BIOASSAY-GUIDED ISOLATION, CHARACTERISATION AND CYTOTOXICITY OF MOSQUITO REPELLENT COMPOUNDS FROM SELECTED MEDICINAL PLANTS

BY

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CERTIFICATION

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DEDICATION

The work is to the exaltation of the Almighty creator

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ABSTRACT

Malaria, a tropical disease caused by *Plasmodium* parasites and spread by the bites of infected female *Anopheles* mosquitoes, remains a major public health issue. Mosquito repellents are used as a control measure.Existing synthetic repellents present challengesof high insecticide resistance, allergic reactions, and non-biodegradable residues. This has necessitated the search for alternative repellent compounds from natural sources.This study was designed to evaluate the repellent activity of selected ethnomedicinal plants against wild adult female *Anopheles gambiae* (s.l.), and characterise isolated bioactive compounds.

Dry powdered plant parts of *Citrus limon* Linn. (seeds), *Citrus paradisi* Macf. (seeds), *Citrus sinensis*L.(seeds), *Jatropha curcas* L.(seeds), *Dennettia tripetala* G. (fruits), and *Afromomum melegueta* K. (fruits) were successively extracted with *n*-hexane (Hex), ethyl acetate (EA) and methanol by cold maceration. Phytochemical screening of the extracts was done using standard methods. The extracts were screened against *A. gambiae* for their repellent activity using human bait method, whileN,N-diethyl-meta-toluamide(DEET) and acetone were used as positive and negative controls, respectively. Extracts with the highest percentage repellency, calculated using standard formula, were subjected to bioassay-guided fractionation and separation using chromatographic techniques. Isolated compounds were evaluated for cytotoxicity using brine shrimp lethality assay (safety cut-off, LC_{50}> 100µg/mL), and characterised by spectroscopic (IR, 1D and 2D NMR) and mass spectrometric analyses. Repellent activity data were analysed using one-way ANOVA at $\alpha_{0.05}$.

Extraction of the plant materials yielded eighteen extracts. Phytochemical screening of the extracts revealed the presence of alkaloids, flavonoids, tannins, glycoside, saponins, and anthraquinones. The screened extracts, tested at 1.5, 2.5, and 5mg/mL, gave percentage repellency of ≤ 68.1, ≤ 74.9, and ≤ 97.5%, respectively (DEET, 100%). At 5 mg/mL, the percentage repellency of the three most active extracts (*C. limon* (Hex, 97.5%), *D. tripetala* (EA, 87.7%), and *C. limon* (methanol, 86.8%) were not significantly different (α<0.05). *Citrus limon* (Hex) fractionation yielded 10 fractions with percentage repellency of ≤ 89.5 %, while*D. tripetala* (EA) yielded eleven fractions which were pooled into four (DTH1, 55.7%; DTH2, 60.6%; DTEA, 77.2%, and DTM, 71.1%). *n*-Hexane-100% and Hex/EA (9:1) fractions of *C. limon*, DTEA and DTM fractions of *D. tripetala* showed the most repellency effects of 89.5%, 71.1%, 77.2% and 71.1%, respectively. Chromatographic purification of *n*-hexane-100%, Hex/EA (9:1) and DTEA gave seven compounds. They were identified as palmitic acid (**a**), 14-oxotricosanoic acid (**b**),*n*-octyl stearate(**c**), 15-(heptanoyloxy) pentadec-9-enoic acid (**d**), 6,8-dimethoxy-3-undecyl-1H-[2]benzopyran-1-one (**e**), 1,2,3-propanetriyl tris(5-eicosenoate) (**f**), and α-linoleic acid (**g**) with percentage repellency of 65.9, 63.6, 51.7, 77.9, 75.2, 37.0, and 57.3%, respectively. Cytotoxicity evaluation of compounds **a-e** gaveLC_{50}ranging from 63.2 to 171.1 µg/mL, with compounds **c** (106.1 µg/mL) and **e** (171.1 µg/mL) being adjudged safe. Compound**d** showed the highest repellent activity against *A. gambiae* mosquito.

Extracts of *Citrus limon* and *Dennettia tripetala*contain potentially useful mosquito repellent compounds. *n*-Octyl stearate and 6,8-dimethoxy-3-undecyl-1H-[2]benzopyran-1-one (both from *C. limon*) may serve as leads for the development of novel bio-degradable and safe mosquito repellent compounds.

Keywords: *Citrus limon*, *Dennettia tripetala*, Repellent activity, *Anopheles gambiae*
Word count: 489

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LIST OF ABBREVIATIONS

UNESCO – Universal Education Social and Cultural Organisation

WHO – World Health Organisation

HPLC – High performance liquid chromatography

CC – Column chromatography

PC- Paper chromatography

TLC – Thin layer chromatography

GLC- Gas-liquid chromatography

R_f– Retardation factor

UV - Ultraviolet

IR - Infrared

NMR – Nuclear Magnetic Resonance

MS – Mass spectrometry

HIV – Human immuno-deficiency Virus

AZT- Azido thymidine

AMCA - The American mosquito control association

DEET - N, N-diethyl-m-toluamide

PMD - *p*-Mentane-3,8-diol

SFE - Supercritical fluids extraction

MAE –Microwave-assisted extraction

2D NMR - Two dimensional (2D) NMR

HMQC -Heteronuclear multiple quantum coherence

HSQC - Heteronuclear single quantum coherence

HMBC - Heteronuclear Multiple Bond Correlation

COSY - ^{1}H-^{1}H Correlation Spectroscopy

GST - Glutathione-S-transferase

IAMRAT - Institute for Advanced Medical Research and Training.

UI/UCH EC- University of Ibadan / University CollegeHospital Ethical Committee

FTIR - Fourier Transform

FRIN - Forest Research Institute of Nigeria

CPT - Complete protection time

CHAPTER ONE

INTRODUCTION

1.1 Background to the study

Plant materials have been used as repellents for long time. They are nontoxic to human or domestic animals and are biodegradable (Das *et al.,* 2003, Regnault-Roger *et al.,* 2012). *Citrus paradisi* peels oil has been reported for its insecticide and antifeedant activities(Tirillini, 2000, Faleye *et al*., 2012).β-phenylnitroethane was identified as the predominant compound from the dried fruits essential oil, containing 78.1% β-Phenylnitroethane (BPNE) (Oyemitan *et al*., 2019). Isobutylamide (neurotoxins in insects, but safe in mammals) was shown to be responsible for repellent effect of *Piper nigrum* and *P.guineense* fruit extracts against insects from five orders (Scot *et al* 2004). *Afromomum melegueta* was insecticidal against*Callosobrochus maculatus*, with paradol being responsible as the major active ingredient (Lale, 2002). *A. melegueta's* water, methanol and hexane extracts were antifeedant against the larva of diamond back moth. Two constituents, gingerol and arylalkanoids 6-shogaol were shown to be responsible for the activity (Ntonifor *et al*., 2010). In China, *Citrus sinensis* oil tested as the most toxic fumigant among 5 citruses. Citral was shown to be responsible for lethality (Yang *et al*., 2005). Traditionally, dried peels of citruses are burnt in the living room at night to deter mosquitoes from biting or entering the house (Ngurukwem *et al*., 2001; Badawy*et al*., 2017).

Human beings have depended on plants for food and medicine for several centuries, and have been able to identify plants with physiological effects on human and environment. Plants have formed the basis of traditional medicinal system for centuries based on the information accumulated over time (Newton *et al*., 2000). Many countries have traditional belief with knowledge of traditional medicine related to different health problems. There is diversity in the medicinal nature, since different communities have different methods of

treatment based on their traditional studies and findings on a disease and its therapy. The Chinese and the Indians have been using plants as their medicine for so long: material medica and Ayuvedic system, respectively (Cragg and Newman, 2005). Also, an African scientist, Ebers papyrus documented hundreds of drugs made from plants and animals as far back as 1500 BC.

Two Greek scientists, Theophrastus and Dioseorides, were able to study the therapeutic quality of plants, their cultivation for increased quantity, and to collect, identify and record the medicinal ones as documentation for modern science (Cavan, 1999).

In Africa, for example in Nigeria, pawpaw (*Carica papaya*) leaves have been used for treating Jaundice. Dongoyaro (*Azadirachta indica*) leaves have also been used as insect repellant and for treating malaria. All these herbs are still in use today. Traditionally, diseases were being treated using local herbal medicine, before the invention of modern medicine and treatment records were normally transferred to the next generation by the herbal medicine practitioners (Wambebe, 1993).

Synthetic drugs have demerits like side effects and high cost of production, making them expensive. People have taken interest in the usage of herbs because they are found to be safe and effective against most diseases (Martins, 2014). Internationally, the Universal Education Social and Cultural Organization (UNESCO) reported that the practice of traditional medicine is the best method to fully satisfy the health need of people globally. (UNESCO, 1994). The World Health Organization (WHO) reported that enhancing broad access to good and enough healthcare will require other sources of healthcare apart from western medicine (WHO, 1978, UNESCO, 1994). The fact that the WHO fully recognises herbal medicine should encourage and increase the interest of world countries, especially in Africa, to exploit all available traditional medicine and healing methods. Herbalists prepare herbal medicine as: essential oils (volatile oils), extracts, powder, tablets and capsules, tinctures, infusions and decoction, and so on.

Phytochemistry is the branch of science that determines what exactly in plants are responsible for their efficacy and therapy. It concerns the study of many organic substances present in plants, their structures, biosynthesis, metabolism, and their pharmacological or therapeutic functions. Carrying out all the operation in phytochemistry

require various chromatographic processes and spectroscopic analysis of plant metabolites.

Plant needs to be initially identified by a taxonomist. Drying of plant prior to extraction, mostly air-drying under shade to avoid degradation, follows immediately. It is then coarsely grinded using an appropriate grinding machine and kept in the freezer prior to usage. Extraction of the powdered plant with organic solvents of varying polarities is done and the liquid extract is concentrated, usually using rotary evaporator, between30 °C to 40 °C.

Separation of crude extract into fractions with different constituents is done using fractionation, before further purification with other chromatography techniques, of which choices depend on the physicochemical properties of the compounds of interest.

Identification of compounds after purification requires knowing the class of the compounds through the measurement of their physical properties and comparing it with the standard values, followed by the determination of their spectra data, using spectroscopic analysis.

1.2Statement of problem

Global mortalities and illnesses are majorly cause by disease transmission from insects, with transmission from mosquitoes alone reaching millions yearly (Adeniran and Fabiyi, 2012). The causative agent of malaria in Africa is *Anopheles gambiae*, and studies have shown that there are five hundred million malaria infections and about four million malaria death annually (WHO 1999). Also, in Nigeria, the level of mosquito population is massive, which make majority of the people to be exposed to infections from mosquitoes. The fact that ailments from insects cause immense health chaos and mortalities in tropical climate does not mean that the rest of the world are safe from their scourge (Adeniran and Fabiyi, 2012).

1.3 Justification

Nigeria has about 76% of the population living in high malaria transmission areas and based on the 2020 World Malaria Report, the most malaria cases in 2019, which led to the greatest number of Malaria mortalitycame from Nigeria (WHO, 2019).

Repellent application is a better replacement to the use of insecticides, because bites from insects could be better prevented with repellents and eradicating infestation (Adeniran and Fabiyi, 2012). In many instances, the only easy way to prevent mosquito bites is the usage of repellents on the skin. It may be used, both, to prevent insects from stored products and food packaging, and also to deter ticks, mites and mosquitoes from biting individuals (Adeniran and Fabiyi, 2012).Plant oil especially the volatile oils has been reported to exhibit insecticidal and repellent effects (Isman *et al*., 2011). Also, saturated andunsaturated fatty acids have shown biting deterrent effects against *Aedes aegypti* (L) mosquito (Ali *et al*,.2012). Plant seeds extractscontaining fixed oil could be a rich source of fatty acids which seem to prevent mosquitobites (Jones *et al*., 2012).

1.4 Aim of the research

This work is aimed at isolating the active metabolites(s) from the extracts of the seeds of *Citrus limon, Citrus paradise, Citrus sinensis, Jatropha curcas* and the fruits of *Dennettia tripetala*, and *Afromomum melegueta*, that are used ethnobotanically as repellents. The extracts, fractions and active compound(s), of the plants will be subjected to repellent bioassay. The active compound(s) isolated will be fully characterized so as to provide useful information for possible development of the active compound(s) into phytomedicine/repellent compounds.

1.5 Objectives of the research

(a.) To investigate successive extracts of six selected plants for repellent activity against adult female *Anopheles gambiae*;
(b.) To isolate and purify bioactive repellent compounds;
(c.) To determine the molecular structure of compounds isolated, and;
(d.) To evaluatethe cytotoxicity of compounds isolated.

CHAPTER TWO

LITERATURE REVIEW

2.1 Biodiversity

Biodiversity is vital in discovering new natural products. The more biodiversity is explored, the more the number of chemical diversitiesthat can be discovered. With all the new bioassay technologies, a lot of new compounds can be discovered from old natural products. This means that all samples which previously showed no activity can be retested (Young, 1997, Cragg *et al*., 2005). Only about 15% of the 250,000 species of plant were already accessed for their active metabolites (Balandrin *et al*., 1999; Lall *et al*., 2020). Also, aquatic organisms seem to be composed of active medicinal constituents because about 3000 new compounds have been isolated from them in the past thirty years (da Rocha *et al*., 2001). The microbial world will have more potential and greatest biodiversity because only about 1% of the world's microbes can be cultivated with the current technology (Brandy *et al*., 2001).

2.2 Drugs developed from plants

Many new drugs produced from botanicals are presented in Figure 2.1a and 2.1b and they have immensely contributed medically to human health (Newman *et al*., 2003, Chin *et al*., 2006). Emetine (1), Figure 2.1a,is an alkaloid, isolated from *pecacuanha* A. Richard, is used in the therapy of abscesses, an infection caused by *Entameoba histolytica*.

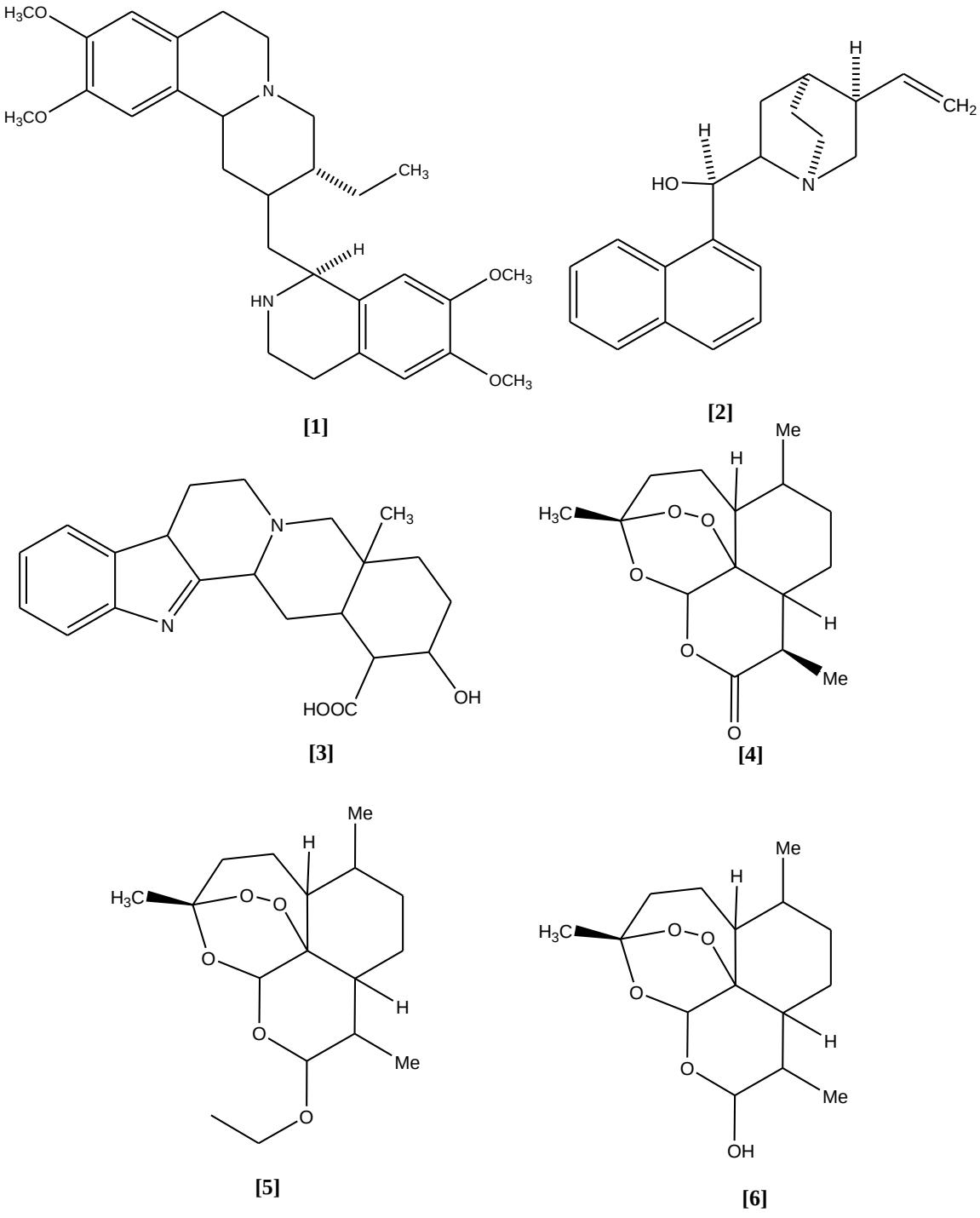


Figure 2.1a: Isolated compounds from plants

Quinine (2), Figure 2.1a, is another vital drug from plant. This alkaloid occursat has been in use for ages. It is present naturally in cinchona plants, and was applied in malaria therapy (Cragg and Newman, 2005).Another drug is yohimbine (3), Figure 2.1a, isolated from *Pausinystalia yohimbe*'s bark (K. Schum.). It has antihypertensive properties.

Investigations done on the data of plants employed in Chinese local therapy led to the examination of *Artemisia annua* L plants. This resulted in the isolation of artemisinin (4), Figure 2.1a, a sesquiterpene endoperoxide (Newman *et al*., 2000).The structure of artemisinin was modified to artemether (5) and dihydroartemisinin (6), Figure 2.1a,so as to make it more potent and safer. This led to the production of the active drug used globally now in the treatment of malaria.

The first bronchodilators, ephedrine (7), Figure 2.1b, was isolated from *Ephedra sinaca* Stapf. (Ephedraceae).The Chinese have used *Ephedra sinaca* stapf. (Ephedraceae) for the treatment of respiratory disorder since 1923 (Newman *et al*., 2003).

Some members of the *Taxus brevifolia*, like the yew tree, with other plants in the same category are used traditionally in America, to heal various non-cancerous ailments. As part of some phytochemical investigations, carried out for the National Cancer Institute, by the US Department of Agriculture, taxol (8),Figure 2.1b, was gotten from*Taxus brevifolia* L. bark (Suffness, 1995).A lot of compounds with related structues to taxol and with biological effects are presently in use (Suffness, 1996).

Galanthus nivalsis L. from the Arnaryllidaceous family has been in use for long time as traditional medicine for central nervous system conditions. Galantamine (9), figure 2.1b, was isolated from the plant, as a selective acetychlorlinesterase inhibitor for Alzhheimer's disease (Yue-Zhong, 1998).

Calanide A (10), figure 2.1b,was gotten from *Calophyllum lanigerum* var. austrocoriaceum (P. F. Stevens), a plant originated from Malaysia. A study on the compound showed its potent activity against HIV, and also the AZT resistant strains and others similar species (Mark, 2004).

A Chinese scientist isolated huperzine (11), Figure 2.1b, from the leaves of Huperzia serrata (Thunb. ex Murray), a plant with outstanding history of use for improvement of memory. This compound is an acetylchorlinesterase selective inhibitor, which is in its final clinical trial in the USA (Ma *et al*., 2007).

2.3 Malaria

Malaria can be defined as a parasitic disease, transmitted by an infected female Anopheles mosquito. The causative agent could be the *Plasmodium falciparum,* which is prevalent in Africa. It damages red blood cells, and causes most deaths globally, or the *P. vivax* which is common to sub-Saharan Africa (WHO, 2016). Anopheles mosquitoes of female types, such as gambiae, funestus, moucheti and arabiensis are the vectors that are responsible for the disease (World Malaria Report, 2015). The infection is on the increase because vectors are resisting most of the insecticides that are applied, and also the malaria parasite, Plasmodium species are resisting the available antimalarial medicines. In Nigeria, about 192,284 Malaria deaths were recorded in 2015, which makes it the leading cause of death. The application of insect repellents and mosquito nets can reduce the transmission of Malaria (Sabina, 2017).

2.4 Mosquitoes

Mosquitoes are midge-like insects. Their female consumes the blood of human beings and some other animals. They cause diseases like malaria, yellow fever, and filariasis (Michigan Mosquito Control Organization). They are members of the order Diptera, called True Flies. They have two wings with scales. The female mouth has proboscis for piercing and sucking, while the male mouth contains feathery antennae. They feed mainly on sugar source like nectar and honey, AMCA (The American Mosquito Control Association). The 3 most common types of mosquito genera are:*Anopheles, Culex*, and *Aedes* as shown in Figure 2.4.

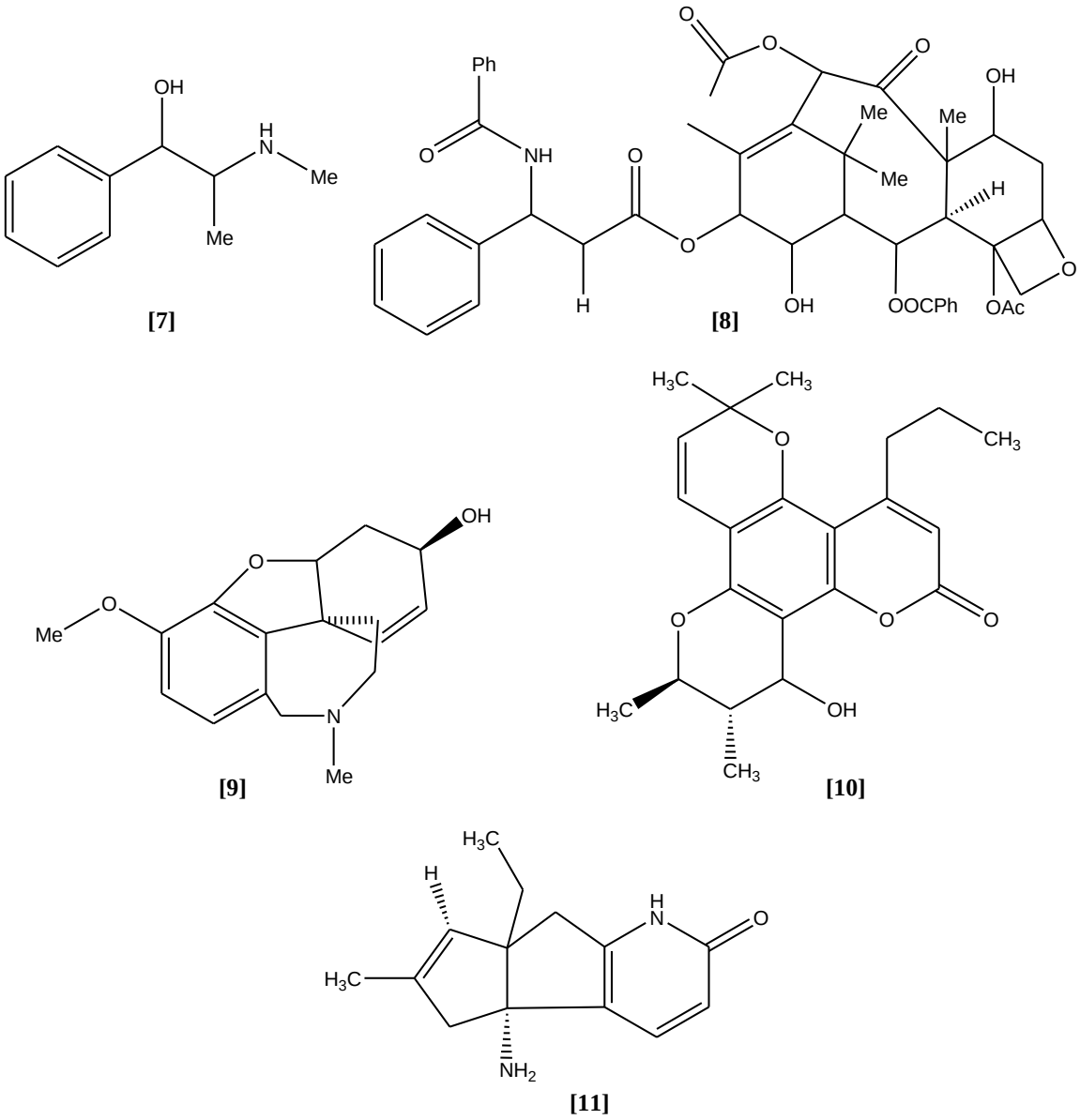


Figure 2.1b: Isolated compounds from plants

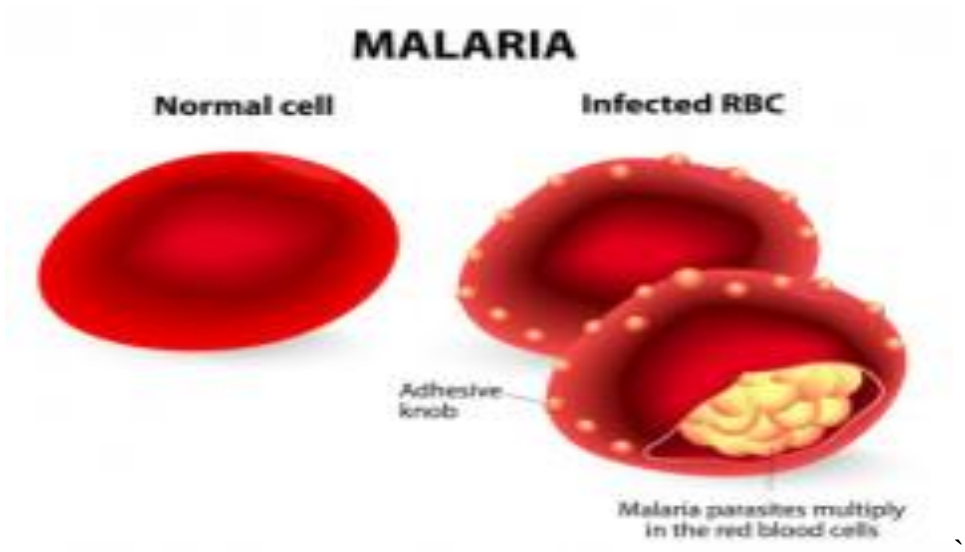


Fig 2.2: *Plasmodium* infected and normal Red Blood cells (Cooke et al., 2001)

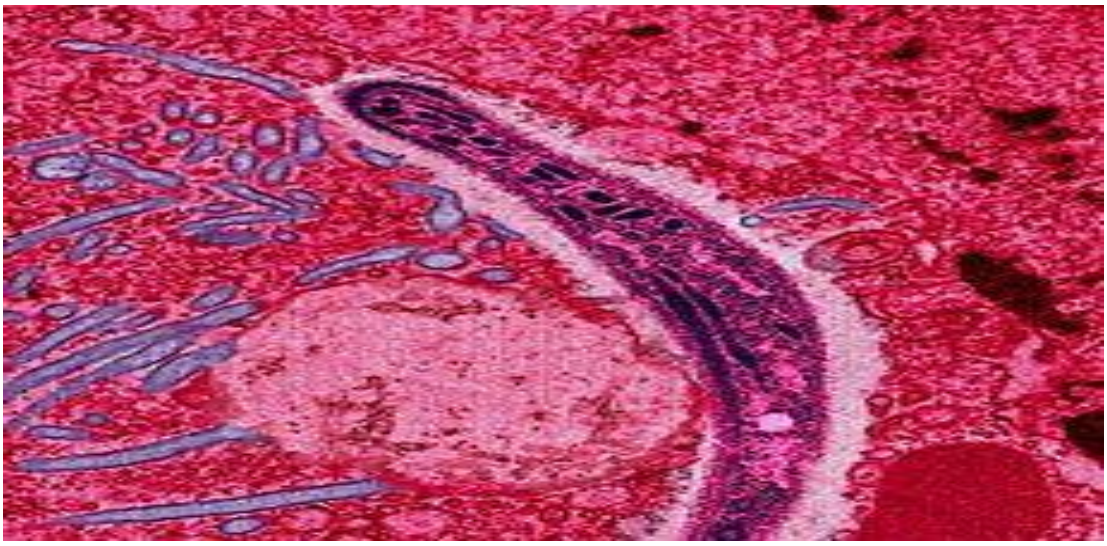


Fig 2.3: A *Plasmodium* moving across mosquito cell (Ryan *et al*., 2010)

Fig 2.4: Images of *Aedes aegypti*, *Anopheles* and *Culex* mosquito (Keswani and Jayesh, 2006)

2.4.1 Mosquito-borne diseases

The ability of mosquitoes to transmit malaria to human beings can lead to a lot of deaths every year. The infection solely resulted in 438,000 mortalities in 2015. The global situation of dengue fever becomes worse every year, with more countries reporting its outbreaks. *Aedes aegypti* mosquito alone causes zika, dengue, chikungunya, and yellow fever. Larger part of world's inhabitants stays around the vicinity of Aedes and other mosquitoes, So, it is vital to make sure that mosquito control is maintained and thus prevent the scourge of malaria. Various kinds of mosquitoes exist and they can cause different diseases (WHO, 2019).

2.4.2 Methods of mosquito control and need for alternatives

Diseases from mosquitoes are rampant globally, with a mosquito bite causing health situations from skin irritation to contacting malaria. There are different products used to prevent mosquito bites, and their potency vary from one to another, so it is good to be aware of the differences in their effectiveness. Mosquitoes are nuisance and harmful, so some control measures employed like emptying stagnant water and wearing covering clothes can only reduce bites, so meaningful control measures like using mosquito repellent are necessary (Elissa *et al*., 2004).

Apparently, resistance to majority of commonly used insecticides in mosquito control programs by Anopheles gambiae, the main vector of malaria, has greatly contributed to the failure of malaria control in various quarters. This is probably due to the abused utilization of synthetic based insecticides for malaria vector management activities, which has negatively affected other system of control like the biological method and therefore leading to surgent in mosquito populations (Croft and Brown, 1978). This underscores the serious demand for the replacement of synthetic mosquito repellents and insecticides (Ranson *et al*., 2000).

Table 2.1: Common mosquito control methods (Patel *et al*., 2012)

S/no	Methods	Sub-types	Examples
1	Chemical	(a) Synthetic Repellent	Mosquito spray (e.g. Baygon), Deet, Permethrin
		(b) Natural repellent	Neem oil, Citronella oil
2	Non-chemical	(a) Physical	Medicated net, Non-medicated net, Mosquito traps
		(b) Mechanical	Electric mosquito zapper, Mosquito Magnet
3	Biological	-	Growing of fish species that feeds on mosquito larvae in the water bodies

2.5 Mosquito repellents

Mosquito repellents are compounds that prevent mosquitoes from landing to take a successful blood meal (Egunyomi *et al*., 2010). It can also be referred to as a chemical that prevents insects and arthropods from landing on a surface on which the chemical is applied (Patel *et al*., 2012).

2.5.1 Merits of Repellents

Repellents seem to be most reliable, affordable and convenient method of personal protection against annoyance and infectious bites from haematophagous insects, thereby minimizing the contact between humans and mosquito vectors (WHO, 1999). Plant repellent materials include phytochemicals of which effects vary largely depending on the parts of the plant, for example, seeds, fruits and flowers (Tavassoli *et al*., 2011).

2.5.2 DEET as standard repellent

N, N-diethyl-m-toluamide (DEET, Figure 2.5) is a prominent and effective repellent. It is a gold standard repellent which protects for up to eight hours and has been in existence since 1957. DEET comes in different brands of liquid and gaseous dosage forms, with strong repellent activities against mosquitoes. The limitations to its uses are due to demerits such as, irritation to mucous membrane, unpleasant odor, and very high concentration that can damage plastics and synthetic rubber, and it also has high skin penetration properties (Qui *et al*., 1998). These side effects indicate the requirements for improved and safe repellents with little or no side effects to the humans adopting them.

2.5.3 Classification of mosquito repellent

Repellents are used personally for protection against bites from mosquito, so as to prevent malaria, dengue fever and other ailments caused by mosquitoes. They are divided into synthetics and plant-based repellents.

2.5.3.1 Synthetic repellents

2.5.3.1.1 IR-3535

IR-3535, fig 2.5 has been in existence for long in Europe, and it is used to prevent bites from mosquitoes and other biting flies. It is an alternative repellent to DEET and it gives effective protection for meaningful duration of time (Feuser *et al*., 2020).

2.5.3.1.2 Picaridin

Picaridin, fig 2.5 has been in use globally since1998, and can also be referred to as KBR3023. It can be used in place of DEET, and it is effective against mosquito bites for long period of time as compared to DEET. Unlike DEET, it does not affect the skin, has no odour and does not have destructive effect on plastics and other products. (Khater *et al*., 2019).

2.5.3.1.3 Permethrin

Permethrin (type1 pyrethroid), fig. 2.5 can be used as a repellent on fabrics, walls of tents and nets, but not on the skin. It can also be used as an insecticide on pests. Permethrin is so effective that even after few launderings, unlike DEET, it can still be potent for like two weeks. The application of both DEET on the skin and permethrin clothing, for protection could be very effective in a mosquito infested area or environment (Banks *et al*., 2015).

2.5.3.1.4 P-menthane-3,8-diol (PMD)

An active ingredient called, p-mentane-3,8-diol (PMD), fig 2.5, is present in the oil of lemon eucalyptus (OLE), the leaves and twigs extracts of eucalyptus plant. PMD activity as repellent is similar to about 20% of DEET, as tested against mosquitoes in the US(Jaenson*et al*.,2006). Children under age three are exempted from using PMD because it has not been tested for their age group. Natural lemon eucalyptus oil (leaves essential oil), is another product which is different from PMD and is yet to be tested and approved for its repellent effect (Jaenson*et al*.,2006).

2.5.3.2 Some plant-based repellents and insecticides

The results of studies on repellent that are plant oil based have shown that some of them only repel mosquitoes for just about 120 minutes, so therefore, plant oil repellent like cajuput, lavender citronella, geranium, basil, garlic and peppermint can only be effective for a short period of time (Mississippi State Department of Health, mosquito repellents. types and recommendations. July 30, 2018). Some botanical repellents and insecticides include:

a. Azadirachtin, fig. 2.5 (Schmutterer*et al*., 1980)

b. Lemon grass oil (*Cymbopogon species*)

c. Peppermint oil (*Mentha piperita*) (Patel *et al*., 2012)

d. Pyrethrin, fig. 2.5 (Macan *et al*., 2006)

e. Citral,fig. 2.5 Ganjewala *et al,* 2012

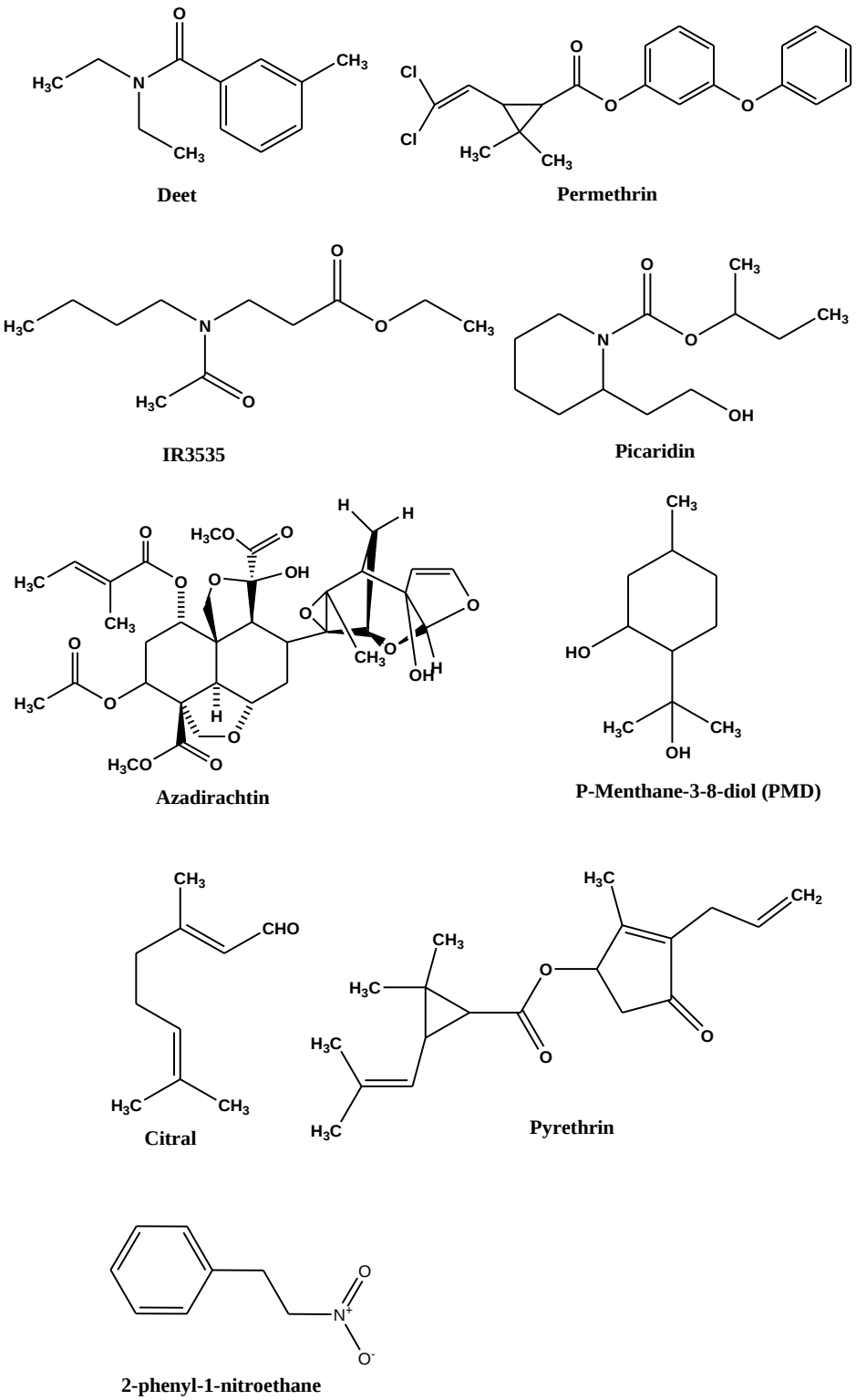


Fig. 2.5: Structures of synthetic and natural repellents.

2.5.3.3 Essential oils as repellent

Essential oils from botanicals have merits such as; low toxicity to animals, biodegradability and insect's specificity, thus they are better alternatives to repellent and insecticides that are synthetic (Amara *et al*., 2018). They are volatile chemicals that contain aldehydes, ketones, ethers, alcohols, terpenoids (Sangwan *et al*., 2001) and they help in protecting plant from insect attack(Bakkali *et al*., 2008).Different species of plants may differ in the repellent or insecticidal effects of their essential oils. While some cause insecticidal effect through contact or ingestion, others like azadirachtin, fig. 2.5 have repellent and feeding inhibition activities (Amaral *et al*., 2018). Neurotoxic effect of some essential oil constituent can also affect respiratory processes and receptors (Fiaz *et al*., 2018a).

