IN VITRO ANTIPARASITIC ACTIVITY OF *Mentha spicata* L. (LAMIALES: LAMIACEAE) IN THE EGGS OF GASTROINTESTINAL HELMINTH PARASITES OF SHEEP

ATIVIDADE ANTIPARASITÁRIA *IN VITRO* DE *Mentha spicata* L. (LAMIALES: LAMIACEAE) EM OVOS DE HELMINTOS GASTROINTESTINAIS DE OVINOS

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ABSTRACT: Phytotherapy has been an alternative to control parasitic resistance. Thus, the objective was to evaluate *in vitro* the anthelmintic activity of the extract of *Mentha spicata* L. in eggs of gastrointestinal helminths of sheep. The leaves were collected for extract preparation and phytochemical analysis. For *in vitro* tests, fecal samples were collected from a sheep herd. Eggs per gram of feces were counted to determine the level of infection, then recovered and hatch test performed. To determine the toxicological effect of the extract, a bioassay was performed with *Artemia salina*. The extract inhibited the hatching of eggs by 79%, 78%, 40% and 23% at concentrations of 80 mg / mL, 40 mg / mL, 20 mg / mL and 10 mg / mL, respectively. The concentrations with the best ovicidal activity were 80 mg / mL and 40 mg / mL, with 80 mg / mL being toxic with an LC_{50} of 59.04 mg / mL, indicating that 40 mg / mL is the most viable concentration. Phytochemical analysis showed the presence of phenols, saponins, steroids and hydrolyzable tannins, which could explain its

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ovicidal activity. The use of *M. spicata* L. shows potential for the control of helminths in sheep.

KEYWORDS: Anthelmintic; Phytotherapy; Sheep farming.

RESUMO: A fitoterapia vem sendo uma alternativa para o controle da resistência parasitária. Dessa forma, objetivou-se avaliar *in vitro* a atividade anti-helmíntica do extrato de *Mentha spicata* L. em ovos de helmintos gastrintestinais de ovinos. As folhas foram coletadas para preparação do extrato e análise fitoquímica. Para testes *in vitro*, amostras fecais foram coletadas de um rebanho ovino. Os ovos por grama de fezes foram contados para determinar o nível de infecção, em seguida recuperados e realizado o teste de eclosão. Para determinar o efeito toxicológico do extrato, foi realizado um bioensaio com *Artemia salina*. O extrato inibiu a eclosão dos ovos em 79%, 78%, 40% e 23% nas concentrações de 80 mg/mL, 40 mg/mL, 20 mg/mL e 10 mg/mL, respectivamente. As concentrações com a melhor atividade ovicida foram de 80 mg/mL e 40 mg/mL, sendo a 80 mg/mL considerada tóxica com uma CL_{50} de 59,04 mg/mL, indicando que 40 mg/mL é a concentração mais viável. Análise fitoquímica demonstrou presença de fenóis, saponinas, esteróides e taninos hidrolisáveis, o que poderia explicar sua atividade ovicida. O uso de *M. spicata* L. mostra potencial para o controle de helmintos em ovinos.

PALAVRAS-CHAVE: Anti-helmíntico; Fitoterapia; Ovinocultura.

INTRODUCTION

Farming is a socially and economically important activity in Brazil, especially in the northeastern and southern regions. According to the Brazilian Institute of Geography and Statistics (IBGE, 2017), there are approximately 13 million sheep being raised in Brazil, the rate of growth in sheep herds being highest in the northeastern region, where the number of sheep increased from 7.7 million in 2006 to just over 9 million in 2017, corresponding to a growth rate of 15.94%. However, the development of these animals may be limited by the presence of gastrointestinal parasites, which can cause weight loss and anemia, together with decreased productive and reproductive potential, having a negative impact on animal production (Vieira *et al*., 2018). Among the main gastrointestinal parasites of sheep are those of the genera *Haemonchus*, *Trichostrongylus*, and *Strongyloides*, as well as those of the species *Oesophagostomum columbianum* (Afonso *et al*., 2013).

