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In vitro ovicidal and larvicidal activity of condensed tannins on gastrointestinal nematode infestations in sheep (*Ovis aries*)

Atividade ovicida e larvicida *in vitro* de taninos condensados em nematódeos gastrintestinais de ovinos (*Ovis aries*)

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Abstract

Infestations of gastrointestinal parasitic nematodes in sheep result in significant costs to farmers. These infestations are controlled using synthetic anthelmintic treatments, which can result in the development of anthelmintic resistance in nematodes. The use of plants rich in condensed tannins (CTs) is a promising alternative for controlling infestations of harmful parasites in sheep, and could allow reduction of the chemical products used. This study investigated the *in vitro* effect of CTs from *Acacia mearnsii* extract (AE) on egg hatching and motility of third-stage larvae. Egg-hatching rate was measured after incubation with extracts for 48 h at 27 °C. The egg hatch test was performed with dilutions of 0.09, 0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 mg mL^{-1}. Distilled water was used as the negative control. The corresponding egg hatching inhibition percentages were 22.3, 32.3, 39.2, 49.1, 56.7, 59.0, 62.3, 77.3, 92.7, 98.3, and 100%. The concentration required to inhibit egg hatching in 50% of eggs (LC_{50}) was 2.85 mg mL^{-1}. The inhibition achieved with the negative control was 7.06%. A larval migration inhibition test was carried out after incubation with the extracts for 48 h at 27 °C, with AE and distilled water used in dilutions of 3.12, 6.25, 12.5, 25, 50, and 100 mg mL^{-1}. The corresponding percentages of migration inhibition were 16.5, 37.0, 56.3, 79.4, 91.8, and 97.1%. The concentration required to inhibit migration of 50% of larvae (LC_{50}) was 12.45 mg mL^{-1}. The inhibition achieved with the negative control was 8.53%. The *in vitro* ovicidal and larvicidal activity of CTs from AE indicate the anthelmintic effect of AE, suggesting the potential of CT extracts to be used as alternatives for controlling gastrointestinal nematode infestations in small ruminants.

Key words: *In vitro* test, helminthes, tannins, phytotherapy, small ruminants

Resumo

O parasitismo gastrintestinal em ovinos acarreta inúmeros prejuízos aos produtores rurais. O controle é realizado basicamente através da administração de anti-helmínticos sintéticos o que estimula a seleção de parasitas resistentes. O uso de plantas ricas em taninos condensados (TC) apresenta-se como uma alternativa promissora no controle de helmintos em ovinos, reduzindo o uso de produtos químicos. Esse estudo avaliou o efeito *in vitro* dos TC provenientes da acácia (*Acacia mearnsii)* sobre a eclosão de ovos e a motilidade de larvas de terceiro estágio de nematódeos gastrintestinais de ovinos. A taxa

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de eclosão foi avaliada após incubação com o extrato por um período de 48 h a 27 ºC. A eclodibilidade foi avaliada nas concentrações de 0,09; 0,19; 0,39; 0,78; 1,56; 3,12; 6,25; 12,5; 25; 50 e 100 mg mL^{-1}. No controle negativo utilizou-se água destilada. As inibições das eclosões foram 22,3; 32,3; 39,3; 49,1; 56,7; 59; 62,3; 77,3; 92,7; 98,3 e 100%, respectivamente. Para a inibição de 50% da eclosão de ovos (CL_{50}) o valor foi de 2,85 mg mL^{-1}. No controle negativo a inibição foi de 7,1%. A inibição da migração larval foi avaliada após incubação com o extrato por um período de 3 h a 27 ºC, nas concentrações de 3,12; 6,25; 12,5; 25; 50 e 100 mg mL^{-1}. As porcentagens de inibição foram: 16,5; 37; 56,3; 79,4; 91,8 e 97,1% respectivamente. Para a inibição de 50% da migração larval (CL_{50}), o valor da concentração foi de 12,45 mg mL^{-1}. No controle negativo a inibição foi de 8,5%. A ação ovicida e larvicida *in vitro* do tanino condensado do extrato de *Acacia mearnsii* encontrada no presente trabalho indica uma ação anti-helmíntica, apresentando potencial para sua aplicação no controle alternativo de nematódeos gastrintestinais de pequenos ruminantes.
Palavras-chave: Teste i*n vitro*, helmintos gastrintestinais, taninos, fitoterapia, pequenos ruminantes

Introduction

Gastrointestinal nematodes are said to be the main limiting factor in sheep production, causing the greatest economic losses for producers worldwide (ADEMOLA; ELLOF, 2010). Currently, helminths are controlled using anthelmintic drugs. However, the global increase in anthelminthic resistance, emergence of resistant isolates (FORTES; MOLENTO, 2013), worrying presence of residues in food, and environmental pollution (ATHANASIADOU; HOUDIJK; KYRIAZAKIS, 2008) have all been an incentive for further research into alternative methods to control helminths (CEZAR; CATTO; BIANCHIN, 2008).

