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Article

Variability in polyphenol content, biological and anthelmintic activity of methanol:water extracts from the leaves of *Gymnopodium floribundum* Rolfe

Guadalupe Isabel Ortíz-Ocampo [a]

Carlos Alfredo Sandoval-Castro [a]

Gabriela Mancilla-Montelongo [b]

Gloria Sarahi Castañeda-Ramírez [a]

José Israel Chan Pérez [a]

Concepción Capetillo Leal [a]

Juan Felipe de Jesús Torres-Acosta [a]*

[a] Universidad Autónoma de Yucatán, Facultad de Medicina Veterinaria y Zootecnia. Km 15.5 Carretera Mérida-Xmatkuil, 97315, Mérida, Yucatán, México.

[b] CONACYT–Universidad Autónoma de Yucatán, Facultad de Medicina Veterinaria y Zootecnia. Km 15.5 Carretera Mérida-Xmatkuil, 97315, Mérida,Yucatán, México.

*Corresponding author: tacosta@correo.uady.mx

Abstract:

The effect of the harvest month and age of the leaves of *Gymnopodium floribundum* on the content of polyphenolic compounds (total phenols (TP), total tannins (TT) and condensed tannins (CT)) of methanol:water extracts was determined. In addition, the biological activity of polyphenols measured as the ability to precipitate protein (PP), inhibit egg hatching (EH), and larval exsheathment (LEI) of *Haemonchus contortus* was determined. *G. floribundum* leaves were harvested in 4 mo of the year: December, March, June and September. Twenty-

four methanol:water extracts (70:30) were obtained, 12 produced from leaves of varied age (VA) and 12 from 90-d-old leaves (A90). All extracts caused similar PP regardless of age and harvest month. EH inhibition was only significant for December VA extract (EC_{50} = 374.4 μg/mL; $P<0.05$). A90 leaf extracts showed a EC_{50} > 1,500 μg/mL in December, June and September. Although all extracts inhibited larval exsheathment (LEI), the lowest EC_{50} was that of the VA leaf extract of June (EC_{50} = 80.4 μg/mL; $P<0.05$). Incubation of extracts with polyvinylpolypyrrolidone (PVPP) limited LEI ($P<0.05$), but polyphenols only explained part of that activity. In conclusion, the CT content of *G. floribundum* leaf extracts depends on their age and harvest month. Polyphenols showed PP activity and were partially associated with LEI. However, polyphenols do not explain the activity against *H. contortus* eggs.

Key words: Polyphenol, Anthelmintic, *Haemonchus contortus*, Protein Precipitation, Extracts, Tannins.

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Introduction

Sheep and goats that browse in the tropical deciduous forest (TDF) of Yucatan consume variable amounts of foliage from a wide variety of tannin-rich plant species[1]. One of the most consumed species is *Gymnopodium floribundum*, which is a low-sized tree abundant in the TDF and has been studied for its content of secondary compounds (SC)[2]. Among SCs reported for *G. floribundum* are the volatile compounds (*E*)-ocimene, 2-ethyl-1-hexanol and linalool present in its flowers[3]. The leaves of this species contain other important SCs such as polyphenols, i.e. total phenols (TP), total tannins (TT) and condensed tannins (CT)[1,2]. Polyphenols may be involved in the defense of plants against infections by phytopathogenic bacteria and fungi and also limit the consumption of the leaves by vertebrate and invertebrate herbivores[4,5,6]. The latter could be related to the astringent properties of polyphenols. The capacity of polyphenols to limit leaf consumption by herbivores has also been described for small ruminants that graze in some ecosystems, causing low animal productivity[7]. However, this effect of reducing consumption has not been found in small ruminants that browse in the TDF[8]. On the contrary, sheep and goats that browse in the TDF seek to consume the foliage of different species of plants with high CT content possibly as a strategy to block excess nitrogen in their diet, favoring a better balance of nitrogen and energy, and reducing the need

to eliminate nitrogen in the urine[2]. This is because polyphenols have the capacity to precipitate protein (PP) in the diet[9,10]. PP is the property of polyphenols to form complexes with proteins and other macromolecules that have carbonyl and amino groups, forming hydrogen bonds with macromolecules susceptible to autooxidation to form covalent bonds[5]. It is unknown whether the PP activity of polyphenols varies throughout the year in *G. floribundum* leaves.

