Veterinary Parasitology 270 (2019) 25–30

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Evaluation of cinnamic acid and six analogues against eggs and larvae of *Haemonchus contortus*⋆

Gabriela Mancilla-Montelongo[a], Gloria Sarahi Castañeda-Ramírez[b], Juan Felipe de Jesús Torres-Acosta[b], Carlos Alfredo Sandoval-Castro[b,*], Rocío Borges-Argáez[c]

[a] CONACYT – Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Yucatán, Km 15.5 Carretera Mérida-Xmatkuil, CP 97100, Mérida, Yucatán, Mexico
[b] Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Yucatán, Km 15.5 Carretera Mérida-Xmatkuil, CP 97100, Mérida, Yucatán, Mexico
[c] Centro de Investigación Científica de Yucatán, Calle 43 No. 130 × 32 Colonia Chuburná de Hidalgo, CP 97205, Mérida, Yucatán, Mexico

ARTICLE INFO

Keywords:
Haemonchus contortus
Nematode
Egg hatch test
Larval exsheathment inhibition test
Cinnamic acid analogues
Chemical standards

ABSTRACT

This study evaluated the *in vitro* anthelmintic (AH) activity of cinnamic acid and six analogues against eggs and larvae of *Haemonchus contortus*. Stock solutions of each compound (*trans*-cinnamic acid, *p*-coumaric acid, caffeic acid, *trans*-ferulic acid, *trans*-sinapic acid, 3,4-dimethoxycinnamic acid, and chlorogenic acid) were prepared in PBS:Tween-20 (1%) for use in the egg hatch test (EHT) and larval exsheathment inhibition test (LEIT) at different concentrations (25–400 µg/mL). The respective effective concentration 50% (EC_{50}) values with 95% confidence intervals were estimated. Mixtures made of all cinnamic acid and its analogues as well as some selected individual compounds were also tested in the EHT. Only ferulic and chlorogenic acids showed AH activity in the EHT (EC_{50}: 245.2 µg/mL (1.26 mM) and 520.8 µg/mL (1.47 mM), respectively) ($P < 0.05$). A higher EC_{50} (1628.10 µg/mL) of the mixture of cinnamic acid and its analogues was required to observe activity against eggs mostly blocking the larvae hatching. The analogues' mixtures tested were less active than ferulic or chlorogenic acid alone. The activity of ferulic and chlorogenic acids against eggs was associated with larvae failing to hatch, and the two compounds exhibited antagonistic effects when evaluated together. All standards had an EC_{50} lower than 0.42 mM in the LEIT. Caffeic acid had the best activity in the LEIT (EC_{50} 0.04 mM), followed by ferulic acid (EC_{50} 0.11 mM) ($P < 0.05$). There was no clear, definitive structure-activity relationship for these non-flavonoid polyphenols against eggs or larvae of *H. contortus in vitro*. This study is the first to directly evaluate cinnamic acid and its derivatives as active compounds against eggs and larvae of *H. contortus*.

1. Introduction

Gastrointestinal nematodes (GINs) such as *Haemonchus contortus* affect animal health and production (Hoste and Torres-Acosta, 2011). Recent studies have revealed the presence of simultaneous resistance against different anthelmintic (AH) classes in small ruminant farms in Latin America (Torres-Acosta et al., 2012). This resistance has spurred interest in alternative GIN control methods, such as bioactive plants as non-conventional AHs (Sandoval-Castro et al., 2012). Many research groups have identified bioactive plants with promising AH activity under temperate and tropical conditions. Studies of the biologically active fractions of these plants have revealed a relationship between various secondary metabolites and *in vitro* AH activity against eggs and larvae of *H. contortus*. Secondary metabolite groups that have been implicated in AH activity include acetogenins, alkaloids and terpenoids (Souza et al., 2008; Marie-Magdeleine et al., 2010a, 2010b). In those studies, the AH activity was not confirmed by testing either molecules isolated from plant extracts or pure compounds obtained as commercial chemical standards.

Other natural products that have been associated with AH activity include polyphenols, both flavonoid and non-flavonoid. For example, *in vitro* AH activity of flavonoids such as condensed tannins (Hernández-Bolio et al., 2018), their monomers (Brunet and Hoste, 2006; Klongsiriwet et al., 2015) and compounds such as quercetin, luteonin (von Son-de Fernex et al., 2015; Castillo-Mitre et al., 2017; Klongsiriwet et al., 2015) and quercitrin (Hernández-Bolio et al., 2018) has been

⋆ This work was supported by CONACYT-México: CB-2013-01/221041
* Corresponding author.
E-mail addresses: maria.mancilla@correo.uady.mx (G. Mancilla-Montelongo), glosahi@hotmail.com (G.S. Castañeda-Ramírez), tacosta@correo.uady.mx (J.F.d.J. Torres-Acosta), carlos.sandoval@correo.uady.mx (C.A. Sandoval-Castro), rborges@cicy.mx (R. Borges-Argáez).

https://doi.org/10.1016/j.vetpar.2019.05.009
Received 14 January 2019; Received in revised form 17 May 2019; Accepted 18 May 2019

reported. Recent studies of tropical plants have also reported *in vitro* AH activity of non-flavonoid polyphenols, including biological activity of arbutin, caffeic acid, chlorogenic acid and derivatives of caffeoyl and coumaroyl acids against *H. contortus* (Hernández-Bolio et al., 2018; Jasso et al., 2017; Castillo-Mitre et al., 2017) and coumarin against *Cooperia punctata* (von Son-de Fernex et al., 2017). In these studies, AH activity was associated with an isolated metabolite found in a fraction rich in a few compounds. Furthermore, pure commercial standards were used to confirm the presence of the suspected active compounds but not to confirm AH activity. Such an approach was used to test a fraction of an *Acacia cochliacantha* extract containing a mixture of compounds (ferulic and coumaric acids) with AH activity against eggs of *H. contortus* (Castillo-Mitre et al., 2017). A recent study also showed the majority presence of coumaric acid in a fraction obtained from *Senegalia gaumeri* with high AH activity against *H. contortus* eggs. However, the ovicidal effect of pure coumaric acid was very low (2.9%) (Castañeda-Ramírez et al., 2019).

