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Caffeoyl and coumaroyl derivatives from *Acacia cochliacantha* exhibit ovicidal activity against *Haemonchus contortus*

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ABSTRACT

Ethnopharmacology relevance: *Acacia cochliacantha* is a small tree whose foliage is traditionally used in Mexico for treatment of kidney pain, gastrointestinal illnesses and to kill intestinal parasites. In recent decades, the study of vegetal extracts has offered other possible alternatives for the control of *Haemonchus contortus*. Considering that this nematode affects dramatically the health and productivity of small ruminants, the aim of this study was to identify the anthelmintic compounds from *A. cochliacantha* hydro-alcoholic extract (HA-E) through an ovicidal test.

Material and methods: In vitro egg hatch assay was conducted to determinate the anthelmintic effects of a HA-E (60 g). Liquid-liquid ethyl acetate/water extraction gave two fractions (EtOAc-F, 1.92 g; Aq-F; 58.1 g). The less polar compounds from ethyl acetate fraction were extracted by addition of dichloromethane offering a precipitate phase (Mt-F, 1.25 g) and a soluble mixture (DCMt-F 1.15 g). All fractions were evaluated for ovicidal activity obtaining the egg hatching inhibition (EHI, 0.07–25 mg/mL). Ivermectin (0.5 mg/mL) was used as a reference drug (positive control), and distilled water, 2.5% DMSO and 2% methanol were used as negative controls. The isolated compounds from the most active fractions were subjected to spectroscopic (^{1}H NMR) Spectrometric (MS) and UV HPLC analysis in order to identify the bioactive compounds.

Results: The less polar treatments (AcOEt-F, DCMt-F, DCMt-P) showed the highest ovicidal activities (98–100% EHI; at 0.62–1.56 mg/mL) and the major compounds found in these fractions were identified as caffeoyl and coumaroyl derivatives, including caffeic acid (**1**), *p*-coumaric acid (**2**), ferulic acid (**3**), methyl caffeate (**4**), methyl-*p*-coumarate (**5**), methyl ferulate (**6**) and quercetin. In case of the less active fractions (Aq-F, Mt-F) were constituted principally by glycosylated flavonoids.

Conclusion: These results show that caffeoyl and coumaroyl derivatives from *Acacia cochliacantha* leaves had promising anthelmintic activity against *Haemonchus contortus*. This leguminous may offer an alternative source for the control of gastrointestinal nematodes of small ruminants.

1. Introduction

Infections caused by gastrointestinal nematodes (GIN) are some of the major concerns for livestock producers since these parasites affect dramatically the health of animals and their productivity (Jackson et al., 2012). *Haemonchus contortus* is one of the most frequent pathogenic nematodes in small ruminants, causing high levels of mortality of these animals (Arosemena et al., 1999). The excessive use of synthetic nematocidal generates a great monetary cost worldwide. On the other hand, *H. contortus* has produced an important drug resistance problem mainly in goats and sheep (Kaplan and Vidyashankar, 2012; Crook et al., 2016; Learmount et al., 2016). Additionally, the health and ecological risk produced by remnants of synthetic nematocides had increased the necessity to evaluate other alternative methods for control of helminthiasis (Waller, 1997, Waller and Thamsborg, 2004). Several medicinal plants used for treatment of

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gastrointestinal illnesses have showed anthelmintic effect (Hammond et al., 1997; von Son-de Fernex et al., 2012; Olmedo-Juárez et al., 2014). Some secondary metabolites like tannins, terpenoids, saponins, flavonoids, hydroxycinnamic derivatives and other polyphenolic compounds have been related to this anthelmintic activity (Williams et al., 2014; von Son-de Fernex et al. 2015). Several of these compounds acted synergistically to achieve higher anthelmintic activity (Klongsiriwet et al., 2015).

Several Acacia species (fruits and leaves) are traditionally used in Mexico for treatment of gastrointestinal illnesses (diarrhoea, gastritis), urinary infections, throat inflammation and headaches (Argueta et al., 1994; Bañuelos, 1999; Yetman and Van Devender, 2002). In case of *Acacia cochliacantha* Humb. & Bonpl. ex Willd (Rudd, 1966), flowers and leaves of this legume displayed antimicrobial activity against gram-positive and gram-negative bacteria (Manríquez-Torres et al., 2007). On the other hand, aqueous extracts from foliage of this plant displayed anthelmintic effects against ruminant parasitic nematodes through the reduction in the eggs per gram of faeces (EPG) in goats artificially infected with *H. contortus* (León-Castro et al., 2015). The objective of this study was to identify the major anthelmintic compounds from *A. cochliacantha* against *H. contortus*.

