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Chemical identification of *Tagetes minuta* Linnaeus (Asteraceae) essential oil and its acaricidal effect on ticks

Caracterização química e efeito acaricida do óleo essencial de *Tagetes minuta* Linnaeus (Asteraceae) em carrapatos

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Abstract

The control of tick species that affect animal production is vital for the economic welfare of the cattle industry. This study focused on testing the acaricidal activity of the essential oil from the leaves and stems of *Tagetes minuta* against several Brazilian tick species, including *Rhipicephalus (Boophilus) microplus*, *Rhipicephalus sanguineus*, *Amblyomma cajennense* and *Argas miniatus*. The chemical composition of the essential oil was determined by chromatography and spectroscopy analyses, which revealed the presence of monoterpenes. The adult immersion test (AIT) and the larval packet test (LPT) were used to evaluate the efficacy of *T. minuta* essential oil in tick management at concentrations of 2.5, 5, 10, 20 and 40%. The results demonstrated that the *T. minuta* essential oil had over 95% efficacy against four species of ticks at a concentration of 20%. These results suggest that the essential oil of *T. minuta* could be used as an environmentally friendly acaricide.

Keywords: *Tagetes minuta*, ticks, phytotherapy, control.

Resumo

O controle de carrapatos que causa impacto na produção de bovinos possui importância econômica para a cadeia produtiva. Neste trabalho objetivou-se testar a atividade acaricida do óleo essencial das folhas e caules de *Tagetes minuta* contra várias espécies de carrapatos brasileiros, incluindo *Rhipicephalus (Boophilus) microplus*, *Rhipicephalus sanguineus*, *Amblyomma cajennense* e *Argas miniatus*. A composição química do óleo foi determinada por GC-MS e análises de espectroscopia de RMN, que revelaram a presença de monoterpenos. Na avaliação destas substâncias no controle do carrapato foram empregados os testes de imersão de adulto (TIA) e o de pacote de larvas (TPL) para o extrato de óleo de *T. minuta* nas concentrações de 2,5%; 5%; 10%; 20% e 40%. Os resultados do TPL e TIA demonstraram que o óleo essencial na concentração de 20% de *T. minuta* apresenta eficácia superior a 95% nas quatro espécies de carrapato. Estes resultados sugerem que o óleo essencial de *T. minuta* pode ser usado como um acaricida eficaz e com baixo impacto ambiental.

Palavras-chave: *Tagetes minuta*, carrapatos, fitoterápico, controle.

Introduction

Ticks are ectoparasites that affect an extensive range of vertebrate hosts and transmit a wide variety of pathogens. They are the most common external parasites of economic importance in livestock production worldwide. Chemical acaricides have been developed to control ticks and are applied as dips, sprays and pour-ons at various application intervals.

There are approximately 61 species of ticks in Brazil (BARROS-BATTESTI et al., 2006). The four species examined in this study represent the ticks that are important in cattle and poultry production and environmental and public health.

There is a considerable economic incentive to control the ticks that affect animal production systems. In Brazil, the annual economic loss in the cattle industry attributed to *Rhipicephalus (B.) microplus* has been estimated at US \$2 billion (GRISI et al., 2002). Over 170 million bovines are managed by the Brazilian cattle industry (ANUALPEC, 2009), and the Brazilian parasiticide

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market has a value of US \$960 million in sales, which accounts for 34% of the Brazilian veterinary product market (SINDAN, 2010).

Rhipicephalus sanguineus is the primary tick species found on dogs in the urban areas of Brazil (SZABÓ et al., 2001); *Amblyomma* sp. are also found on dogs in rural areas (LABRUNA; PEREIRA, 2001). *Amblyomma cajennense* is the primary tick species found on horses and has also been recovered from dogs (LABRUNA et al., 2000).

Argas miniatus Koch (1844) is a tick that affects poultry and other birds. It can cause productivity losses and transmit pathogenic agents (LISBÔA et al., 2008).

