

**MOSQUITOCIDAL ACTIVITY
AGAINST *ANOPHELES ARABIENSIS* OF
PLANTS USED AS MOSQUITO
REPELLENTS IN SOUTH AFRICA**

By

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**Submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy**

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November 2014

STUDENT DECLARATION

Mosquitocidal activity against *Anopheles arabiensis* of plants used as mosquito repellents in South Africa

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We hereby declare that we acted as Supervisors for this PhD student:

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Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the College of Agriculture, Engineering and Science Higher Degrees Office for examination by the University appointed Examiners.

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DECLARATION 2 - PUBLICATIONS

Publication 1: Mavundza, E.J., Maharaj, R., Chukwujekwu, J.C., Finnie, J.F., Van Staden, J., 2014. Screening for adulticidal activity against *Anopheles arabiensis* in ten plants used as mosquito repellent in South Africa. *Malaria Journal* 13, 173.

Contributions: Experimental work and writing of the publication was done by the first author under supervision of the last four authors.

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Contributions: Experimental work and writing of the publication was done by the first author under supervision of the last four authors.

Publication 3: Mavundza, E.J., Maharaj, R., Finnie, J.F., Kabera, G., Van Staden, J., 2011. An ethnobotanical survey of mosquito repellent plants in uMkhanyakude district, KwaZulu-Natal province, South Africa. *Journal of Ethnopharmacology* 137, 1516-1520.

Contributions: Experimental work and writing of the publication was done by the first author under supervision of Prof R. Maharaj, J.F. Finnie and J. Van Staden. The fourth author, Dr G. Kabera helped with statistical analysis.

Signed.....

CONFERENCE CONTRIBUTIONS

1. **39th South African Association of Botanists (SAAB) Annual Conference, 20-24 January 2013, Drakensberg:** Larvicidal activity against *Anopheles arabiensis* of 10 South African plants that are traditionally used as mosquito repellents.
2. **38th SAAB Annual Conference, 15-18 January 2012, University of Pretoria.** An ethnobotanical survey of mosquito repellent plants in uMkhanyakude district, KwaZulu-Natal province, South Africa.

ABSTRACT

Ten plant species that are used as mosquito repellent in South Africa, *Aloe ferox* (leaves), *Atalaya alata* (leaves), *Balanites maughamii* (bark), *Clausena anisata* (leaves), *Croton menyaarthii* (leaves), *Lippia javanica* (leaves), *Melia azedarach* (leaves), *Olox dissitiflora* (bark), *Sclerocarya birrea* (seeds) and *Trichilia emitica* (seeds) were screened for adulticidal, larvicidal and repellent activities against *Anopheles arabiensis*, a potent malaria vector in South Africa. The plant extracts were screened following the WHO standard methods with slight modifications. The plant materials were extracted separately with ethanol (EtOH) and dichloromethane (DCM). All the extracts showed adulticidal activity. The highest activity was observed in the DCM extract of *A. ferox* leaves with an EC₅₀ value of 4.92 mg/ml. With regards to larvicidal screening, all the DCM extracts showed larvicidal activity, while only five EtOH extracts showed activity. The highest larvicidal activity was found in the DCM extract of *O. dissitiflora* bark with an EC₅₀ value of 25.24 µg/ml. All the investigated plants showed no repellent activity. Due to its good larvicidal activity, *O. dissitiflora* was evaluated for antibacterial, antifungal and antiplasmodial activities. The antibacterial activity was evaluated against two Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and two Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacteria using the micro-dilution assay. The micro-dilution assay was also used to evaluate the antifungal activity of *O. dissitiflora* against *Candida albicans*. The antiplasmodial activity was evaluated against a chloroquine-sensitive strain of *Plasmodium falciparum* (D10) using the parasite lactate dehydrogenase assay. Both DCM and EtOH extracts showed good antibacterial activity against all four tested strains with MIC values less than 1 mg/ml. They also showed good antifungal activity with MIC values less than 1 mg/ml. Both DCM and EtOH extracts showed a moderate antiplasmodial activity, with IC₅₀ values of 15.6 and 45 µg/ml, respectively. Good larvicidal activity observed in the DCM extract of *O. dissitiflora* bark prompted an attempt to isolate active compounds. Two active compounds were isolated from *O. dissitiflora* bark, ximeninic acid and a mixture of two closely related compounds (exocarpic acid and octadec-9,11-diynoic

acid). The mixture of exocarpic acid and octadec-9,11-diyonic acid exhibited the highest larvicidal activity with an EC₅₀ value of 17.31 µg/ml compared to ximeninic acid which had an EC₅₀ value of 62.17 µg/ml. The results of the present study showed that the bark of *O. dissitiflora* and leaves of *A. ferox* may have potential to be used as larvicides and adulticides against *An. arabiensis* mosquitoes, respectively. This study also indicated that the bark of *O. dissitiflora* may have potential to be used as an antibacterial, antifungal and antimalarial agent.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
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AmpB	Amphotericin B
ATCC	American Type Culture Collection
CO ₂	Carbon dioxide
CQS	Chloroquine-sensitive
DCM	Dichloromethane
DDT	Dichlorodiphenyltrichloroethane
DEET	N,N-diethyl-3-methylbenzamide
DENV	Dengue virus
DF	Dengue fever
DMSO	Dimethyl Sulphoxide
EC	Effective concentration
EtOH	Ethanol
GMEP	Global Malaria Eradication Programme
HIV	Human Immunodeficiency Virus
IC	Inhibitory concentration
INT	<i>ρ</i> -iodonitrotetrazolium chloride
IRS	Indoor residual spraying
JE	Japanese encephalitis
LF	Lymphatic filariasis
MFC	Minimum fungicidal concentration
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration
RNA	Ribonucleic acid
SAAB	South African Association of Botanists
TLC	Thin Layer Chromatography
WHO	World Health Organisation
YF	Yellow fever
YM	Yeast malt

CHAPTER 1

Introduction and Literature review

1.1. Introduction

Mosquitoes are the most important single group of insects in terms of public health because they are vectors of serious human diseases such as malaria, filariasis, Japanese encephalitis, dengue fever, and yellow fever. Mosquito-transmitted diseases are a major cause of illness and death in the world, particularly in tropical and subtropical countries (**BECKER *et al.*, 2003**). These diseases are also responsible for huge economic losses, both in terms of health care costs and lost productivity (**KARUNAMOORTHY *et al.*, 2009a**). It is estimated that more than 700 million people are infected with mosquito-transmitted diseases annually (**TAUBES, 2000**). Among these diseases, malaria, which is caused by parasites of the genus *Plasmodium* and transmitted by infected mosquitoes of the genus *Anopheles*, continues to be a major public health problem in tropical and subtropical countries. In 2010, the World Health Organization (WHO) estimated that there were 216 million cases of malaria and 655 000 deaths worldwide. About 91% of these deaths occurred in sub-Saharan Africa, and were mostly in children under 5 years of age (**WHO, 2011a**).

Despite significant efforts to control malaria in South Africa since 1930 (**BLUMBERG and FREAN, 2007**), the disease remains a serious health problem (**MAHARAJ *et al.*, 2005**). An estimated 4.3 million people are at risk of contracting malaria (**BLUMBERG and FREAN, 2007**). In 2000, the highest number (61,934) of malaria cases were reported, the worst levels of malaria recorded since the epidemics of the 1930s (**DOH, 2007**). In South Africa, malaria is currently confined to the low-altitude regions of Limpopo, Mpumalanga and KwaZulu-Natal provinces, in the north-eastern part of the country, along the border with Mozambique and Swaziland (Figure 1.1) (**COLEMAN *et al.*, 2008; GERRITSEN *et al.*, 2008; PILLAY *et al.*, 2008**). Malaria transmission in South Africa is distinctly seasonal (**GERRITSEN *et al.*, 2008; PILLAY *et al.*, 2008**), with *Anopheles arabiensis* being the major vector (**MAHARAJ *et al.*, 2005**).

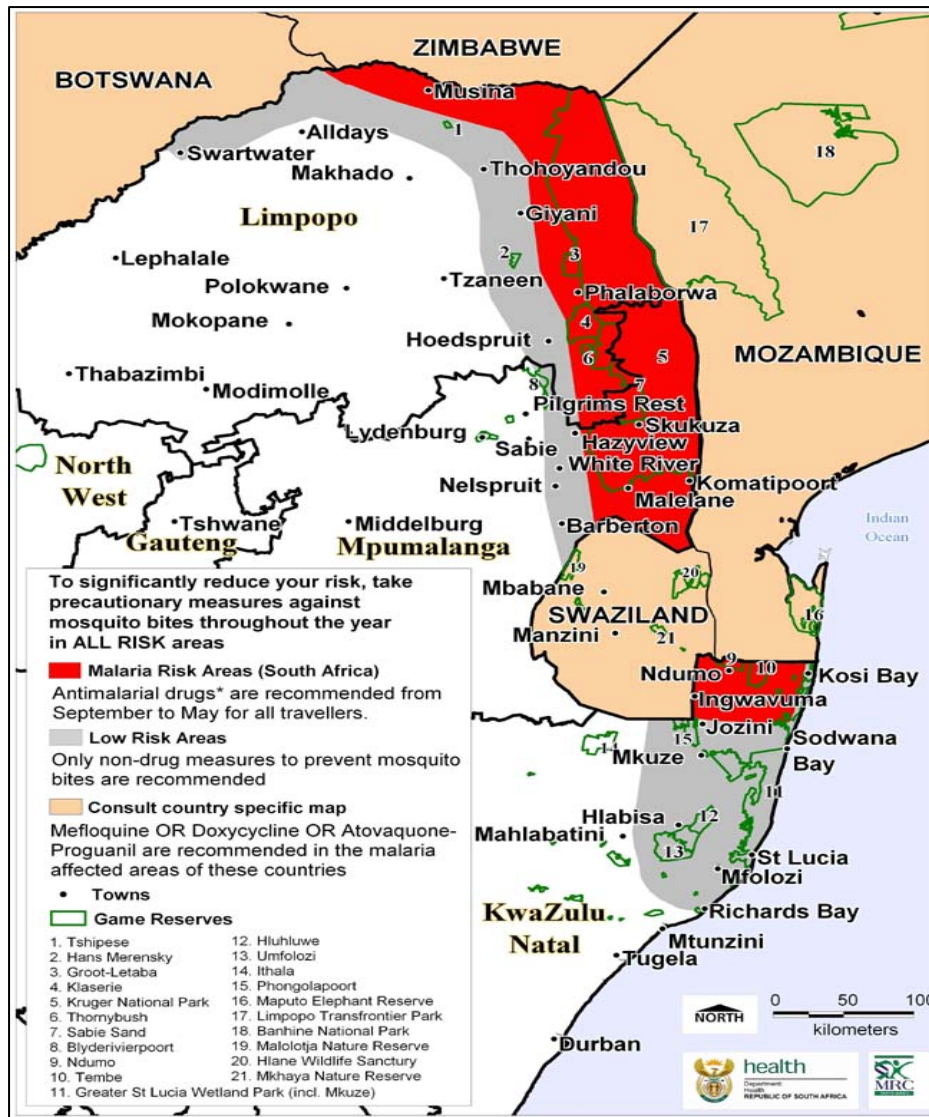


Figure 1.1: Occurrence of malaria in South Africa (MORRIS *et al.*, 2013).

As there is currently no effective vaccine available for the prevention of malaria, vector control is one of the main strategies used to control this disease. Currently, mosquito control strategies depend primarily on the use of synthetic insecticides. Although effective, their repeated use has resulted in many problems, such as toxic effects to humans, toxicity to non-target organisms, pollution of the environment, and the development of resistance in mosquito populations (DAS *et al.*, 2007). These problems highlight the urgent need for development of new insecticides, which are effective, safe, biodegradable and target-specific.

Plants may be an alternative source of mosquito control agents because they constitute a rich source of bioactive chemicals (KAMARAJ *et al.*, 2010; TIWARY *et al.*,

2007). Natural products are generally preferred because of their less harmful nature to non-target organisms and due to their innate biodegradability (**KAMARAJ *et al.*, 2010; RAHUMAN *et al.*, 2008**). Much effort has, therefore, been focused on plant extracts or phytochemicals as potential sources of mosquito control agents or as lead compounds. Today, over 2000 plant species are known to possess insecticidal activities (**TEWARY *et al.*, 2005; CHANSANG *et al.*, 2005; BROUSSALIS *et al.*, 1999**).

1.2. Aims and Objectives

The aim of this study was to screen the plants that are used traditionally as mosquito repellents in South Africa, for mosquitocidal activity. The objectives were:

- To identify mosquito repellent plants;
- To screen the identified plant species for adulticidal, larvicidal, and repellent activity;
- To screen the most active plant species for antibacterial, antifungal and antimalarial activity; and
- To isolate and identify active compounds.

1.3. Literature review

1.3.1. Biology of mosquitoes

Mosquitoes (Figure 1.2) are small, two-winged insects belonging to the Order Diptera and the family Culicidae (**U.S. DEPARTMENT OF HEALTH, EDUCATION AND WELFARE, 1977**). They differ from the other biting Diptera insects in having a long slender body, long legs, and long needle-shaped mouthparts (**ROZENDAAL, 1997**). Mosquitoes are relatively small insects, measuring about 4-6 mm in length, although some species can be as small as 2 mm while others may be as long as 19 mm. They have a worldwide distribution, occurring throughout the tropical and temperate regions and northwards into the Arctic Circle. The only areas from which they are absent are Antarctica and a few islands. There are about 3400 different species of mosquitoes in the world belonging to 42 genera distributed among three

subfamilies; Toxorhynchitinae, Anophelinae and Culicinae. The most important man-biting mosquitoes belong to the genera *Aedes*, *Anopheles*, *Culex*, *Haemagogus*, *Mansonia*, *Sabethes* and *Psorophora* (SERVICE, 2008).



Figure 1.2: Mosquito.

1.3.2. Life cycle of mosquitoes

Mosquitoes have four distinct stages in their life cycle; egg, larva, pupa, and adult (Figure 1.3) (ROZENDAAL, 1997; U.S. DEPARTMENT OF HEALTH, EDUCATION AND WELFARE, 1977; FRADIN, 1998). The first three stages occur in water, but the adult is an active flying insect (U.S. DEPARTMENT OF HEALTH, EDUCATION AND WELFARE, 1977). The female mosquitoes mate only once but produce eggs at intervals throughout their life cycle (ROZENDAAL, 1997). Depending on the species, female mosquitoes lay between 30 and 300 eggs at a time. Many species such as *Anopheles*, *Culex* and some *Mansonia* lay their eggs directly on the water surface. The eggs of *Anopheles* are laid singly, whereas those of *Culex* and some *Mansonia* are laid stuck together in floating rafts. In the tropics, the eggs usually hatch within 2-3 days, but in cooler temperate countries they may take between 7 and 14 days or longer. Other mosquito species such as *Aedes*, *Psorophora* and *Haemagogus*, do not lay their eggs on the water surface but just above the water line or on damp substrates, such as mud, leaf litters, on the inside walls of tree holes and clay pots (ROZENDAAL, 1997; SERVICE, 2008). These eggs can withstand varying degrees of desiccation for periods of weeks, months, or even years, and still remain viable and capable of hatching when they are flooded with water (SERVICE,

2008). The eggs of some species can survive for 3 to 5 years if flooding does not occur (U.S. DEPARTMENT OF HEALTH, EDUCATION AND WELFARE, 1977).

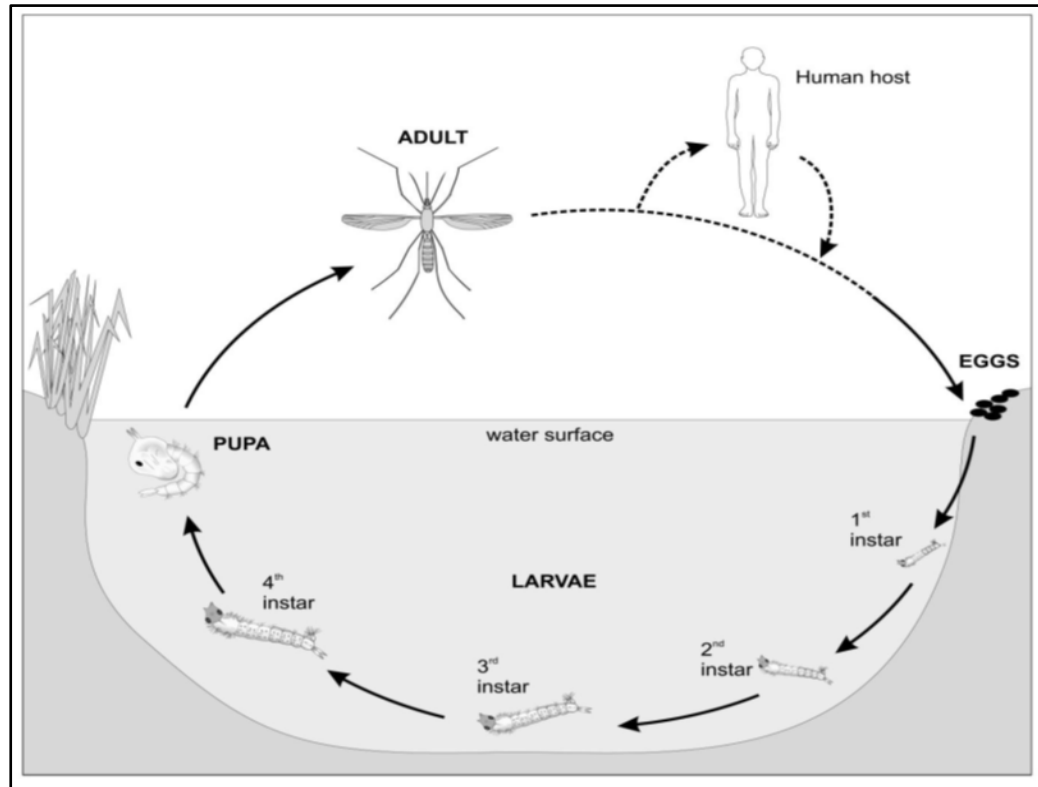


Figure 1.3: The life cycle of the mosquito (STANCZYK, 2011).

Once hatched, the larvae do not grow continuously but in four different stages or instars (ROZENDAAL, 1997). Mosquito larvae assume characteristic positions in the water. *Anopheles* larvae lie parallel to the water surface, while most of other mosquito species hang head down with only the tip of the air tube penetrating the water surface (U.S. DEPARTMENT OF HEALTH, EDUCATION AND WELFARE, 1977). Mosquito larvae feed on yeast, bacteria, protozoa and other small aquatic microorganisms (ROZENDAAL, 1997; SERVICE, 2008). Although mosquito larvae get their food from the water, most of them must come to the water surface to breathe. Other mosquito larvae such as *Mansonia* species do not need to come to the water surface to breathe, because they obtain air from aquatic plants (U.S. DEPARTMENT OF HEALTH, EDUCATION AND WELFARE, 1977).

In warm climates, the larval period lasts about 4-7 days or longer if there is a shortage of food (**ROZENDAAL, 1997**). In temperate areas the larval period may last several weeks or months, and several species overwinter as larvae (**SERVICE, 2008**). The fully grown larva then changes into a comma-shaped pupa, which does not feed but spend most of its time at the water surface taking air through respiratory trumpets (**ROZENDAAL, 1997; SERVICE, 2008**). In the tropics the pupal period lasts 2-3 days, but in cooler temperate regions it may take 9-12 days or longer (**SERVICE, 2008**). At the end of the pupal stage, the pupal skin splits at one end and a fully developed adult mosquito emerges (**ROZENDAAL, 1997; SERVICE, 2008**). After adults have emerged they rest either on the water surface or more usually on nearby vegetation for about an hour to allow their wings to unfold and harden sufficiently for them to fly. Under good conditions, the entire period from egg to adult takes about 7-13 days (**ROZENDAAL, 1997**).

1.3.3. Host-seeking behaviour

Male mosquitoes feed primarily on flower nectar (**FRADIN, 1998; ROZENDAAL, 1997**), whereas female mosquitoes feed on animals and humans (**ROZENDAAL, 1997**). Many mosquito species bite man to obtain their blood meal (**SERVICE, 2008**). Female mosquitoes require a blood meal to produce eggs (**FRADIN, 1998; EDMAN *et al.*, 1992**). Blood is rich in proteins that accelerate development of eggs (**KNOLS AND MEIJERINK, 1997**).

Female mosquitos use olfactory, visual and thermal cues to locate a blood-meal host (**FRADIN, 1998, CLEMENTS, 1999**). Of these, olfactory cues are the most important (**FRADIN, 1998**). Many blood-sucking insects use olfactory cues to locate their blood meal sources (**GEIER *et al.*, 1999**). Ample evidence has shown that host-seeking female mosquitoes mainly locate their hosts by odours produced by the hosts (**OWINO, 2010**). Mosquitoes use their antennae and maxillary palps to detect these odours (**McIVER, 1982**).

