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# Anthelmintic effect of thymol and thymol acetate on sheep gastrointestinal nematodes and their toxicity in mice

## Efeito anti-helmíntico do timol e acetato de timila sobre nematoides gastrintestinais de ovinos e toxicidade em camundongos

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## Abstract

Thymol is a monoterpene and acetylation form of this compound can reduce the toxicity and enhance its biological effects. The objective of this study was to evaluate the effect of thymol and thymol acetate (TA) on egg, larva and adult *Haemonchus contortus* and the cuticular changes, acute toxicity in mice and the efficacy on sheep gastrointestinal nematodes. *In vitro* tests results were analyzed by analysis of variance (ANOVA) and followed by comparison with Tukey test or Bonferroni. The efficacy of *in vivo* test was calculated by the BootStreet program. In the egg hatch test (EHT), thymol (0.5 mg/mL) and TA (4 mg/mL) inhibited larval hatching by 98% and 67.1%, respectively. Thymol and TA (8 mg/mL) inhibited 100% of larval development. Thymol and TA (800 µg/mL) reduced the motility of adult worms, by 100% and 83.4%, respectively. Thymol caused cuticular changes in adult worm teguments. In the acute toxicity test, the $LD_{50}$ of thymol and TA were 1,350.9 mg/kg and 4,144.4 mg/kg, respectively. Thymol and TA reduced sheep egg count per gram of faeces (epg) by 59.8% and 76.2%, respectively. In *in vitro* tests thymol presented better anthelmintic activity than TA. However TA was less toxic and in *in vivo* test efficacy was similar.

**Keywords:** Acetylation, thymol, *Haemonchus contortus*, nematodes, sheep.

## Resumo

Timol é um monoterpeno e a acetilação deste composto pode reduzir a toxicidade e potencializar os seus efeitos biológicos. O objetivo deste trabalho foi avaliar o efeito do timol e acetato de timolila (AT) sobre ovos, larvas e adultos de *Haemonchus contortus* e suas alterações cuticulares, toxicidade aguda em camundongos e a eficácia sobre nematoides gastrintestinais de ovinos. Os resultados dos testes *in vitro* foram analisados por análise de variância (ANOVA) e comparados pelo testes de Tukey ou Bonferroni. A eficácia do teste de redução da contagem de ovos nas fezes (RCOF) foi calculada pelo programa BootStreet. No teste de inibição da eclosão de ovos (TEO), timol (0,5 mg/mL) e AT (4 mg/mL) inibiram a eclosão das larvas em 98% e 67,1%, respectivamente. Timol e AT (8 mg/mL) inibiram 100% do desenvolvimento larval. Timol e AT (800 µg/mL) reduziram a motilidade dos nematoides adultos, em 100% e 83,4%, respectivamente. O timol provocou alterações cuticulares nos nematoides adultos. No teste de toxicidade aguda, a DL50 do timol e AT foi de 1.350,9 mg/kg e 4.144,4 mg/kg, respectivamente. Timol e AT reduziram a contagem de ovos por gramas de fezes (OPG) dos ovinos em 59,8% e 76,2%, respectivamente. Nos testes *in vitro* timol apresentou atividade melhor anti-helmíntica do que AT. Entretanto, AT foi menos tóxico do que o timol e no teste *in vivo* apresentaram eficácia semelhante.

**Palavras-chave:** Acetilação, timol, *Haemonchus contortus,* nematoides, ovinos.

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## Introduction

Gastrointestinal nematodes, especially *Haemonchus contortus*, cause economic losses in sheep and goat production worldwide (PERRY & RANDOLPH, 1999; MARIE-MAGDELEINE et al., 2010; QI et al., 2015). This nematode causes weight loss, decreases milk production, and reduces fertility, and may lead to death in cases of high infection (BESIER et al., 2016). The control of these nematodes is traditionally performed with the administration of anthelmintics, but the inappropriate use of these drugs has favored the selection of resistant and multiresistant populations in some parts of the world (JACKSON et al., 2012; SANTOS et al., 2014). Therefore, new environmentally acceptable and effective methods should be researched (RIBEIRO et al., 2015). Highlights among the alternative gastrointestinal nematode control methods include the use of bioactive plants leading to the reduction of the parasite load and the inhibition of negative pathophysiological changes caused in the host (HOSTE et al., 2015).

