https://doi.org/10.1007/s00436 017 5650 4

Is there a negative association between the content of condensed tannins, total phenols, and total tannins of tropical plant extracts and in vitro anthelmintic activity against *Haemonchus contortus* eggs?

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Abstract In vitro studies using plant extracts suggest a relationship between their polyphenol contents and their anthelmintic (AH) activity against *Haemonchus contortus*. High polyphenol content appears to increase the efficacy of plant extracts against *H. contortus* as assessed by the larval exsheathment inhibition assay (LEIA) while appearing to reduce the AH efficacy measured using the egg hatch assay (EHA). In addition, some plants lack AH activity. Therefore, the present study investigated the relationship between the contents of condensed tannins (CT), total phenols (TP), and total tannins (TT) in methanol:water extracts (70:30) obtained from ten tropical plant species consumed by small ruminants as well as their AH activity against *H. contortus* evaluated by LEIA and EHA. Extracts of *Acacia collinsii*, *Lysiloma latisiliquum*, *Havardia albicans*, *Senegalia gaumeri*, *Mimosa bahamensis*, *Piscidia piscipula*, *Acacia pennatula*, *Gymnopodium floribundum*, *Leucaena leucocephala*, and *Bunchosia swartziana* were examined. Positive correlations were found between the effective concentration 50% (EC_{50}) (EHA) of extracts and their CT ($r = 0.6809$, $P < 0.05$, $n = 10$) and TP ($r = 0.9152$, $P < 0.05$, $n = 10$) content, suggesting that their concentration negatively affected AH activity against eggs. Based on the LEIA, there was no significant association between the EC_{50} and the CT, TP, or TT of all extracts evaluated. Thus, if sheep and goats consume a complex feed mixture with high amounts of CT, TP, and TT, it might be difficult to observe an AH effect against *H. contortus* egg hatching. However, the AH effect upon L_3 establishment might be feasible.

Keywords Egg hatch assay (EHA) · Larval exsheathment inhibition assay (LEIA) · *H. contortus* · Condensed tannins · Total polyphenols · Total tannins

Introduction

The use of several tropical plants have been proposed as a non-conventional method for the control of gastrointestinal nematodes based on their anthelmintic (AH) activity associated with their content of secondary compounds (Torres-Acosta et al. 2012). AH activity has been evaluated using in vitro studies against the eggs or larvae of *Haemonchus contortus*. These previous studies suggested AH activity was related to the presence of polyphenols in acetone:water plant extracts. However, the use of polyphenol-blocking agents with tropical plant extracts has shown three distinct results: (i) a direct relationship between the presence of polyphenols and AH efficacy as measured by larval exsheathment inhibition assay (LEIA) (Ortiz-Ocampo et al. 2016); (ii) increased efficacy of extracts against eggs measured by the egg hatch assay (EHA) in the presence of a polyphenol blocking agent (Vargas-Magaña et al. 2014; Castañeda-Ramírez et al. 2014; Hernández-Bolio et al. 2017); and (iii) a lack of effect from the blocking agent suggesting that secondary compounds different to polyphenols are involved in the AH activity (Hernández-Bolio et al. 2017). Thus, in some cases, the presence of polyphenols was not involved with AH activity

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measured by EHA and LEIA, as reported for the methanol:water extracts of *Annona squamosa*, *A. muricata*, and *A. reticulata* (Castañeda-Ramírez et al. 2014). Given these different scenarios of AH activity against the eggs and larvae of *H. contortus*, we hypothesize there is positive association between the polyphenol content of the extracts and AH activity against L_3 (LEIA), whereas polyphenols have a negative association with AH activity measured by EHA. To test this hypothesis, it is important to use an extraction protocol different to acetone:water (70:30) because this protocol has resulted in a poor AH effect against the eggs of *H. contortus*. An extraction process with a similar polarity to that of acetone:water (70:30), but with a clear AH effect on exsheathment and egg hatching, is necessary. The methanol:water (70:30) extraction process was initially proposed as previous studies using methanolic plant extracts showed ovicidal and L_3 AH activity against *H. contortus* (Souza et al. 2008; Castañeda-Ramírez et al. 2014). Therefore, ten tropical plant species were selected based on previous reports of their intake by small ruminants in browsing (Gónzalez-Pech et al. 2014, 2015) or feeding trials (Galicia-Aguilar et al. 2012; Méndez-Ortíz et al. 2012), to obtain a range of condensed tannins (CT), total phenol (TP), and total tannin (TT). The aim of this study was to identify the relationship between CT, TP, and TT contained in methanol:water (70:30) extracts of the ten plant species and their AH activity against *H. contortus* evaluated by LEIA or EHA to further understand the control of gastrointestinal nematodes by plant secondary compounds.

