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DEPARTMENT OF PARACLINICAL STUDIES

INSECTICIDAL EFFECTIVENESS OF NICOTINE FROM *Nicotiana tabacum* AGAINST SUB-SPECIES OF *Anopheles gambiae* s.l (INSECTA: DIPTERA: CULICIDAE)

BY

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(2019110601)

A DISSERTATION SUBMITTED TO THE UNIVERSITY OF ZAMBIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN ONE HEALTH ANALYTICAL EPIDEMIOLOGY.

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DECLARATION

I, Nicholus Chintu Sande, do hereby declare that this piece of writing is mine and was not copied from any source and that all the work of other people that has been used in this research has been duly acknowledged, and that this work has not been previously presented at any other University for similar purposes.

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ABSTRACT

One of the main issues affecting malaria control initatives in sub-Saharan Africa is the swift and widespread emergence of insecticide resistance. Consequently, there is a need to explore alternative insecticides for the control of malaria vectors. This study investigated the effectiveness of tobacco extract on wild *Anopheles* mosquitoes during the October-November 2021 dry season in Chebele village in Mwense district of Luapula Province in Zambia.

Wild *Anopheles* larvae were collected using pipetting and dipping method along Mwense stream and raised to F1 progeny. Wild *Anopheles* mosquitoes were identified using morphological taxonomic keys. Specimens belonging to the *Anopheles gambiae* complex and *Anopheles funestus* group were further identified by multiplex Polymerase Chain Reaction (PCR). Solvent extraction method was used to extract tobacco extract from tobacco leaves. A protocol was followed to impregnant filter papers with 0.132g/ml clothianidin (SumiShield® 50WG), a chemical used as an indoor residual spraying insecticide in Zambia, as a positive control while distilled water as a negative control. Non-blood-fed 2 to 3 day old female wild *Anopheles* mosquitoes were exposed to clothianidin (0.132g/ml) and tobacco extract at different concentrations (25% v/v, 33.3% v/v, 50% v/v, 62.5% v/v, 71.43 v/v and 83.3% v/v) using WHO bottle bioassay. The laboratory reared Kisumu strain *An. gambiae* sensu stricto mosquitoes were used as reference for insecticide susceptibility tests.

Two hundred and three (203) indoor biting mosquitoes were collected from 73 households using Prokopack Aspirator in order to determine the mosquito species in the study area. Morphological identifications revealed that 96.06% were An. *funestus* s.l (n=195) , 0.99% were *An. gambiae* s.l (n=2) and 0.493% were *Culex* species (n=1). PCR results on wild mosquitoes indicated that *An. funestus* sensu stricto (s.s) (n=93;46%) and *An. gambiae* s.s (n=81;40%) were the dominant species within the *An. funestus* group and *Anopheles gambiae* complex, respectively.

One hundred and forty two (142) wild female *Anopheles* species were exposed to clothianidin for 80 minutes and these were identified morphologically as *An. funestus* sensu lato (s.l) (n=108; 76.1%), *An. gambiae* s.l (n=30; 21.1%), *An. pretoriensis* (n=3;2.1%) and *An. rufipes* (n=1;0.7%). Similarly, 168 wild *Anopheles* species were exposed to tobacco extract and mosquitoes were identified morphologically as *An. funestus* s.l (n=96;57.1%), *An. gambiae* s.l (n=64;38.1%), *An. coustani* (n=1;0.6%) and *An. rufipes* (n=7;4.2%). Tobacco extract and clothinanidin recorded 100% mortality rate at 24 hours post exposure time. These results suggests that the tobacco extract can be considered as an alternative bioinsecticide for the control of malaria vectors.

DEDICATION

This dedication is first given to my lovely parents Christopher Mupeta Sande and Getrude Mwamba Sande for their candid love, patience, support and understanding during my years of studies. Secondly, to my brothers for being good and very encouraging and lastly, my late elder brother (Boas Mwamba Sande) for everything he did to me.

CERTIFICATION OF APPROVAL

This dissertation authored by Nicholus Chintu Sande and entitled "Insecticidal effectiveness of nicotine from *Nicotiana tabacum* against sub-species of *Anopheles gambiae* s.l (Insecta: Diptera: Culicidae)", has been approved as fulfilling the partial requirements for the award of the Master of Science in One Health Analytical Epidemiology degree, by the University of Zambia.

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List of Acronyms or Abbreviation

%	Percent
ACEIDHA	African Center of Excellence for Infectious Diseases of Human and Animals
An.	Anopheles
BDN	Bayesian Decision Network
CHW	Community Health Worker
CI	Confidence Interval
DDT	Dichlorodiphenyltrichloroethane
df	degree of freedom
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
g/ml	grams per milliliter
GCP	Ground Control Point
SPSS	Statistcal Package for Social Sciences
IRS	Indoor Residual Spraying
ITN	Insecticide Treated Net
LC_{50}	Lethal concentration
LLIN	Long Lasting Insecticde Net
LSM	Larval Source Management
MDA	Mass Drug Administration
MIS	Malaria Indicator Survey
MOH	Ministry of Health

NMCC	National Malaria Control Center
NMEC	National Malaria Elimination Center
PATH	Program for Appropriate Technology in Health
PCB	Polychlorinated Biphenyl
PCR	Polymerase Chain Reaction
PMI-AIRS	President’s Malaria Intiative – Africa Indoor Residual Spraying
qPCR	quantitative Polymerase Chain Reaction
SD	Standard deviation
Sig.	Significance level
SOP	Standard Operating Procedure
Std	Standard deviation
s.l	sensu lato
s.s	sensu stricto
UNZA	University of Zambia
UNZABREC	University of Zambia Biomedical Research and Ethics Committee
USAID	United States Agency for International Development
v/v	Volume percentage
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

1.1 Background

Anopheles mosquitoes are crucial to medicine because they spread diseases that affect humans, such as malaria (Oladipupo *et al*., 2019; *Mewara et al.*, 2018). *Anopheles. gambiae* s.l are the primary vectors for human malaria and account for 405,000 deaths worldwide per year, with around 90% occurring in Africa (Deletre *et al*., 2019; Hemming-Schroeder *et al*., 2020). The sub-species of *Anopeheles gambiae* sensu lato (s.l) includes; *An. gambiae* sensu stricto (s.s), *An. arabiensis, An. merus*, *An. melas*, *An. bwambe, An. quadriannulatus*, *An. amharicus*, *An. coluzzii* and *An. fontenillei* (Bassey, 2020; Coetzee, 2020).

In Zambia the spatial dissemination of malaria *Plasmodium* species is not vividly delineated and according to the existing information about 98% of malaria in Zambia is from *Plasmodium falciparum*, 2% from *Plasmodium malariae* and *Plasmodium vivax* is a rare cause of infection (Sitali *et al.*, 2019). Malaria is endemic throughout Zambia and remains a major public health problem in many areas (Nawa *et al*., 2019). Malaria Indicator Survey (MIS) conducted countrywide in 2018 (Figure 1.1), found that Luapula Province was the highest with a prevalence of 30.4% in under five children (MoH et al, 2018).

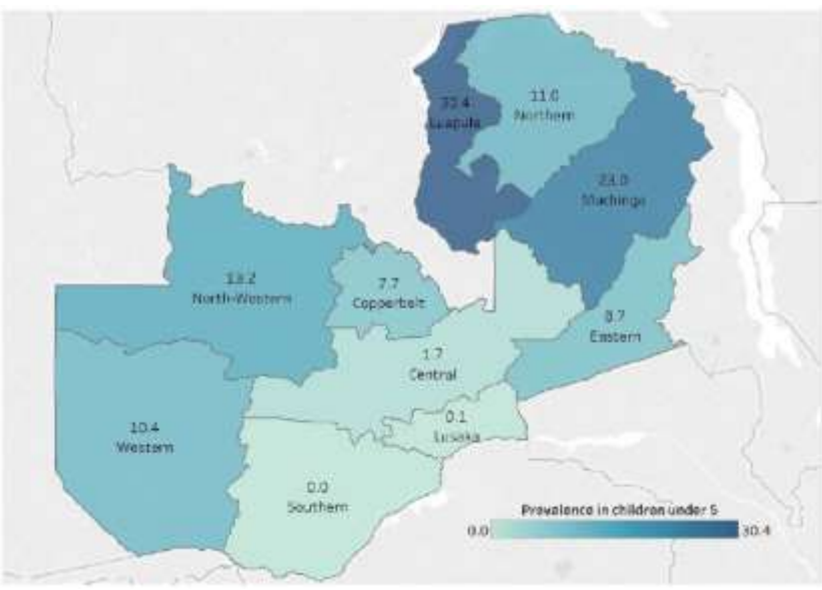


Figure 1.1: Map showing the malaria prevalence by Province among children under age of five years. Source: MoH et al (2018) Malaria Indicator Survey.

For some time, indoor chemical control tools for malaria vector populations have been normally effective (Killeen *et al*., 2016). Fatefully, the fast evolution and spread of insecticide resistance in

the primary malaria vectors in sub-Saharan region have triggered a serious threat to the success of vector control efforts with the extensive use of synthetic insecticides and this has made eco-friendly control tools a priority (Yin *et al*., 2021; Chandramohan *et al*., 2015). Usage of botanical crudes to control disease vectors have advantages due to the presence of numerous bio-active compounds which prevents development of insecticide resistance, low persistence in the environment and less expensive (Tembo *et al*., 2018). Hence, This work aimed at determining the potential of leaf extract of *Nicotiana tabacum* as a future bio-insecticide against malaria vectors.

1.2 Statement of the problem

Insecticide resistance has been reported in nearly all the countries where Indoor Residual Spraying (IRS) and Insecticide Treated Nets (ITNs) distribution have been deployed. These vector control tools are facing great challenges from rapidly escalating insecticide resistance in malaria vector populations (Matiya *et al*., 2019). In Zambia resistance was first detected in *Anopheles gambiae* s.s against the organochlorine insecticide, Dichlorodiphenyltrichloroethane (DDT) and pyrethroids (Chanda *et al*., 2013). Furthermore, studies which were done in Nchelenge district in Zambia have shown widespread insecticide resistance to carbamates and pyrethroids by *Anopheles funestus* (Choi *et al*., 2014). This development possess serious challenges to the success of vector-management in the control of malaria in Zambia.

1.3 Study justification

It has been observed that mosquitoes responsible for malaria transmission are demonstrating either physiological or behavioral resistance to various classes of insecticides and this has exerted pressure on mosquito control programs. The emergence of insecticide resistance in major malaria vectors puts interventions such as IRS and ITNs at risk (Chanda *et al.,* 2020). The options for selecting insecticides for IRS campaigns and insecticide bed nets are running out due to insecticidal resistance mechanisms developed by malaria vectors to synthetic insecticides. In order to overcome the insecticide resistance crisis against malaria vectors, there is a need to continuously explore and introduce new botanical insecticides that have infinitesimal deleterious impact to environmental health at optimum amount or dose. Malaria vector control is further complicated by difficulties in identification of the major vectors in the *Anopheles gambiae* complex which consists of at least seven subspecies that may each exhibit varying levels of susceptibility to insecticides. Therefore, it is imperative that techniques such as Polymerase Chain Reaction (PCR) are readily

available to enable close observations on the possible changes of the malaria transmitting sub-species of *Anopheles*.

1.4 Research question

Does the tobacco extract from *Nicotiana tabacum* have the same Knock Down effect against the sub-species of *Anopheles gambiae* s.l as the conventional clothianidin (SumiShield® 50WG) used as an Indoor Residual Spraying (IRS) insecticide in Zambia?

1.5 General objective

The aim of the study was to determine the effectiveness of tobacco leaf extract in knocking down wild sub-species of *Anopheles gambiae* s.l under field conditions.

1.5.1 Specific objectives

I. To identify *Anopheles gambiae* s.s and *Anopheles funestus* s.s within *Anopheles gambiae* s.l and *Anopheles funestus* s.l respectively.

II. To determine the lethal concentration of tobacco extract to knock down wild *Anopheles gambiae* s.l.

1.6 The ethical clearance

The ethical clearance and approval was sought from the University of Zambia Biomedical Research and Ethics Committee (UNZABREC/Approval number: Ref. 1834-2021)(see appendix 1) and National Health Research Authority (NHRA/Approval number: Ref. NHRA000007/15/10/2021)(see appendix 2). Permission was granted to use the Entomology and molecular laboratories at National Malaria Elimination Center in Lusaka district of Lusaka Province in Zambia (see appendix 3).

CHAPTER 2: LITERATURE REVIEW

2.1 Malaria vectors in Zambia

In Zambia, there are three major malaria vectors that have contributed to high transmission of malaria parasites to all age groups namely; *Anopheles gambiae* (s.s), *Anopheles arabiensis* and *Anopheles funestus* (Chanda *et al.*, 2013). Both *An. funestus* (s.s) and *An. gambiae* (s.s) reveal great levels of endophagy and anthropophagy due to insecticide resistance (Stevenson *et al.*, 2016). On the other hand, the secondary vectors still remain potential vectors in transmitting malaria parasites, this has been a challenge in eliminating malaria disease in Zambia, and although *Anopheles coustani s.l* and *Anopheles squamous* have a zoophilic behavior, both species were found to be anthropophilic from human hosts in Macha in Choma district (Fornadel *et al.*, 2011). A Similar study was conducted in Macha in Choma district of Southern province and *Anopheles squamosus* was detected to have *Plasmodium falciparum* through ELISA and Real-time qPCR methods. Vividly, this has been a significant proof that *Anopheles squamosus* while having exophagic traits, can still be infected with *Plasmodium falciparum* (Stevenson *et al.*, 2016).

2.2 Life cycle of *Anopheles* mosquito

The schematic representation of life cycle of *Anopheles* mosquito, the vector of malaria *Plasmodium* parasites species is shown in figure 2.1.

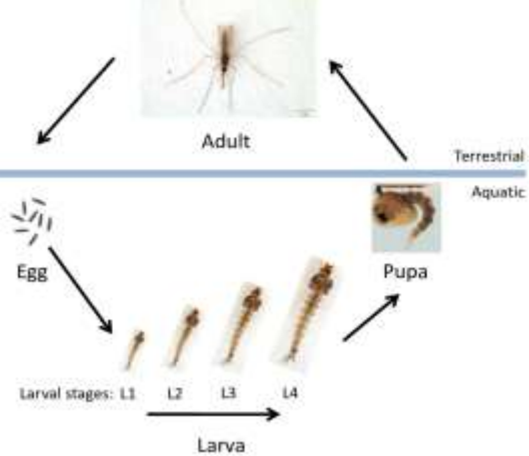


Figure 2.1: Schematic diagram of the life cycle for *Anopheles* species by Williams and Pinto (2012); L1 (instar 1), L2 (instar 2), L3 (instar 3) and L4 (instar 4).

For the *Anopheles* mosquito to develop from an egg to an adult mosquito it usually takes 10 to 14 days and life stages of *Anopheles* species includes; egg, larva, pupa and adult mosquito (CDC, 2022). Human blood is significant for mosquito egg production and cause malaria vector species to breed in high numbers (Zhou *et al.*, 2008). It takes approximately two days for the *Anopheles* mosquitoes' eggs to hatch after the embryo completes its development and the eggs can tolerate drought conditions for several days (Yaro *et al.*, 2006). However, slow embryo development of eggs is induced by environmental and genetic factors (Kaiser et al., 2014).

An experiment conducted in Italy by Porretta *et al.*, (2016) found that intra-instar cannibalism within larvae of second, third and fourth instar (L2, L3 and L4) was greater in *Anopheles gambiae* s.s larvae than in *Anopheles stephensi* larvae. In accordance with Centers for Disease Control and Prevention (2022), within five days, mosquito larva develops into pupa. Studies have shown that mosquito pupa's paddle is used for fast movement in the water to run away from predators (White and Kaufman, 2014). Furthermore, full development of mosquito's body parts occurs at pupal stage and it takes 2-3 days for the mosquito to emerge from the pupa shell (CDC, 2022; White and Kaufman, 2014). Estimates of the life span of wild *Anopheles* mosquitoes vary widely; species-specific life spans range from 3.6 to 20 days, 5.6 to 32 days, or median daily survival rates from 0.68 to 0.98, which corresponds to a life span of 2.6 to 50 days (Matthews *et al*., 2020).

2.3 Primary malaria vector bionomics, abundance and seasonality

The state in which insects are fundamentally resting in dormant state until they feel that conditions for them to develop are impeccable and referred to as diapause (Denlinger and Armbruster, 2014). In the life history of mosquito species, diapause offers a mechanism for linking harsh seasons in temperate and tropical environments and serves to harmonize development within populations, which affect disease transmission cycles (Denlinger and Armbruster, 2014). Water is an important element for the development of all mosquito larvae and no mosquito can endure desiccation, although they may be able to survive short period of time in wet mud (Service, 2012). Climate is a driving factor that strongly influences the geographical distribution of insect vectors (Fouque and Reeder, 2019). Throughout dry season, many *Anopheles* species tend to vanish almost completely. For instance, many larval surveys become more productive towards the end or just after the wet season because breeding sites are temporarily stable for the *Anopheles* larvae to build up to a peak (WHO, 1975). Whilst malaria elimination remains an aspiring goal, hitherto there is

paucity of information regarding to ecology, behavior and distinctiveness of malaria vectors (Massey *et al.*, 2016). Due to spraying of insecticides in the interior walls of households, many malaria vector species demonstrate distinct shift in their bionomics and this leads to for example, mosquitoes to start biting earlier in the evening and resting outdoors (Malaria atlas, 2021).

During the wet season of April 2012, in Nchelenge district of Luapula Province in Zambia, *Anopheles* mosquitoes were collected by Center for Disease Control Light Trap (CDCLT) and Pyrethroid Spray Catch (PSC) which showed both *Anopheles funestus* s.s and *Anopheles gambiae* s.s to be highly anthropophilic (Brieger, 2013).

A Study by Minakawa *et al.* (2002) in West Kenya found that the proportion of *Anopheles funestus* group was greater in dry season than in rainy season and further stated that *Anopheles gambiae* complex species' abundance increase is reliant on climatic factors such as rainfall and temperature. However, the abundance of *Anopheles gambiae* s.l was rainfall dependent in Budiope county in Kamuli district (Uganda) having a high peak in May with a lower peak in October due to minor precipitation recorded in October and the *Anopheles funestus* s.l thrived all year round regardless of the amount of precipitation (Kabbale *et al.*, 2013).

2.4 Control and prevention of malaria

According to WHO (2022), vector control in areas with high risk of malaria disease is an effective way to minimize malaria transmission. Insecticide Treated Nets (ITNs) and IRS are highly recommended to be significant malaria control and elimination strategies. Verdicts from a systematic review done by Sherrard-smith *et al.*, (2018), vividly indicated that mass distribution of IRS and LLINs in Africa prevented nearly 517 million malaria cases from 2000 to 2015.

2.4.1 Indoor Residual Spraying

According to Kandyata (2012), the President's Malaria Initiative Africa Indoor Residual Spraying Project (PMI-AIRS), IRS in Zambia started in 2003 when Konkola Copper Mines succeeded with IRS and this influenced the National Malaria Control Center to adopt the IRS as part of the integrated vector management strategy for malaria control. Since its effective scaling up from 5 districts in 2003 to all districts (72 in 2011, 117 as of 2018) by 2018, IRS has been a pillar of the nation's efforts to eliminate malaria vectors (Chanda *et al*., 2016). Due to the discovery of resistance to DDT and pyrethroids in 2009, the Insecticide Resistance Management Technical Working Group (IRMTWG) was established in 2010 (Chanda *et al*., 2016). Actellic 300CS, a

microencapsulated formulation of the organophosphate insecticide pirimosphos-methyl, was released in 2013 in response to mounting worries about documented resistance to all classes of insecticides in all areas of Zambia, with exception of the organophosphates (Chanda *et al*., 2016). Because of insecticide susceptibility, Actellic 300CS was expected to be more effective than pyrethroid-based insecticides, but it is also significantly more expensive (Mashauri *et al*., 2017). The World Health Organization has ratified nineteen (19) insecticide formulations for IRS from the following five (5) insecticides classes: carbamates, organochlorines, organophosphates, pyrethroids and newly added insecticide class called neonicotinoids (Oxborough *et al*., 2019). Pervasive of resistance in *Anopheles* species in sub Saharan countries have ascended in corresponding expansion in funding and effort of vector control measures (Sougoufara *et al*., 2017).

2.4.1.1 Clothianidin insecticide

The second-generation neonicotinoid insecticide, clothianidin, (E)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine, was created by Sumitomo Chemical Takeda Agro Co./Bayer CropScience in 2001 (Uneme, 2011). Among the Hemiptera, Coleoptera, Thysanoptera, Lepidoptera and Diptera, clothianidin is highly effective biologically against a variety of insects and has low toxicity to mammals (Tomizawa et al., 2005; Dagg et al., 2019). Targeting the nicotinic acetylcholine receptor (nAChR) in the insect central nervous system is the primary mode of action of Clothianidin (Elamathi, et al., 2014).