2. Material and methods

2.1. General

All chemicals used in this work were analytical-reagent grade. Dimethyl sulfoxide (DMSO), ethanol, water, acetonitrile, 2-aminoethyl diphenylborinate, caffeic acid (≥98%), *p*-coumaric acid (≥ 98%) and ferulic acid (≥99%) were purchased from Sigma-Aldrich (MO, USA). Materials and reagents for biological models (EHI %) were purchased from Corning® (New York, USA). ^{1}H NMR spectra were recorded on Varian INOVA-400 at 400 MHz. in $CDCl_3$. Chemical shifts are reported in ppm relative to TMS. Spectrometric analysis was performed on a Waters Xevo TQD mass spectrometer with ESI ion source (Waters Milford, USA). The UV spectra were obtained using a Waters array detector (Waters Co. 2996, Milford, USA). Thin-layer chromatography was performed using TLC Silica gel 60 F254, aluminium sheets 20×20 cm (Merck KGaA, Darmstadt, Germany).

2.2. Plant material

Leaves of *Acacia cochliacantha* (Cubata) were collected from its natural habitat in Salitre Palmarillos village, Amatepec Municipality, State of Mexico, Mexico (18°43′28.4′′ N, 100°17′03.5′′ W). This species was collected between March and April 2016. The plant material was identified by Prof. Rafael Torres-Colin, and a voucher specimen was deposited at the National Herbarium of Mexico in Universidad Nacional Autónoma de México, México, City (under the voucher code number OD07042016). Fresh plant was washed and dried at room temperature in the dark conditions for one week, and then the dry material was ground using an electrical miller (Wiley mill, TS3375E15 model) to reduce the leaf size to 4–6 mm.

2.3. Preparation of hydroalcoholic extract

The milled leaves (1 kg) were extracted by maceration using an aqueous methanol solution (70%, 1:10 ratio, w/v) at room temperature for 24 h. The liquid extract was filtered and the residual solvent was evaporated under low pressure conditions using a rotary evaporator (50–55 °C, Heidolph Laborota 4000, Germany) to obtain a semisolid extract, which was finally freeze-dried giving 120 g (12%) of a brown powder. This integrate extract was stored at −40 °C until pharmacological or phytochemical analysis.

2.4. Hydroalcoholic extract fractionation

The hydroalcoholic extract (HAE, 60 g) was partitioned by liquid-liquid chromatography using immiscible water/ethyl acetate solvents (600 mL each, Merck, Germany). Solutions in both fractions were concentrated by low-pressure distillation to obtain an aqueous fraction (Aq-F, 58.1 g) and an organic phase (AcOEt-F 1.92 g). The less polar mixture was precipitated by addition of dichloromethane (60 mL). After low pressure distillation process, the dichloromethane phase (DCMt-F, 0.83 g) was purified using an open chromatographic column (silica gel 60, 0.04–0.06 mesh, 25 g, 2.0×60 cm, Merck, Germany). The mobile phase consisted of a dichloromethane/methanol gradient system, and 50-mL samples were collected. These fractions were grouped according to their chemical similarity, resulting in 6 final sub-fractions that were labelled as follows: C1F1, C1F2, C1F3, C1F4, C1F5, C1F6. In the case of fraction DCMt-P (0.94 g), this mixture was purified in a chromatographic open column previously packed with 10 g of reverse phase silica gel (Polygoprep 60-50, C-18) and stabilized with methanol. A water/acetonitrile gradient system was used as mobile phase, starting with 100% of H_2O and ending with 100% CH_3CN. 21 samples of 10 mL were obtained which were grouped according to their chemical composition in 4 sub-fractions C2F1 (0.021 g), C2F2 (0.014 g), C2F3 (0.069 g) and C2F4 (0.44 g).

All samples of these two-chromatographic process were analysed by thin-layer chromatography (TLC) on silica gel 60 F254 (Merck, Germany) under UV light at 254 and 360 nm. The natural products-polyethylene glycol reagent (NP-PEG; 1% methanol solution of diphenylboryloxyethylamine, followed by 5% ethanol polyethylene glycol) was used as a chemical detection reagent (Wagner and Bladt, 2001).

2.5. Biological material

2.5.1. Haemonchus contortus eggs recovery test

Haemonchus contortus eggs were obtained from an egg-donor sheep (20.3 kg of bodyweight) previously subjected to a monospecific infection (350 infective larvae/kg, INIFAP strain, Mexico). Sheep were housed indoors on a metabolic floor, feed hay and commercial concentrate and had free access to water. Egg recovery was performed according to the technique described by von Son-de Fernex et al. (2015).

