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Effects of *Mimosa tenuiflora* on larval establishment of *Haemonchus contortus* in sheep

L.M.B. Oliveira[a], I.T.F. Macedo[a], L.S. Vieira[b], A.L.F. Camurça-Vasconcelos[a], A.R. Tomé[a], R.A. Sampaio[a], H. Louvandini[c], C.M.L. Bevilaqua[a,*]

[a] *Programa de Pós-Graduação em Ciências Veterinárias, Universidade Estadual do Ceará, Brazil*
[b] *Empresa Brasileira de Pesquisa Agropecuária/Caprinos e Ovinos, Brazil*
[c] *Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Brazil*

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ABSTRACT

Anthelmintic resistance has limited the ability to control the gastrointestinal nematodes of small ruminants and has therefore awakened an interest in the study of tanniferous plants as a source of anthelmintics. This study was carried out to evaluate the effect of *Mimosa tenuiflora* intake, a tanniferous plant that is fed to small ruminants in northeastern Brazil, on the larval establishment of *Haemonchus contortus* in sheep. In this experiment, 18 nematode-free sheep were divided into three groups ($n = 6$) according to live weight. Group 1 was fed *M. tenuiflora* leaves; Group 2 was fed *M. tenuiflora* stems; Group 3 served as the control group and was fed *Cynodon dactylon*, a plant with low levels of tannins. The animals consumed the plants for 13 days (Day −7 to Day 5). On Day 0, the sheep were experimentally infected with 4500 third-stage *H. contortus* each. Five days after infection (Day 5), the sheep were slaughtered to count the worm burden and perform a histological analysis of the abomasum. The daily plant intake and the live weight gain of the animals were recorded. The groups that ingested *M. tenuiflora* leaves and stems consumed less dry matter than did those that ingested *C. dactylon* ($P < 0.05$). The consumption of *M. tenuiflora* leaves did not reduce the L3 establishment of *H. contortus* compared to the control ($P > 0.05$). The intake of *M. tenuiflora* stems tended toward decreasing larval establishment, but the reduction was not significant ($P > 0.05$). No significant differences were observed in the mucosal cellular response and live weight gain among the groups. These data demonstrated that, with the protocol used, *M. tenuiflora* has no effect on larval establishment of *H. contortus* in sheep.

1. Introduction

Gastrointestinal nematode parasitism remains one of the main threats to the farming of small ruminants in tropical countries. *Haemonchus contortus* is one of the most significant species because of its high prevalence and pathogenicity (Hounzangbe-Adote et al., 2005). Control of these parasites has depended on the administration of synthetic anthelmintics. However, the development of drug-resistant populations to most of the drug families currently used (Torres-Acosta and Hoste, 2008) and concerns regarding drug residues in food and in the environment have stimulated the search for alternative control strategies (Athanasiadou et al., 2008).

An alternative for the control of gastrointestinal nematodes that is currently being researched is the use of bioactive plants that are rich in secondary metabolites. For example, many such bioactive plants with activity against nematodes are rich in polyphenols, particularly tannins (Hoste et al., 2012). Tanniferous plants lead to

* Corresponding author at: Av. Dede Brasil, 1700, Campus do Itaperi, Fortaleza, Ceará, CEP 60740-093, Brazil. Tel.: +55 85 3101 9853; fax: +55 85 3101 9840.
E-mail addresses: claudiamlb@yahoo.com.br, claudia.bevilaqua@pq.cnpq.br (C.M.L. Bevilaqua).

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a reduction of nematode egg excretion or worm burden (Lange et al., 2006; Heckendorn et al., 2007; Minho et al., 2010; Martínez-Ortíz-de-Montellano et al., 2010) and a reduction of infective larvae establishment (Brunet et al., 2008; Joshi et al., 2011) in sheep and goats. However, most studies have focused on legume forage that is adapted to temperate and/or Mediterranean climatic conditions, such as plants belonging to the Fabaceae family: *Hedysarum coronarium*, *Onobrychis viciifolia*, *Lotus corniculatus* and *Lotus pedunculatus* (Hoste et al., 2006). However, in many small ruminant production systems, cultivated forage plants are not the main source of food because browser plants (bushes, trees or shrubs) contribute significantly to the nutrition of these animals during the prolonged dry periods (Manolaraki et al., 2010) that occur in tropical countries (Sanon et al., 2008).