A trial was conducted in Southern Benin in the experimental huts, clothianidin showed a clear delayed expression in the mortality of wild pyrethroid-resistant *An. gambiae* sensu lato that manifested 120 hours after exposure (Ngufor et al., 2017). Moreover, another trial was conducted in Ethiopia and for up to nine months after spraying, clothianidin (SumiShieldTM 50WG) caused mortality rates of more than 80% at 120 hours' post-exposure on all surface types (Yewhalaw et al., 2022). In Democratic Republic of Congo, under semi-field experimental conditions, there was no discernible difference between the two clothianidin doses (200 mg ai/sq m and 300 mg ai/sq m) in terms of mortality over time on all four types of wall surfaces, the mortality rates persisted above 60% for at least 48 weeks (Ngwej et al., 2019).

Since neonicotinoids often exhibit very little toxicity to mammals and have very low affinity for vertebrate nicotinic receptors in comparison to insect nicotinic receptors, they are a promise for application in public health (Ngufor et al., 2017). The alternative use of IRS insecticides and the

creation of IRS co-formulations, the inclusion of clothianidin to the IRS insecticides' product line also offers a chance to reduce the emergence and spread of insecticide resistance in malaria vectors (Fongnikin et al., 2020).

Neonicotinoids have been widely used, which has resulted in the introduction of severely hazardous effects on economically significant insects that are not the target, including honeybees and silkworms (Parte and Kharat, 2019).

Stenotrophomonas maltophilia, *S. maltophilia* CGMCC 1.1788, *Pseudomonas* sp.1G, *Leifsonia* sp., and *Rhodotorula mucilaginosa* strain IM-2 are the bacteria that have previously been shown to have the ability to breakdown clothianidin (Anhalt et al., 2007; Pandey et al., 2009).

Clothianidin degrades aerobically in soil via two primary pathways; the first pathway involves the N-demethylation of clothianidin to produce N-(2-chlorothiazol-5-yl-methyl)-N′-nitroguanidine, and the second pathway involves the breakage of the nitroguanidine molecule (Van der Velde-Koerts et al., 2009).

2.4.2 Long Lasting Insecticide Treated Bed Nets (LLINs)

In sub-Saharan Africa, the most often used vector control strategy to prevent malaria is Long-Lasting Insecticidal Nets (LLINs) (Mosha *et al*., 2020). Insecticide-Treated bed nests (ITNs) were distributed to over 7 million Zambia families as part of the 2020-2021 anti-malaria campaign (USAID, 2022). According to Zambia’s 2012 Malaria Indicator Survey (MIS), 68% of the houses surveyed had at least one insecticide-treated mosquito net (MoH, 2012). In addition, the mass campaigns of LLINs through antenatal clinics have been a significant effort to effectively protect high-risk individuals such as pregnant women and under five children from malaria (Kamuliwo *et al*., 2013).

Malaria control and elimination efforts are hampered by the fact that many people who live in sub-Saharan African nations with high rates of malaria do not use LLINs when they travel (Adam *et al*., 2018). The LLINs may shed residual insecticide more quickly as a result of variations in use and wear in field settings, decreasing their effectiveness (Kroeger *et al*., 2004). Insecticide overuse in ITN, can breed resistant mosquito vector species (Hemingway *et al.*, 2004). However, the ongoing spread of insecticide resistance possess a threat to the progress made by LLINs and could ultimately result in the operational failure of this vector control tool (Dagg *et al.*, 2019).

2.4.3 House screening

Centre of Insect Physiology and Ecology (ICIPE) conducted a study in Zambia (Nyimba district) on using wire mesh screens to mosquito-proofing houses is becoming more and more popular as a useful strategy to lessen exposure to mosquitoes that spread malaria (Ng'Ang'A *et al.*, 2020). Mosquito-proofing houses are fundamental since the primary *Anopheles* species that transmits malaria in Africa bites people while they sleep indoors between dark and dawn (Mburu *et al*., 2018). In endemic areas, open eaves are known to be a risk factor for malaria transmission because they serve as important entryways inside houses for several malaria vector species (Tusting *et al*., 2015). As a result, adding screenings to houses as a physical barrier to keep out mosquitoes has the potential to be a successful method of lowering malaria transmission (Tusting *et al*., 2015). In order to support the development of policy guidelines around house screening for malaria control, the World Health Organization Regional office for Africa (WHOAFRO) and International Centre of Insects Physiology and Ecology (ICIPE) conducted a study on additional impact of house screening on malaria transmission and clinical disease outcomes (Sangoro *et al*., 2021). Furthermore, using non-insecticidal house screens offers the chance to reduce dependency on insecticide-based malaria prevention strategies (Sangoro *et al*., 2021).

According to a study conducted in Gambia, children under the age of ten years who lived in fully screened houses or simply houses with screened ceilings had a 50% lower prevalence of anaemia than those who lived in unscreened houses (Kirby *et al*., 2009). Furthermore, research in Ethiopia, Equatorial Guinea, the Gambia, Kenya, and Tanzania demonstrated that house screening is an efficient and sustainable method of preventing mosquito ingress into houses (Atieli *et al.*, 2009). Historically, the eradication of malaria in the United States of America and the reduction of the disease in Europe were greatly influenced by improved housing (Zhao *et al.*, 2016).

2.4.4 Larval source management

Larval Source Management (LSM) is the management of aquatic habitats (water bodies) that may serve as mosquito larval habitats in order halt the development of the immature stages (WHO, 2013). Larval Source Management (LSM) is divided into four categories: biological control, larviciding, habitat modification, and habitat manipulation (MESA, 2022). The use of *Bacillus thuringiensis israelensis* (Bti), a bacteria species that kills mosquito larvae by releasing a gut paralyzing toxin and starving them, is one common LSM technique (Aronson *et al.*, 1986). The key benefits of Bti are that it is reasonably affordable, simple to use, and environmentally safe (Fuseini *et al*., 2019).

In Burkina Faso, researchers found that Bti significantly reduced the number of malaria vectors by larviciding their breeding habitats (Dambach *et al*., 2019). Moreover, in low-malaria transmission areas of Botswana and Zimbabwe, the use of the microbial larvicide Bti demonstrated to have an effect on larval densities, which reduced adult mosquito numbers and malaria transmission (Mpofu *et al*., 2016). Upon further review of the effectiveness of bio-larvicides, in 2010 the National Larviciding Programme was instigated through a bilateral agreement between the Cuban and the Zambian governments for the eradication of potential vectors of malaria, *Bacillus thuringiensis var. israelensis* and *Bacillus sphaericus* demonstrated to be effective for the control of mosquito larvae in Lusaka urban district (Kandyata, 2012).

2.5 Plant based insect repellents

Insect repellents are considered agents that are used to protect the body from the bites of insects that can cause skin irritation, sickness and possibly death as the insects act as disease vectors (Pinchoff, 2008). According to United States Environmental Protection Agency (2021) insect repellents are not designed to kill insects or pests but makes people less attractive to the biting insects or pests. Insect repellents which are derived from plants are referred to as natural-origin repellents and they exploit their action on mosquitoes, flies, sandflies, horseflies, fleas, mites and ticks (Motta and Monti, 2015). An experiment conducted by Trongtokit *et al* (2005), using an experiment called cage test which was done using human arm and it was found that products with 20% of p-menthane-3, 8-diol (PMD) active ingredient gave a complete repellency for 7-8 hours while products with 10% of p-menthane-3, 8-diol gave repellency for 30 minutes against *Anopheles stephensi*. The natural oils (10%) of clove (*Syzyguim aromatica*) and Makaen (*Zanthroxylum limonella*) provided repellency for 4-5 hours.

Table 2.2: List of repellent products (Trongtokit et al., 2005).

No.	Product	Active ingredient and concentration
1.	Mosi-guard Natural pump spray (Masta, UK)	P-methane diol, 30% from Lemon Eucalyptus
2.	Mosi-guard Natural cream (Masta, UK)	P-methan diol, 20% from Lemon Eucalyptus
3.	Repel Lemon Eucalyptus cream (Jacson, USA)	P-methan diol, 20% from Lemon Eucalyptus
4.	Off! Bontanicles lotion (Johnson. USA)	p-methane diol, 10% synthesized from menthol
5.	Repel Insect Repellent lotion (Boots, UK)	Citronella oil, 5%
6.	Citrepel oil	Citronella, 40%
7.	Autan Active Insect Repellent pump spray (Bayer)	Hydroxyethyl isobutyl piperidine carboxylate[a]
8.	Jungle formula Insect Repellent	DEET, 50%
9.	Mospel (Mahidol university, Thailand)	Clove oil, 10% plus makaen oil (Zanthoxylum limonella), 10%

[a]concentration not specified.

Furthermore, a eucalyptus based insect repellent and N,N-diethyl-3-methylbenzamide(deet) spray repellent were evaluated against *Anopheles gambiae* and *Anopheles funestus* in Tanzania by Trigg (1996), the study result was a non-significant difference between eucalyptus based insect repellent and deet spray repellent in terms of efficacy ($p>0.05$) and longevity ($p>0.05$) of repellency against *Anopheles* species. All repellents provided greater than 6 hours protection from mosquito bites. Due to the scaling up of control measures like bed nets and indoor residual spraying, insecticide resistance in malaria vectors has been found in Rwanda. To address this issue, the use of mosquito repellents to supplement the current indoor vector control measures and to control malaria residual transmission has been approved (Rwanda Standards Board ISO 9001 Certified, 2019). Previous studies by Deletre *et al* (2019), showed that (E)-Cinnamaldehyde, major compound of cinnamon bark *(Cinnamomum zeylanicum*); carvarol, major compound of thyme leaf (*Thymus vulgaris*); geraniol, major compound of citronella leaf (*Cymbopogon winterrianus*); and cuminaldehyde, major compound of cumin seed (*Cuminum cymimum*) could have significant toxic effect, repellent and irritant on *Anopheles gambiae* (Kisumu susceptible strain). Principally, a systematic review research study conducted by Asadollahi *et al* (2019) showed that essential oils emanating from plants like lavender, camphor, catnip, geranium, jasmine, eucalyptus, lemongrass, amyris, carotin, cedarwood, chamomile, cinnamon oil, juniper, cajeput, soya bean, rosemary, niaouli, olive, tagetes, violet, sandalwood, litsea, galbanum and *Curcum longasgave* provided evidence of 8 hours complete repellency against dissimilar *Anopheles* species.

2.5.1 Alternative botanical insecticides

Botanical insecticides are products that are used to exterminate or resist insects that contain dried ground plant material, crude plant extracts or chemicals isolated from plants (Isman, 2004). Nevertheless, Texas A&M University (2021) defines botanical insecticides as natural insecticides extracted from plants and minerals, and they contain active ingredients such as pyrethrins, rotenone, nicotine, linool, d-limonene, sabadilla, neem and ryania.

Aguayo (2021), stated that the first botanical insecticide used was nicotine from tobacco to exterminate plum beetles and around 1850 a new botanical insecticide rotenone was introduced.. During the 1940s, due to massive use of Dichlorodiphenyltrichloroethane (DDT) and because of environmental contamination and insecticide resistance, botanical insecticides were reconsidered as alternatives and crude extracts from *Nicotiana tabacum* are still being used in Thailand to control Arthropod species (Bullangpoti, 2014). Before neonicotinoid formulations were prequalification listed by WHO, only 2 modes of action across 4 insecticides classes were known and these are sodium channel modulation (pyrethroids and DDT) and acetylcholine inhibition (organophosphate and carbamates), and because of dearth of product diversity, there is a possibility of cross resistance between products with same mode of action increases (Oxborough et al., 2019).

Due to over use of synthetic chemicals for the mosquito management and health concerns, people in rural communties embarked on the usage of traditional natural products from plants against insect vectors and parasites (Baranithara et al., 2019). For example in an experiment conducted by Oladipupo *et al* (2019) phytochemical extracts from *Piper guineense* (climbing black paper) and *Eugenia aromatic* (cloves) were exposed to *Anopheles gambiae* complex species and the populations of the mosquitoes demonstrated significant successive knockdowns to the botanicals.

2.6 Use of Nicotiana tabacum as an insecticide for control of mosquitoes

The use of botanical insecticides derived from tobacco leaf components as an insecticide and disease control agent is centuries old (Wuryantini *et al*., 2017). Nicotine, nornicotine, and anabasine, which are found in tobacco extract, are phenolic chemicals that serve as neurotransmitters comparable to organophosphate or carbamate insecticides by mimicking the effects of acetylcholine (Isman, 2006). Nicotine is one of tobacco's key constituents, and it is toxic to insect's nerves because it causes rapid reaction (Afifah *et al*., 2015).

The efficacy of nicotine and its derivatives against mosquitoes forms the basis for a large portion of the hope that tobacco crude can be utilized in mosquito control (Dieng et al., 2014).

One of the studies in Nigeria, found that at 0.5% concentration of leaf extract of *Nicotiana tabacum*, a 100% mortality was observed on adult *Anopheles gambiae* (Ileke *et al.*, 2015). The effectiveness of a tobacco-based anti-*Aedes Aegypti* mosquito paint was tested, and the results revealed that a 5% concentration of tobacco extract could kill half of the mosquito population (LC50) for two hours, a 3-5% concentration for four hours, and a 1-3% and 0-1% concentration for six and twenty-four hours, respectively (Sandralintang et al., 2018). However, it was shown that tobacco extract provided 98% mortality after 72 hours of administration in Pakistan when concentrations (400 ppm, 200 ppm, 100 ppm, 50 ppm, and 25 ppm) were administered to the third instar of the *culex quinguefasciatus* species (Ullah et al., 2018).

2.6.1 Chemical characteristics of *Nicotiana tabacum*

Plants create natural compounds like alkaloids as a defensive mechanism to ward off or kill predators in order to prevent them from being devoured by predator insects and other animals (Wade, 2006). Tobacco plant belongs to the nightshade family, Solanaceae; the common species of tobacco is *Nicotiana tabacum*, and wild tobacco is *Nicotiana rustica* and this remarkable plant is used as a source of a bio-pesticide, as nicotine is considered to be a systemic and contact pesticide (Omara and Attaf, 2014). Nicotine is the major alkaloid found in tobacco leaf at about 96-98% with remaining portion comprising of minor alkaloids namely; nornicotine, anabasine, anatabine, cotinine and myosmine (Clemens *et al.*, 2009). Essentially, nicotine and nornicotine are the alkaloids in *Nicotiana* species that have insecticidal properties of which these insecticidal properties varies depending on the insect species (Zenkner et al., 2019). Nicotine is not only used in cigarette manufacturing industries as an additive but also used in pharmaceutical, fine chemical and agriculture industries (Kheawfu *et al.*, 2021). Other chemical constituents in the tobacco extract which have high economic and medicinal value have been categorized into nine groups: diterpenoids, flavonoids, sterols, isobenzofurans, phylpropanoids, aminoglycosides, terpenes, sesquiterpenes and furan-2-catboxylic acids (Sun and Sun, 2016; Xu *et al.*, 2020).

2.6.2 Chemical structure of nicotine ($C_{10}H_{14}N_2$)

Nicotine has been used as bio-insecticide to harmful insects; it is also regarded as a spring of nicotine sulfate, which is a pesticide used in agriculture and it is eco-friendly; leaves no toxic

residues in the soil, on fruits and on vegetables (Mahendra, 2012). Henceforward, nicotine is also significant for synthesis of nicotinic acid, nicotinamide and nikethamide in pharmaceutical industries (Donald, 2020). Nicotine is a pyridine alkaloid and in the structure of nicotine more nitrogen heterocyclic rings are in place (Figure 2.2) and nicotine is a hygroscopic, oily liquefied substance which is colourless at boiling point of 245.5°C and has molecular mass of 162.12g/mole and melting point of -79°C (CDC, 2021). Moreover, nicotine in water and other solvents (alcohol, ether, chloroform and petroleum ether) is soluble, it has a blistering alkali flavour and when exposed to air it turns brown due to the oxidation process (Kumari and Thangavel, 2016).

Figure 2.2: A model for nicotine molecule developed by McMurry and Fay (2012).

Despite nicotine being volatile, it has a density of 1.007g/ml at 20°C, specific rotation from -166.39° to 168.50° which makes nicotine to be levorotatory and most salts of nicotine e.g. nicotine sulfate are dextrorotatory (Hau and Zeng, 2014).

2.6.3 Bio-degradation of nicotine

White rot fungus (*Phanerochaete chrysosporium*), which reduces organochlorine compounds like Dichlorodiphenyltrichloroethane (DDT), Polychlorinated Biphenyls (PCBs), as well as bio-insecticides in the soil, is an example of cometabolism of toxins (Manahan, 2003). Enzymology assists in twigging how the enzymes biodegrades xenobiotic compounds such as synthetic pesticides and also this implies to the biodegradation of bio-insecticides using microbes such as bacteria which decomposes bio-insecticides to release less toxic residues in the soil (Chowhury et al 2008).

In order to detect the degradation of nicotine, Wang et al (2006) conducted an experiment to study the bio-degradation of nicotine by using the bacterium *Pseudomonas putida* biotype A (Figure 2.3). The alleyways of degradation of nicotine were found from nicotine to 2,5-dihydroxypyridine through the midways N-methlmyosimine, 2´-hydroxynicotine, pseudooxynicotine, 3-

pyridinebutanal, c-oxo, 3-succinoylpyridine and 6-hydroxy-3-succinoylpyridine. Moreover, N-methylmyosmine, 2,5-dihydroxypyridine and succinic acid also were spotted. Again, 1-butanone,4-hydroxy-1-3-pyridinyl (alcohol) was found to be a unique by-product of bio-degradation of nicotine. The following pathway in figure 2.5 displays the eco-friendly bio-degradation of nicotine in the presence of *Pseudomonas putida*:

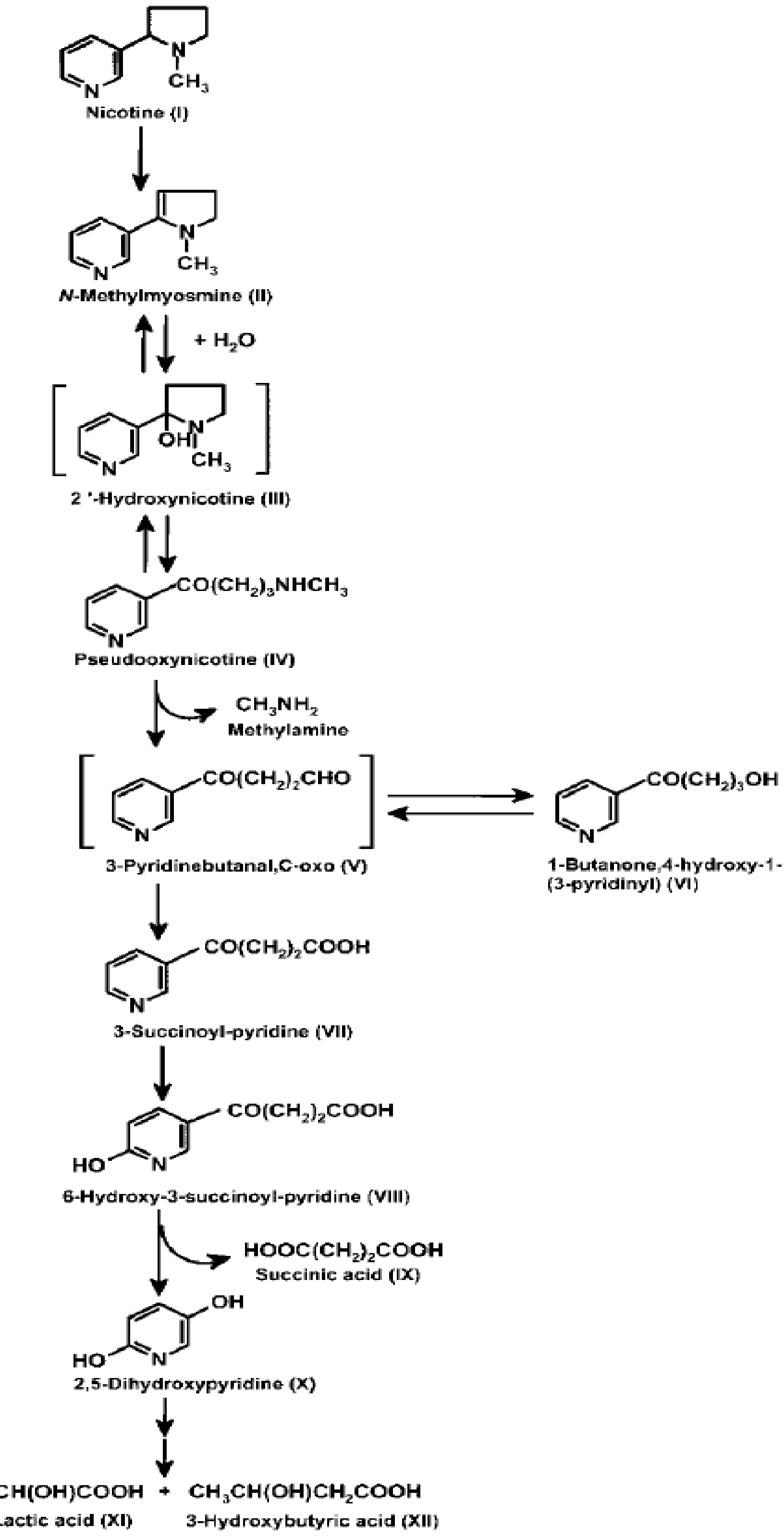


Figure 2.3: Bio-degradation of nicotine by *Pseudomonas putida* Biotype A (Wang *et al*, 2006

CHAPTER 3: MATERIALS AND METHODS

3.1 Study site

This study was conducted in Mwense district located in Luapula province of Zambia (Figure 3.1) between October-November 2021. Mwense district is located in the northern part of Zambia in Luapula Province on coordinates 10°25'0" S and 29°0'0" E (OpenStreetMap, 2021). It shares an international boundary with Democratic Republic of Congo (Smart Zambia Institute, 2022). Mwense town is predominantly a rural area with an estimated population of 1,063 (ZHMIS, 2021). Mwense district's vegetation cover consists of Miombo forest and grassland with *Hyparrhenia* as the dominant species and low-growing grasses with other plant species predominate in the waterlogged areas (Food and Agriculture Organization, 2022). The average annual temperature in the district ranges from 12.8°C to 35°C, rarely falling below 10.6°C or rising over 37.8°C (Cedar Lake Ventures, Inc., 2016). Additionally, the district receives about 89.1 millimeters of rainfall from November to April (Food and Agriculture Organization, 2022; The Global Historical Weather and Climate Data, 2023).

