Efficacy of essential oils from plants cultivated in the Amazonian Biome against gastrointestinal nematodes in sheep

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Abstract The excessive use of anthelmintics to control nematodes has resulted in anthelmintic resistance. Essential oils (EOs) are a rich source of bioactive molecules that can be assessed for their ability to control resistant parasite populations. The aims of this study were to screen EOs from 10 plant species in vitro for anthelmintic activity against *Haemonchus contortus*, evaluate the cytotoxicity of those EOs in a human immortalized keratinocyte cell line (HaCaT), and test the most promising EO candidate in vivo in Santa Inês sheep. The efficacy was investigated in vitro using an egg hatch test (EHT) and a larval development test (LDT). EO cytotoxicity was evaluated with the sulforhodamine-B assay. In the in vivo experiment, 28 Santa Inês sheep naturally infected were distributed into groups: G1—*Mentha arvensis* (EO₅), 200 mg kg⁻¹; G2—menthol, 160 mg kg⁻¹; G3—negative control; and G4—positive control (monepantel). EO₅, from *M. arvensis* (86.7% menthol), had the lowest LC₅₀ and LC₉₀ values in the EHT (0.10, 0.27 mg mL⁻¹,

respectively), good performance in the LDT (0.015, 0.072 mg mL⁻¹, respectively), and the lowest cytotoxicity (190.9 µg mL⁻¹) in HaCaT cells. In the in vivo test, a single dose of the EO₅ (200 mg kg⁻¹ BW) had an efficacy of approximately 50% on days 1, 14, and 21; however, values were not significantly from day 0. Conversely, pure menthol at a dose of 160 mg kg⁻¹ BW showed no in vivo efficacy. This can be attributed to key factors related to bioavailability and pharmacology of terpenes in the host organism, as well as to the fact that menthol is mainly excreted as glucuronides in urine. Thus, further studies should be conducted with formulation systems that deliver bioactives directly to the abomasum, focusing on terpenes, whose excretion route is mainly via faeces.

Keywords Parasitology · Medicinal plants · *Haemonchus contortus* · Control · Sheep

Introduction

The Brazilian livestock population includes approximately nine million goats and 17 million sheep, with approximately 722,000 and 415,000 sheep in the southeast region and in the state of São Paulo, respectively. Between 2000 and 2010, the number of sheep in both regions doubled (IBGE 2014). However, Brazilian sheep producers do not supply the domestic market with efficiency or adequate quality. In 2013, Brazil imported approximately 9000 tons of sheep meat, mainly from Uruguay. Little attention has been paid to improving sheep production despite the considerable potential for development of the industry (De Zen et al. 2014).

Gastrointestinal nematode infection, including *Haemonchus contortus*, is a major problem faced by sheep

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producers worldwide that causes major economic losses (Sun et al. 2012). Gastrointestinal nematodes cause weight loss, affect milk and wool production, and are the leading cause of death among both untreated and treated infected animals due to the development of anthelmintic resistance (Klauck et al. 2014). For more than 50 years, control measures have relied on repeated use of synthetic, chemical anthelmintic drugs that interrupt the life cycle of pathogenic helminths by killing the worms in the hosts (Hoste et al. 2012). The excessive use of anthelmintics to control nematode populations has resulted in substantial problems with anthelmintic resistance, which currently faces the growing concern from consumers about possible contamination in food products and the environmental consequences of veterinary drug use (McBean et al. 2016). So, increased interest has been directed towards the development of new tools to control gastrointestinal parasitism for organic production, including the use of medicinal plants with antiparasitic activity.

The antiparasitic activity of plants is believed to derive mostly from biologically active compounds known as plant secondary metabolites (PSMs). A comprehensive study of PSMs would potentially lead to the detection of novel antiparasitic molecules (Peña-Espinoza et al. 2015). So far, only a small number of essential oils (EOs) have been examined for their activity on helminths in vitro and in controlled in vivo studies. Some botanical families (for example oil from Zingiberaceae, Verbenaceae, Lamiaceae and Euphorbiaceae) are known to exhibit biological activity against *H. contortus* (Camurça-Vasconcelos et al. 2007; Katiki et al. 2011; Carvalho et al. 2012; Ullah et al. 2013). So, in the present study, EOs extracted from both native Amazonian and exotic species were screened in vitro for anthelmintic activity against *H. contortus* eggs and larvae. In addition, the cytotoxicity of the essential oils was evaluated, and the most promising candidate and its major compound were submitted to an in vivo trial in Santa Inês sheep naturally infected with gastrointestinal nematodes.

