Insecticidal Effect of Ethnobotanical Plant Extracts Against *Anopheles Arabiensis* (*Diptera: Culicidae*) Under Laboratory Condition

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Abstract

Background: The emergence and spread of resistant strains of malaria vectors to chemical insecticides are becoming major problem for malaria vector management. Natural plant products play a vital role to resolve the current challenge of malaria control.

Objective: The current study was conducted to evaluate insecticidal effect of ethnobotanical plant extracts against the primary malaria vector, *Anopheles arabiensis* in Northwestern Ethiopia.

Methods: Primarily, ethnobotanical plants used for Anopheles mosquito control was surveyed in Dangur district, Northwestern Ethiopia. Insecticide susceptible strains of *Anopheles arabiensis* mosquito were reared in insectary of tropical and infectious diseases research center, Assosa university. The larvicidal and adulticidal potentials of frequently used plant extracts against susceptible strains of laboratory colony were evaluated.

Result: A total of fifteen plants were identified as ethnobotanical plants helping the local people for mosquito control. *Azadirachta indica, Ocimum lamiifolium, Ocimum americanum, Moringa olifiera* leaf, and *Moringa olifiera* seed species of local plants were found to be frequently used to kill and/or repel mosquitoes in the study district. All the plant extracts were found to have potential larvicidal activity against 4^{th} instar larvae of *An. arabiensis* and only ethanol and methanol extract of *A. indica* and *O. lamiifolium* were found to have potential adulticidal effect against adult of *An. arabiensis*. The highest larvicidal activity was observed in ethanol extract of *A. indica* with 95% larval mortality and lowest LC50 of 40.73 ppm and LC90 of 186.66 ppm. The highest adulticidal activity was observed in methanol extract of *A. indica* with 75% adult mortality at 300 ppm and lowest LC50 of 106.65ppm and LC90 of 1293ppm. The lowest larvicidal and adulticidal activity was observed in methanol extracts of *O. lamiifolium* with 63.35% larval mortality and leaf extract of *M. olifiera* with 50% adult mortality at 300 ppm, respectively.

Conclusion: ethanol extract of *A. indica* exerted a remarkable larvicidal effect against *An. arabiensis* and thus it can be used for botanical mosquito insecticide development.

Introduction

Anopheles arabiensis is the principal malaria vector in Ethiopia having a wide distribution while *An. pharoensis, An. funestus*, *An. nili*, and *An. stephensi* have secondary role in malaria transmission in the country [1, 2, 3]. Vector control is a crucial prevention tool to mitigate the diseases. Long-lasting insecticidal nets (LLINs) and Indoor Residual Spray (IRS) are among the most effective malaria vector management strategies recommended in Africa. However, the wide spread of insecticide resistant strains of Anopheles mosquito is challenging the chemical insecticides-based malaria control strategies. Insecticide susceptibility tests carried out in Ethiopia have shown different levels of resistance by the principal vector to insecticides in use for IRS and/or to treat nets [4, 5]. For these reasons, looking for alternative option is becoming major interest of scientists and policy makers working in the area.

Traditionally or culturally, different communities use different plants in various forms to protect themselves against mosquitoes and other insect bites [6, 7]. Naturally occurring compounds and their derivatives are of increasing interest for the development of new insecticidal compounds against malaria vectors. Plants possess [illegible]at are selective, biodegradable, and have minor or no adverse

effects on non-target organisms and the environment [8]. Reports indicated that the essential oils and extract of local plants have a promising larvicidal, adulticidal, and repellent activities against malaria vectors [9, 10]. Gathering information about ethnobotanical plants used in particular society and evaluating their efficacy is important. Therefore, the main objective of the present study was to investigate the insecticidal effect of ethnobotanical plant extracts against *An. arabiensis* under laboratory condition.

Materials And Methods

Descriptions of the Study Area

Ethnobotanical collection was conducted in in three purposively selected kebeles namely; Manbuk 01, Kitil, and Adipopo of Dangur district, Northwestern Ethiopia. Dandur district is situated 687 km away from the capital city of the country, Addis Ababa, in the Northwest Ethiopia (Fig. 1).

