

Artigo

***In vitro* Acaricidal Activity of *Ocimum gratissimum* Essential Oil on *Rhipicephalus sanguineus*, *Amblyomma sculptum* and *Rhipicephalus microplus* Larvae**

Ferreira, T. P.; Cid, Y. P.; Alves, M. C. C.; Santos, G. C. M.; Avelar, B. R.; Freitas, J. P.; Ożarowski, M.; de Souza, M. A. A.; Chaves, D. S. A.*

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Atividade Acaricida *in vitro* do Óleo Essencial de *Ocimum gratissimum* Sobre Larvas de *Rhipicephalus sanguineus*, *Amblyomma sculptum* e *Rhipicephalus microplus*

Resumo: O uso de óleos essenciais para o controle de ectoparasitos de interesse veterinário pode ser uma excelente alternativa aos compostos sintéticos comercializados como forma de reduzir os impactos negativos na saúde humana e no meio ambiente. A incidência de diferentes espécies de carrapatos em diferentes locais é resultado das características ambientais do território nacional. Entre as espécies de interesse veterinário estão *Amblyomma sculptum*, *Rhipicephalus microplus* e *Rhipicephalus sanguineus*. Óleos essenciais (OEs) de *Ocimum sp* exibem uma gama de atividades biológicas, como inseticida, acaricida e repelência de insetos. O objetivo deste estudo foi avaliar a atividade *in vitro* e estabelecer a CL₅₀ do OE de *Ocimum gratissimum* contra larvas de *A. sculptum*, *R. microplus* e *R. sanguineus*, a fim de explorar o potencial de uso desse OE no controle de carrapatos. Os resultados do bioensaio mostraram que o OE de *O. gratissimum* apresentou grande atividade acaricida contra todas as larvas testadas na faixa de concentração avaliada. A atividade larvicida foi mais pronunciada para *R. microplus* (CL₅₀ = 2,0 mg.mL⁻¹) quando comparada a *A. sculptum* (CL₅₀ = 5,5 mg.mL⁻¹) e *R. sanguineus* (CL₅₀ = 6,2 mg.mL⁻¹). Estes resultados são promissores para o desenvolvimento de produtos fitoterápicos para obtenção de um método alternativo de controle de larvas de carrapatos, minimizando o uso de produtos sintéticos.

Palavras-chave: Eugenol; eucaliptol; CG; carrapato; acaricida.

Abstract

The essential oil use for veterinary ectoparasites control may be an excellent alternative to synthetic compounds marketed, as a way to reduce negative impacts on human health, and the environment. The incidence of different tick species in different locations is a result of the environmental characteristics of the national territory. Among the veterinary interest species are *Amblyomma sculptum*, *Rhipicephalus microplus* and *Rhipicephalus sanguineus*. *Ocimum sp* essential oils (EOs) exhibit a range of biological activities such as insecticide, acaricide and insect repellency. The aim of this study was to evaluate *in vitro* activity and establish the LC₅₀ of *Ocimum gratissimum* EO against *A. sculptum*, *R. microplus* and *R. sanguineus* larvae in order to explore the potential use of this essential oil in tick control. The bioassay results showed that *O. gratissimum* essential oil exhibited great acaricidal activity against all larvae tick tested in the evaluated concentration range. Larvicidal activity was more pronounced for *R. microplus* (LC₅₀= 2.0 mg.mL⁻¹) when compared to *A. sculptum* (LC₅₀= 5.5 mg.mL⁻¹) and *R. sanguineus* (LC₅₀= 6.2 mg.mL⁻¹). These results are promising for the development of herbal products to obtain an alternative method of controlling tick larvae while minimizing the use of synthetic products.

Keywords: Eugenol; eucalyptol; GC; tick; acaricidal.

* Universidade Federal Rural do Rio de Janeiro, Instituto de Ciências Biológicas e da Saúde, Departamento de Ciências Farmacêuticas, CEP 23897-000, Seropédica-RJ, Brazil.

