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SCHOOL OF GRADUATE STUDIES

COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES

DEPARTMENT OF ZOOLOGICAL SCIENCES

Larvicidal and adulticidal effects of some selected plant extracts against *Anopheles stephensi* Liston (Culicidae: Diptera) under laboratory condition

Merdya Muhammed

A thesis submitted to the Department of Zoological Sciences of Addis Ababa University in fulfillment of the requirements for the Degree of Master of Science in Zoological Sciences (Insect Sciences)

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By

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A thesis submitted to the Department of Zoological Sciences of Addis Ababa University in fulfillment of the requirements for the Degree of Master of Science in Zoological Sciences (Insect Sciences)

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Abstract

Mosquitoes that belong to the genus *Anopheles* are among the major vectors of vector-borne diseases (VBDs). Among VBDs, malaria plays a crucial role in public health challenges worldwide. The use of synthetic insecticides to control malaria vectors causes resistance, environmental toxicity and kills non-target animals. Thus, it is imperative to seek a relatively safe and effective alternative tool to the currently available mosquitocidal products. Natural herbal products with insecticidal properties would play an important role in resolving this problem. Therefore, the current study aimed to assess the effects of crude leaf solvent extracts of *Calpurnia aurea*, *Momordica foetida*, and *Zehneria scabra* against the laboratory colony of larvae and adults of *An. stephensi*. Fresh leaves of test plants were collected, air dried, and then separately ground to powder. The powders were soaked in aqueous, hexane, and methanol solvents. The extracts were concentrated, and a stock solution was prepared to desired test concentration. For comparison, temephos (larvicide) and untreated solutions were used as the standard and negative controls, respectively. Mortality was observed after 24 hrs recovery period. The statistical analyses were performed using SPSS software (Kruskal-Wallis test), and R software (a generalized linear model was used to determine LC_{50} and LC_{90} values of the extracts). Larvicidal activity between treatments, negative control, and standard groups were significant differences ($P < 0.05$). Among the test extracts, the lowest LC_{50} and LC_{90} values were recorded in larvae treated with aqueous extract of *M. foetida* with 34.61 ppm and 57.61 ppm, respectively followed by *Z. scabra* (LC_{50} = 35.85 ppm; LC_{90} = 68.26 ppm) and *C. aurea* (LC_{50} = 38.69 ppm; LC_{90} = 108.28 ppm). Larval mortality was not observed from the hexane and control treatments, while the temephos achieved 100%. In addition, aqueous extract of *Z. scabra* showed the most effective adulticidal action in *An. stephensi* with LC_{50} and LC_{90} values of 176.20 ppm and 425.13 ppm, respectively. The results suggest that the leaf extracts of the three test plants have the potential of being used for the control of *An. stephensi* instead of synthetic mosquitocides. Further studies need to be conducted to identify the active ingredients and their mode of action.

Keywords: *Anopheles stephensi*, botanicals, plants extracts, adulticides, larvicides.

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List of Acronyms

DDT	Dichloro-diphenyl-trichloroethane
EPHI	Ethiopian Public Health Institute
FMoH	Federal Ministry of Health
IRS	Indoor Residual Spraying
ITNs	Insecticide Treated Nets
IVM	Integrated Vector Management
LC_{50}	Lethal Concentration that kills 50% of the exposed
LC_{90}	Lethal Concentration that kills 90% of the exposed
PMI	President's Malaria Initiative
PPM	Parts per million
WHO	World Health Organizations

CHAPTER 1: INTRODUCTION

1.1 Background

Mosquitoes are among the most important groups of arthropods with medical significance. They transmit several important parasitic and arboviral diseases such as malaria, filariasis, dengue, yellow fever, and Rift Valley fever (Raghavendra *et al*., 2011; Krishnappa *et al*., 2012). Moreover, malaria which is caused by protozoans (*Plasmodium* spp.), continue to affect the health and wellbeing of humans and domestic animals worldwide. Currently, the global malaria cases that occurred in 2019 were estimated to be 229 million, with 409,000 deaths reported annually in 87 malaria-endemic counties, of which around 215 million (94%) of cases with 384,000 deaths occurred in the African region (WHO, 2020). In Ethiopia, malaria is ranked as the leading communicable disease, accounting for about 30% of the overall Disability Adjusted Life Years lost (Howes *et al*., 2014). About 75% of the country is malarious with about 68% of the total population living in areas at risk of malaria (EPHI, 2016).

Worldwide, approximately 500 species of *Anopheles* mosquito have been described, of which, more than 100 *Anopheles* species are vectors of pathogens that cause diseases in humans (Raghavendra *et al*., 2011; Asadollahi *et al*., 2019). In Africa, *Anopheles gambiae*, *An. coluzzii*, *An. arabiensis*, *An. funestus*, *An. moucheti* and *An. nili* are the main vectors in sub-Saharan tropical Africa, while *An. rivolorum*, *An. pharoensis*, *An. coustani*, *An. ziemanni*, and *An. squamosus* are secondary vectors (Afrane *et al*., 2016). In Ethiopia, there are four species of *Anopheles* mosquitoes, which transmit malaria namely, *An. arabiensis*, *An. pharoensis*, *An. funestus*, and *An. nili*. The former is the major vector, whereas the rest are secondary vectors (PMI, 2016). Additionally, in 2016, the South Asian vector *An. stephensi* was reported from the Somali region for the first time (Carter *et al*., 2018) and was later found to be widely present in urban areas of northeastern Ethiopia (Meshesha Balkew *et al*., 2020). The invasion of this vector in various parts of Ethiopia and other Horn of Africa countries may be the cause of the spread of malaria infection. Prominently, *An. stephensi* from Ethiopia is a more competent vector for *P. vivax* than *An. arabiensis* in the lab (Fitsum Tadesse *et al*. 2021).

Currently, five parasite species are known to cause malaria in humans, including *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi* (Garrido-Cardenas *et al*., 2019). Of these species, *P. falciparum* and *P. vivax* pose the most prominent threat in sub-Saharan Africa (Battle *et al*., 2019; Rondon *et al*., 2019). In Ethiopia, *Plasmodium falciparum* and *P. vivax* are the two most important malaria parasites in the country, in which each accounting for 70% and 30% of cases, respectively (FMoH, 2017).

Globally, malaria control strategies primarily depend on the preventative use of indoor residual spray, insecticide-treated bed nets, and case treatment (WHO, 2019). Following the implementation of IRS and ITN as malaria control strategy, the global malaria cases and deaths have shown a significant reduction (WHO, 2015). However, insecticide resistance occurred by major malaria vectors, the chief weapon of the vector is becoming ineffective, and this posed a challenge to vector control strategy (Kawada *et al*., 2011; Reddy *et al*., 2011; Sindato *et al*., 2011; WHO, 2012).

Insecticide resistance development, particularly for organochlorines and organophosphate by mosquitoes has been noted by WHO (1989), and pyrethroids are now widespread (Zouré *et al*., 2021). Throughout the world, malaria-endemic nations around 60 have reported resistance to at least one class of insecticides, while 50 reported to two or more insecticide classes (WHO, 2017). As evidenced in the recent studies, in different parts of Ethiopia, *An. arabiensis* and *An. stephensi* has developed resistance against synthetic insecticides belonging to all four chemical classes approved for IRS and ITNs, including DDT (organochlorine), pirimiphos-methyl, bendiocarb, malathion (organophosphate), propoxur (carbamates) and alpha-cypermethrin, cyfluthrin, deltamethrin, etofenprox, lambda-cyhalothrin, permethrin (pyrethroids) (Delenasaw Yewhalaw *et al*., 2011; Fekadu Massebo *et al*., 2013; Delenasaw Yewhalaw and Kweka, 2016; Solomon Yared *et al*., 2020). Therefore, chemical-based control strategies are not recommended in terms of environmental safety, the reappearance of various vector populations, and cost-expensiveness (Walker, 2000; Nkya *et al*., 2013; Killeen *et al*., 2017).

The emergence of insecticide resistance and its adverse harmful effects on human health and the environment lead to an urgent search for the development of new and improved vector control

approaches that are cheap, effective, safe, and biodegradable. In this regard, botanical products (plant extracts and essential oils) with insecticidal potential are recognized as potent alternatives to replace synthetic insecticides in mosquito control programs. This is because botanicals showed high larvicidal, pupicidal, adulticidal, oviposition deterrents, repellents, attractants, feeding deterrents/antifeedants, toxicants, growth retardants, and chemosterilants (Shaalan *et al*., 2005; Isman, 2006; Pavela and Benelli, 2016; Stone *et al*., 2018). Ghosh *et al*. (2012) also explained that plant insecticides have several active compounds such as phenolic, alkaloids, terpenoids, rare amino acids, plant amines, and glycosides that act synergistically to provide their activity as opposed to synthetic insecticides that rely on a single constituent, thus decreasing chances of resistance development by the vectors. Generally, biological insecticides are cheap, locally available, and also dynamic against a constrained number of species evoking with typically free from destructive impacts on the environmental ecosystem (Moore *et al*., 2007; Maia and Moore, 2011; Deletre *et al*., 2013).

Currently, throughout the world investigating widely plant-derived essential oils and their extracts for safe use in the control of various malaria vectors (Hardin and Jackson, 2009; Patil *et al*., 2010; Krishnappa *et al*., 2012; Panneerselvam and Murugan, 2013; Sabila *et al*., 2018; Asadollahi *et al*., 2019; Bassene *et al*., 2020). Larvicidal, adulticidal, and repellent activities of plant volatile oils and their extracts from a wide variety have been assessed in Ethiopia against *An. arabiensis*, *An. pharoensis* and some other mosquito vectors (Mamuye Hadis *et al*., 2003; Fekadu Massebo *et al*., 2009; Sisay Dugassa *et al*., 2009; Bezayit Solomon *et al*., 2012; Fekadu Massebo *et al*., 2013; Damtew Bekele *et al*., 2014; Hirpasa Teressa *et al*., 2019; Araya Eukubay *et al*., 2020; Mihretu Tarekegn *et al*., 2020; Sileshi Degu *et al*., 2020; Wondimu Ersino *et al*., 2020). The growing reports indicate that searching for effective and relatively safe insecticides against malaria mosquitoes is still in progress. In line with this, the present study was initiated to evaluate the insecticidal efficacies of some selected plants against *An. stephensi* under laboratory conditions.

1.2 Statement of the problem

Insecticide resistance of mosquitoes is increasing from time to time, not only in developing countries but also in the developed countries throughout the world. In Ethiopia, IRS and LLINs are pillars in malaria prevention and control strategy. However, insecticide susceptibility tests carried out in different parts of the country have shown different levels of resistance by the principal and secondary malaria vectors to insecticides used for IRS and/or LLINs (Fekadu Massebo *et al*., 2013; Delenasaw Yewhalaw and Kweka, 2016; Solomon Yared *et al*., 2020).

A recent report by Solomon Yared *et al*. (2020) indicated that *An. stephensi* collected in eastern and northeast Ethiopia developed resistance to several classes of insecticides, most notably pyrethroids. Thus, the emergence of resistance among *An. stephensi* populations have a critical effect on the malaria control programs designed and implemented in malaria-endemic areas of the country. In addition, the harmful effects of synthetic insecticides on humans and the environment are also of concern. For this, in some parts of the world other than Ethiopia, a relatively safe plant-based control in the form of ovicidal, larvicidal, adulticidal, and repellent activities against immature and adults of *An. stephensi* malaria vectors have been investigated under laboratory and semi field conditions and they gave promising results (Prajapati *et al*., 2005; Rahuman and Zahir, 2010; Xu *et al*., 2020). Moreover, the bioactivities of leave solvent extracts of *Calpurnia aurea*, *Momordica foetida*, and *Zehneria scabra* herbal medicinal plant in Ethiopia was no study on *Anopheles stephensi* mosquito. Therefore, in this study, the insecticidal aspect of *C. aurea*, *M. foetida*, and *Z. scabra* leaves solvent extracts against malaria vector *An. stephensi* was studied.

1.3 Objectives of the study

1.3.1 General objectives

The overall objective of this study was to investigate the insecticidal activities of *Calpurnia aurea* (Ait.) Benth., *Momordica foetida* Schumach, and *Zehneria scabra* (Linn.F.) Sand against *Anopheles stephensi* under laboratory condition.

1.3.2 Specific objectives

- To evaluate the larvicidal activity of different solvent crude leaf extracts of *Calpurnia aurea*, *Momordica foetida*, and *Zehneria scabra* against *An. stephensi*.
- To evaluate the adulticidal activity of different solvent crude leaf extracts of *Calpurnia aurea*, *Momordica foetida*, and *Zehneria scabra* against *An. stephensi*.
- To determine the LC_{50} and LC_{90} values of crude leaf extracts of *Calpurnia aurea*, *Momordica foetida*, and *Zehneria scabra* against *An. stephensi*.

1.4 Significance of the study

The results of this study have paramount importance for strengthening the currently existing vector control strategies, hence reduce malaria transmission. This is because the botanical products with larvicidal and adulticidal activities are environmentally safe, target specific effective, easily available, and cheap alternative vector control methods. The findings of this study also are important to manage the problem of the insecticide resistance phenomenon and its residual effects on humans and the environment. Moreover, this study contributes new knowledge about the role of selected botanical extracts in the scientific community.

CHAPTER 2: LITERATURE REVIEW

2.1 Trends in the burden of malaria

Malaria is a haemoparasitic disease caused by obligate intracellular protozoan parasites of *Plasmodium* species which are transmitted by infected female anopheline mosquitos, causing more than 95% of malaria cases in the world (Vangapandu *et al*., 2007). The burden is higher in some regions of the world partly due to the variations in the geographical distributions of *Plasmodium* parasites. Infections caused by *P. falciparum* are common in sub-Saharan Africa (SSA), East Asia, and South America, as well as in Oceania (Gething *et al*., 2011). Currently, more than 90% of malaria-related deaths are caused by the most pathogenic of all the species, *P. falciparum* (Zekar and Sharman, 2020). In contrast, *P. vivax* is the causative species primarily in South East Asia, Central Asia, Central America, and is also common in Oceania and South America (Gething *et al*., 2012).

2.1.1 Global malaria burden

Previous theories viewed that malaria has a devastating effect on the health and wealth of nations (Hay *et al*., 2004; Tabbabi, 2018). And even still, it continues to pose a threat to the economy, development, environment, biology, agriculture, livelihoods, education, and other social issues in various world countries (Austin, 2013; Heggenhougen *et al*., 2003; Castro, 2017). About 5.4 billion of the world peoples half of the population is at risk from malaria vectors (Mukhtar, 2003). Globally, an estimated 300–500 million range of new clinical cases with 1-4 to 2-6 million deaths are annually reported in more than 90 malaria-endemic countries and territories, among this around 90% of them covered by sub-Saharan tropic African countries, particularly Nigeria, Mozambique, Democratic Republic of Congo, Uganda, Ethiopia, and Tanzania (Karunamoorthi, 2011; Kovendan *et al*., 2012; Krishnappa *et al*., 2012).

Malaria disease is preventable and curable. Worldwide, cases of malaria reduction observed from 2010 to 2014, it was expanded from 2015 to 2017 when 231 million people were reported, but

currently, 229 million malaria cases occurred in 2019, so the damage has shown a slight reduction in the current situation (WHO, 2017, 2020).

