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Evaluation of synergism and development of a formulation with thymol, carvacrol and eugenol for *Rhipicephalus microplus* control

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ABSTRACT

The acaricidal activity of combinations of thymol, carvacrol and eugenol was evaluated on larvae and engorged females of the cattle tick *Rhipicephalus microplus*. The first step assessed the compounds separately, in concentrations of 3.125, 6.25, 12.5 and 25 mg/mL. Then tests were performed with the compounds combined in the ratio of 1:1 at concentrations of 3.125 and 6.25 mg/mL, along with the control group treated with the solvent (3% DMSO). In the second step, combinations were tested incorporated in a formulation at the concentration de 6.25 mg/mL, using the larval packet and adult immersion tests. The associations carvacrol + thymol (3.125 mg/mL), carvacrol + eugenol and thymol + eugenol (6.25 mg/mL) presented synergism, while the other associations had an additive effect. In the experiments with formulation, all combinations caused 100% larval mortality, but the efficacy was under 15% against engorged females. Therefore, the combinations of thymol + carvacrol (3.125 mg/mL) as well as carvacrol + eugenol and eugenol + thymol (6.25 mg/mL) had a synergistic effect on engorged females, but when incorporated in the formulation, the acaricide activity was strong against larvae but weak against engorged females.

1. Introduction

Since the beginning of the last century, researchers have been searching for alternatives to control the cattle tick, *Rhipicephalus microplus*, due to the large economic losses this ixodid causes to the dairy and beef industries. In Brazil, the latest estimate is that this tick causes annual losses of approximately 3.24 billion dollars (Grisi et al., 2014). Reports exist of populations of this tick that are resistant to all the available chemical bases for their control (Reck et al., 2014). This indicates the need to develop new technologies for management and *R. microplus* control.

Compounds found in essential oils (EOs) of aromatic plants have been shown to be promising alternatives for control of these ectoparasites, by presenting biocidal properties while at the same time being considered eco-friendly, since they do not linger in the environment for long periods, with high biodegradability (Regnault-Roger and Philogene, 2008). Thymol and carvacrol are volatile monoterpenes, present in the EOs of plants of Lamiaceae and Verbenaceae families, while, eugenol is a phenylpropanoid, commonly found in plants of the Myrtaceae family. The activities of thymol, carvacrol and eugenol have been demonstrated against different tick species (Senra et al., 2013a, b).

Studies have shown that the combined use of compounds of the OEs (monoterpenes, sesquiterpenes and phenylpropanoids) has a synergistic effect against insects (Pavela, 2010; Lima et al., 2011) and ticks (larvae) (Novato et al., 2015; Araújo et al., 2016), but no studies have been conducted of the effects on other developmental stages of ticks.

To improve the effect of OEs compounds it is important to incorporate the active ingredient in a formulation (Allen et al., 2007). The

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purposes are to guarantee the product's stability, prevent degradation and microbiological contamination, facilitate application, prolong the period the product remains in the animal and enhance the effect of the active compound (Ferreira et al., 2017; Díaz et al., 2019). Therefore, the objective of the present study was to assess the effect of binary combinations between thymol, carvacrol and eugenol on engorged females of *R. microplus*, as well as to develop formulations and evaluate the efficiency of these compounds on larvae and adult females of this tick.

2. Material and methods

2.1. Ticks and essential oil compounds

Were used females of a susceptible strain of *R. microplus*, (strain Porto Alegre - POA) maintained by artificial infestations on *Bos taurus* dairy calves, kept in the Embrapa Dairy research unit (*CEUA 28/2015*). Thymol, carvacrol and eugenol were purchased from Sigma-Aldrich, with purity of 99%.

2.2. Adult female immersion test (AIT) and evaluation of synergism

Ticks were separated into groups (20 per group) with homogeneous weight ($p = 0.9999$), and each group was immersed for 5 min in 5 mL of the solutions. After the immersion, the females were weighed and kept individually in Petri dishes for oviposition (each dish = an experimental unit), and placed in a B.O.D incubator (27 °C and RH > 80%) for 15 days. After this, the egg mass from each female was weighed and placed in a plastic syringe with the distal end cut, and sealed with hydrophilic cotton, and placed in a B.O.D incubator under the conditions for 20 days. Subsequently, the larval hatching percentage were determined. The values of weight of females before oviposition, egg mass weight and larval hatch percentage were used to calculate the control percentage (Drummond et al., 1973).

