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Research paper

In vitro and *in vivo* action of *Piptadenia viridiflora* (Kunth) Benth against *Haemonchus contortus* in sheep

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ABSTRACT

Anthelminthic resistant populations of *Haemonchus contortus* are a major problem in sheep rearing, but plant extracts may offer viable alternative treatments. In our preliminary studies, *Piptadenia viridiflora* was frequently selected by sheep grazing in the Cerrado. The present research evaluated its *in vitro* and *in vivo* anthelmintic activity. The HPLC chromatograms of *P. viridiflora* aqueous extract (AE) and ethanolic extract (EE) showed the presence of flavonoids. The total condensed tannin (proanthocyanidin) was 0.2 and 1.01% in AE and EE, respectively. In an egg hatching inhibition (EHI) test, the LC_{90} of AE was 2.4 mg/mL, and, of EE, was 2.1 mg/mL. After tannin extraction, higher EHI and lower LC_{90} were observed. In a larval development inhibition test, the LC_{90} of AE was 13.66 mg/g of fecal culture. The highest dose of AE administered to mice (203.0 mg/kg bw) was well tolerated, suggesting low toxicity. *In vivo*, AE was orally administered to lambs at 283 mg/kg bw, and, at weeks one, two, and three post-treatment, the mean fecal egg count (FEC) was significantly lower than in untreated lambs ($P < 0.05$). Blood parameters were normal and similar in untreated and treated sheep. For all lamb groups, the mean total serum protein was significantly higher at week two post-treatment than at other evaluated periods ($P < 0.05$). *Piptadenia viridiflora* extracts had low condensed tannin content and exhibited high anthelminthic efficacy *in vitro* and significantly reduced FEC. Tannins were not shown to be the principal components affecting EHI, hence it is necessary to isolate and characterize the principal active *P. viridiflora* compounds, and to assess their possible synergism.

1. Introduction

Sheep are bred worldwide on family farms and in commercial production representing an important part of the global agricultural economy. *Haemonchus contortus* is the most common parasite in sheep reared in tropical areas. Animals with haemonchosis may show anemia and submandibular swelling, with high mortality in young lambs and ewes in the peripartum period. Both sexes at all age levels may be infected, resulting in production losses that include decreased weight gain and feed conversion efficiency; reduced meat, wool, and milk production; and reproductive disorders (Sczesny-Moraes et al., 2010; Taylor et al., 2009).

Anthelminthics provide a rapid method of control, but *H. contortus* has developed resistance on several continents (Schnyder et al., 2005; Cringoli et al., 2007; Duarte et al., 2012; Adiele et al., 2013; Soro et al., 2013). Utilization of plants containing secondary compounds such as condensed tannins may provide alternatives for control of gastrointestinal nematodes (Athanasiadou and Kyriazakis, 2004).

The anthelmintic activity of plant extracts has been reported in several regions of the world, offering a promising strategy for the biotechnology industry and one that will ultimately benefit ruminant breeders (Nery et al., 2010; Qadir et al., 2010; Goswami et al., 2011; Kakar et al., 2013), but there is little research on plants

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naturally selected by sheep that may have potential anthelminthic properties.

Piptadenia viridiflora (Kunth) Benth (Fabaceae), commonly known as "surucucu" (Lorenzi, 2009; Morin, 2015), is widespread in the Cerrado of the Central and South American (Grandtner and Chevrette, 2013; Royal Botanic Gardens Kew, 2016). In the Brazilian Cerrado, Morais-Costa et al. (2015a) identified *P. viridiflora* as naturally selected by grazing sheep during the dry and rainy seasons, with a selectivity index of 2.61% and 3.69%, respectively.

Extracts of *P. viridiflora* leaves have been reported to be associated with significant reduction of *H. contortus* larval development in sheep (Morais-Costa et al., 2015b). To elucidate the anthelminthic potential of this plant, we analyzed the *in vitro* and *in vivo* anthelminthic activity of *P. viridiflora* extracts against *H. contortus*. To identify the main components of anthelminthic action, the extracts were characterized, and their effects on egg hatching inhibition were evaluated after tannin removal.

2. Material and methods

2.1. Study area

The research was conducted in an area of Cerrado vegetation near Montes Claros City in North Minas Gerais State, Brazil (W 43°50′33.56″; S 16°41′10.05″). The climate of the region, classified as tropical wet with dry summer (As) according to the Köppen classification (Alvares et al., 2013), is marked by a dry season from May to September and a rainy period in January and February. The monthly average rainfall and temperature during the dry season are 14.1 mm and 23.2 °C, respectively.

