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Evaluation of the larvicidal effects of *Annona squamosa and Tagetes minuta* essential oils and crude extracts against *Anopheles* mosquito larvae under laboratory and semi field conditions

A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment for the Degree of Master of Science in Zoological Program Units

By
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DECLARATION

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

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ABSTRACT

The larvicidal activity of solvent extracts of *Annona squamosa*, *Tagetes minuta* and essential oils of both plants were evaluated against laboratory colonies and field collected larvae of *Anopheles*. Shade dried and grounded seed from *A. squamosa* and floral part of *T. minuta* were sequentially extracted with acetone, hexane, ethanol and distilled water. A series of concentration of the extracts ranging from 6.25ppm to 100ppm were tested against 3rd-4th instar larvae of *An. arabiensis* and their percentage mortalities were recorded. The acetone and hexane extracted *A. squamosa* showed very high larvicidal activity, where 96% and 98% mortality were recorded respectively in the laboratory and 90% and 87.5% mortality respectively in semi- field at a concentration of 100ppm. In the laboratory, the LC_{50} values of different solvent extracts tested ranged from 13.3ppm to 574.9ppm against *An. arabiensis*. Similarly, the LC_{90} values ranged from 48.35ppm to 916.1ppm. However, acetone and hexane extracted *A. squamosa* with LC_{50} of 13.3ppm; 23.3ppm and LC_{90} 59.9ppm, 48ppm respectively showed strong larvicidal activity against *An. arabiensis* under laboratory condition after 24hrs exposure. On the other hand, the field LC_{50} and LC_{90} values of tested solvent extracted plants ranged from 28.0ppm to 410.2ppm and 82.45ppm to 656.1ppm, respectively and Acetone and Hexane extracted *A. squamosa* was the most effective with LC_{50} 28.0ppm;32ppm and LC_{90} 84ppm;82ppm, respectively. Also *A. squamosa* and *T. minutia* of essential oils in the present study exhibited a larvicidal activity against 3rd - 4th instar larvae of *An. arabiensis*. *Annona squamosa* essential oils exhibited 99% and 71% larval mortality both in the laboratory and semi-field conditions respectively at the concentration of 66.67ppm than *T. minuta* essential oils. Moreover, of the two tested essential oils, *A. squamosa* oil induced highest larvicidal effects both in the laboratory and in field with LC_{50} 41.5ppm; 31.5ppm and LC_{90} 79.2ppm; 32.9ppm, respectively. The results suggest that the investigated plant extracts are promising as larvicides against *An. arabiensis* and could be useful in the search for new and biodegradable plant derived larvicide’s products.

1. Introduction

Malaria is one of the major public health challenges hindering development in the developing countries in the world. From Afghanistan to Zimbabwe—malaria threatens the people of 109 out of 190 countries on Earth (Connie, 2011). The disease is adversely affecting the health of the peoples and the economic development of many developing countries, particularly in sub-Saharan Africa (Wakgari *et al.,* 2006).

About 40% of the world's population, mostly those living in developing countries are threatened by malaria. Of these 2.5 billion people are at risk, more than 500 million become severely ill with malaria every year, and more than 1 million die from the effects of the disease (WHO, 2007).

Over 80% of death due to malaria occurs in Africa, while less than 15% of the deaths occur in Asia and Eastern Europe (WHO and UNICEF, 2005). In endemic African countries, malaria accounts for 25–35% of all outpatient visits, 20–45% of hospital admissions and 15–35% of hospital deaths, imposing a great burden on already fragile health-care systems (WHO, 2005). Each year, approximately 25 million African pregnant women in malaria-endemic areas are at risk of *Plasmodium falciparum* malaria infection during pregnancy (Fred, 2001). Most women in the African Region reside in areas of relatively stable malaria transmission where the principal effects of malaria infection during pregnancy are associated with malaria-related anemia in the mother and with the presence of plasmodium parasites in the placenta (Steketee *et al*., 2001). The resultant impairment of fetal nutrition contributes to low birth weight, which is a leading cause of poor infant survival and development in Africa (Steketee *et al*., 1996).

Malaria is reported to be the number one health problem in Ethiopia, with an estimated more than 65% of the 70 million people exposed to malaria. Each year more than 5 million malaria cases are estimated to occur in the country (Gabriel and James, 2005). Most vulnerable group in the population are children under five and expecting mothers. One child out of 20 is affected by malaria before reaching the age of five years (RBM, 2010). It causes serious health problems to humans and present obstacles to the socioeconomic development of developing countries, particularly in the tropical region (Senthilkumar *et al*., 2008).

Malaria transmission is seasonal in most parts of Ethiopia, which lends itself to the outbreak of epidemics. The transmission patterns and intensity vary greatly due to the variation in altitude, rainfall, and population movement; areas below 2,000 meters above sea level are considered to be malarious or potentially malarious. Those areas are home to approximately 68% (52 million) of the Ethiopian population and cover almost 75% of the country's landmass (Ethiopia MIS, 2007).

Malaria is a highly complex disease caused by a protozoan belonging to the genus *Plasmodium*. Four species are *Plasmodium falciparum, P. vivax, P. malariae* and *P. ovale* infect humans but they differ in many aspects of their biology and geographic distribution. *Plasmodium falciparum* is found in most tropical regions throughout the world, and is the most dangerous of the four in terms of both its lethality and morbidity. All undergo two forms of replication: sexual and asexual. The parasites develop optimally in the vector but cease developing at temperatures 16°C or below. High humidity prolongs the life of the vector and transmission is extended under these conditions. In the human intermediate host, the parasite must function at 37°C or higher, since the infection induces a significant rise in core temperature during the height of the infection (Adugna, 2008).

Malaria parasites are transmitted by approximately 60 species of anopheline mosquitoes (Oaks *et al.*, 1991). Anopheline vectors transmitting human malaria are categorized as main and secondary vectors based on their role in the transmission of the disease. *Anopheles gambiae* complex are the main vectors in tropical Africa and the *An. funestus* having a wide distribution and *An. nili* and *An. moucheti* with limited distribution. *An. pharoensis*, *An. ziemanni*, *An. rufipes* and others are the secondary vectors in tropical Africa. Cytogenetic techniques revealed that the *An. gambiae* complex comprises six named and one unnamed sibling species namely *An. gambiae s.s., An. arabiensis, An. melas, An. merus, An. bwambae, An. quadriannulatus* species *A* and *An. quadriannulatus* species B (Coetzee *et al*., 2000). *An. arabiensis,* and *An. gambiae s.s.* are the most important vectors of human malaria in sub-Saharan Africa (Coetzee *et al*., 2000). The two species occur in sympathry in most parts of the continent. *An. gambiae s.s.* is dominant in the equatorial rain forest and in the more humid areas. It is the most efficient of malaria vector, being more anthrophilic and endophilic in its behavior. It breeds in temporary pools of water exposed to sun light and the adult is present only in or open places. Its density increases with the degradation of forest and thus linked with human activities (Gillies and Demellion, 1968).

An. arabiensis is more concentrated in the lower rainfall zones with annual rainfall less than 1000 mm which represents the drier Savannah areas as compared to *An. gambiae s.s.* which is more concentrated in higher rainfall zones of greater than 1000 mm (Coetzee *et al.,* 2000). *An. arabiensis* though predominant in dry areas and the Savannah belt, is also present in some degraded forest areas of central and West Africa. It is more abundant in the drier inland areas of east Africa and during the dry season. Its presence in the irrigated plains has been confirmed. Being also zoophilic compared to *An. gambiae s.s.,* it is a less efficient vector. *An. arabiensis* is dominant in the Sudan Savannah and further north to the Egyptian border (Gillies and Demellion, 1968).

In Ethiopia, about 42 Anopheles species have been recorded with distribution varying by altitudinal zone and microclimate. Among the identified species, *An. arabiensis* is the major vector but in some areas *An. pharoensis, An. funestus* and *An.nili* also transmit the disease and are considered as secondary vectors (Abose *et al.,* 1998). *An. quadriannulatus* species B in the *An. gambiae* complex is zoophilic and has no role in malaria transmission (Coetzee, 2004).

The clinical symptoms of malaria include periodic fever, varying degrees of anemia and spleen enlargement, and a range of syndromes resulting from the physiological and pathological involvement of certain organs including the brain, liver and the kidneys. Infection with *P. falciparum* and *P. vivax* is the most common, and *P. falciparum* infection can be fatal in the absence of treatment (Webb and Russell, 2007).

1.1. Malaria vector control

Malaria eradication programs launched in the 1950s and 1960s had dramatically decreased the percentage of the world population at risk of the disease from 68% in 1946 to 52% in 1975 (Hay, 2004).

For a number of the vector-borne infections, the most effective method of controlling their transmission is through control of their vectors. Malaria vector control is a difficult task and is becoming even more so due to a variety of factors including the development of insecticide resistance and concern over environmental pollution and many of them are immuno-suppressants (Srivastava and Sharma, 2000). Because of resistance in the vectors, conventional insecticides, are becoming ineffective (Vincent, 2000). Virtually all of the vector and pest control programs depend on the use of insecticides formulated as larvicides, adulticides, baits, or insecticide impregnated bed nets.

Personal protection is one of the most effective control strategy that combines abiotic and biotic (biological) control methods as it works on the behaviors of both people and mosquitoes to minimize human exposure to mosquitoes (Chen *et al*., 2006). Personal protection method included air conditioning, screens, bed nets, topical and air borne repellents and clothing for covering exposed parts of the body during the biting period (Schoepke *et al.,* 1998). In general, Barnard (2000) categorizes personal protection in three categories: avoidance (e.g. staying indoors during the peak biting times), physical barriers (e.g. protective clothing and window screens) and chemical barriers (e.g. insecticide treated bed nets and repellents). Insecticide-treated materials, mainly bed nets and curtains, are reported to be effective for protection against malaria vectors (Gunasekaran and Vaidyanathan, 2008).