The efficiency of the traditional anthelmintic drugs for the treatment of sheep has declined over the years because they have been used indiscriminately, which accelerates the process of parasite selection and resistance to these chemotherapeutic agents (Snyman; Fisher, 2019). Pharmacological treatment for the control of helminths has other

disadvantages, such as the possibility that drug residues will be released into the environment, given that anthelmintics are not normally metabolized completely in the host animal and drug residues can be excreted in manure (Cooke *et al*., 2017). Another factor to be considered is the presence of drug residues in the products derived from these animals, such as milk and meat. That occurs due to the lack of guidance in the administration of these drugs, resulting in overdosing or underdosing, with no regard for the residual period before slaughter, which can cause health problems for consumers (Giannetti *et al*., 2011; Caselani, 2014).

Phytotherapy represents an alternative for the control of gastrointestinal helminths in small ruminants, with the objective of avoiding the problems caused by the use of pharmaceuticals. However, scientific research related to antiparasitic properties of phytotherapeutic agents is scarce (Andrade *et al*., 2018; Fonseca *et al*., 2019). There are *in vitro* tests that allow the antiparasitic properties of plant derivatives to be evaluated, constituting a step prior to the characterization of the active compounds, making it possible to develop alternative treatments for the control of helminthiasis (Costa *et al*., 2002; Nery *et al*., 2009), as well as to validate their use (Rossato *et al*., 2012).

The study of medicinal plants to combat gastrointestinal nematodes in small ruminants should be included in an agroecological scenario, with sustainable natural resource management, together with the preservation of the plant species to be studied (Nery *et al*., 2009). A wide variety of medicinal plants are known to have anthelmintic activity. However, there is need for studies evaluating their effectiveness, analyzing their regional availability, and determining their toxicity in animals (Santos-Lima *et al*., 2016).

The mint species *Mentha spicata* L., belonging to the family Lamiaceae, is an aromatic plant and is among the best known herbs for use in the form of teas, with the purpose of treating diseases such as gastrointestinal disturbances, respiratory disorders, and headaches (Jain *et al*., 2011). It is mainly used in order to obtain essential oil, being widely used as a flavoring and an herb in cooking, as well as an additive in oral hygiene products and pharmaceutical formulations (Santos *et al*., 2013; Nikšić *et al*., 2018). Studies have shown that *M. spicata* L. has antioxidant, antimicrobial, antiparasitic, anxiolytic, bronchodilator, and analgesic properties (Biswas *et al*., 2012; Menezes *et al*., 2012; Chrysargyris *et al*., 2017; Pauli *et al*., 2018). However, there are few data on the effects that *M. spicata* L. has on gastrointestinal parasites in small ruminants.

Given the rise of parasite resistance to traditional anthelmintic drugs, it is necessary to search for new methods of controlling gastrointestinal parasites in small ruminants, especially methods based on the use of medicinal plants, as sustainable and viable alternatives. The use of such methods could reduce environmental contamination, as well as providing a direct return to society by offering a safer consumer product. Therefore, the objective of this study was to evaluate the *in vitro* antiparasitic activity of the leaves of *M. spicata* L. in the eggs of gastrointestinal helminths in sheep.

MATERIAL AND METHODS

Experimental procedures

All experimental procedures were conducted at the Laboratory of Applied Biotechnology for Infectious and Parasitic Diseases of the Federal Rural University of the Semi-Arid Region, in the city of Mossoró, Brazil. The study was approved by the Committee for Ethics in Animal Experimentation of the University (Reference no. 23091.009318/2016-40). All experiments were performed in accordance with the recommendations of the Animal Experimentation Ethics Committee and of the Brazilian College of Animal Experimentation.

Study area

The study was carried out in the city of Mossoró, in the state of Rio Grande do Norte, which is in the northeastern region of Brazil (05°11'16.8"S; 37°20'38.4"W), covering an area of 2,100 km^2. The climate of the region is classified as semi-arid, with two climatic phases: a dry season (June through January) and a rainy season (February through May). The relative humidity is approximately 70%, and the mean annual temperature is 27.4°C (Araújo *et al*., 2012; Silva *et al*., 2018).

Plant material collection

The *M. spicata* L. leaves were collected in the city of Mossoró and were transported to the laboratory, where the extract was prepared. The taxonomic identification of the specie was carried out by the Herbário Dárdano de Andrade-Lima, of the Center for Biological and Health Sciences of the Federal Rural University of the Semi-Arid Region, where an exsiccate number 15,001 was obtained.

