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Ovicidal activity of extracts from four plant species against the cattle nematode *Cooperia punctata*

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Abstract

Bioactive plants might represent an alternative for *Cooperia punctata* control in grazing cattle. The objectives of this study were (1) to assess the ovicidal activity of extracts from 4 plant species against *C. punctata*, (2) to determine the role of the polyphenols in the plants' anthelmintic (AH) activity, and (3) to evaluate the best plant extraction procedure when searching for ovicidal activity. The egg hatch assay was used with different extraction procedures, aqueous (AQ), acetone:water (AW) and acetonic (AC), to evaluate the ovicidal activity of *Leucaena leucocephala*, *Gliricidia sepium*, *Guazuma ulmifolia* and *Cratylia argentea*. Eggs of *C. punctata* were exposed in quadruplicate to 0.6, 1.2, 2.4, 4.8 and 9.6 mg mL^{-1} of each plant extract. The roles of the polyphenols were assessed using polyethylene glycol (PEG). The 12 plant extracts inhibited egg hatching in a dose-dependent manner. Best-fit LC_{50} values were 1.03 ± 0.17 and 7.90 ± 1.19 mg mL^{-1} for *G. sepium*-AC and *L. leucocephala*-AQ, respectively. Differences in AH activity were found among the extraction procedures ($P < 0.05$). At the highest concentration, *L. leucocephala*-AQ inhibited more than 50% of *C. punctata* hatching. The *G. sepium*-AC extract fully inhibited egg hatching. The addition of polyethylene glycol revealed the role of the polyphenols in the bioactivity of most plant extracts; however, for *G. sepium*-AC, the polyphenols were not the main bioactive compounds. Overall, acetone:water extraction represented the best extraction procedure to obtain both ovicidal activity and higher yield. The inhibition rates suggested that *L. leucocephala* and *G. sepium* should be evaluated as a means of reducing larval density in pastures.

Keywords: Cattle; Nematodes; *Cooperia punctata*; Ovicidal effect; Plant extracts.

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Additional information and declarations can be found on page 7

Introduction

Gastrointestinal nematodosis has been ranked as the main endemic parasitic disease in cattle production units (Fitzpatrick, 2013), reducing the productivity and health of livestock (Charlier *et al.*, 2009; Perri *et al.*, 2011). Among the gastrointestinal nematodes (GIN) that affect cattle, *Cooperia* spp. have been highlighted as the nematodes with higher prevalence in grazing cattle around the world (Kenyon

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and Jackson, 2012; Stromberg *et al.*, 2012). These parasites decrease the amount of dry feed consumed and nutrient uptake or utilization (Stromberg *et al.*, 2012). Broad-spectrum anthelmintics (AHs) are a suitable tool for the control of GIN, and the application of these anthelmintics enhances the productivity and performance of animals (Sutherland and Leathwick, 2011). Unfortunately, resistance has become an emerging problem among cattle nematodes (Gasbarre, 2014). Recent studies have reported the emergence of *Cooperia* spp. strains resistant to macrocyclic lactones (ML) (Bartley *et al.*, 2012), benzimidazoles (Arnaud-Ochoa and Alonso Díaz, 2012) and imidazothiazoles (Becerra-Nava *et al.*, 2014). Thus, novel approaches for helminth control in cattle are required before GIN becomes a major problem due to the spread of highly resistant and multi-resistant strains among farms.

One of the most studied novel approaches has been the use of bioactive plants having anthelmintic effects (Hoste *et al.*, 2012). *In vitro* and *in vivo* studies have shown the anthelmintic effect of some plants when using GIN from small ruminants as models (Alonso-Diaz *et al.*, 2008a; Alonso-Diaz *et al.*, 2008b; Alonso-Díaz *et al.*, 2010; Martinez-Ortiz-de-Montellano *et al.*, 2010; Von Son-de Fernex *et al.*, 2012), whereas little research has been carried out with cattle nematodes.

