Bio-guided fractionation to identify *Senegalia gaumeri* leaf extract compounds with anthelmintic activity against *Haemonchus contortus* eggs and larvae

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ABSTRACT

Small ruminants browsing in tropical forests readily consume the foliage of *Senegalia gaumeri*. A *S. gaumeri* methanol:water extract was recently shown to have ovicidal activity against *Haemonchus contortus* eggs *in vitro*. In the present study, the fraction of a *S. gaumeri* methanol:water extract with ovicidal activity against *H. contortus* eggs and the metabolites potentially involved in this activity were identified. Bio-guided fractionation of the *S. gaumeri* methanol:water extract identified high ovicidal activity (80.29%, EC_{50} = 58.9 μg/mL) in the non-polar sub-fraction P1. Gas chromatography-mass spectrometry (GC-MS) identified several fatty acids: pentacosane (18.05%), heneicosane (18.05%), triacontane (30.94%), octacosane (18.05%), and hexanedioic acid bis-(2-ethylhexyl) ester (32.72%). Purification of the polar components of sub-fraction P1 led to the identification of *p*-coumaric acid as a major constituent. In egg hatch tests, 400 μg/mL *p*-coumaric acid resulted in an ovicidal effect of 8.7%, a larvae failing eclosion effect of 2.9%, and of the emerged larvae (88.4%), many were damaged. In conclusion, the low AH activity of *p*-coumaric acid against *H. contortus* eggs indicates that it is not solely responsible for the ovicidal activity of sub-fraction P1 but might act in synergy with other compounds in this fraction. However, *p*-coumaric acid showed potential anthelmintic effects against the larval stage of *H. contortus*.

1. Introduction

Gastrointestinal nematodes (GINs) affect the health and production of grazing small ruminants worldwide (Sackett et al., 2006). Parasite control methods based on the intensive use of conventional anthelmintics (AHs) are losing practical viability for most farms due to the development of resistance among parasite populations. Consequently, various alternative methods for the control of GIN populations are currently being explored. One alternative control method is based on the use of plants with AH activity. The foliage of those plants can be used as nutraceutic feeds that provide both nutrients and AH effects (Hoste et al., 2016). In the tropical deciduous forest (TDF) of Yucatan, Mexico, several plants contain secondary compounds (SCs) with AH activity (Torres Acosta et al., 2016). Early work attributed the AH activity of plant extracts against L_3 larvae of the abomasal parasite *Haemonchus contortus* to its condensed tannin (CT) content (Alonso Díaz et al., 2008). However, follow up studies showed that other plant SCs contributed partially or totally to the AH effect against *H. contortus* eggs (Vargas Magaña et al., 2014). The compounds speculated to be responsible for AH activity against *H. contortus* eggs includes acetogenins (Souza et al., 2008), terpenes, alkaloids (Marie Magdeleine et al., 2010a, 2010b), and, more recently, derivatives of caffeoyl and coumaroyl (Castillo Mitre et al., 2017). In addition, phenolic compounds and flavonoids may have activity against *Cooperia* spp. (von Son de

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Fernex et al., 2015). These studies using bio guided methods to find the most active fraction, suggested the existence of synergism between compounds, or simply attributed the AH activity to the major com pounds. However, most did not confirm the AH activity of the suspected ovicidal compound(s) and the possibility of synergism between com ponents.

Therefore, to the best of our knowledge, the metabolites responsible for the ovicidal effect of plant extracts against *H. contortus* are still unknown. A recent study reported a strong ovicidal effect against *H. contortus* eggs of a methanol:water extract of *Senegalia gaumeri*, a plant of the Fabaceae family used as fodder for ruminants (Castañeda Ramírez et al., 2017). Exposure to this extract prevented the develop ment of eggs beyond the morula phase (56.2% of the AH activity) or the emergence of larvae from the eggs (43.7% of the AH activity). Identi fying the most active fraction and possibly the compound responsible for ovicidal activity requires a bio guided fractionation study. Bio guided assays paired with compound isolation and characterization constitute the basis for identifying the mechanism of action of plant compounds and active agents with AH activity in candidate plants or feeds (Hernández Bolio et al., 2018). In the present study, the bio guided fractionation methodology was used to identify the fraction of a *S. gaumeri* methanol: water extract with ovicidal activity against *H. contortus* and isolate the metabolites involved in that activity.

