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Article

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Mosquito-Repellent Activity of Essential Oils from Five Medicinal Plants and Preliminary Phytochemical Screening of Their n-Hexane Extracts

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ABSTRACT

Background: Synthetic insecticides and chemical repellents may adversely affect human health and the environment, while their prolonged use can also result in the development of insecticide resistance in vector populations. Plant-derived essential oils have emerged as environmentally friendly alternatives for mosquito control. In this study, the mosquito-repellent activity of essential oils from five medicinal plants and preliminary phytochemical profiling of their n-hexane crude extracts was conducted. Essential oils extracted from *Ocimum lamiifolium*, *Lepidium sativum*, *Amaranthus hybridus*, *Ocimum gratissimum*, and *Lippia adoensis* were evaluated against *Anopheles arabiensis*, *Aedes aegypti*, and *Anopheles stephensi* following World Health Organization (WHO) guidelines for mosquito repellency testing. All tested essential oils demonstrated substantial mosquito-repellent activity at a dose of 1 mL. Mean repellency ranged from 82.0% to 95.3% for *O. lamiifolium*, 75.3% to 88.7% for *L. sativum*, 82.7% to 89.3% for *A. hybridus*, 78.0% to 84.7% for *O. gratissimum* and 82.7% to 87.3% for *L. adoensis* against the three mosquito species. Preliminary phytochemical screening of n-hexane crude extract revealed the presence of various phytochemical groups that include glycosides, saponins, tannins, sterols, flavonoids, terpenoids, and alkaloids. The Thin Layer Chromatography (TLC) analysis of the same crude extract under UV illumination at 254 and 366 nm further confirmed the presence of diverse phytochemical constituents, as evidenced by multiple separated spots exhibiting dark bands at 254 nm and fluorescent bands at 366 nm with varying R_f values. The essential oils of the selected medicinal plants exhibited promising mosquito-repellent activity and have the potential to serve as environmentally friendly alternatives for mosquito vector control. Further studies are warranted to isolate and characterize the bioactive compounds responsible for the repellent activity of the essential oils and larvicidal activity of n-hexane crude extracts and to evaluate their safety, efficacy, and persistence under field conditions.

Keywords: *Essential oils; Mosquito repellents; Repellent efficacy; Preliminary phytochemical screening; Thin-layer chromatography; Vector control.*

BACKGROUND

Vector-borne diseases remain major global public health challenge, accounting for approximately 20% of the worldwide burden of infectious diseases and causing an estimated one million deaths annually. The impact is particularly severe in sub-Saharan Africa, where morbidity and mortality rates are disproportionately high[1]. Mosquitoes are among the most important vectors of human diseases, transmitting pathogens responsible for malaria, dengue fever, yellow fever, lymphatic filariasis, chikungunya, and Zika virus infections. These diseases result in substantial health, social, and economic burdens, especially in tropical and subtropical regions[2]. Malaria continues to be one of the most devastating mosquito-borne diseases worldwide. According to recent estimates, approximately 247 million malaria cases and 619,000 deaths occurred globally in 2021, with more than 90% of cases and deaths reported from sub-Saharan Africa[3]. Children under five years of age and pregnant women remain the most vulnerable populations[4]. Among the more than 500 known species of *Anopheles* mosquitoes, about 50 species are capable of transmitting malaria parasites to humans[3, 5]. In addition to malaria, arboviral diseases transmitted by *Aedes aegypti* have emerged as significant public health concerns. *Ae. aegypti* is the principal vector of dengue fever, yellow fever, chikungunya, and Zika virus infections. Dengue fever, in particular, has expanded rapidly in many tropical and subtropical countries, with increasing incidence of severe forms such as dengue hemorrhagic fever and dengue shock syndrome [6].

In Ethiopia, mosquito-borne diseases such as malaria, dengue fever and yellow fever remain major public health problems. *Anopheles arabiensis* is the primary malaria vector, while *Anopheles funestus*, *Anopheles pharoensis*, and *Anopheles nili* serve as secondary vectors[7]. Furthermore, *Anopheles stephensi*, an efficient urban malaria vector originally endemic to South Asia and the Middle East, was first reported in Ethiopia in 2016 and has since spread to several urban centers [8]. Ethiopia reports millions of confirmed malaria cases annually [9, 10], while dengue outbreaks have been documented in eastern and central parts of the country[10]. The continued expansion of mosquito vectors and the emergence of invasive species pose additional challenges to vector control efforts [10,11]. Although vaccines are available for a limited number of mosquito-borne diseases such as yellow fever and, more recently, malaria, vector control and personal protection against mosquito bites remain essential strategies for disease prevention. The use of mosquito repellents has therefore become an important component of integrated vector

management programs[12]. Most commercially available repellents contain synthetic chemicals such as N, N-diethyl-meta-toluamide (DEET), allethrin, dimethyl phthalate (DMP), and N, N-diethyl mandelic acid amide (DEM)[13]. However, concerns regarding environmental contamination, adverse effects on human health and non-target organisms, and the development of insecticide resistance have stimulated interest in alternative mosquito control approaches [14, 15]. Prolonged and frequent use of synthetic repellents in mosquito control has resulted in the development of resistance to insecticides, resurgence in mosquito populations, and adverse impact on non-target organisms [16–19]. Plant-derived repellents have attracted considerable attention as environmentally friendly and sustainable alternatives to synthetic products [20]. Essential oils and plant extracts contain diverse bioactive compounds, including alkaloids, terpenoids, phenolics, proteinase inhibitors, and growth regulators, many of which possess insecticidal, repellent, anti-feedant, or growth-disrupting properties [21-25]. Numerous studies have demonstrated that plant essential oils can effectively repel mosquitoes by interfering with host-seeking behavior and olfactory receptor activity [26]. Compounds such as citronellal, linalool, geraniol, eucalyptol, thujone, and geranyl acetate, which are common constituents of essential oils, have demonstrated strong repellent activity against several mosquito species, including *Anopheles*, *Aedes*, and *Culex* spp. [26, 27]. The use of plants as mosquito repellents has a long history in traditional communities worldwide [28]. In many African countries, including Ethiopia, rural communities commonly use aromatic plants, either by burning plant materials, hanging fresh leaves indoors, or applying plant extracts to the skin and clothing to reduce mosquito bites [29-34]. Despite their widespread traditional use, the repellent efficacy and phytochemical composition of many indigenous Ethiopian medicinal plants have not yet been scientifically validated [35-38].

