has a secondary OH group in its skeleton. Location of the OH group was done by comparison of literature δ values with the OH group on different carbons as shown in the Table 9 (Shashi and Asish, 1994).

Table 9: Comparison of ^{13}C NMR data for lupeol analogs and hydroxy lupeols

	δ_C (ppm)							
Position	48	50	28-OH	3β, 11α-OH	3, 15β-OH	3, 16-OH	3, 24β-OH	3β, 28-OH
1	38.7	38.4	40.2	39.0	38.1	38.9	38.5	38.8
2	27.4	27.5	18.6	27.5	27.4	27.4	27.8	27.2
3	78.9	81.4	42.1*	78.6	78.9	78.8	80.9	78.9
4	38.8	38.7	33.2	39.4	38.8	38.9	42.8	38.9
5	55.3	55.4	56.3	55.6	54.9	55.4	55.9	55.3
6	18.3	18.2	18.6	18.1	18.5	18.3	18.4	18.3
7	34.2	34.2	34.2	35.3	37.8	34.3	34.9	34.3
8	41.8	41.6	41.1	41.1	42.5	41.0	40.9	40.9
9	50.4	50.4	50.4	55.7	51.0	50.0	50.5	50.4
10	37.1	37.1	37.4	37.7	37.4	37.1	38.0	37.2
11	20.9	21.0	20.7	70.5*	21.0	20.9	21.2	20.9
12	25.1	25.5	25.3	27.7	25.2	24.9	25.1	25.3
13	38.0	38.1	37.2	37.7	37.6	37.3	36.9	37.3
14	42.8	43.0	42.7	42.6	47.9	44.1	42.8	42.7
15	29.0	29.3	27.0	27.5	69.7*	36.9*	27.4	42.7
16	35.5	35.6	29.2	35.5	46.5	76.9*	35.6	27.0
17	43.0	43.0	47.7	43.0	43.0	48.6 *	43.0	29.2
18	48.2	48.3	48.7	47.7	48.1	47.7	48.0	47.8
19	47.9	48.0	47.7	47.7	47.4	47.6	48.3	47.8
20	150.9	150.9	150.2	150.2	150.4	149.8	150.9	150.6
21	29.8	29.9	29.8	29.9	30.1	30.0	29.9	29.8
22	40.0	40.1	33.9	39.9	39.7	37.8	40.0	34.0
23	28.0	28.0	33.3	28.3	27.9	28.0	22.4*	28.0
24	15.4	16.0	21.5	15.6	15.4	15.4	64.5*	15.4
25	16.1	16.2	16.1	16.1	16.1	16.1	15.9	16.1
26	16.5	16.6	16.0	17.3	16.6	16.1	16.7	16.0
27	14.5	14.5	14.8	14.5	8.0*	16.1	14.6	14.8
28	18.0	18.2	60.4*	18.1	19.2	11.8	18.0	60.2*
29	109.3	109.3	109.4	109.8	109.7	109.6	109.4	109.6
30	19.3	19.3	19.1	19.4	19.4	19.4	19.3	19.1

*The signals with very high deviation; **48**- lupeol

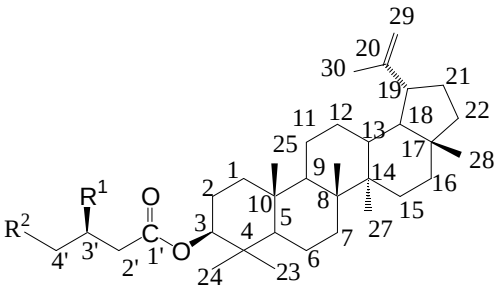
The δ values for the extra OH varied so widely from those of lupeol analogs leading to the conclusion that the OH group is not on the pentacyclic skeleton. Comparing the ^{13}C NMR signals of the compound in the region δ 23.0 - 42.0 groups with those of lupeol, 12 extra signals were found. These signals were confirmed by DEPT (Appendix 1e) analysis to be methylene groups suggesting that the fatty acid moiety involved is linear. This

observation was supported by a strong methylene signal at δ 1.28 and a carbonyl signal at 172.8 in ^{1}H and ^{13}C NMR spectra respectively. By comparing the reported NMR data for lupeol esters (Table 10) and that of the isolated compound it was proposed that the OH group is on C-3' as in **50** due to the presence of the signal at δ 68.3 (Wenkert *et al.*, 1978; Jean *et al.*, 2006).

Table 10: Comparison of ^{13}C NMR data of lupeol esters with lupeol-3-hydroxyoctadecanoate **(50)**

	δ_C ppm				
Carbon	**50**	**51***	**52***	**53**	**54**
1	38.4	40.2	40.1	38.4	38.7
2	27.5	24.3	24.2	23.7	27.4
3	81.4	80.6	80.5	80.6	81.7
4	38.4	38.2	38.1	37.8	39.0
5	55.7	55.7	55.5	55.4	55.7
6	18.2	18.5	18.4	18.2	18.5
7	34.2	34.5	34.4	34.2	34.5
8	41.6	41.1	41.0	40.8	41.9
9	50.4	50.5	50.4	50.3	50.6
10	37.1	37.3	37.2	37.1	37.4
11	21.0	21.1	21.0	20.9	22.7
12	25.4	27.8	27.6	25.1	25.9
13	38.1	38.3	38.2	38.0	38.1
14	43.0	43.1	42.9	42.8	43.2
15	29.3	30.0	29.9	27.4	29.8
16	35.6	35.8	35.7	35.6	35.9
17	43.0	43.2	43.1	43.0	43.2
18	48.3	48.6	48.5	48.3	48.3
19	48.0	48.3	48.2	48.0	48.6
20	150.9	151.0	151.1	151.0	151.1
21	29.9	30.1	29.9	29.8	30.0
22	40.1	38.6	38.5	40.0	41.2
23	28.0	28.2	28.1	28.0	28.8
24	16.0	17.0	17.1	16.7	16.4
25	16.2	16.3	16.2	16.2	16.9
26	16.6	14.3	14.6	16.0	16.5
27	14.5	16.2	16.0	14.5	14.9
28	18.2	18.2	18.1	18.0	18.4
29	109.3	110.0	110.0	109.3	109.6
30	19.3	19.5	19.5	19.3	19.7
1’	172.8	172.2	172.1	173.7	173.0
2’	41.7	44.0	35.0	34.9	41.3
3’	68.3	68.4	25.3	25.2	68.5
4’	38.4	38.2	29.2	29.2	38.4
5’	24.1	25.0	25.0	24.3	24.5
6’	29.4	29.8	29.4	29.3	29.7
7’	29.5	29.8	29.5	29.5	29.6
8’	29.6	29.7	29.6	29.5	29.4
9’	29.6	29.9	29.9	29.3	29.6
10’	29.7	29.7	29.7	29.8	29.5
11’	29.7	29.9	29.9	29.4	29.3
12’	29.7	29.7	29.7	29.7	29.7
13’	29.7	29.8	29.8	29.7	29.7
14	29.7	32.1	29.7	29.7	29.3
15’	29.7	23.0	29.7	29.3	29.3
16’	31.9	14.3	32.0	29.9	29.7
17’	22.7		23.0	29.3	29.7
18’	14.1		14.2	31.9	32.0
19’				22.7	22.3
20’				14.1	14.6

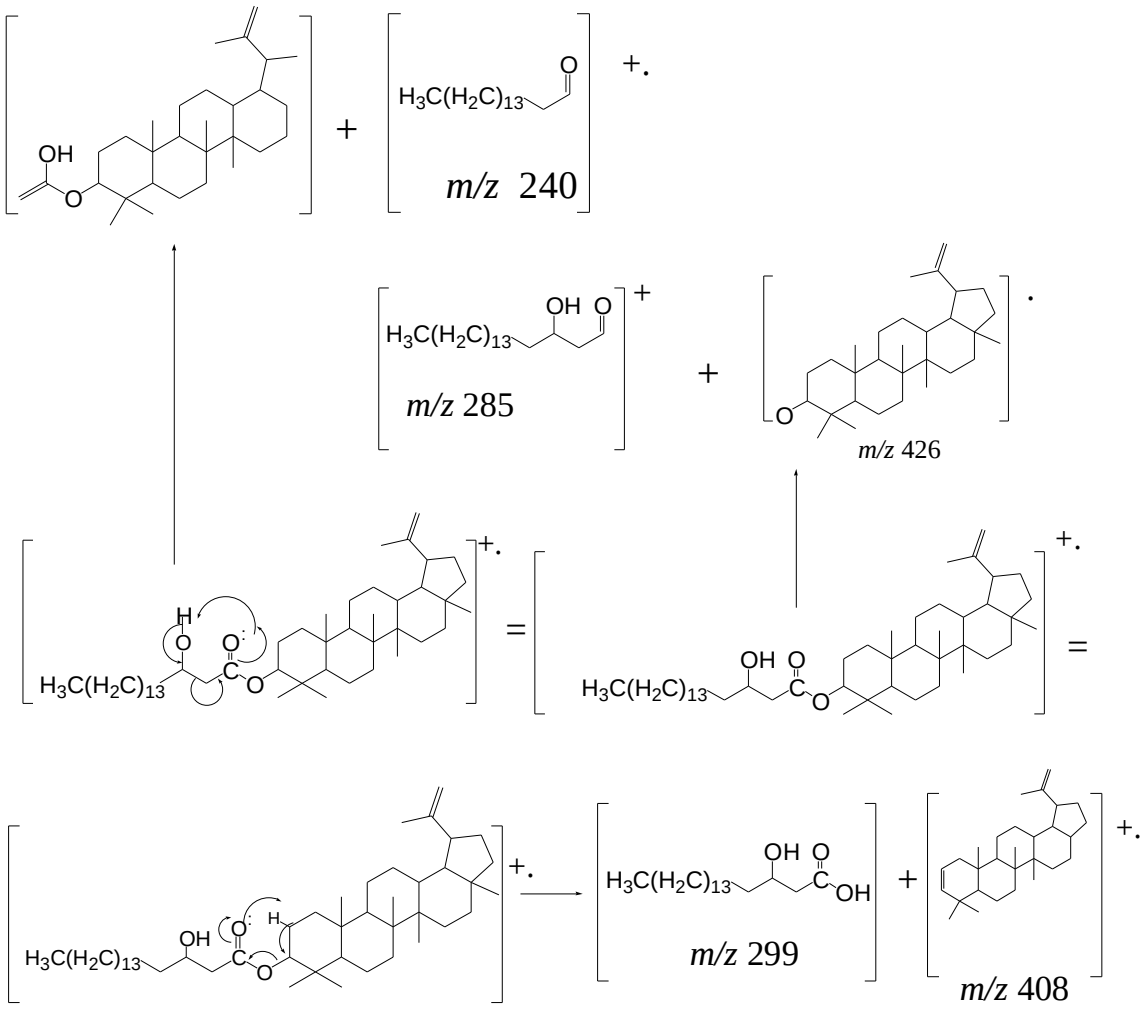
*Solvent used –pyridine d_5



50 R^1= OH; R^2= $CH_3(CH_2)_{13}$
51 R^1 = OH: R^2 = $CH_3(CH_2)_{11}$
52 R^1= H; R^2 = $CH_3(CH_2)_{13}$
53 R^1=H; R^2 = $CH_3(CH_2)_{15}$
54 R^1=OH; R^2 = $CH_3(CH_2)_{15}$

The parent molecular ion peak was observed at *m/z* 709 in the EI-MS spectrum (Appendix 1f). Distinctive fragment ion arising from McLafferty rearrangement was observed at *m/z* 299 $[M\text{-}C_{30}H_{50}]^+$ and *m/z* 408 $[C_{30}H_{48}]$. The MS spectrum also displayed characteristic fragment ion peaks at *m/z* 426 ($C_{30}H_{50}O$, [lupeol]) and *m/z* 425 ($C_{30}H_{49}O$, [lupeol-H]). Other prominent peaks were observed at *m/z* 409 (100% $[M^+ - C_{18}H_{36}O_3]$), 218 (13.3%) and 189 (100%). Some peaks were observed at *m/z* higher than 425: 468 (69% $[C_{30}H_{50}O_2CCH_2\text{-}]$); 516 (2.7%), 566 (16%), 597 (2.5%), 652 (1%) and 693 (7.3%). From the spectral data, the molecular formula of the compound was deduced to be $C_{48}H_{84}O_3$. The MS can be rationalized by the fragmentation pattern in Scheme 2.

Scheme 2: Proposed MS fragmentation for lupeol-3- hydroxyoctadecanoate **(50)**



From the chemical literature information, the ^{13}C NMR data for the compound (Table 10) was similar to that of 3-hydroxyoctadecanoic acid ester of lup-20(29)-en-3β-ol **(50)**. The comparison of spectroscopic data of the isolated compound **(50)** to those of other lupeol esters (Tomosaka *et al.*, 2001; Sholichin *et al.*, 2001) confirmed the structure as lupeol-3-hydroxyoctadecanoate **(50)**.

4.6 Isolation of β-sitosterol (54) and β-amyrin (55)

Periploca linearifolia chloroform extract (52 g) was fractionated by VLC on Kieselgel silica gel 60G (0.040-0.063 mm, Merck, Germany) and 12 fractions collected (Table 11).

Table 11: Column chromatographic fractionation of *Periploca linearifolia* chloroform extract

Solvent	Fraction	Mass (mg)
Hexane: CH_2Cl_2 (90:10)	M_1	25.3
Hexane: CH_2Cl_2 (80:20)	M_2	55.8
Hexane: CH_2Cl_2 (60:40)	M_3	32.0
Hexane: CH_2Cl_2 (50:50)	M_4	61.7
Hexane: CH_2Cl_2 (30:70)	M_5	31.4
Hexane: CH_2Cl_2 (10:90)	M_6	14.8
CH_2Cl_2	M_{11}	37.0
Hexane: EtOAc (90:10)	M_{22}	27.9
Hexane: EtOAc (70:30)	M_{33}	110.3
Hexane: CH_2Cl_2 (60:40)	M_{44}	18.2
Hexane: EtOAc (30:70)	M_{55}	36.5
EtOAc	M_{66}	99.1

Further purification of M_2 and M_{22} yielded β-sitosterol (**54**) and β-amyrin (**55**), respectively.

4.6.1 β-Sitosterol (54)

The compound was isolated as colorless needles, from chloroform extract and recrystallized in *n*-hexane (11.5 mg, R_f 0.19, SiO_2, *n*-C_6H_{14}-CH_2Cl_2 10:90, mp 136-138 ºC. IR spectrum (Appendix 2g) showed peaks at 3405 (-OH str), 2924 (-CH str), 1635 (C=C str), 1455 (C-H bend) cm^{-1}.

^{1}H NMR spectrum (Appendix 2a) revealed 30 signals. The δ values 0.70 (s, 3H), 0.95 (s, 3H), 0.93 (s, 3H), 0.82 (s, 3H) and 0.85 (s, 3H) were attributed to H-18, H-19, H-21, H-26 and H-29, respectively. The multiplet at δ 3.53 (1H) indicated the presence of an OH group at C-3. The signal observed at δ 5.37 (1H) was attributed to olefinic proton (H-6) (Table 12).

