EVALUATION OF LARVICIDAL AND REPELLENT ACTIVITY OF *JUNIPERUS PROCERA* ESSENTIAL OIL AGAINST *ANOPHELES ARABIENSIS*, IN ETHIOPIA

BY

ASKUAL GIRMAY

RESEARCH THESIS SUBMITTED TO DEPARTMENT OF ENVIRONMENTAL HEALTH SCIENCE & TECHNOLOGY, COLLEGE OF PUBLIC HEALTH AND MEDICAL SCIENCE, JIMMA UNIVERSITY; IN PARTIAL FULFILLMENT FOR THE REQUIREMENTS FOR THE MASTERS DEGREE IN ENVIRONMENTAL HEALTH SCIENCE

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ADVISORS: 1. Dr. K. KARUNAMOORTHI (Ph.D)
2. Mr. SAMUEL FEKADU (M.Sc)

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Declaration

I, the undersigned, declare that this is my original work, has not been presented for a degree in this or other university and that all sources of materials used for this thesis have been acknowledged.

Name of the student: Askual Girmay Signature _____

Name of institution: Jimma University

Date of submission: ----------

This thesis has been submitted for examination with my approval as university advisors

Name of the Advisors

Approved by	Signature
Dr. K. Karunamoorthi (Ph.D)	_____
Mr. Samuel Fekadu (M.Sc)	_____

Department Head

Mr. Tadesse Getahun (MSc, PhD candidate) _____ _____

ABSTRACT

Background: *Despite of several decades of control efforts still malaria is considered to be a major public health issue and nearly half of the world population at risk of malaria. Malaria is a major public health problem in Ethiopia.*

Objective *The aim of this study was to evaluate the larvicidal and repellent activity of Juniperus procera essential oil against Anopheles arabiensis, which is major malaria vector in Ethiopia.*

Method: *Experimental study was conducted from June to November 2012, essential oil from Juniperus procera leaves were obtained by hydro-distillation method. The mosquito larvicidal and repellent efficacy were evaluated by using WHO standard procedure in Adama malaria research center under the laboratory conditions, the field study has been carried out by using An. gambiae sensu lato larvae ,from Boye river in Jimma town. Results were analyzed using of probit analysis, SPSS statistical software, Ms Excel 2007 and Repellency calculation.*

Results: *The results showed that essential oil of J. procera exhibited significant larval mortality against An. arabiensis. However, the highest larvicidal activity was observed and the LC_{50} and LC_{90} values were 14.4 and 24.7 ppm and 24.5 and 36.2 ppm for laboratory and field conditions, respectively. The Chi-square value of laboratory and field 6.662 and 4.622 are respectively, and significant at the $P<0.05$ level. The results clearly suggest that the laboratory reared mosquito larvae were more susceptible than field collected anopheles larvae.*

The essential oil showed significant repellency against adult An. arabiensis. Among the different (0.1, 0.15, 0.25 and 0.5 ml/cm^2) concentration of essential oil tested for the repellent activity and the largest protection time330 min was observed in 0.5 ml/cm^2. All the four tested concentrations of J. procera essential oil offered significant protection and independent t test results shows statistically significant (p value=0.0001) [0.1 ml/cm^2 (t=82.7; df=4); 0.15 ml/cm2 (t=80.3; df=4); 0.25 ml/cm2 (t=25.3; df=4); 0.5 ml/cm2 (t=96.8; df=4)] difference between treated and control groups. Overall, both the larvicidal and repellent activities were dose dependent.

Conclusion*: J. procera essential oil showed promising larvicidal and repellent properties against An. arabiensis and it could serve as a natural larvicidal and repellent agent. However, the active principles as well as mode of action must be identified in future studies.*

Key words*: Juniperus procera, Essential oil, larvicidal effect, repellent effect, An. arabiensis*

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Abbreviations

DDT	Dichloro Diphenyl Trichloroethane
FMOH	Federal Ministry Of Health
h	Hour
IRS	Indoor Residual Spraying
ITNs	Insecticide Treated Nets
LLINs	Long Lasting Insecticide Treated Nets
Min	Minute
MOH	Ministry Of Health
PMI	President's Malaria Initiative
ppm	Parts Per Million
SSA	Sub-Saharan Africa
WHO	World Health Organization
WMR	World Malaria Report
v/v	Volume per Volume

CHAPTER ONE: INTRODUCTION

1.1. BACK GROUND

Malaria is one of the most widespread infectious diseases of our time, taking the lives of almost one million people a year, most of them in sub-Saharan Africa and under the age of five. It is the fifth leading cause of death worldwide and almost half the world's population is at risk. Children and pregnant women are among the most vulnerable. The disease is not only a major killer in Africa but a primary cause of poverty. Malaria traps people in poverty and undermines the development of some of the poorest countries in the world. Though the majority of the cases and deaths (85%) from malaria are found in sub-Saharan Africa, malaria is also endemic in Asia and Latin America (WHO, 2010).

Malaria kills a poor African child for every 60 seconds and continues to be a major public health problem in the resource-limited countries of Africa, Asia, and Latin America and beyond (WHO, 2013). Pregnant women are also at high risk of malaria, with illness causing impaired fetal growth and high rates of miscarriage, and significant maternal deaths (up to 50% death rate in cases of severe disease). Malaria during pregnancy often contributes to maternal anemia premature delivery and low birth weight, leading to increased child mortality (Dharani and Yenesew, 2010).

Malaria is ranked as the leading communicable disease in Ethiopia. Approximately 75% of the country is malarious and an estimated 51 million (68%) of the population lives in areas at risk of malaria. According to federal ministry of health (FMOH) reports, approximately 70,000 people die of malaria each year in Ethiopia. Malaria is the leading cause of health problem in the country. *Plasmodium* species of epidemiological importance in Ethiopia are *Plasmodium falciparum* and *Plasmodium vivax* (PMI, 2011).

Mosquitoes go through four stages in their life cycle: egg, larva, pupa and adult. The first three stages live in water and last for 5 to 14 days in tropical settings, depending on the species and environmental factors (IWMI, 2010). *Anopheles arabiensis* is the most important transmitter of malaria vector in Ethiopia and is responsible for more than 95% of transmissions and breeds in small sun exposed pools mainly produced during the rains.

Malaria parasites are transmitted to humans by the bite of infected female mosquitoes of more than 30 *Anopheline* species.

Since, prevention is better than cure, a major strategy of malarial control is to attack the vector with insecticides. The control of mosquito at the larval stage is necessary and efficient in integrated mosquito's management. During the immature stage, mosquitoes are relatively immobile; remaining more concentrated than they are in the adult stage (Elimam *et al.,* 2009; Karunamoorthi and Ilango, 2010).

Since the discovery of dichlorodiphenyltrichloroethane (DDT), mosquito control approach has been almost completely based on synthetic organic insecticides. But the extensive use of synthetic organic insecticides during the last seven decades have resulted in environmental pollution and also in the development of physiological resistance in major vector species in addition to the increased costs of insecticides. This has necessitated the need for search and development of environmentally safer, low cost, indigenous methods for vector control. During the last decade, various studies on natural plant products against mosquito vectors indicate them as possible alternatives to synthetic chemical insecticides (Elimam *et al.,* 2009).

Many approaches have been developed and tried to tackle mosquito menace. The use of larvicides and repellents is an obvious practicality and economical means of preventing the transmission of vector borne diseases to humans (Kumar *et al*., 2011). The common approach for the control of mosquito vectors and reducing the transmission of human pathogens is the use of chemical insecticide-based intervention (Paul *et al*., 2006). However, in the past, the frequent and repeated use of chemical insecticides has resulted in the worldwide development of insecticide resistance, destabilization of the ecosystem and toxic effects on human beings and non-target organisms (Jirakanjanakit *et al.,* 2007). Thus, there is an urgent need to develop new insecticides for controlling mosquitoes which are more environmentally safe, biodegradable and target-specific against the mosquitoes. In past years much effort has therefore been focused on plant extracts or phytochemicals as potential sources of mosquito control agents or as lead compounds (Ansari *et al.,* 2005).

Plant products have been used traditionally by human communities in many parts of the world against the vectors and species of insects. Extracts or essential oils from plants may be alternative sources of mosquito larval control agents, since they constitute a rich source of bioactive compounds that are biodegradable into nontoxic products and potentially suitable for use in control of mosquito larvae .In fact, many researchers have reported the effectiveness of plant extracts or essential oils against mosquito larvae (Amer and Mehlhorn, 2006).

Repellents of plant origin have been used for medicinal purposes for a long time because they do not pose hazards of toxicity to human or domestic animals and are easily biodegradable. Compared to other synthetic compounds, natural products are presumed to be safer for human use (Ansari *et al.* 2000) justifying therefore a broad search for eco-friendly biological materials to be used for the control of vectors of medical importance. Plant products have been used in many parts of the world for killing or repelling mosquitoes either as extracts or as whole plant (Seyoum *et al.,* 2003).

To date there is no study done on the larvicidal and repellent effects of *J. procera* essential oil against *An. arabiensis* in Ethiopia and elsewhere. Thus, this study aimed at evaluating the larvicidal and repellent effects of *J. procera* essential oil against laboratory and wild early fourth instar larvae *An. arabiensis* and Adults of *An. arabiensis*.

1.2. STATEMENT OF THE PROBLEM

The burden of malaria has been increasing due to development of resistance against anti-malaria drugs and insecticides, complex social structures, and rapid environmental changes that have intensified in the last decade (Nathan *et al*., 2005; Snow et *al*., 2005). Consequently, there is no single method of malaria control that is completely effective in high transmission areas (Casmiro et *al.*, 2006). Even the most widely tested interventions, using bed nets treated with pyrethroid insecticides, have proven difficult to implement correctly because of problems related to equity, accessibility, user compliance and insecticide resistance .