Essential oils are among the class of natural products that have anthelmintic activity and may be an alternative treatment for the control of gastrointestinal nematode infection of small ruminants (RIBEIRO et al., 2014). The effect of these oils is associated with its biocomposites that act in synergism and that interact with multiple molecular targets during various stages of nematode development, thus interfering with their biochemical and physiological functions (MACEDO et al., 2013; NORDI et al., 2014).

Thymol (2-isopropyl-5-methylphenol) is a major constituent of the essential oils from plants of the genus *Lippia* and *Thymis* (GOMES et al., 2011; ELANDALOUSI et al., 2013) and presents *in vitro* anthelmintic activity on *H. contortus* (CAMURÇA-VASCONCELOS et al. 2007; ELANDALOUSI et al., 2013; FERREIRA et al., 2016). It is a phenolic compound causing high toxicity due to the presence of the hydroxyl radical. The acetylation of this compound, by replacing the hydroxyl radical with an acetyl group can be an alternative to reduce the toxicity and to enhance its biological effects (ANDRE et al., 2016).

Considering the potential use of bioactive compounds that are isolated from essential oils in the control of helminths, the aim of this study was to evaluate the anthelmintic effect of thymol and thymol acetate (TA) on sheep gastrointestinal nematodes and to evaluate their toxicity in mice.

## Materials and Methods

### *Thymol acetylation*

Thymol (Sigma-Aldrich®, St. Louis, USA) (Figure 1) was acetylated by the addition of acetic anhydride (15 mL) and sodium acetate (1.5 g) to 1 g of thymol. The mixture was refluxed for 1 h, the solution was left at room temperature and 20 mL of cold water was added. The solution was neutralized to pH 7.0 with 5% sodium bicarbonate. The reaction mixture was transferred to a separating funnel and washed three times with chloroform (100 mL). The chloroform layer containing acetylated material was washed with water and then dried with sodium sulfate. The solvent was evaporated under reduced pressure (MATOS, 1997). The yield of TA (Figure 1) was 84.5%. To confirm the acetylation process, TA was subjected to thin layer chromatography and characterized by infrared spectroscopy (FTIR) using a model 8300 (Shimadzu Corporation, Japan).

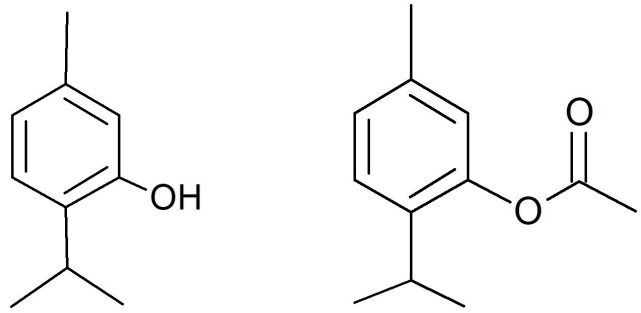


**Figure 1.** Chemical structure of thymol and thymol acetate.

### *Animal welfare*

This study was approved by the Ethics Committee for the Use of Animals at the Universidade Estadual do Ceará (Protocol number: 32228358/2014).

### *Egg hatch test (EHT)*

The egg hatch test (EHT) was performed according to Coles et al. (1992). Briefly, feces were collected directly from the rectum of sheep harboring a *H. contortus* monospecific infection, and the eggs were recovered according to the protocol of Hubert & Kerboeuf (1992). Aliquots (250 µL) of a suspension containing approximately 100 fresh eggs were mixed with 250 µL of the following treatments: G1: 0.03 to 0.5 mg/mL thymol; G2: 0.25 to 4 mg/mL TA; G3: 1% Tween 80 (negative control) and G4: 0.025 mg/mL thiabendazole (positive control). The eggs were incubated for 48 h at 25°C, and a drop of Lugol's iodine was added. The eggs and first-stage larvae (L1) were counted under a light microscope. Three repetitions were performed with five replicates for each treatment and for each control.

### *Larval development test (LDT)*

A larval development test (LDT) was performed according to Camurça-Vasconcelos et al. (2007). For this, *H. contortus* eggs were incubated for 24 h at 28°C to obtain the L1. Next, 500 µL of a suspension containing approximately 250 L1 was incubated for 6 days at room temperature with the same volume of the following treatments: G1: thymol and G2: TA, at the concentrations of 0.5 to 8 mg/mL; G3: 1% Tween 80 (negative control) and G4: 0.008 mg/mL ivermectin (Ivomec®, Merial Saúde Animal, São Paulo, Brazil) in 1 g of feces collected from a gastrointestinal nematode-free sheep. Then, the third-stage larvae (L3) were recovered according to Roberts & O'Sullivan (1950), and a drop of Lugol's iodine were added. The L3 were counted under a light microscope. Three repetitions with five replicates for each treatment and for each control were performed.

