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Short Communication

Ovicidal and larvicidal potential of *Rosmarinus officinalis* to control gastrointestinal nematodes of sheep

Potencial ovicida e larvicida de *Rosmarinus officinalis* para controle de nematódeos gastrintestinais de ovinos

Natália Berne Pinto[1]; Leonardo Mortagua de Castro[1]; Rosária Helena Machado Azambuja[1]; Gabriela de Almeida Capella[1]; Micaele Quintana de Moura[1]; Wesley Douglas Terto[1]; Rogério Antonio Freitag[2]; Sabrina Taiza Jeske[1]; Marcos Marreiro Villela[1]; Marlete Brum Cleff[1]; Fábio Pereira Leivas Leite[3]

[1] Laboratório de Helmintologia, Departamento de Microbiologia e Parasitologia, Instituto de Biologia, Universidade Federal de Pelotas - UFPel, Pelotas, RS, Brasil

[2] Laboratório de Química, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas – UFPel, Pelotas, RS, Brasil

[3] Laboratório de Microbiologia, Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas – UFPel, Pelotas, RS, Brasil

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Abstract

Gastrointestinal Nematode Infection (GIN) are the main constraint to the production of small ruminants. Studies of medicinal plants have been an important alternative in the effort to control these parasites. Therefore, the purpose of this study was to evaluate the in vitro ovicidal and larvicidal activity of essential oil of *Rosmarinus officinalis*. The oil was extracted, analyzed by gas chromatography and tested on GIN eggs and larvae in six concentrations, 227.5mg/mL, 113.7mg/mL, 56.8mg/mL, 28.4mg/mL, 14.2mg/mL and 7.1mg/mL. To determine the ovicidal activity, GIN eggs were recovered from sheep feces and incubated for 48h with different concentrations of the oil. For the evaluation of larval migration, third-stage larvae (L3) were obtained by fecal culture, and associated with the essential oil for 24h at the same concentrations, after which they were left for another 24 hours on microsieves, followed by the count of migrating and non-migrating larvae. The assays of *R. officinalis* oil showed a significant ($p<0.05$) 97.4% to 100% inhibition of egg hatching and a significant ($p<0.05$) 20% to 74% inhibition of larval migration. The main constituent revealed by gas chromatography was Eucalyptol. The results indicate that *R. officinalis* essential oil has ovicidal and larvicidal activity on sheep GINs.

Keywords: Helminths, *in vitro*, phytotherapeutics, ruminants.

Resumo

As infecções por nematódeos gastrintestinais (ING) constituem a maior limitação à produção de pequenos ruminantes. Na busca do controle desses parasitos, estudos com plantas medicinais têm sido uma importante alternativa. Visto isto, o estudo desenvolvido teve como objetivo avaliar a ação ovicida e larvicida *in vitro* do óleo essencial de *Rosmarinus officinalis*. O óleo foi extraído, analisado por cromatografia gasosa e testado sobre ovos e larvas de ING em seis concentrações, 227,5mg/mL; 113,7mg/mL; 56,8mg/mL; 28,4mg/mL; 14,2mg/mL; 7,1mg/mL. Para determinar a ação ovicida, ovos de ING foram recuperados de fezes de ovinos e incubados por 48h com as diferentes concentrações do óleo. Na avaliação da migração das larvas, as larvas de terceiro estágio (L3) foram obtidas por coprocultura, e associadas ao óleo essencial por 24h nas mesmas concentrações, permanecendo por mais 24h em microtamises, seguindo-se a contagem de larvas que migraram e que não migraram. Os testes *in vitro* com o óleo de *R. officinalis* mostraram o nível de significância ($p<0.05$) 97,4% a 100% na inibição da eclodibilidade e 20% a 74% na inibição da migração das larvas. Na análise por cromatografia gasosa o constituinte majoritário foi o eucaliptol. Os resultados apresentados mostram que o óleo essencial de *R. officinalis* possui ação ovicida e larvicida sobre ING de ovinos.