2.2. Sample collection and aqueous and ethanolic extracts

Apparently healthy leaves of *P. viridiflora* were selected and dried to constant weight in a forced air circulating dryer (TE 394/4, Tecnal Equipamentos CientíficosTecnal, Piracicaba, SP, Brazil) at 40 °C for 72 h. Dried leaves were ground in a Wiley mill (CE-430/Macro, Cienlab, SP, Brazil) and stored in paper bags in darkness. Plant samples were deposited in the Montes Claros Herbarium of Universidade Estadual de Montes Claros, as voucher specimen 2283.

The aqueous extract (AE) was produced by placing the ground dried leaves in a distilled water bath (100 g/L) at 40 °C for 60 min. The ethanolic extract (EE) was obtained from macerated dried leaves held in absolute ethanol (100 g/L) in amber-colored glass containers in darkness for seven days. Extracts were filtered through a gauze funnel and dehydrated at 40 °C for 48 h to obtain a residue with constant weight, which was stored in paper bags in darkness (Morais-Costa et al., 2015b).

Subsamples of both extracts were submitted to tannin extraction according to the method proposed by Nyman et al. (1998). Extracts were dissolved at 1 g/20 mL of water at 90 °C and cooled at room temperature. After reaching 30 °C, 0.2 μL 10% NaCl was added, and 1 mL of this solution was combined with 4 mL of 1% gelatin solution and centrifuged at 1800*g* for 6 min. The supernatants were used to assess the effects of tannin-free extracts on egg hatching inhibition (EHI).

2.3. Extract characterization

A Waters Alliance 2695HPLC system composed of a quaternary pump, an auto-sampler, a photodiode array detector (DAD) 2996, and a Waters Empower Pro data handling system (Waters Corporation, Milford, Connecticut, USA) was used for the extract characterization. The analyses were performed on a LiChrospher 100 RP-18 column (5 μm particle size, L × Internal Diameter (ID) 250 × 4 mm) combined with a LiChrospher 100 RP-18 guard column (5 μm particle size, L × ID × 40 × 4 mm) (Merck, Darmstadt, Germany Merck) at 40 °C. Water (A) and acetonitrile (B) were used as eluents, both containing 0.1% (v/v) H_3PO_4 at a flow rate of 1.0 mL min^{-1} as follows: 0 min, 95% A and 5% B and 60 min, 5% A, 95% B, followed by 10 min of isocratic elution. Solvents used were of HPLC grade (Merck, Germany) and were degassed by sonication before use. The chromatograms were obtained at 210 nm, and the UV spectra were recorded on-line from 190 to 400 nm.

The dried extracts were dissolved in methanol (HPLC-grade), ultrapure water, or hydroethanolic solutions, according to their solubility, to concentrations of 10 mg/mL. After centrifugation at 8400*g* for 10 min, 10 μL of sample solutions were automatically injected into the apparatus.

Total condensed tannin (proanthocyanidins) content of extracts was determined by measuring at 540 nm the absorbance of the cyanidin chloride resulting from acid-catalyzed solvolysis with *n*-BuOH/HCl 12 M (95:5), according to the method described by Hiermann et al. (1986). Each sample was analyzed in triplicate with results expressed as mean ± standard deviation. The total condensed tannin content, expressed as cyanidin chloride, was calculated using the following formula:

$$\text{Condensed tannins\%} = \text{Absorbance sample} - \text{Absorbance blank} \times 4.155/\text{Weight sample (g)}$$

2.4. Egg hatching inhibition (EHI)

Two Santa Inês lambs were infected with 2000 L3 of *H. contortus* resistant to albendazole. This strain was from an *H. contortus* female collected from the abomasum of a lamb in North Minas Gerais, Brazil (Duarte et al., 2012). After 22 days, the lambs showed a mean fecal egg count (FEC) >1000/g, determined using the modified McMaster technique, which has minimum sensitivity of 50 eggs/g of feces (Gordon and Whitlock, 1939). Sedimentation in water, filtration, and flotation in saturated saline were conducted to obtain nematode eggs from lamb feces (Coles et al., 1992).

Samples of AE and EE, with and without tannins extracted, were standardized at 24.8 mg/mL. Immediately after dilution, extracts were used in EHI tests. Fifteen experimental groups were used, each with five replicates. Positive controls were exposed to levamisole phosphate (0.3 mg/mL) or ivermectin (16 μg/mL) and the negative control consisted of sterile purified water. Experimental treatments using each *P. viridiflora* extract were performed at 2.4, 1.2, 0.6, 0.3, 0.15, and 0.075 mg/mL, with and without tannins.

