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Repellency properties of oils from plants traditionally used as mosquito repellents in Longido district, Tanzania

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Abstract
Chemical composition and repellency potential of essential oils from *Tagetes minuta* and *Lippia javanica* traditionally used in Longido district to repel mosquitoes when formulated and topically applied as repellents were investigated in this study. The GC-FID analysis of essential oil from *T. minuta* showed presence of limonene (14.31%), camphene (1.95%), and verbenone (5.63%) whereas *L. javanica* essential oils indicated presence of limonene (5.38%), camphene (25.04%), DL-menthol (1.79%) and verbenone (1.41%) as main constituents. A topical formulation of 40% *T. minuta* essential oil in cow butter showed 80.9% protection against mosquito bites for up to 3 hrs 15 min whereas the unformulated oil exhibited only 52% protection for 1 hrs 52 min. Likewise, a formulation of 40% *L. javanica* essential oil in cow butter had 74% protection for up to 2 hrs 50 min, as compared to the unformulated oil that gave 47% protection by 1 hrs 15 min. Topical application of cow-butter-essential oil formulation is more effective in repelling mosquitoes than unformulated essential oils hence can provide alternative prevention measures of expelling mosquitoes during evening time before bedtime.

Keywords: *Tagetes minuta*, *Lippia javanica,* essential oil constituents, Topical application

1. Introduction

Mosquito-borne diseases are major human and animal health problems in tropical and subtropical countries. The diseases transmitted include malaria, filariasis, yellow fever, japa Japanese encephalitis and dengue fever. Despite centuries of control efforts, mosquito-borne diseases are flourishing worldwide with major proportion of children and adolescents contributing to global morbidity and mortality [13]. Among the mosquito-borne diseases occurring in Tanzania, malaria by far is the commonest and of greatest public health concern. About 30% of the disease burden to the people of Tanzania is from acute febrile illness, predominantly caused by malaria [19]. In over 80% of the regions, malaria is the largest cause of hospital attendance, hospital admissions and one of the leading causes of hospital deaths in the country [12]. In Tanzania most of the Malaria attributed cases and deaths occurs in rural villages away from effective diagnostic or treatment facilities.

There has been exploration of various methods over the centuries to combat threats from mosquito-borne diseases. Eliminating the source of infections is believed to be essential components for the control of mosquito-borne diseases [16]. Consequently, the most reliable approach to diminish the disease incidence is to interrupt the vector life cycle [13]. Product containing DEET protect considerably longer than other synthetic and botanical repellents [4]. Despite its excellent repellent property, there are reports of toxicity after application of DEET such as contact urticaria, skin eruption and encephalopathy [16]. Local plants with repellency or insecticidal action have played an important role in Africa region especially in a situation where there are no enough bed nets to cover all the beds in house and mosquito bites in the early evening. However, scientific analysis to identify which bioactive molecules of the repellents plants have greater effect on vector and also evaluate the rate of effectiveness of the application of the repellents plants or their products is crucial. Indeed, the main objective of conducting research of these plants is to build the bridge between laboratories results and the communities eventually benefiting from value added repellents that can be recommended and adopted as mosquito control tools for the community.

2. Materials and Methods

2.1. Chemicals

Chemicals used in GC-coinjections were α-pinene, sabinene, limonene, linalool, camphene, DL-menthol, verbenone, anisaldehyde, carvacrol, eugenol and phytol. All these chemicals were purchased from Sigma-Aldrich (Germany). All chemicals were Analytical grade.

2.2. Collection of plants materials: Collection of the plants was done in the villages of Kitendeni of Longido District. The decision of which plant and/or part of plants to be collected was highly influenced by the information that was given by the surrounding community. Two plant species *Tagetes minuta* (Voucher specimen EI 68) and *Lippia javanica* (Voucher specimen LEP 694) were collected with the help of the botanist from the Department of Botany University of Dar es salaam, authenticated and voucher specimen deposited at ITM herbarium. The collected aerial parts of plants were dried under shade for seven (7) days.

