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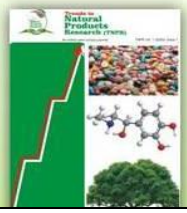

Pharmacognostic Properties and Mosquito Repellent Activity of the Leaf Extract of *Vernonia glaberrima* (Asteraceae) Welw. Ex O. Hoffm against *Aedes aegypti* Mosquito

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Abstract

The world's population is severely burdened by mosquito-transmitted diseases, which claim millions of lives annually. These diseases include malaria, yellow fever, zika virus, dengue fever, and Rift Valley fever. The purpose of this research was to assess the pharmacognostic profile and repellent properties of methanol extract of *V. glaberrima* leaf and its fractions on *Aedes aegypti* adult mosquito. The leaf was microscopically examined, and pharmacognostic evaluation was carried out using the standard method to determine the ash values, extractive values, moisture content, microscopy and phytochemical contents. The bioactive constituents of the leaf were extracted by cold maceration using methanol. The extract was further fractionated with n-hexane, ethyl acetate, and n-butanol using column chromatography. The repellent activity was carried out using WHO protocols at concentrations of 10, 50 and 100% for the various fractions of *V. glaberrima* on adult *Aedes aegypti*. *V. glaberrima* leaf exhibited amphistomatic with anomocytic stomata with stomatal index of 3.63% and 11.06% at the adaxial and abaxial leaf surfaces, respectively. The microscopic results show the presence of oil glands, starch, tannins, cellulose, gum, calcium oxalate, fibre and vessel elements. The mean moisture content of *Vernonia glaberrima* leaf was 9.33±0.57%, while the yield of the methanol extract was 34.60%. The four fractions of *V. glaberrima* leaf exhibited significant repellency activity against *Aedes aegypti* mosquitoes, with percentage repellency ranging from 27.8-98.9%. *Vernonia glaberrima* leaf showed significant repellency against *Aedes aegypti* mosquitoes and might be an effective and safe repellent for malaria control.

Keywords
Vernonia glaberrima Dengue, *Aedes aegypti*, repellent.

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Introduction

Many vector-borne diseases, including illnesses spread by mosquitoes, cause over one million deaths and over one billion infections annually. *(Talapko et al*., 2019) *Aedes aegypti* is the vector of four main arboviral diseases, including dengue fever, chikungunya, zika, and yellow fever. *Aedes aegypti* is a tiny, dark mosquito with banded legs and a white violin-shaped insignia on its back. This vector was first discovered in Africa, but later spread to other parts of the world through trade (Kraemer *et al*., 2015). Because mosquitoes are the primary vector for several serious diseases, the World Health Organisation has designated mosquitoes as "public enemy number one" (WHO, 2022). Insecticide use is one way to keep mosquitoes under control. Thus far, chemical control with synthetic pesticides has proven beneficial due to its quick action and simplicity of usage. Entomologists have extensively investigated the relative toxicity of insecticides to different types of mosquitoes (Muhammed *et al*., 2022). Synthetic insecticides are hazardous and negatively impact the ecosystem because they contaminate soil, water, and air. The growing use of these chemicals may cause irreversible harm to the liver, kidney, and other organs through the food chain. For instance, mosquito coils containing synthetic pyrethroids and other organophosphorus compounds can have several adverse effects on users, including headaches, breathing difficulties, eye irritation, asthma, itching, and sneezing (Hogarh *et al*., 2018). They are even implicated in gene mutations, which take a few generations to manifest as noticeable changes (Powell, 2022). In addition, chemical pesticides are expensive. Researchers are looking into the use of natural products to overcome these challenges of synthetic insecticides. Plant extracts and essential oils could be an alternate source of mosquito control agents due to their rich source of biodegradable bioactive compounds. Other benefits of plant extracts include eco-safety, lack of resistance development, increased acceptability, and applicability for rural locations (Chatterjee *et al*., 2023). Mosquito repellants are volatile chemicals that repel mosquitoes away from human skin, thereby preventing contact and bites (Mapossa et al., 2021). Several studies have documented how well plant extracts or essential oils work to repel mosquitoes (Lee *et al*, 2020; Husna *et al*., 2020).