The peak for malaria transmission in Mwense district is associated with rainy season from November to May with a reported incidence rate of 787 cases per 1000 persons per year (District Health Information System [DHIS]). Previous studies have shown that *Anopheles funestus* s.s and *Anopheles gambiae* s.s are the principal malaria vector species in Luapula province (Stevenson et al., 2016; Jones et al, 2021) .Chebele village is a control sentinel site for the Vector-Link project (Figure 3.1) where IRS Programme is not conducted. Mass distribution of Olyset® Long-Lasting Insecticide Nets (LLINs) incorporating permethrin and a synergist Piperonyl Butoxide is the primary vector control method used in the study area. The houses were geocoded by using the GPS Test application. The selected Chebele village is located along Mwense stream and anthropogenic activities such as brick making have made deep holes on the surface of the soil acting as temporal breeding sites for mosquitoes.

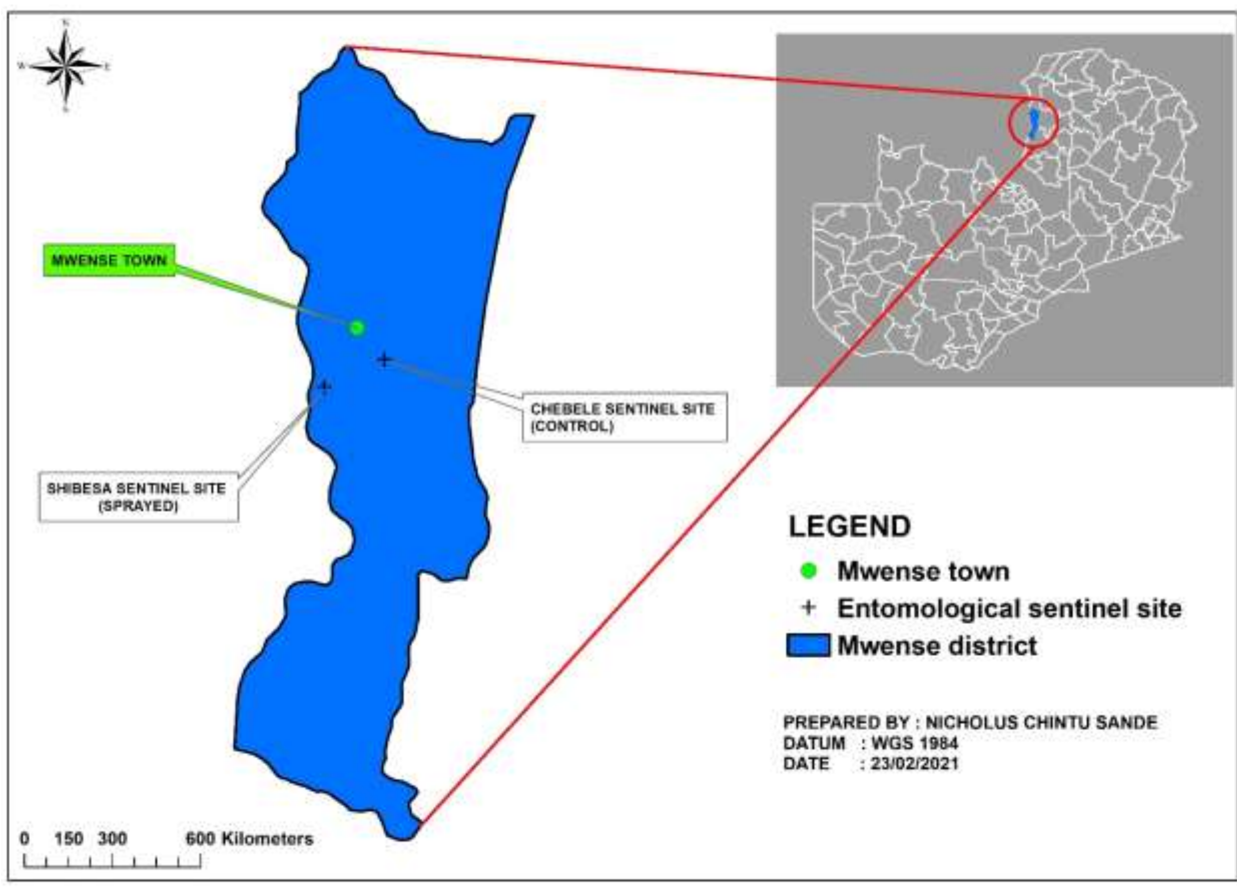


Figure 3.1: Location of entomological sentinel sites in Mwense district in Luapula Province.

3.2 Selection of village households and sample size

Mwense District Health office was engaged in the study and helped to identify a Community Health Worker (CHW) who assisted the researcher to conduct household survey and sampling of *Anopheles* larvae from the breeding sites. Consent to aspirate the adult mosquitoes from inside the households was obtained from the household heads (see appendix 4). This study applied mixed method research design and the number of households in the village were determined using the formula adopted form Almeda et al (2010). Below is the formula that was used to calculate the number of households for Prokopack aspiration:

$$\text{Number of households sampled (n)} = \frac{N}{1 + N\,(e)^{\wedge}2}$$

Where; n is the sample size, N is the total number of households and e is the margin of error taken as 0.1.

Therefore, seventy three (73) households with open eaves were selected from Chebele village by using convient sampling technique. (Figure 3.2).

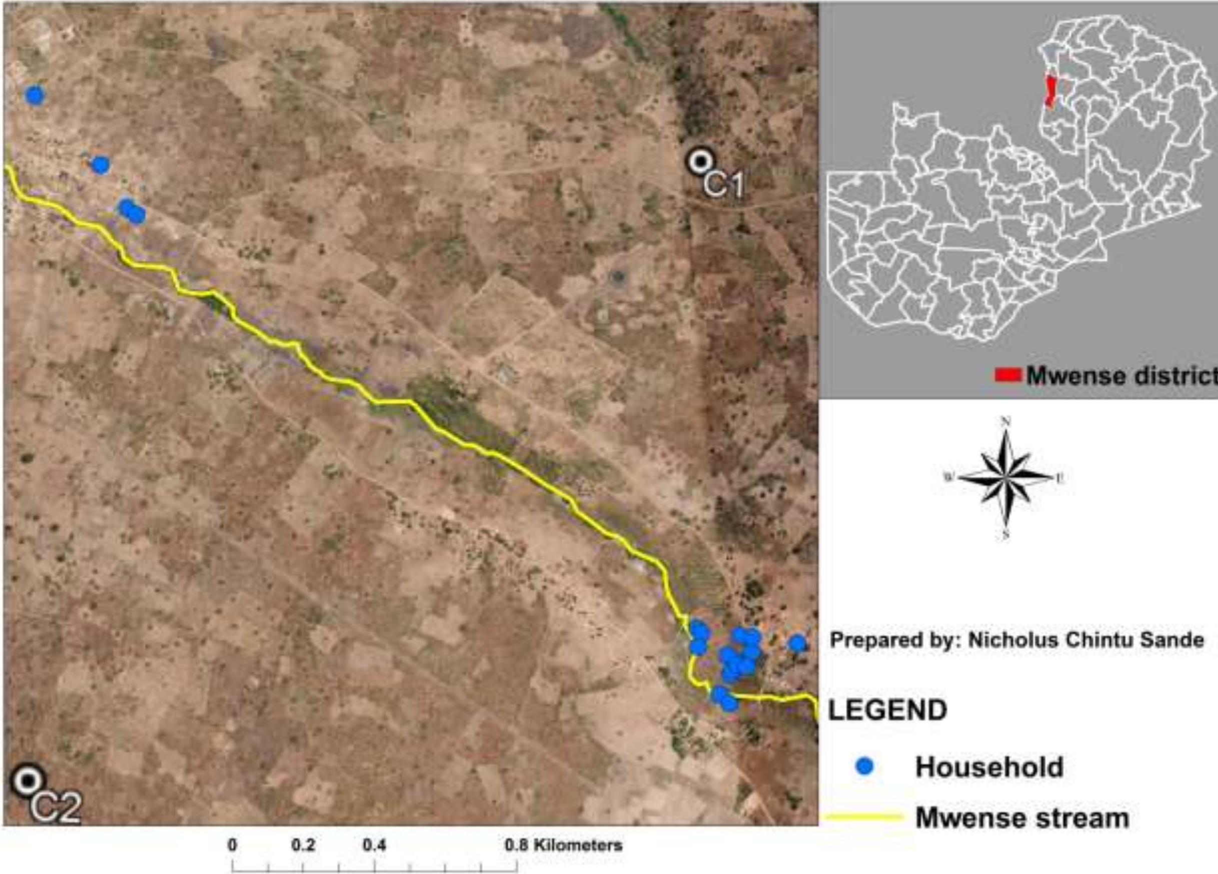


Figure 3.2: Map for households that were selected for Prokopack aspiration in Chebele village. C1 and C2 are Ground Control Points (GCPs) for geo-referencing the satellite image.

3.3 Sample size of mosquitoes for insecticide susceptibility tests

Determination of sample size for the exposure to the insecticides followed the Standard Operating Procedure (SOP) by WHO (2016), where the number of mosquitoes (n) exposed per insecticide is 100. Further, the recommended number of mosquitoes for exposure in each WHO bottle bioassay is 20 to 25.

3.4 Collection of *Nicotiana tabacum* and preparation of tobacco extract

The leaves of *N. tabacum* (2 Kilo grams) were collected from a small scale tobacco farmer in Nkeyema district in Western Province of Zambia. The plant leaves were transported to the field stationed Entomology laboratory in Kaoma district of Zambia for further processing.

The solvent extraction method was used to extract tobacco crude from tobacco leaves as described by Al-ghamdi and Baljoon (2014). The leaves of *N. tabacum* were air dried in the laboratory. When they were crusty, the leaves were pounded into powders using a mortar and pestle. The

powders were further sieved to pass through 1.40mm holes before they were filled into zip lock plastic packs and stored in a refrigerator at 4°C prior to use. About 10g of tobacco powder was soaked in 250ml beaker containing 5% of Sodium Hydroxide (NaOH). The mixtures were stirred vigorously with a spatula for 15 minutes and the filtrate was filtered into a clean 250ml beaker using a Buchner funnel. About, 25ml of petroleum ether was added to the filtrate, the mixture was shaken 30 minutes and left undisturbed until the double layer was formed in the separating funnel (Figure 3.3 B). Filtration was carried out using Whatman No. 1 filter paper with 1 teaspoon of Magnesium Sulphate hepahydrate ($MgO_4.7H_20$) as a drying agent to remove water from tobacco extract. The resulting extract was heated in water bath at 80°C in order to remove traces of petroleum either. Finally, the tobacco extract was kept in glass tubes and preserved in the refrigerator at 4°C till further use (Figure 3.3 A).

Two to three drops of dragendorff reagent were added to the nicotine oil and the formation of red orange precipitate was a positive test for the presence of the alkaloid called nicotine (Arsianti *et al.*, 2020).

(A) **(B)**

Figure 3.3:Tobacco extract stored in a glass tube **(A);** separating funnel used to drain off waste of tobacco extract **(B).**

3.5 Collection of adult mosquitoes

3.5.1 Collection of wild mosquitoes

The method used to aspirate indoor mosquitoes was Prokopack Asipration as described by Williams and Pinto (2012). Prokopack aspiration targets endophagic and anthropophilic mosquitoes. The mechanical aspirator was powered by a 12 volts' battery and aspirated mosquitoes were released in the mosquito cage after being aspirated from the houses (indoors).

Collections were conducted early in the morning between 05:30am and 7:00am when the occupants exited the house. The mosquitoes were to be kept live for a period of 24 hours and evaluation of any knock down effect on the mosquitoes due to stress from high aerodynamic flow of a fluid "air" from the Prokopack aspirator, the knocked down mosquitoes were carefully removed from the cage and put into Eppendorf tubes.

Indoor-resting blood-fed female *Anopheles* mosquitoes were collected from 73 houses in Chebele study site using Prokopack Aspirator (John. W. Hock Company) between 4:00 and 5:00AM. The collected adult mosquitoes were kept in BugDorm-1 cages (Mega View Science Co., Ltd, Taichung, Taiwan) covered with moist cotton towels to support survival. The adult mosquitoes were later transported to the central field station insectary at Grand Palm Lodge (10°23'7.80''S and 28°.40'33.49") in Mwense town for further processing.

3.5.2 Laboratory reared *Anopheles gambiae* s.s mosquitoes

The laboratory strain of mosquitoes used as a reference for the experiment was the *An. gambiae* s.s (Kisumu strain) from National Malaria Elimination Center (NMEC). This strain has never been exposed to any insecticide and therefore considered susceptible.

3.6 Collection of wild *Anopheles* larvae using pipetting and dipping method

Wild *Anopheles* larvae were collected at the larval stage in Mwense district near the banks of Mwense stream (10°23'58.07''S and 28°42'46.77''E) (Figure 3.4). The larvae were collected using dippers, pipettes and white plastic trays. Dippers were used to scoop wild larvae from the breeding sites, pipettes for picking wild larvae from the dippers, and plastics trays for allowing wild larvae to rest during the collection time. Further, wild larvae were transferred using a pipette from plastic trays into small holding breeding cups and transported to the field laboratory where larvae was reared from different instar stages to pupa stage and finally to adult stage.

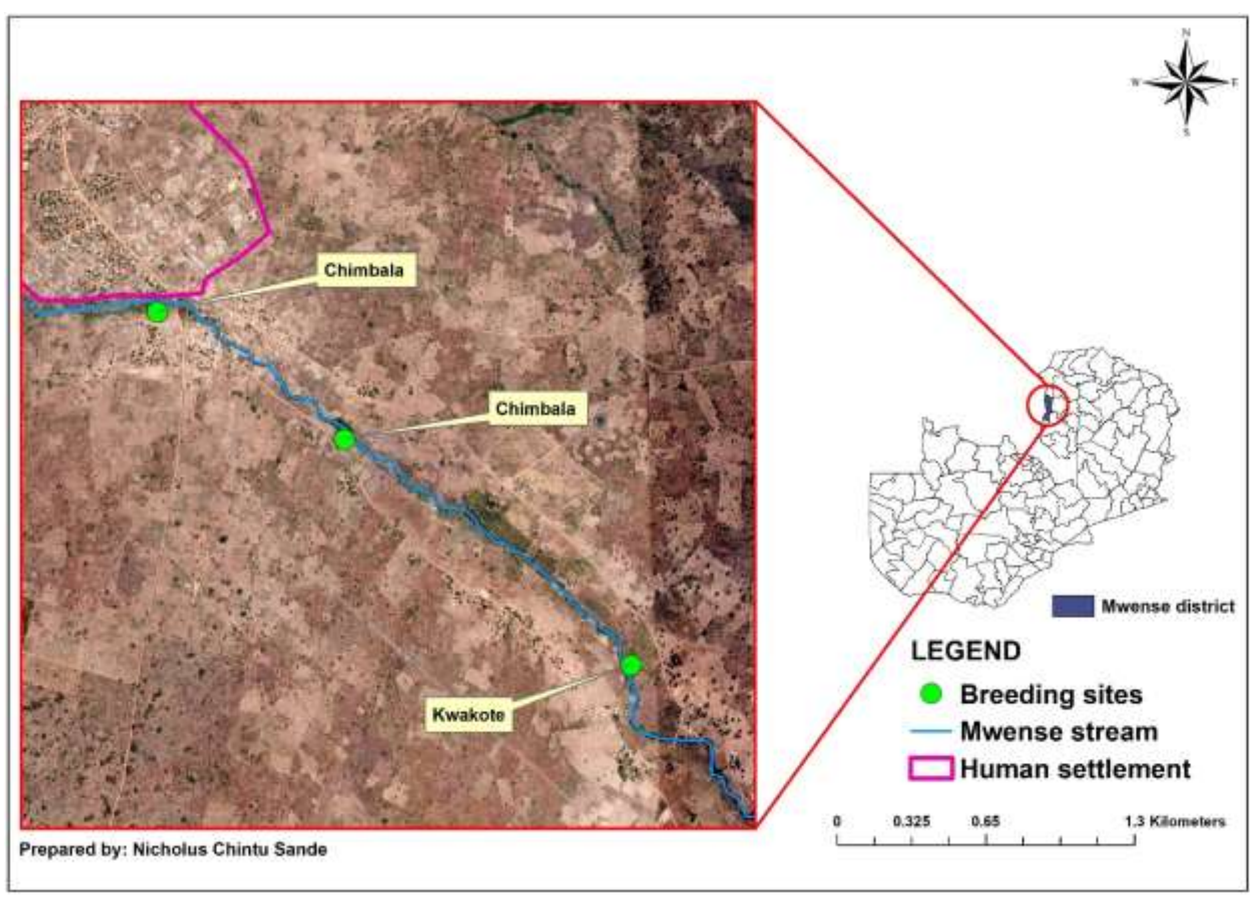


Figure 3.4: Location of breeding sites of wild *Anopheles* mosquitoes in Chebele study site in Mwense district of Luapula Province in Zambia.

3.6.1 Rearing of wild *Anopheles* larvae to adult stage

Larvae were fed with Tetramin flakes fish feed. The pupae were collected in plastic cups and placed in cages, adult emerging from pupae were fed on 10% glucose solution. Rearing of wild *Anopheles* larvae and pupae to F1s were conducted under field climatic conditions with temperature range of 30±2°C and 50-60% relative humidity. While at National Malaria Elimination Centre (NMEC), the insectary reared Kisumu colony (reference species) were maintained continuously at 25-27°C and 75-75% relative humidity and female *Anopheles gambiae* s.s (Kisumu) aged 2-5 days old were used to carry out bioassays.

3.7 Preparation of insecticide-treated papers for clothianidin (SumiShield® 50WG) insecticide bioassays

The Standard Operating Procedure (SOP) which was developed and optimized by PMI-AIRS project (2016) was used to prepare insecticide treated papers for SumiShield® 50WG insecticide. Whatman® No. 1 filter papers measuring 12cm by 15cm were impregnated with a dose of clothianidin (0.132g/ml). Firstly, 2.64g of SumiShield® 50WG (containing 50% clothianidin as active ingredient) was suspended in 20 milliliters of distilled water and the resulting suspension was shaken well before pipetting it onto the filter paper. In order to ensure equitable absorption into the filter paper during insecticide treatment, the filter paper was supported on a bed of nails (hammered into a piece of wood at smilar heights). A target dosage of 0.132g/ml per paper was applied with a pipette to equally distribute 2ml of insecticide solution onto the filter paper until there were no dry areas. After drying overnight, the filter papers were stored in the silled zip lock plastics at 4°C in the mini-bar fridge. Filter paper treated with 2ml of distilled water was used as the negative control.

Insecticide susceptibility tests were conducted on unfed wild adult *Anopheles* mosquitoes aged from 2 to 5-day old. These tests were conducted according to established protocol (WHO, 2016). A total of 142 wild *Anopheles* mosquitoes were exposed for 80 minutes in 6 replicates of 25 mosquitoes against clothianidin (0.132g/ml)(positive control), with an addition 1 replicate of 25 wild *Anopheles* mosquitoes used for the negative control (paper treated with distilled water). Moreover, a total of 116 insectary reared Kisumu strain (used as reference) were exposed for 80 minutes, in 5 replicates of 25 Kisumu strain mosquitoes and 1 additional replicate of 25 Kisumu mosquitoes used for the negative control. After exposure, mosquitoes were transferred to untreated holding tubes and provided with lightly moistened cotton wool containing 10% sugar solution. Knock down was recorded at 10, 15, 20, 30, 40, 50, 60 and 80 minutes (see appendix 5). Mortality was recorded 24 hours after exposure.

