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Original Article

In vitro activity of the essential oil from *Hesperozygis myrtoides* on *Rhipicephalus (Boophilus) microplus* and *Haemonchus contortus*

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ABSTRACT

Commercial antiparasitics have been the main tool to control parasites, but due to the resistance development, plant extracts have been widely investigated to find new molecules. The present study aimed to investigate the *in vitro* acaricide and anthelmintic activities of the essential oil from the aerial parts of *Hesperozygis myrtoides* (A.St.-Hil. ex Benth.) Epling, Lamiaceae. The essential oil was obtained by hydrodistillation analyzed by GC-FID and GC-MS. Four tests were conducted *in vitro* to screen the antiparasitic action of the essential oil. The evaluation on *Rhipicephalus (Boophilus) microplus* was performed with the adult immersion test at concentrations ranging from 0.391 to 25 mg/ml and the larval packet test from 3.125 to 100 mg/ml. For *Haemonchus contortus* the egg hatch test was performed from 0.012 to 25 mg/ml and the larval development test from 0.003 to 0.4 mg/ml. The LC_{50} and LC_{90} values were calculated by Probit. The main components identified in the essential oil were isomenthone (47.7%), pulegone (21.4%), limonene (7.7%), isomenthyl acetate (6.8%) and neoisomenthol (3.9%). In the larval packet test the LC_{50} and LC_{90} were 13.5 and 21.8 mg/ml, respectively. In egg hatch test, the LC_{50} and LC_{90} were 0.249 and 0.797 mg/ml, respectively, while in the larval development test were 0.072 and 0.167 mg/ml, respectively. This is the first report of the *H. myrtoides* evaluation against those parasites. The anthelmintic results proved its efficacy on *H. contortus* encouraging new research with a focus on their main bioactives.

Introduction

Rhipicephalus (Boophilus) microplus, Ixodidae, known as the cattle tick, is an ectoparasite endemic to the tropical and subtropical regions of the world (Rosado-Aguilar et al., 2010; Lopez-Arias et al., 2014). This tick has become included in the genus *Rhipicephalus* after molecular and morphological studies showing the phylogenetic relationship between *Rhipicephalus* and *Boophilus* (Beati and Keirans, 2001; Murrell and Barker, 2003). This parasite has been a major problem for livestock farmers, by setting a very efficient host-parasite relationship. This fact has caused a huge loss and increasing spending on acaricide chemicals to control the infestation. So that control of this parasite is done at farm level and the acaricide products are frequently administrated at a monthly basis throughout the year (Bianchi et al., 2003). The cattle tick infestations cause many economic losses to the livestock farmers due to blood loss, damage in the animal's skin, reduced weight gain and transmission of pathogens such as *Babesia bovis*, *Babesia bigemina* and *Anaplasma marginale*, which cause the disease "Tristeza Parasitária", mainly to the calves (Grisi et al., 2014).

The *Haemonchus* genus belongs to the Trichostrongylidae family, which present several species that parasite ruminants, including *Haemonchus contortus* (Rudolphi 1803) Cobb 1898; *Haemonchus similis* Travassos 1914; *Haemonchus longistipes* Railliet et Henry 1909; and *Haemonchus placei* Place 1893 (Jacquiet et al., 1997). *H. similis* is rarely found in parasitic infections of small ruminants such as sheep and goats but *H. contortus* is prevalent and dominant in terms of intensity of infection (Achi et al., 2003). Therefore, sheep and goats show up highly susceptible, with high establishment rate of infection and large excretion of eggs by females, compared to other ruminant species. Its life cycle includes a free life stage (from egg to the infective larvae L_3) and a parasitic

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stage (from L_3 ingested by the host, to the adult phase) (Jacquiet et al., 1998). Natural populations of *H. contortus* inhabit the United States, Brazil, Argentina, Australia, Europe (Scotland, Ireland and France) and Africa (Congo and Mauritania) (Jacquiet et al., 1997). The pathogenesis caused by *H. contortus* is characterized by a severe anaemia due to blood-sucking habit of the worm, leading to a serious impairment of the animal, severe economic losses and even death (Rodríguez et al., 2015).

