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## Caesalpinia coriaria fruits and leaves extracts possess *in vitro* ovicidal activity against *Haemonchus contortus* and *Haemonchus placei*

### Abstract

The ovicidal effect of hydro-alcoholic extracts from mature fruits and dried leaves of the *Caesalpinia coriaria* tree, against *Haemonchus contortus* and *H. placei* was evaluated by the egg hatching inhibition test (EHIT). Secondary compounds within the extracts were identified by high performance liquid chromatography. Five extract concentrations were assessed in the inhibition tests (25.0, 12.5, 6.2, 3.1 and 1.5 mg/mL), using distilled water and Thiabendazole (0.5 mg/mL) as negative and positive controls, respectively. The results were analysed under a 2 × 2 × 6 completely randomized factorial design, and mean differences were established by the Tukey's post hoc test. The 50% (EC<sub>50</sub>) and 90% (EC<sub>90</sub>) effective concentrations for each extract were determined using a Probit analysis. A concentration-dependent ovicidal effect of hydro-alcoholic extracts from both leaves and fruits was observed, reaching a 100% efficacy against both nematode species with the 25.0 mg/mL concentration (p < 0.05). The EC<sub>50</sub> for the fruit and leaves extracts were established as 1.63 and 3.91 mg/mL, and as 3.98 and 11.68 mg/mL, against *H. contortus* and *H. placei*, respectively. The most important secondary compounds identified in extracts were free-condensed tannins, gallic acid and methyl-gallate. In conclusion, hydro-alcoholic extracts from *C. coriaria* mature fruits and dried leaves inhibit *in vitro* egg hatching of *H. contortus* and *H. placei*.

**Keywords:** *Haemonchus*, *Caesalpinia coriaria*, Secondary compounds, Tannins, Gallic acid

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## Introduction

Gastrointestinal nematodes that infect ruminants cause serious health problems that substantially decrease livestock productivity worldwide.<sup>1</sup> This condition is further complicated by resistance of parasites against existing anthelmintic drugs.<sup>2</sup> Thus, finding alternatives for parasitic control is necessary.

The use of secondary metabolites obtained from legume trees with anthelmintic (AH) properties has become of increasing interest in recent years,<sup>3-5</sup> presenting new natural alternatives for sustainable control of parasitic infections.

Reported secondary compounds with anthelmintic properties include condensed tannins, caffeic acid, ferulic acid and flavonoids.<sup>6</sup> Moreover, nematicidal activity of flavonoids can be enhanced by their interaction with other compounds such as tannins, which produces a synergic effect.<sup>7</sup> Isolated compounds such as caffeoyl and coumaroyl derivatives from a hydro-alcoholic extract of legume *Acacia cochliacantha* leaves, have also been shown to have a high ovicidal activity (close to 100%) against *Haemonchus contortus*.<sup>8</sup>

Plants from the Caesalpiniaceae subfamily, including *C. coriaria*, have analgesic, antibacterial, antifungal and anti-parasitic properties.<sup>9</sup> The objective of this work was to assess the ovicidal activity of mature fruits and leaves hydro-alcoholic extracts from *C. coriaria* against *H. contortus* and *H. placei*, by an egg-hatching inhibition test.

## Materials and methods

### *Plant material*

Mature fruits and leaves were collected from seven *Caesalpinia coriaria* trees at the site of Palmar Grande, Amatepec in the state of Mexico, Mexico (18° 35' 6" latitude north and 100° 24' 59" longitude west, with an altitude of 640 m above sea level). The taxonomic identification of the recovered plant material was performed by an experienced biologist, and a sample was deposited at the herbarium of the Centro de Investigación en Biodiversidad y Conservación from the Universidad Autónoma del Estado de Morelos, Mexico (Voucher code: 35273). Mature fruits and leaves were separately mixed to get a homogeneous sample from all trees, and immediately placed in a portable cooler at 4 °C, for transport to the Laboratory of Animal Nutrition of the University Center UAEM Temascaltepec. Plant material was subsequently left to dry under the shade, until reaching a 100% of dry matter and then ground in a Wiley mill (model 4; Thomas Scientific, Swedesboro, NY, USA), to obtain 2 mm particles.

