

In vitro evaluation of the acaricidal activity of *Syzygium aromaticum* (L.) essential oil and eugenol against non-fed larvae of *Rhipicephalus sanguineus*

Avaliação *in vitro* da atividade acaricida do óleo essencial de *Syzygium aromaticum* (L.) e eugenol contra larvas de *Rhipicephalus sanguineus* não alimentadas

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Abstract

Plant extracts and essential oils have been showing potential for use as acaricides. *Rhipicephalus sanguineus*, the brown tick, mainly parasitizes dogs and is responsible for pathogens transmission. The insecticidal and acaricidal activity of the essential oil (EO) of *Syzygium aromaticum* and eugenol have already been reported, however the activity against *R. sanguineus* is lacked. To evaluate the acaricidal activity of *S. aromaticum* EO and eugenol against larvae of *R. sanguineus*, *in vitro* assays were performed using the larval immersion test. Bioassays were performed in duplicate, within the concentration range of 0.078 to 40 mg.mL⁻¹. Mortality assessment was performed after 24 hours. Mortality above 90% was achieved through using the EO and eugenol at concentrations greater than 5 and 10 mg.mL⁻¹, respectively. The LC₅₀ values of 1.4 and 3.3 mg.mL⁻¹ for eugenol and *S. aromaticum* EO, respectively, while the LC₉₀ values found for eugenol and *S. aromaticum* EO were, respectively, 6.4 and 6.7 mg.mL⁻¹ were calculated. These compounds present activity against *R. sanguineus* larvae and demonstrate potential for use as acaricides.

Keywords: natural product, tick control, clove.

Resumo

Extratos vegetais e óleos essenciais têm apresentado potencial para uso como acaricidas. *Rhipicephalus sanguineus*, o carrapato marrom, parasita principalmente cães, sendo responsável pela transmissão de patógenos. O óleo essencial (OE) de *Syzygium aromaticum* e o eugenol já tiveram atividade inseticida e acaricida relatada, porém faltam ensaios que relatem atividade contra *R. sanguineus*. Com o objetivo de avaliar atividade carrapaticida de OE de *S. aromaticum* e eugenol frente a larvas de *R. sanguineus*, foram realizados ensaios *in vitro* por meio de Teste de Imersão Larval. Os bioensaios foram realizados em duplicata, na faixa de concentração de 0,078 a 40 mg.mL⁻¹. A avaliação da mortalidade foi realizada após 24 horas. Mortalidade acima de 90% foi alcançada para o OE e o eugenol em concentrações superiores a 5 e 10 mg.mL⁻¹, respectivamente. Valores de CL₅₀ de 1,4 e 3,3 mg.mL⁻¹ para eugenol e OE de *S. aromaticum*, respectivamente, enquanto os valores de CL₉₀ encontrados para eugenol e óleo essencial de *S. aromaticum* foram, respectivamente, de 6,4 e 6,7 mg.mL⁻¹. Os compostos têm atividade contra larvas de *R. sanguineus* e demonstram potencial para uso como acaricida.

Palavras-chave: produto natural, controle, carrapato, cravo.

Introduction

The tick *Rhipicephalus sanguineus sensu lato* mainly parasitizes domestic dogs, although it has been described parasitizing other species, such as rabbits, cats, rodents, pigeons, wild canids and



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humans (Dantas-Torres, 2010). It has cosmopolitan distribution and is responsible for transmitting various pathogens to dogs, such as *Babesia* spp., *Rangelia vitalii*, *Ehrlichia canis*, *Anaplasma* spp., *Hepatozoon* spp., *Rickettsia conorii*, *Rickettsia rickettsii* and *Mycoplasma haemocanis* (Dantas-Torres & Otranto, 2015).