The parasite resistance to anthelmintic drugs increasingly affects animals such as cattle, goats and sheep, and is widely spread among several genera and phyla of helminthes (Rose et al., 2015; Paraud et al., 2016). The resistance expression occurs when treatment with the drug allows the survival of resistant parasites, which reproduce and contribute with resistant genes to the new generations. The failure of parasite control provides an increase of individuals able to survive to an antiparasitic dose that would be lethal to the majority of the parasites in a susceptible population of the same species (Geary et al., 1999; Sangster, 1999, 2001). This resistance comes mainly from the intensive use of synthetic antiparasitics with sub-dosages and applications at shorter intervals than necessary (Alves et al., 2012; Lopez-Arias et al., 2014).

Control strategies for parasites that minimize the use of synthetic antiparasitics are of increasing importance, as well as the trend towards non-chemical (ecological, organic, green) farming of livestock (Learmount et al., 2016). Secondary metabolites from plants are the subject of researchers in the screening for products that are less harmful to the environment, therefore more sustainable, more selective and with potent effect on a specific target. *Hesperozygis myrtoides* (A.St.-Hil. ex Benth.) Epling belongs to the Lamiaceae family and is popularly known as "poejo" (pennyroyal). This plant is endemic to southeastern Brazil, growing in the Cerrado and Atlantic Forest biomes, at altitude above 1800 m. It possesses a mint aroma that has been associated with its essential oil rich in monoterpene ketones. The main chemical compounds in the essential oil from the aerial parts of *H. myrtoides* are pulegone (19.8–57.3%), isomenthone (14.3–47.7%), limonene (2.1–22.7%) and isomenthyl acetate (0.3–14.3%) (Martini et al., 2011; Castilho et al., 2016).

The essential oils and terpenoids from many plants rich in monoterpene ketones, such as menthone, isomenthone and pulegone, have been extensively tested against some parasites, showing acaricidal and anthelmintic activity (Facey et al., 2005; Tak et al., 2006; Rosado-Aguilar et al., 2010; Amer et al., 2011; Jeon and Lee, 2011; Kamaraj and Rahuman, 2011; Martinez-Velazquez et al., 2011; Carvalho et al., 2012; Chagas et al., 2012; Molefe et al., 2012; Ferreira et al., 2013; Koc et al., 2013). Therefore, the present study aimed to investigate the *in vitro* acaricide and anthelmintic activities of the essential oil from *H. myrtoides* aerial parts.

Material and methods

Plant origin and extraction

Aerial parts of *H. myrtoides* (A.St.-Hil. ex Benth.) Epling, Lamiaceae, were collected in July 2012 in the "Campo dos poejos" (GPS coordinates: 22° 2 31.83′ S/44° 38 30.20′ W, Aiuruoca, MG, Brazil). Plant identification was performed by Dr. Rosana C. Lopes, and voucher specimens were deposited in the Department of Botany, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil (RFA 39448). Samples of 200 g of fresh aerial parts were subjected to hydrodistillation in a Clevenger-type apparatus for 2 h. The essential oil was separated from the hydrolate by centrifugation and use of manual pipette, and then stored under refrigeration in sealed amber flask. The yield was calculated as ml of oil g^{-1} of fresh weight of the plant material.