### *Hydroalcoholic extracts*

To prepare extracts, 200 g of dry matter from either *C. coriaria* leaves or fruits were independently macerated with a hydro-alcoholic solution (70%, 1:10 ratio, w/v) at room temperature during 24 h. The liquid extracts, free of plant material, were obtained by successive filtration through cotton, four layers of cheesecloth and filter paper (Whatman® 541). Both extracts were then concentrated into a semisolid state, using a rotary evaporator at 50 °C (R-3 Heidolph, Germany). Finally, extracts

were totally dried by lyophilization (Labconco 4.5<sup>®</sup>) and stored at  $-40^{\circ}\text{C}$  until used for bioassays and phytochemical analyses.

### Condensed tannin content

Hydro-alcoholic extracts from fruits and leaves were analysed to quantify total content of condensed tannins (CTC), using the butanol-HCl method.<sup>10</sup> *Lysiloma acapulcensis* free condensed tannins (FCT) were used as internal standards.<sup>11</sup> Free condensed (FCT1), protein-bound (PCT) and fiber-bound (FCT2) tannins were identified following the technique reported by Porter *et al.*<sup>12</sup> Purification was performed using a Shepadex LH-20 column, as described by Hedqvist *et al.*<sup>13</sup>

### Identification of secondary compounds by HPLC analysis

Mature fruits and leaves hydro-alcoholic extracts were analysed using a Waters 2695 separation module HPLC system, equipped with a Waters 996 photodiode array detector and the Empower Pro software (Waters Corporation, USA). Chemical separation was achieved in a supelcosil LC-F column (4.6 mm  $\times$  250 mm i.d., 5- $\mu\text{m}$  particle size) (Sigma-Aldrich, Bellefonte, USA). A 0.5% trifluoroacetic acid aqueous solution (Solvent A) and acetonitrile (Solvent B) were used as the mobile phase. The gradient elution was as follows: 0-1 min, 0% B; 2-3 min, 5% B; 4-20 min, 30% B; 21-23 min 50% B; 24-25 min, 80% B; 26-27 min, 100% B; 28-30 min, 0% B. The flow rate was maintained at 0.9 mL/min, and the injection volume was 10  $\mu\text{L}$ . Absorbance was measured at 280 nm. Gallic acid and methyl gallate were identified by contrasting retention times and UV spectra with reference standards (Sigma-Aldrich, St Louis, Mo, USA).<sup>14</sup>

### Parasite eggs

*Haemonchus contortus* (INIFAP strain) and *H. placei* (wild strain) eggs were collected from feces obtained from an experimentally infected sheep ( $16 \pm 0.2$  kg BW) and calf ( $175 \pm 0.5$  kg BW), respectively. To obtain parasite eggs, feces from donor animals were washed, passed through different sieve sizes (200, 100, 72 and 32  $\mu\text{m}$ ) and spun with a 40% sucrose solution to separate thicker particles. The resulting material was further washed with tap water, subsequently concentrated by spinning (three times at 3500 rpm for 5 min), and finally re-suspended in sterile distilled water eggs were collected and used immediately in the bioassays.<sup>15</sup>

### Egg hatching inhibition test (EHIT)

Tests were conducted using three replicates and four repetitions in 96-well micro-titration plates. Six serial dilutions of both hydro-alcoholic extracts were included: 25.0, 12.5, 6.2, 3.1, 1.5, and 0.0 mg/mL. Distilled water and 0.5 mg/mL of thia-bendazole were used as negative and positive controls, respectively. One hundred eggs contained in 50  $\mu\text{L}$  of distilled water and 50  $\mu\text{L}$  of the corresponding extract (or control) were deposited in each well, resulting in a 100  $\mu\text{L}$  final volume per well. Plates were then incubated at  $28^{\circ}\text{C}$  with 100% humidity (at an incubator Ecoshell, mod CI-80) for 48 hours. Quantification of non-hatched eggs or larvae was per-

formed using an optical microscope (Motic B3<sup>®</sup>), in ten-5 µl aliquots placed on a slide per treatment. The egg hatching inhibition percentage was calculated by the following formula:  $[(\text{number of eggs})/(\text{number of larvae} + \text{number of eggs}) * 100]$ .<sup>15,16</sup>