It has been estimated that the human body release around 300-400 compounds as by-products of metabolism and more than 100 compounds can be detected by mosquitoes in human breath (**FRADIN, 1998**). Of these volatile compounds, only few have been

isolated and characterised. CO₂, which is given off from hosts mainly with breath but also from skin (FRADIN, 1998; GEIER *et al.*, 1999), was the first known mosquito attractant (CLEMETS, 1999). It is an alerting and attractive signal of all mosquito species (GEIER *et al.*, 1999) and can be detected by mosquitoes at distance up to 36 meters (FRADIN, 1998). Lactic acid, a major component in breath and on human skin, is also known as a mosquito attractant (GEIER *et al.*, 1999). It is, however, less attractive when it is alone (SMITH *et al.*, 1970; BERNIER *et al.*, 2003) but acts as an essential synergist when combined with carbon dioxide as well as with other volatiles from the skin (ACREE, 1968; SMITH *et al.*, 1970; EIRAS AND JEPSON, 1991; GEIER *et al.*, 1996; BERNIER *et al.*, 2003). Other compounds that have been found to be mosquito attractants include fatty acids (KNOLS *et al.*, 1997; BOSCH *et al.*, 2000), ammonia (GEIER *et al.*, 1999, BRAKS *et al.*, 2001, SMALLEGANGE *et al.*, 2005), and octenol (TAKKEN AND KLINE, 1989, KLINE *et al.*, 1990, HOEL *et al.*, 2007).

1.3.4. Medical importance of mosquitoes

Besides being an annoyance to humans, mosquitoes are vectors of many pathogens causing severe diseases such as malaria, dengue fever, yellow fever, lymphatic filariasis, and Japanese encephalitis.

1.3.4.1. Malaria

Malaria is a disease caused by single-celled protozoan parasites of the genus *Plasmodium*. There are four species of *Plasmodium* that infect humans: *P. falciparum*, *P. malariae*, *P. ovale*, and *P. vivax* (WHITE, 2009). Among these, *P. falciparum* is the most dangerous, it is associated with almost all of the serious and fatal cases (GKRANIA-KLOTSAS and LEVER, 2007; ALLEN and KEYSTONE, 2004). *Plasmodium* parasites are transmitted from person to person by the bite of infected female mosquitoes of the genus *Anopheles* (GOSONIU *et al.*, 2009; WHITE, 2009). Malaria is widespread in the tropics and also occurs in subtropical and temperate regions (RODENZAAL, 1997). It is endemic in 109 countries (WHITE, 2009). In 2010, the World Health Organization (WHO) estimated that there were 216 million

cases of malaria and 655 000 deaths worldwide. About 91% of these deaths occurred in sub-Saharan Africa, mostly children under 5 years of age (WHO, 2011a).

1.3.4.2. Yellow fever

Yellow fever (YF) is a disease caused by a single-stranded RNA virus that belongs to the genus *Flavivirus*. The virus is transmitted between humans primarily by the bite of infected *Aedes aegypti* mosquitoes (GUBLER, 2004; AMAKU *et al.*, 2011). Yellow fever occurs in tropical regions of Africa and South America (MONATH, 2001), with more than 600 million people living in endemic areas (TOLLE, 2009). The World Health Organisation estimates that there are 200 000 cases of YF and 30 000 deaths each year worldwide. The majority (90%) of these deaths occur in Africa (MUTEBI and BARRETT, 2002; TOLLE, 2009).

1.3.4.3. Dengue fever

Dengue fever (DF) is caused by the dengue virus (DENV) that belongs to the genus *Flavivirus*. There are four closely related DENVs that can cause DF; DENV-1, DENV-2, DENV-3 and DENV-4 (GUBLER, 2004). These viruses are transmitted between people mainly by the bite of infected *Aedes aegypti* mosquitoes; *Ae. albopictus* can also act as a vector (ROZENDAAL, 1997). Dengue fever is endemic in more than 100 countries in Africa, the Americas, the western Mediterranean, South and East Asia, and the western Pacific. The incidence of DF has increased dramatically over the last decade and over 2.5 billion people are at risk (GUBLER, 2004). It is estimated that there are between 50 and 100 million cases of DF and 22 000 deaths each year (WHO, 2012).

1.3.4.4. Lymphatic filariasis

Lymphatic filariasis (LF), commonly known as elephantiasis is caused by nematode parasites of the family Filariodidea. There are three species of nematode parasites that can cause LF, namely, *Wuchereria bancrofti*, *Brugia malayi*, and *B. timori* (BANDYOPADHYAY, 1996). *W. bancrofti* is responsible for 90% of infections. The parasites are transmitted from person to person by the bite of infected mosquitoes. *W.*

bancrofti is mainly transmitted by *Culex quinquefasciatus* and by some *Anopheles* and *Aedes* species. *B. malayi* and *B. timori* parasites are mainly transmitted by *Mansonia* mosquitoes (ROZENDAAL, 1997). Lymphatic filariasis is endemic in 73 countries throughout the tropical and subtropical regions of Asia, Africa, the western Pacific and some parts of the Americas, with more than 120 million people estimated to be infected (OTTESEN *et al.*, 1997).

1.3.4.5. Japanese encephalitis

Japanese encephalitis (JE) is a disease caused by a single-stranded RNA virus that belongs to the genus *Flavivirus*. Japanese encephalitis virus is transmitted to humans through the bite of infected *Culex* mosquitoes, primarily *Cx. tritaeniorhynchus* (DIAGANA *et al.*, 2007; MISRA and KALITA, 2010). Japanese encephalitis occurs in China and south-east Asian countries (ROZENDAAL, 1997). It is estimated that there are 30 000-50 000 clinical cases of JE and 10 000-15 000 deaths every year, most of the victims are children below 10 years of age (DIAGANA, *et al.*, 2007).

CHAPTER 2

An ethnobotanical survey of mosquito repellent plants

2.1. Introduction

Throughout the ages, humans have relied on nature for their basic needs for the production of food, shelter, clothing, means of transportation, fertilizers, flavours, fragrances, and medicines (**GURIB-FAKIM, 2006**). Plants have been used as sources of medicines since ancient times (**MUTHAURA *et al.*, 2007**). A medicinal plant is defined as any plant which can be used to prevent or cure a disease. Fossil records date human use of plants as medicines at least to the middle Paleolithic age, some 60,000 years ago (**FABRICANT and FARNSWORTH, 2001**). These medicines took the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations (**BALUNAS and KINGHORN, 2005**). Despite advances in pharmacology and synthetic organic chemistry, the reliance on plants, remains largely unchanged. According to the World Health Organisation (WHO), about 80% of the world's population, primarily those of developing countries rely on plant-derived medicines for their healthcare needs (**GURIB-FAKIM, 2006**). The specific plants to be used and the methods of application for particular ailments are handed down from generation to generation through oral communication (**BALUNAS and KINGHORN, 2005**).

Plants also play an important role in health care systems of the remaining 20% of the world's population, mainly residing in developed countries (**GURIB-FAKIM, 2006**). About 25% of the drugs prescribed worldwide are derived from plants. Of the 252 drugs considered as basic and essential by the World Health Organisation (WHO), 11% are exclusively derived from plants (**RATES, 2001**). Currently, there are 119 drugs that are derived from 90 plant species and are considered as important drugs

and are in use in one or more countries (**GURIB-FAKIM, 2006**). Examples of drugs derived from plants are aspirin (*Salix* spp.), atropine (*Atropa belladonna*), artemisinin (*Artemisia annua*), codein and morphine (*Papaver somniferum*), colchicine (*Colchicum autumnale*), digoxin (*Digitalis* spp.), ephedrine (*Ephedra sinica*), physostigmine (*Physostigma venenosum*), pilocarpine (*Pilocarpus jaborandi*), quinine and quinidine (*Cinchona* spp.), reserpine (*Rauwolfia serpentine*), taxol (*Taxus brevifolia*), tubocurarine (*Chondodendron tomentosum*), and vincristine and vinblastine (*Catharanthus roseus*) (**RATES, 2001; GILANI and ATTA-UR-RAHMAN, 2005; FABRICANT and FARNSWORTH, 2001**).

2.1.1. Medicinal uses of plants in South Africa

In South Africa, like in other developing countries, traditional medicine still forms the backbone of healthcare (**LIGHT *et al.*, 2005**). Many South Africans still use plants as medicines as an alternative or supplement to visiting a western health care practitioner (**VAN WYK *et al.*, 1997**). It is estimated that around 27 million South Africans rely on traditional plant medicine for their health care needs (**MANDER, 1998**). This is not surprising due to South Africa's cultural diversity as well as its large floral biodiversity. South Africa is home to over 30 000 species of higher plants and 3000 of these species are used as medicines across the country (**VAN WYK *et al.*, 1997**). Around 147 plant families are used traditionally for medicinal purposes in South Africa. The most prominent of these families, with over 50 species each, are the Fabaceae, Asteraceae, Euphorbiceae, Rubiaceae and Orchidaceae (**LOUW *et al.*, 2002**).

Demand for plant-derived medicine has created a trade in indigenous plants in South Africa (**DOLD and COCKS, 2002**). The trade in medicinal plants plays an important role in the South African economy. It is currently estimated that the trade in medicinal plants in South Africa is worth R2.9 billion per year. More than 771 plant species are known to be actively traded for medicinal purposes throughout the country (**MANDER *et al.*, 2007**). Among medicinal plants being traded most frequently are *Warburgia salutaris*, *Siphonochilus aethiopicus*, *Aloe ferox*, *Agathosma* spp., *Harpagophytum procumbens*, *Pelargonium sidoides*, *Elaeodendron transvaalense*,

Alepidea amatymbica, *Erythrophleum lasianthum* and *Xysmalobium undulatum* (FENNELL *et al.*, 2004).

2.1.2. Plants as source of mosquito repellents

Plants have been used to repel blood sucking insects since ancient times (KARUNAMOORTHY *et al.*, 2010). The use of plants against biting insects was first recorded among the ancient Greeks (MOORE *et al.*, 2006). Plant products have been used traditionally to repel or kill mosquitoes in many parts of the world (SEYOUM *et al.*, 2003). The repellent properties of plants to mosquitoes and other insects were well known before the advent of synthetic chemicals (KARUNAMOORTHY *et al.*, 2008). Man has used plants as repellents for thousands of years, most simply by hanging bruised plants in houses. Plants have also been used for centuries in the form of crude fumigants where plants were burnt to drive away mosquitoes and later as oil formulations applied to the skin or clothes (MAIA and MOORE, 2011).

Even today, plant products are still extensively used in many parts of the world to repel mosquitoes. The use of traditional repellents is widespread in different cultures and communities of Africa and beyond (SEYOUM *et al.*, 2002). Different communities use different plants in various forms to protect themselves against mosquitoes and other insect bites (HEBBALKAR *et al.*, 1992). The knowledge and usage customs of the repellent plants has been passed from one generation to another chiefly through word of mouth (KARUNAMOORTHY *et al.*, 2009b). Although there is widespread use of plants as mosquito repellents, scientific understanding of these plants is, however, largely unexplored and therefore there is a need to collect ethnobotanical information on these plants as a first step prior to evaluation of their efficacy and safety as mosquito repellents. In South Africa, an ethnobotanical study of mosquito repellent plants was conducted in the Mpumalanga province (GOVERE *et al.*, 2000). To the best of our knowledge, however, there has been no study in the KwaZulu-Natal province. Comprehensive data on mosquito repellent plants as well as information on efficacy and safety is lacking. The present study therefore was

conducted to document plants that are used to repel mosquitoes in the uMkhanyakude district, KwaZulu-Natal province, South Africa. The specific objectives of the study were to: (1) identify plant species and their parts being used; (2) determine the condition of plant material used and the method of application.

2.2. Materials and Methods

2.2.1. Study area

The study was carried out in five rural villages, namely; Mbadleni, Ndumu, iziPhosheni, Makhanisi and Mziki in the uMkhanyakude district, KwaZulu-Natal province, South Africa. The district is located in the north-eastern part of KwaZulu-Natal, sharing a boundary with Mozambique in the north, Swaziland in the north-west and the Indian Ocean in the east (Figure 2.1). Named after the uMkhanyakude Tree (*Acacia xanthophloea*, Fever Tree which translated to English means “the light in the distance”), the district covers a total land area of approximately 13,859 km². The population of uMkhanyakude district is estimated to be 573,341. The population consist of 45.2% male and 54.8% female (**STATISTIC SOUTH AFRICA CENSUS, 2001**). The major ethnic group in the district is the Zulu. Most of the area is rural, with the majority of people depending on subsistence agriculture and animal husbandry as sources of livelihood. Malaria is one of the leading causes of morbidity and mortality in the study area. Of 4193 malaria cases reported in the 2003/2004 transmission season in KwaZulu-Natal province, 43% were from uMkhanyakude district (**DOH, 2007**). With the majority of people being too poor to afford commercial repellents, they use plant materials to protect themselves from mosquito bites.

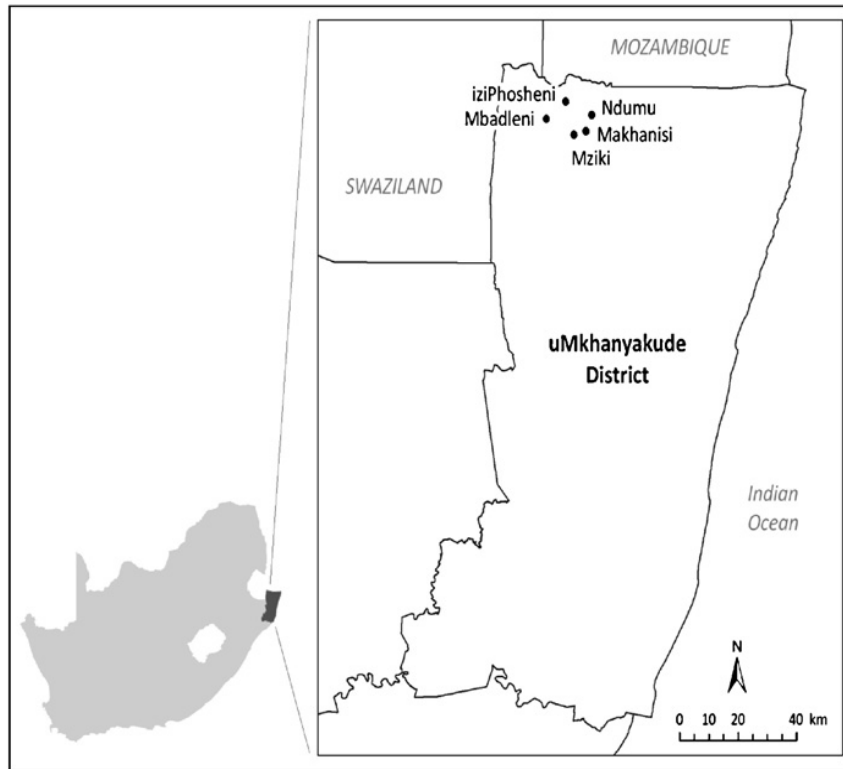


Figure 2.1: Map of South Africa showing uMkhanyakude district within KwaZulu-Natal province and villages where the survey was conducted.

2.2.2. Data collection

The study was undertaken as a descriptive cross-sectional survey between April and May 2011. Before conducting this survey, the leadership of each village was consulted in order to gain their trust and help to identify respondents. Data was collected using a standardised and pre-tested structured questionnaire. In each village, the questionnaire was administered to 12 respondents. One local person conversant with the language and culture of the area, and identified by the leadership of each village was recruited to help locate the selected respondents and to introduce the investigators to them. To ensure clarity and accuracy, questions prepared in English were translated into isiZulu, the principal language spoken in the study area. The questionnaire collected information on locality, sociodemographic data, knowledge of malaria and prevention practices, vernacular plant names, plant parts used, condition of the plant material (dried or fresh), and methods of application. All plant species mentioned by the respondent were collected with the help of traditional healers and the voucher specimens were deposited at the Bews Herbarium, School of Life

Sciences, University of KwaZulu-Natal, Pietermaritzburg Campus. The plants were identified by Dr. C. Potgieter of this Herbarium. Collected data were double entered into EpiData version 3.1 and analyzed using STATA version 11.

2.2.3. Ethical consideration

Ethics clearance for this study was obtained from the Humanities and Social Sciences Research Ethics Committee, University of KwaZulu-Natal (Reference number: HSS/0098/011D). Ahead of data collection, the aim and the objectives of the study were clearly explained and informed consent was obtained from each respondent. Participation in this study was entirely voluntary and participants were assured that they could withdraw at any time without any consequences.

2.3. Results and Discussion

2.3.1. Socio-demographic characteristics of respondents

All selected respondents in each village were interviewed during this survey, yielding a response rate of 100%. Table 2.1 is showing demographic characteristics of respondents. Most (61.7%) of the respondents interviewed were heading households. Of the sixty respondents, 56.7% were male and 43.3% were female. Their ages ranged from 26 to 78 years with a mean of 52 ± 13.3 years. The majority (61.7%) of the respondents were illiterate, while 15.00, 18.33, 3.33, and 1.67% had attained primary, secondary, adult basic education and training, and post matric education, respectively. About 30% of the respondents were unemployed.

Table 2.1: Socio-demographic characteristics of respondents in villages visited in the uMkhanyakude district, KwaZulu-Natal province, South Africa

Characteristics	N	%
Gender		
Male	34	56.67
Female	26	43.33
Age		
26-35	6	10
36-45	18	30
46-55	14	23.33
56-65	11	18.33
66-75	8	13.33
≥76	3	5
Relationship to household head		
Head of household	37	16.67
Spouse	18	30
Children	5	8.33
Highest level of education attained		
No education	37	61.67
Primary	9	15
Secondary	11	18.33
Adult basic education and training	2	3.33
Post matric	1	1.67
Occupation		
Unemployed	18	30
Civil servant	10	16.67
Pensioner	9	15
Traditional healer	17	28.33
Other	6	10

2.3.2. Knowledge of malaria and its prevention measures

Malaria is locally known as “Malaleveve”, meaning malaria fever. Most respondents (95%) associated malaria transmission with mosquito bites. However, few thought that water swamps were responsible for transmitting malaria. All respondents mentioned that malaria transmission can be prevented. Repellents was the most (98%, n = 59/60) mentioned prevention measure, followed by bed-nets (65%, 39/60), indoor residual spraying (25%, 15/60), and creating a clean environment (15%, 9/60). uMkhanyakude district is one of the major malaria endemic areas in KwaZulu-Natal; therefore high numbers of community members have good knowledge of malaria and its prevention measures. Table 2.2 illustrates the respondents’ knowledge about malaria and prevention measures that can be used.

Table 2.2: Respondents’ knowledge of malaria and its prevention measures in villages visited in the uMkhanyakude district, KwaZulu-Natal province, South Africa

Variables	N	%
Mode of transmission		
Mosquitoes	57	95
Water swamps	3	5
Prevention source		
Indoor residual spraying	15	25
Repellents	59	98.33
Chemoprophylaxis	2	3.33
Bed nets	39	65
Other	2	3

2.3.3. Plant species used for repelling mosquitoes

All respondents knew/used plant materials to repel mosquitoes. Seventy percent of the respondents obtained knowledge of repellent plants from their family elders while 30% got it from their ancestors (they are traditional healers). Knowledge of traditional medicines accumulated over a long time is transmitted from one generation to another through oral communication (**WHO, 2000**). The reasons for using plants as repellents were that they were cost-free and accessible. The use of traditional medicines is prevalent in regions where western medicines are inaccessible due to their unavailability and high cost (**LIGHT *et al.*, 2005**).

The survey documented 13 plant species which were used to repel mosquitoes by the local inhabitants in the study area; 2 of these plants remain to be identified (Table 2.3). The identified plant species belong to 11 genera in 9 families. Among these families, Meliaceae and Anacardiaceae were most represented with two species each. **PÅLSSON and JAENSON (1999)** reported that 8 plant species belonging to 8 genera and 6 families were being used to repel mosquitoes in Guinea Bissau. In a similar study, 5 plant species belonging to 4 genera and 4 families were identified in Tanzania (**KWEKA *et al.*, 2008**). **KARUNAMOORTHI *et al.* (2009b)**, in Ethiopia documented 9 mosquito repellent plants belonging to 8 genera and families. The family Meliaceae was represented in all of these studies, indicating its importance as a source of mosquito repellents.