Mimosa tenuiflora (Leguminosae) is a tree known in Brazil under the popular name of jurema-preta. The plant is distributed in areas of tropical deciduous forests in the Americas, from the southeastern regions of Mexico to northern Brazil and Venezuela, growing as secondary opportunistic vegetation (Camargo, 2000). This species has a high level of tannins and is used in the feed of small ruminants (Guimarães-Beelen et al., 2006; Rivera-Arce et al., 2007). A previous study demonstrated the *in vitro* activity of leaf and stem extracts of *M. tenuiflora* on *H. contortus* using a larval artificial exsheathment assay. The role of tannins in this assay was confirmed because the values were similar to those of the control after the addition of a specific metabolite inhibitor, polyvinyl polypyrrolidone (PVPP) (Oliveira et al., 2011). Nevertheless, the *in vivo* anthelmintic activity of *M. tenuiflora* was not evaluated. Therefore, the present study was conducted to evaluate the effect of leaves and stems of *M. tenuiflora* on the larval establishment of *H. contortus* in sheep.

2. Materials and methods

2.1. Plants

The aerial parts of *M. tenuiflora* were collected in December 2011 in Sobral, State of Ceará, located in northeastern Brazil. Prior to the beginning of the trial, samples of the plant were identified and authenticated by botanists at the Herbarium Prisco Bezerra of the Universidade Federal do Ceará, Brazil. A voucher specimen was deposited under the number 50288. The aerial parts of *Cynodon dactylon* were commercially acquired. This vegetal species contains low levels of tannins and was used as a control.

2.2. Nematode infective larvae

Third-stage larvae (L3) of *H. contortus* were obtained from donor sheep harboring a monospecific infection. The isolate of *H. contortus* was benzimidazole-resistant. The larvae were stored at 4 °C until use. A single batch of 2- to 3-month-old larvae was used in the experiment. Twenty-four hours before the experimental infection, the L3 were brought to room temperature. The viability and proportion of exsheathed larvae were found to be acceptable.

2.3. Animals

Eighteen female lambs of undefined breed, which were approximately 2 months old and had an average weight of 16 kg, were kept indoors in individual floored pens and fed with *C. dactylon* hay, mineral salt and water *ad libitum* and concentrated feed (30 g/kg live weight). The concentrated feed was obtained commercially. It contained maize grain, soybean meal, sugar cane molasses, wheat bran and a mineral mix. The protein level of the concentrate was 200 g/kg feed. The animals received three anthelmintics with different active ingredients on alternate days to eliminate any natural gastrointestinal nematode infection. The anthelmintics used were oxfendazole (Systamex®, Schering-Plough, Brazil, 5 mg/kg live weight), levamisole (Ripercol®, Fort Dodge, Brazil, 5 mg/kg live weight) and ivermectin (Ivomec®, Merial, Brazil, 0.2 mg/kg live weight). The withholding period of these anthelmintics is less than 15 days. Fifteen days after treatment, fecal samples from each animal were collected directly from the rectum to determine fecal egg counts by the McMaster technique (Ueno and Gonçalves, 1998). Coprocultures were performed with feces using the Roberts and O'Sullivan (1950) method to confirm their worm-free status.

2.4. Experimental design

The study was based on the method described by Brunet et al. (2008). Sheep were divided into three homogeneous groups ($n = 6$) based on their live weight. The experiment was carried out for 13 days (Day −7 to Day 5). From Day −7 onwards, Group 1 received fresh leaves of *M. tenuiflora*; Group 2 received fresh stems of *M. tenuiflora* and Group 3 (control) received *C. dactylon* hay. On Day 0, all of the sheep were experimentally infected with 4500 *H. contortus* L3. Five days after infection (Day 5), the animals were sacrificed. This study was approved by the ethics committee of the Universidade Estadual do Ceará and registered under the number 09657330-9/02.

2.5. Plant administration, feed intake and live weight

The daily portions of *M. tenuiflora* were harvested early each morning. Fresh leaves and stems of the plant were separated, crushed in forage machine (Eletric Motor, Weg®, Brazil), mixed to obtain a homogeneous distribution of the metabolites, individually weighed before administration and offered to the sheep *ad libitum*. The leftover feed was measured daily. Therefore, the daily plant intake was known for each individual sheep and was used to estimate the consumption of each group. The same protocol was performed for the *C. dactylon* hay. In addition, the live weight of each animal was recorded on the first (Day −7) and last (Day 5) days of the experiment to estimate the live weight gain.

2.6. Plant analysis

From Day 0 to Day 5, samples of the *M. tenuiflora* and *C. dactylon* that were offered daily to the sheep were collected for chemical analysis. The samples were dried separately at 50 °C for 72 h, and pooled samples of each plant were obtained by mixing the same proportion of the daily dried samples. The dry matter (DM), crude protein (CP), fat content, ash, neutral detergent fibers (NDF), acid detergent fibers (ADF) and lignin were determined according to AOAC procedures (1980).

