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Essential oil and composition of *Tagetes minuta* from Uganda. Larvicidal activity on *Anopheles gambiae*

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ABSTRACT

As the search for alternatives to synthetic medicine goes on, several plants have been identified as possible natural insecticides, among which is *Tagetes minuta*. In this study essential oil from *T. minuta* was obtained by hydrodistillation. It was tested against the 3rd and 4th instar *Anopheles gambiae* to determine the larvicidal activity. Six different concentrations of this essential oil were studied and compared with that of methyl parathion, a synthetic organophosphorus insecticide for 24 h. GC–MS results indicated the presence *trans*-ocimene 15.90%, I-verbenone 15% of limonene 8.02%, tegetone 3.56%, and 2-pinen-4-one 7.84% as the major compounds in the essential oil. The LC_{50} was 2.9 mg/l while the LC_{90} was 3.29 mg/l after 2 h of exposure. After 6 h of exposure, the LC_{50} and LC_{90} were 2.31 mg/l and 2.68 mg/l, respectively, while after 12 h, it was 1.49 and 1.82 mg/l, respectively. After 24 h, the LC_{50} and LC_{90} were not determined because the mortality was 100%. Therefore effectiveness of essential oil from *T. minuta* is comparable to that of synthetic insecticides and can be studied further in lieu of becoming a possible alternative.

1. Introduction

Plant essential oils are obtained from non-woody parts of the plant such as leaves, stem and flowers (foliage) (Daizy et al., 2008). The essential oil can be extracted from these plants through steam distillation or hydrodistillation (Gil et al., 2000). They are complex mixture of mainly terpenoids, particularly monoterpenes (C_{10}) and sesquiterpenes (C_{15}) (Daizy et al., 2008). Due to the fact that plants possess these volatile monoterpenes and some essential oils, this provides an important defence strategy to the plants, especially against herbivorous insect pests and pathogenic fungi which tend to attach them (Tholl, 2006). These volatile terpenoids, due to their strong scent also play an important role in plant–plant interactions and help to attract insects for pollination (Tholl, 2006).

Essential oils obtained from aromatic plants serve as potential source of pharmacologically active compounds such as analgesics, anti-inflammatories, anti-tumorals, pesticides, antibiotics and digestives (Zygadlo and Grosso, 1995). Bioassays on a number of essential oils show repellence activity against mosquitoes (Misra and Pavlostathis, 1997).

Essential oils can constitute effective alternatives to synthetic pesticides without producing adverse effects on the environment (Isman, 2000; Isman and Machial, 2006). These oils are being tested as potential candidates for weed, pest and disease management (Isman, 2000). Essential oils are easily extractable, eco-friendly, as they get easily catabolized and degraded in the environment (Zygadlo and Grosso, 1995), and thus do not persist in soil and water (Isman, 2000; Isman and Machial, 2006), possess low or no toxicity against vertebrates such as fishes, birds and mammals (Enan et al., 1998) and play an important role in plant protection against pests (Isman, 2000; Isman and Machial, 2006; Bakkali et al., 2008).

Mosquitoes are nuisance pests and a major vector for the transmission of several life threatening diseases (Rajkumar and Jebanesan, 2010). *Aedes aegypti* is known to carry dengue and yellow fever; malaria is carried by *Anopheles stephensi* and *Anopheles gambiae* and filarial disease is transmitted by *Culex mosquito*.

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Anopheles gambiae is the principal vector of malaria, a disease that afflicts more than 500 million people and causes more than 1 million deaths each year (Rajkumar and Jebanesan, 2010).

The control of mosquito is an important public health concern around the world (Mansour et al., 2012). Due to drug resistance, insecticides against mosquitoes are becoming ineffective (Vincent, 2000). In many parts of the world, plant-derived products have been used to repel or kill mosquitoes and other domestic insect pests. Solvent extracts and essential oils of many plants show varying levels of insect-repellent properties (Omolo et al., 2004). Since adulticides may reduce the adult population only temporarily at the site the insecticide was applied to, most mosquito control programmes target the larval stage at their breeding sites with larvicides, which is a more efficient approach (Omolo et al., 2004; Conti et al., 2010).

