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ORIGINAL PAPER

Acaricidal activity of thymol against larvae of *Rhipicephalus microplus* (Acari: Ixodidae) under semi-natural conditions

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Abstract This is the first study to investigate the activity of thymol on *Rhipicephalus microplus* larvae under semi-natural conditions. For this purpose, tests were conducted in pots with *Brachiaria decumbens* seedlings containing cattle tick larvae. Thymol, diluted in ethanol 50° GL, was tested at concentrations of 2.5, 5.0, 10.0, 15.0, and 20.0 mg/mL, along with the control group treated with the solvent alone. Each treatment was composed of five pots (1 pot=a repetition). The experiment was performed in three steps. On the first day, the larvae were applied at the base of the signalgrass. Twenty-four hours later, approximately 25 mL of the solution was applied with thymol on the top of the vegetation in each pot. The survival of the larvae was measured 24 h after application of the solutions. Each pot was analyzed individually, and the grass fillets contained larvae were cut with scissors, placed in Petri dishes, and taken to the laboratory to count the number of living larvae. At the highest concentrations (10, 15, and 20 mg / mL), the number of live larvae declined by more than 95 % in relation to the control group. The lethal concentration 50 % (LC50) and LC90 values were 3.45 and 9.25 mg/ml, respectively. The application of thymol in semi-natural conditions starting concentration of 10 mg/mL significantly reduced the number of living *R. microplus* larvae.

Keywords Cattle tick · *Brachiaria decumbens* · Semi-natural conditions · Monoterpene

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Introduction

The cattle tick, *Rhipicephalus microplus* (Canestrini, 1888) (Acari: Ixodidae), has serious effects on the productivity of cattle herds because this ectoparasite can cause anemia, weight loss, and decreased milk and meat production, and even animal death in large infestations. Additionally, this tick is responsible for the transmission of pathogenic agents such as the protozoa *Babesia bovis* (Babes, 1888) (Piroplasmida: Babesidae) and *Babesia bigemina* (Smith & Kilborne, 1893) (Piroplasma: Babesidae), as well as the bacterium *Anaplasma marginale* (Jonsson 2006; Andreotti 2010). The losses to breeders also include the expenses for labor and purchase of acaricides and equipment to treat the animals (Furlong et al. 2007; Andreotti 2010). The most recent estimate is that this tick is responsible for total annual losses of over US$ 3 billion in Brazil alone (Grisi et al. 2014).

The use of synthetic acaricides features a significant contribution to control of this tick species. However, the indiscriminate use of these products without proper technical orientation causes multiple problems: contamination of food (beef and milk products) and the environment; intoxication of animals and humans; and selection of resistant tick populations, thus requiring development of new control methods (Mendes et al. 2008).

During the entire life cycle of this tick, only about 5 % of the population is found on host animals, with the other 95 % found in the environment in the form of infesting larvae, eggs, and females in the pre-posture or posture periods. This indicates the need for ongoing control programs, because simple application of acaricides on the animals only affects a small percentage of the overall tick population (Pereira and Labruna 2008). Studies carried out in other countries with other tick species have demonstrated good results from applying pyrethroids and organophosphates in pastures, with reduction of up to 90 % infestation in treated areas. According to Labruna (2008), this initiative is worthy of attention in future research, without obviously failing to consider the risks of environmental contamination.

Thymol, a monoterpene found in the essential oils of various plant species, mainly of the genus *Lippia* (Verbenaceae) (Ntalli et al. 2011), is a substance with potential for this type of application because it has been found to be active against different tick species while having a lesser impact on the environment when compared to commercial acaricides (Isman et al. 2011). Its efficiency on ticks has been demonstrated in vitro against larvae and adult females of *R. microplus* (Novelino et al. 2007; Monteiro et al. 2010; Scoralik et al. 2012); larvae and nymphs of *Amblyomma cajennense* s.l. (Fabricius, 1787) (Mendes et al. 2011; Senra et al. 2013); larvae, nymphs, and females of *Rhipicephalus sanguineus* s.l. (Latreille, 1806) (Monteiro et al. 2009; Senra et al. 2013); and larvae of *Dermacentor nitens* Neumann, 1897 (Daemon et al. 2012).