2.6. Eggs hatching inhibition (EHI) bio-guided test

The assay was carried out in 96-well micro-titration plates. In the assay, 4 wells were set up for each treatment in four experimental units. Treatments were administered in the indicated concentrations as follows: Step 1) hydroalcoholic extract (at 100 mg/mL); Step 2) ethyl acetate fraction (EtOAc-F) and aqueous fraction (Aq-F) at 0.39–25 mg/mL; Step 3) soluble dichloromethane fraction (DCMt-F) and the precipitate dichloromethane fraction (DMCt-P) at 0.07–0.62 mg/mL; Step 4) methanolic fraction (Mt-F) at 1.56–25 mg/mL. First column sub.-fractions: C1F1, CIF2, C1F3, C1F4, C1F5, and C1F6 as well as reversed phase column sub-fractions C1F1, C2F2, C2F3 and C2F4 were tested at concentration of 0.25–1 mg/mL. For each treatment, three proper negative controls were included as follows: 2.5% dimethyl sulfoxide (DMSO), distilled water and 2% methanol. Ivermectin (0.5 mg/mL) was used as the positive control. A total of 50 microliters of an aqueous suspension containing one hundred *H. contortus* eggs was seeded into each well. Then, 50-μL aliquots of the extract, fractions or control were deposited to each well. The plates were then incubated at room temperature for 48 h. The egg hatching process was stopped using Lugol's solution. The criteria for estimating the egg hatching inhibition included counting either the eggs present or the hatched larvae contained in 5-μL aliquots (n=10). The percentage of hatched eggs was estimated for each treatment group using the following formula: % EHI=[(number of eggs)/(number of larvae+number

eggs)]*100.

2.7. HPLC analysis

Chromatographic analysis was performed on a Waters 2695 Separation module system equipped with a Waters 996 photodiode array detector and Empower Pro software (Waters Corporation, USA). Chemical separation was achieved using a Supelcosil LC-F column (4.6 mm×50 mm i.d., 5-µm particle size, (Sigma-Aldrich, Bellefonte, USA). The mobile phase consisted of 0.5% trifluoroacetic acid aqueous solution (solvent A) and acetonitrile (solvent B). The gradient system was as follows: 0–1 min, 0% B; 2–3 min, 5% B, 4–20 min, 30% B; 21–23 min, 50% B 14–15 min; 24–25 min, 80% B; 26–27 100% B; 28–30 min, 0% B. The flow rate was maintained at 0.9 mL min^{-1}, and the sample injection volume was 10 µL. Absorbance was measured at 330 nm, caffeic acid, ferulic acid, *p*-coumaric acid and quercetin were identified by direct comparison of the retention times and UV spectra with those of reference standards (Sigma-Aldrich, St Louis Mo, USA). Methyl esters of caffeic, coumaric and ferulic acids derivatives were established based on their ^{1}H-HMN spectroscopy and spectrometry analysis.

2.8. Statistical analysis

Egg hatching inhibition data were analysed based on a completely random design using ANOVA through a general linear model (GLM) and SAS program. The dependent variables were: hydroalcoholic extract, column 1 sub-fractions, column 2 sub-fraction. A Tukey test was performed to identify significant differences among treatments. Likewise, the lethal concentrations (LC_{50} and LC_{90}) were calculated using Probit analysis in SAS 9.0 (SAS, 2006).

3. Results

3.1. Egg hatching inhibition test

Table 1 and Fig. 1 shows the inhibition of *H. contortus* egg hatching and lethal concentration ($LC_{50,\ 90}$) produced by *A. cochliacantha* hydroalcoholic extract and its first derived fractions. The integrate extract (HA-E, 100 mg/mL) displayed a total *H. contortus* egg hatching inhibition (initial step; Fig. 1A). While the organic fraction (second step, EtOAc-F; Fig. 1C) showed 100% EHI at 3.12 to 25 mg/mL and EHI of 84–98% at 0.78 and 1.56 mg/mL respectively. In case of aqueous fraction (Aq-F; Fig. 1B), this polar mixture displayed only 30% of EHI at the highest tested concentration (25 mg/mL). After bipartition of fraction EtOAc-F (1.5 g) by dichloromethane precipitation (20 mL, third step), the soluble phase (DCMt-F, 0.68 g; Fig. 1D) displayed 95–100% EHI at a concentration range of 0.15–0.62 mg/mL, and precipitated phase (DCMt-P. 0.82 g) showed 91–100% EHI at the same concentrations (0.15–0.62 mg/mL). In all studies, 100% EHI was observed with Ivermectin (0.5 mg/mL). Regarding the lethal concentrations, the LC_{50} for the EtOAc-F was found to be 0.33 mg/mL, while for the dichloromethane fraction (DCMt-F) and the precipitate (DCMt-P), the LC_{50} was 0.06 and 0.04 mg/mL, respectively. The LC_{50} for Aq-F was not estimated due its low anthelmintic effect (30% EHI, 25 mg/mL). The LC_{90} for EtOAc-F was 0.85 mg/mL, and derivative fraction from this mixture displayed better values of nematocidal effect (LC_{90}=0.12 and 0.13 mg/mL for DCMt-F and DCMt-P respectively).