Palavras-chave: Helmintos, *in vitro*, fitoterápicos, ruminantes.

***Corresponding author:** Natália Berne Pinto. Universidade Federal de Pelotas – UFPel, Campus Universitário, s/n, CP 354, CEP 96010-900, Pelotas, RS, Brasil. e-mail: nbernevet@gmail.com

Introduction

Sheep meat production has been strongly promoted and its structure has been modified to meet market demands, aiming for greater productivity in less space. To this end, sheep flocks have been concentrated in small areas and animals selected based on their production of meat. This new reality of production, coupled with inefficient management, has caused gastrointestinal nematodes (GINs) to become the main obstacle to sheep production (AMARANTE et al., 2014).

Attempts to reduce losses have led to significantly increased frequency and doses applied in anthelmintic treatments, causing irreversible resistance of GIN to practically all the available active ingredients (SALGADO & SANTOS, 2016). *Haemonchus contortus* stands out among these parasites, mainly due to its hematophagous nature and high prolificacy of females (GETACHEW et al., 2007).

Worldwide studies that have focused on devising new strategies to combat these nematodes include management procedures involving different ruminant species, rotational grazing, crop-livestock integration, genetic selection of animals, the use of fungi and bacteria in biological control, herbal medicines, and vaccine production (MOLENTO et al., 2013; SINOTT et al., 2012).

In recent years, studies involving plants have stood out among the lines of research that seek alternative methods to control ruminant parasites. These studies have revealed promising anthelmintic activity on gastrointestinal parasites of sheep (CAMURCA-VASCONCELOS et al., 2007; YOSHIHARA et al., 2014; RIBEIRO et al., 2014; MARIE-MAGDELEINE et al., 2014; MACEDO et al., 2015).

Rosmarinus officinalis, which belongs to the family Lamiaceae, is commonly known as rosemary. This plant is widely used in cooking, but studies have shown that its essential oil has antibacterial and antifungal properties (OLUWATUYI et al., 2004; GENENA et al., 2008). Thus, in view of the severe problem of drug resistance allied with the consumer's demand for food devoid of chemical residues, there is an undeniable need to find sustainable alternatives for the control of GIN in sheep. Therefore, this study aims to evaluate the *in vitro* activity of *R. officinalis* oil on eggs and larvae of gastrointestinal parasitic nematodes of sheep.

Materials and Methods

Production of essential oil

Rosmarinus officinalis essential oil was produced from plant material purchased from a commercial distributor (Luar Sul Alimentos) with certified quality and provenance. The dried leaves were subjected to steam extraction in a Clevenger apparatus for 4 hours. The essential oil was then obtained by hydrodistillation (1.5L distilled water/100g plant), dried with P.A. grade anhydrous sodium sulfate, and stored in an amber flask at -18 °C until use.

The *R. officinalis* essential oil was analyzed by gas chromatography coupled to mass spectrometry (GC-MS) (CHIARADIA et al., 2008) and its compounds were analyzed based on the NIST08 GC/MS library. The chemical composition of the essential oil used in this study was determined by a Schimadzu QP2010 GC/MS apparatus, equipped with split/splitless injector with Rtx-5MS RESTEK capillary column (30 mx 0.25 mm x 0.25 μm), graphite stations: Helium carrier gas, electron impact fragments in energy of 70V, flow rate of 1.2 mL/min, 1:10 split, injected volume of 1μL sample. Programmed oven temperature: at an initial temperature of 40 °C, with a heating temperature of 5 °C/min to 280 °C, maximum temperature of 58 °C, the injector temperature being 58 °C and the interface 200 °C. The oil was diluted in hexane, P.A.

In preparation for the tests, essential oil was diluted in distilled water and 1% Tween 80, as follows: 227.5mg/mL, 113.7mg/mL, 56.8mg/mL, 28.4mg/mL, 14.2mg/mL, and 7.1mg/mL corresponding, respectively, to concentrations of 25%, 12.5%, 6.25%, 3.12%, 1.56% and 0.78%.

