Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2022.075

RESEARCH ARTICLE

Biological Activity of the Secondary Compounds of *Guazuma ulmifolia* Leaves to Inhibit the Eggs *Haemonchus contortus* Hatching

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ARTICLE HISTORY (22-235)

Received:	July 16, 2022
Revised:	October 9, 2022
Accepted:	October 14, 2022
Published online:	November 02, 2022

Key words:
Parasites
Extract
Eggs
Phenols

A B S T R A C T

Epidemiologically *Haemonchus contortus* is the most important parasite in the tropics, females can produce up to ten thousand eggs and blood feed. As objective, it was evaluated the *in vitro* effect of the secondary compounds of *Guazuma ulmifolia* leaves extracted with hydroalcoholic (HA) solvent and by fractionation in liquid-liquid layer with ethyl acetate, to inhibit eggs hatching of *H. contortus*, to interrupt the biological cycle of the parasite. The bioactive compounds were identified by high performance liquid chromatography. Challenged doses were in the HA extract 1.25-40 mg/mL, and aqueous and organic fractions of 1.25-10 mg/mL, respectively, distilled water and 2% methanol were negative controls and thiabendazole (0.1 mg/mL) positive control, for a total of seventeen treatments. The treatments were tested in three replicates with four repetitions, the data were analyzed with a GLM in a completely randomized design using ANOVA to an alpha ≤0.05. The effective concentrations (EC_{50} and EC_{90}) were estimated by Probit analysis in the SAS program. The secondary compounds identified were of the phenols group such as flavonols, flavones, coumaroyl derivatives and hydroxycinnamic acid. The HA extract and aqueous fraction showed a similar effect to the positive control from doses of 2.5 mg/mL and the organic fraction from 1.5 mg/mL ($P<0.01$). In HA extract and aqueous fraction, the EC_{50} and EC_{90} were similar, but in the organic fraction the ovicidal effect was at lower EC_{50} (0.86 mg/mL) and EC_{90} (1.67 mg/mL). In conclusion *G. ulmifolia* leaves contain secondary compounds able for interrupting the cycle of *H. contortus* in the egg stage and the content of phenols like hydroxycinnamic acid and coumaroyl derivatives could be responsible for the ovicidal effect.

To Cite This Article: Velázquez-Antunez J, Olivares-Perez J, Olmedo-Juárez A, Rojas-Hernandez S, Villa-Mancera A, Romero-Rosales T, Zamilpa A, Gonzalez-Cortazar M, 2022. Biological activity of the secondary compounds of *Guazuma ulmifolia* leaves to inhibit the eggs *Haemonchus contortus* hatching. Pak Vet J. http://dx.doi.org/10.29261/pakvetj/2022.075

INTRODUCTION

Parasites are microorganisms that affect the health of domestic animals (De Jesús *et al.,* 2020). *Haemonchus contortus* is a gastroenteric nematode that inhabits the abomasum of small ruminants (Baltrusis *et al.,* 2020). Epidemiologically it is the most important species with 71% prevalence (Olivares *et al.,* 2012) and causes the greatest deaths in animals (Gareh *et al.,* 2021). An adult female lays 5,000 to 10,000 eggs per day, causing rapid contamination of pastures (Laca-Megyesi *et al.,* 2020). *H. contortus* has a direct cycle and its transmission depends on environmental conditions (Olivares *et al.,* 2012; Gareh *et al.,* 2021). It is a hematophagous parasite capable of causing losses between 200 to 600 mL of blood per day in the host, which leads to anemia and hypoproteinemia, inflammation and destruction of the intestinal mucosa, decreased intestinal enzymes secretion, diarrhea and death of animals (Ehsan *et al.,* 2020). In less severe conditions, the course can be chronic with loss of appetite, decreased weight gain and weakness, but in all cases the economic losses are evident (Flay *et al.,* 2022). Chemical