There have been 109 Vernonia species reported to have therapeutic benefits (Toyang & Verpoorte, 2013). *Vernonia glaberrima* Welw. Ex.O. Hoffm. (Figure 1), belonging to the Asteraceae family is a shrub commonly found on the hillside grassland of Africa (Burkill, 1985). The folkloric uses of *Vernonia glaberrima* include the treatment of malaria, migraine, diabetes and pain (Burkill, 1985). The pharmacological activities of the plant leaf reported in the literature are anticancer, analgesic, anti-inflammatory, antimalarial, anti-diabetic and antimicrobial activities (Alhassan *et al*., 2018, 2022; Jega, 2020, Odoh *et al*., 2022). In this study, we investigated the repellency activity of *Vernonia glaberrima* leaf on adult *Aedes aegypti* mosquitoes.

Figure.1: Pictorial view of the leaves and flowers of *Vernonia glaberrima* (Alhassan *et al*., 2018).

Materials and Methods

Plant collection and identification

Fresh *Vernonia glaberrima* leaves were collected in November 2022 from the mountainous forest of Eziani Community, Nsukka Local Government Area, Enugu State. The plant was identified and authenticated by Mr. Felix Nwafor, a taxonomist at the University of Nigeria in Nsukka.

Fresh leaf Microscopy

The clearing process was used to prepare the foliar epidermis of the leaves adaxial (upper surface) and abaxial (lower surface) surfaces. Using a pair of forceps, the leaf epidermal strips were carefully scraped, put on a clean slide, dyed with Safranin solution and covered with a cover slip (Nwafor et al., 2019). A light phase contrast microscope (Motic B3, Motic Carlsbad, CA, USA) was used to observe the slides at x40, x100 and x400 magnifications. A Moticam 2.0 image system installed on the microscope with software (Motic Carlsbad, CA, USA) was used to take photomicrographs. The epidermal cell type and quantity were counted, and the stomatal complex types were identified using the methods of Evert (2006). (Evert, 2006). The

stomata's length and width were measured over ten fields of view for every sample using Motic Microscopy software. The quantity of stomata per square millimetre was used to calculate the stomatal density, and the stomatal index (S.I.) was determined as follows:

$$SI = \frac{S \times 100}{S + E}$$

Where S = number of stomata in a field of view; E = number of epidermal cells in the same field of view

The methods used to analyse the stomata were also used to determine the trichome types, sizes, densities, indices, the vein islet termination number and palisade ratio. All parameters were observed on the leaves' adaxial and abaxial surfaces (Nwafor *et al*., 2019).

The transverse section of the leaf was made using a Reichert sledge microtome following the procedures described by Nwosu (200. The sections were microtomed at 10 to 15 unimicrons, and they were carefully transferred into Petri dishes filled with 70% pure alcohol using a camel hair brush from the microtome knife's tip. Safranine and fast green dyes were used to distinguish between lignified tissues.

Extraction of plant material

The leaves were air-dried for about two weeks at room temperature and then pulverized. The powdered leaves (1.8 kg) were extracted by cold maceration using methanol (Barnes, 2010). Briefly, the powdered leaves were immersed in an adequate volume of methanol for 72 h at room temperature with intermittent shaking. The mixture was filtered using a muslin cloth and further filtered using Whatman No. 1 filter papers. The extract was then concentrated using a rotary evaporator at 40 ºC. The concentrate was further heated in a water bath at 40º C for 12 hours to concentrate the extract further. The total yield was recorded.

