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An in vitro approach to evaluate the nutraceutical value of plant foliage against *Haemonchus contortus*

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Abstract

Nutraceutical plants provide nutrients for the animal as well as secondary compounds that can affect the biology and survival of gastrointestinal nematodes (GIN). Current screening of nutraceutical plants is based on in vitro evidence of anthelmintic (AH) activity against different life stages of GIN, but nutritional information is omitted or scarce. This study proposes an integral in vitro screening protocol to identify the nutraceutical value of the foliage from plant species consumed by small ruminants, using *Haemonchus contortus* as a biological model. The leaves from *Acacia collinsii*, *A. pennatula*, *Bunchosia swartziana*, *Gymnopodium floribundum*, *Havardia albicans*, *Leucaena leucocephala*, *Lysiloma latisiliquum*, *Mimosa bahamensis*, *Piscidia piscipula*, and *Senegalia gaumeri* were evaluated for their chemical composition and in vitro digestibility. Acetone:water extracts (70:30) from leaves of each plant were evaluated using the egg hatch assay and larval exsheathment inhibition assay. Respective effective concentrations 50% (EC_{50}) were determined for each assay. The ten plant species showed good nutritional value for ruminants, including crude protein (> 10%), metabolizable energy (> 2.9 MJ/kg DM), and varied CT content (from 1.0 to 37.6%). The best AH activity against *H. contortus* eggs (EC_{50} = 401.8 μg/mL) and L_3 (EC_{50} = 83.1 μg/mL) was observed for *S. gaumeri* extract. Although all the plant species showed in vitro nutraceutical potential, the leaves of *S. gaumeri* had the best values. The proposed in vitro protocol showed to be useful for the integral assessment of the nutraceutical potential of different plant species as it included the nutritional value and the AH activity against eggs and L_3 in the selected plant species.

Keywords Nutraceutical potential · In vitro protocol · Chemical composition · Egg hatch assay (EHA) · Larval exsheathment inhibition assay (LEIA)

Introduction

The use of commercial anthelmintic (AH) drugs for the control of gastrointestinal nematodes (GIN) is threatened by the presence of AH-resistant worm populations. Frequency of sheep farms with multi-resistant worm populations is increasing at an alarming rate in tropical regions (Mahieu et al. 2015; Herrera-Manzanilla et al. 2017; Sepúlveda-Vázquez et al. 2017). As a result, farmers need alternative methods of GIN control to reduce their dependence on commercial drugs. The use of nutraceutical plants has been proposed as an alternative method of GIN control. The leaves of nutraceutical plants contain nutrients to nourish the animal and secondary compounds that can affect the biology and survival of GIN leading to beneficial effects on animal health (Hoste et al. 2015). First studies suggested that the consumption of some plant foliages caused a reduction of the fecal excretion of GIN eggs in naturally infected sheep (Niezen et al. 1998; Marley et al. 2003).

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The AH activity was confirmed under controlled feeding pen studies using small ruminants artificially infected with *Trichostrongylus colubriformis* (Athanasiadou et al. 2000a, b), *Haemonchus contortus* (Shaik et al. 2004) or mixed infections (Paolini et al. 2005). Those studies were performed with temperate plant species of the Fabaceae family with high content of condensed tannins (CT) such as *Hedysarum coronarium* L., *Lotus corniculatus* L., *L. uliginocis* L., *Lespedeza cuneata*, and *Onobrychis viciifolia* (Manolaraki et al. 2010). Nowadays, some commercial feed pellets contain foliage of some of those plant species and are used as adjuvants for GIN control (Gaudin et al. 2016). Such success stories inspired other research groups to search for plants with AH activity in other latitudes and ecosystems. Under tropical conditions, many authors explored the AH effect of plants consumed by animals, mostly with high-CT content (Torres-Acosta et al. 2012). The AH activity of those materials has been studied using in vitro assays targeting different life stages of GIN mainly with the egg hatch assay (EHA; von Samson-Himmelstjerna et al. 2009), the larvae motility inhibition test, and the larval exsheathment inhibition assay (LEIA; Jackson and Hoste 2010). However, the nutritional information is generally explored until plant candidates with good in vitro AH activities are evaluated under in vivo conditions. At that stage maybe the nutritional information suggests that such candidate had poor nutritional quality. Thus, we propose to evaluate the nutritional information of the candidate plants at the earliest stage possible. That could be an important advantage in ecosystems such as the tropical deciduous forest (TDF) in Mexico, where sheep and goats consume > 50 plant species (González-Pech et al. 2015; Ventura-Cordero et al. 2018a), and those plants are consumed at different proportions, from large proportion to occasional consumption. Under such conditions it could be important to use a protocol that helps to speed up the nutraceutical screening process. Such protocol should include the in vitro evaluation of the nutritional value of the plant materials including its crude protein, dry matter and organic matter digestibility, metabolizable energy, neutral and acid detergent fibers, lignin and polyphenol content, together with the in vitro evaluation of the AH activity using in vitro assays that include at least two stages of the parasite model. The nutritional and AH activity results obtained from such in vitro methodological approach could help to direct the in vivo stage of the nutraceutical screening process towards plant materials showing the best nutritional quality as well as good AH activity, rather than wasting time with candidates showing good AH activity but poor nutritional value. The methodology could also help to decide which plant materials should continue further to bio-guided in vitro assays aiming to identify new active compounds causing AH activity (Vargas-Magaña et al. 2014; Chan-Pérez et al. 2016, 2017; Hernández-Bolio et al. 2018a). At present, there are no international guidelines for the in vitro evaluation of the nutraceutical value of plant materials. Hence, this study proposed a new in vitro screening protocol to identify the nutraceutical value of the foliage from plant species consumed by small ruminants, using *H. contortus* as the biological model.

Materials and methods

Production of eggs and larvae of *Haemonchus contortus*

Eggs and larvae were obtained from lamb donors raised free of GIN infections. Animals were kept in individual cages with raised-slatted floors before and during the experiment. Animals were fed a balanced diet based on grass hay and a commercial concentrate feed, as well as ad libitum access to clean water. 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 (Bauer et al. 2010). Once the lambs were confirmed free of any GIN infection, two donors (25 ± 1 kg) were orally inoculated with 4000 *H. contortus* infective L_3. The Paraiso isolate of *H. contortus* obtained from a local farm in Mexico was used. The presence of *H. contortus* eggs was confirmed from fecal samples obtained 28 days after the artificial infection.