Chemical analyzes

Chemical analyses of the *H. myrtoides* essential oil were performed at Embrapa Agroindústria de Alimentos. The identification of the essential oil components was carried out by gas chromatography analyses, performed within 2–3 days after essential oil extraction, using an Agilent 6890N gas chromatograph (Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and HP-5 (5% phenyl – 95% dimethylpolysiloxane) fused silica capillary column (30 m × 0.32 mm × 0.25 μm). The injector temperature was kept at 250 °C and the over temperature programmed from 60 to 250 °C at 3 °C min^{-1}. Detector (FID) was operated at 280 °C. One microliter of a 1% solution of the oil in dichloromethane was injected in split mode (1:50). The percentages of each component were reported as raw percentages without standardization. GC-MS analyses were performed in an Agilent 5973N mass selective detector coupled to an Agilent 6890 gas chromatograph (Palo Alto, CA), fitted with a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm), operating in electronic ionization (EI) mode at 70 eV. Transfer line was maintained at 260 °C, while mass analyzer and ion source temperature were held at 150 °C and 230 °C, respectively. Helium (1 ml min^{-1}) was used as carrier gas. Oven temperature programme, injector temperature and split rate were the same as stated for GC analyses. A standard solution of *n*-alkanes (C_7–C_{26}), injected in the same column and conditions as above, was used to obtain the linear retention indices. Individual volatile components were identified by comparison of their mass spectra (MS) and linear retention indexes (LRI) with those reported in literature (Adams, 2007), as well as to the Wiley Registry of Mass Spectral Data, 6th Edition (1994).

In vitro *assays*

Adult immersion test (AIT)

The *in vitro* tests were carried out in the Animal Health Laboratory of Embrapa Pecuária Sudeste. Engorged females of *R. (B.) microplus* were collected from cattle kept at the experimental farm. According to a resistant test performed in 2016, these ticks are resistant to pyrethroids, organophosphates and amidines. The specimens were selected according to their integrity, motility and maximum engorgement. The ticks were then weighed and separated into groups of ten with homogeneous weights, with three repetitions for each concentration including the control group (immersed in distilled water alone) and blank (2% tween 80).

The engorged females were exposed to the oil in concentrations of 25.0; 12.5; 6.25; 3.13; 1.56; 0.78 and 0.39 mg/ml. Females were immersed in the treatment solutions for 5 min, after which were dried on absorbent paper and placed in sterile Petri dishes and incubated (27 ± 1 °C, RH > 80%) to complete the life cycle. At the end of oviposition, the eggs were weighted and transferred to adapted plastic syringes, identified and sealed with cotton. The eggs were put back into the incubator (Tecnal, TE-391 model) under the same conditions for larval hatching. The data obtained were used to determine the percentage of reduction on egg oviposition and larval hatching, as well as to calculate the reproductive efficiency index (REI) and the efficacy of the essential oil (E), according to Drummond et al. (1973).

Larval packet test (LPT)

The larvae were obtained from engorged females collected from the same source and were incubated as described for the AIT. About 100 larvae, with ages between 14 and 21 days, were placed between two sheets of filter paper (2 × 2 cm) previously moistened with 1 ml of the solutions and then enclosed in packets, made of folded sheets of the same filter paper (Chagas et al., 2002 adapted from FAO, 1971). The concentrations tested were 100.0; 50.0; 25.0; 12.5 and

6.25 mg/ml. The experiment was conducted in triplicate for each dilution, with control group (distilled water) and blank (2% Tween 80). The packets were incubated under the condition previously described. The numbers of dead and living larvae were counted after 24 h incubated (27 ± 1 °C, RH > 80%) with the aid of a vacuum pump with an adapted pipette tip. Larvae that were completely immotile were considered dead.

Egg hatch test (EHT)

The tests were performed using an *H. contortus* isolate resistant to benzimidazole, macrocyclic lactone, and imidazothiazole (Embrapa, 2010; Chagas et al., 2013). Faeces were collected directly from the rectum of the sheep donor and the eggs recovered with the use of sequential sieves according to Coles et al. (1992). Briefly, faeces were mixed with distilled water and filtered through 100, 56, 30 and 25 μm mesh sieves. In the last sieve, the eggs were retained and washed with distilled water and centrifuged in Falcon tubes (50 ml) at 3000 rpm/5 min filled with water. Then the supernatant was removed and a NaCl saturated solution was added. This solution was once again centrifuged under the same conditions and the supernatant was washed through a 25 μm sieve. The eggs were collected and quantified under an inverted microscope (Zeiss, Axiovert 40 CFL model). Approximately 100 eggs were then placed in each well of 24-well plates, along with the treatments, reaching a total volume of 500 μl. All tests were done in six replicates, including control group (distilled water) and blank (2% Tween 80). Then the plates were incubated (27 ± 1 °C, RH > 80%) for 24 h when larvae and eggs were counted in the inverted microscope to calculate hatching inhibition percentage (Chagas et al., 2011).

