INVESTIGATION OF PHYTOCHEMICAL AND ANTIMALARIAL ACTIVITY OF *CAESALPINIA VOLKENSII* HARMS SEEDS

A thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Pharmacy in Pharmaceutical Analysis

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DECLARATION

This research thesis is my original work and has not been presented elsewhere for examination.

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DEDICATION

I dedicate this work to my dad Paul Ngomo and mum Lydia Mueni, for their parental love and sacrifice to see me through in life.

To all my brothers, sisters, inlaws, nephews and nieces for always being there with me.

To my special friend and niece, Jane Wavinya, for her great courage and faith in life.

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LIST OF ABBREVIATIONS

ACT	Artemesinin combination therapy
CD	Cluster of differentiation
$CDCl_3$	Deuterated Chloroform
δ	Delta
ε	Molar extinction
g	Gram
h	Hours
HIV	Human immunodeficiency virus
ICAM	Intercellular adhesion molecule
IR	Infrared
IPT	Intermitted preventive therapy
IC_{50}	Concentration causing 50 % inhibition
$_{P}$LDH	Parasite lactate dehydrogenase
KBr	Potassium bromide
LD_{50}	Dose causing 50 % mortality
mL	Milliliters
m	Multiplet
MHz	Mega Hertz
m/z	Mass to charge ratio
λ_{max}	Maximum Wavelength
nm	Nanometers
NBT	Nitroblue tetrazolium

PES	Phenazine ethosulphate
RBCs	Red blood cells
R_f	Retention factor
str	Stretch
s	Singlet
SP	Sulphur-Pyrimethamine
TLC	Thin layer chromatography
t	Triplet
WHO	World Health Organisation

ABSTRACT

The purpose of the study was to do phytochemical investigation and antimalarial activity tests on the seeds of *Caesalpinia volkensii* Harms. Isolation of compounds, structure elucidation and brine shrimp toxicity assay studies were also done. The seeds were collected at Gatundu in Kiambu County Central province on June 2003. The seeds were extracted using water and sequentially using petroleum ether, chloroform and finally methanol.

Phytochemical tests were done on the seeds for the presence of cardiac glycosides, alkaloids, saponins and anthraquinones. Isolation of compounds was carried out on the extracts using chromatographic methods. Structure elucidation of the isolated compounds was carried out using ultraviolet, infrared, mass spectrometry and nuclear magnetic resonance. *In vitro* antimalarial activity test was carried out on the petroleum extract, chloroform extract, methanol extract, water extracts and one of the isolated compounds. The activities were compared to that of the standard drug chloroquine. Brine shrimp toxicity testing was done on the petroleum extract, chloroform extract, methanol extract, water extract and the isolated compound.

Phytochemical testing of the seeds of *Caesalpinia volkensii* Harms showed the presence of both alkaloids and saponins. The phytochemical tests showed absence of cardiac glycosides, tropane alkaloids and anthraquinones.

From the isolation of compounds, three compounds were isolated and designated as compound 1, 2 and 3. Spectroscopic data strongly suggested that compounds 1, 2 and 3 had some structural similarities to glycerol trilinoleate [1^1,2^1,3^1-propanetriyl tris [cis,cis-9,12-octadecanedienoate],

beta-sitosterol [5-stigmasten-3-beta-ol] and glycerol tridocosanoate [$1^1,2^1,3^1$-propnetriyl tridocosanoate] a long chain carboxylic acid ester respectively.

From the antimalarial activity tests, a plot of inhibition against concentrations for each extract and isolated compound gave 50 % inhibition [IC_{50}] at 250.0 µg/mL, 290.0 µg/mL, 435.0 µg/mL, 625 µg/mL, 757.5 µg/mL and 62.5 ng/mL concentrations for methanol extract, chloroform extract, petroleum ether extract, water extract, isolated compound and standard drug chloroquine respectively. All the tested extracts and isolated compound were shown to have antiplasmodial activity at concentrations much higher than that of standard drug chloroquine. Methanol extract gave the highest activity.

From the brine shrimp toxicity study, a plot of toxicity against concerntrations for each extract and isolated compound gave LD_{50} values at 562.5 µg/mL, 375.0 µg/mL, 375.0 µg/mL, 625.0 µg/mL and 750.0 µg/mL concentrations for methanol extract, chloroform extract, petroleum ether extract, water extract and isolated compound respectively. The water extract used by the traditional folklore was still safe at high concentrations.

The present work gave a scientific basis for the use of *Caesalpinia volkensii* Harms as treatment for malaria and hence recommendations for further studies on qualitative and quantitative analysis and also to ascertain the structures of the isolated compounds.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. Malaria

Malaria is a protozoan disease that is transmitted by the infected female Anopheles mosquito of which the *Anopheles gambiae* is responsible for the transmission in Africa. The specific protozoan organisms causing malaria are from the genus Plasmodium. Only four of the approximately one hundred species cause the disease in humans. The other species affect birds, monkeys, livestock, rodents and reptiles. The four species that affect humans are *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae* and *Plasmodium ovale*. Each of these species has a distinctive morphology, and each of them causes a different onset of symptoms. Concurrent infections by more than one of these species are seen in endemically affected regions of the world [1-4].

Malaria infection may result in a wide variety of symptoms, ranging from very mild symptoms to severe disease and even death. The most common symptoms of malarial infection include chills, fever, sweating, headache, fatigue, anorexia, nausea, vomiting and diarrhea. The malarial infections are known according to the causative parasite [3]. *Plasmodium falciparum* [tertian malaria] is responsible for most malarial death, especially in Africa. The infection has an incubation period of one to three weeks. The infection can develop suddenly and cause several life threatening complications. Recurrence of the symptoms on alternate days is characteristic of *P. falciparum* infection hence tertian malaria. The *P. falciparum* parasite can also cause the human red blood cells to clump and adhere to the walls of blood vessels. Such a phenomenon has been known to cause partial obstruction and sometimes restriction of the blood flow to vital organs such as the brain, liver and kidneys [5,6]. *Plasmodium vivax* [benign tertian infection] is

the most prevalent form of malaria with an incubation period of one to four weeks. The infection produces less severe symptoms but relapses can occur upto three years and the chronic disease is debilitating. This form of malaria can cause spleen rupture and anemia. *Plasmodium malariae* is responsible for quartan malaria, which is the mildest form of malaria and it does not relapse. It has an incubation period of two to four weeks; in addition to the usual malarial symptoms, this form also causes nephritis. The infection can persist in the blood for long periods possibly decades without ever producing symptoms [5,6]. *Plasmodium ovale* [ovale tertian] has an incubation period of nine to eighteen days. Relapses have been known to occur in people infected with this plasmodium [5].

1.2. Epidemiology

1.2.1. Distribution

Malaria occurs in most tropical regions of the world with *P. falciparum* predominating in Africa, New Guinea and Haiti. *Plasmodium vivax* is more common on the Indian sub-continent and Central America with the prevalence of these two infections roughly equal in Asia, Oceania and South America. *Plasmodium malariae* is found in endemic areas especially sub- Saharan Africa, but much less frequently. *Plasmodium ovale* is relatively unusual outside Africa although some cases are now being identified in other regions like the southern states of India. With the relative ease and speed of transport, imported cases of malaria may be present in any country. In addition the so called airport malaria has been identified in such countries like United States of America, United Kingdom, Belgium and Switzerland [4,6-8]. In Kenya *P. falciparum* is the predominant species accounting for 98.2 % while *P. malariae* and *P. ovale* account for 1.8 % often occurring as mixed infections. *Plasmodium vivax* may account for upto 50 % of the infections in the northern and north eastern parts of Kenya.

1.2.2. Transmission

Malaria is transmitted in tropical and subtropical areas where the anopheles mosquito can survive and multiply and where the malaria parasite can complete their growth cycle in the mosquito [extrinsic incubation period]. Temperature is particularly critical. At temperatures below 20 ^{0}C the *P. falciparum* cannot complete its growth cycle in the anopheles mosquito and thus cannot be transmitted even within tropical and subtropical areas at high altitudes, during cooler seasons in some areas and in the desert excluding the oases. Generally transmission is more intense in warmer regions close to the equator [1,4,6]. Malaria is transmitted all year round where the *P. falciparum* predominates with the highest transmission in Africa, South of Sahara. In the cooler regions, transmission is less intense and more seasonal. In these regions, *P. vivax* is more prevalent because it is more tolerant to temperatures below 25 ^{0}C. In many temperate areas, such as Western Europe and the United States of America, economic development and public health measures have succeeded in eradicating malaria. However, most of these areas have anopheles mosquito that can transmit malaria and re-introduction of the disease is a constant risk [1,2,9,10]. In 2009, a model based map of the intensity of *P. falciparum* transmission in Kenya was produced. This was defined by the proportion of infected children aged 2-10 years in the community. Based on the malaria risk map and the eco-epidemiology of malaria in Kenya, districts were stratified into four: Lake stable endemic and coastal seasonal stable endemic [risk class equal to or above 20 %], highland epidemic-prone districts [risk class 5-20 %], seasonal low transmission including arid and semi arid districts [risk class less than 5 %] and low risk districts of central Kenya and Nairobi with risk class less than 0.1 % [7].

1.2.3. Mortality and Morbidity

Worldwide mortality due to malaria threatens the lives of more than one third of world's population. As many as 2.7 million of its victims, mostly infants and children under five years die yearly [2, 4]. In the last decade, the prevalence of malaria has been increasing, especially in Africa. An estimated 300 to 500 million cases are reported each year, of which 90 % are children under five. Malaria has been estimated to be responsible for 2.3 % of all disease conditions globally and 9 % of all disease conditions in Africa ranking third among major infectious disease in Africa. Africa accounts for 90 % of the malaria cases in the world [6, 8]. In Kenya about 70 % [28 million] of the total population is at risk of malaria infection. This accounts for 30 % of all outpatient attendance and 19 % of all admissions to health facilities. About 26,000 children die every year due to malaria infection [7,11]

1.3. Life cycle

The life cycle of the malaria protozoa comprises of the sexual phase [sporogony] in the mosquito and asexual phase [schizogony] in man. Infection in man is caused by sporozoites from bite of an infected female anopheles mosquito. The parasites, in the sporozoite stage, enter the circulatory system and reach the liver in about an hour. These organisms grow and multiply 30,000-40,000 fold by asexual division within the liver cells in 5 - 7 days to form tissue schizonts which mature and release thousands of merozoites in the blood on rupture of the cells. The merozoites enter erythrocytes where they transform into trophozoites. These produce blood schizonts which mature and rupture to release merozoites into circulation, which can infect other erythrocytes. This process results in the patient exhibiting the clinical symptoms such as chills, fever, sweating, headache, fatigue, anorexia, nausea, vomiting and diarrhea. Recurrence of these symptoms on alternate days is characteristic of tertian malaria. Re-infection of the erythrocytes

can occur allowing further multiplication and re-manifestation of the malaria symptoms. Some of the merozoite develop into male and female sexual forms called gametocytes, which can then be acquired by the female mosquito after biting the infected human. Gametocytes enter the mosquito stomach where fertilization occur and go on to form zygotes. The zygote forms migrate to the insects salivary glands where the sporozoite form can be transmitted to another human following a mosquito bite [5,11]. In *P. vivax* and *P. ovale* infections, some of the sporozoites entering the liver cells enter a latent tissue stage in the form of hypnozoites which are responsible for the recurrence of malaria caused by these organisms. Recurrences resulting from the persistence of latent tissue forms are often called relapses while renewed attacks caused by persistent residual erythrocytic forms are called recrudescence [5,12]. The lifecycle of the malaria parasite is shown on figure1.

1.4 Malaria pathogenesis

Malaria sporozoites, the stage transmitted by mosquito bites, have a single antigen on their surface. These sporozoites are released into the blood and within minutes attach to and invade the liver cells by binding to the hepatocyte receptor for the serum proteins thrombospondin and properdin. The sporozoites mature within the liver cells into merozoites and are released into the red blood cells. As the parasites mature within the red blood cells they change morphologic appearance from the ring to schizont form and secrete proteins that form 100 nm bumps on the red blood cells called knobs. Malaria proteins on the surface of the knobs called sequestrins encoded by Var-genes bind to the endothelial cells by ICAM-1, the thrombospondin receptor and the glycophorin CD 46 and cause malaria infected red blood cells to be removed from the circulation. Celebral involvement by *P. falciparum,* which causes upto 80 % of deaths in children, is due to the adhesion of the *P. falciparum* parasite to endothelial cells within the brain.

Patients with celebral malaria have increased amount of ICAM-1, thrombospondin receptor and CD 46 on the celebral endothelial cells to which the malaria infected red blood cells bind [5, 12].

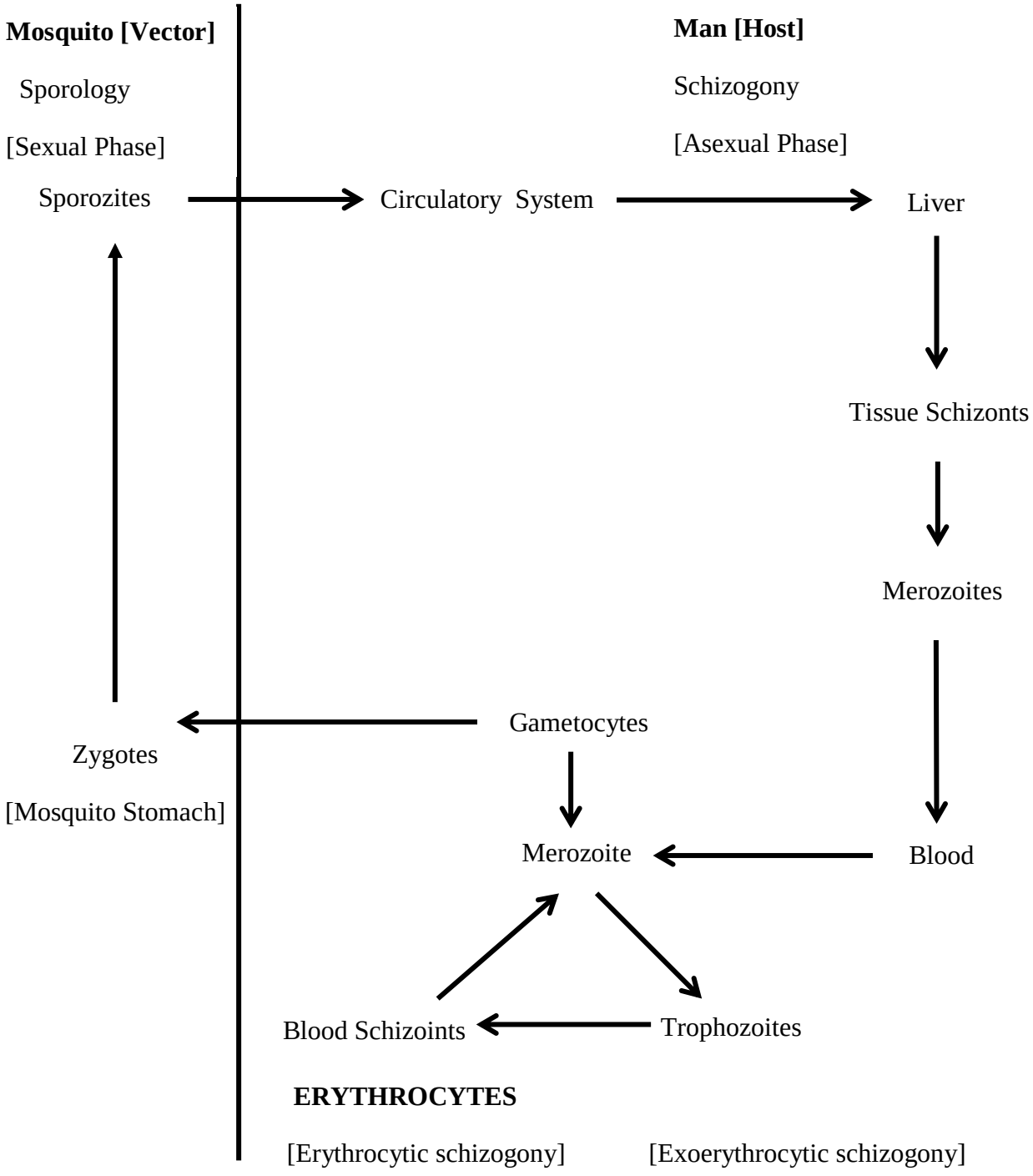


Figure 1. The lifecycle of the Malaria Parasite (Ref 5).

1.5. Malaria diagnosis

1.5.1. Uncomplicated malaria

The classical but rarely observed malaria attack lasts 6-10 hours. It consists of a cold stage [sensation of cold, shivering], a hot stage [fever, headaches, vomiting and seizures in young children] and finally a sweating stage [sweats, return to normal temperature, tiredness]. The attack occurs every second day with the tertian parasites [*P. falciparum, P. vivax and P. ovale*] and every third day with quartan parasite [*P. malariae*]. The patient more commonly presents with a combination of fever, chills, sweats, headaches, nausea and vomiting, body aches and general malaise [3,7,9,12,13].

In countries where cases of malaria are infrequent, these symptoms may be attributed to influenza, a cold or other common infections especially if malaria is not suspected. Conversely, in countries where malaria is frequent, residents often recognize the symptoms as malaria and even treat themselves without seeking laboratory diagnostic confirmation. This is referred to as presumptive treatment. The physical findings may include elevated temperature, perspiration, weakness and enlarged spleen. In *P. falciparum*, additional findings include mild jaundice, enlargement of the liver and increased respiratory rate [7,12,13].

1.5.2. Complicated malaria

Complicated or severe malaria occurs when *P. falciparum* infections are complicated by serious organ failure or abnormalities in the patient blood or metabolism. The manifestations in complicated malaria include cerebral involvement with abnormal behavior, impairment of consciousness, seizures, coma or other neurological abnormalities, severe anemia due to hemolysis, hemoglobinuria, pulmonary edema or acute respiratory syndrome, abnormalities in blood coagulation and thrombocytopenia, cardiovascular collapse and shock, acute kidney failure

and metabolic acidosis often in association with hypoglycemia. Neurological defects may occasionally persist following cerebral malaria especially in children causing such defects like ataxia palsies, speech difficulties, deafness and blindness [5,12].

Malaria in pregnancy may cause severe disease in the mother and may lead to premature delivery or delivery of a low birth weight baby. On rare occasions *P. vivax* malaria can cause rupture of the spleen or acute respiratory distress syndrome. Nephritic syndrome can result from chronic or repeated infections with *P. malariae*. Hyperactive malarial splenomegaly, also called tropical splenomegaly syndrome, occurs infrequently and is attributed to abnormal immune response to repeated malarial infections. The disease is marked by a much enlarged spleen and liver, abnormal immunologic findings, anemia and susceptibility to other infections [6,12,14].

1.5.3. Laboratory diagnosis

The first and most common malaria diagnosis method is the microscopic diagnosis. Malarial parasites can be identified by examining a drop of the patient's blood under a microscope spread out as a blood smear on a microscope slide. Prior to examination, the specimen is stained with Giemsa stain to give the parasite a distinctive appearance. Thick films are recommended for parasite detection and quantification and can be used to monitor treatment outcomes while thin films are recommended for species identification. This technique remains the gold standard for laboratory confirmation of malaria. However it depends on the quality of reagents, the microscope and the experience of the laboratory technologist [12]. Rapid diagnostic tests [RDTs] are immunochromatographic tests based on detection of specific parasite antigens. Tests which detect plasmodial histidine rich protein-2[HRP2] are specific for *P. falciparum* while those that detect parasite specific lactate dehydrogenase [pLDH] or aldolase have the ability to differentiate

between *P. falciparum* and non-*P. falciparum* malaria [12,13,15]. Other techniques include detection of antibodies to malaria parasites and detection of parasite DNA, based on the polymerase chain reaction [PCR].

1.6. Malaria re-emergence and control

A number of factors appear to contribute to the resurgence of malaria. These factors include; a rapid spread of resistance of malaria parasites to the common antimalarials, frequent armed conflicts and civil unrest in many countries forcing large populations to settle under difficult conditions sometimes in areas of high malarial transmission and also routine migration [2,6,10,16]. The goal of malaria control in the malaria endemic countries is to reduce as much as possible the impact of malaria on the population. Its practical implementation requires four main interventions namely, case management [diagnosis and treatment] of the patients suffering from malaria, prevention of infections through vector control, prevention of the disease by administration of antimalarial drugs to particularly vulnerable population groups such as pregnant mothers [focused antenatal care] and Health education.

The Kenya policy on malaria prevention and control during pregnancy in the endemic areas emphasizes on the use of intermittent preventive treatment of malaria in pregnancy [IPTp], long lasting insecticide treated nets [LLITNs], provision of prompt diagnosis and treatment of fever due to malaria and health education. The development of a malaria vaccine is still in laboratories. The focus on vaccine development has been to disrupt the parasite life cycle and blocking of transmission. One example of these vaccines is mosquirix, a joint partnership of Path Malaria Vaccine Initiative and Glaxo Wellcome [17,18].