Tropical browse legumes are one of the most studied forages due to their high content of plant secondary metabolites (PSMs), along with their benefits, which are obtained from their nutritional quality. Anthelmintic activity of PSMs has been mainly associated with the presence of tannins due to their capacity to interrupt specific nematode life-stages, such as inhibiting egg hatching, larval development, larval motility and larval exsheathment (Molan *et al.*, 2000; Athanasiadou *et al.*, 2001; Alonso-Diaz *et al.*, 2008b; Von Son-de Fernex *et al.*, 2012). The AH effect from tannins is related to the ability of the tannins to create chemical bonds with structural proteins present in nematodes morphology (Hoste *et al.*, 2012). However, few reports have identified other bioactive molecules such as flavonol glycosides, flavones and sesquiterpene lactones as being involved in AH effects (Molan *et al.*, 2003; Barrau *et al.*, 2005; Kozan *et al.*, 2013).

Over the last decade, multiple extraction procedures that use different solvents and mixtures for phytochemical extractions have been standardized for *in vitro* AH evaluations. As condensed tannins (CTs) have been the most targeted compounds for investigation, the system proven most efficient for CT recovery is the mixture of acetone:water (Chavan *et al.*, 2001; Chavan and Amarowicz, 2013), although different extraction methods (oleaginous, ethyl acetate, aqueous and acetonic) have also shown bioactivity against different parasites (Katiki *et al.*, 2011; Botura *et al.*, 2013; Kozan *et al.*, 2013). The evaluation of different extraction procedures for tropical plants might help to standardize extracts with possible AH effects against cattle nematodes and to identify the phytochemical classes present. The objectives of this study were (1) to assess the ovicidal activity of extracts from four plant species against *C. punctata*, (2) to state the role of polyphenols in the plants' anthelmintic activity, and (3) to evaluate the best plant extraction procedure when searching for ovicidal activity.

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Materials and methods

Plant material

Fresh leaves of *Leucaena leucocephala*, *Cratylia argentea*, *Gliricidia sepium* and *Guazuma ulmifolia* were harvested during March 2013 from an experimental area located at the Centro de Enseñanza, Investigación y Extensión en Ganadería Tropical (Center for Research, Teaching and Extension in Tropical Livestock) of the Facultad de Medicina Veterinaria y Zootecnia (Faculty of Veterinary Medicine and Animal Science) of the Universidad Nacional Autónoma de México (National Autonomus University of Mexico), located in Martínez de la Torre (20°03' N y 93°03' O; 151 m above sea level), Veracruz, Mexico. These plants were chosen because they have high levels of secondary plant metabolites, and some of the plants have been reported to exhibit AH activity against GIN of small ruminants (Alonso-Díaz *et al.*, 2008 a, b; Von Son-de Fernex *et al.*, 2012). Furthermore, these plants are predominant within the native vegetation of Veracruz and are also distributed in other tropical areas of the world (Flores-Guido, 2001). These fodder trees and shrubs are an important nutritional alternative for animal production.

Extraction procedure

For each plant species, 1 ± 0.15 kg of fresh leaves were air-dried at 60 °C for 72 h and then placed in a grinder to obtain particles of 1 mm in size. This material was then placed in a glass beaker (2 L) containing acetone:water (70:30) with a magnetic stirrer. The mixture was then sonicated for 4 h in a water bath (Branson Sonicator 2510MT®, Emerson Industrial Automation, Danbury, USA). The second extraction was performed by placing 500 ± 36.97 g of dried ground material from each plant species in a mixer with acetone and maintained at room temperature (24 °C) for 24 h. Finally, an aqueous extraction was performed, using the same ground material used for the acetonic extraction, which, after being dried, was placed in distilled water previously heated at 58 °C for 2 hours. For all extraction procedures, the extract was obtained from the filtered material using filter paper (Whatman® qualitative filter paper, Grade 1). Solvents were evaporated from the extracts at 58 °C using a low pressure distillation procedure in a rotovapor machine (Rotovapor® R-3, Büchi®, Switzerland). Extracts were washed 4 times with 500 mL of n-hexane to remove the chlorophyll and lipids, and a separation funnel was used for discarding the n-hexane fraction. Before the n-hexane fraction was discarded, a qualitative chromatographic profile was performed to confirm that only chlorophyll and lipids were removed. Finally, extracts were frozen and lyophilized to obtain the dry ground extracts.