The total phenol (TP) and total tannin (TT) contents were determined using the Folin–Ciocalteu spectrophotometric method (Makkar, 2003). After the initial measurements of TP, PVPP was added and then TT was calculated as the difference between TP measured with or without the addition of PVPP. A tannic acid standard curve was plotted and the results were expressed as tannic acid equivalents. The condensed tannin (CT) was measured using the butanol–HCl assay (Makkar, 2003). A leucocyanidin standard curve was plotted and the results were expressed as leucocyanidin equivalents.

2.7. Parasitological and histological analysis

The sheep were humanely sacrificed, complying with the local regulations on animal welfare. At necropsy, the digestive tract of each sheep was collected immediately. The abomasum was rapidly separated by double ligatures, opened at the major curvature and washed to recover the larvae in the luminal contents. In addition, the mucosa was submitted to incubation in a pepsin solution at 37 °C. The total number of larvae was estimated from a 10% aliquot of the luminal contents and a 10% aliquot of the mucosal digestion. The larvae observed were late L3 and early fourth-stage larvae.

At necropsy, fragments of the abomasum were collected to determine the number of eosinophils and lymphocytes. The mucosal samples were fixed in 10% buffered formalin, embedded in paraffin and sectioned. The sections were then stained with hematoxylin–eosin. The mean cell density for each cellular type was obtained from counts of 10 histological fields that were randomly selected. The results were expressed as the mean number of cells per mm^2 of mucosa.

2.8. Statistical analysis

Data on the plant consumption were subjected to analysis of variance (ANOVA) and compared using the Tukey test. The rate of larval establishment was calculated as the total number of larvae recovered at necropsy divided by the total number of L3 given and multiplied by 100. The data on the worm burden and on the rate of larval establishment were compared among the groups using the non-parametric Kruskal–Wallis test. The comparisons regarding the histological data and live weight were performed using ANOVA and the Bonferroni test. $P < 0.05$ was considered statistically significant. All of the data were analyzed using the GraphPad Prism 5.0 program by GraphPad Software (www.graphpad.com). No statistical analyses were performed on the agronomical and analytical parameters of the plants, as these measurements were only used to describe the forage.

3. Results

3.1. Plants analysis

The results of the bromatological analysis are provided in Table 1. Table 2 describes the TP, TT and CT contents. *M. tenuiflora* presented higher levels of TP, TT and CT than did *C. dactylon*. The TP, TT and CT ratios between the leaves of *M. tenuiflora* and *C. dactylon* were 10.32-, 11.75- and 65.2-fold, respectively. Meanwhile, the ratios between the stems of *M. tenuiflora* and *C. dactylon* were 6.79-, 9.84- and 58.45-fold, respectively. The ratios between the leaves and stems of *M. tenuiflora* were 1.51, 1.19 and 1.11-fold, respectively.

3.2. Feed intake and live weight

During the experiment, the sheep from Groups 1 and 2 (leaves and stems of *M. tenuiflora*) consumed significantly lower quantities of dry matter (average of 267.9 ± 30.1 g/day and 248.2 ± 23.3 g/day, respectively) ($P < 0.0001$) relative to those consumed by the control group (average of 335.2 ± 32.2 g/day). The dry matter intake of Groups 1 and 2 was reduced by 20.1% and 25.9% compared to control-fed sheep. With respect to the intake of CT, the chemical analyses and the consumption of dry matter indicated that the sheep of Groups 1 and 2 consumed an average of 0.5 g and 0.4 g CT/kg live weight/day, respectively, whereas those of Group 3 consumed an average of 0.01 g CT/kg live weight/day.

On the first day of the experiment (Day −7), the mean live weights of the sheep from Groups 1, 2 and 3 were 17.4 ± 5.1 kg, 16.9 ± 4.4 kg and 18.4 ± 3.6 kg, respectively. On the last day of the experiment (Day 5), the mean live weights were 18.5 ± 4.0 kg, 18.6 ± 4.1 kg and 21.2 ± 3.1 kg, respectively. No significant differences were observed in the live weight gain among the groups ($P = 0.082$).

3.3. Parasitological and histological analysis

The effect of the leaves and stems of *M. tenuiflora* on the larval establishment of *H. contortus* in sheep is shown in Table 3. The intake of leaves of *M. tenuiflora* did not reduce the larval establishment rate, as the worm burden was similar to that of the control group ($P = 0.297$). The intake of stems of *M. tenuiflora* caused a tendency toward a decrease in the larval establishment rate [percentage reduction = 27.9%; calculated as: (mean worm burden of the control group − mean worm burden of Group 2)/mean worm burden of the control group × 100)]; however, the reduction was not significantly different, and the worm burden was similar to that of the control group ($P = 0.386$).