Materials and methods

Production of *Haemonchus contortus* eggs and larvae

Two donor lambs (25 ± 1 kg) raised free of gastrointestinal nematode (GIN) infections were used. Animals were kept in individual pens with raised slatted floors before and during the experiment. They were fed a balanced diet based on grass hay and a commercial concentrate feed and had access to clean water ad libitum. Prior to their artificial infection, donors were confirmed free of GIN infection using the centrifuge flotation technique and the McMaster technique on fecal samples obtained directly from the rectum of donors on three consecutive days. The donor lambs (4 months old) were orally inoculated with 4000 *H. contortus* infective L_3 (Paraiso isolate). The isolate was previously characterized as benzimidazole resistant and with low susceptibility to polyphenol-rich plant extracts (Chan-Perez et al. 2016). Fecal samples were obtained 28 days after the artificial infection to confirm the presence of *H. contortus* eggs.

For the EHA, donor feces' were collected directly from the rectum using new airtight plastic bags. Samples were processed within 3 h of collection. Fecal pellets were macerated in purified water using 100 mL for every 10 g of feces to separate *H. contortus* eggs from the fecal material. The egg suspension was filtered with a cheesecloth and the filtrated material was centrifuged (168×*g* for 5 min) using conical tubes (15 mL). Supernatant was discarded and the sediment was mixed with a saturated sugar solution (1.28 density). Once mixed, the sediment was vortexed until homogenized. Suspension was centrifuged at 168×*g* for 5 min. A bacteriological loop was used to collect the superficial portion of the suspension where eggs are present. Eggs were placed in 15-mL tubes containing 10 mL of phosphate-buffered saline (PBS). Egg concentration was determined from ten 0.01-mL aliquots. The suspension was diluted at 150 eggs/mL PBS. For the LEIA, fecal pellets were collected from donor sheep and were washed with tap water to remove grass and other debris. Rinsed pellets were then placed in Petri dishes covered by a larger Petri dish. The fecal cultures were incubated 5 days at 28 °C. Cultures were moisturized daily with a manual water sprinkler. The infective larvae were harvested using a Baermann apparatus. Larvae were stored at 4 °C until their use. Age of larvae ranged from 2 to 5 weeks as suggested by Castañeda-Ramírez et al. (2017).

Production of methanol: water extracts

Based on previous evidence of its consumption by small ruminants, fresh leaves of *Acacia collinsii*, *Acacia pennatula*, *Bunchosia swartziana*, *Gymnopodium floribundum*, *Havardia albicans*, *Lysiloma latisiliquum*, *Leucaena leucocephala*, *Mimosa bahamensis*, *Piscidia piscipula*, and *Senegalia gaumeri* were collected in Yucatán, Mexico (20° 51′ 41″ N 89° 37′ 28″ O). The methanol:water extracts were produced using 75 g of fresh leaves from each plant. Leaves were crushed and incorporated into a methanol:water (70:30) solution containing ascorbic acid (1 g/L) for 24 h. The solution was filtered (paper no. 50) and the solvent (methanol) was evaporated using a rotovap (IKA®, Germany). The aqueous fraction was rinsed with methylene chloride in a portion volume of 1:1 (two washes) to remove chlorophyll and lipids. The extract was again roto-evaporated to eliminate solvent residues. Finally, extracts were lyophilized and stored at 4 °C until use. The extraction yield varied from 1.65 g for *H. albicans* to a maximum of 10.5 g for *G. floribundum*.

The content of TP and TT was determined with the Folin–Ciocalteu procedure. First, TP was determined for each plant extract. Then, polyvinylpolypyrrolidone (PVPP) was used to estimate TT using the following formula: TT = TP − PVPP precipitation. The quantification of TT was made using a diode array spectrophotometer (Agilent 84531). Standard solutions were formulated with the Folin–Ciocalteu reagent and the calibration curve was made with tannic acid. The

quantification of TP and TT was made at 725 nm. The tannins were expressed as tannic acid equivalent.