Preparation of the saline extract

The leaves were washed and oven dried at 35°C, after which they were crushed to a powder. Then the material obtained was subjected to extraction at 10% (w/v) in 0.15 M NaCl solution, stirred for 16 hours, filtered and centrifuged at 8,000 rpm for 20 minutes at 4°C to obtain crude saline extract. Subsequently, serial dilutions were prepared from the crude extract, as previously described (Nelson; Cox, 2014), to obtain concentrations of 10% (80 mg/mL), 5% (40 mg/mL), 2.5% (20 mg/mL), and 1.25% (10 mg/mL).

Egg hatch test

Fecal samples were collected from naturally infected sheep herds in which antiparasitic agents were not used, totaling five collections per month. Initially, we verified parasitic infection by determining the number of eggs per gram of feces (Gordon; Whitlock, 1939) followed by the co-culture technique to identify, after seven days of incubation, which genus of nematodes are present in the infection, resulting in *Haemonchus* sp, *Trichostrongylus* sp, and *Strongyloides* sp (Ueno; Gonçalves, 1998). Eggs were then retrieved according to the methodology devised by Hubert and Kerboeuf (1992), in which the fecal sample is passed through a sequence of sieves with pore sizes of 0.15, 0.10, 0.036, and 0.02 mm. The liquid obtained at the end of this process was distributed into tubes and centrifuged at 4,000 rpm for 5 minutes, after which the supernatant was discarded and the precipitate was dissolved in supersaturated saline. The resulting solution was centrifuged under the same conditions and passed through the 0.02 mm-pore sieve.

The egg hatch test, as described by Coles et al. (2006), was used in order to evaluate the antiparasitic activity of the crude saline extract of *M. spicata* L. in the eggs recovered. Eggs were placed into 24-well plates, with an average of 100 eggs/well, after which they were inoculated with a negative control (0.15 M saline), a positive control (3.2 μg/mL of thiabendazole), and the following concentrations of the *M. spicata* L. extract: 80 mg/mL; 40 mg/mL; 20 mg/mL; and 10 mg/mL. All *in vitro* assays were performed independently in quintuplicate. The plates were incubated in biochemical oxygen demand chambers at 25°C for 48 hours.

Phytochemical analysis

A phytochemical analysis of *M. spicata* L. leaf extract was performed according to the methodology described by Matos (2008), in which the constituents were identified by

metabolic class. The identification of the classes present in the leaf extract was performed by observing the precipitation, colorimetric reactions, and fluorescence responses, in order to identify coumarins (fluorescence test); phenols (ferric chloride test); flavonoids (cyanidin or Shinoda reaction); anthraquinones (Bornträger's test); steroids (Liebermann–Burchard test); triterpenoids (Salkowski's test); saponins (foam test, which involves vigorous agitation); condensed and hydrolyzable tannins (Stiasny's method); and free tannins (gelatin precipitation test).

In vitro tests of acute toxicity

The toxicity of the crude saline extract of *M. spicata* L. leaves was evaluated according to the methodology described by Rodriguez et al. (2012). Eggs of the brine shrimp *Artemia salina* were hatched in a culture solution containing 18 g of NaCl and 5 g of $NaHCO_3$, under constant light and aeration, over a 48-hour period. Hatched nauplii were separated and transferred to well plates (10 nauplii/well) containing 100 μL of *A. salina* culture solution and 400 μL of the crude saline extract of *M. spicata* L. leaves (at 80 mg/mL, 40 mg/mL, 20 mg/mL, and 10 mg/mL) per well, in quintuplicate. The plates were then incubated in an isothermal box at room temperature for 24 hours under illumination, after which the dead and living nauplii were counted.

Statistical analysis

All survey data were tabulated in an Excel spreadsheet. For each experimental group, the proportional inhibition of egg hatching was determined by the following formula:

$$number\ of\ first\ stage\ larvae / (number\ of\ eggs + number\ of\ first\ stage\ larvae) \times 100$$

The Shapiro–Wilk test was applied to verify normality, and analysis of variance followed by Tukey's post hoc test was used in order to identify significant differences among the experimental groups. The level of significance adopted for all tests was 5%. The median lethal concentration (LC_{50}) in the *A. salina* toxicological assay was calculated by nonlinear regression. Statistical analyses were performed with GraphPad Prism, version 8.0 (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

RESULTS

Inhibition of egg hatching

The results of the egg hatching tests with the different concentrations of the *M. spicata* L. extract (80 mg/mL, 40 mg/mL, 20 mg/mL, and 10 mg/mL) are shown in Figure 1.