### *Adult worm motility (AWM)*

Adult worms were collected from an experimentally infected lamb four weeks after infection. Immediately after slaughtering, the abomasum was removed, opened and placed in a saline solution at 37°C. Mobile adult female worms were rapidly collected and put into 24-multiwell plates at a density of 3 worms per well in 1 mL of PBS at 37°C in the presence of 4% penicillin/streptomycin (Sigma-Aldrich® St. Louis, USA). After 1 hour of incubation (37°C, 5% carbon dioxide) 1 mL of 800, 400, 200 and 100 µg/mL of the following treatments were added to the worms: G1: thymol; G2: TA; G3: PBS plus 4% penicillin/streptomycin (negative control) and G4: 100 µg/mL ivermectin (Ivomec®, Merial Saúde Animal, São Paulo, Brazil). The measurements were performed on eight replicates per concentration for each treatment. The motility of adult worms was noted by careful observation under an inverted microscope at a magnification of 40× after 6, 12 and 24 h (HOUNZANGBE-ADOTE et al., 2005).

### *Scanning electron microscopy (SEM)*

*H. contortus* adult females treated with 800 µg/mL TA or thymol for a period of 24 hours were subsequently fixed in a 2.5% glutaraldehyde solution and in a sodium cacodylate buffer 0.1 M (CACO) for 72 h. After three washes in the same buffer, the worms were placed in 2% osmium, and posteriorly in CACO 0.1 M (pH 7.4) buffer fixative for 1 h. Samples were washed two times with CACO and distilled water and were dehydrated in a graded acetone series (30%, 50%, 70%, 90% and 100%). Critical point drying was completed using a CPD 030 (Bal-Tec, Liechtenstein), and samples on metal stubs were coated with a 10 nm layer of gold in a sputter coating machine (Leica SCD 500, Leica Microsystems, Wetzlar, Germany). Parasites were then observed with a scanning electron microscope (FEI QUANTA 200 FEG ESEM, FEI Company, USA) at an accelerating voltage of 20 kV (ANDRE et al., 2016).

### *Acute toxicity for mice*

A total of 90 female Swiss albino mice (*Mus musculus*) were used, with an average weight of 25 g. Mice were kept in polypropylene boxes and were provided commercial food (Labina®, Purina, São Paulo, Brazil) and water *ad libitum*. The mice were randomly divided into treatment groups. G1 to G4 received 250, 500, 1000 and 2000 mg/mL thymol; G5 to G8 received 2000, 2500, 3000 and 3500 mg/kg of TA; G9 received 1% Tween 80. The treatments were administered in a single oral dose. The animals were observed for 15 days and mortality were recorded. The total number of dead animals was verified, and the lethal doses ($LD_{10}$ and $LD_{50}$) were calculated.

### *Fecal egg count reduction test (FECRT)*

The test was performed in two steps. First, the action of the TA on gastrointestinal nematodes of sheep was evaluated in June 2014. A total of 30 crossbred sheep of both sexes was used, which weighed 18 kg on average and were aged between 6 and 16 months. The sheep were reared in a semi-arid region of northeastern Brazil and were fed on native pasture with water *ad libitum*. The sheep were selected according to the number of eggs in feces (epg), over 500, that was assessed using the McMaster technique (UENO & GONÇALVES, 1998). Sheep were divided into 3 homogeneous groups (n = 10) according to the epg and randomly assigned to the following treatments: G1: 250 mg/kg TA; G2: water as negative control and G3: 2.5 mg/kg of monepantel (Zolvix®, Novartis, New Zealand), positive control. In the second phase, one year after the first test, on the same farm, the anthelmintic effect of thymol was evaluated. Thirty crossbred sheep of both sexes, weighing 23 kg on average and whose age ranged between 6 and 16 months were selected. The sheep were divided into 3 homogeneous groups (n = 10) according to the epg and were treated with the following treatments: G1: 250 mg/kg thymol; G2: water as negative control and G3: 2.5 mg/kg of monepantel. Each sheep received a single treatment. Feces were collected directly from the rectum of the animals on days 0, 7 and 14 after treatment to determine the epg. Fecal cultures were performed in accordance with the method of Roberts & O'Sullivan (1950).