### Statistical analysis

Data were analysed by ANOVA.<sup>17</sup> A 2 × 2 × 6 completely randomized factorial design was used.<sup>18</sup> Analysed factors were: 1) Two types of extract (leaves or fruits), 2) two nematode species (*H. contortus* and *H. placei*) and 3) six extract dilution (25.0, 12.5, 6.2, 3.1, 1.5, and 0.0 mg/mL). Mean differences were determined by the Tukey post hoc test ( $p \leq 0.05$ ). Finally, the Probit analysis was used to determine effective concentrations (EC<sub>50</sub> and EC<sub>90</sub>) for both extracts, using the SAS program.

## Results and discussion

### Condensed tannin and other secondary compound content in extracts

The quantity of free, fiber-bound, protein-bound and total condensed tannins for both *C. coriaria* hydro-alcoholic extracts is shown in [Table 1](#). Polyphenolic compounds in the free-condensed tannin form were the most abundant, with 217.1 g/kg found for the mature fruits extract, and 137.1 g/kg for the leaves extract, in a dry matter (DM) basis. Conversely, protein-bound tannins were present in the lowest amounts (31.7 g/kg, for the mature fruits extract, and 21.7 g/kg for the leaves extract in a DM basis).

The major compounds identified in both hydro-alcoholic extracts are shown in [Figure 1](#). The chromatographic analysis in the leaves extract revealed the presence of gallic acid and methyl-gallate ([Fig. 1 A](#)), whilst only methyl gallate was found in the mature fruits extract ([Fig. 1B](#)). [García-Hernández et al.](#)<sup>19</sup> have previously reported the presence of methyl gallate and gallic acid, as well as other galloyl derivatives, in a hydro-alcoholic extract from *C. coriaria* fruits. Likewise, [Sánchez-Carranza et al.](#)<sup>20</sup> found phenolic compounds, including ethyl gallate, gallic acid and tannic acid, in *C. coriaria* pods.