Phytochemical screening provides valuable preliminary information on the bioactive constituents responsible for the biological activities of medicinal plants and serves as an important step in the discovery of novel natural products for vector control. Therefore, the present study aimed to evaluate the mosquito-repellent efficacy of essential oils extracted from five traditionally used medicinal plants, namely *Ocimum lamiifolium*, *Lepidium sativum*, *Amaranthus hybridus*, *Ocimum gratissimum*, and *Lippia adoensis*, against *Anopheles arabiensis*, *Ae. aegypti*, and *An. stephensi* under laboratory conditions and conduct preliminary phytochemical characterization of the n-hexane extracts of the same plant species previously assayed for their larvicidal properties

against the same mosquito species under the same laboratory settings.

MATERIALS AND METHODS

All experimental procedures including the collection, handling and processing of plant materials were performed in accordance with the relevant institutional, national and international guidelines and regulations. The study was conducted following the approval from Institutional Research Ethics Review Committee (ALIPB-IRERC) at Aklilu Lemma Institute of Health Research, Addis Ababa University, with reference number: ALIPB-IRERC/140/2016/2024.

Collection of plant materials

The five plant species evaluated for their mosquito-repellent properties were selected among the ethnobotanically reported medicinal plants and traditionally used as mosquito repellents. Species selection was guided by ethnobotanical knowledge and previous reports on plant families with documented mosquito-repellent activity, as described by Martin [39]. The collected plant materials were identified using an ethno-taxonomic approach, supplemented by standard botanical identification procedures. Five indigenous plant species were selected based on their widespread and longstanding traditional use as insect and mosquito repellents by different communities. They were collected from Asendabo district of Jimma Zone, Oromia Regional State, and Bonga district of the Southwest Ethiopia Peoples' Regional State, Ethiopia. These plant species have historically constituted important components of traditional vector control practices in the respective communities. Field surveys were conducted between August and November 2024 to collect the five selected plant species: *Ocimum lamiifolium* (*O. lamiifolium*), *Lepidium sativum* (*L. sativum*), *Amaranthus hybridus* (*A. hybridus*), *Ocimum gratissimum* (*O. gratissimum*), and *Lippia adoensis* (*L. adoensis*). Voucher specimens were collected for taxonomic identification and herbarium deposition, while bulk fresh plant materials were harvested separately for essential oil extraction and subsequent mosquito repellency bioassays. All collections were carried out following standard botanical sampling and specimen collection procedures to ensure accurate species identification and reproducibility of the study.

Voucher Specimen Identification

The plant specimens were identified by the plant taxonomy specialist, Mr. Melaku Wondafirash and the voucher specimens were deposited at National Herbarium, Department of Plant Biology

and Biodiversity Management, College of Natural and Computational Sciences, Addis Ababa University, Ethiopia for future references under the voucher specimen numbers *Ocimum lamiifolium* Hochst. Ex. Walp (LG-01); *Lepidium sativum* L. (LG10); *Amaranthus hybridus* L.(LG-06); *Ocimum gratissimum* L (LG-02) and *Lippia adoensis* Hochst. (LG-03) to facilitate future verification of the plant species. We also confirm that the collection and use of plant materials complied with applicable conservation and biodiversity regulations, including the relevant principles of the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), where applicable. All the field data were properly recorded, summarized and presented in Table 1.

Table 1 Field data and ethnobotanical information of the plants used in this study

S. N	Species name	Family name	Plant parts	Collection Place	Local name	Habitat	GPS coordinates	Altitude	Ref.
1	*O. lamiifolium Hochst. Ex. Walp* (LG-01)	Lamiaceae	Aerial parts	Asendabo	Damakase (AO)	Home	(07^{0}46′15.155″N) (037^{0}14′03.58″E)	1,761m	[40-45]
2	*L. sativum* L. (LG-10)	Lamiaceae	Seed	Asendabo	Feto (AO)	Stead	(07^{0}43′20.34″N) (36^{0}18′09.79″E)	1,772m	[46-48]
3	*A. hybridus* L. (LG-06)	Amaranthaceae	leaves	Bonga	Asangira (K)	Forest	(07^{0}43′20.34″N) (36^{0}18′09.79″E)	1,772m	[49-51]
4	*O. gratissimum* L (LG-02)	Lamiaceae	Aerial parts	Asendabo	Hancabi (AO)	Herb	(07046′19.41″N (37014′03.29″E)	1,754m	[52-54]
5	*L. adoensis* Hochst. (LG-03)	Verbenaceae	Aerial parts	Asendabo	Koseret (AO)	Home	(07046′13.715″N (037014′04.49″)	1,764m	[55-57]

(AO)- Afan Oromo
(K) - Kafigna

Bulk Sample Collection, Essential oil extraction and preliminary phytochemical characterization of n-hexane crude extracts