## Statistical Analysis

The larvae hatching percentage was determined according to the following equation: (number of hatched eggs /number of hatched larvae + number of eggs) × 100. The inhibition of larval development was calculated based on the following equation: [(L3 control group – L3 treated group)/L3 control group] × 100. Adult worm motility was evaluated as the number of motile worms/total number of worms per well.

The results of EHT were analyzed by analysis of variance (one-way ANOVA) followed by comparison with Tukey's test ($P<0.05$). In LDT a randomized experimental design was performed with two substances (thymol and TA) and 5 levels of concentration and two controls describing a 2 x 5 factorial. In AWM it was performed a randomized experimental design during three different times (6, 12 and 24 h) and 4 concentrations of thymol and thymol acetate and two controls describing a factorial 3 x 4. Then in both tests, TDL and AWM, we performed a two-way ANOVA followed by comparison with Bonferroni test in order to detect signifcant statistical differences ($P<0.05$) using Graph Pad Prism® 5.0.

The effective concentrations for inhibiting 50% ($EC_{50}$) of egg hatching and the larval development and the lethal dose to 10% ($LD_{10}$) and 50% ($LD_{50}$) of the mice in the acute toxicity test were determined by linear regression using SPSS 17.0 program for Windows (IBM, New York, USA). The results of the worm motility inhibition were expressed as the mean ± standard error (SE).

The egg count reduction percentage was calculated by the BootStreet program (OUZIR et al., 2011) through the arithmetic average using the formula 100 (1 – XT/XC), where XT and XC are the average epg in the treatment and control groups, respectively (COLES et al., 1992). The epg of thymol and TA in relation to FECRT were transformed to log (x + 1) and subjected to ANOVA and Friedman tests using GraphPad Prism® 5.0 software. The significance level was $P<0.05$.

## Results

After acetylation, a new band due to an inserted acetyl group appears at 1760 $cm^{-1}$ for the TA. The 3214 $cm^{-1}$ band, which is characteristic of the hydroxyl group, disappeared, while the band at 2963 $cm^{-1}$ attributed to CH remained. The main 1" bands (at 1206 $cm^{-1}$ and 1370 $cm^{-1}$) were also detected (Figure 2).

The results of thymol and TA on EHT are shown in Table 1 and 2. Thymol concentration of 0.5 mg/mL inhibited 98% of larval hatching, while TA inhibited 67.1% at a concentration of 4 mg/mL. The $EC_{50}$ for EHT was 0.08 (0.07–0.09) mg/mL and 1.9 (1.9–2.4) mg/mL for thymol and TA, respectively.

The LDT results are shown in Table 3. At a concentration of 4 mg/mL for both thymol and TA, 100% of larval development was inhibited. These results did not differ statistically from the positive control (P>0.05). The $EC_{50}$ values for LDT were 1.0 (0.9-1.2) mg/mL and 0.8 (0.6-1.2) mg/mL for thymol and TA, respectively.

On the AWT, thymol and TA, each at a concentration of 800 µg/mL, inhibited worm motility by 100% and 83.4%, respectively, at 24 h post-exposure (Table 4 and 5). These results did not differ statistically from the positive control (P>0.05). The percent motility inhibition of the negative control (PBS) and positive control (ivermectin) was 14.3% and 100%, after 24 h of exposure, respectively. The worm motility was dose-dependent.

The parasites exposed to thymol displayed wrinkled cuticles and the presence of bubbles emerging from the integument. TA did not cause changes in the cuticle (Figure 3).

In the acute toxicity test, the $LD_{10}$ and $LD_{50}$ of thymol were 772.1 (224.6-1,056.1) mg/kg and 1,350.9 (1,068.2–1,732.51) mg/kg, respectively, and the $LD_{10}$ and $LD_{50}$ of TA were 2,522.8 (28.1-3,133.0) mg/kg and 4,144.4 (3,356.1 – 191,729.5) mg/kg, respectively.

The results of FECRT are presented in Tables 6 and 7. TA and monepantel reduced epg by 76.2% and 96.7%, respectively, and thymol and monepantel reduced epg by 59.8% and 82.2%, respectively, by 14 days post-treatment. The results of thymol and TA are significantly different from the negative and positive control of the respective tests (P<0.05). However, there was no significant difference between thymol and TA (P>0.05).