Tagetes species have been reported in literature to have many of the above properties and special emphasis has been placed on *Tagetes minuta* as having insecticidal properties (Zoubiri and Baaliouamer, 2011).

T. minuta is an aromatic essential plant with a broad spectrum of biological activities among which are medicinal, antioxidant and antibacterial properties (Daizy et al., 2007). It has rich natural product chemistry and produces highly volatile essential oils that are widely used in the cosmetic, perfumery and as a flavouring agent in food and beverages and as medicine (Vasudevan et al., 1997). *T. minuta* not only exhibits very good medicinal properties but also has strong nematicidal, insecticidal and antimicrobial activity (Daizy et al., 2007).

T. minuta is a widely distributed plant in Uganda and thrives mainly in the rainy season. It is used for different purposes in different regions of the country. Among the Bakiga in south western Uganda, it is used for treatment of head ache, pain and epilepsy. The patient can either smell the strong scent or chew the leaves or rub the herb on the affected part, e.g. head, joints (Hamill et al., 2000; Lacroix et al., 2011).

Tagetes species are sometimes referred to as marigold. They may reduce parasitic plant nematodes (PPN) populations (Cerruti et al., 2010). *T. minuta* is also traditionally used by farmers in parts of Kenya for seed storage. Several papers have been written on the use of extracts of *T. minuta* (Gakuya et al., 2013), and these also report a reasonably high level of effectiveness. The plant essential oil has also been used in the control of *Rhipicephalus microplus* in cattle (Andreotti et al., 2013). *T. minuta* essential oil had significant effect in controlling the spread and reproduction of ticks by affecting their egg production and killing the surviving ones on the bodies of the affected cattle (Andreotti et al., 2013). This indicates that the plant has a strong acaricidal activity against the larvae, nymph and adult tick. Other species of *Tagetes* also have been reported to have insecticidal properties such as *Tagetes patula* (Dharmagadda et al., 2005; Prakash et al., 2012).

The main aim of this study was to assess the larvicidal activity of essential oils from *T. minuta* against *Anopheles gambiae* larvae. It is hoped that this information will help in providing an alternative natural insecticide that is readily available in the environment and provide clues on its effectiveness.

2. Methodology

2.1. Plant material

Fresh *T. minuta* was collected from Mabira Forest in the morning hours in the month of November 2012. It was transported to Makerere University from where essential oils were extracted immediately. A voucher specimen was deposited at Makerere University herbarium (voucher number CK01).

2.2. Isolation of essential oil

Essential oil was extracted by hydro-distillation in a Clevenger type apparatus for 3 h with a separate extraction chamber. 1.5 kg of fresh plant material was extracted with 2 l of distilled water. The resulting essential oils (4 ml/kg fresh weight) were dried over anhydrous sodium sulphate. The oil was kept in refrigerated conditions prior to the larvicidal assay and GC–MS analysis.

2.3. Mosquitoes

The mosquito larvae of *Anopheles gambiae* were obtained from Uganda Virus research institute, Entebbe Offices. The 4th and 3rd instar larvae were selected and were used in the assay.

2.4. Larvicidal activity

Larvicidal activity of the essential oil of *T. minuta* was evaluated according to the WHO protocol (World Health Organisation, 2005). Essential oil was tested at 3, 6, 12, 24, 48, 96 mg/l concentrations in triplicates. Since the essential oil does not dissolve in water it was first dissolved in ethanol (99.8%) (Dharmagadda et al., 2005; Vinayagam et al., 2008; Cheng et al., 2009). The essential oil was dissolved in appropriate quantities to make 1 ml of ethanol solution, and then diluted in 249 ml of distilled water to obtain the desired concentrations. The control was prepared using 1 ml of ethanol in 249 ml of distilled water. The oil–ethanol–water solution was stirred for 30 s with a glass rod. To each of the beakers containing the essential oil, ethanol and water, 25 late third instar larvae of *Anopheles gambiae* were introduced. The experiment was observed over a 24 h period during which no food was given to the larvae (Govindarajan et al., 2013). Preliminary experiments using very low concentrations of the essential oil did not produce significant results while very high concentrations resulted in 100% mortality.