3.7.1 Preparation of treated cotton wool for tobacco extract bioassays

For tobacco extract the concentration were set at 25% v/v, 33.3% v/v, 50% v/v, 62.5% v/v, 71.43% v/v and 83.3% v/v. Due to the high volatility of tobacco extract, an absorbent round cotton wool pieces manufactured by Premier MPCS Limited were used to impregnate tobacco extract with 25% v/v, 33.3% v/v, 50% v/v, 62.5% v/v, 71.43% v/v and 83.3v/v before carrying out susceptibility bioassays. Cotton wool treated with 2ml of distilled was used as the negative control.

Insecticide susceptibility tests were conducted on unfed wild adult *Anopheles* mosquitoes aged from 2 to 5-day old. These tests were conducted according to established protocol (WHO, 2016). In general, a total of 370 wild *Anopheles* mosquitoes were exposed for 80 minutes in 15 replicates of 25 mosquitoes against tobacco extract concentrations (25% v/v, 33.3% v/v, 50% v/v, 62.5% v/v, 71.43% v/v and 83% v/v), with an addition 1 replicate of 25 wild *Anopheles* mosquitoes used for the negative control (paper treated with distilled water). Similarly, a total of 104 insectary reared Kisumu strain (used as reference) were exposed to tobacco extract (83.3% v/v) for 80 minutes in 4 replicates of 25 Kisumu mosquitoes and 1 additional replicate of 25 Kisumu mosquitoes used as the negative control. After exposure, mosquitoes were transferred to untreated holding tubes and provided with lightly moistened cotton wool containing 10% sugar solution. Knock down was recorded at 10, 15, 20, 30, 40, 50, 60 and 80 minutes (see appendix 5). Mortality was recorded 24 hours after exposure.

Tests were conducted on conditions anticipated to be within WHO guidelines of 25°C ± 2°C and relative humidity of 75% ± 10% (Williams and Pinto, 2012). However, temperature and humidity of the room was recorded.

3.7.2 Molecular identification of *Anopheles* species

All wild *Anopheles* mosquitoes were morphologically identified in the field using a dissecting microscope and a standard morphological identification key by Coetzee (2020). Wild *Anopheles* mosquitoes which were susceptible and resistant to the two types of insecticides were well labeled and kept in 1.5ml Eppendorf tubes filled with silica gel and cotton wool at room temperature and transported to the laboratory for molecular analysis in Lusaka at National Malaria Elimination Center (NMEC).

Sufficient DNA was acquired from small crushed mosquito parts such as legs, abdomen and wings from morphologically identified *An. funestus* s.l and *An. gambiae* s.l mosquitoes (Kabbale *et al.*, 2016). Genomic DNA used in this study was extracted using the tween chelex method (Teyssier *et al.*, 2021). The PCR primers used as species identifiers within *An. gambiae* complex includes; UN(forward): 5-GTG TGC CCC TTC CTC GAT GT-3, GA(reverse): 5-CTG GTT TGG TCG GCA CGT TT-3, MR(reverse):5-TGA CCA ACC CAC TCC CTT GA-3, AR(reverse): 5-AAG TGT CCT TCT CCA TCC TA-3 and QD(reverse): 5-CAG ACC AAG ATG GTT AGT AT-3 (Kabbale *et al.*, 2016). Whereas the DNA cocktail primers used for species identification of *An. funestus* group were; Universal primer (UV): 5-TGT GAA CTG CAG ACA T-3, Funestus (FUN):

5-GCA TCG ATG GGT TAA TCA TG-3, Vaneedeni (VAN): 5-TGT CGA CTT GGT AGC CGA AC-3, Rivulorum (RIV): 5-CAA GCC GTT CGA CCC TGA TT-3, Parensis (PAR): 5-TGC GGT CCC AAG CTA GGT TC-3, Rivulorum-Like (RIV-LIKE): 5-CCG CCT CCC GTG GAG TGG GGG-3 and Leesoni (LEES): 5-TAC ACG GGC GCC ATG TAG TT-3 (Koekemoer et al, 2002). Sub-species of *An. funestus* s.l and *An. gambiae* s.l were identified using a multiplex Polymerase Chain Reaction (PCR) assay using 500bp for *An. funestus* s.s and 400 bp for *An. gambiae* s.s. The PCR assay for *An. funestus* s.l is based on species specific single nucleotide polymorphism in the internal transcribed spacer region (ITS2) while for *An. gambiae* s.l is in the intergenic spacer region (IGS) (Fettene and Temu 2003; Besansky et al. 2006; Wilkins et al. 2006; Koekemoer et al, 2002).

3.7.3 Gel electrophoresis of the PCR product

The PCR products were electophoresed on a 1.5% agarose gel with Tris-Borate Ethylene-diamino amplified DNA fragments. The PCR products were loaded into each well and run at 120 V with 100 mA. The gel pictures were taken under ultraviolet light using a gel documentation system equipment. Tris/Borate/EDTA (1X) solution contained Tris-Base (89mM), Boric Acid (89mM) and Ethylene-di amine tetra acetic acid (EDTA) (2mM). The gel was stained with 4µl Midori Advance in 50 ml of TBE to detect the presence of double strand DNA (dsDNA).

3.8 Statistical analysis

Data for susceptibility tests were entered in the Microsoft excel® (Microsoft Corporation, Redmond, WA, 2010) software and later transferred to SPSS statistics 23.0 version for analysis. Efficacy of insecticides against wild *Anopheles* were calculated as percentage mortality following WHO standards to determine phenotypic resistance frequency with 98% - 100% mortality signifying susceptibility; 90% - 97% mortality signifying possible resistance and mortality less than 90% signifying resistance (WHO, 2016). Data from WHO-bottle bioassays was further subjected to probit regression analysis using SPSS statistics 23.0 version and lethal concentrations were calculated with 95% confidence interval.

Assessment of goodness of fit was done using the Chi-square goodness of fit test (Probit Pearson goodness of fit test). Since the significance level was greater than 0.05, no heterogeneity factor was used in the calculation of confidence limits., the data was assumed to fit the probit model used in the analysis.

CHAPTER 4: RESULTS

4.1 Mosquito species composition from households

A total of 203 indoor resting biting mosquitoes were collected in 73 selected houses in Chebele village in Mwense district using Prokopack Aspirator (Figure 4.1), of which 86.7% (n=176) were morphologically identified as fed female *An. funestus* s.l Giles, 8.867% (n=18) as unfed female *An. funestus* s.l Giles, 2.463% (n=5) as unfed male *An. funestus* s.l Giles, 0.493% (n=1) as fed female *An. gambiae* s.l Giles, 0.985% (n=2) as unfed female *An. gambiae* s.l Giles and 0.493% (n=1) as *Culex* species.

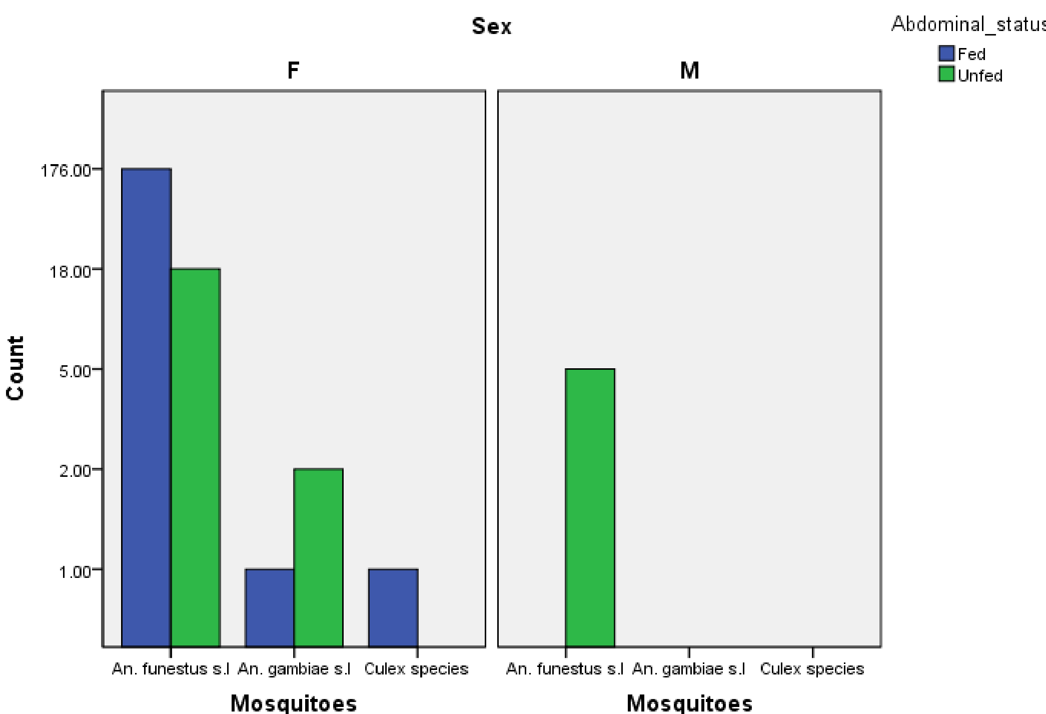


Figure 4.1: Species composition of mosquitoes collected by Prokopack Aspirator in October, 2021 dry season in Chebele village in Mwense district of Luapula Province in Zambia [F=Female and M=Male].

4.2 Mosquito species composition from larval collections

Three hundred and sixty-three (363) wild mosquitoes that emerged from larvae were not exposed to the two insecticides because the mosquitoes died before being exposed to the insecticides

(Figure 4.2). Fifteen point seven percent [15.7%] (n=57) were morphologically identified as female *An. funestus* s.l Giles, 51.791% (n=188) as male *An. funestus* s.l Giles, 4.132% (n=15) as female *An. gambiae* s.l Giles and 22.865% (n=83) as male *An. gambiae* s.l Giles. The remaining 5.510% (n=20) comprised other species namely, *An. pretoriensis* Theobald, *An. rufipes* Gough, and *Culex* species.

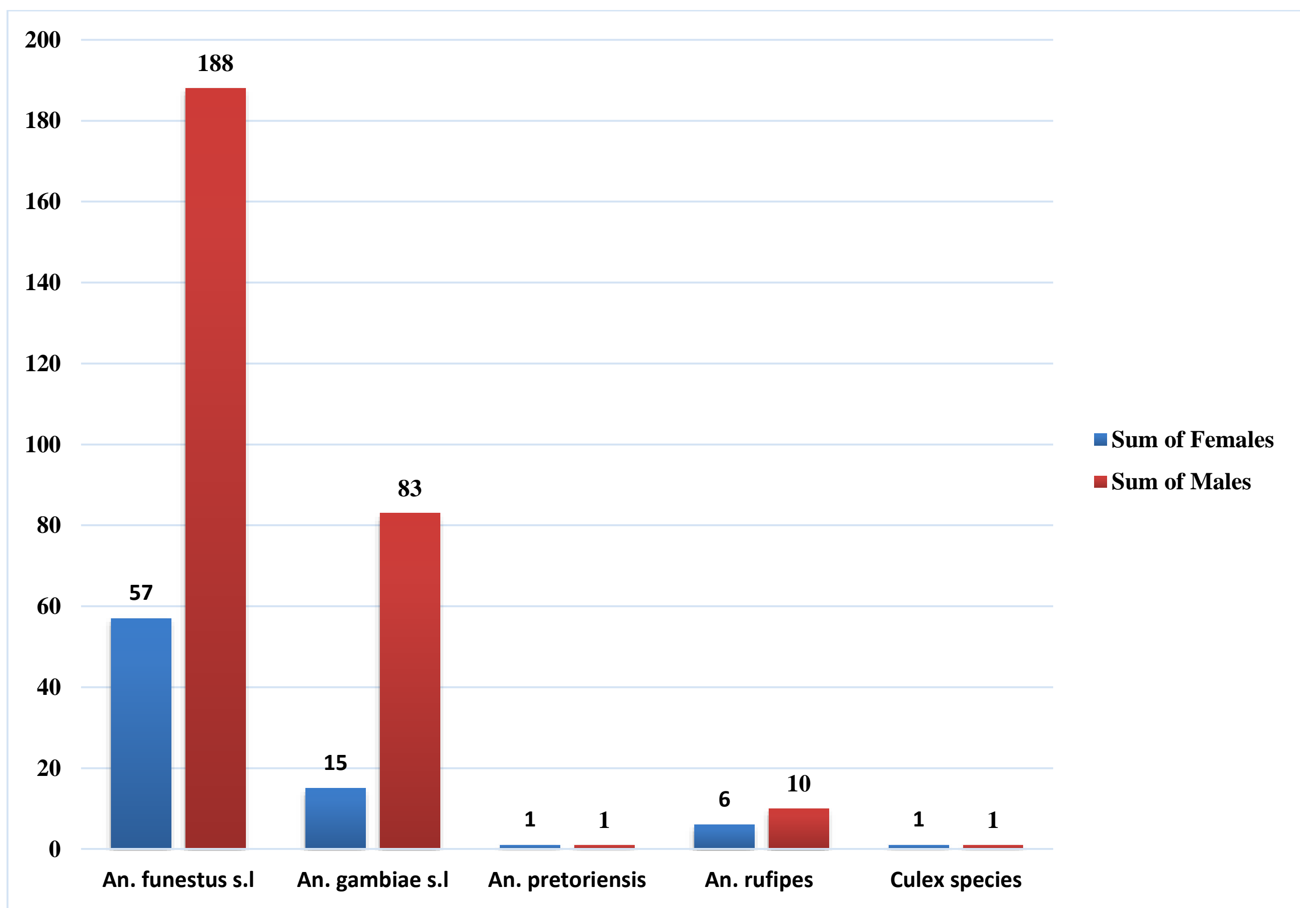


Figure 4.2: Number of mosquito species unexposed to clothianidin (SumiShield® 50WG) insecticide and tobacco extract due to mortalities.

4.3 Species identification

Multiplex Polymerase chain reaction (PCR) was performed on a random subsample (n=202) of wild female *An. funestus* s.l and wild female *An. gambiae* s.l that were exposed to clothianidin (SumiShield® 50WG) and tobacco extract. Molecular analysis confirmed that 40% were *An. gambiae* s.s, the DNA bands of this species was molecularly identified on the gel image with 400

base pair (Figure 4.3) while 14% failed to amplify. About 46% of the samples were confirmed to be *An. funestus* s.s, this was identified with 500 base pair on the gel image (Figure 4.4).

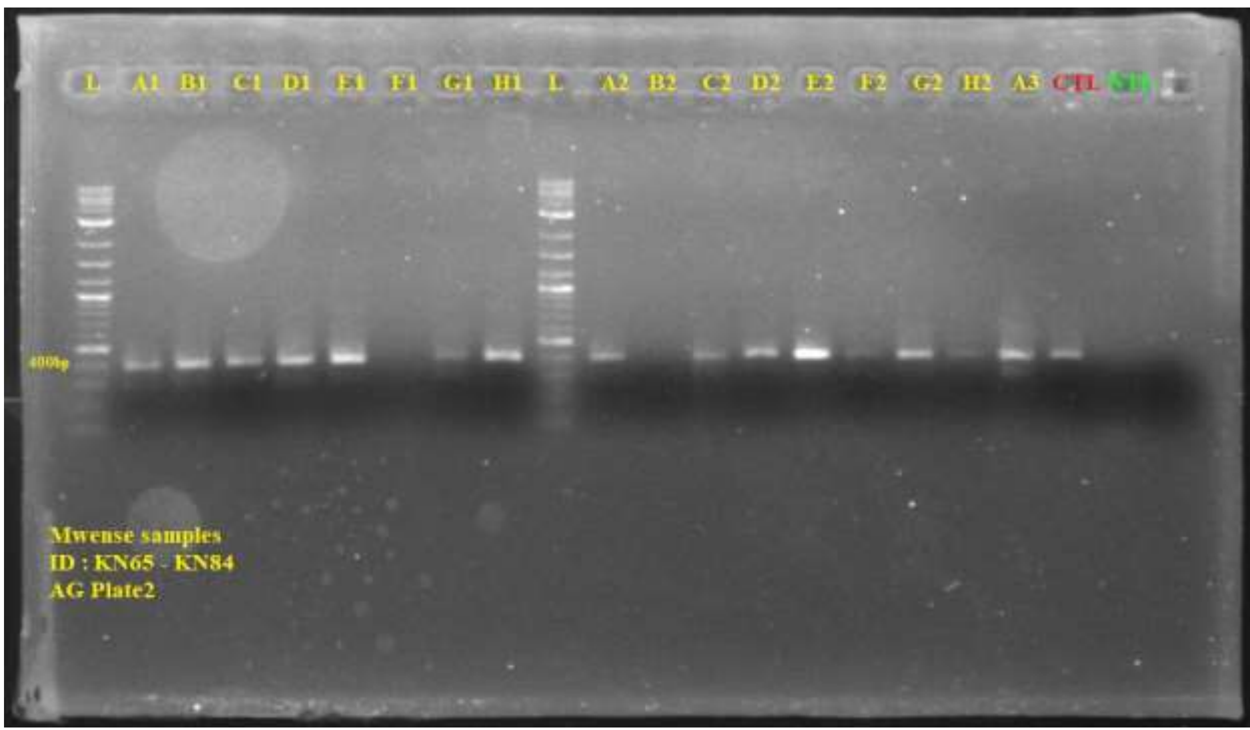


Figure 4.3: Agarose gel of the DNA fragment of amplified PCR of *Anopheles gambiae* complex. Well L represent DNA ladder (100 base pair). Well CTL a positive control, well NTL a negative control, well A1, B1, C1, D1, E1, G1, H1, A2, C2, D2, E2, F2 G2, H2 and A3 represent *Anopheles gambiae* s.s 400 base pair.

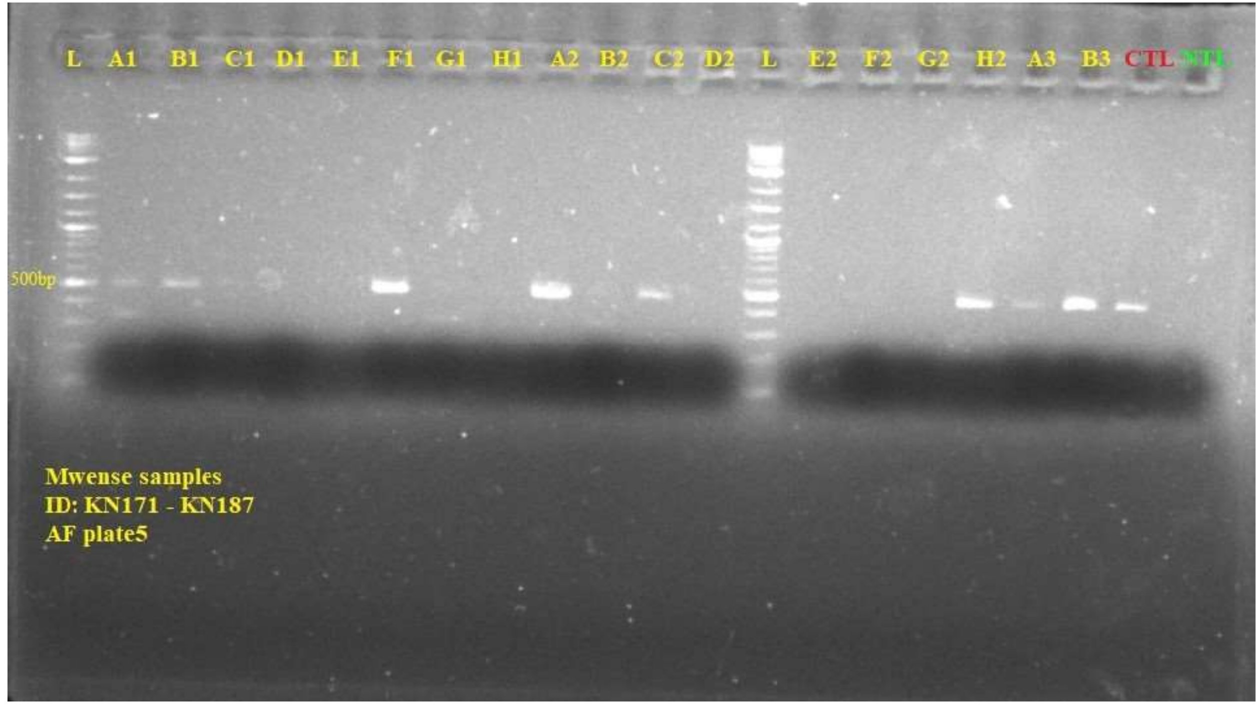


Figure 4.4: Agarose gel of the DNA fragment of amplified PCR of *Anopheles funestus* group. Well L represent DNA ladder (100 base pair). Well CTL a positive control, well NTL a negative control, well A1, B1, F1, A2, C2, H2, A3 and B3 represent *Anopheles funestus* s.s 500 base pair.