Despite intensive efforts to control malaria, the disease continues to be one of the greatest health problems facing Africa. Control strategies deployed in Africa include prompt treatment of clinical attacks of malaria with an effective anti malarial drug combination, vector control using insecticide- treated nets and curtains or indoor residual spraying. In Ethiopia the control of malaria relies on, early diagnosis, treatment of malaria patients, vector control by IRS and use of ITNs and LLINs (MOH, 2004).

Even though adequate prevention measures and effective case management are available, malaria remains one of the most important public health diseases resulting in approximately 300 million cases and an estimated 781 000 deaths annually (WHO, 2010). Adult female *Anopheline* mosquitoes have the ability to transmit malaria from an infected individual to a susceptible person. Vector control measures have, therefore, been established to control the transmission of the disease by targeting the carriers.

Resistance to insecticides is a serious problem threatening malaria control efforts in all regions where insecticides are used to kill mosquitoes. According to the research done in Ethiopia, population of *An. arabiensis* were developed resistance to DDT, permethrin, deltamethrin and malathion (Yewhalaw *et al*., 2010) and drug resistant (Ketema *et al*., 2009). This emergent resistance renders local Indoor residual spraying (IRS) programs using dichlorodiphenyltrichloroethane (DDT) virtually ineffective, and the efficacy of permethrin and deltamethrin diminished. As a defense against the development of such resistance, some

plant species evolve to contain more than one insecticidal chemical, in order to maintain protective effects against invertebrates.

Repeated use of synthetic insecticides for mosquito control has disrupted natural biological control systems and led to resurgence in mosquito populations. It has also resulted in the development of resistance, undesirable effects on non-target organisms, and fostered environmental and human health concern that initiates a search for alternative control measures (Prabhu *et al.*, 2011). This could be one of the potential challenges in the current malaria control efforts in Ethiopia. This situation underlines the need to develop alternative natural plant derived methods of vector control.

A study by Yohannes and Boelee, (2012) conducted in Tigray, northern Ethiopia, indicated that *An. arabiensis* shows a tendency to bite early, before the time most people retire to bed. This situation raises the question about success of control measure of this vector through sleeping under ITNs. Hence, use of plant derived essential oil repellent for repelling vector could be an alternative to overcome the new challenges faced in malaria vector control.

CHAPTER TWO: LITERATURE REVIEW

2.1. GLOBAL MALARIA SITUATION

Arthropod-borne diseases are major causes of morbidity and mortality in many tropical and subtropical countries and principally the devastating nature of malaria is indubitably intolerable (Karunamoorthi and Ilango, 2010). The recent WHO Malaria report estimates that globally 3.3 billion people were at the risk of malaria in 2011, although of all geographical regions, populations living in sub-Saharan Africa (SSA) have the highest risk of acquiring malaria; among 216 million episodes of malaria in 2010, which approximately 81%, or 174 million cases, were reported from Africa. There were an estimated 650,000 of malaria deaths worldwide, 86% of the victims were children under 5 years of age and 90% of malaria deaths occurred in the African with children under five years of age and pregnant women being most severely affected (WHO, 2013).

Malaria is a life-threatening disease caused by the *Plasmodium* parasites that are transmitted through the bites of infected female *Anopheles* mosquitoes. Around half of the world's population is at risk of malaria and there were around 240 million cases in 2008. Most cases (around 85%) and deaths (~ 90%) are in the low-income nations of sub-Saharan Africa (the five main contributors to global deaths are the Democratic Republic of Congo, Ethiopia, Nigeria, Tanzania and Uganda), although Asia, Latin America, the Middle East and parts of Europe are also affected. Malaria is the fifth highest cause of death from infectious diseases globally and second in Africa, after HIV/AIDS. In 2006, malaria was present in 109 countries and territories, and in the future coverage may expand further as climate change allows mosquitoes and the parasite to colonies new areas (Dharani and Yenesew, 2010).

Plasmodium falciparum malaria increased dramatically from 1996 to 2000 despite continued efficient house-spraying for malaria control with pyrethroid insecticides in the two most malarious provinces: KwaZulu-Natal and Mpuma-langa in South Africa (Govere and Durrheim, 2002). Malaria, caused by *P. falciparum* is one of the leading causes of human morbidity and mortality from infectious diseases, predominantly in tropical and subtropical countries (Snow *et al*., 2005).

2.2. MALARIA SITUATION IN ETHIOPIA

Malaria is a leading health problem in Ethiopia. About two-thirds of the population lives in areas where malaria is transmitted; there is little risk of malaria above 2,000 meters. Ethiopia's malaria situation differs from other President's Malaria Initiative (PMI) countries in a number of ways. While the overall risk of malaria is quite low, malaria transmission in Ethiopia is characterized by frequent and often large-scale epidemics, which tend to occur every five to eight years. Because the transmission pattern of the disease is unstable, immunity is low, so all members of the population are at risk of severe disease not just pregnant women and children. Although the majority 60% of malaria infections are due to the malaria parasite *P. falciparum* and a second species, *P. vivax*, is found in up to 40 % of all cases given these factors, surveillance of cases and information management are critical in the country (PMI, 2011).

Vector control measures including selective indoor residual spraying of DDT, distribution of long lasting insecticide treated mosquito nets (LLINs) and source reduction of larval habitats are currently implemented by the Federal Ministry of Health in collaboration with international and non-governmental organizations. The history of utilization of DDT in the country dates back to the mid 1950s with small scale trials followed by wide and extensive application during the malaria eradication period. Thus, DDT has been in use for more than five decades. The emergence of DDT, deltamethrin and permethrin resistance in *An. arabiensis* has been reported from different localities in Ethiopia (Balkew *et al.* 2010) and the highest levels of resistance were recorded from Arba Minch in the South and Gambella in the West (Abose *et al.,* 1998).

2.3. INSECT REPELLENT

Many potential repellents can be considered as barrier to the insect, preventing either landing or penetration of the skin. Repellents contain volatile compounds that rely on vapor pressure and temperature to release specific chemicals that insects avoid (Novak and Gerberg, 2005).

Insect repellents are applied in various forms: directly to the skin, to clothing or other fabrics. They are recommended for people standing or sleeping outdoors at night for work or leisure and those working during day time (Fradin, 1998). There are two kinds of repellents: synthetic and natural (plant-derived) repellents.

2.3.1. SYNTHETIC REPELLENTS

There are many types of synthetic repellents so far manufactured. DEET (N, N-Diethyl-m-methyl benzamide) is the active ingredient of most commercial synthetic repellent formulations which are normally applied directly to skin or clothing (e.g. arm and ankle bands, or mosquito screens). Its efficacy and low toxicity have been proved over many decades of widespread consumer use (Frances and Wirtz, 2005). There are also several synthetic repellents used in addition to DEET such as CIC-4 (2-hydroxymethylcyclohexyl), AI3-37220 (1-(3-cyclohexen-1-yl-carbonyl)-2-methylpiperdine) which are reported to provide >95% protection for 5 hrs after application (Debboun *et al.,* 2000). Bio-Skincare^{TM} (BSC, oils of coconut, jojoba, rapeseed and vitamin E) and Mosiguard^{TM} towelletes with 0.57g quwenling are commercial repellents of *An. arabiensis*, the predominant vector of malaria in South Africa (Govere *et al*., 2000). But synthetic repellents are rarely used to protect communities from malaria and other vector-borne diseases. Cost and sometimes, safety constraints are the main reasons for this situation (Costantini *et al*., 2004). Other disadvantages are associated with the use of DEET in that it acts as a solvent of paints, varnishes, and some plastic and synthetic fabrics which led to the consumer rejection of DEET-based products. There have been also concerns over the toxicity of DEET. It irritates the eyes and mucous membrane when applied on the face (Osimitz and Grothaus, 1995).

2.3.2. BOTANICAL REPLLENTS

Plants and plant-derived substances have been used since ancient times to repel or kill mosquitoes' insects in the human history and, even now, in many parts of the world people are practicing plant substances to repel or kill the mosquitoes and other blood-sucking insects (Karunamoorthi *et al*. 2008a) and other domestic pest insects for a long time before the advent of synthetic chemicals. In different parts of the world, recently research focuses

on plant derived repellents for mosquito and other vector control. Directly the plant parts or their extracts or their essential oils are used as repellents and larvicidal insecticides.

In Germany Amer and Mehlhorn (2006) reported that protection times and percentages of repellency for 20% *Eucalyptus globulus* oil against three mosquitoes as follows: *Aedes aegypti*, 60 minutes and 57.6%, and *Anopheles stephensi,* 330 minutes and 52.4% and *Culex quinquefasciatus,* 480 minutes and 100% against. Fresh branches of *E. globulus* are traditionally and widely used to chase away intruding armies of ants and for treatment of common cold and cough. Additionally in the laboratory, 20% *E. citriodora* oil gave protection times and percentages of repellency of 150 minutes and 59.4%, 480 minutes and 52.4% and 480 minutes and 100% against *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus*, respectively.

In an effort to develop low cost plant-based household protection methods that can be used by communities with minimal external input several plant species were evaluated in terms of their repellent properties under semi-field experimental huts in western Kenya (Seyoum *et al.,* 2002).

In Ethiopia, (Wano, 2006) studied the effects of essential oil of seeds and leaves of some local aromatic plants (*Schinus molle, Ocimum lamiifolium, Ocimum suave, Eucalyptus citriodora, Eucalyptus globulus and Lippia adoensis*) and found some of them (*O. suave and L. adoensis*) significantly repelled endophagic mosquitoes (*An. arabiensis* and *Ae. agepti*).

