Research Paper

Characterization and Larvicidal Potency of Crude *Urtica massaica* Extracts Against *Anopheles gambiae* (Diptera: Culicidae)

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ABSTRACT

Background and Purpose: Plants contain complex bioactive compounds with insecticide properties. The present study was designed to characterize and determine the larvicidal potency of crude methanol and hexane extracts of *Urtica massaica* against laboratory-cultured *Anopheles gambiae* larvae.

Materials and Methods: This research was a laboratory-based experiment. Bioactive compounds were extracted using methanol and hexane, while larvae for bioassay were laboratory stock. The effectiveness of the extracts was evaluated using the World Health Organisation (WHO) >80% mortality threshold and methods, respectively. The extract dose was taken as the independent variable, while observed mortalities were the dependent variable. Dimethyl sulfoxide and distilled water were used as negative controls. Descriptive statistics and one-way analysis of variance were performed for statistical justifications of bioactive quantities and insecticidal properties of the extracts. Significant level was set at $P<0.05$.

Results: Five groups of chemical compounds were extracted: Phenolic > amino acids and nitrogen derivatives>steroids and terpenoids > flavonoids, nitrogen derivatives, and others. Nineteen compounds were from roots, 17 from leaves, and 12 from stems. β-Sitosterol, 3,4-diferuloylquinic, and catechin were more while benzoic acid, L-leucine, N-(m-anisoyl)-, methyl ester, and myricetin were the least abundant in leaves, stem, and roots, respectively. Extracts from the stem killed more than 80% of exposed larvae for doses of 10 mL/100 cm^3 (e/w) than those of the other parts (root and leaves). The observed mortalities, however, were not significantly different ($P>0.05$), irrespective of the dose or control used.

Conclusion: *U. massaica* contains diverse bioactive compounds potent against *A. gambiae* larvae.

Keywords: *Urtica massaica*, *Anopheles gambiae*, Larvae, Crude extracts, Ethanol, Hexane

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Introduction

Plant secondary metabolites or bioactive compounds are products secreted by plants for defense against plant natural enemies. The products serve as repellents, antifeedants, growth and mating inhibitors, anti-moulting, and insecticides [1, 2] against these enemies. The products, however, are environmentally friendly [3] and serve as food and preservatives [4]. The bioactive compounds are diverse with different modes of action. This property gives them great potential for novel biological products [5] and makes it challenging for diseases and pests to develop resistance against them [6]. The tragedy is that most products remain unexplored and serve merely as repository sources [7].

Urtica massaica Mildbr (hereafter *U. massaica*) is a perennial herb [8] belonging to the Urticaceae family [9] and, although considered a weed, is among the most used wild plant species in the world [10, 11]. It is rich in proteins [12], vitamins [13], and polyphenols [14], and it has pharmaceutical [15], antimicrobial [16], and fungicidal [17] potential. It has, therefore, been used as vegetables with high potential to manage food insecurity [18-19] and as an herb to treat various microorganism-caused infections [20-23], stomach ulcers, hypertension, nerve disorders, diabetes, and rheumatism [4, 24].

Though the toxic effect of the extracts has been reported on immature *Anopheles gambiae* (hereafter *A. gambiae*) [25], the chemical profile and toxicity have not been studied. We, therefore, report herein on the characterization and potency of crude methanol and hexane extracts of *U. massaica* against laboratory-cultured *A. gambiae* larvae under controlled conditions.

Materials and Methods

Study site, sourcing for experimental mosquitoes and study design

This research was a laboratory-based experimental bioassay using crude *U. massaica* extracts on *A. gambiae* larvae. We extracted and characterized chemical constituents from parts of *U. massaica* using methanol and hexane in the University of Eldoret's chemistry laboratories. Third larval instars (L3s) of *A. gambiae* mosquitoes were obtained from a laboratory stock at the Centre for Global Health Research/Kenya Medical Research Institute (CGHR/KEMRI), Kisian, Kisumu. Since the effect of treatment meted on the larvae was measured only after the bioassay, an informal 'after-only with control' experimental design [26] was used to determine the larvicidal effect of crude methanol and hexane extracts of *U. massaica* on the larvae.

Plant materials

Fresh leaves, stems, and roots of *U. massaica* (stinging nettle) were sourced from 350 16' 46" E, 00 31' 41" N in Eldoret. The plant was identified, and a voucher specimen number JOY2021/001 was issued and later deposited at the School of Biological Sciences, University of Nairobi, herbarium.