The plant materials and their parts used for essential oil extraction and n-hexane crude extract preparations were collected from natural population (wild) at Asendabo district of Jimma Zone, Oromia Regional State, and Bonga district of the Southwest Ethiopia Peoples' Regional State, Ethiopia. Collection of the plant parts was conducted after obtaining the necessary ethical clearance certificate from Aklilu Lemma Institute of Health Research, Addis Ababa University with reference number (ALIPB-IRERC/140/2016/2024) and permission certificate from Oromia Health Research Ethical Review Committee, Oromia Health Bureau, Oromia Regional State, Addis Ababa, Ethiopia with permission reference number (BFO/HQ/007/16). Fresh plant samples of *O. lamiifolium* Hochst. ex Benth. (aerial parts), *L. sativum* L.(seeds), *A. hybridus* L. (leaves), *O. gratissimum* L. (aerial parts), and *L. adoensis* Hochst. ex Walp. (aerial parts) were

collected from their respective collection sites. The samples were placed separately into clean plastic bags, sealed with rubber bands to prevent cross-contamination, and transported to the Endod and Other Medicinal Plants Research Laboratory, Aklilu Lemma Institute of Health Research, Addis Ababa University, Ethiopia, for further processing and analysis. Prior to extraction of essential oils, moisture content of each sample was determined by drying them at room temperature. Essential oils were extracted from aerial parts, leaves and/or seeds of plants by hydro-distillation method using a Clevenger-type apparatus for 3 hours. Plant materials (aerial parts, leaves or seeds) were shredded and placed in a distillation flask and one liter of distilled water was added. The oil was separated from water and passed through cotton wool containing anhydrous sodium sulphate to remove moisture and kept in light insensitive ambered glass bottles and stored at 4 °C until used for the repellency test. The n-hexane crude extracts obtained from the five selected plants, from which the essential oils were extracted, were subjected to preliminary phytochemical characterization using both chemical and chromatographic methods.

Positive control used for repellency activity test

Arms of volunteers treated with formulated commercial 20% N, N-Diethyl-meta-toluamide (DEET) were used as positive control and solvent (n-hexane)-treated arms were also used as negative control for repellent bioassay.

Mosquito rearing

Insectary-reared female *An. arabiensis, Ae. aegypti*, and *An. stephensi* were used for the repellency assays. All mosquitoes were obtained from the Aklilu Lemma Institute of Health Research (ALIHR), Addis Ababa University, Addis Ababa, Ethiopia, and maintained in a dedicated insectary under standard rearing conditions at a temperature of 27 ± 2 °C, 80 ± 10% relative humidity (RH), and a 12:12 h light photoperiod. The mosquito eggs hatched in distilled water, and the larvae were reared in plastic larval trays containing distilled water. Larvae were fed a standardized, nutritionally balanced larval diet prepared according to the insectary's routine rearing protocol. Upon pupation, pupae were collected and transferred into plastic cups containing distilled water and placed inside standard 30 × 30 × 30 cm screened rearing cages to allow adult emergence.

Following emergence, adult mosquitoes were maintained in the same cages and provided continuous access to a 10% (w/v) sucrose solution supplied on cotton pads placed on the top of

each cage. Adult mosquitoes were maintained at 25–27 °C with a relative humidity of approximately 80 ± 10%, which was maintained using humidification trays containing water within the insectary.

Standardized mosquito rearing and laboratory testing conditions were maintained throughout the study to ensure the reliability and reproducibility of the experimental data. For repellency assays, only non-blood-fed female mosquitoes aged 5–7 days post-emergence were used. Mosquitoes were deprived of the sucrose solution for 12 h before testing while retaining access to water. Repellency experiments were conducted during the peak host-seeking period corresponding to the natural biting activity of each mosquito species**.**

Repellent Bioassay

Mosquito repellency bioassays were conducted against three target mosquito species, *An. arabiensis, Ae. aegypti,* and *An. stephensi*, based on the World Health Organization (WHO) guidelines for mosquito repellent testing[40] and implementing the standard arm-in-cage method previously described[5,41].

For each bioassay, 50 laboratory-reared, blood-starved 5–8 days old female mosquitoes were used [40]. The mosquitoes were deprived of sugar 12h before the tests while access to water was maintained and kept in screened cages (30 × 30 × 30 cm) until the exposure time[40,58].

Bioassays were conducted under lighting conditions appropriate for the biting behavior of each mosquito species. *Ae. aegypti* assays were performed under illuminated conditions because this species is primarily diurnal, whereas *An. arabiensis* and *An. stephensi* assays were conducted under dim-light conditions that simulate their nocturnal host-seeking behavior.

Three different volunteers participated in bioassays where the ethics committee approved the procedure and written informed consent was obtained from all volunteers to minimize bias associated with individual differences in skin attractiveness[40]. The volunteers were non-smoking male adults with no dermatological disease who had not received any medications affecting skin odor[40].They were also monitored throughout the study period for any signs of skin irritation or adverse reactions following application of the plant extracts. They did not apply perfume, lotion, oils, or scented soap on the day of the experiment. Before each bioassay, the forearm and hand of each volunteer were washed with unscented neutral soap, thoroughly rinsed with water, and allowed to air-dry for 10 min before application of the test extract.

For each test, one forearm treated with the plant extract (600 cm^2 while the lower hand section

below the wrist was covered with glove and the upper arm was handled of the cage slit) served as the treatment, while an solvent-treated forearm served as the negative control[40]. A forearm treated with a commercially formulated 20% DEET product served as the positive control. The treated and control arms were introduced simultaneously into separate mosquito cages containing 50 female mosquitoes, and the cages were gently tapped to activate host-seeking behavior. A randomized crossover design was used in which each volunteer tested a new treatment on different days to reduce inter-individual variations. The treatment order was randomized on the first day of each replicate [40,59]. The insectary conditions were maintained at 27 ± 2°C, 70–80% RH during testing[40]. Mosquito landings, probing, and confirmed bites were observed and recorded during a 3-hour exposure period at 30, 60, 90, 120, 150, and 180 min after repellent application.

