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Chemical Composition and Mosquito Repellency of Essential Oil of *Tagetes minuta* from the Southern Slopes of Mount Elgon in Western Kenya

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Abstract: Ethnopharmacologically, *Tagetes minuta* has a lot of applications in the history of human life. The study aimed at characterizing the essential oil from fresh aerial parts of *T. minuta* and evaluating its repellent effect on the host-seeking female *Anopheles arabiensis* mosquitoes, the vector of African malaria. The oil was obtained by hydrodistillation and analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). Compounds were identified by comparison of their mass spectra with those in Wiley NBS and NIST databases and GC retention times to those of authentic samples. The repellent effect of the essential oil was evaluated using a human-bait technique to simulate field situation. The percentage yield of the essential oil of *T. minuta* was 0.00029 % w/w with a specific gravity of 0.8953 mg/ml. The oil showed a complex composition of about 119 hydrocarbon compounds and may be richer in monoterpenes (47.90 %) than in any other type of compounds. The main principal constituents of the essential oil of *T. minuta* included: ocimene, dihydrotagetone, tagetones, ocimenones, piperitenone, 3,9-epoxy-p-metha-1,8(10) diene, β-caryophyllene, bicyclogermacrene and AR-turmerone. Some of these constituents reported in literature have shown insecticidal, acaricidal, pesticidal and/or repellent properties. Although not manifesting a clear general trend, the essential oil however showed a significant dose-response effect of repellency ($p < 0.05$). More mosquitoes significantly landed and bit the control arm treated with vaseline pure petroleum jelly than the arm treated with the essential oil of *T. minuta* ($p < 0.05$), thus showing repellency properties of the oil against *An. Arabiensis* mosquitoes. Nevertheless, the underlying mechanism of repellency remains unknown. However, the oil may represent a potentially new, most practical and economic way and readily available and applicable malaria vector control tool for incorporation into integrated vector management strategies and contribute to the provision of prophylactic measures, particularly at an individual level.

Key word: *Tagetes minuta*; Asteraceae; Essential oil constituents; Repellency; *Anopheles arabiensis* mosquitoes; malaria vector; western Kenya

Introduction

Tagetes minuta L. (also known as wild marigold and/or stinking roger: Family, Asteraceae) is an aromatic and erect annual herb naturalized in mild temperate and temperate forests and mountainous regions of the world in many countries after having originated in South America and spread as a weed [1]. The plant easily grows in disturbed

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areas during early successional stages and because of this affinity for disturbed areas; *T. minuta* has colonized many areas around the world [2]. The plant has deeply divided deep-green feathery leaves with numerous small yellowish-orange carnation-like flowers and may grow to become from 0.6-2 meters tall [2]. The details of the morphology of *T. minuta* have been described [2-4]. In temperate regions, the plant grows naturally from spring and practically disappears with the beginning of winter, having developed its complete life cycle. Nevertheless, this species is commercially cultivated in many regions of the world [4,5].

Tagetes minuta has a long history of human use as cola beverages, alcoholic beverages, frozen dairy desserts, candy, baked goods, gelatins, puddings, condiments, relishes, flavours, perfumes, ornamentals, medicinal decoctions and in ritual and cultural practices, depending on the geographical location and ethnic background of people involved [2,6,7]. Besides, it is well known for its wide range of biocide properties [8-11]. However, the sap of the plant may cause irritation to the skin, thus evoking photodermatitis. On other hand, Chandhoke and Ghatak [12], working with experimental animals, noted that the oil has hypotensive, bronchodilatory, spazmolytic, anti-inflammatory and tranquilizing properties. While in agriculture, the essential oil of *T. minuta* has been reported to have the potential for aphid control and as a natural herbicide for managing rice weeds [13,14]. In South America, *T. minuta* has been reported to help in the retention of humidity in the field [15].

Since ancient times, plant volatile oils have been recognized to possess imperative biological activities such as antibacterial, antifungal and antioxidant properties [16-21]. Some of the essential oils considered to have the greatest bioactivities are those of the plants such as *T. minuta* [8,9,22]. Of the species of *Tagetes*, wild marigold yields the highest amount of essential oil, known as Tagetes oil, and is thus a highly valued species [5]. This essential oil is used in pharmaceutical, agrochemical, food and perfumes industries; formulating high-grade perfumes and as a flavouring agent in food products such as cola and alcoholic beverages, frozen dairy desserts, candy, baked goods, gelatins, puddings and condiments based on the oil's principal constituents: ocimene, dihydrotagetone, tagetones and ocimenones [1].

Anopheles arabiensis, Patton (Diptera: Culicidae) is one of the seven recognized species of *An. gambiae* complex that is the most important vector of human malaria in sub-Saharan Africa, particularly in Sahelian savannas [23]. The malaria situation keeps worsening in tropical Africa, the continent that bears the bulk of the world's malaria burden, with 1 to 3 million deaths a year [24]. Therefore, sustainable alternative strategies are needed to decrease malaria transmission intensity. The use of mosquito repellents appears a practical and an economical way of preventing the transmission of malaria to humans at a personal level, thus providing prophylactic measure [25].

In our studies therefore, an evaluation of the repellency of the essential oil of *T. minuta* against *An. arabiensis* mosquitoes was conducted following the analysis of the chemical composition of the oil. It was hypothesized that essential oil of *T. minuta* may represent a sustainable and cost effective way to confer protection against the host-seeking malaria vector, *An. arabiensis* mosquitoes at an individual level.

