PHYTOCHEMICAL COMPOSITION AND REPELLENCY OF VOLATILES EMITTED BY LIVE POTTED Mentha piperita (PEPPERMINT) PLANTS AGAINST Anopheles gambiae sensu stricto.

JACKSON MATUNDURA OBEGI (B.Ed. Sc.)

I56/CE/24062/2012

A Thesis Submitted in Partial Fulfilment of the Requirements for the Award of the Degree of Master of Science (Chemistry) in the School of Pure and Applied Sciences of Kenyatta University

April, 2017
DECLARATION

I hereby declare that this thesis is my original work and has not been presented for the award of any degree in another University.

Jackson Matundura Obegi
156/CE/24062/2012
Signature.............................. Date 26/04/2017

This thesis has been submitted with our approval as University supervisors.

Dr. Margaret Ng’ang’a
Department of Chemistry
Kenyatta University
Signature.............................. Date 26/04/2017

Dr. David Mburu
Department of Biological Sciences
Pwani University (Kilifi)
Signature.............................. Date 26/04/2017

Prof. Ahmed Hassanali
Department of Chemistry
Kenyatta University
Signature.............................. Date 26/04/2017
DEDICATION

This research is devoted to my wife Lilian Mbete Ndeto, my son Samuel Obegi Jackson and little girl Abigail Nyaboike Jackson who was born in the course of this study, my mother Yunuke Nyaboike Obegi and my late father Samuel Obegi Nyakaru. Likely they may never comprehend the work between, yet their constant, consistence supplications, consolation, mental and profound bolster they stretched out to me amid my whole research period was adequate.
ACKNOWLEDGEMENTS

As a matter of first importance, I give much magnificence and honor to God Almighty for the quality and great wellbeing. He has offered me to do this work from its beginning to the end.

With unassuming and significant profound feeling of commitment, I wish to recognize my supervisors Prof. Ahmed Hassanali, Dr. Margaret Ng’ang’a both from Department of Chemistry, Kenyatta University and Dr. David Mburu from the Department of Biological Sciences, Pwani university for their invaluable contribution and mentorship, throughout the study. I personally acknowledge Dr. Margaret Ng’ang’a for her continuous encouragement when things became difficult, Dr. Mburu for his positive comments and acquisition of semi-field experimental huts, bioassay room and facilities, and Prof Hassanali for bearing up with me, being always at my help when things seemed to hit a deadlock, and his efforts that secured me a working space at the International Center of Insect Physiology and Ecology (ICIPE), Duduville in Nairobi.

I also wish to express gratitude to Prof. Baldwyn Torto, head of Behavioral and Chemical Ecology Department (BCED) unit at ICIPE Duduville for allowing me to conduct part of my research in the Department. I further acknowledge Dr. Xavier Cheseto and Mr. Kipkorir Kirui both from ICIPE for their technical assistance in volatile trapping and Gas Chromatography-Mass Selective Detection sample analysis. I thank Mr. G. Nzai and Mr. D. Shida both from Kenya Medical Research Institute (KEMRI) Kilifi, for their technical assistance towards rearing mosquito larvae to adult stage.
TABLE OF CONTENTS

DECLARATION........................................................................................................... Error! Bookmark not defined.
DEDICATION ............................................................................................................ iii
ACKNOWLEDGEMENTS .............................................................................................. iv
LIST OF TABLES ......................................................................................................... ix
LIST OF FIGURES ....................................................................................................... x
LIST OF PLATES ......................................................................................................... xi
ABSTRACT ................................................................................................................ xiii

CHAPTER ONE ......................................................................................................... 1

INTRODUCTION ......................................................................................................... 1
  1.1 Background Information .................................................................................... 1
  1.2 Problem statement ............................................................................................ 6
  1.3 Justification of the study .................................................................................... 7
  1.4 Research questions ........................................................................................... 8
  1.5 Hypotheses ......................................................................................................... 8
  1.6 Objectives .......................................................................................................... 9
    1.6.1 General objective ......................................................................................... 9
    1.6.2 Specific objectives ....................................................................................... 9
  1.7 Significance of the study .................................................................................... 9
  1.8 Scope and limitation of the study ..................................................................... 10

CHAPTER TWO ......................................................................................................... 11

LITERATURE REVIEW ............................................................................................. 11
  2.1 Malaria parasites ............................................................................................. 11
2.2 Control of malaria........................................................................................................ 12

2.3 Vaccine development.................................................................................................. 15

2.4 Mosquitoes .................................................................................................................. 16

2.5 Mosquito control strategies ......................................................................................... 18

2.5.1 Chemical control .................................................................................................... 18
   2.5.1.1 Adulticides ...................................................................................................... 18
   2.5.1.2 Larvicides ..................................................................................................... 20

2.5.2 Space protection measures .................................................................................... 21

2.5.3 Biological Control .................................................................................................. 22

2.5.4 Genetic Control ...................................................................................................... 24

2.5.5 Habitat management .............................................................................................. 24

2.6 Anopheles gambiae sensu stricto Giles ..................................................................... 26

2.6.1 Occurrence of An. gambiae s.s. ............................................................................. 26

2.6.2 Life cycle of Anopheles gambiae ........................................................................... 28

2.7 Repellents ................................................................................................................... 28

2.7.1 Synthetic repellents ............................................................................................... 29

2.7.2 Mosquito repellent plants ....................................................................................... 31

2.7.3 Plant derived repellent products ............................................................................ 32
   2.7.3.1 Citronella ....................................................................................................... 35
   2.7.3.2 Bite blocker .................................................................................................... 36
   2.7.3.3 Pyrethrins ..................................................................................................... 36
   2.7.3.4 p-Menthane-3,8-diols ..................................................................................... 37

2.8 Application of repellents ............................................................................................ 37
2.8.1 Direct burning .................................................................38
2.8.2 Use of repellent lotions .......................................................39
2.8.3 Vaporizers ........................................................................40
2.8.4 Thermal expulsion ..............................................................40
2.8.5 Potted plants .................................................................41
2.9 Plant under investigation ..........................................................42
2.9.1 Origin and morphology of Mentha piperita .........................42
2.9.2 Bioactivity of the essential oil from Mentha piperita plant ........43
2.9.3 Chemo-types of Mentha piperita ........................................43

CHAPTER THREE ...........................................................................46

MATERIALS AND METHODS ..........................................................46

3.1 Plant propagation and collection ...............................................46
3.2 Rearing and handling of An. gambiae ........................................46
3.3 Semi-field evaluation of the repellency of live potted Mentha piperita ....48
3.4 Ethical considerations ..............................................................51
3.5 Trapping of volatiles from Mentha piperita plants ..................51
3.6 Elution of volatiles from Super-Q traps and storage of samples before analysis ....53
3.7 Analysis of volatile constituents of Mentha piperita plant by Gas Chromatography-Mass Selective Detection (GC-MSD) ..........................................................54
3.7.1 Identification of emitted volatiles constituents ..........................54
3.7.2 Quantification of the emitted volatiles constituents .................56
3.8 Blends and individual constituents tested for repellency against Anopheles gambiae s.s. 57
3.9 Laboratory mosquito repellency assays ........................................58
3.10 Data analyses on the bioassay data ................................................................................................. 60

CHAPTER FOUR ........................................................................................................................................ 61

RESULTS AND DISCUSSION .................................................................................................................. 61

4.1 Time for repellency activity of volatiles from live potted *Mentha piperita* ................................. 61
4.2 Repellency activity of different sets of live potted *M. piperita* plant emitted volatiles .... 63
4.3 Prominent constituents identified in volatiles blends during the day and night.............. 65
4.4 Repellency activity of selected compounds and their blends against *An. gambiae*

mosquitoes .............................................................................................................................................. 73

CHAPTER FIVE ......................................................................................................................................... 79

CONCLUSION AND RECOMMENDATIONS ......................................................................................... 79

5.1 Conclusion ........................................................................................................................................... 79

5.2 Recommendations ........................................................................................................................... 80

REFERENCES ............................................................................................................................................ 81

APPENDICES ........................................................................................................................................... 95
LIST OF TABLES

Table 4.1: Hourly mean percentage repellency (±SE) of different number of potted plants against *An. gambiae* ..............................................................................................................................................61

Table 4.2: Mean percentage repellency (±SE) of different number of potted plants against *An. gambiae* ..............................................................................................................................................63

Table 4.3: Major compounds emitted in the day and night from *M. piperita* volatiles as identified by GC-MSD ..............................................................................................................................................66

Table 4.4: Night and Day prominent compounds identified by the GC-MSD from the emitted volatiles by potted *M. piperita* plant ...........................................................................................................68

Table 4.5: Student t-test analysis of the relative abundances of the prominent compounds in the night and day emitted volatiles ...........................................................................................................70

Table 4.6: Night and Day prominent compounds identified by the GC-MSD from the emitted volatiles by potted *M. piperita* plant ...........................................................................................................71

Table 4.7: Mean percentage repellency (±SE) and RD$_{50}$ of standards and blends at different doses against *An. gambiae* ..............................................................................................................................................73
LIST OF FIGURES

Figure 2.1: Distribution of *Anopheles gambiae* s.s. Giles in tropical Africa ................. 27

Figure 2.2: Life cycle of the mosquito ......................................................... 28

Figure 3.1: Semi-field experimental arena with human volunteer in the experimental hut................................................................. 49

Figure 3.2: The calibration graph of peak area of 1,8-cineole against concentration ........... 56

Figure 3.3: The calibration graph of peak area of β-caryophyllene against concentration ....... 57

Figure 4.1: Hourly SNK mean percentage repellency (±SE) rankings of different number of potted plants against *An. gambiae* ............................................................ 62

Figure 4.2: Mean (±SE) percentage repellency caused by the night emitted volatiles of *M. piperita* plants against *An. gambiae* (bars of the same color are not significantly different (α = 0.05, SNK, 95% CL))............................................. 63

Figure 4.3: The GC-MSD Overlay chromatogram of the emitted volatiles by *M. piperita* plant at night and day time .................................................................................. 65

Figure 4.4: Mean percentage repellency (±SE) of β-myrcene, β-pinene, α-pinene, 1,8-cineole and DEET at different doses ........................................................................... 74

Figure 4.5: Mean percentage repellence (±SE) of DEET and blends at various doses against *An. gambiae* ........................................................................................................... 74
LIST OF PLATES

**Plate 2.1**: The adult female African malaria mosquito, *Anopheles gambiae* Giles........26

**Plate 2.2**: Photo of potted *Mentha piperita* plant ..................................................43

**Plate 3.1**: The semi-field experimental screen houses for night bioassays.................48

**Plate 3.2**: Experimental hut with human bait in a mosquito landing catch ..............50

**Plate 3.3**: Volatile trapping in a manifold system ......................................................52

**Plate 3.4**: Elution of volatiles trapped by super Q adsorbent under ice ..................53

**Plate 3.5**: Gas Chromatography-Mass Selective Detections (GC-MSD) ..................54
ABBREVIATIONS AND ACRONYMS

ACTs  Artemisinin-Based Combination Therapies
ANOVA  Analysis Of Variance
ARS  Analytical Research Supplies
ASAQ  Artesunate-Amodiaquine
ASSP  Artesunate-Sulfadoxine-Pyrimethamine
BCED  Behavioral and Chemical Ecology Department
CDC  Centre for Disease Control
CSP  Circumsporozoite Surface Protein
DDT  Trichloro-2,2-bis-(p-chlorophenyl)ethane
DEET  N,N-diethyl-m-toluamide
DHAP-Q  Dihydroartemisinin Piperaquine
EFAB  Extinguish Fire Ant-Bait
EOs  Essential Oils
GC-MSD  Gas Chromatography-Mass Selective Detector
GMMs  Genetically Modified Mosquitoes
HCH  Hexachlorocyclohexane
HPLC  High Performance Liquid Chromatography
ICIPE  International Centre of Insect Physiology and Ecology
IGRs  Insect Growth Regulators
IGIs  Insect Growth Inhibitors
IPTp  Intermittent Preventive Treatment in Pregnancy
IRS  Indoor Residual Spraying
ITNs  Insecticide Treated mosquito-Nets
IVM  Integrated Vector Management
LLNs  Long Lasting Insecticide Treated bed Nets
MVI  Malaria Vaccine Initiative
MIM  Multilateral Initiative on Malaria
NIST  National Institute of Standards and Technology
PCM  Percentage Control Means
PE  Protective Efficacy
PTM  Percentage Treatment Means
RD50  Dose Response at 50% confidence level
RD75  Dose Response at 75% confidence level
RD90  Dose Response at 90% confidence level
RT  Retention Time
SNK  Student-Newmann-Kuels
SSP-2  Sporozoite Sulfur Protein-2
SSP66  Sporozoite Surface Protein-Based Vaccine
SPSS  Statistical Package of Social Sciences
±SE  Standard Error
UNDP  United Nation Development Program
UNICEF  United Nations Children’s Emergency Fund
VOCs  Volatile Organic Compounds
ABSTRACT

The malaria vector *Anopheles gambiae* sensu stricto remains one of the principal malaria transmission vectors in developing countries, particularly within the tropics and sub-Saharan Africa. Current methods of control are based on synthetic chemical insecticides, but these are costly and inaccessible by poor communities, and large-scale use leads to vector resistance and toxicity to beneficial non-target organisms. Plant-based products have been used for generations in traditional practices, either for space protection to repel the arthropods from distance or for personal protection to deter them from blood feeding on contact. These are eco-friendly, cost effective and readily accessible to low income earners with minimal external input in resource-poor endemic and non-endemic malarial regions of the world. However, there have been limited research and documentation studies undertaken to screen the repellency of different plants and to optimize their deployment to effectively protect human subjects from mosquito bites in households. The aim of the present study was to compare the performance of different numbers of potted *M. piperita* plant(s) against *An. gambiae* s.s. in a choice set-up in a screen-house. In addition, the compositions of volatile emissions of the plant during the day and night (trapped on Super Q adsorbent) were compared and the major constituents identified by Gas Chromatography–linked Mass Selective Detection (GC-MSD). The repellency of the major constituents and blends were evaluated by human landing bait technique in aluminium cages. There was significant incremental rise in repellency from 44.00 % to 70.09 % when the number of potted plants was doubled from 2 to 4 (p<0.05); however, there was no significant further increase with 8 and 16 plants (p>0.05). Thus, the level of emission of volatiles from each plant appears to be negatively affected by the presence of other con-specific plants. There was large quantitative difference between volatile blends emitted during the day and night. Monoterpenes were the major compounds emitted during the night (60.5%) followed by sesquiterpenenes (25%). 1,8-Cineole was the major constituent for both blends (27.68% at night and 23.93% during the day). Of the individual constituents tested, β-Pinene (56.40±6.40) was most repellent. Subtractive assays of the five constituted blends, showed that the blend of 1,8-Cineole, β-Pinene, α-Pinene, and β-Myrcene (143:17:71:19) was most repellent (100.00±0.00) at a dose of 0.1 mg/mL which was comparable to that of the posive control (DEET). This study shows that deployment of a small number of *M. piperita* plants has additive space protection effects. However, further research is needed to achieve significant incremental effects and complete protection from malaria vectors.
CHAPTER ONE

INTRODUCTION

1.1 Background Information

Vector-borne illnesses are vital in general well-being issues and place obstructions to socio-economic development in developing countries, particularly within the tropics (Karunamoorthi et al., 2010). In spite of, just about a century of control endeavors, malaria stays a standout amongst the most vital reason for mortality and morbidity in numerous parts of the tropical nations on the planet. This has been linked to climatic changes like El-nino weather phenomena, global warming, and interference by man on the environment (Mouchet et al., 1988; Lindsay, 1996; Jerten et al., 1996; WHO, 1999). In the battle against the disease, lack of funding for strengthening public health systems facilities, vector and parasites resistance to insecticides and anti-malarial drugs respectively, inaccessibility of ITNs, IRS, ACT for children, and intermittent preventive treatment in pregnancy (IPTp), have obstructed advancement in malaria counteractive action and control in Africa for a long time (WHO, 2015).

Malaria has been associated with long-term suffering in children after recovery from the disease. The WHO (2002) reports that about 2% of children who recover from malaria experience learning impairment and disabilities, epilepsy and spasticity due to brain damage. This has led to neurological damage and developmental problems (Greenwood et al., 2007). The disease exerts a heavy burden on peoples’ lives, working days and government health expenditure in most third world countries (WHO, 1996; WHO, 2015). The yearly worldwide

Mosquitoes are probably the most "dangerous" vectors of ailments confronting mankind. They have been in charge for the transmission of numerous restoratively imperative pathogens and parasites such as viruses, bacteria, protozoa, and nematodes, which cause illnesses. For example, malaria, dengue, yellow and chikungunya fever, encephalitis or filariasis (Becker et al., 2010).

Around 100 viruses infect people and 40 domesticated animals (Monath, 1988). The most imperative viruses transmitted by mosquitoes to people or different vertebrates are found in three families; Togaviridae of the genus Alphavirus (e.g Chikungunya, Sindbis, Equine Encephalitis and Ross river viruses), Flaviviridae of the genus Flavivirus (e.g Yellow fever virus, Dengue 1-4 virus, West Nile virus, Japanese and St. Louis encephalitis viruses), and Bunyaviridae of the genera Bunyavirus (e.g. the California group), and Phlebovirus (Rift Valley fever virus) (Eldrige and Edman, 2000).

