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Antimalarial potential and phytochemical composition of fractions of Lippia kituiensis Vatke (Verbenaceae) growing in Northern Tanzania

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ANTIMALARIAL POTENTIAL AND PHYTOCHEMICAL COMPOSITION OF FRACTIONS OF *Lippia kituiensis Vatke (Verbenaceae)* GROWING IN NORTHERN TANZANIA

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A Dissertation Submitted in Partial Fulfilment of Requirements for Award of a Degree of Master's in Life Sciences of Nelson Mandela African Institution of Science and Technology

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ABSTRACT

Despite past decades of steady advances in reducing severity of Malaria, statistics show that the disease continues to pose a serious threat to human health. Previous successes in development of antimalarial drugs from medicinal plants have fuelled research in this area. However, antimalarial studies to fractionate extracts from such plants have progressed slowly. This study reports antimalarial potential of fractions from *Lippia kituiensis Vatke*, for the first time. Column chromatography was used during fractionation. Antiplasmodial assays against chloroquine-sensitive (D6) and resistant (W2) Plasmodium strains was done using hypoxanthine incorporation assay. A colorimetric assay was done to assess cytotoxicity of fractions against the Vero cell line. Obtained fractions exhibited varied inhibitory concentrations (IC_{50}); with the most efficacious being, Lk-5 (19.45 ± 6.20 µg/ml), Lk-3 (30.43 ± 0.68 µg/ml), Lk-4 (30.82 ± 18.01 µg/ml), and Lk-6 (36.53 ± 14.42 µg/ml) against D6. Generally, lower activity against W2 was obtained with the most active being Lk-4 (24.18±2.50 µg/ml), and Lk-5 (24.42±5.95 µg/ml), while chloroquine (positive control) exerted IC_{50} of 77.86±4.09 ng/ml (W2) and 15.71±6.49 ng/ml (D6) respectively. Fraction LK-4 was the most cytotoxic showing cytotoxic concentration of 46.26 µg/ml. When tested in mice, fractions suppressed *Plasmodium berghei* significantly compared to the negative control with Lk-3 being the most efficacious (80.01±1). Due to substantive efficacy, GC-MS was done on Lk-3 revealing 8 compounds where three have previously been ascribed to antimalarial activity and other pharmacological effects. This study adds to present knowledge of antimalarial efficacy of *L. kituiensis* and provides basis for further work to be done on isolation of compounds from its extracts.

DECLARATION

I, Ng'etich Japheth Kibet, affirm that this thesis is my original work and was never submitted for a degree or any other award in any research or academic institution. All borrowed materials used in preparation of this document have been cited and acknowledged appropriately.

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CERTIFICATION

We hereby certify that we have read and confirmed that this thesis represents a true report of what was done by the student throughout his research process with our supervision.

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DEDICATION

This work is dedicated to my spouse; Jepkorir Emmy and daughter Cheptoo Ng'etich, without whose prayers and immense support, this work wouldn't have been accomplished.

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LIST OF ABBREVIATIONS AND SYMBOLS

>	Greater Than
≤	Less Than or Equal to
ACT	Artemisinin Combination Therapy
CC_{50}	Cytotoxic Concentration (Concentration Required to Cause Alterations in 50% of Intact Cells)
CREATES	Center for Research, Education, Agriculture Training
CTMDR	Center for Traditional Medicine and Drug Research
DMSO	Dimethyl Sulfoxide
DNA	De-Oxyribonucleic Acid
ETOA	Ethyl Acetate
IC_{50}	Inhibitory Concentration
ITN	Insecticide Treated Nets
KEMRI-	Kenya Medical Research Institute
M/Z-	Mass/Charge ratio
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NMAIST	Nelson Mandela African Institute of Science and Technology
PBS	Phosphate Buffered Saline
RBCs	Red Blood Cells
RNA	Ribonucleic Acid
TLC	Thin layer Chromatography
TNF	Tumor Necrosis Factor
TLR	Toll-Like Receptors
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background of the Problem

Malaria remains the most prevalent and fatal-vector-borne disease (Friesen *et al*., 2010). In 2016 alone, the World Health Organization (WHO) reported approximately 216 million cases of malaria globally. This was more than the 211 million cases reported in 2015. Notably, African continues to suffer a disproportionately higher burden of the disease (90% of the global cases). Although there has been a significant decline in the global cases of Malaria over the last decade, recent reports indicate that progress has stalled particularly in the period 2010-2016 (WHO Malaria Report, 2017).

Malaria is spread by the *Anopheles* genus female mosquitoes infected with *Plasmodium spp*. Human malaria can be caused by *Plasmodium falciparum, P. ovale, P. vivax, P. malariae* and *P. knowlesi* (Singh *et al*., 2004; Van Hellemond *et al*., 2009). Humans make excusive mammalian hosts for *P. falciparum* and *P. vivax,* the most common species of plasmodia and are responsible for the largest transmission of the disease (Phillips *et al*., 2017).

The recent discovery of malaria as a zoonotic disease due to *P. knowlesi* transmission, poses obvious complications for elimination (Imwong *et al*., 2011). With currently no approved vaccine, vector control and chemotherapy are the mainstay methods of malaria control (Chen *et al*., 2018). Artemisinin is the cornerstone of malaria chemotherapy (O'Neill *et al*., 2010). However, worrying concerns of failure attributable to resistance have been published (Landier *et al*., 2018); highlighting a pressing need to find alternative treatment options (Pinheiro et al., 2018). Furthermore, the discovery of new effective, well tolerated and safe antimalarial drugs remains an uphill task (Pereira *et al*., 2011). The hunt for new anti-malarial compounds, particularly from plants historically used for malaria treatment, is rife (Batista *et al*., 2009). This is because plants are a rich source of potential leads having new mechanisms of action (Bero *et al*., 2009).

The therapeutic efficacy of two antimalarial drugs derived from plants, (quinine and artemisinin), have led to global consensus that plants possess immense potential as sources of novel drugs (Ziegler *et al*., 2002; Newman *et al*., 2003), hence the current study.

Lippia belongs to the family *Verbenaceae;* a genus of flowering plants known for its characteristic aromaticity from their essential oil (Arthur *et al*., 2011). With approximately 200 species, the family consists of shrubs, herbs, and small trees. These species are primarily distributed throughout tropical African countries and South and Central American territories (Pascual *et al*., 2001). Several studies have reported pharmacological effects of *lippia* including antimalarial, antimicrobial, antifungal, analgesic, antipyretic, anti-inflammatory antispasmodic and larvicidal activities (Stashenko *et al*., 2013).

Lippia kituiensis Vatke is used by local communities in Tanzania to treat malaria. To the best of our understanding, the current study reports antimalarial efficacy of fractions obtained from *L. kituiensis* for the first time. This is a subsequent study guided by previous reports showing a significant chemo-suppressive activity (95.19%) of total extracts of the plant against *Plasmodium berghei* (Mzena *et al*., 2018).

1.2 Statement of the Problem

Despite of there being extensive knowledge of etiology and pathogenesis, malaria continues to be a devastating health concern especially in Africa. According to the World Health Organization, the African continent contains a disproportionately high share of the worldwide protozoal infection. In 2015 alone, 90% of malaria cases and 92% of global malaria deaths were recorded in the region (WHO, 2017). Recent reports indicate that Plasmodium species have developed resistance to Artemisinin, the mainstay antimalarial drug.

In view of these challenges, the need to seek alternative therapies to manage malaria cannot be overemphasized. Plants possess great potential for use in treatment of the disease. This is because of their long history of successful traditional use in developing countries. In order to validate the effectiveness of such herbal remedies, several scientific studies have been done. This study sought to screen fractions from organic extracts of *L. kituiensis* against plasmodium species. *In vitro* cytotoxicity and phytochemical constituents of the fractions was also determined. The medicinal plant is traditionally used to manage Malaria in Tanzania.

1.3 Rationale for the Study

There is evidently a substantial interest in natural products and the possibility for their role as new antimalarials. This is not a new departure considering that three of the mainstay malaria treatment come from natural products: quinine, lapachol (which led to atovaquone) and artemisinin. The traditional utilization of *Lippia kituiensis* to treat malaria in Tanzania is rife. This makes the plant a very important resource with the current scientific interest in exploration of novel antimalarial drugs from plants. This study therefore sought to fractionate organic extracts of *Lippia kituiensis* which has previously been shown to possess strong antimalarial activity.

1.4 Objectives

1.4.1 General Objective

To fractionate *Lippia kituiensis* organic extracts and determine antimalarial efficacy and phytochemical constituents of the fractions.

1.4.2 Specific Objectives

i) To investigate *in vitro* and *in vivo* antiplasmodial activity of fractions of *L. kituiensis*.
ii) To evaluate cytotoxic effects of fractions of *L. kituiensis*.
iii) To determine phytochemical constituents of fractions of *L. kituiensis.*

1.5 Research Questions

This study sought to answer the following questions:

i) Do fractions of *L. kituiensis* possess anti-malaria activity?
ii) What are the cytotoxic levels of fractions of *L. kituiensis?*
iii) What are the phytochemical constituents that can be associated with antimalarial activity of *L. kituiensi*?

1.6 Significance of the Study

This study pioneers the search for novel antimalarial ‘leads’ from *L. kituiensis;* a medicinal plant with reported antimalarial efficacy and locally used to treat malaria in Tanzania.

This study also illustrates secondary metabolites in different fractions that can be associated with their antimalarial efficacy. In this study, the compounds associated with antimalarial efficacy of plant fractions are highlighted, paving the way for further research to be done to isolate the identified compound(s).

Preliminary *in vitro* safety profile of fractions from *L. kituiensis* have been made evidenced in this study. Although further studies are needed on this, the current study has shown that the toxicity of the extracts from the plant by far exceed the therapeutic levels hence the traditional use to manage malaria

In addition, this research adds to the current knowledge on the effectiveness of the plant and thus validates its ethnobotanical value for local communities. This data can extensively contribute to policy development towards the channeling of safe and efficacious medicinal plants to primary healthcare, particularly in Malaria management.

1.7 Delineation of the Study

Previous studies by Mzena *et al*. (2018) demonstrated significant antimalarial activity of total extracts of *L. kituiensis* leading to this study. The samples were harvested on 13th March 2018 and transported to NM-AIST on 14th March 2018 from 8-11 am. Samples of fresh aerial parts of the plant were harvested sustainably from Ugweno village in Kilimanjaro region, Northern Tanzania (3° 39’ 0” South and 37° 39’ 0”), where they are traditionally used to treat Malaria. The plant was authenticated by a resident herbalists in the presence of a professional taxonomist. Voucher specimen was taken and deposited at the Nelson Mandela African Institute of Science and Technology and assigned specimen number LKV-160-2018. Soil and other contaminants were washed off using cold water. The plant material was dried under shade till complete dryness, milled into fine powder and packed in manila bags. The powdered plant material was transported to the Kenya Medical Research Institute, Nairobi where subsequent studies were done.

CHAPTER TWO

LITERATURE REVIEW

2.1 Epidemiology of Malaria

Despite current advances in science and the existing knowledge on pathogenesis, Malaria continues to cause sicknesses and death (Cox, 2010). Whereas the epidemiology of the disease varies geographically the precise numbers may be questionable and sometimes underreported with 395 000 estimated deaths in Africa in 2015 (Nkumama *et al.*, 2017). In 2017, an estimated 435 000 deaths occurred due to Malaria (WHO Malaria Report, 2018). Other than claiming hundreds of thousands of lives of young children yearly, experts observe that malaria may have killed half of the human generation (Whitfield, 2002). The main symptoms of severe Malaria include; metabolic acidosis, renal failure, anaemia, acute kidney injury, cerebral malaria, pulmonary oedema, hypoglycaemia, hypertension and shock (Trampuz *et al*., 2003). Given the wide range of conceivable manifestations, it isn't clear what aspect(s) of the malady can and ought to be measured to monitor the changing epidemiology accurately (Koram & Molyneux, 2007). Sometimes malaria is said to be symptomless, but may be better termed chronic (Chen *et al.*, 2016). The importance of the disease is further demonstrated by the lack of development potential for malaria-endemic countries, the impact on their healthcare systems and their detrimental effects on economic growth (Sicuri *et al*., 2013). The severity of the disease has however declined significantly due to combined efforts by multi-sectoral agencies over time, leading to reduced infection rates up to half and 4.3 million lives saved (Tanner *et al.*, 2013).

2.2 Malaria situation in Tanzania

While malaria estimates in Tanzania are contradictory, reports show that the country has the third largest malaria-risk population in Africa, with 93% of people in mainland Tanzania and the entire population in Zanzibar living in endemic areas of malaria. In 2017, approximately 5.9 million clinical cases of malaria were reported on mainland Tanzania. (President's Malaria Initiative, 2018). Transmission patterns of the disease have been mapped as approximately 20 % of the country experiencing seasonal malaria and another 20 percent facing stable malaria with seasonal variations. The remaining 60% of endemic regions were referred to as stable perennial transmission areas. *Plasmodium falciparum* is

responsible for 96% of malaria cases, while *P. malariae* and *P. ovale* are responsible for the rest (President's Malaria Initiative, 2018).

2.3 The Vector

The Malaria parasites that exclusively cause disease to humans are transmitted by approximately 40 mosquito species of the *Anopheles* genus (Bhatt *et al*., 2015). The most prevalent in Africa include, *Anopheles funestus, Anopheles gambiae sensu stricto* and *Anopheles arabiensis* (Sinka *et al*., 2010). Males of *Anopheles spp* transfer 20-hydroxyecdysone; a steroid hormone, to the females during mating. Availability of the hormone has been related to favourable conditions for development of Plasmodium spp (Mitchell *et al*., 2015). 20-hydroxyecdysone (20 E) induces a cascade of events that prevent further copulation of *A. gambiae* females, while increasing egg production and hence egg laying (Mitchell *et al*., 2015). Since egg production involves blood feeding, the parasite takes advantage of the reproductive needs of the mosquito to achieve its own transmission between vertebrate hosts. The high rate of reproduction seen in *A. gambiae* is a major factor for their ability to transmit high levels of Malaria. After each blood meal, it is assumed that the female mosquito species can lay over one hundred eggs and fertilize them with sperm obtained from a single mating (Baldini *et al*., 2012).

2.4 Plasmodium Parasite

Plasmodium spp. are single-celled eukaryotic organisms of the phylum *Apicomplexa* (Florens *et al*., 2002). Among the five species of parasites responsible for human malaria, *P. falciparum* is attributed to majority of diseases and deaths in Africa (Gething *et al*., 2016). Moreover, *P. falciparum* results in severe malaria, complications during pregnancy and an estimated daily mortality of one thousand two hundred African children aged 5 and below (Maitland, 2016). On the other hand, *P. vivax* is predominant in tropical and temperate regions for example Asia and Ethiopia and is implicated for Malaria sicknesses and deaths in Central and South America. This can be explained by the species' ability to survive in unfavourable climatic regions and majorly its ability to exist dormant as hypnozoites in the human liver for many days or even years. The process of Malaria infection is facilitated by a natural surface protein of erythrocytes called Duffy antigen for which many Africans are innately negative hence conferring natural physiological protection from *P. vivax* malaria

(Miller *et al.*, 1976). *Plasmodium ovale* and *Plasmodium malariae* are also endemic in Africa and Asia, but are specifically adverse in West Africa (Sutherland *et al*., 2010). *Plasmodium knowlesi* previously thought to cause disease to non-human primates has been reported to only cause disease to humans but can also lead to severe complications and even death (Cox-Singh *et al*., 2008).

2.4.1 The Life-Cycle of Plasmodium Species

The Plasmodium parasite develops in two cycles which take place in its two hosts (mosquitos and primates) (Noland *et al*., 2003). During a blood meal, sporozoites (approximately 100) are transferred from the salivary duct of the mosquito to into the venules of the host (Collins & Jeffery, 2007). After 30–60 minutes, the sporozoites are transported by blood into the liver and initiate the hepatic cycle where they invade hepatocytes. The sporozoites develop into schizonts consisting of many hepatic merozoites. The merozoites are then released for systemic circulation hence launching the erythrocytic cycle. This cycle represents the asexual stage of reproduction, and it is at this stage that the symptoms of Malaria are manifested, mostly 4-8 days after initiation (Karunaweera *et al*., 1992). While in the RBCs, the merozoites develop within 36–72 hours between invasion to haemolysis. Thus, with a single bite, episodes of fever happen every 36–72 hours, during which time the infected RBCs lyse and release endotoxins in great mass (Wijesekera *et al.*, 1996). While the merozoites continue their replication within the RBCs, some differentiate into gametocytes. However, *P. vivax* and *P. ovale* have a somewhat unique cycle in its primate hosts where some hepatocellular sporozoites, remain in uninucleate form, called hypnozoites. These hypnozites resume hepatic development facilitated by factors that remain unknown, causing malaria relapses in varied periods after initial exposure (Galinski *et al.*, 2013). Released Merozoites further invade other RBCs , and sometimes they segregate into male or female gametocytes (Baker, 2010) . The gametocytes are then sequestered in the capillaries of the skin from where they are picked by the mosquito in the subsequent bites. The male gametocytes undergo mitosis in the gut of the mosquito producing microgametes; while the female gametocyte grows into a macrogamete and fuses with the motile male microgametes forming a diploid zygote. The zygote grows into an ookinete; which leaves the lumen of the gut a as an oocyst. The sporozoites are formed from the oocysts after several series of reproduction, afterwhich they relocate to the salivary glands of the mosquito. The female mosquito may therefore be virulent to another human within 7–10 days after feeding from blood containing gametocytes

(Annan *et al.*, 2007). Antimalarial drugs exert their pharmacological action at varied critical stages of the parasites' cycle. Drugs that inhibit plasmodial invasion of hepatocytes possess prophylactic effects, while those that prevent erythrocytic stage have curative effects during the symptomatic stage of the disease, The transmission-blocking agents prevent the formation or production of gametocytes in the invertebrate host (including those that kill blood-fed mosquitoes) (Saifi, 2013 & Phillips *et al.*, 2017). The parasite's reproductive cycle and drug action sites are as summarised in Fig. 1.