Experimental

Rearing of Anopheles arabiensis mosquitoes for bioassays

The mosquitoes were reared in the insectary of the School of Biological Sciences, University of Nairobi, Kenya. The mosquitoes were obtained from the International Atomic Energy Agency (I.A.E.A.) Seibersdorf laboratories in Vienna, Austria, but originally from a place called Dongola located in the northern state of Sudan. Eggs were dispensed in larval rearing trays (21 × 15 × 8 cm) containing one litre of distilled water and hatched into first larval instars. Each tray contained about 100 larvae. The larvae were given food three times a day (at 09.00, 13.00 and 17.00 h, respectively) each receiving approximately 0.03 mg of fish food (Tetramin ®Baby) per day. Water in the trays was changed every day, beginning at 9.00 am in the morning. This was done by sieving the larvae using a piece of clean cloth. This process was repeated until larvae moulted to

pupae. The pupae were collected using rubber pipettes and placed in clean beakers, which were placed in mosquito holding cages measuring 30 × 30 × 30 cm and covered with mosquito netting material. A 10 % sucrose solution was placed in each cage on which the newly emerged adults were fed. After three days the mosquitoes were fed on a human forearm, which was placed in the cage through a 15 cm diameter opening for 10 min in complete darkness until the feeding mosquitoes withdrew their proboscis voluntarily. Petri dishes were placed in these cages, which acted as oviposition sites for the mosquitoes. Eggs were collected everday in the morning, dispensed in larval trays containing water and the cycle continued. The temperatures within the insectary were maintained at 28-29°C, relative humidity at 70-80 % with a photoperiod of 12 h light (06.30-18.30 h) alternating with 12 h darkness (18.30-06.30 h). Four to seven day old host-seeking female *An. Arabiensis* were carefully selected and used for repellence tests.

Fig. 1. *Tagetes minuta* L. plant showing the aerial parts used in the extraction of the essential oil for chemical composition evaluation and subsequent bioassay studies on the repellency effect of the oil against host-seeking female *Anopheles arabiensis*, Patton (Diptera: Culicidae).

Plant materials

Plant materials of wild marigold (Fig. 1) were collected from Bungoma County, western Kenya along the southern slopes and foothills of Mount Elgon at altitudes ranging from about 1 300 m (thermal zone 3: 20.0-22.5°C) in the south to about 3 500 m (thermal zone 8: 5.0-10.0 °C) in the north. The County is located between latitude 0° 25'S and 0° 53'N and longitude 34° 21'W and 35° 04'E. The selection of this plant was based on chemotaxonomic (phytochemical) consideration and ethnobotanical information of the Bukusu community in western Kenya. All the aerial parts of the plant were collected and identified in the herbarium at the School of Biological Sciences, University of Nairobi, Kenya. Voucher specimen of the plant species was deposited in the herbarium at the School of Biological Sciences, University of Nairobi, Kenya: *Tagetes minuta* L. (029-BGM-Mwi/2002) (Fig.1).

Extraction of essential oils

The collected plant materials were left in a well-ventilated room for 1-2 weeks before extraction of essential oil by hydrodistillation method. The materials were cut into small pieces and about 1 kg of each plant was hydrodistilled using a Clavenger-type apparatus for 8 h [26]. Pure oil was collected into 2 ml sealed glass vials and stored at –20°C in a freezer until required for analysis and bioassay studies.

Determination of the composition of the essential oils

Both qualitative and quantitative characteristics of the various essential oils were studied using gas-chromatography (GC) and gas-chromatography/Mass Spectrometry (GC-MS) techniques [27]. The constituents of the essential oils were identified by analysis of their mass spectra, direct comparison of their mass spectra to the Wiley NBS and NIST databases or library of mass spectra and co-injection with authentic standards on the GC.

The GC analyses were performed with a Hewlett Packard HP 5890A Gas Chromatography equipped with a flame ionization detector (at 230°C). A fused silica capillary column (Hewlett Packard, 50 m x 0.22 mm x 0.33 mm CD) coated with methyl silicon (0.3 μm film thickness) was

used with nitrogen as the carrier gas. All GC analyses were performed in the splitless mode with the injector temperature at 270°C. The oven temperature was programmed from 60°C isothermal for 7 min, to 120°C at 5°C per min, then to 180°C at 10°C per min and finally to 220°C at 20°C per min, where it was maintained for l0 min. Peak areas were calculated using a Hewlett Packard 3393 B series integrator and together with their GC retention times, compared to those of authentic samples.

The GC-MS analyses were performed with a VG Masslab 12-250 quadruple gas chromatography-mass spectrometer. Chromatographic separations were achieved using a fused silica capillary column (Hewlett Packard, 50 m x 0.32 mm ID) coated with Carbowax 20M (0.3 µm film thickness) with helium as the carrier gas. All the GC-MS analyses were made in the splitless mode with helium as the carrier gas. The GC column temperature was programmed as in the case of GC analysis. Compounds were identified by their electron impact (El) mass spectral data, order of elution and relative GC retention times, and by comparison of their mass spectra and GC retention times to those of authentic samples (where possible).

The computer on the GC-MS system records a mass spectrum for each scan and has a library of spectra that can be used to identify an unknown chemical in the sample. The library compares the mass spectrum from a sample component with mass spectra in the library. It then reports a list of likely identifications along with the statistical probability of the match.

Synthetic chemicals

Synthetic standard chemicals (authentic samples) used in GC co-injections were obtained from Sigma Chemical Company, Poole, UK and Aldrich Chemical Company, Gillingham, UK. All the authentic samples used were over 95 % pure.