Table 12: ^{1}H NMR (125 MHz, $CDCl_3$) data for β-sitosterol **(54)**

Position	δ_H(ppm) $\delta_{(obs)}$	$\delta_{(lit)}$
1	1.26 (m, 2H)	1.28 (m, 2H)
2	1.31 (m, 2H)	1.34 (m, 2H)
3	3.53 (m, 1H)	3.51 (m, 1H)
4	2.31 (m, 2H)	2.30 (m, 2H)
5	-	-
6	5.37 (t, J =18 Hz, 1H)	5.37 (t, J =18.2 Hz, 1H)
7	1.97 (m, 2H)	1.92 (m, 2H)
8	1.54 (m, 1H)	1.54 (m, 1H)
9	0.93 (m, 1H)	0.94 (m, 1H)
10	-	-
11	1.45 (m, 2H)	1.44 (m, 2H)
12	1.58 (m, 2H)	1.59 (m, 2H)
13	-	-
14	1.11 (m, 1H)	1.10 (m, 1H)
15	1.52 (m, 2H)	1.51 (m, 2H)
16	1.46 (m, 2H)	1.47 (m, 2H)
17	1.84 (m, 1H)	1.86 (m, 1H)
18	0.70 (s, 3H)	0.67 (s, 3H)
19	0.95 (s, 3H)	0.98 (s, 3H)
20	1.97 (m, 1H)	1.96 (m, 1H)
21	0.93 (d, J= 3.0 Hz, 3H)	0.92 (d, J = 2.9 Hz, 3H)
22	1.63 (m, 2H)	1.62 (m, 2H)
23	1.65 (m, 2H)	1.65 (m, 2H)
24	1.58 (m,1H)	1.58 (m, 1H)
25	1.56 (m, 1H)	1.56 (m, 1H)
26	0.82 (d, J =7.0 Hz, 3H)	0.80 (d, J =7.0 Hz, 3H)
27	0.80 (d, J = 7.0 Hz, 3H)	0.80 (d, J =7.0 Hz, 3H)
28	1.28 (m, 2H)	1.29 (m, 2H)
29	0.93 (t, 3H)	0.93 (t, 3H)

The ^{13}C NMR spectrum (Appendix 2c) revealed 29 signals.

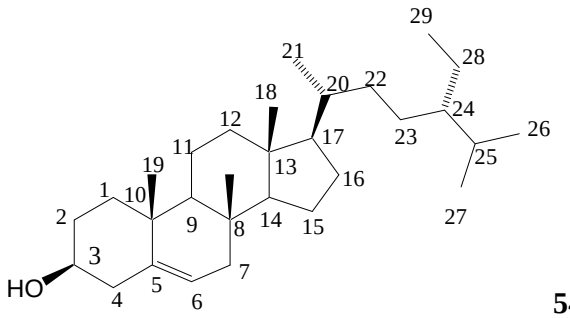
The ^{13}C NMR data is summarized in table 13.

Table 13: ^{13}C NMR (125 MHz, $CDCl_3$) data for β-sitosterol **(54)**

	δ_C (ppm)	
Position	$\delta_{(obs)}$	$\delta_{(lit)}$
1	37.3	37.3 (t)
2	31.7	31.6 (t)
3	71.8	71.1 (d)
4	42.3	42.3 (t)
5	140.8	140.8 (s)
6	121.0	121.6 (d)
7	31.7	31.9 (t)
8	31.9	31.9 (d)
9	50.2	50.2 (d)
10	36.5	36.5 (s)
11	21.1	21.1 (t)
12	39.8	39.8 (t)
13	42.3	42.3 (s)
14	56.8	56.8 (d)
15	24.3	24.3 (t)
16	28.2	28.3 (t)
17	56.1	56.1 (d)
18	11.9	11.9 (q)
19	19.4	19.4 (q)
20	36.1	36.2 (d)
21	18.8	18.8 (q)
22	34.0	33.9 (t)
23	26.1	26.1 (t)
24	45.9	45.9 (d)
25	29.2	29.2 (d)
26	19.8	19.8 (q)
27	19.0	19.1 (q)
28	23.1	23.1 (t)
29	12.0	12.3 (q)

The DEPT (Appendix 2d and 2e) analysis revealed the presence of 3 quaternary centers, 9 methines, 11 methylenes and 6 methyl groups. Two olefinic carbon signals were noted at δ 121.8 and 140.8. Three quartenary carbons at δ 140.8 (C-5), 36.5 (C-10), 42.3 (C-13); six methyls at 11.9 (C-18), 19.4 (C-19), 18.8 (C-21), 19.8 (C-26), 19.0 (C-27), and 12.0 (C-29); 11 methylenes at δ 37.3 (C-1), 31.7 (C-2), 42.3 (C-4), 31.7 (C-7), 21.1 (C-11), 39.8 (C-12), 24.3 (C-15), 28.2 (C-16), 34.0 (C-22), 26.1 (C-23), 23.1 (C-28) and 9 methine carbons at δ 71.8 (C-3), 121.0 (C-6), 31.9 (C-8), 50.2 (C-9), 56.8 (C-14), 56.1 (C-17), 36.1

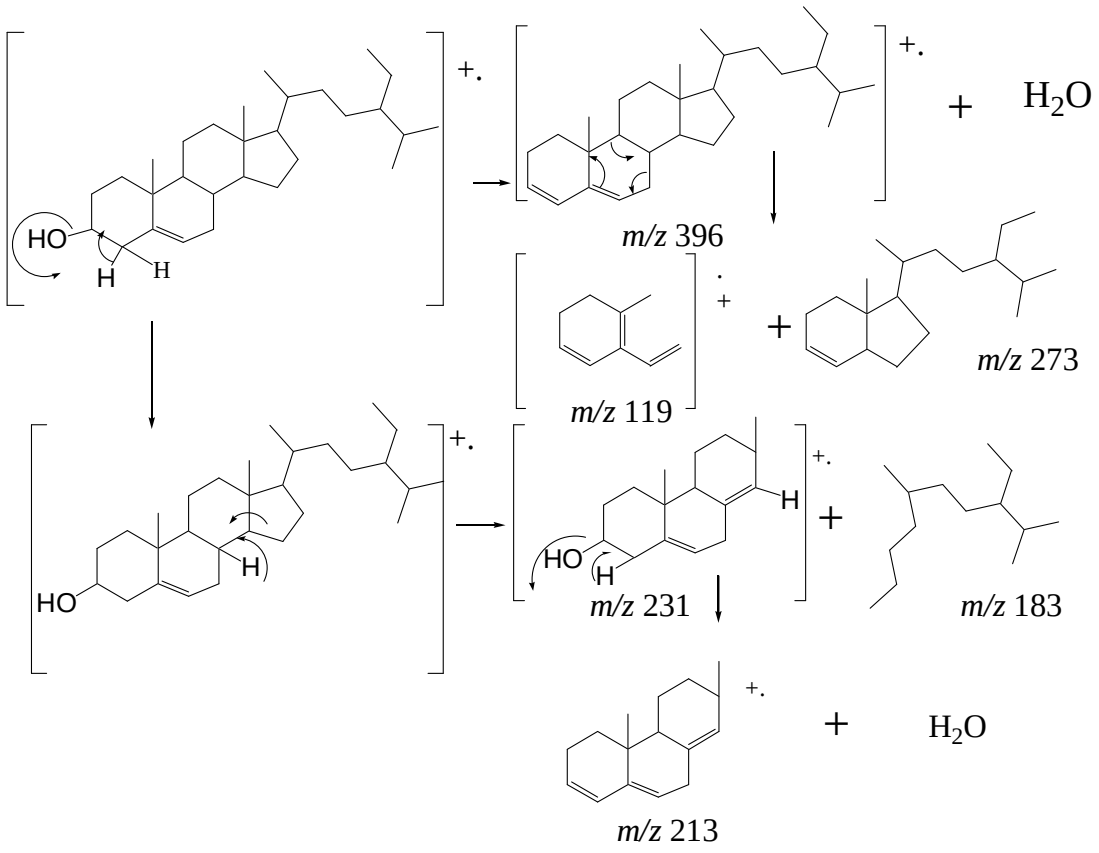
(C-20), 45.9 (C-24), 29.2 (C-25) were observed. The ^{13}C NMR data for the compound was comparable with literature information for β-sitosterol **(54)** (Dey and Harbone, 1991).



54

EI-MS (Appendix 2f) showed characteristic peaks with the molecular ion [M^+] at *m/z* 414 (100%) consistent with the proposed molecular formula $C_{29}H_{50}O$. The peak at *m/z* 396 (88.1 %) was assigned to the fragment formed due to loss of one water molecule [$C_{29}H_{48}$] $^+$. Further fragmentation of the ring results to ions at *m/z* at 381 (67%), 329 (83%), 303 (47%), 273 (39.2%), 231 (55 %), 213 (73.4%), 145 (80 %), 119 (59.3%) and 81 (88%). The MS spectrum can be rationalized by Scheme 3.

Scheme 3: Proposed MS fragmentation pattern for β-Sitosterol **(54)**



By comparison of MS, ^{1}H and ^{13}C NMR data with literature information (Sobrinho *et al.*, 1991; Kojima *et al.,* 1990) the compound was confirmed as β-sitosterol **(54)**.

The compound is known to be active against *Salmonella typhii*, *Corynebacterium diptherie* and *Fusarium spp* at 100 µg/ml with an average inhibition diameter as 10 mm (Kiprono *et al.,* 2000).

4.6.2 β-Amyrin (55)

The compound was isolated from *P. linearifolia* root bark as colorless needle-like crystals (26.1 mg, m.p 162-164 ºC, R_f 0.28, SiO_2, *n*-C_6H_{14}-CH_2Cl_2 10:90. IR spectrum (Appendix 3j) showed peaks at ν_{max} (KBr) 3405 (-OH group), 2924 (-CH, str), 1635 (C=C, str), 1455 (C - H bend) cm^{-1}.

^1H NMR spectrum (Appendix 3a and 3b) revealed 25 signals.

The ^1H NMR data is summarized in table 14.

Table 14: ^1H NMR (125 MHz, $CDCl_3$) data for β-amyrin (**55**)

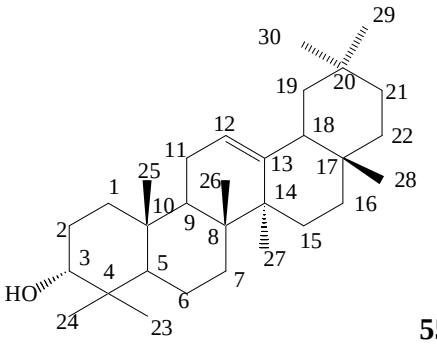
	δ_H(ppm)	
Position	$\delta_{(obs)}$	$\delta_{(lit)}$
1	1.36 (m, 2H)	1.36 (m, 2H)
2	1.60 (m, 2H)	1.59 (m, 2H)
3	3.24 (dd, J=11.5, 5 Hz,1H)	3.15 (dd, J =11.7, 5 Hz, 1H)
4	-	-
5	1.37 (m, 1H)	1.38 (m, 1H)
6	1.40 (m, 2H)	1.40 (m, 2H)
7	1.38 (m, 2H)	1.36 (m, 2H)
8	-	-
9	1.43 (m, 1H)	1.44 (m, 1H)
10	-	-
11	1.92 (m, 2H)	1.92 (m, 2H)
12	5.20 (d, J =3.5 Hz, 1H)	5.21 (d, J =3.5 Hz, 1H)
13	-	-
14	-	-
15	1.25 (m, 2H)	1.27 (m, 2H)
16	1.21 (m, 2H)	1.18 (m, 2H)
17	-	-
18	2.00 (m, 1H)	2.10 (m, 1H)
19	1.38 (m, 2H)	1.37 (m, 2H)
20	-	-
21	1.36 (m, 2H)	1.36 (m, 2H)
22	1.36 (m, 2H)	1.36 (m, 2H)
23	0.85 (m, 3H)	0.87 (m, 3H)
24	0.81 (m, 3H)	0.83 (m, 3H)
25	0.89 (m, 3H)	0.87 (m, 3H)
26	0.96 (m, 3H)	0.94 (m, 3H)
27	1.26 (m, 3H)	1.27 (m, 3H)
28	1.16 (m, 3H)	1.16 (m, 3H)
29	0.99 (m, 3H)	0.97 (m, 3H)
30	1.02 (m, 3H)	1.00 (m, 3H)

The peaks at δ values 0.85 (3H, s), 0.81 (3H, s) 0.89 (3H, s), 0.96 (3H, s), 0.98 (3H, s), 1.02 (3H, s) were attributed to H-24, H-25, H-26, H-29 and H-30, respectively. The multiplet at 3.24 (1H, dd, *J*= 11.5 Hz) indicated the presence of an OH group at C-3. The signal observed at 5.19 (1H, t, *J*= 5.4 Hz) was attributed to olefinic proton H-12. The ^{13}C NMR spectrum (Appendix 3c and 3d) revealed 30 peaks. The ^{13}C NMR data is summarized in table 15.

Table 15: ^{13}C NMR (125 MHz, $CDCl_3$) data for β-amyrin (**55**)

	δ_C (ppm)	
Position	$\delta_{(obs)}$	$\delta_{(lit)}$
1	39.8 (t)	39.7 (t)
2	27.0 (t)	27.3 (t)
3	79.0 (d)	79.0 (d)
4	38.8 (s)	38.8 (s)
5	55.2 (d)	55.3 (d)
6	18.4 (t)	18.5 (t)
7	35.5 (t)	35.7 (t)
8	38.6 (s)	38.8 (s)
9	47.3 (d)	47.7 (d)
10	37.2 (s)	37.6 (s)
11	23.7 (t)	23.6 (t)
12	121.6 (d)	121.8 (d)
13	145.2 (s)	145.1 (s)
14	41.8 (s)	41.8 (s)
15	26.0 (t)	26.2 (t)
16	26.2 (t)	26.7 (t)
17	31.1 (s)	31.5 (s)
18	47.7 (d)	47.4 (d)
19	46.9 (t)	46.9 (t)
20	31.8 (s)	31.1 (s)
21	34.8 (t)	34.8 (t)
22	37.0 (t)	37.2 (t)
23	27.3 (q)	27.2 (q)
24	15.6 (q)	15.5 (q)
25	15.6 (q)	15.6 (q)
26	16.6 (q)	16.8 (q)
27	23.7 (q)	23.5 (q)
28	28.1 (q)	28.4 (q)
29	32.7(q)	33.3 (q)
30	23.5 (q)	23.7 (q)

The DEPT (Appendix 3e) analysis revealed the presence of 7 quaternary centers, 5 methines, 10 methylenes and 8 methyl groups. Two olefinic carbon signals were noted at δ 121.6 and 145.2. Seven quartenary carbons at δ 38.8 (C-4), 38.6 (C-8), 37.2 (C-10), 145.2 (C-13), 41.8 (C-14), 31.1 (C-17), 28.4 (C-20); eight methyls at 27.3 (C-23), 15.6 (C-24), 15.6 (C-25), 16.6 (C-26), 23.7 (C-27), and 28.1 (C-28), 32.7 (C-29), 23.5 (C-30); 10 methylenes at δ 39.8 (C-1), 27.0 (C-2), 18.4 (C-6), 35.5 (C-7), 23.7 (C-11), 26.0 (C-15), 26.2 (C-16), 46.9 (C-19), 34.8 (C-21), 37.0 (C-22) and 5 methine carbons at δ 79.0 (C-3), 55.2 (C-5), 47.3 (C-9), 121.6 (C-12), 47.7 (C-18) were observed. Comparison of ^{13}C NMR data with the literature information confirmed β-amyrin **(55)** (Melo *et al.,* 1974).