Bioassays

Egg recovery

Eggs were obtained from a donor calf infected with the *C. punctata* strain multi-resistant towards macrocyclic lactones, benzimidazoles and imidazothiazoles (CEI-EGT-FMVZ-UNAM strain, Mexico). Adult males of *C. punctata* were identified using taxonomic keys (Gibbons, 1981) and molecular techniques (Von Son de Fernex, unpublished). Calves were housed indoors on a concrete floor, provided with hay and commercial concentrate and allowed free access to water. Feces were collect-

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ed daily using harnesses and polyurethane collection bags; samples were stored and processed at temperature of 23.37 ± 0.21 °C (mean ± SE). Tap water (1 L) was added to 200 g of feces with a fecal egg count (FEC) of 150 eggs per gram of feces (EPGF) and mixed to produce a relatively liquid suspension. Liquid feces were filtered through a household sieve with a 400-µm mesh size to remove coarse plant debris. Then, the suspension was serially filtered through sieves with pore sizes of 1000, 149 and 74 µm, with the eggs finally being trapped on a 24 µm mesh. The material on the 24-µm mesh was washed into 50-mL centrifuge tubes, which were filled with a saturated NaCl solution and centrifuged at 3000 rpm for 15 min. The supernatant was washed in tap water through a 24-µm mesh sieve, on which the eggs were collected. Clean eggs were concentrated and placed in 15-mL centrifuge tubes for counting. The egg concentration was estimated by counting the number of eggs in aliquots of 10 % of the suspension on a microscope slide. A final concentration of 500 eggs/mL was achieved either by concentrating the egg suspension through centrifugation or by diluting it with distilled water. The egg recovery process was standardized for completion in 1.25 ± 0.08 hours (mean ± SE).

Egg hatching assay (EHA)

Approximately 100 eggs/200 µL of egg suspension were pipetted into each well of a 24 well culture plate, and 200 µL of increasing concentrations (1.2, 2.4, 4.8, 9.6 and 19.2 mg mL^{-1}) of the corresponding plant species extract were placed in each test well. Thus, we obtained final concentrations of 0.6, 1.2, 2.4, 4.8 and 9.6 mg mL^{-1}. Levamisole was used as a positive control at a concentration of 10 % to equal the highest plant extract concentration (Dobson *et al.*, 1986). Distilled water was employed as a negative control for the 70:30 and aqueous extracts; whereas 2.5 % dimethyl sulfoxide (DMSO) was used for acetonic plant extracts (because it was employed as a low/non-polar compound solvent for the bioassays). Control wells also contained 200 µL of the egg suspension. Four replicates were run for each dose, extract and control. The plates were incubated at 27.7 ± 0.1 °C (mean ± SE) for 48 h. A drop of Lugol's iodine solution was added to each well to stop further hatching, and all the unhatched eggs and larvae (dead or alive) in each well were counted (Coles *et al.*, 1992). To confirm the role of polyphenolic compounds in an AH effect, another series of incubations was performed for 3 treatments: i) negative control (distilled water or DMSO 2.5 %), ii) the maximum dose of the extract to be tested (9.6 mg of extract/mL) with PEG (19.2 mg mL^{-1}) and a pre-incubation period of 3 h to bind the polyphenolic compounds (before egg exposure), and iii) the maximum dose of incubation without PEG (Makkar *et al.*, 1995).

Statistical analysis

A General Lineal Model (GLM) was used to assess a dose-dependent behaviour within each plant species extract ($Yij = \mu + Tj + Eij$), where the dependant variable was the egg hatching (Yij), which represents the *i*th observation taken under the *j*th treatment; the independent variable was the increasing concentration of each plant extract (Tj); µ represents the general mean; and Eij represents the residual variation or experimental error. Treatment means comparisons were performed with a Least Significant Difference (LSD) test, and the probability value indicative of statistical

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significance was $P < 0.05$ (F-test). No transformation was required because the data had normal distribution and homoscedasticity (STATGRAPHICS, Centurion XVI version 16.1.18, USA). A Kruskal-Wallis test was used to i) compare the egg hatching rates obtained for each plant species extract with and without PEG addition, ii) compare the egg hatching rate among extraction procedures within each plant species, and iii) evaluate extract yields among extraction procedures. Kruskal-Wallis test was employed when assumptions of ANOVA analysis did not met. The probability value indicative of statistical significance was $P < 0.05$ (H-test).

