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Protective action of *Tagetes minuta* (Asteraceae) essential oil in the control of *Rhipicephalus microplus* (Canestrini, 1887) (Acari: Ixodidae) in a cattle pen trial

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ABSTRACT

The *Rhipicephalus microplus* tick is globally regarded as the most economically important ectoparasite of livestock, and the evolution of resistance to commercial acaricides among cattle tick populations is of great concern. The essential oil derived from *Tagetes minuta* may be efficacious against cattle tick infestation, and the results of a cattle pen trial using this essential oil for the control of ticks are reported here. The chemical composition of the essential oil was determined by GC–MS and NMR spectroscopy analyses, which revealed the presence of four major components in the essential oil. These components represent more than 70% of the essential oil: limonene (6.96%), β-ocimene (5.11%), dihydrotagetone (54.10%) and tagetone (6.73%). The results of the cattle pen trial indicated significant differences among the average values of the analyzed biological parameters, including the number of ticks, the average weight of the ticks, the average egg weight per engorged female and larval viability. Treatment with the *T. minuta* essential oil prepared in this study promoted significant effects on all biological indicators analyzed. Based on the biological indicators, the essential oil showed 99.98% efficacy compared to the control group when used at a 20% concentration. The results obtained in this study suggest that the *T. minuta* essential oil is a potential *R. microplus* tick control agent and may be used to mitigate the economic losses caused by tick infestation.

1. Introduction

The southern cattle tick, *Rhipicephalus microplus*, inflicts severe economic losses to the livestock industry. Infestation by *R. microplus* causes losses in productivity directly through the effects of ectoparasitism and indirectly through its role as the vector for *Babesia bovis* and *Babesia bigemina*, which cause bovine babesiosis, and *Anaplasma marginale*, which causes anaplasmosis (Grisi et al., 2002; De la Fuente et al., 2008).

The control of *R. microplus* is achieved primarily through the use of chemical acaricides (Andreotti et al., 2011). However, chemical acaricides have not been utilized judiciously, which has led to the evolution of resistance among populations of *R. microplus* (Furlong, 2004; Rosario-Cruz et al., 2009).

The use of plant extracts as tick control agents has been an area of focused research in several countries (Chungsamarnyart et al., 1991; Williams, 1993; Vatsya et al., 2006; Álvarez et al., 2008). Studies using emulsifiable oils from eucalyptus (*Eucalyptus* spp.: Myrtaceae),

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carotenoids extracted from timbo (*Derris urucu*: Fabaceae) (Veríssimo, 2004), and azadirachtin A, which is present in plants of the family Meliaceae (*Melia azedarach*) (Borges et al., 2003; Sousa et al., 2008), have shown promise in controlling this parasite.

Tagetes minuta (Asteraceae) is an annual perennial herb that belongs to the Asteraceae family. This plant's leaves are slightly glossy, green and pinnately dissected into 4–6 pairs of pinnae (Abdel-Shafy and Zayed, 2002; Bastos et al., 2010). This plant is used in popular medicine and grows in temperate regions of South America (Chamorro et al., 2008). The major components of *T. minuta* oil have been previously found to be α-terpineol, (Z)-β-ocimene, dihydrotagetone, (E)-ocimenone, (Z)-tagetone, and (Z)-ocimenone, which is consistent with the composition observed in the present study (Moghaddam et al., 2007).

Previously, a 20% concentration of *T. minuta* essential oil was shown to be needed for its acaricidal effect and was over 95% effective for controlling the following tick species: *R. microplus*, *Rhipicephalus sanguineus*, *Amblyomma cajennense* and *Argas miniatus*. The efficacy of *T. minuta* essential oil has been assayed using the adult immersion test (AIT) and larval packet test (LPT) (Garcia et al., 2012).

Because the control of *R. microplus* using acaricides is problematic, there is a need to find new control strategies. Our hypothesis is that *T. minuta* essential oil is a potential tool for the control of the ectoparasite *R. microplus*. To support this hypothesis, the efficacy of *T. minuta* essential oil in controlling *R. microplus* infection was tested using a cattle pen trial. The *T. minuta* essential oil prepared in this study was analyzed with regard to its effects on tick biology, and the results of a cattle pen trial using this essential oil to control the *R. microplus* tick are reported here.

2. Materials and methods

2.1. Ticks

Ticks used for cattle infestation were obtained from a six year laboratory colony that was sensitive to pyrethroid (SP), organophosphate (OP) and amitraz (Am) and free of *Babesia* sp. and *Anaplasma* sp. The tick colony was maintained at Embrapa Beef Cattle (20°26′ S, 54°43′ W) in Campo Grande, MS, Brazil (Andreotti et al., 2012).