According to the criteria of the Brazilian Ministry of Agriculture and Food Supply, new tick control products must display an efficacy of at least 95% to be registered. However, an official program for tick control is lacking, and the producers largely define the criteria for tick control. The development of acaricide resistance in ticks continues to be a major driver of new anti-parasitic drug development (ANDREOTTI et al., 2011).

There has been an increase in the amount of research focused on the use of plant extracts for the control of parasites. For example, the oil from neem (*Azadirachta indica*) seeds was evaluated and shown to possess acaricidal properties, such as an inhibitory effect on vitellogenin during the oogenesis of arthropods (WILLIAMS, 1993; KALAKUMAR et al., 2000).

Tagetes minuta is an annual perennial herb that belongs to the Asteraceae family. Its leaves are slightly glossy, green and pinnately dissected into 4 to 6 pairs of pinnae (PRAKASA et al., 1999). This plant is used in popular medicine and grows in temperate regions of South America (MOYO; MASIKA, 2009). Previously, Moghaddam et al. (2007) showed that the main components of *T. minuta* oil are α -terpineol, (Z)- β -ocimene, dihydrotagetonone, (E)-ocimenone, (Z)-tagetonone, and (Z)-ocimenone; the composition was confirmed in this study.

Volatile oils are plant products made by heating with water vapor. These attractive or repellent substances of plants are mainly terpenic in nature, of low molecular weight, and volatile. Such substances are usually referred to as aromatic essential oils, and they accumulate in all plant organs (KNAAK; FIUZA, 2010).

Essential oils are the steam-distilled fraction of the plant, and they are liquid at ambient temperature (ENAN, 2001). The composition of the essential oil of *T. minuta* varies by the part of the plant and its growth stage, but it is consistent at all geographical locations. In the province of Chaco, dihydrotagetonone levels reach a maximum of 42.9% in the leaves of non-bloomed plants, and β -ocimene and tagetenone levels reach a maximum of 45.4% and 32.9%, respectively, in the flowers (CHAMORRO et al., 2008).

T. minuta has been shown to be antimicrobial (SOUZA et al., 2000), insecticidal and acaricidal (TOMOVA et al., 2005; MOYO; MASIKA, 2009). The terpenes in the *T. minuta* oil are toxic to mosquitoes (MACÊDO et al., 1997) and have been reported to have aphicidal properties (TOMOVA et al., 2005).

The purpose of this study was to characterize the essential oil of *Tagetes minuta* by GC-MS analysis and to evaluate its acaricidal properties against four species of Brazilian ticks.

Materials and Methods

1. Study site

The study was conducted in Campo Grande-MS, Brazil, located at 20° 26' S and 54° 42' W at 520 m above sea level.

2. Plant material and extraction

The leaves and stems of *Tagetes minuta* were collected from the garden, dried at 40 °C for 72 hours and ground in a grinder with a 5-mm mesh. A total biomass of 1 kg was submitted to steam distillation to extract 185 mL of essential oil. The herbage was placed on a perforated plate, which served as a support to homogenize the steam flow. The plate was then placed on an extractor, and a pipe (gooseneck) was attached to transfer the vapors to a condenser.

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 process lasted 2 hours inside an extractor at normal atmospheric pressure and 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.

3. Oil extract chromatography (GC-MS)

The essential oil extract was analyzed qualitatively and quantitatively using a Shimadzu GCMS-QP2010 Plus equipped with an Rtx®-WAX Crossbond-Carbowax-polyethylene glycol column (30 m \times 0.25 mm i.d. \times 0.25 μ m film thickness), a split injector, a ratio of 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 from 50 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 μ L. The sample was prepared in CHCl₃. 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) based on an analysis of the fragmentation pattern obtained for each component and on a comparison of their retention indexes (IR) with the Shimadzu GCMS Solution Program, version 2.53.

4. NMR spectroscopy

The NMR spectra were recorded on a Bruker Avance DPX-300 instrument equipped with a 5-mm direct probe with z-gradient field. The ¹H and ¹³C NMR spectra (at 300 and 75 MHz, respectively) were measured at a temperature of 300 K using 10 mg·mL⁻¹ in CDCl₃ solvent. Tetramethylsilane was used as an internal reference.

