In vitro anti-tick properties of the essential oil of *Tagetes minuta* L. (Asteraceae) on *Hyalomma rufipes* (Acari: Ixodidae)

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In this study we examined the anti-tick properties of the essential oil of *Tagetes minuta* L. (Asteraceae: Asterales) against *Hyalomma rufipes* ticks. We obtained the essential oil of *T. minuta* by hydro-distillation of a combination of fresh flowers, leaves and soft stems, and analysed these by using gas chromatography (GC) and gas chromatography-linked mass spectrometry (GC-MS). The oil had a high percentage of monoterpenes and the major compounds identified were cis-ocimene (28.5%), beta-ocimene (16.83%) and 3-methyl-2-(2-methyl-2-butenyl)-furan (11.94%). *Hyalomma rufipes* adults displayed a significant ($P < 0.05$) dose repellent response to the essential oil of *T. minuta*. Probit analysis indicated a repellent EC_{50} of *T. minuta* essential oil for male ticks to be 0.072 mL/mL (CI 0.053 mL/mL to 0.086 mL/mL) and 0.070 mL/mL (CI 0.052 mL/mL to 0.084 mL/mL) for female ticks. There were no significant differences in repellent responses between male and female ticks. The oil also significantly ($P < 0.05$) delayed moulting of 60% of *H. rufipes* engorged nymphs. These results suggest that *T. minuta* may be a potential source of anti-tick agents.

Introduction

The ability of ticks to transmit pathogens to livestock and their direct effect on the health and condition of animals has resulted in great economic losses in various parts of the world. McCosker (1979) estimated the global costs of control and productivity losses as $7000 million annually ($7/head/annum). In a recent study carried out in Tanzania, it was estimated that the total annual national loss as a consequence of tick-borne diseases was $364 million with a mortality of 1.3 million cattle (Kivaria 2006).

The current methods of tick control rely heavily on chemical acaricides and repellents. These chemicals have numerous detrimental effects, including environmental pollution (Bhattacarya, Sarkar & Mukherjee 2003) and acaricide resistance (Li *et al*. 2003), promoting the search for novel compounds from alternative sources such as plants. Plants contain secondary metabolites that are frequently stored in specialised tissues, and which may have biological or repellent properties when extracted (Evans 1989). Some of the plants traditionally used in Africa, for example, neem (*Azadirachta indica*) and *Gynandropsis gynandra* respectively, have effective acaricidal and tick repellent activity (Abdel-Shafy & Zayeb 2002; Lwande *et al*. 1999). In this regard, undiluted neem oil deterred larval and nymphal attachment, inhibited feeding (90% – 100%), reduced fecundity (30% – 45%) and egg hatchability (47% – 55%), decreased larval (22% – 93%) and nymphal (98%) moult of some ixodid ticks (Kaaya & Saxena 1998). Oil extracted from leaves of the tropical shrub *Ocimum suave*, repel and kill all stages of the tick *Rhipicephalus appendiculatus* (Mwangi *et al*. 1995). It has been known for many years that essential oil of *Tagetes minuta* has both repellent and growth inhibitory properties against insect pests (Jacobson 1983). *Tagetes minuta* also has the potential to control ticks (Moyo *et al*. 2009; Wanzala 2009). No experimental evidence, however, on the effects of *T. minuta* against *Hyalomma rufipes* has been found in the literature. In South Africa, *T. minuta* (also known as kakhi bush or mexican marigold) grows as a weed on maize farms, at roadsides and in gardens. The aim of this study was to evaluate the *in vitro* repellent and growth inhibitory bioactivities of *T. minuta* essential oil on *H. rufipes* adults. This tick species is widely distributed in Africa and is capable of transmitting disease-causing pathogens, to people and livestock, such as Crimean-Congo haemorrhagic fever virus and *Babesia* species, respectively (Gray & De Vos 1981; Walker *et al*. 2003).

Materials and methods

Ticks

Hyalomma rufipes used in this study were bred on Himalayan rabbits at the Animal Production unit of the Department of Biology, University of Limpopo (MEDUNSA Campus). For rabbit

infestation, ticks were placed in cotton bags attached to the back of the host. The hosts were shaved on their backs prior to infestation to facilitate attachment (Magano, Els & Chown 2000).