The CT content was determined by the vanillin method (Price et al. 1978). This assay measures not only CT but also flavan-3-ols. A calibration curve was made with a catechin solution. The quantification of CT was performed by spectrophotometry at 520 nm. The CT content was expressed as catechin equivalent (Alonso-Díaz et al. 2008).

In vitro anthelmintic activity against *H. contortus* eggs

The EHA was used to evaluate the in vitro AH activity of the methanol:water leaf extracts of the ten plant species against *H. contortus* eggs. The EHA was conducted following the procedure described by von Samson-Himmelstjerna et al. (2009) and Jackson and Hoste (2010). Stock solutions (10,000 μg/mL of PBS) were prepared for each extract tested. PBS was used as a negative control for all extracts. Briefly, 0.5 mL of stock solution for each plant extract at different concentrations (3600, 2400, 1200, 600, 300, and 150 μg/mL of PBS) was added to 24 well plates. Then 0.5 mL of the egg suspension (150 eggs/mL) was added to each well to a final volume of 1 mL. Six replicates were used for each extract concentration. Multi-well plates were incubated at 28 °C for 48 h. Then, two drops of Lugol's solution were added to each well to kill and dye the eggs and larvae. The number of eggs that failed to form larvae (morulated eggs [ME]), the number of eggs that failed to complete their eclosion (larval failing eclosion [LFE]), and the number of free larvae present in each well were determined as described by Vargas-Magaña et al. (2014).

Larval exsheathment inhibition assay (LEIA)

The LEIA was conducted following the procedure described by Jackson and Hoste (2010). A stock solution was prepared using the methanol:water extract at 5000 μg/mL PBS. Negative controls consisted of larvae exposed only to PBS. Concentrations of methanol:water extracts tested were 1200, 600, 200, 100, and 50 μg/mL PBS. Each concentration was added to 1000 μL of a solution containing approximately 1000 L_3 to obtain the final extract concentrations. Infective larvae were incubated with the plant extract for 3 h at 24 °C. Then, L_3 larvae were centrifuged for 3 min at 168×*g* and washed three times with PBS solution. Then, aliquots of each larva solution were placed in Eppendorf vials (200 μL each). Four repetitions were performed for each concentration and the PBS control. The process of exsheathment was artificially induced by contact with a solution of sodium hypochlorite (2%) and sodium chloride (16.5%) diluted in PBS. The kinetics of exsheathment were assessed with a microscope using the 10× objective and recorded at 0, 20, 40, and 60 min (Jackson and Hoste 2010).

Effect of polyphenols on anthelmintic activity

To determine the role of CT and other polyphenols on the AH effect identified for the extract of each plant species, extracts were incubated with PVPP (0.05 g of PVPP/mL of the respective extract solution) for 3 h at 24 °C. After incubation, solutions were centrifuged at 378×*g* for 5 min and supernatants were used for EHA or LEIA bioassays. For EHA, the extract concentration used for all plant species was 3600 μg/mL PBS with and without PVPP (six replicates for each extract). For LEIA, the extract concentration used for all plant species was 1200 μg/mL PBS with and without PVPP (four replicates for each extract). A previous study using *L. latisiliquum* acetone:water extract reported that incubation with PVPP resulted in a reduction of 66.4% of TP content and 100% reduction of CT content (Hernández-Bolio et al. 2017).

Data processing and statistical analyses

The egg hatch inhibition percentages obtained for each plant extract by EHA was estimated as described by Chan-Pérez et al. (2016). The percentages of ME and LFE were calculated using the formulae reported by Vargas-Magaña et al. (2014). The exsheathment inhibition percentage obtained for each plant extract with LEIA was determined as described by Chan-Pérez et al. (2017).

The egg-hatching inhibition and LEIA results obtained for the different plant extracts tested were analyzed with respective generalized linear models (GLM) to assess differences between the control PBS and the different extract concentrations tested.

Data obtained from PVPP incubations of each extract were analyzed using a completely randomized design (GLM with comparisons performed with the respective control group for each extract). In the case of the EHA results, the percentage values of eggs showing an ovicidal effect, LFE, or emerged larvae and the results obtained for plant extracts at 3600 μg/mL PBS, with and without PVPP, were compared with their respective controls. In the case of the LEIA, a similar procedure was used with larvae at a concentration of 1200 μg/mL PBS, with and without PVPP. For each analysis, and their respective post hoc comparisons, Fisher's least significant difference was analyzed using Statgraphics Centurion XV (Statpoint Technologies Inc. 2005).

