

**Bioefficacy of Selected Indigenous Plant Products against the Malaria Vector Mosquito,
Anopheles arabiensis (Diptera: Culicidae) in Ethiopia**

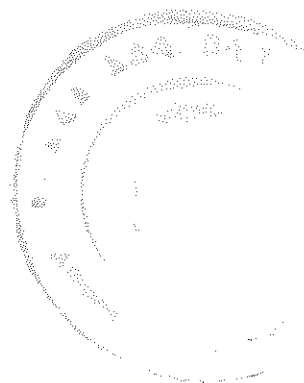
By

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(Applied Biomedical)*

**Addis Ababa University
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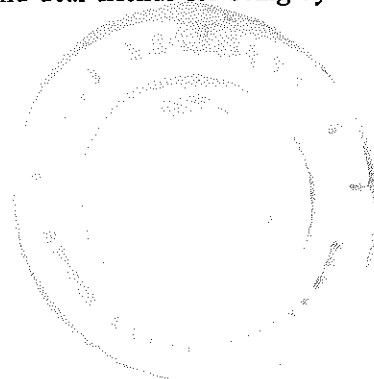
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Abstract

The efficacy of synthetic organic insecticides to control malaria vector mosquitoes is compromised by increased mosquito resistance to insecticides. Furthermore, use of inorganic insecticides raises environmental toxicity concerns. Therefore, plant-based biodegradable insecticides would be ideal alternatives for the control of malaria vector mosquitoes. This study was conducted to evaluate the mosquitocidal bioefficacy of extracts from indigenous plants used in traditional medicine in Ethiopia. The test plants were identified through literature search followed by ethnobotanical surveys based on formal and informal field interviews and discussions with local practitioners. The 80% methanol crude extracts of the plant parts were tested against *An. arabiensis* mosquito larvae and adults. The effects were determined as LC₅₀ and LC₉₀ values based on probit analysis. The larvicidal effects of *Aloe pirottae* and *Acokanthera schimperi* extracts expressed as LC₅₀ and LC₉₀ were 76.34 and 282.76 ppm and 133.39 and 407.93 ppm, respectively. Similarly, 80% methanol extracts of *Aloe pirottae* gel, *Brassica nigra* seed, *Oreosyce africana* leaf and *Piper capense* fruit tested on *Anopheles arabiensis* adults in the laboratory showed significant mortality for *O. africana* (LC₅₀ at 14.88 and LC₉₀ at 44.55 ppm) followed by *P. capense* (LC₅₀ at 25.69 and LC₉₀ at 46.32 ppm). The qualitative phytochemical analysis of *O. africana* and *P. capense* crude extracts revealed the presence of alkaloids, phenols, flavonoids, saponins, glycosides, and chromophores that are known to possess bioactive characteristics. The dichloromethane fraction of *O. africana* and ethyl acetate fraction of *P. capense* had higher adulticidal activities with LC₅₀ values of 4.27 and 10.72 ppm, respectively and were significantly different ($P < 0.05$). The chemical composition of the active purified fractions of *O. africana* were analysed by a combination of TLC, HPLC, NMR and GC-MS methods. Upon purification, the *O. africana* fractions had the most potent adulticidally active

components among the fractions tested. These included the fractions designated as B2'O (LC₅₀ of 2.21 and LC₉₀ of 7.81 ppm) and B2''O (LC₅₀ of 2.62 and LC₉₀ of 11.78 ppm) at 24 hr post-exposure and concentrations were significantly different (P<0.05). Mosquitocidal potency of the impregnated nets lasted for two months. The structural elucidation of the active ingredients in the B2'O fraction was determined using a combination of ¹H- ¹³C-NMR, DEPT-135 and GC-MS measurements. This revealed the presence of linoleic acid (9, 12-Octadecadienoic acid (Z,Z)- (280 m/z) as the major chemical constituent (98.35%) and similar analysis of B2''O fraction showed the presence of dibutyl phthalate (278 m/z) as the major chemical constituent (97.75%). Both compounds have proven insecticidal effects. This study has shown a high potential of *O. africana* leaf extract as an adult mosquito control agent that could be developed into an indigenous malaria control alternative to the expensive and increasingly less effective insecticides in use. The larvicidal potentials of *A. schimperi* and *A. pirottae* that became implicit in this study need further investigation to determine their levels of potency.

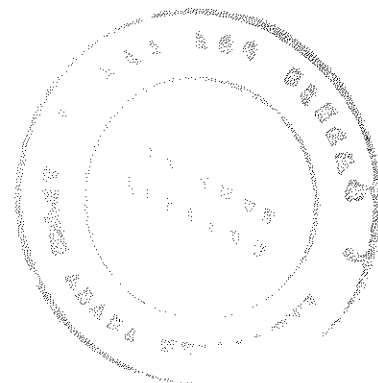
Key words: *Acokanthera schimperi*, *Aloe pirottae*, *Oreosyce africana*, *Piper capense*, *Anopheles arabiensis*, malaria, crude extracts, fractions, larvicidal activity, adulticidal activity, phytochemicals, Ethiopia

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Abbreviations and Acronyms

ANOVA	Analysis of variance
CC	Column chromatography
CDCl ₃	Deuterated chloroform
CHCl ₃	Chloroform
¹³ C-NMR	¹³ C-Nuclear magnetic resonance
Da	molecular mass (dalton)
DCM	Dichloromethane
DOF	Dichloromethane <i>Oreosyce</i> Frction
DPF	Dichloromethane <i>Piper</i> Fraction
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethyl sulfoxide
eV	Electronvolt
EPHI	Ethiopian Public Health Institute
EtOAc	Ethyl acetate
EOF	Ethyl acetate <i>Oreocyse</i> Fraction
EPF	Ethyl acetate <i>Piper</i> Fraction
FMOH	Federal Ministry of Health
F ₂₅₄	Florescence at 254 nm
GC-MS	Gas chromatography-mass spectrometry
H	Proton
HPLC	High performance liquid chromatography
¹ H-NMR	Proton nuclear magnetic resonance
Hz	Hertz
ITN	Insecticide treated nets
LC ₅₀	50% lethal concentration
LC ₉₀	90% lethal concentration
LCL	Lower confidence limit

Abbreviations (continued)

MeOH	Methanol
MS	Mass spectrometry
m/z	mass-to-charge ratio
nm	Nanometer
NMR	Nuclear magnetic resonance
ODS	Octadecylsilane
ppm	parts per million
PTLC	Preparative thin-layer chromatography
RH	Relative humidity
R _f	Retention factor
R _t	Retention time
SD	Standard deviation
SE	Standard error
TLC	Thin-layer chromatography
UCL	Upper confidence limit
UV	Ultraviolet
WHO	World Health Organization
WOF	Water <i>Oreosyce</i> Fraction
WPF	Water <i>Piper</i> Fraction

1. Introduction

Malaria is a disease with wide global prevalence and is caused by protozoan parasites of the genus *Plasmodium*. It is an insect vector transmitted disease with several mosquito species of the genus *Anopheles* involved. World Health Organization (WHO, 2015) estimated about 3.2 billion people to be at risk of malaria globally. Out of these, 89% of the cases and 91% of the deaths occurred in sub-Saharan Africa while the remaining cases and deaths occurred mainly in South-East Asian and Eastern Mediterranean Regions. Thirty-five countries are responsible for the majority of malaria deaths worldwide, of which the five main contributors, Nigeria, Democratic Republic of Congo, Uganda, Ethiopia, and Tanzania accounted for 50% of global deaths and 47% of malaria cases (WHO, 2010). Thus, in spite of available effective preventive measures and case management therapy for a very long time, malaria remains a global public health problem as a leading cause of morbidity and mortality in many tropical countries. Because of its widespread prevalence and disease severity, malaria is the most serious parasitic disease of humankind.

Malaria has a long history in Ethiopia; and the country had experienced the worst malaria epidemics in 1958 with about three million cases and 150,000 deaths (Fontaine *et al.*, 1961). The intensity and distribution of malaria in Ethiopia was documented by studies conducted during the period of Italian invasion 1935-1940 (FMOH, 2003) and the disease continues to be the major health problem in the country. According to Solomon (2013), an estimated 55.7 million people (68% of the population) are at risk of malaria. The malaria transmission pattern by and large is seasonal and unstable and its intensity varies due to differences in altitude, rainfall and population movement. Protective immunity in the population is relatively low due to unstable

transmission and, unlike large parts of sub-Saharan Africa all age groups are at risk of infection and disease.

Parasites that cause malaria belong to protozoa in the Phylum Apicomplexa, Class Aconoidasida, Order Haemosporidia, Family Plasmodiidae, and genus *Plasmodium*. Five species of the genus *Plasmodium* are infectious to humans with *Plasmodium falciparum* Welch, *Plasmodium vivax* Grassi and Feletti, *Plasmodium malariae* Grassi and Feletti, and *Plasmodium ovale* Stephens making up the major human parasites, whereas *Plasmodium cynomolgi* Ceylon is a zoonosis (Loban and Polozok, 1985). According to Collins and Paskewitz (1995), *P. malariae* and *P. ovale* infections cause little morbidity and almost no mortality, but *P. vivax* infections are more severe and debilitating but are usually self-limiting in healthy individuals. Among the malaria parasites, *P. falciparum* is responsible for the vast majority of human deaths. In Ethiopia, the two epidemiologically important species are *P. falciparum* and *P. vivax* (Solomon, 2013).

The life cycle of *Plasmodium* involves several stages both in human host and in mosquito vector (NIAID, 2016). Accordingly, the sporozoite stages of the parasites that first enter human bloodstream infect the liver cells. Upon replication in the liver, merozoites release into the bloodstream and rapidly adhere to and invade erythrocytes. Ultimately the infected erythrocytes lyse and release merozoites into the bloodstream and invade more red blood cells. Some merozoites develop into gametocytes that infect the anopheline mosquitoes during biting and undergo the sexual stage of development. In the mosquito gamete formation and fertilization take place producing ookinetes, which develop into sporozoites. When the mosquito takes next blood meal it can again infect a human host.

Parasite drug resistance particularly due to *P. falciparum* is a major impediment to malaria control in the country. Mengesha *et al.* (1998) reported the possible importation of drug resistant *P. falciparum* strains from their endemic foci in the lowlands to the upland fringe malarious zones, which caused the 1991 malaria epidemics in north-central Ethiopia.

Mosquitoes that transmit malaria parasites belong to the phylum Arthropoda, Class Insecta, Order Diptera, Family Culicidae, and genus *Anopheles*. In Ethiopia, *Anopheles arabiensis* Patton is the primary vector responsible for malaria transmission (White *et al.*, 1980; Hadis *et al.*, 1997) whereas *An. pharoensis* Theobald, *An. funestus* Giles and *An. nili* Theobald are regarded as secondary vectors (Abose *et al.*, 1998).

The life cycle of *Anopheles* mosquito begins as eggs which are laid singly either on water or on a damp surface. *Anopheles* eggs are usually boat shaped with a flattened upper surface. The eggs hatch into larvae and pass through four stages of growth. Mosquito life cycle has a complete metamorphosis, which starts as an egg, hatches into a larva, becomes a pupa and ends up as an adult (Appendix 1). The life cycle of the *Anopheles* mosquito is, therefore, mainly affected by environmental factors, with shorter duration from the egg to the adult at higher temperature; estimated between 7 days at 31°C and 20 days at 20°C. The survival of adult female *Anopheles* is also highly dependent on temperature and humidity of the environment. Under favourable climatic conditions, average temperature below 35°C and humidity over 50%, the average longevity of female *Anopheles* mosquitoes can extend between 2 to 3 weeks (Service, 1993).

Although *Anopheles arabiensis* mainly breeds in small, temporary, and sun-lit water collections such as rain pools, it can also breed in a wide variety of other types of water bodies (Abose *et al.*, 1998). It is usually an indoor-feeding and resting species, which also has an exophagic feeding

habit (Abose *et al.*, 1998), although an incipient tendency to feed indoors and rest elsewhere has been reported (Ameneshewa and Service, 1996) and the highest proportion of this malaria vector fed on human blood were reported from human dwellings (Hadis *et al.*, 1997). Furthermore, it is generally considered to be an anthropophilic as long as the host is available (Adugna and Petros, 1996). Woyessa *et al.* (2004) also reported that populations of *An. arabiensis* are anthropophilic.

According to WHO (2013), the approach to combat malaria relies on diagnostic testing of all suspected malaria and prompt treatment of confirmed cases with effective artemisinin-based combination therapy, intermittent preventive treatment during pregnancy, intermittent preventive treatment in infancy and children and interruption of the disease transmission cycle by targeting the adult mosquitoes using indoor residual spraying (IRS) and long lasting insecticidal nets (LLINs), and by killing mosquito larvae through treatment of stagnant waters with larvicides, as well as through environmental modification to prevent mosquito breeding. The intensification of malaria control efforts includes the delivery of a package of vector control interventions aimed at controlling transmission by promoting integrated vector management principles as set out in the global strategic framework (WHO, 2004).

Vector control measures targeting the larval and adult stages have been established to control the transmission of malaria (FMOH, 2004). Among these, larviciding mosquito control involves the killing of immature stages of mosquitoes by application of various methods including water management, using insecticides and biological control agents to the breeding sites (WHO, 2006a). These control methods are used to stop mosquito larvae from maturing into biting adults that can transmit the disease. For example, the efficacy of a bacterial biological control agent, *Bacillus thuringiensis israelensis* (Bti) and *Bacillus sphaericus* (Bs) against *An. arabiensis* larvae

were evaluated by Seyoum and Abate (1997) in Ethiopia. The LC_{50} value of 1.0 and 1.8 $\mu\text{g/l}$ against the second and third instars larvae, respectively were shown for Bti. However, larval control is realistic only in restricted areas, where the breeding sites are well defined (WHO, 1993). Thus, in most rural areas of Ethiopia where breeding sites are everywhere, larval control measures have short-lived effect, and to be effective, it requires frequent applications (FMOH, 2012). In general, larviciding is effective in localized well accessible breeding sites and may be applied in conjunction with other methods.

Control measures directed against the adult vectors have a more broad applicability than measures directed towards the vector's early stages of development. It requires knowledge of the vector's behaviour and choice of their breeding habitats (WHO, 2006d). One method is that the insecticides impregnated in the net knockdown and often kill mosquitoes that contact the net. If the insecticide treated nets are used by a whole community, the insecticidal impact results in reduced longevity of *Anopheles* mosquitoes so that few survive for the time required for development of the malaria parasite to the sporozoite stage. This reduces malaria transmission (Curtis *et al.*, 1998; 2003).

In sub-Saharan Africa, most transmission of malaria is caused by *Anopheles arabiensis* and *Anopheles funestus* (Collins and Paskewitz, 1995) and the current vector control technique involves the use of residual insecticides which are sprayed on the walls and roofs of houses. This method allows for a lethal dose of an insecticide to adhere to the mosquito once it has rested on a sprayed surface (Walker, 2002). Indoor residual spraying (IRS) is widely used in the control of vectors of malaria by reducing their numbers and has been playing a great role for interrupting malaria transmission (WHO, 2006c). Among insecticides recommended by World Health

Organization, dichloro-diphenyl-trichloroethane (DDT), an organochloride, has the longest residual efficacy for 6 months (WHO, 2006c). However, despite its effectiveness in reducing malaria, the use of DDT has resulted in many problems that cause adverse effects on the environment, human health, and non-target organisms. In addition, the development of resistance to DDT by the mosquito populations in many areas has been a serious limitation to its use (Van den Berg, 2009).

Historically, the use of synthetic insecticides has been very effective in reducing malaria transmission. According to D'Alessandro *et al.* (1995), the community-randomized controlled trials conducted across a range of malaria transmission settings in sub-Saharan Africa have shown ITNs to be associated with up to 30% reduction in all-cause child mortality over the first 1 to 2 years of trials. However, over time, success has been hampered by mosquito resistance to synthetic insecticides and this challenged practical mosquito control around the world (Brown, 1986; Rozendaal, 1997; Gill and Garg, 2014). In addition to resistance to synthetic insecticides in mosquitoes that resulted in the environment with high degree toxicity to non-target organisms has been a major short coming (Liu *et al.*, 2006).

The challenges of malaria control include the complexity of disease control process, the complexity of the vectors and expensive cost of the control program and variations in disease patterns and in the transmission dynamics from place to place. In addition, there is resistance of the parasite to drugs and the increase and spread of insecticide resistance, higher cost of newer insecticides as well as higher costs of spraying operations, high degree of replastering rate of the sprayed houses, and expanded urbanization which lead to the deterioration of vector control operations in Ethiopia (FMOH, 2004).

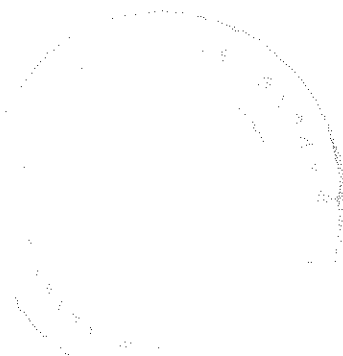
The control of malaria in Ethiopia has a history of more than five decades. Use of chemical insecticides has been the principal method of vector control against the immature as well as the adult stages of mosquitoes since early 1960s. However, the increase and spread of insecticide resistance and higher cost of newer insecticides has necessitated the utilization of all appropriate technological and management techniques in an integrated approach to bring about an effective degree of vector suppression in a cost effective manner (FMOH, 2012). In Ethiopia, malaria control heavily relies on adulticidal vector control interventions including regular deployment of ITNs and IRS. However, their effectiveness has been largely undermined due to the emergence and widespread insecticide resistance to the most commonly used insecticides like DDT and pyrethroids (Karunamoorthi, 2011). As a result, efforts to control *Anopheles* mosquitoes had limited success, although the use of insecticide impregnated bed nets does appear to reduce malaria-related death rates (Yewhalaw *et al.*, 2011).

According to studies conducted in Ethiopia (Abose *et al.*, 1998; Balkew *et al.*, 2003; Yewhalaw *et al.*, 2010; Abate and Hadis, 2011; Yewhalaw *et al.*, 2011), *An. arabiensis* was resistant to an array of insecticides, including dichloro-diphenyl-trichloroethane (DDT), permethrin, deltamethrin and malathion. To counter such widespread vector resistance to synthetic insecticides use of natural products as mosquitocides has been recommended as a useful tool to promote localized control of persistent vector-borne diseases (Hardin and Jackson, 2009). In this regard there is a need for an urgent development of alternative botanical insecticides, that are safe for non-target organisms and do not pose any residue problems to the environment, but are able to suppress pest and vector populations.

The insecticidal plants have been in use in various forms to control mosquitoes. For example, since from the ancient years the plants were being used for the control of mosquitoes in many ways like keeping the plants near window and burning the plant parts to enhance the repellent action of smoke. The use of traditional repellents is widespread among the different cultures and communities of Africa and beyond (Seyoum *et al.*, 2002; Karunamoorthi *et al.*, 2008) as supplementary and complimentary measures for malaria control. This will reduce the chemical burden on the environment.

Other types of applications are spraying the extracts by crushing and grinding the plant parts, hanging and sprinkling the plant leaves on the floor. The basis for the insecticidal effects of the plants are the secondary plant products that are known to be less harmful to non-target organisms and are preferred due to their innate biodegradability (Prabakar and Jebanesan, 2004; Rahuman *et al.*, 2008). For quite sometime the use of indigenous plants has increasingly become a major subject of research as they contain an array of bioactive chemical compounds, which would be used to kill or repel mosquitoes at various life cycle stages (Ahmed *et al.*, 1984; Karunamoorthi *et al.*, 2008). Furthermore, phytochemicals contained in specific plants may act as insecticides/mosquitocides against both the larvae and adult stages of mosquitoes. The mechanism of action of the phytochemicals has been suggested to be growth inhibitors that prevent mosquito larval/adult development (Sivagnaname and Kalyanasundaram, 2004).

Some isolated compounds from plants have been used as insecticides in some countries such as pyrethrum produced from *Chrysanthemum cinerariifolium* (Asteraceae), rotenone from *Derris elliptica* (Fabaceae), azadirachtin from *Azadirachta indica* (Meliaceae), and nicotine from *Nicotiana tabacum* (Solanaceae) (Wood, 2003). According to Abebe *et al.* (2003), pyrethrum,



which is obtained from the dried flower heads of *Chrysanthemum cinerariaefolium* Visiana, is a contact poison in insects which is used largely in the form of powder, but sprays after dissolving the powder in kerosene or other organic solvents are more efficient. Although insecticides of plant origin have been extensively used on agricultural pests, their use against insect vectors of public health importance has been very limited (Das *et al.*, 2007).

Although several compounds of plant origin have been reported as adulticides and larvicides, there is still a wide scope for the discovery of more effective plant products (Saxena and Yadav, 1986) particularly in the indigenous flora of less studied countries like Ethiopia. One of the purposes of identifying plant-derived insecticides is to obtain insight for development of novel synthetic insecticides (Saxena, 1987). Although many crude or refined products have been found to have antimosquito properties only a few have moved to the forefront in malaria vector control (Rozendaal, 1997).

The alternative control measure is screening of locally available indigenous ethnomedicinal plants as mosquito larvicidal and adulticidal agents that would eventually lead to their usage in plant-based mosquito abatement practices (Karunamoorthi and Ilango, 2010). Although the potency of some extracts is less than chemical insecticides, they are safer than the latter (Mann and Kaufman, 2012). This is because they are easily degradable (Alkofahi *et al.*, 1989) and minimize the accumulation of harmful residues in the environment.

Among the Families of plants investigated for their mosquito control potentials are members of Family Piperaceae (Dodson *et al.*, 2000). The chemicals derived from these plants have been projected to function as repellents; oviposition deterrents, by preventing mosquitoes from egg laying; or as growth and reproductive inhibitors, by interfering with the life cycle of the

mosquitoes (Sukumar *et al.*, 1991). Studies carried out so far have shown that some phytochemicals act as general toxicants (insecticide/larvicide) both against adult as well as larval stages of mosquitoes, while others interfere with growth and development (growth inhibitors) or with reproduction (chemosterilant) or produce olfactory stimuli, thus acting as repellents or attractants (Mathur, 2003).

Martin (1995) described that plant species of the same or closely related genera would contain the same or similar bioactive agents. Therefore, piperamides singly, or more importantly in combination, could replace contact insecticides, specifically neurotoxic compounds such as carbamates, organophosphates and pyrethroids, for which resistance has developed. Combination of these amides within a botanical formulation could provide the advantage of attributes such as novel target site, enzyme inhibition and low mammalian toxicity (Scott *et al.*, 2008). In addition, Scott *et al.* (2008) reported that some secondary metabolites from *Piper* species has been described as insecticidal compounds, which can be used as novel insecticides or which are used in traditional control of different insects (vectors of diseases and that damage storage crops).

There are several studies on the larvicidal effects of plant products. Among these, the extracts of *Oligochaeta ramosa* and *Delonix regia* were shown to suppress the population of *Cx. quinquefasciatus* as reported by Saxena and Yadav (1982). Kim *et al.* (2002) studied the larvicidal activity of methanol extracts of 22 Australian and 12 Mexican plants against early fourth instar larvae of *Aedes aegypti* and *Culex pipiens pallens* at 200 ppm, 100 percent mortality on the larvae of *Ae. aegypti* and *Cx. pipiens* was obtained with extracts of *Kigelia pinnata* and *Ruta chalepensis*. Wiesman and Chapagain (2006) reported that chloroform, ethyl acetate, butanol and methanol extracts of fruit mesocarp of *Balanites aegyptiaca* tested against *Ae.*

aegypti mosquito larvae showed larvicidal effects, with the highest larval mortality found with methanol extract. Gianotti and co workers (Gianotti *et al.*, 2008) used powdered seeds of neem trees and applied twice a week to known breeding sites for *An. gambiae* at the rate of 10 gm/m² of pool surface area for effective larval control.

Studies conducted in Ethiopia using essential oils from a local plant, *Chenopodium ambrosioides* L. oil caused high larvicidal activities against *An. arabiensis* and *Ae. aegypti* larvae with an LC₅₀ of 17.5 and 9.1 ppm, respectively (Massebo *et al.*, 2009). Similarly *Ocimum lamiifolium* Hochst oil had an LC₅₀ value of 20.9 and 8.6 ppm against *An. arabiensis* and *Ae. aegypti* larvae, respectively (Massebo *et al.*, 2009). Furthermore, studies on essential oils from *Eucalyptus maculata citrodion*, *Ruta chalapensis*, *Chrysanthemum cinerariaefolium* and *Azadirachta indica* had shown repellent potencies against *Mansonia* species (Hadis *et al.*, 2003).

In a study by Tomass *et al.* (2011), the methanol crude leaf extracts and column chromatographic fractions of *Jatropha curcas* were shown to be toxic to *An. arabiensis* larvae. They reported that the crude extract produced 100% larval mortality at 500 ppm and with fractions obtained from column chromatography, there was an improvement on its potency (99.56% larval mortality at 125 ppm). Matasyoh *et al.* (2008) observed the effect of *Aloe fibrosa* extracts used as larvicides for mosquito control in Kenya, and they demonstrated that the extracts caused mortalities of 61.7 to 78.4%. Nathan *et al.* (2005) had reported that azadirachtin at 1 ppm has 100% larval mortality, and significantly decreases fecundity and longevity of adult *An. stephensi*.

Furthermore, Mullai and Jebanesan (2007) also reported that the methanol leaf extracts of *Citrullus colocynthis* (L.) Schrad. and *Cucurbita maxima* Duchesne ex Lam. exhibited larvicidal activity against *Cx. quinquefasciatus* with an LC₅₀ values of 118.74 and 171.64 ppm. In another

study Mullai *et al.* (2008) showed the benzene crude extract of *Citrullus vulgaris* leaves (Cucurbitaceae) to have an LC₅₀ value of 18.56 and LC₉₀ value of 39.08 ppm against *An. stephensi* larvac. In another study by Hadjiakhoondia *et al.* (2005), the bioassay tests of the chloroform extract of *Tagetes minuta* L., showed higher larvicidal activity against *An. stephensi* at the 2.5 ppm than the other solvent extracts and they suggested that this larvicidal activity might be because of its terpenoids.

The adult mortality was found in methanol extract of *Artemisia nilagirica*, with the LC₅₀ and LC₉₀ values of 205.78 and 459.51 ppm for *An. stephensi*, and 242.52 and 523.73 ppm for *Ae. aegypti*, respectively (Panneerselvam *et al.*, 2012). Govindarajan and Sivakumar (2012) also reported that adult mortality was found in methanol extract of *Andrographis paniculata* against the adults of *Cx. quinquefasciatus* and *Ae. aegypti* with the LC₅₀ and LC₉₀ values of 149.81, 172.37 ppm and 288.12, 321.01 ppm, respectively.

The plants used to control insects contained insecticidal phytochemicals that are predominantly secondary compounds produced by plants to protect themselves against herbivorous insects (Shaalaa *et al.*, 2005). Accordingly, the presence of phytosterols, flavonoids, glycosides, triterpenoids, alkaloids, saponins, tannins and steroids was shown in plants use to control insects.

According to the report by WHO (2002), more than 80% of the population in developing countries relies on traditional medicine, and it is now widely accepted that traditional medicines are more affordable, less toxic, and have a wide acceptance around the world. In Ethiopia, like other developing countries, medicinal plants have been used as remedies for many years and still are used for the treatment of different ailments. In Ethiopia about 1000 higher plant species are

known to be employed in the traditional healthcare; and that 60% of them are indigenous and the same proportion is believed to have healing potentials (Mesfin *et al.*, 2005).

Utilizing indigenous knowledge concerning plants with traditional medicinal value has proven fruitful in identifying potential sources of phyto-extracts with insecticidal activity (Rahuman *et al.*, 2009). Although the application of plant derivatives as larvicides and adulticides to malaria vector control is not common, some reports show that many plant species are traditionally used as repellents and insecticides (Abebe *et al.*, 2003; Berhanu *et al.*, 2006). It is plausible to assume that it is the phytochemicals contained in such plants that act as insecticides against both the larvae and adult stages of mosquitoes.

The most widely used mosquito repellent plant reported in Ethiopia is *Allium sativum* L. followed by *Lepidium sativum* L. and *Capparis tomentosa* Lam. (Abebe *et al.*, 2003; Berhanu *et al.*, 2006). Berhanu *et al.* (2006) also reported that insecticides were made locally to kill insects by spraying all over the walls of the house. These were obtained from *Cyphostema adenanthum*, *Calpurnia aurea*, *Capparis tomentosa*, *Jasminum abyssinicum*, *Momordica foetida* and *Melia azedarach* in their decreasing order. Ethnobotanical surveys by Hunde *et al.* (2004) also showed that *Cryptostegia grandiflora* has killing effects against insects.

Karunamoorthi *et al.* (2009) also reported that the plant species including *Olea europaea* ssp., *Ostostegia intergrifolia*, *Azadirachta indica*, *Silene macrosalen* and *Echinops* spp. are used as mosquito repellents in Addis Zemen Town, north-west Ethiopia. Another study by Bekele *et al.* (2012) showed that people in Akaki District (east-central Ethiopia) used *O. africana*, *B. nigra* and *Aloe* spp. traditionally for mosquito control and for the control of cattle ticks and other arthropod pests.

As part of continued search some plant products available in Ethiopia have been assayed for their larvicidal and adulticidal activities against *An. arabiensis* in combating malaria vectors. As far as literature search could ascertain, no information is available on the larvicidal and adulticidal activities of the experimental plant species given here against *An. arabiensis*.

2. Statement of the Problem

There is currently a scarcity of viable insecticides (larvicides and/or adulticides) to control mosquitoes. There is emergence of resistance to synthetic chemical insecticides which has led to an increase in mosquito population and hence increases in the spread of mosquito-borne diseases like malaria. In addition, there are inadequate products derived from plant products targeting the larval and adult stages of mosquitoes in an effective way. The results of the present study would be useful in closing on the gap of developing new botanicals from indigenous Ethiopian plant source for possible insecticides.

Research hypothesis

Investigation of bioactive compounds from indigenous plant species selected through ethnobotanically guided surveys will lead to detecting plant products with mosquitocidal effects with a potential for malaria vector control in Ethiopia.

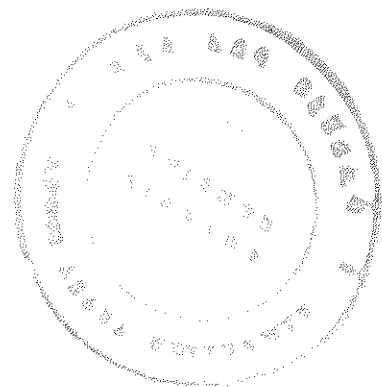
Objectives of the Study

General objective

To investigate bioefficacy of extracts from some traditionally used indigenous plant species for their mosquitocidal activities against *Anopheles arabiensis* Patton and to determine the bioactive chemical components in the plant species for malaria vector control.