The mixtures comprised 100 μL of egg suspension containing an average of 80 fresh eggs and 100 μL of the extracts in 96-well micro plates. Samples were homogenized and incubated at 28 °C for 48 h in a refrigerated incubator (TE 371, Tecnial equipamentos científicos, SP, Brazil). Subsequently, 15 μL of Lugol's solution was added to each tube, which were then stored at 4 °C for counting of unembryonated eggs, embryonated eggs, and larvae (Coles et al., 1992).

The number of L1 relative to the initial number of eggs (remaining eggs plus L1) was determined for each repetition and subjected to variance analysis. The means were compared using Tukey's test ($P < 0.05$). Probit regression was used in the estimation of concentrations sufficient to inhibit 50% (LC_{50}) and 90% (LC_{90}) of egg hatching with SAEG 9.1 software. The formula of Coles et al. (1992) was used to determine the% EHI:

$$\%\text{EHI} = 100 \times (\text{1-L1}/\text{initial number of eggs}).$$

2.5. Inhibition of larval development in fecal culture

Feces were collected from the two lambs described above. To evaluate the activate of AE of *P. viridiflora* leaves, a larval development inhibition (LDI) test was performed by the quantitative coproculture method (Borges, 2003; Nery et al., 2010; Morais-Costa et al., 2015b).

Nine treatments were evaluated, including two positive controls [2 mL of 16 μg/mL ivermectin (LA Ranger, Vallée S.a, MG, Brazil) or 2 mL of 0.1 mg/mL levamisole phosphate (Protall, Vallée S.a, MG, Brazil)] and a negative control of 2 mL sterile purified water. The six experimental treatments consisted of AE of the *P. viridiflora* standardized at 38.62, 19.31, 9.65, 4.83, 2.41, and 1.21 mg dry weight (dw)/g of fecal culture. Each tested solution was added to 2 g feces, with five replicates. After addition of 2 g of industrial sterile vermiculite, the cultures were incubated in a refrigerated incubator at 28 °C for seven days. Infective larvae (L3) were counted in Sedgewick Rafter counting chambers (S52 glass, Pyser-SGI, Edenbridge, UK). The following formula, adapted from Borges (2003), was used to determine the percent reduction of larvae (L3) per gram of feces (LPGF):

$$\%LDI = 100 \times (1\text{-LPGF of the treated group/LPGF of the untreated group}).$$

The data were log transformed, log (x + 1), and submitted to variance analysis. The means were compared by the Duncan's test ($P < 0.05$). The LC_{50} and LC_{90} were determined by probit analysis with SAEG 9.1 software.

2.6. Toxicity in mice

Toxicity testing was performed according to Walum (1998) to determine the maximum tolerated dose in adult mice. Using probe gavage, 22 μL of AE was administered to four (2 males and 2 females) 6–8 week old Balb C mice of mean 22 g body weight (bw). Increasing doses were administered on consecutive days. On days one and two, this extract was administrated at 2.03 mg/kg and 20.3 mg/kg, respectively. On days three and four, the extract was administered at 203.0 mg/kg bw (Walum, 1998). The mice were euthanized by cervical dislocation on day 5 after AE administration. Internal organs and mucosa were clinically examined at necropsy.

2.7. In vivo *test in lambs*

Fecal egg reduction was assessed in twenty-four 4–8 month old Santa Inês lambs of 26.5 kg mean bw (12 male, 12 female). Fourteen days prior to the beginning of the trial, all lambs were administered albendazole (LA Ranger, Vallée S.a, MG, Brazil) (10 mg kg^{-1} bw) and levamisole phosphate (Protall, Vallée S.a, MG, Brazil) (0.6 mg kg^{-1} bw) to ensure zero FEC. During the 14 day adaptation period, the animals were individually confined and fed on a balanced diet containing sorghum silage, concentrate, mineral premix, and water *ad libitum*.

The lambs, which showed zero FEC on two counts, were infected with 800 *H. contortus* L3 (strain previously described) per 10 kg bw. Twenty-eight days post-infection, lambs were assigned to one of three homogeneous groups based on FEC, weight, and sex (4 males and 4 females per group).

A group of untreated sheep served as negative control; a second group, representing the positive control, received a single dose of oral levamisole phosphate at 0.6 mg/kg bw; and a third group was administered 283 mg (dm)/kg bw AE of *P. viridiflora* by esophageal gavage for three consecutive days. The dose was based on the LC_{90} estimated by the LDI test (Nogueira et al., 2012). Treatment was conducted in the morning, following 12 h fasting. Animals were monitored for clinical signs and weighed in the morning before feeding on the day of treatment and on days 7, 14, and 21 post-treatment. The mean weight gain of groups was compared by Tukey's test at 5% significance level.