**Table 1.** Inhibition of hatching (mean ± standard error) of *Haemonchus contortus* eggs exposed to thymol.

| Concentration (mg.mL$^{-1}$) | Thymol |
|---|---|
| 0.5 | 98.0±0.5$^{A}$ |
| 0.25 | 93.8±1.3$^{A}$ |
| 0.12 | 67.5±2.6$^{B}$ |
| 0.06 | 33.4±1.6$^{C}$ |
| 0.03 | 12.5±1.6$^{D}$ |
| Tween 80 (1%) | 4.0±0.3$^{E}$ |
| TBZ (0.025 mg.mL$^{-1}$) | 94.5±0.5$^{A}$ |

Capital letters compare mean in the columns. Different letters indicate significantly different values (P<0.05).

**Table 2.** Inhibition of hatching (mean ± standard error) of *Haemonchus contortus* eggs exposed to thymol acetate.

| Concentration (mg.mL$^{-1}$) | Thymol Acetate |
|---|---|
| 4 | 67.1± 1.4$^{A}$ |
| 2 | 55.2± 1.8$^{B}$ |
| 1 | 30.5± 1.1$^{C}$ |
| 0.5 | 15.4±0.8$^{D}$ |
| 0.25 | 7.5±1.0$^{E}$ |
| Tween 80 (1%) | 3.9±0.3$^{E}$ |
| TBZ (0.025 mg.mL$^{-1}$) | 94.5±0.5$^{F}$ |

Capital letters compare mean in the columns. Different letters indicate significantly different values (P<0.05).

**Table 3.** Larval development inhibition (mean ± standard error) of *Haemonchus contortus* larvae exposed to thymol and thymol acetate.

| Concentration (mg.mL$^{-1}$) | Thymol | Thymol Acetate |
|---|---|---|
| 8 | 100±0.0$^{Aa}$ | 100±0.0$^{Aa}$ |
| 4 | 98.4±0.4$^{Aa}$ | 99.3±0.2$^{Aa}$ |
| 2 | 78.4±2.4$^{Ba}$ | 80.1±2.4$^{Ba}$ |
| 1 | 44.2±2.1$^{Ca}$ | 47.2±1.6$^{Cb}$ |
| 0.5 | 14.0±1.4$^{Da}$ | 29.0±1.5$^{Db}$ |
| Tween 80 (1%) | 5.1±1.7$^{Ea}$ | 5.4±1.5$^{Ea}$ |
| Ivermectin (0.008 mg.mL$^{-1}$) | 99.9±0.0$^{Aa}$ | 99.8±0.0$^{Aa}$ |

Capital letters compare mean in the columns and small letters compare mean in the rows. Different letters indicate significantly different values (P<0.05).