On the matter of environmental contamination, studies have shown that thymol poses a small risk due to its rapid dissipation and low level of residues (Hu and Coats 2008). In the USA, thymol is approved as a food additive that is safe to human health by the Food and Drug Administration (FDA) (Ji et al. 2005). Studies have shown that application of carvacrol, a monoterpene isomer of thymol, on vegetation infested with ticks was able to significantly reduce the nymph population of *Amblyomma americanum* (Linnaeus, 1758) and *Ixodes scapularis* Say, 1821 (Dolan et al. 2009; Jordan et al. 2011). Therefore, the objective of this experiment was to assess the activity of various thymol concentrations on unengorged *R. microplus* larvae, by tests in semi-natural conditions.

Material and methods

Study location

The study was carried out at the Arthropod Parasites Laboratory of Juiz de Fora Federal University, Minas Gerais, Brazil.

Origin of the ticks

The *R. microplus* larvae were obtained by engorged females obtained from artificial infestations on calves maintained at the experimental farm of the Embrapa Cattle Research Unit, located in the municipality of Coronel Pacheco, Minas Gerais, Brazil (Registration in Ethics Committee on Animal Use of Embrapa 11/2013).

Female ticks were washed in distilled water, dried on sheets of paper towel, placed in Petri dishes, and kept within a climate-controlled chamber (27±1 °C and relative humidity (RH) >80 %) for oviposition. After 15 days, the eggs were weighed into 100-mg aliquots, placed in plastic syringes with the distal end cut, and closed with hydrophilic cotton. The syringes were then maintained under the same temperature and humidity conditions mentioned before. For the experiment, larvae aged 15 days after hatching were used, being selected for the experiment syringes with hatching percentage above 95 %.

Obtaining and diluting of the thymol

Thymol crystals were purchased from Henrifarma Químicos e Farmacêuticos Ltda., with 99.9 % purity degree certificate. Thymol was tested at concentrations of 2.5, 5.0, 10.0, 15.0, and 20.0 mg/mL. The dilution of substances was made according to Scoralik et al. (2012), using ethanol 50 % (50 % ethanol and 50 % distilled water).

Preparation of the pots containing *Brachiaria decumbens* seedlings

Plastic pots measuring 25 cm in height and 26 cm in diameter were used to plant the signalgrass seedlings. The pots were filled with 20 kg of soil obtained from pasture areas of the experimental farm of the Embrapa Dairy Cattle Research Unit, located in the municipality of Coronel Pacheco, Minas Gerais, Brazil. Then, approximately six seedlings were planted in each pot.

All the pots were kept in a open area, with direct incidence from rain and sunlight, just outside the Advanced Laboratory of Zoology of Juiz de Fora Federal University, Minas Gerais, Brazil. The pots were watered daily during the period before the experiment, and urea was applied to promote growth of the seedlings. However, 30 days before the start of the experiment, the urea fertilization was interrupted to prevent the presence of residues of this compound, which is toxic to *R. microplus* (Cunha et al. 2008). The grass plants were also pruned so that only the central part of each pot contained plants, with an average height of 40 cm.

Experiment

The pots were prepared and maintained from May to July 2013, and the treatments started in August that year. The entire experiment was carried out in 72 h. On the first day, adhesive tape was affixed around the edges of all the pots to prevent escape of the larvae. Then, the contents (larvae) of a syringe were placed at the base of the grass plants in each of 40 pots.