Chemical control of adult female mosquitoes has been the most widely successful vector control method since the 1940s. The most common practice is indoor residual house spraying (IRS), in which the inside walls, the ceiling, and sometimes the outside eaves, porches, and nearby animal shed are sprayed with a persistent insecticide (Kathleen, 2002). For the control of larvae, larvicides were used but, it's not effective in most rural areas of the African region where breeding sites are ubiquitous and short-lived requiring frequent applications. Chemical control has been relied on as a vector control strategy in the past, and showed clearly if it is properly implemented you can get very good results. But are harmful to other non-target organisms including natural predators of mosquito larvae and may result in environmental pollution if insecticides to be used are not well selected, developing resistance and are expensive (MOH, 2002).

The use of insecticide treated nets (ITNs) is one of the most powerful interventions available for effective reduction of malaria infection incidence (Choi *et al*., 1995), morbidity and mortality (Belay and Deressa, 2008). It provides protection against adult mosquitoes, the proper and regular use of ITNs has resulted in high levels of protection (Msangi *et al*., 2008). However, ITNs have their limitations, such as when vectors bite in the early evening, when people often are not sleeping under their bed net. Also, the ITN protection is geographically limited, providing very little protection in regions where vectors have exophagic (outdoor) feeding habits. In addition, areas of coverage, cost-effectiveness and sustainability of use as well as the necessity of reimpregnation of ITNs have also negative impact on the efficacy of bed nets (Gonzalez *et al*., 2002).

By definition, repellents are substances that act locally or at a distance, deterring an arthropod from flying to, landing on or biting human or animal skin (or a surface in general) (Blackwell *et al.*, 2003; Choochote *et al.*, 2007). Usually, insect repellents work by providing a vapor barrier deterring the arthropod from coming into contact with the surface (Brown and Hebert, 1997). Thousands of synthetic and natural repellent candidates were screened. Diethyl-3-methylbenzamide (DEET) is one of the best and most widely used synthetic insect repellents developed and has been the gold standard for public use, emergencies and military operations (Novak and Gerberg, 2005). It is not only a broad spectrum repellent, but also the most effective and persistent on skin (Isman, 2006). Although DEET and other synthetic repellents are effective, cost is too high for daily use within poor communities in tropical Africa. Moreover, DEET is not always ideal, as few cases of side effects on environmental and human health have been reported (Blackwell *et al.*, 2004). Many plants are known to have repellent properties against a wide variety of biting insects either through traditional use (Seyoum *et al.*, 2003 and Moore *et al.*, 2007) or using their extracts (Hill *et al.*, 2007). Some plant-based repellents that have been developed and commercialized, and are known to be as effective as DEET include, for example, p-menthane-3,8-diol (PMD) obtained from the waste distillate of lemon eucalyptus (*Corymbia citridora*) leaves and citronella oil from Citronell grass, *Cymbopogon nardus* (Curtis *et al.*, 1990). They are believed to be safer and economically more optimal alternatives to synthetic repellents for use in low-income areas of the world where problems with vector-borne infections predominate (Jaenson *et al.*, 2006).

Malaria vector control also include environmental management which refers to planning, organization, carrying out and monitoring of activities for the modification and/or manipulation of environmental factors with a view to preventing or minimizing vector propagation and reducing man-vector-pathogen contact (WHO, 1982). Environmental modification is any physical transformation that is permanent or long-lasting of land, water and vegetation, aimed at preventing, eliminating or reducing the habitats of vectors without causing adverse effects on the quality of human environment. It includes drainage, filling, land leveling and transformation and impoundment margins (WHO, 1982; Yohannes *et al.*, 2005). Yohannes *et al.* (2005) reported that source reduction was carried out by community resulting in

49% relative reduction in *An. arabiensis* adults in the dam village in Tigray compared with pre-intervention period. However, prevention of breeding in and around dwellings has little effect on malaria transmission except when widely applied.

At present, a wide range of organisms help to regulate anopheles populations naturally through predation, parasitism and competition. Biological methods of mosquito control basically involve the utilization of natural enemies of mosquitoes and of biological toxoids to achieve an effective control (WHO, 1982; Yap, 1985). Many field trials have been carried out against vectors, examples include, a fish, *Gambusia affinis* shows a marked preference for mosquito larvae (Rozendaal, 1997). It is a voracious eater of mosquito larvae and, if introduced in sufficient numbers in pools, ponds and marshes, it can destroy large quantities of mosquito eggs, larvae and pupae (WHO, 1982; Rozendaal, 1997). Other fishes used are *Poecilia reticulate, Aplocheilus* species, *Aphanius* and *Tilapia* species (Yap, 1985; Lardeux *et al.,* 2002; Rozendaal, 1997). WHO (1996)
reported that only fish predators (*Gambusia affinis*) of mosquito larvae have been successfully and extensively applied in some areas. Predacious mosquito larvae of *Toxorhynchites* species have been found to be promising and effective for the control of larvae of some mosquito species in plant axils, tree holes, cut bamboos, abandoned containers and similar sites (WHO, 1982).

Among the bacteria, *Bacillus thuringiensis* H-14 (Bti) and *B. sphaericus* have shown high potency for mosquito control without adversely affecting non-target organisms and the environment (Rozendaal, 1997 and Lardeux *et al.,* 2002). Among the fungi the imperfect fungi of the genera Culicinomyces, Tolypocladium, Metarhizium and Beauveria can be readily cultured on artificial media to yield infective stages of mosquito control. Two most known species of protozoa are *Vavraia culicis* which parasitized mainly on *culicines*, and *Nosemia algerae* which appeared to be most effective against *Anopheles* (Yap, 1985). Other organisms showing promise include a number of fungal pathogens, the nematode *Romanomermis culcivorax*, and the aquatic plant *Azolla* (Lacey & Lacey, 1990). In general, Biological control methods have the advantage of target specificity with little effect on non-target organisms and are regarded environmentally safe. However, there are some problems associated importation of predatory fish into any location has serious and complicated environmental impact, and the high cost and difficulties encountered in mass production preclude their wide use, especially in tropical countries (Yap, 1985).

Another biological factor currently in use for mosquito control is insect growth regulators (IGRs), a diverse group of compounds that are highly active against larvae of mosquitoes and other insects (Poopathi and Tyagi, 2006). Two types of IGRs are available, one which inhibits the growth of larvae due to juvenile hormone like action and known as JH mimics or analogues and the other type of IGR compound which interfere with chitin production leading to moulting disturbances, resulting in death of the insect. IGRs differ widely from the commonly used insecticides as they exert their insecticidal effects through their influence on development, metamorphosis and reproduction of the target insects by disrupting the normal activity of the endocrine system. Compared to the conventional larvicides, the IGRs are known to be safer and selective in action (Mulla *et al.,* 1986). IGR compounds such as methoprene, diflubenzuron, pyriproxyfen and triflumuron have been recommended by WHO and diflubenzuron has already passed WHOPES for use against mosquito immature (Chavasse and Yap, 1997). About 90 to 100% emergence inhibition against malaria vector *An. culicifacies* was observed in pools and paddy-fields for five weeks post-application when treated at 1 ppm and therefore, triflumuron can be applied at monthly intervals for longer duration of control of mosquito larvae (Batra *et al*., 2005). Additionally, it has been known that they have shown a good margin of safety for non-target vertebrate biota including fish and birds. Studies indicated that there is no prolonged residual effects of these compounds and these compounds are believed to be environmentally safe with minimal impact on non-target organisms. The major drawback to IGRs is that they are not safe for other aquatic insects (Dong-Kyu, 2002).

Integrated vector management (IVM) is the use of a cost effective combination of vector control measures that are appropriate to local conditions and priorities and relatively safe for human health and the environment. From the vector control stand point, IVM is generally less risky and more effective due to combination of one or more vector control methods (US EPA 1998). Profound in this method is the combination of both chemical and non-chemical methods. However chemicals are used as the last line of intervention. IVM methods include public health measures like drainage of still and swampy waters. In rice growing areas these include, drainage of canals to avoid water stagnation, shifting of planting schedule to avoid optimal mosquito breeding conditions; and introduction of aquatic plants *Azolla*, which is also valuable as natural fertilizer and also covers the whole water surface thereby interfering with mosquito oviposition, larvae and pupae. Clearance of pond algae could have dual benefits since the algae can be used to manufacture fancy paper as well as eliminate mosquito menace (CEAG Africa, 2006).

1.2. Botanical Larvicides

The existing malaria and vector control measures are not sufficient. Unless alternative control strategies are developed, death and illness due to malaria will increase, and the diseases will continue to be a substantial barrier to the economic and social development of malaria endemic regions as well as a threat to the millions of people who travel to those regions each year.

Chemical measures to decrease mosquito populations were initially considered in most public health programs but they have failed because the constant use of chemical insecticides has often led to the disruption of natural biological control systems and outbreak of insects’ species. Moreover, problems created by using synthetic insecticides include the development of mosquito resistance, environmental pollution and undesirable effect on humans, mammals and other non target organisms (Lee *et al.*, 2001).

There is an urgent need to develop new materials for controlling mosquitoes in an environmentally safe way, using biodegradable and target-specific insecticides against them. Natural products are generally preferred because of their less harmful nature to non-target organisms and due to their innate biodegradability (Prabakar and Jebanesan, 2004).

Attention to insecticides of natural origin, particularly plant derived products, has been recently revived. A considerable number of studies have emphasized the research and development of herbal substances for controlling mosquitoes (Sukumar *et al*., 1991 and Jayabalan *et al*., 2003). Monzen *et al.* (1994) reported that some medicinal plants containing natural toxins were effective against mosquito larvae. Not only can medicinal plant extracts be effective but also they may greatly reduce the risk of adverse ecological effects and do not induce pesticide resistance in mosquitoes. Since these chemicals are taken from medicinal plants, they are expected to have low human toxicity and a high degree of biodegradation (Choochote *et al.,* 1999).