2. Materials and methods

2.1. Plant material and extraction

Two kilograms of leaves were collected from four *S. gaumeri* trees in the TDF area around the Faculty of Veterinary Medicine, Universidad Autónoma de Yucatán, México (20°51′41″N, 89°37′28″W). The leaves were collected in April and May (dry season). A sub sample of *S. gau meri* leaves was submitted to the herbarium of the Universidad Autónoma de Yucatán, México, and assigned a voucher number (No. 2010020,100).

Fresh leaves (1 kg) were macerated in a methanol:water solution (70:30, final volume 20 L) (MeOH, Fermont and Edwards®, México) with ascorbic acid (1 g/L) to prevent oxidation. The mixture was left to settle for 24 h. The extract was then filtered, and solvent was eliminated using a reduced pressure rotavapor (Buchi ®). The resulting aqueous fraction was washed with dichloromethane (DCM, Fermont and Edwards®, México) (1:1 v/v) and concentrated again under reduced pressure. Freeze drying of the aqueous extract yielded 49.6 g (4.96% yield), which was stored at 4 °C until use.

2.2. Qualitative phytochemical analysis

Qualitative analyses of the possible secondary metabolites (flavo noids, saponins, triterpenes and alkaloids) in the methanol:water ex tract of *S. gaumeri* leaves were performed using various methodologies. For the Shinoda test for flavonoids, 5 mg of the extract was placed in a test tube and dissolved in 1 mL of MeOH, and 2 3 shavings of magne sium (Mg powder, J.T. Baker®) and 2 3 drops of concentrated hydro chloric acid (HCl A.C.S, Fermont®, México) were added. The develop ment of reddish coloration indicated flavonoids (Lock et al., 2006). For the Salkowski test for saponins, a small amount (5 mg) of the extract was placed in a test tube, and 2 mL of chloroform ($CHCl_3$ A.C.S., Fer mont®, México) and 2 mL of concentrated sulfuric acid (H_2SO_4 RA, Fermont®, México) were added. Orange coloration indicated a positive reaction (Mac Donald et al., 2005). For the Liebermann Burchard re action for triterpenes, a portion of the dry material was dissolved in 1 mL of a reagent prepared with acetic anhydride (Ac_2O, A.C.S., Fer mont®, México), H_2SO_4 and $CHCl_3$ (10:1:25) v/v for 1 2 min. The ap pearance of red, pink, purple, or blue was considered a positive test (Harborne, 1973). To perform thin layer chromatography (TLC) for alkaloids, spots of a mixture of 5 mg of extract in 1 mL of MeOH were deposited on a 10 × 3 cm silica gel 60 F_{254} Merck® TLC plate. The plate was eluted with hexane:ethyl acetate (7:3), dried and sprayed with an iodine potassium iodide acidic solution (1 g of I_2 (resublimed U.S.P., Golden Bell®, México) and 10 g of KI (P.Q.M., México) in 100 mL of 2% glacial acetic acid (CH_3COOH A.C.S., Meyer®, México)). Red spots in dicated a positive test (Merck, 1974).

2.3. Production of Haemonchus contortus eggs

The *H. contortus* L_3 larvae used for the study (Paraiso strain) were previously characterized as resistant to benzimidazole (Chan Pérez et al., 2016). The L_3 larvae were produced in two four month old lambs (25 ± 1 kg LW) raised free of natural GIN infections from birth as described by Chan Pérez et al. (2017). The donor lambs were orally inoculated with 4000 L_3 and maintained in individual metabolic cages. Faecal samples were obtained from the donor lambs on day 28 post infection to confirm the presence of *H. contortus* eggs in the faeces. The lambs were fed a complete diet consisting of commercial concentrate feed and chopped grass (*Pennisetum purpureum*). Water was provided *ad libitum*.

