



Short communication

Anthelmintic activity of plant extracts from Brazilian savanna



Andreia F. Oliveira^a, Livio M. Costa Junior^{a,*}, Aldilene S. Lima^a, Carolina R. Silva^a,
Maria N.S. Ribeiro^b, José W.C. Mesquista^b, Cláudia Q. Rocha^c, Marcelo M.P. Tangerina^d,
Wagner Vilegas^d

^a Universidade Federal do Maranhão, Centro de Ciências Biológicas e da Saúde, CEP 65080-805 São Luís, MA, Brazil

^b Universidade Federal do Maranhão, Departamento de Farmácia, CEP 65080-805 São Luís, MA, Brazil

^c Universidade Federal do Maranhão, Departamento de Química, CEP 65080-805 São Luís, MA, Brazil

^d UNESP – Universidade Estadual Paulista, Instituto de Biociências, Campus do Litoral Paulista, CEP 11330-900 São Vicente, SP, Brazil

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ABSTRACT

Helminth infections represent a serious problem for the production of small ruminants that is currently aggravated by resistance to anthelmintic products and has induced a search for control alternatives, such as natural products. In this study, extracts of *Turnera ulmifolia* L. (leaves and roots), *Parkia platycephala* Benth. (leaves and seeds) and *Dimorphandra gardneriana* Tul. (leaves and bark), which have been cited in ethnoveterinary studies and selected naturally by goats in the cerrado (Brazilian savanna), were tested *in vitro* against *Haemonchus contortus*. Hydroacetic (ACT) and hydroalcoholic (ETH) extracts were evaluated using an Egg Hatching Assay (EHA), a Larval Exsheathment Inhibition Assay (LEIA) and a Larval Development Assay (LDA). A second set of incubations was performed using polyvinylpyrrolidone (PVPP) to determine the influence of polyphenols on the anthelmintic effects of EHA and LEIA. Data from each extract were used to calculate inhibition concentrations (IC₅₀). All tested extracts showed activity against at least one life stage of *H. contortus*. The use of PVPP revealed that the tannins are not the only extracts of secondary metabolites responsible for the anthelmintic effects. The results showed clear *in vitro* anthelmintic activities against *H. contortus* at different stages and indicated the potential use of these species as a promising alternative approach to control helminthic infections of small ruminants.

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1. Introduction

Gastrointestinal nematodes in ruminants are one of the most important causes of animal diseases worldwide, mainly in temperate and tropical areas (Miller et al., 2012; Hoste et al., 2015). The increasing nematode resistance to commercially available drugs, besides the release of residues from these drugs in the environment and in foods of animal origin (Salgado and Santos, 2016), which may affect consumer health, has encouraged studies to develop alternative methods for control. In this context, medicinal plants and their secondary metabolites (PSMs) may be an effective alternative for parasite control (Rochfort et al., 2008; Hoste and Torres-Acosta, 2011). The PSMs could be either used as phytotherapeutic materials or fed as nutraceuticals (Hoste et al., 2015).

To identify anthelmintic activities in plants, different strategies can be proposed as ethnoveterinary and ethological studies

(Huffman, 2003; Diehl et al., 2004). Roots of *Turnera ulmifolia* L. is used as anthelmintic in small ruminants and seed of *Parkia platycephala* Benth and bark of *Dimorphandra gardneriana* Tul. are used to treat diarrhoea in ruminants by farmers in Brazil (Oliveira et al., unpublished results). These plants are naturally consumed by goats in Brazilian savanna, and for these reasons are plants with potential anthelmintic activity.

The objective of this study was to evaluate the anthelmintic (AH) actions *in vitro* of *T. ulmifolia*, *P. platycephala* and *D. gardneriana* extracts which have been cited in ethnoveterinary studies and selected naturally by goats in the Brazilian savanna.

2. Material and methods

2.1. Plant material and extracts preparation

Parts of the *T. ulmifolia* (leaves and roots), *P. platycephala* (leaves and seeds) and *D. gardneriana* (leaves and bark) were collected, identified by the botanist from University of Maranhão State, Brazil and a voucher specimen was deposited at the Herbarium of this institute under numbers 035, 032 and 010, respectively. These

* Corresponding author. Tel.: +55 98 32729547.