Each treatment was evaluated in triplicate using separate mosquito cages. Then, the percentage protection was calculated using the following formula [42]:

$$\frac{[(\text{No: of bites received by control arm}) - (\text{No: of bites received by treated arm})]}{(\text{No: of bites received by control arm})} X100$$

Preliminary phytochemical tests and TLC profiling of the n-hexane crude extracts of five medicinal plants

Preliminary phytochemical screening of the n-hexane crude extracts was carried out using standard qualitative chemical methods to detect the presence or absence of major secondary metabolites. The semi-quantitative phytochemical tests and TLC profiling of crude extracts of targeted plants were carried out in Pharmacogonosy laboratory, Department of Pharmaceutical Chemistry and Pharmacognosy, School of Pharmacy, College of Health Sciences, Addis Ababa University following the methods described by Balbaa[60] and Harborne[61] to investigate the presence of the major phytochemical constituents such as glycosides, Anthraquinone glycosides, cardiac glycosides, saponins, tannins, sterols, flavonoids, terpenoids and alkaloids.

Test for alkaloids: A small quantity of each targeted plant extract was stirred with 5 mL of 1% aqueous HCl in water bath and subsequently filtered. The resulting filtrate (1 mL) was transferred separately into two test tubes. To the first test tube, a few drops of Dragendorff's reagent were added. The formation of an orange-red precipitate was considered a positive

indication of the presence of alkaloids. To the second test tube, 1 mL of Mayer's reagent was added, and the appearance of a buff-colored precipitate was recorded as evidence of the presence of alkaloids [62].

Molisch's test (General test for glycosides): Approximately 2 mL of each extract, dissolved in distilled water, was treated with a few drops of Molisch's reagent. Subsequently, 1 mL of concentrated sulfuric acid (H_2SO_4) was carefully added along the side of the test tube to form a separate layer. The mixture was allowed to stand for 2 min and then diluted with 5 mL of distilled water. The appearance of a red or dull violet ring at the interface of the two layers indicated the presence of glycosides [62].

Barfoed's test (Anthraquinone glycosides): Approximately 0.5 g of each sample was dissolved in distilled water and filtered. One milliliter of the filtrate was mixed with 1 mL of Barfoed's reagent in a test tube and heated in a water bath for 2 min. The formation of a reddish precipitate of cuprous oxide indicated a positive test for anthraquinone glycosides [62].

Keller–Killiani test (Cardiac glycosides): Two milliliters of the acidic filtrate was treated with three drops of ferric chloride ($FeCl_3$) solution prepared in glacial acetic acid. Concentrated H_2SO_4 was then carefully added along the side of the test tube. The formation of a brown ring at the interface, which gradually developed into a bluish-green color upon standing, indicated the presence of cardiac glycosides [62].

Salkowski test (Steroidal glycosides): Each extract was dissolved in chloroform, followed by the careful addition of concentrated H_2SO_4. The mixture was gently shaken. The development of a reddish coloration in the chloroform layer indicated the presence of steroidal glycosides [62].

Liebermann–Burchard test (Steroidal glycosides): Approximately 0.2 g of each sample was mixed with 2 mL of acetic acid and cooled in an ice bath. Concentrated H_2SO_4 was then added carefully along the side of the test tube. The development of a violet, blue, or bluish-green color indicated the presence of the steroidal nucleus (aglycone moiety) of cardiac glycosides [62].

Ferric chloride test (Tannins): Approximately 0.5 g of each sample was stirred with 10 mL of distilled water and filtered. Two milliliters of the filtrate was treated with a few drops of 1% ferric chloride solution. The formation of a blue-black, green, or blue-green coloration or precipitate indicated the presence of tannins [63].

Lead acetate test (Tannins): One milliliter of the plant extract was mixed with 1 mL of 10% lead acetate solution. The formation of a white precipitate indicated the presence of tannins [63].

Sodium hydroxide test (Flavonoids): A small quantity of each sample was dissolved in distilled water and filtered. Two milliliters of 10% aqueous sodium hydroxide solution was added to the filtrate, producing a yellow coloration. The disappearance of the yellow color upon the addition of dilute hydrochloric acid indicated the presence of flavonoids [63].

Froth test (Saponins): One gram of each sample was boiled with 5 mL of distilled water and filtered. Approximately 3 mL of distilled water was added to the filtrate, which was then shaken vigorously for 5 min. The formation of a stable froth that persisted upon warming indicated the presence of saponins [62].

Salkowski test (Terpenoids): A small amount of each sample was dissolved in ethanol. One milliliter of acetic anhydride was added, followed by the careful addition of concentrated H_2SO_4. The appearance of a reddish-brown coloration at the interface indicated the presence of terpenoids [62].

Liebermann–Burchard test (Terpenoids): A small quantity of each sample was boiled with 1 mL of 5% potassium hydroxide (KOH), allowed to cool, and then acidified with concentrated H_2SO_4. The development of a deep green color indicated the presence of terpenoids [62].

Phytochemical Profiling by Thin-Layer Chromatography (TLC)

Thin-layer chromatography (TLC) was performed to obtain the phytochemical profiles following the method described by Shaikh [64] of the non-polar (n-hexane) crude extracts that exhibited the highest larvicidal activity in our previous study [65]. The same plant materials used to prepare the n-hexane extracts were also used for the extraction of essential oils, which were subsequently evaluated for their repellent activity against three mosquito species. The TLC profiles of the n-hexane crude extracts of five plants were analysed using thin-layer chromatography (TLC). Samples (2 µL each) were applied to the TLC plate at the origin with a glass capillary tube and allowed to dry at room temperature for 20 minutes. The solvent system, dichloromethane: methanol (9:1 v/v ratio) was used for TLC development. The solvent system was prepared, placed in a chromatographic tank, covered with lid and allowed to equilibrate for 20 minutes. Then, the TLC loaded with samples, dried with air gun, placed in the developing chamber containing the equilibrated solvent system was allowed to develop chromatogram until the solvent front reached the marked edge of the plates. Afterward, the developed TLC plate was carefully removed from the chamber and dried in a fume hood at room temperature. The

separated spots, representing different compounds, were visualized and examined in daylight, under UV light at shorter wavelength (254 nm) and longer wavelength (366 nm) to identify spots based on their colour and R_f values. The separated components were visualized and documented using a CAMAG® TLC Visualizer 2 under white light and ultraviolet light at 254 and 366 nm**.** The retardation factor (R_f) of each separated spot was determined by measuring the distance traveled by the center of the spot from the origin and dividing it by the distance traveled by the solvent front.