The most frequently mentioned mosquito repellent plants were *Lippia javanica* (91.67%, n = 55/60), followed by *Aloe ferox* (11.67%, n = 7/60), *Sclerocarya birrea* (5%, n = 3/60), *Melia azedarach*, *Balanite maughamii* and *Mangifera indica* (3%, n = 2/60). The use of *L. javanica* as a mosquito repellent has also been reported in the Mpumalanga province, South Africa (**GOVERE *et al.*, 2000**) and Zimbabwe (**LUKWA *et al.*, 1999**) and was found to have repellent activity. In a recent study, **KARUNAMOORTHI *et al.* (2009b)** reported that *M. azedarach* was among the most frequently cited plant species used by the Oromo ethnic group to repel mosquitoes in Ethiopia. However, to the best of our knowledge, 9 plant species, namely, *Aloe ferox*, *Calausena anista*, *Croton menyharthii*, *Sclerocarya birrea*,

Balanite maughamii, *Olax dissitiflora*, *Trichilia emetica*, *Mangifera indica*, and *Atalaya alata* are documented for the first time as mosquito repellents.

Table 2.3: Plant species used to repel mosquitoes in the uMkhanyakude district, KwaZulu-Natal province, South Africa

Family	Species	Voucher number	Local Name (isiZulu)	Frequency	%	Plant part used	Plant condition	Application method
Aloaceae	<i>Aloe ferox</i> Mill.	EM08	iNhlaba	7	11.67	Leaves	Fresh	Smoke
Anacardiaceae	<i>Mangifera indica</i> L.	EM11	Umango	2	3.33	Seeds	Dried	Smoke
Anacardiaceae	<i>Sclerocarya birrea</i> (A.Rich.) Hochst	EM10	Mugano	3	5	Seeds	Dried	Smoke
Balanitaceae	<i>Balanites maughamii</i> Sprague.	EM09	uGobendlovu	2	3.33	Bark	Dried	Smoke
Euphorbiaceae	<i>Croton menyaarthii</i> Pax	EM05	Hubeshani	1	1.67	Leaves	Fresh	Smoke
Meliaceae	<i>Melia azedarach</i> L.	EM01	Umsilinga	2	3.33	Leaves	Fresh	Smoke
Meliaceae	<i>Trichilia emitica</i> Vahl	EM06	Umkhuhlu	1	1.67	Seeds	Dried	Smoke
Olaceae	<i>Olex dissitiflora</i> Oliver	EM04	Mampuzane	1	1.67	Bark	Dried	Smoke
Rutaceae	<i>Clausena anisata</i> (Willd.) Hook. F.	EM02	Umsanga	1	1.67	Leaves	Fresh	Hanging
Sapindaceae	<i>Atalaya alata</i> (Sim) H.H.L. Forbes	EM07	Umnondo	1	1.67	Roots	Dried	Smoke
Unidentified	-----		Umzaneno	1	1.67	Roots	Dried	Smoke
Unidentified	-----		Khokhelo	1	1.67	Roots	Dried	Smoke
Verbanaceae	<i>Lippia javanica</i> (Brum.f) Spreng.	EM03	Umsuzwane	55	91.67	Leaves	fresh	Smoke

2.3.4. Plant parts used, condition of plant material and method of application

The majority (69.2%) of plants used were trees, while 30.8% were shrubs. Leaves were the most (39%) common plant part used in repelling mosquitoes. Similar to the results of this study, leaves were the most common plant parts used to repel mosquitoes in Addis Zemen town, North Western Ethiopia (KARUNAMOORTHI *et al.*, 2009a). Other plant parts used were roots, bark and seeds (Figure 2.2). Interesting is that leaves are frequently used. Harvesting roots and bark can threaten local plant populations unless a sustainable harvesting strategy is developed (CUNNINGHAM, 2001).

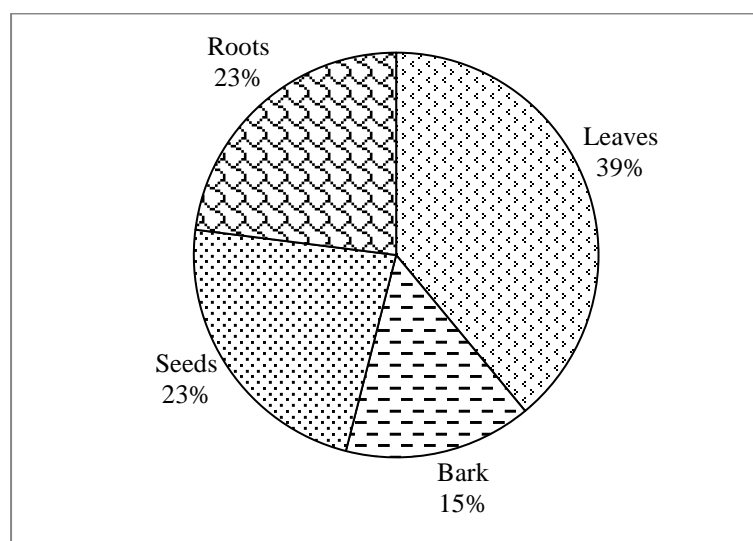


Figure 2.2: Percentage of plant parts used to repel mosquitoes in villages visited in the uMkhanyakude district, KwaZulu-Natal province, South Africa.

The majority (82%) of plant parts were dried before use. The exception being leaves of *L. javanica*, *C. anista*, *M. azedarach*, *C. menyharthii* and *A. ferox* which are used in a fresh state. Burning of plant materials to make smoke was the most (92%) common method of application. Similar results were reported in Ethiopia (KARUNAMOORTHI *et al.*, 2009a, KARUNAMOORTHI *et al.*, 2009b) and Guinea Bissau (PÅLSSON and JAENSON, 1999). Smoke is the most widely used method of repelling biting insects throughout the world (KARUNAMOORTHI *et al.*, 2008). The burning of some herbs such as *Artemisia* (Asteraceae) and *Calmus* species in rural areas in China is used to repel mosquitoes and protect cattle from

blood sucking insects (HWANG *et al.*, 1985). Smoke produced by burning dried leaves of *Azadirachta indica* has been used to repel mosquitoes since ancient times (SUKUMAR *et al.*, 1991). Hanging plants inside the house or sprinkling leaves on the floor is another method used (SANGAT-ROEMANTYO, 1990). In our findings, leaves of *C. anisata* were the only plant part used for hanging inside the house. In East Africa, Lua communities lay the branches of *Ocimum basilicum* (Labiatae) inside the house to drive away mosquitoes (KOKWARO, 1976).

2.4. Conclusions

The present study shows that people of uMkhanyakude district use plant materials to repel mosquitoes. Plant materials are commonly used because they are cost-free and easily accessible. Thirteen plant species were documented and 9 of them are documented for the first time as mosquito repellents. This documentation will provide the basis for further studies in developing new, effective, safe and affordable plant-derived mosquito repellents especially for Africa where malaria is highly prevalent. Knowledge of traditional medicines is passed from one generation to another by oral communication, posing the danger of losing this traditional practice because of no documentation. This study will therefore play an important role in documenting and conserving traditional knowledge of mosquito repellent plants for future use.

CHAPTER 3

Mosquitocidal screening

3.1. Introduction

Since there is currently no effective vaccine available for the prevention of malaria, vector control is one of the main strategies used to control this disease. Mosquito-borne diseases are controlled by interrupting the transmission of disease by either killing or preventing mosquitoes biting human beings (**ICMR BULLETIN, 2003**).

3.1.1. Adulticiding

Indoor residual spraying (IRS) is the application of insecticides on the walls and ceilings of residential structures in order to kill and/or repel the adult vector mosquitoes that land and rest on these surfaces (**WHO, 2006a**). The basic principle behind IRS is that, after biting, the female mosquito eventually rests on sprayed surfaces of the house, where it picks up a lethal dose of insecticide, thus preventing transmission of the parasite. This implies that IRS is most effective against mosquito species that are resting indoors (endophilic mosquitoes) (**PLUESS *et al.*, 2010**). Indoor residual spraying is one of the primary vector control methods for reducing and interrupting malaria transmission (**WHO, 2006a**). There are currently 12 insecticides belonging to four chemical groups recommended by the WHO for IRS, namely, organochlorides, organophosphates, carbamates and pyrethroids. Among these insecticides, DDT (1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane) (Figure 3.1), an organochloride, is the one with the longest residual efficacy (6–12 months depending on dosage and substrate) (**WHO, 2006a; WHO, 2011b**).

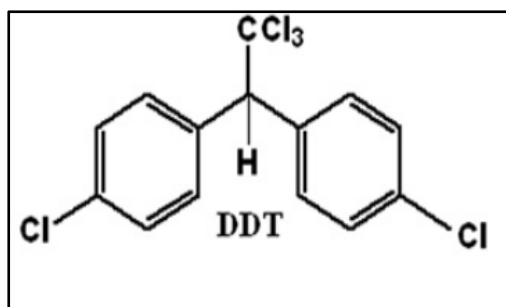


Figure 3.1: Chemical structure of DDT (PURNOMO *et al.*, 2011a).

DDT, the first synthetic organic insecticide (PURNOMO *et al.*, 2011a; PURNOMO *et al.*, 2011b), was synthesised in 1874, but its insecticidal properties were not discovered until 1939 (SMITH, 1991). It was first used by military forces during the Second World War to combat mosquitoes which spread malaria and typhus (BUSVINE 1989; FOGHT *et al.* 2001). Due to its success, DDT was rapidly introduced into malaria control programs in the 1940s (MABASO *et al.*, 2004), and it has contributed to the eradication of malaria in the United States, Japan, Korea, Taiwan, Spain, Italy, the Balkans, Greece and northern Africa during the Global Malaria Eradication Programme (GMEP) of 1955-1969 (MENDIS *et al.*, 2009; GREENWOOD, 2009). It has also been effective in reducing malaria morbidity and mortality in South Africa (CASIMIRO *et al.*, 2006).

3.1.2. Larviciding

Larviciding is a successful strategy of controlling mosquito borne diseases by killing mosquitoes at the larval stage before they emerge into adults. This method dates back to 1899, when Ronald Ross applied kerosene on anopheline larval breeding sites in Sierra Leone (BOCKARIE *et al.*, 1999). The advantages of targeting the larval stages are that mosquitoes are killed before they disperse to human habitations, and that mosquito larvae, unlike adults cannot change their behaviour to avoid control activities targeted at the larval habitat (FILLINGER and LINDSAY, 2006). Larvicides may act as stomach poisons, which must be ingested by the larvae when feeding, or as contact poisons, which penetrate the body wall or the respiratory tract. There are different types of formulations of larvicides. The most commonly used formulations are the emulsifiable concentrates, wettable powders and suspension concentrates. At present, mosquito larviciding depends primarily on the use of

synthetic larvicides. The most commonly used synthetic larvicides are organophosphorus compounds such as temephos, fenthion, chlorpyrifos, fenitrothion, jodfenphos, malathion and primiphos methyl. Among these compounds, temephos is the most widely used larvicide, because it has low toxicity to fish, birds, mammals and humans (ROZENDAAL, 1997).

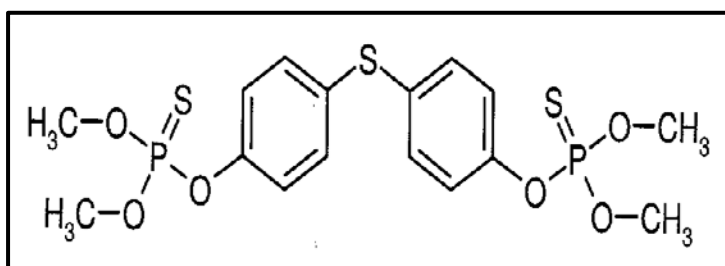


Figure 3.2: Chemical structure of temephos (WHO, 2008).

Temephos (o,o,o'o'-tetramethyl-o,o'-thiodiphenylene phosphorothiorate) (Figure 3.2), also known by the trade name of Abate®, was first registered in 1965 for the control of mosquito larvae (CHEN *et al.*, 2009). It is commonly available as an emulsifiable concentrate (46 and 20% (w/v) active ingredient) and granules (1% active ingredient) (ROZENDAAL, 1997). Temephos has been effective in controlling dengue and dengue haemorrhagic fever, malaria and filariasis vectors. It is also used to control midge, gnat, punkie and sandfly larvae (CHEN *et al.*, 2009).

3.1.3. Repellence

Repellents are substances that act locally or at a distance, deterring insects from flying to, landing on, or biting human or animal skin (BLACKWELL *et al.*, 2003). Usually, insect repellents work by providing a vapour barrier deterring insects from coming into contact with the surface (BROWN and HEBERT, 1997). Repellents are widely used to prevent the transmission of mosquito-borne diseases by minimizing the contact between humans and vectors (PITASAWAT *et al.*, 2003). They are applied directly to the skin or to clothing and other fabrics such as bednets and anti-mosquito screens (ROZENDAAL, 1997). There is a wide range of mosquito repellent formulations on the market, with varying levels of effectiveness and which last for different lengths of time. The majority of these repellents are prepared using synthetic

chemicals such as DEET (N,N-diethyl-3-methylbenzamide), KBR 3023/Picardin [2-(2-hydroxyethyl)-1-piperidinecarboxylic acid 1-methylpropyl ester] and IR3535 (2[N-hydroxy-N-acetyl] amino propionic acid ethyl ester). Among these synthetic chemicals, DEET (Figure 3.3) is the most widely used and most effective repellent currently on the market (VERHULST *et al.*, 2007).

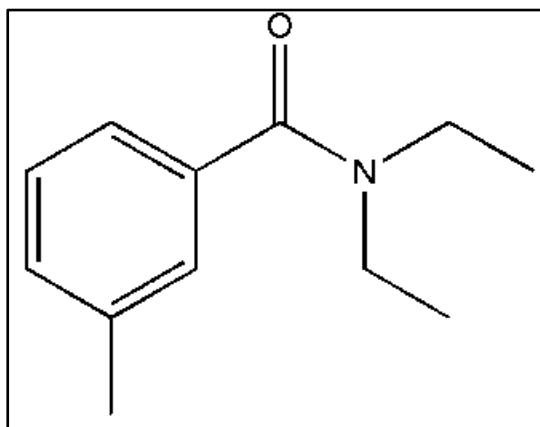


Figure 3.3: Chemical structure of DEET (STANCZYK, 2011).

DEET was developed and patented by the U.S. Army in 1946 and subsequently registered for use by the general public in 1957. It is a broad-spectrum repellent that is effective against mosquitoes, biting flies, chiggers, fleas, and ticks. This insect repellent is available in concentrations ranging from 5 to 100% in various formulations such as solutions, lotions, aerosol and pump sprays, sticks and impregnated towelettes (FRADIN, 1998).

3.1.4. Problems associated with synthetic insecticides

Although these synthetic insecticides are effective in controlling mosquitoes, their repeated use has resulted in many problems, such as toxic effects to humans, non-target organisms, and the environment, and the development of resistance in mosquito populations.

DDT biodegrades slowly, persists for a long time in the environment, and accumulates in the food chain and in the tissues of living organisms. DDT is toxic to freshwater and marine microorganisms, fishes, amphibians, and birds (TURUSOV *et al.*, 2002). Several studies have associated DDT with health effects such as early

pregnancy loss, fertility loss, pancreatic cancer, neurodevelopmental deficits, breast cancer, leukemia and diabetes. Resistance to DDT have been reported in various *Anopheles* species, including, *An. gambiae*, *An. arabiensis*, *An. culicifacies*, *An. sinensis*, and *An. epiroticus* (VAN DEN BERG, 2009).

Temephos is highly toxic to very highly toxic to freshwater and marine/estuarine aquatic invertebrates (HACKENBERGER *et al.*, 2008). Several studies conducted in different countries reported the resistance of *Ae. aegypti* larvae to temephos (OCAMPO *et al.*, 2011; RODRÍGUEZ *et al.*, 2002; FLORES *et al.*, 2006; LIMA *et al.*, 2011; BIBER *et al.*, 2006; RODRIGUEZ *et al.*, 2001; CHEN *et al.*, 2005; TIKAR *et al.*, 2009). Resistance to temephos has also been reported in *Ae. albopictus* larvae (CHEN *et al.*, 2005).

Although highly effective, side effects after the application of DEET vary from mild to severe. DEET has an unpleasant smell, oily feel and high skin penetration, and it can dissolve plastic and synthetic rubber (QUI *et al.*, 1998).

These problems highlight the urgent need for development of new mosquitocides, which are effective, safe, biodegradable and target-specific. Plants may be an alternative source of mosquito-control agents because they constitute a rich source of bioactive chemicals (KAMARAJ *et al.*, 2010; TIWARY *et al.*, 2007). Depending on the type of activity they possess, plant products can be used as mosquito repellents, larvicides and adulticides. Much interest has, therefore, been focused on plant extracts or phytochemicals as potential sources of mosquitocidal agents or as lead compounds. A large number of plant extracts have been reported to possess mosquitocidal activity against different mosquito species (ICMR BULLETIN, 2003).

In view of the recent increased interest in developing plant-derived mosquitocides, the present study was therefore undertaken to screen for adulticidal, larvicidal and repellent activity against *Anopheles arabiensis* in ten plants used traditionally as mosquito repellents in South Africa.

3.2. Materials and Methods

3.2.1. Plant materials

Ten plant species documented as mosquito repellents in Chapter 2 were selected for screening. Table 3.1 lists the plants that were selected. Plant materials were collected from Ndumo Village, in uMkhanyakude district, KwaZulu-Natal province, South Africa. Voucher specimens were prepared and deposited at the Bews Herbarium, University of KwaZulu-Natal, Pietermaritzburg Campus.

Table 3.1: Plant species screened for adulticidal, larvicidal and repellent activities

Family	Plant species	Voucher number	Local name	Plant part used	Medicinal uses
Aloaceae	<i>Aloe ferox</i> Mill.	EM08	iNhlaba	Leaves	Laxative, arthritis, eczema, conjunctivitis (WATT and BREYER-BRANDWIJK, 1962), hypertension and stress (PUJOL, 1990)
Anacardiaceae	<i>Sclerocarya birrea</i> (A.Rich.) Hochst	EM10	Mugano	Seeds	Diarrhoea, dysentery, fever and malaria (WATT and BREYER-BRANDWIJK, 1962; HUTCHINGS, 1996)
Balanitaceae	<i>Balanites maughamii</i> Sprague.	EM09	uGobendlovu	Bark	Emetic (WATT and BREYER-BRANDWIJK, 1962) and coughs (IWU, 1986)
Euphorbiaceae	<i>Croton menyaarthii</i> Pax	EM05	Hubeshani	Leaves	Hepatitis, tapeworm, dysmenorrhoea, intestinal obstruction, influenza malaria, pregnancy and menstruation pains (SCHMELZER and GURIB-FAKIM, 2008)
Meliaceae	<i>Melia azedarach</i> L.	EM01	Umslinga	Leaves	Anthelmintic, leprosy, eczema and asthma (CARPINELLA et al., 1999; MACIEL et al., 2006)
Meliaceae	<i>Trichilia emitica</i> Vahl	EM06	Umkhuhlu	Seeds	Stomach and intestinal complaints, dysentery, kidney problems, indigestion, fever, purgative and eczema (WATT and BREYER-BRANDWIJK, 1962; HUTCHINGS, 1996; PUJOL, 1990)
Olaceae	<i>Olax dissitiflora</i> Oliver	EM04	Mampuzane	Bark	Emetic, gonorrhoea, aphrodisiac, venereal diseases and dental care (NEUWINGER, 2000)
Rutaceae	<i>Clausena anisata</i> (Willd.) Hook. F.	EM02	Umsanga	Leaves	Epilepsy, arthritis, rheumatism, hypertension, schistosomiasis, taeniasis, constipation and abdominal pains, malaria, headaches, body pains, impotence and sterility (OJEWOLE, 2002)

Table 3.1: continuation

Family	Plant species	Voucher number	Local name	Plant part used	Medicinal uses
Sapindaceae	<i>Atalaya alata</i> (Sim) H.H.L. Forbes	EM07	Umnondo	Leaves	No medicinal uses documented
Verbenaceae	<i>Lippia javanica</i> (Brum.f) Spreng	EM03	Umsuzwane	Leaves	Coughs, colds, fever, bronchitis, chest ailments, influenza, measles, rashes, malaria, stomach problems and headaches (WATT and BREYER-BRANDWIJK, 1962; HUTCHINGS, 1996)

3.2.2. Preparation of plant extracts

Plant materials were dried in an oven at 30-60°C. The drying time and temperature varied depending on the nature of the plant material. The dried plant materials were ground into powders by an electric blender and stored in airtight containers under dark conditions at room temperature. The ground plant materials were extracted separately with 20 ml/g of ethanol (EtOH) and dichloromethane (DCM) by sonication for 1 h. The extracts were filtered through Whatman No. 1 filter paper and concentrated under vacuum using a rotary evaporator (Büchi, Germany) at 30°C. The concentrated extracts were dried at room temperature under a stream of cold air. The dried extracts were stored at 4°C in the dark until required for assays.