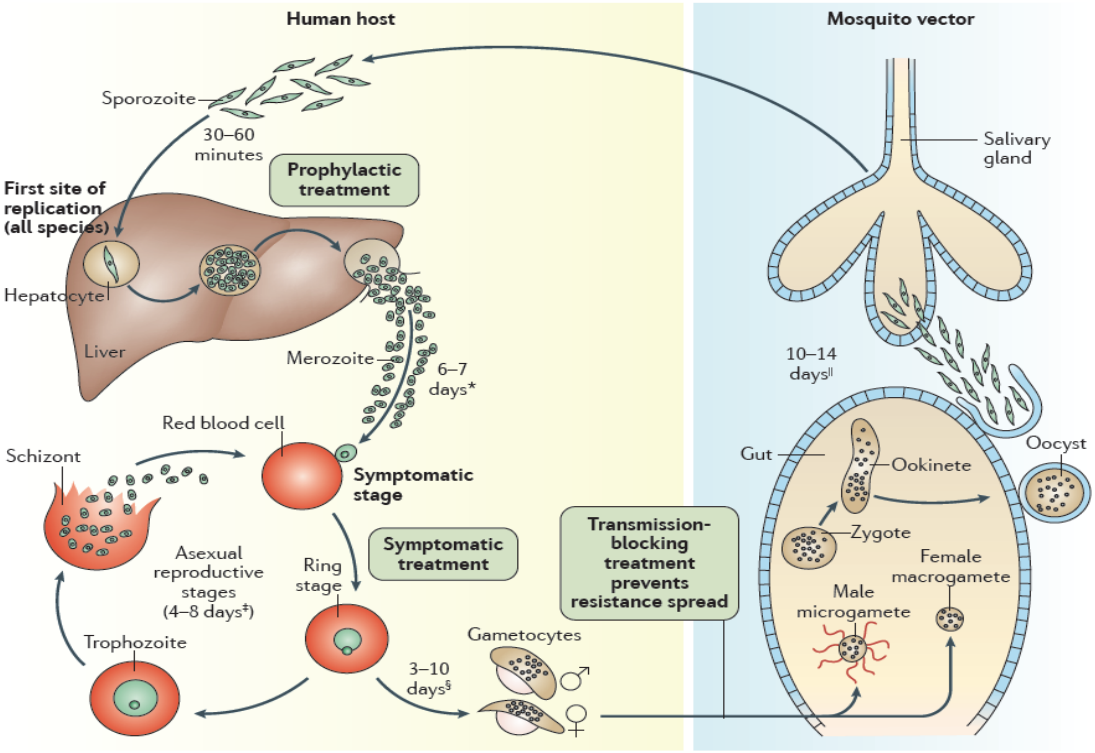

Figure 1: The lifecycle of Plasmodium Parasite highlighting areas of antimalarial drug action (Adopted from Phillips *et al.*, 2017)

2.5 Parasite Invasion and Replication

Plasmodium spp's red blood cell stage marks the symptomatic stage of malaria infection, since erythrocytes are the sites of parasite replication. The parasites enter the red blood cells by precise ligand-receptor association modulated by parasite surface proteins that interact on the host erythrocyte or reticulocyte (Paul *et al.*, 2016). *Plasmodium falciparum* may infect and reproduce in the RBCs and reticulocytes, while *P. vivax* and other species attack reticulocytes which are always less than erythrocytes (Lim *et al.*, 2016). Once in the RBCs, hundreds of proteins are shuttled cell cytoplasm of the host cells and cell surface by parasites which function to modulate nutrient acquisition, cell adhesion and tissue sequestration hence

pathogenesis (Mercier *et al.*, 2005). The parasites' rapid replication may hit > 10^{12} parasites per patient during symptomatic stage of the disease. This proliferation needs sustained nucleotide molecules for DNA and RNA synthesis, and as such, most antimalarials target the biosynthesis of pyrimidine (Phillips *et al*., 2017).

2.6 Pathogenesis

Severe malaria is multifactorial in nature with parasite and host factors contributing to the seriousness of the disease (Miller *et al*., 2013). The infected RBCs which release endotoxins and the parasites (a combination of haemozoin and parasite DNA) highlight plasmodium species' pathogenic mechanisms. This phenomenon triggers increase in Toll-like receptor 9 (TLR9), which modulates the immune system of the hosts against the disease causing agents (Parroche *et al*., 2007). The upsurge in TLR9 results in escalation of tumour necrosis factor (TNF) and eventual symptoms of malaria, especially fever (Vijaykumar *et al.*, 2001). Furthermore, the membrane of infected red blood cells often stiffens, leading to capillary obstruction and this is life-threatening in patients with acute malaria (Parroche *et al*., 2007). The pathogenesis of malaria is aided by surface proteins specific to the parasite. In *P. falciparum*, a large surface antigen encoded by the *var* gene family comprising of approximately 60 genetically classified members of three subgroups A, B and C. The B and C subtypes interfere with the cells of the host through CD36 (a platelet glycoprotein 4), while the subtype A interferes with non-CD36 binding associations responsible for extreme cases of malaria, like cerebral malaria (Smith, 2014; Duraisingh & Horn 2016; Wassmer *et al.*, 2015). The endothelial vasculature cytoadherence of infected RBCs is mediated by *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) encoding *var* genes. Subgroups B and C account for more than 80% of the variants of PfEMP1. In addition, group A variants of PfEMP1 promote the binding of infected red blood cells to the endothelial protein C receptor (EPCR) and the intracellular adhesion molecule 1 (ICAM) of cerebral malaria, hence the pathological characteristics of cerebral malaria (Gillrie *et al*., 2016). Evidence of positive selection of people residing in endemic areas with heterozygous genotypes for haemoglobin-encoding alleles are naturally protected from malaria infection. Nevertheless, with cases of homozygous genotypes, these alleles cause serious disorders of the blood such as thalassaemia and sickle cell disease (López *et al*., 2010; Elguero *et al*., 2015). Invasion of red blood cells by *P. vivax* parasite is modulated by Duffy antigen (a red blood cell-surface proteins) which act as receptors. Genetic mutations in *ACKR1* genes that code for Duffy

antigens have been linked to reduced spread of *P. vivax* caused malaria among people of African origin (Cheng *et al.*, 2016).

2.7 Mainstay Control Measures

In sub-Saharan Africa, the cornerstones of malaria control are long-lasting insecticide nets and indoor insecticide spraying. Together with effective treatment, these approaches are estimated to have decreased malaria morbidity and mortality worldwide by 41% and 62% between 2000 and 2015 (Protopopoff *et al.*, 2018; Killeen & Ranson, 2018). Today's most popular and successful vector control techniques the use of insecticide-treated, long-lasting and wash-proof networks. The fibre of these networks is impregnated with an insecticide that retains its effectiveness after up to 20 lab washes, with 3 years of approved use (Chanda *et al*., 2014). The biological and behavioural diversity of mosquitoes, however, complicates the use of these vector control methods. For example, net treated with insecticides alone is unlikely to be sufficient due to residual malaria transmission (Parker *et al.*, 2015). This leaves chemotherapy as the most reliable alternative to clear residual plasmodium parasites. There are several pharmacological options available for malaria treatment: chloroquine, quinine, primaquine, mefloquine, pyremethamine, derivatives of artemisinine such as artemether and artesunate and amino alcohols such as Halofantrine and Lumefantrine (Bahekar & Kale, 2013). Their respective compounds are as shown in Fig. 2. Uncomplicated Malaria is currently managed by Artemisinin-based combination treatments (ACTs) because of their effectiveness. The drug molecules partnered with artemisinin derivative determines the efficacy of the combination. For example, Artemether,-lumefantrine, artesunate, mefloquine, and dihydroartemisinine, piperaquine, typically exceed 95%. The use of artemisinin derivatives is a serious concern during the first trimester of pregnancy where they should not be used unless viable options exist (Nosten & White, 2007). As with other infectious diseases, the principle behind combination chemotherapy in malaria is that when two drugs are used with different modes of action and different mechanisms of resistance are established, then the per-parasite probability of creating resistance to both drugs in the same cell division is the result of their individual per-parasite probability (Peters, 1987).

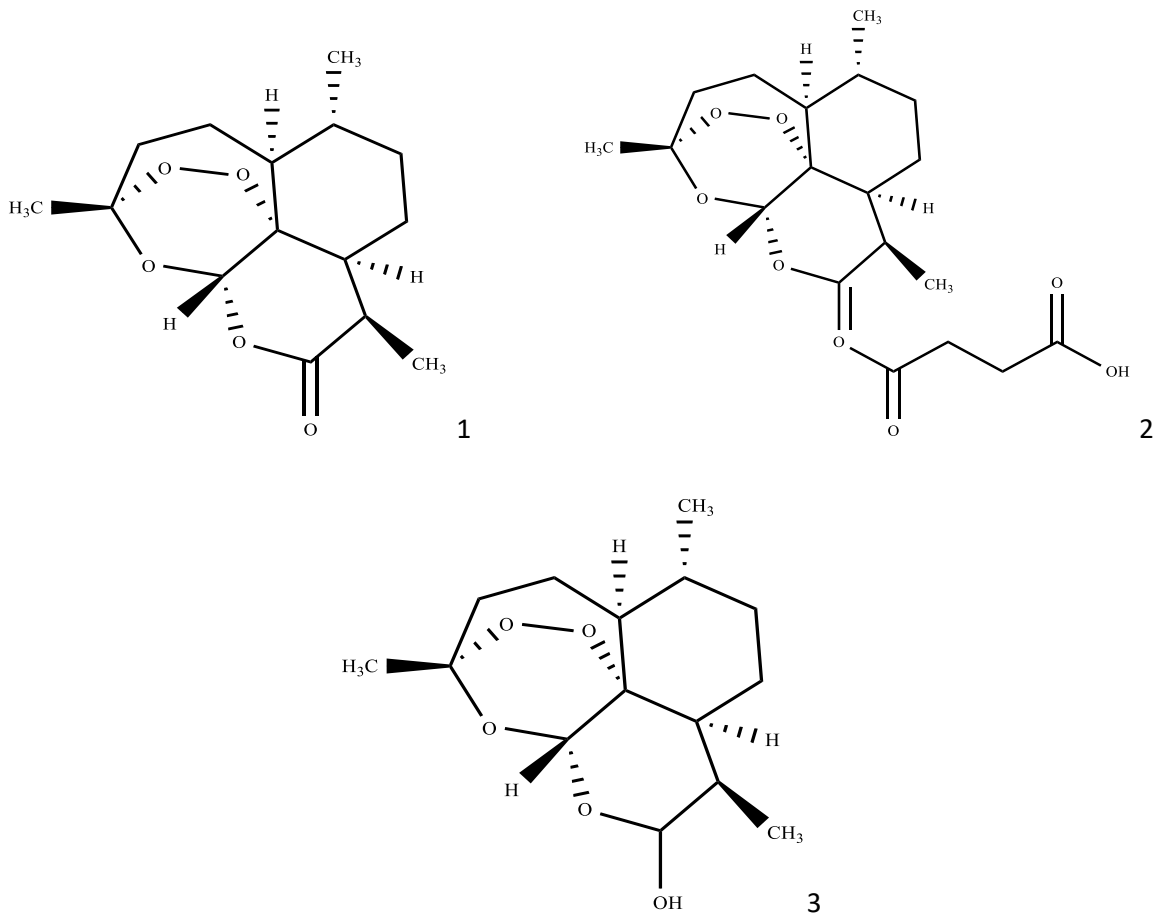

Figure 2: Artemisinin Pharmacophore (1) and its Derivatives, Artesunate (2) and Dihydroartemisisnin (3)

2.8 Antimalarial Drug Resistance

Resistance to most drugs has not only been implicated in enhanced mortality, but it is also an economic burden in the fight against malaria (Bloland, 2001). The resistance of *P. falciparum* to chloroquine (CQ) and sulphadoxine-pyrimethamine (SP) prompted introduction of ACT that has now been adopted by most countries for treatment of malaria (Ishengoma *et al*., 2015). Consequently, the use of Chloroquine as the main option to treat malaria has been discontinued in some countries in Africa, for example Kenya (Gathirwa *et al*., 2008). Recent reports indicate fears of rising cases of decline in efficacy of ACT and artesunate monotherapy, as stated in a case study done in western Cambodia (Alker *et al.,* 2007; Denis *et al.*, 2006; Noedl *et al.*, 2008). These isolates are highly likely to spread to other areas endemic to malaria, including sub-Saharan countries, as was the case with the past drugs for malaria treatment (Mita *et al.,* 2011). Resistance against Artemisinin have been shown to be

propagated by mutations in the propeller region, the Kelch 13 (K13), hence delayed clearance of the parasite both *in vitro* and *in vivo* (WHO status report). Approximately one hundred and eighty six K-13 alleles including one hundred and eight mutations that are not synonymous have been identified. There are rare, but very diverse, non-synonymous mutations in Africa. These mutations have been reported in Chad, Cameroon, Gabon, Comoros, Kenya, Gambia, Madagascar, Mali, Malawi, Togo, Uganda and Zambia (WHO, 2015). Given these rising cases of *P. falciparum* resistance to conventional drugs, there is need to develop alternative control measures for management of malaria. For decades now, medicinal plants have been used to treat pain without resistance development. However, research to isolate bioactive compounds from plants that have shown promising antimalarial activity needs to be done.

2.9 Antimalarial Drug Discovery and Associated Challenges

The WHO and the Bill and Melinda Gates Foundation announced almost a decade ago the long-term goal of malaria eradication. This ambitious plan has had profound achievements attributed to the use of existing therapy compared to the situation at the start of the Millennium (Burrows *et al*., 2017). As such, efforts have been channelled to drug discovery research from natural products. However, the results are yet to bear substantial impact. Of the more than 1300 new drugs developed between 1975 and 1999 for all diseases, only 13 were for tropical diseases. Just about 0.1% of global health research funding was dedicated to the development of drugs for tuberculosis and identified tropical pathologies (malaria, trypanosomiasis and leishmaniosis), which together accounted for an estimated 5% of the global disease burden in the year 2000 (Pink *et al*., 2005). If the fight against malaria is to be won, novel drugs are urgently needed. Sadly, the standard drug discovery pathways have produced few medicines to treat emerging diseases affecting developing countries. Despite this concern, there is hope for retrench. First, the successful genome sequencing of *P. falciparum* presents enormous potential drug targets and the current advances in malaria genetics provide robust techniques of determining potential targets. The discovery and development of new and affordable drugs may be realised with the critical role played by pharmaceutical companies (Fidock *et al*., 2004). Natural products remain a promising source of conventional medicines, thus increasing interest in research in the field (Harvey *et al*., 2015). Since natural products are formed by proteins to interact with proteins, they can therefore be considered by definition as scaffolds (Balamurugan *et al*., 2005). The United States Food and Drug Administration estimates that 34% of approved drugs from 1981 to

2010 were either obtained from natural products or are derivatives there from (Newman & Cragg, 2012). Additionally, a recent report from Novartis suggests that while conducting high-throughput screening, natural products is a source of compounds with unfathomable diversity compared to synthetic and combinatorial molecules, hence a higher hit rate (Bender *et al*., 2009). In light of great interest and potential vested on natural products as sources of new antimalarial drugs, there remain substantial challenges to be overcome. The United Nations Convention on Biological Diversity mandated to oversee global access to natural products, and claims of inability of natural products to match the drug discovery approaches of molecular targets have been raised. Furthermore, the challenges of repeated isolation of non-novel compounds and the complexity of processing natural products in pharmaceutical manufacturing have significantly curtailed the progress of antimalarial drug development from natural products. Finally, the yet to be fulfilled supposition that combinatorial chemistry will avail all the required chemicals for successful discovery of leads has dragged the once great promise of natural products as sources of novel medicines (Newman & Cragg, 2012).

2.10 Lippia Kituiensis Vatke

The genus *Lippia* of the family *Verbenaceae* consists of flowering plants known for their aromaticity based on their high contents of essential oils (Arthur *et al*., 2011). This genus of plants grows in most parts of Countries in South and Central America and parts in Tropical Africa (Nara *et al*., 2016). *Lippia kituensis* has been shown to possess mosquito repellent properties (Kosgei *et al*., 2014). Extensive research has been done on plants of this genus. Leaves of *L. graveolens* are used for culinary purposes (Tucker *et al*., 2009). In other studies, Magano *et al*. (2011) reported that ccamphor oil from *L. kituiensis* possessed a significant repellence against maize weevils compared with NN-diethyltoluamide (DEET) Other ethnobotanical uses of genus *Lippia* include management of malaria, gastrointestinal and respiratory problems apart from possessing, antispasmolitic, hypotensive, sedative and anti-inflammatory activities (Jigam *et al*., 2009). Other plants in the family of *Verbenaceae* including *Lippia javanica* have been used to treat fever, colds, cough, malaria, wounds, chest pains, diarrhoea, asthma, bronchitis, cancer, anti-amoebic, antidiabetic, antimicrobial, antioxidant, antiplasmodial and pesticidal effects (Alfred, 2017). Although plants of the same species *(Lippia)* have shown efficacy against Malaria (Gasquet *et al*., 1993), it is worth noting that claims of antimalarial activity of *L. kituensis* have not been validated scientifically.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Reagents and Chemicals

Silica gel powder (70-230 mesh), pre-coated Thin Layer Chromatography Plates, solvents (Hexane, Ethyl Acetate, Dichloromethane and Methanol) and biological reagents and consumables were purchased from sigma Aldrich. Chloroquine diphosphate and radiolabelled Hypoxanthine Monohydrate salt were generously donated by Dr. Jeremiah Gathirwa of Kenya Medical Research Institute.

3.1.1 Ethical Statement

The KEMRI in-house approval bodies namely, the Scientific and Ethics Review Unit (SERU), and the Animal Care and Use Committee (ACUC) approved the study under approval numbers, KEMRI/SERU/CTMDR/063/3644 and ACUC)-KEMRI/ACUC/01.02.19 respectively.