For the EHA, donor feces' were collected directly from the rectum of the animals using new airtight polyethylene bags. Feces 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 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, the fecal pellets were collected from the donor sheep and washed with tap water to remove grass and other debris. Rinsed pellets were then placed in Petri dishes covered with a larger Petri dish to make a humid chamber. The fecal cultures were incubated for 5 days at 28 °C. Cultures were moistened daily with a manual water sprinkler. The infective larvae were harvested using a Baermann apparatus. Clean larvae were stored at 4 °C until further use. Age of

larvae used for the bioassay ranged from 2 to 5 weeks as suggested by Castañeda-Ramírez et al. (2017a).

Selection of plant species

The in vitro nutraceutical screening protocol was evaluated with plants species that are consumed by sheep and goats in the TDF of Mexico. The information obtained by González-Pech et al. (2015) and Ventura-Cordero et al. (2018a) suggested that animals consumed > 50 plants species. Each plant is consumed at different quantities in relation to the animal's total dry matter intake (DMI): high consumption (> 4% DMI), low consumption (1 to 0.1% DMI), and occasional consumption (< 0.1% DMI). Such differential consumption imposes a difficulty in the decision-making process to select which plants to include in the nutraceutical screening process. We hypothesize that plants with high consumption might display good nutritional value but poor AH activity, while plants with low or occasional consumption might have poor nutritional value but possibly a strong AH activity, which promotes its consumption even when nutritional value is poor. Hence, it was decided to explore the in vitro nutraceutical screening protocol using three plant species with high consumption (*Leucaena leucocephala*, *Mimosa bahamensis*, and *Gymnopodium floribundum*), four plants with low consumption (*Senegalia gaumeri*, *Bunchosia swartziana*, *Lysiloma latisiliquum*, and *Piscidia piscipula*), and three plants with occasional consumption (*Havardia albicans*, *Acacia pennatula*, and *Acacia collinsii*). These plants were included in the nutraceutical evaluation because small ruminants consumed them, and because its ingestion caused no negative impact on animal health or production as recommended by Hoste et al. (2015). Among the selected plants, we included five plant species without prior information of AH activity and five plant species known for having AH activity, either under in vitro or in vivo conditions, which could help to confirm the validity of the proposed approach. Furthermore, nutritional information of all the plant species included in this study has been already reported (Ventura-Cordero et al. 2018a, b). However, the simultaneous evaluation of nutritional value and AH activity has not been attempted before.

Plant extracts

The leaves were collected during the dry season in the Faculty of Veterinary Medicine in Yucatan (FMVZ), Mexico (20° 51′ 41″ N 89° 37′ 28″ W), and received a voucher number at the University Autónoma de Yucatán Herbarium. The plants studied were: *A. collinsii* (voucher no. 21938), *A. pennatula* (voucher no. 20101), *B. swartziana* (voucher no. 20102), *G. floribundum* (voucher no. 20104), *H. albicans* (voucher no. 20107), *L. leucocephala* (voucher no. 20105), *L. latisiliquum* (voucher no. 20103), *M. bahamensis* (voucher no. 20106), *P. piscipula* (voucher no. 20108), and *S. gaumeri* (voucher no. 20100). Respective acetone:water extracts were produced using 75 g of fresh leaves from each plant species. The leaves were crushed and incorporated into an acetone:water (70:30) solution containing ascorbic acid (1 g/L) for 24 h. The solution was filtered (paper no. 50) and the solvent (acetone) was evaporated using a rotavapor (IKA®, Germany). The aqueous fraction was rinsed with methylene chloride in a portion volume 1:1 (two washes) to remove chlorophyll and lipids. The extract was again roto-evaporated to eliminate solvent residues. Finally, the respective plant extracts were lyophilized and stored at 4 °C until use.

Chemical analyses of plants

For each plant species, fresh leaf samples were oven dried at 40 °C until constant weight was reached. Dry samples were milled at 1 mm particle size. The analyses performed were: dry matter (DM) (no. 7.007), crude protein (CP) (no. 2057), ash (no. 7009) (AOAC 1980), neutral detergent fiber (NDF) (Mertens 2002), and acid detergent fiber (ADF) (Van Soest et al. 1991). In vitro dry matter digestibility (IVDMD) and organic matter digestibility (IVOMD) were estimated using the protocol designed for tropical leaves (Barros-Rodríguez et al. 2012). The metabolizable energy (ME) (MJ/kg DM) was estimated as suggested by AFRC (1993). For both leaf samples and extracts, the Folin-Ciocalteu method was used to determine the amount of total phenols (TP) and total tannins (TT) as described by Makkar (2003). The content of condensed tannins (CT) was quantified by the vanillin method (Price et al. 1978).

In vitro tests for evaluation of anthelmintic activity

Egg hatch assay

The EHA was used to evaluate the in vitro AH activity of the acetone:water leaf extracts of the ten plants 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 PBS) were prepared for each plant extract tested. The PBS was used as a negative control, and thiabendazole was used as a positive control (10 μg of the stock solution with 1000 μg of thiabendazole/mL PBS) for the respective extracts. A respective multi-well plate (24 wells) was used to add 0.5 mL of stock solution for each plant extract at different concentrations (3600, 2400, 1200, 600, 300, and 150 μg/mL PBS). Each well was added with 0.5 mL of the egg suspension (150 eggs/mL). Each well had 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. After that time, two drops of lugol solution were added to each well to kill and dye the eggs and

larvae. Then, the number of eggs that failed to form larvae was counted as morulated eggs (MOE). The number of eggs that formed into larvae but failed to complete their eclosion was counted as larvae failing eclosion (LFE). Finally, the number of free larvae present in each well was also counted as described by Vargas-Magaña et al. (2014).