The district has an estimated total population of 68,653. Geographically, the district lies at latitude and longitude of 11°30'N and 35°50'E, respectively with elevation ranging from 672-2731meter above sea level. Based on the information from metrological data in 2021, the area has mean annual rainfall ranging from 700 to 1400 mm per year and mean annual temperature ranging from 26–35°C per year.

Ethnobotanical Data Collection Techniques

Semi-structured interviews were prepared for inhabitants/key informants of the three kebeles (Manbuk 01, Kitil, and Adipopo) and it was used as a guide following Martin [11]. A total of 20 key informants were identified based on the recommendations of local authorities and knowledgeable elders who were suggested by respective kebele elders. Key informants were interviewed to identify plants they use to repel and/or kill insects in general and mosquitoes in particular.

Voucher Specimen Collection and Identification

Voucher specimens of the reported plants as repellant against mosquito vectors were collected and identified with taxonomic keys [12].

Preparation of Crude Plant Extracts

Plant extraction was conducted in the microbiology laboratory, Assosa University. The leaves and seeds of the plant were washed using clean water thoroughly and air-dried under shade at room temperature for 15 days. Then, the dried materials were ground separately to powder using a grinding mill. The powdered material was macerated with ethanol and methanol using Erlenmeyer flasks and placed on an orbital shaker at 60 rpm at room temperature for 72 hours [13]. Then the plant extract was filtered through cotton and subsequently with Whatman filter paper (12.5 cm size). Filtrates were concentrated using a rotary evaporator to remove solvents from the extract. The crude extracts were then collected in small volume beakers and kept in the freezer until used mosquito efficiency tests.

Mosquito Rearing

Larvae of *Anopheles arabiensis* were obtained from Tropical and Infectious Diseases Research Center insectary laboratory, Assosa University. Mosquitoes were reared using the standard procedures. They were maintained at

25 ± 2°C temperature and 80 ± 10% relative humidity and 12:12 light and dark photoperiod. larvae were fed with yeast a 3:1. When Pupae were formed no food was supplied and transferred to the cup that contains water by disposable pipettes and put in screened cages for adult emergence. The adults were fed on 10% glucose solution soaked in cotton pads, in addition to an animal (rabbit with shaved back and belly) blood meal given to the females twice per week. A petri dish lined with a moist cotton piece and covered with filter paper was put inside each cage for eggs lying [14]. By doing so, 4th instar larvae and 2–5 day of adults were continuously available for different bioassay tests.

Larvicidal Activity

Preparation of test and control solutions

200mg of the dried crude ethanol and methanol extract of each plant were placed in a standard measuring flask and distilled water to prepare 20mL of 1% stock solution. 0.1mL of Tween 80 was used as an emulsifier. From the 1% stock solution, concentrations of 50, 100, 150, 200, 250, and 300 ppm were prepared by adding the appropriate volume of dilution. 0.1mL of Tween 80 was made up to 100 mL distilled water to serve as the negative control solution [14].

Larvae bio-assay with crude leaf extract

In the first phase of bio-assay, the mosquito activities of frequently used plants ethanol and methanol of crude extracts of the leaves and seed were screened at 300 ppm concentration. Batches of 20 4th instar larvae of *An. arabiensis* were transferred using a dropper to 200 mL glass beakers each containing 100 mL of water and one batch as a negative control for each concentration. Each test was run three times. The test containers were held at 25 ± 2°C and 80 ± 10% relative humidity with a photoperiod of 12 hours light followed by 12 hours dark. Larvae mortality was recorded after 24 hours of exposure in each concentration of test solutions [14].

Determination of LC50 and LC90 of the crude leaf and seed extract of test plants

Based on the preliminary screening, ethanol and methanol of the leaf and seed of plant extract were selected and subjected for dose-response bioassay at the concentrations of 50–300 ppm. The average mortality after 24 hours was recorded and used to determine LC50 and LC90 values [14].

