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Evaluation of *Eucalyptus citriodora* essential oil on goat gastrointestinal nematodes

Avaliação do óleo essencial de *Eucalyptus citriodora* sobre nematóides gastrintestinais de caprinos

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Abstract

Phytotherapy may be an alternative strategy for controlling gastrointestinal parasites. This study evaluated the anthelmintic efficacy of *Eucalyptus citriodora* essential oil (EcEO). The *in vitro* effects of EcEO were determined through testing the inhibition of egg hatching and larval development of *Haemonchus contortus*. EcEO was subjected to acute toxicity testing on mice, orally and intraperitoneally. The *in vivo* effects of EcEO were determined by the fecal egg count reduction test (FECRT) in goats infected with gastrointestinal nematodes. The results showed that 5.3 mg.mL⁻¹ EcEO inhibited egg hatching by 98.8% and 10.6 mg.mL⁻¹ EcEO inhibited *H. contortus* larval development by 99.71%. The lethal doses for 50% of the mice were 4153 and 622.8 mg.kg⁻¹, for acute toxicity orally and intraperitoneally. In the FECRT, the efficacy of EcEO and ivermectin was 66.25 and 79.16% respectively, on goat gastrointestinal nematodes eight days after treatment. EcEO showed *in vitro* and *in vivo* anthelmintic activity.

Keywords: Phytotherapy, anthelmintic, *Haemonchus contortus*, *Trichostrongylus* spp., toxicity.

Resumo

Fitoterapia pode ser uma estratégia alternativa para o controle de parasitas gastrintestinais. Este estudo avaliou a eficácia anti-helmíntica do óleo essencial de *Eucalyptus citriodora* (OeEc). Os efeitos *in vitro* do OeEc foram determinados através do teste de eclosão de ovos e inibição do desenvolvimento larvar de *Haemonchus contortus*. O OeEc foi submetido ao teste de toxicidade aguda oral e intraperitoneal, em camundongos. Os efeitos *in vivo* do OeEc foram avaliados através do teste de redução da contagem de ovos nas fezes (FECRT) com caprinos infectados com nematóides gastrintestinais. Os resultados mostraram que 5,3 mg.mL⁻¹ OeEc inibiram 98,8% a eclosão de ovos e 10,6 mg.mL⁻¹ OeEc inibiram 99,71% o desenvolvimento larvar de *H. contortus*. As doses letais para 50% dos camundongos foram de 4153 e 622,8 mg.kg⁻¹ pela via oral e intraperitoneal. No FECRT, a eficácia de OeEc e ivermectina foi de 66,25 e 79,16%, respectivamente, em caprinos 8 dias após o tratamento. OeEc mostrou atividade anti-helmíntica *in vitro* e *in vivo*.

Palavras-chave: Fitoterapia, anti-helmíntico, *Haemonchus contortus*, *Trichostrongylus* spp., toxicidade.

Introduction

Gastrointestinal nematode infections are a major factor reducing the economic productivity of livestock throughout the world (VIEIRA, 2008). These parasites are responsible for severe weight loss, diarrhea, anemia and early mortality, and all of these cause production losses, especially in small ruminants (DIEHL et al., 2004). Synthetic anthelmintics have been used

to minimize the losses caused by helminth infections. However, intensive use has led to the global emergence of multiple resistance to anthelmintic drugs in small ruminant nematodes (JACKSON; MILLER, 2006). Therefore, the search for new nematicidal substances remains a priority (GEARY et al., 1999). Substances produced from plants may represent an alternative for controlling gastrointestinal nematodes, since they have the advantage of sustainable supply and are ecologically acceptable (COSTA et al., 2008). The use of medicinal plants has been reported in treating various ailments, thereby increasing the interest in ethnomedical and ethnoveterinary cures (BIZIMENYERA et al., 2006).