The lethal concentrations (LC_{50} and LC_{90}) were then calculated by Probit analysis (Finney, 1971).

2.5. GC–MS analysis

An Agilent GC 6890N/MSD 5973B with a fused silica HP-5ms column (30 m, 0.25 mm, 25 μm) was used. Total flow was 48.7 ml min^{-1} at 44.6 kPa carrier gas pressure, and the resulting column flow was 0.9 ml min^{-1}. A temperature profile that reliably separates most compounds and was previously established as a standard method in this laboratory was used: 50 °C (2 min), 5 °C min^{-1} to 325 °C (20 min). The samples were injected at 230 °C inlet temperature in 50:1 split mode. For MS detection, ionisation was achieved by electron impact at 70 eV. The scan range of the quadrupole was set to 50–950 m/z.

For identification, the GC–MS data was deconvoluted by AMDIS 2.66. A list of probable hits was generated with NIST '08 and Wiley '09 databases. In addition to the match quality of the mass spectra, the retention index of each compound was determined and used as decisive factor. Retention indices were determined relatively to a reference sample of C_7–C_{40} *n*-alkanes according the IUPAC Compendium of Chemical Terminology (McNaught and Wilkinson, 1997). For each peak, the substance with the best match in both mass spectrum and retention index was assigned as most probable compound. This method is an existing method from our laboratory (University of Natural Resources and Life Sciences, Vienna (BOKU)) that has proven over the years to be able to separate most investigated compounds.

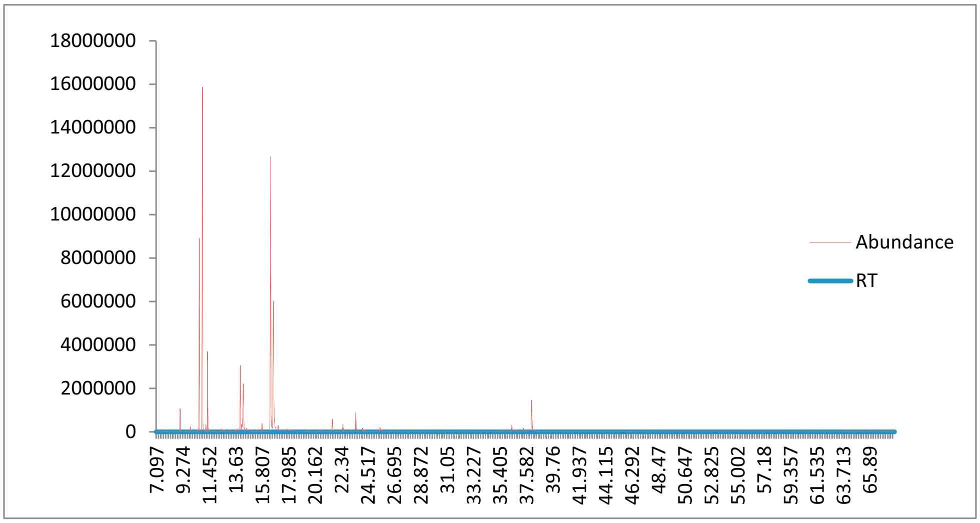

Fig. 1. GC–MS results of the essential oil of *Tagetes minuta*.

3. Results and discussion

The hydro distillation yielded essential oil of 4 ml/kg fresh weight. Fifty-eight compounds were identified by GC–MS (Fig. 1 and Tables 1 and 2). The major compounds were targetone 3.56%, limonene 8.02%, 2-pinen-4-one 7.84%, verbenone 15% and β-*trans*-ocimene 15.90%. However, there were traces of terpinenes, sesquiterpene diol, and araldite 502 in small quantities.