Specific objectives:

- To evaluate the larvicidal and adulticidal activities of extracts from traditionally used indigenous plant species against *An. arabiensis* under laboratory conditions;
- To determine the knockdown effects of the fractionated extracts from the plant species against *An. arabiensis* adults under laboratory conditions;
- To isolate, characterize and elucidate the structure of the constituent chemical compound(s) from bioactive plant extracts with adulticidal effects on *An. arabiensis* mosquitoes; and
- To determine the bioefficacy and persistence of bioactive fraction-impregnated nets against *An. arabiensis* under laboratory condition using the WHO standard procedure.



3. Materials and Methods

3.1. The Candidate Test Plants

The plant species which were screened for mosquitocidal properties were collected following two approaches, namely the ethnotaxonomic approach of searching targeted plant families from the literature and the ethnobotanical search in the manner discussed by Martin (1995). Accordingly, candidate plant species were compiled both from initial literature search following the ethnotaxonomic approach followed by field searching in a series of ethnobotanical survey in Akaki, Babile, Gindeberet, and Qarsa Districts of Oromia Region. During these field trips both voucher specimens of the candidate plants and ethnobotanical descriptions were made by repeatedly conducting unstructured and semi-structured interviews with the local people who used the plants for many years. The distribution of plant species were collected from flora of Ethiopia. Therefore, information on the antimosquito potency of targeted plants was based on ethnobotanical study, existing literature and interview to local community members during the preliminary study. As far as the literature search could ascertain, no information was available on larvicidal and adulticidal activities of the selected plant species given here.

A variety of approaches in the selection of candidate plants with potential as vector control agents exist and range from ethnobotanical studies (Pålsson and Jaenson, 1999; Seyoum *et al.*, 2002) to chemotaxonomic studies in which closely related species of plants with known activities are selected and subjected to experimentation. It is generally acknowledged that sampling based on ethnobotanical approach gives a greater percentage yield of bioactive compounds over the other methods (Kihampa, 2011). Accordingly, five different plant species namely *Acokanthera schimperi*, *Aloe pirottae*, *Brassica nigra*, *Oreosyce africana*, and *Piper*

capense were selected based on traditional use of local plants as insecticides from different localities of Oromia, and taxonomic relationships to known insecticidal plants from literature information. *Acokanthera schimperi* and *Aloe pirottae* were assigned for testing against larvae of *An. arabiensis*, and *A. pirottae*, *B. nigra*, *O. africana* and *P. capense* were assigned for the bioassay test against *An. arabiensis* adults based on literature search and ethnobotanical survey. The larvicidal and adulticidal effect of the selected plants against larvae/adults of *An. arabiensis* have not been reported so far.

3.2. Collection and Identification of Plant Materials

The leaves of *O. africana* Hook.f (Family: Cucurbitaceae) were collected from Yerer Lencho locality of Akaki District (08050.682' N, 038056.630' E); leaves of *A. pirottae* Berger (Aloaceae) were collected from Iftu locality of Babile District (09012.494' N, 042015.228' E); leaves of *A. schimperi* (A.DC.) Schweinf. (Apocynaceae) were collected from Tapisa Medale locality of Gindeberet District (09045.458' N, 037045.687' E); fruits of *P. capense* L.f. (Piperaceae) were collected from Bada Buna locality of Qarsa District (07039.730' N, 036053.213' E); and seeds of *B. nigra* L. Koch (Brassicaceae) were collected from Yerer Lencho locality of Akaki District (08047.144' N, 038053.712' E) of Oromia Region marked on the map (Figure 1). Plant materials (voucher specimens and materials for extraction and testing) were collected during field trips at the sites (Appendix 2). Basic ethnobotanical data focusing on the medicinal uses of the target species were collected by interviewing local traditional herbalists and knowledgeable elders. Ethnobotanical data were collected after explaining the purpose of the research and obtaining their consents provided as blessings by those who volunteered to provide information, typical traditional ethical clearance. Voucher specimens of the species were collected, pressed, dried and authenticity confirmed by taxonomic experts at the Department of Plant Biology and

Biodiversity Management and stored at the National Herbarium of Ethiopia, Addis Ababa University for further reference.

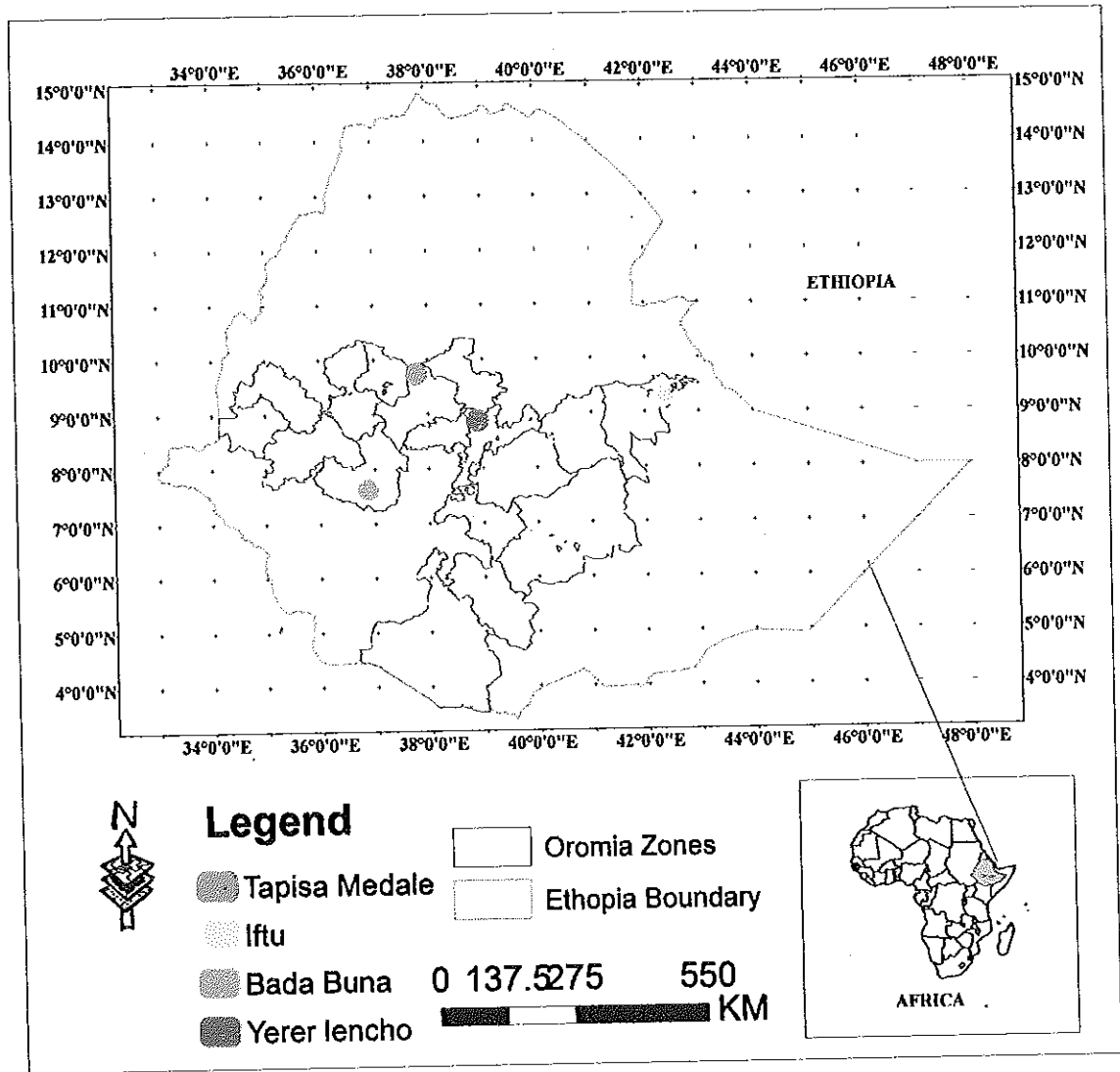


Figure 1. Map of Ethiopia showing areas of plant material collection.

3.2.1. *Acokanthera schimperi* (Appocynaceae)

Acokanthera schimperi (A. DC.) Schweinf. (Appocynaceae) is a shrub or tree of 1-9 m tall and located within woodland and degraded *Podocarpus-Juniperus* forest at altitudes of 800-2100 m asl (Leeuwenberg, 2003). *Acokanthera schimperi* is used to repel insects by smoking (traditional healer, personal communication at Tapisa Medale locality, Gindeberet District, Ethiopia).

3.2.2. *Aloe pirottae* (Aloaceae)

Aloe pirottae Berger (Aloaceae) grows in the drier parts of the country in open *Acacia* woodland, sometimes on dark soil between 1300 and 1820 m asl (Demissew and Gilbert, 1997). *Aloe pirottae* is a member of the group of spotted aloes with a tough skin on the leaves and located in Gamo Gofa, Sidama, Bale, and Harerge floristic regions. Tadesse and Mesfin (2010) reported that among the genera they selected for their study, *Aloe* sp. was hardy, abundant and it grows sufficiently easily and can be sustainably utilized. The mucilaginous fluid from *Aloe* is applied to cuts and wounds to prevent infections and bring about healing and hence this observation is consistent with its traditional application in rural Ethiopia (Tadesse and Mesfin, 2010). The plant extract from *A. pirottae* is reported for its current use to treat malaria and snake bite in Babile District, eastern Ethiopia (Belayneh *et al.*, 2012). *Aloe pirottae* is reported as used in insect repellents in Iftu locality of Babile District in Ethiopia (traditional healer, personal communication).

3.2.3. *Brassica nigra* (Brassicaceae)

Brassica nigra (L.) Koch (Brassicaceae) grows at 1600-2450 m asl and its leaves are used as vegetable and seeds are used to make a condiment and a spicy sauce used particularly during

fasting seasons (Jonsell, 2000). According to ethnobotanical informants *Brassica nigra* crushed seeds are used as insecticides (Bekele *et al.*, 2012).

3.2.4. *Oreosyce africana* (Cucurbitaceae)

Oreosyce africana Hook.f. (Cucurbitaceae) is a slender climber or trailer growing to 3 m and its habitat is in wet or moist *Pouteria* (= *Aningeria*) *adolji-friederici*-*Syzygium guineense* forest margins, grassland and in plantations at an altitude between 1650-2000 m asl (Jeffrey, 1995). The leaf part of *O. africana* is used as an anthelmintic for intestinal worms and for healing burned skin (Yamada, 1999). The water homogenate obtained from *O. africana* was reported to be given through hypodermal injection to treat gonorrhoea (Yineger and Yewhalaw, 2007). This species is also reportedly used as an insecticide traditionally in Ethiopia (Bekele *et al.*, 2012).

3.2.5. *Piper capense* (Piperaceae)

Piper capense L. f. is a small shrub 1-2 m high, possible sometimes sub-scandent, base semi-woody, much branched above and stems are glabrous. It grows in the understorey of moist montane forest at altitude of 1600-2400 m asl (Gilbert, 2000). Among the families of plants investigated to date, one showing enormous potential as insecticides is the Piperaceae Family (Dodson *et al.*, 2000). For example, *Piper longum* and its component piperine have been described as non-toxic immunomodulators which possess antitumor properties (Sunila and Kuttan, 2004). The extract of *Piper nigrum* fruits exhibited the mean knockdown value which varies from 33% at 0.3% extract concentration to 82% at 7.8% extract concentration within three minutes after exposure against adults of *An. gambiae* Giles (Dadji *et al.*, 2011) and Park *et al.* (2002) reported the same effect on *Cx. pipens pallens*, *Ae. aegypti* and *Ae. togoi*. These authors showed the insecticidal effect of *Piper nigrum* extract to be due to isobutylamide compounds and

not due to piperine, which is the principal component of the extract. The dried fruits of some plants belonging to the family Piperaceae are used as flavouring agents in food and are known to have insecticidal properties as well (Su and Horvat, 1981). Thus Piperaceae is considered by many as a potential source of bioactive chemical compounds against mosquito vectors (Chaithong *et al.*, 2006).

3.3. Preparation of Plant Extracts

The flow chart in Figure 2 reveals an outline of the study design following the protocol described by Kamaraj *et al.* (2010), the design was used to evaluate the efficacy of some indigenous Ethiopian plants against *An. arabiensis*. The plant materials collected were washed thoroughly with distilled water and dried at room temperature in the shadow and pulverized. The extraction of plant materials and phytochemical analyses were done in the Department of Traditional and Modern Medicine of the Ethiopian Public Health Institute (EPHI), Addis Ababa. Maceration was done with aqueous methanolic mixture (80:20; v/v) or 80% methanol (MeOH) (SCP, England) in Erlenmeyer flasks containing the powder of the plant materials placed on a rotatory shaker (VWR, USA) at room temperature, and the solution was allowed to stand for 72 hr with vigorous occasional shaking by following the procedures of Phrompittayarat *et al.* (2007) and Sasidharan *et al.* (2008). The suspension was then filtered using Whatman no. 1 filter paper (Chatman Int. Ltd, Kent, UK) and the filtrate was collected into conical flasks.

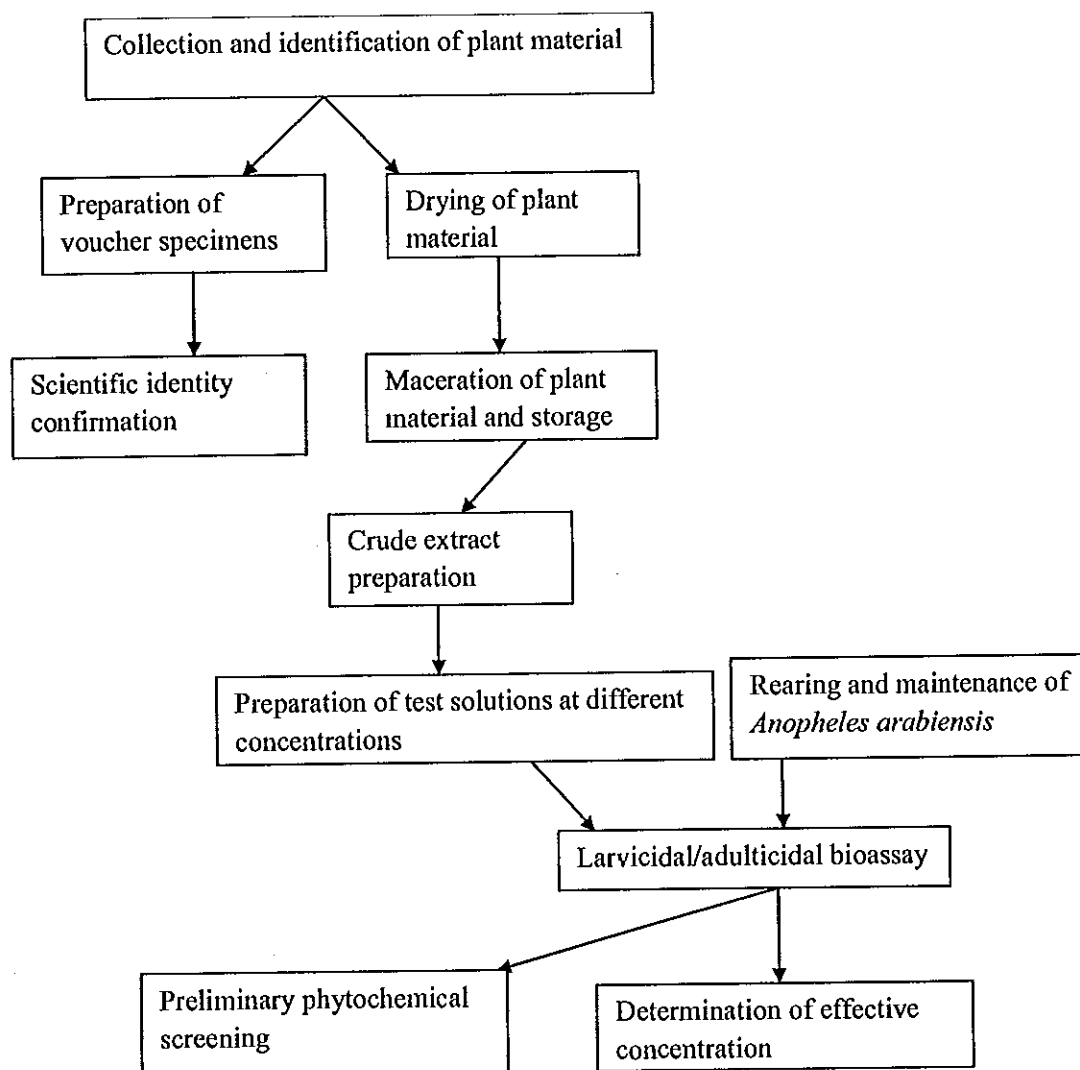


Figure 2. Flow chart showing plant material collection, identification and extraction for bioassay test against *An. arabiensis* (Adopted from Kamaraj *et al.*, 2010).

The residue was macerated again with 80% methanol and the process was repeated three times and filtered. The combined filtrates were concentrated using a rotary evaporator (Stuart®RE300, UK) at 22-26 mm Hg under reduced pressure at 45°C (Appendix 3). The extract thus obtained was concentrated further over water bath (Kottermann, Germany). The extracts obtained were

dark brownish material from *A. schimperi*, *B. nigra*, *O. africana* and *P. capense* and pink from *A. pirottae*. The dried extracts were weighed and stored in a vial at -20°C until used for further bioassay test. The percentage yield of crude extract was determined using the formula $W_2 - W_1 / W_0 \times 100$ as described by Anokwuru *et al.* (2011), where W_2 is the weight of the extract and the vial, W_1 the weight of the vial alone and W_0 the weight of the initial dried sample. Standard stock solutions were prepared at 1% by dissolving the extracts using the universal solvent, 0.05% dimethyl sulfoxide (DMSO, Carlo Erba, France) and diluted in deionized water (deionizer, EASYpureII, USA). From this stock solution different concentrations were prepared.

3.4. Rearing *Anopheles arabiensis* Patton

Anopheles arabiensis, the major malaria vector in Ethiopia, was selected for the testing of larvicidal and adulticidal bioactivities of the test plant extracts. Eggs of *An. arabiensis* for starting the colony were obtained from the Ethiopian Public Health Institute (EPHI) and reared according to the World Health Organization (WHO, 1975) protocol. The colonies were reared and maintained at 25-27°C and 70-80% relative humidity (RH) under a photoperiod cycle of 12 hr light and 12 hr dark in the insectary of the Department of Zoological Sciences, Addis Ababa University, Addis Ababa, Ethiopia. Glass Petri dishes (10.5 cm internal diameter) lined with wet filter paper were kept inside the cages for oviposition. The eggs laid on the filter paper were transferred to plastic and enamel trays containing 3 liters distilled water and allowed to hatch into larvae and kept until the larvae reach the fourth instar stage. The larvae were fed on ground Tetramin® fish food pellets (Tetra holding Inc., Blacksburg, VA, USA); the feed was applied on alternate days for normal development. Water of the larval culture was changed every third day to avoid decay. After attaining pupation, the pupae were transferred to beakers by disposable pipettes and kept inside the mosquito cages for adult emergence (Appendix 4). The mosquito

adults emerged in a netted cage (30 x 30 x 30 cm) and were provided with 10% sucrose solutions soaked in cotton wicks and placed on top of each cage. Adult female *An. arabiensis* were periodically blood-fed on restrained rabbits shaved on the side of their belly. The *An. arabiensis* reared in the laboratory thus served as the source of fourth instar larvae and adults for the different bioassay tests.

3.5. Larvicidal and Adulticidal Bioassays

3.5.1. Larvicidal bioassays

The bioassay tests for the larvicidal effects of 80% methanol extracts of *A. pirottae* gel and *A. schimperi* leaves against *An. arabiensis* fourth instar larvae were carried out in accordance with the WHO standard procedure (WHO, 1981; 2005a). Briefly, the concentrated extracts were first dissolved in 0.02% Tween-80 (Sigma, USA), which was used as an emulsifier to ensure complete solubility of the extract and then diluted with deionized water (Appendix 5). Stock solutions of each extract were prepared and then the test concentrations of 40, 80, 120, 160, 240, 360 and 480 parts per million (ppm) of the plant extracts were serially diluted with deionized water. Serial dilutions were prepared as described in the WHO larvae bioassay protocol (WHO, 2005a). The mixtures were gently stirred to ensure a homogeneous test solution and kept at ambient temperature. Fourth instar larvae of *An. arabiensis* were transferred using fine mesh strainers into separate test cups with respective concentrations of the test solution. Determination of the desired concentration was based on the formula $C_1V_1 = C_2V_2$ as described by Kudom *et al.* (2011).

Three replicates per concentration per test were used to determine the bioactivity of the extracts on the larvae. Tests were carried out simultaneously using four batches of 20 larvae from which

one batch was used for control with a total of 80 larvae for each extract concentration. The set up of the test was assigned in a completely randomized design as reported by Gomez and Gomez (1984). In the test controls the larvae were exposed to 0.02% Tween-80 in deionized water. The larvae in each treatment solution were held for 24 hr, after which they were transferred into 0.02% Tween-80 in deionized water for another 24 hr, as a test for recovery (Edriss *et al.*, 2013; WHO, 2013). Larvae were counted as dead when they were not coming to the surface for respiration and were probe-insensitive (Sivagnaname and Kalyanasundaram, 2004). To prevent decomposition, which may cause rapid death of the remaining larvae, dead larvae were removed as soon as possible when they failed to move after probing with a needle in the siphon or cervical region. The bioassay tests were performed under 25-27°C and 70-80% relative humidity.

3.5.2. Adulticidal bioassays

Adult female *An. arabiensis* mosquitoes were drawn from a stock colony maintained in the laboratory at 25-27°C and 70-80% RH. Beaker bioassays as per WHO (1997) protocol were used at different concentrations to determine the mosquitocidal concentration of the extracts from the selected plants. The respective concentrations of different plant extracts (6.25, 12.5, 25, 50, 100 and 200 ppm) were prepared from the stock solutions by first dissolving in 0.05% DMSO and then diluting with variable amounts of deionized water. Clear 100% polyester fabrics net of 6.5 cm diameter were used for impregnation with each concentration and the nets covered the mouth of the 250 ml glass beakers (Solidex, France) and the inside surfaces of which has been coated with the respective test concentrations. The set-up was left to dry overnight at room temperature prior to testing (Appendix 7).

Two to five days old adult female *An. arabiensis* were aspirated out of rearing net cages and gently introduced into each beaker using a mouth aspirator. The same protocols and conditions as for the extracts was applied to negative control experiments in which mosquitoes were deposited into coated by 0.05% DMSO and deionized water only. Three extract-treated beakers and one control beaker were used for each assay. A total of three replicates for each test concentration were performed in a completely randomized design (Gomez and Gomez, 1984). The triplicate series contained 20 adult females mosquito for each replicate assay plus 20 mosquito in the control, and a total of 80 mosquito adults were assayed for each plant extract by exposing to the extracts in the coated beakers for 1 hr. At the end of exposure time, the adults were transferred to the paper cups and mortality was recorded after 24 hr recovery period (WHO, 2013). Ten percent sucrose solution was provided to both the experimental and the control group as source of food. Mosquito mortality was recorded as dead if it was lying on its back or side and was unable to fly after a gentle tap on the beaker. Mortality was recorded under 25-27°C and 70-80% RH. For different concentrations of *O. africana* and *P. capense*, mortality counts were made at variable post-exposure hours.

3.6. Phytochemical Screening of *Oreosyce africana* and *Piper capense*

The portion of the dry extract of *O. africana* and *P. capense* were subjected to the preliminary phytochemical tests that work on a colour-change basis to determine the presence or absence of plant chemical constituents following the standard procedures as described by different authors (Trease and Evans, 1989; Sofowora, 1993; Harborne, 1998; Sazada *et al.*, 2009). Phytochemical screenings were performed by using solvents and reagents of analytical grade. The tests run were for the secondary metabolites — alkaloids, phenols, tannins, phytosterols, triterpens, flavonoids,

saponins, glycosides, phenolic glycosides, cardiac glycosides, free anthraquinones, anthranoids, and chromophores.

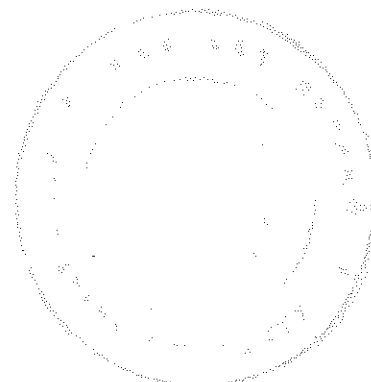
Test procedures for secondary metabolites

To test for alkaloids: 0.5 g from each methanol extract of *O. africana* and *P. capense* were dissolved in 5 ml of 1% HCl and was kept in water bath for 2 min. 1 ml of the filtrate was added to 2 ml of 1% ammonia. Five ml of chloroform was added and shaken gently to extract the alkaloid base; the chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions and Mayer's reagent was added to one portion, while Dragendorff's reagent to the other, the formation of a yellow coloured precipitate with Mayer's reagent or reddish brown precipitate with Dragendorff's reagent was taken as indicator for the presence of alkaloids (Harborne, 1998; Sazada *et al.*, 2009).

Phenol test: 1 g extracts from each of the two plant species were treated with 3-4 drops of ferric chloride solution; formation of bluish black colour indicates the presence of phenols (Trease and Evans, 2002).

Tannin test: 1.5 g of extracts from each plant was stirred with 10 ml of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2 ml of the filtrate and occurrence of a blue black precipitate was taken as a positive test for tannins (Trease and Evans, 2002).

Phytosterol test: 0.5 g extracts were treated with chloroform and filtered, the filtrates were treated with few drops of acetic anhydride, boiled and cooled and conc. Sulphuric acid was



added, formation of brown ring at the junction was taken as an evidence for the presence of phytosterols (Sofowora, 1993).

Triterpene test: 1 g extract from each of the plant was dissolved with chloroform and filtered. To the filtrate on ice, 1 ml of acetic acid was added and then a few drops of conc. sulphuric acid were run down the side of the test tube. The appearance of golden yellow colour was taken as positive for the presence of triterpenes (Harborne, 1998; Sazada *et al.*, 2009).

Flavonoid test: On about 0.2 g extracts from each plant three to five drops of conc. Hydrochloric acid and 1-2 magnesium turnings were added, the development of pink colour was taken as a confirmatory for the presence of flavonoids (Harborne, 1998; Sazada *et al.*, 2009).

Saponin test: 0.5 g of each portion of the plant extract was boiled with 5 ml of distilled water, filtered. To the filtrate, 3 ml of distilled water was further added and shaken vigorously for about 5 minutes. Frothing which persisted on warming was taken as an evidence for the presence of saponins (Sofowora, 1993).

Glycoside test: About 1 g extracts from each plant was treated with ferric chloride solution and immersed in boiling water for about 5 min, the mixture was cooled and extracted with equal volumes of benzene, the benzene layer was separated and treated with ammonia solution, formation of rose-pink colour in the ammonical layer was an indicator of the glycosides (Sofowora, 1993).

Phenolic glycoside test: About 1 g from each plant extract a crystal of ferric sulfate was added, the formation of a dark violet colour that tend to precipitate indicated the presence of phenolic glycosides (Sofowora, 1993).

Cardiac glycoside test: About 0.5 g extracts from each of the plants were dissolved with 3 ml of glacial acetic acid containing 1 drop of 1% FeCl₃. This was underlaid with 1 ml of conc. H₂SO₄. The formation of bright blue colour indicated the presence of cardiac glycosides (Sofowora, 1993).

Free anthraquinone test: About 1.5 g from each of the extracts was shaken vigorously with 10 ml of benzene, the extract was filtered and the filtrate was treated with 5 ml of 10% ammonia solution, the mixture was shaken and the presence of a pink, red or violet colour in the ammonia phase indicated the presence of free anthraquinone (Trease and Evans, 1989).

Anthranoid test: About 0.5 g extracts from the two plant species were boiled for 2 min with 5 ml of 0.5 N KOH and 0.5 ml of 5% H₂O₂ mixtures, after cooling, the suspension was filtered through glass wool, the filtrate was treated with 6 drops of acetic acid and the resulting solution was mixed with 5 ml of toluene, the upper layer was separated and transferred to a test tube and 2 ml of 0.5 N KOH was added, a red colour appears in the aqueous layer was taken as positive for the presence of anthranoids (Harborne, 1998).

Chromophore test: About 1 g from each extract was heated with 10 ml of MeOH for 10 min on a water bath and the solution was filtered through cotton and in a coloured filtrate the change from yellow to red indicated the presence of chromophores (Trease and Evans, 1989).

3.7. Fractionation of *Oreosyce africana* and *Piper capense* Crude Extracts

The dried 80% methanol crude extracts of *O. africana* and *P. capense* were suspended in deionized water and then partitioned with solvents of increasing polarity that is, dichloromethane

(DCM), ethyl acetate (EtOAc) and deionized water using solvent-solvent extraction at room temperature following the methods of Alkofahi *et al.* (1989) (Appendix 8).

The portions of 60 g and 65 g of the 80% methanol crude extracts of *O. africana* and *P. capense* were suspended in 600 ml deionized water in separatory funnel and each of them partitioned with 1200 ml dichloromethane (Appendices 8 and 9). Fractions of each solvent were filtered using Whatman no. 1 filter paper. The mixture was allowed to settle for one day, after which the solvent fraction lower layer was slowly drawn off until only the upper layer remained, and partitions were combined and evaporated at 45°C and labelled as fractions DOF for dichloromethane *Oreosyce* fraction and DPF for dichloromethane *Piper* fraction. Portions of 61 g and 60 g of the crude extracts of *O. africana* and *P. capense* were suspended in 600 ml deionized water in a separatory funnel and each of them partitioned with 1200 ml ethyl acetate (Appendices 8 and 9). The ethyl acetate upper layer filtrates were combined and evaporated to give ethyl acetate *Oreosyce* fraction (EOF) and ethyl acetate *Piper* fraction (EPF). Finally, each of the water residual layer and the solution were evaporated and lyophilized to dryness and labelled as water *Oreosyce* fraction (WOF) and water *Piper* fraction (WPF). Each fraction thus obtained, was filtered and concentrated using rotary vacuum evaporator at 45°C and the dried material was subjected to adulticidal bioassays. The procedure of bioassay-guided fractionation, purification and isolation of *O. africana* leaf and *P. capense* fruit extracts are shown in Figure 3.