E-mail addresses: livio.martins@ufma.br, livioslz@yahoo.com (L.M. Costa Junior).

plants were dried at 40 °C for 72 h and pulverized in an electric mill.

For the acetone extract (ACT), 500 g of material from each plant was extracted with acetone–water (70:30) and sonicated for 20 min in water-bath. The acetone was evaporated, the extract was washed four times with dichloromethane to remove lipids and pigments, and the solvent was evaporated and kept at 4 °C until its use in the *in vitro* assay (Alonso-Díaz et al., 2008). For the ethanolic extract (ETH), fifty grams of material from each plant was extracted in 500 mL of ethanol–water (80:20) and was kept for seven days stored at 4 °C in an amber vial, with periodic agitation of the contents. The solvent was evaporated using a rotary evaporator, the extract was washed four times with dichloromethane, and the solvent was again evaporated using a rotary evaporator and kept at 4 °C until its use in the *in vitro* assay.

2.2. Chemical analysis

Phytochemical screening tests that detect the presence of different secondary metabolites were performed using all extracts as described by Matos (2009). These tests involved visual observation of color modification or formation of precipitates after the addition of specific reagents.

Direct flow infusion of the samples was performed on a Thermo Scientific LTQ XL linear ion trap analyzer equipped with an electrospray ionization (ESI) source, in negative mode (Thermo, San Jose, CA, USA). It was used a stainless-steel capillary tube at 280 °C, spray voltage of 5.00 kV, capillary voltage of –90 V, tube lens of –100 V and a 5 µL/min flow. Full scan analysis was recorded in *M/Z* range from 100 to 1000. Multiple-stage fragmentations (ESI-MSⁿ) were performed using the collision-induced dissociation (CID) method against helium for ion activation. The first event was a full-scan mass spectrum to acquire data on ions in that *M/Z* range. The second scan event was an MS/MS experiment performed by using a data-dependent scan on the $[M - H]^-$ molecules from the compounds of interest at a collision energy of 30% and an activation time of 30 ms. The product ions were then submitted to further fragmentation in the same conditions, until no more fragments were observed.

2.3. Parasitological procedures

2.3.1. Nematodes

Eggs and third stage larvae (L3) were obtained from a donor goat with a monospecific infection of *Haemonchus contortus* isolated from a naturally infected goat. Experimental procedures were performed in accordance with the guidelines of the Animal Ethics Committee of Maranhão Federal University and were approved by this committee under protocol number 23115018061/2011–11.

2.3.2. Egg hatching assay (EHA)

Fresh faeces were collected and mixed with warm water (37 °C); the eggs were then recovered from solution in 25-µm sieves. Recovered eggs were added to a saturated NaCl solution and centrifuged (3000 rpm) for 3 min; floating eggs were recovered using a 25-µm sieve (Coles et al., 1992). Eggs were washed three times to eliminate the remaining salt and were re-suspended in distilled water. A suspension of 100 eggs/well was placed in a plate, and 100 µL of each treatment (methanol at 2% and the extracts) was added. The extracts were diluted in 2% methanol at concentrations that ranged from 10 000 µg/mL to 300 µg/mL, decreasing by halves. Tests were performed with four replicates. The plate was incubated at 27 °C and RH > 80% for 48 h. Larvae and unhatched eggs were counted under an inverted microscope.

2.3.3. Larval exsheathment inhibition assay (LEIA)

This test was performed according to Bahuaud et al. (2006). The extracts were diluted in 2% methanol and evaluated at concentrations that ranged from 1200 µg/mL to 18.75 µg/mL, decreasing by halves. The negative control was performed with 2% methanol and PBS (0.1 M phosphate, 0.05 M NaCl, pH 7.2). The L3 larvae were incubated in the different treatments for 3 h at 22 °C. After incubation, the larvae were washed and centrifuged (3000 rpm) three times with PBS. Approximately 1000 larvae/tubes were subjected to the artificial exsheathment process by contact with sodium hypochlorite (2.0%, w/v) and sodium chloride (16.5%, w/v). Tests were performed with four replicates. The percentages of larval exsheathment process were monitored at 0-, 20-, 40- and 60-min intervals by observation under an inverted microscope.