The chemical shifts are provided on the δ scale. The experiments were performed using standard pulse sequences, as suggested by the equipment manufacturer.

5. Bioassay

The engorged female ticks, including *R. (B.) microplus*, *R. sanguineus*, *A. cajennense* and *A. miniatus*, were collected from naturally infested animals in Campo Grande-MS. The animals were free of acaricide treatments for 45 days prior to tick collection. The ticks were previously diagnosed as resistant to acaricide families, including pyrethroid (SP), organophosphate (OP) and amitraz (Am).

The engorged female ticks were washed with water and dried with paper towels. A group of females was used in an adult immersion test (AIT), and another group was incubated at $27 \pm 1.5^\circ\text{C}$ and 70-80% relative humidity (DRUMMOND, 1973) for two weeks until their eggs were laid. These eggs provided the larvae for the larval packet test (LPT) (FAO, 1984).

6. Adult immersion test

The AIT (DRUMMOND, 1973) was used to test the acaricidal activity of the *T. minuta* crude extracts (leaf and stem), neem oil and acaricides on adult ticks. Ten engorged female ticks of each species were deposited into a petri dish. The tick groups were immersed in *T. minuta* oil extract or neem oil at concentrations of 2.5, 5, 10, 20 and 40%.

Tween 20 (2%) was used to solubilize the essential oil and neem oil in water. A concentration curve was performed to define the optimal concentration of Tween 20 that enabled a stable aqueous solution.

For the established acaricides, the commercially recommended concentrations were evaluated. The assays were performed in triplicate.

The treated groups were immersed for five minutes in diluted crude extract with 2% Tween 20, and the control group was immersed in distilled water with 2% Tween 20, as recommended in a preliminary study (ROSADO-AGUILAR et al., 2008). The ticks were individually plated in a 24-well plate and were incubated for 15 days at the conditions described above. The ticks were analyzed with a stereomicroscope, and the mortality rate and the weight of the eggs produced by each group were recorded.

The mortality rate was recorded daily. The dead ticks were recognized by the presence of cuticular darkness, hemorrhagic skin lesions, a lack of Malpighian tubes and stillness. After 15 days, the number of females laying eggs was recorded, and the eggs of each group were weighed using an analytical scale. Fifty eggs of each batch were placed in 25×95 -mm glass vials, maintained in conditions similar to those in which the adult ticks were maintained and observed for 21 days. The hatching rates of the eggs were then estimated, and they were compared to the control group and the other experimental groups. The percent inhibitions of egg laying and larval hatching were determined for all of the groups (CEN-AGUILAR et al., 1998).

7. Larval packet test

The LPT was used to test the acaricidal activity of the crude extracts (leaf and stem) against the four species of tick larvae (FAO, 1984). Tween 20 was diluted with distilled water to a concentration of 2% and was used to dilute the plant extracts, the *T. minuta* oil extract and the neem oil (to concentrations of 2.5, 5.0, 10, 20 or 40%); the diluted Tween 20 was also included in the control group.

Tick larvae aged 7-14 days were used in this study (FAO, 1984). The vials with the highest larval exclusion rate (90-100%) were selected, and the larvae were placed in the center of a petri dish that was subsequently filled with water and soap to prevent their escape. The diluted plant crude extract (3 mL) was transferred to the petri dishes (60×15 mm in diameter), and ~300-500 larvae were placed between two Whatman No. 1 papers and immersed for 10 minutes. Approximately 100 larvae were selected with a no. 4 paintbrush and gently transferred to clean filter paper packets.

The opened envelopes containing the tick larvae (treated and control) were folded with metallic clips and labeled with the appropriate identification markers (tested solution and concentration). The packets were placed in an incubator at $27 \pm 1.5^\circ\text{C}$ and 70-80% relative humidity for 24 hours. The envelopes were opened 24 hours post-treatment (PT) and observed using stereoscopy. The number of living larvae, the mortality and any other toxic effects were recorded. The larvae that were unable to walk were considered dead, as recommended by FAO (1984), and the corrected mortality was described using the following equation:

$$\text{Corrected mortality} = \frac{\% \text{ Treated mortality} - \% \text{ control mortality}}{100 - \% \text{ control mortality}} \times 100$$

None of the control group bioassays showed a mortality rate of >5%.