anthelmintics used for control normally trigger resistance in the parasite due to exposure (De Jesús *et al.,* 2018). Resistance to anthelmintic drugs such as macrocyclic lactones, imidazothiazoles, and benzimidazoles have been reported (Baltrusis *et al.,* 2020). A study carried out in sheep and goats showed that there is resistance of *H. contortus* to benzimidazole, levamisole, ivermectin and moxidectin (Gainza *et al.,* 2021; Arsenopoulos *et al.,* 2020). This resistance is a global problem (Laca-Megyesi *et al.,* 2020). In this context, the need arises to develop a rational, sustainable and ecological management in small ruminants for the control of nematodes that can reduce the use of anthelmintics and slow down the development of resistance (De Jesús *et al.,* 2020), the use of extracts of plants and their secondary metabolites represents an alternative. Studies have reported that some secondary compounds of leguminous trees have anthelmintic effects to interrupt the life cycle of parasites (De Jesús *et al.,* 2018). Tannins such as flavonoids, glycosylates, flavones, and lactones bind to the structural proteins of nematodes, and can inhibit egg hatching, development, sheathing and larval motility, making anthelmintic activity evident (Von Son-de Fernex *et al.,* 2016). *Guazuma ulmifolia* has pharmacological activity such as antiulcer, antidepressant, antidiabetic, antioxidant, analgesic, antifungal, anticholistenerase and anti-inflammatory activity, due to its chemical compounds such as flavonoids, alkaloids, glycosylated and saponins (Rafi *et al.,* 2020). Another study demonstrated the impact of the hydroalcoholic extract and the fractions (organic and aqueous) of *G. ulmifolia* against *H. contortus* eggs, when added to 10% of the dry matter of the diet in lambs (Le Bodo *et al.,* 2020). The aim of the study was to evaluate *in vitro* the ovicidal effect of secondary compounds in *G. ulmifolia* leaves against *H. contortus* eggs.

MATERIALS AND METHODS

Vegetal Material: *Guazuma ulmifolia* leaves (3000 g) were collected and dehydrated at 45ºC in the shade until constant weight. The dry material was ground in a Willy mill with a 1 mm screen.

Preparation of the extract with hydroalcoholic solvent and its fractionation: The ground leaves were placed in 5 L Erlenmeyer flasks and a hydroalcoholic solution (70% distilled water: 30% methanol) was added using a mass volume ratio of 1:10, for 48 hours. The hydroalcoholic solution (HA) was filtered through different sieves (gauze, cotton and filter paper). The HA solution free of plant material was concentrated by distillation under reduced pressure in a rotary steamer (BUCHI R-300, Switzerland). It was then brought to total dryness by lyophilization processes (LABCONCO FreeZone -1045C 4.5 L Benchtop, U.S.). A part of the HA extract, (10%) was stored at 4°C and its biological effect against *H. contortus* eggs was evaluated *in vitro*, the rest (90%) was re-suspended in distilled water at a ratio of 1 g of extract in 100 mL of water and fractionated in a liquid-liquid layer with ethyl acetate at a ratio of 1:1 (v/v). From this process, the aqueous (F-Aq) and organic (F-AcOEt) fractions were derived, which were purified in the rotary steamer that eliminated the solvents and the total drying was carried out in the lyophilizer. Like the extract, the fractions were stored at 4°C isolated from light, their biological activity were evaluated to inhibit the hatching of parasite eggs (Olmedo-Juárez *et al.,* 2017).

Collection of *Haemonchus contortus* eggs: Eggs were collected from the feces of two sheep (28 kg live weight) artificially infected with a monospecific INIFAP local strain of *H. contortus* orally at a single dose of 350 L_3/kg live weight. The feces collected directly from the rectum were macerated in clean water, 35 mL of the macerated were deposited in 50 mL falcon tubes, plus 15 mL of saline solution (42%), then the tubes were shaken for one minute and centrifuged at 3500 rpm for 5 min and were filtered through 75 µm and 32 µm sieves adding distilled water until clean eggs were obtained, suspended in a solution of 15 mL of distilled water (Coles *et al.,* 1992). Afterwards, the eggs per milliliter were titrated and the dilutions were prepared with distilled water at concentrations of 100 ± 15 eggs in 50 µL and in this way they were used in the bioassay (Zarza-Albarrán *et al.,* 2020).