1.7. Malaria management with modern medicines

1.7.1. Classification of antimalarial drugs

Antimalarial drugs can be classified by the stage of the parasitic life cycle they affect [refer to table 1]. Blood schizontocides act on the erythrocytic stages of the parasite, which are directly responsible for the clinical symptoms of the disease. They can produce a clinical cure or suppression of the infection by susceptible strains of all the four species of malaria parasite, but since they have no effect on the exo-erythrocytic forms they do not produce a radical cure of relapsing forms of ovale and vivax types of malaria. Tissue schizontocides act on the exo-erythrocytic stages of the parasite and are used for causal prophylaxis to prevent invasion of the blood cells, or as antirelapse drugs to produce radical cures of vivax and ovale types of malaria. Gametocytocides destroy the sexual forms of the parasite to interrupt transmission of the infection by the mosquito vector. Sporontocides render gametocytes non-infective by inhibiting the formation of oocyst and sporozoites in infected mosquito. They have no direct effect on the gametocytes in the human host [7,12,19].

Antimalarial drugs can also be classified by the chemical group, to which they belong, which in turn determines the stage of the lifecycle they affect. The principle antimalarial drugs, classified according to the chemical group and activity are shown in table 1. The naphthyridine derivative, pyronaridine is still under investigation for its use as an antimalarial [12,19].

1.7.2. Malaria treatment

1.7.2.1. Treatment of uncomplicated malaria

Treatment of uncomplicated malaria is critical to prevent progression to severe malaria. The changing pattern of the malaria parasite resistance to most of the available antimalarial molecules has led to the change of the recommended first line treatment over the years. Upto

1999, the first line treatment in Kenya was chloroquine. Due to resistance, from the year 2000, the first line treatment was changed from chloroquine to sulphur based-pyrimethamine (SP) drugs. This was used until 2005 when the first line treatment was changed to the artemesinin combination therapy (ACT). In Kenya the recommended first line treatment for uncomplicated malaria is the artemesinin-lumefantrine (AL). The second line treatment is artemesinin-piperaquine and oral quinine, an antipyretic like paracetamol or ibuprofen is administered [7,9,12,19].

1.7.2.2. Treatment of severe malaria

The drug of choice for initiating treatment at peripheral facilities is intramuscular quinine. If quinine is not available to initiate therapy, then rectal artesunate or intramuscular artemether can also be used. If referral is not possible or delayed, treatment for severe malaria with the use of intramuscular quinine should be continued. At the referral center, for both children and adults, parenteral quinine is given until the patient can tolerate oral medication. Thereafter, quinine is continued orally or a complete course of artemether-lumefantrine is given. Concurrent administration of 5 % dextrose is given due to the hypoglycemic effects of quinine [7,19].

Table 1. Principal antimalarial drugs based on chemical classification [Ref 15,19].

Chemical group	Principle drugs	Activity
4-Methaloquinolines	Quinine/Quinidine	Rapid acting blood schizontocides.
	Mefloquine	Some gametocytocidal activity Blood schizontocide
4-Aminoquinolines	Chloroquine	Rapid acting blood schizontocides.
	Amodiaquine	Some gametocytocidal activity
8-Aminoquinolines	Primaquine Tafenoquine	Tissue schizontocides. Also gametocytocidal activity and some activity at other stages of the parasite lifecycle
Biguanides	Proguanil Chlorproguanil	Tissue schizontocides and slow acting blood schizontocides Some sporontocidal activity. Dihydr ofolate reductase inhibitors
Diaminopyrimidines	Pyrimethamine	Tissue schizontocide and slow acting blood schizontocide. Some sporontocidal activity. Dihydrofolate reductase inhibitor.
Dichlorobenzylidines	Lumefantrine	Blood schizontocide
Hydroxynaphthaquinones	Atovaquine	Blood schizontocide
9-Phenanthrenemethanols	Halofantrine	Blood schizontocide
Sesquiterpene lactones	Artemesinin, Artemether Artesunate	Blood schizontocide
Sulfonamides	Sulfadoxine Sulfametopyrazine	Blood schizontocide. Dihydro pteroate and folate synthesis inhibitors. Usually given in combination with pyrimethamine
Tetracyclines	Doxycycline	Blood schizonticide. Some tissue schizontocidal activity.
Lincosamides	Clindamycin	Blood schizontocides. Some tissue Schizontocidal activity.
Sulfones	Dapsone	Blood schizontocide.Folate synthesis inhibitor. Usually given in combination with pyrimethamine.
Bisquinolines	Piperaquine	Blood schizonticide, gametocytocidal activity.

The chemical structures of antimalarial drugs commonly used in Kenya are shown on figure 2.

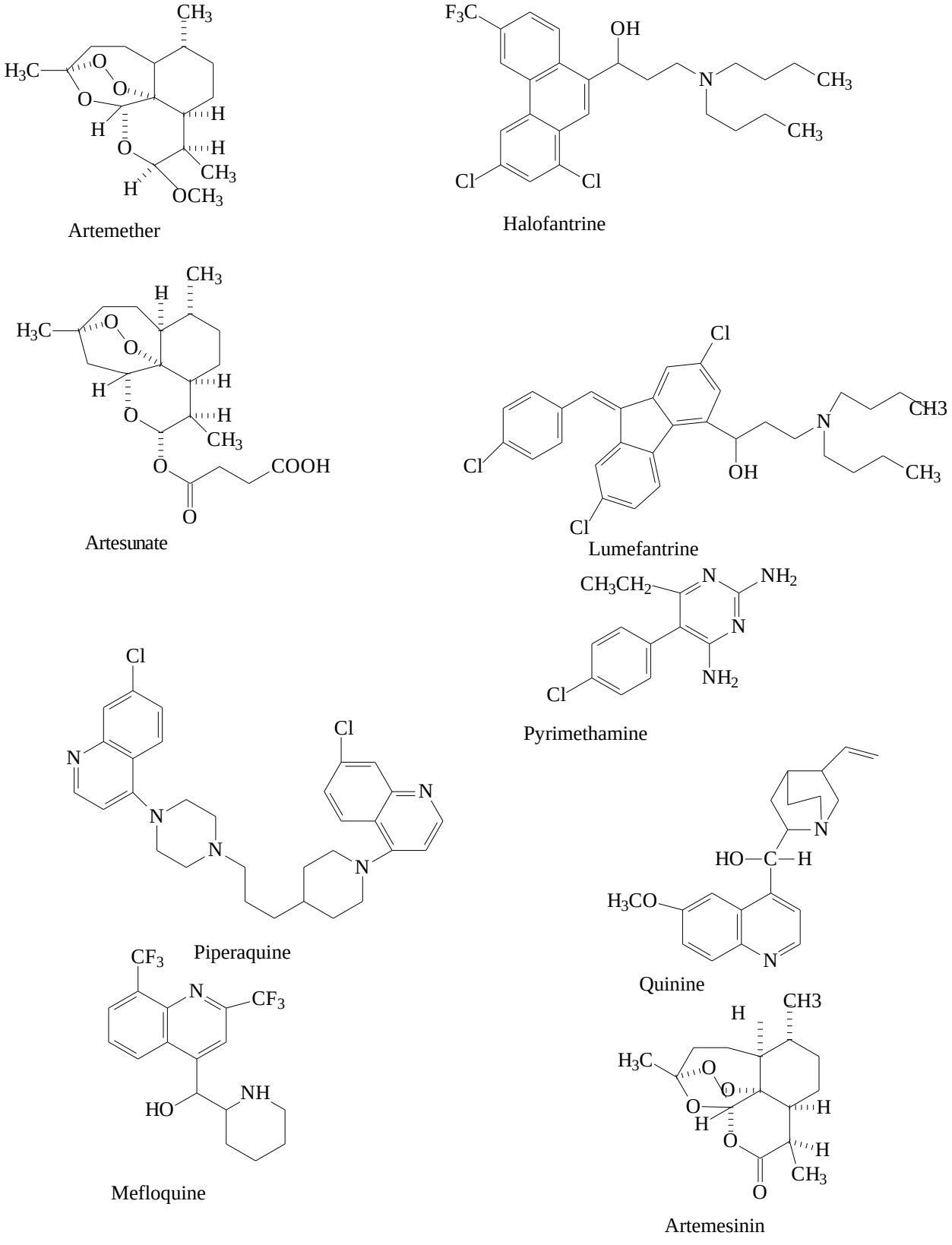


Figure 2. Chemical structures of antimalarial drugs commonly used in Kenya.

1.7.2.3. Treatment of malaria in pregnancy

The recommended treatment of uncomplicated malaria in all trimesters of pregnancy is a 7 day therapy of oral quinine. The artemether-lumefantrine can also be used in the 2nd and 3rd trimester [8, 20].

1.7.3. Malaria prophylaxis

Chemoprophylaxis of malaria refers to absolute prevention of infection [causal prophylaxis] or suppression of parasitaemia and its symptoms [clinical prophylaxis]. Causal prophylaxis is provided by tissue schizontocides, which destroy the exo-erythrocytic forms of the parasite. Clinical prophylaxis is provided by blood schizonticide, which if continued until all forms of the parasite are destroyed will ultimately produce a suppressive cure. In pregnant women, intermittent preventive treatment (IPT) is recommended. The recommended drug for IPT is sulphadoxine/pyrimethamine given as a directly observed therapy in the antenatal clinic. Pregnant women who are HIV positive and also taking daily co-trimoxazole chemoprophylaxis should not be put on IPT [8,9,19,20].

1.7.4. Treatment failure and resistance

Treatment failure is the failure to achieve the desired therapeutic response after the initiation of therapy. The main causes of treatment failure include; poor adherence to treatment, unusual pharmacokinetic properties in that individual, drug resistance and patients misdiagnosis and hence initiation of the wrong treatment. The resistance of the plasmodium parasite and especially the spread of strains of *P. falciparum* resistant to chloroquine is of great concern. Chloroquine resistance in *P. falciparum* is now found in varying degrees in almost all endemic countries of Africa and especially in Eastern Africa. Resistance of *P. vivax* to chloroquine has also been noted. In Kenya parasitic resistance to chloroquine and pyrimethamine-sulfadoxine has been

demonstrated. Knowledge of the extent of resistance in terms of the geographical distribution and the degree of resistance is important in the selection of appropriate control measures and for the development of national policies for the rational use of antimalarial drugs [7,19,21-24].

1.7.5. Adverse reactions to antimalarial drugs.

Most of the antimalarial drugs have been associated with many adverse effects. Frequent adverse reactions of chloroquine and primaquine include headache, convulsions, skin eruptions, pruritis and gastrointestinal disturbances such as nausea vomiting and diarrhea. Retinopathy has also been reported resulting in severe visual impairment. Pyrimethamine causes skin rashes, hypersensitivity reactions and depression of haematopoiesis due to interference with folic acid metabolism. Severe and fatal reactions have been reported when pyrimethamine is used in combination with sulfadoxine, including erythema multiforme, the Steven Johnson's syndrome and toxic epidermal necrosis. Quinine in usual therapeutic doses may give rise to cinchonism, characterized in its mild form by tinnitus, impaired hearing, headache, nausea and disturbed vision. In its more severe manifestations one gets vomiting, abdominal pain, diarrhea and vertigo. Other adverse effects of quinine include hypoglycemia, hypoprothrombinaemia and renal failure. Halofantrine has been associated with transient elevation of transaminases and intramuscular haemolysis. It can adversely affect the heart hence it should not be administered with other drugs that have potential to induce cardiac arrhythmias. Artemesinin and derivatives has been associated with dose related decrease in reticulocyte count for four days after administration [19,22].

1.8. Drugs of plant origin.

1.8.1. Plants as sources of antimalarial drugs

Utilization of traditional medicine is widespread in most countries. Approximately 80 % of the world inhabitants rely mainly on traditional medical remedies [25,26]. Plants have formed a basis for traditional medicine systems that have been used in countries like China and India for thousands of years [26,27]. It is estimated that some 20,000 species of higher plants are used medicinally throughout the world. Many communities in the developing countries especially in the African tropical region have been quite innovative and have come up with various traditional remedies for the treatment of malaria [26,28-32]. In Kenya for example, traditional medicine plays an important role in the treatment of malaria [26,31]. Results of scientific work have yielded highly active antimalarial agent notably quinine and artemesinin. Some of the plants that have been used as traditional remedies for malaria especially in the East Africa region are shown in table 2. Currently, modern pharmaceuticals are not readily available in those areas most affected by malaria particularly in Sub-Saharan Africa and South East Asia. In addition the resistance to the major drugs for malaria treatment has significantly reduced the treatment options. The cost of drugs if available and effective is so high that institutions and patients are increasingly unable to afford them [1, 9, 10].

Scientific studies have been conducted to establish the effectiveness of herbal therapies and they have indicated tremendous symptomatic improvement and reduction in parasite count in patients taking the medication. Such studies could provide a basis for further research in isolation of lead molecules for designing newer and more potent drugs to target the parasite. Two of the most remarkable developments of this kind of venture are artemesinin, quinine and their synthetic derivatives [33-36].

Other molecules like the quassinnoids obtained from Simaroubaceous plants have been tested *in vitro* for antimalarial activity; these were seen to inhibit the incorporation of hypoxanthine into the *Plasmodium falciparum in vitro* at concentrations below 0.41 gm/ml [37,38]. As can be seen from table 2, one of the plants used traditionally for treatment of malaria is *Caesalpinia volkensii* Harms.

1.8.2. *Caesalpinia volkensii* Harms

1.8.2.1. Description

Caesalpinia volkensii Harms belongs to the Caesalpiniaceae family. It is a woody climber 1.8-4 m high. The stems have deflexed prickles to 5 mm and bippinate leaves in 3-6 pairs. Leaflets in 3-6 pairs ovated, apex [obtusely] acuminate 3-8 cm by 1.5-4 cm. The flowers are yellow, petals about 16 mm by 3.5-4.5 mm. The fruit is densely prickly, beaked 7-13 cm by 3.5-6.5 cm. The fruits have seeds that are smooth, shiny and hard to crack [39-41]. The various features of the mature fruit of the plant are shown in figure 3.

1.8.2.2. Distribution

Caesalpinia volkensii Harms is a very rare plant and is found growing in lowland forests. In Kenya, it is mostly a home grown plant in parts of Central, Rift Valley, Coast and Eastern province. This plant is commonly known as Mkomew [Taita], Mkomwe [Swahili], Msoo Miba [Shambaa], Mubuthi [Gikuyu], Muvuthi [Kamba], Mburuga [Digo], Olnkulankula [Maasai] [14,26, 39-41].

1.8.2.3. Traditional uses

The water extract of the seeds has been used to treat malaria among Kenyan and Tanzanian communities. It has also been used to treat stomach disturbances during pregnancy. The water extract of the leaves is used for malaria and pains during pregnancy. This water extract is also

used for conjunctivitis, retinoblastoma, eyelid swellings and refractive errors. The roots are cooked, chewed raw or put in sweet wine and drunk as an aphrodisiac. The leaves are also boiled in soup, allowed to cool and drunk for treatment of malaria [14,26,39].

Table 2. Plants used as traditional remedies for malaria in East Africa [Ref 26,39].

Plant species	Solvent	Part used
Acacia clavigera Brenan	water	root bark
Ajuga remota Benth	water	leaves
Albizia gummifera (JF Gmel.) C.A.Sm.	water	stem bark
Bidens pilosa L.	water	root bark
Caesalpinia volkensii Harms	water	leaves and seeds
Senna didymobotrya Fresen	water	root bark and leaves
Clematis bronchiata Thunb	water	root bark
Clematis simensis Fresen	water	root bark
Clerodendrum myricoides Hochst.	water	leaves
Clerodendrum eriophyllum Gurke	water	leaves
Cordia sinensis Lam	milk	root bark
Croton macrostachyus Delile	water	root
Xanthoxylum chalybea UM Engl.	water	stem bark
Kigelia africana Benth	water	leaves
Lippia javanica Spreng	water	leaves
Ocotea usambarensis Engl.	water	roots
Rauwolfia mombasiana Stapf	water	root bark
Warbugia ugandensis Sprague	water	root bark and leaves

Figure 3. The fruit of *Caesalpinia volkensii* Harms

1.8.2.4. Previous work

The water decoction of the leaves has shown activity against chloroquine strains of *Plasmodium falciparum.* The concentration of the water decoction that gave a 50 % inhibition of the parasite growth was 480.0 µg /ml. The petroleum ether, methanol and ethanol extracts of the leaves have also been demonstrated to have activity against the *Plasmodium falciparum*. The concentrations giving 50 % inhibition were 149.0 µg/ml, 858.0 µg/ml and 960.0 µg/ml respectively. It is only the petroleum ether extract of the leaves that gave a high activity against the *Plasmodium falciparum*. Antibacterial and antifungal activity have also been done for the leaves and shown to be inactive. Phytochemical screening of the leaves has shown presence of alkaloids, flavonoids, tannins and essential oils [14].

1.9. Study justification.

Although nature has been demonstrated to be a source of novel chemotypes and pharmacophores, it is estimated that only about 5-10 % of approximately 250,000 species of

higher plants have been systematically investigated for the presence of bioactive compounds [14,34-39]. There is also an urgent need to validate the safety and efficacy of these herbal remedies to ensure their general acceptance and incorporation into the modern health systems. The determination of the efficacious components of these plants will help in the dose standardization and also their safer packaging will increase the hygiene. Although there is reported wide usage of *Caesalpinia volkensii* Harms, no compounds have been isolated to support the use. Furthermore most of the work that has been done on this plant has been on the leaves only. No scientific work has been done on the seeds, which are also extensively used in the African traditional folklore [39]. The phytochemical work done on this plant could identify molecules which can serve as templates on which safer and efficacious molecules can be developed.

1.10. Objectives of the study

1.10.1. General objective

The general objective of the study was to screen the seeds of *Caesalpinia volkensii* Harms for antimalarial activity and carry out a phytochemical investigation of the various extracts of the seeds.

1.10.2. Specific objectives

1. To carry out phytochemical tests on the seeds.
2. To isolate and characterize compounds from the plant extracts.
3. To carry out antimalarial activity tests on the various extracts and isolated compounds.
4. To carry out brine shrimp toxicity assay on the various extracts and isolated compounds.

CHAPTER 2: EXPERIMENTAL

2.1. Plant materials, reagents and equipment.

2.1.1. Plant collection, identification and preservation.

The plant material was collected at Gatundu in Kiambu county of Central province and identified at the Department of Botany, University of Nairobi. A voucher specimen of the aerial parts of the plant was pressed and preserved at Department of Botany, University of Nairobi. A few of the seeds of the plant were also kept as sample in the School of Pharmacy, University of Nairobi.

2.1.2. Materials and reagents.

The seeds were crushed using pliers and ground into a fine powder using a coffee blender. Normal phase chromatography was done using TLC plates pre-coated with silica gel $60F_{254}$ plates (Merck Darmstadt, Darmstadt, German). Column chromatography was done using silica gel for column chromatography [0.004-0.063 µg] (Merck).

Chloroform (Kobian Kenya Ltd., Nairobi, Kenya), methanol and petroleum ether (Alpha Chemicals Ltd, Nairobi, Kenya) were of general purpose grade and were distilled using glass apparatus before use. Vanillin was from Laboratory Chemicals Ltd (Nairobi, Kenya). Sulphuric acid and hydrochloric acid were from Kanha Laboratories (Nairobi, Kenya). Tween 80 was from BDH Chemicals (Poole, England). Eggs of brine shrimp (*Artemia salina* Leech) were purchased from a pet shop in Nairobi [Kenya]. Malstat™ reagent was from Flow Incorporated (S.W. Corbelt, Portland, U.S.A.). Nitro blue tetrazolium grade 111 (NBT) was from Sigma (N-6876, Aldrich, U.S.A). Phenazine ethosulphate (PES) from Sigma (P-6876, Aldrich, U.S.A). Serum culture of *Plasmodium falciparum* parasites was prepared at Kenya Medical Research Institute (KEMRI, Nairobi, Kenya). Industrial nitrogen gas was obtained from BOC Ltd (Nairobi, Kenya).

2.1.3. Equipment.

Top loading balance H.L.Becker, Belgium was used to weigh samples above 100 g and a Sartorius analytical balance (Goettingen, German) was used to weigh samples below 100 g. A 2 L Soxhlet apparatus (Quickfit, Birmingham, England) was used for the extractions. Solvents were reduced on a Heidolph VV2000 rotary evaporator (Heidelberg, Germany), connected to an Edwards vacuum oil pump (Edwards High Vacuum International, West Sussex, England). Filtration for qualitative analysis was done using filter papers from Whatman Laboratory (Kent, England). A Jaytech fractionating column 100 ml was used and fractions were collected using Superfrac fraction collector from Pharmacia LKB Biotechnology (Uppsala, Sweden).

