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Evaluation of the combined effect of thymol, carvacrol and (*E*)-cinnamaldehyde on *Amblyomma sculptum* (Acari: Ixodidae) and *Dermacentor nitens* (Acari: Ixodidae) larvae

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ABSTRACT

This study aimed at assessing the combined effect of thymol, carvacrol and (*E*)-cinnamaldehyde on *Amblyomma sculptum* and *Dermacentor nitens* larvae. The effects resulting from treatments were evaluated by means of the modified larval packet test. In order to determine the LC_{50}, components of essential oils, the monoterpenes thymol, carvacrol and phenylpropanoid (*E*)-cinnamaldehyde were individually tested at different concentrations. After determining the LC_{50}, each essential oil component was separately evaluated and then combined with another substance at a 1:1 proportion at the LC_{50} concentration and at 1/2 and 1/4 of the LC_{50}. For *A. sculptum,* the lowest LC_{50} value was obtained for (*E*)-cinnamaldehyde (1.40 mg/ml), followed by thymol (2.04 mg/ml) and carvacrol (3.49 mg/ml). The same order of effectiveness was observed for *D. nitens*, with values of 1.68, 2.17 and 3.33 mg/ml, respectively. In the evaluation of component associations of essential oils against *A. sculptum* larvae, only the combinations between carvacrol and thymol (LC_{50}) and carvacrol and (*E*)-cinnamaldehyde (1/4 LC_{50}) presented a moderate synergetic effect. In turn, for *D. nitens* larvae, the combinations between thymol and carvacrol (LC_{50} and 1/2 LC_{50}) presented a synergetic effect, while the others presented an additive or antagonistic effect. Therefore, it can be concluded that the combination of thymol and carvacrol (LC_{50}) has a moderate synergetic effect against *A. sculptum* larvae, while thymol, combined with carvacrol (LC_{50} and 1/2 LC_{50}), has a synergetic effect against *D. nitens* larvae.

1. Introduction

There are about 65 tick species in Brazil (Dantas-Torres et al., 2009). Among them, *Amblyomma sculptum* (Berlese, 1888), and *Dermacentor nitens* (Neumann, 1897), stand out as very important species, both for animals and public health (Nava et al., 2014; Guglielmone et al., 2006).

A. sculptum is distributed in peri-Amazonian regions in Brazil, specifically in the states of Pernambuco, Piauí, Goiás, Mato Grosso, Mato Grosso do Sul, Espírito Santo, Minas Gerais, Rio de Janeiro, São Paulo and Paraná, as well as in Bolivia, Paraguay and northern Argentina (Nava et al., 2014). Equines and capybaras are the preferred hosts of this tick, but its low host specificity, mainly in immature stages, means it can be also found on other animals, such as deer, cattle and canids, as well as on humans (Oliveira, 2004; Guglielmone et al., 2006). Besides pain, its bite may cause inflammation, fever and stress, both in humans and animals (Oliveira, 2004). This tick is also a vector of *Rickettsia rickettsii* bacterium, the etiological agent of Brazilian Spotted Fever (Guedes et al., 2005).

D. nitens can be found from southern Florida and Texas to northern Argentina (Borges and Leite, 1993). It is known in Brazil as "carrapato-da-orelha-do-cavalo" ("horse ear tick") because it

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mainly parasitizes the ears of equines, although, in large infestations, it can also be found on other body regions (Guimarães et al., 2001; Guglielmone et al., 2006). This tick is of great veterinary importance, causing blood loss, stress, transmission of infectious agents, predisposition to myiasis, and secondary bacterial infections in its hosts (Guglielmone et al., 2006; Labruna and Machado, 2006). It is also responsible for the transmission of *Babesia caballi* (Nuttall and Strickland, 1910), the etiological agent of equine babesiosis (Labruna and Machado, 2006).

The most common method to control ticks is the application of acaricide (Borges et al., 2011). However, the indiscriminate use of these products can lead to toxicity, both in animals and humans, as well as soil and water contamination (Monteiro and Prata, 2013). For these reasons, it is of utmost importance to develop new alternatives to control these ticks, which are safer to humans, animals and the environment (Chagas, 2004; Borges et al., 2011).

Essential oils and their components have been indicated as a promising alternative to control these ectoparasites (Regnault-Roger and Philogène, 2008). Thymol and carvacrol are monoterpenes mainly found in the essential oils of Lamiaceae (Martinov, 1820) and Verbanaceae (Saint-Hilaire,1805) plant families, which act against various tick species (Novelino et al., 2007; Coskun et al., 2008; Cetin et al., 2009, 2010; Dolan et al., 2009; Daemon et al., 2009, 2012; Monteiro et al., 2009, 2010; Mendes et al., 2011; Senra et al., 2013a). (*E*)-cinnamaldehyde is a phenylpropanoid present in the essential oil of plants of the *Cinnamomum* (Schafer, 1760) genus, which has proven activity against *Rhipicephalus microplus* (Canestrini, 1888), *D. nitens* (Senra et al., 2013a), *A. cajennense* s.l. and *Rhipicephalus sanguineus* (Latreille, 1806) (Senra et al., 2013b).