Data Analysis

The design of the entire experiment was complete randomized design (CRD), i.e. lottery method was used to decide the treatments' sequence in the bioassays. The data generated in these experiments were recorded in a Microsoft excel spread-sheet, edited, and stored for future analysis. The statistical analysis was conducted using SPSS Version 26 statistical package. The mean percent repellency was calculated from the three replicates. One-way analysis of variance (ANOVA) was used to determine the significance difference in percent repellency exhibited by the various essential oils of each plant species extracts. When a significant difference was observed within 3 h protection time, the Duncan multiple range comparison Post Hoc test was used for mean separation. Bioinsecticide activities were considered to be significantly different when 95% confidence limit levels failed to overlap or if P value< 0.05. Comparisons between mean percent repellency activities that were treated with 1 ml various essential oils of the test plant species (1mg/600 cm^2), negative and positive controls were made by using Turkey's Post Hoc testing. The average mean percent repellency from the three replicates of each test plant essential oil extracts against different female adult mosquito species were calculated and taken as mean percentage. The retardation factor (R_f value) of each separated spot was determined by measuring the distance traveled by the center of the spot from the origin and dividing it by the distance traveled by the solvent front.

RESULTS

The essential oil extracts were weighed and percentage yield of the extracts of the selected plants used for repellency bioassays are summarized in Table 2.

Table 2: Plant material used in the study and percentage yield of Essential oils.

S.N	Species name	Family name	Plant parts	Local name	Yield (%)

1	*Ocimum lamiifolium* Hochst. Ex. Walp (LG-01)	Lamiaceae	Aerial parts	Damakase	4.1mg (0.41%)
2	*Lepidium sativum* L. (LG-10)	Lamiaceae	Seed	Feto	38 mg (3.8%)
3	*Amaranthus hybridus* L. (LG-06)	Amaranthaceae	leaves	Asangira	140 mg (14%)
4	*Ocimum gratissimum* L (LG-02)	Lamiaceae	Aerial parts	Hancabi	13 mg (1.33%)
5	*Lippia adoensis* Hochst. (LG-03)	Verbenaceae	Aerial parts	Koseret	4.9 mg (0.9%)

Laboratory evaluation of repellent plant materials

The results of the laboratory evaluation of the five commonly known traditional insect/mosquito repellent plants, namely *O. lamiifolium*, *L. sativum*, *A. hybridus*, *O. gratissimum*, and *L. adoensis* against adult *An. arabiensis*, *Ae. aegypti*, and *An. stephensi* are presented in Table 3 and Figure 1. The findings demonstrated that all tested plant extracts exhibited significant repellent activity against the three targeted mosquito species under laboratory conditions. Among the evaluated plants, *O. lamiifolium* showed the highest repellent efficacy against *An. arabiensis* (95.3%) and *Ae. aegypti* (93.3%), while *L. adoensis* exhibited the highest repellency against *An. stephensi* (87.3%). The repellent efficacies recorded for *L. sativum* were 88.7%, 84.0%, and 75.3% against *An. arabiensis*, *Ae. aegypti*, and *An. stephensi*, respectively. Similarly, *A. hybridus* demonstrated repellency efficacies of 89.3%, 88.7%, and 82.7%, whereas *O. gratissimum* exhibited efficacies of 84.7%, 81.3%, and 78.0% against the respective mosquito species. Overall, the results indicate that all five traditional repellent plants possess promising mosquito repellent properties with varying levels of efficacies among mosquito species, plant types and their essential oils.

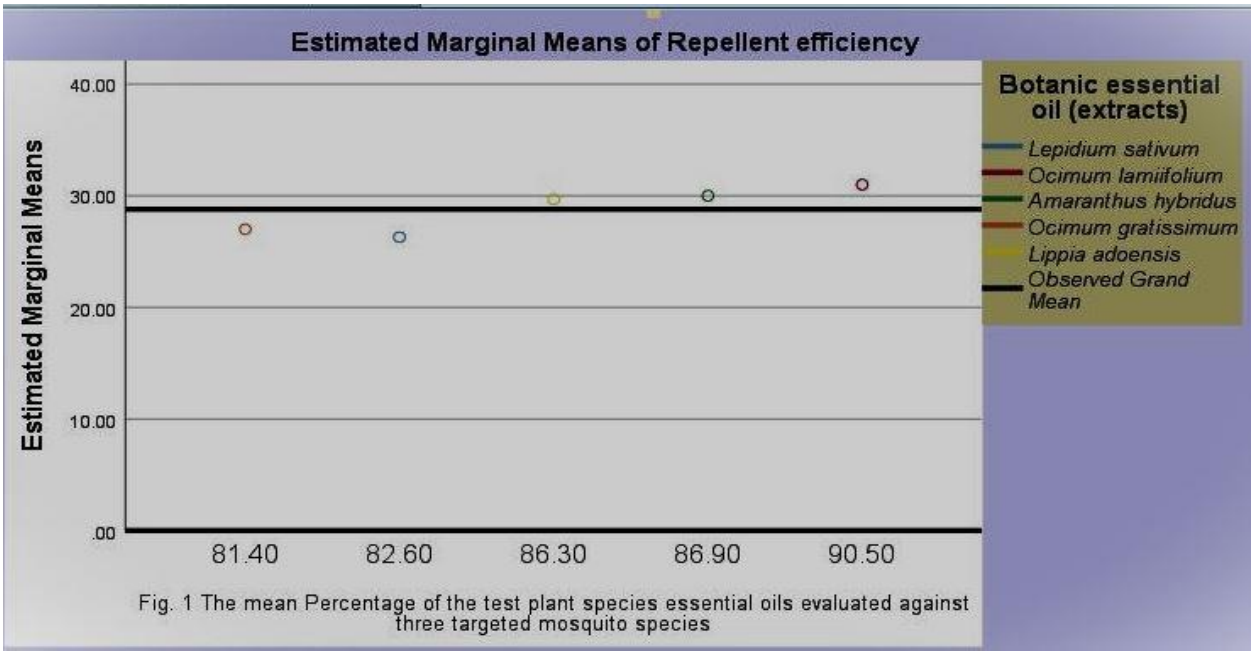
Table 3 Repellent efficacies of essential oils of the tested plant species against different adult mosquito species (*An. arabiensis, Ae. aegypti,* and *An. stephensi*) within 3 h.