Results

1. Oil extract chromatography

Table 1 shows the qualitative and quantitative analyses of a commercial sample of essential oil extract that was obtained from *Tagetes minuta*. Four main components are shown that represent more than 70% of the essential oil. They were identified as limonene (1), β -ocimene (2), dihydrotageton (3) and tageton (4) (Figure 1).

Table 1. The primary chemical composition of *Tagetes minuta* essential oil.

Compound	Retention time (min)	Area (%)
Limonene (1)	9.52	6.96
β -Ocimene (2)	9.62	5.11
Dihydrotageton (3)	9.89	54.21
Tageton (4)	11.60	6.73

Min – minutes. % percentage.

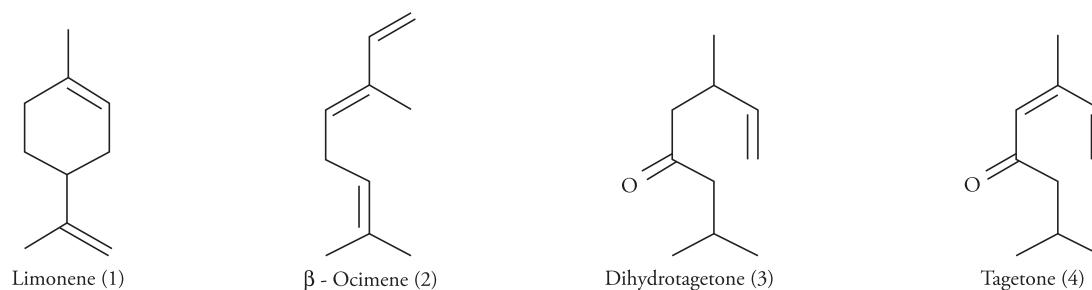


Figure 1. Chemical structures of the compounds identified in the *T. minuta* essential oil.

Table 2. Efficacy of different concentrations of *T. minuta* oil extract against four tick species using the adult immersion test (AIT) and larval packet test (LPT). All protocols were performed in triplicate.

Tick species	Test	Concentrations %							
		0.3	0.6	1.25	2.5	5	10	20	40
<i>R.(B.)microplus</i>	AIT	5	5	9	22	45	79	95	100
	LPT	2	2	3.5	26.5	58	92.5	100	100
<i>R. sanguineus</i>	AIT	2	3	12	27	65	89	100	100
	LPT	3	3	4	8	21	80	100	100
<i>A. cajennense</i>	AIT	2	2	9	26	61	79	100	100
	LPT	3	8.5	9	16	45	62.5	95	100
<i>A. miniatus</i>	AIT	5	12	31	69	97	100	100	100
	LPT	12	32	68	100	100	100	100	100

% Percentage.

2. NMR spectra

The data from the ^1H and ^{13}C spectra of the primary constituents of the essential oil were compatible with those found in the literature (SINGH et al., 2002).

3. Bioassay

The effects of the *T. minuta* essential oil on the four tick species, as determined by the LPT (larval packet test), are described in Table 2. An efficacy of over 95% was observed against *A. miniatus* with a concentration of 2.5%. A similar efficacy was reached against the Ixodidae ticks with a concentration of 20%. In the AIT (adult immersion test) (Table 2), the essential oil showed 95% efficacy against *A. miniatus*, and the ticks from the Ixodidae family had similar results as the LPT results.

No significant acaricidal activity was found against the *R. (B.) microplus* tick larvae by the commercial neem (*Azadirachta indica*) oil or the control sample by either the larval packet test (LPT) or the adult immersion test (AIT) (Table 3).

Four species of ticks were evaluated using the commercial acaricides that are available in the regional market; the data obtained in the assays for the larval packet test (LPT) and the adult immersion test (AIT) are shown in Table 4.