Studies have shown that the association of components of essential oils like terpenes and phenylpropanoid can have a synergetic, antagonistic or additive effect on different target organisms, such as bacteria, nematodes and insects (Didry et al., 1994; Ntalli et al., 2010; Gallardo et al., 2012). However, such research has not been carried out for ticks. Hence, the aim of this study was to assess the combined action of thymol, carvacrol and (E)-cinnamaldehyde on unengorged larvae of *A. sculptum* and *D. nitens*.

2. Material and methods

2.1. Experiment location and tick origin

The study was carried out in the "Laboratório de Artropódes Parasitos" (LAP) of Juiz de Fora Federal University, Minas Gerais, Brazil. *A. sculptum* and *D. nitens* larvae were obtained from the eggs laid by engorged females manually collected on naturally infested horses which had had no recent contact with acaricides, in the municipality of Descoberto, in the state of Minas Gerais. Females of *A. sculptum* were examined for species confirmation according to the new taxonomic proposal of Nava et al. (2014). The larvae of both species submitted to the tests were aged between 15 and 25 days after hatching.

2.2. Origin and dilution of the components of essential oils

The carvacrol and (*E*)-cinnamaldehyde used in this study were purchased from Sigma–Aldrich. The thymol, in the form of crystals, was obtained from Henrifarma Químicos e Farmacêuticos Ltda. All three components of the essential oils tested were 99% pure and were dissolved in 70% ethanol, a solvent with low toxicity to the unengorged larvae of these ticks (Resende et al., 2012).

2.3. Larval packet test

All the tests were carried out using the method proposed by Stone and Haydock (1962) as adapted by Monteiro et al. (2012), in which approximately 100 larvae were placed in the center of a sheet of filter paper (6×6 cm). These sheets were then folded in the middle and their edges were sealed with clips to form packets, which were evenly moistened with 90 μl of the solutions to be tested. The packets (each one an experimental unit) were placed in a climate-controlled chamber (27 °C and relative humidity >80%) for 24 h, after which the mortality rate was measured (percentage of dead larvae).

In order to determine the 50% lethal concentration (LC_{50}) to *A. sculptum* larvae, thymol was tested at concentrations of 0.62, 1.25, 2.5, 5.0, 7.5 and 10.0 mg/ml, while carvacrol and (*E*)-cinnamaldehyde were tested at concentrations of 0.62, 1.25, 2.5, 3.75, 5.0 and 7.5 mg/ml, with 10 repetitions for each group. To determine the LC_{50} to *D. nitens* larvae, thymol, carvacrol and (*E*)-cinnamaldehyde were tested at concentrations of 0.31, 0.62, 1.25, 2.5, 5.0 and 7.5 mg/ml. These concentrations were defined based on previously published results of the acaricidal activity of these components (Daemon et al., 2012; Senra et al., 2013a,b). Control groups were treated with 70% ethanol.

After determining the LC_{50}, thymol, carvacrol and (*E*)-cinnamaldehyde were evaluated separately and in combination with another in 1:1 proportion, at concentrations of LC_{50} and 1/2 and 1/4 of LC_{50}, with 10 repetitions for each treatment. Control groups were exclusively treated with the solvent (70% ethanol). The groups were kept in different climate-controlled chambers at 27 ± 1 °C and RH of $80 \pm 10\%$.

2.4. Analysis of the data

The lethal concentrations for 50% mortality of larvae (LC_{50}) were calculated using POLOPC® software. The combined action of thymol, carvacrol and (*E*)-cinnamaldehyde was measured by calculating the combination index (CI), using the CompuSyn® version 1.0 program (Chou, 2006). This method was chosen due to its sensitivity for the qualitative analysis categorization of the interactions between the substances. In present study, the original categories proposed by Chou (2006) were reduced to facilitate the interpretation of the results obtained (Table 1).

3. Results

The LC_{50} values of the tested substances are presented in Table 2. No mortality was observed for *A. sculptum* or *D. nitens* in any of the control groups. In the tests with a single substance against the *A. sculptum* and *D. nitens* larvae, the substance with the lowest LC_{50} value was (*E*)-cinnamaldehyde, with values of 1.40 and 1.68 mg/ml, respectively, followed by thymol (2.04 and 2.17 mg/ml) and carvacrol (3.49 and 3.43 mg/ml).

Among the nine combinations tested against *A. sculptum* larvae, only the association of carvacrol and thymol (LC_{50}) and (*E*)-cinnamaldehyde (1/4 of LC_{50}) showed moderate synergy. The other associations with thymol and carvacrol exhibited an additive effect, while (*E*)-cinnamaldehyde with thymol and carvacrol presented an antagonistic effect (Table 3).

For the combinations tested against *D. nitens*, thymol and carvacrol, at concentrations of 1/2 of LC_{50} and LC_{50} presented synergetic effect, while at 1/4 of LC_{50} the effect was additive. An antagonistic effect was observed for all of the combinations and concentrations of carvacrol and (*E*)-cinnamaldehyde, as well as of thymol and (*E*)-cinnamaldehyde (Table 4).

4. Discussion

Investigations on the potential of essential oils and their components to control ticks have increased in recent years (Ellse and Wall,