4.4 Insecticide susceptibility tests of tobacco extract with wild *Anopheles* mosquitoes

A total of 512 mosquitoes emerged from the larval collection and were exposed to clothianidin and tobacco extract. Three hundred and seventy (370) *Anopheles* mosquitoes were tested with tobacco extract concentrations (25% v/v, 33.3% v/v, 50% v/v, 62.5% v/v, 71.43% v/v and 83.3% v/v) (see appendix 6). Further, 25 adult wild *Anopheles* mosquitoes (3-5 days old) were aspirated and gently blown into a WHO bioassy tube treated with distilled water as a negative control. Probable resistance to 25% v/v, 33.3% v/v, 50% v/v, 62.5% v/v and 71.43% v/v of tobacco extract was observed in all wild *Anopheles* species and the mortality rates after 24 hours exposure time were 66.67%, 73.91%, 81.08%, 70.37% and 75%, respectively (Table 4.1). The number of wild *Anopheles* mosquitoes that were susceptible and resistant to lower percentage tobacco extract concentrations are presented in figure 4.5. No resistance was detected in wild *Anopheles* species at 83.3% v/v of tobacco extract, and the mortality rate was 100% after 24 hours of exposure. In the negative control, there was no mortality detected after 24 hours of exposure.

Table 4.1: Insecticide resistance for lower tobacco extract concentrations on wild *Anopheles* mosquitoes. S susceptible, R resistance, SR suspected resistance.

Site	Insecticide	concentration (% v/v)	Genus	Sample size (N[a])	%Knock down at 10 min	%Knock down at 30 min	%Knock down at 60 min	%Knock down at 80 min	%Mortality	
									24 hours	Status
Chebele	Tobacco extract	25	Wild *Anopheles*	33	24.24	63.64	66.67	75.76	66.67	*R
Chebele	Tobacco extract	33.33	Wild *Anopheles*	46	13.04	56.52	73.91	84.78	73.91	*R
Chebele	Tobacco extract	50.00	Wild *Anopheles*	37	27.03	40.54	48.65	72.97	81.08	*R
Chebele	Tobacco extract	62.50	Wild *Anopheles*	54	9.26	27.78	57.41	79.63	70.37	*R
Chebele	Tobacco extract	71.43	wild *Anopheles*	32	12.50	59.38	90.63	93.75	75.00	*R
Chebele	Tobacco extracy	83.3	Wild *Anopheles*	168	58.33	83.33	99.41	99.41	100	[λ]S
Chebele	Distilled water	0	Wild *Anopheles*	25	0	0	0	0	0	-

* Resistance, λ Susceptible

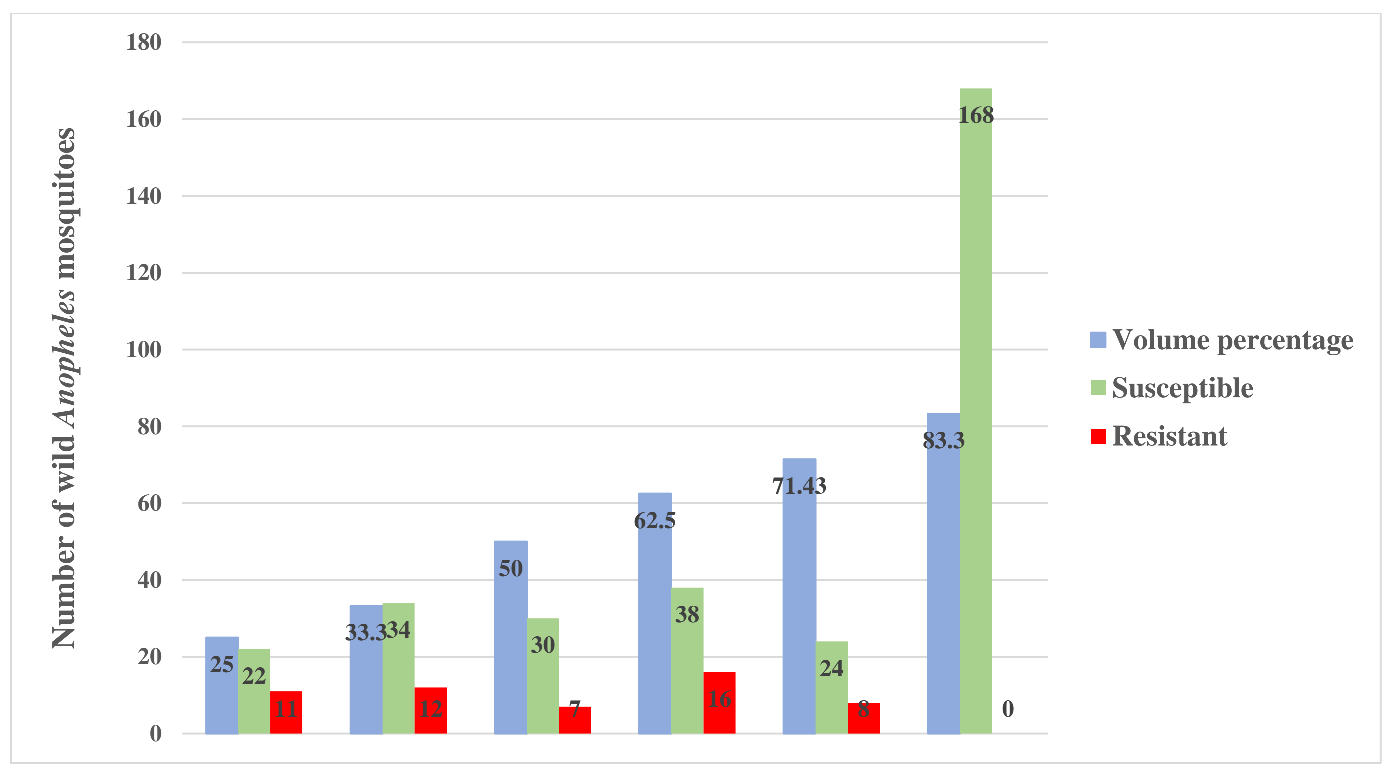


Figure 4.5: Susceptibility insecticide tests of wild *Anopheles* mosquitoes against lower tobacco extract percentage concentrations for 24 hour.

4.5 Efficacy of clothianidin and tobacco extract against malaria vector populations

Hundred percent (100%) mortality was recorded on both wild *Anopheles* mosquitoes (n=142) and Kisumu laboratory reared species (n=116) against clothianidin (0.132g/ml) (Table 4.2). In addition, tobacco extract with the concentration of 83.3%v/v recorded a 100% mortality on wild *Anopheles* mosquitoes (n=168) and Kisumu laboratory reared species (n= 104) after 24-hours post-exposure time (Table 4.2). The mean knock down rate at 80 minutes of wild *Anopheles* mosquitoes exposed to clothianidin (0.132g/ml) and tobacco extract (83.3%) was found to be 61.88% (95% CI: 34.03 – 89.74%) and 85.05% (95% CI: 72.01 – 98.08%) respectively. Twenty-five (25) wild *Anopheles* and Kisumu mosquitoes were put in a separate WHO bioassay tubes as negative control and no mortality or knock downs were recorded. More detailed results for the mean knock down rates are presented in table 4.3.

Table 4.2: Cumulative percentage mortality and knock down percentage of female wild *Anopheles* mosquitoes and Kisumu susceptible strain populations in response to clothianidin (0.132g/ml) and Tobacco extract concentrations (83.3% v/v).

Site/Population	Insecticide	Sample size (N[a])	%Knock down at 10 min	%Knock down at 15 min	%Knock down at 20 min	%Knock down at 30 min	%Knock down at 40 min	%Knock down at 50 min	%Knock down at 60 min	%Knock down at 80 min	%Mortality 24 hours
[b]Chebele Wild *Anopheles*	Clothianidin	142	4.93	26.76	43.66	69.01	78.87	83.1	88.73	100.00	100.00
[b]Chebele Wild *Anopheles*	Tobacco extract	168	58.33	69.64	77.38	83.33	93.45	99.41	99.41	99.41	100.00
[b]Chainama Kisumu Strain	Clothianidin	116	14.66	50.00	64.66	87.93	94.83	96.55	95.69	98.28	100.00
[b]Chainama Kisumu strain	Tobacco extract	104	98.08	98.08	98.08	98.08	98.08	98.08	98.08	98.08	100.00
*Chebele Wild *Anopheles*	Clothianidin	25	0	0	0	0	0	0	0	0	0
*Chainama Kisumu strain	Tobacco extract	25	0	0	0	0	0	0	0	0	0

[a]Total number of mosquitoes exposed to each insecticide.

[b] Source of mosquitoes.

*Negative control

Table 4.3: Clothianidin (SumiShield® 50WG) and tobacco extract knock down effect on wild *Anopheles* mosquitoes.

Group	Insecticide	Sample size	Genus	Knock down Mean (%)	95% Confidence Interval	
					Lower Limit	Upper Limit
Treatment 1	Clothianidin	142	Wild *Anopheles*	61.88	34.03	89.74
Treatment 2	Tobacco extract	168	Wild *Anopheles*	85.05	72.01	98.08
Treatment 3	Clothianidin	116	Kisumu strain	75.33	50.11	100.54
Treatment 4	Tobacco extract	104	Kisumu strain	98.08*	-	-
Negative_Control	Distilled water	25	Kisumu strain	0*	-	-
Negative_Control	Distilled water	25	Wild *Anopheles*	0*	-	-

*Constant mean (%)

4.6 Probit model estimation

The results of the WHO bioassays for wild *Anopheles* mosquitoes tested for tobacco extract susceptibility are summarized in table 4.4. The probit model used in the analysis fitted the data for all assays for the test population of wild *Anopheles* mosquitoes as the Pearson's chi-square test p-value was greater than 0.05. Therefore, the log-dose probit-mortality line of the test population showed a shift to the right as the high values of lethal concentration estimates and an increase in the slope of the test population.

Table 4.4: Summary results of probit analysis

Genus	Wild *Anopheles*
Number	168
Intercept	-1.871
p-value*	0.459
Slope (S.E.)	1.178 (0.489)
X^2 (d.f)	187.3 (186)
LC_{10} (95%CL)	31.65 mg/ml
LC_{20} (95%CL)	74.8 mg/ml
LC_{30} (95%CL)	139.07 mg/ml
LC_{40} (95%CL)	236.24 mg/ml
LC_{50} (95%CL)	387.65 mg/ml

Lethal concentration estimates are presented as percent of active ingredient

S.E, standard error, *d.f*, degree of freedom, 95% CL, 95% confidence limits, X^2, chi-square

* The probit model fitted the data ($p>0.05$)

4.7 Effects of lethal concentrations (LC_{50}) of *N. tabacum* extract on the adult stage of wild *Anopheles* mosquitoes

Figure 4.6 shows the concentration of leaf extract of *N. tabacum* required to knock down 50% of adults of wild *Anopheles* mosquitoes after 40 minutes of exposure. Generally, the concentration of tobacco leaf extract to achieve 50% in knocking down adult wild *Anopheles* mosquitoes was estimated at 388 mg/ml while the lowermost lethal concentration (LC_{10}) was estimated at 31.65 mg/ml. Other lethal concentrations estimates can be seen from appendix 7.

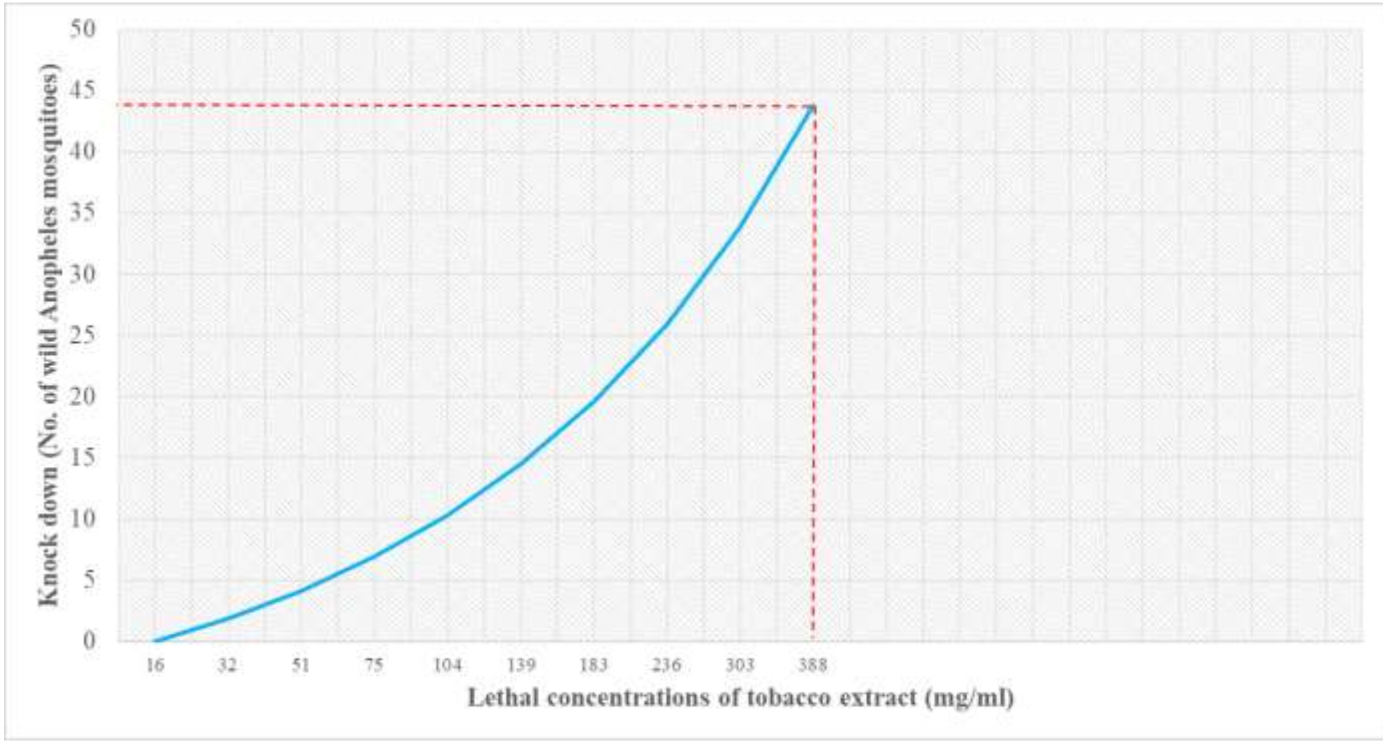


Figure 4.6: Estimated Lethal Concentration (LC_{50}: 388 mg/ml) of tobacco extract at 40 minutes' exposure time.

CHAPTER 5: DISCUSSION

As new bio-insecticides are developed, it is important to find diagnostic concentration in order to decide susceptibility status of malaria vectors and to efficiently monitor insecticide resistance. This study was aimed at determining the insecticidal effectiveness of tobacco extract as an alternative source of a botanical insecticide to control sub-species of *Anopheles gambiae* s.l in Chebele study site of Mwense district of Luapula Province of Zambia. Insecticide resistance has been a great contributor to the resurgence of malaria disease and a threat to sustainable malaria disease control (Chanda *et al.*, 2016). The use of synthetic insecticide in mosquito control has been restricted due to high cost of synthetic insecticides, adverse effect on environmental quality, not biodegradable in nature, deleterious effect on human health and development of resistance (Ahbirami *et al.*, 2014). One of the probable ways to overcome these problems is to use plant derived insecticides as a substitute for synthetic insecticides (Ghosh et al., 2012).

This study revealed that *An. funestus* s.s and *An. gambiae* s.s were the only members of *An. funestus* and *An. gambiae* groups found in Chebele village after screening 202 samples with Polymerase Chain Reaction (PCR). Furthermore, the larval collection results indicated that *An. funestus* s.l was a predominant *Anopheles* species over *An. gambiae* s.l in Chebele village during October-November 2021 dry season. This study findings agree with previous studies that indicated *An. funestus* s.l was the dominant malaria vector over *An. gambiae* s.l with an overall proportion of 87.6% from August 2018 to June 2019 in Mwense district (PMI-VectorLink project, 2019).

According to USAID (2016), Luapula Province is regarded to be confined into an ecological zone called Region III with rainfall amount above 1000mm. Based on this ecological factor, a study which was done in Nchelenge district in Luapula Province by Das *et al* (2016), during dry season reported that *An. funestus* s.s was the species that dominated. In Kasama district (Kalonga sentinel site) and Mwense district (Chebele sentinel site), PMI-AIRS conducted an entomological survey in September-October 2014 dry season and it was reported that an average density of *An. funestus* s.l was higher than that of *An. gambiae s.l* (PMI-AIRS, 2014). In Eastern Democratic Republic of Congo mosquito collections for insecticide susceptibility bioassay tests was collected in November 2015 dry season and confirmed that most mosquitoes tested were *An. funestus* s.l species (Loonen, 2020). With *Anopheles funestus* serving as the primary vector, the Democratic Republic of the Congo (DRC) is known as a holoendemic malaria area (Nardini *et al.*, 2017).

The results obtained in this study demonstrated the knock down effects after exposing wild *Anopheles* mosquitoes to clothianidin (SumiShield® 50WG) and tobacco extract. The mean knock down percentages at 80 minutes exposure time under field conditions for clothianidin (SumiShield® 50WG) and for tobacco extract was found to be different and tobacco extract had better knock down effects. Hundred percent (100%) mortality was also observed at 24 hours post exposure time from clothianidin (SumiShield® 50WG) (0.132g/ml) and tobacco extract (83.3%v/v) against wild *Anopheles* mosquitoes. However, based on the findings of a smilar study in Northern Tanzania, the efficacy of an active ingredient clothianidin with a concentration of 2% exhibited mortality of 80% against wild resistant population of *Anopheles arabiensis* (Kweka *et al*, 2018). Whereas in Benin (West Africa) WHO-Cone bioassay was used to assess the effectiveness of clothianidin against wild *An. gambiae* s.l, 91.7% mortality rate at 120 hours post-exposure time was observed and the mortality rate continued to increase as time was increasing exponentially (Agossa *et al*., 2018). This confirms well the low mortality of wild *Anopheles* mosquitoes against clothianidin (SumiShield® 50WG) with this contemporary research work which supports clothianidin (SumiShield® 50WG) having low mortality on wild *Anopheles* mosquitoes. There is scarce research work documented on toxicity of tobacco extract on adult *An. funestus* s.l and *An. gambiae* s.l but some work has been done on tobacco plant particularly on *Anopheles* mosquito larvae. It is a thought that the results of this extant study will aid virtuous reference material for future researchers. The research work done by the following author is valued: Araka (2018), on the tobacco leaf extract against *An. gambiae* s.s larvae 3rd instar, the author found that the mortality of the larvae upon the use of tobacco extract was 100%.

The results of the probit model explain the relationship between the response variable (Number of knock down wild *Anopheles* mosquitoes) and the predictor variable (Concentration of tobacco extract). A unit change in concentration would cause about 1.178 change in response variable and this result was significant at 5%. Based on this criterion, the LC_{50} was estimated to be 387.65 mg/ml. Ileke *et al.* (2015) reported that higher LC_{50} values of tobacco extract are average and normally the tobacco extract is biodegradable, have low non-target toxicity and eco-friendly.

The intercept was found to be negative and the intercept in this regression is just the mean value of the response variable when the predictor variable in the model is equivalent to zero. The negative intercept revealed that when wild *Anopheles* mosquitoes are not exposed to tobacco extract there could be no wild *Anopheles* mosquito that would be knocked down, holding all else

constant. Intensely, lower concentrations less than 1.6% of tobacco extract can probably cause wild *Anopheles* mosquitoes to develop resistance against tobacco extract.

Nonetheless, different lower concentrations of tobacco extract had knock down effect on the wild female and male *Anopheles* mosquitoes but possible resistance was observed across all lower concentrations (25%, 33%, 50%, 62.5%, and 71.43% v/v). This study has also revealed similar knock down effects on the secondary malaria vectors such as *An. coustani* Lavaran, *An. pretoriensis* Theobald and *An. rufipes* Gough from both insecticides. An experiment done in Indonesia on the larval control of *Aedes aegypti* provided further evidence to show that the tobacco extract is a potential extract to effectively control other mosquito species like the *Aedes aegypti* larvae (Ekapratiwi *et al*., 2019).

Generally, results obtained in this study regarding the slow knock down effect of the clothianidin (SumiShield® 50WG) insecticide against wild *Anopheles* mosquitoes is in an agreement with an experiment that was conducted by Oxborough *et al* (2019), whereby the susceptibility bioassay of insectary reared and wild *Anopheles* mosquitoes results confirmed that clothianidin a chemical ingredient of SumiShiled® 50WG is a relatively slow acting insecticide. The general impression showed that, the insecticidal effects of the tobacco leaf extract on wild *Anopheles* mosquitoes was significant and may be recommended for use as a mosaic insecticide with clothianidin (SumiShiled® 50WG) or with any other conventional Indoor Residual Spraying (IRS) registered insecticide to exterminate the malaria vectors to avoid the development of phenotypic resistance. Further studies are needed to comprehensively understand the ecological succession of malaria vectors in this region. This study used Prokopack aspiration method which was carried out in traditional houses to collect endophilic and endophagic wild *Anopheles* mosquitoes allowing for comparisons of the indoor-resting densities of *Anopheles* mosquitoes in the houses. It is not astonishing that the abundance of *An. funestus s.l* was high in Chebele village. However, this finding permits further studies because in the current study, the abundance of *An. gambiae* s.l was very low, which may have limited the ability to record insecticidal knock down effects of tobacco extract against *An. gambiae* s.l. For future studies, collecting *An. gambiae* s.l larvae in wet season targeting semi-permanent breeding spots for insecticide suscesptibility tests is highly recommended.