### Egg hatching inhibition

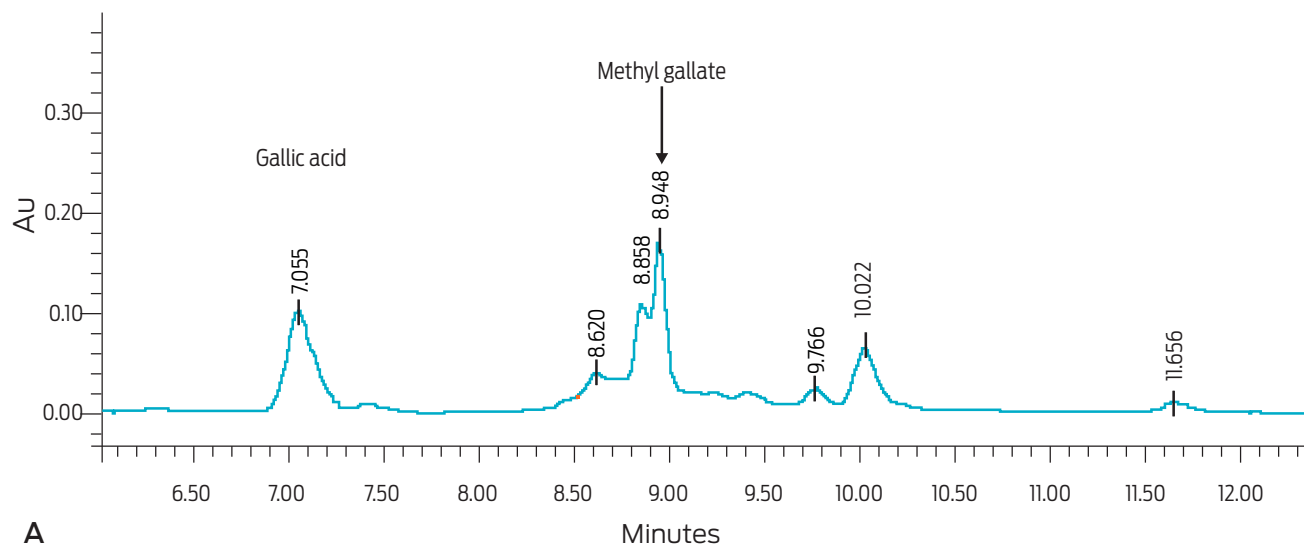
Inhibition of egg hatching in both nematode species increased with greater concentrations of hydro-alcoholic *C. coriaria* mature fruits or leaves extracts, reaching a 100% inhibition with the 25 mg/mL concentration ( $p < 0.01$ ) ([Table 2](#)). When analyzed independently of concentration or nematode spp., the hydro-alcoholic leaves extract seemed to be more efficient for inhibiting hatching of eggs than the extract from mature fruits (79 and 52% EHI respectively,  $p < 0.0001$ ). In addition, EHI percentages were higher for *H. contortus* than for *H. placei* when either type of extract was added, indicating an apparent increased susceptibility to the hydro-alcoholic extracts for the former species of nematode (76% vs. 55% respectively,  $p < 0.01$ ).

*Haemonchus contortus* and *H. placei* have a high prevalence as gastrointestinal nematodes in small ruminants and cattle under tropical grazing conditions.<sup>21,22</sup>

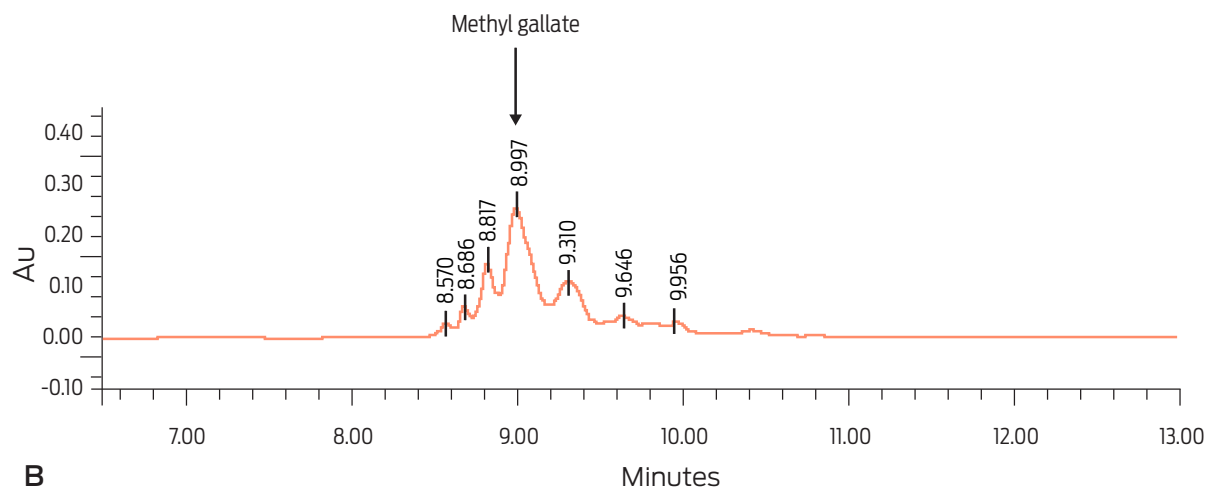
**Table 1.** Free, protein-bound, fiber-bound and total condensed tannin content (g/kg DM) in *Caesalpinia coriaria* hydro-alcoholic extracts obtained from mature fruits or leaves

Tannin content	Mature fruit extract	Leaves extract
Free	217.1	137.1
Protein-bound	31.7	21.7
Fiber-bound	71.8	41.8
Total condensed	320.6	200.6

DM: dry matter.