A Perkin-Elmer Lambda UV/Visible spectrophotometer (Uberlingen, German) was used for ultra-violet (UV) analysis. A UV/Visible box (Desaga GMBH, Heidelberg, Germany) was used for visualizing TLC plates. A VG Platform II gas chromatography-mass spectrometer (Fison instruments, Manchester, England] was used for mass spectrometric analysis. Proton nuclear magnetic resonance spectrometry was done at 300 MHz, $CDCI_3$, temp. 30 ^{0}C. ^{13}Carbon nuclear magnetic resonance spectrometry was done at 303.1 Hz, $CDCI_3$, temp. 30 ^{0}C. An SP3-300 infrared spectrophotometer connected to a Perker Elmer 561 recorder was used for infrared analysis. Gas chromatography was done using a gas chromatograph (GC) fitted with a glass capillary column 2.5 m coated with a stationary phase consisting of 10 % Carbowax (Union Carbido Co., Newyork U.S.A). Injection temperature was set at 100 ^{0}C, column temperature was set at 60 ^{0}C. Detection temperature was set at 150 ^{0}C. The carrier gas was industrial nitrogen.

2.2. Preparation of the plant extracts

2.2.1. Water extract

About 20 g of the seed powder was weighed into a conical flask and 100 mL of distilled water was added. The mixture was subjected to cold maceration with occasional stirring for 6 h and then left overnight. The extract was filtered, reduced to dryness in vacuo, weighed and stored at 4 ^{0}C until further use.

2.2.2. Petroleum ether extract

About 600 g of the seed powder was weighed and transferred into a bag. The bag was fitted into a Soxhlet apparatus and extraction was done for 48 h using petroleum ether. The extract was filtered, reduced to dryness *in vacuo*, the yield was determined and the extract stored at 4 ^{0}C.

2.2.3. Chloroform extract

The material after petroleum ether extraction was sequentially extracted with chloroform for 48 h using the Soxhlet apparatus. The extract was filtered, reduced to dryness *in vacuo*, the yield was determined and the extract stored at 4 ^{0}C.

2.2.4. Methanol extract

The material after the chloroform extraction was further sequentially extracted with methanol for 48 h using Soxhlet apparatus. The extract was filtered, reduced to dryness *in vacuo*, the yield was determined and the extract stored at 4 ^{0}C.

2.3. Visualizing agents

2.3.1. Iodine

About 5 g iodine powder were put in a glass tank and tightly covered. The iodine was allowed to sublime until the glass tank was fully saturated with the iodine vapour. The TLC plates were placed in the tank for about 5 min. to visualize.

2.3.2. Vanillin in concentrated sulphuric acid solution

About 0.5 g of vanillin was weighed into a 100 mL flat bottomed flask and mixed with 50 mL concentrated sulphuric acid to make a 1 % vanillin in conc. H_2SO_4 solution which was used to spray the developed TLC plates and then heated at 110 ^{0}C for 5 min to visualize them.

2.4. Fractionation

2.4.1. Petroleum ether extract

The most suitable mobile phase system as determined by thin layer chromatography was chloroform. About 80 g of silica gel F_{254} was weighed into a 500 mL beaker. About 80 mL of chloroform was added into the silica gel whilst stirring to form thin slurry. The column was then evenly packed with the slurry ensuring no air bubbles. About 8 g of the pet ether extract was weighed and dissolved in 10 mL of chloroform. The dilution was then carefully loaded onto the column which was connected to the superfrac fraction collector at a flow rate of 35-40 drops per min. Monitoring was done on every third tube by TLC on precoated silica gel plates, developed using chloroform and detection of the spots done using UV_{254}, UV_{366}, iodine chamber and finally vanillin in H_2SO_4. Similar fractions were dried *in vacuo* and further to column chromatographed for purification. Similar fractions were further pooled together and reduced *in vacuo* using rotary evaporator to about 2 mL. The remaining solvent was allowed to evaporate slowly at room temperature.

Six pooled fractions were obtained and designated as P-1 to P-6. All the pooled fractions obtained were clear oily liquid at room temperature 25 ^{0}C. The pooled fractions were subjected to normal phase thin layer chromatography using pure chloroform as the mobile phase. The petroleum ether extract was used as the reference. Detection of spot was done using iodine chamber. Pooled fraction designated P-1 gave only one principal spot on detection [appendix 1].

The pooled fraction P-1 was designated as compound 1. The fraction was further purified by adding about 2 mL of hot ethanol. The solvent was left to evaporate for 72 h. The pooled fraction P-1 was stored at the temperature 2 ^{0}C to 8 ^{0}C for further analysis.

The fraction remained clear oily liquid at the stored temperature. The clear oily liquid obtained was further subjected to ultraviolet spectroscopy (UV, $CHCl_3$, nm ε: 243), infrared spectroscopy (IR KBR cm^{-1}), nuclear magnetic resonance spectrometry and mass spectrometry (VG Platform II GC/LC-MS, sample by solid probe) [42-46].

2.4.2. Chloroform extract

The most suitable mobile phase system as determined by thin layer chromatography was chloroform. This gave the best TLC separation profile for the chloroform extract on a normal phase TLC plate. An iodine chamber was used for the detection of the spots on the chromatographic plate [appendix 2]. About 80 g of silica gel F_{254} was weighed into a 500 mL beaker. About 80 mL of chloroform was added into the silica gel whilst stirring to form thin slurry. The column was then evenly packed with the slurry ensuring no air bubbles were trapped. About 3 g of the chloroform extract was dissolved in 5 mL chloroform. The dilution was then carefully loaded onto the column which was connected to the superfrac fraction collector at a flow rate of 35-40 drops per min. Monitoring was done on every third tube by TLC on precoated silica gel plates, developed using chloroform and detection of the spots done using UV_{254}, UV_{366}, iodine chamber and finally vanillin in H_2SO_4. Four columns were run and similar fractions from the columns were pooled together. Similar fractions were dried *in vacuo* and further column chromatographed for purification. The similar fractions were further pooled together and reduced *in vacuo* using the rotary evaporator to about 2 mL. Twelve pooled fractions were obtained and designated as C-1 to C-12 [42,43]. The fractions were left and the remaining solvent in each of

the pooled fractions was allowed to evaporate slowly for 72 h at room temperature. On evaporation of all the solvent, each fraction designated C-1 to C-9 was a clear oily liquid. Fraction designated C-10 was a clear liquid while each of fraction designated C-11 and C-12 was brown liquid. Each fraction was further subjected to normal phase thin layer chromatography using pure chloroform as the mobile phase. The chloroform extract was used as the reference. Detection of the spots was done using iodine chamber. About 2 mL of ethanol was added to each fraction designated C-10, C-11 and C-12 to enhance crystallization. The fractions were further left for 72 h at room temperature for the ethanol to evaporate. On evaporation of all the ethanol, fraction designated C-10 formed clear solid crystals with one principal spot on thin layer chromatography and it was named compound 2. Recrystallization was done for compound 2 by further adding about 2 mL of hot ethanol. The ethanol was allowed to evaporate slowly for 72 h at room temperature. The fraction formed clear round crystals. Each fraction designated C-11 and C-12 formed brown amorphous solid with more than one spot on thin layer chromatography and hence not pure [appendix 3]. Fraction designated C-1 was clear oily liquid with one principal spot on thin layer chromatography and it was named compound 3. Each fraction designated C-2 to C-9 had more than one spot on thin layer chromatography and hence not pure [appendix 3].

The fractions were stored at temperature 2 ^{0}C to 8 ^{0}C for further analysis. Compound 2 and compound 3 were further subjected to ultraviolet spectroscopy (UV, $CHCl_3$ 1g/100mL, nm ε: 242.4, 245.0), infrared spectroscopy (IR KBr cm^{-1}), nuclear magnetic resonance spectrometry and mass spectrometry (VG Platform II GC/LC-MS, sample by solid probe) [44-46].

2.5. *In vitro* antimalarial test

2.5.1. Preparation of the reagents

2.5.1.1. Nitro blue tetrazolium

A stock solution of the nitro blue tetrazolium (NBT) was prepared by dissolving 100 mg of the NBT in 50 mL of distilled water with aid of ultrasonic bath until complete dissolution of the NBT. This stock solution was stored in the dark at 4 ^{0}C.

2.5.1.2. Phenazine Ethosulfate

A stock solution of phenazine ethosulfate (PES) was prepared by dissolving 5 mg of PES in 50 mL of sterilized distilled water with the aid of ultrasonic bath until complete dissolution of the PES. About 10 mL of this solution was dispensed into five 15 mL tubes and covered with aluminium foil. The PES stock solution was then stored in the dark at -20 ^{0}C.

2.5.2. Principle of the *in vitro* antimalarial test

The parasite lactate dehydrogenase ($_{P}$LDH) activity is distinguishable from the host lactate dehydrogenase ($_{h}$LDH) activity using the 3-acetylpyridine adenine dinucleotide analogue of nicotinamide adenine dinucleotide [3-NAD^{+}] and thus an enzymatic method for evaluating antimalarial compounds. The $_{p}$LDH has many amino acids residues that are unique compared to any other known lactate dehydrogenase. This includes residues that define the substrate and co-factor binding sites. The parasite lactate dehydrogenase exhibits an enhanced catalytic efficiency with the 3-NAD^{+} whereas the human isoforms exhibit a lower catalytic efficiency. This differential response to 3-NAD^{+} provides the kinetic basis for the enzyme based detection of antimalarial activity.

2.5.3. Methodology

About 10 mg of the various extracts was weighed. Serial dilutions (250, 125, 62.5, 31.25, 15.25, 7.8125, 3.90625 µg /ml) were prepared for each extract. Chloroquine diphosphate was used as a standard at serial dilutions of 100, 50, 12.5, 6.25, 3.125 and 1.5625 ng for chloroquine diphosphate was also prepared. This was done to compare the inhibition of the plant extracts with the inhibition of chloroquine. A 96 well flat-bottomed microtiter plate was prepared; to each well was added equal cultures of the parasitized red blood cells [$_P$RBCs]. Into four of the wells were added non-parasitized red blood cells as control. The serial dilutions of the plant extracts and the chloroquine diphosphate were added into the appropriate wells of the microtiter plate. The cultures were incubated at 37 ^{0}C for 48 h in a gas mixture 3% O_2, 6% CO_2, 91% N_2. At the incubation period the cultures were carefully re-suspended and aliquots were removed for analysis of the $_P$LDH activity.

The microtiter plate was allowed to thaw at room temperature for about 2 h, 100 µL of the Malstat^{TM} reagent was transferred to another new multi-well plate. Aliquots of 20 µL of the haemolysed parasite suspension were added to the Malstat^{TM} reagent. The plate was then incubated for 15 min at 25 ^{0}C. Nitro blue tetrazolium/Phenazine ethosulfate (1:1) solution 20 µL was added to the haemolysed parasite/Malstat suspension. The plate was incubated for 2 h at room temperature in the dark. The NBT and PES solutions are extremely light sensitive and must be stored permanently in the dark; hence the reaction whereby the blue formazan product is formed during the Malstat/NBT/PES incubation must be protected from light. After the incubation period, the plates were read spectrophotometricaly at 650 nm and the results recorded [47,48].

2.6. Brine shrimp lethality assay

2.6.1. Principle

The eggs of brine shrimp (*Artemia salina* leach) hatch within 24 h when placed in a brine solution, yielding larvae [nuplii]. Toxicity of plant extracts against the larvae is evaluated in the brine shrimp assay.

2.6.2. Method

2.6.2.1. Hatching the shrimps

About 33 g of sea salt and about 5 mg of bakers yeast was dissolved in 500 mL of water. The mixture was stirred using a magnetic stirrer. About 350 mL of this saline solution was transferred into a rectangular plastic box divided into two unequal compartments with several 2 mm holes plastic divider clamped to the box. Approximately 50 mg of brine shrimp eggs were sprinkled to one side of the divided plastic box and the side was covered on top. These were then left to hatch at room temperature.

2.6.2.2. Preparation of the test solutions

Serial dilutions of 1000, 500, 250, 125 and 62.5 were made for each of the plant extracts in distilled water as diluent using a 2 % v/v dimethylsulfoxide to enhance the solubility of the chloroform, Petroleum-ether and P_1 samples. Water was used as a control.

2.6.2.3. Bioassay

Ten shrimps were transferred into each vial containing the drug solutions using a Pasteur pipette. After 24 h in darkness at 25 ^{0}C the contents of each of the vial were mixed gently and emptied into a petridish. The dead and live shrimps were recorded.

2.7. General phytochemical tests on the extracts

2.7.1. Preparation of reagents

2.7.1.1. Mayer's reagent

About 1.36 g of mercuric iodide was dissolved in 60 mL water and about 5 g of potassium iodide was dissolved in 20 mL water. The two solutions were then mixed and made to 100 mL with water to make Mayer's reagent (potassium mercuric iodide) [39].

2.7.1.2. Drangendoff's reagent

About 1.7 g bismuth nitrate, 20 g tartaric acid and 20 mL glacial acetic acid were dissolved in 80 mL of distilled water and made into a solution A. About 16 g potassium iodide were dissolved in 40 mL distilled water to form solution B. Stock solution was obtained by mixing solution A and B in ratio 1:1 (v/v). To make Drangendoff's spray reagent, 5 mL of the stock solution were added to a solution of 10 g tartaric acid in 50 mL water [39].

2.7.2. Procedures for phytochemical tests

2.7.2.1. Test for glycosides

About 1.0 g of the seed powder was extracted with 10 mL of 70 % alcohol by heating on a water bath for about two minutes. The mixture was allowed to cool then filtered. To the filtrate, 10 mL of water and 5 drops of strong solution of lead sub acetate were added. The mixture was filtered and 10 % sulphuric acid was added dropwise until no further precipitate formed. The mixture was filtered and extracted with two successive 5 mL portions of chloroform. The two chloroform extracts were combined and washed with 1 mL of distilled water. The chloroform layer was separated and filtered through a small plug of cotton wool. The chloroform extract was divided into two equal parts for the Kedde test and Keller-Killiani test [39].

2.7.2.1.1 Kedde test for β-unsaturated ring of aglycone

The chloroform extract was evaporated to dryness. One drop of 90 % alcohol and 2 drops of 2 % 3, 5-dinitrobenzoic acid in 90 % alcohol were added. The mixture was made alkaline with 20 % sodium hydroxide solution. Production of a purple colour indicated the presence of β-unsaturated lactone ring of the aglycone.

2.7.2.1.2 Keller-Killiani test for deoxy-sugars.

The chloroform extract was evaporated to dryness, 0.4 mL glacial acetic containing a trace of ferric chloride was added. The mixture was transferred to a test tube and 0.5 mL of concentrated sulphuric acid was added carefully down the side of the tube. Production of a green blue colour in the upper acetic acid layer indicated the presence of a 2-deoxy sugars

2.7.2.2. Test for alkaloids

About 1 g of the seed powder was extracted by warming on a water bath with 10 mL of 10 % sulphuric acid for 5 min. A buffy white precipitate formed and the rest of the extract was made alkaline with dilute ammonia. The mixture was extracted with 2 mL of chloroform. The chloroform layer was separated and washed with little water. This was further filtered through a small plug of cotton wool. The filtrate was divided into two portions and each portion was evaporated to dryness [39].

2.7.2.2.1. General test for alkaloids

One of the residue portion was dissolved in 0.2 mL 1 % sulphuric acid. The solution was divided into two portions; to one 0.1 mL portion was added one drop of mayer's reagent. Production of a buffy white precipitate indicated presence of alkaloids. To the other 0.1 mL portion was added one drop of dragendorff's reagent. Production of orange red precipitate indicated presence of alkaloids.

2.7.2.2.2. Vitali-Morin test for tropane alkaloids

The other residue portion was dissolved by mixing well in two drops of fuming nitric acid. The mixture was evaporated to dryness and moistened with two drops of alcoholic potassium hydroxide solution. Production of a purple colour indicated presence of tropane alkaloids [39].

2.7.2.3. Borntrager's test for anthraquinones

About 0.5 g of the seed powder was boiled with 5 mL dilute sulphuric acid for 5 minutes. The mixture was filtered whilst hot, the filtrate was cooled and shaken with an equal volume of carbon tetrachloride. Production of a rose pink to red colour in the ammoniacal layer indicated presence of anthraquinones [39].

2.7.2.4. Modified Borntrager's test for anthraquinones

About 0.5 g of the seed powder was 5 mL dilute sulphuric acid and 5 % aqueous ferric chloride for 5 min. The mixture was filtered whilst hot and extracted with carbon tetrachloride. Production of a red colour indicates presence of anthracene aglycones in their reduced form.

2.7.2.5. Test for saponins

A little of the powdered drug was shaken with water, a persistent froth suggested presence of saponins. About 0.2 g of the seed powder was extracted with 10 mL of warm water. The extract was filtered and the filtrate retained. About 2 mL of 1.8 % sodium chloride solution was added into 2 test tubes. To one of the test tube was added 2 mL of distilled water. To the other test tube was added 2 ml of the extract. One drop of blood was added to each tube. The tubes were inverted gently to mix the contents. Haemolysis in the tube containing the extract but not in the control tube indicated presence of saponins [39].

CHAPTER 3: RESULTS AND DISCUSSION

3.1. General phytochemical tests

The results for phytochemical screening are shown on table 3.

Table 3. Results for phytochemical screening.

Test	Observation	Inference
Kedde test	no colour change	absence of aglycones
Keller-Killiani test	no green colour in the upper acetic acid layer	absence of 2-deoxy sugars
Tests for alkaloids		
Mayer's reagent	a buffy white precipitate	presence of alkaloids
Dragendorff's reagent	orange red precipitate	presence of alkaloids
Vitali-Morin test	no colour change	absence of tropane alkaloids
Borntrager's test	no colour change	absence of anthraquinones
Modified Borntrager's test	no colour change	absence of reduced anthracene aglycones
Test for saponins	froth produced with persistent shaking	presence of saponins

3.2. Fractionation

The yields obtained from the Soxhlet extractions of the petroleum ether, chloroform, methanol and water solvents were as shown on table 4.

The extraction with the petroleum ether gave the highest yield. The petroleum ether extract remained a liquid at low temperature of 4 ^{0}C. The chloroform, methanol and water extracts were solids even at 25 ^{0}C.

Table 4: Yields obtained from Soxhlet extractions of petroleum ether, chloroform, methanol and water solvents

Solvent	Percentage yield (%)
Petroleum ether	18.7
Chloroform	3.2
Methanol	9.7
Water	85.1

3.2.1. Petroleum ether extract

Column chromatography of the petroleum ether extract gave yields as shown on table 5. Fraction P-1 showed only one principle spot, the other fractions gave several spots as shown in the chromatogram on Appendix 1. Visualization profile of the pooled fractions was as shown on table 6.

Table 5: Yields obtained from pooled fractions of petroleum ether extract

Fraction	percentage (%)
P-1	27.5
P-2	13.8
P-3	8.3
P-4	16.5
P-5	3.8
P-6	7.0

Table 6: Visualization profile for petroleum ether fractions

Fraction	UV at 254_{nm}	UV at 366_{nm}	1 % Vanillin in cH_2SO_4	Iodine
P-1	-	-	-	+
P-2	-	-	-	+
P-3	-	-	-	+
P-4	+	-	+	+
P-5	+	-	+	+
P-6	+	-	+	+

3.2.2 Chloroform extract

Column chromatogram of the chloroform extract gave yields as shown on table 7.

Table 7. Yields obtained from pooled fractions of chloroform extract

Fraction	Percentage (%)
C-1	23.5
C-2	10.8
C-3	6.7
C-4	13.0
C-5	0.8
C-6	2.2
C-7	0.2
C-8	0.3
C-9	0.2
C-10	0.3
C-11	0.6
C-12	0.7

Thin layer chromatograms obtained for chloroform extract and pooled fractions was as shown on appendices 2 and 3. Each pooled fraction designated C-1 and C-10 gave only one principle spot at R_f values of 0.6 and 0.3, respectively and were subjected to further spectroscopic analysis for identification. The visualization profile for the chloroform fractions were as shown on table 8.

Table 8: Visualization profile for chloroform fractions

Fraction	UV at 254_{nm}	UV at 366_{nm}	1 % vanillin in c H_2SO_4	Iodine
C-1	-	-	-	+
C-2	-	-	-	+
C-3	+	-	+	+
C-4	+	-	+	+
C-5	+	-	+	+
C-6	+	+	+	+
C-7	+	+	+	+
C-8	+	+	+	+
C-9	+	-	+	+
C-10	+	-	+	+
C-11	+	-	+	+
C-12	+	-	+	-

3.3. Isolated compounds

3.3.1. Compound 1

Compound 1 was a clear oily liquid at room temperature. It gave a R_f value of 0.61 using chloroform as the mobile phase. The TLC chromatogram gave a yellowish spot when exposed to iodine. The spot was colourless on spraying with 1 % vanillin in conc. sulphuric acid. The compound gave the spectroscopic data shown below.

3.3.1.1. Ultraviolet spectroscopy

UV λ [$CHCl_3$] nm [ε]: 249.7 [2.754]. The UV spectrum is as shown on Appendix 4.

Absorption in the ultraviolet region indicates presence of a chromophore in the compound. The chromophore is due to the pi electrons of the carbonyl group and the isolated carbon carbon double bonds. The ultraviolet absorption at this wavelength indicates conjugated pi bonds and therefore the spectroscopic information was not conclusive. Further UV spectroscopy should be done on the compound [49].