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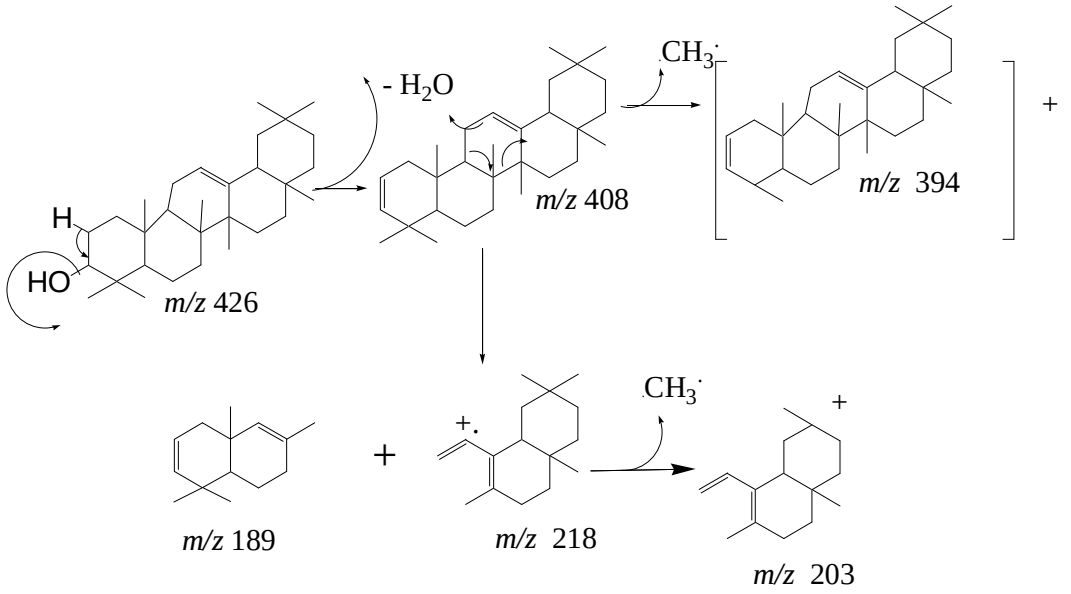
The EI-MS spectra (Appendix 3f) showed signals at *m/z* 426 [$C_{30}H_{50}O$ (M^+)] and 425 [$C_{30}H_{50}O$ (M^+-1)]. The base peaks appeared at *m/z* 218 (RDA 100%), 203 (218 $-CH_3^+$ 100%), 189 (67%) and 175 (46%). Another peak was observed at *m/z* 411 (13%) resulting from loss of a molecule of water.

The MS can be rationalized by the fragmentation pattern in Scheme 4.

Scheme 4: Proposed MS fragmentation pattern for β-amyrin **(55)**



By comparison of mp, MS, ^{1}H and ^{13}C NMR data, with literature information (Sobrinho *et al.*, 1991; Kojima *et al.*, 1990; Chaurasia and Witchtl, 1987) the compound was identified as β-amyrin (**55**).

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The results obtained show that the four plants used by traditional healers for malaria treatment posses anti-plasmodial activity. All the fractions showed high activities towards D6 and W2 strains (IC_{50} < 50 µg/ml) and this supports their use for cultural malaria treatment. All the extracts were not toxic (LC_{50} > 200 µg/ml). The methanol extracts showed anti-oxidant activity dependent on concentration with *P. linearifolia* extract with the highest activity. 3 compounds; **50**, **54** and **55** were isolated from the root bark of *P. linearifolia*. The three compounds belonged to the terpenoid class of compounds

Lupeol, whose structure is related to compound **50**, is known to exhibit cytotoxic activity against some tumor cell lines (Moriarity *et al.*, 1998). It has also been reported to inhibit growth of both the CQ-sensitive (IC_{50} 97µM, FCR-3) and CQ- resistant (IC_{50} 106 µM (3D7). Lupeol esters such as 3-O-(32-hydroxyeicosanoyl) lupeol have also been reported to inhibit growth of both the chloroquine-sensitive (IC_{50} 269 µM, FCR-3) and chloroquine- resistant (282 µM, 3D7) strains (Jean *et al.*, 2006).

Compound **54** (β-sitosterol) has been reported to be active against *Salmonella typhii*, *Corynebacterium diptherie* and *Fusarium spp* at 100 µg/ml with an inhibition diameter as 10 mm (Kiprono *et al.*, 2000).

5.2 Recommendations

- *In vi*vo tests in higher animals are required to confirm efficacy.
- The plant's toxicity should be investigated further in higher animals.
- Since most traditional healers give a mixture of extracts of plants, the blends should also be investigated for their efficacy.
- After detailed *in vivo* anti-malarial evaluation and thorough toxicological studies, some of these plants may find use as anti- malarial drugs in known doses especially in the rural communities where conventional drugs are unaffordable or unavailable.
- It is further recommended that the isolated compounds should be investigated for activity and their potential serve as drugs or templates in the rational development of other drugs established.
- Plants are a potential source of active anti-plasmodial compounds; leaves should be used to reduce destruction of such plants.
- Several modification of all compounds isolated accompanied by bioactivity tests of the resultant compounds are recommendaded to evaluate their suitability as alternative drugs.
- Active extracts should be studied for bactericidal,bacteristatic and bacterilytic.
- Further search for bioactive metabolites from plants belonging to this species needs to be done to boost their use in a wide range of fields like in the control of human, livestock and agricultural disease.

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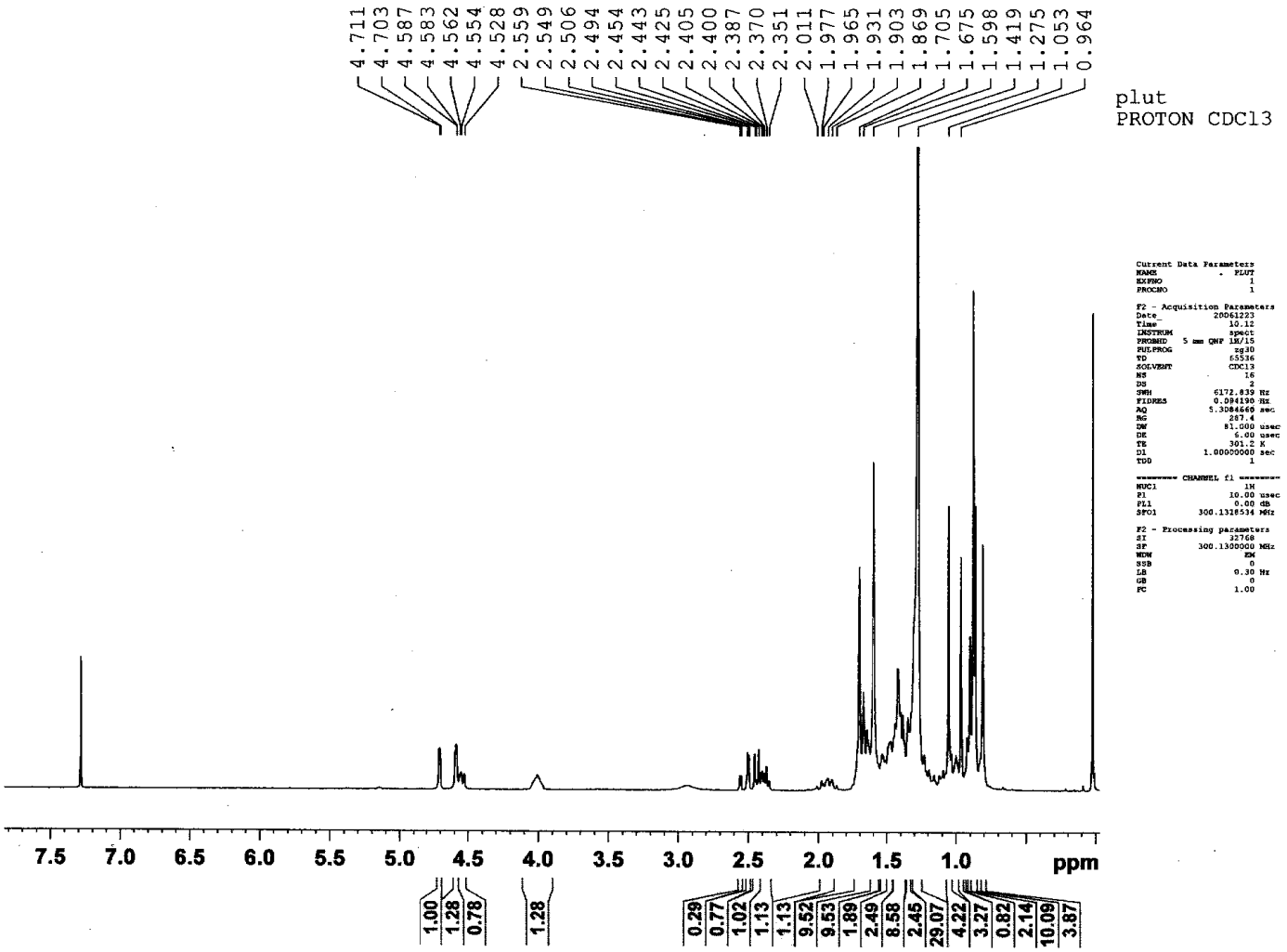
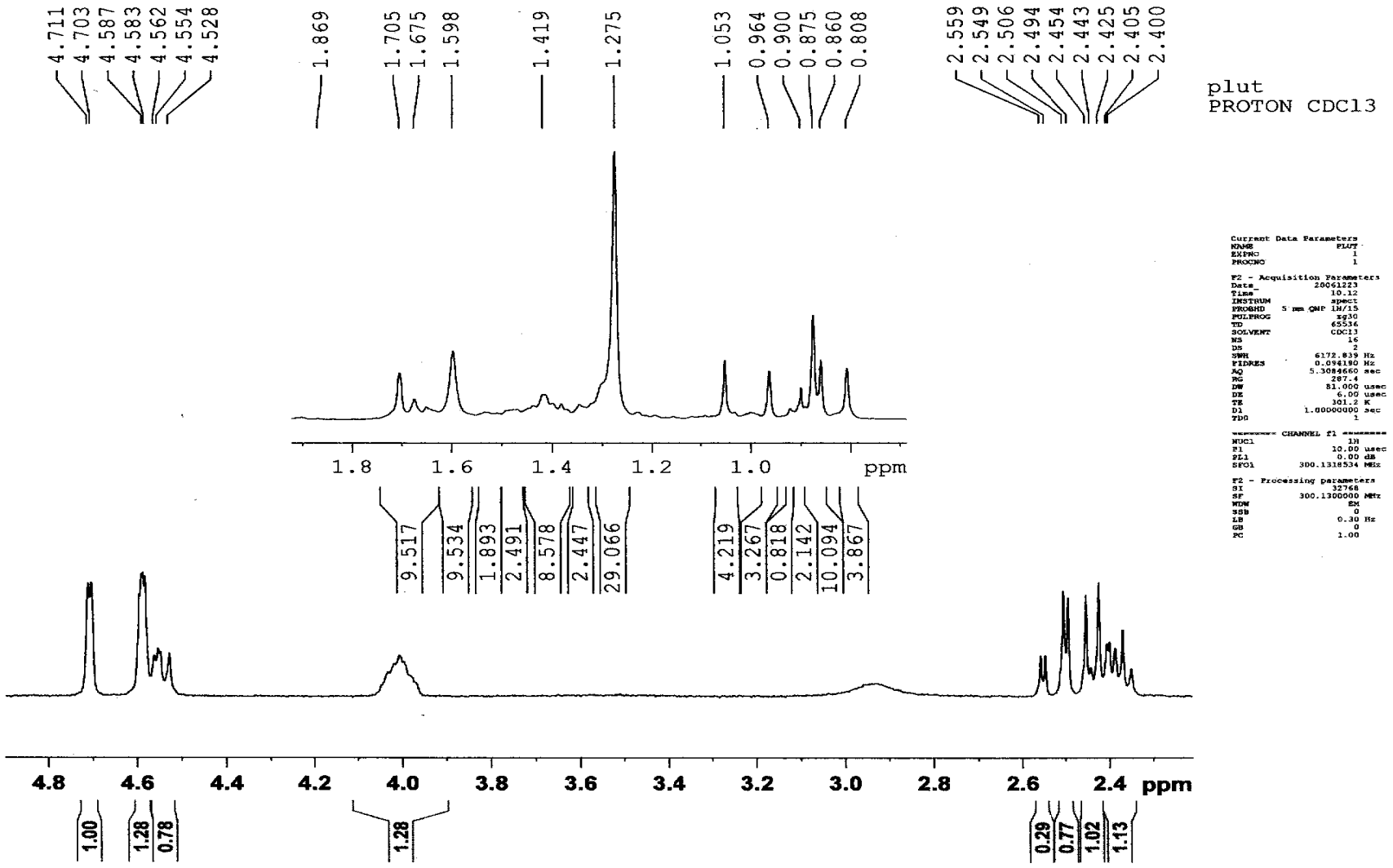
APPENDICES

