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Effects of different extracts of three *Annona* species on egg-hatching processes of *Haemonchus contortus*

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Abstract

This study assessed the *in vitro* anthelmintic (AH) activity of methanol and acetone:water leaf extracts from *Annona squamosa*, *A. muricata* and *A. reticulata* against *Haemonchus contortus* eggs. The egg hatch test was used to determine the effective concentrations required to inhibit 50% of eggs hatching (EC_{50}). The role of polyphenols on AH activity was measured through bioassays with and without polyvinylpolypyrrolidone (PVPP). Methanolic extracts mainly caused the death of eggs at the morula stage (ovicidal activity). Meanwhile, acetone:water extracts caused egg-hatching failure of developed larvae (larvae failing eclosion (LFE) activity). The lowest EC_{50} values against *H. contortus* eggs were observed for the methanolic extracts from *A. reticulata* and *A. muricata* (274.2 and 382.9 μg/ml, respectively). From the six extracts evaluated, the methanolic extracts of *A. muricata*, *A. reticulata* and *A. squamosa* showed the highest ovicidal activity, resulting in 98.9%, 92.8% and 95.1% egg mortality, respectively. When the methanolic extract of *A. squamosa* was incubated with PVPP, its AH activity increased. Similarly, when acetone:water extracts of *A. muriata* and *A. reticulata* were incubated with PVPP, their LFE activity increased. Alkaloids were only evident in methanolic extracts, irrespective of PVPP incubation. The presence of acetogenins was not observed. In conclusion, methanolic extracts obtained from leaves of *A. muricata*, *A. reticulata* and *A. squamosa* showed ovicidal activity affecting the morula of *H. contortus* eggs, with minor LFE activity. Meanwhile, acetone:water extracts showed mostly LFE activity, with a lower proportion of ovicidal activity.

Introduction

The use of plant materials containing secondary metabolites (SMs) with *in vivo* anthelmintic (AH) activity has been proposed as an alternative method for the control of gastrointestinal nematodes (GINs) in veterinary medicine (Sandoval-Castro *et al*., 2012). The selection of plant materials with potential AH activity implies testing several plant extracts obtained through different extraction procedures. Likewise, the AH activity of those plant extracts should be assessed using validated tests (Hoste *et al*., 2015). In recent decades, the evaluation of the AH activity of plant extracts against GIN eggs has been based on an adaptation of the egg hatch test (EHT), which uses thiabendazole to kill eggs at the morula stage (Coles *et al*., 1992). The EHT is an easy to implement and economical tool that is used worldwide to screen the *in vitro* AH activity of plant materials (Jackson & Hoste, 2010). In recent years, several studies showed that extracts of different *Annona* species have an ovicidal activity against *Haemonchus contortus* eggs. For instance, the methanol:water extract (70:30) of *Annona squamosa* seeds produced >80% egg-hatch inhibition (Souza *et al*., 2008). Similarly, the methanol extract from leaves and bark also produced 100% egg-hatch inhibition (Kamaraj & Rahuman, 2011; Kamaraj *et al*., 2011). More recently, an aqueous extract from *A. muricata* leaves was assessed showing 84% egg-hatch inhibition (Ferreira *et al*., 2013). Those studies provided important information about the quantity of extract necessary to inhibit *H. contortus* egg hatching but failed to indicate the mechanism affecting egg hatching. Recent *in vitro* studies evaluating crude plant extracts from different plant species against *H. contortus* eggs revealed two processes explaining the activity related to different mechanisms: (a) death of eggs at the morula stage, also known as ovicidal activity; and (b) the inability of well-developed larvae to hatch from their egg shell, also known as larvae failing eclosion (LFE) (Vargas-Magaña *et al.*, 2014). Recent studies showed that tropical plant extracts inhibit egg hatching by affecting the ability of larvae to hatch from eggs (LFE activity), and very few plant materials show an ovicidal activity (Castañeda-Ramírez *et al.*, 2017, 2018). However, those studies showed that methanolic extracts had higher ovicidal activity against *H. contortus* than the acetone-water extracts from the same plants. We hypothesize that methanolic extracts may allow to obtain more compounds associated with the ovicidal activity or may contain less compounds that antagonize with that activity. Such difference could be related to the polarity of extracts (Cortés-Morales *et al.*, 2019; García-Hernández *et al.*, 2019). Additionally, a recent study showed that the ovicidal activity was less evident for extracts with lower-condensed tannin content (Castañeda-Ramírez *et al.*, 2017). Thus, it is important to determine whether the use of different solvents in the extraction process (acetone:water vs. methanol) can influence the AH activity against nematode eggs. Such comparison can be used to investigate whether leaf extracts from different *Annona* species can affect *H. contortus* eggs at the morula stage. Furthermore, it is also important to identify the developing phase of eggs that is affected by each type of extract. The objective of this study was to assess and compare the AH activity of methanolic and acetone:water leaf extracts from different *Annona* species against *H. contortus* eggs.