SN	Plant species	Concentrations	Mosquito species	Mean %	MD	SD	95% CI		Sig.
							LB	UB	
		Solvent-treated arm		**0.00**	**100.0**	**1.4**	**95.6**	**104.4**	**1.00**
1	*O. lamiifolium* Hochst. Ex. Walp (LG-01).	1 ml without dilution (1ml/600 cm^2)	*An. arabiensis*	95.3	-95.3*	1.4	-99.8	-90.9	.000
			Ae. aegypti	93.3	-93.3*	3.0	-102.6	-84.0	.000
			An. stephensi	82.0	-82.0*	3.2	-92.0	-71.9	.000
2	*L. sativum* L. (LG-10)	1 ml without dilution (1ml/600 cm^2)	*An. arabiensis*	88.7	-88.7*	2.8	-97.5	-79.8	.000
			Ae. aegypti	84.0	-84.0*	0.9	-86.9	-81.1	.000
			An. stephensi	75.3	-75.3*	1.4	-79.8	-70.9	.000
3	*A. hybridus* L. (LG-06)	1 ml without dilution (1ml/600 cm^2)	*An. arabiensis*	89.3	-89.3*	2.4	-96.6	-82.1	.000
			Ae. aegypti	88.7	-88.7*	.54	-90.4	-86.9	.000
			An. stephensi	82.7	-82.7*	3.8	-94.4	-70.9	.000
4	*O. gratissimum* L (LG-02)	1 ml without dilution	*An. arabiensis*	84.7	-84.7*	5.9	-103.0	-66.3	.000
			Ae. aegypti	81.3	-81.4*	5.2	-97.3	-65.4	.000

		(1ml/600 cm^2)	*An. stephensi*	78.0	-78.0*	3.4	-88.4	-67.6	.000
5	*L. adoensis* Hochst. (LG-03)	1 ml without dilution (1ml/600 cm^2)	*An. arabiensis*	86.0	-86.0*	1.9	-91.8	-80.2	.000
			Ae. aegypti	82.7	-82.7*	3.0	-91.97	-73.4	.000
			An. stephensi	87.3	-87.3*	6.3	-106.6	-68.1	.000
		20% DEET		**100.0**	**-100.0***	**1.4**	**-104.4**	**-95.6**	**.000**

*The mean difference is significant at the 0.05 level, **MD** = mean difference, **SD**= Std. Error, 95%, **CI** = 95% Confidence Interval , **LB**= lower boundary limit, **UB**= Upper boundary limit.

Fig. 1 The marginal mean Percentage of the five test plant species essential oil evaluated again adult mosquito species of *An. arabiensis, Ae. aegypti* and *An. stephensi*.



Phytochemical Analysis

Preliminary phytochemical screening revealed the presence of alkaloids, various classes of glycosides, saponins, tannins, sterols, flavonoids, and terpenoids in the n-hexane crude extracts of the test plant species. All n-hexane extracts tested positive for these major phytochemical groups. A summary of the phytochemical screening results is presented in Table 4.

Table 4: Qualitative and semi-quantitative phytochemical analysis of the five test plant crude extracts.

S/N	Constituents Tests	Result				
		O. lamiifolium	***L. sativum***	***A. hybridus***	***O. gratissimum***	***L. adoensis***

1	Alkaloids	Mayer's test	+	++	+	++	+
		Dragendorff's test	+	+++	+	++	+
		Wagner's test	+	++	+	++	+
2	General glycosides Test	Molisch's test	+	++	+++	++	++
2.1	Anthraquinone glycosides	Borntrager's test	+	+	+	+	+
2.2	Cardiac glycosides	Keller–Killiani test	-	+	+	+	+
2.3	Steroidal glycosides	Salko-wski test	++	+	+	+	+
		Lieberman-Burchard's	++	+	+	+	+
3	Tannins	Ferric chloride test	+	++	+	+	+
		Lead acetate test	+	++	+	+	+
4	Flavonoids	Sodium hydroxide test	+	+	+	+	+
5	Saponins	Froth (foam) test	+	+	+	+	+
6	Terpenoids	Salko-wski test	+	+	+	+	+
		Lieberman-Burchard's	+	+	+	+	+

Key: (+) = present (positive result), (-) = absent (negative result), (++ and +++) = strongly present

Thin Layer chromatography (TLC) profiling of the n-hexane crude extracts of selected plants

In the TLC profiling and characterization of the n-hexane crude extracts of plants used in this study, phytochemical constituents were detected as spots with different colours and R_f values under UV light at shorter (254 nm) and longer (366 nm) wavelengths as summarized in Table 5 and Figure 2.

