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**INSTITUTO DE CIÊNCIAS BIOLÓGICAS**  
**PROGRAMA DE PÓS-GRADUAÇÃO EM PARASITOLOGIA**

**Espécies de plantas do cerrado selecionadas por ovinos em pastejo com potencial  
na inibição do desenvolvimento de *Haemonchus contortus***

**FRANCIELLEN MORAIS COSTA**

**Belo Horizonte**

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**FRANCIELLEN MORAIS COSTA**

Tese apresentada ao Programa de Pós-graduação em  
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Doutora em Ciências

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## UNIVERSIDADE FEDERAL DE MINAS GERAIS

### Espécies de plantas do cerrado selecionadas por ovinos em pastejo com potencial na inibição do desenvolvimento de *Haemonchus contortus*

#### Resumo

Espécies vegetais naturalmente selecionadas por ovinos no Bioma Cerrado (W 43°50'33.56" e S 16°41'10.05") foram avaliadas *in vitro* e *in vivo* para o controle de *Haemonchus contortus*. Após um ano de observações, as famílias das espécies que mostraram maior riqueza foram Fabaceae, Rubiaceae, Malpighiaceae, Bignoniaceae, Myrtaceae e Annonaceae. Houve variações do índice de selectividade para nove espécies vegetais selecionadas por ovinos nas estações seca e chuvosa. A coprocultura foi realizada em cinco repetições com 11 tratamentos: ivermectina, água destilada e folhas desidratadas de nove espécies de plantas (333,3 mg g<sup>-1</sup> cultura fecal). Após sete dias, as folhas desidratadas de *Piptadenia viridiflora* e *Ximения americana* foram eficazes em 86,5 e 99,8 % respectivamente reduzindo significativamente o número de larvas infectantes presentes na cultura em comparação com o controle com água destilada estéril. A presença de taninos e flavonóides do extrato aquoso e etanólico foi indicado por HPLC (*High-performance liquid chromatography*). Os extratos de *P. viridiflora* e *X. americana* (0,075-2,4 mg mL<sup>-1</sup>) apresentaram eficácia de 69.6-100% no teste de eclodibilidade. Na inibição do desenvolvimento larval o extrato aquoso de *P. viridiflora* (1,21-38,62 mg g<sup>-1</sup>) foi eficaz entre 55,63-100%, para *X. americana* (1.42-22.70 mg g<sup>-1</sup>) foi eficaz entre 65,63-100% e significativamente menor que o observado para o controle com água (P<0,05) para ambas as espécies. A eficácia antihelmíntica *in vivo* foi entre 32,9-47,2% para *P. viridiflora*, para *X. americana* não houve eficácia. Valores de hemácias e hematócrito ficaram dentro dos padrões fisiológicos normais para ovinos tratados com *P. viridiflora*, para albumina plasmática e proteína total, efeitos significativas entre os tratamentos, não foram encontrados. *P. viridiflora* mostra potencial promissor como tratamento alternativo de haemonchoses. As baixas concentrações de extratos das folhas dessa espécie mostram alta eficácia para eclosão e desenvolvimento larval. O extrato aquoso administrado a 283 mg (ms) kg<sup>-1</sup> de peso corporal durante três dias consecutivos promove eficácia de 47,15% para a redução da contagem de ovos nas fezes e sem distúrbios clínicos e sem alterações no sangue.

**Palavras-Chave:** Savana brasileira. Plantas antihelmínticas. Composição fitoquímica. Toxicidade. Parâmetros sanguíneos. *Haemonchus contortus*.

**Plants of the cerrado selected by grazing sheep with potential for inhibition of larva development of *Haemonchus contortus***

**Abstract**

Plant species naturally selected by sheep grazing in the Cerrado region of Brazil were assessed for *in vitro* or *in vivo* activity against *Haemonchus contortus*. After one year of observations, the plant families showing greatest richness in the region were Fabaceae, Rubiaceae, Malpighiaceae, Bignoniaceae, Myrtaceae and Annonaceae. Variation in the selectivity index for nine commonly plant species selected by grazing sheep was observed with respect to the dry and rainy seasons in the Cerrado. Coproculture was conducted in five replicates of 11 treatments: ivermectin, distilled water, or dehydrated leaves of nine selected plant species administered at 333.3 mg g<sup>-1</sup> fecal culture (FEC). After seven days, the dried powder of *Piptadenia viridiflora* and *Ximenia americana* leaves, were effective at 86.5 and 99.8%, respectively, reducing significantly reduced the number of infective larvae compared to the control with sterile distilled water. The presence of tannins and flavonoids for aqueous and ethanolic extracts was indicated by HPLC (*High-performance liquid chromatography*). The extracts of *P. viridiflora* or *X. americana* (0.075-2.4 mg mL<sup>-1</sup>) was between 69.6-100% efficacy in the egg hatch inhibition (EHI). In larval development inhibition (LDI), the aqueous extract *P. viridiflora* (1.21-38.62 mg g<sup>-1</sup>) anthelmintic efficacies were between 55,63-100%, *X. americana* (1.42-22.70 mg g<sup>-1</sup>) efficacies were between 65,63-100% and showed mean of L3 and significantly lower than those observed for the control with water (P<0.05), for both species. The *in vivo* anthelmintic efficacies were between 32.9 - 47.2% for *P. viridiflora*, no efficacy for *X. americana*. Erythrocyte and hematocrit values were within the normal physiological patterns for lambs for *P. viridiflora*. For plasmatic albumin and total protein, significant effects between treatments were not found. *P. viridiflora* shows promising potential as alternative treatment of haemonchosis. The low concentrations of leaf extracts show high efficacy to EHI and LDI. Aqueous extract administered at 283 mg (dm) kg<sup>-1</sup> bw during three consecutive days promotes efficacy 47,15% to FEC reduction and no clinical or blood disorders.

**Key words:** Brazilian savannah. Anthelmintic plants. Phytochemical composition. Toxicity. Blood parameters. *Haemonchus contortus*.

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## 1 **1 Introdução**

2

3 A ovinocultura pode constituir uma alternativa econômica e viável em regiões do  
4 Brasil, gerando emprego e renda familiar, bem como fixando o homem no campo. No  
5 norte e nordeste de Minas Gerais, pode-se observar a situação precária do manejo  
6 sanitário adotado nos criatórios (Sebrae, 2004). Há dificuldades de realização de exames  
7 laboratoriais, seja pela falta de técnicos ou pela falta de laboratórios regionais. Vieira  
8 (2003), ao pesquisar propriedades no norte de Minas Gerais, verificou que em 94,9%  
9 dessas propriedades nunca tinha sido feito nenhum exame parasitológico nos rebanhos.

10 O principal problema encontrado na ovinocultura, e que limita o aproveitamento  
11 econômico desses animais são, as parasitoses gastrintestinais. A alta prevalência e  
12 grande patogenicidade fazem de *Haemonchus contortus* uma das principais espécies de  
13 endoparasitas de ovinos no Brasil e no mundo. *H. contortus* é o mais patogênico  
14 parasita do abomaso e alimenta-se de sangue durante toda vida parasitária. Os ovinos  
15 com haemoncoses podem apresentar anemia e edema submandibular e casos de  
16 mortalidade em filhotes e fêmeas parturientes ocasionadas por esses parasitos são  
17 relativamente comuns (Bizimenyera et al., 2006).

18 O tratamento frequente dos rebanhos com antihelmínticos tem sido a única  
19 medida de controle da verminose adotada pela maioria dos criadores. A administração  
20 constante e em doses inadequadas favorece a seleção de populações de parasitas  
21 resistentes aos princípios ativos e contribui para a contaminação dos produtos de origem  
22 animal com resíduos das drogas utilizadas (Amarante et al., 1992).

23 Os primeiros estudos referentes à resistência dos helmintos estão relacionados  
24 com o grupo dos benzimidazóis, imidazotiazóis e posteriormente as avermectinas, que  
25 surgiram como uma alternativa de tratamento que tem sido considerada até hoje, como

1 um princípio ativo potente, para o controle das parasitoses de animais domésticos  
2 (Gopal et al., 1999). Muitas propriedades no país já apresentam populações resistentes  
3 a todas as bases disponíveis e esse fenômeno tem inviabilizado a exploração dos ovinos  
4 nessas áreas, pois enfrentam problemas que promovem queda na produção,  
5 disponibilidade e qualidade dos alimentos, deficiência mineral, manejo inadequado e  
6 elevada incidência das helmintoses gastrintestinais, o que ocasiona grandes perdas  
7 econômicas (Araújo e Lima, 2005; Geraseev et al. 2011; Duarte et. al., 2012).

8 A fitoterapia no controle de verminose é uma alternativa que poderá reduzir o  
9 custo com a aquisição de antihelmínticos bem como prevenir o aparecimento de  
10 resistência antihelmíntica e a presença de resíduos nos produtos de origem animal.  
11 Muitas espécies vegetais são tradicionalmente conhecidas como possuidoras de  
12 atividade antihelmíntica, necessitando, entretanto, que suas eficácias sejam  
13 cientificamente comprovadas (Vieira, 2003).

14 Em consequência da intensa exploração econômica do Cerrado, boa parte da  
15 vegetação nativa foi derrubada e muitas espécies estão ameaçadas de extinção, as quais  
16 podem apresentar ampla utilização e manutenção da população local por seu valor  
17 alimentício, medicinal, ornamental, oleaginoso e tanífero. Entretanto, poucos estudos  
18 têm avaliado o efeito antihelmíntico dos compostos químicos das espécies vegetais do  
19 Cerrado para o controle das helmintoses.

20 Os taninos podem exercer ação antihelmíntica direta, ao interferir no ciclo natural  
21 dos helmintos, ou indireto, ao proteger a proteína ingerida da degradação ruminal (com  
22 incremento da disponibilidade protéica no trato gastrintestinal inferior), o que dificulta a  
23 determinação do seu real efeito antiparasitário (Ketzis *et al.*, 2006).

24 A validação científica de novas alternativas de produtos antihelmínticos é uma  
25 etapa inicial e obrigatória para testes *in vitro* e *in vivo* com espécies vegetais, o que vai

1 permitir uma análise da caracterização de novos compostos ativos, possibilitando novas  
2 perspectivas para o controle das endoparasitoses de ovinos ou de outros animais  
3 domésticos.

4 Portanto, o estudo do uso alternativo de extratos de espécies vegetais no controle  
5 de helmintos em pequenos ruminantes pode constituir uma estratégia promissora para a  
6 indústria biotecnológica nacional e conseqüentemente para os criadores.

7

## 8 **2 Justificativa**

9

10 Um dos principais problemas limitantes para a ovinocultura e caprinocultura são as  
11 parasitoses gastrintestinais. Todas as categorias de ovinos podem ser intensamente  
12 parasitadas por helmintos, reduzindo não somente o ganho de peso, mas também a  
13 produção de leite, lã e pele. A alta prevalência e grande patogenicidade fazem de  
14 *Haemonchus contortus* e *Trichostrongylus colubriformis* uma das principais espécies de  
15 endoparasitas de ovinos no Brasil e no mundo.

16 *Haemonchus contortus* parasita a mucosa do abomaso e alimenta-se de sangue  
17 durante toda vida parasitária, ocasiona anemia, edema submandibular e elevadas taxas  
18 de mortalidade em filhotes e em fêmeas parturientes. O tratamento frequente do rebanho  
19 ovino com antihelmínticos sintéticos tem sido uma das únicas medidas de controle dos  
20 nematódeos gastrintestinais adotada pelos criadores de ovinos.

21 A utilização de antihelmínticos, além de elevarem o custo de produção,  
22 compromete o ecossistema através da persistência de seus resíduos no ambiente e nos  
23 produtos de origem animal, que de forma extremamente efetiva induzem a seleção de  
24 cepas de parasitos resistentes.

1 Assim, ao observar a dieta selecionada por ovinos naturalmente em área de  
2 Cerrado, Costa (2010), avaliou pelo índice de seletividade as espécies vegetais mais  
3 consumidas por esses animais durante a estação seca e chuvosa: *Acosmium dasycarpum*  
4 (Vogel) Yakovlev (Fabaceae), *Baccharis tridentata* Vahl. (Asteraceae), *Casearia*  
5 *sylvestris* Sw. (Salicaceae), *Paullinia* sp., (Sapindaceae), *Ximenia americana* L.  
6 (Olacaceae), *Erythroxylum deciduum* A.St.-Hil. (Erythroxylaceae), *Heteropterys*  
7 *byrsonimifolia* A. Juss. (Malphigiaceae), *Lippia sidoides* Cham. (Verbenaceae),  
8 *Schinopsis brasiliensis* Engl. (Anacardiaceae), *Evolvulus* sp. (Convolvulaceae), *Senna*  
9 *spectabilis* (DC.) H.S.Irwin & Barneby (Fabaceae) e *Piptadenia viridiflora* (Kunth)  
10 Benth (Fabaceae).

11 Contudo, poucos estudos têm avaliado o efeito antihelmíntico dos metabólicos de  
12 espécies vegetais, desse modo, emerge a necessidade da utilização de produtos naturais,  
13 a base de espécies vegetais encontradas nas regiões onde ocorre o pastejo de  
14 ruminantes. Sendo assim, torna-se importante testar novas bases farmacológicas para o  
15 controle desses parasitos.

16

### 17 **3 Objetivos**

#### 18 **3.1 Objetivo geral**

19

20 Avaliar o potencial antihelmíntico de espécies vegetais do Cerrado selecionadas  
21 naturalmente por ovinos no norte de Minas Gerais.

22

#### 23 **3.1 Objetivos específicos**

24

- 25 ➤ Realizar o levantamento fitossociológico das espécies vegetais do cerrado.
- 26 ➤ Identificar as espécies selecionadas por ovinos em pastejo no cerrado.

- 1       ➤ Selecionar espécies vegetais do cerrado, com eficácia para inibição de  
2       desenvolvimento larval.
- 3       ➤ Avaliar, *in vitro*, a inibição do desenvolvimento larval de *H. contortus* de  
4       ovinos, sob a concentração de extratos de espécies vegetais do Cerrado.
- 5       ➤ Determinar a DL 90 de dois extratos vegetais para a redução da eclosão de *H.*  
6       *contortus* de ovinos.
- 7       ➤ Identificar e quantificar os compostos químicos presentes em espécies vegetais  
8       do Cerrado, com eficácia antihelmíntica.
- 9       ➤ Testar a toxicidade das espécies vegetais selecionadas em camundongos.
- 10      ➤ Avaliar os parâmetros sanguíneos em ovinos tratados com uma espécie do  
11      Cerrado.
- 12      ➤ Testar, *in vivo*, a inibição do desenvolvimento larval de *H. contortus* de ovinos,  
13      sob a concentração de extratos vegetais do Cerrado.

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1    **CAPTER 1**

2    **Plants from Cerrado for the Control of Gastrointestinal Nematodes of Ruminants**

3

4    **Abstract:** The gastrointestinal helminthes are major limiting factors for the sheep and  
5    goat production in the world and the health of livestock depends of effective control of  
6    nematodes. The constant administration and inadequate doses of chemical anthelmintics  
7    favors the selection of resistant populations and residues these products contribute to the  
8    contamination of animal products and of the ambient. The use of herbal treatment in  
9    veterinary medicine is a promising field of research. Studies in this area require the  
10   insertion into an agroecological context, with the limiting factor to the sustainable  
11   management of natural resources involved. The phytotherapy for the parasite control is  
12   an alternative that can reduce the cost with the purchase of anthelmintics as well,  
13   preventing the emergence of anthelmintic resistance and residues in animal products.  
14   Plant species that have tannins in its constitution are known to possess anthelmintic  
15   activity, requiring, however, that their efficacies are scientifically proven. The Cerrado  
16   is an import biome with high diversity of plants rich in tannins and other metabolic with  
17   potential anthelmintic effect. This study presents a review of research on plant species,  
18   tested in the Cerrado for the control of helminths in ruminants.

19

20   **Keywords:** anthelmintic, nematodes, medicinal plants, Cerrado, ruminants.

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## 1 **1 Introduction**

2       The main problem in the small ruminants and limiting of economic exploitation is  
3 the gastrointestinal parasites. *Haemonchus contortus* is a nematode of abomasum and  
4 feeds of blood throughout, with high prevalence and high pathogenicity (Strong, 1993).  
5 Sheep with haemoncoses may show anemia and submandibular edema, with high  
6 mortality in young lambs and females in peripartum. Both sexes at all age levels may be  
7 intensely affected, reducing weight gain and reproductive capacity, as well as milk,  
8 wool, and hide production (Bizimenyera et al., 2006).

9       The treatment with anthelmintics has been intensely used to control by breeders.  
10 The constant administration and the inadequate dosages can favor the selection of the  
11 parasite populations resistant to the anthelmintics and contributes to the contamination  
12 of animal products with residues of these products (Amarante et al., 1992).

13       The main anthelmintics were developed during the 60's and are actually essential  
14 to control of nematodes. There are currently only three groups of broad spectrum  
15 anthelmintics and two groups of small spectrum used to control these parasites  
16 (Amarante et al., 1992). Early studies reported resistant helminthes to the group of  
17 benzimidazole and levamisoles. With the discovery of a chemical group distinct  
18 anthelmintic, avermectins, was represented an alternative treatment with a potent drug  
19 for the nematode control in domestic animals (Gopal et al., 1999). Multi-resistant  
20 nematodes have been found on several ruminant herds (Molento and Prichard 2001;  
21 Taylor et al., 2009; Wolstenholme et al., 2004; Thomaz-Soccol et al., 2004) . The  
22 possibility of anthelminthic residues in the environment and in animals reared for  
23 consumption (Hammond et al., 1997), as well as the spread of multi-resistant strains  
24 demands research into alternatives for gastrointestinal nematodes (GIN) control.

1           The utilization of plants containing secondary compounds such as condensed  
2 tannins may expand the organic alternatives to controlling GINs (Athanasiadou et al.,  
3 2007; Kahn and Diaz-Hernandez 2000). Phytotherapy in the control of parasitism is an  
4 alternative that can reduce the cost with the purchase of anthelmintics, and prevent the  
5 emergence of anthelmintic resistance and the presence of residues in animal products.  
6 Many plants are traditionally known as having anthelmintic activity, requiring,  
7 however, that their efficacy be scientifically proven (Vieira, 2003). Scientific validation  
8 of the anthelmintic effects and possible side-effects of plant products is necessary prior  
9 to their adoption as novel methods for control (Githiori et al., 2006).

10           The Cerrado biome, which covers 5% of the world flora, is the second largest  
11 source of biodiversity in Brazil (Sano, 2008). However, much of the native vegetation  
12 has been destroyed and many species are threatened of extinction, which would enable a  
13 wide use and maintenance of food, medicinal, ornamental, linseed and tannin  
14 production.

15           However, few studies have evaluated the anthelmintic effect of the plant species  
16 of the Cerrado for the control of GNI. Therefore, the analysis of potential plant species  
17 of this biome for helminthes control for ruminants may represent a promising strategy  
18 for the biotechnology industry and consequently for the breeders of these animals.

19

## 20 **2 The Cerrado**

21

22           Among the vegetation types that cover the American continent, the Cerrado  
23 presents a natural grandeur of plant species, which demonstrates the importance of  
24 education for the conservation and management of this biome. The original vegetation  
25 of the Cerrado has already been reduced by over 37 % (Felfili et al., 2002), prejudicing

1 much of its biodiversity. Mittermeier et al. (1999) estimated that 67 % of the Cerrado  
2 areas are considered “highly modified” and only 20 % are in original condition, since  
3 the changes began with the colonization process, with the introduction of cattle,  
4 associated with rudimentary agricultural practices (Zanetti, 1994).

5 According Eiten (1993), the Cerrado’s flora is composed of two groups of  
6 species: thick stem trees and bushes with an undergrowth layer, consisting in a large  
7 mosaic, which includes a forest canopy formation more or less closed, containing trees  
8 with heights of 12 m tall or more. It has a woodland category, usually around six or  
9 seven meters and undergrowth stratum more or less continuous.

10 The herbaceous and shrub form a thick layer, especially grasses, making it  
11 difficult to distinguish individuals in both the layers as woodlands or as herbaceous due  
12 to many overhead structures being in accordance with shoots from the same root (Felfili  
13 et al., 2002).

14 The Cerrado *sensu strictu* is characterized by the presence of bent and twisted low  
15 trees; the shrub and herbaceous strata exhibits rapid growth during the rainy season  
16 (Ribeiro and Walter, 2008). These authors report that the Cerrado species have bent,  
17 twisted and gnarled timber trunks, leathery and rigid leaves, as adaptations to the dry  
18 environmental conditions. The most common species are represented by the  
19 Vochysiaceae and Fabaceae families, as well as species of Malpighiaceae,  
20 Anacardiaceae, Salicaceae, Rubiaceae (Felfili et al., 2002, Miranda et al., 2006), among  
21 others such as those represented by Caryocaraceae and Annonaceae families (Sales et  
22 al., 2009a; Sales et al., 2009b).

23 In regards climate, the average temperatures in the Cerrado areas vary between  
24 22° C and 27 ° C ( Klink and Machado, 2005) , with average annual rainfall of 1,500  
25 mm , water deficiency ranging from three to seven months of the year, depending on the

1 region's seasonal (Nimer, 1989). The Cerrado's vegetation occurs predominantly in  
2 deep and well drained soil (Reatto et al., 1998), which present a lack of nutrients such as  
3 phosphorus and nitrogen , the pH being between 4.5 and 5.5, with high aluminum  
4 frequency rates (Ribeiro and Walter, 2008).

5

### 6 **3 Anthelmintic Efficacy of Plant Species from Cerrado for Control of** 7 **Gastrointestinal Nematodes**

8

9 In recent years, society has prioritized environmental aspects, directing ample  
10 research towards the discovery of new bioactive substances that may be used in  
11 integrated pest management, with fewer negative effects on the environment (Castro,  
12 1989).