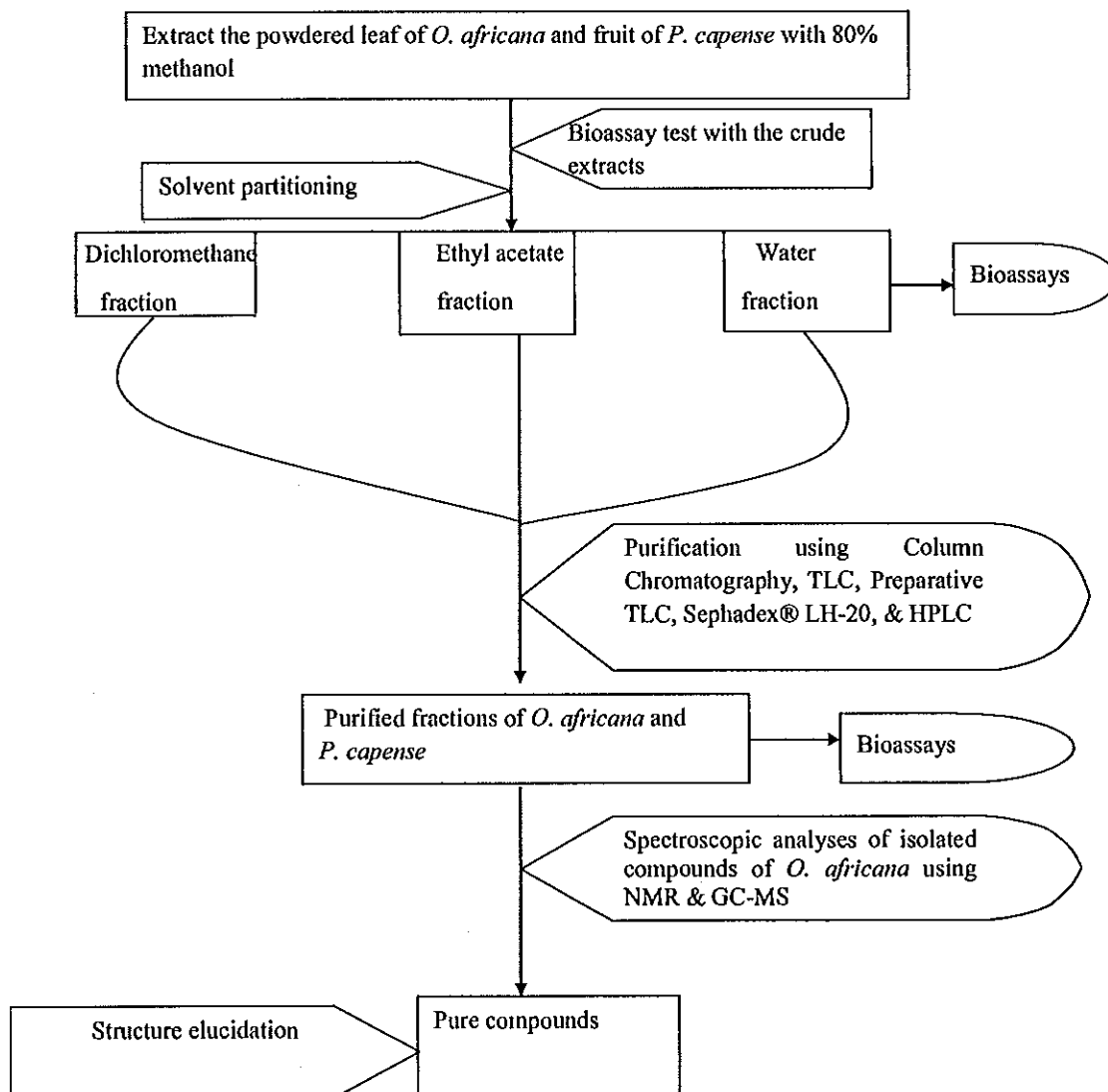


Figure 3. Bioassay-guided fractionation, purification and isolation procedure for the extracts from *O. africana* leaf and *P. capense* fruit (Adopted from Alkofahi *et al.*, 1989).

3.8. Fractionation of *Oreosyce africana* and *Piper capense* following bioassay test

3.8.1. Fractionation procedure

Stock solutions were prepared at 1.0% by dissolving the dried fractions of *O. africana* and *P. capense* in 0.05% DMSO. The stock solutions were then diluted with deionized water to obtain the different test concentrations — 4, 8, 16 and 32 ppm for fractions coded DOF, EOF and WOF and 6, 12, 24 and 48 ppm for fractions coded DPF, EPF, WPF and the positive control (0.05% lambda-cyhalothrin). 0.05% DMSO in deionized water was used as a negative control. Adult *An. arabiensis* were exposed to each test concentration together with the positive and negative controls.

The mosquitocidal activities of the fractions were determined based on WHO (2009) plastic tube bioassay method. These consisted of holding and exposure cylindrical plastic tubes having a length of 125 mm and a diameter of 44 mm. The tests were conducted on uniformly spread impregnated papers (12 x 15 cm) with respective concentrations of *O. africana* and *P. capense* by dipping the papers. The papers were left to dry at room temperature overnight and then inserted into the WHO tubes for test.

3.8.2. Test mosquitoes and experimental design

The test mosquito adults were caught from the rearing net cages with the help of a mouth aspirator (12 mm internal diameter), together with 60 cm of tubing and mouthpiece and released into a plastic holding tube (Appendix 9). The WHO bioassay tubes served to expose the mosquito adults to the papers impregnated with fractions and for holding the mosquito adults before and after the exposure period. The bioassays were performed with non-blood-fed mosquitoes of known age (2 to 5 days old post-emergence of *An. arabiensis* adults) in batches of

20 in each concentration. The mosquitoes were allowed to acclimatize in the holding tube for 1 hr and then exposed to the fractions on the impregnated paper and control for 1 hr. At the end of exposure period, the mosquitoes were transferred back to the holding tube and kept for 24 hr recovery period. A pad of cotton soaked with 10% sucrose solution was placed on the mesh screen during the holding period of 24 hr. Mortality of the mosquitoes was recorded at the end of 24 hr recovery period. Three replicates were maintained at a time in a completely randomized design (Gomez and Gomez, 1984). This adulticidal activity was evaluated at 25-27°C and 70-80% RH. The fraction which exhibited a pronounced adulticidal activity was chosen for further test.

The knockdown effect of the fractions of *O. africana* and *P. capense* against adult *An. arabiensis* was tested by using the standard method of WHO (2006d). The test concentrations used were 4, 8, 16 and 32 ppm for fractions DOF, EOF, WOF and 6, 12, 24 and 48 ppm for fractions DPF, EPF and WPF. Adult mosquito exposed only to 0.05% DMSO in deionized water treated-papers and 0.05% lambdacyhalothrin impregnated papers, were respectively used as negative and positive controls. The number of adult female *An. arabiensis* knockdown in the exposure tube was recorded at 10 min interval period till the last mosquito was knocked down. Knockdown effect of fractions coded DOF and EPF were compared with WHO standard control (0.05% lambdacyhalothrin) was also determined by using the same 10 min interval used for the experimental group. Thus, four concentrations of three replications were tested for the respective study fractions. The temperature and relative humidity of the bioassay tests were maintained at 25-27°C and 70-80%, respectively.

3.9. Purification of Dichloromethane Fraction of *Oreosyce africana* using Flash Column Chromatography

The concentrated 80% methanol crude extract of *O. africana* was fractionated with dichloromethane and deionized water and the fractions were evaporated to dryness by heating under vacuum. 10.68 g of the dried dichloromethane fraction was applied to a column of silica gel (Merck 25-100 mesh size, Germany) and a glass column (40 x 5 cm). The column was vertically clamped to a ring stand and the stopcock end was plugged with cotton wool to hinder the silica gel from spill out. The column was vertically clamped to a ring stand and a few inches of mobile phase solvent (n-hexane, Fisher Scientific, England) was added and the solvents of the mobile phase (chloroform, Fisher Scientific, England) were added to the sample-loaded column following the method of Emilio (2008) and eluted in a step-wise gradient (100:0, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, and 50:50, v/v) from hexane (Fisher Scientific, England) to chloroform (Fisher Scientific, England). In each case, 100 ml of solvent mixture was added and 20 ml fractions were collected using 50 ml conical flasks placed under the stopcock (Appendix 10). Each fraction was concentrated and made up to known ppm solution in a standard flask. After separating the eluate of the dichloromethane fraction by silica gel column chromatography with the hexane and chloroform solvent systems, seven fractions I up to VII was obtained. The fractions were then examined by TLC and that showed similar patterns were combined and stored at -20°C until tested for adulticidal activity.

3.10. Purification of Active Chemical Components from Ethyl Acetate Fraction of *Piper capense* using Sephadex[®] LH-20

Nine grams of EtOAc fraction of *P. capense* was loaded onto a Sephadex[®] LH-20 (Fluka, Switzerland) column chromatography system and this partially purified fraction was successively

eluted with the solvents of the mobile phase as described by Emilio (2008) in a gradient of increasing polarity of n-hexane and chloroform (100:0, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, and 50:50) (Appendix 12). The pooled column chromatographic eluates were based on the method described by Grisi *et al.* (2013).

3.11. Thin-Layer Chromatography Plate Analysis of *Oreosyce africana* and *Piper capense* Fractions

Isolation of the metabolite of *O. africana* and *P. capense* were monitored during the separation stages with thin-layer chromatography (TLC) on Kieselgel gel 60 F₂₅₄ plates (Merck) which has a 0.5 mm thickness of silica sorbent (Merck no. 64271, Darmstadt, Germany) using chloroform-methanol (9:1, v/v) following the method of Okeke *et al.* (1994). The fractions of *O. africana* collected from flash column chromatography and PTLC and fractions of *P. capense* collected from Sephadex[®] LH-20 were analysed using TLC. A small drop of sample was loaded 1 cm above the lower edge of the plate with the help of a small glass capillary tube and adequate time was allowed for the mobile phase to ascend to the marked point. After removing the TLC plate from the TLC tank, it was kept at room temperature for 1 hr, for proper drying. Detection of spots was achieved by spraying the dried chromatogram with 1% sulfuric vanillin solution, then heating on a hot plate at 110°C for 5 min. The TLC plate was visualized under UV 366 nm and 245 nm (Muttenez, Switzerland). The retention factor (R_f) value of the fraction was determined using the formula described by Ettre (1993). Accordingly, the R_f values for the active components visualized were calculated as follows:

$$R_f \text{ value} = \frac{\text{Distance travelled by the spot (cm)}}{\text{Distance travelled by the solvent (cm)}}$$

3.12. Isolation of Chemical Components from Dichloromethane Fraction of *Oreosyce africana* using Preparative Thin-Layer Chromatography

The concentrate obtained by solvent evaporation of dichloromethane fraction of *O. africana* leaf extract was applied to 16 sheets of Kieselgel 60 F₂₅₄ TLC plates (20 x 20 cm, 0.25 mm thickness; Merck, Germany) following the method described by Jiao *et al.* (1994) and Sasidharan *et al.* (2011). These were developed in a chloroform-methanol (5:1) mixed solvent. Each 1.5 cm zone of the distinct bands of purified fractions was scraped off from its plate and extracted eight times with 100 ml of chloroform-methanol (9:1) solvent. The extraction solvent was evaporated under rotary vacuum evaporator and extracted fractions were coded as B2'O and B2''O. These two fractions were stored at -20°C until used in the mosquito bioassay tests.

3.13. Bioassay of Potent Purified Fractions Isolated from *Oreosyce africana* and *Piper capense* against *Anopheles arabiensis*

3.13.1. Evaluation of aerosol spray of potent purified *O. africana* and *P. capense* fractions cage bioassay against *An. arabiensis*

Purified fractions of *O. africana* which include fractions II-IV collected from flash column chromatography, and fractions B2'O and B2''O collected from preparative TLC and fractions II and III of *P. capense* were tested against *An. arabiensis* adults at a concentration of 8 ppm. The test sample concentrations were prepared in 0.05% DMSO in deionized water and shaken vigorously to achieve homogeneity of the contents. Bioassay for the knockdown and the killing effects of the test samples was conducted following the method of WHO (1975) on adult *An. arabiesnsis* held in laboratory cages covered with mosquito netting (19 x 19 x 19 cm) (Appendix 14).

Twenty five non-blood-fed adult 2 to 5 days old, female *An. arabiensis* were transferred into the test cages with a mouth aspirator. The adult mosquitoes were allowed to acclimatize in the cages for 1 hr before treatment. Each treatment was applied using a small hand-held compression sprayer that discharged the purified fractions into mosquito cages, based on the method described by Misni *et al.* (2011) and Abu Bakar *et al.* (2012). The amount of purified fractions sprayed was determined by weighing the compression sprayer on an electronic balance before and after spraying to record the actual weight by following the method described by Misni *et al.* (2011). Two purified fractions of *O. africana* leaf extracts, B2'O and B2''O, were sprayed in separate experiments, inside mosquito cages at a concentration of 8 ppm. The test sample concentrations were prepared in 0.05% DMSO in deionized water and shaken vigorously to achieve homogeneity of the contents. The control cages were sprayed with the same amount of 0.05% DMSO in deionized water. The mosquito knockdown was recorded at 5, 10 and 20 min after treatment following the procedure of WHO (1989; 2006a). 10% sucrose solution soaked on cotton pad, as a source of nutrition, was placed on top of the mosquito netting, in the test as well as in the control group. The Bioassay tests were carried out at 25-27°C and 70-80% relative humidity in the laboratory and mosquito mortality was recorded 24 hr post-treatment.

3.13.2. Test procedures of impregnated mosquito nets with potent purified fractions of *Oreosyce africana* using WHO plastic cone bioassay

Polyester fabrics were purchased from retail shops in Addis Ababa and were cut into 25 x 25 cm size for use in the experiments. The nets were folded and soaked in disposable plastic petri dishes containing solutions of B2'O or B2''O in 0.05% DMSO at concentrations of 4, 8, and 16 ppm. Nets were treated the day before bioassay and stored at room temperature (WHO, 2005b). The impregnated and the control nets were dried in shade by laying them flat on the table. The

impregnated nets were fixed to a cardboard and three standard WHO plastic cones were placed and fixed with tape over the area of the treated net. Ten non-blood-fed, 2 to 5 days old, female *An. arabiensis* each were introduced into the three test cones through a hole on the mosquito nets. The same numbers of non-blood-fed adult female mosquitoes were also introduced into the cone that was fixed over a net impregnated with 0.05% DMSO in deionized water as a negative control. Both test and negative control mosquitoes were exposed for 15 minutes under ambient room temperature.

At the end of the exposure period, the mosquitoes were transferred into recovery paper cups covered with untreated nets and were held for 24 hr with access to 10% sucrose solution. Mortality was determined after 24 hr recovery period (Appendix 13). In case of persistence or residual activity, the adults were introduced in B2'O at concentrations of 4, 8 and 16 ppm through a weekly assessment for three consecutive intervals from April to June 2014 (that is 1, 5 and 9 weeks) post application, as mentioned above, following the procedure of WHO (1975). The treatments were replicated three times and assigned in a completely randomized design (Gomez and Gomez, 1984).

3.13.3. Adulticidal activity of purified fractions of *Oreosyce africana* on *Anopheles arabiensis* using plastic tube bioassay

Bioassay test on the lethal effects with B2'O and B2"O fractions of *O. africana* against adult females mosquitoes were determined based on WHO (2009) test method. Concentrations ranging from 4 to 16 ppm of B2'O and B2"O fractions were applied to WHO papers. Each concentration was prepared in 0.05% DMSO (Carlo Erba, France) in deionized water and shaken vigorously to achieve homogeneity of the contents. The WHO papers treated with the *O. africana* fractions were left to dry at room temperature overnight and then inserted into each exposure tube and the

WHO papers treated with 0.05% DMSO in deionized water served as a negative control. The bioassays were performed at 25-27°C and 70-80% relative humidity with non-blood-fed *An. arabiensis* adult mosquitoes of known age (2 to 5 days post-emergence) in batches of 15 in each concentration. The mosquitoes were allowed to acclimatize in the holding tube for 1 hr and then exposed to the fractions on the impregnated paper and the unimpregnated control for 1 hr. At the end of the exposure period, the mosquitoes were transferred back to the holding tubes and kept for 24 hr recovery period. A pad of cotton soaked with 10% sucrose solution was placed on the mosquito net during the holding period. Each treatment was replicated three times for each concentration.

3.14. High Performance Liquid Chromatography Analysis

The active fractions of *O. africana* were further purified by high performance liquid chromatography (HPLC) using Waters LC-2000 model equipped with Waters 600 pump controller (Milford, MA, USA). 10 µl of each sample fraction - fraction IV, B2'O and B2"O injected manually into a column of Tracer Extrasil ODS2, TR416059 (particle diameter, 5 µm; pore size, 30 nm; column size, 25 x 0.46 cm (Teknokroma, Barcelona, Spain) and eluted by use of solvent system: methanol and water (80:20, v/v) with running time of 0-30 min. Column temperature of 20°C and flow rate of 1.0 ml/min were maintained and the eluate detected at 254 nm using Waters 2487 dual absorbance UV detector operated by Millennium 32 software from WATERS (Milford, MA, USA).

3.15. Characterization of the Mosquitocidal Active Chemical Components of *Oreosyce africana* Extract

The isolated and purified B2'O and B2"O fractions of *O. africana* were analysed for their chemical composition and structure elucidation by using nuclear magnetic resonance (NMR) spectroscopy and gas chromatography-mass spectrometry (GC-MS).

3.15.1. Nuclear magnetic resonance spectroscopy

Proton (^1H), carbon (^{13}C) one-dimensional (1D) Nuclear Magnetic Resonance (NMR) spectra were produced by a Bruker Avance 400 MHz NMR spectrometer. The NMR analysis for the isolated fractions of *O. africana* was made following the method described by Silverstein *et al.* (1991). The spectra for all compounds were recorded at room temperature in deuterated chloroform (CDCl_3). The chemical shifts expressed in ppm (δ) downfield were recorded from the signal of tetramethylsilane (TMS or MeSi_4) internal reference. For the ^{13}C -NMR spectra, multiplicities were determined by Distortionless Enhancement by Polarization Transfer (DEPT) method at the Department of Chemistry, Addis Ababa University. Multiplicities of ^1H -NMR signals were indicated as s (singlet), d (doublet), dd (doublet of doublets), t (triplet) and m (multiplet). Data was acquired from the NMR machine as computer printout. The chemical structures were proposed based on the interpretation of the combined spectra.

3.15.2. Gas chromatography-mass spectrometry

The gas chromatography-mass spectrometry (GC-MS) analyses were performed using the method described by Mohan and Maruthupandian (2011). Gas chromatography measurements were carried out with Hewlett-Packard HP5890 Series II (Agilent Technologies, USA) and GC-MS analysis were conducted with gas chromatograph model 7890B (Agilent Technologies, USA) coupled to Hewlett-Packard MS model 6977A (Agilent Technologies, USA) with mass

selective detector. The analysis was carried out on a GC Clarus 500 Perkin Elmer system and gas chromatograph interfaced to a mass spectrometer (GC-MS) employing the following conditions: Column Elite-1 fused silica capillary column (30 mm x 0.25 mm ID x 1 μ m df, composed of 100% dimethyl poly siloxane). Helium (99.999%) was used as a carrier gas operating in electron impact (EI) mode at a constant flow of 1 ml/min and each sample injected contained a volume of 2 μ l was employed manually (split ratio of 10:1) under injector temperature of 250°C and ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C/min until 200°C and then rose at 5°C/min upto 280°C and was held for the last 9 min (isothermal at 280°C). The GC-MS instrument was used in the electron impact mode under an ionization voltage of 70 eV, a scan interval of 0.5 seconds and fragments from 45 to 450 Da. The detection of the mass spectra was completed in 38 min. Interpretation on mass spectra from GC-MS data was conducted using the database of National Institute of Standards and Technology (NIST) library. The mass spectra of the unknown components were compared to the spectra of the known compounds and determined by searching in the NIST libraries. The relative percentage of each component was calculated by comparing its average peak area to the total areas.

3.16. Data Analysis

Bioassay tests showing more than 20% control mortality were discarded and repeated. However, when control mortality ranged from 5% to 20%, it was corrected using Abbott's formula (Abbott, 1925). Results were expressed as the mean percent mortality with standard deviations. One-way analysis of variance (ANOVA) using SPSS for windows (version 16.0, SPSS software 2007) was used to test differences in mean larval mortality rates between 80% methanol crude extracts of *A. schimperi* and *A. pirottae*. Differences between LC₅₀ concentrations were considered significant at P<0.05 if the respective 95% CL did not overlap. Comparisons between mean percent mortality rates of *An. arabiensis* adults that were treated with 80% methanol crude extracts of *A. pirottae*, *B. nigra*, *O. africana* and *P. capense* and negative controls were made by ANOVA using SPSS for windows (version 16.0, SPSS software 2007). The mean mortality rates of three replicates was taken to determine the percent mortality after 24 hr of exposure by using Tukey's studentized range test (HSD) test.

To test variations in adult mortalities among 80% methanol crude extracts of *A. pirottae*, *B. nigra*, *O. africana* and *P. capense* and among crude extracts of *O. africana* and *P. capense* and their fractions - DOF, EOF, WOF, DPF, EPF, WPF were determined. The chi-square (χ^2) probability for goodness of fit test was used to estimate how well the data of each concentration-mortality curve fit the assumption of the probit model using statistical package PoloPlus (version 2.0, LeOra Software, Petaluma, California, USA; 2007). The 95% confidence limits of upper confidence limit (UCL) and lower confidence limit (LCL) for the lethal concentration (LC₅₀ and LC₉₀) in ppm, and the slope for adulticidal effects were used to measure variations among the *O. africana* and *P. capense* crude extracts and their fractions and lethal concentration ratio confidence limits (95%) that did not include 1.0 were considered significant (P<0.05) (Robertson

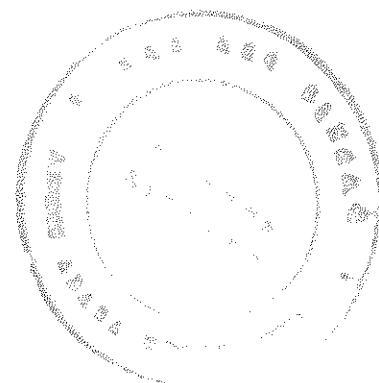
et al., 2007). Hypothesis testing to compare concentration-mortality probit lines was evaluated by likelihood ratio tests for equality and parallelism (χ^2 tests and p-values for comparing the slope and intercept). When the slopes of the concentration-mortality lines were not significantly different, lines were considered parallel, indicating that the two treatments had the same relative potency (i.e., the same variability in response) (Robertson *et al.*, 2007). PoloPlus analysis was also used to determine adulticidal efficacies among purified *O. africana* fractions II, III, B2'O, and B2"O and *P. capense* fractions II and III. The percentage knockdown of mosquitoes was calculated in the test population as described by WHO (1975). Statistically significant differences between the compared LC₅₀ concentrations, the 95% confidence intervals for lethal concentration ratios were calculated. In this pairwise comparison, lethal concentrations were considered significantly different if the value '1' did not fall within the confidence interval for the ratio (Robertson *et al.*, 2007).

The observed percentage mortality was corrected by Abbott's formula (Abbott, 1925):

$$\% \text{ mortality} = \frac{\% \text{test mortality} - \% \text{control mortality}}{100 - \% \text{control mortality}} \times 100$$

Standardized residual plots were generated to show how results fit the log-probit model using PoloPlus (LeOra Software, 2007). Standardized residuals were calculated by taking the difference of the observed value and the expected value and dividing the result by their standard errors. These results were plotted against the lethal concentration estimate for the expected values. Goodness of fit was considered as residuals scattered randomly within a horizontal band around zero and mostly between -2 and 2.

According to Robertson *et al.* (2007), statistical methods used for probit or logit regression analysis and goodness of fit are given by the formula for probit analysis:



$$P_i = \Phi(\alpha + \beta x_i)$$

Where, P_i = probability of response

x_i = dose or a function of that dose

Φ = standard normal distribution function

$\alpha + \beta x_i$ = regression line

For the logit model:

$$P_i = \frac{1}{1 + e^{-(\alpha + \beta x_i)}} = \frac{e^{\alpha + \beta x_i}}{1 + e^{\alpha + \beta x_i}}$$

Where, $e \equiv 2.71828$

$\alpha + \beta x$ = linear predictor

α = intercept

β = slope

For goodness-of-fit test:

A binomial response is standardized with division by their standard errors, $\sqrt{np(1-p)}$.

Where, n = number of subjects tested at the specific concentration

P = probability

4. Results

4.1. Plant Species Selected for Mosquitocidal Testing

Out of several plants reported for their insecticidal potency in the literature and in the field survey only, five, considered most promising, were selected. The crude extracts were weighed for estimating the yield percentages. The scientific, family and local names of the selected plants are provided with parts used for bioassay test and yield of crude extracts as shown in Table 1.

Table 1. List of plant species collected with their local names and yields of 80% methanol crude extracts investigated for larvicidal and adulticidal properties against *Anopheles arabiensis*

Species name	Family name (Specimen №)	Local name*	Plant part used	Net weight (g)	% Yield of crude extract
<i>Acokanthera schimperi</i> (A.DC.) Schweinf.	Appocynaceae (DB38)	Qararo (O), Lemlem (A)	Leaves	596	15.1
<i>Aloe pirottae</i> Berger	Aloaceae (DB37)	Hargeysa (O), Eret (A)	Leaves (Gels)	805	11.4
<i>Brassica nigra</i> L. Koch	Brassicaceae (DB27)	Shinfii (O), Senafech (A)	Seeds	557	12.8
<i>Oreosyce africana</i> Hook.f.	Cucurbitaceae (DB18)	Manabasi (O, A)	Leaves	1700	39.0
<i>Piper capense</i> L. f.	Piperaceae (DB22)	Tinjo (O), Timiz (A)	Fruits	1800	37.2

* (O) = Local name in Afaan Oromo, (A) = Local name in Amharic

4.1.1. Larvicidal test

The 80% methanol extracts of *Aloe pirottae* gel and *Acokanthera schimperi* leaf tested on *An. arabiensis* larvae within 24 hr that gave the median lethal concentration (LC₅₀) and LC₉₀ values are shown in Table 2.

Table 2. Larvicidal activity of 80% methanol crude extracts of *Aloe pirottae* and *Acokanthera schimperi* against fourth instar larvae of *Anopheles arabiensis* after 24 hr exposure (n = 60 in each test)

Plant extract	LC ₅₀ ^a (ppm)	95% confidence limit		LC ₉₀ ^b (ppm)	95% confidence limit		χ^2	Slope±SE
		LCL ^c	UCL ^d		LCL	UCL		
<i>Aloe pirottae</i>	76.344 ^e	31.47	105.42	133.39 ^g	104.59	249.5	3.7109	0.022±0.003
<i>Acokanthera schimperi</i>	282.76 ^f	135.1	338.93	407.93 ^h	399.19	610.02	6.6342	0.01±0.001
Negative control*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

* 0.02% Tween-80 in deionized water.

^aLC₅₀: lethal concentration that kills 50% of the exposed larvae; ^bLC₉₀: lethal concentration that kills 90% of the exposed larvae; ^cLCL: Lower Confidence Limit; ^dUCL: Upper Confidence Limit.

LC₅₀ values with different superscript letters within the same column are significantly different at 95% confidence intervals (Tukey's studentized test).

The 80% methanol extracts of *A. pirottae* exhibited more effect in killing the mosquito larvae than the extracts of *A. schimperi*. The LC₅₀ and LC₉₀ values were 76.34, 133.39 ppm for *A. pirottae* and 282.76, 407.93 ppm for *A. schimperi*, respectively (Table 2).

4.1.2. Adulticidal test

The adulticidal activities of 80% methanol plant extracts were observed with the extracts of *A. pirottae*, *B. nigra*, *O. africana* and *P. capense*. The results were ranked from 1 to 4 based on observed mortality (Table 3).

Table 3. Adulticidal activity of 80% methanol crude extracts of the four indigenous plants in order of relative efficacy on female *Anopheles arabiensis* after 24 hr exposure (n = 60 in each test)

Plant extracts	% mortality (M±SD)						Order of relative efficacy
	Concentration						
	6.25 ppm	12.5 ppm	25 ppm	50 ppm	100 ppm	200 ppm	
<i>Aloe pirottae</i>	0.00±0.00	3.33±2.88	11.67±2.88	20.00±5.00	30.00±5.00	53.33±5.77	4
<i>Brassica nigra</i>	8.33±2.88	8.33±2.88	25.00±0.00	28.33±2.88	35.00±5.00	75.00±5.00	3
<i>Oreosyce africana</i>	20.00±5.00	46.67±2.88	51.67±2.88	100.00±00	100.00±00	100.00±00	1
<i>Piper capense</i>	15.00±5.00	28.33±2.88	45.00±5.00	95.00±00	100.00±00	100.00±00	2
Negative control*	0.0	0.0	0.0	0.0	0.0	0.0	

M = means of three replicates, SD = standard deviation

* 0.05% DMSO in deionized water.

The 80% methanol extract of *O. africana* leaves caused average mortality on mosquitoes, ranging from 20% at 6.25 ppm to 100% at 50 ppm. Similarly, the extracts of *P. capense* fruits caused an average mortality that varied from 15% at 6.25 ppm to 95% at 50 ppm after 24 hr exposure (Table 3), which was more effective than the other two tested plant extracts.

The order of relative efficacy of these crude extracts based on mean mortality values in descending order is: *O. africana* > *P. capense* > *B. nigra* > *A. pirottae* (Table 3).

The adulticidal potencies with their LC₅₀ and LC₉₀ values of these indigenous Ethiopian plants against *An. arabiensis* adults are assessed and presented in Table 4.

Table 4. The LC₅₀ and LC₉₀ values of 80% methanol crude extracts of the four indigenous plants against *Anopheles arabiensis* adults after 24 hr exposure (n = 60 in each test)

Plant extracts	LC ₅₀ (ppm)	95% confidence limit		$\chi^2(P^{**})$	Slope±SE	LC ₅₀ ratio (95% CL)
		LCL-UCL	LC ₉₀ (ppm)			
<i>Aloe pirottae</i>	183.765 (135.410 – 287.745)		1212.157 (644.025 – 3449.028)	1.787 (0.523) ^a	1.564±0.206	-
<i>Brassica nigra</i>	124.489 (84.412 – 230.155)		799.50 (373.41 – 3715.5)	4.995 (0.440) ^a	1.587±0.183	1.476 (0.924-2.358)
<i>Oreosyce africana</i>	14.881 (6.982 – 26.001)		44.553 (25.613 – 286.339)	20.935 (0.419) ^a	2.691±0.27	12.349(8.3-18.375) ^b
<i>Piper capense</i>	25.693 (14.584 – 45.202)		46.327 (40.722 – 363.089)	15.920 (0.488) ^a	2.548±0.220	7.152 (4.811-10.632) ^b
Negative control*		0.0		0.0	0.0	0.0

* 0.05% DMSO in deionized water.

