IDENTIFICATION OF KAIROMONES MEDIATING INTERACTIONS OF THE MALARIA VECTOR *ANOPHELES GAMBIAE* WITH ITS HOST PLANTS

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(**B.Sc., Hons**)

A thesis submitted in partial fulfilment of the requirements for the award of the degree of Master of Science in Medical and Veterinary Entomology of the University of Nairobi.

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

To my son Emmanuel Orina and beloved mother, the late Dourine Akoth

May her soul rest in peace

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LIST OF ABBREVIATIONS AND DEFINATION

DCM – dichloromethane

EAG – electroantennogram

FID – flame ionisation detector

GC-EAD – Gas chromatography-electroantennograghic detection

GC-MS – Gas chromatography-mass spectrometry

GPCR - G-protein coupled receptor

MBTFA – methyl-bis-trifluoro acetate

OPI - olfactometer preference index

ORN- olfactory receptor neuron

SEM – standard error of means

Kairomones- volatile chemicals that act between individuals of different species and benefit the receiver (Dicke and Sabelis, 1988).

ABSTRACT

Malaria remains a major health problem in Africa, with *Anopheles gambiae* Giles as the principle vector. Plant nectar feeding forms an integral part of the vectorial capacity of this mosquito species, yet paucity of information on the nature of this behaviour persists. While it is recognized that olfactory cues play an important role in mediating orientation and attraction of mosquitoes to host plants as sources of sugar, little effort has been made to identify the kairomones involved in this behaviour. This study sought to investigate the effect of *Plasmodium falciparum* Welch infection on the mosquitoes-plant interactions and to identify kairomones involved in mediating the interaction with selected host plants. More than 60% of uninfected, oocyst-stage and sporozoite-stage *Plasmodium*-infected *An. gambiae* mosquitoes responded to plant odours in the dual choice olfactometer with a significant increase in probing response following infection with both stages of the parasite. The interaction between the infection status and the plant species was significant for oocyst-stage and sporozoite-stage *Plasmodium* infected mosquitoes towards *Parthenium hysterophorus.* Further analysis revealed that the vectors ingested plant sugars as well as secondary metabolites. A total of five terpenes and four aldehydes were identified as electrophysiologically active. A blend of these terpenes elicited optimal attraction at a lower dose (2ng/µg) while aldehydes yielded an optimal response at a higher dose (8ng/µg). However, when both terpenes and aldehydes were blended together their natural ratios, a much lower optimal dose of 1ng/µg was obtained. These findings confirm the significance of plant odours in the ecology of the malaria vectors.

CHAPTER ONE: INTRODUCTION

Malaria is increasingly becoming a worldwide threat, with more than three hundred million infections and one million deaths every year (Snow *et al.,* 1999; Snow *et al.*, 2005; World Malaria Report, 2005; WHO fact sheet, 2010). The disease has far reaching debilitating and economic implications in Africa, a continent grappling with many other diseases and poverty. In Africa mosquitoes belonging to the *An. gambiae* complex constitute the principle vectors of *P. falciparum*, the parasite that causes the most severe form of malaria (White 1974; Collins and Paskewitz, 1995).

Conventional methods for malaria control rely on disrupting transmission through vector control and using chemotherapeutic agents to prevent establishment or clear parasites in infected persons. However, all the three major vectors of malaria in Africa namely *An. gambiae, Anopheles arabiensis* and *Anopheles funestus* have shown resistance to one or more of the insecticide classes used in vector control (Hargreaves *et al.,* 2000; Hemingway and Ranson, 2000). This is compounded by development of strains of *P. falciparum* resistant to current antimalarial drugs (Bunnag and Harinasuta, 1987; Bloland, 2001). Recent evidence points to emergence of an exophilic subgroup of *A. gambiae s.s.* that is abundant, lacks differentiation into M and S molecular forms and is highly susceptible to *P. falciparum* infection (Riehle *et al,* 2011). This further complicates the malaria control scenario with the net effect of sustained worldwide epidemics of the disease. Attention is therefore shifting to understanding the natural ecology of malaria vectors which can potentially open up new ways of breaking transmission (Takken and Knols, 1999; Fergurson *et al.,* 2010).

Male mosquitoes have for a long time been known to exclusively depend on plant sugars foraged from plant parts such as floral and extra-floral nectaries, damaged fruits or honeydew for metabolic activities, flight and mating competence (Van Handel, 1984; Foster, 1995), with female mosquitoes feeding on both plant sugars and vertebrate blood (Van Handel, 1984; Foster, 1995). Plant nectar feeding forms an integral pillar in the ecology and behaviour of malaria vectors (Takken *et al.*, 1998), but little is known about the nature of this trait. Evidence points to a discriminative plant feeding exhibited by *An. gambiae* when presented with different plants (Manda *et al.*, 2007a), even though some of the preferred plants do not have enough sugar reserves (Manda *et al.*, 2007b). It has not been established whether sugars are the only products mosquitoes ingest while feeding on plants or if there are other secondary metabolites as well. Besides, *Plasmodium* parasites have been shown to manipulate their mosquito vectors such as to increase transmission success (Wekesa *et al.*, 1992; Anderson *et al.*, 1999; Koella *et al.*, 1998; Koella *et al.*, 2002). The influence of *Plasmodium* parasite on the plant feeding behaviour of *An. gambiae* has not yet been established. More importantly, plant volatiles have been implicated as playing a role in mosquito-plant interactions (Bowen, 1992; Takken and Knols, 1999), but little has been done to identify the compounds involved. This study aimed to investigate the effect of physiological status on mosquito-plant interactions and identify the kairomones mediating this interaction.

CHAPTER TWO: LITERATURE REVIEW

This chapter reviews the current knowledge on the malaria vector species diversity, distribution, biology, existing control strategies, the challenges faced and the prospects for new control methods to help curb the malaria endemic. The chapter also examines the role of olfaction in malaria vectors, the energy budget, nectar feeding behaviour and the behaviour manipulation of the vector following infection with malaria parasites.

2.1 Malaria vectors: species diversity, distribution and biology

Vector ecology and disease transmission are dynamic and complex processes and it is sometimes difficult to draw general conclusions. Specific information about the vector species distribution and behaviour is therefore important in considering surveillance and control tools to be employed. Besides, nectar feeding appear to be an adaptive behaviour rather than intrinsic since there is a great diversity of plants in terms of species diversity and distribution. This section reviews the diversity, distribution and biology of important mosquito species with special attention to *An. gambiae* and its relevance to Africa.

2.1.1 Species diversity and distribution

Mosquitoes belong to the family Culicidae, order Diptera, class Insecta (Hexapoda), phylum Arthropoda. There are three recognized subfamilies, the Anophelinae, Culicinae and Toxorhynchitinae (Harbach and Kitching, 1998; Harbach and Kitching, 2005; Reuda, 2008). The Anophelinae consist of three genera: *Anopheles* Meigen (nearly worldwide distribution), *Bironella* Theobald (Australia only: 11 described species) and *Chagasia* Cruz (Neotropics: 4 described species) (Garros *et al.*,2005; Sedaghat and Harbach, 2005; Rattanarithikul *et al.*, 2006; Walton *et al.*, 2007). Malaria parasites are transmitted by

females of the genus *Anopheles* (White, 1989), which contains 484 species described to date ( ). This genus is further subdivided into seven subgenera: *Anopheles* (189 species), *Cellia* (239 species), *Kerteszia* (12 species), *Lophopodomyia* (6 species), *Nyssorhynchus* (33 species) and *Stethomyia* (5 species) (Harbach *et al.*, 2004; 2007). All the subgenera except *Lophopodomyia* and *Stethomyia* are malaria vectors but the Old World tropical subgenus *Cellia* includes most of the major ones (Fontenille and Simard, 2004; Harbach, 2004). The listing of important malaria vectors differs from publication to publication, reflecting probably the experiences of the authors, changing transmission patterns caused by human movement and environmental modification, and the criteria used for determining importance. While Harwood and James in 1979 wrote that 85 species were incriminated conclusively as vectors, White (1982) identified only 35 species as "important vectors" yet Brown and Nelson, in their 1993 review, listed 51 *Anopheles* species as important vectors.

The global distribution of *Anopheles* species that transmit *Plasmodium* parasites has been described by Kiszewski *et al.* (2004) (Figure 1). For Southeast Asia, three main malaria vector groups are recognized: the *Anopheles dirus* complex, occurring in forested areas; the *Anopheles minimus* complex, widespread in hilly forested regions; and the *Anopheles sundaicus* complex, denizens of brackish water along coastal areas (Garros *et al.*, 2006). The *Anopheles hyrcanus* group consists of several species that are vectors of malaria in Oriental and Palearctic regions. Twenty-seven species of this group are found in Oriental and Eastern Palearctic regions, and only 3 species have a western Palearctic distribution (from western China and south of 50^{o} N) (Ramsdale, 2001).

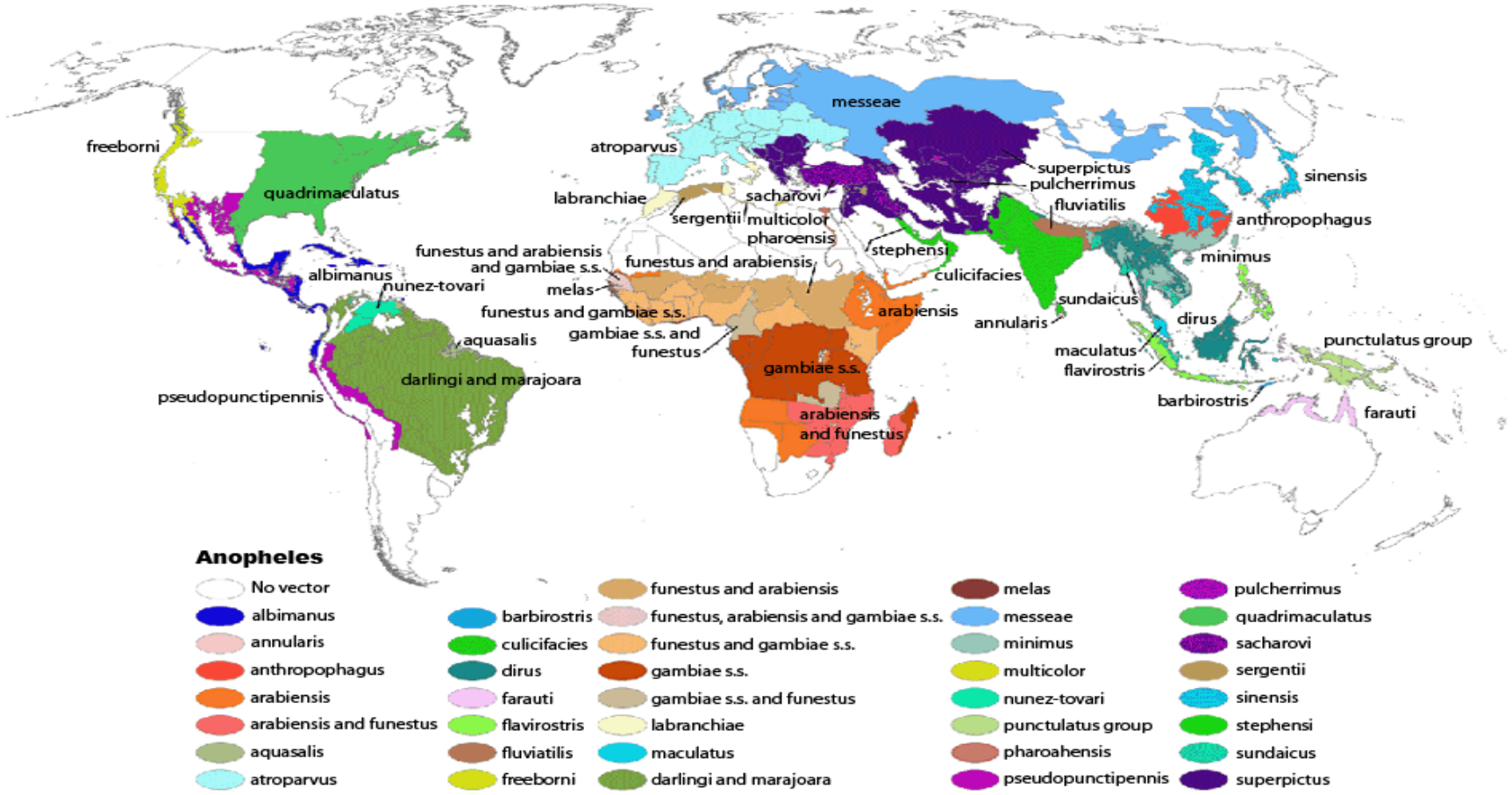

Figure 2.1 Global distributions of dominant or potentially important malaria vectors (Kiszewksi *et al.*, 2004).

Africa has over 140 recorded *Anopheles* species, of which at least eight are considered to be effective vectors of malaria (Gillies and Meillon, 1968; Gillies and Coetzee, 1987). Two of the most efficient vectors of human malaria, *An. gambiae* and *An. arabiensis* (White, 1974) are members of the *An. gambiae* complex. Other recognized species of the complex are *Anopheles merus*, *Anopheles melas*, *Anopheles quadriannulatus*, *Anopheles quadriannulatus* B and *Anopheles bwambae*. *Anopheles merus* and *An. melas* are associated with salt-water with a localized distribution along the eastern and western coasts of Africa, respectively, while *An. bwambae* has only been found breeding in mineral springs in the Semliki forest in Uganda (Coluzzi, 1984). *Anopheles quadriannulatus*, found in south-east Africa (Coluzzi, 1984) and *An. quadriannulatus* B, which has been described in Ethiopia (Hunt *et al*.,1998) are not considered vectors of human malaria as they are generally zoophilic (Coluzzi, 1984; Takken and Knols, 1999).

In addition to the *An. gambiae* complex, other species known to be important in malaria transmission in Africa include *Anopheles funestus, Anopheles nili,* and *Anopheles moucheti* (Gillies and Coetzee, 1987; Harbach, 2004). Other species, such as *Anopheles paludis*, *Anopheles mascarensis* and *Anopheles hancocki* play only a limited, secondary and localized role where they are found (Fontenille and Simard, 2004).

Several of these vector species are found to occur in sympatric in much of Africa and their importance in malaria transmission varies depending on behaviour (e.g. biting activity, feeding and resting preferences), seasonal prevalence and vectorial capacity (Coluzzi, 1984; Fontenille and Simard, 2004). These differences contribute to the varied malaria epidemiological patterns observed in Africa and, subsequently, different areas may require different tools and strategies for optimal vector control.

2.1.2 Vector biology

Anopheles gambiae, alongside *An. arabiensis* and *An. funestus* are the most efficient vectors of malaria in Africa, which shoulders more than 86 percent of the world's malaria infections (WHO, 2010). Both *An. gambiae* and *An. funestus* have a marked preference for human environments and humans as hosts while *An. arabiensis* is mostly zoophilic and only feed on human host opportunistically. They all adapt so rapidly to changes in the environment induced by human habitation and agriculture (Collins and Besansky, 1994) and this makes them efficient vectors of malaria parasites. This genetic plasticity is evident in the often observed ability of the sibling species *An. gambiae* and *An. arabiensis* to rapidly evolve new behavior patterns, such as the shift from indoor to outdoor blood feeding and resting in response to indoor insecticide control programs. The intensity of malaria transmission by these mosquitoes is determined largely by environmental conditions, and where conditions support large mosquito populations and year-round abundance, parasite inoculation rates can exceed 300 infective mosquito bites per person per year (Beier *et al.*, 1990).

Like all mosquitoes, anophelines go through four stages in their life cycle: egg, larva, pupa, and adult. The first three stages are aquatic and last 5-14 days, depending on the species and the ambient temperature. The adult stage is when the female *Anopheles* mosquito acts as malaria vector. The adult females can live up to a month (or more in captivity) but most probably do not live more than 1-2 weeks in nature (Cross, 2004). Male adult mosquitoes forage mainly on plant nectars while females feed on both plant nectars and vertebrate blood and as such are responsible for disease transmission in humans. *Anopheles gambiae s.s.* Giles females are nocturnal, endophilic, and endophagic (Gibson, 1996). The females spend most of their time resting indoors and blood feeding

when human hosts are sleeping. This affords freedom from host defensive behaviour and is probably important to anopheline females, which will continue to blood feed even after the mid-gut is full, concentrating erythrocytes and expelling serous fluid. Vaughan *et al.*, (1991) showed that host-contact time is increased almost twofold beyond that required for gut filling, when *An. gambiae* females are allowed to feed to repletion. This is particularly important when considering that infection with *P. falciparum* lowers a female's blood-feeding efficiency, increasing the number of partial blood meals required to reach repletion (Wekesa *et al.*, 1992).

The gonotrophic cycle of mosquitoes begins with a blood meal and ends with oviposition (Clements 1992). Anophelines may emerge with low teneral metabolic reserves (Briegel 1990). Multiple blood meals taken within the first gonotrophic cycle seem to be necessary for small teneral females. The primary ovarian follicles of undersized female mosquitoes may be arrested in the previtellogenic phase, at the stage 1 gate, until a meal of sugar or blood is taken (Clements 1992). This is sometimes known as the **"**pregravid" state and is linked to juvenile hormone suppression, which is required to stimulate development of follicles to the previtellogenic gate (resting stage) (Clements 1992). After the sugar or blood meal, the follicle develops to the resting stage. Takken *et al.* (1998) found that, unlike *Aedes aegypti* (Feinsod and Spielman 1980), sugar feeding was not sufficient to bring follicles of undersized *An. gambiae* to resting stage, and blood was required. The first blood meal helps build protein reserves and develops follicles to the resting stage. The second meal is then used to initiate vitellogenesis and yolk uptake by the oocytes, to develop them completely (Takken *et al.*, 1998). Even well fed, normal-size females show no inhibition to host seeking during the first oogenesis (Klowden and

Briegel 1994), though they are able to develop oocytes without a second blood meal (Takken *et al.*, 1998).

2.2 The state of malaria vector control

Malaria vector control strategies have been hailed as a viable strategy in breaking the transmission of the malaria parasites and these strategies are largely region-specific (Townson *et al.*, 2005). There is a renewed effort to control malaria and move towards elimination in some countries with current strategies including chemical control (WHO, 1995; Rozendaal, 1997) increasing use of long-lasting insecticide-treated nets (LLINs) (Lengeler, 1998), and artemisinin-based combination therapies (ACTs) (WHO, 2009) as well as integrated environmental management (Killeen *et al.*, 2002). These approaches have shown some promising progress in reduction of the disease burden, with up to 50% reduction in reported cases and deaths in some highly endemic countries such as Eritrea, Rwanda, São Tomé and Príncipe, Zambia as well as Zanzibar, United Republic of Tanzania (WHO, 2009). Some of the insecticides that have been employed in malaria vector control include organochlorides, organophosphates, carbamates and pyrethroids (Pant, 1988; Chavasse and Yap 1997).

