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Short Communication

Anthelmintic effect of *Cassia fistula* and *Combretum leprosum* protein fractions against goat gastrointestinal nematodes

Efeito antihelmíntico das frações proteicas de *Cassia fistula* e *Combretum leprosum* contra nematodeos gastrintestinais de caprinos

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Abstract

In this study, we evaluated the ovicidal and larvicidal activity of protein preparations obtained from *Cassia fistula* L. and *Combretum leprosum* Mart. leaves on the gastrointestinal parasites of goats. Protein preparations were obtained after the extraction of *C. fistula* L. and *C. leprosum* Mart. leaves, followed by protein fractionation (with ammonium sulfate saturation percentages of 30%, 30%-60%, and 60%-90%) and dialysis, which resulted in protein fractions (called F1, F2, and F3, respectively). The fractions were evaluated by egg hatching (the eggs were recovered in stool samples from naturally infected goats) and larval development tests. The results reveled that the inhibition of hatching of eggs caused by the protein fractions of *C. fistula* (38%) were similar to that of the control drug, thiabendazole. In addition, the fractions of *C. fistula* caused significant inhibition (61-69%) of larval development also. However, *C. leprosum* did not reveal significant inhibition of egg hatching and larval development. We conclude that *C. fistula* L. showed better ovicidal and larvicidal activity against endoparasites.

Keywords: Phytotherapy, *Cassia fistula* L., *Combretum leprosum* Mart., larvicidal activity, ovicidal activity.

Resumo

Neste estudo, foram avaliadas as atividades ovicida e larvicida de preparações proteicas de *Cassia fistula* L. e *Combretum leprosum* Mart. em parasitas gastrointestinais de caprinos. As preparações proteicas foram obtidas por extração das folhas de *C. fistula* L. e *C. leprosum* Mart. seguido pelo fracionamento proteico (com porcentagens de saturação de sulfato de amônio de 30%, 30-60%, 60-90%) e diálise, resultando nas frações proteicas (intituladas F1, F2 e F3, respectivamente). As frações foram avaliadas nos testes de eclosão de ovos (os ovos foram recuperados em amostras de fezes de cabras naturalmente infectadas) e de desenvolvimento larvar. Os resultados revelaram que a inibição da eclosão de ovos causada pelas frações proteicas de *C. fistula* (38%) foi semelhante à do fármaco controle, o tiabendazol. Além disso, as frações de *C. fistula* também causaram inibição significativa (61-69%) do desenvolvimento larvar. No entanto, *C. leprosum* não revelou inibição significativa na eclosão dos ovos e no desenvolvimento larvar. Concluiu-se que *C. fistula* L. mostrou uma melhor atividade ovicida e larvicida contra endoparasitas.

Palavras-chave: Fitoterapia, *Cassia fistula* L., *Combretum leprosum* Mart., atividade larvicida, atividade ovicida.

Introduction

Goat breeding is an extremely important activity that constitutes a vital element of the economy in Latin America; however, this industry has been limited by gastrointestinal parasitism (MARIE-MAGDELEINE et al., 2014), where the emphasis is on the gastrointestinal nematode *Haemonchus contortus* due to its high prevalence and pathogenicity. Control methods based exclusively on the use of anthelmintics have led to the emergence of parasite resistance (MOLENTO et al., 2011). In this context, new alternatives of treatment are being researched to control those nematodes, such as the use of phytotherapeutic agents (which are found in various Latin American regions).