Fractionation of plant extract

The dried methanol crude extract was adsorbed on silica gel and sequentially fractionated with n-hexane, n-butanol, methanol, aqueous methanol and ethyl acetate using column chromatography. The fractions were concentrated with a rotary evaporator set at 40 ºC and 120 rpm, and the resultant extracts were further dried at 40 ºC in the oven. The dried extract and fractions were transferred into an airtight container and stored in a refrigerator for further analysis. The percentage yield of the crude extract and the fractions were calculated by determining the ratio of the mass of the dried crude extract obtained to the total yield after fractionation expressed as a percentage (Harborne, 1972).

$$\% \text{ yield} = \frac{\text{Total yield after fractionation}}{\text{Total yield of crude extract}} \times 100$$

Determination of Extractive Yields

The water, alcohol, chloroform and n-hexane extractive yields were determined (Odoh *et al*., 2012). Briefly, the sample was macerated in the solvent system for 24 hours and then filtered. The filtrate was dried to a constant weight dry extract in an oven at 105 ºC. After cooling the dry extracts in the desiccator, their weights were determined and recorded.

Determination of Moisture Content

The leaf sample (2 g) was transferred into a preheated porcelain crucible of known weight (W1). The weight of the crucible and its content was noted as W2. The sample was gradually heated to 105 ºC with intermittent weighing until a constant weight was obtained. The heated sample was cooled in the desiccators and weighed (W3). The moisture content was calculated using the formula:

$$\% \, Moisture \; Content = \frac{W2 - W3}{W2 - W1} \times 100$$

Where W1 is the weight of the crucible, W2 is the weight of the crucible and sample, and W3 is the weight of the sample after drying.

Determination of Ash Values

The total, water-soluble and acid-insoluble ash values of the plant were determined using standard methods (Trease and Evans, 2002).

Chemo microscopy

Using standard procedures, the pulverized leaf was evaluated for the presence of lignin, starch, tannins, gum, oil globules, cellulose, protein and calcium oxalate crystals (Evans, 2002).

Collection and Identification of Mosquito

Each investigation using insects was conducted in accordance with the widely recognized ethical principles on the handling and maintenance of insects used in the study of vector-borne disease (WHO, 2020). Adult *Aedes aegypti* mosquitoes, bred at the National Arbovirus and Vectors Control Research Centre (NAVRC) Enugu State, were obtained and used for the repellency study. Before the investigation, the mosquitoes, which had never received a blood meal, were acclimatized to typical laboratory conditions in their net-enclosed cages, which were furnished with bedding made of softwood shavings (WHO, 2020).

Ethics Approval

The study protocol was approved by the ethics committee of the Enugu State University of Science and Technology, Enugu (Protocol number ESUT/AEC/0253). Five volunteers, with written and informed consent, were enrolled. Only subjects that have the potential to be mosquito baits were included in the study (Colucci and Müller, 2018).

Mosquito Repellency Assays.

Five human subjects were employed in the study. Each of the subjects was treated with 100%, 50% and 10%, respectively, of the extract, while the other two subjects were negative (untreated) and positive control (treated with Odomos) (Luker, 2024). An area of skin ranging from that covering the entire forearm was treated with the sample. Untreated skin was covered with a glove. The treated arm was exposed to 25 caged adults *Aedes aegypti* mosquitoes for 3 mins, followed by repeated exposures every 30 minutes for 2 hours. The mean landing time and the number of mosquitoes that perched on the treated skin were recorded. The repellency was calculated as:

$$Repellency\ (\%) = \frac{(Number\ of\ bites\ on\ control) - (Number\ of\ bites\ on\ treated\ arm)}{Number\ of\ bites\ on\ control} \times 100$$

(Schreck, 1977)

Statistical analysis

The repellency was determined using Abbott's 13 formula for both repellent results (Lacroux *et al*., 2022). The percentage of repellency data was subjected to ANOVA using Statistical Package for Social Sciences (SPSS 17.0). The student-Newman-Keuls (SNK) test at p = 0.05 was used for mean separation. Probit analysis14 was applied to determine 30 minutes interval post-exposure for repellency.