However, use of insecticide treated bed nets pose the threat of selecting for resistance and inducing changes in the biting cycle and indoor/outdoor feeding behaviour of the vectors, which might eventually render bednets useless (Knols and Takken, 1998; Riehle *et al.*, 2011). Besides, chemotherapy as a measure of curbing clinical cases of malaria as well as chemical control of malaria vectors have been hard hit by widespread resistance to most of the currently available antimalarial drugs and insecticides (Brogdon and McAllister,

1998; Bloland 2001; Mittal *et al.*, 2004; Djogbénou, 2009; Ranson, 2011). Moreover, the vector control measures are definitely bound to be complicated with recent discovery of a new subgroup of *An. gambiae s.s.* which bites outdoors and is highly susceptible to *P. falciparum* infection than the indoor biting mosquitoes (Riehle *et al.*, 2011).

Advances in molecular biology have led to the development of new vaccine candidates and identification of genes that code for refractoriness of mosquitoes to infection with *Plasmodium* parasites (Carlson *et al.*, 1995; Collins and Poskewits, 1995, Enger and Godal, 1998). However, majority of malaria candidate vaccines have been discovered and developed based on results from experiments in animal models and in vitro assays as well as epidemiological associations of clinical protection and immune responses in the field (MALVAC Meeting 2004). Efforts to translate these prospects into reality are therefore faced with uncertainty as to the relevance and predictive value of these imperfect screening tools and immune response associations. Besides, the ultimate proof-of-concept for most candidates will require evaluation in clinical efficacy trials which are rather expensive, time consuming and cumbersome.

Hence, integrated approach that relies on early case identification and treatment as well as selective and sustainable prevention measures, including vector control is currently being advocated for (WHO, 1993; Shiff, 2002; Ferguson *et al.*, 2010). Integrated vector control programs which incorporate local information about vector distribution and behavior to identify one or more control techniques that would be effective, affordable, and acceptable to local communities is therefore necessary. It is on the backdrop of these challenges that attempts are being made to understand vector ecology, with the view of

uncovering new approaches to curb the malaria menace (Furguson *et al.,* 2010). The odour-mediated behaviour of the malaria vector offers a promising opportunity for environmentally friendly vector management (Takken and Knols, 1999).

2.3 Mosquito odour bait technology

With the increasing knowledge on the role of olfaction in the behaviour of the malaria vector, the exploitation of odour baits is now widely accepted as potential for incorporation into mosquito traps. Extensive research into the identity of the entire suite of human kairomones and their deployment in traps is underway (Bernier *et al.* 2000, 2002; Smallegange *et al.* 2005; Okumu *et al*, 2010). This technique has been widely fronted as a measure, in combination with other available control strategies, to reduce human-vector contact hence break the malaria transmission cycle and can drastically reduce the number of infectious bites from 200 to 0.2 in high transmission regions (Logan and Birkett, 2007; Takken and Knols, 2009, Jawara *et al.*, 2009; Okumu *et al.* 2010). However, though this technology has been employed with a lot of success in surveillance of agricultural pests (Day and Sjogren, 1994; Pickett *et al.*, 1997; Khan *et al.*, 2000; Cook *et al.*, 2007), its application in insects of public health importance is still limited.

The most effective and efficient traps in current use require suction created by a battery-powered fan, may or may not have a small light, and are baited with volatile chemicals (Foster 2008). Some of the traps in current use for mosquito surveillance include light traps (such as CDC and New Jersey light traps), electric nets, counter-flow geometry trap, mosquito resting box trap and gravid traps (Constantini *et al.*, 1993; Mboera *et al.,* 2000; Killeen and Smith, 2007). Light traps are effective surveillance tools for sampling

mosquito populations of some species and have the advantage of catching males and gravid females besides the blood seeking females. However, they have low capture rates, compared to odour-baited versions, and the congestion of the trap with other flying insects, which damage mosquito morphological features and make the sorting and identification process cumbersome (Foster, 2008). Gravid traps, which make use of volatiles associated with bacterial action on organic matter in water suitable for mosquito oviposition (Beehler *et al.* 1994) also offers a viable option in the fight against the malaria. Recently the use of sugar baited lethal traps has been suggested as another possible upfront in the war against disease transmitting anopheline species (Müller and Schlein, 2006; Schlein and Müller, 2008). Though considerable advances have been made, the development and adoption of odour bait technology in mosquito control still face many challenges including getting highly attractive lures while minimizing the final cost of the traps (Okumu *et al.*, 2010).

Some of the odour baits that have been employed in mosquito traps include whole human, whole cow, human foot odour, cow urine, horney extract, synthetic compounds such as carbon dioxide, ammonia, 1-Octen-3-ol, Octenol, L-lactic acids, butanone, butyric acid, hexanoic acid and phenols (Kline *et al.,* 1990; Kline *et al.,* 1991; Mboera *et al.*, 2000; Njiru *et al.*, 2006). Traps baited with human host odour and carbon dioxide have been found to be as good as those baited with whole human in terms of total catches with the position of these traps in or around experimental huts not affecting the total catch (Jawara *et al.*, 2009). The potential for incorporation of synthetic compounds in the odour baits has made the possibility of the application of this technique even more realistic. However no plant related compounds has been investigated for possible inclusion in these odour baited traps.

2.4 Olfactory reception in mosquitoes

Mosquitoes depend on receptors for a variety of sensory modalities, including vision, hearing, mechanoreception, thermoreception and chemoreception, which transduce environmental information into biologically useful signals. Each modality plays a role in the complex process of identifying and locating appropriate blood-meal hosts and nectar sources (Bowen, 1991). The females' tendency to engage in host-seeking changes in concert with variations in physiological state such as age, reproductive status, and diapause (Klowden, 1988; Klowden *et al.*, 1988; Mitchell, 1988) and these variations thus dictate the vectors response to chemical information in their environment at any given time. Chemical cues play an important role in host location and discrimination by malaria vectors, just as in many other insects (Takken and Knols, 1999; Mukabana *et al.*, 2002). This knowledge has formed the basis of many studies into the physiology and molecular basis of this trait (Zwiebel and Takken, 2004), with the aim of understanding how it influences the transmission of malaria and identifying potential attractant or repellent compounds for mosquito surveillance and abatement efforts (Bernier *et al.*, 2002; Bernier *et al.*, 2007; Qui *et al.*, 2006). Olfactory receptors in the mosquito undergo alterations in sensitivity that is correlated with changes in host-seeking behaviour (Davis, 1984a; Davis, 1984b; Bowen *et al.*, 1988). Although mosquitoes use both visual and olfactory cues to orient toward a host (Allen *et al.*, 1987), the olfactory system plays a prominent role in modulating the response (Takken and Knols, 1999; Bohbot *et al.*, 2010).

Olfactory receptor neurons (ORN) in insects are contained in sensilla, porous cuticular extensions of various shapes predominantly present on their antennae and mouthparts. In female *An. gambiae*, the antenna consists of two types of thick-walled sensilla chaetica;

and four types of thin-walled sensilla: sensilla trichodea, sensilla basiconica (grooved peg sensilla), sensilla coeloconica and sensilla ampullaceal (Figure 2.2). Sensilla chaetica are innervated by a bipolar neuron and are mechanosensilla that are sensitive to touch and air movement. Sensilla coeloconica and sensilla ampullaceal are possibly innervated by thermoreceptor neurons (Davis and Sokolove, 1975; McIver, 1982). Each antenna of female *An. gambiae* mosquitoes bears about 630 trichoid sensilla and 84 sensilla basiconica, both types containing an estimated total of 1500–1600 ORNs per antenna (Ismail 1964; Meijerink *et al*., 2001). Trichoid sensilla are single-walled pore sensilla and sensilla basiconica are double walled with 10-12 hollow finger-like structures fused to each other (Steinbrecht 1997; Pitts and Zwiebel 2006). These two house the major olfactory nerves.

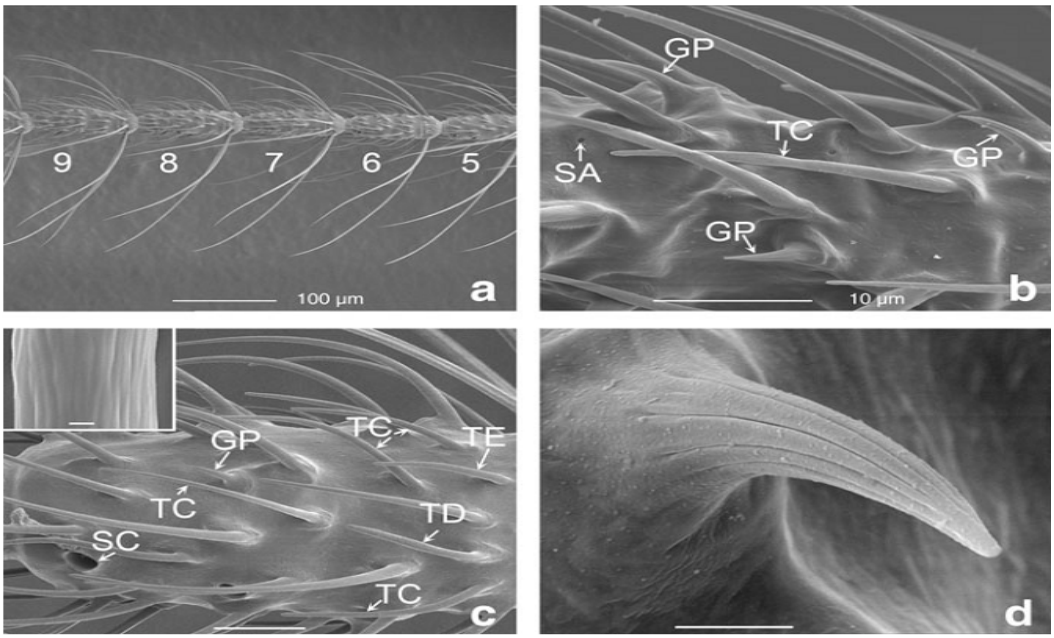

Figure 2.2 Scanning electron micrographs of thin-walled antennal sensilla of a female mosquito of *An. gambiae*. (a) the 5th to 9th antennomeres of a female *An. gambiae*. (b) details showing grooved-peg sensilla (GP), trichoid sensilla C (TC), and sensilla ampullacea (SA). (c) TC, trichoid sensilla D (TD), trichoid sensilla E (TE), and sensilla coeloconica (SC). Inset, wall structure of TC showing slit-like structures on the surface. (d) GP (adapted from Qui *et al*., 2006).

In the Afrotropical malaria vector *An. gambiae* and in other insects, olfactory signal transduction is initiated by G protein-coupled receptors (GPCRs) on the dendrites of olfactory receptor neurons (ORNs), which have, thus far, been characterized in several insect species (Cline *et al.*, 1999; Fox *et al.*, 2001; Krieger *et al.*, 2003). ORNs in trichoid and sensilla basiconica project to different glomerular areas of the antennal lobe, the primary integration center in the deuterocerebrum (Anton *et al.*, 2003; Anton and Rospars 2004). The mosquitoes perceive odour plumes in their environment by olfactory receptor neurons that are mainly housed in antennal trichoid and grooved peg sensilla located in antennae and maxillary palps (Davis and Rebert, 1972; McIver, 1982; Bentley and Day, 1989; Davis and Bowen, 1994). The sensilla in these appendages are composed of odorant-binding proteins (Leal, 2003; Vogt and Riddiford, 1981) and odorant receptors (Hallem *et al.*, 2006) that are sensitive to chemicals originating from skin, breath, oviposition sites and plant nectar. They detect odorant compounds and transduce olfactory signals to the brain through the receptor neurons to mediate insect behaviours.

Long range host detection in mosquitoes starts with interactions between odorants and a distinct subpopulation of odorant receptors embedded in the dendritic membrane of odour receptor neurones. Host location then ensues in a series of steps that encompass orientation, dispersal, attraction, arrestment and feeding. Coupled gas chromatography-electroantennogragh detector utilizes this inbuilt odour detection in insect antenna to isolate compounds that elicit antennal response.

2.5 Olfaction in mosquito-host interaction

Like other interspecific interaction, olfactory cues play an important role in host location by malaria vectors *An. gambiae,* and thus significantly determines the vectoral capacity

of this mosquito species (Takken, 1991; Takken and Knowls, 1999; Zwiebel and Takken, 2004; Gouagna *et al.*, 2010). Male mosquitoes use olfactory cues to locate nectar sources, while females utilize the same cues to locate oviposition site, vertebrate host and nectar sources (Foster, 1995; Foster and Takken, 2004). Human host seeking behaviour of the malaria vectors *An. gambiae* has been widely studied, with a number of chemical compounds identified as responsible for mediating this behaviour. These include carbon dioxide, short chain fatty acids, L-lactic acid, 1-dodecanol and ammonia (Takken *et al.*, 1997; Bernier *et al.*, 1999; Bernier *et al.*, 2000; Braks *et al.*, 2001; Smallegange *et al.*, 2005; Qui *et al.*, 2005; Qui *et al.*, 2007; Okumu *et al.*, 2010). Lactic acid, ammonia and carboxylic acids, present in human sweat, have been found to act in a tripartite synergism in attracting *Ae. aegypti* and *An. gambiae* to human host (Smallegange *et al.*, 2005). Other compounds of human origin which have been reported to be electrophysiologically active include 6-methyl-5-hepten-2-one, 3-methyl-1-butanol, indole, 4-methyl phenol and geranyl acetone (Meijerink *et al.*, 2001; Qui *et al.*, 2005).

Information on olfactory mediation of mosquito-plant interactions is scarce, although previous work *Culex* species have implicated green-leaf volatiles, monoterpenes and sesquiterpenes as responsible for host plant attraction (Bowen *et al.*, 1992, Foster and Hancock, 1994). No attempt has been directed at identifying chemicals involved in mosquito-plant interactions for the malaria vector *An. gambiae*.

2.6 Sources of sugars for mosquitoes

Most mosquito species obtain carbohydrates primarily from flowers and extra-floral nectarines (Haeger 1955; Sandholm and Price 1962; Grimstad and DeFoliart 1974) but also damaged fruits, damaged and intact vegetative tissues, and honeydew widely

available within the vicinity of their habitats (Hocking, 1953; McCrae *et al.*, 1969; Joseph, 1970; Magnarelli, 1979; Yuval 1992). They prefer some plants over others as nectar sources (Grimstad and DeFoliart 1974; Magnarelli 1978; Gadawaski and Smith 1992). Plant fluids also serve to as important sources of water for mosquitoes in arid climates (Reisen *et al.*, 1986). Generally, sugar sources are more attractive than blood-hosts (such as humans) during first foraging periods of females after adult emergence (Foster and Takken 2004) and autogenous as well as anautogenous teneral females prefer sugar over a meal of blood and are preferentially attracted to plant related volatiles (Bowen *et al.*, 1995; Foster and Takken, 2004). In some cases sugar feeding appears to be pre-determined in the sense that host-seeking and blood-feeding cannot take place unless the female first takes a sugar meal (Nayar and Pierce, 1980; Hancock and Foster, 1993). In contrast to females which are strongly attracted to carbohydrates only in specific stages of their life (Foster and Takken, 2004), males are strongly attracted to this food source throughout their whole life as they only ingest carbohydrates (Reisen *et al.*, 1986). In *Ae. aegypti*, which is ecologically similar to *An. gambiae* in that it is anthropophilic and endophilic (Foster, 1995; Gibson, 1996), sugar feeding is more common in the field when nectar sources are abundant (Van Handel *et al.*, 1994; Martinez-Ibarra *et al.*, 1997), despite the presence of human blood hosts (Martinez-Ibarra *et al.*, 1997) and the apparent cost to fecundity (Morrison *et al.*, 1999), suggesting that exclusive blood feeding is a function of sugar scarcity.

Generally, sugar feeding occurs frequently among various mosquito species under natural conditions, particularly prior to mating (Foster, 1995; Gary and Foster, 2004; Garry and Foster 2006; Foster, 2008). There appears to be a range of nectar-host specificity. Even among those generalist mosquito species that feed on a wide variety of plants, there

clearly is a predilection for certain plant species, independent of their availability (Yuval 1992, Foster 1995). Inferences from field data suggest that there is a lot of variation in the frequency of sugar feeding by mosquitoes. Some species feed on small amounts of sugar frequently (Nasci and Edman, 1984), while others may sugar-feed once per gonotrophic cycle (Holliday-Hanson *et al.*, 1997). Whichever the case, many mosquito species do not survive and reproduce as well without sugar in their diet (Nayar and Sauerman, 1971; Briegel and Kaiser, 1973; Nayar and Pierce, 1980).

Anopheles gambiae s.s. is highly anthropophilic, with females depending heavily on human blood for both gonotrophic and metabolic processes (Beier, 1996; Takken *et al.* 1998). However, recent evidence indicates that both males and females of this species equally engage in plant nectar feeding (Impoinvil *et al*., 2004; Gary and Foster, 2004; Müller and Schlein, 2006; Manda *et al*., 2007a; Manda *et al*., 2007b; Müller *et al*., 2010). In semi-field bioassays, preference for certain host plants as nectar sources, accompanied by improved survival and fecundity of females in some cases has been observed (Gary and Foster, 2004; Impoinvil *et al.*, 2004; Manda *et al.*, 2007a; Manda *et al.*, 2007b). For example, of the host plants investigated *Manihot esculenta* Crantz (Euphorbiaceae), *Parthenium hysterophorus* L. (Asteraceae), *Tecoma stans* L. (Bignoniaceae), *Ricinus communis* L. (Euphorbiaceae), and *Senna didymobotrya* Fresen (Caesalpiniaceae), were the most preferred for feeding, while *Lantana camara* L. (Verbenaceae), *B. pilosa* L. (Asteraceae), *Datura stramonium* L. (Solanaceae) and *Flaveria trinervia* Mohr (Asteraceae) among others were the least preferred. These findings have further been confirmed through field studies in which the malaria vectors were shown to be attracted to certain fruits/pods and flowering plants (Gougna *et al*. 2010; Müller *et al*., 2010).