On the other hand, recent studies have shown that extracts from the foliage of *G. floribundum* have anthelmintic (AH) activity *in vitro* against eggs and larvae of *H. contortus*[11,12], and polyphenols have been shown to be involved in such activity[12]. *In vitro* AH activity was recently confirmed in *in vivo* studies using *G. floribundum* foliage in the diet of lambs infected with *H. contortus*[13]. The latter allowed considering *G. floribundum* leaves as a food with nutraceutical potential that could be used in the control of gastrointestinal nematodes (GIN). However, variability in polyphenol content has been reported in the leaves of polyphenol-rich forage trees of the TDF, such as *Acacia pennatula*, *Lysiloma latisiliquum* and *Psicidia piscipula*[14]. Likewise, *G. floribundum* leaves show variation in their polyphenol content, being greater in the rainy season (33.8 %), period of rapid leaf growth, and lower in the dry season (9.5 %), when the trees lose their foliage[2,13]. Recently, an annual study on *G. floribundum* leaves confirmed that the leaf age and the harvest month affect their bromatological composition and polyphenol content[15]. The above suggests that it is essential to study the variability of the content of bioactive compounds in plants to make rational use of these resources as nutraceuticals[6,16,17].

So far there are no studies that identify the variability in the content of polyphenols and their biological activity in tropical trees. This study determined the effect of the harvest month and age of *G. floribundum* leaves on the content of polyphenolic compounds of methanol:water extracts and the biological activity of polyphenols measured as their capacity to precipitate protein (PP), inhibit egg hatching and inhibit larval exsheathment of *H. contortus*.

Material and methods

Place of collection of *Gymnopodium floribundum* material

The study was carried out in the period between December 18, 2017 and December 21, 2018. It was performed in an experimental area of TDF of 12,000 m^2 (50 x 240 m) located in the Faculty of Veterinary Medicine and Zootechnics of the Autonomous University of Yucatan, Mexico (20°51'93.2" N and 89°37'11" W, at 10 m asl). The experimental area has an AW_0

climate (warm subhumid with rains in summer). The soil type is classified as cambisol and luvisol. The average maximum temperature was 32 °C and the minimum 16 °C with an annual rainfall ranging from 984.4 mm to 1,092 mm, distributed from June to November[18].

Collection and production of extracts from *Gymnopodium floribundum* leaves

Vegetative material was harvested by hand quarterly on the following dates: (a) December 18-21, 2017 and 2018, (b) March 18-21, 2018, (c) June 18-21, 2018, and (d) September 18-21, 2018. Three composite samples were formed in the different harvest months. Each sample was formed with all the leaves of four trees. Samples of leaves of varied age (VA) included the leaves of specimens not previously defoliated. Samples of 90-d-old leaves (A90) were obtained from the same specimens at 90 d postharvest. The fresh leaves of each sample were added methanol:water (70:30 v/v) and homogenized with a blender (Oster®, Mexico) for < 1 min, until a homogeneous particle size was achieved. Ascorbic acid was added to the mixture, and it was left to macerate for 24 h. Subsequently, the mixture was filtered using gauze and No. 50 large pore filter paper (Tequimec SDRL, Mexico). To obtain the extract, the solvent (methanol) was evaporated at 50 °C using reduced pressure (rotavapor Ika®, Germany). Chlorophyll and lipids were removed from the aqueous fraction using methylene chloride (1:1 v/v, 3-7 washes). Finally, the rest of the fraction was lyophilized, bottled and kept in refrigeration at 4 °C until use.

Determination of polyphenols in extracts

The quantity of total phenols (TP), total tannins (TT) and condensed tannins (CT) of each extract obtained, of each age and harvest month, was quantified. The Folin-Ciocalteu technique was used to determine TPs[19]. The TT content was determined using the Folin-Ciocalteu technique + PVPP[19]. The CT content of the extracts was determined by the vanillin test[20].