2.3.1. Recovery of H. contortus eggs for the hatching inhibition test

Faeces were collected directly from the rectum of each donor lamb using new plastic bags. The fresh faecal material was used within one hour after collection. The faeces were macerated in purified water (reverse osmosis; 100 mL water:10 g of faeces) in a plastic container. The mixture was filtered and centrifuged (1500 rpm ×5 min) in 15 mL tubes. The sediment was recovered, mixed and homogenized with a saturated sugar solution (1.28 density), and the suspension was cen trifuged (1500 rpm ×5 min). Then, a bacteriological loop was used to recover the *H. contortus* eggs from the surface of the suspension. The eggs were placed inside 15 mL tubes containing 10 mL of phosphate buffered saline (PBS, pH 7.4) (Sigma®, Mexico). The concentration of eggs per millilitre was determined, and the suspension was diluted to achieve a concentration of 150 eggs/mL.

2.4. Bio guided fractionation methodology

A bio guided fractionation methodology was used to identify com pounds in the *S. gaumeri* methanol:water extract, which was previously reported to be active against *H. contortus* eggs and larvae (Castañeda Ramírez et al., 2017). The methodology has been described by Weller (2012) and includes three aspects: (i) reduction of the complexity of the sample by subsequent fractionation steps, (ii) biological testing of each fraction against *H. contortus* eggs, and (iii) identification of the isolated compound and validation by comparison with a commercial standard.

2.4.1. Fractionation of the methanol:water extract

The MeOH:water extract from the leaves of *S. gaumeri* (1 g) was purified using gel permeation chromatography (GPC) with a lipophilic Sephadex (25 100 µm LH20100, Sigma®, Mexico) stationary phase (3.0 cm diameter and 40 cm height) and isocratic MeOH (100%) as the mobile phase. Sixty five fractions of 5 mL each were collected and pooled into 10 fractions (from F1 to F10) according to the similarity of their Rf values on reverse phase TLC (Merck®, México). The fractions obtained were evaluated using the *in vitro* egg hatch test (EHT) as de scribed below.

- *Sub fractionation of F2.* The F2 fraction, which had the highest AH activity, was selected for reverse phase preparatory chromato graphy (TLC, Silica gel 60 RP 18 F_{254}s Merck®, México). A total of 20 aluminium plates were prepared, and 5 mg of sample was applied to each plate. Acetonitrile (ACN, Fermont and Edwards®, México):water (8:2) was used as the elution system, resulting in nine sub fractions (P1 P9). Each sub fraction was evaluated with the EHT to identify the most active sub fraction. The bio guided

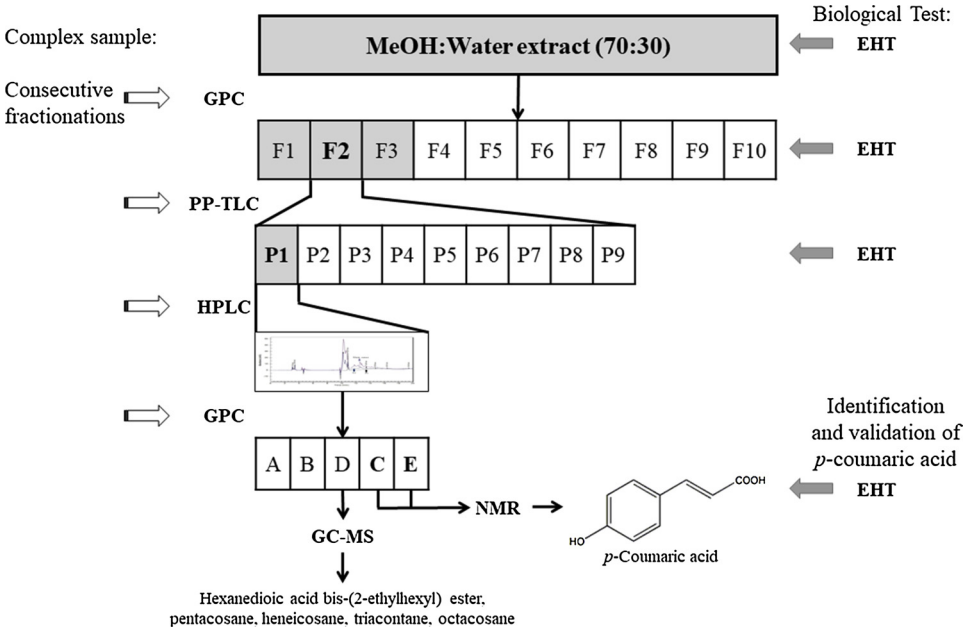

Fig. 1. Bio-guided fractionation scheme of the methanol (MeOH):water extract from *Senegalia gaumeri* leaves.
Grey shadow: active fractions identified with the egg hatch test (EHT). GPC: Gel permeation chromatography PP-TLC: Preparative plate-thin layer chromatography. HPLC: High performance liquid chromatography. GC–MS: Gas chromatography-mass spectrometry. NMR: Nuclear magnetic resonance.

fractionation process is described in Fig. 1.

