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SHORT COMMUNICATION

In vitro larvicidal effect of a hydroalcoholic extract from *Acacia cochliacantha* leaf against ruminant parasitic nematodes

Agustín Olmedo-Juárez[1] · Rolando Rojo-Rubio[2] · Alejandro Zamilpa[3] · Pedro Mendoza de Gives[1] · Javier Arece-García[4] · María Eugenia López-Arellano[1] · Elke von Son- de Fernex[5]

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Abstract The aim of this study was to evaluate the *in vitro* lethal effect of a hydroalcoholic extract (HAE) from *Acacia cochliacantha* leaf against three gastrointestinal nematodes species (*Haemonchus contortus*, *H. placei* and *Cooperia punctata*) of domestic ruminants. The HAE was assessed using five concentrations: 100, 125, 175, 150 and 200 mg/ml; 0.5% Ivermectin was used as a positive control and distilled water, as negative control. The data were normalized using the square root and analysed with a completely randomized design through ANOVA analysis using the general lineal model (GLM) of the SAS program. The HAE tannin content was determined through spectrophotometry (UV-visible) and the other major phenols, were identified by chromatographic processes. The results showed an *in vitro* larvicidal activity of the HAE against the three assessed nematode species with all assessed concentrations. A clear HAE increased concentration dependence effect was observed. The highest activity of the HAE was obtained at the highest concentration (close to 100%, $P < 0.05$). This result was similar to the one obtained with Ivermectin. On the other hand, the chemical analysis of HAE showed the presence of tannins, caffeoyls and coumaroyl derivates and quercetin as the main compounds. The results suggest that the HAE from this plant species possess *in vitro* anthelmintic properties. The identified compounds in this study would good candidates for further *in vivo* researches.

Keywords *Haemonchus* · *Cooperia* · Tannins · Flavonoids · Nematodes · *Acacia cochliacantha*

✉ Agustín Olmedo-Juárez
olmedo.agustin@inifap.gob.mx

[1] Centro Nacional de Investigación Disciplinaria en Parasitología Veterinaria (CENID PAVET). Carretera Federal Cuernavaca-Cuautla, No. 8534 / Col. Progreso/ A.P. 206-CIVAC, C.P. 62550 Jiutepec, Morelos, Mexico

[2] Centro Universitario UAEM-Temascaltepec, Universidad Autónoma del Estado de México, km 67.5. Carr. Fed. Toluca-Tejupilco, CP 51300 Temascaltepec, Estado de México, Mexico

[3] Centro de Investigación Biomédica del Sur, Instituto Mexicano del Seguro Social, Argentina No. 1. Col. Centro, CP 62790 Xochitepec, Morelos, Mexico

[4] Estación Experimental de Pastos y Forrajes Indio Hatuey, Universidad de Matanzas, Central España Republicana, CP 44280 Matanzas, Cuba

[5] Centro de Enseñanza Investigación y Extensión en Ganadería Tropical, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, Km 5.5 Carretera Federal Tlapacoyan-Martínez de la Torre, C.P. 93600 Veracruz, Mexico

Introduction

Gastrointestinal nematode (GINs) parasitic infection is one the major health concern in the ruminant production. The excessive use of chemical anthelmintic drugs is a widespread practice in livestock production worldwide; although their continuous and frequent use triggers a serious problems of anthelmintic resistance (Jabbar et al. 2006; Muñiz-Lagunes et al. 2015). The use of plants with anthelmintic (AH) properties is considered as one possible method for controlling GINs in ruminants. A number of *in vitro* and *in vivo* studies, using plant extracts from Leguminoseae family, have provided information of phenolic compounds such as tannins and flavonoids with AH activity (Olmedo-Juárez et al. 2014; Vargas-Magaña et al. 2014; von Son-de Fernex et al. 2015). *Acacia* is a large genus of the Fabaceae family, with about 1350 species. Most of the species belonging to the *Acacia* genus are rich in

secondary metabolites containing mainly condensed tannins and flavonoids (Seigler 2003; León-Castro et al. 2015). In some Mexican tropical areas, the leaves and fruits from *Acacia cochliacantha* are found scattered in pastures and living fences, where ruminants harvest the leaves and fruits to feed themselves during the dry season. The secondary metabolites identified in this plant species are condensed tannins as main compounds (Olivares-Pérez et al. 2011). *Acacia cochliacantha* showed an *in vivo* anthelmintic effect on *H. contortus* (León-Castro et al. 2016) but more information regarding the metabolites involved and the effect on other parasitic stages is needed. Thus, the objective of this study was to evaluate the *in vitro* effect of a hydroalcoholic extract of *A. cochliacantha* leaves against infective larvae (L_3) of three gastrointestinal parasite species (*Haemonchus contortus*, *Cooperia punctata* and *Haemonchus placei*).