3.2.3. Mosquitoes

The repellent, larvicidal and adulticidal activity of plant extracts were screened against laboratory-reared *An. arabiensis* mosquitoes, a potent malaria vector in South Africa. The mosquitoes were obtained from a permanent colony maintained at $27 \pm 2^\circ\text{C}$ and 85% relative humidity in the insectary of the Malaria Research Unit, Medical Research Council, Durban, South Africa. Larvae were fed on dog biscuits and yeast powder at a 3:1 ratio. Adults were provided with a 10% sucrose solution. Female mosquitoes were periodically blood-fed on restrained albino guinea pigs for egg production. The guinea pigs were reared according to the National Research Council's guidelines for the care and use of laboratory animals (NATIONAL RESEARCH COUNCIL, 1996).

3.2.4. Adulticidal assay

The adulticidal activity of the plant extracts was evaluated following the WHO standard method with slight modifications (WHO, 2006b). Briefly, plant extracts were dissolved in acetone to prepare a testing concentration of 10 mg/ml. Two and half millilitres (2.5 ml) of testing concentration was impregnated into Whatman No 1. filter papers (12 × 15 cm). Acetone was used as a negative control while deltamethrin (K-Othrine) was used as a positive control. The impregnated papers were air-dried for 5 min and then inserted into an exposure tube in the WHO testing kit (Figure 3.4). Twenty, 2-5-day-old, blood-starved female mosquitoes were introduced into the holding tube and held for 1 h to acclimatize. The mosquitoes were then transferred by gentle blowing in the exposure tube. After 1 h in the

exposure tube, mosquitoes were transferred back to the holding tube to recover. A pad of cotton soaked with 10% glucose solution was placed on the mesh screen to feed recovering mosquitoes. At the end of the 24 h recovery period, the number of dead mosquitoes was recorded and the percentage mortality was calculated. Each extract was tested in duplicate and the assay was repeated three times.



Figure 3.4: The WHO adulticidal activity testing kit.

3.2.5. Larvicidal assay

The larvicidal activity of plant extracts was evaluated according to the protocol of the World Health Organization (WHO) with slight modifications (WHO, 2005). Briefly, each extract was dissolved in acetone to prepare a stock solution of 50 mg/ml. One ml of each stock solution was added to 99 ml of distilled water in a 500 ml plastic beaker to obtain a test concentration of 500 $\mu\text{g/ml}$. Twenty five third instar larvae of *An. arabiensis* were then introduced in that beaker. The larvae were fed dry yeast powder. Acetone and distilled water were used as negative controls. Temephos (Abate®) was used as a positive control. The number of dead larvae was recorded after 24 h of exposure and the percentage mortality was calculated. The larvae were considered dead if they did not move when prodded with a needle in the siphon or cervical region. Each extract was tested in triplicate and the assay was

repeated twice. The extracts showing 100% mortality were selected for a dose–response bioassay.

3.2.6. Repellent assay

The repellent activity of plant extracts was evaluated using the rodent-bait technique, following the standard WHO protocol with slight modifications (**WHO, 1996**). The rodent used in this assay was *Mastomys coucha*. Each extract was dissolved in acetone to prepare a stock solution of 10 mg/ml. Before application of the extracts, adult rodents were weighed individually, anaesthetized, shaved on their ventral surface and their exposed skin cleaned thoroughly with acetone. One ml of each stock solution was then applied to the exposed skin. Acetone was used as negative control, while DEET was used as positive control. After air drying for 1 min, the treated rodent was exposed to 20 blood-starved female mosquitoes (4-day-old) in a 500 ml paper cup, which was modified by replacing the base with a mosquito net and covering the mouth with transparent plastic film, for the first 2 min of each 5 min interval. Before the start of each exposure, the readiness of the mosquitoes to bite was confirmed by exposing the untreated rodent first. If at least two or more mosquitoes landed on or bit the untreated rodent, the exposure of treated rodent was then continued. The exposure of treated rodent was continued at 5 min intervals until two or more mosquito bites occurred in a 2 min period, or until a bite in the previous exposure was followed by a confirmatory bite (second bite) in the following exposure. The time between application of the plant extract and the first two consecutive bites was considered as the complete-protection time, which is the criterion used to determine the repellent efficacy of a plant sample. The assay was replicated 3 times for each extract, and in each replicate, a different rodent was used. After each test, the rodent was then returned to the animal facility and allowed to recover from the anaesthetic.

3.3. Results and Discussion

3.3.1. Adulticidal activity

The results of the adulticidal activities against *An. arabiensis* of dichloromethane and ethanol extracts of 10 plants that are used as mosquito repellents in South Africa are presented in Figure 3.5. All the extracts showed adulticidal activity after 24 h of exposure with mosquito

mortality ranging from 4 to 98%. Three levels were used to define the activity of extracts: 1-49% low, 50-69% moderate and 70-100% high. Of the highly active extracts, the DCM extract of *A. ferox* leaves exhibited the highest activity with 98% adult mortality, followed by EtOH extracts of *A. ferox* leaves (86%) and *A. alata* (70%). No activity was observed in the negative control, while the positive control exhibited 100% adult mortality. The high adulticidal activity shown by DCM extract of *A. ferox* leaves against *An. arabiensis* is not surprising since it has been reported as a multipurpose traditional medicine. The plant is traditionally used as a laxative, emetic, to treat arthritis, sinusitis, conjunctivitis, ophthalmia, herpes, shingles, sore throat, red water hypertensions, infertility in women and impotence in men. Furthermore, it has also been reported to possess antioxidant, antimicrobial, anti-inflammatory, anticancer, antimalarial, and anthelmintic activities (CHEN *et al.*, 2012).

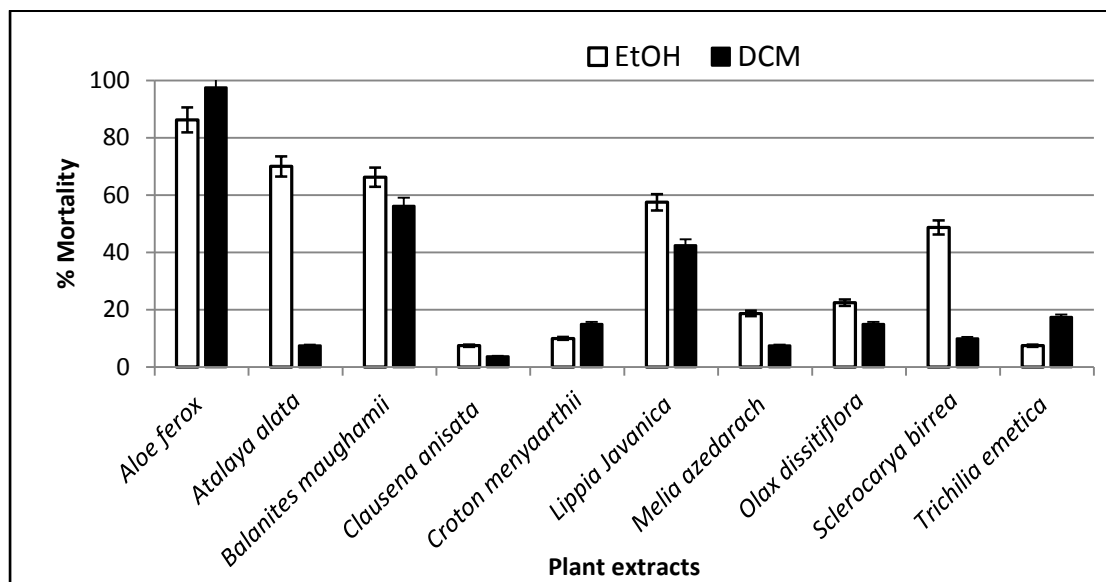


Figure 3.5: Adulticidal activity of DCM and EtOH extracts of 10 plants used to repel mosquitoes in the uMkhanyakude district, KwaZulu-Natal province, South Africa.

These findings are comparable to those of NATHAN *et al.* (2006a), who reported the adulticidal activity of methanol extract of *Dysoxylum malabaricum* leaves against *Anopheles stephensi*. The adulticidal activity of ethanol extract of *Apium graveolens* seeds against *Aedes aegypti* has been reported (CHOOCHOTE *et al.*, 2004). KOVENDAN *et al.* (2013), found the adulticidal activity of methanol extract of *Acalypha alinifolia* leaves against three mosquito species, *Ae. aegypti*, *An. stephensi* and *Culex quinquefasciatus*. Other plant species that are reported to possess adulticidal activity includes: *Curcuma aromatic* against *Ae. aegypti* (CHOOCHOTE *et al.*, 2005); *Aristolocia indica*, *Dolichos biflorus* and *Zingiber*

zerumbet against *Culex gelidus* and *Cx. quinquefasciatus* (KAMARAJ *et al.*, 2010); and *Melia azedarach* against *An. stephensi* (NATHAN *et al.*, 2006b).

Due to its high activity, the DCM extract of *A. ferox* leaves was then subjected to a dose-dependent bioassay to determine the EC₅₀ value. The extract was tested at five concentrations ranging from 0.6 to 10 mg/ml. After 24 h of exposure, mosquito mortality ranging from 5 to 100% was observed (Figure 3.6) and an EC₅₀ value of 4.92 mg/ml was recorded. The activity of this extract may be due to various compounds, such as phenolics, terpenoids, and alkaloids that exist in plants, and they may jointly or individually contribute to the insecticidal, ovicidal, repellent and antifeeding activities against various insect species (YANG *et al.*, 2005). *A. ferox* has been reported to contain compounds such as chromones, anthraquinones, anthrones, and anthone-C-glycosides (CHEN *et al.*, 2012). Therefore, the observed adulticidal activity of the DCM extract of *A. ferox* leaves may be attributed to these compounds. The adulticidal activity of the DCM extract of *A. ferox* against *An. arabiensis* is reported for the first time in this study.

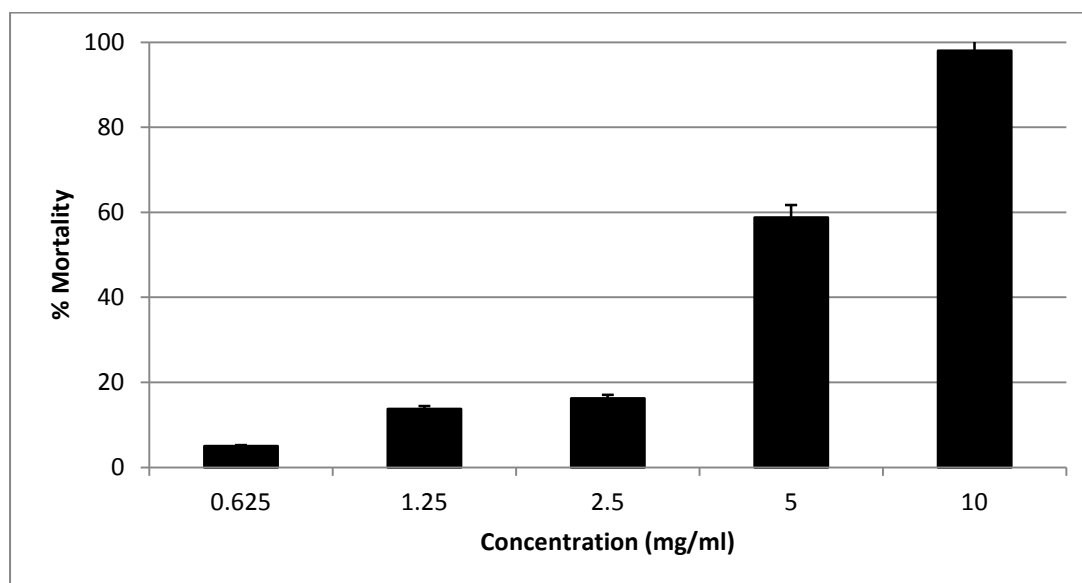


Figure 3.6: Adulticidal activity (Dose-response assay) of DCM extract of *A. ferox* leaves.

3.3.2. Larvicidal activity

Figure 3.7 presents the results of the larvicidal activities against *An. arabiensis* of dichloromethane and ethanol extracts of 10 plants that are used as mosquito repellents in South Africa. All the DCM extracts showed larvicidal activity after 24 h of exposure, while

only five EtOH extracts showed activity. No larval mortality was observed in the negative control, while the positive control exhibited 100% larval mortality. Among the DCM extracts, *Aloe ferox*, *Clausina anisata* leaves and *Olex dissitiflora* bark exhibited the highest larvicidal activity (100%), followed by *Lippia javanica* leaves (45%), *Sclerocarya birrea* seeds (23.8%), *Croton menyarthii* (20%), *Melia azedarach* leaves (18.6%), *Trichilia emetica* seeds (17.5%), *Atalaya alata* and *Balanites maughamii* leaves (7.5%). Of the five active EtOH extracts, *O. dissitiflora* bark and *C. anisata* leaves exhibited the highest larvicidal activity (100%), followed by *T. emetica* seeds (58%), *L. javanica* leaves (21%) and *C. menyarthii* leaves (12%).

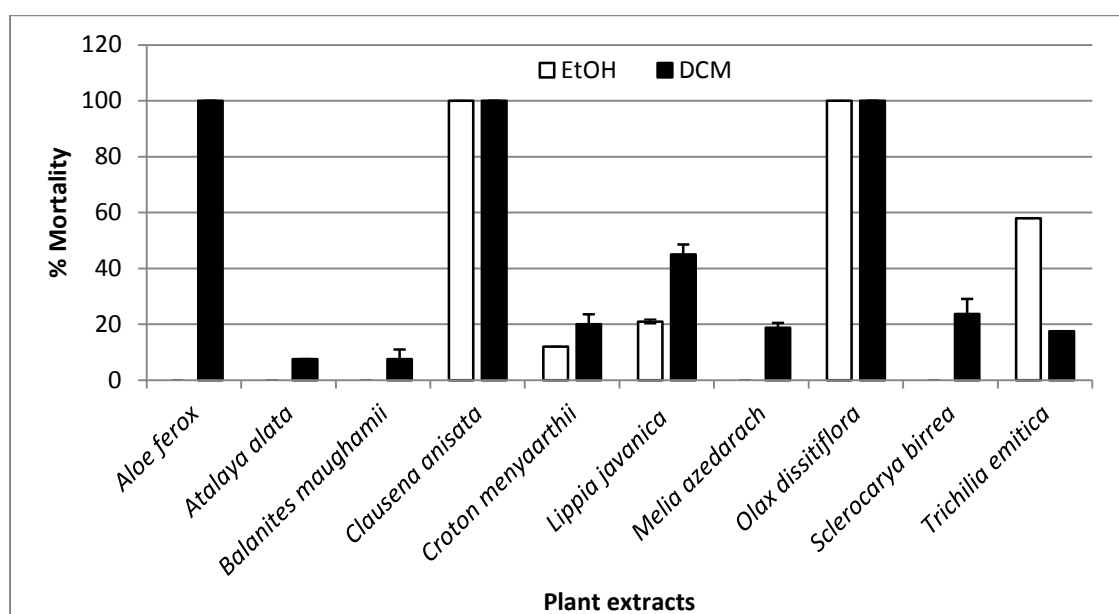


Figure 3.7: Larvicidal activity of DCM and EtOH extracts of 10 plants used to repel mosquitoes in the uMkhanyakude district, KwaZulu-Natal province, South Africa.

Our findings are in agreement to those of **KARUNAMOORTHI and ILANGO (2010)** who reported the larvicidal activity of methanol extract of *Croton macrostachyus* leaves against *An. arabiensis*. Other plant taxa which have been found to have larvicidal activity against *An. arabiensis* include ethanol extracts of *Ricinus communis*, *Catharanthus rosea* and *Lantana camara* leaves (**TAHA et al., 2011**). Larvicidal activity of a water extract of *R. communis* leaves against *An. arabiensis* has also been reported (**ELIMAMA et al., 2009**). The activity of crude plant extracts is often attributed to the complex mixture of active compounds (**KAMARAJ et al., 2011**). Various compounds such as phenolics, terpenoids, and alkaloids,

which are found in plants, jointly or individually contribute to the larvicidal activities against mosquitoes (JANG *et al.*, 2002).

At 500 µg/ml, no larvicidal activity was observed in the EtOH extracts of *A. ferox*, *A. alata*, and *M. azedarach* leaves, *S. birrea* seeds and *B. maughamii* bark. According to JANG *et al.* (2002), most plants with mosquito larvicidal activities belong to the families Apiaceae, Araceae, Magnoliaceae, Piperaceae, Rutaceae, and Zingiberaceae. In contrast to our findings, larvicidal activity of extracts of *M. azedarach* against different species of mosquitoes has been reported (WANDSCHEER *et al.*, 2004; NATHAN *et al.*, 2006b; CORIA *et al.*, 2008). MAHARAJ *et al.* (2012) reported the larvicidal activity of *A. ferox* against *An. arabiensis* mosquitoes. We attribute the absence of larvicidal activities of the plant extracts to natural variation in plant species, the plant part used, its geographical location, as well as methodological differences in application and mosquito species used (SUKUMAR *et al.*, 1991; DAS *et al.*, 2007).

Due to their high activity (100%), DCM and EtOH extracts of *O. dissitiflora* bark and *C. anisata* leaves, and DCM extract of *A. ferox* leaves were then subjected to a dose-response bioassay to determine their EC₅₀ values. The extracts were tested at five concentrations ranging from 15.6 to 500 µg/ml. Figures 3.8 and 3.9 present dose-response assays of DCM extracts of *O. dissitiflora* bark, *A. ferox* and *C. anisata* leaves, and EtOH extracts of *O. dissitiflora* bark and *C. anisata* leaves, respectively. The EC₅₀ values of the plant extracts are shown in Table 3.2. The DCM extract of *O. dissitiflora* bark exhibited the highest larvicidal activity with EC₅₀ value of 17.85 µg/ml, followed by the EtOH extract of *O. dissitiflora*, DCM extract of *C. anisata* leaves, DCM extract of *A. ferox* leaves and EtOH extract of *C. anisata* with EC values of 25.20, 36.66, 75.42 and 112.70 µg/ml, respectively. The larvicidal activity shown by *O. dissitiflora* bark may be attributed to ximeninic acid, a long chain fatty acid, with a double and triple bond (11-octadecen-9-ioic acid), which has been isolated from this plant (SPARAVIGNA *et al.*, 2014). *C. anisata* has been reported to contain many carbazole alkaloids and coumarins (NGADJUI *et al.*, 1989; SONGUE *et al.*, 2012). The larvicidal activity of *C. anisata* could be from these compounds.

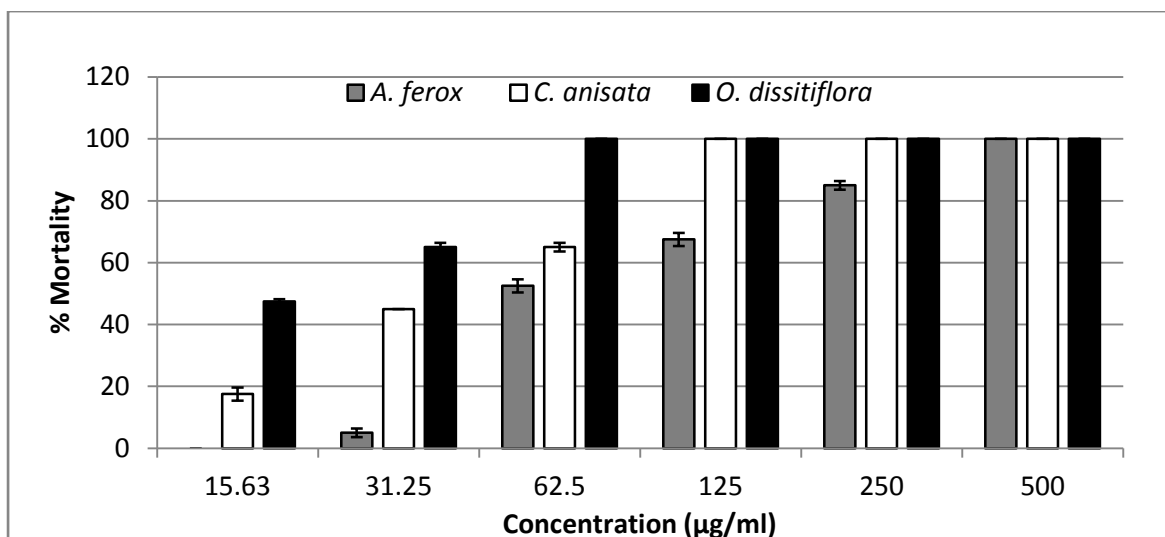


Figure 3.8 Larvicidal activity (Dose-response assay) of DCM extracts of *O. dissitiflora* bark, *A. ferox* leaves and *C. anisata* leaves.