Nectar feedings has been observed to peak in the early evening and early morning (Müller *et al.*, 2010).

The distribution of plant species used in this study overlap since they are all invasive plants. *Parthenium hysterophorus*, which is of South American origin, is considered one of the world's most serious invasive plants, invading Australia, Asia and Africa (Mcconnachie *et al.*, 2011). It invades all disturbed land, including farm, pastures and roadsides. *Ricinus communis*, also commonly known as castor beans, is a native of Africa but has been introduced in other tropical and sub-tropical areas of the world (Kogan and Lattin, 1993; PIER, 2005; Floridata, 2005). It is a perennial shrub that can assume a tree-like status if it establishes in a suitable climate. It is frequently found growing in riparian areas where it displaces the native vegetation (Floridata, 2005). On the other hand, *Bidens pilosa* (Asteraceae), which originated from tropical and temperate America, is less invasive compared to the other two plants and grows on disturbed or waste ground, roadsides and areas that are dry and infertile (Harden, 2002). In Africa, *B. Pilosa* (also known as Black jack) is recorded as a weed in many countries and it is likely to occur in all countries, including Indian Ocean islands (Mvere, 2004).

Preference, survival and fertility of *An. gambiae* on the three plant species used in the current study differ significantly (Impoinvil *et al.*, 2004; Manda *et al.*, 2007a; 2007b). High preference by *An. gambiae* for *R. communis* is observed accompanied by improved survival and fertility. On the contrary, *An. gambiae* show a high preference for *P. hysterophorus* yet the survival and fecundity on this plant is significantly compromised (Manda *et al.*, 2007a; Manda *et al.*, 2007b). Preference and survival of *An. gambiae* on *B. pilosa* is significantly low, though fecundity of the malaria vector on this plant species

has not been investigated. It is, however, not clear what informs the discriminative feeding patterns in these vectors.

2.7 Energetic budget in mosquitoes

For males, and females of some species, nectar is the only food (Van Handel 1984; Foster 1995) and whereas haematophagous females generally utilize protein from blood meals to develop eggs, they still use sugar to help meet their metabolic needs and increase survivorship (Nayar and Sauerman 1975; Van Handel, 1984; Foster, 1995; Foster and Takken, 2004). In addition, sugar provides females with a ready source of flight energy (Nayar and Van Handel 1971; Clement, 1992) and can, in some cases, improve fecundity, both by helping to develop follicles to the resting stage in small females (Magnarelli 1978, Nayar and Sauerman 1975b) and by increasing the number of follicles undergoing vitellogenesis (Mostowy and Foster 2004).

That sugar plays a significant role in the overall fertility of mosquitoes, and by extension their success as vectors of diseases is indisputable. Mosquitoes that feed on blood and sugar have been found to lay more eggs than those that ingest blood alone (Nayar and Sauerman, 1975; Klowden, 1986; Klowden and Chambers, 1989; Andersson, 1992; Manda *et al.*, 2007b), possibly due to the fact that egg production incurs an energy cost, which can be met by sugar intake or because sugar intake reduces the utilization of dietary protein for the synthesis of lipids, which constitute more than 50% of dry weight of mature oocyte (Clement, 1992).

Most parasitic infection have been found to exert energetic cost to their host vectors (Hurd, 1998; Hurd *et al.*, 2001), with resultant loss of reproductive potential and reduced

lifespan (Hacker and Kilama 1974; Freier and Friedman 1976; Hogg and Hurd 1995; Hogg and Hurd, 1997; Ahmed *et al.*, 2001; Ferguson and Read, 2002; Ferguson *et al.*, 2003; Aboagye-Antwi *et al.*, 2010). The loss in these life parameters has been attributed to shift in resource allocation as a result of the parasite intrusion. Rivero and Ferguson (2003) showed that *An. stephensi* infected with oocyst-stages of virulent strains of *Plasmodium chabaudi* showed up to 50% increase in glucose levels. These authors attributed this phenomenon to the possibility of resource reallocation by the vector or increase in the amount of sugars ingested following infection. When faced with metabolic deficiency, the mosquitoes are able to utilize stored molecules such as glycogen and proteins (Clements, 1999). However, Rivero and Ferguson (2003) did not find any change in glycogen and protein contents of infected and uninfected mosquitoes, indicating that the observed increase in glucose levels could have been a result of increased sugar intake. This raises the question as to the role of sugar in *Plasmodium*-mosquito interactions.

2.8 Mosquito-Plasmodium survival tradeoffs

The life cycle of *Plasmodium* parasites is split between a vertebrate host and an insect vector. *Plasmodium* parasites infect susceptible female *Anopheles* mosquitoes when they blood feed on infected vertebrate hosts. Within the insect mid-gut, gametocytes are rapidly activated to produce gametes. Fertilization follows, leading to formation of a motile ookinete which penetrates the epithelial cell monolayer surrounding the gut lumen. The ookinete ceases its migration upon reaching the basal lamina separating the mid gut and hemocoel. Here the ookinete differentiates into an oocyst, which grows over a period of days and produces sporozoites; ultimately these are released into the hemolymph and

migrate to the salivary glands from where they are passed to vertebrate hosts as the mosquito blood-feeds (Touray *et al.*, 1992) (Figure 2.3).

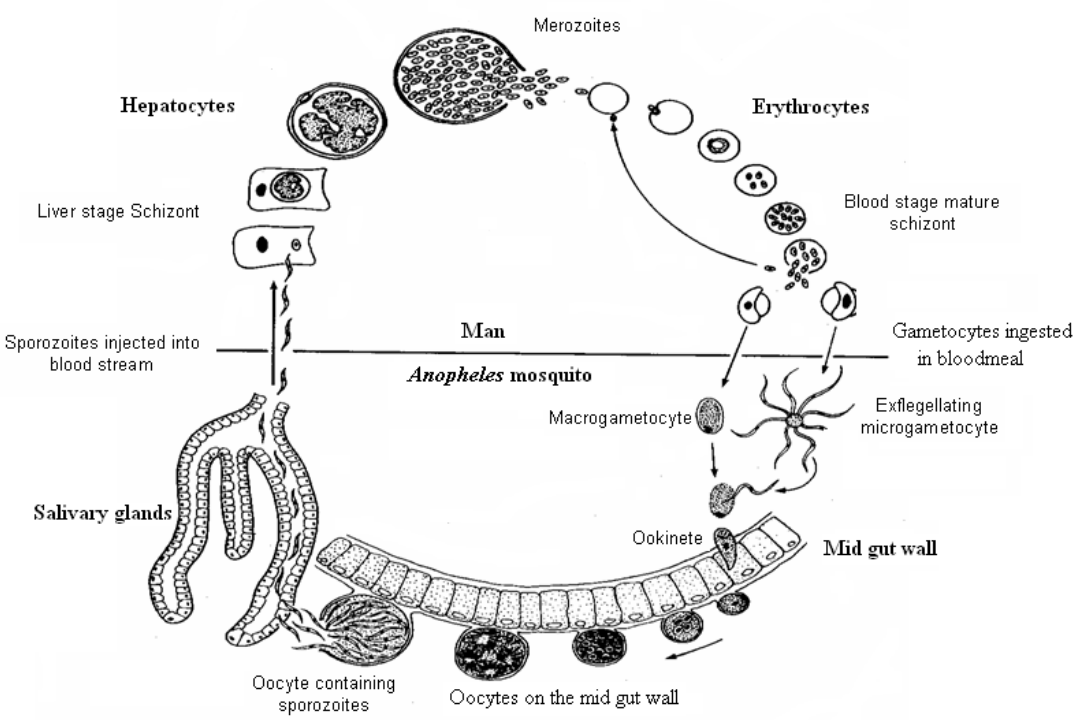

Figure 2.3 Plasmodium life cycle (Adapted from the Atlas of Tropical Parasites – Opperdoes, 1997)

The great majority of *Plasmodium*-mosquito combinations are incompatible, and it is thought that multiple factors contribute to the inherent capacity of a particular *Anopheles* mosquito species to support growth and differentiation of a specific *Plasmodium* parasite (Warburg and Miller, 1991). During the development of *Plasmodium* in the mosquito mid gut, various bottlenecks abound which leads to losses of the parasite load. The three main phases in which development are disrupted involve developmental transitions between gametocyte and ookinete stages, between ookinetes and mature oocysts, and between oocysts and salivary gland sporozoites.

The mechanisms that have been implicated as influencing the gametocyte to ookinete transition involve abortion of macrogamete fertilization and differentiation of zygotes to ookinetes (Beier, 1998). For the ookinete to oocyst transition, two mechanisms that inhibit development include failure of ookinetes to traverse the mid gut and abortion of early-stage oocysts. Similarly, the transition from oocyst to sporozoite can be as a result of oocysts' failure to produce sporozoites, and/or sporozoites' failure to navigate successfully to the salivary glands, invade salivary glands, or survive in the salivary glands. The development is inhibited by physiological or structural barriers, or active innate defence (immune) responses of the insect, which results in the killing of specific stages of the parasite (Warburg and Miller, 1991). The cellular mechanisms against plasmodial infection are two: lysis of migrating ookinetes within the mid gut epithelial cells (Vernick *et al.*, 1995) and melatonic encapsulation of *Plasmodium* parasites at the oocyst stage (Collins *et al.*, 1986). The parasites can also be destroyed by the mosquito immune system peptides.

However, these defence mechanisms are rare in field caught infected mosquitoes and this can be attributed to several factors: this could be due to the fitness cost of immune response, for instance, defence reactions that result in the melanotic encapsulation of parasites are reproductively costly (Hacker and Kilama 1974; Hogg and Hurd 1995, 1997; Ahmed *et al.*, 2001). Both melanization and egg tanning require tyrosine, so a competition for limited resources ensues ultimately resulting in a reproductive cost (lower fertility) for the mosquito (Beernsten *et al.*, 2000). Additionally, successful parasites may circumvent vector defence mechanisms during their developmental progression in a number of ways: by evading recognition, by suppressing the response, or through insensitivity to effector molecules (Loker, 1994). Thus, of the initial infection

load, the vector is able to suppress only a certain fraction of the parasite, the remaining fraction of which is responsible for the maintenance of the transmission cycle. The vectors are, therefore, thought to turn to fauna in their environment for further protection, the role which *P. hysterophorus,* equipped with antiplasmodium active compounds and highly preferred by these malaria vectors, is assumed to play (Manda *et al*., 2007b).

The success of malaria transmission is a product of complex interaction between *Plasmodium* and its mosquito vector. It is a balance between the vector reproductive success and *Plasmodium* transmission success (Koella, 1999). Parasite-vector-vertebrate host interactions have been widely studied, with most evidence pointing to a co-evolution that enhances transmission of the parasite to the vertebrate host while minimizing negative impact to the arthropod vectors. Most studies of altered feeding behaviour mediated by parasites have been done with the malaria system (Wekesa *et al.,* 1992; Anderson *et al*., 1999; Koella *et al.,* 1998, 2002). For instance, the sporozoites of the parasite *Plasmodium gallinaceum* lower the apyrase activity in the salivary glands of infected *Ae. aegypti*, resulting in impaired ability to locate blood and increased time of probing (Rossignol *et al.,* 1984). Laboratory studies indicate that *P. falciparum* sporozoites in naturally infected *An. gambiae* increase not only the duration of probing, but also the number of probes and the likelihood that the mosquitoes will begin to probe on experimental host (hamsters) (Wekesa *et al.*, 1992). These changes in feeding behaviour have also been found to be sensitive to the development stage, with reduced biting persistence at oocyst stage of the parasite as compared to the sporozoite stage of *Plasmodium yoelii* in *Anopheles staphensi* (Anderson *et al*., 1999). However, information on the influence of *Plasmodium* parasite on the plant feeding of *An. gambiae* is scant.

The observed increase in sugar uptake by *Plasmodium*-infected *An. stephensi* can be parasite-driven or host response to parasitism (Rivero and Ferguson, 2003). Evidence indicates that sugar-availability reduces host biting of mosquitoes (Foster and Eischen 1987; Straif and Beier 1996). It is therefore possible that the *Plasmodium* parasites increase sugar uptake by their host vectors during the non-infective stages of their development so as to minimize the mortality risks for mosquitoes, owing to the defensive behaviour of the vertebrate hosts (Day and Edman 1983; Anderson *et al.*, 2000). Alternatively, the parasites could manipulate the sugar-feeding behaviour of mosquitoes to meet the high glucose requirements of the oocysts. It is also possible that *P. chabaudi* induces enhanced glucose uptake to neutralize the mosquito's immune system (Rivero and Ferguson, 2003) by impairing the production of nitric oxide through glucose overload (Prabhakar, 2000; Golderer *et al.*, 2001; Kimura *et al.*, 2001), a molecule that in *An. stephensi* is an important line of defence against *Plasmodium* development (Luckhart *et al.*, 1998; Luckhart and Rosenberg, 1999; Han *et al.*, 2000).

On the other hand, it is also possible that enhanced sugar feeding could be the mosquito's response to parasitism. By increasing the consumption of sugars, which are important precursors of all carbon-based chemical compounds and an essential resource for maintenance in mosquitoes (Clements, 1999), infected females could minimize or compensate for the harm caused by the parasite, such as damage to mid gut epithelial cells and competition for host metabolic products (Maier *et al.*, 1987).

2.8 Justification and significance of the study

Both male and female *An. gambiae* have been observed to discriminately feed on plants of different families for sugars. It has, however, not been established whether *Plasmodium*-infection modifies mosquito-plant interaction, and if sugars are the only benefit mosquitoes derive from plant feeding. Chemical signals from potential host plants play a significant role in host location and host acceptance by nectar-feeding mosquitoes. However, little is known about kairomones involved in mosquito-plant interactions. This study elucidated the kairomones for the malaria vector *An. gambiae* from three host plants: *R. communis, P. hysterophorus,* and *B. pilosa*. Findings of this study can be utilized in the control of the vector through incorporation in mosquito odour-baited traps and attractive toxic sugar baits.

2.9 Hypotheses

1. The attractiveness of *Ricinus communis, Parthenium hysterophorus and Bidens pilosa* to non-infected and *Plasmodium*-infected *An. gambiae* are significantly different.
2. The composition of electrophysiologically active volatile components of *R. communis, P. hysterophorus* and *B. pilosa* are significantly different.
3. There is significant difference in the blends composition and concentration of electrophysiolocally-active components that elicit attractive response the malaria vectors.

2.10 Objectives

2.10.1 Overall objective

To identify kairomones from *R. communis, P. hysterophorus* and *B. pilosa* that mediate interactions of the malaria vector *An. gambiae* with its host plants.

2.10.2 Specific objectives

1. To investigate the attractiveness of *R. communis, P. hysterophorus and B. pilosa* to non-infected and *Plasmodium*-infected *An. gambiae.*
2. To characterize and quantify volatile components from *R. communis, P. hysterophorus* and *B. pilosa* that are electrophysiologically active in *An. gambiae*
3. To determine the blends of plant kairomones that elicit optimal attractive response in malaria vector *An. gambiae*

CHAPTER THREE: MATERIALS AND METHODS

This chapter outlines source and rearing procedure of the mosquitoes; the source and handling of plants used in this study, the source of chemicals used and most importantly the procedures undertaken to achieve the research objectives.

3.1 Rearing of mosquitoes

The mosquitoes were laboratory-reared individuals of the Mbita strain of *An. gambiae s.s.* (established in 2001 from blood-fed and gravid *An. gambiae s.s.* caught in Mbita Point). They were reared in a room maintained at natural ambient mean temperature and relative humidity (mean temperature and RH inside the rearing room: day, 31°C, 52% RH; night, 24°C, 72% RH). Adult mosquitoes were fed on a diet of human blood three times per week, along with glucose (6% solution *ad libitum*) continuously available on filter paper. Approval for feeding the mosquitoes on human subjects was obtained from the Kenya National Ethical Review Board (protocol number KEMRI/RES/7/3/1). Fully engorged females were then allowed to lay eggs in oviposition cups (4 cm diameter, 2 cm depth) placed inside the cages. Eggs were collected the following day and dispensed into plastic trays (25 cm long × 20 cm wide × 14 cm high) filled to a depth of 8 cm with filtered water collected from Lake Victoria. Upon hatching, larvae were reared in these pans at densities of 100-150 per tray and fed fish food (Tetramin®) three times per day (the total amount of food provided was 0.3 grams tetramin /100 larvae/day). Pupae were collected from rearing trays and transferred to an insectary where they were kept in standard 30 × 30 × 30 cm mesh-covered cages in which rolls of filter-paper soaked in 6% glucose solution were provided.

Similar rearing procedures were used in rearing mosquitoes at Duduville campus except that the circadian rhythm was reversed (light: 0301-1500hr, and darkness 1501-0300hr) and water from Kiamombi springs in Kiambu, Kenya used in raising the larval stages. The experimental mosquitoes were allowed access to 6% glucose until they were 3-5 days thereafter fed on gametocyte-positive blood. They were then maintained on glucose solution until day 7 (oocyst-stage *Plasmodium*-infected) and 12 (sporozoite-stage *Plasmodium*-infected) post-infection when bioassays were carried out. Controls for infected mosquitoes consisted non-blood fed mosquitoes of the corresponding ages.

3.2 Gametocyte carriers

School children below the age of 10 years in Mbita area (Gembe East location, Mbita district, Homa Bay County) were screened for gametocytes by preparing thick and thin smears which were stained for 20 minutes with 10% Giemsa and examined with a light microscope (100x oil immersion lens). All gametocyte carriers with malaria species other than *P. falciparum* or with low gametocyte density (<2 gametocytes/200 red blood cells) were excluded. Selected carriers and their parents were informed about the purpose of the study and informed consent obtained. From those who consented, 5ml of venous blood was collected into a heparinized vacutainer tube. The blood was centrifuged to isolate red blood cells to which artificial serum was added. This suspension was then used in infecting the mosquitoes. All malaria positive pupils were treated with antimalarial drugs.

3.3 Experimental infection

These experiments were carried out in Mbita. Three-five day old female *An. gambiae* were used for experimental infection. Four hundred female mosquitoes were allowed to feed for 15 minutes on a membrane feeder with blood from gametocyte carrier (Figure

3.1). Only blood-fed mosquitoes were selected and maintained on 6% glucose solution for subsequent bioassays. Thirty mosquitoes were dissected seven days post-infection for oocyst stage diagnosis as described by Benedict (2007) and the infection rate and density recorded. The infection procedures were repeated three times with blood from three different donors.