Consequently, there is an urgent need to develop alternatives to chemical control of a wide variety of arthropod vectors of human diseases. Many naturally occurring repellents and insecticides have potential for development into useful products because they combine efficacy, biodegradability, and limited risk to mammals and the environment. Plant essential oils and seed pressed oils comprise a significant portion of the market share for natural product-based insecticides, and some have served as the basis of commercial repellent formulations (Dayan *et al.,* 2009).

Mosquitoes are vectors of several diseases affecting humans and domestic animals worldwide. One approach to prevent mosquito-borne diseases is bite prevention through the

application of repellents or physical barriers such as bed nets (WHO, 2006). Repellents are substances that act locally or at a distance, deterring an insect from flying to, landing on or biting human or animal skin. Currently, mosquito repellents available to consumers are based on synthetic chemicals (mainly N, N -diethyl- m–methyl benzamide) and a growing number are derived from plants.

Traditional application methods such as thermal expulsion and direct burning of mosquito repellent plants (*Corymba citriodora, Ocimum suave* among others) have shown to decrease the number of *Anopheles* mosquitoes entering a house (Dugassa *et al.,* 2009). *Cymbopogon citratus (DC) Stapf* and *Croton macrostachyus* are well-known for its medicinal and insect repellent properties among the rural residents of Ethiopia (Karunamoorthi and Ilango, 2010).

Certain natural products have been investigated for repellent activity against mosquitoes. *Ocimum kilimandscharicum* (OK) and *Ocimum suave* (OS) have been reported to possess repellent properties against mosquitoes. The repellent action of plant parts or oil extracts from *Ocimum* species have been evaluated against Afro tropical mosquitoes **(**Seyoum *et al.,* 2003).

Several extract and compounds from different plant families have been evaluated to show new and promising larvicides (Mohan *et al.,* 2007). In the laboratory, *O. suave* caused 81% of *An. gambiae s.s*. and 89% of *An. arabiensis* to be repelled from seeking a blood meal in a tunnel test setup. *O. kilimandscharicum* (camphor scented basil) produced similar levels of repellence and both plant species were also effective at repelling the nuisance mosquito *Cx. quinquefasciatus*. Further, repellency is known to play an important role in preventing the vector borne diseases by reducing man-vector contact. Ethno-botanical studies show that in some village communities, the use of plant repellents to reduce human vector contact is a common practice (Kweka *et al.,* 2008).

Various studies have successfully isolated compounds from plants that display insecticidal properties, commonly used natural insecticide; extracted from the flower heads of *Tanacetum cinerariifolium* (*T. cinerariifolium*) (Asteraceae) is pyrethrum. This has been effective in insect pest control around the world. Due to its rich source of bioactive chemicals, the neem tree (*Azadarachta indica*) (Meliaceae) is one of the most significant

and extensively research do fall medicinal plants (Biswas *et al.,* 2002). Different parts of the tree have been used to treat a wide range of diseases in man and livestock as well as to eradicate disease vectors. Essential oil extracted from *Mentha piperita* possessed excellent larvicidal efficiency against dengue vector. The bioassays showed that LC_{50} and LC_{90} value of 111.9 and 295.18 ppm, respectively after 24 h of exposure. The toxicity of the oil increased 11.8% when the larvae were exposed to the oil for 48 h. The remarkable repellent properties of *M. piperita* essential oil were established against adults *Ae. aegypti*. The application of oil resulted in 100% protection till 150 min. After next 30 min, only 1-2 bites were recorded as compared with 8-9 bites on the control arm. The peppermint essential oil is proved to be efficient larvicidal and repellent against dengue vector (Kumar *et al.,* 2011). As Rajkumar and Jebanesan, (2007) evaluated at three different concentrations (2, 4 and 6%) of these essential oils of *Ipomoea cairica, Momordica charantia* and *Tridax procumbens* exhibited relatively high repellency effect (>300 minutes at 6% concentration), followed by *Centella asiatica* and *Psidium guajava* which showed less effective (<150 minutes at 6 % concentration). However, the ethanol applied arm served as control provided maximum 8.0 minutes repellency in this study. In general, clear dose–response relationships were established in all essential oils, with the highest concentration of 6% provided high repellency effect.

Another study Ansari *et al.* (2000) suggested that the peppermint oil (*M. piperita*) showed strong repellent action against adult mosquitoes when applied on the human skin. The protection obtained against *An. annularis*, *An. culicifacies*, and *Cx. quinquefasciatus* was 100, 92.3, and 84.5%, respectively. Additionally, Ansari *et al.* (2005) reported that Pine oil had strong repellent action against mosquitoes as it provided 100% protection against *Anopheles culicifacies* for 11 h and 97% protection against *Culex quinquefasciatus* for nine hours respectively. Electrically heated mats prepared from Pine oil provided, 94 and 88% protection against *An. culicifacies* and *Cx. quinquefasciatus* for 10 and seven hours respectively.

Similarly, undiluted oil showed the highest protection time in each case. Among the four kinds of oil tested, *Syzygium aromaticum* demonstrated the longest protection time against all three species of mosquito and the order of potency based on the protection time was *Cx*

quinquefasciatus > *An. dirus* > *Ae. aegypti*. The mean durations of protection from bites for *S. aromaticum* were 240, 210 and 120 min against *Cx. quinquefasciatus*, *An. dirus* and *Ae. aegypti*, respectively. At a 50% concentration *S. aromaticum* provided 120 min of complete protection against both *An. dirus* and *Cx. quinquefasciatus*. *Pogostemon cablin and Zanthoxylum limonella* protected for 120 and 130 min, respectively, against *An. dirus*. The protection times of all oils at 10% concentration were less than 120 min against all three species of mosquito (Trongtokit *et al*., 2005). According to Tawatsin *et a1*. (2001) report oils from turmeric, citronella grass and hairy basil, especially with the addition of 5% vanillin, repelled three mosquito vectors, *Ae. aegypti*, *An. dirus* and *Cx. quinquefasciatus* under cage conditions for up to eight hours. The oil from kaffir lime alone, as well as with 5% vanillin added, was effective for up to three hours. With regard to the standard repellent, deet alone provided protection for at least eight hours against *Ae. aegypti* and *Cx. quinquefasciatus*, but for six hours against *An. dirus*. However, DEET with the addition of 5% vanillin gave protection against the three mosquito species for at least eight hours. Barnard and Xue, (2004) reported that Soya bean oil prevented biting mosquitoes for ≥7 hours in the laboratory. However, none of the essential oil of *Juniperus procera* plant found in Ethiopia has been evaluated for its larvicidal and repellency activity against *Anopheles arabiensis*.

According to Barnard *et al*. (1998) cage size and mosquito density are important parameters in repellent bioassays, but the influence of these factors was found to vary between mosquito species. For *Ae*. *aegypti*, the DEET protection period was inversely proportional to cage size but relatively unaffected by mosquito density. For *An*. *quadrimaculatus*, the repellent protection time was shortest in large cages and at high mosquito densities, and longest in medium cages and at low mosquito densities.

2.3. BOTANICAL LARVICIDES

Plants such as *Tagetes* (Asteraceae) species have been shown effective against the adult and immature stages of the mosquito, whilst *Eclipta paniculata* (Asteraceae) displayed significant larvicidal properties and *Polyalthia longifolia* (Annonaceae) exhibited both larvicidal and growth inhibition effects (Mittal and Subbarao, 2003). South Africa possesses

a rich diversity of plant life with over 24,000 plant species, of which approximately 15 % are ethno medicinal (used traditionally for medicinal purposes) (Arnold *et al*., 2002). The importance of ethno medicinal plants lies not only in their chemotherapeutic value in traditional health care but also in their potential as sources of biologically active entities.

Recently study conducted in India to analyses the larvicidal activity of *Eugenia jambolana* leaf extracts by employing against the fourth instar larvae of three medically important species namely *Ae. aegypti*, C*x. quinquefasciatus* and *An. stephensi*, the result shows that among the mosquito species *Ae. aegypti* was found to be most susceptible with the LC_{50} value of 40.97 ppm compared to that of *Cx. quinquefasciatus* and *An. stephensi* with LC_{50} 53.84 and 96.00 ppm, respectively. The crude petroleum ether extract of this plant with good larvicidal efficacy will be considered as a potent candidate for further analysis (Raghavendra *et al.,* 2011)

As Kalaivani *et al.* (2011) reported that the oil extract obtained from the *Mentha piperita*, *Zingiber officinale*, *Curcuma longa* and *Ocimum basilicum* were an effective larvicidal agent against the *Ae. aegypti* larvae; it was highly toxic to mosquito larvae and inhibited the development of pupae. The high rates of larval mortality observed at higher concentrations (80, 100, 200 and 400 ppm of *M. piperita*, *Z. officinale*, *C. longa* and *O. basilicum* oil extract, respectively) within a 48-h exposure indicate the high toxicity of the product.

As Elimam *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*. On the other hand methanolic extracts of leaves and seeds from, *Tribulus terrestris* (Zygophyllaceae) was tested against 3rd instar larvae and adults of mosquito, *Anopheles arabiensis* under laboratory condition. The seeds extract showed high insecticidal activity at all concentrations compared to the leaves extract and the LC_{50} was 36.5 and 123.1ppm for seeds and leaves extract, respectively. All extracts exhibited remarkable effects on the fecundity, fertility and sterility index of adult females resulted from treated larvae, but the seeds extract was more effective than leaves extract. The repellent action of the present plant extracts varied depending on the plant parts and the dose

of extract. The seeds extract was more effective in exhibiting the repellent action (100%) against the mosquito tested as compared with the leaves extract (79.5%) at the dose 1.0 and 2.0mg/cm2, respectively(El-Sheikh *et al*., 2012).