13 In an attempt to contribute with an effective alternative control of gastrointestinal  
14 nematodes in small ruminants, several researchers have attempted to test plants used in  
15 folk medicine, evaluating the efficacy and safety of the same. Plant species rich in  
16 tannins called secondary metabolites have been extensively studied. The tannins  
17 anthelmintic action may act directly by interfering with the natural cycle of helminths,  
18 or indirectly, to protect the protein intake of ruminal degradation (with increased of  
19 protein availability in the lower gastrointestinal tract), which complicates the  
20 determination of its actual antiparasitic effect (Ketzis et al., 2006).

21 Furthermore, the results of *in vivo* tests conducted with these forages can be  
22 influenced by natural variations in the composition of the plant (by environmental  
23 factors or of their own cycle) that alter the concentration of the tannin intake by the  
24 animals (Athanasiadou and Kyriazakis, 2004).

1           The anthelmintic activity, attributed to tannins is present in plant species (Hoste et  
2 al., 2006). Calderon-Quintal et al. (2010) suggest that different strains of *H. contortus*  
3 show different sensitivities to the extracts rich in tannin and further studies are needed  
4 to confirm the *in vivo* results.

5           *Chenopodium ambrosioides* L. (Chenopodiaceae) “erva-de-santa-maria”,  
6 popularly known for its anthelmintic efficacy is a plant of the Chenopodiaceae family,  
7 with stem one meter tall with leaves shaped like spears with sinuous edges. The flowers  
8 are greenish, clustered in a small bouquet. From the leaves and flowers of this plant may  
9 be extracted an essential oil consisting of a mixture of mainly ascaridiol, silvestreno and  
10 safrole, and p-cymene and isohametina. The essential oil contains 60-80 % of ascaridiol  
11 with proven anthelmintic potential, abundant in the fruit, followed by flowers and  
12 leaves (Oliver-Bever, 1983).

13           Ketzi et al. (2002), working with essential oil of *C. ambrosioides* (0.2 mL/Kg<sup>-1</sup> of  
14 body weight) achieved similar thiabendazole efficacy, promoting the impracticability of  
15 all the hatched larvae of *Haemonchus contortus* in sheep. However, Vieira (1992) noted  
16 no effect when administered infused orally to cattle.

17           The Annonaceae family includes about 50 genera and the genus *Annona* being  
18 one of the most important. The Annonaceae is characterized mainly by presenting a  
19 class of acetogenin substances. These substances are derived from long chain fatty  
20 acids, which act as potent inhibitor of mitochondrial respiration (Wang et al., 2002).  
21 The biological activities of *Annona* extracts have been attributed to the occurrence of  
22 annonaceous acetogenins, a class of natural compounds extracted from leaves (Geum-  
23 Soog et al., 1998; Wu et al., 1995) and seeds (Chang and Wu, 2001).

24           *Annona squamosa* L. (Annonaceae), known as the Earl fruit, “pinha” or “ata” are  
25 trees that can reach up to 5 m in height with long, thin and oval leaves. Its flowers have

1 a greenish yellow color, adapting well to climates with little rain and with a well-  
2 defined dry season (Morton, 1987). Amorim et al. (1996) evaluated the aqueous extract  
3 from *A. squamosa* leaves, *in vitro*, on the first larval stages of gastrointestinal  
4 nematodes of cattle, obtaining mortality of 19.4 %. Vieira and Cavalcante (1999) tested  
5 *A. squamosa in vivo* on gastrointestinal nematodes in goats. The plant reduced by 40%  
6 the count of *H. contortus* eggs in feces. With respect to adult forms of the parasites, *A.*  
7 *squamosa* showed reduction rates in the population of *H. contortus* and  
8 *Trichostrongylus columbriformis* of 21.8 % and 31.4 %, respectively, however not  
9 reducing the *Strongyloides papillosus* population. Yet according to the authors, the  
10 extract showed still to be effective against the adult form of *Oesophagostomum*  
11 *columbianum*, reducing by 74 % the parasites. The acute toxicity of plants from the  
12 Annonacea family is still poorly studied and research approaches its *in vitro* cytotoxic  
13 efficacy and with possible emphasis on the anti-tumor effects (Vieira and Cavalcante,  
14 1999).

15 *Annona muricata* L. (Annonaceae), popularly known as graviola, is a medium -  
16 sized fruit tree commonly found in the tropics. The species has been widely used in folk  
17 medicine as an anthelmintic, antipyretic, sedative, antispasmodic, and anticonvulsant  
18 and as a hypotensive agent in humans (Costa et al., 2002). *In vitro* tests to evaluate the  
19 inhibition of egg hatching, larval and adult worm motility are widely used in  
20 prospecting for new anthelmintic agents (Vasconcelos et al., 2007).

21 Ferreira et al. (2013), researching *H. contortus* in sheep, demonstrated that  
22 aqueous extract of *A. muricata* leaves at 50, 25, 12.5 and 6.25% concentrations  
23 inhibited larval hatching in 84, 9 , 79, 1, 66, 9 and 47.42 %, respectively. The authors  
24 also evaluated the effect on the motility of L3 *H. contortus* larvae at the same  
25 concentrations and obtained reduction motility rates at 83.29 %, 89.08 %, 74.62 % and

1 30.47 %, indicating significant activity of *A. muricata* on infective larvae of this  
2 parasite. However, when were evaluated the activity of the extract on the motility of  
3 adult parasites, the response was not dependent on dosage, being able to observe the  
4 extracts activity at different concentrations within the first six hours of exposure.  
5 Phytochemical analysis did not reveal any type of acetogenins or even alkaloids in the  
6 extract but indicated the presence of phenolic compounds in the aqueous leaf extract of  
7 *A. muricata* (Ferreira et al., 2013)

8 Furthermore, since acetogenins have been associated with neurodegeneration in  
9 rats and in humans (Champy et al., 2004) the absence of acetogenins in the *A. muricata*  
10 extract is a somewhat of a motivating fact, because it can make for this aqueous extract  
11 a safe drug to treat targeted animals if compared with plant extracts prepared with  
12 organic solvents presuming the extraction of acetogenin (Ferreira et al., 2013).

13 *Annona crassiflora* Mart. (Annonacea) commonly known as “panã”, “araticum”,  
14 “cabeça de negro” , “cascudo”, “cortiça”, “marolo” ou “pinha do cerrado”, stands out  
15 due to the fruit’s flavor, and is used in alternative medicine for possessing antibacterial  
16 and antifungal properties (Almeida et al., 1998). It is characterized by being a timber  
17 tree species, deciduous in the dry season, hermaphrodite and xerophytic. The phenology  
18 of this species is established by flowering early in the rainy season, which occurs from  
19 September to December, with fruiting having started in November, with ripened fruit  
20 from January to March (Lorenzi and Matos, 2002). The fruits are used as food and  
21 appreciated for having a sweet and yellowish pulp with a strong aroma (Roesler et al.,  
22 2007).

23 Queiroz et al. (2012), using ethanol extract from the leaves of *A. crassiflora*  
24 verified the action of this extract on *H. contortus* larval development in sheep at 100  
25 and 50 mg/mL<sup>-1</sup> mg/mL<sup>-1</sup>. The authors also obtained an anthelmintic efficacy superior



1 to 98.6 % for the larval development of *H. contortus*, using dried leaves of the same  
2 plant at a coproculture concentration of 333.3 mg (ms)/mL<sup>-1</sup>. The aqueous extract from  
3 the seeds and leaves of *A. crassiflora* showed anthelmintic efficacy of 99.43 % and  
4 89.81 %, respectively at 100 mg/mL<sup>-1</sup> (Nogueira, 2009), presenting a promising  
5 alternative for the control of *H. contortus* in sheep.

6 In Southeastern region of Brazil, and especially in the North and Northeast, the  
7 “cajazeira” (*Spondias mombin* L.) also known as “caja-mirim”, “ambaró”, “taperebá”, is  
8 a fruit species belonging to the Anacardiaceae family. Utilized as source of permanent  
9 shading for the cocoa tree, it is also utilized by producing fruits that serve as an  
10 important source of additional income for the producer. The fruits’ juicy, yellow, sour  
11 and aromatic properties are appreciated in refreshments and liquors (Sacramento, 2000).

12 The use of the “cajazeira” in folk medicine and by the pharmaceutical industry has  
13 increased, being utilized in the treatment of fevers, as an antidiarrheal, antidesintérico,  
14 antiblenorrágico and anti-hemorroidiário. According Sacramento (2000), research has  
15 recently revealed that the leaf extract contains ellagic tannins giving the plant antiviral  
16 properties. Ademola et al. (2005), using the *S. mombin* aqueous and ethanol extract  
17 against *H. contortus*, obtained a reduction of approximately 65% of eggs found in the  
18 sheep feces (OPG) at a 500 bw mg/Kg<sup>-1</sup> concentration.

19 *Lippia sidoides* Cham. (Verbenaceae) or alecrim pimenta is a species often used  
20 as herbal medicine in Northeast of Brazil, due to the antiseptic action owing to the high  
21 levels of thymol and carvacrol (Matos and Oliveira, 1998). According Camurça-  
22 Vasconcelos et al. (2007) and Vasconcelos (2006), the essential oil of *L. sidoides*  
23 possess an inhibitory effect in vitro on *H. contortus* eggs in sheep at 0.02 mg/mL<sup>-1</sup> to  
24 1.25 mg/mL<sup>-1</sup>, respectively. Souza et al. (2010), Bevillaqua et al. (2005), and Person  
25 (2001), obtained same results using this oil at 0.5% and 1%, respectively. In tests

1 conducted *in vivo*, Camurça-Vasconcelos et al. (2008), reported an efficacy of 54%  
2 from the oil of *L. sidoides* in the control of *H. contortus* in sheep at a 283 mg/Kg<sup>-1</sup>, 14  
3 days after treatment.

4 The genus *Caryocar*, one of the representatives of the family Caryocaraceae  
5 family, has 16 species that are found in South and Central America (Maya et al., 2008).  
6 *Caryocar brasiliense* Cambess. specie is a tree species native to the Cerrado regions  
7 with wide distribution in the Southeast and Midwest of Brazil (Maia et al., 2008). The  
8 popular name of this plant species may vary according to the region of occurrence, the  
9 most common being: “Pequi”, “Piqui”, “piquiá-bravo”, “amêndoa de espinho”, “grão de  
10 cavalo”, “pequiá”, “pequiá-pedra”, “pequerim”, “Suari” and “piquiá” (Santos et al.  
11 2004). Fruiting is annual and harvesting occurring in the period lasting from September  
12 to February (Vera et al., 2005).

13 The aqueous extract from the *C. brasiliense* fruit peels, at 200 mg/ml<sup>-1</sup>,  
14 significantly inhibited the development of *H. contortus* larvae in sheep. The plant  
15 extracts effectiveness in the inhibition of larval development was of 94.8%. The egg-  
16 hatching inhibition of LC50 and LC90 was of 23.82 and 53.19 mg/mL<sup>-1</sup>, respectively.  
17 The qualitative phytochemical tests performed in this study indicated the presence of  
18 catechins, steroids, flavonoids, saponins, total tannins, xanthones and tannins  
19 catechetical (Nery, 2009).

20 Nogueira et al. (2012) evaluated the aqueous extract of *C. brasiliense* fruit's skin  
21 in the egg hatching inhibition test, with concentrations at 15 and 7.5 mg/ml<sup>-1</sup>, reported  
22 anthelmintic efficacy corresponding to 98.7 % and 91.8 %, respectively. For these  
23 concentrations, the average L1 were significantly lower than treatment with distilled  
24 water or albendazole. The average for unembryonated eggs observed in all the  
25 treatments by extract was not different from the distilled water control and suggests that

1 “Pequi” metabolites do not inhibit the embryogenesis of these nematodes, while they  
2 may reduce hatching. The egg-hatching inhibition of LC50 and LC90 were 3.81 and  
3 7.35 mg mL<sup>-1</sup>, respectively.

4 *In vivo*, the average fecal egg count observed for the groups treated with the  
5 aqueous extract fruit peels of “Pequi” differed from the untreated group at concentration  
6 2 g Kg<sup>-1</sup> bw. During the first and second weeks of post treatment, it was observed a 33  
7 and 32.2 % of anthelmintic efficacy *in vivo*, respectively, compared to pretreatment  
8 when all animals showed high levels of infection (Nogueira et al., 2012).

9 The crude powder derived from the “Pequi” fruit peels and leaves showed high  
10 efficiency (superior to 90 %) for the inhibition of larval development (LPGF) of *H.*  
11 *contortus* in sheep. The average LPGF for the concentrations at 250, 200 and 150  
12 mg/mL were statistically similar to those observed for the control with the commercial  
13 anthelmintic. The aqueous extract from the leaves of the “Pequi” showed higher  
14 anthelmintic action within seven days of incubation. The lethal concentrations of LC50  
15 and LC90 after seven days of incubation were 34.95 and 79.74 mg/mL, respectively, for  
16 the crude powder of the fruit peels and 69.05 and 97.19 mg/mL for the crude powder  
17 from the leaves. For the aqueous extract of the leaves, the LC50 and LC90 were 56.36  
18 and 115.65 mg mL<sup>-1</sup>, respectively (Fonseca, 2012).

19 Morais-Costa et al. (2012) compared the efficacy of *C. brasiliense* from the  
20 northern and central region of Minas Gerais in Brazil. For both regions, the  
21 concentration 333.33 mg/mL<sup>-1</sup> of dried leaves of *C. brasiliense* showed higher efficacy  
22 than negative control with distilled water and showed anthelmintic activity similar to  
23 the control with ivermectin (16 µ mL<sup>-1</sup>). The dried leaves of this plant from northern  
24 and central region had anthelmintic action with efficacy of 98.52 % and 83.09 %  
25 respectively. This difference could be related to vegetation/area where the species were

1 collected, since the area of vegetation in the northern region is a native and preserved  
2 area, which favors better performance and establishment of plant species in the Cerrado.

3 The species *Anacardium occidentale* L. belonging to the Anacardiaceae family, is  
4 popularly known as cashew tree (cajueiro). It is native to Brazil and used in traditional  
5 medicine, especially in northeastern Brazil due to its therapeutic effects. In the  
6 literature, there are proven pharmacological activities, as the cajueiro being anti-  
7 inflammatory plant (Olajide, 2004), ant diabetic (Barbosa-Filho et al., 2005), inhibitor  
8 of acetylcholinesterase (Barbosa-Filho et al., 2006) and antimicrobial (Akinpelu, 2001).

9 Aiming to evaluate the anti-parasitic activity of *Anacardium humile* A. St. - Hil.  
10 (Anacardiaceae). Nery et al. (2010), used aqueous and ethanolic extracts of leaves  
11 against different species of gastrointestinal nematodes in sheep. The aqueous extract  
12 anthelmintic activity showed significantly higher than negative control at all  
13 concentrations. At concentrations of 150 and 187.5 mg/mL<sup>-1</sup>, the percent efficacy was  
14 not significantly different from ivermectin (positive control, 16 mg/mL<sup>-1</sup>). The LD50 in  
15 the inhibition assay for larval development was 10.14 mg/mL<sup>-1</sup>, and for the 5 %  
16 confidence interval it was 13.36-6.83 mg/mL<sup>-1</sup>. Results of the ethanolic extract were not  
17 significantly different from ivermectin at 60 mg/mL<sup>-1</sup>. The LD50 was mg/mL<sup>-1</sup> 23.24.  
18 Larvae of *Haemonchus* spp. (68%), *Strongyloides* spp. (31%) and *Trichostrongylus* spp.  
19 (1%) were identified in the coprocultures of the negative control group. This suggests  
20 that the extracts were effective against the three nematodes considered to be the most  
21 prevalent and pathogenic in sheep (Ueno and Gonçalves, 1998).

22 Morais-Costa et al. (2012), in preliminary study, the activity of anthelmintic was  
23 evaluated to *Paullinea* sp. on gastrointestinal nematodes of sheep. The leaves of this  
24 plant were collected in the city of Montes Claros, Brazil. In this study, *Paullinea* sp. at  
25 333.3 mg/mL<sup>-1</sup> differed from the treatment with distilled water and showed anthelmintic

1 activity of 70.12 %, similar to treatment with ivermectin. In the control group, 100% of  
2 larvae were *Haemonchus* sp.. The anthelmintic activity of the Sapindaceae family and  
3 the species *Paullinea* sp. may be associated with saponin and tannin respectively.

4 The “genipapo” (*Genipa americana* L.), Rubiaceae family, tree that has been used  
5 in folk medicine, foods and animal feed , leather tanning, forestry, and by logging  
6 industries. The species, native to South America, has ecological importance, and is  
7 suitable for planting in degraded areas and wetlands (Epistein, 2001). In the egg  
8 hatching inhibition test, the aqueous extract of *G. american* leaves at 100 mg/mL,  
9 completely inhibited hatching. The relative average number of embryonated eggs was  
10 significantly greater than those of unembryonated eggs at 75 and 100 mg/mL<sup>-1</sup>. This  
11 observation suggests a greater efficacy in inhibiting hatching rather than interfering with  
12 embryo development. The LC50 and LC90 of aqueous extract from *G. American* leaves  
13 were 34.3 and 79.8 mg/mL<sup>-1</sup>, respectively. In the larval development inhibition test,  
14 concentrations  $\geq 30$  mg/mL<sup>-1</sup> showed anthelmintic efficacy above 94 %. The LC50 and  
15 LC90 mg/mL<sup>-1</sup> were 14.6 and 28.7, respectively (Nery, 2009).

16 This suggests that the extracts were effective against the several nematodes  
17 considered to be the most prevalent and pathogenic in sheep (Wood et al., 1995),  
18 showing a wide spectrum of action. However, using the hydro-alcoholic extract from  
19 the leaves of “genipapo”, Krychak-Furtado (2006) found 100 % efficacy for EHI at 50  
20 mg/mL<sup>-1</sup>, thus suggesting the metabolites extracted with alcohol could also show action  
21 against nematode eggs.

22 In an experiment conducted by Costa (2010), the species *Schinopsis brasiliensis*  
23 Engl. (Anacardiaceae), *Baccharis tridentata* Vahl . (Asteraceae), *Ximenia americana* L.  
24 ( Olacaceae ), *Lippia sidoides* Cham. (Verbenaceae), *Paullinea* sp . (Sapindaceae) were  
25 selected by ruminants in the Cerrado, which are considered to be anthelmintic and

1 tanniferous. The animals showed no worm problems during this research, but there is a  
2 need for *in vitro* and *in vivo* to better evaluate the effectiveness of these species.

3 It was reported by Morais-Costa et al. (2012) at a 333.3 mg/mL, the effectiveness  
4 of the dried leaves derived from the plant species *Evolvulus* sp. (Convolvulaceae),  
5 *Acosmium dasicarpum* (Vogel) (Fabaceae Faboideae) *Heteropterys byrsonymifolia* A.  
6 Juss. (Malphiaceae), *Lippia sidoides* Cham. (Verbenaceae), *Erythroxyllum deciduum*  
7 A.St.-Hil. (Erythroxylaceae), *Senna spectabilis* (DC.) H.S. Irwin & Barneby (Fabaceae),  
8 *Baccharis tridentata* Vahl. (Asteraceae), *Casearia sylvestris* Sw (Salicaceae), *Paullinea*  
9 sp. (Sapindaceae), *Piptadenia viridiflora* (Kunth) Benth (Fabaceae) and *Ximenia*  
10 *americana* L. (Olacaceae). After the logarithmic transformation and variance analysis, it  
11 was found that the average LD<sub>50</sub> for treatments with the dehydrated leaves, did not  
12 differ from the control by ivermectin, and the efficacies were: *X. Americana* (99.84%),  
13 *P. viridiflora* (85.77%), *Paullinea* sp. (70.12%) and *C. sylvestris* (43.63%). This effect  
14 can be attributed to condensed tannin concentrations at 10 mg of ethanol extracts from  
15 *C. sylvestris* (7.36%) *Paullinea* sp. (6.37%), *P. viridiflora* (1.75%) and *X. Americana*  
16 (0.36%). This study demonstrates a potent anthelmintic activity *in vitro*, for the ethanol  
17 extract. The fact that some species of this study have low condensed tannin content  
18 highlights the synergism among chemical compounds.

19

#### 20 **4 Final Considerations**

21

22 It is necessary to scientifically validate new alternative anthelmintic compounds,  
23 characterizing them in the control of ruminant GNIs, and to evaluate the toxicity of  
24 these compounds, *in vivo* experiments should be performed, providing some of the plant  
25 species to the animals. Thus, species rich in tannins, catechetic tannins, catechins,

1 steroids, flavonoids, xanthones and saponins have promising potential in the control of  
2 nematodes of ruminants and furthermore, can be active in synergism of these  
3 metabolites.

4 The species *Anacardium occidentale*, *Annona crassiflora*, *A. muricata*, *A.*  
5 *squamosa*, *Caryocar brasiliensis*, *Chenopodium ambrosioides*, *Genipa americana*  
6 *Lippia sidoides*, *Paullinea* sp., *Piptadenia viridiflora*, *Spondias monbin* and *Ximenia*  
7 *Americana* are adapted to the Cerrado and showed very promising results in reducing  
8 the bioactivity in the development of gastrointestinal nematodes of ruminants in Brazil.

9 In order to clarify the mechanisms of action from extracts of plant species, on the  
10 development of larvae using an electronic microscopy, would be a tool to support the  
11 study on reducing the use of chemical products, favoring lower incidences of residues in  
12 products of animal origin, thereby reducing costs and environmental impacts of these  
13 products in the environment.

14 Plant species rich in tannins, saponins and other secondary compounds, are  
15 deserving of further accurate studies to prove the scientific efficacy in controlling  
16 gastrointestinal parasites. Therefore, few studies have evaluated the metabolic  
17 anthelmintic effect of plant species from the Cerrado as well as possible toxicity effects.  
18 Thus, emerges the need to use natural products, based on plant species that are naturally  
19 selected by ruminants in this biome.