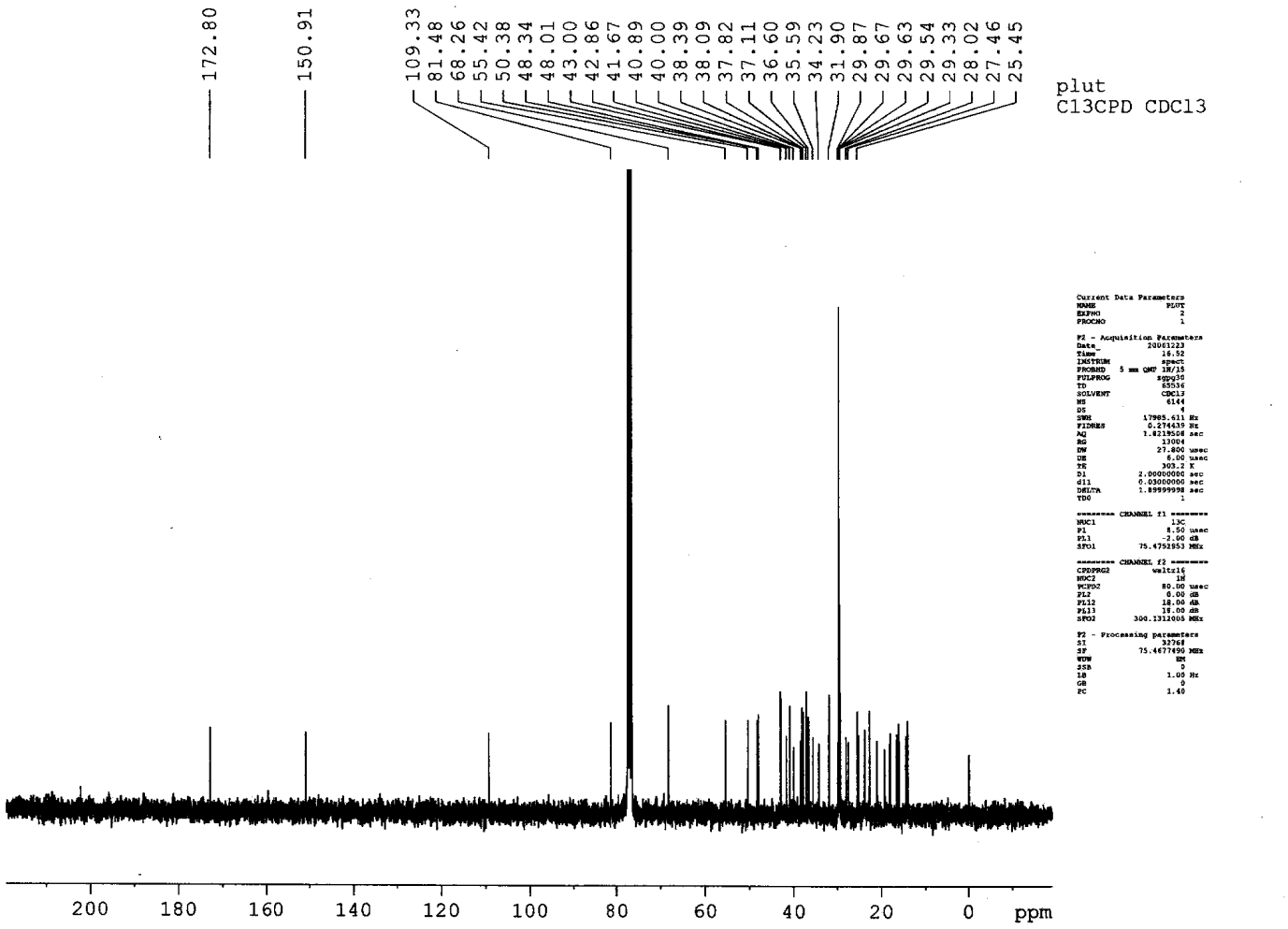
Appendix 1a: ^{1}H NMR spectrum of compound lupeol-3-hydroxyoctadecanoate **(50)**



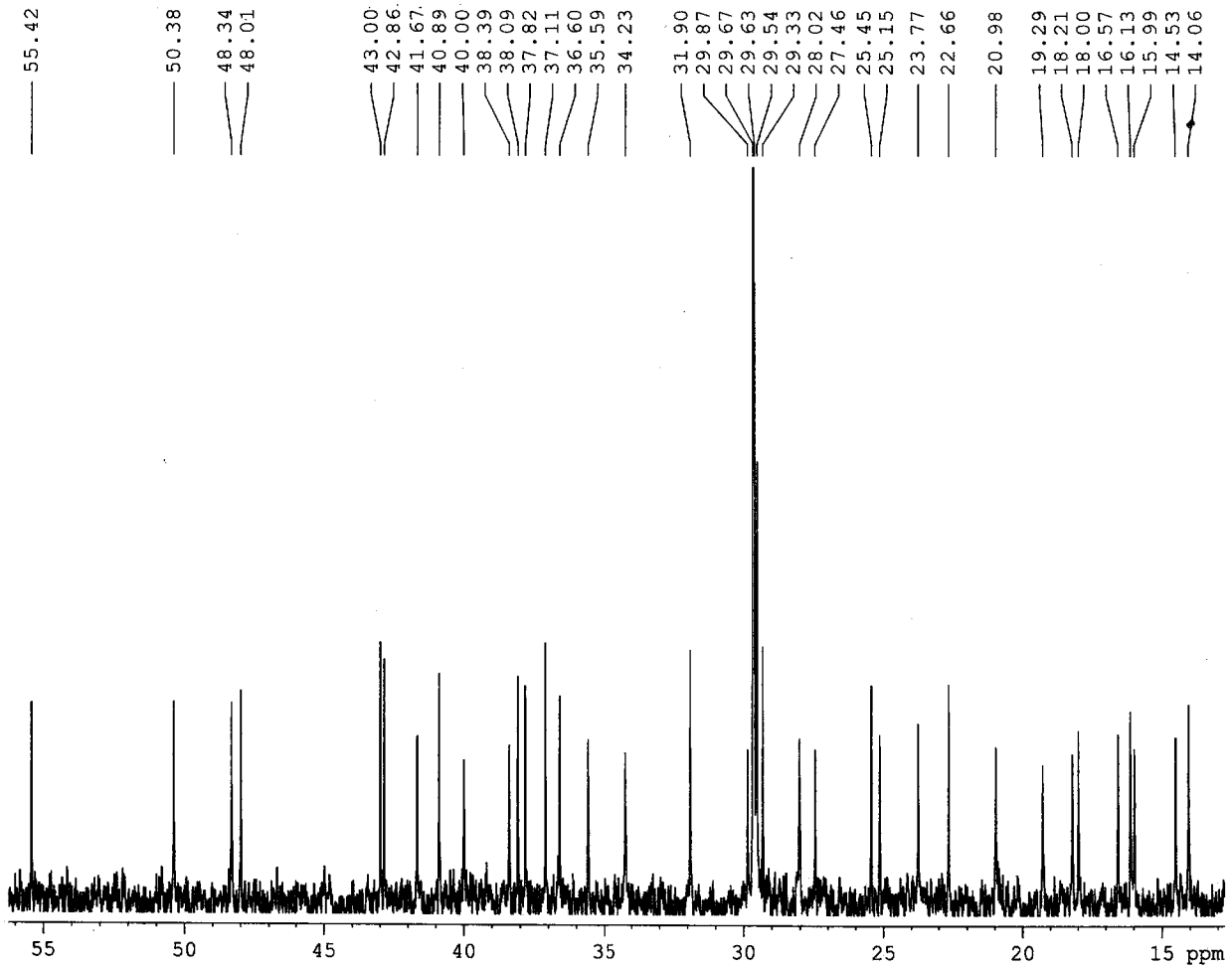
Appendix 1b: ^{1}H NMR spectrum (expanded) of compound lupeol-3-hydroxyoctadecanoate **(50)**



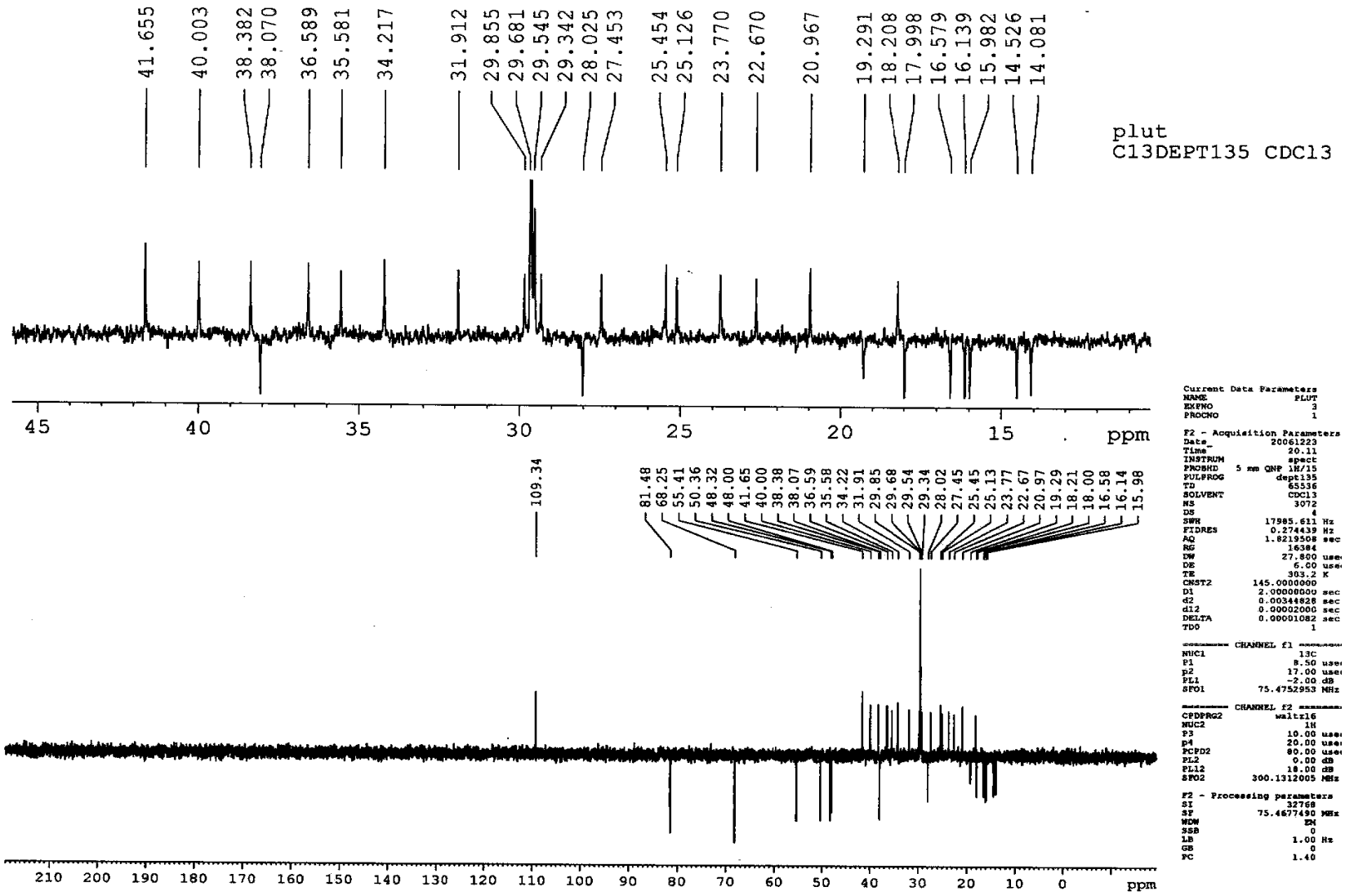
Appendix 1c: ^{13}C NMR spectrum of lupeol-3-hydroxyoctadecanoate **(50)**



Appendix 1d: ^{13}C NMR spectrum (expanded) of lupeol-3-hydroxyoctadecanoate **(50)**



Appendix 1e: DEPT spectrum of lupeol-3-hydroxyoctadecanoate **(50)**

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DS                        4
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FIDRES             0.274439 Hz
AQ                 1.8219508 sec
RG                    16384
DW                   27.800 use
DE                    6.00 use
TE                   303.2 K
CNST2              145.00000000
D1                 2.00000000 sec
d2                 0.00344828 sec
d12                0.00002000 sec
DELTA              0.00001082 sec
TD0                       1

======== CHANNEL f1 ========
NUC1                     13C
P1                      8.50 use
p2                     17.00 use
PL1                    -2.00 dB
SFO1              75.4752953 MHz

======== CHANNEL f2 ========
CPDPRG2               waltz16
NUC2                      1H
P3                     10.00 use
p4                     20.00 use
PCPD2                  80.00 use
PL2                     0.00 dB
PL12                   18.00 dB
SFO2             300.1312005 MHz

