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Research Article

Anthelmintic Activity of *Petiveria alliacea*, *Bursera simaruba* y *Casearia corymbosa* Collected in Two Seasons on *Ancylostoma caninum*, *Haemonchus placei* and Cyathostomins

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Abstract

The aim of the study was to evaluate methanolic extracts from the stem and leaves of *Petiveria alliacea*, bark of *Bursera simaruba* and *Casearia corymbosa* collected in two seasons on eggs of *Ancylostoma caninum*, *Haemonchus placei* and cyathostomin. The egg hatch inhibition assay was used at concentrations of 3600, 2400, 1200, 600 and 300 µg/ml. The extracts with high activity were also evaluated at 300, 150, 75 and 37.5 µg/ml. Lethal concentrations were determined at 50% (LC_{50}) and 99% (LC_{99}), as well as the confidence intervals at 95%. Differences ($p < 0.05$) between control and evaluated concentrations were analyzed. The *P. alliacea* extract collected in the rainy season (CRS) showed a percentage of egg hatch inhibition (PEHI) ≥ 91.6% from 150 µg/ml, and ovicidal effect (≥ 90.1%) from 150 µg/ml with stem and 300 µg/ml with leaves in both parameters on the three genera of gastrointestinal nematodes evaluated. The *B. simaruba* extract CRS (3600 µg/ml) showed a PEHI of 95.4, 25.4 and 56.3% against *A. caninum, H. placei* and cyathostomins, respectively. While the *C. corymbosa* extract at the same season and concentration had the highest PEHI of 55.1, 74.0 and 56.4% against the three nematodes, respectively. The effect of *B. simaruba* and *C. corymbosa* on the eggs was the failure of the L_1 larvae to hatch (23.7 - 95.1% and 30.4 - 60.8%, respectively, at 3600 µg/ml). Additionally, it was observed that *C. corymbosa* extract caused morphological damage to the larvae that hatched (100% from 1200 µg/ml). Extracts from the stem of *P. alliacea* CRS showed the lowest LC_{50} (33.3, 78.9 y 68.6 µg/ml) and LC_{99} (79.5, 178.0 and 277.4 µg/ml) against *A. caninum, H. placei* and cyathostomins, respectively. It is concluded that the methanolic extracts of *P. alliacea, B. simaruba* and *C. corymbosa* collected in rainy season showed the highest anthelmintic activity on eggs of *A. caninum, H. placei* and cyathostomins. The stem of *P. alliacea* CRS has high ovicidal activity on the three nematodes, representing a potential alternative control with a broad spectrum against the main nematodes of domestic animals.

Keywords: Control Alternatives; Gastrointestinal Nematodes; Methanolic Extracts; *Petiveria alliacea; Bursera simaruba; Casearia corymbosa*

Abbreviations

CRS: Collected in the Rainy Season; CDS: Collected in the Dry Season; LC_{50}: Lethal Concentration at 50%; LC_{99}: Lethal Concentration at 99%; GIN: Gastrointestinal Nematodes; WAAVP: World Association for the Advancement of Veterinary Parasitology; PBS: Phosphate Buffer Solution; PEHI: Percentage of Egg Hatch Inhibition; OA: Ovicidal Activity; LFE L_1: Larvae Failing Eclosion

Introduction

In tropical and subtropical regions, gastrointestinal nematodes (GIN) are the main parasites that cause health problems in domestic animals as well as economic losses to production and because of their zoonotic potential, they have a negative impact on public health. Among the GIN with the highest prevalence and high pathogenicity worldwide are *Ancylostoma caninum* in dogs, *Haemonchus placei* in cattle and cyathostomins in horses [1-3].

Due to the importance of GINs and their negative impact, programs have been designed to prevent and control them, and a large amount of money has been invested in the purchase of anthelmintics. However, traditional prevention and control programs have been based on frequent and irrational administration of these chemicals, which has favored the selection of GIN populations that are resistant to most of them [4,5]. Worldwide, there have been reports of anthelmintic resistance of *Ancylostoma* spp. to benzimidazoles, pyrantel and ivermectin [6,7]. In the case of *Haemonchus* spp. reports indicate resistance to salicylanilides, benzimidazoles, imidazothiazole and ivermectin [8-10], while the current situation of resistance by cyathostomins includes benzimidazoles, pyrimidines and macrocyclic lactones [11-13].

Due to the lack of effectiveness of commercial anthelmintics and residues in animal products for human consumption, new control alternatives have been employed. Certain plant extracts have been shown to possess anthelmintic activity by reducing fecal egg count and affecting viability. This has motivated the exploration and search for plants containing secondary metabolites with anthelmintic properties against GIN [14]. In southeastern Mexico, preliminary studies have been conducted to evaluate the anthelmintic potential of *Petiveria alliacea, Bursera simaruba* and *Casearia corymbosa* for nematode control. Arjona-Cambranes., *et al.* [15] (abstract of a congress) observed that *P. alliacea* extract had a percentage of egg hatch inhibition (PEHI) above 90% in *A. caninum* eggs (concentration of 600 µg/ml), while the *B. simaruba* and *C. corymbosa* extracts reported a PEHI of 9.1% and 34.5% (concentration of 3600 µg/ml), respectively. On the other hand, Flota-Burgos., *et al.* [17] evaluated the efficacy of an extract of *P. alliacea* (300 µg/ml) on cyathostomin eggs, finding a PEHI > 90%.

Most studies of extracts against GIN challenge a single genus of nematode, leaving it unclear whether such extracts possess broad anthelmintic spectrum against two or more GIN genera.