Materials and methods

Location

This study was performed in the Faculty of Veterinary Medicine and Zoothecnics (FMVZ-UADY), Universidad Autónoma de Yucatán, Mérida, Mexico.

Biological material

Production of H. contortus eggs

The Paraiso *H. contortus* isolate was used for all the *in vitro* tests. This isolate was previously characterized as benzimidazole-resistant and as having low susceptibility to polyphenol-rich plant extracts (Chan-Pérez *et al.*, 2016).

A four-month-old hair-sheep donor lamb (25 kg) raised free of GIN infections prior to the study was used to produce the *H. contortus* eggs. The donor lamb was fed a balanced diet based on grass hay, a commercial concentrate feed and water *ad libitum*. The donor was kept in an individual pen with raised slatted floors before and during the experiment and was inoculated with 4000 L_3 of the isolate. The presence of eggs in the faeces was confirmed on day 28 post-infection. Faeces were collected three times a week directly from the rectum of the donor using plastic bags. Faeces were processed within 3 h of collection for the EHT. Egg recovery from faeces was carried out as follows: pellets were macerated in purified water, using 100 ml for every 10 g of faeces. The suspension was filtered using cheesecloth, and the filtered material was centrifuged (378 G for 5 min) in conical tubes (15 ml). Supernatant was discarded leaving sediment. A saturated sugar solution (1.28 density) was added and mixed with the sediment using a vortex to get a homogenous suspension. Suspension was centrifuged at 378 G for 5 min. A bacteriological loop was used to collect the supernatant of the suspension, where eggs were present. Eggs were placed in 15 ml tubes containing 10 ml of phosphate-buffered saline (PBS) pH 7.4. Egg concentration was determined, and suspension was diluted to 150 eggs/ml.

Production of methanolic and acetone:water extracts from leaves of Annona species

Fresh leaves from *A. squamosa*, *A. muricata* and *A. reticulata* (Annonaceae) were collected during the rainy season (October) in Yucatan, Mexico (20°56′N, 89°34′W). Specimens of each plant species were deposited in the FVMZ-UADY herbarium (voucher nos 14969, 14967 and 14968, respectively). The methanolic extracts were obtained from 500 g of fresh leaves from each plant species. Leaves were dried at 40°C for 72 h, until reaching a constant weight. Dried leaves were ground (1 mm particle size), weighed and placed in an individual container. Then, 30 ml of methanol was added for every 25 g of dried leaves. Samples remained under the organic solvent for 24 h, and this process was repeated once. The extract was recovered by filtration using filter paper (no. 50) and was concentrated under reduced pressure. Extracts were transferred to respective vials and placed in a laminar flow hood for 24 h to remove residual solvent. Finally, vials containing the respective extracts were closed and refrigerated at 4°C until further use in respective bioassays (Rosado-Aguilar *et al.*, 2010).

The acetone:water extracts were produced using 250 g of fresh leaves from each plant species. Fodder materials were crushed and placed in acetone:water (70:30) solution containing ascorbic acid (1 g/l) to avoid oxidation. The fodder materials were incubated for 24 h. Subsequently, the solution was recovered by filtration (filter paper no. 50). Acetone was removed under reduced pressure. The aqueous phase was rinsed twice with 500 ml methylene chloride to remove chlorophyll and lipids; thus, the remaining fraction was lyophilized and stored hermetically at 4°C until bioassays were conducted (Alonso-Díaz et al., 2008).