Obtaining eggs

GIN eggs were recovered from feces collected directly from the rectal ampulla of sheep with natural mixed infection. The fecal samples were processed immediately, first by maceration and homogenization with distilled water at 40 °C. Next, they were passed through 1mm, 105μm, and 55μm mesh sieves in order to retain the largest particles of fecal matter. The remaining material was sifted through a 25μm mesh sieve where the eggs were retained, recovered and centrifuged three times (203g for 5 minutes) with distilled water, and the supernatant was discarded. A final centrifugation was performed using supersaturated salt solution to float the eggs, and this supernatant was discarded in the 25μm mesh sieve for washing in distilled water (HUBERT & KERBOEUF, 1992).

Inhibition of Hatchability (IH) assay

The hatchability inhibition assay was performed based on the technique described by Coles et al. (1992) with modifications, using approximately 150 eggs from the test solutions in polyethylene 24-well plates. This involved the formation of four groups as follows: Group 1: Rosemary essential oil at concentrations of 227.5mg/mL; 113.7mg/mL; 56.8mg/mL; 28.4mg/mL; 14.2mg/mL; and 7.1mg/mL; Group 2: Negative control with distilled water; Group 3: Positive control with thiabendazole 0.025 mg/mL; Group 4: control of physical activity with Mineral Oil; and Group 5: with 1% Tween 80. All the assays, which involved incubation for 48 hours at 28 °C and 80% relative humidity (RH), were performed in triplicate. After this period, the readings were carried out under an inverted microscope, by counting the eggs and larvae contained in each well. The mean inhibition of hatchability (IH) of each treatment was determined according to the equation described by Camurça-Vasconcelos et al. (2007): % IH = number of larvae/number of larvae + number of eggs X 100.

Obtaining larvae

Fecal samples were collected directly from the rectal ampulla of sheep with natural mixed infection by gastrointestinal nematodes. The samples were processed to identify positive ones by quantifying the eggs using the technique described by Gordon & Whitlock (1939) with modifications. Positive samples were incubated for

7 days at 28 °C and RH higher than 80%, after which third-stage larvae (L3) were recovered (ROBERTS & O'SULLIVAN, 1950), counted and identified (UENO & GONÇALVES, 1988).

Inhibition of Larval Migration (ILM) assay

The IML assay was performed as proposed by Demeler et al. (2010), with modifications. Accordingly, the larvae were first placed on 25µm sieve membranes to migrate for approximately one hour, after which viable larvae were selected and placed in six-well polyethylene plates. After this initial selection, the L3 were placed in 0.6% of the unsheathing solution (2% sodium hypochlorite) for approximately 20min. They were then washed three times by centrifugation in distilled water (203g for 2min), and the finally adjusted to obtain a concentration of 150 larvae in 100µL.

One hundred microliters of the larval solution were then added to the first and third lines of the 24-well plates and incubated for 24 hours at 28 °C and 80% RH, with 900µl of solution with oil at the concentrations of 227.5mg/mL; 113.7mg/mL; 56.8mg/mL; 28.4mg/mL; 14.2mg/mL; and 7.1mg/mL. After this period, the content of each well containing L3 was transferred to 25µm sieve membranes placed in another 24-well polyethylene plate, which was incubated at 28 °C for 24 hours. The membranes were then removed and the content retained on them were placed in the wells of the second and fourth lines of the plate, after which a count was made under an inverted microscope of the larvae that migrated and those that were retained on the sieve membranes. The same procedure was performed with the controls, using distilled water, mineral oil, Tween buffer, and an anthelmintic drug (0.025 mg/mL of Levamisole). All the assays were performed in triplicate.

Analysis of Variance (ANOVA), followed by multiple comparison Tukey's test, was applied at a 5% probability level to analyze the data on inhibition of hatchability (IH) and inhibition of larval migration (ILM), using GraphPad Prism version 7.0 software. The half maximal inhibitory concentration (IC_{50}), which can inhibit larval migration by 50%, was determined from the dose-response curve with a 95% confidence interval, using the program GraphPad Prism v. 5.0 for Windows.