Azadirachtin, the active compound in neem seed oil has been established to be responsible for the biological effects of *Azadiracta indica* seeds extract (Mordue *et al*., 2005). Its agricultural crop protection efficacy wasproven during field trials, likewise, its insecticidal and antifeedant effects against variety of insects were established (Mordue *et al*., 2005). Azadirachtin research could be summarized in the international symposium: Natural Pesticide from the Neem Tree (Azadirachta indica A. Juss). Proceeding of the First International Neem conference. Rottach-Egern, Federal Republic of Germany. 16-18 June, 1980 (Schmutterer *et al*., 1981).

The essential oil from lemongrass (*Cymbopogon citratus*)was effective against agricultural pests (Ketoh *et al*., 2000).Citral (fig. 2.5) a monoterpene aldehyde, is the most vital constituent of lemon grassessential oil. It is also present as major component in citrus fruits. Citral possesses many significant bioactivities such as, mosquito repellent, antimicrobial and anti-inflammatory effects (Ganjewala *et al.*, 2012).

The capillary GC and GC/MS analysis of *Dennettia tripetala* leaf oil led to the identification of forty-eight compounds, out of which 2-phenyl-1-nitroethanehas the highest abundancy of 53% (Adeoti *et al*., 2000). The oil was observed to possess appreciable repellent activity against some pests of stored products and mosquitoes (Palsson *et al*, 1999).

2.5.4 Recent advances in mosquito repellent methods

2.5.4.1 Fogging

An example of this method is the thermal fogger, which is different from the cold type. Thermal fogging operates by heating the solution that has coil inside the fogger. This will produce pesticide smoke that is focused in the direction of the mosquitoes. There are gallons of ready-made solutions for fogging which contain piperonyl butoxide and pyrethrin in the ratio 50% and 0.5% respectively. This method is applicable for usage during outdoor activities and in a situation of serious mosquito threat (Patel *et al*., 2012).

2.5.4.2 Mosquito patch

This is an object that contains only thiamine or vitamin B1. This used based on the fact that thiamine is a natural repellent that is most effective till today, as its odour prevents female mosquitoes from landing on surfaces. The patch operates by transferring or administering a substantial amount of thiamine or vitamin B1 into the circulatory system, and the body then eliminates the ingredients through sweats. The so many sweat glands in the body is tantamount to so many spots for the excretion of thiamine (Patel *et al*., 2012).

2.6 Ethnobotanical (traditional) usage of plants

Ethnobotanical information is a powerful tool which have been used in sourcing for and identifying medicinal plants, and it has helped in the isolation of about 122 compounds from plants, of which 80% is been used as drugs today (Fansworth *et al*., 2001). Organised healing system like Ayurveda, kampo and local Chinese therapy have flourished for thousands of years till today because of their organizational strengths which is based mainly on multi-component mixtures (Bannerman *et al*., 1993).

2.7 Collection of plant materials

The effectiveness of plant materials as medicine changes daily with time and also yearly during different seasons. This is because the phytochemicals produce in plants change

quantitatively depending on which period of the year. The dry season is the best and the most common period of collection of plant. At this period, the plant maturity is fully developed and constituents are at optimum concentration (Arnold, 1969). Collection of contaminated material which gives false results must also be prevented (Paris *et al*., 1960). Table 2.2 shows parts of plants and when they are best collected. The process, begins with the collection of plants from various sources depending on the technique of selection. The authentication of plants to their respective family will have to be done by a specialist (Taxonomist). Also, a voucher specimen which gives information about the place of collection, the date, plant parts and name of plant is prepared by using paper sheets to press the plant for a period of time. The information is recorded and kept safe as reference in the herbarium for future purposes.

Table 2.2: Parts of plant and the best period of collection (Paris *et al*., 1960)

Plant parts	Best collection periods
Roots	Period when the underground parts of plant are acquiring food constituents e.g. November to February.
Rhizomes	Period when the underground parts of plant are acquiring food constituents e.g. November to March.
Leaves	When the plant is about to bloom
Flowers	Morning period when the evaporation of dews is done.
Stems and the bark	When the climate is warm
Seeds and pods	When the fruits are fully ripe

2.8 Adequate storage

The storage of some medicinal plants is very necessary because of the restrictions and difficulties experience in getting them. Some of them are available far away on the mountain or in the forest, so accessibility is not easy. Some plant materials may have to be imported from a different country which may take longer time and may be very expensive. Also many plants come out at different period of the year which makes their availability to be limited and thus makes their storage necessary. Phillipson (1982) confirmed that the herbarium material that was stored in paper for several years contained the same chemical metabolites as the fresh plants collected. Therefore, plant materials which are not to be used immediately may be destroyed by microbial attack and other natural processes, so they are quickly dried so as not to be wasted. The plant materials are dried under shade to avoid sunlight, and then placed inside an airtight plastic, bottles or paper. To avoid insects and microbial attack, storage places need to be large, well aired and dry (WHO, 2003).

2.9 Extraction, chromatographic and spectroscopic techniques in the separation of natural products from plants

Different types of extraction methods are employed for different plant material, but aqueous extraction is more popular among practitioners of traditional medicine. Different solvents with different polarities are employed to capture the solubility of various plant ingridients (Szabolcs, 2004). Extraction by cold maceration is still commonly used among researchers because it is a very simple method. It is carried out by soaking the powdered plant in an airtight container using an appropriate solvent at normal room temperature. The solution is stirred regularly to ensure complete mixing of the preparation and speed up the extraction process. Exhaustive extraction is done by following up the first extraction with the addition of fresh solvent to the plant material and carrying out the process again. All the collected liquid extracts are then pooled together and filtered before concentration into the required extract (Szabolcs, 2004).

Other extraction methods are:

1. Percolation and Maceration (Cannel, 1998).
2. Soxhlet Extraction (Zygmunt and Namiesnik, 2003).

3. Extraction under reflux and steam distillation (Ali *et al*., 2008).

4. Microwave Assisted Extraction (MAE), Supercritical Fluids Extraction (SFE) and Pressurized Liquids Extraction (PLE) (Ali *et al*., 2008).

The crude extracts obtained from any of the extraction procedures contain complex mixtures of compounds that need to be separated by various chromatographic techniques at different stages to isolate the bioactive compounds.Planar Chromatography (Thin layer chromatography TLC) is the process of separation of organic compounds when they are placed on adsorbents. The adsorbent could be coated on aluminum glass or plastic sheets (Nyredy 2000a). TLC is the simplest and the least expensive method for natural product isolation. TLC is done by applying prepared sorbent on a plate and activating it at an appropriate temperature. The sample to be separated is then spotted on the origin (The line drawn on the sorbent at the base of the plate). The spotted sample on the origin must be prevented from entering the solvent mixture or mobile phase. The process of development which happen next is the movement of the solvent front to the top of the plate, through capillary action. Retardation factor (Rf) is the factor used to measure the movement of sample compounds over the sorbent with the mobile phase. The Rf formula as shown in equation 2.1:

$$Rf = \frac{Distance\ travelled\ by\ component}{Distance\ travelled\ by\ solvent} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 2.1$$

Other chromatography methods are:

1. Size- Inclusion/Exclusion Chromatography (Robards *et al*., 1994).
2. Partition Chromatography (Robards *et al*., 1994).
3. Ion Exchange Chromatography (Hostetmann *et al*., 1986).

Some of the spectroscopic identification methods employed in the separation of natural products from plant are:

1. Mass Spectrometry (Loos *et al*., 2016).
2. Nuclear Magnetic Resonance (NMR) Spectroscopy (Derome, 1987).

3. Heteronuclear multiple Quantum Coherence (HMQC) and Heteronuclear Single Quantum Coherence (HSQC) Spectroscopy (Reynolds *et al*., 2012).
4. Heteronuclear Multiple Bond Correlation (HMBC) Spectroscopy (Reynolds *et al*., 2012).
5. 1H-1H Correlation Spectroscopy (COSY) (Reynolds *et al*., 2012).

2.10 Thin layer chromatography (TLC) detection of phytochemicals

It is important to do proper visualization during the process of TLC, whether preparative or analytical stage. Loss of product or low recovery may result due to improper detection. A destructive detection does not allow recovery of compounds from the sorbent because of contamination of compounds by the detector, for example, iodine or spray detection, while non-destructive detection allows the recovery of compounds from the stationary phase, for example, Ultraviolet detection (Robards *et al*., 1994)

2.10.1 Ultraviolet detection

In this method, visualization is done using ultraviolet radiation. A compound that is UV-active is incorporated into the stationary phase on the TLC plate and placed under the UV lamp. The spots of compounds on the plate fluorescence with different colours at both the short wavelength (254nm) and the long wavelength (366nm) of the light, depending as well on the class of the various compounds. For example, furocoumarins, when placed under UV, gives out yellow or blue fluorescence (Robards *et al*., 1994).

2.10.2 Spray detection

This method operates through a reaction of the spray reagent with the spots of compounds on the plates to form a change of colours. The spray reagent is introduced into the spraying container as a mixture. Some spray reagents are universal and they form colour reaction with various phytochemicals, as shown in Table 2.3 (Robards *et al*., 1994).

Table 2.3: Some spraying solution for TLC visualisation (Robards *et al*., 1994)

Spray Reagent	Preparation	Treatment	Notes
H_2SO_4/Vanillin	Dissolve vanillin (6 g) in 1.5 mL Conc. H_2SO_4 and 95 ml of 96% ethanol	Spray plates and heat at 100 °C until colouration appears	A universal spray
Phosphomolybdic acid (PMA)	Dissolve 10 g of PMA in 100 mL ethanol (10% weight/volume) solution	Spray plates and heat at 100 °C until colouration appears	Terpenes are detected as blue spots with yellow background
Ammonium molybdate (VI)	10 g of ammonium molydbate (VI) is dissolved in 100 mL conc. sulphuric acid	Heat at 100 °C after spraying till colour shows.	Spray is universal. Diterpenes gives blue colour
Antimony (III) chloride	Antimony (III) chloride is dissolved in a mixture 20 mL glacial acetic acid and chloroform (60 mL)	Spray and heat at 100 °C for 2-5 min until colouration appears	Diterpenes and triterpenes give a red to blue colouration
Tin (IV) chloride	Add Tin (IV) chloride (10 mL) to 80 mL chloroform mixed with 80 mL glacial acetic acid	Spray onto plates and heat at 100 °C for 5 min until colouration appears	Useful for the detection of flavonoids

Table 2.3 cont.

Dragendrorff's reagent	Add 10 mL of 0.85 g of bismuth substrate solution (basic) in 10mL acetic acid with 10 ml of 40% aqueous solution of KI and 50 mL distilled water. This solution is then diluted with acetic acid and water in the ratio 1:2:10	Generally, no heat is required but if reaction is not spontaneous, heat until colouration appear	Detection of alkaloids
2,4 Dinitro-phenyl hydrazine	Dissolve 2,4-dintiro-phenyhydrazine (0.2 g) in NH_4Cl (50 mL)	Nonspontaneous reaction should be heated until colouration appears.	Carbonyl compounds are detected with yellow to red colouration
Perchloric acid	Aqueous solution of perchloric acid (20% weight by volume)	Heat until colouration detection	Spray is universal. Steroids and triterpenes could also be detected
Borntrager reagent	Solution of KOH in ethanol (10% weight by volume)	Heat till colouration detection.	Anthraquinones and coumarins are detected
Ninhydrin	Add 0.3 g Ninhydrin is added to 3 mL acetic acid mixed with 100mL of butanol	Heat until colouration detection	Amines and amino acids are detected. Alkaloids also give red colour

2.11 Mosquito repellent assay

Literatures (Rios *et al*., 1998) have shown that various technique are available for the evaluation of mosquito repellent effect. There is however no one technique that is been employed by all researchers. Some of the methods employed are:

1. Susceptibility Bio-assays (WHO, 1996).
2. Screened Cage Method (Venkatachalam and Jebanesan, 2001).
3. K&D Module Method (Kiun *et al*., 2000).

2.12 Plants utilized in this study

Repellent activity and phytochemical investigations were carried out on six medicinal plants during the course of this research project. The plants studied are *Dennettia tripetala* G. Baker (Annonaceae), *Jatropha curcas* L. (Euphorbiaceae), *Aframomum melegueta* K. Schum (Zingiberaceae), *Citrus limon* Linn. (Rutaceae), *Citrus sinensis* L. (Rutaceae), and *Citrus paradisi* Macf. (Rutaceae).

2.13 Family Rutaceae

In this family, the most vital economically are the genera citrus. Examples are, *Citrus limon* (lemon), *Citrus aurantifolia* (lime) and *Citrus sinensis* (orange). Apart from using them as food crops, some members of the family like genus citrus are also grown or used to decorate the garden. The family has about 2000 species and 160 genera. Many of the species are aromatic and attractive (*Citrus aurantifolia*) (Tamokou *et al*., 2017).

2.14 Citrus plants

The citrus plants belong to the family Rutaceae. They have very fragrant flowers with petals. Their fruits have segment with juice. They produce fruits which are pulpy with thick skin cover. Most of the member of this genus are economically vital and some examples are orange (*C, sinensis*), grapefruit (*C, paradise*), lemon (*C. limon*). The peel of the fruits which is oily, together with the pulp and seeds could potentially be useful products, but they are treated as waste (El-Adawy *et al*., 1999). The fruit species are of

therapeutical importance, they are utilized as confectionary, toiletry, and in the perfume industries. Citrus plants are grown globally, theyare good sources of vitamin c (Knekt *et al.,* 2004), and they also contain phytochemicals such as phenolics (Rossa *et al*., 2000). Their derived products have a lot of therapeutic benefits, therefore immense attention has been put on them (Wu *et al*., 2007).

2.14.1 *Citrus sinensis*

Citrus sinensis, Fig. 2.5, is a member of family rutaceae. They are the most numerous citrus plants and the height of the tree is about 9-10 m. Commonly known as orange, it is generally cultivated for its edible fruits and has been widely studied for the last decades due to its therapeutic potential. The tree of *C. sinensis* have round leaves that are organized alternatively. The normal orange is differentiated from the bitter orange in that the leaves of the former has winged petioles that are narrow, while the latter has leaves with broad winged petioles. The fruits are round shape with layered fleshy pulp with seeds, and the skin could be orange or green yellow. The tree of orange, which could live for about 100 years is about 6-15 meter tall. Its origin is reported to be from Vietnam-China border. Orange nutrients have shown wide varieties of health importance with one fruit reported to provide more than the needed vitamin c daily(Turnerand Burri, 2013)

2.14.2 *Citrus paradisi*

Citrus paradisi, Fig. 2.6, belongs to the Rutaceae family. Second largest citrus cultivar, perennial shrubs or tree, commonly known as grape fruit is a sub-tropical tree with bitter fruits and limited sweetness. (Amita *et al*., 2013). The height of the tree could be up to six meters which sometimes may reach fifteen meters. The thin long leaves are green and glossy and they always contain four white flowers with petals. The fruits which are spheroid in shape always have yellow orange skin (Gupta *et al*., 2011).

2.14.3 *Citrus limon*

Citrus limon, Fig. 2.7, belongs to the family Rutaceae, evergreen flowering tree, 10m in height. Folk medicine uses include leaves infusion, obesity, diabetes, cancer etc. Research has also shown that hypertension could be treated with *Citrus limon* (Avello, 2014). *Citrus*

limon, has been reported to exhibit numerous pharmacological activities (Hiroyuki *et al.*, 2006). Traditionally, citrus plants dry peels are burnt in the living room at night to deter mosquitoes from biting or entering (Ngurukwem *et al*., 2001). The oil of the peel of lemon and orange are used as a nontoxic insecticide treatment (Hofrichter, 2010). Also, in China, *Citrus sinensis* oil tested as the most toxic fumigant among 5 citruses. Citral was shown to be responsible for lethality (Yang *et al.*, 2005).

2.14.4. Pharmacological Activities of some Citrus Plants

2.14.4.1 Anticancer Properties of Citrus plants

The detoxifying enzyme, Glutathione-S-transferase (GST) which aids the conjugation of glutathione with carcinogens was reported to have been largely induced in the liver and mucosa of the intestine, by some citrus limonoids aglycones e.g. obacunone, ichangin, nomilin (Jacob *et al*.,2000).

2.14.4.2 Antimicrobial Properties of Citrus plants

The EtOAc (ethyl acetate) and the acetone peels extract of *Citrus limon*, together with *Citrus sinensis*, exhibited the highest activities against, *Klebsiella pneumonia, Bacillus subtilis, Escherichia coli* and*Staphylococcus aureus*, when tested against the bacteria. Activities were comparable to that of antibiotics like, methicillin and penicillin (Kumar *et al*., 2011).

2.14.4.3 Antioxidant Activity of Citrus plants

When different fruits, seeds and peels of some domestic and imported citrus fruits were tested for their antioxidant activities, the observed results, in the peels and seeds of white grapes and oranges respectively, were the highest (Aleksandra *et al*., 2007).

Fig 2.6: ***Citrus sinensis***https://www.torbaytreefarmers.com/pics/large/27-1.jpg

Fig 2.7: ***Citrus paradise***
http://viverossoler.com//media/images/news/thumbnails/o_1csm1f6ko1gjre9m09rvr1oigu_1200x800.jpg

Fig 2.8: ***Citrus limon***https://www.gardeningknowhow.com/wp-content/uploads/2021/03/lemon-tree.jpg

2.14.4.4 Anti-ulcer activity of citrus plants

The aqueous extract of *Citrus medica* showed a promising antiulcer activity by significantly reducing ulcer formation in ethanol-induced ulcer rats, with activity comparable to standard drug ranitidine. The presence of flavonoids was reported to be responsible for the antiulcer effect (Nagaraju, *et al*., 2012).

2.14.4.5 Hepatoprotective activity of citrus plants

The hepato-protective effect of the essential oil obtained from bergamot orange, tested with carbon tetrachloride (CCl_4) induced hepatotoxic rats, showed that, the oil had significant activity, by reducing the alanine aminotransferase (ALT) level in the serum, as compared with the carbon tetrachloride group. The aspartate aminotransferase (AST) level in the serum and histopathological findings was not affected. The activity was reported to be a weak one (Mehmet *et al*., 2007).

2.14.5 Compounds isolated from *Citrus limon*

Myriad of compounds have been previously isolated from *C. limon*, these isolated compounds are shown in Figure 2.10a and 2.10b. Kim in 2014, isolated and purified two bioactive compounds from ethyl acetate fraction of lemon seeds using silica gel column chromatography. Limonin (14), a furanoside diterpenoid and β-sitosterol (15); a steroid (Kim *et al*., 2014) were isolated and characterised. The isolation of a number of some Aglycone flavonoids, apigenin (16), luteolin (17), chrysoeriol (18), quercetin (19) amongst others was reported, the separation of the compounds separation was carried out using chromatography on silicic acid (Horowitz and Gentili, 1960). Miyake *et al*, 1997isolated two antioxidative C-glucosylflavones from lemon fruits. The two compounds were characterized to be, 6, 8-di-C-α-glucosyldiosmin (20) and 6-C-α-glucosyldiosmin (21), by Infrared, Ultraviolet, Fast Atomic Bombardment-Mass Spectrometry, Carbon-13 and proton NMR analyses. The compounds 20, 21, narirutin, eriocitrin, hesperidin and diosminfrom *citrus limon* were tested for their antioxidative effects using linoleic acid autoxidation method (Miyake *et al*., 1997).

More glycosides with antioxidant activities were isolated from the juice and peels of lemon, and were characterised as eriodictyol 7-rutinoside (22) of the flavanone glycoside using Carbon-13 NMR, Proton NMR and High Performance Liquid Chromatography(HPLC) analyses (Miyake *et al*, 1997). Nishida and Acree, (1984) reported isolation and characterisation of methyl jasmonate (23) and methyl epijasmonate (24) from lemon peels Their identifications were performed using, Mass spectra, 1H NMR, ozonolysis, hydrogenation and acid-catalysed isomerization. A lemon peel sample analysed using gas chromatography was shown to contained 75 mg of methyl jasmonate isomers, of which greater than ninety-five percent of it, is in the form of the thermodynamically less stable methyl epijasmonate.In another report, isolation of four active antipathogen compounds that have potent effects on dental caries and periodontitis microbes, for example, *Prevotella intermedia* and *Streptococcus mutans*was carried out. The four compounds were characterized to be, 8-geranyloxypsolaren (25), 5-geranyloxypsolaren (26), 5-geranyloxy-7-methoxycoumarin (27), and phloroglucinol 1-β-D-glucopyranoside (phlorin) (28) (Miyake and Hiramitsu, 2011).

Potential antioxidative hydroxyflavones, which include: 8-hydroxyhesperetin (29), Cardamidin (6-hydroxynarigenin) (30) and Isocarthamidin (8-hydroxy narigenin) (31) were also isolated from citrus fruits. Cardamidin (30), was a novel compound confirmed using, Carbon-13 NMR, Proton NMR and Fast Atomic Bombardment(FAB-MS) analyses (Miyake *et al*., 2003).

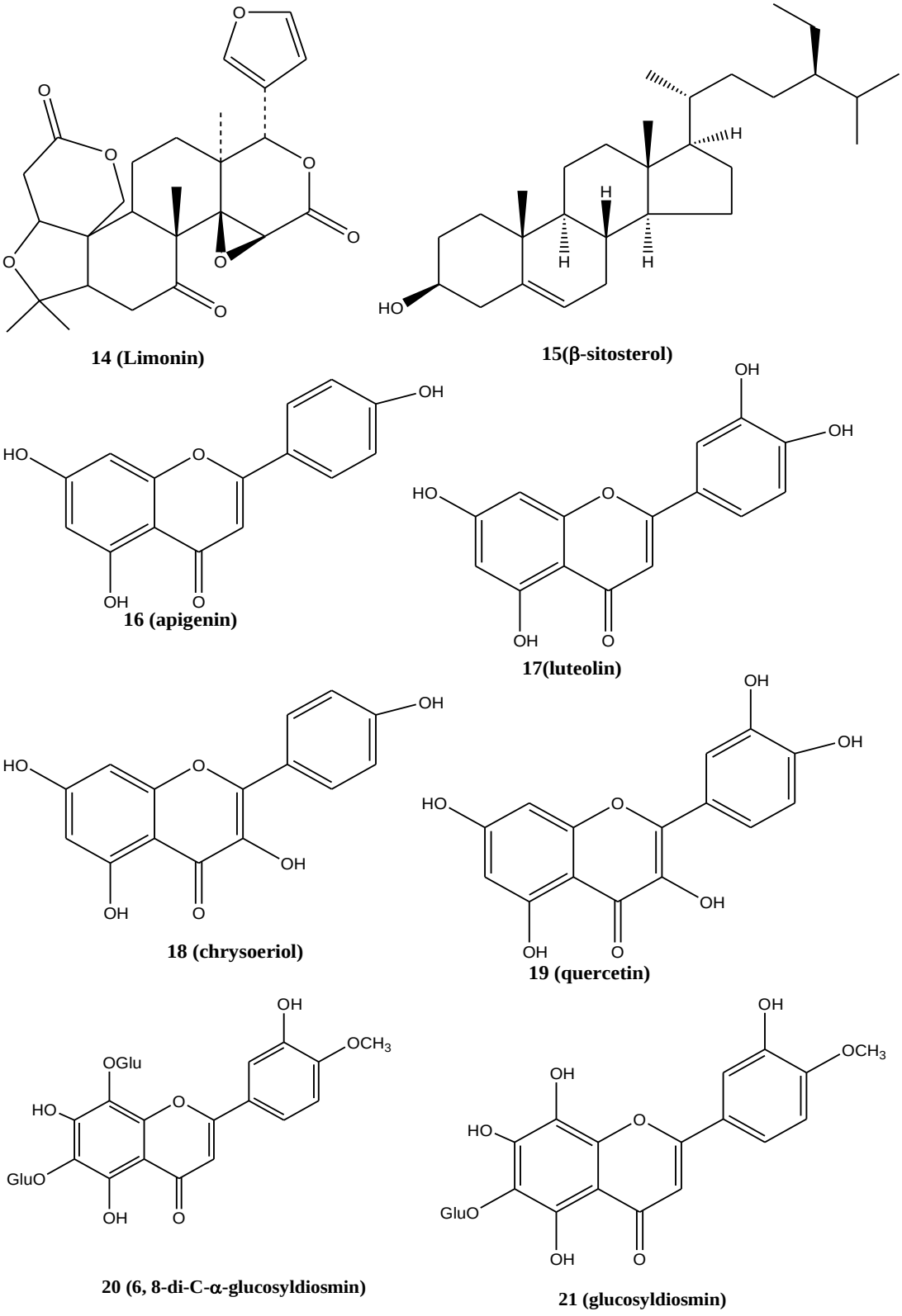
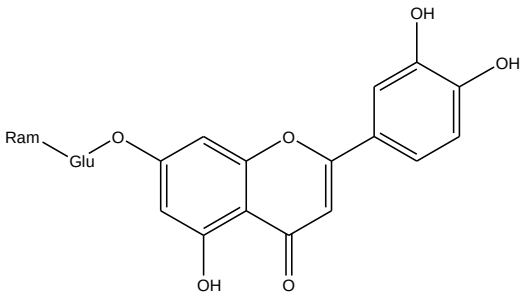
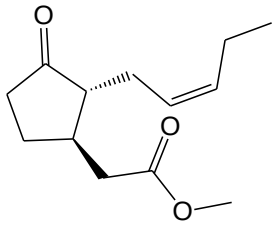


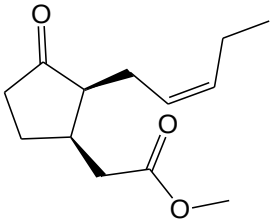
Figure 2.9a: Compounds isolated from *Citrus limon*



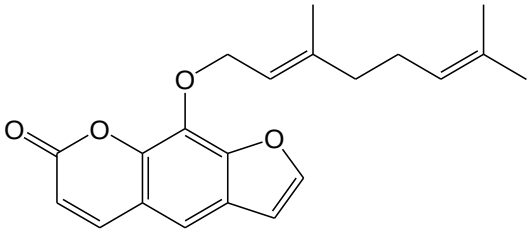
22 (eriodictyol 7-rutinoside)



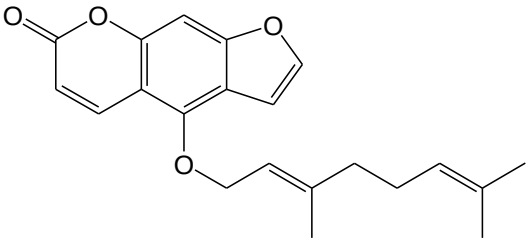
23 (methyl jasmonate)



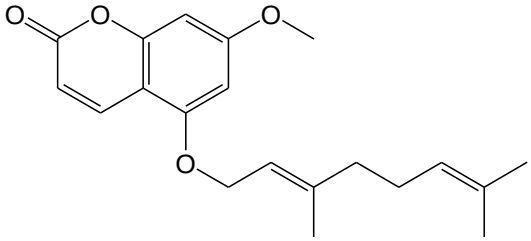
24 (methyl epijasmonate)



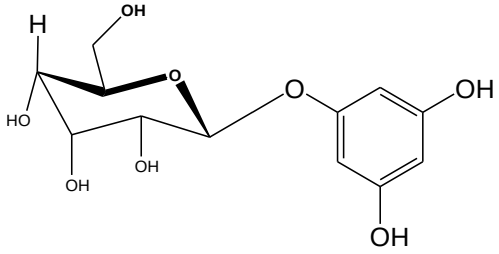
25 (8-geranyloxypsolaren)



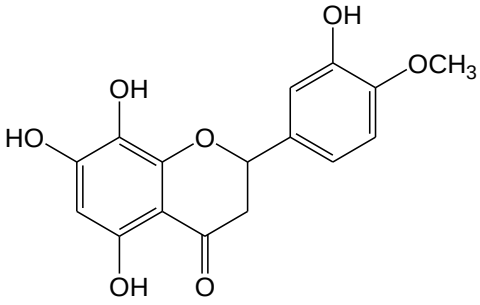
26 (5-geranyloxypsolaren)



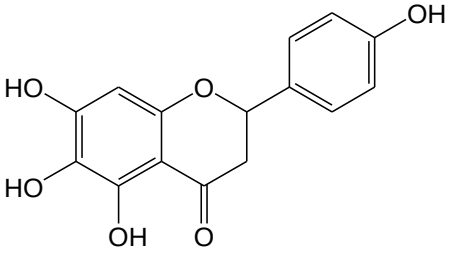
27 (5-geranyloxy-7-methoxycoumarin)



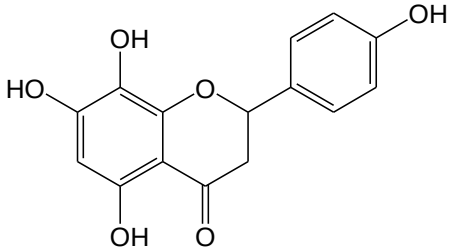
28 (phloroglucinol 1-?-D-glucopyranoside (phlorin))



29 (8-hydroxyhesperetin)



30 (Cardamidin (6-hydroxynarigenin))



31 (and Isocarthamidin (8-hydroxy narigenin))

Figure 2.9b: Compounds isolated from *Citrus limon*

2.15*Afromomum melegueta*

Afromomum melegueta, fig. 2.11, of family Zingiberaceae is herbaceous and can also be referred to as alligator pepper. In ethno medicine, it is used for the treatment of different ailments. Originally, it was grown in West African countries like Nigeria, Ghana, and Cameroun (Rhun *et al*., 2019).

Afromomum melegueta grows to a height of 1.5 m. The flowers are orange in colour and they later grow to form pods, which are about 6 cm long. The pods are eatable and they have many seeds that are reddish in colour, pungent in taste, and have ginger scent. It has short stem which is shielded with scars of leaves. The leaves which have close nerve beneath, are 12 cm wide and around 30 cm long (Rhun *et al*., 2019).

2.15.1. Reported pharmacological activities of *Afromomum melegueta* (AM)

2.15.1.1 Antimicrobial effectof *Afromomum melegueta*

AM has a strong antimicrobial property because it has been used in treating soft tissue and urinary tract infection, which are serious public health problem worldwide (Stamm & Norrby, 2001).

2.15.1.2 Hepatoprotective effectof *Afromomum melegueta*

The water extract of AM seeds has been reported to exhibit hepatoprotective activity, due to its phenolic and flavonoid contents. Their antioxidant effects neutralise free radicals which can cause liver damage (Nwozo & Oyinloye, 2011).

2.15.1.3 Anti-cancer effectof *Afromomum melegueta*

Not much work have been done on anti-cancer flavonoids from Afromomum melegueta, but previously, a study has shown thatthe extracts of AM were potent against pancreatic cancer (Dibwe *et al*., 2012).

Fig 2.10: *Afromomum melegueta*- (OAU, Ile-Ife, Osun State, Nigeria)

2.15.1.4 Anti-inflammatory effectsof *Afromomum melegueta*

Methanol extract and its fractions have been shown to inhibit rat paw oedema induced by egg albumin, thus proving its potential systemic anti-inflammatory activity (Dibwe *et al*., 2012).

2.16*Dennettia tripetala*

Dennettia tripetala also known as pepper fruit, is a member of the annonaceae family, found in the tropical regions of West Africa, where Efik, Niger delta, Ibos and Yoruba people called it, nkarika, imako, mminimi, and igberi respectively. The unripe fruit is greenish while the ripe ones are either red or pink with pepperish, spicy and pungent taste. The consumption could be by eating it raw, through food preparation, and medicinal herbs (Achinewhu *et al*., 1995).The hexanoic extracts of *Dennettia tripetala* has been reported to be active against the larvae of *Aedes aegypti*, with the potency been affected by sunlight and activated under ultraviolet light (Anyaele *et al.*, 2003). Egwunyenga *et al*., in 1998 also reported the repellent activity of the seed powder and solvent extracts of *D. tripetala* against larvae and adults of the leather beetle, *Dermestes maculatus* (F.). Inyang and Emosairue (2005) also reported that aqueous extracts of *D. tripetala* seed elicited antifeedant and repellent activity against the banana weevil, *Cosmopolites sordidus* (Germar). These reports justify the insecticidal activity of the isolated compounds from *D. tripetala*.

2.16.1. Pharmacological studies on *Dennettia tripetala*

2.16.1.1 Anti-sickling properties of *Dennettia tripetala*

The in-vitro anti-sickling activity of the aqueous extract of *Dennettia tripetala*fruits was reported by Okerenta *et al* (2019). The in-vitro red blood cells membrane stabilizing properties was also reported by the same author.

2.16.1.2 Anti-inflammatory effectsof *Dennettia tripetala*

Pepper fruit essential oil relieved inflammation in rodents with oedema, and the effects were comparable with that of dexamethasone, so the oil can be used for reducing body inflammation and pain (Oyemitan *et al*., 2011).

2.16.1.3 Antimicrobial properties of *Dennettia tripetala*

Aderogba *et al*, (2011) reported the in vitro and in vivo growth inhibitory effects of the leaf hexanolic extract of pepper fruit against the rot-causing fungus *Sclerotium rolfsii* in cocoyam. The antimicrobial effect of the leaf extract of pepper fruits has also been reported (Aderogba *et al*., 2011).

2.16.1.4 Bactericidal propertiesof *Dennettia tripetala*

The seed extract of pepper fruit is useful to prevent and inhibit the growth of bacteria and other microorganisms, because study has shown that it has both bactericidal and bacteriostatic purposes (Ogbonna *et al*., 2013). The essential oil and phenolic acid extract also exibits inhibitory activities against *Staphyloccocus aureus*, *Salmonella sp*., and *Escherichia coli* (Ejechi, 2012).

2.16.1.5 Post-partum care

The spices of pepper fruit help in uterus contraction (Okwu and Morah, 2014, Achinewu *et al*., 2013), so the seeds are useful in making soup for newborn mothers (Achinewu, 1995).

2.16.1.6 Anti-ulcer properties of *Dennettia tripetala*

The anti-ulcer effect of pepper fruit seeds ethanol extract was evaluated against aspirin-induced ulcer. The result showed potent and dose-dependent activities. Flavonoids in the pepper fruits were responsible for the anti-ulcer effect (Anosike *et al*., 2016).

2.16.1.7 Anti-glycaemic properties of *Dennettia tripetala*

The lowering effect of*D. tripetala*on glucose level in the plasma of drug-induced hyperglycemic rats is comparable to the level in the normal rats (Ejechi, 2012).

2.16.1.8 Anti-oxidant properties of *Dennettia tripetala*

Comparative antioxidant effects of the unripe pepper fruit aqueous extract and the ripe ones has shown the unripe plant to be more potent, due to its higher inhibition of 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), hydroxyl (OH) and 1,1-diphenyl-2 picrylhydrazyl (DPPH). It also has greater Fe-chelating and reducing potentials (Aderogba, 2011).

2.16.1.9 Glaucoma Intraocular Pressure (IOP) Reduction

Pepper fruit has been shown to significantly reduce intraocular pressure (IOP) up to 25% in people suffering from glaucoma (Ejechi, 2012).

2.16.1.10 Insecticidal properties of *Dennettia tripetala*

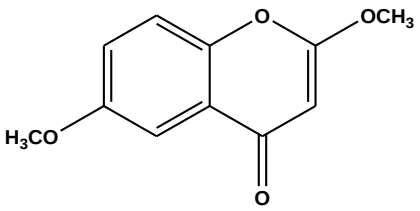
Dennettiatripetala belongs to family Annonaceae which has various members that contain compounds with antifeedants, repellents, and insecticidal properties (Odeyemi et al., 2008). Some solvent extracts of *D. tripetala* have been reported to have activity against different mosquito stages. For example, *Dennettiatripetala*hexanoic extract was effective against the larvae of Aedes aegypti, with the potency being affected by sunlight and activated under ultraviolet light (Anyaele, 2010).Akinbuluma *et al*. (2015) evaluated the insecticidal effect of pepper fruit against *Sitophilus zeamais* (Motsch) and maize weevil, these activities were attributed to the pungency and pepperish nature of plant.

2.16.2 Compounds isolated from *Dennettia tripetala*

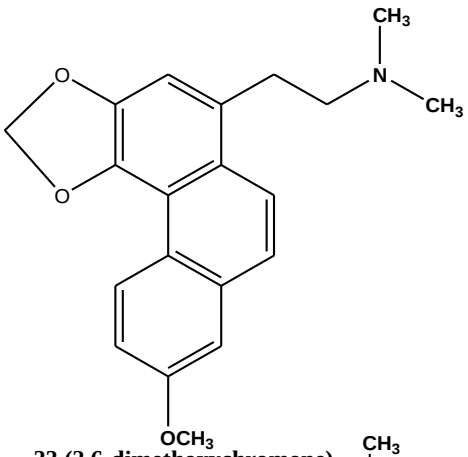
Some of the previously isolated compounds from *D. tripetala* are shown in Figure 2.12. Javier *et al*. (2002) isolated a flavone; Dennettine (32), a new 2,6-dimethoxychromone and three known phenanthrene alkaloids namely; Uvariopsine (33), Stephenanthrine (34), Argentinine (35) and Vanillin (36) were isolated from the roots of *D. tripetala*. Javier *et al*., 2002, did their structural elucidation using one and two dimensional nuclear magnetic

resonce and HMBC.A phenanthrene alkaloid: Ovariopsin (33)was also isolated and the structure determined, from *D. tripetala* ethanolic extract(Morah, 2005).

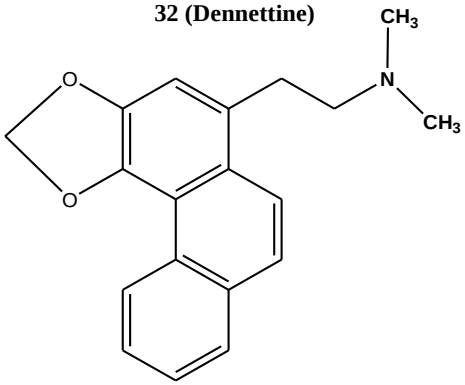
A bio-guided antioxidant isolation and characterization investigation was done onEtOAc fraction from (1:5) aqueous/methanol extract of*Dennattia tripetala*leaves to identify its active constituents, using, DPPH (1,1-diphenyl-2-picrylhydrazine)..Proton and carbon 13 nuclear magnetic resonance, along with Electrospray Ionization Mass-Time-of-Flight spectrometry (ESI-TOF-MS) were used to identify and characterized both, quercetin-3-O-arabinofuranoside (37) and vitexin-2''-O-rhamnoside (38), two flavonoid glycosides (Aderogba *et al*., 2011).2-phenyl-1-nitroethane (39) with good repellent effect against mosquitoes was also isolated from *Dennettia tripetala* (Adeoti *et al*., 2000).