Because of their blood-sucking conduct, mosquitoes get the pathogens from one vertebrate host and pass them to another, if its environment and physiology is suitable for transmission (Becker et al., 2010). The female An. gambiae mosquitoes are in charge of transmission of malaria. About 60 species out of 380 of Anopheline transmit malaria with An. gambiae complex and An. funestus being the fundamental vectors that transmit the Plasmodium parasite. The most efficient malaria vectors in sub-Saharan Africa and worldwide belongs to
Anopheles gambiae Complex (Becker et al., 2010). This complex was perceived in 1960s and incorporates the most essential vectors of malaria in sub-Saharan Africa, tropical Africa, south of the Sahara desert, and southern Arabia (WHO, 1989). Of the eight species of this complex, An. gambiae s.s. and, An. arabiensis (species A and B) are the most imperative vectors of P. falciparum in Africa (Becker et al., 2010).

Vector control has been utilized for a significant long time to control mosquito populace and reduce human vector contacts. This has reduced morbidity and mortality by bringing down the level of malaria transmission through control of Anopheline mosquito populace. The real systems incorporate utilization of larvicides, adulticides, biological control agents, environmental management and repellents (Yeye, 2001; Mayeku, 2006).

Chlorinated synthetic organic compounds that have been used as larvicides have been found to be non-biodegradable, leading to environmental pollution and bio-amplification, threatening human health (Mayeku, 2006). Application of sprays on large scale for a long time is expensive and unaffordable to resource-limited people in developing countries (Fontaine et al., 1979). Therefore, there is need in searching for safe and cost-effective insecticides. Organophosphates have shown adulticidal properties against mosquitoes, but they are non-selective on their mode of action, and their efficiency is reduced by vector resistance (Matsumara and Brown, 1961; Metcalf and Flint, 1962). Insecticides such as, DDT [trichloro-2,2-bis-(p-chlorophenyl)ethane], HCH (hexachlorocyclohexane), dieldrine and lindane have been used. They have been found to contain adverse side effects (WHO, 2003).
Biological larval control methods have become difficult to deploy in many places because mosquitoes have adopted ways that enable their survival in temporary water habitats. Biological control agents such as *Var. israelensis*, *B. thuringiensis* and *B. sphaericus* are expensive and not easy to culture. Larvivorous fish cannot survive in temporary water habitats. All these have resulted in continuous search for sustainable and cost-effective alternative methods for mosquito control (Ndirangu, 2015). Different application methods of repellents have been deployed to reduce human-pathogen-vector contacts in an effort to combat malaria. These methods include direct burning, use of repellent formulated lotions or creams, hanging branches of repellent plants in the houses, vaporizing lamps and thermal expulsion of repellent materials (Gupta and Louis, 1994; Pates et al., 2002; Seyoum et al., 2002; Mark and Fradin, 2003).

Plants have a complex of chemicals with extraordinary natural action (Farnsworth and Bingel, 1977). A large portion of them contain aggravates that keep them from being assaulted by phytophagous (plant eating) insects, by acting either as repellents, feeding deterrents, poisons or development controllers. In the conventional society, plants have been utilized in many parts of the world to secure individuals, domesticated animals and plants against medical vectors and pests by keeping these potential disease vectors away (Seyoum et al., 2002). Several numbers of these plants have been shown to have some level of action against mosquitoes (Kumar and Dutta, 1987; Sukumar et al., 1991).

The following plants have been smouldered to produce compounds that repel mosquitoes; Pyrethrum, pine, *Azadirachta indica*, Eucalyptus spp. (Myrtaceae), *Lantana camara*, *Vitex*
negundo (Verbanaceae), Cymbopogon spp. (Graminae), Mentha piperita (Labiatae), Tagetes minuta (Compositae), Ocimum spp., Ajuga remota, Lippie javanica, and Lippie ukambensis (Omolo et al., 2004; Karunamoorthi et al., 2008). Thermal expulsion of Corymbia citriodora, Ocimum kilimandscharicum and Ocimum suave repelled up to 74% of host-seeking An. gambiae s.s. (Seyoum et al., 2003).

Formulations such as solutions, lotions, creams, gels, aerosol, pump sprays and impregnated towel in United States are mixed with DEET in the concentration range of 5-95% (Curtis, 1986). Sulfur and molasses have been incorporated in lotions making them repellent to mosquitoes in Transvaal and Natal provinces of South Africa (Lindsay and Gibson, 1988). Traditional lamps were used to burn kerosene oil mixed with repellent volatiles that disperses as it burns producing smoke that repelled mosquitoes (Pates et al., 2002). Egyptians and Assyrians are known in using Incense and Joss sticks to control malaria vectors. A leaf infusion of Lippie javanica Spreng and Ocimum canum Sims are applied on the skin to control mosquito bites in Zimbabwe (Lukwa et al., 1994). Kerosene burning lamps (korobois) in Tanzania have been modified to heat and vaporize volatile pyrethroid insecticides to repel host seeking mosquitoes (Pates et al., 2002).

Thermal expulsion of Corymbia citriodora, Ocimum suave, and Ocimum kilimandscharicum repelled up to 74 % of host- seeking An. gambiae s.s. in semi-field experimental huts using modified African traditional stoves in western Kenya (Seyoum et al., 2003). Mosquito repellent plants have been deployed in space protection and their products utilized in personal protection against malaria vectors. Such plants have got some consideration, due to their
ecological and user-friendly nature, low cost, accessibility, local prevalence, and social acceptancy (Karunamoorthi et al., 2008; Karunamoorthi et al., 2009). In western Kenya, three species, *O. americanum*, *L. camara* and *L. ukambensis* emit a blend of volatiles that decrease bitings of *An. gambiae* by 30-40% (Seyoum et al., 2002, 2003). The utilization of potted repellent plants has not yet been fully exploited. However, due to its convenience and simplicity, more broad exploration on screening of a more extensive profile of potted plants needs to be conducted.

1.2 Problem statement

Globally, it is estimated that 3.2 billion people are vulnerable to malaria with 2 billion people living in the tropics (Beier et al., 2008; Manish et al., 2009; WHO, 2015). Malaria caused an estimated 214 million cases globally leading to 438,000 deaths in the year 2015. Approximately 90% and 80% of global malaria cases and deaths respectively, occur in Africa (WHO, 2015). In Kenya 16,000 people die annually from malaria and more than 25 million are at risk (Ratemo, 2010). The actual number of deaths may be significantly higher, as precise statistics are unavailable in many rural areas and many cases are undocumented (Breman, 2001). Currently, there is no affordable drug that provides full proof protection against malaria. The malaria parasites have developed cross resistance to most anti-malarial synthetic drugs (Zucker and Campbell, 1992; WHO, 2000), and more recently to artemisinin and its derivatives (WHO, 2014).
1.3 Justification of the study

Vector control is the most effective means for malaria control (Tawatsin et al., 2001; Odalo et al., 2005). *Anophele*ne mosquitoes have become resistant to DDT, the widely used insecticide that is commercially available. This insecticide has been found to be unfriendly to the environment due to its bio-amplification and toxicity to non-target organisms. DEET which is a broad-spectrum repellent and the main ingredient of most commercially available repellents has been associated with human-health and environmental problems (Lewis et al., 2000). Therefore, they are not safe for public use (Karunamoorthi, 2011).

There is need to find supplemental protective measures for personal and/or space protection against malaria vector which are more potent, selective and biodegradable. This has led to the search for safe alternative synthetic and natural repellents for arthropods (Odalo et al., 2005). Potted plants of *Ocimum americanum*, *Lantana camara* and *Lippie ukambensis* reduced biting of *An. gambiae* by 30-40% (Aklilu et al., 2003). Although the level of repellency of these plants was lower than that required to substantially reduce the incidences of malaria in highly endemic areas, it may usefully contribute to integrated programmes in areas where these plant species are available with the dominant malaria vector being *An. gambiae s.s.* (Seyoum et al., 2002). Due to its convenience and simplicity, a broader profile of potted plants that can be adopted by resource-limited communities needs to be screened.

The essential oil of *M. piperita* has been found to have insecticidal, larvicidal and mosquito repellent properties. The oils are nontoxic to humans (Ansari et al., 1999). The potential of naturally emitted volatile organic blend from this plant in human space protection against
mosquitoes is not well understood. The present study was undertaken to see if indoor deployment of multiple potted *M. piperita* plants can have additive effects in space protection against female *An. gambiae s.s.*

1.4 Research questions

i. What is the percentage repellency of the emitted volatiles by live potted *M. piperita* plant against *An. gambiae s.s.* in a screen house at night?

ii. What is the percentage repellency of multiple uses of live potted *M. piperita* plants in repelling probing female *An. gambiae s.s.* in a screen house at night?

iii. Is there significant quantitative and qualitative difference in the constituent blend profiles of volatiles released from *M. piperita* plants during the day and at night?

iv. Which chemical constituents and/or blends of volatiles emitted by *M. piperita* plant are primarily responsible for repellency?

1.5 Hypotheses

i. Live potted *M. piperita* plant emits a blend of constituents that is repellent to *An. gambiae* during the time the vectors are active.

ii. There is significant difference in phytochemical composition of repellent volatiles emitted by *M. piperita* plant during the day and night.

iii. Multiple deployments of potted *M. piperita* plants (2, 4, 8 and 16) provide incremental increases in repellency against *An. gambiae*.
iv. Repellent property of the emitted volatiles by live potted *M. piperita* plant against *An. gambiae* is due to synergistic effects of different constituents.

### 1.6 Objectives

#### 1.6.1 General objective

To evaluate the repellency of live potted *M. piperita* (peppermint) plants against *An. gambiae* in a screen-house setting, and to characterize the phytochemical composition of emitted volatiles.

#### 1.6.2 Specific objectives

i. To determine the percentage repellencies of multiple (2, 4, 8 and 16) live potted *M. piperita* plants against *An. gambiae* under semi-field conditions in a screen-house using human volunteers as baits.

ii. To characterize the major constituents of the emitted volatile profiles during the day and night using Gas Chromatography- Mass Selective Detection (GC-MSD).

iii. To compare the profiles of volatile emitted by potted *M. piperita* during the day and night.

iv. To undertake individual and subtractive assays of the standards of prominent constituents and blends identified from the emitted volatiles of *M. piperita* plant during the night.

### 1.7 Significance of the study

This study sought to provide information on repellency efficacy of multiple live potted *M. piperita* plants against *An. gambiae* during their active times and the phytochemical composition of the emitted volatiles by the plant grown in the Coast. This information lays down groundwork for more comprehensive field studies and follow-up.
1.8 Scope and limitation of the study

The study focused on repellency of live potted *M. piperita* plants against semi-field reared *An. gambiae* s.s. Evaluation was carried out in a screen-house, and no field studies were carried out. Moreover, use of different species of plants was not investigated.
CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria parasites

It is believed that malaria was brought about by bad air (*mala aria* in Italian) which means ague or marsh fever because of its relationship with swamps and marshlands (Reiter, 2000). After examination, Alphonse Laveran (1889) found the malaria protozoan parasite causing agent (Joy *et al.*, 2003). Through his work in 1887, Ronald Ross found that *Anopheleline* mosquitoes were the main malaria vectors (Ross, 1897). The vector transmits predominantly four malaria human-specific species of the genus *Plasmodium*: *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax* (Ridley, 1997; WHO, 2013), in addition to one Zoonotic species, *P. knowlesi*.

Among all the malaria causing plasmodia, *P. falciparum* is the most virulent and causes malignant tertian level of the disease, *P. vivax* and *P. ovale* causing benign tertian that recurs at 48 h intervals (fever attack at the third day), and *P. malariae* quartan recurring at 72 h intervals (Kettle, 1995; Becker *et al.*, 2010). Approximately 20 *Plasmodium* species occur in other primates and mammals, while about 40 occur in birds and reptiles (Garnham, 1980).

The less virulent *P. vivax* strain is found in the Indian sub-continent, Central America, Asia, Oceania and South America but has a low incidence in sub-Saharan Africa because of protective mutation (Duffy negativity) frequently found in the population. The mildest form of malaria *P. malariae* is found in most regions where malaria is endemic. *P. vivax* is the most common species in Europe with about 10-20% of the world’s cases occurring in Africa, south
of the Sahara (Mendis et al., 2001). In Eastern and Central Africa, *P. vivax* accounts for 10% of malaria cases but less than 1% occur in Western and Central Africa (Mendis et al., 2001). In other parts of the world, *P. vivax* accounts for greater than 50% of all malaria cases with 80-90% occurring in the midle East, Asia and Southern America (Mendis et al., 2001). *P. ovale* is uncommon in areas outside Africa but it has been reported in Central, West Africa and West pacific region (Powells, 1989). *Plasmodium falciparum* is the most prominent, frequent and virulent species occurring in sub-Saharan Africa, Eastern Asia, Oceania and Amazon (WHO, 1997; Ridley, 1997).

### 2.2 Control of malaria

Malaria control includes vector control, chemoprophylaxis, and malaria parasite control. In push to battle malaria, WHO prescribes the accompanying procedures (i) identification of inventive client and natural well disposed option advances and conveyance frameworks (ii) investigation and improvement of novel and effective logical group based mediations (iii) early diagnosis and quick treatment of the disease (iv) arrangingement and execution of specific, manageable, and proficient existing preventive intercessions (v) strengthening of regular assessment of national malaria situations research capacities (WHO, 1998; Karunamoorthi, 2011). The WHO (2015) indicated that malaria control and elimination require continued political commitment, constant vigilance, and massive roll-out of effective preventive and treatment tools.

The worldwide battles against malaria like the Roll Back Malaria (RBM) program driven by real global associations, developed offices for quick case identification and treatment,
prophylaxis, personal protection, indoor residual spraying (IRS) and pandemic readiness (RBM, 2005; Makundi et al., 2007). To address the rising difficulties on malaria control, WHO has developed a worldwide specialized procedure for malaria 2016-2030. The strategy is based on three principle pillars; widespread access to malaria counteractive action, determination and treatment, quickened exertion on malaria elimination and fulfillment of malaria-free status, and change of malaria observation into a center mediation (WHO, 2015). Methodologies, for example, chemotherapy, immunization advancement and vector control are being utilized for malaria management.

Malaria control through use of chemotherapeutic approaches has been in existence since the ancient times. Quinoline containing anti-malarial components drugs like quinine (1), chloroquine (2), amodiaquine (3) and mefloquine (4) were the most effective drugs for malaria chemotherapy used during the post-world war (Baird et al., 1995). Anti-foliates like chloroproguanil (5), pyrimethamine (6) and trimethoprin (7) were also used. Other anti-malarial drugs include halofantrine (8), atovaquone (9), artemisinin (10) and combinations of its derivatives (Baird et al., 1995; CDC, 2010). The malaria parasites have developed cross resistance to most anti-malarial synthetic drugs, and more recently to artemisinin and its derivatives (artesunate, artemotil, artemimol and artemether) (Zucker and Campbell, 1992; WHO, 2000; WHO, 2016). These derivatives are more active and effective on protein synthesis of the malaria parasite than artemisinin itself (Farooq and Mahajan, 2004);
In nations where *P. falciparum* malaria had developed resistance to the convectional anti-malarial, Artemisinin-based Combination Therapies (ACTs) has been prescribed because of its high healing rates and low rate drug resistance (WHO, 2001). Artemisinin-based Combination Therapies use drugs with various components of activity that make a boundary to resistance, constraining the parasite to build up numerous concurrent transformations in order to become resistant (Lin and Juliano, 2010). Treatment of multi-medication resistant strains of *P. falciparum*, Artemisinin and mefloquine blend is being utilized in some parts of South East Asian nations (White *et al.*, 1999).
Artemether-lumefantrine (AL) is the most broadly utilized ACT in Africa since 2004, with more than 30 nations having received it as a first-line treatment (Nosten and White, 2007). In cases of chronic resistance, the following drugs are being used in malaria treatment; proguanil (11), sulphadoxine (12) and 4 (Fredena et al., 1997). This resistance has been attributed to parasite population pressure on previously existing drug resistance, spontaneous mutation and existence of plasmid factors (WHO, 1998). This resistance has led to search for other alternative chemotherapy;

![Chemical structures](image)

2.3 Vaccine development

There has been advancement in creating anti-malaria vaccines with the primary focus being on three types of vaccines anti-sporezoite vaccine, anti-exual blood stage vaccine, and transmission blocking vaccines (Franke et al., 1999). These drugs are designed to prevent infection, severe manifestation of the disease and parasite development through blocking the transformation of gametocytes into sporozoites (WHO, 1998). The sporozoites antigens are the prime candidate antigen for malaria vaccines which are injected into the host to prevent any subsequent infections (Hoffman and Doolan, 2000). The proteins that have been used to stimulate immunity against pre-erythrocytic stages of malaria *in-vitro* experiments include
circumsporozoite surface protein (CSP), sporozoite surface protein-2 (SSP-2), and cytotoxic T-cells (Wakelin, 1996).

The sporozoites surface protein-based antibody 66 (SSP66) has been utilized against *P. falciparum* malaria, with defensive viability of 1.6-48.44% (Margarita *et al.*, 1998). Immuno-stimulants, adjuvant of monophosphoryl A and a saponin subordinate QS21 (SBAS2) formulated with emulsion of oil in water gave malaria antibody RTS defensive resistance to 7 out of 8 volunteers with *P. falciparum* than all other existing antibodies amid field trials (Staute *et al.*, 1998). The current methodologies are concentrating on blending of protein and constricted entire living being vaccines. Despite the fact on clinical trials, they have exhibited immunogenicity in all age groups including children below the age of 2 years (Alonso, 2004). The rise of artemisinin and multi-drug resistant strains particularly, by *P. falciparum* are driving exploration. In this way, there is no effective vaccine that has been brought into clinical practice.