This study was approved by the Ethics Committee for Animal Experimentation (CEEA) of the Federal University of Pelotas – UFPel, under Protocol No. 3897.

Results

The chromatographic evaluation of *R. officinalis* essential oil revealed 18 different compounds (Table 1), and the three major ones were eucaliptol, bornanone and alpha-pinene, which together represent more than 70% of the components of the essential oil.

The inhibitory effect of *R. officinalis* essential oil on hatchability varied from 97.4% to 100% at all the tested dilutions (227.5mg/mL; 113.7mg/mL; 56.8mg/mL; 28.4mg/mL; 14.2mg/mL; and 7.1mg/mL). The oil's inhibitory effect on larval migration ranged from 70% to 74% at the two highest tested concentrations, respectively, and from 20% to 58% at the other concentrations (Table 2). Inhibition of the distilled water, Tween and mineral oil controls, in both tests, was less than 10%.

Table 1. *Rosmarinus officinalis* essential oil compounds identified by gas chromatography coupled to mass spectrometry.

Gas Chromatography (%)	
Eucalyptol (1.8-cineole)	42.11
(+)-2-Bornanone	16.37
alpha.-Pinene	14.76
Isoborneol	5.73
alpha.-Terpineol	5.40
Camphene	3.87
o-Cymene	2.69
Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)	2.08
1,6-Octadien-3-ol, 3,7-dimethyl	1.70
beta.-Myrcene	1.38
Terpinen-4-ol	1.23
Bornyl acetate	0.84
Caryophyllene	1.00
gamma.-Terpinene	0.37
Phenol, 2-methyl-5-(1-methylethyl)	0.19
Humulene	0.15
Methyleugenol	0.07
Thymol	0.05

Table 2. Efficacy of essential oil of *Rosmarinus officinalis in vitro* to inhibit hatchability (IE) and migration of larvae (IM) from gastrointestinal nematodes of sheep.

Treatment	Inhibition of Hatchability (%)	Inhibition of Larval Migration (%)
Control	1.1^{c}	6.9^{f}
Tween 80	5.3^{b}	10^{ef}
Mineral Oil	4.5^{bc}	8.3^{ef}
AH	100^{a}	99.5^{a}
227.5 mg/mL	100^{a}	74^{b}
113.7 mg/mL	100^{a}	70.1^{bc}
56.8 mg/mL	100^{a}	55.3^{c}
28.4 mg/mL	100^{a}	35.6^{d}
14.2 mg/mL	98.3^{a}	23^{de}
7.1 mg/mL	97.4^{a}	20^{def}
MSD	3.45	16.08

AH: antihelmintic; MSD: Minimum Significant Difference Tukey 5%; Values with different lowercase letters in same column indicate significant differences ($p<0.05$) between concentrations.

A statistical analysis of the IH showed no difference between treatments, but all differed statistically from the control. The same analysis of ILM revealed that the effect of the two highest concentrations was statistically the same, but differed from the other treatments and the control. The minimum concentration of *R. officinalis* essential oil needed to inhibit 50% of hatchability (IC_{50}) was 0.5181 mg/mL, while the IC_{50} of larval inhibition was 62.17 mg/mL.

The parasite genera identified in the fecal cultures were *Haemonchus* spp. (79%), *Ostertagia* spp. (18%) and *Trichostrongylus* spp. (3%).

Discussion

Plants of the Lamiaceae family are among the most widely studied for phytotherapeutic purposes, and among the species of this family, *R. officinalis* has shown nematicidal activity on plant nematodes (WANG et al., 2012). The nematicidal effect of essential oils is due to multiple factors that are deleterious to metabolic activity, to alterations of cellular membrane permeability, and to destructuring of the nervous system (OKA et al., 2000).