Hence, there is a need for further studies to determine the insecticide susceptibility status and morphological identification for primary and secondary malaria vectors in the study area to guide

the technical advisory committee of insecticide resistance management for National Malaria Elimination Programme (NMEP) in Zambia.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The results of this study's insecticide susceptibility tests demonstrates that leaf extract from *N. tabacum* led to considerable knock down effect in adult population of wild *Anopheles* mosquitoes (*An. gambiae* s.l, *An. funestus* s.l, *An. rufipes* Gough, and *An. pretoriensis* Theobald). However, the knock down effect for clothianidin and tobacco extract was different, and tobacco extract was found to have a better knock down effects on wild adult *Anopheles* mosquitoes. Therefore, *N. tabacum* leaf extract may be considered as a potential bio-insecticide for the control of *An. gambiae* s.s and *An. funestus* s.s, significant vectors of malaria pathogen.

6.2 Recommendations

From this study, it is recommended that;

1. Future investigation on the tobacco extract to be carried out to examine the effects of optimal diagnostic concentration and lower diagnostic concentrations to the secondary wild *Anopheles* species.
2. Tobacco extract's efficacy needs to be evaluated over long period of time.
3. National Malaria Elimination Program (NMEP) under Ministry of Health may consider tobacco extract to be a potential alternative bioinsecticide under Insecticide Resistance Management Plan.

6.3 Study limitations

This study had some limitations. Sampling of wild *Anopheles gambiae* sensu lato larvae was done during dry the season (October-November 2021 period) and this affected the sample size of *An. gambiae* s.l used for the determination of insecticide susceptibility status using WHO bioassays. This provided information on limited scale to infer the knock down effect and mortality due to high number of *An. funestus* s.l mosquitoes from the breeding sites in Chebele village. More studies with adequate sample size of *An. gambiae* s.l from Chebele village are needed to better understand the insecticide susceptiblity status on *An. gambiae* s.l when exposed to tobacco extract and clothianidin (SumiShiled® 50WG) insecticides.

Moreover, insufficient resources (lack of primers) to analyse all specimens to confirm species identities because not all samples that were exposed to the insecticides underwent Polymerase Chain Reaction (PCR) to determine the species of the *An. gambiae* s.l and *An. funestus* s.l. Though most appeared to be *An. funestus* s.s and *An. gambiae* s.s, few samples did not amplify and it can not be confidently concluded that all *funestus* group were *An. funestus* s.s and *gambiae* group were *An. gambiae* s.s. Also, due to limited resources few households were sampled for adult wild *Anopheles* mosquitoes with Prokopack aspiration method.

References

Adam D.C, Bui CM, Heywood A.E, Kunasekaran M, Sheikh M, Narasimhan P and Maclntyre, C.R (2018) Adherence to anti-vectorial prevention measures among travellers with chikungunya and malaria returning to Australia: comparative epidemiology. BMC Res Notes. 2018;11:590.

Afifah, F., Rahayu, Y.S and Faizah, U., (2015) Efektivitas Kombinasi Filtrat Daun Tembakau (Nicotiana tabacum) dan Filtrat Daun Paitan (Thitonia diversifolia) sebagai Pestisida Nabati Hama Walang Sangit (Leptocorisa oratorius) pada Tanaman Padi (Effectiveness of Combination of Tobacco (Nicotiana tabacum) Leaves Filtrate and Paitan (Tithonia diversifolia) Leaves Filtrate as Botanical Pesticides of Walang Sangit (Leptocorisa oratorius) on Rice Plant). *Lentera Bio*, Volume 4(1), pp. 25–31.

Agossa, F.R., Padonou, G.G., Koukpo, C.Z., Zola-Sahossi, J., Azondekon, R., Akuoko, O.K., Ahoga, J., N'dombidje, B., Akinro, B., Fassinou, A.J.Y.H., Sezonlin, M and Akogbeto, M.C (2018) Efficacy of a novel mode of action of an Indoor Residual Spraying product, SumiShield® 50WG against susceptible and resistance populations of Anopheles gambiae (s.l) in Benin, West Africa, *Parasites & vectors* (2018)11:293.

Aguayo, G. S. (2021) Botanical insecticides [Online] [Available:https://ipmworld.umn.edu/silva-aguayo-botanical] [Accessed: 28th March, 2021]

Akeju, A.V., Olusi, T.A., Adepeju, I and Oke, S. (2022) Molecular identification and wing variations among malaria vectors in Akure North Local Government, *Scientific Reports*, pp. 1–8. Available at: https://doi.org/10.1038/s41598-022-11917-y.

Al-Ghamdi, A and Baljoon (2014) Natural products bioc-342 lab manual[Online] [Available:http://www.kau.edu.sa/search.aspx?Site_ID=0&lng=EN&qr=extraction%20of%20nicotine%20from%20cigaretts] [Accessed: 28th February, 2021].

Almeda, J. V., Capistrano, T. G., Sarte G. M. F (2010) Elementary Statistics, Quezon City:
UP Press

Ahbirami, R., Zuharah, W.F., Thiagaletchumi, M., Subramaniam, S and Sundarasekar, J (2014) Larvicidal Efficacy of Different Plant Parts of Railway Creeper , Ipomoea cairica Extract Against Dengue Vector Mosquitoes , Aedes albopictus (Diptera : Culicidae) and Aedes aegypti (Diptera : Culicidae), *Journal of Insect Science*,doi: 10.1093/jisesa/ieu042.

Anhalt, J.C., Moorman, T.B and Koskinen, W.C (2007) Biodegradation of imidacloprid by an isolated soil microorganism, *Journal of Environmental Science and Health*, Part B, vol. 42, no. 5, pp. 509–514

Araka, G.O (2018) Toxicology efficacy of flower extracts from *Chrysanthemum cinerariifoluim* and leaf extracts from *Eucalyptus camaldulensis* and *Nicotiana tabaccum* on *Anopheles gambiae* s.s Giles mosquito larvae [Online] [Available:http://ir.jkuat.ac.ke/bitstream/handle/123456789/4799/Araka%2C%20Glenn%20Onsomu%2C%20Phd%20Public%20Health%2C%202018.pdf?sequence=1&isAllowed=y

Arsianti, A., Kurniawan, G., Tejaputri, N.A., Qorina, F., Fithrotunnisa, Q., Azizah, N and Fajrin, A.M (2020) Phytochemical Profile , Antioxidant Activity and Cell Line Study of Marine Red Macroalgae Eucheuma cottonii on Lung A-549 Cancer Cells, *Pharmacog J,* 12(2), pp. 276–281.

Aronson, A. I., Beckman, W., and Dunn, P. (1986). Bacillus thuringiensis and related insect pathogens. *Microbiological Reviews*, *50*(1), 1–24. https://doi.org/10.1128/mmbr.50.1.1-24.1986

Asadollahi, A., Khoobdel, M., Ramazani, A.Z., Azarmi, S and Mosawi, S.H (2019) Effectiveness of plant - based repellents against different Anopheles species : a systematic review, *Malaria Journal*. BioMed Central, pp. 1–20. doi: 10.1186/s12936-019-3064-8.

Atieli, H., Menya, D., Githeko, A., and Scott, T. (2009). House design modifications reduce indoor resting malaria vector densities in rice irrigation scheme area in western Kenya. *Malaria Journal*, *8*(1), 1–9. https://doi.org/10.1186/1475-2875-8-108

Baranitharan, M., Tamizhazhagan, V and Kovendan, K (2019) Medicinal Plants as Potent Power for Malaria Control : Review, *Entomology and Applied Sciences Letters,* 5(1), pp. 28–44.

Bassey, B. E., Opara, K.N and Usip, L.P (2020) Morphological and molecular characterization of endophilic Anopheles gambiae complex , in Itu Local Government Area , Akwa Ibom State , Nigeria, pp. 75–81.

Besansky N.J., Collins F.H and Townson, H (2006) A species-specific PCR for the identification of the malaria vector Anopheles bwambae. *Ann Trop Med Parasitol* 100:277-280

Brieger, B (2013) Malaria vector bionomics during the dry season in Nchelenge district, Zambia [Online][Available:http://malariamatters.org/malaria-vector-bionomics-during-the-dry-season-in-nchelenge-district-zambia/[Accessed:1st July, 2021]

Bullangpoti, V. (2014) Botanical Insecticides in Thailand : Hopes and Hindrances Botanical Insecticides in Thailand : Hopes and Hindrances, *Biopestic. Int.* 5(2): 75-99.

Cedar Lake Ventures, Inc (2023) Climate and average weather year round in Mwense, Zambia [Online] [Available: https://weatherspark.com/y/95302/Average-Weather-in-Mwense-Zambia-Year-Round][Accessed: 22nd January, 2023]

Centers for Disease Control and Prevention (2022) Life cycle of *Anopheles* species mosquitoes [Online][Available:https://www.cdc.gov/mosquitoes/about/life-cycles/anopheles.html][Accessed:2nd Febraury, 2023]

Centers for Disease Control and Prevention (2021) Nicotine: Systemic agent [Online] [Available:https://www.cdc.gov/niosh/ershdb/emergencyresponsecard_29750028.html] [Accessed: 14th September, 2021].

Chanda, E., Kandyata, A., Chanda, J., Phiri, F.N., Muzia, L and Kamuliwo, M (2013) The Efficacy of Vectron 20 WP , Etofenprox , for Indoor Residual Spraying in Areas of High Vector Resistance to Pyrethroids and Organochlorines in Zambia, *ISRN Preventive Medicine*, Hindwawi publishing corporation,pp.1-6.

Chanda, E., Thomsen, E.K., Musapa, M., Kamuliwo, M., Brogdon, W.G., Norris, D.E., Masaninga, F., Wirtz, R., Sikaala, C.H., Muleba, M., Craig, A., Govere, J.M., Ranson, H., Hemingway, J., Seyoum, A., Macdonald, M.B and Coleman, M (2016) An Operational Framework for Insecticide Resistance Management Planning, *Emerging Infectious Diseases,* 22(5), pp. 773–779. Doi:http://dx.doi.org/10.3201/eid2205.150984

Chanda, J., Saili, K., Phiri, F., Stevenson, J.C., Mwenda, M., Chishimba, S., Muleba, C., Mambwe, B., Lungu, C., Earle, D., Bennett, A., Eisele, T.P., Kamuliwo, M., Steketee, R.W., Keating, J., Miller, J.M and Sikaala, C (2020) Pyrethroid and Carbamate Resistance in Anopheles funestus Giles along Lake Kariba in Southern Zambia, *American Society of Tropical Medicine and Hygiene*, pp. 90–97. doi: 10.4269/ajtmh.19-0664.

Chandramohan. B., Murugan, K., Madhiyazhagan, P., Kovendan, K., Kumar, P.M., Panneerselvam, C., Dinesh,D., Subramanlam, J., Rajaganesh, R., Nicoletti, M., Canale, A. and Benelli, G. (2015) 'Neem by-products in the fight against mosquito- borne diseases: Biotoxicity of neem cake fractions towards the rural malaria vector Anopheles culicifacies (Diptera: Culicidae)', *Asian Pacific Journal of Tropical Biomedicine*. Elsevier B.V., 6(6), pp. 472–476. doi: 10.1016/j.apjtb.2015.11.013.

Choi, K. S., Christian, R., Nardin, L., Wood, O.R., Agubuzo, E., Muleba, M., Munyati, S., Makuwaza, A., Koekemoer, L.L., Brooke, B.D., Hunt, R.H and Coetzee, M (2014) Insecticide resistance and role in malaria transmission of *Anopheles funestus* populations from Zambia and Zimbabwe, *Parasites & Vectors*,BioMed Central, pp. 1–8.

Chowhury, A., Pradhan, S., Sanyal, N and Saha, M (2008) Impact of pesticides on soil microbiological parameters and possible bioremediation strategies, *Indian Journal microbiology*, 48:114-127.

Clemens, K.J., Caillé, S., Stinus, L and Cador, M (2009) The addition of five minor tobacco alkaloids increases nicotine-induced hyperactivity, sensitization and intravenous self-administration in rats, *International Journal of Neuropsychopharmacology*, pp. 1355–1366. doi: 10.1017/S1461145709000273.

Coetzee, M. (2020) Key to the females of Afrotropical Anopheles mosquitoes (Diptera : Culicidae), *Malaria Journal*. BioMed Central, pp. 1–20. doi: 10.1186/s12936-020-3144-9.

Dagg, K., Irish, S., Wiegand, R. E., Shililu, J., Yewhalaw, D., and Messenger, L. A. (2019). Evaluation of toxicity of clothianidin (neonicotinoid) and chlorfenapyr (pyrrole) insecticides and cross-resistance to other public health insecticides in Anopheles arabiensis from Ethiopia. *Malaria Journal*, *18*(1), 1–11. https://doi.org/10.1186/s12936-019-2685-2

Das, S., Muleba, M., Stevenson, J.C and Norris, D.E (2016) Habitat Partitioning of Malaria Vectors in Nchelenge District , Zambia, *American Society of Tropical Medicine and Hygiene,* 94(6), pp. 1234–1244. doi: 10.4269/ajtmh.15-0735.

Dambach, P., Baernighausen, T., Traoré, I., Ouedraogo, S., Sié, A., Sauerborn, R., Becker, N., and Louis, V. R. (2019). Reduction of malaria vector mosquitoes in a large-scale intervention trial in rural Burkina Faso using Bti based larval source management. *Malaria Journal*, *18*(1), 1–9. https://doi.org/10.1186/s12936-019-2951-3

Deletre, E., Martin, T., Dumênil, C and Chandre, F (2019) 'Insecticide resistance modifies mosquito response to DEET and natural repellents', *Parasites & Vectors*. BioMed Central, pp. 1–10. doi: 10.1186/s13071-019-3343-9.

Denlinger, D.L and Armbruster, P.A (2014) Mosquito diapause. Annu Rev Entomol.,doi: 10.1146/annurev-ento-011613-162023. Epub 2013 Oct 18. PMID: 24160427.

Dieng, H., Rajasaygar, S., Ahmad, A. H., Md. Rawi, C. S., Ahmad, H., Satho, T., Miake, F., Zuharah, W. F., Fukumitsu, Y., Saad, A. R., Abdul Hamid, S., Vargas, R. E. M., Ab Majid, A. H., Fadzly, N., Abu Kassim, N. F., Hashim, N. A., Ghani, I. A., Abang, F. B., & AbuBakar, S. (2014) Indirect effects of cigarette butt waste on the dengue vector *Aedes aegypti* (Diptera: Culicidae). *Acta Tropica*, *130*, 123–130. https://doi.org/https://doi.org/10.1016/j.actatropica.2013.11.001

Donald, A. N. (2020) Extraction and Estimation of the Amount of Nicotine in a Tobacco Leaf, International Journal of Creative Research Thoughts, 8(8), pp. 3229–3237.

Elamathi N, Verma V, Sharma P, Uragayala S and Kamaraju R. (2014) Neonicotinoids in vector control: in silico approach. *Asian J Biomed Pharm Sci*.

Ekapratiwi, Y., Rachmadiva., Virgine, K.A., Fauzantoro, A., Gozan, M and Jufri, M (2019) The effect of tobacco extracts based biolarvicide emulsion formulation against Aedes aegypti larvae [Online] [Available: https://aip.scitation.org/doi/abs/10.1063/1.5096713][Accessed:27th March, 2022]

Febrianto, A., Wijana, S. and Wahyudi, A. S. (2020) 'Optimization of Nicotine Extraction In Tobacco Leaf (Nicotiana tabacum L .) : (Study : Comparison of Ether and Petroleum Ether) The International Conference on Chemical Engineering UNPAR 2013 Optimization of Nicotine Extraction In Tobacco Leaf (Nicotiana tabacum L .) :(Study : Comparison of Ether and Petroleum Ether)', (May 2014). doi: 10.13140/RG.2.1.2290.7926.

Fettene M. and Temu, E.A (2003) Species-specific primer for identification of *Anopheles quadriannulatus* sp. B (Diptera: Culicidae) from Ethiopia using a multiplex polymerase chain reaction assay. *J. Med. Entomol*. 40:112-115

Fongnikin, A., Houeto, N., Agbevo, A., Odjo, A., Syme, T., N'Guessan, R., and Ngufor, C. (2020). Efficacy of Fludora® Fusion (a mixture of deltamethrin and clothianidin) for indoor residual spraying against pyrethroid-resistant malaria vectors: Laboratory and experimental hut evaluation. *Parasites and Vectors*, *13*(1), 1–11. https://doi.org/10.1186/s13071-020-04341-6

Food and Agriculture Organization (2023) Luapula Province[Online][Available: https://www.fao.org/3/AD011E/AD011E06.htm][Accessed: 18th January, 2023]

Fornadel, C. M., Norris, L., Glass, E.G and Norris, E.D (2010) Analysis of Anopheles arabiensis Blood Feeding Behavior in Southern Zambia during the Two Years after Introduction of Insecticide-Treated Bed Nets Analysis of Anopheles arabiensis Blood Feeding Behavior in Southern Zambia during the Two Years after Introduction of Insecticide-Treated Bed Nets, *American Journal Tropical Medicine Hygiene,* doi: 10.4269/ajtmh.2010.10-0242.

Fornadel, C. M., Norris, C.L., Franco, V and Norris, D.E (2011) Unexpected Anthropophily in the Potential Secondary malaria vectors *Anopheles coustani* s.l. and *Anopheles squamosus* in Macha, Zambia, 11(8). doi: 10.1089/vbz.2010.0082, *Vector-Borne and Zoonotic Diseases*, Doi:10.1089/vbz.2010.0082

Fouque, F. and Reeder, J. C. (2019) Impact of past and on-going changes on climate and weather on vector-borne diseases transmission : a look at the evidence, *Infectious Diseases of Poverty*, 1, pp. 1–9.

Frérot, M. *et al.* (2018) 'What is epidemiology? Changing definitions of epidemiology 1978-2017', *PLoS ONE*, 13(12). Available at: https://doi.org/10.1371/journal.pone.0208442.

Fuseini G, Nguema R.N., Phiri W.P., Donfack O.T., Cortes C, Von Fricken M.E., Meyers, J.I., Kleinschmidt., I., Garcia, G.A., Mass, M., Schwabe, C and Slotman, M.A (2019) Increased biting rate of insecticide-resistant Culex mosquitoes and community adherence to IRS for malaria control in Urban Malabo, Bioko Island. Equatorial Guinea. *J Med Entomol.* 2019;56:1071–7.

Ghosh, A., Chowdhury, N. and Chandra, G. (2012) 'Plant extracts as potential mosquito larvicides', (May), pp. 581–598.

Hemingway J, Hawkes NJ, McCarroll L and Ranson H (2004) The molecular basis of insecticide resistance in mosquitoes. Insect Biochem Mol Biol. 2004, 34: 653-665. 10.1016/j.ibmb.2004.03.018.

Hemming-Schroeder, E., Zhong, D., Machani, M., Nguyen, H., Thong, S., Kahindi, S., Mbogo, C., Atieli, H., Githeko, A., Lehmann, T., Kazura, J. W., and Yan, G. (2020). Ecological drivers of genetic connectivity for African malaria vectors Anopheles gambiae and An. arabiensis. *Scientific Reports*, *10*(1), 1–12. https://doi.org/10.1038/s41598-020-76248-2

Hau, J and Zeng, Z (2014) Feellife's research survey on nicotine[Online][Available: http://www.feellife,com] [Accessed: 17th September, 2021].

Id, J.L.S. *et al.* (2021) 'Malaria risk factors in northern Namibia : The importance of occupation , age and mobility in characterizing high-risk populations', pp. 1–23. Available at: https://doi.org/10.1371/journal.pone.0252690.

Ileke, K. D., Oyeniyi, E.A., Ogungbite, O.C and Adesina, J.M (2015) Nicotiana tabacum a prospective mosquitocide in the management of *Anopheles gambiae* (Giles), *International Journal of Mosquito Research*, 2(4), pp. 19–23.

Isman M.B. (2004) Botanical Insecticides. In: Encyclopedia of Entomology. Springer, Dordrecht. https://doi.org/10.1007/0-306-48380-7_573

Isman, M.B. Botanical insecticides, deterrents and repellents in modern agriculture and an increasingly regulated world. Annu. Rev. Entomol. 2006, 51, 45–66.