✉ gnosv.ufrj@gmail.com

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***In vitro* Acaricidal Activity of *Ocimum gratissimum* Essential Oil on *Rhipicephalus sanguineus*, *Amblyomma sculptum* and *Rhipicephalus microplus* Larvae**

Thais P. Ferreira,^a Yara P. Cid,^b Melina A. Cardilo,^c Gabriela C. M. dos Santos,^c Barbara R. Avelar,^c Juliana P. Freitas,^c Marcin Ożarowski,^d Marco A. A. de Souza,^e Douglas S. A. Chaves^{b,*}

^a Universidade Federal Rural do Rio de Janeiro, Programa de Pós-Graduação em Química, Instituto de Química, BR 465, km 7, CEP 23897-000, Seropédica-RJ, Brazil.

^b Universidade Federal Rural do Rio de Janeiro, Departamento de Ciências Farmacêuticas, Instituto de Ciências Biológicas e da Saúde, BR 465, km 7, CEP 23897-000, Seropédica-RJ, Brazil.

^c Universidade Federal Rural do Rio de Janeiro, Programa de Pós-Graduação em Ciências Veterinárias, Instituto de Veterinária, BR 465, km 7, CEP 23897-000, Seropédica-RJ, Brazil.

^d Department of Biotechnology, Institute of Natural Fibres and Medicinal Plants, Wojska Polskiego 71b, 60-630 Poznań, Poland.

^e Universidade Federal Rural do Rio de Janeiro, Departamento de Bioquímica, Instituto de Química, BR 465, km 7, CEP 23897-000, Seropédica-RJ, Brazil.

* gnosy.ufrj@gmail.com

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1. Introduction

Essential oils are natural products obtained from plants, mainly by hydrodistillation or steam distillation, principally containing monoterpenes, sesquiterpenes and phenylpropanoids.¹ Essential oils' chemical complexity gives them a broad spectrum of biological action, so they have been investigated for the control of pests and diseases that affect crops and human health.^{2,3} In this context, different studies have demonstrated essential oils' toxic effect against ectoparasites of veterinary interest.^{4,5} Indeed, the use of essential oils for veterinary ectoparasite control can be an excellent alternative to the use of synthetic compounds, by reducing negative impacts on human health and the environment,⁶ besides minimizing the development of tick resistance.⁷

The wide geographic dispersion of different tick species is a result of the environmental characteristics of the endemic regions.⁸ Among the species of veterinary interest are *Amblyomma sculptum*, *Rhipicephalus microplus* and *Rhipicephalus sanguineus*. *Amblyomma sculptum*, popularly called the "star tick", has low parasitic specificity, so many species of animals can serve as hosts, including humans.^{8,9} It is an important public health problem since it is main transmitter of the bacterium *Rickettsia rickettsii*, the etiological agent of Brazilian macular fever.¹⁰ *Rhipicephalus microplus* is the main cattle tick and contributes to the spread of various diseases in livestock.¹¹ It is estimated that this tick causes losses of up to US\$ 3.2 billion in the Brazilian livestock sector, considering treatment costs and reduced productivity.^{12,13} *Rhipicephalus sanguineus* has high prevalence in dogs, but human infestations have been reported¹³, and it can transmit pathogens such as *Babesia canis* and *Ehrlichia canis*.¹⁴

The use of parasiticides is the main strategy for control of these ectoparasites. The immature stages (eggs and larvae) of ticks are more sensitive, so reducing them leads to

a decrease in the number of adults.¹⁵ The search for sustainable alternatives to reduce the economic and environmental impact includes investigation of essential oils (EOs) for the control of parasites.¹⁶

The *Ocimum* genus, belonging to the Lamiaceae family,^{17,18} exhibits a high degree of polymorphism, so a large number of subspecies exist (over 150) that produce EOs of varying chemical composition, offering variable levels of potential activity.¹⁶ In particular, *Ocimum sp* EOs have a range of biological activities, such as insecticidal, acaricidal, insect repellency, antimicrobial, antioxidant and anti-inflammatory.¹⁷ *O. gratissimum* occurs spontaneously throughout Brazil,¹⁸ and three chemotypes are known: thymol, geraniol and eugenol (the most common), which has an essential oil rich in two compound (eugenol and eucalyptol).¹⁹

To ensure the efficacy and safety of using EOs to control ticks, it is first necessary to determine effective concentrations. *In vitro* activity evaluation and LC₅₀ establishment are important tools at this stage. Therefore, the aim of this study was to evaluate the *in vitro* activity and establish the LC₅₀ of *Ocimum gratissimum* EO against *A. sculptum*, *R. microplus* and *R. sanguineus* larvae in order to explore the potential use of this essential oil for tick control.