2.3. Extraction of essential oils

Essential oils from the dried plants were extracted using Hydro-distillation method by Clevenger type apparatus. The oils were separately collected in airtight containers, dried over anhydrous sodium sulphate and stored at 4°C for further experiment uses.

2.4. Mosquito bioassay

Mosquito repellency bioassay was carried out by topical application using human bait. Mosquitoes were reared at National Institute for Medical Research, UBWARI- station insectary. Disease free and blood naive female *Anopheles gambiae,* Kisumu strain, aged 2-5 days old were used in the experiments. These mosquitoes were reared 12 hrs light and 12 hrs dark photoperiod at 27ºC and 80% humidity. Mosquitoes were fed with blood meal but maintained at 10% sucrose overnight while also starved for a period of 6 hours before experiments.

2.5. Human volunteers

Four volunteers who were used throughout the study were recruited and signed a written informed consent form. They were instructed not to consume alcohol or smoke at least 5 hours prior to the experiment as well to avoid deodorants and colognes during the study period. The volunteers were asked to wear protective clothing exposing only their forearms from elbow to wrist to prevent generalized discomfort caused by mosquito bites. All volunteers were of age between 25 - 45 years been used throughout the entire experiment to avoid individual biasness.

2.6. Repellency activity by topical application

The formulated essential oils were made by dissolving appropriate weight of essential oil to make up 10g in cow butter. In this experiment the repellency activity of unformulated essential oil and formulated essential oil in cow-butter carriers were assessed using human bait technique [26]. Test was performed between 19:00-22:00 hrs since the mosquitoes are nights biting. Evaluation was carried out by four volunteers using cages (dimension of 12 × 12 × 26 cm^3) with four concentrations to the treated and untreated hand. The right hand was applied the treatments starting with the lower dose and DEET that was used as positive control applied last. The left hand was used as negative control being applied with no or with cow butter only that was used as a formulation carrier. Hundred (100) starved blood seeking adult female mosquitoes were placed in the experiment cage to acclimatize. The mosquitoes were assessed for host seeking behaviour prior to the experiment by counting the number of mosquitoes that landed on hand within 3 minutes. Mosquitoes were regarded as host seeking if at least five mosquitoes landed on the hand. During experiment, volunteers counted the number of mosquitoes that probed or landed the treated area of the arm while a researcher confirmed and record. Experiment was invariably terminated when mosquito bites were reported twice on two consecutive exposures. The percentage protection for a particular time of exposure was calculated using the equation $(B_C-B_T)/B_C$ whereby B_C represent the number of mosquito bite in control arm while B_T represents number of mosquito bite in treated arm [18].

2.7. Gas chromatography (GC-FID)

Gas chromatography (GC) analysis of the oil was performed on a Shimadzu GC 17A, using HP-5, (30 m× 0.32mm × 025µm), equipped with Flame Ionization Detector. Helium was used as carrier gas at a flow rate of 1.2 ml/min. Oven temperature was programmed at 60 ºC, holds for 3 minutes, then raised to 260 ºC at rate of 6ºC/min, and then held isothermal at 260 ºC for 36 minutes; injector temperature 270 ºC; detector temperature, 280 ºC; 1 µl of sample injected in a split ration of 50%.

2.8. Identification of the compounds

Compounds of the essential oil were identified by comparison of the retention time of authentic standards injected under the same condition as sample. Percentage composition was calculated from the sum of the peaks areas of the total oil composition.

2.9. Statistical analysis

The Student`s *t-test* was used to determine statistical significance of the repellency for the treatment and control. Standard errors of the means and 95% confidence intervals (CIs) of the fitted parameters were estimated by maximum likelihood analysis. All values were expressed as mean ±S.E and whenever the *p*-value was <0.05, the correlation was considered statistically significant.

2.10. Ethical clearance

The scientific protocols and ethical clearance to conduct the study was approved by the University Senate (MU/DRP/AEC/Vol. XVIII/67), upon recommendation from the Senate committee of Research and Publication-MUHAS, Tanzania.