Assessment of the in vitro lethal effect of Annona spp. against H. contortus eggs

The EHT was used to evaluate the *in vitro* AH activity of the methanolic and acetone:water leaf extracts of the three *Annona* species against *H. contortus* eggs. The EHT was conducted following the procedure described by von Samson-Himmelstjerna et al. (2009) and Jackson & Hoste (2010). Preparation of stock solutions (10 mg/ml) of extracts were made in PBS prepared with purified water plus 2% Tween-80 for methanolic extracts and only PBS for acetone:water extracts. A multi-well plate (24-wells) was used containing PBS and the respective volume of stock solution of extracts. The PBS + 2% Tween-80 and PBS were used as negative controls for the respective extracts. Subsequently, 1 ml of the *H. contortus* egg suspension (150 eggs/ml) was added to each well to obtain the final extract concentrations (150, 300, 600, 1200, 2400 and 3600 μg/ml). Six replicates were used for each extract concentration. The multi-well plates were placed in an incubator at 28°C. After 48 h of incubation, 100 μl of Lugol's solution was added to kill and dye eggs and larvae (L_1). The number of eggs that failed to form larvae (morulated eggs (MEs)), the number of eggs that failed to complete their hatching (LFE) and the number of free larvae present in the sample were determined (Vargas-Magaña *et al.*, 2014).

To determine the role of condensed tannins and other polyphenols on the AH activity reported, solutions of different extracts were incubated with polyvinylpolypyrrolidone (PVPP; Fluka Analytical, Germany) (Makkar *et al.*, 1995) (0.05 g of PVPP/ml of solution) for 3 h at 24°C. The PVPP was used as a polyphenol-blocking material. The PVPP is a polymer able to form a tannin capture net and it is commonly used to detect and quantify the total tannin content (Hernández-Bolio *et al.*, 2018). After incubation, solutions were centrifuged at 1849 G for 5 min. The supernatant was used for testing at 3600 µg/ml, with and without PVPP, for the respective extracts in the same manner as described above. Six replicates were used for each extract concentration for the EHT.

Thin-layer chromatography (TLC)

The methanolic and acetone:water extracts from the *Annona* spp. were used to determine the metabolic profiles by TLC. Tests included extracts with and without PVPP incubation. The eluting system for TLC consisted of chloroform/MeOH/H_2O + 50 µl of formic acid, using the following detection reagents: (a) phosphomolybdic acid for oxidizable compounds; (b) DPPH (1,1-diphenyl-2-picrilhidrazil) for phenolic compounds with antioxidant activity; (c) Kedde's reagent for acetogenins; and (d) Dragendorff's reagent for alkaloids. The ultraviolet visualization of TLC plates (254 and 365 nm) was also used to detect SM-containing chromophore groups (Jork, 1990).

Data analysis

Means of MEs, eggs containing trapped larvae and hatched larvae were analysed through the Generalized Linear Model (GLM) analysis and compared with their respective controls. Post-hoc analysis was performed with Fisher's least significant difference (LSD) test, using Statgraphics Centurion XV software (Statpoint Technologies, 2005).

The extract activity on the assessed parameters – (a) MEs, (b) number of LFE and (c) the egg-hatching rate (EHR) – was determined using the following formulas (Chan-Pérez *et al.*, 2016):

(1) The percentage of MEs was estimated as:

$$\%\ \text{ME} = \frac{\text{Number of morulated eggs}}{\text{Number of morulated eggs} + \text{number of eggs containing a larva} + \text{number of larvae}} \times 100$$

(2) The percentage of LFE was calculated as:

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

(3) The EHR expressed as percentage was calculated as:

$$\%\ \text{EHR} = \frac{\text{Number of larvae}}{\text{Number of morulated eggs} + \text{number of eggs containing a larva} + \text{number of larvae}} \times 100$$

Data for ME, LFE and EHR were compared with their respective control (PBS) and with the respective extract at 3600 µg/ml with or without PVPP. Data were analysed using a GLM procedure in each methanolic and acetone:water leaf extract from each *Annona* spp. For each parameter, a respective post-hoc analysis was performed with Fisher's LSD using Statgraphics Centurion XV software (Statpoint Technologies, 2005).