Aim of the Study

The aim of the present study was to evaluate the anthelmintic activity of the methanolic extract of *P. alliacea*, *B. simaruba* and *C. corymbosa* on eggs of *A. caninum*, *H. placei* and cyathostomins affecting canines, cattle and horses, respectively.

Materials and Methods

Place of study

This study was carried out at the Laboratory of Parasitology (Diagnostic Unit) of the Faculty of Veterinary Medicine and Zootechny, Universidad Autónoma de Yucatán, located in Yucatan, Mexico (19°30′, 21°35′ N; 87°30′, 90°24′ W). The state of Yucatan is characterized by a predominantly warm sub-humid climate (Aw), with an average annual temperature of 26°C and an annual rainfall of 1,000 mm. There are mainly two seasons: dry (November-May) and rainy (June-October) [18].

Methanolic plant extracts

The evaluation was carried out on extracts of stem and leaves of *P. alliacea*, bark of *B. simaruba* and bark of *C. corymbosa*, which were collected during the dry season (March-April) and the rainy season (August-September). Once the plant material was collected, it was separated according to the plant and structures mentioned above and placed in a drying oven at 40°C for two days. The dry material was then ground with the aid of an electric mill to reduce particle size to 5 mm. Two extractions were performed, with a duration of 24h each, using methanol (MeOH) as solvent at a rate of 30 ml per 25g of ground material. At the end of each extraction period, the solvent of the ground material was decanted through a filter paper and deposited in glass flasks. The concentration of the extract was achieved by removing the solvent at reduced pressure in a rotary evaporator (Rotavapor Büichi®). The methanolic extracts were stored in glass vials (4°C) until they were used [19].

Collection of nematode eggs

Feces were collected from canines, cattle and horses naturally infected with *A. caninum*, *H. placei* and cyathostomins, respectively. Stool cultures were performed to identify morphologically the genera of L_3 larvae from each animal donor. Prior to sample processing, each sample was centrifugally floated to ensure that the eggs were in the morula phase and that larval formation had not yet begun [20]. The feces were macerated with purified water and the mixture was filtered through non-sterile gauze. The liquid obtained was placed in 45 ml plastic tubes and centrifuged at 1500 rpm for

5 minutes. Afterwards, the supernatant was discarded, and a sugar saturated solution was added (density: 1.280). With the help of a vortex, the mixture was homogenized and centrifuged again. The eggs from the surface portion were collected with an inoculation loop and suspended in a phosphate buffer solution (PBS). Three washings with PBS were performed to remove any traces of the saturated sugar solution that might have been present in the suspension. The concentration of the recovered eggs per milliliter was determined and the suspension was diluted to a solution of 400 eggs/ml [17,21].

Egg hatch inhibition assay

The egg hatch inhibition assay was performed following the guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAVP) [4]. Concentrations of 3600, 2400, 1200, 600 and 300 µg/ml were evaluated, and extracts showing ≥ 90% of anthelmintic activity at concentrations of 600 and 300 µg/ml were evaluated at lower concentrations (150, 75 and 37.5 µg/ml). Three repetitions were made for each concentration. A mixture of 95% PBS + 5% absolute ethanol was used as solvent. This mixture was used as a negative control and did not affect the hatching of the eggs (≥ 90%). Thiabendazole (0.1 µg/ml) was used as a positive control. An ultrasonic bath (Branson®) was used to optimize the dilution of the extracts at the different concentrations mentioned. In the culture plates of 24 wells, 0.5 ml of the egg solution (approximately 200 eggs) and 0.5 ml of the diluted extract were deposited in each well (total volume of each well: 1.0 ml). The culture plates were incubated at 28°C for 48h. After the incubation time, two drops of lugol were added to stop the hatching process. Only those plates whose negative controls obtained a hatching percentage equal to or greater than 80% were analyzed. The contents of each well were deposited in McMaster cameras, examined with a microscope (10×) to count the morulated eggs (ovicidal activity), eggs containing a larva (larvae failing eclosion) and L_1 larvae [17].

Results analysis

Extract activity and effect

Hatching rate and egg hatch inhibition were calculated with the following formula [17,22]:

- % hatch: (larvae L_1/morulated eggs + eggs containing larvae + larvae L_1) x 100.
- % egg hatch inhibition: 100 - % hatch.

The formulas proposed by Vargas-Magaña., *et al*. [20] were employed to calculate the percentage of eggs that failed to form larvae (ovicidal activity; %OA) and the percentage of L_1 larvae failing eclosion (%LFE):

- % OA: (morulated eggs/morulated eggs+ eggs containing a larva+ L_1 larvae) × 100
- % LFE: (eggs containing a larva/morulated eggs + eggs containing a larva + L_1 larvae) × 100

Statistical analysis

An analysis of variance (generalized linear models) was used to identify statistically significant differences between controls, evaluated concentrations and collection seasons (Statgraphics 5.1).

Lethal concentrations at 50% (LC_{50}), 99% (LC_{99}) and their 95% confidence intervals were determined using concentration-response analysis (Probit Analysis) [23].

Results and Discussion

Several studies have reported on the anthelmintic activity of plants against GIN; most of them address this potential by focusing on one genus only. The use of plant extracts as an alternative to control GIN should consider the search for plants with a broad antiparasitic spectrum. In this study, the extracts collected in the rainy season (CRS) of the three plants evaluated showed greater anthelmintic activity against *A. caninum, H. placei* and cyathostomins. The extracts of *P. alliacea* demonstrated the highest anthelmintic activity against the three different nematode genera.