Study conducted in Pakistan the insecticidal nature of seed extracts of M*oringa Oleifera* showed greater impacts on the development of second and fourth instar larvae of *Cx. quinquefasciatus* and hence displays significant mortality. Severe toxic effects were observed showing an increasing trend towards higher concentration in dose dependent manner. A complete control over the larvae was recorded in case of higher dose of extract and overall larval mortality ranged from 9.36 to 98.89% for second instar which was significantly higher in all treatments when compared with control. The highest dose rate of water extracts of *M. oleifera* seeds. (120 mg/L) caused highest mortality at all intervals and showed 25.94±4.15, 50.31±2.83 and 71.66±1.90% larvicidal effect after 6, 12 and 18 h, respectively which approached to98.89±0.54% after 24 h of treatment (Ashfaq *et al.,* 2012).

As Karunamoorthi *et al.* (2008b) suggested that *Vitex negundo* leaf extract served as a potential larvicidal agent against Japanese encephalitis vector *Cx. tritaeniorhynchus* and additionally acted as a promising repellent against various adult vector mosquitoes.

Another study carried out to evaluate the repellent efficacy of a methanol-leaf extract of Ethiopian traditionally used insect repellent plant viz., Lomi sar (*Cymbopogon citratus* (DC) Stapf.(Poaceae) against *An. arabiensis* at four different concentrations viz 1.0, 1.5, 2.0, and 2.5 mg/cm2. The percentage protection in relation to the dose method was performed. C. citratus extract has shown various degrees of repellency impact against *An. arabiensis*. It provided the maximum total percentage protection of 78.83% at 2.5 mg/cm^2 and followed 68.06% at 2.0 mg/cm^2 for 12 h. All four tested concentrations of C. citratus extract offered significant protection and Student's t test results shows statistically significant (*p value*=0.001) difference between treated and control groups (Karunamoorthi *et al.,* 2010).

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.

Similar study in Ethiopia as Tomass *et al.* **(**2011) suggested that LC_{50} and LC_{90} values of crude methanol leaf extract of *Jatropha curcas* against laboratory reared late third instar larvae of *An. arabiensis* were found to be 92.09 and 241.09 ppm, respectively. Furthermore, oil of *Annona 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***.** Similarly, larvicidal activity of *T*agetes minuta oil extract showed 29.4ppm and 49.9ppm of LC_{50} and LC_{90} values, respectively (Assefa, 2011). According to Shaalan *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. 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*., 2003 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.

2.4. Description of the test plant

Juniperus procera leaves

Taxonomy

Current name**:** ***Juniperus procera***

Family: **Cupressaceae**
Common names **(**Amharic**): Tid**

Juniperus procera is a medium-sized tree reaching 25–30 m (rarely 40 m) tall, with a trunk up to 1.5–2 m diameter and a broadly conical to rounded or irregular crown. The leaves are of two forms, juvenile needle-like leaves 8–15 mm long on seedlings, and adult scale-leaves 0.5–3 mm long on older plants, arranged in decussate pairs or whorls of three. It is largely dioeciously with separate male and female plants, but some individual plants produce both sexes. The cones are berry-like, 4–8 mm in diameter, blue-black with a whitish waxy bloom, and contain 2-5seeds; they are mature in 12–18 months. The male cones are 3–5 mm long, and shed their pollen in early spring. *Juniperus procera* is native to the Arabian Peninsula (in Saudi Arabia and Yemen), and northeastern, eastern, west-central, and south tropical Africa (in the Democratic Republic of theCongo (CongoBrazzaville); Kinshasa); (CongoDjibouti; Eritrea; Ethiopia;Kenya; Malawi ; Mozambique; Somalia; Sudan; Tanzania; Uganda; Zambia; and Zimbabwe). The specific name, 'procera', is Latin for tall or high. *J. procera* is heavily used for building houses, construction poles, furniture and fuel wood by different peoples in those countries (GRIN, 2010).

Traditional medicinal uses: The stem is used as tooth brush leaves are used to treat or cure tonsillitis (Seshathri *et al*., 2011). It uses also to treat intestinal worm. The vapour from a leaf decoction is inhaled several times a day for treatment of flu. In addition to treat diarrhea, diabetes, stomach aches and ulcers and mixing *Juniperus procera* with lemon to treat malaria.

2.5. Significance of the study

To the best of our knowledge there is no study done to see the effectiveness of *Juniperus procera* essential oil regarding larvicidal and repellent activity against *An. arabiensis*. Finding of this study can be used to show potential of *J. procera* essential oil and its possibility of using this plant leaves extract against *Anopheles* larvae and adult stages of *An. arabiensis*. It uses to enhance eco-friendly insecticides which are environmental safe, biodegradable and low cost larvicides, to promote sustainable utilization of locally available bio resources. It provides evidence based information about plant products for the national malaria control program and policy makers for malaria prevention and control. As well as could be used as one component in integrated vector management (IVM) in areas where malaria is endemic. Therefore, this study finding could be also used as a baseline data for future study.

CHAPTER THREE: OBJECTIVES

3.1. General objectives

The main objective of this study was to evaluate larvicidal and repellency activity of *Juniperus procera* essential oil against *Anopheles arabiensis*.

3.2. Specific objectives

1. To evaluate the larvicidal activity of essential oil of J. *procera* against fourth instar larvae of *An. arabiensis* under laboratory condition.

2. To evaluate the larvicidal activity of essential oil of *J. procera* against fourth instar larvae of *An. gambiae sensu lato* under semi field condition.

3. To determine the repellent activity of *J. procera* essential oil against adults *An. arabiensis* under laboratory conditions.

Hypothesis

1. A *Juniperus procera* leaves has larvicidal and repellent effect against *An. arabiensis* larvae and adult *An. arabiensis*.

CHAPTER FOUR: MATERIALS AND METHODS

4.1. Study Area and period

Laboratory investigations were carried out at Adama malaria research center and field evaluation of larvicidal activity were conducted in Boye river around Jimma town from June to November, 2012.

4.2. Study Design

Experimental study design was conducted to evaluate essential oil of *J. procera* against *An. arabiensis* based on the WHO standard procedure and techniques.

4.3. Collection of mosquito larvae and rearing

The larva of *An. arabiensis* was obtained from Adama Malaria Research Center, Ethiopia. The colony was reared under laboratory conditions at 27±1°C and 70±10% relative humidity. The eggs were placed in Petri dishes containing distilled water. The larvae were fed by adding powdered yeast on the surface of the water. The larvae were reared up to early fourth-instar by following WHO standard methods. Fourth instar larvae of *An. arabiensis* were used continuously for the experiments.

4.4. Collection of the test plant

Leaves of *J. procera* were collected from a tree growing in Jimma university campus, Taxonomy of the plant was verified and confirmed by Dr Remsh, in the Herbarium, Department of Biology, College of Natural science, Jimma University, Ethiopia. Voucher specimens were deposited at the Department of Biology, Jimma University, Ethiopia.

4.5. Extraction of essential oil

After collection of the test plant *J. procera* from their natural habitats, it's essential oil was extracted by hydro-distillation method with the help of the Drug Quality Control Laboratory, Pharmacy Department, Jimma University. In this process, the fresh leaves were washed with tap water and then ground by using mortar and pestle or cut into small pieces. Fresh

leaves was (200 gm) of tid (*J. procera*) placed into a distillation flask and extracted with much water by hydro-distillation for over 5 hour using a Clevenger apparatus. The distillation chamber was heated at about 100^0C and allowed to boil until the distillation process was completed.

4.6. Larvicidal bioassay under laboratory condition

Larvicidal activity of *J. procera* essential oil against *An. arabiensis* was assessed by using the WHO standard procedure (WHO, 2005). 1ml of the solution were dissolved in 9ml using acetone and then to obtain a final concentration of 5 ppm to 30 ppm. Various concentrations of dissolved oils were prepared in distilled water. After, 25 active larvae of early fourth instar were transferred in to 250 ml glass beaker. 25 larvae were transferred in to glass beaker contained distill water with 1 ml acetone but not extract that provide as a control. Three replicates for the treatments and three replicates for controls were carried on for each concentration. Dead and moribund larvae in three replicates were combined and expressed as a percentage of larval mortality in each concentration.

4.7. Larvicidal bioassay under semi field condition

Field trials were conducted according to the methods of (WHO, 2005). Artificial containers of 10 cm wide (diameter) by 4.5 cm depth of 200 ml capacity were used for larvicidal bioassays in the field. The containers were buried into the ground. Water from the natural breeding habitats of the larvae was added into the container. Following the above procedure 10ml stock solution was prepared. Each container was then treated with various concentrations. Concentrations ranging from 15 to 40 ppm were used. Batch of 25 wild collected early fourth instar *Anopheles gambiae sensu lato* larvae were released into each container and for each test concentration. Three replicates were conducted at a time for the treatments and for the control also as described in section 4.6.

Plate 1: Collection of *Anopheles* larvae (photograph taken during larvae collection)

4.8. Repellent activity under laboratory condition

The repellent study was done following the method .Blood-starved, 5–7 days old female *An. arabiensis* (n=120) were kept in a net cage (45 × 40× 45 cm) tested throughout the night (at night time). Volunteer had no contact with lotions, perfumes or perfumed soaps on the day of the assay. The hand and forearm of the human volunteer were washed with unscented soap, thoroughly rinsed and allowed to dry for 5 minute before the oil application. The whole forearm (wrist and elbow) skin on each arm was exposed; glove was worn on the hand (wrist and fingers) during each test to prevent biting. The essential oil was used for treated forearm and for control which was untreated forearm (negative control) without any application was used.