3.3.1.2. Infrared spectroscopy

IR ν_{max} [KBr] cm^{-1} 2880[C-H str], 1720[C=O str], 1460[C-C str], 1340[C-H bend], 980[=C-H bend], 720 [C-H rocking]. Absorption at 2880 is characteristic of C-H bond stretch of a CH_2 group, Absorption at 1720 is characteristic of the carbonyl group C=O of the ester. Absorption at 1460 is characteristic of C-H deformation of CH_2 group. Absorption at 1340 is characteristic of C-H deformation of C-H group. Absorption at 980 is characteristic of =C-H deformation of CH_3 group. Absorption at 720 is characteristic of $–CH_2-$ skeletal stretch [49,]. The IR spectrum is as shown on Appendix 5.

3.3.1.3. Mass spectrometry

The mass spectrum is shown on Appendix 6.

MS m/z[relative intensity]: 39[72], 41[100], 43[85], 44[88], 45[40], 55[98], 57[92], 60[25], 67[30], 71[40], 72[25], 73[20], 81[40], 83[20] 85[10], 95[10], 99[20], 109[10], 125[10], 127[5], 149[5], 152[5], 165[5], 167[5], 177[5], 179[3], 193[3], 207[3], 239[2], 250[5] 257[2], 288[1.5], 299[1]. Comparison of the mass spectrometry for compound 2 with that of literature for $1^1,2^1,3^1$-propanetriyl tris[cis,cis-9,12-octadecadienoate] is shown on table 9. The fragment ion with m/z 41 was the most abundant ion and is characteristic of compounds with ester linkages [49-55].

Table 9: Comparison of mass spectrometric data of compound 1 with that of $1^1,2^1,3^1$ -propanetriyl tris[cis,cis-9,12-octadecadienoate] from literature* [53].

Compound 1 m/z	$1^1,2^1,3^1$-propanetriyl tris [cis,cis-9,12-octadecadienoate] m/z	Compound 1 m/z	$1^1,2^1,3^1$-propanetriyl tris[cis,cis-9,12-octadecadienoate] m/z
38 (1)	38 (1)	109 (10)	109 (31)
39 (72)	39 (14)	125 (10)	125 (6)
41 (100)	41 (53)	127 (5)	127 (5)
43 (85)	43 (35)	149 (5)	149 (13)
44 (88)	44 (5.2)	152 (5)	152 (4)
45 (40)	45 (3.3)	165 (5)	165 (7)
55 (98)	55 (84)	167 (5)	167 (3)
57 (92)	57 (25)	177 (5)	177 (6)
60 (25)	60 (6)	179 (3)	179 (3)
67 (30)	67 (100)	193 (3)	193 (3)
71 (40)	71 (10)	207 (3)	207 (2)
72 (25)	72 (1)	288 (2)	288 (2)
73 (20)	73 (5)	299 (1)	299 (1)
81 (40)	81 (83)		
83 (20)	83 (27)		
85 (10)	85 (8)		
95 (10)	95 (61)		
99 (20)	99 (7)		

* The figures in parentheses represent relative intensity.

3.3.1.4. Nuclear magnetic resonance.

^{1}H-NMR [300 MHz, $CDCl_3$] δ 5.31[12H, s, -CH=], 4.29[2H, t, HC-O-], 4.14[2H, t, CH-O-], 2.77[6H, t, =C-CH_2-], 2.31[6H, s, C=C-CH_2-], 2.28[6H, s, O=C-CH_2-], 2.25[12H, s, -CH_2-C=], 2.05[6H, t, -CH_2-], 1.58[6H,s,-CH_2-] 1.27[12H, t, -CH2-], 1.28 [4H, m, CH_2-], 1.23[6H, m, -CH_2-], 0.87[3H, s, CH_3].

^{13}C-NMR [300MHz, $CDCl_3$] δ 173.17[2C-1], δ 172.73[C-1], δ 130.09[3C-13], δ 129.88[3C-9], δ 127.99[3C-10], δ 127.82[3C-12], δ 68.85[C-2^1], δ 62.03[C-1^1, C-3^1], δ 34.10[C-2], δ 33.94[2C-2], δ 31.82[3C-16], δ 29.66[3C-7], δ 29.37[3C-15], δ 29.16[3C-6], δ 29.06[3C-5], δ 29.01[3C-4], δ 27.20[3C-8], δ 27.10[3C-14], δ 25.55[3C-11], δ 24.88[3C-3], δ 22.58[3C-17], δ 13.99[3C-18].

Carbons numbered C-1 are the carbonyl carbons of the ester group, carbons numbered C-9, C-10, C-12 and C-13 are the doubly bonded carbons. Carbons 1^1, 2^1, and 3^1 are the carbons of the propane moiety, carbons numbered C-2 are the carbons attached to the ester group. The chemical shift at δ 172.72, 173.13, and 173.17 is attributed to the ester carbonyl carbons atom. The ^{1}H-NMR and ^{13}C-NMR spectra are as shown on Appendices 7 and 8.

Comparison of the ^{13}C-NMR spectrum with that in literature for 1^1,2^1,3^1-propanetriyl tris[cis,cis-9,12-octadecadienoate] is shown on table 10 [54]. The ^{1}H-NMR and ^{13}C-NMR spectra from literature for 1^1,2^1,3^1-propanetriyl tris[cis,cis-9,12- octadecadienoate] are as shown on Appendices 9 and 10.

Table 10. Comparison of ^{13}C NMR spectrum of compound 1 with that of $1^1,2^1,3^1$-propanetriyl tris[cis,cis-9,12-octadecadienoate] from literature [53].

Carbon Number	Compound 1	$1^1,2^1,3^1$-propanetriyl tris [cis, cis-9,12-octadecadienoate]
1, 1	173.17	173.17
1	172.73	172.76
13, 13, 13	130.09	130.21
9, 9, 9	129.88	129.99
10, 10, 10	127.99	128.12
12, 12, 12	127.82	127.95
2^1	68.85	69.00
1^1, 31	62.03	62.14
2	34.10	34.21
2, 2	33.94	34.05
16, 16, 16	31.82	31.54
7, 7, 7	29.66	29.63
15, 15, 15	29.37	29.36
6, 6, 6	29.16	29.12
5, 5, 5	29.06	29.06
4, 4, 4	29.01	29.01
8, 8, 8	27.20	27.22
14, 14, 14	27.10	27.10
11, 11, 11	25.55	25.68
3, 3, 3	24.88	24.87
17, 17, 17	22.58	22.58
18, 18, 18	13.99	14.04

From literature, $1^1,2^1,3^1$-propanetriyl tris[cis,cis-9,12-octadecadienoate] is a clear oily liquid at room temperature.

From all spectroscopic data, it strongly suggests that compound 1 is $1^1,2^1,3^1$-propanetriyl tris[cis,cis-9,12-octadecadienoate]. The chemical structure of $1^1,2^1,3^1$-propanetriyl tris[cis,cis-9,12-octadecadienoate] is shown on figure 4 below.

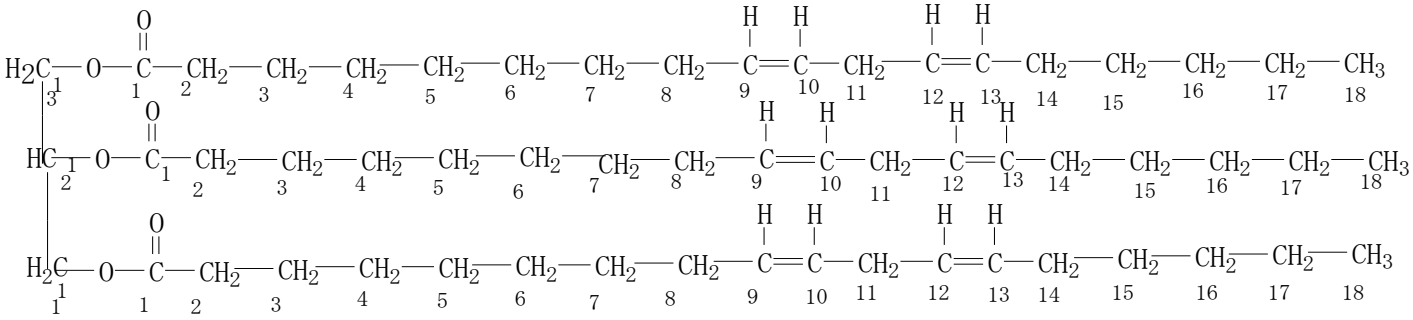


Figure 4. Structure of $1^1,2^1,3^1$-propanetriyl tris[cis,cis-9,12-octadecadienoate] $C_{57}H_{98}O$

3.3.2. Compound 2

The compound was clear solid crystals at room temperature. It gave a R_f value of 0.3 using chloroform as the mobile phase. The compound gave a yellowish spot when exposed to iodine and a pinkish spot when sprayed with 1 % vanillin in conc. sulphuric acid. A yellow spot on exposure to iodine indicates presence of an unsaturated organic compound. A pinkish spot with 1 % vanillin in conc. sulphuric acid indicates presence of steroid. The melting point for the compound was not determined due to little sample size. The compound gave the spectroscopic data given below.

3.3.2.1. Ultraviolet spectroscopy

C-2; UV λ max [$CHCl_3$] nm [ε]: 242.4 [0.631]. The spectrum is shown on Appendix 11.

Absorption in the ultraviolet region indicates presence of a chromophore in the compound. Absorption at these wavelengths indicates conjugation of the chromophore to single bonds that leads to a bathochromic shift of the absorption [49,54,55]. This UV spectroscopic data was not

conclusive and hence further UV analysis should be done on the compound. The compound further gave the spectroscopic data given below.

3.3.2.2. Mass spectrometry

The mass spectrum of compound 2 [C-10] is shown on Appendix 12

MS m/z [relative intensity]: 37[1], 38[20], 40[60], 42[100], 54[75], 56[50], 66[23], 68[40], 78[15], 80[25], 82[25], 84[10], 94[10], 96[10], 106[6], 108[5], 132[5], 144[7], 148[9], 157[10], 158[30], 170[5], 177[5], 185[5], 199[5], 213[9], 214[10], 239[10], 255[4], 261[4], 276[4], 289[3], 303[3], 315[3], 329[5], 381[5], 396[5], 412[7], 414[10], The ion of m/z 414 corresponds to the molecular formula $C_{29}H_{50}O$. This may suggest the molecular mass for 5-stigmasten-3-beta-ol, a phytosterol compound. The chemical structure of 5-stigmasten-3-beta-ol is shown on figure 5 [49-55]. Comparison of the mass spectrometry with that of literature for the 5-stigmasten-3-beta-ol is as shown on table 11.

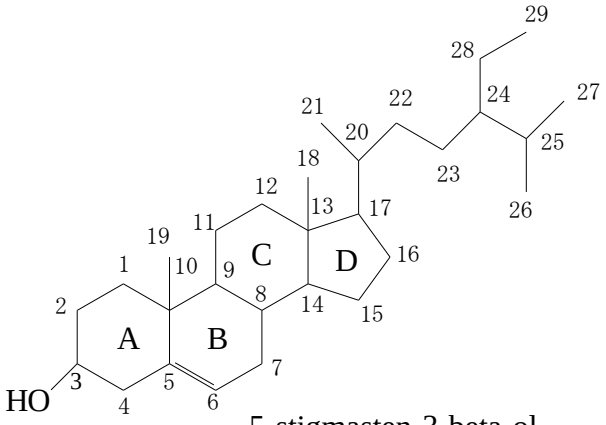
3.3.2.3. Nuclear magnetic resonance

^{1}H-NMR [300.1 MHz, $CDCl_3$]: δ 0.80[3H, s, CH_3-18], 0.82[6H, d, CH_3-26, CH_3-27], 0.85[3H, s, CH_3-29], 0.86[3H, s, CH_3-21], 0.88[3H, s, CH_3-19], 0.90[2H, s, CH_2-28], 1.01[2H, s, CH_2-23], 1.11[2H, s, CH_2-22], 1.17[2H, s, CH_2-16], 1.20[2H, s, CH_2-15], 1.21[2H, s, CH_2-12], 1.25[2H, s, CH_2-11], 1.30[2H, s, CH_2-1, CH_2-2], 1.61[1H, s, CH-25], 1.98[1H, s, CH-24], 2.02[2H, d, CH-20, CH-17], 2.07[3H, t, CH-14, CH-8, CH-9], 2.31[1H, s, CH-3], 4.19[1H, t, CH_2-7], 4.25[1H, t, CH_2-4], 5.25[1H, d, CH-6].

The ^{1}H-NMR spectrum is shown on Appendix 13.

The difference in chemical shifts in phytosterols is due to stereoisomerism. This occurs at the ethyl group attached to C-24. Rings A, B, C and D of the sitosterols exist either in the boat or chair conformations. This further gives phytosterols a variation in chemical shift at C-2 and C-4

[53-57]. The chemical shift at δ 5.25 a doublet is comparable to that of the proton attached to carbon at position 6 of sitosterols. The chemical shift is a doublet due to the double bond at carbon 5-6 of ring B of sitosterol. Mass spectrometric and nuclear magnetic resonance studies done suggest that compound 2 could probably be 5-stigmasten-3-beta-ol. Further spectroscopic and spectrometric data is needed to confirm this.



5-stigmasten-3-beta-ol m/z 414

Figure 5. Structure of 5-stigmasten-3-beta-ol [52-55,57].

Table 11: Comparison of mass spectrometric data of compound 2 with that of 5-stigmasten-3-beta-ol from literature* [53].

Compound 2 m/z	5-stigmasten-3-beta-ol m/z	Compound 2 m/z	5-stigmasten-3-beta-ol m/z
42 (100.0)	42 (2.0)	213 (9.0)	213 (43.0)
43 (20.0)	43 (32.8)	214 (10.0)	214 (12.9)
56 (50.0)	56 (2.0)	239 (10.0)	239 (2.5)
68 (30.0)	68 (2.5)	255 (4.0)	255 (44.1)
80 (20.0)	80 (3.2)	261 (4.0)	261 (10.0)
82 (20.0)	82 (4.3)	276 (4.0)	276 (4.4)
94 (11.0)	94 (7.3)	289 (3.0)	289 (26.6)
96 (10.0)	96 (4.6)	303 (3.0)	303 (31.7)
106 (6.0)	106 (9.5)	315 (3.0)	315 (29.7)
108 (6.0)	108 (11.2)	329 (5.0)	329 (37.7)
132 (6.0)	132 (6.5)	381 (5.0)	381 (31.3)
144 (7.0)	144 (7.1)	396 (10.0)	396 (60.1)
148 (9.0)	148 (9.6)	412 (7.0)	412 (8.2)
157 (10.0)	157 (11.7)	414 (10.0)	414 (100.0)
158 (30.0)	158 (13.6)		
170 (5.0)	170 (1.1)		
177 (5.0)	177 (11.1)		
185 (5.0)	185 (11.8)		
199 (5.0)	199.0 (15.8)		

* The figures in parentheses represent relative intensity.

3.3.3. Compound 3

Compound 3 was a clear oily liquid at room temperature. It gave a R_f value of 0.61 using chloroform as the mobile phase. The compound gave one yellowish spot when exposed to iodine indicating presence of unsaturated organic compound. No colour was produced on spraying with 1 % vanillin in conc.H_2SO_4. It gave one principle spot on thin layer chromatography and was thus subjected to further spectroscopic analysis for identification. The compound gave the spectroscopic data shown below.

3.3.3.1. Ultraviolet spectroscopy.

UV λ max [$CHCl_3$] nm [ε]: 245 [1.133]. The UV spectrum is as shown on Appendix 14.

The absorption at this wavelength is attributed to the chromophore of pi electrons of the carbonyl group. Conjugation of the chromophore to single bonds leads to a bathochromic shift of the absorption. The UV spectroscopic data was not conclusive and further UV analysis should be done on the compound [50-56].

3.3.3.2. Infrared spectroscopy

IR V_{max} [KBr] cm^{-1} 2850 [C-H str], 1720 [C=O str], 1380 [C-H asymmetrical str], 1220 [C-O str], 1140 [C-H skeletal], 980 [C-H deformation], 720 [C-H skeletal]. Absorption at 2850 is characteristic of C-H bond of a CH_2 group. The absorption at 1720 is characteristic of a carbonyl group of a saturated ester compound. Absorption at 1380 is characteristic of asymmetrical stretch of a C-H bond of a CH_3 group. Absorption at 1220 is characteristic of the C-O of the ester group. Absorption at 1140 is characteristic of the CH_3 skeletal stretch. Absorption at 980 is characteristic of CH_3 deformation. Absorption at 720 is characteristic of the CH_2 skeletal stretch. The infrared spectrum is characteristic of an ester compound [50-56]. The IR spectrum is as shown on appendix 15.

3.3.3.3. Mass spectrometry

MS m/z[relative intensity]: 37[5], 38[50], 40[100], 42[90], 43[20], 50[10], 52[25], 54[40], 56[30], 57[5], 64[12], 66[25], 68[15], 78[10], 80[95], 82[20], 90[7], 94[10], 95[10], 108[6], 122[5], 136[4], 148[5], 151[5], 164[5], 165[20], 166[4], 177[2], 203[2], 205[3], 430[10], 431[4].

The fragment ion with molecular weight 40 was the most abundant and is characteristic of compounds with ester linkages and long chain hydrocarbons. Fragment ion of molecular weight 42 is due to the carbonyl ion [$CH_2C=O$] characteristic of compounds with ester linkage. Fragment ions of molecular weight 43 and 56 are due to fragmentation of the long hydrocarbon chain [49,51-55]. The mass spectrum is shown on appendix 16. The mass spectrum of compound 3 is comparable to that of $1^1,2^1,3^1$-propanetriyl tridocosanoate with molecular mass 1054.

3.3.3.4. Nuclear magnetic resonance

^{1}H-NMR [300.1 MHz, $CDCl_3$]: δ 5.35[1H, s, -O-CH], δ 4.29[2H, d, CH-O], δ 4.14[2H, d, CH-O], δ 2.3[2H,t, CH_2-C=O], δ 1.60[2H, d, CH_2-], δ 1.31[4H, m, CH_2-CH_2-], δ 1.25[32H, s, -$[CH_2]_{16}$], δ 0.87[3H, t, -CH_3].

^{13}C-NMR [300MHz, $CDCl_3$]: δ 173.20[C-1^1,C-1^3], 172.79[C-1^2], 68.92[C-2], 62.10[C-1,C-3], 34.19[C-2^2], 34.02[C-2^1,C-2^3], 31.92[C-20^1,C-20^2,C-20^3], 29.76[C-7^1-C-18^1,C-7^2-C18^2,C-7^3-C18^3], 29.52[C-6^1,C-6^2,C-6^3], 29.32[C-19^1,C-19^2,C-19^3], 29.26[C-5^1,C-5^2,C-5^3], 29.17[C-4^1,C-4^2, C-4^3], 24.87[C-3^1, C-3^2, C-3^3], 22.67[C-21^1,C-21^2,C-21^3], 14.08[C-22^1,C-22^2,C-22^3].

The chemical shift at δ 5.35, δ 4.29 and δ 4.14 are due to the protons of the glycerol moiety of the ester compound. Chemical shift at δ 2.30 is due to the protons of the carbon attached to the carbonyl group. Chemical shift at δ 1.60, δ 1.29, δ 1.26 are due to the protons of the hydrocarbon chain. Chemical shift at δ 0.87 is due to the protons of the terminal methyl group of the hydrocarbon chain. Chemical shifts at δ 173.21 and δ 172.80 are due to the carbonyl carbons of

the ester linkage. Chemical shifts at δ 69.02 and δ 62.16 are due to the carbons of the glycerol moiety. The ^{1}H-NMR and ^{13}C-NMR spectra are as shown on Appendices 17 and 18. Comparison of the ^{13}C-NMR spectrum of compound 3 with that of literature for $1^{1},2^{1},3^{1}$-propanetriyl tridocosanoate is shown on table 12. The ^{1}H-NMR and ^{13}C-NMR spectra of literature for $1^{1},2^{1},3^{1}$-propanetriyl tridocosanoate are as shown on Appendices 19 and 20.

Table 12. Comparison of ^{13}C-NMR spectrum of compound 3 with that of $1^{1},2^{1},3^{1}$-propanetriyl tridocosanoate from literature [53].

Carbon number	**Compound 3 δ values**	**$1^{1},2^{1},3^{1}$-propanetriyl tridocosanoate δ values**
1, 1	173.19	173.21
1	172.79	172.80
2^{1}	68.92	69.02
1^{1}, 31	62.10	62.16
2^{2}	34.18	34.28
2, 2	34.02	34.12
20, 20, 20	31.91	31.96
7-18, 7-18, 7-18	29.76	29.74
6, 6, 6	29.52	29.52
19, 19, 19	29.33	29.39
5, 5, 5	29.26	29.32
4, 4, 4	29.16	29.17
3,3,3	24.87	24.93
21, 21, 21	22.66	22.71
22, 22, 22	14.07	14.09

From the ^{1}H-NMR spectrum, the chemical shifts not accounted for were as follows, δ 7.2, δ 2.8, δ 2.28 and 2.03. From the ^{13}C-NMR spectrum, the chemical shifts not accounted for were as follows, δ 130.22, δ 129.97, δ 128.07, δ 127.90, δ 77.42, δ 77.00, δ 76.58, 31.58, δ 31.51, δ 29.46, δ 29.16, δ 29.08, δ 27.19, δ 25.63, δ 22.55 and δ 14.03.