Jones, C. M., Ciubotariu, I. I., Muleba, M., Lupiya, J., Mbewe, D., Simubali, L., Mudenda, T., Gebhardt, M. E., Carpi, G., Malcolm, A. N., Kosinski, K. J., Romero-Weaver, A. L., Stevenson, J. C., Lee, Y., and Norris, D. E. (2021). Multiple Novel Clades of Anopheline Mosquitoes Caught Outdoors in Northern Zambia. Frontiers in Tropical Diseases, 2(December), 1–14. https://doi.org/10.3389/fitd.2021.780664

Kabbale, F. G., Akol, A.M., Kaddu, J.B., Matovu, E., Kazibwe, A., Yadouleton, A and Onapa, W (2016) Molecular identification of *Anopheles gambiae* sensu stricto Giles (formerly *Anopheles gambiae* Savannah Form) in Kamuli District , Uganda. *African Journal of Biotechnology*, 15, 2124–2131.

Kabbale, F. G., Akol, A.M., Kaddu, J.B and Onapa, A.W (2013) Biting patterns and seasonality of anopheles gambiae sensu lato and anopheles funestus mosquitoes in Kamuli District, Uganda,, *BioMed Central,* pp. 1–9.

Kaiser, M.L., Duncan, F.D. and Brooke, B.D. (2014) 'Embryonic Development and Rates of Metabolic Activity in Early and Late Hatching Eggs of the Major Malaria Vector *Anopheles gambiae'*, pp. 1–15. Available at: https://doi.org/10.1371/journal.pone.0114381.

Kamuliwo M, Chanda E, Haque U, Mwanza-Ingwe M, Sikaala C, Katebe-Sakala C, Sikaala, C., Mukonka, V.M., Norris, D.E., Smith, D.L., Glass, G.E and Moss, W.J (2013) The changing burden of malaria and association with vector control interventions in Zambia using district-level surveillance data, 2006–2011. Malar J. 2013;12:437.

Kandyata, A. (2012) 'Impacts of Bacillus thuringiensis var. israelensis and Bacillus sphaericus insect larvicides on mosquito larval densities in selected areas of Lusaka urban district, Zambia', *Medical Journal of Zambia*, 39(4).

Kheawfu, K., Kaewpinta, A., Chanmahasathien, W., Rachtanapun, P and Jantrawut, P.(2021) Extraction of Nicotine from Tobacco Leaves and Development of Fast Dissolving Nicotine Extract Film. *Membranes*,11, 403. https:// doi.org/10.3390/membranes11060403

Killeen, G. F., Govella, N. J., Lwetoijera, D. W. and Okumu, F. O. (2016). Most outdoor malaria transmission by behaviourally-resistant Anopheles arabiensis is mediated by mosquitoes that have previously been inside houses. *Malaria Journal*, *15*(1), 1–10. https://doi.org/10.1186/s12936-016-1280-z

Kirby, M. J., Ameh, D., Green, C., Jawara, M., Milligan, P. J., Snell, P. C., Conway, D. J., and Lindsay, S. W. (2009). Efficacy of two different house screening interventions against exposure to malaria and anaemia in children in The Gambia: a randomized controlled trial. *Lancet*, *374*(9694), 998–1009. https://doi.org/10.1016/S0140-6736(09)60871-0.Efficacy

Koekemoer, L. L., Kamau, L. and Coetzee, M. (2002) Cocktail polymerase chain reaction assay to identify members of the Anopheles funestus (Diptera : Culicidae) group a cocktail Polymerase Chain Reaction Assay to idenify members, doi: 10.4269/ajtmh.2002.66.804.

Kroeger A, Skovmand O, Phan QC and Boewono DT (20040 Combined field and laboratory evaluation of a long-term impregnated bednet, PermaNet. Trans R Soc Trop Med Hyg. 2004, 98: 152-155. 10.1016/S0035-9203(03)00038-5.

Kumari and Thangavel. (2016) Microbial reduction of nicotine and effect on oral flora, *European Journal of Pharmaceutical and Medical Research,* 3(4), pp. 315–321.

Kweka, E., Mahande, A., Ouma, J., Karanja, W., Msangi, S., Temba, V., Lyaruu, L and Himeidan, Y (2018) Novel Indoor Residual Spray insecticide with extended mortality effect: A case of SumiShield 50WG against wild resistance populations of *Anopheles arabiensis* in Northern Tanzania, *Global Health: Science and practice journal*.

Lobo, N.F., Sikaala, L.B., Hamainza, B., Chanda, J., Chinula, D., Kishnankutty, S., Mueller, J., Deason, N., Hoang, Q., Boldt, H., Thumloup, J., Stevenson, J., Seyoum, A and Collins, F (2015) Unexpected diversity of *Anopheles* species in Eastern Zambia : implications for evaluating vector behavior and interventions using molecular tools, *Nature Publishing Group*, (July), pp. 1–10. Available at: https://doi.org/10.1038/srep17952.

Loonen, J.A.C., Dery, D.B., Musaka, B.Z., Bandibabone, J.B., Bousema, T., Lenthe, M.V., Stefanija, B.P., Fesselet, J.F and Koenraadt, C.J.M (2020) Identification of main malaria vectors and their insecticide resistance profile in internally displaced and indigenous communities in Eastern Democratic Republic of the Congo (DRC), *Malaria journal*, 19,425(2020).

Lukwa, N., Sande, S., Makuwaza, A., Chiwade, T., Nesta, M., Asamoa, K., Vazqueza,-Prolopec, G., Reithinger, R and Williams, J. (2014) Nationwide assessment of insecticide susceptibility in *Anopheles* gambiae populations from Zimbabwe, *Malaria Journal,* pp. 1–9.

Mburu, M. M., Juurlink, M., Spitzen, J., Moraga, P., Hiscox, A., Mzilahowa, T., Takken, W., & McCann, R. S. (2018). Impact of partially and fully closed eaves on house entry rates by mosquitoes. *Parasites and Vectors*, *11*(1), 1–9. https://doi.org/10.1186/s13071-018-2977-3

McMurry, J.E & Fay, R.C (2012) Chemistry, sixth edition (Pearson Education, Inc.

Manyando, C., Njunju, E.M., Chileshe, J., Siziya, S., Shiff, C. (2014). Rapid diagnostic tests for malaria and health workers' adherence to test results at health facilities in Zambia. BioMed Central Ltd.

Malaria atlas project (2021) About mosquito bionomics [Online] [Available: https://malariaatlas.org/about-bionomics/ [Accessed: 20th August, 2021]

Matthews, J., Bethel, A. and Osei, G. (2020) An overview of malarial Anopheles mosquito survival estimates in relation to methodology. *Parasites and Vectors*, *13*(1), 1–12. https://doi.org/10.1186/s13071-020-04092-4

Manahan, E.S (2003) Toxicological chemistry and biochemistry. 3rd Ed. New York Washington D.C: Lewis publisher.

Mahendra, S (2012) Characterization of nicotine sulfate from tobacco as bio-pesticide, *Journal of environmental monitoring* ,14, 2317-2326.

Mewara, A., Sharma, M., Kaura, T., Zaman, K., Yadav, R., and Sehgal, R. (2018). Rapid identification of medically important mosquitoes by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Parasites and Vectors, 11(1), 1–9. https://doi.org/10.1186/s13071-018-2854-

Mashauri, F. M., Manjurano, A., Kinung'hi, S., Martine, J., Lyimo, E., Kishamawe, C., Ndege, C., Ramsan, M. M., Chan, A., Mwalimu, C. D., Changalucha, J., & Magesa, S. (2017). Indoor residual spraying with microencapsulated pirimiphos-methyl (Actellic® 300CS) against malaria vectors in the Lake Victoria basin, Tanzania. PLoS ONE, 12(5), 1–21. https://doi.org/10.1371/journal.pone.0176982

Massey, N. C., Garrod, G., Wiebe, A., Henry, A.J., Huang, Z., Moyes, C.L and Sinka, M.E (2016) A global bionomic database for the dominant vectors of human malaria, *Scientific Data,* pp. 1–13, Doi:10.1038/sdata.2006.14.

Matiya, D. J., Philbert, A.B., Kidima, W and Matowo, J (2019) Dynamics and monitoring of insecticide resistance in malaria vectors across mainland Tanzania from 1997 to 2017 : a systematic review, *Malaria Journal*. BioMed Central, pp. 1–16. doi:10.1186/s12936-019-2738-6

MESA (2022) Larval Source Management [Online][Available: https://mesamalaria.org/mesa-track/deep-dives/larval-source-management#:~:text=There%20are%20four%20types%20of,%2C%20larviciding%2C%20and%20biological%20control.

Minakawa, N., Sonye, G., Mogi, M., Githeko and Yan, G (2002) The Effects of Climatic Factors on the Distribution and Abundance of Malaria Vectors in Kenya, pp. 833–841.

Ministry of Health, Central Statistics Office, PATH-MACEPA, USAID, CDC, UNCF, W. (2018) Malaria Indicator Survey [Online] Available: https://www.path.org/resources/zambia-natl-malaria-indicator-survey-mis-2018/ [Accessed: 26th February, 2021].

Mosha, J. F., Lukole, E., Charlwood, J. D., Wright, A., Rowland, M., Bullock, O., Manjurano, A., Kisinza, W., Mosha, F. W., Kleinschmidt, I., & Protopopoff, N. (2020). Risk factors for malaria infection prevalence and household vector density between mass distribution campaigns of long-lasting insecticidal nets in North-western Tanzania. Malaria Journal, 19(1), 1–11. https://doi.org/10.1186/s12936-020-03369-4

Motta, Stefania & Monti, Marcello. (2015). Insect Repellents [Online] [Available: https://www.researchgate.net/publication/283526547_Insect_Repellents][Accessed:15th September, 2021]

Mpofu, M., Becker, P., Mudambo, K., and De Jager, C. (2016). Field effectiveness of microbial larvicides on mosquito larvae in malaria areas of Botswana and Zimbabwe. *Malaria Journal*, *15*(1), 1–8. https://doi.org/10.1186/s12936-016-1642-6

Nawa, M., Hangoma, P., Morse, A. P., and Michelo, C. (2019). Investigating the upsurge of malaria prevalence in Zambia between 2010 and 2015: A decomposition of determinants. *Malaria Journal*, 18(1), 1–10. https://doi.org/10.1186/s12936-019-2698-x

Nardini, L., Hunt, R., Dahan-Moss, Y., Christie, N., Christian, R and Coetzee, M (2017) Malaria vectors in the Democratic Republic of the Congo : the mechanisms that confer insecticide resistance in *Anopheles* gambiae and *Anopheles* funestus, *Malaria Journal*, pp. 1–15. Available at: https://doi.org/10.1186/s12936-017-2099-y

Ng'Ang'A, P. N., Okoyo, C., Mbogo, C., and Mutero, C. M. (2020). Evaluating effectiveness of screening house eaves as a potential intervention for reducing indoor vector densities and malaria prevalence in Nyabondo, western Kenya. *Malaria Journal*, *19*(1), 1–12. https://doi.org/10.1186/s12936-020-03413-3

Ngufor, C., Fongnikin, A., Rowland, M., and Guessan, R. N. (2017). Indoor residual spraying with a mixture of clothianidin (a neonicotinoid insecticide) and deltamethrin provides improved control and long residual activity against pyrethroid resistant *Anopheles gambiae* sl in Southern. 1–14.

Ngwej, L. M., Hattingh, I., Mlambo, G., Mashat, E. M., Kashala, J. C. K., Malonga, F. K., and Bangs, M. J. (2019). Indoor residual spray bio - efficacy and residual activity of a clothianidin - based long persistence on various wall surfaces for malaria control in the Democratic Republic of the Congo. *Malaria Journal*, 1–18. https://doi.org/10.1186/s12936-019-2710-5

Oladipupo S.O., Callaghan A., Holloway G.J and Gbaye O.A (2019) Variation in the susceptibility of Anopheles gambiaeto botanicals across a metropolitan region of Nigeria. PLoS ONE 14(1): e0210440. https://doi.org/10.1371/journal. pone.0210440

Omara, A.H and Attaf, M.M (2014) Spectrometric determination of nicotine in cigarette tobacco and biological samples of smokers. Vol 3. Journal of pharmacy and pharmaceutical sciences. Issue 8, 1327-1340. ISSN 2278-4357.

OpenStreetMap (2021) Mwense district [Online] [Available: https://latitude.to/articles-by-country/zm/zambia/198682/mwense-district [Accessed:30th August, 2021]

Oxborough, R. M., Seyoum, A., Yihdego, Y., Dabire, R., Gnanguenon, V., Wat'senga, F., Agossa, R.F., Yohannes, G., Coleman, S., Samdi, L.M., Diop, A., Faye, O., Magesa, S., Manjurano, A., Okia, M., Alyko, E., Masendu, H., Baber, I., Sovi, A., Rakotoson, J.D., Varela, K., Abong'o, B., Lucas, B., Fornadel, C and Dengela, D (2019) Susceptibility testing of Anopheles malaria vectors with the neonicotinoid insecticide clothianidin ; results from 16 African countries , in preparation for indoor residual spraying with new insecticide formulations, *Malaria Journal*. BioMed Central, pp. 1–11. doi: 10.1186/s12936-019-2888-6.

Pandey, G ., Dorrian,S.J., Russell, R.J and Oakeshott, J.G (2009) Biotransformation of the neonicotinoid insecticides imidacloprid and thiamethoxam by *Pseudomonas* sp. 1G, B*iochemical and Biophysical Research Communications*, vol. 380, no. 3, pp. 710–714.

Parte, S. G., and Kharat, A. S. (2019). Aerobic Degradation of Clothianidin to 2-Chloro-methyl Thiazole and Methyl 3- (Thiazole-yl) Methyl Guanidine Produced by *Pseudomonas stutzeri smk.*

Pinchoff, J., Chaponda, M., Shields, T.M., Sichivula, J., Muleba, M., Mulenga, M., Kobayashi,

Ngan, V (2008) Insect repellent [Online] [Available: https://dermnetnz.org/topics/insect-repellent] [Accessed: 30th August, 2021]

President's Malaria Initiative - African Indoor Residual Spraying Project (2014) The PMI AIRS Project [Online] [Available: http://www.africairs.net/about-airs/] [Accessed: 28th August, 2021].

Porretta, D., Mastrantonio, V., Crasta, G., Bellini, R., Comandatore, F., Rossi, P., Favia, G., Bandi, C., & Urbanelli, S. (2016). Intra-instar larval cannibalism in *Anopheles gambiae* (s.s.) and *Anopheles stephensi* (Diptera: Culicidae). Parasites and Vectors, 9(1), 1–9. https://doi.org/10.1186/s13071-016-1850-5

President's Malaria Initiative - African Indoor Residual Spraying Project (2016) Treatment of filter papers for WHO susceptibility testing (tubes tests), SOP001/01 [unpublished].

President's Malaria Initiative - VectorLink (2019) Entomological Annual report August 2018 - June 2019 [Online] [Available: https://d1u4sg1s9ptc4z.cloudfront.net/uploads/2021/03/zambia-2019-entomological-monitoring-final-report-1.pdf [Acessed: 25th July, 2021]

Rwanda Standards Board ISO 9001 Certified (2019) [Online] [Available: https://www.rsb.gov.rw/fileadmin/user_upload/files/pdf/Standards_Journal_issue_15.pdf] [Accessed:5th September, 2021]

Sandralintang, T. C., Fauzantoro, A., Hermansyah, H., Jufri, M and Gozan, M. (2018). Use of Nicotiana tabacum L extract for anti-Aedes Aegypti mosquito paint. AIP Conference Proceedings, 1933(1), 30010. https://doi.org/10.1063/1.5023957

Sangoro, O. P., Fillinger, U., Saili, K., Nkya, T. E., Marubu, R., Masaninga, F., Trigo, S. C., Tarumbwa, C., Hamainza, B., Baltazar, C., Mberikunashe, J., Chisanga, B., Menale, K., Chanda, E., and Mutero, C. M. (2021). Evaluating the efficacy, impact, and feasibility of community-based house screening as a complementary malaria control intervention in southern Africa: a study protocol for a household randomized trial. *Trials*, *22*(1), 1–16. https://doi.org/10.1186/s13063-021-05768-7

Santi, V.P. De and Khaireh, B.A., Chiniard, T., Pradines, B., Taudon, N., Larreche, S., Mohammed, A.B., de Laval, F., Berger, F., Gala, F., Mokrane, M., Benoit, N., Malan, L., Abdi, A.A and Briolant, S (2021) Role of Anopheles stephensi mosquitoes in malaria outbreak, Djibouti, 2019, *Emerging Infectious Diseases* Available at: https://doi.org/10.3201/eid2706.204557.

Sensenta, M. (2017) The Association of Vector Control By Indoor Residue Spraying And Insecticide Treated Nets, with the Prevalence of Malaria in Luapula Province, Zambia: A Retrospective Analysis of Surveillance Data [Online] [Available: http://palevel.unza.zm/handle/123456789/18/browse?rpp=20&sort_by=1&type=title&offset=349&etal=-1&order=ASC [Accessed: 10th September, 2021]

Service, M. (2012) Medical Entomology for Students, Fifth Edition, Cambridge University Press, New York.

Sherrard-Smith, E., Griffin, J. T., Winskill, P., Corbel, V., Pennetier, C., Djénontin, A., Moore, S., Richardson, J. H., Müller, P., Edi, C., Protopopoff, N., Oxborough, R., Agossa, F., N'Guessan, R., Rowland, M., & Churcher, T. S. (2018). Systematic review of indoor residual spray efficacy and effectiveness against Plasmodium falciparum in Africa. Nature Communications, 9(1). https://doi.org/10.1038/s41467-018-07357-w

Sitali, L., Miller, J.M., Mwenda, M.C., Bridges, D.J., Hawela, M.B., Hamainza, B., Kawesha, E.C.,

Eisele, T.P., Chipeta, J and Lindtjørn, B (2019) Distribution of Plasmodium species and assessment of performance of diagnostic tools used during a malaria survey in Southern and Western Provinces of Zambia, *Malaria Journal*. BioMed Central, pp. 1–9. doi: 10.1186/s12936-019-2766-2.

Smart Zambia Institute (2022) Luapula [Online] [Available: https://www.lua.gov.zm/?page_id=1637][Accessed: 28th April, 2022].

Sougoufara, S., Doucouré, S., Backé Sembéne, P. M., Harry, M., & Sokhna, C. (2017). Challenges for malaria vector control in sub-Saharan Africa: Resistance and behavioral adaptations in *Anopheles* populations. *Journal of vector borne diseases*, *54*(1), 4–15.

Stevenson, J. C., Simubali, L., Mbambara, S., Musonda, M., Mweetwa, S., Mudenda, T., Pringle, J. C., Jones, C. M., & Norris, D. E. (2016). Detection of *Plasmodium falciparum* Infection in *Anopheles squamosus* (Diptera: Culicidae) in an Area Targeted for Malaria Elimination, Southern Zambia. *Journal of medical entomology*, *53*(6), 1482–1487. https://doi.org/10.1093/jme/tjw091

Stevenson, J. C., Pinchoff, J., Pinchoff, J., Muleba, M., Lupiya, J., Chilusu, H., Mwelwa, I., Mbewe, D., Simubali, L., Jones, C.M., Chaponda, M., Coetzee, M., Mulenga, M., Pringle, J.C., Shields, T., Currier, F.C and Norris, D.E (2016) Spatio-temporal heterogeneity of malaria vectors in northern Zambia : implications for vector control, *Parasites & Vectors*.BioMed Central, pp. 1–15. doi: 10.1186/s13071-016-1786-9.

Stinus, L. *et al.* (2009) 'The addition of five minor tobacco alkaloids increases nicotine-induced hyperactivity , sensitization and intravenous self-administration in rats', pp. 1355–1366. doi: 10.1017/S1461145709000273.

Sun, H. and Sun, M. (2016) Study on Chemical Constituents from Tobacco Leaves, *International Conference on Electrical Engineering and Automation*, pp. 44–46, ISBN: 978-1-60595-407-3.

Tembo, Y., Mkindi, A. G., Mkenda, P. A., Mpumi, N., Mwanauta, R., Stevenson, P. C., Ndakidemi, P. A., and Belmain, S. R. (2018). Pesticidal plant extracts improve yield and reduce insect pests on legume crops without harming beneficial arthropods. *Frontiers in Plant Science*, *9*(September), 1–10. https://doi.org/10.3389/fpls.2018.01425

Teyssier, N. B., Chen, A., Duarte, E.M., Sit, R., Greenhouse, B and Tessema, S.K (2021) Optimization of whole - genome sequencing of Plasmodium falciparum from low - density dried blood spot samples, *Malaria Journal*. BioMed Central, pp. 1–8. doi:10.1186/s12936-021-03630-4.

Texas A&M University (2021) Landscape IPM [Online] [Available:https://landscapeipm.tamu.edu/types-of-pest-control/chemical-control/organic/botanical/[Accessed:2nd September, 2021]

Tomizawa M and Casida J.E (2005) Neonicotinoid insecticide toxicology: mechanism and selective action. Annu Rev Pharmacol Toxicol. https://doi.org/10.1146/annurev.pharmtox.45.120403. 095930 PMID: 15822177

Trigg, J. K. (1996) Evaluation of a Eucalyptus-based repellent against Anopheles spp. in Tanzania, *Joumal of the Ameican Mosquito Control Association*, 12(2), pp. 243–246.