Several non-flavonoid polyphenols reported to have ovicidal activity against nematode eggs in *in vitro* studies are members of the cinnamic acid family, suggesting that it could be important to study structural analogues of cinnamic acid. Structural analogues share a common base structure but differ in substituents. Studies of the behaviour of a family of compounds in the context of a certain activity or reaction can be used to analyse potential structure-activity relationships (SAR). Cinnamic acid analogues (cinnamic, coumaric, caffeic, ferulic, sinapic, dimethoxycinnamic and chlorogenic acids) (Fig. 1) differ in the presence of hydroxyl groups, free or methoxylated, on the aromatic ring. Therefore, the objective of the present study was to assess the *in vitro* AH activity of cinnamic acid and six structural analogues against *H. contortus* eggs and infecting larvae and to identify a relationship between structure and biological activity.

2. Materials and methods

2.1. Production of Haemonchus contortus eggs

The *H. contortus* Paraiso isolate, which was previously characterized as resistant to benzimidazoles and polyphenols, was used in the present study (Chan-Pérez et al., 2016). Two four-month-old lambs (25 ± 1 kg) were used as donors. The donor animals were previously confirmed to be free of GINs and were housed individually in metabolic cages during the experimental period. The donors were orally infected with *H. contortus* (6000 L_3). Faecal samples were obtained from the donors on day 28 after infection to confirm the presence of *H. contortus* eggs. All procedures performed on donor animals complied with the ethical standards of the Bioethics Committee of the Faculty of Veterinary Medicine, Universidad Autónoma de Yucatán, México (license No. CB-CCBA-D-2014-003). Donor animals were fed a commercial concentrated feed (300 g fresh basis/d) and chopped *Pennisetum purpureum* grass (750 g fresh basis/d). Water was provided *ad libitum*.

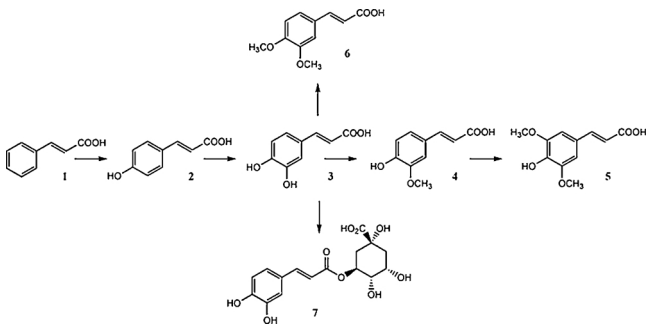

Fig. 1. Chemical compounds derived from cinnamic acid via the shikimic acid pathway. (1) *trans*-Cinnamic acid, (2) *p*-Coumaric acid, (3) Caffeic acid, (4) *trans*-Ferulic acid, (5) *trans*-Sinapic acid, (6) 3,4-Dimethoxycinnamic acid, and (7) Chlorogenic acid.

2.2. Recovery of H. contortus eggs for the egg hatch test

Following MAFF procedures (1986), the faecal samples were collected directly from the rectum of donors into the plastic bag which was then turned inside out, the bag was tied in the neck and was labeled with the corresponding animal number. Fresh samples were processed within three hours after collection. The faeces were macerated in a plastic container with purified water (100 mL/10 g of faeces). The mixture was filtered and centrifuged (453 *g* for 5 min) in 15-mL tubes. The sediment was recovered, re-suspended in a saturated sugar solution (SG = 1.28), and centrifuged at 453 *g* for 5 min. With the aid of a bacteriological loop, eggs of *H. contortus* were recovered from the surface and placed in 15-mL tubes containing 10 mL of phosphate-buffered saline (PBS, pH 7.4, Sigma®). The concentration of eggs/mL of PBS was determined, and the suspension was diluted to obtain a concentration of 150 eggs/mL.

2.3. Production of H. contortus L_3

The faeces were collected in a plastic mesh located below the slatted floor of each metabolic cage where donor animals were kept. The plastic mesh was cleaned in the afternoon, and the total amount of faeces was collected overnight. The collected faeces were mixed with some concentrate food, pieces of grass and hair debris from the donor animals. The food and hair debris were removed from the faeces in the parasitology laboratory by rinsing the faeces with tap water in a plastic kitchen colander. The rinsed faeces were placed in plastic cylindrical containers (1 L of capacity), filled up to three quarters of their capacity. Containers with faeces were labeled with date of coproculture and were covered with a gauze. The coproculture was incubated at 28 °C for 4 days. The faecal cultures were mixed daily to maintain oxygenation. At the end of the incubation period, the L_3 were recovered using the Baermann technique (Ministry of Agriculture, Fisheries and Food (MAFF, 1986).

2.4. Egg hatch test

The egg hatch test (EHT) was used to evaluate the *in vitro* AH activity of the pure compounds, against eggs of *H. contortus* as described by von Samson-Himmelstjerna et al. (2009) and Jackson and Hoste (2010) with some modifications as described below.

