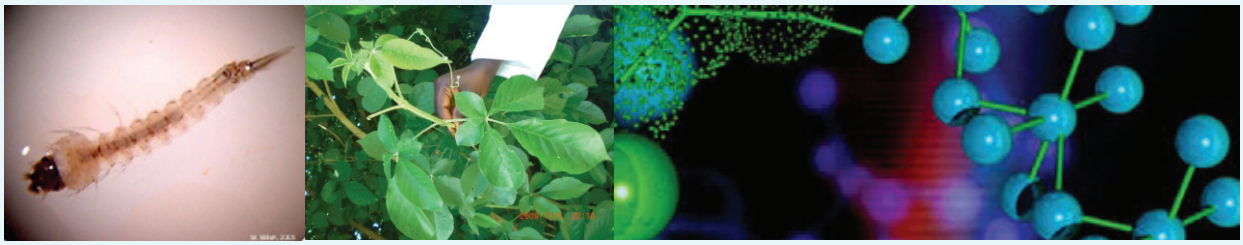
Laboratory and semi-field evaluation of some *Vitex* species for larvicidal activity against *Anopheles gambiae* larvae

Mokua Gladys Nyamoita



Thesis for doctoral degree (PhD)

Natural Products Development and Formulation

Institute of Traditional Medicine

2014

Muhimbili University of Health and Allied Sciences (MUHAS

Institute of Traditional Medicine
Muhimbili University of Health and Allied Sciences

Laboratory and semi-field evaluation of some *Vitex* species for larvicidal activity against *Anopheles gambiae* larvae

By

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Dar-es-Salaam
2014

Published and printed by Kenya Literature Bureau

ISBN 978-9966-065-19-3

The work reported in this thesis has been supported by the following institutions:

Muhimbili University of Health and Allied Sciences (MUHAS)
P.O. Box 65001, Dar es Salaam, Tanzania

International Centre of Insect Physiology and Ecology, P.O. Box 30772-00100, Nairobi, Kenya

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Dedication

To my dear husband, Dr. Geoffrey Mokua Maroko, our children Brenda F. Kemunto, Marian Z. Moraa, Allan M. Mecha and Emmanuel H. Obegi.

Also in the memory of my late father Benjamin Onsongo Anuri, mum Agnes Kerubo Onsongo, parents-in-law Paul Maroko Ombogo and Mary Kwamboka Maroko.

Abstract

Background: Over two billion people in tropical countries are at risk from mosquito-borne diseases such as dengue fever, hemorrhagic fever, malaria and filariasis. It is estimated that US$ 2 billion is spent annually on malaria control and treatment programmes in sub-Saharan Africa alone, where 90% of all malaria-related deaths occur. The problem has become increasingly difficult to manage because of the emergence of drug-resistant parasites to the currently available anti-malarial drugs. Personal protection from mosquito bites with synthetic insecticides is currently the dominant measure to control the bites from mosquitoes. However, vector resistance to insecticides is a recurring problem and a threat to malaria control programmes. To address these problems, attention to insecticides of natural origin, particularly botanical products, has been the subject of current research.

Objectives: The study set out to investigate the larvicidal and insect growth regulatory (IGR) potential of Vitex schiliebenii, V. payos, and V. trifolia against Anopheles gambiae larvae under laboratory and simulated semi-field conditions. Phytochemical tests of the extracts were carried out to compare their constituents and the larvicidal results. Toxicity test for the active extracts was evaluated on Brine shrimp larvae and compounds isolated from the extracts were also evaluated for their activity.

Materials and methods: The present study was designed to determine the mosquito larvicidal/Insect Growth Regulatory (IGR)/adult inhibition activities of acetone, methanol and aqueous extracts from three *Vitex* species belonging to the family Verbenaceae *viz, V. payos, V. schiliebenii* and *V. trifolia.* Plant materials were collected from the coastal region of Kenya and tested on 3rd and early 4th instar larvae of a malaria vector A*nopheles gambiae* s.s. in a dose-dependent manner. Different parts of the plants (root bark, stem bark, leaves and seeds) were air-dried, ground, extracted and concentrated to dryness using a rotary evaporator at 40^{o}C and the combined extract stored at 4^{o}C. This procedure was repeated with methanol in the same proportion and for the same periods while the aqueous extracts were obtained using soxhlet extraction. The extracts were filtered and then freeze dried to obtain the dry powder which was then stored at 4^{o}C for further chemical and biological analysis. The crude solvent extracts were tested for their biological activity under laboratory and simulated semi-field conditions. The extracts were then subjected to column chromatography and the fractions and pure compounds thereof obtained were also tested for their biological activity. Lethal concentrations of each test sample was calculated using probit analysis. The structural elucidation of the isolated compounds was done using physical properties, (melting point) and spectroscopic methods [infra red (IR), nuclear magnetic resonance (NMR) and mass spectroscopy (MS)].

Results: Bioassay of the extracts gave different levels of mortality of the larvae. Methanol extract of *V. trifolia* leaves, acetone extracts of stem bark and leaves of *V. schiliebenii* and acetone extract of root bark of *V. payos* caused 100% mortality at 100 ppm

in 72 hours, with those of *V. schiliebenii* and *V. payos* having shorter mortality time (LT_{50}=8 h) than that of *V. trifolia* (LT_{50}=14 h). At < 50 ppm, most of the larvae failed to transform to normal pupae but gave larval-pupal intermediates between 4-14 days of exposure. Some pupated normally but the adults that emerged appeared to be weak and died within 48 hours. Larvae exposed to extracts of the stem bark of *V. payos* were relatively hyperactive compared to those in control treatments. They later became stretched, inactive, died and floated in clusters on the surface. These observations suggest some interesting growth-disrupting constituents in the plants, with possible application in the practical control of mosquito larvae in aquatic ecosystems. The results of the simulated semi-field conditions revealed that *An. gambiae* larvae were susceptible to the *Vitex* extracts with the percentage inhibition of emergence of adult mosquitoes falling below the threshold value of 80% at concentrations ≥ 25 ppm. Phytochemical screening revealed the presence of flavonoids, terpenoids, steroids, alkaloids, saponins and tannins in the extracts.

The isolation and purification of bioactive compounds resulted into four compounds two from *V. payos*: 20-hydroxyecdysone-20, 22-monoacetonide **(166)** and 20-hydroxecdysone **(80)**; and three from *V. schiliebenii:* 20-hydroxyecdysone **(80)**, stigmasterol **(168)** and γ-sitosterol **(167)**.

The isolated phytosteriods showed good larvicidal activity against *An. gambiae* s.s. larvae when evaluated individually. When tested in blends, three variants were noted. First, production of a less active blend from active constituents; secondly, enhancement of the activity of an active compound by an inactive constituent and thirdly, synergism between moderately active compounds to give a mixture that is more active than the individual activities of the constituents. The first variant was illustrated by the high lethal activity of compounds 20-hydroxyecdysone-20, 22-monacetonide **(166)** and 20-hydroxyecdysone **(80)** with LD_{50} value of less than 1 ppm. The second variant was illustrated by the enhancement of the activity of compound **168** in blends with 20-hydroxyecdysone-20,22-monacetonide **(166)** and 20-hydroxyecdysone **(80)** and compound **167** in blends with 20-hydroxyecdysone-20,22-monacetonide **(166)** and 20-hydroxyecdysone **(80)** (LD_{50} < 1 ppm). The third variant was illustrated by the combination of compounds stigmasterol **(168)** and γ-sitosterol **(167)** (LD_{50} = 1 ppm). These findings have important practical implication in the strategy adopted in the search for and use of plants and their phytochemicals for mosquito larvae control.

Conclusion: In summation, results of this study show interesting larvicidal and/or growth-disrupting effects of *Vitex* extracts and the isolated compounds. Enriched extracts of the plants may have potential for controlling malaria vectors in breeding sites around human dwellings. This will also go along with a reduction in the annual entomological inoculation rate and consequently lead to a reduction in malaria incidences.

List of publications

I. Mokua G.N., Innocent E., Mbwambo H. Z., Lwande W., Ochola J. B., Hassanali A. (2013). Comparison of the Effects of Extracts from Three *Vitex* Plant Species on *Anopheles gambiae* s.s. (Diptera: Culicidae) Larvae. *Acta Tropica* 127: 199-203.

II. Mokua G.N., Innocent E., Mbwambo H. Z., Lwande W., Ochola J. B., Hassanali A. (2013). Time-course effects of *Vitex schiliebenii* (Varbenaceae) solvent extracts on *Anopheles gambiae* giles s.s. larvae under simulated semi-field conditions. *Novus Natural Science Research.* 2 (1): 1-8.

III. Mokua G.N., Innocent E., Mbwambo H. Z., Lwande W., Ochola J. B., Hassanali A. (2013). Larvicidal and brine shrimp activities of *Vitex schiliebenii* extracts and isolated phytoecdysteroids on *Anopheles gambiae* giles s.s larvae. *Journal of Applied Pharmaceutical Science*. 3 (5): 91-95.

IV. Mokua G.N., Mbwambo H. Z., Ochola J. B., Lwande W., Innocent E., Hassanali A. (2013). Chemical composition and evaluation of mosquitolarvicidal activity of *Vitex payos* extracts against *Anopheles gambiae Giles* S.S larvae. *Spatula Drug Discovery.* 3 (3): 113-120.

List of abbreviations

ANOVA	Analysis of Variance
B.t	*Bacillus thuringiensis*
CC	Column Chromatography
COSY	Correlation Spectroscopy
CNB	*Culex nigripalpus* baculovirus
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
CSEA	Central and South East Asia
CQ	Chloroquine
DOMC	Division of Malaria Control
DDT	Dichlorodiphenyl-trichloroethane
DEET	*N, N*-Diethyl-*m*-toluamide
DEPT	Distortionless Enhancement Spectroscopy
DHF	Dengue Hemorrhagic Fever
DHFR	Dihydrofolate Reductase
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of Congo
EI	Electron Impact
EIMS	Electron Ionization Mass Spectroscopy
GC	Gas Chromatography
GDP	Gross Domestic Product
HCH	Hexachlorocyclohexane
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Quantum Coherence
^{1}H NMR	Proton Nuclear Magnetic Resonance
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
ICIPE	International Center of Insect Physiology and Ecology
IE	Inhibition of Adult Emergence
IGR	Insect Growth Regulator
IR	Infra red
IVM	Integrated Vector Management

ITM	Institute of Traditional Medicine
ITN	Insecticide Treated Net
LC_{50}	Lethal Concentration at which 50 % of the test organism are dead
LT_{50}	Lethal Time at at which 50 % of the test organism are dead
MDR	Multi-drug Resistance
MS	Mass Spectroscopy
MUHAS	Muhimbili University of Health and Allied Sciences
NMR	Nuclear Magnetic Resonance
OP	Organophosphate
PABA	Paraaminobenzoic acid
PHC	Primary Health Care
ppm	Parts per million
PTLC	Preparative Thin Layer Chromatography
RBC	Red Blood Cells
RBM	Roll Back Malaria
SP	Sulfadoxine Pyrimethamine
SSA	Sub-Saharan Africa
TLC	Thin Layer Chromatography
ULV	Ultra-low-volume
WHO	World Health Organization

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CHAPTER 1: INTRODUCTION

1.1. Background information

The mosquito is the principal vector of many vector-borne diseases affecting human beings and other animals. Mosquitoes constitute a major public health problem that causes morbidity, mortality, economic loss and social disruption (Becker *et al.,* 2003). Several mosquito species belonging to genera *Anopheles, Culex,* and *Aedes* are vectors for the pathogens of various diseases like malaria, filariasis, Japanese encephalitis, dengue fever, dengue hemorrhagic fever (DHF) and yellow fever (Hubalek and Haluzka, 1999). *Culex pipiens* is the vector of West Nile virus which causes encephalitis or meningitis which is known to affect the brain tissue, finally resulting in permanent neurological damage (Hubalek and Haluzka, 1999). *Aedes aegypti* is the principle vector of dengue fever and DHF is reported to infect more than a hundred million people every year in more than 110 countries in the tropics and subtropics (Halstead, 2000). At present, there is no effective vaccine which is available for dengue and malaria; therefore, the main way of reducing the incidence of the diseases is by mosquito control, which is frequently dependent on applications of conventional synthetic insecticides (Malavige *et al.,* 2004).

Chemical measures in public health programs were initially considered likely to decrease mosquito populations but this has been reported to have failed because the indiscriminate use of chemical insecticides has led to the disruption of natural biological control systems and outbreaks of insect species (Chaithong *et al*., 2006). Majority of the chemical pesticides have been reported not to be easily degradable; hence, spreading toxic effects to humans, mammals and non-target organisms (Lee *et al*., 2001). The increased use of these insecticides may enter into the food chain and thereafter the liver and the kidney causing irreversible damage but also may result in mutation of genes and the changes may become

prominent after a few generations (Ghosh, 1991).

In larval mosquito control, application of insecticides in ponds, wells and other water bodies may cause health hazards. Mosquito coils containing synthetic pyrethroids and other organophosphorus compounds have been reported to cause side effects such as breathing problems, eye irritation and headache, asthma, itching and sneezing to the user. Govindarajan *et al*. (2011) reported that with the use of mosquito repellents, people have complained of ill health effect and sometimes require medical treatment. In addition, chemical insecticides are very costly and the mosquitoes have developed resistance. Indoor residual sprays stain walls and leave a long lasting unpleasant odour. In an attempt to resolve these problems, the search for new strategies for selective mosquito control particularly of plant origin need to be developed. Accordingly, a considerable number of studies have emphasized the development of herbal substances for controling mosquitoes (Debboun *et al,* 2006; Govindarajan *et al*, 2008; Innocent *et al.*, 2008; Amalraj *et al*., 2009; Birkett *et al.*, 2011,). Although results vary, botanical phytochemicals with mosquitocidal potential may be a possible altenative to or used along with other insecticides as they are eco-friendly, target specific, do not develop resistance, are highly accepted and suitable for rural areas (Govindarajan *et al*., 2011). Extracts or essential oils from plants have been used as alternative sources of mosquito larval control agents since they constitute a rich source of bioactive compounds that are biodegradable into nontoxic products and potentially suitable for use in the control of mosquito larvae (Amer and Mehlhorn, 2006a, b, and c; Govindarajan *et al.,* 2011).

1.2. Global distribution of malaria and populations at risk

More than 40% of the people of the world are at risk of malaria (WHO, 2008). It occurs in over 100 developing tropical countries and territories and its control is a major goal for improvement of worldwide health (Govindarajan *et al.*, 2011).

Large areas of Central and South America, Hispaniola (Haiti and Dominican Republic), Africa, the Indian subcontinent, Southeast Asia, the Middle East and the Oceanic are considered malaria risk areas. Malaria is a major killer in Africa where it also presents major obstacles to social and economic development. It has been estimated to cost Africa more than US$ 2 billion every year in Gross Domestic Product (GDP) even though it can be controlled (Akande and Musa, 2005). At least 300 million acute cases of malaria occur each year globally, resulting in more than a million deaths. Approximately 90% of these deaths occur in Africa and mostly in young children (WHO, 2008).

Malaria is Africa's leading cause of under-five mortality and constitutes 10% of overall disease burden. The other groups of people at most risk of dying from malaria are pregnant women (whose immunity is depressed to protect the fetus from rejection as foreign tissue); populations in regions of borderline transmission (which leads to unstable epidemic malaria); people moving from non-malarious to malaria zones for reasons of work, migration, refuge, war or tourism; people from the industrialized, non-malarious world who catch malaria and return home with the disease.

Malaria accounts for 40% of public health expenditure, 30-50% of in-patient admissions and up to 50% of out-patient visits in areas with high malaria (WHO, 2008). It is caused by a parasitic infection, transmitted by four different parasites: *Plasmodium falciparum P. vivax, P. ovale* and *P. malariae* (Oaks *et al.*, 1991). Recently, one more species P. knowlesi (Bronner *et al.*, 2009) vectored by anopheles mosquitoes has been discovered. In endemic regions such as sub-Sahara Africa (SSA), Asia, Oceanic and Amazons *P. falciparum* is the most virulent parasite (Snow et al. 2005). In 2007, the global area at risk of P. *falciparum* malaria was 29.73 million km^2, distributed between the America (6.03 million km^2, 20.30%), Africa (18.17 million km^2, 61.10%) and Central and South East Asia (CSEA) regions (5.53 million Km^2, 18.60%) (Guerra *et al*., 2008). Among

the other species, *P. vivax* is the most common and predominates in Southern, Northern and Central Asia, Eastern Europe and most of South America. The parasite threatens almost 40% of the world's population resulting in 132-391 million clinical infections each year (Price *et al*., 2009). *P. ovale,* which is less common occurs mostly in West Africa and occasionally in Southeast Asia and Papua New Guinea. *P. malariae* occurs at low frequency and is found in most endemic areas especially in SSA. *P. knowlesi*, similar morphologically to *P. malariae*, has been identified by molecular methods in patients in Malaysia, the Philippines, Thailand and Myammar. However, this species has not yet been proven to be transmitted from humans to mosquitoes (White, 2008)

Malaria outbreaks are being reported in some other locations of Africa that had been previously thought to be at elevations too high for malaria transmission, such as the highlands of Kenya. Some scientists hypothesize that this is due to climate change, while others hypothesize that this is due to human migration. In addition, malaria has resurged in certain locations of Africa that had previously had effective control programs, such as Madagascar, South Africa and Zanzibar (Lindsay and Martens, 1998). Figure 1 shows the distribution of malaria in the world.

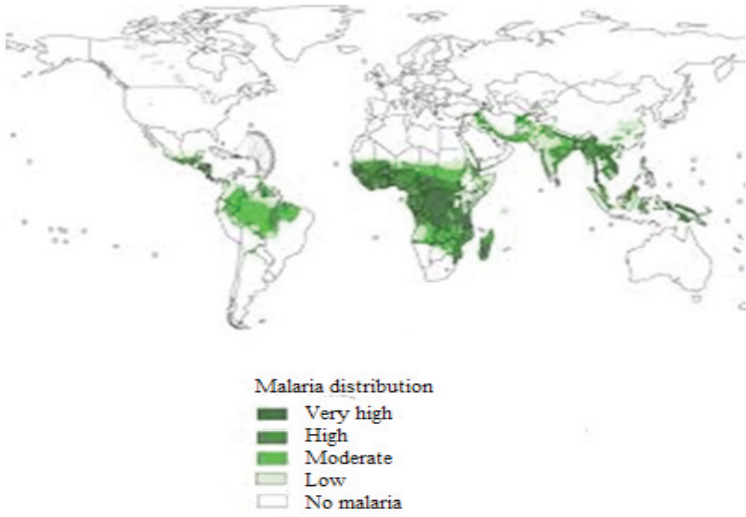


Figure 1.1: Global distribution of malaria
Source: http://upload.wikimedia.org/wikipedia/en/d/d5/Malaria_map.PNG

1.3. Worldwide malaria control efforts

Control of malaria represents one of the world's greatest public health challenges, especially in SSA where most of the disease occurs (Hay and Snow, 2006). Countries like Uganda, Tanzania, Malawi and Mozambique are among the most affected in the tropical and sub-tropical countries and thus carry enormous economic burdens (WHO, 2005). In the past, efforts to control malaria have been met with mixed success (Becker *et al*., 2003). The strategy for malaria control is based on breaking the chain of transmission of the parasites between humans and mosquitoes (Lambrecht and Challier, 1987). It therefore involves three living beings; host-pathogen-vector.

Currently, there are two approaches that are important and vital for the elimination or control of malaria. These involve control of parasites by chemical drugs known as chemotherapy, chemoprophylaxis and the control of vector population by chemical insecticides, repellents, screens such as bed nets, window gauzes and curtains, or environmental management (The Wellcome Trust, 1999). Discoveries of chemical insecticides and drugs have provided a powerful weapon against malaria control (Service, 1996). A long-hoped-for third arm, an effective malaria vaccine has not yet materialized.

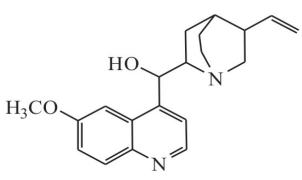
1.3.1. Parasite control

1.3.1.1. Chemotherapy

Many anti-plasmodial drugs have been developed in the course of time. Quinine (1) was identified as a useful drug from the South American plants of the genus Cinchona since 1645. The extracts of the species were used for a long time without cases of parasite resistance being observed. The first case

of quinine (1) resistance was reported from South America (Thai-Cambodian border) in mid 1960s (Pickard and Wernsdorfer, 2002). Clinical resistance to quinine therapy has been noticed sporadically in Southeast Asia and western Oceania. However, quinine **(1)** resistance is less frequent in South America (Zalis *et al*., 1998) and Africa (Jelinek *et al*., 2001). Due to the fact that quinine **(1)** exhibited relatively high mammalian toxicity and issues of ready availability, its synthetic analogues were developed. Therefore, inexpensive and less toxic drugs like chloroquine **(2)** and mefloquine **(3)** were synthesized. Other synthetic single compound drugs available for curative or prophylactic purposes include: amodiaquine **(4),** primaquine **(5),** mepacrine **(6),** sulfadiazine **(7)**, sulfadoxine **(8)**, dapsone **(9),** proguanil **(10)**, atovaquone **(11),** halofantrine **(12),** lumefantrine **(13)** and quinacrine **(14).**

However, in many cases, these synthetic anti-malarial drugs have not eradicated malaria due to the emergence of drug resistance particularly in *P. falciparum* (Wellemss and Plowe, 2001), high cost and adverse side effects they cause.



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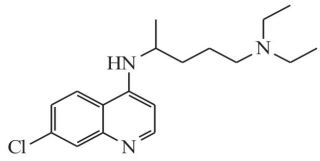

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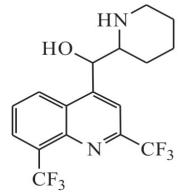

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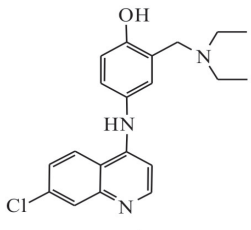

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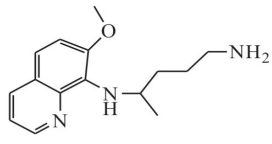

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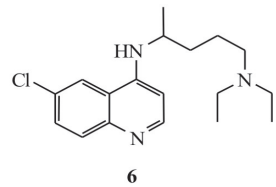

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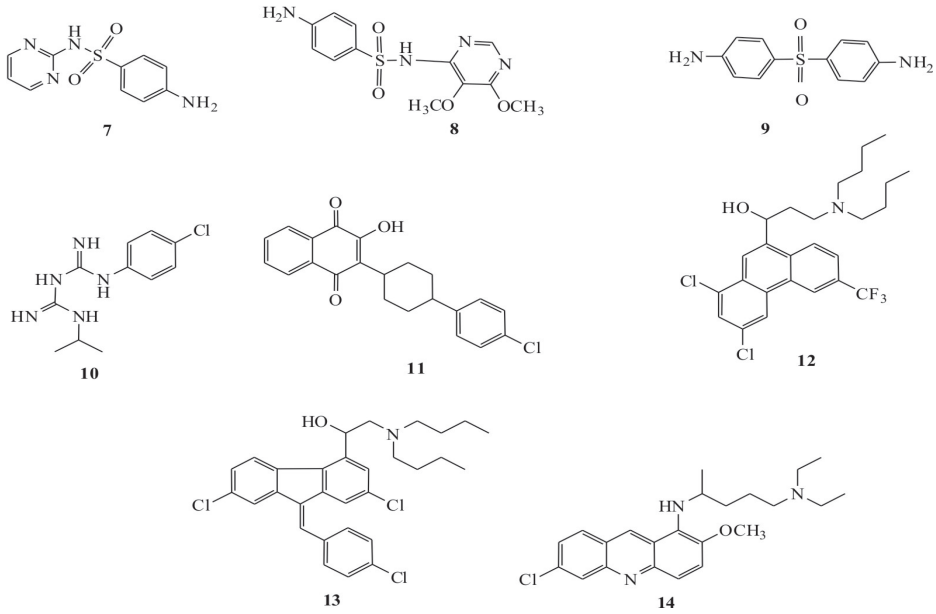


Since 1950s, the treatment of malaria principally relied on chloroquine **(2),** which was safe for use in pregnancy, inexpensive and available worldwide which formed the pillars of worldwide campaign to eradicate malaria parasites (Wellemss and Plowe, 2001). Chloroquine **(2)** is a synthetic quinoline with anti-malarial and anti-inflammatory properties. It has also been used in the treatment of rheumatoid, arthritis, systemic lupus erythematosus and in systemic therapy of amoebic liver diseases. Although the mechanism of action is not fully understood, chloroquine **(2)** is shown to inhibit the parasitic enzyme heme polymerase that converts the toxic heme to non-toxic hemazoin, thereby resulting in the accumulation of toxic heme within the parasite. Chloroquine **(2)** may also interfere with the biosynthesis of nucleic acids. After the emergence of chloroquine **(2)** resistance in Southeast Asia (Thai-Cambodian border) and South America (Colombia) (Young, 1961; Spencer, 1985; Wernsdorfer and Payne, 1991), other cases caused by chloroquine **(2)** resistant *P. falciparum* have been observed globally, contributing to the abandonment of the eradication effort and leading to large increase in malaria morbididty and mortality (Trape, 2001). Chloroquine **(2)** resistance was reported in Thailand-Cambodia border in the

early 1960s (Harinasuta *et al*., 1965). Since then, chloroquine **(2)** resistance has spread to several parts of the world where falciparum malaria is endemic except Central America (Hastings and D'Alessandro, 2000). In Africa, chloroquine **(2)** resistance was first reported in the eastern part in 1978 which then spread to the central and southern parts before being reported in West Africa. By 1989, chloroquine **(2)** resistance was wide spread in SSA (Pickard and Wernsdorfer, 2002). Resistance from Senegal (Trape, 2001), Ghana (Landagraf *et al*., 1994), Cameroon (Rigwald *et al.*, 2000) and Mali (Djimde *et al*., 2001) have also been reported.

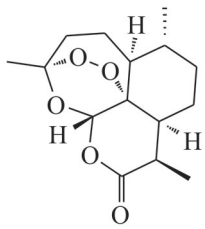
Earlier studies have shown that quinoline drugs and the next generation of drugs, sulfadoxine-pyrimethamine (SP) and then mefloquine **(3)**, quickly followed their introduction and shortly compromised their efficacy (WHO, 1992; Conway 2007). Resistance to SP was reported from Thai-Cambodian border in 1960s (Bjorkman and Philipps-Howard, 1990). Since then, SP resistance has been reported from several parts of Southeast Asia, sourthern China and Amazon basin (Aramburu et al., 1999; Vasconcelos *et al.*, 2000; WHO, 2001). Some resistance has also been reported from Pacific Coast of South America, Southern Asia, east of Iran and western Oceania (Bloland, 2001). In Africa, SP resistance was detected in late 1980s which has since spread more in the East than in the West (Ronn *et al*., 2001). Mefloquine **(3)** resistance was first observed in late 1980s near Thai-Cambodian border (Wongsrichanalai *et al.*, 2001). It is frequent in some parts of South East Asia and has been reported from Amazon region of South America and sprodically in Africa (Muckenhaupt, 1995). Resistance in non-immune individuals has been reported in Thailand (Boudreau *et al.*, 1982), Tanzania (Bygbjerg *et al*., 1983) and West Africa (Ringwald *et al.*, 1990). Mefloquine **(3)** is used for prophylaxis or treatment of malaria. It is a preferred agent in chloroquine **(2)** resistant areas. It is a phospholipid interacting anti-malarial drug. It is effective against P. falciparum with very few side effects.

Resistance to dapsone **(9)** and pyrimethamine **(10)** has also been reported (WHO 2001). Resistance to amodiaquine **(4)** was reported in the early 1990s (Philips-Howard and Björkman, 1990). Halofantrine **(13)** has been effective against *P. falciparum* with multi-drug resistances (MDR) (Watt *et al.*, 1994), but studies in Thailand revealed lower cure rates than expected (Olliaro and Trigg, 1995), Halofantrine **(13)** has also been reported to exhibit serious toxicity at doses required for treatment of resistant strains (Ter Kuile *et al.*, 1993). Lumefantrine **(14)** has been reported as an effective anti-malarial drug but has the disadvantage of being slow in action during monotherapy (Wendsorfer, 1994).

Following the rapid development of resistance to single line therapy and a high likelihood of resistance development to new anti-malarial agents, combination therapies have been targeted and have become the standard measure for treatment of P. falciparum infection world wide. Fansidar® [sulfadoxine -pyrimethamine] Also known as: daraprim; Malarone® [proguanil **(14)** and atovaquone (12)] and Coartem® [lumefantrine (14) and artemether **(15)**] are available for malaria treatment (Kamya *et al.*, 2001). However, resistance of *P. falciparum* to Fansidar® has been reported from various parts of the world, including SSA (Ogutu *et al*., 2000). Fansidar® is a combination product containing pyrimethamine **(10)** and sulfadoxine **(8)** (SP). Pyrimethamine (10) is a competitive inhibitor of dihydrofolate reductase (DHFR). DHFR is a key enzyme in the redox cycle for production of dinucleic acid (DNA) and proteins. Sulfadoxine (8) competitively antagonizes paraaminobenzoic acid (PABA), resulting in disruption of folic acid synthesis and ultimately DNA synthesis. Sulfadoxine **(8)** acts synergistically with pyrimethamine **(10)**.

Despite the activity of artemisinin **(16)** and its derivatives, monotherapy with these agents has been associated with high relapse hence monotherapy with artemisinin **(16)** drugs is not recommended (Teuscher *et al*., 2010). In addition, suspected resistance to artemisinin has been identified in four countries in

the Greater Mekong subregion: Cambodia, Myammar, Thailand and Vietnam (World Malaria Report, 2011). The combination of oral artemisinin **(16)** derivatives usually artesunate **(17)** and mefloquine **(3)** has become standard treatment for MDR falciparum malaria (Angus *et al.*, 2002).



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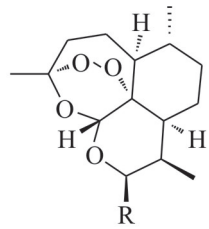


15 R = $CHOCH_3$

17 R = $CHCH_2CO_2(CH_2)_2CO_2Na$

The potential use of Malarone® [proguanil **(11)** and atovaquone **(12)**] also known as: mepron, for the treatment of multi-drug resistant *P. falciparum* malaria has been reported in Thailand and Brazil (Blanchard *et al.*, 1994; Looareesuwan *et al*., 1996). However, there is evidence that the parasites may develop resistance to proguanil **(11)** and atovaquone **(12)** when used separately (Looareesuwan *et al.*, 1996). A case of in vivo resistance to proguanil **(11)** and atovaquone **(12)** in non-immune individuals has been reported (Fivelman *et al*., 2002).

Thus, the looming failure of two-compound anti-malarial drug combinations leaves triple therapy as a viable approach to fighting drug resistant *P. falciparum* malaria. Three compound combinations slow down development of resistance against the individual drugs (McIntosh and Greenwood, 1998). This approach is likely to promote the use of the currently withdrawn cheap anti-malarial agents like chloroquine **(2)** in the treatment of drug resistant malaria using combination therapy regimes. Chloroquine**(2)** plus SP and amodiaqune **(4)** in combination with SP have been used (Winstanley *et al.*, 2002). Due to the development of drug resistance by the *P. falciparum* malaria parasite strains, more attention is being focused on the development of anti-malarial agents that do not have the quinoline skeleton found in order to avoid the emergence of parasite cross

resistance to the new drugs (Nkunya, 2004).

Table 1.1 shows the number of years it took before some preferred anti-malarial drugs experienced resistance from parasites. There is a difference between the natural drug quinine (1) and the synthetics in that it took much longer for quinine **(1)** to develop resistance as compared to the synthetics. The problem of resistance makes the search for anti-malarial drugs elusive and a continuous undertaking. Due to this problem, the situation of malaria therapy is worse now than it was 20 years ago.

Table 1.1: Time taken by various anti-malarial drugs to experience resistance from the parasite

Anti-malarial drug	Introduction	1st resistance reported	Difference
Quinine	1645	1910	278
Chloroquine	1945	1957	12
Proguanil	1948	1949	1
Sulfadoxin/Pyrimethamine	1967	1967	0
Mefloquine	1977	1982	5
Atovaquone	1996	1996	0

Source: (Aspects of African Biodiversity, Midiwo, 2010)

The other natural compound and its derivatives that is now being widely constituted into anti-malarial drugs is artemisinin **(16).** This compound was isolated from the age-old Chinese anti-malarial drug, *qinq hao su*, or *Artemisia annua* (Asteraceae), which is now a widely grown crop in Kenya and East Africa in general. Artemisinin **(16)** and its derivatives such as sodium artesunate **(17)** and artemether **(15)** are now commercial anti-malarial drugs and have not shown any cross-resistance (Marina *et al*., 2010). Presently, artemisinin **(16)** is used for the treatment of malaria in many countries including Kenya.

Due to the challenges in the search for effective anti-malarial drugs, there has

been need for research to inform people on the use of innovative interventions to prevent and cure the disease (WHO 2002b). Clinical testing of new compounds with possible anti-malarial potency has been greatly accelerated and hundreds of such compounds are already in trials. However, some of these trials have not been successful. The failure has been attributed to mutation in parasite genome which may bring resistance, resulting to natural selection of drug resistant parasites and this reduces the efficacy of a drug (Mauro, 2002). In addition, some trials have been found to have severe and frequent side effects (neuropschitric toxicity) especially when used as prophylactics (Mauro, 2002). This implies that a combination of at least two anti-malarial drugs should always be used as curative treatment which poses another problem of cost of treatment especially to the poor people. Based on these challenges, the current study seeks to look for an alternative strategy of using botanical larvicides and blend of insect growth regulators (IGRs) in controlling the malaria vector at the larval stages in the laboratory and semi-field set-up.

1.3.1.2. Vaccine development

Although progress has been made in the last 10 years towards developing malaria vaccines, there is not yet a licensed malaria vaccine in the market. The complexity of the malaria life cycle could be a hindrance towards the development. The vaccines currently under evaluation target the four stages of the malaria parasite's life cycle and hence are classified as pre-erythroctic blood stage (Graves and Gelband, 2006), sexual stage vaccines and transmission-blocking vaccines.

The pre-erthrocytic vaccines are intended to stop the parasite life cycle from progressing from the sporozoite or liver stage. They aim to generate antibody response against sporozoites and thus prevent the infection of hepatocytes, or T cells against the antigens expressed by infected hepatocytes, which prevent

merozoite release by killing infected hepatocytes or interfering with parasite development. More than a dozen vaccine candidates are now in clinical development and one, Glaxo Smith Kline Biologicals' RTS,S/AS01, is in Phase III clinical testing and the results are promising (World Malaria Report 2011). This vaccine comprises portions of the circumsporozoite protein (CSP), containg antibody and T-cell epitopes, linked to components of the hepatitis B surface antigen such that immunogenic particles are formed. It has been reported that this vaccine given as three doses over several months gave rise to sterile protection in 41% of individuals in sporozoite challenge studies. Subsequent field trials have also been reported to show significant protection in adults (34% protection against infection for several months in Gambia). From Mozambique, 30% protection in children from the disease in one out of two cohorts studied has also been reported (Alonso *et al*., 2004) with the efficacy extending to 18 months (Alonso *et al.*, 2005). However, protection from infection is not complete and hence the development of additional candidates that elicit significant protection and can be feasibly administered to at-risk populations is essential.

Other malaria vaccine candidates are also being developed but their development is far behind RTS,S/AS01 vaccine. For instance, SPf66 was one of the earliest vaccines developed. It was first formulated and tested in Colombia (Patarroyo, 1988) and later in the USA. It is a synthetic peptide vaccine containing antigens from the blood stages of malaria linked together with an antigen from the sporozoite stage, and is targeted mainly against blood (asexual) stages. The vaccine has had 10 trials in Africa, Asia and South America. The results were initially promising but further trials showed only a small effect in some trials, and no effect in Africa. There was a modest reduction in attacks of *P. falciparum* malaria following vaccination with SPf66 in South Africa. However, it did not reduce episodes of *P. vivax* (Graves and Gelband, 2009). Peptide based vaccines have been successfully employed but face the challenge of toxicity (Bojan *et al*., 2001).

The FMP2.1/AS02A vaccine has been evaluated in a Phase 1 dose escalation clinical trial in malaria–naïve North American adults. Polhemus *et al*., (2007) report that the vaccine was tolerated and strongly immunogenic, inducing both humoral and cellular immune response. The vaccine is also being evaluated in Phase I and II clinical trials in children aged 1-6 years at Bandigara Malaria project in Mali.

Erthrocytic vaccines aim to reduce infection, rather than to eliminate it, in order to protect against clinical and particularly severe disease. A phase IIb trial in 5-9 year old children of an erythrocytic vaccine known as combination B comprising of merozoite surface proteins 1 and 2 (MSP-1 and -2) with *P. falciparum* ring stage-infected erythrocyte surface Antigen (RESA) has been reported to elicit 62% reduction in parasite density in Papua New Guinea. However, there was no reduction in disease incidence (Genton *et al*., 2003).

Transmission-blocking aims to reduce antibodies against antigens that are expressed by the sexual stage of the parasite; for example, gametocytes and thus stop their subsequent combination in the mosquito gut and development into infective sporozoites. The main antigens assessed as vaccine candidates are the surface antigens Pfs25, Pfs28, Pfs48/45 and Pfs230 with the intention of protecting communities from infection rather than the individual. Other candidate trial vaccines include nucleic acids that target asexual and pre-erthrocytic stages of malaria parasite and several others are currently in clinical trials.

1.3.2. Malaria vector control

Historically, vector control has been a central strategy in most settings for malaria control in various parts of the world (Trape *et al*., 2002). The options available for vector control efforts mainly include chemical, biological, natural plant

products and environmental management.