The effective concentration required to inhibit 50% of hatching (EC_{50}) was estimated with data obtained from the EHR for each extract on the *H. contortus* eggs using the Polo-Plus 1.0 software (LeOra Software, 2004). The respective 95% confidence intervals (CIs) were also calculated. The EC_{50} values were considered significantly different when the 95% CIs did not overlap.

Results

Activity of Annona species leaf extracts against H. contortus eggs

Figure 1 shows the activity of methanolic and acetone:water leaf extracts of the three *Annona* species. The methanolic extracts of *A. muricata* (fig. 1a) and *A. reticulata* (fig. 1c) showed a significant reduction in egg hatching, starting from 300 µg/ml PBS, while the methanolic extract of *A. squamosa* only showed significant activity from 1200 µg/ml PBS (fig. 1e) ($P < 0.05$). The *A. muricata* and *A. reticulata* extracts showed activity against the morula stage of eggs from 600 µg/ml PBS. Meanwhile, the methanolic extract of *A. squamosa* showed activity against the morula stage from 1200 µg/ml PBS.

The acetone:water extracts of *A. reticulata* (fig. 1d) showed a significant reduction in egg hatching, starting at 600 µg/ml PBS, while the acetone:water extracts of *A. muricata* (fig. 1b) and *A. squamosa* (fig. 1f) only showed significant activities from