Identification of secondary compounds by HPLC analysis: For the identification of secondary compounds in the extract elaborated with hydroalcoholic solvent and the fractions (aqueous and organic) they were analyzed by High Performance Liquid Chromatography (HPLC) equipped with a 2695 water separation module and a 996 photodiode array detector of water and the empower Pro software (Waters Corporation, USA). Chemical separation was performed on a SUPELCOSIL LC-F column (4.6 mm x 250 mm i.d., 5 µm particle size) (Sigma-Aldrich, Bellefonte, USA). In the mobile phase, 0.5% aqueous solution of trifluoroacetic acid (Solvent A) and acetonitrile (Solvent B) were used. Titrated gradient systems were as follows: 0–1 min, 0% B; 2–3 min, 5% B; 4–20 min, 30% B; 21–23 min 50% B; 24–25 min, 80% B; 26–27 min, 100% B and 28–30 min, 0% B. The flow rate was kept at 0.9 mL/min and the injection volume was 10 µL. The absorbance was measured at 330 nm. The compounds obtained from the fractions were identified by comparison of the retention times and UV spectra with the reference standards (Sigma-Aldrich, St Louis Mo, USA) (Wagner and Bladt, 2001).

Egg Hatching Inhibition (EHI) Assay: The ovicidal activity of the secondary compounds of *G. ulmifolia* present in the extract prepared with HA solvent and the aqueous and organic fractions was determined separately through three replicates with four repetitions (n=12) for each concentration dose used. The study was performed *in vitro* in 96-well microtiter plates. In the extract prepared with HA solvent, the concentrations challenged were 1.25, 2.5, 5, 10, 20 and 40 mg/mL, in the aqueous fraction 1.25, 2.5, 5, 10 mg/mL, in the organic fraction 0.65, 1.25, 2.5, 5 and 10 mg/mL and distilled water and 2% methanol were used as negative controls and Thiabendazole (0.1 mg/mL) as positive control. Concentration doses and negative and positive controls were considered as treatments in a completely randomized design. In each replica and repetition (each well of the plate) 50 µL of the treatments and 50 µL of the aqueous suspension with 100 ± 15 parasite eggs were deposited. Subsequently, the plates were

Table 1: Egg hatching inhibition (EHI) of *H. contortus* exposed to secondary compounds extracted from the *G. ulmifolia* leaves with hydroalcoholic solvent

Treatments	Number		%EHI
	Inhibited eggs	Eggs hatched to Larvae	
Distilled water	4.80	86.60	4.72c
Methanol 2%	6.45	89.3	6.51[c]
Thiabendazole (0.1%)	98.35	0.1	99.65[a]
Hydroalcoholic extract (mg/mL)			
40	80.125	0.5	98.85[a]
20	72.50	0.13	99.85[a]
10	105	0	100.0[a]
5	76.88	0.50	99.31[a]
2.5	94.75	0.25	99.77[a]
1.25	14.25	59.75	19.02[b]
Aqueous fraction (FAq, mg/mL)			
10	87	0.5	99.56[a]
5	81.1	2.1	98.09[a]
2.5	53.57	5.71	92.88[a]
1.25	2.57	81.14	2.75[c]
Organic fraction (FAc-OEt, mg/mL)			
10	112.44	2.38	96.72[a]
5	113.81	0.25	99.78[a]
2.5	105.19	1.50	97.70[a]
1.25	115.60	0.20	97.77[a]
Variation coefficient			2.63
R^2			0.98
P-value			<0.01

incubated at room temperature (25-28°C) for 48 h, after the incubation time, the hatching of the eggs was stopped by applying 10 µL of Lugol's solution to each well. Finally, the plate was read to count the total number of eggs and larvae (L_1 or L_2) in an optical microscope (LABOMED, USA) with a 10X objective. The percentage reduction in egg hatching was estimated using the following formula:

$$\text{Egg hatching inhibition (EHI\%)} = \frac{\text{egg number}}{\text{egg number} + \text{larvae}} x100$$

Statistical analysis: EHI percentage data were analyzed by ANOVA in a completely randomized design. The comparison of means between treatments was performed with the Tukey test at a minimum confidence level of 95%. For the treatments that resulted in concentration-dependent effects, the effective concentrations 50 and 90 (EC_{50} and EC_{90}) were estimated using the PROC PROBIT procedure of the SAS statistical package (SAS, 2014).