Petiveria alliacea extracts

Since *P. alliacea* extracts showed percentages of inhibition > 90% at concentrations of 600 and 300 µg/ml, they were evaluated at concentrations of 150, 75 and 37.5 µg/ml (Table 1). The highest PEHI of *P. alliacea* were obtained from the CRS extracts. When each of the plant parts were evaluated, it was found that the stem showed higher anthelmintic activity at a lower concentration ($p < 0.05$) than the leaves, independently of the nematode genus evaluated. The stem extract of *P. alliacea* CRS showed a PEHI >91% from the concentration of 150 µg/ml (98.4%, 91.6% and 94.6%, against eggs of *A. caninum*, *H. placei* and cyathostomins, respectively), while the extract belonging to the leaves CRS reached a similar PEHI with double the concentration (99.2%, 94.3% and 94.0% at 300 µg/ml, respectively). With respect to *A. caninum*, it was observed that the stem and leaf extracts of *P. alliacea* CRS showed a PEHI >97% at 75

µg/ml and 150 µg/ml, respectively. This represents half of the concentration required to obtain a similar PEHI against *H. placei* and cyathostomin eggs (150 µg/ml for stem and 300 µg/ml for leaf). Using the analysis of variance, it was determined that the PEHI (from 75 µg/ml against *A. caninum* eggs and 150 µg/ml against *H. placei* and cyathostomin eggs) of the *P. alliacea* stem extracts CRS showed no statistical difference ($p > 0.05$) against the PEHI of the positive control (Thiabendazole), whereas with the leaf extract, this statistical similarity was observed using twice the concentration (150 µg/ml against *A. caninum*. eggs and 300 µg/ml against *H. placei* and cyathostomin eggs).

Structure	Concentration (µg/ml)	*Ancylostoma caninum*		*Haemonchus placei*		Cyathostomins	
		Dry	Rainy	Dry	Rainy	Dry	Rainy
Leaf	Negative control	0.1 (0.9)^{a}	2.2 (0.6)^{a}	3.2 (0.9)^{a}	3.9 (2.3)^{a}	6.8 (2.7)^{a}	6.0 (3.6)^{a}
	Positive control	98.1 (1.4)*^{b}	98.2 (1.5)*^{b}	97.2 (3.1)*^{b}	98.1 (1.7)*^{b}	98.6 (0.9)*^{b}	98.3 (0.7)*^{b}
	600	98.9 (0.3)*^{b}	99.8 (0.2)*^{b}	96.7 (2.2)*^{b}	94.9 (2.1)*^{b}	98.6 (0.8)*^{b}	100.0 (0.0)*^{b}
	300	91.7 (0.6)*^{c}	99.2 (0.3)*^{b}	93.1 (2.1)*^{b}	94.3 (1.8)*^{b}	86.5 (5.2)*^{c}	94.0 (2.6)*^{b}
	150	88.7 (1.5)*^{d}	98.9 (0.5)*^{b}	88.1 (4.4)*^{c}	65.5 (3.0)*^{c}	84.3 (3.0)*^{d}	88.0 (0.4)*^{c}
	75	27.6 (1.2)*^{e}	38.9 (0.8)*^{c}	32.3 (6.0)*^{d}	17.9 (6.3)*^{d}	62.0 (7.9)*^{e}	63.5 (3.5)*^{d}
	37.5	14.4 (0.9)*^{f}	18.2 (0.8)*^{d}	5.2 (1.4)^{e}	8.4 (0.6)^{e}	31.7 (8.2)*^{f}	44.2 (2.2)*^{e}
Stem	Negative control	4.1 (0.7)^{a}	2.2 (2.3)^{a}	0.8 (0.6)^{a}	2.2 (0.8)^{a}	5.8 (4.8)^{a}	4.3 (3.1)^{a}
	Positive Control	98.2 (1.5)*^{b}	98.6 (1.2)*^{b}	99.4 (0.5)*^{b}	97.8 (1.0)*^{b}	98.9 (1.1)*^{b}	98.5 (0.4)*^{b}
	600	99.6 (0.3)*^{b}	99.3 (0.5)*^{b}	97.8 (0.7)*^{b}	94.5 (0.3)*^{b}	98.7 (1.4)*^{b}	97.7 (0.2)*^{b}
	300	99.6 (0.2)*^{b}	99.0 (0.7)*^{b}	97.0 (1.0)*^{b}	91.9 (6.1)*^{b}	98.2 (0.7)*^{b}	97.6 (2.1)*^{b}
	150	97.8 (0.9)*^{b}	98.4 (0.7)*^{b}	77.2 (3.0)*^{c}	91.6 (4.7)*^{b}	70.7 (3.1)*^{c}	94.6 (0.9)*^{b}
	75	17.5 (0.5)*^{c}	97.1 (2.3)*^{b}	34.0 (1.1)*^{d}	70.6 (3.0)*^{c}	27.9 (0.5)*^{d}	56.8 (1.9)*^{c}
	37.5	4.5 (1.1)^{d}	40.4 (5.5)*^{c}	2.1 (0.6)^{e}	17.6 (3.7)*^{d}	20.0 (8.2)*^{e}	36.4 (5.1)*^{d}

Table 1: Mean and standard deviation (±) of the percentage of egg hatch inhibition obtained with *Petiveria alliacea* extracts on eggs of *Ancylostoma caninum, Haemonchus placei* and cyathostomins.

* Significant difference with the negative control.

Different letters between columns mean statistical difference ($p < 0.05$).