The composition of the essential oil was already hinting towards its usefulness as nematocide. For example, verbenone is a natural terpene which is found naturally in a variety of plants. The chemical has a pleasant characteristic odour. Besides being a natural constituent of plants, it and its analogues are insect pheromones. In particular, verbenone has an important role in the control of the southern pine bark beetle (Santoyo et al., 2005).

Essential oil composition of *T. minuta* in this study is comparable to other studies conducted in other countries (Tereschuk et al., 1997; Gil et al., 2000; Daizy et al., 2007; Andreotti et al., 2013). There are four major compounds, limonene ocimene (*cis* and *trans*), dihy-drotagetone, and tagetone that represent more than 70% of the essential oil in most of the reports above although the concentrations vary. This may be due to the different seasons when the sampling took place in addition to the geographical location of the country.

3.1. Statistical analysis

The results of the larvicidal assay were analysed statistically using SPSS 20. The Probit analysis model (Finney, 1971) was used to determine the LC_{50} and LC_{90} at different times of the experiment. Since the experiment was done in triplicate, the average mortality was used for every concentration (Tables 3 and 4).

At 24 h, the mortality was 100% using the essential oil. Therefore, no statistical evaluation was conducted. A positive correlation was observed between the essential oil concentration and mortality. Also there was a positive correlation between length of exposure and mortality. A longer exposure at the same oil concentration caused higher mortality. At higher concentrations (24–96 mg/l), the larvae became weak as soon as the essential oil was introduced to the beaker. They settled at the bottom of the beaker, some of them dying immediately. At higher essential oil concentrations, the larvae became less mobile. The larvae in the control experiment developed into adults on the third day (after 48 h) while in the rest of the experiments (3–12 mg/l), some pupae developed but died before becoming adults. In the positive control experiment where methyl parathion was used, it was observed that some larvae could survive even after 36 h of exposure which was not the case with *T. minuta*. It was noted that methyl parathion was slow in action but still effective in killing the mosquito larvae. None was able to grow to maturity.

The results of this study are comparable to the previous reports on larvicidal activities of plant essential oils (Enan et al., 1998; Isman, 2000; Dharmagadda et al., 2005; Isman and Machial, 2006; Daizy et al., 2008) although *T. minuta* essential oil appears to be more toxic according to the results of this study. In a study on the essential oil of *T. patula*, the larvicidal bioassay results showed that it was most toxic against *A. aegypti* (LC_{90} 38 mg/l) followed by *A.*

Table 1
Summary of the major compounds identified by gas chromatography–mass spectrometry (GC–MS), in the essential oil of *Tagetes minuta*.

Sn	Cas number	Name	Synonym	Retention time	Abundance (%)
1	138-86-3	Cyclohexene, 1-methyl-4-(1-methylethenyl)-	Limonene	10.667	8.02
2	3779-61-1	1,3,6-Octatriene,3,7-dimethyl-(E)-	β-*Trans*-ocimene	10.933	15.90
3	360-34-9	>2-Hexanone, 1,1,1-trifluoro-	1,1,1-trifluoro-2-hexanone	11.537	3.09
4	6752-80-3	2,6-Dimethylocta-5,7-dien-4-one	Tagetone	14.293	3.56
5	94250-29-0	1h,4h-3a,8a-Ethanoazulen-4-ol, hexahydro-(3aα,4α,8aα)-	Endo-tricyclo[5.3.2.0(1,7)]dodecan-2-ol	14.296	1.26
6	6752-80-3	2,6-Dimethylocta-5,7-dien-4-one	Tagetone	14.3	3.83
7	1196-01-6	Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl-(1s)-	l-Verbenone	16.558	15.00
8	80-57-9	>Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl-	2-Pinen-4-one	16.781	7.84
9		4,6-Dimethyl-2-[2-(trimethysilyl)ethynyl]aniline		38.07	1.51

Table 2
All compounds identified in the essential oil of *Tagetes minuta* by GC–MS including retention indices.