Materials and methods

Plant material

The following plant species were tested in the in vitro study: Euphorbiaceae: *Croton cajucara* Benth (white and red morphotypes) and *Croton sakaquinha* Croizat; Zingiberaceae: *Curcuma longa* L. and *Zingiber officinale* Roscoe; Verbenaceae: *Lippia alba* (Mill) N. E. Brown, *Lippia gracilis* Schauer, *Lippia origanoides* Kunth, and *Lippia sidoides* Cham; Lamiaceae: *Mentha arvensis* L. and *Mentha piperita* L. Plants were grown in 2014 at Embrapa

Amazônia Ocidental, Manaus (AM), Brazil. Botanical samples were deposited at the EAFM Herbarium of the Federal Institute of Amazonas, Manaus (Exsiccate Numbers: 13880, 13879, 13881, 13888, 13887, 13886, 13883, 13885, 13882, 13895, 13896, respectively). The field plots were located at 03°06'23.04"S and 60°01'35.14"W at a mean altitude of 50 m. The area has a mean temperature of 25.6 °C and mean annual rainfall of 2200 mm and the climate is characterized as equatorial (Af).

Oil extraction

After harvest in July 2014, the parts of the plants were separated and put to dry in a covered shed for a week at an average temperature of 27 °C. The EOs were extracted by hydrodistillation in a modified Clevenger apparatus for 4 h. After separation was complete, oils were dried over anhydrous sodium sulfate and kept refrigerated (8 °C) in closed vials until used. EO chemical analyses were performed according to Chagas et al. (2016), where the main components and other details of the plants' EOs are given.

In vitro tests

Essential oil concentrations

The concentrations tested in the egg hatch test (EHT) were determined in a geometric progression of two: 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, and 0.19 mg mL⁻¹. In turn, the respective concentrations in the larval development test (LDT) were 0.4, 0.2, 0.1, 0.05, 0.025, 0.0125, 0.006, and 0.003 mg mL⁻¹ (lower concentrations because larvae are more susceptible than eggs). In both tests, the lowest concentrations were determined when the hatching rate and larval development rate were similar to readings in the negative control. The highest concentrations were determined to attain 100% efficacy. Thus, the estimate of the lethal concentration of the EOs would be more reliable. In the cytotoxicity test, EOs were evaluated at 6.25, 12.5, 25.0, 50.0, 100 and 200 µg mL⁻¹. These concentrations are relatively standardized for HaCaT cells, in which EOs are considered cytotoxic when IC₅₀ ≤ 30 µg mL⁻¹ (Geran et al. 1972).

Egg hatch test (EHT)

Eggs of the Embrapa 2010 isolate of *H. contortus* were used in the EHT. This strain is resistant to benzimidazoles, macrocyclic lactones, and imidazothiazoles (Chagas et al. 2013). Faecal samples were obtained directly from the rectum of a donor animal. Eggs were recovered with sequential use of sieves (Bertel) (Coles et al. 1992). Briefly, the faecal samples were mixed with tepid water (37 °C)

and filtered through sieves with mesh sizes of 1 mm, 106, 53, and 25 μm . The eggs retained in the last sieve were washed with distilled water and centrifuged (Fanen Excelsa II 206 BL) with water at 3000 rpm for 5 min, after which the supernatant was discarded and a saturated NaCl solution was added to resuspend the sediment. After another centrifugation cycle under the same conditions, the supernatant was washed through a 25 μm sieve and the eggs were collected.

Approximately 100 eggs in suspension (25 μL) were added to each well of a 24-well plate (TPP 92006) together with one of the 11 EOs. For each EO, eight dilutions were performed in distilled water and 2% Tween 80 (Synth). The negative controls also consisted of distilled water and distilled water plus 2% Tween 80, and there were six replicates of all treatments and controls. The plates were placed in an incubator (Ethik 411D) for 24 h at 27 ± 1 °C and > 80% humidity. Next, the eggs and larvae (L_1) were counted using an inverted microscope (Zeiss Axiovert 40 CFL).