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Eucalyptus citriodora (Myrtaceae) is a plant native to Australia, and is widely cultivated around the world. It is the most common species used in Brazil for extraction of essential oil (HASEGAWA et al., 2008). It is used in anti-inflammatory and antipyretic remedies for the symptoms of respiratory infections, such as colds, flu and sinus congestion (SILVA et al., 2003). The essential oil from the leaves is purported to have multiple medicinal applications, including use as antifungal and antibacterial agents (CIMANGA et al., 2002; RAMEZANI et al., 2002). There have also been reports demonstrating its activity against the tick *Boophilus microplus* (CHAGAS et al., 2002), against Coleoptera such as *Acanthoscelides obtectus* (MAZZONETTO; VENDRAMIM, 2003), *Zabrotes subfasciatus* and *Callosobruchus maculatus* (BRITO et al., 2006), and against the phytonematode *Meloidogyne incognita* (PANDEY et al., 2000).

The aim of the present study was to evaluate the potential anthelmintic effect of *E. citriodora* essential oil on goat gastrointestinal nematodes.

Materials and Methods

1. Obtaining the essential oil

Eucalyptus citriodora essential oil (EcEO) was purchased from Dierberger Óleos Essenciais Ltda (Barra Bonita, State of São Paulo, Brazil). To increase the aqueous solubility, the oil was diluted in 3% polysorbate 80 (Tween 80) (Dinâmica®).

2. Egg hatch test

The egg hatching test was performed based on the methodology described by Coles et al. (1992). Sheep experimentally infected with 5000 infective larvae of *Haemonchus contortus* were used as a source of fresh eggs of this parasite. *H. contortus* eggs were recovered as described by Hubert and Kerboeuf (1992). Briefly, 10 g of feces collected directly from the rectum, were mixed with distilled water and filtered through 590, 149, 101 and 30 µm mesh sieves. 250 µL of egg suspension, containing approximately 100 fresh eggs, and 250 µL of EcEO at concentrations of 0.33, 0.66, 1.32, 2.65 and 5.3 mg.mL⁻¹ were incubated in test tubes for 48 hours at 25 °C. After this time, drops of Lugol were added to the tubes. The eggs and first larval stage (L1) were counted under a microscope. This test had two controls: a negative containing the diluent (3% Tween 80) and a positive control with 0.025 mg.mL⁻¹ of thiabendazole. Three repetitions with five replicates for each *E. citriodora* essential oil concentration and for each control were performed.

3. Larval development test

The larval development test (LDT) was performed using an aliquot of egg suspension obtained as described by Hubert and Kerboeuf (1992). The suspension was incubated for 24 hours at 37 °C to obtain the L1. Following this, 1 mL of larval suspension containing approximately 250 L1, and 1 mL of EcEO at concentrations of 0.66, 1.32, 2.65, 5.3 and 10.6 mg.mL⁻¹,

were incubated with 2 g of feces from a nematode-free sheep for 6 days at 25 °C. The third-stage larvae (L3) were then recovered as described by Roberts and O'Sullivan (1950) and were counted under a microscope. This test had two controls: a negative with 3% Tween 80 and a positive with 0.008 mg.mL⁻¹ ivermectin. Three repetitions with five replicates for each oil concentration and for each control were conducted.

4. Acute toxicity test in mice

The care and handling of the animals used in the acute toxicity test were in accordance with the internationally accepted standard guidelines for use of animals, and the protocol was approved by the Ethics Committee of Ceará State University (number: 08332518-2).

For the acute toxicity tests, Swiss albino mice (n = 96) of both sexes, with average weight of 27.5 g, were kept in polypropylene boxes and fed with commercial feed and water *ad libitum*. The mice were randomly divided into 12 groups (n = 8): G1 to G5 received 2000, 3000, 4000, 5000 and 6000 mg.kg⁻¹ of EcEO via oral administration; G6 received 3% Tween 80 via the same route; G7 to G11 received 300, 400, 500, 600, and 700 mg.kg⁻¹ of EcEO via intraperitoneal administration; and G12 received 3% Tween 80 via intraperitoneal administration. The animals were observed for general behavioral changes, signs of toxicity and mortality for 6 hours after treatment. After 24 hours, the total number of dead animals was ascertained and the lethal doses were calculated (LD10 and LD50).