RESULTS

Results on the of the eggs *Haemonchus contortus* hatching inhibition caused by exposure to secondary compounds extracted from the leaves of *G. ulmifolia* with the hydroalcoholic solvent, as well as secondary compounds derived in the aqueous and organic fractions are presented in Table 1. It is observed that both the secondary compounds extracted by the hydroalcoholic solvent, as well as those partitioned in the aqueous fraction (FAq), showed a similar effect to thiabendazole (positive control) at a concentration dose of 2.5 mg/mL with inhibitions of 99.77 and 92.88% respectively. The secondary compounds partitioned in the organic fraction (FAc-OEt) showed the same effect (97.77% inhibition) from the concentration doses of 1.5 mg/mL.

Fig. 1 shows the effective concentrations (EC) shown by the secondary compounds extracted from the *G. ulmifolia* leaves to inhibit the eggs hatching of *H. contortus*. In the extract elaborated with hydroalcoholic solvent, EC_{50} (1.59 mg/mL) and EC_{90} (2.55 mg/mL) were similar to those expressed by the compounds carried in the aqueous fraction (EC_{50} = 1.80; EC_{90} = 2.93 mg/mL), however, the compounds derived in the organic fraction showed an ovicidal effect at lower EC_{50} (0.86 mg/mL) and EC_{90} (1.67 mg/mL).

The secondary compounds identified in the extracts elaborated from the *G. ulmifolia* leaves are shown in Fig. 2, 3 and 4. In the extract elaborated with a hydroalcoholic solvent (Fig. 2), the main active compounds responsible for inhibiting the fertility of the parasite eggs were, the phenols observed in Fig. 2, peaks 1, 2 and 6 correspond to flavonols, peaks 3 and 5 belong to flavones and finally peaks 4 and 7 correspond to derivatives of hydroxycinnamic acid and coumaroyl derivatives respectively. The compounds derived in the aqueous fraction (Fig. 3) were from the group of phenols such as flavonols (peaks 1 and 2) and, to a lesser extent, those derived from flavones (peak 3) and coumaroyl derivatives (peak 4). In the organic fraction (Fig. 4) the secondary compounds identified were flavonols (peaks 1, 2 and 8), flavones (3, 5 and 7) and derivatives of hydroxycinnamic acid (peaks 4 and 10) and coumaroyl derivatives (peak 9).

DISCUSSION

The results showed that the secondary compounds extracted by the HA solvent as well as those fractionated in water and ethyl acetate showed biological activity to eggs hatching inhibit of the *H. contortus* and thereby break the life cycle of the parasite, a phenomenon that results of epidemiological interest in animal production systems (Olivares *et al.,* 2012). Olmedo *et al.* (2020) also

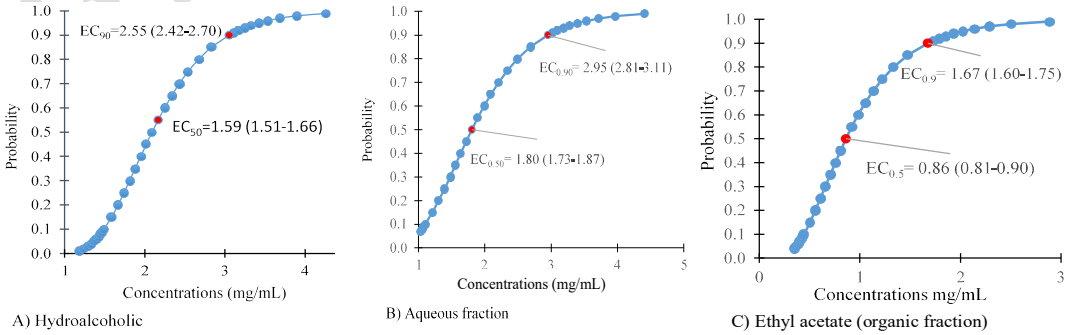

Fig. 1: Effective concentrations (EC) of secondary compounds extracted from *G. ulmifolia* leaves with different solvents.