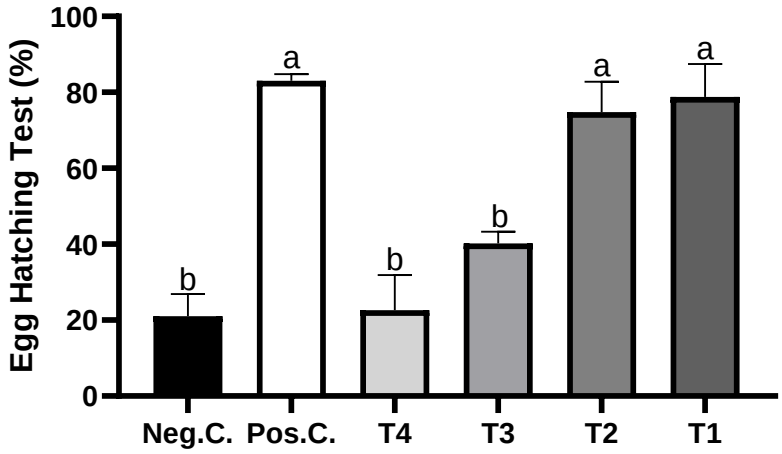

Figure 1. Proportion of inhibition of hatching eggs of gastrointestinal helminths of sheep inhibited from hatching after treatment with the crude saline extract of leaves of *Mentha spicata* L. at concentrations of 80 mg/mL (T1), 40 mg/mL (T2), 20 mg/mL (T3), and 10 mg/mL (T4), as well as with a negative control (Neg.C., 0.15 M saline solution) and a positive control (Pos.C., 3.2 µg/mL of thiabendazole). Different lowercase letters indicate significant differences ($p < 0.05$).

The anthelmintic thiabendazole, used as the positive control, inhibited hatching by 83%, in comparison with 21% for the negative control. For the *M. spicata* L. extract, the inhibition rates were highest at the concentrations of 80 mg/mL (79%) and 40 mg/mL (78%), and those rates were not statistically different from those observed for the positive control. However, the results obtained for the concentrations of 20 mg/mL and 10 mg/mL showed no statistical difference in comparison with those obtained for the negative control.

Phytochemical analysis

In the phytochemical analysis, the crude saline extract of *M. spicata* L. leaves was found to contain phenols, saponins, steroids, and hydrolyzable tannins.

***In vitro* acute toxicity**

The results of the toxicity test with *A. salina* are shown in Figure 2. The crude saline extract of *M. spicata* L. leaves presented an LC_{50} of 59.04 mg/mL (R^2 = 0.6096).

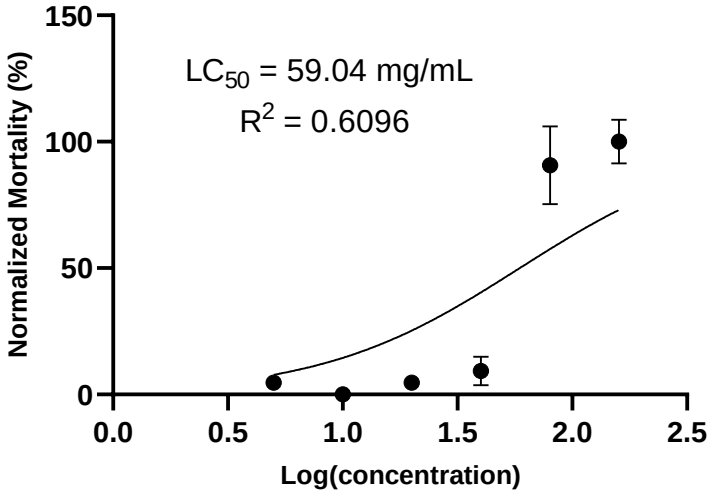

Figure 2. Representation of the toxicological effect that the tested concentrations of the saline extract of the leaves of *Mentha spicata* L. have on *Artemia salina*.