Stock solutions of each of the seven analogues (*trans*-cinnamic acid, *p*-coumaric acid, caffeic acid, *trans*-ferulic acid, *trans*-sinapic acid, 3,4-dimethoxycinnamic acid, and chlorogenic acid; purity > 98%, Sigma®) were prepared at 1500 µg/mL in PBS:Tween-20 (1%). Subsequently, 0.5 mL containing different concentrations of each analogue (from 25 to 400 µg/mL) was added to each well of a 24-well plate. Then, 0.5 mL of *H. contortus* egg suspension (150 eggs/mL) was added to obtain a final volume in each well of 1 mL. Each analogue concentration was tested in six replicates. The plates were incubated for 48 h at 28 °C, followed by the addition of 100 µL of Lugol's iodine solution to each of the wells to stop the egg hatching process.

The contents of each well were counted with the aid of a compound microscope, including the number of morulated eggs (ME), which was considered to indicate the true ovicidal activity, the number of eggs containing larvae failing to hatch (LFH, named as LFE for larvae failing eclosion in other studies) and the number of L_1 present in the sample (Vargas-Magaña et al., 2014).

2.5. Evaluation of combinations of structural analogues in the EHT

After each structural analogue of cinnamic acid was tested individually, further evaluations of the following analogue mixtures (M) were performed: M1, all compounds together; M2, analogues blocking

larval hatching; M3, analogues with any type of activity against eggs; M4, analogues damaging L_1 larvae outside the egg; and M5, analogues with ovicidal activity. The mixtures of analogues were evaluated in the EHT at concentrations ranging from 40 to 2793 µg/mL.

2.6. Larval exsheathment inhibition test

The larval exsheathment inhibition test (LEIT) was used to evaluate the AH activity of the analogues against L_3. A stock solution (5000 µg/mL PBS) of each analogue was prepared. Larvae suspended in PBS were used as a negative control. One ml solutions of each analogue were prepared to obtain different concentrations in separate 15-mL tubes (1200, 600, 400, 200, 100 and 30 µg/mL). One ml of suspension containing L_3 of *H. contortus* (1000 L_3/mL) were added to each tube and incubated for 3 h at 23 °C. After the incubation period, the tubes were centrifuged at 453 *g* for 5 min, and the L_3 were washed three times by removing 1 mL of supernatant and adding 1 mL of PBS. The remaining suspension in each tube was divided into 4 aliquots of 200 µL each in 1.5-mL vials. Each vial was labelled with the concentration evaluated and refrigerated overnight.

After incubation, larval exsheathment of the L_3 was induced by the addition of a solution of sodium hypochlorite (2% w/v) and sodium chloride (16.5% w/v). Each analogue was evaluated individually at different concentrations (control PBS, 1200, 600, 400, 200, 100 and 30 µg of analogue/mL). The exsheathment process was observed, and the number of larvae with or without a sheath was counted, starting from time zero (before contact with the chlorine solution) and continuing at 20 min intervals (20, 40 and 60 min) (Jackson and Hoste, 2010).

2.7. Data analysis

All concentration values, 50% and 90% effective concentrations (EC_{50} and EC_{90}) and confidence intervals (CI) were expressed as µg/mL, as commonly reported when a mixture of compounds (*i.e.* fraction or extract of a plant) is evaluated. Individually evaluated analogues were also expressed as millimolar (mM). This unit enables a more precise estimation of AH activity by allowing active molecules to be compared independently of their molecular size.

For each EHT, the ME, LFH and L_1 present in the sample were recorded for all pure tested analogues and their mixtures at the respective concentrations previously described. This information was used to determine the egg-hatching rate (EH) as follows (Vargas-Magaña et al., 2014; Chan-Pérez et al., 2016):

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

$$LFH = \frac{\text{Number of eggs containing larvae}}{\text{Number of morulated eggs} + \text{number of eggs containing larvae} + \text{number of larvae}} \times 100$$

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

The number of eggs (ME + LFH) and L_1 were used to determine the effective concentration required to inhibit 50% of hatching. The EC_{50} and EC_{90} in the EHT were estimated for each standard and the mixtures using PoloPlus 1.0 software (LeOra software, 2004). The respective 95% CIs were also calculated. When the respective 95% CIs of the EC_{50} or the EC_{90} did not overlap, then these values were considered significantly different (Schenker and Gentleman, 2001).

The median values of the raw ME, LFH and L1 data were analysed using a Kruskal-Wallis test by comparing the tested concentrations with their respective controls to evaluate significant differences ($P < 0.05$). One-way ANOVA was used in the case of caffeic acid, for which the data were normally distributed. The data for all other compounds and mixtures were analysed with Fisher's least significant difference in Statgraphics Centurion 18 software (Statgraphics Technologies Inc., 2018).

The exsheathment inhibition percentage (EI%) was calculated for each pure analogue and their mixtures as described by Chan-Pérez et al. (2017):

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

$$\text{EI}\,\% = 100 - \text{Exsheathment}\,\%$$

The LEIT results for the different standards at 60 min were used to determine their EC_{50} and EC_{90} (both µg/mL and mM concentrations) with corresponding 95% CIs against *H. contortus* larvae (L_3) with PoloPlus 1.0 software.

3. Results

3.1. AH activity of the analogues in the EHT

The percentages of ME, LFH and L_1 at each concentration of the different analogues are presented in Fig. 2. Ferulic acid showed significant AH activity, with > 74% egg hatch inhibition at 300 µg/mL and > 65% at 400 µg/mL (Fig. 2D). Meanwhile, at that concentration chlorogenic acid showed > 39% inhibition (Fig. 2G). Both compounds had clear LFH activity and negligible ovicidal activity. Cinnamic, coumaric and caffeic acids did not show significant hatching inhibition effects at the concentrations evaluated. However, these three compounds produced structural damage (degradation of the cuticle, the intestinal cells) and death of the emerged larvae.