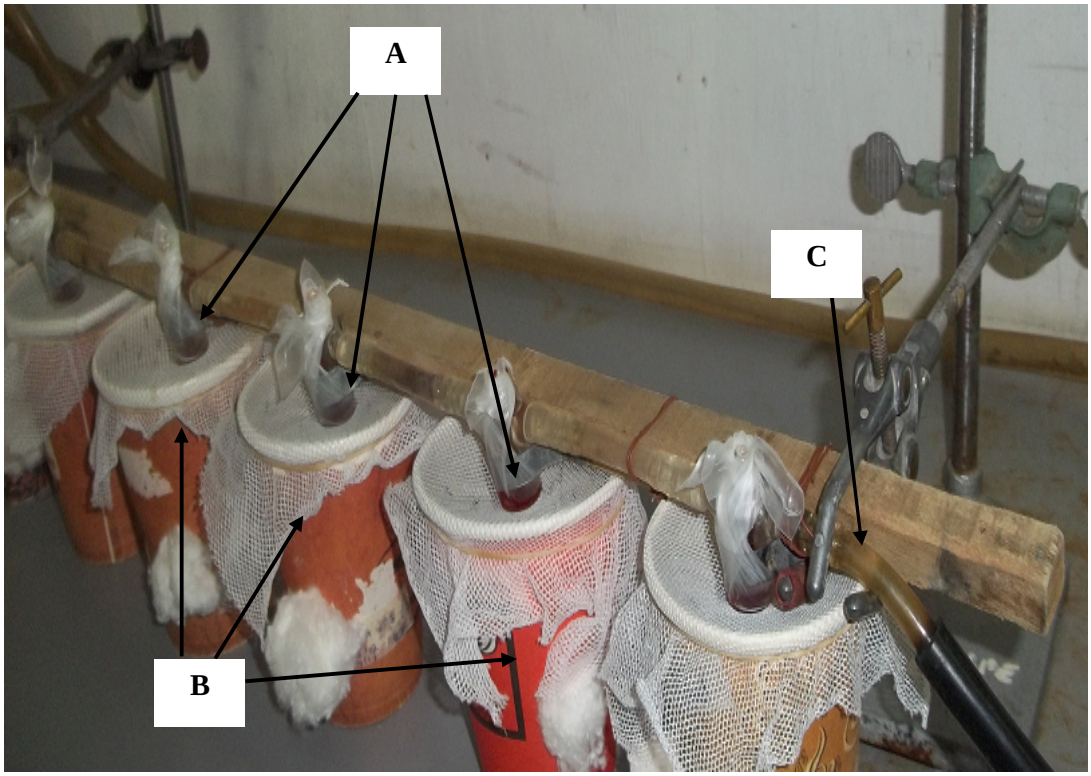

Figure 3.1 Membrane feeder infection set up showing membrane feeder containing gametocyte-positive blood (A), plastic cups containing 50 female *An. gambiae* each (B) and teflon tube (C) supplying water at 37 ^{o}C.

3.4 Plant species

Parthenium hysterophorus, *R. communis* and *B. pilosa* seedlings (Figure 3.2) were collected in Mbita Point located on the southern shore of the Winam gulf of Lake Victoria in Nyanza Province, Kenya (00^{o}25' S, 34^{o}13' E). The three plants were identified

with the aid of scientists at the National Museum, Kenya. The plant seedlings were transplanted into potted soil and then maintained in a screen house under ambient conditions. They were watered daily and used at flowering stage. The plants were transferred to the laboratory at least 3 hours prior to bioassays and allowed to acclimatize under red fluorescent light.

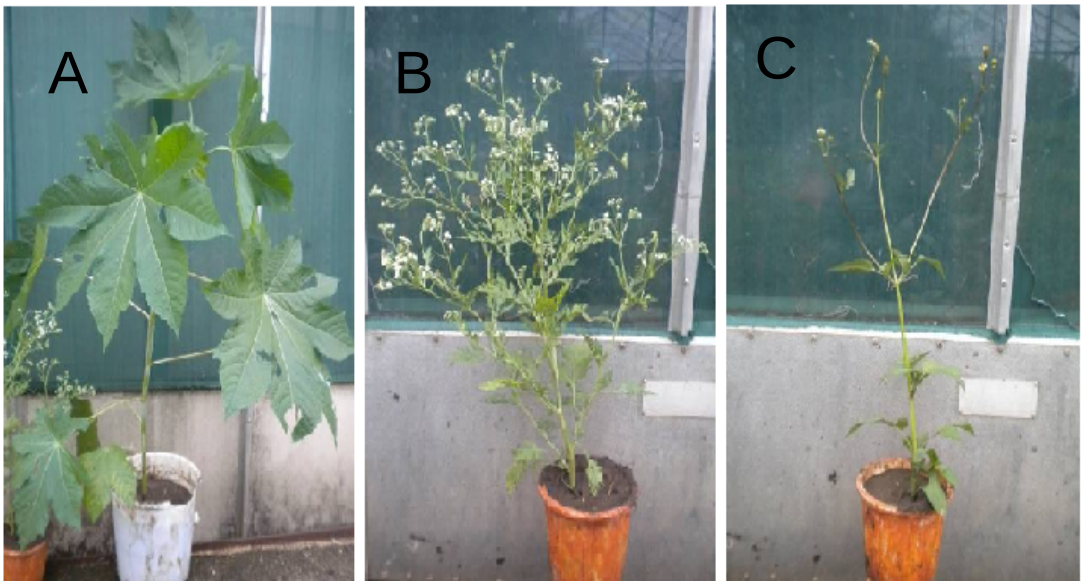

Figure 3.2 Plant species used in the study which include R. communis (A), P. *hysterophorus* (B) and *B. pilosa* (C).

3.5 Dual choice olfactometer assay

Bioassay was done using a dual choice olfactometer, similar to that described by Torto *et al.* (2010) (Figure 3.3). Briefly, air from a compressed air tank was first purified by passing it through activated charcoal and then it was delivered into each arm of the olfactometer at 260 ml/minute flow rate. A vacuum line powered by a fan pulled air from the centre of the olfactometer at 710 mm/minute. Two 40-W red fluorescent light bulbs placed above the centre of the olfactometer illuminated the test arena evenly. Uninfected,

oocyst-stage and sporozoite-stage *Plasmodium*-infected *An. gambiae* were assayed separately on host plant attraction to all the three plant species. The experimental plants were assayed separately against blank control (consisting of purified air only, hereafter referred to as control) and against each other. The positions of the experimental plants and the control in the olfactometer arms were alternated between the two arms of the olfactometer to prevent positional effect. Ten female mosquitoes were used in each bioassay, the bioassays replicated five times with fresh potted plant in each replicate. All bioassays were conducted at a temperature of 24°C, and relative humidity of 72%. The study was conducted between 1900-2100 hours under red fluorescent light (40 W) and each bioassay lasted 10 minutes.

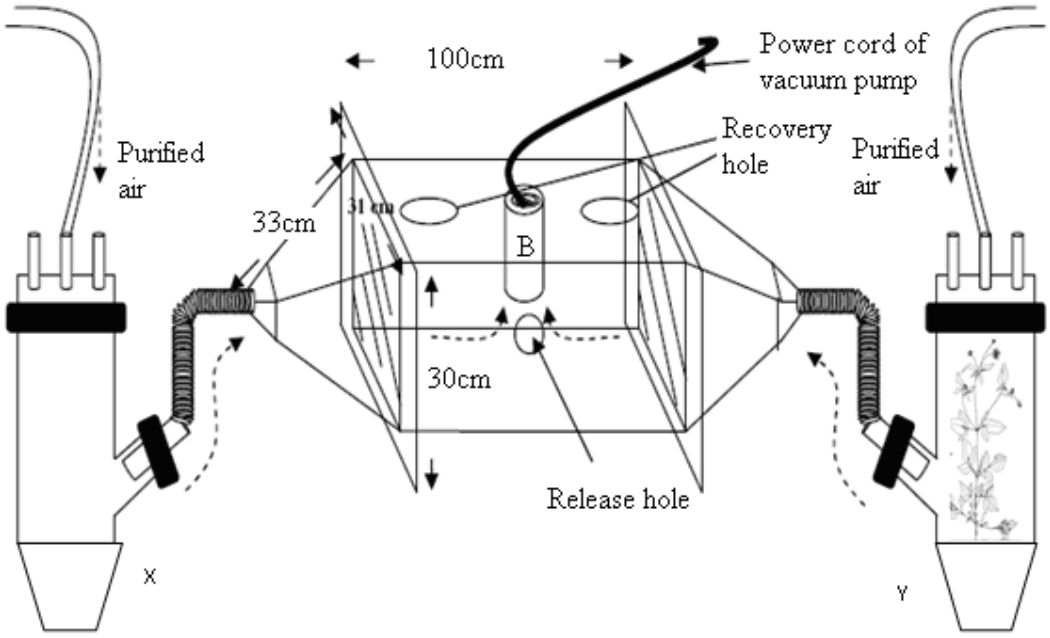

Figure 3.3 Dual choice olfactometer set up (Torto *et al.*, 2010). The broken arrows point the direction of flow of air in the olfactometer while B is the vacuum pump.

3.6 Plant probing assay

This experiment was used to study the responsiveness of malaria vectors, *An. gambiae,* to the experimental plants. Each of the three plant species were assayed separately for each of the three groups of mosquitoes (uninfected, oocyst-stage *Plasmodium*-infected and sporozoite-stage *Plasmodium*-infected mosquitoes). Ten mosquitoes were released into a 60 x 60 x 60 cm cage containing one potted plant. They were observed for 30 minutes for landing and probing activity. The experiments were conducted between 1900-200 hours under rearing environmental conditions of mean temperature of 24 °C and relative humidity of 72%. Data was collected for the number of mosquitoes landing and probing the leaves and flowers at 10 minutes intervals (Figure 3.4). The peak probing response within the 30 minutes was used in the final analysis and the data replicated three times. The mosquitoes used in the bioassay and parts of the plants were preserved for further chemical analysis using coupled gas chromatography-mass spectrometry (GC-MS).

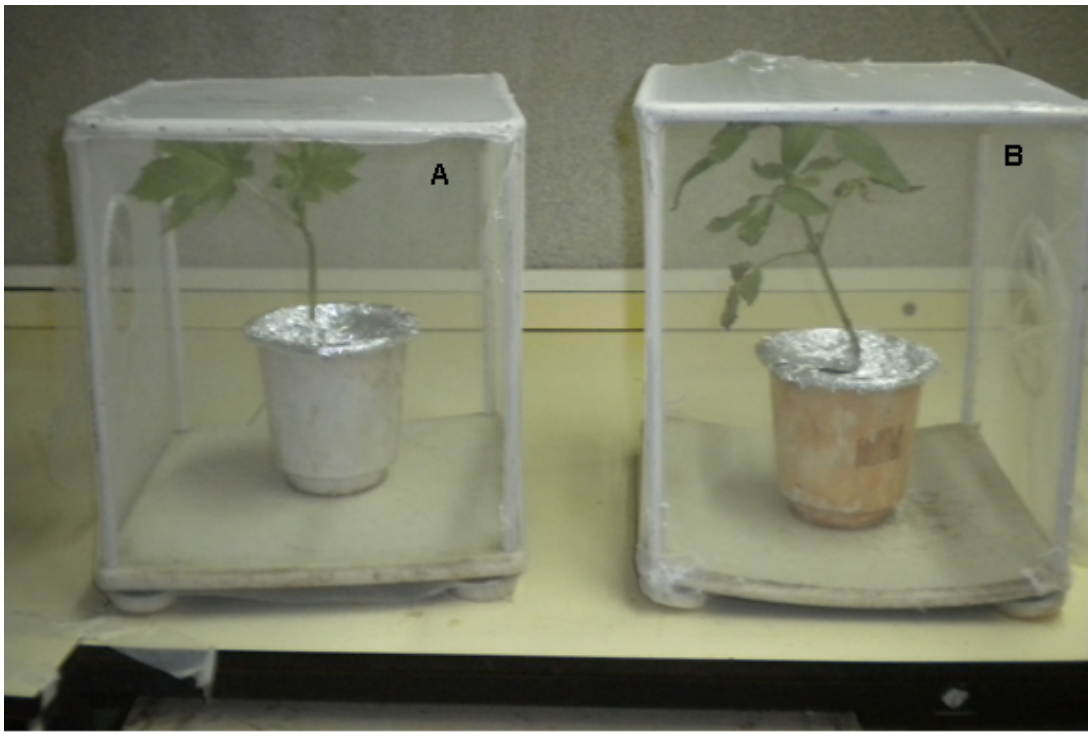

Figure 3.4 Set up of plant-probing assay showing potted *R. communis* (A) and *B. pilosa* (B) in netted cage.

3.7 Sugar analysis

Five mosquitoes from each set of probing assay (totalling fifteen for each plant species) were macerated in 2 ml pyridine (Sigma®) for 3 days. They were then derivatized with 100 µl pyridine and 100 µl *N*-Methyl-bis trifluoro acetamide (MBTFA) (Sigma®) at 60 ^{o}C for 1 hour. The product was analysed on GC-MS. Similar procedures were used to analyse sugar content in one gram of leaves and one gram of flowers from each plant species used in the probing assay. Controls included fifteen mosquitoes fed on 6% glucose alone and one gram of glucose used to prepare their solution. HP-6890 GC coupled to an HP5973 mass spectrometer (EI, 70 eV, Agilent, Palo Alto, California, USA) equipped with an HP-1 column (30 m . 0.32 mm internal diameter . 0.25 µm, Agilent, Palo Alto, California, USA) was used. The oven temperature was held at 35 °C for 3 minutes, then programmed at 10 °C /minute to 280 °C and maintained at this temperature for 10 minutes.

3.8 Identification of secondary metabolites

Five mosquitoes from each set of probing assay (totalling fifteen for each plant species) were dissected and the mid-gut extracted in dichloromethane. The mid gut extracts as well as one gram of fresh flowers and one gram of leaves of each plant species were separately analysed by GC-MS for secondary metabolites under the operating conditions described in section 3.7.

3.9 Collection of volatiles

Volatiles emanating from the aerial parts of potted plants of *R. communis, P. hysterophorus* and *B. pilosa* were collected by aeration and adsorption using Super-Q traps (30 mg, Alltech, Nicholasville, KY). An automated volatile entrainment system

(Talento 800 time switch {Grässlin}) programmed for 12 hours entrainment during the day (0700-1859 hours) and night (1900-0659 hours) was employed in capturing volatiles released (Figure 3.5). The Super-Q trappings were eluted using 200 µL GC-MS-grade dichloromethane (Burdick and Jackson, Muskegon, Michigan, USA), and elute stored at -80 °C. An aliquot of the eluted volatiles was isolated for electrophysiological activity using coupled gas chromatography-electroantenographic detection (GC-EAD) while the remaining part was analyzed on GC-MS to identify and quantify the various-electrophysiologically-active compounds. The volatile collection was replicated three times.

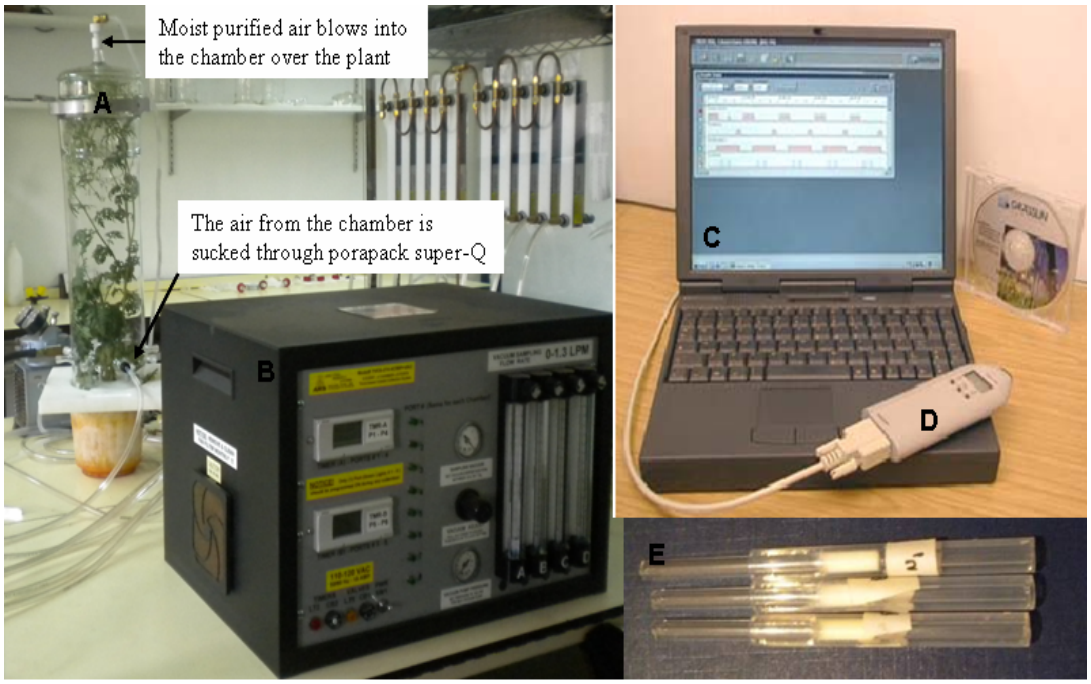

Figure 3.5 Volatile collection using talento volatile collection system. The components include potted plant in air-tight glass chamber (A), talento time switch (B), personal computer installed with programming software (C), Taxxi unit for transferring programme from personal computer into talento time switch (D) and porapack super-Q for trapping volatiles emanating from the potted plant.

3.10 Volatile analysis

Analysis of volatiles from the three plant species was carried out using a coupled GC-EAD Hewlett-Packard (HP) 5890 Series II gas chromatograph equipped with an HP-1 column (30 m x 0.32 mm ID x. 0.25 µm, Agilent, Palo Alto, California, USA) with nitrogen as the carrier gas (Figure 3.6). Volatiles were analysed in split mode at an injector temperature of 280 °C and a split valve delay of 5 minutes. The oven temperature was held at 35 °C for 3 minutes, then programmed at 10 °C/minute to 280 °C and maintained at this temperature for 10 minutes. The column effluent was split 1:1 for simultaneous detection by flame ionisation detector (FID) and EAD. For EAD detection, silver-coated wires in drawn-out glass capillaries (1.5 mm I.D.) filled with Ringer saline solution (Kugel, 1977) served as reference and recording electrodes. Antennal preparations were made by first cutting the base of the head and distal end of antenna with a scalpel. The reference electrode was connected to the base of the head, and the recording electrode was connected to the cut apical tip of the antenna. Chlorinated silver-silver chloride junctions were used to maintain electrical contact between the electrodes and input of preamplifier. The analog signal was detected through a probe (INR-II, Syntech_, Hilversum, the Netherlands), captured and processed with a data acquisition controller (IDAC-4, Syntech, the Netherlands), and later analyzed with a software (EAG 2000, Syntech) on a personal computer. An aliquot (5 µl) of the Super Q extract of plant volatiles was analyzed with fresh female antenna. This was replicated three times using fresh antenna.