Formulation of the essential oil of *Tagetes minuta*

Vaseline pure petroleum jelly (a non-perfumed product from Unilever Kenya Limited) was melted and maintained in a water bath at 80°C. Using a pipette, 9 ml of the melted vaseline pure petroleum jelly was put into a 20 ml glass vial, where 1 ml of pure essential oil was added. This solution was uniformly mixed to make the stock solution. From this stock solution, 1 ml was drawn and added to 9 ml of the melted vaseline pure petroleum jelly and uniformly mixed to make the first test dilution. Following the determination of the weight of 1 ml of pure essential oil as 0.8953 mg in the stock solution, this procedure was repeated to make five other serial dilutions using the formula: Mass 1 × Volume 1 P% Mass 2 × Volume 2, so that test dilutions ranging from 1:10 (with 0.08953 mg of pure essential oil) to 1:1000000 (with 0.0000008953 mg of pure essential oil) were prepared for use in the bioassays.

Experimental procedure

All experiments were conducted in an acclimatized bioassay room located at the School of Biological Sciences, University of Nairobi, Kenya. During the experimental period, room temperature and relative humidity were maintained between 27-30°C and 70-80 %, respectively. Identical aluminium cages measuring 30 × 30 × 30 cm and uniformly covered with a meshed transparent fabric were used for repellence tests. Experimental mosquitoes, 4-7 days old host-seeking female *An. arabiensis*, which had not received any blood meal before, were only fed on a 10 % sucrose solution, which was offered to them through soaked cotton wool. Six hours prior to the start of experiments, the sucrose solution was withdrawn from the mosquitoes in the breeding cages. The test mosquitoes were carefully selected from the breeding cages to the experimental ones using a sucking tube, 1 h before the start of the experiments. The experiments were carried out between 6.30 and 9.30 pm (East African time) under red light. Each test concentration of the essential oil was assayed on a separate day to avoid any possibilities of contamination. As a control test agent (diluent), vaseline pure petroleum jelly (non-perfumed product from Unilever Kenya Limited) was the first one to be assayed. The diluent was evenly applied round on a specified section of the human forearm using a clean worn rubber glove to avoid any possibilities of contamination. After 3 min of post

application of the diluent, the arm was introduced into the cage containing one mosquito through a 15 cm diameter hole on the cage. Once in the cage, the arm continuously remained still as the mosquito was allowed to land, probe and bite it. The number of mosquitoes landing and biting the arm (biting was realized through noting any slight pain) were recorded. The arm was withdrawn after 3 min and washed in warm soapy water and then rinsed in warm clean water and wiped with clean paper towels. The repellent was then applied to the arm using a glove and evenly spread just like the diluent. The arm was introduced into a different cage with a new mosquito and the same procedure followed. After the tests, the experimental mosquitoes were pressed independently between clean white filter papers to score the feeding success by sign of red blood showing on the filter papers. This was a confirmatory test of any one given mosquito having obtained a blood meal. Mosquitoes were only used once for each assay and only one volunteer was used for all the assays to avoid discrepancies in the results.

Data management and analysis

Data were entered in excel, database structure constituted, cleaned and entered into SPSS database for Windows and made usable as SPSS data sets for analysis. The data were transformed and subjected to analysis of variance (ANOVA) [28]. Backward logistic regression was used to analyze the significance of the effect of each concentration of the essential oil. The proportion of mosquitoes that landed and bit was analyzed by logistic regression. By considering only those mosquitoes that actually chose to bite in a given 3-min period, the probability of biting on the treatment arm and the control arm were estimated. Similar models were used to determine the effect of each concentration on the time taken for a mosquito to land and time taken to first bite. Student–Newman–Kuels (SNK) test was used to compare the mean values of repellency obtained of different doses [28].

Legal use of experimental animals and humans

All the procedures requiring experimental animals were approved by the International Centre of Insect Physiology and Ecology (ICIPE)'s Institutional Animal Care and Use Committee and were performed in compliance with guidelines published by the Kenya Veterinary Association and the Kenya Laboratory Animal Technician Association [29]. The research involving human volunteers used for mosquito repellency bioassays, followed guidelines of the Declaration of Helsinki and Tokyo for humans and the research was conducted in accordance with the ICIPE's ethical rules on scientific research and development. In addition, informed consent was obtained from the human volunteers used for mosquitoes' repellency bioassays.

Results and discussion

Yield and properties of the essential oil of *Tagetes minuta*

The percentage yield of the essential oil of the fresh aerial parts of *T. minuta* was 0.00029 % w/w, which was much lower than that obtained variably from *T. minuta* cultivated in sub-Tropical plains of North India (2.14 % and 1.89 % by dry weight basis) [30]. This difference may be attributed to both abiotic and biotic factors existing in the soil and in the external and internal environments of the growing *T. minuta* plant [5, 30-34]. The oil had a wild, sweet, fruity almost citrus-like smell and was yellowish-green to reddish-amber in color, depending on the duration of exposure to light, storage conditions and nature/mechanisms of extraction of the oil from the aerial parts of the plant [3-4]. The oil exhibited a specific gravity of 0.8953 mg/ml, which compared favourably with that obtained by Singh and co-workers [5] (0.8405 mg/ml) but relatively higher than that obtained by EL-Deeb and co-workers [31] (0.688 mg/ml). During the experimentation, the oil was observed to be soluble in dichloromethane (DCM), ether and ethanol and insoluble in water [3, 31] and did not cause either skin irritation or hot sensation on human skin upon contact. The oil was of medium viscosity that could turn thick and even gel-like if exposed to the air for a long time.

Chemical composition of the essential oil of *Tagetes minuta*

The essential oil showed a varied composition of chemical constituents occurring in different proportions and, some, reported to have insecti-

cidal, acaricidal, pesticidal and/or repellant properties in literature (Table 1). In this essential oil, majority of compounds were monoterpenes (47.90 %) followed by sesquiterpenes (30.25 %), hemiterpenes (15.13 %) and diterpenes (1.68 %) in that order (Table II). Within these sub-classes of terpenes, oxygen-containing terpenes were found in the highest proportion (56.30 %) followed by hydrogen-carbon-containing terpenes (36.97 %), nitrogen-containing terpenes (5.04 %) and chlorine-containing terpenes (0.84 %). Oxygenated monoterpenes occurred in the highest proportion (33.62 %) followed by hydrogen-carbon-containing sesquiterpenes (18.49 %), hydrogen-carbon-containing monoterpenes (15.13 %), oxygenated hemiterpenes (11.76 %) and oxygenated sesquiterpenes (10.08 %) amongst others (Table 2).