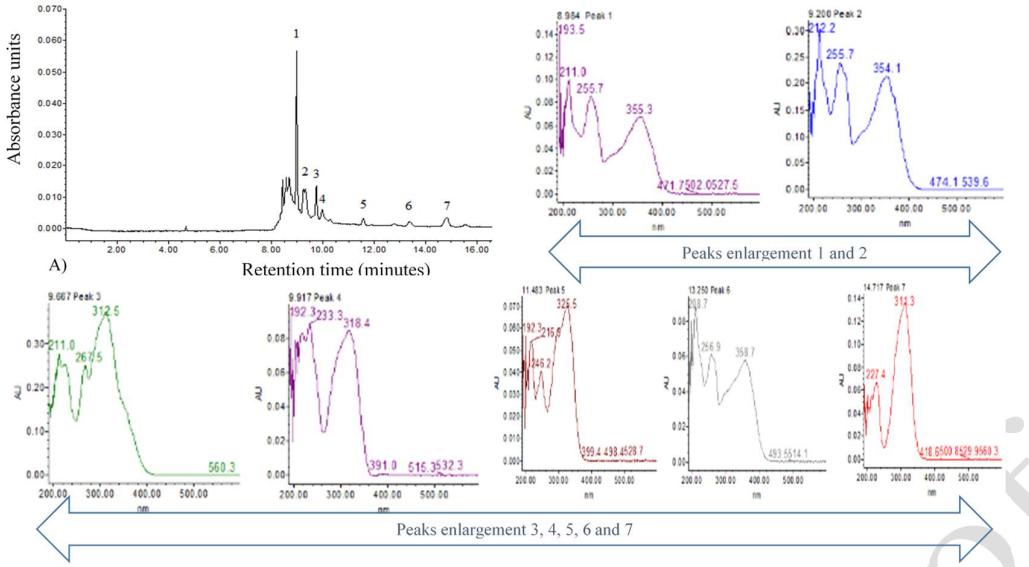

Fig. 2: Secondary compounds (A) identified in the extract of *G. ulmifolia* leaves with hydroalcoholic solvent (HPLC analysis).