DISCUSSION

Our finding that thiabendazole at 3.2 µg/mL inhibited hatching in only 83% of the helminth eggs recovered indicates that the parasite has developed drug resistance. In a study conducted by Coles et al. (2006), a dose of 0.1 µg/mL of thiabendazole was found to prevent the hatching of 99% of eggs from susceptible species of *Haemonchus contortus*, *Teladorsagia circumcincta*, and *Trichostrongylus colubriformis*. Therefore, our findings suggest that the efficiency of this anthelmintic has decreased over the last ten years. Helminths have many biological and genetic characteristics that favor the development of drug resistance, such as short life cycles, high reproduction rates, and extremely large population sizes. The combination of those characteristics confers an exceptionally high level of genetic diversity (Idris *et al*., 2019; Kaplan, 2020).

Given that the saline extract of the leaves of *M. spicata* L., at the concentrations of 80 mg/mL and 40 mg/mL, showed inhibition of helminth egg hatching comparable to that of thiabendazole, *M. spicata* L. extract can be considered an alternative for the control of gastrointestinal helminthiasis and its use could delay the development of parasitic resistance

by reducing the use of anthelmintics. In an ethnobotanical study, Andrade et al. (2018) showed that the leaves and flowers of *Mentha* spp. can be used in the treatment of gastrointestinal helminthiasis. Pauli et al. (2018) showed that *M. spicata* L. is commonly used in the treatment of parasitic infections, as well as other diseases, by the population in the Brazilian state of Mato Grosso. Almeida et al. (2007) found that the aqueous extract of *M. piperita* L. leaves (at 115.9 mg/mL) reduced the number of infective larvae of *H. contortus* and *Trichostrongylus* sp. in goats by more than 95% *in* vitro. However, there have been few studies investigating the anthelmintic activity of *M. spicata* L., especially in parasites of sheep.

The ovicidal activity of *M. spicata* L. observed in the present study might be related to the presence of secondary metabolites such as phenols, saponins, steroids, and hydrolyzable tannins. Plants with anthelmintic properties that contain phenolic compounds (tannins, flavonoids, and saponins) show promise as a means of combating gastrointestinal nematodes, as well as being food supplements with potential nutritional value (Silva *et al*., 2016).

Tannins, which are natural phenolic compounds, are generally classified as condensed or hydrolyzable (Laurichesse; Avérous, 2014). Their mechanism of action might involve their affinity to bind to parasite proteins, causing changes in the cuticles, as well as degeneration of the muscle and intestinal cells. The metabolic changes resulting from the structural damage to the cuticle can reduce the motility of nematodes. The destruction of the oviduct can also impede oviposition by gastrointestinal nematodes (Borges; Borges, 2016). Alonso-Díaz et al. (2012) confirmed the anthelmintic effect of tannins by studying the extract of *Onobrychis viciifolia*, which they found to inhibit 76.93% of gastrointestinal parasite oviposition.

Saponins act on the integrity of the helminth membrane, modifying its permeability and promoting the entry of molecules due to differences in osmolarity, resulting in the formation of cytoplasmic vacuoles and disintegration of the integument (Poolperm; Jiraungkoorskul, 2017). Those changes can prevent the development of the parasite (Gomes *et al*., 2016).

In the present study, we found that a 10% concentration of the saline extract of *M. spicata* L. leaves (80 mg/mL) had an LC_{50} of 59.04 mg/mL, whereas a 5% concentration (40 mg/mL) had a lower LC_{50}, making it the ideal concentration for use.

Our findings indicate that *M. spicata* L. is a target species for the development of a new herbal formulation with beneficial characteristics for the treatment of gastrointestinal helminthiasis in sheep. At the ideal concentration, the *M. spicata* L. extract proved to be efficient and non-toxic. However, despite the fact that *in vitro* tests of acute toxicity are widely accepted and well regarded, *in vitro* and *in vivo* assessments of chronic toxicity are needed in order to determine the reliability of the results.

CONCLUSION

A saline extract of the leaves of *M. spicata* L. appears to inhibit the oviposition of gastrointestinal helminth eggs in sheep. That makes the plant a possible source of an alternative treatment for the control of gastrointestinal helminths. Its use could reduce parasite drug resistance as well as avoiding the environmental impacts of the use of anthelmintic drugs.

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