The effect of the compounds combinations was assessed by the AIT, in two steps. Firstly, the female ticks were immersed in 5 mL of each compound separately, at different concentrations, to determine the efficacy of each one. Thymol and carvacrol were tested at concentrations of 3.125, 6.25 and 12.5 mg/mL, while eugenol was tested at concentrations of 3.125, 6.25, 12.5 and 25 mg/mL. A control group was formed with solvent dimethyl sulfoxide (DMSO) 3% (v/v).

In the second step, the compounds were tested in binary combinations (1:1 ratio), at the concentrations of 3.125 mg/mL and 6.25 mg/mL (thymol + carvacrol; thymol + eugenol; carvacrol + eugenol).

2.3. Formulations development and efficacy evaluation

The results of the combinations were used to develop a formulation, that was prepared using the combinations that had presented 100% efficacy against the engorged females: thymol + carvacrol (6.25 mg/mL); thymol + eugenol (6.25 mg/mL); and carvacrol + eugenol (6.25 mg/mL). The base formula for incorporation of the active compounds was a 30% ethanol solution combined with a preservative, humectant, penetration agent, surfactant and co-surfactant (Ferreira et al., 2017), listed in Table 1.

We used the larval packet test to evaluate the efficacy of the formulations on the larvae (Monteiro et al., 2012). In this procedure, groups of approximately 100 larvae were placed in the center of a sheet of filter paper (6 × 6 cm). Then each sheet was folded and the sides were sealed with binder clips. The packets were moistened with 90 µL on each side of the solution tested, for a total volume of 180 µL. The packets were placed in B.O.D incubator (27 ± 1 °C and RH > 80 ± 10%) and after 24 h, the mortality rate of the larvae was measured.

Three treatment groups were formed: thymol + carvacrol (6.25 mg/mL + 6.25 mg/mL); thymol + eugenol (6.25 mg/mL + 6.25 mg/mL); and carvacrol + eugenol (6.25 mg/mL + 6.25 mg/mL). Two control groups were also formed, one exposed to the base formula without the active compounds and the other exposed to distilled water. There were 10 repetitions for each group (control or treated).

To evaluate the engorged females, we used the method described in topic 2.2, with formation of groups with the same concentrations used in the test with unfed larvae.

2.4. Data analysis

To evaluate the effect of the combinations, we used the control percentages of the treatments with the isolated and associated compounds to calculate the combination index (CI), using the software CompuSyn® version 1.0 (Chou and Martin, 2005). The effects of the combinations were classified according to the CI values by the categories proposed by adapted by Novato et al. (2015): < 0.70 = synergism; 0.70–0.90 = moderate synergism; 0.90–1.10 = additive effect; 1.10–1.45 = moderate antagonism; > 1.45 = antagonism.

The statistical analysis was performed using Biostat software (version 5.0). The mean values of each treatment were submitted to analysis of variance (ANOVA) followed by the Tukey test, except for nonparametric data, which were submitted to the Kruskal-Wallis and Student-Newman-Keuls tests ($p < 0.05$).

3. Results

3.1. Evaluation of synergism

In the test performed with the compounds separately, carvacrol and thymol presented similar activity, with control percentage of 36.9 and 35.1% at the concentration of 3.125 mg/mL, respectively, reaching 100% at the concentration of 12.5 mg/mL, while eugenol, at the lowest concentration, had efficacy of 2.4%, reaching 100% only at the concentration of 25 mg/mL (Table 2). In the experiments using binary combinations (1:1), the association of carvacrol + thymol had the best activity, with a control percentage of 96.1% at the concentration of 3.125 mg/mL, while the associations of carvacrol + eugenol and thymol + eugenol presented control percentage below 70% at this same concentration. All the combinations tested at the concentration of 6.25 mg/mL were 100% effective (Table 2).