2.2. Plant material and extraction

T. minuta leaves and stems were cultivated at Embrapa Beef Cattle (20°26′ S, 54°43′ W) in Campo Grande, MS, Brazil, dried at 40 °C for 72 h and ground in a grinder with a 5-mm mesh. The total biomass was subjected to steam distillation to extract the essential oil. The plant material was placed on a perforated plate, which served as a support and also allowed for the homogeneous flow of steam. The plate containing the plant material was placed on an extractor, and a gooseneck pipe was attached to transfer the vapors to a condenser (Prakaso et al., 1999).

The steam carried the volatile organic compounds (essential oil) that were present in the plant material into the vapor phase. A container was placed at the end of the condenser to separate the essential oil from the water. The extraction was conducted for 2 h inside an extractor at normal atmospheric pressure and at a temperature of 96–97 °C. The residual water from the essential oil isolation was removed by filtration with anhydrous sodium sulfate. The essential oil was stored in amber flasks.

2.3. Oil extract chromatography (GC/MS)

The essential oil extract was analyzed qualitatively and quantitatively using a GCMS-QP2010 Plus (Shimadzu, Tokio, Japan) equipped with an Rtx®-WAX Crossbond-Carbowax-polyethylene glycol column (30 m × 0.25 mm i.d. × 0.25 μm film thickness – Restek, Bellefonte, PA, USA), a split injector (split ratio 50:1), an automatic injection system and a selective mass detector. The test was performed at 250 °C, and the oven temperature was programmed to increase the temperature from 50 °C to 210 °C at 10 °C/min using He as the carrier gas. The gas flow was 0.7 mL/min at a constant speed of 30 cm/s and an interface of 250 °C. The injector temperature was 200 °C, and the injection volume was 1.0 μL. The sample was prepared in $CHCl_3$. The peak area percentages were calculated without correction factors or internal standards. The peaks were identified by comparison of their mass spectra (MS) to the mass spectral data from the National Institute of Standards and Technology (NIST) and Wiley's FFNSC (Flavor and Fragrance Natural and Synthetic Compounds). The results obtained through this method were also based on an analysis of the fragmentation pattern obtained for each component and comparison of their retention indexes (IR) with the Shimadzu GCMS Solution Program, version 2.53.

2.4. T. minuta (Asteraceae) essential oil

T. minuta essential oil was prepared at a 20% concentration because previous studies showed that this concentration is sufficient for the acaricidal effect, and this concentration is also over 95% effective on the *R. microplus* tick in the adult immersion test (AIT) and larval packet test (LPT) (Garcia et al., 2012). The solution applied to the animals was prepared as a pour-on formulation (*T. minuta* essential oil 20%, isopropanol 72%, DMSO 8%, v/v).

2.5. Cattle pen trial and efficacy assessment

One-year-old Holstein calves were randomly distributed into two groups of six animals each. The bovines were infested with 5000 larvae at three separate time points, specifically, on days 0, 9 and 18. The infestations were performed on these days to produce larvae, nymphs and adults on the day of treatment. All of the animals were infested with ticks on the same day. On the 20th day after the beginning of infestation, one group was treated with 20% *T. minuta* (Asteraceae) essential oil. The solution was applied onto the skin of each animal using approximately 50 mL of solution. The application of the *T. minuta* essential oil was done using a 50 mL falcon tube. The oil formulation was distributed longitudinally on the dorsum of the animals, uniformly. Did not formed clumps in the hair coat, and was not there dripping. The second group (control group) was evaluated for tick production. The tick collection period started at the 1st day and lasted until the 25th day after the last infestation, when the ticks in the control group stopped falling. The oviposition was measured from the day 15 after the beginning of the egg laying, and the fertility was assessed at day 45 after initiation of larval hatching (Drummond et al., 1973). Cattle were cared for in accordance with standards specified in the Guide for Care and Use of Laboratory Animals.

2.6. Statistical analysis

Tick biology was assessed following a previously described method (Andreotti, 2006). The percent reduction was calculated in relation to the following control, untreated group: DT, adult female ticks; DO, egg-laying capacity; and DF, fertility. The efficacy (%) = 100 × [1 − (CRT × CRO × CRF)], where CRT, CRO and CRF represent the reduction in the number of engorging ticks, the egg-laying capacity, and the fertility, respectively, compared to the control group. Student's *t*-test was used to compare the means between the groups.

3. Results and discussion

3.1. Oil extract chromatography

The qualitative and quantitative analyses of the essential oil extract obtained from *T. minuta* showed that there are four major components in the extract. The four compounds, limonene (6.96%), β-ocimene (5.11%), dihydrotagetone (5421%) and tagetone (6.73%), represent more than 70% of the essential oil (Garcia et al., 2012). The results of this study are consistent with those found by Moghaddam et al. (2007).