** Probability of good fit of the probit model.

^a Good fit of the data to the probit model (P>0.05).

^b LC₅₀ ratio significant at P<0.05; 95% confidence interval did not comprise the value 1.0.

LC₅₀: lethal concentration that kills 50% of the exposed adults; LC₉₀: lethal concentration that kills 90% of the exposed adults; LCL: Lower Confidence Limit; UCL: Upper Confidence Limit.

Negative control - No mortality.

The hypothesis test for parallelism was rejected ($\chi^2 = 23.14$; P = 0.001) showing that slopes differed significantly. Among the crude extracts tested, *O. africana* with LC₅₀ at 14.88 ppm and

LC₉₀ at 44.55 ppm and *P. capense* with LC₅₀ at 25.69 ppm and LC₉₀ at 46.32 ppm elicited potent adulticidal effect than *A. pirottae* and *B. nigra* (Table 4). The 80% methanol crude extracts concentrations of *O. africana* and *P. capense* at which 100% adult mortality was achieved were further subjected to bioassay tests to determine the time and concentration values (Figures 4 and 5).

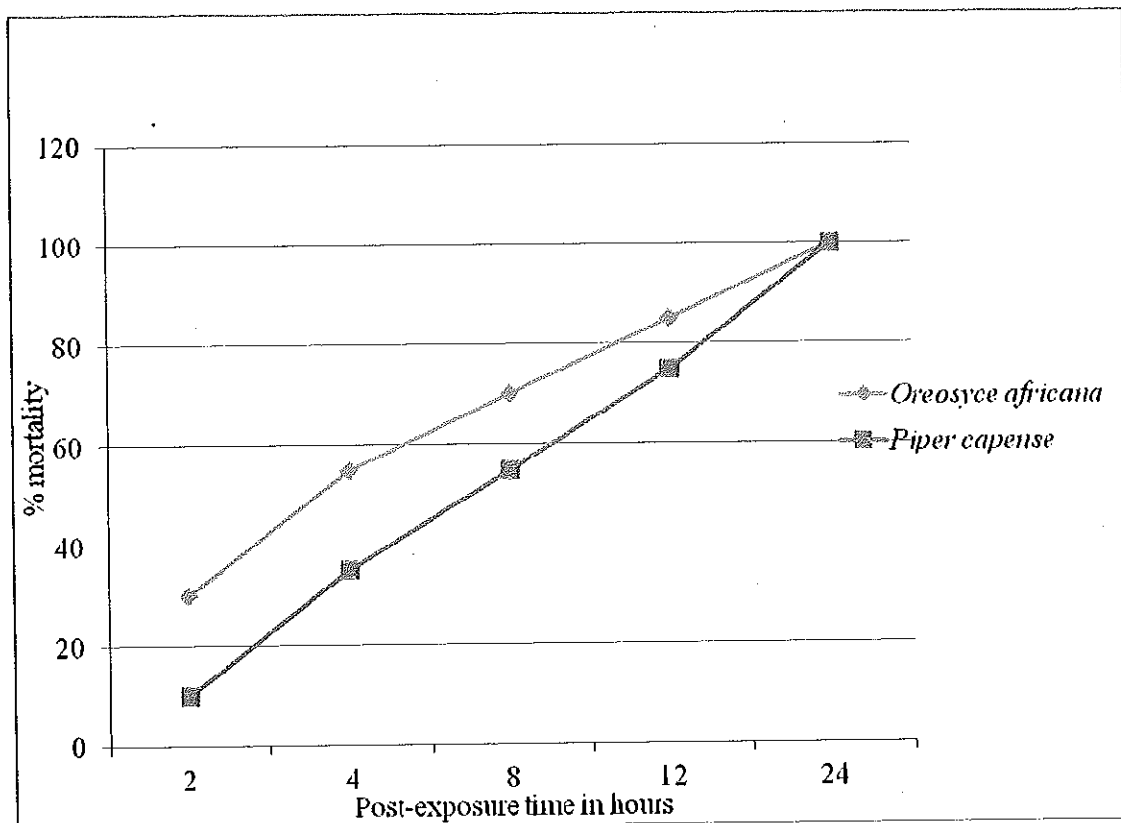


Figure 4. Effect of 80% methanol crude extracts of *Oreosyce africana* and *Piper capense* at 55 ppm on adult *An. arabiensis* at variable post-exposure hours.

The data presented in Figure 4 reveals that the 80% methanol extracts of *O. africana* and *P. capense* induced 100% mortality against *An. arabiensis* adults at ≥ 55 ppm after 24 hr exposure period.

Figure 5. Concentrations effect of *Oreosyce africana* and *Piper capense* extracts at 100% mortality on adult *Anopheles arabiensis* in six hours time intervals.

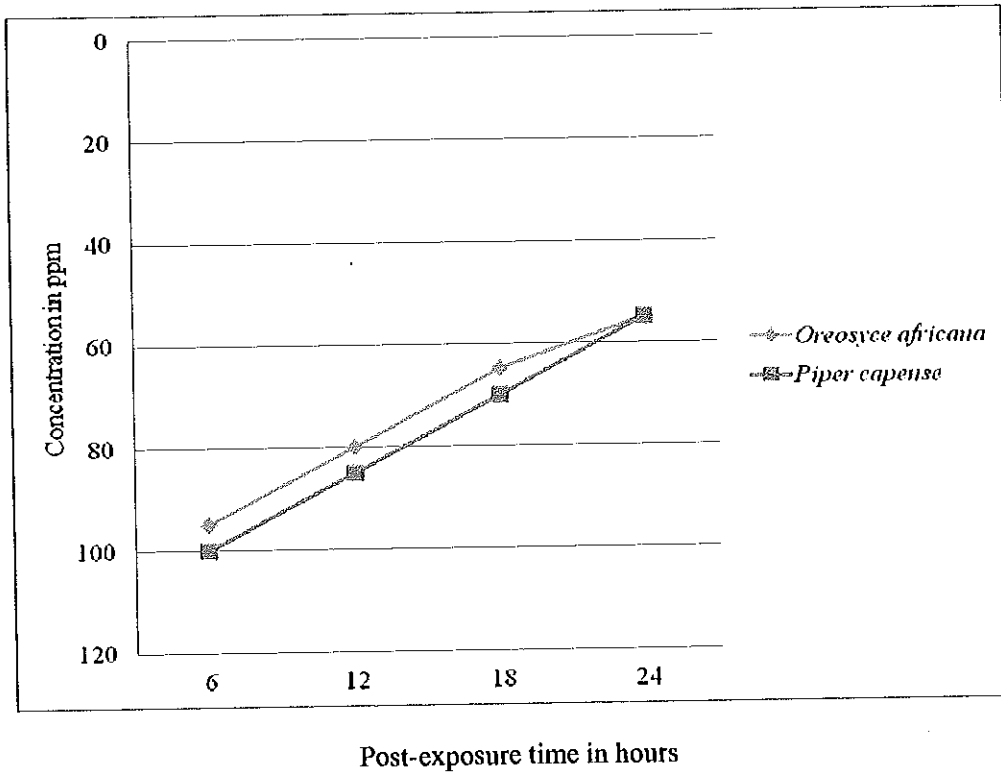


Figure 5. Effect of *Oreosyce africana* and *Piper capense* extracts at 100% lethality on adults of *Anopheles arabiensis* in six hours time intervals.

The current results of bioassay tests by the methanol extract of *O. africana* and *P. capense* against *An. arabiensis* adults caused 100% mortality at concentrations of 80 and 85 ppm, respectively after 12 hr exposure period. This proved that the 80% methanol crude extracts of *O. africana* and *P. capense* were more effective plant extract treatments which showed the best mortalities and had concentration activities at 80 and 85 ppm of *O. africana* and *P. capense* against the adults of *An. arabiensis*, as shown in Figure 5.

4.2. Preliminary Phytochemical Analysis

Based on the highest adulticidal activity obtained from Table 4, *O. africana* and *P. capense* were chosen for phytochemical screening. The qualitative analysis of the phytochemicals was carried out on 80% methanol crude extracts of *O. africana* and *P. capense*. Table 5 shows the results of preliminary qualitative phytochemical analysis and the presence of a variety of secondary metabolites was revealed in the crude extracts.

Table 5. Phytochemical screening for secondary metabolites in 80% methanol crude extracts of *Oreosyce africana* leaf and *Piper capense* fruit

S. No.	Plant constituents	Test performed	Plant extracts		References
			<i>Oreosyce africana</i>	<i>Piper capense</i>	
1	Alkaloids	Mayer's test	+	+	Harborne (1998); Sazada <i>et al.</i> (2009)
		Dragendorff's test	+	+	
2	Phenols	Ferric chloride test	+	+	Trease and Evans (2002)
3	Tannins	Gelatin test	-	-	Trease and Evans (2002)
4	Phytosterols	Liebermann Burchard's test	+	-	Sofowora (1993)
5	Triterpenes	Salkowski's test	+	-	Harborne (1998); Sazada <i>et al.</i> (2009)
6	Flavonoids	Alkaline reagent test	+	+	Harborne (1998); Sazada <i>et al.</i> (2009)
7	Saponins	Froth test	+	+	Sofowora (1993)
		Foam test	+	+	
8	Glycosides	Test for glycosides	+	+	Sofowora (1993)
8a	Phenolic glycosides	Test for phenolic glycosides	+	-	Sofowora (1993)
8b	Cardiac glycosides	Keller-Killiani test	+	+	Sofowora (1993)
9	Free anthranquinones	Borntrager's test	-	+	Trease and Evans (1989)
10	Anthranoids	Test for anthranoids	-	-	Harborne (1998)
11	Chromophores	Test for chromophores	+	+	Trease and Evans (1989)

Key: + = present; - = absent

The results for the preliminary phytochemical analysis of 80% methanol extracts of *O. africana* and *P. capense* indicated that most of the constituents were found to be alkaloids, phenols, flavonoids, saponins and chromophores, whereas tannins, and anthranoids seem to be absent. The secondary metabolites like phytosterols, triterpenes, and phenolic glycosides were detected in the 80% methanol extract of *O. africana* leaves but not found in *P. capense* fruits. Free anthraquinones were found in *P. capense*.

4.3. Fractionation and Bioassay Test of Bioactivity of *Oreosyce africana* and *Piper capense*

The percentage yields of these fractions with the solvents used are presented in Table 6.

Table 6. Percentage yields of *Oreosyce africana* and *Piper capense* crude 80% methanol fractions obtained using solvents of different polarities

Plant species	Plant part (g)	Solvent used	Percent yield of fraction
<i>Oreosyce africana</i>	Leaves (905)	Dichloromethane	24.5% DOF ^a
		Ethyl acetate	12.8% EOF ^b
		Water	15.7% WOF ^c
<i>Piper capense</i>	Fruits (815)	Dichloromethane	16.3% DPF ^d
		Ethyl acetate	26.9% EPF ^e
		Water	15.6% WPF ^f

Note: ^a Dichloromethane *Oreosyce* fraction.

^b Ethyl acetate *Oreosyce* fraction.

^c Water *Oreosyce* fraction.

^d Dichloromethane *Piper* fraction.

^e Ethyl acetate *Piper* fraction.

^f Water *Piper* fraction.

The percentage yields of ethyl acetate and water fractions were lower in *O. africana*, whereas the percentage yields of water and dichloromethane fractions in *P. capense* were lower as compared with dichloromethane fraction of *O. africana* and ethyl acetate fraction of *P. capense*. The EtOAc fraction yields for the *P. capense* was the highest followed by DCM fraction yields for *O. africana* (Table 6). The yields of DOF, EOF and WOF were 24.5%, 12.8%, and 15.7% and the yields of DPF, EPF and WPF were 16.3%, 26.9%, and 15.6% (Table 6). These fractions were subjected to adulticidal test against *An. arabiensis* adults and performed before embarking on purification of the extracted fractions.

The overall test of 80% methanol crude extracts and their various solvent fractions, against *An. arabiensis* adults after 24 hr exposure period, showed the various solvent fractions to exhibit better adulticidal potencies than the crude extracts. However, the efficacy of the fractions varied from one fraction to another (Tables 7 and 8 and Figures 6 and 7).

Table 7. Evaluation of the effect of 80% methanol crude extract and solvent fractions of *Oreosyce africana* on *An. arabiensis* adults after 24 hr exposure (n = 60 in each test)

Extract Tested*	LC ₅₀ ppm (95% CL)	LC ₉₀ ppm (95% CL)	Slope ±SE	χ ² (P ^c)	LC ₅₀ ratio (95% CL)
Crude extract	13.019 (9.981-16.982)	68.369 (41.861-111.656)	1.779±0.207	5.90 (0.56) ^a	-
DOF	4.267 (1.325-6.451)	14.123 (9.268-49.248)	2.466±0.289	3.127 (0.47) ^a	3.051(2.3-3.980) ^b
EOF	14.562 (13.720-25.292)	82.57 (69.094-98.728)	1.701±0.206	5.086 (0.53) ^a	0.894(0.6-1.154)
WOF	16.973(13.456-17.209)	117.877(88.475-157.372)	1.523±0.204	6.65 (0.48) ^a	0.767 (0.580-1.1014)
Negative control**	0.0	0.0	0.0	0.0	0.0

* The codes used for the fraction are the same as in Table 6.

** DMSO (0.05%) in deionized water.

^a Good fit of the data to the probit model ($P > 0.05$).

^b LC₅₀ ratio significant at $P < 0.05$; 95% confidence interval did not comprise the value 1.0.

^c Probability of good fit of the probit model.

The hypothesis test for parallelism was rejected ($\chi^2 = 9.43$; $P = 0.024$) showing that slopes differed significantly. Quantitative estimation in all the solvent fractions of *O. africana* and *P. capense* were also carried out to determine the lethal concentrations (LC₅₀ and LC₉₀) of a particular fraction. All chi-square values were not significant ($\alpha = 0.05$) in goodness of fit test on the probit model, indicating a good fit of regression line (Tables 7 and 8). Among the extracts, dichloromethane fraction of *O. africana* with LC₅₀ at 4.267 and LC₉₀ at 14.123 ppm showed potent adulticidal effect than crude extract, EOF and WOF (Table 7).

The adulticidal effect of 80% methanol crude extract of *O. africana* leaves and fractions DOF, EOF and WOF were evaluated on *An. arabiensis* at 4, 8, 16 and 32 ppm after 24 hr exposure on impregnated papers using WHO test tubes (Figure 6).

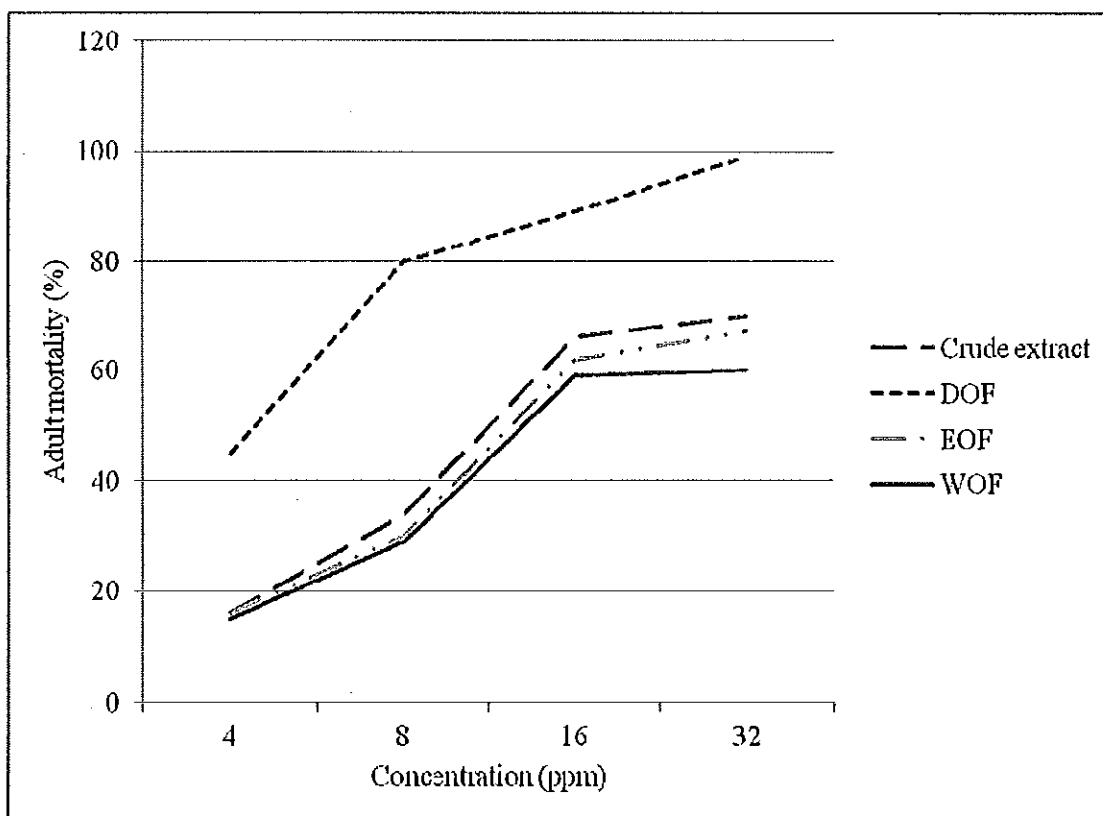


Figure 6. Adulticidal effect of 80% methanol crude extract of *O. africana* and its solvent fractions on *Anopheles arabiensis* adults 24 hr post-exposure on impregnated papers in WHO test tubes (n = 60 in each test).

Comparison of the various solvent fractions with 80% methanol crude extract of *O. africana* showed dichloromethane fraction of *O. africana* (DOF) had more adulticidal effect against *An. arabiensis* (99% mortality at 32 ppm) than the crude extract, EOF and WOF (Figure 6).

Comparison of the various solvent fractions with regard to mean LC₅₀ and LC₉₀ values in *O. africana* extracts showed the dichloromethane fraction with 4.267 and 14.12 ppm, respectively to be significantly the most potent than the other solvents. Furthermore, the ethyl acetate fraction with 14.562 and 82.57 ppm showed a distant second to the dichloromethane fraction while the water fraction was least potent. The adulticidal effect of 80% methanol crude extract of *O. africana* leaves and fraction DOF were evaluated on *An. arabiensis* at 4, 8, 16 and 32 ppm after 24 hr exposure on impregnated papers using WHO test tubes. Comparison of DOF with 80% methanol crude extract of *O. africana* showed dichloromethane fraction of *O. africana* had more adulticidal effect against *An. arabiensis* (99% mortality at 32 ppm) than the crude extract, EOF and WOF (Figure 7). The hypothesis test for parallelism was rejected ($\chi^2 = 8.46$; P = 0.003) showing that slopes differed significantly.

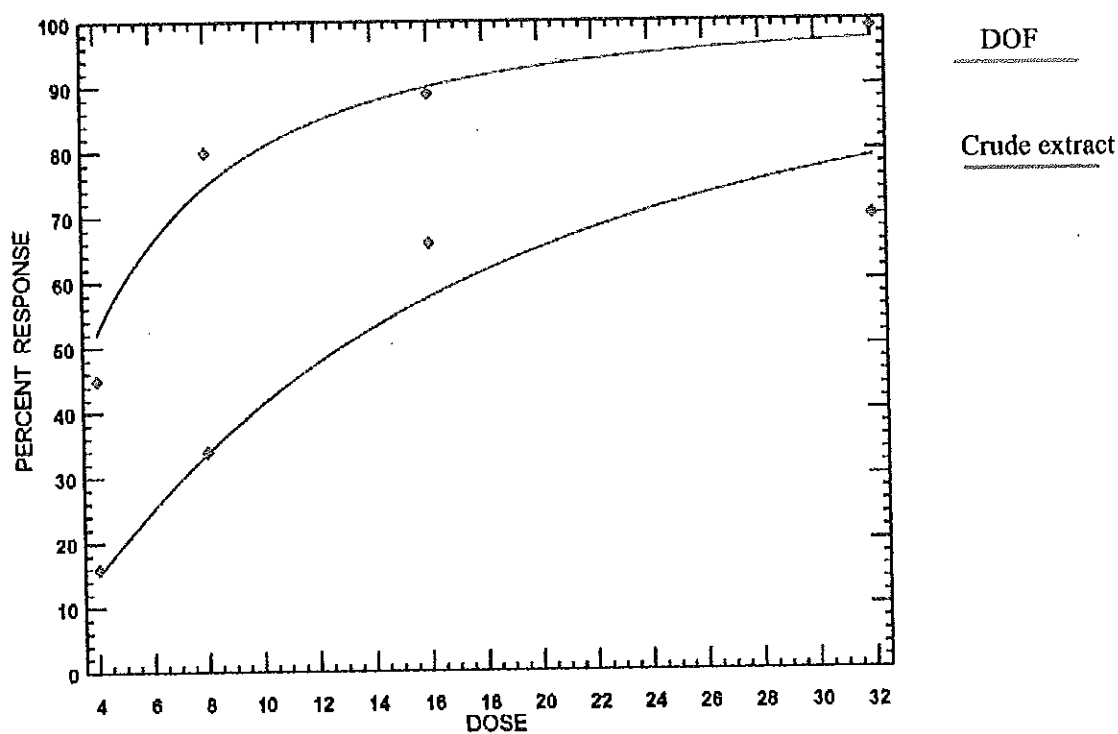


Figure 7. Percentage mortality rates of *An. arabiensis* after treatment with different concentrations of crude extract and dichloromethane fraction of *O. africana*.

With regard to *P. capense* extracts, the ethyl acetate fraction with LC_{50} at 10.715 and LC_{90} at 30.591 ppm showed more potency than the dichloromethane and water fractions (Table 8).

Table 8. Evaluation of the effect of 80% methanol crude extracts and solvent fractions of *P. capense* on *An. arabiensis* after 24 hr exposure on impregnated papers in WHO test tubes (n = 60 in each test)

Extracts tested*	LC ₅₀ ppm (95% CL)	LC ₉₀ ppm (95% CL)	Slope±SE	χ^2 (P ^c)	LC ₅₀ ratio (95%CL)
Crude extract	25.518 (21.618-31.083)	121.395 (83.862-213.379)	1.892±0.215	0.775 (0.48) ^a	-
DPF	27.661 (22.75-35.504)	173.493 (107.212-380.843)	1.607±0.207	1.815 (0.46) ^a	0.923 (0.695-1.224)
EPF	10.715 (9.349-12.100)	30.591 (25.796-38.413)	2.813±0.261	0.569 (0.56) ^a	2.382 (1.908-2.972) ^b
WPF	28.196 (21.534-36.876)	150.614 (131.959-208.212)	1.761±0.213	0.988 (0.45) ^a	0.905 (0.692-1.185)
Negative control**	0.0	0.0	0.0	0.0	0.0

* The codes used for the fractions are the same as in Table 6.

** DMSO (0.05%) in deionized water.

^a Good fit of the data to the probit model (P > 0.05).

^b LC₅₀ ratio significant at P < 0.05; 95% confidence interval did not comprise the value 1.0.

^c Probability of good fit of the probit model.

The hypothesis test for parallelism was rejected ($\chi^2 = 8.46$; P = 0.03) showing that slopes differed significantly. Among the extracts, ethyl acetate fraction of *P. capense* (EPF) with LC₅₀ at 10.715 and LC₉₀ at 30.591 ppm showed potent adulticidal effect than crude extract, DPF and WPF (Table 8). The adulticidal effect of 80% methanol crude extract of *P. capense* fruits, DPF,

EPF and WPF were evaluated at 6, 12, 24 and 48 ppm after 24 hr exposure period against *An. arabiensis* adults (Figure 8).

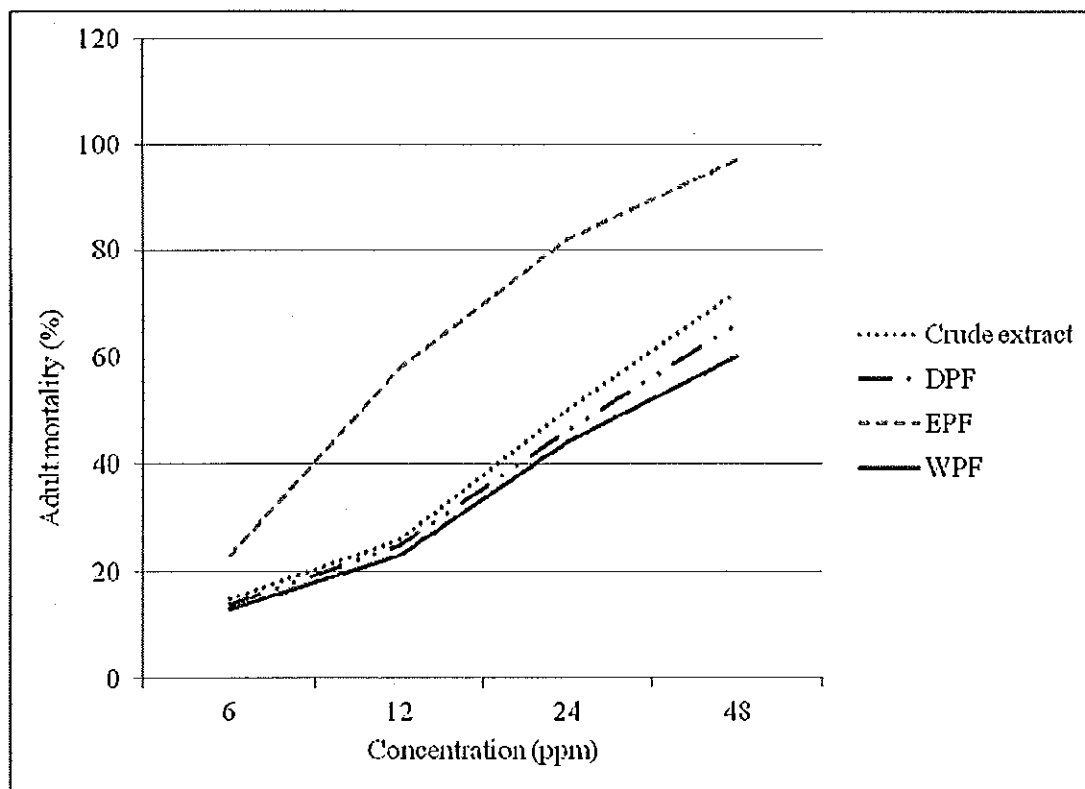


Figure 8. Adulticidal effect of 80% methanol crude extract of *P. capense* and its solvent fractions on *An. arabiensis* adults after 24 hr exposure on impregnated papers in WHO test tubes (n = 60 in each test).

Comparison of the various solvent fractions with 80% methanol crude extract of *P. capense* showed ethyl acetate fraction of *P. capense* (EPF) had more adulticidal effect against *An. arabiensis* (97% mortality at 48 ppm) than the crude extract, DPF and WPF (Figure 8). Overall, the results showed that the DCM fraction of *O. africana* leaves extract (DOF) and the EtOAc fraction of *P. capense* fruits extract (EPF) were higher in adulticidal activity against *An.*

arabensis than the different fractions obtained. The adulticidal effect of 80% methanol crude extract of *P. capense* fruits and fraction EPF were evaluated at 6, 12, 24 and 48 ppm after 24 hr exposure period against *An. arabiensis* adults (Figure 9). A comparison of the 80% methanol crude extract of *P. capense* with EPF showed ethyl acetate fraction of *P. capense* had more adulticidal effect against *An. arabiensis* (97% mortality at 48 ppm) than the crude extract and the hypothesis test for parallelism was rejected ($\chi^2 = 3.84$; $P = 0.04$) showing that slopes differed significantly (Figure 9).

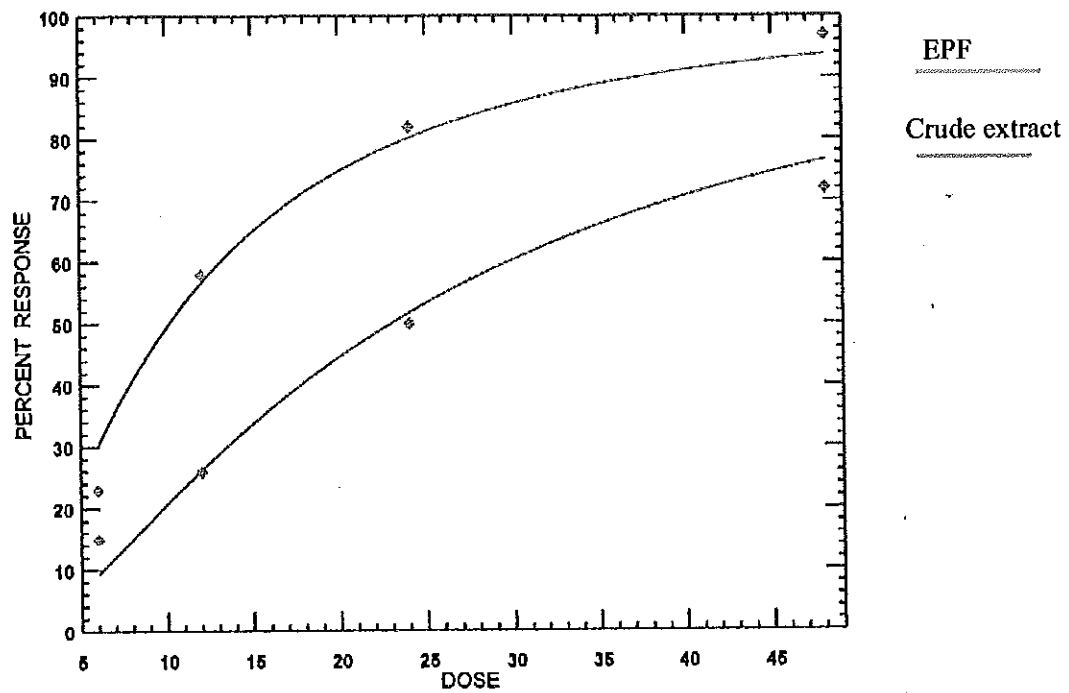


Figure 9. Percentage mortality rates of *An. arabiensis* after treatment with different concentrations of crude extract and ethyl acetate fraction of *P. capense*.

As a result, the DCM fraction of *O. africana* and EtOAc fraction of *P. capense* were subjected to a bioassay to determine the knockdown values. The fractions were tested at four concentrations ranging from 4 to 32 ppm for DOF and 6 to 48 ppm for EPF. Results of knockdown mosquitoes were recorded at 10 min interval up to 60 min. The knockdown potency of the extract fractions was also compared with the insecticide Lambdacyhalothrin (positive control) and with 0.05% DMSO in deionized water, the negative control (Table 9).