The extract of *P. alliacea* showed ovicidal activity on the eggs treated (Figure 1), regardless of plant structure, season of collection and nematode genus. The highest ovicidal activity percentage (OA%) was obtained from the stem extract CRS, on average a OA% of 91.4% was obtained against *A. caninum*. eggs at 75 µg/ml. Regarding *H. placei* and cyathostomins, the OA% was 90.3% and 90.1% at 150 µg/ml, respectively (Table 2). On the other hand, the lowest LC_{50} and LC_{99} were obtained from the extracts CRS compared to the extracts collected in the dry season (CDS) ($p < 0.05$) (Table 3). The lowest LC_{50} and LC_{99} (µg/ml) were obtained from the stem extract CRS compared to leaf extract. When evaluating the stem extract of *P. alliacea* against *A. caninum* eggs, a significant statistical difference ($p < 0.05$) was found between LC_{50} and LC_{99} CRS (33.3 and 79.5 µg/ml) and the extract CDS (91.3 and 186.3 µg/ml, respectively). In the case of *H. placei* no significant difference was observed between the seasons of collection of the *P. alliacea* stem. However, the LC_{50} obtained from CRS (78.9 µg/ml) was lower compared to the CDS (92.3 µg/ml). The LC_{50} of the stem CRS obtained for cyathostomins was statistically different with respect to the CDS. However, in LC_{99} no significant difference was found between the two collection seasons.

Structure	Concentration (µg/ml)	*Ancylostoma caninum*		*Haemonchus placei*		Cyathostomins	
		Dry	Rainy	Dry	Rainy	Dry	Rainy
Leaf	Negative control	0.0 (0.0)	1.5 (0.6)	1.5 (1.3)	2.2 (1.1)	5.5 (0.8)	5.8 (3.5)
	Positive control	96.9 (1.9)	96.6 (3.1)	95.5 (4.0)	96.9 (0.4)	97.4 (2.6)	97.8 (0.7)
	600	97.3 (0.4)	95.1 (4.4)	92.4 (1.4)	93.8 (0.8)	98.3 (0.8)	99.2 (0.6)
	300	84.4 (4.4)	98.1 (1.5)	89.7 (1.9)	94.1 (1.7)	82.8 (7.5)	90.3 (3.1)
	150	54.4 (1.7)	93.1 (0.5)	84.4 (5.1)	61.3 (3.5)	82.1 (5.2)	87.7 (0.8)
	75	24.2 (1.9)	33.9 (1.7)	28.8 (7.2)	16.8 (6.3)	58.1 (8.6)	58.9 (6.1)
	37.5	9.6 (2.4)	17.0 (0.9)	4.3 (2.0)	7.4 (0.2)	27.4 (7.7)	36.62 (4.4)
Stem	Negative control	2.9 (1.3)	0.9 (1.5)	0.4 (0.1)	1.4 (0.2)	3.0 (2.1)	3.9 (3.5)
	Positive control	98.2 (1.5)	98.6 (1.2)	99.2 (0.7)	97.5 (1.5)	98.1 (2.3)	97.6 (1.4)
	600	98.3 (0.3)	96.1 (1.1)	97.3 (1.7)	93.9 (1.3)	98.7 (1.4)	97.7 (0.2)
	300	98.6 (0.5)	96.9 (2.5)	95.1 (0.9)	90.0 (7.1)	97.4 (0.5)	97.6 (2.1)
	150	95.4 (2.1)	90.5 (3.0)	74.1 (1.1)	90.3 (4.3)	64.8 (3.6)	90.1 (0.2)
	75	16.4 (1.5)	91.4 (1.3)	28.6 (0.9)	59.3 (3.4)	26.2 (0.7)	48.1 (6.6)
	37.5	2.2 (0.5)	35.1 (4.7)	1.9 (0.8)	12.2 (2.8)	18.9 (6.7)	32.9 (4.5)

Table 2: Mean and standard deviation (±) of the percentage of ovicidal activity obtained with *Petiveria alliacea* extracts on eggs of *Ancylostoma caninum, Haemonchus placei* and cyathostomins.

Extract	Seasons	*Ancylostoma caninum*		*Haemonchus placei*		Cyathostomins	
		LC 50	LC 99	LC 50	LC 99	LC 50	LC 99
Stem of *P. alliacea*	Rainy	33.3^{a} (18.8-41.3)	79.5^{a} (70.0-98.2)	78.9^{a} (66.8-93.4)	178.0^{a} (149.9-229.3)	68.6^{a} (31.8-96.8)	277.4^{a} (219.9-402.7)
	Dry	91.3^{b} (70.3-104.4)	186.3^{b} (160.8-246.0)	92.3^{ab} (85.8-98.9)	159.9^{ab} (148.8-174.1)	113.6^{b} (98.3-129.1)	301.8^{ab} (265.2-357.6)
Leaf of *P. alliacea*	Rainy	85.6^{bc} (80.9-90.1)	154.6^{bc} (145.8-165.9)	138.3^{c} (122.7-158.3)	321.4^{c} (277.6-390.8)	73.1^{ac} (64.1-81.5)	230.2^{bc} (212.3-253.0)
	Dry	97.8^{bd} (91.0-105.1)	207.0^{bd} (189.3-231.1)	129.1^{bcd} (94.1-180.8)	308.1^{cd} (236.0-488.6)	89.8^{abcd} (47.8 -158.6)	475.1^{bd} (339.8-909.2)
Bark of *B. simaruba*	Rainy	2027.5^{e} (1775.1-2371.8)	4961.4^{e} (4223.8-6140.9)	4524.5^{e} (3718.5-8279.7)	9268.5^{e} (6633.9-23858.0)	3138.4^{e} (2715.1-3725.2)	6639.0^{e} (5578.4-8555.5)
	Dry	3023.6^{f} (2662.2-3843.4)	5293.7^{ef} (4735.1-6177.3)	ND	ND	$3,201.3^{ef}$ (2555.1-4319.6)	7317.7^{ef} (5687.6-11198.0)
Bark of *C. corymbosa*	Rainy	3541.4^{fg} (2924.1-4372.1)	$12,670.0^{g}$ (10268.0-17080.0)	1627.8^{f} (1321.5-2075.5)	4634.3^{f} (3772.2-6155.7)	5191.7^{fg} (4306.0-6080.6)	12032.0^{fg} (10031-15658.0)
	Dry	4442.6^{fgh} (3458.9-6232.4)	$13,880.0^{gh}$ (10464.0-21978.0)	2536.0^{g} (2114.5-3083.4)	6095.8^{fg} (5109.1-7736.5)	4656.1^{fgh} (4262.9-5337.9)	9557.5^{fgh} (8045.1-12585.0)

Table 3: Lethal concentrations at 50% and 99% (µg/ml) and 95% confidence intervals obtained with extracts of *Petiveria alliacea, Bursera simaruba* and *Casearia corymbosa* on eggs of *Ancylostoma caninum, Haemonchus placei* and cyathostomins.