The *T. minuta* essential oil used in this study was rich in terpenes, as determined by GC and GC–MS analyses. Chemical analysis performed on different species of *Tagetes* grown in northern Italy indicated that dihydrotagetone, tagetones, ocimenones and piperitone occur in *Tagetes erecta*, *T. minuta*, *Tagetes patula* and *Tagetes tenuifolia* (Marotti et al., 2004).

T. minuta oil reportedly has aphicidal properties (Tomova et al., 2005). The terpenes in *T. minuta* oil are responsible for the toxic effects observed in mosquitoes (Seyoun et al., 2002), and the insecticidal activity of *T. minuta* oil has also been observed against stored product pests (Sarin, 2004).

The results obtained in this study indicate that the *T. minuta* essential oil prepared in this study includes β-ocimene, a tick repellent. *Hyalomma rufipes* adults display a significant dose-repellent response to *T. minuta* essential oil (Nchu et al., 2012). However, individual compounds were not evaluated by bioassays for their repellent activity. The compound tagetone may be related to a delayed molting effect in the engorged nymphs of *H. rufipes* (Nchu et al., 2012).

3.2. Cattle pen trial

The results of the cattle pen trial showed significant differences between the averages of the analyzed biological parameters, including the number of ticks, the average weight of the ticks, the average egg weight per engorged female and the viability of the larvae. This finding suggests that treatment with the *T. minuta* essential oil had a significant effect on all of the biological indicators analyzed. Engorged ticks that detached in the first 2 days of the tick collection period reflected the effect of treatment on engorged ticks. Engorged ticks that detached from the 3rd to 13th day reflected the effect on the nymph instar at the moment of the treatment. As there was no evidence of detaching ticks after the 13th day after treatment, the *T. minuta* essential oil likely had a 100% effectiveness on the larvae instar at the time of treatment.

The greatest number of ticks that detached from the control groups of animals was recorded on the 11th day of collection. There was a significant effect on the ability of the female ticks to lay eggs. The eggs collected from the female ticks that detached from the cattle appeared to hatch at a high rate (data not shown). This observation is reflected in the fertility index used to calculate the overall *T. minuta* essential oil efficacy (Table 1). Based on the biological indicators DT, DW, DO and DF, the *T. minuta* essential oil was 99.98% effective compared to the control group (Table 2). This result indicates acaricidal activity against the larvae, nymph and adult tick.

Previous studies have reported the ineffectiveness of *T. minuta* essential oil against ticks (Moyo et al., 2009). A satisfactory efficacy of the *T. minuta* essential oil against *R. microplus* infestation in bovines is reported in this study for the first time. A single spray treatment was sufficient to effectively treat the *R. microplus* infestation. The *T. minuta* essential oil was 99.98% effective and may represent a new biological acaricide for the control of cattle ticks.

Dermal irritation of animals subjected to a 20% *T. minuta* essential oil treatment was not observed in this study. Dermal irritation was assessed according to the method of Moyo et al. (2009).

Our findings are in contrast with those obtained by Moyo et al. (2009). In that study, the researchers investigated the effects of *T. minuta* essential oil and did not see a visible reduction in the tick load. Additionally, neither of the *T. minuta* essential oil concentrations that were tested had an effect on the magnitude of the tick burden of the treated animals compared to the control group. Moyo et al. (2009) tested the flowthrough produced from *T. minuta* leaves after a simple maceration process in water and filtration using only water as a diluent and vehicle for the leaf extract. In this paper, leaves and stems of *T. minuta* were used for the extraction of the essential oil. The purification process of the essential oil was more complex than that used by Moyo et al. (2009); thus, we obtain a concentrated extract of the major molecules present in the plant (Garcia et al., 2012). This essential oil, used for the spray formulation, is most likely much more concentrated than the simple extract produced by Moyo et al. (2009) and is also more pure and does not contain the presence of other substances that could interfere with the action of the essential oil.

The differences in the observations may also be due to differences in the environment in which the plant grew, which may affect the chemical composition of

Table 1
Effect of *T. minuta* treatment on the biological performance of cattle ticks from each individual animal.