Trongtokit, Y., Curtis, C. F. and Rongsriyam, Y. (2005) Efficacy of repellent products against caged and free flying *Anopheles Stephensi* mosquitoes, *Southeast Asian Journal Tropical Medicine Public Health*, 36(6), pp. 1423–1431.

The Global Historical Weather and Climate Data (2023) Zambia climatic classificstion[Online][Available: https://tcktcktck.org/zambia][Accessed:4th March, 2023]

Tusting, L. S., Ippolito, M. M., Willey, B. A., Kleinschmidt, I., Dorsey, G., Gosling, R. D., and Lindsay, S. W. (2015). The evidence for improving housing to reduce malaria: A systematic review and meta-analysis. *Malaria Journal*, *14*(1). https://doi.org/10.1186/s12936-015-0724-1

Ullah, Z., Ijaz, A., Mughal, T. K., and Zia, K. (2018). Larvicidal activity of medicinal plant extracts against Culex quinquefasciatus Say . (Culicidae ,. 5(2), 47–51.

Uneme H (2011) Chemistry of clothianidin and related compounds. J Agric Food Chem. 13;59(7):2932-7. doi: 10.1021/jf1024938. Epub. PMID: 20873771

United States Agency International Development (2016) Climate change risk profile Zambia [Online][Available:https://www.climatelinks.org/sites/default/files/asset/document/2016%20CRM%20Fact%20Sheet%20-%20Zambia.pdf [Accessed:16th May, 2022]

United States Agency International Development (2022) The journey of 7 million bed nets[Online][Available: https://www.usaid.gov/zambia/news/journey-7-million-bed-nets#:~:text=During%20the%202020%2D2021%20anti,ITNs)%20to%20protect%20from%20malaria.[Accessed: 8th February, 2023]

United States Environmental Protection Agency (2021) Repellents: Protection against Mosquitoes, Ticks and other Arthropods [Online] [Available:https://www.epa.gov/insect-repellents] [Accessed:14th September, 2021]

Van der Velde-Koerts, T., Van Hoeven-Arentzen, P.H and Mathieu, (2009) Clothianidin, National Institute of Public Health and the Environment Centre for Substances and In- tegrated Risk Assessment, Netherlands.

Wade, L.G (2006) Organic Chemistry, Second Edition, Pearson, New Jersey, ISBN 0-43-147871

Wang, S.N., Liu, Z., Tang, Z.H., Meng, J and Xu, P (2006) Characterisation of environmentally friendly nicotine degradation by *Pseudomonas putida* biotype A strain S16, *Journal of microbiology,* DOI 10.1099/mic.02006/005223-0.

White, S.A and Kaufman, P.E (2014) African malaria mosquito *Anopheles gambiae* Giles (Insecta: Diptera: Culicidae)[Available: https://edis.ifas.ufl.edu/publication/IN1048][Accessed:30th March, 2023]

Wilkins E.E,. Howell P.I and Benedict M.Q (2006) IMP PCR primers detect single nucleotide polymorphisms for Anopheles gambiae species identification, Mopti and Savanna rDNA types, and resistance to dieldrin in Anopheles arabiensis. Malar J 5:125

Williams, J. and Pinto, J. (2012) Training Manual on Malaria Entomology For Entomology and Vector Control Technicians (Basic level) [Online] [Available: https://www.paho.org/en/documents/training-manual-malaria-entomology-entomology-and-vector-control-technicians-basic-0 [Accessed: 13th August, 2021]

World Health Organization (1975) Manual on prractical entomology in malaria [Online] [Available:http://apps.who.int/iris/bitstream/handle/10665/42481/WHO_OFFSET_13_(part1).pdf;jsessionid=ED5808F349356B6750F9C95D490A950A?sequence=1[10th August, 2021].

World Health Organization (2016) Test procedures for insecticide resistance monitoring in malaria vector mosquitoes 2nd edition[Online][Available:https://apps.who.int/iris/bitstream/handle/10665/250677/9789241511575-eng.pdf [Accessed:15th January, 2022].

WHO. (2013). Larval source management: A supplementary measure for malaria control. *Outlooks on Pest Management*, *25*(1), 41–43. https://doi.org/10.1564/v25_feb_13

World Health Organization (2022) Vector control [Online] [Available: https://www.who.int/teams/global-malaria-programme/prevention/vector-control] [Accessed: 18th August, 2022].

Wuryantini S, Harwanto and Rizki A Y (2017) Response of citrus aphids *Aphis gossypii* to several botanical insecticides. Proceedings of the 2017 National Seminar and Perhorti Congress. ISBN:970-602-702-094-8.

Xu, K., Liu, Q., Ni, L., Du, Y.M and Zhang, Z.F (2020) An Update on Chemical Constituents from Nicotiana tabacum L and Their Bioactivities from 2017 to 2020, *Medical research,* pp. 1–6. doi: 10.21127/yaoyigc20200019.

Yaro, A.S., Dao, A., Adamou, A., Crawford, J.E., Ribeiro, J.M.C., Gwadz, R, Traore, S.F and Lehmann, T (2006) The distribution of hatching time in *Anopheles gambiae*, 7, pp. 1–7. Available at: https://doi.org/10.1186/1475-2875-5-19.

Yewhalaw, D., Simma, E. A., Zemene, E., Zeleke, K., and Degefa, T. (2022). Residual efficacy of SumiShield TM 50WG for indoor residual spraying in Ethiopia. Malaria Journal, 1–10. https://doi.org/10.1186/s12936-022-04395-0

Yin, J., Yamba, F., Zheng, C., Zhou, S., Smith, S. J., Wang, L., Li, H., Xia, Z., and Xiao, N. (2021). Molecular Detection of Insecticide Resistance Mutations in Anopheles gambiae from Sierra Leone Using Multiplex SNaPshot and Sequencing. *Frontiers in Cellular and Infection Microbiology*, *11*(August), 1–7. https://doi.org/10.3389/fcimb.2021.666469

Yukich, J. O., Bennett, A., Albertini, A., Incardona, S., Moonga, H., Chisha, Z., Hamainza, B., Miller, J.M., Keating, J., Eisele, T.P and Bell, D (2012) Reductions in Artemisinin-Based Combination Therapy Consumption after the Nationwide Scale up of Routine Malaria Rapid Diagnostic Testing in Zambia, 87(3), *American Society of Tropical Medicine and Hygiene,* pp. 437–446. doi: 10.4269/ajtmh.2012.12-0127.

Zambian Ministry of Health (2012) Malaria Indicator Survey[Online][Available: https://www.malariasurveys.org/documents/Zambia_MIS_2012.pdf][Accessed: 8th February, 2023].

Zambia Health Management Information System (2021) [Online] Available: https://dhis2.moh.gov.zm/hmis/dhis-web-commons/security/login.action [Accessed: 24th February, 2021].

Zenkner, F. F., Margis-Pinheiro, M and Cagliari, A. (2019) Nicotine biosynthesis in Nicotiana : A metanolic overview, *Tobacco science*, 56: 1–9

Zhao, X., Smith, D. L and Tatem, A. J. (2016). Exploring the spatiotemporal drivers of malaria elimination in Europe. *Malaria Journal*, *15*(1), 1–13. https://doi.org/10.1186/s12936-016-1175-z

Zhou, G., Kohlhepp, P., Geiser, D., Frasquillo, C, Vazquez, L and Winzerling, J.J (2008) Fate of blood meal iron in mosquitos, *Journal of Insect Physiology* 53(11), pp. 1169–1178.

APPENDICES

Appendix 1: Ethical clearance

UNIVERSITY OF ZAMBIA
BIOMEDICAL RESEARCH ETHICS COMMITTEE

Telephone: +260 977925304
Telegrams: UNZA, LUSAKA
Telex: UNZALU ZA 44370
Fax: + 260-1-250753
Federal Assurance No. FWA00000338

Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia
E-mail: unzarec@unza.zm
IRB00001131 of IORG0000774

24th September 2021

Your REF. No. 1834-2021

Mr. Nicholus Chintu Sande,
University of Zambia,
School of Veterinary Medicine,
P.O Box 32379,
Lusaka.

Dear Mr. Sande,

RE: INSECTICIDAL EFFECTIVENESS OF NICOTINE FROM NICOTIANA TABACUM AGAINST ANOPHELES GAMBIAE S.L (INSECTA: DIPTERA: CULICIDAE) (REF. NO. 1834-2021)

The above-mentioned research proposal was presented to the Biomedical Research Ethics Committee on 18th August, 2021. The proposal is **approved**. The approval is based on the following documents that were submitted for review:

a) **Study proposal**
b) **Questionnaires**
c) **Participant Consent Form**

APPROVAL NUMBER : REF. 1834-2021

This number should be used on all correspondence, consent forms and documents as appropriate.

- **APPROVAL DATE : 24th September 2021**
- **TYPE OF APPROVAL : Ordinary**
- **EXPIRATION DATE OF APPROVAL : 23rd September 2022**
  After this date, this project may only continue upon renewal. For purposes of renewal, a progress report on a standard form obtainable from the UNZABREC Offices should be submitted one month before the expiration date for continuing review.
- **SERIOUS ADVERSE EVENT REPORTING**: All SAEs and any other serious challenges/problems having to do with participant welfare, participant safety and study integrity must be reported to UNZABREC within 3 working days using standard forms obtainable from UNZABREC.
- **MODIFICATIONS**: Prior UNZABREC approval using standard forms obtainable from the UNZABREC Offices is required before implementing any changes in the Protocol (including changes in the consent documents).

- **TERMINATION OF STUDY**: On termination of a study, a report has to be submitted to the UNZABREC using standard forms obtainable from the UNZABREC Offices.
- **NHRA:** You are advised to obtain final study clearance and approval to conduct research in Zambia from the National Health Research Authority (NHRA) before commencing the research project.
- **QUESTIONS**: Please contact the UNZABREC on Telephone No. +260977925304 or by e-mail on unzarec@unza.zm.
- **OTHER**: Please be reminded to send in copies of your research findings/results for our records. You are also required to submit electronic copies of your publications in peer-reviewed journals that may emanate from this study. Use the online portal: unza.rhinno.net for further submissions.

Yours sincerely,

Munsaka.

Sody Mweetwa Munsaka, BSc., MSc., PhD
CHAIRPERSON
Tel: +260977925304
E-mail: s.munsaka@unza.zm

Appendix 2: Ethical clearance

Ref No: NHRA000007/15/10/2021 Date: 15th October, 2021

The Principal
Investigator, Mr.
Nicholus Chintu
Sande,University
of Zambia,
School of Veterinary Medicine,
Lusaka, Zambia.

Dear Mr Sande,

Re: Request for Authority to Conduct Research

The National Health Research Authority is in receipt of your request for authority to conduct research titled **"Insecticidal Effectiveness of Nicotine from Nicotiana Tabacum Against sub-species of Anopheles Gambiae S.L (Insecta: Diptera: Culicidae)."**

I wish to inform you that following submission of your request to the Authority, our review ofthe same and in view of the ethical clearance, this study has been **approved** on condition that:

1. The relevant Provincial and District Medical Officers where the study is being conducted are fully appraised;
2. Progress updates are provided to NHRA quarterly from the date of commencementof the study;
3. The final study report is cleared by the NHRA before any publication or dissemination within or outside the country;
4. After clearance for publication or dissemination by the NHRA, the final study reportis shared with all relevant Provincial and District Directors of Health where the study was being conducted, University leadership, and all key respondents.

Yours sincerely,

Prof. Godfrey
Biemba
Director/CEO
National Health Research Authority

Appendix 3: Permission to use laboratories at National Malaria Elimination Centre

All Correspondence should be addressed to the
Permanent Secretary
Telephone: +260 211 253040 5
Fax: +260 211 253344

In reply please quote:
MOH/101/9/19

REPUBLIC OF ZAMBIA
MINISTRY OF HEALTH

NDEKE HOUSE
P. O. BOX 30205
LUSAKA

10th January, 2022

Prof. Enala Tembo-Mwase,
The University of Zambia,
School of Veterinary Medicine,
P.O Box 32379,
LUSAKA

RE: REQUEST TO USE FACILITIES AT THE NATIONAL MALARIA ELIMINATION CENTRE (NMEC)

Reference is made to the subject matter above.

In response to your letter dated 19th November, 2021 requesting to utilise the National Malaria Elimination Centre (NMEC) facilities for academic research project purposes, the NMEC has no objection and permits Mr. Sande to utilise the facilities for the completion of his research project.

Mr. Sande can thus report to the Vector Control Specialist, Dr. Emmanuel Kooma, for placement to commence his activities. You are also expected to share the research report with the NMEC.

Yours faithfully,

Dr. Mutinta Mudenda Chilufya
Director NMEC
For/Permanent Secretary – Technical Services
MINISTRY OF HEALTH

Appendix 4: Consent form for aspirating adult mosquitoes from the households using Prokopack aspiration method.

I declare having read (or had read to me) and understood the present research, the nature and extent of my participation, as well as the risks and benefits related to it, as described in the information letter above that has been given to me. I had the opportunity to ask all questions regarding the various aspects of the study and to receive satisfactory responses. I understand that I can withdraw at any time without any penalty.

<table>
<tr><td rowspan="2">Today's study: The above has been explained to me and I agree to take part in the study. I understand that I am free to choose to be in this study and that saying "NO" will have no effects for me or my household. I understand that my house may be used for mosquito collection using Prokopack aspiration device, and that I will be asked to participate in a survey.</td><td colspan="2">If you agree, circle "YES," if you do not agree, circle 'NO'.</td></tr>
<tr><td>YES</td><td>NO</td></tr>
</table>

Household Head	Name:	Signature or thumb print: ….	Date…………………
Witness*	Name:	Signature:	Date………………….

*A witness can be another adult member of the household or of the community

Name of Household Head (Print)

____

Name of the field worker obtaining the consent:

____

Signature: __________________________ Date: ________________

Appendix 5: Insecticide susceptibility form

USAID

PRESIDENT'S MALARIA INITIATIVE

CDC

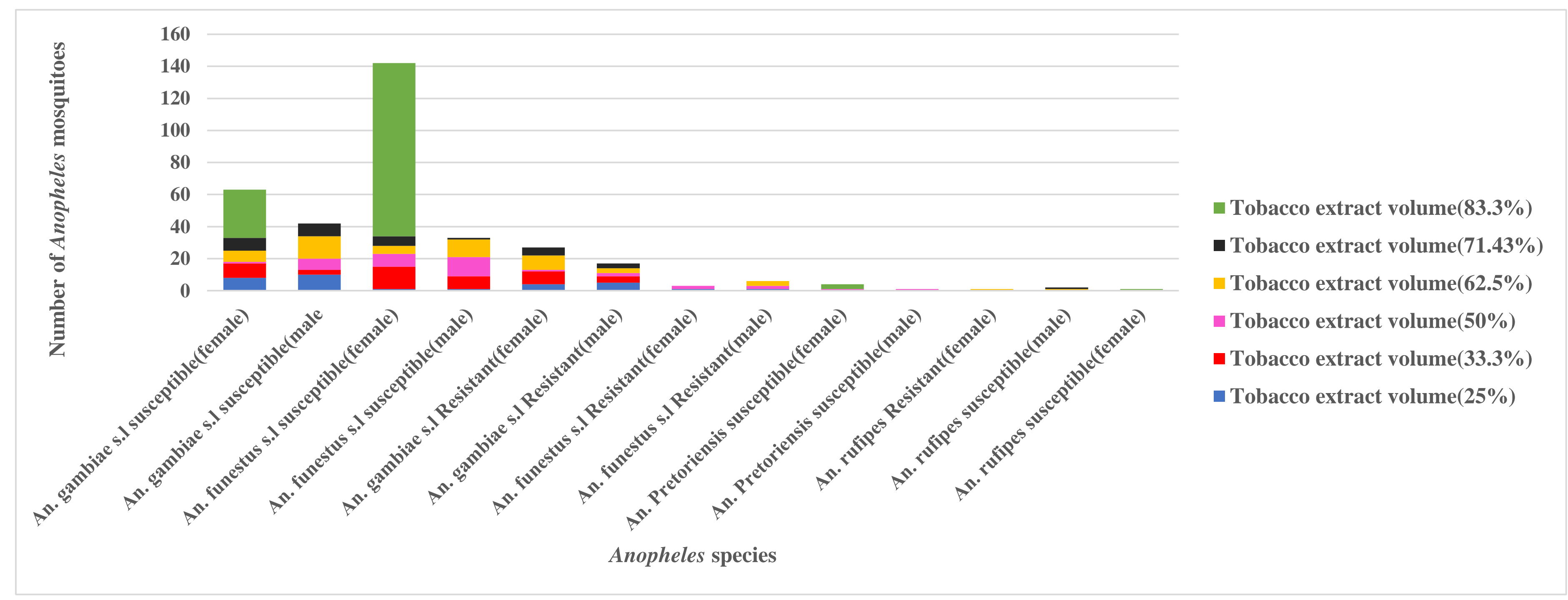
WHO tube Susceptibility test record form

1	Country________ Region________ District________ Name of study village/site________
2	Spray status of the study area: Sprayed ☐ | Unsprayed ☐ | If sprayed last spray date________ (DD/MM/YYYY) Insecticide sprayed________
3	Mosquito origin and Species: Reared from field collected larvae and pupae:________ | F1 progeny of field collected mosquitoes:________ | F2 progeny of filed collected mosquitoes:________ | An. species tested:________
4	Insecticide tested: ________ Concentration: ________ date of impregnation: ________ date of expiry:________ Number of times the paper previously used: ________ storage condition room at: ________ refrigerated at: ________
5	Temperature: Exposure period Max________ Min________ Holding period: Max________ Min________
6	Relative humidity: Exposure period________% holding period ________%
7	Insecticide resistance results

Description	*Replicate1*	*Replicate2*	*Replictoe3*	*Replicate4*	*Control 1*	*Control2*	*Total Test*	*Total Control*
No of mosquitoes tested								
No mosquito knock down :10 min								
15 min								
20 min								
30 min								
40 min								
50 min								
60 min								
80 min								
No dead at the end of 24hrs holding								
Observed mortality (%)								
Corrected mortality (%)								
Resistance Mechanism								

Remark:________

Appendix 6: Insecticide susceptibililty status of wild *Anopheles* mosquitoes against tobacco extract



Appendix 7: Probit analysis results for lethal concentrations for tobacco extract

Confidence Limits

	Probability	95% Confidence Limits for Concentration			95% Confidence Limits for log(Concentration)[a]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT	.010	.411	.000	3.593	-.387	-9.452	.555
	.020	.700	.000	4.830	-.155	-8.217	.684
	.030	.981	.000	5.830	-.008	-7.433	.766
	.040	1.265	.000	6.717	.102	-6.843	.827
	.050	1.556	.000	7.538	.192	-6.363	.877
	.060	1.856	.000	8.317	.268	-5.955	.920
	.070	2.165	.000	9.067	.336	-5.597	.957
	.080	2.486	.000	9.797	.396	-5.277	.991
	.090	2.819	.000	10.512	.450	-4.986	1.022
	.100	3.165	.000	11.218	.500	-4.717	1.050
	.150	5.111	.000	14.697	.709	-3.608	1.167
	.200	7.480	.002	18.255	.874	-2.727	1.261
	.250	10.371	.011	22.043	1.016	-1.972	1.343
	.300	13.907	.051	26.202	1.143	-1.296	1.418
	.350	18.252	.213	30.924	1.261	-.672	1.490
	.400	23.624	.824	36.549	1.373	-.084	1.563
	.450	30.322	2.990	43.897	1.482	.476	1.642
	.500	38.765	9.992	55.906	1.588	1.000	1.747
	.550	49.559	26.513	89.662	1.695	1.423	1.953
	.600	63.610	43.951	235.616	1.804	1.643	2.372
	.650	82.332	56.876	833.336	1.916	1.755	2.921
	.700	108.055	69.349	3395.082	2.034	1.841	3.531
	.750	144.899	83.685	15866.658	2.161	1.923	4.200
	.800	200.890	101.880	89452.453	2.303	2.008	4.952
	.850	294.001	127.177	676661.370	2.468	2.104	5.830
	.900	474.733	167.181	8679446.788	2.676	2.223	6.938
	.910	532.981	178.499	16083346.361	2.727	2.252	7.206
	.920	604.388	191.631	31439228.139	2.781	2.282	7.497
	.930	693.993	207.151	65708976.329	2.841	2.316	7.818
	.940	809.859	225.932	149715651.855	2.908	2.354	8.175

	.950	965.797	249.388	383044600.380	2.985	2.397	8.583
	.960	1187.779	280.010	1155251635.211	3.075	2.447	9.063
	.970	1531.772	322.767	4489448551.992	3.185	2.509	9.652
	.980	2147.978	389.730	27290784395.947	3.332	2.591	10.436
	.990	3659.831	524.238	469567860997.474	3.563	2.720	11.672

a. Logarithm base = 10.