Larvae exsheathment inhibition assay

The LEIA were conducted following the procedure described by Jackson and Hoste (2010). A stock solution was prepared using respective acetone:water extracts at 5000 μg/mL PBS. The negative controls consisted of L_3 exposed only to the PBS, and the positive controls consisted of L_3 exposed to levamisole (20 μg of the stock solution with 3000 μg levamisole/mL water). Concentrations of acetone:water extracts tested were 1200, 600, 200, 100, and 50 μg/mL PBS. Each concentration was added with 1000 μL of a solution containing 1000 L_3 to obtain the final extract concentrations. Infective larvae were incubated with the plant extract for 3 h at 24 °C. After that time, the L_3 were centrifuged for 3 min at 168 g and washed three times with a PBS solution. Then, aliquots of each larvae solution were placed in Eppendorf vials (200 μL each). Four replicates were performed for each concentration and the PBS control. The process of exsheathment was artificially induced by contact with a stock solution of sodium hypochlorite (2% *w*/*v*) and sodium chloride (16.5% *w*/*v*) diluted in PBS (30 μL of the stock solution in 6 mL PBS). The resulting sodium hypochlorite concentration was 0.006%. The kinetics of exsheathment was assessed with a microscope using the × 10 objective and recorded at 0, 20, 40, and 60 min at 24 °C (Jackson and Hoste 2010). The exsheathment process was stopped at each time point by flaming the respective slides that contained the L_3. Then, the number of sheathed and exsheathed larvae were recorded.

Effect of polyvinylpolypyrrolidone

The polyvinylpolypyrrolidone (PVPP) has a high affinity for tannins, and its removal using centrifugation following the PVPP treatment removes tannins from the extract (Makkar et al. 1995) and may also remove flavonoids (Doner et al. 1993) and polyphenols (Laborde et al. 2006). To determine the role of tannins and other polyphenols on the AH activity 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 incubations, solutions were centrifuged at 378 g for 5 min. The supernatant was used in each EHA or LEIA bioassays. For EHA, the extract concentration used for all the plant species was 3600 μg/mL PBS with or without PVPP (six replicates for each extract). For the LEIA, the extract concentration used for all the plant species was 1200 μg/mL PBS with or without PVPP (four replicates for each extract).

Data analyses

For the respective EHA, the number of morulated eggs (MOE), eggs with LFE, and the number of larvae that emerged from eggs were recorded for the different extracts at the respective concentrations previously described. That information was used to determine the egg-hatching rate (EH) as follows (Vargas-Magaña et al. 2014; Chan-Pérez et al. 2016):

$$\mathrm{MOE} = \frac{\text{number of morulated eggs}}{\text{number of morulated eggs} + \text{number of eggs containing a larvae} + \text{number of larvae}} \times 100$$

$$\mathrm{LFE} = \frac{\text{number of eggs containing a larvae}}{\text{number of morulated eggs} + \text{number of eggs containing a larvae} + \text{number of larvae}} \times 100$$

$$\mathrm{EH} = \frac{\text{number of larvae}}{\text{number of morulated eggs} + \text{number of eggs containing a larvae} + \text{number of larvae}} \times 100$$

Meanwhile, the exsheathment inhibition percentage (EI) was calculated for each plant extract as described by Chan-Pérez et al. (2017):

$$\text{Exsheathment}\% \quad \frac{\text{larvae L3 without sheath}}{\text{larvae with sheath} + \text{larvae without sheath}} \times 100$$

$$\mathrm{EI}\% = 100 - \text{exsheathment}\%$$

Egg hatching and LEIA results of different extracts were analyzed with respective GLM to assess differences between results of the control PBS and those from different extract concentrations tested. Post hoc analysis was performed using the Fisher's LSD. The GLM and post hoc analyses were performed with Statgraphics Centurion XV (Statpoint Technologies Inc. 2005). Data obtained from PVPP

incubations of respective extracts was analyzed using a completely randomized design with respective GLM (comparison performed with the corresponding control group).

In the case of the EHA results, the percentage of eggs showing either MOE, LFE, or emerged larvae between PBS controls and results obtained for plant extracts at 3600 μg/mL PBS, with and without PVPP were compared.

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, a respective post hoc analysis was performed using the Fisher's LSD of Statgraphics Centurion XV.

The number of eggs (MOE + LFE) and emerged larvae were used to determine the effective concentration required to inhibit 50% of hatching (EC_{50}) with respective 95% confidence intervals (95% CI). The latter was estimated for each plant extract tested using the PoloPlus 1.0 software (LeOra software 2004). Exsheathment data obtained from LEIA for each plant extract on the 60th minute were used to determine respective EC_{50} and 95% CI for each plant extract tested using the same software.

Results

Chemical analysis of plants and extracts

The nutritional value of the evaluated plants is shown in Table 1. The values of neutral detergent fiber (NDF) and acid detergent fiber (ADF) ranged from 31.7% (*L. leucocephala*) to 50.1% (*L. latisiliquum*) and from 17.0% (*S. gaumeri*) to 33.1% (*L. latisiliquum*) respectively. The crude protein (CP) content was higher than 10% in all cases and up to 21.4% for *S. gaumeri*. The in vitro dry matter digestibility (IVDMD) varied from 26.0% (*H. albicans*) to 54.7% (*B. swartziana*). The highest values of metabolizable energy (ME) were found for *B. swartziana* (9 MJ/kg DM) and *S. gaumeri* (8.6 MJ/kg DM). The condensed tannin (CT) content of experimental plants varied from 1% (*S. gaumeri*) to 37.6% (*G. floribundum*). The extracts of leaves with the highest content of total tannins (TT) was 20.5% (*A. pennatula*) and the highest percentage of CT was 95.8% (*G. floribundum*) (Table 2).

The effect of tropical plant extracts on egg hatching

The proportions of the morulated eggs (MOE), larvae failing eclosion (LFE), and the larvae that hatched in the presence of each plant extract are shown in Table 3. The latter was observed when eggs were exposed to high-extract concentrations (3600 μg/mL) with or without PVPP. This strategy helped to identify differences in the mechanism of action against egg hatching for the extracts evaluated. Extracts of *P. piscipula* and *M. bahamensis* showed clear ovicidal activity (MOE) of 40 and 13.7% respectively. Furthermore, the ovicidal activity of these extracts increased to 64.5 and 30.5%, respectively, when removing the polyphenols with the PVPP. Meanwhile, extracts of *L. leucocephala* and *G. floribundum* only showed ovicidal activity when polyphenols were removed with PVPP (14.2 and 18.6%, respectively).