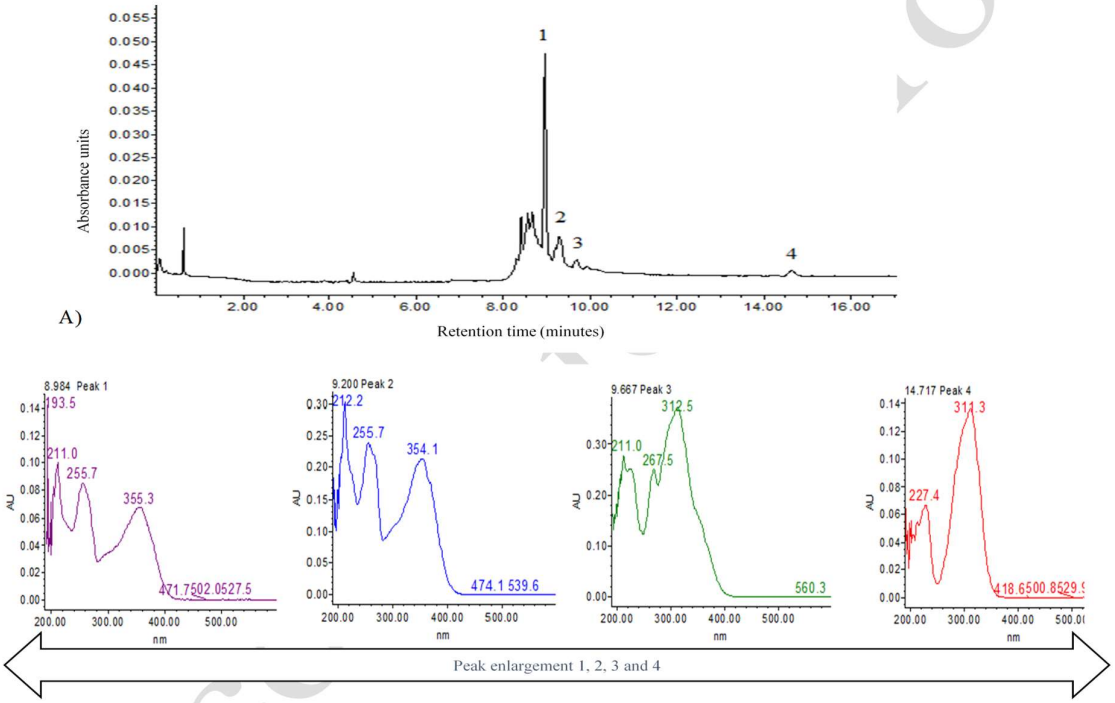

Fig. 3: Secondary compounds (A) identified in aqueous fraction of the extract of *G. ulmifolia* leaves (HPLC analysis).

interrupted the cycle of *H. contortus* in lambs with the oral use of the organic fraction of a hydroalcoholic extract of *Acacia farnesiana* with an effectiveness between 54 and 68%, Castañeda-Ramírez *et al.,* (2020) inhibited the development of *H. contortus* eggs in the morula stage by *in vitro* exposure to a methanolic extract of *Annona* tree, Birhan *et al.,* (2020) demonstrated a similar response against parasite eggs and larvae when exposed to extracts made from *Rhus glutinosa*, *Syzygium guineensa* and *Albizia gumifera*. It was also evident that the effect of the secondary compounds present in the plant extracts were dependent on the concentration doses, with a determination coefficient of 0.98 (Table 1), this explained that the unhatched parasite eggs were attributed in a 98% at the concentration doses of the extracts, results with the same trend were reported by De Jesús *et al.* (2020) and Olmedo *et al.* (2022) with secondary compounds extracted from the *Caesalpinia coriaria* fruits against the same parasite and in the same phase of the life cycle. The HPLC analysis revealed both in the hydroalcoholic extract and in the aqueous and organic fraction, the presence of the same secondary compounds of the phenol group such as flavonols, flavones and coumaroyl and hydroxycinnamic acid derivatives, this was related to the similarity in the EC_{50} and EC_{90} observed between the secondary compounds present in the hydroalcoholic extract and the derivatives in the aqueous fraction (Fig. 1, letters A and B). The EC_{50} and EC_{90} in the secondary compounds derived from the organic