Materials and methods

Plant material

Acacia cochliacantha leaves Humb. & Bonpl. (Cubata) were collected from a Salitre Palmarillos village, Amatepec Municipality, in the State of Mexico, Mexico (18°43′28.4″ N, 100°17′03.5″ W). Plants were collected between March and April 2016. The plant was taxonomically identified by Prof. Rafael Torres-Colin and deposited at the Herbario Nacional de México at Universidad Nacional Autónoma de México, México, City (Voucher code number OD07042016). Fresh material was washed and dried at room temperature in the dark for one week. Plant leaves were milled using an electrical miller (Wiley mill, TS3375E15 model), so as to reach a size of 4–6 mm.

Preparation of the hydroalcoholic extract

One kg of dried and ground leaves were used to obtain the extract by maceration with an aqueous methanol solution (70%, 1:10 ratio, *w*/*v*) at room temperature during 24 h. The liquid extract was paper-filtered and the residual solvent was evaporated using a rotary evaporator (Heidolph Laborota 4000, Germany) under reduced pressure at 50–60 °C to obtain a semisolid extract, which was finally freeze-dried to get 120 g (12%). The dry extract was stored at −40 °C until bioassays and phytochemical analysis.

Condensed tannin content

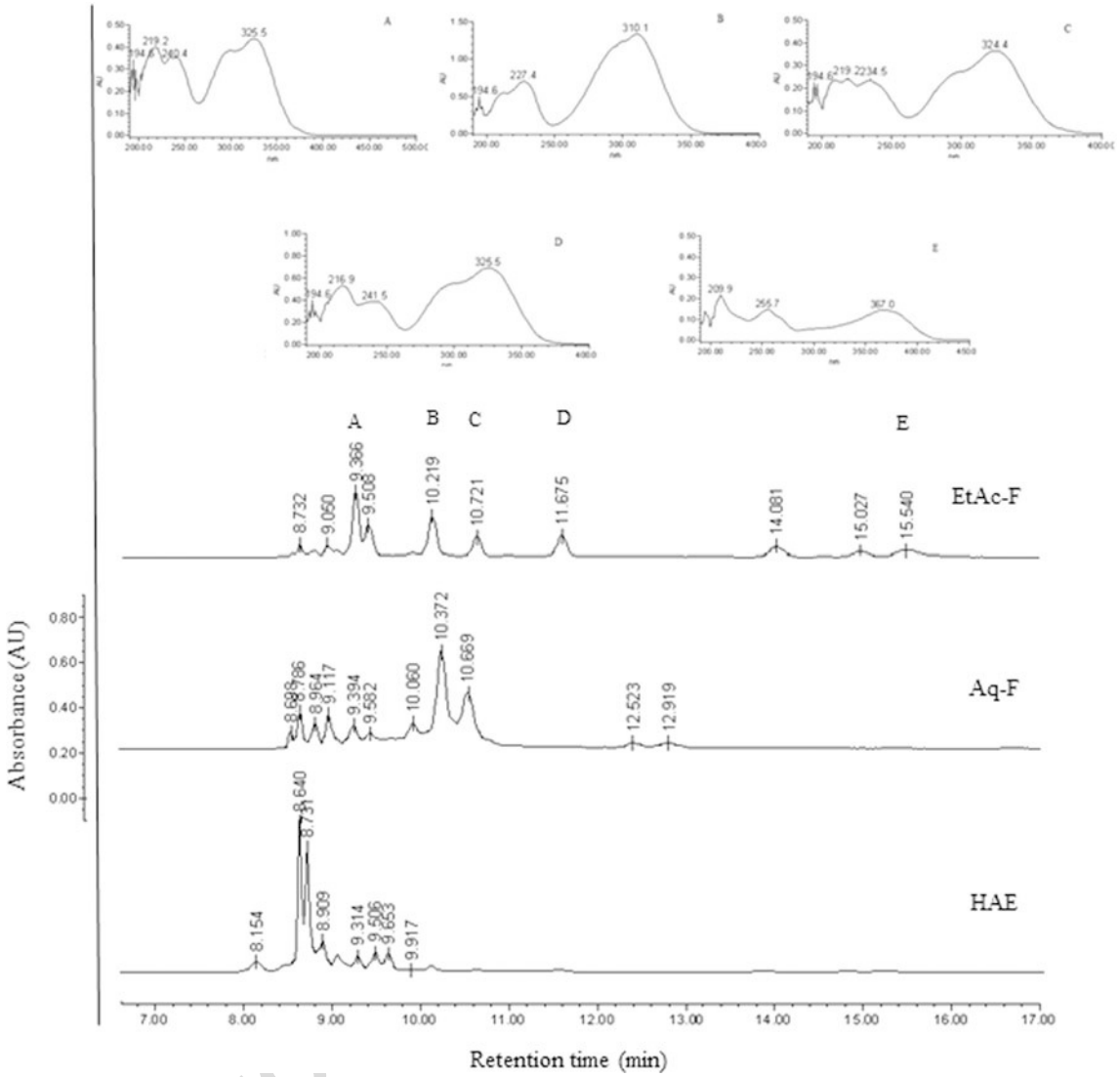
The hydroalcoholic extract (HAE) was analysed to determine the total condensed tannin content (TCT) by using of the butanol-HCL method (López et al. 2004); the *Lysiloma acapulcensis* free condensed tannins (FCT) were used as internal standards (Olmedo-Juárez et al. 2014). The free (FCT1), the protein- (PCT) and fiber- (FCT2) bound CT analyses were conducted following the technique reported by Porter et al. (1986). Purification was performed using a Shepadex LH-20 column, as described by Hedqvist et al. (2000).