Table 9. Knockdown time efficacy of dichloromethane fraction of *O. africana* and ethyl acetate fraction of *P. capense* against *An. arabiensis* at 10 min interval of exposure period (n = 60 in each test)

Fraction code	Conc. (ppm)	% Knockdown					
		10 min	20 min	30 min	40 min	50 min	60 min
DOF ^a	4	6.7	15	25	35	41.7	46.7
	8	11.7	30	45	56.7	70	81.7
	16	18.3	41.7	60	70	80	91.7
	32	33.3	53.3	71.7	85	100	100
Positive control ^c		26.7	35	53.3	68.3	81.7	93.3
Negative control ^d		0.0	0.0	0.0	0.0	0.0	0.0
EPF ^b	6	3.3	10	18.3	26.7	31.7	31.7
	12	8.3	16.7	26.7	41.7	58.3	63.3
	24	20	31.7	40	60	73.3	86.7
	48	31.7	45	56.7	75	88.3	96.7
Positive control ^c		26.7	35	53.3	68.3	81.7	93.3
Negative control ^d		0.0	0.0	0.0	0.0	0.0	0.0

Note: ^a Dichloromethane *Oreosyce* fraction.

^b Ethyl acetate *Piper* fraction.

^c 0.05% Lambdacyhalothrin.

^d 0.05% DMSO in deionized water.

The knockdown tests showed the DOF fraction to be the most potent (100% at 32 ppm in \geq 50 minutes), which was more potent than the standard insecticide, Lambda-cyhalothrin (81.7% in 50 minutes). However, the negative control showed no knockdown effect in the concurrent assay (Table 9).

4.4. Adulticidal Activity of *Oreosyce africana* and *Piper capense* against Adult *Anopheles arabiensis* upon Fractionation

After separating the eluate by silica gel column chromatography with the hexane and chloroform solvents, a total of 28 separable fractions were eluted and the fractions pooled. Visualization of spots that indicate a constituent of each eluate was examined under UV light (366 and 254 nm) and those fractions of *O. africana* with similar TLC patterns were combined in one pool and concentrated to dryness under reduced pressure at 45°C and the dry weight yields determined in milligrams (Table 10). Thin-layer chromatography of 7 pooled fractions (I-VII) was developed with chloroform: methanol (9:1). Following the method of Grisi *et al.* (2013) aliquots of DCM fraction of *O. africana* were applied onto the columns and fractions were combined and designated as fractions I to VII as follows: O₁₋₅ as fraction I; O_{6&7} as fraction II; O₈₋₁₀ as fraction III; O₁₁₋₁₄ as fraction IV; O₁₅₋₁₉ as fraction V, O₂₂₋₂₅ as fraction VI and O₂₆₋₂₈ as fraction VII. The dry weight determinations showed higher yield for fraction IV followed by fractions III and II. Further bioassay tests against *An. arabiensis* adults were made with these purified active fractions.

Table 10. The dry weight of seven fractions of *O. africana* obtained from flash column chromatography by combining groups from twenty eight sub-fractions based on similarity in their TLC profile

Fraction number	Solvent used	Fractions combined	Yield (mg)
I	Hexane (100:0)	O ₁₋₅	17
II	Hexane-chloroform (95:5)	O _{6&7}	23
III	Hexane-chloroform (90:10)	O ₈₋₁₀	27
IV	Hexane-chloroform (80:20)	O ₁₁₋₁₄	30
V	Hexane-chloroform (70:30)	O ₁₅₋₁₉	21
VI	Hexane-chloroform (60:40)	O ₂₂₋₂₅	19
VII	Hexane-chloroform (50:50)	O ₂₆₋₂₈	22

4.4.1. Thin-layer chromatography analysis of *Oreosyce africana* fractions

The purified fractions of *O. africana* collected from flash column chromatography were analysed using TLC (Figure 10).

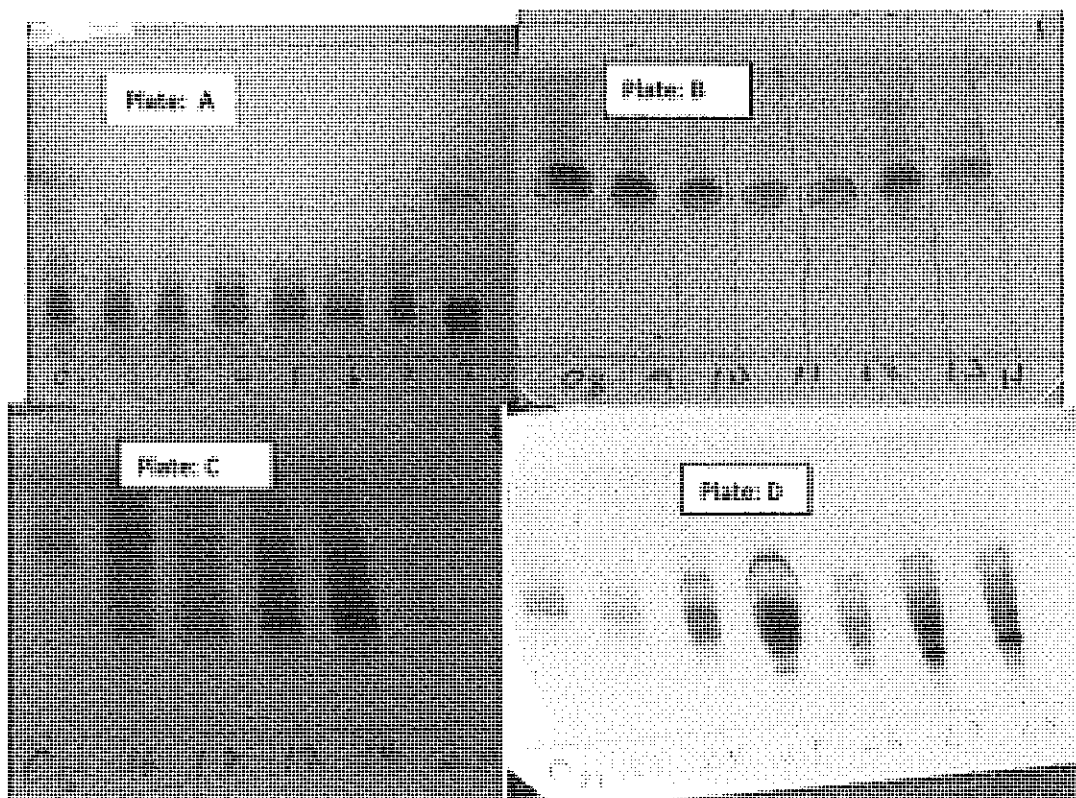


Figure 10. Thin-layer chromatograms showing the products released from *Oreosyce africana* leaf fractions collected from flash chromatography. 5 μ l of each fraction were spotted on TLC plate from left to right: O₁₋₈ was labelled as plate A, O₈₋₁₄ was labelled as plate B, O₁₅₋₂₀ was labelled as plate C and O₂₂₋₂₈ was labelled as plate D; plates A-D were developed with methanol-chloroform (1:9) solvent.

The dichloromethane fraction of *O. africana* leaf extract was dissolved with chloroform and then applied to PTLC for purification. The purification of the fractions by PTLC yielded two major yellow bands (Appendix 11). From the combined fractions two active purified fractions B2'O and B2''O were obtained after separation by PTLC on silica gel plates with chloroform-methanol

(5:1). In TLC analysis, two yellow spots corresponding to B2'O ($R_f = 0.5$) and B2''O ($R_f = 0.46$) were observed by spraying with 1% sulfuric vanillin reagent (Figure 11).

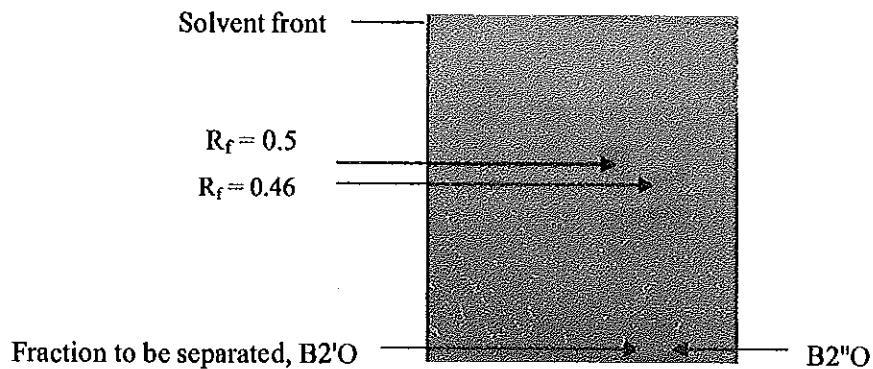


Figure 11. Purified fractions of *Oreosyce africana* (B2'O and B2''O) collected from PTLC on TLC plate developed by chloroform (100%).

4.4.2. Thin-layer chromatography analysis of *Piper capense* fractions

The ethyl acetate fraction of *P. capense* was fractionated by chromatography on Sephadex® LH-20 column chromatography and fractions with similar TLC patterns were combined in one pool and concentrated to dryness under reduced pressure at 45°C. These were combined in 3 pools. The TLC of three pooled fractions (I-III) was developed with chloroform-methanol (9:1) solvent (Figure 12). The pooled column chromatographic eluates Pc_{1,3} were designated as fraction I; eluates Pc_{4,6} as fraction II, and eluates Pc₇₋₁₂ as fraction III.

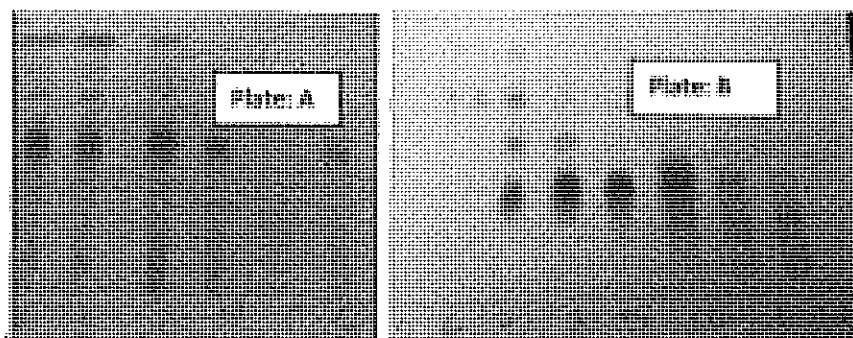


Figure 12. Thin-layer chromatograms showing the products released from *Piper capense* fruit fractions collected from Sephadex® LH-20 column chromatography. 5 μ l of fractions were spotted on each TLC plate from left to right: Pc₁₋₆ labelled as plate A, developed with methanol-chloroform (1:9) solvent and Pc₅₋₁₂ labelled as plate B, developed with chloroform (100%).

The Pc₄₋₆ fraction of *P. capense* collected from Sephadex® LH-20 (middle row of plate A in Figure 12) was again subjected to TLC using a solvent system chloroform-methanol in the ratio 1:1 and showed a violet color spot on TLC when visualized under UV light and by spraying with 1% sulfuric vanillin reagent (Figure 12).

The dry weight determinations in the fractions of *P. capense* showed higher yield for fraction I followed by fractions II and III (Table 11).

Table 11. The dry weight of three pooled fractions of *P. capense* based on their TLC profile

Fraction number	Solvent used	Fractions combined	Yield (mg)
I	Hexane (100:0)	Pc ₁₋₃	32
II	Hexane-chloroform (95:5)	Pc ₄₋₆	20
III	Hexane-chloroform (80:20)	Pc ₇₋₁₂	18

The higher yields were in fractions I (yield, 32 mg).

4.4.3. Activities of isolated components of *Oreosyce africana* and *Piper capense* on *Anopheles arabiensis* adults using impregnated papers in WHO test tubes

Comparison of the adulticidal activities of purified potent fractions of *O. africana* collected following flash column chromatography (FCC) and PTLC and the potent bioactive fractions of *P. capense* collected following Sephadex[®] LH-20 against the malaria vector, *An. arabiensis* adults are presented in Table 12.



Table 12. Adulticidal activity of different fractions of *O. africana* (fraction II, III and IV using flash column chromatography (CC); B2'O and B2''O using preparative TLC) and *P. capense* (fraction II and III from Sephadex® LH-20) against *An. arbiensis* adults 24 hr post-exposure on impregnated papers in WHO test tubes (n = 45 in each test)

Fractions*	Method used	LC ₅₀ ppm (95% CL)	LC ₉₀ ppm (95% CL)	Slope±SE	χ ² (P ^c)	LC ₅₀ ratio (95% CL)
<i>P. capense</i>						
II	Sephadex® LH-20	8.890 (7.063-12.073)	83.975 (44.390-266.239)	1.314±0.200	0.552 (0.48) ^a	ND
III	Sephadex® LH-20	13.136 (10.175-19.369)	107.348 (55.200-354.087)	1.405±0.210	1.465 (0.56) ^a	0.677(0.453-1.011)
<i>O. africana</i>						
II	Flash CC	6.906 (6.047-7.950)	23.731 (18.580-33.420)	2.391±0.229	1.666 (0.56) ^a	1.287(0.961-1.725)
III	Flash CC	7.388 (6.329-8.776)	32.366 (23.443-52.165)	1.997±0.215	1.687 (0.53) ^a	1.203(0.887-1.633)
IV	Flash CC	2.206 (1.734-2.632)	7.811 (6.470-10.173)	2.334±0.273	0.571 (0.53) ^a	4.030(2.898-5.603) ^b
B2'O	Prep. TLC	2.206 (1.734-2.632)	7.811 (6.470-10.173)	2.334±0.273	0.571 (0.53) ^a	4.030(2.898-5.603) ^b
B2''O	Prep. TLC	2.620 (1.031-3.952)	11.779 (7.263-47.258)	1.963±0.232	2.480 (0.41) ^a	3.393(2.428-4.742) ^b
Negative control**		0.0	0.0	0.0	0.0	0.0

* The codes used for the fractions; ** DMSO (0.05%) in deionized water.

^a Good fit of the data to the probit model ($P > 0.05$)

^b LC_{50} ratio significant at $P < 0.05$; 95% confidence interval did not comprise the value 1.0.

^c Probability of good fit of the probit model.

ND = Not determined

The hypothesis test for parallelism was rejected ($\chi^2 = 22.90$; $P = 0.001$) showing that slopes differed significantly. Among the fractions tested, the highest adulticidal activity was observed on post-treatment with fractions of B2'O with an LC_{50} and LC_{90} values of 2.206 and 7.811 ppm followed by fraction of B2''O with an LC_{50} and LC_{90} values of 2.62 and 11.779 ppm. The fraction III of *P. capense* was the least potent among the fractions tested with an LC_{50} and LC_{90} values of 13.136 and 107.348 ppm (Table 12). The purified fractions of *O. africana* and *P. capense* exhibited a significant difference in their adulticidal activity against *An. arabiensis*.

The order of adulticidal potencies of the purified fractions of *O. africana* and *P. capense* on *An. arabiensis* adults based on the LC_{50} and LC_{90} values in descending order is: *O. africana* B2'O > B2''O > fraction IV > fraction II > fraction III > *P. capense* fraction II > fraction III (Table 12).

4.4.4. Evaluation of the efficacy of active components of *Oreosyce africana* and *Piper capense* on adults of *Anopheles arabiensis* using aerosol sprayed inside the cage

Five purified fractions of *O. africana* and two fractions of *P. capense* exhibited different levels of adulticidal activity as shown in Figure 13.

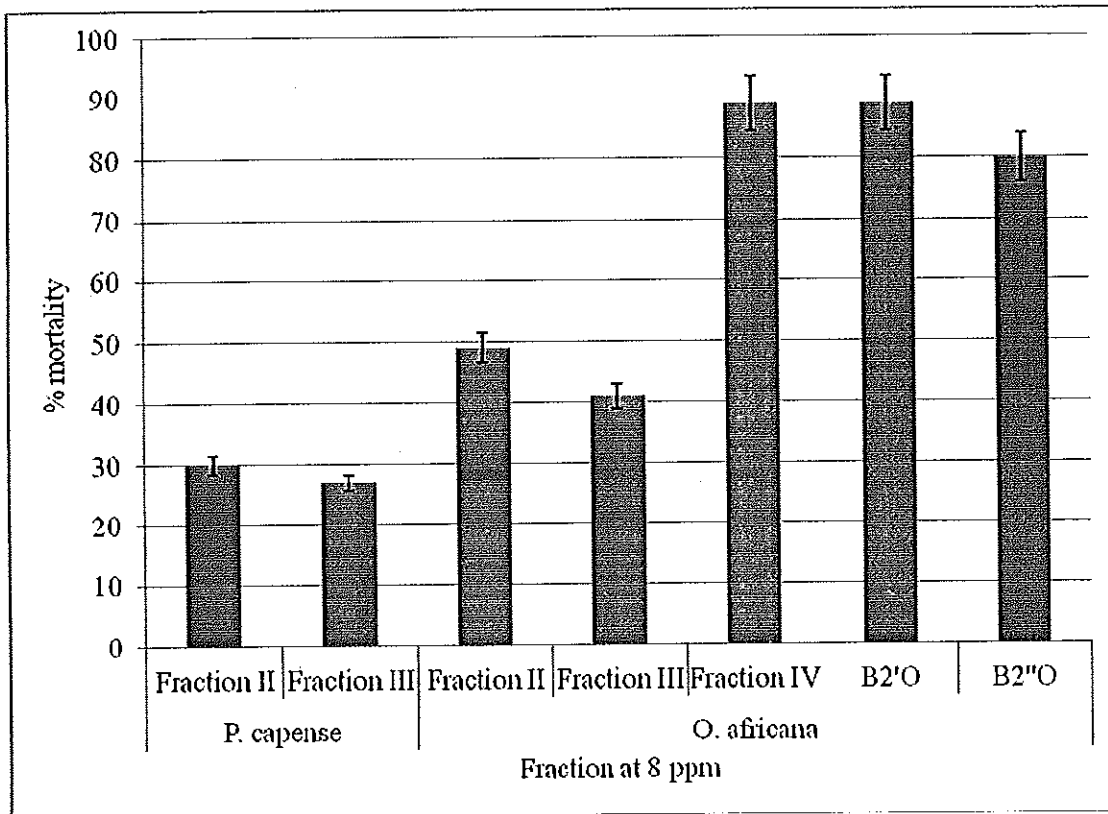


Figure 13. Mortality rates of *Anopheles arabiensis* adults following aerosol spray treatment with fractions of *Oreosyce africana* and *Piper capense* after 24 hr exposure period (n = 75 in each test).

Treatment by aerosol sprayed inside the cage against adult *An. arabiensis* with purified fractions *P. capense* fractions II and III and *O. africana* fractions II, III, IV, B2'O and B2''O at 8 ppm concentration each resulted in 30, 27, 49, 41, 89, 89, and 80 % mortality, respectively (Figure 13).

The knockdown effect of *An. arabiensis* after exposure to B2'O and B2''O potent purified fractions of *O. africana* sprayed onto mosquito cages were evaluated at 8 ppm (Figure 14).

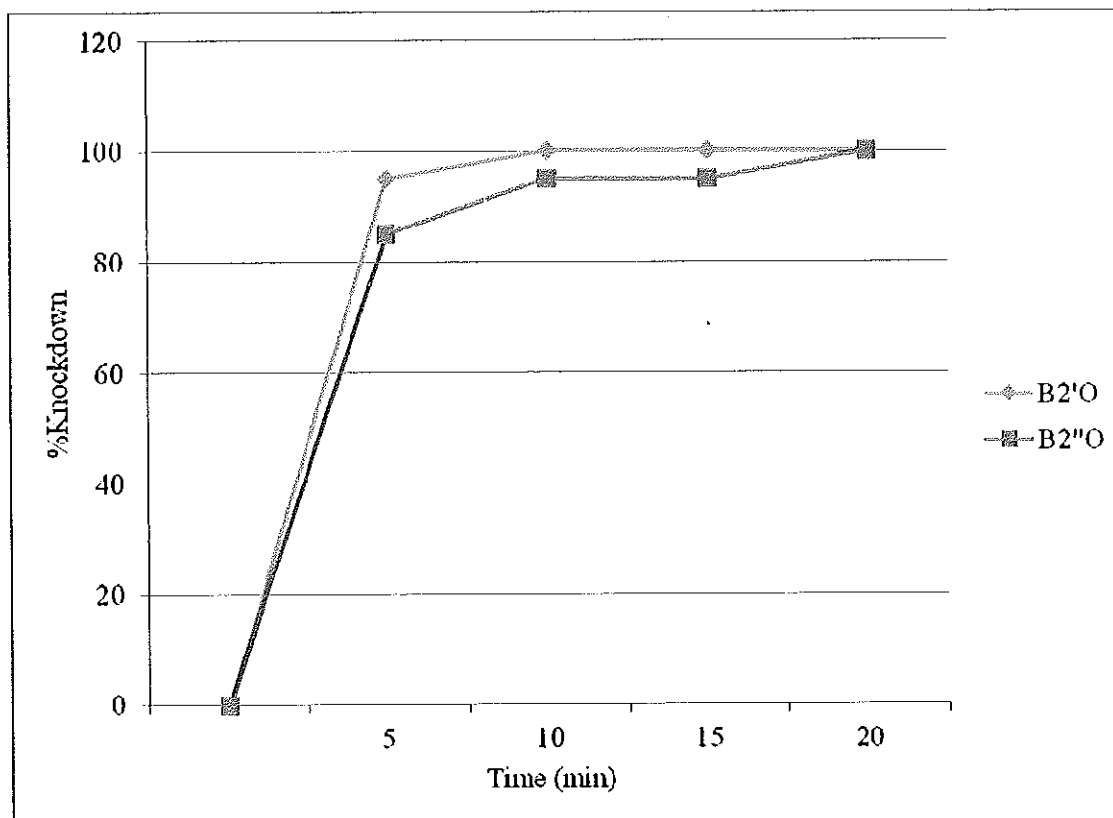


Figure 14. Knockdown effects of B2'O and B2''O purified fractions of *Oreosyce africana* at 8 ppm by using aerosol sprayed onto the net cages containing *An. arabiensis* adults after exposure at different intervals of time (n = 75 in each test).

The B2'O and B2''O of *O. africana* fractions induced 95% and 85% knockdown, respectively against *An. arabiensis* adults at a concentration of 8 ppm after 5 min of exposure. The B2'O and B2''O caused 100 and % 95% knockdown, respectively after 10 min exposure. However, the two purified fractions showed 100% knockdown after 20 min exposure (Figure 14).

Percentage mortality of *An. arabiensis* adults were determined after exposed to the isolated fraction of B2'O of *O. africana* using a direct sprayed application at 8 ppm by different weight of aerosol spray (Figure 15).

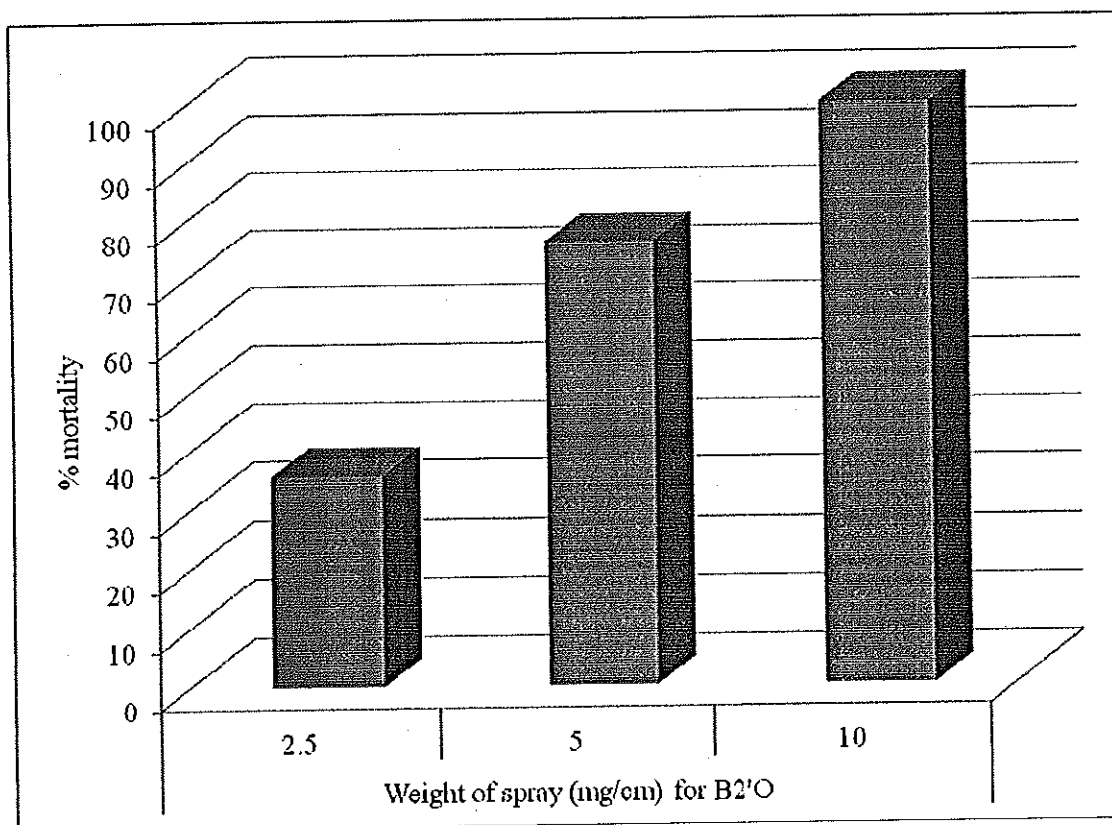


Figure 15. Effect of purified fraction B2'O sprayed onto the cage at different weight on *An. arabiensis* adults at 8 ppm after 24 hr exposure (n = 75 in each test).

The 100% mortality of *An. arabiensis* was observed in B2'O fraction of *O. africana* at 10 mg aerosol sprayed inside the cage on *An. arabiensis* adults after 24 hr exposure period while 76 and 36% mortality were observed at 5 and 2.5 mg aerosol sprayed, respectively (Figure 15). Percentage mortality was dependent on weight of aerosol sprayed.

4.4.5. Evaluation of the effects of purified potent fractions of *Oreosyce africana* against adults of *Anopheles arabiensis* using impregnated papers in WHO test tubes

The effect of the purified potent fractions, B2'O and B2''O isolated from *O. africana* were compared with that of 0.05% Lambdacyhalothrin (positive control) against adult *An. arabiensis* (Figure 16).

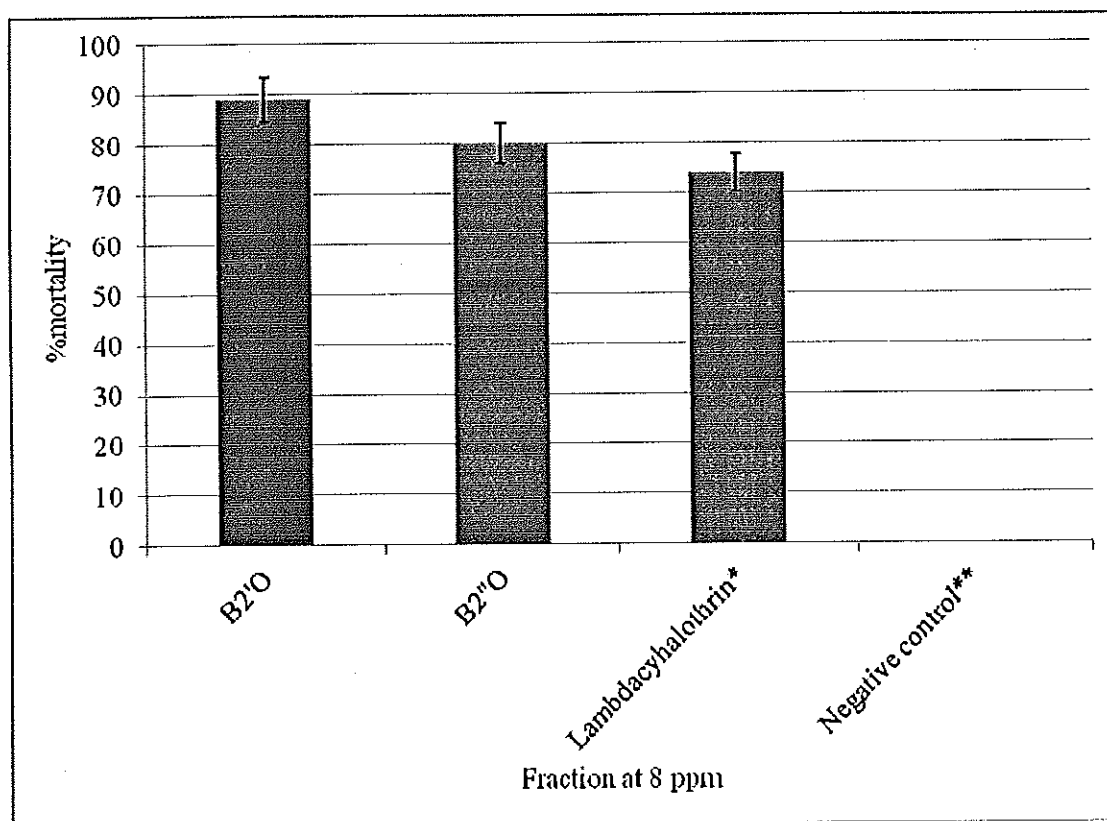


Figure 16. Comparison of activities of potent fractions of *Oreosyce africana* (B2'O and B2''O) at 8 ppm with 0.05% Lambdacyhalothrin (positive control) on *Anopheles arabiensis* adults in WHO test tubes after 24 hr exposure (n = 45 in each test).

* 0.05% Lambdacyhalothrin (positive control).

** Negative control (0.05% DMSO in deionized water).

The adulticidal activities of the purified fractions, B2'O and B2''O, at a concentration of 8 ppm each against *An. arabiensis*, caused 89 and 80% adult mortality, respectively while 0.05% Lambdacyhalothrin caused 74% adult mortality (Figure 16). There was no mortality in the untreated control group.

4.4.6. Adulticidal activities of B2'O and B2''O fractions of *Oreosyce africana* against *Anopheles arabiensis* adults using impregnated net in cone bioassay

Based on high adulticidal potency of fractions B2'O and B2''O of *O. africana*, dose-response bioassays for the adulticidal activities of the two fractions against *An. arabiensis* were conducted and mortality was observed after 24 hr exposure period. The percentage adult mortality against the logarithm of concentrations was plotted based on the mean of three replicates (Figures 17 to 19).