Adulticidal Activity

Adult bio-assay with crude leaf extract

The triplicate series contained twenty females of *An. arabiensis* in each tube. In the first phase of bio-assay, the mosquito activities of ethanol and methanol crude plant extracts were screened at 300 ppm concentration. This concentration was impregnated into filter papers (12 × 15 cm). Only distilled water is used as a negative control. The impregnated papers were air-dried for 5 minutes and then inserted into an exposure tube in the WHO testing kit. Twenty 2–5 days of old blood-starved female mosquitoes were introduced into the holding tube and held for 1 hour to acclimatize. The mosquitoes were then transferred by gentle blowing in the exposure tube. After 1 hour in the exposure tube, mosquitoes were then transferred back to the holding tube to recover. A pad of cotton soaked with 10% glucose solution was placed on the mesh screen to feed recovering experimental and control

mosquitoes. At the end of the 24 hours recovery period, Mosquito mortality was recorded and the percentage mortality was calculated [15].

Then Percentage of mosquito mortality was calculated by using the formula:

$$\text{percentageofmortality} = \frac{\text{Numberofdeadmosquitoes}}{\text{numberofmosquitoestested}} * 100$$

Determination of LC50 and LC90 of the crude leaf extracts of test plants

Based on the preliminary screening ethanol and methanol leaf of plant extract were selected and subjected for dose-response bioassays at the concentrations of 50–300 ppm. The average mortality after 24 hours was recorded and used to determine LC50 and LC90 values [14].

Phytochemical screening of methanol and ethanol of crude extracts of test plants

Crude methanol and ethanol extracts of all test plants were subjected to test the presence of major secondary metabolites (alkaloids, flavonoids, terpenoids, tannin, saponin, and phenols following the procedures described by Madike *et al*., [16] and Bandiola *et al*., [17].

Data Analysis

Data from all replications were pooled and mean percent mortalities of larva and adult mosquitoes that are treated with crude leaf extract of the plants were determined by analysis of variance (ANOVA) using SPSS version 20. Fishers Least Significant Difference (LSD) was used to investigate statistical significance between the different test plants against mosquito mortality. The difference between means was considered statistically significant at $P < 0.05$. LC50% and LC90% and other statistics at 95% fiducial limit of lower confidence limit and upper confidence limit and Chi-square values were determined using dosage mortality probit regression analysis of SPSS program version 20 to determine their larvicidal and adulticidal efficacies [14].

Results

Ethnobotanical Plant Species that used to repel Mosquito in the Study Area

Fifteen local plant species used to repel mosquitoes were reported from the study area and identified (Table 1). From fifteen local plant species, the most frequently used plant was *Azadirachta indica* A. (75%), *Ocimum lamiifolium* Hochst ex. Benth. (65%), *Ocimum americanum* L. (70%), leaf of *Moringa olifiera* L. (50%), and seeds of *Moringa olifiera* L. (50%) and selected to test the larvicidal and adulticidal effect of these plant extract against *An. arabiensis.*

Table 1
Plant species collected from the three kebeles of Dangur district

Scientific Name	Vernacular Name	Family Name	Plant type	Part used and methods of application	Usage frequency (%)
Allium sativum L.	Nech Shenkurt (A)	Alliaceae	Herbs	Crushing the 3–5 bulb and applying the juice to the body	5%
Azadirachta indica A.	Meem(A)Lemima(G)	Meliaceae	Tree	10–15 Leaves put in the house	90%
Carica papaya L.	Papayaa(A)	Caricacae	Tree	5 leaves crushing and apply the juice to the exposed parts of the body.	10%
Citrus sinensis L.	Birtukan(A)	Rutaceae	Tree	The fruit peeled and burning to generate smoke	15%
Crotonmacrostachyus Hochst. Ex Del.	Bissana(A)	Euphorbiaceae	Tree	Burning the dried 8–10 leaves to generate smoke.	5%
Cymbopogon citratus Stapf.	Tej sar(A)	Poaceae	Herb	Burning 10 of the leaf plant to generate smoke	10%
Echinops keberich Mesfn.	Kebericho(A) Asitana(G)	Asteraceae	Herb	3 Dried root parts and burned to generate smoke	25%
Eucalyptus globulus L.	Nech bahirzaf (A	Myrtaceae	Tree	Burning the whole plant to generate smoke	10%