Plant extracts obtained from leaves of *S. gaumeri*, *H. albicans*, *B. swartziana*, *A. collinsii*, and *A. pennatula* showed LFE activity, which was not reduced with the addition of PVPP (Table 3). However, when polyphenols of *L. latisiliquum* extract were blocked with PVPP, the extract

Table 1 Chemical analysis of fresh leaves of *Acacia collinsii*, *Acacia pennatula*, *Bunchosia swartziana*, *Gymnopodium floribundum*, *Havardia albicans*, *Leucaena leucocephala*, *Lysiloma latisiliquum*, *Mimosa bahamensis*, *Piscidia piscipula*, and *Senegalia gaumeri*, which are plants consumed by small ruminants in the tropics of Yucatán, México

Plant species	DM (%)	Ash (%)	NDF (%)	ADF (%)	CP (%)	CT* (%)	TP (%)	TT (%)	IVDMD (%)	IVOMD (%)	ME (MJ/kg DM)
Acacia collinsii	47	5.6	41.2	24.6	16.4	7.0	4.4	1 5	32.8	29.9	4.8
Acacia pennatula	48	3.7	36.5	17.8	15.9	17.8	10.9	6.0	31.9	21.9	3.5
Bunchosia swartziana	36	10.9	32.0	25.5	16.5	3.8	3.8	3.8	54.7	56.4	9.0
Gymnopodium floribundum	36	6.1	43.4	25.0	10.1	37.6	8.9	5.4	30.6	23.0	3.7
Havardia albicans	43	4.4	44.7	31.4	15.8	19.6	6.7	3.4	26.0	18.3	2.9
Leucaena leucocephala	34	8 3	31.7	22.4	19.8	5.7	1.3	1 3	43.3	34.4	5.5
Lysiloma latisiliquum	24	5 5	50.1	33.1	16.2	1.3	3.8	2.9	33.1	22.5	3.6
Mimosa bahamensis	44	4.7	44.0	25.0	13.5	19.8	5 2	2.0	27.4	19.5	3.1
Piscidia piscipula	26	9.0	38.1	27.5	15.9	1.1	1.3	0.8	46.4	34.0	5.4
Senegalia gaumeri	42	6.1	34.9	17.0	21.4	1.0	3.8	3.6	53.9	53.5	8.6

DM dry matter, *NDF* neutral detergent fiber, *ADF* acid detergent fiber, *CP* crude protein, *CT* condensed tannins, *TP* total phenols, *TT* total tannins, *IVDMD* in vitro dry matter digestibility, *IVOMD* in vitro organic matter digestibility, *ME* metabolizable energy

*Cathequin equivalent

Table 2 Total phenols (%), total tannins (%), and condensed tannins (%) determined on the acetone:water extracts obtained from the fresh leaves of ten tropical plant species from Mexico

Extracts of plants	Botanical families	Total phenols	Total tannins	Condensed tannins*
Acacia collinsii	Fabaceae	18.4	15.2	21.3
Acacia pennatula	Fabaceae	24.7	20.5	38.2
Bunchosia swartziana	Malpighiaceae	22.7	18.5	40.3
Gymnopodium floribundum	Polygonaceae	26 5	19.7	95.8
Havardia albicans	Fabaceae	25.4	18.6	83.4
Leucaena leucocephala	Fabaceae	13.9	9.8	23.7
Lysiloma latisiliquum	Fabaceae	21	20.1	20.8
Mimosa bahamensis	Fabaceae	20.7	17.2	88.3
Piscidia piscipula	Fabaceae	10.9	10.6	7.8
Senegalia gaumeri	Fabaceae	16.4	16.1	7.1

TP total phenols measurement with Folin Ciocalteu; *TT* total tannins measurement with Folin Ciocalteu + PVPP; *CT* condensed tannins (vanillin method)

*Cathequin equivalent

Table 3 Effect of the addition of polyvinylpolypyrrolidone (PVPP) to block tannins, flavonoids, and other polyphenols on the proportion (%) of morulated eggs (MOE), larvae failing eclosion (LFE), and larvae (L1) of *Haemonchus contortus* as a result of incubations with acetone:water extracts of ten tropical plant species at the concentration of 3600 μg/mL phosphate buffered saline (PBS)

Plant species	Life stage	PBS	3600 μg/mL	PVPP	SE
Acacia collinsii	MOE	2.7^{a}	3.1^{a}	5.3^{a}	0.9
	LFE	1.6^{a}	96.9^{b}	94.5^{b}	0.9
	L1	95.6^{a}	0.0^{b}	0.1^{b}	0.4
Acacia pennatula	MOE	1.8^{ab}	0.5^{a}	3.4^{b}	0.7
	LFE	1.8^{a}	98.8^{b}	95.6^{c}	0.7
	L1	96.4^{a}	0.61^{b}	0.8^{b}	0.4
Bunchosia swartziana	MOE	0.7^{a}	3.6^{a}	2.5^{a}	1
	LFE	0.2^{a}	95.5^{b}	95.6^{b}	1.3
	L1	99.0^{a}	0.8^{b}	1.7^{b}	0.5
Gymnopodium floribundum	MOE	1.2^{a}	0.7^{a}	18.6^{b}	1.9
	LFE	3.6^{a}	98.3^{b}	80.1^{c}	2.2
	L1	95.1^{a}	0.9^{b}	1.18^{b}	1.1
Havardia albicans	MOE	1.2^{a}	2.3^{a}	7.8^{a}	3.2
	LFE	1.8^{a}	97.6^{b}	91.9^{b}	3.3
	L1	96.8^{a}	0.0^{b}	0.2^{b}	0.4
Leucaena leucocephala	MOE	0.6^{a}	0.6^{a}	14.2^{b}	1.5
	LFE	5.1^{a}	99.1^{b}	85.4^{c}	2
	L1	94.2^{a}	0.2^{b}	0.3^{b}	1.3
Lysiloma latisiliquum	MOE	1.7^{a}	3.3^{a}	1.5^{a}	0.7
	LFE	3.7^{a}	45.2^{b}	98.4^{c}	0.9
	L1	94.5^{a}	51.5^{b}	0.0^{c}	1.1
Mimosa bahamensis	MOE	0.6^{a}	13.7^{b}	30.5^{c}	3.1
	LFE	5.8^{a}	84.1^{b}	66.6^{c}	3.7
	L1	93.5^{a}	2.1^{b}	2.8^{b}	1.3
Piscidia piscipula	MOE	2.7^{a}	40.2^{b}	64.5^{c}	2.8
	LFE	6.1^{a}	59.2^{b}	35.4^{c}	2.8
	L1	91.1^{a}	0.4^{b}	0^{b}	1.1
Senegalia gaumeri	MOE	3.28^{a}	9.3^{a}	8.0^{a}	2
	LFE	1.9^{a}	90.6^{b}	91.7^{b}	2.1
	L1	94.8^{a}	0.0^{b}	0.25^{b}	0.7

SE standard error

^{a,b,c} Different letters in the same row means a significant difference ($P < 0.05$).

increased its LFE activity, changing from 45.2 to 98.4% (with and without PVPP respectively).