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1   **CAPTER 2**

2

3   **Plants of the Cerrado selected by grazing sheep with potential for inhibition of**  
4   **larva development of *Haemonchus contortus***

5

6   **Abstract** Plant species naturally selected by sheep grazing in the Cerrado region of  
7   Brazil were assessed for *in vitro* activity against *Haemonchus contortus*. After one year  
8   of observations, the plant families showing greatest richness in the region were  
9   Fabaceae, Rubiaceae, Malpighiaceae, Bignoniaceae, Myrtaceae and Annonaceae.  
10   Variation in the selectivity index for nine commonly plant species selected by grazing  
11   sheep was observed with respect to the dry and rainy seasons in the Cerrado.  
12   Coproculture was conducted in five replicates of 11 treatments: ivermectin, distilled  
13   water, or dehydrated leaves of nine selected plant species administered at 333.3 mg /g  
14   fecal culture. The dried powder of *Piptadenia viridiflora* and *Ximenia americana* leaves  
15   significantly reduced the number of infective larvae compared to the control with sterile  
16   distilled water. These species showed efficacy of over 85%, despite their low  
17   concentrations of proanthocyanidin. High-performance liquid chromatography analyses  
18   showed major peaks with UV spectra characteristic of flavonoids to extracts of these  
19   plants. Those naturally selected vegetal species with high anthelmintic efficacy show  
20   promise for use in diet as an alternative control of *Haemonchus contortus* in sheep.

21

22   **Keywords** Cerrado · vegetation structure · sheep grazing · phytochemical composition ·  
23   Semiarid · *Haemonchus contortus*

24

25

## 1 **1 Introduction**

2

3 The Cerrado is a region of savannah-type vegetation found in central Brazil,  
4 including the state of Minas Gerais. It is characterized by a high degree of diversity and  
5 endemism of species, accounting for 5% of the plant species on earth (Sano et al. 2008).  
6 Plant screening is recognized as a valuable strategy to identify new bioactive agents to  
7 develop new drugs for human or veterinary use.

8 Gastrointestinal nematode infections in sheep are a major cause of loss of  
9 production. *Haemonchus contortus* is the most prevalent and the most pathogenic  
10 species, being hematophagous in the abomasum (Arosemena et al. 1999). Infection can  
11 result in hemorrhagic anemia, dark-colored feces, edema, weakness, reduced production  
12 of wool and muscle mass, and sometimes sudden death (Taylor et al. 2007). Treatment  
13 with anthelmintics has been the only available measure to control haemonchosis (Paiva  
14 et al. 2001; Rangel et al. 2005). Their intensive use, often in sub-therapeutic doses  
15 associated with incorrect diagnosis, and the consistent use of a single pharmacological  
16 base, has favored the selection of multiresistant nematodes (Souza et al. 2008).

17 Plants extract can potentially be used as an alternative for reduction of costs and  
18 drug residues in animal products as well as to prevent the emergence of antihelmintic  
19 resistance. Some plants are traditionally believed to show antihelminthic activity, but  
20 their efficacy needs to be scientifically evaluated (Vatta et al. 2009).

21 Polyphenols, such as proanthocyanidins and tannins, are bioactive compounds  
22 found in several plant species that account for their pharmacological and nutraceutical  
23 properties. Antihelminthic activity of plants is credited to the presence of tannins (Hoste  
24 et al. 2006). To explore the potential of plants from the Cerrado for alternative

1 helminthosis control it is necessary to conduct bioassays and quantify chemical  
2 constituents of the species, especially polyphenols.

3 The objectives of this study were to identify plant species naturally selected by  
4 sheep grazing in the Cerrado and to assess *in vitro* antihelminthic efficacy of these  
5 species on *Haemonchus contortus*.

6

## 7 **2 Material and methods**

8

### 9 2.1 Study area

10

11 The research was conducted from January 2009 through March 2010 in a rural  
12 area in Montes Claros City of North Minas Gerais state, Brazil (W 43°50'33.56"; S  
13 16°41'10.05"). The climate of the region, tropical wet and dry (Aw) according to the  
14 Köppen classification, is marked by a long dry season from May to September and a  
15 rainy period in January and February. The monthly average rainfall and temperature  
16 during the dry season was 14.16 and 23.24°C, respectively. In the rainy period, the  
17 monthly average rainfall and temperature was 201.32 mm and 24.32°C. The area  
18 originally presents Cerrado vegetation, but actually is composed by patches of Cerrado  
19 in different stages of succession, due to the grazing of different domestic animals.

20

### 21 2.2 Collection and identification of plant species

22

23 The collection of tree, shrub, and herbaceous and trailing species in the area based  
24 on Müller-Dombois and Ellenberg (1974) and Menino et al (2012). Identification was  
25 carried out according to morphological characteristics described by Lorenzi (2000,

1 2002) and Lorenzi and Matos (2002). Phytosociological parameters such as relative  
2 density, relative dominance, relative frequency, and importance value (IV) were  
3 calculated according to Müeller-Dombois and Ellenberg (1974).

4

### 5 2.3 Management of animal and plant species selected by sheep

6

7 Three adult male Santa Inês sheep of mean weight 35.4 kg were used. The  
8 animals were adapted to the study area and to the observers over the course of two  
9 months. They were released into the Cerrado for 3 h each morning and afternoon. All  
10 procedures were performed according to principles of animal experimentation approved  
11 by protocol 23/2009 by the Ethics Committee on Animal Experimentation of the  
12 Federal University of Minas Gerais, Brazil.

13 The observations of plant selection were conducted after a 30 minute grazing  
14 adaptation period. For the verification of selected plants in the diet of three sheep,  
15 grazing was monitored on the first day of each month, observing the behavior for one  
16 hour in the morning and one hour in the afternoon. Three observers recorded the  
17 consumed plants every 5 minutes, totaling 12 observations daily for each animal.  
18 Considering the three animals, 432 observations were made over the course of one year  
19 (six observations/h x 2 h =12 observations per animal per month x 3 animals = 36  
20 observations x 12 months = 432 observations). This method preserves animal welfare  
21 and allows good visualization of the selected vegetal species (Costa et al. 2009).

22 The calculation of selectivity index (SI) of plant species was adapted from the  
23 methodology used by Heady (1975), and consisted of the division of the percentage of  
24 selected and consumed plant species by the percent of those species in the area plant  
25 cover, multiplied by 100.



## 1 2.4 Dehydration and obtaining extracts of selected plant species

2

3 Healthy leaves were selected and dried to constant weight in a forced air  
4 circulating drier at 40°C for 72 h. Dried leaves were ground and stored in paper bags in  
5 darkness. Aqueous extracts were obtained using the method of Nery et al. (2010) with  
6 modifications. Ground dried leaves were held in a distilled water bath at 40°C for 60  
7 min, hot filtered through a gauze funnel, and the resulting extract was dehydrated at  
8 40°C for 48 h.

9

## 10 2.5 High-performance liquid chromatography (HPLC) analyses of the extracts

11

12 A Waters Alliance 2695 HPLC system composed of a quaternary pump, an  
13 autosampler, a photodiode array detector (DAD) 2996, and a Waters Empower Pro data  
14 handling system was used (Waters Corporation, Milford, USA). The analyses were  
15 performed on a LiChrospher 100 RP-18 column (250 × 4 mm i.d., 5 µm; Merck,  
16 Darmstadt, Germany) combined with a LiChrospher 100 RP-18 guard column (4 × 4  
17 mm i.d., 5 µm; Merck) at 40 °C. Water (A) and acetonitrile (B) were used as eluents,  
18 both containing 0.1% (v/v) of H<sub>3</sub>PO<sub>4</sub> at a flow rate of 1.0 ml min<sup>-1</sup> as follows: 0 min,  
19 95% A and 5% B; 60 min, 5% A, 95% B, followed by 10 min of isocratic elution.  
20 Solvents used were of HPLC grade (Merck, Germany) and were degassed by sonication  
21 before use. The chromatograms were obtained at 210 nm, and the UV spectra were  
22 recorded on-line from 190 to 400 nm.

23 The dried aqueous extracts were dissolved in methanol (HPLC-grade), ultrapure  
24 water, or hydroethanolic solutions, according to their solubility, to concentrations of 10

1 mg /ml. After centrifugation at 8 400 x g, the sample solutions (10 µL) were  
2 automatically injected into the apparatus.

3

#### 4 2.6 Spectrophotometric quantification of total proanthocyanidins

5

6 Total proanthocyanidin content of the dried aqueous extracts was determined by  
7 measuring at 540 nm the absorbance of the cyanidin chloride resulting from acid-  
8 catalyzed solvolysis with *n*-BuOH/HCl 37% (95:5), according to the method described  
9 by Hiermann et al. (1986). Each sample was analyzed in triplicate and the results  
10 expressed as mean ± standard deviation. The total proanthocyanidin content, expressed  
11 as cyanidin chloride, was calculated using the following formula:

12

$$13 \quad \text{Proanthocyanidin \%} = \frac{\text{Absorbance sample} - \text{Absorbance blank} \times 4.155}{14 \quad \text{Weight sample (g)}}$$

15

#### 16 2.7 Inhibition of larva development

17

18 To evaluate the effectiveness of the dried leaves on larval development inhibition  
19 (LDI), the adapted coproculture quantitative methodology (Borges, 2003; Nery et al.  
20 2010) was employed using feces of sheep with *Haemonchus contortus* mono-infection.  
21 All procedures performed were approved under protocol 25/2013 by the Ethics  
22 Committee on Animal Experimentation of the Federal University of Minas Gerais,  
23 Brazil.

24

25 Eleven treatments were conducted, each with five replicates, including a positive  
control: 2 ml of solution 16 µg ml<sup>-1</sup> ivermectin (LA Ranger, Vallée, Minas Gerais,

1 Brazil) added to 2 g feces, and a negative control of 2 ml of sterile purified water added  
2 to 2 g feces. The nine plant treatments consisted of dehydrated and ground leaves of  
3 *Cerrado* species at final concentration of 333.3 mg of (dw) g<sup>-1</sup> of feces culture. The  
4 materials were incubated in a BOD incubator at 28°C for seven days and assessed for  
5 presence of infective larvae (L3). The following formula, adapted from Borges (2003),  
6 was used to determine the percent reduction of larva /g of feces (LPGF):

7

$$8 \quad \% \text{ efficacy} = 100 \times (1 - \text{LPGF of the treated group} / \text{LPGF of the treated group})$$

9

10 The data were log transformed, log (x+ 1) and submitted to variance analysis. The  
11 means were compared with the Duncan test at 5% probability using the SAEG 9.1  
12 Program (2007).

13

### 14 **3 Results**

15

16 The area contained 1288 arboreal, 102 shrubs, and 1388 herbaceous species. A  
17 total of 94 plant species were grouped into 72 genera and 33 families. The  
18 phytosociology of 10 species arboreal, shrubs, and herbaceous species were ordered  
19 according to the IV values (Table 1). Voucher specimens were stored at the Montes  
20 Claros Herbarium (HMCMG) of Universidade Estadual de Montes Claros.

TABLE 1

Families and species recorded in vegetation of the Cerrado of North Minas Gerais, Brazil, with VN = voucher number; N = number of individuals; RD = relative density (%); Rdo = relative dominance (%); RF = relative frequency (%); and IV importance value (%)

Arboreal species	Family	N	RD	Rdo	RF	IV
<i>Tachigali rugosa</i> (Mart. ex Benth.) Zarucchi & Pipoly	Fabaceae	39	3.03	83.54	3.59	90.16
<i>Heteropterys byrsonimifolia</i> A.Juss.	Malpighiaceae	275	21.35	2.16	8.03	31.54
<i>Astronium fraxinifolium</i> Schott	Anacardiaceae	168	13.04	1.81	8.03	22.88
<i>Machaerium opacum</i> Vogel	Fabaceae	132	10.25	2.24	6.34	18.83
<i>Copaifera langsdorffii</i> Desf.	Fabaceae	87	6.75	2.81	4.23	13.79
<i>Tabebuia aurea</i> (Silva Manso) Benth & Hook.f. ex S.Moore	Bignoniaceae	65	5.05	0.83	5.07	10.95
<i>Curatella americana</i> L.	Dilleniaceae	55	4.27	1.22	3.38	8.87
<i>Antonia ovata</i> Pohl	Loganiaceae	47	3.65	0.65	4.23	8.53
<i>Terminalia argentea</i> Mart.	Combretaceae	41	3.18	0.49	4.44	8.11
<i>Schwartzia adamantium</i> (Cambess) Bedell ex Gir-Canãs	Marcgraviaceae	34	2.64	0.22	3.38	6.24
Shrubs species	Family	N	RD	Rdo	RF	IV

<i>Lantana fucata</i> Lindl.	Verbenaceae	14	13.73	6.75	11.54	32.02
<i>Heteropterys byrsonimifolia</i> A.Juss	Malpighiaceae	10	9.80	10.73	8.97	29.50
<i>Astronium fraxinifolium</i> Schott	Anacardiaceae	8	7.84	12.64	8.97	29.45
<i>Tachigali rugosa</i> (Mart. ex Benth.) Zarucchi & Pipoly	Fabaceae	12	11.76	6.47	8.97	27.20
<i>Curatella americana</i> L.	Dilleniaceae	4	3.92	6.10	5.13	15.15
<i>Erythroxylum deciduum</i> A.St.-Hil.	Erythroxylaceae	7	6.86	1.87	5.13	13.86
<i>Guapira tomentosa</i> (Casar.) Lundell	Nyctaginaceae	3	2.94	6.14	2.56	11.64
<i>Byrsonima pachyphylla</i> A. Juss.	Malpighiaceae	2	1.96	7.76	1.28	11.00
<i>Cheiloclinium cognatum</i> (Miers.) A.C.Sm.	Celastraceae	1	0.98	7.18	1.28	9.44
<i>Banisteriopsis</i> sp. 1	Malpighiaceae	3	2.94	3.66	2.56	9.16
Herbaceous species	Family	N	RD	Rdo	RF	IV
<i>Evolvulus</i> sp.	Convolvulaceae	507	36.52	36.41	35.8	108.73
<i>Rhynchospora</i> sp.	Cyperaceae	460	33.14	33.16	34.5	100.80
<i>Hyptis</i> sp.	Lamiaceae	131	9.43	9.46	10.7	29.59
<i>Andropogon</i> sp.	Poaceae	56	4.03	4.04	3.51	11.58
<i>Zornia</i> sp.	Fabaceae	48	3.45	3.46	4.29	11.20
<i>Mascagnia</i> sp.	Malpighiaceae	44	3.17	3.17	3.28	9.62
<i>Stylosanthes</i> sp.	Fabaceae	41	2.95	2.96	1.47	7.38
<i>Chamaecrista</i> sp.	Fabaceae	38	2.73	2.74	1.90	7.37
<i>Coursetia</i> sp.	Fabaceae	15	1.08	1.08	1.08	3.24
<i>Belucia</i> sp .	Melastomataceae	9	0.64	0.65	1.11	2.41

$$RD_i = (RD_i / \sum (RD_i \dots RD_n)) \times 100.$$

$$Rdo_i = (Rdo_i / \sum (Rdo_i \dots Rdo_n)) \times 100.$$

$$RF_i = (RF_i / \sum (RF_i \dots RF_n)) \times 100$$

$$IV = RD_i + RF_i + Rdo_i$$

RD = indicates the percentage of each species of plant in relation to the whole plant community:

$$RD_i = (RD_i / \sum (RD_i \dots RD_n)) \times 100.$$

So:

RD<sub>i</sub> = Relative density by species.

Rdo = allows calculating the percentage of dominance of each plant species in relation to the others:

$$Rdo_i = (Rdo_i / \sum (Rdo_i \dots Rdo_n)) \times 100$$

So:

Rdo<sub>i</sub> = relative dominance of species 'i'.

RF = Indicates the percentage frequency of plants of each species in relation to the plant community

$$RF_i = (RF_i / \sum (RF_i \dots RF_n)) \times 100$$

So:

RF<sub>i</sub> = relative frequency of the species 'i'.

1           After one year of monitoring, the selection of species was calculated from the  
2 selectivity index (SI) to determine the native plants most commonly consumed by  
3 grazing sheep. Considerable variation between the dry and rainy seasons was observed  
4 in these indices for nine species (Table 2).

TABLE 2

Selectivity index of the main plant species selected by grazing sheep in the Cerrado of North Minas Gerais, Brazil

Plants	2009									2010		
	Apr	May*	Jun*	Jul*	Aug*	Sep*	Oct	Nov	Dec	Jan*	Feb *	Mar
<i>Baccharis cognata</i>	10.74	0.00	11.57	29.57	0.00	0.00	8.10	0.00	0.00	0.00	0.00	0.00
<i>Casearia sylvestris</i>	4.75	0.00	23.00	5.23	36.72	40.01	3.58	7.38	3.10	3.52	0.00	3.42
<i>Erythroxylum deciduum</i>	2.80	3.43	1.88	0.77	3.31	0.55	0.79	4.90	0.00	3.52	14.77	6.84
<i>Evolvulus</i> sp.	4.05	5.26	4.91	3.56	3.03	2.79	1.55	4.57	5.38	7.42	7.32	4.03
<i>Heteropterys byrsonimifolia</i>	0.29	0.08	0.08	0.24	0.12	0.92	0.33	0.00	0.00	0.00	0.45	0.10
<i>Paullinia</i> sp.	29.15	0.00	0.00	0.00	12.53	30.72	0.00	0.00	9.52	32.47	0.00	0.00
<i>Piptadenia viridiflora</i>	0.00	0.00	0.00	2.61	0.00	0.00	0.00	0.00	0.00	0.00	3.69	0.00
<i>Schinopsis brasiliensis</i>	0.00	0.00	0.00	48.15	50.13	53.76	10.99	0.00	0.00	0.00	0.00	21.01
<i>Ximenia Americana</i>	0.00	0.00	0.00	2.34	0.00	0.00	16.03	13.23	0.00	6.31	3.31	3.06
Rainfall (mm)	64.9	5.45	0.00	0.00	0.20	48.4	323.9	135.8	219.5	27.8	17.3	262.5

\* Dry period, rainfall &lt; 50 mm

SI = Selectivity index: % consumed species / % species in the area × 100

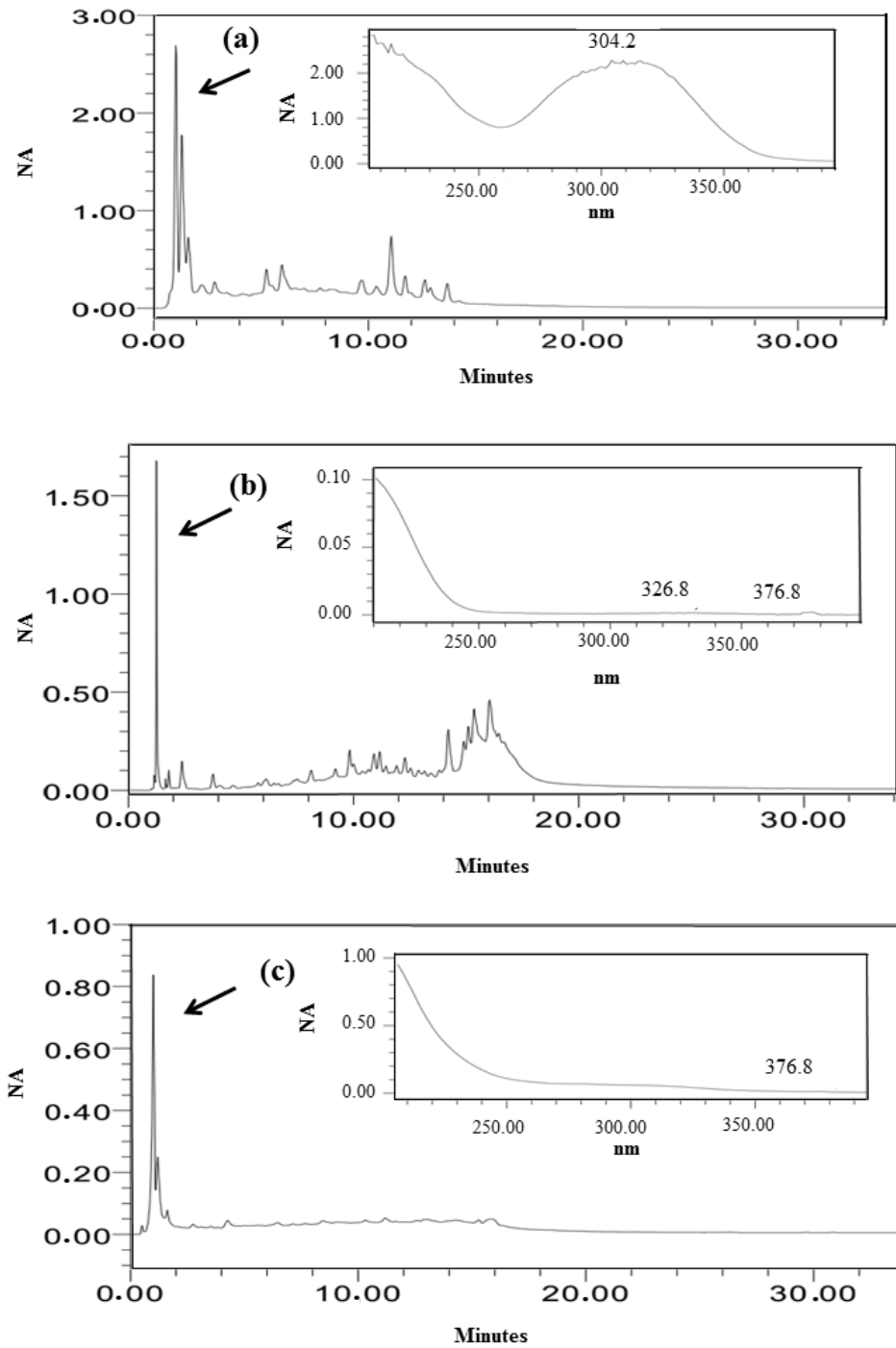




- 1 Efficacy: % efficacy = 100 x (1 - LPGF of the treated group/LPGF of the treated group).  
2 NA: not assayed (below the limit of quantitation of the method)  
3 NE: Not effective  
4