Plants synthesize secondary metabolites that may possess insecticidal, antimicrobial (Leeja and Thoppil, 2007), herbicidal and other biological activities (Setia *et al.*, 2007; Tonk *et al.*, 2006). By using plant parts in early historical times and plant extracts and concentrated components in more recent times, man has been able to control certain pests with these remedies quite successfully. The current use and future potential of plants for pest control on farms and homes are detailed in a FAO document (FAO, 1999).

Wood (2003) listed some important phytochemical products such as pyrethrum, derris, quassia, nicotine, hellebore, anabasine, azadirachtin, dlimonene, camphor, and terpens; all of which have been used as insecticides. They are major groups of insecticides of plant origin that were used in developed countries before the advent of synthetic organic insecticides (Casida and Quistad, 1998). Over one thousand plant species contain bioactive substances with many of these containing phytoecdysones, phytojuvenoids and anti-juvenile hormones, which act as IGRs (Varma and Dubey, 1998).

Phytochemicals can be extracted from either whole plants or specific parts of the plant, depending on the activity of the derivatives. Some plants accumulate bioactive chemicals differentially in the various parts of the plant, such as leaves, fruits, flowers, roots and bark (Trongtokit, 2004). According to Shaalaan *et al*. (2005) the bioactivity of phytochemical against mosquito larvae can vary significantly depending on plant species, plant part, solvent used in extraction and mosquito species. In addition to these Marcard *et al*. (1986) reported that the mortality of the insect was directly related to the concentration of the insecticide and exposure time. Moreover, with the same mosquito species, variations in susceptibilities between laboratory and field strains are expected. George and Vincent, 2005; Sun *et al.*, 2006; and Kabir *et al*., 2006 noted that the field strain larvae were more resistant than laboratory reared strain. The possible reasons are that the field strains were genetically more heterogeneous.

Investigators have found that the effectiveness of chemicals derived from specific plant parts often varies with the mosquito species. Some phytochemicals act as general toxicants to all life stages of mosquitoes, whereas others interfere with growth and reproduction, or act on the olfactory receptors, eliciting responses of attractancy or repellency (Sukumar *et al*., 1991).

In the search for environmentally safe and relatively inexpensive methods to control mosquitoes, plant extracts have received much interest as potential bioactive agents against the mosquito larvae. Most mosquito control programs target the larval stage at their breeding sites with larvicides (Knio *et al*., 2008). Bagavan *et al*. (2009) reported that the acetone, chloroform ethyl acetate and methanol extracts of *Annona squamosa* against *An. subpictus* with the LC_{50} values of 17.4, 76.04, 18.60, 119.93ppm respectively. Studies conducted by Macedo *et al*. (1997) showed that ethanol extracted *Tagete patula* was effective at higher concentration (100ppm) against three mosquito species (*Aedes aegypti, An. stephensi* and *Culex quinquefasciatus*). Brahim *et al.* (2006) evaluated that the activity of aqueous extracted *Ricinus communis* against 4th instar larvae of four mosquito species with the range of 200mg/l-1090mg/l.

In Ethiopia, Massebo *et al*. (2009) evaluated 11 local plants for larvicidal activities against laboratory colonies of *An. arabiensis* and *Ae. aegypti*. It was found that the LC_{50} values of the oils ranged from 17.5 to 85.9 ppm against *An. arabiensis* under laboratory condition.

The essential oil of Ocimum species had shown to be quite potent as larvicidal agents against Anopheles mosquito larvae and caused 100% mortality at concentration range of 300-500µl/l for 3hrs (Azhari *et al.*, 2009). Abdela *et al*. (2009) investigate the larvicidal, adult emergence inhibition and oviposition deterrent activity of aqueous leaves extract of Calotropis against *An. arabiensis* and *Cx. quinquefasciatus*. It was found that, LC_{50}-LC_{90} values were 454.99-1224.62ppm for 4th larval instar of *An. arabiensis*. Though several plants from different families have been reported for their mosquitocidal activity, only a few botanicals have moved from the laboratory to field use, like neem based insecticides, which might be due to the light and heat instability of phytochemicals compared to synthetic insecticides (Green *et al.,* 1991).

Singh *et al.* (2001) reported the larvicidal activity of *Solanum nigrum* extract against *An. culicifacies, Ae. egypti* and *Cx. quinquefasciatus* in the laboratory with LC_{50} values of 0.027, 0.032 and 0.0275ml% in water, respectively. Pushpalatha and Muthukrishnan (1999) reported the larvicidal activity of seed and leaf extracts of *Calophyllum inophyllum* and leaf extract of *Rhinacanthus nasutus* against *Cx. quinquefasciatus, Ae. aegypti* and *An. stephensi* larvae. The leaf extract of *Agave americana* showed strong larvicidal activity against *Anopheles, Aedes* and *Culex* larvae (Dharmshaktu *et al*., 1987). Singh and Bansal (2003) tested the larvicidal properties of *Solanum xanthocarpum* against vector of malaria and dengue larvae. Amer and Mehlhorn (2006) evaluated the larvicidal effect of essential oils of 41 plants against *Aedes, Anopheles* and *Culex* mosquitoes and reported 13 plant oils (*Cinnamomum* *camphora, Thymus serpyllum, Amyris balsamifera, Citrus limon, Juniperus virginiana, Boswellia carteri, Anethum graveolens, Myrtus communis, Juniperus communis, Piper nigrum, Lippia citriodora, Helichrysum italicum* and *Santalum album*) to have significant effect.

The larvicidal and mosquitocidal activities of ethanolic water mixture extract of *Annona squamosa* and *Centella asiatica* (Senthilkumar *et al*., 2009); the leaves methanolic extract (Jaswanth *et al*., 2002) and the seeds petroleum ether extract showed larvicidal activity were tested against *Anopheles stephensi, Culex quinquefasciatus* and *Aedes aegypti.*

The leaf acetone, chloroform, ethyl acetate, hexane, and methanol extracts of *Aegle marmelos, Andrographis lineata, Andrographis paniculata, Cocculus hirsutus, Eclipta prostrata,* and *Tagetes erecta* (Elango *et al*., 2009); extracts of leaf, flower and seed of *Cassia auriculata, Leucas aspera, Rhinacanthus nasutus, Solanum torvum* and *Vitex negundo* (Kamaraj *et al*., 2009) and the leaf extracts of Citrus *sinensis, Ocimum canum, O. sanctum and R. nasutus* (Bagavan *et al*., 2009) were tested against the fourth instar larvae of *An. subpictus, Cx. tritaeniorhynchus* and feeding deterrence to nymphs of *Aphis gossypii*.

2. OBJECTIVES OF THE STUDY

2.1. General objective

- To evaluate bioactivity of Custard Apple (*A. squamosa*) and Marigold (*T. minuta*) crude extracts and essential oils as alternative integrated vector management against *An. arabiensis* under laboratory and *Anopheles* mosquito under semi-field conditions.

2.2. Specific objectives

- To evaluate the larvicidal activity of essential oils and solvent extracts of *A. squamosa* and *T. minuta* plants against third to fourth instar larvae of *An. arabiensis* under laboratory condition.

- To evaluate the larvicidal activity of essential oils and solvent extracts (distilled water, ethanol, hexane and acetone) of *A. squamosa and T. minuta* plants against third to fourth instar larvae of *Anopheles* mosquito under semi-field condition.

- To determine mortality-time rates of essential oils and solvent extracts of *A. squamosa* and *T. minuta* against third to fourth instars larvae of *Anopheles* mosquitoes (*An. arabiensis*).

3. MATERIALS AND METHODS

3.1. Description of the test plants

3.1.1. Custard Apple (*Annona squamosa L.*)

More than 90 species of small trees and shrubs are known in the Annonaceae family. They occur mostly in tropical America but some are also found in Asia and Africa. Custard Apple (*Annona squamosa* L.) is much branched shrub or small tree 3-6m tall. Fruits are globose or broadly conical, 5-10cm long and wide. The leaves alternate, simple and oblong-lanceolate.

Annona squamosa is widely cultivated throughout the tropics at low to medium altitudes; also naturalized near the coast in east Africa. When ripe, pulp is creamy, very sweet and pleasantly flavoured. It is usually eaten as a dessert fruit and finds immense applications in the preparations of beverages and ice creams. The seeds are scattered through the fruit flesh; the seed coats are blackish-brown, 12–18 mm (0.47–0.71 in) long, and hard and shiny (Chikhalikar *et al.*, 2000).

These species are most frequently found being sold under the name of custard Apple (English) or GISHTA (Amharic). It does not require special conditions of soil or water, but thrive best in places where there is a clear division between the rainy and dry season, and generally prefer dry sites. It is cultivated between 900 and 1700 m in Ertirea west above 1000m contour to the Sudan on the west, Showa region above and below to the west of the 1000m contour, Ethiopia /Hararge region of Ethiopia , Gamu Gofa region and Sidamo region of Ethiopia.

Antifeedant, growth inhibitory and toxic effects of crude seed extracts of *Annona squamosa* were evaluated against the cabbage looper, *Trichoplusia ni* (Hubner) (Lepidoptera: Noctuidae) using different bioassays. Powdered seeds and leaves of *Annona* squamosa have been used traditionally to treat head and body lice (Tiangda *et al*., 2000). Aqueous seed extracts of *A. squamosa* toxic to the cabbage looper, Trichoplusia ni (Leatemia and Isman, 2004). Kamaraj *et al*., 2009 calculated the percentage mortality for acetone, ethyl acetate, methanol and hexane extracts of dried bark of *A. squamosa* were in the range of 85 to 100% on 4th instar larvae of *An. stephensi*. However, the essential oils and solvent extracts of *A. squamosa* have show incecticidal and/or growth inhibition activity against mosquitoes (Saxena *et al*., 1993). The essential oils and crude extracts of distilled water, hexane, ethanol and acetone extract of this plant have not been evaluated against *An. arabiensis.*

3.1.2. Marigold (*Tagetes minuta L.)*

The herb (*Tagetes minuta*) is a member of the family Compositae (Asteraceae). It is an erect annual herb, 15-150cm high, strongly aromatic; with stems glabrous, green or purplish, densely branched, woody at base. Leaves 3-15cm long, imparipinnate; leaflets 9-17, linear- lanceolate, 1.5-10cm long, 2-7mm wide, margins serrate. The phytochemical analysis of aerial parts of *T. minutia* exhibited the presence of Saponins, Terpenoides, Tannins, Alkaloids and Flavonods (Abbas *et al*., 2005).

