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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 ± 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,

Table 1
Adaptation of the qualitative categories defined by Chou (2006) for description of the combined effect of different substances and their respective combination index values.

Original description (Chou, 2006)	Range of combination index of original description	Adapted description (Combination index)
Very strong synergism	<0.1	Synergism
Strong synergism	0.1–0.3	(<0.7)
Synergism	0.3–0.7	
Moderate synergism	0.7–0.85	Moderate synergism
Slight synergism	0.85–0.90	(0.7–0.9)
Additive	0.90–1.10	Additive (0.9–1.10)
Slight antagonism	1.10–1.20	Moderate antagonism
Moderate antagonism	1.20–1.45	(1.10–1.45)
Antagonism	1.45–3.3	Antagonism
Strong antagonism	3.3–10	(>1.45)
Very strong antagonism	>10	

Table 2
Lethal concentration (LC_{50}) of component of essential oils, thymol, carvacrol and (*E*)-cinnamaldehyde on unengorged larvae of *Amblyomma sculptum* and *Dermacentor nitens*.

Tick species	Components of essential oils	LC_{50} (mg/ml)	Confidence interval (95%)
Amblyomma sculptum	Carvacrol	3.49	3.18–3.82
	Thymol	2.04	1.76–2.37
	(*E*)-cinnamaldehyde	1.40	1.24–1.57
Dermacentor nitens	Carvacrol	3.33	2.87–3.86
	Thymol	2.17	1.92–2.45
	(*E*)-cinnamaldehyde	1.68	1.47–1.92

Table 3
Evaluation of paired combinations of components of essential oils, carvacrol, thymol and (*E*)-cinnamaldehyde on *Amblyomma sculptum* larvae.

Fraction of LC_{50}	Componet	Component	Larval mortality (%)				
	A	*B*	*A* (mean ± S.D)	*B* (mean ± S.D)	*A* + *B* (mean ± S.D)	CI	Effect CompuSyn modified
1/4	Carvacrol	Thymol	1.1 ± 1.5	0.0 ± 0.0	10.5 ± 6.7	1.01	Additive
1/2	Carvacrol	Thymol	14.1 ± 6.6	2.81 ± 2.9	58.3 ± 33.5	1.09	Additive
LC_{50}	Carvacrol	Thymol	89.2 ± 7.1	48.6 ± 18.2	99.5 ± 1.3	0.79	Moderate synergy
1/4	Carvacrol	(*E*)- cinnamaldehyde	1.1 ± 1.5	1.5 ± 1.1	14.1 ± 13.3	0.85	Moderate synergy
1/2	Carvacrol	(*E*)- cinnamaldehyde	14.1 ± 6.6	0.9 ± 0.9	19.8 ± 17.4	1.50	Antagonism
LC_{50}	Carvacrol	(*E*)- cinnamaldehyde	89.2 ± 7.1	37.4 ± 18.9	65.4 ± 36.6	1.76	Antagonism
1/4	Thymol	(*E*)- cinnamaldehyde	0.0 ± 0.0	1.5 ± 1.1	0.0 ± 0.0	4.30	Antagonism
1/2	Thymol	(*E*)- cinnamaldehyde	2.8 ± 2.9	0.9 ± 0.9	2.1 ± 1.6	2.88	Antagonism
LC_{50}	Thymol	(*E*)- cinnamaldehyde	48.6 ± 18.2	37.4 ± 18.9	16.7 ± 8.5	2.54	Antagonism

Combination index (CI) <0.70: synergy; 0.70–0.90: moderate synergy; 0.90–1.10: additive; 1.10–1.45: moderate antagonism; >1.45: antagonism. The control (70% ethanol) produced zero mortality.

Table 4
Evaluation of paired combinations of components of essential oils, carvacrol, thymol and (*E*)-cinnamaldehyde on *Dermacentor nitens* larvae.