- *HPLC chemical profile of sub fraction P1*. The active fractions were analysed by high performance liquid chromatography (HPLC) using Perkin Elmer Flexar™ equipment and a C18 column (Phenomenex 250 × 4.6 mm) at 25 °C. The mobile phase consisted of ACN (A) (HPLC grade, Jalmek®, Mexico) and H_2O (B) with the following gradient profile: 0 1 min, 100% B; 1 12 min, 50% B; 12 20 min, 0% B. A constant flow rate was used (0.7 mL/min), and the injection volume was 20 µL. The absorbance was measured at 254 nm. The purity of sub fraction P1 (1.4 mg) was analysed by HPLC using the ACN:H_2O gradient profile described above, which revealed 5 signals named A through E. The HPLC chromatograms of sub fraction P1 and fraction F2 were compared with the whole extract graphically according to their retention times (Fig. 2).
- *Purification of the active sub fraction P1*. Sub fraction P1 (21 mg) was purified via GPC using Sephadex LH 20 as the stationary phase (2.0 cm diameter and 45 cm height). The mobile phase was isocratic MeOH (100%). A total of 310 fractions (1 mL each) were collected and pooled into 5 fractions according to similar Rf values from re verse phase TLC, corresponding to the A E signals detected by HPLC.

2.4.2. *Evaluation of biological activity*

The EHT was used to evaluate the *in vitro* AH activity of the plant extract, its fractions, sub fractions and the chemical standard. The methodology has been described by von Samson Himmelstjerna et al. (2009) and Jackson and Hoste (2010). A stock solution of *S. gaumeri* leaf extract in PBS was prepared, and different concentrations (from 150 to 3600 µg/mL) in PBS were added to the wells of 24 well plates. Subsequently, an equal volume of egg suspension (300 eggs/mL) was

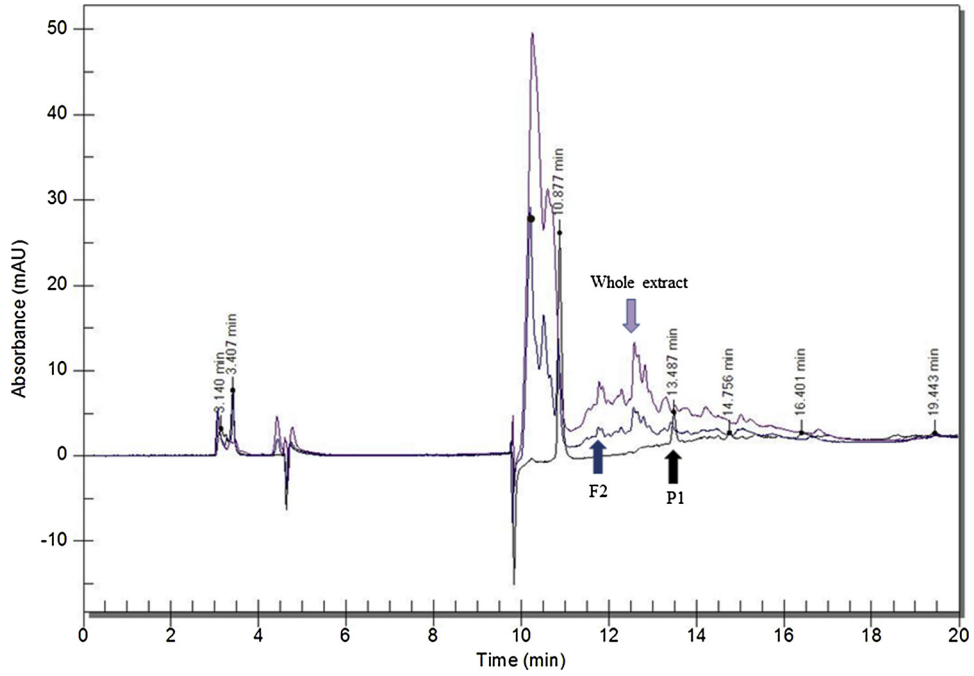

Fig. 2. Comparison of the chromatograms from whole extract, fraction F2 and sub-fraction P1.

added to obtain a final volume of 1 mL. Six replicates were used for each extract concentration. The plates were incubated for 48 h at 28 °C, followed by the addition of Lugol's solution to each well to stop hatching.