The FEC was evaluated over four time periods at weekly intervals. Each period covered an average of three days, obtaining two counts each day, totaling 24 FECs per animal (Nogueira et al., 2012). Fecal egg counts were recorded on the two days prior to treatment and on the day of treatment, with the mean used to standardize levels for each lamb group. Subsequently, mean FEC was calculated on days 7, 8, and 9 (second period); days 14, 15, and 16 (third period); and days 21, 22, and 23 (fourth period). The modified McMaster technique was performed with saturated NaCl with minimum sensitivity of 50 eggs/g of feces (Gordon and Whitlock, 1939). The efficacy of treatments was calculated by formula adapted from Coles et al. (1992):

$$\%FEC\ reduction = 100 \times [1\text{-(FEC mean of treated group/FEC mean of untreated group)}]$$

The FEC data obtained were transformed to $\log^{10}$ (x + 10) and subjected to analysis of variance in a split plot design with respect to the four evaluated periods. Means were compared by the Scott–Knott's test ($P < 0.05$).

2.8. Lamb blood parameters

Blood samples were collected from the jugular vein on days 0, 7, 14, and 21 into tubes containing ethylenediaminetetraacetic acid (EDTA) and stored at 4 °C. Erythrocyte numbers and hematocrit were evaluated in an electronic automatic analyzer (2.800 BCE Vet®, Mindray Medical International Ltd., Shenzhen, China). Protein and albumin values were analyzed with Bioclin (Quibasa Basic Chemicals Ltd., Belo Horizonte, MG, Brazil) by colorimetric spectrophotometry (Automatic system for biochemical, BIOPLUS BIO 2000, Shenzhen, China). Data were subjected to analysis of variance with split plots, and means were compared by Scott–Knott's test ($P < 0.05$), using the SAEG (2007).

3. Results

3.1. Extract characteristics

The chemical composition of the AE and EE extracts of *P. viridiflora* leaves was determined by their chromatographic profiles recorded by HPLC-DAD. The chromatograms showed similarity, with the predominance of peaks corresponding to polar compounds, with UV spectra compatible with polyphenols (Fig. 1). The two major peaks found in both chromatograms showed retention times of 1.35 and 1.59 min and 1.27 and 4.77 min for AE and EE, respectively. The UV data were compatible with flavonoids (λ 262.7 and 376.8 nm and λ 278.1 and 375.6 nm for AE and EE, respectively). The concentrations of total condensed tannins, assayed spectrophotometrically, were 0.2% ± 0.01 and 1.01% ± 0.2 for AE and EE, respectively.

3.2. In vitro *anthelmintic activity*

Egg hatching inhibition increased with increased concentration of the AE and EE extracts and at 1.2 mg/mL, EHI was >95% (Table 1). The LC_{90} values obtained for AE and EE were 2.4 and 2.1 mg/mL, respectively. At all tested concentrations, the embryonated eggs showed degraded embryos with structural malformations. Extracts without tannins, at similar concentrations, promoted higher EHI values ($P < 0.05$) and lower LC_{90} were

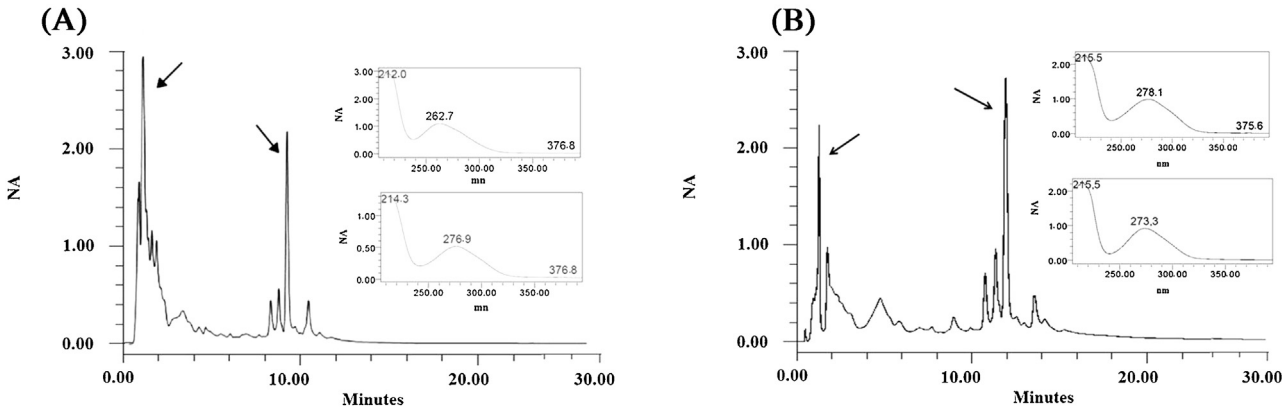

Fig. 1. HPLC-DAD chromatogram of aqueous (a) and ethanolic (b) extract of the dried leaves of *Piptadenia viridiflora* in the anthelminthic assay.