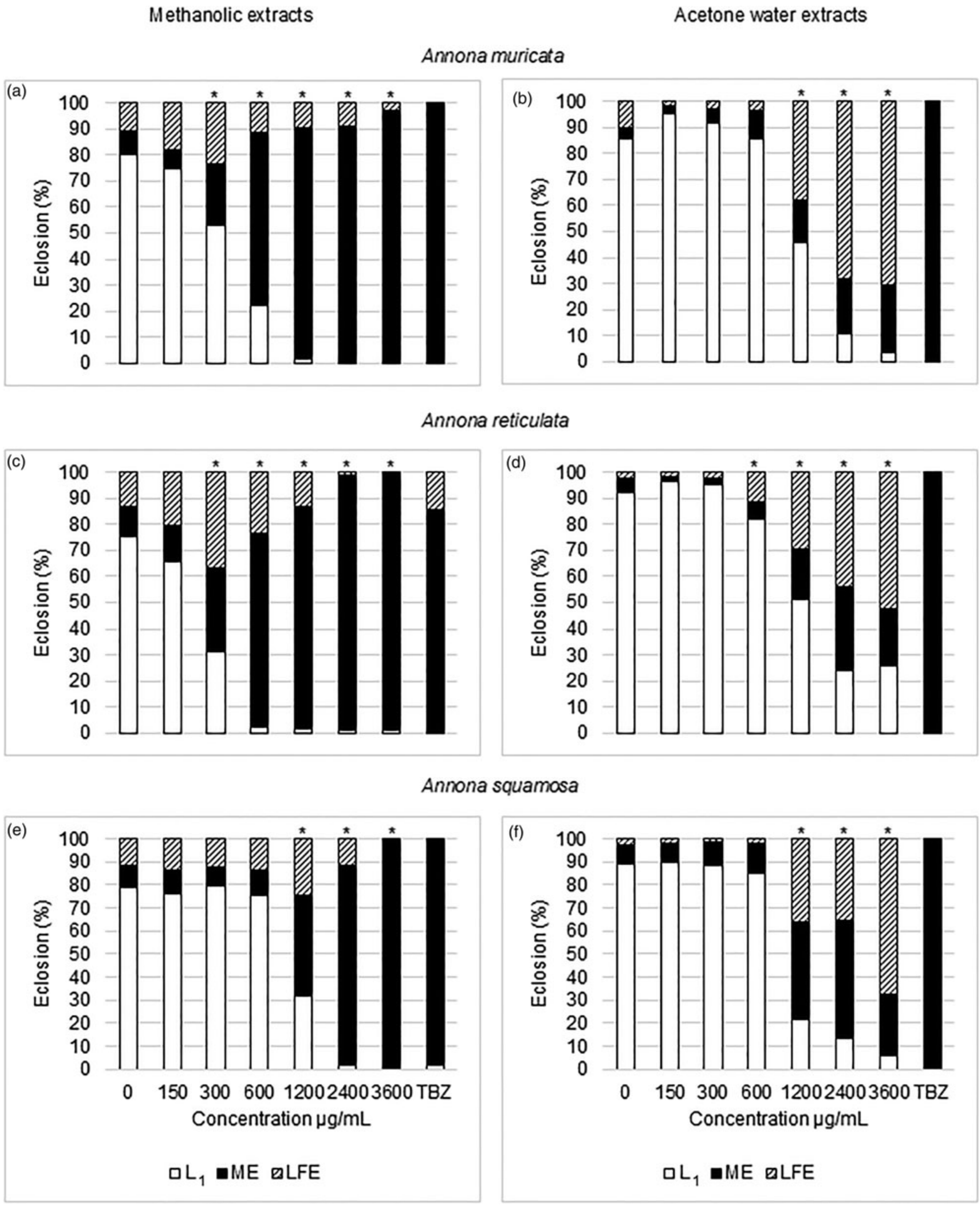

Fig. 1. Effect of different concentrations of methanol and acetone:water leaf extracts of *Annona muricata*, *A. reticulata* and *A. squamosa* on *Haemonchus contortus* egg-hatching inhibition. Graphs on the left correspond to methanolic extracts: (a) *A. muricata*; (c) *A. reticulata*; (e) *A. squamosa*. Graphs on the right correspond to acetone:water extracts: (b) *A. muricata*; (d) *A. reticulata*; (f) *A. squamosa*.

1200 µg/ml PBS ($P < 0.05$). The AH activity of acetone:water leaf extracts from *Annona* species was mainly related to block eclosion of larvae formed inside the egg (LFE activity), and a small proportion of eggs were killed at the morula stage.

Table 1 shows that the lowest EC_{50} values against the *H. contortus* eggs were found with the methanolic extracts of *A. muricata* and *A. reticulata*. The acetone:water extract of *A. reticulata* was the least active against *H. contortus* eggs.

Table 1. Effective concentration 50% (EC_{50}) and respective 95% CIs of methanolic and acetone:water leaf extracts of *Annona* species against eggs of *Haemonchus contortus*.

Plant species	Type of extract	EC_{50} μg/ml	95% CI μg/ml
Annona muricata	Methanolic	382.9^{b}	322.2–441.6
	Acetone:water	$1354.1^{d,e}$	1176.0–1533.1
Annona reticulata	Methanolic	274.2^{a}	239.3–306.0
	Acetone:water	1610.9^{f}	1424.8–1824.4
Annona squamosa	Methanolic	869.8^{c}	659.6–1048.0
	Acetone:water	$1042.0^{c,d}$	908.2–1179.2

$^{a-e}$Different letters in the same column mean significant difference ($P < 0.05$).

The effect of PVPP on the assessed parameters (proportion of ME, LFE and hatched eggs) of *H. contortus* after exposure to methanolic and acetone:water leaf extracts is shown in table 2. Methanolic extracts of *A. squamosa*, *A. muricata* and *A. reticulata* showed high ovicidal activity (92.8–98.9% ME). Pre-incubation of the *A. reticulata* methanolic extracts with PVPP increased the proportion of ME to 99.0% ($P < 0.05$).

On the other hand, the acetone:water extracts showed low ovicidal activity(12.4–20.7% ME). The acetone:water extracts showed a high LFE activity against *H. contortus* eggs.

Lesions on H. contortus eggs and L_1

The normal morula stage of eggs incubated in PBS is shown in fig. 2a, and the ovicidal activity of extracts is shown in fig. 2b. In the latter, it is evident that the egg contains more liquid in comparison to the egg exposed to PBS. Furthermore, the morula cells appeared shrunk and rough in the egg exposed to extracts, in contrast to the turgid morula cells of the egg in PBS.