The percentage of egg hatching inhibition (EHI) was calculated using the following formula: Inhibition (%) = 100 (1 - P_t / P_c),where 1 represents the total number of eggs, P_t is the number of eggs hatched in a treatment group, and P_c is the respective number in water or DMSO control groups (Bizimenyera *et al.*, 2006). The lethal concentration to inhibit 50 % of egg hatching (LC_{50}) was calculated for each extract using a Probit Analysis Program (Minitab® 17.1.0, Minitab Inc., USA).

Results and discussion

Egg hatching assay (EHA)

The mean egg hatching (± SE) of *C. punctata* in negative and positive control groups ranged from 92.48 ± 1.97 % to 95.29 ± 0.76 % and 31.17 ± 4.69 % to 35.49 ± 4.37 %, respectively. Egg hatching showed a dose-dependent behaviour when exposed to each of the 12 extracts ($P < 0.01$) (Figures 1 to 3). *Leucaena leucocephala*-AQ inhibited more than 50 % of the *C. punctata* egg hatching ($P < 0.05$; $r^2 = 69.51$ %) (Figure 1). For *G. ulmifolia* and *C. argentea*, the highest inhibition rate was obtained with the AW extraction procedure: 45.42 ± 2.3% ($P < 0.01$; $r^2 = 95.71$ %) and 35.07 ± 1.40 % ($P < 0.01$; $r^2 = 97.46$ %), respectively (Figure 2). At the highest concentration, the *G. sepium*-AC fully inhibited hatching of the *C. punctata* eggs ($P < 0.01$; $r^2 = 94.42$ %) (Figure 3). *Cooperia* spp. is responsible for one of the GINs with higher prevalence in grazing cattle. Increasing reports of nematode resistance to chemotherapy notes the need to develop effective and secure strategies of control (Bartley *et al.*, 2012; Demeler *et al.*, 2013). This work provides evidence of the ovicidal effect of bioactive plant extracts against the egg and free-living stages of *C. punctata*. Reports on the novel technologies available to control free-living stages of cattle nematodes are scarce (Novobilsky *et al.*, 2011). Previous *in vitro* assessments have shown temperate legumes to be active against the infective larvae of *C. oncophora* (Novobilsky *et al.*, 2011), but few reports exist on the novel technologies available for other free-living stages such as egg hatching. The nematode egg is a GIN biological stage with a relatively thick tri-layered shell (Mansfield *et al.*, 1992), which provides resistance to adverse environmental conditions (temperature, moisture, UV radiation and trampling). These characteristics complicate the development of effective control strategies.

The lethal concentrations required for 50 % of hatching inhibition calculated for all 12 extracts are presented in Table 1. Best-fit LC_{50} values were 1.03 ± 0.17 and 7.9 ± 1.19 mg mL^{-1} for *G. sepium*-AC and *L. leucocephala*-AQ, respectively. For the AW extracts, the LC_{50} ranged from 8.84 to 15.12 mg mL^{-1}. The significant dose-dependent effect observed for most of the plant extracts indicates a toxicological response of the phytochemicals present in the 4 plant species evaluated (Hoste *et*