Table 1
Egg hatching inhibition of aqueous and ethanolic extracts of *Piptadenia viridiflora* leaves in *Haemonchus contortus.*

Treatments	Unembryonated eggs mean	Embryonated eggs mean	L1 mean	Eggs + L1	Efficacy[a] (%)
Aqueous extract (mg/mL)					
2.4	7.2^{b}	35.8^{a}	0.0^{e}	43.0	100.0
1.2	2.0^{c}	43.8^{a}	1.8^{e}	47.6	97.85
0.6	2.5^{c}	48.8^{b}	20.2^{d}	71.5	75.84
0.3	1.4^{c}	42.2^{c}	46.0^{c}	89.6	44.98
0.15	1.4^{c}	13.0^{d}	72.6^{b}	87.0	13.16
0.075	1.8^{c}	17.6^{d}	74.2^{ab}	93.6	11.24
Ethanolic extract (mg mL^{-1})					
2.4	39.2^{a}	0.0^{c}	0.0^{e}	39.2	100.0
1.2	36.0^{a}	0.0^{c}	0.0^{e}	36.0	100.0
0.6	49.0^{b}	0.0^{c}	7.6^{d}	56.6	90.9
0.3	54.2^{b}	0.0^{c}	11.4^{d}	65.6	86.4
0.15	49.8^{c}	4.0^{b}	21.6^{c}	75.4	74.2
0.075	25.2^{d}	0.2^{b}	25.4^{b}	50.6	69.6
Levamisole phosphate (0.3 mg mL^{-1})	78.0^{a}	0.0^{c}	0.0^{e}	78.0	100.0
Ivermectin (16 μ mL^{-1})	78.8^{a}	0.0^{c}	2.6^{e}	81.4	96.88
Sterile distilled water	0.0^{e}	0.0^{c}	83.6^{a}	83.6	–
Variation coefficient (%)	5.93	43.63	20.17		

Different letters in columns (a, b, c, d, e) indicates significant differences by Tukey's test ($P < 0.05$).
[a] % efficacy = 100 × (1-L1/initial number of eggs).

observed (0.39 and 0.08 mg/mL for AE and EE without tannins, respectively).

The treatments with *P. viridiflora* EE, levamisole phosphate, and ivermectin showed mean numbers of unembryonated eggs significantly higher ($P < 0.05$) than with the distilled water control and all concentrations of AE. However for AE, the mean number of embryonated eggs was significantly higher ($P < 0.05$) than for the distilled water controls, levamisole phosphate, ivermectin, and all concentrations of EE (Table 1).

In the larval development inhibition test, the AE at ≥1.2 mg/g resulted in mean numbers of infective larvae significantly lower than observed for the distilled water control ($P < 0.05$), and anthelmintic efficacy increased with increasing concentration of this extract to the LC_{50} and LC_{90} of 1.0 and 13.66 mg/g, respectively (Table 2).

3.3. Toxicity test in mice

No clinical signs of toxicity in mucosal tissue, changes in animal behavior, or mortality were observed during the four days of extract administration. The maximum dose of 203.0 mg/kg bw was tolerated by both male and female mice. At necropsy, macroscopic examination revealed no abnormalities of liver, kidney, spleen, lung, or other viscera.

Table 2
Mean numbers of infective *Haemonchus contortus* larvae exposed to aqueous extract of the *Piptadenia viridiflora* leaves in fecal culture.

Extract concentrations (mg/g)	LPGF	Efficacy (%)
38.62	0 ^{f}	100.0
19.31	55 ^{e}	93.13
9.65	105 ^{d}	86.88
4.83	180 ^{c}	77.05
2.41	285 ^{b}	64.38
1.21	385 ^{b}	55.63
Levamisole phosphate (0.1 mg/mL)	0 ^{f}	100.0
Ivermectin (16 μg/mL)	0 ^{f}	100.0
Sterile distilled water	800 ^{a}	–

Different letters in the columns (a, b, c, d, e, f) indicate significant differences by Duncan's test ($P < 0.05$).
Coefficient ofvariation LDI: 4.67%.
LPGF: number of larvae (L3)/g of feces.
Efficacy: % efficacy = 100 × (1-LPGF of the treated group/LPGF of the untreated group).