Larval development test (LDT)

A suspension containing approximately 100 eggs (25 μL) was added to each well of a 24-well microplate together with 80 μL of a nutrient solution [containing *Escherichia coli* strain W (ATCC 9637, Sigma-Aldrich™ E9637), yeast extract (Fluka 92144), Amphotericin-B (Sigma-Aldrich™ A9528)] and distilled water to a volume of 250 μL (Hubert and Kerboeuf 1992). The plates were placed in an incubator for 24 h at 27 ± 1 °C and > 80% humidity to obtain the L_1 larvae. Next, a 250 μL aliquot of one of the 11 EOs was added to the well at dilutions previously determinate. Distilled water and distilled water plus 0.5% DMSO (Synth) were used as negative controls, and there were six replicates for all treatments and controls. After addition of the EOs, the plates were incubated for 6 days, and the L_1 , L_2 , and L_3 larvae in each well were counted using an inverted microscope.

Cytotoxicity test

Because the *Croton* genus performed poorly in the EHT and LDT, and there was an insufficient quantity of *C. longa* EO available, the cytotoxicity test was performed with the other seven essential oils using the sulforhodamine B (SRB) assay (Skehan et al. 1990). Briefly, HaCaT cells were pre-incubated in 96-well plates with DMEM culture medium (Sigma-Aldrich™) supplemented with 10% fetal bovine serum (Sigma-Aldrich™), 100 μL /well, for 24 h at 37 °C and 5% CO_2 atmosphere, in order to enable the growth and adhesion of the cells. Subsequently, the medium was discarded and the EOs and controls [doxorubicin

(Sigma-Aldrich™) 20 $\mu\text{g mL}^{-1}$ as positive control and a vehicle as negative control] were added to the wells to achieve a cell count of 1.4×10^4 cells mL^{-1} . After 24 h of incubation, the cells were fixed with trichloroacetic acid [TCA (Sigma-Aldrich™), 100 $\mu\text{L well}^{-1}$] and refrigerated for 1 h at 4 °C. The TCA was removed and 0.4% SRB dye (Sigma-Aldrich™) was added (50 $\mu\text{L well}^{-1}$). After 20 min, the plates were washed with 1% acetic acid solution and dried at 25 °C. The unbound dye was removed by washing and dyes bound to the protein were solubilized in a basic medium [Tris base (Sigma-Aldrich™), 10 mM, pH 10.5], after which optical density was determined at 570 nm on an iMark Microplate Reader (Bio-Rad Laboratories, Hercules, CA, EUA).

In vivo anthelmintic test

The EO of *M. arvensis* was selected for use in the in vivo experiment because of its superior performance in vitro, especially against eggs, which are a very resistant stage in relation to L_1 (Katiki et al. 2011; Molan et al. 2002); in addition, the selected EO had the highest IC_{50} value in the cytotoxicity test (5 times less toxic than *L. origanoides*). The choice of *M. arvensis* also took into consideration the ease with which this species is cultivated, its EO yield (3.24%), and the fact that menthol (86.7% of the sample used in the in vitro test and 80.0% of the sample used in the in vivo test), can be easily acquired and could be monitored in future screening tests for residues in meat and milk. Menthol was also evaluated in order to compare its efficacy when used as a pure compound (Ferreira et al. 2016). Twenty eight 7-month-old female Santa Inês sheep with an average weight of 32 kg were maintained on *Panicum maximum*. Faecal samples were collected directly from the rectum of each animal and the number of eggs per gram (EPG) of faeces was counted using a modified McMaster technique (Gordon and Whitlock 1939).

The animals were assigned to one of four groups (N = 7) based on the EPG and live weight, as follows: G1: *M. arvensis* essential oil (EO_5), 200 mg kg^{-1} BW; G2: menthol, 160 mg kg^{-1} BW (80% of 200 mg, its composition in EO_5 ; L-menthol, natural, > 99%, Sigma-Aldrich 2216-51-5); G3: negative control (water); and G4: positive control (monepantel, Zolvix®, 2.5 mg kg^{-1} BW). The G1, G2, and G3 treatments contained 4% (v/v) polysorbate 80 (Synth) and 1.5% (v/v) hydrogen peroxide (Vetec) according to Squires et al. (2010). EPG was counted on days 0, 1, 3, 7, 14, and 21 after treatment. From day 14, animals with EPG values > 4000 received anthelmintic treatment. Mass faecal culture for the determination of GIN genera was performed (Roberts and O'Sullivan 1950) and then it was verified that the animals were naturally infected with 93% *H. contortus* and 7% *Trichostrongylus* spp.