In the comparison of the control percentages of the isolated and associated compounds for calculating the synergism, three

Table 1
Components present in the base-formula for the incorporation of the compounds (thymol, carvacrol and eugenol) for development of the formulation.

Components	Suppliers for base compounds	Function	Concentrations
Tween 80	Labsynth® (Diadema, São Paulo, Brazil)	Surfactant	1 mL
Dimethyl sulfoxide (DMSO)	Labsynth® (Diadema, São Paulo, Brazil)	Penetration agent	2.5 mL
Glycerin	Labsynth® (Diadema, São Paulo, Brazil)	Humectant	2.5 mL
Lauryl	Labsynth® (Diadema, São Paulo, Brazil)	Co-surfactant	0.05 g
Nipagin	Labsynth® (Diadema, São Paulo, Brazil)	Preservative	0.05 g
Ethanol 30%	Dinâmica® (Indaiatuba, São Paulo, Brazil)	Base solution	q.s.p. 50 mL

The combined compounds were previously solubilized in 3% DMSO and incorporated shortly after the preparation of base formula.

Table 2
Effect of binary combinations of thymol, carvacrol and eugenol against *Rhipicephalus microplus* engorged females, under laboratory conditions (27 ± 1 °C and UR > 80 ± 10%).

Compound A	Compound B	Concentration (mg/mL)	[a]Percent control of compounds (thymol, carvacrol and eugenol) associated, or not			C.I	Effect
			A	B	A + B		
Carvacrol	Thymol	3.125	36.9	35.1	96.1	0.68	Synergism
Carvacrol	Thymol	6.25	77.1	80.9	100	0.94	Additive
Carvacrol	Eugenol	3.125	36.9	2.4	68.3	0.95	Additive
Carvacrol	Eugenol	6.25	77.1	34.9	100	0.68	Synergism
Thymol	Eugenol	3.125	35.1	2.4	70.2	0.94	Additive
Thymol	Eugenol	6.25	80.9	34.9	100	0.69	Synergism

Combination Index (CI) - < 0.70 = synergism; 0.70–0.90 = moderate synergism; 0.90–1.10 = additive; 1.10–1.45 = moderate antagonism; > 1.45 = antagonism.
[a] Percent control obtained according to Drummond et al. (1973).

combinations produced CI < 0.70, being classified as synergistic: carvacrol + thymol (3.125 mg/mL); carvacrol + eugenol (6.25 mg/mL); and thymol + eugenol (6.25 mg/mL). The other associations had additive effect (Table 2).

3.2. Evaluation of the formulations efficacy

In the larval packet tests performed with the base formulation, the mortality rate was 100% in all the treatments and was significantly different ($p < 0.01$) in relation to the two control groups, in which no larval mortality occurred (Table 3). In test with engorged females, no significant differences ($p > 0,05$) were observed between the treated and control groups for any of the biological parameters evaluated (egg mass weight and larval hatching percentage). In relation to the control percentages, values near 10% were observed for all the treatments (Table 3).

4. Discussion

All the combinations assessed in this study resulted in positive effects (additive or synergistic). These results are in agreement with the observations in the studies with larvae of different tick species. For unfed *R. microplus* larvae, Araújo et al. (2016) observed synergistic interactions in all combinations (1:1) between carvacrol, thymol eugenol, and synergistic and moderate synergistic effects for *R. sanguineus* s.l. Novato et al. (2015) found a synergistic or additive effect for combinations of thymol + carvacrol, depending on the concentration, against *D. nitens* larvae, while for *A. sculptum* larvae they observed a moderate synergistic or additive effect.