3.2. Bioguided fractionation of EtOAc-F

Fingerprints and anthelmintic activity (1 mg/mL) of sub-fractions from dichloromethane soluble mixture are shown in Fig. 2 and Table 2. Fractions with the highest ovicidal effects were C1F1, which is principally constituted by caffeic acid (**1**, 98.25% EHI) and C1F2 (97% EHI), which contains a mixture of *p*-coumaric (**2**) acid and ferulic acid (**3**). The methyl ester derivatives: methyl caffeate (**4,** C1F3), methyl-*p*-coumarate (**5,** C1F4) and methylferulate (**6**, C1F5) displayed values of EHI of 88.2%, 88.2% and 71.5% respectively at 1 mg/mL. The mixture al methyl ferulate and quercetin (C1F6, 1 mg/mL) displayed a 94% of EHI. Finally, the chemical separation of fraction DCMt-P gave three non-active fractions C2F1 (flavone, 5.2% EHI), C2F2 (flavonol, 6.2% EHI), C2F3 (flavones and flavonols, 7.0% EHI) and a bioactive mixture of compounds **1**–**6** (C2F4, 98% of EHI)

Table 1
Results of the *Haemonchus contortus* egg hatching inhibition effect caused by an *Acacia cochliacantha* hydroalcoholic extract and five aqueous and organic fractions and its lethal concentration ($LC_{50,\ 90}$).

Initial step	Hydroalcoholic extract (HA-E 100 mg/mL) as a whole extract=100%[a] EHI		
Second step	HA-E bipartition with ethyl acetate		
Fraction (mg/mL)	Egg hatching Inhibition %	LC_{50}	LC_{90}
Organic fraction (EtOAc-F)			
25.00	100.00[a]	0.33 mg/mL	0.85 mg/mL
12.50	100.00[a]		
6.25	100.00[a]		
3.12	100.00[a]		
1.56	98.00[a]		
0.78	84.00[a]		
0.39	67.25[b]		
Aqueous fraction (Aq-F)			
25.00	30.50[c]	–	–
12.50	29.25[cd]		
6.25	16.00[cdef]		
3.12	13.25[def]		
DMSO 2.5%	3.00[ef]		
Distilled water	0.00[f]		
Ivermectin (0.5 mg/mL)	100.00[a]		
Third step	EtOAc-F bipartition with dichloromethane		
Dichloromethanoic fraction (DCMt-F)		0.06 mg/mL	0.13mg/mL
0.62	100.00[a]		
0.31	99.24[a]		
0.15	95.63[a]		
0.07	71.67[b]		
Precipitate (DCMt-P)			
0.62	100.00[a]	0.04 mg/mL	0.12 mg/mL
0.31	99.47[a]		
0.15	91.30[a]		
0.07	71.60[b]		
Methanol at 4%	0.00[d]		
Ivermectin (0.5 mg/mL)	100.00[a]		
Fourth step	Aq-F bipartition with methanol		
Methanolic fraction (Mt-F)			
25.0	20.00[b]		
12.5	8.00[c]		
6.25	4.75[cd]	–	–
3.12	5.25[cd]		
1.56	8.00[c]		
Methanol at 4%	0.00[d]		
Ivermectin (0.5 mg/mL)	100.00[a]		

Means with different letters within each fractionation step represent significant differences $P < 0.05$.

3.3. Identification of bioactive compounds

Fingerprints comparison of bioactive fractions with those commercial references (caffeic acid, coumaric acid, ferulic acid, and quercetin, allowed to identify these polyphenols. Methyl caffeate, methyl-*p*-coumarate and methylferulate (Fig. 3) were identified by comparison of their ^{1}H NMR and mass spectrometry previously described (Hatfield et al., 2008; Jazly et al., 2013; Znati et al., 2014) HPLC analysis of the 4 fractions derived from DCMt-P chromatographic fractionation indicated that non-active fractions C2F1, C2F2 and C2F3 were constituted