Malaria in developing countries still often affects pregnant women, adolescents, and children (Meremikwu *et al*., 2009; Bango *et al*., 2020). In areas with high transmission of malaria, children mainly under age five are susceptible to infection, illness, and death (Phillips, 2001). It also disrupted school-aged children, mainly 5–15 years from their education (Nosten and McGready, 2013). In Africa, among the newborns, an estimated 16% (822, 000) endure complications from low birth weight including death emerging from malaria disease during pregnancy (WHO, 2020). Currently, an estimated 24 million cases of malaria infection with 67% (274, 000) of deaths occurred globally in children under age five in 2019, among this large present of deaths covered by sub-Saharan tropical African countries (UNICEF, 2020).

Malaria and poverty are mutually supportive, as malaria affects the power of producers such as farmers and other people with family responsibilities of householders, thus it leads to distorting the families' income and impedes development, including saving and investment, worker productivity, absenteeism, population growth, and premature mortality (Sachs and Malaney, 2002; Ricci, 2012). Malaria imposes significant costs to people, society, and the government, moreover, the use of the continent economy directly and/or indirectly in treating or preventing malaria illness leads to high levels of economic burden, particularly in Africa, currently an estimated 25% of economic disruption (Xia *et al*., 2016; WHO, 2020). Willis and Hamon (2019) predicted malaria outbreak in sub-Saharan Africa over the next two decades would affect the incomes of agricultural households. WHO (2020) also reported the annual budget for mosquito control and elimination in 2019 exceeds $ 3.0 billion, among these commitments from governments of endemic countries, amounted to $ 900 million, representing 31% of add up to financing, so the world growth rate fell to $ 5.6 billion below expectations.

2.1.2 Malaria burden in Ethiopia

Malaria in Ethiopia is still one of the top three leading causes of morbidity and mortality in all age groups (Brhane Berhe *et al*., 2019). Areas found less than 2,000 meters above the sea level is

more suitable for malaria development, thus two-thirds of the population is at risk, currently, an estimated 5-6 million of malaria infection with 70,000 deaths occurs every year (Aynalem Adugna, 2014; Brhane Berhe *et al*., 2019). Among the five *Plasmodium* species known to contaminate human creatures in Ethiopia, *Plasmodium falciparum* and *P. vivax* malaria are by distant the foremost transcendent and broadly distributed in different regional states, particularly in Amhara, Oromiya, Tigray, Gambella, Afar, Somali, Harari, and South regions (Solomon Tesfaye *et al*., 2011; Teshiwal Deress and Mekonnen Girma, 2019). Currently, these two parasite species respectively contribute to nearly 70% and 30% of malaria diseases infections (FMoH, 2017).

The epidemiology of malaria has always been geographically specific, but mainly in Ethiopia malaria transmission peaks twice per year during harvesting time between September to December and April to May, thus it causes a serious economic problem (Aschalew Alelign and Tadesse Dejene, 2016). Losses of productivity, school absenteeism, direct and indirect costs are the major financial burdens of malaria in Ethiopia (Amsalu Woldie, 2020). The cost of malaria in Ethiopia is about $ 200 million every year, which is 10% of the total health costs (FMoH, 2017). Currently, Lelisa Fekadu *et al*. (2020) reported that Ethiopia spends $ 4,627,800 for four interventions of households. Mebrate Dufera *et al*. (2020) also indicated that in a rural district of western Ethiopia, the economic burden of malaria is indirectly estimated to be 78% of the country's average income, among them 55% of costs directly related to pharmaceutical services.

2.2 Malaria vectors

Throughout the world, approximately 4,500 mosquito species with 42 genera of the Culicidae family have been recorded (Tandina *et al*., 2018; Shirzadi, 2019; Ali and Venkatesalu, 2020. Among the genera, *Anopheles*, *Aedes*, *Culex*, *Mansonia*, *Psorophora*, *Coquillettidia*, *Haemagogus*, *Sabethes,* and *Ochlerotatus* are the most common vector species that transmit several diseases to humans and animals throughout the world, of which, the genes *Anopheles* is one of the most major vectors to transmit malaria (Souza *et al*., 2012; Naseem *et al*., 2016). Of more than 500 recently reported species of *Anopheles* mosquitoes, over 100 of the species are expected to transmit malaria to humans (Kamareddine, 2012; Pimenta *et al*., 2015; Asadollahi *et*

al., 2019). Among these, malaria vector *An. stephensi* in Afghanistan, Bahrain, Bangladesh, China, Africa, India, Iran, Iraq, Myanmar, Nepal, Oman, Pakistan, Saudi Arabia, and Thailand currently has become a wide concern for human health and the economy (Verardi *et al*., 2002). Moreover, in the Horn of Africa, this species has been found to play a key role became one of the most malaria transmit parasite species both *P. falciparum* and *P. vivax* (Sinka *et al*., 2020).

2.2.1 Major Afro-tropical malaria vectors

Africa is home to over 140 species of the genus *Anopheles*, among which the seven are efficient vectors of malaria (Sinka *et al*., 2010). The well-documented dominant vector species in the continent are *Anopheles gambiae*, *An. arabiensis*, *An. funestus*, *An. meles*, *An. merus*, *An. nili* and *An. moucheti* (Sinka *et al*., 2010, 2012). *An. stephensi*, an Asian urban vector, was reported from Djibouti, Ethiopia, and the Republic of Sudan (Seyfarth *et al*., 2019; Sinka *et al*., 2020). In Djibouti, it was associated with an unusual resurgence of malaria cases (Seyfarth *et al*., 2019). Its importance as an emerging threat was notified by WHO in August 2019 (WHO, 2019). Sinka *et al*. (2020) reported that recently over 126 million (40%) of people live in the Horn of African countries, in which they are at risk by malaria infection of *An. stephensi*.

2.2.2 Malaria vectors in Ethiopia

In Ethiopia, 44 anopheline species and subspecies have been recorded (Kyalo *et al*., 2017). However, only a few species are incriminated as vectors of malaria to date. *An. arabiensis* is the most efficient primarily malaria vectors, whereas *An. funestus*, *An. pharoensis*, and *An. nili* plays a much lesser role in transmission (Tulu, 1993; PMI, 2017). In the central part of Ethiopia, especially *An. pharoensis* is mostly distributed and usually transmits *P. vivax* to humans (Afrane *et al*., 2016). In addition, the South Asian urban malaria vector *An. stephensi* was first reported in 2016 from the Somalia Regional State, and currently, this vector has expanded to wider geographic areas of Ethiopia (Meshesha Balkew *et al*., 2020; Carter *et al*., 2021). It has been shown that *An. stephensi* is an established vector in Awash Sebat Killo, in eastern Ethiopia, and is highly permissive for local *P. vivax* and *P. falciparum* isolates (Temesgen Ashine *et al*., 2020; Fitsum Tadesse *et al*., 2021).

2.3 Biology and ecology of *Anopheles stephensi*

Anopheles (Cellia) *stephensi* Liston belongs to the Class Insecta, Order Diptera, Family Culicidae, and Genus: *Anopheles* (Nathan, 2007). Morphologically, adult *An. stephensi* resembles adult *An. maculatus* unless some unique characteristics such as *An. stephensi* is medium sized and having speckled palpi with two broad apical bands, and also the thorax is beset with broad scales, as well as the hind legs are not conspicuously marked in white (Gillies and Coetzee, 1987; Dharmasiri *et al*., 2017; WHO, 2019). Based on their egg morphological characteristics *An. stephensi* exist in three different ecological forms as type, intermediate, and mysorensis form, in which the type form of *An. stephensi* is an effective vector of malaria in urban areas, whereas the intermediate and mysorensis are considered to be rural as well peri-urban species with poor vectors capacity because mysorensis is essentially zoophilic in nature (Oshaghi *et al*., 2006; Tyagi *et al*., 2017).

Biologically, *An. stephensi* is holometabolous, they undergo complete metamorphosis such as egg, larva, pupa, and adult within two weeks under favored environmental conditions, unless it may be expanded within three weeks (Taylor *et al*., 2020). After once mated only adult females *An. stephensi* feed on animals and other alternatives host-specific blood to provide well development. Adult females *An. stephensi* laid approximately 50 to 200 eggs separately on the surface of the water regularly after 48 hours of adequate blood meal. The eggs are black boat-shaped, and also drifting to avoid sinking (Malhotra *et al*., 2009). After 72 hours of blood meal hatching first instar larvae. The larvae are active filter feeders and also they undergo three molts with four instars between 7 to 10 days, and there after immediately change into the pupal stage. The immature stages usually, larvae and pupa; complete their life cycle parallel to the water surface. Then, after two days (24-48 hours) adults emerge in the temperate areas and also immediately begin to living arboreal modes for 27 to 32 days of their life span (Thompson and Bell, 1968).

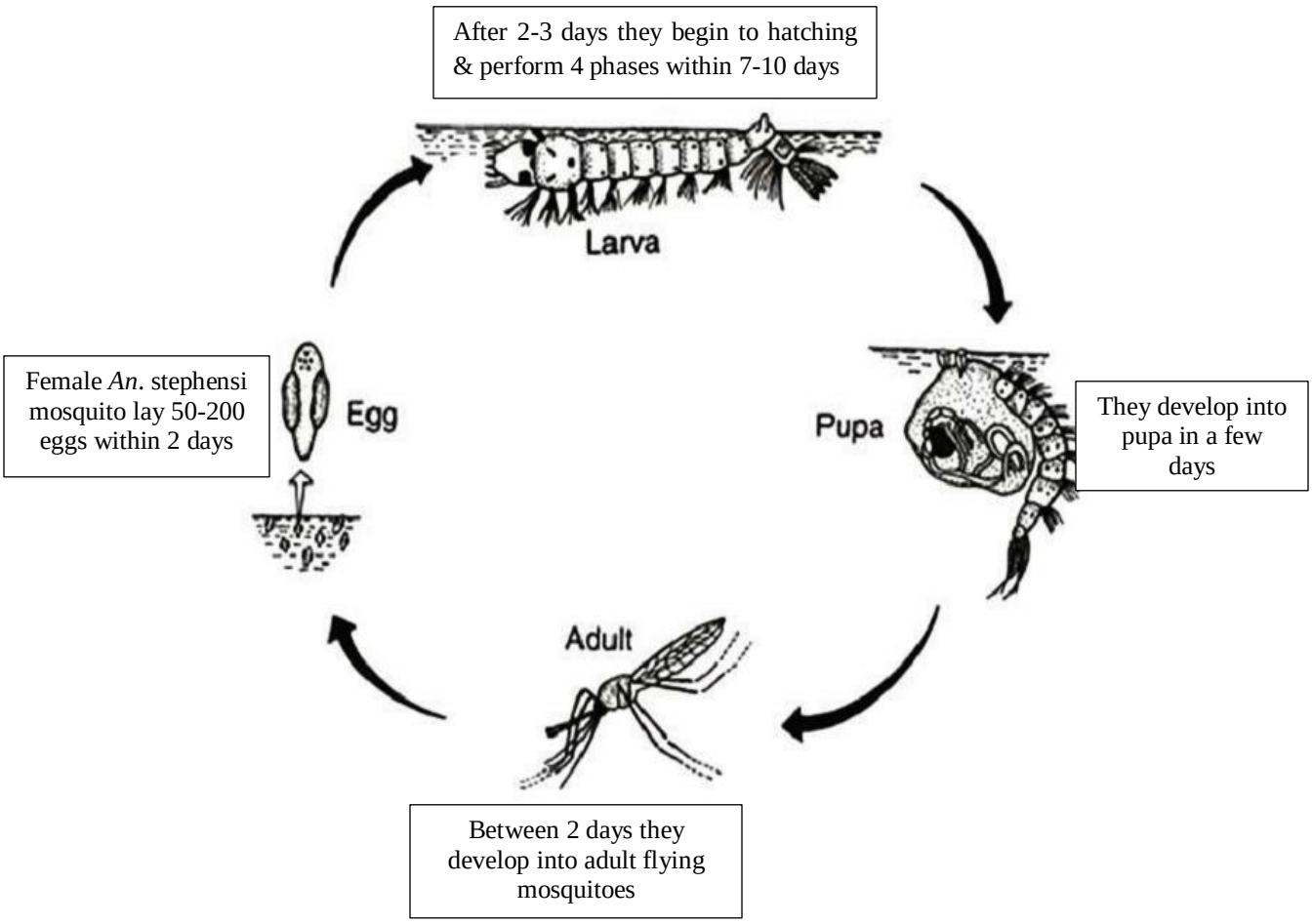

Plate 1: Life cycle of *Anopheles stephensi* (Source, Baranitharan, 2018)

An. stephensi more prefer to develop in pure and settled sunlight water bodies which are having some vegetative and organic matter (Thomas *et al*., 2016). However, where occur unfavored environmental conditions, like cryptic habitats with extremely cold in winter and hot in summer, polluted and high salinity water bodies *An. stephensi* has a great ability to quickly adapt and survive, but it may be the major cause for the decline in activity and the growth rate of the candidates (Vatandoost *et al*., 2006). *An. stephensi* survive long years in spring and also seasonally more spread during the summer June to August (Mehravaran *et al*., 2012). The immature larvae and pupa of *An. stephensi* coexist in the same breeding sites of the immature stage of *Aedes aegypti* and *Culex quinquefasciatus* (Yadav *et al*., 2017). Usually the immature stages of *An. stephensi* inhabit urban areas in various man-made containers, cisterns, wells,

tubes, and fountains, while in rural areas with shaded stream margins, pools, seepages, irrigation ditches, springs, and other water storage (Manouchehri *et al*., 1976; Sinka *et al*., 2011; Meshesha Balkew *et al*., 2021).

2.4 Behavior of *Anopheles stephensi*

2.4.1 Biting rhythm

Understanding the biting behavior of mosquito vectors is important to develop effective vector control methods (Damb ach *et al*., 2018). The biting activity of malaria vector, *An. stephensi*, varied in different season and across regions, its happens mostly in the evening and at night, as well as possible to occur at the daytime, but the great indoor biting activity of the vector occurred just before midnight usually between 20:00 to 24:00 hours, in addition, peak biting after midnight has also been observed between 03:00 to 06:00 hours (Korgaonkar *et al*., 2012; Dev and Sharma, 2013).

2.4.2 Feeding and resting behavior

The feeding preferences of *Anopheles* species are concerned with different hosts. *An. stephensi* appears to have some level of plasticity in its behavior, specifically in host choice, with rural *An. stephensi* tending to be more zoophilic than urban populations (Thomas *et al*., 2017; Mojahedi *et al*., 2019). After blood-feeding, the engorged females seek resting places and spend so much time until their eggs get to be matured (Rowland, 1989; Sumodan *et al*., 2004). This behavioral pattern of the vector is more critical for arranging satisfactory vector control techniques. Ghosh *et al*. (2010) and; Nagpal *et al*. (2012) noted that adult *An. stephensi* is primarily exophagic and exophilic malaria vector, which mainly rests in the rural areas near to human settlements like in cattle sheds, barracks, poorly constructed houses, stream beds, seepage canals, and other wells when warm weather conditions prevail. Unless, in the urban areas it is enophagic and endophilic mostly rest in the human dwellings like in clothes, hanging threads, cupboards, furniture, goods stacks on lofts, cobwebs, ceilings, floors, and walls (Dev and Sharma, 2013).