The EC_{50} with respective 95% CI obtained with EHA for each plant extract is presented in Table 4**.** Extracts with lower EC_{50} were those of *A. pennatula* and *S. gaumeri* (EC_{50} 415.5 and 401.8 μg/mL respectively). Plant extracts showing highest EC_{50} were *H. albicans* and *L. latisiliquum* (EC_{50} 2572 μg/mL and 2528 μg/mL respectively). For the extract of *B. swartziana* it was not possible to calculate the EC_{50} against eggs at the concentrations tested. Figure 1 shows the concentration-response relationship in the EHA for each plant extract.

Effect of plant extracts against L_3

The exsheathment inhibition activity of plant extracts, with or without PVPP is shown in Fig. 2. Only the *A. pennatula* and *G. floribundum* extract returned to PBS values when incubated with PVPP. Extracts of *A. collinsii*, *B. swartziana*, *L. latisiliquum*, and *M. bahamensis* significantly reduced its AH activity when incubated with PVPP, compared to incubations without PVPP. Meanwhile, extracts of *H. albicans*, *L. leucocephala*, *P. piscipula*, and *S. gaumeri* were not affected in their AH activity when the polyphenols were removed with PVPP (Fig. 3).

The EC_{50} with respective 95% CI obtained with LEIA for each plant extract is presented in (Table 5). The lowest EC_{50} were those of *S. gaumeri* and *M. bahamensis* (EC_{50} = 83.1 and 93.5 μg/mL, respectively) ($P < 0.05$). In contrast, the *P. piscipula* extract had the highest EC_{50} (708.0 μg/mL) compared to other plant extracts ($P < 0.05$).

Table 4 Effective concentration 50% (EC_{50}) with respective 95% confidence intervals (95% CI) of the acetone:water (70:30) extracts obtained from fresh leaves of ten tropical plant species against eggs of *Haemonchus contortus*

Plant species	EC_{50} μg/mL	95% CI μg/mL
Acacia collinsii	1119.3^{cd}	737.5 1448 2
Acacia pennatula	415.5^{ab}	278.1 581.2
Bunchosia swartziana		
Gymnopodium floribundum	946.9^{cd}	629.0 1240 3
Havardia albicans	2572.1^{e}	2034.4 3346.5
Leucaena leucocephala	1340.2^{de}	816.9 2140.9
Lysiloma latisiliquum	2528.0^{e}	1874.7 3825.3
Mimosa bahamensis	1214.5^{d}	1075.0 1356.3
Piscidia piscipula	832.7^{bc}	576.3 1064.4
Senegalia gaumeri	401.8^{a}	282.1 514.5

^{a,b,c,d,e} Different letters in the same column means significant difference ($P < 0.05$)

Discussion

The in vitro screening methodology used in the present study allowed to rank all ten plant species according to their nutraceutical potential. The nutritional value was confirmed in all the tested materials, as well as the AH activity against *H. contortus* egg hatching or L_3 exsheathment. This methodology also allowed identifying the role of polyphenols on the activity against eggs or L_3.

Nutritional value of plant species

As it was mentioned before, the plants included in the present study were selected because goats and sheep consumed their leaves in their daily diet while browsing the TDF (González-Pech et al. 2014, 2015; Ventura-Cordero et al. 2017). Thus, these plants fulfilled the first criteria for any nutraceutical material. The present evaluation included features that are essential for the evaluation of ruminant feedstuffs (AFRC 1993), including dry matter (DM), crude protein (CP), ash, neutral detergent fiber (NDF), acid detergent fiber (ADF), in vitro dry matter digestibility (IVDMD), in vitro organic matter digestibility (IVOMD), the condensed tannins (CT), total tannins (TT), and total phenols (TP). Furthermore, the metabolizable energy (ME) was also estimated. According to the present study, the leaves of all the plant species had a nutritional profile that enables its use for ruminant nutrition. However, the leaves of *B. swartziana* and *S. gaumeri* had the best nutritional quality, with IVDMD and IVOMD higher than 50%. Consequently, these two plant species showed highest ME content, with good CP content and medium to low CT, TT, and TP contents. Other two species showed good nutritional quality (*L. leucocephala* and *P. piscipula*) with CP content > 15% and ME > 5.4 MJ/kg DM. The other plant species can be considered with medium quality for ruminants. The plants with lowest quality were *M. bahamensis* and *H. albicans*. However, recent studies showed that the latter were consumed and selected by sheep and goats (Méndez-Ortíz et al. 2012; Ventura-Cordero et al. 2017). Thus, the present results suggest that, from the nutritional point of view, any of the plant species tested in the present study could be used as a nutraceutical material. Although it was evident that some plants had better quality than others, it is important to consider that animals might not prefer consuming plants according to their nutritional value per se, but depends also on the plant mix that is consumed. For example, goats may refuse to consume a good quality fodder such as *L. leucocephala* because of its high-CP content. Meanwhile, goats may consume a medium quality fodder such as *M. bahamensis* or *G. floribundum*, with low CP and ME content and high CT, as those plants possibly help blocking the excess protein from other plants such as *L. leucocephala* (Ventura-Cordero et al. 2017).