3. Results and Discussion

When the 100 g of air dried sample of *T. minuta* was extracted by hydro-distillation, about 1.5 mls (1.5%) of essential oil were obtained after 3 hrs while dried *L. javanica* yielded 2.5 mls (2.5%) from 100 g of sample distilled. Presence of linalool (0.45%), limonene (14.31%), camphene (1.95%) and 1s-verbenone (5.63%) were confirmed from *Tagetes minuta* while analysis of *L. javanica* essential oil showed that, the plants contains limonene (5.38%), camphene (25.04%), DL-menthol (1.79%) and 1s-verbenone (1.41%) (Table 1).

Previous studies indicate that limonene, camphene and verbenone have insecticidal activity against insects [23]. The observed and reported repellency of these plants as used by

Maasai in Longido District may be attributed by these chemical constituents. The composition of the essential oils of *T. minuta* from different countries has been reported previously and showed significant differences in their composition [8]. The analyses of essential oils of chemotypes (genetic and epigenetic) of *T. minuta* indicates that the composition of the oils varies according to a number of factors: the harvesting location [1, 2, 6], stage of harvest, plant parts distilled, soil type, nutrient status [8] and the climatic condition under which the plant grows [20]. The essential oil of *T. minuta* was formulated by mixing essential oil in cow-butter to make 10, 20, 30 and 40% while also testing the unformulated essential oils topically. The essential oil of the aerial parts of the *T. minuta* exhibited various degrees of repellency against female *An. gambiae*. The results of mean protection time and percentage protection in relation to different doses of *T. minuta* essential oil and the formulations showed remarkable results against *An. gambiae* (Table 2).

The formulated essential oils of *T. minuta* exhibited various degrees of repellency against female *An. gambiae* with protection time ranging from 10 minutes to 195 minutes (Table 2). However, at low concentration of formulated product (10 - 20%) the efficacy of oil constituents seemed to be masked by the cow butter since the repellency was lower than of formulated oils (Table 2). The students *t*-test results showed statistically significant difference ($p<0.05$) between treatment and control with the degree of repellency of *T. minuta* improved significantly when the oil concentration increases at 30% and above rather than being used with lower concentrations or when used unformulated essential oil (Table 2). Likewise, the results for *L. javanica* essential oil repellency against *An. gambiae* mosquitoes as summarized in Table 3 showed significant differences in repellency among different doses.Tested concentrations of the essential oil of *L. javanica* exhibited various degrees of repellency against female *An. gambiae* with protection time ranging from 40 minutes to 170 minutes (Table 3). Likewise, better repellency was observed when formulation was above 30% where protection time went up to 145 minutes ($p<0.05$). This indicate the significance of blending labile essential oil constituents with heavy non-volatile carriers such as cow butter that were used in this study to aid slow release of repellent constituents while also protecting mosquito bites for long time.

Mosquito repellency properties of the two plant species or some related plants in the same genus have been supported by other studies done for plants growing in Tanzania and elsewhere in East Africa [14, 17, 22, 24]. The present investigations demonstrated that, repellency efficacy could be improved significantly by blending appropriately plants essential oils in appropriate carriers. The level of mosquito repellency of *T. minuta* and *L. javanica* essential oil formulation was lower than the standard repellent DEET but could significantly protect for some time which is enough to protect Maasai at evening time before bed time. The essential oil formulation is likely to offer a cheaper and effective tool that can be used to prevent as well as drive away vector mosquitoes from the human dwelling hence reducing diseases incidences and nuisance bites. Results of mosquito repellency of essential oil of *T. minuta* and *L. javanica* show that they have low protection time compared to formulated essential oil an observation which has been reported by other studies [3, 15].