### 2.4 Mosquitoes

Mosquitoes are members of a family of nematocerid flies the Culicidae (from the Latin *culex*, *genitive culicis*, meaning “midge” or “gnat”). They are classified under the class insecta, and belong to the order of two winged flies known as diptera, which include house flies and others (Gillet, 1971). More than 3,500 species of culicidae have been described from various parts of the world (Leisnham, 2012). The family is further divided into three sub-families; anophelinae, taxorrhynchitinae and culicinae which comprises of 43 genera. The anophelinae and culicinae sub-families differ in their significance as vectors of different classes of diseases.
(Gillet, 1971). Many species of the Anopheline under the genus anopheles are responsible for the transmission of malaria and culicines are responsible for transmission of arboviral diseases including yellow and dengue fever (Gillet, 1971; Dawes, 1973).

Both male and female mosquitoes feed on nectar, plant juices and other sources of sugar but only the female takes blood (Marquardt and Demance, 1985). The mouth parts of many female species except members of Chironomidae and Tipulidae are adapted for piercing the skin of animals to suck blood as ectoparasites. This makes them to be the most dangerous human disease vectors. The female lives 1-2 weeks while the male live for about 5-7 days. Their live span depends on temperature, humidity, and their ability to successfully obtain a blood meal while avoiding host defenses and predators (Arrow et al., 2004; CDC, 2010).

The mosquito lands on the exposed body parts of the human host, imbibes, and bites as it tries to locate blood capillaries. This leads to irritation and itching in the host due to an immune response towards the binding of IgG and IgE antibodies to antigens in the mosquito’s saliva. Some of the sensitizing antigens are common to all mosquito species, whereas others are specific to certain species (Clements, 1992).

There are both immediate hypersensitivity reactions (types I and III) and delayed hypersensitivity reactions (type IV) to mosquito bites that result to itching, redness and swelling. Immediate reactions develop within a few minutes and lasts for a few hours after the bite. Delayed reactions take around a day to develop, and lasts for up to a week (Clements, 1992). Several anti-itch medications are commercially available, including those taken orally,
such as Benadry (13), or topically applied antihistamines. For severe cases, corticosteroids, such as hydrocortisone (14) and triamcinolone (15) are used;

\[ \text{Chemical structures} \]

2.5 Mosquito control strategies

2.5.1 Chemical control

2.5.1.1 Adulticides

Adulticides are used to control the population of adult mosquitoes. They are applied in the form of indoor and outdoor residual spraying. The indoor residual spraying (IRS) involves application of long-lasting insecticide inside walls of residential houses and other structures, with an aim of killing mosquitoes that enter houses and rest on sprayed surfaces (Enayati and Hemingway, 2010). The commonly used insecticides include; chlorinated hydrocarbons, organophosphates, organocarbamates, synthetic pyrethroids and organic insecticides of plant origin (Roll Back Malaria, 2010).

Chlorinated hydrocarbons have been used for a long time due to their low toxicity (Matsumara and Brown, 1961). Although, organochlorides have adverse effects, it has been reported that propoxur (2-isopropoxyphenyl-N-methylcarbamate) the main constituent of
organocarbamate, produces limited effects on malaria transmission even when combined with mass drug administration (Fontaine et al., 1979).

Organophosphates such as Malathion (16), diazinon (17), phosphorothioic acid (18), methoxyphosphorothioic acid (19) and methylparathion (20) have been used as adulticides, though they are highly toxic to animals and other non-target organisms. Due to their non-biodegradability, these chemicals have been found to accumulate in food chains, water bodies and soil. Some species of mosquito have shown resistance to most of them (Matsumara and Brown, 1961; Metcalf and Flint, 1962; Motoyama et al., 1997).

Carbamates and its derivatives have been deployed in adult mosquito control (Matsumara and Brown, 1961). 2,2-dimethyl-1,3-benzodioxol carbamate (21), and methyl carbamate (22) have been effective in the control of mosquito. Application of sprays on a large scale for a long time is expensive and unaffordable to resource limited people in developing countries (Fontaine et al., 1979). Therefore, there is need in searching for safe and cost effective insecticides;
2.5.1.2 Larvicides

Mosquito larvae control dates back to 1379 AD when they were killed by application of oil on stagnant water surface (Wiggleworth, 1976). The synthetic inorganic compounds such as Paris green \([\text{Cu(H}_2\text{O})_2.3\text{Cu(AsO}_2\text{)_2}]\) and copper metarsenite \([\text{Cu(AsO}_2\text{)_2}]\) were used in the eradication of \textit{Anopheles gambiae} larvae in Brazil in the 1930s. These arsenicals were found to be too toxic to comply with modern standards due to their high toxicity to humans and domestic animals, phytotoxicity to plants, and extreme environmental persistence (Metcalf and Flint, 1962; Kirk and Othmer, 1981). Chlorinated synthetic organic compounds that have been used as larvicides include lindane (23), dieldrin (24), chlordane (25) and DDT (26) (Kirk and Othmer, 1981). These larvicides have been found to be non-biodegradable, leading to environmental pollution and bio-amplification threatening human health. Methoprene (27) has been found to hinder egg and larvae developmental stages of mosquitoes, making it suitable for deployment in areas with standing water and permanent mosquito breeding habitats (Metcalf and Flint, 1962);
2.5.2 Space protection measures

*Anopheles* mosquitoes that prefer staying inside (endophilic) after a blood meal are reduced by Insecticide Treated bed Nets (ITNs) and improved housing construction to prevent entry of mosquitoes (e.g. window screens) (Takken and Knols, 2009). The ITNs are currently widely used form of vector control with an increase of 2\% in the year 2000 to 59\% in 2014 of the population accessing them (WHO, 2015). They have been found to be more reliable, cheap and selectively kill those insects that attack humans without affecting the general ecology of the area (Roll Back Malaria, 2002).

Among the compounds used to treat the nets, deltamethrin (28) is the most widely used compound, constituting about 60\% of the global usage, followed by permethrin (29) (22\%) (Zaim and Jambulingam, 2007). The ITNs reduces child mortality by 20\%, saving an average of six lives for every 1000 children below the age of 5 years every year (Lengeler, 2002). In an area of intense perennial transmission in western Kenya, the ITNs have reduced malaria and anemia in infants by more than 60\% (Ter *et al.*, 2003), and 33\% of sick child incidences visiting health facilities (Phillips *et al.*, 2003);
Mosquitoes have developed resistance against pyrethroids the widely used chemical for net treatment since the year 2010, posing great risk against ITN’s effectiveness (WHO, 2015). These ITNs only protect people when they are asleep. However, people can still suffer from the mosquito bites at night before retiring to the confines of the nets. Therefore, they can contract malaria as the vectors are active at this time (Seyoum et al., 2002). The smoke produced by mosquito coils burnt at night to repel mosquitoes has been associated with irritation and breathing related respiratory problems with asthmatic people.

2.5.3 Biological Control

Biological control is the introduction or manipulation of organisms to suppress vector populations through predation, parasitism, and competition (Chandra et al., 2008). Larvivorous fish have been used for more than 100 years in mosquito control. *Gambusia affinis* has been widely used to control the immature stages of various malaria vectors. Other species include Tilapia spp., *Poecilia reticulata*, and Cyprinidae (Lacey and Lacey, 1990). Various small fishes, such as species of Galaxias and members of the *Poeciliidae*, such as guppies (*Poecilia*), and banded killifish (*Fundulus diaphanus*), cyprinids carps and minnows eat mosquito larvae (Krumholz, 1948). Other fish that consume mosquito larvae include bass, bluegills, piranhas, arctic char, salmon, trout, catfish, and gold fish (Fradin, 1998).
The bacillus-based mosquito larvicides popularly known as biocides or biolarvicides have been deployed in vector control. Certain types of bacteria, especially *Bacillus thuringiensis*, *Var. israelensis* and *Bacillus sphaericus* have been found to be highly effective for the control of mosquito larvae at very low doses (Marquardt *et al.*, 1985; Mittal, 2003). Mermithid nematodes such as *Romanomermis culicivorax* have been used to suppress mosquito populations. They get into the insect through the breathing holes, mouth, anal part or cuticle and release special bacteria, which produce toxins that kill the host after a short while (Zaim *et al.*, 1988; Lacey, 1998). Microsporidia such as *Nosema algerae* (Undeen and Dame, 1987), and several entomopathogenic fungi (Federici, 1995) among them *Oomycete lagenidium giganteum* has been used for vector control in rice fields (Hallmon *et al.*, 2000).

Dragonfly naiads, consume mosquito larvae in the breeding waters, and some species of lizard and gecko eat adult mosquitoes (Canyon and Hii, 1997). Predators such as birds, bats, and frogs, have been used, but their effectiveness is only anecdotal. Larvae of the non-biting *Toxorhynchites* mosquitoes are natural predators of other culicidae. Each larva can eat 10 to 20 mosquito larvae per day while during its entire development, it can consume up to 5,000 first-instars larvae (*L*₁) or 300 fourth-instars larvae (*L*₄) (Steffan and Evenhuis, 1981; Focks *et al.*, 1982). Introducing large numbers of sterile males is another approach in reducing mosquito numbers (Jennifer, 2011).

Biological mosquito control methods have become difficult to deploy in places where the mosquitoes have adapted ways that enable their larvae to survive in temporary water habitats such as tree trunks, hoof prints, old tyres and tins (Ndirangu, 2015). Biological control agents
such as *Var. israelensis*, *B. thuringiensis* and *B. sphaericus* are expensive and not easy to culture. Larvivorus fish cannot survive in temporary water habitats. All these have resulted to continuous search for sustainable and cost effective alternative methods for mosquito control (Yeye, 2001; Ndirangu, 2015).

### 2.5.4 Genetic Control

Genetic control involves use of Genetically Modified Mosquitoes (GMMs). Experimental genetic methods including cytoplasmic incompatibility, chromosomal translocations, sex distortion and gene replacement have been used because they are cheaper and not subject to vector resistance (Webb, 2014). Site-specific gene recombination technologies insert the anti pathogen effect or genes in the integration sites of the genome, making it more effective (Amenya *et al.*, 2010). Sterile insect technique is a species-specific and environmentally non-polluting methodology that relies mainly on the release of large numbers of sterile insects (Knipling, 1955). The reared sterile mosquitoes are released into the environment (WHO, 2009). Mating of released sterile males with native females leads to a decrease in the females’ reproductive potential, resulting to elimination or suppression of the vector population. For instance, *Anopheles albimanus* was successfully controlled by the use of chemo sterilized mosquitoes during a trial in El Salvador (Lofgren *et al.*, 1974).

### 2.5.5 Habitat management

Habitat management involves source reduction and manipulation of mosquito breeding sites through engineering measures such as land leveling and filling, tree planting, improved irrigation structures such as drip irrigation. Takken and Knols (2009) reported that periodic
drainage of rice fields and fish ponds was a very effective method in reducing mosquito population in Java and Indonesia. Source reduction can also be through building of settlements away from vector sources, mosquito-proofing of houses, personal protection and hygienic measures against vectors, and provision of mechanical barriers (Takken and Knols, 2009).

Sanitation measures include waste water and excreta disposal, laundry, bathing and recreation to prevent human contact with infested waters, and zooprophylaxis. Mechanical barriers involves strategic placement of cattle as a buffer between mosquito breeding places, and areas of human habitation (WHO, 1980). Manipulation of breeding sites include; temporary removal or disturbance of larval habitat formation, such as making water unsuitable for mosquitoes to breed in by changing its salinity, stream flushing, intermittent irrigation, regulation of the water level in reservoirs through de flooding of swamps or boggy areas, vegetation removal, and exposure to sunlight (Mouchet et al., 1988; Patz et al., 2000).

The malaria vectors have adopted ways that enable their larvae to survive in temporary water collections. They prefer breeding sites that are small, numerous, scattered and shifting. Each species has its own idiosyncratic preferences. Thus, detailed knowledge of the specific kinds of water exploited by the various local vectors is necessary (Enayati et al., 2009). This calls for multi-dimensional vector integration methods to manipulate mosquito populations.
2.6 *Anopheles gambiae* sensu stricto Giles

2.6.1 Occurrence of *An. gambiae* s.s.

*Anopheles gambiae* (Plate 2.1) is a complex of morphologically indistinguishable species of mosquitoes in the genus *Anopheles*, broadly divided as fresh water and salt water *gambiae*. This species complex is made up of eight reproductively isolated species, which include *Anopheles arabiensis*, *Anopheles bwambiae*, *Anopheles merus*, *Anopheles melas*, *Anopheles quadriannulatus*, *Anopheles gambiae* sensu stricto, *Anopheles amharicus*, and *Anopheles coluzzi* (White, 1975; Coetzee et al., 2013). *Anopheles gambiae* has a medium size, irregular speckled legs, and pale spots in the 3rd dark area of the wings’ vein 1 and lower branch vein 5. It rests inside and outside houses, and breeds on open exposed ground pools of all sizes, brick pits, foot prints, and tyres (Gillet, 1972). Occasionally, it breeds in man-made containers such as wheel barrows, motor pans and at times during heavy infestation, they have been found in domestic ant-traps within houses (Gillet, 1972).

![Image of Anopheles gambiae](Plate 1.1: The adult female African malaria mosquito, *Anopheles gambiae* Giles)
The sibling species of the complex are collectively known as *Anopheles gambiae* sensu lato. Despite being morphologically indistinguishable, individual species of *An. gambiae* complex exhibits different behavioral traits. *An. quadriannulatus* is generally considered to be zoophilic (taking its blood meal from animals), whereas *An. gambiae* sensu stricto is generally anthropophilic (taking blood meal from humans) (White, 1975; Coetzee *et al.*, 2013). This complex was recognized in 1960s and includes the most important vectors of malaria in sub-Saharan Africa. Members of this complex are found throughout tropical Africa, south of the Sahara desert, with *Anopheles arabiensis* extending across southern Arabia. *An. gambiae* s.s. is distributed throughout sub-Saharan Africa, including Madagascar (Figure 2.1) (WHO, 1989).

**Figure 2.1:** Distribution of *Anopheles gambiae* s.s. Giles in tropical Africa
2.6.2 Life cycle of *Anopheles gambiae*

Like all other mosquitoes, *Anophelines* undergo complete metamorphosis. They go through four distinct developmental stages during their lifetime (Figure 2.2). The first three stages are aquatic and lasts for 5-14 days depending on the species and the ambient temperature.

![Life cycle of the mosquito](image)

**Figure 2.2:** Life cycle of the mosquito

2.7 Repellents

Repellents are natural or synthetic chemical compounds that make arthropod make oriented movements away from plants, animals and materials such as fabrics, grains and timber by making them unattractive, unpalatable or offensive either through masking the organism or blocking the olfactory system of the arthropod (Metcalf and Flint, 1962). The potency of a repellent depends on its ability to cause sensory stimulation other than that responsible for locomotion or feeding (Dethier, 1956). Apart from these, the repellent must possess the following qualities; long protection period of the affected area, non-toxic, free of irritation,
pleasant smell, cheap, and readily available (Shambaugh and Brown, 1958). The first repellent was used in 1379 AD (Kreier, 1983).

2.7.1 Synthetic repellents

Chemical repellents protect people from blood-feeding insects and other arthropods. They are applied on the skin or clothing to give short-term protection against mosquito bites. This reduces transmission of malaria and other arthropod-borne diseases (Brown and Hebert, 1997). Synthetic repellents that have been used to control mosquito bites include 2-butyl-2-ethyl-1,3-propanediol (30), 2-phenylcyclohexanol (31) and dimethyl phthalate (32) (Knox et al., 2003);

![Chemical structures](image)

The Centre for Disease Control (CDC) recommends the following synthetic repellents; 28, 29, 30, 31, 32 and Picaridin (Icaridin) (33). These synthetic repellents have several limitations, such as reduced efficacy owing to sweating, costs, allergic reactions, drug resistance and toxicity to non-target organisms. Therefore, they are not safe for public use (Zadikoff, 1979; Ronald et al., 1985).

Repellent-treated fabrics might obviate some of these limitations, since many species of bloodsucking insects bite predominantly around the ankles and wrists. The N,N-diethyl-m-
toluamide (DEET) (34) impregnated anklets, wristbands, shoulder and pocket fabric strips at a concentration of 2 mg/cm² has been reported to provide 5 h of complete protection against mosquito bites (Karunamoorthi and Sabesan, 2009). Use of these early synthetic repellents was overshadowed by the discovery of compound 34, which gradually became the gold standard for arthropod repellents (Strickman, 2007);

![Chemical structure of compound 33 and 34](image)

Compound 34 was formulated as an arthropod repellent in 1946 and registered for commercial use in 1957 (Xue et al., 2007). It is the active ingredient in most of the commercially available mosquito repellents used on human skin today and is effective against several mosquito species, biting flies, jiggers, ticks and fleas (Schreck et al., 1995). It poses no risks to humans if administered as per the guidelines and directives. However, the repellent has been associated with human and environmental problems (Lewis et al., 2000). This has led to search for safe and alternative repellents for arthropods (Seo et al., 2005). A number of other synthetic alternatives are available. Compound 33 has the advantage of being odorless and causes no damage to plastics, with comparable protection to 34, and is effective against equal range of insects.