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In the semiarid region of Northeast Brazil, the Caatinga biome is seen as a region of great plant diversity with yet unexplored biological potential (ALBUQUERQUE et al., 2007). In the present study, *Cassia fistula* L. and *Combretum leprosum* Mart. are evaluated. The first belongs to a genus with potential anthelmintic activity, as demonstrated by the species *Cassia occidentalis* and *C. alata* (ADEMOLA & ELOFF, 2011), and its activity is influenced by the presence of compounds like triterpenes and flavonoid derivatives (KASHIWADA et al., 1990). The methanolic extract of *C. fistula* leaves has also demonstrated ovicidal and larvicidal activity against *Culex quinquefasciatus* and *Anopheles stephensi* (GOVINDARAJAN et al., 2008). In addition, the ethanolic extract of leaves of this species completely inhibited eggs hatching in *Rhipicephalus (Boophilus) annulatus* when applied in high concentrations of 80 mg/mL (SUNIL et al., 2013).

Combretum has some secondary metabolites, such as saponins and alkaloids, that are involved in the anthelmintic effects (GITHIORI et al., 2006); in particular, *C. leprosum* Mart. was highlighted for its efficient leishmanicidal action in blood (LIMA et al., 2011).

In this context, this experiment aimed to evaluate the *in vitro* ovicidal and larvicidal activity of protein preparations obtained from *C. fistula* L. and *C. leprosum* Mart. leaves on the gastrointestinal nematodes of naturally infected goats.

Materials and Methods

Extraction and obtaining protein preparations

Specimens of *C. fistula* L. and *C. leprosum* Mart. were collected in the semiarid region of the Caatinga biome and submitted to taxonomic identification; they were cataloged under registration numbers 14.514 and 10.195, respectively, in the Dárdano de Andrade Lima Herbarium, Department of Plant Sciences at the Semiarid Federal Rural University.

Following identification, the collected leaves were washed, dried, and ground. The obtained material was extracted in 0.15 M of NaCl solution 10% (w/v), followed by filtration and centrifugation to obtain the crude extract. The extracts were subjected to protein precipitation using increasing ammonium sulfate saturations: 30%, 30%-60%, and 60%-90%. After each saturation, the samples were incubated under constant agitation, followed by centrifugation to obtain protein fractions (precipitated protein) referred to as F1, F2, and F3, respectively.

The samples were subjected to dialysis, protein quantification (LOWRY et al., 1951), and hemagglutinating activity assays in microtiter plates (CORREIA & COELHO, 1995) to detect lectin activity. The specific hemagglutinating activity was determined by the hemagglutinating activity/protein concentration (mg/mL) ratio. Then, the samples were subjected to a dilution (in 0.15 M NaCl) that was minimal enough to standardize the protein concentration of the *C. fistula* L. (4 mg/mL) and *C. leprosum* Mart. (20 mg/mL) fractions.

The selection of rural properties and the collection of fecal samples

This study was conducted in the countryside of the city of Mossoró, Rio Grande do Norte, Northeastern Brazil, at south latitude coordinates of 5º11'15", west longitudinal coordinates of 37º20'39", and at an altitude of 16 m in a predominantly semiarid climate; here, the average temperature is 27.4°C, the rainy season is concentrated between summer seasons with irregular rainfalls. Feces were collected directly from the rectum of naturally infected goats; this was followed by the counting of eggs per gram of feces (EPG) (GORDON & WHITLOCK, 1939; CHAGAS et al., 2011). Fecal samples from the animals of five different farms were collected in a pool for each farm. The selected animals had reached the end of 12 weeks of a residual effect period following their last deworming (COLES et al., 1992) and they exhibited a fecal pool with ≥100 EPG of feces (CHAGAS et al., 2011), regardless of breed, gender, or animal class. All experiments were performed in accordance with the recommended procedures of the National Council for the Control of Animal Experimentation. The study was approved by the Ethics Commission on Animal Experimentation of the Semiarid Federal Rural University (number 23091.009318/2016-40).