The contents of each well were counted with the aid of a compound microscope, including the number of morulated eggs (ME) to indicate the true ovicidal activity; the number of eggs containing larvae failing eclosion (LFE); and the number of L_1 present in the sample (Vargas Magaña et al., 2014). These L_1 were also observed to identify any ab normal shape or form.

Similar protocols were performed on the fractions and sub fractions obtained from the leaf extract and for the chemical standard *p* coumaric acid (C9008 1 G Sigma Aldrich©) at different concentrations (25 400 µg/mL for the fractions and *p* coumaric acid and 25 150 µg/mL for the sub fractions).

2.4.3. Identification of compounds from the simplest fraction

Fraction D from sub fraction P1 was analysed by gas chromato graphy mass spectrometry (GC MS). This methodology was used to separate and identify the volatile, semi volatile and thermally stable molecules (metabolites or compounds) in the mixture. An Agilent Technologies® 6890 N GC coupled with a selective mass detector 5973 was used for GC MS using the following chromatographic conditions: split injection of 1 mL of a 1% concentration sample; HP5 MS column of phenyl methyl silicone 30 m × 0.25 mm; and a flow rate of 1 mL/min (helium as the carrier gas). Fractions C and E were analysed by nuclear magnetic resonance (NMR). The NMR analysis was performed in $CDCl_3$ with a 500 MHz probe (Bruker®) at Fundación Medina (Granada, Spain). The NMR data included ^1H, ^{13}C and bi dimensional HMBC, HMQC and COSY to elucidate the structures of potentially active mo lecules.

2.5. Data analysis and statistical analyses

The proportions (%) of eggs (morulated + containing larvae) and emerged larvae were used to determine the effective concentrations of whole extract required to inhibit 50% of hatching (EC_{50}) and 90% of hatching (EC_{90}) with 95% confidence intervals (95% CIs) using PoloPlus 1.0 software (LeOra Software 2002). The same procedure was used for the fractions and sub fractions.

The proportions of ME and eggs with LFE were calculated using the formulas reported by Vargas Magaña et al. (2014). The percentage values of ME, LFE or hatched larvae from the PBS controls were com pared with the results obtained at the highest concentration (extract, F1 F3 fractions, P1 sub fraction, or *p* coumaric acid) in PBS. The re spective generalized linear models (GLMs) were used to assess differ ences in egg hatching between the PBS controls and the different con centrations of the tested extracts and standard.

3. Results

3.1. Qualitative phytochemical analysis

The qualitative analysis of the methanol:water extract of the leaves of *S. gaumeri* showed the presence of saponins and flavonoids but not triterpenes or alkaloids.

3.2. Anthelminthic activity of the whole extract of Senegalia gaumeri and its fractions

The results of the EHT evaluations using the whole extract or frac tions were used to determine EC_{50} and EC_{90} values (Table 1). The whole extract and fractions F1, F2 and F3 showed AH activity against eggs. However, only the EC_{50} and EC_{90} values of fraction F1 were similar to those of the whole extract. The remaining fractions (F4 to F10) had no AH effects.

Table 1
Effective concentration 50% (EC_{50}) and 90% (EC_{90}) with their respective 95% confidence intervals (95% CI) of the ten fractions (F1 to F10) of the methanol:water extract (70:30) of *Senegalia gaumeri* leaves against *Haemonchus contortus* eggs.