Synthetic pyrethroids that have been used to control mosquitoes include allethrin (35), bioallethrin (36), cymethrin (37) and tetramethrin (38) (Knox et al., 2003). These repellents
accumulate in the environment and transform into poisonous substances that are toxic to non-target organisms. Therefore, search for safe and cheap natural products with the same properties is inevitable;

\[
\text{35} \quad \text{36} \\
\text{37} \quad \text{38}
\]

### 2.7.2 Mosquito repellent plants

Most plants are used as mosquito repellents because they have been found to exhibit insecticidal and/or mosquito repellent properties. The following plants have been documented to possess repellency activity against mosquitoes: Pyrethrum, pine, *Azadirachta indica*, Eucalyptus spp. (Myrtaceae), *Lantana camara*, *Vitex negundo* (Verbanaceae), *Cymbopogon* spp. (Gramineae), *Mentha piperita* (Labiatae), *Tagetes minuta* (Compositae), *Ocimum* spp., *Ajuga remota*, *Lippie javanica*, and *Lippie ukambensis* (Karunamoorthi et al., 2008). These plants are usually smouldered to produce compounds that repel mosquitoes (White, 1975). In Nigeria *Ocimum gratissimum* “mosquito” plant emits strong fragrance that repels mosquitoes and other insects when propagated around residential areas (Dalziel, 1937).
Ethno-botanical studies of traditionally used mosquito repellent plants in western Kenya showed that thermal expulsion of *Coryombia citriodora*, *Ocimum kilimandscharicum* and *Ocimum suave* repelled up to 74% of host-seeking *An. gambiae* s.s. Branches of some plants were cut and hanged inside houses, particularly around beds for space-fumigation. Intact and live potted plants of *Ocimum americanum*, *Lantana camara* and *Lippie ukambensis* reduced biting by *An. gambiae* by 30-40% (Aklilu *et al*., 2003).

Rural population in China burn *Artemisia* and *Calamus* herbs to repel mosquitoes and other blood-sucking arthropods (Marbiah *et al*., 1988). The Luo community in Kenya hangs branches of *Ocimum basillicum* (Labitae) in houses to keep away mosquitoes and other blood-sucking arthropods (Seyoum *et al*., 2002). *Ocimum* and *Hyptis* species are being used by women in Tanzania as natural repellents (Edoh *et al*., 1997).

### 2.7.3 Plant derived repellent products

The potential of plants as sources of essential oils or fumigants that are repellent to mosquitoes and other biting arthropods is well known (Odalo *et al*., 2005). Plant-based products have been used to repel/kill blood-sucking insects in many parts of the world, either as larvicides/adulticides or repellents for space and personal protection against mosquito. Natural products were used as poisons reflecting the advantage over synthetic molecules in terms of ecological safety and biodegradability (Mawada *et al*., 2005). Arthropod repellent properties of plants are due to their volatile organic compounds (VOCs). The relative amount of repellent VOCs determines the degree of repellency of a certain plant.
The mixture of *Curcoma longalar* mixed with vegetable oil has been used for protection against mosquitoes and other blood-sucking insects in India (Gupta and Louis, 1994). Mixture of *Bixa orelana* with either vegetable or animal oil made by women in Mexico has been used by men to protect themselves from mosquito bites whenever they go out for hunting (Gupta and Louis, 1994).

Plant extracts from repellent plants have been used and even their constituents incorporated in lotions or formulations of the whole plant oil. Extracts from plants such as *Azadirachta indica*, *Cymbopogon nardus*, *Syzyalum aromaticum*, and *Thymus vulgaris* were found to be repellent against mosquito (Banard, 1999).

Essential oils from *Ocimum* spp., *Cymbopogon* spp., Eucalyptus (*Maculate citriodora*), *Pelargonium citrosum*, *Artemisia vulgaris*, *Lantana camara*, *Mentha piperita*, *Vitex rotundofolia*, *Curcuma* spp., *Conyza newii*, *Plectranthus marrubioides*, *Tetradenia riparia*, *Tarchonanthus camphoratus*, *Lippie javanica* and *Lippie ukambensis* have been found to repel mosquitoes (Odalo et al., 2005). The most commonly used plant derived repellents include citronellal (39), geraniol (40), pyrethrins (41a-f) and *p*-menthane-1, 8-diol (42) (Wright, 1975; Kirk and Othmer, 1981). Other plant derived repellents include *α*-pinene (43), linalool (44), cineol (45), *p*-menthane-1,3-diol (46), camphor (47), *p*-menthane-3,8-diol (48), and eugenol (49) (Barasa et al., 2002);
The *iso*-pulegol (50), citronella (51) and citronellol (52) compounds from Lemon eucalyptus have been found to exhibit mosquito repellence properties (Collin and Brady, 1993; Fradin and Day, 2002). The essential oil from cloves, *Syzygium aromaticum*, Merrill and perry, contains compound 49 and β-caryophyllene (53) which are more repellent against *Anopheles albimanus* than DEET but non-repellent against *Ae. Aegypti* (Bernard, 1999). Studies have
shown that some plants and their products are comparable to DEET as repellents and can even be better (Mayeku, 2006);

2.7.3.1 Citronella

Compound 51 is one of the common ingredients in most mosquito repellents found in herbal insect repellents in many parts of the world (Wright, 1975). The compound was first extracted from lemon grass *Cymbopogon nardus*. It has a strong aroma that masks other scents, and keeps mosquitoes away from being attracted to things within its vicinity. The compound has short protection efficacy as compared to synthetic DEET-based repellents (Wright, 1975). During a 4-minute test, Natrapel which is a commercial repellent containing 10% citronella reduced mosquito bites by 84% (Wright, 1975).

Formulation of Buzz Away® with 5% citronella, provided 1.9 h protection time against *Ae. aegypti* during field evaluation (Wright, 1975). Candles made from citronella have been found to be effective in repelling mosquitoes in the backyard (Wright, 1975). The main phytochemicals that have been identified in the essential oil containing compound 51 include 52, 39, 40, nerol (55), citral (56), camphene (57) and limonene (58) (Lindsay *et al.*, 1990);
2.7.3.2 Bite blocker

The oil of geranium has shown to exhibit repellent activity against mosquitoes (Lindsay et al., 1990). The oil has been found to contain several organic compounds which include 40, 52, citronellyl formate (59), 44, 49, myrtenol (60) and terpineol (61) (Lindsay et al., 1990). Geraniol (40) is used in the production of Bite blocker, a commercial repellent containing 90% geraniol, soybean and coconut oil. This repellent has shown protective efficacy of up to 93% for 3 h against An. gambiae during field experiments (Wright, 1975). It provides percentage protection efficacy of 97% for 3.5 h after application against Aedes mosquito during field trials (Lindsay et al., 1990);

2.7.3.3 Pyrethrins

The pyrethrins are obtained from the flowers of Chrysanthemum cinerariaefolium L. and Chrysanthemum coccineum plants through solvent extraction of the grounded and dried
flowers harvested shortly after blooming. The pyrethrins ducts and extract have active ingredient content of about 30% and 65%, respectively. The insecticidal components of pyrethrum are collectively known as pyrethrins which result from six esters, the pyrethrins I and II \(41a, b\), cinerins I and II \(41c, d\), and jasmolin I and II \(41e, f\), (Kirk and Othmer, 1981; Sukumar et al., 1991). Natural pyrethrins quickly penetrate the insect nervous system due to their contact poison property. Pyrethrin and pyrethroid insecticides are biodegradable, non-toxic to mammals and birds but slightly toxic to young children and bees (Kirk and Othmer, 1981).

2.7.3.4 \(p\)-Menthane-3,8-diols

Among the essential oils, \(p\)-menthane-3,8-diol has a lower vapor pressure than volatile monoterpenes (Carroll and Loyce, 2006). Hence, it is less volatile and gives long protection time making it to be the active ingredient in most insect repellents. It is extracted from the essential oil of Corymbia citriodora (Eucalyptus citriodora). The extract from this plant has been found to be repellent against An. gambiae s.s. (Barasa et al., 2002).

2.8 Application of repellents

Different application methods of repellents have been deployed to reduce human-pathogen-vector contacts in an effort to combat malaria. These methods include direct burning, use of repellent formulated lotions or creams, hanging branches of repellent plants in the houses, vaporizing lamps and thermal expulsion of repellent materials. The deployment of live intact potted repellent plants in the control of An. gambiae s.s. is of main concern (Odalo et al., 2005; Mayeku et al., 2006).
2.8.1 Direct burning

This method involves direct burning of either, repellent plant material, plant derived repellent products or synthetic repellents. Smoke produced by burning of dried leaves of various plants has been used for protection against mosquitoes since ancient times (Karunamoorthi et al., 2008; Karunamoorthi et al., 2009). Plants such as Cyperus articulate, Hyptis spicigera, Citrus sinensis peel and Ocimum spp., when burnt produced smoke that repelled mosquitoes (Dalziel, 1937). The smoke produced by burning a mixture of charcoal and plant powder from Azadirachta or Ocimum santum, serves as a good mosquito repellent and an adulticide (Adebayo, 2001).

Traditional lamps were used to burn kerosene oil mixed with repellent volatiles that dispersed as it burned producing smoke that repelled mosquitoes (Pates et al., 2002). Egyptians and Assyrians are known in using Incense and Joss sticks to control malaria vectors. These are made from natural pyrethrum; and used in repelling of mosquitoes and killing midges, both indoors and outdoors. Citronella candles have been found to be effective in repelling mosquitoes. Research conducted by Wright (1975) under field conditions on the efficacy of citronella candles found that people using 3% citronella candles had 42% fewer bites from Aedes mosquitoes than those using 5% citronella incense and plain candles (reduced by 23%).

The composition of several resins and wood mainly from the tree D. oliveri (Churai) produced smoke when burned that reduced the number of mosquito bites. Coils have also been used to control malaria vectors. They are composed of insecticide/repellent, organic filler, binder and additives such as synergists, dyes and fungicide (Robert et al., 2003). The repellents
commonly used are compound 34, 40, and 51. Coils containing synergists such as octachlorodipropyl ether as active ingredient have been used to repel mosquitoes in China (Robert et al., 2003).

2.8.2 Use of repellent lotions

The use of formulated repellent lotions material to reduce the number of mosquitoes landing on human hosts has been a widespread malaria control strategy in most parts of the world (Gupta and Louis, 1994). They are either, applied directly on the skin or released in the air to turn mosquitoes away before or just after they land, hence, interrupting their landing and feeding behavior. Sulfur and molasses have been incorporated in lotions making them repellent to mosquitoes in Transvaal and Natal provinces of South Africa (Lindsay and Gibson, 1988).

Compound 34 and ethylhexanediol (62) when applied on the skin or clothing has been effective in repelling mosquitoes and other insects (Marquardt et al., 1985);

\[
\begin{align*}
\text{OH} & \\
\text{CH}_2 & \\
\text{CH}_2 & \\
\text{OH} & \\
\end{align*}
\]

Formulations such as solutions, lotions, creams, gels, aerosol, pump sprays and impregnated towel in United States are mixed with DEET in the concentration range of 5-95% (Curtis, 1986). DEET has been effective in repelling mosquitoes and other insects. A 100% protection efficacy against mosquitoes has been achieved with standard mosquito repellents containing
up to 75% DEET in an alcohol base. About 35% of DEET has been used to make a mosquito repellent polymer based lotion (Curtis, 1986).

A leaf infusion of *Lippia javanica* Spreng and *Ocimum canum* Sims are applied on the skin to control mosquito bites in Zimbabwe (Lukwa *et al*., 1994). The use of lotions containing repellent substances, are now being used to offer protection in the evening before bed time (Gupta and Louis, 1994). This has reduced malaria transmission incidences and irritations from the mosquito bites (Ndirangu, 2015).

2.8.3 Vaporizers

This method is similar to thermal expulsion of plant materials. It releases smokeless natural pyrethrin into the space, killing indoor and repelling outdoor flying mosquitoes. Kerosene burning lamps (*korobois*) in Tanzania have been modified to heat and vaporize volatile pyrethroid insecticides to repel host seeking mosquitoes (Pates *et al*., 2002). A mixture of 0.1% Transfluthrin and vegetable oil heated up to 120°C in a modified lamp tin held above the flame provided reduction rate of 50-75% in the number of bites from *Cx. quinquefasciatus* say (Pates *et al*., 2002).

2.8.4 Thermal expulsion

Research shows that thermally expelled repellents have relatively substantial repellency both during application of plant treatments and residual effect in post application periods. Thermal expulsion of *Corymbia citriodora*, *Ocimum suave*, and *Ocimum kilimandscharicum* repelled up to 74% of host-seeking *An. gambiae* s.s. in semi-field experimental huts using modified
African traditional stoves in western Kenya (Seyoum et al., 2003). Highest repellency both during and after application of plant treatments was observed with *C. citriodora*, followed by *O. kilimandscharicum* and *O. suave* (Seyoum et al., 2003). Thermal expulsion of oil obtained from *Conyza newii* was shown to be repellent to mosquitoes, with steam distilled oil and directly burned oil having highest and lowest protection efficacy respectively, (Mayeku, 2006).

2.8.5 Potted plants

Potted intact repellent plants are currently receiving attention, owing to their environmental and user-friendly nature (Karunamoorthi et al., 2009). They are cheap, readily available, locally known, and culturally acceptable (Karunamoorthi et al., 2008). Three species (*O. americanum*, *L. camara* and *L. ukambensis*) from a pool of nine plants evaluated emitted blend of volatiles that was repellent against *An. gambiae* under semi-field conditions. Although this was lower than required to substantially reduce the incidences of malaria in endemic areas, it can usefully contribute to integrated programmes (Seyoum et al., 2002, 2003).

Due to its convenience and simplicity, more extensive research on screening of a broader profile of potted plants needs to be conducted. The use of potted repellent plants has not yet been fully exploited due to low knowledge on plant use (Odalo et al., 2005). Therefore, there is need to study scientifically the ethno-botanical practices on mosquito control (Odalo et al., 2005). This study reports the repellent activity of emitted volatiles by live intact potted *M. piperita* plants propagated in the coastal region of Kenya against *An. gambiae* s.s.
2.9 Plant under investigation

2.9.1 Origin and morphology of *Mentha piperita*

Peppermint’s Latin name, *Mentha piperita*, comes from the Greek mintha, the name of a mythical nymph thought to have metamorphosed into the plant and the Latin piper, meaning pepper. *Mentha piperita* (Plate 2.2) is a peppermint species from the genus mentha and it belongs to the family of lamiaceae (mint family), commonly known as peppermint (English), brandy mint, candy mint, lamb mint, balm mint, curled mint, amenta, *Mentha balsamea* (wild lam mint), Vilayati pudina, or Papara minta. The plant is a strongly scented, rhizomatous, perennial and glabrous herb that grows 50-60 cm (3-4 feet) high.

Propagation of the plant takes place through the runners which grow above and below the ground. Peppermint plant thrives in moist habitats, including stream sides, drainage ditches, shaded locations, and expands by underground rhizomes in sandy, loamy soils rich in humus with humid and temperate climate. The plant is cultivated in temperate regions of Europe, Asia, North America and Australia (Mckay and Blumberg, 2006). It is grown in India, Kashmir, Nilgiris, Mysora, and Delhi.
Plate 2.2: Photo of potted *Mentha piperita* plant

2.9.2 Bioactivity of the essential oil from *Mentha piperita* plant

Most plants are used as mosquito repellents because they have been found to exhibit insecticidal and/or mosquito repellent properties. Essential oil of *M. piperita* has been found to have insecticidal, larvicidal and mosquito repellent properties. The oils are nontoxic to humans (Ansari *et al*., 1999; Odalo *et al*., 2005; Karunamoorthi *et al*., 2008). The plant is usually smouldered to produce compounds that repel mosquitoes (White, 1975). The potential of naturally emitted volatile organic blend from this plant in human space protection against mosquitoes is not well understood. The present study was undertaken to see if indoor deployment of multiple potted *M. piperita* plants can have additive effects in space protection against female *An. gambiae s.s*.

2.9.3 Chemo-types of *Mentha piperita*

Certain small variations in the environment, geographical location, gene etc. which have little or no effect on a morphological level can produce big changes in chemical phenotypes. The
composition of the essential oils of *M. piperita* from different countries has been reported previously and showed significant differences in their composition. GC-MS analysis of the essential oils of *M. piperita* from Australia had the following prominent constituents: menthol (33-60%) (63), menthone (5-32%) (64), menthy acetate (2-11%) (65), pulegone (0.5-1.6%) (66), limonene (1-7%) (58), 1,8-cineole (5-13%) (67), menthofuran (1-10%) (68), β-myrcene (0.1-1.7%), β-caryophyllene (2-4%) (53), isomenthone (2-8%) and carvone (1%) (Mckay and Blumberg, 2006);

![Chemical structures](image)

Analysis of the essential oils of *M. piperita* from India had the following compounds; menthylacetate (1.6-4.3%) and cineole (0.5-6.5%) (Dwivedi et al., 2004). From Portugal the oil had linalyl acetate (72%) and linalool (12.3%) as the main compounds (Martins et al., 2004). Essential oils from Iran contained α-terpinene (19.7%), isomenthone (10.3%), trans-carveol (14.5%), piperitenone oxide (19.3%) and β-caryophyllene (7.6%) (Rasooli et al., 2008). Moreover, in Morocco major compounds were; linalool (60.7%), linalylacetate (20.7%), geraniol (3.2%), 1,8-cineol (2.3%) and limonene (1.5%) (Debbab et al., 2007).

The analysis of the essential oils from chemo-types (genetic and epigenetic) of *Mentha piperita* plant indicate that its composition depends on a number of factors, for example,
harvesting location, stage of harvest, plant parts distilled, method of distillation, kind of 
storage, time of harvest, season, soil type, soil nutrient status and the climatic condition under 
which the plant grows (Verma et al., 2010).
CHAPTER THREE

MATERIALS AND METHODS

3.1 Plant propagation and collection

The identity of *M. piperita* plant was initially confirmed by plant botanist from Kenyatta University botany department and plant taxonomist from Jomo Kenyatta University of Science and Technology (JKUAT). The labeled plant voucher specimens (JMO/MP01/15, JMO/MP02/15, and JMO/MP03/15) were deposited at Kenyatta University herbarium. The plant rooted suckers were taken from old vigorous plants, grown at JKUAT botanical garden and propagated at Pwani University and Kenyatta University. The plants used in the repellency test were planted in plastic pots containing soil, sand, and farmyard manure mixed in the ratio 3:1:2 respectively.