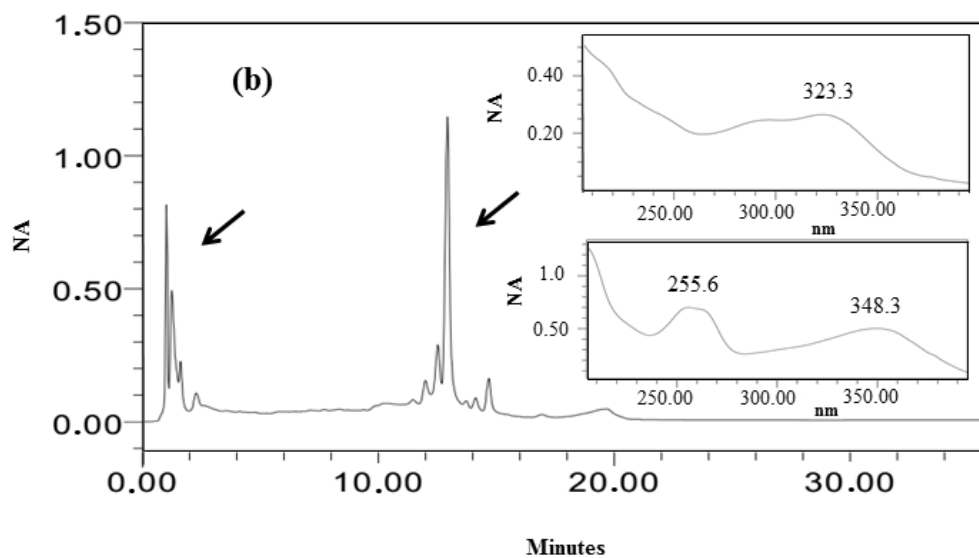
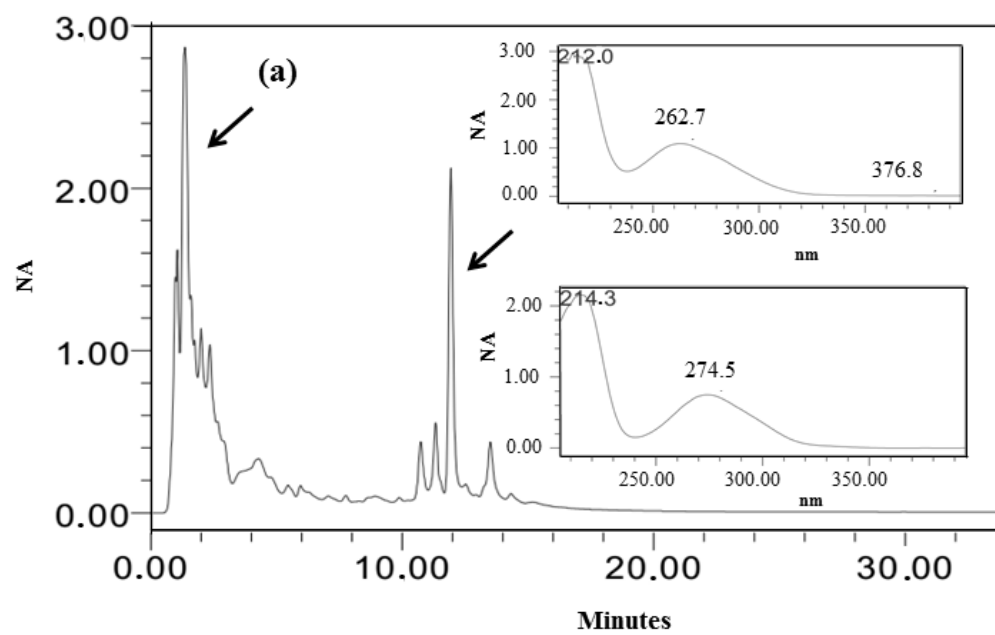
5           The chemical composition of the selected species was accessed by their contents  
6 of proanthocyanidins, quantified using spectrophotometry, as well as by the  
7 chromatographic profiles registered by HPLC-DAD. *Ximenia americana* and *Piptadenia*  
8 *viridiflora* showed efficacy of over 85%, but presented low concentrations of  
9 proanthocyanidin (Table 3). The occurrence of polyphenols in all active species is  
10 suggested by their HPLC-DAD chromatograms, which indicated the predominance of  
11 peaks corresponding to polar compounds, with UV spectra compatible with polyphenols  
12 (Fig. 1). High-performance liquid chromatography analyses showed major peaks with  
13 UV spectra characteristic of flavonoids to extracts of *P. viridiflora* ( $\lambda$  262.7 and 376.8  
14 nm) and *X. americana* ( $\lambda$  255.6 and 348.3 nm) (Fig. 2).

15  
16



1

2 **Fig. 1** HPLC-DAD chromatograms obtained for the aqueous extracts of the species  
 3 active in the antihelminthic assay. (a) *Casearia sylvestris*, (b) *Schinopsis brasiliensis*  
 4 and (c) *Paullinia* sp.



1

2 **Fig. 2** HPLC-DAD chromatograms obtained for the aqueous extracts of the species  
 3 active in the antihelminthic assay. (a) *Piptadenia viridiflora*, and (b) *Ximenesia*  
 4 *americana*.

5

6

## 1 4 Discussion

2

3 The plant families showing greatest richness were Fabaceae with 15 species,  
4 Rubiaceae and Malpighiaceae, with eight, Bignoniaceae and Myrtaceae, with seven, and  
5 Annonaceae with four (Table 1). These families were comparable to those reported by  
6 Cerrado of Miranda et al. (2006), Sales et al. (2009a, b) and Menino et al. (2012).

7 The absolute values of selectivity index were based on a scale having the central  
8 point value of one, indicating the balance between the percentage of a species selected  
9 by the grazing animal and the percentage of that plant in the cover of the grazing area.  
10 An index of less than one indicates that a species was seldom selected. An index greater  
11 than one indicates a higher level of selection (Santos et al. 2008).

12 The commonly selected species *Erytroxylum deciduum*, *Astronium fraxinifolium*,  
13 *Machaerium opacum*, *Tabebuia aurea*, *Copaifera langsdorffii*, *Senna spectabilis*,  
14 *Ximenia americana*, and *Schinopsis brasiliensis* (Table 2) were also identified by Sales  
15 et al. (2009a, b) in other Cerrado areas.

16 *Baccharis cognata*, *Casearia sylvestris*, *Paullinia* sp., and *Schinopsis brasiliensis*  
17 showed higher SI during the dry season (Table 2). This period is critical for animal  
18 feeding, because the supply of forage is compromised in quantity and quality.

19 Assessment of nutritive value and toxicological analyzes may show a potential of  
20 these plants for supplementation of the diet of sheep and for a role of grazing on these  
21 plants in the strategic control of nematode infection, which is commonly concentrated in  
22 the dry season (Niezen et al. 2002; Taylor et al. 2007). *Evolvulus* sp., *Erytroxylum*  
23 *deciduum*, and *Heteropterys byrsonimifolia* were selected throughout the evaluation  
24 period (Table 2), indicating the potential for use in both dry and rainy seasons.

1        *Paullinia* sp. and *Piptadenia viridiflora* and *Ximenia americana* promoted  
2 significant reduction of the LDI average with efficacies more than 70% (Table 3).  
3 *Casearia sylvestris*, and *Paullinia* sp. showed the highest contents of proanthocyanidins  
4 but were not among those with the highest anthelmintic efficacy *in vitro* (Table 3).

5        *Piptadenia viridiflora* was selected only in July 2009 and February 2010.  
6 February represented the end of the rainy season, and the end of new growth. Possibly  
7 in this period *P. viridiflora* was not harmful to sheep, although it has been reported to be  
8 toxic at 4.43 g kg / bw (Tocarnia et al. 1999). This species contains hydrocyanic acid  
9 bonded to carbohydrates called cyanogenic glycosides that is released upon hydrolysis  
10 (Vetter, 2000).

11        *Ximenia americana* was selected by the animals in January 2010, in the plant  
12 fruiting period. The seeds are considered purgative (Pio Correa 1984). The species has  
13 been shown to exhibit healing properties, which can be credited to the presence of  
14 tannins (Monte et al. 2012). In general, the compounds found in *X. americana* were  
15 saponins, glycosides, flavonoids, tannins, phenolics, alkaloids, quinines, and terpenoids.  
16 In addition, the plant is rich in fatty acids and glycerides, and the seeds contain cyanide  
17 derivatives (Monte et al. 2012).

18        Among the five active species, only *C. sylvestris* and *Paullinia* sp. presented a  
19 high content of condensed tannins, suggesting that other compounds may account for  
20 the antihelminthic properties of *S. brasiliensis*, *P. viridiflora*, and *X. americana*. HPLC  
21 chromatograms of *P. viridiflora* and *X. americana*, species with low proanthocyanidin  
22 content present major peaks with UV spectra characteristic of flavonoids (Fig. 2).

23        Tannins can exert direct antihelminthic action, interfering with the natural cycle of  
24 helminths, or indirectly, by inhibiting the degradation of ruminal protein (Ketzis et al.  
25 2006). Some Cerrado species possessing these compounds have been shown to inhibit

1 larval development of gastrointestinal nematodes in ruminants (Paolini et al. 2005;  
2 Oliveira et al. 2011; Nery et al. 2010; Nogueira et al. 2012; Ferreira et al. 2013; Cala et  
3 al. 2014).

4 The aqueous extract of *Caryocar brasiliense* fruit specie occurring in the  
5 Cerrado biome, peel tested at 200 mg /ml , significantly inhibited the development of *H.*  
6 *contortus* larvae in sheep (Nogueira et al. 2012). *In vitro* tests of extracts of this plant  
7 for inhibition of larval development showed 94.8% effectiveness (Nogueira et al. 2012).  
8 The qualitative phytochemical tests performed indicated the presence of catechins,  
9 steroids, flavonoids, saponins, xanthones, and tannins (Nogueira et al. 2012).

10 In another study, the aqueous extract of seeds and leaves of *Annona crassiflora*  
11 species occurring in the Cerrado biome showed antihelminthic efficacy of 99.43 % for  
12 seeds and 89.81% for leaves at 100 mg /mlin LDI with quantitative coproculture  
13 (Nogueira et al. 2009). Nery et al. (2010) evaluated extracts of leaves of *Anacardium*  
14 *humile* A. St.-Hil (Anacardiaceae) against several species of gastrointestinal nematodes  
15 in sheep. The aqueous extract showed antihelminthic activity significantly higher than  
16 that of the negative control at all concentrations evaluated. At 150 and 187.5 mg/ml, the  
17 percent efficacy was not significantly different from ivermectin at 16 mg /ml . Larvae of  
18 *Haemonchus* spp. (68%), *Strongyloides* spp. (31%), and *Trichostrongylus* spp. (1%) were  
19 identified in the coprocultures of the negative control group. This extracts were  
20 effective against the three nematodes most prevalent and pathogenic in sheep (Wood et  
21 al. 1995).

22 Variation in the selectivity index for nine main plant species selected by grazing  
23 sheep was observed with respect to the dry and rainy seasons in the Cerrado. The dried  
24 leaves of *C. sylvestris*, *S. brasiliensis*, *Paullinia* sp., *P. viridiflora*, and *X. americana*  
25 show LDI efficacy ranging from 46.6% to 99.6%. Despite having low levels of

1 condensed tannins, *P. viridiflora* and *X. americana* showed higher antihelminthic  
2 efficacy than did other selected plants and are promising for use in diet and alternative  
3 control of *Haemonchus contortus* infections in sheep.

#### 4 5 **5 Statement of Animal Rights**

6 All procedures were performed according to principles of animal experimentation  
7 approved by protocol 23/2009 by the Ethics Committee on Animal Experimentation of  
8 the Federal University of Minas Gerais, Brazil.

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1   **CAPTER 3**

2

3   *Piptadenia viridiflora* (Kunth) Benth selected from Cerrado to control of

4   *Haemonchus contortus* in lambs

5

6   **Abstract**

7   Resistance to anthelmintics has been common in different continents. *Piptadenia*  
8   *viridiflora* is an plant from Cerrado selected naturally by sheep and could be source of  
9   bioactive compounds for development of new farmacs. In this study, *in vitro* and *in vivo*  
10   efficacy this vegetal was evaluated for lambs naturally infected with *Haemonchus*  
11   *contortus*. The presence of tannins and flavonoids for aqueous and ethanolic extracts of  
12   the leaves was indicated by HPLC-DAD. These extracts at 2.4 and 1.2 mg mL<sup>-1</sup>,  
13   respectively, showed 100% efficacy to egg hatch inhibition (EHI). In larval  
14   development inhibition (LDI) test with quantitative cultures, the aqueous extract ≥ 1.2  
15   mg mL<sup>-1</sup> promoted L3 mean lower than those observed for the control with water  
16   (P<0.05) and the estimated LC90 was 2.28 mg g<sup>-1</sup> of feces. Maximum tolerated dose  
17   for male and female mice was > 12.87 mg kg<sup>-1</sup> bw in intraperitoneal via. Aqueous  
18   extract was orally administered at 283 mg (dm) kg<sup>-1</sup> bw during three consecutive days  
19   and the anthelmintic efficacies observed up to three weeks pos-treatment were between  
20   32.9 - 47.2% with fecal egg counts (FEC) lower averages than untreated lambs  
21   (p<0.05). For all sheep erythrocyte and hematocrit values were within the normal  
22   physiological patterns and plasmatic albumin and total protein were similar between *in*  
23   *vivo* treatment groups. Low concentrations of extracts promoted high efficacy for EHI  
24   and LDI. The oral treatment with aqueous extract is promising since shows moderated  
25   *in vivo* anthelmintic efficacies, not interference with normal clinical standards and no  
26   toxicity to blood parameters evaluated.

27

28   **Keywords:** Brazilian savanna, sheep, antihelminthic plant, phytochemical composition,  
29   toxicity, blood parameters.

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## 1 **1 Introduction**

2

3 *Haemonchus contortus* is parasite of the abomasum and responsible for most of  
4 losses in the sheep creations. Lambs with haemonchosis can show anemia and  
5 submandibular swelling, with high mortality in young lambs and females in peripartum.  
6 Both sexes at all age levels may be intensely infected, reducing weight gain and  
7 reproductive capacity, as well as milk, wool, and hide production (Bizimenyera et al.  
8 2006).

9 Anthelmintics have quick solution for the control of this nematode, but resistance  
10 have been observed in diferente countries such as New Zealand (Leathwick et al. 2001),  
11 Switzerland (Schnyder et al. 2005), Italy (Cringoli et al. 2007), Africa (Soro et al.  
12 2013), Nigeria (Adiele, et al. 2013) and Brazil (Araújo e Lima, 2005; Duarte et. al.,  
13 2012).

14 The constant administration and the inadequate dosages can favor selection of  
15 the populations resistant to the anthelmintics (Cooper et al. 2011; Power et al. 2013) and  
16 contributes to the contamination of animal products with residues of these products and  
17 environment (Power et al. 2013). The utilization of plants containing secondary  
18 compounds such as condensed tannins has represented organic alternatives to  
19 controlling for gastrointestinal nematodes (Athanasiadou et al. 2004).

20 Therefore, the analysis of potential plant species from Cerrado biome for  
21 helminthe control can represent promising strategy for the biotechnology industry and  
22 consequently for the breeders (Nogueira et al., 2012; Nery et al. 2010; Iqbal 2005).

23 *Piptadenia viridiflora* (Kunth) Benth. (Fabaceae) it is popularly known as  
24 “surucucu” (Lorenzi, 2009), is found frequently in the Cerrado (Fig. 1) and shows, has

1 medicinal properties with compounds such as tannins and flavonoids (Lorenzi, Matos,  
2 2002).



3  
4 Figure 1 – *Piptadenia viridiflora* (Fabaceae)

5  
6 However species contains hydrocyanic acid bonded to carbohydrates called  
7 cyanogenic glycosides that is released after hydrolysis (Vetter, 2000), which may be  
8 toxic if used in an amount above 9 g kg / bw (Tokarnia, et al. 1999).

9 In preliminary study of Cerrado Biome, *P. viridiflora* was naturally selected by  
10 lambs grazing during the dry and rainy seasons, with selectivity index 2.61% and 3.69%  
11 respectively (Morais-Costa, et al., 2014 ), when this ratio is  $\geq$  one indicates that this  
12 vegetal species is important in the animal's diet . Then the purpose in this study was to  
13 analyze *in vitro* and *in vivo* extracts of *Piptadenia viridiflora* to control *Haemonchus*  
14 *contortus*.



1 **2 Material and methods**

2

3 2.1 Study area

4

5 The research was conducted in a rural area of Montes Claros of North of Minas  
6 Gerais state, Brazil (W 43°50'33.56"; S 16°41'10.05"). The climate of this region is  
7 tropical wet and dry (Aw) according to the Köppen classification and marked by long  
8 dry season from May to October and rainy period in November to April.

9

10 2.2 Production of vegetal extratcts

11

12 Healthy leaves of *P. viridiflora* from Cerrado Biome were selected and dried to  
13 constant weight in a forced air circulating drier at 40°C for 72 h. Dried leaves were  
14 grinded in a wiley mill and then were stored in paper bags, free of light incidence.  
15 Samples of the plant were stored in the Montes Claros Herbarium (HMCMG) of the  
16 Universidade Estadual de Montes Claros-Brazil, with voucher specime, 2283.

17 Aqueous extract of dried leaves were held in a distilled water bath at 40°C for 60  
18 min. Ethanolic extract was obtained by maceration of the dried leaves in absolute ethyl  
19 alcohol (PA), in glass amber containers, kept in a dark place and stored for seven days.  
20 After this extraction, filtration was held through a gauze funnel. The extracts were  
21 dehydrated at 40°C for 48 h, until obtaining the residue with constant weight and stored  
22 in paper bags in darkness and refrigerated at ~4°C until use (Adapted Nery et al., 2010).

23 2.3 High-performance liquid chromatography (HPLC) analyses and proanthocyanidin  
24 quantification.

25

1 A Waters Alliance 2695 HPLC system composed of a quaternary pump, an  
2 autosampler, a photodiode array detector (DAD) 2996, and a Waters Empower Pro data  
3 handling system was used (Waters Corporation, Milford, USA). The analyses were  
4 performed on a LiChrospher 100 RP-18 column (250 × 4 mm i.d., 5 μm; Merck,  
5 Darmstadt, Germany) combined with a LiChrospher 100 RP-18 guard column (4 × 4  
6 mm i.d., 5 μm; Merck) at 40 °C. Water (A) and acetonitrile (B) were used as eluents,  
7 both containing 0.1% (v/v) of H<sub>3</sub>PO<sub>4</sub> at a flow rate of 1.0 mL min<sup>-1</sup> as follows: 0 min,  
8 95% A and 5% B; 60 min, 5% A, 95% B, followed by 10 min of isocratic elution.  
9 Solvents used were of HPLC grade (Merck, Germany) and were degassed by sonication  
10 before use. The chromatograms were obtained at 210 nm, and the UV spectra were  
11 recorded on-line from 190 to 400 nm.

12 The dried aqueous and ethanolic extracts were dissolved in methanol (HPLC-  
13 grade), ultrapure water, or hydroethanolic solutions, according to their solubility, to  
14 concentrations of 10 mg mL<sup>-1</sup>. After centrifugation at 8 400 × g, the sample solutions  
15 (10 μL) were automatically injected into the apparatus.

16 Total proanthocyanidin content of the dried aqueous extracts was determined by  
17 measuring at 540 nm the absorbance of the cyanidin chloride resulting from acid-  
18 catalyzed solvolysis with *n*-BuOH/HCl 37% (95:5), according to the method described  
19 by Hiermann et al. (1986). Each sample was analyzed in triplicate and the results  
20 expressed as mean ± standard deviation. The total proanthocyanidin content, expressed  
21 as cyanidin chloride, was calculated using the following formula:

$$22 \quad \% = \frac{A_{\text{sample}} - A_{\text{blank}} \times 4.115}{m_{\text{sample}}}$$

23 *A* is the measured absorbance at 420 nm, and *m*<sub>sample</sub> is the sample weight in g.

24

1 2.4 Egg hatching inhibition (EHI)

2

3 The aqueous and ethanolic extract of the dried leaves was at 4.8 mg mL<sup>-1</sup> and  
4 diluted in sterile distilled water. These extracts were used in EHI tests immediately after  
5 dissolution as described Coles et al. (1992).

6 Flotation, sedimentation, and filtration techniques in saturated NaCl solution were  
7 conducted to obtain nematode eggs from feces of two Santa Inês lambs infected only  
8 with *Haemonchus contortus* and with an average fecal egg count (FEC) >1000 g<sup>-1</sup>,  
9 determined using the modified McMaster technique (Gordon and Whitlock, 1939).

10 Experimental mixtures contained: 100 µl fecal suspension with an average of 80  
11 hanging eggs, and 100 µl of the extract, at final concentrations 0.15-2.4 mg mL<sup>-1</sup> or two  
12 positives controls with solution levamisole phosphate (0.3 mg mL<sup>-1</sup>) or ivermectin (16 µ  
13 mL<sup>-1</sup>) or a negative control with sterile distilled water. The samples were homogenized  
14 and incubated in a BOD incubator at 28°C for 48 h. Subsequently, 15 µl Lugol's  
15 solution was added to each tube, which were then stored at 4°C for subsequent counting  
16 of unembryonated eggs, embryonated eggs, first stage larvae (L1; Coles et al. 1992).

17 The number of L1 relative to the total number of eggs plus L1 was determined for  
18 each repetition and subjected to variance analysis. The means were compared using the  
19 Tukey test at 5% significance. Probit regression was employed to determine the  
20 concentrations sufficient to inhibit 90% (lethal concentration, LC90) of egg hatching  
21 using the statistical package, Saeg 9.1 (2007).