It is a weed of cultivated land, waste places and roadsides naturalized in Acacia woodland; 1350-2200m, Ertirea west above 1000m contour to the Sudan on the west, Tigray region, Gondor region of Ethiopia, Wolo region of Ethiopia, Showa region above and below to the west of the 1000m contour, Ethiopia, Kefa region, Sidamo region of Ethiopia, Bale region of Ethiopia, and Hararge region of Ethiopia ; native of west central, south America, now introduced and naturalized in many tropical and warm- temperate countries. It is a multipurpose plant which used as pigment, repellent, biocidal, bactericidal, fungicidal, larvicidal and also has medicinal value.

Tagetes minuta has showed insecticidal and/or growth inhibition activity against *An. stephensi* and *Ae. aegypti* (Green *et al*. (1991) and Perich *et al.* (1994)). Singh *et al*. (2001) and Wells, (1993) demonstrated that insecticidal activity and chemical composition of marigold species vary considerably. The essential oils and distilled water, hexane, ethanol and acetone extracts of this plant have not been evaluated against *An. arabiensis.*

Nowadays, the search for alternative, simple and cost effective tools for malaria vector control is underway in different parts of the world. One field of research interest is the search for new larvicidal plant derived extracts. Plant based extracts that reduce the adult emergence and have the advantage that they can be produced locally. Thus reducing cost and environmentally safe. It is based on this point of view that this study was conducted in the laboratory and on semi field environment to evaluate the extracts of *Annona squamosa* and *Tagetes minuta* plants found in Ethiopia against *Anopheles* mosquito larvae. Hence, this study was proposed and undertaken based on the following objectives.

3.2. Description of the study area

The field study was conducted in Tolay settlement areas which is one of the major military training sites located, 264km from Addis Ababa around Gibe Valley at Western Showa Zone in Oromia Regional State (Fig.1). It has a military hospital, a government clinic, a private clinic and schools. A total of 1000 households (about 5000 people) have been resettled from various parts of Ethiopia due to food insecurity, drought, flood and other natural calamities. Most of their activities are related to agriculture and small scale trade. The main agricultural crops cultivated are maize, sorghum, pepper and some horticultural products.

The area is sparsely populated because of the high risk of diseases like trypanosomiasis to cattle and malaria to people in the area. The upper Ghibe Valley has open and woody grass land vegetation with riparian forest along the banks of the river. The area is very fertile and is estimated to be about 3000 km^2 (ICIPE, 2009). The valley is covered by wood land and indigenous trees mainly *Acacia* species. There are several tributaries that join the main Ghibe River which finally becomes part of the Omo River system. The average annual rainfall of the area is between 1,100-1,400mm. The average maximum temperature is 40 ^{o}C and the minimum average temperature ranges between 0 and 4 ^{o}C (ICIPE, 2009).

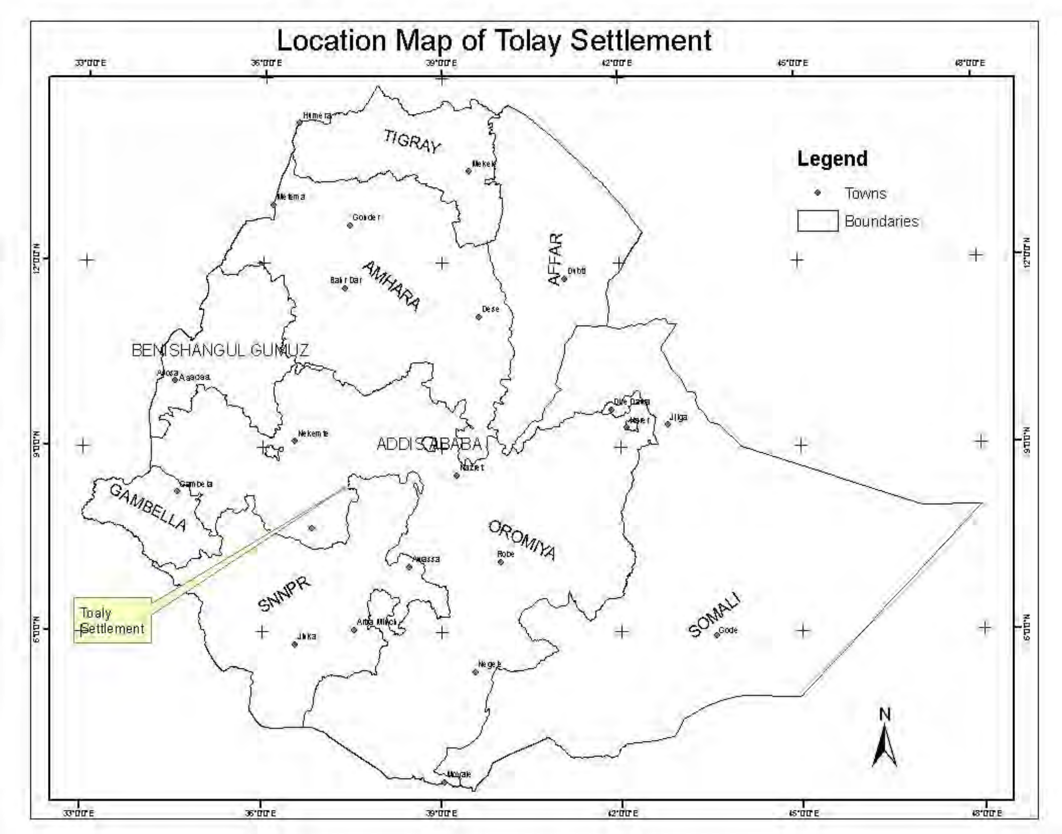

Figure 1. Location of the study area

Source: ICIPE/ Ethiopia malaria project annual report, 2009.

3.3. Sampling immature stage of Anopheles mosquitoes

In the study period, mosquito breeding habitats were identified and larval sampling were conducted using WHO standard dipper (WHO, 2003) and the collected larvae were transferred to separate vials using a dropper (Plate 1). During sampling, larval habitats were characterized, and major breeding sites were recorded.

Plate1. Anopheles larval sampling at breeding sites in the study area.

3.4. Rearing of *An. arabiensis* in the laboratory

The laboratory culture of *An. arabiensis* eggs were obtained from the Insectory of Aklilu Lemma Institution of Pathobiology (ALIPB) and reared in the Insect Science Laboratory of Addis Ababa University at a temperature of 25-27^{o}C and 60-70% relative humidity. The laboratory cultures of *An. arabiensis* were reared and maintained following recommended mosquito rearing procedures (WHO, 2005). Larvae were fed on a mixture of powdered dried animal food pellets, dry milk and yeast were added to rearing trays every other day. The media were changed every three days. Adults were maintained in mosquito cages and were fed with sterile 10% sugar solution soaked in cotton pads and placed in Petri dishes inside rearing cages. 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 paper in cups was placed inside rearing cages for oviposition by gravid female mosquitoes. The eggs were washed off with distilled water on to larval rearing trays and allowed to hatch into neonate larvae in the laboratory. Third to fourth instars larvae of *An. arabiensis* were used continuously for the experiments (Plate 2).

Plate 2. Rearing of *An. arabiensis* in the laboratory

3.5. Collection of the test plants

Marigold (*Tagetes minuta* L.) and Custard Apple (*Annona squamosa* L.) (Plate 3 and 4), respectively were selected based on their known toxic effects (Green *et al*. (1991) and Perich *et al. (1994)* and Saxena *et al*. (1993), respectively. *Tagetes minuta* L. was collected around Awassa, south Ethiopia and *Annona squamosa* L. was collected around Dire Dawa, eastern Ethiopia. The plants were identified by a taxonomist at the National Herbarium (Biology Department, AAU).

Plate 3. *Tagetes minuta* L. with intact floral parts

Plate 4. *Annona squamosa* L. fruit and seeds

3.6. Extraction of essential oils from *Tagetes minuta* floral parts and *Annona squamosa* seeds

After collection of the test plants from their natural habitats within the country, their essential oils were extracted by hydro-steam distillation in a Clevenger type of apparatus at the insect science laboratory of Addis Ababa University. In this process, the seed of *A. squamosa* L. and floral part of *T. minuta* L. were dried in the shade at the environmental temperatures. The dried seed and floral parts were powdered mechanically using commercial electrical stainless steel blender and the powdered seed and floral part (250g) were placed into a distillation flask with Clevenger type apparatus (Plate 5) and approximately three times water in volume than the plant material were added. The distillation chamber was heated from 80-90^{0}C and allowed to boil until the distillation process was completed for 3 hours. The distillate was then collected from the separating funnel in which the aqueous portion was separated from the essential oil. The water layer was slowly drawn off until only the oil layer remained. The oil was collected in Vials and stored at about 4^{0}C until it was used for the different treatments. The yield obtained from *A. squamosa* and *T. minuta* were 2.75ml and 6.5ml, respectively.

3.7. Extraction of crude oil from *Tagetes minuta* floral parts and *Annona squamosa* seeds

After collection of the test plants from their natural habitats within the country, their crude oils were extracted by Rotary evaporator at the insect science laboratory of Addis Ababa University. In this process, the seed of *A. squamosa* L. and floral parts of *T. minuta* L. were dried in the shade at the environmental temperature. The dried seed and floral parts were powdered mechanically using commercial electrical stainless steel blender and the powdered seed and floral parts (150g) were dissolved in 300ml of acetone, ethanol, hexane and distilled water separately for 24 hours. The mixtures were filtered by Whatman's No.1 filter paper with the diameter of 11 cm. The extracts were concentrated in a rotary evaporator (Plate 6) under reduced pressure at 56°C for acetone, 69°C for hexane, 79°C for ethanol and 100^{o}C for distilled water and the residue obtained was stored at 4°C until it was used for different treatments. The extracts of the *A. squamosa* seed powder with distilled water, hexane, acetone and ethanol yielded 4.3g, 8.7g, 9.9g and 11.9g respectively and 3.9g, 3.58g, 4.9g and 4.86g of *T. minuta* floral parts respectively.