1.3.2.1. Adult control

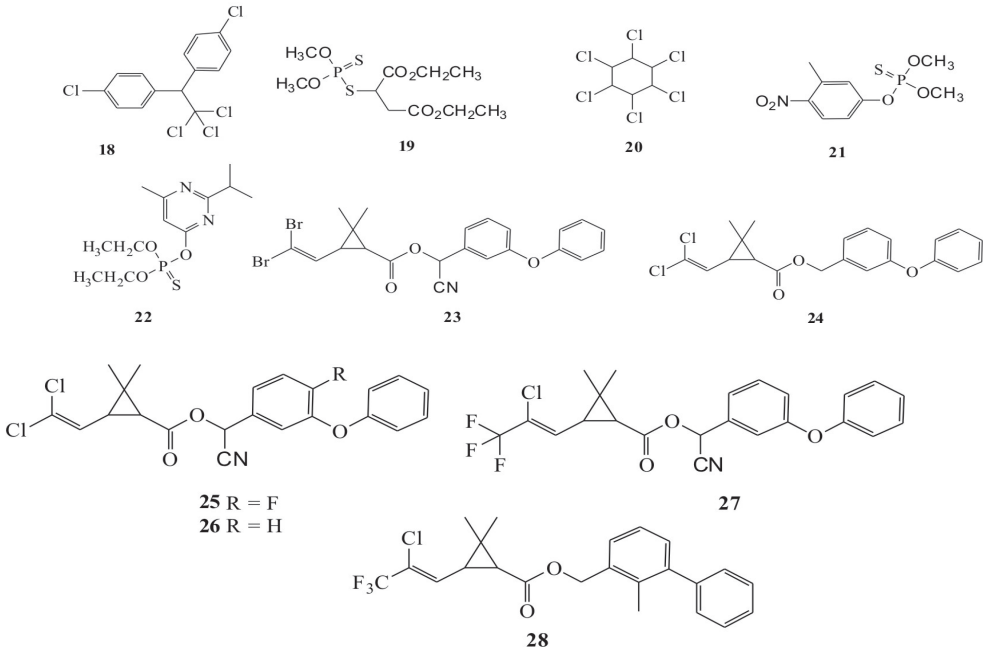
Killing of adult mosquitoes or preventing access to the human host can control the spread of malaria. This can include use of indoor residual sprays (IRS) with insecticide, insecticide treated nets (ITNs) and use of repellents and attractants. Insecticides play a central role in controlling mosquitoes (Breman *et al.*, 2001). In 1955, the WHO assembly proposed the global eradication of the most prevalent vector-borne human disease, malaria, by use of residual house-spraying of DDT **(18)**. However, the insecticide euphoria ended in 1976 and WHO reverted from malaria eradication to malaria control due to the appearance of DDT **(18)** resistance in a broad range of mosquito vectors. This resitance has presented a threat to the future success of malaria vector control programmes. Hemmingway and Ranson (2000) have reported resistance in many insecticides such as organochlorides, organophosphates, carbamates and pyrethroid.

Convectional insecticides such as Malathion **(19)**, (DDT) **(18)**, hexachlorocyclohexane (HCH) **(20)**, fenitrothion (21), diazinon **(22)** and synthetic pyrethyroids that are generally used for mosquito control are known to cause the problem of environmental pollution, residual effects and resistance by their indiscriminate use. Pates and Curtis (2005) have reported reduced effectiveness of these convectional control methods due to behavioral changes of the adult mosquitoes. Similarly, development of resistance to Malathion **(19)** (Karunaratne and Hemingway, 2001) and to deltamethrin **(23)** (Trape *et al.,* 2011) in adult *An. gambiae* has been reported.

Synthetic chemical larvicides continue to be applied for controlling mosquitoes in most parts of the world. But many of these chemicals are toxic to human, plant and animal life and resistance can be problematic in maintaining control. With increasing environmental concerns, synthetic pyrethroids have replaced

organochlorines, organophosphates and organocarbamates in mosquito control (WHO, 1997). Use of permethrin **(24)** treated bed-nets has had tremendous impact on malaria cases in Kenya (MacIntyre *et al*., 2003). Other examples of synthetic pyrethroids used as insecticides include; cyfluthrin **(25)**, cypermethrin **(26)**, λ-cyhalothrin (27) and bifentrin **(28)** (WHO, 1997).

There has been serious concern about the use of chemical-based mosquito larvicides and repellents and their persistence in the environment, as well as development of physiological resistance in the insects. As a result, natural products of plant origin with insecticidal properties have been tried as an indigenous method for the control of a variety of insect pests and vectors (Khanna *et al*., 2011; Peeyush *et al*., 2011).

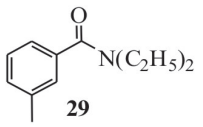


Insecticides for reduction of man-vector contact approach have been used in the control of adult mosquitoes. The approach involves clothing, netting, screening, use of repellents and insecticides. Clothing, bed nets and curtains impregnated with pyrethroids have been used against biting insects in various parts of the

world (Herber and Kroeger, 2003). The effectiveness of permethrin **(24)** impregnated bed nets in Tanzania (Lines *et al.*, 1987) and Kenya (Gimnig et al., 2003) has been documented. Permethrin **(24)** deposits have a long shelf life but frequent washing and handling reduce efficacy (Rafinejad *et al.*, 2008). Use of insecticide treated nets (ITNs) reduced malaria inoculation rate by 90% in Tanzania (Magesa *et al.*, 1991). Similarly, 5.5 deaths are prevented each year for every 1,000 children using ITNs in Kenya (Lengeler, 2004). Although pyrethroid impregnated bed-nets and curtains are widely employed to reduce the risk of malaria transmission, its resistance is becoming more prevalent among malaria vectors (Awolola *et al*., 2009; Enayati and Hemingway, 2006).

Repellents are among the most commonly used methods to prevent adult mosquitoes from biting. They provide short-lived protection due to their volatile nature (Debboun *et al.*, 2007). The most widely used commercial repellent is N,N-diethyl-m-toluamide (DEET) **(29).** It repels a wide variety of insects, ticks and mites, and lasts longer than other repellents (Curtis *et al.*, 1987).

Other synthetic repellents include: dimethyl phthalate **(30)**, 2-ethyl-2-butyl-1,3-propanediol **(31)**, *O*-chloro-*N,N*-diethylbenzamide **(32)**, 2-ethyl-1,3-hexanediol **(33)** and *n*-propyl-*N,N*-diethyl-succinamate **(34)**.



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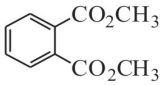

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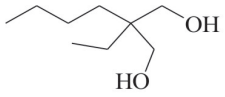

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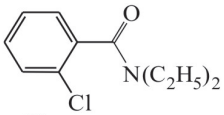

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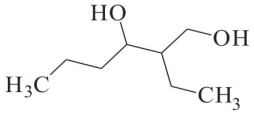

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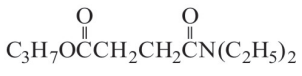

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Allergy and other serious reactions to DEET **(29)** have rarely been reported (Goodyer and Behrens, 1998). Since children's is more sensitive, it is recommended that DEET **(29)** exposure be kept to a minimum by applying to clothing

and not skin (WHO, 1991a). Other modes of administration of repellents include use of mosquito coils, which is popular in many tropical countries (Deepak, 2011), candles, incense, creams, foams, soap, lotion, sprays among others. Housing improvements that reduce access of mosquitoes to the occupants such as screening of windows and doors may have a major impact on malaria transmission control if rigorously promoted (Kirby *et al*., 2009).

Attractants have also been used to control adult mosquitoes. Host-attractants baited traps and targets are promising technologies currently being considered for mosquito control (Mathenge *et al*., 2002). It is widely used for the surveillance of mosquitoes in the centre for disease control (CDC) trap. The trap uses carbon dioxide and a light source to attract mosquitoes (Kline and Shreck, 1994).

Mosquitoes use chemical attractants to locate their mates and hosts for blood meals (Takken *et al*., 2001). Host attractants therefore appear to offer potential application for use in traps and targets in the effort to minimize the threat of human diseases transmitted by mosquitoes. However, attractants of disease vectors and human pests are not adequately studied (WHO, 1996) and therefore not well understood.

Traditionally, plants and their derived substances were used to repel or kill mosquitoes (Innocent *et al.*, 2010; University of Mississippi, 2006). Smoke from burning cattle or goat dung was used in some communities to keep mosquitoes away from human dwellings. It has been reported that repellent effect of smoke may be increased by burning certain materials such as aromatic wood containing resins (Snow *et al*., 1987). During the World War II, the military used oils like citronella, bergamot, eucalyptus, peppermint, turpentine and spirit of camphor in various formulations to repel biting insects. However, several plants and products that are traditionally used to kill or repel mosquitoes and other

blood sucking insects lack scientifically reliable data that would demonstrate their efficacy under uncontrolled field conditions (Curtis *et al*., 1991). In Southern India, leaves of *Vitex negundo* are burned to repel mosquitoes from houses (Curtis, 1991). In Africa, Asia and Latin America, leaves of the neem tree (*A. indica*) are sometimes burned providing unpleasant odour for the mosquitoes (WHO, 1997). It is believed that the neem tree keeps mosquitoes away but the Environmental Protection Agency (EPA) has not approved it for use as a topical insect repellent (Reutemann and Ehrlich, 2008).

The repellent effect of *Lantana camara* flowers has been evaluated against Aedes mosquitoes. *Lantana* flower extract in coconut oil provided 94.5% protection from Aedes albopictus and *Aedes aegypti* (Dua *et al*., 1996). Studies have shown *Ocimum* sp to be repellent against mosquitoes, either by burning or hanging fresh leaves in occupied houses or by brushing foliage on the exposed arms and legs of humans (Waka *et al*., 2004). *Ocimum forskolei* has been reported as a beneficial repellent in reducing vector biting if used in communities in areas with partially zoophilic mosquito species such as *An. stephensi* and where animals are present (Waka *et al.*, 2006). Ethno botanical survey in two communities in western Kenya revealed that the most commonly known repellent plants were *Ocimum americanum, Lantana camara, Targetes minuta, Azadirachta indica, Hyptis suaveoleus* and *Ocimum basilicum*. Direct burning of the plants is the most common method of application. The repellency of these plants was evaluated against the *An. gambiae s.s*. Giles in experimental huts within a screen walled green house (Seyoum *et al*., 2002). Plant essential oils and terpenoids have also been reported to show repellency to adult mosquitoes (Birkett *et al.*, 2011).

The most efficient approach to control the vector is to target the immature stages of the life cycle. But WHO (2004a) has observed that larval control is an often overlooked control method which can be extremely useful either by itself or in

an integrated vector management (IVM) program. Killeen *et al*., (2002) suggest that the limitations of larval control in SSA are 'practical rather than functional' and that; because of the limited mobility of the immature mosquito stages, they can be effectively controlled.

1.3.2.2. Larval control

Methods used for larval control include: environmental management, synthetic chemical larvicides, biological control agents, botanical anti-pest agents and insect growth regulators (IGRs). Environmental management approach to vector control aims at modifying the environment to deprive the target vector of its requirements for survival (mainly breeding, resting and feeding). According to WHO, environmental management for vector control involves planning, organization, carrying out and monitoring of activities for the modification and/or manipulation of environmental factors or their interaction with man with a view to preventing or minimizing vector propagation and reducing man-vector-pathogen contact (WHO, 2004b). These include building dams at high altitudes or far away from settlements, draining water in permanent (swamps) and temporary breeding habitats (tryes, tins, water storage tanks), intermittent irrigation, dessication by planting trees and improved housing (Ghebreyesus *et al*., 1999).

In Malaysia, the breeding of the *An. maculata* in streams has been controlled by periodical flushing of small dams with siphons and sluice gates. In Indonesia, changing the salinity of the breeding habitats of *An. sundaicus* has been used as an environmental management tool for vector control (WHO, 1995). In India (Watts, 1999) and Tanzania (Marcia *et al*., 2009), community-based environmental management projects for vector control have been undertaken. However, environmental control has suffered a major setback because some vectors have undergone adaptations that make them survive in temporary water collections in tree-holes, animal hoof prints, old disused tins, clay pots, tyres and

mashy grounds (Becker *et al.*, 2010). Additionally, most of these environmental control strategies are not universally applicable and should be designed with close attention to the local ecological, socio-economic, political and cultural factors in mind. Environmental management practices are labour intensive and repetitive while some drains require engineering works and is costly (Marcia *et al*., 2009). Therefore, environmental management should not be a replacement of other control strategies but one of the several optional components that will make up an intergrated vector management (IVM) approach to vector control programmes (Beier *et al.*, 2008). Hence, there is need for other control strategies like larvicides for effective mosquito control.

Biological larvicides have also been used in mosquito control. This involves introduction of natural enemies such as parasites, pathogens and predators into the environment. *Bacillus thuringiensis* serotype H-14 (B.t. H-14) produces toxins (δ-toxin) which kill larvae after ingestion and is effective against mosquito strains resistant to chemical larvicides (Subbiah and Abidha, 2010). Major drawbacks of *B.t.* are lack of residual activity, persistence for months in larval habitats and it is difficult to culture (Jeff, 2009). *Bacillus sphaericus* var israeliensis also produces a larvicidal toxin but is more effective in polluted water while *B.t.* H-14 is more effective in clean water (Yousten *et al.*, 1992). Bacteria agents have a slow mode of action as compared to chemical larvicides and are sensitive to ultra violet light (Yousten *et al*., 1992). The more recent strategies in vector control is the use of bacterium biopesticides, *Wolbachia* (Xi *et al*., 2005) and densovirus (Gu *et al*., 2011) that manipulate the life cycle of the mosquitoes. These strategies have been reported to be effective but need more research.

Inert spores of the fungus, *Beauveria bassiana*, kill mosquitoes when sprayed on walls and bed nets (Donald, 2005). Other fungal bio-control like *Entomopghag maimaiga* (Ann and Ruth, 2010), *Mediga saviva* and *Cordyceps militaris*

(Li *et al*., 1995) have also been used for larval control. *Tolypocladium cylindrosporum* Gams, is a potential microbial control agent of mosquitoes (Bandani et al., 2000). It is effective against *Culex* and *Psorophoria* with variable activity against *Aedes* species (Scholte *et al*., 2004).

There have been tremendous advancements in the ability to transmit some mosquito pathogenic viruses as well as new molecular tools and capabilities to understand and manipulate viruses at the molecular level. *Culex nigripalpus* baculovirus (C.N.B) has been studied for possible mosquito control. It is highly pathogenic to *C. nigripalpus* and *Culex quinquefascitus* both of which are important vectors of St. Louis and Eastern encephalitis virus. It is also responsible for the reduction of field populations of C. nigripalpus larvae (Becnel, 2006); *Romanomermis culicivorax* (RC), an entomopathogenic nematode; parasites, *C. quinquefasciatus* and *An. pseudopunctipennis* larvae (Santamarina *et al.*, 1996). Larvae of *Toxorhynchites, Culex* and *Lutzia* species predate on other mosquito larvae, thus reducing their populations. This may be a way that natural population control may be taking place.

Genetically manipulated vectors have also been used as bio-control agents (Sparagano and De Luna, 2008). However, their application may present serious operational problems. Proposed improvements in sterile insect techniques include release of insect carrying dominant lethal genes that may make it a practical bio control method (Thomas *et al.*, 2000). However, mass application may present serious operational problems.

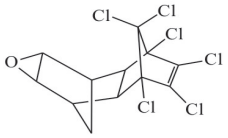
Vertebrate bio-control agents include fishes, like *Gambusia affinis* and *Poe cilia reticulate,* which feed on mosquito larvae (Ghosh and Dash, 2007). However, mass breeding and introduction in unstable habitats present serious challenges. Bio-control agents being members of a balanced ecosystem cannot be an alternative to insecticidal control. However, they can be used as adjuncts in logical

and necessary components of integrated vector management (IVM) measures as population stabilising agents. They are slow acting and cannot be used in emergencies. Alternative control strategies can be sought from chemical larvicides.

Larvicides are applied to mosquito breeding sites to kill larvae. They act as stomach poisons, which must be ingested by the larvae while feeding or as contact poisons, which penetrate the body wall or the respiratory tract. Application of petroleum oil on stagnant water is one of the oldest methods of killing mosquito larvae in large-scale operation (Thevasagayam *et al.*, 1979). The larvae are killed in two ways when they rise to the surface to breath: by suffocation and by poisoning with toxic vapour. Because of the relatively high cost of petroleum oils compared with some other larvicides, environmental pollution, toxic effect on non-target organisms and development of resistance by mosquito larvae, their use for mosquito control has decreased (Wigglesworth, 1976). They are of special interest in situations where mosquitoes have developed resistance to insecticides. However, for small-scale applications, they offer the advantage of wide availability. Monomolecular organic surface films alter water surface tension and thus disrupt behaviour and normal development of immature mosquitoes. The role of surface tension agents (surfactants) and their effects on immature stages of mosquitoes has been studied by several workers. For instance, White *et al*. (1978) reported how changes in surface tension of water by the addition of surfactants and monomolecular organic films produce substantial mortality in mosquito larvae and pupae or results in the drowning of emerging adults in treated water.

Synthetic inorganic compounds such as paris green, $(Cu(H_2O)_2.3Cu(AsO_2)_2$ and copper metarsenite, $Cu(AsO_2)_2$ have also been used as larvicides (Metcalf and Flint, 1962). Organochlorines like DDT **(18)** and dieldrin **(35)**, organophosphates such as temephos **(36)**, Malathion **(19)**, parathion **(37),** fenitrothion **(21)**, chlorpyrifos **(38)** and pirimiphos methyl **(39)**; and organocarbamates such

as propoxur **(38)** and bendiocarb **(41)** have been used to control insect larvae (WHO, 2009). Resistance of anopheline mosquitoes to organochlorine (DDT (1) has been reported (Philippe *et al.*, 2009). Organochlorines have progressively been replaced by other insecticedes partly because of emergence of insecticide resistance in target species (Trigg and Kondrachine, 1998). Consequently, organophosphates and organocarbamates have been recommended for many situations including household use because of low toxicity to non-target organisms and low effective dosage (Boobis *et al*., 2008). Due to biodegradability, low soil persistence and low toxicity to fish, phosphates and carbamates have also found use as mosquito larvicides. However, resistance to phosphates and carbamates have been reported (Ahoua *et al.*, 2010).



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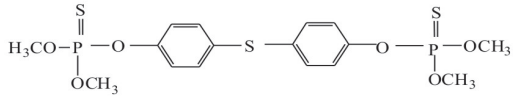

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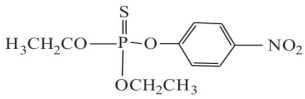

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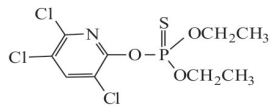

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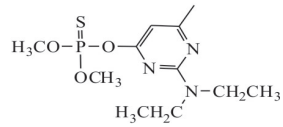

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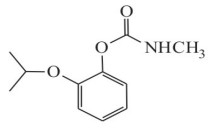

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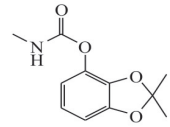

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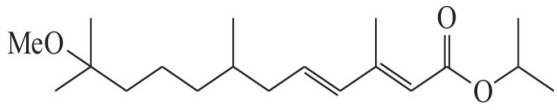
In response to strong selective pressures of herbivorous insects, plants produce toxic secondary metabolites known as allelochemicals that often affect insect nerve function and behavior (Freeman and Beattie, 2008). Studies on the natural plant products for larvicidal activity suggest potential sources of compounds for mosquito control (Sukumar *et al.*, 1991; Singh *et al*., 2006; de Omena *et al.*, 2007; Kihampa *et al.*, 2009; Githua et al., 2010). In view of the fact that mosquitoes develop resistance to synthetic larvicides (Tikar *et al.*, 2011) and even bio-pesticides such as *B. sphaebicus* (Lacey, 2007), the application of eas-

ily degradable botanicals for larval control is recommended (Alkofahi *et al.*, 1989). Studies from India (Joish and Pathipati, 2009; Remia and Logaswamy, 2010), North America (Bergeron et al., 1996), Argentina, Bolivia, Brazil and Peru (Ciccia *et al*., 2000), Trinidad and Tobago (Chariandy *et al.*, 1999), Mali (Diallo *et al*., 2001), Negev Desert (Sathiyamoorthy *et al*., 1997) and Nigeria (Mgbemena, 2010) among others have revealed numerous examples of plant extracts, representing diverse taxonomic groups, that are active against *Ae. aegypti.* More systematic and directed studies have revealed many active plant extracts, essential oils, and isolated larvicidal phytochemicals (Park *et al.*, 2002).

Although no traditional use of plant products targeting vector control has been documented, the possible deployment of natural products from readily accessible plants in community participation programmes to substantially reduce mosquito larval populations has been recognized, and plants belonging to the families Asteraceae, Verbenaceae, Meliaceae, and Rutaceae have been reported as potential sources of secondary metabolites for larval control (Innocent *et al.*, 2008; Katade *et al.*, 2006; Ndung'u *et al.*, 2004). Vitex species belonging to the family Verbenaceae, have been reported to exhibit larvicidal activities against a number of mosquito species (Rahman and Talukda, 2006; Yuan *et al.*, 2006; Kannathasan *et al.*, 2007; Rodríguez-Lopéz *et al.*, 2007; Karunamoorthi *et al*., 2008). Plants of this genus occur in both tropical and temperate regions of the world (Mabberley, 1997).

Methanolic extract of the leaves of *Atlantia monophylla* (Rutaceae) was evaluated for mosquitocidal activity against immature stages of three species, *C. quinquefasciatus, An. stephensi* and *Ae. aegypti* in the laboratory. Larvae of *C. quinquefasciatus* and pupae of *An. stephensi* were more susceptible (LC_{50} 140 and 50 µg/l, respectively) to the extract. IGR activity of the extract was more pronounced in *Ae. aegypti* (EI_{50} 2 µg/l). The extract was safe to larval predators: *G. affinis, P. reticulata* and *Diplonychus indicus* (LC_{50} 23.4, 21.3 and 5.7

mg/l) respectively. The larvicidal effect of the extract is comparable to that of neem extract and synthetic chemicals like fenitrothion **(21)** and methoprene **(42)** (Sivagnanam and Kalyanasundaram, 2004).

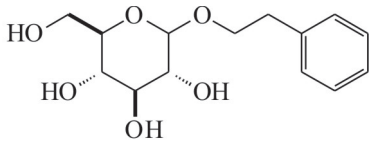


42

Udom *et al.* (2006) reported that ethanolic extracts derived from three species of Piperaceae (pepper) family, *Piper longum* L., *P. ribesoides* Wall and *P. sarmentosum* Roxy. Ex Hunt evaluated for efficacy against early 4th instar larvae of *Aedes aegypti* mosquitoes possesses high mosquito larvicidal activity with lethal concentrations (LD_{50}) ranging from 2.23 to 8.13 ppm. The leaf extract of *V. negundo, Nerium oleander* and seed extract of *Syzgium jambolanum* exhibited larvicidal activity against *C. quinquefasciatus* and *An. stephensi* (Pulshpalatha and Muthukrishnan, 1995). Crude extracts containing saponins from fruits pods of *Swartzia madagascariensis* produced higher mortality in *An. gambiae* larvae than *Ae. aegypti* and no effect in *C. quinquefasciatus* (Minijas and Sarda, 1986). Methanol leaf extract of *Cassia fistula* exhibited high larvicidal and ovicidal activity against *Culex quinquefasciatus* and *An. stephensis* (Govindarajan *et al.*, 2008).

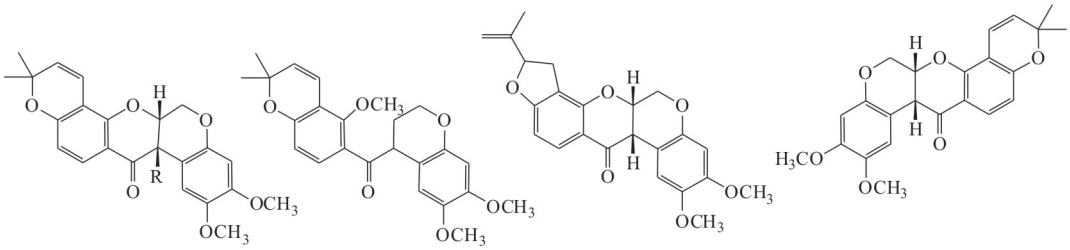
Alcoholic extracts of leaves and stems of *Vanilla fragrans* fractionated with ethyl acetate and aqueous butanol exhibited mosquito larvicidal activity (Sun *et al*., 2001). Extracts of different parts of neem tree are also potential mosquito larvicides (Howard *et al*., 2009; Okumu *et al.*, 2007). Petroleum ether extract of thyme plant, *Thymus capitatus*, was also found to be toxic to the larvae and adults of *Culex pipiens* (Mansour *et al.*, 2000). Phytochemicals derived from various botanical sources have provided numerous beneficial uses ranging from

pharmaceuticals to insecticides (Sukumar *et al*., 1991). *O*-phenylethyl-*D*-glucopyranoside **(43)** isolated from the stem bark of *Sida rhombifolia*, was evaluated for larvicidal activity against common filarial vector, *C. quinquefasciatus,* under laboratory conditions. The activity (LC_{50} 36.22, 43.94; 44.92, 58.34; and 60.40, 63.32, 70.72, 82.52 ppm) of the compound against 1st, 2nd, 3rd and 4th instar larvae after 24 and 48 h, respectively, has been reported (Ekramul *et al*., 2003). Larvicidal activity of a neem tree extract (Neemarin) has been reported to exhibit larvicidal activity against *An. stephensi* and *Culex quinquefascitus* under laboratory and field conditions with LC_{50} values of 0.35 and 0.69 mg/L respectively (Vetandoost and Viziri, 2004).



43

Chloroform extract of seeds of *Millettia dura* Dunn (Leguminosae) showed high larvicidal activity (LC_{50} 3.5 µg/ml after 24 h) against 2nd instar larvae of *Ae. aegypti* (Yenesew *et al*., 2003). Deguelin **(44)** and tephrosin **(45)**, isolated from the extract showed potent larvicidal acivity (LC_{50} 1.6 and 1.4 µg/ml, respectively, after 24 h). Saturation at the B/C ring junction and the presence of the methoxy groups at C-2 and /or C-3 in deguelin **(44)** and tephrosin **(45)** were suggested to be important for the observed larvicidal activity. Acetone extract of the seeds of *Derris trifolia* (Leguminosae) has also been reported to exhibit high toxicity against second instar larvae of the mosquito *C. quinquefasciatus* (LC_{50} 1.35 µg/ml) (Yenesew *et al.*, 2005). From this extract, a novel isoflavonoid derivative (7a-*O*-methyldeguelol) **(46)** together with three known compounds [(-)-rotenone **(47)** and (-)-deguelin **(48)**] were isolated (Yenesew *et al.*, 2005).

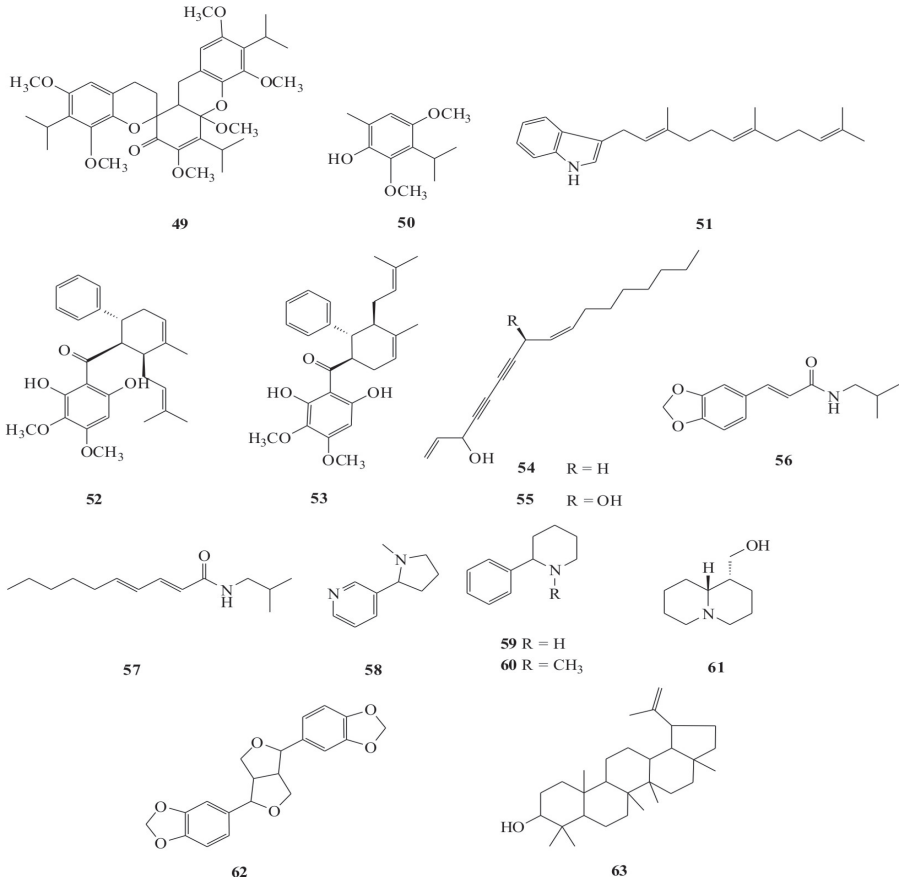


44 R = H
45 R = OH

46 **47** **48**

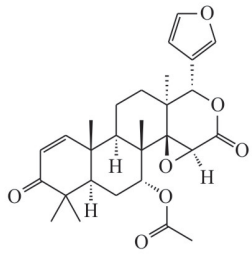
From the root bark of *Uvaria scheffleri*, a mild mosquito larvicide (±)-schefflone (49) and the anti-protozoal compound (espintanol) **(50)** have been isolated (Nkunya *et al.*, 2004). *U. scheffleri* is used traditionally for treating fevers (Kokwaro, 1993), and previous phytochemical investigations of the stem bark yielded 3-farnesylindole **(51)** as the anti-malarial constituent and the condensed chalcones (±)-schefflerion **(52)** and (±)-isoschefflerin **(53)** (Nkunya *et al*., 1990). From *Cryptotaenia canadensis*, an umbellifer frequently encountered in moist woodlands in North America, two acetylenic compounds: falcarinol (54) and falcarindiol (55) (LC_{50} 3.5 and 6.5 ppm, respectively, against *C. pipiens*), were isolated (Eckenbach *et al.,* 1999).

Isobutyl amides like fagaramide **(56)** and pellitorine **(57)** (LC_{50} 14.92 and 3.69 µg/ml, respectively have been reported to display good mosquito larvicidal activity against *An. gambiae* (Weenen *et al.*, 1990). Nicotine **(58),** anabasine **(59)**, methylanabasine **(60)** and lupinine **(61)** have also been reported to be effective against culicine mosquito larvae. Sesamine **(62)** and lupeol (63) have also been reported to exhibit some larvicidal activity against *An. gambiae* (Okinyo, 2002).



Limonoids from Meliaceae have anti-insect activity and low toxicity (Cespedes *et al*., 2004). Two non-azadirachtin limonoids, Gedunin **(64)** and epoxyazadiradione **(65)**, isolated from neem seed kernel oil were evaluated against fourth instar larvae of *A. eagypti* and *C. quinquefasciatus.* Gedunin **(64)** exhibited 100% toxic action against both mosquito larvae at 50 and 100 ppm while epoxyazadiradione **(65)** showed significant toxicity ≥ 50% against larvae of both mosquito species at 50 ppm (Gurulingappa *et al*., 2009). Wandscheer *et al.*, (2004) have also assayed limonoid extracts of *Azadirachta indica* and *Melia azedarach* against the larvae of dengue mosquito *Ae. aegypti* and suggested that by improving the extraction and fractionation of the crude limonoids, the larvicidal activity can be enhanced. Methanol extract from the aerial parts of *Roldana barba-johannis* (Asteraceae) afforded sargachromenol **(66),** sargahydroquinoic acid **(67)**, and sargaquinolic acid **(68).** These compounds show in-

secticidal and IGR activities against *Spodoptera frugiperda* (LC_{50} 20-35 ppm) (Cespedes *et al.*, 2004).



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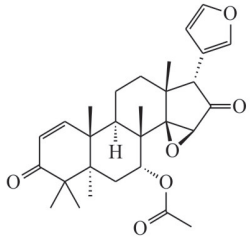

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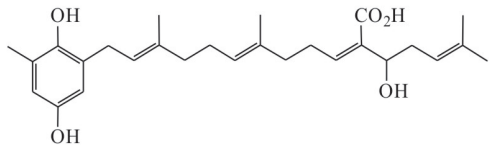

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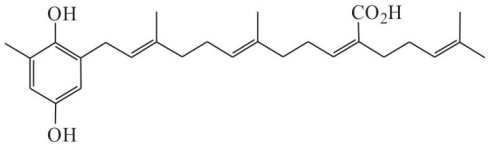

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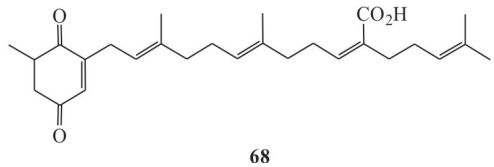

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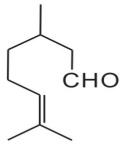
Other examples of botanical larvicides used include: citronellal (69), citrus oil extracts limonene **(70)** and linalool **(71)**] and sabadilla **(72)** (http://hgic.clemson.edu) among many others.



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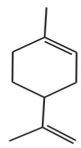

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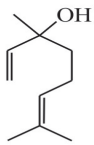

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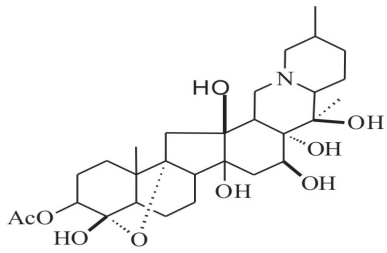
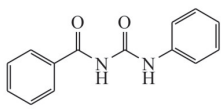


72

The problems posed by the use of conventional larvicides have been overcome by IGRs. Compounds containing growth-regulating properties are found in terpenoids, benzamides, carbamates, triazines, benzylurea and several other classes of organic compounds (Mulla, 1995). Synthetic IGRs including juvenoids and chitin synthesis inhibitors have shown promising results for the control of insects of public health importance (Williams, 1967). The efficacy of many

IGRs has been studied against various mosquito species (Tyagi *et al.*, 1987; Amalraj *et al.*, 1988). Laboratory and field investigations with methroprene **(42)** have shown high efficacy in controlling the immature stages of the dipterans of medical importance (Das *et al*., 1981).

Benzoyl phenylurea **(73)** causes various morphogenetic abnormalities in larvae, pupae and adult mosquitoes (Amalraj *et al*., 1988). The application of diflubenzuron **(74)** has shown high larval mortality in blackflies (Lacey and Mulla, 1977). Successful control of DDT-resistant strains of mosquitoes by the application of IGRs has been demonstrated in the laboratory (Schaefer *et al*., 1975). Methoprene **(42)** and diflubenzuron **(74)** have low mammalian toxicity (WHO, 1984). Fenoxycarb **(75)**, a non-neurotoxic carbamate, exhibits IGR activity on many insects (Grenier and Grenier 1993). IGRs are generally species specific, biodegradable, non-toxic to man and non-target organisms (Miura and Takahashi, 1973).



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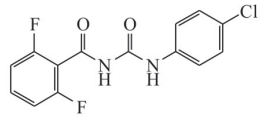

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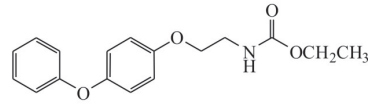
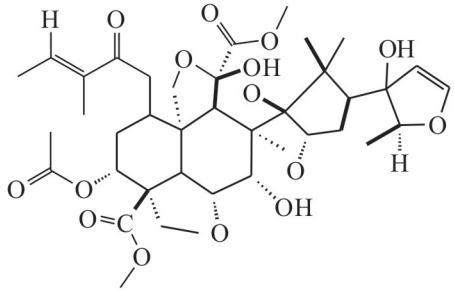


75

Previous studies with petroleum ether on six plants (*Acorus calamus, Ageratum conyzoides, Annona squamosa, Bambusa arundanasia, Madhuca longifolia* and *Citrus medica*) showed remarkable IGR activity, at 5 ppm, against *An. gambiae* larvae (Sujatha *et al.,* 1988). Similar observations were made by Raghavendra et al. (2009) who tested the larvicidal efficacy of the extracts of ripe fruits of *Solanum nigrum* against An. culicifacies species and An. Stephensi. Laboratory evaluation of crude extract of fruit of *Solanum xanthocarpum* (Singh and Bansal 2003) and aqueous extract from the roots of *Hibiscus abelmoschus* (Dua *et al*. 2006) were found to be toxic to the larvae of *An. culicifacies*. Plant-derived IGR substances are known to be safe to man and environment, and have a potential for intergrated pest management programmes (Williams, 1967).

So far, azadirachtin **(76)** is the most potent tetranotriterpenoid antifeedant and IGR isolated from neem tree (*A. indica*) that is being commercialised and marketed for managing insect pests (Devakumar and Sukh, 2000). It was isolated from the seeds of *A. indica* (Butterworth and Morgan, 1968) with the structural determination completed 17 years later (Kraus *et al*., 1985). 23-*O*-methylnimocinolide [7α-acetoxy-6α-hydroxy-23ξ-methoxy-3-oxo-24,25,26, 27-tetranorapotirucalla (apoeupha)-1,14, 20-trieno-21,23-lactone] **(77)** and 7-*O*-acetyl-23-*O*-methyl-7α-*O*-senecioylnimotrieno-21, 23-lactone **(78)** isolated from the methanolic extract of the fresh leaves of *A. indica* (neem) show IGR effect on Ae. aegypti (LC_{50} 53 and 2.14 ppm, respectively). The senecioyloxy substituent at C-7 in 78 results in a significant increase of activity (Bina *et al*., 1999). A number of limonoids isolated from several other plant species have also demonstrated larvicidal activity against larvae of *Anopheles* mosquito (Aliero, 2003; Ndung'u *et al*., 2004).