Methanol and hexane extracts of *U. massaica*

Two hundred grams of ground leaves of *U. massaica* were soaked in 400 mL of absolute methanol for 1 hour. The suspension was then filtered using Whitman's No. 1 filter paper, and the filtrates were freeze-dried using the Edwards Modulyo freeze-drying machine. The result is a paste taken as stock material [27]. This procedure was repeated for ground stem and roots and hexane solvent.

Gas chromatography-mass spectrometry (GC-MS) analysis

Sample preparation

This procedure was done as described elsewhere [28]. Briefly, 1 mg of each crude extract was weighed (in triplicates) and dissolved in 1 mL dichloromethane. The samples were vortexed for 10 s, ultra-sonicated for 1 hour, and centrifuged at 14000 rpm. The supernatant was dried using anhydrous Na_2SO_4 and centrifuged at 14000 rpm before GC-MS analysis.

Instrument conditions

This activity was conducted as described elsewhere [29]. Briefly, the samples were analyzed using a 7890A gas chromatograph connected to a 5975C mass selective detector. A temperature of 270 ˚C was set as the inlet and 280 ˚C as the transfer line. The oven temperature was programmed at between 35 ˚C and 285 ˚C, with the initial temperature being maintained for 5 minutes, adjusted to 10 ˚C/minute, and progressively brought up to and held at 280 ˚C for half an hour. A low bleed capillary column (HP-5 MS, 30 m × 0.25 mm i.d., 0.25 µm) and helium were employed as the carrier gas with a 1.25 mL/min flow rate. The detector's ion source and quadrupole temperatures were maintained at 230 ˚C and 180 ˚C, respectively. Electron impact of mass spectra was obtained at 70 eV. About 1.0 µL and the analyte injected in split/splitless mode and fragmented ions analyzed, in full scan mode, over 40 to 550 m/z range. The filament

delay time for the analytes was set at 3.3 min. The compounds were identified by comparing their fragmentation patterns with reference spectra from Library–MS databases. This process also included the National Institute of Standards and Technology (NIST) 11, 08, and 05, Adams and Chemical mass spectral databases.

Liquid chromatography with tandem mass spectrometry analysis

The analysis was conducted as described elsewhere [30]. Three replicates of 1 mg of each crude methanolic extract were weighed and constituted in 1 mL methanol. The samples were then vortexed for 10 s ultrasonicated for 1 h before centrifugation at 14000 rpm. Thereafter, the supernatant was filtered and analyzed qualitative tandem liquid chromatography quadrupole time of flight mass spectrometry (MeOH) under the following conditions: Ultra-performance liquid chromatography (UPLC) (Waters ACQUITY I-Class system); UPLC column (Waters ACQUITY UPLC BEH C18 column, 2.1×50 mm, 1.7-µm particle size Waters Corporation, Dublin, Ireland); Column temperature of 25 ˚C; mobile phase of water (A) and methanol (B), each with 0.01% formic acid; flow rate of 0.3 mL/min, gradient from 95% A to 100% B and back to starting solvent proportion; with the run time being 25 min. A positive Q-tof ion mode with a nitrogen desolvation flow rate of 500 L/h and an accuracy of <5 ppm was used. The quantitative analysis of the secondary metabolites present was based on a standard curve of apigenin (y=10288x–11117; R^2=0.999).

Larvicidal bioassay

Preparation of serial dilutions

The dilutions were prepared according to Vloemans et al. [31]. Briefly, 1 g of crude methanol stock's extracts of *U. massaica* leaves were weighed and dissolved in 1% of dimethyl sulfoxide (100 mL) and then serially diluted. Also, 80 mL of this solution was then topped up with 20 mL of distilled water to make 100 mL. The mixture was then serially diluted to 40, 20, 10, 5, and 2.5 mL/100 cm^3 (e/w) of distilled water.

Larviciding

One hundred newly transformed L3s were placed in 3 plastic containers, each measuring 6×5.7×3.5 cm, using a plastic pipette. Two sets of the containers held 33, while the third had 34 larvae each. All the containers contained similar doses of a particular treatment. Each container held approximately 33 mL of a particular dose. Doses were either 80, 40, 20, 10, 5, or 2.5 mL/100 mL (e/w). The larvae were left exposed overnight, after which the experiment was stopped, and the number of dead or live individuals was noted and recorded. All larvae (live, moribund, and dead) were then put in a pail of hot water and dispensed in a septic tank. The experiment was replicated four times. Larvicidal activities were tested following the World Health Organization (WHO) procedure [32] and standards [33] for insecticidal effectiveness. Larval mortality was calculated using the Equation 1:

1.

$$\% \text{ Larval mortality} = \frac{\text{Number of dead larvae}}{\text{Total number of exposed larvae}} \times 100$$

Furthermore, observed larval mortality of between 5% and 20% was corrected using Abbot's formula [34] (Equation 2):

2.

$$\% \text{ Corrected mortality} = \frac{\text{Percent mortality in test-Percent mortality in contro}}{\text{100-Percent in control}} \times 100$$

The extract dose was taken as the independent variable, while observed mortalities were the dependent variable. Dimethyl sulfoxide and distilled water were used as negative controls. The temperature and humidity regime at the laboratory was maintained at 28 – 30 ˚C and 70% - 80%, respectively, and a photoperiod at 12 h light (06.30 – 18.30 hours) alternated with 12 h darkness (18:30 – 06:30 hours).

Statistical analysis

Data on characterization and bioassay on the effect of the crude extracts of *U. massaica* on larvae of *A. gambiae* were entered in Excel spreadsheets and appropriately organized for processing. Descriptive statistics were used to determine the quantity of bioactive compounds and the effect of the solvent of extraction, dose, and part of the plant used on exposed larvae. A one-way analysis of variance was used to determine the significance of the impact of extract as a larvicide. All statistical analysis was performed using IBM SPSS statistics software for Windows, version 22 (IBM Corp, Armonk, NY, 2013).

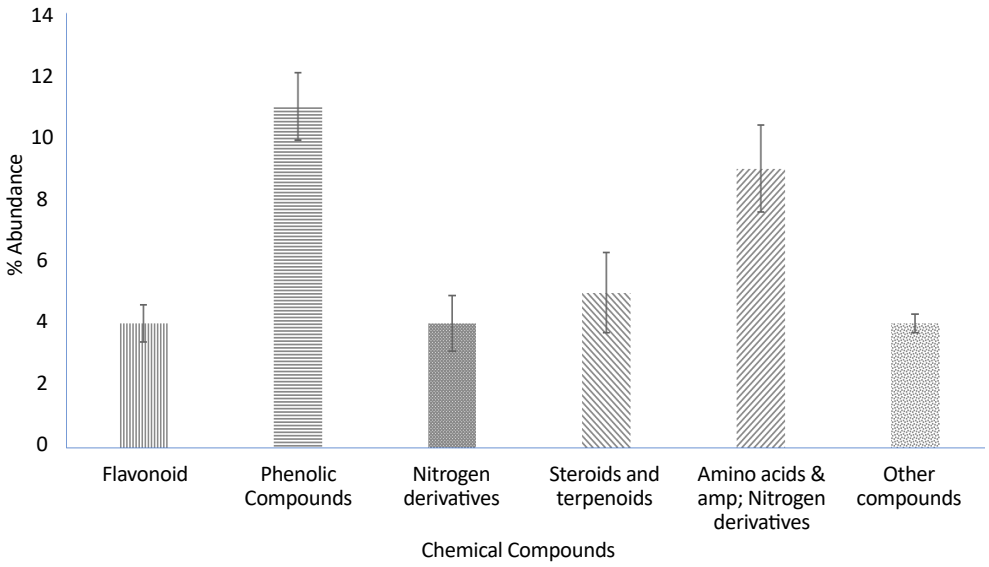

Figure 1. Percentage abundance of major chemical constituents in *U. massaica*

Results

Five major chemical constituents were extracted from *U. massaica* parts. Those that could not be classified were placed under "other groups." The order of abundance was phenolic > amino acids and nitrogen derivatives > steroids and terpenoids > flavonoids, nitrogen derivatives, and other groups (Figure 1).

Roots produced the highest quantity of constituents [12], followed by leaves [16] and then stems [12]. All the observed abundance, however, did not significantly differ ($P<0.05$) except for the flavonoids (Table 1).

Individual chemical constituents, however, differed in abundance per chemical group and plant part. Luteolin 6-C-glucoside (flavonoids), neoergosterol and β-sitosterol (steroids and terpenoids), isoleucine (amino acid and nitrogen derivatives), 3,4-diferuloylquinic acid (phenolic compounds) and p-isopropyl aniline (nitrogen derivatives) (Figure 2) were abundant per group and catechin (10.00) in roots, β-sitosterol (7.84) in leaf and 3,4-diferuloylquinic acid (11.43) in stem (Figure 3).