New alternative methods of controlling gastrointestinal parasites have been researched, some of which involve tanniniferous plants, which may be a promising option to reduce the severity of nematode infections in small ruminants and the use of anthelminthic drugs. The incubation of eggs and larvae with plant extracts rich in tannins has produced positive results with regard to egg eclosion, and the mobility and exsheathment of larvae (BARRAU et al., 2005; BRUNET et al., 2007; MOLAN; FARAJ, 2010; OLIVEIRA et al., 2011a). A decrease in the parasitic load and number of eggs eliminated has also been described in sheep and goats when using tanniniferous plants (LANGE et al., 2006; HECKENDORN et al., 2007; MINHO et al., 2010; PARRA et al., 2011).

Tannins are secondary plant metabolites, classified into hydrolysable tannins (HT) and condensed tannins (CTs). CTs are most commonly found in forest legumes, trees, and bushes (MIN; HART, 2003). Various species of plants and extracts have been studied to determine if they possess anthelminthic properties, particularly with regard to the action of CTs (OLIVEIRA et al., 2011a; SHALDERS et al., 2013), in an attempt to prove the direct and indirect effects of CTs on gastrointestinal nematodes in sheep. The *in vitro* anthelminthic activity of CTs can be assessed at different stages of nematode development, with the effects recorded being reduction in eclosion, larval exsheathment, and motility. The main advantages of *in vitro* tests are their low cost and the fact that data can be rapidly collected (GITHIORI; ATHANASIADOU; THAMSBORG, 2006). The exact mechanism of action of tannins is still unknown and may differ according to parasite, stage of development, and biochemical characteristics of the plant (HOSTE et al., 2006). This study aimed to assess the *in vitro* effects of CTs from the acacia (*Acacia mearnsii)* plant on the eggs and third-stage larvae (L_3) of gastrointestinal sheep nematodes.

Materials and Methods

Source of CTs

The source of the CTs was a commercial extract of *A. mearnsii* bark (universal use acacia extract,

Seta Sun®. Seta S.A.), containing 72.5% tannins. This extract contained 15% CTs as per the HCl-butanol method described by Porter, Hrstich and Chan (1986).

Animals

Two naturally infected sheep, from the region of Londrina, PR, Brazil, were kept in a stable in the Isolation Unit of the Veterinary Hospital at the Londrina State University and fed a tannin-free diet. Isolation and identification of the fecal matter revealed the presence of four genera of nematodes frequently found in naturally infected animals in the Londrina region. The genera found were *Haemonchus* (84%), *Trichostrongylus* (13%), *Oesophagostomum* (2%), and *Cooperia* (1%). These animals were used as the source of helminth larvae.

Isolation of eggs and third-stage larvae

The feces were collected directly from the rectum of the animals, and the eggs were isolated and a fecal culture was prepared to obtain third-stage larvae (L_3). The eggs were recovered according to the methodology described by Coles et al. (1992) and adapted by Bizimenyera et al. (2006). The feces were homogenized in distilled water and filtered using a series of sieves, with the eggs being removed using a 25 mm sieve, washed with distilled water, and centrifuged at 1,100 x *g* for 5 min in 50-mL tubes containing water. The supernatant was discarded and saturated saline solution was added to resuspend the sediment. After another centrifugation using the same conditions as described above, the supernatant was washed in a 25 mm sieve. The collected eggs were stored in a sedimentation beaker for 2 h. After syphoning, the eggs were counted in five aliquots of 100 mL.

Egg eclodibility assay (EEA)

The test was based on the method described by Von Samson-Himmelstjerna, Coles, and Jackson (2009). Dilutions of the *A. mearnsii* extract (EA) were prepared using distilled water. The final concentrations of the extract were: 0.09, 0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 mg mL^{-1}. Subsequently, a 100-µL suspension of the eggs, containing approximately 150 eggs, was incubated in 24-well cell culture plates for 48 hours at 27 ºC with 400 µL of each dilution. The test was carried out using two controls: the negative control comprised distilled water, and the positive comprised albendazole sulfoxide (50 µg mL^{-1}, Ricofarm 10®, Biofarm). The test was carried out with six repetitions for each concentration tested. After the incubation period, the ecloded eggs and larvae were quantified to calculate the percentage inhibition of larval eclodibility. Reading was performed using an inverted microscope Motic AE 31 with a 10x resolution.