The effective concentration required to inhibit 50% of egg hatching or L_3 exsheathment (effective concentration 50% (EC_{50})) was estimated with data obtained from EHA and LEIA for each plant extract tested using PoloPlus 1.0 software (LeOra Software 2004). The respective 95% confidence intervals (95% CI) were also calculated.

In addition, the CT, TP, and TT contents of each plant extract were correlated against the EC_{50} for LEIA and EHA and % ovicidal activity using Spearman's correlation. The correlation was performed using the Statgraphics Centurion XV program (Statpoint Technologies Inc.).

Results

Polyphenolic components of plant extracts

Table 1 shows the CT, TP, and TT content of the methanol:water extracts obtained from the leaves of ten plant species from the tropical deciduous forest of Yucatán, México. Plant extracts with a high content of TT were *H. albicans* and *L. latisiliquum* (24.31 and 23.16%). A high CT content was found for *H. albicans* and *G. floribundum* (48.5 and 48.32%) extracts, and a high TP content was recorded for *G. floribundum* and *A. pennatula* (27.65 and 27.72%). The *P. piscipula* and *S. gaumeri* extracts showed no detectable levels of CT. *P. piscipula* extract also showed the lowest TP content (0.75%) and TT content (0.63%).

Egg-hatching assay (EHA)

The methanol:water extracts of the ten plants studied showed different EC_{50} values (Table 2). The lowest EC_{50} values were observed for *L. leucocephala*, *S. gaumeri*, *P. piscipula*, and *A. collinsii* ($P < 0.05$), while the highest EC_{50} values were recorded for *A. pennatula* and *B. swartziana* ($P < 0.05$).

The incubation of extracts with PVPP showed a broad variability of AH activity on the proportions of ME, LFE, and larvae, as assessed by EHA (Table 3). Extracts with the highest ovicidal effect were from *S. gaumeri* and *P. piscipula* (56.2 and 17% ME at 3600 μg/mL). Extracts of *L. leucocephala* and *H. albicans* showed higher ovicidal effect when polyphenols were removed with PVPP. Extracts of *L. latisiliquum*, *A. pennatula*, *M. bahamensis*, *G. floribundum*, *B. swartziana*, and *A. collinsii* showed no ovicidal activity when evaluated at 3600 μg/mL or when polyphenols were removed. The effect of these extracts was mainly to inhibit LFE.

The larval exsheathment inhibition assay (LEIA)

The EC_{50} and 95% CI obtained for the different methanol:water extracts tested by LEIA are presented in Table 4. The lowest EC_{50} values were found for *H. albicans* and *G. floribundum* extracts ($P < 0.05$) whereas extracts with the highest EC_{50} values were from *A. collinsi* and *A. pennatula* ($P < 0.05$). Table 5 shows the exsheathment inhibition percentage recorded for L_3 larvae exposed to different plant extracts with or without PVPP. Extracts of *A. collinsii*, *A. pennatula*, *B. swartziana*, *G. floribundum*, *M. bahamensis*, and *P. piscipula* had significantly decreased AH activity when polyphenols were removed with PVPP and evaluated at a concentration of 1200 μg/mL PVPP ($P < 0.05$). However, the removal of polyphenols with PVPP did not reduce the exsheathment inhibitory effect for extracts of *H. albicans*, *L. latisiliquum*, *L. leucocephala*, and *S. gaumeri*.

Table 1 Content of condensed tannins (CT), total phenols (TP), and total tannins (TT) of the methanol:water extract obtained from fresh leaves of ten plants species evaluated for their in vitro anthelmintic activity against eggs and L3 larvae of *Haemonchus contortus*

Plant extracts	CT (%)	TP (%)	TT (%)
Acacia collinsii	26.64	19.72	8.01
Acacia pennatula	34.41	27.72	14.91
Bunchosia swartziana	35.28	24.27	10.39
Gymnopodium floribundum	48.32	27.65	19.95
Havardia albicans	48.50	25.59	24.31
Lysiloma latisiliquum	14.63	21.79	23.16
Leucaena leucocephala	15.69	14.63	10.48
Mimosa bahamensis	35.03	19.81	16.41
Piscidia piscipula	ND	0.75	0.63
Senegalia gaumeri	ND	14.04	14.01