Interestingly, extracts from stems were not only more potent than those from leaves or roots, but their effects equaled the WHO threshold of >80% mortality

Table 1. Major chemical compounds in different parts of *U. massaica*

Major Chemical Compound	Mean±SEM			df	F	P
	Plant Parts					
	Roots	Stem	Leaves			
Flavonoid	10±0[a]	0±0[a]	3.03±0.6[a]	2	19.067	0.020
Phenolic CPDS	4.84±1.56[b]	6.88±1.52[b]	1.42±0[b]	2	1.196	0.351
Nitrogen derivatives	5.49±0.88[b]	0.71±0[b]	2.5±0.27[b]	2	5.079	0.080
Steroids and terpenoids	6.35±1.4[b]	0±0[b]	2.98±2.43[b]	2	1.112	0.435
Amino acids and nitrogen derivatives	5.36±0[b]	5.35±1.4[b]	2.9±0.68[b]	2	1.793	0.208
Other compounds	2.61±0.25[b]	3.49±0[b]	2.79±0[b]	2	1.214	0.411

$P<0.05$.

Notes: The mean abundance of chemical components followed by superscripts of the same letters differ significantly.

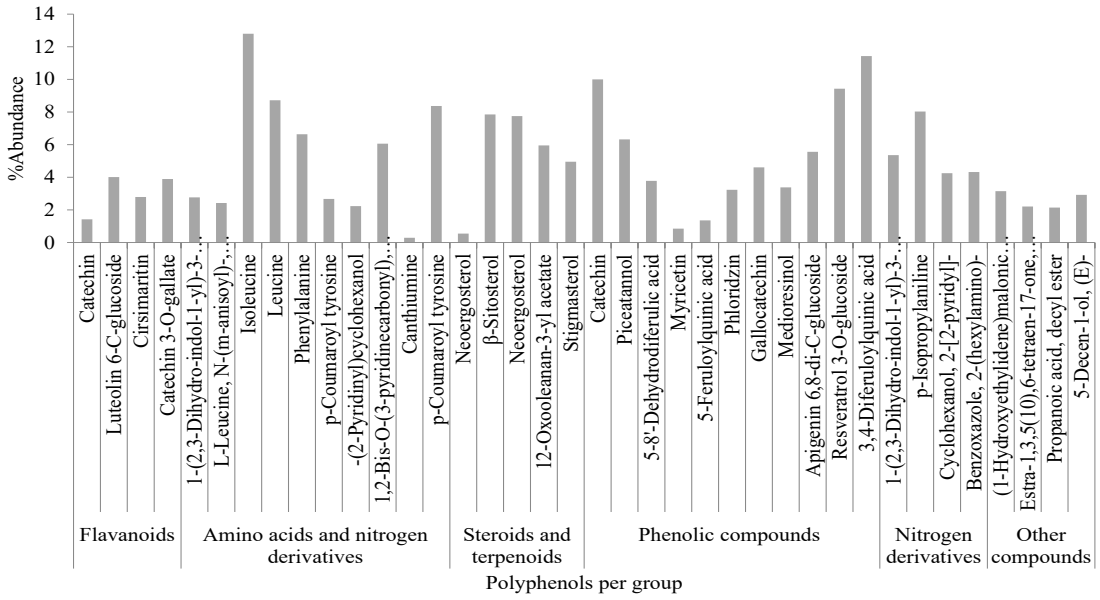

Figure 2. Polyphenol abundance per chemical group

for insecticidal effectiveness for doses of 10 mL/100 cm^3 (e/w) and above (Figure 4).

Observed mortalities, however, were not significantly different (P>0.05) irrespective of dose or control (Table 2).

Discussion

Different parts of plants (leaves, fruits, seeds, roots, and bark) contain polyphenols or secondary metabolites (flavonols, anthocyanins, and phenolic acids) that are responsible for mosquitocidal properties [35]. Indeed, much of the research on malaria intervention is on the search for novel chemical agents that have equivalent potency as synthetic insecticides but whose activity is specific and friendly to humans and the environment [35, 36]. The study reported herein was one of such, and herein we demonstrate that *U. massaica* contains several chemical agents that are toxic to *A. gambiae* larvae. The findings are similar to *Justicia adhatoda* L. [37] and *Acacia nilotica* [38] against different mosquito species.