Effective concentrations (EC_{50} and EC_{90}) were only determined for two compounds: ferulic acid, with an EC_{50} of 245.2 µg/mL (95% CI 195.3–316.5), and chlorogenic acid, with an EC_{50} of 520.8 µg/mL (95% CI 473.8–564.9). Ferulic acid had an EC_{90} of 462.9 µg/mL (95% CI 348.0–960.1), while chlorogenic acid had an EC_{90} of 891.9 µg/mL (95% CI 756.9–1306.3). The EC_{50} and EC_{90} of ferulic and chlorogenic acids differed significantly when the concentrations were expressed in µg/mL but were similar when expressed in millimolar (mM). The EC_{50} was 1.26 mM (95% CI 1.0–1.6) for ferulic acid and 1.47 mM (95% CI 1.33–1.59) for chlorogenic acid. The EC_{90} was 2.37 mM (95% CI 1.79–4.94) for ferulic acid and 2.5 mM (95%CI 2.13–3.68) for chlorogenic acid.

3.2. AH activity of different mixtures of analogues in the EHT

Five different mixtures of analogues were tested in the EHT: M1 included all seven analogues; M2 included the two analogues with LFH activity (ferulic and chlorogenic acids); M3 included the two analogues with activity against eggs (ferulic and chlorogenic acids) plus the three compounds damaging L_1 (cinnamic, coumaric and caffeic acids); M4 included only three analogues damaging larvae outside the egg (cinnamic, coumaric and caffeic acids); and M5 included the two analogues with more obvious ovicidal activity (coumaric and cinnamic acids). The AH activities of the different mixtures against eggs are presented in Table 1. The mixtures with significant AH activity were M2 (EC_{50}: 1628.10 µg/mL), M3 (1143.75 µg/mL) and M4 (923.37 µg/mL). M1 and M5 did not present significant AH activity against eggs.

3.3. AH activity of the analogues in the LEIT

The EC_{50} and EC_{90} values of the analogues in the LEIT are summarized in Table 2. Caffeic acid had the best AH activity, with an EC_{50} of 0.04 mM, followed by ferulic acid with an EC_{50} of 0.11 mM. The EC_{50}

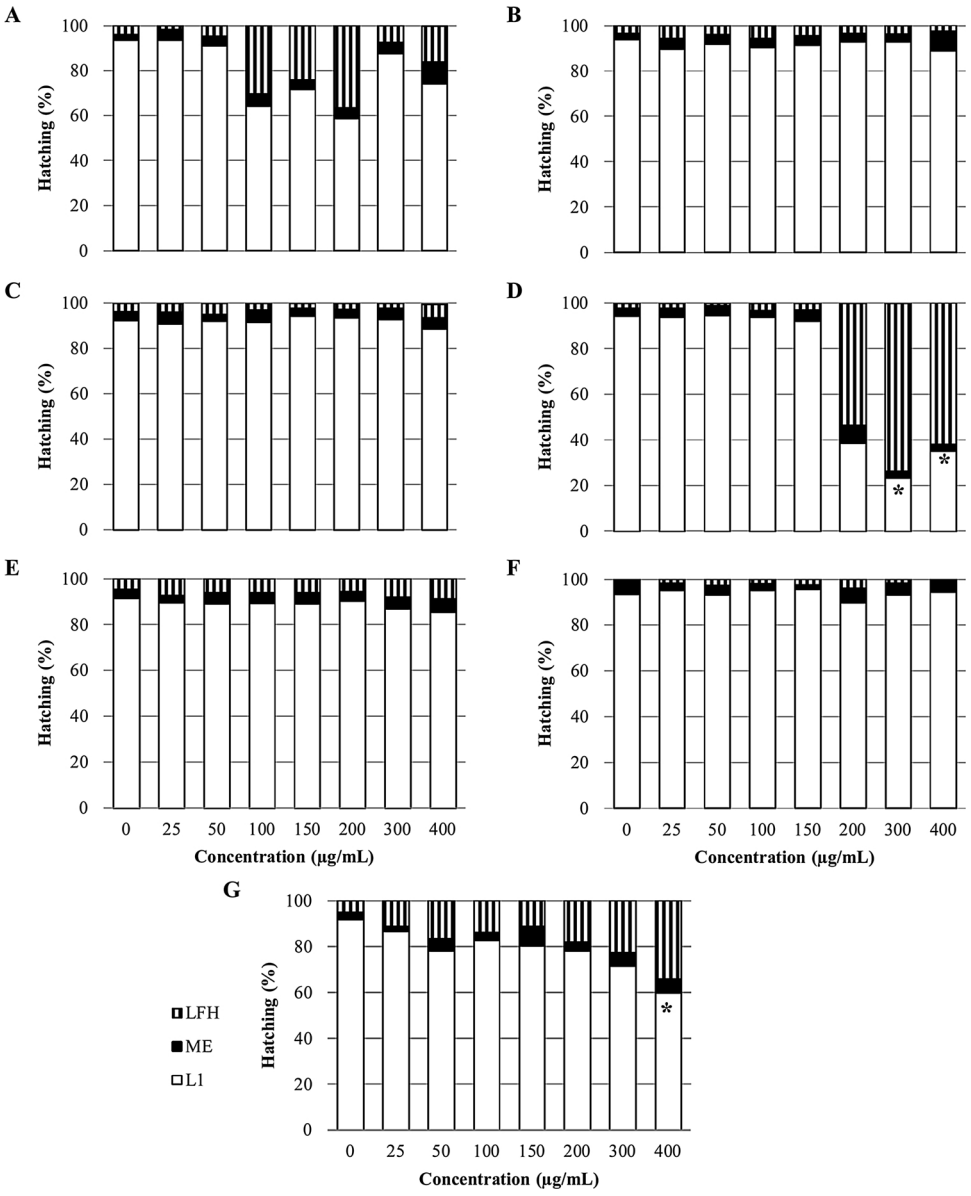

Fig. 2. Percentages of *Haemonchus contortus* morulated eggs (ME), larvae failing to hatch (LFH) and total larvae (L_1) at different concentrations of the different analogues of cinnamic acid. (A) *trans*-Cinnamic acid, (B) *p*-Coumaric acid, (C) Caffeic acid, (D) *trans*-Ferulic acid, (E) *trans*-Sinapic acid, (F) 3,4-Dimethoxycinnamic acid, and (G) Chlorogenic acid.
* Significantly different ($P < 0.05$) according to the Kruskal-Wallis test.