22 The formula of Coles et al. (1992) was used to determine the EHI effectiveness:

23 
$$\% \text{ effectiveness} = 100 \times (1 - \text{mean of L1} / \text{mean eggs} + \text{L1})$$

24

25

## 1 2.5 *In vitro* larval development inhibition (LDI)

2

3 The effectiveness of the aqueous extract was evaluated by adapted coproculture  
4 quantitative methodology (Borges, 2003; Nery et al. 2010, Nogueira, 2012) using fresh  
5 feces of lambs with *Haemonchus contortus* mono-infection.

6 Nine treatments were performed, each with five replicates, including two  
7 positive control: 2 mL of solution 16 µg g<sup>-1</sup> ivermectin (final concentration) or 2 mL of  
8 solution 0.1 mg g<sup>-1</sup> levamisole phosphate (final concentration) and a negative control of  
9 2 mL of sterile purified water, both added to 2 g feces.

10 The six treatments with *P. viridiflora* was standardized at 1.21-38.62 final  
11 concentration mg of dw g<sup>-1</sup> of fecal culture. The samples were incubated in a BOD  
12 incubator at 28°C for seven days and assessed for presence of infective larvae (L3). The  
13 following formula, adapted from Borges (2003), was used to determine the percent  
14 reduction of larva g<sup>-1</sup> of feces (LPGF):

$$15 \quad \% \text{ efficacy} = 100 \times (1 - \text{LPGF of the treated group} / \text{LPGF of the treated group})$$

16 The data were transformed in log (x + 1) and submitted to variance analysis. The  
17 means were compared through the Duncan test at 5% probability and LC<sub>90</sub> was  
18 determined by probit analysis using the statistical package SAEG® 9.1 (2007).

19

## 20 2.6 Toxicity in mice

21

22 The mouse toxicity testing was performed as Walum (1998) to determine the  
23 maximum tolerated dose (MTD) for an adult mouse. Using probe gavage 22µL of  
24 aqueous extract of *P. viridiflora* was added for four Balb C mice (2 males and 2  
25 females) with average 22g of body weight and 6 to 8 weeks.

1 For the first and second days the extract was diluted at 100 and 10 times  
2 respectively in 1x PBS. In the third and fourth days the extract was administered at  
3 38.62 mg ml<sup>-1</sup>. The mice were euthanized by cervical dislocation on day 5 after extract  
4 administration.

5

## 6 2.7 *In vivo* anthelmintic test

7

8 The analyses were performed on 24 Santa Inês lambs with average 26.5 kg bw of  
9 both sexes, with 4-to-8-month-old. Prior to the beginning of the trial, all sheep were  
10 administered albendazole (10 mg kg<sup>-1</sup> bw) and phosphate levamisole (0.6 mg kg<sup>-1</sup> bw),  
11 to ensure that were worm-free.

12 During 10 days of adaptation, the animals were individually confined which fed  
13 balanced diet containing sorghum silage, concentrate, mineral premix and water *ad*  
14 *libitum*, according to the age category requirement.

15 The animals, with zero fecal egg count (FEC), were infected with 800 L3 for  
16 10kg<sup>-1</sup> bw from lambs naturally contaminated in *H. contortus*. Twenty-eight days post-  
17 infection, sheep were assigned to one of three homogeneous groups based on FEC,  
18 weight, and sex.

19 One untreated animals served as the negative control, other group was orally  
20 administered phosphate levamisole (0.6 mg kg<sup>-1</sup> bw) via subcutaneous and represented  
21 the positive control, the third group was administered aqueous vegetal extract of *P.*  
22 *viridiflora*, by esophageal gavage, at 283 mg (dm) kg<sup>-1</sup> bw during three consecutive  
23 days.

1           The mean of two counts of FEC was obtained for each collection periods. The  
2 McMaster technique was utilized with the addition of saturated NaCl for FEC in  
3 duplicate (Gordon and Whitlock, 1939).

4           Initial FEC value was recorded based on the mean values of three days prior to  
5 initiation of treatment. Subsequently, mean FEC was determined for three periods post-  
6 treatment to efficacy analysis: 7, 8 and 9 days (first period), 14, 15 and 16 ( second  
7 period) and 21, 22 and 23 (third period). Fecal samples were cultured to obtain larvae  
8 for identification and confirmation of mono-infection by *H. contortus* (Ueno &  
9 Gonçalves, 1998).

10           The data relating FEC values were previously transformed into  $\log(x + 10)$  and  
11 subjected to analysis of variance and the means were compared by Duncan test  
12 ( $p < 0.05$ ). A formula adapted from Coles et al. (1992) was used to determine the  
13 percentage efficacy in FEC reduction:

$$14 \quad \text{Efficacy} = 100 \times (1 - \text{mean FEC of treated group} / \text{mean FEC of control group})$$

15

## 16 2.8 Blood parameters of sheep

17

18           Blood samples were collected from jugular vein on days 0, 7, 14 and 21 into  
19 tubes containing (EDTA) and transported at 4°C. Erythrocytes and hematocrit were  
20 evaluated in an automatic analyzer (2.800 BC Vet®, Mindray Medical International  
21 Ltd. in Shenzhen, China). The protein and albumin values were analyzed in enzymatic  
22 kits (Bioclin ® -. Quibasa Basic Chemicals Ltd., Belo Horizonte, MG, Brazil) by  
23 colorimetric spectrophotometer (Automatic system for biochemical - BIOPLUS BIO  
24 2000, Shenzhen, China). Data were subjected to variance analysis with split plots.

1 Means were compared by the Scott-Knott test at 5% probability using the SAEG 9.1  
2 package.

3 All procedures were performed in accordance with the principles of animal  
4 experiments approved in the 275/2013 protocol of the Ethics Committee on the use of  
5 animals (CEUA) of the Federal University of Minas Gerais, Brazil.

6

### 7 **3 Results and discussion**

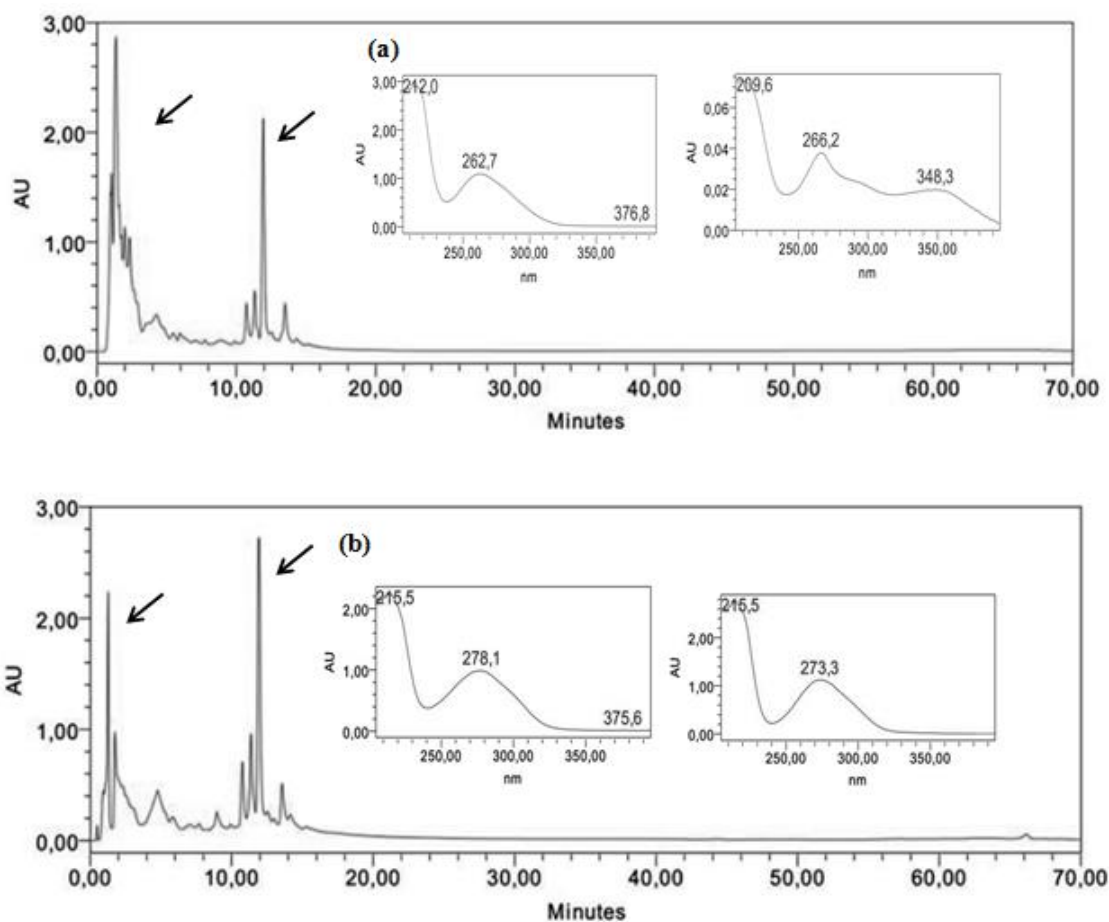
8

#### 9 3.1 Extracts characterization

10

11 The presence of polyphenols for both extracts was indicated by HPLC-DAD  
12 chromatograms, which clearly indicate the predominance of peaks corresponding to  
13 polar compounds, with UV spectra compatible with polyphenols (Fig. 2).

14



1

2

3 Figure 2 - HPLC-DAD chromatogram obtained for the aqueous (a) and ethanolic (b)

4 extract of the dried leaves of the *P. viridiflora* in the antihelminthic assay.

5 Chromatographic conditions: see Experimental section.

6

7 In addition, the HPLC chromatograms both extracts showed present major peaks

8 with UV spectra characteristic of flavonoids ( $\lambda$  262.7 and 376.8 nm aqueous and  $\lambda$  278.1

9 and 375.6 to ethanolic). The quantification total de proanthocyanidin concentrations for

10 aqueous and ethanolic extracts were  $0.23\% \pm 0.2$  and  $1.75\% \pm 0.3$  respectively. It

11 showed that proanthocyanidin from *P. viridiflora* was better extracted by ethanol.

12 Flavonoids and tannins can present beneficial effects in animals infected with

13 gastrointestinal nematodes (Barrau et al., 2005) and has been used to improve the



1 growth performance of animals (Niezen et al 1998, Athanasiadou et al 2001, Paolini et  
2 al 2004, Valderrábano et al 2010).

3 This study showed *P. viridiflora* with low content of condensed tannin when  
4 compared to other Cerrado species *Ouratea semiserrata* (6.63%) and *Ouratea*  
5 *spectabilis* (9.99%) (Valadares et al., 2003). Thus other compounds of *P. viridiflora* as  
6 flavonoids could be acting in the control of *H. contortus*.

7 Between Cerrado species with anthelmintic effects, phytochemical tests indicated  
8 the presence of tannins and flavonoids for *Anacardium humile* (Nery et al. 2010) and  
9 for *Cariocar brasiliense* were identified condensed tannins, hydrolysable tannins,  
10 flavonoids, terpenoids (Bezerra et al. 2002; Paula-Junior et al. 2006), and saponins  
11 (Paula-Junior et al. 2006). Flavonoids also were observed to aqueous extract of  
12 immature mango that showed *in vitro* and *in vivo* inhibition of *Haemonchus contortus*  
13 (Camurça-Vasconcelos et al. 2007, Nogueira et al. 2012).

14

### 15 3.2 Egg hatching inhibition

16

17 The effectiveness was directly related with increasing concentration of the  
18 aqueous extract of *P. viridiflora* leaves. The aqueous and ethanolic efficacies were at  
19 13.16-100% and 69.6-100% respectively (Table 1), and LC<sub>90</sub> for these extracts were 2.62  
20 and 2.70 mg mL<sup>-1</sup> respectively.

21 All concentrations tested for both extracts promoted significant reduction for  
22 hatched larvae mean when compared to the negative control with distilled water. The  
23 aqueous extracts showed greater inhibition of larval hatching, whereas ethanol extract  
24 showed the best inhibition of early embryonic development (Table 1). These differences  
25 could be attributed to the higher concentration of proanthocyanidin in the ethanol

1 extract (1.75%) compared to aqueous (0.23%).The extract treatments promoted  
 2 embryonated eggs with degraded larvae and lesions to cuticle of L1 (Fig. 3a, b).

3

4 Table 1- Aqueous and ethanolic extracts of *Piptadenia viridiflora* leaves in egg hatching  
 5 of *Haemonchus contortus*

Treatments	Unembryonated egg mean	Embryonated egg mean	L1 mean	Eggs + L1	Efficacy* (%)
Aqueous extract (mg mL <sup>-1</sup> )					
2.4	7.2 <sup>b</sup>	35.8 <sup>b</sup>	0.0 <sup>c</sup>	35.8	100.0
1.2	2.0 <sup>b</sup>	43.8 <sup>ab</sup>	1.6 <sup>c</sup>	45.4	98.08
0.6	2.5 <sup>b</sup>	48.8 <sup>a</sup>	20.2 <sup>c</sup>	69.0	75.84
0.3	1.4 <sup>b</sup>	42.2 <sup>ab</sup>	46.0 <sup>b</sup>	88.2	44.98
0.15	1.4 <sup>b</sup>	13.0 <sup>c</sup>	72.6 <sup>a</sup>	85.6	13.16
Variation coefficient (%)	59.44	22.60	42.98		
Ethanolic extract (mg mL <sup>-1</sup> )					
1.2	36.0 <sup>bc</sup>	0.0 <sup>b</sup>	0.0 <sup>b</sup>	36.0	100.0
0.6	49.0 <sup>bc</sup>	0.0 <sup>b</sup>	7.6 <sup>b</sup>	56.6	90.9
0.3	54.2 <sup>abc</sup>	0.0 <sup>b</sup>	11.4 <sup>b</sup>	65.6	86.4
0.15	49.8 <sup>bc</sup>	4.0 <sup>b</sup>	21.6 <sup>b</sup>	75.4	74.2
0.075	25.2 <sup>cd</sup>	0.0 <sup>b</sup>	25.4 <sup>b</sup>	50.6	69.6
L. phosphate (0.3 mg mL <sup>-1</sup> )	78.0 <sup>ab</sup>	0.0 <sup>a</sup>	2.6 <sup>b</sup>	80.6	100.0
Ivermectin (16µ mL <sup>-1</sup> )	78.8 <sup>a</sup>	0.0 <sup>a</sup>	0.0 <sup>b</sup>	0.0	96.88
Sterile distilled water	0.0 <sup>d</sup>	0.0 <sup>ab</sup>	83.6 <sup>a</sup>	83.6	—
Variation coefficient (%)	30.12	119.39	76.45		

6

7 Mean followed by a different letter in the columns indicates significant differences by  
 8 tukey test (P < 0.05)

9 \*% efficacy = 100 x (1 - L1/initial egg + L1).

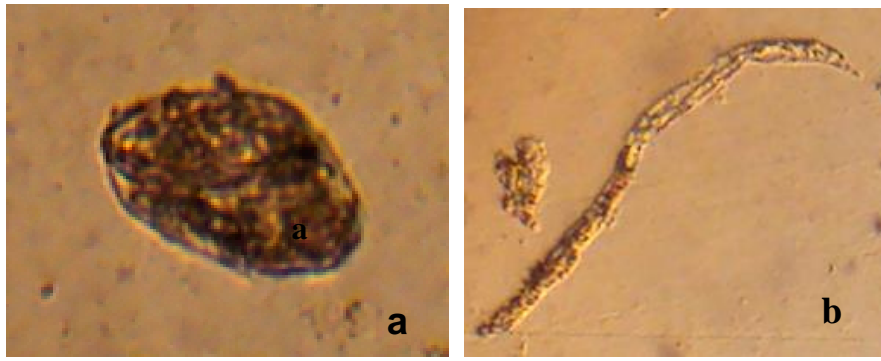
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11

12

13

14



1

2

3 Fig. 3 - *Haemonchus contortus* with embryonated egg degradation (a) and L1 with  
4 cuticle alteration (b) by the action of leaves ethanolic extract of *Piptadenia viridiflora*  
5 (objective 10×)

6

7 Research with other Cerrado plants has also reported high efficacies in EHI tests.  
8 The aqueous extract from fruit's skin of *Caryocar brasiliense*, popularly named as  
9 “pequi, in the EHI test at 15 mg ml<sup>-1</sup> presented anthelmintic efficacy of 98.7 and LC<sub>90</sub>  
10 was 7.35 mg ml<sup>-1</sup> (Nogueira et al. 2012a). Aqueous extract of *Annona muricata* at 50%  
11 inhibited 84.91% the egg hatching of *H. contortus* (Ferreira et al., 2013).

12

### 13 3.3 Larval development inhibition

14

15 The aqueous extract  $\geq 1.2$  mg mL<sup>-1</sup> showed mean of L3 significantly lower than  
16 those observed for the control with water (P<0.05). There was increase to effectiveness  
17 of LDI with increased concentration (Table 2) and estimated LC<sub>90</sub> was 2.28 mg g<sup>-1</sup> of  
18 feces.

19

20

21

1 Table 2 - Mean *Haemonchus contortus* larvae per gram of feces (LDPG) in quantitative  
 2 cultures treated with different concentrations of the aqueous extract of the *Piptadenia*  
 3 *viridiflora* leaves  
 4

Treatments	LPGF*	Efficacy (%)
Aqueous extract (mg g <sup>-1</sup> )		
38.62	0 <sup>f</sup>	100.00
19.31	55 <sup>e</sup>	93.13
9.65	105 <sup>d</sup>	86.88
4.83	180 <sup>c</sup>	77.05
2.41	285 <sup>b</sup>	64.38
1.21	385 <sup>b</sup>	55.63
Levamisole phosphate (0.1 mg g <sup>-1</sup> )	0 <sup>f</sup>	100.00
Ivermectin (16µ mL <sup>-1</sup> )	0 <sup>f</sup>	100.00
Sterile distilled water	800 <sup>a</sup>	---

5 Means followed by different letters in the columns indicate significant differences (P< 0.05),  
 6 by Duncan test.  
 7 Coefficient of variation LDI: 4.67%  
 8 \*LPGF: number of larvae (L3) per gram of feces  
 9 Efficacy: % efficacy = 100 x (1 - LPGF of the treated group/LPGF of the treated group).  
 10

11 In other research with Cerrado species has reported LDI efficacies at higher  
 12 concentration than *P. viridiflora*. The aqueous extract from *Caryocar brasiliense* fruit  
 13 peels at 200 mg mL<sup>-1</sup> significantly inhibited the development of *H. contortus* larvae  
 14 from sheep with efficacy of 94.8%. The qualitative phytochemical tests performed  
 15 indicated the presence of catechins, steroids, flavonoids, saponins, xanthonenes and  
 16 tannins that could promote mixed action (Nogueira et al. 2012). Ferreira et al.  
 17 (2013), demonstrated that aqueous extract of *Annona muricata* (Painã) leaves at 50%  
 18 concentration inhibited larval hatching in 83.29%. Nery et al. (2010), evaluated extracts  
 19 of leaves *Anacardium humile* A. St.-Hil. (Anacardiaceae), against different species of

1 gastrointestinal nematodes in sheep. The aqueous extract at 150 mg mL<sup>-1</sup> showed LDI  
2 significantly higher than negative control at all concentrations evaluated.

3 In other study, the aqueous extract of immature fruits at 50.0 mg ml<sup>-1</sup> showed  
4 effective anthelmintic activity for LDI of 90 % and the fresh juice of mango showed  
5 100 % efficacy at 59.2 mg ml<sup>-1</sup> (Nery et al. 2012).

6

### 7 3.4 Toxicity test

8

9 For evaluated concentrations, no clinical signs of toxicity for mucosal tissues or  
10 changes in animal behavior or deaths during were observed in the four days of extract  
11 administration. During the autopsy, macroscopical alterations were no observed in the  
12 liver, kidneys, spleen, lungs or other viscera. Then maximum tolerated dose for male  
13 and female mice was > 38.62 mg kg<sup>-1</sup> bw.

14

### 15 3.5 *In vivo* anthelmintic activity

16

17 The FEC was influenced by treatments and periods. Oral administration of *P.*  
18 *viridiflora* extract promoted reduction of FEC means for the three pos-treatments  
19 periods that was significantly lower than the observed for the control group in its  
20 respective periods. The *in vivo* anthelmintic efficacies were between 32.9 - 47.2%  
21 (Table 3) and the animals showed no behavioral changes, submandibular edema,  
22 weakness or lack of appetite during the experiment.

23

24

25

Table 3 - Mean of fecal egg count (FEC) from sheep and anthelmintic efficacy after oral administration of aqueous extract of the *P. viridiflora* leave at 283 mg (dm) kg<sup>-1</sup> bw or levamisole phosphate at 0.6 mg kg<sup>-1</sup> bw

Treatments	Initial period	First week		Second week		Third week	
		FEC g <sup>-1</sup>	Efficacy (%)	FEC g <sup>-1</sup>	Efficacy (%)	FEC g <sup>-1</sup>	Efficacy (%)
Control	5601,75 A	3950,0 Aa	--	2675 Ba	--	2650 Ba	--
Levamisole	4483,00 A	66,7 Bc	99.99	29,25 Cc	98.90	18,75 Dc	97.48
<i>P. viridiflora</i>	5962,37 A	2088,0 Bb	47.15	1469,00 Cb	45.10	1781,00 Cb	32.86

Means followed by different uppercase letters in rows and lowercase letters in columns differ by Scott-Knott test with values of P<0.05

Efficacy = 100 x (1 – mean FEC of treated group/mean FEC of control group)

Variation coefficient of = 8.85%

1       The aqueous extract of *P. viridiflora* leaves produced lower reduction when  
2 compared to synthetic anthelmintics recommended by World Association for the  
3 Advancement of Veterinary Parasitology (WAAVP). However these results are  
4 promising, since it was used at low doses and administered in three single doses and  
5 could reduce multi-resistant nematode populations. Future studies with a larger dose at  
6 greater frequency may indicate better anthelmintic efficacy *in vivo*.