In this study, the roots contained more bioactive compounds than the leaves or stem, though extracts from the stem were more potent than those of the leaves or roots. These findings were similar to those of Baz et al. [39], Anupam et al. [40], and Yugi and Kiplimo [41], who reported on the differential distribution and con-

Table 2. Levels of significance on mortality of aquatic stages *A. gambiae* exposed to different doses of crude extracts from different parts of *U. massaica*

Dose (mL/100 mL)	df	F	P
80	2	1.899	0.184
40	2	1.723	0.212
20	2	1.558	0.243
10	2	1.705	0.215
5	2	1.294	0.303
2.5	2	0.693	0.515
Dimethyl sulfoxide	2	0.542	0.592
Distilled water	2	1.958	0.176

P<0.05.

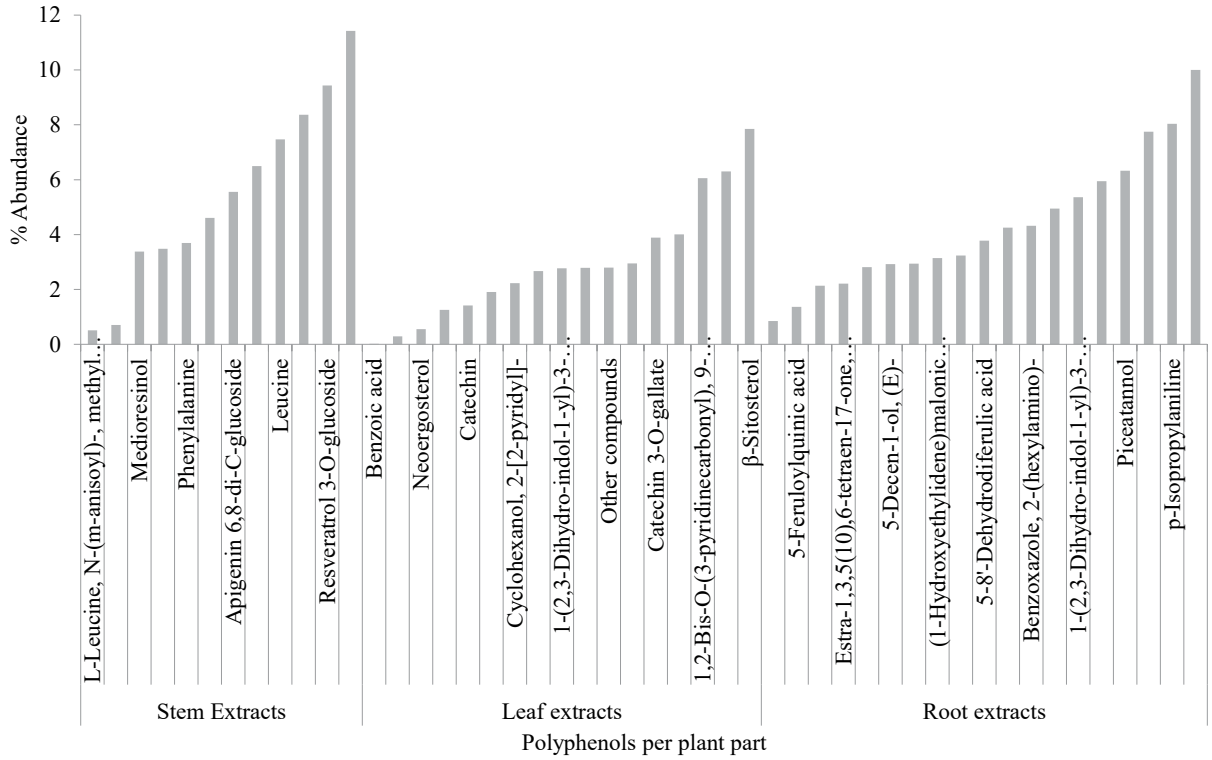

Figure 3. Polyphenol abundance per plant part

centration of bioactive compounds in different plant parts. These findings come when insecticide resistance and environmental challenges [42] posed by synthetic insecticides are a thorn in the flesh against the race to reduce, and if not pacified, malaria infection must be welcomed. Indeed, it adds to the cumulated stock and use of natural products that are not only rich in bioactive compounds but are target-specific and safe for the environment [3, 36]. As such, the findings of this study put extracts of *U. massaica* among plants that can provide alternative sources of green insecticides [43] for mosquito-borne disease control [33].