Production of *Haemonchus contortus* eggs and larvae

The eggs and infective larvae (L_3) of *H. contortus* were obtained from donor animals artificially infected with *H. contortus* (Paraíso isolate, Yucatán, Mexico). Fresh eggs were

collected from the feces of each donor animal. The donors' feces were collected directly from their rectum, using new plastic bags and the feces were processed within 3 hours after collection. Approximately 10 g of feces were macerated in 100 ml of purified water. The suspension was filtered with gauze. The filtered material was centrifuged (168 xg/5 min/21 °C) using 15 ml conical tubes. The supernatant was discarded, and the sediment was mixed with a saturated solution made from commercial cane sugar (relative density 1.28). Once mixed, the sediment was homogenized by a vortex. The suspension was centrifuged (168 x*g*/5min/21 °C). The surface layer of the solution was recovered with an inoculation loop. The eggs were washed three times with purified water to remove the remaining sugar and were resuspended in 15 ml tubes containing 10 ml of phosphate saline solution (PBS 0.01 M: NaCl 0.138 M, KCl 0.0027 M, KH_2PO_4 0.001M, Na_2HPO_4 0.0081M; pH 7.4; Sigma® USA). Egg concentration was determined and the suspension was diluted to 150 eggs/ml of PBS for use in the egg hatch (EH) test[12].

For the larval exsheathment inhibition (LEI) assay, feces were collected from the donor animals and rinsed in a strainer with running water to remove grass or other debris. The feces were placed in Petri dishes (15 cm in diameter), incubated for 5 d at 28 °C and hydrated daily manually with a water spray. L_3 larvae were harvested using Baermann's technique and stored at 4 °C until use. The age of the larvae used in LEI was between 2 and 5 wk[12,21].

In vitro anthelmintic activity against *Haemonchus contortus* eggs

Stock solutions (10,000 µg/ml of PBS) were prepared for each extract tested. PBS was used as a negative control. Respective 0.5 ml aliquots of the different dilutions (3,600, 2,400, 1,200, 600, 300 and 150 µg/mL of PBS) were prepared from the stock solution of each plant extract in 24-wells plates. Point five milliliters of the egg suspension (150 eggs/mL) were added to each well until a final volume of 1 mL was achieved. Six replicates were used for each extract concentration. The multi-well plates were incubated at 28 °C (48 h). At the end of this process, two drops of Lugol were added to each well to stop hatching, in addition to staining the eggs and larvae[22,23].

The non-larvated eggs, larvated eggs and L_1 larvae of each well were counted, and the percentage of hatching was calculated with the formula:

Egg hatch %= (100) (L_1 larvae) / (larvated eggs + eggs + L_1 larvae)

To determine the role of polyphenols in the AH effect of extracts, a tannin inhibitor, polyvinylpolypyrrolidone (PVPP), was used[11,19]. These bioassays included only the

concentration of 3,600 µg of extract / ml of PBS (with and without PVPP) and their respective PBS controls[24].

Haemonchus contortus larval exsheathment inhibition (LEI) test

One thousand microliters of L_3 suspension (~1,000 /ml) were added to each tube to obtain the final extract concentrations (1,200, 600, 400, 200, 100, 30 µg/ml) from the respective stock solutions of *G. floribundum*. A tube containing 1,000 µl of PBS without extract was used as a negative control. The larvae were incubated for 3 h (24 °C). Aliquots of each larval suspension were placed in microvials (200 µl in each.) with four repetitions for each concentration and PBS control. The exsheathment of L_3 was artificially induced with a solution of hypochlorite (2.2 %) and sodium hydroxide (0.7 %) (Clorox®) diluted to 1/300, 1/343, 1/400 and 1/480. The kinetics of the exsheathment was estimated by counting sheathed and unsheathed larvae with a microscope (10x), and the exsheathment was recorded at 0, 20, 40 and 60 min[23]. The percentage of L_3 larval exsheathment for each measurement point was calculated using the following formula:

Exsheathment (%) = (100) (total L_3 without sheath) / (L_3 with sheath + L_3 without sheath)

To determine the role of polyphenols in the AH effect of extracts, a tannin inhibitor, PVPP[12,19], was used. For each extract, only the dose of 1200 µg/ml of PBS (with and without PVPP) and their respective PBS controls were included.