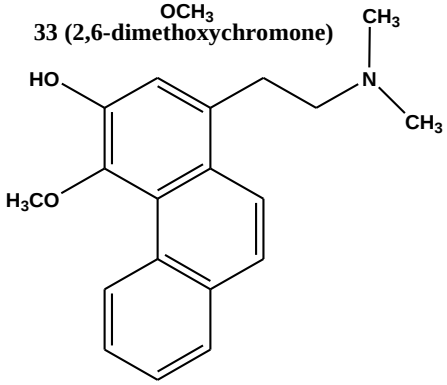
32 (Dennettine)



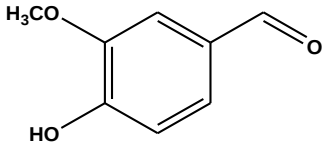
33 (2,6-dimethoxychromone)



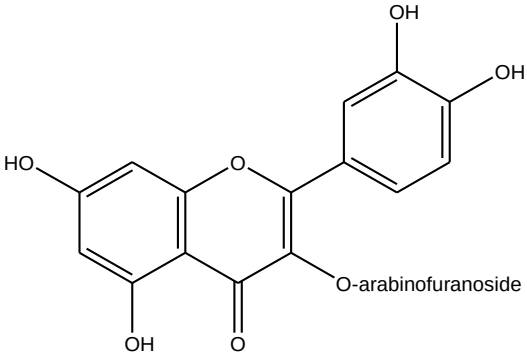
34 (Stephenanthrine)



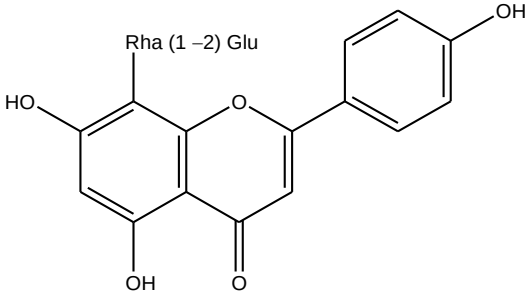
35 (Argentinine)



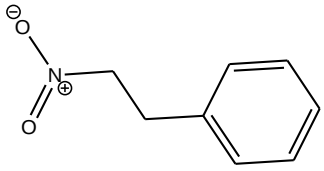
36 (Vanillin)



37 (quercetin-3-O-arabinofuranoside)



38 (vitexin-2''-O-rhamnoside)



39 (2-Phenylnitroethane)

Figure 2.11: Compounds isolated from *Dennettia tripetala*

2.17*Jatropha curcas*

Jatropha curcas L. Fig. 2.13, belongs to Euphorbiaceae family. It is also called physic nut or purging nut in English. Its genus, called Jatropha contains about 170 known species. Jatropha curcas is planted as fence globally, because animal do not browse it and it grows to a height of about 5 meters (Reinhard, 2007). Jatropha curcas originated from Central America, but it is now cultivated in almost all tropical and subtropical countries as protection hedge around gardens and fields (Reinhard, 2007). Ethno-medicinal uses of Jatropha curcas include treating medical conditions like: yellow fever, cough, ulcers, inflammation, jaundice and pneumonia (Setty *et al*., 2013).

2.17.1. Pharmacological activity of *Jatropha curcas*

2.17.1.1 Anti-inflammatory activity of *Jatropha curcas*

Jatropha curcas root methanol extract showed anti-inflammatory effect on carrageenan-induced rat paw edema. Activities was also exhibited on other anti-inflammatory models. Treatment was done orally and activities was reported to be due to prostaglandin formation from the effects of extracts on several mediators and the metabolism of arachidonic acid involving cyclo-oxygenase pathway (Mujumdar *et al*., 2004).

2.17.1.2 Anti-metastatic effects of curcusone B

Curcusone B is a diterpene isolated from *Jatropha curcas*. At non-cytotoxic doses, it exhibited an in vitro Anti-metastatic effects on human cancer cell lines, by reducing the invasion,motility, and secretion of matrix-metalloproteinases of the cancer cells (Muangman *et al*., 2005).

Fig 2.12: *Jatropha curcas*- (Olode village, Adegbayi, Ibadan, Nigeria)

2.17.1.3 Coagulant and anticoagulant activity of *Jatropha curcas*

Jatropha curcas was confirmed when whole latex significantly ($p<0.01$) reduced the blood clotting period, while the latex that was diluted, and the one of high dilution gave prolonged clotting time and no clotting at all, respectively. The butanol fraction had better anticoagulant activity than the ethyl acetate fraction, while the effect of aqueous fraction was reported as insignificant (Osoniyi *et al*., 2003).

2.17.1.4 Insecticidal properties of *Jatropha curcas*

Serial dilutions were done on *Jatropha curcas* seed oil to produced concentrations ranging from 0% to 2% (v/w) at 0.5% intervals. The doses were tested for their long-term protective and anti-ovipositional effects on treated cowpeas against*Callosobruchus maculatus*. At all concentration 2% (v/w), *Jatropha curcas*seed oil significantly ($p< 0.05$) reduced ovipositional activity of *Callosobruchus maculatus* against cowpea. Three months' protection was also observed on the treated seeds due to no seed damage and non-adult insect emergency in treated seeds (Adedire *et al*., 2003).

2.17.1.5 Pregnancy terminating effect of *Jatropha curcas*

The abortifacient properties of Jatropha curcas fruit was observed at an early stage of pregnant rat. After implantation, the methanol, petroleum ether and dichloromethane extracts administered on pregnant rats was done orally, loss of body weight, ranging from slight to severe body weight loss was observed during the dosing period. (Goonasekera *et al.*, 1995).

2.17.1.6 Antidiarrheal activity of *Jatropha curcas*

In albino mice, the methanol fraction of *Jatropha curcas* root extract showed antidiarrheal activity against castor oil induced diarrhea and intraluminal accumulation of fluid, after successive extraction of the fluid content. Also, a charcoal meal administration was performed, in which gastrointestinal motility was reduced. These effects were ascribed to both intestinal reduced propulsive movement and the inhibition of elevated prostaglandin biosynthesis (Mujumdar *et al*., 2000).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Ethical Approval for repellent assay using human volunteers

Ethical approval was obtained from the College of medicine, University of Ibadan, Nigeria's Institute for Advanced Medical Research and Training (IAMRAT), with registration number NHREC/05/01/2008a and Ethics Board assigned number UI/EC/17/0308.

3.2 Materials

The solvents used were analytical grade and included methanol, dichloromethane, n-Hexane, chloroform, ethyl-acetate, acetone, and dimethyl sulfoxide, Silica gel (kieselgel Merck 200-400 mesh) was used for column chromatography and pre-coated TLC aluminium plates (20×20 cm) was employed for thin layer chromatography. The Thin Layer Chromatography were visualised under short and long wavelength ultraviolet light at (254 and 365 nm respectively), vanillin and sulphuric acid reagent and inside iodine vapour.

The Bruker Avance Spectrophotometer at the temperature of 25 $^{\circ}$C in methanol (MeOD-*d*), chloroform ($CDCl_3$-*d*) and dimethyl sulfoxide (DMSO-d_6) was used to conduct the proton and carbon-13 NMR experiments, at frequencies 600 MHz and 150 MHz. The internal reference used was Tetramethylsilane (TMS), while all chemical shifts were measured in ppm.

The Fourier Transform Infrared spectrum was recorded on PerkinElmer spectrophotometer. Reichert Austria 281313 model was used for the melting point. The Mass spectra; high and low resolutions were recorded on JEOL-JMS 600-H spectrometer.

The brine shrimp lethality assay was conducted using Artemia salina eggs.

3.3 Mosquito collection and culture

Adult female Anopheles gambiae mosquitoes (5-6 days old) which have not been blood-fed for 24 hours were used for the repellency assay (Dua *et al*., 1996). The positive and negative controls were DEET and acetone.

Mosquito larvae were collected from various habitats in Ibadan metropolis, Nigeria, and were authenticated at the Zoology Department of the Science Faculty, University of Ibadan, Nigeria. They were left to hatch into adults inside the (30×30×30 cm) netted cage, at the Insectary of the Department of Zoology, University of Ibadan, Nigeria. Full-grown female mosquitoes were maintained on 10% sugar solution at 27±2 °C and 68±2% Relative humidity.

3.4 Reagents for cell lines and culture

Originally, Cell lines (HeLa, SH-SY5Y) were acquired from the European Collection of Authenticated Cell Cultures. Cell culture reagents and viability materials were procured from Thermo Fisher Scientific; Dulbecco's Modified Eagle Medium (DMEM), L-glutamine, penicillin/streptomycin/amphotericin B antibiotic-antimycotic solution (anti-anti), phosphate-buffered saline (PBS), recombinant trypsin (TrypLE), alamar blue, from Sigma-Aldrich (Foetal Bovine Serum (FBS), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)). Specialised plates were obtained from Greiner Bio-One (UK).

3.5 Cell culture and treatments

Cells were cultured as adherent monolayers in growth medium comprising, FBS, glutamine, anti-anti solution and a modified eagle medium. They were grown in tissue culture (T25) flasks in a saturated vapour atmosphere of 37 °C and 5% carbon (IV) oxide in an incubator. When they reached 80% confluency the growth medium was pour out, the cultures were rinsed with PBS which was then removed before trypsinising the culture. The medium was used to submerged the cells and the suspension was triturated to break up any cell clumps and achieve a single cells suspension before cell mass was determined with the aid of haemocytometer-assisted counting under a microscope. The density was then adjusted to 7.5 x 10^4 cells/milliliter and 100 µL of the suspension was transfered into a sterile microclear 96-well plate (7500 cells/well).

The plate was placed in the incubator for 24 h before treatment with samples Dilutions of extracts were prepared from their DMSO stock solutions using the growth medium. Cultures were then treated with the different concentrations of the extracts for 48 h. Negative control cultures treated with the vehicle only (DMSO) were included. Each treatment was done in triplicate. The positive control utilized was penicillin/streptomycin/amphotericin B antibiotic-antimycotic solution (anti-anti). The concentration of DMSO to which cultures were exposed did not exceed 0.1% v/v (Fatokun *et al*., 2013).

3.6 Plant collection, authentication and preparation

3.6.1 *Dennettia tripetala*

Fresh fruits of the *D. tripetala* were purchased from Benin, Edo state of Nigeria, and was authenticated at Forest Research Institute of Nigeria (FRIN) Herbarium, Jericho, Ibadan Nigeria, with voucher number: FHI-112112. It was dried for two weeks, and then pulverized into fine powder thereafter stored inside the deep freezer before extraction.

3.6.2 *Afromomum melegueta*

The fruits of *A. melegueta* were bought at a local market in Ore, Ondo state of Nigeria. The identity was confirmed at the herbarium section of the Department of Botany, Obafemi Awolowo University (OAU), Osun state of Nigeria. The voucher number was: IFE-17741. The fruits were air-dried under shade for a fortnight period and then grinded into powder, which was store inside the deep freezer pending extraction.

3.6.3 *Jatropha curcas*

Dried and opened fruits of *Jatropha curcas* were gotten from Olode Village, Adegbayi, Ibadan, Nigeria. Identification was done at the Herbarium, Department of Botany, Obafemi Awolowo University (OAU), Osun state of Nigeria. The number for the voucher was: IFE-17740. The seeds were separated from the fruits and shade-dried for a fortnight period. They were pulverized and then stored at room temperature until the time of extraction.

3.6.4 *Citrus limon*

Seeds of *Citrus limon* were purchased from juice seller at a market (Oje) in Ibadan, Nigeria and identified atFRIN, Jericho, Ibadan, Nigeria, with voucher number: FHL112112. The seed were dried under shade for a fortnight, ground and was stored inside the deep freezer until the time of extraction.

3.6.5 *Citrus sinensis*

Fruits of orange were gotten from Bodija market, Ibadan, Oyo state of Nigeria. Identification was done at the Federal Research Institute of Nigeria (FRIN), Jericho, Ibadan, Oyo state of Nigeria, with voucher number: FHL112111. The seed were removed from the fruit, air-dried under shade for a fortnight, and then ground into powder. It was then stored inside the deep freezer until the time of extraction.

3.6.6 *Citrus paradise*

Grapefruits were gotten from Bodija market, Ibadan, Nigeria. Identification was done at the Federal Research Institute of Nigeria (FRIN), Jericho, Ibadan, Nigeria, with voucher number: FHL112113. The seeds were separated from the fruit and air-dried under shade for a fortnight period. It was then grinded into powder and stored inside the deep freezer until the time of extraction.

3.7 Drying

All plant materials were dried in a room equipped with an air conditioner and the plant parts were spread at interval. The dried plant parts were ground on trap grinding machine TRF 80 with different sieve sizes to separate bigger plant marc from the powdered ones. The powder collected were stored inside sample bottle in the fridge pending extraction.

3.8 Extraction of plants materials

The air-dried and pulverized seeds (800 g) of *C. limon, C. paradise, C. sinensis*, and *J. curcas* were taken through extraction using, ethyl acetate, hexane and methanol, by soaking each plant materials in the appropriate solvent for a period of three days. Rotary evaporator (Buchi R215, USA) was used in concentrating the extracts, which were further kept in the vacuum desiccator at 40 °C prior to use.

Also, air-dried and pulverised fruits (850 g) of *A. melegueta* and *D. tripetala* were cold-macerated using, n-hexane, ethyl acetate and MeOH in successive order to obtain the respective crude extract samples. The concentration of the extracts was carried out using rotary evaporator (Buchi R215, USA) at 40°Cand they were preserved in the refrigerator (4 °C) prior to use (Avila *et al.*, 2021).

3.9 Thin Layer Chromatography (TLC)

Analytical TLC was done on a commercial pre-coated plates of silica-gel GF254 0.25 mm thick (Merck Ltd, Germany). The developing solvents for the TLC plates were chosen from different and varying solvent systems ratio of increasing polarities as shown in Table 3.1.

3.9.1 TLC Visualisation

The analytical plates were mostly visualised using spray reagents (vanillin and sulphuric acid), UV-Visible light and iodine vapour.

3.10 Identification tests for the extracts

Initial screening for the phytochemicals was done on the crude extracts to identify different classes of phytochemical present in them. This was carried out using approved techniques by; Trease and Evans (1989) and Harborne (1998). The tests carried out are hereby outlined.

3.10.1 Tannins

200 mg of each sample was dissolved in distilled water. This was mixed very well and filtered. About 0.2 mL of $FeCl_3$ was added after filtration. The appearance of a blue-green or blue-black precipitate indicated or confirmed that Tannin was present (Trease and Evans, 1989).

3.10.2 Saponins

400 mg of each of the sample was mixed very well with distilled water. It was heated until boiling point is reached and then filtered. Distilled water (about 20 mL) was added to the filtrate and then shaken strongly for sixty seconds. This was left at room temperature for half an hour. Continuous foaming confirmed that saponins was present (Trease and Evans, 1989).

3.10.3 Anthraquinones

0.5 mL of the sample was mixed with benzene and the resulting solution was properly mixed until precipitation occurred. The precipitate was filtered. To the filtrate, 100% ammonia was introduced and then properly shaken. The appearance of violet, red or pink colouration in the water layers showed that anthraquinone was present (Harborne, 1998).

3.10.4 Alkaloids

3.10.4.1 Dragendroff Test

0.5 g of each sample was mixed with 10 mL of one percent dilute hydrochloric acid. This was warmed for 120 second and filtration was done. Four drops of Dragendroff's reagent (potassium bismuth iodide solution) were introduced. The formation of brown colouration with turbidity showed that alkaloid was present (Harborne, 1998).

3.10.4.2 Mayer Test

0.5 g of each sample was mixed with 10 mL of one percent dilute HCl. This was warmed for 120 second and filtered. 1 mL of Mayer's solution was added. The formation of reddish brown colour showed that alkaloid was present (Harborne, 1998).

3.10.4.3 Wagner Test

0.5 g of each sample was mixed with 10 mL of one percent dilute HCl. This was warmed for 120 second and filtered. Two milliliter volume of Wagner's solution was mixed with the filtrate. The formation of red colouration showed that alkaloid was present (Harborne, 1998).

3.10.5 Cardiac glycosides Test

About 0.5mL of each extract was mixed with acetic anhydride (2mL). The mixture was placed in ice to cool down. Few drops of conc. H_2S0_4 was then introduced. The changes of colour to blue from violet and then, to green showed the presence of steroidal nucleus. This cardiac glycoside part is called aglycone (Trease and Evans, 1989).

3.10.6 Flavonoids test

Distilled water was mixed about 0.5 mL of each extract. This was stirred and then filtered. Some drops of Concentrated potassium hydroxide were added to the filtrate. The formation of deep yellow colour showed that flavonoid was present (Harborne, 1998)

3.10.7 Test for steroid (Salkowski's test)

About 0.5 g of the extract was dissolved in about 2 mL of chloroform. Concentrated sulphuric acid (H_2S0_4) was added carefully, forming a lower layer. The formation of reddish-brown colour in between the layers showed that steroidal ring was present (Trease and Evans, 1989).

3.11 Column Chromatography for *Citrus limon*

Sample: Hexane extract of *C. limon*

Length of column: 45 cm

Diameter of column: 5 cm

Silica gel mass: 150 g

Extract loaded: 10 g

Type of Sillica gel: 200-400 mesh (Kieselgel Merck)

Glass column were packed with silica gel (200-400 mesh) in a ratio of 1.5 g of extract to 10 g of silica gel. The column size was highly dependent on the amount of material to be separated.

3.11.1 Fractionation of the hexane extract of *Citrus limon*

The hexane extract (18 g) of the seeds of *C. limon* was adsorbed on 30 g of silica gel (200-400 mesh) and introduced into a column packed with the same adsorbent (150 g) in hexane. The column was eluted using a step gradient of hexane, ethyl acetate, and methanol, starting with 100% hexane stepped to 10, 20, 30, 40, 60, 80, 100% ethyl acetate, 50% methanol in ethyl acetate, and 100% methanol (Weiming *et al*., 1982). 100 mL of each fraction was collected. The procedure was repeated three times to build up the yield. The fractions obtained were coded as CLE1-CLE10 and were all screened for their repellent activities. All fractions with percentage repellencies of greater than or equal to 70% were considered for further purification in order to isolate the most active compounds.

3.11.2. Purification of CLE1 to give compounds a and b

The fraction coded CLE1 obtained from step gradient column chromatography of the n-Hexane extract at mobile phase of 100% hexane was further subjected to column chromatography (CC) on silica gel. 5 g of CLE1 was dissolved in dichloromethane and adsorbed on 5 g of silica gel inside a glass Petri dish. The petri dish was covered with perforated foil paper and the solvent was evaporated overnight at room temperature. The dried powder obtained was introduced into a column containing the stationary phase in hexane. Elution was done using a mixture of hexane/ethyl acetate (100:0) and (95:5). 568 fractions (20 mL each) were collected and pooled into two fractions 1-235 and 236-568, coded CLE1* and CLE2* respectively. CLE1* and CLE2* were also screened for repellency activities. CLE1*, which showed four (4) spots on TLC, and better repellency activity was purified further with a column, using 100% hexane and 2% ethyl acetate in n-hexane to yield two white compounds coded compound a and b.

3.11.3 Purification of CLE2

The fraction CLE2 eluted with 10% ethyl acetate in hexane (10:90) was a whitish yellow oily paste that showed four (4) major spots on TLC plate. This fraction (4 g) was loaded on silica gel column and eluted using a mixture of 7.5% ethyl acetate in n-hexane as the mobile phase. A total of 158 fractions, 20 mL each were collected and combined into four sub-fractions labeled CLE2-A to CLE2-D, based on the TLC profile. These sub-fractions were tested for their repellent activities, and sub-fractions CLE2-C (1.35 g) and CLE-D (0.86 g) that showed 67.50% and 70.50% activities respectively were further purified to give compound c, d and e

3.11.4 Isolation of compounds c and d from CLE2-C

The sub-fraction CLE2-C (1.35 g) that showed six (6) spots on TLC plate was subjected to both preparatory TLC and mini-column for purification. The sample was prepared with dichloromethane and spread on the origin of the prep TLC plate (3 plates were used). The plates were developed inside the TLC tank using a mixture of acetone in n-hexane (20:80). Eight bands were collected from the three plates altogether, their TLC profiles were done and the bands with similar profile were pooled together to give 2 sub-fractions CLE2-Ca and CLE2-Cb that weighed 0.90 g and 0.25 g respectively. The sub-fraction CLE2-Ca that weighed 0.90 g, and showed three (3) spots on TLC was later purified with a mini-column, using 100% hexane and 0.5 % acetone in hexane to yield compound c and d.

3.11.5 Isolation of Compound e from CLE2-D

The sub-fraction CLE2-D (0.86 g) that showed three (3) spots on TLC plate was subjected to both preparatory TLC and mini column for purification: The sample was prepared with dichloromethane and spread on the origin of the prep TLC plate (3 plates were used). The plates were developed inside the TLC tank using a mixture of acetone in n-hexane (20:80). Three bands were collected from the three plates, the TLC was done and the similar ones pooled together to give one sub-fraction that weighed 0.85 g. This sub-fraction that showed one (1) major spot on a TLC, was purified further with a mini-column using 0.5% acetone in hexane (A/H) to yield compound e.

3.12 Vacuum Liquid Chromatography (VLC) fractionation of *Dennettia. tripetala* fruits ethyl acetate crude extract

Sample: Ethyl acetate extract of *Dennettia tripetala*

Sintered glass funnel Length: 10 cm

Sintered glass Diameter: 5 cm

Mass of Silica gel: 70 g

Mass of Extract loaded: 5 g

Type of Silica gel: (60-200 µM) mesh (Kieselgel Merck, Germany)

Sintered glass funnel was packed with silica gel (60-200 µM) mesh size in a ratio of 1 g of extract to 14 g of silica gel. The funnel size was highly dependent on the amount of material to be separated.

3.12.1 Fractionation of the active ethyl acetate extract

The ethyl acetate extract (5 g, semi solid) was pre-absorbed with silica gel, left exposed at room temperature overnight and then subjected to Vacuum Liquid Chromatography on TLC-grade silica gel (60-200 µM) mesh for fractionation. The solvents used were n-hexane, ethyl acetate and methanol. The gradients of the mobile phase elution involved: n-hexane in ethyl acetate mixture and then ethyl acetate in methanol mixture. Eleven fractions labelled F1-F11 (100% n-hexane, 10, 20, 30, 40, 50, 60, 80% ethyl acetate in n-hexane, 100% ethyl acetate, 50% methanol in ethyl acetate and 100% methanol) were collected. The fractions were spotted on pre-coated plates of silica gel GF254 (20 x 20, 0.5 mm thick; E. Merck) using capillary tubes. The spotted TLC plates were developed with the best solvent mixtures (mobile Phases) of 20% and 30% acetone in n-hexane. Similar fractions were pooled together based on their Rf to give four sub-fractions labelled: DTH1 (Hexane), DTH2 (Hexane), DTEA (Ethyl acetate) and DTM (Methanol). The four fractions were all evaluated for their mosquito repellent effects.

3.12.2 Purification of *Dennettia tripetala* ethyl acetate fraction (DTEA) to give compounds f and g

The sub-fraction coded DTEA obtained from the vacuum liquid chromatography of the active ethyl acetate, with 77.20% repellent activity was further subjected to vacuum liquid chromatography (VLC) on silica gel. 5 g of DTEA was dissolved in dichloromethane and adsorbed on 5 g of silica gel inside a glass Petri dish. The petri dish was covered with perforated foil paper and the solvent was evaporated overnight at room temperature. The dried powder obtained was introduced into a sintered glass funnel packed with TLC grade silica gel, and eluted with a mixture of n-hexane/ethyl acetate, starting with 100% hexane, with an increment of 5% ethyl acetate, until 100 % ethyl acetate. Fourteen (14) fractions (F1-F14) were collected and combined into three fractions (DTEA1, DTEA2 and DTEA3) using their TLC profiles. The DTEA2 which showed two (2) major spots on TLC, and best repellent activity was purified further with preparatory thin layer chromatography (PREP TLC) to yield two brownish compounds coded compounds f and g.

3.13 Bioassays

3.13.1 Repellency Bioassay for the extracts

Human bait technique (Dose-percentage protection relationship) (Murugan *et al*., 2003), which simulates skin condition to which repellents would be eventually applied was used. 5-6 days old, 50 blood starved mosquitoes were kept in the cage (30×30×30 cm) for each test. The untreated forearm of the volunteer was inserted to confirm mosquito readiness to bite (when about 10 mosquitoes were observed landing within 30 secs), and the hand was removed. The arm was then covered with a glove, with only about 35 cm^2 dorsal side exposed. Acetone (Control) was applied to the exposed skin first, dried for 1min, and dipped inside the cage. The number of mosquitoes that landed (shaken off), over 5 min period, at every 15 min break was observed and noted. Each extracts at 1.5, 2.5, and 5 mg/mL was then applied separately, starting with the lowest concentration (Venkatachalam & Jebanesan, 2001). Three (3) replicates were done for each test. Tests were conducted at room temperature and normal atmospheric pressure. Percentage repellency was calculated using formula from Schreck *et al*, (1977) as shown in equation 3.1.

$$\%\ repellency = \frac{Ta - Tb}{Ta} \times 100 \quad ---------- \quad 3.1$$

Ta = Number of mosquitoes in the control, Tb=Number of mosquitoes in the treated

3.13.2 Repellency bioassay for fractions

Human bait technique (Dose-percentage protection relationship) (Murugan *et al*., 2003;), which simulates skin condition to which repellents would be eventually applied was used to determine the repellency bioassay for the various fractions obtained from the extracts. 5-6 days old, 50 blood starved mosquitoes were kept in the cage (30×30×30 cm) for each test. The untreated forearm of the volunteer was inserted to confirm mosquitoes' readiness to bite (when about 10 mosquitoes were observed landing within 30 secs), and the hand was removed. The arm was then covered with a glove, with only about 35cm^2 dorsal side exposed. Acetone (Control) was applied to the exposed skin first, dried for 1min, and dipped inside the cage. The number of mosquitoes that landed (shaken off), over 5min period, at every 15min break for 2 h were observed and noted. Each fraction at an optimized dose of 5mg/ml was then applied separately (Venkatachalam and Jebanesan, 2001). 3 replicates were done for each test. Tests were conducted at room temperature and normal atmospheric pressure. Percentage repellency was calculated using formula from (Schreck *et al*., 1977), presented in equation 3.1.

3.13.3 Cytotoxicity assay

Brine shrimp lethality test

The method of McLaughlin *et al*. (1991) was used. This process was done for the hexane fraction (100%), which was the most active fraction of the *C. limon* seeds n-hexane extract and the isolated compounds.

3.13.3.1 Hatching of Eggs

The seawater collected from the Bar Beach, Lagos was transferred into a soap case that has been divided, using a slide, to make a dam. The case was half covered. Brine shrimp eggs were added into the covered (dark) side and the whole case was exposed to light. The hatching of eggs to larva occurred after 2 days and the larva moved to the open or exposed side of the case, they were attracted to light (phototaxis).

3.13.3.2 Preparation of Samples

The samples utilized for this study were n-hexane fractions (100%) and isolated compounds. The samples were prepared in concentrations of 1000, 100 and 10 µg/mL.

The stock solution was prepared by weighing 0.002 g (2 mg) of either fraction or isolated compound and dissolving in 2 mL of dimethyl sulfoxide in a test tube (labelled as test tube A) to give a 1 mg/mL solution. From this, 0.5 mL was taken and added to 3 separate test tubes. To each of the test tubes, 4.5 mL of seawater containing 10 nauplii (Artemia salina) were added using a micropipette making the drug concentration now 1000 ppm.

From the stock solution (test tube A), 0.2 mL was taken and 1.8 mL of sea water was added into a test tube (labelled as test tube B). From this, 0.5 mL was taken and added to 3 separate test tubes. To each of the test tubes, 4.5 mL of seawater containing 10 nauplii were added using a micropipette making the drug concentration now 100 ppm.

From test tube B, 0.2 mL was taken and 1.8 mL of sea water was added into a test tube (labelled as test tube C). From this, 0.5 mL was taken and added to 3 separate test tubes. To each of the test tubes, 4.5 mL of seawater containing 10 nauplii were added using a micropipette making the drug concentration now 10 ppm. For the control, 5.0 mL of seawater containing 10 nauplii were added to 3 separate test tubes.

3.13.3.3 Assessment of brine shrimp for mortality

After 24 Hours incubation at room temperature, all the set-ups were accessed and the dead and surviving larva were counted and noted, by pouring the content into petri dishes and observed under light. The values were used for % mortality estimations. The LC_{50} values were also calculated using Finney's Probit Test Analysis for Quantal Data (Meyer, 1982; Aboaba *et al.,* 2010).

3.14 The MTT assay for HeLa (cancer) cell line and SHSY5Y neuronal (normal) cell line

The absorbance-based MTT assay was used to assess the viability of the two cells. This was conducted as previously reported (Ajibade *et al*., 2020). At the end of the treatments,5 mg/mL of sterile MTT was prepared in Phosphate Buffer Saline, and ten microlitter of it was added to each well (10% v/v), after which incubation of three hours was done on the plate. Each well content was extractedbefore adding one hundred microlitter of dimethyl sulphoxide to solubilize the formed formazan. The product was then placed on a plate shaker for about 5 minutes and absorbance was read at 570nm on a microplate reader (CLARIO star, BMG LABTECH, UK). The process was done in triplicate and the mean was calculated; the mean of the negative control treatment was set to 100% and the mean of every other process was normalized to that of the control treatment (set to one hundred percent) to obtain the percentage viability for that treatment.

3.15 Statistical analysis for Repellency and MTT assay

Statistical analysis of the experimental data was performed using the computer software SPSS 14 version and MS EXCEL 2003. One-way ANOVA in SPSS was used to calculate and compared the percentage repellency of the extracts at each concentration on plant basis. Results were also calculated as Mean ± SD.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Repellency assay-guided successive extraction of the fruits of *Dennettia tripetala,* and *Afromomum melegueta,* seeds of *Jatropha curcas, Citrus limon, Citrus paradise,* and *Citrus sinensis* and their percentage repellencies

The dried powdered parts of the studied plants were extracted successively using N-hexane, EtOAc, and then methanol. Crude extracts obtained were then coded as: DTH, DTE, and DTM (*Dennettia tripetala*), AMH, AME, and AMM (*Afromomum melegueta*), JCH, JCE, and JCM (*Jatropha curcas*), CLH, CLE, and CLM (*Citrus limon*), CPH, CPE, and CPM (*Citrus paradisi*), and CSH, CSE, and CSM for hexane, ethyl acetate, and methanol respectively.

The phytochemical constituents of the plant extracts are as presented in (Tab. 4.1). Phytochemical analysis showed the occurrence of only alkaloid in all the plants extracts.
All the extracts were screened for their repellent activities against the malaria vector, adult female Anopheles mosquito. The repellent activity was evaluated on plant basis, and for each plant, activities varied according to solvent extract and concentration. The hexane extract of *C. limon* coded CLH, at 5mg/mL, had the highest protection, with percentage repellency of 96.24%, followed by the Ethyl acetate extract of *D. Tripetala*, DTE, at 5mg/mL (87.67%). There were no significantly differences between the two at $p \leq 0.05$, as shown in the Table 4.2.

Table 4.1. Results of phytochemical screening and yield of extracts

Plants	Extracts	% Yield	Phytochemicals					
			Alkaloid	Flavonoid	Tannins	Saponins	Glycosides	Antraq-uinones
D. tripetala	Hexane	15.0	+	-	+	+	-	-
	E. acetate	20.0	+	-	+	+	-	-
	Methanol	31.0	+	-	+	+	-	+
A.	Hexane	18.4	+	++	+	+	-	-
melegueta	E. acetate	21.6	+	++	+	+	+	-
	Methanol	28.0	+	++	+	+	+	-
J. curcas	Hexane	27.0	++	+	-	-	-	-
	E. acetate	25.5	++	+	++	++	-	-
	Methanol	20.0	++	+	++	+	-	-
C. limon	Hexane	36.5	+	++	-	++	++	++
	E. acetate	25.0	++++	++	++	-	++	++
	Methanol	19.0	+	++	++	++	++	++
C. sinensis	Hexane	30.4	++	++	++	++	++	++
	E. acetate	28.0	+++	-	++	-	-	-
	Methanol	20.0	++	++	++	++	++	++
C. paradise	Hexane	28.5	++	++	-	++	-	++
	E. acetate	20.0	+++	-	++	-	-	++
	Methanol	18.0	++	++	++	++	++	-

\+ means present of detectable amount,

\- means absent of detectable amount,

++ means present of concentrated amount.

E. acetate – Ethyl acetate

Table 4.2: Showing the percentage repellency of the Hexane, Ethyl acetate, and Methanolic extracts of *A. melegueta, D. tripetala, J. curcas, C. limon, C. paradise*, and *C. sinensis* at 1.5. 2.5, and 5mg/ml. No significant difference between values with superscript a in each plant at $p \leq 0.05$

Plants	Extracts	Conc(mg/mL)	Mean landed mosquitoes(3 replicates)±SD			% Repellency
			20 min	40 min	1hr	
A. melegueta	Hexane	1.5	5.67±0.58	9.00±1	12.00±1	15.49
		2.5	1.33±0.58	3.33±0.58	10.00±1	27.67
		5.0	0.33±0.58	1.00±0	3.00±1	79.08[a]
	Control	Acetone	3.67±0.58	6.67±1.16	14.33±0.58	0.00
	E. acetate	1.5	0.33±0.58	1.00±1	7.67±1.53	3.76
		2.5	0.00±0	0.33±0.58	2.00±1	74.90[a]
		5.0	0.00±0	0.00±0	1.00±1	87.45[a]
	Control	Acetone	1.33±0.58	2.97±1	7.97±1	0.00
	Methanol	1.5	1.00±1	2.00±2	5.33±3.06	0.19
		2.5	0.33±0.58	1.33±0.58	6.33±1.53	18.54
		5.0	0.33±0.58	0.67±1.16	3.67±1.53	31.46
	Control	Acetone	2.00±0	2.67±0.58	5.34±1.16	0.00
D. tripetala	Hexane	1.5	2.67±0.58	4.67±0.58	6.67±1.52	31.02
		2.5	0.33±0.58	1.00±1	4.00±2	58.63
		5.0	0.00±0	1.00±0	4.00±1	58.63
	Control	Acetone	1.67±0.58	4.33±0.58	9.67±1.52	0.00
	E. Acetate	1.5	3.33±0.58	7.33±0.58	15.00±1	44.44
		2.5	2.67±0.58	6.00±1	12.67±0.58	53.07
		5.0	0.00±0	0.67±0.58	3.33±1.52	87.67
	Control	Acetone	5.67±0.58	10.67±1.52	27.00±1	0.00
	Methanol	1.5	1.00±1	2.33±2.08	10.67±3.51	58.43
		2.5	0.67±0.58	4.00±1	12.00±2.64	53.25
		5.0	0.33±0.58	3.67±1.15	13.33±1.52	48.07
	Control	Acetone	7.33±0.58	13.33±1.52	25.67±1.52	0.00
J. curcas	Hexane	1.5	0.67±0.58	3.33±0.58	7.67±1.52	61.65[a]
		2.5	0.67±0.58	2.33±0.58	6.33±1.15	68.35[a]

		5.0	0.33±0.58	1.67±1.15	6.33±2.08	68.35[a]
	Control	Acetone	7.33±0.58	12.33±1.53	20.0±3.60	0.00
	E. Acetate	1.5	26.0±1.15	39.67±1.15	53.33±0.58	-62.26
		2.5	12.67±1.52	28.33±1.53	52.00±1.73	1.89
		5.0	11.33±1	13.00±1.00	26.33±2.08	50.32
	Control	Acetone	20.67±1.52	36.33±1.53	53.00±1	0.00
	Methanol	1.5	6.67±1.15	16.67±2.08	36.67±3.21	15.79
		2.5	11.00±1.73	23.33±2.30	46.00±1	45.25
		5.0	7.67±1.52	10.00±2.00	19.33±3.51	38.96
	Control	Acetone	10.67±1.15	20.67±0.58	31.67±1.52	

Table 4.2. Cont.

Plants	Extracts	Conc.(mg/ml)	Mean landed mosquitoes(3 replicates)±SD			% Repellency
			20 min	40 min	1hr	
C. limon	Hexane	1.5	3.00±0	6.66±0.58	14.33±0.58	7.69
		2.5	2.33±0.58	4.33±1.53	9.00±1	35.05
		5.0	0.33±0.58	0.33±0.58	0.33±0.58	97.52[a]
	Control	Acetone	2.33±1.16	7.66±1.53	13.33±3.06	0.00
	E. acetate	1.5	0.33±0.58	1.33±0.58	5.00±1	68.07
		2.5	0.00±0	5.67±0.58	15.33±1.53	-2.11
		5.0	3.00±0	4.33±0.58	9.00±0	29.757
	Control	Acetone	3.33±0.58	8.33±0.58	15.33±0.58	0.00
	Methanol	1.5	1.00±0	2.33±0.58	9.67±0.58	58.84
		2.5	4.00±0	7.00±1	11.33±0.58	51.48
		5.0	0.33±0.58	1.33±0.58	3.00±1	86.77[a]
	Control	Acetone	7.00±1	13.67±1.53	22.67±2.52	0.00
C. paradise	Hexane	1.5	2.33±0.58	4.33±0.58	8.67±1.15	29.68
		2.5	1.67±0.58	4.00±1	7.67±1.15	37.79
		5.0	1.33±0.58	2.33±0.58	5.67±0.58	54.01[a]
	Control	Acetone	3.33±1.15	7.67±0.58	12.33±0.58	0.00

	E. Acetate	1.5	7.67±0.58	12.33±1.52	18.67±1.15	-36.58
		2.5	3.00±0	6.00±1.23	13.33±0.58	2.55
		5.0	4.67±0.58	10.67±0.58	18.33±1.52	-34.09
	Control	Acetone	4.67±0.58	6.00±1	13.67±0.58	0.00
	Methanol	1.5	5.67±0.58	8.33±0.58	17.33±0.58	-36.78
		2.5	4.67±0.58	7.67±0.58	15.67±0.58	-23.68
		5.0	3.00±1	6.33±1.52	14.33±0.58	-13.10
	Control	Acetone	4.33±1.15	7.33±1.15	12.67±0.58	0.00
C. sinensis	Hexane	1.5	1.33±0.58	3.33±0.58	6.67±1.15	66.09[a]
		2.5	0.67±0.58	2.00±1	8.00±1.72	59.33[a]
		5.0	0.00±0	0.33±0.58	4.00±1	79.66[a]
	Control	Acetone	2.67±0.58	6.67±0.58	19.67±1.15	0.00
	E. Acetate	1.5	6.67±0.58	11.33±1.52	19.67±1.15	-40.50
		2.5	2.00±0	5.00±1.73	13.67±1.15	2.36
		5.0	3.67±0.58	11.33±0.58	21.33±2.08	-52.36
	Control	Acetone	3.67±0.58	5.00±1	14.00±1	0.00
	Methanol	1.5	4.67±0.58	8.00±1	16.33±2.51	-25.61
		2.5	2.67±0.58	7.00±1	14.00±1	-7.69
		5.0	2.00±1	5.33±1.52	10.67±3.06	17.92
	Control	Acetone	2.67±0.58	6.33±1.15	13.00±2.54	0.00

Superscript a = Values with superscript a for each plant contain the highest values and similar ones

E. acetate – Ethyl acetate

The mosquitoeshad probably been diverted because their attraction by lactic acid receptor cells has been masked due to inhibition by the active metabolites from the lemon seeds extracts (Ansari and Razdam, 1995), and therefore preventing or repelling the mosquitoes from biting. Therefore, mosquitoes could not smell the host for blood feeding and could not bite, because the sample has prevented them from smelling the lactic acids attractant, which indicates a sign of deterrent by the active ingredients. These constituents in themetabolites oflemon seeds extracts is believed to evaporate with carbondioxide from the skin, thus converting the carbondioxide sign to the plants type, which mosquitoes perceived instead of that of human (Jacobson, 1990).