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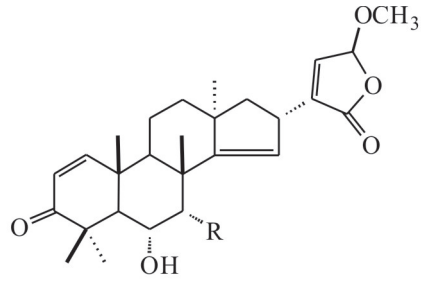


77 R = OAc

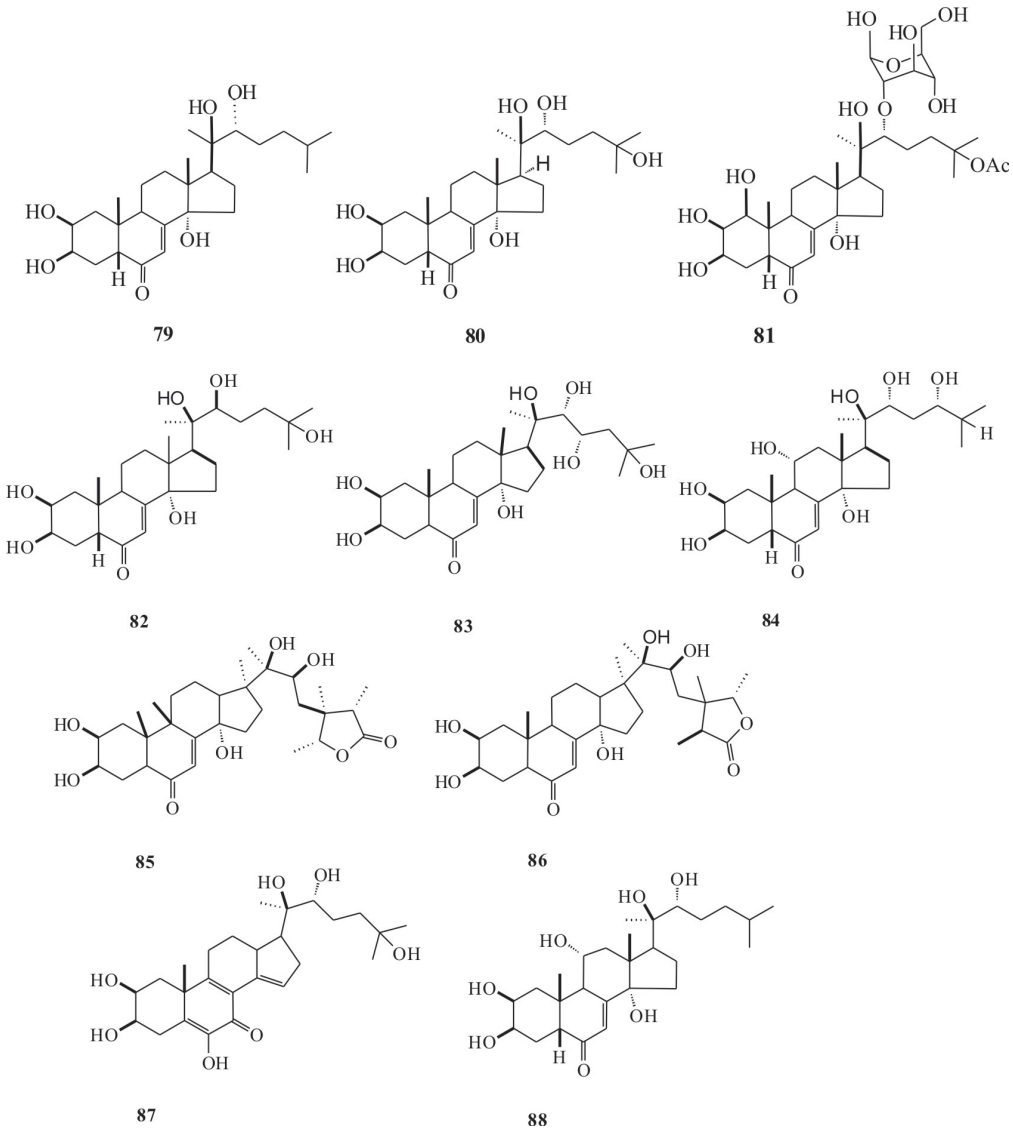
78 R=$O_2CCHC(CH_3)_2$

Ecdysteroids represent a widespread family of steroids found in both animal (zooosteroids) and plant (phytosteroids) kingdoms (Bergamasco and Horn, 1980). They were initially defined as moulting hormones. However, this definition appears to be too restricted, since they are present at all stages of insect development: in newly laid eggs, during embryonic and post-embryonic development, and in adult insects (Whiting and Dinan, 1988). The fortuitous discovery in 1966 of large amounts of ponasteroid A **(79)** in the bark of *Podocarpus nakaii*

was the starting point for highly fruitful research which has led to the discovery of more than 100 ecdysteroids in plants (Camps, 1992). In plants, ecdysteroids are widely distributed as secondary metabolites, and often reach concentrations of several orders of magnitude greater than in insects (Bergamasco and Horn, 1980). For example, flowers of *Serratula inermis* contain up to 2% of 20-hydroxyecdysone **(80)** (Yatsyuk and Segal, 1970). The roots of the Chinese herb, *Cyanotis arachnoidae*, afforded 2.9% of 20-hydroxyecdysone **(80)**, while the mature stem of *Disploclisia glaucesens* yielded 3.2% of 20-hydroxyecdysone **(80)**. Ecdysteroid concentrations vary with the plant part, climatic conditions, the season and habitat of the plant (Chou and Lu, 1980). The presence of ecdysteroids in plants represents a clear case of defense mechanism against insects using chemical mimicry.

From the results of phytochemical screening, ecdysteroids have been found in vascular plants: 27 families of *Pteridophyta*, 10 families of the *Gymnospermae,* and 74 families of the *Angiospermae.* The probability of finding ecdysteroids in ferns is high. The probability of finding active plant species in *Angiospermae* is typically much lower than in *Pteridophyta or Gymnospermae*. Among the Angiosperms: Verbenaceae, Labiatae (*Ajuga*), Asteraceae (*Serratuea*), Amaranthaceae (*Achyranthes, Cyathula*), Ranuculaceae (*Helleborus*) and Caryophyllaceae are more likely sources than others (Volodin *et al*., 2002; Dinan *et al*., 2001). Sileneoside H, a phytoecdysteroidglycoside, was isolated from the roots of *Silene brahuica* (Caryophyllaceae) and identified as 22-*O*-α-D-galactosylintegristerone A 25-acetate **(81)** (Sadikov *et al*., 2000). Two minor plant ecdysteroids, 22-epi-20-hydoxyecdysone **(82)** and gerardiasterone **(83)**, were isolated from Serratula tinctoria L (Compositae) (Bathori *et al.*, 1998). Punisterone [(20R,24S)-25-deoxy-11α,20,24-trihydroxyecdysterone] **(84)** has been isolated from the seeds of *Blandfordia punicea* (Satyajit *et al*., 1996) while cyasterone **(85)**, 25-epi-cyasterone **(86)** and 25-epi-28-*epi*- calonysterone **(87)** were recently isolated from *Cyathula officinalis* (Okuzumu *et al*., 2005). From

Ajuga (Lamiaceae), 20-hydroxyecdysone **(80)**, cyasterone **(85)** and ajugasterone C **(88)** were isolated (Santos *et al.,* 2001).



In this study, larvicidal and insect growth regulatory (IGR) compounds from *Vitex* species against *An. gambiae* were investigated.

1.4. Malaria vectors and life cycle of *An. gambiae*

Mosquitoes of the genus *Anopheles* are the exclusive vectors of malaria in hu-

mans. About 100 species of *Anopheles* are known vectors of malaria and the number is expected to increase as more sibling species of various complexes continue to be identified within the genus. Most of the malaria cases in tropical Africa are transmitted by three malaria vectors: *An. gambiae* sensu stricto, *An. funestus* and *An. arabiensis*. *An. gambiae* s.s. is the most efficient vector due to its high anthropophilic character. Due to the high densities of *An. gambiae* during wet seasons, it is an important vector in specific locations (Charlwood and Jolly, 1984). *An. funestus* is important in malaria transmission during dry periods since it breeds in permanent water bodies. The combination of anthropophilicity and endophilicity of *An. gambiae* and *An. funestus* puts them in an advantageous position over other malaria vectors (Gillies and Coetzee, 1987). *An. arabiensis* is known to vary from being anthropophilic to zoophilic depending on geographical location, climatic conditions and host availability. It has lower vectorial capacity than *An. gambiae* s.s (Brack *et al*., 1994).

The life cycle of mosquitoes can be completed in 1.5-3 weeks. They will mate during flight and once mated the female searches out a blood meal. After feeding, she seeks out a resting place to digest her meal in order to allow the ovaries to develop and then lay eggs (typically in 2 days after sufficient blood meal). Usually, the female lays a batch of 50-200 eggs in the shape of floating raft on the surface of the water at night. In tropical temperature, the eggs hatch in two to three days into larval stages in aquatic environment. The larvae will lie just below the surface of the water and feed on microorganisms and typically after 7-14 days (9 days in controlled environment at 28^{o}C) they will turn into pupae. *Anopheles* are unlike *Culex* and *Aedes* larvae since they do not have a breathing tube; they must lie parallel to the water surface in order to get a supply of oxygen through a breathing opening. The 1st instar undergoes 1st molting, and then 2nd instar undergoes 2nd molting and finally 3rd and 4th instars.

Pupal stage takes 2 days at 28^{o}C to several weeks to complete developing on

lower temperatures. The pupa is coma-shaped and is the least active stage of the *Anopheles* life cycle. After two to four days the pupa metamorphoses into an adult mosquito, in which both male and female mosquitoes feed on nectar and damaged fruits, but only the female feed on animal blood too to provide proteins for their eggs. The adult mosquito survives for between one week (in natural habitat) and one month (in captivity).

1.5. Statement of the problem

Malaria is a serious public health problem particularly to resource-poor people in rural Africa. Therefore there is need to develop an environmentally friendly and locally available larvicide that can be used in the control of mosquito larvae. From the reported literature, larvicidal principles of *Vitex* species found in Kenya that may be useful for the control of mosquito larvae have not been identified and evaluated. In previous work, while the less polar extracts had been worked on (Mokua *et al.*, 2010, Hernández *et al.*, 1999), the isolated compounds were not evaluated. Two important species in this study are *V. payos* and *V. schiliebenii.* Locally, the decoction of *V. payos* is used as remedy for stomach problems, the ground bark is administered to treat threadworm and skin problems, while boiled leaves are taken by patients to improve their appetite for food. The burnt leaves of *V. schiliebenii* are used as insect repellents. This study identifies and bioassays isolated compounds from these species with particular focus on aqueous extract which is more applicable to rural situations where malaria causes the greatest burden.

1.6. Significance of the study

Mosquitoes are responsible for more human diseases than any other group of arthropods. Malaria has been of real concern for centuries and it threatens the well being of more than 40% of the world's population. Mosquitoes kill between

1.5-2.7 million people each year while 300-500 million others fall ill of malaria. Furthermore, the parasites have become increasingly resistant to most commonly used and affordable anti-malarial drugs. An effective malaria vaccine would constitute a powerful control tool but none is currently available. Vector control based on chemical insecticides is a major technique to combat malaria in many parts of the world. However, the emergence and spread of insecticide resistance to new types of chemical insecticides and environmental pollution have necessitated the search for affordable, safe, biodegradable and effective alternatives. In the last 30 years of research, few insect growth regulators (IGRs) have been registered for use in mosquito control, a situation indicating the need for further evaluation and development of other effective and more affordable IGRs. The findings of this study will therefore contribute towards the development of more natural product-based IGRs that may be used as possible products for *An. gambiae* larval control.

CHAPTER 2: AIMS OF THE STUDY

2.1. General objective

The general objective was to investigate the larvicidal and IGR potential of *Vitex schiliebenii, V. payos* and *V. trifolia An. gambiae* larvae.

2.2. Specific objectives

The study set out to achieve the following specific objectives:

i. To carry out phytochemical tests of the extracts and compare their constituents with larvicidal results;
ii. To evaluate the larvicidal/IGR activity of the extracts under simulated semi-field conditions;
iii. To evaluate the effect of the extracts on Brine shrimp and An. gambiae larvae;
iv. To carry out bioassay guided isolation of compounds from the most active larvicidal extracts and assay them separately and as blends;
v. To elucidate the structures of the isolated compounds using spectroscopic and physical techniques.

CHAPTER 3: LITERATURE REVIEW

3.1. The family verbenaceae

The family Verbenaceae consists of shrubs or trees, sometimes climbing shrubs, rarely herbs. Indumentum of simple, stellata, and/or other complex hairs. The leaves are opposite or rarely whorled, without stipules, simple or 3 foliolate, less often palmately compound. They have inflorescence terminal or axillary, racemose, cymose, and spicate or thyrses. The flowers are bisexual or polygamous by arbotion, zygomorphic or rarely actinomorphic. The calyx is persistent. The corolla is 4 or 5 or more lobes and the lobes are usually spreading, aestivation overlapping. Their fertile stamens are inserted on corolla tubes, alternate with lobes; the filaments are free and the anthers are dorsifixed, 1 or 2- locular, dehiscing by longitudinal slits or pendulous. The style is terminal, simple, entire or 2-cleft. The fruits are drupe or indehiscent capsules, sometimes breaking up into nutlets. They have (1 or) 2-4 seeds without an endosperm and the seed coat is thin. Their embryo is straight which is as long as the seed and the radicle short which is inferior. The classification of Verbenaceae is in a state of flux, especially regarding its relationship to Lamiaceae (Mabberley, 1997).

3.2. The genus *Vitex*

The genus *Vitex* consists of over 270 species predominantly trees, shrubs and lianas with square branches. They grow to a height of 1-35 m tall and occur naturally in tropical and sub-tropical regions although few species may be found in temperate zones. The origin of the genus is obscure (Eagles, 1986) or may be from a Latin word meaning “to blind” (Bruce, 1985) referring to the flexible twigs (Mathews, 1962).

3.3. The Kenyan *Vitex* species

In Kenya, different *Vitex* species are found growing naturally at different ecological settings, including the coast, the dry woodlands, Mount Kenya area, and across the Rift valley to the shores of Lake Victoria (Figure 3.1; Beentje, 1994; Ruffo *et al.*, 2002). They are used in the local system of folk medicine by different communities for the treatment of a range of diseases (Kimondo *et al.*, 2010). Kenyan *Vitex* species include: *Vitex doniana* (black plum, mfudu in Kiswahili, mufuku in Gikuyu and mutahuru in Ekegusii); *V. mombassae* (mfudumaji in Kiswahili, mfududu in Giriama and mfudukoma in Digo); *V. payos* (chocolate berry, mfufu in Kiswahili and mfudu in Digo); *V. ambonienesis* (mufudu in Giriama); *V. fischeri* (mohutu in Nandi and mufutumwe in Ateso); *V. keniensis* (Meru Oak, muhuru in Gikuyu and muuru moru in Kimeru); *V. schiliebenii; V. madiensis; V. ferruginea; V. strickeri* (mukichano in Giriama, mvumbain in Kiswahili and Mukakinga in Gikuyu); *V. zanzibariensi* (vifuu in Kiswahili) and *V. tangensis* (mgegi in Kiswahili and mfududu/mufudumaji in Giriama). The distribution of the *Vitex* species in Kenya is shown in Figure 3.1.

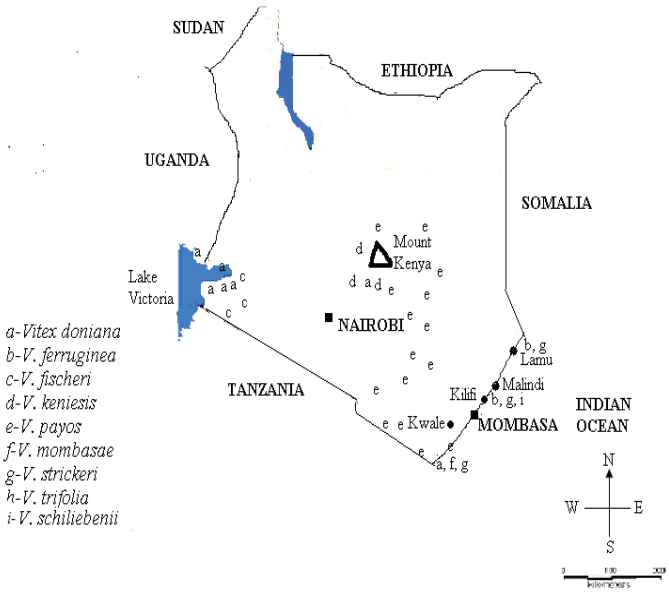


Figure 3.1: Distribution of *Vitex* species in Kenya: Source: Author's Literature

3.3.1 *Vitex doniana*

The common names of *Vitex doniana* (Figure 3.2) are: English-Black plum; Kikuyu-Muhuru; Swahili-Mfudu, Mfulu, Mfundu, Mfuru, Mfuu; Meru-Muur; Kisii-Mutahuru; Luo-Jwelu, Kalemba; Luyha-Muholu, Omuhutu; Pokot-Tirkiwa (Beentje, 1994). *Doniana* is a medium-sized deciduous tree, 8-18 m high with a heavy rounded crown. The bark is rough, pale brown or grayish-white. The bases of old trees have oblong scales. Leaves lie opposite to each other, glabrous, 14-34 cm long, usually with five leaflets on stalks 6-14 cm long. The leaf tips rounded. The leaves are dark green above, pale grayish-green below, thickly leathery with a few scattered stellate hairs on the upper surface. The flower petals are white except on the largest lobe which is purple in dense opposite and auxillary cymes. The flowers are small, blue or violet, 3-12 cm in diameter, only a few being open at a time. The fruits are oblong about 13 cm long; green when young, turning purplish-black on ripening and with a starchy black pulp. Each fruit contains one hard, conical seed, 1.5-2 cm long and 1-1.2 cm wide.

The fruits of *V. doniana* are sweet and tasty. They are occasionally sold. They contain Vitamins A and B and can be made into jam. The leaves are often used as herb for cooking. The leaves, pods and seeds are good fodder. *V. doniana* is a favorite tree for hanging bark beehives. It is also used for firewood and charcoal. The tree produces teak-like termite-resistant timber which is hard and suitable for light building, furniture, carvings and making boats.

The fruit can be made into wine. The pounded leaves can also be added to warm filtered grain beer and then taken as alcohol. The bark yields a dye that can be used for dyeing clothes. The fruit is also used to improve fertility and to treat anemia, jaundice, leprosy and dysentry. The roots are used to treat backache in women and the young tender leaves are pounded and the juice squeezed into the eyes to treat eye troubles (Kokwaro, *et al.,* 1993). Root extract is used locally

as curative drug for vaginal thrush. In Uganda and Ethiopia, the roots and stem bark are used as medicine (Katende *et al.*, 1995). The heavy rounded crown of *V. doniana* provides good shade. The tree has nitrogen fixing roots. The leaves can be used for mulch which is a soil improver. The tree is used in fields along boundaries.

Figure 3.2: *Vitex doniana*

3.3.2 *Vitex ferruginea*

Vitex ferruginea (Figure 3.3) is known as Mgegi-Swahili; Mfududu, Mfudum-aji-Giriama. It is a shrub or tree of 1.5 – 6 m. The leaves are 3-5 foliolate with leaflets which are elliptical. The apex is shortly acuminate with the terminal leaflet 5-14 by 2-4 cm. The leaves are finely pubescent beneath, sometimes only on the veins. The flowers are whitish with one large mauve lobe, in axillary dichasis 1.5-6 cm long. The flowers are 8-10 mm long. The fruits are grayish, speckled with white, globose, 15-25 mm long.

Figure 3.3: *Vitex ferruginea*

3.3.3. *Vitex fischeri*

Vitex fischeri (Figure 3.4) is synonymous to *V. keniensis.* Local names are Jwe-lo-Luo; Mufutumwe-Luhya; Omuhuruhuru-Teso (Beentje, 1994; Ruffo *et al*., 2002). It is a medium sized tree that grows up to 3-15 m high. The leaves have five leaflets which are elliptical or slightly obovate with the apex shortly acuminate. The terminal leaflets are 9-12 by 4-9 cm which are slightly sandpapery or rarely glabrous above but densely pubescent beneath. The flowers are white or pale mauve with lower lip darker mauve, in axillary dichasis 5-24 cm long. The flowers are 6-8 cm long. The fruits are purple to black, oblong-globose, 8-12mm long.

Its wood is used for furniture, coffin board, paneling and veer. In Kenya, it is in high demand for furniture and for making tool handles, and oxen yokes. The wood is suitable for light construction, flooring, joinery, interior trim, ship building, vehicles bodies, toys, novelties, boxes, crates, carvings turnery, draining boards, hardboard, particle boards and pulpwood. It is used as firewood and for charcoal production. The blackish pulp of the fruits is edible and eaten raw. It has been reported that the honey produced by bees visiting *V. fischeri* flowers is of superior quality and fetches high prices in the market. *V. fischeri* is planted

as a shade tree for crops such as coffee and yams and may be retained in maize and cassava fields. It is grown as an ornamental and windbreaker. It produces useful mulch and serves as a soil improver.

Figure 3.4: *Vitex fischeri*

3.3.4 *Vitex keniensis*

Vitex keniensis, (Figure 3.5) is also called Meru Oak-Trade name; Muuru moru-Meru (Ruffo *et al*., 2002; Beentje, 1994). It is endemic to Kenya and it is threatened by habitat loss. It is a magnificent tree when mature and also one of the largest trees that is native to Kenya. Due to its use as a source of wood that is both durable and has an attractive gain, it has been severely over exploited and is now very rare. It has a tall straight trunk and light green leaves with five leaflets arranged in a vaguely star-like formation. Each leaflet can be up to 25cm long with a prominent midrib. Young trees are particularly susceptible to a disease of the leaves that produces 'bubbles' all over the upper surface of the leaves but which does not appear to affect the growth of the tree.

Figure 3.5: *Vitex keniensis*

3.3.5 *Vitex mombassae*

Vitex mombassae (Figure 3.6) is also called Mfundumaji-Swahili; Mdudumadzi, Mfudukoma-Digo; Mkufu, Mfududu-Galla. It is a shrub or tree of 1.8–6 m. Leaves 3-5 foliolate, leaflets elliptical or slightly obovate. The apex sort-acuminate or acute and the terminal leaflet 4-12 by 1.5-4.5 cm, pubescent especially beneath. The flowers are white with lower lobe mauve in axillary dichasis 3-5 cm. The flowers are 8-14 mm long. The fruits are black, blobose, 23-30 mm across.

Figure 3.6: *Vitex mombassae*

3.3.6 *Vitex payos* (Chocolate berry)

V. payos (Figur 3.7) is known as black plum in English; Swahili- Mfudu; Kamba- Kimuu; Embu- Muburu; Giriama- Mfudu. It is a tree with round leather-like leaves and fruits that resemble black olives. The strong smelling fruits are surprisingly pleasant to taste, something like crumbled chocolate. The fruit tree has been ranked number one among the ten indigenous fruit trees species in Kenya (Muok *et al.*, 2002). In Kenya it grows in semi-arid parts of Eastern, Coastal and Central Kenya for example Kitui, Embu, Machakos, Kilifi, Kwale, Tharaka, Mbeere and Mwingi areas.

Figure 3.7: *Vitex payos* (Chocolate berry)

Nutritional composition of *V. payos* fruit pulp per 100 g of raw edible portion contains 63 kJ energy, 70.6% water, 27.4 g carbohydrates, 27 g fiber, and 0.7g proteins. Other parameters include 5.5 g ash, 0.8 g fat and 1.96 mg vitamins. Inorganic elements reported in the fruit include 34.0 mg calcium, 50 mg phosphorus and 2.7 mg iron (Muok *et al.*, 2002).

Fresh fruits are eaten by human beings, monkeys, gorillas, chimpanzees and elephants. The blackish pulp of the fruits is edible and eaten raw. It is used to

make jam. A beverage is used from the fruit juice and boiled fruits are the basis for an alcoholic liquor and wine. The seeds inside the fruit stone are also edible. Cooked young leaves are eaten as a vegetable or in sauces.

Pounded bark is administered to treat threadworm and skin problems. Leaf sap is used as eye drop to treat conjunctivitis and other eye problems. Leaves are boiled and drunk by patients who have lost appetite. A paste of pounded leaves and bark are applied to wounds and burns. Infusions are added to alcoholic drinks to make them stronger. A root decoction is administered orally to treat stomach problems or gastro-intestinal disorders. Powdered bark added to water, is taken to treat stomach complaints and kidney troubles. The bark is also used to treat leprosy, liver diseases and to control bleeding after childbirth. Dried and fresh fruits are eaten to control diarrhoea. The twigs are used as chewing sticks for teeth cleaning.

The blackish extract obtained by boiling leaves, bark, roots and/or fruits is used as ink and as dye for clothes. The flowers serve as a source of nectar for honey bees. Cattle eat foliage as fodder. The heavy rounded crown of *V. payos* provides good shade. The tree has nitrogen fixing roots; hence, it fixes nitrogen to the soil. The leaves can be used for mulching which improves the soil fertility. It can also be planted along boundaries.

The wood is popular for house building, vats, furniture, stools, carvings, tool handles, gunstocks, bowls, spoons and beehives. It is also suitable for light construction, light flooring, joinery, hardboard, particle board, wood-wool and pulpwood. The wood is used for firewood and charcoal production.

3.3.7 *Vitex schiliebenii*

Vitex schiliebenii (Figure 3.8) is a scrambling shrub or tree. The bark is grey to brown with young branch-lets four angled. The leaves are five foliolate, lower

most usually much smaller. The leaflets are elliptical to ovalate dark green, densely covered with hair below. Petiolules are short or absent. The flowers are short, dense axillary clusters, white with cream to pale yellow. The fruits are almost round, 4-6 mm in diameter, fleshy, white to purple black.

Figur 3.8: *Vitex schiliebenii*

3.3.8. *Vitex strickeri*

V. strikeri (Figure 3.9) is also called Mvumba-Swahili; Mfudu koma-Digo; Mukichano-Giriama; Mwalika-Kamba; Mukakinga-Kikuyu; Mugomba-Taita. It is a shrub or climber of 1 – 9 m. The leaves are trifoliate with the leaflets ovate. The apex acuminate with the margin toothed. The terminal leaflet is 3-10 by 1.3-5.5 cm. The leaves are sandpapery above, more or less pubescent and glandular beneath to almost glabrous. The flowers are white pale yellow near tip of lobes, in terminal and axillary panicles 1-8 cm long. The flowers are 3-5mm long. The fruits are black, globose, 3-8 mm long. The fruits are edible and the wood is used to make arrows by the Taita.

Figure 3.9: *Vitex strickeri*

3.3.9. *Vitex trifolia*

The plant *Vitex trifolia* L. (Figure 3.10) is commonly known as common chaste tree in English. It is an exotic plant from Asia occasionally grown wild in shore vegetation. In Kenya, it is found in Kilifi, Mombasa, Diani and Shimoni (Ruffo *et al*., 2002; Beentje, 1994). It is a shrub or tree of 1–9 m. The leaves are 3-5 foliolate and the leaflets are silvery-green beneath, apex acuminate. The terminal leaflet is 5-10 by 1.5-3 cm, minutely but densely pubescent beneath. The flowers are mauve, in terminal (sometimes also axillary) panicle 3-10 cm long. The flowers are 3-5mm and the fruits are 3-5 mm long.

The leaves are commonly used as poultice for rheumatic pains, in inflammations, sprains and fever. Roots are used to as febrifuge, to treat painful inflammations, cough and fever. Flowers are used in treating fever and fruits in amenorrhoea (Chopra *et al.*, 1956). This plant is known to possess various active constituents *viz*., essential oil (Pan *et al.*, 1989), halimane-type diterpenes, vitetrifolins (Ono and Nohara , 2001) and several pharmacological properties have been studied

viz., antipyretic (Ikram *et al*., 1987), antibacterial (Hossain *et al.*, 2001), against asthma and allergic diseases (Ikwati *et al*., 2001).

Figure 3.10: *Vitex trifolia*

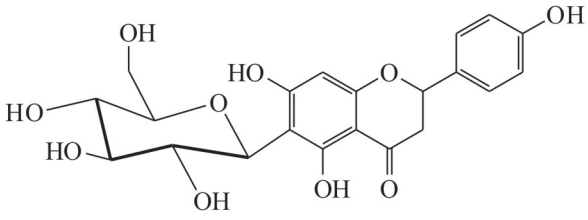
3.3.10. *Vitex zanzibarensis*

Vitex zanzibarensis is a large forest tree. The leaves are trifoliate with winged petiole. The petiole is auriculated at the base. In Kenya, it is found at Gogoni and Kinondo forests (Maundu *et al.*, 1999; Beentje, 1994).

3.4. Ethnopharmacological importance of the genus *Vitex*

The genus has been traditionally attributed to immense medicinal value. Various species of *Vitex* have been used to treat a range of human ailments, particularly related to insects, fungi, snakes and poisonous spiders and diseases associated with mensturation and gynaecological problems (Meena *et al.*, 2010). Vitexin **(89)** and β-sitosterol **(90)** from *Vitex lucens* have been reported to be a potent inhibitor of thyroid peroxidase (Harbone and Baxter, 1993). *V. leucoxylon L*. has been reported to possess anti-microbial and anti-inflammatory properties (Sarma *et al.*, 1990). The methanolic extracts of the *V. leucoxylon* leaf and stem bark has been reported to exhibit good anti-bacterial activity against Gram-pos-

itive (+ve) and negative (-ve) bacteria but low for Bacillus subtilis compared to other bacteria (Ganapaty *et al.*, 2005). The hot water extract of the fruits is used as vermifuge (Chopra *et al*., 1956). Phytochemical studies on the plant have revealed the presence of flavonoids, iridoids and sterols (Sarma *et al.*, 1990; Krishnaro *et al.*, 1997).



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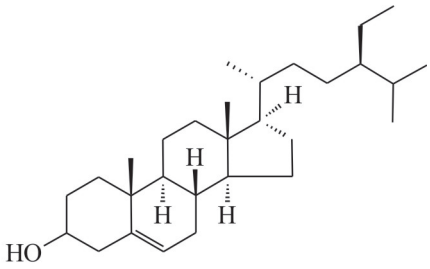

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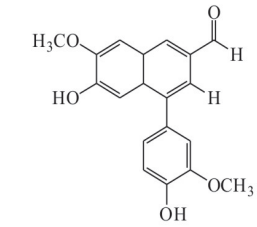
The leaf extract of *V. altissima* is used against fungal infections and in inflammatory conditions. The fruit has been reported to treat stomatitis, cardiac diseases, anorexia, blindness, leprosy and worm infestations and heart-wood. Anti-microbial properties have been reported by Ganapaty *et al.*, (2005). The flavonoids, triterpenoids, lignans and iridoids have also been reported to be present in this plant (Sridhar *et al.*, 2004, 2005; Parrotta, 2001). The leaves and bark have been reported to contain the flavonoid-vitexin **(89)** (Yoganarasimhan, 2000).

Extracts from leaves and roots of *V. negundo* have been reported to be important in the field of medicine and are sold as drugs. A decoction of leaves is used as tonic, vermifuge and is given along with long pepper in catarrhal fever (Chandramu *et al.*, 2003). Aqueous mature fresh leaves of *V. negundo* taken orally have been reported to have anti-inflammatory, pain suppressing, antihistamine, membrane-stabilizing, antioxidant activites (Dharmasiri *et al.*, 2003) and it is also used in the treatment of colds and coughs (Damayanti *et al.*, 1996). Anti-fungal activity against *Trichophyton metagrophytes* and *Cryptococcus neoformans* and anthelmintic activity against the Indian earthworm *Pheritima post-*

huma has been reported from *V. negundo* ethanolic leaf extract (Sathiyamoorthy *et al*., 2007). The methanolic root extract of the plant has also been reported to possess antisnake venom activity (Alam and Gomes, 2003). Tyrosinase inhibitory lignins have also been found in the extract (Mary and Villasenor, 2006). Umamaheswari *et al.*, (2007) report that the methanol root extracts (*V. negundo* and *Emblica officinalis*) antagonize the *Vipera russellii* and *Naja kaouthia* venom-induced lethal activity both in vitro and in vivo. The plant extracts therefore possess potent snake venom neutralizing capacity.

Classes of compounds found in *V. Negundo* include volatile oil (Singh *et al*., 1999), triterpenes (Chawla *et al*., 1992), diterpenes (Chawla *et al*., 1991), sesquiterpenes, flavonoids and stilbene derivative (Banerji *et al*., 1988), flavones gylcosides (Misra and Subramanian, 1980), iridoid gylcosides (Dutta et al., 1983; Sehgal *et al.*, 1982; 1983) and lignin (Azhar-ul-Haq *et al*., 2006). A number of phytochemicals have been isolated from the plant including vitrofolal E **(91)**, (+)-lyoniresinol **(92)**, (+)-olyoniresinol-3α-*O*-β-D-glucoside (93), (+)-(-)-pinoresinol **(94),** (+)-diaasyringaresinol **(95)** (Azhar-ul-Haq *et al.*, 2006; Yamasaki *et al.*, 2008). From an ethanolic leaf extract of *V. negundo*, two flavonoid glycosides were isolated namely 5-hydroxy-3,6,7-trimethoxy-2-(3,4-dimethoxyphenyl)-4H-chromen-4-one **(96)** and 5,7-dihydoxy-2-(3,4-dihdroxyphenyl)-4H-chromen-4-one **(97)**. The methanol extract has been reported to contain negundoside **(98)**, agnuside **(99)** and vitegnoside **(100)**. From the bark, p-hydroxybenzoic acid **(101)** and β-sitosterol **(90)** have been identified and isolated from the methanol and hexane extracts. In the acetoacetate fraction of the seeds of *V. negundo*, two pheylnaphtha-lene-typelignans have been obtained and were identified as 6-hydroxy-4-(4-hydroxy-3-methoxy)-3-hyroxymethyl-7-methoxy-3,4-dihyro-2-naphthaledehyde **(102)** and vitedoamine A **(103)**. It is used to treat dyspepsia, colic, rheumatism, worms, boils and leprosy (Mary and Villasenor, 2006).



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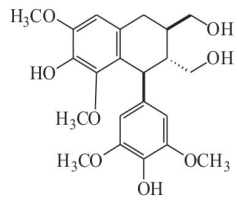

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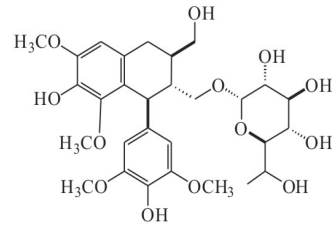

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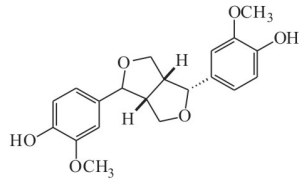

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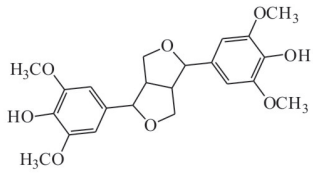

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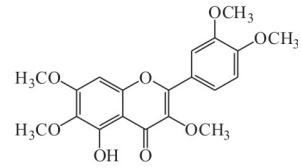

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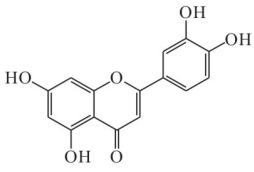

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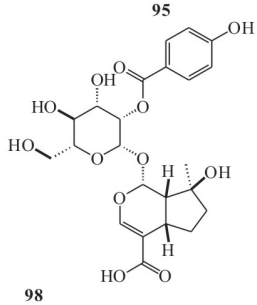

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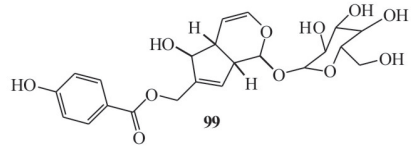

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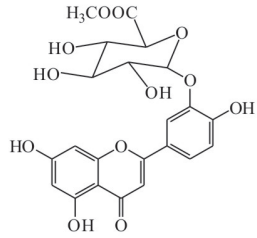

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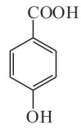

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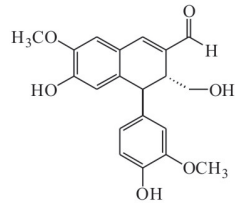

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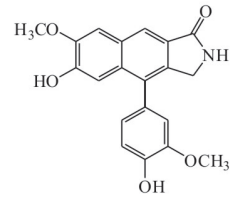

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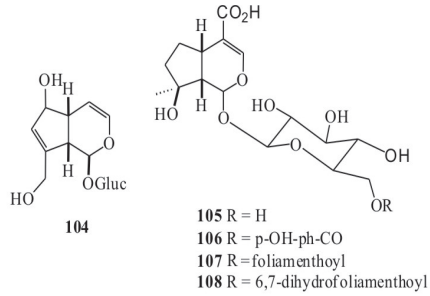
Vitex agnus-castus is renowned for its medicinal properties (Padmalatha *et al*., 2009). It is popularly used in folk medicine to treat ovarian insufficiency, uterine bleeding, premenstrual syndrome, fibroid cysts, infertility, hyperprolactinemia and acne in teenagers (Berger *et al*., 2000; Danielel *et al*., 2005; Hu *et al.*, 2007). It has also been traditionally used as a digestive aid, sedative and anti-infective (Christie and Walker, 1997). The plant has been reported to exhibit action against P388 leukemia cells, inhibition of prolactin synthesis and an inhibitor for dopamine D_2 and opiod receptors. There have been sev-

eral reports on its chemical constituents. It includes iridoids [agnuside **(99)**; aucubin **(104)**; mussaenosidic acid **(105)**; 6'-*O*-p-hydroxybenzoylmussanosidic acid **(106)**; 6'-*O*-foliamethoxylmussaenosidic acid (agnucastoside A) **(107)**; 6'-*O*-(6,7-dihydrofoliamethoxyl mussaenosidic acid (agnucastoside B) **(108)** and (agnucastoside C) **(109)**] (Kuruuzum *et al.*, 2003); flavonoids [vitexin **(89)**, kaempferol **(110)**, casticin **(111)**, quercetagetin **(112)**]; progestins [progesterone **(113),** hydroxyprogesterone **(114),** and rostenedione **(115)**]; volatile oil [1,8-cineole **(116),** α-pinenes **(117),** β-pinenes **(118),** sabinene **(119)**, (Z)-β-farnesene **(120)**, β-caryophyllene **(121)**, α-terpinenyl acetate**(122)**] (Sorensen and Katsiotis, 2000) and essential fatty acids [palmitic acid **(123),** oleic acid **(124)**, stearic acid **(125),** linoleic acid **(126)]** (Liu *et al.*, 2004).