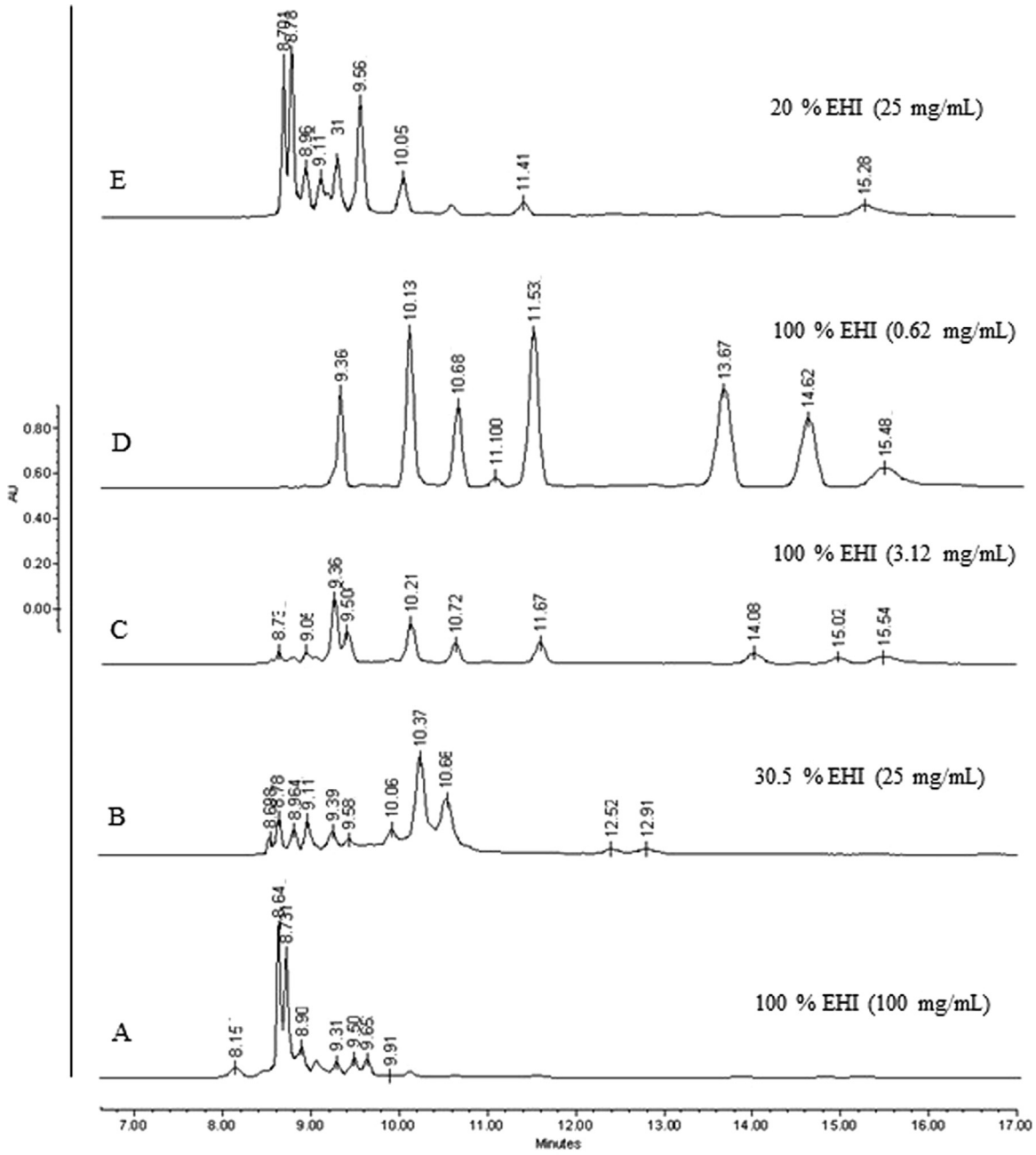


Fig. 1. Results of the HPLC analysis showing *Haemonchus contortus* egg hatching inhibition (EHI) of 5 different fractions from *Acacia cochliacantha* leaves: A) hydroalcoholic extract (HA-E), B) Aqueous fraction (Aq-F), C) Ethyl acetate fraction (EtOAc-F), D) dichloromethane fraction (DCMt-F) and E) methanolic fraction (Mt-F).

by glycosylated flavonoids principally (Table 2). While the C2F4 contained a mixture of all hydroxycinnamic derivatives (1–6) and displayed a high nematode inhibition (98% EHI).

4. Discussion

The Leguminosae family comprises a group of plants that are well known as producers of secondary metabolites with medicinal properties (Olmedo-Juárez et al., 2014; Sibaja-Hernández et al., 2015). Several species from genus *Acacia* have been described as antiparasitic traditional herbals and recently it has been demonstrated that goat kids previously fed with *A. cochliacantha* leaves were protected against the artificially infection with *H. contortus*, generating a significant reduction in egg count per gram of faeces elimination (Akkari et al., 2008; León-Castro et al., 2015). In this case, the anthelmintic effect was attributed to tannins content. (Costa-Júnior et al., 2014; León-Castro et al., 2015).

In this work, the nematocidal activity of the hydroalcoholic extract from *A. cochliacantha* (100 mg/mL, Table 1) was confirmed using free living stages of *H. contortus* nematode. This pharmacological tool, allowed to identify the major bioactive compounds through a bio-guided chemical fractionation of the integrate extract.