3.4. In vivo *anthelmintic activity*

All lambs initially showed high FEC. After treatment, the AE of *P. viridiflora* leaves was associated with significant reduction in mean FEC compared to the untreated group ($P < 0.05$, Table 3). At

Table 3
Mean fecal egg count (FEC) in lambs after oral administration of aqueous extracts of *Piptadenia viridiflora* leaves at 283 mg (dm)/kg bw or levamisole phosphate at 0.6 mg/kg bw and untreated (control group).

Treatments	Before treatments	First week		Second week		Third week	
	FEC/g	FEC/g	Efficacy (%)	FEC/g	Efficacy (%)	FEC/g	Efficacy (%)
Control	5601.75 ^{Aa}	3950.00 ^{Aa}	–	2675.00 ^{Ba}	–	2650.00 ^{Ba}	–
Levamisole phosphate	4483.00 ^{Aa}	66.70 ^{Bc}	99.99	29.25 ^{Cc}	98.90	18.75 ^{Dc}	97.48
P. viridiflora	5962.37 ^{Aa}	2088.00 ^{Bb}	47.20	1469.00 ^{Cb}	45.10	1781.00 ^{Cb}	32.90

Different uppercase letters in rows and lowercase letters in columns indicate significant differences by Scott–Knott's test ($P < 0.05$).
Efficacy = 100 × (1-mean FEC of treated group/mean FEC of control group).
Coefficient of variation = 8.85%.

weeks one, two, and three post-treatment, 47.2, 45.1, and 32.9% anthelminthic efficacy was observed, respectively. Treatment with AE resulted in mean FECs significantly lower at weeks one, two, and three post-treatment than at baseline ($P < 0.05$).

After 21 days of treatment, lambs treated with AE gained an average of 4.0 kg ± 1.5, more ($P < 0.05$) than was observed in untreated animals (2.28 ± 0.9 kg) and those treated with levamisole phosphate (1.9 ± 0.91 kg). The animals treated with *P. viridiflora* did not exhibit behavior changes, submandibular edema, weakness, or lack of appetite during the experiment.

3.5. Blood parameters

Erythrocyte count and hematocrit were similar among the three groups, and lambs treated with *P. viridiflora* AE showed blood counts according to reference values for sheep (Kahn and Line, 2010, $P > 0.05$ (Table 4). Both the treatment and the untreated lambs (control) showed average total serum protein significantly higher 14 days post-treatment (Table 4).

4. Discussion

Significant negative impact on the productivity of sheep infected with *H. contortus* has been reported, and resistance to anthelminthics can increase production costs (Miller et al., 2012). *Piptadenia viridiflora*, common in Cerrado and naturally selected by sheep (Morais-Costa et al., 2015a), gave leaf extracts with high EHI and LDI as well as moderate and significant FEC reduction in lambs infected with *H. contortus*.

Condensed tannin content was low for both extracts of *P. viridiflora*. This was expected, since it is a plant species readily consumed by sheep (Morais-Costa et al., 2015b). Plants having condensed tannins contents higher than 60 g/kg (dm) are less palatable and digestible by sheep than those with lower concentrations (López et al., 2004).

The HPLC analyses revealed flavonoids as the major components of *P. viridiflora* extracts, which showed high *in vitro* EHI after removal of tannins, suggesting that flavonoids were responsible for this anthelminthic effect.

Few studies have investigated the specific role of flavonoids and their synergism with other plant compounds in inhibiting nematode development. The anthelminthic action of flavonoids can be attributed to their effects on enzyme activity and metabolic processes in parasites (Kerboeuf et al., 2008).

The single tannins arbutin, vanillic acid, and flavanone taxifolin have not been reported to larval exsheathment inhibition (LEI) in *H. contortus*, and gallocatechin, a monomeric component of condensed tannins, demonstrated such activity only at 1000 μM. However, the flavonoids naringenin, quercetin, and luteolin were reported highly effective in LEI at 250 μM (Klongsiriwet et al., 2015). These results suggested that flavonoids are more active than tannins in LEI. In addition, our findings indicate that tannins are not the most active metabolites of *P. viridiflora* in inhibition of *H. contortus* hatching.