7       Other researches with plants from Cerrado have reported *in vivo* efficacies similar  
8 with the observed in this study. *Pterocaulon interruptum* aqueous extract were  
9 administered orally at 33.34 mg kg<sup>-1</sup> bw reduced to 47% of the FEC in sheep  
10 (Krychak-Furtado, 2006). Significant FEC reductions were observed in lambs treated  
11 with the “traditional” of the dried bark *Albizia anthelmintica* - Fabaceae (Coração-de-  
12 nego) preparation with efficacy of 34% (Githiori et al., 2003).

13       In sheep naturally infected with mixed species of gastrointestinal nematodes in the  
14 Pakistan was administered aqueous methanolic extract of *Caesalpinia crista* (L) seed  
15 and *Chenopodium album* (L) whole plant, the maximum FEC reductions were and 93.9  
16 and 82.2% with *C. album* and *C. crista* at 3.0 g kg<sup>-1</sup> bw, on day 13 and 5 post-  
17 treatment, respectively.

18       In experiments *in vivo*, the extracts of plants often do not reach the level proposed  
19 for commercial anthelmintic products by the WAAVP (Githiori et al. 2006). In this  
20 study was verified high *in vitro* efficacies e a lower *in vivo* efficiency. Differences of  
21 anthelmintic efficacies of plant treatment between *in vitro* and *in vivo* tests have been  
22 reported (Peneluc et al., 2009, Nogueira et al., 2012). Report that the efficacy of the  
23 plant extracts may vary with bioavailability of plant compounds in different  
24 compartments of the gastrointestinal tract of ruminates infected and with the parasite

1 specie, producing divergent results study between *in vitro* and *in vivo* with the same  
2 plant species (Athanasiadou et al., 2007; Eguale et al., 2007).

### 3 4 3.6 Blood parameters of sheep

5  
6 Erythrocyte and hematocrit values were within the normal physiological patterns  
7 for lambs (Table 4). The hematocrit is good indication of the anemia level and  
8 represents important parameter for the assessment hematophagous parasites (Sotomaior  
9 et al. 2007). In cases of acute haemonchosis, anemia is characterized by progressive  
10 decrease in hematocrit (Taylor et al. 2010). Values below 15% are concomitant with  
11 weakness and indicate bad prognosis for the animal (Bowman 2010). Costa et al. (2011)  
12 found hematocrit values ranging from 24.7 to 29.6%, respectively, for the treated sheep  
13 with different anthelmintics or not treated. These values were similar to those observed  
14 in this study (Table 4).

15 For plasmatic albumin and total protein, significant effects between treatments  
16 were not found (Table 4). In the early period there was decrease of protein values,  
17 which could be justified by the spoliation of *H. contortus* in the acute phase, at the  
18 experiment beginning. However, after the 3rd week it was observed normal values for  
19 this variable (Table 4).



Table 4 – Average of erythrocyte, hematocrit, protein and albumin plasmatic from lambs infected with *Haemonchus contortus* and treated orally with *Piptadenia viridiflora* (283 mg kg<sup>-1</sup> bw) or levamisole phosphate (0.6 mg kg<sup>-1</sup> bw)

Treatments	Collection period (days)				Reference <sup>a</sup>	CV (%)
	0	7	14	21		
	Erythrocyte (×10 <sup>6</sup> μL <sup>-1</sup> )					
Control	11.57 a	11.00 a	10.91 a	10.11 a		
Levamisole	11.24 a	9.08 a	9.15 a	9.72 a	9-15	17.35
<i>P. viridiflora</i>	10.84 a	11.87 a	11.46 a	9.73 a		
* Interaction period	11.22 A	10.64 A	10.63 A	9.85 A		
	Hematocrit (%)					
Control	34.67 Aa	31.75 Aa	34.97 Aa	30.35 Aa		
Levamisole	34.00 Aa	29.35 Aa	30.85 Aa	29.85 Aa	27-45	18.11
<i>P. viridiflora</i>	33.22 Aa	35.35 Aa	35.12 Aa	35.17 Aa		
	Total protein (%)					
Control	5.73 a	7.29 a	7.91 a	6.71 a		
Levamisole	5.70 a	6.76 a	7.35 a	5.66 a	6-7.9	16.25
<i>P. viridiflora</i>	5.67 a	5.36 a	6.44 a	6.12 a		
* Interaction period	5.70 B	6.47 B	7.23 A	6.17 B		
	Albumin (g dL <sup>-1</sup> )					
Control	4.02 a	3.60 a	4.10 a	4.17 a		
Levamisole	2.87 a	3.42 a	2.87 a	4.27 a	2.4-3	26.68
<i>P. viridiflora</i>	3.27 a	3.42 a	5.30 a	3.52 a		
* Interaction period	3.39 B	3.48 B	3.99 B	4.09 B		

Different letters in uppercase and lowercase line in columns differ significantly by the Scott-Knott test (P < 0.05)<sup>5a</sup> Reference range for sheep (Sharp, Corp., 2015)

6

7           The plasmatic albumin values can be indicator of protein deficit or chronic  
8 lesions in the live (Reis et al. 2007). The albumin values found in this study were within  
9 the normal range and probably did not occurred protein deficit, despite infection with  
10 this nematode. Possibly adequate diet administrated to the animals might has  
11 contributing to the maintenance of plasma albumin. In another study in sheep treated  
12 with albendazole, ivermectin and levamisole, closantel, moxidectin was also reported

1 similar concentrations of serum albumin between treated or not treated groups  
2 (Holsback et al. 2013).

3 The results indicated that aqueous extract of *P. viridiflora* administered at 283  
4 mg (dm) kg<sup>-1</sup> bw during three consecutive days did not interfere with normal standards  
5 showing no toxicity to blood parameters evaluated.

6

#### 7 **4 Conclusion**

8

9 *Pipitadenia viridiflora* shows promising potential as alternative treatment of  
10 haemonchosis. The low concentrations of leaf extracts show high efficacy to EHI and  
11 LDI. Aqueous extract administered at 283 mg (dm) kg<sup>-1</sup> bw during three consecutive  
12 days promotes moderated efficacy to FEC reduction and no clinical or blood disorders.  
13 Futures analyses with higher doses, other extraction processes and higher administration  
14 frequency could promote better *in vivo* efficacy to alternative control of *Haemonchus*  
15 *contortus*.

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1 **CAPTER 4**

2 *Ximения americana* L. (Olacaceae), selected from Cerrado to control of  
3 *Haemonchus contortus* in lambs

4 **Abstract**

5 Resistance to anthelmintics has been common in different continents. *Ximения*  
6 *Americana* is an plant from Cerrado selected naturally by sheep and could be source of  
7 bioactive compounds for development of new farmacs. In this study, *in vitro* and *in vivo*  
8 efficacy this vegetal was evaluated for lambs naturally infected with *Haemonchus*  
9 *contortus*. The presence of tannins and flavonoids for aqueous and ethanolic extracts of  
10 the leaves were indicated by HPLC-DAD. These extracts at 0.6 and 1.2 mg mL<sup>-1</sup>,  
11 respectively, showed 100% efficacy to egg hatch inhibition (EHI). In larval  
12 development inhibition (LDI) test with quantitative cultures, crude powder ≥ 333.3 mg  
13 g<sup>-1</sup> or aqueous extract ≥ 22.70 mg mL<sup>-1</sup> promoted L3 mean lower than those observed  
14 for the control with water (P<0.05) and the estimated LC90 were 41.96 and 8.1 mg kg<sup>-1</sup>  
15 bw of feces respectively. Maximum tolerated dose for male and female mice was >  
16 22.70 mg kg<sup>-1</sup> bw in probe gavage via. *In vivo* of lambs crude powder and aqueous  
17 extract were orally administered at 157.35 and 30 mg (dm) kg<sup>-1</sup> bw respectively during  
18 three consecutive days. There was no efficacy. Low concentrations of extracts promoted  
19 high efficacy for EHI and LDI. The oral treatment with crude powder and aqueous  
20 extract is not promising *in vivo* anthelmintic efficacies not interference with normal  
21 clinical standards.

22 **Keywords:** Brazilian savanna, sheep, antihelmintic plant, phytochemical composition,  
23 toxicity.

24

25

## 1 **1 Introducion**

2

3 *Ximenia americana* L. (Olacaceae) it is popularly known as “ameixa-do-cerrado”  
4 (Lorenzi, 2009) is is found frequently in the Cerrado and Africa India, New Zeland,  
5 Central America and Sul America (Sacande; Vautier, 2006). *X. americana* is  
6 characterized as shrub 3-4 meters high, with a prickly small tree (Matos, 2007). It has  
7 healing action, which can be justified by the presence of certain substances, such as  
8 tannins (Veras; Morais, 2004).

9 In preliminary study of Cerrado Biome, *X. americana* was naturally selected by  
10 lambs grazing during the dry and rainy seasons, with selectivity index 2.34-16.03%  
11 (Morais-Costa, et al., 2014 ), when this ratio is  $\geq$  one indicates that this vegetal species  
12 is important in the animal's diet . Then the purpose in this study was to analyze *in vitro*  
13 and *in vivo* extracts of *Ximenia americana* to control *Haemonchus contortus*.

14

## 15 **2 Material and methods**

16

### 17 2.1 Study area

18 The research was conducted in a rural area of Montes Claros of North of Minas  
19 Gerais state, Brazil (W 43°50'33.56”; S 16°41'10.05”). The climate of this region is  
20 tropical wet and dry (Aw) according to the Köppen classification and marked by long  
21 dry season from May to October and rainy period in November to April at 2013.

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1 2.2 Production of vegetal extratcts

2

3 Healthy leaves of *Ximenaia americana* (Fig. 1) from Cerrado Biome were  
4 selected and dried to constant weight in a forced air circulating drier at 40°C for 72 h.  
5 Dried leaves were grinded in a wiley mill and then were stored in paper bags, free of  
6 light incidence. Samples of the plant were stored in the Montes Claros Herbarium  
7 (HMCMG) of the Universidade Estadual de Montes Claros-Brazil, with voucher  
8 specime, 211.

9



10

11 Figure 1 - *Ximenaia Americana* L. (Olacaceae)

12

13

14 Aqueous extract of dried leaves were held in a distilled water bath at 40°C for 60  
15 min. Ethanolic extract was obtained by maceration of the dried leaves in absolute ethyl  
16 alcohol (PA), in glass amber containers, kept in a dark place and stored for seven days.  
17 After this extraction, filtration was held through a gauze funnel. The extracts were

1 dehydrated at 40°C for 48 h, until obtaining the residue with constant weight and stored  
2 in paper bags in darkness and refrigerated at ~4°C until use (Adapted Nery et al., 2010).

3

4 2.3 High-performance liquid chromatography (HPLC) analyses and proanthocyanidin  
5 quantification

6

7 A Waters Alliance 2695 HPLC system composed of a quaternary pump, an  
8 autosampler, a photodiode array detector (DAD) 2996, and a Waters Empower Pro data  
9 handling system was used (Waters Corporation, Milford, USA). The analyses were  
10 performed on a LiChrospher 100 RP-18 column (250 × 4 mm i.d., 5 µm; Merck,  
11 Darmstadt, Germany) combined with a LiChrospher 100 RP-18 guard column (4 × 4  
12 mm i.d., 5 µm; Merck) at 40 °C. Water (A) and acetonitrile (B) were used as eluents,  
13 both containing 0.1% (v/v) of H<sub>3</sub>PO<sub>4</sub> at a flow rate of 1.0 mL min<sup>-1</sup> as follows: 0 min,  
14 95% A and 5% B; 60 min, 5% A, 95% B, followed by 10 min of isocratic elution.  
15 Solvents used were of HPLC grade (Merck, Germany) and were degassed by sonication  
16 before use. The chromatograms were obtained at 210 nm, and the UV spectra were  
17 recorded on-line from 190 to 400 nm.

18 The aqueous and ethanolic extracts were dissolved in methanol (HPLC-grade),  
19 ultrapure water, or hydroethanolic solutions, according to their solubility, to  
20 concentrations of 10 mg mL<sup>-1</sup>. After centrifugation at 8 400 x g, the sample solutions  
21 (10 µL) were automatically injected into the apparatus.

22 Total proanthocyanidin content of the dried aqueous extracts was determined by  
23 measuring at 540 nm the absorbance of the cyanidin chloride resulting from acid-  
24 catalyzed solvolysis with *n*-BuOH/HCl 37% (95:5), according to the method described  
25 by Hiermann et al. (1986). Each sample was analyzed in triplicate and the results

1 expressed as mean  $\pm$  standard deviation. The total proanthocyanidin content, expressed  
2 as cyanidin chloride, was calculated using the following formula:

3

$$4 \quad \% = \frac{A_{\text{sample}} - A_{\text{blank}} \times 4.115}{m_{\text{sample}}}$$

5  $A$  is the measured absorbance at 420 nm, and  $m_{\text{sample}}$  is the sample weight in g.

6

#### 7 2.4 Egg hatching inhibition (EHI)

8

9 The aqueous and ethanolic extract of the dried leaves was standardized at 1.2 mg  
10  $\text{mL}^{-1}$  and diluted in sterile distilled water. These extracts were used in EHI tests  
11 immediately after dissolution as described Coles et al. (1992).

12 Flotation, sedimentation, and filtration techniques in saturated NaCl solution were  
13 conducted to obtain nematode eggs from feces of two Santa Inês lambs infected only  
14 with *Haemonchus contortus* and with an average fecal egg count (FEC)  $>1000 \text{ g}^{-1}$ ,  
15 determined using the modified McMaster technique (Gordon and Whitlock, 1939).

16 Experimental mixtures contained: 100  $\mu\text{l}$  fecal suspension with an average of 80  
17 fresh eggs, and 100  $\mu\text{l}$  of the extract, at final concentrations 1.2-0.037  $\text{mg mL}^{-1}$  or two  
18 positives controls with solution levamisole phosphate (0.3  $\text{mg mL}^{-1}$ ) or ivermectin (16  $\mu$   
19  $\text{mL}^{-1}$ ) or a negative control with sterile distilled water. The samples were homogenized  
20 and incubated in a BOD incubator at 28°C for 48 h. Subsequently, 15  $\mu\text{l}$  Lugol's  
21 solution was added to each tube, which were then stored at 4°C for subsequent counting  
22 of unembryonated eggs, embryonated eggs, first stage larvae (L1; Coles et al. 1992).

23 The number of L1 relative to the total number of eggs plus L1 was determined for  
24 each repetition and subjected to variance analysis. The means were compared using the

1 Tukey test at 5% significance. Probit regression was employed to determine the  
2 concentrations sufficient to inhibit 90% (lethal concentration, LC<sub>90</sub>) of egg hatching  
3 using the statistical package, Saeg 9.1 (2007).

4

5 The formula of Coles et al. (1992) was used to determine the EHI effectiveness:

6

7  $\% \text{ effectiveness} = 100 \times (1 - \text{mean of L1} / \text{mean eggs} + \text{L1})$

8

9 2.5 *In vitro* larval development inhibition (LDI)

10

11 The effectiveness of the crude powder and aqueous extract were evaluated by  
12 adapted coproculture quantitative methodology (Borges, 2003; Nery et al. 2010,  
13 Nogueira, 2012) using fresh feces of lambs with *Haemonchus contortus* mono-  
14 infection.

15 Eight treatments were performed, each with five replicates, including two  
16 positive control: 2 mL of solution 16  $\mu\text{g g}^{-1}$  ivermectin (final concentration) or 2 mL of  
17 solution 0.1  $\text{mg g}^{-1}$  levamisole phosphate (final concentration) and a negative control of  
18 2 mL of sterile purified water, both added to 2 g feces.

19 The five treatments for crude powder for standardized at 83.3-333.3  $\text{mg of dw g}^{-1}$   
20 of fecal culture final concentration and and five treatments aqueous extract, was  
21 standardized at 1.42-22.70  $\text{mg dw g}^{-1}$  of fecal culture. The samples were incubated in a  
22 BOD incubator at 28°C for seven days and assessed for presence of infective larvae  
23 (L3). The following formula, adapted from Borges (2003), was used to determine the  
24 percent reduction of larva  $\text{g}^{-1}$  of feces (LPGF):

25  $\% \text{ efficacy} = 100 \times (1 - \text{LPGF of the treated group} / \text{LPGF of the treated group})$



1           The data were transformed in  $\log(x + 1)$  and submitted to variance analysis. The  
2 means were compared through the Duncan test at 5% probability and  $LC_{90}$  was  
3 determined by probit analysis using the statistical package SAEG® 9.1 (2007).

#### 4 5 2.6 Toxicity in mice

6  
7           The mouse toxicity testing was performed as Walum (1998) to determine the  
8 maximum tolerated dose (MTD) for an adult mouse. Using probe gavage 22 $\mu$ L of  
9 aqueous extract of *X. americana* was added for four Balb C mice (2 males and 2  
10 females) with average 22g of body weight and 6 to 8 weeks.

11           For the first and second days the extract was diluted at 100 and 10 times  
12 respectively in 1x PBS. In the third and fourth days the extract was administered at  
13 22.70 mg ml<sup>-1</sup>. The mice were euthanized by cervical dislocation on day 5 after extract  
14 administration.

#### 15 16 2.7 *In vivo* anthelmintic test

17  
18           Two experiments were performed crude powder or aqueous extract. For each  
19 analyses were used on 24 Santa Inês lambs with average 26.5 kg bw of both sexes, with  
20 4-to-8-month-old. Prior to the beginning of the trial, all sheep were administered  
21 albendazole (10 mg kg<sup>-1</sup> bw) and phosphate levamisole (0.6 mg kg<sup>-1</sup> bw), to ensure that  
22 were worm-free.

23           During 10 days of adaptation, the animals were individually confined which fed  
24 balanced diet containing sorghum silage, concentrate, mineral premix and water *ad*  
25 *libitum*, according to the age category requirement.

1           The animals, with zero fecal egg count (FEC), were infected with 800 L3 for  
2 10kg<sup>-1</sup> bw from lambs naturally contaminated in *H. contortus*. Twenty-eight days post-  
3 infection, sheep were assigned to one of three homogeneous groups based on FEC,  
4 weight, and sex.

5           One untreated animals served as the negative control, other group was orally  
6 administered phosphate levamisole (0.6 mg kg<sup>-1</sup> bw) via subcutaneous and represented  
7 the positive control, the third group was administered crude powder of *X. americana*,  
8 at 157.35 mg (dm) kg<sup>-1</sup> bw during three consecutive days, the third group was  
9 administered aqueous vegetal extract of *X. americana*, by esophageal gavage, at  
10 30.375 mg (dm) kg<sup>-1</sup> bw during three consecutive days and the positive control.

11           The mean of two counts of FEC was obtained for each collection periods. The  
12 McMaster technique was utilized with the addition of saturated NaCl for FEC in  
13 duplicate (Gordon and Whitlock, 1939).

14           Initial FEC value was recorded based on the mean values of three days prior to  
15 initiation of treatment. Subsequently, mean FEC was determined for three periods post-  
16 treatment to efficacy analysis: 7, 8 and 9 days (first period), 14, 15 and 16 ( second  
17 period) and 21, 22 and 23 (third period). Fecal samples were cultured to obtain larvae  
18 for identification and confirmation of mono-infection by *H. contortus* (Ueno &  
19 Gonçalves, 1998).

20           The data relating FEC values were previously transformed into log (x + 10) and  
21 subjected to analysis of variance and the means were compared by Duncan test  
22 (p<0.05). A formula adapted from Coles et al. (1992) was used to determine the  
23 percentage efficacy in FEC reduction:

24           Efficacy = 100 x (1 – mean FEC of treated group/mean FEC of control group)

25

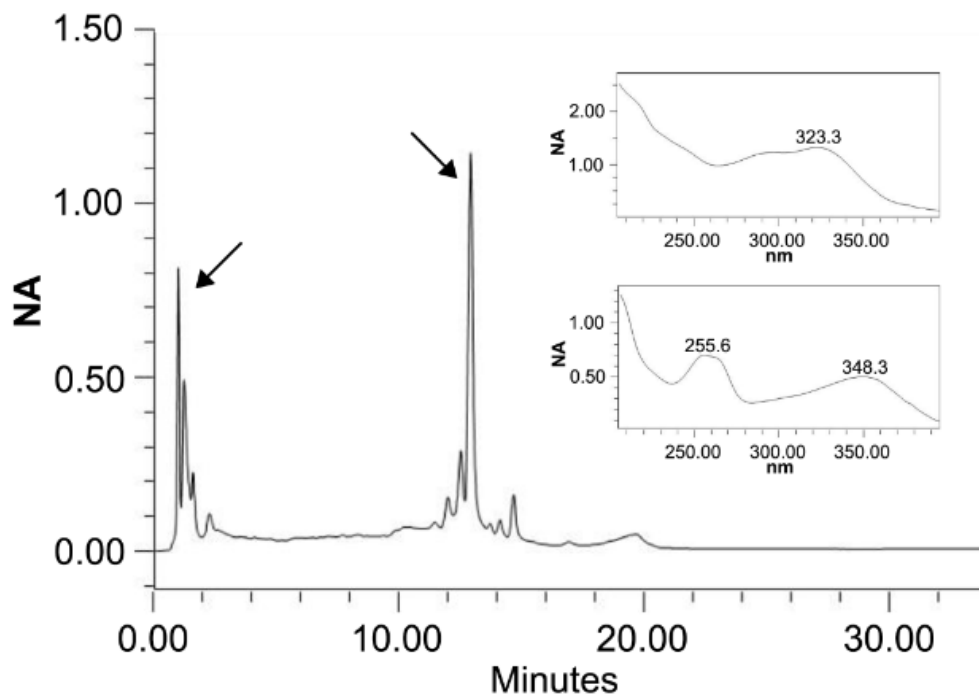
1 All procedures were performed in accordance with the principles of animal  
2 experiments approved in the 275/2013 protocol of the Ethics Committee on the use of  
3 animals (CEUA) of the Federal University of Minas Gerais, Brazil.

