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In Vitro Toxicity Levels of *Urtica massaica* Mildbr (Family: *Urticaceae*) on *Anopheles gambiae* Giles (Diptera: Culicidae) Mosquitoes

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Abstract Botanicals are targets for green insecticides and alternatives to synthetic insecticides. In this study, a randomized experimental design with control was used to evaluate *in vitro* toxicity level (LC_{50} and LC_{90}) of crude methanol and hexane *Urtica massaica* leaf, stem and root extracts on immature stages of *Anopheles gambiae*. 100 eggs, larvae or pupae were exposed to doses of 80 mg/100mls (e/w), 40 mg/100mls (e/w), 20 mg/100mls (e/w), 10 mg/100mls (e/w), 5 mg/100mls (e/w), 2.5 mg/100mls (e/w) of the extracts in clear plastic containers measuring 6 cm × 5.7 cm × 3.5 cm. Each container held 33 mls of a dose and either 33 eggs, larvae or pupae. The experiments were replicated four times. The set ups were left to stand overnight except that of eggs that stood for 48hrs. Mortality was assessed at the end of the period. It was found that methanol extracts were more toxic than hexane and leaf and root extracts were more toxic than stem extracts. Dose and solvent of extraction significantly influenced mortality ($p < 0.05$) of all stages for methane and hexane extracts except for hexane root extracts ($p > 0.05$) used against L3s. Since calculated goodness of fit was greater than the critical value ($\chi^2 = 22.4$; df = 22; $p < 0.05$) for all cases, the null hypothesis was rejected and the conclusion that *U. massaica* crude extracts was toxic to immatures of *An. gambiae* in vitro was adopted. It is concluded that *U. massaica* crude extracts are toxic against immatures of *An. gambiae* in vitro.

Keywords *Urtica massaica*; Methanol; Hexane; Lethal effect; *Anopheles gambiae*

1 Background

Mosquitoes are vectors of global public health importance as the mosquito borne infections (MBI) (WHO, 2020) for which they are famous are of global public health concern (WHO, 2022). Indeed, the infections threaten more than 40% of the world's population (Franklinos et al., 2019), with malaria accounting for the highest reported cases of morbidity and mortality (Maharaj et al., 2019; WHO, 2021; Oladipo et al., 2022; Li et al., 2024). Most cases of malaria infections occur in sub-Saharan Africa (WHO, 2019; WHO, 2020). Intervention against malaria is largely through vector management due to lack of effective medication and vaccination and though RTS, S/AS01 vaccine has been endorsed against malaria (WHO, 2021, Ogieuhi et al., 2024), it is faced with a myriad of challenges (Sallam et al., 2025). Additionally, the vaccine is mainly meant for children and regions with moderate to high *Plasmodium falciparum* malaria transmission (WHO, 2021).

The first line mitigative measure against malaria vector over the years has been the use of synthetic insecticides on various platforms (WHO, 2018; WHO, 2019). These have successfully managed the vector densities (Derua et al., 2018; Derua et al., 2019), interfered with their host-seeking behaviour, reduced their contacts with humans and reduced malaria disease transmission (Cibulskis et al., 2016, Govindarajan et al., 2016). However, their continued indiscriminate use has led to resistance in mosquito populations (Oduola et al., 2019; WHO, 2020; Peng et al., 2022) in addition to unwarranted environmental toxicity (Deng et al., 2019; Semenza et al., 2022; Wafula et al., 2023). These challenges are expected to escalate with emerging issue with climate change, alien vector spp. and the ever-growing threat of resistance to antimalarial drugs and insecticides (Mordecai et al., 2020; Li et al., 2024). Envisaged solution is bringing on board new inventions and strategies (Richards et al., 2020).