TP total phenols determined by the Folin Ciocalteu method (expressed as tannic acid equivalent), *TT* total tannins determined with the Folin Ciocalteu method + PVPP (expressed as tannic acid equivalent), *CT* condensed tannins determined with the vanillin method (expressed as percentage of catechin equivalents per gram of extract), *ND* not detected (< lowest standard concentration used)

Table 2 Effective concentration 50% (EC_{50}) and respective 95% confidence intervals (95% CI) of methanol:water leaf extracts of ten plants species tested with the egg hatch assay against *Haemonchus contortus*

Plant	EC_{50} μg/mL	95% CI μg/mL
Acacia collinsii	263.7[a]	185.0 344 5
Acacia pennatula	8180.8[c]	3478.5 169,791.6
Bunchosia swartziana	3180.8[c]	2885.8 3584.5
Gymnopodium floribundum	946.9[b]	629.0 1240.3
Havardia albicans	664.7[b]	549.5 800.6
Lysiloma latisiliquum	641.1[b]	549.7 736.1
Leucaena leucocephala	139.9[a]	49.3 226.4
Mimosa bahamensis	560.4[b]	372.9 854.6
Piscidia piscipula	252.9[a]	191.3 315.8
Senegalia gaumeri	186.3[a]	60.6 285.3

Mean values with different superscript letters in the same column are significantly different ($P < 0.05$)

Table 3 Effect of the addition of polyvinylpolypyrrolidone (PVPP) on the proportion (%) of morulated eggs (ME), larval failing eclosion (LFE), and larvae (L1) of *Haemonchus contortus* resulting from incubations with methanol:water leaf extracts from ten plant species at a concentration of 3600 μg extract/mL PBS

Plant	Life stage				
		PBS	Extract	Extract + PVPP	SE
Acacia collinsii	ME (%)	0.4[a]	4 5[b]	6.9[b]	0.8
	LFE (%)	1.0[a]	91.6[b]	89.7[b]	1.0
	L1 (%)	98.5[a]	3.8[b]	3.3[b]	0.7
Acacia pennatula	ME (%)	0 2[a]	4.6[b]	5.1[b]	0.8
	LFE (%)	0.9[a]	46.5[b]	86.3[c]	4.0
	L1 (%)	98.8[a]	48.7[b]	8.5[c]	3.7
Bunchosia swartziana	ME (%)	0.1[a]	5.1[a]	4.3[a]	1.8
	LFE (%)	0[a]	85.5[b]	93.1[c]	2.4
	L1 (%)	99.8[a]	9 3[b]	2.4[c]	0.9
Gymnopodium floribundum	ME (%)	0 5[a]	2.6[a]	3.6[a]	1.1
	LFE (%)	1.9[a]	97.0[b]	95.7[b]	1.4
	L1 (%)	97.6[a]	0 3[b]	0.7[b]	0.8
Havardia albicans	ME (%)	0.1[a]	4 3[a]	12.1[b]	2.1
	LFE (%)	0 2[a]	95.6[b]	87.7[c]	2.1
	L1 (%)	99.5[a]	0[b]	0.1[b]	0.1
Lysiloma latisiliquum	ME (%)	1.7[a]	2.1[b]	7.9[b]	0.7
	LFE (%)	0[a]	85.9[b]	91.6[c]	1.8
	L1 (%)	98.2[a]	11.9[b]	0.3[c]	1.7
Leucaena leucocephala	ME (%)	1.9[a]	11.8[b]	25.9[c]	2.3
	LFE (%)	6.4[a]	88[b]	73.6[c]	4.2
	L1 (%)	91.6[a]	0.1[b]	0.3[b]	4.2
Mimosa bahamensis	ME (%)	0[a]	2.9[b]	8.1[b]	1.0
	LFE (%)	3 5[a]	97[b]	89.8[c]	1.8
	L1 (%)	96.5[a]	0[b]	2.0[b]	1.6
Piscidia piscipula	ME (%)	0 2[a]	17.0[b]	31.7[c]	3.0
	LFE (%)	0[a]	82.9[b]	68.2[c]	3.0
	L1 (%)	99.7[a]	0.0[b]	0[b]	0.1
Senegalia gaumeri	ME (%)	0[a]	56.2[b]	51.7[b]	3.4
	LFE (%)	0 2[a]	43.7[b]	48[b]	3.6
	L1 (%)	99.8[a]	0[b]	0.2[b]	0.1