2.3.4. Larval development assay (LDA)

A larval development assay (LDA) was performed according to Demeler et al. (2010). One hundred eggs were added to wells with distilled water in a total volume of 200 µL and incubated for 24 h at 27 °C to obtain L1 larvae. Nutritive medium (a mixture of yeast extract: Earle's solution: amphotericin B (Sigma A2942, 0.5 mg/mL) at 2:2:1) and 1.5 mg/mL of autoclaved *Escherichia coli* were added to each well, and the treatments (water, methanol 2%, and plant extracts) were evaluated at concentrations that ranged from 2500 µg/mL to 250 µg/mL, decreasing by halves and added to a total volume of 500 µL. The plate was incubated at 27 °C for 7 days. After incubation, the proportions of unhatched eggs and L1–L3 larvae in each well were counted under an inverted microscope.

2.3.5. Influence of polyphenols

To determine the influence of polyphenols in the larval exsheathment and egg hatching processes, PVPP (polyvinylpyrrolidone) was added to the extracts at concentrations of 1200 µg/mL for LEIA and 5000 µg/mL for EHA in a 1:50 ratio per 2 h to both tests (Barrau et al., 2005; Chan-Pérez et al., 2016). These solutions were then centrifuged at 3000 rpm (10 min, 20 °C). After centrifugation, the supernatant and the extracts without PVPP were used for testing the respective extracts in the same manner as described above.

EHA evaluations with PVPP were performed by determining the percentage of morulated eggs (ME), eggs containing larvae but failing to complete their eclosion (LFE) and larvae (L1) present in the sample according to Vargas-Magaña et al. (2014).

2.4. Statistical analysis

The results were used to determine the concentration required to inhibit 50% of hatching, larval exsheathment and larval development (IC₅₀) with respective 95% confidence intervals (95% CI) using PoloPlus 1.0 software (LeOra Software, 2002). The extract was considered to be significantly ($P < 0.05$) more (or less) efficient than another extract when there was no overlap between the 95% confidence limits of the IC₅₀ values (Roditakis et al., 2005). Data obtained from the PVPP incubations of extracts were analysed with the respective GLM to assess differences in the percentage values of the results of the PBS control and those obtained for the extract solutions with and without PVPP.

3. Results

3.1. Chemical analysis

Initial phytochemical analysis of *T. ulmifolia* roots and leaves showed the presence of phenols and condensed tannins in the ACT and ETH extracts. The leaves extract showed cumarins, flavones,

phytosteroids and saponins, whereas the roots extracts were positive for flavanones, catechins and triterpenoids. *P. platycephala* extracts showed phenols, flavones and phytosteroids. The seeds extracts showed condensed tannins, and the leaves extracts were positive for flavones and saponins. *D. gardneriana* extracts showed the presence of phenols, condensed tannins, flavones, flavanones, phytosteroids and saponins.

Mass spectrometry data shows that each species predominate one class of compounds. *D. gardneriana* presents as major flavonoid quercetin derivatives, while *P. platycephala* shows in its majority composition of the flavonoids catechin derivatives. The species *T. ulmifolia* has the majority composition of the flavonoid with basic skeletons of apigenin and luteolin (Supplementary material).

3.2. Egg hatching assay (EHA)

The ETH extract of *T. ulmifolia* leaves showed the highest egg hatching inhibition with the lowest IC₅₀ value of 430 µg/mL (95% CI 400–460 µg/mL), followed by the ETH extract of *P. platycephala* seeds (IC₅₀ 1340, 95% CI 1170–1550 µg/mL) (Table 1). Seven different extracts did not show activity with low egg hatching inhibition at 10 000 µg/mL (Table 1).

3.3. Larval exsheathment inhibition assay (LEIA)

We observed inhibitory effects of exsheathment in all extracts tested in the present study (Table 1); however, the extracts of *T. ulmifolia* roots were not dose-dependent. The ACT and ETH extracts of *P. platycephala* leaves showed a significant inhibition of larval exsheathment with the lowest IC₅₀ values of 80 and 70 µg/mL, respectively (Table 1).