### 4 5 **3 Results and discussion**

#### 6 7 3.1 Extracts characterization

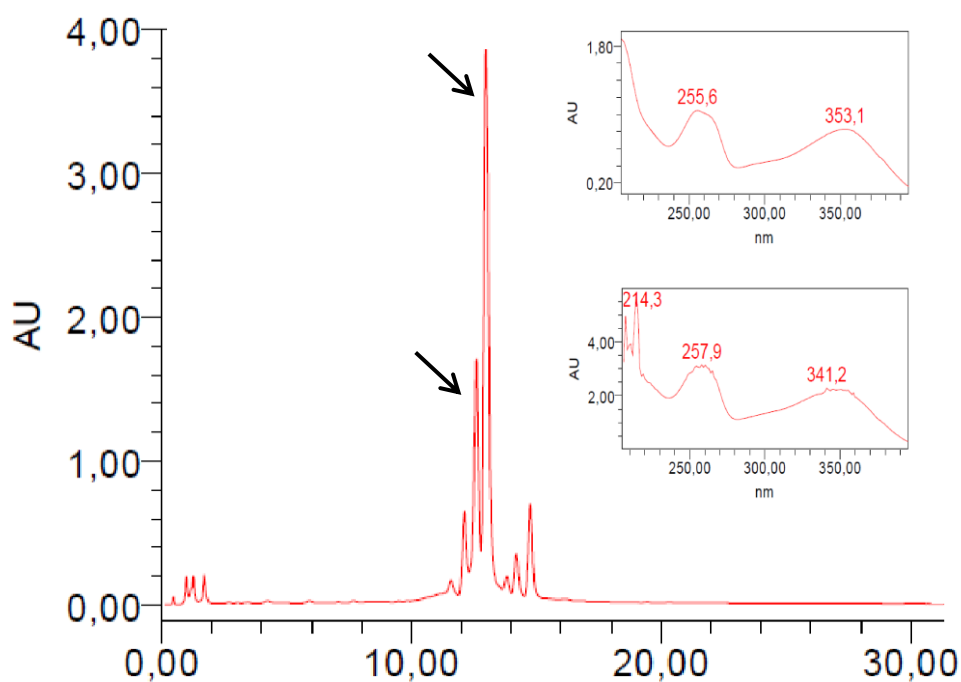
8  
9 The presence of polyphenols for both extracts was indicated by HPLC-DAD  
10 chromatograms, which clearly indicate the predominance of peaks corresponding to  
11 polar compounds, with UV spectra compatible with polyphenols (Fig. 2, 3). In addition,  
12 the HPLC chromatograms both extracts showed present major peaks with UV spectra  
13 characteristic of flavonoids ( $\lambda$  255.6 and 348.3 nm aqueous and  $\lambda$  255 and 353.1 to  
14 ethanolic). The quantification total de proanthocyanidin concentrations for aqueous and  
15 ethanolic extracts were  $0.3\% \pm 0.1$  and  $0.4\% \pm 0.2$  respectively.

16 In general, the compounds found in *X. americana* were saponins, glycosides,  
17 flavonoids, tannins, phenolics, alkaloids, quinines, and terpenoids. In addition, the plant  
18 is rich in fatty acids and glycerides, and the seeds contain cyanide derivatives (Monte et  
19 al. 2012). Tannins can exert direct antihelminthic action, interfering with the natural  
20 cycle of helminths, or indirectly, by inhibiting the degradation of ruminal protein  
21 (Ketzis et al. 2006).



1

2 Figure 2 - HPLC-DAD chromatograms obtained for the aqueous extracts of the *Ximenia*  
 3 *americana*



4

5 Figure 3 - HPLC-DAD chromatograms obtained for the aqueous ethanolic of the *Ximenia*  
 6 *americana*.

7

8

9

1 3.2 Egg hatching inhibition

2

3 The effectiveness was directly related with increasing concentration of the  
 4 aqueous extract of *X. americana* leaves. The aqueous and ethanolic efficacies were at  
 5 42.58-100% and 57.41-100% respectively (Table 1), and LC<sub>90</sub> for these extracts were 2.17  
 6 and 2.10 mg mL<sup>-1</sup> respectively. All concentrations tested for both extracts promoted  
 7 significant reduction for hatched larvae mean when compared to the negative control  
 8 with distilled water. The aqueous extracts showed greater inhibition of larval hatching,  
 9 whereas ethanol extract showed the best inhibition of early embryonic development  
 10 (Table 1).

11 Table 1 - Aqueous and ethanolic extracts of *Ximonia americana* leaves in egg hatching of  
 12 *Haemonchus contortus*

13

Treatments	Unembryonated egg mean	Embryonated egg mean	L1 mean	Eggs + L1	Efficacy* (%)
Aqueous extract (mg mL <sup>-1</sup> )					
0.6	1.4 <sup>b</sup>	27.8 <sup>a</sup>	0.0 <sup>e</sup>	29.2	100.0
0.3	1.8 <sup>b</sup>	23.6 <sup>a</sup>	3.0 <sup>e</sup>	28.4	96.41
0.15	2.2 <sup>b</sup>	25.4 <sup>a</sup>	11.6 <sup>d</sup>	39.2	86.12
0.075	0.8 <sup>b</sup>	14.6 <sup>b</sup>	38.2 <sup>c</sup>	53.8	54.30
0.037	0.8 <sup>b</sup>	2.33 <sup>c</sup>	48.0 <sup>b</sup>	51.13	42.58
Variation coefficient (%)	8.12	21.15	14.61		
Ethanolic extract					
1.2	0.4 <sup>d</sup>	2.4 <sup>bc</sup>	0.0 <sup>f</sup>	2.8	100.0
0.6	42.6 <sup>c</sup>	0.0 <sup>c</sup>	8.2 <sup>de</sup>	50.8	90.19
0.3	55.0 <sup>b</sup>	0.0 <sup>c</sup>	12.0 <sup>d</sup>	67.0	85.65
0.15	44.8 <sup>b</sup>	5.4 <sup>b</sup>	21.4 <sup>c</sup>	71.6	74.40
0.075	0.0 <sup>d</sup>	13.2 <sup>a</sup>	35.6 <sup>b</sup>	48.8	57.41
Levamisole (0.3 mg mL <sup>-1</sup> )	78.0 <sup>ab</sup>	0.0 <sup>a</sup>	2.6 <sup>b</sup>	80.6	100.0
Ivermectin (16µ mL <sup>-1</sup> )	78.8 <sup>a</sup>	0.0 <sup>a</sup>	0.0 <sup>b</sup>	0.0	96.88
Sterile distilled water	0.0 <sup>d</sup>	0.0 <sup>ab</sup>	83.6 <sup>a</sup>	83.6	—
Variation coefficient (%)	9.53	72.20	15.47		

14 Mean followed by a different letter in the columns indicates significant differences by Tukey test  
 15 ( $P < 0.05$ ).

16 % efficacy = 100 x (1 - L1/initial egg + L1).

1 Research with other Cerrado plants has also reported high efficacies in EHI tests.  
 2 The Aqueous extract of *C. brasiliense* fruit's skin in the EHI test at 15 mg ml<sup>-1</sup>  
 3 presented anthelmintic efficacy of 98.7 and LC<sub>90</sub> was 7.35 mg ml<sup>-1</sup> (Nogueira et al.  
 4 2012). The essential oil from *Chenopodium ambrosioides* (Erva-de-Santa-Maria)  
 5 administered in goats at 0.2 mg ml<sup>-1</sup> bw reduced EHI of *H. contortus* but had no effect  
 6 (P>0.05) on the reduction of number of eggs or adult nematodes (Ketzis et al. 2002).  
 7 Aqueous extract of *A. muricata*, cerrado specie inhibited the hatching of *H. contortus*  
 8 eggs by 84.91% with for dilution 50%.

9  
 10 3.3 Larval development inhibition  
 11

12 The crude powder 333.3 mg g<sup>-1</sup> showed mean of L3 significantly lower than  
 13 those observed for the control with water (P<0.05). There was increase to effectiveness  
 14 of LDI with increased concentration (Table 2) and estimated LC<sub>90</sub> was 41.96 mg g<sup>-1</sup> of  
 15 feces.

16  
 17 Table 2 - Mean *Haemonchus contortus* larvae per gram of feces (LPGF) in quantitative  
 18 cultures treated whit different concentrations of the crude powder of the *Ximения*  
 19 *americana* leaves  
 20

Tratamentos (mg g <sup>-1</sup> )	LPG*	Eficácia (%)
333,3	0,0 <sup>c</sup>	100
250,0	6,6 <sup>c</sup>	99,1
166,7	6,6 <sup>bc</sup>	99,1
83,3	26,5 <sup>b</sup>	96,3
Levamisol (0.1 mg g <sup>-1</sup> )	0,0 <sup>c</sup>	100.0
Ivermectin (16 μ mL <sup>-1</sup> )	0 <sup>c</sup>	100.0
Sterile distilled water	713,3 <sup>a</sup>	---

21 Means followed by different letters in the columns indicate significant differences (P<  
 22 0.05), by Duncan test.

23 Coefficient of variation LDI: 20.10%

24 \*LPGF: number of larvae (L3) per gram of feces

25 Efficacy: % efficacy = 100 x (1 - LPGF of the treated group/LPGF of the treated group).  
 26

1           The aqueous extract 22.70 mg mL<sup>-1</sup> showed mean of L3 significantly lower than  
 2 those observed for the control with water (P<0.05). There was increase to effectiveness  
 3 of LDI with increased concentration (Table 3) and estimated LC<sub>90</sub> was 8.1 mg g<sup>-1</sup> of  
 4 feces.

5  
 6 Table 3 - Mean *Haemonchus contortus* larvae per gram of feces (LDPG) in quantitative  
 7 cultures treated with different concentrations of the aqueous extract of the *Ximenia*  
 8 *americana* leaves  
 9

Treatments	LPGF *	Efficacy (%)
Aqueous extract (mg g <sup>-1</sup> )		
22.70	0 <sup>e</sup>	100
11.34	25 <sup>d</sup>	96.88
5.67	165 <sup>c</sup>	79.38
2.84	190 <sup>c</sup>	76.25
1.42	275 <sup>d</sup>	65.63
Levamisole phosphate (0.1 mg g <sup>-1</sup> )	0 <sup>f</sup>	100.00
Ivermectin (16µ mL <sup>-1</sup> )	0 <sup>f</sup>	100.00
Sterile distilled water	800 <sup>a</sup>	---

10 Means followed by different letters in the columns indicate significant differences (P< 0.05),  
 11 by Duncan test.

12 Coefficient of variation LDI: 3.04%

13 \*LPGF: number of larvae (L3) per gram of feces

14 Efficacy: % efficacy = 100 x (1 - LPGF of the treated group/LPGF of the treated group).  
 15  
 16

17           The aqueous extract from *Caryocar brasiliense* fruit peels, popularly named as  
 18 “pequi”, assayed at 200 mg mL<sup>-1</sup> significantly inhibited the development of *H.*  
 19 *contortus* larvae from sheep with efficacy of 94.8%. Ferreira et al. (2013), demonstrated  
 20 that aqueous extract of *Annona muricata* (Panã) leaves at 50% concentration inhibited  
 21 larval hatching in 84.9%. Nery et al. (2010), evaluated extracts of leaves *Anacardium*  
 22 *humile* A. St.-Hil. (Anacardiaceae), against different species of gastrointestinal  
 23 nematodes in sheep.

24

25

### 1 3.4 Toxicity test

2

3 For evaluated concentrations, no clinical signs of toxicity for mucosal tissues or  
4 changes in animal behavior or deaths during were observed in the four days of extract  
5 administration. During the autopsy, macroscopical alterations were no observed in the  
6 liver, kidneys, spleen, lungs or other viscera. Then maximum tolerated dose for male  
7 and female mice was  $> 22.70 \text{ mg kg}^{-1} \text{ bw}$ .

8

### 9 3.5 *In vivo* anthelmintic activity

10

11 There was no efficacy for crude powder or aqueous extract. This may be due  
12 because significant physiological differences are expected in conditions *in vitro* and in  
13 *vivo* (Githiori et al. 2006). Eguale et al. (2007), reported that the lower effect *in vivo* can  
14 be attributed the biotransformation of some of the active components inside the  
15 digestive tract of the host.

16

### 17 **Conclusion**

18 *Ximения americana* shows promising potential as alternative treatment of  
19 haemonchosis. The low concentrations of leaf extracts show high efficacy to EHI and  
20 LDI. Crude powder administered at  $157 \text{ mg (dm) kg}^{-1} \text{ bw}$  or aqueous extract at  $30.375$   
21  $\text{mg (dm) kg}^{-1} \text{ bw}$  during three consecutive days dont promoted efficacy to FEC  
22 reduction. Futures analyses with higher doses, other extraction processes and higher  
23 administration frequency could promote better *in vivo* efficacy to alternative control of  
24 *Haemonchus contortus*.

25



1 **References**

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## 1 Conclusões finais

2

3 1) As principais espécies vegetais selecionadas por ovinos da Raça Santa Inês em pastejo  
4 em área de Cerrado foram: *Casearia silvestres*, *E. deciduum*, *Evolvulus* sp., *H.*  
5 *byrsonimifolia*, *Paullinea* sp. *Piptadenia viridiflora*, *Schinopsis brasiliensis* e *Ximения*  
6 *americana*. A ação de extrato de folhas desidratadas dessas espécies foi testada *in vitro*  
7 a fim de se observar o potencial antihelmíntico frente *Haemonchus contortus*.

8

9 2) *Piptadenia viridiflora* e *Ximения americana* apresentam respectivamente, baixos teores  
10 de taninos condensados (0,2 e 0,3%), embora, apresentem potencial antihelmíntico  
11 alternativo promissor no tratamento de hemoncoses. Já que extratos brutos das folhas de  
12 tais plantas são eficientes em testes *in vitro* contra larvas infectantes de *Haemonchus*  
13 *contortus* de 86.6 a 99.6%.

14

15 3) Baixas concentrações dos extratos aquoso e etanólico das folhas de *P. viridiflora* e *X.*  
16 *americana* mostram elevada eficácia em testes de eclodibilidade de ovos e larvas de  
17 *Haemonchus contortus*.

18

19 4) O extrato aquoso de *P. viridiflora* administrado *in vivo* à concentração de 283 mg (ms)  
20  $\text{kg}^{-1}$  de peso corporal durante três dias consecutivos promove moderada eficácia para a  
21 redução da ovos nas fezes sem promover sintomas de toxicidade e alterações nos  
22 padrões fisiológicos do sangue de ovinos da Raça Santa Inês experimentalmente  
23 infectados por *Haemonchus contortus*.

24

25 5) As folhas desidratadas de *X. americana* administrada em 157 mg (ms)  $\text{kg}^{-1}$  de peso  
26 corporal ou extrato aquoso em 30,375 mg (ms)  $\text{kg}^{-1}$  de peso corporal durante três dias

- 1 consecutivos não promovem reduções das contagens de ovos nas fezes de ovinos da
- 2 Raça Santa Inês experimentalmente infectados por *Haemonchus contortus*.
- 3
- 4

WILLIAM QUICK  
EDITOR

# Anthelmintics

CLINICAL PHARMACOLOGY,  
USES IN VETERINARY  
MEDICINE AND EFFICACY



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## **ANTHELMINTICS**

# **CLINICAL PHARMACOLOGY, USES IN VETERINARY MEDICINE AND EFFICACY**

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**ANTHELMINTICS**

**CLINICAL PHARMACOLOGY,  
USES IN VETERINARY MEDICINE  
AND EFFICACY**

**WILLIAM QUICK  
EDITOR**

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FROM A DECLARATION OF PARTICIPANTS JOINTLY ADOPTED BY A COMMITTEE OF THE AMERICAN BAR ASSOCIATION AND A COMMITTEE OF PUBLISHERS.

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1 *Chapter*

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5 **Plants from Cerrado for the Control of Gastrointestinal**  
6 **Nematodes of Ruminants**

7

8

9 *Franciellen Morais-Costa<sup>1</sup>, Viviane de Oliveira Vasconcelos<sup>2</sup>, Eduardo*  
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18

19

20 ***Abstract***

21

22 The gastrointestinal helminthes are major limiting factors for the sheep and goat production in  
23 the world and the health of livestock depends of effective control of nematodes. The constant  
24 administration and inadequate doses of chemical anthelmintics favors the selection of resistant  
25 populations and residues these products contribute to the contamination of animal products and  
26 of the ambient. The use of herbal treatment in veterinary medicine is a promising field of  
27 research. Studies in this area require the insertion into an agroecological context, with the  
28 limiting factor to the sustainable management of natural resources involved. The phytotherapy  
29 for the parasite control is an alternative that can reduce the cost with the purchase of  
30 anthelmintics as well, preventing the emergence of anthelmintic resistance and residues in  
31 animal products. Plant species that have tannins in its constitution are known to possess  
32 anthelmintic activity, requiring, however, that their efficacies are scientifically proven. The  
33 Cerrado is an import biome with high diversity of plants rich in tannins and other metabolic with  
34 potential anthelmintic effect. This study presents a review of research on plant species, tested in  
35 the Cerrado for the control of helminths in ruminants.

36

37 **Keywords:** anthelmintic, nematodes, medicinal plants, Cerrado, ruminants

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**Introduction**

The main problem in the small ruminants and limiting of economic exploitation is the gastrointestinal parasites. *Haemonchus contortus* is a nematode of abomasum and feeds of blood throughout, with high prevalence and high pathogenicity (Strong, 1993). Sheep with haemoncoses may show anemia and submandibular edema, with high mortality in young lambs and females in peripartum. Both sexes at all age levels may be intensely affected, reducing weight gain and reproductive capacity, as well as milk, wool, and hide production (Bizimenyera et al., 2006).

The treatment with anthelmintics has been intensely used to control by breeders. The constant administration and the inadequate dosages can favor the selection of the parasite populations resistant to the anthelmintics and contributes to the contamination of animal products with residues of these products (Amarante et al., 1992).

The main anthelmintics were developed during the 60's and are actually essential to control of nematodes. There are currently only three groups of broad spectrum anthelmintics and two groups of small spectrum used to control these parasites (Amarante et al., 1992). Early studies reported resistant helminthes to the group of benzimidazole and levamisoles. With the discovery of a chemical group distinct anthelmintic, avermectins, was represented an alternative treatment with a potent drug for the nematode control in domestic animals (Gopal et al., 1999). Multi-resistant nematodes have been found on several ruminant herds (Molento and Prichard 2001; Taylor et al., 2009; Wolstenholme et al., 2004; Thomaz-Soccol et al., 2004) . The possibility of anthelminthic residues in the environment and in animals reared for consumption (Hammond et al., 1997), as well as the spread of multi-resistant strains demands research into alternatives for gastrointestinal nematodes (GIN) control.

The utilization of plants containing secondary compounds such as condensed tannins may expand the organic alternatives to controlling GINs (Athanasiadou et al., 2007; Kahn and Diaz-Hernandez 2000). Phytotherapy in the control of parasitism is an alternative that can reduce the cost with the purchase of anthelmintics, and prevent the emergence of anthelmintic resistance and the presence of residues in animal products. Many plants are traditionally known as having anthelmintic activity, requiring, however, that their efficacy be scientifically proven (Vieira, 2003). Scientific validation of the anthelminthic effects and possible side-effects of plant products is necessary prior to their adoption as novel methods for control (Githiori et al., 2006).

The Cerrado biome, which covers 5% of the world flora, is the second largest source of biodiversity in Brazil (Sano, 2008). However, much of the native vegetation has been destroyed and many species are threatened of extinction, which would enable a wide use and maintenance of food, medicinal, ornamental, linseed and tannin production.

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However, few studies have evaluated the anthelmintic effect of the plant species of the Cerrado for the control of GNI. Therefore, the analysis of potential plant species of this biome for helminthes control for ruminants may represent a promising strategy for the biotechnology industry and consequently for the breeders of these animals.

## **The Cerrado**

Among the vegetation types that cover the American continent, the Cerrado presents a natural grandeur of plant species, which demonstrates the importance of education for the conservation and management of this biome. The original vegetation of the Cerrado has already been reduced by over 37 % (Felfili et al., 2002), prejudicing much of its biodiversity. Mittermeier et al. (1999) estimated that 67 % of the Cerrado areas are considered “highly modified” and only 20 % are in original condition, since the changes began with the colonization process, with the introduction of cattle, associated with rudimentary agricultural practices (Zanetti, 1994).

According Eiten (1993), the Cerrado’s flora is composed of two groups of species: thick stem trees and bushes with an undergrowth layer, consisting in a large mosaic, which includes a forest canopy formation more or less closed, containing trees with heights of 12 m tall or more. It has a woodland category, usually around six or seven meters and undergrowth stratum more or less continuous.

The herbaceous and shrub form a thick layer, especially grasses, making it difficult to distinguish individuals in both the layers as woodlands or as herbaceous due to many overhead structures being in accordance with shoots from the same root (Felfili et al., 2002).

The Cerrado *sensu strictu* is characterized by the presence of bent and twisted low trees; the shrub and herbaceous strata exhibits rapid growth during the rainy season (Ribeiro and Walter, 2008). These authors report that the Cerrado species have bent, twisted and gnarled timber trunks, leathery and rigid leaves, as adaptations to the dry environmental conditions. The most common species are represented by the Vochysiaceae and Fabaceae families, as well as species of Malpighiaceae, Anacardiaceae, Salicaceae, Rubiaceae (Felfili et al., 2002, Miranda et al., 2006), among others such as those represented by Caryocaraceae and Annonaceae families (Sales et al., 2009a; Sales et al., 2009b).

In regards climate, the average temperatures in the Cerrado areas vary between 22 ° C and 27 ° C ( Klink and Machado, 2005) , with average annual rainfall of 1,500 mm , water deficiency ranging from three to seven months of the year, depending on the region’s seasonal (Nimer, 1989). The Cerrado’s vegetation occurs predominantly in deep and well drained soil (Reatto et al., 1998), which present a lack of nutrients such as phosphorus and nitrogen , the pH being between 4.5 and 5.5, with high aluminum frequency rates (Ribeiro and Walter, 2008).



1 **Anthelmintic Efficacy of Plant Species from Cerrado for Control of Gastrointestinal**  
2 **Nematodes**

3

4 In recent years, society has prioritized environmental aspects, directing ample research towards  
5 the discovery of new bioactive substances that may be used in integrated pest management,  
6 with fewer negative effects on the environment (Castro, 1989).