2. Material and Methods

2.1. Plant material

Leaves of *Ocimum gratissimum* L. (Clove basil) were collected at the Botanical garden of the Universidade Federal Rural do Rio de Janeiro - 2018 (GPS 22°31'36.23 S; 44°04'31.62 W), dried in an oven chamber at 37 °C during 72 h and manually pulverized. The specimen voucher was deposited in the Herbarium of the Institute of Botany (UFRRJ, Brazil) number RBR 36382.

2.2. Extraction, content and chemical characterization of the essential oil

Essential oil from dried leaves was obtained by hydrodistillation in a Clevenger apparatus for 3 h, then, dried with anhydrous Na₂SO₄. GC analysis was carried out on a Hewlett-Packard 5890 II (Palo Alto, USA) apparatus equipped with flame ionization detection (FID) and a split/splitless injector. Injected volume was 1 µL on a 1:20 split ratio. Helium was used as the carrier gas at a flow rate of 1.0 ml min⁻¹. Substances were separated into a fused silica capillary column HP-5 (30 m × 0.25 mm i.d., 0.25 µm, Agilent J & W). The column temperature was programmed as follows: 60 °C for 2 min followed by heating at 5 °C min⁻¹ to 110 °C, followed by heating at 3 °C min⁻¹ to 150 °C and finally followed by heating at 15 °C min⁻¹ until 290 °C and holding constant for 15 min. The injector temperature was 220 °C and the detector temperature was 290 °C as reported by Adams (2007).¹⁶ Percentage of essential oil compounds were calculated from the relative area of each peak analyzed by GC-FID. Essential oil was also analyzed on a GC-MS QP-2010 Plus (Shimadzu, JPN). Carrier gas flow, capillary column and temperature conditions for GC/MS analysis were the same as those described for GC-FID and reported by Adams (2007)²⁰. The temperature of the injector was 220 °C and the temperature of the interface was 250 °C. Mass spectrometer operating conditions were ionization voltage at 70 eV and mass range 40-400 *m/z* and 0.5 scan/s. The compounds retention index were calculated based on co-injection of samples with a C₈-C₂₀ hydrocarbon mixture as reported by van Den Dool and Kratz (1963)²¹. Constituents were identified by comparison of their mass spectra with the NIST library (2008) and with those reported by Adams (2007).²⁰

2.3. *In vitro* larvicidal assay

Bioassays were performed using Larval Immersion Test (LIT) method (Shaw 1966)²² adapted for Leite (1988)²³ and Chagas *et al*, 2002.²⁴ Stock solutions at a concentration of 200 mg.mL⁻¹ of essential oil from *Ocimum gratissimum* was prepared using a mixture of acetone (Synth, code: 219385), distilled water and Tween-80 (Vetec, code: 0803511) as diluent, an innocuous solvent able to dissolve the essential oil constituents, which was also used as negative control. Fipronil at 400 µg.mL⁻¹ was used as positive control to ensure viability of the colony. Serial dilutions (1:2) were performed from stock solutions allowing for ten solutions in a concentration range varying from 40 to 0.0781 mg.mL⁻¹.

For each concentration approximately 100 non-fed 21 days old larvae of each tick (*A. sculptum*, *R. sanguineus* and *R. microplus*) were deposited on a 2 cm x 2 cm filter paper sandwich, which was impregnated with 0.5 mL of the solution under test. The filter paper sandwich was wrapped in a filter paper envelope (6 cm x 6 cm) properly sealed with binder clips. The envelopes were kept in a climatized chamber at 27 ± 1 °C and relative humidity of 80 ± 10 % for 24 h for subsequent mortality assessment. The evaluation criterion used was motility, any larva that presented minimal movement was considered alive. The mean number of live larvae per concentration was evaluated in periods of mainly 24 hours with the help of a stereoscopic microscope. The tests were performed in duplicates for each concentration. Mortality was calculated according to the following formula: Mortality (%): dead larvae x 100 / total larvae

The experiments followed the standards established by the Ethics Committee for Animal Use of the Institute of Veterinary (CEUA). Tick larvae of *Amblyomma sculptum* (CEUA/IV n° 7699190418), *Rhipicephalus microplus* (CEUA/IV n° 4667181218) and *Rhipicephalus sanguineus* (CEUA/IV n° 092/14) used in the experiment were obtained from colonies maintained in the

Laboratory for Experimental Chemotherapy in Veterinary Parasitology of Federal Rural University of Rio de Janeiro (UFRRJ).