Hydroalcoholic extract major compounds identification

The hydroalcoholic extract (HAE, 60 g) was processed for bipartition via liquid-liquid chromatography using water/ethyl acetate solvents (600 mL each, Merck, Germany). Two fractions, an aqueous fraction (Aq-F) and an organic fraction (EtAc-F) were obtained. The solvents in both fractions were eliminated using low-pressure distillation. Fraction yields were as follows: Aq-F = 58.1 g and EtAc-F = 1.92 g. Chromatographic analysis was developed by HPLC using a Waters 2695 separation module HPLC system equipped with a Waters 996 photodiode array detector and Empower Pro software (Waters Corporation, USA). Chemical separation was achieved in a supelcosil LC-F column (4.6 mm × 250 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 obtained 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 and the injection volume was 10 μl. The absorbance was measured at 330 nm. Caffeic acid and coumaric acid were identified by comparison of the retention times and UV spectra with the reference standards (Sigma-Aldrich, St Lous Mo, USA). Other caffeoyl and coumaroyl derivatives were established based on their UV spectra (Wagner and Bladt 2001).

Biological material

Haemonchus contortus infective larvae (L_3) (strain, INIFAP), were obtained from a donor sheep artificially infected with 350 L_3 larvae per kg BW. Likewise, infective larvae from *H. placei* (wild strain) and *C. punctata* (Cp de Fernex-MEX strain) were obtained from two young cattle. Faecal cultures were prepared by mixing faeces with polystyrene particles in plastic bowls. Water was added to the faecal cultures and mixed with a wooden spoon for obtaining an adequate oxygenation to promote a better egg hatching. The faecal cultures were covered with foil and incubated for 7 days at room temperature (25–31 °C). The infective larvae were extracted from faecal material using the Baermann funnel technique (Liebano-Hernández 2004). The L_3 were cleaned by density gradient (40% Sacharose) and centrifugation; the larvae were later exsheathed with sodium hypochlorite at 0.187%. Finally, the exsheathed larvae were used for the mortality assay.