Table 1
Effective concentration 50% (EC_{50}) and 90% (EC_{90}) with their respective 95% confidence intervals (95% CI) of the mixtures of analogues against *Haemonchus contortus* eggs. Mixtures: M1, all compounds together; M2, analogues blocking larval hatching (ferulic and chlorogenic acids); M3, analogues with any type of activity against eggs (ferulic, chlorogenic, cinnamic, coumaric and caffeic acids); M4, analogues damaging L_1 larvae outside the egg (cinnamic, coumaric and caffeic acids); and M5, analogues with ovicidal activity (coumaric and cinnamic acids).

Mixture	EC_{50} µg/mL	95% CI µg/mL	EC_{90} µg/mL	95% CI µg/mL
M1	1628.10[B]	1535.97-1729.10	2138.90[B]	1987.37-2362.36
M2[†]	—			
M3	923.37[A]	651.72-1359.89	2538.37[B]	1635.88-7047.17
M4	1143.75[A]	1107.80-1186.43	1409.21[A]	1331.72-1539.95
M5[†]	–	——	—	——

[A, B] Different letters in [the] same column means a significant difference.
[†] EC_{50}, and EC_{90} with their corresponding CI were not estimated due to lack of AH activity.

values of the remaining compounds did not differ significantly and ranged from 0.23 to 0.41 mM. The EC_{90} values ranged from 0.43 to 0.91 mM. Caffeic and ferulic acids had similar EC_{90} values (0.18 and 0.26 mM, respectively).

4. Discussion

The seven cinnamic acid structural analogues investigated in this study have been reported to have antimicrobial (Guzman, 2014), antioxidant (Bratt et al., 2003), and anticancer properties (De et al., 2011), as well as multiple effects on human health (Naveed et al., 2018). Two of these phenolic compounds have been identified in plant extract fractions with AH activity against *H. contortus* eggs (Castillo-Mitre et al., 2017; Jasso et al., 2017; Castañeda-Ramírez et al., 2019), but whether these two or other phenolic compounds of the shikimic acid pathway are active against eggs or L_3 of *H. contortus* is unknown. Consequently, this study investigated the *in vitro* AH activity of these seven commercially available cinnamic acid analogues against *H.*

Table 2
Effective concentration 50% (EC_{50}) and 90% (EC_{90}) with their respective 95% confidence intervals (95% CI) of the seven cinnamic acid analogues against *Haemonchus contortus* larvae (L_3).

Analogue	EC_{50} μg/mL (mM)	95% CI μg/mL (mM)	EC_{90} μg/mL (mM)	95% CI μg/mL (mM)
trans-Cinnamic acid	34.4^{C} (0.23^{C})	27.1 - 42.9(0.18-0.29)	101.4^{CD} (0.68^{B})	74.4 - 175.3(0.50-1.18)
p-Coumaric acid	39.5^{C} (0.24^{C})	31.1-47.9 (0.19-0.29)	86.3^{C} (0.53^{B})	69.6-118.9 (0.42-0.72)
Caffeic acid	7.8^{A} (0.04^{A})	1.7-13.8 (0.01-0.08)	32.4^{A} (0.18^{A})	21.7- 42.7 (0.12-0.24)
trans-Ferulic acid	20.6^{B} (0.11^{B})	15.3-24.7 (0.08-0.13)	49.9^{B} (0.26^{A})	43.1- 62.1 (0.22-0.32)
trans-Sinapic acid	91.2^{D} (0.41^{C})	65.3-124.4 (0.29-0.55)	204.7^{D} (0.91^{B})	146.1-385.2 (0.65-1.72)
3,4-Dimethoxycinnamic acid	55.2^{CD} (0.26^{C})	30.8-85.1 (0.15-0.41)	107.4^{C} (0.52^{B})	71.9-292.4 (0.34-1.40)
Chlorogenic acid	92.4^{D} (0.26^{C})	80.2 -117.2 (0.23-0.33)	153.6^{D} (0.43^{B})	119.8 - 359.7 (0.34-1.02)

$^{A, B, C, D}$ Different letters in the same column means a significant diference according to concentration units: μg/mL or (mM).

contortus eggs and L_3.

4.1. Activity of the cinnamic acid analogues against eggs

Ferulic and chlorogenic acids showed significant LFH effects against *H. contortus* eggs (Fig. 2D and 2 G). AH activity has previously been suggested for *trans*-ferulic acid in a fraction of an *A. cochliacantha* extract (Castillo-Mitre et al., 2017) and for chlorogenic acid in a *Tagetes filifolia* extract (Jasso et al., 2017).

The five remaining analogues did not have AH activity against *H. contortus* eggs. This result is consistent with findings by Castañeda-Ramírez et al. (2019) that coumaric acid in a fraction of *S. gaumeri* extract had no significant AH activity against *H. contortus* eggs despite the significant ovicidal activity of that fraction. This discrepancy highlights the importance of confirming that a suspected compound is the active molecule by using a pure compound/standard before concluding that its presence is responsible for AH activity.