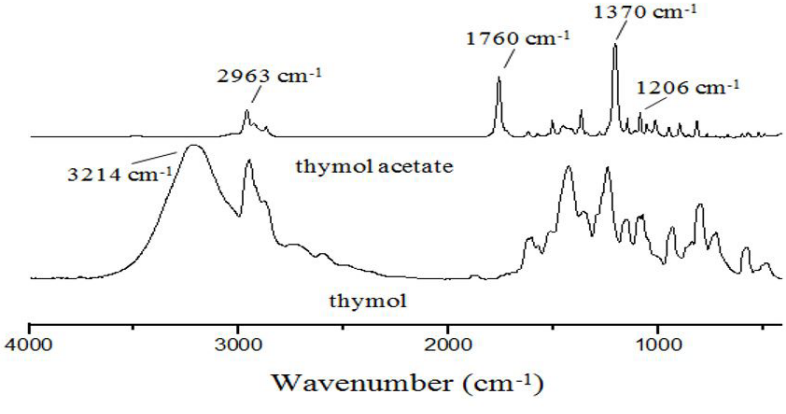


**Figure 2.** Infrared spectra of the sample of thymol and thymol acetate.

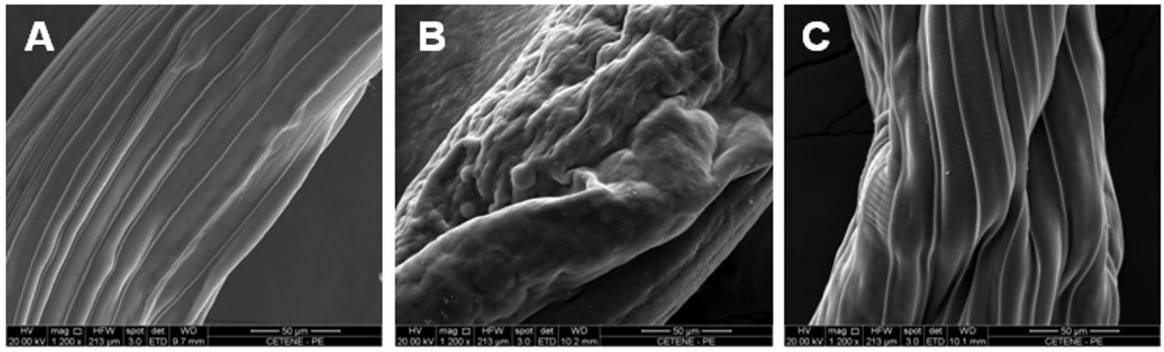


**Figure 3.** Scanning electron microscopic image of cuticle of *Haemonchus contortus* adult female after incubation with PBS (A), thymol (B) or thymol acetate (C).

**Table 4.** Effect (mean ± standard error) of thymol on motility of adult worms of *Haemonchus contortus.*

| Treatment | Concentrations (µg/mL) | Exposure time of adult worms to treatments (hours) | | |
|---|---|---|---|---|
| | | 6 h | 12 h | 24 h |
| | | % inhibition of motility ± SE | % inhibition of motility ± SE | % inhibition of motility ± SE |
| Thymol | 800 | 70.9±0.2$^{Aa}$ | 83.4±0.16$^{Aa}$ | 100±0.0$^{Ab}$ |
| | 400 | 58.4±0.2$^{Aa}$ | 83.4±0.16$^{Ab}$ | 95.9±0.72$^{Ac}$ |
| | 200 | 0.0±0.0$^{Ba}$ | 0.0±0.0$^{Ba}$ | 58.4±0.75$^{Bb}$ |
| | 100 | 0.0±0.0$^{Ba}$ | 0.0±0.0$^{Ba}$ | 33.4±0.14$^{Cb}$ |
| Ivermectin (positive control) | 100 | 100±0.0$^{Ca}$ | 100±0.0$^{Aa}$ | 100±0.0$^{Aa}$ |
| PBS (negative control) | | 0.0±0.0$^{Ba}$ | 0.0±0.0$^{Ba}$ | 12.5±0.18$^{Ba}$ |

Capital letters compare mean in the columns and small letters compare mean in the rows. Different letters indicate significantly different values (P<0.05).

**Table 5.** Effect (mean ± standard error) of thymol acetate on motility of adult worms of *Haemonchus contortus.*

| Treatment | Concentrations (µg/mL) | Exposure time of adult worms to treatments (hours) | | |
|---|---|---|---|---|
| | | 6 h | 12 h | 24 h |
| | | % inhibition of motility ± SE | % inhibition of motility ± SE | % inhibition of motility ± SE |
| Thymol acetate | 800 | 20.9±0.1$^{Ba}$ | 83.4±0.16$^{Ab}$ | 83.4±0.16$^{ABc}$ |
| | 400 | 4.2 ±0.0$^{Ba}$ | 8.4±0.12$^{Ba}$ | 66.7±0.07$^{BCb}$ |
| | 200 | 0.0±0.0$^{Ba}$ | 0.0±0.0$^{Ba}$ | 45.9±0.15$^{CDb}$ |
| | 100 | 0.0±0.0$^{Ba}$ | 0.0±0.0$^{Ba}$ | 25±0.15$^{Db}$ |
| Ivermectin (positive control) | 100 | 100±0.0$^{C}$ | 100±0.0$^{A}$ | 100±0.0$^{A}$ |
| PBS (negative control) | | 0.0±0.0$^{B}$ | 0.0±0.0$^{B}$ | 12.5±0.18$^{Ba}$ |

Capital letters compare mean in the columns and small letters compare mean in the rows. Different letters indicate significantly different values (P <0.05).

**Table 6.** Mean efficacy and egg counts per gram of feces (epg±standard deviation) of thymol acetate and monepantel on sheep gastrointestinal nematodes.

| Treatments | Day 0 | Day 7 | Day 14 |
|---|---|---|---|
| Thymol acetate | | | |
| Mean epg | 2660±2408$^{Aa}$ | 1590±1309$^{ABab}$ | 735±561,3$^{Ba}$ |
| Efficacy (%) | - | 35.4 | 76.2 |
| Monepantel | | | |
| Mean epg | 2670± 2387$^{Aa}$ | 165.3± 141,1$^{Ba}$ | 100 ± 122,1$^{Bb}$ |
| Efficacy (%) | - | 93.2 | 96.76 |
| Mean epg (negative control) | 2570±2022$^{Aa}$ | 2465±1707$^{Ab}$ | 3090±2026$^{Ac}$ |

Capital letters compare mean in the rows and small letters compare mean in the columns. Different letters indicate significantly different values (P<0.05).