Results

Microscopic Examination of Vernonia glaberrima Leaf

The leaf exhibited amphistomatic (stomata occur on both the upper and lower surfaces) with anomocytic (lack of subsidiary cells with epidermal cells directly associated with the guard cells) type of stomata (Figure 2). The stomata were more on the abaxial (lower) surface than the adaxial (upper) surface of the leaf. The epidermal cells were irregularly shaped with undulated anticlinal cell walls on both the adaxial and abaxial surfaces. Unicellular covering types of trichomes were present on the petiole. The microscopic results showed the presence of oil glands, calcium oxalate, fibre and vessel elements. The stomata indices were 3.63% and 11.06% at the adaxial and abaxial leaf surfaces, respectively (Table 1).

Yields of the Vernonia glaberrima leaf extract and fractions

The yield of the extract was 34.60% (138.46 g). The aqueous methanol fraction gave the highest yield of 12.70%, while the hexane fraction had the lowest yield of 2.35 % (Table 2).

Results of the moisture content, ash and extractive values of Vernonia glaberrima leaf

The mean moisture content of the extract was 9.33±0.57%. Water gave the highest extractive value of 23.60±2.23%, while n-hexane gave the lowest extractive value of 2.30±0.98%. Ash values of 6.63 ± 0.32%, 4.17 ± 0.29% and 0.33 ± 0.28% were obtained as the total ash, water-soluble ash and acid-insoluble ash, respectively (Table 3).

Chemo microscopic Study

Chemo microscopy study of the leaf of *V. glaberrima* revealed the presence of starch grains, lignified tissues, calcium oxalate crystals, cellulose, gums/mucilage and oil globules while proteins were absent (Table 4)

Repellency activity of the extract and its fractions against Aedes aegypti mosquitoes

The extract gave a percentage repellency of 36.8 - 53.5%. The four fractions also exhibited significant repellency activity against *Aedes aegypti* mosquitoes, with percentage repellency ranging from 27.8 – 98.9%. The highest repellency activity was obtained from 50% ethyl acetate fraction, while the least activity was obtained from 10% aqueous methanol fraction (Table 5).