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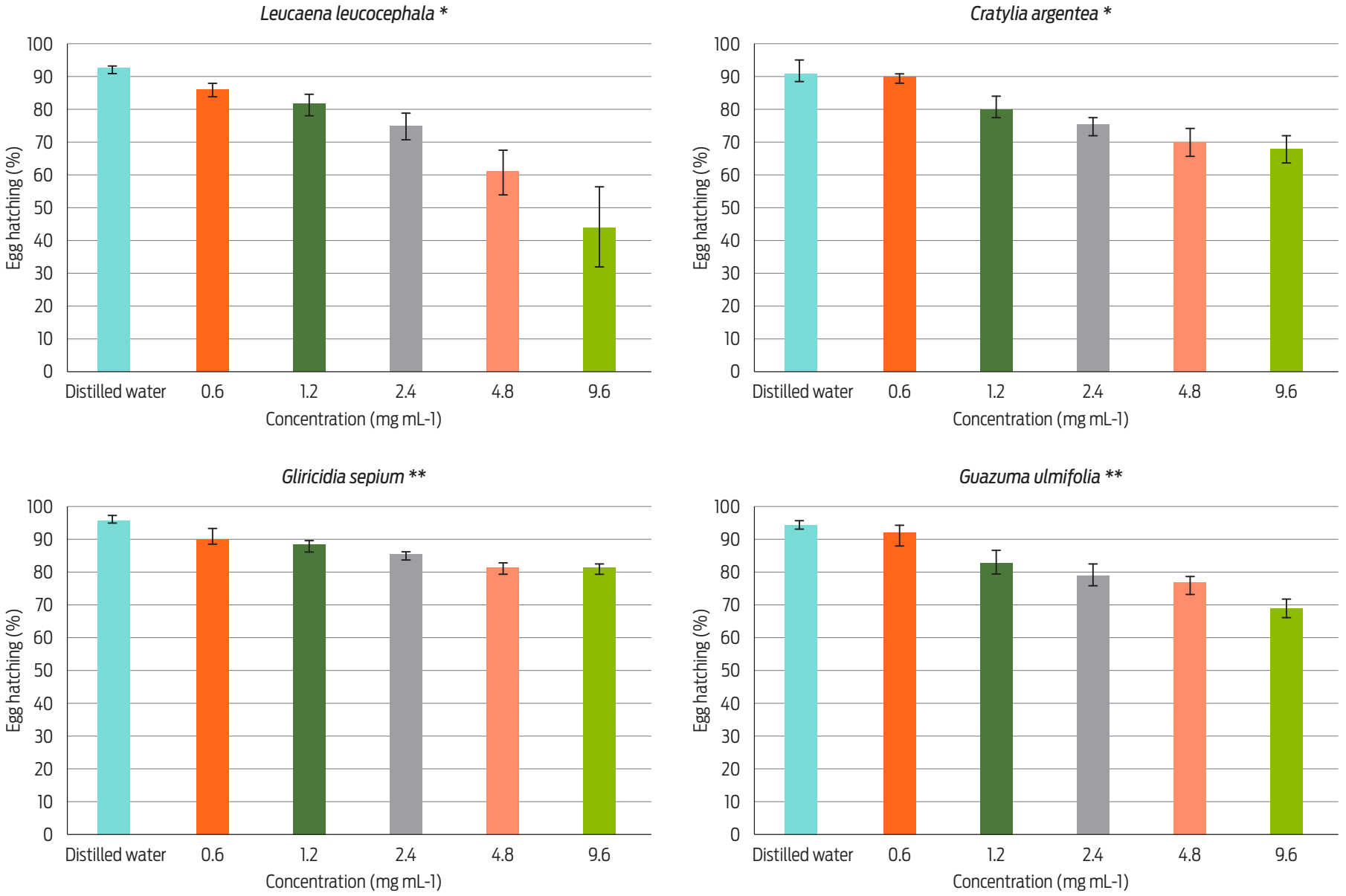

Figure 1. *Cooperia punctata* egg hatching after incubation in aqueous plant extracts (* $P < 0.05$; ** $P < 0.01$).