Mass spectrometric and nuclear magnetic resonance studies show that compound 3 has some structural similarities to 1^{1},2^{1},3^{1}-propanetriyl tridocosanoate. However, the data is not conclusive and further spectroscopic and spectrometric studies need to be done to ascertain the full structure of compound 3. The chemical structure of 1^{1},2^{1},3^{1}-propanetriyl tridocosanoate on figure 6.

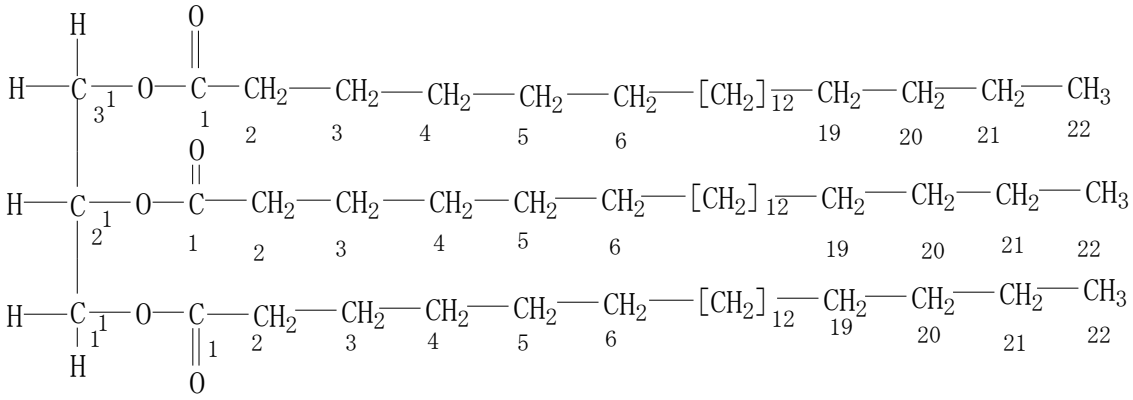


Figure 6. Structure of 1^{1},2^{1},3^{1}-propanetriyl tridocosanoate

3.4. Antimalarial *in vitro* test

Antimalarial *in vitro* test was done for all the extracts and also for the compound 3 isolated from the petroleum ether extract. The concentrations of the test samples that gave a 50 % inhibition [IC_{50}] are shown on table 13.

Table 13. Concentrations of test samples that gave IC_{50}

Extract	IC_{50} µg /ml
Chloroquine	0.0625
Chloroform	290.0
Petroleum ether	435.0
Methanol	250.0
Water	625.0
Compound 3	757.5

The concentration of chloroquine that gave a 50 % inhibition was 62.5 ng/ml. From these results, the methanol extract was the most active. The extracts gave a significant activity compared to chloroquine.

3.5. Brine shrimp lethality assay

The concentrations that gave 50 % lethality were determined. These were the concentrations at which 50 % of the shrimps died [LD_{50}]. These were as shown on table 14.

Table 14. LD_{50} profile for the brine shrimp lethality assay

Extract	LD_{50} [µg/ml]
Water	625.0
Methanol	562.5
Chloroform	375.0
Petroleum ether	375.0
Compound 3	750.0

The water extract used by the traditional folklore was still safe at very high concentrations. The chloroform and petroleum ether extracts are the most lethal giving the lowest LD_{50}

CHAPTER 4: CONCLUSIONS AND FURTHER WORK

4.1. Conclusion

Caesalpinia volkensii Harms is a woody climber plant found growing in lowland forests and in homesteads. The water extract of the seeds and the leaves has traditionally been used to treat malaria and stomach disturbances in pregnancy among Kenyan and Tanzanian communities. The purpose of the study was to carry out phytochemical testing, isolation of compounds, structure elucidation, antimalarial activity testing and brine shrimp toxicity assay studies on the seeds of the plant.

Phytochemical testing of the seeds of *Caesalpinia volkensii* Harms showed the presence of cardiac glycosides in the petroleum ether, chloroform and methanol extracts. The petroleum ether extract also showed presence of alkaloids and saponins. All the extracts showed absence of any anthraquinones.

Isolation of compounds from the petroleum ether extract yielded compound 1 and from chloroform extract yielded compound 2 and compound 3. Structure elucidation of the isolated compounds was carried out using ultraviolet spectroscopy, infrared spectroscopy, mass spectrometry nuclear magnetic resonance spectrometry. From the structure elucidation, the obtained data suggested that compound 1 had structural similarities with glycerol trilinoleate [1^1,2^1,3^1-propanetriyl tris [cis,cis-9,12-octadecanedienoate]. Compound 2 had some common structural features to beta-sitosterol [5-stigmasten-3-beta-ol]. Compound 3 had partial structural similarities to glycerol tridocosanoate [1^1,2^1,3^1-propanetriyl tridocosanoate] a long chain carbon ester. From literature, beta-sitosterol, a common phytosterol, has previously been isolated from

various plants including *Jatropha podagrica* and it has been demonstrated to have several bioactivities including *in vitro* cytotoxic activity on cervical carcinoma cell lines [58,59].

In vitro antimalarial activity test was carried out on the petroleum extract, chloroform extract, methanol extract, water extract and isolated compound 3. The activities were compared to that of the standard drug chloroquine. From the antimalarial activity tests, a plot of inhibition against concentration for each extract and the isolated compound gave 50 % inhibition [IC_{50}] at 250.0 µg/mL, 290.0 µg/mL, 435.0 µg/mL, 625 µg/mL, 757.5 µg/mL and 62.5 ng/mL for methanol extract, chloroform extract, petroleum ether extract, water extract, isolated compound and standard drug chloroquine respectively. All the tested extracts and the isolated compound were shown to have antimalarial activity at concentrations much higher than that of chloroquine. Methanol extract gave the highest activity.

Brine shrimp toxicity testing was done on the petroleum extract, chloroform extract, methanol extract, water extract and the isolated compound. From the brine shrimp toxicity study of the extracts and the isolated compound, a plot of toxicity against concentration gave LD_{50} values at 562.5 µg/mL, 375.0 µg/mL, 375.0 µg/mL, 625.0 µg/mL and 750.0 µg/mL concentrations for methanol extract, chloroform extract, petroleum ether extract, water extract and the isolated compound respectively. The water extract used by the traditional folklore was still safe at high concentrations.

4.2. Further work

From phytochemistry studies, it is clear that the plant extracts contain more classes of compounds with important bioactivity. Further work therefore can be done to isolate more of these compounds and study their uses. This can be important like the isolation of cardiac glycosides from this plant can be used as a drug or a template for synthesis of drugs useful in

the management of cardiac related diseases. Further work can also be carried out on the petroleum ether extract to isolate the alkaloids and the saponins noted present in the plant extract. Structural elucidation of the isolated compounds avails more information on the key chemical groups present in the elucidated compounds. Some of the chemical moieties may be crucial groups on lead compounds in synthesis of new medicinal compounds. Demonstration of antimalarial activity in the water extract justifies its traditional use for the treatment of malaria. Further work should be carried out on all the extracts to probably isolate more compounds with antimalarial activity. The isolated compounds can be used as lead molecules for synthesis of new, more effective and efficacious antimalarial drugs able to handle the perennial challenges encountered in effective management of malaria.

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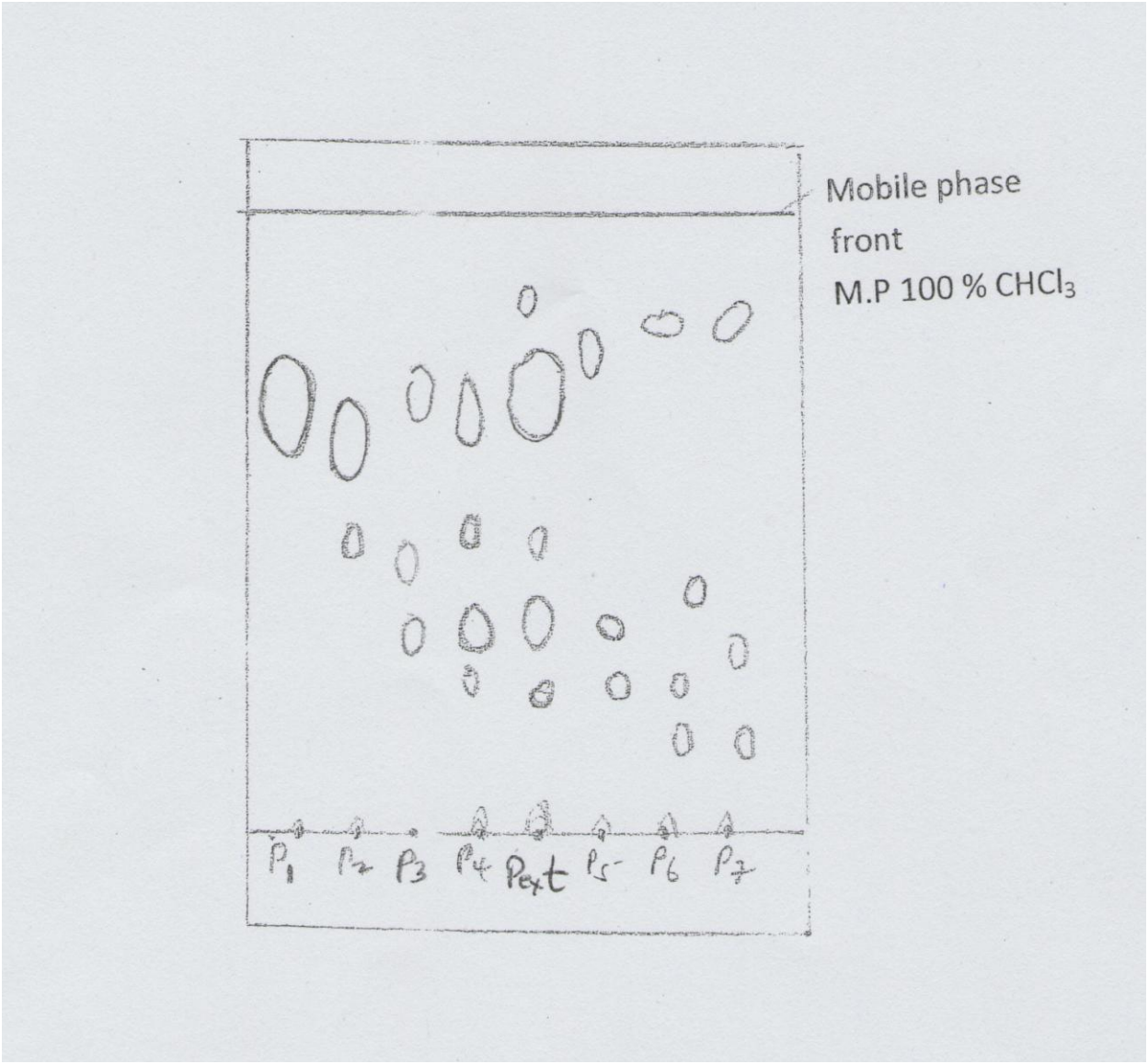
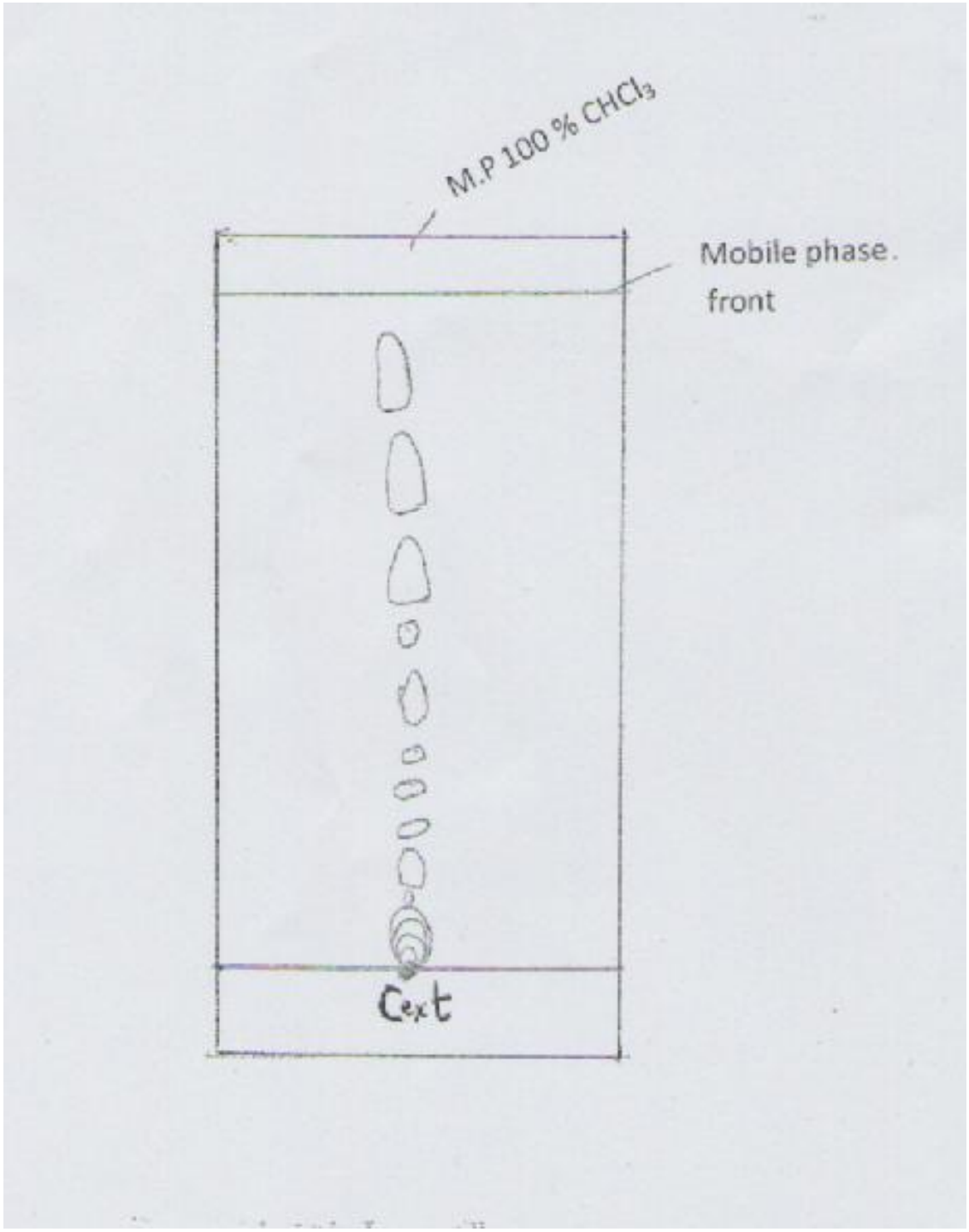
APPENDICES

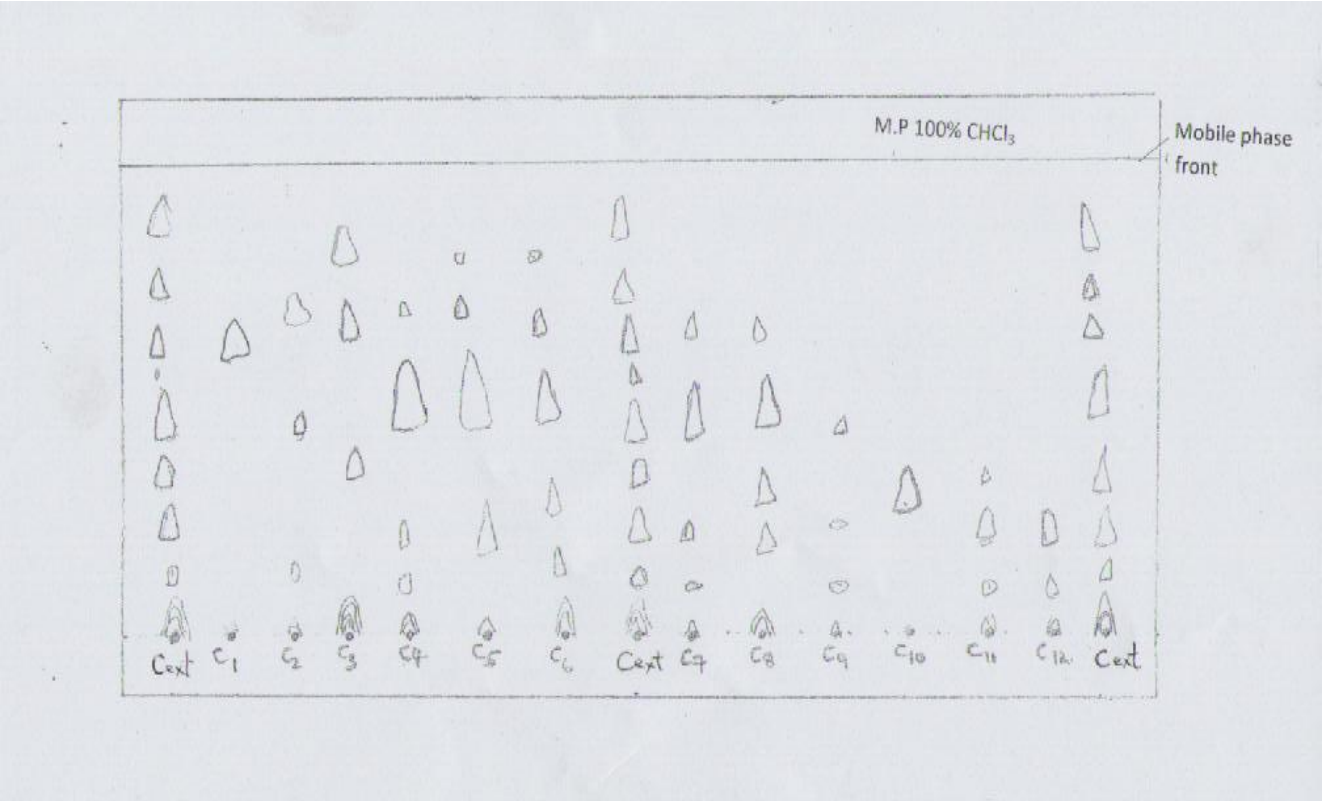
Appendix 1. Thin layer chromatogram for petroleum ether extract



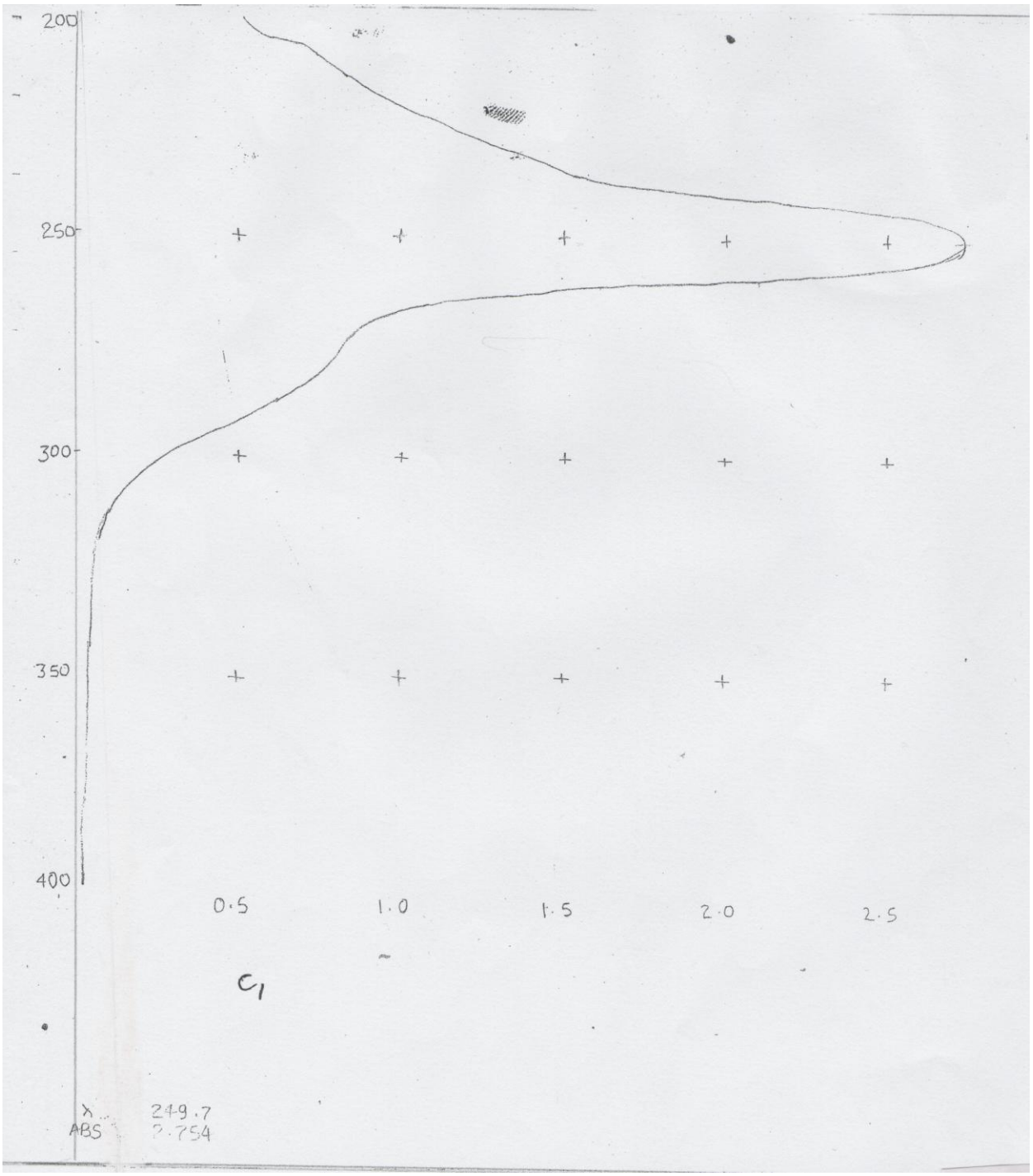
Appendix 2. Thin layer chromatogram for chloroform extract