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WDW                        EM
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LB                      1.00 Hz
GB                          0
PC                      1.40
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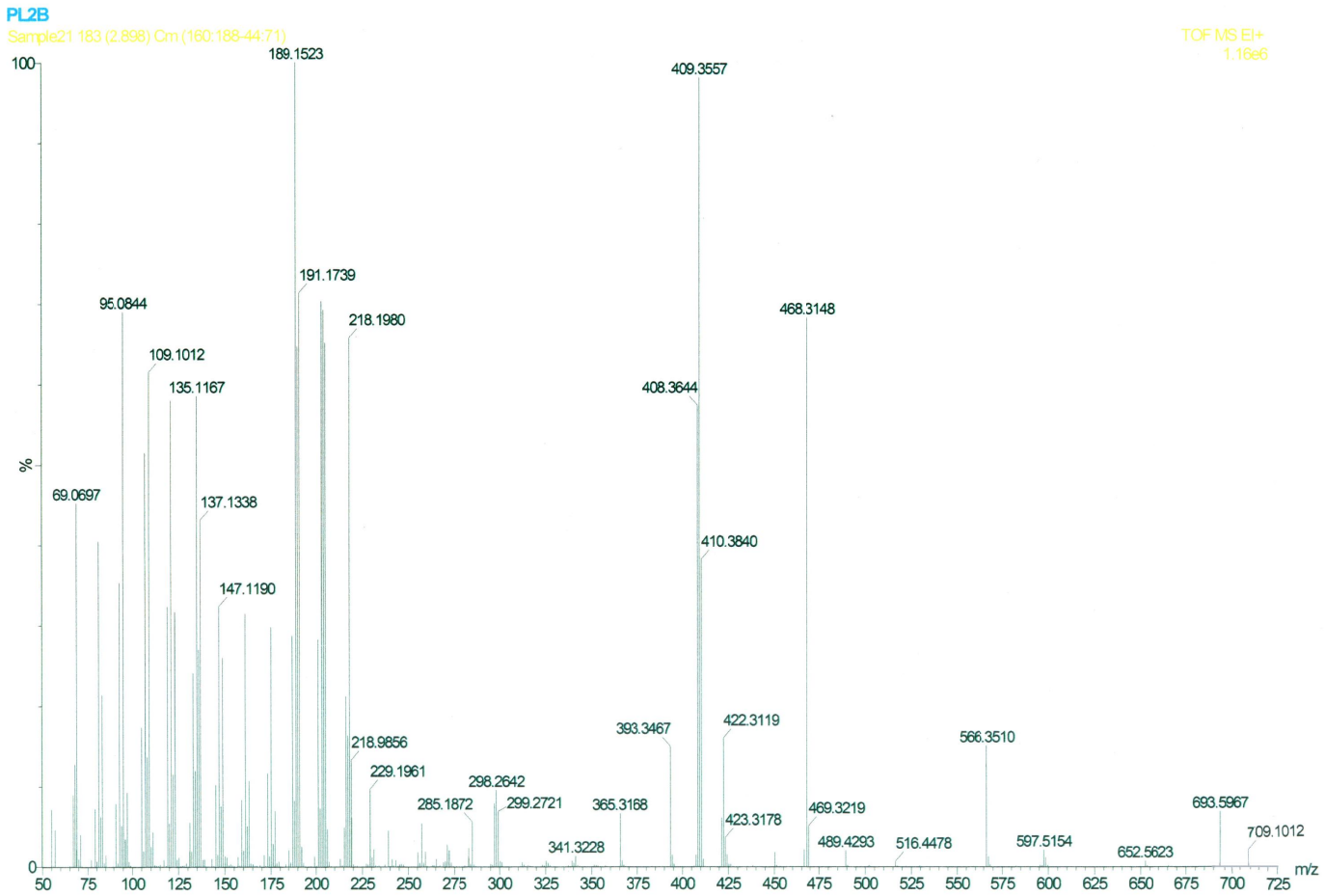
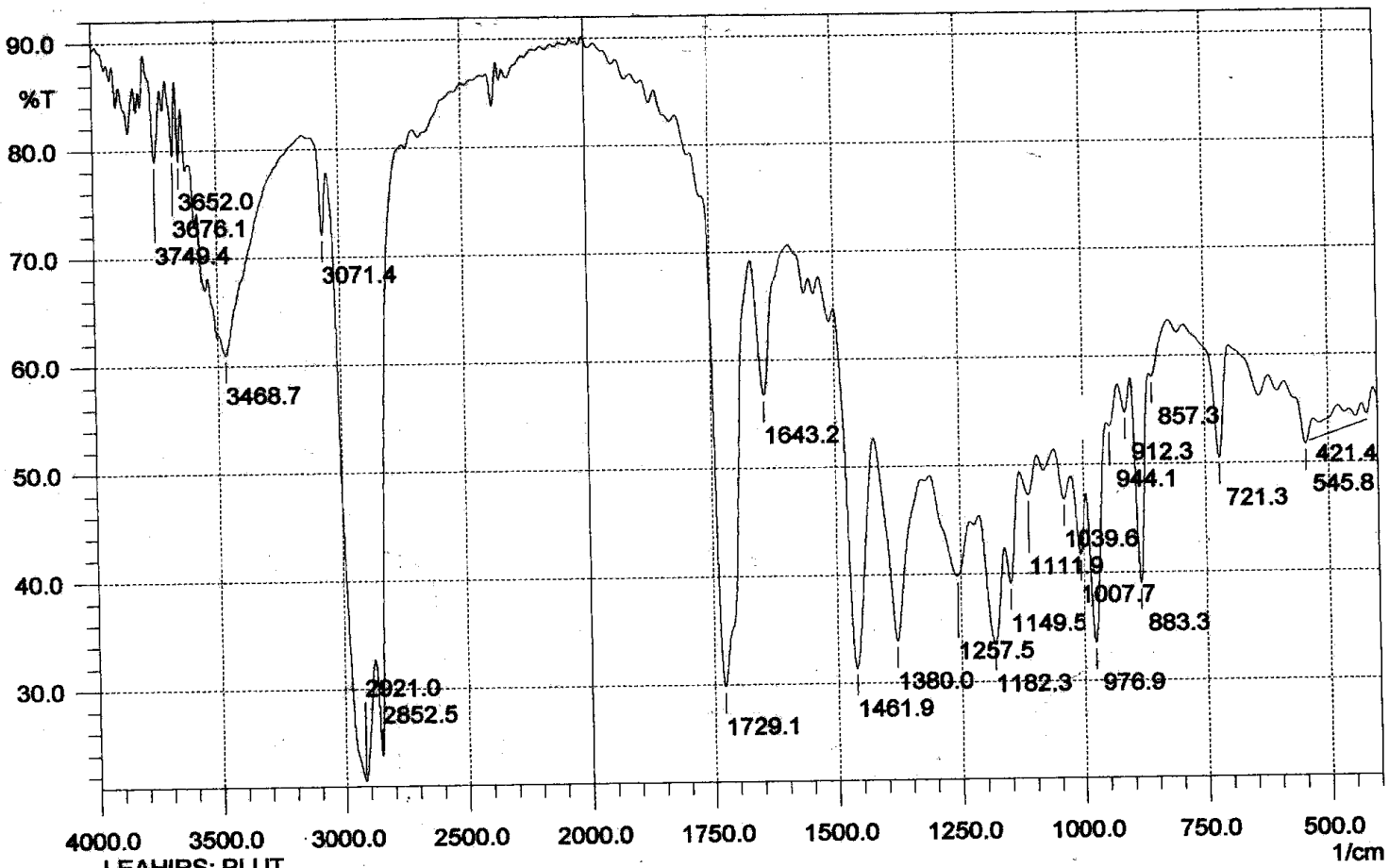
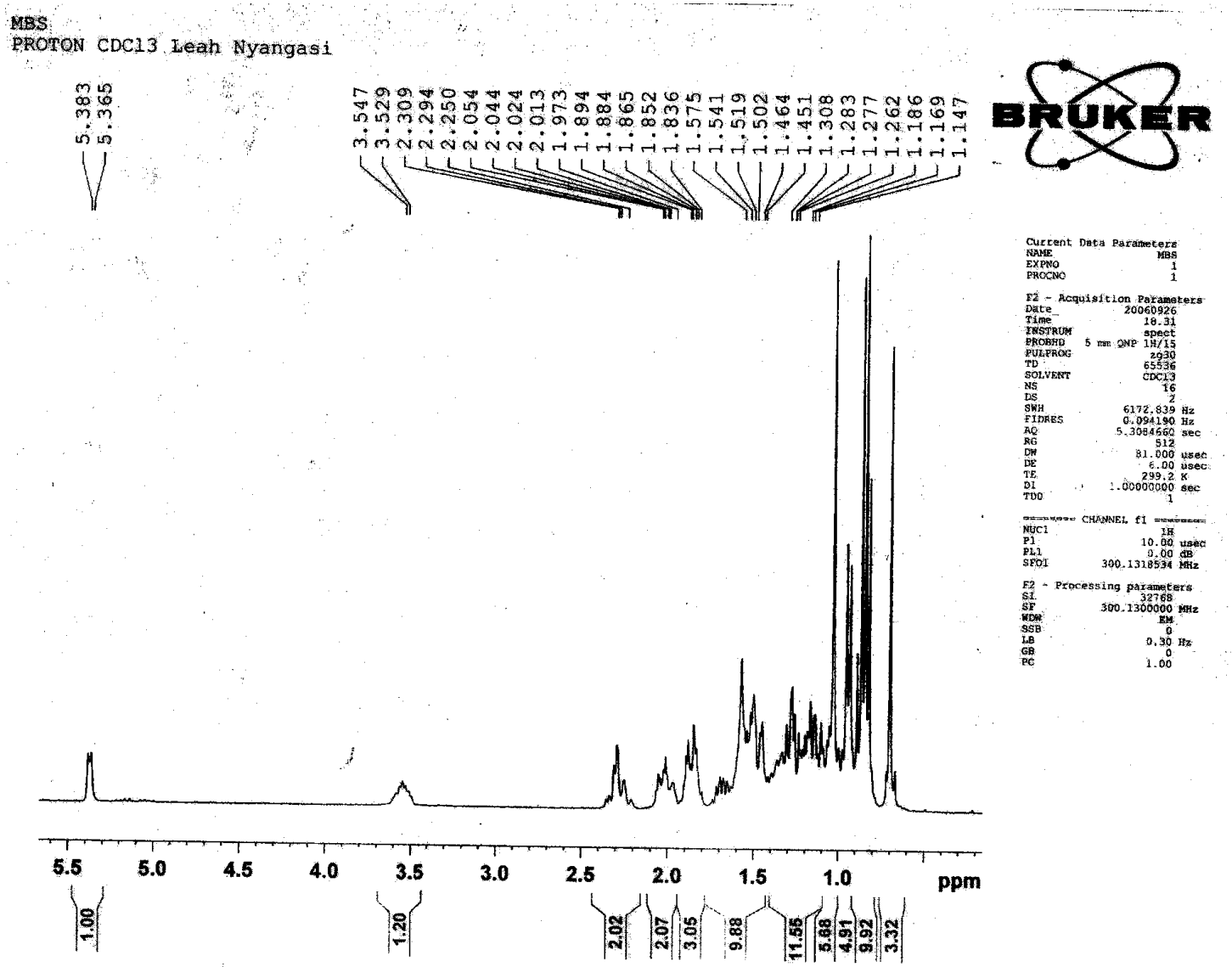
Appendix 1f: Mass spectrum of lupeol-3-hydroxyoctadecanoate **(50)**



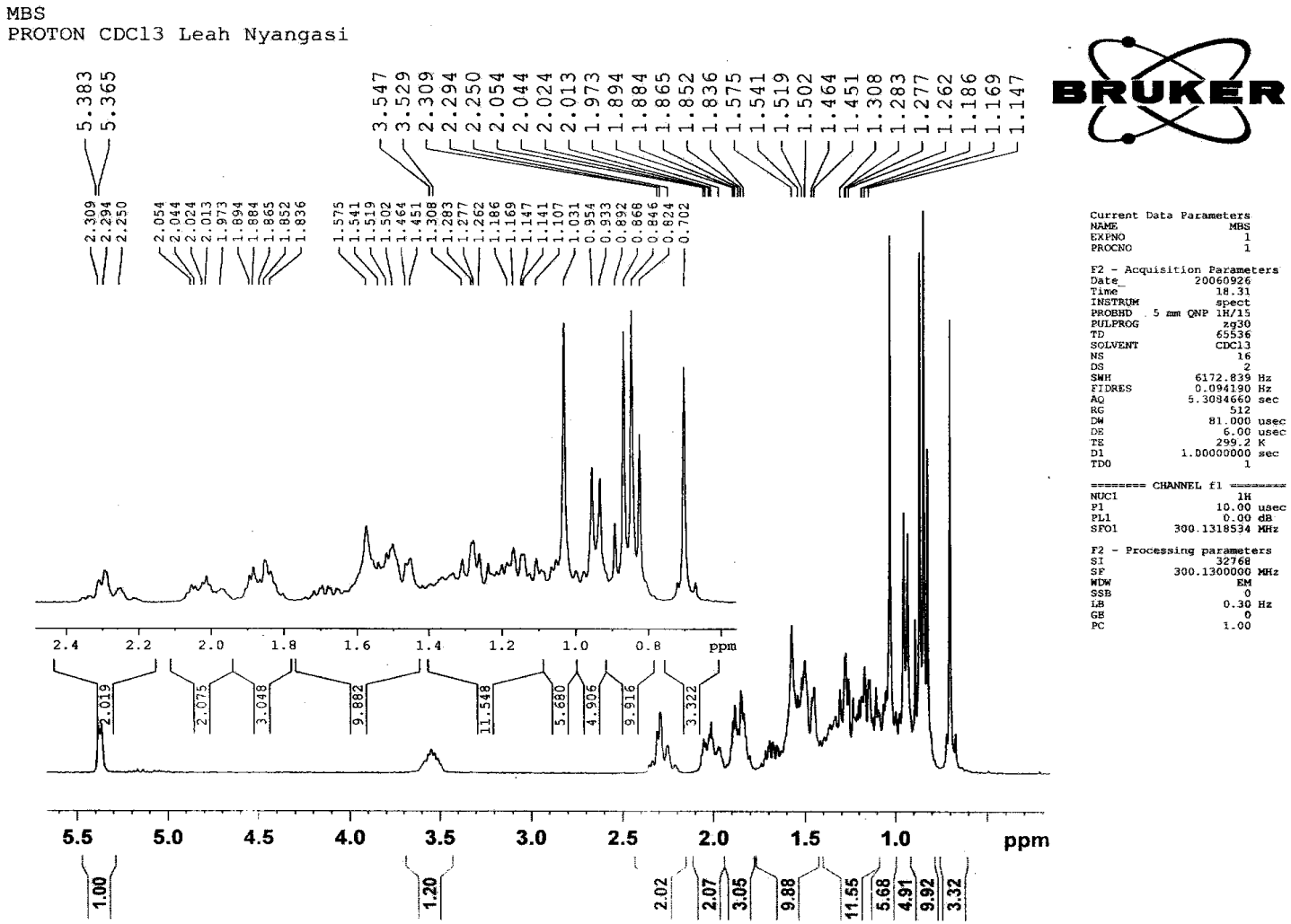
Appendix 1g: IR spectrum of lupeol-3-hydroxyoctadecanoate **(50)**



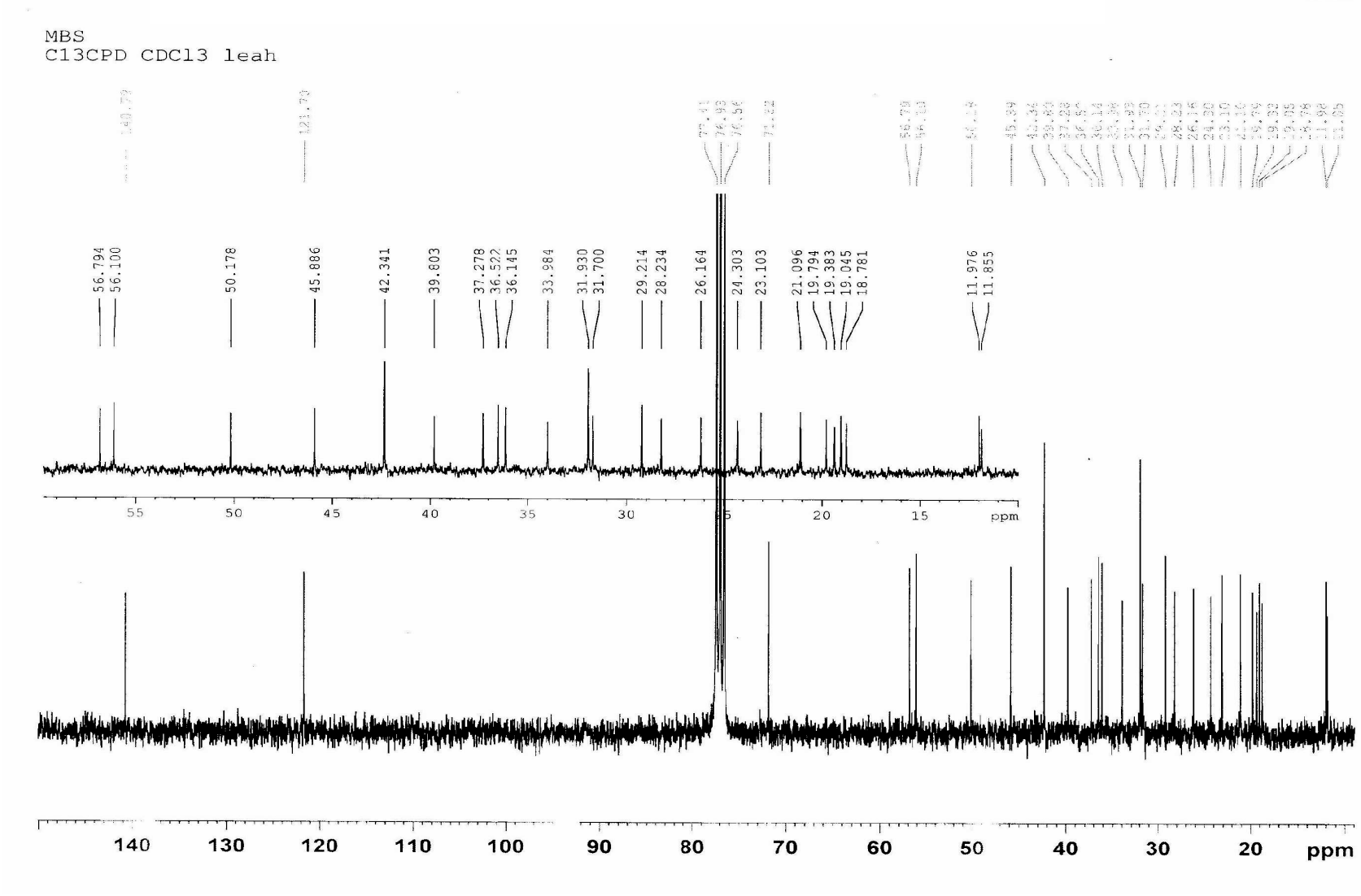
Appendix 2a: ^{1}H NMR spectrum of β-sitosterol **(54)**