In the recent years, the use of natural products from plants has witnessed a lot of attention as the derived biopesticides also known as secondary metabolites, have shown abilities of killing or repelling mosquitoes (Youmsi et al., 2017; Ali et al., 2023). It has also been found to be target specific, non-toxic to valuable natural enemies, fully biodegradable (Vivekanandhan et al., 2018; Vivekanandhan et al., 2020), of broad spectrum (Ebadollahi et al., 2020), and a promising alternative to synthetic insecticides (Yohana et al., 2022). Their use is envisaged to be sustainable as they are readily regenerative, of low cost and environmentally safe (Borges, 2016; WHO, 2020). The use of extracts of *U. massaica*, though proven as botanicals with promising biospesticide potential (Khatoro et al., 2021; Owiti et al., 2025), the demonstration has not been exhaustive. This study therefore demonstrates in vitro toxicity levels of *Urtica massaica* on *Anopheles gambiae* mosquitoes.

2 Results

It was found that methanol extracts were required in smaller doses as compared to hexane extracts of stem or roots for the LC_{50} regardless of plant parts or immature stage exposed. However, the trend for the LC_{90} was different. Dose did not influence ($p > 0.05$) mortality of exposed aquatic stages (Table 1). For hexane solvent, extracts from the roots were more toxic than those of stem and leaves for LC_{50} regardless of immature stage exposed. No singular trend was observed for the LC_{90}. Dose and solvent of extraction significantly influenced mortality ($p < 0.05$) of all exposed aquatic stages except for L3s exposed to root extract ($p > 0.05$) (Table 2). However, because all observed calculated goodness of fit were greater than the critical value ($\chi^2 = 22.4$; df = 22; $p < 0.05$) for all cases, the null hypothesis was rejected and the alternative adopted.

Table 1 Lethal concentration (LC_{50} & LC_{90}) of methanol extracts of *U. massaica* plant parts against different aquatic stages of *An. gambiae* mosquitoes

Life stage	Plant part	LC_{50}			LC_{90}			Chi-Square Tests		
		Estimate	95% confidence limit for log $_{10}$ (Concentration)		Estimate	95% confidence limit for log $_{10}$ (Concentration)		χ^2	df	p
			Lower	Upper		Lower	Upper			
Eggs	Leaves	4.86[a]	0.42	0.86	48.39[a]	1.46	2.10	269.13	22	0.000
	Stem	4.56[a]	0.60	0.71	19.48[a]	1.22	1.37	49.28	22	0.001
	Roots	11.91[a]	0.92	1.22	50.69[a]	2.33	3.37	98.54	22	0.000
L3	Leaves	1.86[a]	2.20	0.57	9.05[a]	0.68	2.39	901.19	22	0.000
	Stem	-	-	-	-	-	-	-	-	-
	Roots	3.06[a]	0.45	0.52	5.64[a]	0.66	0.75	46.96	22	0.001
Pupae	Leaves	5.22[a]	0.68	0.76	8.61[a]	0.88	1.02	129.74	22	0.000
	Stem	6.12[a]	0.69	0.91	9.33[a]	0.86	1.24	704.08	22	0.000
	Root	12.46[a]	0.46	1.61	21.02[a]	2.93	13.50	175.40	22	0.000

Notes: df = degree of freedom; χ^2 = the chi-square factor; P = probability for the level of significance. P was taken as significant at $p<0.05$; LC = refers to lethal concentration, LC_{50} & LC_{90} concentration that kills 50% & 90% of exposed experimental aquatic stage; L3 = third larval instar. Rows having LC estimates superscripted with letter "a" denotes no significant influence of dose on exposed *An. gambiae* aquatic stages

3 Discussion

In the study herein, it was demonstrated that leaf and root extracts of methanol and hexane extracts respectively were required in smaller amounts and therefore more toxic than extracts of the other parts of *U. massaica* regardless of aquatic stage exposed or dose administered. This could be explained by differential concentration of bioactives in different parts of a plant. Indeed, secondary metabolites also known as botanicals are distributed differently in different plant parts. That is some plant parts have higher and others lower concentrations. This could be judged from the activity of extracts from different parts of a plant in a bioassay. The amount therein being directly proportional to activity. The more the concentration, the more potent the extracts (Yugi and Kiplimo, 2017). It is assumed therefore that the leaves and roots for methanol and hexane extracts contained the highest concentrations of botanicals respectively, a finding that was consistent with those of Anupam et al., (2012) and Yugi and Kiplimo, (2017), for methanol extracts but inconsistent with that of Thouri et al., (2017) for hexane extracts.