Discussion

T. minuta oil was shown to be very effective against both hard and soft ticks. Its efficacy at a concentration of 20% was comparable

to the referenced conventional acaricides, and it reached over 95% efficacy as required by the ministry of agriculture in Brazil.

According to the criteria of the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA), new acaricides must provide an efficacy of at least 95% to become registered (BRASIL, 1997).

Plant extracts could provide a viable alternative to conventional acaricides because they are available locally at a low cost, and communities generally accept them. The concentration of *T. minuta* had an effect on the magnitude of the tick burden of the treated animals compared to the negative control when an infusion was used (MOYO et al., 2009). Ayacko (2008) reported an efficacy of 55% when using a decoction.

Importantly, the level of acaricidal activity of such compounds may vary depending on the plant species, growing conditions and the form of extraction, which directly affects the activity of essential oils. For this reason, it is necessary to conduct insightful research on the chemical composition of plant-derived substances and their effects. The process of extracting solvents, the applications of the techniques in the field, conservation and plant selectivity also need to be further evaluated (KNAACK; FIUZA, 2010).

Essential oils are distributed in a limited number of families, such as Asteraceae, and accumulate in all types of plant vegetative organs such as the flowers, leaves, barks, roots, rhizomes, fruits and seeds. In most cases, the biological function of the essential oil terpenoids remains obscure. It is conceivable, however, that they have an ecological role. The toxic mechanism of action of terpenoids has not been uncovered and is still unknown. Observations clearly suggest that the insecticidal activity and potency of monoterpenoids, as with the other insecticides, depend

Table 3. Efficacy of commercial neem oil (*Azadirachta indica*) using the adult immersion test (AIT) and larval packet test (LPT). All protocols were performed in triplicate.

Tick Species	Concentrations %								
	Test	0.3	0.6	1.25	2.5	5	10	20	40
<i>R. (B.) microplus</i>	AIT	0.1	1.3	2.6	2	3	16	34	46
	LPT	0	0.3	1	1.2	2.8	19	36	55
<i>R. sanguineus</i>	AIT	1.1	2	2.9	5.9	7.8	19	42	55
	LPT	0.3	0.9	1.4	2.9	6	15	39	45
<i>A. cajennense</i>	AIT	0.6	2.1	3	3.4	5.3	16	38	41
	LPT	1.1	1.0	2.9	2.8	4	15	37	56
<i>A. miniatus</i>	AIT	0.3	10	53	74	96	100	100	100
	LPT	9	26	61	79	100	100	100	100

% Percentage.

Table 4. Efficacy values for commercial acaricides that were used at the commercially recommended concentrations for ticks and tested using the adult immersion test (AIT) and the larval packet test (LPT). All protocols were performed in triplicate.

Tick Species	Test	Chemical base					
		Cypermethrin	Amitraz	Cpt			
				Cpf		Ddvp	
				Ct		Dmt(*)	
				Bupi (*)		Cpt (*)	
<i>R. (B.) microplus</i>	AIT	14	39	98	28	16	100
	LPT	36	26	100	35	28	100
<i>R. sanguineus</i>	AIT	54	100	100	95	98	100
	LPT	53	100	100	96	95	100
<i>A. cajennense</i>	AIT	95	99	100	97	95	100
	LPT	100	98	100	100	99	100
<i>A. miniatus</i>	AIT	100	100	100	100	100	100
	LPT	99	99	100	100	100	100

(*) Associations. Abbreviations: Cypermethrine: Cpt; Chlorpyrifos: Cpf; Deltamethrin: Dmt; Citronella: Ct; Piperonylbutoxide: Bupi; Dichlorvos (DDVP): Ddvp.

on several factors, including dose, species, application surface, route of penetration, and method of application (ENAN, 2001).

There is evidence that the octopaminergic system is a target of several monoterpenoids, whether they are antagonists or agonists. Such monoterpenoids are highly selective to insects and are safer to the environment than the currently used insecticides, such as organophosphorus, carbamates and synthetic pyrethroids (ENAN, 2001).