Both *T. minuta* and *L. javanica* essential oils contained limonene, verbenone and camphene that have previously been described as components of essential oils functioning as mosquito repellents [7, 10, 11]. Verbenone is a non toxic anti-aggregation pheromone that affects the mountain pine beetle (*Dendroctonus ponderosae*) [9]. Limonene has been reported to have repellency property against different arthropods such as mosquitoes, fleas, ticks and cockroaches [10]. The findings of the present studies, therefore, are in agreement with the rest that report presence of limonene in essential oils of *T. minuta*[6, 20] and in *L. javanica*[21, 25].However in this study the *T. minuta* essential oil is effective than *L. javanica* essential oil due to higher content of limonene in *T. minuta* (14.3%) compared to *L. javanica* (5.38%)

Table 1: Compounds confirmed by co-injection with authentic sample and their percentage composition in each of *T. minuta* and *L. javanica* essential oils

S/N	Compound identified in oils by Co-injection	Authentic compound purity/grade	Batch no.	Retention time (RT)	#Abundance of compounds in the essential oils (%)	
					T. minuta	***L. javanica***
1	α-pinene	98	147524	2.89	0.06	-
2	Sabinene	75	W530597	3.38	0.49	0.43
3	Limonene	97	183164	4.73	14.3	5.38
4	Linalool	97	L2602	6.94	0.45	0.06
5	Camphene	95	456055	7.78	1.95	25.04
6	DL-menthol	≥95	W266507	8.62	0.06	1.79
7	Verbenone	≥93	W506907	10.03	5.63	1.41
8	Anisaldehyde	98	A88107	10.93	0.55	0.09
9	Carvacrol	≥98	W224502	12.35	0.07	0.04
10	Eugenol	99	E51791	12.85	0.03	0.01
11	Phytol	≥97	W502200	26.53	0.76	0.02

#Identification by co-injection was done to constituents in the essential oils whose commercial authentic samples were available.

Table 2: Repellent activity by topical application of *T. minuta* unformulated essential oil, DEET and formulated essential oil against *An. gambiae* at various concentrations

Concentrations of formulation(%)	Mean number of bites received		Mean protection time (minutes)[a]	Percentage protection (%)	*p*-value
	(treated)[a]	(control)[a]			
0 (Carrier)	19.50±2.02	22.50±2.02	0.00	13.3	0.020
10	3.333±0.988	23.167±0.703	10.0±6.324	85.6	0.384
20	5.500±1.522	22.667±1.116	47.50±8.138	75.7	0.003
30	4.333±0.558	24.167±0.749	150±10.950	82.0	0.003
40	4.500±1.258	23.500±1.522	195.0±6.708	80.9	0.003
Unformulated essential oil	7.25±1.109	15.25±2.016	112±7.500	52	0.001
DEET	2.00±0.516	20.00±2.394	315±16.88	90	0.000

[a]Values are mean of four replicates ± S.E at $p<0.05$ level by *t*-test

Table 3: Repellent activity by topical application of *L. javanica* essential oil, DEET and formulated butter-essential oil against the malarial vector *An. gambiae* at various concentrations

Concentrations of formulation (%)	Mean number of bites received		Mean protection time (minutes)[a]	Percentage protection (%)	*p*-value
	(treated)[a]	(control)[a]			
0 (Carrier)	19.50±2.02	22.500±2.020	0.00	13.3	0.020
10	9.333±2.906	22.667±1.115	0.00	58	0.000
20	8.333±2.027	23.500±1.522	40.0±10.00	65	0.003
30	6.667±1.585	24.667±1.382	145.0±5.00	73	0.003
40	6.167±1.682	24.000±0.775	170.0±6.325	74	0.000
Unformulated essential oil	7.75±1.493	14.750±0.478	75±8.660	47	0.003
DEET	2.00±0.516	20.000±2.394	315±16.88	90	0.000

[a]Values are mean of six replicates ± S.E at $p<0.05$ level by *t*-test

4. Conclusion

The study validated the claimed traditional use of these plants in insect-pest control by the people of Longido District whereby the findings suggest that, the use of the formulated essential oil from the *T. minuta* and *L. javanica* improves repellency protection time than unformulated essential oil. Such protection exhibited by formulated essential oils when used with other vector-borne control measures are likely to yield significant reductions of vector-borne disease burden in endemic settings, especially to Maasai people whose house and dress codes constantly expose them to contacts with mosquitoes. The cow butter is less converted in liquid form by the body temperature thus leaving the repellent compounds being released slowly providing much more protection time to mosquito bites, an experience that can be extended to testing other carriers like petroleum and aqueous base creams.

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