Larval development test (LDT)

Approximately 100 eggs obtained according to the technique described above, were placed in each well. Each one also received 50 μl of *Escherichia coli* lyophilized in suspension (ATCC 9637), 10 μl of amphotericin B and 20 μl of nutrient medium (Hubert and Kerboeuf, 1992). Distilled water was added to reach a total volume of 250 μl. The material was homogenized and incubated (27 °C ± 1 °C and RH > 80%) for 24 h. After this period 250 μl of the solutions were added to each well. All tests were done in six replicates, including the control group (distilled water) and the solvent blank (0.5% DMSO). Plates were incubated for 6 days, when L_1, L_2 and L_3were counted to calculate larval development inhibition percentage (Chagas et al., 2011).

Data analyses

In the AIT the reproductive efficiency index (REI) and the efficacy of the essential oil (E) were calculated according to the formulas proposed by Drummond et al. (1973):

$$REI = \text{egg mass weight} \times \%\,\text{egg hatching/engorged females weight} \times 20{,}000^{*}$$

* Constant that indicates the number of eggs present in 1 g

$$E = (REI\ \text{control} - REI\ \text{treated})/REI\ \text{control} \times 100$$

In the EHT the Hatching Inhibition Percentage was calculated according to the formula: [(number of eggs/number of eggs + larvae in the well)] × 100. In the LDT the Larval Development Inhibition Percentage = [(number of $L_1 + L_2$/number of $L_1 + L_2 + L_3$ in the well)] × 100. The LC_{50} and LC_{90} were determined by statistical analysis using the Probit procedure of the SAS program (SAS 2002/2010) and the data on concentrations were compared within each treatment with the Tukey test ($p < 0.05$).

Results and discussion

Chemical analyzes

The essential oil of *H. myrtoides*, obtained from plants collected in July 2012 with 0.74% yield, was used in four different *in vitro* tests to determine their antiparasitic activity. In previous works from our group we have determined that the essential oil yields of this plant varies considerably (0.74–2.99%), depending upon the collection site, altitude, and time of year (Martini et al., 2011; Castilho et al., 2016). The analysis of the oil allowed the identification of 99.2% compounds (Table 1) and the main ones were as follows: isomenthone (47.7%), pulegone (21.4%), limonene (7.7%), isomenthyl acetate (6.8%) and neoisomenthol (3.9%), respectively, as depicted in the chromatogram (Fig. 1). The main substances identified in the present study are also the major compounds in the *H. myrtoides* oil reviewed by Martini et al. (2011), featuring well this plant species.

Acaricidal activity

Laboratory tests were conducted to determine the efficacy of the *H. myrtoides* essential oil on engorged female and larvae of *R. (B.) microplus*. In the adult immersion test (AIT) the action of the oil was evaluated by measuring the inhibition of egg hatching, reproductive efficiency index and efficacy of the oil (E),

Table 1
Chemical composition and relative percentages of the identified compounds in the essential oil of *Hesperozygis myrtoides*.