Protein precipitation using the radial diffusion technique

The PP was determined as an indicator of the biological activity of polyphenols. It was performed with the radial diffusion assay[25]. The technique identifies the ability of polyphenols to bind to protein molecules (e.g., bovine hemoglobin) on a plate with agar. One percent agarose gel (Baker®, Germany) was prepared in acetate and bovine hemoglobin buffer (Sigma®, Germany) (100 mg/L agar). The pH was adjusted to 5.0 with NaOH. Ten milliliters of agar were placed in Petri dishes 10 cm in diameter. Five wells of 4 mm in diameter each were formed in the agar of each Petri dish (one in the center and the remaining four in the positions of 0, 90, 180 and 270 degrees). In the latter, 15 µl of a solution of each extract were added and incubated for 48 h at 25 ºC. At the end of that time, the halo that was formed around each well was measured. This halo is the result of the precipitation of hemoglobin by the action of the polyphenols of each extract. PP was weighted by the

concentration of TT, TP and CT contained in each extract evaluated. For this, the formula described by Hagerman[25] was used:

PP= (($D2^2$-$D1^2$) /T); where: D1: smaller diameter of the well (mm); D2: larger diameter (mm); T: Total phenols or total tannins or condensed tannins (mg).

Data processing and statistical analyses

The effect of leaf age (VA or A90) and harvest month, as well as their interaction on polyphenol composition (TP, TT, CT) were determined using respective generalized linear models (GLM). Subsequently, the comparison of means was performed using Tukey's test with $\alpha<0.05$[26].

For the EH test, the number of eggs that remained in the morula stage (MOE), eggs that developed a larva but did not hatch (LNH), and the number of larvae that emerged from the eggs as a result of their exposure to different extracts at the respective concentration previously described were recorded. This information was used to determine egg hatching rate (%EH) and egg hatching inhibition (%EHI) as follows [24,27]:

$$\%EH = \frac{\text{Number of larvae}}{\text{number of morulated eggs} + \text{eggs with larva} + \text{number of larvae}} \times 100$$

$$\%EHI = 100 - \%EH$$

The percentage of morulated eggs that did not form larvae (ovicidal effect) was calculated as follows:

$$\%MOE = \frac{\text{Number of morulated eggs}}{\text{number of morulated eggs} + \text{eggs with larva} + \text{number of larvae}} \times 100$$

The percentage of eggs with larva that did not hatch (%LNH) was calculated as follows:

$$\%LNH = \frac{\text{Number of eggs that contain larvae}}{\text{number of morulated eggs} + \text{eggs with larva} + \text{number of larvae}} \times 100$$

The percentage of exsheathment (%E) and that of exsheathment inhibition (%LEI) were determined with the following formulae[28]:

$$\%E = \frac{L_3 \text{ Larvae with sheath}}{\text{larvae with sheath} + \text{larvae without sheath}} \times 100$$

$$\%LEI = 100 - \%E$$

EH inhibition and LEI results obtained for the different extracts were analyzed with the respective generalized linear models (GLM) to evaluate the differences between the PBS control and the different extract concentrations analyzed. Data obtained from PVPP incubations of each extract were analyzed using a completely randomized design (GLM with comparisons made with the respective control group for each extract)[26].

The effective concentration required to inhibit 50 % of egg hatching, or 50 % of L_3 exsheathment (effective concentration 50 %; EC_{50}) was estimated with data obtained from EH and LEI tests, respectively, for each plant extract tested using PoloPlus 1.0 software[29].

The Shapiro-Wilk test was performed to assess the normality of the PP, EH and LEI data. The respective biological activity (PP, EH and LEI) was analyzed by means of a GLM and the main effects of leaf age (VA and A90) and harvest month (four harvest months), as well as their interaction. The comparison of means was performed using Tukey's test with $\alpha<0.05$. Additionally, respective Pearson correlations were performed to determine the association between the content of polyphenols (TP, TT and CT) and PP, as well as the EC_{50} of EH and LEI, respectively[26].

Results

Table 1 shows the content of TP, TT and CT, in the extracts of the composite samples of *G. floribundum* leaves of different ages. The content of TP and TT was not modified by the harvest month, age or interaction ($P>0.05$). However, significant differences in the CT content due to the interaction between leaf age and harvest month were found, as can be seen for the March (dry) and June (rainfall) extracts of VA leaves ($P<0.05$). Likewise, in June (rainfall), a higher CT content was observed in the VA leaves than in the A90 leaves ($P<0.05$).