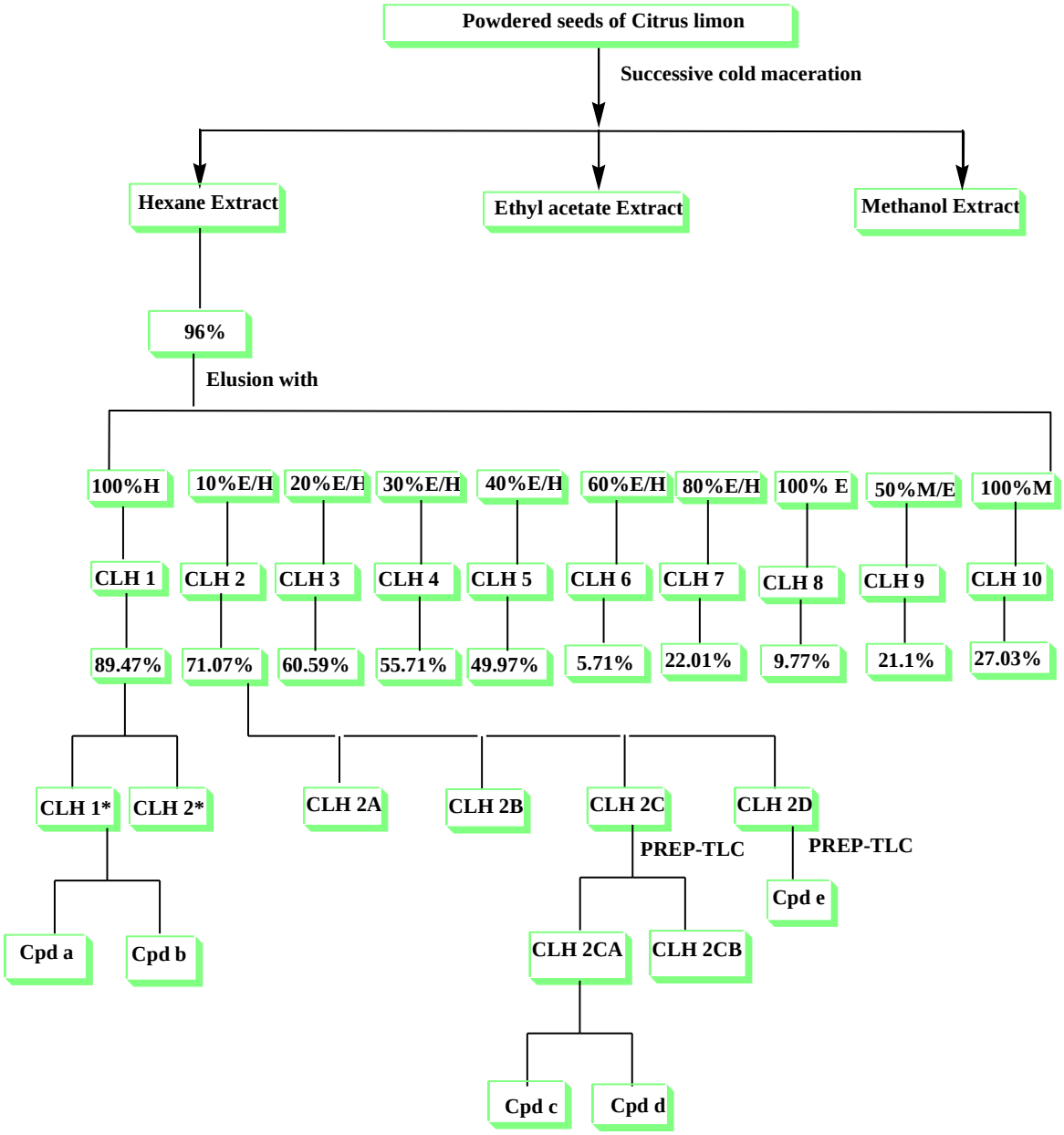


Fig 4.1: Scheme of extraction and isolation of compounds from *Citrus limon*

Table 4.3: The fractions from the hexane extract of *Citrus limon* and their percentage repellencies. No significant difference between values with superscript a at $p \geq 0.05$.

Volunteers	Fractions	Conc. (mg/mL)	Mean landed mosquitoes (Triplicate) Mean±SD				% Rep
			25 minutes	45 minutes	80 minutes	120 minutes	mean±SEM
1	100% Hex	5.00	0.33±0.06	1.33±0.58	2±0	3.67±0.58	89.47±1.74[a]
	10% E/H	5.0	4.33±3.21	6±2.65	7.33±1.53	10±2	71.07±6.20[b]
	-ve Control	Acetone	11±1	18.33±1.15	28±1.73	34.67±0.58	0.00
	20% E/H	5.00	2.33±0.58	6.33±1.16	9.67±1.53	13±2	60.59±8.63[bc]
1	30% E/H	5.00	3.33±0.577	6.67±0.58	10.33±0.58	15.33±1.53	55.71±5.15[c]
	-ve Control	Acetone	9.67±0.577	14.33±0.58	20±1	29.33±1.53	0.00
	40% E/H	5.00	5.33±1.155	9±1.73	12±2	17.33±2.08	49.97±6.25[c]
1	60% E/H	5.00	11±1	15.67±1.53	22±1.73	28.67±2.52	5.71±5.72
	-ve Control	Acetone	10.67±1.53	16±1.73	22.33±2.08	33±2	0.00
	80% E/H	5.00	7±1	11.33±1.53	17.33±1.55	24.67±1.53	22.01±8.99
1	100% EA	5.00	8±1.732	12.67±2.08	20±2	28.67±2.52	-9.77±15.08[e]
	-ve Control	Acetone	11.33±1.16	17.67±1.16	24±1.73	35.33±0.58	0.00
1	50% M/E	5.00	16.33±1.53	23±2	30.67±2.52	39±3.61	-21.12±4.15[e]
	100% M	5.00	16.33±1.53	23.67±2.08	32.33±2.52	40.67±3.05	-27.03±9.81[e]
	-ve Control	Acetone	9.33±1.53	14.33±0.58	19.33±1.16	31.67±1.53	0.00
	ODOMOS	DEET(20 %)	0.00	0.00	0.00	0.00	100.00[a]

Superscript a = Values with superscript a are similar
Superscript b = Values with superscript b are similar
Superscript c = Values with superscript c are similar
Superscript e = Values with superscript e are similar
E/H – Ehyl acetate in hexane, EA – Ethyl acetate, ME – Methanol in ethyl acetate, M - Methanol

4.2 Characterisation of Isolated Compounds from *Citrus limon*

4.2.1 Characterisation of compound a

Compound a (20 mg) was obtained as a white oily paste. Mp: 61-63 ˚C. Composition: $C_{16}H_{32}O_2$, Molecular weight: 256. LC-MS: (Positive ion probe): 279 $[M+Na]^+$. The proton and C-13 NMR are presented in Table 4.4. The assignments justify that reported by Bulama *et al*. (2014).

The IR spectrum [KBr, Vmax (cm^{-1})] shows the following bands 3485 (OH), 2920 and 2854 (-C-Hstretch), 1701 (-C=O) of a carboxylic acid, 1463, 1365, 1300 and 1120 (C-O). The IR spectrum shows a peak at 3485 cm-1, this suggests the presence of an -OH group of a carboxylic acid. The two peaks at 2920 and 2854 cm^{-1} indicate the –C-H stretch. The absorption at 1701 cm^{-1} suggest the presence of a carbonyl group of an acid. The peaks at 1463, 1365, 1300 and 1120 indicate the presence of C-O stretch of a carbonyl group. Figure 4.2.

1H NMR spectrum (400 MHz, $CDCl_3$) of compound a (Fig. 4.3) contains a total number of 32 protons. The spectrum showeda prominent multiplet signal at δH 1.19, and also 24 H, being C-3 to C-14, prominent CH_2 peak. The proton nuclear magnetic resonance (NMR) displayeda tripletatδ 2.29 (J = 8.0 Hz),representing the methylene at position C-2. The multiplet signal at δ 1.56 corresponds to a methylene group at C-15. At position δ 9.8, there is a singlet representing hydroxy in COOH. Also, the triplet integrating for three protons at δ 0.81 (J = 8.0 Hz) is fot the CH_3 at C-16.

Compound a possesses a total of sixteen carbons, a quaternary carbon δC 179.3 (C1), fourteen methylene sp^2 hybridized carbons at δC 33.9 (C2), 31.9 (C3), 29.7 (C4), 29.6 (C5, C6 and C8), 29.5 (C7), 29.3 (C9), 29.5 (C10), 29.4 (C11, C12 and C13), 24.7 (C14), 31.9 (C15) and a methyl carbon δC 14.1 (C16) (Figure 4.4).

The HMBC spectrum, showed a correlation between δH 2.29 and δC 179.3 (C1)/ δC 33.9 (C2)/ 29.7 (C4). δH 1.19 and δC 33.9 (C2)/ 22.7 (C3) and 29.7 (C4). This confirms the presence of a carbonyl carbon at C1. Another correlation of δH 0.81 and δC 24.7 (C14)/

31.9 (C15). This confirms a methyl group at the terminal part of the aliphatic chain shown in Figure 4.5.

The proton-proton COSY relationships of compound a shows a strong correlation between two protons H2 (δH 2.29) and H3 (δH 1.56), also H3 (δH 1.56) and H4 (δH 1.19), these correlations further indicate that a carbonyl carbon at C1 is present.

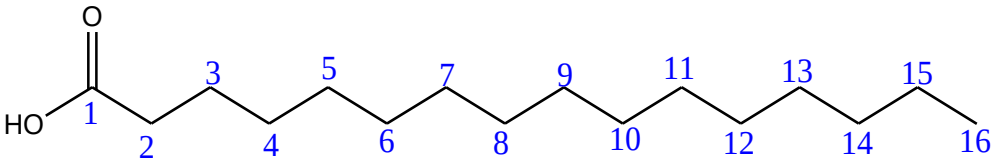
The ESI-MS experiment of the compound a was performed. The peak at 279 $[M+Na]^+$, confirms the molecular ion $[M]^+$ at *m/z* 256 suggesting the formula $C_{16}H_{32}O_2$ (Fig. 4.6). This information is in line with the report of Bulama *et al*. (2014).

The entire spectra information suggests the compound to be a fatty acid.

Table 4.4: Proton and carbon-13 NMR (400 MHz) spectroscopic data of compound a in Chloroform as compared with existing literature*

S/N	^{1}H NMR	^{13}C NMR	HMBC
1	--	179.3, Qc	
2	2.29 (t, *J* = 8.0)	33.9, CH_2	179.3 (C1), 33.9 (C2), 29.7 (C4)
3	1.19, m	22.7, CH_2	33.9 (C2), 22.7 (C3), 29.7 (C4)
4	1.19, m	29.7, CH_2	
5	1.19, m	29.6, CH_2	
6	1.19, m	29.6, CH_2	
7	1.19, m	29.5, CH_2	
8	1.19, m	29.6, CH_2	
9	1.19, m	29.3, CH_2	
10	1.19, m	29.5, CH_2	
11	1.19, m	29.4, CH_2	
12	1.19, m	29.4, CH_2	
13	1.19, m	29.4, CH_2	
14	1.19, m	24.7, CH_2	
15	1.56, m	31.9, CH_2	
16	0.81 (t, *J* = 8.0)	14.1, CH_3	24.7 (C-14), 31.9 (C-15)

* Reported literature by Bulama *et al*. (2014). quaternary carbon = Qc, singlet = s, broad singlet = bs, triplet = t, multiplet = m, coupling constant (Hertz) = *J*



Compound a – Palmitic acid

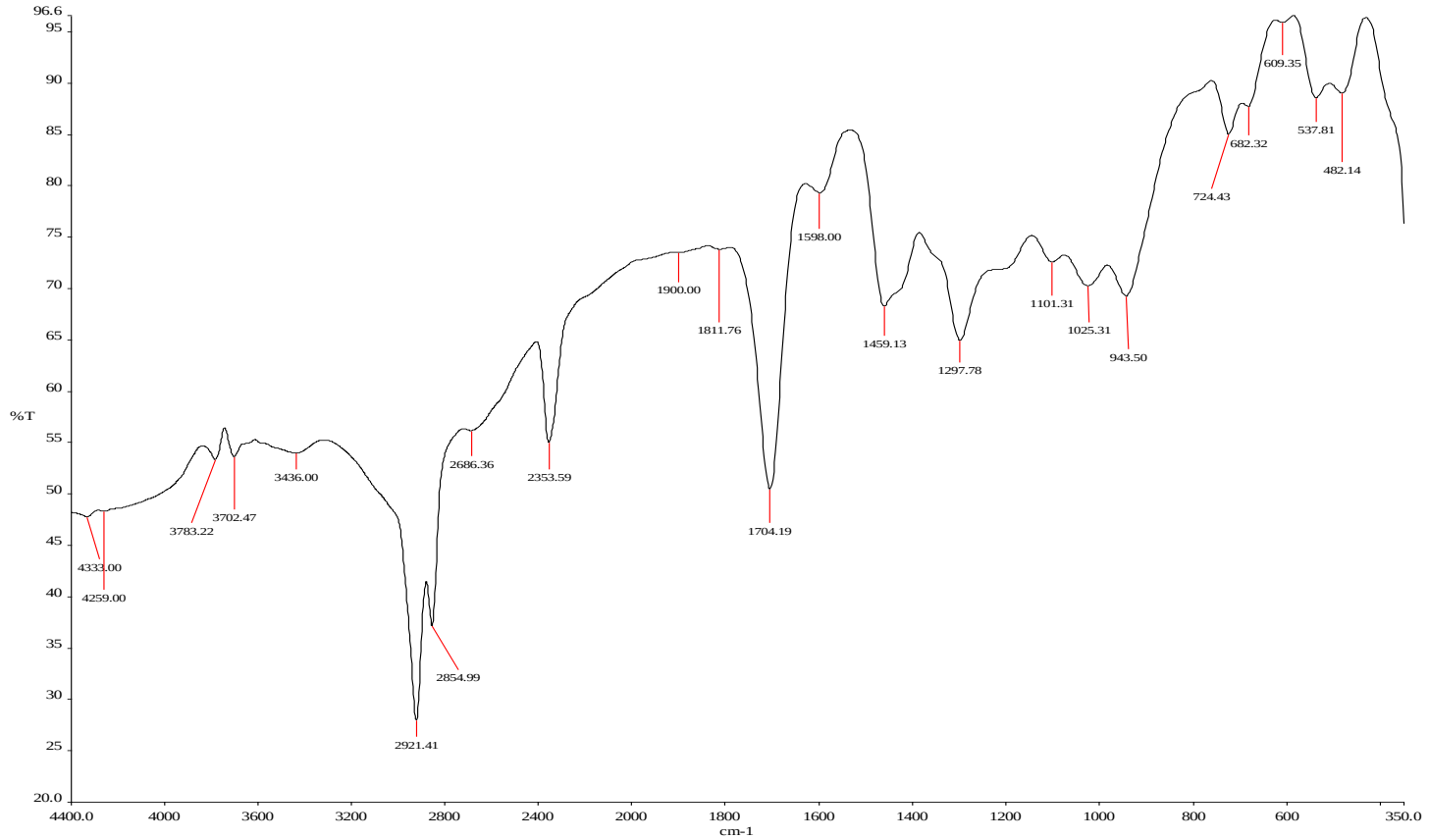


Fig 4.2: IR spectrum[KBr] of compound a

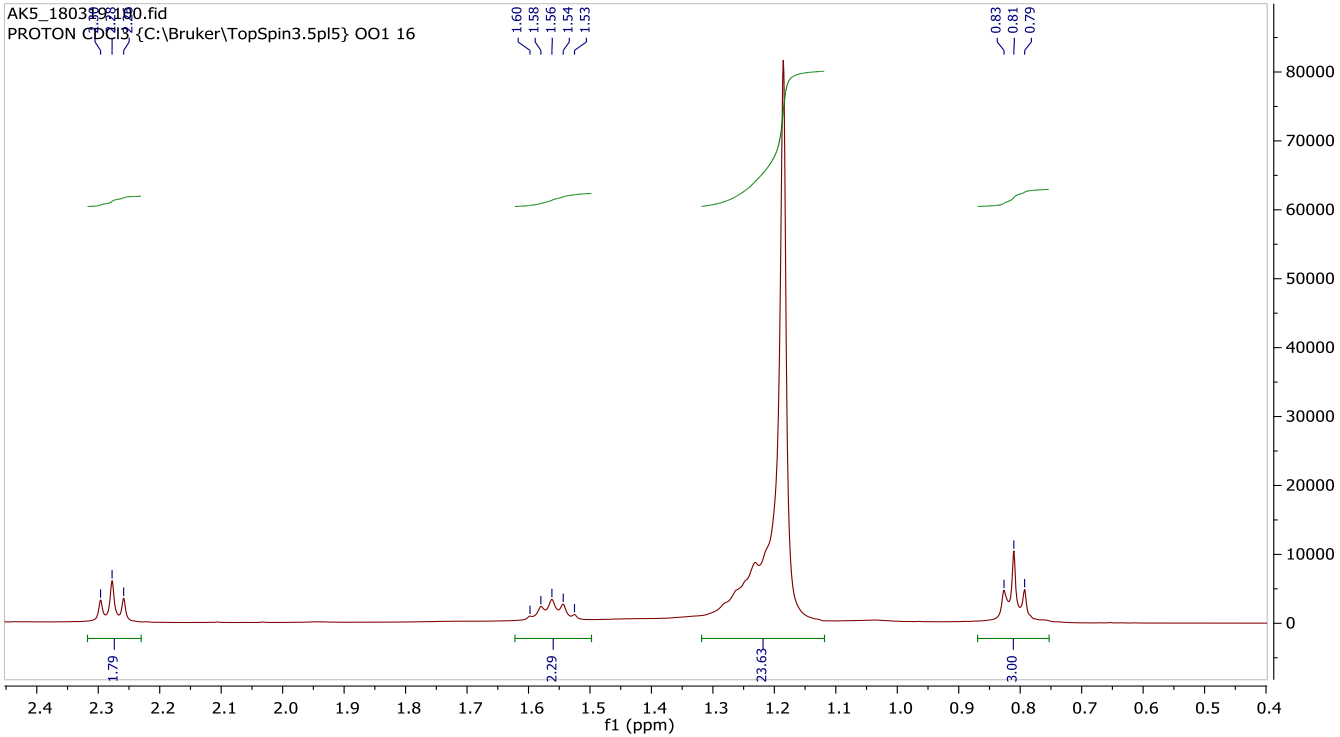


Figure 4.3: Proton NMR of a

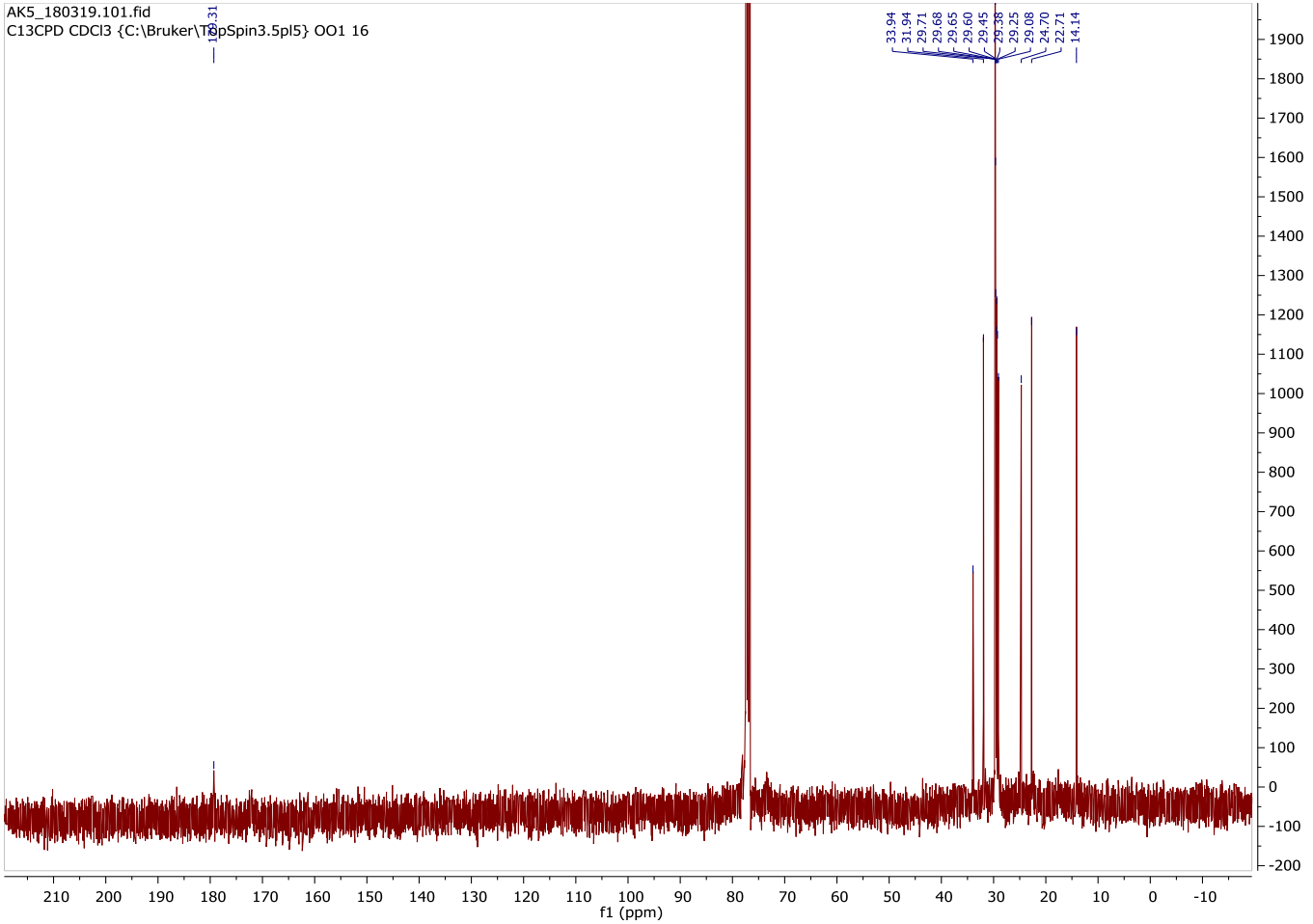


Fig 4.4a: Carbon-13 NMR) of a

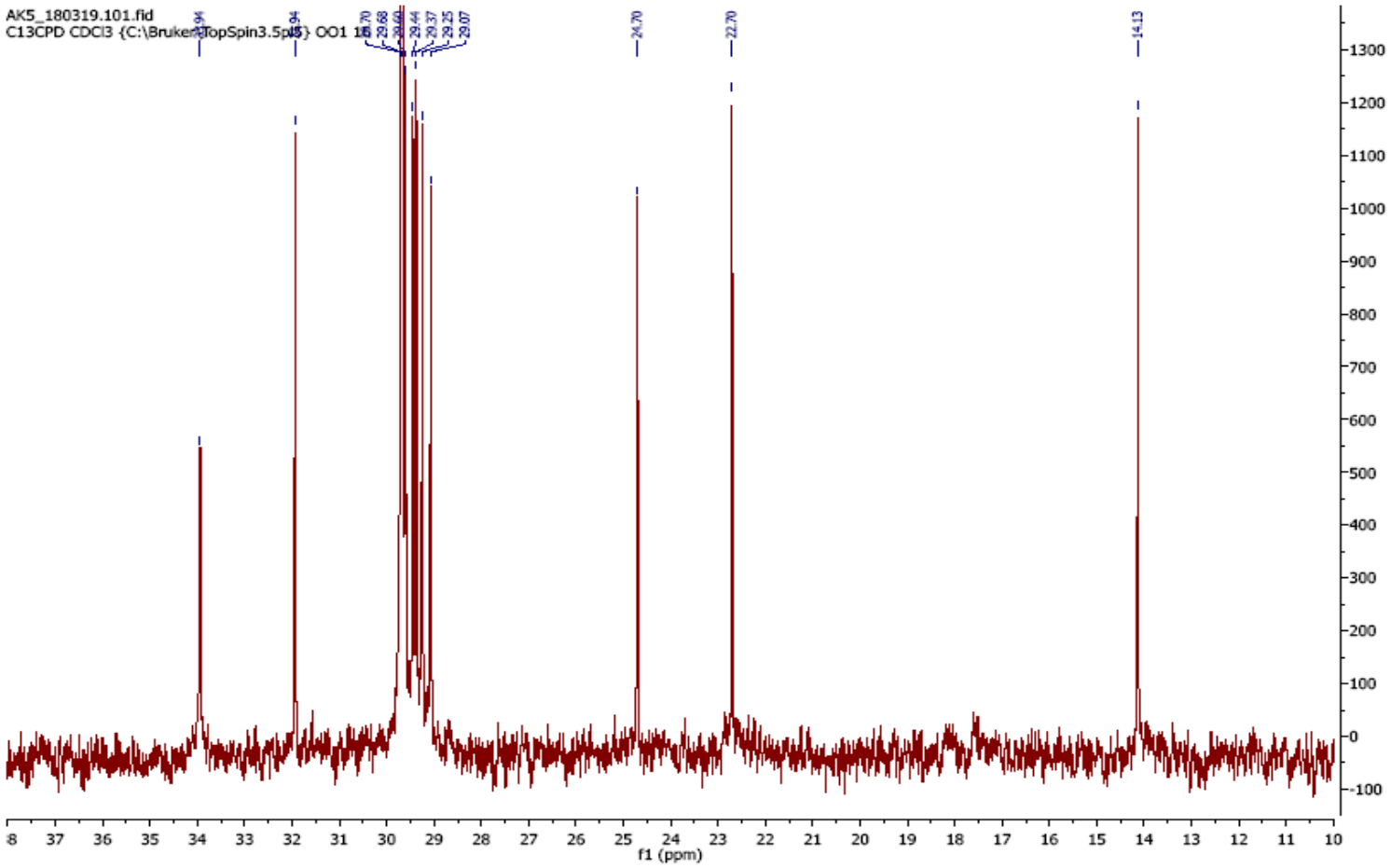


Fig 4.4b: Expanded (10-40 ppm) Carbon-13 NMR) of a

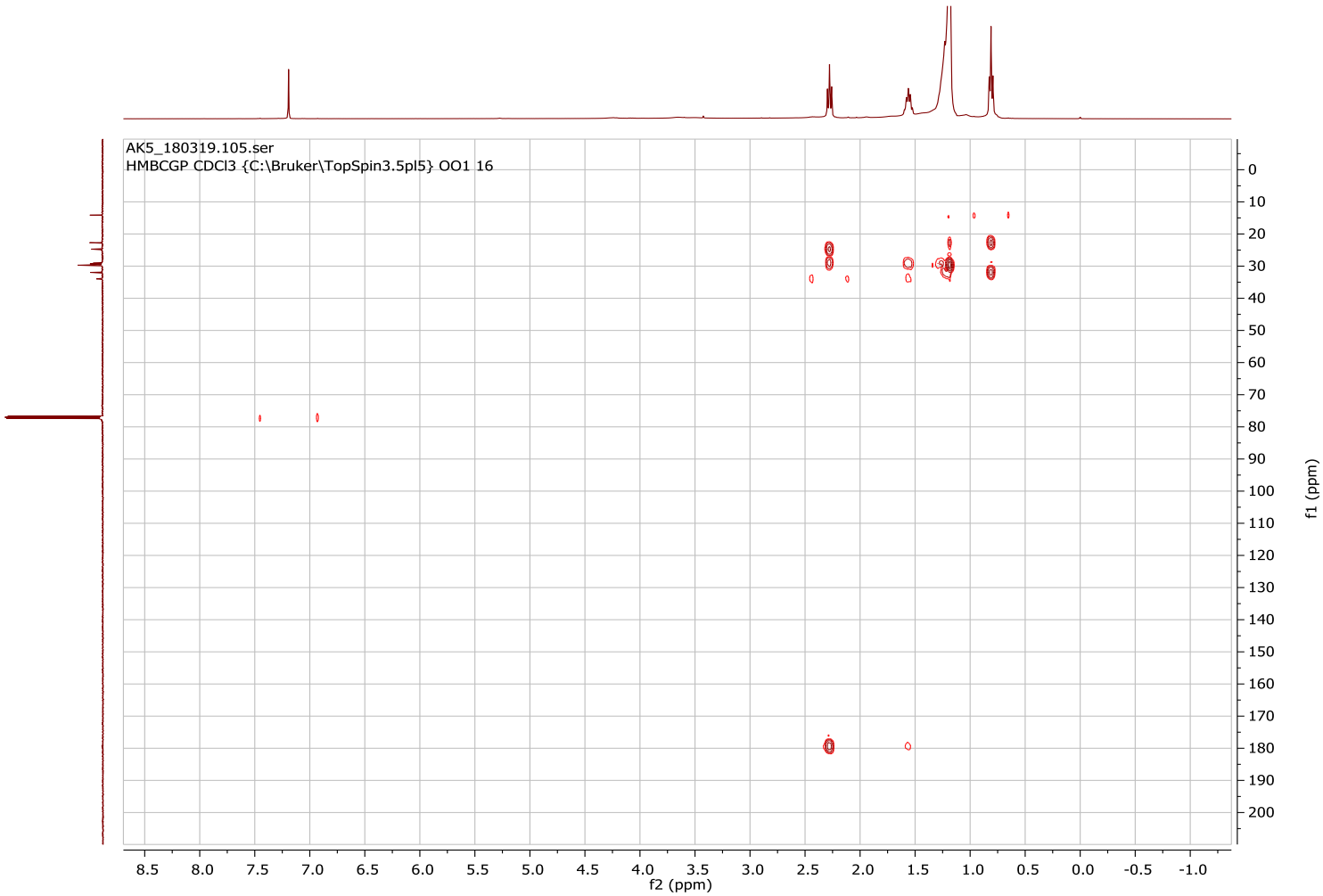


Fig 4.5: HMBC of a

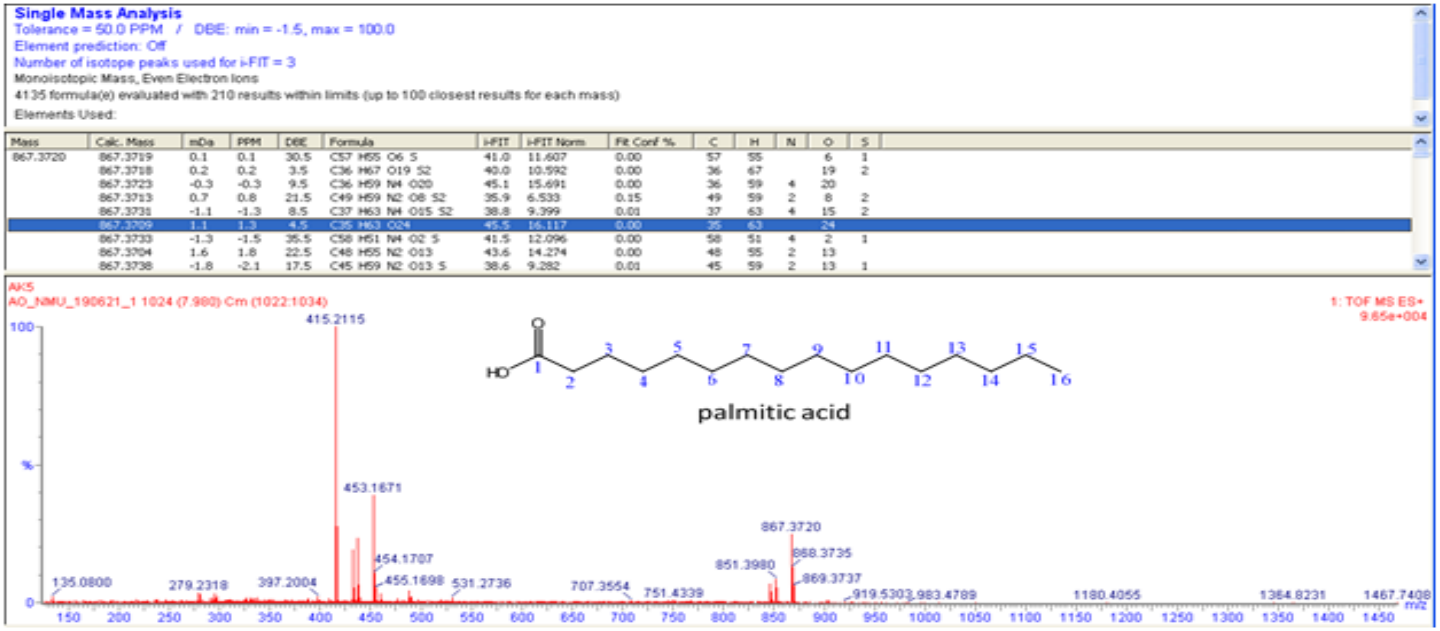


Figure 4.6: Mass spectrometry of a

4.2.2 Characterisation of compound b

Compound b (40 mg) obtained in the form of white pasty-solid. Mp: 73-75°C. Composition $C_{23}H_{44}O_3$, Molecular weight: 368. LC-MS: (Positive ion probe): 391 $[M+Na]^+$. Proton and carbon -13 nuclear magnetic resonance are presented in Tab. 4.5. The complete structural elucidation of compound b is in consistent with that reported by Gufta *et al*. (1985).

IR spectrum [KBr, Vmax (cm^{-1})]: 3779.7, 3699.0 (OH), 2921.2 and 2854.6 (-C-Hstretch), 1702.0 (-C=O) of a ketone and acid carbonyl bonds, overlapping, 1298.8 and 1107.5 (C-O). The IR spectrum show two peaks at 3779.7 and 3699.0 cm^{-1}, this suggest the presence of an -OH group of a carboxylic acid. The two peaks at 2921.2 and 2854.6 cm^{-1} indicate that –C-H stretching vibration is present. Maximum absorption which showed at 1702.0 cm^{-1} suggest the presence of a carbonyl group of a ketone and carboxylic acid. The peaks at 1298.8 and 1107.5 indicate the presence of C-O stretch of a carbonyl group. Shown in Figure 4.7.

Compound b possesses a total of twenty-three carbon atoms, two quaternary carbon δC 206.9, (C-14, keto carbonyl) and 179.3 (C-1, acid carbonyl), twenty sp^2-hybridized carbons at δC 33.7 (C13 and C15), 31.9 (C2 and C16), 30.9 (C4), 29.4 (C5, C6, C7, C8, C9, C10, C11, C17, C18, C19, C20 and C21), 24.7 (C3, C8 and C12), 22.7 (C22) and a methyl carbon δC 14.1 (C23) (Fig. 4.9).

The proton Nuclear Magnetic Resonance spectrum (400 MHz, $CDCl_3$) of b showed a multiplet signal at position δ 1.27, which represent 40 H ofnumerous CH_2. The proton Nuclear Magnetic Resonance spectrum revealed slightly downfield signals appearing as multiplet at δ 2.36 ppm which corresponds to proton at C-13 and C-15 respectively. Also, a doublet peak signal next to the carboxylic acid with δ 2.19. The multiplet peaks at δ 1.65 representing protons at position 3, 12 and 16 respectively. The triplets of three proton intensity at δ 0.90 (J = 8.0 Hz) represent the CH3 at position C-23 (Fig. 4.8)

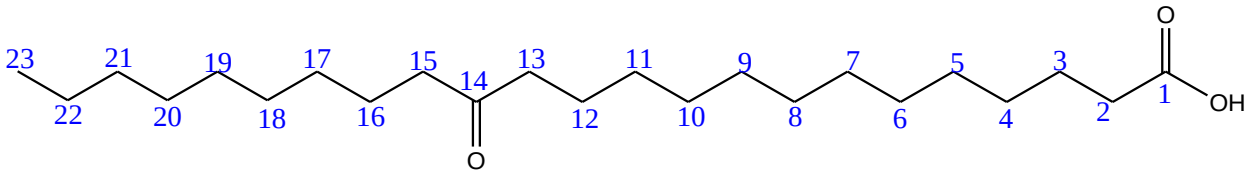
The ^1H-^1H-COSY correlations of Compound b shows a strong correlation between two protons H13 (δH 2.36) and H12 (δH 1.65), this suggests the presence of a carbonyl group

at position 10 (C-14), also H23 (δH 0.90) and H22 (δH 1.27), these correlations suggest the presence of the terminal sp^3 methyl carbon at position 23 (C23). Shown in Figure 4.10. The ESI-MS experiment of the compound was also performed. The peak at 391 $[M+Na]^+$, confirms the molecular ion signal; $[M]^+$ at mass-to-charge ratio 368, this suggests the formula $C_{23}H_{44}O_3$ (Fig. 4.11). This is in agreement with the report of Gufta *et al*. (1985).

Table 4.5: The proton and carbon-13 Nuclear Magnetic Resonance spectrum (400 MHz) spectroscopic data of compound b as compared with existing literature

	Chemical shifts [ppm] /Coupling constants [Hz]				
S/N	^{1}H NMR	^{13}C NMR	S/N	^{1}H NMR	^{13}C NMR
1	-	179.3, Qc	16	1.65, m	31.9, CH_2
2	2.19(d, *J* = 4.0)	31.9, CH_2	17	1.27, m	29.4, CH_2
3	1.65 (t, *J* = 8.0)	24.7, CH_2	18	1.27, m	29.4, CH_2
4	1.27, m	30.9, CH_2	19	1.27, m	29.4, CH_2
5	1.27, m	29.4, CH_2	20	1.27, m	29.4, CH_2
7	1.27, m	29.4, CH_2	21	1.27, m	29.4, CH_2
8	1.27, m	24.7, CH_2	22	1.27, m	22.7, CH_2
9	1.27, m	29.4, CH_2	23	0.90 (t, *J* = 4.0 Hz)	14.1, CH_3
10	1.27, m	29.4, CH_2			
11	1.27, m	29.4, CH_2			
12	1.65, m	24.7, CH_2			
13	2.36, m	33.7, CH_2			
14	--	206.9, Qc			
15	2.36, m	33.7, CH_2			

* Reported literature by Gufta *et al*. (1985). Qc = quaternary carbon, s = singlet, bs = broad singlet, t = triplet, m = multiplet, *J* = coupling const. (Hertz).