The essential oil at 0.1, 0.15, 0.25 and 0.5 ml/cm^2 concentration was applied separately in different. In case if during the observation period no mosquitoes landed on the control arm or attempted to bite, the trial was discarded, and the test was repeated with a new batch of mosquitoes to ensure that lack of bites was due to repellence and not because mosquitoes are not predisposed to get a blood meal.

The number of bites were counted over 3 min every 30 min interval. If no mosquitoes bite or landed during the 3 minute study period, the arm was withdrawn from the cage and we

waited 30 minutes before attempting to conduct the test again. The experiment was conducted triplicate for each concentration in different days. The percentage protection was calculated using of the following formula

$$\% \text{ Repellency} = C\text{-}T/C \times 100$$

Where, C is the number of mosquito bites in the control group, and

T is the number of mosquito bites in the treated group

Plate 2: Human volunteers exposing forearms in the cage (photographs taken during laboratory test)

4.9. Study variables

4.9.1. Dependent variables

- Number of dead larvae
- Number of mosquitoes Repelled

4.9.2. Independent variables

- Concentration
- Time

4.10. Data Analysis

The average larval mortality data were subjected for calculating LC_{50}, LC_{90} and other statistics at 95% confidence limits of upper confidence limit and lower confidence limit and chi-square values were calculated using the SPSS 16.0 (Statistical Package of Social Sciences) software, MS Excel 2007. Results with $p<0.05$ were considered to be statistically significant. The total of each hour count of each replicate test (treated and control) were summed and percentage of repellency reduction was calculated by comparing the number of bites for control against the number of bites for treated human volunteers. Then results were summarized and subsequently presented by tables and graphs.

4.11. Data quality assurance

To assure data quality, bioassays was replicated three times per trial, for each trial using fresh stock solution and use fresh batches of larvae at different time. The same for repellent also triplicates in different days by using new batch of adult mosquitoes.

4.12. Ethical consideration

Ethical clearance was obtained from research and ethics committee of Jimma University, College of Public Health and Medical Sciences. The purpose of study was elaborated to the volunteers before the test and informed consent from volunteers was obtained.

4.13. Dissemination of study result

The final result of this study was presented to Department of Environmental Health science and Technology, Jimma University, College of Public Health and Medicinal sciences.To federal ministry of health, NGOs which are concerns in health.

4.14. Materials

- BioQuip breeders
- Mortar and pestle
- Clevenger apparatus
- Droppers with rubber suction bulbs

- Plastic container
- Four 1-2 ml pipettes for essential oil and one for the control
- One pipette delivering 100–1000 µl.
- Strainer or a loop of plastic screen may be used to transfer test larvae into test cups or vessels
- Data recording forms
- Disposable cups/ beakers of two capacities: 150 ml (holding 100 ml) and 250 ml (holding 200 ml).
- Nylon mesh screen
- Mosquito cage
- Torch light

CHAPTER FIVE: RESULTS

5.1. Larvicidal effects of essential oil under laboratory conditions

The results of larvicidal bioassays performed upon the early fourth instar larvae of *An. arabiensis* with the distillate essential oil of *J. procera* leaves are presented in Table 1. The results obtained proved and established the efficacy of the *J. procera* essential oil against the mosquito larvae. The control or untreated groups did not show any mortality within 24 h exposure. In terms of lethal concentrations for 50% and 90% mortality *J. procera* essential oil appeared to be most effective against *An. arabiensis* under laboratory condition was (LC_{50}=14.4 ppm) and (LC_{90}=24.7 ppm) respectively as shown in figure 1. The effect on larval mortality was dose dependent.

Table 1. Efficacy of *J. procera* essential oil against laboratory reared fourth instars *An. arabiensis* larvae in 24 h under laboratory conditions

Concentration in ppm	Mean Mortality (%) (±SD)	Larvicidal activity(95% C.L, ppm)	
		LC_{50} (ppm) (LCL–UCL)	LC_{90} (ppm) (LCL–UCL)
Control	0±0.00		
5	0±0.00		
10	28.4±0.69		
15	49.6±0.69	14.4(12.6–16.1)	24.7(21.6– 30.3)
20	64.4 ±0.69		
25	96±0.69		
30	100±0.00		
Chi square(X^2)	6.662		
R^2	0.844		
Slope	5.50		

Mortality values in 24 h are means of three replicates
LC_{50} lethal concentration that kills 50% of the exposed larvae, LC_{90} lethal concentration that kills 90% of the exposed larvae, UCL upper Confidence limit, LCL lower confidence limit,

From the range of concentration used, the mortality effect observed was from 5 ppm to 30 ppm in distilled water. After 24 hours exposure, the mean mortality percentage ranged from 0 to 100 % for *An. arabiensis*. Concentration response non overlapping confidence limits showed that there were statistically significant differences in LC_{50} and LC_{90} values ($P< 0.05$). The larvicidal activity of essential oil *J. procera* leaves showed 0, 28, 48, 64, 96 and 100% of mortality with the use of 5,10,15,20, 25 and 30 ppm concentrations, respectively.

As we observe from the result as the concentration of the plant extract increased, the total larval mortality of the mosquitoes was also found to be increased. As well as the results of larvicidal activity clearly indicates that the percentage of mortality being directly proportional to concentration of the extract. At higher concentration the larvae showed restless movement for some time and then settled at the bottom of the cup and died slowly.

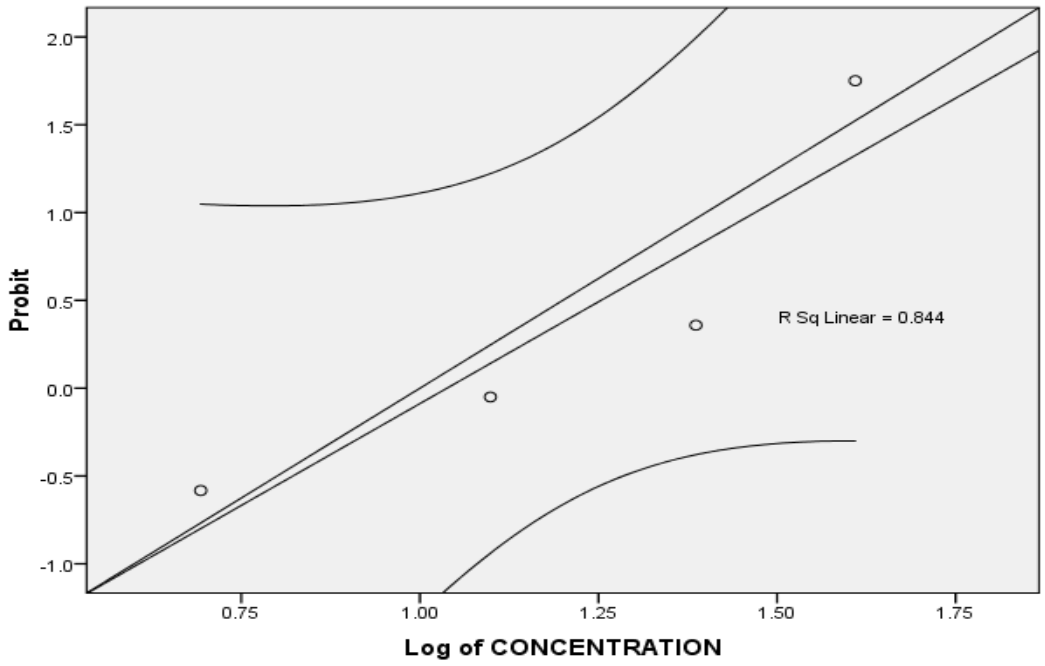

Fig.1. Probit concentration curve of *J. procera* essential oil against *An. arabiensis* 4th instar larvae under laboratory conditions

The result of regression analysis indicates that the mortality rate is positively correlated with concentration having a regression value 0.844 as shown in Figure 1.

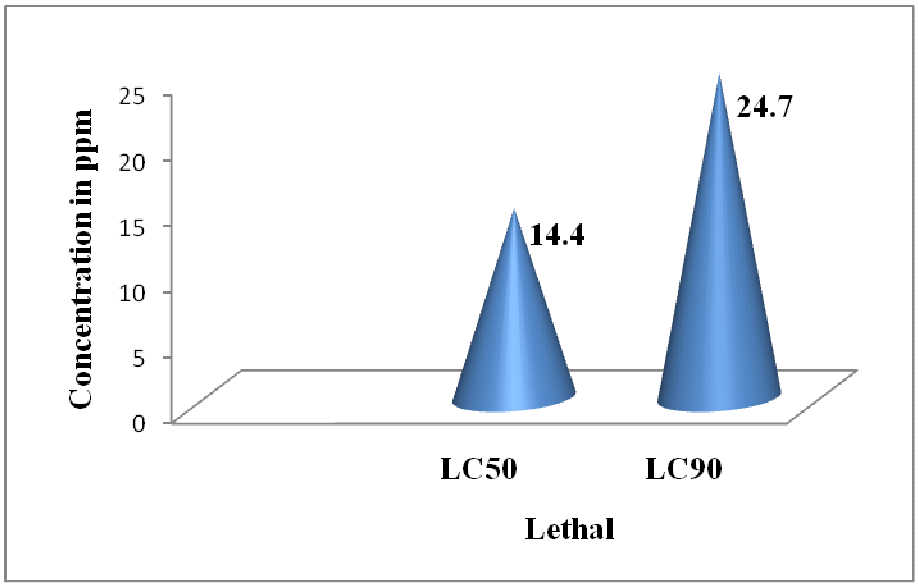

Fig. 2. LC_{50} and LC_{90} values of *J. procera* essential oil against early fourth instar larvae of *An. arabiensis* under laboratory conditions