Varying results were obtained in the evaluation of the different mixtures of cinnamic acid analogues. The mixture of all analogues together (M1) showed AH activity (LFH) against *H. contortus* eggs (Table 2), and the EC_{50} of M1 against eggs was higher than the values of the two analogues with activity (ferulic or chlorogenic acid). This result suggested that the AH activity of M1 was mainly due to the two active analogues. However, the AH activity against eggs was lost when a mixture containing only these two active analogues (M2) was tested. This latter result suggests that the two compounds are antagonistic or that M1 contained one or more synergistic compounds.

M3, which excluded only the two non-active analogues, sinapic and dimethoxycinnamic acids, exhibited a significant increase in LFH activity compared with M1, suggesting that sinapic and dimethoxycinnamic acids contributed only their masses to the EC_{50} value of M1.

The next mixture (M4) contained only cinnamic, coumaric and caffeic acids and exhibited an EC_{50} similar to that of M3. Both M3 and M4 had lower EC_{50} values than M1. The AH activity of M4 suggests that cinnamic, coumaric or caffeic acid might act as a synergistic compound.

Finally, when caffeic acid was removed (M5), AH activity against eggs was lost, suggesting that caffeic acid acts as a synergistic compound, even though this acid is not active by itself against *H. contortus* eggs. Synergistic AH activity of caffeic acid has been previously described in combination with quercetin in EHTs of an extract of *Leucaena leucocephala* enriched in caffeic acid and/or quercetin against eggs of *Cooperia* spp. (von Son-de Fernex et al., 2015). Similar to the results of the present study, no AH activity against eggs of *Cooperia* spp. was observed when caffeic acid was evaluated individually.

In a previous study, the SAR between different molecules and AH activity against adult *Bursaphelenchus xylophilus* nematodes was associated with the molecules' functional groups, bond saturation and carbon structures (Jeong-Ok et al., 2007). In the case of condensed tannin monomers, the SAR depended on the number of hydroxyl groups in the aromatic ring; the higher the number of hydroxyl groups, the higher the AH activity against *H. contortus* L_3 (Brunet and Hoste, 2006). The analogues evaluated in the present study differed in the number of hydroxyl groups, including both free and protected in the form of methoxy groups. However, no clear, definitive SAR between the structures of the tested non-flavonoid polyphenols and their AH activity against *H. contortus* eggs could be identified in the present study. The two compounds that showed LFH effects (ferulic and chlorogenic acids) presented similar EC_{50} values (< 1.5 mM), and both compounds contained two substituents in the benzene ring. The three additional hydroxyl groups in chlorogenic acid did not result in greater activity compared to ferulic acid. Caffeic acid was expected to have some AH activity, given the presence of two free hydroxyl groups, but was not active by itself against eggs. Sinapic and dimethoxycinnamic acids, in which the hydroxyl groups are blocked (as methoxy), were not active against eggs of *H. contortus*.

Although we could not define a SAR, our results suggest that the activity of the active compounds is related to the inhibition of processes related to hatching, such as proteolysis, lipolysis and activation of matrix metalloproteinases (Raghavendra et al., 2007; Qiang, 2011; Sullivan and Zeller, 2013). The AH activity of the phenolic molecules against *H. contortus* eggs could also be explained by their ability to bind to proteins to form a complex film (Ndhlala et al., 2015). Thus, it is possible that binding of the active analogues to eggshell proteins prevented the hatching of larvae from eggs, leading to the LFH effect.

4.2. Activity of cinnamic acid analogues against larvae

All tested compounds showed AH activity in the LEIT, and caffeic acid had the lowest EC_{50}. All other tested analogues had EC_{50} < 93 μg/mL (or < 0.41 mM). Thus, the AH activities of the tested analogues suggest a need to further confirm their effects against other stages of the parasite under *in vitro* (*i.e.* L_4 or adults) or *in vivo* conditions. Only two previous studies have investigated the AH activity of non-flavonoid phenolic compounds against the exsheathment of *H. contortus* L_3. Klongsiriwet et al. (2015) showed that two non-flavonoid compounds of synthetic origin (vanillic acid and arbutin) were not active against L_3. However, another study reported AH activity of arbutin against L_3 of *H. contortus* (Hernández-Bolio et al., 2018). The discrepancy between these two studies could be related to the nature of the compound (synthetic *vs*. natural) and the origin of the *H. contortus* isolates, as the first study used a temperate isolate and the second used a tropical isolate that has been reported to have low susceptibility to polyphenols (Chan-Pérez et al., 2016).

The AH activity of cinnamic acid analogues against L_3 exsheathment has not been studied previously. The activity of these analogues might be associated with the transthyretin-type proteins of *H. contortus* infective larvae. The main role of these proteins is to transport lipophilic substances (Cantacessi et al., 2010), such as the cinnamic acid analogues evaluated in this study. Transport proteins may facilitate the entrance of the evaluated compounds through collagen networks, which are common in the sheath of *H. contortus*. Once the compounds are present in the sheath, they may interact to prevent exsheathment of L_3 at low EC_{50} (0.04 to 0.4 mM). The cinnamic acid analogues might also interact with/inhibit the function of proteinases, lipases and

aminopeptidases, all of which are involved in the exsheathment process (Rogers, 1982; Gamble et al., 1989). Further studies are necessary to improve our understanding of the interactions between these analogues and to design adequate strategies for the *in vivo* evaluation of these compounds or mixtures of compounds with greater AH activity.