After 24 h, each pot was examined to observe the migration of larvae for the apex of the leaves of *B. decumbens*. Following, the application of thymol was performed at different concentrations. For each concentration (treatment), five pots were chosen at random. A manual sprayer was used to apply about 25 mL of the solution on the plants in each pot, directing the spray to the upper part of the plants where the larvae were agglomerated. At the moment of spraying, each pot was taken to an isolated area. After application, the pots were placed in rows outside the laboratory, with 50 cm between rows (Fig. 1). The solution was applied by the same person in all the pots to prevent any variation in the form of application.

It also formed a control group with the application of ethanol 50 %, solvent having low toxicity to larvae of *R. microplus* (Chagas et al. 2003; Gonçalves et al. 2007). Due to the volatility of thymol, the pots of the control group were kept away from the pots of the treated groups.

The survival of the larvae was determined 24 h after applying the solutions. Each pot was analyzed individually, and the grass blades that contained larvae were cut with scissors, placed in Petri dishes (Fig. 2a, b), and was taken to the laboratory to count the living larvae with a vacuum pump (Fig. 2c). Larvae that remained immobile or did not respond to stimuli such as exposure to carbon gas were considered dead.

The number of living larvae found in the control group was compared against the numbers in each treatment group. The treatment efficacy was calculated by the following formula: $\% \text{ efficacy} = \frac{(A-B\times 100)}{A}$, according to Bittencourt et al. (2003), where A=average number of larvae in the control group and B=average number of larvae in each treated group. Probit analysis was used to calculate the lethal concentrations (LC 50 % and LC 90 %), with the POLOPC program (Finney 1971).

Results

The results are presented in Table 1. In the treatments with the highest thymol concentrations (10.0, 15.0, and 20.0 mg/mL), there were on average fewer than 50 living larvae in each treatment, differing significantly (p<0.05) from the control group (on average, 1079) (Table 1). In the treatments with concentrations of 2.5 and 5.0 mg/mL, the average number of live larvae was 631.6 and 483.4, being statistically similar to the control group (p>0.05).

At the lowest concentrations tested (2.5 and 5.0 mg/mL), the efficacy values of the treatments were 41.49 and 55.22 %, respectively. In the other treatments, the values were above 95 %, reaching 99 % in the treatment with a concentration of 20.0 mg/mL (Table 1), and the lethal concentration (LC50 % and LC90 %) values of the treatments were 3.45 and 9.25 mg/mL, respectively (Table 2).

Discussion

Essential oils and their constituents are promising alternatives to the use of synthetic chemical compounds to control plant and animal pests, aiming to diminish the negative effects of synthetic pesticides (Bakkali et al. 2008). The acaricidal activity of thymol, a monoterpene found in essential oil of plants of the families Lamiaceae and Verbenaceae (Pengelly 2004), has already been demonstrated in vitro studies on larvae (Novelino et al. 2007; Scoralik et al. 2012) and engorged

Fig. 1 Pots containing *Rhipicephalus microplus* larvae, each corresponding to a treatment with a different concentration to thymol (mg/mL)