The anthelminthic effect induced by a combination of condensed tannins and flavonoids was evaluated by Klongsiriwet et al. (2015) who showed evidence of synergistic effects of condensed tannins and the flavonoids quercetin and luteolin in *in vitro* LEI. In contrast, the extracts of *P. viridiflora* assayed after removing tannins were more effective than the crude extracts, suggesting the absence of synergism with flavonoids.

Rates (2001) reported anthelminthic effects of flavonoids and alkaloids and suggested a synergistic effect between them. Synergism between flavonoids and other compounds of leaf extracts of *P. viridiflora* should be investigated, since its extracts without tannins showed high efficacy *in vitro*.

Despite the low levels of condensed tannins, both *P. viridiflora* extracts were associated with high inhibition of *H. contortus* embryogenesis and hatching. The mean number of unembryonated eggs observed with *P. viridiflora* EE treatment was significantly higher than in the distilled water control and at all concentrations of AE, suggesting that EE inhibited *H. contortus* embryogenesis (Table 1). In contrast, when using AE, the mean number of embryonated eggs was significantly higher than seen with the distilled water control, levamisole phosphate, ivermectin, or *P. viridiflora* EE (Table 1). The mean number of blastomered eggs was significantly lower than that of embryonated eggs with AE treatment, indicating an inhibitory effect on hatching as opposed to embryogenesis.

In another study, the main anthelmintic effect of acetone-water extracts of *Lysiloma latisiliquum*, *Laguncularia racemosa*, *Rizophora mangle*, *Avicennia germinans*, and *Theobroma cacao* was found to block eclosion of embryonated eggs. The tannins of *L. racemosa* extract were reported to be responsible for the EHI. However, tannins did not appear to be the source this activity in extracts of *L. latisiliquum*, *A. germinans*, *T. cacao*, suggesting that interactions between tannins and other secondary compounds may promote EHI (Vargas-Magana et al., 2014).

High EHI was observed after removal of tannin from *P. viridiflora* extracts, indicating that other metabolites must act to inhibit embryogenesis and hatching in *H. contortus*. Flavonoids were the principal components of these extracts and could be responsible for the anthelminthic effects. In contrast, aqueous extracts of *Lantana camara* leaves at 10 mg/mL demonstrated EHI that may have been related to action of tannins, since the removal of this metabolite reversed the inhibitory effect (Macedo et al., 2012).

Other Cerrado plants have exhibited EHI properties in *H. contortus*. The AE of *Cariocar brasiliense* (epicarp and mesocarp) at 7.5 and 15 mg/mL promoted EHI of 91.8% and 98.7%, respectively (Nogueira et al., 2012). The methanol extract of *Annona squamosa* bark at 6.25 mg/mL demonstrated 77.4% EHI (Kamaraj and Rahuman, 2011) and Ferreira et al. (2013) reported 84% EHI for AE of *A. muricata* leaves at 50% final dilution.

In fecal cultures, the LDI LC_{90} of *P. viridiflora* AE was 13.6 mg g^{-1}, evidence of its effectiveness at low concentrations. Among other Cerrado species, *Ximenia americana* has been reported to be

Table 4
Mean erythrocyte count, hematocrit value, and serum concentration of protein and albumin in lambs infected with *Haemonchus contortus* and treated orally with *Piptadenia viridiflora* (283 mg/kg bw) or levamisole phosphate (0.6 mg/kg bw) or untreated (control).

	Collection time (days)				Reference[a]	
Treatments	0	7	14	21		CV (%)
Erythrocytes ($\times 10^6/\mu L$)						
Untreated	11.57	11.00	10.91	10.11		
Levamisole phosphate	11.24	9.08	9.15	9.72	9–15	17.35
P. viridiflora	10.84	11.87	11.46	9.73		
Hematocrit (%)						
Untreated	34.67	31.75	34.97	30.35		
Levamisole phosphate	34.00	29.35	30.85	29.85	27–45	18.11
P. viridiflora	33.22	35.35	35.12	35.17		
Total protein (g/dL)						
Untreated	5.73	7.29	7.91	6.71		
Levamisole phosphate	5.70	6.76	7.35	5.66	6–7.9	16.25
P. viridiflora	5.67	5.36	6.44	6.12		
Mean in interaction period	5.70 [b]	6.47 [b]	7.23 [a]	6.17 [b]		
Plasma albumin (g/dL)						
Untreated	4.02	3.60	4.10	4.17		
Levamisole phosphate	2.87	3.42	2.87	4.27	2.4–3	26.68
P. viridiflora	3.27	3.42	5.30	3.52		

Values with different letters in the line (a, b) differ significantly by Scott–Knott's test ($P < 0.05$).
CV = Coefficient of variation.
[a] =Reference range for sheep (Kahn and Line, 2010).

selected by sheep during its fruiting period (Morais-Costa et al., 2015a). Aqueous extract of this species has low condensed tannin content (0.3%) and major peaks of UV spectra in HPLC chromatograms characteristic of flavonoids. The extract AE of *X. americana* at 333.3 mg dw/g of fecal culture demonstrated 99.8% LDI (Morais-Costa et al., 2015b), reinforcing the suggestion that flavonoids may act as inhibitors of nematode larva development.