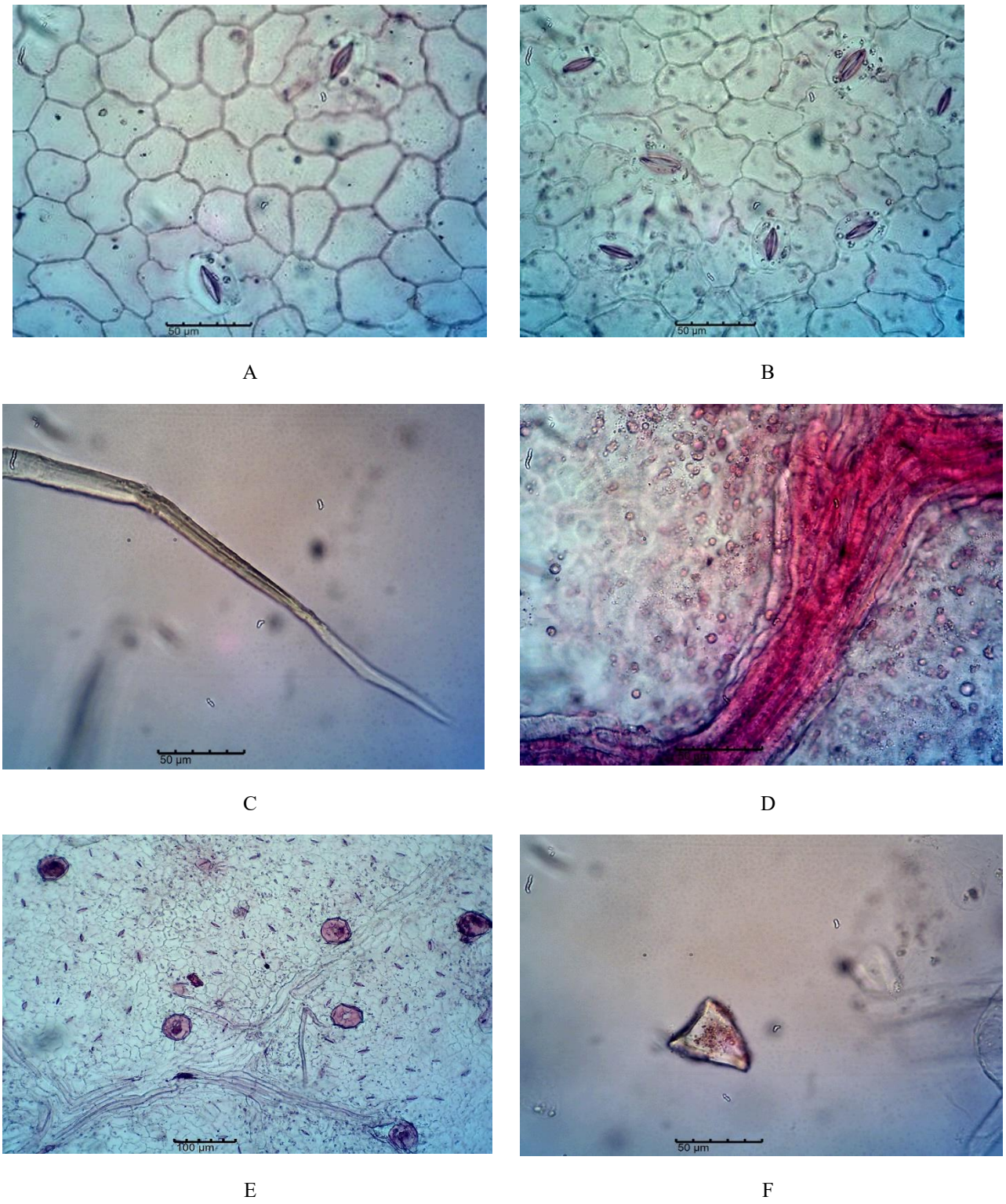

Figure 2: Photomicrographs of the *V. glaberrima* leaf (×400) showing A) Adaxial surface of the leaf with anomocytic type of stomata and undulated epidermal cell B) Abaxial surface of the leaf with anomocytic type of stomata and undulated epidermal cell C) an isolated fragmented fibre element D) bundle of lignified fibre and vessel elements E) oil glands and unicellular covering trichome F) prism-shaped calcium oxalate crystal.

Table 1: Stomatal parameters of *V. glaberrima* leaf

Parameters	Adaxial surface of the leaf	Abaxial surface of the leaf
Stomata number	2.00 ± 0.00	8.00 ± 0.41
Stomata density (mm^{-2})	11.70 ± 0.00	47.06 ± 2.40
Stomata length (µm)	40.33 ± 0.52	33.80 ± 0.80
Stomata width (µm)	28.02 ± 0.05	24.94 ± 0.74
Stomata size (µm)	1129.50 ± 12.53	844.28 ± 41.91
Stomata index (%)	3.63 ± 0.08	11.06 ± 0.46

Table 2. Yield of the fractions of the extract

Fractions	Weights (g)	Yield (%)
N-hexane	3.25	2.35
Ethyl acetate	15.45	11.16
N-butanol	11.63	8.40
Aqueous methanol	17.58	12.70

Table 3. Analytical evaluation of the powdered leaf sample

	Parameter	Mean(%)±SD
Ash values	Total ash	6.63±0.32
	Water soluble ash	4.17±0.29
	Acid insoluble ash	0.33±0.28
Extractive values	Water	23.60±2.23
	Methanol	14.67±3.23
	Chloroform	3.17±0.98
	N-Hexane	2.30±0.98

Values expressed in mean ± S.D., n = 3.