2.4.3 Breeding site

Anopheles mosquito vectors mainly prefer to breeds in small, numerous, scattered, and shifting fresh watery areas containing some organic matter, vegetables, and algae (Thomas *et al*., 2017; Zogo *et al*., 2019). Knowing such breeding habitats of malaria vectors would help to develop the best vector control options to reduce the spread of malaria disease (Arthiyan *et al*., 2018). Studies revealed that the breeding sites of *An*. *stephensi* found in various water bodies in both urban and rural areas, typically they breed in the urban areas particularly with artificial water containers, evaporation coolers, cisterns, overhead water tanks, building construction sites, cement mortar pond, domestic wells, pots, roof gutters, and other industrial areas (Tiwari *et al*., 2010; Nagpal *et al*., 2012; Biswas *et al*., 2015). Whereas in rural areas, particularly the immature stages of *An*. *stephensi* mainly found in freshwater pools, stream margins, stream beds, catch basins, seepage water holding, wells, and other domestic water storage receptacles (Surendran *et al*., 2019; Meshesha Balkew *et al*., 2020).

2.5 Malaria vector control strategies

The purpose of vector control is to prevent the transmission of diseases and nuisance in humans and their livestock, as well as limit or eradicate the spread of vector population to reduce vector morbidity and mortality all over the world (Berg *et al*., 2012). They require specific and feasible preventive measurements to decrease the incidence and prevalence of human infection and disease from malaria vectors as well as reduced distortion of countries' economies and satisfaction. Globally, effective methods used for malaria vectors control strategies are IRS and LLINs, space spraying, larval control, biological control, botanical control, environmental control, and personal protection (Ahmed *et al*., 2019). When applied, the combination of these different vector control interventions (IVM) on malaria vectors are according to international health standards targeted at a different life of stage within their ecological influence (WHO, 2006; Gueye *et al*., 2016). This will help to achieve the goals of objectives for malaria global technical strategies (MGTS) 2016-2030, which aimed at malaria elimination by 2030 for all malaria-endemic countries (Killeen *et al*., 2017; Gracio and Gracio, 2019).

2.5.1 Larval sources management

Larval source management (LSM) is the most potential and efficient control method for reducing the spread of malaria disease by killing or eliminating the immature stages of mosquito larvae and pupa from the breeding sites to disrupt the completion of vector life stages (Olalubi and Chinwe, 2016). Throughout the world, LSM is one of the main components of integrated vector management (IVM), particularly in North America, Europe, and tropical Africa (Fillinger and Lindsay, 2011). Once the immature stage of malaria vectors is removed from the breeding habitats of standing water bodies, by synthetic and/or natural larvicides, they neither emerge nor appear, so we can get rid of malaria incidence by using the larval control method (Keiser *et al*., 2005). WHO (2013) recommended that LSM as a supplement malaria control intervention to long-lasting insecticidal bed nets and indoor residual spraying where mosquito larval habitats are few, fixed, and findable. Generally, four types of LSM are applied in the water bodies to control the immature stage of mosquitoes, such as habitat modification, habitat manipulation, chemical larviciding, and biological control (McCann *et al*., 2017).

2.5.1.1 Habitat modification and manipulation

Community participation in environmental management targeted for mosquito breeding habitats is an effective and relatively inexpensive method of reducing and/or eliminating the incidence of malaria. Habitat modification is a permanent alternation of land and water environment that are potential vector breeding habitats to create unfavorable conditions for vector development (Walker and Lynch, 2007). Possible to control large scale area of malaria vector by habitat modification including landscaping, surface water drainage, filling and land reclamation, and complete coverage of water surfaces and other water storage containers with a material that's invulnerable to mosquitoes (Tusting *et al*., 2013). Another option is habitat manipulation, which changes environmental conditions temporarily to disrupt the vector breeding habitat (Bond *et al*., 2004). Recurrent activates in the malarious environment like water-level manipulation, fleshing of streams, drain clearance, source reduction, and shading or exposure of habitats to the sun can effectively reduce the spread of malaria (Wilson *et al*., 2020).

2.5.1.2 Biological control

A biological control method is preferable and effective from chemical control, as it is suitable for host-specific, long-term effective at relatively low doses, safe to human and non-target organisms, easy to handle, low cost of production in some cases, low risk of resistance development, and ideal for malaria vector control intervention (Das and Amalraj, 1997). Currently, among biological control agents, mainly entomopathogenic bacteria, fungi, and viruses, as well as natural enemies, are introduced in the water bodies to control the immature stage of larva and pupa of mosquito vectors is applied in various parts of malaria risk world countries (Fasil Adugna, 2020).

Entomopathogenic bacteria, especially *Bacillus thuringiensis* (*Bti*) and *Bacillus sphaericus* (*Bs*) are the most effective mosquitocidal bacteria worldwide, particularly in Europe and Africa, as well as in India mainly employed for controlling mosquito larvae (Kamareddine, 2012). *Bacillus* spp. control malaria vector without external influence due to releasing toxic crystals, receptor binding, and pore formation internally within the larval vector stomach to disturbed the osmoregulatory mechanism of the cell membrane (Becker, 2006). Other microbial agents, like *Wolbachia* spp. are induced male sterility and decreased adult lifespan of malaria vectors due to infection in the reproductive organs of the vectors by the formation of cytoplasmic incompatibility (Walker and Moreira, 2011).

Fungal pathogens belonging to the genera *Lagenidium*, *Coelomomyces*, *Culicinomyces*, *Beauveria*, *Metarhizium*, *Entomophthora*, *Lagenidium*, *Entomophaga*, *Neozygites*, *Erynia*, *Aschersonia*, *Verticillium*, *Nomuraea*, *Hirsutella*, and *Paecilomyces* widely infect the proportion of surviving mosquito vector populations by adhesion of the spores on the vector cuticle (Samuels *et al*., 2016).

Mosquito viral pathogens such as baculoviruses, cytoplasmic polyhedrosis viruses, densoviruses, cypoviruses, entomopoxviruses, and iridoviruses have shown promise as vector larvicides by interacting themselves with the immature stages of the vectors immune system (Agboli *et al*.,

2019). Even if these different viral infections progressed to the vector adult stage, the disease transmitted vertically to the subsequent generation (Bukhari *et al*., 2013).

Natural enemies, mainly predatory fishes were used as a biological control tool against immature mosquito vectors in the pre-DDT era (Louca *et al*., 2009). And even still, larvivorous fishes are the main components of biological vector control strategy in various parts of malaria risk world countries, particularly in Greece, Italy, Georgia, Spain, India, Malaysia, Madagascar, and Papua New Guinea (Chandra *et al*., 2008; Kamareddine, 2012). It is possible to reduce the density of the target vector population by introducing indigenous and/or exotic predatory fishes into certain vector breeding water habitats (Lou and Zhao, 2016). Walshe *et al*. (2017) noted that different types of predatory fishes had been introduced so far for controlling mosquito larvae, but mainly *Gambusia affinis*, *Poecilia reticulate*, *Tilapia*, and *Aphanius dispar* larvivorous fishes are used frequently around the world in attempts to control malaria vectors.

2.5.1.3 Chemical larviciding

Chemical control remains the most important and widely used strategy against mosquito vectors. Throughout the world, synthetic insecticide was the first to use for malaria vector control in the late 1940s. After World War II thus expanding the notoriety of insecticide-based control additionally ended up to victory amid in 1947 especially pyrethroids and organophosphates because of their low cost and high effectiveness (Wilson *et al*., 2020). Until 1972 where some chemicals before being banded, insecticides extract from the main chemical classes such as carbamates, organochlorines, organophosphates, and pyrethroids have been used for mosquito control (Okumu and Moore, 2011; Gebrehiwet Tesfahuneygn and Gebremichael Gebreegziabher, 2019).

Regular application of synthetic or natural insecticides to water bodies in the form of oils, monolayer films, wettable powders, or as formulations like tablets, granules, emulsifiable concentrates, and water-dispersible granules against the immature stage of mosquito is effective in reducing malaria (WHO, 2016; Wilson *et al*., 2020). Larvicides decrease malaria transmission consequently by affecting the larval nervous system (Senthil-Nathan, 2020), among the synthetic

chemical compounds, mainly temephos, fenthion, malathion, chloropyrifos, pyrethroids, and insect growth regulators (IGRs) like methoprene, pyriproxyfen, diflubenzuron, and triflumeron as well as naturally occurring petroleum oils and alcohol-based surface product insecticides have been used widely for controlling larval anopheline mosquitoes (Zewdneh Tomass *et al*., 2011; Choi *et al*., 2019). Of these, mainly the use of temephos, menthoprene, and pyriproxyfen in potable water bodies is recommended by the WHO, however, because of environmental safety and resistance development currently like chloropyrifos, deltamethrin, permethrin, and malathion larval synthetic insecticides, as well as petroleum products, are not recommended by WHO for the control of immature stage malaria vectors (Bukhari *et al*., 2013; WHO, 2013).

2.5.2 Adult mosquito control

Control of adult mosquitoes by mechanical barriers and natural or synthetic insecticides with different modes of mechanisms such as indoor residual spraying, insecticide-treated bed net, and space spraying is an essential facet to reduce the infection and transmission of malaria (Raghavendra *et al*., 2011).

2.5.2.1 Indoor residual spraying

Indoor residual spraying (IRS) is the application of long-lasting residual insecticides, which is one of the most core effective control interventions against adult indoor feeding (endophagic) and indoor-resting (endophilic) malaria vectors (WHO, 2015). Internationally, the use of IRS vector control intervention tool is as old as humanity and still widely used primarily in many malarious countries (Gebrehiwet Tesfahuneygn and Gebremichael Gebreegziabher, 2018). The World Health Organization (WHO) and other international organizations have launched The Malaria Eradication Program in 1955, out of this program, IRS and ITNs have been the most aggressive control interventions that contributed to reducing malaria transmission and achieving the ambitious vector elimination goals in various parts of the world (Gebrehiwet Tesfahuneygn and Gebremichael Gebreegziabher, 2018; Tizifa *et al*., 2018).

IRS with long-acting chemical insecticides takes place before the onset of the peak transmission season both in the urban and rural vector resting areas such as internal walls and roofs of households, public buildings, storerooms, barns, or animal dwellings (Gracio and Gracio, 2019). It involves spraying on a large scale in the high transmission of the inside housing structures with an insecticide once or twice a year should be rapidly reduced the vector density and longevity as well as human contacts before they can become infective and transmit the diseases, particularly among older female vector mosquitoes, therefore, the IRS is very effective and efficient in reducing the severity of malaria as long as people are willing to accept and receive permanent housing (WHO, 2006).

Previously, indoor residual spraying with different chemical compounds such as carbamates (like propoxur), organochlorides (like DDT and dieldrin), and organophosphates (like malathion and fenitrothion) with alternative formulations had been used for antimalaria programs in various malaria-endemic countries, particularly in Asia, Russia, Europe, Iran, Latin America, and some Southern African countries like in Mozambique, Swaziland, and Zimbabwe (Bayoh et al., 2010; Killeen, 2014; Choi *et al*., 2019). In African regions IRS against malaria vectors is endemic, but protection coverage is limited, particularly in some East African malarious countries (Tangena *et al*., 2020).

Some synthetic chemical compounds, mainly pirimiphos-methyl, propoxur, chlorfenapyr, and bendiocarb, adulticides still used for the IRS on a limited scale in some malarious areas (Choi *et al*., 2019). The persistent or residual impact varies with the kind of insecticide, its formulation, dosage applied, type of surfaces sprayed, and climatic conditions (WHO, 2015). Currently, chemical compounds like dichloro-diphenyl-trichlorethane (DDT) are banned in various countries due to the subsequent development of resistance by the major malaria vectors and environmental hazards (Yive and Knols, 2020). Universally, IRS protection percentages declined from a peak of 5% in 2010 to 2% in 2019, in 2018, about 93 million households were sprayed with insecticides (IRS), of which more than 87% were in sub-Saharan Africa (WHO, 2019). Similarly, in Ethiopia, 595,618 households with 44 districts were conveyed to IRS (PMI, 2018). Currently, more than 97 million peoples are at risk of malaria infection were protected by IRS in 2019 (WHO, 2020).

2.5.2.2 Insecticide-treated mosquito nets

Around the world, ITNs including, LLINs, are another current vector control intervention and effective tool for preventing and controlling the spread of malaria, as well as significantly reducing the burden and nuisance of other vectors by barrier the physical contact between the infected vectors and human beings (Hill *et al*., 2006; Messenger *et al*., 2017; Tizifa *et al*., 2018). Bed nets impregnated with insecticide are ready to use multiple washes and stay viable to stand the late-night and indoor-biting malaria vectors for up to three years (Soleimani-Ahmadi *et al*., 2012; Dahmana and Mediannikov, 2020). ITNs reduced indoor biting malaria vectors by repels, disables, or kills when the vector comes into contact with the impregnated insecticide netting material (Okumu and Moore, 2011).

Of the four chemical compounds that are used to control malaria vectors worldwide, the only pyrethroid is licensed for LLINs (Raghavendra *et al*., 2011). However, currently, pyrethroids such as permethrin and deltamethrin insecticides are resisted by *Anopheles* mosquitoes in various parts of malaria risk areas, particularly in sub-Saharan Africa (Riveron *et al*., 2018). WHO (2007) recommends all households to have distributed and sleep under LLINs, but primarily focused and targeted children under age five and pregnant women, who are at high risk of malaria infection. According to the WHO malaria reports in 2019, the percentage of ITNs within the household's protection coverage increased 3% to 52%, but the use of awareness is still diminished mainly in African countries. In 2018, 72% of the populations were protected by long-lasting insecticidal nets (LLINs) (WHO, 2019). Recently, the prevalence of ITNs throughout the world was estimated to be 253 million in 2019, of which 84% of them were covered by countries in sub-Saharan Africa thus 46% of the peoples are at risk of malaria infection was protected by ITNs (WHO, 2020).

2.5.2.3 Space spraying

Space spraying, either aerial or ground application techniques, is more effective for decrease disease transmission from *Anopheles* and other certain exophilic and exophagic vectors that are not affected by IRS and LLINs (WHO, 2003). Throughout the world, space spraying is another integral component of vector control intervention, particularly in low and middle-income

countries like Turkey, Mauritius, and Sir Lanka (Gueye *et al*., 2016). Presently, in India, Tanzania, and El Salvador widely applied ground-based space-sparing against adult malaria vectors (Killeen *et al., 2017).* Besides, it has been used regularly in public health and pest control programs (Pryce *et al*., 2018).

WHO (2019) advises not to prioritize space spraying before IRS or ITNs for malaria vector control, but if it intends to be spray during an outbreak by insecticides may be carried out following the technical guidance provided by WHO. Outdoor smoking or fine droplets with regular insecticides early in the morning or late in the evening, particularly aerosol against adult malaria vector by using handheld, ground vehicle, or aircraft-mounted equipment to produce a fog, is highly effective in epidemic situations where rapid reduction the existence of mosquito vectors (WHO, 2006). Previously, pyrethrum and pyrethroid compounds, dichlorovos, and other similar insecticides were widely used in aerosol to kill flying malaria vectors (Ngufor *et al*., 2020). Applied one time of space-sparing, either ULV (cold) or thermal types of fogging with suitable insecticides into the air, it can treat or control a large scale of vector resting areas, therefore, rapidly destroy the mass of adult outdoor biting and outdoor flying vector population (Gari and Lindtjørn, 2018).