Egg-hatch test

The recovery of nematode eggs was performed according to Hubert & Kerboeuf (1992) methodology. The egg hatch test (EHT) was performed according to the methodology from Coles et al. (2006); a 100 µL solution containing approximately 100 eggs and 400 µL of protein fractions or control solutions was added in each well of 24-well plates. For each experiment, one plate contained the controls: negative (eggs in distilled water) and positive (eggs and Thiabendazole 32 µL/mL), and another plate contained the assays with the protein fractions: T1 (eggs and F1), T2 (eggs and F2), and T3 (eggs and F3). The EHT assays were performed in quintuplicate with three replicates for each treatment, as well as with the control group. Two independent studies were performed. Each one evaluated the activity of the fractions of one of the plants, *Cassia fistula* or *Combretum leprosum.* The plates were incubated at 27°C for 48 hours, after which point Lugol's solution was added; then, the eggs and L1 (larval development stage 1) were counted and the egg-hatching inhibition percentage was determined in accordance with the following formula:

$$\text{Egg hatching inhibition percentage} = \frac{\text{Unhatched eggs}}{\text{Unhatched eggs + L1}} \times 100 \quad (1)$$

Larval development test

For the larval development test (LDT) (HUBERT & KERBOEUF, 1992; BIZIMENYERA et al., 2006), 24-well plates were used containing approximately 100 eggs/well in 100 µL; 80 µL of suspension containing lyophilized *Escherichia coli* (ATCC 9637; Sigma-Aldrich Co., St Louis, MO, USA), 10 µL of amphotericin B (Sigma-Aldrich Co., St Louis, MO, USA), and 20 µL of nutrient

media were added. Then, 100 μL of distilled water was added and the plates were incubated in an oven incubator for 48 hours. After incubation, 220 μL of F1, F2, or F3 was added. In separate plates, the assay with the positive control (larvae suspension and ivermectin) and the negative control (larvae suspension and distilled water) were carried out. The tests were performed in five replicates. Two independent studies were performed. Each one evaluated the activity of the fractions of one of the plants, *Cassia fistula* or *Combretum leprosum*. The plates were incubated for 7 days, after which Lugol's solution was added to the wells; then, L1 and L3 (the third larval stage of development) were counted to calculate the percentage of larval development inhibition, in accordance with the following formula:

$$\text{Larval development inhibition} = \frac{L1}{L1+L3} \times 100 \qquad (2)$$

Statistical analysis

The tests were conducted in a completely randomized design; for each test, there were 5 samples. Each experimental unit consisted of 100 eggs or larvae. The data were tabulated in an electronic spreadsheet and submitted to the Kruskal-Wallis test and Mann-Whitney pairwise comparations with Bonferroni correction, and significance level equal to 5% ($\alpha = 0.05$). The data were expressed as the percentage inhibition (± standard deviation), and the inhibition was represented as percentage of inhibition per milligram of protein.

Results

Protein preparation characterization

From the extraction processes, which were followed by salt fractionation, the protein fractions F1, F2, and F3 obtained from *C. fistula* L. and *C. leprosum* Mart. after dialysis exhibited protein contents of 5.06 mg/mL, 4.62 mg/mL, and 5.53 mg/mL, and 66.47 mg/mL, 54.55 mg/mL, and 42.04 mg/mL, respectively. These protein fractions from *C. fistula* L. and *C. leprosum* Mart. presented hemagglutinating activity (HA) titers of 64, 256, and 256 and 32,768, 8,192, and 8,192, respectively, revealing the presence of lectins. The specific HA (HA/protein concentration in mg/mL) in these samples was 12.64, 55.41, and 46.29 and 492.95, 150.19, and 194.88, respectively.

Egg-hatching and larval development inhibition

The results of the EHT and LDT using the *Cassia fistula* L. and *Combretum leprosum* Mart. protein fractions (F1, F2, and F3) (and compared to controls) are shown in Table 1 and 2. Through the EHT assays, all *C. fistula* L. protein fractions yielded the same percentage of egg-hatching inhibition (38%), which was not statistically significantly different compared to the positive control. The EHT investigating the *C. leprosum* Mart. protein fractions F1, F2, and F3, revealed low egg-hatching inhibition percentages (8%, 10%, and 9%, respectively), and these were also not statistically significantly different when compared to the negative control ($P \geq 0.05$).