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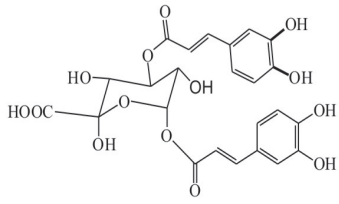
105 R = H
106 R = p-OH-ph-CO
107 R = foliamenthoyl
108 R = 6,7-dihydrofoliamenthoyl



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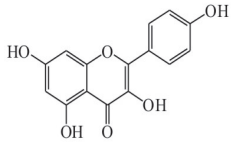

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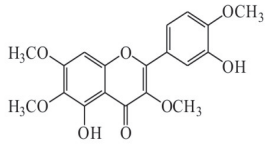

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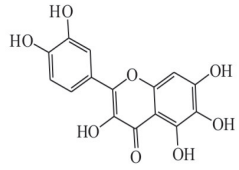

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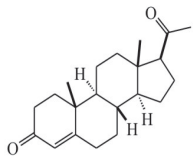

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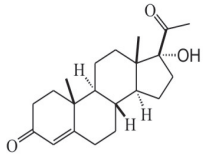

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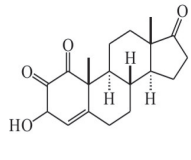

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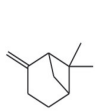

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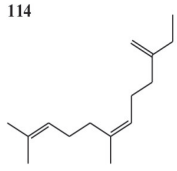

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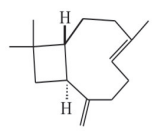

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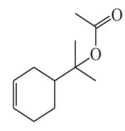

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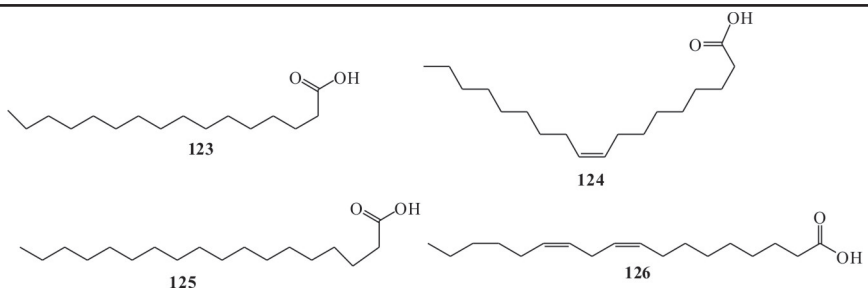
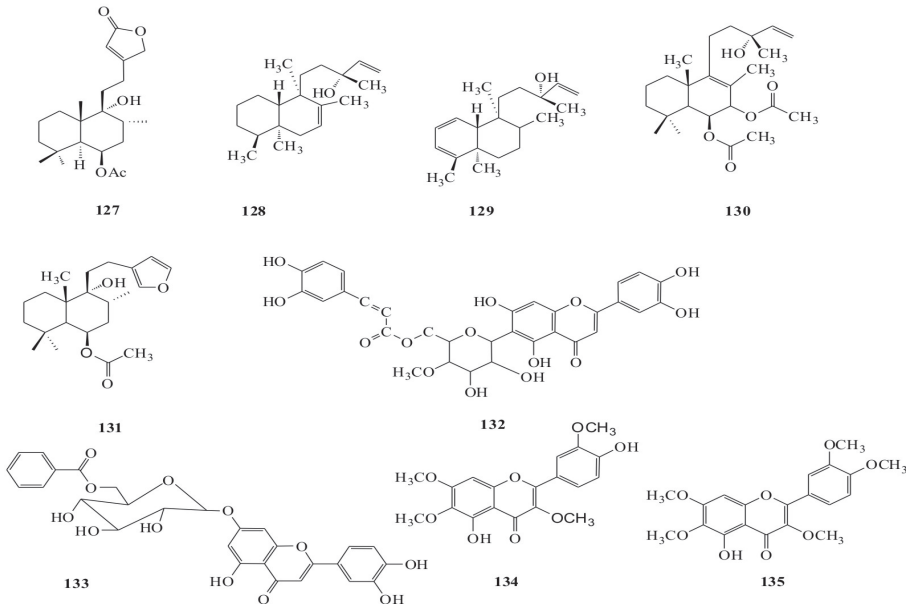
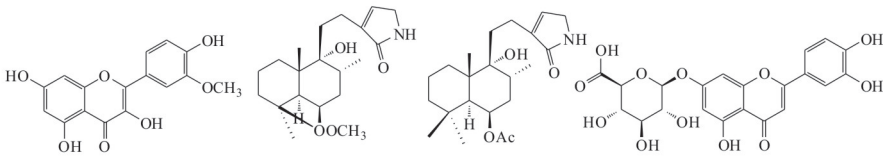


Bioactive constituents reported from the plant include vitexilactone **(127)** which has been reported as an anti-oxidant (Kondo *et al.*, 1986), clerodadienols, clero-da-7,14-dien-13-ol **(128)** and cleroda-1,3,14-trine-13-ol **(129)** implicated to be responsible for pre-mensturation management (Wuttke *et al.,* 2004) and 6β,7β-diacetoxy-13-hydroxy-labda-8,14-diene **(130)** and rotundifuran (**131**) reported as a prolactin secretion stimulant (Artz, 2006). Other phytochemicals reported from the plant as tumor inhibitors are luteolin 6-C-(4''-methyl-6''-O-trans-caffeoylglucoside **(132),** luteolin-7-*O*-(6-p-benzoylglucoside) **(133)**, 5, 4'-dihyrdoxy-3,6,7,31, tertamethoxyflavone, **(134)** artemetin **(135)** and isorhamnetin **(136),** vitexlactam A (137), 6β-acetoxy-9α-hydroxy-13 (14)-labden-16,15-amide **(138)** (Hirobe *et al*., 1997). Artemetin **(135)** and luteolin-7-*O*-β-glucuronide **(139)** have been reported to control haemorrhages (Alam and Gomes, 2003) and as anti-oxidants (Denney *et al.*, 2006).

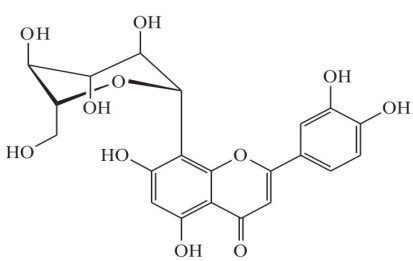




136 137 138 139

V. pinramidata, V. pubescens, V. gaumeri are folk remedies for diarrhoea, gastrointestinal infections, malaria, colds and cough spells (Argueta *et al.*, 1994; Ahmad and Holdsworth, 1995; Bajpai et al., 1995; Hernandez *et al.*, 1999). In Europe, fruits from the chaste-tree, *V. agnus-cactus* L., were used to treat pain in the limbs and general weakness (Grieve, 1931). Although not scientifically proven, Grieve (1931) also states that the fruits of the Australasian species, *V. trifolia* have similar medicinal properties to the chaste tree.

The leaf tea of *V. polygama* Cham. which is a Brazilian species popularly known as Tarumã has been reported to treat kidney diseases. Gonçalves *et al*. (2001) reported the anti-viral activity of flavonoids from *V. polygama*. *O*-glycosidic-flavones, orientin (**140**) and isoorientin **(141)** as well as C-glycosylflavones, schaftoside (**142**) and carlinoside **(143)** along with their isomers, known as potent anti-inflammatory, antinociceptive and antioxidant agents, identified as the active constituents have been isolated from the hydro alcoholic extract of leaves (Arokiyaraj *et al.*, 2009). The bark and fruits of this plant are reported to be traditionally used as emenagogues and diuretics. Phytoecdysteroids, 20-hydroxyecdysone (**80**), ajugasterone C (**88**), and turkesterone (**144**) have been isolated from the branches of *V. polygama* (Suksamrarn and Sommechai, 1993).



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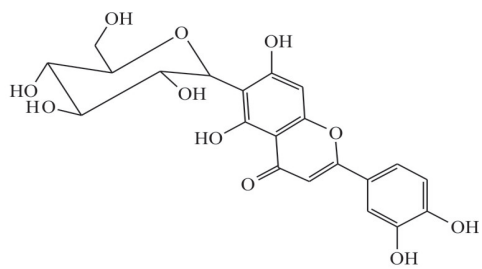

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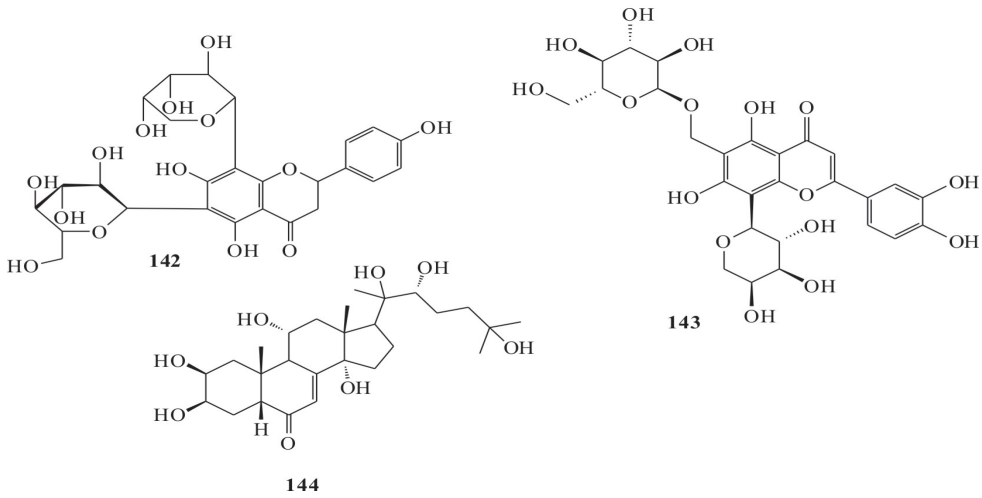
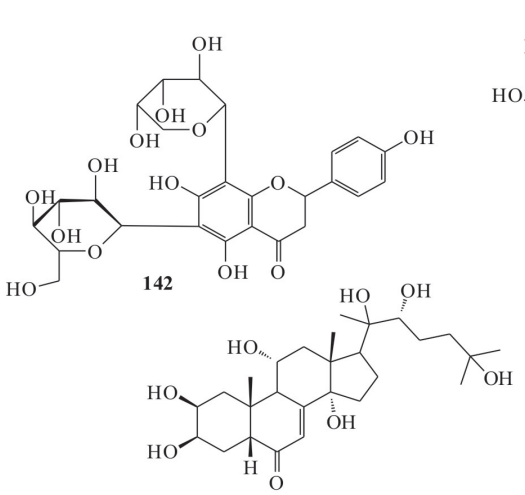


Biological assays of *V. trifolia* organic extracts have shown larvicidal activities. The petroleum ether and ethanol leaf extracts have been reported to exhibit moderate inhibition activity against both Gram-positive and Gram-negative bacteria (Hossain *et al*., 2001). In another study, the hexanic and dichloromethane extracts were shown to be very toxic against several cancer cell lines and also exhibited antifeeding activity against the insect pest *frugiderda* (Lepidoptera: Noctuidae). The hexanic leaf extract was reported to completely inhibit the growth of the fungal plant pathogen *Fusaruim* sp. (Hernández *et al*., 1999). Alcoholic and hexane extracts of *V. trifolia* have been reported to contain compounds that inhibit mast-cell degranulation and hence provide insight into the deveplopment of new drugs for treating asthma, and/or allergic disease (Ikawati *et al*., 2001).

The fruits of *V. trifolia* accommodate an interesting range of diterpenes including abietane-type diterpenes, named vitetrifolin A (**145**), and labdane-type diterpenes, named vitetrifolins B **(146)** and C (**147**), diterpenes, named rotundifuran (**131**), dihydrosolidagenone (**148**) and abietatriene-3ß-ol (**149**) which were isolated from the acetone fruit extract. These phytochemicals have been reported to exhibit anti-oxidant activity (Ono *et al*., 2000, 2001), treatment of amenorrhoea (Daniele *et al.*, 2005; Manjunatha and Vidya, 2008), and leuco-

derma (Rajendran *et al*., 2003; Tiwari and Tripathi, 2007).

Essential oils like limonene (**150**), humulene oxide (**151**), caryophyllene oxide (**152**) and α-humulene (**153**) have been isolated from the plant. These phytoconstituents have been reported to treat tooth and skin diseases, intestinal bilharzia, headache, amoebiasis (Nebie *et al*., 2005), alleviate dysentry, as an analgesic, treat stomach ache, relieve scorpion sting pain and inflammation (Argueta *et al.*, 1994). An ecdysteroid, 20-hydroxyecdysone (**80**) isolated from *V. trifolia* and *V. magapotamica* has been reported in the management of glycemic condition (Zannata *et al.*, 2007).



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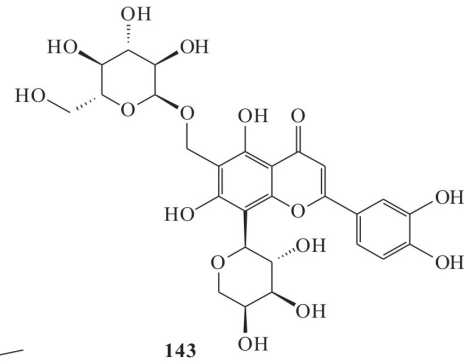

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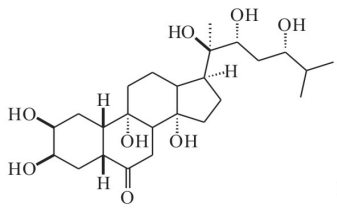
V. glabrata produces an edible fruit which is an important part of the diet for Aboriginal communities in the Kimberley area (Isaacs, 1987). Suksamrarn *et al.*, (1998, 1999) reported the presence of the insect growth hormone pterosterone (**154**), 20-hydroxyecdysone (**80**) and dihydroxyecdysone (turkesterone) (**144**) in *V. glabrata* and *V. diversifolia.* These ecdysteroids have been reported to be used as astringents, anthelmintics and in the treatment of gastrointestinal disorders.

Iridoids, viteoid 11 (**155**) and agnuside (**99**) have been isolated from *V. cymosa*. Ecdysteroids, 26-hydroxypinnatasterone (**156**) and 20-hydoxyecdysone **(80)** have been isolated from the stem bark of *V. cymosa* (Correa, 1926). In Nigeria, *V. doniana* is used as a nutritive sweetener, rich in vitamin C (Egbekun *et al*., 1996) and in the treatment of diarrhoea (Ladeji *et al*., 2004).

In Orissa, a decoction of the leaves of *V. peduncularis* leaves is taken as tea during cold season. Girach *et al.* (1994) reported that an infusion of leaves administered intramuscularly or orally to rabbits increase the osmotic resistance of cells and inhibits haemolysis produced by saponins, cobra venom, bile salt or saline solution. Peduncullariside (**157**) and the iridoid agnuside **(99)** have been isolated from *V. peduncularis* and have been reported to exhibit anti-infammatory and COX-2 inhibition activities (Suksamrarn *et al*., 2002). From the dried plant, ursolic acid (**158**), 2α-hydroxyursolic acid (**159**) and vitexin (**89**) have been isolated. Anti-bacterial, leukaemia, anti-carcinogenic and anti-mutagenic activities have been reported from *V. rotundifolia* (Kawazoe *et al*., 2001). In Brunei, the young leaf shoots of *V. pinnata* are eaten raw to treat hypertension and fever. A root-tea is taken for backache, bodyache and fatigue (Kok, 2008). The fruits of *V. mollis* have been reported to treat diseases such as fever, diarrhoea, dysentery and abdominal colic. It has also anti-inflammatory and analgesic properties. The plant has also been suggested for the treatment of scorpion stings and to alleviate menstrual pains. The fruits have been reported to be an important source of minerals, fiber and vitamins and they provide essential dietary nutrients (Herrera *et al*., 2004).



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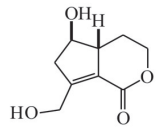

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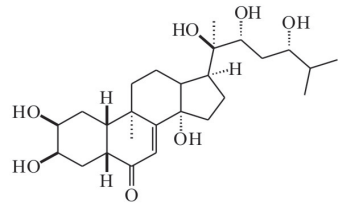

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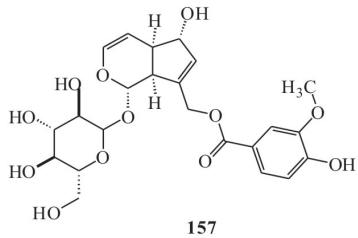

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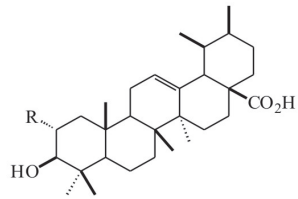


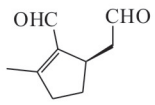
158 R = H
159 R = OH

In medical implication, the genus *Vitex* has been used in several features. Literature available reveals that the plants in the genus *Vitex* contain many bioactive agents ranging from antioxidants, anti-inflammatory, anti-microbial, hepatoprotective, analgesic antihistamine, anti-implantation and antiasthmatic effects. Thus, the genus may be an important source for the development of new drugs which may be cost-effective in treating other diseases.

3.5. The genus *Vitex* as a source of insecticides

The genus *Vitex* has also been reported to exhibit larvicidal activities against a number of mosquito species (Rahman and Talukda, 2006; Yuan *et al.*, 2006; Kannathasan *et al*., 2007; Rodriguez-Lopez *et al*., 2007; Karunamoorthi *et al.*, 2008). *Vitex negundo* extracts exhibit larvicidal activity against *C. quinquefasciatus* and *An. stephensis* (Pulshpalatha and Muthukrishnan, 1995) and antifeeding deterrent to *Ae. Aegypti* larvae (Hebbalkar *et al*., 1992). *Vitex rotundifolia* also shows feeding deterrent properties towards *Ae. Aegypti* larvae while insecticidal substances were reported to be present in the leaf tissue (Watanabe *et al.*, 1995). Rotundial (**160**), isolated from *Vitex* rotundifolia, exhibited insect

repellent properties (Ono *et al*., 1997). Leaf extract of *V. trifolia* leaf showed insect antifeedant activity against the insect pest *Spodoptera frugiperda* (Hernández *et al.*, 1999). Also, of particular interest is the presence of the insect growth hormone pterosterone (**154**) in *Vitex glabrata* (Suksamrarn *et al.*, 1999). The genus *Vitex* is therefore a good source of IGR compounds and this could be due to the presence of several bioactive compounds, which include ecdyteroids [24-Epi-pinnatasterone (**161**), scabrasterone (**162**), calonysterone (**163**), pterosterone **(154)**, 24-epi-makisterone A (**164**), polypodine B (**165**)] (Suksamrarn *et al.*, 2000).



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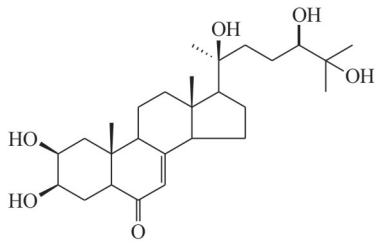

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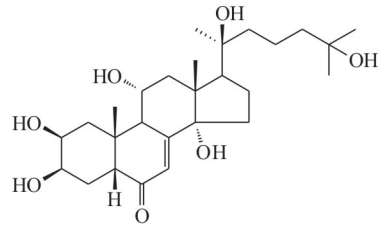

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CHAPTER 4: MATERIALS AND METHODS

4.1. General procedures

All solvents used were analytical grade (E- Merck, D-6100 Darmstadt, F.R. Germany). Analytical TLC was performed on 60 F_{254} plates 5 X 10 cm, 0.20 mm thickness) with fluorescence indicator. The spots were viewed using a multi-band UV- 254/366 nm lamp (UV GL-58). The TLC plates were then sprayed with *p*-anisaldehyde reagent (solution of anisaldehyde, 0.6% ethanol and 5% sulphuric acid) (reagents obtained from Sigma Aldrich, Germany) and kept in the oven at 110^{o}C until the spots appeared. Compounds containing unsaturated bonds especially those with conjugated systems became visible as quenching spots under UV light at 254 nm. Spots of organic compounds gave specific colors with this reagent after heating at 100^{o}C for 2-5 minutes.

Column chromatography was carried out using silica gel, mesh 0.06 mm (70-230 Merck-Germany) and eluted with varying concentrations of dichloromethane, acetone and methanol mixture.

4.2. Instruments

Melting points of the isolated crystallized compounds were determined on Sanyo Gallen Kamp electronic melting point apparatus and are not corrected.

^{1}HNMR spectra were run on Varian Gemini 300, 500 MHZ in $CDCl_3$ CD_3OD.

The chemical shift values (δ) were recorded in parts per million (ppm) and residual solvent peaks as reference. Proton noise decoupled (pnd) ^{13}C NMR, DEPT, COSY were determined on Varian-Germini/Bruker 600 MHz in $CDCl_3$

and CD_3OD. HSQC and HMBC were run in 500 and 600 MHz NMR machines. The multiplicities of ^{13}C signals were determined from DEPT analyses.

EI-MS of solid samples were obtained from Fissions VG Platform 11 model 12-250 MS upgraded (Manchester, UK), at 70 eV and 180^{o}C ion source temperature. Computer software (Mass lynx) and MS libraries (J. Willey, NIST) were used to match the spectra.

4.3. Plant collection and treatment

The investigated plant species were chosen based on their ethnobotanical and chemotaxonomical literature regarding their use as anti-mosquito plant species. Different plant parts (Table 4.1) were collected from different areas along the Kenyan coastal region. They were authenticated at the field by a Mr. Simon Mathenge, a botanist from the National Museum of Kenya (NMK) where voucher specimens were preserved at the Herbarium. *Vitex trifolia* and *V. payos* plant materials were collected from the South Coast of Kenya at Msambweni and Kaya Muhaka in the Kaya forest in Kwale district, respectively (voucher specimen Ref. Nos. GMN/20 and GMN/21) respectively while *V. schiliebenii* plant materials were collected from the North Coast at Kenya Forest Research Institute (KEFRI) near Gedi in Kilifi along Mombasa-Malindi road, 18 km from Malindi town (voucher specimen Ref. Nos. GMN/22).

Table 4.1: Investigated plant species

Name of Plant	Part collected	Place of collected
V. trifolia	Seeds, leaves, stem bark, roots	Msambweni
V. payos	Root bark, stem bark	Kaya Muhaka
V. schiliebenii	Leaves, stem bark, roots bark	Near Gedi

The materials were air-dried at room temperature for three weeks and ground into powder using an electric miller. Each powdered material was extracted three times in acetone (5-fold volume) for 24 h with occasional stirring. The extracts were filtered and concentrated to dryness using a rotary evaporator at 40°C and the combined extract stored at 4°C. This procedure was repeated with methanol in the same proportion and for the same periods.

The aqueous extracts were obtained using soxhlet extraction. The extracts were filtered and then freeze dried to obtain the dry powder which was then stored at 4°C in airtight containers for further chemical and biological analysis.

4.4. Mosquito larvae

Larvae of *An. gambiae* s.s. Giles used in bioassays were obtained from a colony maintained at the International Centre of Insect Physiology and Ecology (ICI-PE) at the Insect Mass Rearing Unit. This strain of mosquitoes originated from Njage village, 70 km from Ifakara, south eastern Tanzania and had been reared under laboratory conditions since April 1996. Larvae were allowed to emerge from eggs in plastic containers filled with distilled water and were transferred to larger pans (37 × 31 × 6) cm at densities of 200-300 at 2nd instar stage. Larvae were fed on Tetramin fish food and the water temperature was maintained at 28±2°C throughout larval development.

4.5. Bioassays

4.5.1. Laboratory larvicidal assay and IGR effect

Laboratory bioassays were conducted in accordance to the World Health Organization standard method (WHO, 2005). Crude acetone, methanol and aqueous extracts of each plant material (5 mg) was dissolved in 1 ml distilled water or absolute ethanol containing 5% dimethyl sulphoxide (DMSO) to prepare a stock solution of 5000 ppm. Different doses (25, 50, 100, 250 and 500 ppm) were prepared by serial dilution and the volume made up to 100 ml in 250 ml glass beakers. Twenty freshly molted late 3rd and early 4th instar larvae of An. gambiae from different rearing batches were tested in three replicates with two controls running simultaneously. The controls received absolute ethanol or DMSO-distilled water. Larval mortality (at higher doses), abnormal behavior and morphological deformations (at low doses) were observed at time intervals of 24 h until the death of the last larvae or emergence to adult as described in the WHO technical report series. The bioassay experiment room was kept at a temperature of 30^{o}C and an average humidity of 78 % and a photo period of 12 h of light and 12 h of darkness. The larvae were fed on Tetramin® fish food (Terta GmbH, Germany) at about 1 mg per beaker every 24 h.

Crude extracts that displayed strong larvicidal activity were further subjected to column chromatography fractionation. The fractions available in adequate amounts were assayed for larvicidal activity against *An. gambiae* larvae.

4.5.2. Simulated semi-field assay

Following Phase I (laboratory assay) carried out on larvicidal activity of *Vitex* species at the International Center of Insect Physiology and Ecology (ICIPE),

the efficacy of the extracts that exhibited significant larvicidal activity were validated in a simulated semi-field trial in a screened house. Bioassays were conducted following the standard procedures of World Health Organization (WHO, 2005b) at 500, 250, 100,50, 25 and 12.5 ppm based on Phase I trials and the total volume made up to 1000 ml chlorine free tap water in white plastic basins.

Figure 4.1: White artificial containers of water placed in a screened house under simulated field conditions for experimental set up.

White artificial containers of water were used (Figure 4.1). These containers were placed in holes in a screened house. Each test solution was done in triplicate with three negative controls running simultaneously. The water filled containers were left for 24 h for conditioning and then batches of 50 freshly molted late 3rd and early 4th instar larvae of *An. gambiae* were added together with food and left for 2-3 h to allow the larvae to get acclimatized with the environment. The larvae were then treated with test samples. Sampling method was based on 3 dips and live larvae were counted to score post-treatment larval mortality. All the treated and control basins containing pupae were kept separately in a netted cage to prevent successfully emerged adult from escaping into the environment.

The percent adult emergence inhibition (% EI) was based on the number of moribund and dead larvae, pupae that did not develop successfully into viable adults and adult mosquitoes not completely separated from the pupal case. During the experiment, the larvae were fed on Tetramin® fish food (Terta GmbH, Germany) and the adults 10% glucose solution.

4.5.3. Brine shrimp test (BST)

In vitro brine shrimp lethality test of the extracts was used to detect cell toxicity. Brine shrimp eggs were purchased from Aquaculture innovations (Grahamstown 6140, South Africa) and sea salt was prepared locally by evaporating water collected from the Indian Ocean, along the Dar es Salaam Coast. Brine shrimp eggs were placed in sea water (38 g of sea salt in one liter of distilled water) and incubated infront of a lamp. Eggs were hatched within 48 h providing a large number of brine shrimp larvae (nauplii). Stock solutions (40 mg/ml) of each extract were prepared by dissolving them in 5% DMSO. Different concentration levels were prepared by serial dilution and 10 nauplii were added into 10 ml vials. The volume was then adjusted to 5 ml with artificial sea water (3.8% w/v sea salt in distilled water). Each concentration was tested in triplicates with two negative controls running simultaneously containing 10 nauplii, sea water and 0.5% DMSO only for comparison. The vials were incubated for 24 h under the same conditions used for rearing the brine shrimp larvae. The dead larvae were counted and percentage mortality was calculated.

4.5.4. Toxicity of individual and blends of pure compounds

Larvicidal and IGR activity of the individual and blends of the isolated pure compounds were determined using $3^{rd}/4^{th}$ instar larvae of *An. gambiae.* The test larvae were treated with solutions containing the phytosteroids of concentration

1, 5 and 10 ppm. The blends were prepared in the ratio of 1:1. Accordingly, the control larvae were analogously treated except that their solutions did not contain steroids. Cumulative mean % mortality was calculated after 24 h until the death of the last larva or emergence of the adult.

4.6. Statistical analysis

4.6.1. Larvicidal activity

The average larval mortality (± standard error) resulting from each dose of each extract was calculated and the data was subjected to probit analysis for calculating the lethal concentrations of the crude plant extracts at LC_{50} at 95% confidence limit of upper and lower levels. The values were calculated using GenStat Discovery edition 4. Results with $p<0.05$ were considered to be statistically significant.

4.6.2. Adult emergence inhibition activity

The average number of larvae or pupae collected per dip for each replicate of each treatment and the control were calculated after 24 h. The percentage inhibition of adult emergence (% IE) was estimated (WHO, 2005b) for each treatment according to the formula:

$$IE\ \% = 100 - \frac{(T \times 100)}{C}$$

Where T = Percentage emerging in treated

C = Percentage emerging in control

Bioassay tests showing more than 10% control mortality were discarded and repeated. However, for those where control mortality ranged between 5-10%,

the corrected mortality was calculated using Abbott's formula (Abbott, 1925) as follows:

$$\text{Corrected percentage mortality} = \frac{\%\text{ mortality in treated} - \%\text{ mortality in control}}{100 - \%\text{ mortality in control}} \times 100$$

4.7. Phytochemical screening

Phytochemical tests were carried out on the acetone, methanol and aqueous extracts using standard procedures.

4.7.1. Alkaloids

Dragendorff's reagent was added to about 20 mg of the extract and the presence of orange red precipitate indicated the presence of alkaloids.

4.7.2. Tannins

A small quantity of the extract (2 mg) was mixed with water and heated on a water bath. The mixture was filtered and ferric chloride was added to the filtrate. A dark green solution indicated the presence of tannins.

4.7.3. Saponins

About 0.2 g of the extract was shaken with 5 ml of distilled water and then heated to boil. Frothing (appearance of creamy miss of small bubbles for about 15 min) shows the presence of saponins.

4.7.4. Flavonoids

An extract of about 0.2 g was dissolved in dilute NaOH and HCl was added. A yellow solution that turns colorless indicates the presence of flavonoids.

4.7.5. Steroids

About 2 ml of acetic anhydride was added to 0.5 g of the extract of each with 2 ml of H_2SO_4. The color changed from violet to green, indicating the presence

of steroids.

4.7.6. Terpenoids (Salkowski test)

About 0.2 g of the extract was mixed with 2 ml of chloroform and 3 ml concentrated H_2SO_4 was carefully added to form a layer. A reddish brown coloration of the interface was formed to indicate positive results for the presence of terpenoids.

4.8. GC-MS analysis

The extracts were analyzed by GC-MS on a 7890A stand-alone gas chromatograph (Agilent Technologies, Inc., Beijing, China) and a 5975 C mass selective detector (Agilent Technologies, Inc., Santa Clara, CA, USA) by using the following conditions: Inlet temperature of 270°C, transfer line temperature of 280°C, and column oven temperature programmed ranging from 35 to 280°C with the initial temperature maintained for 5 min then 10 °C/min to 280 °C for 10.5 min and then 29.9 min 50 °C/min to 285 °C. The GC was fitted with a HP-5 MS low bleed capillary column (30 m × 0.25 mm i.d., 0.25-µm) (Restek, Bellefonte, PA, USA). Helium at a flow rate of 1.25 ml/min served as carrier gas. The Agilent 5973 mass selective detector maintained an ion source temperature of 250°C and a quadruple temperature of 180°C. 230°C was set as the MS ion source temperature. Electron impact (EI) mass spectra were obtained at acceleration energy of 70 eV. A 1.0 µl aliquot of extract was automatically injected in the splitless mode using an auto sampler 7683 (Agilent Technologies, Inc., Beijing, China). Fragment ions were analyzed over 40-550 m/z mass range in the full scan.

4.9. Bioassay guided isolation of compounds

4.9.1. Acetone extract of *V. payos* root bark

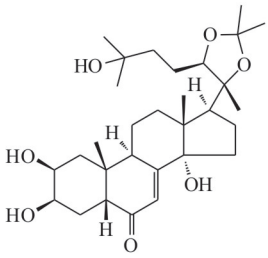
Powdered root bark (30g) of *V. payos* was extracted using acetone and the extract (8.0g) was subjected to column chromatography on silica gel (105g) (column diameter of 7 cm and silical gel height of 46 cm) eluting with 100% dichloromethane and gradually increasing acetone to 100% then methanol to 30%. The 60% acetone eluent gave a mixture of two compounds, which were separated by repeated preparative thin layer chromatography (PTLC) on silica gel (eluting with dichloromethane-acetone, 1:1) to give 20-hydroxyecdysone-20, 22-monacetonide **(166)** (16 mg of a white amorphous powder which was soluble in methanol, with melting point 257-259^{o}C; lit. 256°C, (Jiaju et al., 2011) and impure 20-hydroxyecdysone **(80)**. This was further subjected to repeated PTLC (eluting with dichloromethane-acetone, 4:1) followed by sephadex LH_{20} (DCM: MeOH 1:1). Ten milligrams of need-like crystals of melting point 234-236°C (lit. 230-233°C, (Kavel *et al*., 1998) were obtained which were soluble in methanol.

4.9.2. Acetone extract of *V. schiliebenii* leaves

The acetone extract (10g) was subjected to column chromatography on silica gel (column diameter of 10 cm with silical gel height of of 50 cm eluting with 100% dichloromethane and gradually increasing acetone to 100%). The 40% acetone eluent gave a mixture of two compounds, which were subjected to repeated column chromatography using DCM: MeOH to yield γ-sitosterol **(167)** (6 mg with a melting point of 142-144°C) which was then re-crystallized using the same solvent into a white star shaped crystal soluble in DCM. The other compound was too little to be analyzed.

4.9.3 Acetone extract of *V. schiliebenii* stem Bark

Sixteen grams (16 g) of the extract was subjected to column chromatography on silica gel with Dichloromethane: Acetone gradient (100:0 - 0:100). Column used was of diameter 12 cm and silica gel height of 55 cm.. The 50% acetone eluent gave a mixture of two compounds (47 mg), which were separated by repeated column chromatography followed by PTLC. The fraction yielded two compounds 20-hydroxyecdysone **(80)** (16 mg) isolated as a crystalline solid and stigmasterol **(168)** (25 mg of white crystals with a melting point of 165-7°C) lit. 163-6°C,



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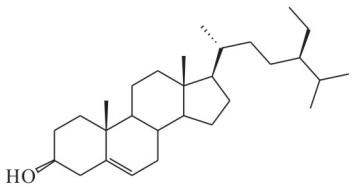

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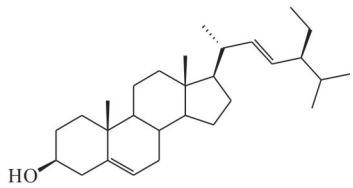

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CHAPTER 5: RESULTS AND DISCUSSION

5.1. Paper I

Comparison of the effects of extracts from three *Vitex* plant species on *Anopheles gambiae* s.s. (Diptera: Culicidae) larvae

5.1.1. General characteristics of crude extracts

Acetone, methanol and aqueous extracts of the three *Vitex* species, *V. trifolia, V. payos* and *V. schiliebenii* provided semi-solid or solid materials with various average percentage yields (w/w) (Table 5.1). The average yields ranged between 0.17-12.00 percent (w/w). The aqueous yields of most of the plant parts used were low compared to the acetone and methanol extracts. However, the amount of aqueous extracts from *V. schiliebenii* was relatively high.

Botanical name	Part used	Solvent	Physical characteristics	w/w % yield
V. trifolia	Seeds	Acetone	Oily green solid	3.37
		Methanol	Brown sticky solid	3.02
		Aqueous	Straw berry black	2.28
	Leaves	Acetone	Green semi-solid	12.00
		Methanol	Green solid	10.63
		Aqueous	Dark brown solid	6.25
	Stem bark	Acetone	Dark green solid	5.24
		Methanol	Greenish black solid	4.70
		Aqueous	Light yellow	3.59
V. payos	Root bark	Acetone	Shiny dark green solid	3.34
		Methanol	Shiny black gummy solid	5.87
		Aqueous	Light green solid	0.76
	Stem bark	Acetone	Green brown solid	0.16
		Methanol	Green brown solid	0.71
		Aqueous	Brown solid	0.68
V. schiliebenii	Root bark	Acetone	Brown yellow gummy solid	3.67
		Methanol	Yellow gummy solid	5.71
		Aqueous	Brown yellow solid	5.62
	Stem bark	Acetone	Sticky green solid	3.70
		Methanol	Green yellow sticky solid	2.48
		Aqueous	Yellow solid	0.17

5.1.2. Larvicidal activity against *An. gambiae*

Larvicidal activity of different solvent crude extracts of three *Vitex* species (*V. schiliebenii, V. payos* and *V. trifolia*) extracted from various plant parts were presented in Table 1 appendix 1. The results indicated that larvicidal activities varied according to solvent polarity and plant parts. However, the highest larval mortality was found in the acetone leaf extract of *V. schiliebenii* followed by acetone root bark extract of the *V. payos* , acetone stem bark extract of *V. schiliebenii* and then methanol leaf extract of *V. trifolia.* The aqueous extracts had negligible larvicidal activity except *V. schilebenii* stem bark and *V. trifolia* leaves. The extracts gave different levels and rate of mortality of the larvae. Some (methanol extract of *V. trifolia* leaves, acetone extracts of stem bark and leaves of *V. schiliebenii*, acetone extract of root bark of *V. payos*) caused 100% mortality at 100 ppm in 72 hours, with those of *V. schiliebenii* and *V. payos* showing faster rate of mortality (LT_{50}=8 h) than that of *V. trifolia* (LT_{50}=14 h).

In all the test experiments, mortality values were significantly greater than the value of the control experiments. In the case of the control experiments, nil larval mortality was observed within 24 h and the larvae developed into pupae and then adults within 48-72 h. The efficacy of the plants was not significantly different at p = 95 %.

5.1.3. Growth disruption effects at lower doses of *Vitex* extracts

Generally, at lower doses the larval stage was prolonged. The average developmental period of 10 days during control was prolonged to 13, 14, and 15 days at 25, 50, and 100 ppm, respectively. It was observed that at lower doses, *viz* 50 ppm and below, extracts of *V. payos* caused interesting physiological and neuro-physiological effects to the exposed larvae. In 8-10 days, about 50% of the larvae died and floated on the surface of the solution in clusters, some of

which were black (Figure 2A appendix I). The other 50% either transformed into lighter-colored larval-pupal intermediates or emerged as weak adults. Similar observations were also made with extracts of *V. schiliebenii* root bark extracts at 100 ppm. Morphological abnormalities were observed 48 h post-treatment. About 30% of the larvae failed to transform to normal pupae, and instead produced larval-pupal intermediates (Figure 2B and C appendix I). Approximately 60% successfully pupated, but the resulting pupae either failed to emerge as adults or emerged as weak adults that died within 48 h.

Lower doses (<50 ppm) of *V. trifolia* leaf extracts did not appear to cause any morphological deformities, but led to retardation and 100% mortality in 7-8 days.

5.2 Paper II

Time-course effects of *Vitex schiliebenii* (Varbenaceae) solvent extracts on *Anopheles gambiae* giles s.s. larvae under simulated semi-field conditions

5.2.1. Developmental disruption and mortality

The study aimed at determining the growth-disrupting effects of polar phyto-extracts of *V. schilibenii* on *An. gambiae* larvae in a simulated semi-field condition and to undertake phytochemical screening of constituents present.

The tested plant extracts showed a promising activity of crude plant extracts which is often attributed to the complex mixture of active compounds. Phytoextracts that exhibited high larvicidal activity in laboratory bioassays were considered to determine adult inhibition emergence (IE) potentiality.