These greatly diversified proportions of compounds confirmed the previous reports that *T. minuta* is rich in many secondary compounds including monocyclic and bicyclic monoterpenes, sesquiterpenes, flavournoids, thiophenes and aromatics [2,5,31,32]. In this study, monoterpenes occurred in the highest proportion in the essential oil of *T. minuta*, and a higher proportion of monoterpene constituents were confirmed in literature to have repellence property (Tables 1 & 2), hence the repellent property of the essential oil demonstrated in this study against the host-seeking female *An. arabiensis* mosquitoes. These results on the composition of the essential oil of *T. minuta* compared favourably with the results obtained previously from other countries such as Egypt and Argentina where the main components of the essential oil were monoterpenes.

In Egypt, *trans*- and *cis*-tagetone together comprised 52.3-64.2 % [33], whereas in Argentina, depending on the parts of the plant and its growth stage, essential oil from leaves of non-bloomed plants was rich in dihydrotagetone (42.9 %) while that from flowers was rich in β-ocimene (45.4 %) and tagetenone (32.9 %), but the essential oil generally comprised: - β-phelandrene, limonene, β-ocimene, dihydrotagetone, tagetone and tagetenone [11,34]. While in Iran, the main components of the essential oil were found to be: - limonene (13.0 %), piperitenone (12.2 %), α-terpinolene (11.0 %), piperitone (6 %), (E)-tagetone (5.7 %) and (Z)-ocimenone (5.1 %) in that order [4]. The results from this study and those from the literature further confirms generally, the main principal constituents of the essential oil of *T. minuta* outlined previously to be: - ocimene, dihydrotagetone, tagetones and ocimenones as providing the basis of using this oil in the pharmaceutical, agricultural, food and perfumery industries, hence its high demand [1,5,35]. This evaluation is of course no wonder as in the recent past, the monoterpenes have too, been identified to offer an attractive alternatives to chlorofluorocarbons (CFCs) in many industrial applications [36] since CFCs were phased out by the Montreal Protocol because of their contribution to ozone depletion [37]. A number of these monoterpenes such as α-pinene, limonene, γ-terpinene, terpinolene, arbanol, α-terpineol, linalool and plinol (some also found in this study (Table I), have already been evaluated on their biological and physical properties to predict their most likely fate in the environment while in use [36]. This therefore shades more light on the likelihood application of the constituents of the essential oil of *T. minuta* in the industrial development.

From literature study, the many biological properties, which have been reported to be exhibited by the essential oil of *T. minuta*, may be as a result of the synergistic effects of the chemical constituents combined but not attributed to a single chemical compound [38]. This pheno-menon is nevertheless widespread in phyto-chemicals [39-43]. Further, these chemical consti-tuents may change their biological properties depending on the concentration(s) in use or composition proportion in which they occur in a given sample [43]. This specific important biological aspect needs further investigations using subtraction bioassays in order to understand the underlying scientific mechanisms of the observed wide range of the biological properties of the essential oil particularly the envisaged widespread synergism phenomenon in the phytochemicals shown in Table I so that sustainable application of this essential oil is achieved. Studies of synergism effects have important implications for the way bioprospecting for useful plant natural products is carried out and how these products are exploited for practical and sustainable use [40,44,45,46,47].

Table I. The GC-MS identified constituents in the essential oil of *Tagetes minuta* plant from Bungoma County, western Kenya