On the other hand, the acetone:water extracts damaged the larvae formed inside the egg. The larvae trapped inside eggs had a compressed aspect and wrinkled architecture (fig. 3a). Likewise, the few L_1 that were able to hatch from eggs once exposed to acetone:water extracts were dead, and presented a generalized body swelling characterized by a separation of the shrivelled digestive tract, which seemed separated from the external cuticle compared to larvae exposed to PBS (see fig. 3b, c).

Groups of compounds identified by the Thin Layer Chromatography

The methanolic extracts showed the presence of compounds with a wide polarity range (from non-polar to polar), while the acetone:water extracts revealed the presence of compounds with medium to high polarity. In spite of these findings, both extraction systems showed a similar profile of polar metabolites. Phosphomolybdic acid identified the presence of flavonoids in those leaf extracts not incubated with PVPP, while DPPH revealed the presence of phenolic compounds with antioxidant activity in the acetone:water extracts that was less evident in methanolic extracts. Non-phenolic compounds with antioxidant activity were detected in the PVPP-incubated extracts. The use of Kedde's reagent did not show evidence of acetogenins in the tested extracts, with or without PVPP incubation. Finally, Dragendorff's reagent suggested the presence of alkaloids, and that was only evident for the methanolic extracts, irrespective of the PVPP incubation.

Table 2. Effect of the addition of PVPP on the proportion (%) of MEs, LFE and L_1 of *Haemonchus contortus* resulting from incubations with methanolic and acetone:water leaf extracts from three *Annona* species at a concentration of 3600 μg/ml PBS.

Plant species	Life stage	PBS + Tween	3600 μg/ml	PVPP	SE
Methanolic extract					
Annona squamosa	ME	8.4^{a}	95.1^{b}	93.0^{b}	1.8
	LFE	19.7^{a}	4.8^{b}	6.5^{b}	1.8
	L_1	71.9^{a}	3.6^{b}	0.5^{b}	0.4
Annona muricata	ME	8.3^{a}	98.9^{b}	97.8^{b}	0.6
	LFE	19.9^{a}	1.0^{b}	2.1^{b}	0.5
	L_1	71.7^{a}	0.1^{b}	0.0^{b}	0.3
Annona reticulata	ME	9.5^{a}	92.8^{b}	99.0^{c}	1.3
	LFE	16.0^{a}	6.8^{b}	1.0^{c}	1.4
	L_1	74.5^{a}	0.4^{b}	0.0^{b}	1.1
acetone:water extract					
Annona squamosa	ME	1.7^{a}	12.4^{b}	8.0^{a}	2.1
	LFE	8.7^{a}	20.2^{b}	40.2^{b}	3.5
	L_1	89.6^{a}	67.4^{b}	51.8^{b}	4.9
Annona muricata	ME	3.7^{a}	20.2^{b}	21.6^{b}	2.6
	LFE	4.1^{a}	70.1^{b}	76.1^{c}	2.5
	L_1	92.2^{a}	9.7^{b}	2.3^{c}	1.1
Annona reticulata	ME	4.2^{a}	12.5^{b}	15.2^{b}	2.5
	LFE	3.7^{a}	52.9^{b}	67.8^{c}	3.7
	L_1	92.1^{a}	34.6^{b}	17.0^{c}	4.6

$^{a-c}$Different letters in the same row mean significant difference ($P < 0.05$).
SE, standard error.

Discussion

This study showed that the extracts obtained from leaves of the tested *Annona* species possess strong *in vitro* activity against *H. contortus* eggs. The latter confirm previous *in vitro* AH activity against *H. contortus* egg hatching obtained with extracts from different parts of plants of the Annonaceae family, as described below. In the present study, low concentrations of the methanolic plant extracts (2.4 mg/ml) were sufficient to achieve almost 100% egg-hatching inhibition (fig. 1). The activity of *A. muricata* and *A. squamosa* acetone:water extracts also reduced egg hatching >90% from a concentration of 3.6 mg/ml (fig. 1). In comparison, the lowest extract concentrations previously tested were reported for the methanol:water extract of *A. squamosa* seeds, where 5 and 2.5 mg/ml reduced egg hatching by 81.9% and 81.5%, respectively (Souza *et al.*, 2008). Further studies using acetonic, methanolic and ethyl acetate extracts of *A. squamosa* bark required 25 mg/ml to reduce egg hatching >90% (Kamaraj *et al.*, 2011). When using the same organic solvents for the leaves of *A. squamosa*, the egg-hatch inhibition was <88.6% at 25 mg/ml (Kamaraj & Rahuman, 2011). The last study reported an 84.9% egg-hatch inhibition for the *A. muricata* organic extracts, but the concentrations tested were not defined (Ferreira *et al.*, 2013). The stronger AH activity of the *Annona* species tested in the present study could be attributed to different factors, such as the influence of