5. Chemical analysis

The chemical composition of the EcEO used in this study was determined by means of gas chromatography (GC) and mass spectrometry (MS). The oil was analyzed in a Hewlett-Packard 5971 instrument using the following experimental conditions: DB-1 coated fused silica capillary column (30 m × 0.25 mm); carrier gas: helium; injector temperature: 250 °C; detector temperature: 200 °C; column temperature program: 35 to 180 °C at 48 °C/min and then 180 to 250 °C at 10 °C/min. For MS, the electron impact was 70 eV.

Compounds were identified according to their GC retention time, expressed through Kovat's index, which was calculated by means of the Van den Dool and Kratz equation using a hydrocarbon homologous series and by comparing the test compound mass spectra with those present in the National Institute for Standard Technology computer database (NIST; 62,235 compounds) and published spectra (ADAMS, 2001).

6. Fecal egg count reduction test (FECRT)

To evaluate the efficacy of EcEO on the gastrointestinal nematodes of goats, the fecal egg count reduction test (FECRT) was performed. Thirty goats of both sexes, aged from 12 to 16 months and weighing 30 kg on average, from the Embrapa research farm in the municipality of Sobral, were used. Individual fecal samples were collected to determine the level of gastrointestinal nematode infection using a modified McMaster technique

(UENO; GONÇALVES, 1998). The goats were divided into three homogeneous groups (n = 10), in which the mean egg count per gram (epg) in each group was 6000. The groups were administered the following treatments orally: G1 (untreated animals) received water; G2 received 0.2 mg.kg⁻¹ of ivermectin (Ivomec®; Merial) in accordance with the manufacturer's instructions; and G3 received 500 mg.kg⁻¹ of EcEO for three consecutive days. Fecal samples from each animal were collected on treatment day 0 and on days 8, 15 and 22 post-treatment to determine the epg. Fecal cultures were performed in accordance with the method of Roberts and O'Sullivan (1950). The egg count percentage reduction (ECR) was calculated using the following formula (IQBAL et al., 2006):

$$\text{ECR (\%)} = \frac{\text{Pretreatment egg count per gram} - \text{Post-treatment egg count per gram}}{\text{Pretreatment egg count per gram}} \quad (1)$$

7. Statistical analysis

For the statistical analysis, the results from the *in vitro* tests were analyzed using ANOVA and compared by means of the Tukey test ($P < 0.05$) (OLIVEIRA et al., 2009). The effective concentrations for inhibiting 50% (EC50) of egg hatching and larval development were determined using the probit method (COSTA et al., 2008). The lethal doses required to kill 50% (LD50) and 10% (LD10) of the mice were calculated for each administration route from the acute toxicity analysis using the probit method (OLIVEIRA et al., 2009). The efficacy of EcEO in relation to the FECRT results was assessed by means of nonparametric one-way Kruskal-Wallis analysis of variance, followed by Dunn's test.

Results

The inhibition of egg hatching and larval development was dose dependent. Table 1 shows the mean efficacy according to the egg hatching and larval development tests using EcEO. The EC50 for inhibition of egg hatching and larval development was 1.14 (0.67 – 2.01) and 2.71 (2.00 – 3.69) mg.mL⁻¹, respectively.

In the acute toxicity tests, the LD10 and LD50 calculated for oral administration were 2609 (689.7 – 3466.4) and 4153.2 (2861.8 – 5849.2) mg.kg⁻¹, respectively. In the test with intraperitoneal administration, LD10 and LD50 were 478.3 (439.8 – 505) and 622.8 (603.3 – 645.5) mg.kg⁻¹, respectively.