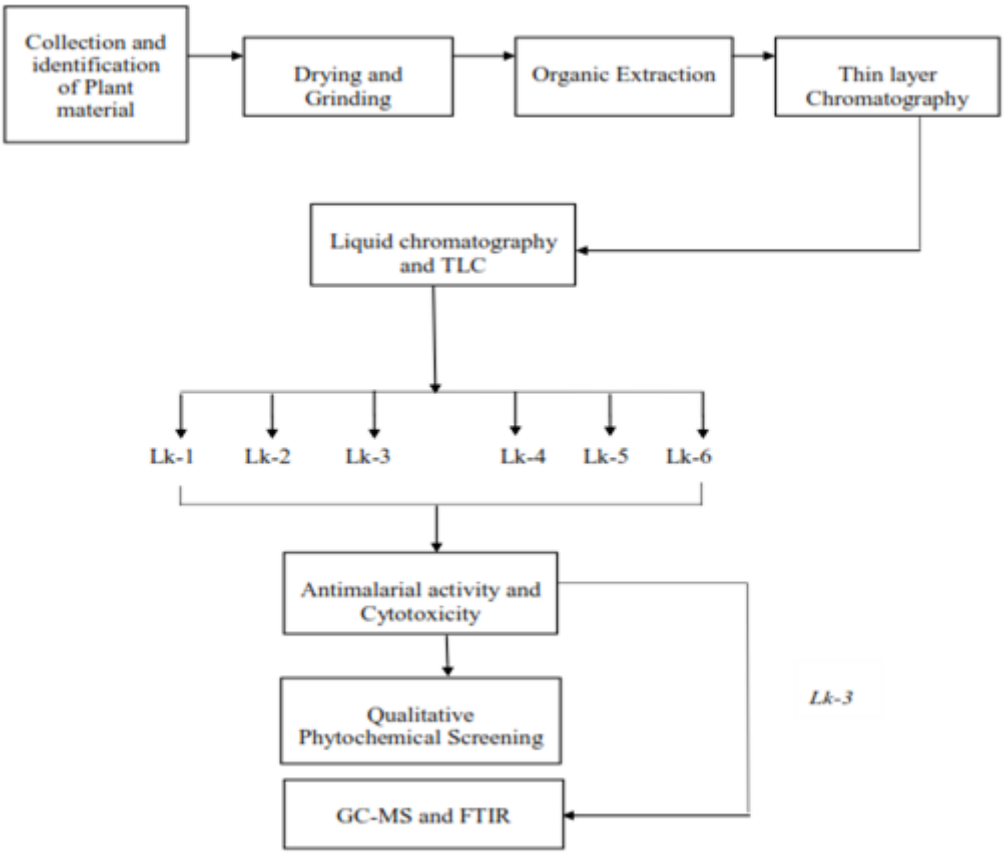

Figure 3: Hypothetical Outlook of the Study

3.2 Extraction of the Processed Plant Material

Organic extraction was done with brief modifications of the method described by Gathirwa *et al*. (2008). Briefly, a top pan balance (Slater model-323, China), was used to weigh 1 kilogram of plant powder into a 5-litre flat-bottomed flask. Methanol (MeOH) and Dichloromethane (DCM) mixture in the ratio 1:1 was used to macerate the plant material. The powdered product was extracted at room temperature for 24 hours, which was repeated twice. The material was filtered using the whatman filter paper and concentrated using a rotary evaporator at reduced pressure (BUCHI Rotavapor model No. R-114; Switzerland) giving a dark green gum of 87.8 g, hence a percentage yield of 8.78 %.

3.3 Thin Layer Chromatography

A small portion of the total extract of *L. kituiensis* was reconstituted in MeOH: Dichloromethane mixture and spotted on cut pieces of pre-coated Thin layer chromatography plates (TLC). The plates were then developed in different solvent pairs (Sule *et al*., 2011). Upon development, assessment of plates was done by observation on open light, under UV (254 nm and 365 nm), and after vanillin spray with plates heated to 110^{o}C for 5 minutes. Based on retention factors (Rf) obtained from developed plates, the best solvent mixture to run a column was identified. Hexane and Ethyl acetate mixed in the ratio 4:1 gave the best separations and hence the pair was established as ideal mobile phase in liquid column chromatography.

3.4 Column Chromatography

The open column chromatography technique was adopted to separate the total extract where briefly, a glass column of diameter 10 centimetres was packed with a slurry of 300 g of Silica powder (70-230 mesh, using 100 % Hexane) (Walker *et al.*, 2015). The slurry was immediately transferred into the column with intermittent swirling to prevent solidification. The wetness of the gel was maintained by several elutions of 100% hexane. After 24 hours of undisturbed packing, Hexane was drained and 60 g of the total extract of *L. kituiensis* was mixed with 70 grams of silica and 100% hexane. The mixture was swirled to homogeneity and transferred into the packed column. Hexane (500 ml) was then loaded into the column to begin fractionation as fractions were collected in different flasks. The polarity of the mobile phase was progressively increased until no further separations could be identified as guided by TLC profiles gathered in the process.


1


2


..3

Figure 4: Liquid chromatography showing progressive development of fractions (1, 2 and 3 respectively)

Figure 5: Fractions after column chromatography and fractions after combination as guided by TLC plates

3.5 Drug Preparation for Bioassays

For *in-vitro* antiplasmodial assays, stock formulations of the fractions were constituted at concentrations 1 mg/ml in DMSO diluted by RPM1640 to attain a final 0.2 % content of DMSO. Based on *in vitro* cytotoxicity results, where fractions exhibited moderate to low toxicity, and guided by Mzena *et al*. (2018), two dosages (500 mg/kg and 1000 mg/kg) were chosen for *in vivo* assay. Dosages for *in-vivo* assay were prepared in a vehicle composed of 10% Tween 80, 3% DMSO and PBS (Basir *et al.*, 2012). Calculation for dosages preparation were as illustrated below. The vehicle served as the negative control in each case respectively. Calculations were done as follows:

3.5.1 *In vivo* Drug Preparation Per Dogase-1000 mg/kg of Body Weight

1000 mg → 1000 g

? → 20 g

= (20/1000) ×1000 mg

= 20 mg per mouse

Therefore, a four-day dose for 5 animals per group,

= 20 mg×5 animals ×4 days

400 mg of drug was weighed into centrifuge tubes

3.5.2 Drug Dissolution

Each animal was to receive an oral 0.2 ml of drug solution

Therefore, 0.2 × 5 animals per group= 1 ml

Since they were organic extracts, they were dissolved in 3% DMSO and topped up to 1ml using 10% Tween 80 and PBS

3.6 *In vitro* Screening of Fractions for Antiplasmodial Activity

Antiplasmodial activity of fractions was done through a semi-automated technique on two culture-adapted strains of *P. falciparum* namely, chloroquine sensitive (D6) and chloroquine resistant (W2) as earlier described by Desjardins *et al*. (1979). Briefly, culture media consisted of RPMI 1640, 25 MM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)at pH 7.4, supplemented with 12.5% human serum (obtained from pooled blood types A, AB and O and inactivated by heating). Haematocrit was prepared at 6 % human erythrocytes obtained from donors of blood group O^+ making 5 ml total volume in culture. Cultures were kept in sealed flasks at 37°C, and gassed by gas mixed as 92% N_2 , 3% O_2 and 5% CO_2. Fractions were prepared at 100 mg/ml by DMSO whose concentration was diluted by RPM1640 to attain a final 0.2% content of DMSO. Test samples were constituted in DMSO (diluted to < 0.2% DMSO content) while Chloroquine was disolved in distilled water. The wells of the sterile, flat-bottomed 96-well plates were filled with 25 µl of screening medium (Corning Incorported, Costar®, U.S.A). Constituted test samples (50 µl) at starting concentration of 100 µg/ml was seeded to wells of row B in duplicate. After proper mixing, 25 µl of drug solution at row B was transferred to wells at row C successively to row H 200 µl of 1.5% hematocrit at parasitemia level of 0.5% was dispensed to the wells of the plates

excluding wells A9 to A12, where 200 µl of healthy erythrocytes at 1.5% hematocrit) were seeded and taken to be the negative controls. Plates were then incubated for 48 hours at 37 ^{0}C in a chamber of mixed gas (92% N_2, 3% O_2 and 5% CO_2) at 95% humidity. The radiolabel (hypoxanthine-25 µl) was then seeded into the plates at 0.5 µlCi in every well. The plates were further incubated for 18 hours after which they were frozen to negative 20 °C. The plates were then thawed and the contents transferred onto filter mats (A 1450-421; Perkin Elmer) by use of a cell harvester (Wallac Perkin Elmer; 1295-004 Betaplate). The filter mats were then oven-dried and saturated in 10 ml scintillation fluid (Perkin Elmer, Beta Scintilla®; Schwerzenbach, CH) while covered in special foil (1450-432; Perkin Elmer). A Betaplate scintillation counter (Wallac Perkin Elmer, Betaplate) was then used to determine icorported hypoxanthine [3H] yielding results as counts per minute (cpm). The results of each well were reported as counts per minute and recorded as a percentage of untreated (positive) control (wells from A1 to A8; no test compound). The background effect was substracted from the reading of the negative control. Linear interpolation was important in calculation of IC_{50}s (concentration of the drug at which 50% of radiolabelled hypoxanthin was inhibited relative untreated controls).

3.7 Cell Culture and Cytotoxicity Assay

A calorimetric-based assay that uses 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, which measures conversion of MTT into formazan crystals by living cells, hence determining mitochondrial activity was used to asses cytotoxic levels of fractions as earlier described by Kaur *et al*. (2018). Vero (E6) cell line was cultured in supplimented Dulbecco's Eagle's media supplemented with 10% heat inactivated Fetal bovine serum (Himedia), l-Glutamine and treated with penicillin/streptomycin (100 µgml−1), gentamycin (100 µgml−1). Culture flasks were incubated in a CO_2 incubator (90 % Relative Humidity and 5% CO_2) at 37 °C. Upon attainment of maximum confluence (90-100 %) viewed under an inverted microscope (Fig 6), the cell monolayer was trypsinized and seeded at 2×10^5 cells/ml in each well of the 96 well plates and incubated for 24 hours. Drug concentrations were prepared (1000 µg/ml), seeded at the highest concentration and serial diluted to afford different concentrations across the plate. Plates were then re-incubated for fourty eight hours. Thereafter, 10 µl of MTT solution (5 mgml−1) was then added into all wells and incubated for 4 hours. The plates were emptied of media and the formazan crystals formed were treated with 100 µl of neat DMSO seeded per well. Using a microplate reader set at 550 nm,

(Thermofisher, MultiscanGo Spectrophotometer), absorbances of each well were determined. Dose response curves was plotted from inhibition data and used to determine CC_{50} values (concentration required to inhibit/alter 50% of the cells). Selectivity index ($SI=IC_{50}/CC_{50}$) which measures the significance of drugs by comparing their toxic levels and their discriminatory inhibitory effect on *P. falciparum* was determined (Wright & Phillipson, 1990).

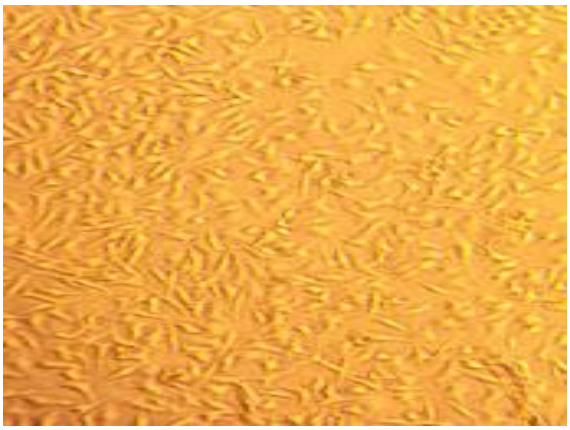

Figure 6: Morphological appearance of Vero E6 cell line at almost 100% confluence (Magnification ×400)

3.8 *In-vivo* Antimalarial Assays

Dosages for the *in vivo* assay were prepared in a vehicle composed of 10% Tween 80, 3% DMSO and PBS. The vehicle served as the negative control in each case respectively. We adopted a model involving the use of *Plasmodium berghei* NK65 strain to study *in vivo* antimalarial activity on Swiss albino mice according to Peter's suppressive test (Peters, 1975). Upon attainment of ethical approvals, Naïve Swiss albino mice weighing 20±2g were acclimatized for 48 hours. Frozen stabilates of *P. berghei* were thawed and injected into a group of 3 animals for propagation. Assessment of parasite growth was done microscopically and upon attainment of approximately 20% parasitaemia, blood was drawn from the passage mice (parasite donors) by cardiac puncture and reconstituted to 2% in phosphate saline glucose. Each of the 40 mice were injected in the peritoneal cavity with 0.2 ml of blood (infected) at an estimated $2x10^7$ *P. berghei* on day 0. Animals were then randomized into cages of 5 each i.e.; positive control (chloroquine 10mg/kg), negative control and the 6 fraction-test groups which received 1000 mg/kg and 500 mg/kg of test groups. All cages were well labelled with experimental details and animals supplied with clean water and standard

rodent pellets. A 22-gauge cannula (Harvard Apparatus; length = 25.4 mm, ball diameter = 1.25 mm) was used for oral administration of drugs 2 hours after infection. This continued on days 1 to 3 (24, 48 and 72 hrs. Drug administration was stopped after 96 hours since infection. On day 4, microscopic slides were made by drawing blood through the tail veins. Slides were fixed in methanol and stained using 10% Giemsa for 15 minutes. Parasitemia in each group was assessed microscopically under high magnification and oil immersion. Percentage chemosuppression of fractions was calculated with mean parasitemia on day 4 as described by Tona *et al*. (2001). Raw data on parasitemia and chemosuppression per concentration are illustrated in Appendix B.

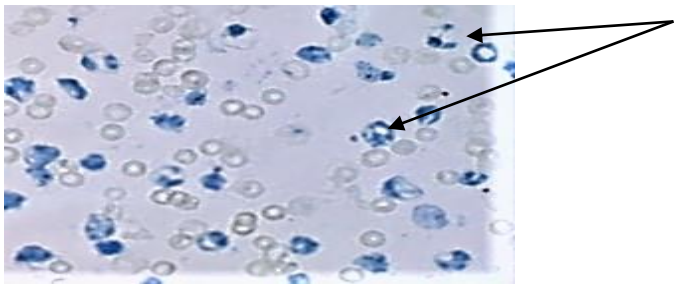

Figure 7: Microscopic appearance of heavily parasitized RBCs (arrows illustrate parasitized RBCs). Slide prepared from blood obtained from a donor mouse. Magnification (×1600) under oil immersion

3.9 Preliminary Qualitative Phytochemical Screening

Phytochemicals refers to a group of compounds found in plants which have found applications in various aspects of human life, particularly phmaceutical uses (Igwe, 2015). Assessment of secondary metabolites in the fractions was done using standard procedures and tests as cited. Availability or absence of alkaloids, tannins, saponins, phenols and glycosides was analysed and qualitatively indicated as (+) when present and (-) when absent. Fractions were constituted in their respective solvent mixtures of fractionation prior to each phytochemical test.

3.9.1 Alkaloids

The constituted fractions (5 ml each) was added to tubes of 2 ml dilute Ammonia and 5 ml of Chloroform added. 1 ml of acetic acid was then added and separated into two portions based on the formation of chloroform layer. To one tube containing one portion, Draggendorff's

solution was added while Mayer's reagent was added to the other. The appearance of a cream color on the portion in Mayer's reagent and a brown precipitate in the Draggendorff's reagent confnirmed presence of alkaloids (Radhia *et al*., 2018).

3.9.2 Phenols

Phenols are a group of compounds that possess hydroxyl groups attached to an aromatic ring. They are the most abundant compounds in the plant kingdom (Lattanzio, 2013). To 2 ml of each fraction, 5% aqueous ferric chloride (2 ml) was added. Appearance of blue colour confirmed for phenols content (Ayoola *et al.*, 2008).

3.9.3 Saponins

Aliquots of each fraction at 5.0 ml was diluted in 20 ml of deionized water, shaken vigorously and observed. Persistent foaming indicated the presence of saponins (Nkwocha, 2015).

3.9.4 Terpenoids

To 5 ml of each fraction, 2.0 ml of chloroform was added and boiled with 3 ml concentrated sulfuric acid. Formation of grey colour showed availability of terpenoids (Ukoha *et al*., 2011).

3.9.5 Tannins

Ferric chloride (0.1 %) of volume 1 ml was dispensed in drops to tubes containing 3 ml of each fractions and observed for blue-black colouration (gallic tannins) or blue-green (cathechic tannins) (Bargah, 2015).

3.9.6 Glycosides

A volume of 1 ml concentrated sulphuric acid was poured on walls of an inclined tube containing 1ml each of the fractions separately. Ten percent of Ferric chloride solution was added in drop wise and a brown ring was observed indicating presence of glycosides (Andrew *et al*., 2016).

3.10 Fourier Trans-*Infrared* Spectroscopy

Fraction *Lk-3* (dark yellow paste) exhibited outstanding antimalarial efficacy against *Plasmodium berghei* at an oral dose of 1000 mg/kg. A sample from this fraction was therefore subjected to FTIR at Jommo Kenyatta University of Agriculture, Science and Technology (JKUAT). The Shimadzu FTIR spectrophotometer (FTS- 8000, Japan) was used to analyse the functional groups as described by Memon *et al*. (2015). The standard KBr disc method was used, where briefly, crystal sample was ground with KBr in the ratio 1 to 10 milligrams respectively. The mixture was pressed into a pellet using a hydraulic press and scanned with a spectral resolution set at 4 cm^{-1} and the scanning ranged from 400 to 4000 cm^{-1}.

3.11 Gas Chromatography-Mass Spectroscopy Analysis

Gass chromatography couled to a mass spec housed at JKUAT was used to further determine compounds in fraction *Lk-3.* This was done as previously described by Igwe (2015). A Shimadzu QP 2010-SE GC-MS coupled to an auto sampler was used for analysis. Ultrapure Helium was used as the carrier gas at a flow rate of 1 ml per minute. A BPX5 (non-polar column, 30 m; 0.25 mm ID; 0.25 µm film thickness, composed of 100 % Dimethyl poly siloxane) was used for separation. The GC was programmed as follows: 50 ˚C (1 minute); 5 ˚C /min to 250˚ C (1 minute). Total runtime was 42 minutes. Injection temperature was set to 200^0 C, while the interface temperature was set at 250^0C. The electron impact ion source was set at 200^0 C and mass analysis was done in full scan mode, 50-550 a.m.u. The compounds were then identified from the GC-MS peaks upon compariosn with the National Institute of Advanced Industrial Science and Technology (NAIST) 2014 library of mass spectra.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Thin Layer Chromatographic Analysis of Total Organic Extracts of *Lippia kituiensis*

A total of 32 fractions were obtained and concentrated as described previously. Thin layer Chromatography (TLC) profile was used to determine fractions that contained similar compounds, based on their retention factors. Such fractions were pooled to make single entities. The TLC plate was run in a mobile system consisting of Hexane and Ethyl acetate in the ratio 8:2. Eventually, 6 fractions were obtained as summarised in Table 1. When observed under UV 254 nm, fractions Lk-4 and largely Lk-5 contained fluorescent spots, Fig 6.