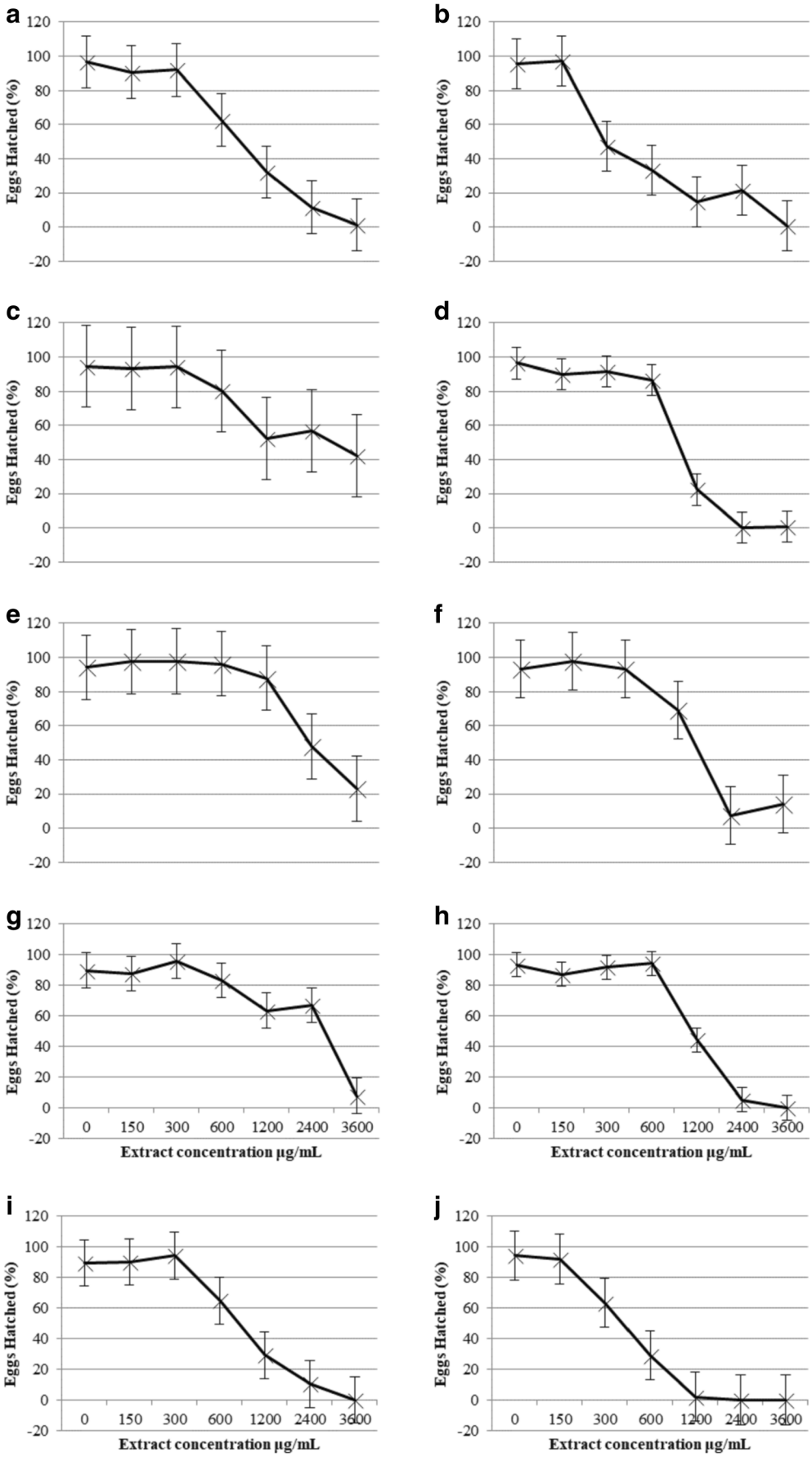

Fig. 1 Effect of different concentrations of acetone:water extracts of *Acacia collinsii* (**a**), *A. pennatula* (**b**), *Bunchosia swartziana* (**c**), *Gymnopodium floribundum* (**d**), *Havardia albicans* (**e**), *Leucaena leucocephala* (**f**), *Lysiloma latisiliquum* (**g**), *Mimosa bahamensis* (**h**), *Piscidia piscipula* (**i**), and *Senegalia gaumeri* (**j**) on the proportion of *Haemonchus contortus* eggs hatched in vitro. Each point represents the mean (± SD) of six replicates per extract concentration