Table 1: Effect of leaf age and harvest month on the polyphenol content in methanol:water extracts from *Gymnopodium floribundum* leaves

	Total phenols (%)	Total tannins (%)	Condensed tannins (%)*
Varied age (VA) leaf extracts			
December	19.3[a]	2.9[a]	65.9[ab]
March	20.2[a]	4.4[a]	48.7[b]
June	28.2[a]	6.7[a]	131.7[a]
September	26.1[a]	10.4[a]	106.9[ab]
90-d-old (A90) leaf extracts			
December	20.0[a]	5.4[a]	69.5[ab]
March	20.8[a]	9.0[a]	65.9[ab]
June	24.5[a]	6.4[a]	49.8[b]
September	27.6[a]	12.9[a]	99.2[ab]
Standard error	1.83	2.80	13.50

[a,b] Different literals in the same column indicate differences at $P<0.05$.
*: Equivalent to catechin.

Egg hatching (EH) test

The extract from VA leaves harvested in December was the only one that showed activity on the EH of *H. contortus* (EC_{50} = 374.4 µg/ml). In Table 2, it can be seen that A90 leaf extracts of *G. floribundum* of December, June and September showed low activity on EH (EC_{50} > 1,500 µg/ml), while for the extract of March, it was not possible to calculate the EC_{50}.

Table 2: Effect of leaf age and harvest month on effective concentration (EC_{50}) and confidence interval of methanol:water extracts from *Gymnopodium floribundum* leaves on the hatching of *Haemonchus contortus* eggs

	EC_{50} (µg/ml)	95%CI (µg/ml)
Varied age (VA) leaf extracts		
December	374.4[a]	282.08 - 473.66
March	No activity	-
June	No activity	-
September	No activity	-
90-day-old (A90) leaf extracts		
December	3088.3[b]	2262.45 - 4192.55
March	No activity	-
June	1907.5[b]	1783.75 - 2029.55
September	1575.0[b]	981.26 - 2395.96

[a,b] Different literals in the same column indicate differences at $P<0.05$.

Table 3 shows the effect of blocking polyphenols with PVPP on the proportion of MOE, LNH and L_1 of eggs incubated with the different *G. floribundum* extracts. The different extracts showed an activity more oriented to retain the L_1 larvae inside the eggs (LNH). However, by blocking polyphenols, increased ovicidal activity was observed for VA leaf extracts (December and March). Correlation analyses showed no association between the content of TP, TT or CT, and the EC_{50} of egg hatching inhibition (P>0.05).

Table 3: Effect of incubation of *Haemonchus contorrtus* eggs in different extracts of *Gymnopodium floribundum* at the concentration of 3,600 µg/ml, with and without polyvinylpolypyrrolidone (PVPP), on the proportion (%) of eggs that remained in the morula stage (MOE), larvae that did not hatch from their eggs (LNH) and larvae (L_1)

	Life stage	**PBS (%)**	**3,600 µg/ml (%)**	**3,600 µg/ml + PVPP (%)**	**Standard error**
Varied age (VA) leaf extracts					
December	MOE	5.14[a]	8.60[b]	12.97[a]	4.40
	LNH	1.60[a]	66.69[b]	86.33[c]	2.65
	L_1	93.25[a]	24.71[b]	0.70[c]	2.52
March	MOE	4.91[a]	7.72[a]	13.67[a]	3.56
	LNH	0.62[a]	28.60[b]	82.80[c]	0.79
	L_1	94.46[a]	63.69[b]	3.54[c]	3.97
June	MOE	2.35[a]	4.62[a]	29.27[a]	8.13
	LNH	1.22[a]	22.25[a]	33.37[b]	8.41
	L_1	96.42[a]	73.13[a]	35.37[b]	8.12
September	MOE	7.37[a]	9.86[a]	10.16[a]	1.11
	LNH	0.14[a]	25.11[b]	26.58[b]	0.94
	L_1	92.50[a]	65.03[b]	63.27[b]	1.69
90-day-old (A90) leaf extracts					
March	MOE	7.37[a]	9.86[b]	10.16[c]	0.77
	LNH	0.14[a]	25.11[b]	26.58[c]	0.82
	L_1	92.50[a]	65.03[b]	63.27[b]	1.37
June	MOE	0.37[a]	3.48[b]	41.09[c]	0.77
	LNH	9.21[a]	25.83[b]	83.55[c]	2.46
	L_1	90.42[a]	70.69[b]	2.36[c]	3.11
September	MOE	11.66[a]	15.91[a]	15.28[a]	3.14
	LNH	0.14[a]	37.61[b]	34.11[b]	0.90
	L_1	88.20[a]	46.47[b]	50.61[b]	1.90
December	MOE	10.95[a]	19.52[b]	11.74[a]	2.02
	LNH	0.48[a]	33.13[b]	34.85[b]	0.96
	L_1	88.57[a]	47.35[b]	53.41[c]	2.06