Plate 5. Clevenger type apparatus used for essential oil extraction

Plate 6. Rotary evaporator apparatus used for solvent extraction

3.8. Test procedures for essential oils and crude oils

3.8.1. Larvicidal bioassay of essential oils and crude oils in the laboratory

The plant oils and crude extracts from *Annona squamosa* L. seed and *Tagetes minuta* L. floral parts larval toxicity tests were carried out based on the World Health Organization standard larval bioassay test method (2005) and (1963), respectively. The stock solutions were prepared over different concentration series. To prepare 5ml of fresh stock solution of 0.25% (2500 ppm), 0.5% (5000 ppm), and 1% (10,000 ppm): 0.0125 ml, 0.025 ml and 0.05 ml of the oil were dissolved in 4.9875ml, 4.975 ml and 4.95 ml of acetone, respectively. Then 1ml was taken from each stock solution and added to three enamel cups, each enamel cups containing 149ml of distilled water to obtain a final concentration of 16.67 ppm, 33.33 ppm, and 66.67 ppm, respectively. For solvent extract 150mg (0.15g) of each crude oil were dissolved in 10 ml of acetone to prepare stock solution. From these stock solutions different concentrations (6.25ppm, 12.5ppm, 25ppm, 50ppm 100 ppm) were prepared in to five different enamel cups containing 150 ml distilled water.

Twenty five active 3rd-4th instars larvae from colonies of *An. arabiensis* were transferred in to 350ml of white enamel cups containing oils and crude extracts independently with different concentrations (16.67, 33.33 and 66.67ppm) for oils and (6.25ppm ,12.5ppm, 25ppm, 50ppm 100ppm) for crude extract. Twenty five larvae were transferred in to white enamel cups contained distil water with 1 ml acetone but not extract that provide as a control (Plate 7). The cups containing the exposed larvae were maintained at room temperature of 25-27 °C and 60-70% relative humidity. Four replicates for the treatments and four replicates for controls were carried on for each concentration during which no food was offered to the larvae. The mortalities of mosquito larvae were recorded in 24 and 48 hours, if moribund larvae were found incapable of rising to the surface or of showing the characteristic diving reaction when the water was disturbed or they showed discoloration, unnatural position or rigor.

Plate 7. Larvicidal bioassay in the laboratory using white enamel cups

3.8.2. Larvicidal bioassay of essential oils and crude oils in semi-field conditions

The field trials were conducted according to the methods recommended by World Health Organization (WHO, 2005 and Mwakiko and Savaeil, 1994). Artificial containers or plastic bowels with shallow depth were used for the larvicidal bioassay in the field. The containers were buried into the ground. Water from the natural breeding habitats of the larvae was added into the container. The essential oils were applied and evaluated against the field population of *Anopheles* larvae in such a way that a batch of 30 field population of *Anopheles* larvae were released into each bowel containing 298ml of water. Following the above procedure 5ml stock solution was prepared. After an hour of larval acclimatization, in each container 2 ml of the stock solution was added to make different concentrations. Then closed with mosquito net to prevent other mosquitoes from laying eggs and falling debris (Plate 8).

For solvent extract, 300mg (0.3g) of each crude oil were dissolved in 10 ml of acetone to prepare stock solution and from it different concentrations (6.25ppm, 12.5ppm, 25ppm, 50ppm and 100 ppm) were prepared in 300 ml distilled water and the larvae treated following the above procedure. Then all containers were examined at 5 and 24 hours and data were recorded.

Plate 8. Larvicidal bioassay under semi field condition

3.9. Data analysis

Data from laboratory and field experiments were subjected to two way analysis of variance (ANOVA) and one way analysis of variance using computer soft ware programs (SPSS Inc., version 15, 2006). When significant differences were observed, the means were separated with LSD test. Percentages of mortality counts were made using Abbott's formula (Abbott, 1925).

$$\text{Mortality } \% = \frac{X-Y}{X} \times 100$$

Where X= number surviving in the untreated control and Y= number surviving in the treated group. Percentage of larval mortality was means of four replicates (WHO, 2005). The whole set was discarded, if the mean mortality in the control was greater than 20%.

After 5hr field treatment and 24hr laboratory treatment their LC_{50} (the concentration at which 50% of test larvae were died), LC_{90} (the concentration at which 90% of test larvae were died) and the 95% confidence limit of upper confidence limit (UCL) and lower confidence limit (LCL) and chi-square values were calculated by probit analysis using SPSS computer software programs version 15.0 in order to compare the larvicidal potency of the plants and susceptibility of the test mosquito larvae.

4. RESULTS

4.1. The effect of different solvent extracts of *Annona squamosa* seed and *Tagetes minuta* floral parts against *An. arabiensis* larvae under laboratory conditions

Results of mean percentage mortality of *Anopheles arabiensis* treated with *A. squamosa* seed and *T. minuta* flower extracts in the laboratory with different concentrations at different exposure time periods are presented in Table 1 and 2.

Analysis of variance showed that there were highly significant differences ($P<0.05$) between solvent extract of *Annona squamosa* seed and *Tagetes minuta* flower ,among the solvents used and different levels of concentrations under laboratory conditions in both 24 and 48hrs, respectively (Annex 1 and 2). *Annona squamosa* seed powder extracted with acetone at 100ppm resulted in high percent larval mortality followed by 100ppm *A. squamosa* hexane extract at 24hr exposure time (Table 1). Acetone and hexane extracts of *A. squamosa* at 50 ppm and hexane extracted *T. minuta* at 100ppm also showed a marked level of mortality against 3rd-4th instar larvae of *An. arabiensis* under laboratory conditions (Table 1 and 2).

The result in Table 2 indicates that hexane extract of *A. squamosa* seed powder at 50ppm caused 100% mortality of the 3rd-4th instar larvae of *An. arabiensis* at 48hrs exposure time. Similar result was also observed in acetone and hexane extracts of *A. squamosa* at 100ppm. *Tagetes minuta* extracted with hexane resulted in higher percent larval mortality at 100ppm compared to those extracted with ethanol, acetone and distilled water at 24 and 48hrs exposure time. However, both botanicals (*A. squamosa* and *T. minuta*) extracted with distilled water did not cause any significant **(**$P > 0.05$) larvicidal activity after exposure for 24 and 48hrs and were the least effective as compared to all other tested extracts under laboratory conditions**.**

Table 1. Mean Percentage mortality of *An. arabiensis* larvae treated with *A. squamosa* and *T. minuta* crude extracts under laboratory condition at 24hrs exposure time

Botanicals	Solvents	Concentrations					
		6.25ppm	12.5ppm	25ppm	50ppm	100ppm	Control
Annona squamosa	Acetone	61.0±4.5 Ad	63.0±4.8Ad	71.0±4.5Ac	85.0±3.6Bb	100.0±0.0Aa	0.0±0.0 Ae
	Ethanol	19.0±3.9Be	24.0±4.3Cd	47.0±5.0Bc	73.0±4.4Cb	76.0±4.3Ba	0.0±0.0 Af
	Hexane	19.0±3.9Be	29.0±4.5Bd	70.0±4.6 Ac	93.0±2.5Ab	98.0±1.4Aa	0.0±0.0 Af
	Distilled water	1.0±1.0Ca	3.0±1.7Da	3.0±1.7 Ca	3.0±1.7Da	3.0±1.7Ca	0.0±0.0 Aa
	Control	0.0±0.0 Ca	0.0±0.0 Da	0.0±0.0Ca	0.0±0.0Da	0.0±0.0Ca	
Tagetes minuta	Acetone	2.0±1.4Bd	3.0±1.7Bd	8.0±2.7Cc	48.0±5.0Bb	57.0±4.9 Ba	0.0±0.0 Ad
	Ethanol	5.0±2.2Bc	8.0±2.7Bc	10±3.0 B b	10±3.0Cb	12.0±3.2Ca	0.0±0.0 Ac
	Hexane	17.0±3.8Ae	28.0±4.5Ad	61.0±4.9Ac	72.0±4.5Ab	76.0±4.3Aa	0.0±0.0 Af
	Distilled Water	3.0±1.7Ba	5.0±2.2Ba	6.0±2.4Ca	6.0±2.9D a	7.0±2.6Da	0.0±0.0 Aa
	Control	0.0±0.0Ca	0.0±0.0Ca	0.0±0.0Da	0.0±0.0 Ea	0.0±0.0Ea	

Means followed by the same letter (s) within a column (upper case) and within a row (lower case) along with botanical are not significantly different at α=0.05 (LSD)

Table 2. Mean Percent mortality of *An. arabiensis* larvae treated with *A. squamosa* and *T. minuta* crude extracts under laboratory condition at 48hrs exposure time

Botanicals	Solvents	Concentrations					
		6.25ppm	12.5ppm	25ppm	50ppm	100ppm	Control
Annona squamosa	Acetone	86.0±3.5Ac	89.0±3.1Ac	94.0±2.4Ab	98.0±1.4Aa	100.0±0.0Aa	0.0±0.0Ad
	Ethanol	72.0±4.5Be	78.0±4.1Bd	88.0±3.2Bc	93.0±2.5Bb	98.0±1.4Aa	0.0±0.0Af
	Hexane	36±4.8Cd	60±4.9Cc	92±2.7 Ab	100±0.0Aa	100±0.0Aa	0.0±0.0Af
	Distilled Water	5.0±2.2Db	8.0±2.7Db	9.0±2.9Cb	10.0±3.0Cb	14.0±3.5Ba	0.0±0.0Ac
	Control	0.0±0.0Ea	0.0±0.0Ea	0.0±0.0Da	0.0±0.0Da	0.0±0.0Ca	
Tagetes minuta	Acetone	6.0±2.4Ce	20.0±4.0Bd	43.0±4.9Bc	88.0±3.2Bb	90.0±3.0Ba	0.0±0.0 Af
	Ethanol	14.0±3.5Bc	15.0±3.8 Cc	15.0±3.8Cc	21.0±4.1Cb	29.0±4.5Ca	0.0±0.0 Ad
	Hexane	80.0±4.0Ad	81.0±3.9Ad	89.0±3.1Ac	94.0±2.4Ab	99.0±1.0Aa	0.0±0.0 Ae
	Distilled Water	6.0±2.4Cc	7.0±2.6Dc	9.0±2.9Db	9.0±2.9 Db	17.0±3.6Da	0.0±0.0 Ad
	Control	0.0±0.0Da	0.0±0.0Ea	0.0±0.0Ea	0.0±0.0Ea	0.0±0.0Ea	

Means followed by the same letter (s) within a column (upper case) and within a row (lower case) along with botanical are not significantly different at α=0.05 (LSD).