2.5.3 Botanical control

Vectors control by natural products can reduce the impact on human health, the environment, and the economy. In humans, plants have always been present as a source of health throughout history, according to different fossil records indicates that humans used plants for medicinal and vector control properties during the Middle Paleolithic age (Karunamoorthi *et al*., 2014). Traditionally, many plants have been used by the ancient peoples hierarchically in numerous parts of the world to protect themselves and their livestock from troublesome species such as anthelmintics (Feyisa Kuma *et al*., 2015), haematophagous (particularly for mosquitoes, fleas, lice, ticks, and mites) (Wanzala, 2017; Bullitta *et al*., 2018), and phytophagous pests (Pavela, 2016). Moreover, in ancient times mainly in Rome, Europe, North America, Asia, and Africa country certain herbs and barks of some trees were smoked, fragrant plants were hanging in houses, rubbing vinegar on the body, and oil formulation applied to the skin, clothes, or other

surfaces to killed and/or repelled against mosquitoes and other biting vector species (Kihampa, 2011; Maia and Moore, 2011; Pavela and Benelli, 2016).

Among the plant family, mainly Asteracae, Cladophoraceae, Labiatae, Meliaceae, Oocystacae, and Rutaceae, with several representative species have been reported to have great potential for mosquitoes (Mohankumar *et al*., 2016). Traditionally, the use of herbs for cultural and other disease control properties is one of the century-old practices in Africa, particularly in sub-Saharan tropic Africa vector endemic countries, commonly peoples have used indigenous medicinal plant *Allium sativum* (Nchu *et al*., 2004), *Azadirachta indica* (Tiwari *et al*., 2014), *Chrysanthemum cinerariaefolium* (Palsson and Jaensona, 1999), *Commiphora myrrha* (Mulgeta Lemenith and Demel Teketay, 2003), *Eucalyptus citriodora*, *Hyptis suaveolens* (Isman, 2006; Kihampa, 2011), *Rosmarinus officinalis*, *Juniperus communis* (Nabti and Bounechada, 2019), *Nicotiana tabacum* (Sarker and Lim, 2018), and *Ocimum* spp. (Kishore *et al*., 2014), were reported to protect themselves from mosquito bites and insect pests.

In Ethiopia, around 80% of the human population and 90% of animals depend on traditional medicines for treating ailments (Tadesse Birhanu *et al*., 2015). Conventionally, many plants have been used by various cultures and local communities in different forms of human and animal treatments, as well as pest control during their lifetime (Dawit Kidane *et al*., 2013; Genene Bekele and Reddy, 2015). Regarding this, Getachew Alebie *et al*. (2017) reviewed that large number of local medicinal plants are traditionally used for the treatment of malaria in various regions of Ethiopia, particularly in the western lowlands of Oromia, Amhara, Tigray, Southern Region, and almost the entire areas of Benishangul Gumuz and Gambella.

The role of medicinal plants traditionally wellbeing care framework will not reduce with the future because they are both culturally viable and expected to remain affordable and service of modern health care is both restricted and costly (Sileshi Degu *et al*., 2020). Ethnobotanical insecticide derivatives from various medicinal plants have rich phytochemical compounds such as phenolic, alkaloids, terpenoids, rare amino acids, plant amines, and glycosides (Bilal and Hassan, 2012) that are suitable candidates for the development of defense mechanisms viz., ovicidal, larvicidal, pupicidal, and adulticidal activities against various vectors (Gebrehiwet

Tesfahuneygn and Gebremichael Gebreegziabher, 2018). Without external influences, some secondary metabolites that accumulate from various parts of the plant such as barks, leaves, roots, flowers, fruits, seeds, cloves, rhizomes, and stems have good potential to inhibit vector development and reproduction, while others act as repellents, attractants, feeding deterrents or antifeedants, and toxicants (Hikal *et al*., 2017). The efficiency of botanical insecticides depends on their chemical composition and target vectors (Lengai *et al*., 2020). Mainly, essential oils extracted from various volatile plants consist of complex mixtures of active substances like alcohols, esters, ether, aldehyde, ketones, lactones, phenols, terpenes, and sesquiterpenes are high repellency against mosquitoes and other biting vectors (Afzal *et al*., 2018).

The effectiveness of bio-pesticide against mosquito vectors without posing hazards of toxicity on the non-target organisms has been reported previously by many researchers (Tehri and Singh, 2015). To date, many investigations are in progress for finding operative, environmentally friendly, and relatively inexpensive plant compounds for mosquito control, mainly in the form of ovicidal, larvicidal, adulticidal, and repellent activities.

Several herbal products are effective in killing the immature stages of mosquito vectors. In recent years, Dey *et al*. (2020) tested the larvicidal and ovicidal potentiality of petroleum ether, chloroform, methanol, and aqueous leaf extract of *Piper longum* against three important mosquito larvae of *An*. *stephensi*, *Ae*. *aegypti*, and *Cx*. *quinquefasciatus* in the laboratory condition and found an aqueous extract of *P*. *longum* had the highest larval mortality after 24h exposure. Kazempour *et al*. (2021) also found the larvicidal activities from the essential oils of *Mentha pulegium*, *Satureja hortensis*, and *Thymus vulgaris* (LC_{50} = 40.1324, 34.1527, and 53.1063 ppm, respectively) against *An*. *stephensi* larvae.

Likewise, Araya Eukubay *et al*. (2020) in Ethiopia, found good (LC_{50} = 84.85 ppm) larvicidal activity from *Calpurnia aurea* methanol crude leaf extract against 3rd instar *An. arabiensis* larvae in laboratory condition. Similarly, Mihretu Tarekegn *et al*. (2020) also reported larvicidal properties of *Parthenium hysterophorus* petroleum ether root extract against 4th instar *An. arabiensis* larvae. Wondimu Ersino *et al*. (2020) also showed the larvicidal potential of *Juniperus procera* leaves extract with chloroform, petroleum ether, ethanol, and acetone against

3rd to 4th instar *An. arabiensis* larvae. Another study, Hirpasa Teressa *et al*. (2019) investigated petroleum ether, chloroform, acetone, and ethanol leaf extracts of *Olea europaea* against 3rd to 4th instar larvae of *An. arabiensis*. Ayalew Jejaw *et al*. (2017) showed that *Phytolaca dodecandra* seed powder and its extract were promising larvicidal activity against 3rd instar larvae of *An. arabiensis* both in laboratory and field conditions. In the same way, Damtew Bekele *et al*. (2014) also reported that methanol leaf extract of *Aloe pirottae* and *Acokanthera schimperi* had quite potency (LC_{50} = 76.344 and 282.76 ppm, respectively) against 4th instar larvae of *An. arabiensis*. Moreover, previously, Fekadu Massebo *et al*. (2009) tested the larvicidal activity of 11 indigenous medicinal plants essential oils against the early 4th instar *An. arabiensis* and *Ae. aegypti* larvae within different concentrations and found from *Ocimum lamiifolium, Chenopodium ambrosioides, Schinus molle, Ocimum suave,* and *Piper nigrum* essential oils to have a strong larvicidal effect on the anopheline vector in simulated field conditions.

Phytochemical plant products are also known to have adulticidal activities against *Anopheles* mosquitoes. Zahir *et al*. (2010) conducted an experimental study of adulticidal activities and its inhibition of hexane, chloroform, ethyl acetate, and acetone leaves extract from seven medicinal plants of *Anisomeles malabarica, Euphorbia hirta, Gloriosa superba, Ocimum basilicum, Ricinus communis, Splanum trilobatum* and *Tridax procumbens* against *An. stephensi* and found effective adulticidal activities from the chloroform and acetone extract of *G. superba, T. procumbens, R. communis, S. trilobatum,* and ethyl acetate extract of *O. basilicum* and also observed quite potent to inhibition of adult emergence from the ethyl acetate extract of *A. malabarica*, chloroform extract of *O. basilicum, S. trilobatum,* acetone extract of *R. communis, T. procumbens* and seed extract of *G. superba*.

In the same way, Fekadu Massebo *et al*. (2013) from Ethiopia, conducted a CDC bioassay of *Chenopodium ambrosioides, Eucalyptus citriodora, Eucalyptus globules, Lippia adoensis, Mentha spicata, Nigella sativa, Ocimum lamiifolium, Ocimum suave, Piper nigrum, Schinus molle*, and *Thymus vulgaris* essential oils against adult *An. arabiensis* in a laboratory colony and found essential oil from *O. suave* could induce 100% of adult mortality with LC_{50} and LC_{90} values of 0.0014 and 0.0027 ml% v/v, respectively. Besides, Damtew Bekele *et al*. (2014) also

found high (LC_{50} = 18.74 and 24.30 ppm, respectively) adulticidal activity from the *Oreosyce africana* methanol leaf extract and *Piper capense* fruit extracts against *An. arabiensis*.

Essential oils extracted from mainly volatile plants such as citronella, clove, lemongrass, rosemary, dill, eucalyptus, lavender, soybean, chrysanthemum, castor, tulsi, camphor, limeone, geranium, neem, galbanum, pepper mint, cedar, and basil have known to be quite potent on mosquito repellents (Naseem *et al*., 2016; Asadollahi *et al*., 2019). Moreover, Moemenbellah-Fard *et al*. (2020) conducted the experimental study of repellent activity from the essential oils of nine medicinal plants viz., clove, dill, eucalyptus, lemon, myrtle, orange, peppermint, tarragon, and zingiber against *An. stephensi*, and found 100% repellent effect for 1-60 min at a concentration of 0.5% (v/v), of this the most potent, was recorded from the essential oil of clove.

Likewise, Sisay Dugassa *et al*. (2009) in Ethiopia found around 78.69% of repellency effects from some aromatic plants against the main malaria vector *An. arabiensis* and *An. pharoensis* in the field condition. Besides, Bezayit Solomon *et al*. (2012) have also obtained more than 70% of repellent protection for 3 and 4h at a concentration of 10% and 20%, respectively from the essential oils of *Cymbopogon citratus*, *Cymbopogon nardus*, and *Eucalyptus citriodora* against *An. arabiensis*. Another study, Ephrem Abiy *et al*. (2015) recorded 70% of repellent effects for 3-1h, respectively from the 20% of neem and chinaberry essential oils against *An. arabiensis*.

2.5.4 Personal protection

Self-defense from mosquito bites is one of the prophylactic tools of malaria prevention. Personal control during the biting periods of anopheline vectors by using a variety of protective methods like preferably insecticide-treated nets, wearing clothes that cover most of the body, and insect repellent on exposed skin are also possible to reduce malaria incidence (Plourde *et al*., 2005). WHO (2019) recommended that when the peoples are not within housing stricture and basic intervention methods such as the IRS and/or ITNs are not available it, use topical repellents, insecticide-treated clothing, and spatial/airborne repellents to protect oneself from insect bites, but, mainly special or airborne repellents on malaria vectors are not recommended by WHO until more studies assessing malaria epidemiological outcomes have been conducted.

CHAPTER 3: MATERIALS AND METHODS

3.1 Description of test plants

3.1.1 *Calpurnia aurea* (Ait.) Benth.

Calpurnia aurea is a flowering plant in the family Fabaceae. It has various vernacular names in Amharic such as Digita, Lytta, and Zikita (Hedberg and Edwards, 1989). The habit of this plant is either shrub or small tree 1-10 m tall. Young branches and inflorescences densely pubescent, *Calpurnia aurea* situated in forest margins, bush-land or grassland, favored by overgrazing about 1650-3000m altitudes in most Ethiopian regions, in Eritrea, westwards to the Central Africa Republic and south to South Africa and also in India (Zeven and De Wet, 1982). In Ethiopia, *C. aurea* is used for treating various diseases such as coughs, ameba, syphilis, leishmaniasis, malaria, rabies, diabetes, lung TB, tapeworm, scabies, elephantiasis, abscesses, dandruff, and wounds as well as stomach ache, and eye diseases (Fekadu Fullas, 2001; Shemsu Umer *et al*., 2013). It also used both wound healing and anti-inflammatory effects of mice (Getachew Ayal *et al*., 2019), antioxidant and antibacterial activities (Wondimu Gebre, 2019). Abiyot Berhanu *et al*. (2006) reported that *C. aurea* leaf and seed extracts act as insecticides and repellents activity. Besides, to prevent stored maize weevils (Berhanu Hiruy and Emana Getu, 2018). Commonly, *C. aurea* extracts in southern Ethiopia use to kill ticks and head lice (Zorloni *et al*., 2010).

Plate 2: Leaves of *Calpurnia aurea* (Source, Brhane Hadera and Eyasu Alemseged, 2019).

3.1.2 *Momordica foetida* Schumach. Et Thonn

Momordica foetida is classified under the family Cucurbitaceae, and it has the vernacular names of Yekura-hareg and Marqura in Amharic (Edwards *et al*., 1995). The habits of this angiosperm mostly exist as trailing or climbing herbs up to 3m or more. It is mainly occurring in the forest and thicket margins, wooded grassland, and cultivated places within the altitudinal range of 530-3450 m above sea level (Madala *et al*., 2016). Moreover, it is distributed in most Ethiopian regions of tropical Africa (Acquaviva *et al*., 2013). *M. foetida* medicinal herb uses naturally for food in various African countries (Chacha and Laswai, 2020). Different parts of this plant are mainly used in traditional medicine to treat smallpox, dropsy, malaria, pancreatic, hypertension, peptic ulcers, diabetes mellitus, stomach ache, headache, and earache (Ngarivhume *et al*., 2015). In addition, it has antioxidant and antibacterial activities (Froelich *et al*., 2007). In Ethiopia, the root part of *M. foetida* is mainly taken internally for asthma, leaves for toothache, and fruits for scabies (Gelahun Abate, 1989; Nigussie Amsalu *et al*., 2018). It has been reported that this plant species is toxic to weevils, moths, and ants and as an insect repellent (Watt and Breyer-Brandwijk, 1962). Furthermore, this plant product is found to be effective against insect bites and tick infestation (Damtew Bekele *et al*., 2012).

Plate 3: Leaves of *Momordica foetida* (Source, Chacha and Laswai, 2020).

3.1.3 *Zehneria scabra* (Linn. f.) Sond.

Zehneria scabra (Amharic name: Aregresa) is a climber plant species classified under the family Cucurbitaceae. The habit of this plant is a slender climber or trailer up to 10m high, and it is found in wet or moist forest and on forest margins, riverine fringes, and exotic plantations within the altitudinal range of 900-2100m (Bizuneh Woldeab *et al*., 2018). Furthermore, it is widely distributed in Ethiopian regions, mountains of tropical Africa, Arabia, India, Java, and the Philippines (Bereket Abew *et al*., 2014). *Z. scabra* has been used in various African countries for local healing. Mainly, in Ethiopia, this plant is commonly utilized in conventional medication to treat skin diseases, gonorrhea, alopecia, syphilis, diarrhea, malaise, mumps, fever, taeniasis, constipation, conjunctivitis, swelling, rabies, and fever as well as uterus cleansing, headache, eye infection, evil eye, and michi role were also accounted for this herb (Ejigu Bayu *et al*., 2018). Moreover, their antimalarial and antimicrobial activity has been reported (Admassie Endalkachew and Wubshet Hailu, 2014). The plant is also found to be effective for protecting stored maize grains from insect weevil infestations (Matovu and Olila, 2009).