Table 1

Effective concentration of *Turnera ulmifolia*, *Parkia platycephala* and *Dimorphandra gardneriana* extracts (µg/mL) required for achieving 50% inhibition of egg hatching (EHA), larval exsheathment (LEIA) and larval development (LDA) in *Haemonchus contortus* (IC₅₀) with respective 95% confidence intervals (95% CI).

Plant	Part	Extract	Assay		
			EHA	LEIA	LDA
<i>T. ulmifolia</i>	Roots	ACT	>10000	1200 [*]	190 (160–220)c
		ETH	>10000	1200 [*]	520 (450–610)e
	Leaves	ACT	3200 (2600–3900)d	150 (140–160)c	120 (100–130)b
		ETH	430 (400–460)a	310 (270–350)e	310 (290–340)d
<i>P. platycephala</i>	Seeds	ACT	>10000	460 (420–510)g	740 (700–770)f
		ETH	1300 (1200–1500)b	750 (690–820)i	880 (850–920)g
	Leaves	ACT	2400 (2000–2900)c	80 (70–90)a	40 (36–48)a
		ETH	>10000	70 (50–90)a	54 (48–60)a
<i>D. gardneriana</i>	Barks	ACT	>10000	360 (330–400)f	160 (150–170)c
		ETH	>10000	530 (480–570)h	180 (150–210)c
	Leaves	ACT	>10000	120 (110–130)b	130 (100–160)b,c
		ETH	3400 (2600–5100)d	230 (220–250)d	530 (490–580)e

ACT: Acetone extract; ETH: Ethanolic extract. Different letters in the same column indicate significant differences ($P < 0.05$).

^{*} Treatments showed no dose-dependent effects.

Table 2

Means and standard errors of the proportion of morulated eggs (ME), larvae failing eclosion (LFE) and larvae (L) with *Haemonchus contortus* resulting from incubations with extracts of *Turnera ulmifolia*, *Parkia platycephala* and *Dimorphandra gardneriana* at a concentration of 5000 µg/mL (methanol 2%) and the addition of PVPP (polyvinylpyrrolidone).