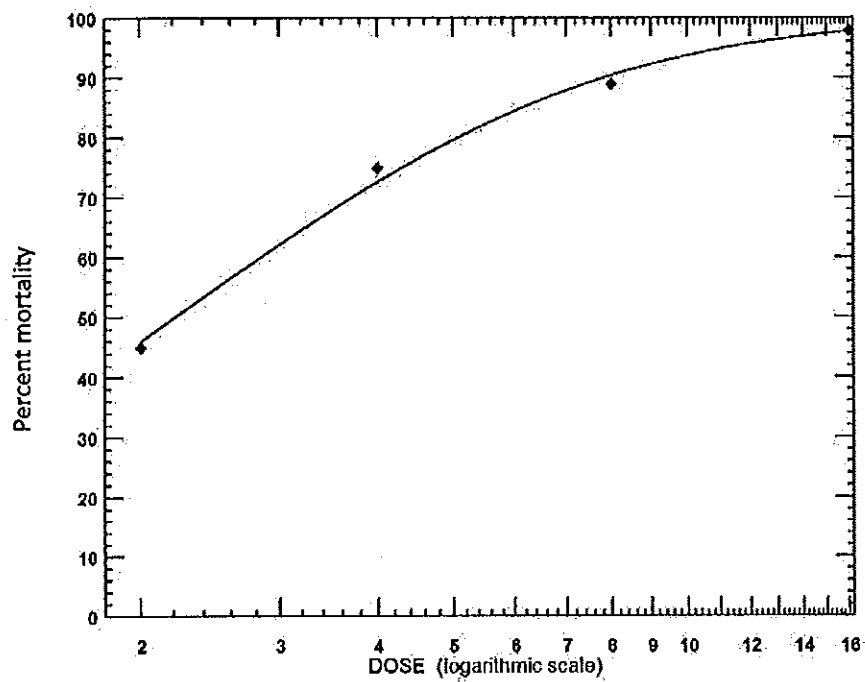


Figure 17. Probit regression plot showing percent mortality of adult *Anopheles arabiensis* after exposure to different concentrations of B2'O, purified fraction of *Oreosyce africana* (n = 45 in each test).

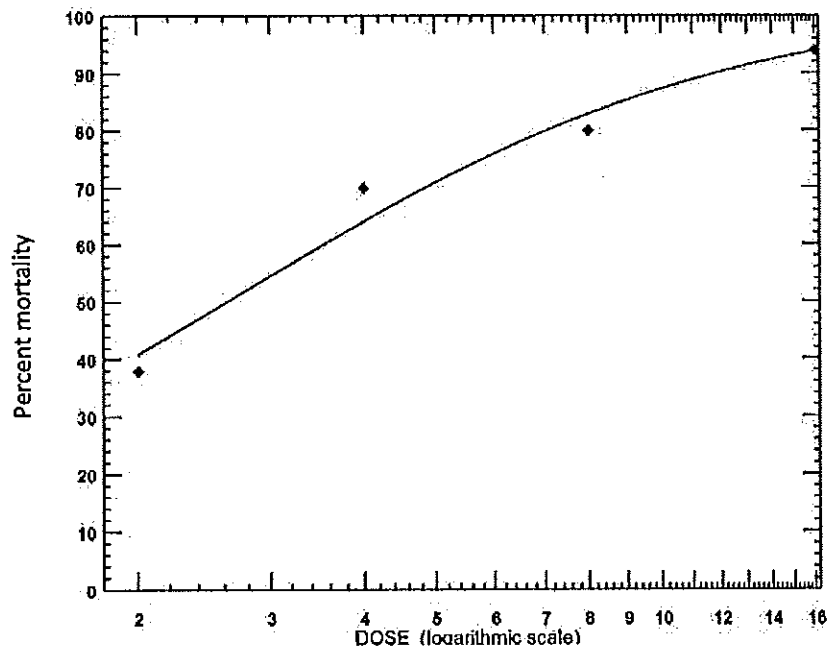


Figure 18. Probit regression plot showing percent mortality of adult *Anopheles arabiensis* after exposure to different concentrations of B₂O, purified fraction of *Oreosyce africana* (n = 45 in each test).

The determination of adulticidal activities of B₂O and B₂O (Figures 17 and 18) clearly indicated the percentage of *An. arabiensis* adult mortality to be directly proportional to the concentration of the fractions.

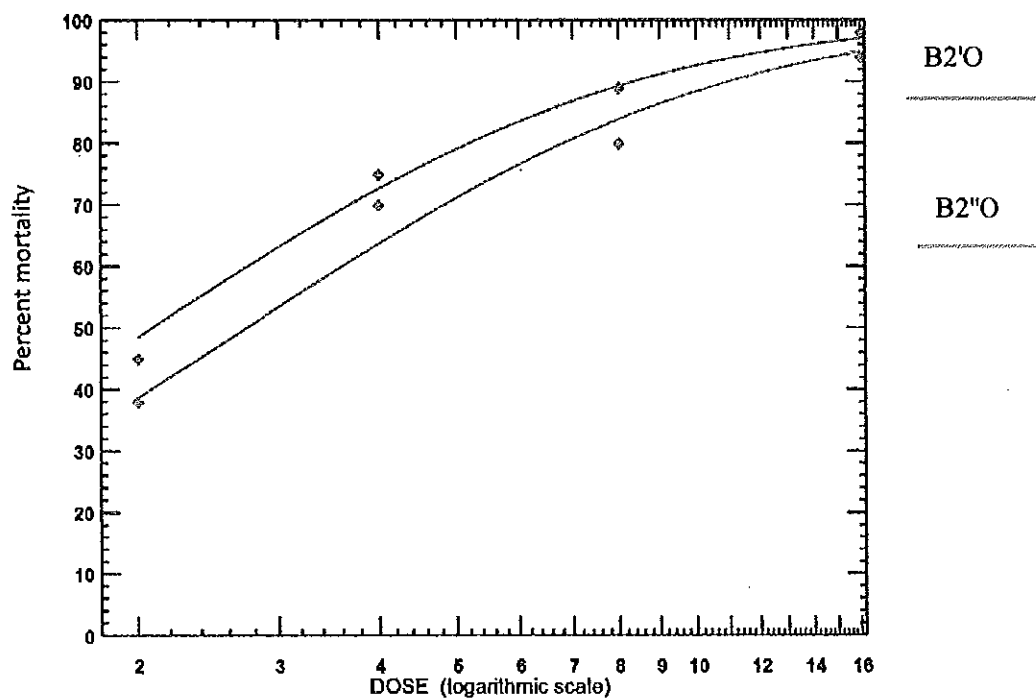


Figure 19. Parallel case regression plot showing percent mortality of adult *Anopheles arabiensis* after exposure to different concentrations of B2'O and B2''O, the purified fractions of *Oreosyce africana* (n = 45 in each test).

Plot of residuals against predicted values were between -2 and 2, which indicated its fitted plot (Figures 20 and 21).

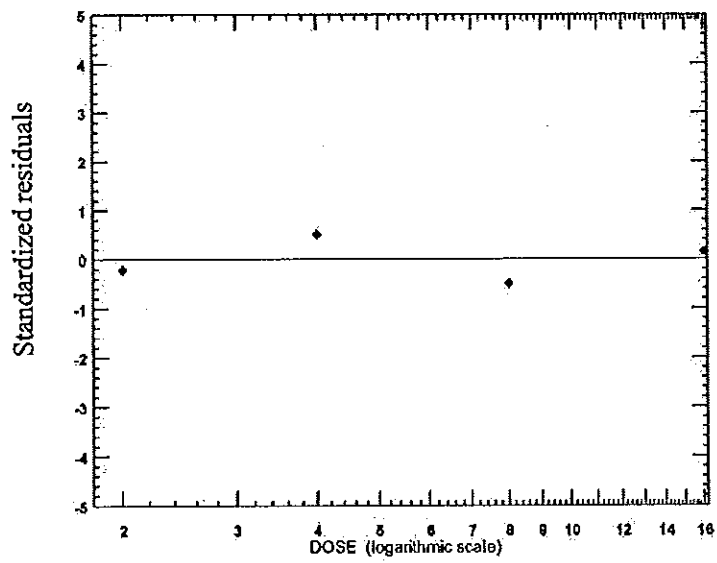


Figure 20. PoloPlus plot showing standardized residual of B2'O fraction of *Oreosyce africana* at different concentrations on *Anopheles arabinesis* adults (n = 45 in each test).

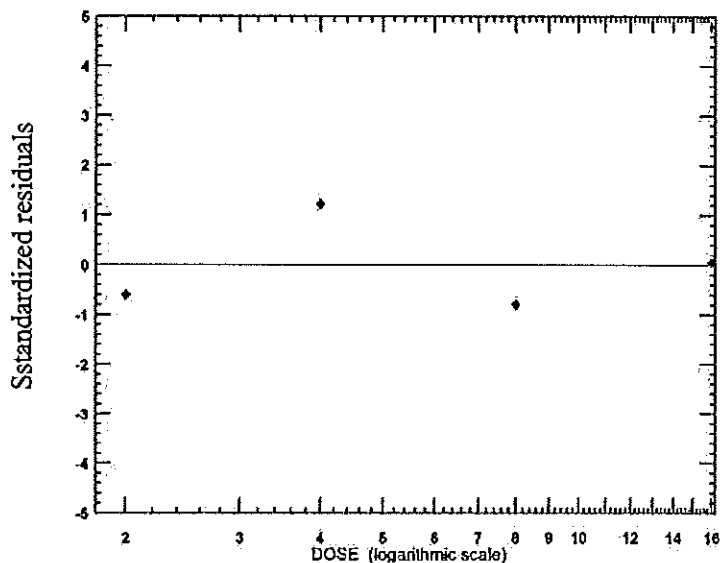


Figure 21. PoloPlus plot showing standardized residual of B2'O fraction of *Oreosyce africana* at different concentrations on *Anopheles arabiensis* adults (n = 45 in each test).

4.4.7. Evaluation of the B2'O purified fraction of *Oreosyce africana* on *Anopheles arabiensis* adults using impregnated net cages

The B2'O, purified fractions isolated from *O. africana* using PTLC were tested for adulticidal activity against *An. arabiensis*. The persistence or the residual effect of the fraction B2'O of *O. africana* against *An. arabiensis* was also demonstrated through a weekly assessment for three consecutive months starting from April, 2014 to June, 2014 (Figure 22).

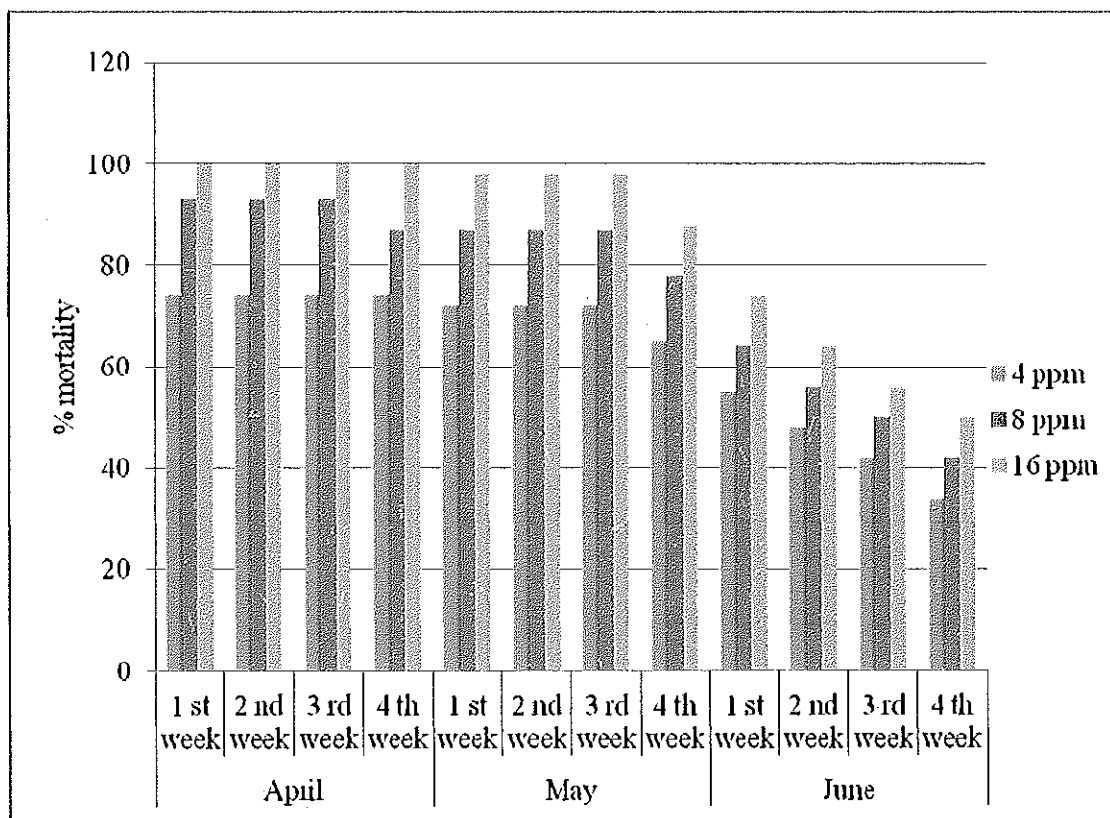


Figure 22. Percentage mortality of *Anopheles arabiensis* adults at various concentrations in ppm after 24 hr exposure with fraction B2'O of *Oreosyce africana* at one week intervals from April, 2014 to June, 2014.

* At each concentration three replicates of 30 *An. arabiensis* adults per test were run and no mortality was observed in the negative control.

The active fraction of B2'O of *O. africana* leaves exhibited the mean *An. arabiensis* adult mortality which varied from 74% at 4 ppm to 100% at 16 ppm in April 2014, 72% at 4 ppm to 98% at 16 ppm in May 2014 and the % mortality was decreased in June 2014 after 24 hr exposure period against adults of *An. arabiensis* (Figure 22). With regard to the perisistence of

activity of the active fraction of B2'O on impregnated nets, the presented data showed the active fractions were stable as their adulticidal activity persisted up to two months (Figure 22).

4.5. High Performance Liquid Chromatography Analysis of *Oreosyce africana* Fractions

The purity of the isolated fractions of *O. africana* including fraction IV collected from flash column chromatography, fractions B2'O and B2''O collected from preparative TLC were determined by HPLC analysis using ODS2 column (Figures 23 to 25).

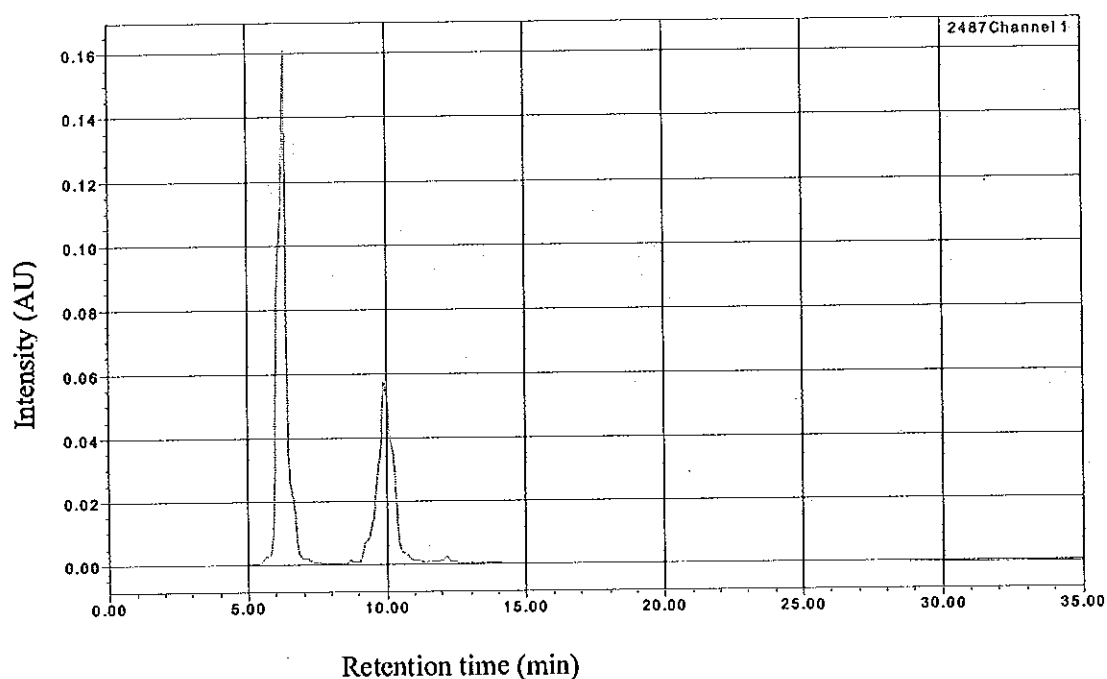


Figure 23. High performance liquid chromatograms for fraction IV of *O. africana*, detection at 254 nm.

As shown in Figure 23, the HPLC profile of fraction IV of *O. africana* gave the first peak at the retention time (R_t) of 6.329 minute with a % of 61.43 and the second peak at retention time of 9.921 minute with a % of 37.52.

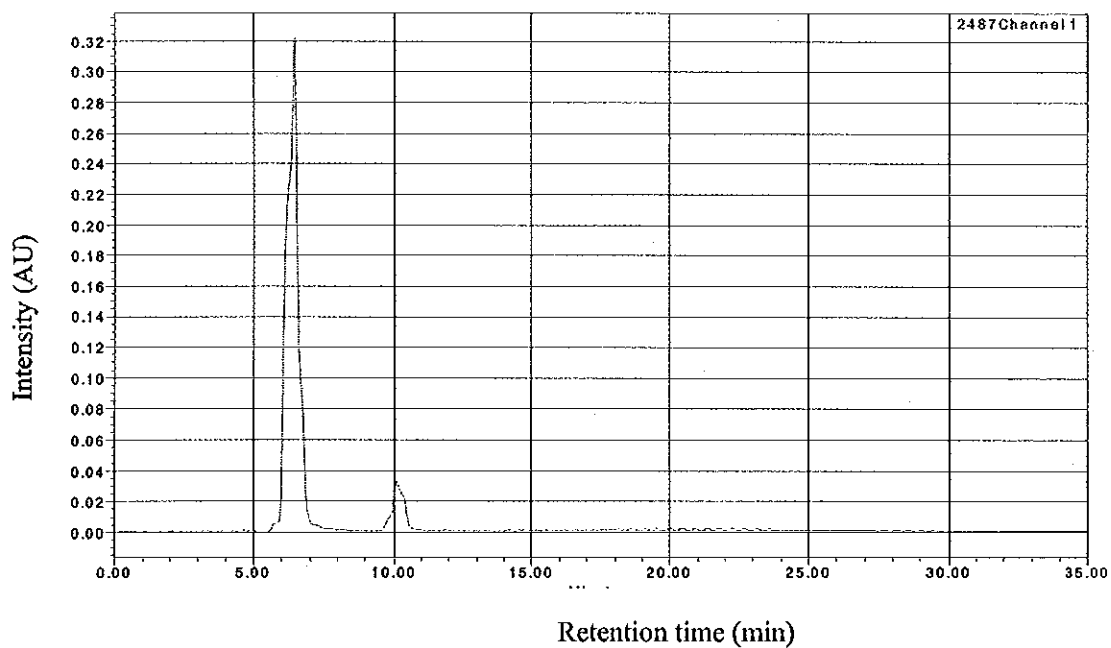


Figure 24. High performance liquid chromatograms for B2'O fraction of *O. africana*, detection at 254 nm.

High performance liquid chromatograms analysis showed that the retention times (R_t) of the two peaks were 6.398 and 10.471 minute with 91 and 5.4% area under the peak, respectively (Figure 24).

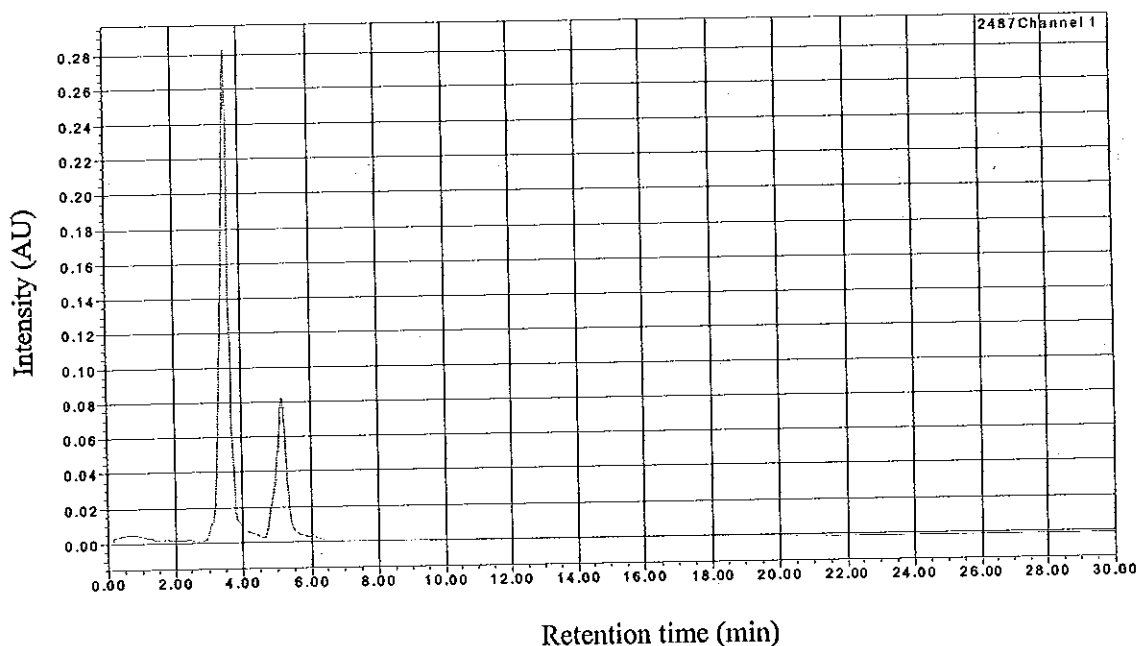


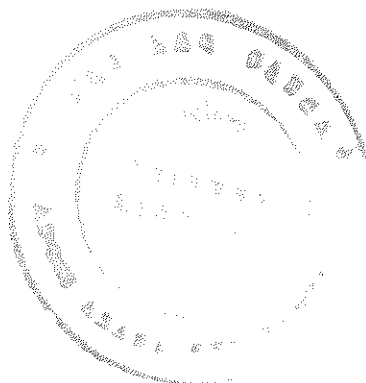
Figure 25. High performance liquid chromatogram for B2'O fraction of *O. africana*, detection at 254 nm.

The HPLC chromatogram showed two peaks of which the first one was the major peak and the second was lesser peak (Figure 25). The first peak presented at retention time (R_t) 3.565 minute with area % of 72.30 and the second peak appeared at R_t 5.175 minute with a % of 24.76.

4.6. Chemical Identification of Active Components of *Oreosyce africana*

4.6.1. Identification of compounds from B2'O fraction of *Oreosyce africana*

Structures of purified adulticidal active compounds of *O. africana* were elucidated using NMR spectroscopic technique. Proton (^1H)-NMR, ^{13}C -NMR and DEPT-135 spectra for the isolated B2'O fraction from *O. africana* are showed, respectively (Figures 26 to 28). Among isolated fractions of *O. africana* the one which was labelled as B2'O was submitted for NMR analysis



and obtained complete spectra for structural elucidation. Observation of $^1\text{H-NMR}$ spectrum of purified fraction B2'O showed distinct signals (Figure 26).

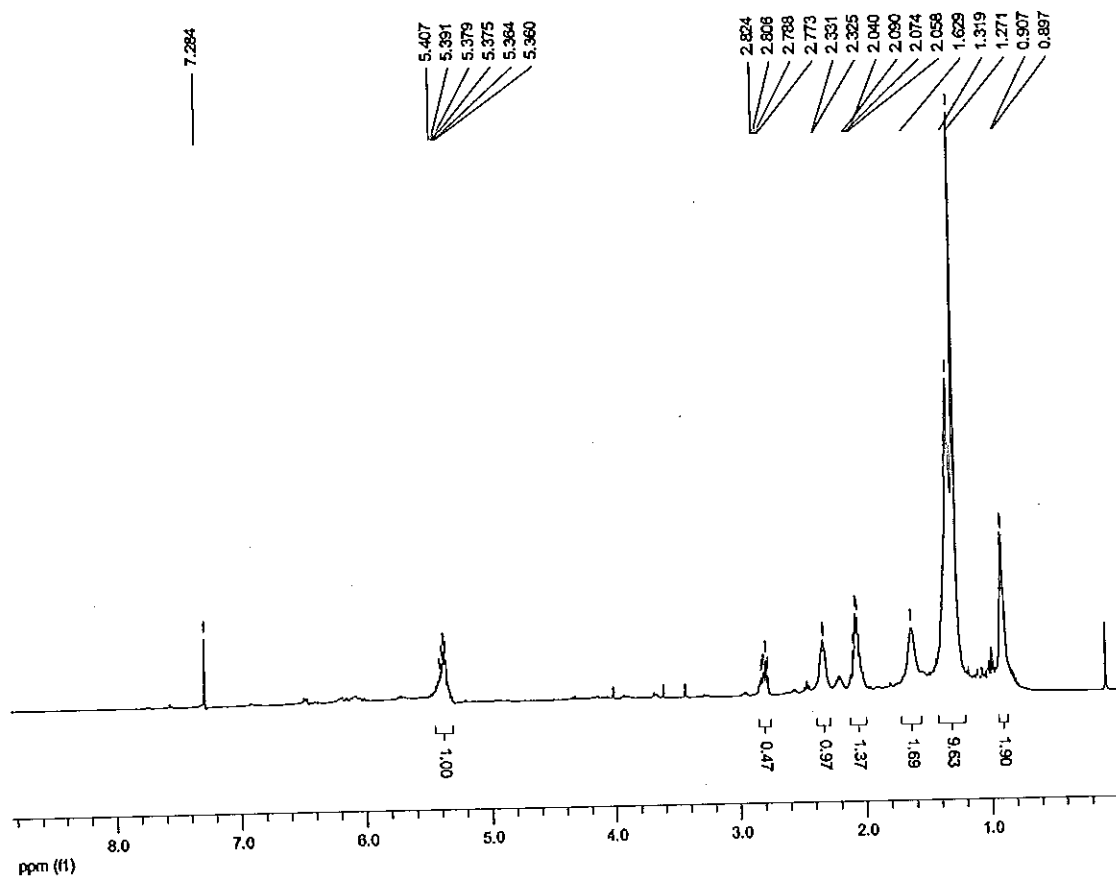


Figure 26. $^1\text{H-NMR}$ spectra of major fractions of B2'O from *Oreosyce africana*.

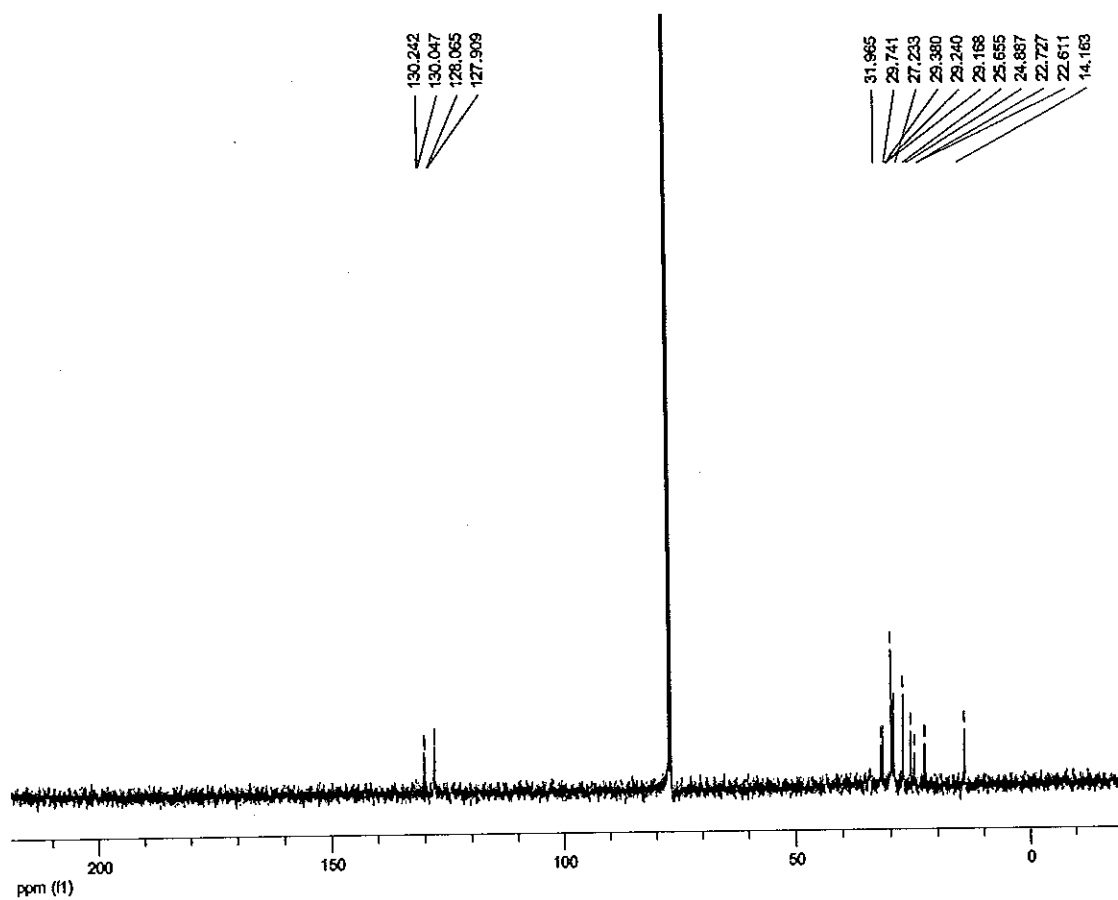


Figure 27. ^{13}C -NMR spectra of major fractions of B2'O from *Oreosyce africana*.