Scientific Name	Vernacular Name	Family Name	Plant type	Part used and methods of application	Usage frequency (%)
Eucalyptus camaldulensis Dehn.	Key beharzaf (A)	Myrtaceae	Tree	Burning the whole plant to generate smoke	10%
Moringa olifiera L. leaf	Sheferaw(A) Chehwie(G)	Moringaceaea	Tree	15–20 Leaves put in the house	50%
Moringa olifiera L. seed	Sheferaw(A) Chehwie(G)	Moringaceaea	Tree	20–30 Seeds crushed and rubbed on the skin	50%
Ocimum lamiifolium Hochst, ex Benth.	Damakasse(A)Akawaya(G)	Lamiaceae	Shrub	About 5 leaves of the juice applied to the skin	65%
Ocimum americanum L.	Yezenjero Zekakebie(A)Omasiya(G)	Lamiaceae	Shrub	About 5 leaves of the juice applied to the skin	70%
Olea europaea L.	Woira(A)	Oleaceae	Tree	3 stem parts of the plant burned to generate smoke	20%
Otostegia integrifolia Benth.	Tnjut(A)	Lamiaceae	Shrub	Dried 10 leaf and smoke	25%
Ruta chalepensis L.	Tena Adam (A)	Rutaceae	Herb	Thermal expulsion and direct burning of seeds	20%
* A- Amharic, G- Gumzegna					

Larvicidal Activity of Crude Leaf and Seed Extracts of the Test Plants

The highest mortality was recorded in ethanol leaf extract of *A. indica* (95%). However, the methanol leaf extract of *O. lamiifolium* recorded the lowest activity with mortality of 63.35% (Table 2).

Table 2
Larvicidal activity of ethanol and methanol crude leaf and seed plant extract of test plants against 4th instar of larvae of *An. arabiensis* at 300ppm

% Mean mortality ± SE		
Plant species	Solvent	
	Ethanol	Methanol
Azadirachta indica	95 ± 0.577^{Aa}	65 ± 1.528^{Ba}
Ocimum lamiifolium	90 ± 0.577^{Aab}	63.35 ± 3.480^{Bab}
Ocimum Americanum	88.25 ± 0.333^{Aabc}	76.65 ± 0.667^{Babc}
Moringa olifiera leaf	86.65 ± 0.667^{Abc}	91.65 ± 0.882^{Ac}
Moringa olifiera seed	83.35 ± 0.333^{Abc}	90 ± 0.557^{Ac}
Negative Control	0.00 ± 0.000^{Ad}	0.00 ± 0.000^{Ad}
* Each value (% mean ± SE) represents mean value of three replicates.		
* Means followed by the same letters within the same row (Upper case) and within the same column (Lower case) are not significantly different ($p > 0.05$)		

There was no statistically significant ($p > 0.05$) difference in the larvicidal potential between ethanol and methanol extract of *M. olifiera* leaf and *M. olifiera* seed. However, there was a significant difference ($p < 0.05$) in the larvicidal efficacy between ethanol and methanol leaf extracts of *A. indica, O. lamiifolium*, and *O. americanum.*

Determination of LC50 and LC90 of Leaf and Seed Crude Plant Extract

Ethanol leaf extract of *A. indica, O. lamiifolium, O. americanum*, and methanol extract of *M. olifiera* leaf and *M. olifiera* seed were subjected (Table 3). Ethanol leaf extract of *A. indica* caused the highest larvicidal activities against the 4th instar larvae of *An. arabiensis* with the lowest LC50 of 40.73 ppm and LC90 of 186.66 ppm. Ethanol crude leaf extract of *O. americanum* relatively showed the highest LC50 and LC90 (LC50 = 92.42 ppm, LC90 = 551 ppm)

Table 3
LC50 and LC90 of test plant extract against larvae of *An. arabiensis.*

Solvent	Plant name	LC 50	LCL	UCL	LC90	LCL	UCL	X^2 (df^b=4)
Ethanol	*Azadirachta indica*	40.73	10.38	65.31	186.66	131.97	378.50	0.339
	Ocimum lamiifolium	40.93	5.35	69.23	230	170.5	944.18	0.744
	Ocimum americanum	92.42	45	129.22	551	318	3092	1.419
Methanol	*Moringa olifiera* leaf	45.43	11.93	72	260	159	558	0.390
	Moringa olifiera seed	62.35	19.1	94.01	388	239.75	1792.11	2.831

*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, X^2 –chi-square, df- degree of freedom

Adulticidal Activity of Crude Leaf and Seed Extracts of the Test Plants

The highest mortality was recorded in methanol leaf extract of *A. indica (*75%) (Table 4). However, the ethanol and methanol leaf extract of *M. olifiera* lowest mortality with 50% (Table 4). No adult mortality was observed in the negative control during the experiment.