With regard to the cell counts in the abomasal mucosa, no significant differences were observed in the number of eosinophils and lymphocytes among the experimental groups (Fig. 1).

4. Discussion

This study was carried out to identify a bioactive plant that could be used to help control gastrointestinal nematodes in small ruminants. In particular, interest has turned

Table 1
Bromatological compositions of *Mimosa tenuiflora* and *Cynodon dactylon*.

	Leaves of *Mimosa tenuiflora*	Stems of *Mimosa tenuiflora*	*Cynodon dactylon* hay
Dry matter	41.90	59.90	96.05
Crude protein	15.05	3.77	6.90
Fat content	7.46	1.47	2.23
Ash	4.61	2.03	8.29
Neutral detergent fiber	38.63	75.83	71.51
Acid detergent fiber	23.44	60.15	36.19
Lignin	9.94	17.68	4.51

Results expressed as g/kg dry matter.

Table 2
Total phenol, total tannin and condensed tannin contents of *Mimosa tenuiflora* and *Cynodon dactylon*.

	Leaves of *Mimosa tenuiflora*	Stems of *Mimosa tenuiflora*	*Cynodon dactylon* hay
Total phenols[a]	99.29	65.37	9.62
Total tannins[a]	65.57	54.93	5.58
Condensed tannins[b]	34.56	30.98	0.53

[a] Results expressed as g tannic acid equiv./kg dry matter.
[b] Results expressed as g leucocyanidin equiv./kg dry matter.

Table 3
Individual worm burdens, mean worm burdens ± S.D. and larval establishment rates (%) ± S.D. of *Haemonchus contortus* in sheep after intake of *Mimosa tenuiflora* or *Cynodon dactylon*.

	Leaves of *Mimosa tenuiflora*	Stems of *Mimosa tenuiflora*	*Cynodon dactylon* hay
Individual worm burdens	133	88	116
	156	105	120
	166	108	145
	173	110	150
	182	126	224
	188	177	236
Mean worm burdens ± S.D.	166.3 ± 19.8[a]	119.0 ± 30.8[a]	165.2 ± 52.1[a]
Larval establishment rates (%) ± S.D.	3.6 ± 0.4[a]	2.6 ± 0.6[a]	3.6 ± 1.1[a]

Small letters compare the means between columns. Different letters indicate significant differences ($P < 0.05$).

to tannin-rich plants, whose negative effects on gastrointestinal nematodes have been supported by relatively consistent experimental results (Hoste et al., 2012). The possible antiparasitic role of tannins has been supported by most *in vitro* assays and has usually been confirmed by the results of *in vivo* tests. These studies with tanniferous plants have been performed in two ways: administering the plant extracts to animals as a drench or feeding animals with the plant as a nutraceutical (Alonso-Díaz et al., 2010). The main effect of tanniferous plants is probably on larval establishment when incoming larvae are ingested and have contact with a tannin-rich environment in the digestive tract (Hoste et al., 2006). Unlike tannin-rich plants some conventional anthelmintics act primarily on adults and have a lowered efficacy against larval stages.

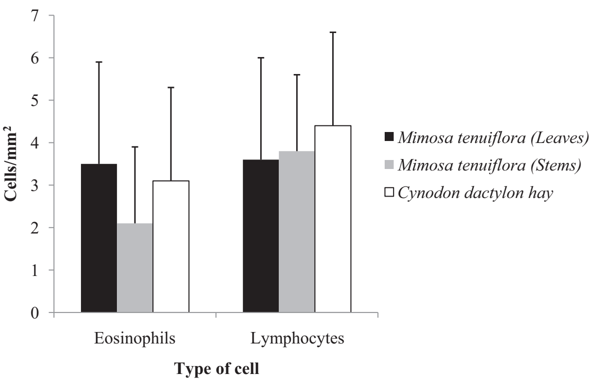

Fig. 1. Mean levels of eosinophils and lymphocytes ± SD in the mucosa of sheep abomasum after intake of *Mimosa tenuiflora* or *Cynodon dactylon*.