Mean values with different superscript letters in the same row are significantly different ($P < 0.05$)

SE standard error

Relationship between polyphenol content and anthelmintic activity

A positive correlation ($r = 0.6809$; $P < 0.05$, $n = 10$) was found between the CT content of extracts and the respective effect of extracts on egg hatching (EC_{50}). Higher CT concentrations in the extracts resulted in higher EC_{50} values required to inhibit the hatching of *H. contortus* eggs. Similarly, a positive relationship was also found between TP content and the EC_{50} to inhibit egg hatching ($r = 0.9152$; $P < 0.05$, $n = 10$). However, a negative relationship was found between TT content and the ovicidal effect ($r = -0.7091$; $P < 0.05$, $n = 10$). No relationship was found between CT, TP, and TT content and exsheathment inhibition measured by LEIA ($P > 0.05$).

Discussion

The results of the present study showed positive relationships between the EC_{50} values obtained for the EHA using extracts from different tropical plants and their CT content ($r = 0.6809$; $P < 0.05$, $n = 10$) or TP content ($r = 0.9152$; $P < 0.05$, $n = 10$).

Table 4 Effective concentration 50% (EC_{50}) and respective 95% confidence intervals (95% CI) of methanol:water leaf extracts of ten tropical plant species tested against *Haemonchus contortus* using the larval exsheathment assay

Plant	EC_{50} μg/mL	95% CI μg/mL
Acacia collinsii	297.7[b, c]	228.4 362.9
Acacia pennatula	426.0[c]	353.4 517.3
Bunchosia swartziana	205.0[b]	148.2 252.2
Gymnopodium floribundum	66.9[a]	46.9 82.9
Havardia albicans	63.5[a]	32.7 90.2
Lysiloma latisiliquum	251.5[b]	186.4 313.8
Leucaena leucocephala	269.1[b]	223.8 322.3
Mimosa bahamensis	244.6[b]	89.0 313.8
Piscidia piscipula	271.8[bc]	175.7 358.2
Senegalia gaumeri	184.7[b]	114.1 270.9

Mean values with different superscript letters in the same column are significantly different ($P < 0.05$)

Both results suggest a direct link between these compounds (CT and TP) and the AH activity of extracts against egg hatching, where a high concentration of CT and TP in the extracts resulted in a higher amount of plant extract required to obtain an AH effect. Furthermore, the high CT and TP content of extracts mainly reduced the number of eggs with LFE, which was the main AH effect for most extracts evaluated in the present study (Table 3). In addition, a higher TT content in the extracts reduced the ovicidal effect (eggs remaining as morula) ($r = -0.7091$; $P < 0.05$, $n = 10$).

Table 5 Mean exsheathment inhibition percentage of *Haemonchus contortus* infective larvae resulting from incubations with methanol:water extracts from ten tropical plant species at 1200 μg/mL of PBS with and without incubation with polyvinylpolypyrrolidone (PVPP)

Plant	Exsheathment inhibition (%) with PBS	Exsheathment inhibition (%) at 1200 μg extract/mL	Exsheathment inhibition (%) at 1200 μg extract/mL + PVPP	SE
Acacia collinsi	4.8[a]	100.0[b]	13.7[a]	3.8
Acacia pennatula	2.7[a]	100.0[b]	26.3[c]	3.2
Bunchosia swartziana	3.8[a]	100.0[b]	26.0[c]	6.8
Gymnopodium floribundum	5.7[a]	100.0[b]	15.2[c]	3.3
Havardia albicans	7.6[a]	100.0[b]	96.9[b]	1.1
Lysiloma latisiliquum	2.9[a]	100.0[b]	100.0[b]	1.0
Leucaena leucocephala	2.2[a]	100.0[b]	100.0[b]	0.6
Mimosa bahamensis	2.9[a]	98.8[b]	15.2[c]	2.1
Piscidia piscipula	6.4[a]	93.9[b]	10.3[a]	3.2
Senegalia gaumeri	3.5[a]	100.0[b]	96.6[b]	1.5

Mean values with different letters in the same row indicate a significant difference ($P < 0.05$)