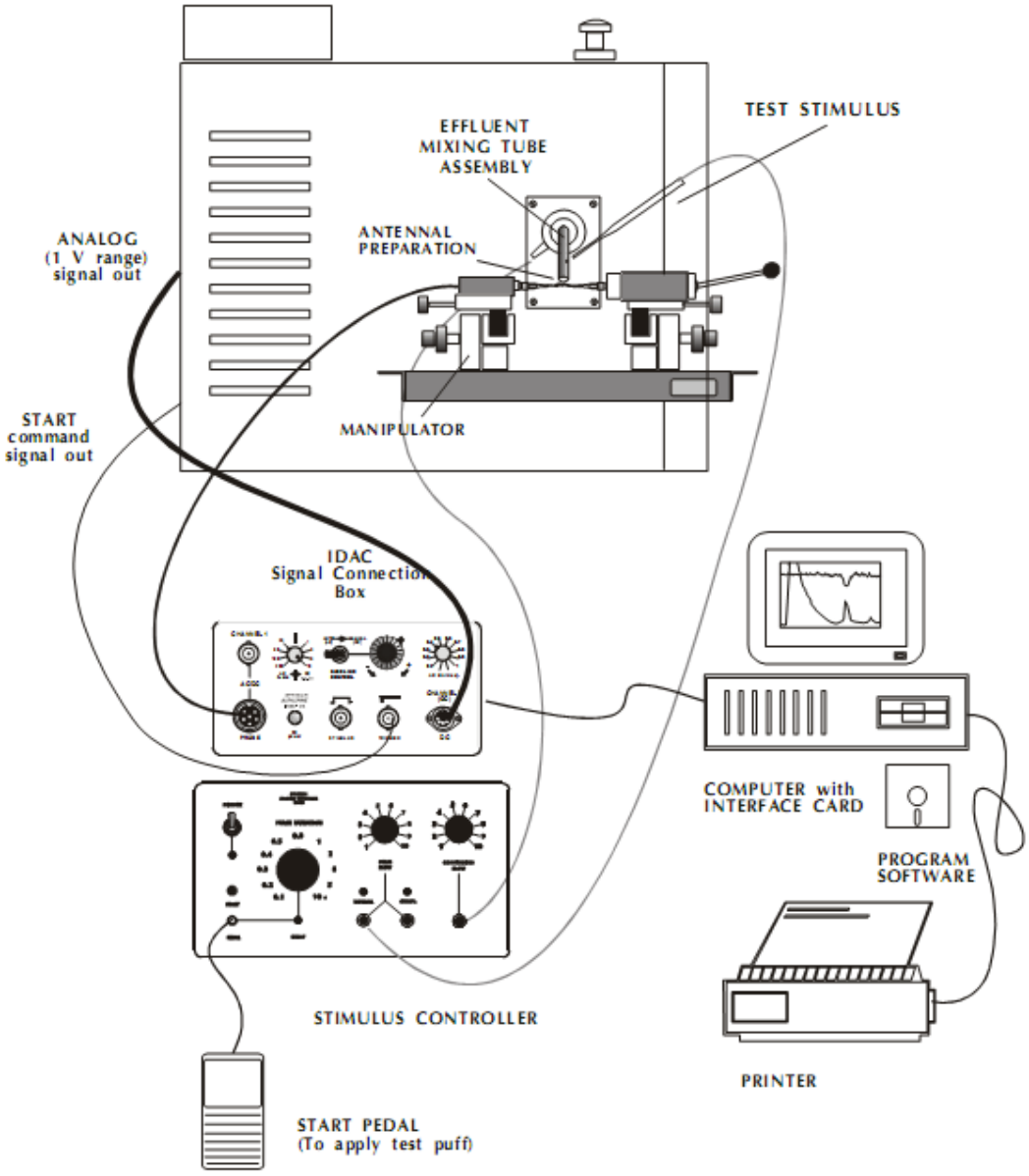

Figure 3.6 Typical Syntech GC-EAD recording arrangement showing how the antenna is positioned for detection of separated compounds from the gas chromatography.

Super Q extracts were also subjected to GC-MS analyses on an HP-6890 coupled to an HP5973 mass spectrometer (EI, 70 eV, Agilent, Palo Alto, California, USA) equipped with an HP-1 column (30 m . 0.32 mm internal diameter . 0.25 µm, Agilent, Palo Alto, California, USA) with helium as the carrier gas. The oven temperature was held at 35 °C

for 3 minutes, then programmed at 10 °C/minute to 280 °C and the maintained at this temperature for 10 minutes. For analysis, a 1 µL containing 29.35 ng of internal standard (methyl salicylate) was prepared and added to 39 µL of each volatile extract and 1 µL analyzed injected into GC-MS. EAD-active compounds were identified by comparing their mass spectral data with those in the library (NIST, 98K) of the mass spectrometer and by retention times and GC-EAD analysis with authentic commercial samples.

3.11 Chemicals

The following standards of the identified active plant compounds were used: β-pinene (Chemika, 99.5%), *E*-β-ocimene (Chemika, 98%), E-linalool oxide (Aldrich), linalool (Fluka, 97%), and *E*-β-farnesene (Bedoukian Research, CT, USA), hexanal (Aldrich, 98%), heptanal (Aldrich, 99%), nonanal (Aldrich, 99%), and decanal (Aldrich 99%).

3.12 Bioassay with different blends

A blend of the EAG-active compounds was prepared in a ratio simulating that of *P. hysterophorus* (the most attractive plant species among the three experimental plants) to make a stock solution. Three different stocks of blends were prepared comprising of 74 µg of terpenes only (β-pinene, *E*-β-ocimene, *E*-linalool oxide, linalool, and E-β-farnesene in the ratio 32:14:6:11:37); 85 µg of aldehydes only (hexanal, heptanal, nonanal, and decanal in the ratio 18:7:47:28); and 100 µg of both aldehydes and terpenes (hexanal, heptanal, nonanal, decanal, β-pinene, E-β-ocimene, linalool oxide, linalool and E-β-farnesene in the ratio 2:1:28:11:5:10:7:4:32) dissolved in pentane and made up to 1000 µl. The stock solution was diluted to give 0.5 ng/µl, 1 ng/µl, 2 ng/µl, 4 ng/µl, 8 ng/µl, and 16 ng/µl concentrations, 200 µl of which was dispensed in100 mg of Luna dental roll (Roeko®, Langenau, Germany) and allowed to set for 1 hour at room temperature for the

solvent to evaporate. This was tested against blank control (treated similarly with solvent only) in the dual choice olfactometer. A dual choice olfactometer set up similar to the one described by Torto *et al*. (2010) was used with 10 uninfected female mosquitoes tested per assay and the assays replicated five times. The mosquitoes responding to the test and control sources were counted and recorded for each dose. The most attractive doses of each blend of compounds were then assayed against potted *P. hysterophorus* in a dual choice olfactometer.

3.13 Statistical analysis

The dual choice assay data was subjected to two tail proportional analyses at 95% confidence interval. The no-choice assay data was subjected to square root transformation and analysed using ANOVA and means separated using Tukey's HSD post hoc test. Differences in the amount of sugar ingested and that in the leaves and flowers were compared using ANOVA and Tukey's HSD post hoc test. Day and night volatile release rate was compared using paired sample t test.

A preference index (PI) for the responses to different blend preparations data was calculated according to the formula:

$$PI = [(SS - NSS)/(SS + NSS)] \; x \; 100$$

where *SS* is the number of mosquitoes on the test odour zone and *NSS* the number of mosquitoes on the control odour zone (Carlsson *et al.*, 1999). The PI would be zero if equal numbers of mosquitoes were found on each side of the chamber and ± 100 if all mosquitoes preferred one side of the chamber. A positive value indicates a majority of the mosquitoes on the odour zone, while a negative value indicates the converse.

Within each group a single sample t test was performed on the PI to test if the response to the odour differed from zero. For differences between the doses, analysis of variance (ANOVA) test was used to detect interaction between the different blends of standards and the concentration, comparing means of preference indices. All statistical analysis was carried out using R software (R Development Core Team, 2010).

CHAPTER FOUR: RESULTS

4.1 Experimental infection

An average infection rate of 53.73% (Mean oocyst density 5.15 ± 0.87) was achieved from the three experimental infections of *An. gambiae* with *P. falciparum.* See figure 4.1 showing mid-gut stage of the parasite.

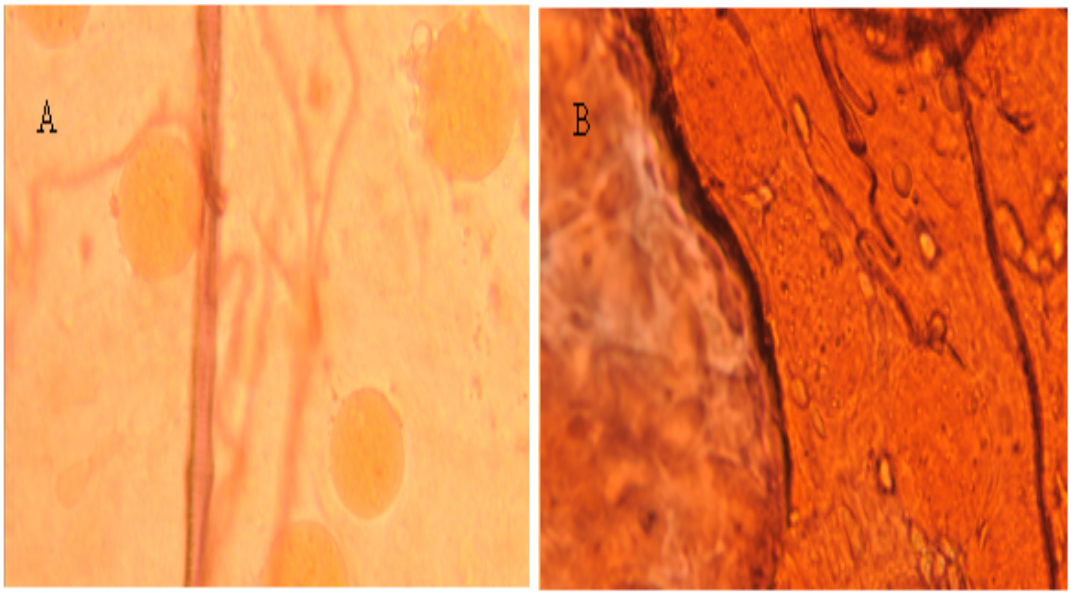

Figure 4.1 Normal (A) and encapsulated (B) oocyst of *P. falciparum* in *An. gambiae* mid gut basement membrane. Mosquito mid guts were examined 7 days post-infection

4.2 Dual choice olfactometer response

Over 60% of all the three groups of mosquitoes (uninfected, oocyst-stage *Plasmodium*-infected and sporozoite-stage *Plasmodium*-infected mosquitoes) flew upwind towards plant odour source in the olfactometer. However, the responses by the uninfected mosquitoes to odours from *R. communis* and *B. pilosa* were not significantly different from the controls (Figure 4.2). When the three plant species were assayed against each other, no significant difference was detected, but *P. hysterophorus* was the most preferred and *B. pilosa* the least preferred (4.3).

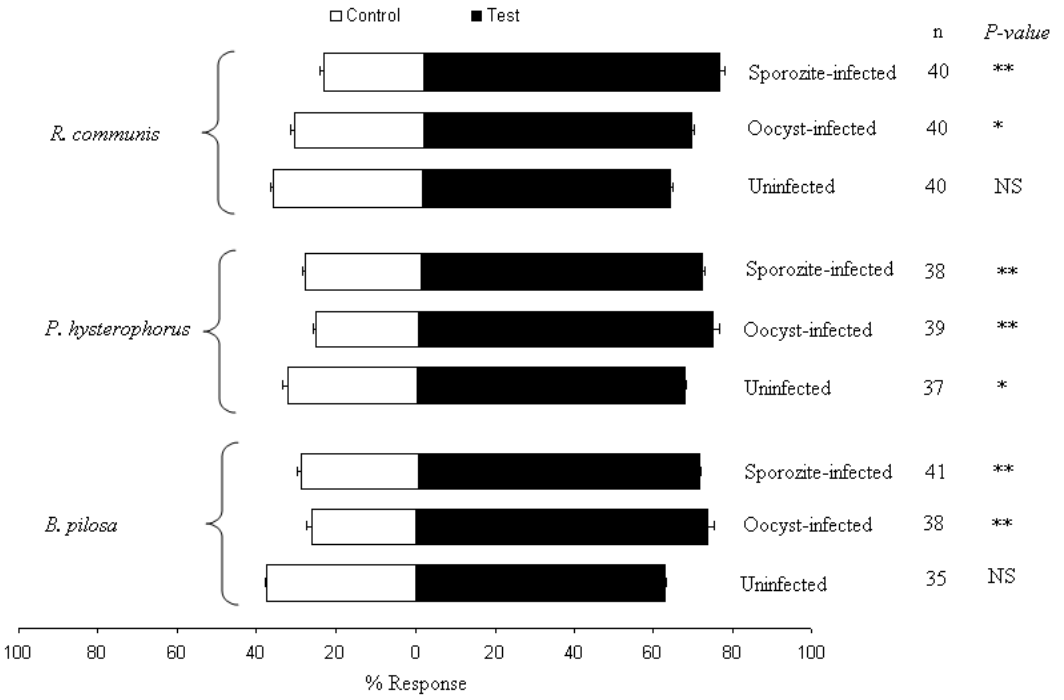

Figure 4.2 Plant odour responses by uninfected and Plasmodium-infected *An. gambiae*. Control = without plant, test = with plant, n = total number of respondents, NS = not significant, * = significant at $P < 0.05$, and ** = significant at $P < 0.01$.

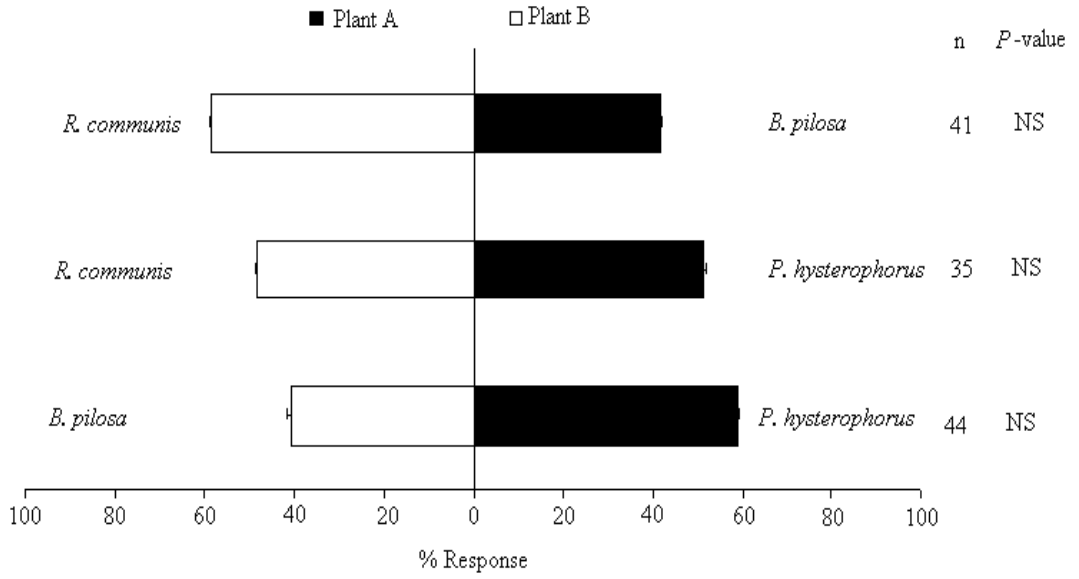

Figure 4.3 Olfactometric response of *An. gambiae* to different plant species. n = total number of respondents, and NS = not significant at 0.05.

4.3 Plant probing assay

Plant species and infection rate significantly influenced the probing activity of *An. gambiae* at sporozoite stages (F = 13.3365, df = 2, P <0.001; and F = 6.1796, df = 1, P <0.05 respectively), but not at oocyst stage of infection (F = 1.2478, df = 2, P = 0.2938 and F = 0.9892, df = 3, P = 0.4032 respectively). Probing was more intense on *P. hysterophorus* than *R. communis and B. pilosa* at sporozoite-stage of infection (Figure 4.4).