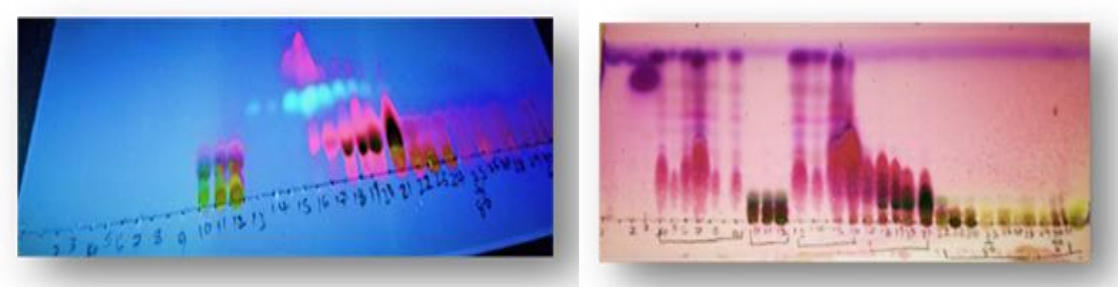

Figure 8: TLC plates when observed under 254 nm UV and appearance after Vanillin spray

Table 1: Fractions of *L. kituiensis* and their characteristics

Fraction Code	Number of fractions combined	Weights of fractions (grams)	Percentage yield of fractions	Physical Appearance
Lk-1	3	3.483	3.97	Dark green gum
Lk-2	6	5.072	5.78	Dark green gum
Lk-3	3	2.051	2.34	Dark Yellow paste
Lk-4	4	3.603	4.10	Dark green paste
Lk-5	5	3.347	3.81	Dark green paste
Lk-6	11	5.910	6.73	Dark green paste

4.2 Antiplasmodial Activity of Fractions of *L. kituiensis* and their Cytotoxicity

Preliminary *in-vitro* antiplasmodial assay was performed on P. falciparum strains of W2 (chloroquine-resistant) and D6 (chloroquine-sensitive). The positive control was chloroquine diphosphate, while the negative control was DMSO. *In vitro* antiplasmodial activity of fractions is shown in Table 2.

Table 2: Antiplasmodial Activity of Fractions against W2 and D6 Plasmodium strains

Fraction	IC_{50} of Anti-plasmodial Assay (M±SD)	
	W2 (µg/ml)	D6 (µg/ml)
Lk-1	>100	79.41±16.61
Lk-2	68.42±5.55	78.85±5.82
Lk-3	45.16±9.05	30.43±0.68
Lk-4	24.18±2.50	30.82±18.01
Lk-5	24.42±5.95	19.45±6.20
Lk-6	56.75±5.12	36.53±14.42
Cq	77.86±4.09 ng/ml	15.71±6.49 ng/ml

IC_{50} - effective concentration that inhibits 50% of the cultured parasite from hypoxanthine uptake.

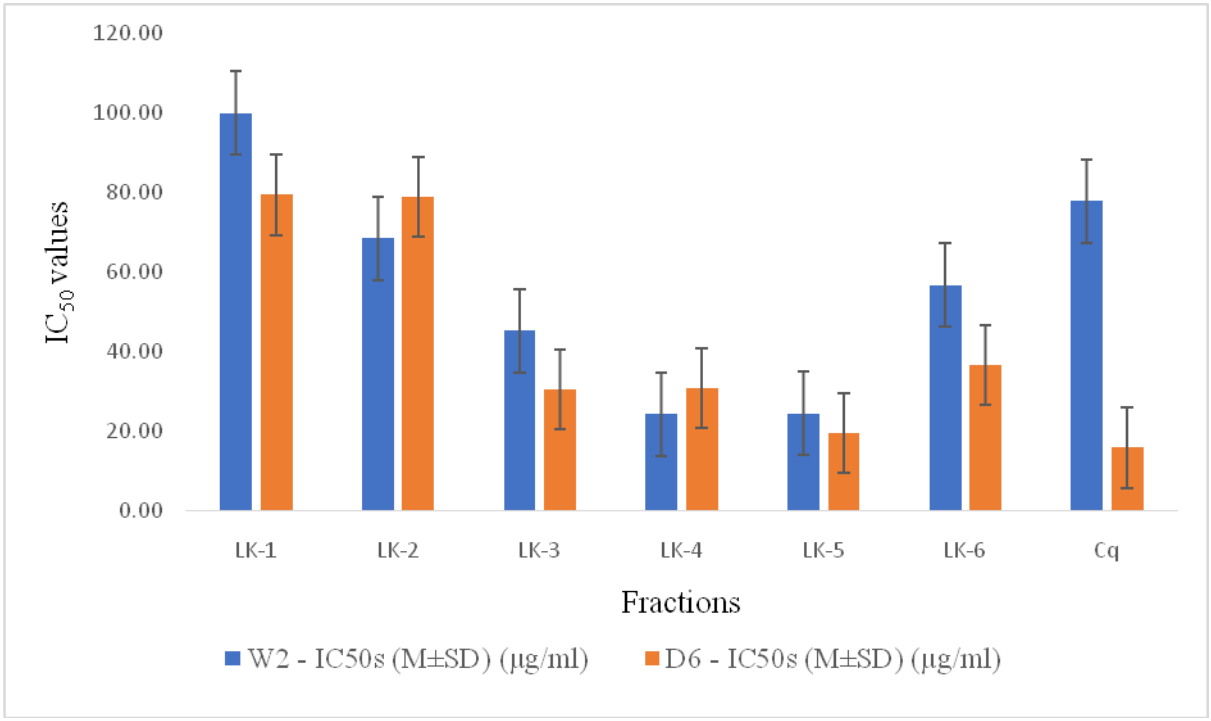

Figure 9: Graphical representation of Antiplasmodial Activity (IC_{50} values) of Fractions against W2 and D6 Strains of Plasmodium

The fractions displayed medium to low activity; where efficacy was graded as IC_{50} µg / ml strong, 10–50 µg / ml low, 50–100 µg / ml low and > 100 µg / ml inactive (Berthi *et al.*,

2018). Generally, fraction LK-5 showed the highest antiplasmodial activity against chloroquine-sensitive strain (D6) with IC_{50} of 19.45 µg/ml and second highest activity against chloroquine-resistant strain (W2) IC_{50} 24.42 µg/ml respectively. Notably, efficacy increased with increasing polarity of the mobile phase except in fraction Lk-6. Judgement of cytotoxic levels was made where a fraction was considered highly toxic when CC_{50}< 10 µg/ml, moderately cytotoxic with CC_{50} ranging from 11–30 µg/ml, slightly cytotoxic when CC_{50}, 31–50 µg/ml, and preferably non-toxic with CC_{50}> 50 µg/ml (Berthi *et al*., 2018). Hence, all fractions were found to be potentially non-toxic, showing CC_{50} values >50, except Lk-4 (46.26 µg/ml). Selectivity indices (SI) were calculated as previously explained by Rocha *et al*. (2012). The fractions generally depicted lower selectivity indices against D6 compared to W2 plasmodium strains. However, with SI > 2 against both parasite strains, Lk-5, which also exerted the best antiplasmodial efficacy showed specific activity rather than toxicity.

Table 3: Cytotoxicity and Selectivity indices of fractions

Fraction	CC_{50} (µg/ml) against Vero E6 Cell line	Selectivity Indices of Fractions	
		W2	D6
Lk-1	71.21	0.71	0.89
Lk-2	55.00	0.80	0.69
Lk-3	64.90	1.44	2.13
Lk-4	46.26	1.91	1.50
Lk-5	67.13	2.75	3.45
Lk-6	69.96	1.23	1.92
Cq	n/d	n/d	n/d

CC_{50}- Drug concentration that reduces cell viability (Vero E6 cell line) by 50%. **Selectivity index**- Ratio of IC_{50} to CC_{50}-measure of drug's ability to target parasite, **Cq**: Chloroquine **n/d**: not done.

4.3 Antimalarial Activity of Fractions of *L. kituiensis*

Two dosages of each fraction (500 mg/kg and 1000 mg/kg) were screened in a mouse model following Peter's suppressive test (Peters, 1975) (results are as shown in Table 4). Drug dosages were administered orally. Chloroquine (10 mg/kg) was used as the positive control, while the vehicle served as negative control. Chemo-suppression was used as a measure of the fractions' effectiveness in reducing parasite growth over the period tested.

Table 4*: In vivo* antimalarial activity of fractions of *L. kituiensis* against *P. berghei (Anka 65)*

Fractions	Parasite density per dose (Mean±SD)		Chemo-suppression per dose (Mean±SD)	
	500 mg/kg	**1000 mg/kg**	**500 mg/kg**	**1000 mg/kg**
Lk-1	2.30±0.43	3.99±0.07	78.64±4.01^{b}	73.04±044^{b}
Lk-2	5.47±0.65	4.10±0.52	49.24±6.06^{b}	72.27±3.51^{b}
Lk-3	3.99±0.82	2.96±0.16	62.24±7.61^{b}	80.01±1.07^{b}
Lk-4	5.33±0.44	4.39±0.55	50.57±4.16^{b}	70.35±3.72^{b}
Lk-5	4.14±0.42	4.01±0.89	61.59±3.95^{b}	72.91±6.06^{b}
Lk-6	5.74±0.59	4.09±0.55	46.77±5.51^{b}	72.37±3.71^{b}
Cq -(10 mg/kg)	0.56±0.37	1.10±0.31	94.72±3.43^{a}	92.57±2.11^{a}
Placebo	10.78±0.70	14.82±0.90	0.00^{b}	0.00^{b}

Cq-Chloroquine-was the positive control administered at 10mg/kg, **Placebo**-Negative control- (vehicle), **Parasite density**-Denotes percentage of infected erythrocytes, $^{a, b}$ P≤ 0.05 level of significant difference between groups against positive control and negative control respectively. Same letters are not significantly different.

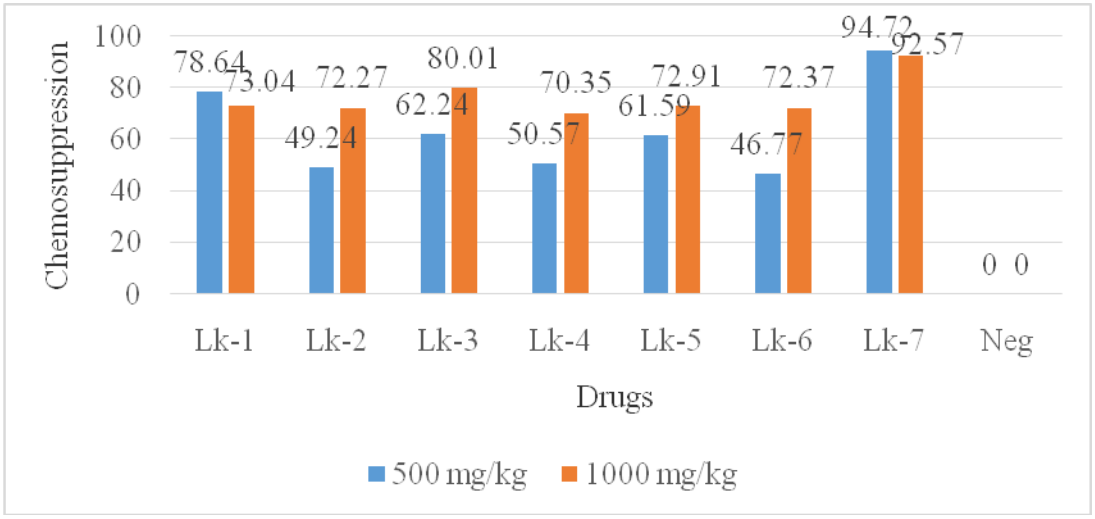

Figure 10: Chemosuppression of fractions against *P. berghei* at 500 mg/kg and 1000 mg/kg

Generally, all fractions exhibited significant antimalarial activity when compared to the negative control. Their efficacy was however insignificant on comparison with the positive control. A dose dependent activity was observed with the highest efficacy seen in animals treated with 1000 mg/kg. However, 500 mg/kg of Lk-1 was more efficacious than it was at 1000 mg/kg. This scenario can be linked to toxicity since mortality was seen day 2 post-infection. Additionally, the fraction showed low selectivity index alluding to cytotoxicity.

Fraction Lk-3 was found to possess the most pronounced efficacy during *in vivo* testing with percentage chemosuppression of 80.01 at 1000 mg/kg. This was consistent with *in vitro* data in Table 2, particularly against D6 strain.

4.4 Qualitative Phytochemical Screening of *L. kituiensis*

To determine classes of secondary metabolites in each fraction, qualitative phytochemical screening was conducted. Results are shown in Table 5.

Table 5: Qualitative phytochemical screening of *L. kituiensis*

Fractions	Secondary Metabolites Screened				
	Alkaloids	**Terpenoids**	**Phenols**	**Tannins**	**Glycosides**
Lk-1	+	-	-	-	+
Lk-2	+	-	-	-	+
Lk-3	+	+	+	-	-
Lk-4	+	-	-	+	+
Lk-5	+	-	+	-	+
Lk-6	-	+	+	-	+

+ = Presence - = Absence

Comparatively, *Lk-3 and Lk-5* demonstrated the richness of different secondary metabolites, hence the efficacy observed from the fractions. Notably, all the fractions except *Lk-6* indicated presence of alkaloids. These results are consistent with phytochemical screening of total extracts of *L. kituiensis* growing in Kenya, where alkaloids, Tannins and Glycosides were found (Omwenga *et al*., 2011).

4.5 Spectral Patterns and their Interpretations for Fraction *Lk-3*

Fourier transform-infrared spectral data complement the qualitative phytochemical screening of LK-3 depicted in Table 4. Presence of different classes of phytochemical species were obtained and suggestion of compounds given by gas chromatography coupled to mass spectrophotometer. Fourier transform-infrared results of *Lk-3* are as indicated in Fig. 9 and Table 5, while compounds available in fraction *Lk-3* are as indicated in Table 7.

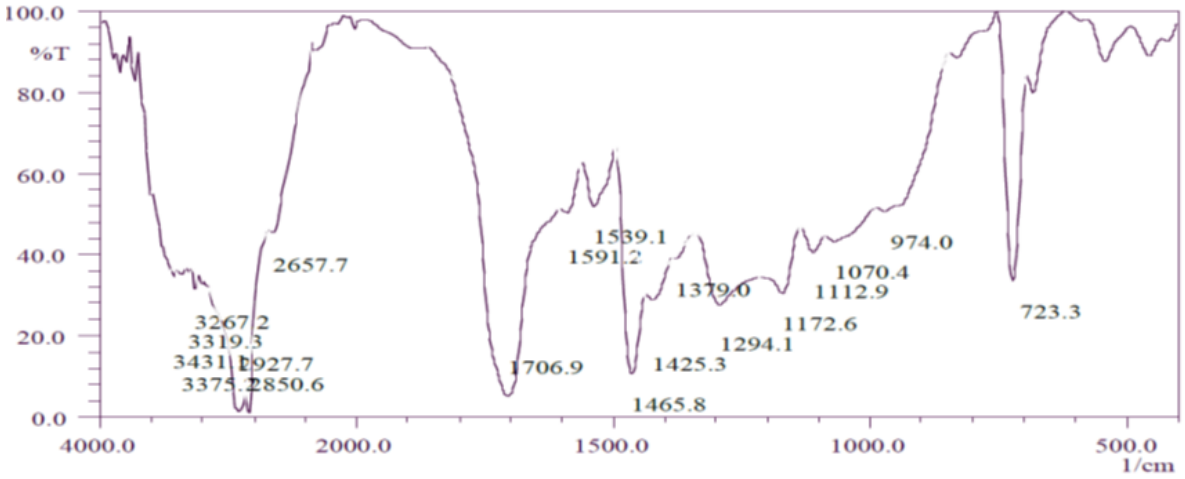

Figure 11: Fourier transform-infrared Spectral pattern of Lk-3

Table 6: Functional groups present in fraction *Lk-3*

S/No	Frequency range (cm^{-1})	Functional Group identified
1	3329.3-2657.7	O-H carboxylic acid
2	1706.9	C=O saturated carbonyl group
3	1465.8	C-H methylene group from an alkane
4	1591.2 and 1425.3	C=C stretching in aromatic ring
5	1172.6, 1112.9, 1070.4	C-H weak bending in the aromatic ring
6	1379.0	O-H bending due to Phenol
7	974.0, and 723	Long chain Alkanes

4.6 Compounds Present in Fraction *Lk-3* Following Interpretations of GC-MS Spectra

Eight compounds were identified where 3 have previously been reported for antimalarial activity and other pharmacological properties (structures are indicated below). Hexatriacontane (28.75%) was the major compound identified followed by Hentriacontane, 3-methyl (7.13%) and Tetratetracontane (6.13%). The first compound to be eluted (retention time 6.758 minutes) needs further identification using nuclear magnetic resonance which was beyond the scope of the current study. Although the NIST library suggested 1, 3-Butanediol, the similarity index is so low to imply so.

Table 7: Compounds in Lk-3 identified by GC-MS

S0/N	Retention time (mins)	Name	Molecular Formula	Molecular Weight	Peak Area %
1	6.758	Unidentified	$C_8H_{14}O_4$	174	0.99
2	10.772	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	206	0.98
3	13.417	2-Pentadecanone, 6,10,14-trimethyl	$C_{18}H_{36}O$	268	2.37
4	14.02	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	1.91
5	14.33	l-(+)Ascorbic acid2,6 dihexadecanoate	$C_{38}H_{68}0_8$	652	5.88
6	23.055	Tetratetracontane	$C_{44}H_{90}$	618	6.31
7	26.435	Hentriacontane, 3-methyl	$C_{32}H_{66}$	450	7.13
8	21.594	Hexatriacontane	$C_{36}H_{74}$	506	28.75

Three compounds identified have previously been reported to possess antimalarial activity and other pharmacological properties (structures and processes of their splitting are as shown below, while GC-MS chromatograms of all compounds are as indicated in Appendix 1)

4.6.1 Hexadecanoic Acid, Methyl Ester ($C_{17}H_{34}O_2$)

The base peak at m/z 74 and m/z 87 were due to McLafferty rearrangement. Loss of a Methoxy group gave a peak at m/z 239 ($C_{15}H_{31}O_1^+$). The peaks at m/z 115, 129, 143, 157, 171, 185, 199, 213 and 227 follows fragmentation pattern of $C_nH_{2n-1}O_2^{+\cdot}$, corresponding to $C_6H_{11}O_2^{+\cdot}$, $C_7H_{13}O_2^+$, $C_8H_{15}O_2^+$, $C_9H_{17}O_2^+$, $C_{10}H_{19}O_2^+$, $C_{11}H_{21}O_2^+$, $C_{12}H_{23}O_2^+$, $C_{13}H_{25}O_2^+$ and $C_{14}H_{27}O_2^+$ respectively.