**Table 7.** Mean efficacy and egg counts per gram of feces (epg± standard deviation) of thymol and monepantel on sheep gastrointestinal nematodes.

| Treatments | Day 0 | Day 7 | Day 14 |
|---|---|---|---|
| Thymol | | | |
| Mean epg | 3350±1488$^{Aa}$ | 2110±1454$^{ABa}$ | 1195±1492$^{Ba}$ |
| Efficacy (%) | - | 25.5 | 59.8 |
| Monepantel | | | |
| Mean epg | 3250± 3250$^{Aa}$ | 965± 1278$^{Bb}$ | 530 ± 682,0$^{Ba}$ |
| Efficacy (%) | - | 66.1 | 82.2 |
| Mean epg (negative control) | 2800±3843$^{Aa}$ | 2825±3525$^{Aa}$ | 3220±3631$^{Ab}$ |

Capital letters compare mean in the rows and small letters compare mean in the columns. Different letters indicate significantly different values (P<0.05).

The larvae recovered by fecal cultures of sheep before treatment were identified as *Haemonchus* spp. (90%), *Trichostrongylus* spp. (7%) and *Oesophagostumom* spp. (3%), while those recovered 14 days after treatment with TA were *Haemonchus* spp. (72%), *Trichostrongylus* spp. (19%) and *Oesophagostumom* spp. (9%). In the second FECRT, larvae recovered from sheep stool cultures treated with thymol were identified as *Haemonchus* spp. (66%), *Trichostrongylus* spp. (31%) and *Oesophagostumum* spp. (3%), while those recovered 14 days after treatment were *Haemonchus* spp. (61%), *Trichostrongylus* spp. (35%) and *Oesophagostumum* spp. (4%).

## Discussion

Medicinal plants are used to control parasites, and their administration is based in ethnoveterinary knowledge. Studies are needed to validate compounds derived from plants that have antiparasitic activity because they are safer and cheaper than commercial anthelmintics, besides to reduce veterinary drug residues in products of animal origin (CHAGAS, 2008; HOSTE et al., 2015).

The anthelmintic action of essential oils is associated with major components, justifying the investigation of the anthelmintic action of these compounds for the development of new anthelmintic drugs (MACEDO et al., 2013; RIBEIRO et al., 2013). Thymol is a monoterpene phenol with many pharmaceutical properties including the following: anthelmintic (CAMURÇA-VASCONCELOS et al., 2007), acaricide (ARAÚJO et al., 2015), anti-*Leishmania infantum* (MORAIS et al. 2014), anti-*Trypanosoma cruzi* (ESCOBAR et al., 2010) and anti-*Echinococcus multilocularis* (MARÍA & CELINA, 2014). However, thymol is more toxic than many esters, therefore, the synthesis of TA was performed to obtain a derivative of thymol with improved biological activity and less toxicity (MORAIS et al., 2014).

*In vitro* tests are used to select compounds that have anthelmintic activity (HOUNZANGBE-ADOTE et al., 2005). Thymol showed superior efficacy than TA, probably because the acetylated compound could not penetrate the three layers (outer vitelline, average chitin and inner layer of lipids) that form the *H. contortus* eggshell (MANSFIELD et al., 1992). The fibrils of chitin in the intermediate layer hinders the penetration of acetylated compounds because the higher liposolubility conferred by the acetyl radical only allows greater penetration in lipid membranes. Carvacryl acetate (CA), a derivate acetylated from carvacrol, presented lower ovicidal activity than the origin compound (ANDRE et al., 2016), providing evidence that acetylation did not potentiate the ovicidal activity of these monoterpenes. Thymol presented ovicidal activity ($EC_{50}$ = 0.1 mg/mL) similar to citral ($EC_{50}$ = 0.1 mg/ml) and higher than the compounds isolated from essential oils that exhibit anthelmintic activity, such as carvacrol ($EC_{50}$ = 0.17 mg/mL), anethol ($EC_{50}$ = 0.6 mg/mL), Terp-4-ol ($EC_{50}$ = 0.63 mg/mL), borneol ($EC_{50}$ = 1.5 mg/mL), and camphor ($EC_{50}$ = 1.8 mg/mL) (CAMURÇA-VASCONCELOS et al., 2007; QI et al., 2015; MACEDO et al., 2015; ANDRE et al., 2016). The ovicidal effect of thymol is probably related to the presence of hydroxyl radical in its chemical structure, considering that thymol acetate presented significantly lower results at the EHT. Thymol is a secondary metabolite of plants and several hypotheses have been made to explain the action of these substances on larval hatching, either preventing changes in the egg shell permeability through the binding to lipoproteins of the eggshell membranes (PERRY, 2002) or inhibiting the activation of egg hatching enzymes (MOLAN & FARAJ, 2010) or competitively binding to hatching factors in the eggshell and consequently altering the hatching process (DONCASTER & SHEPHERD, 1967).