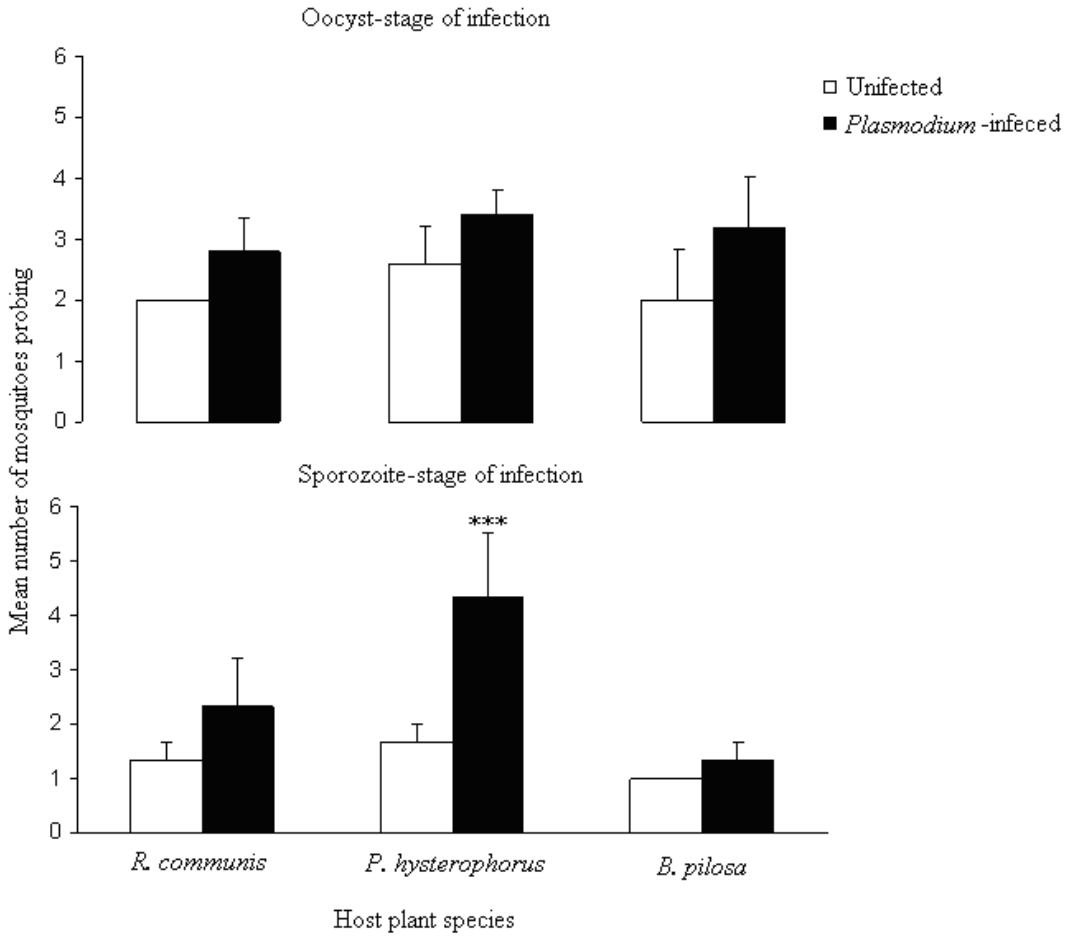

Figure 4.4 Probing responses by uninfected and *Plasmodium*-infected *An. gambiae*.

4.4 Sugar analysis

Six plant sugars including glucose, galactose, fructose, rhamnose, sucrose and maltose (identified using GC-MS library (Adams2.L, Chemecol.L and NISTO5a.L) and synthetic standards) were detected in the mosquito extracts and their peak areas used to quantify the amounts present in mosquitoes and corresponding plant species. There was significant difference in the mean total amount of sugars in *R. communis*, *P. hysterophorus* and *B. pilosa* ($F = 15.86$, $df = 5$, $P < 0.001$ respectively). *Ricinus communis* extraflorals had the highest amount of all the sugars while *B. pilosa* leaves had the least amount (Table 1). Maltose was the most abundant sugar among all the three plants while rhamnose was the least abundant. Between the three plant species, maltose was significantly higher in *R. communis* than *P. hysterophorus* and *B. pilosa* ($F = 35.30$, $df = 2$, $P < 0.001$). In terms of sugar ingestion, the amount of sugars ingested by the mosquitoes were significantly different ($F = 3.927$, $df = 2$, $P < 0.05$), the highest amount being obtained from *R. communis* and the least from *B. pilosa* (Table 1).

Table 1 Mean sugar contents of plant leaves and flowers of *R. communis*, *P. hysterophorus* and *B. pilosa* and the amount ingested by *An. gambiae* following probing assays. The values denoted with letter a, b and ab is significantly different between the three plants.

Plant species	**Sugar**	**Amount in leaves ± SEM (ng/mg)**	**Amount in flowers ± SEM (ng/mg)**	**Amount ingested ± SEM (ng/mg)**
R. communis	Glucose	129.51 ± 33.65	365.93 ± 65.67	76.35 ± 19.85
	Galactose	701.24 ± 119.08	17.46 ± 5.73	16.14 ± 6.04
	Rhamnose	9.46 ± 2.08	44.57 ± 11.29	20.63 ± 1.53
	Fructose	198.63 ± 50.36	196.65 ± 53.76	57.73 ± 12.74
	Sucrose	225.03 ± 51.81	170.93 ± 33.01	24.61 ± 5.31
	Maltose	4826.39 ±345.55 (xy)	6785.31 ± 462.99 (x)	130.19 ± 28.02
	Total	**6084.26 ± 164.47 (a)**	**7580.85 ± 842.40 (a)**	**325.65 ± 0.64**
P. hysterophorus	Glucose	392.11 ± 55.61	392.11 ± 46.61	43.80 ± 3.32
	Galactose	491.73 ± 33.81	463.83 ± 48.95	35.45 ± 5.58
	Rhamnose	43.69 ± 4.97	42.59 ± 10.94	20.94 ±3.35
	Fructose	202.79 ± 50.77	84.08 ± 15.42	31.14 ± 5.51
	Sucrose	85.68 ± 15.65	79.75 ± 24.87	7.42 ± 3.21
	Maltose	3500.91 ± 242.33(xy)	1382.71 ± 168.21 (y)	80.96 ± 13.97
	Total	**4716.91 ± 265.33(ab)**	**2445.07 ± 101.49 (b)**	**219.71 ± 5.31**
B. pilosa	Glucose	112.29 ± 40.28	334.52 ± 53.31	30.33 ± 8.12
	Galactose	381.85 ± 68.12	102.24 ± 34.87	22.41 ± 5.68
	Rhamnose	49.09 ± 11.63	47.43 ± 15.66	15.44 ± 1.20
	Fructose	113.13 ± 37.28	192.50 ± 51.88	20.89 ± 3.49
	Sucrose	89.93 ± 22.71	87.32 ± 5.20	5.56 ± 0.06
	Maltose	1037.05 ± 291.53 (y)	1623.07 ± 202.81 (y)	37.35 ± 8.55
	Total	**1783.34 ± 228.35 (b)**	**2387.08 ± 119.80 (b)**	**131.98 ± 4.03**

4.5 Secondary metabolites

The secondary metabolites detected in mosquitoes following probing assays were identified as copaene (1), germacrene D (2), parthenin derivatives (3, 4, 5), parthenin (6) and decahydro-6a-hydroxy-6,9a-dimethyl-azuleno [4,5-b] furan-2,9-dione (dihydroparthenin) (7) from those probing on *P. hysterophorus* (Figure 4.5); and benzene acetaldehyde (1), b-elemene (2), copaene (3), germacrene D (4) and 1-phenyl-hepta-1,3,5-triyene (5) from those probing on *B. pilosa* (Figure 4.6). None of the *R. communis* secondary metabolites was recovered from the mid-gut and whole insect extract of mosquitoes probing on this plant species (Figure 4.7).

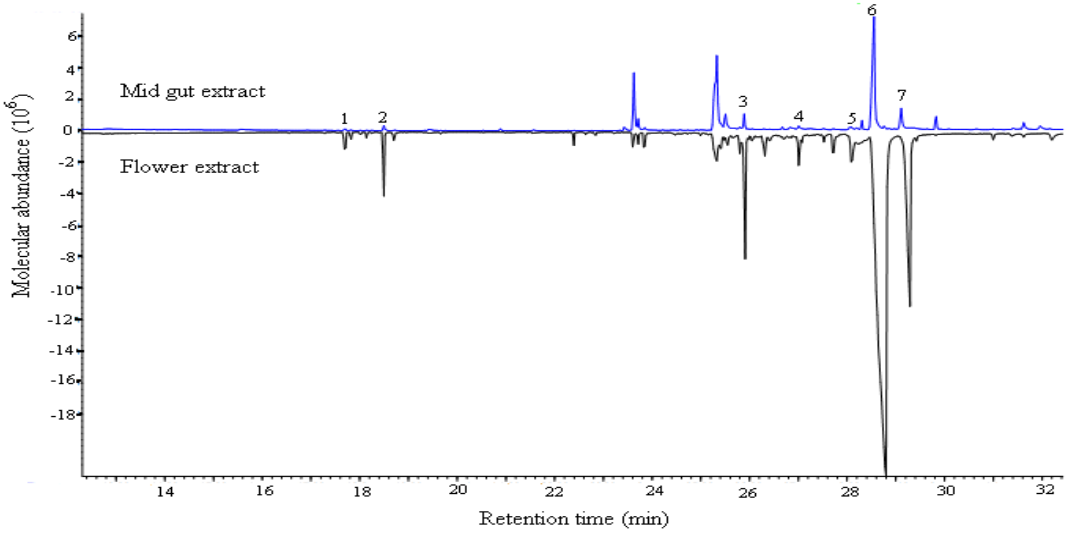

Figure 4.5 GC profile of mosquito mid-gut and *P. hysterophorus* flower extract following probing assay. The target peaks are copaene (1), germacrene D (2), parthenin derivatives (3, 4, 5), parthenin (6) and dihydroparthenin (7).

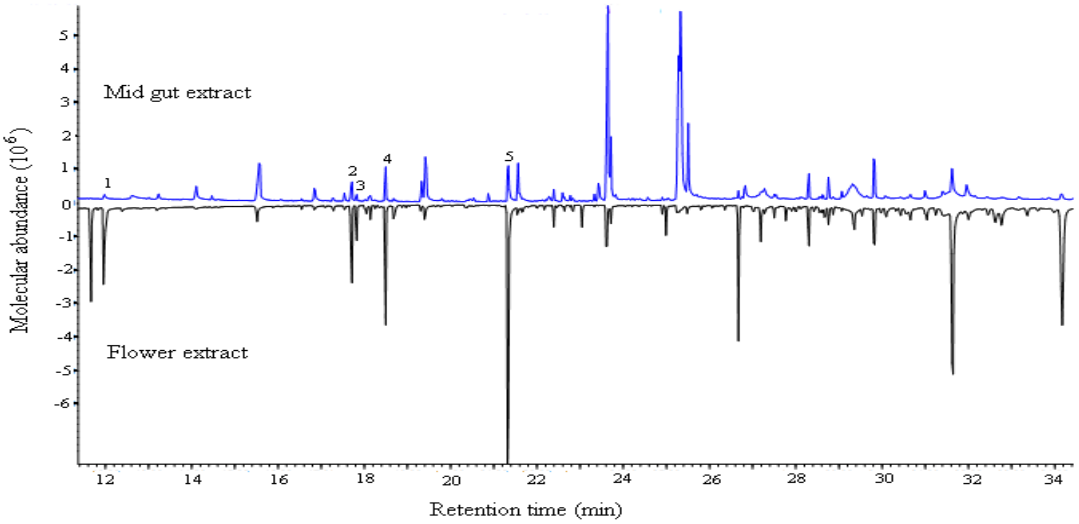

Figure 4.6 GC profile of mosquito mid-gut and *B. pilosa* flower extract following probing assay. The target are benzene acetaldehyde (1), b-elemene (2), copaene (3), germacrene D (4) and 1-phenyl-hepta-1,3,5-triyene (5).

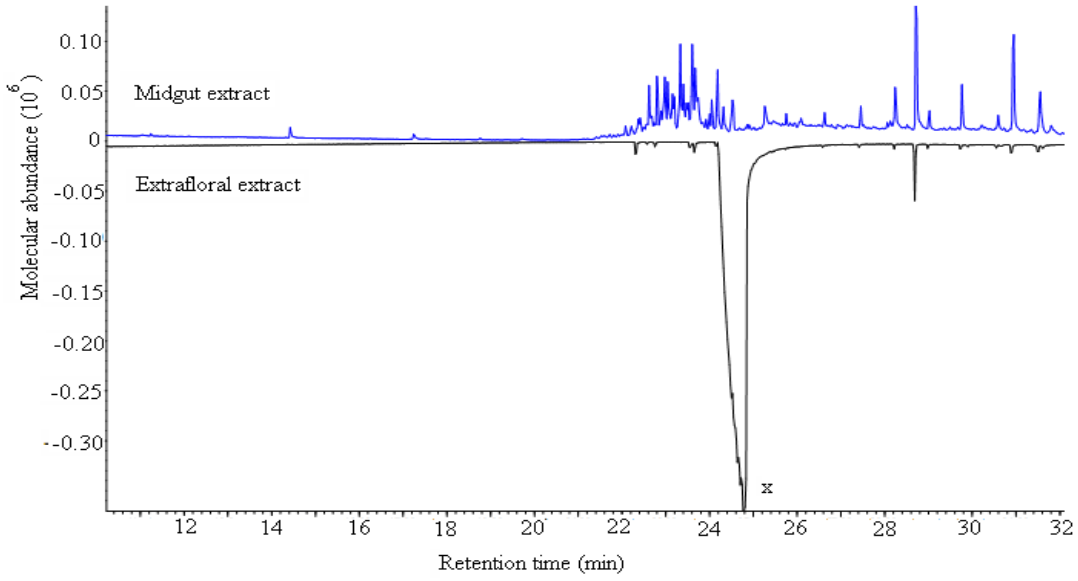

Figure 4.7 GC profiles of mosquito mid-gut and *R. communis* extrafloral extract following probing assay. None of *R. communis* secondary metabolites, including the most abundant (recinin (x)) was not detected in the mid gut of mosquitoes.

4.6 Electrophysiologically active compounds

Electrophysiological studies with plant volatiles of the three plant species recorded strong EAD responses to an array of components, most of which were aldehydes and terpenes. A total of 15 components were identified as EAD-active from *P. hysterophorus* volatiles, 12 from *R. communis,* and 8 from *B. pilosa* (Figure 4.8: A, B, and C). Hexanal, heptanal, α-pinene, camphene, β-pinene, *E*-β-ocimene, *E*-linalool oxide, and nonanal were common among all the three plant species. In addition, *P. hysterophorus* and *R. communis* had linalool and decanal in common. However, some compounds were unique to each plant species. These include 2-methyl-(E)-2-butenoic acid, δ-elemene and *E*-β-farnesene for *P. hysterophorus*; borneol and α-muurolene for *R. communis*; and 2-epi-α-funebrene and caryophellene oxide from *B. pilosa*. Electrophysiological activity of 9 of these compounds was confirmed by testing with authentic synthetic standards (Figure 4.8 D). These included β-pinene (Chemika, 99.5%), *E*-β-ocimene (Chemika, 98%), *E*-linalool oxide (Aldrich), linalool (Fluka, 97%), and *E*-β-farnesene (Bedoukian Research, CT, USA), hexanal (Aldrich, 98%), heptanal (Aldrich, 99%), nonanal (Aldrich, 99%), and decanal (Aldrich 99%).

Except for α-pinene in *R. communis,* the rate of volatile release of the identified EAD active components did not vary significantly between day and night (Figure 4.9: A, B, and C).

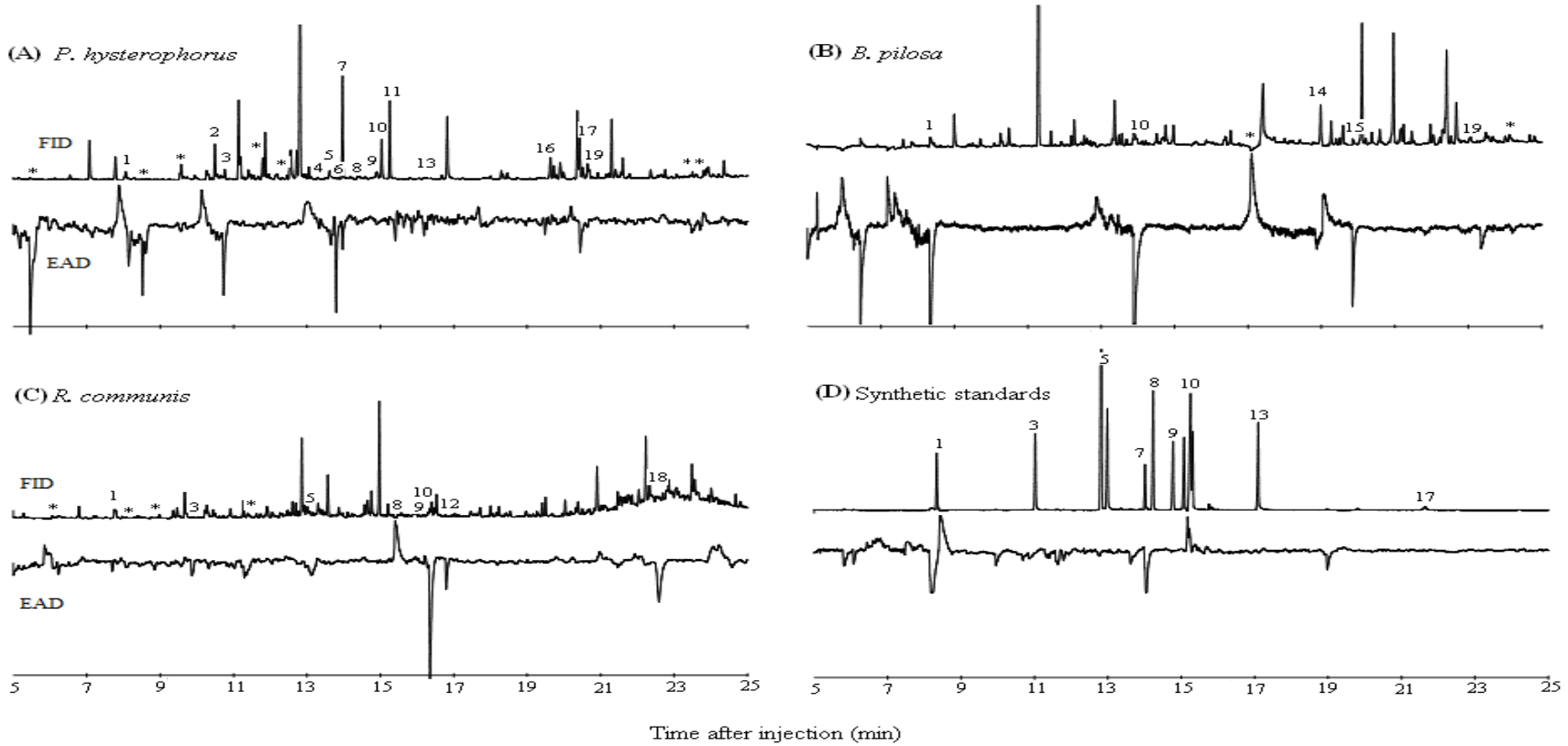

Figure 4.8 Representative flame ionization detector (FID) and electroantennographic detector (EAD) traces of female *An. gambiae* antennal responses to components from aerial volatile extracts of the three host plants and the synthetic standards. The components were identified as: hexanal (1), octanol (2), heptanal (3), octanal (4), β-pinene (5), Z-β-ocimemene (6), E-β-ocimemene (7), *E*-linalool oxide (8), linalool (9), nonanal (10), 2-methyl-(E)-2-butenoic acid (11), borneol (12), decanal (13), Hexenyl hexanoate (14), 2-epi-funebrene (15), δ-elemene (16), *E*-β-farnesene (17), α-muurolene (18), δ-cadinene (19), and unidentified (*).