4.2. The effect of essential oils of *Annona squamosa* seed and *Tagetes minuta* floral parts against 3rd-4th instar *An. arabiensis* larvae under laboratory conditions

The results of essential oils of *Annona squamosa* seed and *Tagetes minuta* flower powder against 3rd-4th instar *An. arabiensis* larvae at different exposure times under laboratory conditions are presented in Table 3. Analysis of variance showed that there were highly significant differences ($P<0.05$) between the oils of *Annona squamosa* seed and *Tagetes minuta* flower and different concentrations under laboratory conditions in 24 and 48hrs respectively (Annex 5 and 6).

The essential oils of *A. squamosa* seed and *T. minuta* flower at 66.67ppm exhibited the highest larvicidal activity against 3rd-4th instar *An. arabiensis* larvae both after 24hrs and 48 hrs exposure under laboratory conditions (Table 3). Moderately high larvicidal effects were also exhibited by essential oils of both *A. squamosa* seed and *T. minuta* flower powder at 33.33 ppm against *An. arabiensis* under laboratory conditions (Table 3).

Table 3. Mean Percent mortality of *An. arabiensis* larvae under laboratory condition treated with *A. squamosa* and *T. minuta* essential oils

Botanicals	Concentrations							
	16.67ppm	33.33ppm	66.67ppm	Control	16.67ppm	33.33ppm	66.67ppm	Control
	24hrs				48hr			
Annona squamosa	45.0±4.9c	70.0±4.6b	99.0±1.0a	0.0±0.0d	60.0±4.9c	83.0±3.8b	100.0±0.0a	0.0±0.0d
Tagetes minuta	24.0±4.3c	65.0±4.8b	97.0±1.7a	0.0±0.0d	33±4.7c	76±4.3b	100±0.0a	0.0±0.0d

Means followed by the same letter (s) within a row along with botanicals and exposure time are not significantly different at α=0.05 (LSD).

4.3. The effect of different solvent extracts of *A. squamosa* seed and *T. minuta* floral parts powder against 3rd-4th instar larvae of *Anopheles* mosquito under semi – field conditions

Data on the percent mortality of 3rd -4th instar larvae of *Anopheles* mosquito treated with different concentrations of the extracts of *A. squamosa* seed and *T. minuta* floral parts under semi field conditions at the end of 5hrs are presented at (Table 4 and 5). Analysis of variance showed that there were significant differences between botanicals, solvent and concentrations under semi field condition both in 5 and 24hrs respectively ($P<0.05$) (Annex 3 and 4). However, there were no significant differences among botanicals, solvents and concentrations under semi field conditions in 5hrs ($P>0.05$) (Annex3).

The acetone extracted *A. squamosa* showed a greater larvicidal effect at 100 ppm when compared to other solvent extracted tested botanicals after 5hrs exposure. The larvae of *An. arabiensis* were also susceptible to hexane extracted *A. squamosa* followed to acetone extracted *A. squamosa* with similar concentration. Acetone and hexane extracted *A. squamosa* at 50 ppm and hexane extracted *T. minuta* at 50 ppm and100 ppm exhibited a moderate activity. However, water extracted *A. squamosa* and *T. minuta* were the least effective in all tested concentrations.

As time increased, the effectiveness of the tested botanical also increased as shown in Table 4 and 5. *Annona squamosa* dissolved in acetone caused 100% mortality of anopheline larvae in simulated field condition at 100 ppm after 24hrs exposure. Similarly, hexane extracted *A. squamosa* showed approximately 100% mortality exhibited in similar concentration.

Table 4. Mean Percent mortality of *Anopheles* larvae treated with *A. squamosa* and *T. minuta* crude extracts under semi field condition at 5hrs exposure time

Botanicals	Solvents	Concentrations					
		6.25ppm	12.5ppm	25ppm	50ppm	100ppm	Control
Annona squamosa	Acetone	39.2±4.5 Ae	47.5 ±4.6 Ad	58.3±4.5Bc	72.5 ±4.1Bb	90.0±2.7Aa	0.0±0.0 Af
	Ethanol	31.7 ±4.2Bd	36.7±4.4Cc	55.8±4.5Cb	58.3±4.5Cb	66.7±4.3Ca	0.0±0.0 Ae
	Hexane	20.0±3.7Ce	40.0±4.5Bd	60.0±4.5Ac	80.0±3.7Ab	87.5±3.0Ba	0.0±0.0 Af
	Distilled Water	5.0±2.0Db	5.8±2.1Db	5.8±2.1Db	6.7±2.3Db	10.8±2.8Da	0.0±0.0 Ac
	Control	0.0±0.0Ea	0.0±0.0 Ea	0.0±0.0 Ea	0.0±0.0Ea	0.0±0.0Ea	
Tagetes minuta	Acetone	5.0±2.0Be	11.7 ±2.9Bd	22.5 ±3.8 Bc	38.3 ±4.4Bb	55.0 ±4.5Ba	0.0±0.0 Ae
	Ethanol	2.5 ±1.4Cc	5.0 ±1.9Cb	5.0 ±1.9Cb	5.8 ±2.1Cb	8.3 ±2.5Ca	0.0±0.0 Ac
	Hexane	34.2 ±4.3Ae	45.0 ±4.5Ad	55.8 ±4.5Ac	76.7 ±3.9 Ab	81.7 ±3.5Aa	0.0±0.0 Af
	Distilled Water	1.7 ±1.2Cb	1.7±1.2Db	4.2 ±1.8Ca	4.2±1.8C a	4.2±1.8Ca	0.0±0.0 Ab
	Control	0.0±0.0Ca	0.0±0.0Da	0.0±0.0Da	0.0±0.0Da	0.0±0.0Da	

Means followed by the same letter (s) within a column (upper case) and within a row (lower case) along with botanical are not significantly different at α=0.05 (LSD).

Table 5. Mean Percent mortality of *Anopheles* larvae treated with *A. squamosa* and *T. minuta* crude extracts under semi -field condition at 24hrs exposure time

Botanicals	Solvents	Concentrations					
		6.25ppm	12.5ppm	25ppm	50ppm	100ppm	Control
Annona squamosa	Acetone	75.8±3.9Ae	82.5±3.5Ad	88.3±2.9Ac	96.6 ±1.6Ab	100.0±0.0Aa	0.0±0.0 Af
	Ethanol	56.7±4.5Bc	76.7±3.9Bb	80±3.6Ba	80.8±3.6Ba	81.7±3.5Ba	0.0±0.0 Ad
	Hexane	50.0±4.6Be	71.7±4.1Bd	85.0±3.3Ac	94.2 ±2.1Ab	99.2±0.8Aa	0.0±0.0 Af
	Distilled Water	7.5±3.5Cc	8.3±2.5Cc	9.2±2.6Cc	11.7±2.9Cb	17.5±3.5Ca	0.0±0.0 Ad
	Control	0.0±0.0Da	0.0±0.0 Da	0.0±0.0Da	0.0±0.0Da	0.0±0.0Da	
Tagetes minuta	Acetone	13 .3 ± 3.1Be	25.8 ± 3.9Bd	60.8 ±4.5Bc	81.7 ±3.5Bb	94.2 ±2.1Ba	0.0±0.0 Af
	Ethanol	7.5 ±2.4Cc	8.3 ±2.5Cc	9.2 ±2.6Cc	12.5 ±3.0 Cb	20±3.7Ca	0.0±0.0 Ae
	Hexane	83.3 ±3.4Ac	86.7± 3.1Ac	90 ± 2.7Ab	97.5 ± 1.4Aa	98 ± 1.2Aa	0.0±0.0 Ad
	Distilled Water	3.3 ± 1.6Dd	7.5 ± 2.4Cc	7.5±2.4Cc	10.8± 2.8Cb	13.3 ± 3.1Da	0.0±0.0 Ad
	Control	0.0±0.0 Da	0.0±0.0Da	0.0±0.0D a	0.0±0.0Da	0.0±0.0Ea	

Means followed by the same letter (s) within a column (upper case) and within a row (lower case) along with botanical are not significantly different at α=0.05 (LSD).

4.4. The effect of essential oils of *Annona squamosa* seed and *Tagetes minuta* floral parts against 3rd-4th instar *Anopheles* mosquito larvae under semi-field conditions

Analysis of variance showed that there were no significant differences between botanicals and concentrations interaction in the oil of *Annona squamosa* seed and *Tagetes minuta* flower under semi field condition in 5hrs (P>0.05) (Annex 7). However, there were significant differences in the larvicidal effect of botanicals and concentration under semi-field condition in both 5 and 24hrs respectively (P<0.05) (Annex 7 and 8).

Data on the effect of *A. squamosa* seed and *T. minuta* flower powder essential oils against larvae of *Anopheles* mosquito under semi field conditions are presented in Table 6. The results from simulated field experiment show that there were statistically significant difference in percentage of larval mortality of *Anopheles* larvae treated with *A. squamosa* and *T. minuta* oils at different concentrations at 5hrs and 24hrs exposure time Table 6.