Table 2 presents the results from the FECRT. On day 8 post-treatment, EcEO ($P < 0.05$) and ivermectin ($P < 0.05$) reduced epg significantly. The maximum FECR observed was 79.16% for ivermectin and 66.25% for EcEO on day 8. The results relating to larvae identified in fecal cultures are presented in Table 3.

The results obtained by means of gas chromatography indicated the following main constituents and respective concentrations: alpha-pinene (1.1%); beta-citronellol (2.9%); (-) isopulegol (7.3%); eucalyptol (0.8%); beta-citronellal (71.77%); and isopulegol (4.3%).

Discussion

The *in vitro* model reported in this study demonstrated the ovicidal and larvicidal effects of essential oil of *E. citriodora* against *H. contortus*. EcEO inhibited egg hatching and larval development

Table 1. Mean percentage efficacy ± standard error of *Eucalyptus citriodora* essential oil on *Haemonchus contortus* egg hatching and larval development.

Concentrations (mg.mL ⁻¹)	Egg hatching	Larval development
10.6	-	99.71 ± 0.11 ^A
5.3	98.8 ± 0.43 ^A	87.92 ± 1.90 ^B
2.65	97.78 ± 0.40 ^{AE}	46.93 ± 2.14 ^C
1.32	48.72 ± 3.26 ^B	7.08 ± 1.68 ^D
0.66	14.31 ± 0.99 ^C	3.35 ± 1.32 ^D
0.33	7.78 ± 1.08 ^D	-
Tween 80 (3%)	3.12 ± 0.45 ^D	3.5 ± 0.57 ^D
Positive control*	92.7 ± 1.28 ^E	99.81 ± 0.1 ^A

Letters compare means in the lines. Different letters indicate significantly different values ($P < 0.05$). *Positive control for egg hatching was 0.025 mg.mL⁻¹ of thiabendazole and for larval development, 0.008 mg.mL⁻¹ of ivermectin.

at a lower concentration than done by other plants studied previously. Ethyl acetate extract of *Cocos nucifera* at concentrations of 5 and 80 mg.mL⁻¹ inhibited 100% and 97.7% of egg hatching and larval development (OLIVEIRA et al., 2009). Ethyl acetate extract of *Spigelia anthelmia* inhibited 83.8% of egg hatching and 83.1% of larval development at concentrations of 25 mg.mL⁻¹ (ASSIS et al., 2003). Aqueous extract of *Annona senegalensis* leaves inhibited 11.5% of egg hatching at a concentration of 7.1 mg.mL⁻¹ (ALAWA et al., 2003). The maximum effectiveness of essential oil of *Eucalyptus globulus* on eggs was 99.3% at a concentration of 21.75 mg.mL⁻¹ and on larvae was 98.7% at a concentration of 43.5 mg.mL⁻¹ (MACEDO et al., 2009).

Since the *in vitro* tests presented good results, the essential oil was subjected to toxicological assessment to evaluate its effect and to estimate the dose on live organisms. Substances with an LD50 value of 1000 mg.kg⁻¹ via the oral route are regarded as being safe or of low toxicity (CLARKE; CLARKE, 1977). EcEO presented low acute toxicity via the oral route but its LD50 via the intraperitoneal route was higher. The observed difference in LD50 values between oral and intraperitoneal administration may be explained by the fact that in oral administration, less of the substance is absorbed from the gastrointestinal tract, or the substance can become detoxified during liver passage. On the other hand, intraperitoneal absorption is systemic and the toxic effects are produced faster and more intensely (LOOMIS; HAYES, 1996). The high LD50 values obtained indicate that EcEO can be administered with a high degree of safety.

Investigation of chemical compounds from natural products is important for developing new anthelmintic drugs, especially in view of the vast worldwide flora (ASSIS et al., 2003). Chemical analysis on EcEO has identified substances that may be responsible for its anthelmintic activity. In essential oils, some components have a higher concentration and are known as major components. The main component of EcEO was citronellal, which has been shown activity against insects such as houseflies (LEE et al., 2003) and also against females, males and juveniles of the phytonematode *Bursaphelenchus xylophilus* (CHOI et al., 2007).