A



B

**Figure 1.** Major secondary compounds of two *Caesalpinia coriaria* hydro-alcoholic extracts identified by HPLC. A) Hydro-alcoholic extract from leaves. B) Hydro-alcoholic extract from fruits

**Table 2.** *Haemonchus contortus* and *H. placei* egg hatching inhibition test values after incubation with one of two *Caesalpinia coriaria* hydro-alcoholic extracts (from fruits or leaves) for 48 h

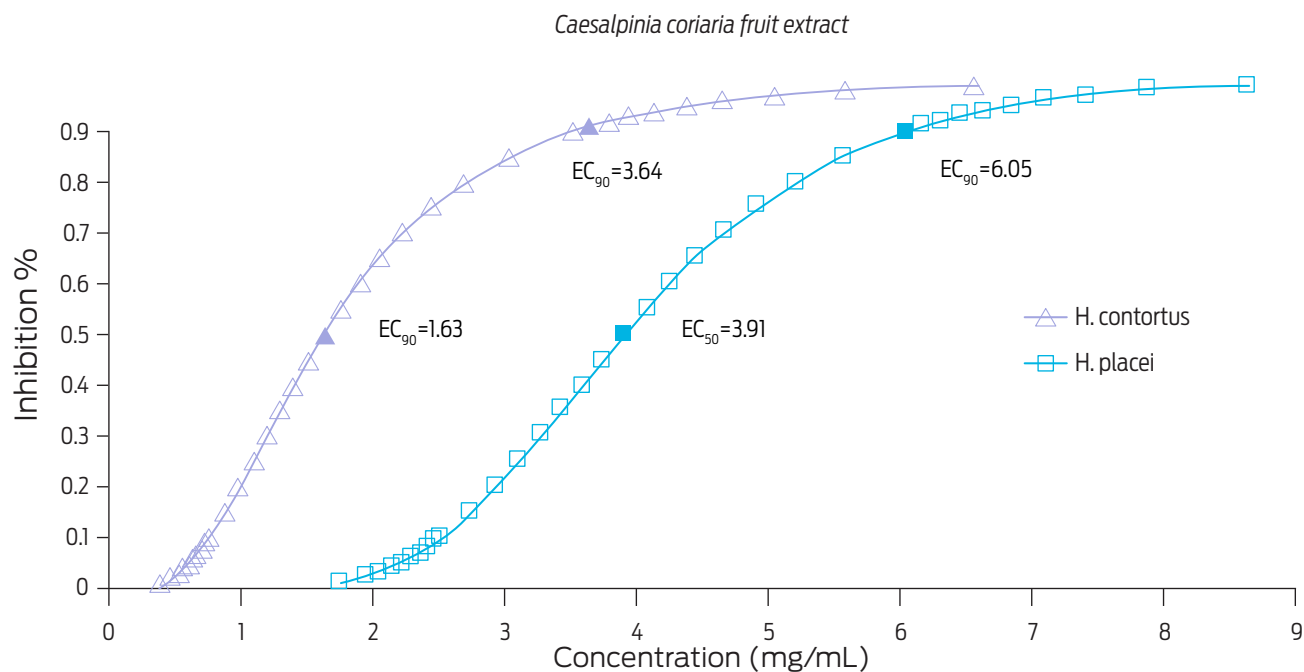
Treatments	Egg hatching inhibition (%)	
	<i>Haemonchus contortus</i>	<i>Haemonchus placei</i>
Mature fruits extract (mg/mL)		
25.0	100 <sup>a</sup>	100 <sup>a</sup>
12.5	100 <sup>a</sup>	100 <sup>a</sup>
6.2	99.5 <sup>a</sup>	92.75 <sup>a</sup>
3.1	89.25 <sup>a</sup>	33.75 <sup>cd</sup>
1.5	58.75 <sup>b</sup>	17.75 <sup>e</sup>
0.0	3.25 <sup>fg</sup>	2.75 <sup>g</sup>
Leaves extract		
25.0	95.25 <sup>a</sup>	92.50 <sup>a</sup>
12.5	90.00 <sup>a</sup>	60.75 <sup>b</sup>
6.2	60.00 <sup>b</sup>	22.75 <sup>de</sup>
3.1	40.75 <sup>c</sup>	19.75 <sup>de</sup>
1.5	27.50 <sup>cde</sup>	16.25 <sup>efg</sup>
0.0	3.25 <sup>fg</sup>	2.75 <sup>g</sup>
Thiabendazole (0.5 mg/mL)	100 <sup>a</sup>	100 <sup>a</sup>
P-value (Extract*Concentration*Nematode)		<0.0001
SEM		0.10