Annona squamosa oil at 66.67 ppm resulted in the highest larval mortality (71%). On the other hand, the least effect (17.5%) observed at 16.67% ppm of *T. minuta* oil after 5hrs exposure Table 6. *Annona squamosa* and *Tagetes minuta* oils exhibited similar effect (83.3%) at the concentration of 66.67 ppm after the exposure of 24hrs in the field.

Table 6. Mean Percent mortality of *Anopheles* larvae under semi-field condition treated with *A. squamosa* and *T. minuta* essential oils

Botanicals	Concentrations							
	16.67ppm	33.33ppm	66.67ppm	Control	16.67ppm	33.33ppm	66.67ppm	Control
	5hrs				24hrs			
Annona squamosa	20.8±3.7c	57.5±4.5b	71.7±4.1a	0.0±0.0d	34.2±4.3c	69.2±4.2b	83.3±3.4a	0.0±0.0d
Tagetes minuta	17.5±3.3c	52.5±4.6b	60±4.5a	0.0±0.0d	32.5±4.3c	65.0±4.4b	83.3±3.4a	0.0±0.0d

Means followed by the same letter (s) within a row along with botanicals and exposure time are not significantly different at α=0.05 (LSD).

4.5. Dose response effect of *A. squamosa* seed *and T. minuta* floral part extracts on *An. arabiensis* under laboratory conditions

Table 7 shows the LC_{50} and LC_{90} values of the solvent extracts of *A. squamosa and T. minuta* against 3rd-4th instar larvae of *An. arabiensis* in the laboratory. The LC_{50} and LC_{90} values against *An. arabiensis* larvae ranged from 13.3 to 574.9 ppm and 48.3 to 643.3 ppm, respectively. From the tested plants, acetone extracted *A. squamosa* seed extracts exhibited the highest larvicidal activity (LC_{50} = 13.3ppm) and hexane extracted *A. squamosa* extracts showed the highest LC_{90} value (LC_{90}= 48.35). Both water extracted *A. squamosa and T. minuta* were the weakest in larvicidal activity against *An. arabiensis*. The dose response effects showed that there were statistically significant differences among the concentrations ($P<0.05$), except *A. squamosa* seed extracted by water and *T. minuta* extracted by ethanol and water. Furthermore, oil of *A. squamosa* showed strong larvicidal activity after the exposure of 24 hrs with LC_{50} values 23.7ppm and LC_{90} values 43.4ppm against *An. arabiensis* Table 8**.** Similarly, larvicidal activity of *T. minuta* oil extract showed 29.4ppm and 49.9ppm of LC_{50} and LC_{90} values, respectively (Table 8).

Table 7. LC_{50} and LC_{90} values of *A. squamosa* and *T. minuta* solvent extracts against 3^{rd}-4^{th} instar larvae of *An. arabiensis* after 24hrs exposure in the laboratory.

Source of botanicals	Solvent used	LC_{50}values (95%CI)	LC_{90} values (95% CI)	X^2 (df=22)	P-value
A. squamosa seed powder	Acetone	13.3(-0.381-23.5)	59.9(44.5-97.7)	114.3	0.000*
	Ethanol	45.8(35.5-59.1)	106.8(86.8-143.5)	73.247	0.000*
	Hexane	23.386 (†)	48.355 (†)	1953.956	0.000*
	Water	574.913 (†)	916.089 (†)	19.005	0.645
T. minuta flower powder	Acetone	79.9(68.9-95.6)	134.8(114.9-168.0)	4.647	0.000*
	Ethanol	286.4(182.6-879.7)	508.0(313.4-1633.4)	17.637	0.727
	Hexane	42.5(29.6-59.4)	105.7(81.9-157.9)	109.7	0.000*
	Water	382.5(212.3-11241.832)	643.3(345.6-19788.5)	22.707	0.418

Numbers in parenthesis are the 95% confidence intervals

*=There were significant difference among the concentrations at α=0.05 (Probit regression analysis).

(†)=There was no remarkable difference between the upper and lower values

Table 8. LC_{50} and LC_{90} values of the essential oils of *A. squamosa* seed and *T. minuta* floral parts against 3rd- 4th instar larvae of *An. arabiensis* after 24hrs (laboratory based experiment).

Botanicals	LC_{50}values (95%CI)	LC_{90} values (95% CI)	X^2 (df= 14)	P-value
A. squamosa seed powder	23.7(19.6-28.2)	43.4(37.2-53.9)	30.538	0.006*
T. minuta flower powder	29.4(25.4-33.9)	49.9(43.8-59.5)	25.461	0.030*

Numbers in parenthesis are the 95% confidence intervals.

*=There were significant difference among the concentrations at $\alpha=0.05$ (Probit regression analysis).

4.6. Dose response effect of *A. squamosa* seed and *T. minuta* floral part extracts on *Anopheles* mosquito larvae under semi-field conditions

The LC_{50} and LC_{90} results of *A. squamosa* and *T. minuta* with different solvent extracts for laboratory and field strains were not the same. The LC_{50} value of acetone extracted *A. squamosa* for field strain *Anopheles* mosquito after 5hrs exposure was 28 ppm. *Annona squamosa* and *Tagetes minuta* extracted with hexane, LC_{50} value was almost similar (LC_{50}= 32 ppm). On the other hand, the LC_{90} value of hexane extracted *A. squamosa* was the highest (82ppm) whereas the LC_{90} values of acetone extract *A. squamosa* was 84ppm (Table 9). Water extracted *A. squamosa and T. minuta* had exhibit the least LC_{50} and LC_{90} value when compared with the other solvents. Based on the probit analysis, the 5hrs LC_{50} and LC_{90} values of the *A. squamosa* oil for *Anopheles* mosquito larvae were found to be 41.5ppm and 79.2ppm, respectively (Table 10). Dose response effects showed that there were statistically significant differences in LC_{50} and LC_{90} values among the concentrations ($P< 0.05$).

Table 9. LC_{50} and LC_{90} values of *A. squamosa* and *T. minuta* solvent extracts against 3rd-4th instar larvae of *Anopheles* mosquito after 5hrs exposure in semi- field conditions.

Source of botanicals	Solvent used	LC_{50}values (95%CI)	LC_{90} values (95% CI)	X^2 (df= 22)	P- value
A. squamosa seed powder	Acetone	28.007(17.364-39.297)	84.753(66.665-121.187)	102.833	.000*
	Ethanol	48.960(34.717-70.153)	143.037(108.176-226.062)	79.952	.000*
	Hexane	32.209(23.043-42.659)	82.456(66.609-111.570)	99.330	.000*
	Water	293.022 (193.070- 745.793)	499.410 (319.539-1322.375)	13.079	.931
T. minuta flower powder	Acetone	82.479 (71.116-98.996)	153.794 (130.378-191.656)	40.754	.009*
	Ethanol	310.505(199.740-902.111)	515.020(321.681-1557.252)	13.414	.921
	Hexane	32.330(20.216-45.658)	98.036(76.402-143.441)	104.550	.000*
	Water	410.224(225.738-34353.239)	656.083(350.095-57328.177)	15.514	.839

Numbers in parenthesis are the 95% confidence intervals.

*=There were significant difference among the concentrations at α=0.05 (Probit regression analysis)

Table 10. LC_{50} and LC_{90} values of essential oils of *A. squamosa* and *T. minuta* against 3^{rd}-4^{th} instar larvae of *Anophlese* mosquito after 5hrs exposure (semi-field based experiment).

Botanicals	LC_{50}values (95%CI)	LC_{90} values (95% CI)	X^2 (df= 14)	p-value
A. squamosa seed powder	41.5(34.7-49.9)	79.2(67.1-100.3)	37.401	0.001*
T. minuta flower powder	48.8(40.2-61.3)	93.1(76.2-127.0)	41.847	0.000*

Numbers in parenthesis are the 95% confidence intervals.
*=There were significant difference among the concentrations at $\alpha=0.05$ (Probit regression analysis).

5. DISCUSSION

In the present study, the effects of *Annona squamosa* seed and *Tagetes minuta* flower extract and their essential oils on *An. arabiensis* 3rd-4th instar larvae were compared in terms of their relative potential as source of botanicals for malaria vectors (*An. arabiensis*) control. As the result indicated that *A. squamosa* and *T. minuta* extracted with acetone, ethanol, hexane and distilled water applied in different concentration have variable larvicidal effects against the 3rd- 4th instar *Anopheles* mosquito larvae under laboratory and semi-field conditions. At higher concentration the larvae showed restless movement for some time and then settled at the bottom of the enamel cup and died slowly.

After 24hrs exposure in the laboratory, the hexane and acetone extracts obtained from the seed of *A. squamosa* at 100ppm have shown higher percent mortality while the ethanol extract of *A. sqamosa* had moderate percentage mortality. The mean percentage mortality of 3rd-4th instar larvae of *An. arabiensis* treated with hexane and acetone extracted *A. squamosa* at 100ppm were 98% and 100% respectively where as ethanol extracted *A. squamosa* with the same concentration was 76% under laboratory condition after 24 hrs exposure. The percentage larval mortality of 3rd-4th instar larvae of *An. arabiensis* were increased as time of exposure prolonged. Hexane extracted *A. squamosa* at 50ppm, 100ppm and acetone extracted *A. squamosa* at 100ppm showed 100% mortality after 48hrs exposure under laboratory condition.

As Kamaraj *et al.* (2010) reported that hexane and chloroform extracted bark of *A. squamosa* had significant larvicidal effect against 4th instar *An. stephensi* with 84 and 100 percentage mortality respectively at 1000 ppm after 24hrs exposure. Sukumar *et al.* (1991) reported that the existence of variations in toxicity of phytochemical compounds against the larval instars varied with the plant part used, solvent used in extraction and the concentration of the extract.