Table 2 Lethal concentration (LC_{50} & LC_{90}) of hexane extracts of *U. massaica* plant parts against different aquatic stages of *An. gambiae* mosquitoes

Life stage	Plant part	LC_{50}			LC_{90}			Chi-Square Tests		
		Estimate	95% confidence limit for log $_{10}$ (Concentration)		Estimate	95% confidence limit for log $_{10}$ (Concentration)		χ^2	df	*p*
			Lower	Upper		Lower	Upper			
Egg	Leaves	103.30[a]	1.73	2.62	39.11[a]	2.88	5.266	179.96	22	0.000
	Stem	7.81[a]	0.80	0.98	41.87[a]	1.49	1.799	133.24	22	0.000
	Roots	6.89[a]	0.69	0.96	61.19[a]	1.60	2.079	167.86	22	0.000
L3	Leaves	8.53[a]	0.85	1.02	17.99[a]	1.15	1.430	314.57	22	0.000
	Stem	3.88[a]	0.54	0.64	7.88[a]	0.83	0.994	113.84	22	0.000
	Roots	1.05[b]	-0.30	0.16	2.67[b]	0.35	0.486	33.79	22	0.052
Pupae	Leaves	70.05[a]	1.77	1.96	13.91[a]	1.99	2.383	295.15	22	0.000
	Stem	4.94[a]	0.63	0.76	9.69[a]	0.91	1.108	193.38	22	0.000
	Roots	31.34[a]	1.25	1.92	56.28[a]	2.90	6.056	138.86	22	0.000

Notes: As described (Table 1) except for "b" to denote significant influence of dose on the aquatic stages

In this study methanol extracts were more potent than hexane extracts. Methanol is more polar than hexane and according to the findings of Thouri et al. (2017), Borges et al. (2020) and Nguyen et al., (2021) possess optimal extraction ability as well as capacity to conserve the stability of the chemical structure of desired compounds. This finding was similar to others that demonstrated the influence of solvent type on extracted bioactives as well as larviciding potency (Anupam et al., 2012). However, it is noted that there is not a single standard solvent for optimal bioactive extraction as different solvents react differently for different plant matrices (Ngo et al., 2017).

Mosquitoes are a very important group of arthropods based on their role in the transmission and impact of mosquito borne infection (MBI) to humanity (WHO, 2020) and thus have been under constant human surveillance. Such has today yielded the best possible approaches of mosquito attack some of which include oviciding (Khatoro et al., 2021), larviciding (Yohana et al., 2022), pupiciding (Khatoro et al., 2021) and adulticiding (Muhammed et al., 2022) targeting the life stages of the vector. But again, the success observable today in managing malaria vector population is only possible because the ontogeny of the vector is predictable. As the adage goes, 'a chain is as strong as its weakest link'. The vector's aquatic stages (eggs, larvae and pupae) are the weakest link in the chain (life cycle). This is because their movement is restricted to the breeding ground (stagnant water) and are unable to escape to avoid "invasion or attack" by natural enemies (predators) or anthropogenic neutralization (through insecticides). It follows therefore that programmes that target mosquito immature stage are highly impactful (Chung et al., 2009; Conti et al., 2010). Indeed, larviciding has been the most preferred malaria vector control tool (Thomas, 2018) as the statistics on reduced malaria incidence and mortality due to reduced larval and adult mosquito abundance (Afrane et al., 2016; Ingabire et al., 2017) has largely been due to targeting the larvae (William et al., 2018; Zhou et al., 2020). When the larvicide is a biopesticides as is in this study, the programme transforms into the use of green biopesticide and biodiversity conservancy. This is because the biopesticide is not only lethal but since extract is a composition of varied acting bioactives, the vectors don't get to mount effective resistance against them. Additionally, since plants from which the biopesticides are derived are easily accessible and regenerative, the programme is not only sustainable and safe (Govindarajan et al., 2016), it also leads to conservation of the plant resources. It is therefore a solution to insecticide resistance mosquitoes, sustainable use and conservation of resources (Rahimi et al., 2019; Rahimi et al., 2020).