Table 4
Adulticidal activity of ethanol and methanol crude plant extract of test plants against adult of *An. arabiensis* at 300ppm

% Mean mortality ± SE		
Plant species	**Solvent**	
	Ethanol	**Methanol**
Azadirachta indica	71.65 ± 0.333^{Aa}	75 ± 0.577^{Aa}
Ocimum lamiifolium	70 ± 0.577^{Aa}	65 ± 1.201^{Aa}
Ocimum americanum	55 ± 0.577^{Ab}	51.5 ± 0.333^{Ab}
Moringa olifiera leaf	50 ± 0.577^{Ab}	50 ± 0.577^{Ab}
Moringa olifiera seed	55 ± 0.881^{Ab}	53.3 ± 1.4529^{Ab}
Negative Control	0.00 ± 0.000^{Ac}	0.00 ± 0.000^{Ac}
* Each value (% mean ± SE) represents mean value of three replicates.		
* Means followed by the same letters within the same row (Upper case) and within the same column (Lower case) are not significantly different ($p > 0.05$)		

There was no statistically significant ($p > 0.05$) difference in the adulticidal potential between ethanol and methanol extract of all test plants. However, there was a significant difference ($p < 0.05$) in the adulticidal efficacy between ethanol leaf extracts of *A. indica and O. americanum* and

Determination of LC50 and LC90 of Crude Leaf Plant Extracts

Methanol leaf extract of *A. indica* caused the lowest LC50 of 106.655ppm and LC90 of 1293ppm and Ethanol crude leaf extract of *A. indica* showed the highest LC50 and LC90 (LC50 = 151.033ppm, LC90 = 1059 ppm) (Table 5).

Table 5
LC50 and LC90 of test plant leaf extracts against adult *An. arabiensis*

Solvent	Plant name	LC50	LCL	UCL	LC90	LCL	UCL	$X^2(df^b=4)$
Ethanol	*Azadirachta indica*	151.033	96.794	232.69	1059	486.47	20498.55	0.360
	Ocimum Lamiifolium	125.48	50.472	206.825	1468.15	526.74	88685.0	0.471
Methanol	*Azadirachta indica*	106.655	30.232	168.537	1293	481.79	751031	1.099
	Ocimum lamiifolium	158.50	105.391	245.687	1054.1	491.37	17355.7	0.045

*LC50-Lethal concentration that kills 50% of the exposed adult, LC90-Lethal concentration that kills 90% of the exposed adult, UCL = Upper confidence limit, LCL = Lower confidence limit, X^2 –chi-square, df- degree of freedom

Phytochemical Screening of Methanol and Ethanol Crude Leaf and Seed Extracts of Test Plants

Phytochemical screening was conducted for all methanol and ethanol crude plant extract to test the presence of alkaloids, flavonoids, terpenoids, tannin, saponin, and phenols and the results were presented in Table 6.

Table 6: Phytochemical screening of methanol and ethanol crude extracts of test plants

Plant name	Solvent	Secondary metabolite					
		Alkaloids	Flavonoids	Terpinoids	Tannin	Saponin	Phenols
Azadirachta indica	Ethanol	+	+	+	+	+	+
	Methanol	+	+	+	+	+	+
Ocimum lamiifolium	Ethanol	+	–	+	+	+	+
	Methanol	+	–	+	+	+	+
Ocimum americanum	Ethanol	–	–	+	–	–	+
	Methanol	–	–	+	–	–	+
Moringa olifiera leaf	Ethanol	+	+	+	+	+	–
	Methanol	+	+	+	+	+	+
Moringa olifiera seed	Ethanol	+	+	+	–	–	–
	Methanol	+	+	+	–	–	+

+= Present, - = absent

Discussions

A total of fifteen local plant species were identified to be used for mosquito control in Dangur district. The presence of several plant species to be used for mosquito control by the local people is a good indication of the deep-rooted culture of local plants used in the study area. This result is similar to other studies conducted in Ethiopia in two plants [18, 19]. The current result showed that the local communities had more indigenous knowledge and give emphasis to use the local plants to repel mosquitoes. Out of all local plant species used for mosquito control the most frequently used plants were *Azadirachta indica* A.(75%), *Ocimium lamiifolium* Hochst ex. Benth. (65%), *Ocimium amiricanum* L. (70%), leaf of *Moringa olifiera* L.(50%), and seeds of *Moringa olifiera* L.(50%). Some of the repellant plants recorded in current study were also reported in other parts of Ethiopia [9, 20, 21].