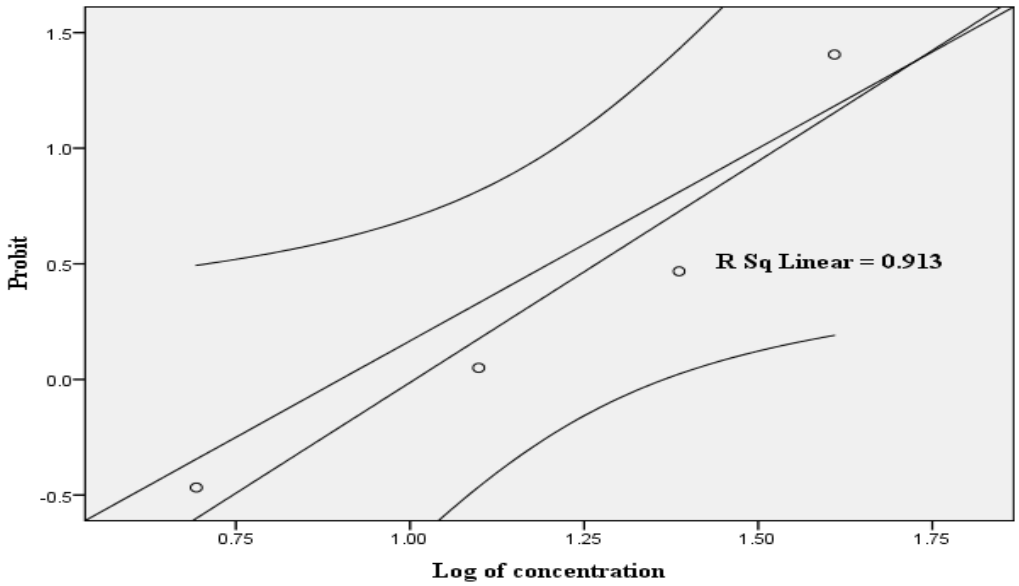

Fig. 3. Probit concentration curve of *J. procera* essential oil against *An. gambiae sensu lato* 4th instar larvae under semi field conditions

The result of regression analysis indicates that the mortality rate is strong relationship with concentration having a regression value of 0.913 shown in Figure 3.

5.2. Larvicidal effects of essential oil in semi field conditions

The toxicity of *J. procera* essential oil against fourth stage wild-collected *Anopheles gambiae sensu lato* larvae in semi field conditions is shown in Table 3 and Figure 4. The LC_{50} and LC_{90} results of *J. procera* essential oil with different concentrations for laboratory and field strains were not the same. However, wild-collected *Anopheles gambiae sensu lato* larvae had higher LC_{50} and LC_{90} values of the essential oils than laboratory reared *An. arabiensis* larvae. For the tested essential oil, it was evident that larval mortality was dosage dependent. Generally, results of this experiment showed that essential oil extract of the plant material were effective in controlling *Anopheles gambiae sensu lato* larvae.

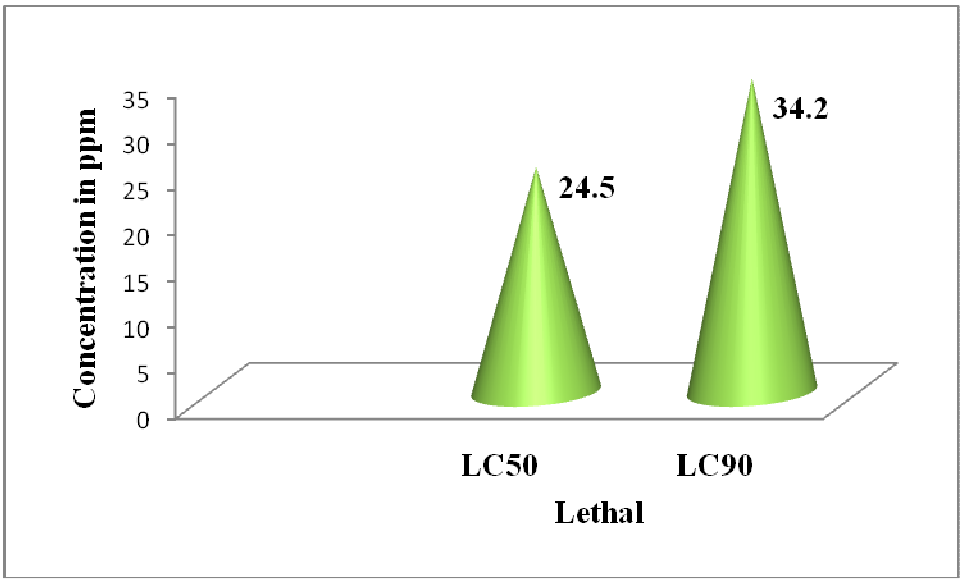

Fig. 4. LC_{50} and LC_{90} values of the early fourth instar larvae of *Anopheles gambiae sensu lato* under semi field conditions

In the above Figure 4 indicated that comparison of LC_{50} and LC_{90} of *J. procera* essential oil as the efficient larvicidal agent against *Anopheles gambiae sensu lato larvae*. On exposure to the early fourth instar larvae showed as LC_{50} and LC_{90} values against *Anopheles gambiae sensu lato* larvae were from 24.5 to 34.2 ppm respectively. Based on the calculation of LC_{50}

and LC_{90} values wild *Anopheles gambiae sensu lato* larvae were tolerance to the essential oil than laboratory reared *An. arabiensis* larvae.

Table 2. Efficacy of *J. procera* essential oil against fourth instars wild *Anopheles gambiae sensu lato* larvae in 24hrs under semi field conditions

Concentration in ppm	Mean Mortality (%) (±SD)	Larvicidal activity(95% C.L, ppm)	
		LC_{50} (ppm) (LCL–UCL)	LC_{90} (ppm) (LCL–UCL)
Control	0.0±0.00		
20	32.1±1.84		
25	51.9±1.40		
30	68.4 ±0.69	24.5(22.7–26.2)	34.2(31.4–38.8)
35	93.3±1.40		
40	100±0.0		
Chi square(X^2)	4.615		
R^2	0.913		
Slope	8.85		

Each value (mean ± SD) represents mean value of three replicates
LC50 lethal concentration that kills 50% of the exposed larvae, LC90 lethal concentration that kills 90% of the exposed larvae, UCL upper Confidence limit, LCL lower confidence limit,

Data on the percent mortality of 4th instar larvae of *Anopheles gambiae sensu lato* mosquito treated with different concentrations of the extracts of *J. procera* leaves under semi field conditions at the end of 24 hrs are presented at (Table 3). As concentration increased, the effectiveness of the tested botanical also increased as shown in Table 3. Dose response effects showed that there were statistically significant differences in LC_{50} and LC_{90} values among the concentrations ($P< 0.05$).

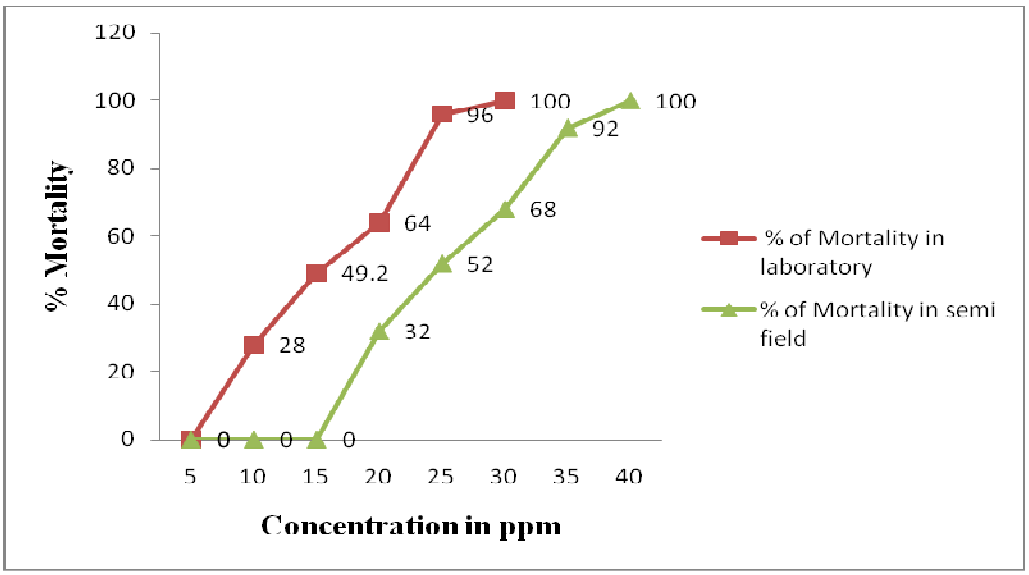

Fig. 5. Larvicidal activity of *J. procera* essential oil against 4th instar larvae of *An. arabiensis* under laboratory and semi field conditions

The results in Figure 5 indicate that in both laboratory and semi field conditions larval percentile mortality were increase as the concentration increase. It is directly proportional to dose.

5.3. Repellent activity under laboratory condition

The result of the repellent activity revealed that excellent repellent properties of *J. procera* essential oil against adults *An. arabiensis* as shown in Table 3. The result obtained shows that 0.1 ml oil had 100% repellency until 80 min protection for the mosquitoes. The second concentration was at 0.15 ml it shows also 100 % repellency until 120 min protection. The third concentration at 0.25 ml shows 100% repellency until 180 min protection. The last concentration 0.5 ml shows also 100 % repellency until 330 min protection time. The highest protection time found at 0.5 ml with 330 min protection time. The complete protection times of *J. procera* essential oil based repellents correlated positively with the concentration.