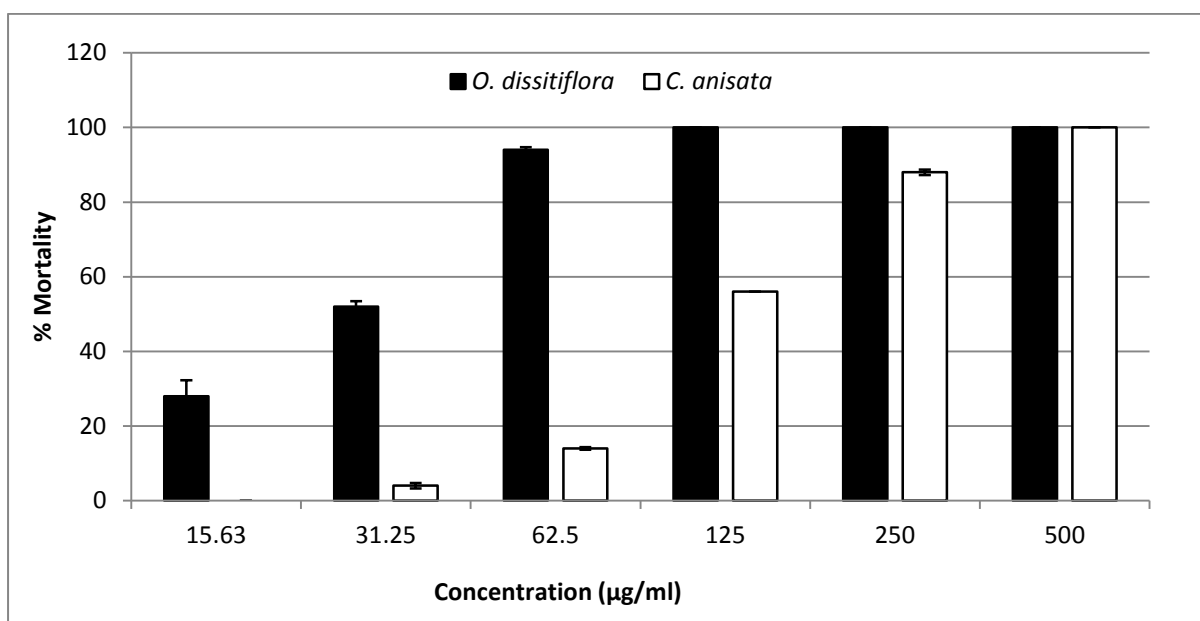


Figure 3.9 Larvicidal activity (Dose-response assay) of EtOH extracts of *O. dissitiflora* bark and *C. anisata* leaves.

Table 3.2: Larvicidal activity (EC₅₀ values) of DCM and EtOH extract of *A. ferox* leaves, *C. anisata* leaves and *O. diisitiflora* bark

Plant species	Solvent	EC ₅₀ (µg/ml)
<i>Aloe ferox</i>	DCM	75.42
	EtOH	NT
<i>Clausena anisata</i>	DCM	36.66
	EtOH	112.7
<i>Olax dissitiflora</i>	DCM	17.85
	EtOH	25.2

NT- EC₅₀ could not be calculated because there was no activity in EtOH extract.

3.3.3. Repellent activity

All the tested plant extracts did not show repellent activity. In contrast to our findings, repellent activity of *L. javanica* against *An. arabiensis* has been reported (GOVERE *et al.*, 2000). MAHARAJ *et al.* (2010) reported the repellent activities of *A. ferox*, *M. azedarach*, *C. anisata* against *An. arabiensis*. Repellent activity of *M. azedarach* has also been reported against *An. stephensi* (NATHAN *et al.*, 2006b). We can also attribute the absence of repellent activities of the plant extracts to natural variation in plant species, the plant part used, its geographical location, as well as methodological differences in the application and mosquito species used (SUKUMAR *et al.*, 1991; DAS *et al.*, 2007).

3.4. Conclusions

The DCM extract of *A. ferox* leaves exhibited the highest adulticidal activity. The highest larvicidal activity was observed in DCM extract of *O. dissitiflora* bark. These plants have the potential to be used as an adulticide and larvicide against *An. arabiensis* mosquitoes. The results of the present study could be useful in promoting research aimed at the development of new agents for mosquito control based on bioactive chemical compounds from indigenous plant sources.

CHAPTER 4

Antimicrobial activity of *Olax dissitiflora*

4.1. Introduction

Infectious diseases remain a leading cause of morbidity and mortality in the world. These diseases kill approximately 15 million annually (MORENS *et al.*, 2004). Pneumonia, diarrhoea, tuberculosis and malaria are leading causes of these deaths (LOUW, 2002). The burden of infectious diseases is felt mostly in developing countries (OKEKE *et al.*, 2005). Infectious diseases are caused by viruses, bacteria, fungi, and parasites (MORENS *et al.*, 2004).

4.1.1. Bacterial infection

Bacteria are microscopic, single-celled organisms. They have a prokaryotic cell type with a rigid wall which protects the cell against osmotic damage. Bacteria are capable of adapting in a diverse range of environments such as soil, water and air (SLEIGH and TIMBURY, 1998). They are classified according to their shape, reaction to the Gram stain, and need for oxygen. Bacteria may be classified as one of three shapes: spherical (cocci), rod-like (bacilli), and comma-shaped (vibrio). Those that stain purple when they are treated with Gram stain are classified as “Gram-positive” bacteria, while those that stain red are called “Gram-negative” bacteria. Bacteria that need oxygen to live and grow are called aerobic, while those that do not need oxygen are called anaerobic (FESKE, 2006).

Bacteria are both useful and harmful to humans. Some bacteria (actinomycetes) produce antibiotics such as streptomycin and nocardicin (BLACK, 2002). Others live symbiotically in the gut of animals including humans, or on the roots of some legumes converting nitrogen into a usable form. They also help to break down dead organic matter and form the base of the food web in our environment (BLACK, 2002).

Some bacteria cause diseases by producing toxins. *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* are some of the bacteria that are known to

cause diseases. *Bacillus subtilis* is a rod-shaped, endospore-forming, and Gram-positive bacterium that is found in soil and dust. This bacterium causes human eye infections (FUERST, 1978). *Escherichia coli*, commonly found in the intestinal tract of humans and other animals, is a Gram-negative bacterium that causes diarrhoea, wound and urinary infections (SLEIGH and TIMBURY, 1998). *Klebsiella pneumoniae* is a Gram-negative, rod-shaped and non-motile bacterium which is commonly found in water, soil, and occasionally food and can form part of the intestinal flora of humans and animals (HUGO, 1992). It causes pneumonia, urinary tract infections, bacteremia, septicaemia, chronic pulmonary disease, soft tissue infections, diarrhoea and other infections (RYAN and RAY, 2004; HARYANI *et al.*, 2007). *Staphylococcus aureus* is a spherical Gram-positive pyogenic bacterium that causes illnesses such as boils, conjunctivitis, pneumonia, skin infections, meningitis and septicaemia (LOWY, 1998).

4.1.2. Fungal infection

Fungi are eukaryotic, filamentous, and mostly spore-bearing microorganism, which exist as saprophytes or as parasites of animals and plants (KURUP *et al.*, 2000). They are either single-celled (yeast) or multicellular (moulds) (BASTERT *et al.*, 2001). Fungi are both useful and harmful to humans. Some are edible (mushrooms) and antibiotic producers (*Penicillium notatum*), while some are disease causing (BORDON-PALLIER *et al.*, 2004). Fungal infections are associated with increasing morbidity and mortality in immunocompromised and severely ill patients (LEHRNBECHER *et al.*, 2010). Candidiasis is the most common fungal infection in immunocompromised patients, with *Candida albicans* being the most frequently isolated species (LIU *et al.*, 2011). *C. albicans* is the major causative agent of fungal infections (ROHITASHW and SHUKLA, 2010). It accounts for more than 80% of candidiasis cases (GREENE and CHANDLER, 1998). *C. albicans* forms part of the flora living in the gastrointestinal tract, including the mouth and the gut. Overgrowth of *C. albicans* results in candidiasis mostly in patients with endocrine disorders, immunosuppression, malignant disorders, HIV and AIDS (MARTÍNEZ *et al.*, 1998).

4.1.3. Malaria infection

Malaria infection was explained as described in **Section 1.3.4.1.** in **Chapter 1**

4.1.4. Development of resistance to antimicrobial agents

The treatment of infectious diseases continues to be a major global public health problem due to the development of resistance of pathogens to currently available antimicrobial drugs. Antimicrobial resistance is defined as the ability of a microorganism to withstand a normally active concentration of an antimicrobial agent (**WITTE, 1998**). It is mainly caused by the indiscriminate use and abuse of antimicrobial drugs (**DEBRUYNE, 1997**).

Staphylococcus aureus strains are resistant to several antibiotics, including, penicillin, methicillin, benzylpenicillin, thethincillin, and vancomycin (**CASAL et al., 2005; CHAMBERS, 2001; BONTEM et al., 1998**). Resistance of *Escherichia coli* to sulphonamide and ampicillin-sulbactam have been reported (**ENNE et al., 2001; KAYE et al., 2000**). *Klebsiella pneumoniae* strains are resistant to carbapenems antibiotics (**SANCHEZ et al., 2013**).

There are several effective drugs currently available for the treatment of fungal diseases, for example, amphotericin B (AmpB), 5-flucytosine, fluconazole, ketoconazole, and itraconazole (**RAID and MARES, 2003**). However, their widespread and incorrect usage has led to the development of drug resistant strains. *C. albicans* strains have developed resistance to AmpB and fluconazole (**WHITE et al., 2002**).

There are a handful of accepted, affordable and effective drugs that are used to treat malaria (**WHO, 2006c**). Despite the existence of an array of antimalarial drugs, the control of this ancient infection is increasingly threatened by the development of resistance to these drugs (**CRAFT, 2008**). The development of resistance, particularly in *P. falciparum*, has been a major contributor to the global resurgence of malaria in the last three decades (**WHITE, 2004**). Resistance of *P. falciparum* have been observed in all currently used antimalarial

drugs, including, amodiaquine, chloroquine, mefloquine, quinine, sulfadoxine-pyrimethamine and more recently, in artemisinin derivatives (WHO, 2006c).

With the development of resistance of pathogens to the current antimicrobial drugs, there is, therefore an urgent need for new, effective, affordable and safe antimicrobial agents to control infectious diseases. Medicinal plants remain an important source of new drugs. In the search for new plant-derived antimicrobial drugs, *Olax dissitiflora*, a plant which showed the best larvicidal activity against *An. arabiensis* mosquitoes, was evaluated for antibacterial, antifungal and antiplasmodial activity. To the best of our knowledge, antibacterial, antifungal and antiplasmodial activity of this plant was reported for the first time in this study.

4.2. Materials and Methods

4.2.1. Plant collection

Plants were collected as described in **Section 3.2.1.** in **Chapter 3.**

4.2.2. Preparation of plant extracts

Plant extracts were prepared as described in **Section 3.2.2.** in **Chapter 3.**

4.2.3. Antibacterial assay

Minimum inhibitory concentration (MIC) values for antibacterial activity of DCM and EtOH extract of *O. dissitiflora* bark were determined using the microdilution bioassay in a 96-well (Greiner Bio-one GmbH, Germany) microtitre plate (ELOFF, 1998). One hundred microlitres of redissolved extract (50 mg/ml) in 80% ethanol were two-fold serially diluted with sterile distilled water, in duplicate in the microtitre plates, for each of the four bacteria used. A similar two-fold serial dilution of neomycin (Sigma) (0.1 mg/ml) was used as a positive control while 80% ethanol and water were used as solvent and negative controls, respectively. Overnight Mueller-Hinton (MH) broth cultures (incubated at 37 °C in a water bath with shaking) of four bacterial strains: two Gram-positive (*Bacillus subtilis* ATCC 6051 and *Staphylococcus aureus* ATCC 12600) and two Gram-negative (*Escherichia coli* ATCC 11775 and *Klebsiella pneumoniae* ATCC 13883) were diluted with sterile MH broth (1 ml

bacteria/50 ml MH) resulting in a final inoculum of approximately 10^6 cfu/ml. One hundred microlitres of each bacterial culture were added to each well. The plates were covered with parafilm and incubated overnight at 37 °C. Bacterial growth was tested by adding 50 µl of 0.2 mg/ml *p*-iodonitrotetrazolium chloride (INT) to each well and the plates incubated at 37 °C for 1 h. Bacterial growth in the wells was indicated by a red-pink colour, whereas clear wells indicated inhibition of growth by the tested sample. MIC values were recorded as the lowest concentration of extract showing a clear well. Each assay was repeated twice with two replicates.

4.2.4. Antifungal assay

The antifungal activity of *O. dissitiflora* was evaluated against *Candida albicans* (ATCC 10231) using the micro-dilution assay (ELOFF, 1998) modified for an antifungal assay (MASOKO *et al.*, 2007). An overnight fungal culture was prepared in 10 ml yeast malt (YM) broth. Four hundred microliters of the overnight *Candida* culture were added to 4 ml of sterile saline solution. The absorbance was read at 530 nm and adjusted with sterile saline solution to match that of a 0.5 M McFarland standard solution. From this prepared stock, a 1:1000 dilution with sterile YM broth was prepared to give an approximately 10^6 cfu/ml culture. One hundred microlitres of extract were resuspended in 80% ethanol to a concentration of 50 mg/ml and two-fold serially diluted with sterile distilled water, in duplicate down a 96-well microtitre plate. A similar 2-fold serial dilution of AmpB (Sigma) (2.5 mg/ml) was used as a positive control while water and fungal free broth were used as negative controls. One hundred microliters of the *Candida* cultures were added to each well and incubated for 24 h at 37 °C. To indicate fungal growth, 50 µl of 0.2 mg/ml INT was added to each well, and the plates were re-incubated for a further 24 h. The wells which displayed no change in colour represented antifungal activity. The MIC was taken as the lowest concentration of plant extract to inhibit growth of the tested fungus after 48 h. After noting the MIC, 50 µl YM broth was added to the clear wells to determine whether the inhibition was fungicidal. The microtitre plates were re-covered with parafilm and incubated for a further 24 h after which the MFC (minimum fungicidal concentration) values were noted. In the cases where there was no growth of fungus in the last clear well, even after addition of YM broth to clear wells, this was taken as the MFC. The assay was repeated twice with two replicates per assay.

4.2.5. Antiplasmodial assay

The antiplasmodial activity of *O. dissitiflora* was evaluated against a chloroquine-sensitive (CQS) strain of *Plasmodium falciparum* (D10). Continuous *in vitro* cultures of asexual erythrocyte stages of *P. falciparum* were maintained using a modified method of **TRAGER and JENSEN (1976)**. Quantitative assessment of antiplasmodial activity *in vitro* was determined via the parasite lactate dehydrogenase assay using a modified method described by **MAKLER *et al.* (1993)**. The test samples were prepared to a 20 mg/ml stock solution in 100% DMSO and sonicated to enhance solubility. Stock solutions were stored at -20°C . Further dilutions were prepared on the day of the experiment. Chloroquine diphosphate and artesunate were used as the reference drugs. A full dose-response was performed to determine the concentration inhibiting 50% of parasite growth (IC_{50} -value). The same dilution technique was used for all samples. Chloroquine and artesunate were tested at a starting concentration of 1000 ng/ml. The highest concentration of solvent to which the parasites were exposed to had no measurable effect on the parasite viability (data not shown). The IC_{50} values were obtained using a non-linear dose-response curve fitting analysis via GraphPad Prism v.4.0 software.

4.3. Results and Discussion

4.3.1. Antibacterial activity

The antibacterial MIC values of DCM and EtOH extracts of *O. dissitiflora* bark are presented in Table 4.1. Both extracts showed good activity against the four bacterial strains used, with MIC values ranging from 0.195 to 0.78 mg/ml. According to **ALIGIANNIS *et al.* (2001)**, crude extracts with MIC values less than or equal to 1 mg/ml are considered to have good activity. The DCM extract showed the best antibacterial activity with a MIC value of 0.195 mg/ml against *B. subtilis*. According to **RABE and VAN STADEN (1997)**, Gram-negative bacteria are more resistant than Gram-positive ones. The reason for the differences in sensitivity between Gram-positive and Gram-negative bacteria is attributed to the morphological differences between them (**PALOMBO and SEMPLE, 2001; TADEG *et al.*, 2005**). Gram-negative bacteria have an outer phospholipidic membrane that carries structural lipopolysaccharide components making the cell walls impermeable to lipophilic solutes. Gram-positive bacteria, on the other hand, are more susceptible having only an outer

petidoglycan layer which is not an effective permeable barrier (TADEG *et al.*, 2005). Therefore, the cell walls of Gram-negative organisms which are more complex than the Gram-positive ones acts as a diffusional barrier making them less susceptible than Gram-positive bacteria to the antimicrobial agents (PALOMBO and SEMPLE 2001).

Table 4.1: Antibacterial activity (MIC) of DCM and EtOH extracts of *O. dissitiflora* bark

Bacteria strains	MIC (mg/ml)	
	DCM	EtOH
<i>Bacillus subtilis</i>	0.195	0.39
<i>Escherichia coli</i>	0.39	0.78
<i>Klebsiella pneumoniae</i>	0.39	0.78
<i>Staphylococcus aureus</i>	0.78	0.78

The MIC values ($\mu\text{g/ml}$) of Neomycin (positive control) were: *B. subtilis* = 1.531×10^{-3} ; *E. coli* = 3.063×10^{-3} ; *S. aureus* = 6.125×10^{-3} ; *K. pneumoniae* = 6.125×10^{-3} .

4.3.2. Antifungal activity

Table 4.2 presents the antifungal activity of DCM and EtOH extracts of *O. dissitiflora* bark against *C. albicans*. Both extracts showed good antifungal activity with MIC values ranging from 0.195 to 0.39 mg/ml and MFC (minimal fungicidal concentration) values ranging from 0.39 to 0.78 mg/ml. According to MULAUDZI *et al.* (2012), MIC and MFC values < 1 mg/ml of crude extracts are considered to having good activity. The DCM extract exhibited the highest antifungal activity with MIC and MFC values of 0.195 and 0.78 mg/ml, respectively. According to POLAK (1999) “ideal antifungal drugs have not yet been discovered”. However, the observed results from DCM extract of *O. dissitiflora* bark suggested a potential lead to the isolation of a fungicidal drug.

Table 4.2: Antifungal activity (MIC and MFC) of DCM and EtOH extracts of *O. dissitiflora* bark

Solvent	Antifungal activity (mg/ml)	
	MIC	MFC
DCM	0.195	0.78
EtOH	0.39	0.39

The MIC and MFC values ($\mu\text{g/ml}$) of Amphotericin B ($\mu\text{g/ml}$) (positive control) were 9.77×10^{-3} and 7.81×10^{-2} respectively.

4.3.3. Antiplasmodial activity

The results of the antiplasmodial activity of DCM and EtOH extracts of *O. dissitiflora* bark against chloroquine-sensitive strain of *P. falciparum* are shown in Table 4.3. Both DCM and EtOH extracts showed a moderate antiplasmodial activity, with IC_{50} values of 15.6 and 45 $\mu\text{g/ml}$ respectively. According to MUREGI *et al.* (2003), antiplasmodial activity of extract is considered high, moderate, mild, and inactive if the IC_{50} value is <10 , 11 to 50, 51 to 100, and >100 respectively.

Table 4.3: Antiplasmodial activity of DCM and EtOH extracts of *O.dissitiflora* bark

Solvent	IC_{50} ($\mu\text{g/ml}$)
DCM	45
EtOH	15.6

The IC_{50} values (ng/ml) of Chloroquine diphosphate and Artesunate (positive controls) were 4.80 and 2.89 respectively.

4.4. Conclusions

Both DCM and EtOH extracts of *O. dissitiflora* bark showed good antibacterial and antifungal activities with MIC values less than 1 mg/ml . These extracts also showed a moderate antiplasmodial activity. The observed activities indicate that *O. dissitiflora* has potential to be used as antibacterial, antifungal and antimalarial agent. However, further studies aimed at isolation and identification of active compounds as well as evaluating their toxicity effect are required.

CHAPTER 5

Isolation and identification of larvicidal compounds from *Olax dissitiflora* Oliver (Olacaceae)

5.1. Introduction

5.1.1. Description and distribution of *O. dissitiflora*

Olax dissitiflora (Figure 5.1), commonly known as Bastard Sourplum, is a large shrub to small or medium sized deciduous tree of about 3 to 10 m in height. The bark is grey and smooth. The leaves are alternate, ovate to narrowly ovate and folded upwards along the midrib. The flowers are white, solitary or loosely grouped into axillary clusters. The fruits are a small round drupes, initially orange but turn red at maturity (SCHMIDT *et al.*, 2002). *Olax dissitiflora* is widely distributed throughout Africa, it occurs in Madagascar, Tanzania, Mozambique, and northern South Africa (ROGERS *et al.*, 2006). It is found in low-altitude bushveld, often along drainage lines and on rocky outcrops (SCHMIDT *et al.*, 2002).