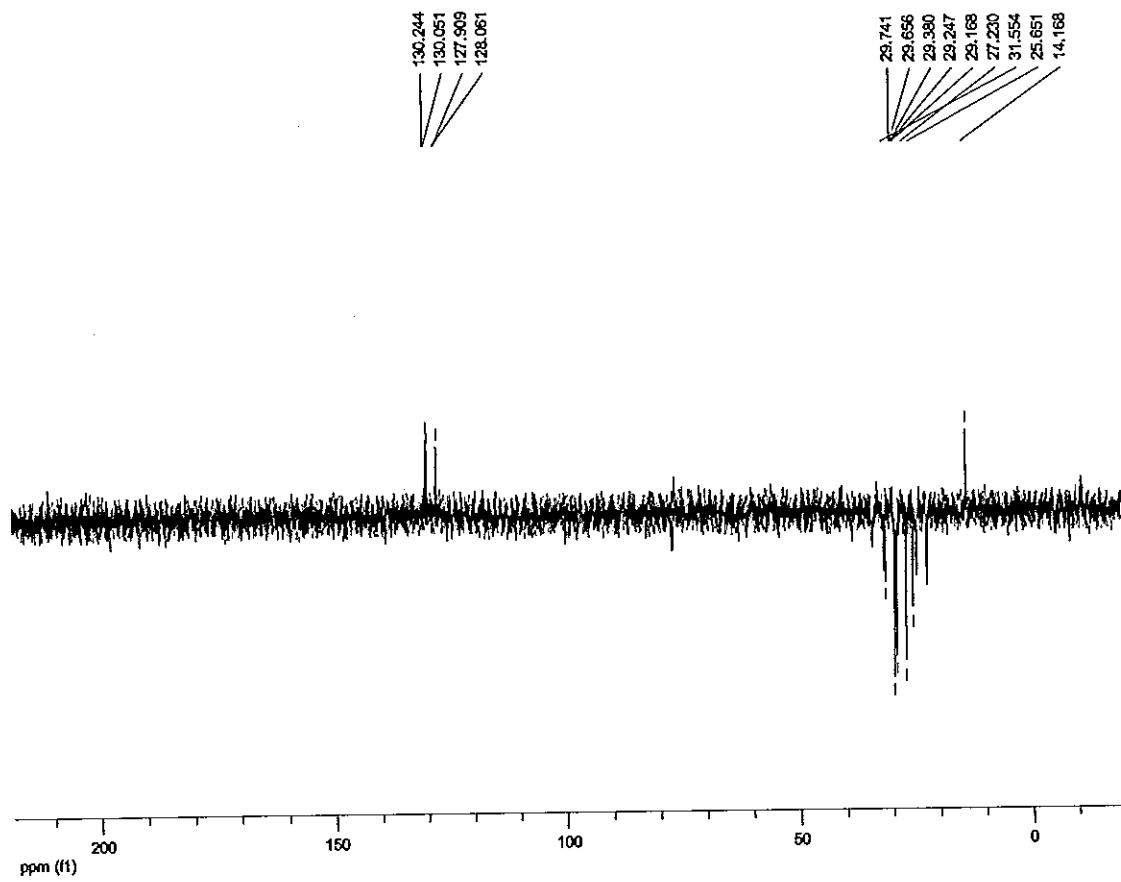


Figure 28. DEPT-135 NMR spectra of major fractions of B2'O from *Oreosyce africana*.

The proton NMR spectrum of compound B2'O as presented in Figure 26 showed proton signals that belong to alkenic and alkanic protons. The signal at δ 5.4 ppm (4H, multiplet) is ascribed to the presence of four olefinic protons in the structure of the compound. This was confirmed by the appearance of four peaks at δ 130.2, 130.0, 128.0 and 127.9 ppm in the carbon NMR spectrum of the compound (Figure 27). The presence of multiplet at δ 2.8 ppm which integrates two protons is due to protons allylic to two double bonds. The additional peak in the double bond region confirmed the presence of double bonds. The presence of signal adjacent to carbonyl carbon is ascertained by the presence of signal at δ 2.3 ppm in its proton NMR spectrum. The presence of many overlapping carbons was also observed from the broad signals appearing at δ 1.3 ppm. The presence of terminal methyl signal is confirmed by the appearance of signal at δ 0.89 ppm.

The DEPT spectrum of this compound depicted signals in the positive mode (Figure 28). One of them belongs to methyl at δ 14.1 ppm and the remaining are due to methine carbon.

The gas chromatogram of fraction coded B2'O of *O. africana* obtained is presented in Figure 29.

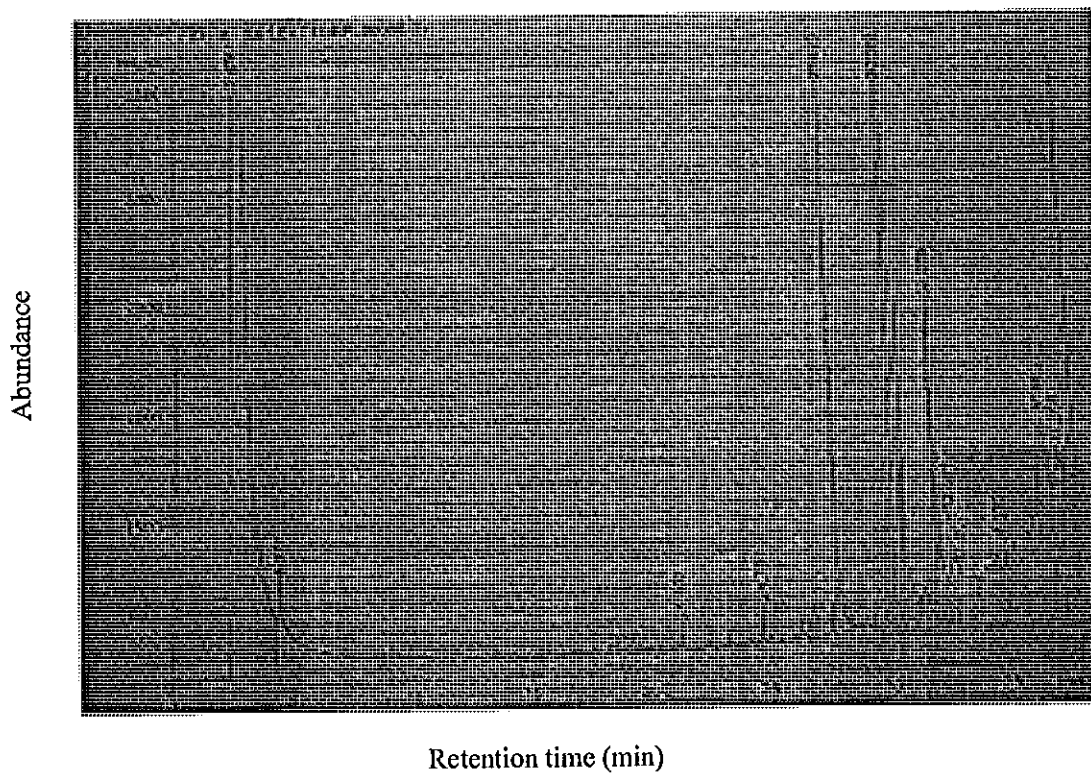


Figure 29. Gas chromatogram of B2'O fraction obtained from the isolated extract of *Oreosyce africana* leaf.

As presented in Figure 29, 12 peaks or compounds were separated by gas chromatogram. Among peaks observed in the GC chromatogram, peak appearing at retention time of 2.476 min was subjected to mass spectroscopy (MS) analysis. The spectrum of this peak at scan time of 28.304 min is shown in Figure 30.

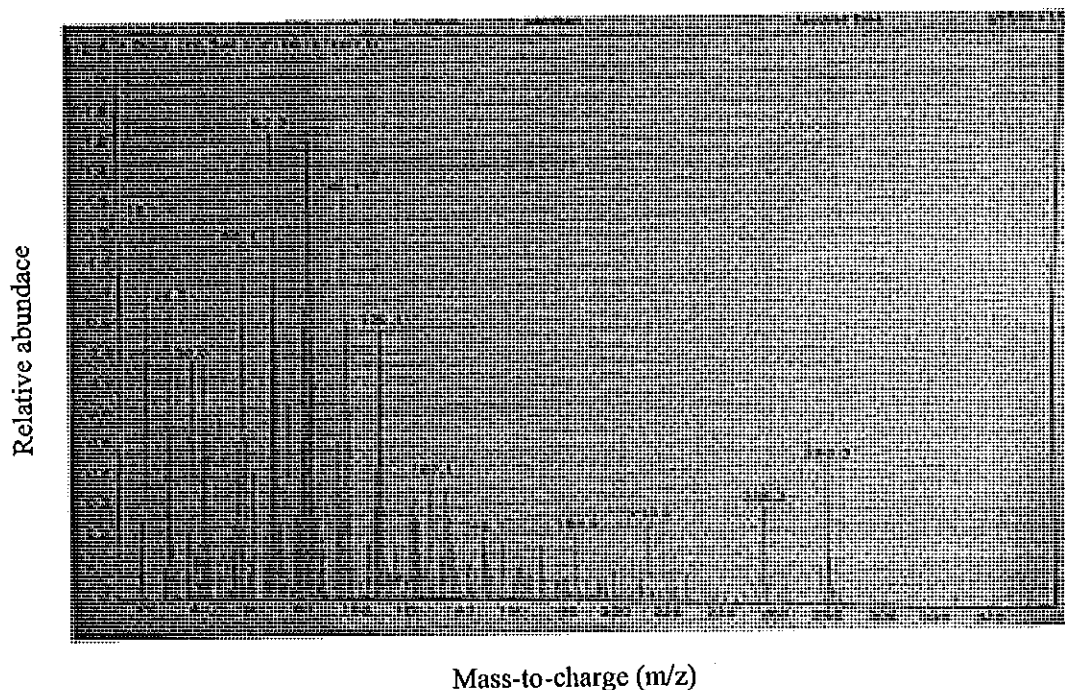


Figure 30. Mass spectra of the 9,12-Octadecadienoic acid (Z,Z)- and fragmentation patterns of the peak at scan time of 28.304 min for B2'O fraction isolated from *Oreosyce africana* leaf.

The mass spectrum presented in Figure 30 reveals the structure of 9,12-Octadecadienoic acid (Z,Z)- more commonly known as linoleic acid, was isolated and identified in the B2'O of *O. africana* extract (Appendix 15). The mass spectrometer analysis showed a parent peak at m/z 280 and corresponded to a molecular formula of $C_{18}H_{32}O_2$. Also, observed fragments are peaks at m/z 256.3, 129.1, 109.1, 95.1, 67.0, 55.1, 36.0, 28.0 and 18.1 in the mass spectra (Figure 30). With the above fragmentation patterns observed for the compound, in comparison to library values, the name, molecular weight, structure of the component of the test material was ascertained (Table 13). The molecular formula was deduced from the mass spectra in combination with the 1H - and ^{13}C -NMR and DEPT spectra presented above.

Most main peaks were identified by comparing their mass spectra and retention time from GC with those of reference compounds. Accordingly, in the present study, twelve compounds have been identified from purified extract of the leaves of *O. africana* by GC-MS analysis. The active principles, retention time (R_t), molecular formula, molecular weight and the approximate relative percentages of this B2'O fraction, analysed by GC-MS are listed in Table 13.

Table 13. Compounds identified in the isolated fraction of B2'O from *O. africana* by GC-MS

S.No.	Retention time (min)	Name of the compound	Molecular formula	Molecular weight	Peak area % ^a
1	2.476	9, 12-Octadecadienoic acid (Z,Z)-	C ₁₈ H ₃₂ O ₂	280	98.34847
2	4.377	Isopropyl linoleate	C ₂₁ H ₃₈ O ₂	322	0.06866
3	21.392	9,12-Octadecadienoic acid(Z,Z),2-hydroxy-1-[hydroxymethyl]ethyl ester	C ₂₁ H ₃₈ O ₄	354	0.00452
4	24.791	9, 12-Octadecadienoic acid, ethyl ester	C ₂₀ H ₃₆ O ₂	308	0.00736
5	27.923	E,E,Z-1,3,12-Nonodecatriene-5,14-diol	C ₁₉ H ₃₄ O ₂	294	0.15081
6	30.382	Ethanol,2-(9,12-Octadecadienyloxy)-, (Z,Z)-	C ₂₀ H ₃₈ O ₂	310	0.57465
7	32.078	10,13-Octadecadienoic acid, methyl ester	C ₁₉ H ₃₄ O ₂	294	0.1329
8	32.681	cis-13,16-Docosadienoic acid, methyl ester	C ₂₃ H ₄₂ O ₂	316	0.03463
9	33.067	9,12-Octadecadienoic acid (Z,Z)-, methyl ester, (E,E)-	C ₁₉ H ₃₄ O ₂	294	0.07472
10	34.839	10-Octadecenoic acid, methyl ester	C ₁₉ H ₃₆ O ₂	296	0.11585
11	36.663	9,12-Octadecadienoic acid, methyl ester, (E,E)-	C ₁₉ H ₃₄ O ₂	294	0.37539
12	37.265	9,12-Hexadecadienoic acid, methyl ester	C ₁₇ H ₃₀ O ₂	266	0.11205

^a Relative percentage of total sample.

The results pertaining to GC-MS analysis confirmed the identification of twelve compounds with different retention times from the GC fractions of the purified extract of *O. africana* leaves (Table 13). Gas chromatogram showed that the major compounds in the B2'O of *O. africana* was

found to be 9,12-Octadecadienoic acid (Z,Z)-, which constituted 98.34847% as the most intense peak, followed by E,E,Z-1,3,12-Nonodecatriene-5,14-diol (0.15081%), Ethanol,2-(9,12-Octadecadienyloxy)-, (Z,Z)- (0.57465%), 10,13-Octadecadienoic acid, methyl ester (0.1329%), 10-Octadecenoic acid, methyl ester (0.11585%), and 9,12-Hexadecadienoic acid, methyl ester (0.11205%).

4.6.2. Identification of compounds from B2"O fraction of *Oreosyce africana*

Structures of purified adulticidal active compound for B2"O were elucidated using GC-MS spectroscopic technique (Figures 31 and 32).

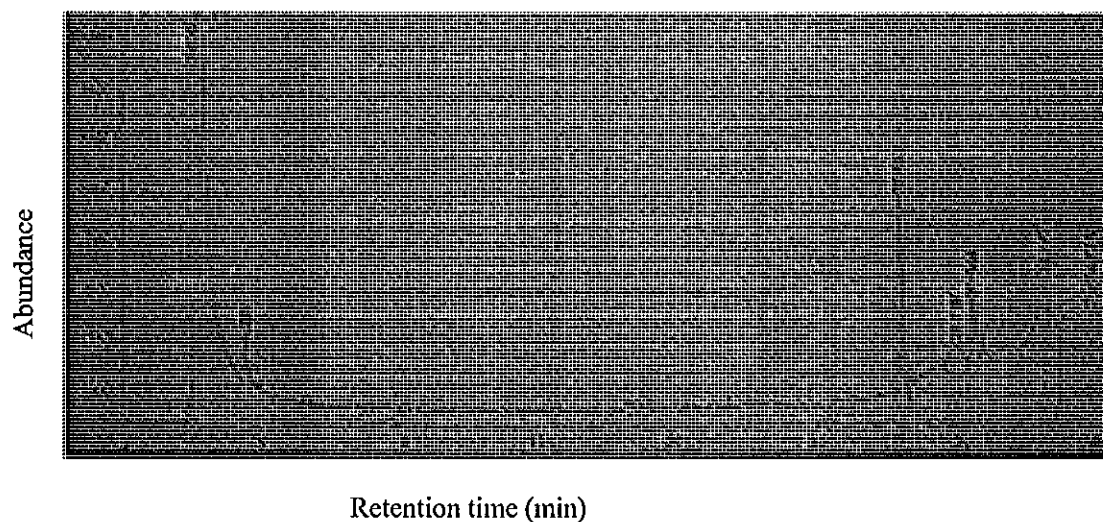


Figure 31. Gas chromatogram of B2"O fraction obtained from the isolated extract of *O. africana* leaf.

As presented in Figure 31, seven peaks or compounds were separated in gas chromatogram. Among peaks observed in the GC chromatogram, peak appearing at retention time of 2.506 min was subjected to mass spectroscopy (MS) analysis. The spectrum of this peak at scan time of

25.376 min is shown in Figure 32.

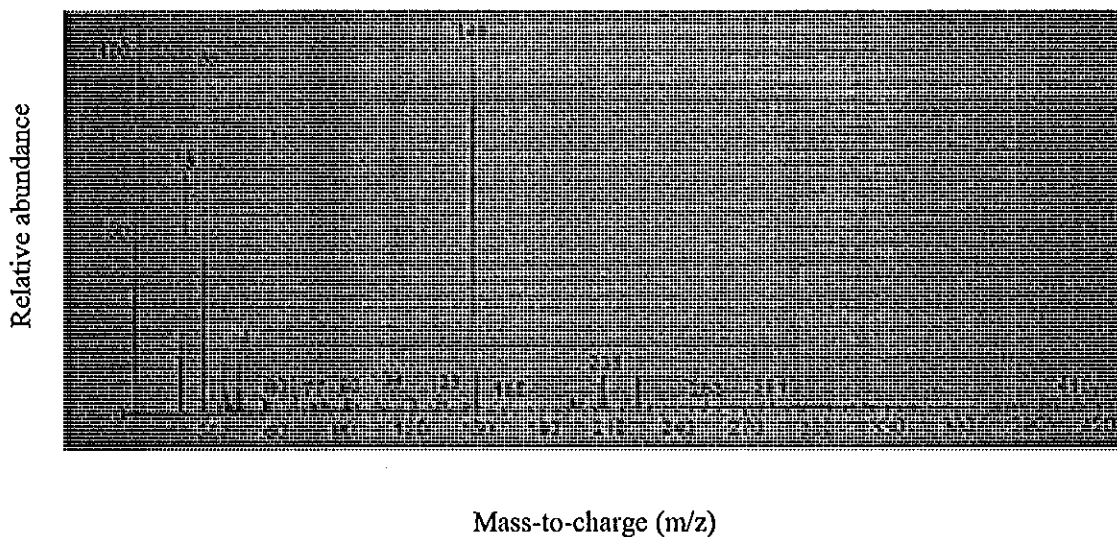


Figure 32. Mass spectra of the Dibutyl phthalate and fragmentation patterns of the peak at scan time of 25.376 min for B2"O fraction isolated from *Oreosyce africana* leaf.

Based on the mass spectral data, a parent peak was detected at m/z 278 and fragments at m/z 18, 28, 44, 109, 149, and 208 (Figure 32). The mass spectrum of B2"O fraction gave a molecular formula of $C_{16}H_{22}O_4$ and the compound was determined as dibutyl phthalate.

Seven compounds have been identified from B2"O purified fraction of the leaf extracts of *O. africana* by GC-MS analysis. The active principles, retention time (R_t), molecular formula, molecular weight and the approximate relative percentages of this B2"O fraction, analysed by GC-MS are presented in Table 14.

Table 14. Compounds identified in the isolated fraction of B2"O from *O. africana* by GC-MS

S.No.	Retention time (min)	Name of the compound	Molecular formula	Molecular weight	Peak area % ^a
1	2.506	Dibutyl phthalate	C ₁₆ H ₂₂ O ₄	278	97.7535
2	4.399	1,2-Benzenedicarboxylic acid, butyl octyl ester	C ₂₀ H ₃₀ O ₄	334	0.053
3	27.891	Ethaneperoxoic acid, 1-cyano-1-[2-(2-phenyl-1,3-dioxolan-2-yl)ethyl]pentyl ester	C ₁₉ H ₂₅ NO ₅	347	0.38466
4	29.796	Phthalic acid, butyl dodecyl ester	C ₂₄ H ₃₈ O ₄	390	0.05628
5	30.344	Phthalic acid, butyl tetradecyl ester	C ₂₆ H ₄₂ O ₄	418	0.32408
6	32.795	Phthalic acid, butyl nonyl ester	C ₂₁ H ₃₂ O ₄	348	0.30852
7	34.515	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	C ₁₆ H ₂₂ O ₄	278	0.30972

^a Relative percentage of total sample.

In GC-MS analysis of the B2"O fraction seven compounds were identified, among which Dibutyl phthalate constituted the largest proportion (97.7535%) followed by Ethaneperoxoic acid, 1-cyano-1-[2-(2-phenyl-1,3-dioxolan-2-yl)ethyl]pentyl ester (0.38466%), Phthalic acid, butyl tetradecyl ester (0.32408%), and 1,2-Benzenedicarboxylic acid, butyl octyl ester had the least proportion of 0.053% (Table 14).

Structural elucidation of B2"O fraction isolated from *O. africana* leaf extracts was achieved by GC-MS spectral data (Appendix 16) and the mass spectrum of B2"O gave a molecular ion peak at m/z 278 for C₁₆H₂₂O₄.

5. Discussion

The rationale for considering plant materials for anti-mosquito effects was premised on the fact that plants are rich sources of bioactive secondary metabolites and offer an advantage over synthetic insecticides as their extracts are less toxic, less prone to development of resistance, and easily biodegradable thereby reducing the possible accumulation of toxic residues in the environment (Prabakar and Jebanesan, 2004). The information supplied by local traditional medicine practitioners and knowledgeable elders from the geographical areas where the plants were collected were the most critical guide in the study. The ethnobotanically guided approach lead to identifying *Aloe pirottae* and *Acokanthera schimperi* with larvicidal effects and *B. nigra*, *O. africana* and *P. capense* with different levels of adulticidal potencies against *An. arabiensis*.

Earlier reports showed on the multiple medicinal values of the extracts from *A. pirottae*. *Acokanthera schimperi* was also included in the study because of the ethnobotanical information about its use as a repellent against mosquitoes (Belayneh *et al.*, 2012). Reports on *O. africana* showed that it had diverse effects, such as anthelmintic (Yamada, 1999), anti-gonorrhoeal (Yineger and Yewhalaw, 2007) and anti-malarial (Chifundera, 2001).

The use of methanol as a solvent to extract mosquitocidal chemical components was justified on the basis of earlier works (Mwangi and Mukiama, 1988; Sivagnaname and Kalyanasundaram, 2004). Similar to the mosquito (*Ae. aegypti*) larvicidal effect of methanol extracts of *Ocimum canum* (Lamiaceae) leaves (LC₅₀ at 72.40 ppm) (Bagavan *et al.*, 2009) and the root extracts of *Balanites aegyptiaca* (L.) Del (Balanitaceae) (LC₅₀ at 289.59 ppm) (Patil *et al.*, 2010), methanol extracts of *A. pirottae* gel and *A. schimperi* leaves were also larvicidal. The effect of *A. pirottae* gel extract was more potent than that reported by Karunamoorthi and Ilango (2010) for the

methanol leaf extracts of *Croton macrostachyus* (LC₅₀ of 89.25 and LC₉₀ of 224.98 ppm) against late third instar larvae of *An. arabiensis*. However, a much more higher potency compared to *A. pirottae* and *A. schimperi* methanol extracts, was reported for *Cassia fistula* L. (Fabaceae) against *Cx. quinquefasciatus* and *An. stephensi*, with LC₅₀ values of 17.97 ppm and 20.57 ppm, respectively (Govindarajan *et al.*, 2008). On the other hand, the larvicidal effects of *A. pirottae* and *A. schimperi* extracts against *An. arabiensis* was comparable to what Kumar and Maneemegalai (2008) reported for the methanol leaf extracts of *Lantana camara* L. (Verbanaceae) which caused 100% mortality at a concentration of 2.0 mg/ml on fourth instar larvae of *Cx. quinquefasciatus*.

As suggested by Mwangi and Mukiana (1988), the larvicidal effects of methanol extracts of *A. pirottae* and *A. schimperi* are most likely caused by some neuro-muscular effects of the active principles. The lack of significant effect of these extracts on the adult mosquitoes may indicate the larval stage specificity of the active principles contained in the extracts.

Of the four plant extracts tested initially against the adults of *An. arabiensis*, *A. pirottae* and *B. nigra* extracts were dropped from further testing since their methanol crude extracts had very low lethal effects. On the other hand, the finding that methanol extracts of the leaves of *O. africana* and fruits of *P. capense* were effective against adult *An. arabiensis* at lower crude extract concentrations prompted further detailed investigation of the effects of their fractionated extracts.

The demonstrated high adulticidal activity of methanol extract of *O. africana* against adult *An. arabiensis* was a confirmation of the ethnobotanical information provided for its use as an insecticide against various ectoparasitic pests on cattle (Bekele *et al.*, 2012). The adult mosquitocidal effects of *O. africana* and *P. capense* methanol extracts were about 2 to 4-fold

more than what was reported for other botanicals with adult mosquitocidal activity. This includes the extracts of *Azadirachta indica* (Meliaceae) (LC₅₀ of 37.75 and LC₉₀ of 166.83 ppm) and *Juniperus procumbens* (Cupressaceae) (LC₅₀ of 85.66 and LC₉₀ of 429.33 ppm) against adult of *Culex gelidus* Theobald (Kamaraj *et al.*, 2010). The mosquitocidal effect of *P. capense* demonstrated in the present study is comparable to the earlier reports from other *Piper* spp. extracts, that is, *P. longum* (fruit) (Piperaceae), *P. ribesoides* (wood) (Piperaceae), and *P. sarmentosum* (whole plant) (Piperaceae), against *Stegomyia aegypti* (Diptera: Culicidae), the dengue fever vector mosquito in Thailand (Choochote *et al.*, 2006). This indicates that the mosquitocidal effect is the characteristic of several members of the genus *Piper*.

The biological activities of the phytochemicals that include alkaloids, terpenoids, steroids, phenols, saponins and tannins extracted from several tropical plants have been receiving the attention of many researchers as potential sources of mosquitocides and for treatment of various diseases (Shaaan *et al.*, 2005; Chowdhuri *et al.*, 2007). The demonstration of phytochemical secondary metabolites in *O. africana* and *P. capense*, whose crude extracts caused high mortality to adult mosquitoes, was a further indicator of insecticidal active compounds as the alkaloids, phenols, flavonoids, saponins, cardiac glycosides, detected are among substances with insecticidal effects (Bell *et al.*, 1990; Rajkumar and Jebanesan, 2005). Therefore, the high mosquitocidal potencies of methanol crude extracts of the two plants was the basis for considering further screening studies on the different solvent partitioned fractions against adult *An. arabiensis*.

The finding of the present study that dichloromethane (DCM) partitioned fraction of 80% methanol crude extract had superior toxicity against adult *An. arabiensis* is similar to the report

of Joseph *et al.* (2004) who showed DCM extract of the plant *Neorautanenia mitis* (Fabaceae) to have the highest (LC₅₀ at 3.05 ppm) anti-adult *An. gambiae* activity. Therefore, it is to be expected that since different phytochemical constituents dissolve in specific solvents (Sukumar *et al.*, 1991), the DCM fraction of *O. africana* and ethyl acetate (EtOAc) fraction of *P. capense* contained constituents with demonstrable adulticidal activity in the present study.

Furthermore, since the yield of the active ingredients, in addition to their bioactivity, would determine the suitability of plant extracts for mosquito control, further purification of the DMC and EtOAc respective fractions, that had higher yields of the active components was considered appropriate. The enhanced adulticidal activity of purified fractions of *O. africana* and *P. capense* compared to their respective crude extracts and partially purified extracts showed that purification of plant extracts would significantly improve the bioefficacy of the active constituents in an extract. This was shown by the increased larvicidal potency of methanol crude extract of *P. longum* and *P. nigrum* fruits upon purification of piperonaline (Yang *et al.*, 2002) and piperide (Park *et al.*, 2002) from the hexane fraction.

As the purpose of fractionating crude extracts for bioactivity is to extract as many potentially active constituents as possible, the observed weak to very strong adulticidal effects of the different solvent fractions was an indication that the plant extracts consisted of different phytochemicals with varying adulticidal potencies. Such variation in the bioactivities of different solvent fractions of crude extracts has been reported by Sun *et al.* (2001) who screened ethyl acetate, n-butyl alcohol and water fractions of alcoholic extracts of leaves and stems of *Vanilla fragrans* against *Cx. pipiens* larvae. They reported that both n-butyl alcohol and ethyl acetate

fractions were potent in the bioassays, while the water fraction appeared to contain no substance that inhibited larval growth.

The demonstration in the present study that the mosquitocidal activity of DCM fraction of *O. africana* crude methanol extract was much higher compared to the methanol crude extract and the ethyl acetate and water fractions implies that the potency of the active constituents in the crude extracts may have been masked by other, less active or completely inactive, minor constituents. This was further evident from *P. capense* extracts whereby the ethyl acetate fraction was more potent as a mosquito adulticide as compared to its methanol crude extract and the dichloromethane and water fractions. This suggestion is in agreement with a study reported by Kihampa (2011) who showed that crude extracts are less active than partitioned fractions.

The rationale for extract fractionation by using solvents with different polarity gradients to water, the most polar (polar index, $P = 9.0$), ethyl acetate ($P = 4.4$), and dichloromethane ($P = 3.1$) was because different organic solvents show difference in dissolving the bioactive components present in the plant materials. Use of different solvents with differing polarities was necessary because different solvents can significantly affect the potency of extracted plant compounds (Shaalán *et al.*, 2005). This has also been shown by Aivazi and Vijayan (2009) in oak gall extraction and Mulla and Su (1999) in neem plant extraction, whereby a converse relationship between extract effectiveness and solvent polarity was observed. Hidayatulfathi *et al.* (2004) had also reported the extraction of more bioactive components from *Acorus calamus* (Acoraceae) that had more lethal effect on adult mosquitoes with certain solvents than others.

Data from *O. africana* and *P. capense* leaf and fruit extracts also showed similar converse relationship between extract efficacy and solvent polarity where efficacy increased as polarity

decreases. This is consistent with earlier reports (Overgaard *et al.*, 2014) that showed decline in mortality of mosquitoes with increasing solvent polarity of a mosquitocidal plant extract. These authors showed that water extracts of *Zanthoxylum heitzii* (Rutaceae) produces low adult mortalities whereas its ethyl acetate and hexane extracts produced higher mortalities on *Anopheles gambiae*. From this, it is clear that the bioactive components responsible for the lethal effect on mosquitoes are extracted in greater measures with certain solvents only and not with all.

The high mosquitocidal activity of DCM fraction of *O. africana* is consistent with the broad insecticidal activities of DCM plant extracts shown for *Tagetes erecta* L. (Fabaceae/Compositae), which showed a significant pesticidal activity against the rice weevil, *Sitophilus oryzae* (L.) (Curculionidae) (Broussalis *et al.*, 1999). A study by Ajaegbu *et al.* (2016) also showed the DCM fraction of *Spondias mombin* L. (Anacardiaceae) to possess the most effective adulticidal activity among the fractions tested against *Ae. aegypti*. These indicate that the mosquitocidal bioactive components in these plants were better soluble in DCM than in other solvents. Thus, comparison of solvents for effective extraction of mosquitocidal chemical components has enabled determination of DCM for *O. africana* and ethyl acetate for *P. capense* as the best solvents.