Statistical analysis

The calculation of the lethal concentration (LC) of each EO was performed by fitting the Probit linear regression using the normal distribution, and generalized linear models were used for binary data (logistic regression), with equation parameters estimated by maximum likelihood (SAS 2002/2010). The results of the cytotoxic assay are reported as the mean $\pm$ standard error (SE) of three separate experiments. Statistical analyses of this test were performed using GraphPad Prism™.

The EPG data were analyzed by analysis of variance using the GLM procedure of SAS considering a split-plot design with animal as plots and time as sub-plots. The statistical model used was $Y_{ijk} = \mu + \beta_i + \tau_{j(i)} + \gamma_k + (\tau\gamma)_{ik} + \varepsilon_{ijk}$, where Y_{ijk} is EPG measured at day k on the j animal assigned to the i treatment, μ is the average overall effect, β_i is the effect of the i treatment, $\tau_{j(i)}$ is the random effect of the j animal within the i treatment, γ_k is the fixed k day effect when the measurement was taken, $(\tau\gamma)_{ik}$ is the fixed interaction effect between the i treatment and day k , and ε_{ijk} is the random error NID (0, σ^2). To meet the assumptions of the variance analyses, i.e., that residues are independent, identically distributed, and with homogeneous variance among treatments, the data were transformed as $\log(\text{EPG} + 1)$ as suggested elsewhere (Sampaio 1998). The log-transformed data satisfied the assumption of normality. The lsmeans procedure of SAS followed by the Tukey test ($\alpha = 0.05$) was used for the comparison of least square means for treatments within days, as well as for days within treatments.

Results

The highest yields were obtained for the EOs from *L. sidoides* (4.36%) and *M. arvensis* (3.24%). The *C. longa* EO was rich in sesquiterpenic ketones such as α , β , and ar-turmerone (62% in total), whereas carvone, a monoterpenic ketone, was the main constituent in the EO of *L. alba*. Carvacrol was the major compound in the EOs of both *L. gracilis* (61.7%) and *L. origanoides* (40.4%). Its isomer, thymol, was predominant in the EO of *L. sidoides* (64.5%). Menthol was the main compound in *M. arvensis* (86.7%) and *M. piperita* (30.5%). The EO of *C. saccuinha* contained the largest number of compounds (57), but for only one (germacrene) its content was greater than 10%.

LC₅₀ and LC₉₀ values were calculated for most EOs in the EHT (Table 1). The best results were obtained for EOs from the following species (LC₅₀, LC₉₀): *M. arvensis* (0.10, 0.27 mg mL⁻¹), *Z. officinale* (0.11, 0.40 mg mL⁻¹), *L. sidoides* (0.15, 0.34 mg mL⁻¹), *L. alba* (0.24, 0.59 mg mL⁻¹), *L. origanoides* (0.25, 0.70 mg mL⁻¹), *C.*

longa (0.36, 2.49 mg mL⁻¹), *L. gracilis* (0.42, 1.27 mg mL⁻¹), and *M. piperita* (1.44, 4.62 mg mL⁻¹). *Croton cajucara* (white and red morphotypes) and *C. saccuinha* had maximum efficacies of 3.3, 33.8, and 41.0%, respectively.

The eight EOs with the best results in the EHT were evaluated in the LDT. In general, efficacy was good at concentrations lower than those obtained in the EHT, especially in the genus *Lippia*, resulting in lower LC₅₀ and LC₉₀ values: *L. origanoides* (0.004, 0.013 mg mL⁻¹), *L. alba* (0.007, 0.028 mg mL⁻¹), *L. sidoides* (0.006, 0.044 mg mL⁻¹), *L. gracilis* (0.005, 0.058 mg mL⁻¹), *M. piperita* (0.010, 0.046 mg mL⁻¹), *M. arvensis* (0.015, 0.072 mg mL⁻¹), *Z. officinale* (0.014, 0.080 mg mL⁻¹), and *C. longa* (0.104, 0.169 mg mL⁻¹).

In the cytotoxicity assay, the EOs of *L. alba* (EO₁), *L. gracilis* (EO₂), *L. origanoides* (EO₃), *L. sidoides* (EO₄), *M. arvensis* (EO₅), *M. piperita* (EO₆), and *Z. officinale* (EO₇) had IC₅₀ values of 68.76, 46.83, 47.74, 48.69, 190.9, 92.44, and 93.06 $\mu\text{g mL}^{-1}$, respectively. The percentage of viable immortalized human keratinocytes treated with EOs is shown in Fig. 1. EO₅, from *M. arvensis*, had the lowest cytotoxicity, even at 100 $\mu\text{g mL}^{-1}$. This was also observed at 50 $\mu\text{g mL}^{-1}$, in which the cytotoxicity of the EOs in decreasing order was *M. arvensis* < *M. piperita* < *Z. officinale* < *L. alba*, confirming the lower toxicity of the first one.