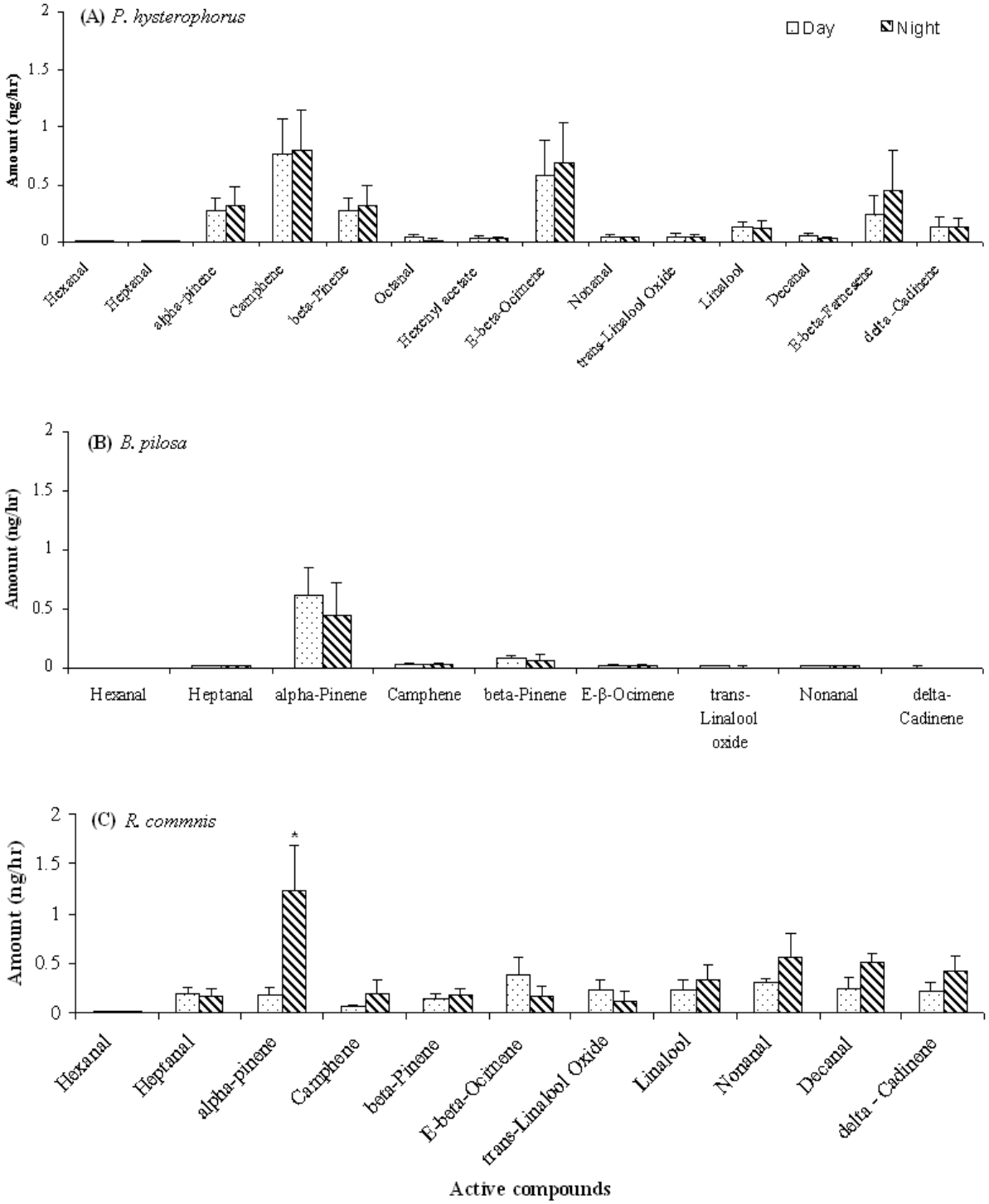

Figure 4.9 Relative amount and standard error of means (error bars) of volatiles released per hour per plant from: *R. communis*, *P. hysterophorus* and *B. pilosa*.

4.7 Bioassay with blends

Behavioural activity of 9 of these compounds was confirmed by testing with authentic synthetic standards (Figure 4.10). Optimal attractive responses for terpenes and a mixture of terpenes and aldehydes were recorded at lower doses (2ng/µl and 1ng/µl respectively) while aldehydes gave an optimal attractive response at 8ng/µl. Higher doses of terpenes only or when mixed with aldehydes resulted in repulsion of the mosquitoes.

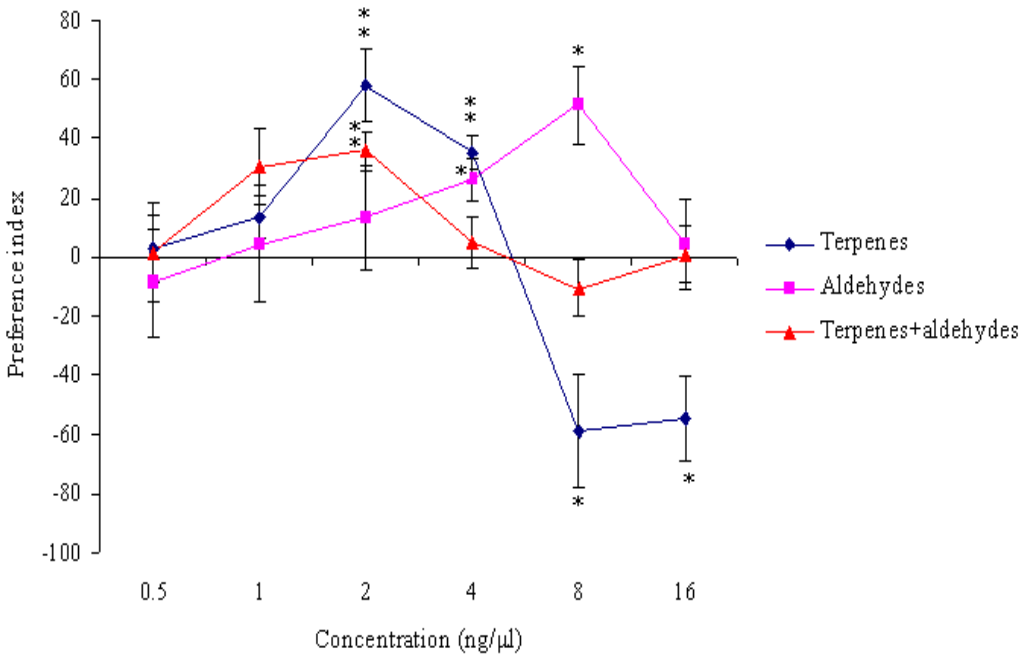

Figure 4.10 Response of uninfected *An. gambiae* to synthetic standards at different doses expressed as mean PI ± SEM. Bars capped by asterisk differ significantly from reference point (PI = 0). * = significant at 0.05 and ** = significant at 0.01.

CHAPTER FIVE: DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

More than 60% of all the three groups of mosquitoes were attracted to the odours of the three tested plants, indicative of a strong recognition of the plant odours. It was also evident that infection with *P. falciparum* significantly altered the behaviour of *An. gambiae* towards host plants in both assays. These results corroborates with reported altered vector behaviour towards vertebrate host following infection with *Plasmodium* parasites (Koella, 1999), and is important in understanding the intricate of malaria parasite development in the host vector and its transmission success. Previous studies found that the malaria parasite reduces host seeking behaviour of its host vector during the non-transmissible stages (oocyst), causing them to be more strongly attracted to human host when they reach infective stage (sporozoite) (Anderson *et al.*, 1999; Koella, 1999; Lacroix *et al.*, 2005). While this is logical in that it reduces the risk of vector mortality through vertebrate host aggression, it also poses a challenge to the vector which heavily relies on vertebrate blood for gonotrophic as well as metabolic activities. It would therefore be normal to expect the vector to turn to other alternative sources of energy to compensate for resultant deficit. This is in tandem with the results of sugar analyses showing that the mosquitoes ingest plant sugars when they probe on the plants.

Besides, the *Plasmodium* parasites have been found to impose adverse physiological cost to their host vectors (Ferguson and Read, 2002; Hurd, 2003; Tripet *et al.*, 2008), and the immune system of the vector is only able to neutralize a fraction of the initial infection (Locker, 1994; Beernsten *et al.*, 2000). Rivero and Ferguson (2003) attributed the observed increase in sugar intake by *Plasmodium* infected *An. staphensi* to possible

manipulation by the parasite to overcome the vector immune system by impairing the production of nitric oxide, an important line of defence in these species. The results here showed an overall increase in plant probing following infection with both the oocyst and sporozoite stages of the malaria parasite. Besides, the mosquitoes also ingested other secondary metabolites from the plants such as parthenin and 1-phenyl-hepta-1, 3, 5-tryne from *P. hysterophorus* and *B. pilosa* respectively which have been found to have antiplasmodial activity *in vitro* (Sharma and Bhutani, 1988; Brandão *et al.*, 1997; Oliveria *et al.*, 2004).

While *R. communis* was richer in sugar contents, *P. hysterophorus* recorded the highest probing activity with the strongest preference for this plant showing at sporozoite stage of infection. This observation augments that made by Manda *et al.* (2007b) and lends support to her suggestion that there could be plant constituents of *P. hysterophorus* that is of benefit in an unrecognized way to the vector. The detection of plant sugars and other secondary metabolites further suggest that the infected mosquitoes could be turning to this plant not only as energy source but also to boost their immune protection against the malaria parasite or at least heal the wounds left by the migrating parasite. It is also possible that the marked plant aggression at sporozoite stage could be as a result of the vector attempting to clear the parasite load from its salivary ducts. Olfactory cues are likely to play an important role in the observed preference, as was evident in this study.

This study demonstrated electrophysiological activity to a number of terpenes by the malaria vectors, suggesting the existence of terpene receptor in *An. gambiae.* However, not all the components showed activity in a single run and a number of early and late eluting active components could not be identified either as a result of masking by the

solvent peak or due to the low amounts in which they were present. Trans-*β*-farnesene, *E*-linalool oxide and δ-cadinene consistently elicited electrophysiological responses. Bowen (1992) demonstrated an olfactometer attraction of *Culex pipien* to thujone and verbenone, and was able trace their receptors in the antennae. Besides the study by Bowen (1992), no studies have been conducted to isolate mosquito attractive compound from host plants. Further, the results here showed that the malaria vectors detected plant aldehydes (hexanal, heptanal, nonanal and decanal). Aldehydes have not previously been reported to have any activity in the malaria vectors, but have been reported for other species such as *Culex quinquefascioutus* and *Ae. Aegypti* (Puri *et al.* 2006; Ghaninia *et al.*, 2008). This study therefore presents a significant bench mark in the search for more attractive odour baits that target both nectar-searching and blood-searching vectors.

The results also show variation in volatile release rates between the three plant species. *Parthenium hysterophorus* has the highest release rate as compared to *R. communis* and *B. pilosa*. It can therefore be inferred that plant odours, besides sugars and other metabolites, play a crucial role in mosquito plant interaction. Plant odours have been widely implicated as responsible for floral host location by mosquitoes with a number of mosquito species demonstrating an upwind flight towards plant odour source in olfactometer experiments (Thorsteinson and Brust, 1962; Whensler, 1972; Vargo and Foster, 1982; Healy and Jepson, 1988; Jepson and Healy, 1988; Kline *et al.*, 1990; Bowen, 1992; Hancock and Foster, 1993; Foster and Takken, 2004). However, the role and chemical nature of the plant volatiles has not been established in malaria vectors *An. gambiae* (Takken and Knols, 1999). Present study has demonstrated that these vectors respond to plant related odours, albeit under laboratory conditions. Foster and Hancock (1994) attributed the mosquito attraction to plant odour to terpenes, phenols, aliphatic

esters and aldehydes which are associated with plant fragrance. The study elucidated, for the first time, some of the specific compounds responsible for mosquito-plant interaction.

Müller *et al.* (2010) observed that the foraging activity of *An. gambiae* in the wild peaked in the early evening and early morning and attributed this to the possibility of the interactions between mosquito behaviour and the timing of volatile release by plants. Though there was no significant variation in day and night volatile release rates for all the three plant species, the dose-response studies attested to the significance of concentration in the overall mosquito-plant interactions, with terpenes being highly attractive at lower doses and aldehydes at a higher dose.

5.2 Conclusions

1. *Parthenium hysterophorus, R. communis* and *B. pilosa* release volatiles that are attractive to both uninfected and *Plasmodium*-infected *An. gambiae.* Sporozoite-stage *Plasmodium*-infected *An. gambiae* responds more strongly to *P. hysterophorus*. This response is related to the sugar content, secondary metabolites and the odour plumes of the plant.
2. The malaria vectors *An. gambiae* use *E*-β-ocimene, β-pinene, *E*-linalool oxide, linalool, *E*-β-farnesene, hexanal, heptanal, octanal, and decanal in locating *P. hysterophorus, R. communis* and *B. pilosa*. The variation in volatile release rates between these plants could partly be responsible for the discriminative feeding behaviour.
3. A blend of terpenes at a concentration of 2ng/µl, aldehydes at a concentration of 8ng/µl and a mixture of terpenes and aldehydes at a concentration of 1ng/µl give optimal olfactometric attraction of *An. gambiae*.

5.3 Recommendations

1. Further studies should be conducted to determine the role of secondary metabolites identified in this study in the fecundity, survival and vector-competence of malaria vectors.
2. The identified behaviourally active compounds should be studied individually to determine their olfactory role in these malaria vectors.
3. The blend of plant compounds identified in this study should be tested with available mosquito traps in the field to evaluate the catch efficacy and considered for possible inclusion in available odour baits to improve trap catches, particularly of outdoor resting *An. gambiae.*

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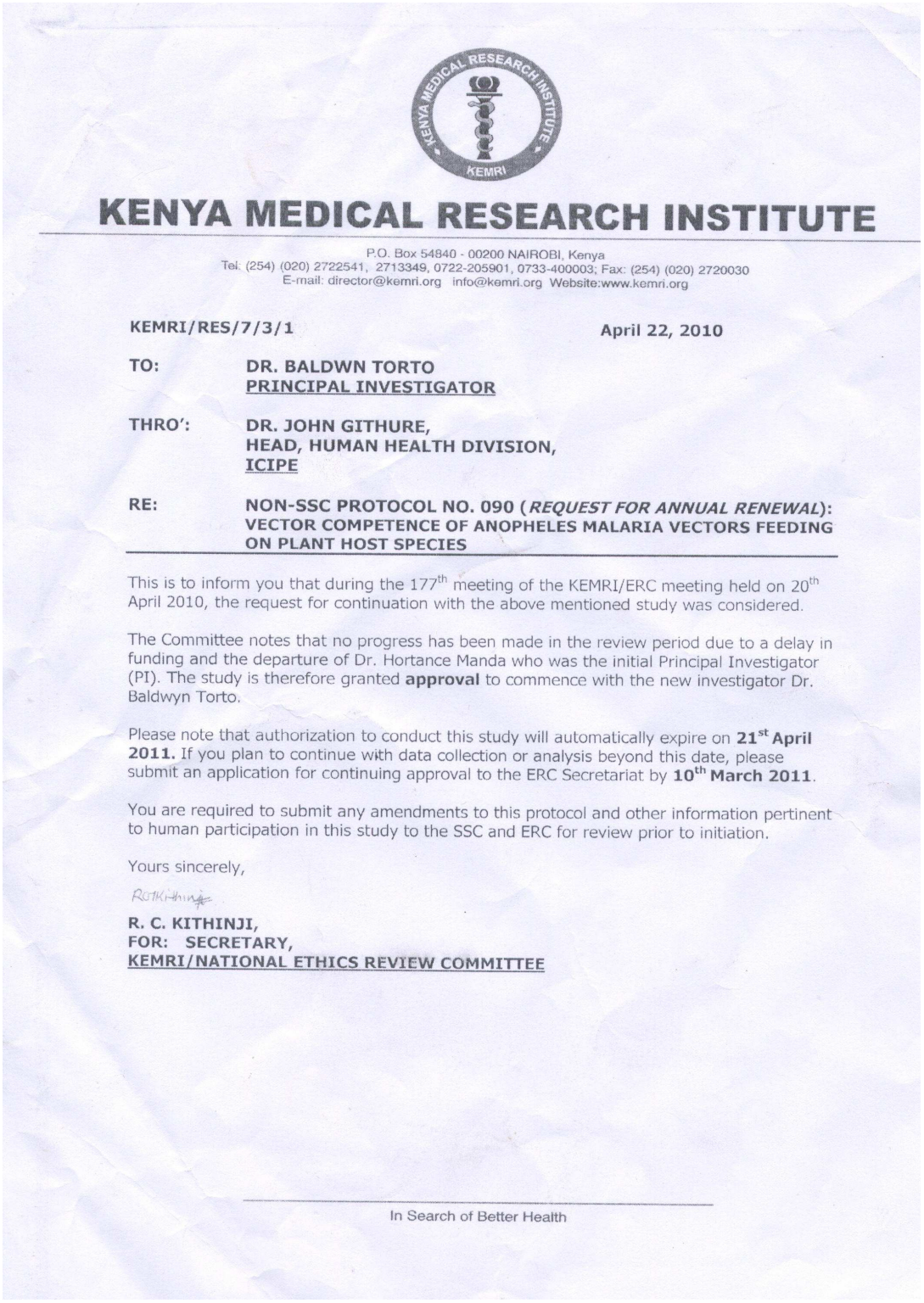
APPENDICES

Appendix 1 Screening of pupils of Nyawiya primary School for *P. falciparum* gametocytes

Appendix 2 Ethical clearance letter from Kenya Medical Research Institute



KENYA MEDICAL RESEARCH INSTITUTE

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Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **April 22, 2010**

TO: **DR. BALDWN TORTO**
PRINCIPAL INVESTIGATOR

THRO': **DR. JOHN GITHURE,**
HEAD, HUMAN HEALTH DIVISION,
ICIPE

RE: **NON-SSC PROTOCOL NO. 090 (*REQUEST FOR ANNUAL RENEWAL*): VECTOR COMPETENCE OF ANOPHELES MALARIA VECTORS FEEDING ON PLANT HOST SPECIES**

This is to inform you that during the 177th meeting of the KEMRI/ERC meeting held on 20th April 2010, the request for continuation with the above mentioned study was considered.