Synergistic effects can also be observed in insects. Lima et al. (2011) found a synergistic effect for combination between thymol + carvacrol on *Tenebrio molitor*. The same was observed in a study of *Spodoptera littoralis*, that where reported synergistic effect of the carvacrol and eugenol association (Pavela, 2010). However, the association of thymol, carvacrol and eugenol has not always has a synergistic effect. Koul et al. (2013) reported an antagonistic effect of associating thymol and carvacrol on larvae of *Helicoverpa armigera*, *Spodoptera litura* and *Chilo partellus*. Singh et al. (2009) did not observe a synergistic effect of combinations containing carvacrol and eugenol on *C. partellus*. These differences found in the effects of combining compounds can be related to the target species studied, indicating that responses are species-specific. Besides this, factors such as concentrations and solvents used can also interfere. Therefore, various aspects must be considered when investigating the interaction of EOs compounds.

Research has shown that compounds found in EOs have a neurotoxic effect, however, the mode of action of each compost can vary (Enan, 2005; Tong et al., 2013; Price and Berry, 2006). Thymol stimulates GABA receptors (Enan, 2005), carvacrol binds to nicotinic acetylcholine receptors (Tong et al., 2013) and eugenol stimulates octopamine receptors (Price and Berry, 2006). These differences in mechanisms of action may be related with the synergistic and additive effect.

In this study the formulation maintained the activity on unfed *R. microplus* larvae, enabling 100% mortality for the three associations. Larvae are the first phase that has contact cattle, starting the parasitic phase of *R. microplus*. The application of the formulation on cattle, eliminating larvae may represent an important reduction in infestation by this tick.

However, in the tests with the engorged females, the formulation did not present a satisfactory result, with efficacy values near 10%. Ferreira et al. (2017) evaluated the activity of the essential oil of *Syzygium aromaticum* and eugenol diluted in 50% ethanol or incorporated in a similar formulation as used here (glycerin + DMSO + polysorbate + lauryl + methylparaben) on engorged *R. microplus* females. They also observed better activity for the oil and eugenol when diluted in ethanol.

According to Ferreira et al. (2017) and Díaz et al. (2019), the development of formulations has the advantage of assuring the stability

Table 3
Mean values and standard deviation of larval mortality, weight of females before oviposition (mg), egg mass weight (mg), larval hatch (%) and percent control (%) of *Rhipicephalus microplus* treated with binary associations (1:1) of thymol, carvacrol and eugenol incorporated in formulations, under laboratory conditions (27 ± 1 °C and RH > 80 ± 10%).

Treatments	Larval packet test	Adult immersion test			
	Mortality (%)	Weight of females before oviposition (mg)	Egg mass weight (mg)	Larval hatching (%)	Percent control (%)
Control 1 - H_2O	0.0[a] ± 0.0	147.7[a] ± 16.4	81.9[a] ± 13.8	95.4[a] ± 5.9	...
Control 2 – Base-formulation	0.0[a] ± 0.0	148.2[a] ± 14.6	80.3[a] ± 12.6	97.8[a] ± 0.3	- 0.3
Formulation - thymol + carvacrol (6.25 mg/mL)	100.0[b] ± 0.0	147.9[a] ± 27.8	80.6[a] ± 21.0	87.4[a] ± 30.7	10.1
Formulation – thymol + eugenol (6.25 mg/mL)	100.0[b] ± 0.0	147.8[a] ± 19.9	76.2[a] ± 15.4	88.7[a] ± 27.6	13.8
Formulation - carvacrol e eugenol (6.25 mg/mL)	100.0[b] ± 0.0	148.9[a] ± 21.7	79.7[a] ± 13.6	88.7[a] ± 28.0	10.6

Means followed by the same letter in the same column do not differ significantly at 5%.

and preserving the active compounds, but in some cases certain components of the formulation or mixtures of these components can reduce the activity of the EOs compounds. To better understand the results, it is necessary to conduct tests by isolating the components of the formulation, to identify which one(s) might cause reduced activity.

We can conclude that the associations of thymol + carvacrol, carvacrol + eugenol and thymol + eugenol, at the concentrations tested, presented synergistic effect on engorged *R. microplus* females. However, when these compounds were incorporated in the formulation developed, there was strong action only on the unfed larvae. Future studies, with new formulation development efforts, with the association of these compounds, may allow the development of contact acaricides for *R. microplus* control.

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