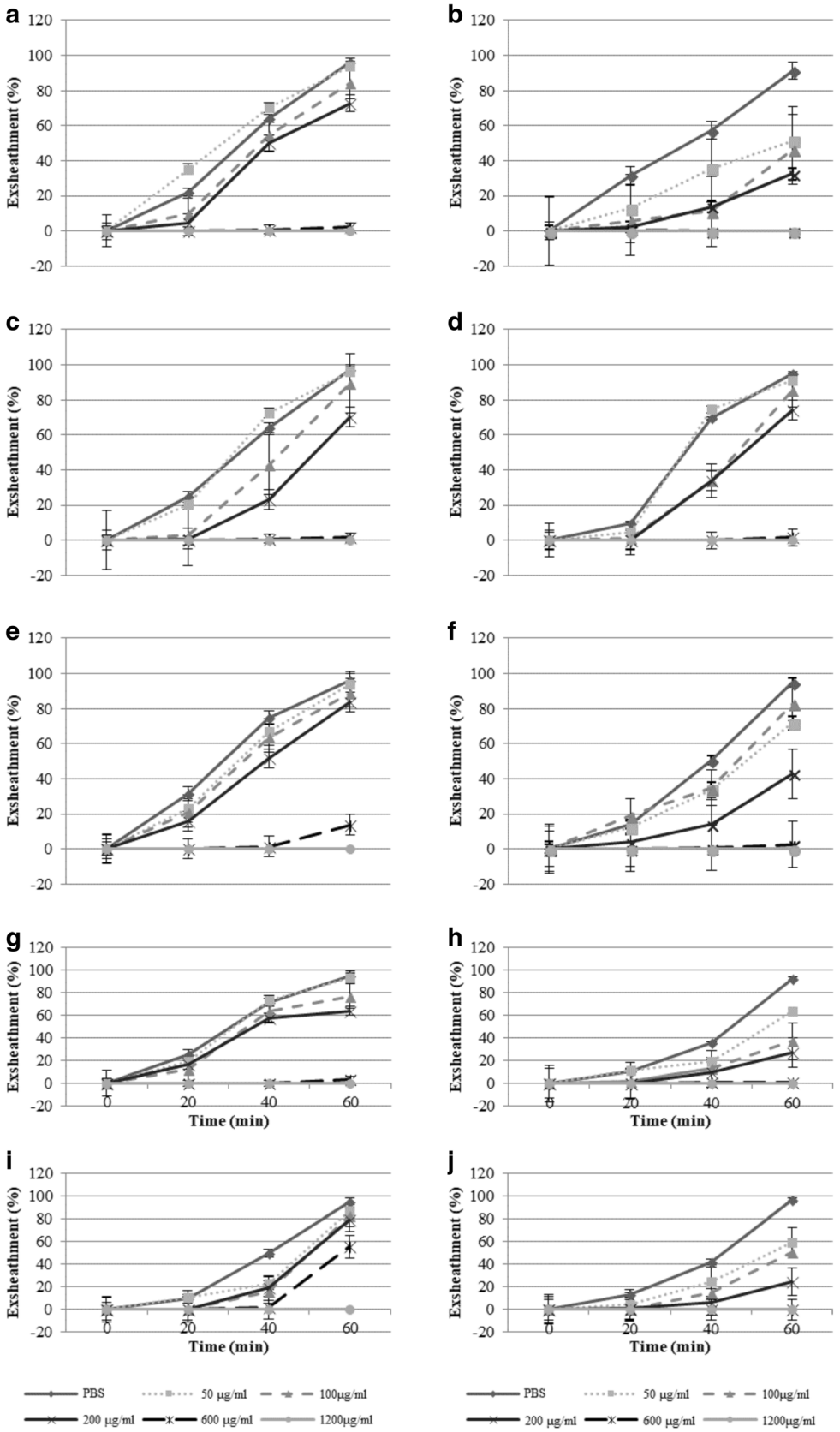

Fig. 2 Effect of different concentrations of acetone:water extracts of *Acacia collinsii* (**a**), *A. pennatula* (**b**), *Bunchosia swartziana* (**c**), *Gymnopodium floribundum* (**d**), *Havardia albicans* (**e**), *Leucaena leucocephala* (**f**), *Lysiloma latisiliquum* (**g**), *Mimosa bahamensis* (**h**), *Piscidia piscipula* (**i**), and *Senegalia gaumeri* (**j**) on the process of artificial in vitro exsheathment of *Haemonchus contortus* L_3

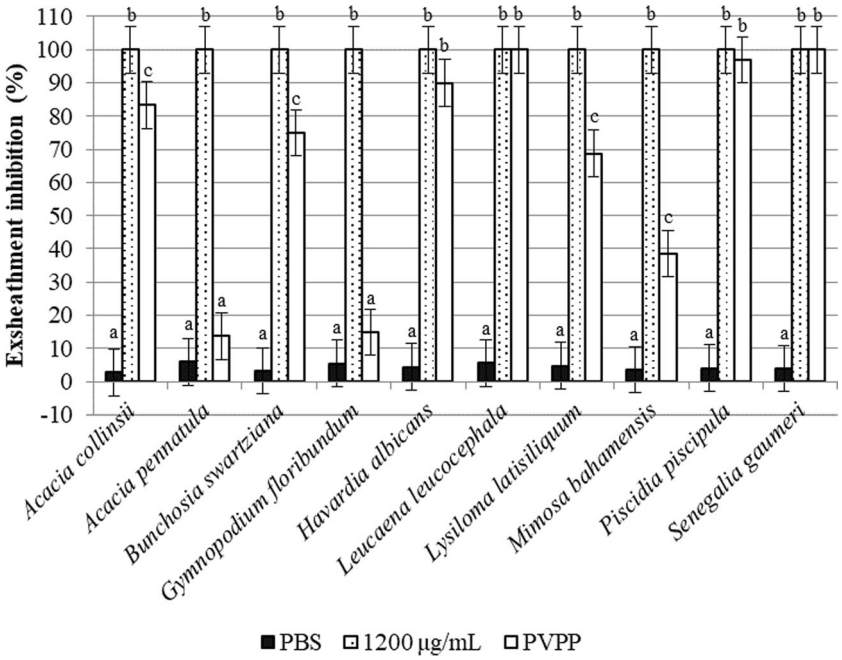

Fig. 3 Mean exsheathment inhibition percentage of *Haemonchus contortus* infective larvae resulting from incubations with acetone:water extracts from ten tropical plants species at a concentration of 1200 µg/mL phosphate buffered saline (PBS) with and without incubation with polyvinylpolypyrrolidone (PVPP). Different letters between bars of the same plant extract in dicate significant difference ($P < 0.05$)

Anthelmintic activity of acetone:water extracts against eggs and L_3

The next stage to confirm the nutraceutical potential of the experimental plant species was to confirm their AH activity. Nine of the plant extracts evaluated in this study showed AH activity against *H. contortus* eggs (Fig. 1). The present nutraceutical screening approach confirmed the AH activity of *A. pennatula* fresh leaves (Chan-Pérez et al. 2016), while all the other materials were tested for the first time in the present study. The best EC_{50} value was found for *S. gaumeri* extract, and the lowest EC_{50} value was found for *H. albicans* extract (Table 4).

Table 5 Effective concentration 50% (EC_{50}) with respective 95% confidence intervals (95% CI) of acetone:water leaf extracts of 10 tropical plant species tested against *Haemonchus contortus* using the larvae exsheathment inhibition assay. Data used for respective calculations were obtained on the 60th minute

Plant species	EC_{50} µg/mL	95% CI µg/mL
Acacia collinsii	273.8^{c}	235.7 311.8
Acacia pennatula	258.0^{c}	231.1 277.8
Bunchosia swartziana	260.8^{c}	221.9 300.6
Gymnopodium floribundum	283.3^{cd}	245.8 322.3
Havardia albicans	372.5^{d}	308.5 426.3
Leucaena leucocephala	218.3^{bc}	168.7 267.6
Lysiloma latisiliquum	186.7^{b}	155.9 226.1
Mimosa bahamensis	93.5^{a}	46.5 140
Piscidia piscipula	708.0^{e}	621.8 778.6
Senegalia gaumeri	83.1^{a}	64.6 101.7