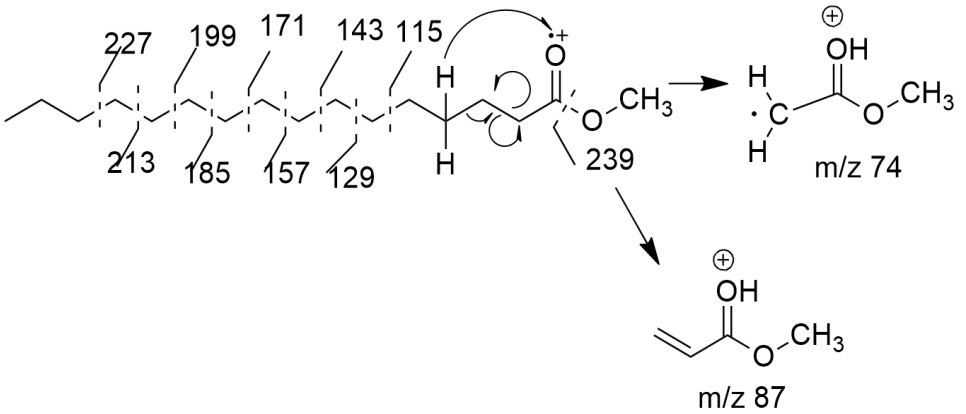

Figure 12: The 2, 4-Di-tert-butylphenol

The molecular ion peak is m/z 206 corresponding to the formula $C_{14}H_{22}O$. The base peak at m/z 191 ($C_{13}H_{19}O^+$) was due to loss of methyl group M-CH_3. Subsequent loss of water gave a

peak at m/z 175 ($C_{13}H_{19}^+$). The loss of three methyl groups (1, 1-dimethylethyl) gave a peak at m/z 163 ($C_{11}H_{15}O^+$). The loss of water followed by 1, 2-propadiene gave peaks at m/z 147 ($C_{11}H_{15}^+$) and $C_8H_{11}^+$ (Ethyl benzene) respectively. Similarly the peak m/z 107, $C_7H_7O^+$ (*p*-cresol) was also due to loss of 2-butene from m/z 163. Peak at m/z 91 corresponds to $C_7H_7^+$ (hydroxyl tropylium ion) is as a result of benzylic cleavage. Other peaks noted at m/z 74, 57, 41 and 29 were observed and corresponded to the following ions: $C_4H_{10}O^+$, $C_4H_9^+$, $C_3H_5^+$ and $C_2H_5^+$ respectively.

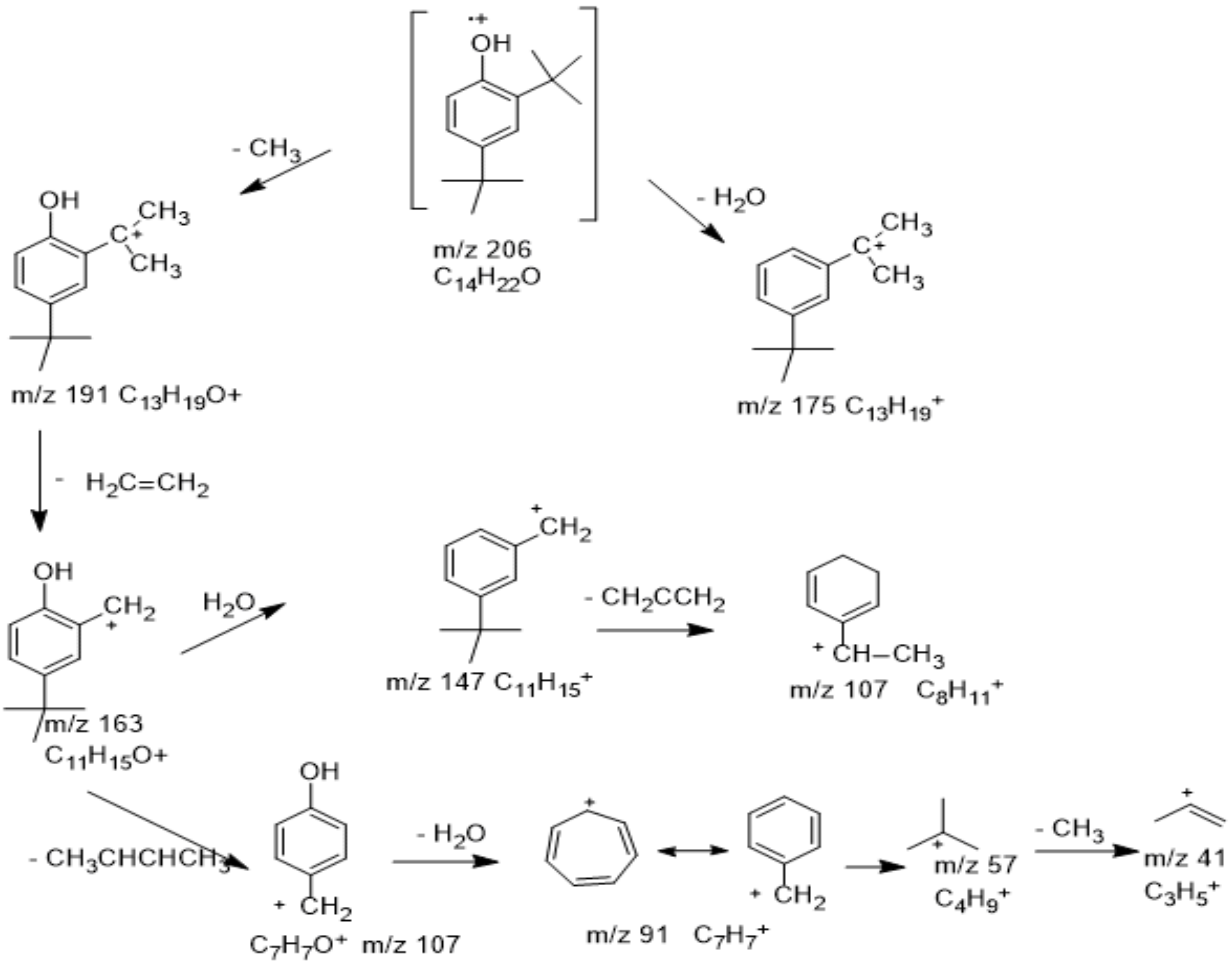

Figure 13: Hentriacontane, 3-methyl

The molecular ion peak is m/z 450 corresponding to the formula $C_{32}H_{66}$. The compound was identified as a hydrocarbon due to its fragmentation pattern. The mass spectrum of saturated hydrocarbons has a fragmentation pattern characterized by clusters of peaks and corresponding cluster are 14 mass units (CH_2) apart. The most abundant fragments are at C_4

and C_5 and the fragment abundances decrease smoothly. The base peak at m/z 57 was due to $C_4H_9^+$ and the peaks at m/z 99, 85, 71 were due to $C_7H_{15}^+$, $C_6H_{13}^+$ and $C_5H_{11}^+$, respectively.

4.7 Discussion

4.7.1 Fractions of Total Organic Extracts of *Lippia kituiensis*

The antimalarial activity of fractionated organic extracts of *L. kituiensis* and their cytotoxic levels have been revealed in this study. Moderate to low antiplasmodial activity was seen (Table 2). This can be linked to instability of the fractionated extracts which are normally protected by chlorophyll and/or other components in their native forms (Zani *et al*., 1997). The concerted antimalarial effects of the total extracts of *L. kituiensis* as depicted in previous reports by Mzena *et al*. (2017) could have also been compromised by the semi-purification process during fractionation

4.7.2 Antimalarial Activity of the Fractions

Outstanding efficacy observed during *in vivo* studies rather than *in vitro* suggests immunomodulatory activity of drugs which may be due to localization of specific phytochemicals including fatty acids such as linoleic acids, squalene and tannins which have previously been reported to possess immune-stimulating properties (Kumaradevan *et al.*, 2015; Chakrabarti *et al.*, 2012). These class of compounds (except Tannins) were found to be present in fraction Lk3 as illustrated by GC-MS data in Table 7.

To the best of our understanding, this study reports for the first time the antimalarial potential of fractionated extracts of *L. ktuiensis.* Nevertheless, the genus *Lippia* has been assigned a number of pharmacological properties, including antimalarial, antimicrobial, antimicrobial, larvicidal, antispasmodic, analgesic, anti-inflammatory and antipyretic activities (Santos *et al*., 2016). Numerous studies on this genus have focused on their essential oils (Stashenko *et al.*, 2013). The promising antimalarial activity observed is consistent with other reports on the use of plants in the same family for the management of Malaria. Among other pharmacological properties, *Lippia javanica* has been reported for use to cure fever or malaria (Maroyi, 2017). Ludere *et al*. (2013) isolated a compound (Lippialactone) with antiplasmodial effects from aerial parts of *L. javanica*. In a different study involving plants

collected from Nigeria, *Lippia multiflora* was among plant samples from 11 families collected based on antimalarial ethnopharmacology (Ajaiyeoba *et al*., 2006).

4.7.3 Qualitative Antimalarial Activity of Fractions of *L. kituiensis*

Presence of secondary metabolites, especially alkaloids, phenolic compounds, and terpenoids could be responsible for the observed antimalarial activity. Although the particular study reports antimalarial efficacy of fractionated extracts of *L. kituiensis (V)* for the first time, reports of the medicinal value of the plant to the local communities have been reported previously. For example, Onyango (2013) reported lervicidal activity of extracts of *L. kituiensis.*

4.7.4 Compounds Identification in LK-3 Using GC-MS

Most of the compounds in fraction Lk-3 identified by GC-MS have been reported previously. Hexadecanoic acid, methyl ester was earlier identified in whole plant extracts of *Phyllanthus amarus* a medicinal plant used to treat malaria in Eastern Nigeria (Igwe, 2015) Hexadecanoic acid, methyl ester, is a polyunsaturated fatty acid (PUFA) that has been involved in antiplasmodial activity and has been documented to increase with unsaturation, (Melariri *et al.*, 2012). The suggested compound 2,4-Di-tert-butylphenol (DTBP) which is characteristically volatile is a phenolic compound reported to possess antioxidant properties (Choi *et al.*, 2013) and *in vitro* antimalarial activity at 100 mM (Kusch *et al*., 2011; Kulangara *et al*., 2015). Furthermore, DTBP isolated from a *Lactococcus sp* demonstrated antifungal, antioxidant and cytotoxicity against cancer cell lines (Jayaseelan *et al*., 2017). The rest of the compounds have not been reported for antimalarial activity but other pharmacological importance where Hexatriacontane identified in essential oils of *Cassia fistula* showed significant activity against the standard and clinical isolates of *Candida* species (Irshad *et al*., 2012). The observed antimalarial efficacy of *Lk-3* and by extension the other fractions can be attributed to individual or synergistic activity of compounds with antimalarial activity evidenced herein.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Plasmodium resistance to Artemisinin threatens global efforts in the elimination or eradication of malaria. Despite past decades of steady advances in the fight against the disease, statistics show that the disease is still a threat to public health. The need for continued research towards development of new therapies for Malaria cannot be overstated. The potential of medicinal plants to serve as the source of the next novel antimalarial drug formed the basis of this study. Total organic extracts of *Lippia kituiensis* were subjected to fractionation using Chromatographic techniques. Substantive antimalarial efficacy which was more pronounced *in-vivo* rather than *in-vitro* was revealed for the first time in this study. The *in-vitro* cytotoxicity results of the fractions of *L. kituiensis,* suggest their potential safety.

The classes of compounds attributable to the efficacy have also been made evident in this study. Compounds that have been reported previously to possess antimalarial activity were identified in the most efficacious fraction (LK-3) from the plant. Therefore, results from this study validate traditional use of extracts of the plant to manage malaria.

5.2 Recommendations

Based on this study the following recommendations are warranted. Given the revealed background antimalarial efficacy of some fractions from *L. kituiensis* further studies are needed in the following areas:

i) Isolation and purification of novel compound(s) from fractions, particularly Lk-3.
ii) Screening of compounds isolated from the fractions against Plasmodium parasite
iii) Comprehensive *in-vivo* safety studies (sub-acute and chronic toxicity assays) from fractions and compounds isolated therefrom.

These activities were beyond the scope of the current study based on budgetary limitations.

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APPENDICES

Appendix 1: Antimalarial activity of fractions

500 mg/kg

Drug Name	Mouse No.	RBC	PRBC	Parasitemia	%chemosuppression
LK-1	1	397	11	2.770780856	74.31883336
	1	393	9	2.290076336	78.77427517
	1	405	7	1.728395062	83.98025542
	1	371	9	2.425876011	77.51560686
LK2	2	397	19	4.785894207	55.64162125
	2	345	21	6.086956522	43.58263865
	2	396	22	5.555555556	48.50796385
LK3	3	360	18	5	53.65716746
	3	381	16	4.199475066	61.07688606
	3	395	12	3.037974684	71.8423296
	3	399	15	3.759398496	65.15576501
LK4	4	353	21	5.949008499	44.86121908
	4	349	17	4.871060172	54.85225484
	4	380	20	5.263157895	51.21807102
	4	324	17	5.24691358	51.36863252
LK5	5	371	14	3.773584906	65.02427733
	5	362	17	4.696132597	56.4735827
	5	371	15	4.043126685	62.52601143
	5	375	14	3.733333333	65.39735171
	5	380	17	4.473684211	58.53536036
LK6	6	384	22	5.729166667	46.89883772
	6	366	19	5.191256831	51.88449081
	6	365	20	5.479452055	49.21333421
	6	350	23	6.571428571	39.09227724
Negative control	M1	469	47	10.02132196	0
	M2	449	49	10.91314031	0
	M3	446	45	10.0896861	0
	M4	469	54	11.51385928	0
	M5	412	47	11.40776699	0
Positive control	M1	450	5	1.111111111	89.70159277
	M2	436	1	0.229357798	97.87418199
	M3	453	3	0.662251656	93.86187649

Drug Name	Mouse No.	RBC	PRBC	Parasitemia	%chemosuppression
	M4	478	3	0.627615063	94.18290805
	M5	470	1	0.212765957	98.02796457

1000 mg/kg

Drug Name	Mouse No.	RBC	PRBC	%Prasitemia	%chemosuppresion
LK1	1	356	14	3.93258427	73.47116308
	1	426	17	3.990610329	73.07972484
	1	443	18	4.063205418	72.59000533
LK2	2	445	19	4.269662921	71.19726277
	2	331	15	4.531722054	69.42943696
	2	482	17	3.526970954	76.20739167
LK3	3	568	17	2.992957746	79.80979363
	3	394	11	2.791878173	81.16625718
	3	322	10	3.105590062	79.04998682
LK4	4	457	23	5.032822757	66.0490596
	4	517	21	4.061895551	72.59884156
	4	538	22	4.089219331	72.41451796
lk5	5	443	17	3.837471783	74.11278281
	5	404	13	3.217821782	78.29288238
	5	501	25	4.99001996	66.33780317
LK6	6	444	21	4.72972973	68.09369614
	6	474	18	3.797468354	74.38264211
	6	426	16	3.755868545	74.66327044
Negative control	7	412	66	16.01941748	0
	7	440	62	14.09090909	0
	7	313	47	15.01597444	0
	7	367	52	14.16893733	0
					0
Positive control	8	149	2	1.342281879	90.94509497
	8	144	2	1.388888889	90.63068855
	8	133	1	0.751879699	94.92789155
	8	435	4	0.91954023	93.79686966

Appendix 2: GC-MS Results

Library

Line#:1 R.Time:6.757(Scan#:645) MassPeaks: 304
RawMode: Averaged 6.751-6.763(644-646) BasePeak:71.05(6109)
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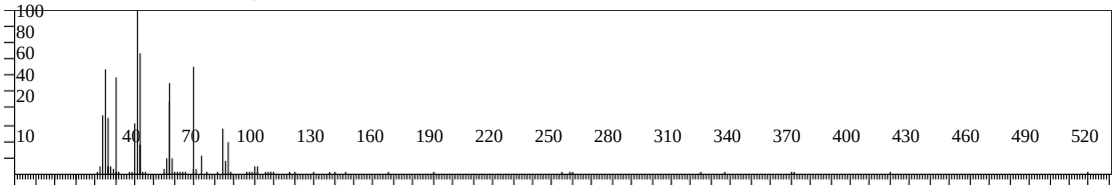
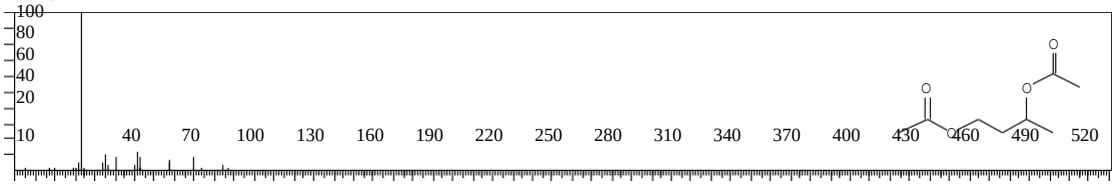
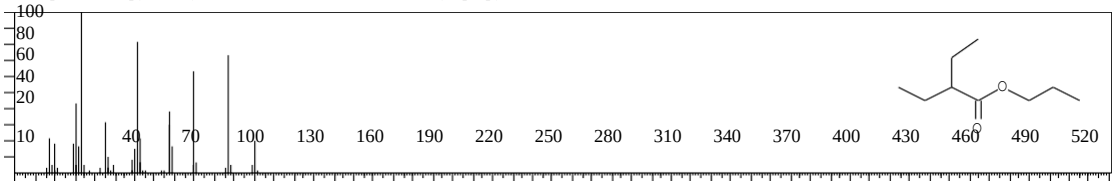


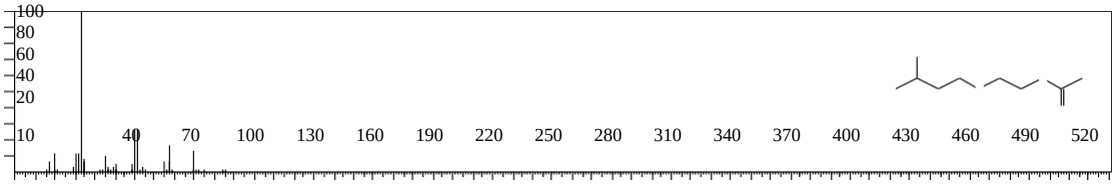
Hit#:1 Entry: 29102 Library:NIST14.lib
SI:77 Formula:C8H14O4 CAS:1117-31-3 MolWeight:174 RetIndex:1087
CompName:1,3-Butanediol, diacetate $$ 1,3-Butylene diacetate $$ 1,3-Butylene glycol diacetate $$ 1,3-Diacetoxybutane $$ 1,3-Butandiol diacetate $$ 3-(A