Figure 5.1: *Olax dissitiflora*

5.1.2. Medicinal uses of *O. dissitiflora*

Leaf saps are eaten with coconut as an emetic. Root decoctions are drunk to treat gonorrhoea and hernias. The powdered roots are added to beer and taken as an aphrodisiac. They are also added in porridge and eaten to treat venereal diseases. The twigs are chewed for dental care (NEUWINGER, 2000). The leaves are used to treat wounds (RIBEIRO *et al.*, 2010) and as a purgative (ROGERS *et al.*, 2006).

5.1.3. Chemical constituents of *O. dissitiflora*

Four triterpenoid sapogenins, 21-epimachaerinic acid, oleanolic acid, oleanolic acid 3-O-glucuronide, and hederagenin have been isolated from the roots of *O. dissitiflora* (GABETTA *et al.*, 1974). Ximeninic acid has also been isolated from this plant (SPARAVIGNA *et al.*, 2014).

This study was aimed at isolating and identifying active compounds from the DCM extract of *O. dissitiflora* bark using larvicidal assay-guided fractionation.

5.2. Materials and Methods

5.2.1. Plant extraction

Olox dissitiflora bark was collected and dried as described in **Section 3.2.1.** and **3.2.2.** in **Chapter 3.** The dried powdered bark (800g) was extracted with 3L of dichloromethane by sonication for 1h, and left overnight with constant stirring using a magnetic stirrer. The extract was filtered through a Buchner funnel and Whatman No. 1 filter paper. The bark residue was further extracted two times with 0.5L dichloromethane. The filtered extracts were combined and concentrated under vacuum using a rotary evaporator (Rotavapor-R, Büchi, Switzerland) at 30 °C. The dried residue achieved was 32g.

5.2.2. Bioassay-guided fractionation for isolation of active compounds

5.2.2.1. Vacuum Liquid Chromatography (VLC)

The dichloromethane crude extract (32 g) was first re-dissolved in acetone, mixed with silica gel (20g) and allowed to dry. The dried mixture was then fractionated by Vacuum Liquid Chromatography (VLC) (Merck 9385 (silica gel), 150 g, 6 cm x 30 cm) using a hexane: ethyl acetate (1:0 - 0:1) and subsequently ethyl acetate: methanol (1:0 - 0:1) gradient solvent system. A volume of 300 ml of each solvent combination was used for each elution. Twenty (20) fractions of about 200 ml each were collected manually. The collected fractions were then spotted onto TLC plate and developed in a hexane: ethyl acetate (7:3) solvent system. The twenty fractions were reduced to six fractions (B1, B2, B3, B4, B5 and B6) by pooling together fractions with similar TLC profiles. The six fractions were subsequently assayed for larvicidal activity as described in **Section 3.2.6.** in **Chapter 3.** The fractions were tested at 250 µg/ml. Fraction B1 was the most active, exhibiting 100 % larval mortality

5.2.2.2. Gravity Column Chromatography

Fraction B1 (12 g) was further fractionated by gravity column chromatography (Merck 9385 (silica gel), 130 g, 2.5 cm x 73 cm) using a hexane: ethyl acetate (1:0 - 0:1) and subsequently ethyl acetate: methanol (1:0 - 0:1) gradient solvent system. A volume of 300 ml of each solvent combination was used for each elution. A fraction collector (Gilson FC 203B) was used to collect fractions. This process yielded eighty four fractions which were subsequently reduced to two fractions (C1 and C2) according to their TLC profiles. The TLC was performed as previously described in **Section 5.2.2.1**. The two fractions were then tested for larvicidal activity. Fraction C1 exhibited a better larvicidal activity and was later subjected to preparative TLC.

5.2.2.3. Preparative Thin Layer Chromatography

About 20 mg of fraction C1 was applied to each of three TLC plates (Merck glass plates, 20 x 20 cm, Silica gel 60 F254, 0.25 mm thick). The TLC plates were developed in a hexane: ethyl acetate (7:3) solvent system. Two very prominent bands with different R_f s were detected under UV (254 and 366 nm) light. They were each scraped off the TLC plates individually and eluted with absolute ethanol. Two compounds (D1 and D2) were isolated. The flow diagram in Figure 5.2 outlines the procedure followed. The isolated compounds were then assayed for larvicidal activity.

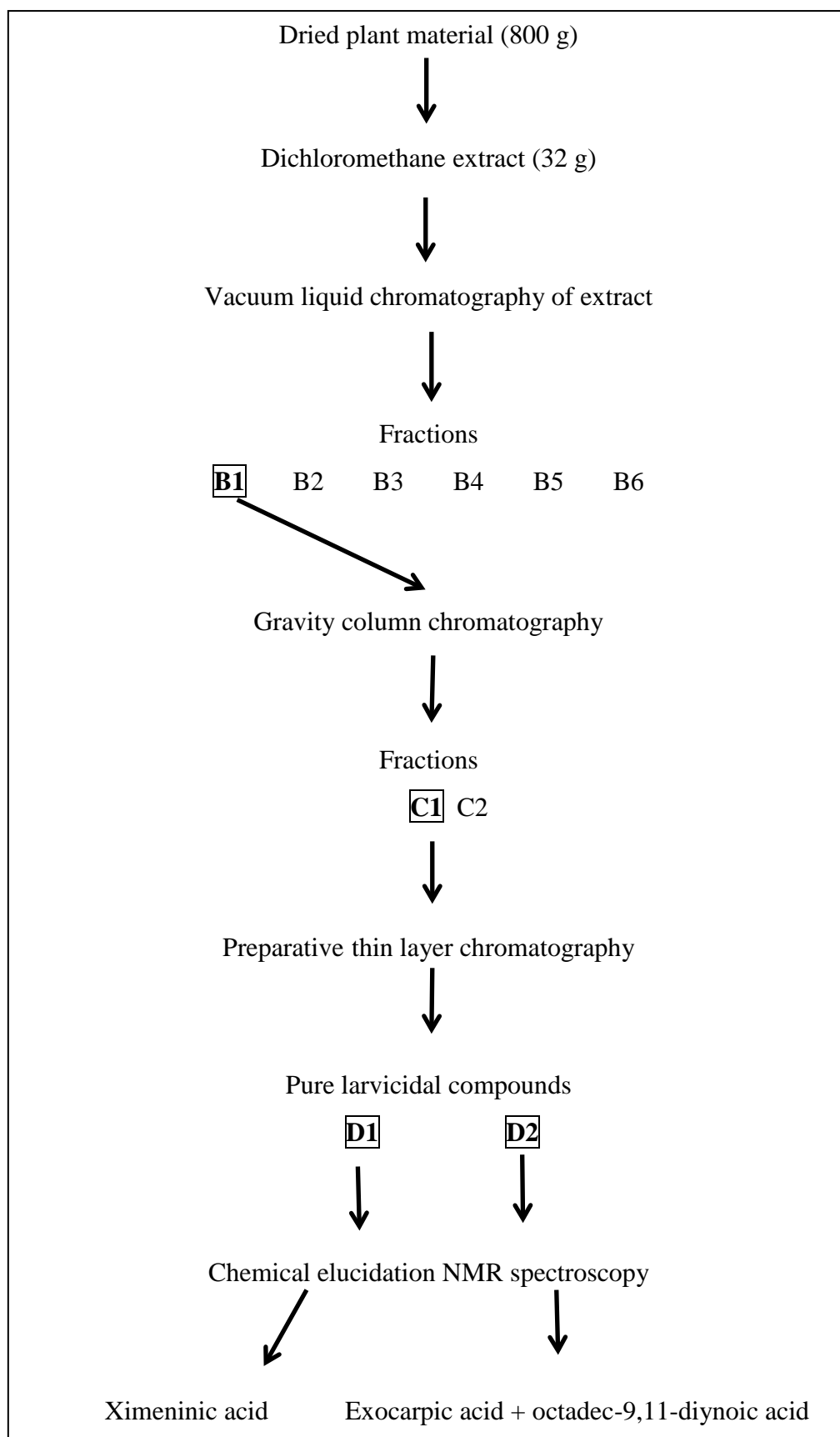


Figure 5.2: Flow chart outlining the isolation procedure for larvicidal compounds from DCM extract of *O. dissitiflora* bark

5.2.3. Identification of purified active compounds

Nuclear Magnetic Resonance Spectroscopy (NMR) (^1H , ^{13}C , COSY, HMBC, HMQC and NOESY) was performed at the School of Chemical and Physical Sciences, University of KwaZulu- Natal, Pietermaritzburg, to authenticate the structure of the isolated compounds. The NMR spectra were analyzed with the help of Professor Fanie van Heerden. Nuclear magnetic resonance spectra were recorded on a Bruker Avance III 400 spectrometer operating at 400 MHz for ^1H and 100 MHz for ^{13}C nuclei. The standard operating temperature was 30 °C. Spectra were referenced to residual solvent peaks (^1H , δ 3.31; ^{13}C , δ 49.00). Mass spectra (MS) were obtained on a Waters LCT Premier time-of-flight mass spectrometer. Electrospray ionization (ESI) in the negative mode was used to ionize molecules.

5.3. Results and discussion

5.3.1. Plant extraction

The dried and powdered bark (800 g) of *O. dissitiflora* yielded 32 g of crude extract.

5.3.2. Bioassay-guided fractionation for isolation of active compounds

5.3.2.1. Vacuum Liquid Chromatography (VLC)

The VLC of the dichloromethane crude extract yielded 20 fractions. The fractions were pooled into six (B1 - B6) fractions based on the similarity of their TLC profiles. They were subsequently subjected to larvicidal activity assay. The results are shown in Figure 5.3. Fraction B1 exhibited the highest larvicidal activity (100%) followed by B5 (80%), B6 (80%), B2 (65%), B4 (55%) and B3 (40%). Fraction B1 was further fractionated using gravity-assisted column chromatography

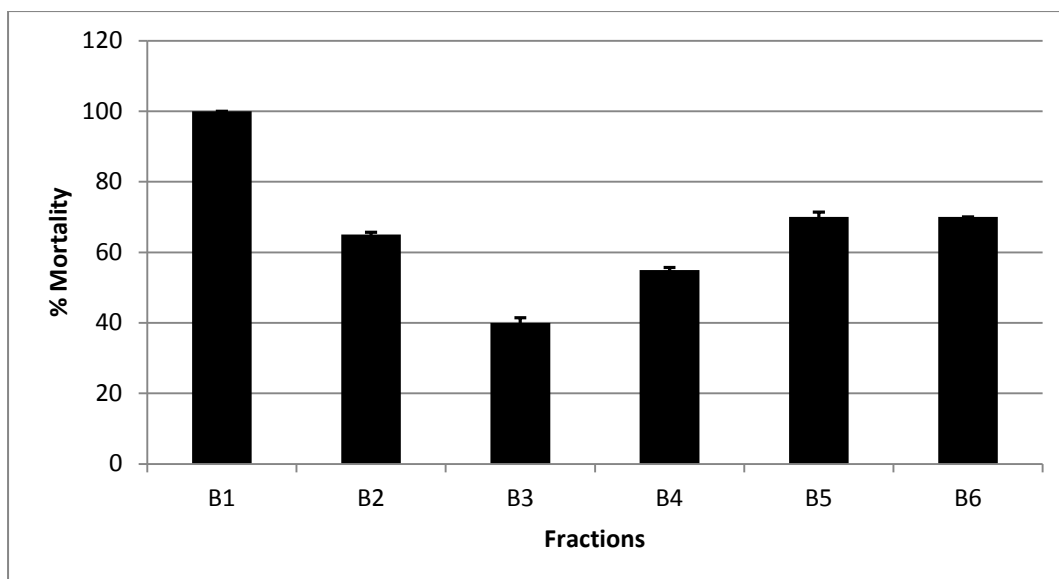


Figure 5.3: Larvicidal activity of fractions obtained from Vacuum Liquid Chromatography of DCM extract of *O. dissitiflora* bark.

5.3.2.2. Gravity Column Chromatography

Gravity assisted column chromatography of fraction B1 yielded 84 fractions. The fractions were pooled into two fractions (C1 and C2) based on similarity of their TLC profiles and were subjected to larvicidal activity assay. The results are presented in Figure 5.4. Fraction C1 exhibited the highest larvicidal activity (100%) compared to C2 (75%). Fraction C1 was further purified using preparative TLC.

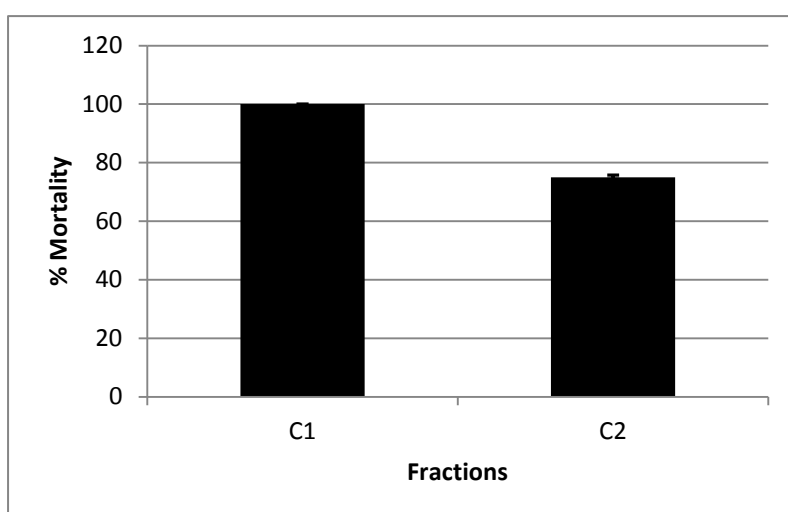


Figure 5.4: Larvicidal activity of fractions obtained from Gravity Column Chromatography of DCM extract of *O. dissitiflora* bark.

5.3.2.3. Preparative Thin Layer Chromatography

Preparative TLC of fraction C1 yielded two active pure compounds, D1 and D2. Figure 5.5 shows the larvicidal activity of isolated compounds. Compound D2 exhibited the highest larvicidal activity with an EC₅₀ value of 17.31 µg/ml compared to compound D1 which had an EC₅₀ value of 62.17 µg/ml.

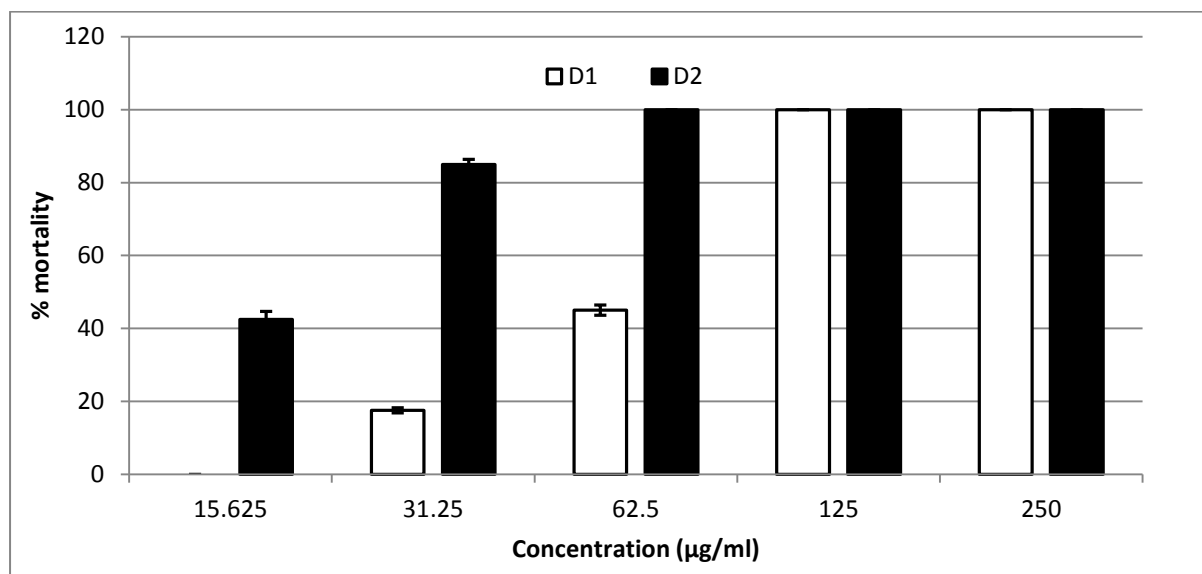


Figure 5.5: Larvicidal activity of isolated compounds from DCM extract of *O. diisitiflora* bark.

5.3.3. Identification of pure compounds

5.3.3.1. Identification of compound D1

The ¹H NMR spectrum (Figure 5.6) indicated that compound D1 is a fatty acid containing a *trans* double bond. The coupling constants of the double bond protons indicated that on the one side of the double bond there was a methylene (CH₂) group and on the other side a non-protonated carbon. The ¹³C NMR spectrum (Figure 5.7) shows the presence of two acetylenic carbons and that there is a triple bond in conjugation with the double bond. The DEPT-135 spectrum (Figure 5.8) enabled us to assign the ¹³C NMR signals as C, CH₂ or CH₃ signals. The COSY spectrum (Figure 5.9) correlated ¹H NMR signals with each other; whereas the HSQC spectrum (Figure 5.10) correlated the ¹H NMR signals with the ¹³C NMR signals. The

structure of compound D1 was assigned as *trans*-11-octadecen-9-ynoic acid, also known as santalbic acid or ximeninic acid (Figure 5.11), an acetylenic fatty acid that occurs widely in the Santalaceae family (AIZETMÜLLER, 2012). The NMR data of compound D1 is in close agreement with those reported for santalbic acid (WANG *et al.*, 2012).

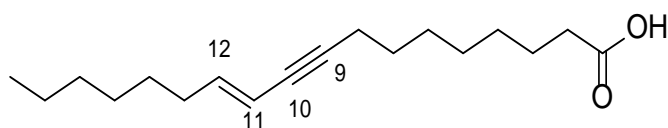


Figure 5.11: Structure of ximeninic acid.

5.3.3.2. Identification of compound D2

Both the NMR and the mass spectra indicated that compound D2 is a mixture of two closely related compounds. In the ESI (-)-mass spectrum, two peaks were observed at m/z 273 and 275, which correspond with molecular masses of 274 ($C_{18}H_{26}O_2$) and 276 ($C_{18}H_{28}O_2$), respectively. In the 1H NMR spectrum (Figure 5.12), the *trans*-double bond was still observed, but the integral indicated that it is present in only one of the compounds. In the ^{13}C NMR spectrum (Figure 5.13), one compound with a *trans*-alkene in conjugation with an acetylene was observed, whereas it was clear that the other compound contains two triple bonds. This observation is in agreement with the mass spectral data. The structures of the two compounds were assigned as exocarpic acid (Figure 5.14) and octadec-9,11-diynoic acid (Figure 5.15). The NMR data of exocarpic acid is in agreement with published data (NAIDOO *et al.* 1992). The DEPT, COSY and HSQC spectra of compound D2 are presented in Figure 5.16, 5.17 and 5.18, respectively.

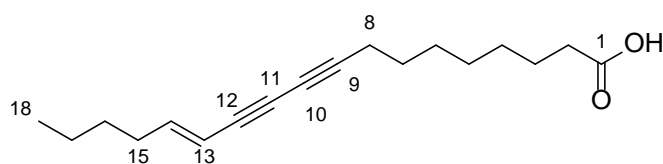


Figure 5.14: Structure of exocarpic acid.

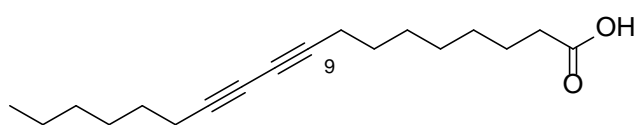


Figure 5.15: Structure of octadec-9,11-diynoic acid.