S. No.	Compound	Biological property from literature	Molecular formula	RT	M^+ g/mol	Base peak	Major peaks	Relative % abundance	GC-MS co
1	3-Hexanol	p	$C_6H_{14}O$	7.350	102.20	59	43,55,73	0.02	*
2	2-Butanone,3-methyl	Characteristic odour	$C_5H_{10}O$	7.775	86.13	43	41,86	0.03	-
3	Butane,2-chloro-2-methyl-.	Flavours and Fragrances	$C_5H_{11}CI$	8.050	106.60	71	41,76,85	0.02	*
4	Ethyl-2-methylbutyrate		$C_7H_{14}O_2$	14.775	130.18	57	41,74,85,102	0.07	*
5	5-ethyl-4-methyl-2 H-pyran-2-one		$C_8H_{10}O_2$	15.350	138.00	43	39,67,95,110,138	0.003	-
6	1-Butanol 2-methyl-Acetate		$C_7H_{14}O_2$	15.950	114.00	43	41,55,70	0.01	-
7	2,3,5, Trimethylfuran		$C_7H_{10}O$	17.225	110.00	67	43,109,110	0.003	-
8	2,3,5, Trimethylfuran		$C_7H_{10}O$	17.525	110.00	67	39,43,95,110	0.15	-
9	2,3,5, Trimethylfuran		$C_7H_{10}O$	17.525	110.00	67	39,43,95,110	0.003	-
10	α-Pinene	I, P, R	$C_{10}H_{16}$	18.800	136.20	93	39,77,79,121,136	0.06	*
11	Camphene	R^{PmAg}	$C_{10}H_{16}$	19.375	136.20	93	41,67,79,121,136	0.02	*
12	Sabinene	R^{PmAg} [51]	$C_{10}H_{16}$	20.225	136.20	93	41,77,79,1360.36		*
13	β-Pinene	P, R, I [51]	$C_{10}H_{16}$	20.450	136.20	93	39,41,69,121, 136	0.02	*
14	N-Octanol		$C_8H_{16}O$	20.775	128.00	41	43,57,84	0.19	-
15	3-Hexene-1-ol,Acetate	P [51]	$C_8H_{14}O_2$	20.976	142.00	41	41,67,82	0.01	-
16	α-Phellandrene	R^{PmAg}	$C_{10}H_{16}$	21.426	136.20	93	39,77,136	0.06	-
17	*cis*-Ocimene		$C_{10}H_{16}$	22.701	136.20	93	41,79,105,121	43.78	-
18	Dihydrotagetone		$C_{10}H_{18}O$	23.126	154.00	85	41,57,69,97	16.71	-
19	Tricyclene		$C_{10}H_{16}$	23.526	136.20	93	41,79,121,136	0.03	-
20	α-Terpinolene	R^{tRa}	$C_{10}H_{16}$	23.626	136.20	91	41,43,77,121,136	0.001	*
21	α-Citral		$C_{10}H_{16}O$	23.726	152.20	69	239,41,84	0.03	*
22	2,6-octadienal		$C_{10}H_{16}O$	23.801	152.20	69	39,41,84,94,109	0.08	-
23	2,4,6-octatriene,2,6- dimethyl		$C_{10}H_{16}O$	24.176	152.20	121	79,105,136	0.02	-
24	2-methyl-4(1-methylethyl-2-cyclohexenone		$C_{10}H_{16}O$	24.476	152.20	110	41,81,95,152	0.25	-
25	Geraniol formate		$C_{11}H_{18}O_2$	24.551	182.00	69	41,92,136	0.06	-
26	Linalool	A, I, P, R, R^{tRa}, R^{PmAg}	$C_{10}H_{18}O$	24.651	154.25	71	41,43,93,121	0.09	*
27	Artemesia ketone		$C_{10}H_{16}O$	25.001	152.00	83	39,55	0.06	*
28	2,3,4,6-Tetramethylphenol		$C_{10}H_{14}O$	25.101	150.00	135	39,77,91,150	0.02	-
29	Chysanthenone		$C_{10}H_{14}O$	25.401	150.00	107	79,91,122,150	0.08	*

table 1. (contined).

S. No.	Compound	Biological property from literature	Molecular formula	RT	M^+ g/mol	Base peak	Major peaks	Relative % abundance	GC-MS co
30	3-cyclohexene-1-methanol		$C_7H_{12}O$	25.601	112.00	79	41,53,94	0.1	*
31	β-Ocimene	I, R^{PmAg}	$C_{10}H_{16}$	25.851	136.20	121	41,79,105,136	3.25	*
32	*cis*-Tagetone		$C_{10}H_{16}O$	26.126	152.00	95	41,67,109,152	1.95	*
33	*trans*-Tagetone		$C_{10}H_{16}O$	26.476	152.00	95	41,67,109,137,152	8.67	*
34	Bicyclo 2.2-1 Heptane,2 -(propethyl)		$C_{10}H_{16}$	26.601	136.20	95	39,41,67,150	0.09	-
35	1,5,7-octatrien-3-one,2,6 dimethyl		C_9H_{14}	27.026	122.20	101	41,83,89,135,150	0.001	-
36	Endo-Borneol		$C_{10}H_{18}O$	27.226	154.25	95	41,55,69,110, 139	0.03	-
37	3,4-octadiene,7-methyl		C_9H_{16}	27.451	124.00	82	39,41,67,95,109	0.07	-
38	3-cyclohexen-1-ol,4 methyl		$C_{10}H_{18}O$	27.576	154.25	71	43,69,86,93,111,154	0.09	-
39	Carvone	R^{PmAg}	$C_{10}H_{14}O$	27.676	150.00	82	39,54,93,108, 150	0.07	*
40	α-Terpineol	I, P, R, A [19], R^{PmAg} , R^{Ras}	$C_{10}H_{18}O$	27.926	154.3	59	43, 93, 121, 136	0.03	*
41	N- Decanal		$C_{10}H_{20}O$	28.101	156.00	41	44,57,82,112	0.10	*
42	Cyclohexene-1-sopropthyl -2, 4		$C_{10}H_{16}O$	28.326	152..00	150	41,82,93,107,135	0.44	-
43	5-(2-cyclohexylethyl)-2-pyridine carboxylic acid		$C_{14}H_{19}NO_2$	28.501	233.31	55	55,93,106	0.03	-
44	Urea,N-nitroso-N-phenyl- (http://www.lookchem.com/cas-626/6268-32-2.html)	Toxic	$C_7H_7N_3O_2$	28.501	165.17	77	65,93,135	0.03	-
45	3,9 epoxy-p-metha-1,8 (10) diene		$C_{10}H_{14}O$	29.076	150.00	135	41,79,122,150	6.47	*
46	Piperitenone		$C_{10}H_{16}O$	29.401	152.00	150	41,91,107,135	10.15	*
47	Carvacrol	R^{Ras} ,R^{tRa} , R^{PmAg}	$C_{10}H_{14}O$	29.876	150.00	135	77, 91, 150	0.15	*
48	2-Cyclohexen-1-one,3-methyl-6-(methylethenyl)		$C_{10}H_{16}O$	30.251	152.00	82	39,54,135,150	0.32	-
49	Bornyl acetate	R^{PmAg} P, R, I [51]	$C_{12}H_{20}O_2$	31.051	180.00	95	41,43,121,136	0.03	-
50	Azulene,4,5,6,7,8,7A Hexahydro-		$C_{10}H_{14}$	31.201	-	39		0.01	-
51	Bicyclo 4.1.0 Heptane,7-(1-Methylethylidene)-		$C_{10}H_{16}$	31.201	136.12	93	77,91,107	0.01	-
52	Azulenone 4,5,6,7,8,8, A-Hexahydro-8A-Methyl-,(S)-		$C_{10}H_{140}$	31.226	-	39	33,77,79,93	0.003	-
53	*cis*-3-Acetoxy-3,6,6-Trimethylbicyclo Heptan-2-One		$C_{10}H_{16}O$	31.301	152.23	43	55,83,107	0.002	-
54	Dihydroedulan II		$C_{13}H_{22}O$	31.551	194.00	69	43,84,107,179	0.009	-
55	Dihydroedulan I		$C_{13}H_{22}O$	31.676	194.00	179	43,55,107,179	0.02	-
56	8-oxo-Neoisolongifolene		$C_{15}H_{22}O$	32.951	218.00	175	41,105,147,218	0.001	-
57	Bicycloelemene		$C_{15}H_{24}$	33.076	204.00	125	49,93,107,136	0.08	-

table 1. (contined).