In the in vivo trial, monepantel had the best efficacy, whereas EPG values did not change significantly in either the menthol treatment or the negative control (Fig. 2). The efficacy of the *M. arvensis* EO in relation to the day of the treatment (D) was 61.6% on D₁, 48.1% on D₁₄, and 44.9% on D₂₁ (Table 2); however, the means of the log-transformed EPG values of these time points were not significantly different from that of D₀ ($p > 0.05$). On day 14, two animals in the control group, three animals in the menthol group, and one animal in the *M. arvensis* EO group had to be dewormed (EPGs > 4000). No toxic symptoms were observed in animals treated with either *M. arvensis* EO or menthol at the doses tested.

Discussion

The EO of *M. arvensis* was selected for use in the in vivo experiment because of its superior performance in the EHT, the highest IC₅₀ in the cytotoxicity test, and its good yield (3.24%). The use of in vitro assays to select promising EOs minimized the extensive use of animals in experiments, in keeping with the strategy of reducing the reliance on in vivo tests.

The EO of *M. arvensis* (86.7% menthol) had the lowest LC₅₀ and LC₉₀ values in the EHT and good performance in

Table 1 LC₅₀ and LC₉₀ (mg mL⁻¹) for essential oils (EO) evaluated in *Haemonchus contortus* by the egg hatch test (EHT) and larval development test (LDT)

Plant species	LC ₅₀ -SD	LC ₉₀ -SD
Egg hatch test (EHT)		
<i>Curcuma longa</i>	0.36 (0.33–0.40)	2.49 (2.37–2.43)
<i>Zingiber officinale</i>	0.11 (0.10–0.12)	0.40 (0.37–0.43)
<i>Lippia alba</i>	0.24 (0.23–0.26)	0.59 (0.55–0.64)
<i>Lippia gracilis</i>	0.42 (0.40–0.44)	1.27 (1.17–1.39)
<i>Lippia organoides</i>	0.25 (0.24–0.26)	0.70 (0.64–0.76)
<i>Lippia sidoides</i>	0.15 (0.14–0.16)	0.34 (0.32–0.36)
<i>Mentha arvensis</i>	0.10 (0.08–0.11)	0.27 (0.25–0.29)
<i>Mentha piperita</i>	1.44 (1.37–1.51)	4.62 (4.26–5.05)
Larval development test (LDT)		
<i>Curcuma longa</i>	0.104 (0.101–0.107)	0.169 (0.162–0.179)
<i>Zingiber officinale</i>	0.014 (0.013–0.015)	0.080 (0.062–0.075)
<i>Lippia alba</i>	0.007 (0.007–0.008)	0.028 (0.026–0.031)
<i>Lippia gracilis</i>	0.005 (0.005–0.006)	0.058 (0.052–0.066)
<i>Lippia organoides</i>	0.004 (0.003–0.004)	0.013 (0.012–0.014)
<i>Lippia sidoides</i>	0.006 (0.005–0.007)	0.044 (0.040–0.050)
<i>Mentha arvensis</i>	0.015 (0.014–0.016)	0.072 (0.065–0.081)
<i>Mentha piperita</i>	0.010 (0.009–0.011)	0.046 (0.042–0.052)

Fig. 1 Percentage of viable cells of immortalized human keratinocyte (*HaCaT*) treated with 7 essential oils as measured by the sulforhodamine B assay. Results are expressed as the mean $\pm$ standard error (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. vehicle-treated control). PC positive control (doxorubicin), VC vehicle control **Fig. 2** Averages of the eggs per gram of faeces (EPG) counts performed for the control, monepantel (2.5 mg kg⁻¹ BW), menthol (160 mg kg⁻¹ BW) and *M. arvensis* essential oil (200 mg kg⁻¹ BW) groups on the treatment day (D0) and from D1 to D21 post treatment, in Santa Inês sheep