Fraction of LC_{50}	Componet	Component	Larval mortality (%)				
	A	*B*	*A* (mean ± S.D)	*B* (mean ± S.D)	*A* + *B* (mean ± S.D)	CI	Effect CompuSyn modified
1/4	Carvacrol	Thymol	5.9 ± 3.6	0.9 ± 1.5	9.4 ± 9.1	1.00	Additive
1/2	Carvacrol	Thymol	9.9 ± 3.5	3.7 ± 4.8	43.9 ± 13.7	0.54	Synergy
LC_{50}	Carvacrol	Thymol	28.5 ± 10.6	34.9 ± 15.8	100 ± 0	0.07	Synergy
1/4	Carvacrol	(*E*)- cinnamaldehyde	5.9 ± 3.6	1.8 ± 3.0	3.6 ± 4.6	2.00	Antagonism
1/2	Carvacrol	(*E*)- cinnamaldehyde	9.9 ± 3.5	8.3 ± 5.2	11.7 ± 7.6	2.01	Antagonism
LC_{50}	Carvacrol	(*E*)- cinnamaldehyde	28.5 ± 10.6	55.8 ± 19.6	52.7 ± 19.8	1.37	Antagonism
1/4	Thymol	(*E*)- cinnamaldehyde	0.9 ± 1.5	1.8 ± 3.0	2.2 ± 2.4	1.65	Antagonism
1/2	Thymol	(*E*)- cinnamaldehyde	3.7 ± 4.8	8.3 ± 5.2	3.3 ± 4.1	2.82	Antagonism
LC_{50}	Thymol	(*E*)- cinnamaldehyde	34.9 ± 15.8	55.8 ± 19.6	47.3 ± 12.3	1.57	Antagonism

Combination index (CI) <0.70: synergy; 0.70–0.90: moderate synergy; 0.90–1.10: additive; 1.10–1.45: moderate antagonism; >1.45: antagonism. The control (70% ethanol) produced zero mortality.

2014) due to the potential advantages they have when compared to chemical products, such as low toxicity to mammals and short persistence in the environment (Miresmailli and Isman, 2006). Besides this, a number of components obtained from the essential oils of plants have been shown to be effective against various tick species, thus demonstrating the potential of developing new acaricidal formulations. However, this is the first study of the effect of the association of monoterpenes (thymol, carvacrol) and a phenylpropanoid (*E*)-cinnamaldehyde on these two tick species.

According to the LC_{50} values against *A. sculptum* and *D. nitens* larvae, (*E*)-cinnamaldehyde had the greatest activity, followed by thymol and carvacrol. The activity of these substances on these ticks' larvae has been reported in other studies (Daemon et al., 2012; Senra et al., 2013a,b), however, in those studies, these

components of essential oils caused high mortality (>80%) starting from the lowest concentrations tested, making it impossible to calculate the LC_{50} values, as achieved in this study. With respect to the order of activity and efficacy, Senra et al. (2013b) also found that (*E*)-cinnamaldehyde was the most active substance against *A. cajennense* s.l. larvae, followed by thymol and carvacrol. In experiments with bacteria of the genera *Streptococcus* and *Prevotella*, (*E*)-cinnamaldehyde performed by Didry et al. (1994), was also the most active substance, followed by thymol and carvacrol. However, Friedman et al. (2002), assessing the bactericidal effect of essential oils on *Campylobacter jejuni, Escherichia coli, Listeria monocytogenes* and *Salmonella enterica*, observed that carvacrol had the strongest activity, followed by (*E*)-cinnamaldehyde and thymol. The differences between our results and those presented in the literature indicate that organisms have varied responses to different natural compounds.

Comparison of the activity of each substance alone against the two tick species showed that *A. sculptum* and *D. nitens* had similar sensitivity to (*E*)-cinnamaldehyde, thymol and carvacrol, with proximate LC_{50} values and overlapping confidence intervals. Daemon et al. (2012) evaluated the activity of thymol on *R. sanguineus* and *D. nitens* larvae and also observed that these two tick species had similar sensitivity to this monoterpene when tested at concentrations of 10, 15 and 20 ul/ml, with respective mortality rates of 96.7, 95.9 and 98.1% against *R. sanguineus* and 90.2, 90.3, 99.5% against *D. nitens*.