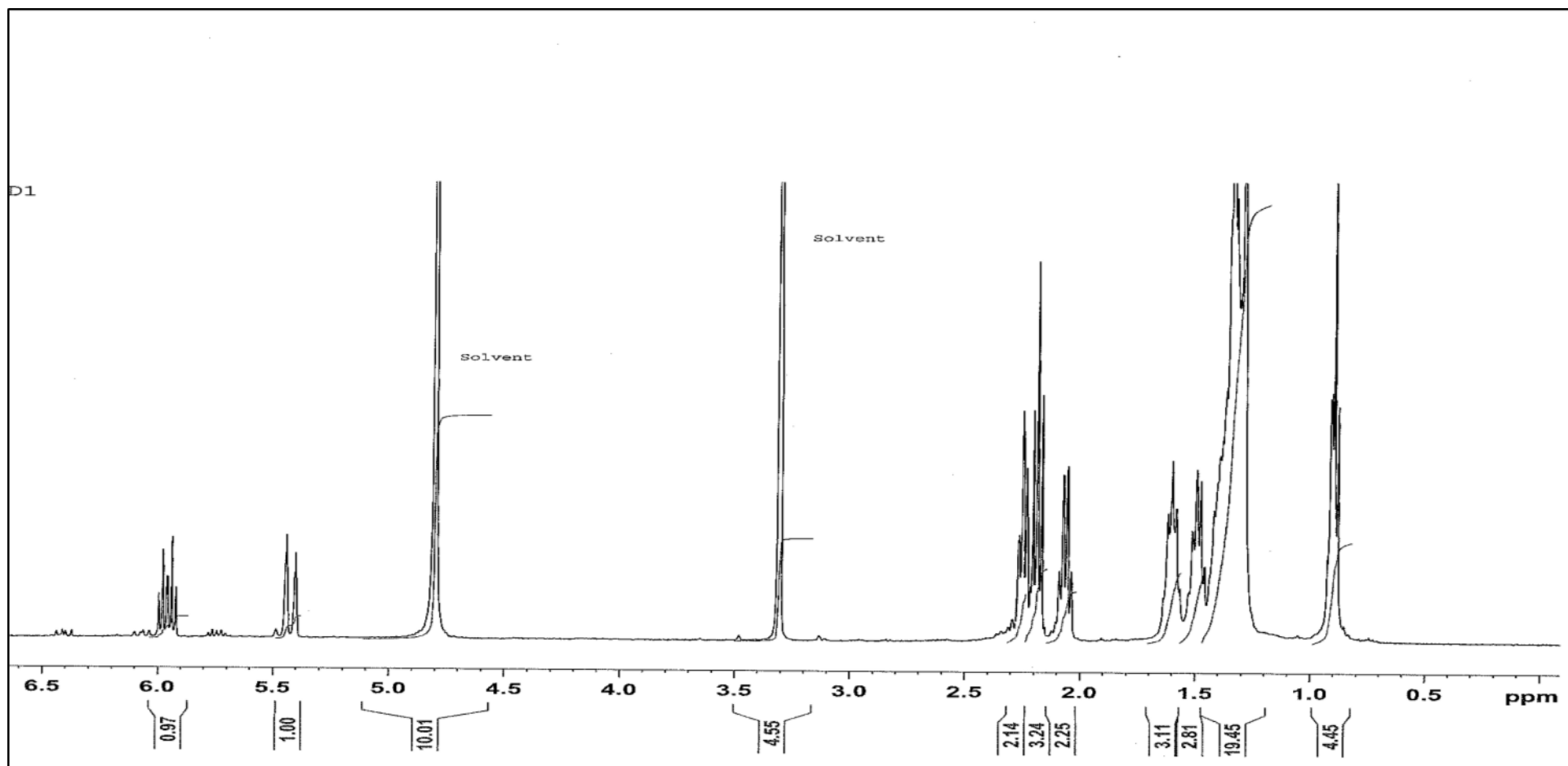


Figure 5.6: ^1H NMR spectrum of compound D1 isolated from DCM extract of *O. dissitiflora* bark.

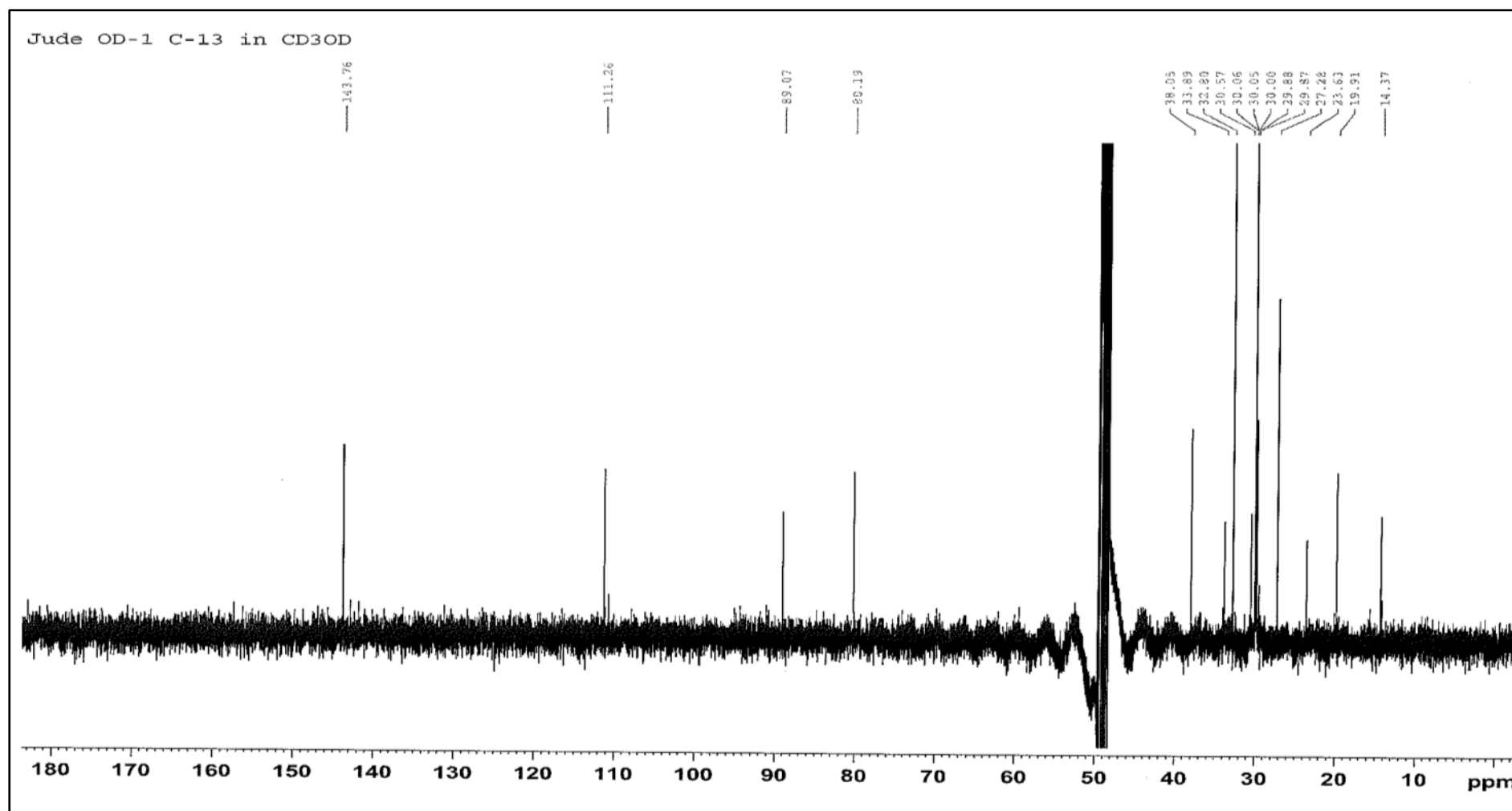


Figure 5.7: ^{13}C spectrum of compound D1 isolated from DCM extract of *O. dissitiflora* bark.

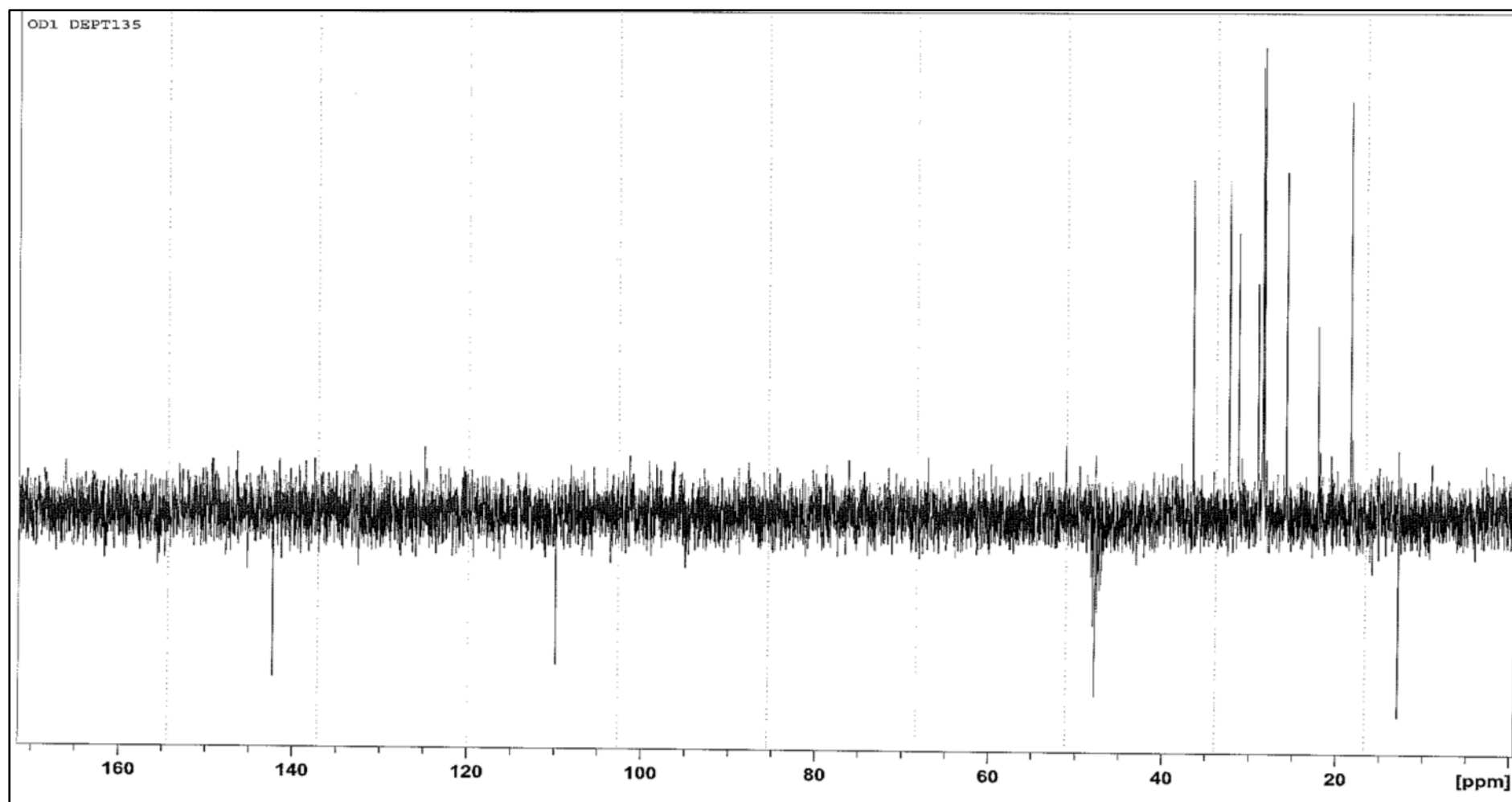


Figure 5.8: DEPT spectrum of compound D1 isolated from DCM extract of *O. dissitiflora* bark.

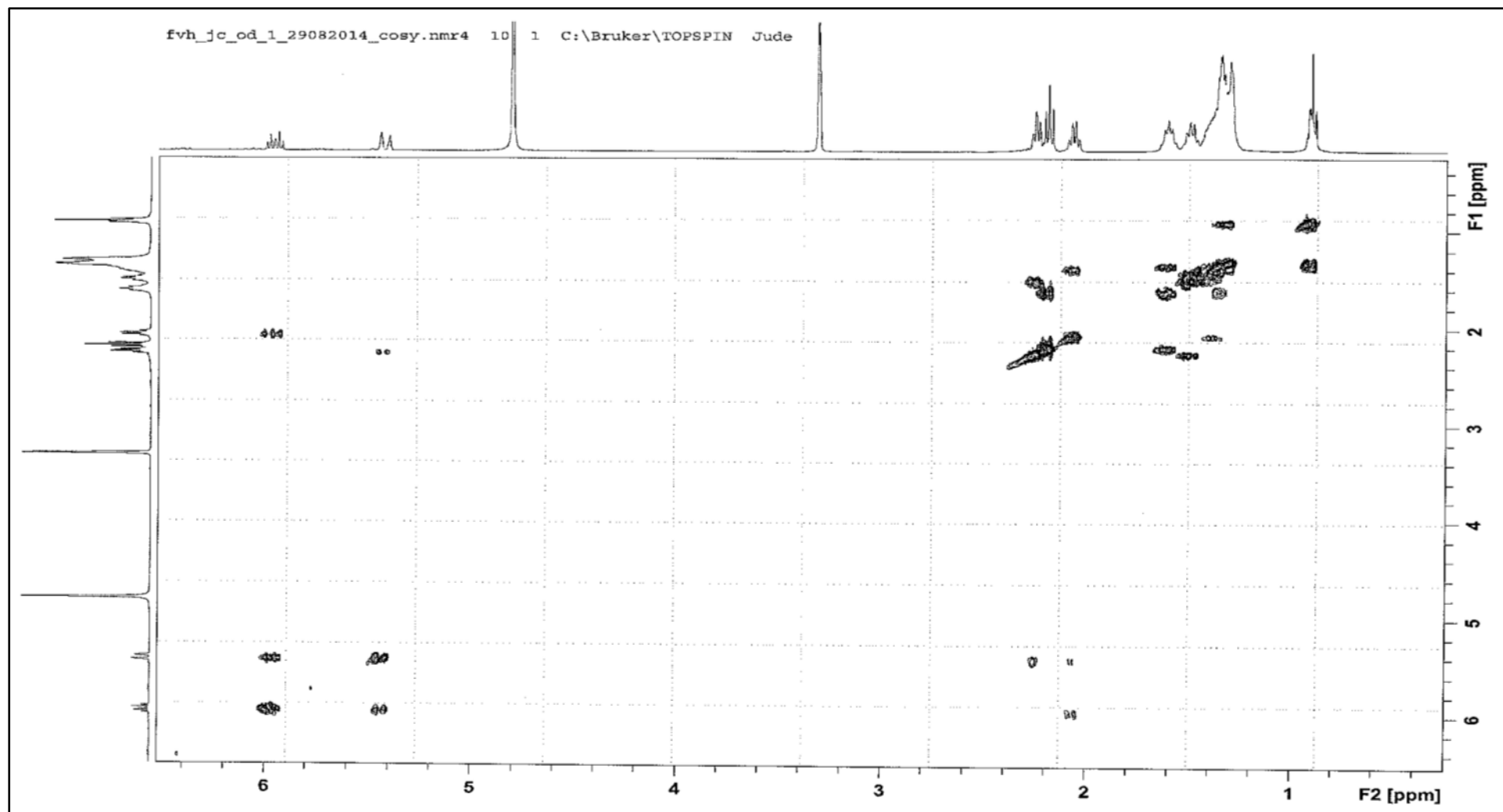


Figure 5.9: COSY spectrum of compound D1 isolated from DCM extract of *O. dissitiflora* bark.

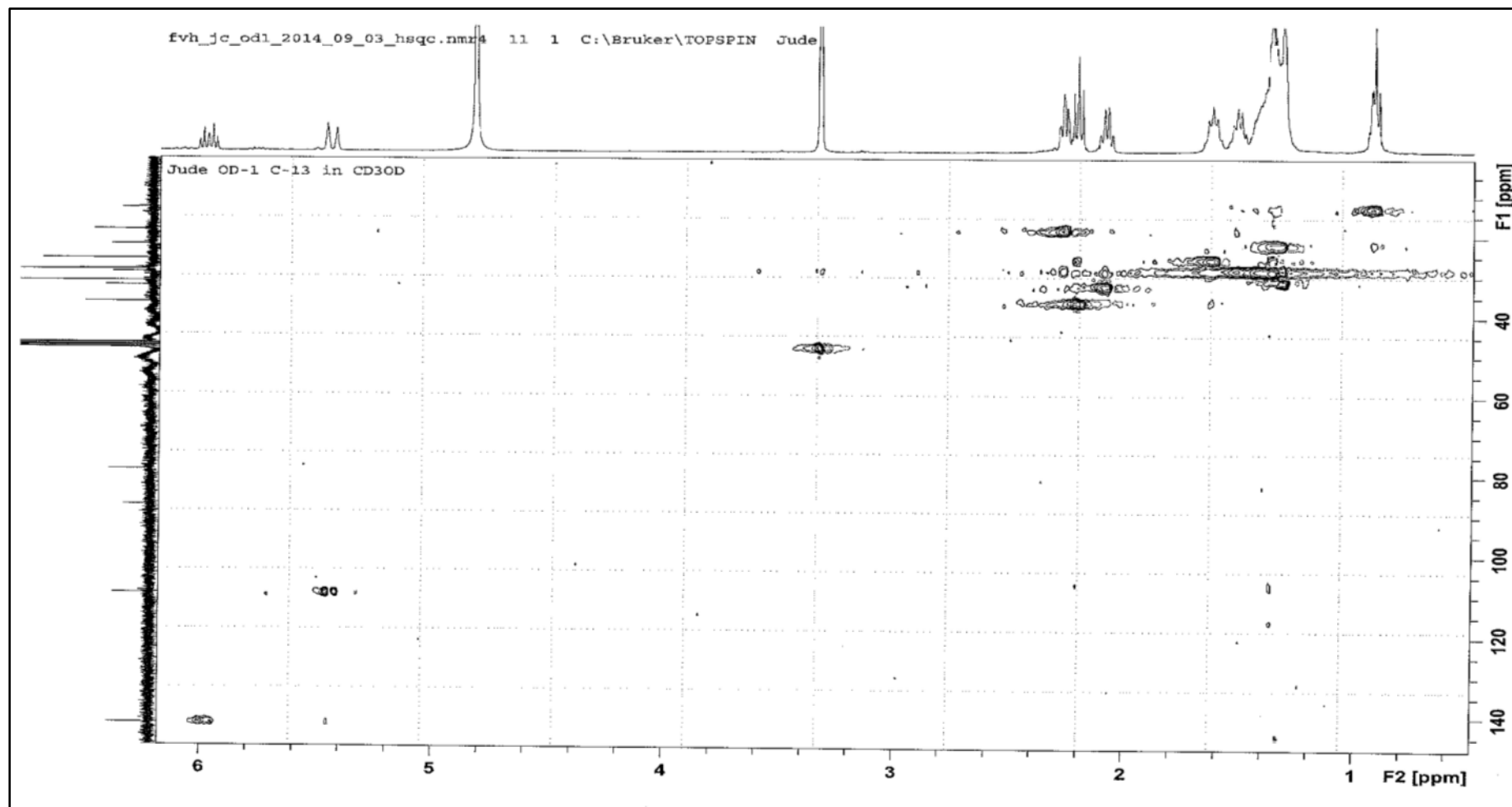


Figure 5.10: HSQC spectrum of compound D1 isolated from DCM extract of *O. dissitiflora* bark.

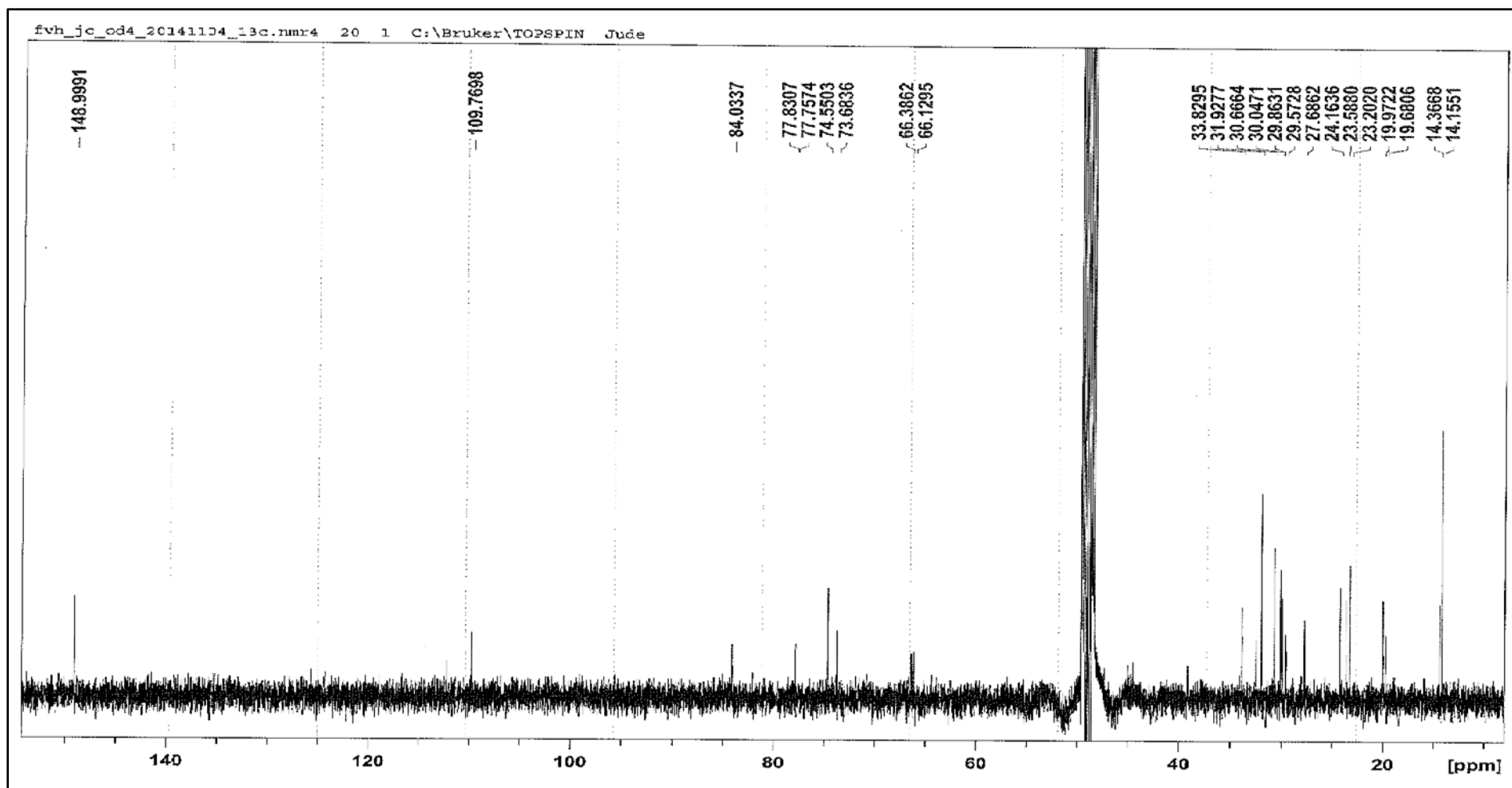


Figure 5:12: ^1H NMR spectrum of compound D2 isolated from DCM extract of *O. dissitiflora* bark.