Different letters between columns mean statistical difference ($p < 0.05$).

LC50: lethal concentration at 50%; LC99: lethal concentration at 99%. ND: Not determined

Figure 1: Effect of stem extract from *Petiveria alliacea* (collected in the rainy season) on nematode eggs: (A) negative control, (B) positive control, (C) *Ancylostoma caninum*, (D) *Haemonchus placei*. and (E) cyathostomins.

The species *P. alliacea* has antimicrobial, analgesic, anti-inflammatory, anticancer, antifungal, ixodicidal and antiparasitic properties [24,25]. The anthelmintic activity of this plant has been evaluated, individually, in previous studies against *Ancylostoma* spp., *Haemonchus* spp. and *Trichostrongylus* spp. reporting activity at higher concentrations (300 to 3600 µg/ml) and no ovicidal effect [15-17]. Due to this fact, the technique used in the present study was modified to ensure the complete dilution of the methanolic extracts evaluated and to enhance their efficacy. This modification consisted in the addition of absolute ethanol at 5% to the extract's solvent and the use of an ultrasonic bath. Additional measures can be used when the extracts are not homogeneously dissolved, ethanol is often used to help break down the thermodynamics of the extract components [26-28].

Arjona-Cambranes., *et al*. [15] evaluated the methanolic extract of the stem and leaves of *P. alliacea* CRS and CDS, against *Ancylostom*a spp. eggs at concentrations from 3600 to 300 µg/ml, and generally, they obtained PEHI higher than 98% at 600 µg/ml. In this study, no significant difference ($p>0.05$) was found between the PEHI obtained (300 µg/ml) on the stem and leaf extracts from the rainy and dry seasons. On the other hand, this study was unable to determine the LC_{50} and LC_{99} due to the high PEHI obtained at the lowest dose (300 µg/ml). It was reported that the predominant effect of *P. alliacea* extract was "L1 larvae failing eclosion", because more than 80% of the eggs treated managed to develop the L_1 larvae inside them, in all the concentrations evaluated. In contrast to the reports of Arjona-Cambranes., *et al*. [15] this study found a significant difference ($p < 0.05$) with the plant parts of *P. alliacea* and the season of collection. The stem CRS obtained the best PEHI (97.8% at 75 µg/ml) against *A. caninum*, four times less concentration was required to obtain the same efficacy as Arjona-Cambranes., *et al*. [15]. Ovicidal effect was also observed from 91.4% to 75 µg/ml in contrast to the reports (larvae failing eclosion) of Arjona-Cambranes., *et al*. [15]. Moreover, the extracts evaluated showed higher anthelmintic activity in contrast to those reported for the ethanolic extract of *Canthium mannii* (PEHI of 90% at 1000 µg/ml) and the ethanolic extracts of *Mikania laevigata, M. glomerata* and *Euterpe edulis* (PEHI of 21.8%, 25.9 and 21.1%, respectively, at 10,000 µg/ml) [26,28]. De Aguiar-Santos., *et al*. [29] evaluated hydroalcoholic extracts from 10 plants with insecticide properties from Brazil; however, they did not show anthelmintic activity against *A. caninum* eggs even at high concentrations (12,500 µg/ml).

Rosado-Aguilar., *et al*. [16] (abstract of a congress) evaluated the effect of the methanolic extract from the stem and leaf of *P. alliacea* CRS and CDS on eggs of *Haemonchus* spp. and *Trichostrongylus* spp. found in cattle. A 100% PEHI at a concentration of 1200 µg/ml was obtained for the stem CRS and the leaves CRS and CDS. No statistical difference was found between the above-mentioned extracts and the LC_{50} and LC_{99} were not reported. The effect reported on the eggs treated was the development of L_1 larvae failing eclosion. In the present study, the stem CRS presented the best PEHI (91.6% at 150 µg/ml), while Rosado-Aguilar., *et al*. [16] required four times the dose to obtain the same efficacy (91.8% at 600 µg/ml). In addition, an ovicidal effect against *H. placei* was observed from 90.3% to 150 µg/ml, unlike prior studies. *H.* spp. has been one of the most studied GIN regarding the use of plant extracts as a control alternative in ruminants; however, there are scarce studies carried out in southeastern Mexico reporting plant extracts with ovicidal effect against this GIN, mainly observing the effect of "L_1 larvae failing eclosion" [21,30,31].