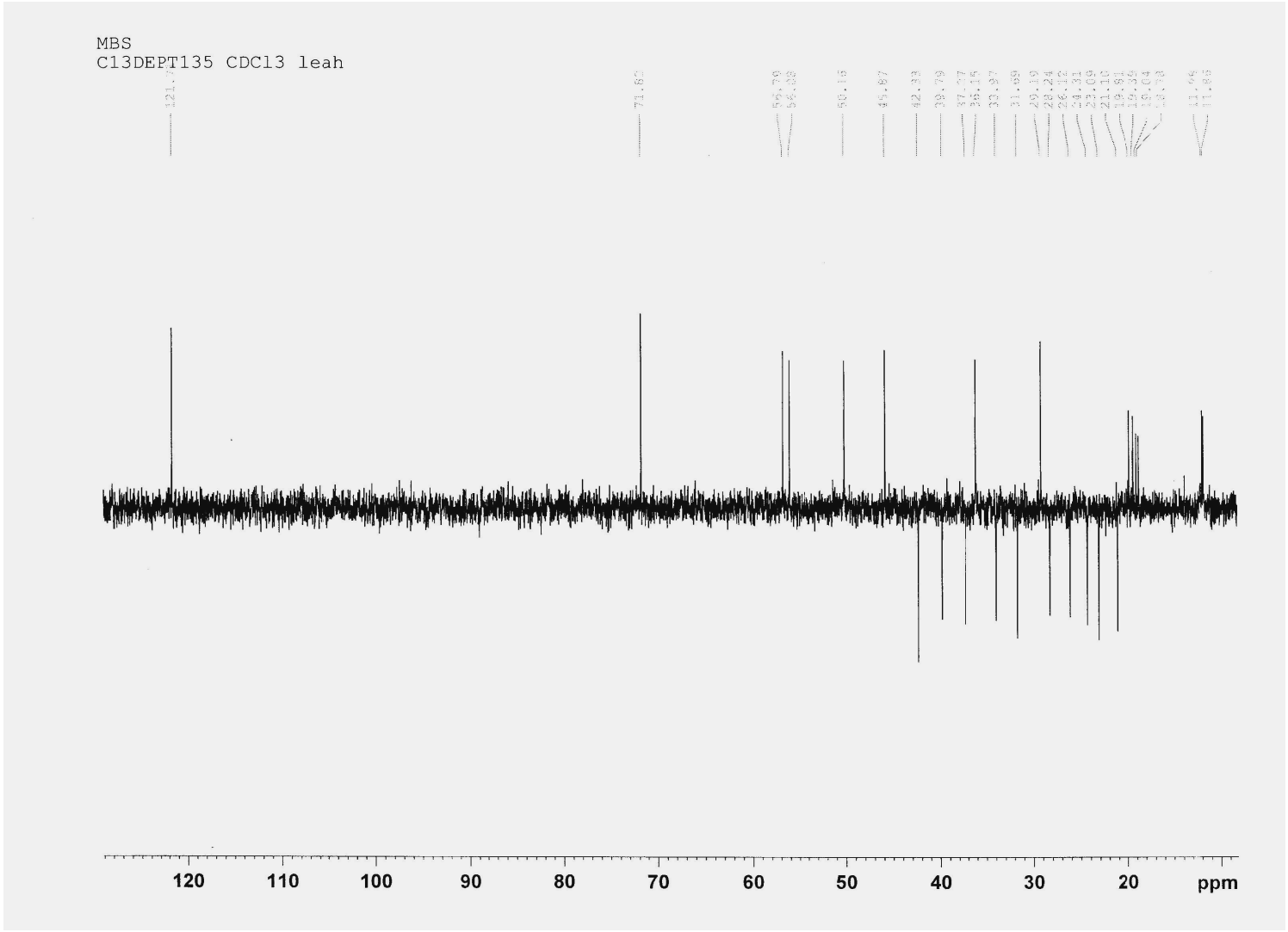
Appendix 2 b: ^{1}H NMR spectrum (expanded) of β-sitosterol **(54)**



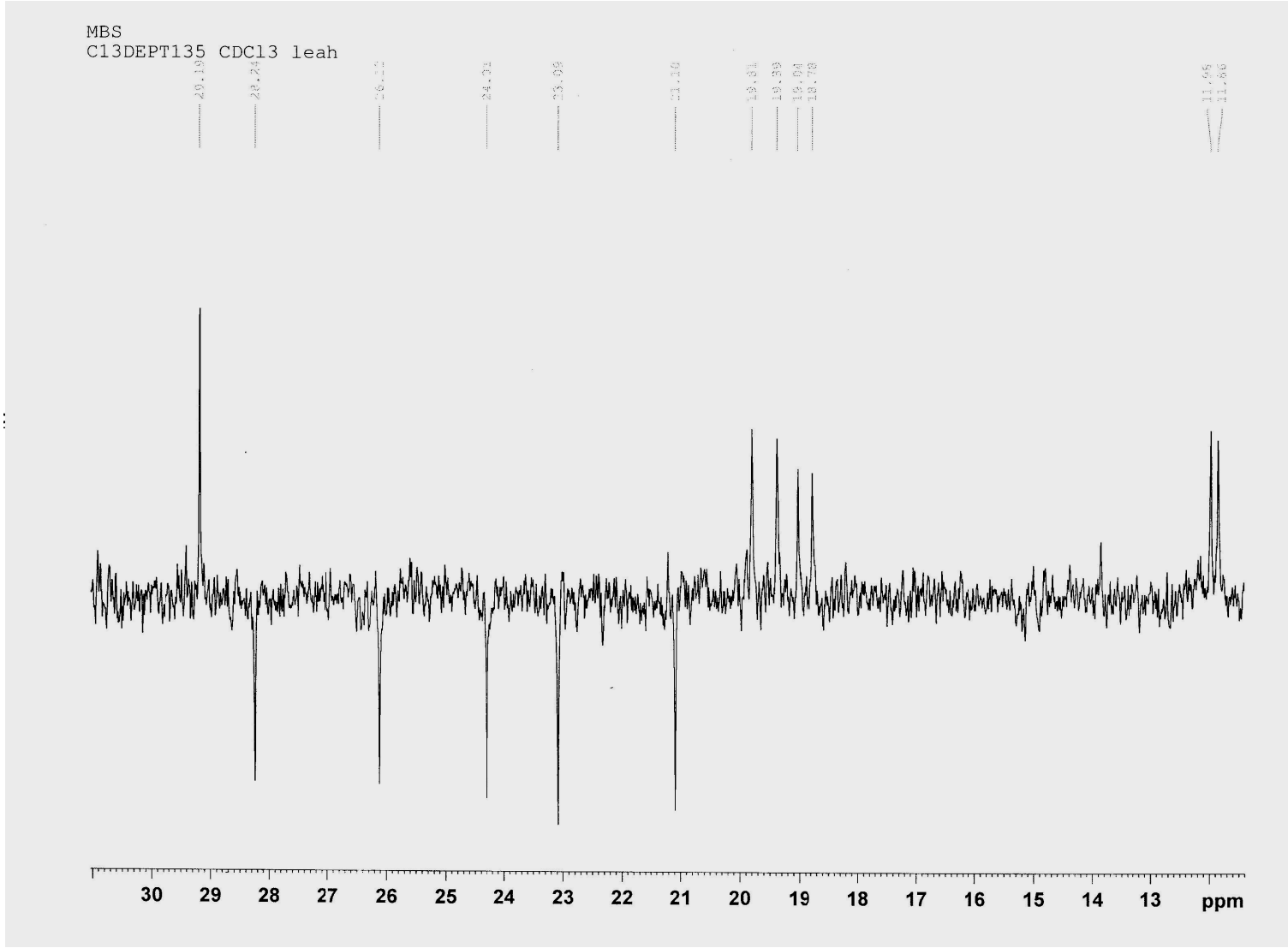
Appendix 2c: ^{13}C NMR of β-sitosterol **(54)**



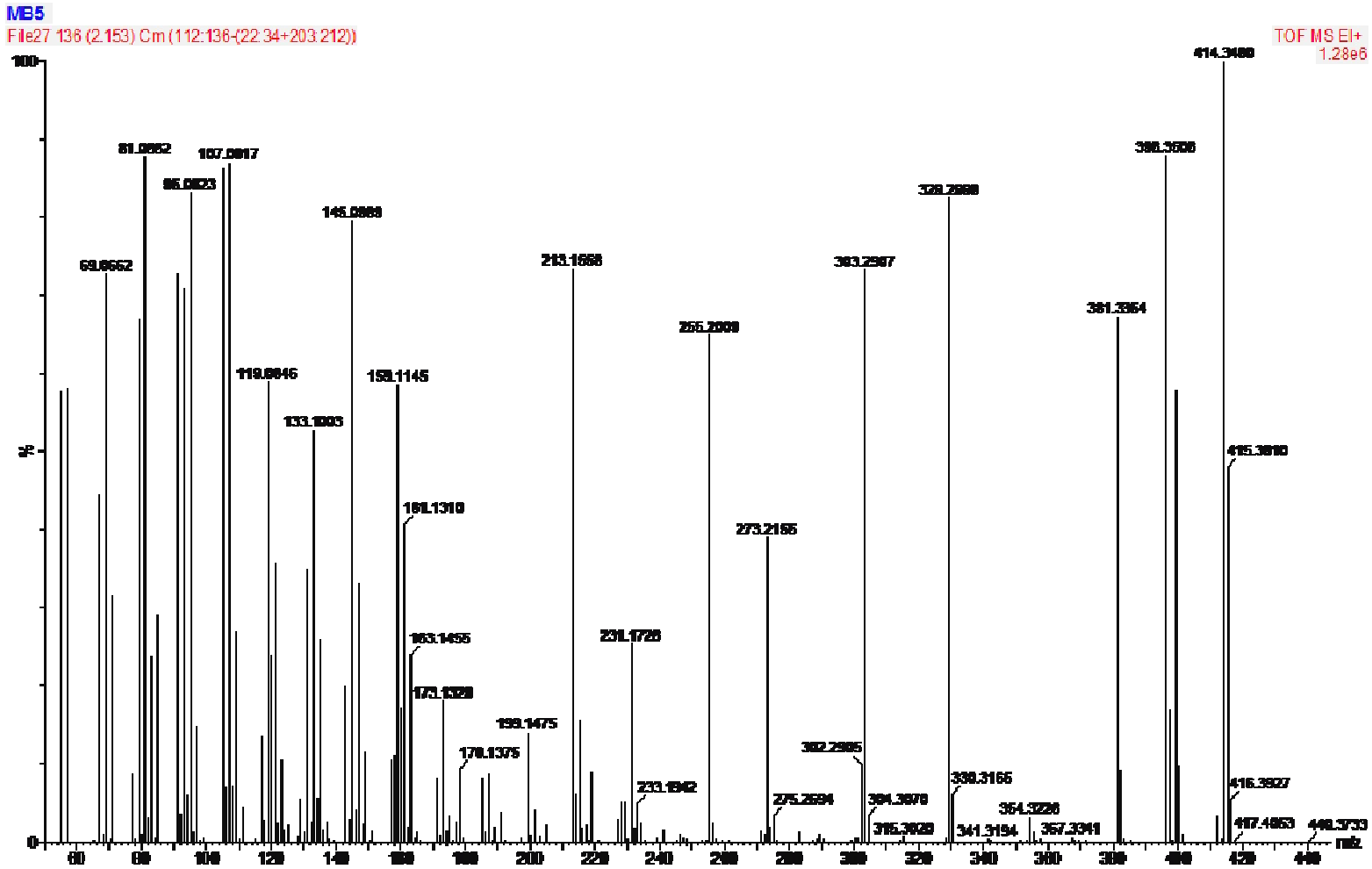
Appendix 2d: DEPT spectrum of β-sitosterol **(54)**



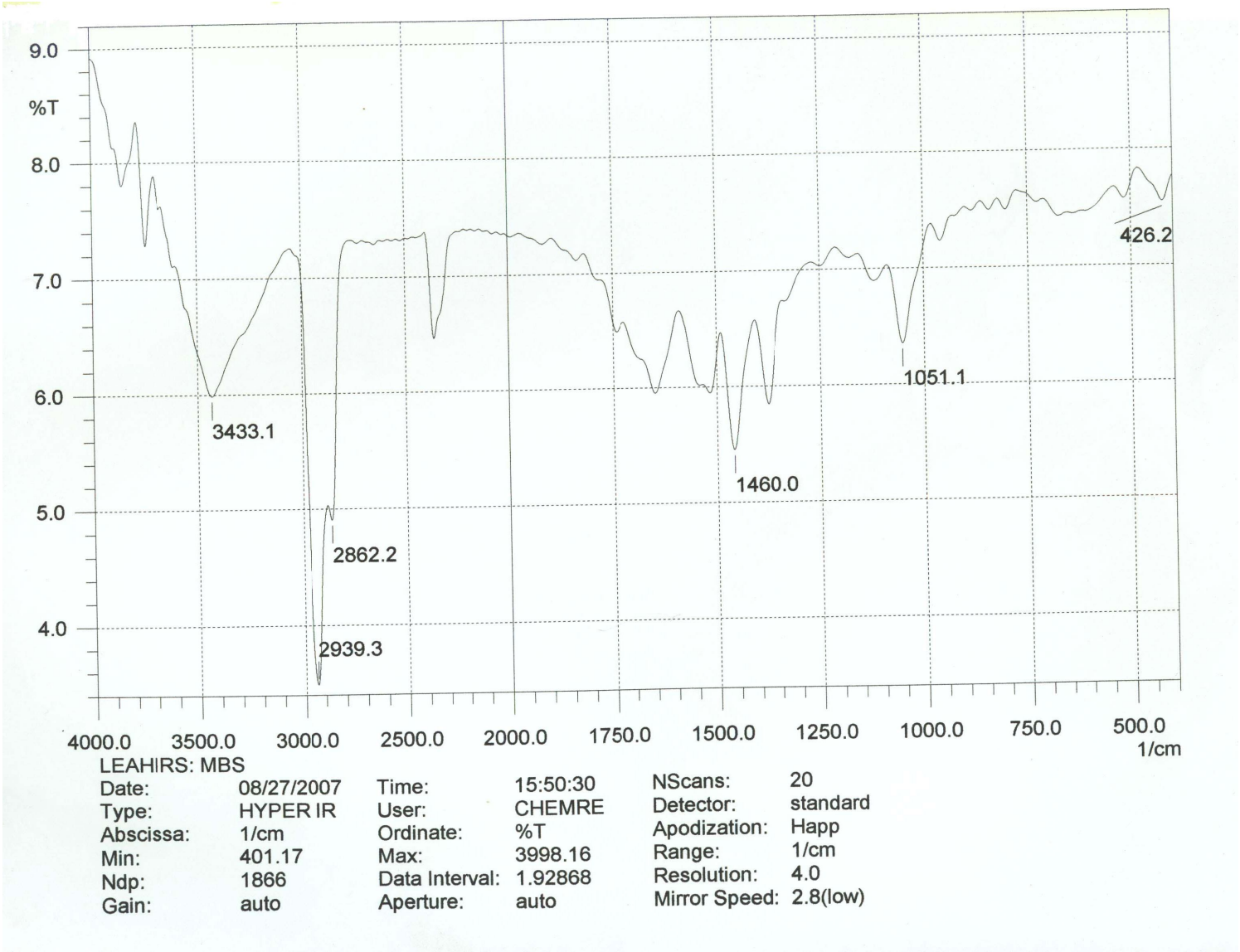
Appendix 2e: DEPT spectrum (expanded) of β-sitosterol **(54)**