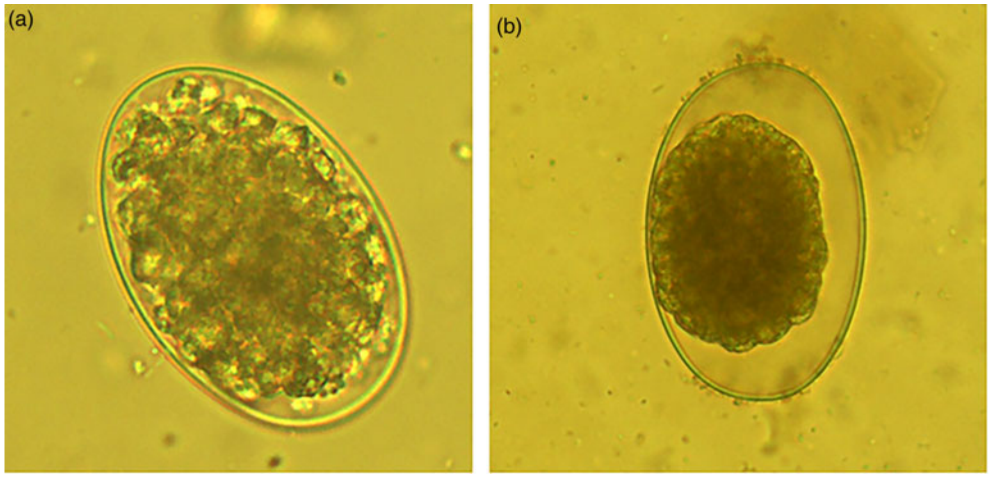

Fig. 2. Normal morulated *Haemonchus contortus* egg (a) exposed to PBS and (b) with a death morula resulting from the incubation with extracts of different *Annona* spp.

Fig. 3. (a) *Haemonchus contortus* egg incubated for 48 h in acetone:water extract of *Annona* spp. leaves showing a larva inside the egg; (b) image of a normal larva obtained from a control PBS incubation; (c) image of a deformed death larva that emerged from an egg exposed to acetone:water extracts.

environmental conditions in Yucatan, with a warm and sub-humid tropical climate, the plants' surrounding soil microbiota, the age of plants at collection time, the physiological stage of plants, micro-environmental conditions, sunlight exposure, soil water, fertility and salinity, damage caused by herbivores, among others factors, as all of those factors can influence the SM profile of plants (Badri *et al.*, 2013; Yang *et al.*, 2018). The latter could also affect their expected anti-parasitic activity (Arceo-Medina *et al.*, 2016; Hoste *et al.*, 2016). The plant extraction procedure, including the drying of plant materials as well as the volume and type of solvents used, could also influence the concentration of bioactive metabolites causing the AH activity (Hoste *et al.*, 2016; Hernández-Bolio *et al.*, 2018).