Plate 4: Leaves of *Zehneria scabra* (Source, Bizuneh Woldeab *et al*., 2018).

3.2 Collection of plant samples

The fully developed fresh leave samples of *Calpurnia aurea* and *Momordica foetida* were collected around Bahir Dar University campus located at 11°34'28.0"N, 37°23'53.4"E, altitude

1801 m, while the leaves of *Zehneria scabra* were collected from Akaki district, Addis Ababa (8°49'40.5"N, 38°50'23.6"E, altitude 2117 m). The plant species were authenticated by a plant taxonomist from the National Herbarium (Department of Plant Biology and Biodiversity Management), Addis Ababa University. The voucher specimens (MM-01 of *M. foetida*, MM-02 of *C. aurea*, and MM-03 of *Z. scabra*, respectively) were deposited at the National Herbarium, College of Natural and Computational Sciences of Addis Ababa University, Ethiopia.

3.3 Preparation of plant samples

The leaves were washed with tap water and air-dried separately under shade at room temperature for two weeks in the Insect Sciences Lab. Department of Zoological Sciences, Addis Ababa University. Finally, the dried leaves were manually ground by pestle and mortar and sieved until it becomes a fine powder. Each of the leaves powders was then kept at room temperature in labeled plastic bags until used (see Annex 1).

3.4 Extraction of the plant samples

The extraction processes were conducted in the Organic Chemistry Laboratory of the Department of Chemistry, College of Natural and Computational Sciences, Addis Ababa University. Each, powdered leaf of the test plant was soaked in 500 ml of Erlenmeyer flask with 99.9% of hexane and methanol, and distilled water at a ratio of 1:10 (W/V). Afterward, the mixtures were sonicated by ultrasonic cleaner apparatus (USC-T, Malaysia) at 20 kHz frequency with 11W power for two days twice per day for 15 minutes. The mixtures were allowed to settled and then cooled at room temperature at least for 10 minutes. The resulted from the supernatant of hexane and methanol crude extracts was filtered through Whatman No.3 (Whatman International Ltd., Maidstone, England) filter paper, while the aqueous extracts using suction filtered through a Buchner funnel with Whatman filter paper No.1. The filtrates of hexane and methanol crude extracts were concentrated using a vacuum rotary evaporator (Rota-vapor-RE, Switzerland) under reduced pressure at 40^{o}C, while the aqueous crude extracts were evaporated to dryness using a lophilizer. The residue obtained from each plant extracts was left to cool at room temperature to remove traces of solvent, and then finally, were collected separately in a Wheaton

glass bottle containers and were preserved a refrigerated at 4°C until used for experimentation (see Annex 2).

3.5 Rearing of *An. stephensi*

Larvicidal and adulticidal bioassays were conducted with colony larvae and adults of *An. stephensi* (susceptible strain), maintained at 28±3^{o}C temperature with 70±10% relative humidity in the Aklilu Lemma Institute of Pathobiology (ALIPB) insectary, Addis Ababa University.

The larvae were kept in plastic trays containing de-chlorinated water and were maintained at under 12:12h light and dark photoperiod cycles in the laboratory following the standard mosquito rearing procedure WHO (2005). The larvae were fed yeast (Saf-Instant yeast). The media were changed every three days. The pupae formed were collected by a glass beaker and transferred to the cloth cages for adult emergence. Newly emerged adults were maintained in mosquito cages under identical conditions and fed sterile 10% sugared solution soaked in cotton pads within Petri dishes. The cotton was always kept moist with the solution and changed every day. In addition to sugar feeding, female mosquitoes were allowed to take blood meals from a restrained rabbit three times a week for egg development and oviposition. Moist filter papers in cups were placed inside rearing cages for oviposition by gravid female mosquitoes. The eggs were washed off with deionized water onto larval rearing trays and allowed to hatch into neonate larvae in the laboratory. Third to fourth instars larvae and starved 2 to 5 days old adult female *An. stephensi* was used continuously for larvicidal and adulticidal tests, respectively.

3.6 Preparation of stock solution

One gram of each crude plant extract was placed separately in 250 ml Wheaton glass bottles and dissolved in 100 ml of distilled water for methanol and aqueous extracts, while the hexane extracts were first dissolved in 4 ml of acetone and then added 96 ml of distilled water to prepare finally 100 ml of 1% stock solution. In the stock solution, one drop of emulsifier (Tween 80) was added to each extract at a concentration of 0.05%. From each stock solution of crude extracts, 1 ml with concentrations of 25, 50, 100, 150, 200, 250, and 300 ppm for the larvae, whereas 1 ml

with a concentration of 20, 40, 80, 160, and 320 ppm for the adult were prepared for exposure of the target mosquito.

3.7 Larvicidal bioassay

The larvicidal activities of *An. stephensi* in each leaf crude extract were performed according to WHO standard procedure (WHO, 2005). From the stock solution, further dilutions, constituting 25, 50, 100, 150, 200, 250, and 300 ppm of test concentrations were prepared (see Annex 3). The bioassay was initiated in four batches of 20 late 3^{rd} - early 4^{th} instar larvae and they were introduced in 300 ml capacity of enamel cups with 99 ml volume of distilled water. After a minute, unhealthy or damaged larvae were removed and replaced. Then, to each test cap were added 1 ml of desired crude extract test concentration to prepare the final 100 ml volume of test solution. Besides, a mixture of 4 ml of acetone and one drop Tween 80 were made up to 100 ml in a standard test cups by adding distilled water to serve as the negative control solution. In addition, temephos at the rate of 0.25 ppm within a similar volume of test cups was used as a positive control (Tikar *et al*., 2011). The experiment was conducted for 24 hrs at the optimum conditions of 28±3°C temperature and 70±10% relative humidity with under 12:12 light and dark photoperiod in the insectary. During the exposure period, no food was offered to the larvae. Each experiment was replicated three times on different days along randomly set up with appropriate control and standard. The numbers of dead larvae were counted after 24 hrs of exposure, and the percentage of mortality was reported from the averages of twelve replicates. The dead larvae included moribund, those incapable of rising to the surface in each concentration of treatments, and the standards were combined separately and expressed as the average mortality to determine LC_{50} and LC_{90} values (see Annex 5).

3.8 Adulticidal bioassay

The adulticidal activities of each crude extract were assessed using the CDC bottle bioassay method (Brogdon and McAllister, 1998; CDC, 2010). Almost all synthetic insecticides were reported to be resisted by adults of malaria vector *An. stephensi*, thus, in this bioassay does not use it as a standard comparison. From 1% of each plant extracts stock solution, were prepared 1

ml with acetone contained 20, 40, 80, 160, and 320 ppm to desired different test concentrations (see Annex 4), and coated in cleaned and dried 250 ml of wheaton glass bottles (Sigma-Aldrich). Additionally, 1 ml with a mixture of acetone and emulsifier were added to the control bottle handled as before. The bottles and their cups were rotated gently to make all sides covered with the test solution. After that, the caps were removed and rolled continuously on their side and were left horizontally overnight within aluminum foil in the insectary until the solution was completely dried. Three batches of 20, 2–5 days old, adult sucrose-feeding and blood-starved female *An. stephensi* was introduced into each treatment and control coated glass bottle for two hours. After two hours of exposure, the treated mosquitoes were transferred into 250 ml capacity plastic cups, where the test mosquitoes were kept for 24 hrs. During the 24 hrs held period, the test mosquitoes were provided with 10% of sugar solution in a pad of cotton soaked in sugar solution. Mortality was assessed both in the treatments and the control assay after 2 hrs introduced up to 24 hrs after the treatment performance (see Annex 6). The percentage of adult mortality was corrected using Abbott's formula (Abbott, 1925) when needed.

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

3.9 Data analysis

The mean percentage mortality was analyzed using SPSS version 25.0 software (SPSS Inc, Chicago, IL, USA), and dose-dependent mortality also were performed by R software version 4.0.5. The percentage mortality of larvae and adult were checked for normality by 1-Sample Kolmogorov-Smirnov Z test (K-S). When trapping data did not confirm to the normal distribution, the non-parametric equivalent tests of Kruskal-Wallis were followed. P-values were adjusted with the Bonferroni correction to adjust for the inflation of type I errors when several Mann–Whitney tests are performed (Dytham, 2011). The LC_{50} and LC_{90} values were calculated using a generalized linear logistic regression binomial model, considering a 95% significance interval.

CHAPTER 4: RESULTS

4.1 Larvicidal activity of plant extracts against *An. stephensi*

The results of larval mortality were obtained from bioassays of aqueous and methanol extracts of *Calpurnia aurea*, *Momordica foetida*, and *Zehneria scabra* leaves against *An. stephensi* larvae after 24 hrs exposure periods are presented in Tables 1-3 and Figure 1. All aqueous and methanol test plant extracts showed low, moderate, and high larvicidal activities tested between 25 ppm-300 ppm treatments against the late 3rd to early 4th instar larvae of *An. stephensi*. Within the same exposure period, larval mortality was not recorded for the hexane crude leaf extracts and the negative control, while the standard (temephos) achieved 100% of larval mortality.

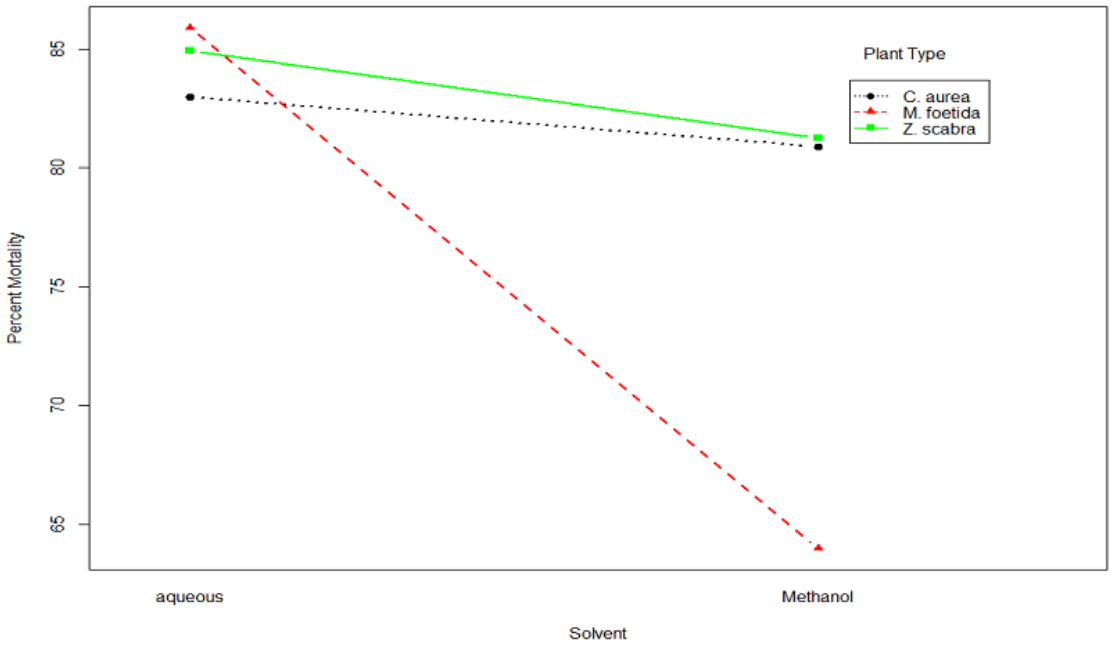

Figure 1: The effects and relationship of aqueous and methanol test plant extract exposure at 25-300 ppm of treatments against the late 3rd and early 4th instar *An. stephensi* larvae.

There was significant difference and interaction effects between all aqueous and methanol test plant extract against the tested larvae (Kruskal-Wallis test, $P < 0.05$; Fig 1). Besides, the mean percent larval mortality between treatments, control, and the standard groups had a statistically

significant difference (Kruskal-Wallis test, $P < 0.05$).The mortality effect of the test plant extracts against *An. stephensi* larvae were dose and extraction solvent dependent.

In the *C. aurea* test plant, the highest (100%) larval mortality was found both in aqueous and methanol extracts at 200 ppm and 250 ppm of treatments, respectively (Table 1). There were no significant differences detected in mean percent mortality between aqueous and methanol extracts of *C. aurea* at ≥ 150 ppm, which caused more than 95.00% mortality and standard check (Mann-Whitney U test, $p > 0.002$; Table 1). However, all *C. aurea* treatments had a significant effect on larval mortality compared to the negative control (Mann-Whitney U test; $p < 0.002$).

Table 1: Mean % larval mortality of *C. aurea* crude leaf solvent extracts at different rates against the late 3rd and early 4th instar *An. stephensi* larvae after 24 hours of exposure.

Concentration(ppm)	**% Mean mortality ± SE**	
	Solvents	
	Aqueous	**Methanol**
25	35.00±2.6$^{\mathbf{Aa}}$	23.75±3.65$^{\mathbf{Aa}}$
50	60.00±3.54$^{\mathbf{Ba}}$	35.00±0.00$^{\mathbf{Bb}}$
100	86.25±2.23$^{\mathbf{Ca}}$	90.42±1.4$^{\mathbf{Ca}}$
150	99.58±0.42$^{\mathbf{Da}}$	95.00±2.46$^{\mathbf{DCa}}$
200	100.00±0.00$^{\mathbf{Da}}$	96.67±1.42$^{\mathbf{Da}}$
250	100.00±0.00$^{\mathbf{Da}}$	100.00±0.00$^{\mathbf{Da}}$
300	100.00±0.00$^{\mathbf{Da}}$	100.00±0.00$^{\mathbf{Da}}$
Temephos (0.25)	100.00±0.00$^{\mathbf{Da}}$	100.00±0.00$^{\mathbf{Da}}$
Control	0.00±0.00$^{\mathbf{Ea}}$	0.00±0.00$^{\mathbf{Ea}}$

*Each value of % mean mortality ± SE represents the value of twelve replicates

*Means values followed by the same letters across column (Upper case) and row (Lower case) are not statistically significantly different (Multiple-Mann Whitney U-test; $P > 0.002$).

Aqueous and methanol extracts of the *M. foetida* test plant showed 100% larval mortality at 200 ppm and 250 ppm of treatments, respectively (Table 2). Moreover, larvicidal activity found from aqueous extracts of *M. foetida* at ≥ 100 ppm, while its methanol extracts at ≥ 200 ppm caused

mortality (ranging 95% to 100%) was not significantly ($p > 0.002$; Mann-Whitney U test) different from that the mortality achieved in temephos exposed on *An. stephensi* larvae (Table 2). Statistical differences were observed between all *M. foetida* treatments and negative control ($p < 0.002$; Mann-Whitney U test).