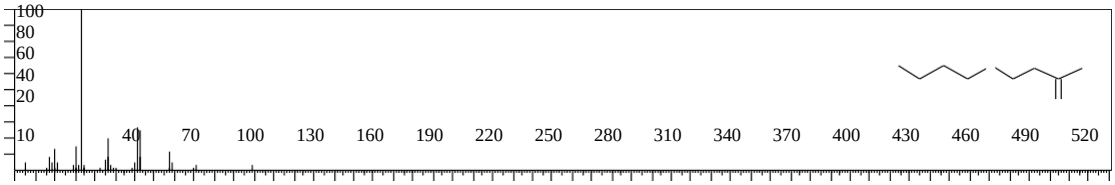
Hit#:2 Entry:20017 Library:NIST14.lib
SI:75 Formula:C9H18O2 CAS:5129-46-4 MolWeight:158 RetIndex:1019
CompName:Propyl 2-ethylbutanoate $$ Butanoic acid, 2-ethyl, propyl ester $$



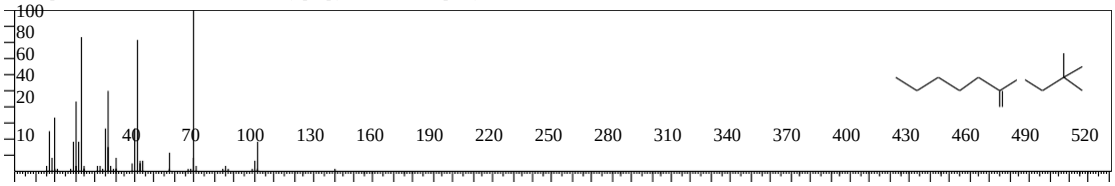
Hit#:3 Entry:29247 Library:NIST14.lib
SI:74 Formula:C9H18O3 CAS:204652-53-9 MolWeight:174 RetIndex:1095
CompName:Isopentyloxyethyl acetate $$ 2-(Isopentyloxy)ethyl acetate # $$ 2-Isopentoxyethyl acetate $$



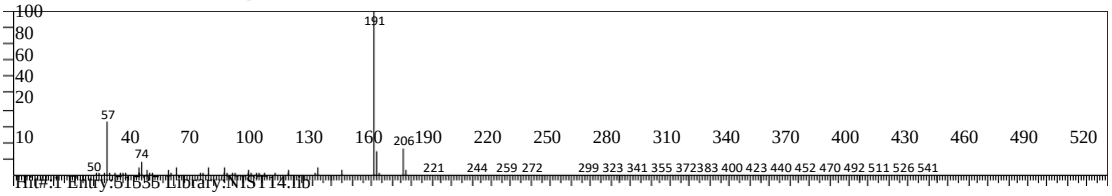
Hit#:4 Entry:13239 Library:NIST14.lib
SI:74 Formula:C8H16O2 CAS:30536-44-8 MolWeight:144
RetIndex:1028 CompName:4-Butoxy-2-butanone



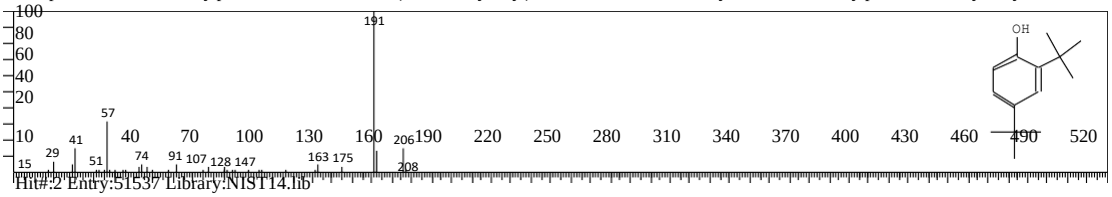
Hit#:5 Entry:37228 Library:NIST14.lib
SI:74 Formula:C11H22O2 CAS:65127-75-5 MolWeight:186 RetIndex:1197
CompName:Hexanoic acid, 2,2-dimethylpropyl ester $$ Neopentyl hexanoate # $$



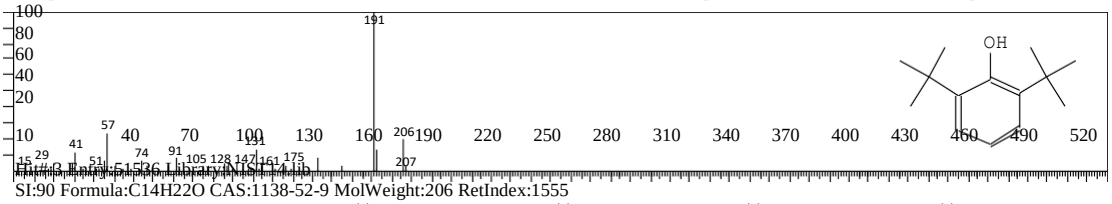
<< Target >>
Line#:2 R.Time:10.770(Scan#:1333) MassPeaks:273
RawMode:Averaged 10.764-10.776(1332-1334) BasePeak:191.15(15770)
BG Mode:Calc. from Peak Group 1 - Event 1 Scan



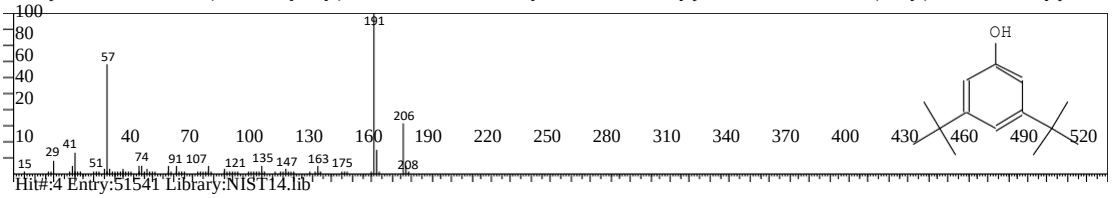
Hit#:1 Entry:51535 Library:NIST14.lib
SI:96 Formula:C14H22O CAS:96-76-4 MolWeight:206 RetIndex:1555
CompName:2,4-Di-tert-butylphenol $$ Phenol, 2,4-bis(1,1-dimethylethyl)- $$ Phenol, 2,4-di-tert-butyl- $$ 2,4-di-t-Butylphenol $$ 1-Hydroxy-2,4-di-tert-bu



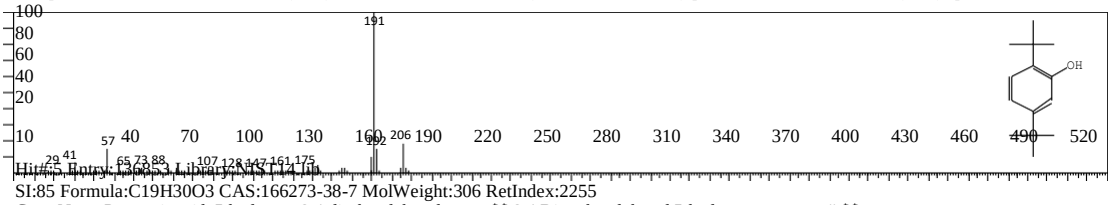
Hit#:2 Entry:51537 Library:NIST14.lib
SI:91 Formula:C14H22O CAS:128-39-2 MolWeight:206 RetIndex:1555
CompName:Phenol, 2,6-bis(1,1-dimethylethyl)- $$ Phenol, 2,6-di-tert-butyl- $$ 2,6-Bis(tert-butyl)phenol $$ 2,6-Bis(1,1-dimethylethyl)phenol $$ 2,6-Di-tert



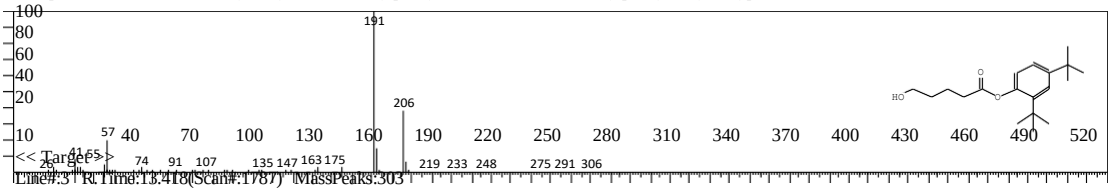
Hit#:3 Entry:51536 Library:NIST14.lib
SI:90 Formula:C14H22O CAS:1138-52-9 MolWeight:206 RetIndex:1555
CompName:Phenol, 3,5-bis(1,1-dimethylethyl)- $$ Phenol, 3,5-di-tert-butyl- $$ 3,5-Di-tert-butylphenol $$ Phenol, 3,5-bis(t-butyl) $$ 3,5-Di-t-butylphenol $



Hit#:4 Entry:51541 Library:NIST14.lib
SI:87 Formula:C14H22O CAS:5875-45-6 MolWeight:206 RetIndex:1555
CompName:Phenol, 2,5-bis(1,1-dimethylethyl)- $$ Phenol, 2,5-di-tert-butyl- $$ 2,5-Di-tert-butylphenol $$ 2,5-bis(1,1-Dimethylethyl)phenol $$



Hit#:5 Entry:[illegible] Library:NIST14.lib
SI:85 Formula:C19H30O3 CAS:166273-38-7 MolWeight:306 RetIndex:2255
CompName:Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters $$ 2,4-Ditert-butylphenyl 5-hydroxypentanoate # $$



<< Target >>
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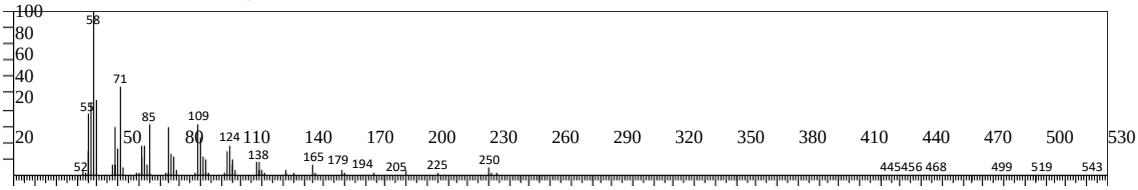
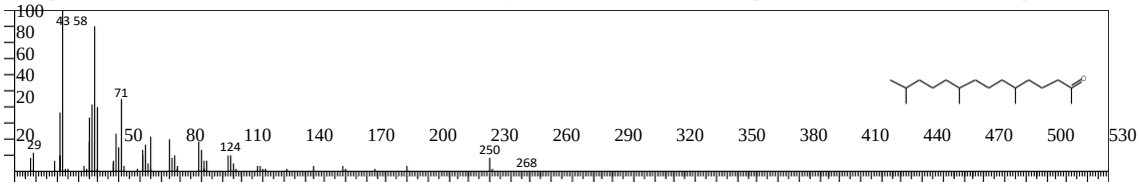
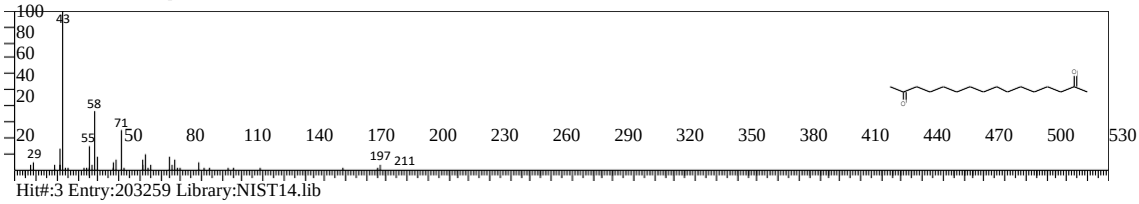
RawMode:Averaged 13.413-13.424(1786-1788) BasePeak:58.05(14751)
BG Mode:Calc. from Peak Group 1 - Event 1 Scan



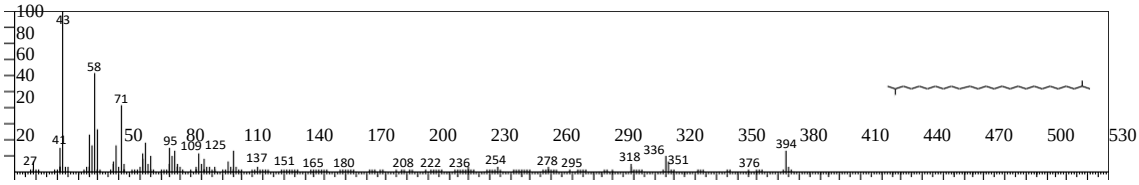
Hit#:1 Entry:102848 Library:NIST14.lib
SI:95 Formula:C18H36O CAS:502-69-2 MolWeight:268 RetIndex:1754
CompName:2-Pentadecanone, 6,10,14-trimethyl- $$ Hexahydrofarnesyl acetone $$ 6,10,14-Trimethyl-2-pentadecanone $$ 6,10,14-Trimethylpentadecan-2-



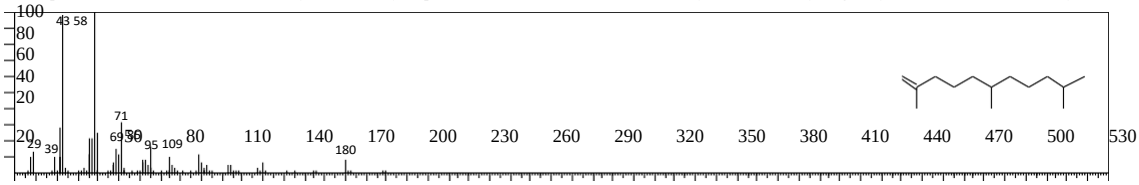
Hit#:2 Entry:90545 Library:NIST14.lib
SI:82 Formula:C16H30O2 CAS:18650-13-0 MolWeight:254
RetIndex:1884 CompName:2,15-Hexadecanedione



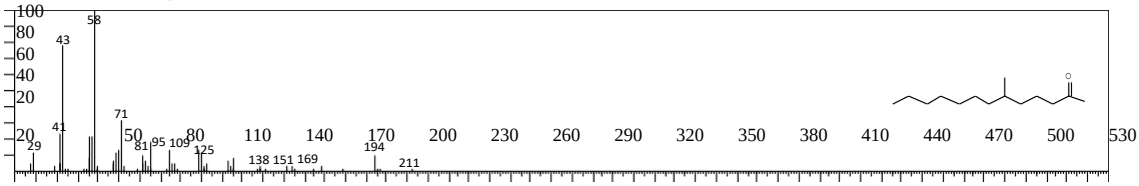
Hit#:3 Entry:203259 Library:NIST14.lib
SI:82 Formula:C26H50O2 CAS:0-00-0 MolWeight:394
RetIndex:2878 CompName:Hexacosa-2,25-dione $$ 2,25-
Hexacosanedione # $$



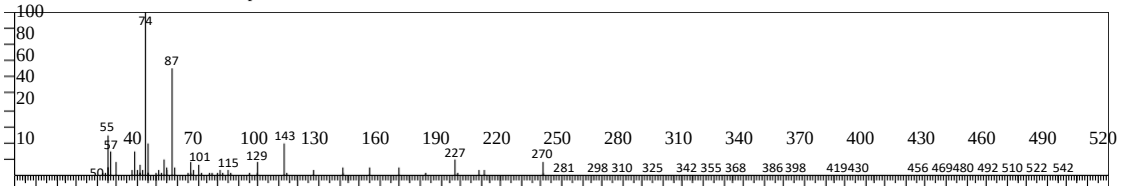
Hit#:4 Entry:45740 Library:NIST14.lib
SI:82 Formula:C13H26O CAS:1604-34-8 MolWeight:198 RetIndex:1321
CompName:2-Undecanone, 6,10-dimethyl- $$ Hexahydropseudoionone $$ Pseudoionone, hexahydro- $$ Tetrahydrogeranylacetone $$ 6,10-Dimethyl-2-un



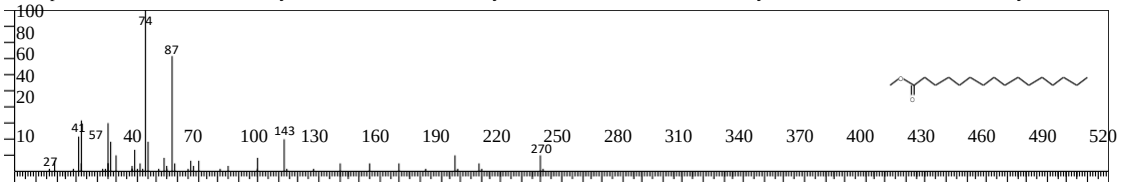
Hit#:5 Entry:56451 Library:NIST14.lib
SI:81 Formula:C14H28O CAS:73105-73-4 MolWeight:212
RetIndex:1485 CompName:6-Methyl-2-tridecanone



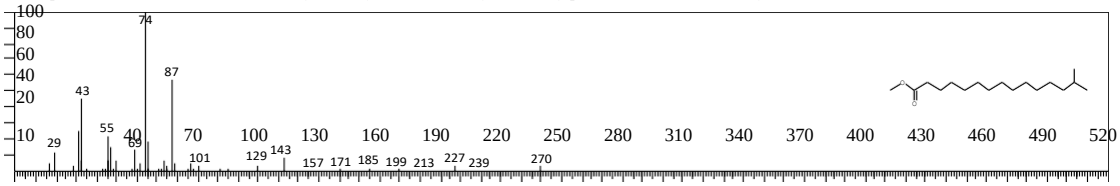
<< Target >>
Line#:4 R.Time:14.019(Scan#:1890) MassPeaks:294
RawMode:Averaged 14.013-14.025(1889-1891) BasePeak:74.05(20175)
BG Mode:Calc. from Peak Group 1 - Event 1 Scan



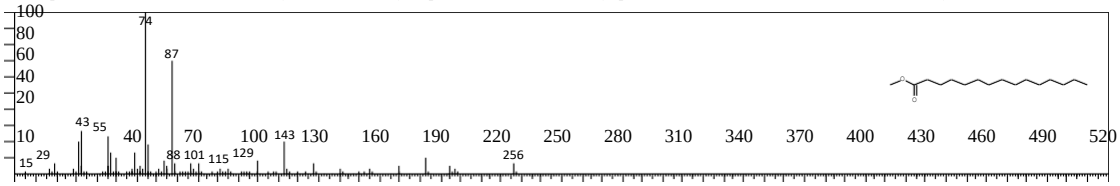
Hit#:1 Entry:104648 Library:NIST14.lib
SI:96 Formula:C17H34O2 CAS:112-39-0 MolWeight:270 RetIndex:1878
CompName:Hexadecanoic acid, methyl ester $$ Palmitic acid, methyl ester $$ n-Hexadecanoic acid methyl ester $$ Metholene 2216 $$ Methyl hexadecano