The results of mean protection time and total percentage protection in relation to dose of *J. procera* leaves extract are given in Table 3. Skin repellent test at 0.1, 0.15, 0.25, and 0.5 ml/cm^2 concentration of *J. procera* offered 100% protection up to 1.19±0.01h, 2.05±0.05h,

3.10±0.03h, and 5.27±0.02, respectively. There was a statistically significant difference in complete protection time between each concentration based repellent

Table 3. Mean number of *An. arabiensis* bites per test ± standard error at various concentrations with *J .procera* essential oil

Concentrations (ml/cm^2)	Mean number of landing /bites received		Mean complete protection time (h)	Total protection for 12 h (%)	t value
	Treated	Control			
0.1	62.7±1.20	173±0.58	1.19±0.01	58	82.7*; *df*=4
0.15	58.3±1.33	175±0.57	2.05±0.05	64.3	80.3*; *df*=4
0.25	57.3±3.18	171.3±3.18	3.10±0.03	68.4	25.3*; *df*=4
0.5	27.3±0.33	173.7±1.20	5.27±0.02	80.5	96.8*; *df*=4

Represents mean of three values ± S.E.

*Statistically significant at p<0.0001 level

Table 4 shows the percentage protection in relation to dose and time (h). All tested four concentrations of *J. procera* essential oil provided significant protection and the independent t test results shows that statistically significant (p value=0.0001) [0.1 ml/cm^2 (t=82.7; df =4); 0.15 ml/cm^2 (t=80.3; df =4); 0.25 ml/cm^2 (t=25.3; df=4); 0.5 ml/cm^2 (t=96.8; df=4)] difference between treated and control groups.

The essential oil of *J. procera* exhibited various degrees of repellent efficacy against female *An. arabiensis.* The results of protection time and total percentage protection in relation to concentration of *J. procera* essential oil are given in the above Table 3. Repellent test at 0.1 0.15, 0.25, and 0.5 ml/cm^2 concentration of *J. procera* essential oil offered 100% protection up to 80 min, 120 min, 180 min, and 330 min, respectively. *J. procera* essential oil provided the highest total percentage protection of 82.14% at 0.5 ml/cm^2 and followed 69.2% at 0.25 ml/cm^2, 65.6% at 0.15 ml/cm^2 and 59.5% at 0.1 ml/cm^2 for 12 h. As evidenced from the above table, generally increased protection was observed with increased concentration of the extract tested against *An. arabiensis.* The protection provided by *J. procera* essential oil is proportional to the concentration; higher concentrations of *J. procera* provide longer protection.

Based on this study the percentage of repellency of *J. procera* essential oil for *An. arabiensis* species of mosquitoes is presented in Figure 6. Likewise the percentage repellency of *J. procera* increased when the concentration of this essential oil increased, additionally, biting or landing rates decreased when the concentration increased. The results showed significant differences in both the percentage of repellency and the number of mosquitoes biting or landing ($P<0.05$).

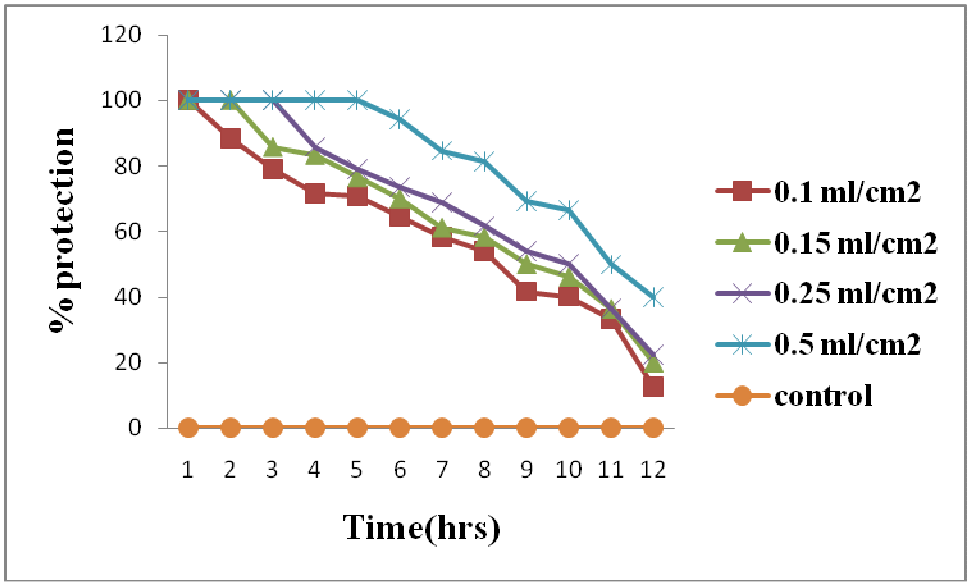

Fig.6. Repellency of *J. Procera* essential oil against *An. arabiensis* at various concentrations

Data presented in Fig 6 indicate that the relationship of % protection time and concentrations with relation to observation time, thus, as we see from the figure it shows as the concentration increase the protection time also increase and also in similar manner as the concentration decrease the protection time also decreases. On the other hand biting rates decreased when the concentration of *J .procera* essential oil increased **(**Figure 6).

CHAPTER SIX: DISCUSSION

Essential oils from plants may be an alternative source of mosquitoes larval control, since they are rich sources of bioactive compounds that are biodegradable rapidly in the environment, low mammalian toxicity and potentially suitable for use in integrated vector management programs. To cite a few examples, the Essential oil extracted from different plants have been reported to have both larvicidal and repellent properties against different mosquito species (Ansari *et* al., 2005; Amer and Mehlhorn, 2006; Maharaj *et al*., 2012). The present study was performed to assess the potential of *J. procera* essential oil as larvicidal and repellent activity against *Anopheles.*

In the present study, the essential oil of *J. procera* leaves possesses excellent larvicidal efficiency against *Anopheles arabiensis* under laboratory as well as semi field conditions. The laboratory and field bioassays performed resulted in LC_{50} value of 14.4 ppm which significantly increased to 24.7 ppm at LC_{90} value and values of LC_{50} 24.5 ppm and LC_{90} 34.2 ppm after 24 hours of exposure respectively. 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 extract of *J. procera* leaves both in laboratory and on semi-field conditions.

The larvicidal activities of *J. procera* obtained in this study are in agreement with previous reported data. Massebo *et al*. (2009) essential oils of *C. ambrosioides* had an excellent effect against the fourth instar larvae of *An. arabiensis* under laboratory condition with LC_{50} values of 17.5 ppm and values of LC_{90} also 33.2 ppm; and under field condition with LC_{50} values of 47.3 ppm and values of LC_{90} 97.9 ppm respectively. Similarly the result agree with the finding of Assefa,(2011) who had reported that the laboratory reared larvae of *An. arabiensis* was more susceptible than field anopheles larvae to the essential oil extracted from *Annona squamosa* plant ,with LC_{50} 41.5ppm; 31.5ppm and LC_{90} 79.2ppm; 32.9ppm, respectively. Besides this, the *J. procera* essential oil LC_{50} values of the laboratory were different from the result of the semi- field.

Based on the LC_{50} and LC_{90} values, it can be seen (Figure 1 and 4) laboratory reared *An. arabiensis* larvae were found to be more susceptible to essential oil than field population of *Anopheline* larvae. Essential oils from *J. procera* showed the higher toxicity against laboratory reared *An. arabiensis* larvae than field population of *Anopheline* larvae. This might be due to using of heterogeneous mosquitoes. Thus; the presence of *Anopheles gambiae suns lato* species in the test solution may have resulted in higher tolerance to the oils since variations in susceptible to toxic products exist between species mosquitoes. However, even with the same mosquito species, variations in susceptible between laboratory and field strains are expected. This study in line with the finding of Sun *et al.* (2006) evaluated the larvicidal effects of ethanol extract of *Ginkgo biloba* L. against laboratory and field strain of *Cx. 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*., 2003) and are routinely exposed to diverse insecticides. Therefore; they probably have a higher general tolerance to toxic compounds.

Moreover, George and Vincent, (2005) evaluated the larvicidal activity of petroleum ether seed extract of *Annona 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. In an earlier study by Maharaj *et al*. (2012) found that the crude extract obtained from Rhizomes of *Zingiberaceae* and leaves, stem of Rutaceae displayed promising larvicidal activity when tested against *An. arabiensis* third instar larvae.

The present finding was better as compared with study done by Tomass *et al.* **(**2011) crude methanol leaf extract of *Jatropha curcas* have larvicidal activities with the LC_{50} values of 92.09 ppm on the late third instar larvae of *An. arabiensis*. Similarly, El-Sheikh *et al*. (2012) reported that methanolic extract leaves and seeds of *Tribulus terrestris* demonstrated toxic effect against *An. arabiensis larvae* with LC_{50} values of 123.1ppm and 36.5 ppm respectively. Moreover, larvicidal effects in 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* (Elimam *et al.,* 2009).

Another finding reported by Kalaivani *et al*. (2011) Compared to the leaf of essential oil from four plants were screened for larvicidal activity against fourth instar larvae of *Aedes aegypti*. The oil from *Z. officinale* was the most potent, giving 50% mortality at 40.5 ppm while oils from *M. piperita*, *C. longa* and *O. basilicum* resulted in 50% mortality at 47.54, 115.6 and 148.5 ppm respectively. Since, the present investigation found that low LC_{50} value against *An. arabiensis* than those reported by a previous study it could serve as a potential larvicidal activity in the future. As reported by Shaalan *et al.* (2005) bioactivity of phyto chemicals against mosquito larvae can vary significantly depending on plant species, plant parts, age of plant and mosquito species.