Plant	Part	Control/Extract	Life stage		
			ME	LFE	L
<i>T. ulmifolia</i>	Roots	Methanol (2%)	2.2 ± 1.2 ^a	6.7 ± 3.0 ^a	91.1 ± 1.6 ^a
		ACT	2.3 ± 0.8 ^a	2.5 ± 1.1 ^a	95.2 ± 1.7 ^a
		ACT + PVPP	4.0 ± 3.1 ^a	4.0 ± 3.3 ^a	92.0 ± 2.6 ^a
		ETH	1.1 ± 0.02 ^a	3.3 ± 0.06 ^a	95.6 ± 0.08 ^a
	Leaves	ETH + PVPP	2.2 ± 0.6 ^a	3.9 ± 0.3 ^a	93.9 ± 0.3 ^a
		ACT	32.3 ± 3.2 ^b	60.5 ± 4.5 ^b	7.1 ± 1.0 ^b
		ACT + PVPP	8.4 ± 2.4 ^c	36.7 ± 3.4 ^c	54.9 ± 3.9 ^c
		ETH	22.5 ± 1.1 ^b	70.8 ± 1.0 ^b	6.7 ± 1.5 ^b
<i>P. platycephala</i>	Seeds	ETH + PVPP	11.9 ± 4.4 ^c	56.1 ± 3.4 ^c	36.0 ± 4.4 ^c
		ACT	21.2 ± 3.1 ^b	71.0 ± 3.4 ^b	7.8 ± 0.5 ^b
		ACT + PVPP	4.0 ± 1.0 ^a	0.9 ± 0.5 ^a	95.1 ± 0.7 ^a
		ETH	29.5 ± 3.3 ^b	65.7 ± 3.9 ^b	4.8 ± 2.1 ^b
	Leaves	ETH + PVPP	8.3 ± 2.5 ^c	85.0 ± 2.5 ^c	6.7 ± 0.7 ^b
		ACT	5.1 ± 1.8 ^a	49.9 ± 3.5 ^b	45.0 ± 3.9 ^b
		ACT + PVPP	3.4 ± 2.1 ^a	0.8 ± 0.4 ^a	95.8 ± 2.5 ^a
		ETH	6.2 ± 1.9 ^a	29.3 ± 2.4 ^b	64.5 ± 3.8 ^b
<i>D. gardneriana</i>	Bark	ETH + PVPP	5.4 ± 0.7 ^a	0.5 ± 0.3 ^a	94.1 ± 0.6 ^a
		ACT	5.8 ± 0.4 ^a	12.3 ± 3.6 ^b	82.3 ± 4.1 ^b
		ACT + PVPP	2.0 ± 1.3 ^a	4.2 ± 3.5 ^d	93.7 ± 4.0 ^a
		ETH	7.8 ± 3.1 ^b	4.6 ± 3.1 ^d	87.6 ± 4.0 ^b
	Leaves	ETH + PVPP	8.1 ± 1.0 ^b	7.3 ± 4.1 ^d	84.6 ± 4.1 ^b
		ACT	3.8 ± 0.6 ^a	1.4 ± 1.0 ^a	94.8 ± 1.5 ^a
		ACT + PVPP	4.1 ± 1.15 ^a	1.7 ± 0.8 ^a	94.2 ± 1.77 ^a
		ETH	20.3 ± 3.8 ^b	78.5 ± 3.5 ^b	1.2 ± 0.5 ^b
		ETH + PVPP	12.4 ± 4.5 ^c	83.8 ± 4.5 ^b	3.8 ± 1.0 ^b