Means with different letters represent significant differences  $p < 0.05$ .

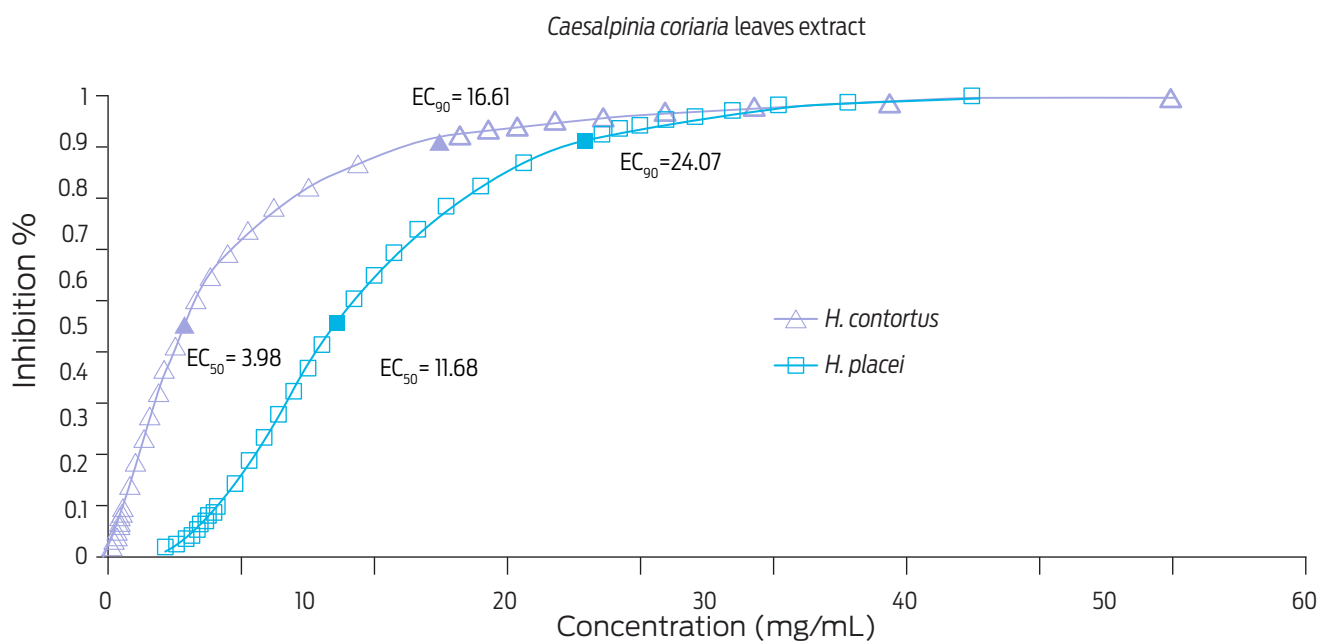
This work shows evidence of the ovicidal effect of two *C. coriaria* tree extracts against both parasites. Plants belonging to Caesalpiniaceae subfamily have been shown to possess different medicinal properties. Anthelmintic effects have been particularly found in the *Caesalpinia* genus.<sup>23,24</sup> These results coincide with those recorded in the present study, and could be related to the content of free condensed tannins, which is higher than in other leguminous species from the same region, such as *Lysiloma acapulcensis*<sup>11</sup> and *Acacia cochliacantha*.<sup>4,8</sup>