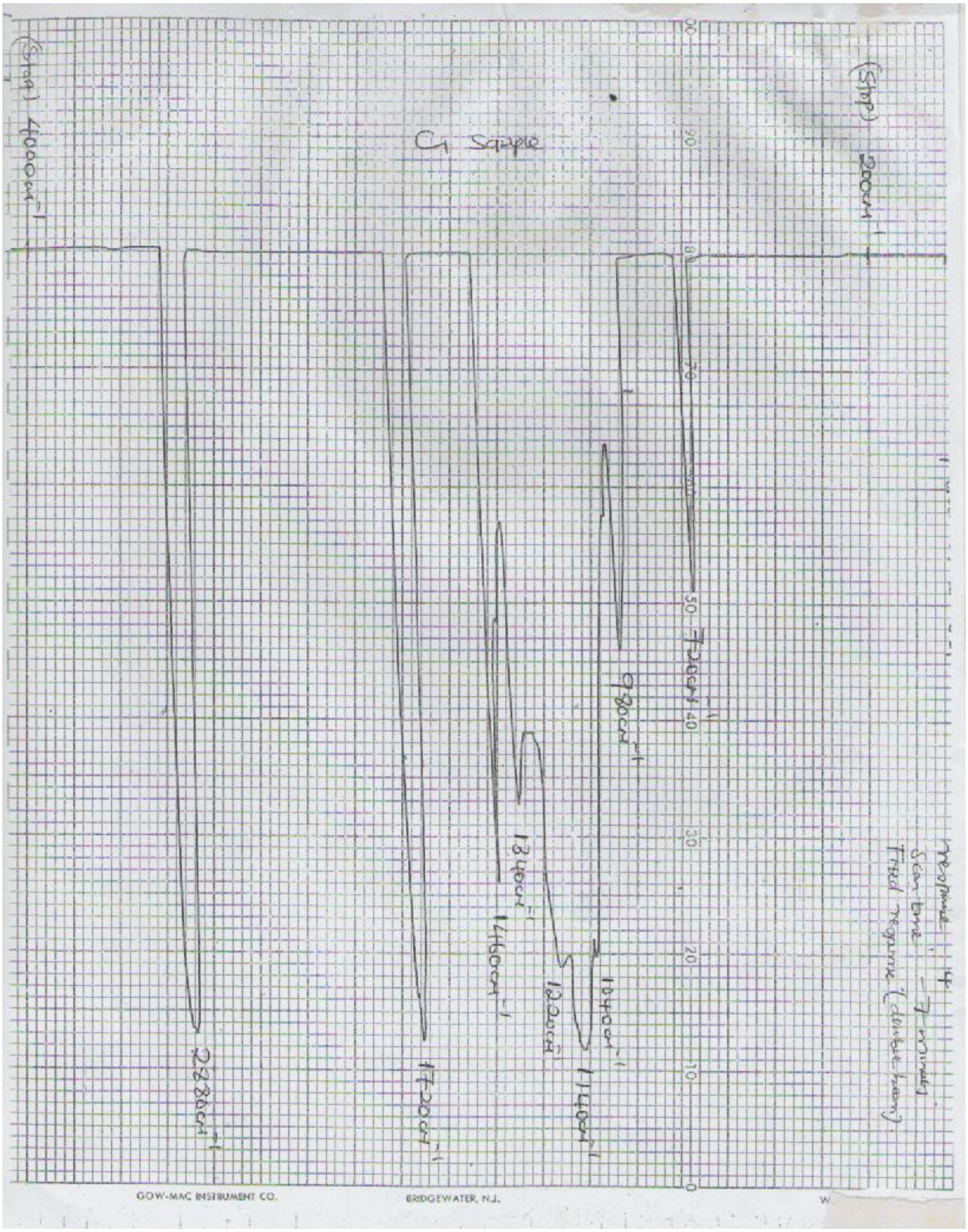
Appendix 3. Thin layer chromatogram for chloroform pooled fractions



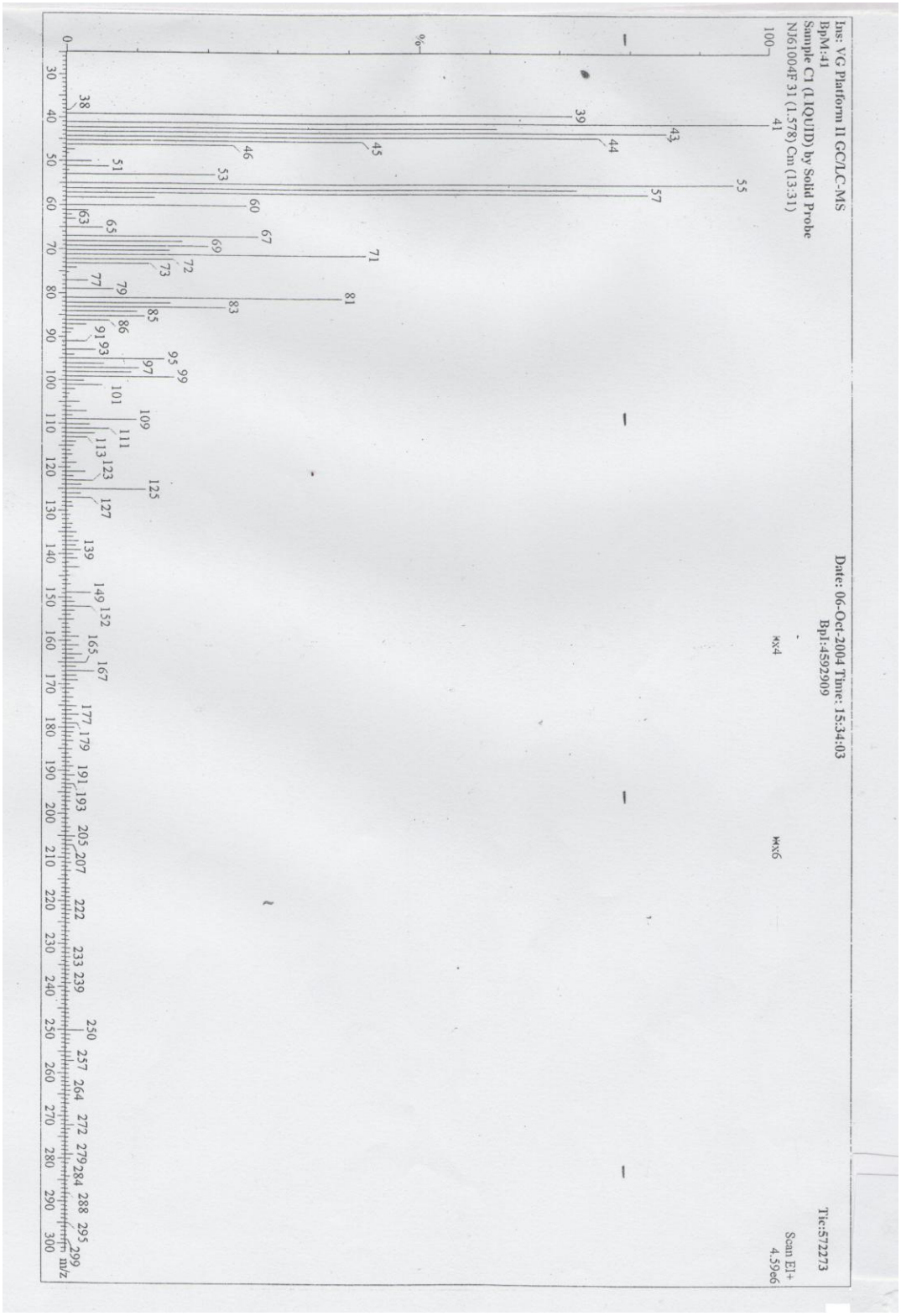
Appendix 4. Ultra-violet spectrum for compound 1



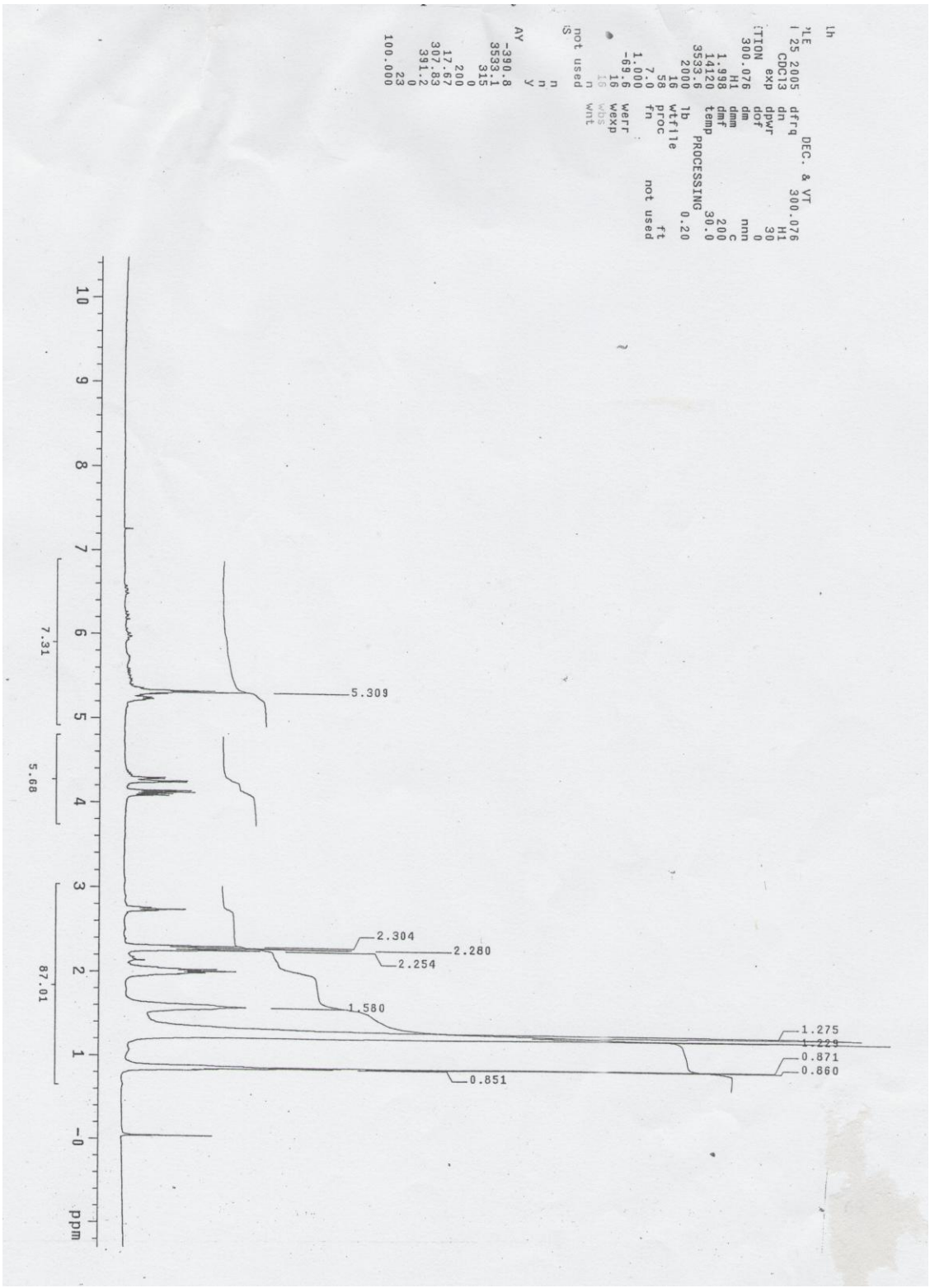
Appendix 5. Infrared spectrum for compound 1



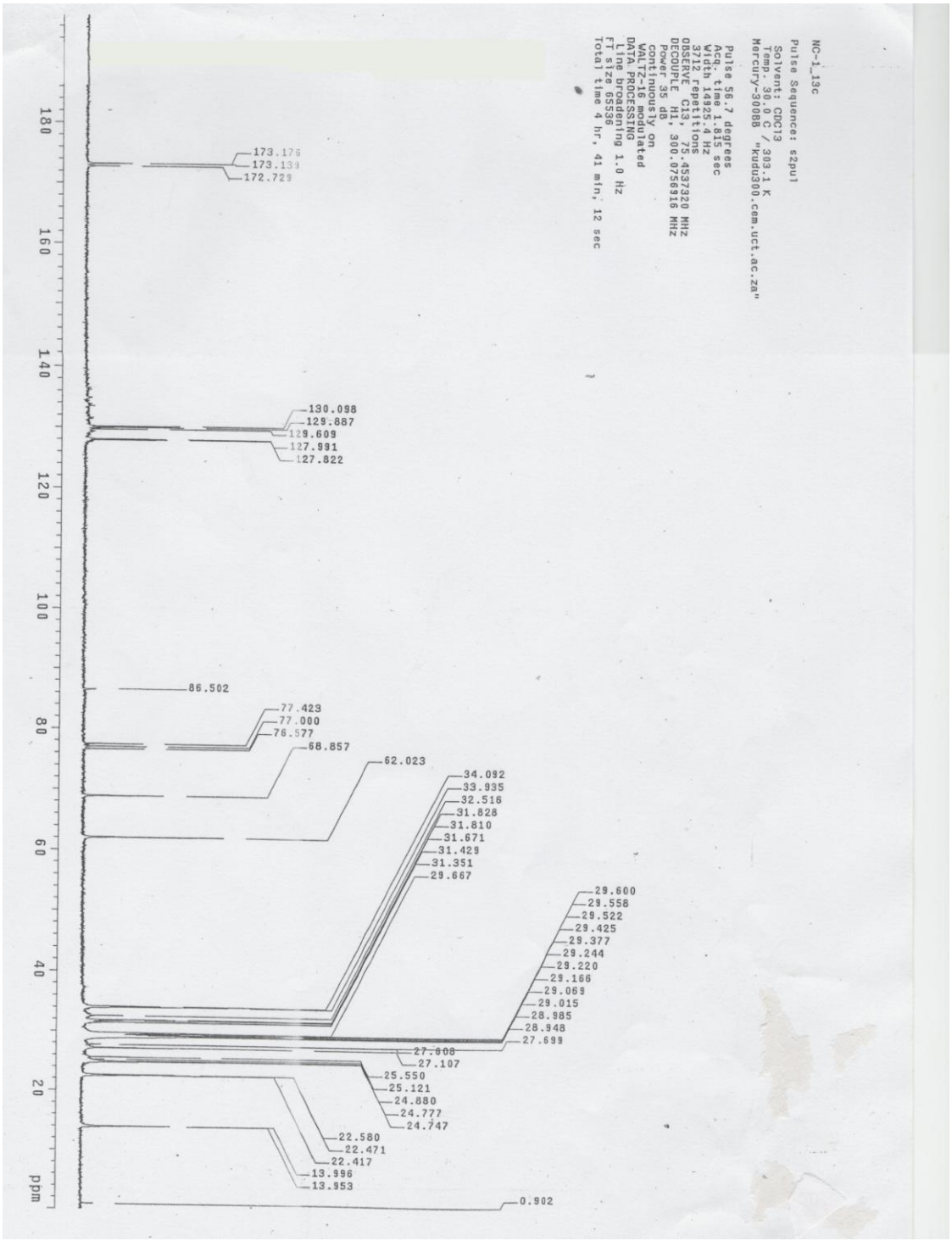
Appendix 6. Mass spectrum for compound 1



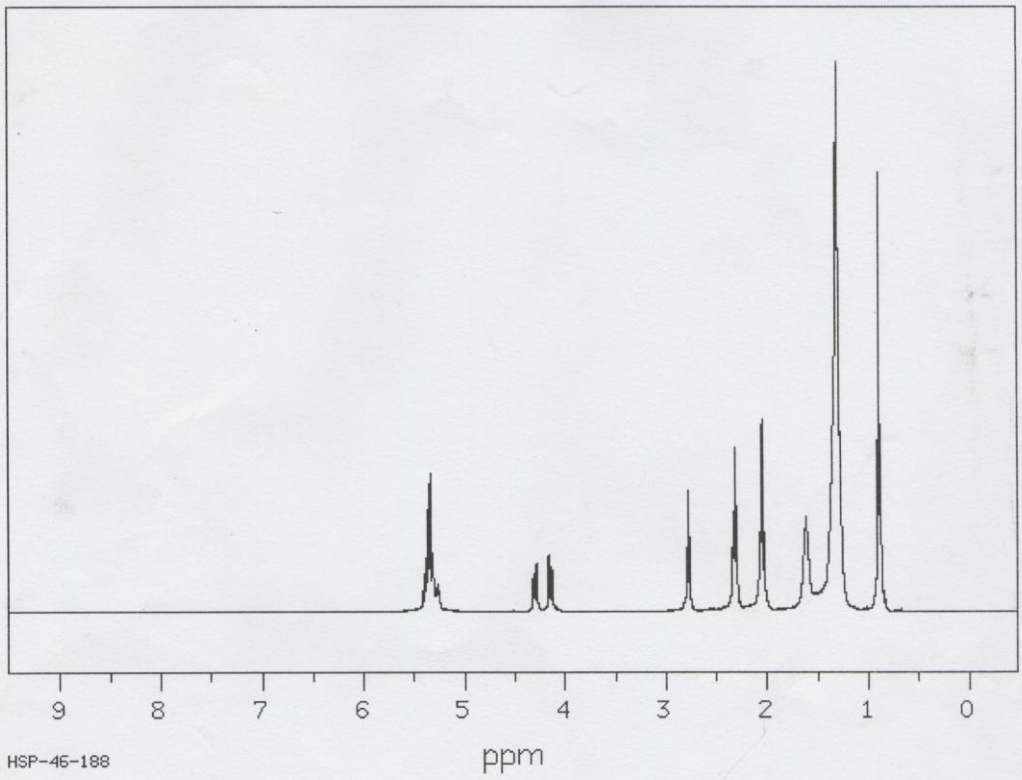
Appendix 7. [1]Proton nuclear magnetic spectrum for compound 1