[abc] Different letters in the same row indicate significant differences between groups PBS, extract and extract+PVPP (P<0.05).

Larval exsheathment inhibition (LEI) test

The EC_{50} obtained for the different extracts of *G. floribundum* from VA and A90 leaves with the LEI test is presented in Table 4. A significant effect of the interaction between leaf age and harvest month was observed. In the case of VA leaf extracts, all harvest months showed different activity, with the extract of June being the most active and that of March being the least active ($P<0.05$). On the other hand, the extracts from A90 leaves were also different for each month ($P<0.05$), with that of September being the most active and that of June being the least active.

Table 4: Effect of leaf age and harvest month on effective concentration (EC_{50}) and confidence interval of *Gymnopodium floribundum* leaf extracts on the exsheathment of *Haemonchus contortus* L_3

	EC_{50} (µg/ml)	**95%CI (µg/ml)**
Varied age (VA) leaf extracts		
December	199.9^{ef}	136.67 - 279.12
March	283.5^{gh}	207.27 - 382.01
June	80.4^{a}	55.83 - 104.55
September	146.1^{bc}	119.93 - 175.37
90-day-old (A90) leaf extracts		
December	168.3^{de}	134.10 - 205.21
March	146.1^{cd}	119.93 - 175.37
June	263.6^{fg}	245.33 - 281.28
September	108.4^{ab}	81.41 - 139.02

^{abcdefgh} Different letters in the same column indicate a significant difference ($P<0.05$).

Table 5 presents the effect of extracts of *G. floribundum* from leaves of different age and harvest month on the LEI percentages of *H. contortus* L_3, with or without the addition of PVPP to block polyphenols. The use of PVPP showed that inhibition of exsheathment is partially due to polyphenols and makes it evident that other SCs participate in LEI. In addition, correlation analyses showed no association between TP, TT or CT contents, and the EC_{50} of LEI.

Table 5: Effect of incubation of *Haemonchus contortus* L_3 in different methanol:water extracts of *Gymnopodium floribundum* with and without polyvinylpolypyrrolidone (PVPP) on the percentage of exsheathment inhibition

	PBS (%)	1,200 µg/ml (%)	1,200 µg/ml+ PVPP (%)	Standard error
Varied age (VA) leaf extracts				
December	0.2[a]	100.0[b]	60.0[c]	6.75
March	0.0[a]	100.0[b]	65.5[c]	9.94
June	3.4[a]	100.0[b]	45.7[a]	20.80
September	2.9[a]	100.0[b]	79.3[b]	13.25
90-day-old (A90) leaf extracts				
December	2.1[a]	92.0[b]	36.6[a]	9.24
March	3.1 [a]	100.0[b]	53.5[b]	11.18
June	0.3[a]	100.0[b]	76.1[c]	3.34
September	0.4[a]	100.0[b]	86.7[b]	2.51

[abc] Different letters in the same column indicate a significant difference ($P<0.05$).

Radial diffusion test to measure protein precipitation (PP)

The PP obtained with *G. floribundum* leaf extracts showed no difference due to leaf age or harvest month (Table 6). Correlation analysis showed that a higher content of TP, TT or CT in the extracts did not influence PP.