Extracts evaluated in the present study also showed stronger AH activity against *H. contortus* eggs compared to previous results with other plants species. For example, a methanolic extract obtained from *Tagetes filifolia* required 10 mg/ml to achieve 100% egg-hatching inhibition (Jasso-Díaz *et al.*, 2017). Likewise, 100 mg/ml of a methanol:water extract from *Acacia cochliacantha* leaves was required to achieve 100% egg-hatching inhibition (Castillo-Mitre *et al.*, 2017). On the other hand, the tested *Annona* extracts showed similar AH activity to that of methanol: water and acetone:water extracts from several plant species of the tropical deciduous forest. For instance, methanol:water extracts obtained from *Gymnopodium floribundum*, *Havardia albicans*, *Leucaena leucocephala*, *Mimosa bahamensis*, *Piscidia piscipula* and *Senegalia gaumeri* leaves caused >98% egg-hatching inhibition at 3.6 mg/ml (Castañeda-Ramírez *et al.*, 2017). Also, the acetone: water extracts from the leaves of those plants and from *Acacia collinsi*, *A. penatula* and *Bunchosia swartziana* provoked 98% egg-hatching inhibition at 3.6 mg/ml (Castañeda-Ramírez *et al.*, 2018).

Most extracts produced from plants of the tropical deciduous forest inhibited egg hatching by blocking the larvae eclosion (LFE activity); however, the *Annona* leaf extracts showed strong ovicidal activity (methanolic extracts; fig. 2a, b), as well as LFE activity (acetone:water extracts; fig. 3a–c). Previous studies evaluating the AH activity of *Annona* extracts only reported an egg-hatching inhibition but failed to clarify whether that activity was directed against the morula stage, or was associated with an LFE activity. The strong ovicidal activity reported in the present study for the methanolic extracts of *Annona* leaves is an interesting target of AH activity.

The physiological mechanism causing the ovicidal activity with these plants extracts is currently unknown. The inhibition of embryonation of freshly collected nematode eggs (ovicidal activity) was observed for the commercial drug thiabendazole (Coles *et al.*, 1992). Thiabendazole causes the inhibition of the fumarate reductase enzyme, which is specific to helminths (Prichard, 1973; Lacey, 1988).

The difference in AH activity between the methanol and the acetone:water extracts could be due to differences in the SM obtained with the two solvents. It is possible that the methanolic extracts have smaller SM, that could penetrate the eggshell, causing a higher lethal effect compared to SM present in acetone:water extracts. Additionally, the Dragendorff's reagent showed that the methanolic extracts contained alkaloids, which were not present in the acetone:water extracts. Those compounds could have an implication in the AH activity against the morula inside the egg.

Alkaloids present in *A. muricata* have cytotoxic and neurotoxic effects (Coria-Téllez *et al.*, 2018), and perhaps those compounds could cause death of eggs at the morula stage.

Meanwhile, the LFE activity could involve the presence of SM that are present in many plant species (Vargas-Magaña *et al.*, 2014). Different compounds affecting *H. contortus* egg hatching have been studied, including P-coumaric acid (Castillo-Mitre *et al.*, 2017; Castañeda-Ramírez *et al.*, 2018). Such compounds obtained from polyphenol-rich plant extracts allowed L_1 development inside the egg, but the eggshell could not be broken, similar to the egg shown in fig. 3a.

Blocking polyphenols with PVPP did not affect the AH activity of *A. squamosa* and *A. muricata* methanolic extracts (table 2), while the incubation with PVPP improved the AH activity of *A. reticulata* methanolic extract. The latter suggests that polyphenols were not involved in the AH activity of *A. squamosa* or *A. muricate*, but could limit the ovicidal activity of *A. reticulata.* The antagonism between the SM causing ovicidal activity and polyphenols contained in leaf extracts was already reported in previous studies (Vargas-Magaña *et al.*, 2014; Castañeda-Ramírez *et al.*, 2017).

The *Annona* leaf extracts evaluated in the present study warrant further investigation aiming to identify the active SMs causing the ovicidal activity. Meanwhile, these extracts can also help to identify compounds causing the LFE activity. Once these compounds are identified, commercial standards can be used to confirm those findings.

Conclusion

Methanol extracts obtained from leaves of *A. muricata*, *A. reticulata* and *A. squamosa* showed ovicidal activity affecting the morula stage of *H. contortus* eggs, with minor LFE activity. Meanwhile, the acetone:water extracts from the leaves of those same plants showed mostly LFE activity, with a smaller proportion of ovicidal activity.

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Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals. All procedures performed on donor animals complied with the Ethical Standards of the Bioethics Committee of the Faculty of Veterinary Medicine, UADY, Mexico.

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