S. No.	Compound	Biological property from literature	Molecular formula	RT	M^+ g/mol	Base peak	Major peaks	Relative % abundance	GC-MS co
58	γ-Guisunene		$C_{15}H_{24}$	33.526	204.00	122	81,107,161,204	0.12	-
59	α-Guaiene		$C_{15}H_{24}$	33.626	204.00	91	81,105,147,204	0.03	*
60	Photoneol B			33.876		83		0.008	-
61	Cyclohexene,4-(1,5-Dimethyl-4-Hexadienyl)-1-methyl-methyl 7		$C_{15}H_{24}$	34.001	204.00	109	41,93,119,161	0.01	-
62	3-octen-5-yne,2,2,7,3, Tetra methyl		$C_{12}H_{20}$	34.226	164.00	164	41,77,91,107,149	0.15	-
63	1H-cyclopropa-A Naphthalene		$C_{12}H_{24}$	34.676	168.00	189	41,91,105,161,204	0.46	-
64	A,2,3,5,6,7,7A,7B octahydro-1,1,7,7A tetramethyl		$C_{15}H_{24}$	34.851	204.00	162	41,91,105,189,204	0.05	-
65	δ-Guaine		$C_{15}H_{24}$	35.501	204.00	108	41,79,93,135,189	0.04	-
66	Caryophyllene (*trans*) β	P,R,I R^{PmAg}	$C_{15}H_{24}$	35.701	204.00	41	69,93,133	0.84	*
67	5-(6-methyl-2-pyridy) tetrazore		-	35.876	-	161		0.03	-
68	Phenol,2-(5-isoxazolyl)-4-(1-methylethyl)-		$C_{12}H_{13}NO_2$	35.876	203.24	161	39,65,93,105,121	0.03	-
69	*iso*-caryophyllene	R^{PmAg}	$C_{15}H_{24}$	36.251	204.40	41	69,93,133,161	0.26	*
70	α-Humulene (á-Caryophyllene)		$C_{15}H_{24}$	36.626	204.40	93	41,80,121,147	0.26	-
71	Germacrene-D	R^{PmAa}	$C_{15}H_{24}$	37.301	204.40	161	79,91,105,204	0.17	*
72	Bicyclogermacrene		$C_{15}H_{24}$	37.726	204.40	121	41,93,136,161	0.62	-
73	ä-Cadinene	P, R [51]	$C_{15}H_{24}$	38.151	204.40	151	105, 119, 134,204	0.01	-
74	1,4-Benzenediol,2-(1,1-Dimethylethyl)-		$C_{15}H_{22}O_2$	38.326	234.00	123	77,151,166	0.03	-
75	*iso*-Bornyl butyrate		$C_{14}H_{24}O_2$	38.401	224.34	43	71,95,109,136	0.02	-
76	17-Methyl-5-á-androst-2-en-17-β-ol		$C_{20}H_{32}O$	38.401	288.47	43	29,55,81,109	0.02	-
77	Valerenol		$C_{15}H_{22}$	39.176	202.00	91	41,79,105,189,220	0.03	-
78	Spathulenol		$C_{15}H_{22}$	39.726	202.00	43	69,91,119,205	0.26	*
79	1H cycloprope Azulene 7-ol Decahydro-1,1,7,72		$C_{15}H_{22}$	39.876	202.00	43	91,119,159,205	0.16	-
80	Viridiflorol		$C_{15}H_{26}O$	40.001	222.36	161	43,69,109,121	0.07	-
81	(-)-Globulol		$C_{15}H_{26}O$	40.001	222.37	161	43,69,109,122	0.07	-
82	Ledol		$C_{15}H_{26}O$	40.001	222.37	109	43,69,81,122,161	0.07	-
83	(+) - Aromadendrene		$C_{15}H_{24}$	40.251	204.35	43	41,77,93,107,149	0.04	-
84	Elemol		$C_{15}H_{26}O$	40.251	222.37	43	41,69,93,107,161	0.04	-
85	1H-Indene,1-Ethy Lideneoctahydro-7A-Methyl-,*cis*-		-	40.351	-	43	41,69,93,105,165	0.03	-

table 1. (contined).