When larvae were treated with *V. schiliebeinii* methanol stem bark extract at 25 ppm, 80% died at the larval stage, 4% at the pupal/adult stage(s) and the per-

centage adult emergence was 16% over a period of 9 days. Overall corrected % IE was 83% (Figure I appendix II). The corresponding methanol extract caused 43% larval mortality, 14% pupal/adult mortality while another 43% emerged as adults. The overall corrected % IE was 52% over a period of 6 days (Figure I appendix II). One hundred percent reduced adult emergence was observed in the experiment treated with *V. schiliebenii* acetone and methanol leaf extracts. The extracts caused 88 and 86% larval mortality and 12 and 14% pupal/adult mortality for the aacetone and methanol extracts respectively (Figure I appendix II). At concentration ≥ 100 ppm, larvae exposed to *V. schiliebenii* methanol stem bark extract, *V. schiliebanii* acetone leaf extract and *V. payos* methanol root bark extract produced no live or dead pupae. Percentage adult emergence inhibition of adult *An. gambiae* fell below the threshold value of 80% at concentration ≥ 25 ppm in all the test experiments except in *V. schiliebenii* methanol stem bark at 25 ppm (52%).

Morphogenetic variations and behavioral changes seen were similar to those observed in the laboratory experiments. The dead larvae had abnormally large heads with a ‘question mark-like’ structure. The emerged pupae had their thorax and abdomen tucked together forming a straight structure. The larvae that pupated were reared in the laboratory and monitored through their life cycle until the emergence of adults. The morphological appearance of the emerged adults was similar to those from the control experiments though they were weak. The males and females were allowed to mate and then fed on human blood. The females produced very little batches of eggs suggesting that the extracts could have interfered with the reproductive system of the mosquitoes.

5.2.2. Phytochemical screening

The phytochemicals present in various extracts of the three *Vitex* species are presented in Table 5.2. The qualitative analysis revealed the presence of terpenoids, steroids, tannins, flavonoids, saponins and alkaloids in the extracts.

Table 5.2: Qualitative analysis of phytochemicals of acetone, methanol and aqueous crude extracts of four *Vitex* species

Extract code	Alkaloids	Tannins	Saponins	Flavonoids	Steroids	Terpenoids
VSRB-221	-	++	-	++	-	++
VSRB-222	+++	ND	-	++	-	++
VSRB-223	-	++	++	++	-	++
VSSB-221	-	++	-	++	++	++
VSSB-222	++	-	-	++	++	++
VSSB-223	-	++	++	++	-	++
VSL-221	++	++	+++	++	++	+
VSL-222	++	++	+++	++	+	++
VSL-223	-	++	+	++	--	++
VPRB-211	++	-	+++	+	++	++
VPRB-212	++	-	+++	++	++	++
VPRB-213	++	++	++	++	-	++
VPSB-211	++	ND	-	++	-	++
VPSB-212	+	ND	-	++	-	++
VPSB-213	++	++	-	+	-	++
VTS-201	++	-	++	++	++	++
VTS-202	-	-	-	-	-	++
VTS-203	++	++	+	-	-	++
VTL-201	-	++	-	-	-	++
VTL-202	++	++	-	-	-	++
VTL-203	+	+	-	++	-	++
VTSB-201	++	-	++	++	-	++
VTSB-202	-	++	-	-	-	++
VTSB-203	++	++	++	-	-	++

+++: Present in large amount, ++: Present, +: Present in trace amount -: Absent, ND: Not done

5.3. Paper III

Larvicidal and Brine Shrimp Activities of *Vitex schilebenii* Extracts and Isolated Phytoecdysteroids on *Anopheles gambiae* Giles s.s Larvae

5.3.1. Bioassays

Acetone, methanol and aqueous extracts of the leaves, stem bark and root bark of *V. schiliebenii* belonging to the family Verbenaceae were evaluated for their larvicidal activity against late 3rd/early 4th *An. gambiae* Giles s.s. larvae (Diptera: Culicidae). The extracts of the acetone leaves and stem bark were active with LC_{50} values of 14.6 and 17.4 ppm respectively at 24 hrs. These extracts exhibited low toxicity to brine shrimps with LC_{50} values of 180.9 and 154.4 ppm respectively (Table 1 appendix III). The constituents in these extracts were isolated and evaluated and the phytoecdysteroids 20-hydroxyecdysone **(80)** and stigmasterol **(168)** were identified as the active principles in the acetone stem bark while γ-sitosterol **(167)** was the active principle of the acetone leaf extract. The methanol leaf extract, the stem bark aqueous extract and the acetone root bark also showed potency against the mosquito species.

5.3.2. Structure elucidation

20-hydroxyecdysone-20, 22-monoacetonide (166)

TLC analysis of the acetone extracts of *V. payos* roots gave eight spots (DCM: Acetone, 3:2). Column chromatography with 100% DCM and gradually increasing acetone to 100% then methanol to 30% afforded 22 fractions.

To obtain 20-hydoxyecdysone-20, 22-monoacetonide **(166)**, fraction 8 was subjected to repeated preparative thin layer chromatography (Dichloromethane: Acetone 1:1). It produced 16 mg of a white amorphous powder which was soluble in methanol with melting point 257-259°C; lit. 256°C. Qualitative analysis

of the compound indicated it to be steroid. The ^{1}H NMR data for 20-hydoxyecdysone-20, 22-monoacetonide **(166)** is summarized in table 5.3.

Table 5.3: ^{1}H NMR (500 MHz, MeOD) data for 20-hydroxyecdysone-20, 22-monoacetonide **(166)**

Position	$\delta_{obs.}$	Multiplicity	Integral	$\delta_{lit.}$
1a	1.89	br s	1H	1.78 (m)
1b	1.45	s	1H	
2	3.97	br s	1H	3.82 (m)
3	3.86	M	1H	3.94 (m)
4a	2.05	d	1H	1.73 (m)
4b	1.76	m	1H	1.69 (m)
5	2.40	d	1H	2.37 (m)
7	5.83	S	1H	5.80 (br s)
9	3.17	t	1H	3.13 (m)
11a	1.82	m	1H	1.79 (m)
11b	1.23	s	1H	1.67 (m)
12a	2.05	d	1H	2.09 (m)
12b	1.98	m	1H	1.82 (m)
15a	2.00	m	1H	1.94 (m)
15b	1.50	m	1H	1.60 (m)
17	2.32	m	1H	2.28 (m)
18	0.85	s	3H	0.81 (s)
19	0.98	s	3H	0.95 (s)
21	1.20	s	3H	1.15 (s)
22	3.70	q	1H	3.68 (dd)
23a	1.55	m	1H	1.47 (m)
23b	1.64	m	1H	-
24a	1.80	m	1H	1.68 (m)
24b	1.50	m	1H	1.15 (m)
26	1.45*	s	3H	-
27	1.21*	s	3H	-

*- Signals may be interchanged, Literature data for inokosterone-20, 22-acetonide measured in C5D5N

Five methyl singlets were observed at δ 0.85 (Me-18), 0.98 (Me-19), 1.20 (C-21), 1.21 (Me-27) and 1.45 (Me-26). The signals at δ 1.21 and 1.45 (6H, s) indicated that the side chain C-26/C-27 was intact. The signal at δ 5.83 (*s*, 1H), was assigned to an olefinic proton at C-7 due to the double bond between C-7 and C-8. The signals at δ 3.97 (br *s*), 3.86 (br *s*) and 3.70 (*q*, 9) suggested the presence of hydroxylated carbons. A series of proton signals at δ 0.8-2.5 were

attributed to resonances of overlapping of methylenes and methines of framework of steroid.

Table 5.4: ^{13}C NMR (500 MHz, MeOD) data for 20-hydroxyecdysone-20, 22-monoacetonide **(166)**

Position	$\delta_{(obs.)}$	$\delta_{(lit.)}$	DEPT
1	36.0	38.0	CH_2
2	67.3	68.1	CH
3	67.1	68.0	CH
4	31.5	31.7	CH_2
5	50.4	51.3	CH
6	205.1	203.5	C
7	120.7	121.8	CH
8	165.3	165.4	C
9	33.7	34.4	CH
10	37.8	38.6	C
11	21.0	21.0	CH_2
12	30.9	32.4	CH_2
13	48.6	47.8	C
14	84.5	84.1	C
15	30.2	31.6	CH_2
16	20.1	22.1	CH_2
17	49.1	50.0	CH
18	16.3	17.2	CH_3
19	23.1	24.4	CH_3
20	83.9	82.1	C
21	21.2	22.4	CH_3
22	81.9	85.5	CH
23	23.3	24.3	CH_2
24	40.8	42.1	CH_2
25	69.7	69.2	C
26	28.1*	30.0	CH_3
27	27.9 *	29.8	CH_3
O-C-O	106.6	106.9	C
Me	27.6	27.1	CH_3
Me	25.8	29.4	CH_3

*- signals may be interchanged, Literature data δ (lit.) measured in C5H5N taken from Sena Filho et al., (2008)

The ^{13}C-NMR (DEPT) of 20-hydoxyecdysone-20, 22-monoacetonide **(166)** revealed signals due to seven methyls, eight methylenes, seven methines and seven quartenary carbons. The signal at δ 106.6 was assigned to O-C-O bond which is fully consistent with that reported in the literature (Sena Filho *et al.*, 2008) for C-28 in the structure 20-hydoxyecdysone-20, 22-monoacetonide **(166)**. Two olefinic carbon signals observed at δ 120.7 (C-7) and 165.3 (C-8)

and one carbonyl at δ 205.1 (C-6) suggested an α, β unsaturated ketone. Presence of two quartenary hydroxyl groups at C-14 and C-25 was confirmed by the signals at δ 84.5 and 69.7. The ^{13}C NMR further suggested the presence of secondary hydroxyl groups by signals at δ 67.3 and 67.1 for C-2 and C-3. One oxygen-bearing quarternary carbon signal was revealed at δ 83.9 ppm (C-20) and a secondary one at δ 81.9 ppm (C-22). The signals at δ 27.6 and 25.8 were assigned to the two methyl groups attached to quarternary carbon which is dioxygenated carbon. All the protonated carbons were assigned with the aid of HSQC spectrum. In the HMBC spectrum, the correlations between δH 5.83 (1H, *s*, H-7) and δC 33.7 (C-9), 50.4 (C-5) and 84.5 (C-14) supported the presence of 6-keto-7-ene unit.The proton signal at δH 3.70 (H-22) correlated with the signals at δC 21.2 (C-21), 40.8 (C-24), 49.1 (C-17) and 83.9 (C-20).

The electron ionization mass spectrum of 20-hydoxyecdysone-20, 22-monoacetonide **(166)** revealed a significant peak at *m/z* 363 (37%) which corresponded to the formula [M^+ -$C_{21}H_{31}O_5$], suggesting the presence of hydroxyl group on C-14 and a 6-keto-7-ene moiety (as shown in Figure 5.1). This also confirmed that the carbon-bearing group and one hydroxyl group were located on the side chain which was also supported by the presence of a fragment ion at *m/z* 310 (21%) due to loss of two water molecules and a hydroxyl group. The peaks at *m/z* 301 (27%) and 283 (51%) were attributed to fission between C-17 and C-20 and then loss of one and two water molecules respectively. An additional bond cleavage occurred between C-24/C-25 and it gave rise to the peak at 59 (89%).

The ^1H NMR and ^{13}C NMR data of 166 were consistent with the reported spectral data for 20-hydroxecdysone-20, 22-monoacetonide. The previously reported compound was from *V. strickeri* (Zhang *et al.*, 1992) a member of the family Verbenaceae.

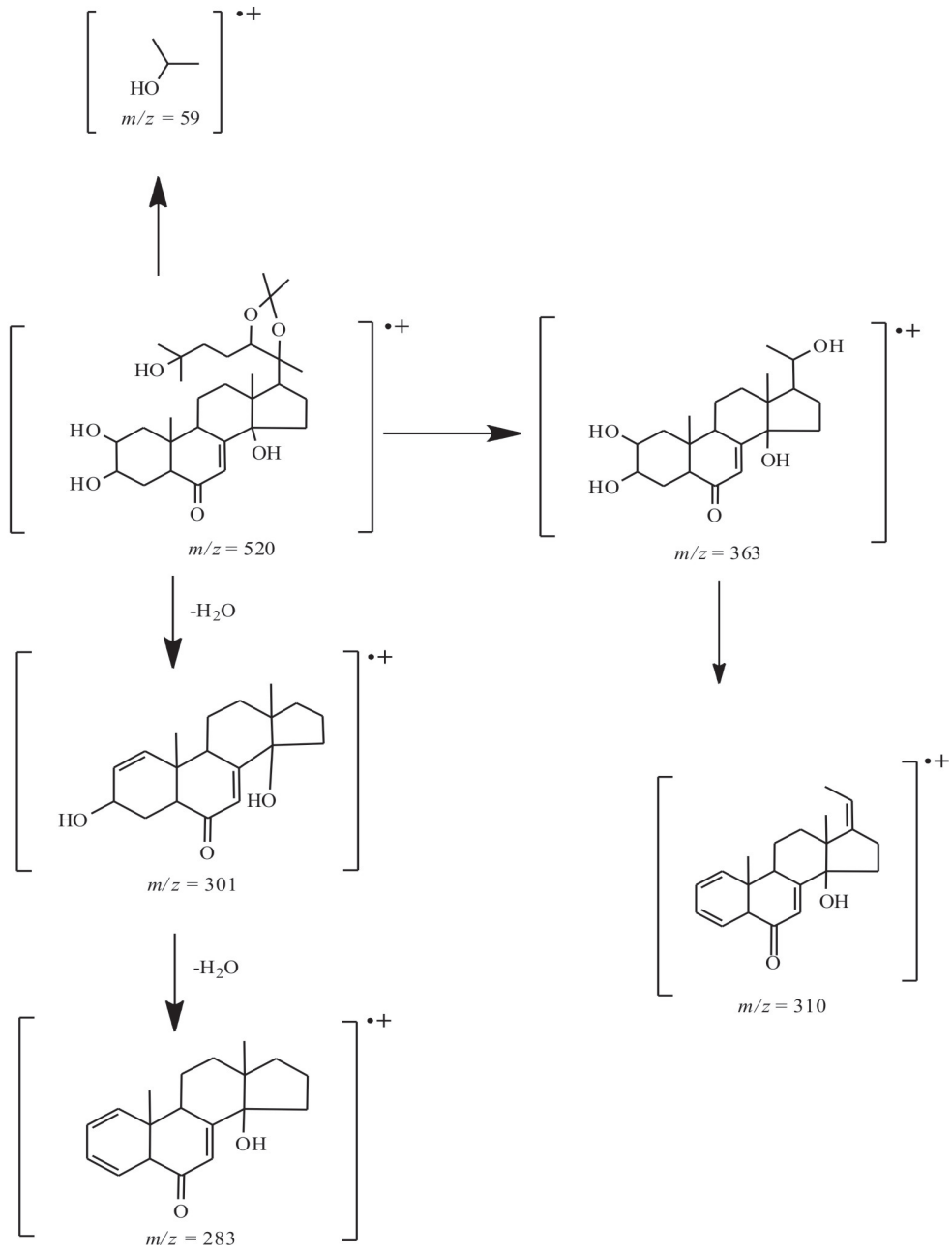


Figure 5.1: Mass spectral fragmentation pattern of 20-hydroxecdysone-20,22-monoacetonide **(166)**

20-hydroxyecdysone (80) from *V. payos* acetone root bark and *V. schiliebenii* acetone stem bark

To obtain compound 20-hydroxyecdysone **(80)**, (3:2 acetone: methanol) was subjected to preparative thin layer chromatography (Dichloromenthane: Acetone 8:2) followed by sephadex LH_{20} (DCM: MeOH 1:1). Ten milligrams-*V. payos* and 16 mg-*V. schiliebenii* crystals of melting point 232-234^{o}C (lit. 230-233^{o}C), were obtained which were soluble in methanol. Qualitative analysis indicated it to be a steroid. The ^{1}H NMR spectrum of 20-hydroxyecdysone **(80)**, displayed a vinyl signal at δH 5.79 (1H, *s,* H-7) which is consistent with those reported for ecdysteroids (Mei *et al.*, 2012). The ^{1}H NMR indicated the presence of five methyl groups at δH 0.89 (3H, *s*, CH_3-due to C-18), 0.95 (3H, *s*, CH_3-due to C-19), 1.25 (3H, *s*, CH_3-due to C-21), 1.22 (6H, *s*, CH_3-for C-26 and C-27). A series of proton signals at δ 0.89-2.4 (*m*) were attributed to resonances of overlapping of methylenes and methines of framework of steroid.

The ^{13}C-NMR revealed 27 signals (Table 2 appendix III). The DEPT-135 of 20-hydroxyecdysone **(80)**, revealed signals due to five methyls, eight methylenes, seven methines and seven quarternary carbons. All the protonated carbons were assigned by HSQC experiment. The presence in the ^{1}H-NMR spectrum of a methyl singlet at δ 1.25 (C-21) suggested the location of one hydroxyl function at C-20, and this was supported by a quarternary carbon signal at δ 76.5 ppm in the carbon spectrum which did not appear in the DEPT spectrum. Additionally, in the HMBC experiment, the methyl proton signals at δ_H 1.25 (H-21) correlated with the signals at δ_C 49.1 (C-17), 76.5 (C-20) and 77.0 (C-22) and that at δ_H 1.22 (6H, *s*, H-26, 27) correlated with the signals at δ_C 41.0 (C-24) and 69.9 (C-25). Thus two hydroxyl groups were assigned to C- 20 and C-25 respectively.

The EIMS of 20-hydroxyecdysone **(80)**, gave a series of peaks 363/345/327

which is typical of all ecdysones containing no extra OH at C-5 or C-11 (Koreeda *et al.*, 1968) (Figure 5.2). The EI mass spectral peak of 80 at *m/z* 363 was associated with a fragmentation between C-20 and C-22. Loss of one and two molecules of water from this ion would give ions observed at *m/z* 345 and 327 respectively. The peaks at m/z 301 and 283 were attributed to ions produced by fission between C-17 and C-20 followed by loss of one and two molecules of water respectively. The mass spectral data confirmed the existence of the same tertracyclic ring system in 20-hydroxyecdysone **(80)**, as that in 20-hydoxyecdysone-20, 22-monoacetonide **(166)**and thus the observed differences were due to the side chain. The MS could be accounted for by the fragmentation pattern in Figure 5.2. The ^{1}H-NMR and ^{13}C-NMR spectral data of 20-hydroxyecdysone **(80)**, were consistent with the reported spectral data for 20-hydroxyecdysone. The compound was therefore identified as the ecdysteroid 20-hydroxyecdysone **(80)**.

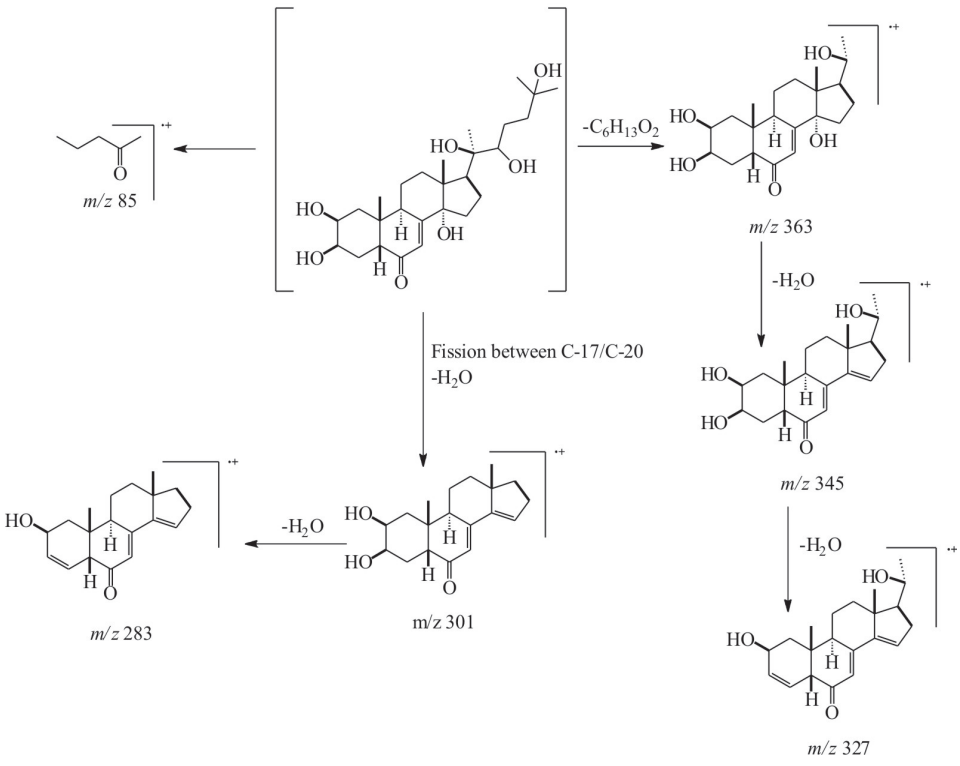


Figure 5.2: Mass spectral fragmentation pattern of 20-hydroxecdysone **(80)**

Plants from the genus *Vitex* have been reported to contain many types of ecdysteroids which may be used as taxonomic markers. Literature reports indicate that 20-hydroxyecdysone is present in many *Vitex* species (Sena Filho *et al*., 2008). Some Solanaceae species (Savchenko *et al.*, 2000) for example *Browallia speciosa, Nierembergia hippomanica var.violacea, N. solanaceae* and *Solanum nigrum;* Chenopodiaceae (Dinan *et al*., 1999) for example *Rhagodia baccata*; Labiatae, Asteraceae, Amaranthaceae, Ranuculaceae and Caryophyllacea (Laosooksathit *et al*., 2003) species also contain ecdysteroids, but other than these sources, ecdysteroids are not commonly found in many other plant families. The phytoecdysteroid 20-hydroxyecdysone has been associated with many pharmacological activities including stimulating hepatic function, prevention of myovardial ischemia, acting as an antioxidant and anti-free-radical properties (Lafont and Dinan, 2003).

Stigmasterol (168)

Repeated chromatography of an acetone fraction of *V. schiliebenii* stem bark yielded stigmasterol. This compound was isolated as white crystals (25 mg, Rf 0.5 EtOAc/Hexane, SiO_2) and the melting point was 165-167^{o}C (Lit. 163-167^{o}C, Greca *et al.*, 1990).

Methyl signals were observed at δ 0.68 (*s*, 3H, H-18), 1.01 (*s*, 3H, H-19), 0.93 (*s*, 3H, H-21), 0.85 (*s*, 3H, H-26), 0.80 (*s*, 3H, H-27) and 0.65 (*s*, 3H, H-29). The signal at δ 3.50 (*m*, 1H) was assigned to C-3 which suggested the presence of an α-proton typical of hydroxylated sterols. The signal at δ 5.32 (*d*, 1H) was assigned to an olefinic proton. The multiplet at 5.0-5.2 (2H) suggested presence of two olefinic protons. The region between 0.80-2.22 representing methyl, methylene and methine protons closely compared to the signal pattern of phytosterols. Furthermore, the ^{13}C NMR data (Table 2 appendix III) suggested a

triterpenoidal/steroidal skeleton.

^{13}C NMR revealed 29 signals of which four olefinic carbons at δ 121.7 (C-6), 129.3 (C-23), 138.3 (C-22) and 140.8 (C-5) suggested the presence of two double bonds. Six methyl groups appeared at δ 11.9 (C-29), 12.0 (C-18), 19.0 (C-27), 19.4, (C-19), 21.1 (C-26) and 21.1 (C-26) and 9 methylene groups δ 37.3 (C-1), 31.9 (C-2), 42.3 (C-4), 33.9 (C-7), 21.1 (C-11), 39.7 (C-12), 24.3 (C-15), 28.4 (C-16) and 25.4 (C-28) as revealed by DEPT-135. Similarly, 11 methine carbon resonances were observed at δ 138.3, 129.3, 121.7, 71.8, 56.8, 56.7, 50.1, 51.2, 40.5, 31.7 and 29.7. Signals at δ 140.8, 36.5 and 42.2 revealed quartenary carbons. The signal at δ 71.8 suggested oxygenation which according to biosynthesis pathway, it shouldn't.

The ^{13}C NMR and ^{1}H NMR spectra were identical with those previously reported for stigmasterol (Maria *et al.*, 2004) which had been previously isolated from a number of higher plants (Morale *et al*., 2003; Forgo and Köver, 2004, Maria *et al.,* 2004).

γ-sitosterol (167)

The acetone extract of *V. schiliebenii* leaves (10 g) was subjected to column chromatography on silica gel (eluting with 100% dichloromethane and gradually increasing acetone to 100%). The 40% acetone eluent gave a mixture of two compounds, which were subjected to repeated column chromatography using DCM: MeOH to yield γ-sitosterol **(167)** (6 mg with a melting point of 142-144^{o}C) which was then re-crystallized using the same solvent into a white star shaped crystal soluble in DCM. The other compound was too little to be analyzed.

The ^{1}H NMR revealed a signal at δ 5.35 (*d*, 1H), which was assigned to an olefinic proton at C-6 owing to the double bond between C-5 and C-6. The signal at δ 3.50 (*m*, 1H) suggested the presence of an α-proton typical of sterols hydrox-

ylated at C-3. Some methyl singlets at δ 0.98, 0.9, 0.82, 0.85, 0.8 and 0.7 were observed and assigned to H-18, H-21, H-26, H-29, H-27 and H-19, respectively. The doublet at δ 5.35 and the broad tripplet at δ 3.50 were assigned to olefinic proton and hydroxyl group, respectively. The rest of the protons were in complex continuous multiplets spread between δ 1.0-2.2.

^{13}C NMR revealed 29 signals (Table 2 appendix III). From ^{13}C NMR, two olefinic carbons appeared at δ 121.7 (C-6) and 140.8 (C-5). The carbon signal at δ 71.8 (C-3) suggested oxygenation. From DEPT analysis, six methyls at δ 11.9, 11.8 18.8, 19.1, 19.4 and 19.8, eleven methylenes at δ 21.1, 23.3, 24.3, 26.1 26.2, 29.4, 31.7, 37.3, 37.3, 39.8 and 42.3, nine methines at δ 28.2, 29.2, 34.0, 50.2, 50.2, 56.1, 56.8, 71.8 and 121.7 were noted. The other three remaining signals at δ 36.5 (C-10), 45.9 (C-13) and 140.8 (C-5) were due to quaternary carbons. From ^{1}H and ^{13}C NMR analysis, the structure stigmast-5-en-ol **(167)** was proposed.

5.3.3. Toxicity of pure and blends of pure compounds isolated from *Vitex* species to *An. gambiae* larvae

The results revealed that the evaluated phytosteroids were toxic to *An. gambiae* larvae. The compounds 20-hydroxyecdysone-20, 22-monacetonide (**166**) and 20-hydroxyecdysone **(80)** caused 100 % mortality at 10 ppm. Similarly, high mortality was obtained at 1 and 5 ppm (Table 5.5). Stigmasterol **(168)** and γ-sitosterol (**167**) caused moderate mortality at 10 ppm. A blend of 20-hydroxyecdysone-20, 22-monacetonide (**166**) and 20-hydroxyecdysone **(80)** was moderately active (65±2.1 %) at 1 ppm but the activity was high at 5 and 10 ppm but not 100 %. This result therefore indicated a slight drop in the activity of the resulting blend. On the other hand, a combination of stigmasterol (**168**) and γ-sitosterol (**167**) which were less active improved the activity than the individual compounds. Addition of 20-hydroxyecdysone-20,22-monacetonide (**166**)

to stigmasterol (**168**) and γ-sitosterol (**167**) improved the activity too. It was also interesting to note that addition of 20-hydroxyecdysone **(80)** to stigmasterol **(168)** and γ-sitosterol **(167**) improved their activity (Table 5.5), although a slight drop in activity of compound 80 was noted when in the mixture.

Table 5.5: Percentage mortality of *An. gambiae* larvae exposed to pure compounds isolated from *V. payos* and *V. schiliebenii* individually and in blends (1:1) at 1, 5, and 10 ppm for 72 h

S/No.	Blend/Compound(s) name	Mean % mortality/Concentration (ppm)		
		1 ppm	5 ppm	10 ppm
1.	20-hydroxyecdysone-20,22-monacetonide (**166**)	90±2.1	95±2.2	100±0.0
2.	20-hydroxyecdysone (**80**)	80±2.4	90±2.0	100±0.0
3.	Stigmasterol (**168**)	25±2.4	40±2.1	55±2.5
4.	γ-sitosterol (**167**)	35±2.5	45±1.5	65±2.4
5.	**166** + **80**	65±2.1	85±2.9	90±2.5
6.	**166** + **168**	90±3.3	90±2.1	100±0.0
7.	**166** + **167**	90±2.0	90±2.2	100±0.0
8.	**80** +**168**	80±2.1	85±2.0	100±0.0
9.	**80** + **167**	65±2.1	80±2.9	90±2.5
10.	**167** + **168**	50±2.4	70±2.0	85±2.4
11.	Control	0±0.0	0±0.0	0±0.0

From the GC-MS analysis, a number of steroids were found to be present in the extracts including the above isolated steroids. The aim of this experiment was to elucidate the role and relative importance of steroids in *Vitex* species in controlling *An. gambiae* larvae and to use this information in guiding effective development of formulations to be used in integrated pest management programmes. It was therefore interesting to note the synergistic effect of the steroids and the contrasting ways in which the two most active compounds (20-hydoxyecdysone-20, 22-monoacetonide **(166)** and 20-hydroxyecdysone **(80)**) were contributing to the toxicity of their blends. But blend effects of compounds 20-hydoxyecdysone-20, 22-monoacetonide **(166)** and 20-hydroxyecdysone **(80)** were noted to be less active than the individual compounds (Table 5.5). Synergism between moderately active compounds to give a mixture that is more active than the individual activities of the constituents was illustrated by the combi-

nation of compounds stigmasterol **(168)** and γ-sitosterol **(167)** (Table 5.5). A number of studies on steroids as insecticides have focused on the identification of active components rather than mixtures (Zolotar *et al*., 2001). Certainly, there is accumulating evidence of adaptive value of phytochemical diversity in ecological interactions among plants and their associated herbivores and pathogens (Berenbaum and Zangerl, 1996; Cates, 1996). These findings have important practical implication in the strategy adopted in the search for and use of plants and their phytochemicals for mosquito larvae control.

5.4. Paper IV

Chemical composition and evaluation of mosquito larvicidal activity of *Vitex payos* extracts against *Anopheles gambiae* s.s. Giles larvae

The aim of this study was to evaluate acetone and methanol root bark extracts and different fractions thereof, following acetone extractions and column chromatographic separation for their immediate toxicity and long term effects on *An. gambiae* s.s Giles larvae under simulated semi-field conditions.

5.4.1. Bioassay results

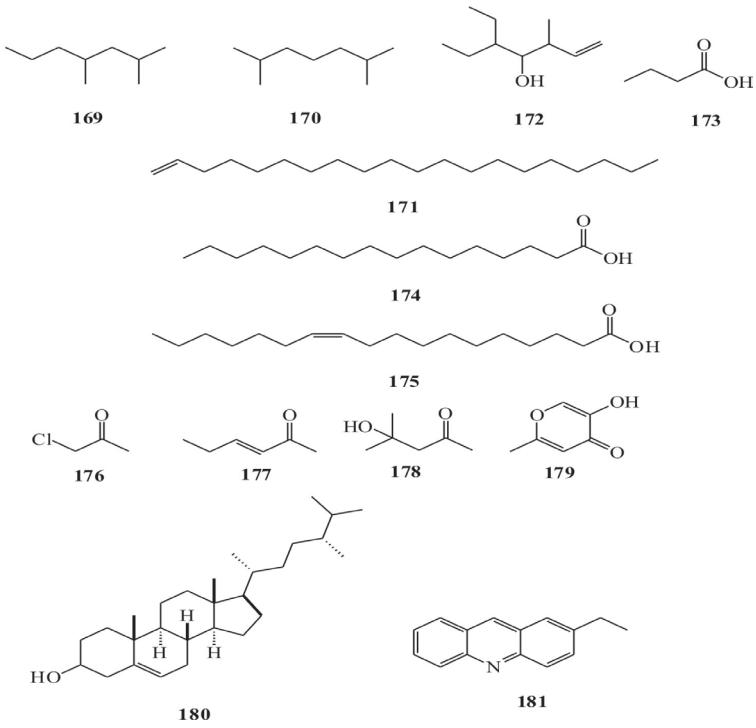
The tested acetone and methanol extracts and the acetone column chromatography fractions thereof exhibited larvicidal activity against larvae of *An. gambiae* within 72 h (Table 1 and Figure 2 appendix IV).

The active fractions tested were all positive for steroids and saponins with larval mortality ranging between 85-100 % at 25 ppm and above after 72 h of exposure to the fractions. It was also noted that the fractions caused similar larval deformities as the crude extracts. The larvae attained black, brown and white coloration with the fractions.

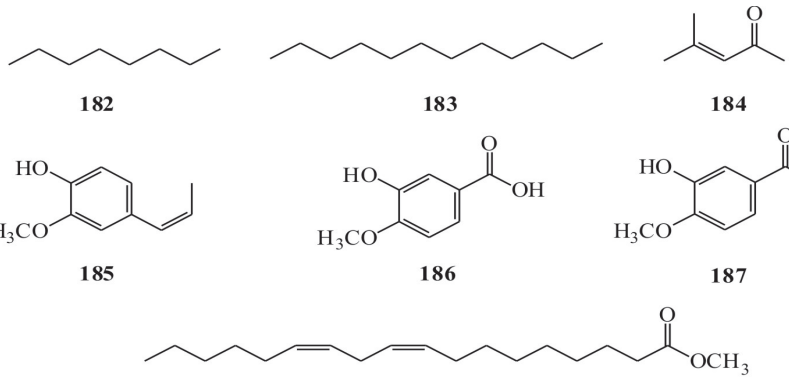
5.4.2. GC-MS analysis of *V. payos* crude extracts

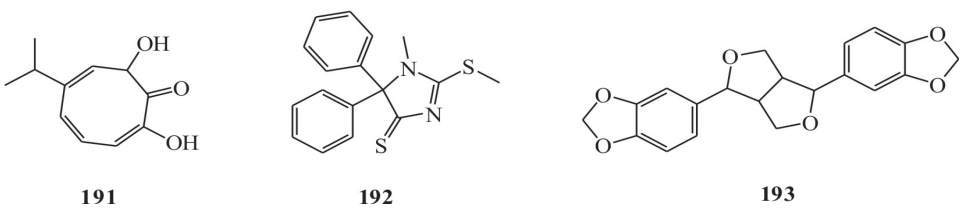
The GC-MS analysis of compounds in the *V. payos* acetone and methanol root extracts are presented in Table 2 appendix IV.

From the GC-MS analysis of *V. payos* acetone root extract, 74 compounds were detected out of which 15 had more than 1 % peak area. Generally, the extract showed a variety of compounds. Hydrocarbons present in the extract include 2,4-dimethylheptane **(169),** 2, 6-dimethyl-Heptane **(170)** and eicosene **(171).** The aliphatic alcohol, 5-Ethyl-3-methylhept-1-en-4-ol **(172)** was found to be present in the extract. The extract also contained fatty acids, namely butanoic acid **(173)**, hexadecanoic acid **(174)** and cis-vaccenic acid **(175)**. Carbonyl compounds present in the extract included 1-chloro-2-propanone **(176),** 3-Hexen-2-one **(177)** and 4-hydroxy-4-methyl-2-pentanone **(178).** 5-hydroxy-2-methyl-4H-pyran-4-one **(179)**, a member of the pyrone family was also detected in the exctract. Phytoecdysteroids campesterol **(180)**, stigmasterol **(168)** and γ-sitosterol **(167)** and the volatile 2-ethylacridine **(181)** were also detected.