Table 2 Minimum square averages and standard errors (SE) for *Haemonchus contortus* eggs per gram (EPG) counts performed in control, monenpantel (2.5 mg kg⁻¹ BW), menthol (160 mg kg⁻¹ BW),and *Mentha arvensis* essential oil (200 mg kg⁻¹ BW) groups on the day of treatment (D₀) and from D1 to D21 post treatment, in Santa Inês sheep

Treatment	D ₀		D ₁		D ₃		D ₇		D ₁₄		D ₂₁	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	3050.00 ^{Aa}	623.74	2921.43 ^{Aa}	623.74	2571.43 ^{Aa}	623.74	2189.64 ^{Aa}	683.48	1948.26 ^{Aa}	683.48	2169.53 ^{Aa}	759.44
Monenpantel	3250.00 ^{Aa}	738.01	3350.00 ^{Aa}	738.01	930.00 ^{Aab}	738.01	840.00 ^{Ab}	738.01	530.00 ^{Ab}	738.01	702.50 ^{Ab}	841.47
Menthol	2978.57 ^{Aa}	623.74	2080.71 ^{Aa}	686.16	3822.38 ^{Aa}	686.16	4092.55 ^{Aa}	683.67	2635.71 ^{Aa}	623.74	2706.00 ^{Aa}	863.22
<i>M. arvensis</i>	2850.00 ^{Aa}	623.74	1092.86 ^{Aa}	623.74	2257.14 ^{Aa}	623.74	2490.06 ^{Aa}	683.33	1478.57 ^{Aa}	623.74	1569.37 ^{Aa}	683.33

Mean values followed by different uppercase letters in the columns (treatments) and different lowercase letters in the rows (days) are significantly different by the Tukey's test ($p \leq 0.05$)

the LDT. To the best of our knowledge, this is the first report of anthelmintic activity of *M. arvensis* against *H. contortus*. For *M. piperita* oil, the content of menthol was considerably lower (30.5%), which probably explains its lower performance in the EHT. Katiki et al. (2011) assessed this oil and reported LC₉₉ values of 1.0 mg mL⁻¹ in the EHT and 0.91 mg mL⁻¹ in the LDT against gastrointestinal nematodes (95% *H. contortus*). In the current study, LC₉₀ values for *M. piperita* in the EHT and LDT were 4.62 and 0.046 mg mL⁻¹, respectively. In addition, Carvalho et al. (2012) found for the EO of *M. piperita* LC₉₀ values of 0.10 and 0.03 mg mL⁻¹ in the EHT and LDT, respectively. The EO they tested had similar amounts of menthol, menthyl acetate, pulegone, and menthone as the EO used in the present study, but it also contained 22.5% of menthofuran. Menthol's influence on lipid organization in membranes has been reported (Kupisz et al. 2015). Among the suggested modes of action of terpenes are inhibitory effects on growth, parasite enzymes or plasma membrane pumps, and interference with metabolic pathways (Kaur et al. 2009; Squires et al. 2010).

Within the genus *Lippia*, *L. sidoides* contained thymol as its major component (64.5%), whereas different compounds predominated in *L. alba* (61.7% carvone), *L. organoides* (49.7% carvacrol), and *L. gracilis* (40.4% carvacrol). The anthelmintic activity of *Lippia* EOs in the EHT in decreasing order was *L. sidoides* > *L. alba* > *L. organoides* > *L. gracilis*. Carvalho et al. (2012) found LC₅₀ and LC₉₀ values of 0.04 and 0.13 mg mL⁻¹ in the EHT and 0.02 and 0.04 mg mL⁻¹ in the LDT, respectively, for the EO of *L. sidoides*. Ferreira et al. (2016) evaluated the EO of *Thymus vulgaris* (50.2% thymol) as well as pure thymol against *H. contortus* in vitro and in sheep artificially infected at doses of 300, 150, and 75 mg kg⁻¹. For the EHT, the LC₅₀ values were relatively low for the EO (0.446 mg mL⁻¹) and pure thymol (0.442 mg mL⁻¹); in the LDT, the corresponding values were 0.013 and 0.022 mg mL⁻¹, and 0.338 and 0.497 mg mL⁻¹ in the

larval motility inhibition test. Thus, our LC₅₀ values for *L. sidoides* were superior in both the EHT and the LDT. Studies confirmed the modulatory action of thymol at invertebrate glutamate receptors associated with the chloride (GluCl) and demonstrated the interaction of the hydroxyl radical present in thymol by hydrogen bonds along the GluCl receptors, as well as for ivermectin. This may explain at least partially the mode of action of thymol against *H. contortus* (Ferreira et al. 2016).