Compound b: 14- Oxotricosanoic acid

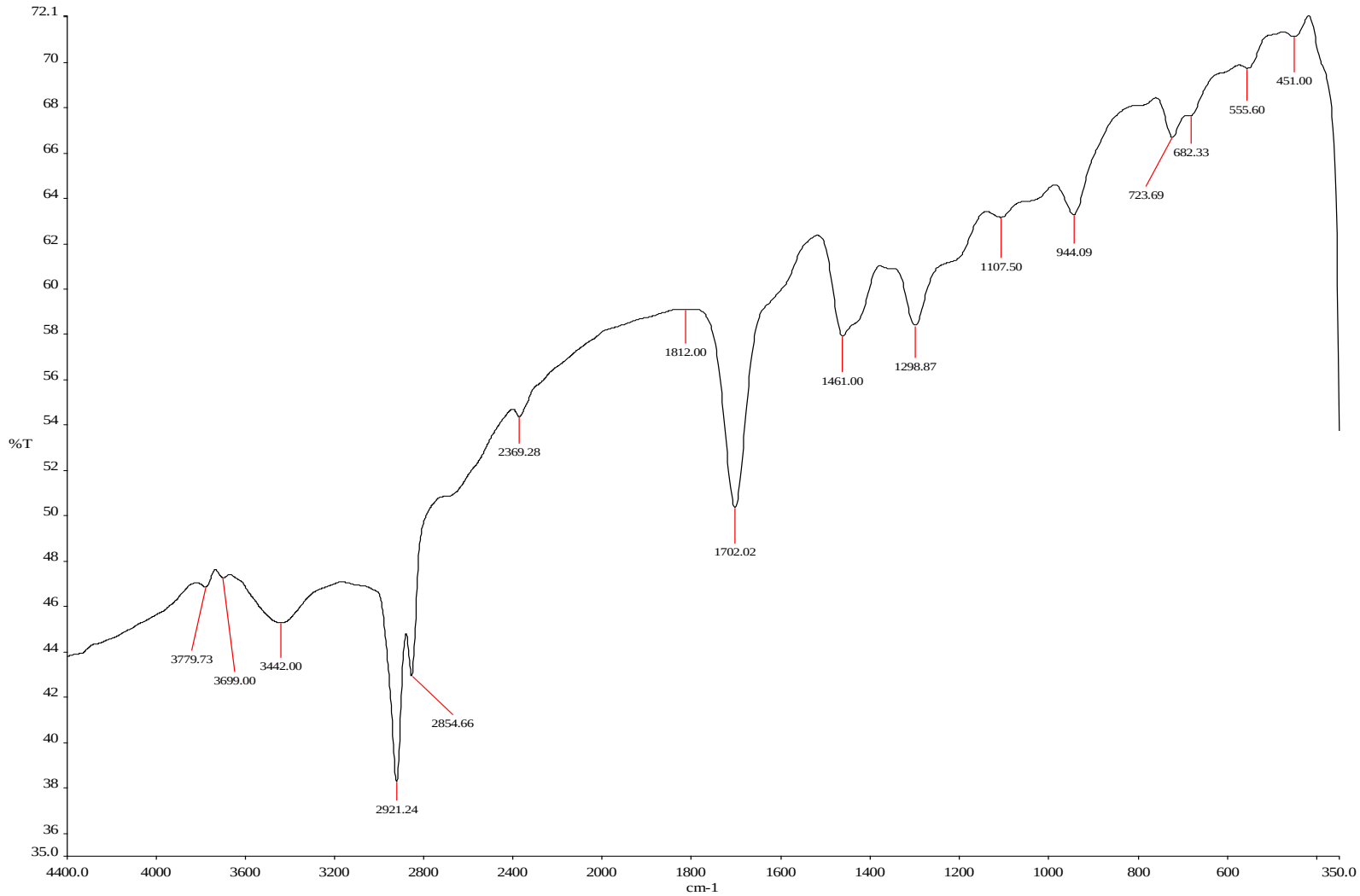


Fig 4.7: Infrared [KBr] of b

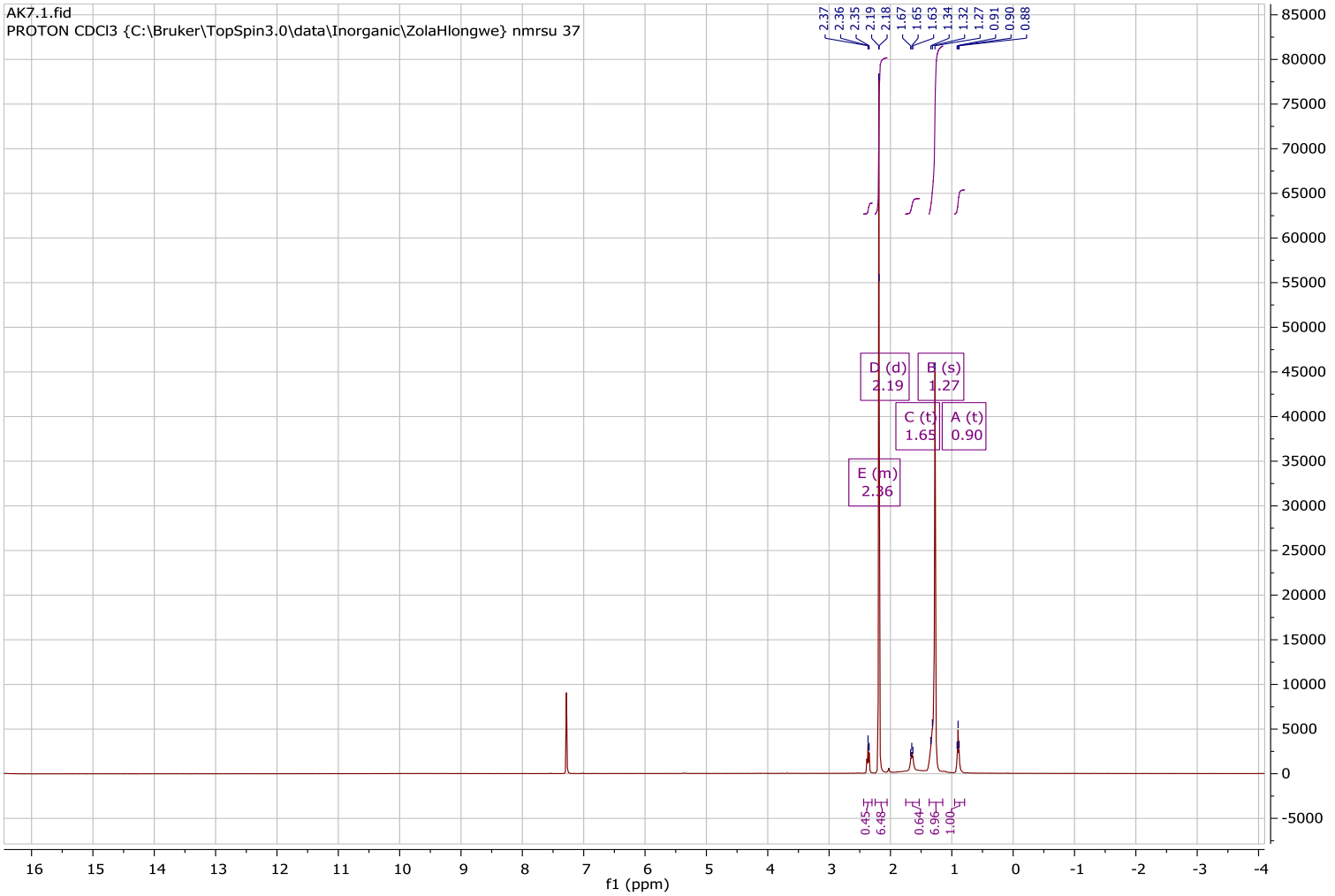


Figure 4.8: Proton Nuclear Magnetic Resonance spectrum of Compound b

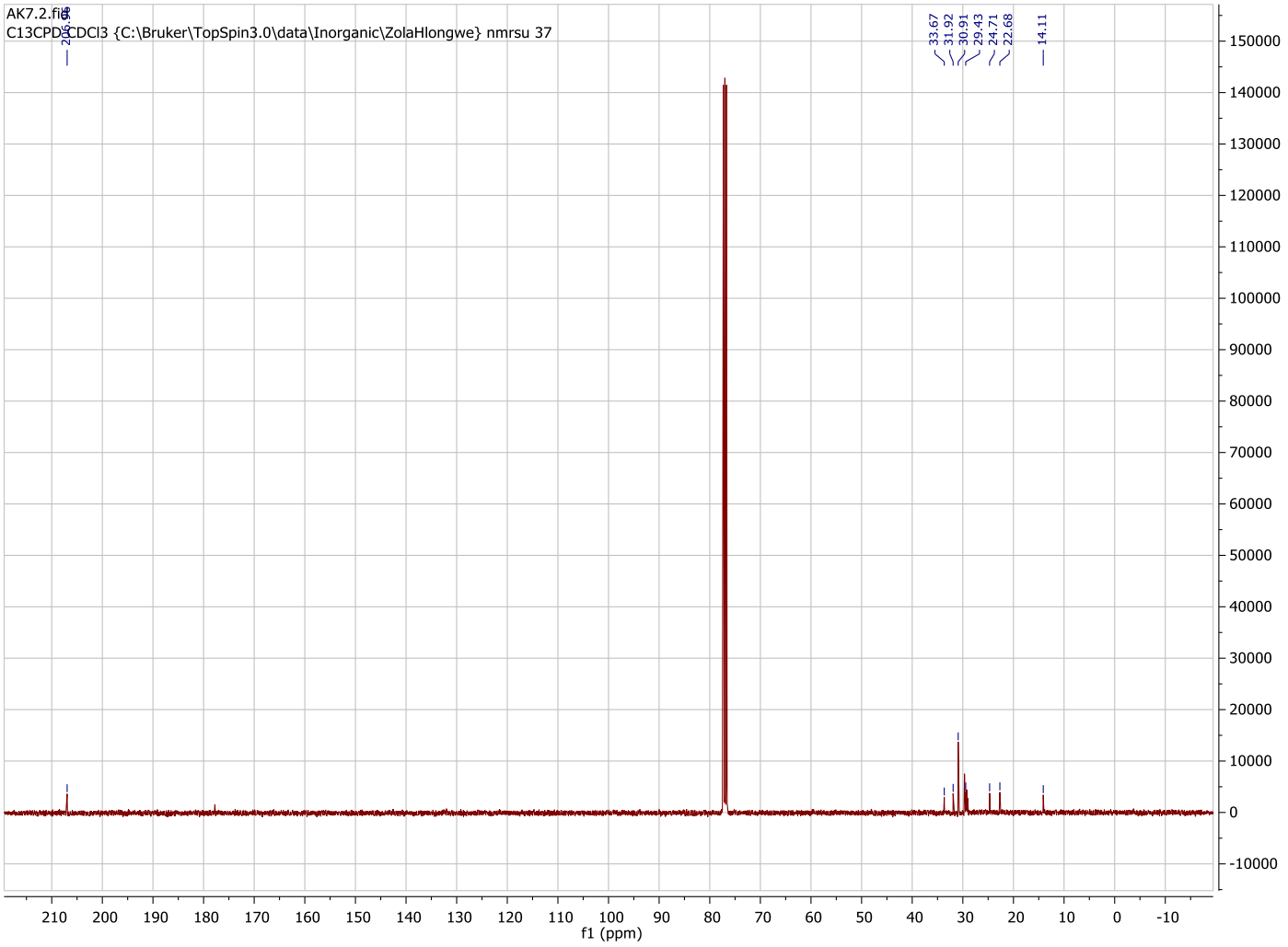


Fig 4.9: Carbon-13 NMR of b

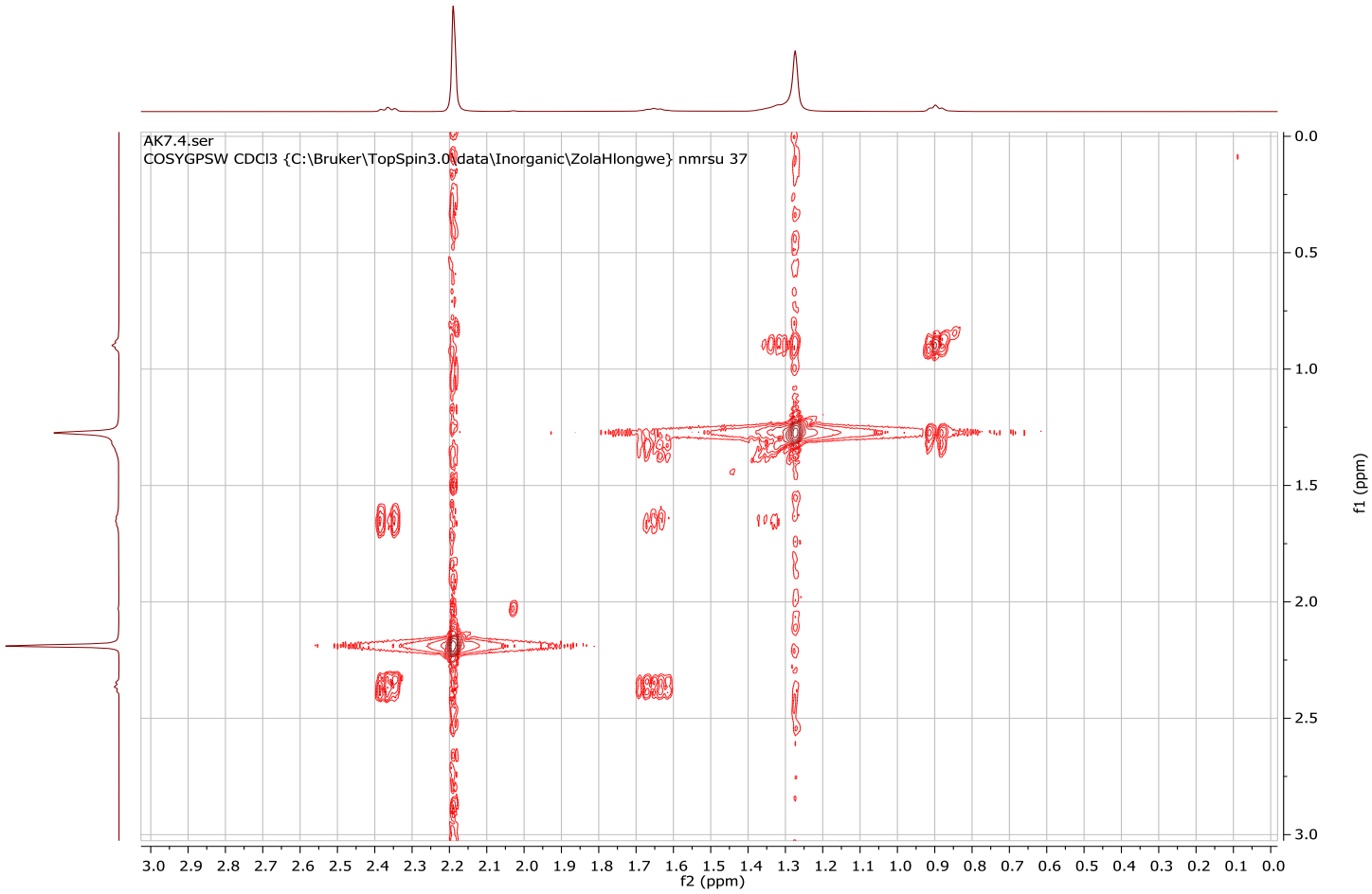


Figure 4.10: 1H-1H Correlation Spectroscopy (COSY) of b

Single Mass Analysis

Tolerance = 50.0 PPM / DBE: min = -1.5, max = 100.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

109 formula(e) evaluated with 12 results within limits (up to 100 closest results for each mass)

Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	O
517.1790	517.1804	-1.4	-2.7	25.5	C37 H25 O3	49.4	8.417	0.02	37	25	3
	517.1769	2.1	4.1	3.5	C19 H33 O16	50.7	9.673	0.01	19	33	16
	517.1862	-7.2	-13.9	16.5	C30 H29 O8	45.5	4.463	1.15	30	29	8
	517.1710	8.0	15.5	12.5	C26 H29 O11	41.0	0.013	98.67	26	29	11
	517.1921	-13.1	-25.3	7.5	C23 H33 O13	48.0	6.994	0.09	23	33	13
	517.1651	13.9	26.9	21.5	C33 H25 O6	48.9	7.892	0.04	33	25	6
	517.1956	-16.6	-32.1	29.5	C41 H25	52.6	11.563	0.00	41	25	
	517.1616	17.4	33.6	-0.5	C15 H33 O19	54.2	13.223	0.00	15	33	19
	517.1980	-19.0	-36.7	-1.5	C16 H37 O18	53.9	12.882	0.00	16	37	18

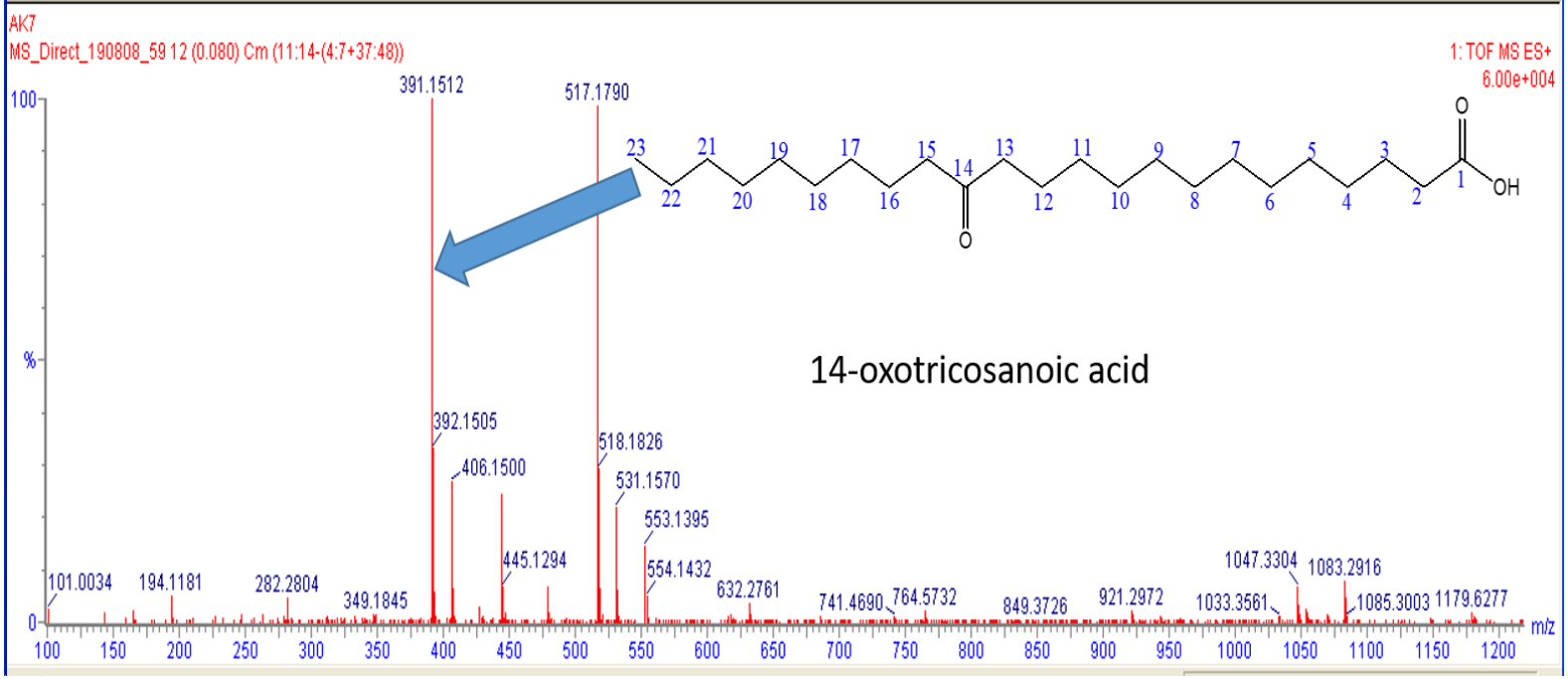


Figure 4.11: Mass spec. of b

4.2.3 Characterisation of Compound c

Compound c (30 mg) was gotten as a crystalline white solid. Mp: 85-87°C. Composition $C_{26}H_{52}O_2$, Molecular weight: 396.

Proton and carbon-13 Nuclear Magnetic Resonance are presented in Tab. 4.6. Structural elucidation was done using DEPT HSQC. The assignments is in line with that reported Mir *et al*. (2018).

IR spectrum [KBr, V̄max (cm^{-1})]: 2907 and 2842 (-C-Hstretch), 1738 (-C=O) of a carboxylic ester, 1460, 1167 and 1097 (C-O). The two peaks at 2907 and 2842 cm^{-1} indicate the presence of –C-H stretch. Maximum absorption shown at 1738 cm^{-1} indicates the presence of a carbonyl of an ester. Absorption signals at 1460, 1167 and 1097 indicate the presence of C-O stretch of a carbonyl group (Figure 4.12).

Compound c possesses a total of twenty-six carbons, one quaternary carbon δC 174 (C1), twenty-three methylene carbons δC 63 (C1′), 34.1 (C2), 31.9 (C6′ and C16), 29.7 (C4, C5, C6, C7, C8, C9, C10, C11, C12, C13 and C14), 29.6 (C2′, C3′, C4′, C5′ and C15), 24.9 (C3), 22.7 (C7′ and C17) and two sp^3 carbons δC 14.1 (C8` and C18) shown in Fig. 4.14.

The proton nuclear magnetic resonance spectrum (400 MHz, $CDCl_3$) of the compound. (Fig. 4.13) revealed an intense multiplet peak at δ 1.27, this is typical of 38 H of the long chain of CH_2. The ^1H NMR spectrum revealed two double doublets peaks at δ 4.32 and 4.14 (J = 4.0 Hz) which are slightly upfield, representing the two methylene protons linked to an oxygen atom at C-1′. A multiplet signal at δ 2.33 represents the CH_2at C2, next to the carbonyl carbon. Also, δ 1.25 and 1.66, corresponds to the C-3 and C-4, CH_2 protons respectively. The two triplets of three proton intensity at δ 0.90 (J = 4.0 Hz) is assiignedto the CH_3 at C18 and C8′ respectively.

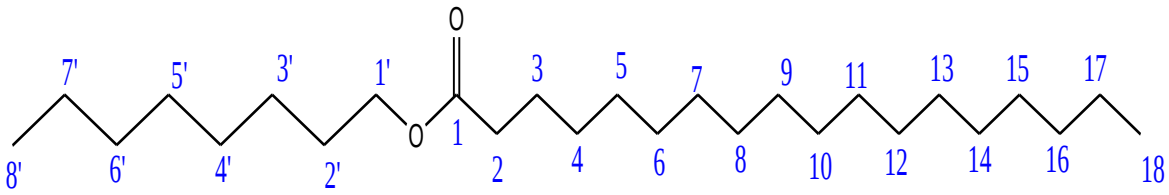
The Heteronuclear Nuclear Magnetic data show correlations were observed between δH 2.33 proton at position 2 and δC 174.0 (C1)/ δC 34.1 (C2)/ 29.7 (C4)/ 24.7 (C3). Another correlation was observed between δH 1.25 and δC 34.1 (C2)/ 24.9 (C3) and 29.7 (C4). This confirms a carbonyl carbon group at C1. Another correlation of δH 0.90 and δC 31.9 (C16)/ 22.7 (C17) and 14.1 (C18). This confirms a methyl group at the terminal part of the aliphatic chain shown in Fig. 4.15.

Table 4.6: Proton and C-13 NMR (400 MHz) data of compound c as compared with existing literature*

1	--	174.0,Qc	17	1.27 (m)	22.7, CH_2
2	2.33 (m)	34.1, CH_2	18	0.90 (t, *J* = 4.0)	14.1, CH_3
3	1.25 (m)	24.9, CH_2	1′	4.32 (dd, *J* = 4.0 Hz), 4.17 (dd, *J* = 4.0 Hz)	63.0, CH_2
4	1.66 (m)	29.7, CH_2	2′	1.27, m	29.6, CH_2
5	1.27 (m)	29.7, CH_2	3′	1.27, m	29.6, CH_2
6	1.27 (m)	29.7, CH_2	4′	1.27, m	29.6,CH_2
7	1.27 (m)	29.7, CH_2	5′	1.27, m	29.6,CH_2
8	1.27 (m)	29.7, CH_2	6′	1.27, m	31.9,CH_2
9	1.27 (m)	29.7, CH_2	7′	1.27, m	22.7,CH_2
10	1.27 (m)	29.7, CH_2	8′	0.90 (t, *J* = 4.0 Hz)	14.1, CH_3
11	1.27 (m)	29.7, CH_2			
12	1.27 (m)	29.7, CH_2			
13	1.27 (m)	29.7,CH_2			
14	1.27 (m)	29.7,CH_2			
15	1.27 (m)	29.6,CH_2			
16	1.27 (m)	31.9,CH_2			

* Reported literature Mir *et al*. (2018). Qc = quaternary carbon, s = singlet, bs = broad singlet, t = triplet, m = multiplet, J = coupling constant (Hertz).

Compound c: n-Octyl stearate



n-octyl stearate

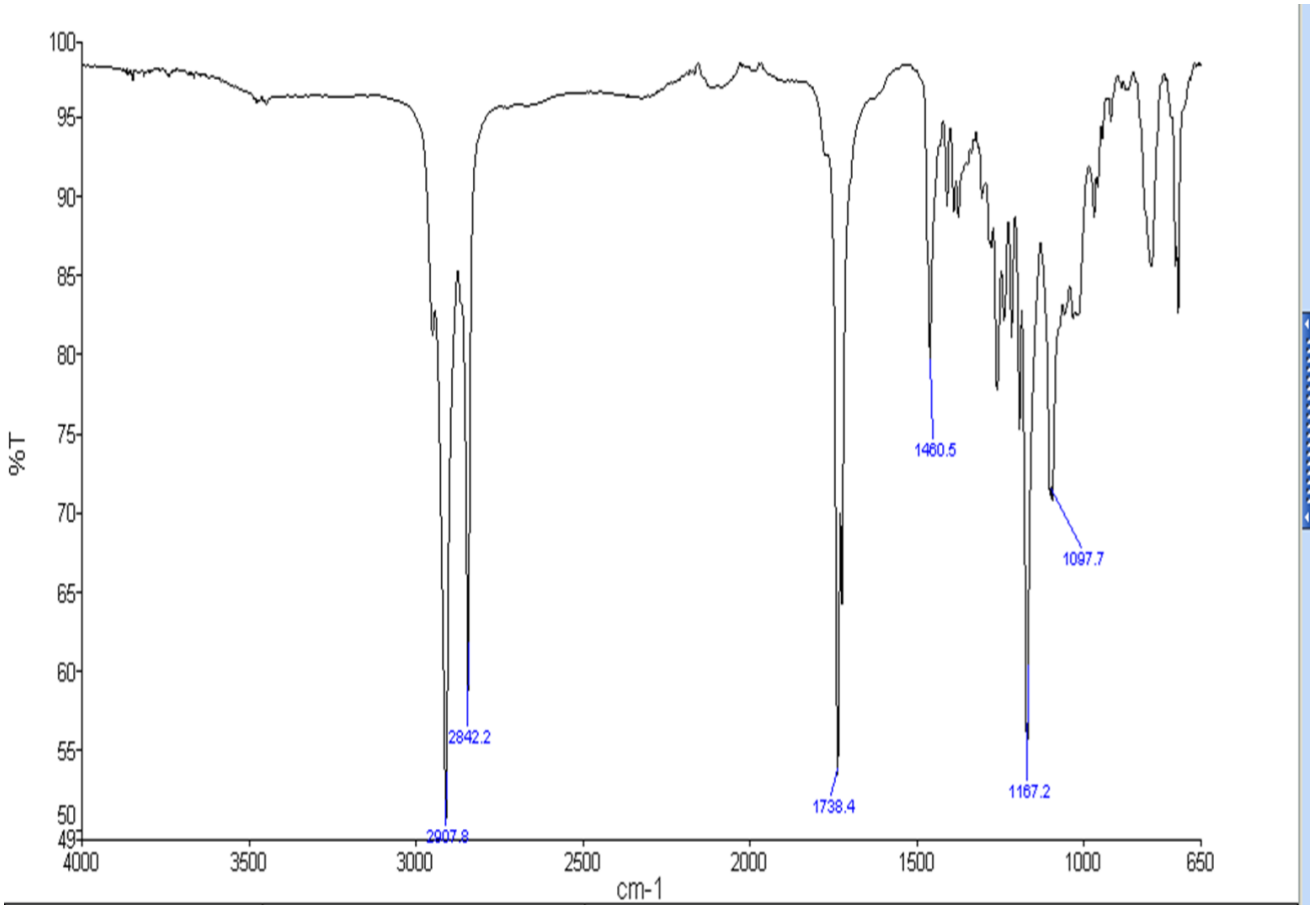


Fig 4.12: IR[KBr] of c

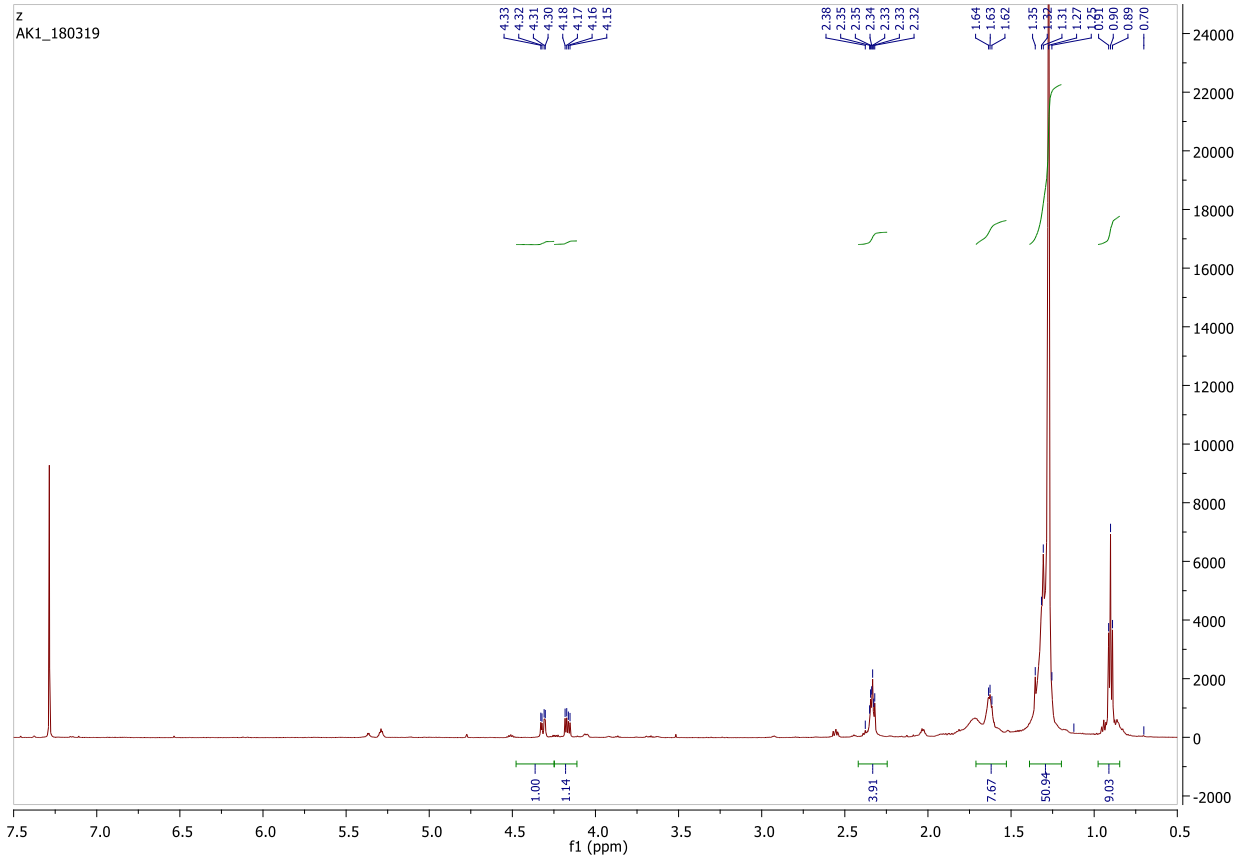


Figure 4.13: Proton NMR of c

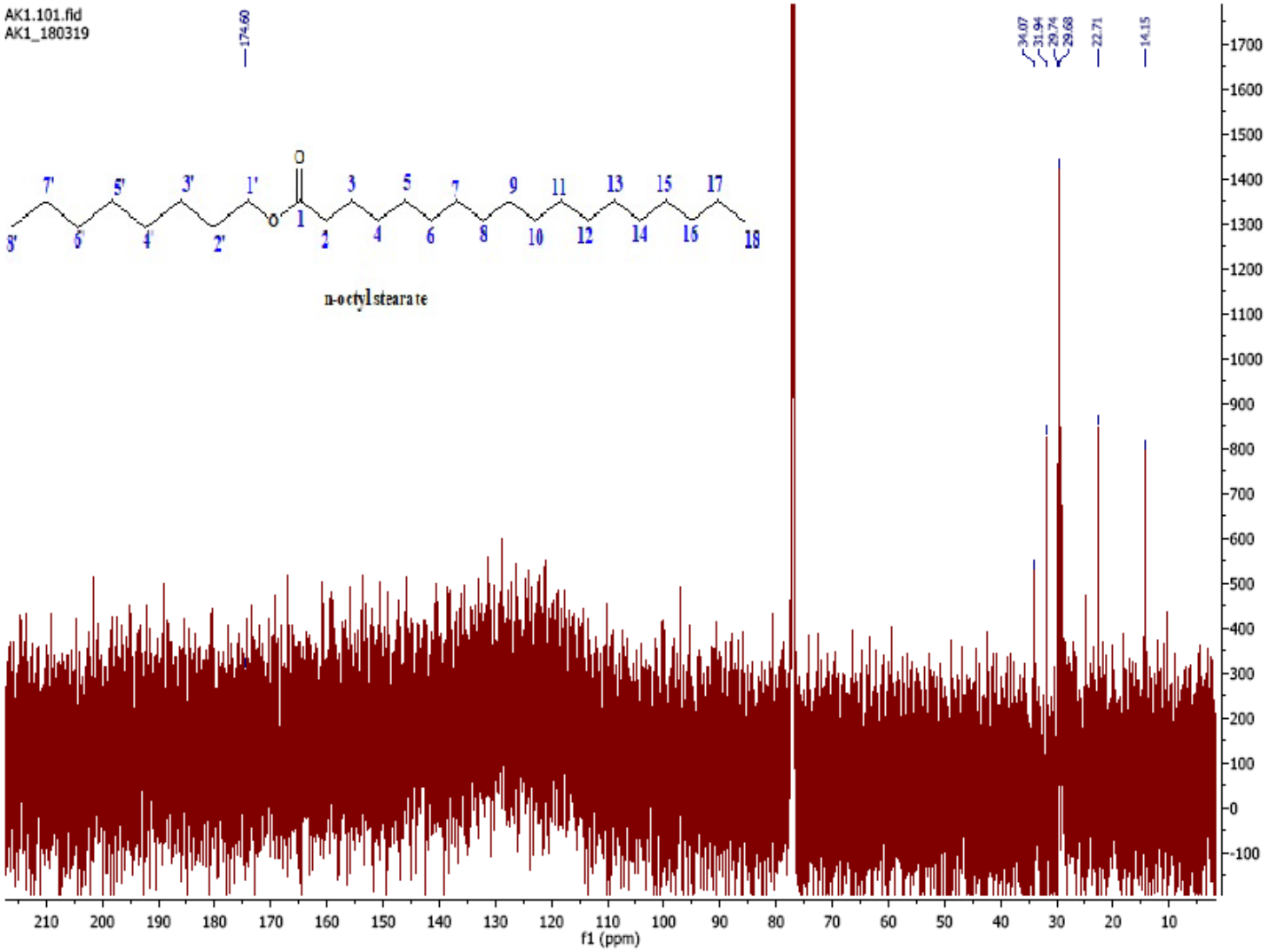


Figure 4.14: Carbon (13C) Nuclear Magnetic Resonance spectrum of compound c

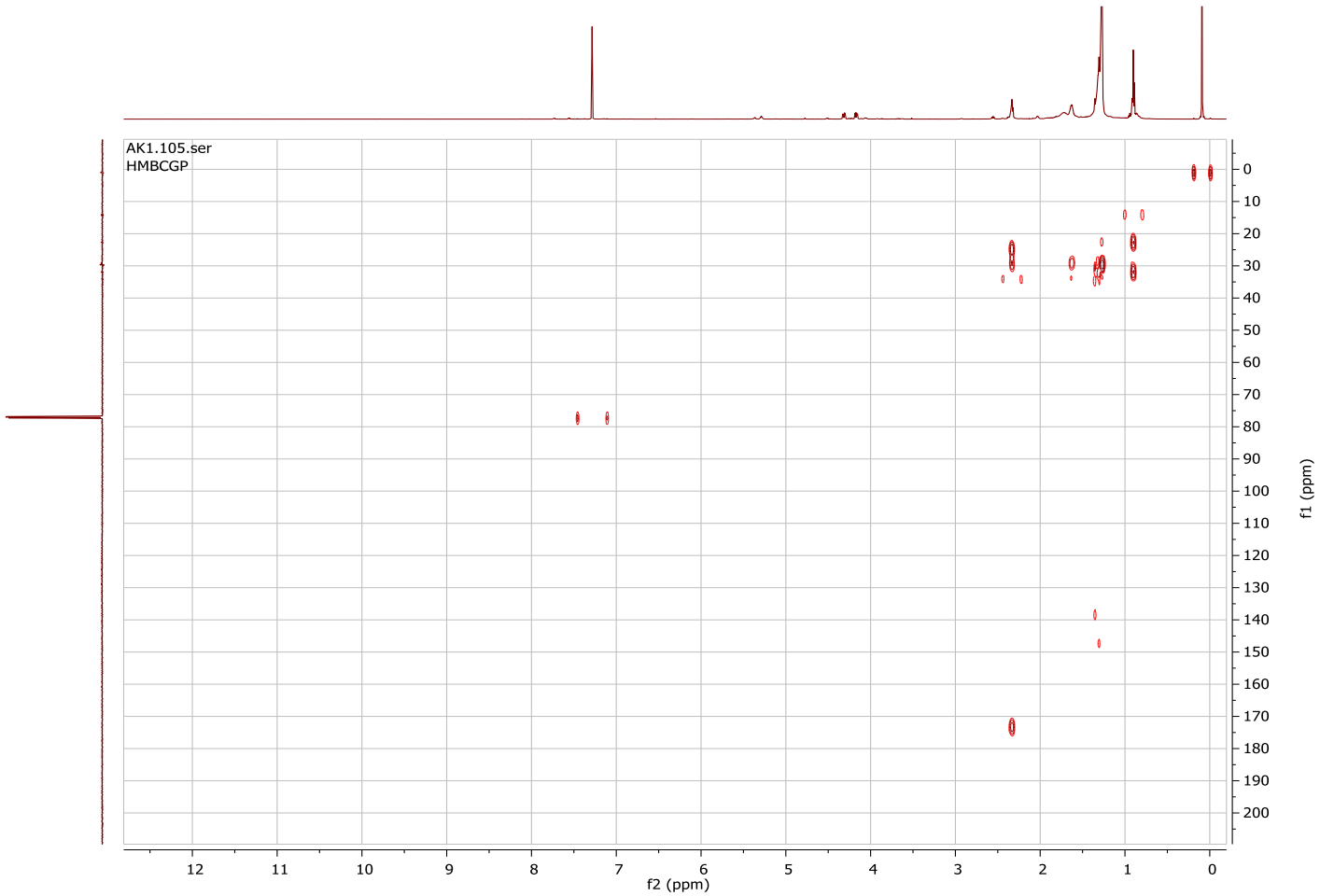


Figure 4.15: HMBC of c

4.2.4 Characterisation of compound d

Compound d (20 mg) was gotten from column as a colorless oily paste. Composition $C_{22}H_{40}O_4$, Molecular weight: 368. ESI: (Positive ion probe): 391 $[M+Na]^+$ Proton and caron-13 NMR are presented in Tab. 4.7. The structural elucidation is in line with that reported by Jang *et al*. (2016).