^{a,b,c,d} Different letters in the same column means significant difference ($P < 0.05$)

The incubation with PVPP allowed identifying two modes of action of the tested plants against *H. contortus* eggs: (a) eggs that remain at the morula stage (MOE) or ovicidal activity, and (b) larvae that fail eclosion from the egg (LFE) (Table 3). The first type of activity was evident for extracts of *P. piscipula*, *M. bahamensis*, *G. floribundum*, and *L. leucocephala.* Thus, it is possible that those plant species contain similar plant secondary compounds that cause such activity, and those compounds might not be present in the other plant species. However, the MOE activity was enhanced for *G. floribundum*, *L. leucocephala*, *M. bahamensis*, and *P. piscipula* extracts when polyphenols were removed with PVPP, thus, polyphenols seem to partially block that activity. The present results support previous evidence suggesting that polyphenols antagonize with the secondary compounds causing the in vitro MOE activity (Vargas-Magaña et al. 2014; Castañeda-Ramírez et al. 2017b). The LFE activity was the most evident activity against eggs in the plant species evaluated in this study. Thus, plant extracts seemed to contain secondary compounds that can stop the rupture of the egg by the larvae. Interestingly, the *L. latisiliquum* extract showed a potent LFE activity when eggs were incubated in PVPP, suggesting that polyphenols were blocking the LFE activity in that extract. The presence of LFE activity has already been reported for other tropical plant extracts (Vargas-Magaña et al. 2014; Chan-Pérez et al. 2016). A recent study by von Son-de Fernex et al. (2015) using *L. leucocephala* against *Cooperia* spp. eggs suggested that quercetine was a majoritary compound of the extract affecting LFE, but did not confirm its activity in a bio-

guided assay. Thus, it is still unknown on what secondary compounds are causing the LFE activity in these completely different plants species.

All the plant materials tested in the present study showed clear in vitro exsheathment inhibition activity against *H. contortus* L_3 (Fig. 2). Best EC_{50} against L_3 was observed for the extracts of *S. gaumeri* and *M. bahamensis* ($P < 0.05$) (Table 5). The use of PVPP showed that the in vitro exsheathment inhibition of *H. contortus* can be associated with the polyphenol content in some tested plants (i.e., *A. pennatula* and *G. floribundum*), while for other plants there was a weak association with polyphenols (*A. collinsii*, *B. swartziana*, *L. latisiliquum*, and *M. bahamensis*) or no association with polyphenols (*H. albicans*, *L. leucocephala*, *P. piscipula*, and *S. gaumeri*) (Fig. 3). The lack of association between polyphenols and in vitro exsheathment inhibition was already reported for other plant materials and by-products (Ortiz-Ocampo et al. 2016; Hernández-Bolio et al. 2018a). It is still unknown what other secondary compounds are responsible for the exsheathment inhibition activity against *H. contortus* under in vitro conditions, but recent studies suggest that the in vitro AH activity is at least partially caused by flavonoid monomers (Klongsiriwet et al. 2015). Furthermore, metabolomics analyses performed on polyphenol-free *L. latisiliquum* extracts suggested that the in vitro activity was associated with glycosylated metabolites of high polarity (quercitrin and arbutin) (Hernández-Bolio et al. 2018b).

How viable is the use of the in vitro nutraceutical evaluation protocol?

The present in vitro protocol for the nutraceutical evaluation of plant species is proposed to help in the selection of plant candidates that should continue towards the in vivo nutraceutical evaluation or further bio-guided assays to identify secondary compounds with AH activity. It is important to realize that the AH activity observed for some plant species under in vitro conditions do not always translate into in vivo AH effects. Furthermore, the AH effect may also depend on the animal species. For example, the consumption of pellets containing sainfoin did not reduce fecal egg counts, worm burdens, or female worm fecundity in rabbits (Legendre et al. 2017) or horses (Collas et al. 2018), while the same type of pellets led to in vivo AH activity in sheep (Gaudin et al. 2016).

The present in vitro protocol is suitable to screen plants in those regions of the world where there is no previous information on the nutritional value or AH activity of local plant species. However, in the present study, it was possible to generate new information for Mexican plant species that already had some information on AH activity from previous studies. For example, this protocol produced the first EC_{50} values against *H. contortus* eggs in nine of the ten plant species evaluated, with the mechanism of action involved in that activity. It also generated the EC_{50} values for six plant species using the exsheathment inhibition assay. According to the present protocol, the first plant species that should have been evaluated under in vivo conditions is *S. gaumeri* and not those plants with high-CT content. However, the results of the present study confirmed that the plants already evaluated under in vivo conditions in México (Galicia-Aguilar et al. 2012; Martínez-Ortíz-de-Montellano et al. 2010; Méndez-Ortíz et al. 2012) were likely to produce nutraceutical effects. Those authors reported in vivo AH effects such as the reduction in the fecal GIN egg excretion or the reduction in the size and fecundity of female adult worms.

To implement the proposed protocol in other latitudes of the world, researchers should first identify the plant species that are consumed by small ruminants in the local vegetation. Botanists with the relevant expertise should confirm the identification of plant specimens and store them with its voucher number. Then, fresh leaves of the plants should be collected to produce the extract of interest. Although most nutrition laboratories worldwide should be able to determine the nutritional information for the nutraceutical evaluation, the research groups should implement in vitro assays to evaluate the AH activity against eggs, L_3, and if possible adult worms when that methodology is technically feasible. Due to phenotypic variation between GIN strains (Chan-Pérez et al. 2016, 2017) it is recommended to use local strains produced in donor animals with mono-specific GIN infections. The staff involved in this protocol should have strong technical expertise with donor animal management, production of parasite stages, and in vitro assays.

Conclusion

The proposed in vitro protocol showed to be useful for the integral assessment of the nutraceutical potential of different plant species as it included the nutritional value and the AH activity against eggs and L_3 in the selected plant species. This protocol helped to rank different plant species for the respective nutritional value as well as the activity against eggs or L_3. Furthermore, the protocol helped to confirm that polyphenols were not the sole compounds responsible for the in vitro AH activities.

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Compliance with ethical standards

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

Ethics statement The authors assert that all procedures contributing to this work complied with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals (license no. CB CCBA D 281 2014 003).

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