S. No.	Compound	Biological property from literature	Molecular formula	RT	M^+ g/mol	Base peak	Major peaks	Relative % abundance	GC-MS co
86	Trichothecolone		$C_{15}H_{20}O_4$	40.351	264.32	43	55,67,95	0.03	-
87	2-Naphthalenemethanol,Decahydro		$C_{15}H_{26}O$	40.376	222.37	59	41,79,121,133	0.03	-
88	*iso*-Spathulenol		$C_{15}H_{22}$	41.126	202.00	43	93,119,162,220	0.08	-
89	δ-Elemene	I [51]	$C_{15}H_{22}$	41.526	202.00	121	41,93,136,161	0.02	-
90	Neophytadiene		$C_{20}H_{38}$	45.676	278.00	68	43,57,82,95,123	0.23	-
91	Benzofuran,3-methyl-2-(-1 methylethenyl)		$C_{12}H_{12}O$	47.226	172.00	172	128,131,157	0.04	-
92	7-Methyl-3-Octyne (CAS)		C_9H_{16}	47.451	124.22	41	67,95,109	0.007	-
93	-1,6-Dimethyl-10-Ethoxy-11-Oxtricyclo [5.3.0.1(2,5)Undec-6-Ene		-	47.476	-	207	77,79,91,107,121	0.006	-
94	*trans*-Pinocarveol		$C_{10}H_{16}O$	47.601	152.23	83	41,55,70,92	0.010	-
95	α-Farnesene		$C_{15}H_{24}$	47.601	204.35	83	41,55,69,93	0.010	-
96	*iso*-Pinocarveol		$C_{10}H_{16}O$	47.626	152.23	83	40,41,55,70,91,92	0.010	-
97	β-Atlantone		$C_{10}H_{16}O$	47.951	152.00	83	41, 55, 69, 168	0.018	*
98	Hexamethylcyclohexane		$C_{12}H_{24}$	48.326	168.32	83	41,55,69,168	0.013	-
99	1,3,7,7-Tetramethyl-11- Oxatricyclo (4.4.1.1**2,8) Dodecane		$C_{14}H_{30}$	48.451	198.39	82	41,43,122,137	0.004	-
100	3,7-Cyclodecadiene-1-one,3,7-Dimethyl-10-(Methylethyldene)-		$C_{15}H_{22}$	48.551	218.33	135	55,91,107,150	0.036	-
101	4-Heptyn-3-ol		$C_7H_{12}O$	48.776	112.00	83	29,55	0.008	-
102	3,3,6-trimethyl-1,5-heptadien-4-one		$C_{10}H_{16}O$	48.776	152.24	83	39,41,55	0.008	-
103	2(1h)-Napthalenone,4A,5,8,8a-Tetrahydro-4A-Dimethyl-,TRAS		$C_{10}H_{16}O$	48.776	152.24	83	41,55,136	0.008	-
104	α-Cedrol		$C_{15}H_{26}O$	49.076	222.00	95	43,87,135,150	0.032	-
105	1H-Indene,2,3,3A,4,7,7A-Hexahydro 2,24,47,7-Hexamethyl		$C_{15}H_{26}$	49.276	206.00	83	41,55,69,121,191	0.095	-
106	1,5,7-Octatrien-3-one,2,6-Dimethyl		$C_{15}H_{22}$	49.551	218.33	150	41,89,135,	0.028	-
107	1-Phenyl-2-(2-Methyl-1-Propenyl)Carbonyl) Cyclopropane		-	49.901	-	83	116,157,202	0.009	-
108	4-(3-Methyl-1h-Pyrazol-1-YL) Benzonoesaure-Ethylester		-	49.901	-	185	116,157,202,230,231	0.007	-

table 1. (contined).

S. No.	Compound	Biological property from literature	Molecular formula	RT	M+ g/mol	Base peak	Major peaks	Relative % abundance	GC-MS co
109	2,6,-Nonadien-4-one,9-(3-Furanyl)-2,6-Dimethyl		$C_{15}H_{20}O_2$	49.926	232.00	83	55,134	0.038	-
110	Propanedinitrile,dicyclohexyl-(CAS)		$C_{15}H_{22}N_2$	49.926	230.35	83	41,55,84	0.038	-
111	E-Ocimenone	I	$C_{10}H_{14}O$	50.151		150	41,91,107,135	0.221	*
112	Cinnamyl tiglate		$C_{14}H_{16}O_2$	50.301	216.28	83	91,105,135	0.018	-
113	Ar-Turmerone		$C_{15}H_{20}O$	50.626	216.32	83	55,108,122,150,206	0.501	*
114	4,4-Dimethyltricyclo 6.3. 0(1,7)Undecane2,6-Dione		$C_{12}H_{26}$	51.176	170.34	83	55,108,122,150,206	0.214	-
115	2,6-Nonadiene-4-one, 9-(3-furanyl)-2,6-dimethyl		-	51.326	-	83	55,69,123,190	0.182	-
116	Cinamyl tiglate		$C_{15}H_{20}O_2$	51.326	232.00	83	55,134	0.18	-
117	α-Atlantone		$C_{10}H_{16}O$	53.301	152.00	83	55,123,135	0.188	-
118	4,4-Dimethyl-3-ethylidene-2-(2-methyl-1-Propenyl) cyclohexanone		$C_8H_{16}O_2$	52.702	144.21	135	43,91,107,191,206	0.031	-
119	Propanedinitrile, dicyclohexyl-,2,2-dic		$C_{15}H_{22}N_2$	52.927	230.35	83	55,70,123,193	0.111	*

RT : Retention Time in Minutes

GC-MS co : Gas Chromatography-Mass Spectrometry Co-injection

R^{pmAg} : Repellent to *Anopheles gambiae* mosquitoes [38]

R^{Ag} : Repellent to *An. gambiae* mosquitoes [38]

R^{tRa} : Repellent against *Rhipicephalus appendiculatus* ticks [47,48]

R^{Ras} : Repellent against *R. appendiculatus* ticks[49]

R^{PIr} : Part of repellent essential oil to *lxodes ricinus* nymphs [50]

R^{PmAa} : Part of repellent essential oil to mosquitoes, *Aedes aegypti* [51]

R^{tRa}: Repellent against ticks-*R. appendiculatus* [47,48]

According to Duke [52] and/or other source(s) as referred to in Table I. or other references as indicated by the author(s) in the Table 1 above.

A : acaricidal; I : insecticidal

R : insect repellant (insectifuge); P : pesticidal

R^{Tm} -Repellent against *Tetranychus* mites [53,54]

* - Gas Chromatography-Mass Spectrometry Co-injection was conducted.