IR spectrum [KBr, Vmax (cm^{-1})]: 2907.8 and 2842.2 (-C-Hstretch), 1703 (-C=O) of a carboxylic acid, 1468.2 and 1182.6 (C-Ostretch). The IR spectrum show two peaks at 2907.8 and 2842.2 cm^{-1} indicate the presence of carbon-to-hydrogen stretching vibration. The maximum absorption shown at 1703 cm^{-1} suggest the presence of a carbonyl group of an acid. The peaks at 1468.2 and 1182.6 indicate the presence of C-O stretch of a carbonyl group (Fig. 4.19).

The proton Nuclear Magnetic Resonance spectrum (400 MHz, $CDCl_3$) of compound d (Fig. 4.16) revealed deshielded protons of methine group representing the unsaturation of the compound appearing at δ 5.38 as a quartet, J = 4.0 Hz. Slightly downfield methylene protons at δ 4.17, appearing as quartet J = 4.0 Hz, belong to H-6. Another methylene peak at δ 2.37, which appears as a triplet, J = 4.0 Hz, representing H-2. A methylene peak at δ 1.65, which was found to appear as a quartet, J = 4.0 Hz, belongs to H17 and H21.At δ 1.27 is a multiplet signal representing34 H, being H3, H4, H6 to H11, H14 to H18 and H19 to H20.

A terminal methyl proton integrating as 3H, appearing at δ 0.90 (J = 4.0 Hz) could represent the terminal CH_3 at C22.

Compound d (Figure 4.17), possesses a total of twenty-two carbons, two quaternary carbon δC 179.0 (C1) and 173.9 (C5), two methine carbons at δC 131.2 (C12) and 129.3 (C13), other slightly downfield carbon at δC 68.4 (C-6), 34.1 (C-2), 31.9 (C-14 and C-20), 24.9 (C-3), 29.3 (C-9), a long chain of methylene carbon at δC 27.2 appearing as multiplet, representing (C7 to C8, C10 and C15 to C19), 22.7 (C21). A methyl carbon at δC 14.1; which could be given to the terminal sp^3 hybridized carbon at C22.

The HMBC spectrum, (Figure 4.18), correlations were observed between δH 2.37 and δC 179.1 (C1)/ δC 31.9 (C-4)/ 24.9 (C-3)/27.2 (C-7). Also, a correlation exists between a proton at δH 1.27 and δC 27.2 (C-19)/ 22.7 (C-21)/ 14.1 (C-22). This confirms the occurrence of a carbonyl carbon group at C1. Another correlation between δH 0.81 and δC 24.7 (C-14)/ 31.9 (C15) and 14.1 (C16). This confirms a sp^3 hybridized carbon at the terminal part of the aliphatic chain. The last HMBC correlation was observed between δH 0.81 and δC 14.1 (C22)/ δC 22.7 (C21/ 31.9 (C20), which suggest the presence of a methyl group at the terminal of the aliphatic carbon chain.

The ESI peak at 391 represents $[M+Na]^+$, which confirms the compound 15-(heptanoyloxy)pentadec-9-enoic acid, with molecular mass of 368, with the formula $C_{22}H_{40}O_4$. (Figure 4.20)

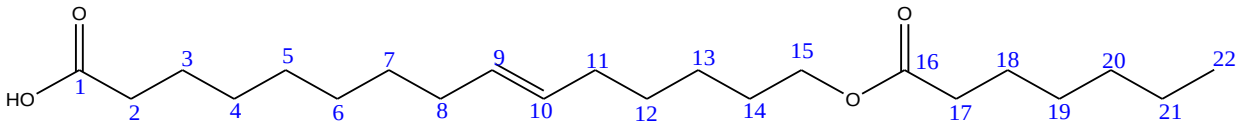
The assignments of carbon to proton was done using DEPT/HSQC, the compound; 15-(heptanoyloxy)pentadec-9-enoic acid, resembles a synthetic compound reported by Jang *et al*. (2015), in terms of the functionality, however, there is little variation only in the chain length of the compound. The compound d is being isolated for the very first time from natural source.

Tab. 4.7: Proton and carbon-13 NMR (400 MHz) spectroscopic data of compound d as compared with existing literature*

	Chemical shifts [ppm] /Coupling constants [Hz]				
S/N	1H NMR	13C NMR	S/N	1H NMR	13C NMR
1	--	179.1,Qc	16	1.27, m	27.2, CH_2
2	2.37 (t, *J* = 4.0)	34.1, CH_2	17	1.65 (q, J =4.0 Hz)	22.7, CH_2
3	1.27, m	24.9, CH_2	18	1.27, m	27.2, CH_2
4	1.27, m	31.9, CH_2	19	1.27, m	27.2, CH_2
5	--	174.0,Qc	20	1.27, m	31.9, CH_2
6	4.17 (q, *J* = 4.0)	68.4,CH	21	1.65 (q, *J* =4.0 Hz)	22.7, CH_2
7	1.27, m	27.2, CH_2	22	0.90 (t, *J* = 4.0 Hz)	14.1, CH_3
8	1.27, m	27.2, CH_2			
9	1.27, m	29.3, CH_2			
10	1.27, m	27.2, CH_2			
11	1.27, m	31.9, CH_2			
12	5.38 (q, *J* = 4.0)	131.2,CH			
13	5.38 (q, *J* = 4.0)	129.3,CH			
14	1.27, m	31.9, CH_2			
15	1.27, m	27.2, CH_2			

* Reported literature Jang *et al*. (2015). Qc = quaternary carbon, s = singlet, bs = broad singlet, t = triplet, m = multiplet, *J* = coupling constant (Hertz).

Compound d: 15-(heptanoyloxy)pentadec-9-enoic acid



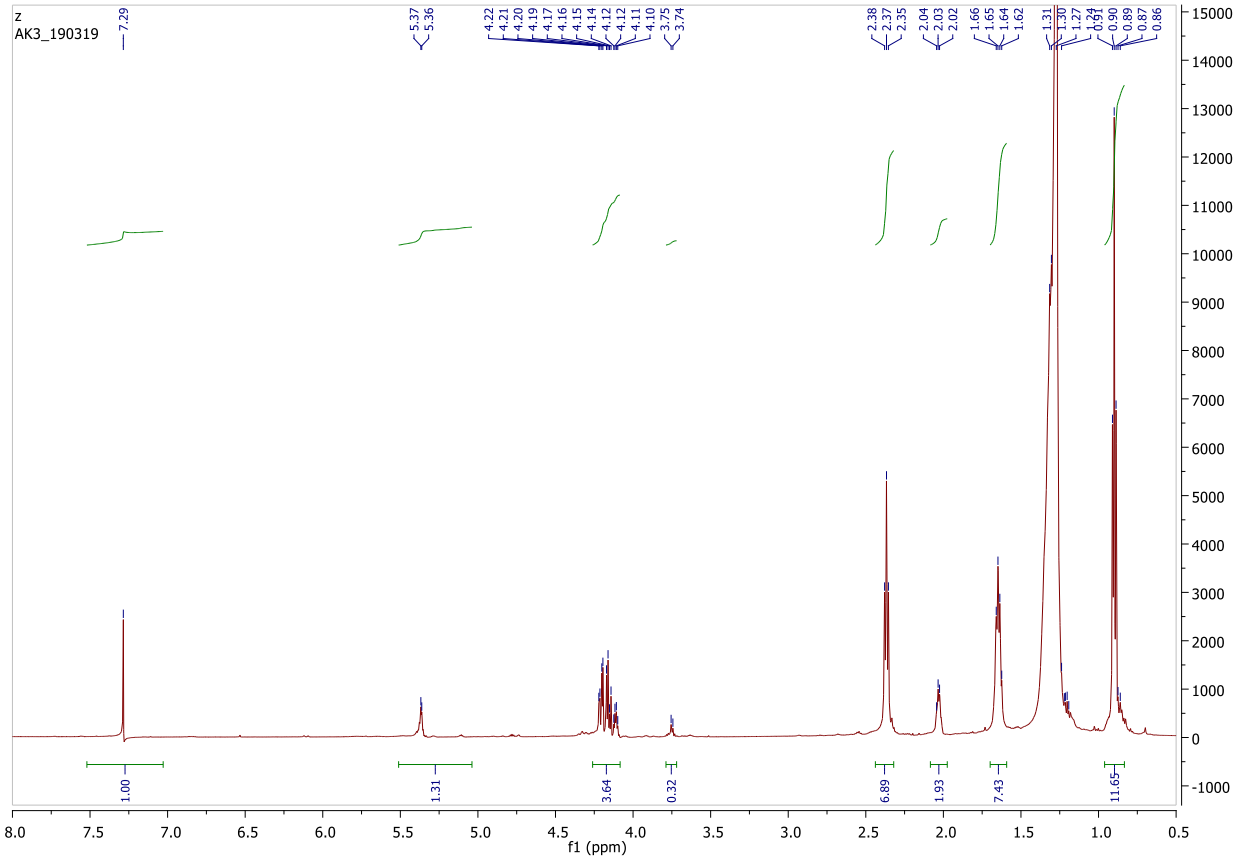


Figure 4.16: Proton NMR of d

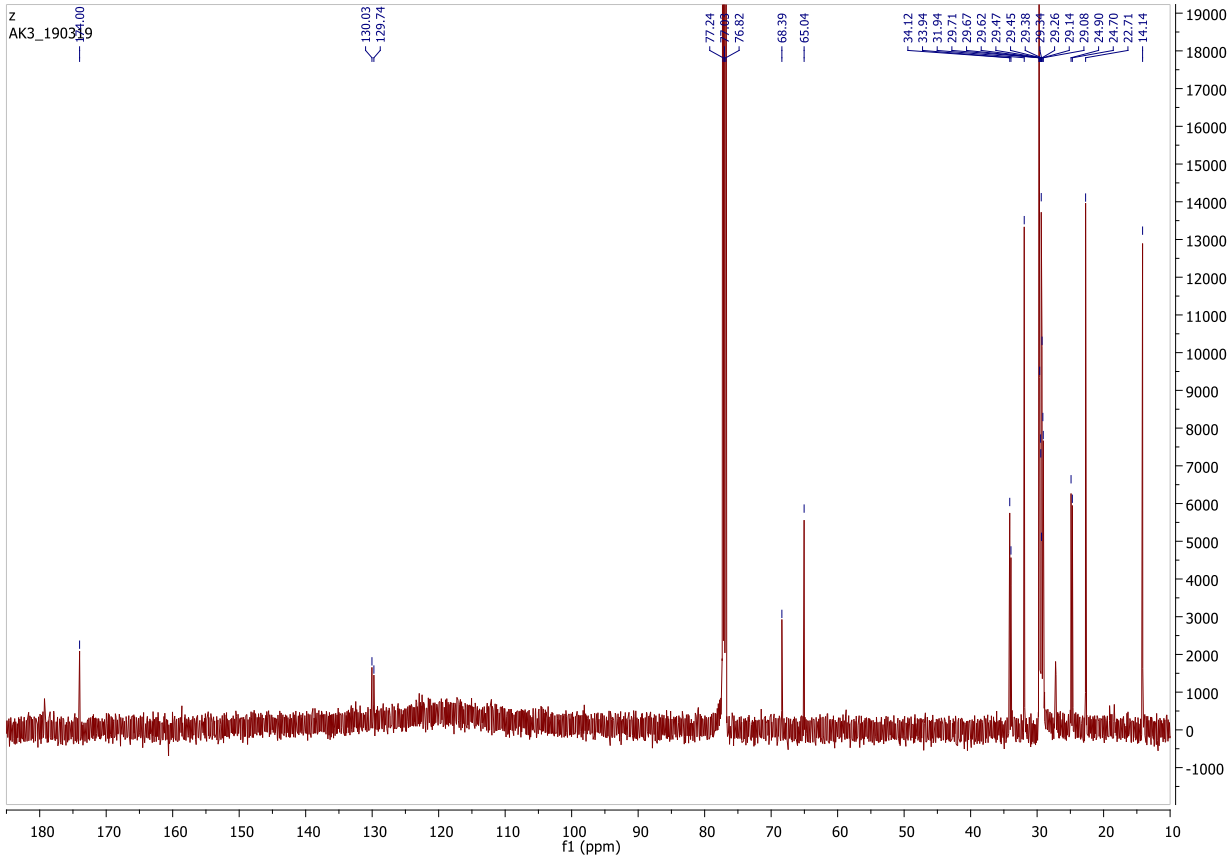


Figure 4.17: Carbon-13 NMR of d

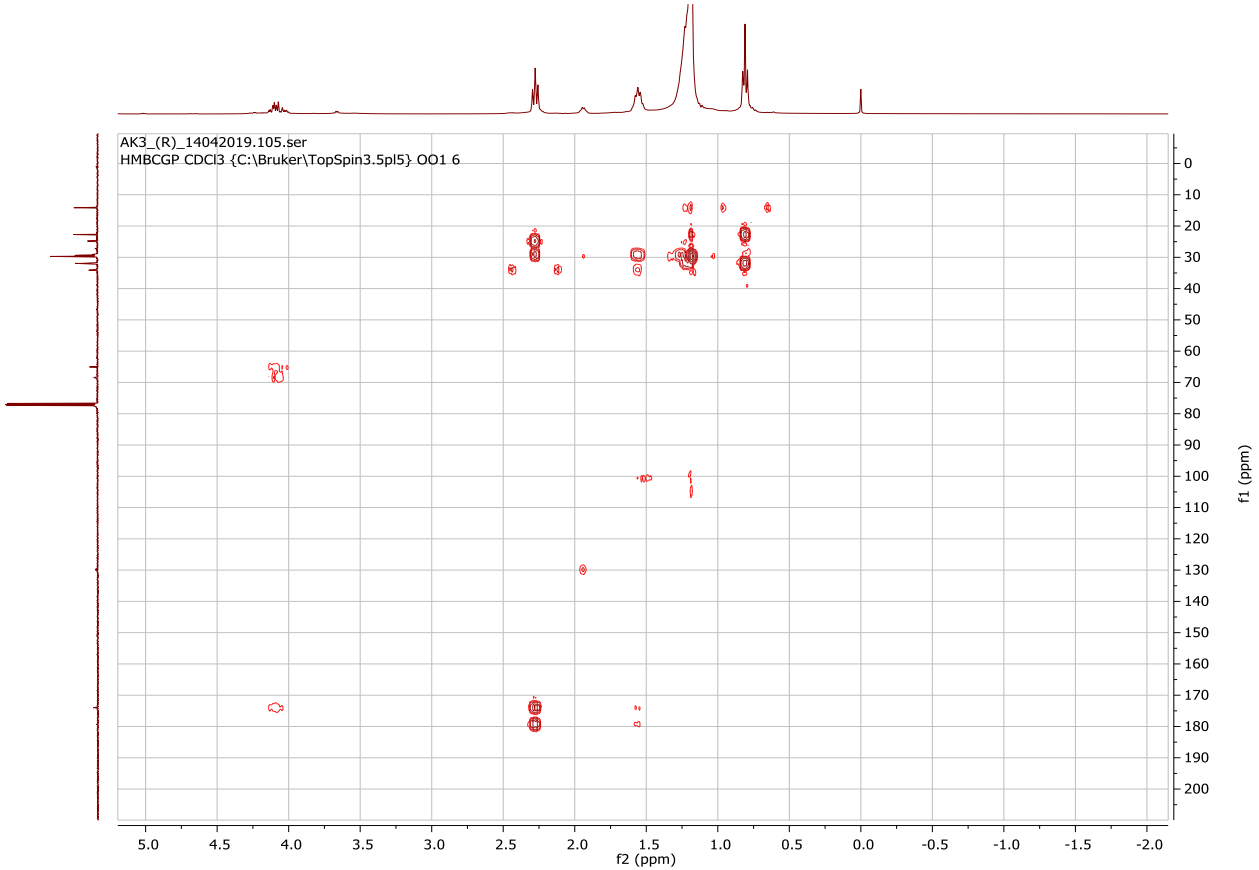


Fig 4.18: HMBC of d

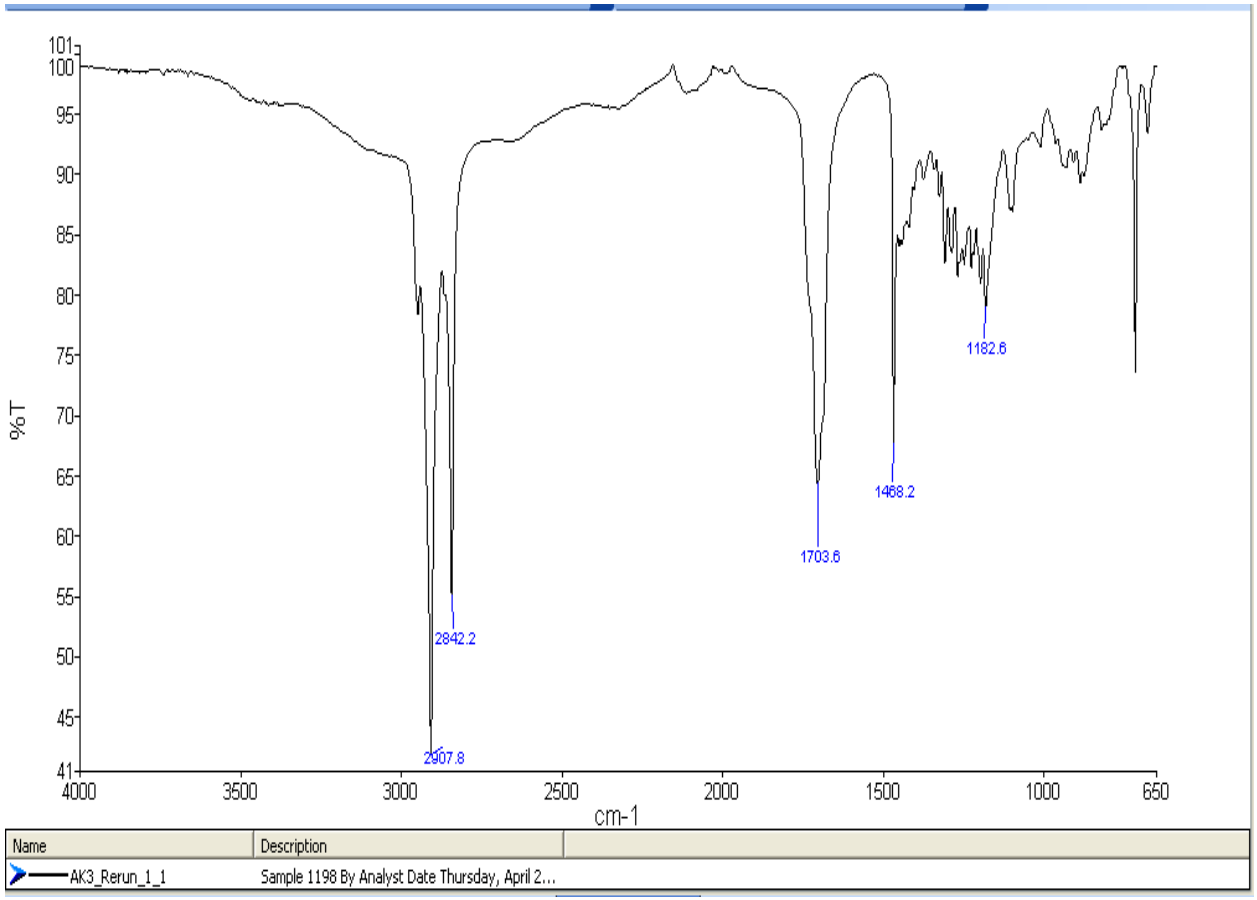


Figure 4.19: IR [KBr] of d

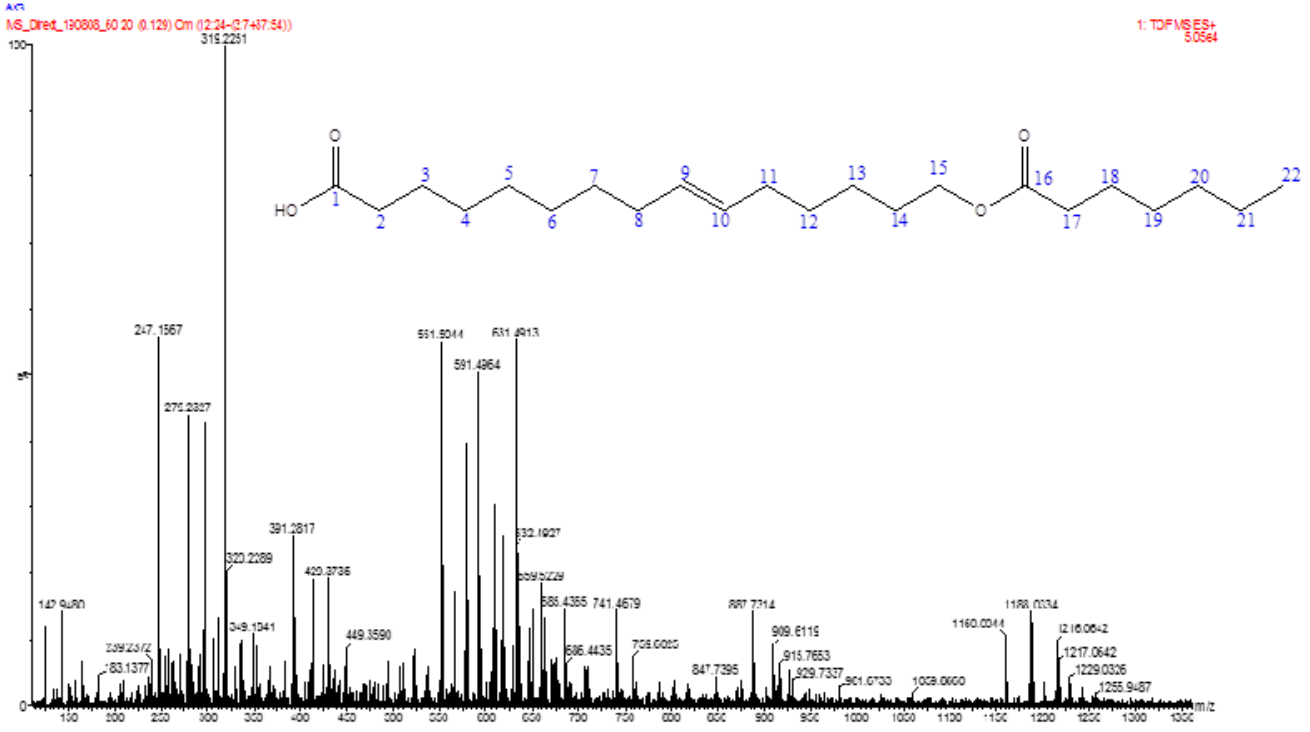


Fig 4.20: Mass spec. of d

4.2.5 Characterisation of compound e

Compound e (35 mg) was obtained as a white oily paste. Composition $C_{22}H_{32}O_4$, Molecular weight: 360. LC-MS: (Positive ion probe): 383 $[M+Na]^+$.Proton and carbon-13 NMR are presented in Tab. 4.8. Assignments of the compound is in agreement with that reported by Aamer, (2003).

IR spectrum (Figure 4.25), [KBr, Ѵmax (cm^{-1})]: 2949, 2843 and 2908(-C-$H_{stretch}$), 1710 (-C=Ostretch), 1425 and 1358 (CH_2 bending vibration), 1250 (C-O-C stretch), 890, 845, 824, 794, 716 (=C-H out of plane). The IR spectrum shows a peak at two peaks at 2949, 2843 and 2908(-C-Hstretch) indicate the existence of a carbon-to-hydrogen stretching vibration. Absorption maximum at 1710cm^{-1} suggest the presence of a carbonyl moiety of a conjugated ester unit. The peaks at 1425 and 1358 (CH_2 bending vibration), represents a long straight chain aliphatic system at the side chain at position C-3 on the flavone ring. 1250 (C-O-C stretch) indicating the presence of an ester group), 890, 845, 824, 794, 716 (=C-H out of plane) of aromatic substituted ring system. Which confirms the substitution at point C3, C6 and C8 of the chromene unit.

Compound e, Figure 4.22, possesses a total of twenty-two carbon atoms, six quaternary carbon δC 177.6 (C1), 162.7 (C3), 160.5 (C6), 156.1 (C8), 138.8 (C10) and 110.9 (C9), three methine carbon atoms δC 110.9 (C4), 94.9 (C7) and 92.8 (C5), ten methylene carbons δC [36.9 (C1′), 31.9 (C9′), 30.9 (C2′), 29.7 (C3′, C-4′, C5′, C6′, C7′ and C8′) 23.5 (C10′)] and a methyl carbon δC 14.1 (C11′). Two methoxy carbons δC 55.9 (C-6) and 57.2 (C-8).

The proton Nuclear Magnetic Resonance spectrum (400 MHz, $CDCl_3$) of e, (Fig. 4.21) showed a multiplet signal at δ 1.23, which is for 16 H of the aliphatic system. Three slightly downfield proton CH_2 signals appearing at δ 2.28 (H-1′), 1.94 (H-2′) and 1.56 (H-9′). The proton Nuclear Magnetic Resonance revealed a downfield aromatic signals at δ 6.36 ppm, representing H-7, which meta coupled with H-9. Another aromatic peak at δ 6.22 (H-9), δ 6.11 (H-4). A methyl proton at δ 0.81 (H-11′). Two methoxy peaks making an integration of 6H directly attached to C6 and C8 positions respectively.

HMBC correlations of Compound e (Figure 4.23) shows a strong correlation of δH 1.56 (H-10′) with δc 31.9 (C-9′) and 23.5 (C-10′), 2.28 (H-1′) with 177.6 (C-1), 3H with δH 3.81 at position C-6 correlated with 160.5 (C-6), and another 3H with δH 3.81 at position C-6 correlated with 156.1 (C-8).This indicates the presence of two methoxy groups at position C-6 and C-8 respectively.

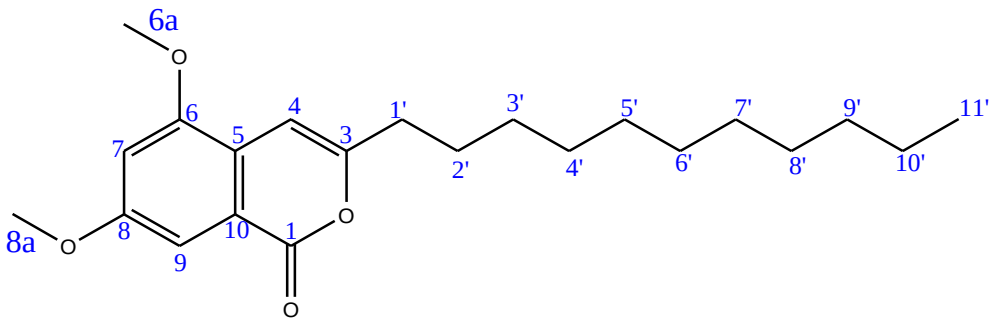
Liquid Chromatogaraphy-Mass Spectrometry (LC-MS) experiment of the Compound e was also performed. The peak at 383 $[M+Na]^+$, confirms the signal of $[M]^+$ at molecular mass*m/z* 360, which suggests $C_{22}H_{32}O_4$. Compound e appeared at 383.2087, which represents the $[M+Na]^+$ at Rt 9.74 min shown in (Fig. 4.24). This is in agreement with the reported, Aamer, (2003). (Figure 4.24).

Tab. 4.8: Proton and Carbon-13 Nuclear Magnetic Resonance (400 MHz) spectroscopic data of Compound e as compared with existing literature*

	Chemical shifts [ppm] /Coupling constants [Hz]		
S/N	**1H NMR**	**13C NMR**	**HMBC**
1	--	177.6,Qc	
3	--	162.7,Qc	
4	6.11 (d, J=12.0 Hz)	110.9,CH	
5	--	138.8, Qc	
6	--/3.81, s	160.5/55.9,Qc/CH_3	160.5 (C6)
7	6.36 (d, J = 4.0 Hz)	92.8,CH	
8	--/3.81,s	156.1/57.2Qc/CH_3	156.1 (C8)
9	6.22 (d, *J* = 4.0 Hz)	94.9,CH	
10	--	110.9,Qc	
1′	2.28 (t, *J* = 8.0 Hz)	36.9, CH_2	177.6 (C1)
2′	1.94,	30.9, CH_2	
3′	1.23, m	29.7, CH_2	
4′	1.23, m	29.7, CH_2	
5′	1.23, m	29.7, CH_2	
6′	1.23, m	29.7, CH_2	
7′	1.23, m	29.7, CH_2	
8′	1.23, m	29.7, CH_2	
9′	1.23, m	31.9, CH_2	
10′	1.56, m	23.5, CH_2	31.9 (C9′), 23.5 (C10′)
11′	0.81 (*J* = 8.0, 16.0 Hz)	14.1, CH_3	

* Reported literature; Aamer, (2003). Qc = quaternary carbon, s = singlet, bs = broad singlet, t = triplet, m = multiplet, *J* = coupling constant (Hertz).