2.4. Statistical analysis

The calculation of LC₅₀ (concentration that kill 50 % of the treated population) was performed by probit analysis using Minitab® 16 (2013, Minitab Inc. LEADTOOLS, LEAD Technologies, Inc.). Statistical significance was set at 5 % ($p < 0.05$).

3. Results and Discussion

3.1. Content and chemical characterization of the essential oil

Ocimum gratissimum essential oil content obtained after hydrodistillation was 0.9 % (w/w) and the analysis showed the phenylpropanoid eugenol (74.5 %) and the oxygenated monoterpene eucalyptol (14.8 %) as the major compounds (Table 1).

Ocimum gratissimum presents three important chemotypes (CT): CT eugenol, CT thymol and CT geraniol (Lima *et al.*, 2018; Hue *et al.*, 2015; Silva *et al.*, 2015).^{25, 26-19} In our work, we identified eugenol as the major compound (74.5 %), followed by eucalyptol (1,8-cineol). The relationship between *O. gratissimum* EOs chemical composition and their acaricidal efficacy against *R. microplus* was reported by Hue *et al.* (2015).²⁶

3.2. Larvicidal activity

The bioassay results showed that *O. gratissimum* essential oil exhibited strong acaricidal activity against all tick larvae tested in the evaluated concentration range (Table 2). The best results were found against *R. microplus* and *A. sculptum*, with 100 % efficacy at the concentration of 20 mg.mL⁻¹. The results were also good against *R. sanguineus*, with 100 % and 96.53 % efficacy at 40 and 20 mg.mL⁻¹ respectively.

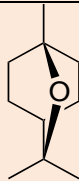
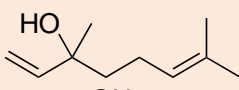
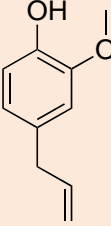
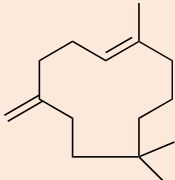
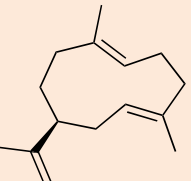
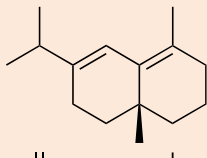
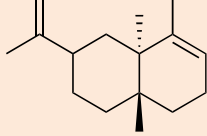
3.3. LC₅₀ establishment

LC₅₀ and slope values of *O. gratissimum* EO for all larvae of the three tick species are reported in the Table 3.

Larvicidal activity was more pronounced for *R. microplus* (LC₅₀= 2.0 mg.mL⁻¹), with relative potency up to 2.7-fold higher than *A. sculptum* (LC₅₀= 5.5 mg.mL⁻¹) and 3.1-fold higher than *R. sanguineus* (LC₅₀= 6.2 mg.mL⁻¹). Our results corroborate the data reported by Monteiro *et al.* (2012)²⁷, in which *R. microplus* larvae showed higher sensitivity to eugenol compared to *Dermacentor nitens* larvae. Previous studies have reported that *R. microplus* is more sensitive to the monoterpene thymol compared to *R. sanguineus* and *D. nitens*.²⁸⁻³⁰ These differences can be attributed to the variation in tick cuticle permeability and thickness.²⁷

The control of *R. microplus* using *O. gratissimum* EO was reported by Hocayen *et al.* (2013)³¹, but very high concentrations of EO were required to achieve mortality. Our results (LC₅₀= 2.0 mg.mL⁻¹) are 4.8-fold higher than previously reported by Hue *et al.* (2015)²⁶, with LC₅₀ of 9.8 mg.mL⁻¹ for *O. gratissimum* EO (CT eugenol) and 1.25-fold higher than described by Lima *et al.* (2018)²⁵, with LC₅₀ of 2.6 mg.mL⁻¹ for *O. gratissimum* EO (CT thymol).