In the methanol root extract, 123 compounds were detected but only 15 compounds had ≥ 1 % peak area. Compounds detected include the hydrocarbons, octane **(182)** and dodecane **(183)**; carbonyls, 4-methyl-3-penten-2-one **(184)** and 4-hydroxy-4-methyl-2-pentanone **(178)** and simple aromatic volatile, Z-Isoeugenol **(185)**, 2,4-dihydroxy-benzaldehyde **(186)** along with 2-ethylacridine **(181)**. 3-Hydroxy-4-methoxybenzoic acid **(187),** methyl esters, methyl linoleate **(188)** and methyl hexadecanoate **(189)** were also detected in the extract together with the ester silver butanoate **(190)**. A simple volatile aromatic compound ß-Thujaplicinol **(191)** and the fatty acid cis-vaccenic acid **(175)** were also detected in the extract. Phytoecdysteroids campesterol **(180)**, stigmasterol **(168)** and γ-sitosterol **(167)** and the volatile 2-ethylacridine **(181)** were also detected in the extract. Other compounds present included 1,5-dihydro-1-methyl-2-(methylthio)-5,5-diphenyl-4H-imidazole-4-thione **(192)** and 2,6-bis(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo(3.3.0)octane **(193).**





The biosynthesis of phytosterols in plants is shown in Figure 5.3

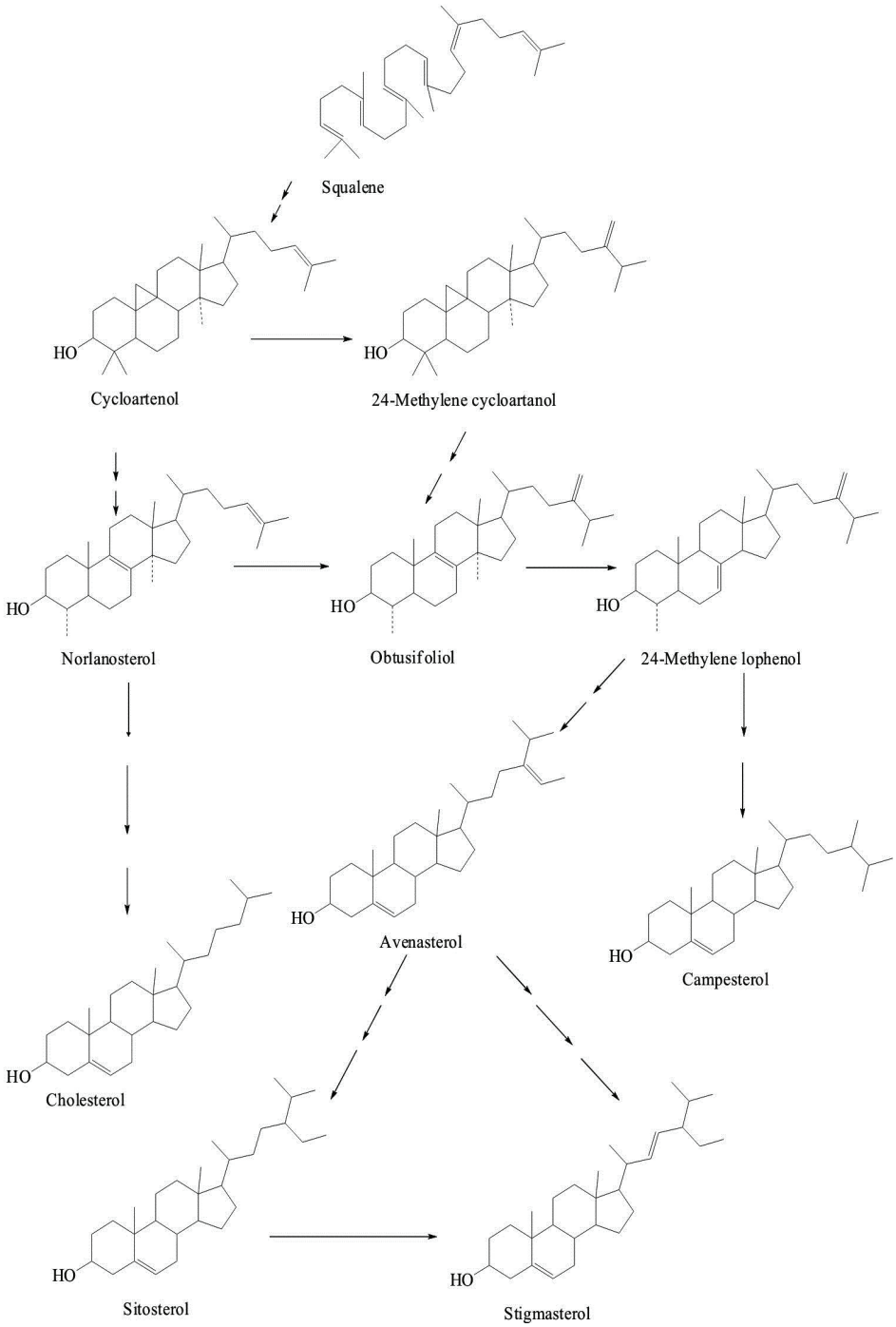


Figure 5.3: Pathway of sterol biosynthesis in plants

5.5. Discussion

In the integrated pest management program, environmental safety of insecticides is of paramount concern. It has been reported that an insecticide does not have to cause high mortality on target organism for it to be accepted (Kabaru and Gichia, 2001). In the search for an eco-friendly pesticide, researchers have considered pesticides of plant origin as a replacement for organic pesticides. Phytochemicals may therefore be deployed as complimentary insecticides as they are relatively safe, inexpensive, selective and are readily available in many areas of the world.

The larvicidal activity of the three *Vitex* species showed that *An. gambiae* larvae were susceptible to the test extracts. The LC_{50} values for *V. schiliebenii* acetone stem bark extracts was 17.4 ppm; *V. schiliebenii* acetone and methanol leaf extract were (14.6 ppm, 136.3 ppm) respectively; *V. payos* acetone root bark was 15.6 ppm and *V. trifolia* methanol leaf extract was 76.6 ppm, all at 24 h. The other extracts tested were however not very effective as larvicides with comparatively higher LC_{50} values (Table 1 appendix I). The low activity of the aqueous extracts could be attributed to the method of extraction used. The phytochemicals in the extracts might have been destroyed by the high temperature used in the extraction process. The larvicidal effect of the active extracts were comparable with the LC_{50} values of methanol leaf extracts of *V. negundo* (212.57 ppm), *V. trifolia* (41.41 ppm), *V. peduncularis* (76.28 ppm) and *V. altissima* (128.04 ppm) against the early fourth-instar larvae of *Cx. quinquefasciatus* after 24 h (Kannathasan *et al.*, 2007) hence the extracts can also be used as botanical larvicides.

The biological activity of the experimental extracts may be attributed to the chemical composition of the extracts which include alkaloids, tannins, saponins, flavonoids, steroids, volatile oils and terpenes (diterpenes, triterpenes and sesquiterpenes). The leaf extracts of *V. schiliebenii* and the root bark extracts of

V. payos which displayed higher larvicidal activity were rich in saponins. Several authors have shown the defensive role of saponins by plants. They have been reported to protect plants from phytopathogenic microorganisms, phytophagus mammalsand insects (Oleszek *et al*., 1999; Harmatha, 2000). Test solutions of *V. payos* acetone root bark formed a thick jelly on the surface, which is a characteristic property of sponins and the thickness increased with concentration of the solutions and time of the extract in the water. The surface of the solution behaved as though it was covered by a thinly stretched membrane. This could have possibly led to the mortality of the larvae by suffocation. In the case of *V. schiliebenii* actone leaf extract, the presence of cholesterol may have led to the formation of insoluble complexes formed between saponins and cholesterol. Mitra and Dungan (2002) have shown that there is a formation of micelle between cholesterol and saponin molecules and this hypocholesterolemic activity interferes with the biosynthesis of ecdysone and various other ecdysteroids. The disturbance of the moulting process has been observed following the ingestion of *Cestrum parqui* leaves (Barbouche *et al*., 2001) or by incorporation of the extracts in the insect diet (Chaieb *et al.*, 2001) which contains saponins. Leaves of *V. schilebenii* and root bark of *V. payos* were found to be equally effective against *An. gambiae* larvae. However, the advantage of using *V. schiliebenii* leaf extracts is that the leaves are easier to harvest, especially at an earlier stage of the tree development, and they are more able to regenerate.

Several other authors suppose a possible saponin/cholesterol interaction causing cholesterimic deficit in insects, disturbing the ecdysone synthesis. They suggest that the complexation can occur in food, hemolymph or inside the insect cell (Harmatha *et al*., 1987; Pracros, 1988 and Weissenberg *et al*., 1998). Studies trying to react in-vitro cholesterol with saponins still remain unfruitful though others have reported formation of precipitate with similar reactions (Gestener *et al*., 1972; Takagi *et al.*, 1982).

Entomotoxicity effect of saponins has also been observed in other insect spe-

cies. Nozzolillo *et al* (1997) reported 100 % mortality of *Ostrinia nubilalis* larvae caused by introducing alfalafa saponins into the food of the larvae and mortality was also recorded for the nymphal stage with only 60 % of the treated chrysalis emerging to adults. *Spodoptera littoralis* treated with 100 ppm saponin of alfalafa leaves caused 90 % cumulative mortality of the larval and nymphal stages. The commercial saponins extracted from *Quillaja saponaria* have been reported to exhibit larvicidal activity against larvae of two mosquito species namely *Ae. aegypti* and *Cx. pipiens*; 100 % mortality was obtained by using amounts of 1000 mg/l during 5 days (Pelah *et al*., 2002). Similar results were obtained by Chaieb (2005) who evaluated the toxic effect of saponins of *Cestrum parqui* on various insects (*Schstocera greagria, S. littoralis, Tribolium confusum and Cx. pipiens*). The most significant toxicity was observed on the mosquito larvae of *Cx. pipiens.*

Though the extract of *V. payos* methanol root bark extract was rich in saponins, the activity after 24 h was relatively low compared to the other extracts which were also rich in saponins. However, the LC_{50} decreased from 377.8 ppm to 30.7 ppm after 72h. This suggested that the phytochemicals present in the extract were slow acting and most probably the saponins in the extract acted antagonistically with the other chemicals present. In a similar study, antagonistic effect of saponins and other chemical constituents has been evaluated (Chaieb *et al*., 2004).

It was interesting to note that larvae treated with *V. payos* methanol root bark extract were smaller than the larvae in the control experiment. This suggested anti-feeding activity of the extract. Some saponins have been reported to exhibit antifeeding activity. A spirostanic saponin isolated from Solanaceae (*Solanum laxum)* has been reported to exhibit anti-feeding activity against *Shizaphis graminum* aphid (Soule *et al*., 2000). Saponins extracted from *Balanites roxburghii, Agave cantala* and *Phaseolus vulgaris* exhibited anti-feeding activity

against *Spilosoma* larvae (Soule *et al*., 2000). Similar results have been reported on *S. littoralis* larvae treated with fifteen various purified extracts obtained from different plants (Adel *et al.,* 2000). Synthesis of tritepenic saponins by damaged alfalfa leaves has been reported to inhibit the consumption of the leaves by *S. littoralis* larvae (Agrell *et al.*, 2003).

It is worth noting that although acetone and methanol extracts produced encouraging results, they are impractical to produce and use by resource-poor people in rural Africa and elsewhere in the world. The aqueous extract is more applicable to rural situations where malaria causes the greatest burden. The aqueous extracts of *V. schiliebenii* stem bark and *V. trifolia* leaves displayed good larvicidal potential with LC_{50} values decreasing from 182.6 ppm to 49.1 ppm for the former and 319.4 ppm to 188.9 ppm for the latter at 24 h and 72 h. *V. trifolia* is well known and grows all over Africa and in the absence of *V. schiliebenii, V. negundo* can be used. Vasanth *et al.*, (2009) evaluated aqueous extract of *V. negundo* against mosquito larvae of *Cx. quinquefasciatus*, *An. stephensis* and *Ae. aegypti* and the extract was found to be effective with LC_{50} values of 167.88 ppm, 167.88 ppm and 231.17 ppm respectively.

Apart from plants being potential sources of mosquito larvicides, they also exhibit insect growth regulatory activity. Insect growth regulators have been reported to offer significant potential for vector control and a number of synthetic compounds and plant derivatives are being examined for IGR activity. In this study, it was observed that at lower doses *viz,* 25 ppm and below, extracts of *V. payos* seemed to have caused interesting physiological and neuro-physiological effects to the exposed larvae. Abnormal behaviors like excitation, paralysis, convulsions, coiling and stretching, retardation and eventually death were observed. For the case of *V. payos* extracts, the dead larvae floated on the surface of the solution in clusters. This observation was attributed to increase in surface tension of the test solution as the density of the solution increased

with concentration. The observed behavioral changes suggest that the extract had substances noxious to larvae which probably, through the neuromuscular system, led to structural deformation, dysfunction and consequently their death. These findings corroborate with previously reported findings (Amalraj *et al.*, 2008; Chaithong *et al.*, 2006; Dharmagadda *et al.*, 2005; Chochoote *et al.*, 2005, 2004) who evaluated various phytoextracts against different mosquito species. The extract treated larvae were observed to move sluggishly and spent most of their time on the surface of the water in the beaker rather than diving to the bottom or being on the sides. The observed increase in developmental period would mean exposing the larvae to the predators for a longer period, thus increasing the chances of being predated.

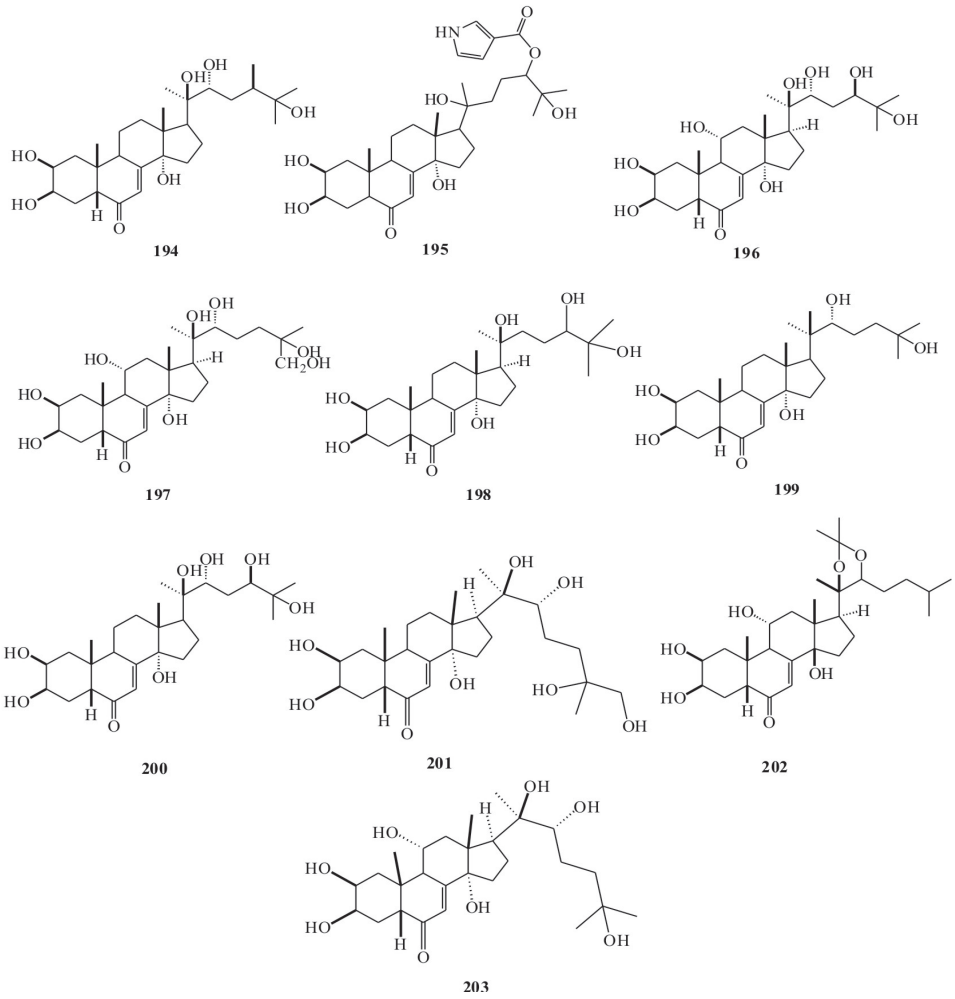
The results also showed that there was improved larvicidal activity of the fractions compared to their respective crude extracts. This observation suggests that there were some inactive components in the crude extracts which were interfering with the activity of the extracts. So far, there are no phytochemical investigations already done on the polar extracts of the two plants.

Phytochemical screening and GC-MS analysis of the test extracts revealed the presence of a wide range of phytoecdysteroids. Presence of these phytochemicals might have contributed to the delayed larval and pupal development. A similar study was conducted by Budowski *et a*l (2012) on the larvae of *Dermestes maculates* which reported that plant sterols inhibit larval development by interfering with the uptake of cholesterol by the insect. This had been observed earlier by Zolotar *et al* (2001) who reported that exogenic ecdysteroids ingested with food cause significant disruptions of the insect hormonal system and eventually death. Ecdysteroids being biodegradable, environmentally friendly and relatively non-toxic to warm blooded animals and humans should be considered as principles in the development of modern safe means of insects controlling agents. In the family Verbeneceae, the genus *Vitex* has been identified as one of

the best sources of ecdysteroids (Sena Filho *et al*., 2008).

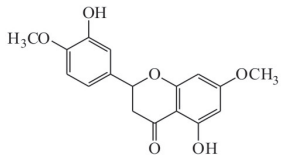
Previously, from *V. canescens* stem bark, four ecdysteroids were isolated namely 20-hydroxecdysone **(80)**, makisterone A (**194**), 24-*epi*-makisterone A (**164**) and canescensterone (**195**); from the root bark, two highly oxygenated ecdysteroids, (24R)-11α-20,24-trihydroxyecdysone (**196**) and 11α-20,26-trihydroxyecdysone (**197**) were isolated from the polar fraction (Sena Filho *et al*., 2008). Thirteen ecdysteroids, 24-epi-pinnatasterone (**161**), scabrasterone **(162)**, calonysterone (**163**), pterosterone (**154**), 24-epi-makisterone A (**164**), 20-hydroxyecdysone (**80**), polypodine B (**165**), ajugasterone C (**88**), pinnatasterone (**198**), 11α-hydroxyecdysone (**199**), 24-epi-abutasterone (**200**), 20,26-dihydroxyecdysone (**201**), and turkesterone (**144**), were isolated from the stem bark of *V. scabra* (Suksamrarn *et al.,* 2002). Zhang *et al*. (**1992**) isolated ajugasterone C (**88**) from *V. strickeri.*

The compound 26-hydroxypinnatasterone (**156**), together with 20-hydroxyecdysone, were isolated from the stem barks of *V. cymosa*. 20-Hydroxyecdysone **(80)**, ajugasterone C (**88**), ajugasterone C-20,22- monoacetonide **(202)** and turkesterone **(144)** were isolated from the branches of *V. polygama* (dos Santos *et al*., 2001). Phytochemical investigation of *V. glabrata* reported the presence of 20-hydroxyecdysone (**80**) and 11α, 20-hydroxyecdysone **(203)** in the plant (Hemendra *et al*., 2012). Pinnatasterone (**198**) together with 20-hydroxyecdysone (**80**) and turkesterone (**144**) were isolated from the root bark of *V. pinnata* Linn (Davis, 1982).



In addition, the observed biological activity of the extracts may also be due to the alkaloids and flavonoids present in them. This observation concurs with earlier investigation on some *Vitex* species by Hernández *et al* (1999). Ayse *et al* (2008) isolated four flavone glycosides from methanol extract of the flowering stems of *V. agnus-castus* L. growing in Turkey. Six flavonoids, persicogenin (**204**), artemetin **(135)**, luteolin (**205**), penduletin (**206**), vitexicarpin (**207**) and chrysosplenol-D (**208**), were isolated form *V. trifolia* L (Li *et al*., 2005). In another study, five phenolic compounds namely 4-hydroxybenzoic acid methyl ester (**209**), vanillic acid methyl ester (**210**), 4-hydroxybenzaldehyde (**211**), 4-hydroxybenzoic acid and ferulic acid (**213**) and four flavonoids, artemetin **(135)**, luteolin (**205**), vitexcarpin (**207**) and 5,5-dihydroxy-4',6,7-trimethoxy-

flavone (**214**) were isolated from fruits and leaves of *V. rotundifolia* (Takeo *et al.*, 2004). Several flavonoids have also been isolated from *V. negundo* leaves (Dayal *et al.*, 2003; Diaz *et al*., 2003; Maurya *et al*., 2007); leaves and twigs (Banerji *et al*., 1988; Kosankar *et al.*, 2000); roots (Khare, 2004; Dayal, 2004; Haq and Khan, 2004); seeds (Chawla *et al*., 1991) and from the stem bark (Rao *et al*, 1977).



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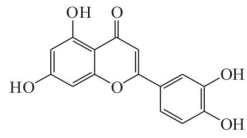

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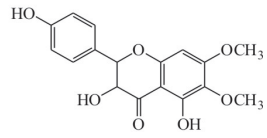

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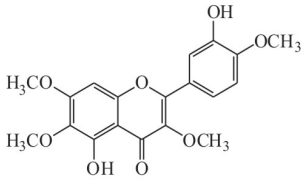

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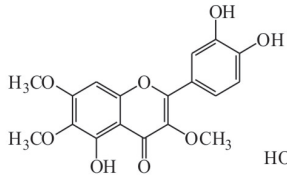

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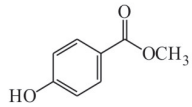

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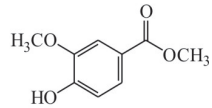

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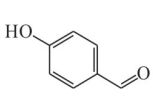

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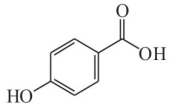

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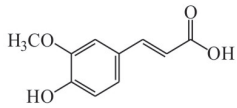

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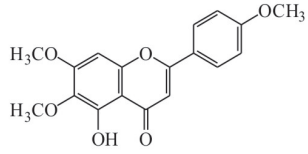

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Earlier studies have reported flavonoids as potential larvicidal agents. Methanol leaf extract of *Ervatamia coronaria* exhibited larvicidal activity against *Cx. quanquefasciatus*, *Ae. Aegyptia* and *An. stephens* is larvae after 24 hour post-treatment with LC_{50} values of 72.41 mg/l, 65.67 mg/l and 62.08 mg/l respectively. In another study on larvicidal activity, results of preliminary phytochemical analysis of the leaf extract revealed the presence of alkaloids, saponins, tannins, flavonoids and steroids (Mathivanan, 2010). A study conducted by Abiy *et al* (2009) reported that different plant parts of *Derris trifoliate* showed larvicidal activity against *Ae. aegypti* larvae and rotenoids were identified as the ac-

tive principles. Rajkumar and Jabanesan (2008) reported the larvicidal effect of four flavonoid compounds obtained from Poncirus trifoliata against *Ae. aegypti* fourth instar larvae. The compounds exhibited remarkable effect against the larvae.

Gas chromatography-mass spectroscopy of the extracts revealed the presence of volatile components. Although these compounds could, in principle, be responsible for the biological activity of the extracts, the occurrence of synergistic and/or additive effects between these constituents could be possible. Furthermore, studies have shown that these volatiles carry huge potential as mosquito larvicides (Ditasawat *et al*., 2008; Inder and Aarti, 2012).

The number of adults emerging from the breeding site determines the significance of the threat of mosquitoes in disease transmission. The crude extracts of the three *Vitex* species tested in the simulated semi-field conditions were found to possess adult emergence inhibition activity. Larval mortality started decreasing after sometime which is a typical response of plant based insecticides which do not generally persist in the environment. However, the extracts were found to have a residual activity of two weeks implying that one has to apply them frequently in order to bring down the mosquito larvae level. The lowest concentration that was found to be used to avoid causing any problem was 12.5 ppm. Results obtained from the study was not a surprise since the extracts were found to possess chemical substances like terpenoids (diterpenoids, triterpenoids, sesquiterpenoids), saponins, flavonoids, tannins, alkaloids and steroids among others which have been found to have reasonable efficacy against a range of mosquito species (noted earlier). The reduction in the adult emergence suggests the insect growth regulatory action of the test extracts on pupae.

In order to ascertain the toxicity of the extracts, brine shrimp toxicity test was performed. Results revealed that there was mortality at higher concentrations

and therefore doses higher than 50 ppm are not safe to non-target organisms. It was observed that the extracts possess remarkable larvicidal activity against *An. gambiae.*

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

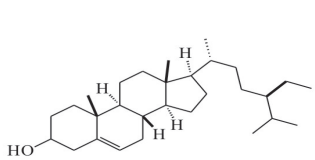
6.1. Conclusion

Biological evaluation of acetone, methanol and aqueous extracts of the *Vitex* species showed remarkable quantitative differences in their larvicidal activity against An. gambiae larvae. Phytochemical and GC-MS analyses provided an insight into the qualitative and quantitative differences in the components of the individual extracts. The general larvicidal activity was in the order of acetone > methanol > aqueous. This trend could be attributed to the solubility of the active component in the various solvent systems. The results revealed that the aqueous extracts had significantly low larvicidal activity except *V. schiliebenii* stem bark and *V. trifolia* aqueous leaf extract and therefore these two can be used to control *An. gambiae* larvae in aquatic environment.

A relationship was observed between dosage, time of exposure to the extract, mortality and the number of deformed larvae. Similar observations were observed in the simulated semi-field experiment. The extracts generally exhibited IGR activity at lower doses (≤ 25 ppm) while at higher doses the extracts were toxic to the larvae. Acetone leaf extract of *V. schiliebenii* exhibited the highest larvicidal activity with LD_{50} = 14.6 ppm. The results also revealed that mortality was directly proportional to the time of exposure of the larvae to the test extracts. Adult emergence inhibition was also noted in the test experiments with the longevity of the emerged adults reduced to not more than 48 h. This suggested that the extract had potential to be used in the development of botanical larvicides and IGRs in the control of *An. gambiae* larvae.

GC-MS analysis of the extracts showed that the extracts contained many components, majority of which have been reported to be present in other *Vitex* spe-

cies. Results revealed that phytoecdysteroids were the main components in the extracts and these included 20-hydroxyecdysone (**80**), stigmasterol (**168**), campesterol (**180**), β-sitosterol (**215**), γ-sitosterol (**167**), 20-hydroxyecdysone-20, 22-monoacetonide (**166**), 24-methyl-5-cholestene-3-ol (**216**), 3β, 16β, 18α, 19α, 20-ursane-3-ol (**217**), cholesterol (**218**) and vitamin E (**219**).



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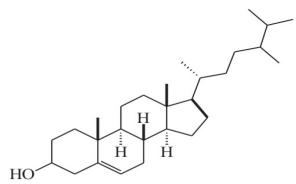

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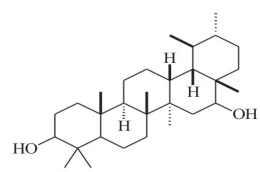

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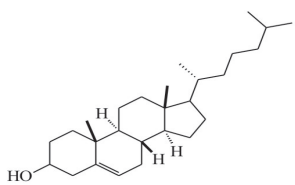

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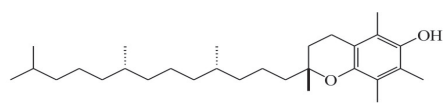

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Four steroids were isolated and identified. The ecdysteroid 20-hydroxyecdysone (**80**) was found to be present in the active extracts (acetone *V. payos* root bark and *V. schiliebenii* stem bark which is a common steroid in the genus *Vitex* (Sena Filho *et al*., 2008). Therefore the qualitative and quantitative differences in the composition of the extracts may explain the differences in their biological activity.

The steroids 20-hydroxyecdysone (**80**), stigmasterol (**168**), γ-sitosterol (**167**) and 20-hydroxyecdysone-20,22-monoacetonide (**166**) showed good biological activity against *An. gambiae* larvae suggesting that these constituents contributed towards the observed activity of the extracts either singly or synergically. The results revealed that 20-hydroxyecdysone-20, 22-monoacetonide (**166**) exhibited the highest activity and hence the activity of acetone extract from *V. payos* root bark could be attributed to this compound. It was also interesting to note that the compound exhibited both larvicidal and IGR activity. Therefore

this desirable characteristic can be used for the development of a commercial product in vector control.

In this study, various blends were formulated to determine if the constituents had any sygnergistic and/or antagonistic effect. Larvicidal activities of blends containing 20-hydroxyecdysone-20,22-monoacetonide **(166)** were higher than the individual components (Table 5.5). The larvicidal/IGR activity of these phytoecdysteroids might therefore be much more improved than when only a few components are used. Furthermore, the activity of these phytoecdysteroids may be influenced by their percentage composition and their complex interactions with the test organism. These finding therefore demonstrated the ability of 20-hydroxyecdysone-20,22-monoacetonide (**166**) to quantitatively enhance the larvicidal activity of certain compounds against *An. gambiae* larvae. This sygnergistic effect demonstrated by the isolated phytosteroids may give rise to other highly effective larvicidal/IGR activity of *Vitex* extracts. It was evident from the study that larvicidal/IGR activity of the *Vitex* extracts was due to single compound(s) as well as a result of synergistic action of the components present. This opens ways of using phytoecdysteroids as larvicides/IGRs.

6.2. Implications of the study

Fewer IGR compounds have been evaluated against mosquitoes, compared to the conventional larvicides. So far, three IGR products: neem, methroprene (a juvenoid-JH mimic) and diflubenzuron (chitin synthesis inhibitor), are available for use in the control of vectors of public health importance. However, they are not recommended for use in drinking water sources. In addition, they are difficult to produce and use by resource-poor people in rural Africa particularly. The aqueous extract is more applicable to rural situations where malaria causes the greatest burden. Consequently, evaluation of more polar IGRs against malaria vectors is essential for the development of new tools for vector control

programme. The mode of action of the larvicidal and IGR activity exhibited by the *Vitex* species need to be investigated further for the control of mosquitoes at the larval stage. This will be of importance in the control of the malaria vectors which have developed insecticide resistance.

6.3. Limitation of the study and Recommendations

The low activity of aqueous extracts could be attributed to the method of extraction used. However, due to time constraint the research did not use a different method to compare this effect. Apparently, not all the bioactive principals were isolated from the active extracts in this study. These larvicidal principals need to be isolated and identified in future work.

It was evident from this study that there were several essential oil components in the *Vitex* species that could offer protection against mosquitoes like *Anopheles* species. Further research should therefore be focused on the repellency and larvicidal effects of these oils against *An. gambiae.*

Behavioural differences may occur between simulated semi-field populations of *An. gambiae* larvae and natural populations. Testing of the promising extracts and compounds against *An. gambiae* under natural field conditions at several locations within a defined ecological range is required to determine if the extracts, isolated compounds and blends can effectively control mosquito larvae.

Acknowledgements

First and foremost, I would like to thank the Almighty God for giving me the power to believe in myself and pursue my dreams. I would never have done this without the faith in you, the Almighty.

I would also like to sincerely express my appreciation to my supervisors Dr. Ester Innocent and Prof. Zakaria Mbwambo both of Muhimbili University of Health and Allied Sciences (MUHAS), Institute of Traditional Medicine (ITM), Prof. Ahmed Hassanali of Kenyatta University, Department of Chemistry and Dr. Wilber Lwande of the International Center of Insect Physiology and Ecology (ICIPE-Kenya), Bioprospecting Unit for their guidance, patience, encouragement, insightful advice and cooperation at various stages of this study. I also offer my special thanks to them for providing the necessary materials for the work such as analytical facilities, mosquito larvae and working space.

I also offer my profound gratitude to the ITM staff and Muhimbili University at large for their critiques, suggestions and encouragement that added significantly to my research progress and the quality of the work. Your comments during seminar presentations were valuable in shaping this study.

I would like to thank my colleagues; Philip Mayeku and David Ywaya for conveying compounds for this work to various destinations for MS and NMR analysis. I am also thankful to Mr. Simon Mathenge of the National Museum of Kenya (NMK) for locating and identifying the investigated plant species. My thanks also go to Mrs. Agnes Wangila, Mr. Karimi, and Mr. Maina of Kenyatta University for their technical support at various points of the study. Assistance

offered by the ICIPE staff including Mr. John Bwire, Mrs. Gitau Milka, Mr. Jeremiah Ojude and Mr. Ochieng Kodhek is also highly appreciated.

In addition, there are several institutions that contributed significantly to the study also need special mention. First, I would like to thank DAAD-NAPRECA for financing my studies at MUHAS-ITM. Secondly, I am thankful to MUHAS-ITM for admitting me into a PhD programme, guiding and mentoring me throughout my study period and also for providing the chromatographic instruments during fractionation and purification of the plant extracts. Thirdly, my appreciation goes to ICIPE-Kenya for the three years' support as a Graduate Research Fellow. Last but not least, I acknowledge Kenyatta University for providing me with a conducive working environment during the study period.

All the wisdom, love and support I receive from my late father, Benjamin Onsongo, mother Agnes Onsongo and my parents-in-law, Paul Maroko and Mary Kwamboka are highly appreciated. They have always been my well wishers. I take this moment to express my never ending love and respect towards them for their blessings.

I am deeply indebted to my dearest husband, Dr. Geoffrey M. Maroko who saw me through all joys and frustrations of this study while helping me stay focused on the work. His assurance and good-will in every way is highly appreciated. I thank you for always believing in me and reminding me that the journey to a goal is one step at a time. To our children Brenda Farida, Marian Zawadi, Allan Manasseh and Emmanuel Hamadi, your moral support, good humour, love, prayers and patience during the tenure of my course is greatly appreciated. Last but not least, I wish all of you God's blessings.

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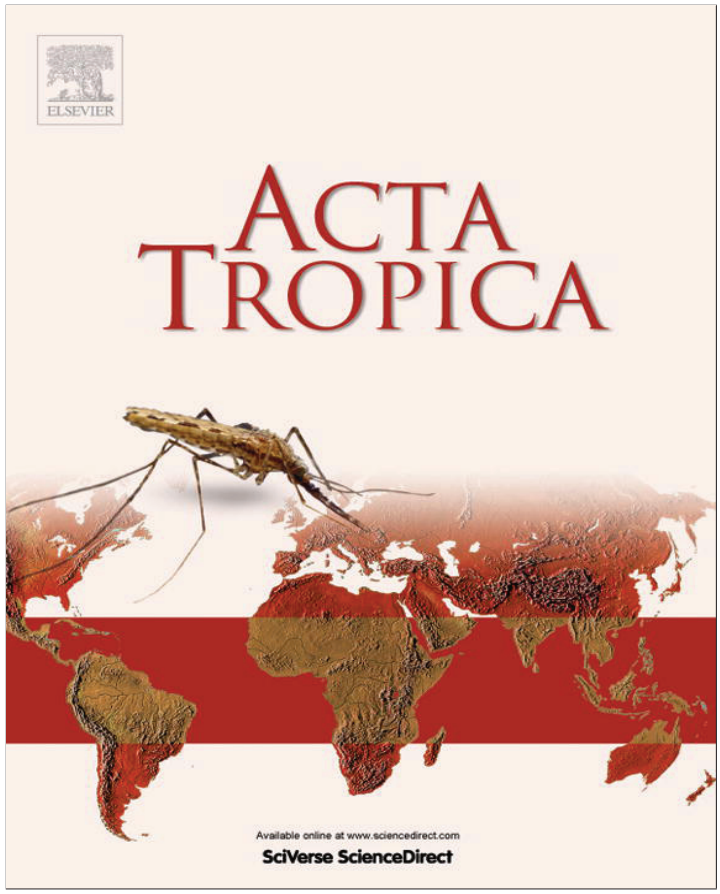
Appendices (Papers I-IV)

PAPER 1



Acta Tropica 127 (2013) 199–203

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Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Comparison of the effects of extracts from three *Vitex* plant species on *Anopheles gambiae* s.s. (Diptera: Culicidae) larvae

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ARTICLE INFO

Article history:
Received 14 February 2012
Received in revised form 2 May 2013
Accepted 12 May 2013
Available online xxx

Keywords:
Vitex schiliebenii
Vitex payos
Vitex trifolia
Larvicidal potential
Anopheles gambiae

ABSTRACT

Acetone and methanol extracts of different parts of three *Vitex* species (leaves and stem bark of *Vitex trifolia*, leaves, stem bark and root bark of *Vitex schiliebenii* and stem and root bark of *Vitex payos*) were evaluated for their potential to control *Anopheles gambiae* Giles s.s. larvae (Diptera: Culicidae). The extracts gave different levels and rate of mortality of the larvae. Some (methanol extract of *V. trifolia* leaves, acetone extracts of stem bark and leaves of *V. schiliebenii*, acetone extract of root bark of *V. payos*) caused 100% mortality at 100 ppm in 72 h, with those of *V. schiliebenii and V. payos* showing faster rate of mortality (LT_{50} = 8 h) than that of *V. trifolia* (LT_{50} = 14 h). At lower doses of these extracts (≤50 ppm), most of the larvae failed to transform to normal pupae but gave larval–pupal intermediates between 4 and 14 days of exposure. Some pupated normally but the adults that emerged appeared to be weak and died within 48 h. Extracts of the stem bark of *V. payos* showed interesting effects on the larvae. Initially, the larvae were relatively hyperactive compared to those in control treatments. Later, the ones that did not transform to larval–pupal intermediates became stretched and inactive and died and floated in clusters on the surface. These observations suggest some interesting growth-disrupting constituents in the plants, with possible application in the practical control of mosquito larvae in aquatic ecosystems.

1. Introduction

Plants have been recognized as rich sources of bioactive secondary metabolites with potential in the control of disease vectors and/or the diseases they transmit (e.g. de Omena et al., 2007; Githua et al., 2010; Khalid et al., 1989; Kihampa et al., 2009; Mackinnon et al., 1997; Singh et al., 2006; Sukumar et al., 1991). Given the high incidence of malaria in Africa and other tropical countries, the search for alternative tools and tactics for the control of mosquitoes has assumed special importance. Two types of plant products have been sought: volatile repellent blends for personal or space protection to reduce human-vector contacts (Birkett et al., 2011; Debboun et al., 2006; Omolo et al., 2005; Seyoum et al., 2002, 2003), and largely non-volatile plant constituents that are toxic or growth-disruptive to the larval or adult stages of mosquitoes (Govindarajan et al., 2008; Innocent et al., 2008; Kamaraj et al., 2009; Mwangi and Rembold, 1988; Ndung'u et al., 2004; Sharma et al., 2006). The use of plant repellents is widespread in different communities in the tropics and the performance of some has been evaluated experimentally (Curtis et al., 1991; Pålsson and Jaenson, 1999a,b; Seyoum et al., 2003). No similar traditional use of plant products targeting vector control has been documented. However, the possible deployment of natural products from readily accessible plants in community participation programmes to substantially reduce mosquito larval populations has been recognized, and plants belonging to the families Asteraceae, Verbenaceae, Meliaceae, and Rutaceae have been reported as potential sources of secondary metabolites for larval control (Innocent et al., 2008; Katade et al., 2006; Mwangi and Rembold, 1988; Ndung'u et al., 2004). *Vitex* species belonging to the family Lamiaceae (formally classified as Verbenaceae, Mabberley, 1997), have been reported to exhibit larvicidal activities against a number of mosquito species (Kannathasan et al., 2007; Karunamoorthi et al., 2008; Rahman and Talukder, 2006; Rodríguez-Lopéz et al., 2007; Yuan et al., 2006). Plants of this genus occur in both tropical and temperate regions of the world (Mabberley, 1997). In Kenya, different *Vitex* species are found growing naturally at different ecological settings, including the coast, the dry woodlands, Mount Kenya area, and across the Rift valley to the shores of Lake Victoria (Fig. 1; Beentje, 1994; Ruffo et al., 2002).

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0001-706X/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.actatropica.2013.05.003

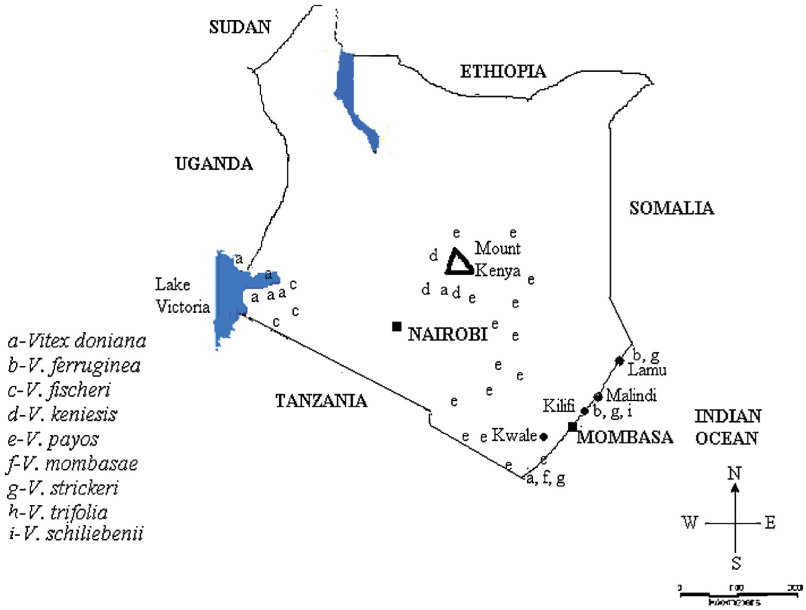

Fig. 1. Distribution of *Vitex* species in Kenya.