The anthelmintic activity of *P. alliacea* on the eggs of cyathostomins was reported by Flota-Burgos., *et al*. [17], in that study, a PEHI higher than 98% was obtained at 150 µg/ml, while the best PEHI was obtained with the stem extract CRS (97.7% at 75 µg/ml). The results of the present study matched those reported by Flota-Burgos., *et al*. [17] in terms of the extract with the highest PEHI; however, the best PEHI was obtained at 150 µg/ml (94.6%) and at 75 µg/ml the PEHI dropped to 56.8%. Flota-Burgos., *et al*. [17], reported L_1 larvae failing eclosion, contrary to the ovicidal effect of 90.1% at 150 µg/ml observed in the present study. Studies on the anthelmintic activity of plants such as *Acacia baileyana, A. melanoxylon, A. podalyriifolia, Alectryon oleifolius, Duboisia hopwodii, Eucalyptus gomphocephala* and *Santalum spicatum* against these nematodes have reported that a higher concentration (1400 µg/ml) is required to obtain a PEHI of 100% similar to *P. alliacea* [22,32]. In the present study it was observed that *P. alliacea* extract caused an ovicidal effect on treated eggs of the three GIN genera studied. The ovicidal effect refers to the inhibition of larval development, so the treated eggs remain in the morula phase and do not develop into larvae L_1. This effect could be attributed to a more homogeneous dilution of the *P. alliacea* extract with the addition of 5% of absolute ethanol and the use of the ultrasonic bath, which probably allowed the secondary compounds responsible for the anthelmintic activity to penetrate in greater proportion the membrane that covers the eggs affecting the development of the morula causing the ovicidal effect not present in previous studies with the same nematodes [17,33]. Chagas [34] mentions that it is important to choose a suitable solvent for the in vitro evaluation of the plant extracts of interest, since an inadequate one may cause toxicity to the GIN eggs under evaluation, generating false positives or masking the true effect of the extract. The effect of a potent extract may be underestimated or ruled out by factors outside the extract itself (poor dilution, inadequate techniques, unsuitable solvents), thus losing a potential source for the development of new control alternatives [35].

The ovicidal effect observed with *P. alliacea* extract is similar to that caused by thiabendazole, therefore, when using this extract, it is likely to obtain efficacies and effects similar to anthelmintics currently available on the market and that these GINs have been reported as resistant [36].

Bursera simaruba extracts

B. simaruba extract CRS showed significant anthelmintic effect on *A. caninum* eggs. (95.4% PEHI at 3600 µg/ml) and showed no significant difference with respect to the positive control ($p>0.05$). On the other hand, the anthelmintic activity (PEHI) of this extract against *H. placei* (25.4% to 3600 µg/ml) and cyathostomins (56.4% to 3600 µg/ml) was low (Table 4). Using the bark extract of *B. simaruba*, it was observed that the LC_{50} and LC_{99} were lower in the collection in the rainy season. Statistically, the LC50 of both collections was found to be different against eggs of *A. caninum*, while no difference was found between the LC_{50} and LC_{99} of both collection seasons against cyathostomin eggs. Due to the low PEHI against *H. placei* eggs obtained from *B. simaruba* extract CDS, the LC_{50} and LC_{99} could not be determined in this study (Table 3). On the other hand, the extracts to *B. simaruba* caused the effect of "L_1 larvae failing eclosion" (LFE) on the eggs treated (Figure 2), regardless of the season of collection and the genus of nematode against which they were evaluated. The highest percentages of LFE were obtained from the extracts CRS compared to CDS. The *B. simaruba* extract CRS showed, on average, 95.1%, 23.7% and 51.4% LFE against *A. caninum, H. placei* and cyathostomins, respectively, at the concentration of 3600 µg/ml.

B. simaruba bark has antifungal, antidiarrheal, anti-inflammatory, analgesic, antipyretic and antispasmodic properties [37]. Other species of the genus *Bursera* have antiparasitic activity against Mexican Leishmania, *Entamoeba histolytica* and infective larvae of *H. contortus* [38-40]. In particular, the anthelmintic activity *of B. simaruba* against nematode eggs has been scarcely studied. Arjona-Cambranes., *et al*. [15] found a PEHI of less than 10% when evaluating the methanolic extract of *B. simaruba* against *A. caninum* (3600 µg/ml), they attributed the low activity to the poor solubility of the extract. Due to the complete dilution of the extract, in the present study it was obtained a PEHI of up to 95.4% and 56.3% at the dose of 3600 µg/ml against *A. caninum* and cyathostomins, respectively. Despite the fact that the nematodes evaluated belong to the order Strongylida, the eggs of the nematodes studied may have structural differences that would explain the different susceptibility to the metabolites present in the extracts studied [41,42]. It was observed that the *B. simaruba* extract presented the effect of L_1 that failed eclosion in the three evaluated GIN genera, coinciding with the reports of a study conducted against *A. caninum* [15]. This effect suggests that the metabolites responsible for the activity may interfere with the permeability of the membrane that covers the egg, preventing changes that are necessary for the hatching process, inhibiting the enzymes responsible for hatching or competing with the receptors for hatching factors present in this structure of the egg [20].

Extract	Season	Concentration (µg/ml)	*Ancylostoma caninum*	*Haemonchus placei*	Cyathostomins
Bursera simaruba	Dry	Negative control	1.1 (0.7)[a]	2.4 (1.0)[a]	8.9 (5.3)[a]
		Positive control	98.9 (1.3)*[b]	97.2 (0.8)*[b]	98.9 (0.3)*[b]
		3600	79.8 (7.0)*[c]	2.3 (2.7)[c]	48.1 (2.1)[c]
		2400	21.6 (3.0)*[d]	2.4 (1.3)[d]	39.9 (1.2)[d]
		1200	8.5 (2.1)*[e]	2.9 (1.5)[e]	25.1 (3.0)*[e]
		600	1.3 (0.6)[f]	2.8 (0.6)[f]	17.1 (2.3)*[f]
		300	1.0 (1.1)[g]	2.8 (1.6)[g]	7.1 (1.3)[g]
	Rainy	Negative control	3.1 (1.7)[a]	3.0 (2.7)[a]	8.3 (3.1)[a]
		Positive control	99.7 (0.2)*[b]	95.7 (1.8)*[b]	100.0 (0.0)*[b]
		3600	95.4 (3.7)*[b]	25.4 (3.5)*[c]	56.3 (4.1)*[c]
		2400	44.1 (2.7)*[c]	25.2 (3.7)*[d]	35.7 (7.8)*[d]
		1200	27.0 (5.8)*[d]	2.1 (0.6)[e]	21.4 (0.9)*[e]
		600	15.8 (2.4)*[e]	5.0 (4.5)[f]	7.5 (1.9)[f]
		300	6.4 (2.0)[f]	3.4 (1.1)[g]	3.0 (0.6)[g]