The use of DCM for isolating linoleic acid from the leaves of *Helichrysum pedunculatum* (Asteraceae) (Dilika *et al.*, 2000) for its antibacterial activity, is good evidence that bioactive substances with a broad activity can be extracted with DCM. The mosquitocidal effect of DCM fraction of *O. africana*, of which linoleic acid is the major component, is proof to its broad spectrum of bioactivity. Furthermore, the broad spectrum of bioactivity of linoleic acid is evident

from its inhibition of parasitemia in mice infected with *Plasmodium vinckei* and *Plasmodium yoelii* in a 4-day suppressive test (Krugliak *et al.*, 1995).

Overall, mosquitocidal bioactive substances are multiple in nature and could belong to different plant species as the present study showed for the DCM fraction of *O. africana* and ethyl acetate fraction of *P. capense*, both of which had superior knockdown effects on adult mosquitoes.

The demonstration in the present study that purified fractions of *O. africana* (B2'O and B2''O) were superior in their mosquitocidal effects as aerosol sprays in knockdown assays against *An. arabiensis* compared to the WHO standard insecticide, Lambda-cyhalothrin, is a promising discovery for its potential use in malaria vector control. This level of mosquitocidal potency against *An. arabiensis* is much higher than what has been reported for extracts from other plants. This includes the 50% adult mortality (Maharaj *et al.*, 2011) in a cone bioassay test with DCM extracts of *Ptaeroxylon obliquum* (Ptaeroxylaceae) and *Pittosporum viridiflorum* (Pittosporaceae). Other reports on adult mosquito exposure to plant extract impregnated paper show variable effects. These include essential oil of *Lantana camara* leaves that showed percent mortalities that ranged from 93 to 100% against *Ae. aegypti*, *Cx. quinquefasciatus*, *An. culicifacies*, *An. fluviatilis* and *An. stephensi* (Dua *et al.*, 2010) and a study by Sharma *et al.* (2011) by exposing adult *Ae. aegypti* females to filter paper treated with ethanolic plant extracts of *Annona squamosa* and *Artemisia vulgaris* caused 63% and 79% mortality after 24 hours.

The basis for the mosquitocidal potency of the B2'O and B2''O containing fractions appears to be the chemical components, octadecadienoic acid (linoleic acid) in B2'O and dibutyl phthalate in B2''O that are known to possess insecticidal potencies. Ramos-López *et al.* (2012) had reported that linoleic acid isolated from *Ricinus communis* has insecticidal activity against *Spodoptera*

frugiperda (Noctuidae). The extracts of *Annona squamosa* and *Annona muricata* (Annonaceae) contained fatty acids including linoleic acid, palmitic acid and oleic acid among others against adults of *Aedes albopictus* and *Culex quinquefasciatus* had significant insecticidal effects compared to mortality induced by deltamethrin (Ravaomanarivo *et al.*, 2014).

Specific evidence for linoleic acid, one of the primary chemical compounds identified as adulticide in this study, as a larvicide exists from *Citrullus colocynthis* (L.) Schrad (Cucurbitaceae) with an LC₅₀ value of 9.79 ppm and LC₉₀ value of 37.42 ppm against fourth instar larvae of *An. stephensi* Liston (Sayed *et al.*, 1973; Basalah *et al.*, 1985; Rahuman *et al.*, 2008). In addition, a study by Ramsewak *et al.* (2001) showed that linoleic acid isolated from *Dirca palustris* (Fabaceae), exhibited activity against fourth instar larvae of *Ae. aegypti* with LC₅₀ values of 100 µg/ml after 24 hr exposure. Edriss *et al.* (2013) and Gutierrez *et al.* (2014) also showed the presence of linoleic acid in the seed extracts of *Jatropha curcas*, a plant that has been shown to possess larvicidal effects against *An. arabiensis* (Tomass *et al.*, 2011).

Probable mode of action of linoleic acid and dibutyl phthalate compounds are axonic poisons. As the products of *O. africana* have a knockdown effect the mode of action may be on the sodium ion gate channel by keeping the channels open they affect their nervous system and the mosquitoes lose normal posture and locomotion. Although *O. africana* fractions were more potent than lambda-cyhalothrin, they resemble in their mode of action.

The adulticidal activity of linoleic acid containing product (B2'O), on cage bioassay against *An. arabiensis* with residual activity persisting for up to two months, is a promising discovery. Furthermore, the existence of evidence for organic insecticides such as Azadirachtin (Shivakumar *et al.*, 2010) as a replacement for organic/synthetic insecticides, is a good indication

for considering the linoleic acid and dibutyl phthalate containing products identified from *O. africana* in the present study.

In the use of mosquito nets for protection against malaria, physical barrier provided by the nets, usually needs to be supplemented by a long-lasting deposit of insecticides on the netting. Treating mosquito nets with active fractions of *O. africana* extracts would also be a reasonable proposition as fabrics treated with B2'O, which contains linoleic acid from *O. africana* exhibited high adulticidal activity against *An. arabiensis*. Such use for botanical extracts has been reported by Kihampa *et al.* (2010) for the phenylpropanoid compounds from *Uvariadendron pycnophyllum* (Annonaceae), where long term lethal effect on adult *An. gambiae* was achieved. However, for its use as a mosquito net treatment agent, the uptake and retreatment rates need to be assessed among several other considerations (WHO, 2003). Therefore, it will be necessary to determine these rates and other factors relating to ecotoxicity for B2'O containing *O. africana* extract fraction before its potential as a replacement to the existing IRS can be fully appreciated.

The broad bioactive spectrum of the chemical components identified by using the GC-MS from *O. africana* leaves has been reported variously. Among the identified components, 9,12-octadecadienoic acid(Z,Z), 2-hydroxy-1-[hydroxymethyl) ethyl ester and 9, 12-Octadecadienoic acid ethyl ester have been used as nematocidal, hepatoprotective and antieczemic agents (Saral and Gnanavel, 2013). Also, Ethanol,2-(9,12-Octadecadienyloxy)-(Z,Z)- and 10,13-Octadecadienoic acid methyl ester have been reported to possess anti-inflammatory, anticancer and antihistaminic effects (Lakshmi *et al.*, 2014), while 9,12-Octadecadienoic acid, methyl ester, (E,E)- and 9,12-Hexadecadienoic acid, methyl ester are used as hypocholesterolemic, insectifuge, antiacne and 5- α reductase inhibitor (Baumgard *et al.*, 2000). Furthermore, 9,12-

Octadecadienoic acid (Z,Z)- was reported to possess anti-inflammatory, anticancer, nematocidal, insectifuge, antihistaminic, antiandrogenic and antiarthritic effects (Mohan and Maruthupandian, 2011). And, 10-Octadecenoic acid, methyl ester is used as immune enhancing agent (Yamada *et al.*, 2009). Hence, the potent insecticidal activities demonstrated for the purified fractions of *O. africana* leaf extracts fall within the broad spectrum of bioactivities characteristic of this plant.

The chemical components of B2^o containing fraction also have been reported to possess antimicrobial and insecticidal bioactivities. This included the ethaneperoxoic acid, 1-cyano-1-[2-(2-phenyl-1,3-dioxolan-2-yl)ethyl] pentyl ester that was identified from *Peristrophe bicalyculata* (Retz.), whole plant (Janakiraman *et al.*, 2012). The other major compound is dibutyl phthalate, which is a high production chemical and it has a wide spectrum of industrial and commercial applications, including plasticizers, solvents and in flexible plastics (ATSDR, 2001). The bioactivity of dibutyl phthalate extracted from leaves of *Urtica dioica* (Urticaceae) has been reported to have antimicrobial activity (Singh *et al.*, 2012). When used as a solvent, it is in oil-soluble dyes, insecticides, peroxides and other organic compounds (European Chemicals Agency (ECHA), 2009). Dibutyl phthalate in the extract from *Tinospora smilacina* Benth (Menispermaceae) was reported to possess anti-inflammatory activity (Li *et al.*, 2004). It is also used as peroxisome proliferator (O'Brien *et al.*, 2001), which is an effective compound against demodicidosis (Yuan *et al.*, 2001), as well as an endocrine disruptor with estrogenic activity (Ohtani *et al.*, 2000).

In addition, dibutyl phthalate isolated from *Ipomoea carnea* (Convolvulaceae) stem extracts possessed mosquito larvicidal activities (Khatiwora *et al.*, 2014). Dibutyl phthalate and permethrin impregnated clothing provides good protection against chigger mite - the vectors of

scrub typhus (Francis *et al.*, 1992). This compound isolated from *Stellaria chamaejasme* L. (Caryophyllaceae) extract showed insecticidal activity against *Locusta migratoria manilensis* (Wu *et al.*, 2014). Therefore, it is possible that the compounds from *O. africana* could synergistically or independently cause mosquitocidal effects.

The mosquitocidal effects of *O. africana* and *P. capense* crude extracts and purified fractions against adult *An. arabiensis* may require further research to determine formulations with enhanced activity and may lead to an environmentally acceptable insecticide that could replace the conventional synthetic insecticides for mosquito control.

6. Conclusions

1. The present study has shown that among the four plant extracts tested against adult *An. arabiensis*, the extracts of *O. africana* and *P. capense* had effective adult mosquitocidal potency. The nearly 100% mosquito mortality obtained with 55 ppm crude extracts of *O. africana* and *P. capense* is an indication of a very good potential that the two plants, that can be easily cultivated by farmers, could be used to extract products for use as mosquito control agents.
2. The findings of this study showed that the purified extract fractions of *O. africana* that contain linoleic acid (B2'O) and dibutyl phthalate (B2"O) caused high mortality on adult mosquitoes.
3. The persistence of the purified *O. africana* extract fractions (B2'O and B2"O) upon impregnation of mosquito nets possessed high adult mosquitocidal effects at a level superior to lambda-cyhalothrin, the WHO standard insecticide, that can reduce man-vector contact, leading to interrupting malaria transmission.
4. The findings are indicative of the potential of traditional insecticidal indigenous plants as sources of organic antimosquito agents in Ethiopia.



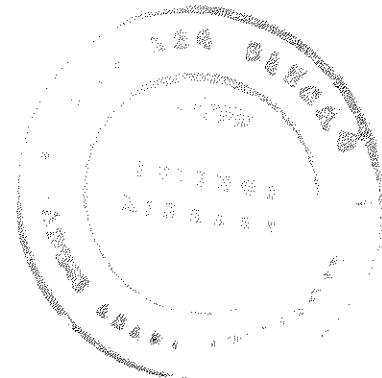
7. Recommendations

1. The purified extracts of *O. africana* and *P. capense* need to be evaluated under field conditions and for their effect on non-target insects and other organisms.
2. The development of the products of *O. africana* and *P. capense*, indigenous plants to Ethiopia, for the impregnation of mosquito nets, must be seriously considered to augment the malaria control and elimination efforts currently underway in Ethiopia.
3. The mosquito net uptake and retreatment rates for B2'O and B2"O must be determined to assess their potential as a replacement to the existing residual spray.
4. Further characterization of the active chemical components must be conducted on *A. pirottae* and *A. schimperi* to better determine their potential as larvicides.

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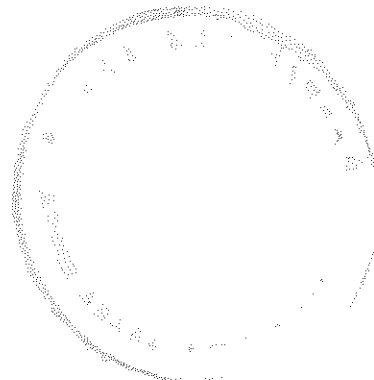
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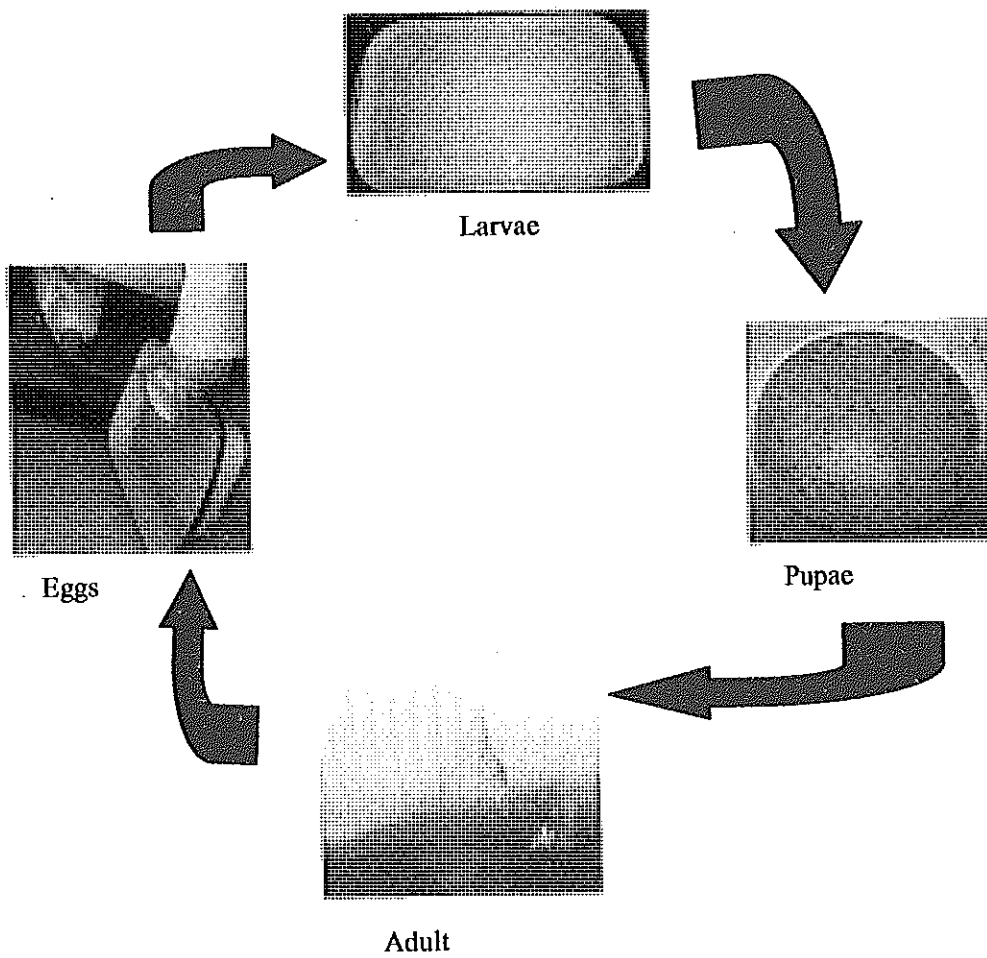
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Appendices

Appendix 1. The life cycle of *Anopheles* mosquito used in the present study



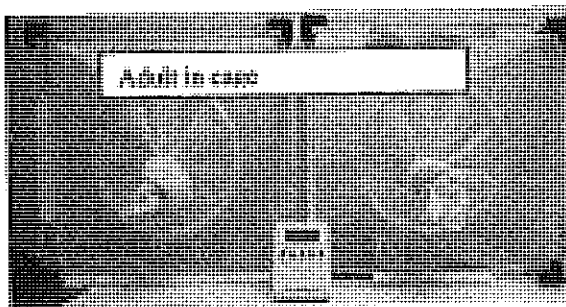
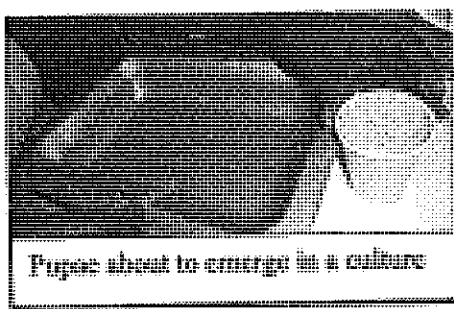
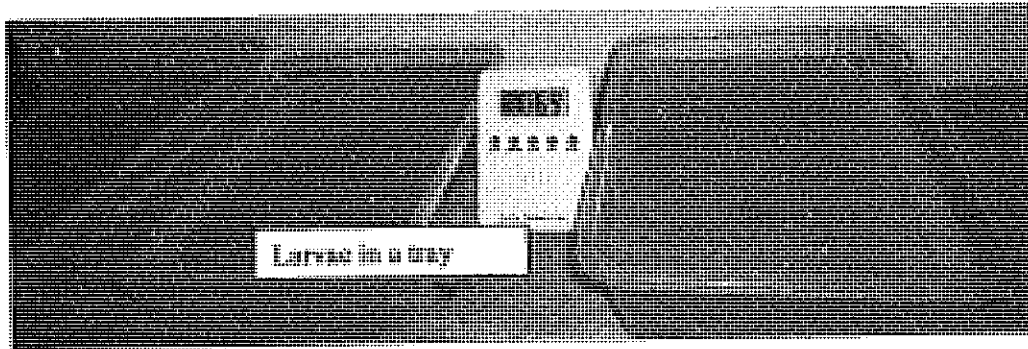
Appendix 2. The plants tested for bioactivity, from left to right: *Acokanthera schimperi*, *Aloe pirottae*, *Brassica nigra*, *Oreosyce africana* and *Piper capense*



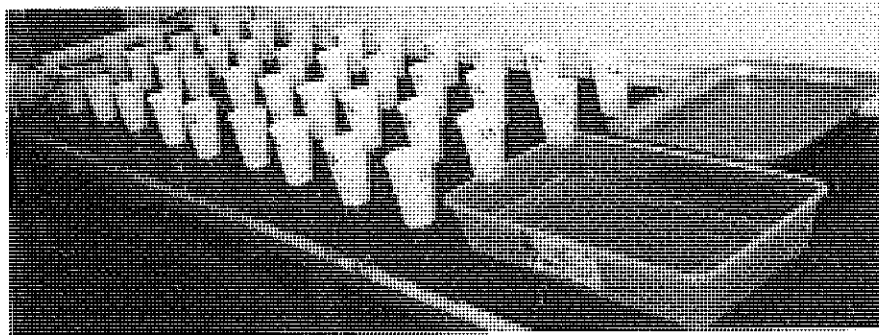
Appendix 3. Removal of solvents from plant extracts using rotary vacuum evaporator at the Centre for Traditional Medicine and Drug Research, Ethiopian Public Health Institute, Feb. 2013



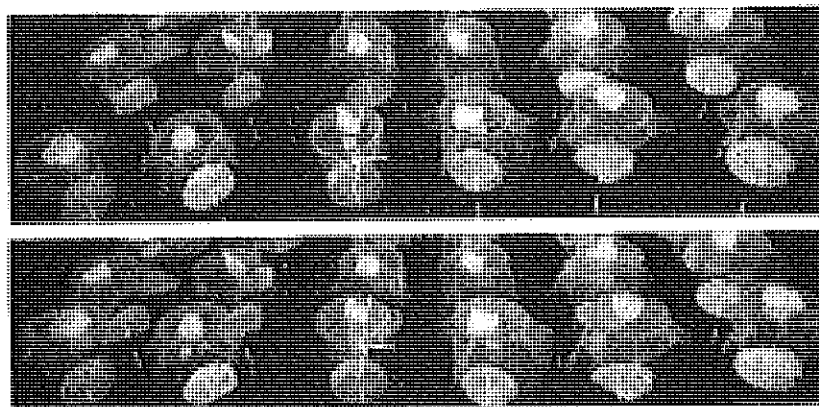
Appendix 4. Rearing and maintaining larvae, pupae and adults of *An. arabiensis* at the insectary of the Department of Zoological Sciences, College of Natural Sciences, Addis Ababa University



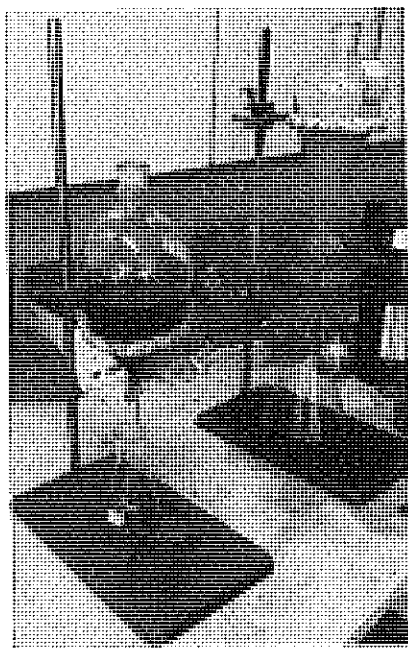
Appendix 5. Exposure of *Anopheles arabiensis* fourth instar larvae to different concentrations of 80% methanol crude extracts of *Aloe pirottae* and *Acokanthera schimperi*



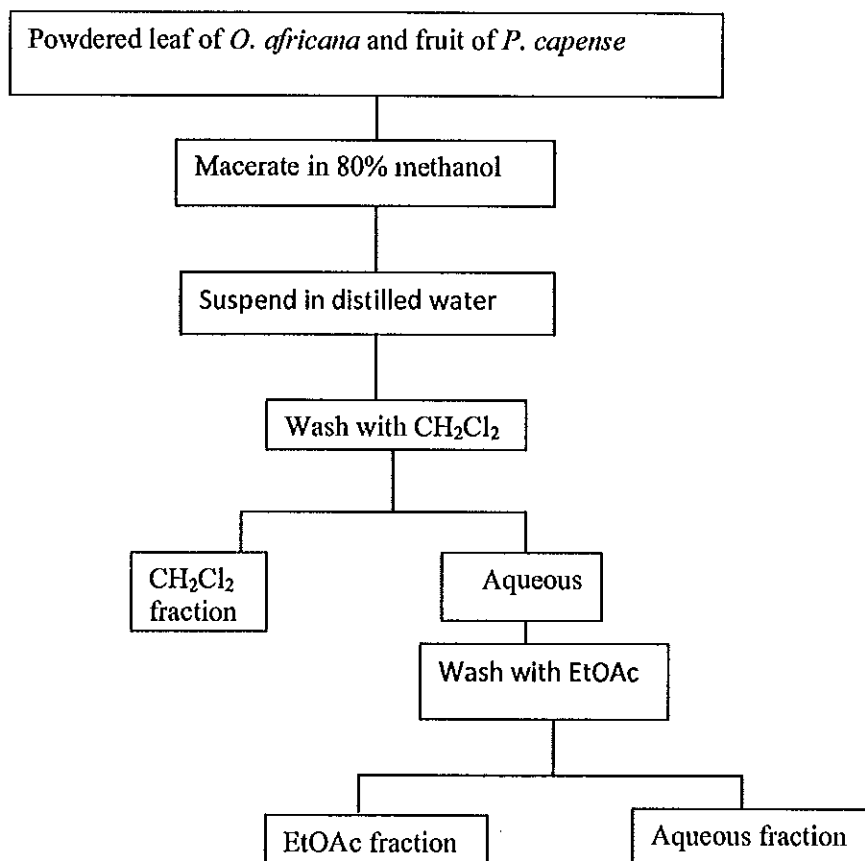
Appendix 6. Beaker bioassay with different concentrations of 80% methanol crude extracts of *Aloe pirottae*, *Brassica nigra*, *Oreosyce africana* and *Piper capense* against *Anopheles arabiensis* adults



Appendix 7. Extraction of fractions of *Oreosyce africana* (right), and *Piper capense* (left) using separatory funnel



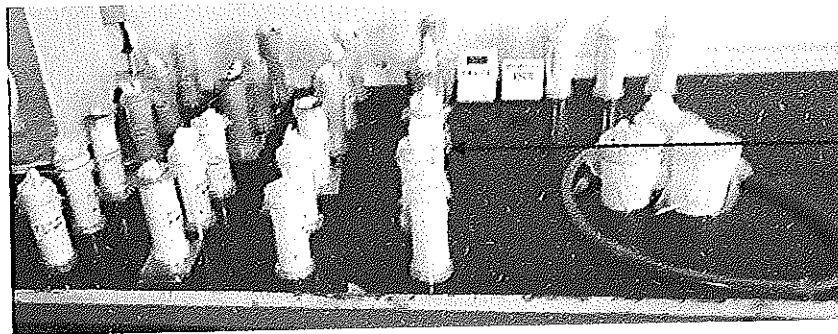
Appendix 8. Bioassay-guided fractionation, purification, and isolation procedure for the extracts from *O. africana* leaf and *P. capense* fruit (Adopted from Alkofahi *et al.*, 1989)



Appendix 9. Testing the effect of dichloromethane fraction of *Oreosyce africana* and ethyl acetate fraction of *Piper capense* by using holding and exposure tubes against *Anopheles arabiensis* adults



Exposure tubes

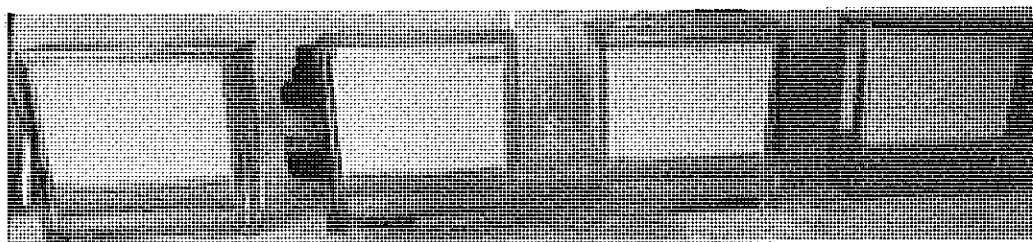


Holding tubes

Appendix 10. Picture representation of flash column chromatography packed with silica gel and eluent of dichloromethane fraction of *Oreosyce africana*



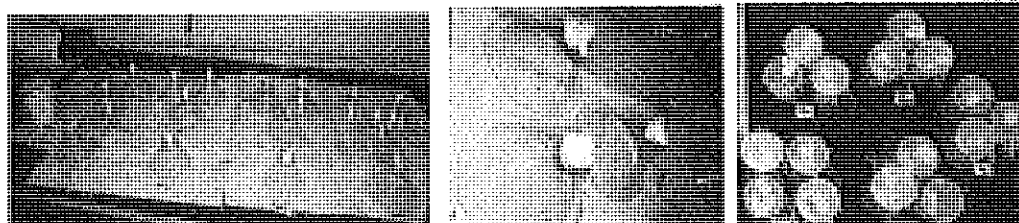
Appendix 11. Picture of development of PTLC for dichloromethane fraction of *Oreosyce africana*



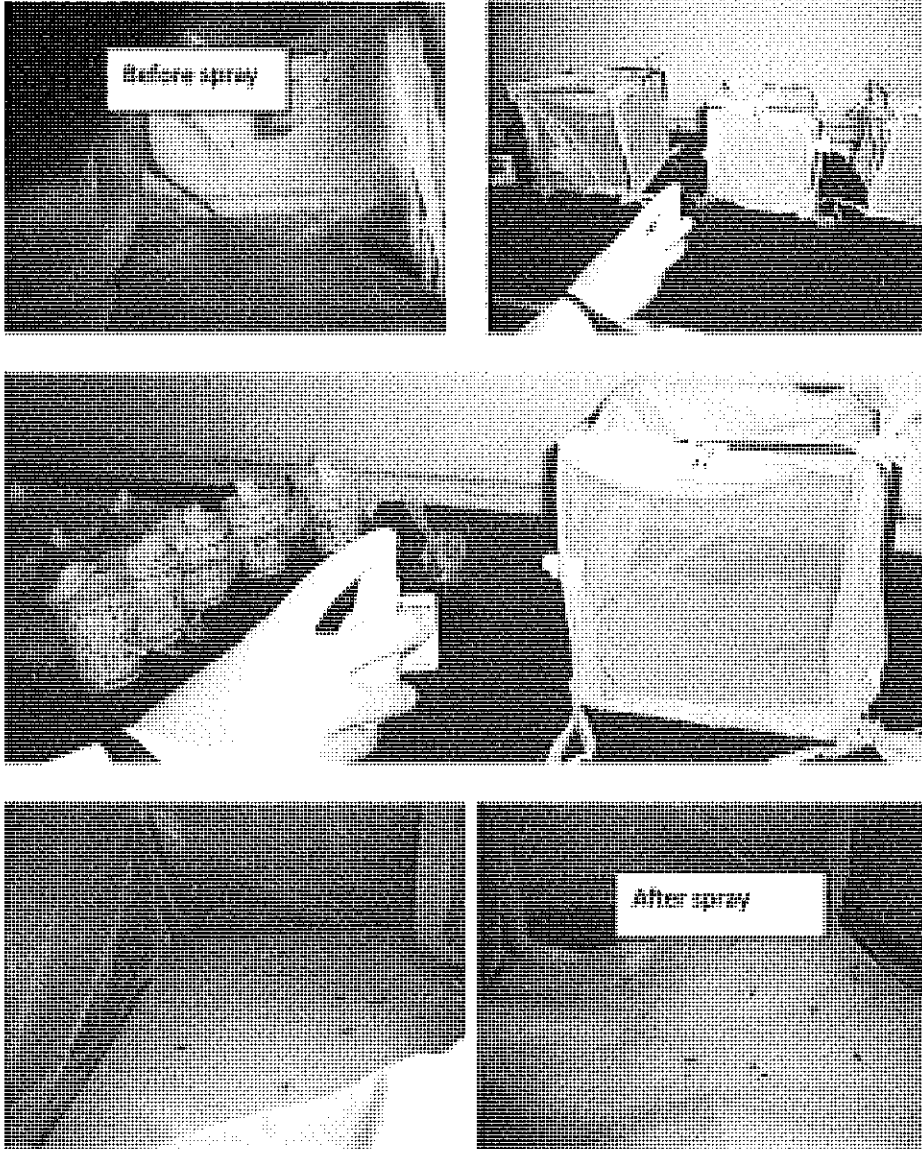
Appendix 12. Picture representation of Sephadex® LH-20 with silica gel and eluent of ethyl acetate fraction of *Piper capense*



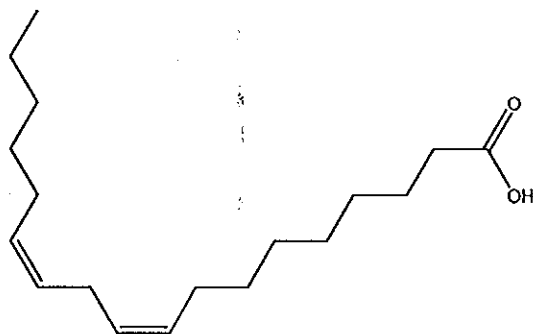
Appendix 13. Cone bioassay on mosquito nets treated with purified *Oreosyce africana* fraction and the paper cups used for *Anopheles arabiensis* recovery period



Appendix 14. Effect of B2'O fraction of *Oreosyce africana* as aerosol sprayed inside the cage on *Anopheles arabiensis* adults after 15 min exposure period



Appendix 15. Chemical structure of 9,12-Octadecadienoic acid (Z,Z)- in B2'O fraction isolated from *Oreosyce africana*



Appendix 16. Chemical structure of Dibutyl phthalate in B2''O fraction isolated from *Oreosyce africana*