The Committee notes that no progress has been made in the review period due to a delay in funding and the departure of Dr. Hortance Manda who was the initial Principal Investigator (PI). The study is therefore granted **approval** to commence with the new investigator Dr. Baldwyn Torto.

Please note that authorization to conduct this study will automatically expire on **21st April 2011.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **10th March 2011**.

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the SSC and ERC for review prior to initiation.

Yours sincerely,

R.C. Kithinji

R. C. KITHINJI,
FOR: SECRETARY,
KEMRI/NATIONAL ETHICS REVIEW COMMITTEE

In Search of Better Health

Appendix 3 Informed consent agreement

Informed consent agreement

Title of Study: Vector competence of the African malaria vector *Anopheles gambiae* feeding on host plant species

Scientists in charge of this research study are: Dr. Baldwyn Torto and Dr. Patrick Sawa of *icipe*, Kenya

Purpose: We are conducting a study to better understand how environmental factors including plants affect the development of the parasites taken by mosquitoes from an infected person, and how that contribute to malaria transmission.

Screening for parasites: We are asking you/your child to participate in this research study. We would like to take a small drop of blood from your finger, by finger-prick, to examine for malaria parasites. If malaria parasites are seen on the slide, you/your child will be treated whether or not you/your child agree to participate in this study and you/your child will be followed up by the medical doctor until all the malaria parasites are cleared from the blood.

Recruitment of volunteers: If you/your child are found to have the form of the parasites (gametocytes) that infect mosquitoes, we will request you/your child to volunteer to participate in the study by allowing us to take a small amount of blood by venipuncture (6 ml which is about a teaspoon full) from you/your child arm. You/your child will be required to come to our *icipe* Centre where your blood will be taken by a clinical officer associated to our project. We will provide transport and the time taken to and from here will be about 45-60 minutes. The blood taken will be given to mosquitoes to feed on to see whether they become infected with malaria when feeding on different diets. The rest of the blood collected will be used to characterize the type of the malaria parasite present.

Risks: The taking of blood from you/your child's arm may cause a little discomfort from the stick of the needle, and may cause a small bruise. After receiving medication from the Hospital staff, we will take you/your child home or back to the school. We may request you/your child to come a week later or we would contact you/your child at home where we shall take a small drop of blood from your finger to check whether parasites are still present. If malaria parasites are seen on the slide, the Hospital staff will provide you/your child with another treatment that works better. In case you/your child have to come back during appointment visit, a transport fee will be given to you/your child. If any problems develop as a result of drawing blood, the same hospital staff will provide prompt and appropriate medical attention.

Benefits: You/your child will receive no direct benefit from participating in this study. The results of the study, however, may help the scientific community learn about malaria in mosquitoes and how malaria transmission can be prevented

Appendix 4 Informed consent form for minors

Informed consent form for minors

Confidentiality: The medical information will be kept private and your blood sample that is fed to the mosquitoes will have no bearing to you/your child' name

Payment: You/your child will not receive any payment for your participation in this study. We may however provide some money for your transportation costs and for meals if necessary.

I,.....(name of parent/guardian) being lawful parent/guardian for my child do hereby consent for the child named.....to participate in the research project titled: "Vector competence of the malaria vector *Anopheles gambiae* feeding on host plant species"

I have been given the opportunity to ask questions concerning this project. Any such questions have been answered to my full satisfaction. Should any further questions arise concerning the right of this child, I may contact Dr. Patrick Sawa, *icipe*, P.O. Box 30, Telephone 05922218, Mbita; or Dr. Baldwyn Torto, *icipe*, P.O. Box 30772, Telephone 861680, Nairobi; or KEMRI/NERC, P.O. Box 54840-00200, Telephone 2722541, Nairobi.

I also understand that I may revoke this consent at any time without penalty or loss of benefits, if any.

Parent/guardian signature and date.....

ID No.....

Village address.....

Witness name, signature and date.....

Investigator's name, signature and date.....

Appendix 5 Parasite load/gametocyte count in children at Kaugege beach.

VECTOR COMPETENCE OF THE AFRICAN MALARIA VECTORS

MALARIA PREVALENCE DATA SHEET

DATE OF SCREENING 10/12/2010 LOCALITY KAUGEGE BEACH

Serial No	Age	Sex	Species	Parasitaemia	Gametocytaemia
KG001	8YRS	M	—	—Ve	—
KG002	8YRS	M	Pf	+	—
KG003	7YRS	M	—	—Ve	—
KG004	7YRS	M	—	—Ve	—
KG005	2YRS	F	—	—Ve	—
KG006	5YRS	F	—	—Ve	—
KG007	7YRS	"	—	—Ve	—
KG008	7YRS	"	—	—Ve	—
KG009	6YRS	"	—	—Ve	—
KG010	7YRS	"	—	—Ve	—
KG011	6YRS	"	Pf	+	—
KG012	5YRS	M	—	—Ve	—
KG013	5YRS	F	Pf	+	—
KG014	5YRS	"	—	—Ve	—
KG015	6YRS	F	Pf	+	—
KG016	8YRS	"	—	—Ve	—
KG017	11YRS	M	Pf	+	—
KG018	12YRS	"	Pf	+	—
KG019	9YRS	"	Pf	+	—
KG020	3YRS	F	—	—Ve	—
KG021	9M	"	—	—Ve	—
KG022	3YRS	M	—	—Ve	—
KG023	1YR	"	—	—Ve	—
KG024	1YR	"	Pf	+	—
KG025	3YRS	F	—	—Ve	—
KG026	10YRS	M	—	—Ve	—
KG027	10YRS	F	—	—Ve	—
KG028	6YRS	"	Pf	+	—
KG029	5YRS	M	Pf	+	—
KG030	5YRS	F	—	—Ve	—
KG031	3YRS	"	—	—Ve	—
KG032	6YRS	"	—	—Ve	—
KG033	4YRS	"	—	—Ve	—
KG034	7YRS	"	—	—Ve	—
KG035	3YRS	"	—	—Ve	—
KG036	4YRS	M	—	—Ve	—
KG037	1YR	"	Pf	+	16/200
KG038	4YRS	F	Pf	—Ve	—
KG039	2YRS	M	Pf	+	—
KG040	4YRS	F	Pf	+	—
KG041	1YR	"	—	—Ve	—

Appendix 6 Parasite load/gametocyte count in pupils in Nyawiya primary school in November, 2010

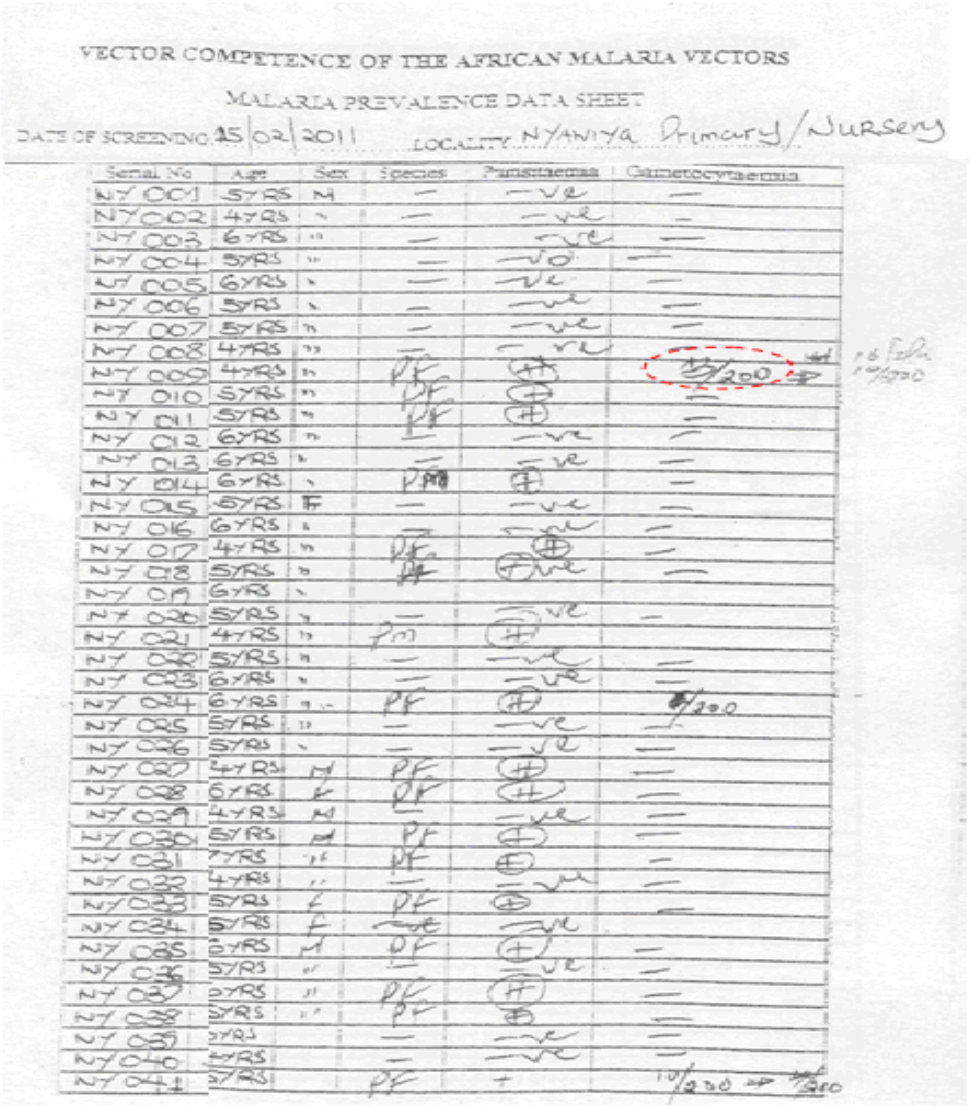
VECTOR COMPETENCE OF THE AFRICAN MALARIA VECTORS

MALARIA PREVALENCE DATA SHEET

DATE OF SCREENING 3RD NOV 2010 LOCALITY NYAWIYA PRI. C-IT

Serial No	Age	Sex	Species	Parasitaemia	Gametocytaemia
NW001	8YRS	F	—	-Ve	1/200
NW002	8YRS	"	—	-Ve	—
NW003	7YRS	"	Pf	+ve	1/200
NW004	9YRS	"	—	-Ve	—
NW005	8YRS	"	—	-Ve	—
NW006	7YRS	"	Pf	(+)	4/200
NW007	8YRS	"	—	-Ve	—
NW008	9YRS	"	—	-Ve	—
NW009	8YRS	"	Pf	(++)	1/200
NW010	8YRS	"	—	-Ve	—
NW011	7YRS	"	Pf	(++)	—
NW012	8YRS	"	—	-Ve	—
NW013	9YRS	"	Pf	(++)	—
NW014	9YRS	"	Pf	(+)	—
NW015	7YRS	"	—	-Ve	—
NW016	8YRS	"			
NW017	8YRS	"	Pf	(++)	1/200
NW018	7YRS	"			
NW019	8YRS	M	—	-Ve	—
NW020	7YRS	M	Pf	(++)	—
NW021	10YRS	"			
NW022	8YRS	"	—	-Ve	—
NW023	9YRS	"	—	-Ve	—
NW024	8YRS	"	—	-Ve	—
NW025	10YRS	"	Pf	(+)	—
NW026	9YRS	"	—	-Ve	—
NW027	9YRS	"	—	-Ve	—
NW028	9YRS	"	Pf	(+)	—
NW029	10YRS	"	—	-Ve	—
NW030	8YRS	"	Pf	(++)	—
NW031	10YRS	"	Pf	(+)	—
NW032	9YRS	"	Pf/m	(++)	—
NW033	10YRS	"	—	-Ve	—
NW034	9YRS	"	Pf/m	(++)	—
NW035	9YRS	"	Pm	(+)	—
NW036	8YRS	"	—	-Ve	—
NW037	10YRS	"	Pf	(++)	—
NW038	10YRS	"	—	-Ve	—
NW039	10YRS	"	Pm	(+)	—
NW040	10YRS	"	Pf	(+)	—
NW041?	7YRS	"	Pf	(++)	—

Appendix 7 Parasite load/gametocyte count in pupils in Nyawiya primary school in February, 2011

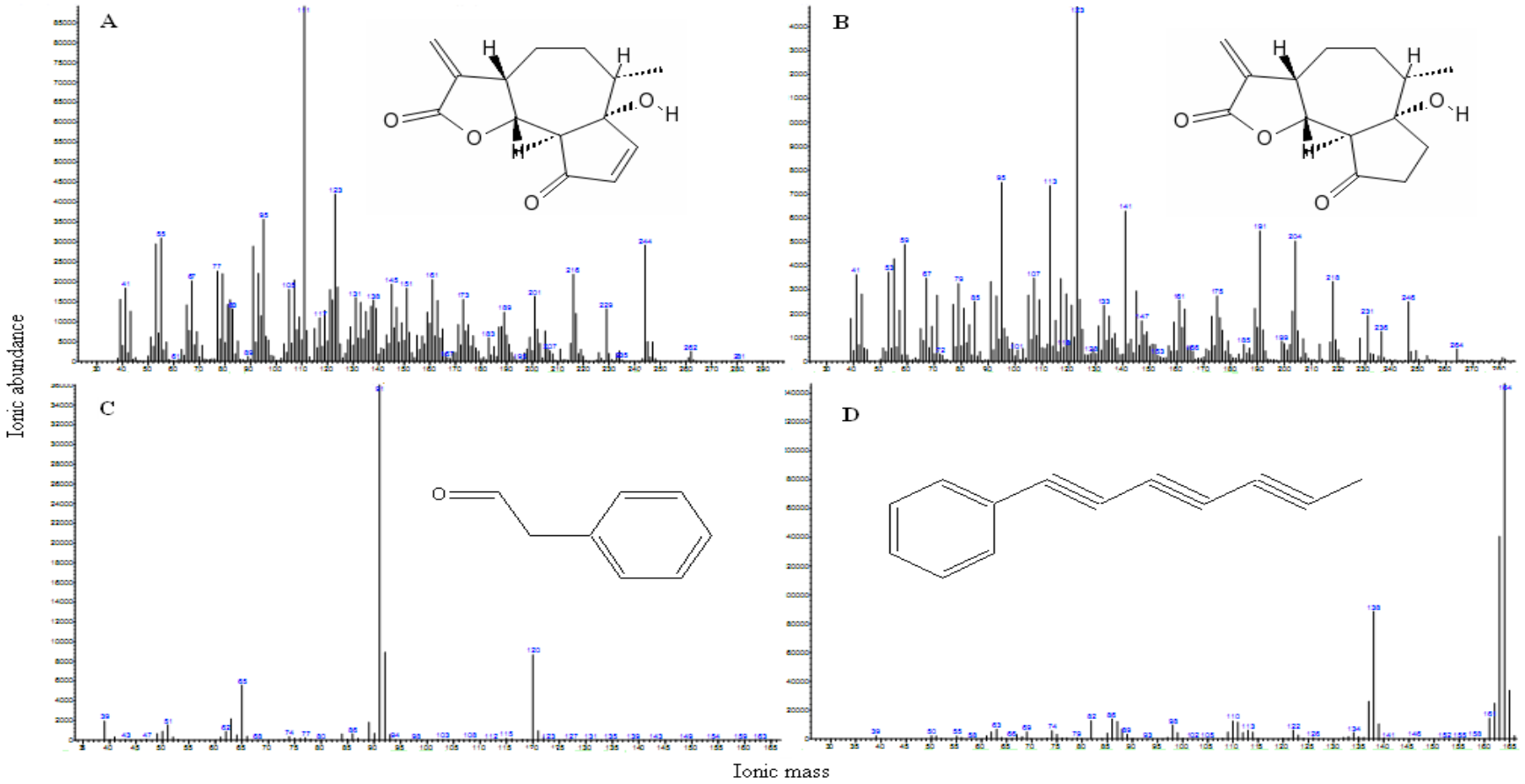


VECTOR COMPETENCE OF THE AFRICAN MALARIA VECTORS

MALARIA PREVALENCE DATA SHEET

DATE OF SCREENING 15/02/2011 LOCALITY NYANIYA Primary/Nursery

Serial No	Age	Sex	Species	Parasitaemia	Gametocytaemia
NY 001	5YRS	M	—	-ve	—
NY 002	4YRS	"	—	-ve	—
NY 003	6YRS	"	—	-ve	—
NY 004	5YRS	"	—	-ve	—
NY 005	6YRS	"	—	-ve	—
NY 006	5YRS	"	—	-ve	—
NY 007	5YRS	"	—	-ve	—
NY 008	4YRS	"	—	-ve	—
NY 009	4YRS	"	PF	+	1/200
NY 010	5YRS	"	PF	+	—
NY 011	5YRS	"	PF	+	—
NY 012	6YRS	"	—	-ve	—
NY 013	6YRS	"	—	-ve	—
NY 014	6YRS	"	PM	+	—
NY 015	5YRS	F	—	-ve	—
NY 016	6YRS	"	—	-ve	—
NY 017	4YRS	"	PF	+	—
NY 018	5YRS	"	PF	+	—
NY 019	6YRS	"			
NY 020	5YRS	"	—	-ve	—
NY 021	4YRS	"	PM	+	—
NY 022	5YRS	"	—	-ve	—
NY 023	6YRS	"	—	-ve	—
NY 024	6YRS	"	PF	+	1/200
NY 025	5YRS	"	—	-ve	—
NY 026	5YRS	"	—	-ve	—
NY 027	4YRS	M	PF	+	—
NY 028	6YRS	F	PF	+	—
NY 029	4YRS	M	—	-ve	—
NY 030	5YRS	M	PF	+	—
NY 031	7YRS	"	PF	+	—
NY 032	4YRS	"	—	-ve	—
NY 033	5YRS	F	PF	+	—
NY 034	5YRS	F	—	-ve	—
NY 035	6YRS	M	PF	+	—
NY 036	5YRS	"	—	-ve	—
NY 037	5YRS	"	PF	+	—
NY 038	5YRS	"	PF	+	—
NY 039	5YRS		—	-ve	—
NY 040	4YRS		—	-ve	—
NY 041	5YRS		PF	+	1/200



Appendix 8 Some of the ingested secondary metabolites which include parthenin (A) and dihydro-parthenin (B) from *P. hysterophorus*; benzene acetyladehyde (C) and 1-phenyl-hepta-1,3,5-triyne (D) from *B. pilosa.*