The liquid-liquid fractionation of hydroalcoholic extract produced a non-polar ovicidal phase (EtOAc-F, 100% EHI, 3.12 mg/mL) and an inactive aqueous phase (Aq-F, 13.25% EHI, 3.12 mg/mL). It is interesting that DCMt-F showed sixfold higher ovicidal activity than EtOAc-F (LC_{50}=0.33 and LC_{90}=0.85 mg/mL, Table 1). This finding indicates that the non-polar compounds from the hydroalcoholic extract may be responsible for this biological activity. Even if a previous chemical analysis stablished that *A. cochliacantha* contains polyphenols: tannins and flavonoids (Olivares-Pérez et al., 2013). This investigation allowed to confirm that caffeoyl and coumaroyl derivatives including their

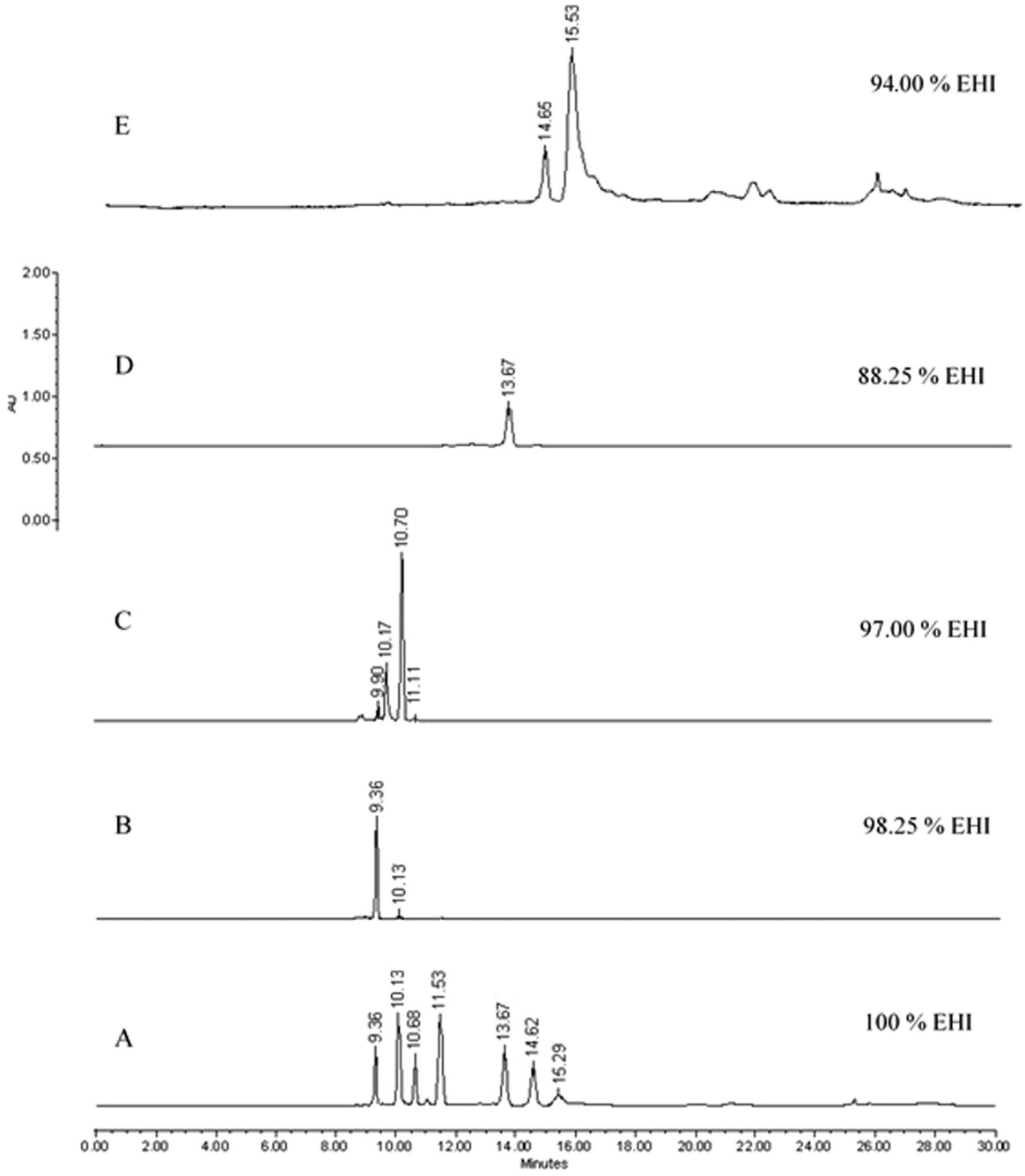


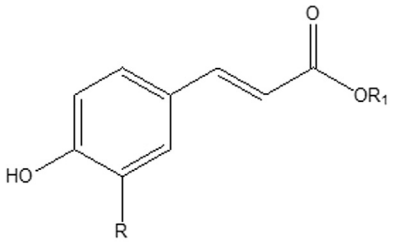
Fig. 2. Results of the HPLC analysis showing *Haemonchus contortus* egg hatching inhibition (EHI) of 6 different fractions from *Acacia cochliacantha* leaves assessed at 1 mg/mL: A) dichloromethane fraction (DCMt-F), B) fraction C1F1, C) fraction C1F2, D) fraction C1F4, and E) fraction C1F6.