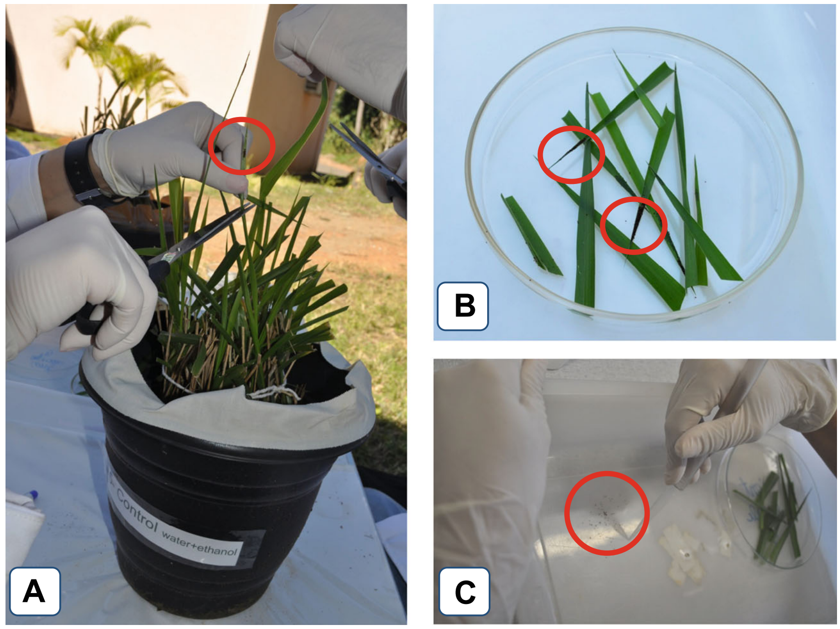

Fig. 2 **a** Cutting the signalgrass blades containing *Rhipicephalus microplus* larvae. **b** Grass blades containing larvae. **c** Count the living larvae

females of *R. microplus* (Monteiro et al. 2010; Matos et al. 2014). However, this article reports the first experiment of the application of thymol in semi-natural conditions.

The mortality observed here was above 95 % starting at a thymol concentration of 10 mg/mL, while in the in vitro study by Scoralik et al. (2012), the same mortality percentage was observed starting at a concentration of 2.5 mg/mL. These differences can be related to the method used and the greater dissipation of thymol in open environments. The experiments of Scoralik et al. (2012) were conducted in controlled laboratory conditions, using the larval packet test, that assures continuous contact of the test organisms with the active ingredient, and the groups were all kept in a climate-controlled chamber, in contrast to the conditions in this study.

Although no studies have yet been published on application of thymol in the environment to control ticks, this approach has been investigated in studies using carvacrol, a monoterpene isomer of thymol that is also very common in essential oils of plants of the genus *Lippia* (Botelho et al. 2007; Cavalcanti et al. 2010), results being observed similar to what was found in this study. Dolan et al. (2009) observed 100 % control of nymphs of *I. scapularis* and *A. americanum* 1 day after applying carvacrol at a concentration of 5.0 % with a sprayer on a layer of burlap containing these ticks. Seven days after the treatment, the control values for *I. scapularis* and *A. americanum* decayed to 82.7 and 65.6 %, respectively. In another study, Jordan et al. (2011) observed a rapid decline in the population of *I. scapularis* and *A. americanum* nymphs when applying carvacrol at a concentration of 2 % in a pasture, with mortality greater than 90 % 1 day after the treatment. However, 14 days after treatment, the mortality rate decayed to 76.7 %, so it was necessary to apply the product again. These results reinforce the potential of these compounds to tick control.

Although there have been no studies with ticks, the environmental application of thymol has been investigated for control of other pests. In a study to control the bacterium *Ralstonia solanacearum* on tomato crops, Ji et al. (2005) reported that thymol at a concentration of 0.7 % reduced the percentage of plants infected by this bacterium to 12 %, while in the control group (treated with 70 % ethanol), the infection rate was 65.5 %. Besides this, they observed greater tomato yield in the treated plots (increase in both the number and size of fruits). Products based on thymol have also been used with

Table 1 Mean of living *Rhipicephalus microplus* larvae recovered from the pots treated with thymol at different concentrations and efficacy of the treatments (%) (mean±standard deviation)

Treatments	Mean of living larvae	Efficacy of the treatments (%)
Control (ethanol 50° GL)	1079.6a±514.35	
Thymol 2.5 mg/mL	631.6a±229.36	41.49
Thymol 5.0 mg/mL	483.4a±133.78	55.22
Thymol 10.0 mg/mL	41.0b±15.06	96.20
Thymol 15.0 mg/mL	49.6b±39.51	95.40
Thymol 20.0 mg/mL	1.4bc±0.89	99.87

Means followed by different letters in the column differ from each other at 5 % significance