Name	Synonym	RT	RI	Amt (%)
α-Pinene	Bicyclo[3.1.1]hept-2-Ene, 2,6,6-trimethyl-	7.986	930.5	0.04
Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-	(+)-Sabinene	9.080	969	0.83
β-Myrcene	1,6-Octadiene, 7-methyl-3-methylene-	9.557	985.8	0.04
α-Phellandrene	1,3-Cyclohexadiene, 2-methyl-5-(1-methylethyl)-	9.953	999.8	0.19
Benzene, 1-methyl-2-(1-methylethyl)-	O-Cymene	10.540	1020.4	0.08
Cyclohexene, 1-methyl-4-(1-methylethenyl)-	Limonene	10.667	1024.9	8.03
1,3,6-Octatriene, 3,7-dimethyl-(E)-	β-*Trans*-ocimene	10.933	1034.2	15.90
1,3,6-Octatriene, 3,7-dimethyl-	β-Ocimene	11.212	1044.1	0.25
2,6-Dimethyloct-7-en-4-one	Dihydrotagetone	11.356	1049.1	3.10
1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	Γ-Terpinene	11.537	1055.5	0.04
2-Cyclohexen-1-one, 6-methyl-3-(1-methylethyl)-		12.483	1088.8	0.10
2-Cyclohexen-1-one, 6-methyl-3-(1-methylethyl)-		12.487	1088.9	0.10
5,7-Octadien-4-one, 2,6-dimethyl-(E)-	(E)-Tagetone	14.043	1142.9	2.43
5,7-Octadien-4-one, 2,6-dimethyl-(E)-	(E)-Tagetone	14.048	1143.1	2.57
3-Methylbut-2-enoic acid, 4-cyanophenyl ester		14.162	1147	0.22
5,7-Octadien-4-one, 2,6-dimethyl-(E)-	(E)-Tagetone	14.293	1151.5	3.56
Vanillin		14.296	1151.6	1.26
5,7-Octadien-4-one, 2,6-dimethyl-(E)-	Tagetone	14.300	1151.8	3.84
Sorbic acid vinyl ester	Vinyl (2e,4e)-2,4-hexadienoate	14.578	1161.4	0.21
3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-	1-Terpinen-4-ol	15.026	1176.8	0.06
Bicyclo[3.1.1]hept-2-en-6-one, 2,7,7-trimethyl-	Chrysanthenone	15.404	1189.9	0.07
3-Methyl-6-(1-methylethylidene)-2-cyclohexen-1-one	3-Terpinolenone	15.832	1204.9	0.41
Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl-(1s)-	L-Verbenone	16.558	1231	15.00
Tropidine, 2-acetyl-		16.749	1237.9	0.75
Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl-(1s)-	L-Verbenone	16.772	1238.7	0.34
Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl-	2-Pinen-4-one	16.781	1239.1	7.84
Phenol, 2,3,4,6-tetramethyl-	1.2,3,4,6-Tetramethylphenol	17.161	1252.7	0.20
2-Cyclohexen-1-one, 3-methyl-6-(1-methylethenyl)-(S)-		17.902	1279.4	0.16
Caryophyllene	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-[1r-(1r*,4e,9s*)]-	21.649	1421.7	0.53
α-Caryophyllene	1,4,8-cycloundecatriene, 2,6,6,9-tetramethyl-(E,E,E)-	22.507	1456.1	0.30
Γ-Elemene	1-Methyl-2,4-bis(1-methylethylidene)-1-vinylcyclohexane	23.569	1498.6	0.85
1r,4s,7s,8r,11r-2,2,4,8-Tetramethyltricyclo[5.3.1.0(4,11)]Undecan-7-Ol		24.131	1522.3	0.15
(−)-Spathulenol	1h-Cycloprop[E]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-[1as-(1aα,4aα,7β)]	25.567	1583.1	0.23
1h-Cycloprop[E]azulen-7-Ol, decahydro-1,1,7-trimethyl-4-methylene-[1ar-(1aà,4aà,7á,7aá,7bà)]-	(+)-Spathulenol	25.571	1583.2	0.22
Caryophyllene oxide	(−)-5-Oxatricyclo[8.2.0.0(4,6)]dodecane,12-trimethyl-9-methylene-	25.706	1588.9	0.12
Cyclopropanecarboxylic acid, 2-methyl-, 2,6-di-t-butyl-4-methylphenyl ester	2,6-Ditert-butyl-4-methylphenyl 2-methylcyclopropanecarboxylate	36.434	2042.9	0.30
3-Methylbut-2-enoic acid, 4-cyanophenyl ester		36.815	2059.1	0.04
2(1h)Naphthalenone, 3,5,6,7,8,8a-hexahydro-4, 8a-dimethyl-6-(1-methylethenyl)-		37.384	2083.1	0.08
Acetic acid, 3-hydroxy-6-isopropenyl-4, 8a-dimethyl-1,2,3,5,6,7,8,8a-octahydronaphthalen-2-Yl ester		37.390	2083.4	0.15
3-Pyridineacetic acid, 1,2,5,6-tetrahydro-1-methyl-2-oxo-4-phenyl-	3-Carboxymethyl-1-methyl-4-phenyl-5,6-dihydro-2-pyridone	38.065	2112	0.29
Cyclopropanecarboxylic acid, 2-Methyl-, 2,6-di-t-butyl-4-methylphenyl ester		38.070	2112.2	1.51
2(3h)-Furanone, 5-(2,5-dimethylphenyl)-4-methyl-	5-(2,5-Dimethylphenyl)-4-methyl-2(3h)-furanone	38.077	2112.5	0.18