They are used in the local system of folk medicine by different communities for the treatment of a range of diseases (Kimondo et al., 2010).

In the present study, the effects of different doses of polar extracts of three *Vitex* species (*V. payos, V. schiliebenii* and *V. trifolia*) on *Anopheles gambiae* Giles s.s. were investigated with the overall aim of evaluating their potential as sources of anti-larval agents for community-based control of malaria vectors. *V. payos* (Louri) Merr. (commonly known as black plum in English, Mfudu in Kiswahili, Kimuu in Kikamba, Muburu in Embu, and Mfudu in Giriama) grows in semi-arid parts of eastern, coastal and central Kenya. It has round leathery leaves (Beentje, 1994; Ruffo et al., 2002). *V. schiliebenii* is a scrambling shrub that grows in the north coast around Watamu. The leaves are five foliolate. Its use in traditional medicine, if any, has not been documented. *V. trifolia* L. is commonly known as chaste tree (English). It is an exotic from Asia occasionally grown wild in shore vegetations. In Kenya, it is found in Kilifi, Mombasa, Diani and Shimoni, near the banks of Indian Ocean (Beentje, 1994; Ruffo et al., 2002). It is a shrub of 1–9 m with 3–5 foliolate leaves.

2. Materials and methods

2.1. Plant collection and treatment

The plant materials were collected from different parts of the Kenyan coastal region. They were authenticated by Simon Mathenge of the National Museum of Kenya (NMK). Preliminary screening of extracts of *V. payos* leaves showed no significant anti-larval activities. Accordingly, in the present study the following plant parts were used in the study: leaves and stem bark of *Vitex trifolia*, leaves, stem bark and root bark of *Vitex schiliebenii* and stem and root bark of *Vitex payos*. The materials were air-dried at room temperature in shade for three weeks and ground into powder in an electric miller. Each powdered material was extracted three times in acetone (5-fold volume) for 24 h with occasional stirring. The extracts were filtered and concentrated to dryness using a rotary evaporator at 40 °C and the combined extract stored at 4 °C. This procedure was repeated with methanol in the same proportion and for the same periods.

2.2. Mosquito rearing

Larvae of *A. gambiae* Giles s.s. used in bioassays were obtained from a colony maintained at the international Centre of Insect Physiology and Ecology (ICIPE) Insect Mass Rearing Unit. This strain of mosquitoes originates from Njage village, 70 km from Ifakara, south eastern Tanzania and has been reared under laboratory conditions since April 1996. Larvae were allowed to emerge from eggs in plastic containers filled with distilled water and were transferred to larger pans ($37 \times 31 \times 6$) at densities of 200–300 at 2nd instar stage. Larvae were fed on Tetramin fish food and the water temperature was maintained at 28 ± 2 °C throughout larval development.

2.3. Biossays exposing larvae to phytoextracts

Laboratory bioassays were conducted in accordance to the World Health Organization method (WHO, 1996). Crude extracts of each plant material (5 mg) was dissolved in 1 ml distilled water (*V. schiliebenii* leaves) containing 5% dimethyl sulphoxide (DMSO) or in 1 ml absolute ethanol (all other *Vitex* extracts) to obtain stock solutions each of 5000 ppm. For each extract, 100 ml of different doses (25, 50, 100, 250 and 500 ppm) were prepared by serial dilutions. Three replicates of twenty freshly moulted late 3rd and early 4th instar larvae of *A. gambiae* s.s. were exposed to each dose of each extract with two negative controls (treated with absolute ethanol or DMSO-distilled water). Larval mortality (at higher doses), abnormal behavior and morphological deformations (at lower doses) were recorded at 24 h intervals until the death of the last larva or emergence to adult (WHO, 1996). The bioassay room was kept at a temperature of 30 °C, an average humidity of 78% and a photo

Table 1
Mean percentage mortality induced by the phytoextracts of *Vitex* species against 3rd/4th instar larvae of *Anopheles gambiae* Giles s.s. after 24 h exposure.

Extract code	Mean mortality (% ± SE)/concentration (ppm)					Lethal (ppm)	Concentration values
	500	250	100	50	25	LC_{50}	95% CL
VSR-1	96.7 ± 1.6a	43.3 ± 3.3c	3.3 ± 1.6e	0.0 ± 0.0e	0.0 ± 0.0e	252.1	225.0–281.8
VSR-2	56.7 ± 1.6bc	15.0 ± 2.9de	5.0 ± 2.0e	0.0 ± 0.0e	0.0 ± 0.0e	444.0	392.0–505.0
VSS-1	100.0 ± 0.0a	100.0 ± 0.0a	100.0 ± 0.0a	100.0 ± 0.0a	78.3 ± 6.0b	17.4	14.6–20.3
VSS-2	43.3 ± 1.7b	6.7 ± 1.7d	0.0 ± 0.0e	0.0 ± 0.0e	0.0 ± 0.0e	522.6	462.8–594.9
VSL-1	100.0 ± 0.0a	96.7 ± 3.3a	100.0 ± 0.0a	87.0 ± 4.4b	83.3 ± 0.3b	14.6	11.9–17.6
VSL-2	100.0 ± 0.0a	100.0 ± 0.0a	31.6 ± 3.3d	10.0 ± 2.9e	6.7 ± 1.7e	136.3	120.8–154.8
VPR-1	100.0 ± 0.0a	100.0 ± 0.0a	100.0 ± 0.0a	100.0 ± 0.0a	85.0 ± 1.7b	15.6	12.9–18.6
VPR-2	55.0 ± 1.7cd	36.7 ± 4.4d	0.0 ± 0.0e	0.0 ± 0.0e	0.0 ± 0.0e	377.8	334.7–427.1
VPS-1	45.0 ± 1.6b	27.5 ± 1.6d	15 ± 3.3de	0.0 ± 0.0e	0.0 ± 0.0e	511.6	451.2–581.0
VPS-2	40.0 ± 1.7b	25.3 ± 1.6d	0.0 ± 0.0e	0.0 ± 0.0e	0.0 ± 0.0e	587.5	478.5–597.6
VTL-1	45.0 ± 1.6c	43.3 ± 1.7c	0.0 ± 0.0e	0.0 ± 0.0e	0.0 ± 0.0e	513.2	455.0–583.2
VTL-2	100.0 ± 0.0a	100.0 ± 0.0a	91.7 ± 4.4b	0.0 ± 0.0e	0.0 ± 0.0e	76.6	68.2–86.0
VTS-1	8.3 ± 3.3e	0.0 ± 0.0e	0.0 ± 0.0e	0.0 ± 0.0e	0.0 ± 0.0e	925.9	753.4–1164.6
Control	2.3 ± 3.3e						

VS: *V. schiliebebenii*; VP: *V. payos*; VT: *V. trifolia*; R: root bark; S: stem bark; L: leaves; 1: acetone; 2: methanol; means with the same letters within a column are not significantly different at 5% level.

period of 12 h of light and 12 h of darkness. The larvae were fed on Tetramin® fish food (Terta GmbH, Germany) at about 1 mg per beaker every 24 h.

2.4. Analyses of results

The average larval mortality (±SE) resulting from each dose of each extract was calculated and the data was subjected to probit analysis for calculating the lethal concentrations of the extracts at LC_{50} at 95% confidence limit of upper and lower levels. The values were calculated using GenStat Teaching edition version. Results with $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Larval mortality at higher doses

Table 1 presents a summary of the mean mortality ± SE of *A. gambiae* larvae, LD_{50} of the extracts after 24 h exposure. In all the experiments, mortality in treated tests was significantly higher than in control treatments. The highest larval mortality was obtained with the acetone leaf extract of *V. schiliebeniii* (LC_{50} 14.6 ppm), followed by acetone root extract of *V. payos* (LC_{50} 15.6 ppm), acetone stem bark extract of *V. schiliebenii* (LC_{50} 17.4 ppm) and methanol leaf extract of *V. trifolia* (LC_{50} 76.6 ppm). At 100 ppm, the first three caused 100% mortality of the larvae within 24 h (Table 1) while that of *V. trifolia* caused this level of mortality in 72 h (result not included in Table 1).

3.2. Growth disruption effects at lower doses

Generally, at lower doses the larval stage was prolonged (7–10 days post-treatment) (In press) compared to that in control (4–5 days). It was observed that at lower doses, viz. 50 ppm and below, extracts of *V. payos* caused interesting physiological and neurophysiological effects to the exposed larvae. The larvae were smaller in size compared with those in control treatments. Gentle introduction of a pipette into the beaker triggered a series of abnormal behaviors, including initial coiling followed by stretching and loss of mobility. In 8–10 days, about 50% of the larvae died and floated on the surface of the solution in clusters, some of which were black (Fig. 2A). The other 50% either transformed into lighter-coloured larval–pupal intermediates or emerged as weak adults. Interesting observations were also made with extracts of *V. schiliebenii* root bark extracts at 100 ppm. Although mortality was <10% in 24 h, the surviving larvae appeared smaller in size compared with those in the control. Moreover, they were sluggish and failed to move toward deeper sections of the solution. Morphological abnormalities (Fig. 2B and C) were observed 48 h post-treatment. About 30% of the larvae failed to transform to normal pupae, and instead produced larval–pupal intermediates. Approximately 60% successfully pupated, but the resulting pupae either failed to emerge as adults or emerged as weak adults that died within 48 h. Lower doses (<50 ppm) of *V. trifolia* leaf extracts did not appear to cause any morphological deformities, but led to retardation and 100% mortality in 7–8 days.

4. Discussion

No previous studies on bioactivities of phytochemical extracts of *V. payos* and *V. schiliebenii* have been reported. In the present study, acetone extracts of different parts of the two plants exhibited potent larvicidal effects at higher doses (≥100 ppm). Of particular interest, are the longer term growth-disrupting effects of the extracts at lower doses. Larvae exposed to the extracts exhibited structural deformation and dysfunction, which resulted either in

Fig. 2. Phytoextracts induced morphological deformities (A) black larva; (B) and (C) partially formed pupae (larval/pupal intermediates).

their death, or short-lived adults. Similar structural deformations were previously reported with *A. gambiae* larvae exposed to root bark extracts of Meliaceae species (Ndung'u et al., 2004). These deformities were associated with blends of several limonoids, which are structurally related to ecdysteroids and have been specifically considered as potential ecdysteroid agonists or antagonists. Indeed, two limonoids from *Turraea obtusifolia* have been shown to antagonize 20-hydoxyecdysone action in a *Drosophilia* cell line (Sarker et al., 1997). Limonoids have also been implicated as possible disruptants of the endocrine system of other insects by different authors (Jayaprakasha et al., 1997; Lopez-Olquin et al., 1997; Roel et al., 2002 and Rembold, 1995). Previous phytochemical screening of *V. agnuscastus* revealed the presence of different classes of constituents, including iridoid glycosides, flavonoids, alkaloids and terpenoids (Artz, 2007). Of particular interest is demonstration of an insect growth hormone pterosterone in *V. glabrata* (Suksamrarn et al., 1999) and related ecdysterioids, including (24R)-11 alpha, 20, 24-trihydroxyecdysone and 11 alpha, 20, 26-trihydoxyecdysone from *V. canescens* root bark (Suksamrarn et al., 2000). We are currently undertaking bioassay-guided studies to isolate and characterize the active compounds from the acetone extracts of *V. payos* and *V. schiliebenii* responsible for the observed activities, and it would be interesting to see if the growth-disrupting effects observed with the extracts of these plants are due to similar or different constituents.

In the present study, unlike the extracts of *V. payos* and *V. schiliebenii*, methanol leaf extract of *V. trifolia* did not show growth-disrupting effects on *A. gambiae* larvae, but physical retardation and delayed mortality occurred at lower doses. Previously, extracts of the plant were shown to have anti-feedant effects on the insect pest *Spodoptera frugiperda* (Hernández et al., 1999). The plant has also been reported to have pharmacological properties, including antipyretic (Ikram et al., 1987) and antibacterial (Hossain et al., 2001), as well as asthma and allergy relieving effects (Ikwati et al., 2001). Phytochemical studies have shown the presence of monoterpenes (Pan et al., 1989), halimane-type diterpenes and vitetrifolins (Ono et al., 2001). It would be interesting to see which of these or other phytoconstituents of the plant are responsible for the effects observed on *A. gambiae* in the present study.

In summation, results of this study show interesting larvicidal and/or growth-disrupting effects of *V. trifolia*, *V. payos* and *V. schiliebenii* extracts. The active constituents responsible for these effects remain to be characterized. Enriched extracts of the plants may have potential for controlling malaria vectors in breeding sites around human dwellings.

Acknowledgements

The authors are grateful to Natural Product Research Network for Eastern and Central Africa (NAPRECA-DAAD) for financial assistance and the International Centre for Insect Physiology and Ecology (ICIPE) for providing the insects and necessary facilities to carry out this research work successfully. Acknowledgement is extended to Muhimbili University of Health and Allied Sciences (MUHAS), Institute of Traditional Medicine (ITM) for its technical support. We also thank Mr. Simon Mathenge for identifying the plants under the study.

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PAPER 2

ISSN 2278-6929

Novus Natural Science Research
2013, Vol. 2, No. 1

www.novusscientia.org

Accepted on: February 7, 2013

Time-course effects of Vitex schiliebenii (Varbenaceae) solvent extracts on Anopheles gambiae giles s.s. larvae under simulated semi-field conditions

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ABSTRACT

***Objectives:** To determine the growth-disrupting effects of polar phyto-extracts of V. schilibenii on Anopheles gambiae larvae in a simulated semi-field condition and to undertake phytochemical screening of constituents present.*
***Materials & Method:** 3rd and early 4th instars of An. gambiae larvae were exposed to acetone and methanol extracts of stem bark and leaves of V. schiliebenii and their effects on larval, pupal and adult stages recorded. Phytochemical screening of the extracts was undertaken using standard methods.*
***Results:** The results revealed that Anopheles gambiae larvae were susceptible to V. schiliebeinii extracts with less than 20 % adult emergence at concentrations ≥ 25 ppm except for methanol extract of stem bark. About 11 % pupae emerged in V. schiliebenii acetone leaf extract (VSL 1) between day 6 and 10 but they did not transform into viable adults. Phytochemical screening revealed the presence of flavonoids, terpenoids, steroids, alkaloids, saponins and tannins in the extracts.*
***Conclusion:** Eco-friendly polar extracts of V. schiliebenii show potential for mosquito control in small breeding habitats, which may be a useful component in integrated control of malaria vectors. Characterization of the active constituents of the extracts of the plant is in progress.*

KEYWORDS: *Anopheles gambiae* s.s., *Vitex schiliebenii*, Verbenaceae, larvicidal effects, Photochemical

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INTRODUCTION

Over two billion people in tropical countries are at risk from mosquito-borne diseases such as dengue fever, hemorrhagic fever, malaria and filariasis. It is estimated that US$ 2 billion is spent on malaria control and treatment programmes in Africa annually[1]. The problem has become increasingly difficult to manage because of the spread of resistance to anti-malarial drugs by the parasites resulting in increased severity of the disease[2]. Personal protection using

synthetic and plant repellents from mosquito bites and control of mosquitoes using mosquito nets impregnated with synthetic insecticides are currently the dominant measures of reducing mosquito bites[3]. However, vector resistance to synthetic insecticides is a recurring problem[4]. A number of studies have shifted focus to botanical substances for either personal protection and/or controlling mosquitoes at immature stages[5-11]. Promising plant families studied for larvicidal effects include Meliaceae, Rutaceae, Labiatae, Piperaceae, Verbenaceae, Asteraceae, Cladophoraceae, Oocystaceae, and Annonaceae[12].

Besides their popular use in traditional medicines in many countries, *Vitex* species have been reported to exhibit activities against a variety of mosquito species, such as *Culex trataeniorhnchus*, *Anopheles gambiae*, *Culex quinquefasciatus*, *Plutella xylostella*, and *Callosobruchus maculates*[9, 13-15] *V. schiliebenii* is a branched shrub with multiple square-shaped stems and low height (4-8 m). The leaves have 3-leaflets with a smooth surface, about 10-12 cm long. In Kenya, it grows in the coastal region at Watamu located in a low altitude, semi-arid area[16-17]. In an initial laboratory study, polar extracts of *V. schiliebenii* showed interesting growth-disrupting activities on immature of *An. gambiae* (Mokua *et al*., submitted). In the present study, the potential of the extracts to control the vector was investigated under simulated semi-field conditions.

MATERIAL AND METHODS

Plant material collection

The leaf and stem bark of *V. schiliebenii* were collected from the North Coast of Kenya (at Kenya Forest Research Institute , KEFRI) near Gede along Mombasa-Malindi road 18 km from Malindi town. The plant species was authenticated at the field by Simon Mathenge of the National Museum of Kenya (NMK) and a voucher specimen Ref. No. GMN/22 deposited at the NMK herbarium.

Preparation of plant extracts

The plant materials were air-dried under shade at room temperature (25±2^{o}C) for three weeks, ground into powder in an electric miller and soaked in different solvents to obtain the extracts. Powdered leaves (400 g) and stem bark (800g) were separately soaked in acetone (2.0 and 4.0 L, respectively) for 24 h with occasional stirring, and then filtered. This was repeated three times. The process was repeated with similar quantities of methanol for both plant materials. Each set of solvent extracts were pooled together and concentrated to dryness using a rotary evaporator at 40^{o}C.

Mosquito species

Larvae of *An. gambiae* s.s. Giles used in bioassays were obtained from a colony maintained at the Insect Mass Rearing Unit of International Centre of Insect Physiology and Ecology (ICIPE). This strain of mosquitoes originated from ICIPE's Thomas Odhiambo Campus (Mbita Point) near Lake Victoria in 2003. Larvae were allowed to emerge from eggs in plastic containers filled with distilled water and were transferred to larger pans (37 × 31 × 6) at densities of 200-300 at 2nd instar stage. They were fed on Tetramin® fish food (Terta GmbH, Germany) and the water temperature was maintained at 28±2^{o}C throughout larval development.

Simulated field assays

Bioassays were conducted in accordance to World Health Organization procedure[18] in 1000 ml chlorine-free water in white plastic containers of internal diameter 21.5 cm and 13 cm. These containers were placed in holes (10 cm deep) in a screen house arranged in three rows and 13 columns with a distance of 50 cm in between. The containers were filled with 400 ml

of water and left for 24 h for conditioning and then batches of 50 freshly molted late 3rd and early 4th instars larvae of *Anopheles gambiae* were added together with food and left for 2 h to allow the larvae get acclimatized with the environment. Five milligrams of each extract was dissolved in 1 ml distilled water containing 5 % dimethyl sulphoxide (DMSO), which gave a homogenous solution. Stock solution of each plant extract was successively diluted to give concentrations of 12.5, 25, 50, 100, 250, and 500 ppm in 500 ml. The effect of each test solution together with a negative control (treated with DMSO-distilled water) was replicated three times. Sampling was carried out using a 350 ml dipper at different sides of the containers. Three dips were taken for each test sequenced in such a way that triplicates of similar concentrations were sampled consecutively with the same dipper. One dip was taken from each container at a time returning to it after completion of the cycle for that particular treatment until all the four samples were taken. Mortality was recorded after every 24 hours until the death of the last larva or emergence of adult[19]. Mortality in control treatments was corrected using Abbott's[20] formula when it ranged between 5-10 percent. All the treated and control containers containing pupae were kept separately in a netted cage to prevent successfully emerged adult from escaping into the environment. The percent adult emergence inhibition (% EI) was based on the number of moribund and dead larvae, pupae that did not develop successfully into viable adults and pupal-adult intermediates. The average screen house temperature was 32 ± 4°C and the humidity was 65 ± 10 % RH. The larvae were fed on Tetramin® fish food (Terta GmbH, Germany) and the adults 10 % glucose solution.

Analyses

The average number of larvae or pupae collected per dip for each replicate of each treatment and the control were recorded after 24 h. Percentage inhibition of adult emergence (% IE) from the data on mortality at all stages was estimated for each treatment according to the formula [18]:

$$\% \text{ IE} = 100 - (T / C \times 100)$$

Where T = number of adults emerging in treated test and C = number of adults emerging in control test.

Where mortality in control tests ranged between 5-10 %, the corrected mortality was calculated using Abbott's formula [19]:

$$\% \text{ mortality} = \left[\frac{\text{mortality in treated - mortality in control}}{100 - \text{mortality in control}}\right] \times 100$$

However, where control tests showed more than 10 % mortality (two cases out of sixteen), they were discarded and the whole experiment repeated.

Phytochemical screening

The following phytochemical tests [20] were carried out on the acetone and methanol extracts to identify the major classes of constituents.

Alkaloids: about 20 ml of Dragendorff's reagent was added to about 20 mg of the extract; the formation of orange red precipitate suggested the presence of alkaloids;

Tannins: a small amount of the extract was mixed with water, heated on the water bath, filtered and ferric chloride added; a dark green solution suggested the presence of tannins;

Saponins: about 200 mg of the extract was shaken with 5 ml distilled water heated until it boiled, transferred into a test tube and shaken vigorously and left to stand for about 10 minutes; a thick persistent froth suggested presence of saponins;

Flavonoids: Extract of about 200 mg was dissolved in NaOH followed by addition of HCl; yellow solution that turned colorless suggested the presence of flavonoids;

Terpenoids (Salkowski test): About 200 mg of the extract was mixed with 2 ml of chloroform and 3 ml concentrated sulphuric acid was carefully added; a reddish brown coloration at the interface suggested the presence of terpenoids;

Steroids: About 2 ml of acetic anhydride was added to about 200 mg of the extract followed by 2 ml of sulphuric acid; change from violet to green indicated the presence of steroids.

RESULTS

Yields and phytochemical profiles

Acetone and methanol crude extracts of *V. schiliebenii* produced semi-solid materials of varying yields between 2.48-4.11 % w/w. The percentage yield obtained from acetone extracts (3.67-4.11) was generally higher than from methanol extracts (2.48-3.05). Phytochemical screening revealed the presence of flavonoids, terpenoids, steroids, alkaloids, saponins and tannins in both sets of extracts.

Developmental disruption and mortality

Prolonged developmental time was observed in the treated cohorts as compared with control tests. The total larval period lasted 9-10 days (6-8 days in control) and pupal period lasted 2-3 days (1 day in control) resulting in total developmental period (larval + pupal development) of 11-13 days (7-9 days in control).

Most of the mortality was in larval and pupal stages and only a few were dead at the adult stage. Cumulative mean percentage mortality of larvae exposed to different extracts at 12.5 ppm ranged between 23-52 %, and that of the pupae and pupal-adult intermediates ranged between 2-15 %. At 25 ppm, % larval mortality ranged between 43-90 % while that of pupae and pupal-adult intermediates ranged between 4-14 %. There were no significant differences ($P < 0.05$) in mortalities between treatments at 50 and 100 ppm with different extracts. Larvae exposed to *V. schiliebenii* methanol stem bark extract and *V. schiliebanii* acetone leaf extract at 100 ppm and above produced no live or dead pupae. The few adults that emerged from treated cohorts appeared weak and unable to fly effectively. They rested for longer periods on the surface of the water compared with the control adult mosquitoes and died within 48 h after emergence. In control treatments, during the four-day exposure mortality increased from 2 to 8 % and the rest developed into pupae and then adults within 2-4 days.

Inhibition of adult emergence

The main indicator of the treatment response was the percentage inhibition of emerged adults. At 12.5 ppm, acetone extracts of *V. schilebenii* stem bark and leaves were found to be most effective, inhibiting adult emergence by 56-57 %, At 25 ppm, no adult emerged in treatments involving both *V. schiliebenii* acetone and methanol leaf extracts (88 and 86 % larval mortality and 12 and 14 % pupal/adult mortality, respectively; Fig 1).

DISCUSSION

In the present study, the effects of leaf and stem bark extracts of *V. schiliebenii* on *An. gambiae* s.s. larvae were evaluated for their potential as sources of botanicals for the control of malaria vectors under semi-field conditions. Prolonged developmental time was observed at the lowest concentration (12.5 ppm), suggesting a subtle physiological effect of the phytochemicals. Suppression in pupation was also observed at 12.5 ppm and in some cases resulting in larval-pupal intermediates. This could be attributed to hormonal balance disruption with internal levels of ecdysone. One of the main effects of ecdysteroids on insects

is their interference with development and the important role they play in the transition from one developmental stage to another, especially during metamorphosis.At higher concentrations (≥25 ppm), remarkable larval mortality was observed suggesting that *V. schilibenii* can be used as an insecticidal agent. Results revealed that *V. schiliebenii* is rich in phytochemicals and the observed biological activity might be due to the presence of these bioactive compounds which could synergistically, antagonistically or independently contribute to the activity of the crude extracts.

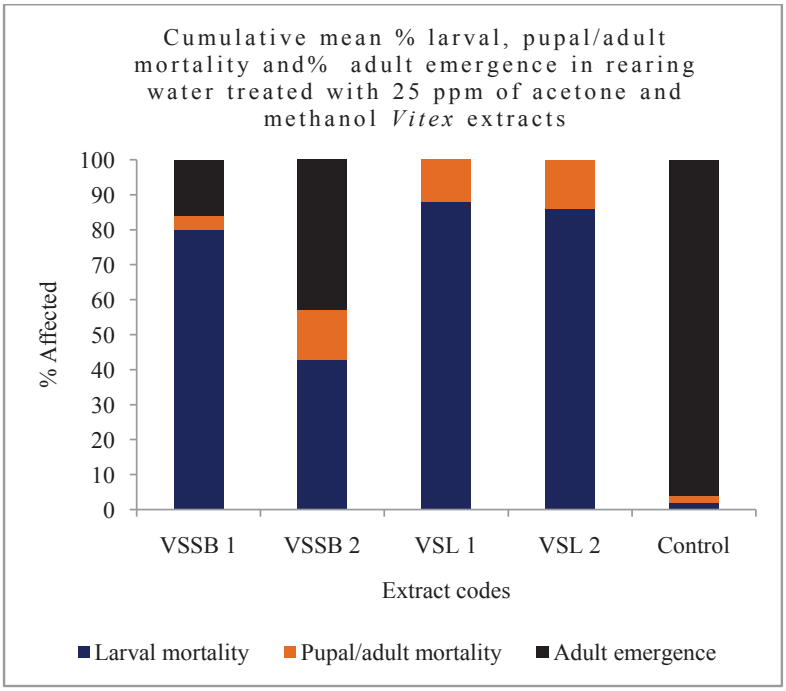

Figure 1: Percent affected at different stages
VSL 1: acetone leaf extract; VSL 2: methanol leaf extract;
VSSB 1: acetone stems bark extract, VSSB 2: methanol stem bark extract

Interestingly, a similar study conducted by[21] with plant sterols on the larvae of *Dermestes maculates* reported that the phytosterols interfere with the utilization of cholesterol by the insect. In the family *Verbeneceae*, the genus *Vitex* has been identified as one of the best sources of phytoecdysteroids [22]. Phytochemical investigation of the plant in the current study also revealed the presence of phytoecdysteroids in the extracts. Significant disruptions of the insect hormonal system by exogenic ecdysteroids ingested with food and eventual death have also been reported by[23]. Some of the *Vitex* species like *V. negundo*, *V. trifolia*, *V. rotundifolia* and *V. agnus-castus* and their bioactive constituents have been reported to exhibit larvicidal activity against various mosquito species such as *Anopheles subpictus*, *Culex tritaeniorhynchus*, *Culex quinquefasciatus*, *Anopheles stephensi*,[7, 13-14, 24-27]. Bioactive constituents like methyl-*p*-hydroxybenzoate isolated from the leaves of *V. trifolia*[28], lauric acid, palmitic acid, steric actid and oleic acid extracted from *V. altissima*, *V. negundo* and *V. trifolia*[29] have been reported to be responsible for their potential as larvicides. However, in the present study the specific constituents or blends associated with the observed effects on the mosquito larvae are yet to be unveiled.

In conclusion, *V. schiliebenii* showed promising larvicidal activity and hence may be considered as a source of a botanical agent which can be developed into eco-friendly chemicals for control of vector-borne diseases and more particularly, *An. gambiae*. Identification of the principle constituents responsible for the observed effects is underway to help in throwing further light on the mode of action.

ACKNOWLEDGEMENT

The authors are grateful to Natural Product Research Network for Eastern and Central Africa (NAPRECA-DAAD) for financial assistance and the International Centre for Insect Physiology and Ecology (ICIPE) for providing the insects and necessary facilities to carry out this research work successfully. We also thank Mr. Simon Mathenge of the National Museum of Kenya for identifying the plants under the study. Acknowledgement is extended to Muhimbili University of Health and Allied Sciences (MUHAS), Institute of Traditional Medicine (ITM).

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PAPER 3

Journal of Applied Pharmaceutical Science Vol. 3 (05), pp. 091-095, May, 2013
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2013.3517
ISSN 2231-3354

Larvicidal and Brine Shrimp Activities of *Vitex Schiliebenii* Extracts and Isolated Phytoecdysteroids on *Anopheles gambiae* Giles S.S Larvae

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ARTICLE INFO

Article history:
Received on: 12/04/2013
Revised on: 29/04/2013
Accepted on: 15/05/2013
Available online: 30/05/2013

Key words:
Vitex schiliebenii, *Verbenaceae*, *Anopheles gambiae*, phytoecdysteroids, 20-hydroxyecdysone, stigmasterol, γ-sitosterol, Larvicide

ABSTRACT

Acetone, methanol and aqueous extracts of the leaves, stem bark and root bark of *Vitex schiliebenii* belonging to the family Verbenaceae were evaluated for their larvicidal activity against late 3rd/early 4th *Anopheles gambiae* Giles s.s. larvae (Diptera: Culicidae). The extracts of the acetone leaves and stem bark were active with LC_{50} values of 14.6 and 17.4 ppm respectively at 24 hrs. These extracts exhibited low toxicity to brine shrimps with LC_{50} values of 180.9 and 154.4 ppm respectively. The constituents in these extracts were isolated and evaluated and the phytoecdysteroids 20-hydroxyecdysone (**1**) and stigmasterol (**2**) were identified as the active principles in the acetone stem bark while γ-sitosterol (**3**) was the active principle of the acetone leaf extract. The methanol leaf extract, the stem bark aqueous extract and the acetone root bark also showed potency against the mosquito species.

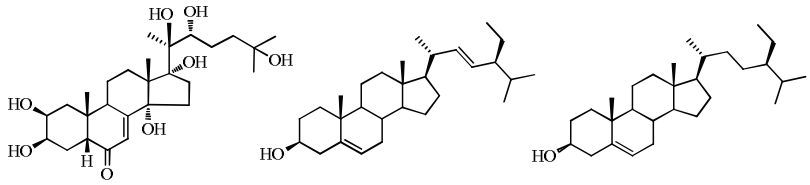

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Fig. 1: Isolated Phytoecdysteroids.

INTRODUCTION

Several mosquito species belonging to the genera *Anopheles*, *Culex* and *Aedes* thriving in the tropical and sub-tropical climates are vectors for pathogens transmitting diseases that affect the health of both man and livestock. A*nopheles* species, the malaria vector (Sinka et al., 2010), *Aedes aegypti*, the vector for yellow fever and dengue (Reiter, 2010) and *Culex pipiens*, the vector of West Nile virus (Diaz-Badillo et al., 2011) are responsible for most of tropical diseases. Malaria alone kills over half a million people each year (WHO, 2011).

Most of these insects are controlled using conventional chemical insecticides which are harmful to the environment, humans and many beneficial organisms. In addition, the chemicals have developed resistance to these synthetic insecticides. Consequently, this has indicated a need for additional or alternative approaches for controlling the proliferation of mosquito population. In response to this, several efforts have been made to identify alternative insecticides that are target-specific, biodegradable, environmentally safe, and botanicals in origin for integrated pest management (IPM) programmes. Presently, several products of botanical origin, especially the secondary metabolites, have received significant renewed attention as potentially bioactive

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agents used in insect vector management (Baraza *et al*., 2008; Kiran and Devi, 2007; Ndungu et al., 2004; Maniafu *et al*., 2009; Ribeiro, *et al*., 2009). Plant derived insecticides comprise of an array of chemical compounds which act in concert on both behavioral and physiological processes. Hence, the chances of pests developing resistance to such substances are less likely. Moreover, botanical insecticides are less likely to bioaccumulate as they are biodegradable (Saxena, 1987).

Among the botanical insecticides, phytoecdysteroids are present in the genus *Vitex* belonging to the family Verbenaceae and may be used as taxonomic markers. An extensive literature search on the genus *Vitex* revealed the presence of ecdysteroids in a number of *Vitex* species (Sena *et al*., 2008) found in tropical and subtropical regions (Correa, 1926).

The genus *Vitex* has been reported to exhibit larvicidal activities against a number of mosquito species (Rahman and Talukda, 2006; Yuan et al., 2006; Kannathasan et al., 2007; Rodriguez-Lopez et al., 2007; Karunamoorthi et al., 2008). In the current study, larvicidal and brine shrimp activities of *V. schiliebenii* extracts and isolated phytoecdysteroids on *An. gambiae* are presented.

MATERIALS AND METHOD

Plant materials

The leaves, stem bark and root bark of *V. schiliebenii* used in the study were collected from Watamu (Malindi County) along the Kenyan coast in 2009. The plant species was authenticated at the field by a botanist from the National Museum of Kenya (NMK) and a voucher specimen Ref. No. GMN/22 deposited at the NMK herbarium. The plant materials were dried under the shade to constant weight and ground into powder in an electric miller.

Chemicals and instruments

All solvents used were analytical grade (E-Merck,D-6100 Darmastadt, F.R. Germany). Analytical thin layer chromatography (TLC) was performed on 60 F_{254} plates 5 X 10 cm, 0.20 mm thickness) with fluorescence indicator. The spots were viewed using a multi-band UV- 254/366 nm lamp (UV GL-58). The TLC plates were then sprayed with *p*-anisaldehyde reagent (solution of anisaldehyde, 0.6% ethanol and 5% sulphuric acid) (reagents obtained from Sigma Aldrich, Germany) and kept in the oven at 110^{o}C until the spots appeared. Compounds containing unsaturated bonds especially those with conjugated systems became visible as quenching spots under UV light at 254 nm. Spots of organic compounds gave specific colors with this reagent after heating at 110^{o}C for 2-5 minutes. Column chromatography was carried out using silica gel mesh 0.06 mm (230-400 Merck-Germany) and eluted with varying concentrations of dichloromethane, acetone and methanol mixture. Melting points of the isolated crystallized compounds were determined on Sanyo GallenKamp electronic melting point apparatus. ^{1}H NMR spectra were run on Varian Gemini 300, 500 MHZ in $CDCl_3$ and CD_3OD and ^{13}C NMR spectra in Varian-Germini/Bruker 600 MHz in $CDCl_3$ and CD_3OD.

Extraction of plant materials

Each powdered material was extracted three times in acetone (5-fold volume) for 24 h with occasional stirring. The extracts were filtered and concentrated to dryness using a rotary evaporator at 40^{o}C and the combined extract stored at 4^{o}C. This procedure was repeated with methanol in the same proportion and for the same periods. The aqueous extracts were obtained using soxhlet extraction. The extracts were filtered and then freeze dried to obtain the dry powder which was then stored at 4^{o}C for further chemical and biological analysis.

Larvicidal assay

Larvae of *An. gambiae* Giles *s.s.* used in bioassays were obtained from a colony maintained at the International Centre of Insect Physiology and Ecology (ICIPE) Insect Mass Rearing Unit. This strain of mosquitoes originated from ICIPE's Thomas Odhiambo Campus (Mbita Point) near Lake Victoria in 2003. The larvae were fed on Tetramin® fish food (Terta GmbH, Germany) at about 1mg per beaker every 24- h and the water temperature was maintained at 28±2^{o}C throughout larval development. Larvicidal and insect growth regulatory (IGR) activities were conducted in accordance to the World Health Organization method (WHO, 1996). Batches of twenty freshly moulted late 3rd and early 4th instar larvae of *An. gambiae* s.s. were transferred by means of dropper to glass beakers containing 100 ml of tap water. Appropriate volume of stock solution where the crude or pure compounds were dissolved in 5% dimethylsuphoxide (DMSO) was added to 100 ml water in the glass beakers to obtain 25, 50, 100, 250 and 500 ppm for the crude extracts and 1, 5, 10 ppm dose levels for the pure compounds. Three replicates were set up for each concentration and two negative controls (treated with DMSO-distilled water) were set up simultaneously. Larval mortality, abnormal behavior and/or morphological deformations were recorded at 24-h intervals until the death of the last larva or emergence to adult. The bioassay room was kept at a temperature of 30^{o}C, an average humidity of 78 % and a photo period of 12 hours of light and 12 hours of darkness.

Brine shrimp test (BST)

In vitro brine shrimp lethality test of the extracts was used to detect larval toxicity. Brine shrimp eggs were purchased from Aquaculture Innovations (Grahamstown 6140, South Africa) and sea salt was prepared locally by evaporating water collected from the Indian Ocean, along the Dar es Salaam Coast. Brine shrimp eggs were placed in sea water (3.8 g of sea salt in one liter of distilled water) and incubated infront of an electric lamp. Eggs were hatched within 48 h, providing a large number of brine shrimp larvae (nauplii). Stock solutions (40 mg/ml) of each extract were prepared by dissolving them in DMSO. Different concentration levels were prepared by serial dilution and 10 nauplii were added into 10 ml vials. The volume was then adjusted

to 5ml with artificial sea water (3.8% w/v sea salt in distilled water). Each concentration was tested in triplicates with two negative controls running simultaneously containing 10 nauplii, sea water and 5% DMSO only for comparison. The vials were incubated for 24 h under the same conditions used for rearing the brine shrimp larvae.