Table 2. Mean efficacy and eggs per gram (epg) (\pm standard error) of 500 mg.kg⁻¹ of *Eucalyptus citriodora* essential oil and 0.2 mg.kg⁻¹ of ivermectin based on fecal egg count reduction test in goats.

Treatments	Day 0	Day 8	Day 15	Day 22
<i>E. citriodora</i>				
Mean epg	5266 \pm 475.9 ^{Aa}	1777 \pm 274.1 ^{Ab}	2088 \pm 247.1 ^{Ab}	2188 \pm 325.4 ^{Ab}
Efficacy (%)	-	66.25	60.34	58.45
Ivermectin				
Mean epg	5280 \pm 1343 ^{Aa}	1100 \pm 187.4 ^{Ab}	5230 \pm 1147 ^{Ba}	1640 \pm 415.9 ^{Ab}
Efficacy (%)	-	79.16	00.94	68.93
Water				
Mean epg	3550 \pm 732.2 ^{Aa}	2370 \pm 478 ^{Aa}	5000 \pm 647.4 ^{Ba}	3100 \pm 734.8 ^{Aa}

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

Table 3. Percentages of larval helminths in fecal cultures from goats before and after treatment with 500 mg.kg⁻¹ of *Eucalyptus citriodora* essential oil or 0.2 mg.kg⁻¹ of ivermectin or water.

Genera	<i>E. citriodora</i>		Ivermectin		Water	
	Day 0	Day 22	Day 0	Day 22	Day 0	Day 22
<i>Haemonchus</i> sp.	22	7	28	10	24	24
<i>Oesophagostomum</i> sp.	12	10	4	9	2	11
<i>Trichostrongylus</i> sp.	66	83	68	81	74	65
Total	100	100	100	100	100	100

In the goat fecal cultures, there was a high prevalence of *Trichostrongylus* spp. This may be explained by the intensive use of selective treatment of animals through application of the FAMACHA method, which is focused on management of *H. contortus* infections in sheep and goats, based on clinical identification of anemia in individual animals within a flock (REIS, 2004).

The efficacy of FECRT produced by EcEO shows that this substance is promising for use against gastrointestinal nematodes. This result is similar to the findings from other studies on essential oils. *Lippia sidoides* essential oil at a dose of 283 mg.kg⁻¹ reduced epg by 54% (CAMURÇA-VASCONCELOS et al., 2008); 500 mg.kg⁻¹ of *Eucalyptus staigeriana* essential oil reduced epg by 76.57% (MACEDO et al., 2010); and 600 mg.kg⁻¹ of orange oil reduced epg by 94.9% (SQUIRES et al., 2010).

Ivermectin produced a small FECR, thus indicating the presence of resistant nematodes. This is a common finding in northeastern Brazil and elsewhere (SARGISON et al., 2007; KUMSA; ABEBE, 2009). The use of essential oil would be justified even with effectiveness below 95%, in situations in which synthetic anthelmintics are not recommended, such as in organic breeding or dairy production, or when the cost outweighs the benefit. Therefore, the alternative use of plants may be a useful tool, in association with other methods for controlling the gastrointestinal nematodes of small ruminants (CAMURÇA-VASCONCELOS et al., 2008).

Use of plants with moderate anthelmintic activity should still be considered, perhaps not as a total alternative to anthelmintic drugs, but as part of an integrated approach specifically designed to achieve sustainable parasite control in ruminant production systems. The role of plants in extending the use and increasing the efficacy of existing anthelmintics thus warrants study (GITHIORI et al., 2006).

E. citriodora essential oil presented promising results, but the efficacy did not reach the therapeutically required level. Further investigation of isolated fractions, at different dose levels, should be pursued.

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