Compounds[a]	$LRI_{calc.}$[b]	$LRI_{lit.}$[c]	%
α-Pinene	932	932	0.4
Sabinene	972	969	0.3
β-Pinene	976	974	0.5
Myrcene	990	988	1.0
p-Cymene	1024	1022	0.1
Limonene	1027	1024	7.7
1,8-Cineole	1030	1026	0.8
cis-Ocimene	1036	1032	0.2
trans-Ocimene	1046	1044	1.3
Linalool	1103	1095	0.4
Octen-3-yl acetate	1113	1110	0.2
Menthone	1153	1148	0.5
Isomenthone	1165	1158	47.7
Isopulegone	1176	[d]	0.4
Terpinen-4-ol	1178	1174	0.1
Neoisomenthol	1185	1184	3.9
α-Terpineol	1193	1186	0.1
Pulegone	1240	1233	21.4
2-Phenyl ethyl acetate	1259	1254	0.1
Isomenthyl acetate	1308	1304	6.8
Neoiso-pulegyl acetate	1312	1312	0.3
α-Terpinyl acetate	1349	1346	0.3
α-Copaene	1373	1374	0.1
β-Bourbonene	1381	1387	0.1
β-Caryophyllene	1415	1417	1.8
n.i. (MW:166)	1421	n.i.	1.4
α-Humulene	1449	1452	0.2
Germacrene D	1477	1480	0.7
Bicyclogermacrene	1492	1500	0.5
Spathulenol	1576	1577	0.2
Caryophyllene oxide	1578	1582	0.2
Monoterpenes			87.1
Sesquiterpenes			3.8
Total of identified compounds			99.2

[a] Components were reported according their elution order on SE-52.
[b] Linear retention index based on a series of n-hydrocarbons reported according to their elution order on HP-5MS. Adapted from Castilho et al. (2016).
[c] Identification confirmed by comparing mass spectra and retention times with those obtained from the literature (Wiley, 1994; Adams, 2007).
[d] Tentatively identified by comparing mass spectra; t, traces (<0.1%).
n.i., not identified; MW, molecular weight.

Table 2
Average of the females' weight (FW), egg mass weight (EW), percentage of egg hatching (%H), reproductive efficiency index (REI), and efficacy of the essential oil (E) in engorged females of *Rhipicephalus (Boophilus) microplus*, tested by means of emersion in different concentrations (mg/ml) of *Hesperozygis myrtoides* oil.

Concentrations	FW (g)	EW (g)	%H	REI	E (%)
25.0	2.05 ± 0.02^{A}	0.85 ± 0.06^{A}	76.67 ± 3.33^{A}	63.90 ± 5.86^{A}	24.97
12.5	2.06 ± 0.00^{AB}	0.84 ± 0.07^{A}	83.33 ± 3.33^{AB}	68.15 ± 7.86^{A}	19.98
6.25	2.03 ± 0.00^{A}	0.90 ± 0.04^{A}	80.00 ± 0.00^{AB}	70.79 ± 3.36^{A}	16.89
3.12	2.07 ± 0.01^{AB}	0.88 ± 0.03^{A}	86.67 ± 3.33^{AB}	74.03 ± 3.37^{A}	13.08
1.56	2.07 ± 0.01^{AB}	0.92 ± 0.03^{A}	85.00 ± 2.89^{AB}	75.36 ± 2.84^{A}	11.52
0.78	2.06 ± 0.01^{AB}	0.89 ± 0.06^{A}	90.00 ± 0.00^{B}	77.83 ± 5.49^{A}	8.62
0.39	2.11 ± 0.03^{B}	0.95 ± 0.05^{A}	86.67 ± 3.33^{AB}	78.16 ± 6.46^{A}	8.23
Control	2.05 ± 0.01^{A}	0.97 ± 0.08^{A}	90.00 ± 0.00^{B}	85.17 ± 7.02^{A}	0.0

Different letters in the column indicate statistically significant difference ($p < 0.05$).

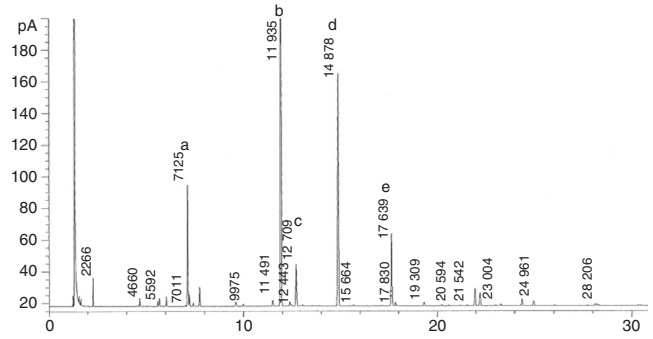

Fig. 1. Representative chromatogram (GC-FID) of the essential oil from fresh aerial parts of *Hesperozygis myrtoides*. [a]limonene; [b]isomenthone; [c]neoisomenthol; [d]pulegone; [e]isomenthyl acetate.

demonstrating low hatching inhibition and low effect on reproductive efficiency and therefore, low efficacy (Table 2).