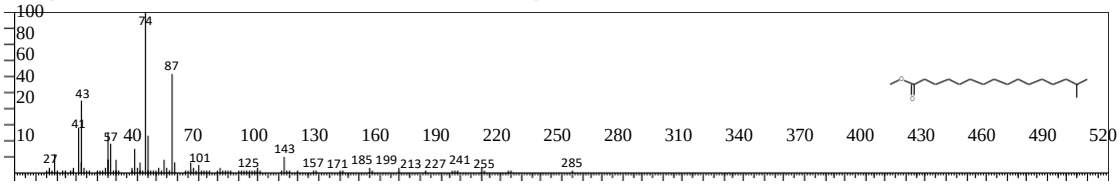
Hit#:2 Entry:104649 Library:NIST14.lib
SI:91 Formula:C17H34O2 CAS:5129-60-2 MolWeight:270 RetIndex:1814
CompName:Pentadecanoic acid, 14-methyl-, methyl ester $$ Methyl 14-methylpentadecanoate # $$ Methyl isohexadecanoate $$



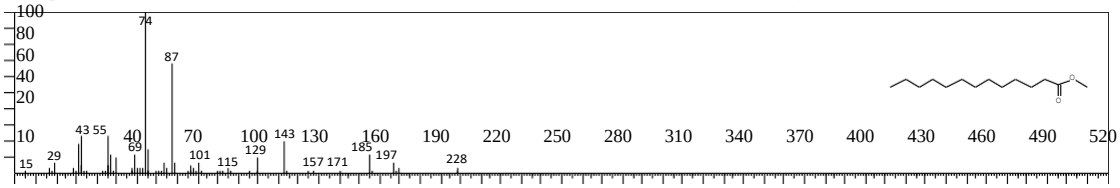
Hit#:3 Entry:92472 Library:NIST14.lib
SI:91 Formula:C16H32O2 CAS:7132-64-1 MolWeight:256 RetIndex:1779
CompName:Pentadecanoic acid, methyl ester $$ Methyl n-pentadecanoate $$ Methyl pentadecanoate $$ n-Pentadecanoic acid methyl ester $$



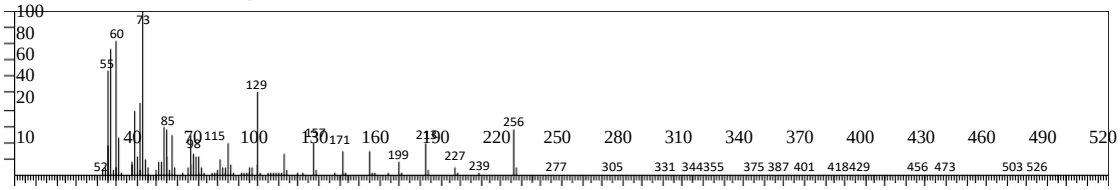
Hit#:4 Entry:117104 Library:NIST14.lib
SI:90 Formula:C18H36O2 CAS:6929-04-0 MolWeight:284 RetIndex:1914
CompName:Hexadecanoic acid, 15-methyl-, methyl ester $$ Methyl isoheptadecanoate $$ Methyl 15-methylhexadecanoate $$



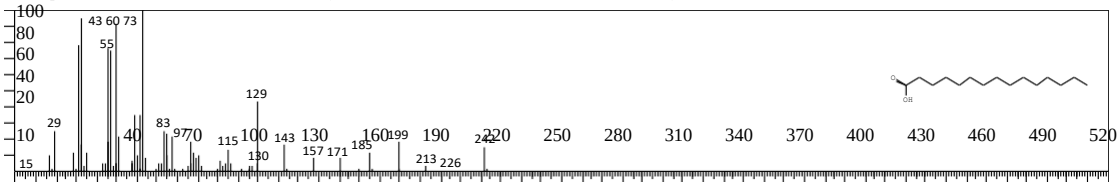
Hit#:5 Entry:69324 Library:NIST14.lib
SI:90 Formula:C14H28O2 CAS:1731-88-0 MolWeight:228 RetIndex:1580
CompName:Tridecanoic acid, methyl ester $$ Methyl tridecanoate $$ n-Tridecanoic acid methyl ester $$ Methyl ester of tridecanoic acid $$ Methyl n-tridec



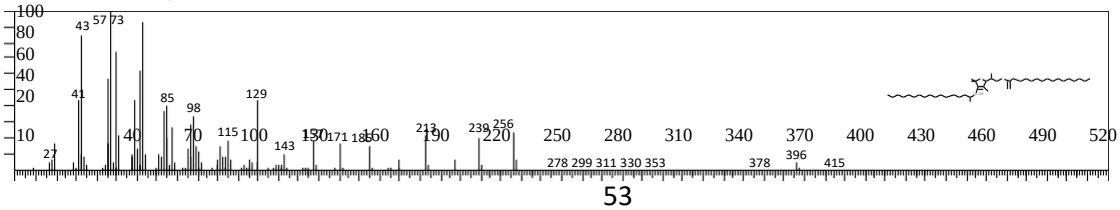
<< Target >>
Line#:5 R.Time:14.328(Scan#:1943) MassPeaks:322
RawMode:Averaged 14.323-14.334(1942-1944) BasePeak:73.00(16877)
BG Mode:Calc. from Peak Group 1 - Event 1 Scan



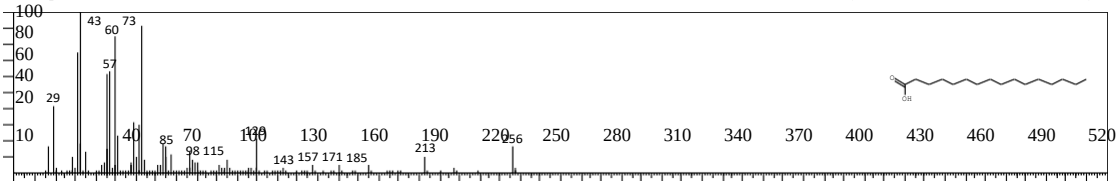
Hit#:1 Entry:80643 Library:NIST14.lib
SI:92 Formula:C15H30O2 CAS:1002-84-2 MolWeight:242 RetIndex:1869
CompName:Pentadecanoic acid $$ Pentadecylic acid $$ n-Pentadecanoic acid $$ n-Pentadecylic acid $$



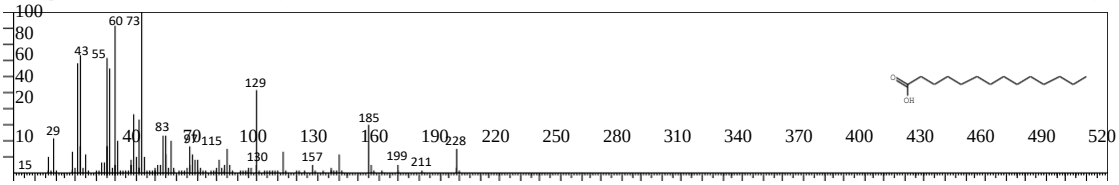
Hit#:2 Entry:240687 Library:NIST14.lib
SI:91 Formula:C38H68O8 CAS:28474-90-0 MolWeight:652
RetIndex:4765 CompName:l-(+)-Ascorbic acid 2,6-dihexadecanoate



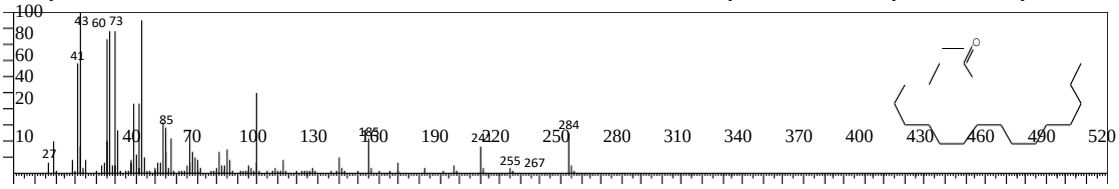
Hit#:3 Entry:92441 Library:NIST14.lib
SI:90 Formula:C16H32O2 CAS:57-10-3 MolWeight:256 RetIndex:1968
CompName:n-Hexadecanoic acid $$ Hexadecanoic acid $$ n-Hexadecoic acid $$ Palmitic acid $$ Pentadecanecarboxylic acid $$ 1-Pentadecanecarboxylic



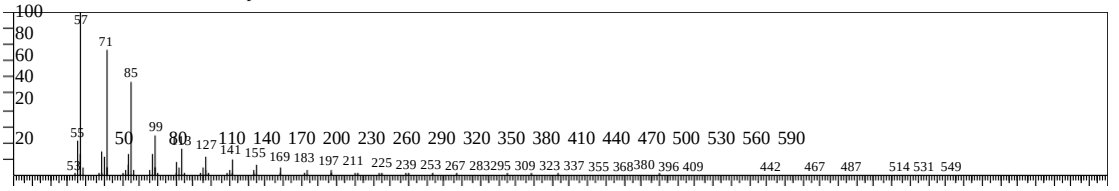
Hit#:4 Entry:69322 Library:NIST14.lib
SI:90 Formula:C14H28O2 CAS:544-63-8 MolWeight:228 RetIndex:1769
CompName:Tetradecanoic acid $$ Myristic acid $$ n-Tetradecanoic acid $$ n-Tetradecoic acid $$ Neo-Fat 14 $$ Univol U 316S $$ 1-Tridecanecarboxylic



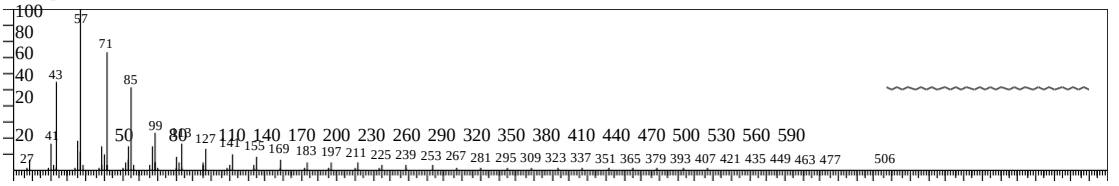
Hit#:5 Entry:117078 Library:NIST14.lib
SI:89 Formula:C18H36O2 CAS:57-11-4 MolWeight:284 RetIndex:2167
CompName:Octadecanoic acid $$ Stearic acid $$ n-Octadecanoic acid $$ Humko Industrene R $$ Hydrofol Acid 150 $$ Hystrene S-97 $$ Hystrene T-70 $



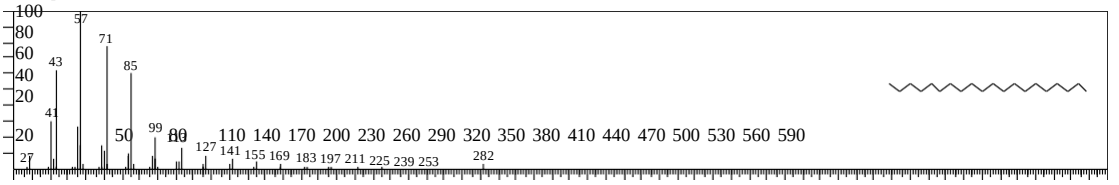
<< Target >>
Line#:7 R.Time:19.368(Scan#:2807) MassPeaks:283
RawMode:Averaged 19.363-19.374(2806-2808) BasePeak:57.05(55797)
BG Mode:Calc. from Peak Group 1 - Event 1 Scan



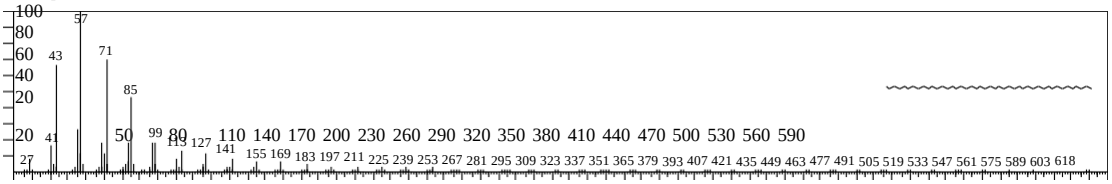
Hit#:1 Entry:233779 Library:NIST14.lib
SI:96 Formula:C36H74 CAS:630-06-8 MolWeight:506 RetIndex:3600
CompName:Hexatriacontane $$ n-Hexatriacontane $$



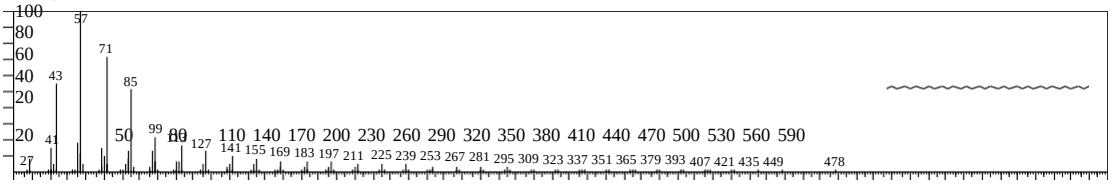
Hit#:2 Entry:115221 Library:NIST14.lib
SI:96 Formula:C20H42 CAS:112-95-8 MolWeight:282 RetIndex:2009
CompName:Eicosane $$ n-Eicosane $$ Icosane # $$ n-Icosane $$



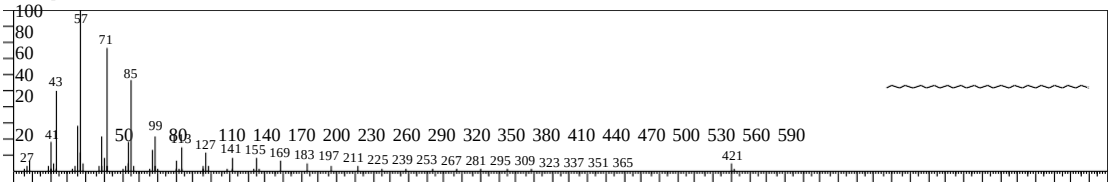
Hit#:3 Entry:239932 Library:NIST14.lib
SI:95 Formula:C44H90 CAS:7098-22-8 MolWeight:618 RetIndex:4395
CompName:Tetratetracontane $$ n-Tetratetracontane $$



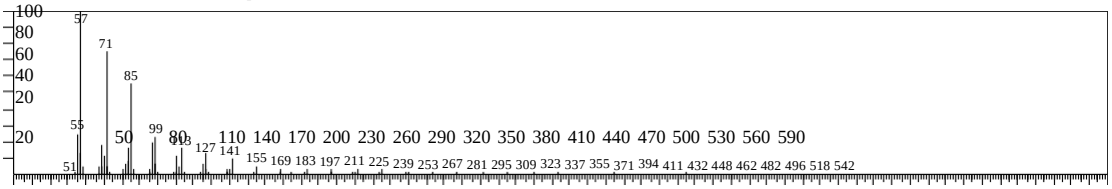
Hit#:4 Entry:229701 Library:NIST14.lib
SI:95 Formula:C34H70 CAS:14167-59-0 MolWeight:478 RetIndex:3401
CompName:Tetratriacontane $$ n-Tetratriacontane $$



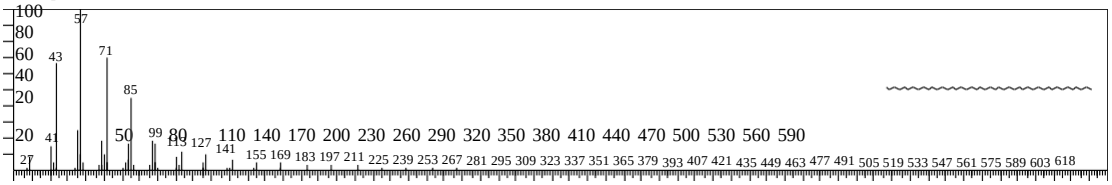
Hit#:5 Entry:237196 Library:NIST14.lib
SI:94 Formula:C30H61I CAS:0-00-0 MolWeight:548 RetIndex:0
CompName:Triacontane, 1-iodo-



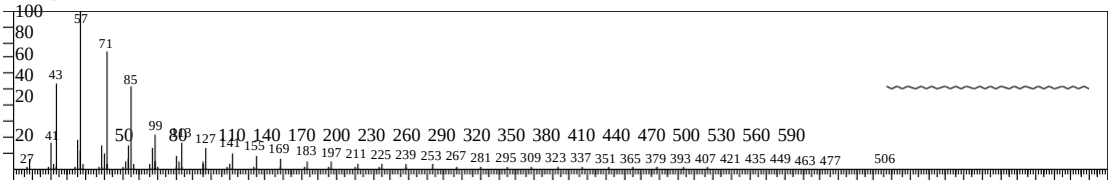
<< Target >>
Line#:8 R.Time:20.378(Scan#:2980) MassPeaks:322
RawMode:Averaged 20.372-20.383(2979-2981) BasePeak:57.05(21968)
BG Mode:Calc. from Peak Group 1 - Event 1 Scan



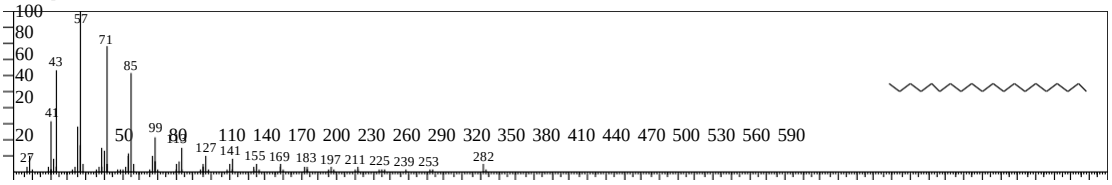
Hit#:1 Entry:239932 Library:NIST14.lib
SI:96 Formula:C44H90 CAS:7098-22-8 MolWeight:618 RetIndex:4395
CompName:Tetratetracontane $$ n-Tetratetracontane $$



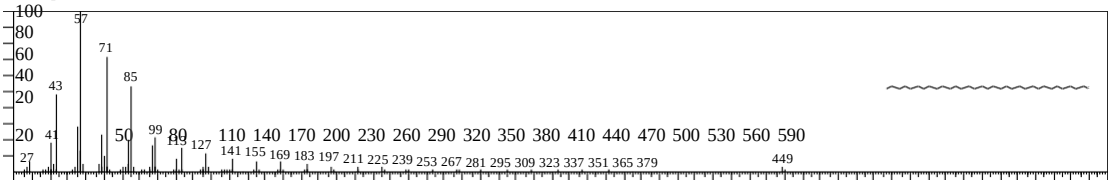
Hit#:2 Entry:233779 Library:NIST14.lib
SI:94 Formula:C36H74 CAS:630-06-8 MolWeight:506 RetIndex:3600
CompName:Hexatriacontane $$ n-Hexatriacontane $$