ACT: acetone extract; ETH: ethanolic extract. Different letters in the same column in each category of each extract indicate significant differences ($P < 0.05$).

3.4. Larval development assay (LDA)

The ACT and ETH extracts of *P. platycephala* leaves showed the highest inhibition of larval development with the lowest IC50 values of 40 and 50 µg/mL, respectively (Table 1). We observed activities for all of the extracts tested in the present study (Table 1).

3.5. Role of polyphenols on AH effects

The effects of leaves extract of *T. ulmifolia* and *P. platycephala* were blocked by PVPP and permitted the highest levels

of larval development and egg hatching (Table 2). These same effects were evident when using the ACT extract of *P. platycephala* seeds and *D. gardneriana* bark. However, the ETH extract of *P. platycephala* seeds plus PVPP showed an interesting effect: the number of larvae failing eclosion increased ($P < 0.05$) (Table 2). The PVPP blocked the inhibition of larval exsheathment with the ACT and ETH extracts of roots and leaves from *T. ulmifolia*, the ACT extract of *P. platycephala* seeds, and the ETH extracts of *P. platycephala* and *D. gardneriana* leaves (Figs. 1 and 2).

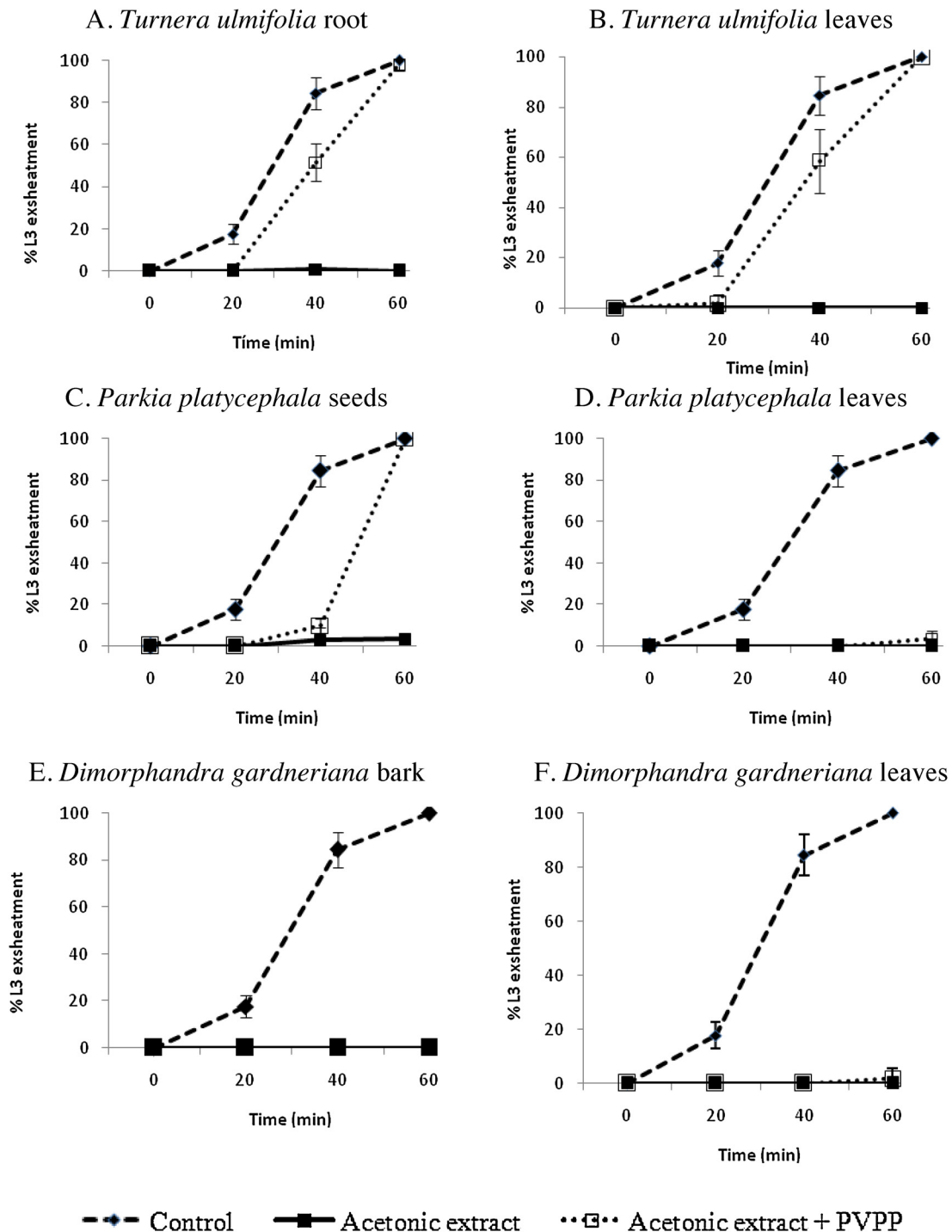


Fig. 1. Larval exsheathment of *Haemonchus contortus* in the presence of acetonic extracts of (A) roots and (B) leaves of *Turnera ulmifolia*, (C) seeds and (D) leaves of *Parkia platycephala*, and (E) bark and (F) leaves of *Dimorphandra gardneriana* at a concentration of 1200 µg/mL and their combinations with PVPP (polyvinylpyrrolidone).