Our results showed that the *A. miniatus* tick of the Argasidae family is at least five times more sensitive to essential oil than the ticks of the Ixodidae family. Thus, acaricide efficacy may vary by tick species. For the *T. minuta* essential oil to become an alternative acaricide, it is important to consider a formulation that is efficient and low cost. Plant products can be used in association with, or as replacements for, synthetic compounds. Therefore, follow-up studies are needed to validate this strategy. The advantage of using plant extracts may be slow resistance development because the extracts are usually composed of a mixture of different active agents with different mechanisms of action (OLIVO et al., 2009).

In this work, the effect of commercial neem oil was compared to *T. minuta* essential oil. At a 5% concentration, the *T. minuta* essential oil displayed only partial effectiveness against Ixodidae ticks. However, it achieved satisfactory results against *A. miniatus*.

Several studies have been conducted on the effect of neem extracts on cattle ticks, such as *Amblyomma hebraeum*, *Rhipicephalus*

evertsi, *Hyalomma truncatum*, *R. (B.) decoloratus*, *R. (B.) microplus* and *Hyalomma anatolicum excavatum* (WILLIAMS, 1993; KALAKUMAR et al., 2000; WEBB; DAVID, 2002).

A study by Benavides et al. (2001) showed that the treatment of naturally infested animals with a 5% soapy, aqueous neem extract every 21 days was as effective against *R. (B.) microplus* as an amitraz-based commercial acaricide. The authors also demonstrated high *in vitro* efficacy (100% inhibition of reproduction) by an ether extract, while alcohol extracts resulted in a 70% reduction in tick reproduction. The ethanol neem extracts were found to be effective in inhibiting oviposition. Abdel-Shafy and Zayed (2002) and Williams (1993) also concluded that neem oil could be used for tick control at economical concentrations of 1.6 to 3.2%.

Souza et al. (2000) produced a concentrated emulsion of green fruits that was tested at concentrations of 0.25 and 0.5% and showed efficacies ranging from 46.7 to 82.6% and from 16.6 to 89.0%, respectively. Broglio-Micheletti et al. (2010) analyzed the efficacies of a neem hexane extract and a 2% oil concentration against *R. (B.) microplus* and found *in vitro* efficacies of 73.2 and 65%, respectively.

These results were found in different laboratories under different conditions, and the extract efficacies did not reach 95%, which is required by the ministry of agriculture in Brazil.

The data from the AIT and LPT assays corroborated previous results that demonstrated the resistance of *R. (B.) microplus*. Only

acaricides containing a mixture of cypermethrin, chlorpyrifos, citronella, piperonyl butoxide and a mixture of dichlorvos and clorpyrifos reached efficacies of over 95% against hard ticks (ANDREOTTI et al., 2011).

A. miniatus and *A. cajennense* ticks were sensitive to all of the tested chemicals, while *R. sanguineus* was only resistant to cypermethrin, an acaricide with wide commercial usage for this species. The data reinforce the notion that selective pressures through the use of different products on tick species lead to the low efficacy of each individual chemical in the control of these tick species.

The results of Knaak and Fiuza (2010) showed that the production of essential oil is viable and profitable. However, to obtain high quality products that are competitive in the market, incentives are necessary to develop modern techniques of cultivation, selection and plant breeding. Thus, we must continue to search for new control methods, such as the use of new compounds extracted from wild and cultivated medicinal plants, which can be used seamlessly with other methods. Ultimately, such new discoveries can lead to a reduction in the impact of chemical use.

Conclusion

This study shows that the essential oil of *T. minuta* has potential as a larval and adult acaricide for the control of four tick species in Brazil when compared to neem oil. In addition to being an effective larvicide, the oil is environmentally friendly; the active components are atoxic terpenoids, which are generally considered safe and are used in fragrances and as food additives.

The recommended concentration of *T. minuta* oil to be used as an acaricide is 20%, and its use as a phytotherapeutic can contribute to the trend of integrating natural products into tick management in Brazil.

Future research efforts should be geared toward synthesizing chemical analogs of the active compounds of this essential oil and verifying their acaricidal activity.

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