Table 1. Essential oil chemical profile from *Ocimum gratissimum* obtained by hydrodistillation

Order of Elution	Compounds	Al _c	Al _τ	<i>Ocimum gratissimum</i>	Chemical structure
1	Eucalyptol	1033	1026	14.8	
2	Linalool	1102	1095	0.6	
3	Eugenol	1379	1356	74.5	
4	β -caryophyllene	1420	1417	2.6	
5	Germacrene D	1483	1484	1.6	
6	δ -selinene	1493	1491	3.5	
7	α -selinene	1501	1498	1.2	
Monoterpene hydrocarbons				-	
Oxygenated monoterpenes				15.4	
Sesquiterpene hydrocarbons				8.9	
Oxygenated sesquiterpenes				-	
Phenylpropanoid				74.5	
Total				98.8	

The chemical composition was analyzed by GC-MS and organized in the table by order of elution (EO) in the chromatographic column. The concentration (%) was calculated based on the total area of the peak by GC-FID. Tabulated arithmetic index (Al_τ). Calculated arithmetic index (Al_c)

Table 2. *In vitro* larvicidal activity (% mortality) of *O. gratissimum* essential oil against *Amblyomma sculptum*, *Rhipicephalus microplus* and *Rhipicephalus sanguineus*

Essential oil (mg.mL ⁻¹)	Tick species		
	<i>Amblyomma sculptum</i>	<i>Rhipicephalus microplus</i>	<i>Rhipicephalus sanguineus</i>
40	100	100	100
20	100	100	96.53
10	68.18	93.10	91.11
5	50.94	49.73	33.53
2.5	32.64	25.15	2.78
1.25	32.03	26.92	0
0.625	13.28	12.84	0
0.312	5.79	26.64	0
0.156	0	28.02	0
0.0781	0	12.99	0
Control (+)	98.53	100	100
Control (-)	0	0	0

Table 3. LC₅₀ (mg.mL⁻¹) and slope of *Ocimum gratissimum* essential oil against larvae of *Amblyomma sculptum*, *Rhipicephalus microplus* and *Rhipicephalus sanguineus*

Tick species	LC ₅₀ (mg.mL ⁻¹) (95 % CI)	Slope (SE)
<i>Amblyomma sculptum</i>	5.533 (4.054 – 8.742)	3.909 (0.258)
<i>Rhipicephalus microplus</i>	2.042 (1.572 – 2.605)	1.280 (0.103)
<i>Rhipicephalus sanguineus</i>	6.253 (5.704 – 6.827)	4.841 (0.399)

Probit analyses were performed for all data using Minitab® 16 (2013, Minitab Inc., LEADTOOLS, LEAD Technologies, Inc.); LC₅₀ (mg. mL⁻¹) (95 % CI): 50 % lethal concentration values together with their 95 % confidence interval; Slope (SE): slope of the concentration curve and Standard Error

Eugenol has already been reported in the literature as a natural insecticide^{33,26} and acaricide.³³ The mortality³⁰ and repellence³⁴ activity against *R. microplus* of EOs containing eugenol as their major constituent have been described, mainly against *R. microplus*.^{25, 26, 31, 34} However, activity against *Dermacentor nitens*²⁷ and *R. appendiculatus*³⁵ has also been reported. Nevertheless, there is a lack of reports evaluating the acaricidal activity of *Ocimum gratissimum* EO as well as its major constituents' effects against *A. sculptum* and *R. sanguineus*.

The results obtained in the present work point to the potential use of *Ocimum gratissimum* essential oil for tick control. However, further research is needed, including the formulation of standardized veterinary products, to apply the active compounds efficiently and with a prolonged residual effect on the host's skin, as well as toxicology tests to evaluate possible effects on the host.

4. Conclusion

Ocimum gratissimum essential oil showed larvicidal efficacy in *in vitro* assays against larvae of *Amblyomma sculptum*, *Rhipicephalus microplus* and *Rhipicephalus sanguineus*. These findings, together with the chemical composition data, can support studies aiming at the development of products for the control of these ticks.

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