7 In an attempt to contribute with an effective alternative control of gastrointestinal nematodes in  
8 small ruminants, several researchers have attempted to test plants used in folk medicine,  
9 evaluating the efficacy and safety of the same. Plant species rich in tannins called secondary  
10 metabolites have been extensively studied. The tannins anthelmintic action may act directly by  
11 interfering with the natural cycle of helminths, or indirectly, to protect the protein intake of  
12 ruminal degradation (with increased of protein availability in the lower gastrointestinal tract),  
13 which complicates the determination of its actual antiparasitic effect (Ketzis et al., 2006).

14 Furthermore, the results of *in vivo* tests conducted with these forages can be influenced by  
15 natural variations in the composition of the plant (by environmental factors or of their own cycle)  
16 that alter the concentration of the tannin intake by the animals (Athanasiadou and Kyriazakis,  
17 2004).

18 The anthelmintic activity, attributed to tannins is present in plant species (Hoste et al., 2006).  
19 Calderon-Quintal et al. (2010) suggest that different strains of *H. contortus* show different  
20 sensitivities to the extracts rich in tannin and further studies are needed to confirm the *in vivo*  
21 results.

22 *Chenopodium ambrosioides* L. (Chenopodiaceae) “erva-de-santa-maria”, popularly known for  
23 its anthelmintic efficacy is a plant of the Chenopodiaceae family, with stem one meter tall with  
24 leaves shaped like spears with sinuous edges. The flowers are greenish, clustered in a small  
25 bouquet. From the leaves and flowers of this plant may be extracted an essential oil consisting  
26 of a mixture of mainly ascaridiol, silvestreno and safrole, and p-cymene and isohamentina. The  
27 essential oil contains 60-80 % of ascaridiol with proven anthelmintic potential, abundant in the  
28 fruit, followed by flowers and leaves (Oliver-Bever, 1983).

29 Ketzis et al. (2002), working with essential oil of *C. ambrosioides* (0.2 mL/Kg-1 of body weight)  
30 achieved similar thiabendazole efficacy, promoting the impracticability of all the hatched larvae  
31 of *Haemonchus contortus* in sheep. However, Vieira (1992) noted no effect when administered  
32 infused orally to cattle.

33 The Annonaceae family includes about 50 genera and the genus *Annona* being one of the most  
34 important. The Annonaceae is characterized mainly by presenting a class of acetogenin  
35 substances. These substances are derived from long chain fatty acids, which act as potent  
36 inhibitor of mitochondrial respiration (Wang et al., 2002). The biological activities of *Annona*  
37 extracts have been attributed to the occurrence of annonaceous acetogenins, a class of natural  
38 compounds extracted from leaves (Geum-Soog et al., 1998; Wu et al., 1995) and seeds (Chang  
39 and Wu, 2001).

40 *Annona squamosa* L. (Annonaceae), known as the Earl fruit, “pinha” or “ata” are trees that can  
41 reach up to 5 m in height with long, thin and oval leaves. Its flowers have a greenish yellow  
42 color, adapting well to climates with little rain and with a well-defined dry season (Morton, 1987).  
43 Amorim et al. (1996) evaluated the aqueous extract from *A. squamosa* leaves, *in vitro*, on the  
44 first larval stages of gastrointestinal nematodes of cattle, obtaining mortality of 19.4 %. Vieira  
45 and Cavalcante (1999) tested *A. squamosa in vivo* on gastrointestinal nematodes in goats. The  
46 plant reduced by 40% the count of *H. contortus* eggs in feces. With respect to adult forms of the

1 parasites, *A. squamosa* showed reduction rates in the population of *H. contortus* and  
2 *Trichostrongylus columbriformis* of 21.8 % and 31.4 %, respectively, however not reducing the  
3 *Strongyloides papillosus* population. Yet according to the authors, the extract showed still to be  
4 effective against the adult form of *Oesophagostomum columbianum*, reducing by 74 % the  
5 parasites. The acute toxicity of plants from the Annonaceae family is still poorly studied and  
6 research approaches its *in vitro* cytotoxic efficacy and with possible emphasis on the anti-tumor  
7 effects (Vieira and Cavalcante, 1999).

8 *Annona muricata* L. (Annonaceae), popularly known as graviola, is a medium -sized fruit tree  
9 commonly found in the tropics. The species has been widely used in folk medicine as an  
10 anthelmintic, antipyretic, sedative, antispasmodic, and anticonvulsant and as a hypotensive  
11 agent in humans (Costa et al., 2002). *In vitro* tests to evaluate the inhibition of egg hatching,  
12 larval and adult worm motility are widely used in prospecting for new anthelmintic agents  
13 (Vasconcelos et al., 2007).

14 Ferreira et al. (2013), researching *H. contortus* in sheep, demonstrated that aqueous extract of  
15 *A. muricata* leaves at 50, 25, 12.5 and 6.25% concentrations inhibited larval hatching in 84, 9 ,  
16 79, 1, 66, 9 and 47.42 %, respectively. The authors also evaluated the effect on the motility of  
17 L3 *H. contortus* larvae at the same concentrations and obtained reduction motility rates at 83.29  
18 %, 89.08 %, 74.62 % and 30.47 %, indicating significant activity of *A. muricata* on infective  
19 larvae of this parasite. However, when were evaluated the activity of the extract on the motility  
20 of adult parasites, the response was not dependent on dosage, being able to observe the  
21 extracts activity at different concentrations within the first six hours of exposure. Phytochemical  
22 analysis did not reveal any type of acetogenins or even alkaloids in the extract but indicated the  
23 presence of phenolic compounds in the aqueous leaf extract of *A. muricata* (Ferreira et al.,  
24 2013)

25 Furthermore, since acetogenins have been associated with neurodegeneration in rats and in  
26 humans (Champy et al., 2004) the absence of acetogenins in the *A. muricata* extract is a  
27 somewhat of a motivating fact, because it can make for this aqueous extract a safe drug to treat  
28 targeted animals if compared with plant extracts prepared with organic solvents presuming the  
29 extraction of acetogenin (Ferreira et al., 2013).

30 *Annona crassiflora* Mart. (Annonaceae) commonly known as “panã”, “araticum”, “cabeça de  
31 negro” , “cascudo”, “cortiça”, “marolo” ou “pinha do cerrado”, stands out due to the fruit’s flavor,  
32 and is used in alternative medicine for possessing antibacterial and antifungal properties  
33 (Almeida et al., 1998). It is characterized by being a timber tree species, deciduous in the dry  
34 season, hermaphrodite and xerophytic. The phenology of this species is established by  
35 flowering early in the rainy season, which occurs from September to December, with fruiting  
36 having started in November, with ripened fruit from January to March (Lorenzi and Matos,  
37 2002). The fruits are used as food and appreciated for having a sweet and yellowish pulp with a  
38 strong aroma (Roesler et al., 2007).

39 Queiroz et al. (2012), using ean thanol extract from the leaves of *A. crassiflora* verified the  
40 action of this extract on *H. contortus* larval development in sheep at 100 and 50 mg/mL<sup>-1</sup>  
41 mg/mL<sup>-1</sup>. The authors also obtained an anthelmintic efficacy superior to 98.6 % for the larval  
42 development of *H. contortus*, using dried leaves of the same plant at a coproculture  
43 concentration of 333.3 mg (ms)/mL<sup>-1</sup>. The aqueous extract from the seeds and leaves of *A.*  
44 *crassiflora* showed anthelmintic efficacy of 99.43 % and 89.81 %, respectively at 100 mg/mL<sup>-1</sup>  
45 (Nogueira, 2009), presenting a promising alternative for the control of *H. contortus* in sheep.

46 In Southeastern region of Brazil, and especially in the North and Northeast, the “cajazeira”  
47 (*Spondias mombin* L.) also known as “caja-mirim”, “ambaró”, “taperebá”, is a fruit species  
48 belonging to the Anacardiaceae family. Utilized as source of permanent shading for the cocoa

1 tree, it is also utilized by producing fruits that serve as an important source of additional income  
2 for the producer. The fruits' juicy, yellow, sour and aromatic properties are appreciated in  
3 refreshments and liquors (Sacramento, 2000). The use of the "cajazeira" in folk medicine and by  
4 the pharmaceutical industry has increased, being utilized in the treatment of fevers, as an  
5 antidiarrheal, antidesintérico, antiblenorrágico and anti-hemorroidiário. According Sacramento  
6 (2000), research has recently revealed that the leaf extract contains ellagic tannins giving the  
7 plant antiviral properties. Ademola et al. (2005), using the *S. mombin* aqueous and ethanol  
8 extract against *H. contortus*, obtained a reduction of approximately 65% of eggs found in the  
9 sheep feces (OPG) at a 500 pc mg/Kg<sup>-1</sup> concentration.

10 *Lippia sidoides* Cham. (Verbenaceae) or alecrim pimenta is a species often used as herbal  
11 medicine in Northeast of Brazil, due to the antiseptic action owing to the high levels of thymol  
12 and carvacrol (Matos and Oliveira, 1998). According Camurça-Vasconcelos et al. (2007) and  
13 Vasconcelos (2006), the essential oil of *L. sidoides* possess an inhibitory effect in vitro on *H.*  
14 *contortus* eggs in sheep at 0.02 mg/mL<sup>-1</sup> to 1.25 mg/mL<sup>-1</sup>, respectively. Souza et al. (2010),  
15 Bevilaqua et al. (2005), and Person (2001), obtained same results using this oil at 0.5% and  
16 1%, respectively. In tests conducted *in vivo*, Camurça-Vasconcelos et al. (2008), reported an  
17 efficacy of 54% from the oil of *L. sidoides* in the control of *H. contortus* in sheep at a 283 mg/Kg<sup>-1</sup>  
18 <sup>1</sup>, 14 days after treatment.

19 The genus *Caryocar*, one of the representatives of the family Caryocaraceae family, has 16  
20 species that are found in South and Central America (Maya et al., 2008). *Caryocar brasiliense*  
21 Cambess. specie is a tree species native to the Cerrado regions with wide distribution in the  
22 Southeast and Midwest of Brazil (Maia et al., 2008). The popular name of this plant species  
23 may vary according to the region of occurrence, the most common being: "Pequi",  
24 "Piqui", "piquiá-bravo", "amêndoa de espinho", "grão de cavalo", "pequiá", "pequiá-pedra",  
25 "pequerim", "Suari" and "piquiá" (Santos et al. 2004). Fruiting is annual and harvesting occurring  
26 in the period lasting from September to February (Vera et al., 2005).

27 The aqueous extract from the *C. brasiliense* fruit peels, at 200 mg/ml<sup>-1</sup>, significantly inhibited the  
28 development of *H. contortus* larvae in sheep. The plant extracts effectiveness in the inhibition of  
29 larval development was of 94.8%. The egg-hatching inhibition of LC50 and LC90 was of 23.82  
30 and 53.19 mg/mL<sup>-1</sup>, respectively. The qualitative phytochemical tests performed in this study  
31 indicated the presence of catechins, steroids, flavonoids, saponins, total tannins, xanthonés and  
32 tannins catechetical (Nery, 2009).

33 Nogueira et al. (2012) evaluated the aqueous extract of *C. brasiliense* fruit's skin in the egg  
34 hatching inhibition test, with concentrations at 15 and 7.5 mg/ml<sup>-1</sup>, reported anthelmintic  
35 efficacy corresponding to 98.7 % and 91.8 %, respectively. For these concentrations, the  
36 average L1 were significantly lower than treatment with distilled water or albendazole. The  
37 average for unembryonated eggs observed in all the treatments by extract was not different  
38 from the distilled water control and suggests that "Pequi" metabolites do not inhibit the  
39 embryogenesis of these nematodes, while they may reduce hatching. The egg-hatching  
40 inhibition of LC50 and LC90 were 3.81 and 7.35 mg/mL<sup>-1</sup>, respectively.

41 *In vivo*, the average fecal egg count observed for the groups treated with the aqueous extract  
42 fruit peels of "Pequi" differed from the untreated group at concentration 2 g/Kg<sup>-1</sup> bw. During the  
43 first and second weeks of post treatment, it was observed a 33 and 32.2 % of anthelmintic  
44 efficacy *in vivo*, respectively, compared to pretreatment when all animals showed high levels of  
45 infection (Nogueira et al., 2012).

46 The crude powder derived from the "Pequi" fruit peels and leaves showed high efficiency  
47 (superior to 90 %) for the inhibition of larval development (LPGF) of *H. contortus* in sheep. The  
48 average LPGF for the concentrations at 250, 200 and 150 mg/mL were statistically similar to

1 those observed for the control with the commercial anthelmintic. The aqueous extract from the  
2 leaves of the “Pequi” showed higher anthelmintic action within seven days of incubation. The  
3 lethal concentrations of LC50 and LC90 after seven days of incubation were 34.95 and 79.74  
4 mg/mL, respectively, for the crude powder of the fruit peels and 69.05 and 97.19 mg/mL for the  
5 crude powder from the leaves. For the aqueous extract of the leaves, the LC50 and LC90 were  
6 56.36 and 115.65 and mg/mL<sup>-1</sup>, respectively (Fonseca, 2012).

7 Morais-Costa et al. (2012) compared the efficacy of *C. brasiliense* from the northern and central  
8 region of Minas Gerais in Brazil. For both regions, the concentration 333.33 mg/mL<sup>-1</sup> of dried  
9 leaves of *C. brasiliense* showed higher efficacy than negative control with distilled water and  
10 showed anthelmintic activity similar to the control with ivermectin (16 mg/mL<sup>-1</sup>). The dried leaves  
11 of this plant from northern and central region had anthelmintic action with efficacy of 98.52 %  
12 and 83.09 % respectively. This difference could be related to vegetation/area where the species  
13 were collected, since the area of vegetation in the northern region is a native and preserved  
14 area, which favors better performance and establishment of plant species in the Cerrado.

15 The species *Anacardium occidentale* L. belonging to the Anacardiaceae family, is popularly  
16 known as cashew tree (cajueiro). It is native to Brazil and used in traditional medicine,  
17 especially in northeastern Brazil due to its therapeutic effects. In the literature, there are proven  
18 pharmacological activities, as the cajueiro being anti-inflammatory plant (Olajide, 2004), ant  
19 diabetic (Barbosa-Filho et al., 2005), inhibitor of acetylcholinesterase (Barbosa-Filho et al.,  
20 2006) and antimicrobial (Akinpelu, 2001). Aiming to evaluate the anti-parasitic activity of  
21 *Anacardium humile* A. St. - Hil. (Anacardiaceae). Nery et al. (2010), used aqueous and  
22 ethanolic extracts of leaves against different species of gastrointestinal nematodes in sheep.  
23 The aqueous extract anthelmintic activity showed significantly higher than negative control at all  
24 concentrations. At concentrations of 150 and 187.5 mg/mL<sup>-1</sup>, the percent efficacy was not  
25 significantly different from ivermectin (positive control, 16 mg/mL<sup>-1</sup>). The LD50 in the inhibition  
26 assay for larval development was 10.14 mg/mL<sup>-1</sup>, and for the 5 % confidence interval it was  
27 13.36-6.83 mg/mL<sup>-1</sup>. Results of the ethanolic extract were not significantly different from  
28 ivermectin at 60 mg/mL<sup>-1</sup>. The LD50 was mg/mL<sup>-1</sup> 23.24. Larvae of *Haemonchus* spp. (68%),  
29 *Strongyloides* spp. (31%) and *Trichostrongylus* spp. (1%) were identified in the coprocultures of  
30 the negative control group. This suggests that the extracts were effective against the three  
31 nematodes considered to be the most prevalent and pathogenic in sheep (Ueno and  
32 Gonçalves, 1998).

33 Morais-Costa et al. (2012), in preliminary study, the activity of anthelmintic was evaluated to  
34 *Paullinea* sp. on gastrointestinal nematodes of sheep. The leaves of this plant were collected in  
35 the city of Montes Claros, Brazil. In this study, *Paullinea* sp. at 333.3 mg/mL<sup>-1</sup> differed from the  
36 treatment with distilled water and showed anthelmintic activity of 70.12 %, similar to treatment  
37 with ivermectin. In the control group, 100% of larvae were *Haemonchus* sp.. The anthelmintic  
38 activity of the Sapindaceae family and the species *Paullinea* sp. may be associated with  
39 saponin and tannin respectively.

40 The “genipapo” (*Genipa americana* L.), Rubiaceae family, tree that has been used in folk  
41 medicine, foods and animal feed , leather tanning, forestry, and by logging industries. The  
42 species, native to South America, has ecological importance, and is suitable for planting in  
43 degraded areas and wetlands (Epstein, 2001). In the egg hatching inhibition test, the aqueous  
44 extract of *G. american* leaves at 100 mg/mL, completely inhibited hatching. The relative average  
45 number of embryonated eggs was significantly greater than those of unembryonated eggs at 75  
46 and 100 mg/mL<sup>-1</sup>. This observation suggests a greater efficacy in inhibiting hatching rather than  
47 interfering with embryo development. The LC50 and LC90 of aqueous extract from *G. American*  
48 leaves were 34.3 and 79.8 mg/mL<sup>-1</sup>, respectively. In the larval development inhibition test,  
49 concentrations  $\geq 30$  mg/mL<sup>-1</sup> showed anthelmintic efficacy above 94 %. The LC50 and LC90

1 mg/mL<sup>-1</sup> were 14.6 and 28.7, respectively (Nery, 2009). This suggests that the extracts were  
2 effective against the several nematodes considered to be the most prevalent and pathogenic in  
3 sheep (Wood et al., 1995), showing a wide spectrum of action. However, using the hydro-  
4 alcoholic extract from the leaves of “genipapo”, Krychak-Furtado (2006) found 100 % efficacy  
5 for EHI at 50 mg/mL<sup>-1</sup>, thus suggesting the metabolites extracted with alcohol could also show  
6 action against nematode eggs.

7 In an experiment conducted by Costa (2010), the species *Schinopsis brasiliensis* Engl.  
8 (*Anacardiaceae*), *Baccharis tridentata* Vahl. (*Asteraceae*), *Ximenia americana* L. (*Olacaceae*),  
9 *Lippia sidoides* Cham. (*Verbenaceae*), *Paullinea* sp. (*Sapindaceae*) were selected by  
10 ruminants in the Cerrado, which are considered to be anthelmintic and tanniferous. The animals  
11 showed no worm problems during this research, but there is a need for *in vitro* and *in vivo*  
12 to better evaluate the effectiveness of these species.

13 It was reported by Morais-Costa et al. (2012) at a 333.3 mg/mL, the effectiveness of the dried  
14 leaves derived from the plant species *Evolvulus* sp. (*Convolvulaceae*), *Acosmium dasicarpum*  
15 (Vogel) (*Fabaceae* Faboideae) *Heteropterys byrsonymifolia* A. Juss. (*Malphigiaceae*), *Lippia*  
16 *sidoides* Cham. (*Verbenaceae*), *Erytroxylum deciduum* A.St.-Hil. (*Erythroxylaceae*), *Senna*  
17 *spectabilis* (DC.) H.S. Irwin & Barneby (*Fabaceae*), *Baccharis tridentata* Vahl. (*Asteraceae*),  
18 *Casearia sylvestris* Sw (*Salicaceae*), *Paullinea* sp. (*Sapindaceae*), *Piptadenia viridiflora* (Kunth)  
19 Benth (*Fabaceae*) and *Ximenia americana* L. (*Olacaceae*). After the logarithmic transformation  
20 and variance analysis, it was found that the average LD<sub>50</sub> for treatments with the dehydrated  
21 leaves, did not differ from the control by ivermectin, and the efficacies were: *X. Americana*  
22 (99.84%), *P. viridiflora* (85.77%), *Paullinea* sp. (70.12%) and *C. sylvestris* (43.63%). This effect  
23 can be attributed to condensed tannin concentrations at 10 mg of ethanol extracts from *C.*  
24 *sylvestris* (7.36%) *Paullinea* sp. (6.37%), *P. viridiflora* (1.75%) and *X. Americana* (0.36%). This  
25 study demonstrates a potent anthelmintic activity *in vitro*, for the ethanol extract. The fact that  
26 some species of this study have low condensed tannin content highlights the synergism among  
27 chemical compounds.

28

29

### 30 **Final Considerations**

31

32 It is necessary to scientifically validate new alternative anthelmintic compounds, characterizing  
33 them in the control of ruminant GNIs, and to evaluate the toxicity of these compounds, *in vivo*  
34 experiments should be performed, providing some of the plant species to the animals. Thus,  
35 species rich in tannins, catechetic tannins, catechins, steroids, flavonoids, xanthonenes and  
36 saponins have promising potential in the control of nematodes of ruminants and furthermore,  
37 can be active in synergism of these metabolites.

38 The species *Anacardium occidentale*, *Annona crassiflora*, *A. muricata*, *A. squamosa*, *Caryocar*  
39 *brasiliensis*, *Chenopodium ambrosioides*, *Genipa americana*, *Lippia sidoides*, *Paullinea* sp.,  
40 *Piptadenia viridiflora*, *Spondias monbin* and *Ximenia Americana* are adapted to the Cerrado and  
41 showed very promising results in reducing the bioactivity in the development of gastrointestinal  
42 nematodes of ruminants in Brazil.

43 In order to clarify the mechanisms of action from extracts of plant species, on the development  
44 of larvae using an electronic microscopy, would be a tool to support the study on reducing the  
45 use of chemical products, favoring lower incidences of residues in products of animal origin,  
46 thereby reducing costs and environmental impacts of these products in the environment.

1 Plant species rich in tannins, saponins and other secondary compounds, are deserving of  
2 further accurate studies to prove the scientific efficacy in controlling gastrointestinal parasites.  
3 Therefore, few studies have evaluated the metabolic anthelmintic effect of plant species from  
4 the Cerrado as well as possible toxicity effects. Thus, emerges the need to use natural  
5 products, based on plant species that are naturally selected by ruminants in this biome.

6

7

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