Compound e: 6,8 dimethoxy-3-undecyl-1H- [2]benzopyran-1-one



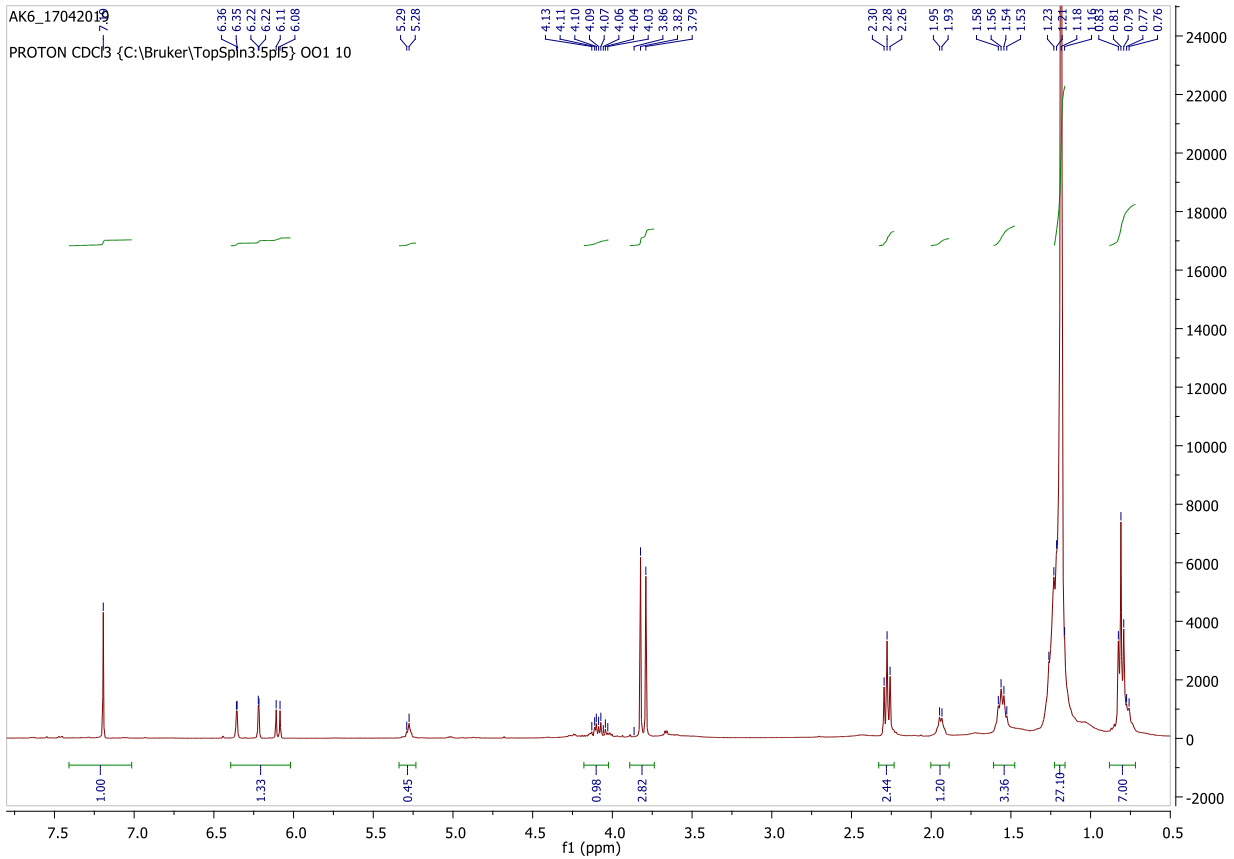


Fig 4.21: Proton NMR of e

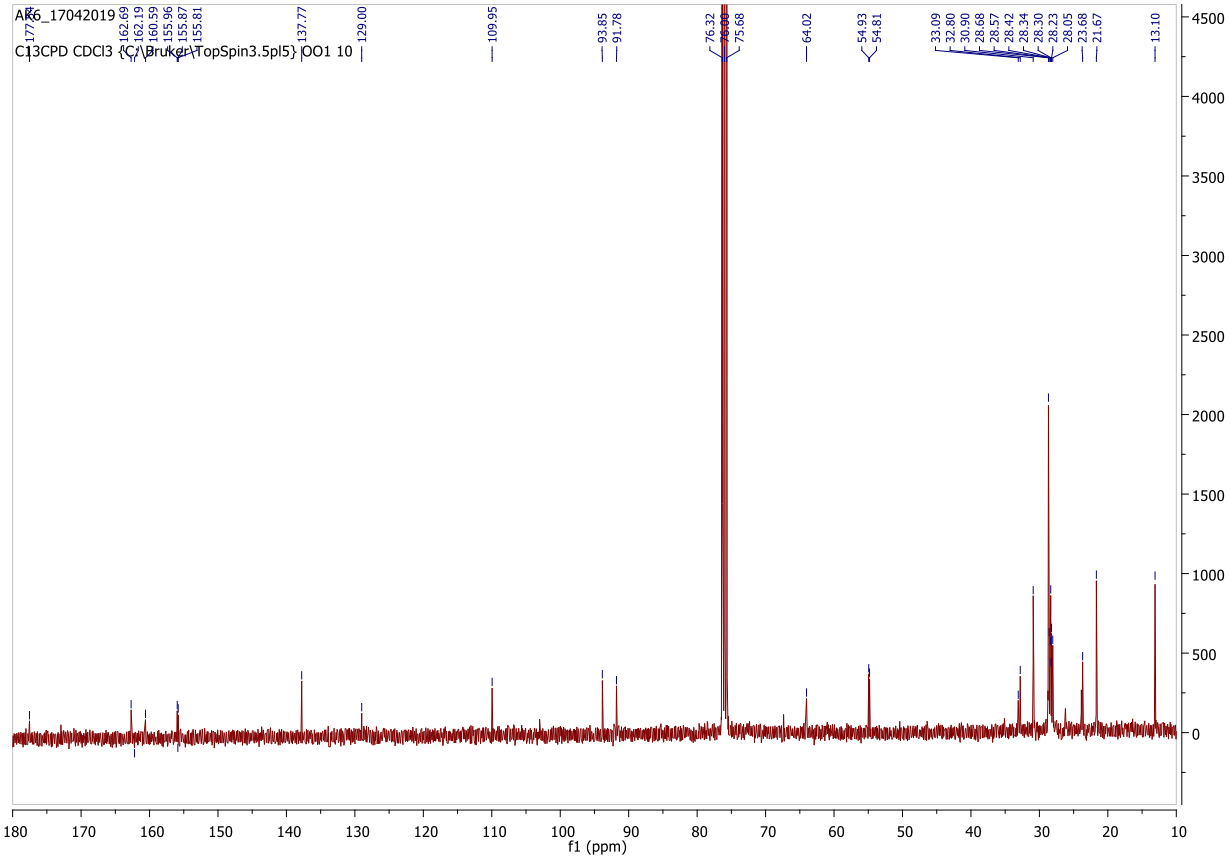


Figure 4.22: Carbon-13 NMR of e

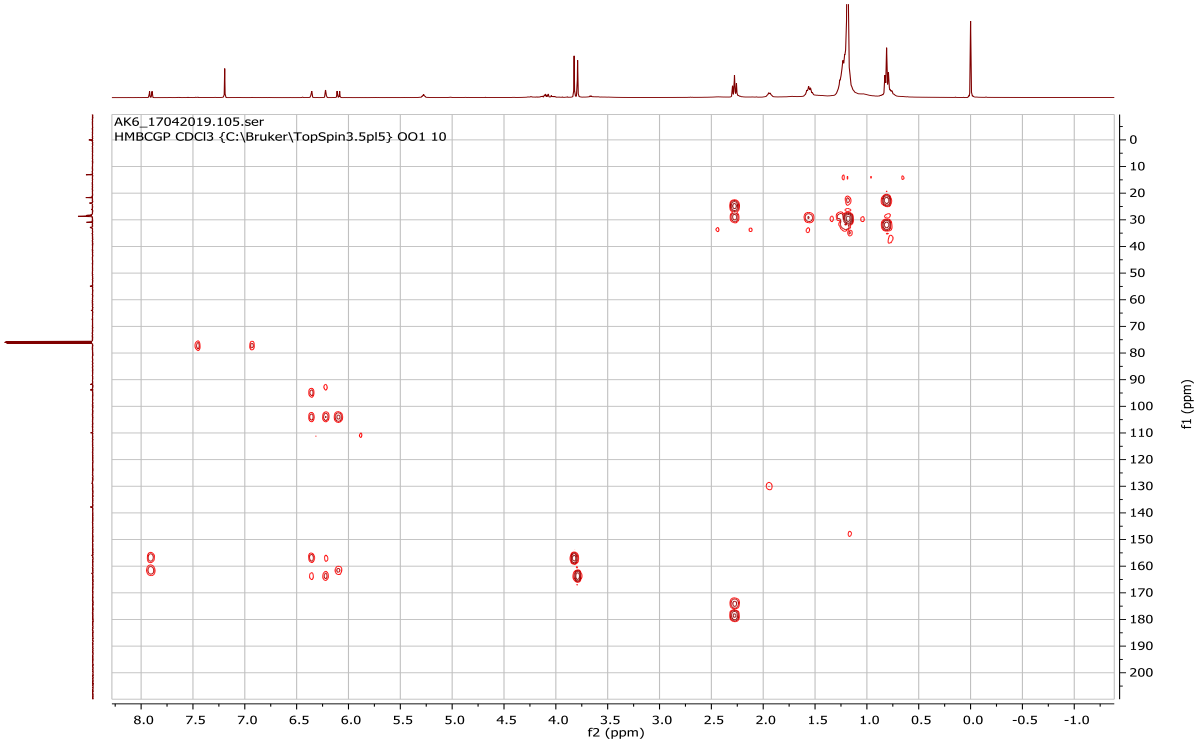


Figure 4.23: HMBC of e

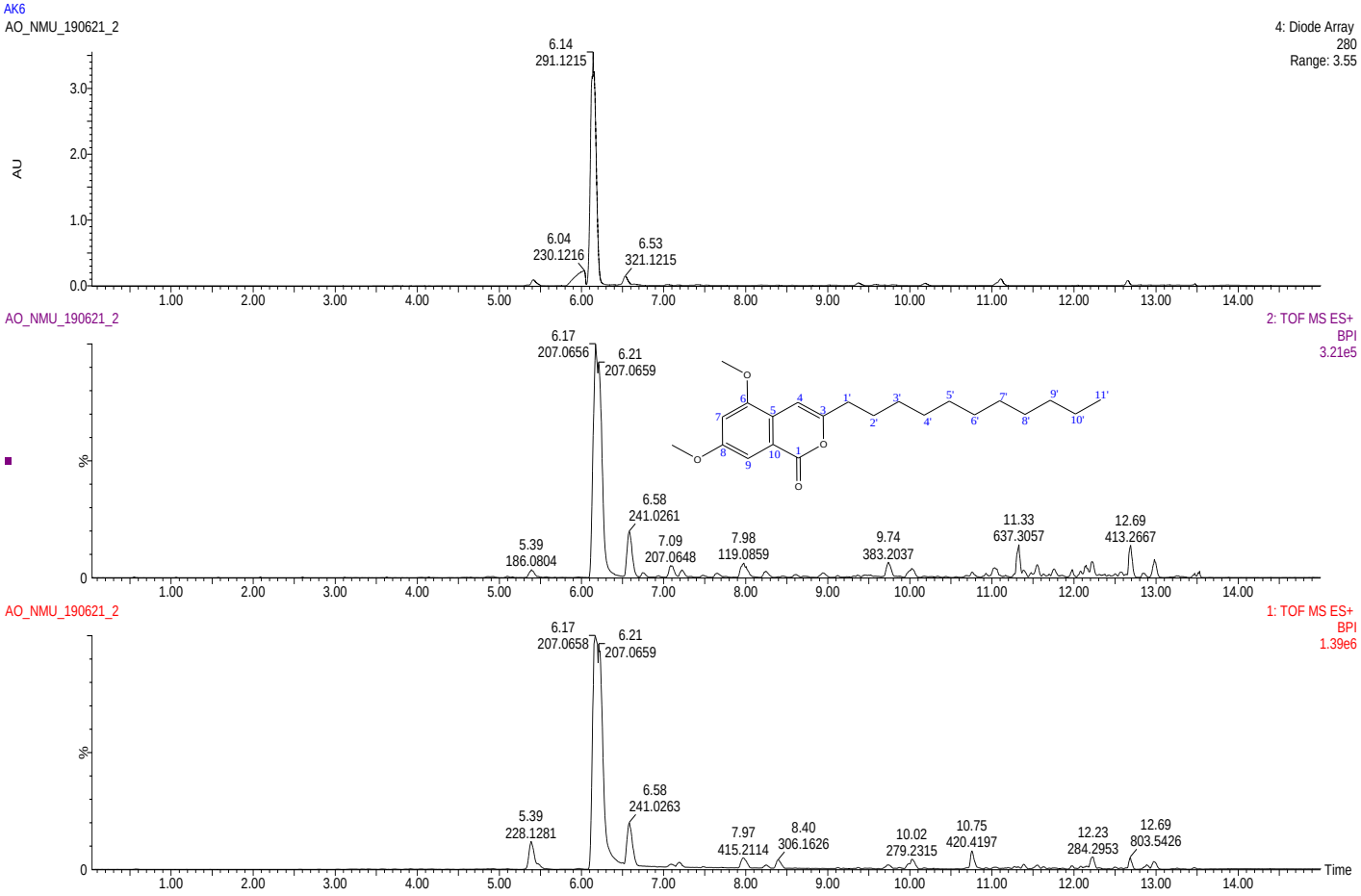


Fig 4.24: Mass spectrum of Compound e

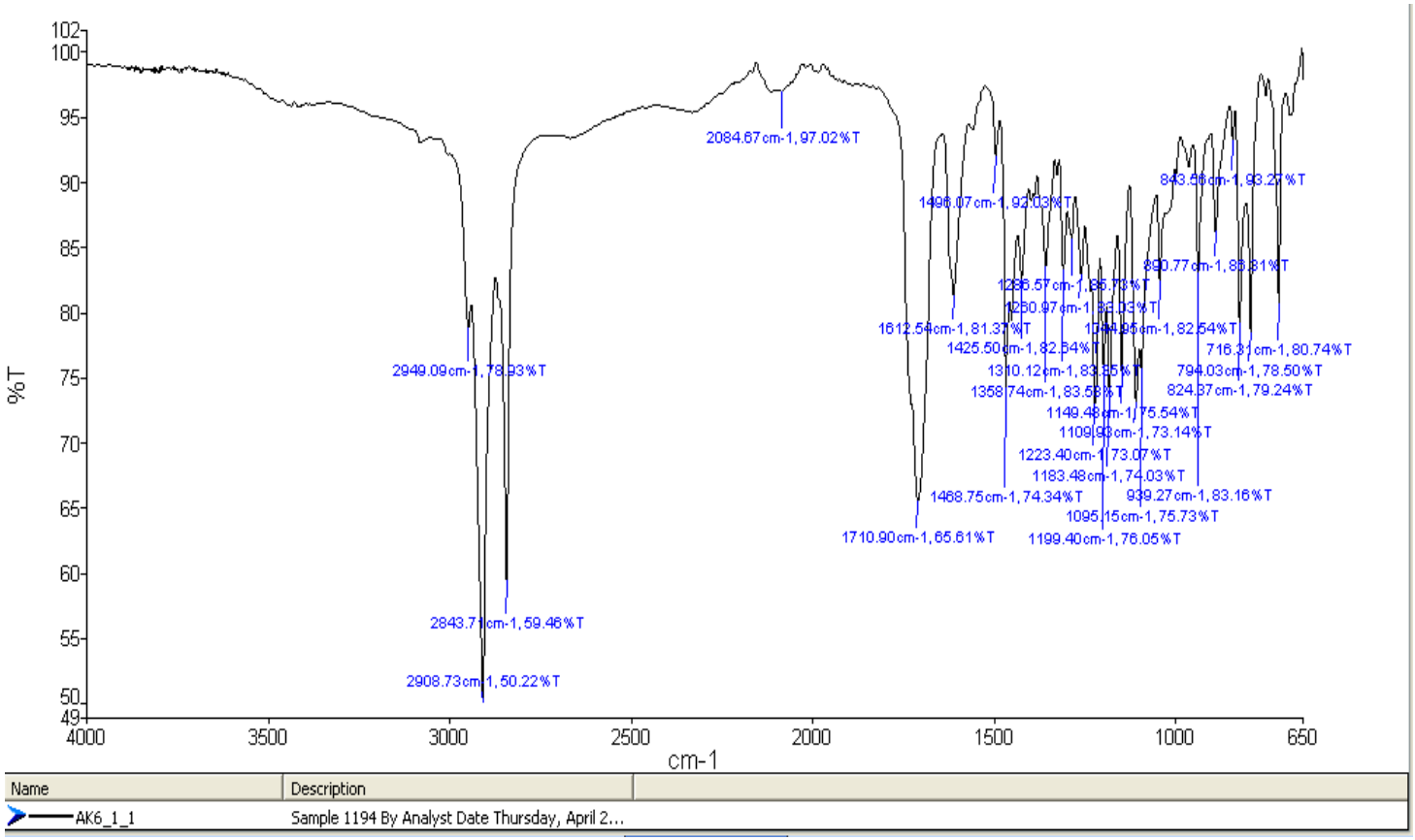


Fig. 4.25: IR [KBr] **of e**

4.2.6 Discussion of *Citrus limon* fractionation

Citrus limon n-hexane extract were subjected to open chromatography in an attempt to separate the active constituents. Fractionation of the hexane extract of *C. limon* resulted in fractions CLH1, CLH2, CLH3, CLH4, CLH5, CLH6, CLH7, CLH8, CLH9, CLH10 with percentage repellent activities as shown in Table 4.3. The fractions, CLH1 and CLH2 were further purified leading to the isolation of a, b and c, d, e respectively. The isolated compounds were tested against adult female Anopheles gambiae mosquitoes, and the percentage repellency results are shown in Tab. 4.9. The repellencies of compound d (77.87%) and e (75.15%) are the most promising at 5 mg/mL optimum concentration. Compound e, a flavonoid, 6,8 dimethoxy-3-undecyl-1H-[2]benzopyran-1-one, has been reported for its in vitro antifungal activity against some human, animal and plant pathogenic moulds (Kazmi *et al*., 2001, Aamer, 2003), while compound d, 15-(heptanoyloxy)pentadec-9-enoic acid. a new compound being isolated for the first time from natural product has not been reported for any biological activities, except its derivatives like pentadecanoic acid, which is present as a gas chromatography- mass spectrometry constituent (gcms) in the *Cenchrus ciliaris* L. leaf extract, and is employed by pharmaceutical companies and traditional healers to producemedicinal drugs, both plant based and synthetics (Arora *et al*, 2017). Palmitic acid (compound a) with repellent effect of 65.91% and stearic acid were reported to be among the constituents in *Carica papaya* seeds extract responsible for its insecticidal activities against *Spodoptera frugiperda* (Puangsri *et al*., 2005).Saturated and unsaturated fatty acids have been reported to show biting deterrent effects against *Aedes aegypti* (L) mosquito (Ali *et al*,.2012). Plant seeds extracts containing fixed oil could be a rich source of fatty acids which seem to prevent mosquito bites (Jones *et al*., 2012).

Table 4.9: Percentage Repellency of Compounds isolated from *Citrus limon*

Volunteers	Compounds	Conc. (mg/mL)	Mean landed mosquitoes (Triplicate) Mean ± SD				% Rep
			25 minutes	45 minutes	80 minutes	120 minutes	
	a	5.00	6±1	9.33±1.53	13.33±2.52	20.67±3.06	65.91%
1	b	5.0	5±0	10±1.73	15.33±2.31	23±2.64	63.63%
	-ve Control	Acetone	15.67±3.06	29.33±3.21	54.33±11.54	70.33±10.50	0.00
	c	5.00	6.67±1.53	11±2	16.67±1.53	24±2.65	51.69%
	d	5.00	2±1	3.33±1.55	6.33±1.55	10.67±2.08	77.87%
1	e	5.00	3±1	5±1	8.67±1.55	12.33±1.53	75.15%
	-ve Control	Acetone	15.33±0.71	24.67±0.58	36.33±1.53	49.67±2.08	0.00
+ve Control		DEET (20%)	0.00	0.00	0.00	0.00	100.00

a – Palmitic acid

b – 14-Oxotricosanoic acid

c – n-Octyl stearate

d – 15-heptanoyloxy pentadec-9-enoic acid

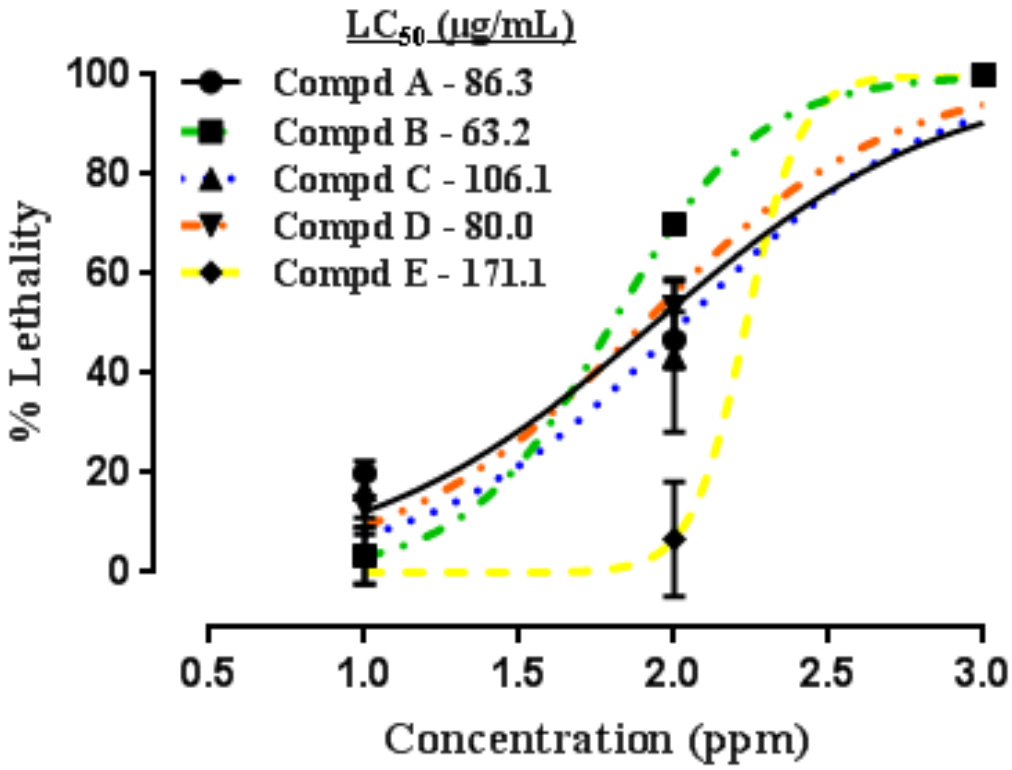
e – 6,8-dimethoxy-3-undecyl-1H-(2)benzopyran-1-one

4.3 Brine shrimp lethality test (Cytotoxicity)

The n-hexane fraction (100%) and the isolated compounds were assayed for their brine shrimp lethality. The assay showed that compounds (a, b and d), showed some level of toxicity with LC_{50} values less than 100 µg/mL, while compounds c and e are considered safe because of their LC_{50} values, that are greater than 100 µg/mL as shown in Table 4.10. Preliminary assessment of plants and their isolated compounds for their cytotoxicity can be carried out using brine shrimp lethality assay. This can be done to access their safety to biological cells prior to human consumptions. The assay can also be used to detect medicinal properties such as antimicrobial and antitumor activities of plants and their compounds, based on the LC_{50} values they exhibited (Anderson *et al*., 1988). The hexane fraction and all the isolated compounds showed varying cytotoxic activities on thelarva. The observed mortality was found to be directly proportional to the concentration. Toxicity level shown by compounds a, b and d (LC_{50} value< 100 µg/mL) could be an indication of the existence of some cytotoxic compounds in the extracts (Meyer *et al*., 1982). However, the LC_{50} ranges from 63.2 to 171.1 µg/mL, so compounds c and e with LC_{50} values of 106,1 and 171.1 µg/mL showed some safety margin (LC_{50} value > 100 µg/mL), hence they can be potentially recommended as safe repellent compounds.

Table 4.10: Brine shrimp cytotoxic mortality (%) and LC_{50} values of n-Hexane fraction (100%) and compounds

	(%Mortality) Concentrations			
Samples	1000 µg/mL	100 µg/mL	10 µg/mL	LC_{50} µg/mL
Hexane fraction (100%)	(100%)	(80%)	(23.33%)	29.24
Compound a	(100%)	(46.67%)	(20%)	86.3
Compound b	(100%)	(70%)	(0%)	63.2
Compound c	(100%)	(43.33%)	(16.67%)	106.1
Compound d	(100%)	(53.33%)	(13.33%)	80.0
Compound e	(100%)	(3.33%)	(0%)	171.1



Percentage lethality of compounds A-E against brine shrimp

Fig. 4.26: Percentage Lethality of Compounds A-E against brine shrimp

4.4: Scheme for bio-guided isolation of repellent compounds from *Dennettia tripetala* fruits

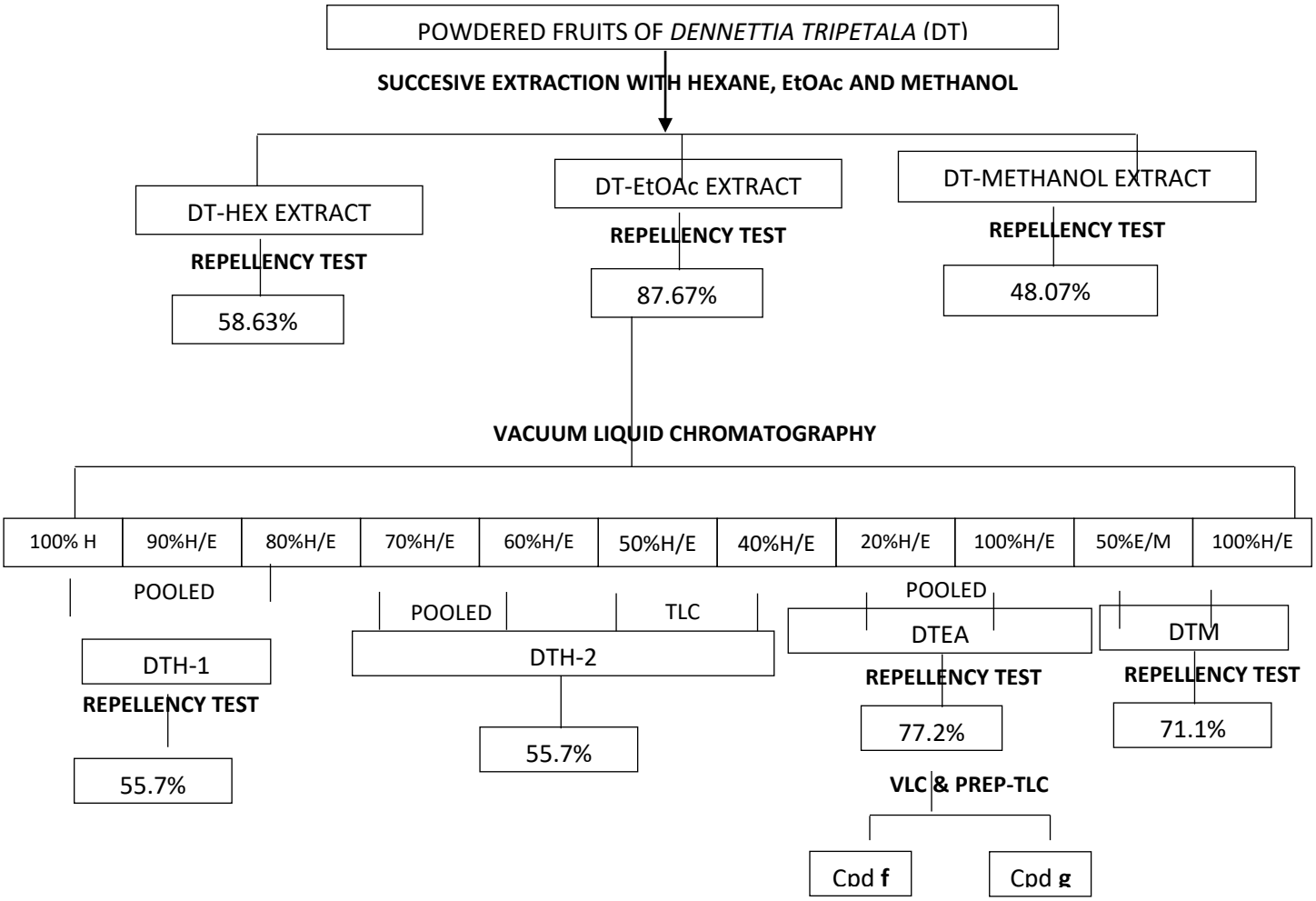


Fig 4.27: Scheme of extraction and isolation of compounds from *D. tripetala*

Table 4.11: Percentage repellencies of fractions from *Dennettia tripetala*

Volunteers	Fractions	Conc. (mg/mL)	Mean landed mosquitoes (Triplicate) Mean±SD				% Rep (2hr)
			25 min	45 min	80 min	120 min	mean±SEM
1	+ve ctrl	Deet (20%)	0.0	0.0	0.0	0.0	100.0[a]
	-ve ctrl	Acetone	11.0	18.3±1.2	28.0±1.7	34.7±0.6	0.0
	DTEA		0.3±0.1	1.3±0.6	2±0	7.9±0.6	77.2±1.7[b]
	DTM	5.0	4.3±3.2	6±2.7	7.3±1.5	10.0±2.0	71.1±6.2[b]
1	-ve ctrl	Acetone	9.7±0.6	14.3±0.6	20.0±1.0	29.3±1.5	0.0
	DTH2	5.0	2.3±0.6	6.3±1.2	9.7±1.5	13.0±2.0	60.6±8.6[bc]
1	DTH1	5.0	3.3±0.6	6.7±0.6	10.3±0.6	15.3±1.5	55.7±5.2[c]

No significant difference between values with superscript b at $p \geq 0.05$.

-ve control = Acetone, +ve Control = Deet, DTH1 = First hexane fraction, DTH2 = second hexane fraction, DTEA = Ethyl acetate fraction, DTM = Methanol fraction

a = Value with highest activity and similar ones

b = Value with second highest activity and similar ones

c = Value with third highest activity and similar ones

4.5 Characterisation of isolated compounds from *Dennettia tripetala*

4.5.1 Characterisation of compound f

Compound f (30 mg), obtained as a white solid crystalline, with Mp = 80–82 °C. Composition: $C_{63}H_{116}O_6$ and molecular weight was found to be 968 g/mol. TOF-MS-ES+ 969 $[M + H]^+$.

C-13 and 1H-NMR assignments shown in Table 4.12 justify that reported by Hamid *et al*. (2014).

The proton Nuclear Magnetic Resonance ($CDCl_3$, 300 MHz): δ 0.89 (t, 9 H, 3 × CH3-20′), 1.25 (s, 3 × 26 H, 3 × 13 CH_2), 1.61 (bs, 3 × 7-CH_2), 2.15 (bs, 3 × 4CH_2), 2.70 (m, 3 × CH_2), 4.00 (m, 4 H, 2 × –OCH_2 of three fatty esters), 4.31 (m, 1H, –OCH 1 of three fatty esters), 5.34-5.41 (m, 2 H, OCH in CH=CH of three fatty esters, CH5 and CH6). Shown in Figure 4.27. (Hamid *et al*., 2014).

Carbon-13 Nuclear Magnetic Resonance ($CDCl_3$, 600 MHz): *δ* 14.8 (C20′), 24.7 (C18′), 25.6 (C7′), 29.5 (C8′), 27.2 (C4′), 29.7 (C9′), 130.0 (C5′), 131.9 (C6′), 29.8 (C3′), 29.7 (C17′), 29.8 (C10′), 30.1 (C11′), 29.7 (C12′), 29.8 (C13′), 29.8 (C14′), 29.8 (C1′), 29.8 (C16′), 31.9 (C19′), 34.1 (C2′), 65.1 (2 × OCH2 of three fatty esters), 68.4 (–OCH of three fatty esters), 173.9, 173.9 (C-1′, acetate moiety); (Figure 4.28), mass (in methanol): 969 $[M + H]^+$, 646 $[M-14Na]^+$, representing $C_{63}H_{116}O_6$, M.M. 968 (Hamid *et al*., 2014).

The 1H NMR revealed deshielded protons of 6 methine protons representing the unsaturation of the oleifinic protons appearing at δ 5.38 as a multiplet belonging to H-5′ and H6′ of the three branches of three fatty esters. Slightly downfield methine proton at δ 4.31, appearing as multiplet, belonging to H-2. Another methylene peak at δ 4.00, which appears as a multiplet, representing the two oxy-methylene protons belonging to H1 and H3. A methylene peak at δ 2.70, which was found to appear as a multiplet, belonging to H-4′ and 7′ respectively. At δ 1.25 is a long multiplet signal, representing 13 methylene peaks. A terminal methyl proton integrating as 3H, appearing as a triplet at δ 0.89 (J = 4.0 Hz) is for the three CH3 groups at C20′ positions.

The compound possesses a total of sixty-three carbons, three quaternary carbon δC 173.9 (C1′) and 173.9 (C1′), one downfield methine carbon δC 68.4 (C2), two oxy-methylene carbons δC 65.1 (C1 and C3), other slightly downfield carbon at δC 24.7 (C18′), 25.6 (C7′), 29.5 (C8′), 27.2 (C4′), 29.7 (C9′), 130.0 (C5′), 131.9 (C6′), 29.8 (C3′), 29.7 (C17′),

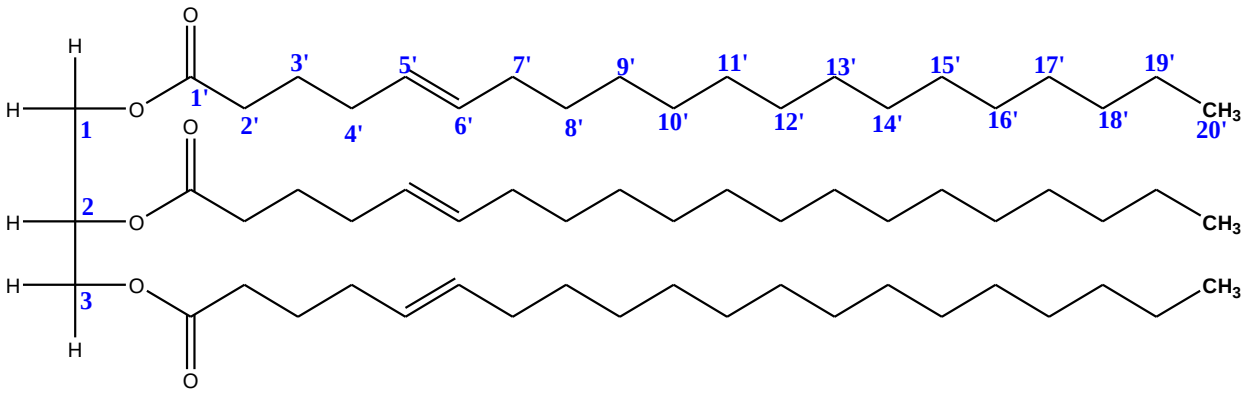
29.8 (C10′), 30.1 (C11′), 29.7 (C12′), 29.8 (C13′), 29.8 (C1′), 29.8 (C15′), 29.8 (C16′), 31.9 (C19′), 34.1 (C2′) representing a long chain of methylene carbons of the fatty ester. A methyl carbon at δC 14.8; is for the CH_3group positioned at C-22.

HMBC spectrum of compound f shows correlations of H1 (δH 4.00) with C1 (δC 65.1)/ C2 (δC 68.4)/ C1′ (δC 173.9), and this suggests the presence of an oxy-methine and two oxy-methylene carbons connected to a carbonyl ester of the triglyceride. The HMBC correlations of H-2′ (δH 2.70) with C-4′ (δC 27.2)/ C-1′ (δC 173.9) suggests the presence of a repeating methylene unit attached to an ester functional group. H-18′ (δH 1.61) correlate with C-20′ (δC 14.8)/ C-18′ (δC 24.7) and C-19′ (δC 31.9) which suggests the presence of a terminal methyl group at the fatty end of the triglyceride. The HMBC correlation furthermore reveal correlation of H4′ (δH 2.15) with C5′ (δC 130.0) suggests the presence of an olefinic double bond at C5′ (Figure 4.29).

The proton-proton COSY spectrum reveals that the olefinic signal δH 5.38 (H-6′) coupled with two methylene protons δH 2.70 (H-7′); this suggest the occurrence of an olefinic double bond between C-6′ and C-7′. Additionally, an olefinic δH 5.26 (H-5′) coupled with another methylene protons at δH 2.1 (H4′); this indicates the presence of a double bond between C-4′ and 5′. Also, a terminal methyl peak at δH 0.89 (H-20′) coupled with δH 1.25 (H-19′); this confirms the presence of the terminal methyl group which ends the fatty ester chain (Figure 4.30).

TOF-MS-ES^+ spectrum (Figure 4.31), shows a peak at 969; this represents a pseudomolecular ion peak, hence, justify $[M+H]^+$, which indicates the compound as 1,2,3-Propanetriyl tris(-5-eicosenoate), with molecular mass of 968 and formula $C_{63}H_{116}O_6$.

Compound f: 1,2,3-Propanetriyl tris(-5-eicosenoate)



Tab. 4.12: ^{1}H and ^{13}C NMR of compound f (1,2,3-Propanetriyl tris(-5-eicosenoate) ($CDCl_3$-*d*, 600Mz) as compared with existing literature*

S/N	1H NMR (ppm)	13C NMR (ppm)	S/N	1H NMR (ppm)	13C NMR (ppm)
1	4.00, m	65.1, CH_2	10′	1.25, s	29.8, CH_2
2	4.31, m	68.4, CH	11′	1.25, s	30.1, CH_2
3	4.00, m	65.1, CH_2	12′	1.25, s	29.7, CH_2
1′	--	173.9, Qc	13′	1.25, s	29.8, CH_2
2′	2.15, bs	34.4, CH_2	14′	1.25, s	29.8, CH_2
3′	2.70, m	29.8, CH_2	15′	1.25, s	29.8, CH_2
4′	1.61, bs	27.2, CH_2	16′	1.25, s	29.8, CH_2
5′	5.34-5.41, m	130.0, CH	17′	1.25, s	29.7, CH_2
6′	5.34-5.41, m	131.9, CH	18′	1.25, s	24.7, CH_2
7′	1.25, s	25.5, CH_2	19′	1.25, s	31.9, CH_2
8′	1.25, s	29.5, CH_2	20′	0.89 (t, J = 4.0 Hz)	14.8, CH_3
9′	1.25, s	29.7, CH_2			

*Reported literature Hamid *et al*. (2014). Qc = quaternary carbon, s = singlet, bs = broad singlet, t = triplet, m = multiplet, J = coupling constant (Hertz).