Table 4: Mean and standard deviation (±) of the percentage of egg hatch inhibition obtained with *Bursera simaruba* extracts on eggs of *Ancylostoma caninum, Haemonchus placei* and cyathostomins.

* Significant difference with negative control.

Different letters between columns mean statistical difference ($p < 0.05$).

Figure 2: Effect caused by *Bursera simaruba* on the development of L1 larvae within treated eggs that failed to hatch, (A) *Ancylostoma caninum* (B) *Haemonchus placei* and (C) cyathostomins.

Casearia corymbosa extracts

In the case of *C. corymbosa* extract CRS (Table 5) a moderate anthelmintic activity was obtained on *H. placei* eggs. (74.0% to 3600 µg/ml) compared to the results obtained against the eggs of *A. caninum* and cyathostomins (55.1% and 56.6% at the same concentration, respectively). The effect of *C. corymbosa* extracts was "L_1 larvae failing eclosion" (LFE) on the eggs treated (Figure 3). The extract of *C. corymbosa* of CRS obtained, on average, 54.3%, 60.8% and 30.4% of LFE against *A. caninum, H. placei* and cyathostomins, respectively, at the concentration of 3600 µg/ml. In addition to the effect caused against the eggs, the extract of *C. corymbosa* CRS (1200 µg/ml) caused damage to the internal structures in 100% of the larvae that were able to hatch in comparison with the larvae that emerged from the negative control (Figure 4, 5 and 6). Additionally, no significant difference was found between the LC_{50} and LC_{99} of the different collection seasons when evaluating the bark extract of *C. corymbosa* against eggs of *A. caninum* and cyathostomins. In the evaluation against *H. placei* eggs, a difference was found between the LC_{50} obtained in CRS (1627.8 µg/ml) compared with CDS (2536.0 µg/ml); however, there was no significant difference in the LC_{99} of both collections (Table 3).

Extract	Season	Concentration (µg/ml)	*Ancylostoma caninum*	*Haemonchus placei*	Cyathostomins
Casearia corymbosa	Dry	Negative control	10.0 (1.4)[a]	4.6 (1.2)[a]	8.9 (5.3)[a]
		Positive control	95.8 (2.1)*[b]	100.0 (0.0)*[b]	98.9 (0.3)*[b]
		3600	45.0 (1.3)*[c]	65.4 (1.3)*[c]	48.1 (2.1)[c]
		2400	44.0 (0.6)*[d]	61.6 (4.2)*[d]	45.7 (0.2)[d]
		1200	38.7 (1.7)*[e]	31.7 (5.6)*[e]	28.8 (5.3)*[e]
		600	26.1 (2.6)*[f]	18.9 (6.1)*[f]	22.1 (3.9)*[f]
		300	22.3 (2.0)*[g]	13.0 (3.6)*[g]	20.5 (2.8)*[g]
	Rainy	Negative control	9.2 (1.6)[a]	3.5 (3.0)[a]	8.3 (3.1)[a]
		Positive control	95.3(2.7)*[b]	100.0 (0.0)*[b]	100.0 (0.0)*[b]
		3600	55.1 (0.8)*[c]	74.0 (4.0)*[c]	56.4 (4.5)*[c]
		2400	46.0 (1.3)*[d]	73.7 (6.6)*[d]	35.7 (7.8)*[d]
		1200	42.1 (3.1)*[e]	66.6 (4.0)*[e]	21.4 (0.9)*[e]
		600	31.2 (1.5)*[f]	23.9 (4.4)*[f]	7.5 (1.9)*[f]
		300	28.3 (2.3)*[g]	19.3 (3.6)*[g]	3.0 (0.6)[g]

Table 5: Mean and standard deviation (±) of percentage of egg hatch inhibition obtained with *Casearia corymbosa* extracts on eggs of *Ancylostoma caninum, Haemonchus placei* and cyathostomins.

* Significant difference with negative control.

Different letters between columns mean statistical difference ($p < 0.05$).

Figure 3: Effect caused by *Casearia corymbosa* on the formation of L_1 larvae within treated eggs that failed to hatch (A) *Ancylostoma caninum*, (B) *Haemonchus placei* and (C) cyathostomins.

Figure 4: L_1 larvae of *Ancylostoma caninum*, treated with *C. corymbosa* (40x): (A) control; anterior part (B) and posterior part (C and D) of the larvae with degeneration and vacuolization of the esophagus and intestinal cells.

Figure 5: Effect of *C. corymbosa* on L_1 larvae of *Haemonchus placei* (40x): Negative control larvae (A and B); anterior part (C and D) and posterior (E and F) of larvae with vacuolization of the esophagus and intestinal cells.

Figure 6: Damage caused by *C. corymbosa* in cyathostomin larvae: (A) negative control at 40x; (B and C) degeneration of the esophagus, intestinal cells and cuticle seen at 10x; vacuolization and degeneration of esophagus (D) and intestinal cells (E and F) seen at 40x.