The collected plants were left for two months to acclimatize with the external environmental conditions. They were then transferred into experimental screen houses where they were maintained at day time temperature of 34±7ºC and relative humidity of 61±22%. The diurnal temperature was maintained at 27.9±2.2ºC and relative humidity at 71±5%. They acclimatized at these conditions for 2 weeks before the assays were carried out.

3.2 Rearing and handling of *An. gambiae*

The semi-field reared *An. gambiae* mosquitoes were used for repellency test. The identification of the larvae was based on the morphological features which include; lying parallel on the water surface, well developed head with mouth brushes, lack of respiratory siphons and their body movement either by jerky or propulsion by the mouth brushes.
Moreover, the adults were confirmed by polymerase chain reaction in the department of biological sciences of Pwani University. The mosquito larvae were collected from river Sabaki, Burangi village in Malindi 3.2-10.8 meters above sea level (S3° 9’ 47”, E40° 4’44” and S3° 9’ 49”, E40° 4’ 44”). They were transported in Nasco whirl-PAK® stand-up bags to Pwani University.

The larvae were reared according to the WHO (1996) protocol in a special room (S3° 37’ 14”, E39° 50’ 42”) at an altitude of 146.6 meters. They were placed in white plastic plates (internal diameter of 28 cm and depth of 3 cm), filled to a depth of 1.5 cm with fresh water and fed with biscuit powder (WHO, 1996). Plastic pipettes were used to transfer them into plates with fresh water after every two days. The emerged pupae were collected on a daily basis using plastic pipettes and placed into plastic pupae rearing cages 20 cm high and 11.5 cm in diameter containing fresh water. The room was maintained at a temperature of 27±1°C and relative humidity of 77±8%.

The emerged adults were carefully transferred into adult mosquito rearing cages (12 by 12 by 12 inches) and fed with 10% glucose solution in cotton wool substrate. The room was maintained at 27±1°C and 77±8% of relative humidity under a photoperiod 12 h light: 12 h dark photo cycle.
3.3 Semi-field evaluation of the repellency of live potted *Mentha piperita*

The effect of potted *M. piperita* emitted volatiles on human-vector contact of *An. gambiae* was determined under semi-field conditions inside two screen houses (Figure 3.1 and Plate 3.1), measuring 3.1 M by 4.2 M by 1.6 M (S3º 37’ 1‘’, E39º 50’ 39’’ and S3º 36’ 59’’, E39º 50’ 39’’) at an altitude of 39 M and 43.1 M, each with an experimental hut in the department of biological sciences of Pwani University.

**Plate 3.1:** The semi-field experimental screen houses for night bioassays.
The two experimental arenas (Figure 3.1 and Plate 3.1) were constructed with 1’’ by 2’’ inches pine frames, with the walls, the roof and the doors covered with polyvinyl green house paper. The front phase of each of the arena was covered with a cream coloured plastic mosquito net (16×16 netting per inch) covering an area of 2.52 M$^2$. Only 0.504 M$^2$ of the rear phase was netted. The two rectangular experimental huts (1.5×1.5 M) were made from polyvinylchloride green house paper on the walls and their roofs covered with black nylon cloth inside and Makuti (palm tree branches) outside (Seyoum et al., 2002). One of the experimental huts acted as an arena for the control experiment. The experimental arenas were switched to eliminate any experimental bias.

In the first night, two potted test plants were placed in one of the huts while in the other two pots full of a mixture of soil, farm yard manure and sand without the potted plant were placed in similar positions to serve as a negative control (Plate 3.1). The experimental assays were replicated four times with four, eight and sixteen potted test plants. The control and the treatment were exchanged between the huts using cross-over design. Each night at 7 pm,
twenty, 3-5-day old, unfed female An. gambiae which had never received a blood meal were starved for six hours (Shin-Ho et al., 2009). They were then released from a paper cup at the centre of each experimental hut for repellency test, and recaptured at mid-night by use of a mechanical aspirator (John W. Hock 412 and 612), transferred into a paper cup and placed inside a freezer.

The bioassays were carried out according to Akililu et al., 2002 method with some modification. During the bioassays, the human volunteers sat on the white polyethylene chairs (ACME CH-001) placed at the centre of each experimental hut. A white mosquito net covered the whole body of the volunteer, except the area between the toes and knees (Plate, 3.2).

Plate 3.2: Experimental hut with human bait in a mosquito landing catch
The volunteers counted only the mosquitoes that landed on the exposed area as they probed using a hand held counter and a torch to illuminate the area of landing. After counting, the mosquitoes were shaken off before tearing the skin and imbibing any blood. After one and half hours, the volunteers exchanged positions between the huts to minimize differential bias due to attractiveness and counting skills. The experiment was replicated 4 times on 4 different nights in both control and treatment in each test night.

3.4 Ethical considerations.

A certificate of ethical approval (ERC/MSc /019/2015; Appendix 8) was obtained from Ethical Review Committee of Pwani University for the involvement of human subjects in this study. Trained human volunteers with age of 30, 33 and 36 years old signed a standard ethical clearance consent form before participating in the exercise (Appendix 9). Only males were used who had no parasitemia and were from the locale. No pregnant or lactating women were to be used. The volunteers must be between 18 and 45 years of age.

3.5 Trapping of volatiles from Mentha piperita plants

Volatile released from the intact aerial parts of M. piperita plants were trapped using head space entrainment with a super Q trap as an adsorbent and transparent oven bags (355 mm ×508 mm, Classic Consumer Products, Inc, Englewood, NJ, USA) pre-sterilized at 100°C for 12 hours. The adsorbent traps were made of a 3 cm long Teflon® tube filter trap packed with 30 mg of super Q polymer (80–100 mesh size; ARS) held in place between two plugs of glass wool (Mburu et al., 2010).
Before headspace entrapping, each adsorbent trap was cleaned by flushing it ten times with 1mL of dichloromethane (HPLC grade, 99.9%, Sigma-Aldrich). Purified nitrogen gas (BOC Gases, Nairobi, Kenya) was passed through each trap for 3 minutes to dry them, and then sealed with Teflon® thread tape on both ends to prevent contamination as described by Mburu et al. (2010). The volatiles were collected on an air entrainment system; using purified medical air (BOC Gases) that passed through activated carbon filters (ARS) with continuous flow rate of 170 mLmin⁻¹. The aerial foliage parts of the plant were enclosed in pre-sterilised oven bag and tied with a string slightly above the pot’s soil level. The adsorbent trap was firmly held in place at the open end of a tube connected to the vacuum pump (Vacuum Brand, MZ 2C, Wertheim, Germany). The vacuum and the supply tubes were then pushed into the oven bag from the bottom so that both were above the headspace of the plant (Plate 3.3).

Plate 3.3: Volatile trapping in a manifold system

The oven bag was supplied with a stream of purified and humidified air at a flow rate of 170 ml/min at room temperature. The mixture of air and volatiles in the oven bag was passed into Super Q adsorbent traps (30 mg, Analytical Research System, Gainesville, Florida, USA) for
volatile collection and then sucked out through Teflon® tubes by a vacuum pump (Vacuum Brand, MZ 2C, Wertheim, Germany). The air flow was regulated into the headspace oven volatile collecting bag by a flow meter at a reading scale of 30 (Aalborg, Orange burg, NY, USA). The traps were carefully removed from the oven bags in the manifold volatile trapping system, immediately sealed at both ends with Teflon thread tape, and carried under ice to elution point. The same procedure was applied for trappings from the controls set-ups. Trapping of the volatile blends was done for 12 h at night and 12 h daytime.

3.6 Elution of volatiles from Super-Q traps and Storage of samples before analysis

Volatile trapped by each super-Q trap were eluted with 200 µl of dichloromethane (Analytical grade, Sigma Aldrich, St, Louis, MO, USA) under ice and stream of pure nitrogen gas into 1.5 mL brown ambered Teflon corked vials with inserts (Sigma-Aldrich) (Plate 3.4).

Plate 3.4: Elution of volatiles trapped by super Q adsorbent under ice

The vials were tightly corked with Teflon corks, sealed with Teflon® tape, wrapped with aluminium foil, labeled and stored in a freezer (New Brunswick Scientific Freezer, U725-86G, Eppendorf company, Hamburg, Germany) at -80°C a waiting GC-MSD analysis. Each
used super-Q trap was cleaned by flushing 1mL of dichloromethane (HPLC grade, 99.9%, Sigma-Aldrich) ten times through it using Pasteur pipettes with a bulb, and dried by passing purified nitrogen gas. They were then sealed with Teflon® tape on both ends and wrapped with aluminum foil to prevent contamination.

### 3.7 Analysis of volatile constituents of Mentha piperita plant by Gas Chromatography-Mass Selective Detection (GC-MSD)

#### 3.7.1 Identification of emitted volatiles constituents

Volatile of live potted *M. piperita* and that of the controls were eluted and analyzed using an HP 7890B series GC (Agilent Technologies, Wilmington, DE, USA) coupled to a gas chromatogram 5977A series mass selective detector (GC-MSD) (Agilent technologies) that was fitted with an 7693 series auto sampler detector (Plate 3.5).

![Plate 3.5: Gas Chromatography-Mass Selective Detections (GC-MSD)](image)

The GC column stationary phase was made of a non-polar ultra inert capillary material HP-5 Methyl silicone (HP-5ms, Agilent 19091S-433UI: 001), measuring 30 M long, 250 μM
internal diameter and 0.25 µM film thickness (Agilent, Palo Alto, CA). For the first oven, the GC was programmed at temperature of 35°C held for 5 minutes, followed by a temperature ramp rate of 10°Cmin⁻¹ up to 280°C and maintained at this final temperature for 5.5 minutes. The second oven was programmed at a rate of 50 °C/min to 285 °C for 14.9 minutes with total run time of 50 minutes. 1 µL of each sample was injected into the injector port on splitless mode with septum purge flow of 3 mL/min at an injector temperature of 270 °C. Helium was used as a carrier gas at a constant flow rate of 1.2 mLmin⁻¹.

Dichloromethane was used as a solvent in all the analysis with the delay set for 3 minutes before injection purging. The energy of electron impact (EI) of the ionizer was operated at 70eV with the temperature of the Mass Spectrometry (MS) source and the MS Quad interface being held between 230-250°C and 150-200°C, respectively. The GC was coupled to an HP monitor (HP Compaq LA 2206 Xc) to display chromatographic data, which were evaluated using 3365 MSD ChemStation software (G1701EA E.02.00.493, Agilent Technologies) (plate 3.5). The mass scan range m/z was set at 38-550 g. The mass spectra for the peaks obtained were compared with mass spectral libraries (Adams 2.L /NIST 08.L/Chemecol. L) registries of mass spectral data. Each peak represented the signal produced when a compound in the injected emitted volatiles elute through the GC column to the detector. The identity of the constituents was based on comparison with the mass spectral fragmentation patterns provided in the libraries.
3.7.2 Quantification of the emitted volatiles constituents

Quantification was done using external standards and calibration graphs (Figure 3.2 and 3.3). The quantification was based on comparison of the peak area of each component to that of the external standards 1,8-cineole and β-caryophyllene compounds (Sigma® Chemicals Co, St Louis, MO, USA) for identification of monoterpenoids and sesterquiterpenes, respectively. Different concentrations of 100 ngµl⁻¹, 75 ngµl⁻¹, 25 ngµl⁻¹, 1 ngµl⁻¹, 0.1 ngµl⁻¹, 0.001 ngµl⁻¹ of 1,8-cineole and β-caryophyllene were prepared and ran under the same GC-MSD conditions as described in Section 3.7.1. The graph of concentration against time was plotted and applied in finding the unknown concentrations.

**Figure 3.2:** The calibration graph of peak area of 1,8-cineole against concentration
Figure 3.3: The calibration graph of peak area of β-caryophyllene against concentration

3.8 Blends and individual constituents tested for repellency against Anopheles gambiae s.s.

The four prominent compounds from M. piperita that were commercially available were procured and assayed individually at concentration of $10^{-5}$ to $10^{-1}$ g/mL range. The synthetic blends of these compounds were constituted sequentially starting with the highest concentration according to their relative percentage abundance in the emitted volatiles 1,8-cineole (27.68±2.27), α-pinene (3.43±0.24), β-pinene (14.86±0.77) and β-myrcene (3.56±0.34), as follows: 1,8-cineole (143µl) + α-pinene (17µl)+ β-pinene (71µl) + β-myrcene (19µl) (blend 1), 1,8-cineole (155µl)+ α-pinene (18µl)+ β-pinene (77µl) (blend 2), 1,8-cineole (200µl)+ α-pinene (23µl)+ β-myrcene (27µl) (blend 3), 1,8-cineole (153µl)+ β-pinene (77µl) + β-myrcene (20µl) (blend 4), β-pinene (167µl) + α-pinene (39µl)+ β-myrcene (44µl) (blend
5) and DEET (250µl) as a positive control. This was followed by serial dilution to obtain the consecutive concentrations (Odalo et al., 2005).

### 3.9 Laboratory mosquito repellency assays

The WHO (1996) protocols for laboratory and field evaluation of insecticides and repellents were used for all mosquito repellency assays. The assay room was kept dark with only red light as source of light. The temperature and relative humidity (RH) were maintained at 27-35ºC and RH≥65%, respectively so as to mimic the feeding conditions of the female *An. gambiae*s.s. Bioassay of the synthetic standards and their blends was carried out in aluminium-frame cages (50 cm × 50 cm × 50 cm). The cages were made of aluminium sheet on the bottom, window screen (mesh size 256) on top and back, clear acrylic screen (for viewing) on the right and left sides, and a cotton stockinet sleeve for access on the front.

The repellency was evaluated using the human-bait technique to simulate the condition of human skin to which repellents will be eventually applied (WHO, 1996). Three human volunteers aged 30, 33 and 36 years who had under gone through ethical consenting process as authorized by Ethical Review Committee of Pwani University (Appendices 8 and 9) were used in the bioassays. They had no contact with lotions, perfumes or perfumed soaps on the day the bio-assays were carried out. In each repellency assay, 5-7 day-old female *An. gambiae*s.s. mosquitoes that were reared at ICIPE under standard conditions were used. They had been fed on 6% glucose solution and later starved for 18 hours before the experiments (Omolo et al., 2004).
Fifty female mosquitoes were released into each of the five bioassay cages using an aspirator and left for 10 minutes to acclimatize. The synthetic standards and their blends were prepared in percentage concentrations levels of 0.001, 0.01, 0.10, 1 and 10% in HPLC grade acetone. 1 mL of the test solution was dispensed with a syringe on one of the forearm of each volunteer from the elbow to the wrist while the rest of the arm was covered with a glove. Acetone (1 mL) was dispensed on the other arm to act as a negative control.

The control arm was introduced into the cage first, and kept there for 1 minute. The sides of the experimental cage were gently tapped to activate the mosquitoes and those that landed on the hand were recorded and then handled as previously described. The test arm was introduced into the cage for the same period of time as the control arm and the number of mosquito that landed on it recorded. Repellency of DEET (a positive control) was also undertaken at similar concentrations.

The arms were washed before application of the next concentration with soap, rinsed with tap water and dried using tissue paper for 10 minutes. The different sample concentrations were tested sequentially starting with the lowest. Each concentration was screened with a fresh batch of mosquitoes after which they were sacrificed. Although the number of mosquito bites was low due to short exposure duration time, the volunteers were provided with insect bite cream in case of any minor bites and associated irritation (WHO, 2013).
3.10 Data analyses on the bioassay data

Percentage protective efficacy (PE) data from the four replicates during semi-field experiments (Section 3.3) was calculated for each number of test plant(s) using the formula \( \text{Repellency} = \frac{C-T}{C} \times 100 \). Where \( C \) represents the number of mosquito landings on the control, and \( T \) represents the number of mosquito landings on the treated arm, respectively (Sharma and Ansari, 1994; Yap et al., 1998). The data was transformed and subjected to analysis of variance (ANOVA) and the means compared using the Student–Newman–Kuels (SNK) test (IBM, SPSS software version 21). Significant variation for phytochemical relative composition of the emitted volatiles by the plant during the day and night were analyzed using student t-test at 95% confidence level.

The % protective efficacy (PE) data from laboratory bioassays (Section, 3.9), was calculated using the formula \( \text{PE} = \frac{\text{PCM} - \text{PTM}}{\text{PCM}} \times 100 \). Where PCM and PTM is the % control and treated means, respectively (WHO, 1996). Dose-response relationships values \( (\text{RD}_{50}, \text{RD}_{75} \text{ and } \text{RD}_{90}) \) were determined by probit analysis using the formular \( \text{Probit} [P (\text{Dose}1)] = \beta_0 + x\beta_1 + \hat{\epsilon} \) (Busvine, 1971; Finney, 1971).

Where:
\[ \beta_0 = \text{Coefficient of the model representing y-intercept} \]
\[ \beta_1 = \text{Coefficient of the model representing dose 1} \]
\[ \text{Dose } 1 = \log_{10} (\text{dose}) \]
\[ \hat{\epsilon} = \text{Error term in the data set of the predictor (Regressor) variable (x)} \]
\[ P = \text{Repellency probability}. \]
CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Time for repellency activity of volatiles from live potted *Mentha piperita*

Repellency of volatiles emitted by live potted *M. piperita* plants in combination (2, 4, 8 and 16) against *An. gambiae* s.s. were determined. The hourly mean percentage activity is as shown in table 4.1 and figure 4.1.