SE standard error

Regarding the AH activity of the different methanol:water extracts tested against eggs of *H. contortus*, we found two main effects: (i) all ten extracts (one for every plant species) blocked the larvae from breaking the eggshell and emerging (LFE). Such a mechanism of action was reported for other tropical plant extracts either with acetone:water or methanol (Vargas-Magaña et al. 2014; von Son de Fernex et al. 2015; Castañeda-Ramírez et al. 2014; Chan-Perez et al. 2016; Castillo-Mitre et al. 2017); (ii) nine out of ten plants showed a slight ovicidal effect ranging from 2.1% ME in *L. latisiliquum* to 17.0% ME in *P. piscipula*, and a significantly high effect (56%, $P < 0.05$) for the *S. gaumeri* extract. Such an ovicidal effect considers all the eggs that remained at the morula stage after being exposed to the plant extract (Vargas-Magaña et al. 2014; Castañeda-Ramírez et al. 2014; Chan-Perez et al. 2016). This is the first report of significant ovicidal effects using methanol:water extracts of *S. gaumeri.* Recent studies suggested that caffeic acid, coumaric acid, and ferulic acid are involved in the ovicidal effect of tropical plants (Castillo-Mitre et al. 2017. Quercetin has also been proposed to be an active metabolite involved in ovicidal effects affecting eggs of *Cooperia* spp. (von Son-de Fernex et al. 2015) and *H. contortus*. (Castillo-Mitre et al. 2017). However, quercetin might not cause ovicidal activity in the present study as it binds to PVPP. The addition of PVPP to block CT and other polyphenols increased the ovicidal effect of plant extracts such as *L. leucocephala*, *H. albicans*, and *P. piscipula* ($P < 0.05$). The latter finding is consistent with the positive correlation between TT content and the ovicidal effect of the plant extracts.

Regarding the effect of extracts on eggs failing eclosion (eggs with trapped larvae), incubation with PVPP caused a significant increase in the AH effect for *L. latisiliquum*, *A. pennatula*, and *B. swartziana* extracts. However, the addition of PVPP significantly reduced the AH effect for *L. leucocephala*, *H. albicans*, *M. bahamensis*, *and P. piscipula*. These results support earlier studies showing that blocking polyphenols of plant extracts with PVPP resulted in different hatching inhibitory effects for each plant extract (Vargas-Magaña et al. 2014). Thus, it is likely that several secondary compounds interact differently for each plant extract, making it unlikely to find a single effect when adding PVPP to different plant extracts.

No association was found between the CT, TP, or TT content of the extracts evaluated and the EC_{50} of LEIA. This suggests that CT, TP, and TT cannot be used as the only predictors of AH activity on exsheathment for the methanol:water

plant extracts tested. Only extracts from five tropical plants tested in the present study showed AH activity associated with polyphenols (Table 5). Although it was not evaluated, it is possible that factors such as CT molecule size, complexity/polymerization, or the ratio of prodelphinins and procyanidins might explain the differences in biological activity of the extracts evaluated against *H. contortus* exsheathment (Hoste et al. 2016).

We observed that the addition of PVPP did not block the inhibition of exsheathment of larvae exposed to the methanol:water extracts of *L. leucocephala*, *H. albicans*, *L. latisiliquum*, and *S. gaumeri*. Therefore, in these plant extracts, the active compounds causing exsheathment inhibition might be different to polyphenols, or these were not blocked by PVPP as used in the methodology of the present study. Thus, studies should test different PVPP quantities, different incubation periods, or even different polyphenol blocking protocols to confirm these possibilities (Hernández-Bolio et al. 2017). Furthermore, the findings of the present study need to be assessed and confirmed in vivo.

Conclusion

The present study showed that a higher CT and TP content limited the AH effect of methanol:water plant extracts measured by EHA. In addition, the TT content of plant extracts inhibited the percentage of eggs showing an ovicidal effect. No clear relationship was found between the content of CT, TP, and TT from the plant extracts evaluated and inhibition of the exsheathment of L_3 larvae.

Acknowledgements This work financed by CONACYT, Mexico (Project CB 2013 01 221041). The support of CONACYT PCP No. 229330 Mexico France collaboration project is acknowledged. The first author (G.S. Castañeda Ramirez) acknowledges a scholarship from CONACYT for their PhD study (Reference number 7464535353).

Compliance with ethical standards

Conflict of interest statement The authors declare that they have no conflict of interest.

Ethical standards The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals (License No. CB CCBA D 2014 003).

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