To date, no study reporting the acaricide activity of *H. myrtoides* essential oil on the cattle tick *R. (B.) microplus* has been described in the literature. There is only one study of Ribeiro et al. (2010) with the species *Hesperozygis ringens*, in which its acaricide activity was evaluated against the *R. (B.) microplus* engorged females, by evaluating the egg production. It seems that it did not cause females' mortality, but the authors observed that the oil at concentrations of 50 and 25 μl/ml, inhibit the egg laying of the females in 76.4 and 48.0%, respectively. In addition, the egg hatching was inhibited in 95% and 30% in the same concentrations. The isolated pulegone was also tested and its effect was similar to that of the essential oil (Ribeiro et al., 2010). In the present study, the greater acaricide efficacy was 24.97% on engorged females and there was some action on the larval hatching of the females treated with the oil.

The LPT evaluates the mortality of the *R. (B.) microplus* larvae after contact with the *H. myrtoides* essential oil, where larvae without movement were considered dead. It can be observed in the present study that up from the concentration of 25 mg/ml the *H. myrtoides* oil eliminated 100% of the larvae (Table 3). This concentration may be considered too high, because according to Ribeiro et al. (2010), the essential oil of *H. ringens* and the isolated pulegone, in concentrations of 20.0–0.625 μl/ml were lethal to the totality of the larvae. The authors suggest that the monoterpene pulegone is responsible for the powerful acaricide activity of *H. ringens* oil to females and larvae tick of *R. (B.) microplus*.

The LC_{50} and LC_{90} here obtained were 13.50 and 21.80 mg/ml, respectively, and it can be best viewed in Table 4. These high LCs suggest that the *H. myrtoides* oil is not a potent acaricide. It is possible that this result has correlation with the pulegone level detected in the oil (21.4%) compared to that on *H. ringens* oil (86.0%) (Ribeiro et al., 2010). According to the discussion of Ribeiro et al. (2010), attempts to evaluate penetration and cutaneous absorption of the oil components are needed, mainly considering the consumption of meat and dairy products from cattle. Pulegone has been associated with severe hepatotoxicity and death in dogs and mouse, as well as menthofuran, its metabolite produced by oxidation *via* Cytochrome P450 (Sudekum et al., 1992; Sztajnkrycer et al., 2003).

The acaricidal activity of many pure terpenes against *R. (B.) microplus* has been shown in previous studies, due mainly to oxygenated compounds, such as 1,8-cineole, citronellol, linalool, isopinocamphone, camphor (Prates et al., 1998), carvacrol (Cruz et al., 2013), thymol (Monteiro et al., 2010; Scoralik et al., 2012; Cruz et al., 2013; Araújo et al., 2015), cadina-4,10(15)-dien-3-one (Porter et al., 1995), eugenol (Valente et al., 2014), carvone (Peixoto et al., 2015), nerolidol (Lage et al., 2013) and non-oxygenated compound, like limonene (Ferrarini et al., 2008; Peixoto et al., 2015).

Several species of the Lamiaceae family had their acaricide activity tested, especially those from the genus *Cunila*. Species of the genus are also known in Brazil as Poejo, because of its minty aroma (Apel et al., 2009). The essential oil from *Cunila angustifolia*, *Cunila incana* and *Cunila spicata* caused 100% of mortality to the *R. (B.) microplus* larvae at 5 μl/ml, evaluated in the Larval Immersion test. In these oils, the main compounds were the monoterpenes sabinene (32.1%); α and β-pinene (26.7 and 27.5%); and limonene, menthonfuran and borneol (12.0, 34.8 and 19.7%, respectively). In the oils from these species, no pulegone was detected.