Larval migration inhibition assay (LMI)

The LMI assay was carried out according to the methodology described by Rabel, McGregor, and Douch (1994). Larvae were obtained from the fecal culture of naturally infected sheep. Approximately 150 L_3 were incubated at 27 ºC in Eppendorf tubes containing 1 mL of the plant extract diluted in distilled water to concentrations of: 3.12, 6.25, 12.5, 25, 50, and 100 mg mL^{-1}. The test was carried out using two controls: the negative comprising distilled water and the positive comprising levamisole phosphate (40 µg mL^{-1}, Ripercol®, Fort Dodge). After 3 h of incubation, the tubes were centrifuged at 1,100 x *g* for 2 min, and the supernatant was discarded leaving 200 µL. A 1,800-µL aliquot of each AE dilution was added to each of the 24 wells on the cell culture plate, with this test being carried out in sextuplicate. Next, a 22-µm mesh filter was added to the wells and 200 µL of the larval suspension was added to the top of the filter in the respective concentrations tested. The plate was covered and placed in a B.O.D. incubator chamber for 2 h at 27 ºC. The number of larvae that remained or migrated was counted using an inverted microscope Motic AE 31 at a 10x resolution.

Statistical analysis

In the EEA and LMI assays, the effectiveness of both treatments was determined based on the percentage of eclosion or migration, according to the following equation: Inhibition (%) = $100(N_{test}/N_{total})$, where N_{test} corresponds to the number of non-ecloded eggs, EEA, or number of larvae that did not migrate in the LMI, and N_{total} corresponds to the number of eggs + L_1 in the EEA test or the number of migrated larvae + remaining larvae in the LMI test.

The 50% lethal concentration (LC_{50}), which is the concentration that can effectively inhibit 50% of eclosion or migration, was determined using the dose-response curve, with a confidence interval of 95%, using the program GraphPad Prism for Windows, version 5.0.

Ethics Committee

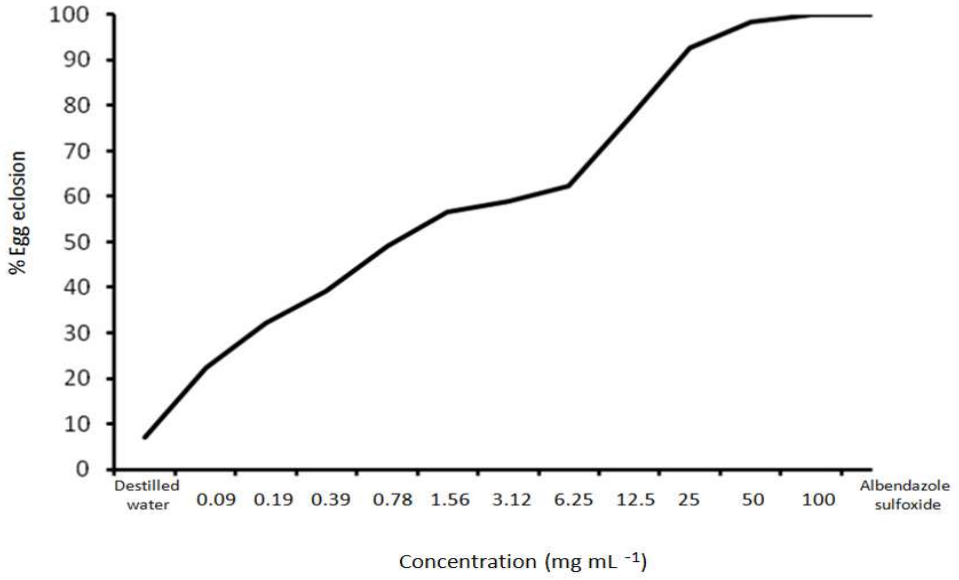
This article was approved by the Ethics Committee of Animal Research at Londrina State University, recorded in the CEEA with the no. 87/09, and was carried out under the ethical principles stated by the Brazilian College of Animal Experimentation (COBEA).