Table 1. Inhibitory effect of *Cassia fistula* L. protein fractions (F1, F2, and F3) on the egg-hatching test (EHT) and on the larval development test (LDT) of gastrointestinal nematodes in goat.

Treatments		EHT (%)* ± SD (%)**	LDT (%)* ± SD (%)**
C. fistula	F1	38 ± 15 [a]	61 ± 14 [a]
	F2	38 ± 17 [a]	62 ± 26 [a]
	F3	38 ± 17 [a]	69 ± 23 [a]
Controls	H_2O	8 ± 7 [b]	18 ± 7 [b]
	Thiabendazol	30 ± 15 [a]	-
	Ivermectin	-	81 ± 11 [a]

Notes: *Inhibition (%); **The values represent the mean ± standard deviation (SD). F1, F2, and F3: protein fractions obtained by the saturation of plant extracts with ammonium sulfate (at 30%, 30%-60%, and 60%-90%, respectively), followed by centrifugation and dialysis. Different letters indicate statistically significant differences ($P<0.05$) when compared with the control.

Table 2. Inhibitory effect of *Combretum leprosum* Mart. protein fractions (F1, F2, and F3) on the egg-hatching test (EHT) and on the larval development test (LDT) of gastrointestinal nematodes in goat.

Treatments		EHT (%)* ± SD (%)**	LDT (%)* ± SD (%)**
C. leprosum	F1	8 ± 23 [a]	18 ± 16 [a]
	F2	10 ± 21 [a]	16 ± 9 [a]
	F3	9 ± 24 [a]	18 ± 10 [a]
Controls	H_2O	4 ± 3 [a]	13 ± 3 [a]
	Thiabendazol	49 ± 30 [b]	-
	Ivermectin	-	57 ± 7 [b]

Notes: *Inhibition (%); **The values represent the mean ± standard deviation (SD). F1, F2, and F3: protein fractions obtained by the saturation of plant extracts with ammonium sulfate (at 30%, 30%-60%, and 60%-90%, respectively), followed by centrifugation and dialysis. Different letters indicate statistically significant differences ($P<0.05$) when compared with the control.

The LDT results showed that the *C. fistula* L. protein fractions F1, F2, and F3 inhibited larval development (61%, 62%, and 69%, respectively). There were no significant differences in the effect of the three preparations and that of the positive control, which characterized F1, F2, and F3 as efficient. The *C. fistula* L. protein fractions showed more intense larvicidal action than ovicidal action. Cuticular damage was observed, which was characterized by degeneration and retraction of the larval cuticle, which were suggested to be related to the larvicidal activity of the *C. fistula* L. preparations (Figure 1). The LDT performed with the *C. leprosum* Mart. preparations showed a low larval development inhibition percentage (18% for F1 and F3, and 16% for F2), which were not statistically different ($P \geq 0.05$) when compared to the negative control.

Discussion

The results reinforce the potential ovicidal and larvicidal action that was already reported in species of the *Cassia* genus, as verified by Ademola & Eloff (2011), in which *C. alata* extracts showed a