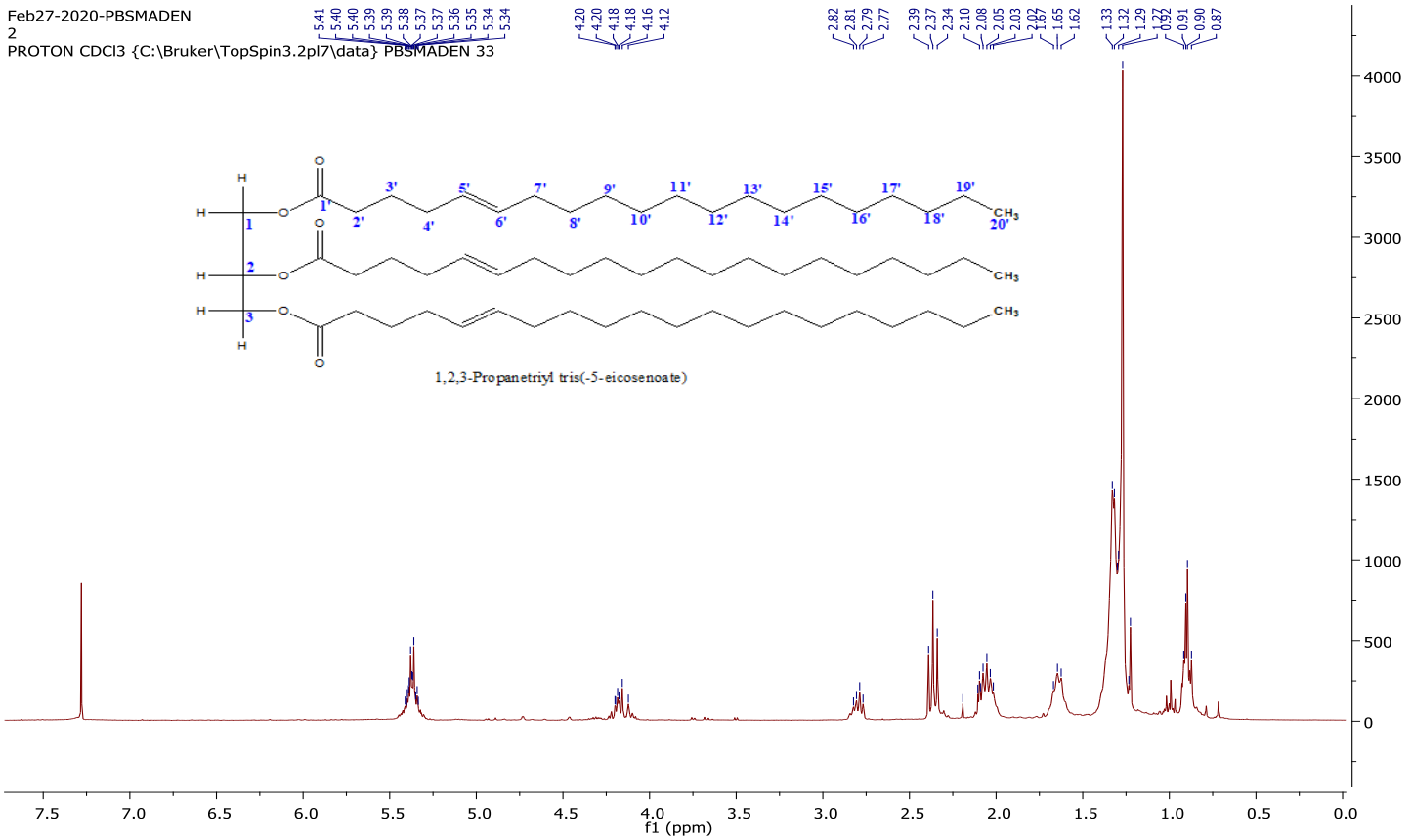


Figure 4.28: Proton NMR of f

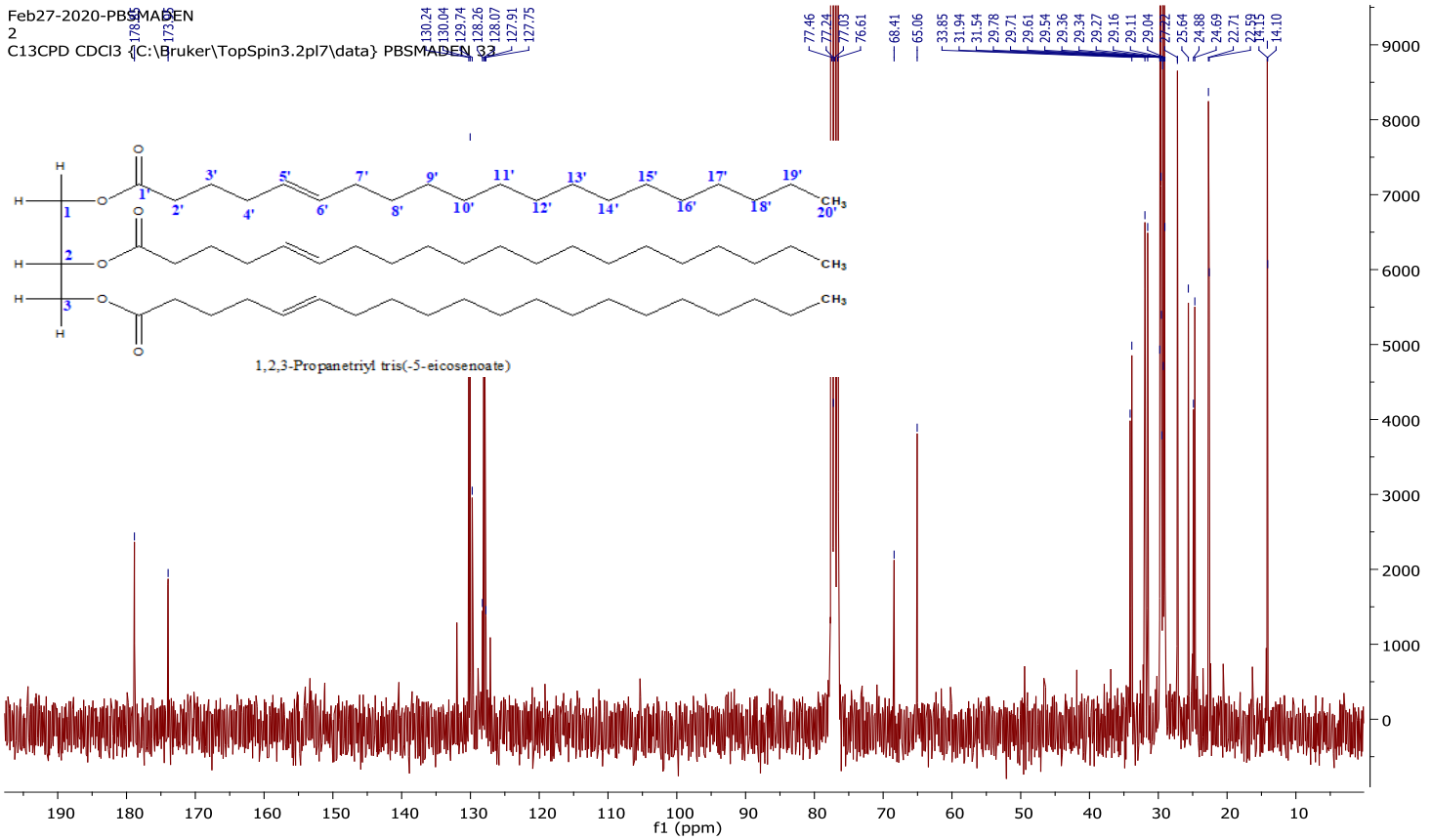


Fig. 4.29: Carbon-13 NMR f

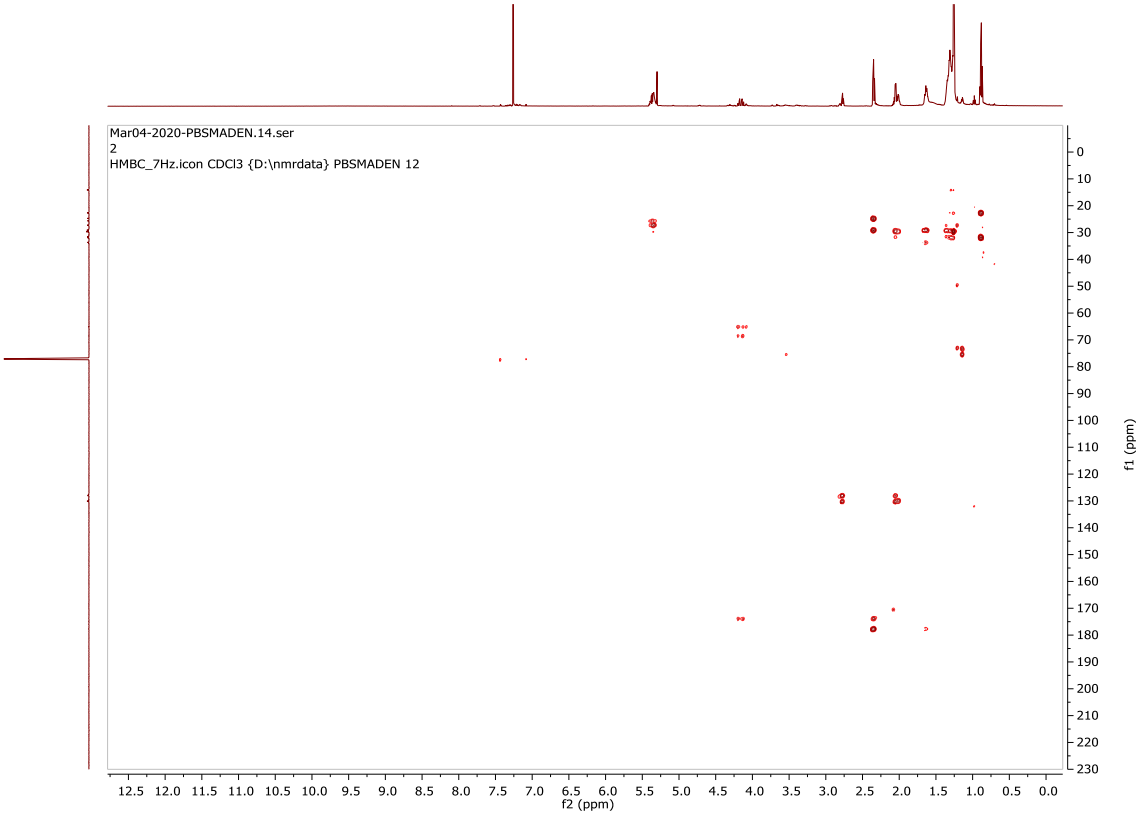


Figure 4.30: HMBC of f

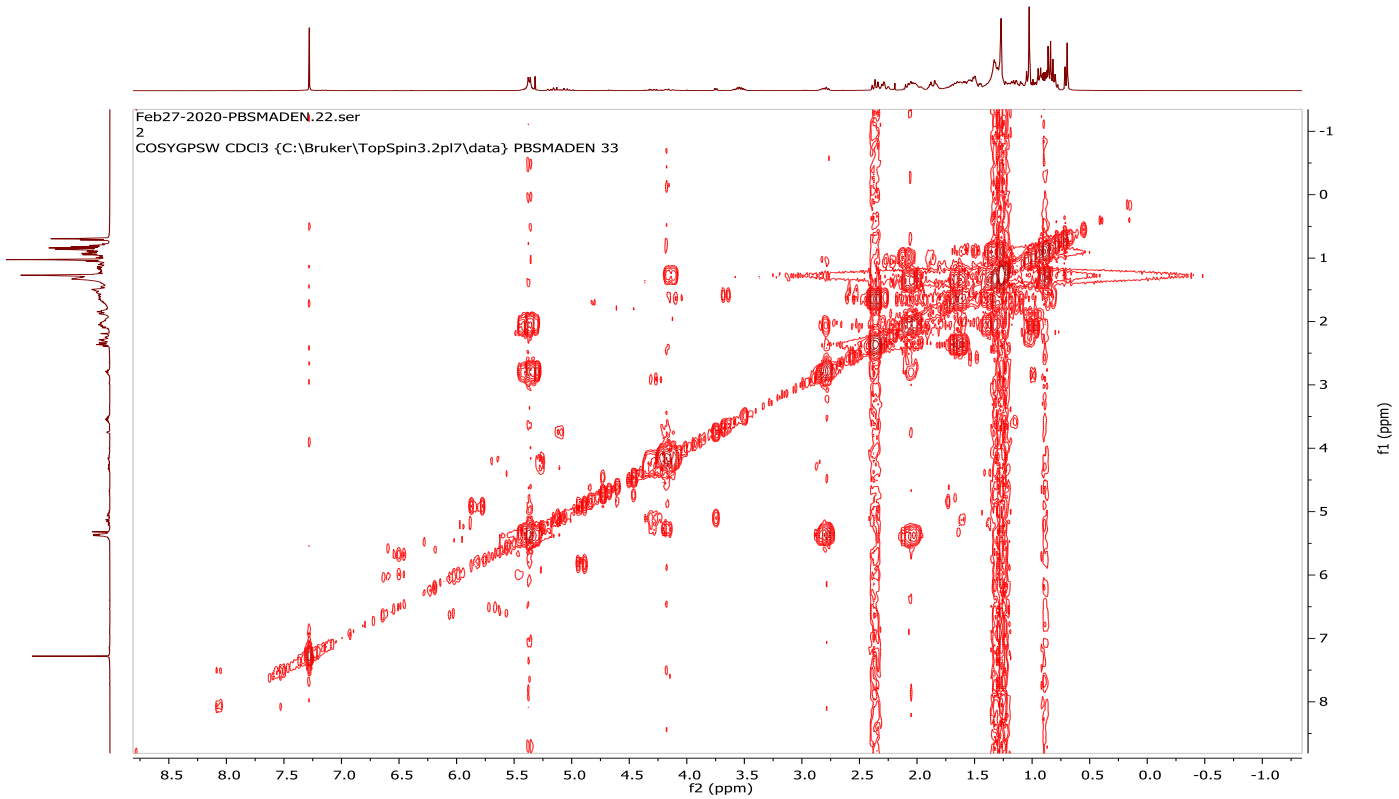
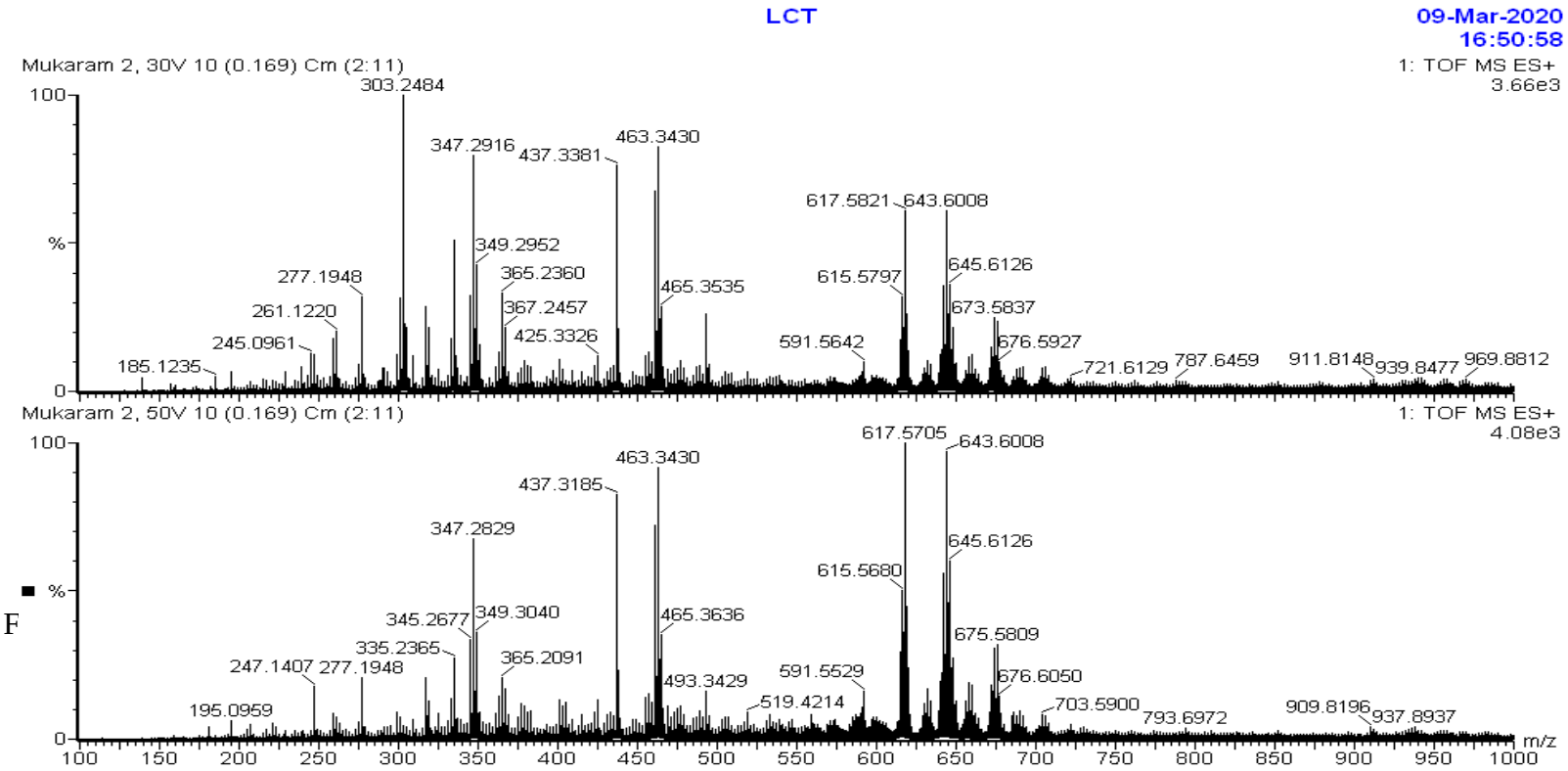


Fig. 4.31: Correlation Spectroscopy of f



F

Fig. 4.32: Mass spec. of f

4.5.2 Characterisation of compound g

Compound g (35 mg), was obtained as a brown paste, with Mp. = 60 – 62 °C, with atomic composition of $C_{18}H_{30}O_2$ and molecular weight: 278. TOF-MS-ESI+: 301 $[M + Na]^+$, with 4 degree of unsaturation; one for the carbonyl and the remaining three for the oleifinic double bonds at C6, C9 and C12 positions.

Proton and carbon-13 NMR assignments shown in Table 4.13 is in line with the report by Alamsjah *et al.* (2005) and Loreau *et al*. (2013).

Proton Nuclear Magnetic Resonance spectrum (Fig. 4.32) reveals the existence of 6 deshielded methine protons representing the unsaturation of the oleifinic protons appearing at δ 5.37 as a multiplet belonging to H6, H7, H9, H10 with H12 and H13. An upfield methylene protons at δ 2.39, appearing as multiplet, belonging to CH2 at 8 and CH2 at 11. Other methylene peaks at δ 2.81 and 2.03, which appear as a triplet with coupling constant of 12.0 Hz, representing the two methylene at positions 8 and 12 respectively. An intense multiplet peak at *δ* 1.25, typical of three 6 methylenes. A terminal methyl proton integrating as 3H, appearing as a triplet at δ 0.82 with coupling constant of 4.0 Hz was unambiguously allocated to the terminal methyl groups at C18 position.

Compound g possesses a total of eighteen carbons (Figure 4.33), one quaternary carbon δC 179.3 (C-1), which represents the carbonyl of the acid group. Oleifinic carbons in the range of 127.3-132.3 represent an sp3 hybridized double bond carbons at position C6, C7, C9, C10 with C12 and C13. Some other slightly downfield carbon at δC 36.1 (C2), 31.9 (C16), 29.7 (C15), 27.9 (C14), 27.2 (C5), 25.6 (C8) and 22.8 belonging to C17. A methyl carbon at δC 14.6; which could be allocated to the sp^3 hybridized CH_3 group at C22.

HMBC spectrum of compound g (Figure 4.34) show correlations of H10 (δ_H 5.37) with C10 (δ_C 25.6)/ C11 (δ_C 24.8) and this suggests the presence of an oleifinic double bond at C10 connected to a methylene at C8. H12 (δ_H 5.37) with C14 (δ_C 27.8)/ C11 (δ_C 24.8) and this suggests the presence of another oleifinic double bond at C12. The Heteronuclear Multiple Bond Correlations of H11 (δH 2.39) with C13 (δ_C 132.2) and C9 (δ_C 128.8) indicate the existence of a methine carbon of an oleifinic double bond at C13 connected to a methylene at C11. Also, H-8 (δH 2.39) with C6 (δC 130.2) and C10 (δC 128.9) suggests the presence of a methine carbon of an oleifinic double bond at C10 connected to a

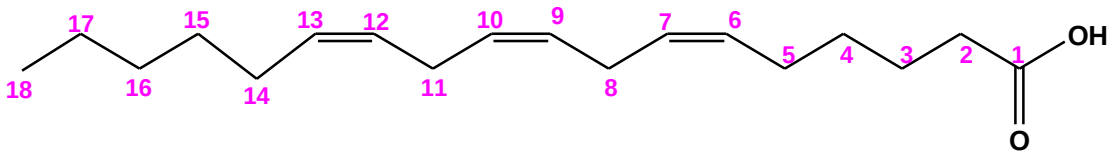
methylene at C8. The HMBC correlation furthermore reveal correlation of H18 (δH 0.82) with C16 (δC 31.9) and C17 (δC 22.8); suggests the presence of a terminal sp3 hybridized group at the end of the fatty acid chain.

The Time of Flight-Electrospray and Ionisation (TOF-ES^+) MS spectrum shows a peak at 301 which represents $[M+K]^+$, which confirms the compound Octadeca-6,9,12-trienoic acid, with molecular mass of 278 and formula $C_{18}H_{30}O_2$ (Figure 4.36).

Tab. 4.13: ^{1}H and ^{13}C NMR of compound g (α-linolenic acid) ($CDCl_3$-*d*, 600Mz) comparable with existing literatures*

S/N	^{13}C NMR (ppm)	^{1}H NMR (ppm)	S/N	^{13}C NMR (ppm)	^{1}H NMR (ppm)
1	179.3,qC	-	12	127.3, CH	5.37-5.41, m
2	36.1, CH_2	2.03 (t, *J* = 12.0 Hz)	13	132.2, CH	5.37-5.41, m
3	24.6, CH_2	2.81(t, *J* = 12.0 Hz)	14	27.8, CH_2	1.25, m
4	29.4, CH_2	1.25, m	15	29.7, CH_2	1.25, m
5	27.2, CH_2	1.25, m	16	31.9, CH_2	1.25, m
6	130.2, CH	5.37-5.41, m	17	22.8, CH2	1.25, m
7	127.9, CH	5.37-5.41, m	18	14.6, CH3	0.82 (t, *J* = 6.0 Hz)
8	25.6, CH_2	2.39, m			
9	128.8, CH	5.37-5.41, m			
10	128.9, CH	5.37-5.41, m			
11	24.8, CH_2	2.39, m			

* Reported literature; Alamsjah *et al*. (2005) and Loreau *et al*. (2013). Qc = quaternary carbon, s = singlet, bs = broad singlet, t = triplet, m = multiplet, *J* = coupling constant (Hertz).



Compound g: Octadeca-6,9,12-trienoic acid

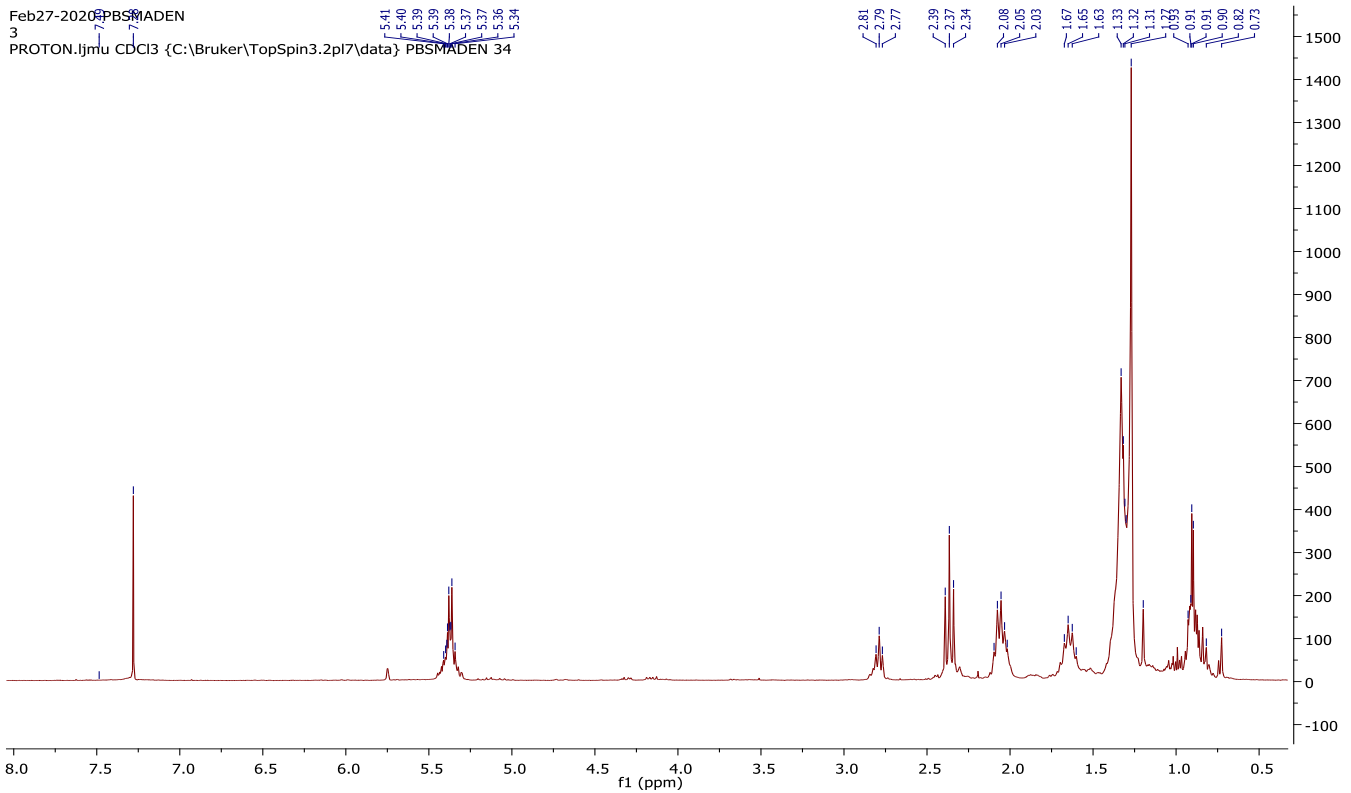


Figure 4.33: Proton NMR of g

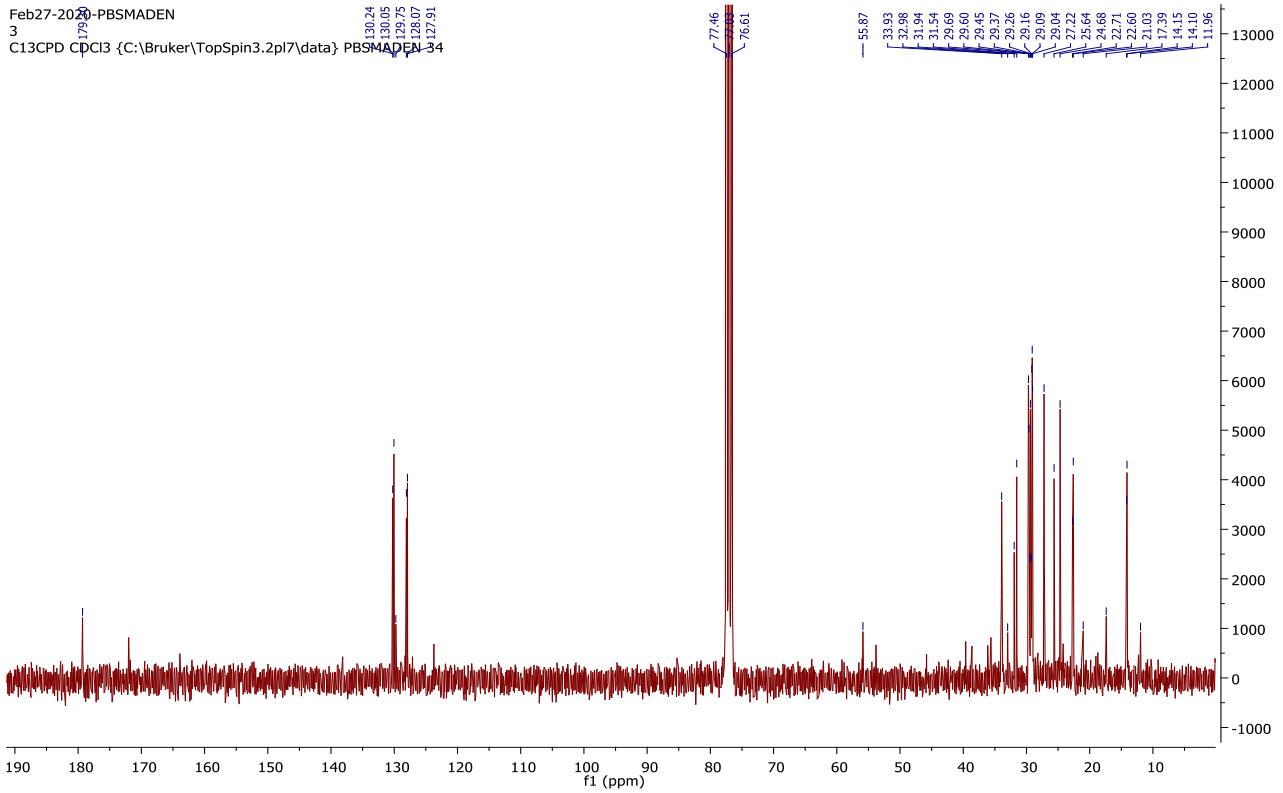


Fig. 4.34: Carbon-13 NMR of g

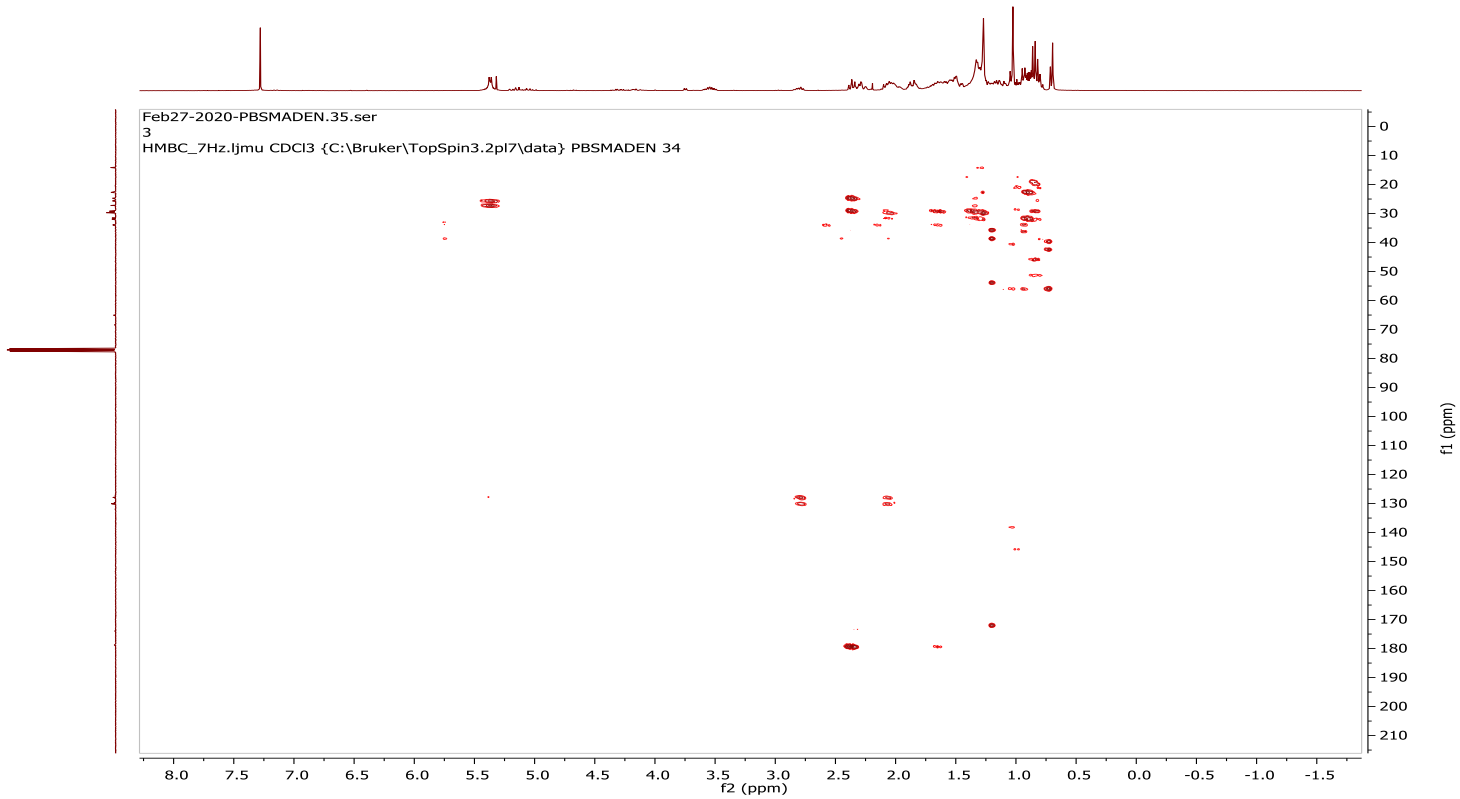


Fig. 4.35: HMBC of g

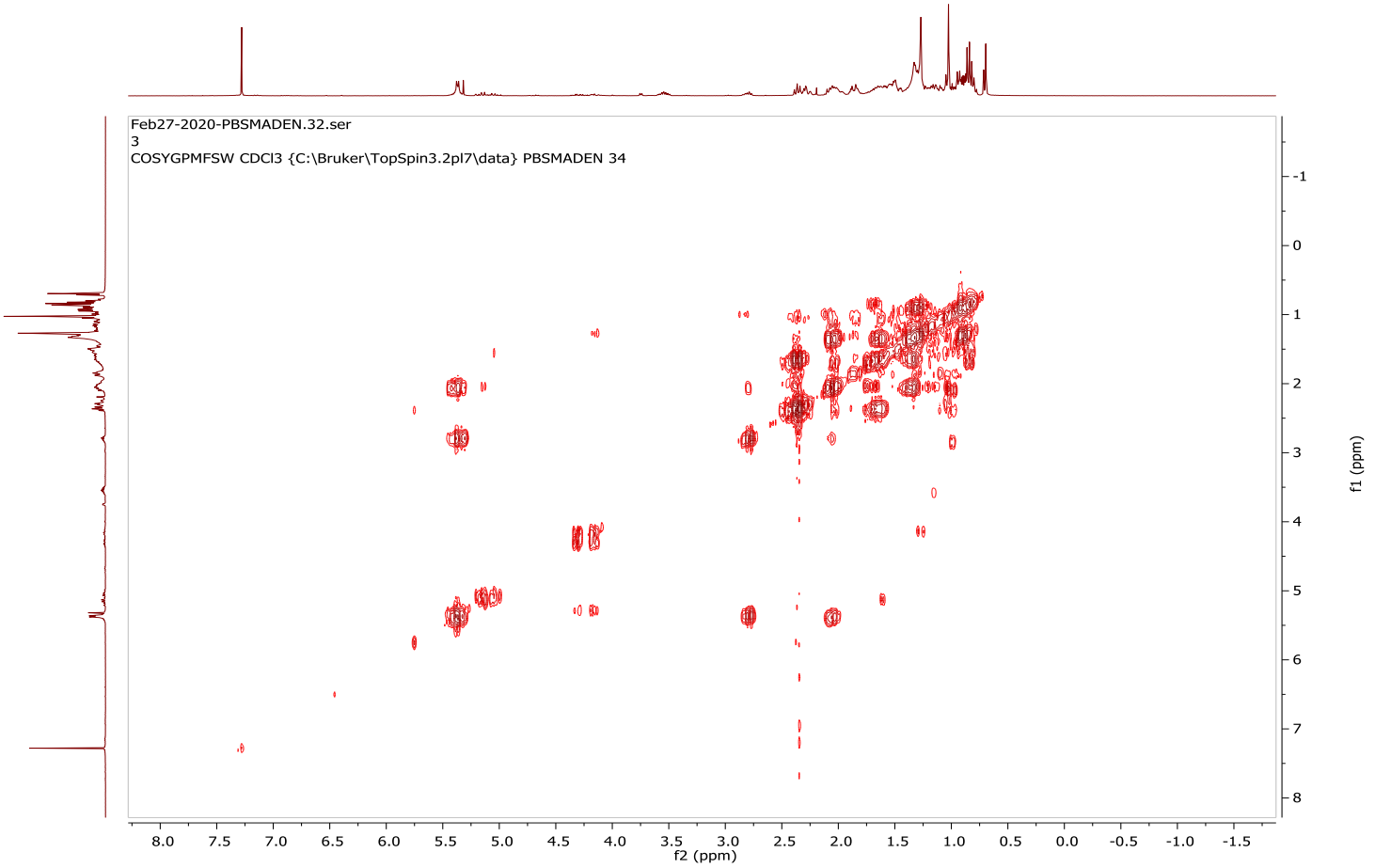


Fig. 4.36: proton-proton Correlation Spectroscopy of g

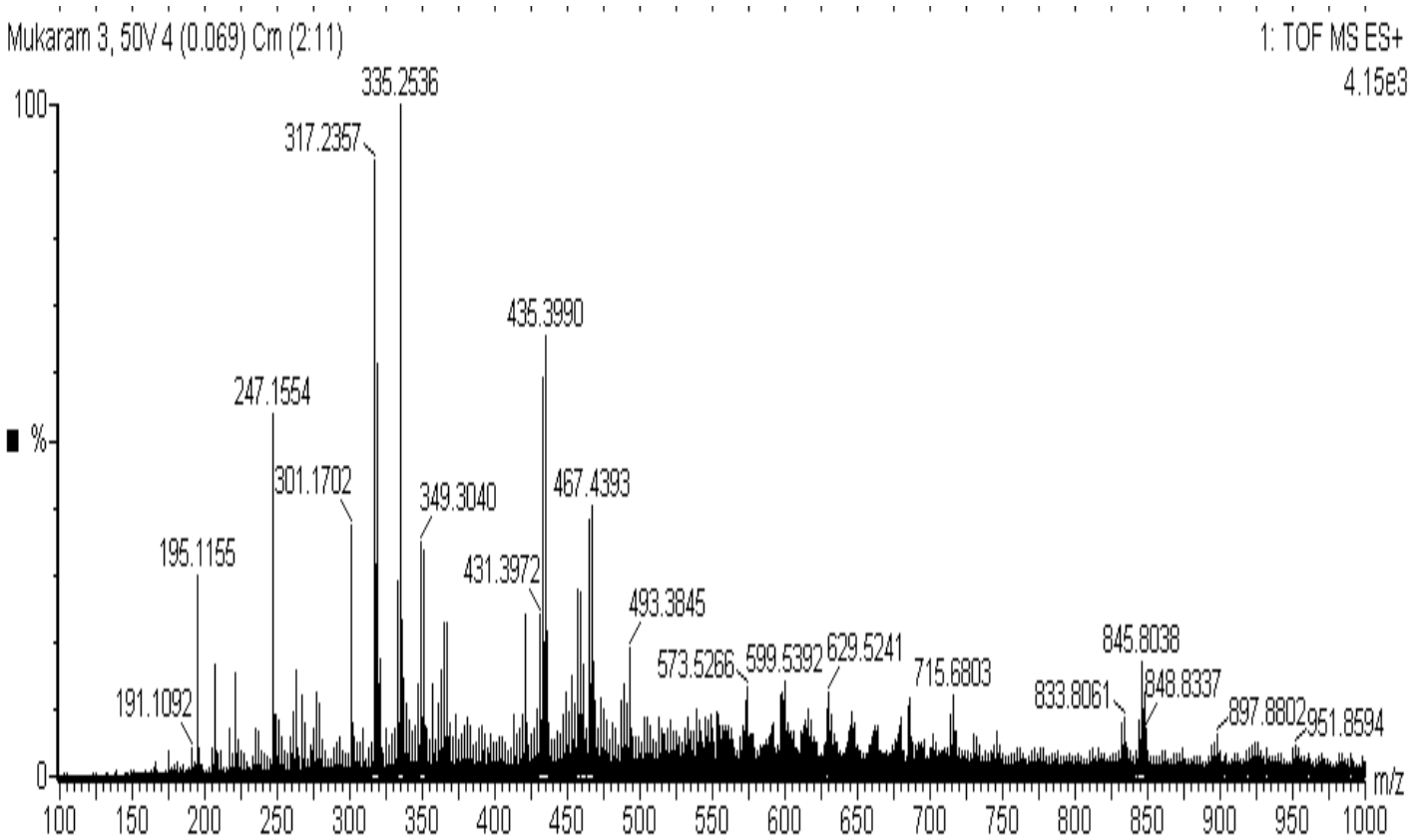


Fig. 4.37: Mass Spec. of g

Table 4.14: Percentage repellency of compound f (1,2,3-Propanetriyl tris(-5-eicosenoate)) and compound g (α-linoleic acid) from *D. tripetala* fruits at 5 mg/mL for a 2 h period

Plants	Compds	Conc.(mg/ml)	Mean landed mosquitoes (3 replicates) ± SD			% Rep. (2 h)
D. tripetala			40 min	80 min	120 min	
	Control	Acetone	7.00±1	13.67±1.53	22.67±2.52	0.00
	1,2,3-Propanetriyl tris(-5-eicosenoate)	5.00	3.00±0	6.66±0.58	14.33±0.58	37.00
	α-linoleic acid	5.00	1.00±0	2.33±0.58	9.67±0.58	57.27

Table 4.15: The MTT assay %-viability and IC_{50} of Crude ethyl acetate extract and fractions of *Dennettia tripetala*against SHSY5Y cell line

Concentration (mg/mL)	% Viability (mean of triplicate ± SD)			
	Crude	Hexane	Ethyl acetate	Methanol
10	99.55 ± 1.38	98.69 ± 2.27	91.39 ± 2.64	75.675 ± 0.76
50	92.56 ± 4.12	97.79 ± 2.10	82.19 ± 0.78	68.19 ± 4.24
100	74.07 ± 8.96	90.75 ± 0.67	76.80 ± 4.82	48.98 ± 4.15
200	60.50 ± 6.90	86.27 ± 5.09	67.49 ± 2.21	18.41 ± 5.30
IC_{50}	239.83	711.46	337.17	99.8025

Table 4.16: The MTT assay %-viability and IC_{50} of Crude ethyl acetate extract and fractions of *Dennettia tripetala* against HeLa cell line

Concentration (mg/ml)	% Viability (mean of triplicate ± SD)			
	Crude	Hexane	Ethyl acetate	Methanol
10	79.55 ± 8.23	94.94 ± 4.19	84.25 ± 3.69	62.68 ± 2.37
50	66.84 ± 6.84	92.00 ± 4.19	71.58 ± 2.08	50.36 ± 1.46
100	60.06 ± 4.61	100 ± 0.00	52.17 ± 3.98	42.64 ± 2.53
200	53.39 ± 5.11	83.02 ± 2.55	40.75 ± 0.91	39.05 ± 4.16
IC_{50}	207.91	840.69	143.33	78.60

The hexane extracts *C. limon* and the ethylacetate extract of *D. tripetala* was most potent with repellent activities of 97.52% and 87.67% respectively, compared to Deet positive control 100% activity. They were both further investigated for fractionation and separation of active compounds. The ethylacetate extract of *D. tripetala* was subjected to vacuum liquid chromatography (VLC) in order to isolate the active constituents. The fractionation of the ethyl acetate extract of *Dennettia tripetala* resulted in eleven (11) fractions, DT1 – DT11, which were analysed on Thin layer chromatography, and pooled to give four fractions labelled as DTH1 (Hexane), DTH2 (Hexane), DTEA (Ethyl acetate) and DTM (Methanol).

The four fractions were all evaluated for their mosquito repellent effects, with the The percentage repellent activities shown in Table 4.11. The fraction with the most potent repellent effect (DTEA) of 77.20% was further purified to isolate compound f and g, while DTEA and DTM with repellent activities greater than 70% were also tested on human cell lines for their cytotoxicity effect.

The isolated compounds were tested against adult female Anopheles gambiae mosquitoes, and the percentage repellencies are presented in Table 4.13. The repellent activities of α-linoleic acid (57.27%) was the most promising at 5 mg/mL optimum concentration.

Compound g (α-linoleic acid) was reported to exhibit high significant growth inhibition potential against 2nd and 4th instar creepy-crawlies of *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae), among other constituents like methyl ester of oleic acid and un-derivatised oleic acid as the main components of essential oil from the ripe fruits of *Melia azedarach*. They are mainly responsible for the insecticidal and growth inhibition activity against *Spodoptera littoralis* (Farag *et al*., 2011).

Ovicidal activity of the acetone based solution of linoleic acid, using egg-infested seeds was effectively toxic, with LC_{50} of 26 mLlitre-1, even more toxic when compared to acetone extracts of traditional coconut oils and groundnut. This supported the possibility of using fatty acids and their derivatives in the small and large scale traditional food storage systems (Don-Pedro, 1990).

α-linolenic acid was identified using spectroscopy methods, as the compound that produced coiling in the tendrils of *Bryonia dioica* jacq. α-Linoleic acid and its metabolites has been proven, through further investigations, to effectively induce tendril coiling in *Bryonia dioica* (Falkenstein *et al*., 1991).

Compound f (1,2,3-Propanetriyl tris(-5-eicosenoate)) has been previously reported for its antiproliferative activity among other fatty esters in order to support its ethnomedicinal application (Hamid *et al*., 2017).

From the results obtained in the cell line cytotoxic assays (Table 4.14 and 4.15) only the methanol fraction of the ethyl-acetate extract of *D. tripetala* displayed meaningful cytotoxic activities against HELLA and SHSY5H cell lines, with LD_{50} values of 78.60 mg/mL and 99.80 mg/mL respectively using the MTT assay.

The cytotoxic effect shown by the methanol fraction corroborates the reports of Graf *et al*, (2005) and Neuhouser, (2014) in which *D. tripetala* is said to be effective for reducing cancer attacks onset and cancer risk respectively. Alkofahi *et al*, (1988) reported the cytotoxic and insecticidal activities of the Annonaceae family alongside with other authors, due to their inhibition of the development of crown-gall tumors formed on potato. Activities was reported to be due to the presence of annonacin. This result is also an indication and warning of the risk of toxicity that may arise from the abusive consumption of *D. tripetala*, and the need for caution while eating it.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 Summary

The hexane extract of *Citrus limon* and the ethyl acetate extract of *Dennettia tripetala*, both at 5mg/mL, gave the highest repellent effects of 97.52% and 87.67% respectively, out of the six plants **(***Citrus limon, Citrus sinensis, Citrus paradise, Dennettia tripetala*, *Afromomum melegueta* and *Jatropha curcas*) evaluated, and the seven compounds isolated from the two extracts had varying repellent effects in decreasing order: 15-(heptanoyloxy) pentadec-9-enoic acid (77.87%),6,8 dimethoxy-3-undecyl-1H- [2]benzopyran-1-one (75.15%) > Palmitic acid (65.91%)> 14-Oxotricosanoic acid (63.63%)>α-linoleic acid (57.27%)>n-Octyl stearate (51.69%) >1,2,3-Propanetriyl tris(-5-eicosenoate) (37.00%).

5.2 Conclusion

The six plants studied, *Citrus limon, Citrus sinensis, Citrus paradise, Dennettia tripetala*, *Afromomum melegueta* and *Jatropha curcas* were selected based on ethnobotanical uses, and their repellent activity results were in line with the traditional claims that they are used as mosquitoes and insects' repellent. Investigation of their repellent activities showed *Citrus limon* and *Dennettia tripetala* to be most effective while the other plants had low activities.

The Bio-guided activity chemistry of the seeds of *Citrus limon* and fruits of *D. tripetala* resulted in the isolation of n-Octyl stearate, 15-(heptanoyloxy) pentadec-9-enoic acid, Palmitic acid, 6,8 dimethoxy-3-undecyl-1H- [2]benzopyran-1-one, 14-Oxotricosanoic acid, 1,2,3-Propanetriyl tris(-5-eicosenoate) and α-linoleic acid with percentage repellencies of 51.69%, 77.87%, 65.91%,75.15%, 63.63%, 37.00% and 57.27%, respectively. Compound d (15-(heptanoyloxy) pentadec-9-enoic acid) is being isolated from natural source for the first time.

All the isolated compounds have some repellent activities, with pronounced activities in compound d (15-(heptanoyloxy) pentadec-9-enoic acid) and compound e (6,8 dimethoxy-3-undecyl-1H- [2]benzopyran-1-one). The repellent activity of *C. limon* seeds could be as a result of the synergistic effects of the isolated compounds, while compound g (α-linoleic acid) could be responsible, at least in part, for the repellent activity of *Dennettia tripetala*.

The results of the brine shrimp lethality test gave further information concerning the cytotoxicity of the compounds isolated with compounds c (n-Octyl stearate) and e (6,8 dimethoxy-3-undecyl-1H- [2]benzopyran-1-one) shown to be potentially safe (LC_{50}> 100 µg/mL) while the other compounds have varying level of cytotoxic effects (LC_{50}< 100 µg/mL).

5.3 Recommendations

1**.** The active compounds isolated could be utilized in synergy with the existing synthetic and plant based repellents against mosquito bite.

2**.** The newly isolated Compound d (15-(heptanoyloxy) pentadec-9-enoic acid), with the highest activity is recommended to undergo further studies, e.g. on agricultural pests and other insects.

5.4 Contributions to knowledge

1. The repellent activities of the seven compounds (six existing and one novel compounds) are being reported for the first time.
2. The brine shrimp cytotoxic evaluation of the five compounds from *C. limon* is also being reported for the first time.
3. Compound d (15-(heptanoyloxy) pentadec-9-enoic acid) is being isolated from natural source for the first time.

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