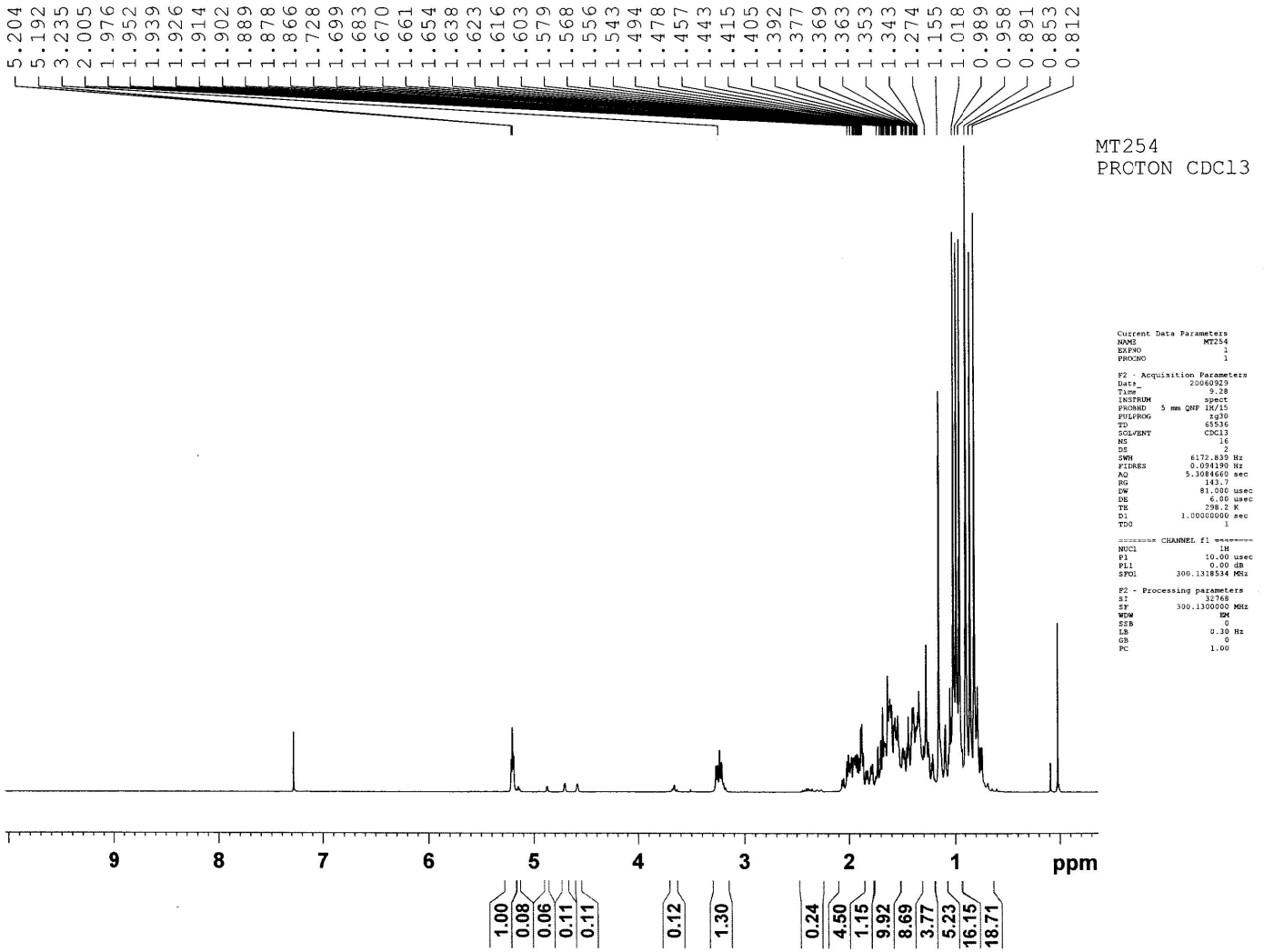
Appendix 2f: Mass spectrum of β-sitosterol **(54)**



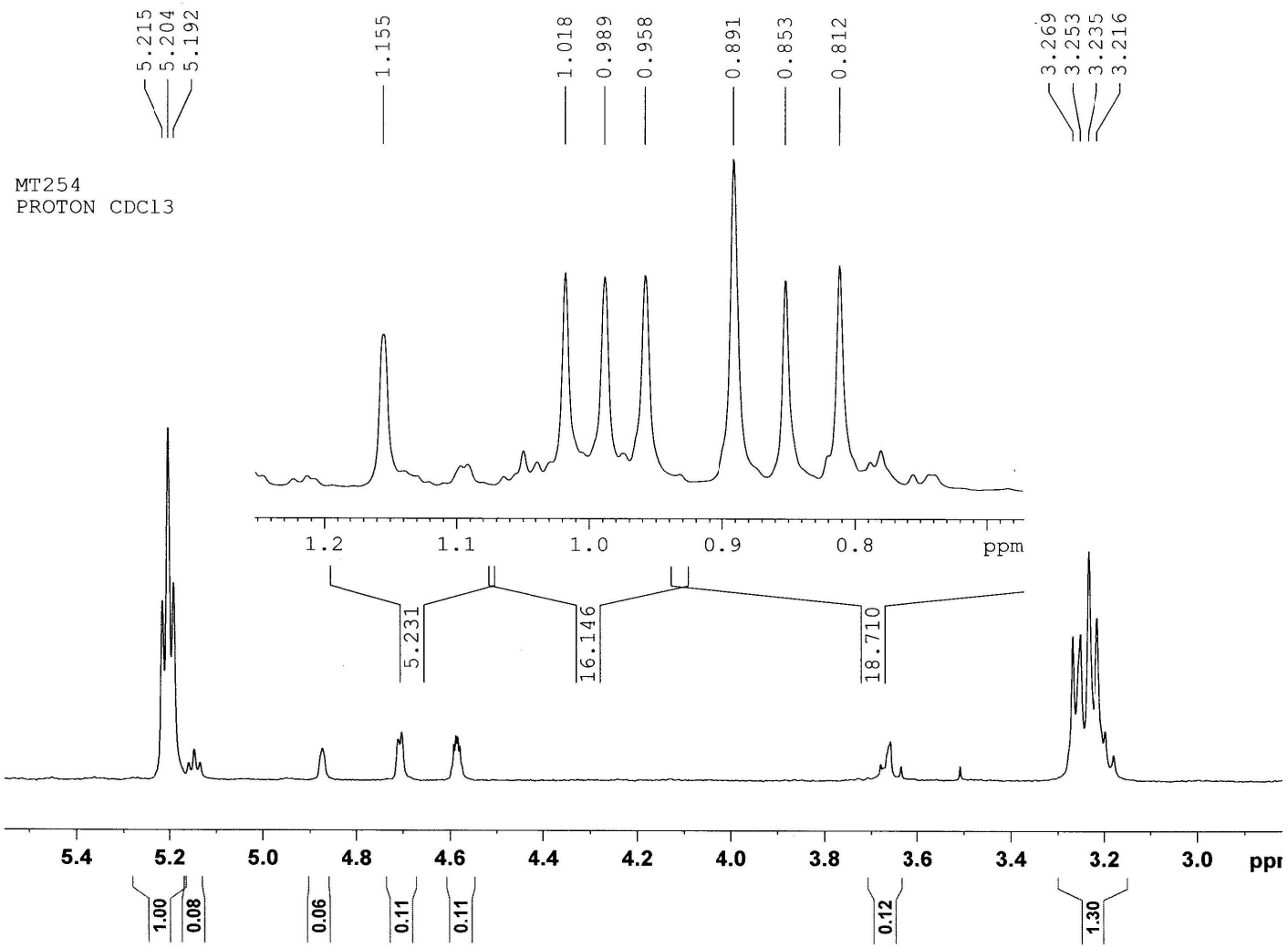
Appendix 2g: IR spectrum of β-sitosterol **(54)**



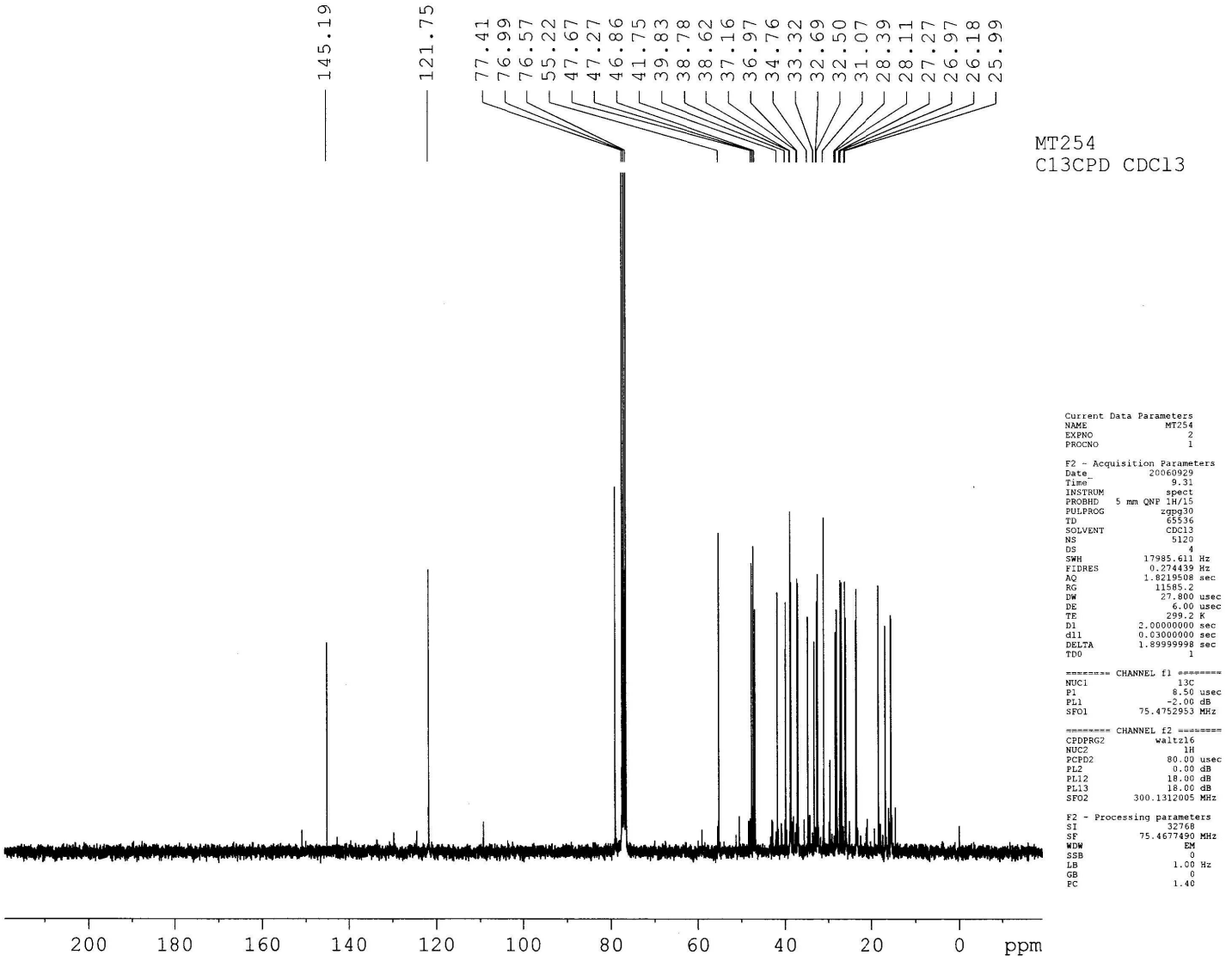
Appendix 3a:[1]H NMR spectrum of β-amyrin **(55)**