In the in vivo test, a single dose of the EO of *M. arvensis* had an efficacy of approximately 50% on days 1, 14, and 21. Conversely, pure menthol (160 mg kg⁻¹ BW) showed no in vivo efficacy. Probably the other 25 compounds contained in the *M. arvensis* EO had an associative effect. Only studies testing very large (seemingly impractical) doses of EOs have reported significant EPG reduction. For instance, a single dose of 600 mg kg⁻¹ of Valencia orange oil reduced EPG by 97.4% in *H. contortus*-infected sheep (Squires et al. 2010). Similarly, efficacy of 76.57% was observed in goats 15 days post-treatment with *Eucalyptus staigeriana* EO (500 mg kg⁻¹ BW) (Macedo et al. 2010). A combination of extracts from *C. longa*, *Citrullus colocynthis*, and *Peganum harmala* (4 g kg⁻¹ BW) was evaluated in sheep naturally infected with gastrointestinal nematodes, and 98.7% efficacy was observed 18 days post-treatment (Ullah et al. 2013). At that dosage, it would be necessary to administer up to 120 g of the extract per animal (30 kg BW) to achieve the observed EPG reduction. *M. arvensis* presented slight efficacy in vivo and pure menthol none. This can be attributed to key factors related to bioavailability and pharmacology of those compounds in the host organism, destruction of active compounds by gut flora metabolism in rumen and ruminal pH (Chagas 2015; Ferreira et al. 2016).

In the cytotoxicity test, all EOs had IC₅₀ values higher than 30 µg mL⁻¹. These results do not indicate that EOs do not pose a risk to the health of animals, but can be an indication of low toxicity to other biological systems

(Geran et al. 1972). Of course, more complete toxicity testing would be necessary to reach better in vivo efficacy. Some studies have reported toxicity symptoms from the oral administration of EOs to small ruminants, like signs of discomfort, apathy, lethargy, and drowsiness in sheep treated with *C. schoenanthus* at a dosage of 360 mg kg⁻¹ (Katiki et al. 2012). Ascaridole, the major component of the EO of *Chenopodium ambrosioides* (LD₅₀: 200 mg kg⁻¹), was related to high toxicity effects in goats (Ketziş et al. 2002). In the current study, no toxicity symptoms were observed in sheep. Menthol and *M. arvensis* EO are generally recognized as safe by the U.S. Food and Drug Administration, which has approved menthol for food use (Opdyke 1976; FDA 2014). All menthol isomers are of very low acute oral toxicity (LD₅₀: > 2000 mg kg⁻¹ BW). In fact, no toxicity was observed in rats receiving diets providing up to 200 mg kg⁻¹ BW per day of either L- or D/L-menthol for 5.5 weeks. L-menthol is well absorbed by the oral route and is mainly excreted as glucuronides in rats' urine, with minor quantities in faeces (OECD SIDS 2003). Substances excreted mainly in faeces would be more interesting in future studies because they affect parasites at all development stages throughout the gastrointestinal tract (including eggs).

Conclusions

Albeit several of the EOs studied showed good efficacy in vitro against *H. contortus*, this activity was not reproduced in in vivo tests. This can be attributed to key factors related to bioavailability and pharmacology of terpenes in the host organism. Moreover, since menthol is excreted mainly as glucuronides in urine, it appears not to be a good anthelmintic candidate. Thus, further studies should be conducted with formulation systems delivering bioactives directly to the abomasum, focusing in terpenes, whose excretion route is mainly via faeces.

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Authors' contribution ACSC organized data, set statistical analyses and the manuscript final writing. AF performed the in vitro and in vivo trials. FGSP and IJM performed the cytotoxicity test. SNE provided and monitored the experimental animals. HRB and PEG performed chemical analyses and contributed to critical reading of the manuscript. FCMC contributed in collecting plant sample and extracting the essential oil.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All experimental protocols were approved by the CPPSE Animal Care and Use Committee (No. 02/2014). Animals were under the care of a veterinary medical assistant during all 21 days of the experiment. The number of eggs per gram of faeces of the donor animal and the animals of the in vivo anthelmintic test was counted to monitor the level of infection during the experiment. After treatment, the animals were also observed for 1 h to monitor any toxicity symptoms caused by the essential oils, such as signs of discomfort, apathy, lethargy and/or drowsiness. None died or was in poor physical condition.

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