On the other hand, the essential oils from *Cunila incisa* and *Cunila microcephala* at 10 μl/ml killed 18 and 5% of the larvae, respectively, *i.e.*, presented low larvicidal activity. The main compounds found in these oils were the monoterpenes 1,8-cineole (55.4%) and menthofuran (72.7%), respectively (Apel et al., 2009). The essential oil of *Hyptis verticillata*, also belonging to the family Lamiaceae, was quite

Table 3
Percentage of mortality of *Rhipicephalus (Boophilus) microplus* larvae (±standard deviation) submitted to the Larval Packet Test (LPT) caused by *Hesperozygis myrtoides* essential oil in different concentrations.

mg/ml	%
100.0	100 ± 0.0^{E}
50.0	100 ± 0.0^{E}
25.0	100 ± 0.0^{E}
12.5	57.25 ± 0.38^{D}
6.25	13.79 ± 0.11^{C}
3.12	5.60 ± 0.40^{B}
Control	0.0 ± 0.0^{A}

Different letters in the column indicate statistically significant difference ($p < 0.05$).

Table 4
CL_{50} and CL_{90} (±95% confidence limits) obtained in the Larval Packet Test (LPT) with *Rhipicephalus (Boophilus) microplus* larvae, Egg Hatch Test (EHT) and Larval Development Test (LDT) with *H. contortus* submitted to the *Hesperozygis myrtoides* essential oil.

In vitro test	LC_{50} (mg/ml)	LC_{90} (mg/ml)
LPT	13.50 (12.05–15.23)	21.80 (18.76–27.43)
EHT	0.249 (0.238–0.261)	0.797 (0.743–0.860)
LDT	0.072 (0.069–0.075)	0.167 (0.157–0.180)

more effective for tick females in relation to the present study. The oil at 4 mg/ml caused mortality of *R. (B). microplus* adult females in 96 h (45%), interruption of oviposition (87%) and of the egg hatching of this tick (90%) (Facey et al., 2005). However, it's chemical composition is quite different from those monoterpene rich oils of *Hesperozygis* and *Cunila* species, being rich in sesquiterpenes, with cadina-4,10(15)-dien-3-one (15.1%) and aromadendr-1(10)-en-9-one (30.7%), as major sesquiterpene-ketones, followed by the minor compounds viridiflorol (4.3%) and spathulenol (2.2%).

Anthelmintic activity

The EHT assessed the percentage of hatching inhibition on *H. contortus* eggs, after being treated with the of the essential oil solution at concentrations from 25.0 to 0.012 mg/ml. In this test, up from the concentration of 3.12 mg/ml, 100% of egg hatching inhibition was detected (Table 5). The LC_{50} and LC_{90} results in the EHT were 0.249 and 0.797 mg/ml, respectively (Table 4).

Concentrations from 0.40 to 0.0125 mg/ml were used in the LDT and the oil demonstrated better efficacy at this parasite stage because 100% of the larval development was inhibited at 0.40 mg/ml (Table 6). The LC_{50} and LC_{90} results were also lower than in the EHT, where the values obtained were of 0.072 and 0.167 mg/ml, respectively (Table 4, Fig. 2).

The *H. myrtoides* essential oil evaluated in the present study is rich in monoterpenes, which are a class of secondary metabolites with a vast array of biological activities, including antimicrobial, acaricide and anthelmintic (Katiki et al., 2011; Chagas et al., 2012; Chagas et al., 2014). In this oil, 36 constituents were identified, being the major ones isomenthone, pulegone, limonene, isomenthyl acetate and neoisomenthol. These secondary metabolites are known to present a broad action against various organisms and synergism that can be effective for different parasites, since essential oils are a complex mixture of substances that can interact with multiple targets in various stages of their development (Marie-Magdeleine et al., 2009).