Aqueous extract of the peel of *C. brasiliense* at 200 mg/mL inhibited development of 94.8% of *H. contortus* larvae in fecal cultures, and phytochemical tests indicated the presence of catechins, steroids, flavonoids, saponins, xanthones, and tannins (Nogueira et al., 2012).

Morais-Costa et al. (2015b) found dried leaves (333.3 mg/g) of *Casearia sylvestris* and *Paullinia* sp. to show the highest condensed tannin content (7.4 and 6.4%) of tested species, but lower LDI, at 46.6 and 71.6%, respectively, compared to leaves of *P. viridiflora* (86.5%) and *X. americana* (99.8%), which display lower concentrations of condensed tannins (0.2 and 0.3%, respectively).

In this study, the oral administration of AE of *P. viridiflora* leaves significantly reduced mean FEC, showing moderate anthelminthic efficacy. These results are promising, since the dose of 283 mg (dm)/kg bw over three consecutive days was low. A higher, more frequent, dose may enhance the anthelminthic effects. The extract contains substances with inhibitory action against adult *H. contortus* and can be easily produced, since this plant is common and widespread in the Cerrado biome, making it a viable and sustainable control for this nematode in sheep reared in the region.

. Such differences between *in vitro* and *in vivo* results with plant treatments have been previously reported (Peneluc et al., 2009; Nogueira et al., 2012) and could be associated with bioavailability of plant compounds in different segments of the ruminant gastrointestinal tract (Athanasiadou et al., 2007; Eguale et al., 2007). Adult nematodes may also be more resistant to the active components, or rumen microbiota may reduce the activity of metabolites (Nogueira et al., 2012).

The best weight gain of lambs treated with AE in this research needs to be investigated. Possibly this extract would have promoted best conditions in the rumen environment, selecting favorable microbiota, which should be carefully analyzed in future studies.

No changes were observed in blood parameters after *P. viridiflora* administration. The mean hematocrit was >35% for all lambs treated with *P. viridiflora* extract. With levamisole treatment, the hematocrit mean was 31.0%, similar to those observed by Costa et al. (2011) for lambs treated with anthelminthic.

Erythrocyte count and hematocrit are good indicators of anemia and represent important parameters for the assessment of infection by hematophagous nematodes (Sotomaior et al., 2007). In acute hemonchosis, anemia is characterized by progressive decrease in hematocrit and erythrocyte count (Taylor et al., 2009). Hematocrit values below 15% are concomitant with weakness and indicate a poor prognosis (Bowman, 2010). However, in this study the lambs were not anemic and possibly the provided diet could have favored clinical resistance for the blood spoliation.

The maximum oral dose of 203 mg/kg bw *P. viridiflora* AE administered to mice was tolerated, indicating low toxicity (Walum, 1998). Total serum protein values in sheep were lower than reference values (Kahn and Line, 2010) for all lambs during the initial infective period. Hematophagy of *H. contortus* and abomasitis with reduced protein digestion in the acute phase could explain the low concentrations. After day 14, protein levels were significantly elevated compared to the initial period, suggesting recovery.

Plasma albumin values can be an indicator of protein deficit or chronic lesions in the liver (Reis et al., 2007). Albumin concentrations observed in this study were within normal limits. The adequate diet provided to the animals may have contributed to the maintenance of plasma albumin.

5. Conclusions

Leaf extracts of *P. viridiflora*, a plant naturally selected by grazing sheep, show effective *H. contortus* EHI and LDI. Phytochemical analyses of the extracts indicate the presence of flavonoids. Aqueous and ethanolic extracts have low condensed tannins content, and, after tannin removal, showed high anthelminthic efficacy *in vitro*. In lambs, the AE was not toxic at 283 mg/kg bw and exhibited moderate and significant anthelminthic activity *in vivo*.

Conflict of interest

The authors of this manuscript have no financial or personal relationship with individuals or organizations that could influence or bias the content of the paper.

All procedures were performed in accordance with the principles of animal experimentation approved in the 275/2013 protocol

of the Ethics Committee on the use of animals (CEUA) of the Federal University of Minas Gerais, Brazil.

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