Animal	Tick number		Tick mean weight (mg)		Egg weight (mg)		Larval viability	
	Control	*Tagetes*	Control	*Tagetes*	Control	*Tagetes*	Control	*Tagetes*
1	2473	79	225	197	119	29	91	3
2	3171	95	228	165	121	52	98	12
3	2596	0	227	0	130	0	89	0
4	1937	0	225	0	130	0	89	0
5	2017	0	219	0	116	0	96	0
6	2045	29	226	181	127	47	91	15
Mean	2373[a]	34[b]	225[a]	91[b]	124[a]	21[b]	92[a]	5[b]
Standard deviation	473.5	43.0	3.2	99.7	6.0	24.6	3.8	6.8
Coefficient of variation	20.0%	127.1%	1.4%	110.1%	4.8%	115.3%	4.1%	135.1%
p-Value*	$2.80385E^{-07}$		0.0079545		$1.71E^{-06}$		$8.88E^{-11}$	

Different letters indicate means in the same line with different $\alpha < 0.01$.
* The *p*-value refers to the Student's *t* test to compare two means.

the plant, or differences in the extraction or purification methods. López et al. (2011) extracted the essential oil of *T. minuta* and reported concentrations of 1.3% limonene, 16.3% ocimene, 10.3% dihydrotagetone, and 65.3% tagetone. In our research, we found concentrations of 6.96%, 5.11%, 54.21%, and 6.73% for the same chemical compounds, respectively. We especially highlight the differences between dihydrotagetone and tagetone, which varied 5.7- and 9.7-fold, respectively. These observed variations are supported by Chamorro et al. (2008), who reported wide variations between these four compounds. These authors found variations of 259%, 199%, 216%, and 181% in the concentrations of limonene, ocimene, limonene, and tagetone, respectively, with respect to the collection point of the plant.

T. minuta essential oil, at a concentration lower than 20%, has a demonstrated variation in its efficacy depending on the tick species studied. A concentration of 2.5% is enough to kill *A. miniatus* in the LPT. At a concentration of 20%, this essential oil was able to kill more than 95% of *A. miniatus, A. cajennense*, *R. sanguineus* and *R. microplus* at both LPT and AIT, as reported previously (Garcia et al., 2012).

Njoroge and Bussmann (2006) have documented that in Kenya, when the leaves of *T. minuta* are boiled, the resulting solution can be used to kill ticks. Therefore, the farmers in the study area may benefit from boiling the *T. minuta* leaves. This method is much more cost-effective than distilling the oil from the plants. This information could be used to guide acaricide discovery research efforts involving the use of biological agents. Although the mechanism of *T. minuta* essential oil is unknown, the decrease in the biological rates observed on the treated cattle may be a result of one or more active ingredients acting on a biological function of *R. microplus*.

Based on the results of this work, *T. minuta* essential oil at a concentration of 20% may be an option for the control of *R. microplus.* The essential oil is a viable option for controlling the cattle tick because the oil caused a 98.57% increase in tick mortality and interfered with reproduction, suggesting that the oil may be an alternative to the acaricides that are normally used for tick control.

The results of this work indicate that *T. minuta* essential oil is more promising than other phytotherapic agents of interest for the control of the cattle tick. The results obtained by Broglio-Micheletti et al. (2010) differ from those obtained by Oliveira et al. (2007). In the former study, phytotherapic agents were used, and the mortality of ticks ranged from 20% to 96% with azadirachtin A treatment. In the latter study, only solutions prepared with commercial neem oil at concentrations above 25% were effective at eliminating adult females. For oviposition, treatments resulted in a reduction of the egg mass from 11% to 22.8%. Borges et al. (2003) used 0.25% *M. azedarach* (Meliaceae) and observed results similar to those of Broglio-Micheletti et al. (2010), who observed in vitro inhibition of total oviposition in females; however, the extract did not kill the adult females.

The results observed using the AIT for acaricide evaluation indicate that the efficacy of single ingredients, such as alphacypermethrin, cypermethrin and amitraz, was generally poor. The *T. minuta* essential oil presented AIT results that were similar to DDVP + chlorfenvinphos and cypermethrin + citronella + chlorpyrifos + piperonyl butoxide (Garcia et al., 2012; Andreotti et al., 2011).

Table 2
Percent reduction for each biological indicator investigated in the group treated with *T. minuta* compared to the control group.

	% Reduction[a] (treated/control)				Efficacy (%)[b]
	DT	DW	DO	DF	
T. minuta essential oil	98.57 (34/2373)	59.77 (91/225)	82.77 (21/124)	94.58 (5/92)	99.98

[a] Percent reduction was calculated in relation to the control untreated group: DT, adult female ticks; DW, engorged tick weight; DO, egg laying capacity; DF, fertility.
[b] Efficacy (%) = 100[l − (CRT × CRW × CR0 × CRF)], where CRT is the reduction in the number of adult female ticks, CRW is the reduction in tick weight, CRO is the reduction in the egg laying capacity, and CRF is the reduction in fertility.

The benefits of using *T. minuta* essential oil as part of an integrated control program include reducing the use of chemical acaricides, extending the useful life of acaricides by delaying the onset of resistance, reducing the incidence of *R. microplus*-borne diseases, and decreasing production costs. The results obtained in this study suggest that *T. minuta* essential oil is a potential *R. microplus* tick control agent that can be used to mitigate the economic losses associated with tick infestation.

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