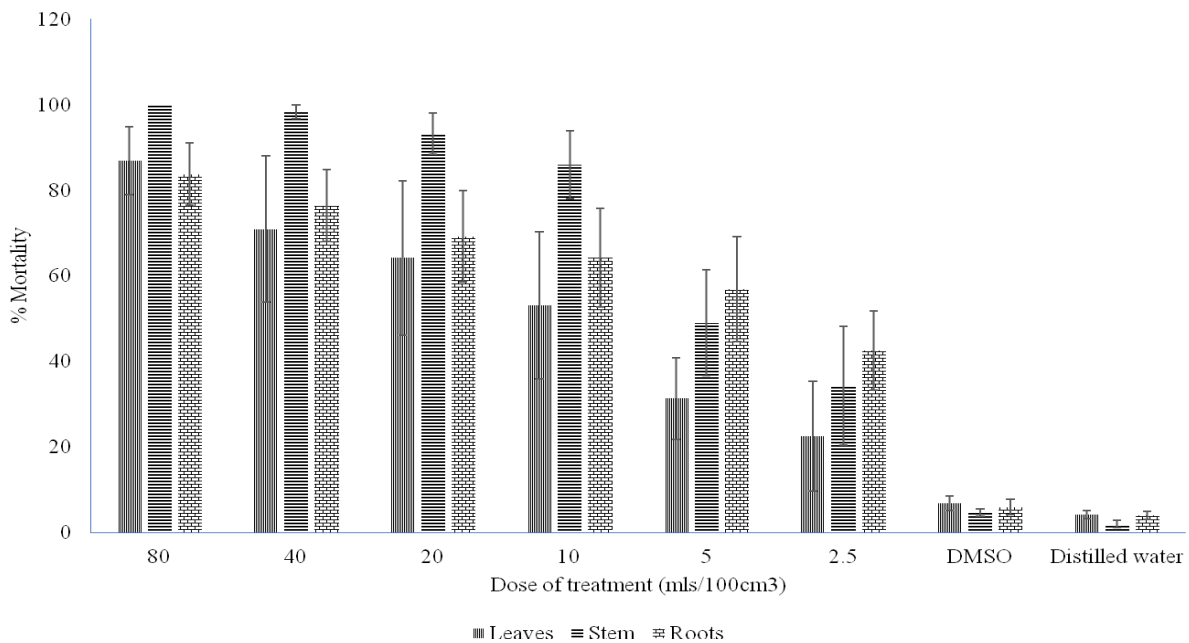

Figure 4. % Mortality of larvae exposed to different doses of extracts from different parts of *U. massaica*

The study showed that *U. massaica* contains secondary metabolites identified as tannins, terpenes, saponins, flavonoids, alkaloids, and phenols. A total of 47 bioactive compounds were extracted in different quantities (roots, 19; leaves, 16; and stem, 12) of the *U. massaica* plant. This amount was moderate but still higher than recently reported elsewhere [14]. This finding is promising as we believe that with refining, we are likely to realize more compounds. Though the exact nature and the specific bioactive compound responsible for the observed activity were not expressly determined, it is evident from an earlier demonstration that phenolic compounds, flavonoids, and tannins possess insecticidal properties [44] and may have been responsible for the observed toxicity. These findings are comparable with earlier works by Hoesain et al. [45] of plant metabolite potential against *Spodoptera litura*, Hillary et al. [46] of efficacy of plant products, Bassey et al. [47] of *Allium sativum* and *Murraya koenigii,* and Folawewo et al. [48], Oboho et al. [49] of *Hippocratea africana* with demonstrable mosquitocidal [50] activities. The bioactive compounds' toxicity was believed to be due to their impairing mitochondrial function in the exposed insect vectors [51].

The present study demonstrates the toxicity of extracts of *U. massaica* against *A. gambiae* larvae with activity being dependent on dose, solvent, and part of plant extracted. Though the time of exposure was not tested, the observations reported here agree with those reported by Ubulom et al. [51], Opara et al. [52], and Ghosh et al. [40] on the count of increasing concentration of bioactive compounds in different parts of the plant.

Conclusion

U. massaica bioactive compounds are diverse and have toxicants against *A. gambiae* larvae. They qualify for exploitation as green insecticides to target and protect against the anthropophilic, endophilic, and endophagic mosquitoes in areas where they are endemic and resist synthetic insecticides.

Study limitations

The study was limited to L3 of laboratory-reared *A. gambiae* mosquitoes.

Ethical Considerations

Compliance with ethical guidelines

This research was a laboratory-based experimental bioassay. It involved the use of laboratory-sourced and membrane-fed mosquitoes only. There was, therefore, no need for ethical consent.

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Authors contributions

Conceptualization, data analysis, and writing the original draft: Jared Yugi Owiti, supervision, methodology, investigation, data collection, review and editing: All authors.

Conflict of interest

The authors declared no conflict of interest.

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