Q4 **Fig. 1** HPLC chromatogram of a hydroalcoholic extract (HAE), an aqueous fraction (Aq-F) and an ethyl acetate fraction (EtAc-F) indicating the presence of phenols (showing *UV-spectral*); as caffeoyl derivatives displayed λmax = 325 nm (peaks *A*, *C*, *D*,); coumaroyl derivatives gave λmax =310 nm (peaks *B*) and quercetin displayed λmax = 360 nm (peak *E*)

Larval mortality assay

The assay was carried out using 96-well micro-titration plates ($n = 12$) for each treatment. Treatments were designed with the HAE concentration at 100, 125, 150,175 and 200 mg/ml, respectively. Each treatment was tested using a negative control (water) and anthelmintic (0.5% ivermectin) as the positive control. Fifty microliters of an aqueous suspension containing 150 nematode (*H. contortus, H. placei, C. punctata*) larvae were distributed in each well. Then, 50-µl aliquots of the extract and controls were added to each well. The plates were incubated at room temperature (18–25 °C) during 48 h. Ten aliquots of 10 µl were taken from each well to count dead or living larvae; the larval mortality was assessed if mobility was not observed during 20 s. When larvae remained motionless but their aspect caused confusion about if they were death or alive; a physical stimulus was applied touching their coat with a metal needle and the final decision was based on their motility. Finally, the larval mortality percentage was determined using the following formula: % mortality = [(number of living larvae)/ (number of dead larvae + number of living larvae)]*100.

Statistical analysis

The data of larval mortality were normalized using the square root transformation and it was analysed through a completely randomized design through ANOVA analysis using the general lineal model (GLM) of the SAS program. Differences among means were assessed by the Tukey's test. Likewise,

Q5 **Table 1** Mortality percentages of infective larvae (L_3) of three different ruminant parasitic nematodes exposed to an *Acacia cochliacantha* hydroalcoholic extract at different concentrations

Mortality percentage of infective larvae (%)			
Treatment	*Haemonchus contortus* (INIFAP strain)	*Cooperia punctata* (de Fernex-MEX strain)	*Haemonchus placei* (wild strain)
Distilled water (C^-)	1.00^{f}	0.75^{d}	0.00^{e}
Ivermectin (C^+)	100.00^{a}	100.00^{a}	100.00^{a}
A. cochliacantha hydro-alcoholic extract (mg/ mL)			
200	97.75^{ab}	99.25^{a}	97.00^{a}
175	89.50^{b}	77.50^{b}	92.75^{a}
150	73.00^{c}	37.00^{b}	76.00^{b}
125	46.25^{d}	10.00^{c}	39.00^{c}
100	25.00^{e}	8.50^{c}	16.00^{d}
SEM	1.75	2.90	2.20

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

SEM standard error of mean

the lethal concentrations (LC_{50} and LC_{90}), were estimated through a Probit analysis (SAS 2006).

Results

Condensed tannin content and other main compounds

The TCT, PCT and FCT2 bound resulted in 140.0, 26.0 and 36.6 g/kg of dry matter, respectively. On the other hand, the chromatographic analysis in the HAE revealed the presence of caffeoyl derivates (Fig. 1 ACD) and coumaroyl derivatives (Fig. 1B) as well as some flavonoids (Fig. 1E) such as quercetin as the main compounds.

Infective larvae (L_3) mortality test

Table 1 shows the results of the GIN mortality percentages from cattle and sheep exposed to the extract at the different assessed concentrations and at their proper controls. A larvicidal effect ($P < 0.05$) was observed in all the nematode species as well as a concentration/dependence. Mortality percentages close to 100% were achieved ah the HAE highest concentration (200 mg/ml). On the other hand, the HAE mean lethal concentrations (LC_{50} and LC_{90}) for the three nematode assessed species are show in Table 2. The HA extract LC_{50} and LC_{90} against *H. placei*, were: 126.53 and 172.59 mg/ml, respectively; meanwhile, these values were 129.39 and 177.88 mg/ml, for *H. contortus,* respectively and 136.90 and 174.7 mg/ml for *C. punctata,* respectively.

Discussion

The use of plants with medicinal properties represents a sustainable alternative for controlling diseases with important repercussions on livestock health, such as internal parasitic infections. The leaves of some leguminous trees like *Lysiloma acapulcensis* and *Leucaena leucocephala* have shown possessing anthelmintic activity against ruminant parasitic nematodes in a number of *in vitro* and *in vivo* studies (Mejía-Hernández et al. 2014; Olmedo-Juárez et al. 2014; von Son-de Fernex et al. 2015; García-Hernández et al. 2017). It is common to find a miscellaneous GIN fauna

Table 2 Fifty and ninety lethal concentrations of a hydroalcoholic extract from *Acacia cochliacantha* leaves against *Haemonchus contortus*, *H. placei* and *Cooperia punctata* infective larvae after 48 h *in vitro* exposure