All the test plants were found to have potential larvicidal activities against the 4th instar larvae of *An. arabiensis* at the test concentration. The highest mortality was recorded in ethanol leaf extract of *A. indica*, the methanol leaf extract of *M. olifiera*, the ethanol leaf extract of *O. lamiifolium*, and methanol seed extract *M. olifiera* with mortality of 95%, 91.65%, 90%, and 90%, respectively. However, the methanol leaf extract of *O. lamiifolium, A. indica*, *O. americanum* followed by ethanol seed extract of *M. olifiera*, the leaf extract of *M. olifiera*, and leaf extract of *O. americanum* recorded the lowest activity with mortality of 63.35%, 65%, 76.65%, 83.35%,86.65%, and 88.25%, respectively. The toxicity difference among test plants and extraction solvent suggested that different plants have different phytochemicals which can be extracted by different solvents.

High larval mortality was caused by ethanol leaf extract of *A. indica* with mortality 95%. This finding is similar to the report of Okumu *et al*. [22]. where hexane leaf extract of *A. indica* was found to cause 100 % mortality against larvae of *An. gambiae* at 1000 ppm although the concentrations are different. This could be due to the presence of excess bioactive secondary metabolites.

However, this finding disagrees with finding of Vilayatakar *et al*. [23] that reported the aqueous extract of *M. oleifera* leaves against larvae of *Anopheles* which has shown 60% mortality at 500ppm. Similarly, acetone extract of *O. lamiifolium* oil was found to have high larvicidal activity against *An. arabiensis* [24]. Various factors might be responsible for the larvicidal activity, but the difference in larvicidal activities in the current finding could be due to locality of the plants, and different solvents.

Methanol leaf extract of *A. indica cause* adult mortality with mortality 75% which could be due to the presence of bioactive secondary metabolites. This finding is in line with the earlier finding of Kamaraj *et al.* [25]. who reported adulticidal efficacy of methanol against *Cx. gelidus Theobald*.

Ethanol leaf extract of *O. lamiifolium* and *O. americanum* were found to have 71.65 % and 55 % mortality respectively at 300 ppm against adults of *An. arabiensis* after 24 hours' exposure. This result is lower compared to the finding of Messebo *et al*. [26]. where ethanol leaf extract of *O. lamiifolium* showed 90 % mortality against the adult *An. arabiensis* after 1 hour exposure 0.25(v/v) in other part of Ethiopia. The difference in adulticidal activities with the current finding could be due to species of plants, extraction solvent, and locality of the plants.

Conclusions

Ethnobotanical plants are widely used in Dangur district, northwestern Ethiopia. The *Azadirachta indica, Ocimum lamiifolium, Ocimum americanum, Moringa olifiera* leaf, and *Moringa olifiera* are plant species frequently used for mosquito prevention in Northwest Ethiopia. They have effective larvicidal potential against 4th instar larvae of *Anopheles arabiensis.* Ethanol leaf extract of *A. indica* has higher larvicidal potential and promising for further botanical insecticide developments. Ethanol and methanol extract of *A. indica* and *O. lamiifolium* have potential adulticidal activity with mortality > 60% mortality against adult of *An. arabiensis*. The phytochemical analysis of leaf extract of *A. indica* has secondary metabolites such as alkaloid, flavonoid, terpenoid, tannin, saponin, and phenols which have potential insecticidal activities. Finally, we recommend further study to identify the active ingredient of ethanol and methanol extracts of *A. indica* and their mode of action in the study area and other parts of Ethiopia.