These ticks are difficult to control, due to their heterogeneous life cycle, since only about 5% of the total population of *R. sanguineus* is on animals, while the remainder is in the environment. Controlling these ticks is usually done using conventional acaricides based on chemicals, which are applied to the environment and/or to dogs (Dantas-Torres, 2008). Over the years, many ticks have managed to survive treatments and there have been reports that some ectoparasiticides, such as pyrethroids, amitraz and organophosphates, are no longer as effective for controlling *R. sanguineus* as they used to be (Eiden et al., 2015; Rodriguez-Vivas et al., 2017).

Plant-based products with ectoparasiticide activity have shown significant results *in vitro* over recent years (Ellse & Wall, 2014). The diversity of these plants, combined with their properties of biodegradability, low cost of production, low toxicity and low environmental waste, confirms the potential for use of these parasite control products (Barreto et al., 2006; Chagas et al., 2012).

Syzygium aromaticum (L.) Merr. & L.M. Perry (Myrtaceae), is popularly known as clove. Eugenol (EG) is the major compound in its essential oil and can reach a proportion of up to 95% (Mbaveng & Kuete, 2017). Both the essential oil (EO) of *Syzygium aromaticum* (EOSA) and EG have already been evaluated regarding their insecticidal activity (Bagavan et al., 2011; Jairoce et al., 2016) and also regarding their pulicidal activity (Lambert et al., 2020). There is a lack of reports about the use of EOSA against *R. sanguineus* in the literature. However, the acaricidal activity of EOSA and EG and of other EOs containing EG as the major component has already been described (Araújo et al., 2016; Ferreira et al., 2019).

Therefore, the aim of this study was to evaluate the *in vitro* activity of EOSA and EG against *R. sanguineus* larvae.

Material and methods

Essential oils from the flower buds of *S. aromaticum* (Kitano[®], São Paulo, Brazil) were obtained by means of hydrodistillation in a Clevenger apparatus for 3 h and they were dried over anhydrous Na₂SO₄. And chemical characterization was performed through gas chromatography (GC-FID and GC-MS) analysis, either proceedings described by Lambert et al. (2020). Technical-grade eugenol (99%) was purchased from Sigma-Aldrich[®], code E51791 (St. Louis, USA).

The experiments followed the standards established by the Ethics Committee for Animal Use (CEUA) of the Veterinary Institute of the Federal Rural University of Rio de Janeiro (UFFRJ), under the approval number CEUA/IV 090/14. The larvae of *R. sanguineus* used in the experiment were obtained from colonies that were maintained in rabbits in the Laboratory for Experimental Chemotherapy in Veterinary Parasitology of UFRRJ.

To carry out bioassays, stock solutions of EG and EOSA were prepared at a concentration of 40 mg.mL⁻¹ using as diluent a solution containing 20% of acetone (Synth) and 3% of Tween-80 (Vetec) in distilled water. Serial dilutions (1:2) were performed from stock solutions, using the same diluent mentioned above, which gave rise to ten solutions over a concentration range from 0.0781 to 40 mg.mL⁻¹. The diluent was also used as negative control. To ensure viability of the colony, fipronil at 0.400 mg.mL⁻¹ was used as a positive control.

The bioassays were performed using the larval immersion test (LIT) method adapted for Chagas et al. (2002). Approximately 100 non-fed 21 days old larvae of *R. sanguineus* were deposited on a 2 cm × 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 × 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:

$$\text{Mortality (\%)}: \text{dead larvae} \times 100 / \text{total larvae} \quad (1)$$

Data obtained for both EG and EOSA were submitted to Probit analysis to estimate Lethal Concentrations (LC₅₀ and LC₉₀) using the IBM SPSS Statistics program, version 26.0 April, 2019. The evaluation of the relative potency of EOSA and eugenol activity on *R. sanguineus* was also calculated out in the same statistics program. All analyzes were calculated considering the 95% confidence interval (p ≤ 0.05).

Results

The major compounds described by Lambert et al. (2020) and found in present study to EOSA, were eugenol (1, 61.42%), beta-caryophyllene (2, 19.95%) and eugenol acetate (3, 14.86%).