3.1 Conclusion

It is concluded that leaf, stem and root methanol and hexane crude extracts of *U. massaica* are required in small amount to kill immature *An. gambiae* mosquitoes. Their toxicity levels are promising as candidates for natural mosquito control strategies.

4 Materials and Methods

4.1 Study area, experimental mosquitoes, study design and laboratory conditions

This was an *in vitro* designed study conducted at the Centre for Global Health Research (CGHR) entomology laboratory where immature stages (Eggs, third larval instars (L3s) and pupae) of *An. gambiae* were sourced. The experimental design used was as described (Kothari, 2004; Yugi and Kiplimo, 2017). Briefly, a completely randomized informal 'after-only with control' experimental design was used with the solvent, dose and biopesticide extracts taken as independent while mortalities as dependent variables. Distilled water was taken as negative control. The laboratory temperatures and humidity were 28 ºC~30 ºC and 70%~80% respectively. Photoperiod was 12 hrs light (06.30~18.30 hrs) and 12 hrs darkness (18.30~0630 hrs).

4.2 *Urtica massaica* plant parts source, extraction and stocks solution preparation

U. massaica plant (leaves, stem and roots), voucher specimen number JOY2017/001 were sourced from 35º16' 46'' E, 0º31' 41'' N in Eldoret, Kenya. The extraction and processing of the biospesticide was done as described by Khatoro et al., (2021). Briefly, two grams of crude biopesticide stock's extracts was dissolved in 200 millilitres (mls) of dimethyl sulfoxide (DMSO). 160 mls (with 160 mls (v/v) of extract) of this solution was obtained and topped up with 40 mls of distilled water to make 200 mL (with 160 mls (v/v) of extract). This solution was then aliquoted in two beakers of equal capacity (100 mL) each to give a concentration of 80 ml/100ml (s/w). One of this was picked and 100ml distilled water added to top it up to 200 mls and then aliquoted in equal units of 100 mls to give a concentration of 40 mL/100 mL (s/w). This procedure was repeated until serial dilution of 80 mL/100 mL (s/w), 40 mL/100 mL (s/w), 20 mL/100 mL (s/w), 10 mL/100 mL (s/w), 5 mL/100 mL (s/w), 2.5 mL/100 mL (s/w) were obtained for the leaf, stem and root extracts.

4.3 Toxicity bioassay

Toxicity bioassays was conducted as described by Khatoro et al., (2021) and insecticidal potency of the biopesticide determined following the WHO, (2005) procedures. Briefly, 100 freshly transformed third larval instars (L3) were transferred by means of a dropper to plastic containers measuring 6 cm × 5.7 cm × 3.5 cm and left exposed for 24 hours after which the experiment was stopped. The experiments were replicated four times. This procedure was repeated for eggs and pupae for both methanol and hexane extracts. Eggs were however exposed for 48 hours. Mortality was calculated (i) and corrected (ii) using Abbot's (1925) formula for mortality of 5 % larvae in the control.

$$\% \text{ Mortality} = \frac{\text{Number of dead aquatic stage}}{\text{Total number of aquatic stage introduced}} \times 100 \quad \text{.....(i)}$$

$$\% \text{ Corrected mortality} = \frac{\text{Percent mortality in test-Percent mortality in control}}{\text{100-Percent in control}} \times 100 \quad \text{.....(ii)}$$

4.4 Statistical Analysis

Data was organized in excel spreadsheets and analysed using regression (probit) statistics to determine levels of toxicity (LC_{50} and LC_{90}) for the 50% and 90% respectively for dose and solvent of extraction. Levels of significance were adopted at 95% confident interval (CI) (that is at $p \leq 0.05$). Calculated goodness of fit was compared with critical value ($\chi^2 = 22.4$) at the same CI to inform on the relationship with the hypothesis of no relation. All statistical analysis was performed using statistical package for social scientists (SPSS) version 22.

Authors' contributions

Conceptualization, data analysis, and writing of the original draft done by JOY, supervision, methodology, investigation, data collection, review and editing done by all authors.

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Competing interests

None

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