Declarations

Ethical approval and consent to participant:

not applicable

Consent for publication:

not applicable

Availability of data and materials:

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests:

The authors declare that they have no competing interests"

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Authors' contributions

AA conducted the laboratory work and interpreted the data and wrote the first draft of the manuscript. DE and AA designed the field collection protocols and oversaw the collection. DE provided a critical revision of the manuscript. All authors have read and approve the content of the submitted manuscript.

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Figures

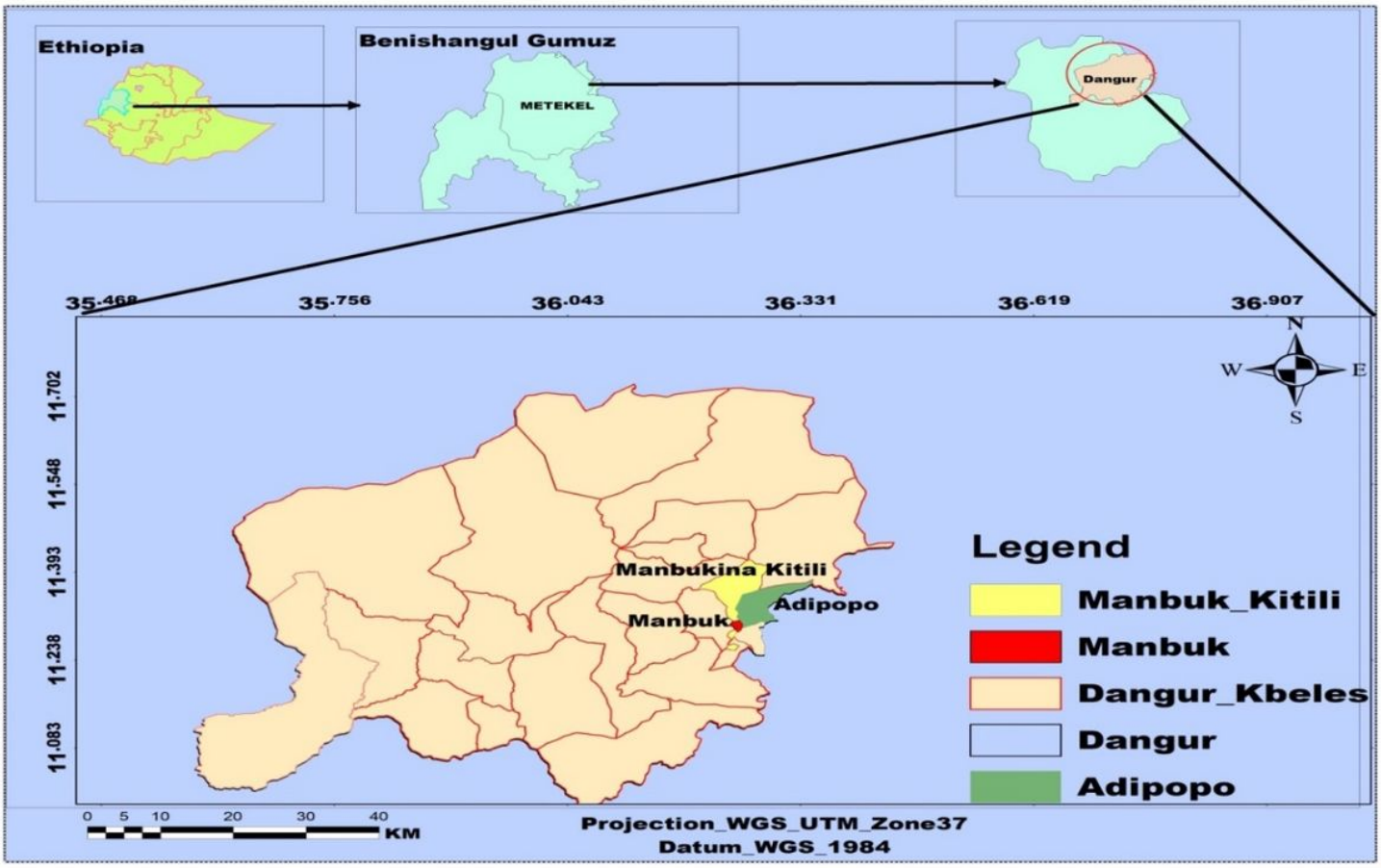

Figure 1

Map of the study area