Table 2 Lethal concentration 50 % (LC50) and 90 % (LC90) of thymol on unengorged larvae of *Rhipicephalus microplus* kept under semi-natural conditions

LC50 (mg/mL)	CI (95 %)	LC90 (mg/mL)	CI (95 %)
3.45	2.89–4.11	9.25	7.71–13.16

CI confidence interval

high efficacy to control the mite *Varroa destructor* Anderson & Trueman, 2000, a parasite of the honeybee *Apis mellifera* Linnaeus, 1758 (Baggio et al. 2004). Castagnino and Orsi (2012), assessing the effects of thymol alone on colonies of Africanized *A. mellifera* bees, observed that this substance reduced infestation by 67.1 %.

In other countries, studies of the application of synthetic acaricides in the environment to control of other tick species demonstrated that some products of pyrethroids and organophosphates are highly effective (Labruna 2008). Mount et al. (1999) evaluated the application of an acaricide in non-agricultural areas to control the tick *A. americanum*. This method resulted in reductions of 81, 76, and 68 % in the populations of larvae, nymphs, and adults, respectively. When employed together with vegetation reduction, the population declines were 95, 92, and 87 % for larvae, nymphs, and adults. However, the application of pesticides in fields has the drawback of possible environmental contamination. This risk could be minimized by the use of thymol. Miñambres et al. (2010) evaluated the in vitro impact of different thymol doses on the microbial communities in the soil, when applied alone or in combination with a fungicide, and observed that the use of thymol did not significantly alter the microbial activity and biomass, and only posed a risk to the survival of gram-negative bacteria. However, the application of the fungicide in the soil caused a greater reduction in the microbial activity and fungal biomass than thymol. The authors also observed that when the fungicide was mixed with thymol, these negative effects were minimized.

In the USA, the Environmental Protection Agency (EPA) recognizes thymol as a safe compound due to its plant origin, considering that its use in registered pesticides will not cause adverse effects to the environment or human health (Environmental Protection and United States 1993). Hu and Coats (2008) demonstrated that thymol is rapidly dissipated both in water and in soil under aerobic conditions. They measured the time for 50 % to dissipate TD50 in these two environments and observed that in water, the TD50 was 16 days while in soil, it was only 5 days, with volatilization being the main dissipation route.

According to Isman et al. (2011), with rare exceptions, essential oils and their main constituents are relatively nontoxic to mammals, with acute oral LD50 values in rodents between 800 and 3000 mg kg^{-1} for pure compounds and 5000 mg kg^{-1} for formulated products. Besides this, due to their volatility, oils and their constituents are generally not environmentally persistent.

In the present study, thymol was applied on the apex of the forage grass, a method that can minimize the possibility of environmental impacts even more. In this context, the application on this layer of vegetation would be aimed to combat infesting larvae, whose natural behavior of climbing to the apex would bring them into contact with the substance. With regard to environmental impacts, this type of application presents as a favorable aspect; the fact reduces the chance of the substance causing some kind of interference on beneficial organisms on the basis of vegetation and soil. Furthermore, the application in the apex of the forage grass can make it easier to implement this method in management programs of this and other tick species.

This method can also reduce infestation of cattle due to the alteration of the larvae's behavior, since an in vitro test demonstrated that thymol also acts as a repellent on this stage of *R. microplus* (Novelino et al. 2007). Therefore, application at the apex of the vegetation can kill the larvae concentrated there (as indicated in this study) and repel the larvae that are still at the base of the vegetation.

The results obtained in this study show that thymol when applied on *B. decumbens* under semi-natural conditions, in concentrations starting at 10 mg/mL, is effective on *R. microplus* larvae, significantly reducing the number of larvae. These results further emphasize the potential of thymol as an alternative for control of ticks. However, further studies are necessary under natural conditions to assess the effect of this substance on non-target organisms in pastures and to verify the residual action of this monoterpene.

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