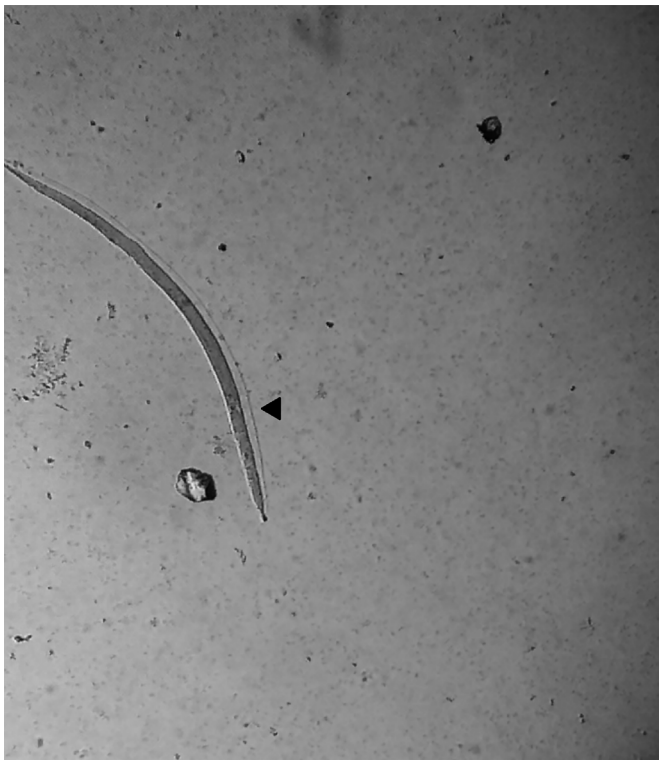

Figure 1. Larvicidal effect of protein extract preparations. The arrow indicates damage to the tegument accompanied by retraction of the nematode cuticle.

lethal dose (LD_{50}) of 0.191 mg/mL (in acetone) and 0.040 mg/mL (methanol:water) against *H. contortus* infective larvae. The ability of the preparations obtained from the *C. fistula* L. species to inhibit egg hatching may be associated with the presence of condensed tannins (BRÍGIDA et al., 2015). Tannins have promoted reductions in the emergence and development of nematodes in ruminants (IQBAL et al., 2007). However, it has been suggested that tannins act by a direct mechanism of action, which may affect the parasite's biological processes (HOSTE et al., 2006) or it may be related to egg-hatching inhibition. It may even have an indirect mechanism of action, where it may improve the host's protein usage and, consequently, its immune response (BUTTER et al., 2000).

The larvicide action promoted by *C. fistula* L. protein preparations may be related to the presence of lectins, which are found in the preparations through the HA test; the larvicidal action is intrinsic to this class of proteins. Three possible mechanisms are suggested for these protein preparations: inhibition of the larvae's feeding behavior (which involves lectin's ability to recognize and bind to carbohydrates from the intestinal cells of larvae); interruption of larval migration (by the binding of lectins to the parasite's chemical sensors); and induction of visible cuticle damage, as observed in the extracts obtained from plants rich in cysteine proteases (RÍOS-DE ÁLVAREZ et al., 2012). However, additional studies are needed to elucidate the active component in the ovicidal and larvicidal action of *C. fistula* and its probable mechanism of action.

The results obtained with the *C. leprosum* Mart. protein preparations showed low ovicidal and larvicidal action. The ovicidal effect may be related to the presence of saponins, which may be present in the preparations; it may also be linked to the ability of this compound to destabilize membranes (such as those in eggs) and make them more permeable, thus preventing egg development (MANSFIELD et al., 1992). The action of *C. leprosum* Mart. preparations on parasite larvae may also be associated with lectins and their likely mechanisms of action, which have already been described.

The evaluation of protein preparations made from plant extracts as potential anthelmintics have allowed them to become incorporated in ethnobotanical concepts, which explore the knowledge accumulated by traditional communities, such as indigenous tribes (GARÍ, 2001), and it showed that the *C. fistula* L. preparations are an efficient alternative for parasite control. Since these preparations are obtained from renewable resources, are rapidly degradable, do not persist in the environment, and feature low production costs, their use is extremely advantageous when compared to synthetic drugs (ROEL, 2001).

It was concluded that protein preparations obtained from *C. fistula* L. and *C. leprosum* Mart. have ovicidal and larvicidal activity against goat endoparasites. Particular emphasis was placed on *C. fistula* L., which showed higher inhibition percentages. This study thus revealed that *C. fistula* L. is a target species for the development of a new formulation that has plant-based protective benefits against gastrointestinal nematodes in goat. However, further studies are needed to evaluate *in vitro* and *in vivo* cytotoxicity.

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