RT – retention time; RI – retention index; Amt – amount.

Table 3
LC_{50} and LC_{90} values at 2, 6, 12, and 24 h for the essential oil from *Tagetes minuta* against *Anopheles gambiae*.

Time (h)	LC_{50} mg/l 95% FCL	LC_{90} mg/l 95% FCL
2	2.9 (1.523, 2.924)	3.29 (3.219, 4.609)
6	2.31 (0.598, 2.633)	2.68 (1.929, 3.904)
12	1.49 (0.384, 1.522)	1.82 (1.800, 3.013)

FCL: fiducial confidence interval.
LC_{50}: lethal concentration 50.
LC_{90}: lethal concentration 90.

Table 4
LC_{50} and LC_{90} values at 2, 6, 12, 24, and 36 h for methyl parathion against *Anopheles gambiae*.

Time (h)	LC_{50} mg/l 95% FCL	LC_{90} mg/l 95% FCL
2	4.10 (3.79, 5.57)	4.90 (3.342, 6.39)
6	2.77 (2.24, 3.92)	3.74 (3.09, 5.64)
12	2.36 (2.11, 3.84)	2.94 (2.52, 4.94)
24	1.99 (1.54, 2.44)	2.40 (2.14, 2.62)
36	1.60 (1.04, 2.54)	1.84 (1.36, 2.67)

stephensi (LC_{90} 58 mg/l) and *Culex quinquefasciatus* (LC_{90} 72 mg/l) (Prakash et al., 2012). Compared to these finding, the essential oil of *T. minuta* clearly has a lower LC_{90}. Accordingly, it is more toxic.

4. Conclusion

This study indicates that essential oil from *T. minuta* is a potent natural insecticide. It is environmental friendly and has probably no negative effects on humans since it can also be used in food as a spice (Vasudevan et al., 1997). Its effectiveness is comparable to that of methyl parathion and it can be used for the same purpose. The study therefore recommends the use of essential oil formulation from *T. minuta* as a natural larvicide to curb mosquitoes when they are at larvae stage. It will be a helpful tool to reduce the malaria epidemic which is a tremendous problem in Uganda (Lacroix et al., 2011).

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