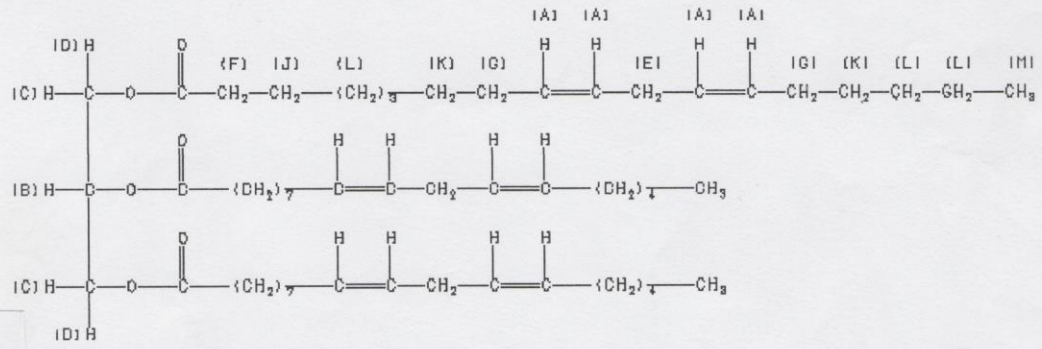


Appendix 8. ^{13}Carbon nuclear magnetic spectrum for compound 1



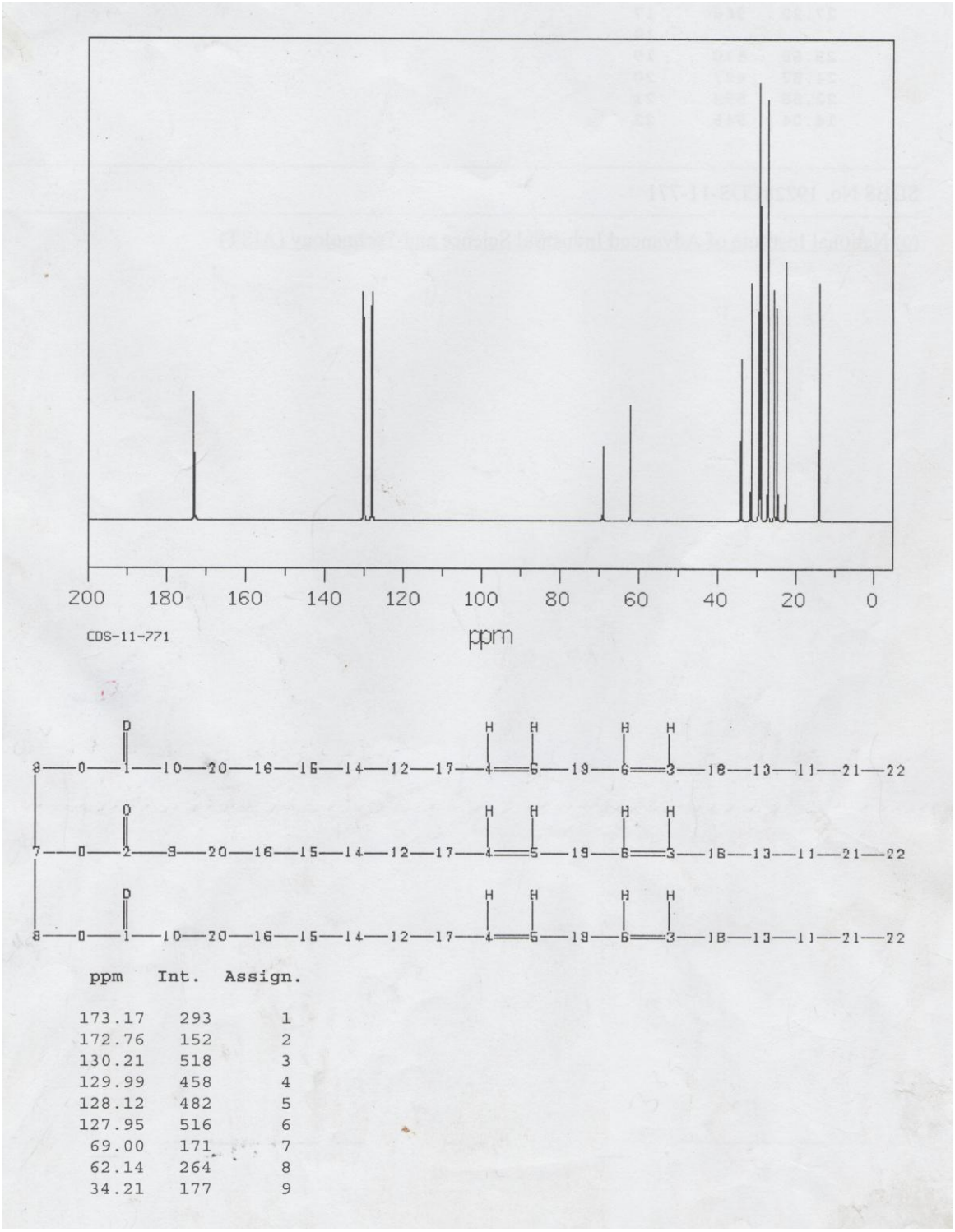
Appendix 9. [1]Proton nuclear magnetic spectrum for $1^1,2^1,3^1$-propanetriyl tris[cis,cis-9,12-octadecadienoate]





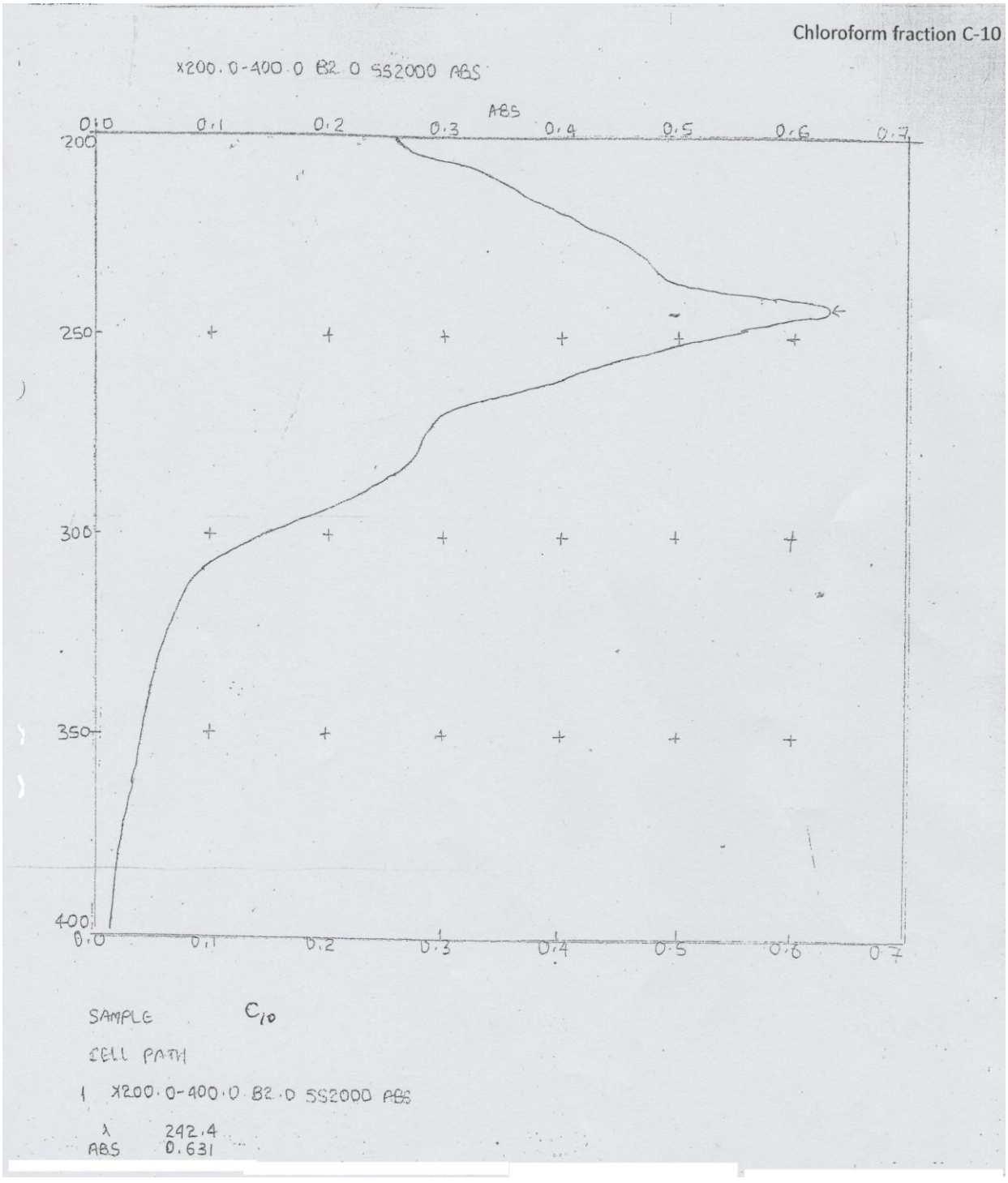
Assign.		Shift (ppm)
A		5.35
B		5.27
C	*1	4.295
D	*1	4.147
E		2.769
F		2.315
G		2.049
J		1.61
K		1.36
L		1.30

Appendix 10. ^{13}Carbon nuclear magnetic spectrum for 1^{1},2^{1},3^{1}-propanetriyl tris[cis,cis-9,12-octadecadienoate]

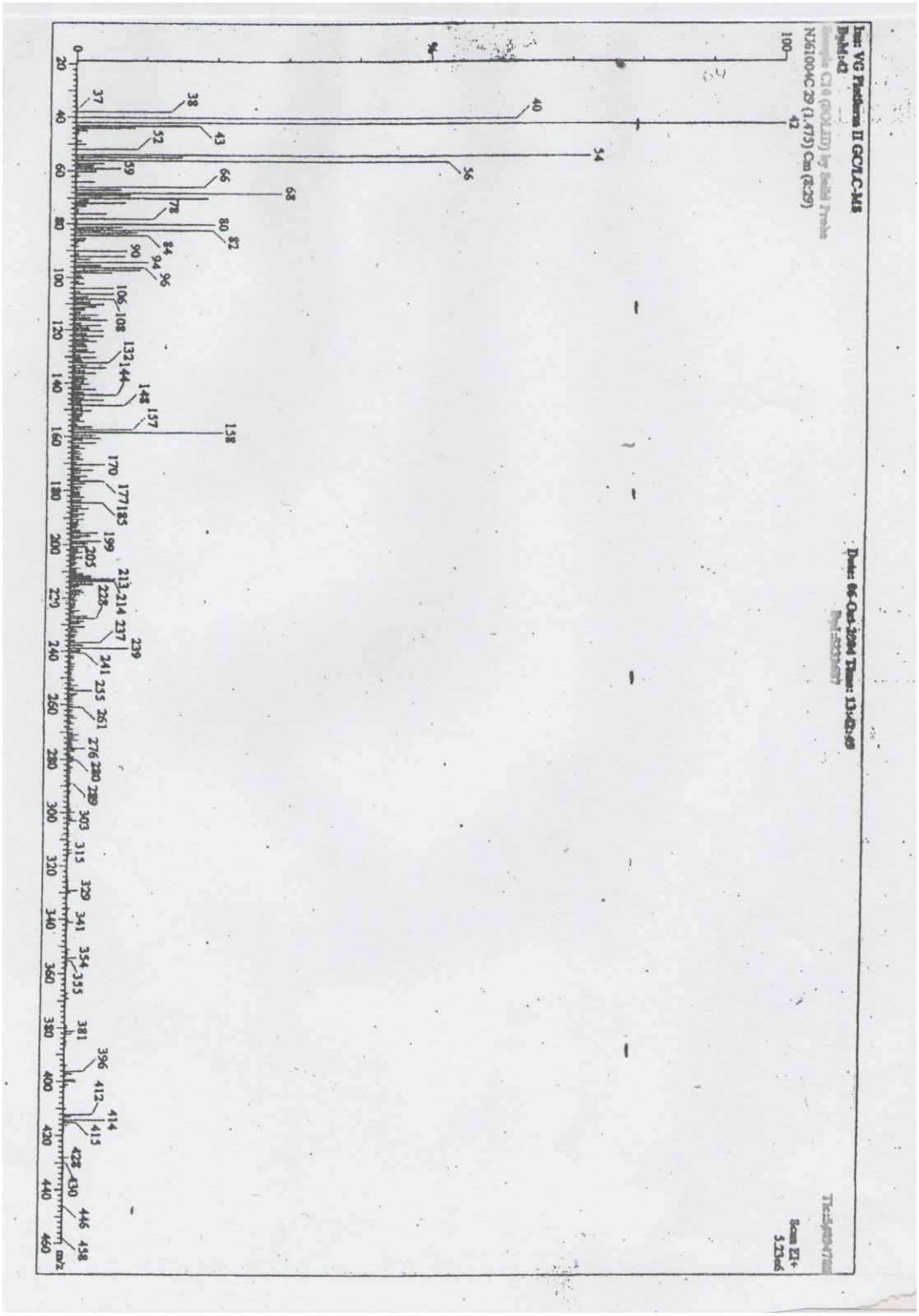


ppm	Int.	Assign.
173.17	293	1
172.76	152	2
130.21	518	3
129.99	458	4
128.12	482	5
127.95	516	6
69.00	171	7
62.14	264	8
34.21	177	9

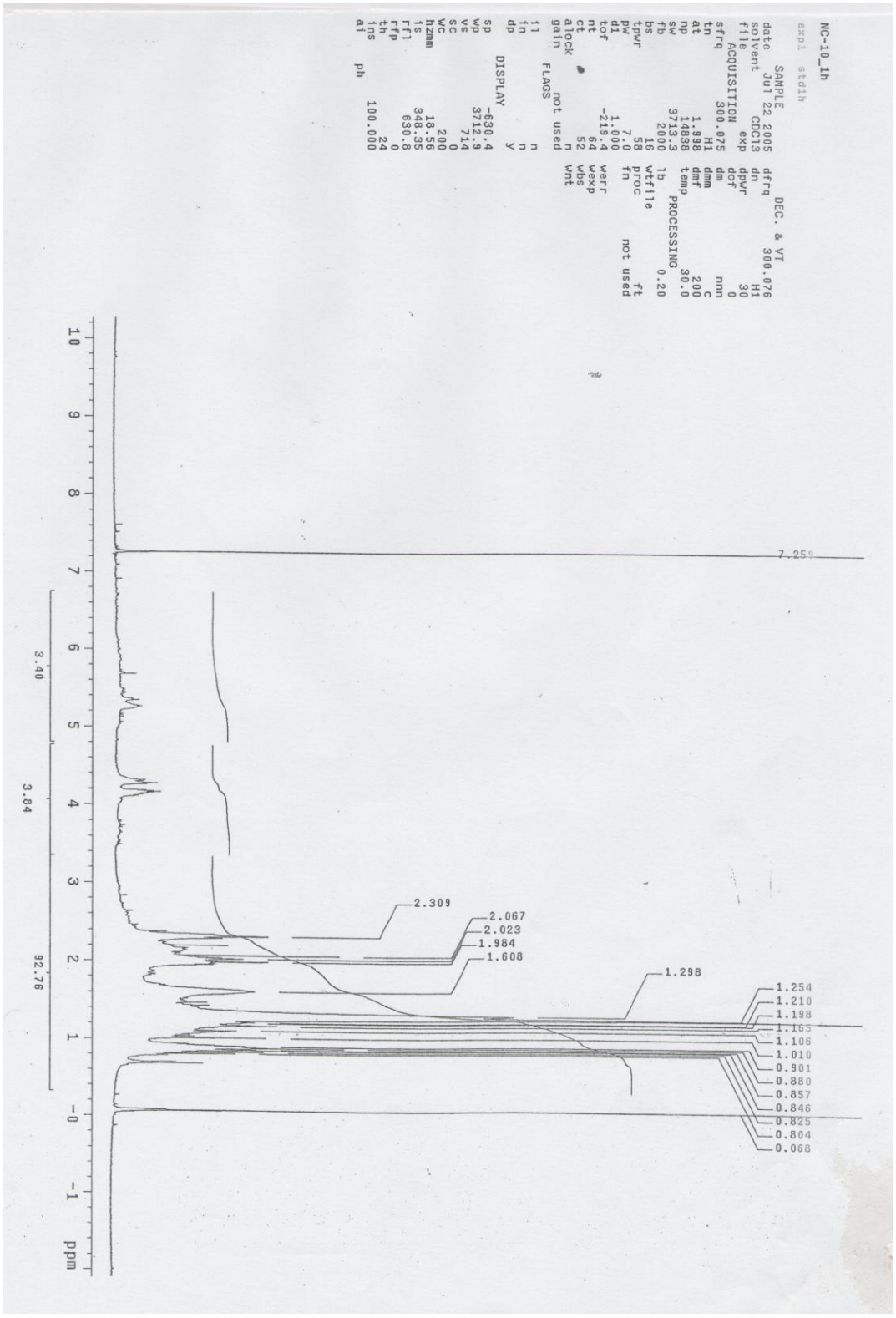
Appendix 11. Ultra Violet spectrum for compound 2



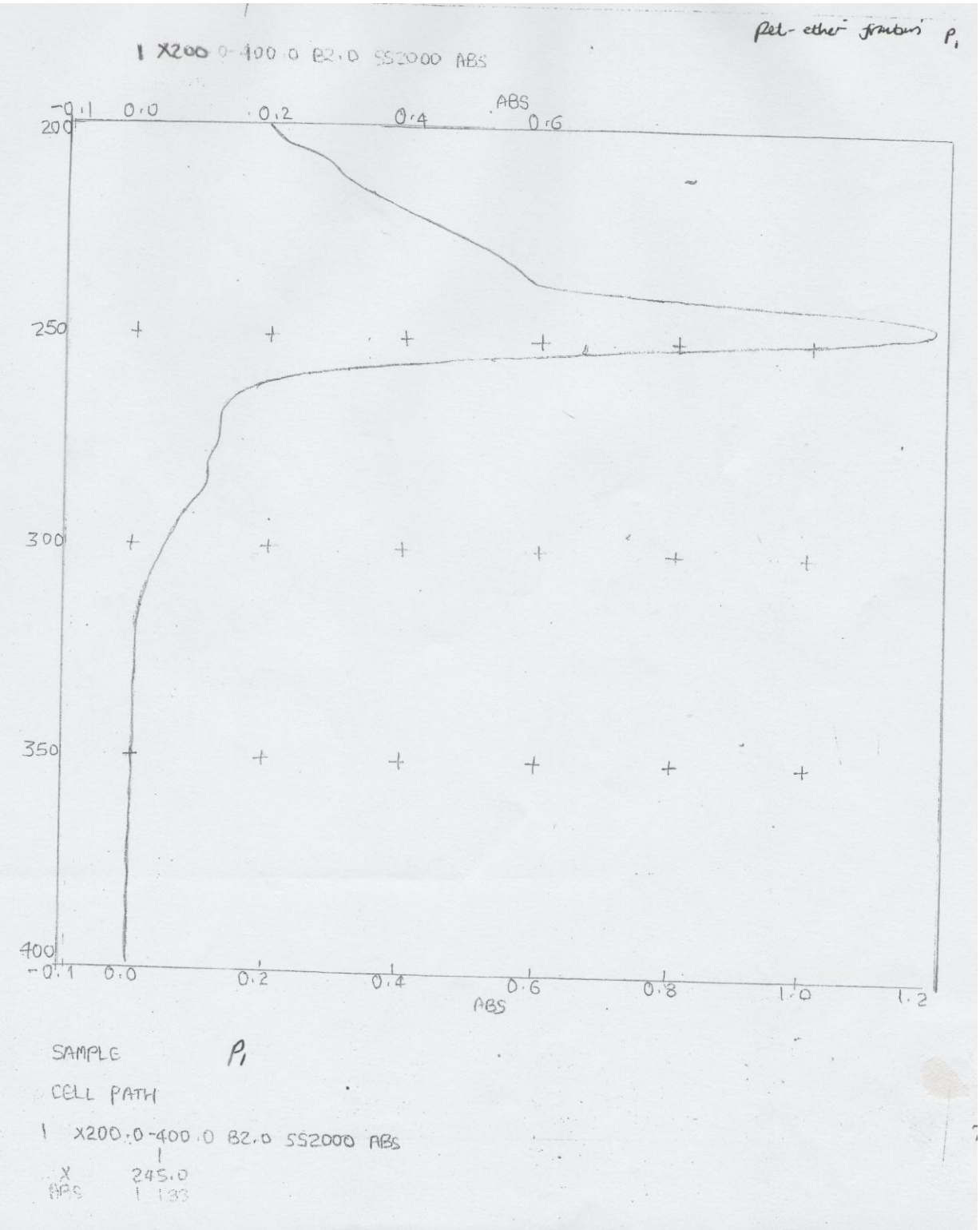
Appendix 12. Mass spectrum for compound 2



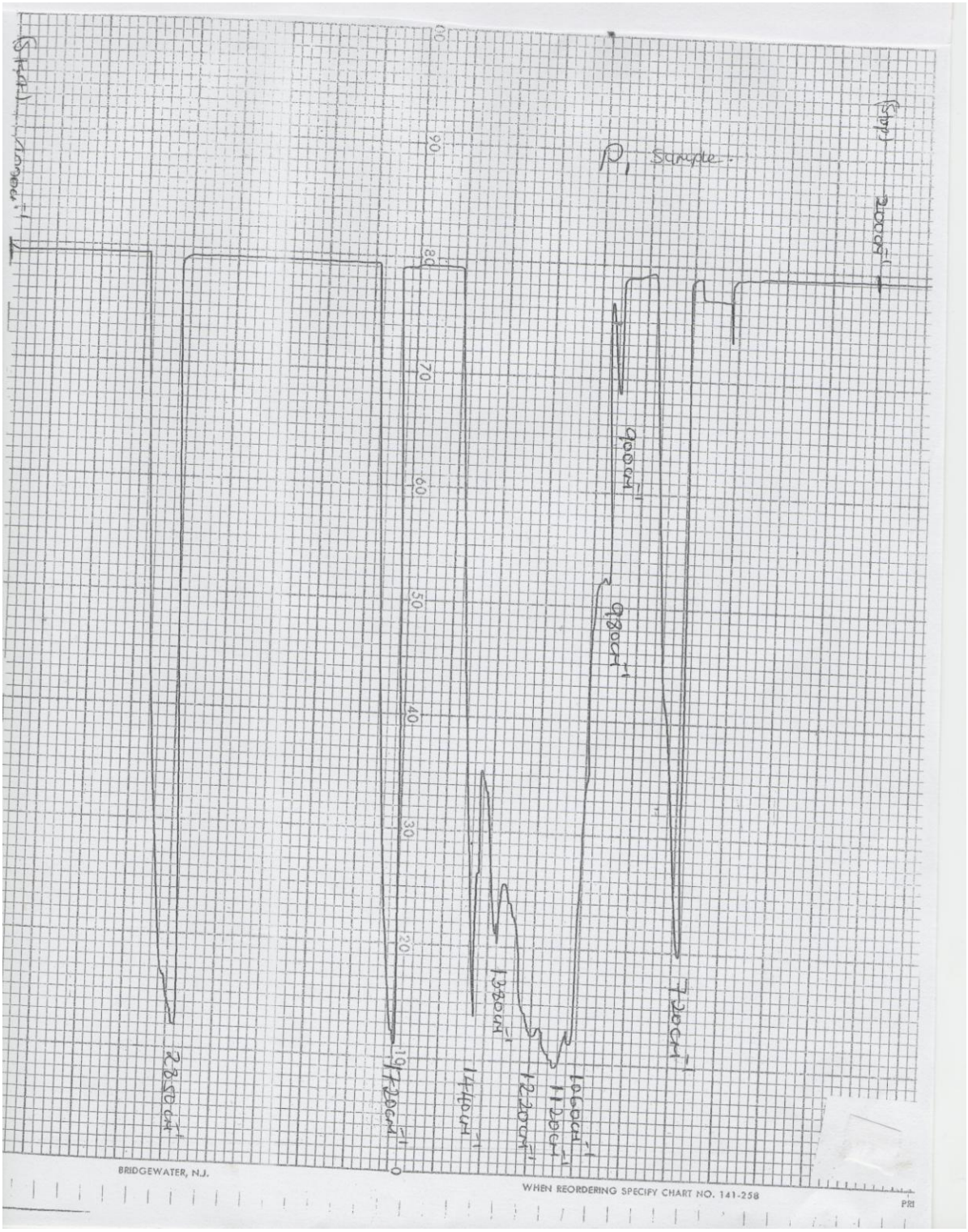
Appendix 13. [1]Proton nuclear magnetic spectrum for compound 2