The mortality results from non-fed larvae of *R. sanguineus* that were observed for EOSA and EG are shown in Table 1. EOSA showed increasing acaricide activity starting from the concentration of 1.25 mg.mL⁻¹, and reached approximately 90% mortality at 5 mg.mL⁻¹ and 100% at 40 mg.mL⁻¹. The mortality results for EG did not shown linearity in the concentration range between 0.15 and 2.5 mg.mL⁻¹, with rates ranging from 11.49% to 32.54%. There was a substantial increase in mortality from 2.5 to 5.0 mg.mL⁻¹, to reach 86.78% at the latter concentration. Linear increases were observed thereafter, until reaching mortality of 99.42% at the highest concentration tested (40 mg.mL⁻¹). In both tests, no mortality was observed in the negative controls (0%), while in the positive control mortality was higher than 98%, thus demonstrating that the method was applied correctly.

Table 1. Percentage mortality of non-fed larvae of *Rhipicephalus sanguineus* exposed to the essential oil of *Syzygium aromaticum* and eugenol.

Concentration (mg.mL ⁻¹)	Mortality (%)	
	<i>S. aromaticum</i> essential oil	Eugenol
0.078	0	0
0.15	0	32.54
0.31	0	19.22
0.62	0	24.02
1.25	1.08	11.49
2.5	29.27	15.92
5	91.4	86.78
10	96.3	96.4
20	98.59	98.95
40	100	99.42
Control (-)	0	0
Control (+)	100	98.18

The Probit analysis on the acaricidal activity for EOSA and EG is described in Table 2. The LC₅₀ and LC₉₀ estimates found were 3.29 and 6.74 mg.mL⁻¹ and 1.42 and 6.39 mg.mL⁻¹ for EOSA and EG, respectively. Pearson correlation coefficient (r) values were calculated for both assays (EOSA and EG) and were close to 1 (r > 0.9). This indicated that there was a strong linear correlation over the concentration range tested. EOSA showed higher slope values (4.127) than EG (1.965), thus showing greater sensitivity. However, the analysis on relative potency for LC₉₀ (1.05) showed that there was no significant difference between EG and EOSA, regarding activity against these non-fed larvae of *R. sanguineus*.

Table 2. Probit analysis on *Rhipicephalus sanguineus* larvae exposed to different concentrations of the essential oil of *Syzygium aromaticum* and eugenol after 24 hours: LC₅₀ (mg.mL⁻¹), LC₉₀ (mg.mL⁻¹), R² and slope.

	LC ₅₀ (mg.mL ⁻¹) (95% CI)	LC ₉₀ (mg.mL ⁻¹) (95% CI)	R ²	Slope (SE)
<i>S. aromaticum</i> essential oil	3.29 (2.43-4.38)	6.74(4.97-11.70)	0.902	4.127 (0.27)
Eugenol	1.42(1.24-1.63)	6.39 (5.46-7.61)	0.987	1.965 (0.088)

Probit analyses were performed for all data using IBM SPSS Statistics software (version 26.0 April, 2019); LC₅₀ (mg.mL⁻¹) (95% CI): 50% lethal concentration values followed with their 95% confidence interval; LC₉₀ (mg.mL⁻¹) (95% CI): 90% lethal concentration values followed with their 95% confidence interval; R: Pearson correlation coefficient; Slope (SE): slope of the concentration curve and Standard Error.

Discussion

Eugenol is the chemotype of *S. aromaticum* and it shows several properties such as pulicidal and insecticidal activity (Lambert et al., 2020). However, the activity of EOSA against *R. sanguineus* has not yet been reported. The activity of EOSA and EG against *R. microplus* larvae was described by Ferreira et al. (2018), who showed that they caused 100% mortality at concentrations of 2.5 mg.mL⁻¹ and 5 mg.mL⁻¹ for EG and EOSA, respectively. Our results demonstrated that higher concentrations (10, 20 and 40 mg.mL⁻¹) were necessary to achieve *R. sanguineus* larvae mortality above 95% for EOSA and EG.