Isolation of compounds from the acetone stem barks and leaves of *V. schiliebenii*

Sixteen grams (16 g) of the acetone stem bark extract was subjected to column chromatography on silica gel with Dichloromethane: Acetone gradient (100:0 - 0:100). The 50% acetone eluent gave a mixture of two compounds (47 mg), which were separated by repeated column chromatography followed by preparative thin layer chromatography (PTLC). The fraction yielded two compounds 20-hydroxyecdysone (**1**) (16 mg) isolated as a crystalline solid melting point 234-236 ^{o}C (lit. 230-233 ^{o}C, (Kavel *et al*., 1998) which was soluble in methanol and stigmasterol (**2**) (25 mg of white crystals with a melting point of 165-7^{o}C (lit. 163-6^{o}C (Greca *et al*., 1990). The acetone leaf extract (10 g) was subjected to column chromatography on silica gel (eluting with 100% dichloromethane and gradually increasing acetone to 100%). The 40% acetone eluent gave a mixture of two compounds, which were subjected to repeated column chromatography using DCM: MeOH to yield γ sitosterol (**3**) (6 mg with a melting point of 142-144^{o}C) which was then re-crystallized using the same solvent into a white star shaped crystal soluble in DCM. The other compound was too little to be analyzed.

Statistical analysis

The average larval mortality (± standard error) resulting from each dose of each extract/compound was calculated and the data was subjected to probit analysis for calculating the lethal concentrations of the crude plant extracts/pure compounds at LC_{50} at 95% confidence limit of upper and lower levels. The values were calculated using GenStat Discovery Edition 4. Results with $p<0.05$ were considered to be statistically significant.

RESULTS

Acetone, methanol and aqueous extracts of the leaves, stem bark and root bark of *V. schiliebenii* were tested for lethality against the late 3rd and early 4th instar larvae of *An. gambiae*. Acetone leaves and stem bark extracts showed significant activities ($p<0.05$), with LC_{50} values of 14.6 and 17.4 ppm respectively at 24 h with the former extract causing 100% mortality at all doses tested while the latter achieved 90% mortality at the lowest dose at 72 h (Table 1).

The two extracts exhibited low toxicity to the brine shrimp larvae with LC_{50} values of 180.9 and 154.4 ppm respectively.

Other active extracts included methanol leaf, aqueous stem bark and acetone root bark extracts with LC_{50} values of 136.3, 182.6 and 252.1 ppm respectively while methanol stem bark exhibited relatively weak activity (LC_{50} = 522.6 ppm) at 24 h. The control cohorts showed a maximum percentage mortality of 8.3±1.7 at 72 h. The larvae developed into pupae and then adults within 48–72 h.

Table. 1: Laboratory activity of crude extracts of *Vitex schiliebenii* against 3rd/4th Instar larvae of *Anopheles gambiae* s.s Giles after 24h, 48h and 72h exposure

Extract code	Timee (Hr)	Cumulative mean mortality (% ± SE)/Concentration (ppm)					Lethal concentration values (ppm)	
		500 ppm	250 ppm	100 ppm	50 ppm	25 ppm	LC_{50}	95% CL
VSRB-221	24	96.7±1.6	43.3±3.3	3.3±1.6	-	-	252.1	225.0-281.8
	48	100.0±0.0	96.6±3.3	15.0±0.0	0.3±0.0	0.3±03	136.0	120.1-154.0
	72	100.0±0.0	100.0±0.0	100.0±0.0	83.3±4.4	4.7±1.7	38.5	33.9-43.6
VSRB-222	24	56.7±1.6	15.0±2.9	5.0±2.0	-	-	444.0	392.0-505.0
	48	71.7±3.3^{ab}	38.3±1.6	18.3±1.6	-	1.7±0.0	295.9	260.8-335.7
	72	100.0±4.4^{a}	100.0±0.0	68.3±4.4	10.0±1.7	5.0±2.9	80.6	71.6-90.9
VSRB-223	24	-	-	-	-	-	3652.6	50.2-284895
	48	-	-	-	-	-	-	-
	72	10.00±0.0^{a}	28±1.6	-	-	-	307.5	256.9-370.7
VSSB-221	24	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	78.3±6.0	17.4	14.6-20.3
	48	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	81.7±4.4	15.0	12.3-18.1
	72	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	90.0±5.8	11.9	9.1-15.1
VSSB-222	24	43.3±1.7	6.7±1.7	-	-	-	522.6	462.8-594.9
	48	100.0±0.0	98.3±1.7	50.0±1.7	6.7±3.3	10.0±2.7	103	91.7-117.1
	72	100.0±0.0	100.0±0.0	90.0±5.8	65.0±5.7	30.0±0.0	39.9	35.2-45.2
VSSB-223	24	100.0±0.0	57±3.3	30±3.3	-	-	182.6	161.8-206
	48	100.0±0.0	88±1.7	86±2.7	-	-	84	67.9-104
	72	100.0±0.0	100±0.0	100±0.0	50.8±1.7	-	49.1	41.2-58.2
VSL-221	24	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	96.7±1.7	14.6	11.9-17.6
	48	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	13.1	10.4-16.2
	72	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	10.6	7.9-13.9
VSL-222	24	100.0±0.0	100.0±0.0	31.6±3.3	10.0±2.9	6.7±1.7	136.3	120.8-154.8
	48	100.0±0.0	100.0±0.0	60.0±5.8	48.3±1.7	10.0±0.0	63.1	56.0-71.1
	72	100.0±0.0	100.0±0.0	95.0±6.0	63.3±2.9	28.3±1.7	40.1	17.0-28.2
VSL-223	24	3.0±3.3	-	-	-	-	1672.9	1222.1-2389
	48	15.0±3.3	-	-	-	-	-	-
	72	25.0±1.7	-	-	-	-	-	-
Control	24	2.3±3.3	-	-	-	-	-	-
	48	8.3±1.7	-	-	-	-	-	-
	72	8.3±1.7	-	-	-	-	-	-

Chromatographic separation of the acetone stem bark extract resulted in the isolation and identification of two phytoecdysteroids [20-hyrdoxecdysteroid (**1**) and stigmasterol (**2**)] and γ-sitosterol (**3**) was isolated from the acetone leaves. The identification was done by physical and spectroscopic techniques and also comparison with literature data (Table 2). These compounds were evaluated for larvicidal activities where compounds **1** and **2** were found to be responsible for the activity of the stem bark and compound **3** was responsible for the activity of the leaves. Among the three compounds, 20-hyrdoxecdysteroid (**1**) showed the highest activity with LC_{50} value of less than 1 ppm followed by stigmasterol (**2**) with LC_{50} = 8.145 ppm and then γ-sitosterol (**3**) with LC_{50} = 8.609 ppm at 72 h.

Table. 2: ^{13}CNMR spectral data for compounds 1, 2, and 3.

Compounds	**1**		**2**		**3**	
Position	$\delta_{(obs.)}$	$\delta_{(lit.)}$	$\delta_{(obs.)}$	$\delta_{(lit)}$	$\delta_{(obs.)}$	$\delta_{(lit.)}$
1	36.0	37.3	37.3	37.2	37.3	37.0
2	67.3	68.6	31.9	31.8	29.4	29.5
3	67.1	68.5	71.8	71.5	71.8	71.8
4	31.5	32.7	42.3	42.2	42.3	42.3
5	50.4	51.7	140.8	140.7	140.8	140.8
6	205.1	206.6	121.7	121.6	121.7	121.7
7	120.7	122.1	33.9	33.6	31.7	31.9
8	166.5	168.1	29.7	29.6	29.2	29.2
9	33.7	35.0	50.1	50.1	50.2	50.2
10	37.9	39.3	36.5	36.4	36.5	36.5
11	20.1	21.5	21.1	21.1	21.1	21.1
12	30.4	32.4	39.7	39.7	26.1	26.1
13	47.4	48.6	42.2	42.2	45.9	45.9
14	83.4	84.2	56.8	56.1	56.8	56.7
15	31.1	31.7	24.3	24.1	24.3	24.1
16	20.1	21.5	28.4	28.3	39.8	39.8
17	49.1	50.5	56.7	56.0	56.1	56.1
18	16.7	18.1	12.0	12.1	11.9	12.2
19	23.0	24.4	19.4	19.4	18.8	18.8
20	76.5	78.0	40.5	40.3	34.0	34.0
21	19.7	21.1	19.8	20.5	19.1	19.1
22	77.0	78.4	138.3	138.5	37.3	37.3
23	25.9	27.3	129.3	129.4	26.2	26.6
24	41.0	42.3	51.2	51.2	51.6	50.1
25	69.9	71.4	31.7	31.9	28.2	28.3
26	28.3	29.1	21.1	21.2	19.4	19.4
27	27.6	29.7	19.0	19.8	19.8	19.8
28			26.1	25.4	23.3	23.3
29			11.9	11.9	11.8	12.0

*- signals may be interchanged
Compounds 1 and 2: (500 MHz, MeOD); Compound 3: (300 MHz, $CDCl_3$);

DISCUSSION

Currently, numerous products of botanical origin have received considerable renewed attention as potentially bioactive agents used in mosquito control (Marimuthu et al., 2012; Kovendan et al., 2012; Paneerselvan & Murugan, 2013). More than 2000 plant species have been known to produce chemical factors and metabolites of value in pest control programmes. Members of the plant families-Solanaceae, Asteraceae, Cladophoraceae, Labiatae, Miliaceae, Oocystaceae, Verbenaceae and Rutaceae have various types of larval, adulticidal or repellent activities against different species of mosquitoes (Anupam et al., 2012). In addition, the genus *Vitex* (Verbenaceae) has been reported to have a plethora of ethnopharmacological uses (Padmalatha et al., 2009). This suggests that natural products from plant species in the genus have low human toxicity but can still be used as potential agents against mosquitoes. This is confirmed by the observation made in the current study where the two active acetone extracts were found to exhibit good larvicidal activity but very low toxicity to the brine shrimp larvae. It is also worth noting that although acetone and methanol extracts produced encouraging results, they are difficult to produce and use by resource-poor people in rural Africa particularly. The aqueous extract is more applicable to rural situations where malaria causes the greatest burden. The stem bark aqueous extracts displayed good larvicidal potential with LC_{50} values decreasing from 182.6 ppm to 49.1 ppm at 24 h and 72 h (Table 1).

Previously, Vasanth Raj et al (2009) had evaluated aqueous extract of *V. negundo* against mosquito larvae of *Cx. quinquefasciatu, An. stephensis* and *Ae. aegypti* and the extract was found to be effective with LC_{50} values of 167.88 ppm, 167.88 ppm and 231.17 ppm respectively. Hence in the absence of *V. schiliebenii*, *V. negundo* can be used. Other than phytoextracts being used as larvicides, several groups of phytochemicals have been reported for their insecticidal activity and among them are the phytocdysteroids (Zolotar et al 2001). In the present study, the high larvicidal activity noted in compound **1** is in agreement with those of other phytoecdysteroids having a long side chain with hydroxyls (Zolotar et al 2001, Radi et al., 2011). According to literature, the steroidal side chain with hydroxyls is necessary in producing high insect hormonal activity in ecdysteroids (Zolotar et al 2001).

CONCLUSION

The results of the present study indicate a potent effect of phytoextracts from *V. schiliebenii* and the isolated phytoecdysteroids on *An. gambiae*. The plant therefore holds great promise as a locally available larvicidal plant. Further work in this area should focus on the exploration of the efficacy of the isolated phytoecdysteroids, under field conditions, with the aim of formulating a botanical larvicide.

ACKNOWLEDGEMENTS

The authors are grateful to the International Centre of Insect Physiology and Ecology (ICIPE-Kenya) for providing the necessary bioassay requirements. Muhimbili University of Health and Allied Sciences is also acknowledged for providing the necessary chromatographic equipment for fractionation and purification of the crude plant materials. The authors also acknowledge DAAD-NAPRECA for funding the work reported in this paper.

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How to cite this article:

Mokua G. Nyamoita, Innocent Ester, Mbwambo H. Zakaria, Lwande Wilber, Bwire J. Ochola, Hassanali Ahmed., Larvicidal and Brine Shrimp Activities of *Vitex Schiliebenii* Extracts and Isolated Phytoecdysteroids on *Anopheles gambiae* Giles S.S Larvae. J App Pharm Sci, 2013; 3 (05): 091-095.

PAPER 4

Original Article | *Spatula DD. 2013; 3(3):113-120*

Chemical composition and evaluation of mosquito larvicidal activity of *Vitex payos* extracts against *Anopheles gambiae* Giles S.S larvae

Anopheles gambiae larvalarına karşı *Vitex payos* ekstrelerinin kimyasal bileşimi ve larvasidal etkilerinin değerlendirmesi

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SUMMARY

AIM: The aim of this study was to evaluate the acetone and methanol root bark extracts of *Vitex payos* and different fractions thereof, following acetone extraction and column chromatographic separation, for their immediate toxicity and long term effects on *Anopheles gambiae* Giles sensu stricto larvae under simulated semi-field conditions.

METHODS: In the present study, acetone and methanol extracts and acetone chromatographic fractions were investigated against late third/early fourth-instar larvae of laboratory reared *A. gambiae*. The tests were conducted according to the method of the World Health Organization (WHO) on guidelines for laboratory and field testing of mosquito larvicides. In addition, chemical compositions of the extracts were carried out using gas chromatography-mass spectrometry (GC-MS). The study was conducted in 2012.

RESULTS: Acetone chromatographic fractions showed significant larvicidal activity against *A. gambiae* larvae with LC_{50} values ranging between 0.7 - 0.9 ppm. Dose-response relationships were established with the highest dose of 500 ppm causing over 85% mortality in all the test extracts. Long-term (11-14 days) post-exposure observations at lower doses showed that the extracts achieved over 90% adult growth inhibition with morphogenetic variations and behavioral changes. In acetone and methanol extracts, carbonyl compounds were determined as the main constituents with percentage compositions of 34.26% and 23.53% respectively. The other class of compounds determined was the phytoecdysteroids where gamma sitosterol (6.43%) was the major sterol in acetone extract while stigmasterol (2.83%) was major in methanol extract.

CONCLUSION: Extracts and chromatographic fractions from *V. payos* possess larvicidal and/or insect growth regulatory (IGR) principles which can be used in aquatic ecosystems by the local communities where most of the people cannot afford synthetic larvicides.

Key words: *Anopheles gambiae, Vitex payos,* larvicidal activity, phytoecdysteroids, saponins, growth inhibition.

ÖZET

AMAÇ: Bu çalışmanın amacı, simüle edilmiş yarı-alan koşulları altında, *Anopheles gambiae* Giles sensu stricto larvaları üzerindeki uzun dönem etkileri ve erken toksisiteleri açısından aseton estraksiyonu ve kolon kromatografisi ayrıştırmasını takiben *Vitex payos* kök kabuklarının aseton ve methanol ekstrelerini değerlendirmekti.

YÖNTEM: Bu çalışmada, aseton ve metanol ekstreleri ile aseton kromatografik fraksiyonları, laboratuvarda yetiştirilen *An. gambiae* geç üçüncü ve erken dördüncü ever larvalarına karşı araştırıldı. Testler, Dünya Sağlık Organizasyonu (WHO) metodlarına göre sivrisinek larvisidleri alan testi ve laboratuvarları rehberi eşliğinde yapıldı. Ayrıca, ekstrelerin kimyasal bileşimi, gaz kromatografisi-kütle spektrometrisi (GC-MS) kullanılarak gerçekleştirildi. Çalışma 2012 yılında yapıldı.

BULGULAR: Aseton kromatografik fraksiyonları, 0,7-0,9 ppm değerleri arasında değişen LC_{50} değerleri ile, *An. gambiae* larvasına karşı belirgin larvisidal etki gösterdi. Doz bağımlı ilişkiler, tüm test ekstrelerinde % 85'in üzerinde mortaliteye neden olan 500 ppm'lik en yüksek doz ile belirlendi. Düşük dozdaki uzun dönem (11-14 gün) maruziyet sonrası gözlemler, morfogenetik değişimler ve davranışsal değişikliklerle birlikte, ekstrelerin % 90'ın üzerinde erişkin büyüme inhibisyonuna ulaştığını göstermiştir. Aseton ve metanol ekstrelerinde, sırasıyla %34,26 ve %23,53 yüzde bileşimler temelinde ana karbonil bileşikleri olarak karbonil bileşikleri saptandı. Saptanan diğer sınıf bileşikler aseton ekstresinde ana sterol gama sitosterol (%6,43) iken metanol ekstresinde stigmasterolün (%2,83) olduğu fitoekdisteroidlerdi.

SONUÇ: *V. payos* ekstre ve kromatografik fraksiyonları, sentetik larvisidlere ulaşamayan yerel topluluklarca sulu ekosistemlerde kullanılabilecek larvisidal ve/veya insekt büyüme düzenleyici (IGR) özelliklere sahiptir.

Anahtar kelimeler: *Anopheles gambiae, Vitex payos,* larvisidal etki, fitoektisteroidler, saponinler, büyüme inhibisyonu.

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Received May 15, 2013; accepted August 06, 2013
DOI 10.5455/spatula.20130806054707
Published online in ScopeMed (www.scopemed.org).
Spatula DD. 2013; 3(3):113-120.

INTRODUCTION

Mosquitoes of the genus *Anopheles* are the exclusive vectors of malaria in humans [1]. Most of the malaria cases in tropical Africa are transmitted by three malaria vectors: *Anopheles gambiae* sensu stricto, *An. funestus* and *An. arabiensis* [1]. *An. gambiae* s.s. is the most efficient vector due to its high anthropophilic and endophilic character [2]. The use of synthetic insecticides in public health programs were initially considered likely to control mosquito populations but this has currently been reported to have failed and often confronted with challenges like disruption of natural biological control systems, outbreaks and development of resistance in many mosquito species, causing toxic hazards to humans and other non-target organisms and are not easily biodegradable [3]. Furthermore, conventional insecticides have been found to be very expensive for the majority of the targeted population thus calling for concerted efforts in exploiting the vector control potential of natural origins [3, 4]. The increased use of these insecticides may enter into the food chain and thereafter the liver and the kidney, causing irreversible damage; but, also may result in mutation of genes whose changes may become prominent after a few generations [5].

In an attempt to resolve these problems, the search for new strategies for selective mosquito control particularly of plant origin need to be developed. Accordingly, a considerable number of studies have emphasized the development of herbal substances for controlling mosquitoes [6-10]. Although results vary, botanical phytochemicals with mosquitocidal potential may be a possible alternative to, or used along with other insecticides as they are eco-friendly, target-specific, do not develop resistance, are highly accepted and are suitable for rural areas [10].

Studies on the natural plant products for larvicidal activity suggest potential sources of compounds for mosquito control [11-14]. In view of the fact that mosquitoes develop resistance to synthetic larvicides [15] and even bio-pesticides such as B. sphaericus [16], the application of easily degradable botanicals for larval control has been recommended [17].

Plants belonging to the families *Asteraceae*, *Verbenaceae*, *Meliaceae*, and *Rutaceae* have been reported as potential sources of secondary metabolites for larval control [9, 18-20]. *Vitex* species belonging to the family *Verbenaceae* have been reported to exhibit larvicidal activities against a number of mosquito species [21-25]. Plants of this genus occur in both tropical and temperate regions of the world [26].

Vitex payos, also known as black plum in English, is a tree with round leather-like leaves and fruits that resemble black olives. In Kenya, it grows in semi-arid parts of Eastern, Coastal and Central Kenya for example Kitui, Embu, Machakos, Kilifi, Kwale, Tharaka, Mbeere and Mwingi areas [27]. According to the local people along the Kenyan coast, pounded bark is administered to treat threadworm and skin problems. Leaf sap is used as eye drop to treat conjunctivitis and other eye problems. Leaves are boiled and decoction drunk by patients who have lost appetite. A paste of pounded leaves and bark are applied to wounds and burns. A root decoction is administered orally to treat stomach problems or gastro-intestinal disorders. Powdered bark added to water is taken to treat stomach problems and kidney ailments. The bark is also used to treat leprosy, liver diseases and to control bleeding after childbirth. Dried and fresh fruits are eaten to control diarrhoea. The twigs are used as chewing sticks for teeth cleaning.

Its acetone and methanol extracts have been reported to possess larvicidal activity against *An. gambiae* under laboratory conditions [28]. Two phytoecdysteroids (20-hydroxyecdysone-20, 22-monacetonide and 20-hydroxyecdysone) isolated from acetone extract of *V. payos* root bark have been reported to cause 100% mortality to *An. gambiae* larvae at 10 ppm [29]. Based on this literature report, the plant was selected for investigation against *An. gambiae* under semi-field conditions. This study focuses on its chemical composition and the larvicidal activities of its extracts and chromatographic fractions against *An. gambiae*.

MATERIAL AND METHODS

Plant materials

The root bark of *V. payos* was collected in 2009 from South Coast of Kenya at Kaya Muhaka in the Kaya forest, Kwale County. The plant species was authenticated at the field by a botanist from the National Museum of Kenya (NMK) and a voucher specimen Ref. Nos. GMN/21) was preserved at the NMK Herbarium.

Mosquito larvae

Larvae of *An. gambiae* s.s. Giles used in bioassays were obtained from a colony maintained at the Insect Mass Rearing Unit of International Centre of Insect Physiology and Ecology (ICIPE). This

strain of mosquitoes originated from ICIPE's Thomas Odhiambo Campus (Mbita Point) near Lake Victoria in 2003. Larvae were allowed to emerge from eggs in plastic containers filled with distilled water and were transferred to larger pans (37 × 31 × 6) at densities of 200-300 at 2nd instar stage. They were fed on Tetramin® fish food (Terta GmbH, Germany) and the water temperature was maintained at 28±2°C throughout larval development.

Extraction and fractionation

The root bark of the plant was air-dried at room temperature under the shade for three weeks and ground into powder in an electric miller. The powdered material (30 g) was extracted three times using acetone (1.5 litres) for 24h with occasional stirring at room temperature. The extract was filtered and concentrated to dryness using a rotary evaporator at 40°C. The extraction process was repeated three times and the combined extracts (10 g) were stored at 4°C. This procedure was repeated sequentially with the same material using methanol in the same proportions and for the same periods of time.

The acetone extract (8.0g) was subjected to column chromatography on silica gel (105g) eluting with 100% dichloromethane and gradually increasing acetone to 100% then methanol to 30% (Fig 1). Separation was monitored by thin layer chromatography (TLC). The TLC plates were developed with acetone-dichloromethane (2:3). The plates were sprayed with p-anisaldehyde reagent (solution of anisaldehyde, 0.6% ethanol and 5% sulphuric acid) (reagents obtained from Sigma Aldrich, Germany) and kept in the oven at 110°C until the spots appeared. The fractionation was done as illustrated in (Fig. 1).

Semi-field larvicidal assay

Bioassays were conducted in accordance to World Health Organization procedure [30] in 1000 ml chlorine-free water in white plastic containers of internal diameter 21.5 cm and 13 cm. These containers were placed in holes (10 cm deep) in a screen house arranged in three rows and 13 columns with a distance of 50 cm in between. The containers were filled with 400 ml of water and left for 24 h for conditioning and then batches of 50 freshly molted late 3rd and early 4th instars larvae of *An. gambiae* were added together with food and left for 2 h to allow the larvae to get acclimatized with the environment. Five milligrams of each extract was dissolved in 1 ml distilled water containing 5 % dimethyl sulphoxide (DMSO), which gave a homogenous solution. Stock solution of each plant extract was successively diluted to give concentrations of 12.5, 25, 50, 100, 250, and 500 ppm in 500 ml. The effect of each test solution together with a negative control (treated with DMSO-distilled water) was replicated three times. Sampling was carried out using a 350 ml dipper at different sides of the containers. Three dips were taken for each test sequenced in such a way that triplicates of similar concentrations were sampled consecutively with the same dipper. One dip was taken from each container at a time, returning to it after completion of the cycle for that particular treatment, until all the four samples were taken. Mortality was recorded after every 24 hours until the death of the last larva or emergence of adult. All the treated and control containers containing pupae were kept separately in a netted cage to prevent successfully emerged adults from escaping into the environment. The percent adult emergence inhibition (% EI) was based on the number of moribund and dead larvae or pupae that did not develop successfully into viable adults and pupal-adult intermediates. The average screen house temperature was 32 ± 4°C while the humidity was 65 ± 10 % RH. The larvae were fed on Tetramin® fish food (Terta GmbH, Germany) and the adults on 10 % glucose solution.

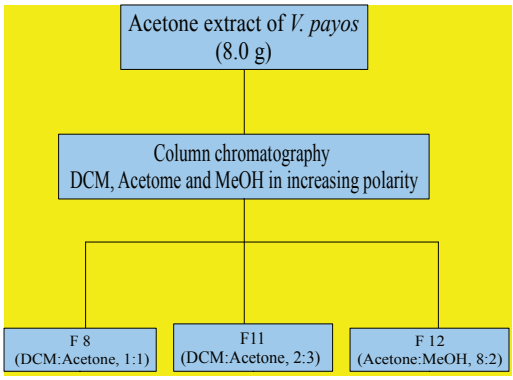

Figure 1. Bioassay guided fractionation of the acetone root bark extract of V. payos.

Statistical analysis

Data was collected and analyzed in 2012. To determine the adult emergence inhibition activity, the average number of larvae or pupae collected per dip for each replicate of each treatment and the control were calculated after 24 h. The percentage inhibition of adult emergence (% IE) was estimated for each treatment according to the formula:

IE % = 100 - (T × 100/C) [30]

Where T = Percentage emerging in treated

C = Percentage emerging in control

Bioassay tests showing more than 10% control mortality was discarded and repeated. However, for those where control mortality ranged between 5-10%, the corrected mortality was calculated using Abbott's formula [31] as follows:

Corrected % mortality = (T – C) / (100 – C) × 100

Where T = % mortality in treated

C = % mortality in control

The average larval mortality (+ standard error) resulting from each dose of each chromatographic fraction was calculated and the data was subjected to probit analysis for calculating the lethal concentrations of the semi-pure fractions at LC_{50} at 95% confidence limit of upper and lower levels. The values were calculated using GenStat Discovery Edition 4. Results with $p<0.05$ were considered to be statistically significant.

GC-MS analysis

The extracts were analyzed by GC-MS on a 7890A stand-alone gas chromatograph (Agilent Technologies, Inc., Beijing, China) and a 5975 C mass selective detector (Agilent Technologies, Inc., Santa Clara, CA, USA) by using the following conditions: Inlet temperature of 270°C, transfer line temperature of 280°C, and column oven temperature programmed ranging from 35 to 280°C with the initial temperature maintained for 5 min then 10 °C/min to 280°C for 10.5 min and then 29.9 min 50 °C/min to 285 °C. The GC was fitted with a HP-5 MS low bleed capillary column (30 m × 0.25 mm i.d., 0.25-µm) (Restek, Bellefonte, PA, USA). Helium at a flow rate of 1.25 ml/min served as carrier gas. The Agilent 5973 mass selective detector maintained an ion source temperature of 250°C and a quadruple temperature of 180°C. MS ion source temperature was set at 230°C. Electron impact (EI) mass spectra were obtained at acceleration energy of 70 eV. A 1.0 µL aliquot of extract was automatically injected in the split/splitless mode using an auto sampler 7683 (Agilent Technologies, Inc., Beijing, China). Fragment ions were analyzed over 40-550 m/z mass range in the full scan.

RESULTS

Larval mortality after 72 h

The acetone and methanol extracts of *V. payos* root bark and the acetone column chromatography fractions thereof exhibited larvicidal activity against larvae of *An. gambiae* within 72 h. The fractions tested caused larval mortality ranging between 85-100 % (not shown) at the dose of 25 ppm and above after 72 h of exposure and the LD_{50} values ranged between 7.0-19.3 ppm after 24 h (Table 1). These fractions were all positive for steroids as confirmed by comparing their TLC stains with those of phytoecdysteroids previously characterized from the same plant [29]. Saponins also tested positive for these fractions.

Table 1. Activity of steroid and saponin rich fractions of *V. payos* root bark against 3rd/4th instar larvae of *An. gambiae* s.s.Giles

Fraction	Time (h)	LC_{50} (ppm)	95 % CL
F_8	24	7.0	2.2-14.6
	48	1.8	0.25-5.5
	72	0.8	0.0-4.0
F_{11}	24	13.4	5.2-25.1
	48	5.6	1.2-13.2
	72	0.7	0-4.0
F_{12}	24	19.3	8.3-34.2
	48	7.6	1.9-16.5
	72	0.9	0.0-6.0

Long term effects of lower doses for the crude extracts

The acetone and methanol extracts caused 93% corrected adult growth inhibition each; 87 and 90% larval mortality and 10% each pupal/adult respectively (Fig.1). Consequently the methanol extract produced no live or dead pupae. Prolonged developmental time was observed in the treated cohorts as opposed to control experiments. The total larval period lasted 9-10 days (control 6-8 days) and pupal period lasted 2-3 days (control 1) resulting to a total developmental period (larval + pupal development) of 11-13 days (control 7-9 days) (data not shown). The results also revealed a decrease in adult longevity with the resulting adults dying after 48 h of emergence. It was noticed that treated adults were weak, could not fly high and rested for a longer period on the surface of the water unlike the control adult mosquitoes.

Morphogenetic variations and behavioral changes were also observed. The dead larvae had abnormally large heads with a 'question mark-like' structure (larval-pupal intermediates). The emerged pupae had their thorax and abdomen tucked together forming a straight structure. The larvae that pupated were reared in the laboratory and monitored through their life cycle until the emergence of adults. The

morphological appearance of the emerged adults was similar to those from the control experiments though they were weak. The males and females were allowed to mate and then fed on human blood. The females produced very little batches of eggs suggesting that the extracts could have interfered with the reproductive system of the mosquitoes.

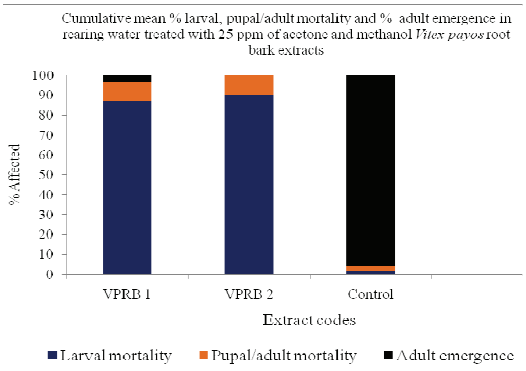

Figure 2. Percent affected at different stages.**VPRB 1**: Acetone extract; **VPRB 2**: Methanol extract

GC/MS analysis

Constituents of the extracts were identified based on NIST Library/Wiley Library and the literature. The identified compounds and relative quantitative distribution are shown in Table 2. In the acetone extract, a total of 74 compounds were detected out of which 15 had more than 1 % peak area, representing 78.97% of the extract (Table 2). Generally, the extract showed a variety of compounds. Carbonyl compounds (34.26%) were determined as the first major constituent in the extract followed by hydrocarbons (16.93%) and then phytoecdysteroids (11.01%) whereby gamma sitosterol (6.43%) was identified as the major sterol. The extract also contained fatty acids (9.60%) 5-hydroxy-2-methyl-4H-Pyran-4 one (1.62%), a member of the pyrone family and the volatile 2-ethylacridine (5.52%).

In the methanol extract, a total of 123 compounds were detected out of which 18 compounds had ≥ 1 % peak area representing 60.59% of the extract. Like in the acetone extract, carbonyl compounds (23.53%) were determined as the first major constituent in the extract though less by 10.73% compared to the quantity in the acetone extract. Simple aromatic volatile (8.08%) was the second group followed by hydrocarbons (7.87%) then phytoecdysteroids (6.38%) whereby stigmasterol (2.83%) was identified as the major sterol. Other classes of compounds detected include, methyl esters (3.11%) together with the ester silver butanoate (3.44%) and fatty acids (3.76%). In addition to the major mentioned functional groups, 1,5-dihydro-1-methyl-2-(methylthio)-5,5-diphenyl-4H-Imidazole-4-thione (2.14%) and 2,6-Bis(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo(3.3.0)octane (1.47%) compounds were also identified.

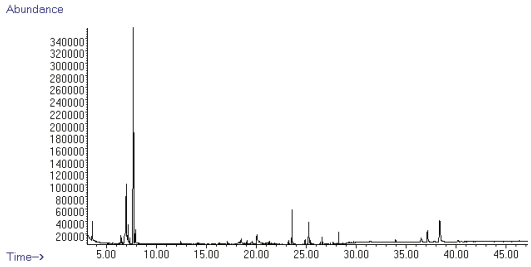

Figure 3. GC-MS profile of the *V. payos* acetone root extract

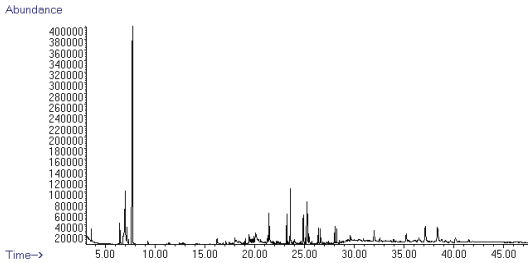

Figure 4. GC-MS profile of the *V. payos* methanol root extract

DISCUSSION

Results of the present study with the extracts and acetone fractions showed good larvicidal and/or insect growth regulatory (IGR) activity against *An. gambiae*. However, the crude extracts were less active than the fractions suggesting that the activity of the active compounds in the crude mixtures were masked by the other less active or completely inactive minor constituents. This observation should therefore be considered when using a botanical larvicide and/or IGR in crude form or as semi-purified fractions. The observation could be supported by some evidence that semi-purified natural mixtures [20] or blends of pure compounds [29] do act synergistically as larvicides. Considering the challenges involved in isolating pure compounds including the isolation of small amounts of pure compounds which are not sufficient to conduct bioassay tests, semi-purified fractions can be used.

Table 2. Major peak report total Ion chromatogram of of *V. payos* root bark extracts by GC/MS

Peak No.	Retention Time	Compound Name (Number)	% in Acetone extract	% in Methanol extract
1.	3.58	1-chloro-2-propanone	1.61	-
2.	6.45	3-hexen-2-one	1.30	-
3.	6.45	4-methyl-3-penten-2-one	-	1.60
4	7.01	2,4-dimethyl-heptane	12.46	-
5.	7.01	Octane	-	6.32
6.	7.19	2,6-dimethyl-heptane	2.92	Dodecane (1.55)
7.	7.71	4-hydroxy-4-methyl-2-pentanone	31.35	21.93
8.	7.88	5-Ethyl-3-methylhept-1-en-4-ol	1.65	-
9.	18.00	Z- Isoeugenol	-	1.60
10.	18.47	5-hydroxy-2-methyl-4H-pyran-4-one	1.62	-
11.	19.44	3-Hydroxy-4-methoxybenzoic acid	-	1.07
12.	19.59	Eicosene	1.55	-
13.	20.06	Butanoic acid	3.34	-
14.	20.11	Silver butanoate	-	3.44
15.	21.43	Beta-thujaplicinol	-	3.18
16.	23.20	Methyl hexadecanoate	-	1.10
17.	23.56	Hexadecanoic acid	3.50	-
18.	24.83	Methyl linoleate	-	2.01
19.	25.24	cis-Vaccenic acid	2.76	3.76
20.	29.99	2-Ethylacridine	5.52	1.16
21.	30.46	1,5-dihydro-1-methyl-2-(methylthio)-5,5-diphenyl-4H-imidazole-4-thione	-	1.88
21.	31.98	2,4-dihydroxy-benzaldehyde	-	2.14
22.	35.20	2,6-Bis(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo(3.3.0)octane	-	1.47
23.	36.48	Campesterol	1.53	1.11
24.	37.13	Stigmasterol	3.05	2.83
25.	38.36	Gamma-Sitosterol	6.43	2.44

- = Not detected beyond 1%,

In other studies, crude extracts from the plant species in the genus *Vitex* were reported to exhibit larvicidal activity and/or IGR activities against a number of mosquito species [6, 22, 32] under laboratory and/or simulated semi-field conditions. The findings of this study corroborate with these earlier researchers. However, the study needs to evaluate the larvicidal activity of these extracts against other mosquito species under different ecological zones. The observed biological activity may be due to the presence of the various classes of compounds present in the extracts as revealed in the GC/MS analysis, which could synergistically, antagonistically or independently contribute to the activity of the crude extracts and the fractions.

Of particular interest were the phytoecdysteroids and the fatty acids. These classes of compounds had been previously reported to be present in some *Vitex* species and were reported to cause growth and developmental disruptions in a number of insect species [29, 33]. From the GC/MS analysis, phytoecdysteroids and fatty acids constituted a significant percentage in the extract. The observed morphogenetic variations and behavioral changes could then be attributed to the presence of these groups of compounds. A similar study [29] isolated two phytoecdysteroids (20-hydroxyecdysone-20, 22-monacetonide and 20-hydroxyecdysone) from the plant and these compounds exhibited larvicidal activity against *An. gambiae* in the laboratory conditions.

CONCLUSION

Extracts and chromatographic fractions from *V. payos* possess larvicidal and/or IGR principles which can be used in the local communities where most of the people cannot afford synthetic larvicides. These phytoextracts could be utilized in the formulation of

botanical larvicides either alone or in combinations. However, there is need for further investigation on the activity of these extracts in the natural environment at different ecological zones and on a wide variety of pests. The mode of action of the active constituents also needs to be evaluated.

ACKNOWLEDGEMENTS

The authors are grateful to Natural Product Research Network for Eastern and Central Africa (NAPRECA-DAAD) for financial assistance and the International Centre for Insect Physiology and Ecology (ICIPE) for providing the insects and necessary facilities to carry out this research work successfully. We also thank Mr. Simon Mathenge of the National Museum of Kenya for identifying the plants under the study. Acknowledgement is also extended to Muhimbili University of Health and Allied Sciences (MUHAS), Institute of Traditional Medicine (ITM) where fractionation of the crude extract was carried out.

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This thesis focuses on the control of *Anopheles gambiae*, a malaria vector at the immature stages of the life cycle using botanical larvicides. Acetone, methanol and aqueous extracts of different parts of three *Vitex* species (*Vitex payos*, *Vitex schiliebenii* and *Vitex trifolia*) were evaluated for their potential to control *An. gambiae* Giles s.s. larvae (Diptera: Culicidae) under laboratory and simulated semi-field conditions. The extracts and isolated compounds gave different levels and rates of mortality of the larvae. In all the experiments, mortality in treated tests was significantly higher than in control cohorts. Most of the larvae failed to transform to normal pupae but gave larvalpupal intermediates at between 4 and 14 days of exposure. Some larvae pupated normally but the adults that emerged appeared to be weak and died within 48 h. The thesis suggests that there are interesting growth-disrupting constituents in the selected plants, with possible application in the practical control of mosquito larvae in aquatic ecosystems.

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KENYA LITERATURE BUREAU
PUBLISHERS AND PRINTERS
P.O.Box 30022-00100, Nairobi
Tel:020 6005595, 6008305/6
Fax: 020 6001474

ISBN 978-9966-065-19-3
9 789966 065193

Laboratory and semi-field evaluation of some *Vitex* species for larvicidal activity against *Anopheles gambiae* larvae • Mokua Gladys Nyamoita