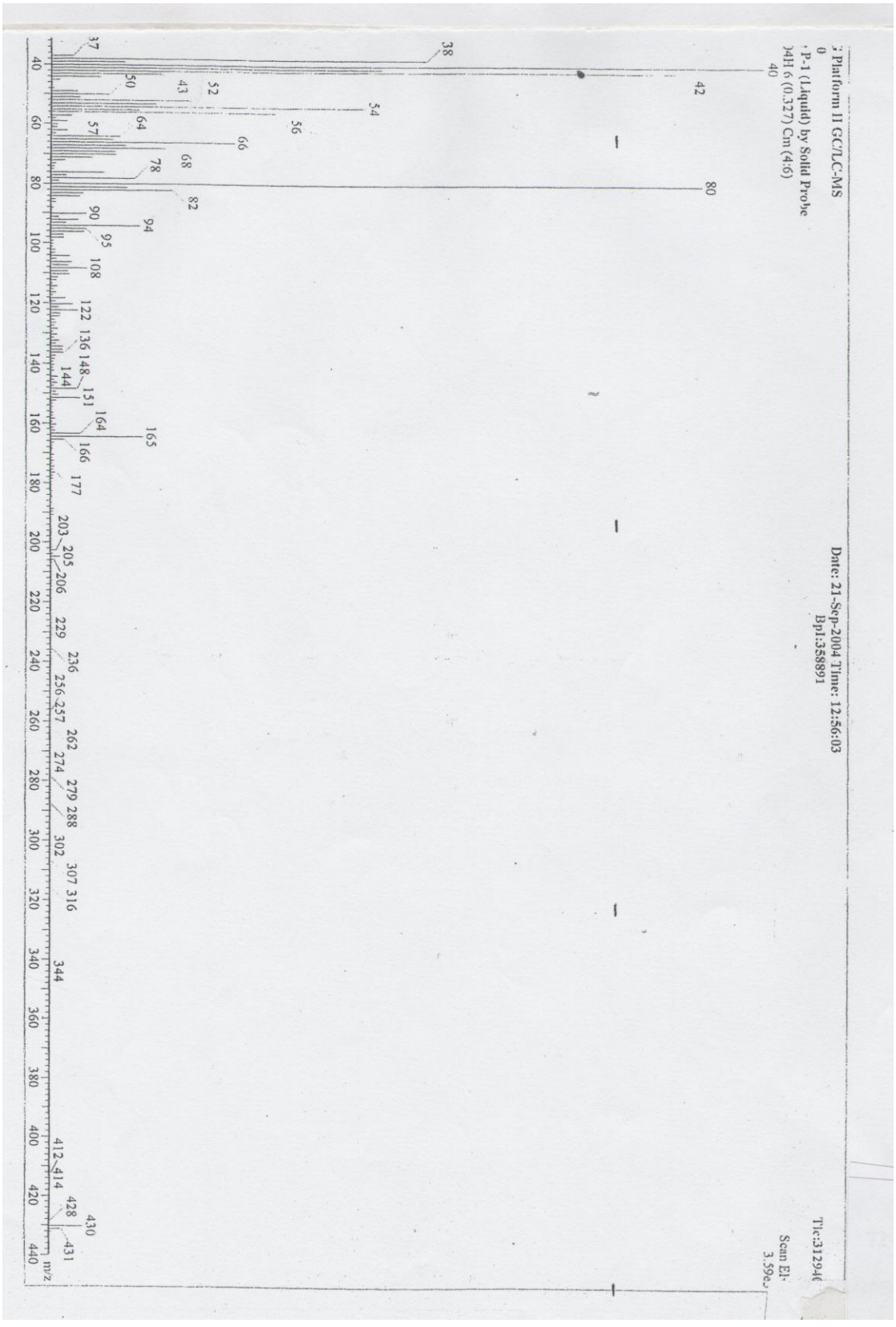
Appendix 14. Ultra-violet spectrum for compound 3



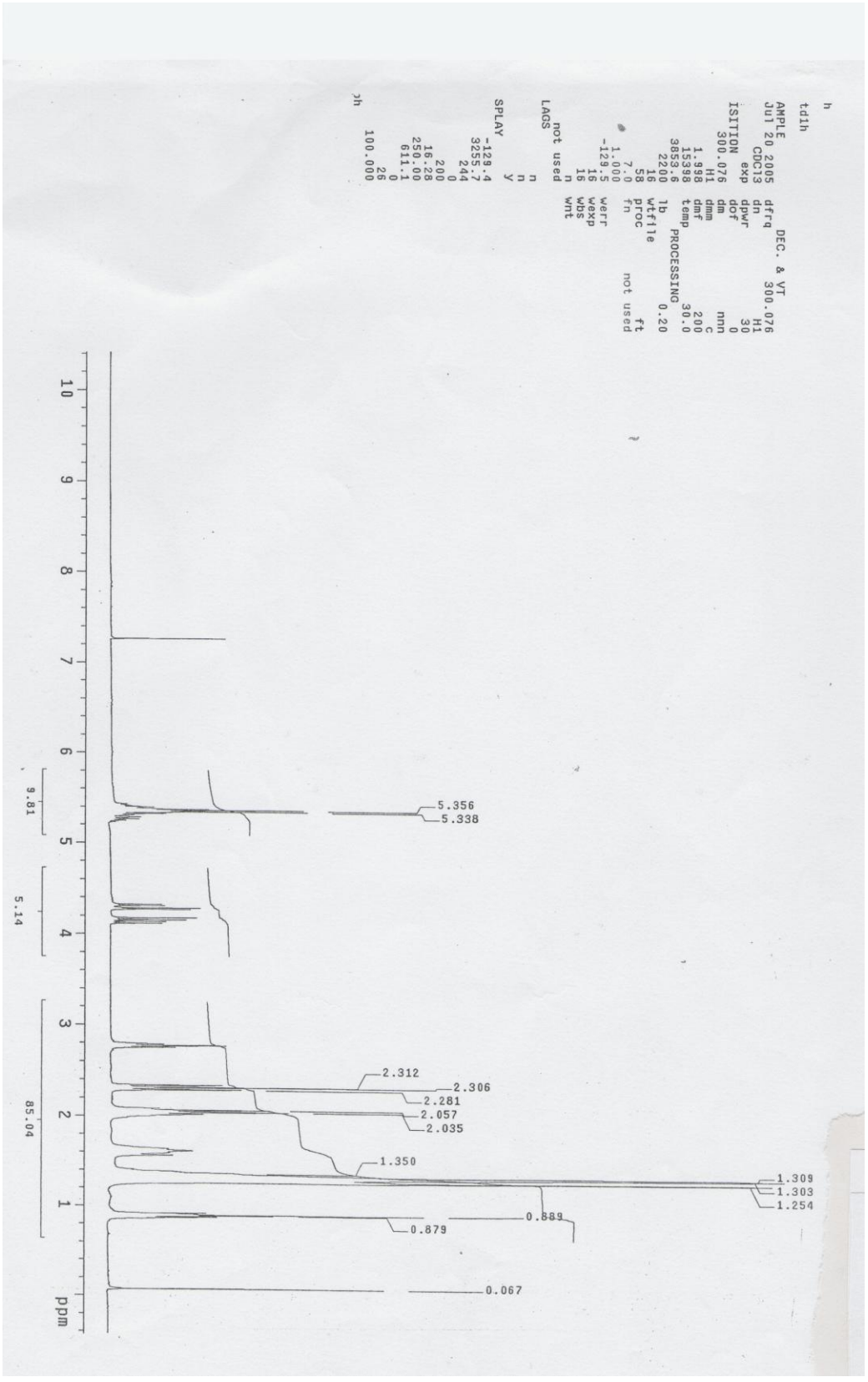
Appendix 15. Infrared spectrum for compound 3



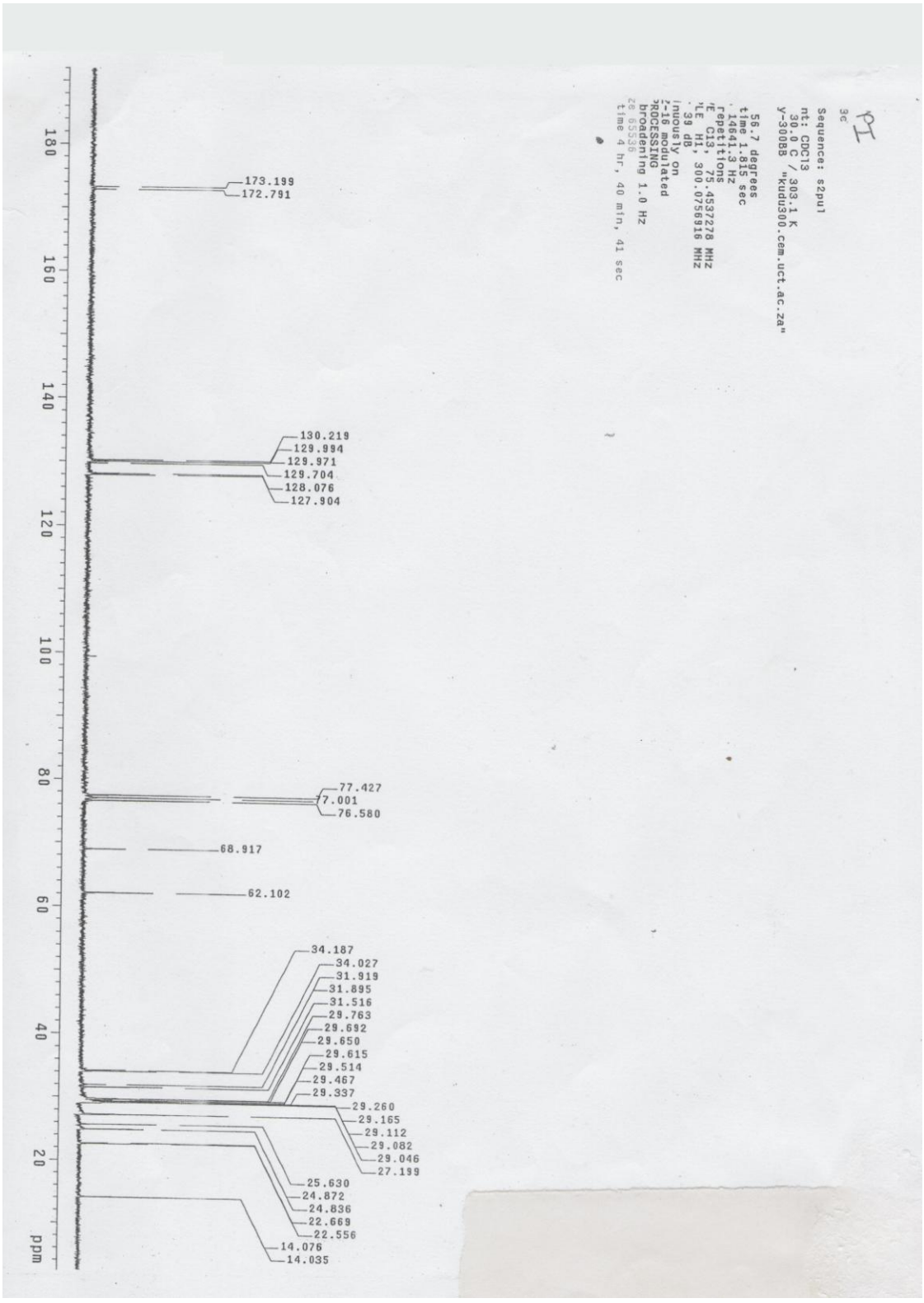
Appendix 16. Mass spectrum for compound 3



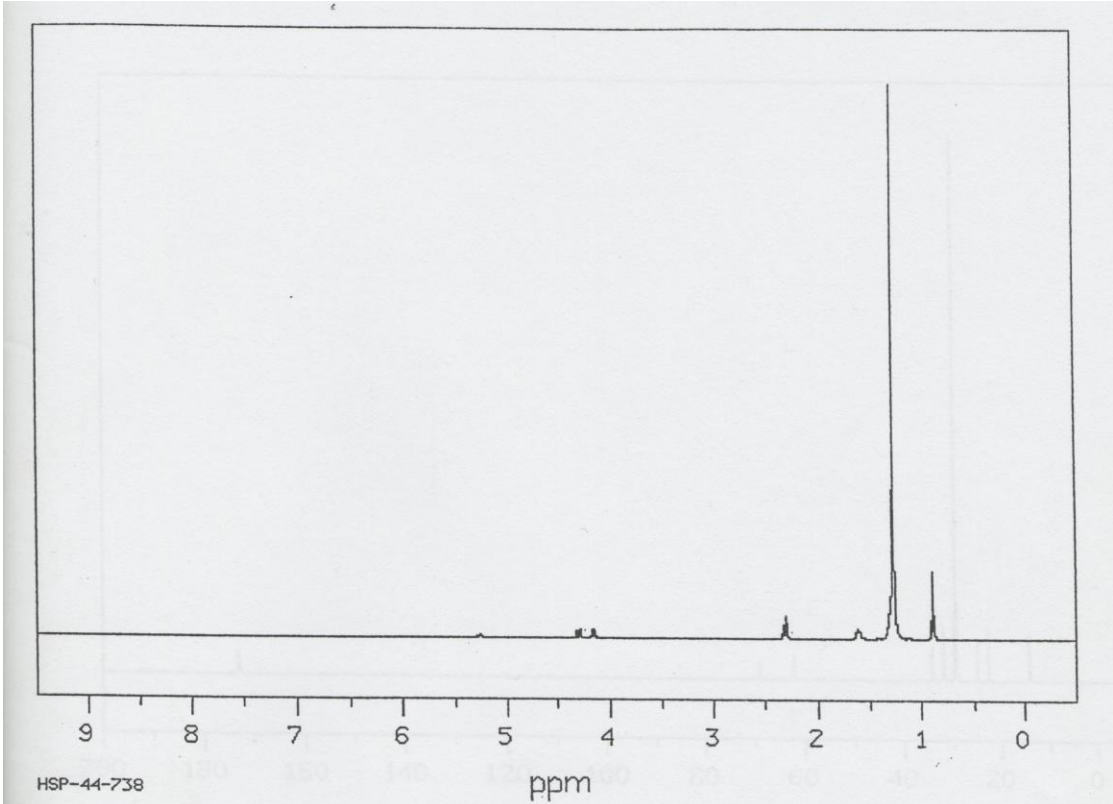
Appendix 17. [1]Proton nuclear magnetic spectrum for compound 3

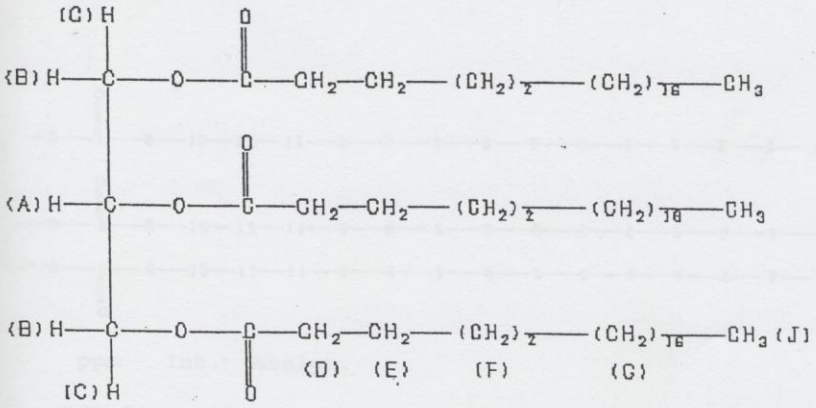


Appendix 18. ^{13}Carbon nuclear magnetic spectrum for compound 3



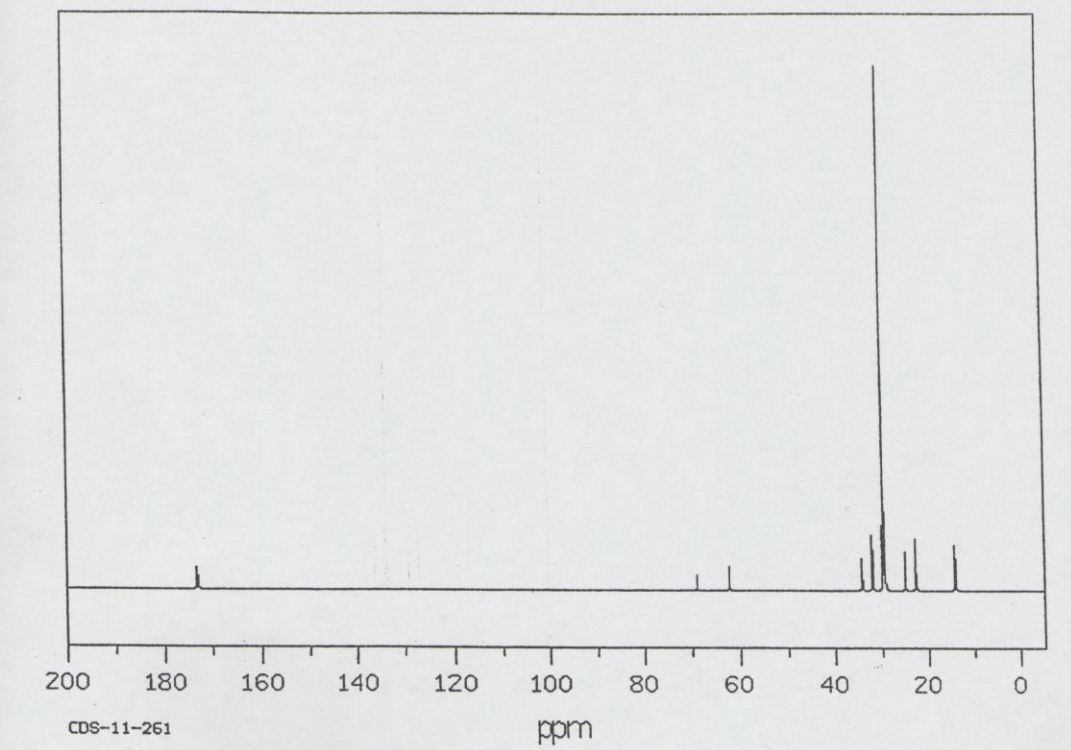
Appendix 19. [1]Proton nuclear magnetic spectrum for 1[1],2[1],3[1]-propanetriyl tridocosanoate

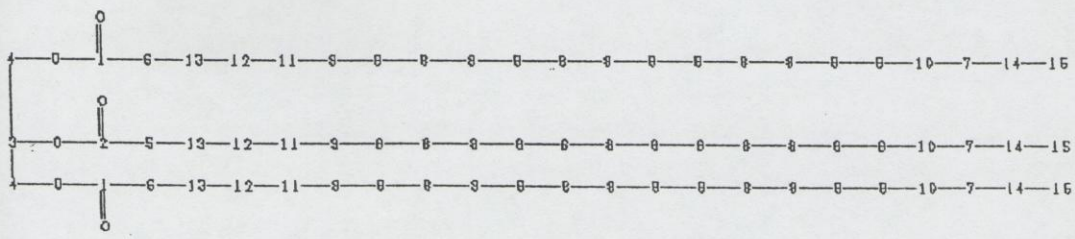




Assign.		Shift (ppm)
A		5.255
B	*1	4.290
C	*1	4.145
D		2.302
E		1.60
F		1.29
G		1.26
J		0.879

Appendix 20. ^{13}Carbon nuclear magnetic spectrum for 1^{1},2^{1},3^{1}-propanetriyl tridocosanoate





ppm	Int.	Assign.	
173.21	42	1	
172.80	24	2	*
69.02	29	3	
62.16	43	4	
34.28	32	5	
34.12	59	6	
31.96	106	7	
29.74	1000	8	*
29.52	100	9	*
29.39	135	10	*
29.32	94	11	*