Table 2: Mean % larval mortality of *M. foetida* crude leaf solvent extracts at different rates against the late 3rd and early 4th instar *An. stephensi* larvae after 24 hours of exposure.

Concentration(ppm)	% Mean mortality ± SE	
	Solvents	
	Aqueous	**Methanol**
25	27.5 ± 2.85^{Aa}	4.55 ± 1.4^{Ab}
50	80.83 ± 4.51^{Ba}	20.83 ± 2.53^{Bb}
100	95.00 ± 1.74^{Ca}	45.83 ± 2.20^{Cb}
150	97.92 ± 0.96^{Ca}	71.67 ± 3.39^{Db}
200	100.00 ± 0.00^{Ca}	97.5 ± 2.50^{Ea}
250	100.00 ± 0.00^{Ca}	100.00 ± 0.00^{Ea}
300	100.00 ± 0.00^{Ca}	100.00 ± 0.00^{Ea}
Temephos (0.25)	100.00 ± 0.00^{Ca}	100.00 ± 0.00^{Ea}
Control	0.00 ± 0.00^{Da}	0.00 ± 0.00^{Fa}

*Each value of % mean mortality ± SE represents the value of twelve replicates

*Means values followed by the same letters across column (Upper case) and row (Lower case) are not statistically significantly different (Multiple-Mann Whitney U-test; P>0.002).

Among the two solvent extracts of *Z. scabra*, statistically non-significant differences were observed (Mann-Whitney U test, $p > 0.002$; Table 3). However, their larvicidal activity had significantly different from negative control (Mann-Whitney U test; $p < 0.002$). At the highest concentrations (≥ 250 ppm) both aqueous and methanol *Z. scabra* crude leaf extracts resulted in 100% larval mortality (Table 3). Moreover, aqueous extract of *Z. scabra* at ≥ 100 ppm, while its methanol extracts at ≥ 200 ppm, caused larval mortality of more than 95.42% had no significant effect compared to mortality achieved in temephos treatment ($p>0.002$; Mann-Whitney U test).

Table 3: Mean % larval mortality of *Z. scabra* crude leaf solvent extracts at different rates against the late 3rd and early 4th instar *An. stephensi* larvae after 24 hours of exposure.

Concentration(ppm)	% Mean mortality ± SE	
	Solvents	
	Aqueous	**Methanol**
25	29.17±3.68^{Aa}	23.75±2.69^{Aa}
50	73.33±3.9^{Ba}	66.25±5.04^{Ba}
100	95.42±1.44^{Ca}	87.08±2.17^{Ca}
150	97.08±1.44^{Ca}	92.50±1.44^{Da}
200	99.50±0.42^{Ca}	99.17±0.83^{Ea}
250	100.00±0.00^{Ca}	100.00±0.00^{Ea}
300	100.00±0.00^{Ca}	100.00±0.00^{Ea}
Temephos (0.25)	100.00±0.00^{Ca}	100.00±0.00^{Ea}
Control	0.00±0.00^{Da}	0.00±0.00^{Fa}

*Each value of % mean mortality ± SE represents the value of twelve replicates

*Means values followed by the same letters across column (Upper case) and row (Lower case) are not statistically significantly different (Multiple-Mann Whitney U-test; P > 0.002).

The lethal toxicity doses of aqueous and methanol crude leaf extracts of *C. aurea*, *M. foetida*, and *Z. scabra* against larvae of *An. stephensi* are shown in Figure 2. The dose-response curves showed that all crude leaf extracts had a good performance. Moreover, the aqueous *M. foetida* crude leaf extract showed strong larvicidal activity, having an LC_{50} value of 34.61 ppm and LC_{90} value of 57.61 ppm, followed by *Z. scabra* (LC_{50} = 35.85 ppm; LC_{90} = 68.26 ppm) and *C. aurea* (LC_{50} = 38.69 ppm; LC_{90} = 108.28 ppm). Similarly, methanol leaf extract of *Z. scabra* had good larvicidal activities with LC_{50} and LC_{90} values of 41.32 ppm and 99.07 ppm followed by *C. aurea* (LC_{50} = 43.25 ppm; LC_{90} = 96.02 ppm). However, the crude methanol leaf extract of *M. foetida* had lower larvicidal toxicity against the larvae of *An. stephensi* (LC_{50}= 99.50 ppm; LC_{90}= 188.76 ppm, Figure 2).

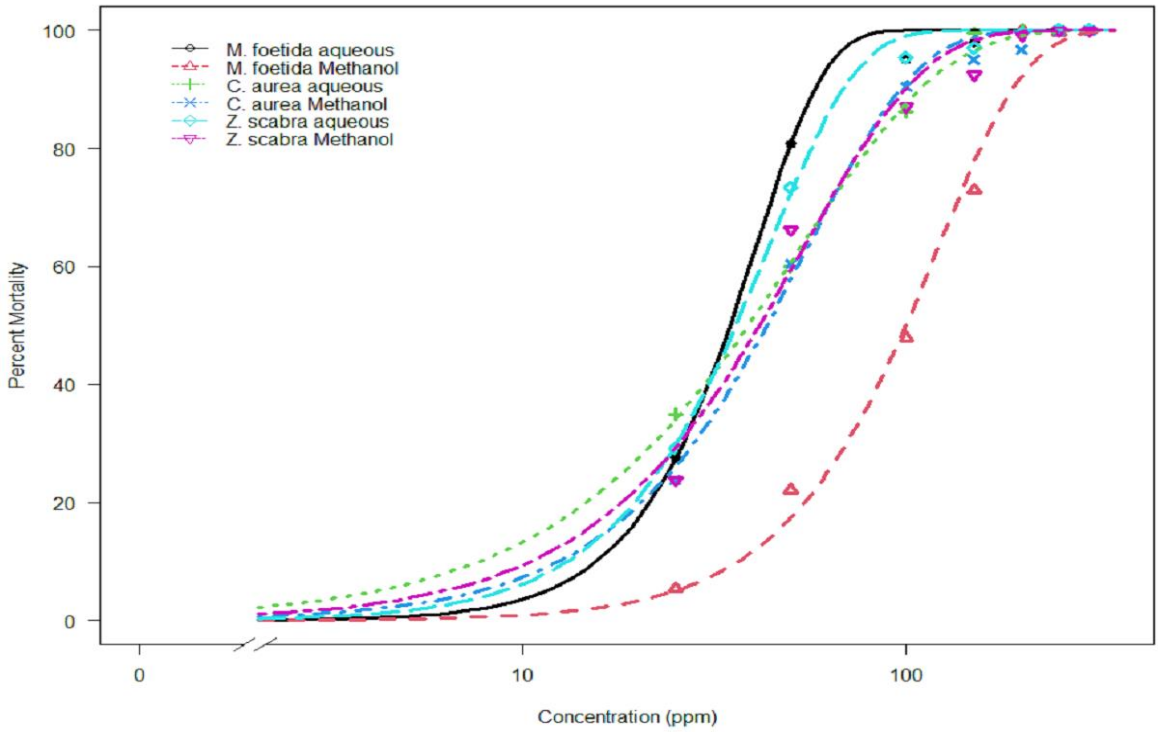

Figure 2: Dose-response curve mortality of *An. stephensi* larvae after 24 hrs exposure to aqueous and methanol extracts of *C. aurea*, *M. foetida*, and *Z. scabra*. The LC_{50} and LC_{90} were calculated using a generalized linear model, considering a 95% significance level

4.2 Adulticidal activity of plant extracts against *An. stephensi*

Tables 5-7 show the mean percentage mortalities of adult *An. stephensi* after 24 hrs exposures to different solvent extracts of *C. aurea*, *M. foetida*, and *Z. scabra*. Statistically significant differences in mean percentage mortality among the different extracts and the negative control were observed (Kruskal-Wallis test, $P < 0.05$). The adulticidal data showed that the mortality rate of the vector was directly proportional to the concentration and extraction solvents. Between the different solvent leaf extracts of *C. aurea*, only aqueous extract gave 55% adult mortality of *An. stephensi* at 320 ppm, whilst other crude solvent leaf extracts had lower adulticidal activity, having adult mean % mortalities ranging from 0-48% (Table 4).

Table 4: Mean % adult mortality of *C. aurea* crude leaf solvent extracts at different treatments against *An. stephensi*

Concentration(ppm)	% Mean mortality ± SE		
	Solvents		
	Aqueous	**Methanol**	**Hexane**
20	1.67 ± 1.67^{Aa}	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Aa}
40	6.67 ± 1.67^{Aa}	6.67 ± 1.67^{Ba}	0.00 ± 0.00^{Ab}
80	16.67 ± 1.67^{Ba}	16.67 ± 1.67^{Ca}	0.00 ± 0.00^{Ab}
160	21.67 ± 1.67^{Ba}	26.67 ± 3.33^{Da}	6.67 ± 3.33^{Bb}
320	55.00 ± 5.77^{Ca}	48.33 ± 1.67^{Ea}	18.33 ± 1.67^{Cb}
Control	0.00 ± 0.00^{Da}	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Aa}

*Each value of % mean mortality ± SE represents the value of three replicates

*Means values followed by the same letters across column (Upper case) and row (Lower case) are not statistically significantly different (Multiple-Mann Whitney U-test; $P > 0.003$).

Different solvent (aqueous, methanol, and hexane) crude leaf extracts of *M. foetida* resulted in lower adult mortalities of *An. stephensi* (< 24%) at all treatments tested in the bioassays, and were not that effective against adults of malaria vector *An. stephensi* (Table 5).

Table 5: Mean % adult mortality of *M. foetida* crude leaf solvent extract at different concentrations against *An. stephensi*

Concentration(ppm)	% Mean mortality ± SE		
	Solvents		
	Aqueous	**Methanol**	**Hexane**
20	0.00 ± 0.00^{ABa}	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Aa}
40	1.67 ± 1.67^{Ba}	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Ab}
80	5.00 ± 2.89^{Ba}	1.67 ± 1.67^{Ba}	0.00 ± 0.00^{Ab}
160	13.33 ± 1.67^{Ca}	13.33 ± 1.67^{Ca}	0.00 ± 0.00^{Ab}
320	18.33 ± 4.41^{Ca}	23.33 ± 1.67^{Da}	1.67 ± 1.67^{Bb}
Control	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Aa}

*Each value of % mean mortality ± SE represents the value of three replicates

*Means values followed by the same letters across column (Upper case) and row (Lower case) are not statistically significantly different (Multiple-Mann Whitney U-test; P > 0.003).

There were statistically significant differences in mean percentage mortality among different concentrations of aqueous, methanol, and hexane leaf extracts of *Z. scabra* and with negative control (Kruskal-Wallis test; P < 0.05; Table 6). Within the given time intervals adult mortality was recorded for the control treatment. However, the highest adult mortality (84%) at higher concentration 320 ppm against *An. stephensi* was recorded in aqueous crude leaf extract of *Z. scabra* followed by its methanol extracts (65%) and their adulticidal potential was statistically different from the negative control (Mann Whitney U-test; P < 0.003, Table 6).

Table 6: Mean % adult mortality of *Z. scabra* crude leaf solvent extracts at different concentrations against *An. stephensi*

Concentration(ppm)	**% Mean mortality ± SE**		
	Solvents		
	Aqueous	**Methanol**	**Hexane**
20	4.33±2.6$^{\mathbf{Aa}}$	11.67±3.33$^{\mathbf{Aa}}$	0.00±0.00$^{\mathbf{Aa}}$
40	11.67±1.67$^{\mathbf{Ba}}$	20.00±2.89$^{\mathbf{ABa}}$	0.00±0.00$^{\mathbf{Ab}}$
80	26.67±3.33$^{\mathbf{Ca}}$	23.33±3.33$^{\mathbf{Ba}}$	0.00±0.00$^{\mathrm{Ab}}$
160	36.67±6.00$^{\mathbf{Ca}}$	41.66±4.40$^{\mathbf{Ca}}$	15.00±2.89$^{\mathbf{Bb}}$
320	84.00±2.89$^{\mathbf{Da}}$	65.00±5.77$^{\mathbf{Db}}$	20.00±2.89$^{\mathbf{Bc}}$
Control	0.67±0.45$^{\mathbf{Ea}}$	65.00±5.77$^{\mathbf{Db}}$	0.67±0.45$^{\mathbf{Aa}}$

*Each value of % mean mortality ± SE represents the value of three replicates

*Means values followed by the same letters across column (Upper case) and row (Lower case) are not statistically significantly different (Multiple-Mann Whitney U-test; P > 0.003).

The lethal effects of different crude leaf extracts against adults of *An. stephensi* are shown in Figure 3. Due to the fact, all hexane and *M. foetida* crude leaf extracts caused lower adulticidal activities, only aqueous and methanol *C. aurea* and *Z. scabra* leaf extracts were subjected to dose-response bioassay to detect the lethal concentrations because. From the dose-response curves, lowest LC_{50} (= 176.20 ppm and 205.41 ppm) and LC_{90} (= 425.13 and 883.11 ppm) values

were demonstrated from the aqueous and methanol *Z. scabra* crude leaf extracts respectively (Figure 3), and its relative toxicities against adult *An. stephensi* was much higher than the rest test plant extracts. While the highest LC_{50} (= 297.75 ppm and 333.27 ppm) and LC_{90} (=762.63 ppm and 1031.74 ppm) values were revealed from the aqueous and methanol *C. aurea* crude leaf extracts, respectively, and its relative toxicities on adult malaria vector *An. stephensi* was much lower than *Z. scabra* crude extracts.

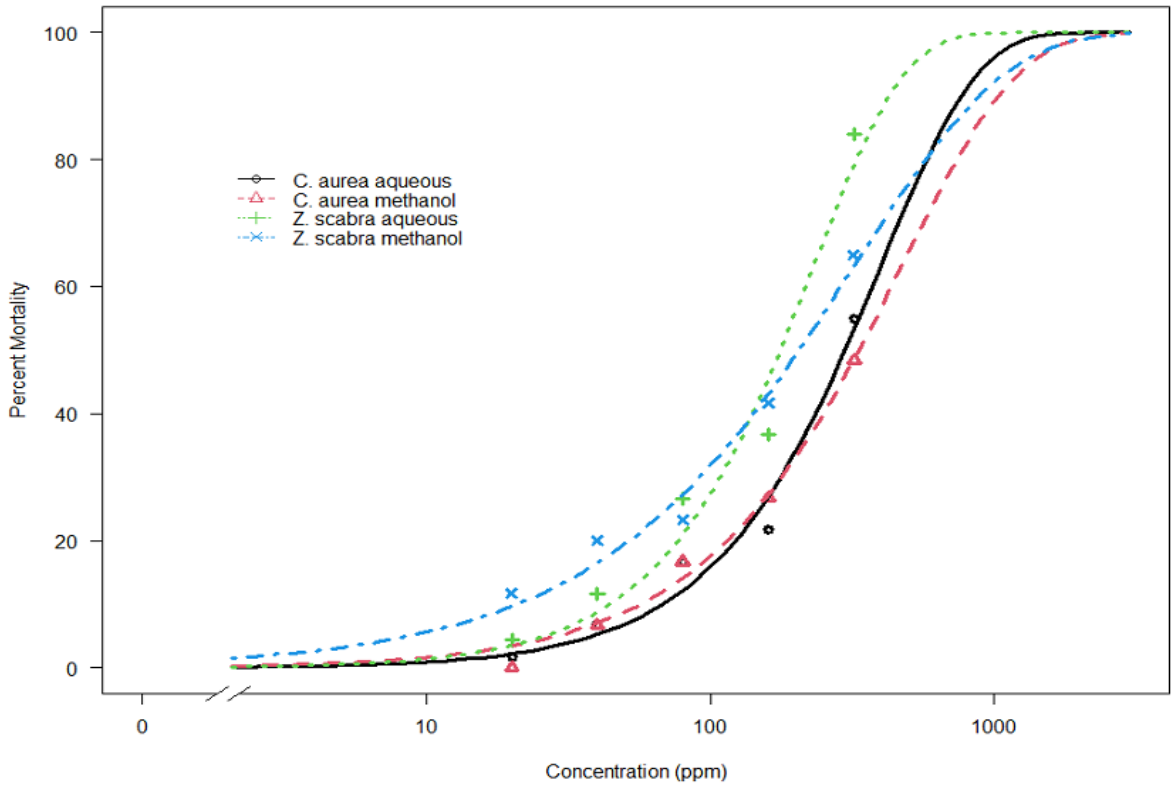

Figure 3: Dose-response curve of mortality *An. stephensi* adult exposed to aqueous and methanol extracts of *C. aurea* and *Z. scabra*. The LC_{50} and LC_{90} were calculated using a generalized linear model, considering a 95% significance level.