Leguminosae is the third largest family of angiosperms, comprising 650 genera and approximately 18,000 species, including three subfamilies: Caesalpinioideae, Mimosoideae and Papilionoideae (Bouchenak-Khelladi et al., 2010). Most studies have examined the species of the Papilionoideae subfamily, whereas the Mimosoideae subfamily has not been well studied. *M. tenuiflora* was selected because of its local availability, high tannin content, voluntary consumption by small ruminants and *in vitro* efficacy against *H. contortus*. Therefore, to follow up on the *in vitro* anthelmintic effects of *M. tenuiflora*, this *in vivo* test was carried out to confirm or refute its activity. For example, *Lysiloma latisiliquum*, a tropical tanniferous plant, exhibited *in vitro* reduction of the larval exsheathment of *H. contortus* (Alonso-Díaz et al., 2008). An *in vivo* test then confirmed its activity on L3 establishment of *H. contortus* in goats (Brunet et al., 2008). The exsheathment of L3 is a key process in the life cycle of trichostrongylid nematodes because it marks the transition from the free-living to the parasitic stage. Interrupting the process of sheath loss prevents the establishment of L3 in the host and consequently blocks the infection (Hertzberg et al., 2002). Although the exact mechanism of tannins on L3 establishment remains unknown, the assumption is that condensed tannins complex with the sheath proteins of nematodes, and their high proline content prevents exsheathment (Brunet et al., 2007) and therefore larval establishment. This hypothesis suggests a direct mode of action due to interactions between the plant metabolites and the nematode structures (Hoste et al., 2012). However, the effect of tannins on improving the immune response of the host cannot be ruled out. Therefore, the indirect effect of these secondary metabolites was also evaluated in this study.

In the present experiment, the intake of leaves or stems of *M. tenuiflora* did not significantly reduce the establishment of *H. contortus* infective larvae in sheep, refuting the anthelmintic effect observed in the previous *in vitro* study. Leaf and stem extracts of *M. tenuiflora* totally blocked larval exsheathment of *H. contortus* (Oliveira et al., 2011). A number of factors can explain this discrepancy between the *in vitro* and *in vivo* results. One possible explanation could be the applied chemical composition of the plants. Genetic factors, climate, soil, harvest time and solar radiation can influence the composition and concentration of secondary metabolites (Gobbo-Neto and Lopes, 2007). The concentration and structure of the condensed tannins could modulate their antiparasitic efficacy (Hoste et al., 2012). The influence of the tannin concentration was confirmed in studies performed *in vitro* and *in vitro*. The dose-dependent effect of extracts rich in tannins was demonstrated on egg hatching, larval motility and the exsheathment of nematodes (Molan et al., 2003; Alonso-Díaz et al., 2008). Moreover, this effect was confirmed in experimental studies performed either in sheep or in goats receiving various levels of tanniferous plants (Athanasiadou et al., 2001; Terrill et al., 2009). However, the diversity of methods used to measure tannins in plants can sometimes make comparisons among studies difficult (Hoste et al., 2012). As previously mentioned, the structure of condensed tannins can also influence their anthelmintic efficacy (Molan et al., 2003; Brunet and Hoste, 2006). The host species could also justify the discrepancy in the results. In certain host species (*e.g.*, goat or deer *vs.* sheep or cow), the presence of tannin-binding salivary proteins might not only reduce the potential negative effects of tannins but also affect the anthelmintic properties by increasing the level of activity of these metabolites relative to their concentration (Hoste et al., 2012). Moreover, according to Hoste et al. (2012), the nutraceutical effects of plants are usually achieved following relatively long-term consumption. The evidence behind a long-term feed and the consequent antiparasitic effect was confirmed in studies performed either in sheep or goats receiving plants rich in tannins (Athanasiadou et al., 2005; Akkari et al., 2008). The short-term consumption that was carried out in this experiment could be responsible for the lack of an antiparasitic effect of *M. tenuiflora*. In addition, the diet was available *ad libitum* to the sheep. This type of availability increases the flow rates of digesta (Grovum and Hecker, 1973), which reduces the retention time of the CT in the digestive tract and renders them less effective against parasites (Dawson et al., 2011). Increased food intake has also been considered responsible for the reduced efficacy of anthelmintic drugs in sheep due to an increased digesta passage rate (Ali and Hennessy, 1995).

Despite the lack of an effect from *M. tenuiflora* on the establishment of nematode L3 in this experiment, its utilization for small ruminant farming should not be discarded. This plant can be used in situations of feed scarcity, for example, during dry periods. Although differences in live weight between the groups were noted, these differences were not reflected by significant differences in the live weight gain between the feeding groups. However, studies should be conducted to evaluate the toxicity of this plant after a longer term of treatment than in the present study. Further tests to evaluate the anthelmintic activity of *M. tenuiflora* in more long-term courses of treatment or extract administration should be considered before discarding this plant as a possible alternative for nematode gastrointestinal control in small ruminants.

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