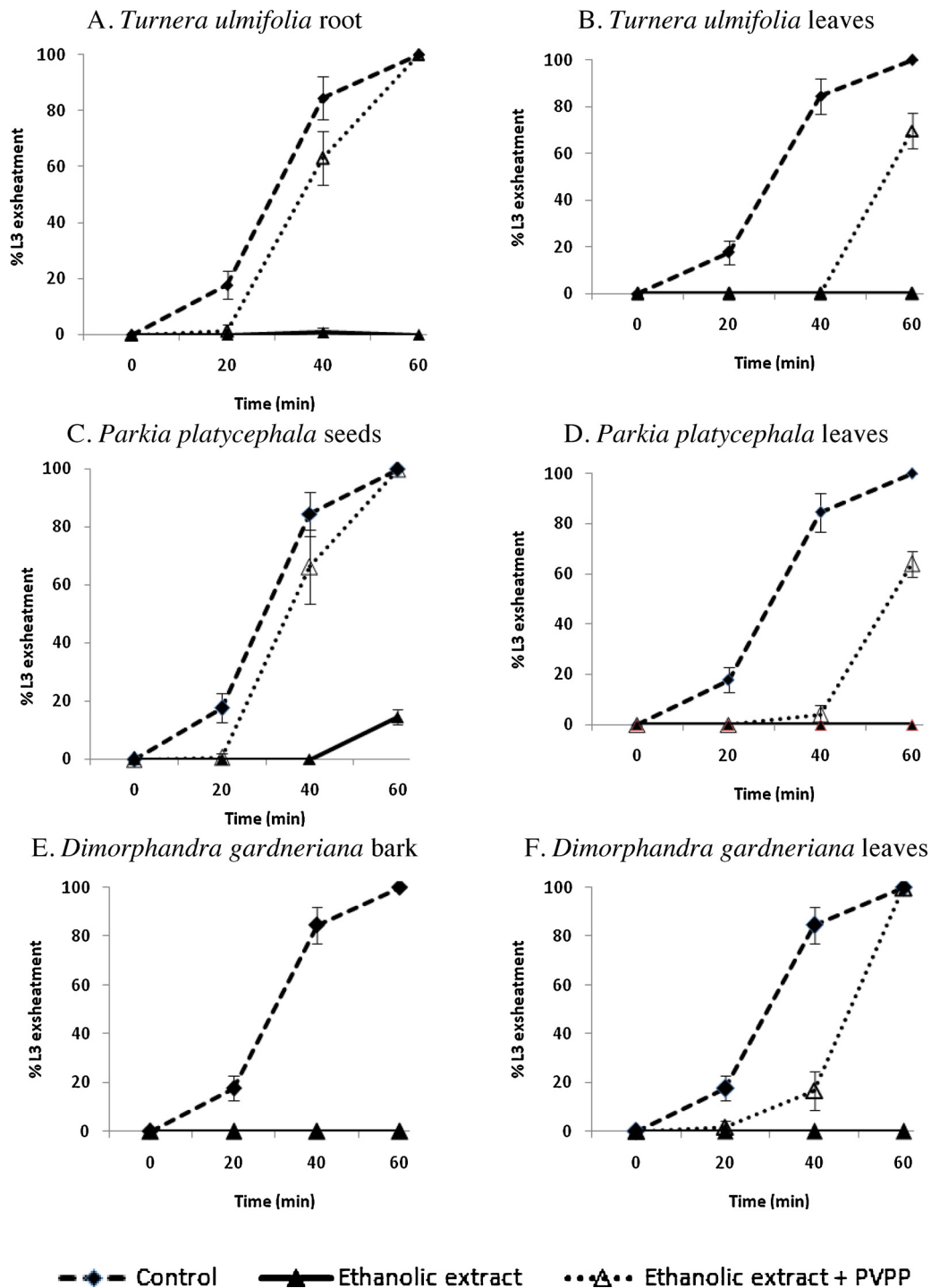


Fig. 2. Larval exsheathment of *Haemonchus contortus* in the presence of ethanolic extracts of (A) roots and (B) leaves of *Turnera ulmifolia*, (C) seeds and (D) leaves of *Parkia platycephala*, and (E) bark and (F) leaves of *Dimorphandra gardneriana* at a concentration of 1200 $\mu\text{g/mL}$ and their combinations with PVPP (polyvinylpyrrolidone).

4. Discussion

Ethnoveterinary and ethological approaches are two ways to select bioactive plants (Huffman and Seifu, 1989; Diehl et al., 2004). The combined use of these techniques can increase the possibility of selecting bioactive plants. In the present study, we selected three plants that have been used by humans to treat animals and that were naturally grazed by goats. *T. ulmifolia*, *P. platycephala* and *D. gardneriana* are highly used by humans to treat many infections. Moreover, *P. platycephala* is the main leguminous plant used by

the farmers in the nutritional diets of small ruminants in parts of northeast of Brazil (Machado et al., 1999; Alves et al., 2007).

All tested extracts showed activity against at least one life stage of *H. contortus*. Different IC_{50} values were calculated in the assays performed. Generally, these differences in values are attributed to the sensitivity of each stage. L1 is the most sensitive stage because the larva's pharynx is more sensitive to the paralysis caused by drugs, eggs are more resistant than L1 due their hard and resistant shell, and L3 larvae are more resilient due to their double sheath (Molan et al., 2002). These facts lead to the requirements for high or

low contents of active compounds to achieve IC₅₀ values for each test.