Table 5
Average percentage of hatching inhibition of *Haemonchus contortus* eggs (±standard deviation) submitted to different concentrations (mg/ml) of *Hesperozygis myrtoides* essential oil, evaluated in the Egg Hatch Test (EHT).

mg/ml	%
5.0	100 ± 0.0^{G}
12.5	100 ± 0.0^{G}
6.25	100 ± 0.0^{G}
3.12	100 ± 0.0^{G}
1.56	99.3 ± 1.0^{G}
0.78	92.0 ± 1.9^{F}
0.39	59.3 ± 3.1^{E}
0.19	39.4 ± 4.5^{D}
0.098	16.9 ± 1.6^{C}
0.049	4.5 ± 1.8^{B}
0.024	1.7 ± 0.69^{AB}
0.012	0.74 ± 0.87^{A}
Control	1.4 ± 1.3^{AB}

Different letters in the column indicate statistically significant difference ($p < 0.05$).

Table 6
Average percentage of development inhibition of *Haemonchus contortus* larvae (±standard deviation) submitted to different concentrations (mg/ml) of *Hesperozygis myrtoides* essential oil, evaluated in the Larval Development Test (LDT).

mg/ml	%
0.40	100.0 ± 0.0^{F}
0.20	94.1 ± 1.9^{E}
0.10	67.4 ± 4.6^{D}
0.05	26.6 ± 1.9^{C}
0.02	6.2 ± 1.1^{B}
0.01	2.0 ± 0.4^{A}
Control	2.0 ± 0.7^{A}

Different letters in the column indicate statistically significant difference ($p < 0.05$).

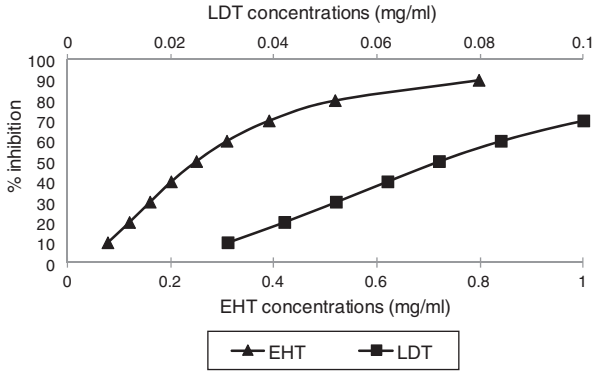

Fig. 2. Inhibition of the egg hatching and larval development (LC_{50} and LC_{90}) of *H. contortus* of sheep submitted to the *Hesperozygis myrtoides* essential oil concentrations.

In general, the action of essential oils is the result of combined effects that activate or inactivate structures. They possibly act by breaking or disrupting membranes by action on the lipophilic compounds, causing loss of several enzymes and nutrients through the cell membrane (Cowan, 1999; Cox et al., 2000). According to Sikkema et al. (1993, 1995), the cell membrane can be significantly different between one organism and another, affecting permeability and hence the response of the same inhibitory compounds. Thus, the differences between the performances of the essential oil on the parasites can be attributed to interactions between these compounds and parasitic structures, being more efficient in *H. contortus*.

Based on the results obtained, the essential oil from aerial parts of *H. myrtoides* presented *in vitro* anthelmintic property against the gastrointestinal nematode *H. contortus*. Those tests are the most used to detect *in vitro* anthelmintic activity and resistance in nematodes of small ruminants. They are interpreted based on the lethal concentrations, because high LC_{50} suggest the presence of parasitic resistance (Coles, 2005; Várady et al., 2009). According to our knowledge, this is the first report of the anthelmintic activity of the *H. myrtoides* essential oil on this important parasite.

Conclusion

In sum, from the significant results obtained in this work, especially against the parasite *H. contortus*, the possibility of using *H. myrtoides* essential oil to control gastrointestinal nematodes encourages further research focusing on bioactive constituents for veterinary use in small ruminants and cattle. Suggestion of a possible protection of ruminants against parasites requires careful studies and consideration due to intake of meat and milk from those animals.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

CVVC extracted the essential oil and drafted the paper. RRF, YAG performed the bioassay work and HRB performed GC analyses. NSB, SGL participated in the results, discussion and writing the paper. ACSC organized data, set statistical analyses and the manuscript final writing.

Conflicts of interest

The authors declare no conflicts of interest.

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