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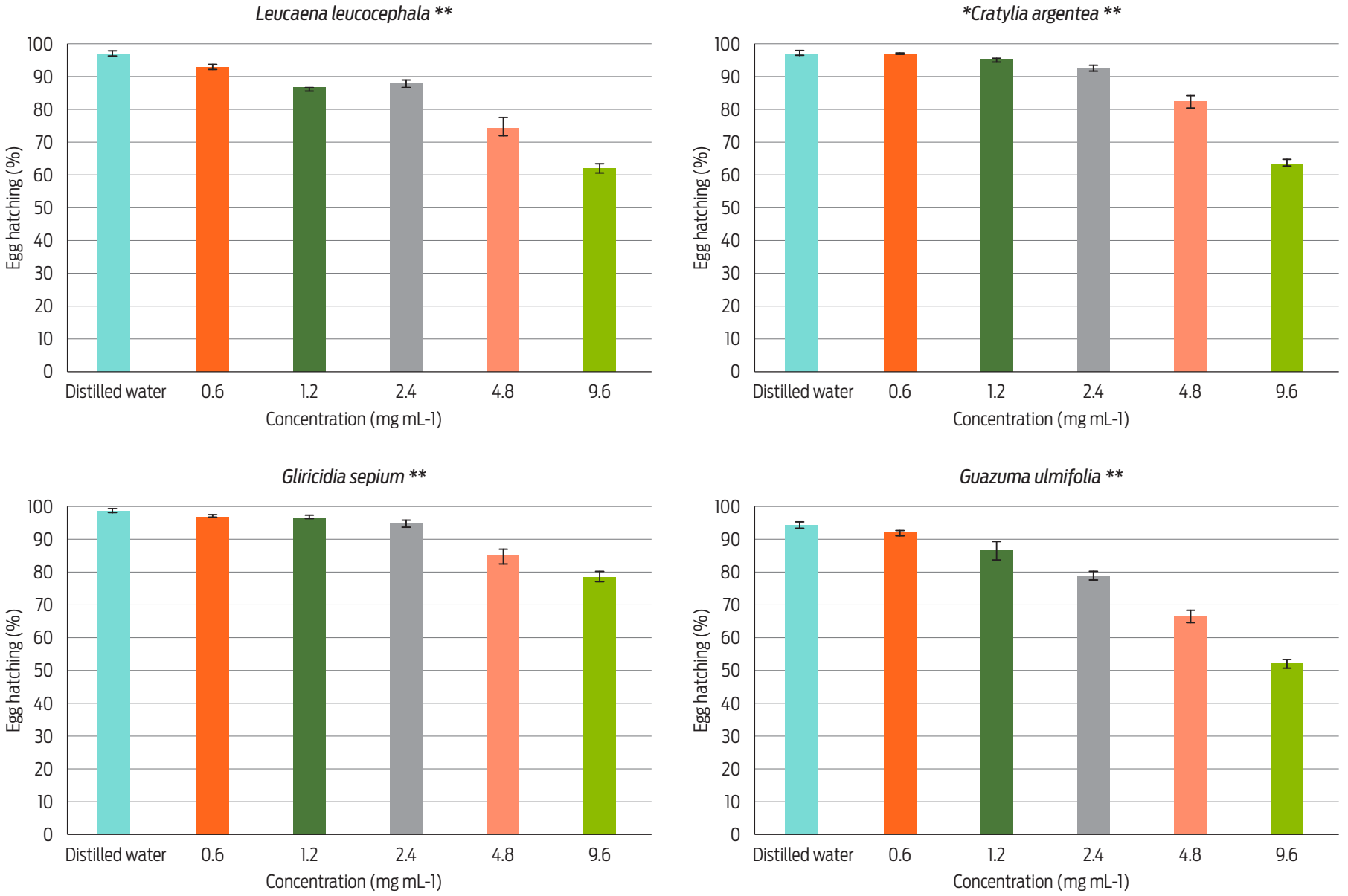

Figure 2. *Cooperia punctata* egg hatching after incubation in acetone:water plant extracts (* $P < 0.05$; ** $P < 0.01$).