Phenols, such as tannins, may negatively impact nematode egg hatching by binding to the egg cuticle which is rich in glycoproteins, forming a tannin-protein complex that may alter embryo development and eventually the hatching process.<sup>25,26</sup> Parasitic load in *H. contortus* infected hair sheep was decreased when animals were fed with leaves of the *L. acapulcensis* tree, with an added beneficial effect in productive performance.<sup>27</sup> Authors found a high content of condensed tannins (116 g/kg of dry matter) in these leaves. Both *C. coriaria* extracts (leaves and mature fruits) used in this study had a higher content of condensed tannins than that found for *L. acapulcensis*.

The effective concentrations EC<sub>50</sub> and EC<sub>90</sub> obtained with both *C. coriaria* mature fruits and leaves extracts are shown in Figures 2 and 3, respectively. The EC<sub>50</sub> and EC<sub>90</sub> for the *C. coriaria* fruits extract were: 1.63 and 3.64 mg/mL respectively, for *H. contortus* eggs, and 3.91 and 6.05 mg/mL respectively, for *H. placei* eggs. Effective concentrations using leaf extracts were: EC<sub>50</sub> > 3.98 and EC<sub>90</sub> > 16.61 mg/mL for *H. contortus* eggs, and EC<sub>50</sub> > 11.68 and EC<sub>90</sub> > 24.07 mg/mL, for *H. placei* eggs (Fig. 3).



**Figure 2.** Effective concentrations (EC<sub>50</sub> and EC<sub>90</sub>) required to inhibit hatching of *H. contortus* and *H. placei* eggs after a 48 h incubation with a *Caesalpinia coriaria* mature fruits extract, as determined by Probit analysis.



**Figure 3.** Effective concentrations (EC<sub>50</sub> and EC<sub>90</sub>) required to inhibit hatching of *H. contortus* and *H. placei* eggs after a 48 h incubation with a *Caesalpinia coriaria* leaves extract, as determined by Probit analysis.

A stronger ovicidal effect was observed when the fruits extract was used on both parasite species. In fact, the EC<sub>90</sub> value for the fruits extract was fivefold than that obtained when the leaf extract was used on *H. contortus* eggs or fourfold on *H. placei* eggs. Differences for effective concentrations found for leaves and fruits extracts could be associated with content of phenolic compounds.<sup>7</sup> A report of a 93% reduction in the number of *Trichostrongylus* eggs per gram of feces of infected sheep, was recorded when a methanolic extract from a leguminous of the same genus (*Caesalpinia crista*) than the plant used for this study, was administered orally (3 g/kg live weight). This same study found an EC<sub>50</sub> of 0.134 mg/mL through the EHIT.<sup>28</sup>

Other specific compounds derived from secondary metabolism of *Caesalpinia* spp. leaves, such as ellagocatechins and galloocatechins,<sup>28,29</sup> have also been found to have nematicidal effects.<sup>29-31</sup> Both gallic acid and methyl gallate, which were found in extracts used in the present study, are considered as the basic chemical structure of hydrolysable tannins.<sup>32</sup> The nematicidal activity of different plants has also been attributed to these two compounds.<sup>7</sup> Indeed, a recent study by De Jesús-Martínez *et al.*,<sup>33</sup> found a high ovicidal activity of a methanolic extract of *C. coriaria* fruits harvested in Guerrero, Mexico, and the anthelmintic effect was associated with gallotannin content (methyl gallate). Similarly, another study showed that gallic acid and other galloyl derivatives obtained from *C. coriaria* fruits were associated with hatching inhibition of five gastrointestinal parasitic nematode eggs (*Haemonchus* spp., *Cooperia* spp., *Ostertagia* spp., *Trichostrongylus* spp. and *Oesophagostomum* spp.). However, these authors also showed that methyl gallate had no activity against these parasites.<sup>19</sup> Therefore, the increased efficiency in ovicidal activity (against both parasites species) observed for the hydro-alcoholic extract from *C. coriaria* leaves in the present study could be associated with its gallic acid content.