Fractions	EC_{50} (µg/mL)	95% CI (µg/mL)	EC_{90} (µg/mL)	95% CI (µg/mL)
Whole extract	42.6^{A}	30.2-50.4	75.2^{A}	68.2-83.6
F1	49.2^{A}	32.9- 59.5	97.5^{A}	84.3-123.0
F2	208.9^{B}	149.3-288.0	733.8^{B}	450.7-3215.1
F3	279.8^{B}	225.6- 355.9	1153.1^{B}	746.4-2757.6
F4 -F10	–	–	–	–

$^{A;B}$ The different letters make a significant difference ($P < 0.05$).

The whole extract and active fractions F1, F2 and F3 were re eval uated at 400 µg/mL to obtain the percentages of ME, LFE and hatched larvae (L) (Fig. 1). These bioassays indicated that the fraction with the greatest ovicidal effect was F2 (33.5%); this fraction also reduced hatching by 94% ($P < 0.05$). The effects of fraction F2 were greater than those of the whole extract, which had an ovicidal effect of 17% and reduced hatching by 98% ($P < 0.05$). Fraction F1 exhibited a powerful inhibitory effect on hatching (99%, $P < 0.05$) but a low ovicidal effect (< 10%). By contrast, fraction F3 showed low hatching inhibition (44%, $P < 0.05$) and a low ovicidal effect (< 5%).

3.3. Anthelmintic activity of sub fractions obtained from fraction F2

Preparatory chromatography of the active fraction F2 yielded 9 sub fractions (P1 to P9). Sub fraction P1 showed AH activity in the EHT, with an EC_{50} of 58.9 µg/mL (95% CI 13.1 104.0) and EC_{90} of 263.3 µg/mL (95% CI 164.0 547.4). The other sub fractions had no AH effects against *H. contortus* eggs.

Sub fraction P1 was re evaluated in the EHT at 150 µg/mL, which showed that the eggs were mainly affected in the morula phase (ME = 53.4%, $P < 0.05$), although some LFE (23.6%, $P < 0.05$) and larvae effects (23.05%, $P < 0.05$) were observed. Sub fraction P1 in hibited egg hatching by 77.0% ($P < 0.05$).

3.4. Purification of the active sub fraction P1

In the HPLC chromatograms, the signals at 3.14, 3.41, 10.88 and 13.49 min were present in both active fractions, that is, fraction F2 and sub fraction P1. Some signals (10.25, 10.60, 11.78, 12.58 min) were present in the whole extract but not in the active fractions (F2 and P1) (Fig. 2).

After GPC, TLC analyses showed that sub fraction P1 had five chromatographic signals (A to E). The fractions from signals C and E were analysed by NMR, which showed that the spectral data of both fractions were consistent with *p* coumaric acid. The spectral data for *p* coumaric acid were similar to those reported in the literature (Alavi et al., 2009). Co injection of sub fraction P1 and a commercial standard showed that these analytes had the same retention time on HPLC.

GC MS analysis of the fraction from signal D allowed the identifi cation of pentacosane (18.05% abundance), heneicosane (18.05% abundance), triacontane (30.94% abundance), octacosane (18.05% abundance), and hexanedioic acid bis (2 ethylhexyl) ester (32.72% abundance).

3.5. Anthelmintic activity of p coumaric acid

To confirm the involvement of *p* coumaric acid in the AH activity of sub fraction P1 against *H. contortus* eggs, a commercial chemical stan dard was used at a concentration of 400 µg/mL. An ovicidal effect of 8.7% was observed, as was a low proportion of eggs with LFE (2.9%). In addition, up to 50% of the larvae that emerged from the eggs (88.4%)