The difference in mortality rates obtained can be explained in terms of differences in susceptibility between tick species. Greater susceptibility of *R. microplus* than of *R. sanguineus* has already been shown by Monteiro et al. (2012) and Araújo et al. (2016) to EG, and Ferreira et al. (2019) to *Ocimum gratissimum* EO, whose major compound is EG.

The mortality activity of EOs containing EG against *R. sanguineus* larvae has already been described (Coelho et al., 2020; Ferreira et al., 2019). Ferreira et al. (2019) evaluated the EO of *O. gratissimum* (74.5% EG) and despite the largest proportion of EG, the LC₅₀ that they found was higher (LC₅₀ = 6.2 mg.mL⁻¹) than what we found for EOSA (61% EG) (LC₅₀ = 3.29 mg.mL⁻¹).

The larvicidal activity of EG alone against *R. microplus* (Araújo et al., 2016; Ferreira et al., 2018; Monteiro et al., 2012; Novato et al., 2018; Valente et al., 2014) and *R. sanguineus* (Araújo et al., 2016; Coelho et al., 2020; Senra et al., 2013) have already been described. The concentrations with which 100% mortality against *R. microplus* was achieved ranged from 3 mg.mL⁻¹ (Valente et al., 2014) to 5 mg.mL⁻¹ (Ferreira et al., 2018; Monteiro et al., 2012) and 15 mg.mL⁻¹ (Novato et al., 2018).

The LC₅₀ values ranged from 1.7 mg.mL⁻¹ (Valente et al., 2014) to 2.77 mg.mL⁻¹ (Novato et al., 2018) and 4.76 mg.mL⁻¹ (Araújo et al., 2016). Mortality above 99% was reached at higher concentrations for *R. sanguineus* than for *R. microplus* (Araújo et al., 2016; Coelho et al., 2020; Senra et al., 2013). We found mortality above 99% at a higher concentration (40 mg.mL⁻¹) than what was reported by Senra et al. (2013) (10 mg.mL⁻¹) and Coelho et al. (2020) (20 mg.mL⁻¹). On the other hand, our LC₅₀ values were lower (1.42 mg.mL⁻¹) than those reported by Araújo et al. (2016) (5.19 mg.mL⁻¹). Although EG is the major compound of EOSA, we did not find any significant superiority of EG activity against non-fed larvae of *R. sanguineus*, compared with EOSA, thus corroborating the findings of Ferreira et al. (2018) in relation to *R. microplus*. The activity of essential oils on arthropods is generally related to the major compound, however there is evidence that oil compounds may have synergistic activities when together, this fact is described by Ellse and Wall (2014).

Conclusion

The essential oil of *S. aromaticum* and eugenol showed larvicidal efficacy on *R. sanguineus* larvae *in vitro*. Even though *in vivo* efficacy and safety assessments are still needed, these findings are promising for development of products to control these ticks.

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Ethics statement

The experiments followed the standards established by the Ethics Committee for Animal Use (CEUA) of the Veterinary Institute of the Federal Rural University of Rio de Janeiro (UFRFRJ), under the approval number CEUA/IV 090/14.

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Conflict of interests

No conflict of interests.

Authors' contributions

MML and BRA - Writing, Review & Editing. MML, BRA, DRC, DAB, LOM and GAP - Methodology Development. DSAC, YPC and KC - Investigation Conducting a research and investigation process; Writing, Review and Editing. FBS - Resources Provision of study materials, reagents.

Availability of complementary results

All results are present in the manuscript.

This study was conducted in Laboratório de Quimioterapia Experimental em Parasitologia Veterinária, Departamento de Parasitologia Animal, Instituto de Veterinária, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, Brasil.

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