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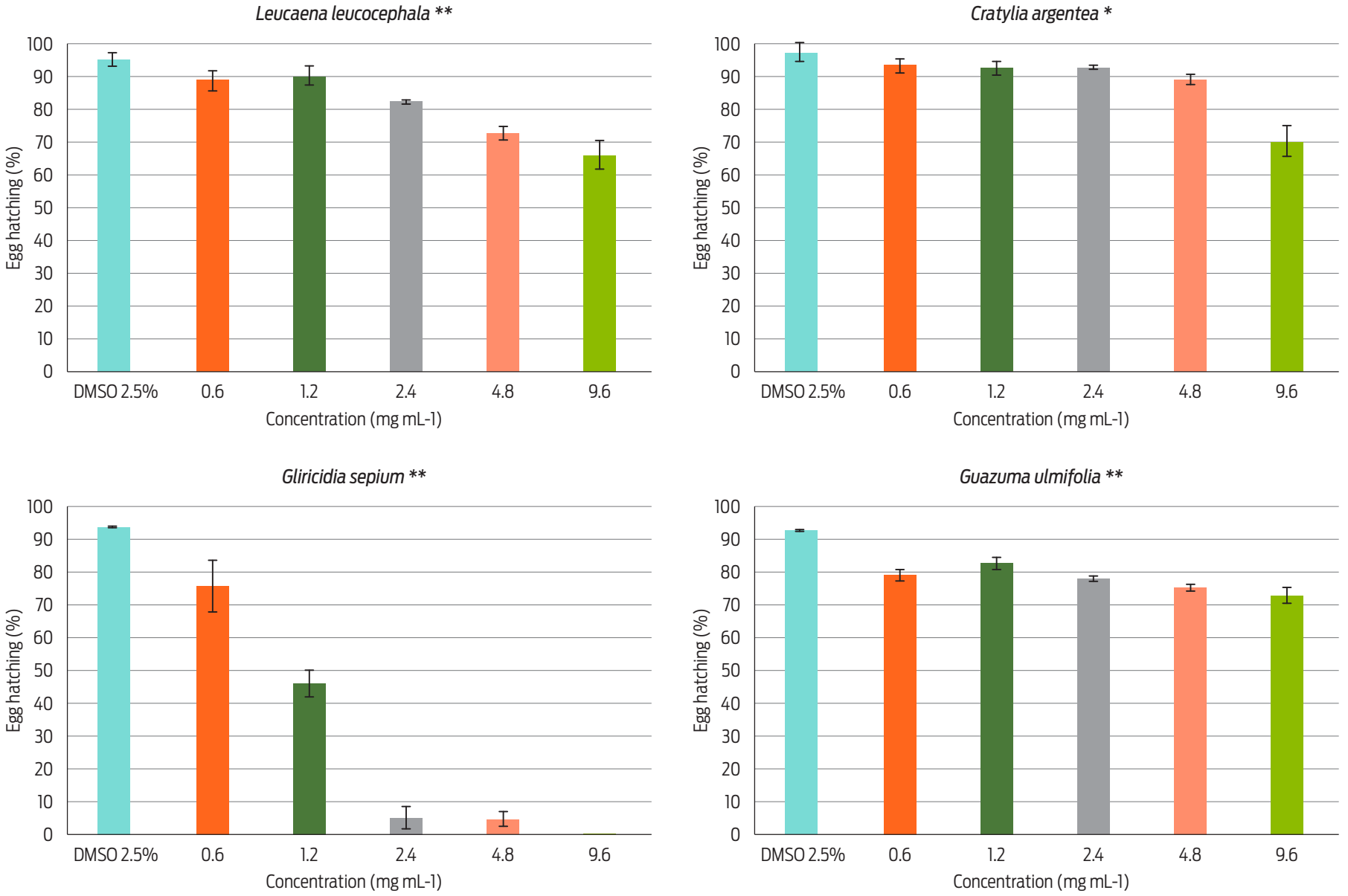

Figure 3. *Cooperia punctata* egg hatching after incubation in acetonic plant extracts (* $P < 0.05$; ** $P < 0.01$).

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Table 1. Lethal concentrations required to inhibit 50% of *Cooperia punctata* egg hatching (LC_{50}), after a 48-h incubation period with bioactive extracts (mg mL^{-1}).

Probit analysis (LC_{50}) for *C. punctata* egg hatching inhibition (95% Normal CI)					
Plant material	Extraction	Mean	Std. Error	Limits	
				Lower	Upper
L. leucocephala	Aqueous	7.93	1.19	5.59	10.27
	70:30	11.77	1.19	9.42	14.12
	Acetonic	13.84	1.19	11.51	16.16
C. argentea	Aqueous	15.05	4.44	6.34	23.75
	70:30	11.31	0.79	9.78	12.86
	Acetonic	14.79	2.51	9.88	19.71
G. sepium	Aqueous	32.63	16.41	0.47	64.79
	70:30	15.12	1.86	11.47	18.76
	Acetonic	1.03	0.17	0.69	1.37
G. ulmifolia	Aqueous	16.41	5.35	5.92	26.89
	70:30	8.84	0.89	7.09	10.59
	Acetonic	29.08	9.31	10.83	47.34

al., 2012). However, further studies are necessary for the identification and isolation of the AH-like molecules present in the most active extracts. Phytochemical identification could help to understand the ongoing mechanisms involved in their activity.