5. Conclusion

The cinnamic acid analogues ferulic and chlorogenic acids showed significant hatching inhibition effects. This activity was associated with larvae failing eclosion from eggs, but a structure-activity relationship could not be defined. All mixtures of compounds tested were less active than ferulic and chlorogenic acids, which were mutually antagonistic, while caffeic acid acted as a synergistic agent. In addition, all evaluated analogues blocked the exsheathment of L_3, but the concentration corresponding to AH activity was lowest for caffeic acid, followed by ferulic acid.

Conflictof interest statement

None

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was financed by CONACYT-México (project number: CB-2013-01/221041). S.C.R. thanks CONACYT-México for granting her a scholarship to pursue PhD studies (Reference number 7464535353). G.M. thank the support of the Catedras CONACYT-México Programme (Project number 692).

References

Bratt, K., Sunnerheim, K., Bryngelsson, S., Fagerlund, A., Engman, L., Andersson, R.E., Dimberg, L.H., 2003. Avenanthramides in oats (Avena sativa L.) and structure – antioxidant activity relationships. J. Agric. Food Chem. 51, 594–600.

Brunet, S., Hoste, H., 2006. Monomers of condensed tannins affect the larval exsheathment of parasitic nematodes of ruminants. J. Agric. Food Chem. 54, 7481–7487.

Cantacessi, C., Campbell, B.E., Young, N.D., Jex, A.R., Hall, R.S., Presidente, P.J., Zawadzki, J.L., Zhong, W., Aleman-Meza, B., Loukas, A., Sternberg, P.W., 2010. Differences in transcription between free-living and CO_2-activated third-stage larvae of *Haemonchus contortus*. BMC Genomics 11, 266.

Castañeda-Ramírez, G.S., Mancilla, G., Torres-Acosta, J.F.J., Sandoval-Castro, C.S., Borges-Argáez, R., Cáceres-Farfán, M., Mathieu, C., 2019. Bio-guided fractionation to identify the anthelmintic compounds of *Senegalia gaumeri* leaf extract against *Haemonchus contortus* eggs and larvae. Vet. Parasitol. 270, 13–19. https://doi.org/10.1016/j.vetpar.2019.05.001.

Castillo-Mitre, G.F., Olmedo-Juárez, A., Rojo-Rubio, R., González-Cortázar, M., Mendoza-de Gives, P., Hernández-Beteta, E.E., Reyes-Guerrero, D.E., López-Arellano, M.E., Vázquez-Armijo, J.F., 2017. Caffeoyl and coumaroyl derivatives from *Acacia cochliacantha* exhibit ovicidal activity against Haemonchus contortus. J. Ethnopharmacol. 204, 125–131.

Chan-Pérez, J.I., Torres-Acosta, J.F.J., Sandoval-Castro, A.C., Hoste, H., Castañeda-Ramírez, G.S., Vilarem, G., Mathieu, C., 2016. *In vitro* susceptibility of ten *Haemonchus contortus* isolates from different geographical origins towards acetone:-water extracts of two tannin rich plants. Vet. Parasitol. 217, 53–60.

Chan-Pérez, J.I., Torres-Acosta, J.F.J., Sandoval-Castro, C.A., Castañeda-Ramírez, G.S., Vilarem, G., Mathieu, C., Hoste, H., 2017. Susceptibility of ten *Haemonchus contortus* isolates from different geographical origins towards acetone: water extracts of polyphenol-rich plants. Part 2: infective L_3 larvae. Vet. Parasitol. 240, 11–16.

De, P., Baltas, M., Bedos-Belval, F., 2011. Cinnamic acid derivatives as anticancer agents – a review. Curr. Med. Chem. 18, 1672–1703.

Gamble, H.R., Lichtenfels, J.R., Purcell, J.P., 1989. Light and scanning electron microscopy of the ecdysis of *Haemonchus contortus* infective larvae. J. Parasitol. 75, 303–307.

Guzman, J.D., 2014. Natural cinnamic acids, synthetic derivatives and hybrids with antimicrobial activity. Molecules 19, 19292–19349.

Hernández-Bolio, G.I., Kutzner, E., Eisenreich, W., Torres-Acosta, J.F.J., Peña Rodríguez, L.M., 2018. The use of 1H–NMR Metabolomics to optimise the extraction and preliminary identification of anthelmintic products from the leaves of Lysiloma latisiliquum. Phytochem. Anal. 29, 413–420.

Hoste, H., Torres-Acosta, J.F.J., 2011. Non chemical control of helminths in ruminants: adapting solutions for changing worms in a changing world. Vet. Parasitol. 180, 144–154.

Jackson, F., Hoste, H., 2010. *In vitro* methods for primary screening of plant products for direct activity against ruminant nematodes, *in vitro* methods for primary screening of plant products for direct activity against ruminant nematodes. In: Vercoe, P.E., Makkar, H.P.S., Schlink, A.C. (Eds.), *In Vitro* Screening of Plant Resources for Extra-Nutritional Attributes in Ruminants: Nuclear and Related Methodologies. Springer, pp. 25–45 252 pp.

Jasso, G.D., Hernández, G.T., Zamilpa, A., Becerril, C.P., Ramírez, J.B., Hernández, O.M., Sánchez, H.A., González, M.C., de Gives Mendoza, P., 2017. *In vitro* assessment of Argemone mexicana, Taraxacum officinale, Ruta chalepensis and *Tagetes filifolia* against *Haemonchus contortus* nematode eggs and infective (L_3) larvae. Microb. Pathog. 109, 162–168.

Jeong-Ok, K., Sang-Myung, L., Yil-Seong, M., Sang-Gil, L., Young-Joon, A., 2007. Nematicidal activity of cassia and cinnamon oil compounds and related compounds toward *Bursaphelenchus xylophilus* (Nematoda: parasitaphelenchidae). J. Nematol. 39, 31–36.