Based on the calculated percentage mortality for both laboratory and field conditions as indicated in Table 2 and 3 and figure 2, generally increased larval mortality was observed with increased concentration of the essential oil tested against *An. arabiensis* under laboratory and semi field conditions. This result consistent with the early study finding of treatment increased mortality with increased dose rate were also reported by Ashfaq *et al.* (2012) who found 98.89±0.54% against *Cx*. *quinquefasciatus* larval mortality within 24 h at 120 ppm of water extract of *Moringa oleifera* at the highest application rate. In the present finding, the dose response effects showed that there were statistically significant differences among the concentrations ($P<0.05$) in both laboratory and field conditions, As it indicated in figure 3 and 5 from regression equation it was evident that for the fourth larval stages mortality had relationship to its corresponding dose and the value of R^2 for both laboratory and field conditions 0.844 and 0.913 respectively. This result indicates that the rate of mortality linearly increases with the increasing dose. Though, chemical control of vectors is increasingly becoming difficult because of the development of insecticide resistance in many groups that serve as vectors of diseases. Our result suggests that the investigated plant extract are promising as larvicides against *An. arabiensis* and could be useful in the search for new and biodegradable plant derived larvicides products.

Our study showed that excellent repellent properties of *J. procera* essential oil against adults laboratory colonies of *An. arabiensis*. Dose of 0.1, 0.15, 0.25 and 0.5 ml concentrations were used to compare the efficacy of the essential oil. At 0.5ml essential oil of *J. procera*

has shown 82.14% repellency after 330 minute protection time of application and the other concentration oil provided less than 330 minute protection time against *An. arabiensis.*

Based on this finding it can be therefore suggested that as the concentration of essential oil increase the protection time also increase. In this study as well, it can be seen that repellency could be improved when the oils are increase rather than used with lower concentration application at 0.1, 0.15, 0.25 and 0.5ml concentration as shown in Figure 6. This finding is consistent with study done by Wano, (2006) although the same trend of increased repellency was observed when the concentration was increased to 20%. When *Ocimum suave* was mixed with *Lippia adoensis* at 10% concentration, protection time was only for 2 hours with about 92-100% repellency. Increasing the concentration of this mixture to 20%, protection was also increased to 3 hours with about 88%-100% repellency against *An. arabiensis*.

El-Sheikh *et al.* (2012) reported that seeds extract of *Tribulus terrestris* was more effective in exhibiting the repellent action (100%) against *An. arabiensis* at the dose 1.0 and 2.0mg/cm^2, respectively. Furthermore, to evaluate repellent efficacy of a methanol-leaf extract of Ethiopian traditionally used insect repellent plant *Cymbopogon citratus* (DC) Stapf (Poaceae) against *An. arabiensis* at four different concentrations viz 1.0, 1.5, 2.0, and 2.5 mg/cm^2. It provided the maximum total percentage protection of 78.83% at 2.5 mg/cm^2 and followed 68.06% at 2.0 mg/cm^2 for 12 h. results shows statistically significant (*P*-value=0.001) difference between treated and control groups (Karunamoorthi *et al*., 2010).

The results indicated that *J. procera* essential oil can be useful in preventing mosquitoes bite and has potential use as a repellent. In Kenya, essential oils of *Ocimum forskolei* and *Ocimum fischeri* were evaluated for repellency on forearms of human volunteers against *An. gambiae s.s.* and were found to be more repellent than DEET (Odalo *et al*., 2005). Research has shown that only product containing DEET offer long lasting protection (80 to 90% protection for about 360 minutes when the concentration is about 20% or less) after a single application. Although, the repellent effects of herbal essential oils do not usually last as long as synthetic chemical this can protect from mosquito bite for up to 6 h (Debboun *et al.*, 2000).

The low duration of protection for low concentration might be there is faster loss of repellent effect due to faster volatilization of compounds of the essential oil rather than lack of effectiveness. To extend the protection time, at lower concentration appropriate formulations may be used. Tawatsin *et a1*. (2001) confirmed that the repellency of volatile oils was improved dramatically when they were formulated with vanillin.

In addition the protection time of *Eucalyptus globulus* was prolonged from 3-5 hours after adding 5% vanillin against *Ae. albopictus* under laboratory condition in china (Yang and Ma, 2005). Formulation technology, therefore, plays an important role for long lasting repellents.

In the present study we used undiluted oil with various concentrations the result showed as at 0.5ml concentration *J. procera* oil *provided* 82.14% repellency after 330 minutes protection time of application, 0.25 ml is provided 69.6% repellency after 180 min and 0.15 ml oil provided 65.4% repellency after 120 min and for 0.1 ml provided 59.5% after 80 min protection time against *An. arabiensis.* This result in agreement with the study finding of Trongtokit *et al*. (2005) reported that the undiluted oil showed the highest protection time in each case in the laboratory studies. The mean durations of protection from bites for *Syzygium aromaticum* were 240, 210 and 120 min against *Cx. quinquefasciatus*, *An. dirus* and *Ae. aegypti* respectively. At a 50% concentration *S. aromaticum* provided 120 min of complete protection against both *An. dirus* and *Cx quinquefasciatus*.

Moreover, experiments made on other *Pinu longifolia* species exhibited high repellency 1ml of undiluted Pine oil had strong repellent action against mosquitoes as it provided 100% protection against *Anopheles culicifacies* for 11 h and 97% protection against *Cx. quinquefasciatus* for 9 hours respectively in the field studies (Ansari *et al* ., 2005).

Similar results were obtained by Rajkumar and Jebanesan, (2007) essential oils of *Ipomoea cairica, Momordica charantia* and *Tridax procumbens* exhibited relatively high repellency effect (>300 minutes at 6% concentration), followed by *Centella asiatica* and *Psidium guajava* which showed less effective (<150 minutes at 6 % concentration) against *An. stephensi.*

Many factors such as differences in plant species, different mosquito densities in the cages and cage size (Barnard *et al*., 1998) and different test mosquito species with different sensitivity to repellent oils (Robert *et al*., 1991) may contribute for these variations in the repellent activity of the oils.

CHAPTER SEVEN: CONCLUSION AND RECOMMENDATIONS

CONCLUSIONS

Malaria is the most important of the vector borne diseases both in terms of its geographical distribution, incidence and the extent of the morbidity and mortality it causes in Ethiopia. Due to increased insecticide resistance by vector mosquitoes, anti-malarial drug resistance by malaria parasites and environmental changes, the problems caused by malaria is increasing and forcing people to search for alternative control methods. Hence, there is a constant need to search for plant-derived materials as larvicides and mosquito-repellents, 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 present study revealed that essential oil *J. procera* (Cuperceacea) leaves has very potent larvicidal and repellent properties against *Anopheles arabiensis*, the dominant malaria vector in Ethiopia as well as much of Africa. In laboratory and semi field conditions the treatments resulted complete mortality without any pupal emergence. In addition control or untreated groups did not show any mortality within 24 h exposure. The current study prove the potential of *J. procera* essential oil has been found to be more effective larvicidal activity against larvae of *An. arabiensis* at lower LC_{50} 14.4 and 24.5 ppm values in both laboratory and semi field conditions respectively. However, it revealed that laboratory reared mosquito larvae were more susceptible than wild-collected *Anopheles gambiae sensu lato* larvae.

Based on the repellent activity revealed excellent repellent properties of *J. procera* essential oil against adults *An. arabiensis* under laboratory condition. In repellent activity we were found the highest protection time 330 min at 0.5 ml applied on the forearms of human volunteers. In all the control treatment did not provide any protection even during the first trial.

RECOMMENDATIONS

The gap between repellent results from the laboratory studies and what actually happens in the field can be large. Therefore, field experiment for repellent is essential to validate this laboratory finding. To confirm the efficacy of *J. procera* essential oil to combat *Anopheles arabiensis* mosquitoes at the large scale.

Plant derived compounds are easily degradable and their effect in the environment is least compared to conventional insecticides. Thus, development of bio-insecticides using these products should be encouraged to ensure the safety of our environment and our health. Further studies are needed to develop appropriate formulations including a fixative, which would increase their efficacy and cost effectiveness.

Further studies are also recommended to find out if the repellents from plants have no ill effects on the health of human beings. While generally plants are regarded as safe, toxicity studies are necessary to ensure that the products would be safe. In addition, this appropriate strategy affords the opportunity to minimize chemical repellents usage and the risks associated with adverse side effects. This would offer an eco-friendly and less expensive way to reduce the problem of the *An. arabiensis* especially *J .procera* leaves of the examined plants are commonly available in our country.

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ANNEX 1.

GUIDELINES FOR DEVELOPMENT OF THE INFORMED CONSENT FORM

Name of principal investigator:

Name of organization:

Name of proposal:

PART I: Information sheet

1. Introduction

State briefly who you are and explain to the participants that you are inviting them to take part in research that you are doing.

2. Purpose of the research

Explain why you are doing the research.

3. Type of research intervention

State briefly the type of intervention that will be undertaken.

4. Participant selection

State why this participant has been chosen for this research (adult males or females will preferably be recruited among the inhabitants of the study site, after having announced in the district, through oral advertisements, that the project is looking for volunteers.

5. Voluntary participation

Indicate clearly that volunteers can choose to participate or not.

6. Information on the repellent [name of the repellent]

Explain to the participant why you are testing a repellent product. Provide as much information as is appropriate and understandable about the repellent product,

7. Participant protection against malaria or other vector-borne diseases

Explain to each participant the safeguards that will be provided (e.g. chemoprophylaxis, where relevant) to protect them from malaria or other vector-borne diseases

8. Description of the process, procedures and protocol

Describe or explain to the participant the exact procedures that will be followed on a step-by-step basis and the tests that will be done.

9. Duration

Include a statement about the time commitments of the research for the participant, including the duration of the research and volunteer follow-up.

10. Right to refuse or withdraw

This is a reconfirmation that participation is voluntary and includes the right to withdraw.

Part II: Certificate of Consent

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

Print name of participant: _____

Signature of participant: _____

Date: _____