The use of plants as an alternative for the control of gastrointestinal nematodes of sheep has been reported in different regions of the world (RAJESWARI, 2014). The essential oils are prominent in this line of research and are characterized by being complex compounds that can contain from 20 to 60 compounds in different concentrations, being the major components the probable determinants of the biological action of the oil (SINTIM et al., 2015).

In chromatographic analyses, different compounds have been identified in *R. officinalis* essential oil. Among them, eucaliptol (1,8-cineole) have been described as major compounds (42.8%), according to different authors Jiang et al. (2011), Santoyo et al. (2005) and Okoh et al. (2010). Bozin et al. (2007) and Gachkar et al. (2007). The monoterpene 1,8-cineol (eucalyptol) has been reported to have action on larvae of the *Anisakis simplex* nematode (NAVARRO et al., 2008) and also against *Dactylogyrus minutus* (Monogenea) parasites in fish (ZORAL et al., 2017).

In the present study, where *Haemonchus* was the most prevalent nematode (79%), suggest that the essential oil of *R. officinalis* acts on gastrointestinal nematodes with 100% inhibition of hatchability (IH) at the highest concentration (227.5 mg/mL) ($p < 0.05$) to the concentration of 7.1 mg/mL (97.4%). Similar results were reported by Macedo et al. (2009), studying *Eucalyptus globulus* essential oil, whose major constituent is also 1,8-cineol (eucalyptol), observed 99.3% egg hatching was inhibited at concentration of 21.75 mg/mL.

In the literature, there are reports of the toxic effect of *R. officinalis* essential oil against protozoa (PEREIRA et al., 2017) and ectoparasites (MARTINEZ-VELAZQUEZ et al., 2011) however, studies on the action of nematode of domestic ruminants are still scarce.

The results of the essential oil effect of *R. officinalis* on inhibition of larval migration (ILM) show that the concentration required to obtain this activity satisfactorily is higher than that required for inhibition of hatchability (IH), the maximum of ILM (74%) was achieved in the concentration of 227.5 mg/mL, while in the HI the lowest concentration was already efficient (97.4%). Similar effect was observed with *Melaleuca alternifolia*, which at a concentration of 56mg/mL and 3.5mg/mL inhibited 88% larval migration and 100% hatchability of *H. contortus*, respectively (GRANDO et al., 2016). The concentration required to inhibit larval migration is higher than that needed to inhibit hatchability (GRANDO et al., 2016; YOSHIHARA et al., 2014; QI et al., 2015). This is probably due to the fact that infecting larvae can survive in the environment exposed for long periods to deleterious weathering. The survival of these larvae in the environment is attributed to their double cuticle and the process they undergo, called anhydrobiosis, which severely reduces their metabolic activity, thereby prolonging their survival (AMARANTE et al., 2014). The LC_{50} for hatchability was similar, i.e., 0.43 mg/mL for *M. alternifolia* and 0.51 mg/mL for *R. officinalis*.

Macedo et al. (2015), who evaluated the potential of essential oil of *Cymbopogon citratus* inhibiting the hatchability of *H. contortus*, reported 99.5% of inhibition at a concentration of 1.25 mg/mL. Their result is close to that of *R. officinalis* essential oil, observed in this study, which was 97.4% when tested at the highest concentration (7.1 mg/mL). One can suggest that the dissimilar results of these studies can be attributed to fact that they involved different plants, as well as different parasites. In our study, we evaluated three genera of gastrointestinal nematodes, similar to those found in the field, since sheep commonly have mixed infestations, whereas the effect of *Cymbopogon citratus* essential oil was evaluated only on *H. contortus*. This difference in product efficacy among genera of gastrointestinal parasitic nematodes of ruminants has been described even with commercial anthelmintics, suggesting that the species *Haemonchus* is more sensitive than other nematodes (NOVA et al., 2014).

Conclusions

Rosmarinus officinalis essential oil showed ovicidal and larvicidal activity *in vitro* on gastrointestinal nematodes, constituting a promising alternative for use as a phytotherapeutic agent for the control of intestinal nematodes of sheep.

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