For the LDT results, thymol and TA exhibited no significant difference (P>0.05). The TA ($EC_{50}$ = 0.8 mg/mL) larvicide activity was superior to citral ($EC_{50}$ = 1.3 mg/mL), anetol ($EC_{50}$ = 2.1 mg/mL), camphor ($EC_{50}$ = 7.8 mg/mL) and borneol ($EC_{50}$ = 1.9 mg/mL) (MACEDO et al., 2015; QI et al. 2015) and lower than CA ($EC_{50}$ = 0.3 mg/mL).

The larvicidal action of thymol may be related to its interaction with SER-2 tyramine receptor, since this monoterpene acts on this receptor in *Caenorhabditis elegans* (LEI et al., 2010). This receptor modulates a number of key processes in nematodes, including pharyngeal pumping, locomotion and egg laying (SMITH et al., 2007), besides being expressed in all phases of the life cycle of *H. contortus* (RAO et al., 2010), justifying the possible interaction of this monoterpene with SER-2 tyramine receptor and its larvicidal action. Both thymol and TA showed larvicidal action indicating that replacement of the hydroxyl radical by the acetyl group did not change the larvicidal activity of TA, proving that the action of thymol is not related to the hydroxyl radical.

AWM is an *in vitro* test that evaluates the action of compounds isolated from natural products and that can be used to assess the interaction of bioactive compounds with the cuticle of adult nematodes in a short time by SEM (ANDRE et al., 2016;

CAVALCANTE et al., 2016). Thymol showed superior action to TA in inhibiting motility of the adult worms. This can be related to the formation of bubbles in the cuticles of these worms, due to the monoterpene that affects the organization and the electrostatic property of the membrane surface, changing the permeability and inhibiting the activity of membrane proteins, such as ATPases (SÁNCHEZ et al., 2004). Thymol and TA both have a low molecular weight (150.24 g/mol and 192.25 g/mol, respectively), and this property may lead to increase penetration of the compounds via transcuticular diffusion, which is a common way for non-nutrient and non-electrolyte substances to gain entry to helminths (EGUALE et al., 2007). *In vitro* tests evaluating the action of thymol on *Echinococcus granulosus* found the formation of blebs on the tegument and the presence of numerous vacuoles in the inner tissue of these cestodes (ELISSONDO et al., 2008). The acetyl radical does not potentiate the efficacy of TA in relation to thymol on the motility of the adults, differing from the CA that caused *H. contortus* injuries (ANDRE et al., 2016), but this compound probably penetrates the cuticle of the parasite and causes internal ultrastructural lesions.

The reduction of toxicity of the acetylated compound was observed when comparing the toxicity of TA and thymol in mice. In the macrophage cytotoxicity assay, thymol decreased cell survival to 36.5%, whereas the TA was non-toxic to the cells (MORAIS et al., 2014).

Thymol and TA did not present statistically different results ($P > 0.05$) in FECRT. However, studies suggest that acetylated substances have higher liposolubility (ANDRE et al., 2016), which facilitates metabolism, distribution in gastrointestinal tissues and penetration through the cuticle of the parasites (LANUSSE & PRICHARD, 1993). Despite the higher liposolubility of thymol acetate, thymol caused greater damage to the adult nematode cuticle and inhibited parasite motility *in vitro*, being the possible justification for the similar effects obtained in FECRT. Further studies should be performed to determine the pharmacokinetics of these biocompounds in sheep.

## Conclusions

Thymol and TA showed ovicidal, larvicide and adulticide activity. The TA has low toxicity in mice and good effects against sheep gastrointestinal nematodes. This result suggests that the acetylation of thymol was effective. Nonetheless, it is necessary to increase the effectiveness of TA, and the use of encapsulation techniques may be one feasible solution.

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