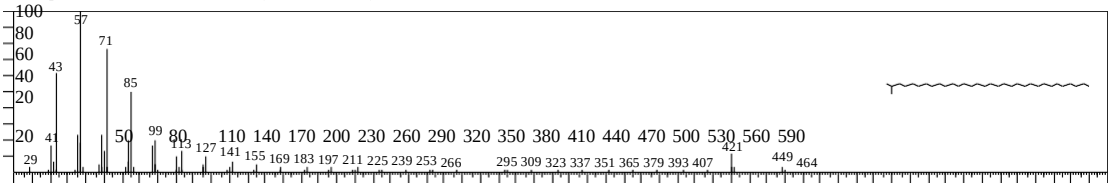
Hit#:3 Entry:115221 Library:NIST14.lib
SI:94 Formula:C20H42 CAS:112-95-8 MolWeight:282 RetIndex:2009
CompName:Eicosane $$ n-Eicosane $$ Icosane # $$ n-Icosane $$



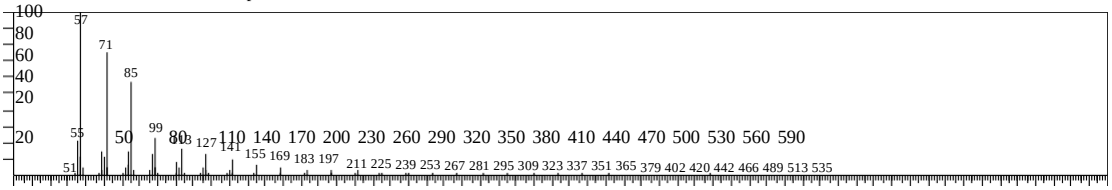
Hit#:4 Entry:238607 Library:NIST14.lib
SI:94 Formula:C32H65I CAS:0-00-0 MolWeight:576 RetIndex:0
CompName:Dotriacontane, 1-iodo-



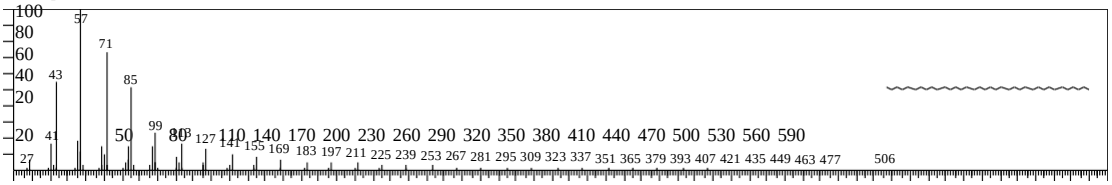
Hit#:5 Entry:226987 Library:NIST14.lib
SI:93 Formula:C33H68 CAS:1720-11-2 MolWeight:464 RetIndex:0
CompName:Dotriacontane, 2-methyl- $$ 2-Methyldotriacontane $$



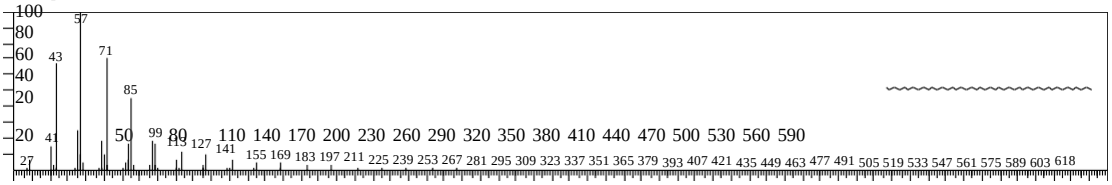
<< Target >>
Line#:9 R.Time:21.597(Scan#:3189) MassPeaks:318
RawMode:Averaged 21.591-21.602(3188-3190) BasePeak:57.05(120270)
BG Mode:Calc. from Peak Group 1 - Event 1 Scan



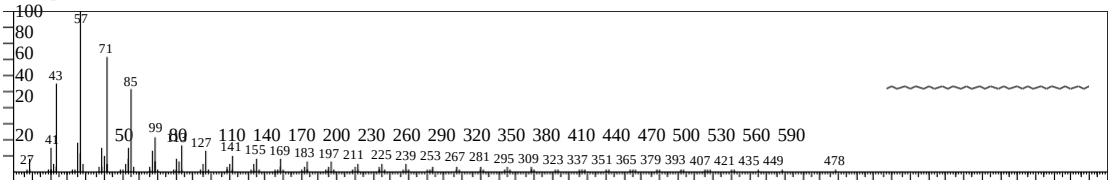
Hit#:1 Entry:233779 Library:NIST14.lib
SI:96 Formula:C36H74 CAS:630-06-8 MolWeight:506 RetIndex:3600
CompName:Hexatriacontane $$ n-Hexatriacontane $$



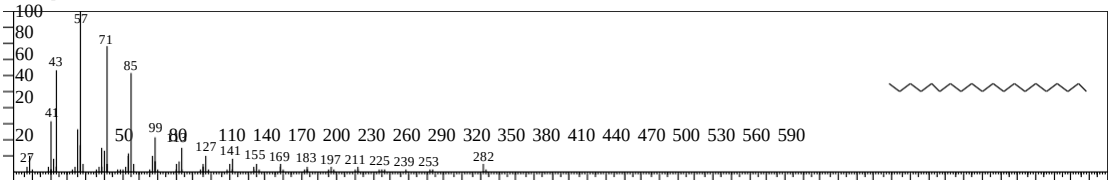
Hit#:2 Entry:239932 Library:NIST14.lib
SI:96 Formula:C44H90 CAS:7098-22-8 MolWeight:618 RetIndex:4395
CompName:Tetratetracontane $$ n-Tetratetracontane $$



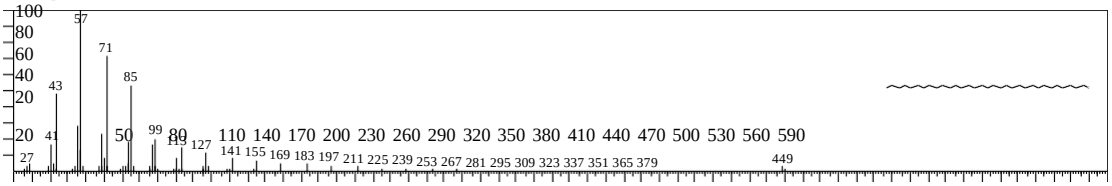
Hit#:3 Entry:229701 Library:NIST14.lib
SI:95 Formula:C34H70 CAS:14167-59-0 MolWeight:478 RetIndex:3401
CompName:Tetratriacontane $$ n-Tetratriacontane $$



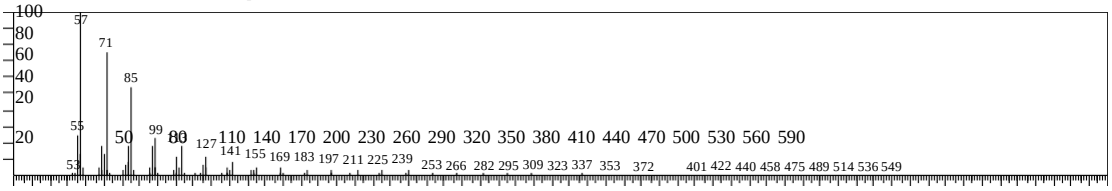
Hit#:4 Entry:115221 Library:NIST14.lib
SI:95 Formula:C20H42 CAS:112-95-8 MolWeight:282 RetIndex:2009
CompName:Eicosane $$ n-Eicosane $$ Icosane # $$ n-Icosane $$



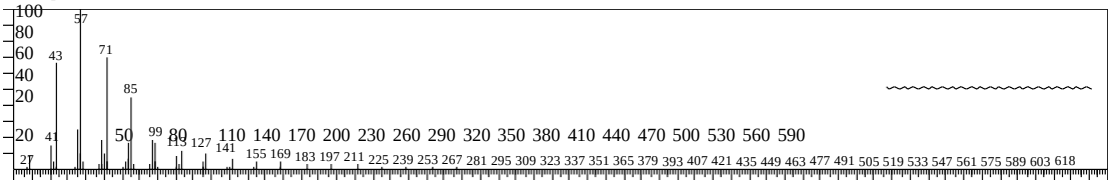
Hit#:5 Entry:238607 Library:NIST14.lib
SI:94 Formula:C32H65I CAS:0-00-0 MolWeight:576 RetIndex:0
CompName:Dotriacontane, 1-iodo-



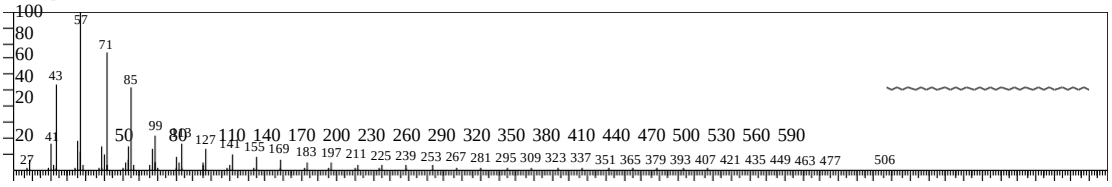
<< Target >>
Line#:10 R.Time:23.055(Scan#:3439) MassPeaks:302
RawMode:Averaged 23.049-23.061(3438-3440) BasePeak:57.05(19287)
BG Mode:Calc. from Peak Group 1 - Event 1 Scan



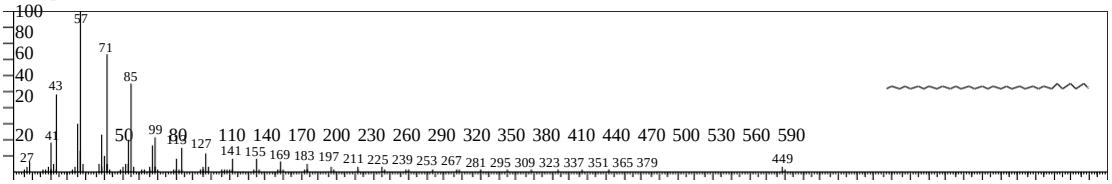
Hit#:1 Entry:239932 Library:NIST14.lib
SI:95 Formula:C44H90 CAS:7098-22-8 MolWeight:618 RetIndex:4395
CompName:Tetratetracontane $$ n-Tetratetracontane $$



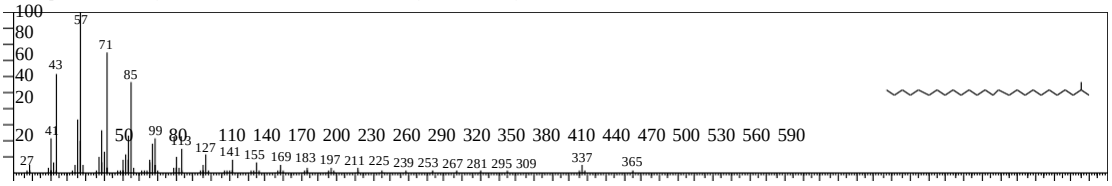
Hit#:2 Entry:233779 Library:NIST14.lib
SI:94 Formula:C36H74 CAS:630-06-8 MolWeight:506 RetIndex:3600
CompName:Hexatriacontane $$ n-Hexatriacontane $$



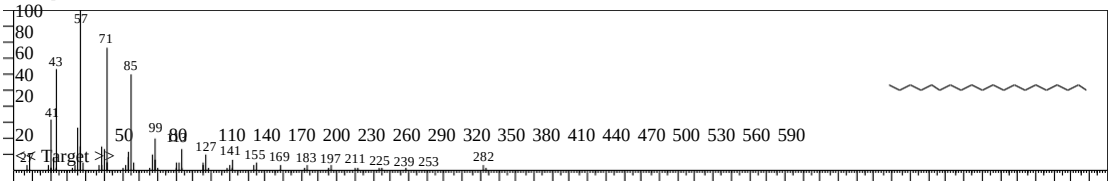
Hit#:3 Entry:238607 Library:NIST14.lib
SI:94 Formula:C32H65I CAS:0-00-0 MolWeight:576 RetIndex:0
CompName:Dotriacontane, 1-iodo-



Hit#:4 Entry:195535 Library:NIST14.lib
SI:93 Formula:C27H56 CAS:1561-02-0 MolWeight:380 RetIndex:0
CompName:2-Methylhexacosane $$ Hexacosane, 2-methyl- $$ 2-Methyl-n-hexacosane $$

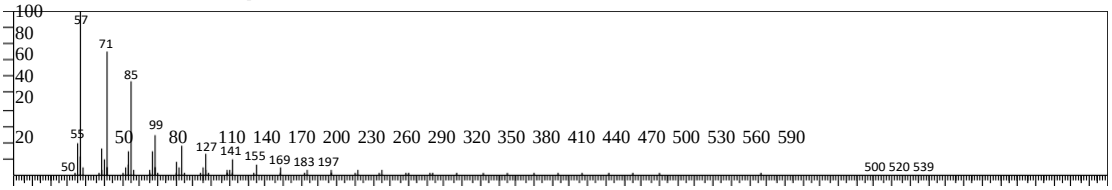


Hit#:5 Entry:115221 Library:NIST14.lib
SI:93 Formula:C20H42 CAS:112-95-8 MolWeight:282 RetIndex:2009
CompName:Eicosane $$ n-Eicosane $$ Icosane # $$ n-Icosane $$

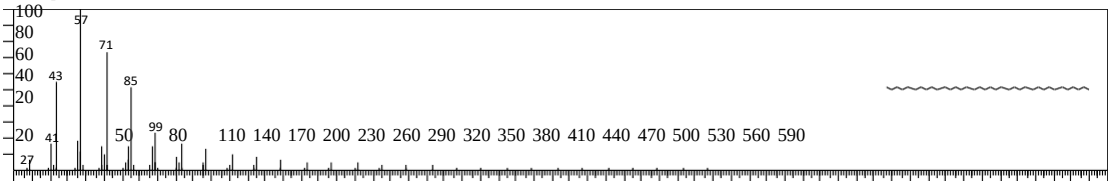


<< Target >>

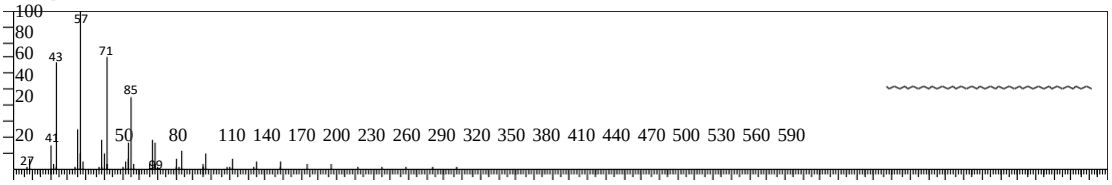
Line#:11 R.Time:24.846(Scan#:3746) MassPeaks:332
RawMode:Averaged 24.840-24.852(3745-3747) BasePeak:57.05(72267)
BG Mode:Calc. from Peak Group 1 - Event 1 Scan



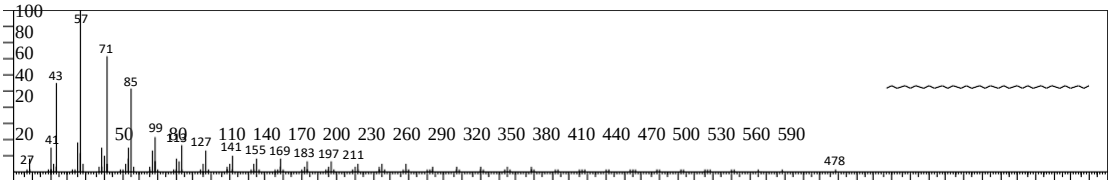
Hit#:1 Entry:233779 Library:NIST14.lib
SI:96 Formula:C36H74 CAS:630-06-8 MolWeight:506 RetIndex:3600
CompName:Hexatriacontane $$ n-Hexatriacontane $$



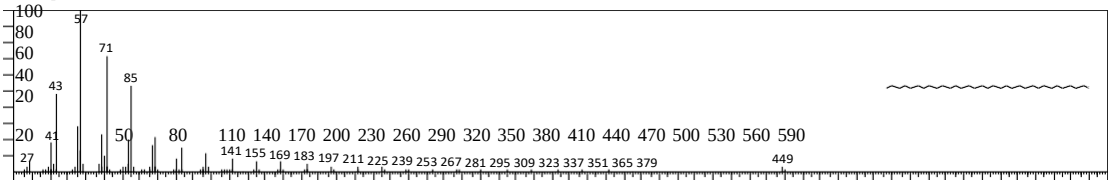
Hit#:2 Entry:239932 Library:NIST14.lib
SI:96 Formula:C44H90 CAS:7098-22-8 MolWeight:618 RetIndex:4395
CompName:Tetratetracontane $$ n-Tetratetracontane $$



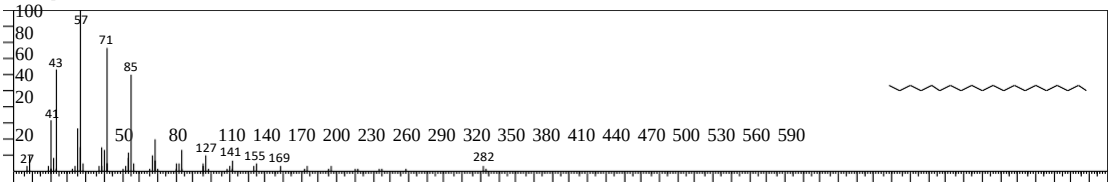
Hit#:3 Entry:229701 Library:NIST14.lib
SI:95 Formula:C34H70 CAS:14167-59-0 MolWeight:478 RetIndex:3401
CompName:Tetratriacontane $$ n-Tetratriacontane $$



Hit#:4 Entry:238607 Library:NIST14.lib
SI:94 Formula:C32H65I CAS:0-00-0 MolWeight:576 RetIndex:0
CompName:Dotriacontane, 1-iodo-



Hit#:5 Entry:115221 Library:NIST14.lib
SI:94 Formula:C20H42 CAS:112-95-8 MolWeight:282 RetIndex:2009
CompName:Eicosane $$ n-Eicosane $$ Icosane # $$ n-Icosane $$



<< Target >>
Line#:12 R.Time:26.433(Scan#:4018) MassPeaks:319
RawMode:Averaged 26.427-26.438(4017-4019) BasePeak:57.05(15755)
BG Mode:Calc. from Peak Group 1 - Event 1 Scan