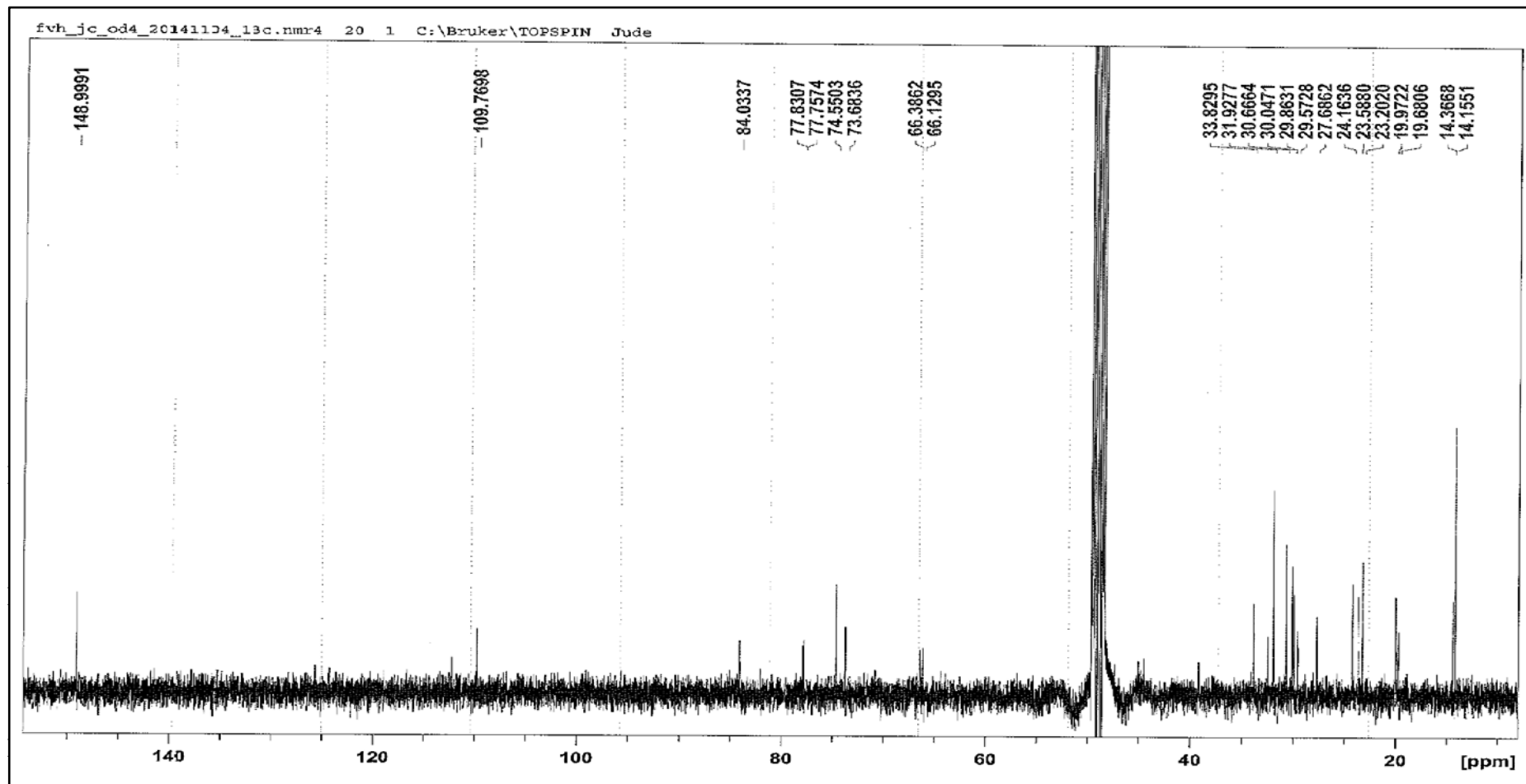


Figure 5.13: ^{13}C spectrum of compound D2 isolated from DCM extract of *O. dissitiflora* bark.

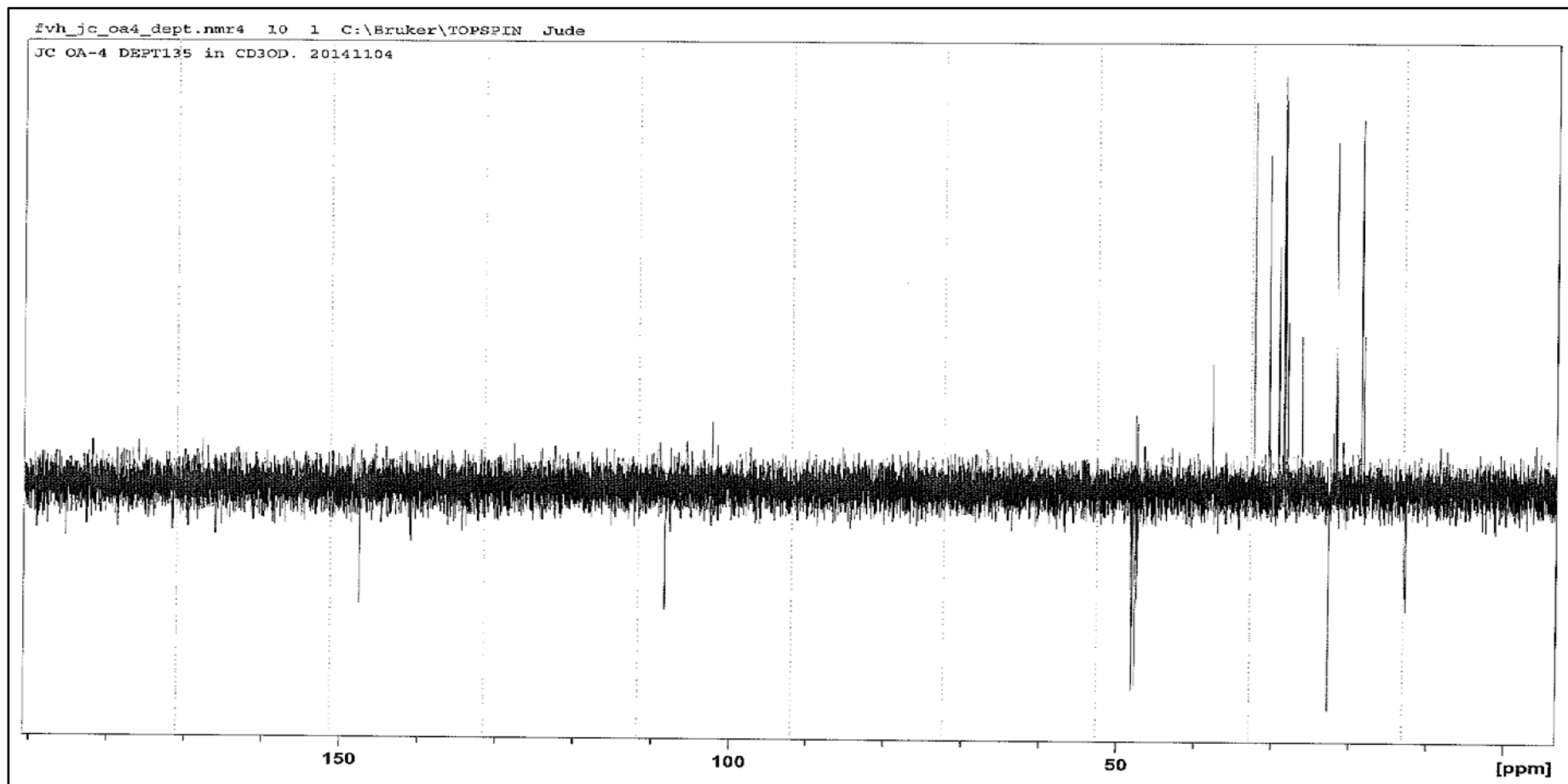


Figure 5.16: DEPT spectrum of compound D2 isolated from DCM extract of *O. dissitiflora* bark

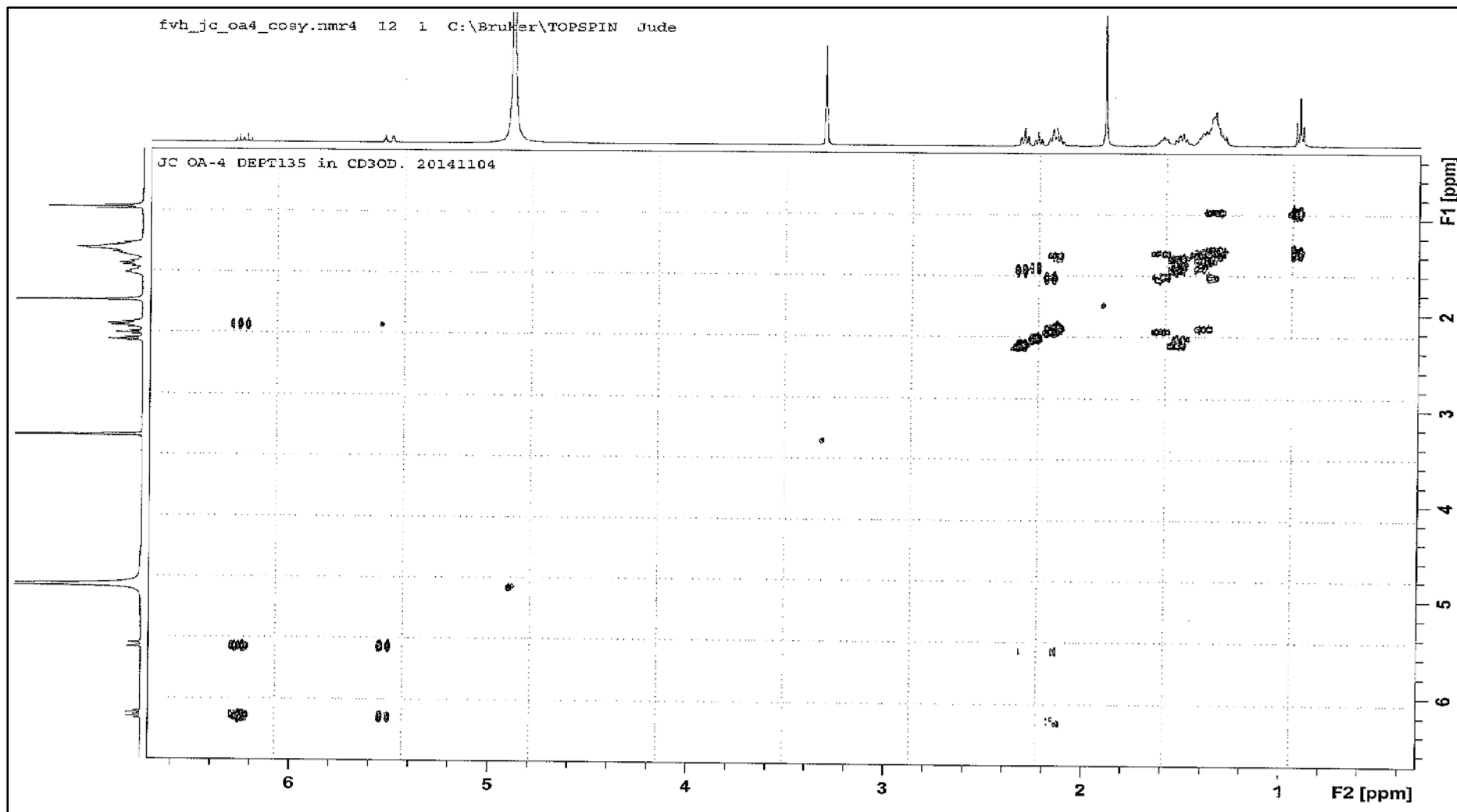


Figure 5.17: COSY spectrum of compound D2 isolated from DCM extract of *O. dissitiflora* bark.

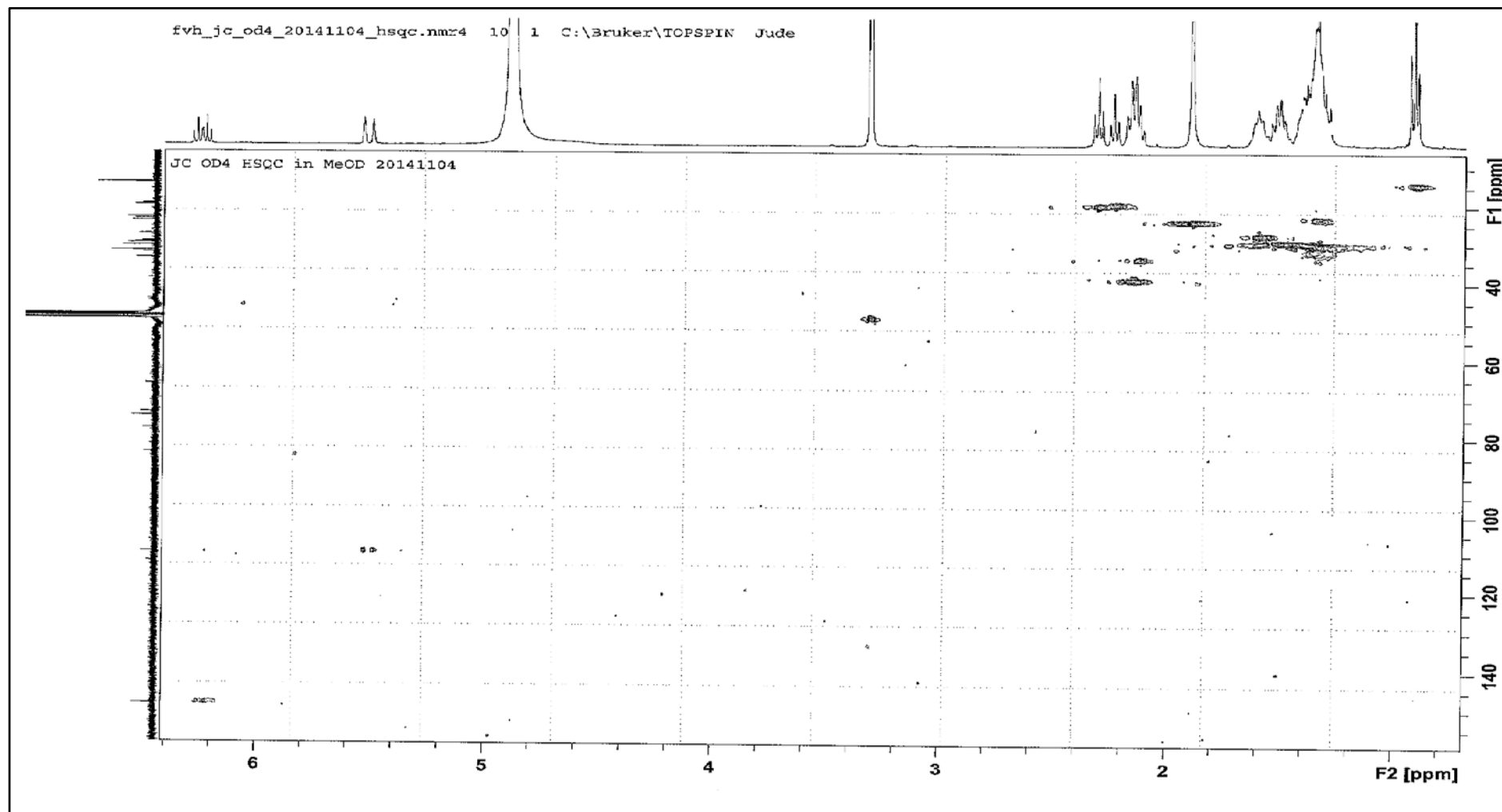


Figure 5.18: HSQC spectrum of compound D2 isolated from *O. dissitiflora* bark.

5.4. Conclusions

Bioassay-guided fractionation of the dichloromethane extract of *Olax dissitiflora* has led to the isolation of two compounds, ximeninic acid and the mixture of two closely related compounds (exocarpic acid and octadec-9,11-diynoic acid). Ximeninic acid has previously been isolated from *O. dissitiflora* (SPARAVIGNA *et al.*, 2014). Exocarpic acid and octadec-9,11-diynoic acid are isolated for first time from *O. dissitiflora*. The mixture of exocarpic acid and octadec-9,11-diynoic acid exhibited the highest larvicidal activity with an EC₅₀ value of 17.31 µg/ml compared to the crude extract which had an EC₅₀ value of 17.85 µg/ml. The larvicidal activity exhibited by ximeninic acid was lower (EC₅₀ value of 62.17 µg/ml) than that of the crude extract. In view of the activity shown by the isolated compounds, it can be concluded that *O. dissitiflora* bark contains compounds which may be used as larvicidal agents against *Anopheles arabiensis* mosquitoes. Further studies to evaluate the larvicidal activity of these compounds in field conditions are required. Studies to evaluate the toxicity effects of these compounds to non-target organisms are also needed.

CHAPTER 6

General Conclusions

Mosquitoes are a major public health concern around the world. They transmit diseases to more than 700 million people annually (TAUBES, 2000). Mosquito-borne diseases are a major cause of morbidity and mortality in the world, particularly in tropical and subtropical countries. Among these diseases, malaria is the most important, affecting 300-500 million people and killing over 1 million persons annually throughout the world (SNOW *et al.*, 2005). Because there is currently no effective vaccine available for the prevention of malaria, vector control is one of the main strategies used to control this disease. Current mosquito control strategies depend primarily on synthetic insecticides. However, widespread use of these insecticides has created many problems such as toxic effects to humans, toxicity to non-target organisms, pollution of the environment, and the development of resistance in mosquito populations. Therefore, there is an urgent need to develop new insecticides, which are effective, safe, biodegradable and target-specific. Plants may be an alternative source of mosquito control agents because they constitute a rich source of bioactive chemicals.

This study was aimed at screening the plants that are used traditionally as mosquito repellents in South Africa, for mosquitocidal activity. Ten plant species were screened for adulticidal, larvicidal and repellent activities against *Anopheles arabiensis*, a potent malaria vector in South Africa. All the extracts showed adulticidal activity. The highest activity was observed in DCM extract of *A. ferox* leaves. With regards to larvicidal screening, all the DCM extracts showed larvicidal activity, while only five EtOH extracts showed activity. The highest larvicidal activity was found in DCM extract of *O. dissitiflora* bark.

Due to its good larvicidal activity, *O. dissitiflora* was also screened for antibacterial, antifungal and antiplasmodial activities. Both DCM and EtOH extracts showed good

antibacterial activity against all four tested strains. They also showed good antifungal activity against *Candida albicans*. With regards to antiplasmodial screening, both DCM and EtOH extracts of *O. dissitiflora* bark showed a moderate activity.

Due to good larvicidal activity showed by DCM extract of *O. dissitiflora* bark, an attempt was made to isolate possible active compounds. Two compounds were isolated. The isolated compounds showed good larvicidal activity. The results of the present study showed that the bark of *O. dissitiflora* and leaves of *A. ferox* may have potential to be used as larvicides and adulticides against *An. arabiensis* mosquitoes, respectively.

However, further studies to evaluate their toxicity and effects on non-target organisms and the environment need to be conducted. This study also indicated that the bark of *O. dissitiflora* may also have potential to be used as an antibacterial, antifungal and antimalarial agent. The results of this study could also be useful in promoting research aimed at the development of new agents for mosquito control based on bioactive chemical compounds from indigenous plant sources.

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