Table 5: The colour and Rf values of spots separated on TLC in dichloromethane: methanol (9:1 v/v ratio) and visualized under UV light at 254 and 366 nm

Plant crude extracts	**At 254 nm UV light**		**At 366 nm UV light**	
	Spot color	**Rf value**	**Spot color**	**R_f value**
L. sativum	Dark	0.3	Purple	0.28
	Dark	0.5	Yellow	0.64
	Dark	0.74	Blue	0.84
O. gratissimum	Tailed dark	0.3	Tailed yellow	0.3
	Dark	0.96	Blue	0.84
O. lamiifolium	Dark	0.28	Blue	0.28
	Tailed dark	0.5	Purple	0.5

			Light Yellow	0.7
L. adoensis	Deep dark	0.22	Yellow	0.4
	Dark	0.96	Blue	0.8
A. hybridus	Dark near origin	0.04	Light purple	0.38
			Blue	0.64

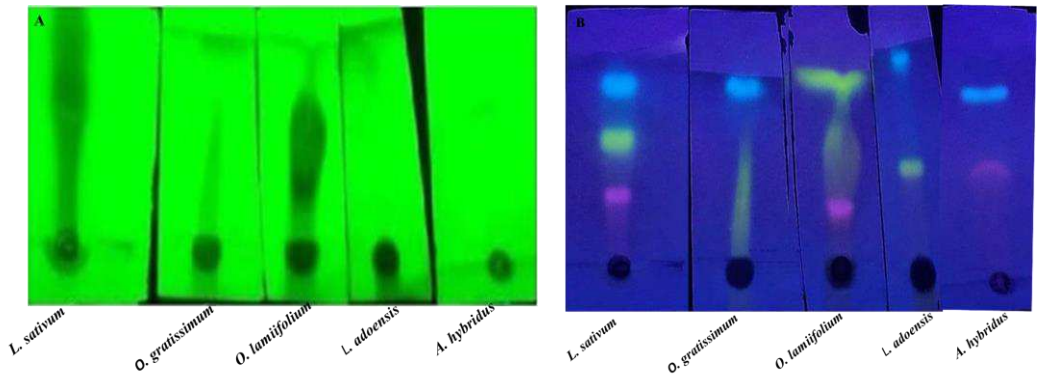


Figure 2. TLC chromatogram of n-hexane crude extracts developed in dichloromethane: methanol (9:1 v/v ratio) and visualized under UV-light at 254 nm (A) and at 366 nm (B). .

The TLC profiles of n-hexane crude extracts of the selected plants revealed the presence of a lot of primary and secondary metabolites that might be responsible for the promising mosquitocidal activity observed against the respective larvae of *An. arabiensis, Ae. aegypti, and An. stephensi* in our previous study. The presence of these metabolites was detected from the TLC chromatogram by visualizing in day light and examining under UV-light (at 254 nm and 366 nm) to identify spots with different colors (as dark spots at 254 nm, and fluorescent spots with purple, yellow and blue-colored spots at 366 nm). However, this study tested the five selected plant species essential oils for their mosquito-repellent activity, these plant species n-hexane crude extracts were tested and demonstrated significant bioinsecticidal activity against the respective larvae of *An. arabiensis*, *Ae. aegypti*, and *An. stephensi* in our previous study [65].

DISCUSSION

The extensive and prolonged use of chemical insecticides for mosquito control has resulted in widespread insecticide resistance, thereby reducing the effectiveness of conventional vector control interventions. Consequently, there is an increasing need to identify alternative,

sustainable, and environmentally friendly approaches for mosquito management[66]. Plant-based repellents have attracted considerable attention because they are generally regarded as safer for humans, biodegradable, environmentally compatible, and culturally acceptable [67]. Furthermore, their potential for local cultivation and production makes them cost-effective alternatives, particularly in resource-limited settings. Essential oils and plant-derived extracts have demonstrated promising repellent activity against medically important mosquito vectors, including *Anopheles arabiensis*, *Aedes aegypti*, and *Anopheles stephensi*, making them attractive candidates for incorporation into integrated vector management (IVM) programs[68].

In the present study, the repellent efficacy of essential oils extracted from five indigenous plant species (*Ocimum lamiifolium*, *Lepidium* sativum, *Amaranthus hybridus*, *Ocimum gratissimum*, and *Lippia adoensis*) was evaluated against *An. arabiensis*, *Ae. aegypti*, and *An. stephensi*. All five essential oils exhibited significant repellent activity for up to 3 h against the three mosquito species. Under the laboratory conditions used, the test repellents consistently reduced mosquito landings and bites without causing visible skin irritation or other adverse reactions in the volunteers. A commercial 20% DEET formulation served as the positive control, whereas solvent-treated forearms were used as the negative control to compare the protective efficacy of the plant-derived repellents.

Among the five tested plants, *O. lamiifolium* demonstrated the highest overall repellency, providing 82.0–95.3% protection against the three mosquito species. This was followed by *A. hybridus* (82.7–89.3%), *L. adoensis* (82.7–87.3%), *L. sativum* (75.3–88.7%), and *O. gratissimum* (78.0–84.3%). Species-specific differences in repellency were also observed. *O. lamiifolium* produced the highest repellency against *An. arabiensis* (95.3%) and *Ae. aegypti* (93.3%), whereas *L. adoensis* showed the greatest activity against *An. stephensi* (87.3%). These findings suggest that the repellent efficacy of plant essential oils may vary according to mosquito species, reflecting differences in mosquito olfactory responses to volatile phytochemicals[69].

The superior repellent activity of *O. lamiifolium* may be associated with its rich composition of biologically active secondary metabolites, including alkaloids, flavonoids, tannins, steroids, and phenolic compounds, which have previously been reported in this species[70]. In addition, species of the genus *Ocimum* are known to contain abundant volatile constituents, particularly

monoterpenes and phenylpropanoids that possess insect-repellent and antimicrobial properties[71]. The high repellency observed in the present study is therefore likely attributable to the combined effects of these bioactive compounds.

The results of this study are generally consistent with previous reports demonstrating the mosquito-repellent potential of indigenous plant species, although the level of protection observed here was comparatively higher. For example, a study conducted in Eritrea reported that hanging fresh leaves and shoots of *Ocimum forskolei* above beds reduced indoor entry of *An. arabiensis* by approximately 53% [72]. Likewise, alcoholic extracts of *Lippia javanica* provided 76.7% protection against *An. arabiensis* for up to 4 h, while *C. excavatus* and *P. reniforme* afforded 66.7% and 63.3% protection, respectively, for 3 h. After 5 h, only *L. javanica* maintained appreciable repellency (59.3%)[73]. The higher repellency recorded in the present study may be related to differences in plant species, extraction methods, essential oil composition, application rate, experimental conditions, or mosquito populations tested. These factors have been shown to substantially influence the biological activity of plant-derived repellents.