In contrast to the present study, Brahim *et al*. (2006) reported that preliminary evaluation of larvicidal activity of aqueous extracts from *Ricinus communis* showed strong toxic activity against 4th instar larvae of four mosquito species *Culex pipiens* (L.), *Aedes caspius*, *Culiseta longiareolata* and *Anopheles maculipennis*. The present study results indicate that the water extracted *A. squamosa* and *T. minuta* had little or no effect against 3rd-4th instar larvae of *An. arabiensis*. The difference between the current findings and that by Brahim *et al*. (2006) could hence lie due to different in plant species. As reported by

Shaalan *et al.* (2005) bioactivity of phytochemicals against mosquito larvae can vary significantly depending on plant species, plant parts, age of plant and mosquito species.

In this study, hexane extracted *T. minuta* exhibited relatively moderate larvicidal activity against *An. arabiensis* larvae with the percentage mortality of 76% at 100ppm after 24hrs exposure next to *A. squamosa*. Abbas *et al.* (2005) reported that the larvicidal activity of *T. minuta* was possibly because of its terpenoids, aglycon, flavonoids and saponins which may be the effective components that have larvicidal effect.

According to Marcard *et al.* (1986) total mortality was consistently positively correlated with insecticide concentrations and duration of exposure. Similarly this study showed that, as the concentration of hexane extracted *A. squamosa* increased from 6.25ppm to 100ppm, the percentage mortality exhibited almost five-fold of the initial concentration percentage mortality. The percentage larval mortality of 3^{rd}-4^{th} larvae of *An. arabiensis* were also increased as time of exposure prolonged. The mortality of Hexane extracted *A. squamosa* at 50 ppm was 100% after 48hrs exposure under laboratory condition.

The LC_{50} and LC_{90} values of larvicidal effect of the dose responses in the present study showed that there were significant differences ($P< 0.05$) in lethal concentrations of extracts of *A. squamosa* seed and *T. minuita* flower pod both in laboratory and on semi- field conditions. The exceptions were *A. squamosa* seed extracted by water and *T. minuta* flower extracted by ethanol and water in which the LC_{50} and LC_{90} were insignificant
(P ☜ 0.05).

In the present study, the dose response result showed that, the LC_{50} values for *A. squamos*a seed extracts were achieved at concentrations lower than 50ppm against 3^{rd}-4^{th} instar Anopheles larvae both in the laboratory and on semi-field conditions after 24 hrs and 5hrs exposure respectively. The LC_{50} result of *A. squamosa* seed extracted with acetone, hexane and ethanol were 13.3ppm, 23.3ppm and 45.8ppm respectively under laboratory condition with in 24hrs exposure time. Similarly, 28ppm, 32ppm and 48.9ppm LC_{50} value were observed for acetone, hexane and ethanol extracts respectively under semi-field condition after 5hrs exposure time. Of all results the LC_{50} value of acetone extracted *A. squamosa* had the highest larvicidal activity than other extracts against 3rd-4th instar *An. arabiensis* larvae after 24 hrs laboratory exposure.

Earlier studies involving the aqueous leaf extract of *Calotropis procera* was calculated with the LC_{50} values of 273.53, 366.44 and 454.99 ppm against 2nd, 3rd, and 4th instar larvae respectively of *An. arabiensis* (Abdalla *et al.,* 2009). Similarly, Bagavan *et al.* (2009) reported larvicidal activity of acetone leaf extracted *A. squamosa* against *An. subpictus* with LC_{50} value 17.48ppm after 24hrs of exposure. Moreover, Larvicidal effects in leaf acetone of *Adhatoda vasica*, bark ethyl acetate of *A. squamosa* against the fourth instar larvae of *An. stephensi* after 24hrs exposure were (LC_{50} = 18.20 and 25.18 ppm; LC_{90} = 96.33 and 94.04 ppm) (Kamaraj *et al.,* 2010). However, the effects of acetone extracted *A. squamosa* tested in the present study were much lower with LC_{50} values of 13.3ppm against *An. arabiensis*.

In this study bioassay showed a moderate effect of hexane and acetone extracted *T. minuta* against 3rd-4th instar larvae of *An. arabiensis* with the LC_{50} value of 42.5 ppm and 79.9 ppm, respectively. Conversely, studies conducted by Macedo *et al.* (1997) showed that ethanol extract of *T. patula* was less active and only 50% larvae were killed at higher concentration (100ppm) against *A. aegypti*, *An. stephensi* and *C. quinquefasciatus*. It has been demonstrated that insecticidal activity and chemical composition of marigold (*Tagetes*) species vary considerably depending on geographic location, growing conditions, plant parts from which they are extracted, developmental stage of plant, solvent used for extraction, photosensitivity of some of the compounds in the extract, and the methods used to isolate the essential oils (Singh *et al.,* 2001; Wells, 1993).

In this study also, the larvicidal activity of essential oils from *A. squamosa* and *T. minuta* was tested against 3rd-4th instar larvae of *An. arabiensis*. As the results indicate that essential oils extracted by hydro distillation applied with different concentration have variable larvicidal effects against *An. arabiensis* larvae. Among the two tested plants, *A. squamosa* was generally effective. Treatment on *An. arabiensis* larvae with *A. squamosa* oil at a concentration of 66.67 ppm caused 99% mortality after 24hrs exposure under laboratory condition.

The results somewhat are similar with earlier studies on essential oils, Azhari *et al.* (2009) reported that larvicidal activity of *Ocimum basilicum* essential oil against Anopheles mosquito larvae caused 100% mortality at a concentration range of 300-500ppm. The slight differences can be attributed to many factors; perhaps the most obvious is the difference in plant species, concentration or amount of essential oil and obtaining method.

The LC_{50} and LC_{90} values for *A. squamosa* oil were 41.5 ppm and 79.2 ppm, respectively against 3rd-4th instar *An. arabiensis* larvae in the laboratory after 24 hrs exposure. The larvicidal activities of *A. squamosa* obtained in this study are in agreement with previous reported data. Massebo *et al.* (2009) reported larvicidal activity of essential oils of 11 local plants against 3rd-4th instar larvae of *An. arabiensis* and *Aedes aegypti* and the LC_{50} values of the oils ranged from 17.5 - 85.9 ppm against *An. arabiensis*. The effects of the essential oil (*Annona squamosa)* of the present study were much similar to the information explained by Massebo *et al.* (2009) and probably the *A. squamosa* will be an additional source of biologically active agents against mosquito control in the future in Ethiopia.

The laboratory reared *An. arabiensis* larvae were found to be more susceptible to the tested botanical extracts than field population of Anopheles larvae. No tested plant material in this study exhibited similar activity against laboratory reared *An. arabiensis* and field population of Anopheles mosquito. The LC_{50} value of solvent extracted *A. squamosa* in the laboratory was in the range of 13.3 ppm - 574.9 ppm while the LC_{50} values of *A. squamosa* in the field was in the range of 28 ppm - 293 ppm. Besides this, the *A. squamosa* oil LC_{50} values of the laboratory were different from the result of the semi- field.

Recently, George and Vincent (2005) evaluated the larvicidal activity of petroleum ether seed extract of *A. squamosa* L. and *Pongamia glabra* L. against field collected and laboratory reared *Cx. quinquefasciatus* larvae and noted that the field collected larvae were apparently better adapted to adjust to stress variations in the environment and hence required a higher concentration of extract to bring about the required mortality. Moreover, Sun *et al.* (2006) evaluated the larvicidal effects of ethanol extract of *Ginkgo biloba* L. against laboratory and field strain of C*ulex pipiens* and reported that the field strain were more resistant than laboratory reared strain. The possible reasons are that the field strains were genetically more heterogeneous (Kabir *et al.*, 2006) and are routinely exposed to diverse insecticides. Therefore, they probably have a higher general tolerance to toxic compou

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. CONCLUSION

Synthetic insecticides have created a number of ecological problems, such as the development of resistant insect strains, ecological imbalance and harm to mammals. Hence, there is a constant need for developing biologically active plant materials as larvicides, which are expected to reduce the hazards to human and other organisms by minimizing the accumulation of harmful residues in the environment. Natural products are generally preferred because of their less harmful effect on non-target organisms and due to their biodegradable and non persistent in the environment.

The toxicity of the botanicals in this study investigated in laboratory and semi field conditions. The results indicate that acetone and hexane crude extract of the seed of *A. squamosa* shows very high larvicidal activity in the laboratory and semi field condition. In addition to this both plant essential oils are excellent larvicidal property against 3rd-4th instar larvae of *An. arabiensis*.

The toxicity of these botanicals under laboratory and semi field condition is that the solvent extract of *A. squamosa* seed and *T. minuta* floral parts and their essential oils have remarkable larvicidal properties and their use as larvicide against *Anopheles* mosquito should be explored, as these plant grows in some parts of Ethiopia. Regarding the effectiveness of the plant material, hexane and acetone extracted *Annona squamosa* and its oil were showing the most promising larvicidal activity both in the laboratory and on semi field conditions. Nevertheless, differences among individual extracts were found. Therefore, the larvicidal activity of *T. minutia* was less than *A. squamosa*. However, results suggest that *A. squamosa* and *T. minuta* having the potential to be used as larvicides against larvae of *Anopheles* mosquito.

6.2. RECOMMENDATIONS

- The efficacy of *A. squamosa* and *T. minuta* extracted by acetone and hexane and their essential oils promising in the laboratory and under semi field conditions. Therefore, wide range of field study should be made particularly in areas where Anopheles species is highly prevalent to determine the practical potential of the botanical extracts.

- As the study showed that *A. squamosa* and *T. minuta* extracts posses promising larvicidal effect on Anopheles mosquito larvae. Therefore, further study should be made on the evaluation of these plants extract on insect growth and development inhibition.

- Further detailed study on the mode of action of *A. squamosa* and *T. minuta* against larvae of *Anopheles* mosquitoes may pave the way for the development of botanical larvicidal for the control of *Anopheles* larvae in Ethiopia.

- This study suggest that the active ingredient of the plant extracts responsible for causing mortality of Anopheles larvae should be identified, utilized and approved not to cause toxic effects in non target organisms. If possible, it can be prepared as a commercial product or formulation for possible control of Anopheles mosquitoes larvae as a part of the integrated vector management program.