**Table 4.1:** Hourly mean percentage repellency (±SE) of different number of potted plants against *An. gambiae*

<table>
<thead>
<tr>
<th>No of plants</th>
<th>1st hour Mean±SE</th>
<th>2nd hour Mean±SE</th>
<th>3rd hour Mean±SE</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>43.78±13.63aA</td>
<td>59.43±9.01aB</td>
<td>56.44±25.20aD</td>
<td>0.799</td>
</tr>
<tr>
<td>4</td>
<td>65.52±17.81bA</td>
<td>57.18±14.07bB</td>
<td>86.60±7.96cD</td>
<td>0.347</td>
</tr>
<tr>
<td>8</td>
<td>60.31±14.19dA</td>
<td>86.84±13.16dC</td>
<td>72.36±12.02dD</td>
<td>0.399</td>
</tr>
<tr>
<td>16</td>
<td>61.64±7.64eA</td>
<td>59.53±24.63eB</td>
<td>86.96±8.00eD</td>
<td>0.623</td>
</tr>
<tr>
<td>p-value</td>
<td>0.528</td>
<td>0.542</td>
<td>0.505</td>
<td></td>
</tr>
</tbody>
</table>

Means (±SE) with same small and capital letters in a row and within a column respectively, are not significantly different (α = 0.05, p<0.05, SNK).
Figure 4.1: Hourly SNK mean percentage repellency (±SE) rankings of different number of potted plants against *An. gambiae*

There was no significance difference in terms of repellency activity of different number of potted plants with time (SNK, $p \geq 0.05$, 95% CL). This is similar to the results reported by Aklilu *et al.* (2003), which indicated that live potted plants do not appreciably vary in their repellent properties at different times of the night.
4.2 Repellency activity of different sets of live potted *M. piperita* plant emitted volatiles

Repellence activity of the emitted volatiles of different number sets of plants was determined and the results are given in the table 4.2 and figure 4.2.

**Table 4.2:** Mean percentage repellency (±SE) of different number of potted plants against *An. gambiae*

<table>
<thead>
<tr>
<th>No. of potted plants</th>
<th>PE ( %R) Mean±SE</th>
<th>Significant levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>44.00±11.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>70.09±8.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td><em>P</em>&lt;0.05</td>
</tr>
<tr>
<td>8</td>
<td>72.53±8.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>66.00±15.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td><em>P</em>&gt;0.05</td>
</tr>
</tbody>
</table>

Figures with the same letters are not significantly different (*α* = 0.05, *p*<0.05, SNK).

**Figure 4.2:** Mean (±SE) percentage repellency caused by the night emitted volatiles of *M. piperita* plants against *An. gambiae* (bars of the same color are not significantly different (*α* = 0.05, SNK, 95% CL)
From table 4.2 and figure 4.2 it’s evident that there is a general trend in increase of repellency as the plants increased from two (2) to eight (8). Among the plants there was significant \( p<0.05 \) increase in repellency between the exposure of the cohort replicate mosquitoes when two (2) and four (4) plants were used. However, there was a drop of repellency with the exposure of the cohort of mosquitoes to 16 plants. Thus, the level of emission of volatiles from each plant appears to be negatively affected by the presence of other con-specific plants.
4.3 Prominent constituents identified in volatiles blends during the day and night

The GC-MSD analysis of volatiles emitted during the day and night gave the mass spectra as shown in figure 4.3 and the chemical composition of the major compounds, retention time and relative abundance as given in the table 4.3 and 4.5.

![GC-MSD chromatogram of emitted volatiles by M. piperita plant]

**Figure 4.3:** The GC-MSD Overlay chromatogram of the emitted volatiles by *M. piperita* plant at night and day time
**Table 4.3:** Major compounds emitted in the day and night from *M. piperita* volatiles as identified by GC-MSD

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>Compound Name</th>
<th>Relative %</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.235</td>
<td>4-Methyl-2-hexanol (69)</td>
<td>1.95</td>
<td>Non-terpenoid alcohol</td>
</tr>
<tr>
<td>9.37</td>
<td>1,2,3,4,5-Pentamethylcyclopentane (70)</td>
<td>1.38</td>
<td>Non-terpenoid hydrocarbon</td>
</tr>
<tr>
<td>9.703</td>
<td>α-Pinene (71)</td>
<td>3.51</td>
<td>Mono-terpenoid hydrocarbon</td>
</tr>
<tr>
<td>9.715</td>
<td>Tricyclene (72)</td>
<td>3.51</td>
<td>Mono-terpenoid hydrocarbon</td>
</tr>
<tr>
<td>10.616</td>
<td>β-Pinene (73)</td>
<td>14.72</td>
<td>Mono-terpenoid hydrocarbon</td>
</tr>
<tr>
<td>10.622</td>
<td>β-Phellandrene (74)</td>
<td>14.83</td>
<td>Mono-terpenoid hydrocarbon</td>
</tr>
<tr>
<td>10.943</td>
<td>β-Myrcene (75)</td>
<td>3.82</td>
<td>Mono-terpenoid hydrocarbon</td>
</tr>
<tr>
<td>11.71</td>
<td>1,8-Cineole (76)</td>
<td>26.41</td>
<td>Mono-terpenoid ether</td>
</tr>
<tr>
<td>12.365</td>
<td>cis-Thujan-4-ol (77)</td>
<td>1.12</td>
<td>Mono-terpenoid alcohol</td>
</tr>
<tr>
<td>12.371</td>
<td>α-Ocimene (78)</td>
<td>1.11</td>
<td>Mono-terpenoid hydrocarbon</td>
</tr>
<tr>
<td>15.372</td>
<td>trans-Piperitone epoxide (79)</td>
<td>4.0</td>
<td>Mono-terpenoid ether</td>
</tr>
<tr>
<td>15.378</td>
<td>1-Cyclopentylethanone (80)</td>
<td>1.21</td>
<td>Non-terpenoid hydrocarbon</td>
</tr>
<tr>
<td>15.383</td>
<td>3-Ethyl-2,5-dimethyl-3-hexene (81)</td>
<td>1.19</td>
<td>Non-terpenoid hydrocarbon</td>
</tr>
<tr>
<td>16.945</td>
<td>Piperitenone oxide (82)</td>
<td>2.08</td>
<td>Mono-terpenoid ketoether</td>
</tr>
<tr>
<td>17.507</td>
<td>2-Butyl-1,3-cyclopentanedione (83)</td>
<td>1.39</td>
<td>Non-terpenoid hydrocarbon</td>
</tr>
<tr>
<td>17.513</td>
<td>4-(Dimethylamino)-3-penten-2-one (84)</td>
<td>1.38</td>
<td>Non-terpenoid ketone</td>
</tr>
<tr>
<td>17.527</td>
<td>Bicyclo[2.2.2]octane-1-carboxylicacid (85)</td>
<td>1.39</td>
<td>Non-terpenoid carboxylic acid</td>
</tr>
<tr>
<td>17.683</td>
<td>β-Cubebene (86)</td>
<td>1.14</td>
<td>Sesquiterpenoid hydrocarbon</td>
</tr>
<tr>
<td>17.7</td>
<td>γ-Cadinene (87)</td>
<td>1.23</td>
<td>Sesquiterpenoid hydrocarbon</td>
</tr>
<tr>
<td>17.812</td>
<td>β-Copaene (88)</td>
<td>1.32</td>
<td>Sesquiterpenoid hydrocarbon</td>
</tr>
<tr>
<td>18.496</td>
<td>Germacrene D (89)</td>
<td>1.82</td>
<td>Sesquiterpenoid hydrocarbon</td>
</tr>
</tbody>
</table>
The following are the structures of the most prominent compounds identified by the GC-MSD in the emitted volatiles from *M. piperita* potted plants both at night and day as listed in table 4.3:

From the GC-MSD analysis, 38 and 43 compounds were identified in the day and night emitted volatiles, respectively (Appendices 5 and 6). 1,8-Cineole was the prominent compound emitted both at night and day time with percentage relative abundance of 26.41% and 23.19% respectively. The major components of the emitted volatiles were monoterpenes 60.5% and 54.5% during the night and day respectively; this is in agreement with Para et al. (2013) who reported that monoterpenes are more volatile and constitute the larger relative percentage abundance of any plant emitted volatiles. The sesquiterpenes were only 23.3% and 25% during the day and night respectively. Non-terpenoids were only in trace amounts. Twenty compounds were common both during the day and at night with combined relative
percentage abundance of 82.39% and 83.6% at night and day consecutively, (Table 4.3). Twenty two and twenty three different compounds were emitted during the day and night with relative abundance of 16.4% and 17.61%, respectively (Table 4.4).

**Table 4.4:** Night and Day prominent compounds identified by the GC-MSD from the emitted volatiles by potted *M. piperita* plant

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>DAY Relative %</th>
<th>NIGHT Relative %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(2r,4r)-p-Mentha-[1(7),8]-diene,2-hydroperoxide</td>
<td>3.41</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Nortricyclene</td>
<td>1.95</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>β-Myrcene</td>
<td>1.75</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>1-Cyclopentylethanone</td>
<td>1.21</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>3-Ethyl-2,5-dimethyl-3-hexene</td>
<td>1.19</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>2,3,6-Trimethylphenol</td>
<td>0.55</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>(cis)-β-Terpineol</td>
<td>0.49</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>(+)-3-Carene</td>
<td>0.49</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>(trans)-Sabinenehydrate</td>
<td>0.47</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>Tetradecane</td>
<td>0.47</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>β-Elemene</td>
<td>0.45</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>β-Gurjunene</td>
<td>0.44</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>α-Caryophyllene</td>
<td>0.43</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>trans-Muurola-4(14),5-diene</td>
<td>0.41</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>(+)-epi-Bicyclosesquiphellandrene</td>
<td>0.35</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>(e)-β-Ocimene</td>
<td>0.25</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>3-Pinanol</td>
<td>0.25</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>2,6-Dimethyl-7-octen-2-ol</td>
<td>0.22</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>2-Hydroxypiperitone</td>
<td>0.14</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Compound</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---------------------------------</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>20</td>
<td>Carvone</td>
<td>0.10</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>Pinocarvone</td>
<td>0.09</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td>Sabinene</td>
<td>0.06</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>4-Methyl-2-hexanol</td>
<td></td>
<td>1.95</td>
</tr>
<tr>
<td>24</td>
<td>Bicyclo[2.2.2]octane-1-carboxylic acid</td>
<td></td>
<td>1.39</td>
</tr>
<tr>
<td>25</td>
<td>2-Butyl-1,3-cyclopentanediol</td>
<td></td>
<td>1.39</td>
</tr>
<tr>
<td>26</td>
<td>4-(Dimethylamino)-3-penten-2-one</td>
<td></td>
<td>1.38</td>
</tr>
<tr>
<td>27</td>
<td>1,2,3,4,5-Pentamethylcyclopentane</td>
<td></td>
<td>1.38</td>
</tr>
<tr>
<td>28</td>
<td>β-Cubebene</td>
<td></td>
<td>1.14</td>
</tr>
<tr>
<td>29</td>
<td>5,7-Diethyl-5,6-decadien-3-yne</td>
<td></td>
<td>0.95</td>
</tr>
<tr>
<td>30</td>
<td>Germacrene D</td>
<td></td>
<td>0.94</td>
</tr>
<tr>
<td>31</td>
<td>Phenylethanediol</td>
<td></td>
<td>0.72</td>
</tr>
<tr>
<td>32</td>
<td>Germacrene B</td>
<td></td>
<td>0.71</td>
</tr>
<tr>
<td>33</td>
<td>α-Terpinolene</td>
<td></td>
<td>0.71</td>
</tr>
<tr>
<td>34</td>
<td>β-Ylangene</td>
<td></td>
<td>0.61</td>
</tr>
<tr>
<td>35</td>
<td>Buccocamphor</td>
<td></td>
<td>0.59</td>
</tr>
<tr>
<td>36</td>
<td>2,5,5-Trimethyl-1,3,6-heptatriene</td>
<td></td>
<td>0.55</td>
</tr>
<tr>
<td>37</td>
<td>2,5,6-Trimethyl-1,3,6-heptatriene</td>
<td></td>
<td>0.55</td>
</tr>
<tr>
<td>38</td>
<td>1,5,9-Trimethyl-1,5,9-cyclododecatriene</td>
<td></td>
<td>0.46</td>
</tr>
<tr>
<td>39</td>
<td>(+) -Valencene</td>
<td></td>
<td>0.38</td>
</tr>
<tr>
<td>40</td>
<td>2, 3-Dihydro-4-methylfuran</td>
<td></td>
<td>0.35</td>
</tr>
<tr>
<td>41</td>
<td>(trans)-2-Methyldecalin</td>
<td></td>
<td>0.35</td>
</tr>
<tr>
<td>42</td>
<td>cis-Pinocamphene</td>
<td></td>
<td>0.34</td>
</tr>
<tr>
<td>43</td>
<td>α-Terpineol</td>
<td></td>
<td>0.26</td>
</tr>
<tr>
<td>44</td>
<td>β-Cadinene</td>
<td></td>
<td>0.26</td>
</tr>
<tr>
<td>45</td>
<td>δ-Cadinol</td>
<td></td>
<td>0.22</td>
</tr>
<tr>
<td>46</td>
<td>Terpinolene</td>
<td></td>
<td>0.16</td>
</tr>
</tbody>
</table>
There were both qualitative and quantitative significant differences in the day and night emitted volatiles. The relative abundance of the prominent compounds in day and night emitted volatiles, showed that 60% of the compounds were significantly different \((p<0.05)\) and 40% were not \((t\text{-test, } \alpha=0.05, p>0.05)\), (Table 4.4, 4.5 and 4.6).

**Table 4.5:** Student t-test analysis of the relative abundances of the prominent compounds in the night and day emitted volatiles

<table>
<thead>
<tr>
<th>Compound</th>
<th>Day Mean±SE</th>
<th>Night Mean±SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,8-Cineole</td>
<td>23.93±1.59</td>
<td>27.68±2.27</td>
<td>0.200</td>
</tr>
<tr>
<td>Tricycylene</td>
<td>11.26±1.23</td>
<td>3.42±0.25</td>
<td>0.001</td>
</tr>
<tr>
<td>β-Phellandrene</td>
<td>11.15±1.27</td>
<td>15.11±0.78</td>
<td>0.024</td>
</tr>
<tr>
<td>β-Pinene</td>
<td>9.28±1.60</td>
<td>14.86±0.77</td>
<td>0.008</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>17.91±3.91</td>
<td>3.43±0.24</td>
<td>0.010</td>
</tr>
<tr>
<td>Myrcene</td>
<td>2.24±0.12</td>
<td>3.56±0.34</td>
<td>0.003</td>
</tr>
<tr>
<td>Germacrene D</td>
<td>0.67±0.30</td>
<td>0.79±0.12</td>
<td>0.723</td>
</tr>
<tr>
<td>β-Copaene</td>
<td>0.63±0.19</td>
<td>0.41±0.07</td>
<td>0.301</td>
</tr>
<tr>
<td>trans-Piperitone epoxide</td>
<td>1.08±0.45</td>
<td>2.90±0.95</td>
<td>0.111</td>
</tr>
<tr>
<td>((z))-β-Ocimene</td>
<td>0.53±0.06</td>
<td>0.77±0.06</td>
<td>0.013</td>
</tr>
<tr>
<td>δ-3-Carene</td>
<td>0.53±0.06</td>
<td>2.19±0.73</td>
<td>0.062</td>
</tr>
<tr>
<td>Bicyclogermacrene</td>
<td>0.33±0.10</td>
<td>0.67±0.09</td>
<td>0.027</td>
</tr>
<tr>
<td>allo-Ocimene</td>
<td>0.23±0.08</td>
<td>0.56±0.07</td>
<td>0.008</td>
</tr>
<tr>
<td>cis-Sabinene hydrate</td>
<td>0.77±0.15</td>
<td>1.22±0.27</td>
<td>0.177</td>
</tr>
<tr>
<td>cis-Thujane-4-ol</td>
<td>0.77±0.14</td>
<td>1.39±0.22</td>
<td>0.033</td>
</tr>
<tr>
<td>γ-Cadinene</td>
<td>0.23±0.07</td>
<td>0.94±0.18</td>
<td>0.006</td>
</tr>
<tr>
<td>1,5,5-Trimethyl-6-methylene-cyclohexene</td>
<td>0.27±0.05</td>
<td>0.59±0.07</td>
<td>0.003</td>
</tr>
<tr>
<td>5,9,9-Trimethylspiro[3.5]non-5-en-1-one</td>
<td>0.29±0.04</td>
<td>0.60±0.06</td>
<td>0.002</td>
</tr>
</tbody>
</table>
The means (±SE) for day and night with \(p>0.05\) have no significant difference, those with \(p<0.05\) are significantly different (\(\alpha=0.05\), Student-t test).