methyl esters as well as quercetin were responsible for anthelmintic effect of this organic fraction (DCMt-F, 0.62 mg/mL, EHI 100%, LC_{50}=60 and LC_{90}=130 μg/mL respectively). Pharmacological analysis of these compounds: caffeic acid (**1**), *p*-coumaric acid (**2**) and ferulic acid (**3**), methyl caffeate (**4**), methyl-*p*-coumarate (**5**), methylferulate (**6**) and quercetin (**7**) indicated that in general, the hydroxycinnamic derivatives were more active than quercetin. On the other hand, the carboxylic free compounds (**1**, **2, 3**) displayed a higher nematocidal effect than those methyl ester derivatives (**4**, **5**, **6)**. All compounds tested at 0.39–1 mg/mL displayed ovicidal activity in the range of 67.25–100% in the *H. contortus* egg hatching inhibition. Even though these compounds displayed a high level of bioactivity, none of them (evaluated at 1 mg/mL) produced a better anthelmintic effect than fractions which they were obtained (DCMt-F and DCMt-P, 1 mg/mL). This observation allows to suppose that there may be an additive biological effect of each hydroxycinnamic compound. Despite the evidence of several plants from Acacia genus have shown anthelmintic activity in vitro an in vivo, these effects were lower than those produced by commercial drugs (Kahiya et al., 2003; Minho et al., 2008). *A. cochliacantha* produced a reduction of *H. contortus* eggs in goats previously feed with foliage of this legume (León-Castro et al., 2015). This finding is relevant because the identification of the chemical structure of the anthelmintic compounds allow to propose the use of a standardized extract of *A. cochliacantha* (more practical than isolated polyphenols) as adjuvant in the control of parasites in ruminants. Although there is no information about the mechanism of action of these compounds or the damage that each exerts on the egg hatching process, when *H. contortus* eggs were exposed to the most active fractions, these treatments produced damage to the egg affecting the embryo and larva development process (Fig. 4). In this study caffeic

Table 2
Results of the *Haemonchus contortus* egg hatching inhibition test using *Acacia cochliacantha* dichloromethanolic sub-fractions and precipitate, major identified compounds.

Evaluated fractions (1 mg/mL)	Major identified compounds (retention time, min)	% EHI
C1F1	Caffeic acid (**1**; 9.36)	98.25[a]
C1F2	*p*-coumaric acid, Ferulic acid (**2**,**3**; 10.1, 10.7)	97.00[a]
C1F3	Methyl caffeate (**4**; 11.52)	88.25[b]
C1F4	Methyl-*p*-coumarate (**5**; 13.6)	88.25[b]
C1F5	Methylferulate (**6**; 14.6)	71.5[c]
C1F6	Quercertin (**6**, 14.6, 15.5)	94.00[ab]
C2F1	Flavonoids	5.25[d]
C2F2	flavonoids	6.25[d]
C2F3	flavonoids	7.00[d]
C2F4	**1**, 2, **3**, **4**, **5**,**6** (9.36, 10.1, 10.7, 11.52,13.6, 14.6)	98[a]
Methanol 4%	–	3.00[d]

Means with different letters in the same column represent statistically differences $P < 0.05$.



	R	R_1
1	OH	H
2	H	H
3	OH	H
4	OH	CH_3
5	H	CH_3
6	OCH_3	CH_3

Fig. 3. Chemical structure of anthelmintic hydroxycinnamic derivatives from *A. cochliacantha* leaves.