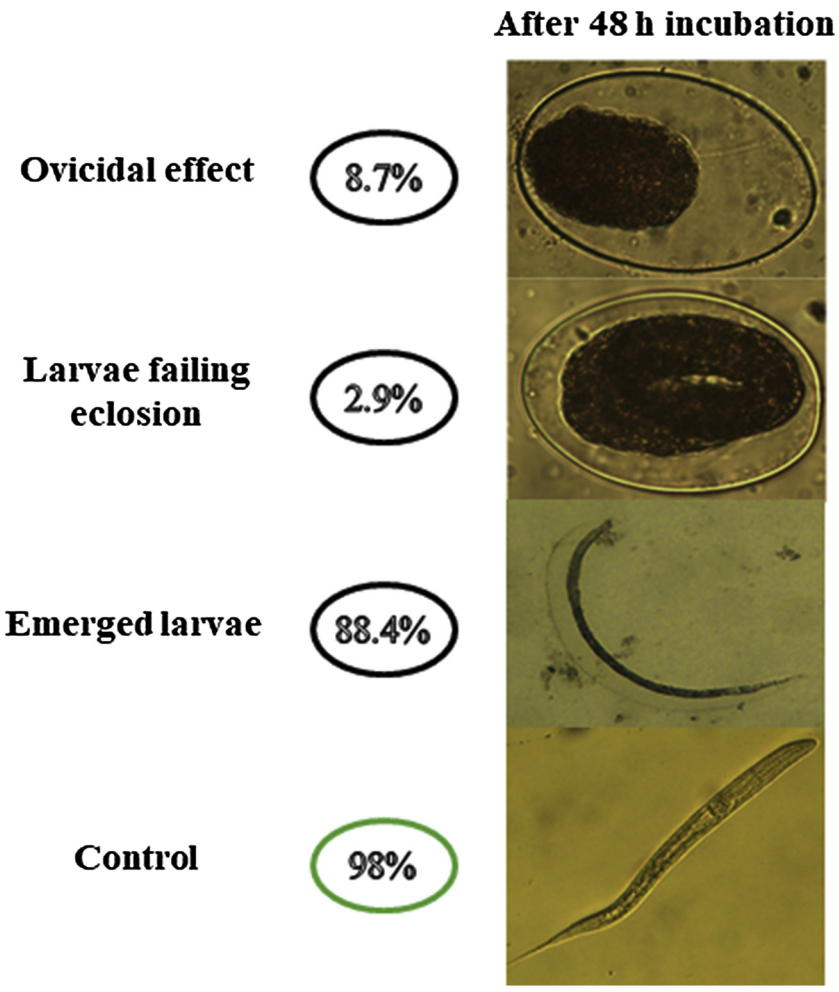

Fig. 3. Anthelmintic activity of *p*-Coumaric acid against *Haemonchus contortus* eggs and emerged larvae after 48 h incubation.

were swollen, with altered cell structure (Fig. 3).

4. Discussion

Several plants in TDFs are consumed by sheep and goats (González Pech et al., 2015), and many of these plants have potential as nu traceutics (Torres Acosta et al., 2016), with *in vitro* AH activity against eggs of GINs such as *H. contortus* (Castañeda Ramírez et al., 2017). In that study it was evident that polyphenols were not responsible for the *in vitro* AH activity against eggs. Hence, the present study aimed to identify the secondary metabolites potentially involved in that activity.

The inhibition of hatching by polar extracts from tropical plants has been associated with two mechanisms of action (Vargas Magaña et al., 2014; Chan Pérez et al., 2016): (i) true ovicidal activity that stops eggs from developing beyond the morula stage similar to that observed for benzimidazole (BZ) drugs, in which shrinking and damage of the morula are observed compared to negative controls; (ii) larvae failing eclosion, in which a fully developed larva is unable to hatch from the egg. Both modes of action ultimately result in a reduction of the number of larvae hatched from eggs, and both should be assessed to understand the mechanism of AH activity associated with SCs from plant extracts.

Most plant extracts tested so far affect the ability of larvae to emerge from the eggshell (high LFE activity), whereas ovicidal effects are generally less common and correspond typically to evaluation with BZ (EC_{50} < 0.1 µg/mL in non resistant isolates). We do not know whether an extract from a nutraceutical plant might ever show the level of *in vitro* efficacy of a commercial AH product such as a BZ. However, a recent study showed that a MeOH:water extract from the leaves of *S. gaumeri* had unusually high ovicidal activity (Castañeda Ramírez et al., 2017) with an EC_{50} < 300 µg/mL. Thus, although secondary com pounds contained in some nutraceutical plants can reduce the GIN in fection in a natural (and probably cheaper) way for producers as shown under *in vivo* conditions (Martínez Ortíz de Montellano et al., 2010; Méndez Ortiz et al., 2019), further investigation to enhance their effi cacy is needed.