Table 4. Chemomicroscopy of the leaf of *V. glaberrima*

Parameter	Result
Starch grains	+
Lignified tissues	+
Calcium oxalates	+; Prism-shaped
Tannin	+
Cellulose	+
Gum/Mucilage	+
Protein	-
Oil globules	+

+ = present, - = absent

Table 5. Repellency activity of methanol extract and its fractions

	Sample Conc (%)	Mean Landing Time (min)	Number of Perching (Mean)	Repellency (%)
Methanol	Control (-)	45.9	11.4	0.0
Extract	10	52.3	7.2	36.8
	50	49.0	5.3	53.5
	100	53.1	6.9	39.5
	Control (+)	60.0	0.0	100.0
Ethyl acetate	Control (-)	5.5	9.4	0.0
Fraction	10	17.1	1.2	87.2
	50	2.8	0.1	98.9
	100	6.1	1.9	83.0
	Control (+)	60.0	0.0	100.0
Aqueous	Control (-)	11.0	10.8	0.0
methanol	10	10.50	7.80	27.8
fraction	50	11.40	5.40	50.0
	100	6.90	6.90	36.1
	Control (+)	60.00	0.00	100.0
N-Hexane	Control (-)	9.4.00	13.6	0.0
fraction	10	12.90	0.6	95.6
	50	9.50	0.4	97.1
	100	17.90	1.2	91.2
	Control (+)	60.00	0.0	100.0
N-butanol	Control (-)	10.00	7.50	0.0
fraction	10	2.00	0.50	93.3
	50	6.50	0.20	97.3
	100	10.80	0.30	96.0
	Control (+)	60.00	0.00	100.0

Discussion

Vernonia glaberrima is a medicinal plant commonly used for treatment of skin cancer, diabetics, pain and other ailments in Africa. We investigated the mosquito repellency activity of the leaf on *Aedes aegypti* mosquitoes. The macroscopic and microscopic examinations revealed a green, fragrant leaf with asymmetrical, undulating anticlinal cell walls on both the upper and lower surfaces. The leaf is amphistomatic (stomata occur on both the upper and lower surfaces) with anomocytic (lack of subsidiary cells with epidermal cells directly associated with the guard cells) type of stomata. The leaf had more stomata on the abaxial surface than on the adaxial surface, as shown by the stomatal indices. The microscopic results revealed the presence of oil glands, calcium oxalate, fibre and vessel elements. These characteristic features of *Vernonia glaberrima* leaf might be helpful in assessment and authentication of the plant during collection (Obinna *et al*., 2023).

The solvent used in extraction influences the nature and amount of phytochemicals extracted (Dirar et al., 2019). Water gave the highest extractive value, followed by methanol, chloroform, and n-hexane. However, the extraction of the bioactive compounds of the leaf was carried out using methanol instead of water. This was due to reports of low extraction yield of secondary metabolites when water was used as the extracting solvent. In addition, water extracts are vulnerable to microbial contamination. Thus, we chose methanol as the extracting solvent.

The powdered leaf had a low moisture content of 9%, which means that it will have minimal hydrolytic degradation in storage. The leaf also showed low total ash, acid insoluble, and water-soluble ash values. This finding means there was a negligible quantity of extraneous earth matter in the powdered leaf, such as siliceous earth matter, sand, and water-soluble salts. The negligible amount of these extraneous matters suggests that the plant sample was appropriately handled. These parameters can assist in identifying any adulteration in the plant sample (Simmler *et al*., 2018).

The antimalarial activity of the methanol extract of *V. glaberrima* leaf was previously reported by Abdullahi *et al*. (2015). However, there is no previous report on the plant's mosquito repellency activity. In this study, the repellency of the methanol extract and different fractions of *V. glaberrima* leaf extract were assessed by comparing the number of bites on the treated arm against the untreated arm. The four fractions exhibited significant repellency activity against *Aedes aegypti* mosquitoes, with percentage repellency ranging from 27.8 – 98.9%. The highest repellency activity was obtained from 50% ethyl acetate fraction, while the least was obtained from 10% aqueous methanol fraction. No significant difference exists in the repellency activity exhibited by ethyl acetate, n-hexane and n-butanol fractions of *V. glaberrima* leaf.

Conclusion

In this study, *Vernonia glaberrima* leaf has been shown to have significant repellency activity against *Aedes aegypti* mosquitoes and might be an effective and safe repellent in malaria control.

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