**Table 4.6:** Night and Day prominent compounds identified by the GC-MSD from the emitted volatiles by potted *M. piperita* plant

<table>
<thead>
<tr>
<th>No.</th>
<th>RT</th>
<th>Compound name</th>
<th>NIGHT Relative %</th>
<th>DAY Relative %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.81</td>
<td>Tricyclene</td>
<td>3.51</td>
<td>13.1</td>
</tr>
<tr>
<td>2</td>
<td>9.715</td>
<td>(\alpha)-Pinene</td>
<td>3.51</td>
<td>8.9</td>
</tr>
<tr>
<td>3</td>
<td>10.616</td>
<td>(\beta)-Pinene</td>
<td>14.72</td>
<td>12.78</td>
</tr>
<tr>
<td>4</td>
<td>10.622</td>
<td>(\beta)-Phellandrene</td>
<td>14.83</td>
<td>13.08</td>
</tr>
<tr>
<td>5</td>
<td>10.943</td>
<td>Myrcene</td>
<td>3.82</td>
<td>1.96</td>
</tr>
<tr>
<td>6</td>
<td>11.71</td>
<td>1,8-Cineole</td>
<td>26.41</td>
<td>23.18</td>
</tr>
<tr>
<td>7</td>
<td>11.821</td>
<td>(z)-(\beta)-Ocimene</td>
<td>0.86</td>
<td>0.72</td>
</tr>
<tr>
<td>8</td>
<td>12.207</td>
<td>(\delta)-3-Carene</td>
<td>0.78</td>
<td>0.69</td>
</tr>
<tr>
<td>9</td>
<td>12.359</td>
<td>cis-Sabinene hydrate</td>
<td>1.08</td>
<td>0.49</td>
</tr>
<tr>
<td>10</td>
<td>12.365</td>
<td>cis-Thujan-4-ol</td>
<td>1.14</td>
<td>0.49</td>
</tr>
<tr>
<td>11</td>
<td>13.4</td>
<td>allo-Ocimene</td>
<td>0.78</td>
<td>0.5</td>
</tr>
<tr>
<td>12</td>
<td>15.372</td>
<td>trans-Piperitene epoxide</td>
<td>4</td>
<td>1.93</td>
</tr>
<tr>
<td>13</td>
<td>16.548</td>
<td>1,5,5-Trimethyl-6-methylenecyclohexene</td>
<td>0.55</td>
<td>0.43</td>
</tr>
<tr>
<td>14</td>
<td>16.553</td>
<td>5,9,9-Trimethyl-spiro[3.5]non-5-en-1-one</td>
<td>0.55</td>
<td>0.42</td>
</tr>
<tr>
<td>15</td>
<td>16.945</td>
<td>Piperitenone oxide</td>
<td>2.08</td>
<td>0.34</td>
</tr>
<tr>
<td>16</td>
<td>17.238</td>
<td>(\beta)-Bourbonene</td>
<td>0.35</td>
<td>0.3</td>
</tr>
<tr>
<td>17</td>
<td>17.7</td>
<td>(\gamma)-Cadinene</td>
<td>1.23</td>
<td>0.46</td>
</tr>
<tr>
<td>18</td>
<td>18.256</td>
<td>(\beta)-Copaene</td>
<td>0.46</td>
<td>1.32</td>
</tr>
<tr>
<td>19</td>
<td>18.496</td>
<td>Germacrene D</td>
<td>0.95</td>
<td>1.82</td>
</tr>
</tbody>
</table>
The qualitative and quantitative differences in diurnal and nocturnal emitted volatiles from *M. piperita* plant could be regulated by storage organs, their rate of synthesis and volatility (Dudareva *et al.*, 2004). Terpene accumulation and glandular trichome development in this plant is controlled by the rate of biosynthesis, which is regulated by individual pathway enzymes and structural genes (Mc Conkey *et al.*, 2000). Monoterpene volatilization rate varies with light, temperature, time of day, and stage of development. Light decreases the diffusion resistance of stomata during the day and the availability of glyceraldehyde-3-phosphate, a precursor of terpenes formed during photosynthesis (Niinemets *et al.*, 2004). Non-flowering, 6-week-old plants which were measured under light and temperature had lower volatilization rate during the light period than the dark period (Loreto *et al.*, 1996).

Light intensity has been reported to have an effect on the quality and quantity of the induced odor blend. The relative amount of β-myrcene, (Z)-3-hexenyl acetate, and β-bisabolene significantly decreased with increases in light intensity (Mc Conkey *et al.*, 2000). Nocturnal emission of floral volatiles is more often found to be controlled by endogenous factors than diurnal emission which may be more influenced by prevailing light and temperature conditions (Hansted *et al.*, 1994). From previous studies increased night-time concentrations of limonene and β-phellandrene within boronia flower tissues have been observed (MacTavish, 1995). The rapid increase in emission of both volatiles in the dark phase of the alternating light: dark treatment is a clear indication that production and emission of these volatiles is controlled by light. Diurnal changes in tissue concentrations of particular volatiles
also showed relatively higher concentrations of ionone at night-time, the emission of volatiles is influenced to a great extent by the emission of major volatiles, ionones and dodecyl acetate. (Mac Tavish, 1995).

4.4 Repellency activity of selected compounds and their blends against An. gambiae mosquitoes

The results of the dose-dependent responses of selected compounds, blends and DEET against female An. gambiae s.s. are given in table 4.7, figure 4.4 and 4.5 and appendices 7a and b.

**Table 4.7:** Mean percentage repellency (±SE) and RD<sub>50</sub> of standards and blends at different doses against An. gambiae

<table>
<thead>
<tr>
<th>Sample</th>
<th>10&lt;sup&gt;-5&lt;/sup&gt;</th>
<th>10&lt;sup&gt;-4&lt;/sup&gt;</th>
<th>10&lt;sup&gt;-3&lt;/sup&gt;</th>
<th>10&lt;sup&gt;-2&lt;/sup&gt;</th>
<th>10&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>RD&lt;sub&gt;50&lt;/sub&gt; (gcm&lt;sup&gt;2&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Myrcene</td>
<td>0</td>
<td>6.87±2.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.93±5.95&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>38.73±3.21&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>39.07±5.89&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.204</td>
</tr>
<tr>
<td>β-Pinene</td>
<td>8.00±3.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.67±2.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.10±5.96&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>50.00±5.53&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>56.40±6.40&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.023</td>
</tr>
<tr>
<td>1,8-Cineole</td>
<td>6.27±2.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.63±0.83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.00±3.21&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>41.37±0.84&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>52.23±4.00&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.043</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>0</td>
<td>11.03±3.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.33±4.41&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>22.73±12.83&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>53.70±4.29&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.133</td>
</tr>
<tr>
<td>BLEND 1</td>
<td>0</td>
<td>8.40±0.70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.20±3.00&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>40.05±11.45&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>100.00±0.00&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.006</td>
</tr>
<tr>
<td>BLEND 2</td>
<td>7.00±1.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.35±5.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.00±1.40&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>66.70±0.00&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>95.45±4.55&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.003</td>
</tr>
<tr>
<td>BLEND 3</td>
<td>6.15±2.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.25±0.75&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>30.50±5.50&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>53.85±7.65&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>79.65±3.65&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.006</td>
</tr>
<tr>
<td>BLEND 4</td>
<td>0</td>
<td>15.35±3.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.50±1.20&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>49.05±4.25&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>83.55±4.65&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.008</td>
</tr>
<tr>
<td>BLEND 5</td>
<td>0</td>
<td>7.40±3.70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.00±9.90&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>37.6±0.50&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>44.95±0.55&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.212</td>
</tr>
<tr>
<td>DEET</td>
<td>3.80±2.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.85±7.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47.05±5.85&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>94.75±5.25&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>100.00±0.00&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.001</td>
</tr>
<tr>
<td>P-value</td>
<td>0.732</td>
<td>0.063</td>
<td>0.032</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>
Means (±SE) values with different small letters in a column and capital letter(s) within a row are significantly different (α = 0.05, p<0.05, SNK).

**Figure 4.4:** Mean percentage repellency (±SE) of β-myrcene, β-pinene, α-pinene, 1,8-cineole and DEET at different doses.

**Figure 4.5:** Mean percentage repellence (±SE) of DEET and blends at various doses against *An. gambiae*
For a given dose, there were varying degrees of dose-dependent responses. None of the individual components (1,8-cineole, α-pinene, β-pinene and β-myrcene) that were tested had significant repellent effect against *An. gambiae*. However, a blend of these compounds had significant repellent effect (Table 4.7, Figure 4.4, 4.5 and Appendices 7a, b). Among the individual compounds assayed the most repellent was β-pinene with a repellence of 15.67% and 56.40% at concentration of 0.0001 g/mL and 0.1 g/mL respectively, against that of DEET of 25.85% and 100.00% at the same concentrations.

Of the five blends, blend-1 (1,8-Cineole, α-Pinene, β-Pinene and β-Myrcene) at 0.1g/ mL was the most repellent with its action comparable to that of DEET of 100%. However, it exhibited lower repellence at 8.40% against 25.85% of DEET at a concentration of 0.0001 g/mL and RD50 of 0.006 gcm⁻². This was lower than that of DEET at 0.001 gcm⁻² (Table 4.7, Figure 4.5 and Appendix 7 b).

When one compound from this four component blend was removed, there was a reduction in percentage repellency with the highest drop observed when 1,8-cineole (76) was absent giving 44.95%. When β-pinene (73) was absent from the mixture, there was 79.65% repellency against *An. gambiae*. When α-pinene (71) was removed the repellency was 83.55%. Subtraction of β-myrcene (75) led to 95.45% repellency (table 4.7). Therefore, 1,8-cineole was the most active component in the blend followed by β-pinene. Removal of β-myrcene led to the significant rise in repellency of the resulting blends. Thus, this compound contributes negatively to the repellence of the full blend. At the lowest dose of 0.00001 g/mL, β-myrcene (-1.50%), α-pinene (-1.20%), blend 1 (-0.80%) and blend 5 (-6.15%) had negative repellency
against that of DEET (3.80%). This can be attributed to low concentration of active compounds which could have been too low to be detected by the mosquitoes.

1,8-Cineole (76) was responsible for 47.77% repellency activity within the blend while β-pinene (73) contributed 43.6% repellency activity in relative amount present in the four component blend. Therefore, repellent effect of the emitted volatiles is attributed to the higher percentage of 1,8-cineole. Addition of each major individual component resulted to an increase in the repellency of the full blend against An. gambiae. This shows that active compounds gain synergism between themselves resulting to an increase in repellency. Thus, subtractive assays provide additional insight into the relative contributions of these compounds to the repellency of the four component blend.

A number of studies on the effect of the essential oils on mosquitoes and other biting arthropods focused on the application of essential oil and identification of the active components rather than the free emitted volatile blends (Mathu et al., 2015). Ndirangu et al. (2015) working on the essential oil of Nigella sativa L. seeds and Ywaya et al. (2013) working on the essential oils of three plant species (Ocimum gratissimum L, Hyptis suaveolens L. and Vitex keniensis Turill) reported that α-pinene and β-pinene repels An. gambiae.

Previous studies have focused largely on anti-microbial activities of crude products and specific constituents of M. piperita plant. Various crude extracts of this plant showed significant activity against all the bacteria tested. The leaf extract was reported to have the
highest activity against Bacillus subtilis, Staphylococcus aureus and Proteus vulgaris than Escherichia coli, Streptococcus pneumonia and Klebsiella pneumonia (Para et al., 2013).

Menthol and peppermint oil were found to be fungicidal against Candida albicans, Aspergillus albus and Dermatophytic fungi (Para et al., 2013). Semi-field experiments showed that M. piperita plant extracts has repellent and insecticidal activity against cabbage aphid (Brevicoryne brassicae) (Mersha et al., 2014), rice Weevil (Sitophilus oryzae), rice moth (Corcyra cephalonica) (Khani et al., 2012) and larvicidal activity against dengue vector, Aedes aegypti (Kumar et al., 2011). The essential from this plant has been reported to present a good activity against the following important postharvest deteriorating fungi: Aspergillus flavus, Aspergillus glaucus, Aspergillus niger, Aspergillus ochraceous, Colletotrichum gloesporioides, Colletotrichum musae, Fusarium oxysporum and Fusarium semitectum (Marcelo et al., 2012).

M. piperita plant leaves have been reported to contain alkaloids, flavonoids, steroids, tannin and phenols. The phenolic compounds interfere with the feeding of insects. Development of Spodoptera litura was inhibited by a phenolic compound from the wild groundnut. Phenolics in plant tissues may change the rate of consumption of tissues by a geometrid caterpillar (Epirrita autumnata) (Para et al., 2013). From this, it is clear that phenolic compounds from the M. piperita will not allow insects to feed on treated environment (Para et al., 2013).

The study shows that multiple deployment of potted M. piperita plant can provide space protection against An. gambiae up to a certain level, after which no further enhancement in
repellency occurs. Thus, the level of emission of volatiles from each plant appears to be negatively affected beyond four con-specific plants. This could be due to intra-plant communication that leads to suppression of emission of volatiles. Although, the repellency is below substantial levels that can significantly repel host seeking arthropods, potted plants due to their simplicity may provide an alternative of reducing malaria incidences in resource-limited people.
CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Different research has been conducted in various countries to determine the efficacy of ethno-botanical plants for space fumigation against human biting arthropods. This research was conducted to determine the repellency of the volatiles emitted by different numbers of potted M. piperita against An. gambiae s.s. and the phytochemical composition of the plant. The study concludes the following:

i. There is significant incremental rise in repellency when the number of potted plants is doubled from 2 to 4. However, there is no significant further increase beyond 4 potted plants.

ii. Live potted M. piperita plants emit blends of volatiles that are repellent to An. gambiae s.s. during its active times. Hence, the plant should be placed indoors to offer protection against mosquito bites before people retire to the confines of mosquito nets.

iii. The day and night phytochemical compositions of the emitted volatiles showed significantly large qualitative and quantitative differences. The monoterpenes and sesquiterpenes were 60.5% and 25%, 54.5% and 23.3% during night and day respectively.

iv. Repellency of blends (blend 1; 100.00±0.00) of four or three commercially available major components of volatiles emitted by M. piperita plants against An. gambiae s.s. was higher than those of the individual components (β-Pinene;
56.40±6.40), suggesting that the repellence of volatile emission of the plant may be due to additive or synergistic effects of individual constituents.

5.2 Recommendations

The following are recommended from this research and for further research:

i. The *M. piperita* plant should be combined with different species of plants emitting different volatiles blends to check whether if there is any additive repellent effect of multiple combinations. This may provide substantially significant space protection in households against *An. gambiae* s.s. for resource-limited people.

ii. The mosquito repellency was carried using *M. piperita* plant propagated in one place, and it will be interesting to see if the plants grown in different agro-ecological zones contain different phytochemical profiles, which may confer different level of activity.

iii. The repellence of the emitted volatiles was evaluated in a choice set ups in two screen houses. Full field trials need to be undertaken to rule out any possible differences in repellency due to the overlap of repellency range of the treatment with that of the control and behavior of mosquitoes when they are constrained.

iv. Gas Chromatography linked Electroantennography (GC-EAD) analysis of the volatiles should be conducted so as to identify all compounds perceived by the mosquito antennae, which can then be assayed as a full blend to determine its repellency, and in subtractive modes to determine the relative contribution of each component.
REFERENCES


Pates, H. V., Lines, D. J. and Miller, E. J. (2002). Personal protection against mosquitoes in Dar- es salaam, Tanzania, by using a kerosene oil lamp to vaporize transfluthrin Medical and Veterinary Entomology, 16, 277-284.


piperita) Cultivars at different stages of plant growth from kumaon region of Western Himalaya. Journal of Medicinal and Aromatic Plants, 1, 13-18.


Appendix 1: The mass spectrum of compound 76 from the sample collected from the emitted volatiles by *M. piperita* plant (A-Sample mass spectrum, B-Adams 2.L library mass spectrum)
Appendix 2: The mass spectrum of compound 73 from the sample collected from the emitted volatiles by *M. piperita* plant (A-Sample mass spectrum, B-Adams 2.L library mass spectrum)
Appendix 3: The mass spectrum of compound 71 from the sample collected from the emitted volatiles by *M. piperita* plant (A-Sample mass spectrum, B-Adams 2.L library mass Spectrum)
Appendix 4: The mass spectrum of compound 75 from the sample collected from the emitted volatiles by *M. piperita* plant (A-Sample mass spectrum, B-Adams 2.L library mass Spectrum)
Appendix 5: GC-MSD prominent constituents identified in the emitted volatiles by potted *M. piperita* plant during the Night