Klongsiriwet, C., Quijada, J., Williams, A.R., Mueller-Harvey, I., Williamson, E.M., Hoste, H., 2015. Synergistic inhibition of *Haemonchus contortus* exsheathment by flavonoid monomers and condensed tannins. Int. J. Parasitol. Drugs Drug Resist. 5, 127–134.

LeOra Software, 2004. Polo Plus. Probit and Logit Analysis. Berkeley. LeOra Software, California, U.S.A.

Ministry of Agriculture, Fisheries and Food (MAFF), 1986. Manual of Veterinary Parasitological Laboratory Techniques (Reference Book; 418), 3rd ed. Her Majesty's Stationery Office (HMSO), London 160 p.

Marie-Magdeleine, C., Mahieu, M., D'Alexis, S., Philibert, L., Archimede, H., 2010a. *In vitro* effects of *Tabernaemontana citrifolia* extracts on *Haemonchus contortus*. Res. Vet. Sci. 89, 88–92.

Marie-Magdeleine, C., Udino, L., Philibert, L., Bocage, B., Archimede, H., 2010b. *In vitro* effects of Cassava *(Manihot esculenta)* leaf extracts on four development stages of *Haemonchus contortus*. Vet. Parasitol. 173, 85–92.

Naveed, M., Hejazi, V., Abbas, M., Kamboh, A.A., Khan, G.J., Shumzaid, M., Ahmad, F., Babazadeh, D., FangFang, X., Modarresi-Ghazani, F., WenHua, L., 2018. Chlorogenic acid (CGA): a pharmacological review and call for further research. Biomed. Pharmacother. 97, 67–74.

Ndhlala, A.R., Ghebrehiwot, H.M., Ncube, B., Aremu, A.O., Gruz, J., Šubrtová, M., Doležal, K., du Plooy, C.P., Abdelgadir, H.A., Van Staden, J., 2015. Antimicrobial, anthelmintic activities and characterisation of functional phenolic acids of *Achyranthes aspera* Linn.: a medicinal plant used for the treatment of wounds and ringworm in East Africa. Front. Pharmacol. 6, 1–8.

Qiang, Z., 2011. Bioavailability and Metabolism of Botanical Constituents and Enhancement of Intestinal Barrier Function by Caffeic Acid Derivatives in Caco-2 Cells. Iowa State University, Ames. Iowa.

Raghavendra, M.P., Jumar, P.R., Prakash, V., 2007. Mechanism of inhibition of rice bran lipase by polyphenols: a case study with chlorogenic acid and caffeic acid. J. Food Sci. 72, E412–E419.

Rogers, W.P., 1982. Enzymes in the exsheathing fluid of nematodes and their biological significance. Inter. J. Parasitol. 12, 495–502.

Sandoval-Castro, C.A., Torres-Acosta, J.F.J., Hoste, H., Salem, A.Z.M., Chan-Pérez, J.I., 2012. Using plant bioactive materials to control gastrointestinal tract helminths in livestock. Anim. Feed Sci. Technol. 176, 192–201.

Schenker, N., Gentleman, J.F., 2001. On judging the significance of differences by examining the overlap between confidence intervals. Am. Stat. 55, 182–186.

Souza, M., Belvi, C., Morais, S.M., Costa, C., Silva, A., Braz-filho, R., 2008. Anthelmintic acetogenin from *Annona squamosa* L. Seeds. An. Da Acad. Bras. Ciãªncias 80, 271–277.

Statgraphics Technologies Inc, 2018. Statgraphics Centurion 18 Warranton, VA, U.S.A.

Sullivan, M.L., Zeller, W.E., 2013. Efficacy of various naturally occurring caffeic acid derivatives in preventing post-harvest protein losses in forages. J. Sci. Food Agric. 93, 219–226.

Torres-Acosta, J.F.J., Mendoza-de-Gives, P., Aguilar-Caballero, A.J., Cuéllar-Ordaz, J.A., 2012. Anthelmintic resistance in sheep farms: update of the situation in the American continent. Vet. Parasitol. 189, 89–96.

Vargas-Magaña, J.J., Torres-Acosta, J.F.J., Aguilar-Caballero, A.J., Sandoval-Castro, C.A., Hoste, H., Chan-Pérez, J.I., 2014. Anthelmintic activity of acetone-water extracts against *Haemonchus contortus* eggs: interactions between tannins and other plant secondary compounds. Vet. Parasitol. 206, 322–327.

von Samson-Himmelstjerna, G., Coles, G.C., Jackson, F., Bauer, C., Borgsteede, F., Cirak, V.Y., Demeler, J., Donnan, A., Dorny, P., Epe, C., Harder, A., Höglund, J., Kaminsky, R., Kerboeuf, D., Küttler, U., Papadopoulos, E., Posedi, J., Small, J., Várady, M., Vercruysse, J., Wirtherle, N., 2009. Standardization of the egg hatch test for the detection of benzimidazole resistance in parasitic nematodes. Parasitol. Res. 105, 825–834.

von Son-de Fernex, E., Alonso-Díaz, M.A., Mendoza-de Gives, P., Valles-de la Mora, B., González-Cortazar, M., Zamilpa, A., Epigmenio-Castillo, G., 2015. Elucidation of *Leucaena leucocephala* anthelmintic-like phytochemicals and the ultrastructural damage generated to eggs of *Cooperia* spp. Vet. Parasitol. 214, 89–95.

von Son-de Fernex, E., Alonso-Díaz, M.A., Valles-de la Mora, B., Mendoza-de Gives, P., González-Cortazar, M., Zamilpa, A., 2017. Anthelmintic effect of 2H-chromen-2-one isolated from *Gliricidia sepium* against *Cooperia punctata*. Exp. Parasitol. 178, 1–6.