Results

Inhibition of egg eclosion

The LC_{50} value for the inhibition of egg eclosion by AE was 2.85 mg mL^{-1} (95% CI: 2.45–3.31 mg mL^{-1}). The positive control was 100% effective in inhibiting eclosion, and with the negative control, the mean inhibition was 7.1%. The percentage eclosion inhibition of gastrointestinal nematode eggs using concentrations between 0.09 and 100 mg mL^{-1} of AE varied between 22.3 and 100% inhibition, respectively (Figure 1). With concentrations below 0.09 mg mL^{-1}, the degree of inhibition was similar to that with the negative control. There was 100% inhibition with the positive control, albendazole sulfoxide (50 µg mL^{-1}, Ricofarm 10®, Biofarm).

Figure 1. Effect of the *Acacia mearnsii* extract on the egg hatchability of gastrointestinal nematode of sheep

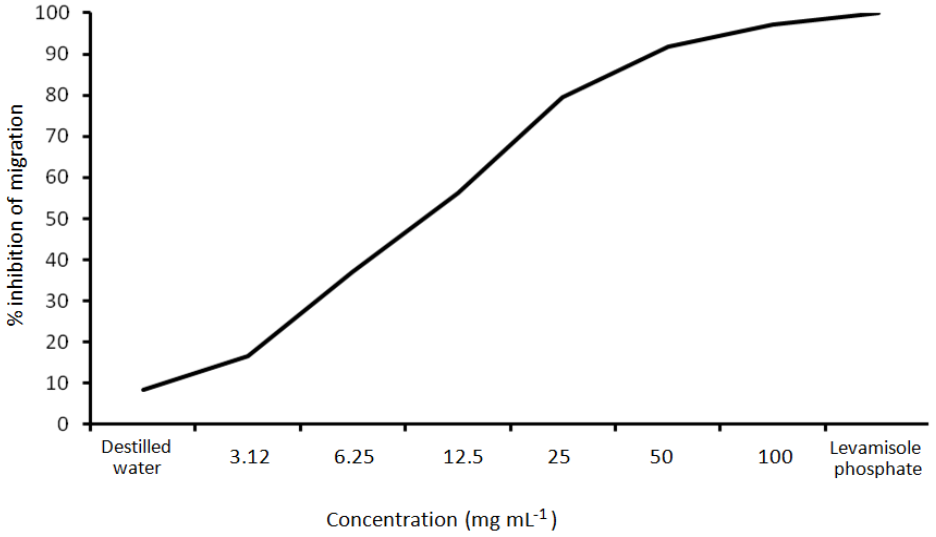


Source: Elaboration of the authors.

Inhibition of larval migration

The LC_{50} value of AE for the inhibition of larval migration was 12.45 mg mL^{-1} (95% CI: 11.49–13.47 mg mL^{-1}). The positive control was 100% effective in inhibiting larval migration and the negative control had a mean inhibition of 8.5%. The percentage of larval migration inhibition using concentrations of AE between 3.12 and 100 mg mL^{-1} varied between 16.5 and 97.1% inhibition, respectively (Figure 2). There was 100% inhibition with the positive control, levamisole phosphate (40 µg mL^{-1}, Ripercol®, Fort Dodge).

Figure2. Effect of the *Acacia mearnsii* extract on the inhibition of larval migration (infective larvae) of gastrointestinal nematodes of sheep.



Source: Elaboration of the authors.

Discussion

In the *in vitro* tests carried out in this study, the inhibitory action of AE was demonstrated by the inhibition of egg eclosion and of the larval migration of gastrointestinal nematodes in naturally infected sheep. Two hypotheses, based on the direct and indirect effects of tannins, have been used to explain this anthelminthic action. However, the exact mechanism of action of these metabolites is still not fully understood and may differ according to the parasite, stage of development, and possibly, on the biochemical characteristics of the plants studied (HOSTE et al., 2006). Both *in vitro* and *in vivo* tests were carried out to assess the anthelminthic action of AE. The efficiency of AE was tested *in vivo* by Cenci et al. (2007) and Max (2010) using naturally infected sheep, and showed a drop in the eggs per gram of faeces (EPG) count and a decrease in parasitic load. Minho et al. (2008) assessed the *in vitro* inhibitory effect of AE on the feeding of the stage one larvae (L_1) of *Haemonchus contortus, Trichostrongylus colubriformis,* and *Teladorsagia circumcincta*. According to the authors, AE inhibited L_1 feeding. In another study, Minho et al. (2010) observed a decrease in the elimination of eggs and parasitic load of *T. colubriformis* in experimentally infected sheep. This study showed the inhibitory effect of CTs present in the AE, consistent with the results found by other authors, when they assessed the anthelminthic action of the AE as a source of CTs.