<table>
<thead>
<tr>
<th>No.</th>
<th>RT</th>
<th>Compound name</th>
<th>Molecular formula</th>
<th>M+</th>
<th>Relative %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.71</td>
<td>1,8-Cineole</td>
<td>C_{10}H_{18}O</td>
<td>154</td>
<td>26.41</td>
</tr>
<tr>
<td>2</td>
<td>10.622</td>
<td>β-Phellandrene</td>
<td>C_{10}H_{16}</td>
<td>136</td>
<td>14.83</td>
</tr>
<tr>
<td>3</td>
<td>10.616</td>
<td>β-Pinene</td>
<td>C_{10}H_{16}</td>
<td>136</td>
<td>14.72</td>
</tr>
<tr>
<td>4</td>
<td>15.372</td>
<td>trans-Piperitone epoxide</td>
<td>C_{10}H_{16}O_{2}</td>
<td>168</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>10.943</td>
<td>Myrcene</td>
<td>C_{10}H_{16}</td>
<td>136</td>
<td>3.82</td>
</tr>
<tr>
<td>6</td>
<td>9.715</td>
<td>Tricyclene</td>
<td>C_{10}H_{16}</td>
<td>136</td>
<td>3.51</td>
</tr>
<tr>
<td>7</td>
<td>9.703</td>
<td>α-Pinene</td>
<td>C_{10}H_{16}</td>
<td>136</td>
<td>3.51</td>
</tr>
<tr>
<td>8</td>
<td>16.945</td>
<td>Piperitenone oxide</td>
<td>C_{10}H_{16}O_{2}</td>
<td>166</td>
<td>2.08</td>
</tr>
<tr>
<td>9</td>
<td>8.235</td>
<td>4-Methyl-2-hexanol</td>
<td>C_{7}H_{16}O</td>
<td>116</td>
<td>1.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bicyclo[2.2.2]octane-1-carboxylic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>17.527</td>
<td>acid</td>
<td>C_{6}H_{13}O_{2}</td>
<td>154</td>
<td>1.39</td>
</tr>
<tr>
<td>11</td>
<td>17.507</td>
<td>2-Butyl-1,3-cyclopentanedione</td>
<td>C_{6}H_{14}O_{2}</td>
<td>154</td>
<td>1.39</td>
</tr>
<tr>
<td>12</td>
<td>17.513</td>
<td>4-(Dimethylamino)-3-penten-2-one</td>
<td>C_{7}H_{13}NO</td>
<td>127</td>
<td>1.38</td>
</tr>
<tr>
<td>13</td>
<td>9.37</td>
<td>1,2,3,4,5-Pentamethylcyclopentane</td>
<td>C_{10}H_{20}</td>
<td>140</td>
<td>1.38</td>
</tr>
<tr>
<td>14</td>
<td>17.7</td>
<td>γ-Cadinene</td>
<td>C_{15}H_{24}</td>
<td>204</td>
<td>1.23</td>
</tr>
<tr>
<td>15</td>
<td>17.683</td>
<td>β-Cubebene</td>
<td>C_{15}H_{24}</td>
<td>204</td>
<td>1.14</td>
</tr>
<tr>
<td>16</td>
<td>12.365</td>
<td>cis-Thujan-4-ol</td>
<td>C_{10}H_{16}O</td>
<td>154</td>
<td>1.12</td>
</tr>
<tr>
<td>17</td>
<td>12.371</td>
<td>α-Ocimene</td>
<td>C_{10}H_{16}</td>
<td>136.2</td>
<td>1.11</td>
</tr>
<tr>
<td>18</td>
<td>17.811</td>
<td>5,7-Diethyl-5,6-decadien-3-ynе</td>
<td>C_{14}H_{32}</td>
<td>190</td>
<td>0.95</td>
</tr>
<tr>
<td>19</td>
<td>18.496</td>
<td>Germacrene D</td>
<td>C_{15}H_{24}</td>
<td>204</td>
<td>0.94</td>
</tr>
<tr>
<td>20</td>
<td>11.821</td>
<td>(z)-β-Ocimene</td>
<td>C_{10}H_{16}</td>
<td>136.2</td>
<td>0.86</td>
</tr>
<tr>
<td>21</td>
<td>12.207</td>
<td>δ-3-Carene</td>
<td>C_{10}H_{16}</td>
<td>136</td>
<td>0.78</td>
</tr>
<tr>
<td>22</td>
<td>18.683</td>
<td>Bicyclogermacrene</td>
<td>C_{15}H_{24}</td>
<td>204</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>18.613</td>
<td>Phenylethanediol</td>
<td>C_8H_{10}O_2</td>
<td>138</td>
<td>0.72</td>
</tr>
<tr>
<td>24</td>
<td>13.4</td>
<td>allo-Ocimene</td>
<td>C_{10}H_{16}</td>
<td>136.2</td>
<td>0.71</td>
</tr>
<tr>
<td>25</td>
<td>18.689</td>
<td>Germacrene B</td>
<td>C_{15}H_{24}</td>
<td>204</td>
<td>0.71</td>
</tr>
<tr>
<td>26</td>
<td>13.394</td>
<td>α-Terpinolene</td>
<td>C_{10}H_{16}</td>
<td>136</td>
<td>0.71</td>
</tr>
<tr>
<td>27</td>
<td>17.671</td>
<td>β-Ylangene</td>
<td>C_{15}H_{24}</td>
<td>204</td>
<td>0.61</td>
</tr>
<tr>
<td>28</td>
<td>16.033</td>
<td>Buccocamphor</td>
<td>C_{10}H_{16}O_2</td>
<td>168</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>16.547</td>
<td>1-one</td>
<td>C_{12}H_{16}O</td>
<td>178</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>16.548</td>
<td>Cyclohexene</td>
<td>C_{10}H_{16}</td>
<td>136</td>
<td>0.55</td>
</tr>
<tr>
<td>31</td>
<td>16.553</td>
<td>2,5,5-Trimethyl-1,3,6-heptatriene</td>
<td>C_{10}H_{16}</td>
<td>136</td>
<td>0.55</td>
</tr>
<tr>
<td>32</td>
<td>16.518</td>
<td>2,5,6-Trimethyl-1,3,6-heptatriene</td>
<td>C_{10}H_{16}</td>
<td>136</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>17.29</td>
<td>Cyclododecatrione</td>
<td>C_{12}H_{18}</td>
<td>162</td>
<td>0.46</td>
</tr>
<tr>
<td>34</td>
<td>18.256</td>
<td>β-Copaene</td>
<td>C_{15}H_{24}</td>
<td>204</td>
<td>0.38</td>
</tr>
<tr>
<td>35</td>
<td>18.139</td>
<td>(+)-Valencene</td>
<td>C_{15}H_{24}</td>
<td>204</td>
<td>0.38</td>
</tr>
<tr>
<td>36</td>
<td>14.167</td>
<td>2,3-Dihydro-4-methylfuran</td>
<td>C_8H_8O</td>
<td>84</td>
<td>0.35</td>
</tr>
<tr>
<td>37</td>
<td>14.178</td>
<td>(trans)-2-Methyldecalin</td>
<td>C_{11}H_{20}</td>
<td>152</td>
<td>0.35</td>
</tr>
<tr>
<td>38</td>
<td>14.172</td>
<td>cis-Pinocamphone</td>
<td>C_{10}H_{16}O</td>
<td>152</td>
<td>0.34</td>
</tr>
<tr>
<td>39</td>
<td>17.238</td>
<td>β-Bourbonene</td>
<td>C_{15}H_{24}</td>
<td>204</td>
<td>0.34</td>
</tr>
<tr>
<td>40</td>
<td>14.412</td>
<td>α-Terpineol</td>
<td>C_{10}H_{16}O</td>
<td>154</td>
<td>0.26</td>
</tr>
<tr>
<td>41</td>
<td>18.882</td>
<td>β-Cadinene</td>
<td>C_{15}H_{24}</td>
<td>204</td>
<td>0.26</td>
</tr>
<tr>
<td>42</td>
<td>18.812</td>
<td>δ-Cadinol</td>
<td>C_{15}H_{26}O</td>
<td>222</td>
<td>0.22</td>
</tr>
<tr>
<td>43</td>
<td>12.722</td>
<td>Terpinolene</td>
<td>C_{10}H_{16}</td>
<td>136</td>
<td>0.16</td>
</tr>
</tbody>
</table>

5,9,9-Trimethyl-spiro[3.5]non-5-en-1,5,5,6-trimethyl-1,3,6-heptatriene-2,5,5-trimethyl-1,3,6-heptatriene-1,5,9-trimethyl-1,5,9-cyclododecatriene
**Appendix 6:** Prominent constituents identified by the GC-MSD in the emitted volatiles by potted *M. piperita* plant during the day

<table>
<thead>
<tr>
<th>No.</th>
<th>RT</th>
<th>Compound</th>
<th>Molecular formula</th>
<th>M+ (g/mol)</th>
<th>Relative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.721</td>
<td>1,8-Cineole</td>
<td>C\textsubscript{10}H\textsubscript{16}O</td>
<td>154</td>
<td>23.19</td>
</tr>
<tr>
<td>2</td>
<td>10.606</td>
<td>Tricyclene</td>
<td>C\textsubscript{10}H\textsubscript{16}</td>
<td>136</td>
<td>13.1</td>
</tr>
<tr>
<td>3</td>
<td>10.622</td>
<td>β-Phellandrene</td>
<td>C\textsubscript{10}H\textsubscript{16}</td>
<td>136</td>
<td>13.08</td>
</tr>
<tr>
<td>4</td>
<td>10.616</td>
<td>β-Pinene</td>
<td>C\textsubscript{10}H\textsubscript{16}</td>
<td>136</td>
<td>12.78</td>
</tr>
<tr>
<td>5</td>
<td>9.715</td>
<td>α-Pinene</td>
<td>C\textsubscript{10}H\textsubscript{16}</td>
<td>136</td>
<td>8.89</td>
</tr>
<tr>
<td>6</td>
<td>18.496</td>
<td>Germacrene D</td>
<td>C\textsubscript{15}H\textsubscript{24}</td>
<td>204</td>
<td>1.82</td>
</tr>
<tr>
<td>7</td>
<td>10.949</td>
<td>β-Myrcene</td>
<td>C\textsubscript{10}H\textsubscript{16}</td>
<td>136</td>
<td>1.75</td>
</tr>
<tr>
<td>8</td>
<td>17.812</td>
<td>β-Copaene</td>
<td>C\textsubscript{15}H\textsubscript{24}</td>
<td>204</td>
<td>1.32</td>
</tr>
<tr>
<td>9</td>
<td>15.366</td>
<td>trans-Piperitone epoxide</td>
<td>C\textsubscript{10}H\textsubscript{16}O\textsubscript{2}</td>
<td>168</td>
<td>1.22</td>
</tr>
<tr>
<td>10</td>
<td>15.378</td>
<td>1-Cyclopentylethanone</td>
<td>C\textsubscript{7}H\textsubscript{12}O</td>
<td>112</td>
<td>1.21</td>
</tr>
<tr>
<td>11</td>
<td>15.383</td>
<td>3-Ethyl-2,5-dimethyl-3-hexene</td>
<td>C\textsubscript{10}H\textsubscript{20}</td>
<td>140.3</td>
<td>1.19</td>
</tr>
<tr>
<td>12</td>
<td>11.815</td>
<td>(z)-β-Ocimene</td>
<td>C\textsubscript{10}H\textsubscript{16}</td>
<td>136</td>
<td>0.72</td>
</tr>
<tr>
<td>13</td>
<td>11.821</td>
<td>δ-3-Carene</td>
<td>C\textsubscript{10}H\textsubscript{16}</td>
<td>136</td>
<td>0.69</td>
</tr>
<tr>
<td>14</td>
<td>18.683</td>
<td>Bicyclogermacrene</td>
<td>C\textsubscript{15}H\textsubscript{24}</td>
<td>204</td>
<td>0.69</td>
</tr>
<tr>
<td>15</td>
<td>13.4</td>
<td>2,3,6-Trimethylphenol</td>
<td>C\textsubscript{9}H\textsubscript{12}O</td>
<td>136</td>
<td>0.55</td>
</tr>
<tr>
<td>16</td>
<td>13.383</td>
<td>allo-Ocimene</td>
<td>C\textsubscript{10}H\textsubscript{16}</td>
<td>136</td>
<td>0.5</td>
</tr>
<tr>
<td>17</td>
<td>12.359</td>
<td>(cis)-β-Terpineol</td>
<td>C\textsubscript{10}H\textsubscript{16}O</td>
<td>154</td>
<td>0.49</td>
</tr>
<tr>
<td>18</td>
<td>12.369</td>
<td>(+)-3-Carene</td>
<td>C\textsubscript{10}H\textsubscript{16}</td>
<td>136</td>
<td>0.49</td>
</tr>
<tr>
<td>19</td>
<td>12.315</td>
<td>cis-Thujane-4-ol</td>
<td>C\textsubscript{10}H\textsubscript{16}O</td>
<td>154</td>
<td>0.49</td>
</tr>
<tr>
<td>20</td>
<td>12.359</td>
<td>(trans)-Sabinene hydrate</td>
<td>C\textsubscript{10}H\textsubscript{16}O</td>
<td>154</td>
<td>0.47</td>
</tr>
<tr>
<td>21</td>
<td>18.139</td>
<td>γ-Cadinene</td>
<td>C\textsubscript{15}H\textsubscript{24}</td>
<td>204</td>
<td>0.47</td>
</tr>
<tr>
<td>22</td>
<td>17.296</td>
<td>Tetradecane</td>
<td>C\textsubscript{14}H\textsubscript{10}</td>
<td>198</td>
<td>0.47</td>
</tr>
<tr>
<td>23</td>
<td>17.293</td>
<td>β-Elemene</td>
<td>C\textsubscript{15}H\textsubscript{24}</td>
<td>204</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>24</td>
<td>18.139</td>
<td>(\beta)-Gurjunene</td>
<td>C_{15}H_{34}</td>
<td>204</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,5,5-Trimethyl-6-methylene-cyclohexene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>16.548</td>
<td>cyclohexene</td>
<td>C_{10}H_{16}</td>
<td>136</td>
<td>0.43</td>
</tr>
<tr>
<td>26</td>
<td>16.553</td>
<td>(\alpha)-Caryophyllene</td>
<td>C_{15}H_{34}</td>
<td>204</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5,9,9-trimethyl-spiro[3.5]non-5-en-1-one</td>
<td>C_{12}H_{18}O</td>
<td>178</td>
<td>0.42</td>
</tr>
<tr>
<td>27</td>
<td>18.25</td>
<td>trans-Muurola-4(14),5-diene</td>
<td>C_{15}H_{34}</td>
<td>204</td>
<td>0.41</td>
</tr>
<tr>
<td>28</td>
<td>18.256</td>
<td>((+))-epi-Bicyclesquiphellandrene</td>
<td>C_{15}H_{34}</td>
<td>204</td>
<td>0.35</td>
</tr>
<tr>
<td>29</td>
<td>16.946</td>
<td>Piperitenone oxide</td>
<td>C_{10}H_{14}O_{2}</td>
<td>166</td>
<td>0.34</td>
</tr>
<tr>
<td>30</td>
<td>17.238</td>
<td>(\beta)-Bourbonene</td>
<td>C_{15}H_{34}</td>
<td>204</td>
<td>0.3</td>
</tr>
<tr>
<td>31</td>
<td>12.014</td>
<td>((e))-(\beta)-Ocimene</td>
<td>C_{10}H_{16}</td>
<td>136</td>
<td>0.25</td>
</tr>
<tr>
<td>32</td>
<td>14.173</td>
<td>3-Pinanol</td>
<td>C_{10}H_{18}O</td>
<td>154</td>
<td>0.25</td>
</tr>
<tr>
<td>33</td>
<td>12.43</td>
<td>2,6-Dimethyl-7-octen-2-ol</td>
<td>C_{10}H_{20}O</td>
<td>156</td>
<td>0.22</td>
</tr>
<tr>
<td>34</td>
<td>15.565</td>
<td>2-Hydroxypiperitone</td>
<td>C_{10}H_{16}O_{2}</td>
<td>168</td>
<td>0.14</td>
</tr>
<tr>
<td>35</td>
<td>13.693</td>
<td>Carvone</td>
<td>C_{7}H_{12}O_{2}</td>
<td>128</td>
<td>0.1</td>
</tr>
<tr>
<td>36</td>
<td>13.986</td>
<td>Pinocarvone</td>
<td>C_{10}H_{14}O</td>
<td>150</td>
<td>0.09</td>
</tr>
<tr>
<td>37</td>
<td>10.575</td>
<td>Sabinene</td>
<td>C_{10}H_{16}</td>
<td>136</td>
<td>0.06</td>
</tr>
</tbody>
</table>

**KEY**

\[ M^+ = \text{Molecular Weight} \]
Appendix 7a: Mean percentage repellency of β-myrcene, β-pinene, α-pinene, 1,8-cineole and DEET at different doses

Appendix 7b: Mean percentage repellency of blend 1, blend 2, blend 3, blend 4, blend 5 and DEET at different doses
Appendix 8: Standard ethical certificate of Ethical Review Committee of Pwani University

NACOSTI ACCREDITED

ETHICS REVIEW COMMITTEE
ACCREDITED BY THE NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY
AND INNOVATION (NACOSTI, KENYA)

CERTIFICATE OF
ETHICAL APPROVAL

THIS IS TO CERTIFY THAT THE PROPOSAL SUBMITTED BY:

OBEGI J. MATUNDURA

REFERENCE NO:
ERC/MSc/019/2015

ENTITLED:
Phytochemical composition and repellence of volatiles emitted by live potted Mentha piperita (Peppermint) plants against Anopheles gambiae s.s.

TO BE UNDERTAKEN AT:
KENYA

FOR THE PROPOSED PERIOD OF RESEARCH

HAS BEEN APPROVED BY THE ETHICS REVIEW COMMITTEE
AT ITS SITTING HELD AT PWANI UNIVERSITY, KENYA
ON THE 20TH DAY OF APRIL 2015

CHAIRMAN

SECRETARY

LAY MEMBER

Pwani University, www.pw.ac.ke, email: erc@pwaniversity.co.ke, tel: 0719 857436.
The ERC: Giving Integrity to Research for Sustainable Development
NOTICE:

This decision is subject to the information available at the time of APPROVAL. The Committee may on its own motion and/or by application by a Party, review its decision on the grounds of discovery of new and important information which was not reasonably within its knowledge at the time of decision or on account of mistake or error apparent on the face of the record, or for any other sufficient reason, provided the researcher shall be given prior opportunity to be heard.
Appendix 9: Certificate of Consent

This research involves phytochemical composition and repellency of volatiles emitted by live potted *Mentha piperita* (peppermint) plants against *Anopheles gambiae* s. s. Potted *M. piperita* plant(s) will be placed in one of the experimental huts inside a screenhouse, a human volunteer will be allowed to sit in the hut from 7:00 p.m to mid-night. The legs of the participant from the toes to the knee will be exposed for 3 hrs to mosquitoes; the number of mosquitoes that land on the exposed area will be counted and then shaken off before imbibing any blood. The mosquitoes that will be used will not have any disease causing agent. An insect bite cream will be provided to the participants in case of any minor bites and associated irritations. The results are expected to form the basis of downstream development for development of appropriate potted plants with potent repellent volatile blends for space protection against malaria vectors.

*I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it, and any questions that I have asked has been answered to my satisfaction. I consent voluntarily to participate as a participant in this research and understand that I have the right to withdraw from the research at any time without in any way affecting my medical care.*

Print name of participant: _______________________

Signature of participant: _______________________

Date: ___________________________