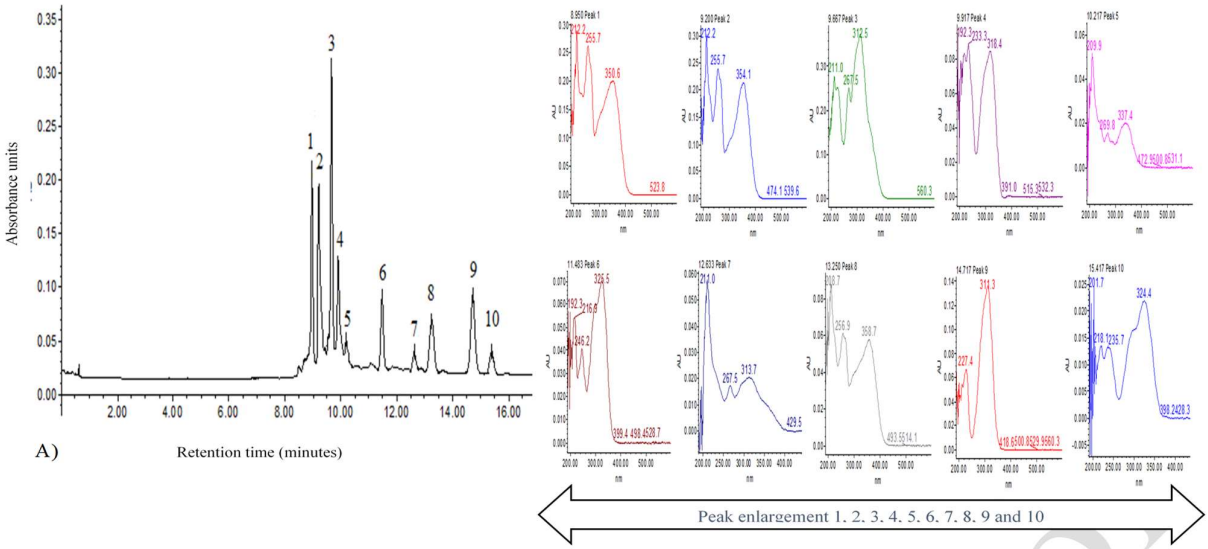

Fig. 4: Secondary compounds (A) identified in the organic fraction of *G. ulmifolia* leaves with ethyl acetate solvent (HPLC analysis).

fraction demonstrate effectiveness at lower doses (Fig. 1, letter C), even when the secondary compounds identified belong to the same group (Fig. 4), these results could indicate that the observed effect is attributed at a higher concentration per unit volume of the extract. Olmedo *et al.* (2022) observed that during the elaboration of the extract a higher concentration of secondary compounds could be derived in response to the solvent used and linearly with the lower EC_{50} and $_{90}$; Al-Rawahi *et al.* (2013) and Alara *et al.* (2021) explained that phenols reacted differently depending on the solvent used. Olmedo *et al.* (2020) concluded in a study with *Acacia farnesiana* that the secondary compounds with the greatest biological activity to inhibit eggs and larvae of *H. contortus* were found in the organic fraction. Ahmed *et al.,* (2020) also reported differences between crude extracts of two plants and their parts on the inhibition of *H. contortus* eggs. In addition, in Fig. 4 of the organic fraction, the HPLC recorded the presence of ten phenolic compounds, compared to four in the aqueous fraction (Fig. 3) and seven in the hydroalcoholic extract (Fig. 2), which indicated a greater synergistic action of the secondary compounds derived in the organic fraction and therefore the lethal concentrations to inhibit the eggs hatching of the parasite were expressed at lower concentration doses, this action was also described by Beltrão *et al.* (2020) in extracts used for the control of hectoparasites , Younoussa *et al.* (2020) in extracts used for insect control and by Escareño-Díaz *et al.* (2019) in combinations between various phenolic compounds such as quercetin, coumaric acid, caffeic acid and rutin, where they reported reductions up to 43 and 64% in the EC_{50} to inhibit the eggs hatching and up to 68 to 83% in the unsheathing inhibition of the L_3 larvae of *Cooperia punctata*. In the mechanism of action, there is evidence that phenols, such as those identified in this study, are a group of secondary compounds that affect the development of the parasite cycle in a similar way to anthelmintics (Muhammad *et al.,* 2020). Escareño-Díaz *et al.* (2019) and Zarza-Albarrán *et al.* (2020) identified that phenolic compounds adhere to the egg shell to inhibit the development of the embryo and to the cuticle of the larvae to break cell integrity and prevent the unsheathing. In this study, the inhibition of eggs hatching of the *H. contortus* could be attributed to the interruption in the development of the embryo inside the parasite egg due to the biological action of the phenolic compounds extracted and identified in the foliage of the plant.

Conclusions: *Guazuma ulmifolia* leaves contain secondary compounds able for interrupting the cycle of *H. contortus* in the egg stage and the content of phenols like hydroxycinnamic acid and coumaroyl derivatives could be responsible for the ovicidal effect. These results justify continuing the investigation on *G. ulmifolia* leaves *in vivo* under controlled conditions to verify the biological activity recorded in the *in vitro* bioassays.

Authors contribution: JVA, JOP, AOJ, ZA and GCM developed the project. SRH, AVM and TRR performed the statistical analyzes on the variable data. All authors reviewed and approved the structure and writing of the manuscript. There is no conflict of interest among authors

Acknowledgments: The authors thank CONACYT as an institution that grants scholarships to postgraduate students and thus allowed the planning and execution of this research project that allowed the formation of high-quality human resources.

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