The phytochemical profiles of the n-hexane crude extracts from the five selected medicinal plants were further investigated using Chemical and thin layer chromatography (TLC) test methods. These plants had previously demonstrated significant larvicidal activity, while their essential oils exhibited strong repellent activity in the present study. The chemical test method revealed that the n- hexane crude extracts of the five selected medicinal plants contained all the major phytochemical groups responsible for the mosquitocidal activities (Table 4). TLC analysis under ultraviolet light (254 and 366 nm) also revealed multiple UV-active compounds in all extracts. Dark spots observed at 254 nm and fluorescent purple, yellow, and blue spots detected at 366 nm indicated the presence of diverse classes of secondary metabolites. Variation in spot colour and retention factor (R_f) values among the extracts suggests qualitative differences in their phytochemical composition, consistent with previous findings[74]. These phytochemical differences may partly explain the variation in repellent activity observed among the tested plant species.

Essential oils are complex mixtures of volatile compounds obtained from aromatic plants, primarily through hydrodistillation or related extraction techniques[16]. They typically contain numerous constituents, with monoterpenes and sesquiterpenes representing the predominant

chemical classes. These terpenoid compounds are well documented for their insecticidal and repellent properties and have been identified as the principal bioactive components responsible for mosquito repellency[16]. The strong repellency (>75%) demonstrated by all five essential oils in the present study is therefore likely due to the combined or synergistic effects of multiple volatile constituents rather than the activity of a single compound.

Globally, approximately 17,500 higher plant species are regarded as aromatic and nearly 3,000 essential oils have been identified, of which around 300 are commercially utilized in the pharmaceutical, cosmetic, and fragrance industries[75]. The high repellent efficacy demonstrated by the essential oils evaluated in this study further supports their potential as environmentally friendly alternatives or complements to synthetic mosquito repellents. Because essential oils comprise multiple bioactive constituents with different modes of action, they may also reduce the likelihood of rapid resistance development compared with single active ingredients.

Generally, the findings indicate that these indigenous medicinal plants possess considerable potential for reducing human–mosquito contact, particularly in rural and resource-limited communities where access to electricity and commercially manufactured repellents may be limited. Their local availability, affordability, cultural acceptance, and environmental safety make them promising candidates for incorporation into community-based mosquito control programmes and integrated vector management strategies. Although the laboratory results are encouraging, further investigations are required before these plant-derived repellents can be recommended for operational use. Future studies should evaluate their efficacy under semi-field and field conditions, identify and characterize the major bioactive constituents responsible for repellency, investigate possible synergistic interactions among individual compounds, and develop stable formulations such as lotions, creams, sprays, and mosquito-repellent candles. In addition, comprehensive toxicological, dermatological, and residual efficacy assessments will be essential to establish their safety, effectiveness, and long-term applicability for mosquito vector control.

CONCLUSION

The essential oils of the indigenous plant species evaluated in this study demonstrated significant mosquito repellent activity against *Anopheles arabiensis*, *Aedes aegypti*, and *Anopheles stephensi*. Qualitative phytochemical screening of the *n*-hexane crude extracts further confirmed the presence of alkaloids, various classes of glycosides, saponins, tannins, sterols, flavonoids, and terpenoids. Thin-layer chromatography (TLC) analysis of the test plant extracts, examined under UV light at wavelengths of 254 and 366 nm, revealed distinct phytochemical profiles, indicating the presence and relative distribution of bioactive constituents. These bioactive compounds, detected across the five indigenous plant species by chemical and chromatographic methods, are likely to contribute to their observed mosquito repellent activity. Overall, the findings highlight the potential of these locally available plant species as environmentally friendly, sustainable, and cost-effective botanical repellents for personal protection and integrated mosquito vector management. Nevertheless, further research is required to validate their efficacy under semi-field and field conditions, optimize formulations for improved stability and effectiveness, evaluate their safety for human use and non-target organisms, and identify the specific active compounds responsible for the repellent activity. Such studies will facilitate the development and practical application of these botanical repellents in mosquito-borne disease prevention and control programs.

Abbreviations

- ANOVA Analysis of variance
- DEET N, N-diethyl-meta-toluamide
- DEM N, N-diethyl mendelic acid amide
- DMP dimethyl phthalate
- SPSS Statistical Package for the Social Sciences
- LC Lethal concentration

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Authors' Contributions

N.G. designed the research, conducted data collection and analysis, and wrote both the initial and final drafts of the manuscript. S.D. conceived the idea, designed the research, secured funding, and participated in the writing process. K.T. designed the research, acquired funding, supervised data collection, confirmed plant identification, and contributed to the manuscript write up, reviewing, editing and submission. A.G. took part in the research design, contributed to the writing, and reviewed the manuscript. E.A. was involved in the research design and manuscript review. D.B. and B.K. participated in the planning and execution of the research. All authors read and approved the final manuscript.

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Availability of Data and Materials

The datasets used and/or analyzed during the current study may be obtained from the corresponding author on reasonable request.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics approval and consent to participate

The field data collection was conducted after receiving ethical clearance certificate from Institutional Research Ethics Review Committee (ALIPB-IRERC) at Aklilu Lemma Institute of Health Research, Addis Ababa University, with reference number: ALIPB-IRERC/140/2016/2024) and permission certificate from Oromia Health Research Ethical Review Committee, Oromia Health Bureau, Oromia Regional State, Addis Ababa, Ethiopia with permission reference number (BFO/HQ/007/16). A few brief statements about the research for

the first was given and followed by a statement similar to the one in bold below. The participant was given gave oral consent, a witness was signed. A researcher was signed also the informed consent on each consent form.

A few brief statements of the informed consent are as follows:

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.

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