Among the biological properties of *C. corymbosa*, there are reports of anti-cancer, anti-inflammatory, antitussive and antibacterial effects [43,44]. Arjona-Cambranes., *et al*. [15] evaluated the bark extract of *C. corymbosa* collected in the rainy season and obtained a 34.5% (3600 µg/ml) PEHI in *Ancylostoma* spp. eggs, compared to this work, in which it was obtained a PEHI > 50% in the eggs of the three evaluated GINs. The ethanolic extract of *Casearia acuelata* was evaluated against *H. placei* eggs obtaining low PEHI even using higher concentrations than this study: 11.3%, 7.3% and 10.2% at 1000, 10,000 and 50,000 µg/ml, respectively [45]. There is no information about the anthelmintic potential of this plant or others of its genus against cyathostomins; in the present work a moderate anthelmintic effect was observed (56.4%) at lower concentrations than those reported in previous studies with other nematodes. The effect of the *C. corymbosa* extract on the three nematodes was L_1 larvae failing eclosion, an explanation for this has already been described above as for the *B. simaruba* extract.

Despite not having a high anthelmintic effect on the treated eggs, from the concentration of 1200 µg/ml, the extract of *C. corymbosa* caused morphological damage in 100% of L_1 larvae that managed to hatch and that were not observed in larvae that hatched in the negative controls. Degeneration of intestinal cells was observed, similar to those reported in L_1 larvae of *H. contortus* exposed to secondary metabolites present in the extracts of *Acacia pennatula* and *Onobrychis viciifolia*, as well as the presence of numerous vacuoles in the internal structures [21,46].

Secondary metabolites involved in the anthelmintic activity of plant extracts

Secondary metabolites reported in *P. alliacea* include alkaloids, coumarins, saponins, flavonoids, steroids, polyphenols, tannins, triterpenes and sulfur compounds, with different concentrations in the leaves and stem [47,48]. The presence of sulphur compounds stands out as being responsible for their antimicrobial, anti-cancer and anti-fungal activity [49-52]. Arceo-Medina., *et al*. [24] also identified sulphur compounds (dibenzyl disulphide and dibenzyltrisulphide) as responsible for the acaricidal activity of *P. alliacea* against *Rhipicephalus microplus.* Kubec and Musah [47] mention that the concentration of sulphur compounds is approximately four times higher in the stem than in the leaves of the plant. In addition, an essential oil called petiverin, has been reported to be found in higher concentrations in the stem than in the leaf [47,48]. The aforementioned could explain the differences observed in the PEHI and LC of the stem and leaf extracts.

Only the LC_{50} of *B. simaruba* CRS, evaluated against *A. spp*. showed significant difference when compared to the LC_{50} CDS. In the evaluation against *H. placei*. and cyathostomins no differences were found between the LC_{50} and LC_{99} of the different collection seasons. In the genus *Bursera*, the presence of essential oils has been reported as well as triterpenes, steroids, bilignans, lignans, alkaloids, saponins, quinones and flavonoids, particularly in the plant resin [53,54]. Maldini., *et al*. [53] mention that in the bark of *B. simaruba* there are abundant phenolic and lignan-derived compounds, which are related to antimicrobial, fungistatic and insecticidal activity.

On the other hand, only LC_{50} of *C. corymbosa* CRS proved to be statistically significant when evaluated against *H. placei*. Terpenoids, particularly clerodal dipertenoids, are the predominant secondary metabolites in the genus *Casearia*, as well as phenylpropanoids, sesquiterpenoids, flavonoids, steroids and essential oils [55-57]. Antiparasitic activity of the genus *Casearia* against *Trypanosoma cruzi, Leishmania donovani* and *Plasmodium falciparum* has been demonstrated with promising results, attributed to the presence of clerodane-type dipertenoids. It is important to mention that these secondary compounds are significantly present in the bark, so they could be responsible for the observed anthelmintic activity [58-61].

The significant difference in anthelmintic activity (PEHI and LC) observed in this study when comparing extracts CRS versus CDS may be explained by the seasonal variation that influences the amount, composition and expression of the secondary metabolites present in the plants evaluated [62,63]. Several studies have found a higher concentration of secondary metabolites with biological activity in the months corresponding to the rainy season compared to the dry season [64,65]. Kubec and Musah [47] mention that the content of secondary compounds is variable and depends on several factors including the weather and the date of collection. Arceo-Medina., *et al*. [24] reported higher acaricidal activity with *P. alliacea* extracts collected in the dry season, which was different from the results of this study. There are no studies on the seasonal variability of secondary compounds present in *B. simaruba* and *C. corymbosa*.

Extracts from the stem of *P. alliacea* and the *bark of B. simaruba*, CRS, have been shown to possess a broad spectrum of activity against eggs of the three GIN genera studied, so future studies should focus on identifying the secondary metabolites responsible

for their anthelmintic activity. Concerning the *C. corymbosa* bark extract and due to the effect observed on larvae of the evaluated genera, in addition to the identification of secondary active metabolites, it is required to evaluate it with more appropriate tests on larval stages, such as the nematode larval mortality assay, larval migration inhibition test or larval exsheathment inhibition assay [66,67]. Furthermore, *in vivo* studies are needed to establish the pharmacodynamics and pharmacokinetics of the active compounds found in the extracts with anthelmintic activity, as well as the appropriate doses and to ratify their broad spectrum of activity under field conditions.

Conclusion

The methanolic extracts of *P. alliacea, B. simaruba* and *C. corymbosa* collected in rainy season showed the highest anthelmintic activity on eggs of *A. caninum, H. placei* and cyathostomins. The stem of *P. alliacea* CRS has high ovicidal activity on the three nematodes, representing a potential alternative control with a broad spectrum against the main nematodes of domestic animals.

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Conflict of Interest

None.

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