Plant material and extract preparation

Fresh leaves, branches and flowers of *T. minuta* were collected in April 2004 from a nursery and a maize field managed by the Department of Plant Production and Soil Sciences, University of Pretoria. Fresh plant material was sliced and hydro-distilled by using a clevenger-type apparatus with slight modifications (Evans 1989). Heat was provided by a heating-mantle equipped with a thermostat and the temperature was maintained at 90 °C. Two hundred grams of plant material mixed with 400 mL of distilled water was placed into a round bottomed flask and hydro-distilled for 2 hours. The distillate was collected as the essential oil band above the water. The essential oil obtained was stored in a refrigerator at 4 °C until used. A mixture of n-hexane and the distillate was prepared and the following concentrations were used: 0.107 mL/mL, 0.053 mL/mL and 0.027 mL/mL. The components of the essential oil were determined by Gas chromatography (GC) and Gas chromatography-linked mass spectrometry (GC-MS), (QP 20-10 Shimadzu GC-MS instrument).

Gas chromatography conditions

The column temperature was programmed to rise from 50 °C to 300 °C at 10 °C/min. The injector temperature was 250 °C. The total flow rate was 24 mL/min and the column flow rate was 1 mL/min.

Gas chromatography-linked mass spectrometry conditions

One µL of essential oil was analysed by using a GC-MS instrument equipped with a Supelco equity 1 column with a film thickness of 30 m x 0.25 µm. Ultra high purity helium was used as the carrier gas with an injector split ratio of 20:1. The ion source and interphase temperatures were 200 °C and 250 °C, respectively. The solvent cut time lasted 4 min and the detector gain was 0.70 kV. A Wiley 229 library search was conducted on major peaks of each sample in order to identify the components of the sample. The relative percentage of each compound was determined by area normalisation methods, whereby the area under the peak was calculated as (width at half height) x height (Houghton & Raman 1998).

Repellency bioassay

The tick-climbing repellency bioassay used in this study was a modification of that described by Carroll (1998). Two glass rods of similar length were each fixed vertically and firmly on a polystyrene platform ($L = 5$ cm, $W = 5$ cm, $H = 3.5$ cm). A height of 21 cm of each glass-rod was exposed above its platform. The two platforms with inserted glass rods were fixed separately on the inside of a plastic container ($L = 35$ cm, $W = 24$ cm and $H = 8$ cm). Water was added to the container in such a way that it completely surrounded each of the platforms and almost reached the height of each of the platforms. One hundred µL of the test solution (n-hexane plus distillate of *T. minuta*) was placed on a test filter paper strip (Whatman No. 1), (2.5 cm x 5 cm). A control filter paper strip of the same kind and size was impregnated with n-hexane only. After evaporating the solvent by air-drying, the filter paper strips (test and control) were used to cover the top 5 cm of the respective glass-rods. Two additional neutral filter paper strips of the same size (2.5 cm x 1.5 cm) were each fixed below the test and control filter papers on the glass rods so that the adjacent edges of the filter papers met. Ten adults of *H. rufipes* of both sexes were released separately on the test and control platforms, and five replicates for each treatment (male and female) group were performed. The positions of ticks on the glass rods were recorded 1 hour after their release. Ticks that were found on the upper filter paper were considered not to be repelled. Those on the bottom neutral filter paper, on the naked part of the glass-rod, and on the platform were considered repelled. Ticks that moved into the water were dried and replaced. The repellent effect was calculated as percentage repellency, according to the formula:

> Percentage repellency = 100 - [(Mean no. of ticks on the upper filter paper on test rods) / (mean no. of ticks on upper filter paper on control rods)] x 100. (Jantan & Zaki 1998)

Growth inhibition bioassay

In this bioassay, 10 µL of *T. minuta* essential oil was applied on a 1 cm x 1 cm filter paper (Whatman no. 1). The filter paper was introduced into a glass vial (height = 7.2 cm and diameter = 2.3 cm) containing 10 engorged nymphs held in a plastic net (25 mesh) to prevent direct contact with the essential oil. The control had untreated filter paper. The top of the glass vial was plugged with cotton wool (weight 0.99 g), held tight in tissue paper (3 cm x 3 cm) allowing the air in the test glass vial to be saturated with volatiles from the essential oil. The bioassay was replicated five times in the test and control treatments. Glass vials containing ticks were carefully kept in chambers at 75 ± 5% relative humidity, 25 ± 1 °C and a natural day-night regime. Test and control vials were kept in separate chambers, but laboratory conditions for both groups were the same. The number of ticks that completely moulted 25 days post-treatment was counted and percentage inhibition calculated with the formula:

> Percentage inhibition = 100 x [(1 - percentage moult in treated group) ÷ (percentage moult in control group)]. (Lok, Pollack & Donnelly 1987)

Data analysis

Repellency bioassay

Probit analysis (EPA 2006) was used to determine the effective concentration needed to repel 50% (EC_{50}) of ticks as well as the Confidence Interval (CI) of the mean number of ticks repelled by the plant extract. Each replication was considered independently. Confidence intervals (95%) of EC_{50} were used to determine the difference in the response

between male and female ticks (Lerdthusnee *et al*. 2003). Data were normalised by transformation into the arc sin square root of the proportion of ticks repelled or inhibited from moulting prior to subjecting it to one-way independent ANOVA (analysis of variances), (Hammer, Harper & Ryan 2001). The repellent responses of male and female ticks for each concentration were pooled together for ANOVA, because no significant differences were found between male and female ticks at all concentrations for *T. minuta* essential oil; however, the mean (± s.e.) of untransformed data are reported.

Growth inhibition bioassay

The number of nymphs that moulted completely to adults was counted and data were presented as percentage inhibition of moulting. The Student's *t*-test was used to determine significance of the differences ($P < 0.05$) between the treatments.

Ethical considerations

This study was approved by the Animal Ethics Committee, University of Limpopo, MEDUNSA Campus. Rabbits used in the study were treated humanely.

Results

The yield of the essential oil of *T. minuta* obtained, following distillation, was 1 mL per 200 g of fresh plant material. The GC-MS analysis of the distillate of the aerial parts of *T. minuta* revealed that the oil is rich in terpenes (Table 1). The major constituents of *T. minuta* essential oil were cis-ocimene (28.50%), beta-ocimene (16.83%) and 3-methyl-2-(2-methyl-2-butenyl)-furan (11.94%). In the tick-climbing repellency bioassay, *H. rufipes* showed a significant ($P < 0.05$) dose repellent response in the climbing repellency bioassay (Table 2). Probit analysis indicated a repellent EC_{50} of *T. minuta* essential oil for male ticks to be 0.07 mL/mL and 0.07 mL/mL for female ticks. With a density of ± 0.87 mg/mL (Azafran 2004), this equates to an EC_{50} of ± 0.06 mg/mL. The repellent responses between male and female ticks did not differ significantly (Table 3). Furthermore, the essential oil of *T. minuta* delayed moulting in 60% (s.e. ± 4.7) of nymphs after 25 days, compared to the control group.

TABLE 1: Constituents of the essential oil of *Tagetes minuta* and their relative amounts (%) according to Gas chromatography-linked mass spectrometry analysis.

Compound	Relative Percentage
2,3,5-trimethylfuran	0.65
Linalyl acetate	0.72
Cis-ocimene	28.50
Dihydrotagetone	6.42
Carvenone	0.63
Beta-ocimene	16.83
Cis-tagetone	1.58
2-Butanone	4.97
Bicyclo(2.2.1)heptane	0.54
Piperitenone	7.96
3-methyl-2-(2-methyl-2-butenyl)-furan	11.94
2,3,4,6-Tetramethylphenol	0.36
2-cyclohexen-1-one	2.71
Unknown	1.64

TABLE 2: Dose-dependent repellent response of *Hyalomma rufipes* (male and female ticks pooled) to *Tagetes minuta* essential oil.

Concentration mL/mL	Mean proportion of ticks repelled ± s.e.	% Repellency
0	0.22 ± 0.03	0.00
0.027	0.23 ± 0.04	2.04
0.053	0.33 ± 0.04	14.81
0.107	0.95 ± 0.02	93.61

Significant difference of dose-repellent-response of ticks was observed.
s.e., standard error.
$df = 3.23$; $F_{observed} = 70.21$; $F_{critical}$ (at 0.05) = 3.03.

TABLE 3: Effective concentration needed to repel 50% of ticks (EC_{50}).

Plant	Sex of Ticks	EC_{50} (mL/mL)	Lower CI (95%)	Upper CI (95%)
Tagetes minuta	Male	0.07	0.05	0.09
	Female	0.07	0.05	0.08
	Both	0.07	0.06	0.08

CI, indicates confidence interval.