CHAPTER 5: DISCUSSION

Due to environmental concerns and the development of insect resistance to synthetic insecticides, the recent trend is to explore plants to obtain extracts that are safe for non-target animals and do not pose any residue problem but are still able to suppress vector populations. In view of this, the present study tested the larvicidal and adulticidal activities of crude leaf solvent extracts of *C. aurea*, *M. foetida*, and *Z. scabra* against *An. stephensi*. In this study, all hexane crude leave extracts showed no insecticidal activity on malaria vector *An. stephensi*, while aqueous and methanol extracts of the test plants achieved lower, moderate, and high mosquitocidal activity. This suggests the presence of more polar solvent-soluble phytochemicals in leaves of *C. aurea, M. foetida,* and *Z. scabra,* which are responsible for the observed bioactivities of these plants against *An. stephensi*. These findings are in agreement with that of Chore *et al*. (2014) who suggested that solvent polarities used for extraction could influence the mortality of mosquito vectors.

Among the tested crude leaf extracts on the late 3rd to early 4th instar *An. stephensi* larvae, aqueous extracts of *M. foetida* and *Z. scabra* at ≥ 100 ppm, while *C. aurea* at ≥ 150 ppm of treatments caused larval mortality ranging 95% to 100% after 24 hrs of exposure had similar effects to the standard larvicide temephos which also induced 100% of larval mortality at a similar exposure period. In addition, methanol *C. aurea* test plant extracts at ≥ 150 ppm, while *M. foetida* and *Z. scabra* at ≥ 200 ppm of treatments which caused more than 95.00% larval mortality, had also a comparable effect with the standard check during this study.

Ghosh *et al*. (2012) reported that insecticidal effects of plant extracts can vary due to the difference in plant species, mosquito species, geographical variation, extraction methodology, and polarity of solvents used during extraction. In addition, Abubakar and Haque (2020) also reported that the quantity and quality of phytochemical extracts from plants are decided by the type of solvent and its extraction methods. Similarly, the finding of the current study varies among concentrations and extraction solvents. Therefore, in agreement with this study, all aqueous crude leaf extracts proved to be sufficiently effective on larvae of *An. stephensi*. Interestingly, the aqueous crude leaf extract of *M. foetida* was found to be an extremely potent

larvicidal activity with low LC_{50} and LC_{90} values of 34.61 and 57.61 ppm, respectively, compared to the rest two plant extracts assayed concurrently. However, aqueous extract of *M. foetida* caused larvicidal activity against *An. gambiae* and *An. coluzzii* larvae with LC_{50} values of 593.96 and 505.19 ppm, respectively (Njuabe *et al*., 2021). This high difference in larvicidal activities with the current finding could be due to differences in the mosquito species, extraction methods, locality of the plant, preparation of test concentration, and parts of the plant which are different from those used in this study.

No previous reports on the insecticidal activity of *Z. scabra* crude extracts against mosquito vectors were reported elsewhere. This study demonstrated the effective larvicidal activity from the *Z. scabra* aqueous (LC_{50} = 35.85 ppm; LC_{90} = 68.26 ppm), and methanol (LC_{50} = 41.32 ppm; LC_{90} = 99.06 ppm) crude leaf extracts against *An. stephensi* larvae. In addition, their larvicidal activities also showed better results than *C. aurea* extracts. Previously, the extract of *Z. scabra* was found to be effective against maize weevils (Matovu and Olila, 2009). Moreover, Sangeetha *et al*. (2013) reported that its extracts at a low dose were potent against crown rot pathogens. These indicate that the plant has bioactive secondary metabolites that can be used to control insects. The presence of secondary metabolites claimed to have insecticidal activities such as phenol, tannins, flavonoids, and glycosides were revealed in methanol extract of *Z. scabra* leaf (Admassie Endalkachew and Wubshet Hailu, 2014; Wondmagegn Tamiru *et al*., 2014). Therefore, the strong larvicidal activity from this plant against *An. stephensi* larvae could be associated with the presence of these active secondary metabolites.

In this study, crude aqueous and methanol extracts of *C. aurea* leaf were also shown to have high larvicidal activity against *An. stephensi* larvae with respective LC_{50} and LC_{90} values of (38.69 ppm, 108.28 ppm), and (43.25 ppm, 96.02 ppm), respectively. This finding is comparable with the methanol extract of *C. aurea* leaves caused high larval mortality against *An. arabiensis* with LC_{50} and LC_{90} values of 84.85 and 192.29 ppm, respectively (Araya Eukubay *et al*., 2020). However, aqueous crude extract of *C. aurea* leaf caused strong larvicidal activity against Mediterranean fruit fly larvae *Ceratitis capitata* with LC_{50} value of 4.49 ppm (Abaynesh Birhanu and Abebe Asale, 2015). Secondary metabolites mainly alkaloids, flavonoids, terpenoids, tannin, saponin, and phenols compounds which are expected to have potential activity on larval

mortality were recorded from the aqueous and methanol crude leaf extracts of *C. aurea* (Araya Eukubay *et al*., 2020; Getachew Mulatu, 2020).

Methanol extract of *M. foetida* leaf showed to have relatively lower larvicidal efficacy than other test extracts against *An. stephensi* larvae with LC_{50} = 99.50 and LC_{90} = 188.76 ppm, in the present study. However, this result is preferable to methanol *M. foetida* extract against the larvae of *An. gambiae* and *An. coluzii* with LC_{50} values of 276.32 and 235.31 ppm, respectively (Njuabe *et al*., 2021). A previous study demonstrated that *M. foetida* is known for its insecticidal and repellent activities against mosquitoes (Abiyot Berhanu *et al*., 2006). Moreover, the application of its extracts in the prevention of mosquito bites and tick infestation was reported in Eastern Shewa Zone, Ethiopia (Damtew Bekele *et al*., 2012). In addition, the crude aqueous extract of *M. foetida* leaf revealed the presence of high phenolic and flavonoid secondary metabolites during the study of Molehin and Adefegha (2014).

The values of LC_{50} and LC_{90} obtained by the larvae in this study are lower than those reported by Dey *et al*. (2020) who found that the maximum larvicidal activity from the *Piper longum* aqueous (LC_{50} = 133.42 ppm; LC_{90} = 279.61 ppm) and methanol (LC_{50} = 134.71 ppm; LC_{90} = 464.73 ppm) crude leaf extracts against *An. stephensi*. In another study, Mohankumar *et al*. (2016) showed insecticidal promising from the methanol extract of *Annona reticulate* leaf against *An. stephensi* larvae with LC_{50} and LC_{90} values of 262.71 and 636.94 ppm, respectively. Besides, Mathew *et al*. (2009) also found less larvicidal efficacy from the methanol *Clitoria ternatea* leaf and *Nyctanthes arbortristis* flower crude extracts with LC_{50} values of 555.6 ppm and 679.4 ppm, respectively against larvae of *An. stephensi*. The difference in efficacy with the current study might be occurred probably since leaves of *C. aurea*, *M. foetida*, and *Z. scabra* have high quality and quantity of phytochemical compounds that cause insecticidal activity. Therefore, this study suggests that the three plant species can be preferable and have a beneficial role in the control of mosquitoes.

Plant extracts can be used either as insecticides for killing larvae or adult mosquitoes or as repellents for protection against mosquito bites, depending on the sort of activity they have (Sukumar *et al*., 1991). In this study, the effects of test plant extract on adults of *An. stephensi*

are remarkably less than those recorded on larvae. The adulticidal activity of *M. foetida* crude leaf extracts showed less effectiveness against *An. stephensi* and it caused no more statistically significant difference from the control treatment. This low activity can be due to lack of toxic bioactive secondary metabolites that causes adult mortality in the plant.

In the present study, low LC_{50} values against the adults of *An. stephensi* was found from the aqueous (LC_{50} = 176.20 ppm) and methanol (LC_{50} = 205.41 ppm) crude leaf extracts of *Z. scabra*, as compared other test plant extracts. This result was in line with finding of Matovu and Olila (2010) where the extracts of *Z. scabra* had effective insecticidal activities against aphids, glowworms, and mill bugs. In contrast, aqueous and methanol extracts of *C. aurea* showed less mortality against adults of *An. stephensi* with LC_{50} values of 297.75 and 333.28 ppm, respectively, in the present study. However, their larvicidal activity showed that greater effects than the *M. foetida* extracts. This suggests that the chemical constituents present in the leaf extracts arrest the metabolic activities of adult *An. stephensi*. In a related study, Berhanu Hiruy and Emana Getu (2018) obtained potent adulticidal activity from the aqueous, ethanol, and acetone extracts of *C. aurea* against adult of maize weevils. Although, the WHO does not specify any criteria for classifying the larvicidal and adulticidal potentialities of plant products, some authors use LC_{50} values as a criterion for activity level determination. Specifically, considered the product is very active if LC_{50} <50 ppm, the product is active if LC_{50} <100 ppm, the product is moderate if LC_{50} is between 100 ppm-200 ppm, and the product is weakly effective if LC_{50} is between 200 ppm-750 ppm, while the product is inactive if LC_{50} >750 ppm (Komalamisra *et al*., 2005; Dias *et al*., 2014). Accordingly, the adulticidal activity of *C. aurea* extract showed that weakly effective against *An. stephensi*.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study demonstrated that crude aqueous and methanol extracts of *C. aurea*, *M. foetida*, and *Z. scabra* leaf have high insecticidal efficacy against the late 3rd to early 4th instar larvae of *An. stephensi*. In addition, their larvicidal properties between the higher concentrations (100-300 ppm) were similar to the effect of the standard larvicide temephos. Furthermore, crude leaf extracts of *C. aurea* and *Z. scabra* are potential candidates for insecticidal activity against the adults of the tested mosquito. Among the test extractions, aqueous extracts yield more potent insecticidal activity than methanol extracts. Therefore, the results of the current study could be valuable sources for the development of effective, safe, target-specific, biodegradable, and cheap botanical insecticides for vector control, potentially leading to improved resistance management.

6.2 Recommendations

Based on the above conclusion, the following recommendations have been forwarded:-

- ✓ Further study must be conducted to the identification and isolation of bioactive compounds and fractionation of the three test plant extracts for further mosquito insecticide development.
- ✓ Insecticidal activities and residual efficacy of the test extracts should be evaluated under field condition.
- ✓ Studies should be conducted to assess toxicity of the test plant extracts to non-target organisms.
- ✓ Further investigations are also needed on the three plant extracts for developing effective formulations to be utilized in IVM measuring through community participation.

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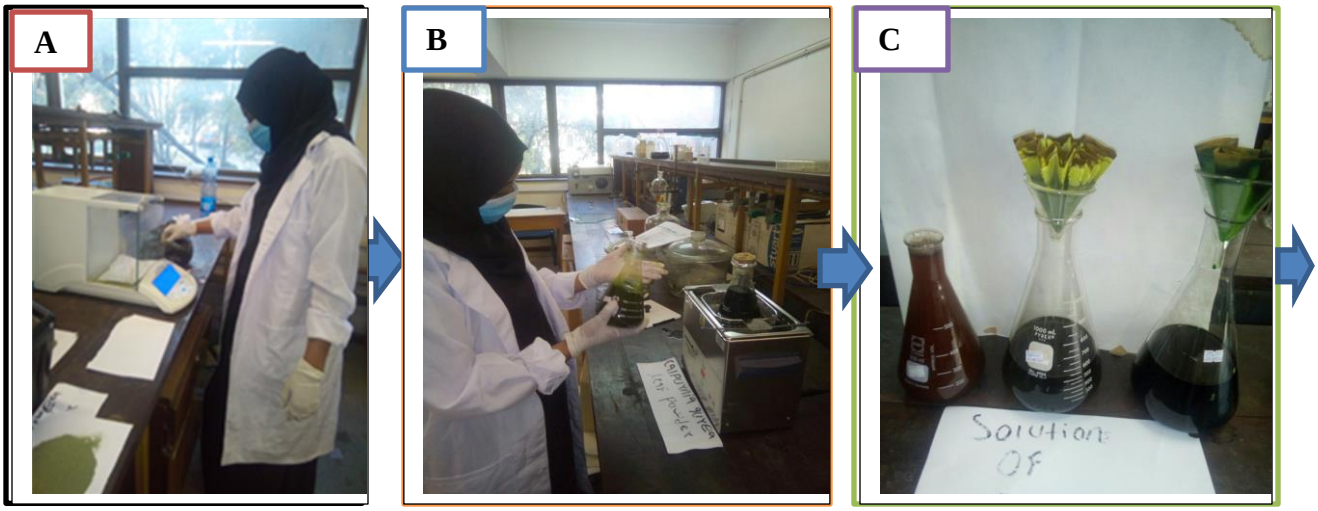
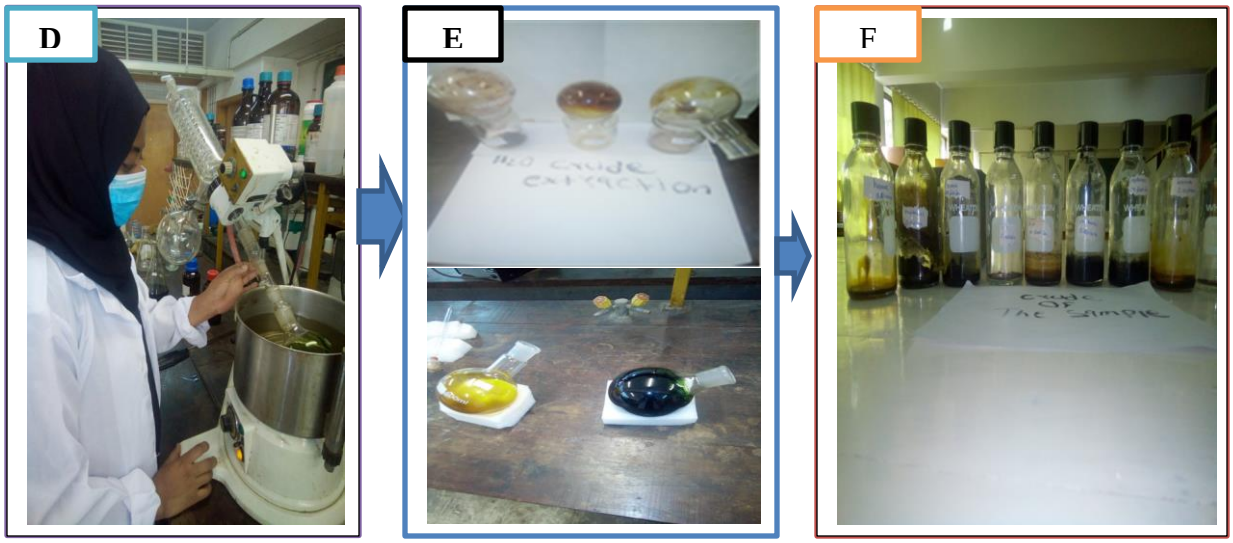
Appendices

Leaf of *Calpurnia aurea* (**A**), *Momordica foetida* (**B**), and *Zehneria scabra* (**C**) separately under shade at room temperature for drying, and their powders (**D**)

Annex 1: Pictures of Test plants preparations





Scaling of the samples powder (**A**), extracting (**B**), the mixture of the solutions (**C**), exposing (**D**), the concentration of the remaining supernatants (**E**), and all sample crude extracts (**F**).