N/B - Unknown compounds had very low ionic current and hence could not be found easily in the Wiley NBS and NIST databases.

Table 2. Percentage group composition and classification of terpene constituents in the essential oil from the aerial parts of *Tagetes minuta*

Group composition of the essential oil / Type of terpene	Classification of terpene constituents A	B	C	D	E
Hemiterpenes	18(15.13%)	3(2.52%)	14(11.76%)	1(0.84%)	1(0.84%)
Monoterpenes	57(47.90%)	18(15.13%)	40(33.61%)	3(2.52%)	-
Sesquiterpenes	36(30.25%)	22(18.49%)	12(10.08%)	2(1.68%)	-
Diterpenes	2(1.68%)	1(0.84%)	1(0.84%)	-	-
Unknown	6(5.04%)	-	-	-	-
Total	119	44 (36.97%)	67 (56.30%)	6 (5.04%)	1 (0.84%)

A: Proportion of the terpene in the composition
B: Hydrogen-carbon terpenes only
C: Oxygen-containing terpenes
D: Nitrogen-containing terpenes
E: Chlorine-containing terpenes
- No compound was identified in the essential oil

Repellency assays of the essential oil of *Tagetes minuta*

The repellent effect of the essential oil was evaluated using the human-bait technique to simulate field situation. The assays focused on the analysis of the proportion of host-seeking female *An. arabiensis* mosquitoes landing on the human forearm and biting it following its subsequent treatment with either the essential oil of *T. minuta* or vaseline pure petroleum jelly, a non-perfumed product from Unilever Kenya Limited being used as a diluent as well as a control agent. The relative repellency of the various concentrations of the essential oil against the mosquitoes is shown in Table 3. Unlike the control experiment, there were significant differences in repellency amongst the concentrations against the mosquitoes, which landed on the treated arm with the essential oil of *T. minuta* ($p < 0.05$). Although not manifesting a clear general trend, the essential oil however showed a significant dose-response effect of repellency ($p < 0.05$). A similar pattern was observed with the biting behavior of the mosquitoes (Table 3). For all the concentrations, there was a significant difference in the proportion of the mosquitoes landing and biting the control arm treated with vaseline pure petroleum jelly and the test arm treated with the essential oil of *T. minuta* ($p < 0.05$). This therefore implied that more mosquitoes landed and bit the control arm treated with vaseline pure petroleum jelly than the arm treated with the essential oil of *T. minuta*, thus showing the repellency properties of the oil against *An. arabiensis* (Table 3). This was however, contrary to the results obtained when *T. minuta* potted plants were evaluated of their repellency against the malaria vector, *An. gambiae* sensu stricto Giles in experimental huts under semi-field conditions inside a screen-walled green house [55]. Nevertheless, significant comparable results may be achieved if experimental conditions and design were made the same. The experimental set up utilized by allowing the use of one mosquito per cage for every observation made, eliminated the overlap of the number of mosquitoes, which landed and those ones, which bit as it was observed previously with Tawatsin *et al*.[56] and Fradin *et al*.[57] who used 250 and 10 mosquitoes per cage, respectively, for every observation made. In future, our experimental model and those described in literature need to change to the standardized procedure of testing repellency in a choice assay [3], in which case, both the test and control arms need to be presented to the candidate host-seeking female *An. arabiensis* mosquitoes in order to make a choice on whose arm to land.

Table 3. Comparison between the proportion of mosquitoes that landed and bit the test arm treated with different concentrations of the essential oil of *Tagetes minuta* and the control arm treated with the same concentrations of the diluent (vaseline pure petroleum jelly, a non-perfumed product from Unilever Kenya Limited).

Dose (mg)	Number of mosquitoes used	Proportion of mosquitoes landing			Proportion of mosquitoes biting		
		A	B	P-value	A	B	P-value
0.08953	54	0.167[a]	0.741[a]	0.001	0.148[a]	0.741[a]	0.001
0.008953	53	0.283[ba]	0.660[a]	0.001	0.283[ba]	0.642[a]	0.001
0.0008953	50	0.160[a]	0.760[a]	0.001	0.140[a]	0.760[a]	0.001
0.00008953	51	0.314[b]	0.765[a]	0.001	0.275[ba]	0.745[a]	0.001
0.000008953	50	0.340[b]	0.640[a]	0.002	0.300[b]	0.620[a]	0.003
0.0000008953	60	0.270[ba]	0.635[a]	0.001	0.238[b]	0.603[a]	0.001

A: Treated with essential oil
B: Treated with vaseline (control)
For a given column, figures with the same superscript alphabetical letters are not significantly different at $\alpha = 0.05$ level of significance (Student-Newman-Keuls H test).

In conclusion therefore, the essential oil of *T. minuta* aerial parts shows a complex composition of hydrocarbon compounds and may be richer in monoterpene hydrocarbons than in any other type of compounds. Nevertheless, both the physical and chemical properties of the oil are constantly changing, thus affecting its biological functions and hence its envisaged industrial applications. The results indicated that the essential oil of *T. minuta* shows the potential to repel mosquitoes to a variable degree and this bioactivity may be dose-dependent. However, the underlying mechanism of repellency remains unknown and therefore, warrants further research. The phytochemical diversity and the potential repellent property of the essential oil in addition to its use in the perfumery, agriculture, food and therapeutic industries, presents useful property that help to add economic value to the plant for its sustainable utilization and conservation reasons. As such, the essential oil of *T. minuta* may represent a potentially new, most practical and economic way and readily available and applicable malaria vector control tool for incorporation into integrated vector management strategies and contribute to the provision of prophylactic measures, particularly at an individual level [3,25,58,59,61].

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