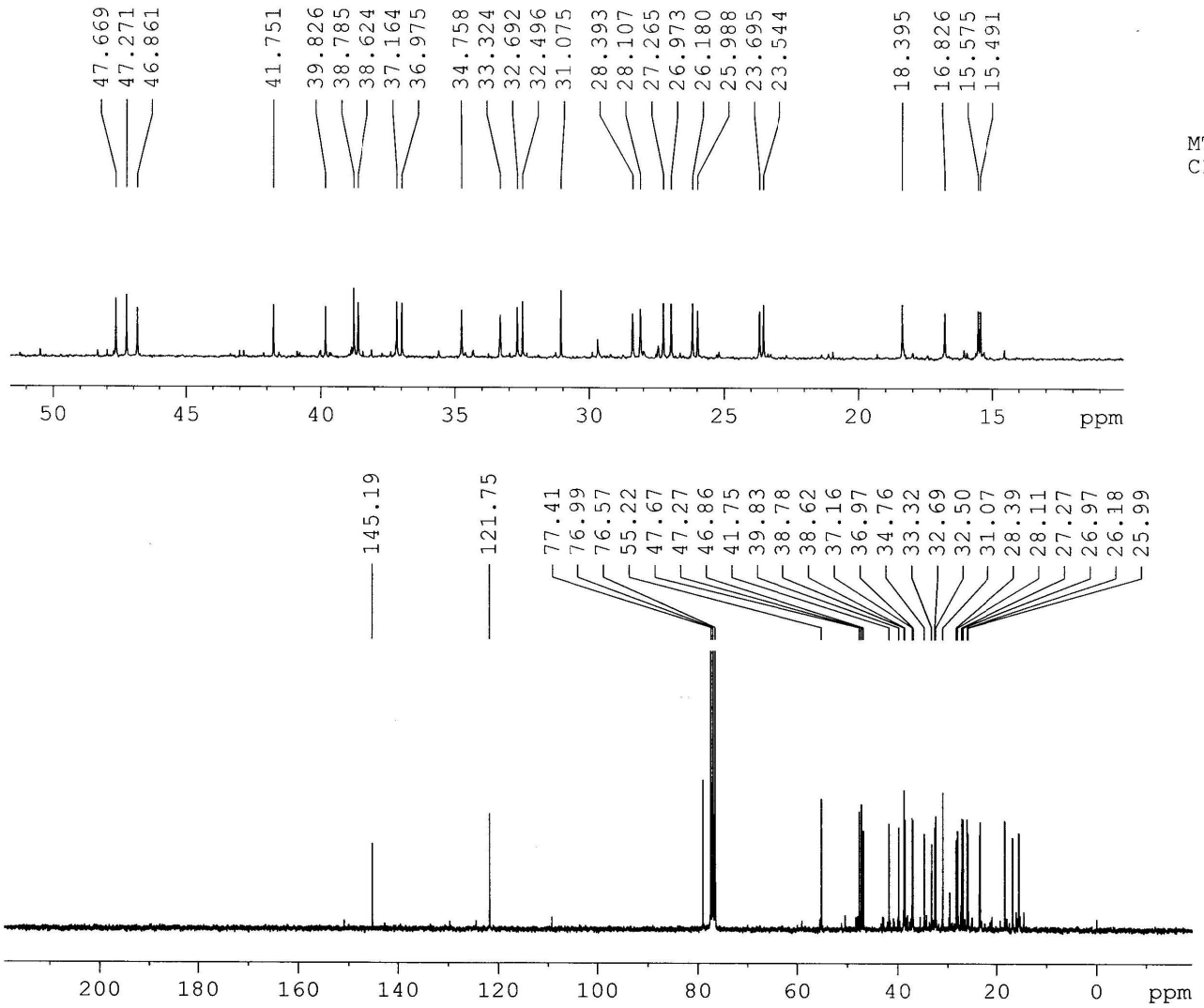
Appendix 3b:[1]H NMR spectrum (expanded) of β-amyrin **(52)**



Appendix 3c:^{13}C NMR of β-amyrin **(55)**



Appendix 3d: ^{13}C NMR spectrum (expanded) of β-amyrin **(55)**

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FIDRES             0.274439 Hz
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DW                    27.800 usec
DE                     6.00 usec
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PL1                   -2.00 dB
SFO1             75.4752953 MHz

======== CHANNEL f2 ========
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NUC2                     1H
PCPD2                 80.00 usec
PL2                    0.00 dB
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PL13                  18.00 dB
SFO2            300.1312005 MHz

F2 - Processing parameters
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PC                      1.40
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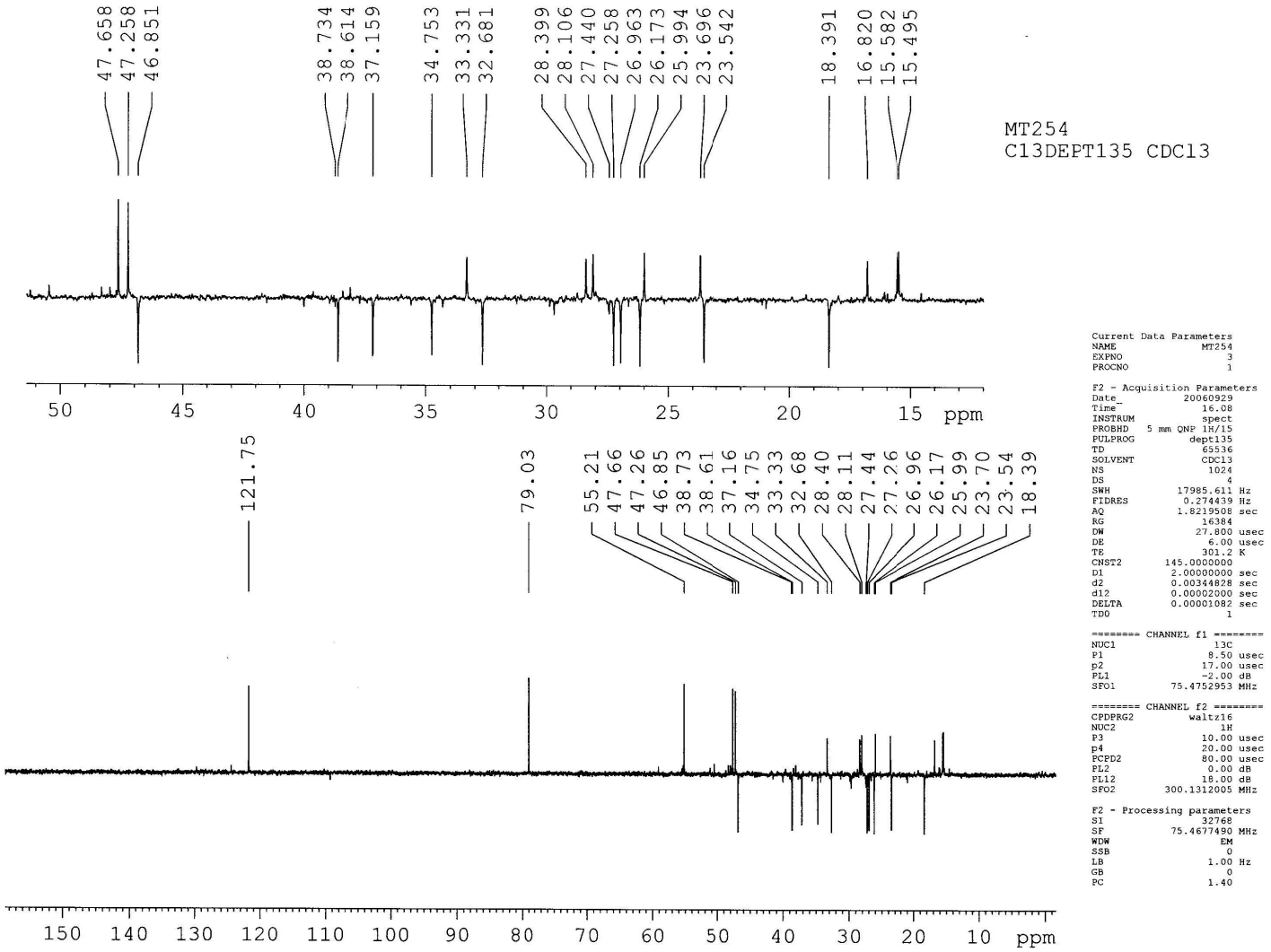
Appendix 3e: DEPT spectrum of β-amyrin **(55)**

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======== CHANNEL f1 ========
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======== CHANNEL f2 ========
CPDPRG2            waltz16
NUC2                    1H
P3                   10.00 usec
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PCPD2                80.00 usec
PL2                   0.00 dB
PL12                 18.00 dB
SFO2          300.1312005 MHz

F2 - Processing parameters
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GB                         0
PC                     1.40
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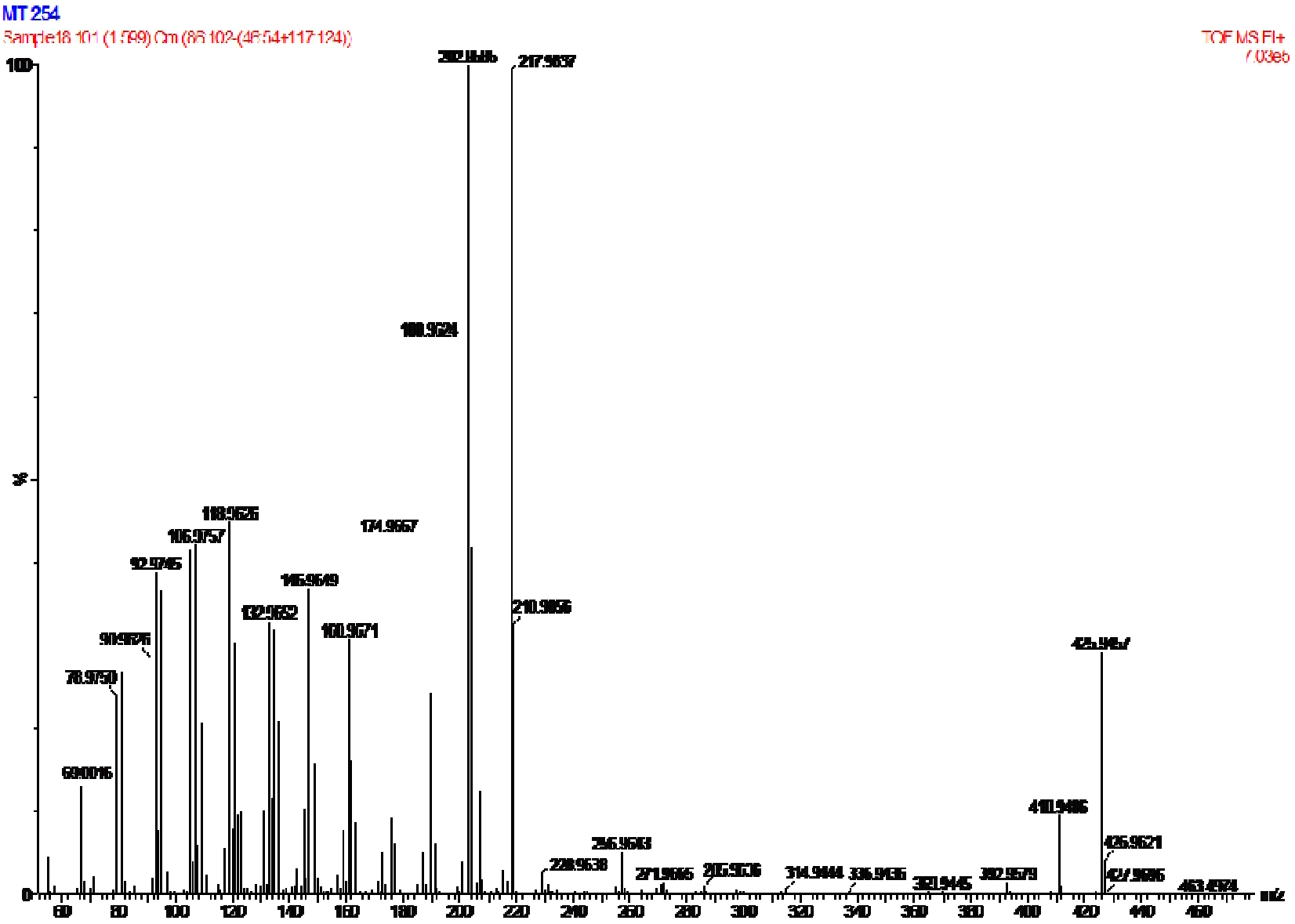
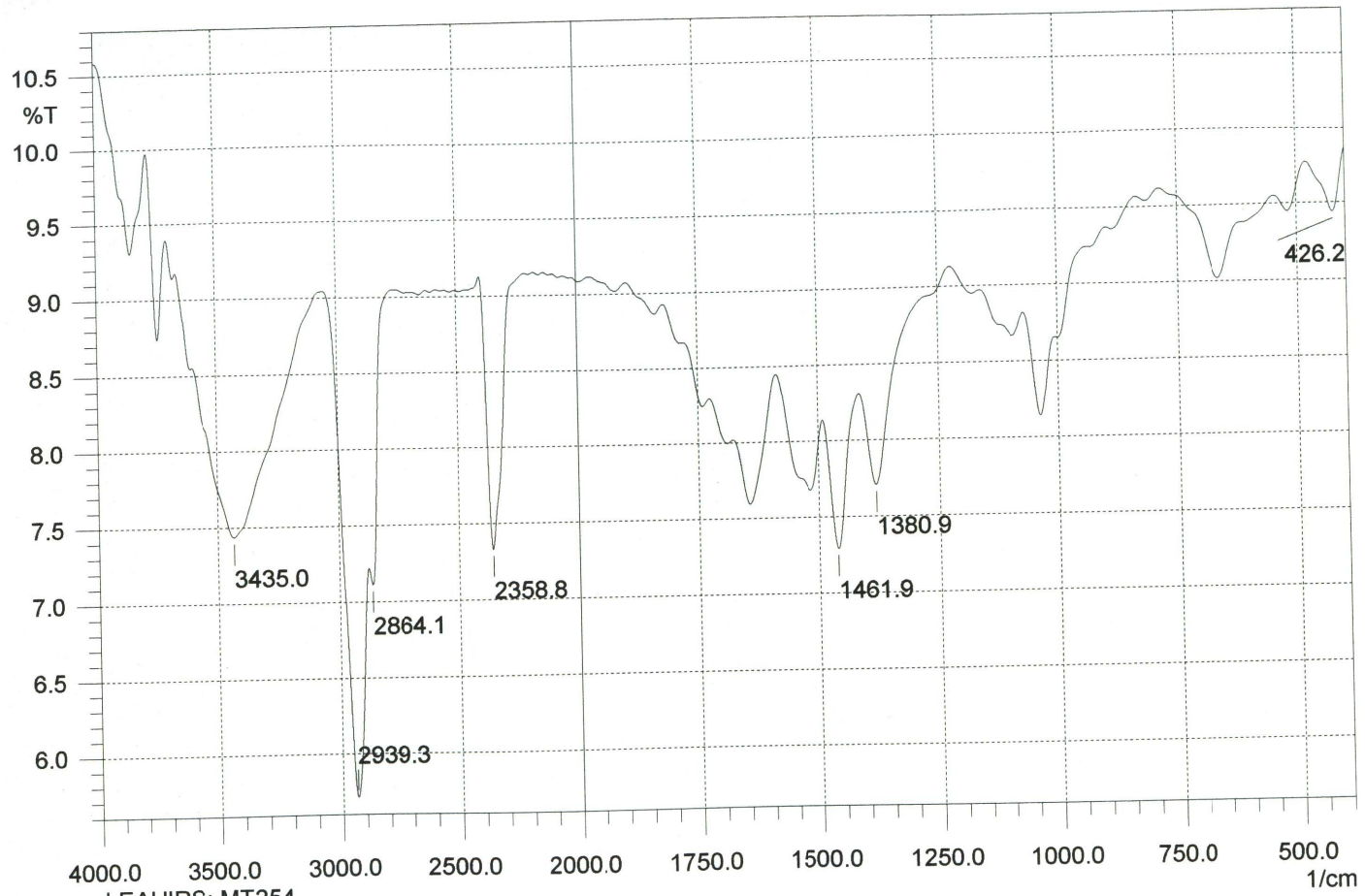
Appendix 3f: Mass spectrum of β-amyrin (**55**)



Appendix 3g: IR of β – amyrin **(55)**



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Ndp:	1866	Data Interval:	1.92868	Resolution:	4.0
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