Regarding the extracts used, the hydroalcoholic extracts had better inhibitory activities than hydroacetic extracts for EHA, while the IC₅₀ values were generally lower with the hydroacetic extracts than with the hydroalcoholic extracts in larval tests. As it is known, the solvents and protocols used for extraction promote variation in concentrations and the classes of secondary metabolites present in extracts (Marie-Magdeleine et al., 2009), which could have large effects on the activities of botanical compounds (Eloff, 1998). For instance, acetone:water mixtures are used for the extraction of phenolic compounds, such as condensed tannins and its monomers as catechin, besides flavonoids (Cork and Krockenberger, 1991; Nikousaleh and Prakash, 2016), which have anthelmintic actions related to their capacity to form complexes with proteins, such as proteins rich in proline and hydroxyproline in the sheath, cuticle and fluid, unsheathing nematode larvae and changing their physical and chemical properties (Alonso-Díaz et al., 2011). Flavonoids are modulators of P-glycoprotein and improve the anthelmintic potential of macrocyclic lactones and condensed tannin *in vitro* and bioavailability of macrocyclic lactones in lamb (Conseil et al., 1998; Dupuy et al., 2003; Klongsiriwet et al., 2015). Egg hatching could have been inhibited by a hydro-alcoholic extract due the saponins and/or other molecules that can inhibit *H. contortus* egg hatching (Camurca-Vasconcelos et al., 2007; Eguale et al., 2007).

In our study, a second set of EHA and LEIA using the hydroacetic and hydroalcoholic extracts with and without PVPP was performed to attempt to identify the possible role of polyphenols in the AH activity observed. The results of these assays indicate that different extracts show variabilities in the roles of tannins in terms of ovicidal effect, %LFE, larvae, or larval exsheathment.

In the EHA, the addition of PVPP was not associated with significant reductions of the ovicidal effect for both *T. ulmifolia* leaves extracts, both *P. platycephala* seeds extracts and the *D. gardneriana* leaves ETH extract. In the %LFE for *H. contortus*, the different extracts showed variations after incubation with PVPP that included different scenarios depending on the extract tested. Incubation with PVPP was associated with reductions in this effect with both *T. ulmifolia* leaves extracts, but they remained different to PBS control values. Other extracts did show reductions of the AH effect when incubated with PVPP (the *P. platycephala* seeds ACT extract and both leaves extracts). Finally, some isolates further increased the AH effect observed with the extracts alone. The latter finding suggests that polyphenols might have negative interactions with other PSMs, reducing the AH effect on egg hatching.

In the LEIA, after the addition of PVPP to the 1200 µg/mL extracts, different extracts showed variations in the rate of exsheathment of *H. contortus* larvae. For the *D. gardneriana* bark extracts, incubation with PVPP was not associated with increased exsheathment values. Some extracts, after the addition of PVPP, showed partial restoration towards the control values, as in the case of the *T. ulmifolia* leaves ACT extract and the *P. platycephala* leaves ETH extract, or a weak restoration (*P. platycephala* and *D. gardneriana* leaves ACT extracts). Further, six extracts (both *T. ulmifolia* roots extracts and the leaves ETH extract, both *P. platycephala* seeds extracts and the *D. gardneriana* leaves ETH extract) showed complete reversion of the inhibitory effect of larval exsheathment.

These results suggest that condensed tannins are not the only PSM involved in these antiparasitic effects in all extracts. Other studies suggest that tannins are not the only PSM responsible for anthelmintic activity on *H. contortus* with synergistic effect of condensed tannin and flavonoid monomers showed (Azando et al., 2011; Vargas-Magaña et al., 2014; Chan-Pérez et al., 2016; Klongsiriwet et al., 2015).

The considerable activity levels of the extracts observed in the different tests suggest a possible synergistic relationship of compounds that can interact with multiple molecular targets in various developmental stages of the parasite (Marie-Magdeleine et al., 2009).

To our knowledge, the current study is the first one assessing the anthelmintic activities of *T. ulmifolia*, *P. platycephala* and *D. gardneriana*. The results showed clear *in vitro* anthelmintic activities against *H. contortus* at different stages and indicated the potential use of these species as a promising alternative approach to control helminthic infections of small ruminants.

Conflict of interest statement

The authors declare that they have no competing interests.

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