Table 6: Effect of the age and harvest month of *Gymnopodium floribundum* leaves on protein precipitation (PP) measured by the radial diffusion method and its relationship with the content of total phenols (TP), total tannins (TT) and condensed tannins (CT)

	PP-TP (mm/mg)	PP-TT (mm/mg)	PP-CT (mm/mg)
Varied age (VA) leaf extracts			
December	9.29[a]	64.65[a]	2.75[a]
March	9.23[a]	43.15[a]	4.38[a]
June	8.29[a]	34.96[a]	1.87[a]
September	9.37[a]	40.69[a]	2.29[a]
90-day-old (A90) leaf extracts			
December	10.05[a]	42.65[a]	2.09[a]
March	11.95[a]	40.69[a]	3.79[a]
June	7.93[a]	38.14[a]	4.68[a]
September	9.57[a]	28.34[a]	2.68[a]
Standard error	1.03	11.05	0.63

[a] Values in columns with the same literal do not differ significantly $P>0.1$

Discussion

Composition of polyphenols in the *Gymnopodium floribundum* extracts

The values reported in the present study for TP and TT are similar to those previously reported for methanol:water and acetone:water extracts made with leaves of the same plant species[11,12]. An interesting aspect of the TP and TT content is that they remained relatively constant for the different extracts regardless of the leaf age or harvest month. In the case of TPs, this could be because the plant needs a constant amount of these compounds since they are intermediaries of different biosynthetic pathways of the plant[30]. In the case of TTs, which are more complex compounds, the similarity in their content could be due to the fact that they are affected by variables other than the two evaluated in the present study.

As for the CT content, there is only one previous study of an extract of *G. floribundum* made with VA leaves obtained in the dry season[11] and in this, a value similar to that of VA leaves of March of the present study was reported. However, this study showed that there are differences in CT content due to the interaction between leaf age and harvest month. The variation in the CT content of *G. floribundum* leaves due to the harvest month had already been previously suggested[1,13,31]. The difference in CT content was only evident between the VA leaves of March (drought month) and June (rainy month), and of the latter with respect to the A90 leaves of June. The higher CT content in the VA leaves of June could be due the fact that plants use CTs as a tool to defend themselves against fungi and bacteria that proliferate in the rain. On the other hand, A90 leaves did not have a higher CT content, compared to VA leaves. This could be because the trees from which the A90 leaves were harvested were completely defoliated 90 days earlier. Therefore, the A90 leaves, which were growing, perhaps, could not invest more plant resources in producing defense substances.

Anthelmintic activity of methanol:water extracts

Egg hatching (EH) inhibition test

Extract from VA leaves of December significantly inhibited the hatching of *H. contortus* eggs and that inhibition was achieved at an EC_{50} lower than that previously reported for the same type of VA leaf extract[11]. On the other hand, three of the A90 leaf extracts (June, September, and December) inhibited EH, although these extracts had an EC_{50} higher than

that reported for VA leaves in this study and the previous report[11]. With the exception of the VA leaf extract of March, the zero or low activity on EH of methanol:water extracts of *G. floribundum* is similar to that reported for other polyphenol-rich plants and it has been suggested that this low activity against eggs is due to the SCs obtained using methanol or acetone as organic solvents[11,24,27]. The present study also confirmed that the activity against *H. contortus* egss in methanol:water extracts of *G. floribundum* manifests itself as the presence of larvae trapped inside the eggs (LNH), as had been reported[12,24,27]. Likewise, the use of PVPP confirmed that polyphenols do not explain the EH inhibition activity, but that the blocking of polyphenols increased the effect of LNH in the months of December, March and June for VA leaf extracts ($P<0.05$), and in the months of March and June for A90 leaf extracts ($P<0.05$).

Haemonchus contortus larval exsheathment inhibition (LEI) test

All *G. floribundum* leaf extracts inhibited the exsheathment of *H. contortus* L_3. These results coincide with previous studies that used extracts of *G. floribundum*, either methanol:water[11] or acetone:water[12,13]. The best EC_{50} was observed for the VA leaf extract of June ($P<0.05$), which in turn was the extract with the highest concentration of CT. This increased AH activity coincides with the time when *G. floribundum* begins its highest leaf production (rainy season)[15]. As mentioned, in the rainy season, the plant could use the CTs of its leaves to defend itself against the attack of insects, fungi and bacteria[4]. The high CT content in the rainy months could also limit the attack of vertebrate herbivores such as ruminants, since recent studies show that small ruminants consume less *G. floribundum* foliage in the rainy season compared to the dry season[1,2]. Coincidentally, it is in the dry season when *G. floribundum* leaves contain less CT[15].