Discussion

The essential oil of *T. minuta* used in this study was rich in terpenes based on GC and GC-MS analysis. Chemical analysis carried out on different species of *Tagetes* grown in Northern Italy indicated that dihydrotagetone, tagetones, ocimenones and piperitone occurred in *Tagetes erecta*, *T. minuta*, *Tagetes patula* and *Tagetes tenuifolia* (Marotti *et al.* 2004). These compounds were also present in the essential oil of *T. minuta* evaluated in this study.

The results obtained in this study indicate that the essential oil of *T. minuta* has tick repellent and growth inhibitory properties. Tick repellency by the essential oil of *T. minuta*, corroborates studies by Lwande *et al*. (1999) who further showed that this was because of one of its constituents, beta-ocimene. Even though it is important to evaluate individual compounds in suitable bioassays for repellency (Lwande *et al*. 1999), whole oil, such as the one used in this study, may cause increased bioactivity compared to individual compounds because of synergistic effects. Ticks have highly efficient sensory organs. The tick's sensory organ, the Haller's organ, is situated on the dorsal surface of each foreleg and it has both olfactory and gustatory chemosensilla (Sonenshine 1991). Olfactory chemoreceptors or sensilla perceive volatiles, whilst gustatory chemoreceptors perceive stimuli on contact (McMahon, Kröber & Guerin 2003).

Despite the necessity to explore plant based repellents as tick control agents, there is still a future need to improve on the longevity of effective, yet extremely volatile repellents in order to compete with registered compounds. Several studies deal with the improvement of formulations of plant oils to increase their longevity through the development of nano-emulsions, improved formulations and fixatives (Maia & Moore 2011). Kaaya and Saxena (1998) used petroleum jelly as a carrier for plant extract during an *in vivo* study. A

further approach that could be relevant in the sustainable management of ticks is through the disruption of their life cycle by targeting engorged immature stages. This may result in the reduction of tick infestations to low and controllable levels, hence reducing the tick population during favourable climatic conditions. During this study, moulting of engorged nymphs of *H. rufipes* was significantly ($P < 0.05$) delayed by 60%. This could be attributed to tagetone, one of the identified constituents of *T. minuta* used in this study, possessing growth inhibitory properties (Jacobson 1983). These results are in agreement with findings of another study; the essential oil of a variety of *T. minuta* (genotype TM-1) deterred oviposition in *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) by 81% and suppressed its egg hatchability by 91% when applied at a dosage of 70 000 ppm on filter paper (Alok *et al*. 2005). The bioactive compound(s) are very likely to be of a volatile nature as no direct contact was established between ticks and the extracts. The rate at which the volatiles diffuse from the glass vials could not be determined, but the delay in moulting indicates that the bioactive constituents of *T. minuta* should be very effective to produce such results with a single dose. Other herbal products that contain essential oils such as citronella oil or *Chrysanthemum* spp. (containing pyrethrum), are available as commercial arthropod repellents (Fradin & Day 2002).

The use of botanicals for the control of ticks is compatible with traditional practices in Africa and Asia, where most resource-poor farmers use plant materials to treat endoparasites and ectoparasites of livestock (Lans & Brown 1998; Madge 1998). Traditional knowledge about the use of these plants is transferred through successive generations, especially in rural communities. Knowledge about the use of individual plant species, however, varies between localities in Africa, and scientific validation of their uses may increase the range of plants available for tick control. This may reduce the burden substantially on plant species that are at risk of extinction.

Conclusion

The results obtained in this study suggest that *T. minuta* is a potential source of tick control agents. Although *T. minuta* is a common weed in rural areas, it is unlikely that high enough concentrations of the volatile oils would be reached to affect ticks when animals are housed closely together with certain plants scattered on the ground. The extracted essential oil of *T. minuta*, however, may be of use in the integrated control of *H. rufipes* or other insects.

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Competing interests

The authors declare that they have no financial or personal relationship(s) which may have inappropriately influenced them in writing this paper.

Authors' contributions

S.R.M. (University of South Africa) and J.N.E. (University of Pretoria) were the project leaders and made conceptual contributions and edited the final manuscripts, F.N. (Cape Peninsula University of Technology) made conceptual contributions, performed the experiments and wrote the first draft of the manuscript.

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