acid and methyl caffeate (fractions: C1F1, C1F3; Table 2) were the most active against eggs of *H. contortus*. These results are similar to those reported by von Son-de Fernex et al. (2015), who identified quercetin and caffeic acid from an aqueous extract of *Leucaena leucocephala*. The mixture of these compounds (85% of caffeic acid) showed a 90.49% ± 2.85 EHI on *Cooperia* spp eggs generating ultrastructural damage of *Cooperia* spp eggs. On the other hand, fractions with high amounts of glycosyl flavonoids (Aq-F, Met-F, C2F1, C2F2 and C2F3) showed lowest ovicidal effect (7.0–20%, Tables 1 and 2) and less polar fraction C2F4 (constituted by compounds **1–6**) showed 97% and 88% EHI corroborating the ovicidal activity of these polyphenolic compounds. Therefore, the nematocidal activity against other exogenous and endogenous stages of *H. contortus* and other nematodes could be essential for use of this plant or its isolated compounds as antiparasitic agents. Up to now the anthelmintic effect of Fabaceae species (*Onobrychis viciifolia, Acacia nilotica* and *Lysiloma acapulcensis*) has been related to the tannins content principally (Desrues et al., 2016; Paswan et al., 2016; García-Hernández et al., 2017) or a mixture of quercetin and caffeic acid in *Leucaena leucocephala* (von Son-de Fernex et al., 2015). Recently it was found that caffeic and *p*-coumaric acids found in *Ipomea batatas* were responsible for the antioxidant and anti-carcinogenic activities (Nasr et al., 2015; Peng et al., 2015; Zhang et al., 2016). On the other hand, a synergic anthelmintic effect (*H. contortus* larvae exsheathment inhibition) between a mixture of tannins when commercial flavonoids (luteolin and quercetin) were added (Klongsiriwet et al., 2015). Although the tannins have been considered as responsible for this nematicidal activity (Williams et al., 2014; Saric et al., 2015; García-Hernández et al., 2017), this is the first time that hydroxycinmmic derivatives group (caffeic acid, coumaric acid, ferulic acid, methyl caffeate, methylcoumarate and methyl ferulate) are described as anthelmintics from *A. cochliacantha*.

5. Conclusions

The results of this work show an anthelmintic that a hydroalcoholic extract of *A. cochliacantha* leaves contains compounds with high in vitro ovicidal activity against *H. contortus*. The bio-guided separation demonstrated that the organic fraction (AcOEt-F, 1 mg/mL) had higher activity than the aqueous soluble compounds. Selective extraction of the less polar compounds with dichloromethane produced the highest bioactive mixture (DCMt-F, 1 mg/mL). The main constituents in this fraction were caffeoyl and coumaroyl derivatives as well as quercetin. All isolated compounds from this mixture, exhibited a high level of nematocidal activity. Studies focused on identifying potential anthelmintic compounds from plants used to feed small ruminants can generate new ecologically friendly alternatives for the control of livestock parasitic nematodes.

This chemical identification of the anthelmintic compounds from *A. cochliacantha* leaves, will allow to obtain standardized extracts useful in the developing of new veterinary (fodder, dried extracts, fractions or isolated compounds) or pharmacological treatments for the control of helminthiasis.

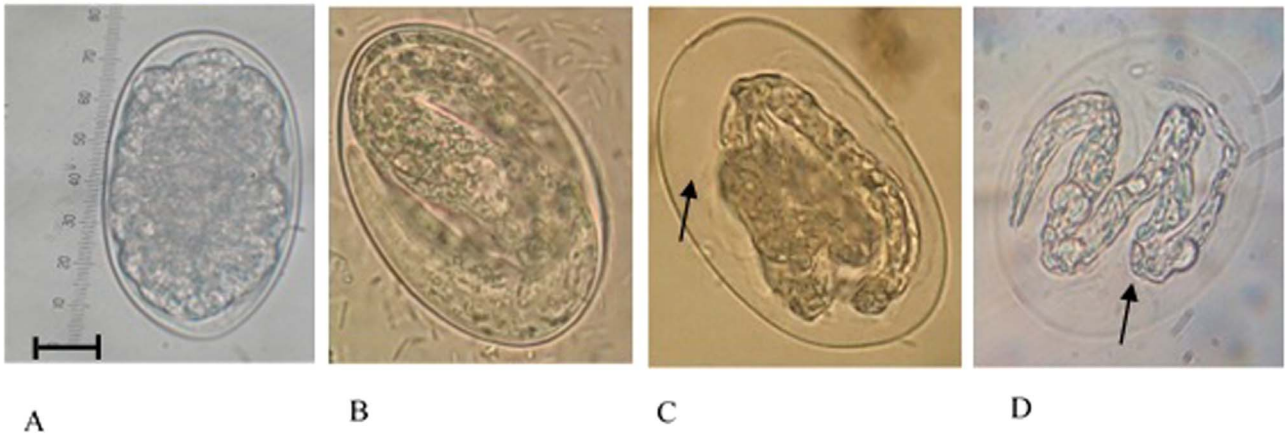


Fig. 4. Images taken through an optical microscope showing *Haemonchus contortus* eggs (40×): A) morulated egg; B). embryonated egg (control), C and D) *H. contortus* eggs after exposure to a *Acacia cochliacantha* DCMt-F fraction. Bar scale (40 μM).

Conflict of interest statement

The authors declare that they have no competing interests.

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