Annex 2: Crude extractions processes

Annex 3: Preparation of different concentrations from 1% stock solutions for larvicidal test

Initial solution (%) (10,000 ppm)	**Aliquot (ml; ppm)**	**Final concentration in each test cup when 1ml (PPM) of stock solution added in 100 ml of distilled water**
1.0	0.25	25.0
	0.5	50.0
	1.0	100.0
	1.5	150.0
	2.0	200.0
	2.5	250.0
	3.0	300.0

PPM = parts per million

Annex 4: Preparation of different concentrations from 1% stock solutions for adulticidal test

Initial solution (%) (10,000 ppm)	Aliquot (µg/bottle)	Final concentration (1ml; PPM) of stock solution coated in a CDC bottle
1.0	20	20
	40	40
	80	80
	160	160
	320	320

Using a formulation of;-

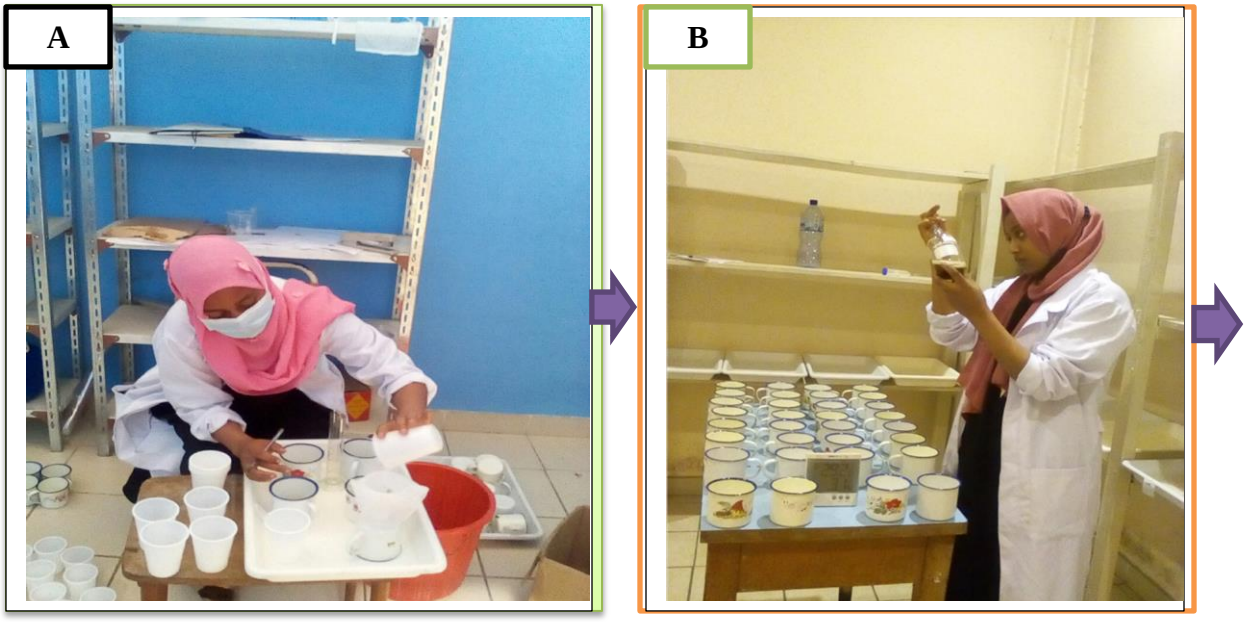
C1V1 = C2V2 → C1, initial concentration = (1% stock solution) 10,000 ppm

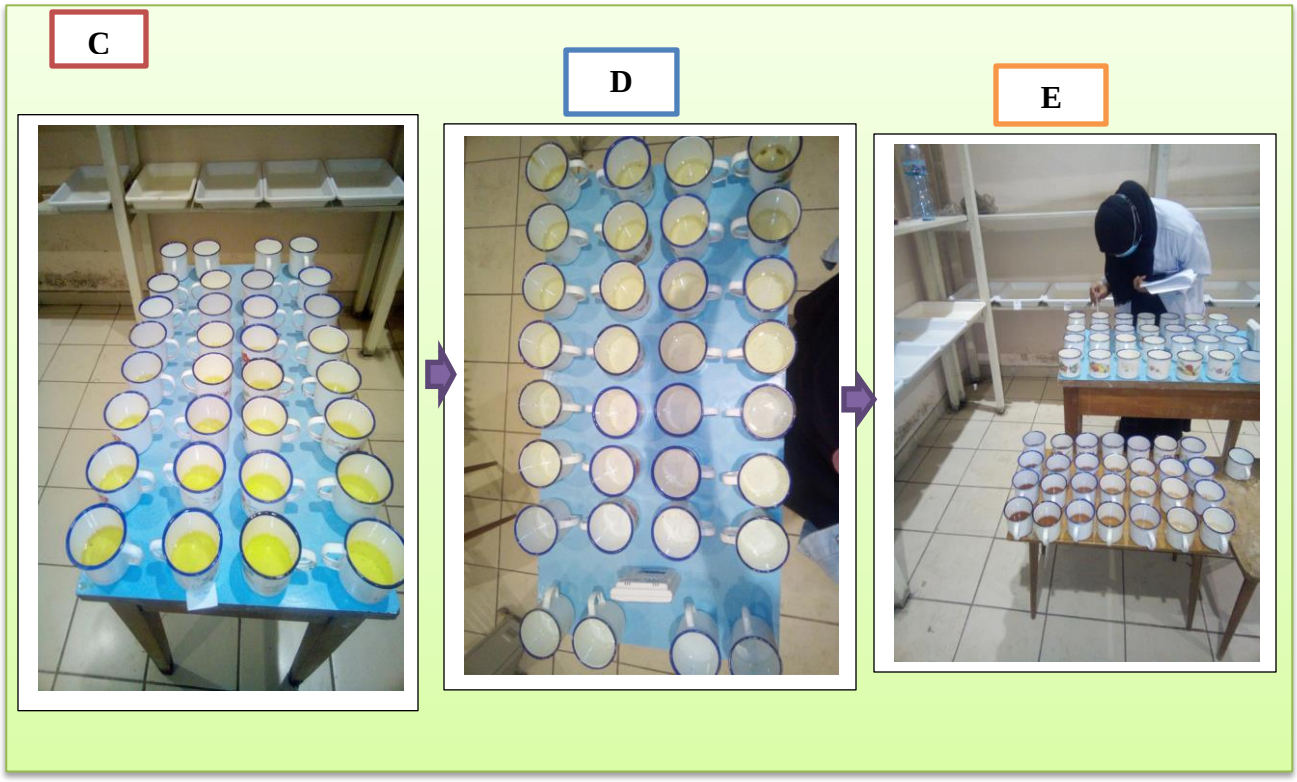
V1, volume needed =?

C2, finale concentrations = 20, 40, 80, 160, and 320 ppm

V2, final volume prepared = 10 ml

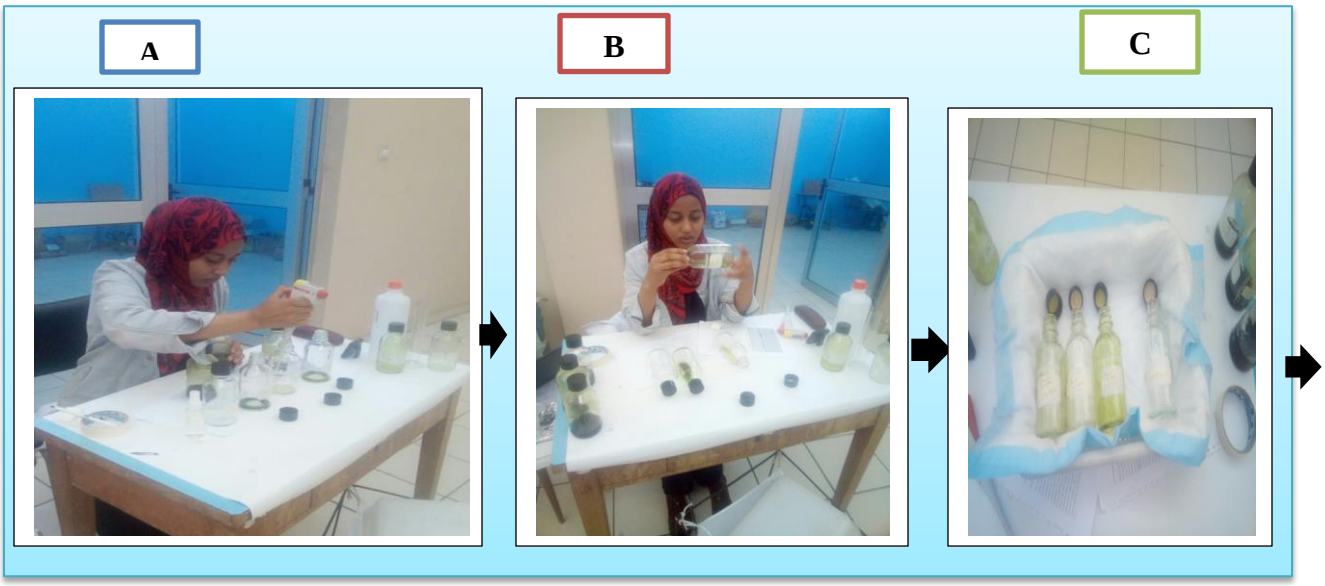
Re: 1 ppm = 1000 µg/bottle

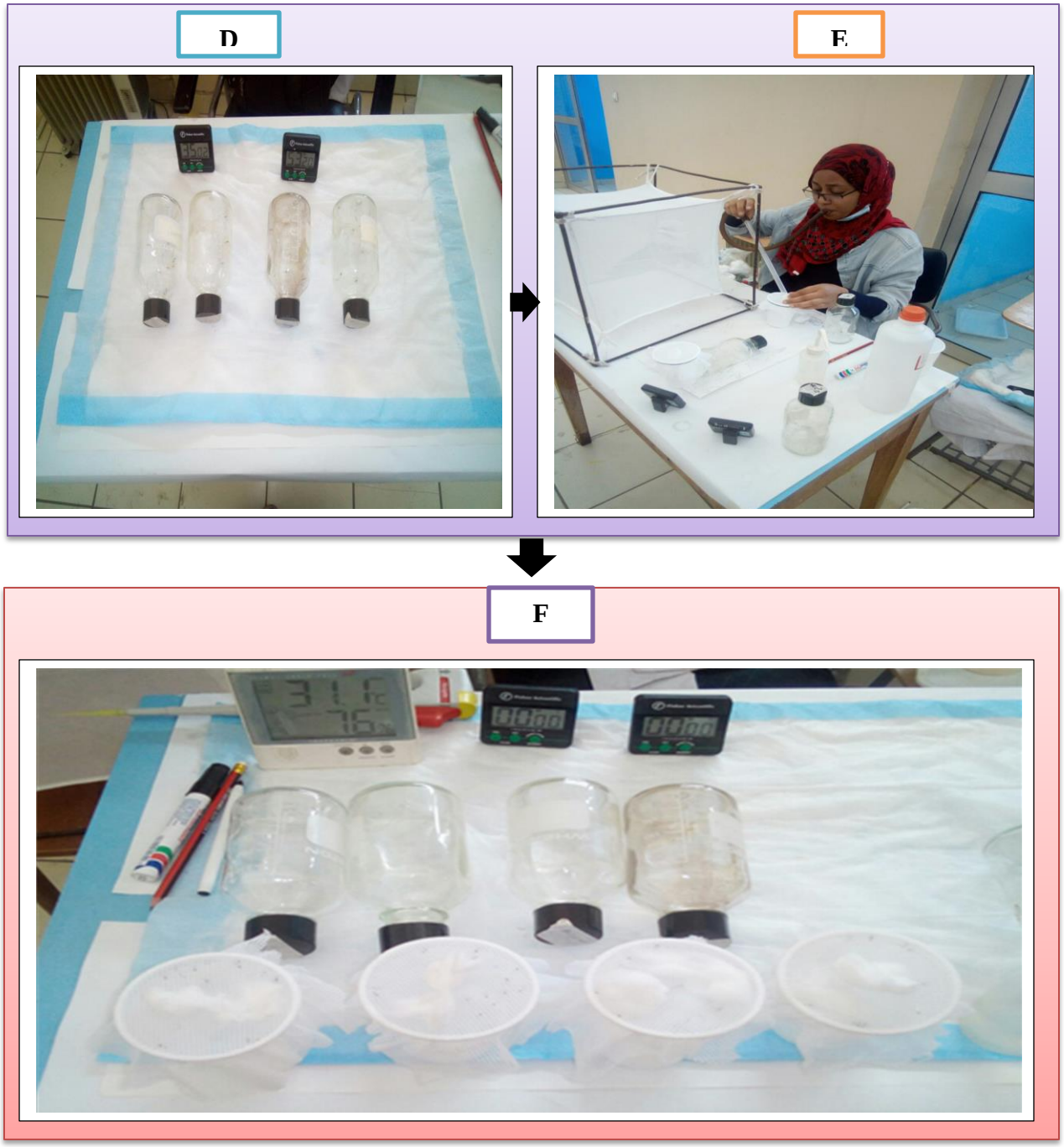




Larval filtering (**A**), adding aliquot stock solution (**B**), and larval exposing with extract of hexane (**C**), methanol (**D**), and aqueous extract with dead larvae counting process (**E**).

Annex 5: Larvacidal bioassay with enamel cups





Adding aliquot stock solution in the bottles (**A**), coating the sides of the bottles (**B**), drying the bottles (**C**), exposing (**D**), transferring (**E**), and holding (**F**) processes with treatments against adults of *An. stephensi*.

Annex 6: Adulticidal bioassay with a CDC glass bottle