Nematode specie	LC_{50}	95% CI limits		LC_{90}	95% CI limits	
		Lower	Upper		Lower	Upper
Haemonchus contortus	127.39	123.99	130.33	177.88	172.90	183.77
Haemonchus placei	126.53	121.26	131.17	172.59	167.53	178.33
Cooperia punctata	136.90	134.61	139.06	174.07	170.79	177.84

Values are expressed as mg/ml

CI confidence interval

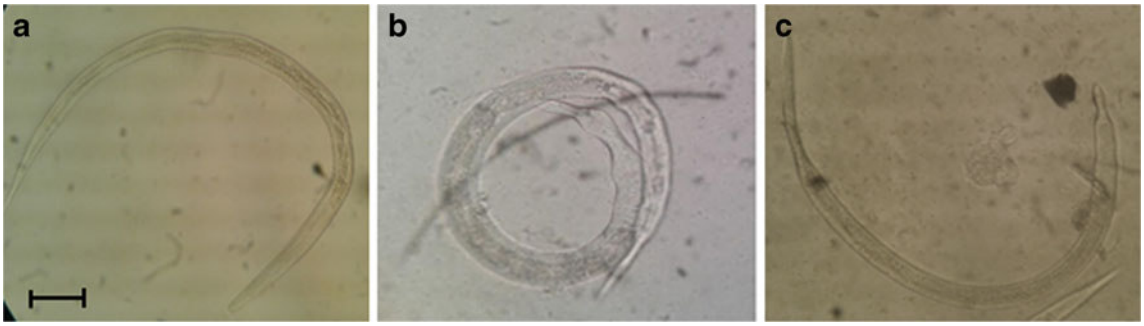


Fig. 2 Photographies taken through an optical microscope showing the aspect of *Haemonchus contortus* infective larvae (L_3) (40 x): **a** Normal larvae (control), **b** and **c** infective larvae after 48 h exposure to an *Acacia cochliacantha* hydroalcoholic extract. *Bar scale* (40 μM, ⊢⊣)

infecting grazing animals simultaneously. However, some genera/species are more pathogenic than others. The GIN *Haemonchus contortus, H. placei* and *C. punctata*, are considered as the main genera of parasitic nematodes affecting ruminants under tropical grazing conditions (Howell et al. 2008; Vlaminck et al. 2015). The present research demonstrated that the HAE from *A. cochliacantha* leaves had an important larvicidal effect against the infecting larvae L_3 of three different nematode species. Such effect is likely related with a secondary metabolite profile, especially associated with condensed tannins (Brunet and Hoste 2006; Martínez-Ortíz-de-Montellano et al. 2013; Williams et al. 2014). Nevertheless, Klongsiriwet et al. (2015) demonstrated that tannins are not the only plant secondary metabolites responsible for affecting the gastrointestinal nematodes of ruminants; these authors reported a synergism of tannins with other compounds, such as flavonoids, which enhance their nematicidal effect. In the present study, some phenols such as flavonoids and coumaroyl and caffeoyl derivates were identified through chromatographic techniques (Fig. 1). These compounds could also be related to the biological activity of this plant. In another study, an anthelmintic effect of quercetin and caffeic acid obtained from *L. leucocephala* leaves was found through a bio-guided egg hatching inhibition assay (von Son-de Fernex et al. 2015). On the other hand, significant structural changes on the larvae bodies were observed (Fig. 2). Such morphological changes were observed in the larvae exposed to the two highest HAE concentrations (175 and 200 mg/ml). A slimming of either the anterior and posterior parts of the larvae bodies was observed in most of the HAE exposed larvae at these concentrations. The slimmed extremes of the larval body looked like finger-shape (Fig. 2b, c). Unfortunately, in our study was not possible to identify the metabolite responsible of this structural change. In another study, some phenols such as caffeoyl and coumaroyl derivates as well as the flavonoid quercetin were identified as responsible for inhibiting the *H. contortus* egg hatching (Castillo-Mitre et al. 2016).

According to the above-explained facts, the larvicidal effects of the HAE in our study could be related to those identified metabolites; although this will need to be demonstrated in future studies.

Conclusion

The results of this research show that the HAE of *A. cochliacantha* leaves possess larvicidal properties against *H. contortus, H. placei* and *C. punctata* infective larvae. Thus, this plant species could be an option for the control of nematode infestations in ruminants under an environment-sustainable approach. Nevertheless, *in vivo* studies with experimental cattle infected with GINs are required in order to evaluate the effect.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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