G. floribundum extracts decreased their LEI activity when polyphenols were blocked with PVPP ($P<0.05$). However, PVPP only partially blocked the LEI activity of the extracts. This could be due to two phenomena: (a) not enough PVPP was used to block all polyphenols in the solution, and (b) there are other SCs that are partially responsible for LEI activity. Either of the two phenomena could explain the absence of correlation between LEI activity and TP, TT and CT contents. This suggests that increasing doses of PVPP should be explored when performing the LEI test to confirm that the dose used does block most or all polyphenols. On the other hand, it would be necessary to explore what other SCs could help explain the activity of LEI not associated with polyphenols. This would require a bio-guided fractionation process. This type of process has made it possible to identify the activity of chromenone[32] and phenolic acid derivatives (caffeic, coumaric) on the inhibition of the hatching of GIN eggs of ruminants[33,34].

Protein precipitation assay

It was observed that all extracts precipitated the hemoglobin protein. This corroborates the PP activity that has been reported for other polyphenol-rich forage trees in Yucatan[14]. However, these authors determined that the acetone:water extract of *A. pennatula* had a strong association between TP and PP. In the case of *G. floribundum*, no correlation between polyphenol content and PP was found. This could represent an opportunity for future studies to help select individuals that give rise to plant varieties with different polyphenol content or with different biological activity of PP. It is necessary to identify which additional factors influence the expression of polyphenols in the leaves or their capacity for PP. In this study, it was confirmed that *G. floribundum* extracts precipitate proteins regardless of the harvest month, age of leaves or their polyphenol content. Therefore, sheep and goats could take advantage of the biological activity (PP) of *G. floribundum* leaves as part of a strategy to survive in an environment where protein-rich plants (legumes) predominate. This is consistent with the hypothesis that sheep and goats could consume the foliage of *G. floribundum* to block some of the protein in the diet and help reduce the pathway of elimination of nitrogen in the urine, which is very costly for the animal[2,8].

Since the extracts showed good PP measured with hemoglobin, it is suggested to evaluate this PP activity using other proteins that may have closer relationship with the AH activity against *H. contortus*. For example, proteins could be obtained directly from *H. contortus* L_3 (with or without sheath) since these stages of life would be in contact with polyphenols in the gastrointestinal tract. This contact with polyphenols occurs from the moment they enter the ruminant's mouth and remains in contact along the esophagus, reticulum-rumen, omasum and abomasum, until they invade the abomasal mucosa to settle and pass to L_4. The PP could also be evaluated using protein of *H. contortus* eggs, as these are in contact with polyphenols throughout the entire transit from their exit from the uterus of the female worm, through the abomasum, small and large intestines until reaching the feces. These assessments could serve as a model for studying the parasite-host-plant interaction.

Conclusions and implications

There are differences in CT composition associated with the interaction between leaf age and harvest month in methanol:water extracts from *G. floribundum* leaves. Egg hatching inhibition activity was evident only in the VA leaf extract of December, and three A90 leaf extracts exhibited activity at high concentrations. All extracts showed L_3 larval exsheathment inhibition activity, with the VA leaf extract of June having the best activity. The polyphenols

of the extracts showed PP activity and were associated with the inhibition of the larval exsheathment of *H. contortus*. However, they do not explain the activity against *H. contortus* eggs. The main implication of the present work was to demonstrate for the first time that the TP and TT of *G. floribundum* leaf extracts are not significantly modified by the age of the leaves and harvest month, while CTs do vary. In addition, the biological activity of polyphenols was shown to be strong for PP, partial for LEI, and independent of EH. This information serves as a basis for decision-making regarding the application of *G. floribundum* leaves in ruminant nutrition and for the evaluation of nutraceutical potential against *H. contortus*. The variability found also indicates that there is potential for the selection of individuals of this species that are oriented towards a greater or lesser content or activity of CT.

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Conflict of interest

The authors declare that they have no conflict of interest with the publication of this study.

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