Several authors have assessed the anthelminthic effect of extracts of different tannin-containing plants using *in vitro* techniques. The effects of the extracts of six tannin-rich tropical plants were tested by Oliveira et al. (2011b), who showed the inhibition of larval development in *H. contortus* indicating anthelminthic activity in *in vitro* tests. Son-de Fernex et al. (2012) assessed the anthelminthic activity of five plants containing CTs, showing that there was an inhibitory effect on the motility and exsheathment of infected *H. contortus* larvae.

The concentration required to inhibit 100% eclosion and the LC_{50} (100 mg mL^{-1} and 2.85 mg mL^{-1}, respectively) in this study were higher than the values stated in previous studies, where Molan, Waghorn, and McNabb (2002) reported total inhibition of egg eclosion in *T. colubriformis* at concentrations between 200 and 500 μg mL^{-1}, depending on the plant analyzed. In another study, Molan et al. (2003) found 100% inhibition of egg eclosion in *T. colubriformis* at a concentration of 1000 μg mL^{-1}. Bizimenyera et al. (2006) tested extracts of different parts of *Peltophorum africanum*, and found that a concentration of 25 μg mL^{-1} of all extracts caused 100% inhibition of egg eclosion in *T. colubriformis*, with LC_{50} values of 0.619, 0.383, and 0.280 mg mL^{-1} for leaf, bark, and root extracts, respectively. Maciel et al. (2006) also showed 100% inhibition of egg eclosion in *H. contortus* with a 25-μg mL^{-1} concentration of the ethanolic extract of leaves, whereas the hexanic extract, at a concentration of 50 μg mL^{-1}, inhibited eclosion by only 16.92%. The LC_{50} values were 35.8 and 2.2 μg mL^{-1} for the hexanic and ethanolic leaf extracts, respectively. In the same study, extracts of seeds were tested; there was total inhibition with the ethanolic extract at a concentration of 3.12 μg mL^{-1}, whereas the chloroformic extract, at a concentration of 50 μg mL^{-1}, showed an inhibition of 92.39%. The LC_{50} values were 0.36 and 7.26 μg mL^{-1} for the ethanolic and chloroformic extracts, respectively. Oliveira et al. (2011a) tested *Myracrodruon urundeuva* extracts on *H. contortus* larvae, and found that there was 100% inhibition at a concentration of 2.5 μg mL^{-1} of the leaf extract and 83.56% inhibition at 5 μg mL^{-1} of the stem extract.

The values of 97.1% inhibition of larval migration with the 100 mg mL^{-1} concentration and an LC_{50} of 12.85 mg mL^{-1} shown in this study are higher than those found in the literature. Alonso-Díaz et al. (2008) assessed the action of four tannin-rich plants in stage three *H. contortus* larvae and found different degrees of inhibition, varying between 49.1 and 63.8%, when they used an extract at a concentration of 1,200 μg mL^{-1}. In another study, Alonso-Díaz et al. (2011) found migration inhibitions of 48.5 and 20.9% for *Havardia albicans* and *Acacia gaumeri*, respectively, at a concentration of 1,200 μg mL^{-1}. Furthermore, with a concentration of 1,200 μg mL^{-1}, Manolaraki et al. (2010) tested seven plants and found inhibition of migration to vary between 38.9 and 79.1%.

The concentrations that inhibited 100% of egg eclosion varied between 3.12 and 1000 μg mL^{-1} in the literature. With regard to the inhibition of larval migration, there are studies that show different degrees of inhibition at a concentration of 1,200 μg mL^{-1}, varying between 20.9 and 79.1%. The values stated for a 50% inhibition of egg eclosion (CL_{50}) in studies carried out by different authors vary between 0.36 μg mL^{-1} and 7.26 mg mL^{-1}. The values found in this study are higher than those found in previous studies on CT sources. The differences in the values found in this study may be due to the source of CT, extraction methods, and the strains of parasites assessed.

In the long term, the main contribution that this compound could offer would be to decrease the number of anthelminthic applications required and reduce the contamination of pastures, increasing the time between chemical treatments. This would decrease selection pressure on the parasite population, thereby minimizing the risk or delaying the development of isolates resistant to the drugs used.

Conclusions

The results obtained in this study reveal the *in vitro* anthelminthic action of the AE on egg eclosion and the migration of stage-three larvae of gastrointestinal nematodes of naturally infected sheep.

Further studies are necessary to define some aspects associated with the extraction methods and therapeutic dose so that that there is more consistency in production, in order to make the use of these compounds viable in the alternative control of nematodes, either together with chemical products, on properties with anthelminthic resistance, or for use on properties that adopt an organic production system.

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