The present study confirmed the strong *in vitro* AH activity of the whole MeOH:water extract from *S. gaumeri* leaves, with an EC_{50} of 42.6 µg/mL (95% CI: 30.2 50.4). The present bio guided fractionation protocol identified three fractions with egg hatching inhibitory activity (F1 F3); one had a clear ovicidal effect (F2), and the two other active fractions (F1 and F3) had LFE effects. These latter two fractions were not further explored in this study because of their poor ME effects but could hold promise for identifying compounds with LFE effects on eggs. Fraction F2 was subjected to further fractionation, and of the 9 sub fractions (P1 P9) identified, sub fraction P1 showed the greatest ac tivity against ME, reaching 80% at a concentration of 150 µg/mL. This ovicidal effect of sub fraction P1 was greater than that of the original fraction (F2).

Sub fraction P1 yielded five TLC signals (A E), of which only one was pure enough to be identified by NMR, resulting in *p* coumaric acid. The ovicidal activity of *p* coumaric acid, either on ME or LFE, was very low. Thus, the ovicidal activity of sub fraction P1 cannot be attributed solely to a single compound. Sub fraction P1 may contain other uni dentified compounds that act synergistically to enhance the ME effect. Synergism between plant metabolites has been described for *Cooperia* spp. eggs exposed to different polyphenols (von Son de Fernex et al.,

2015), ticks exposed to benzyl sulphides (Arceo Medina et al., 2016) and *H. contortus* L_3 exposed to flavonoid monomers and CTs (Klongsiriwet et al., 2015).

The metabolites responsible for the AH activity of plant extracts, including those of *S. gaumeri*, remain unknown. Vargas Magaña et al. (2014) hypothesized that the ovicidal (ME) effect could be caused by bioactive compounds that are small enough to penetrate and kill the morula within the egg. In the present study, NMR analysis led to the identification of *p* coumaric acid. Castillo Mitre et al. (2017) identified this same compound as potentially responsible for the hatching inhibition of *H. contortus* eggs by an ethyl acetate extract (medium polarity) from dry leaves of *Acacia cochliacantha* together with other caffeoyl and coumaroyl derivatives (including caffeic acid, ferulic acid, methyl caffeate, methyl *p* coumarate, methyl ferulate and quercetin). However, in that study, the role of *p* coumaric acid was not tested to verify its activity against eggs. In the present study, the ovicidal activity of *p* coumaric acid was evaluated using a bioassay against *H. contortus* eggs. In the present study, *p* coumaric acid showed low ovicidal activity (8.7%) and low LFE activity (2.1%). Therefore, other compounds with likely synergy with *p* coumaric acid are involved in the ovicidal activity of sub fraction P1. The search for compounds responsible for ovicidal activity is complex and limited by the difficulty of separating each fraction or sub fraction.

An important finding was the effect of *p* coumaric acid on the structure of the emerged larvae, which was abnormal. Such an effect has not been reported previously for this compound and was more evident than the ovicidal activity mentioned above. The mechanism of action against larvae warrants further research.

GC MS analysis of sub fraction P1 showed the presence of many lipidic compounds. However, these compounds were not investigated, as previous studies have not provided evidence suggesting any AH activity against *H. contortus* eggs. Moreover, although qualitative phytochemical analysis revealed the presence of saponins and flavonoids and confirmed the absence of triterpenes and alkaloids, these findings could not be related to the AH activity of the whole extract.

The present study is the first attempt to evaluate the ovicidal activity of a pure compound (> 98%) in the form of either ME (death at morula stage) or LFE effects in *H. contortus* eggs. Further efforts are needed to identify the compounds or mixture of compounds responsible for the AH effects observed in this and other plant extracts.

5. Conclusion

Sub fraction P1 obtained from fraction F2 of the MeOH:water extract of *S. gaumeri* leaves showed an ovicidal effect on the morula phase. Purification and characterization of sub fraction P1 by HPLC, followed by GC MS and NMR, revealed the presence of *p* coumaric acid, pentadecane, pentacosane, heneicosane, triacontane, octacosane, and hexanedioic acid bis (2 ethylhexyl) ester. *p* Coumaric acid alone had low ovicidal activity against *H. contortus* eggs, suggesting synergy of this compound either with the hydrocarbons or fatty acid ester present in sub fraction P1.

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 (licence No. CB CCBA D 2014 003).

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Conflict of interest statement

None.

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