With respect to the combinations of thymol and carvacrol, there was a moderate synergetic effect on *A. sculptum* larvae when using the concentration of the $LC_{50,}$ and synergy for *D. nitens* when using the concentration of the LC_{50} and 1/2 of LC_{50}. The synergetic action of thymol combined with carvacrol has already been reported for a variety of organisms, such as bacteria and nematodes (Didry et al., 1994; Ntalli et al., 2010) as well as for other arthropods, such as beetles (Lima et al., 2011) and lepidopterans (Pavela et al., 2010). Lima et al. (2011) observed a synergetic effect of the interaction of thymol and carvacrol against *Tenebrio molitor* (Linnaeus, 1758) and Pavela (2010) observed the same effect in tests with *Spodoptera littoralis* (Boisduval, 1833) larvae. Studies have shown that the toxicity of thymol and carvacrol to different organisms is partly related to the effect of these monoterpenes on the cells of target organisms, since they cause disorganization in the cell membrane, leading it to lose permeability (Medeiros et al., 2011). That effect has been demonstrated both for eukaryotic (Medeiros et al., 2011) and prokaryotic cells (Lambert et al., 2001). In the case of thymol, this effect has also been verified on oocytes of the cattle tick *R. sanguineus* (Matos et al., 2014). Hence, it can be inferred that the synergetic effect may be related to the joint action on the cell membrane of the ticks, with enhanced action in combination. However, histological tests are necessary to confirm this hypothesis.

In insects, thymol and carvacrol have action on the GABA receptor, acting on the neuromuscular system, deregulating motor activity (Waliwitiya et al., 2010; Tong and Coasts, 2010) and on the octopamine receptor, causing cell membrane disruption and compromising the tracheal system (Isman et al., 2007; Khanikor et al., 2013). It can be inferred that these monoterpenes also act on these receptors in ticks, although there are no studies about it in the literature.

The associations between carvacrol and (*E*)-cinnamaldehyde presented an antagonistic effect in all combinations tested against *D. nitens*, while in the case of *A. sculptum*, only the combination with a concentration of 1/4 of LC_{50} did not present an antagonistic effect (showing moderate synergism instead). Pavela (2014), in assessing the effect of some aromatic compounds on *S. littoralis*, found an antagonistic effect of the combination of carvacrol and (*E*)-cinnamaldehyde. However, in a study with bacteria, Didry et al. (1994) observed a synergetic effect when combining carvacrol with (*E*)-cinnamaldehyde against *Streptococcus milleri* and *Prevotella intermedia*. The same result was reported by Ye et al. (2013) based on tests with the bacteria *E. coli*, *Staphylococcus aureus*, *S. intermedius* and *S. haemolyticus,* in which the association of carvacrol and (*E*)-cinnamaldehyde presented a synergetic effect. This fact indicates that different species can respond distinctly to the association of components of essential oils, so that an effect found against one organism cannot be extrapolated to others. These differences can also be related to the testing methods, concentrations tested and source of the substances.

The combination of thymol and (*E*)-cinnamaldehyde had an antagonistic effect at all the concentrations of each substance, for both tick species. The same effect was observed by Didry et al. (1994) in tests applying thymol together with (*E*)-cinnamaldehyde against the bacteria *Streptococcus mitis* and *Prevotella oris*. Pavela (2014) also found an antagonistic effect of thymol combined with (*E*)-cinnamaldehyde against *S. littoralis*. Therefore, it can be assumed that this antagonistic effect is related to the possible reaction of (*E*)-cinnamaldehyde (aldehyde) with thymol and carvacrol (phenolic alcohol), forming a hemiacetal or acetal, which normally does not have biological activity due to the unavailability of an active functional group (phenol and enone) in both compounds.

Thus, it is possible to conclude that the combination of carvacrol and thymol, at the LC_{50}, is more effective in controlling the two tick species, because there is a synergetic effect for both species, thus enabling the use of these monoterpenes in the development of commercial acaricidal formulations aimed at controlling mixed infestations. In turn, combinations containing (*E*)-cinnamaldehyde with thymol and carvacrol are not appropriate for controlling these ectoparasites, since we observed an antagonistic effect in the majority of the associations tested. In cases of synergetic effect, it will be possible to reduce the concentrations of the components of essential oils applied without losing efficiency. This knowledge can serve as basis for developing new acaricidal products. However, further studies are necessary on other stages of these tick species and under natural conditions, as well as toxicity evaluation in the hosts.

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