The anthelmintic activity of condensed and hydrolysable tannins has also been documented.<sup>34,35</sup> To ascertain the anthelmintic effect of condensed tannins in particular, addition of polyethylene glycol (PEG) to the tests is imperative, since PEG has the ability to bind and inactivate tannins.<sup>36,37</sup> Since PEG was not used in this study, the observed ovicidal effect of *C. coriaria* extracts cannot be directly attributed to their condensed tannin content, but it could be associated with found gallotannins.<sup>19</sup> Hence, further *in vitro* studies are warranted (using PEG) to verify if condensed tannin content in extracts from *C. coriaria* fruits and leaves does in fact have an ovicidal activity.

Other studies ascribe nematicidal activity to free condensed tannins. For instance, Williams *et al.*<sup>6</sup> evaluated acetone extracts of diverse tanniniferous plants (*Ribes nigrum*, *Tillia cordata*, *T. vulgaris*, *Salix* spp. and *Triflorium repens*) by the determination of EHT and larval development (L<sub>3</sub>) of *Oesophagostomum dentatum* (a pig nematode), and found a 90% inhibition of larval development at a 125-µg/mL concentration of all extracts used. The ovicidal effect of the studied extracts could also be related to the combined effect of phenols such as methyl gallate, gallic acid and flavonoids, which could interfere with the processes of embryo development of the larva within the egg. Indeed, ultrastructural damage on the cuticle of *Cooperia* spp. eggs has been observed through transmission electronic microscopy, after being exposed to phenols (quercetin and caffeic acid) isolated from *L. leucocephala* leaves.<sup>37</sup> On the other hand, Brunet *et al.*<sup>30</sup> observed damage of inter-



nal and external structures of infective larvae of *H. contortus* and *Trichostrongylus colubriformis* (such as structural changes in the hypodermis, cytoplasmic vesicles and muscular and intestinal cell degeneration), after a 3 h contact with *Onobrychis viciifolia* condensed tannins.

Genetic diversity of each nematode parasite could also affect the efficiency of the ovicidal activity of fruits and leaves extracts from *C. coriaria*. In their adult stage, both nematode species can be distinguished by their morphological structures such as the spicules and copulatory bursa in males, or the synlophe in males and females.<sup>38</sup> A tri-layer complex cuticule has been described for *H. contortus* eggs, with the external and middle layers composed of protein fibrils and chitin, respectively, as well as a semipermeable internal layer composed of lipids and glycoproteins.<sup>39</sup> Information for *H. placei* egg composition is however very limited. The greater ovicidal activity found for *H. contortus* when compared to *H. placei*, independently of the type of extract used in this study could therefore be due to structural biochemical or molecular differences between these parasites. Future studies to establish and identify these factors are warranted.

## Conclusions

Hydro-alcoholic extracts from mature fruits and leaves of *Caesalpinia coriaria* decreased *H. contortus* and *H. placei* egg hatching *in vitro*.

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## Conflicts of interest

The authors declare that they have no conflict of interest.

## Author contributions

R. R.-R., M. G.-C. and A. O.-J. designed the study and wrote the manuscript; A. Z. performed the plant extraction processes and executed phytochemical identification of secondary compounds; J. A.-G., G. D. M.-M., H. A. L. and J. F. V.-A. analysed the data; P. M.-de G. designed the study and revised the manuscript.

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