Role of polyphenols in the ovicidal activity of bioactive plant extracts

The addition of polyethylene glycol revealed the role of polyphenolic compounds in the ovicidal activity of most plant extracts, restoring egg hatching to values similar to those for control groups (distilled water or DMSO 2.5 %) (Table 2). However, almost no reestablishment was achieved with *G. sepium*-AC (EHI of 79.85 ± 1.2 %), discarding polyphenols as the main bioactive compound present in those extracts (Table 2). The hydrophilic polymer PEG was utilized to test for the role of polyphenols in the extracts regarding bioactivity (Makkar, 2003). When PEG was added, the inhibition values of most extracts were restored to values similar to those obtained with negative controls indicating the predominate role of polyphenols (Table 2). Nevertheless, inhibition values of 79.85 ± 1.2 % were obtained after PEG addition for *G. sepium*-AC, which suggests the possible involvement of other phytochemicals in the ovicidal activity of the acetonic extracts. Thin layer chromatography analysis is one of the most frequently used techniques when evaluating herbal medicines for the identification and differentiation of phytochemical classes (Rafi *et al.*, 2011). The chromatographs obtained for each plant extract, as well as the use of PEG, suggest that medium-polar flavonoids have ovicidal activity against *C. punctata*. This is in agreement with previous authors who have reported flavonols, such as quercectin, rutin and kempherol, as having AH properties (Barrau *et al.*, 2005). On the other hand, fingerprint analysis of *G. sepium*-AC extract show the predominant constituent to be a low polar phytochemical visible under UV

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Table 2. Egg hatching inhibition (EHI) obtained with the highest concentration tested (9.6 mg mL^{-1}) with or without addition of polyethylene glycol (PEG), among plant species and extraction procedures. Different small letters in the same row and different capital letters amongst extraction procedures of the same plant, represent statistically significant differences ($P < 0.05$).

Plant material	Extraction	*Cooperia punctata* egg hatching inhibition (%)	
		EHI (%)	PEG EHI (%)
L. leucocephala	Aqueous	52.01 ± 12.4 [a]	12.59 ± 2.5 [b]
	70:30	35.75 ± 1.53 [a]	0.82 ± 0.90 [b]
	Acetonic	29.80 ± 1.50 [a]	2.76 ± 1.50 [b]
C. argentea	Aqueous	25.73 ± 4.10 [a]	5.86 ± 3.40 [b]
	70:30	35.07 ± 1.40 [a]	0.16 ± 0.20 [b]
	Acetonic	26.76 ± 4.90 [a]	0.67 ± 1.60 [b]
G. sepium	Aqueous [A]	15.40 ± 1.40 [a]	1.78 ± 0.90 [b]
	70:30 [A]	19.59 ± 1.70 [a]	2.40 ± 0.50 [b]
	Acetonic [B]	100.0 ± 0.00 [a]	79.85 ± 1.2 [b]
G. ulmifolia	Aqueous [A]	27.26 ± 3.40 [a]	2.48 ± 3.10 [b]
	70:30 [B]	45.42 ± 2.30 [a]	0.95 ± 1.20 [b]
	Acetonic [A]	21.46 ± 3.00 [a]	1.59 ± 1.50 [b]

short wave (254 nm) but non-reactive when sprayed with AS and NEU reagents. Additionally, PEG failed to restore egg hatching inhibition to control values (79.85 ± 1.2 %), thus supporting the suggestion of a non-flavonoid phytochemical with AH-like activity but disagreeing with Wabo Poné *et al.* (2011), who linked the role of CT in *G. sepium*-AC extracts to *H. contortus* egg hatching inhibition. It was not possible to elucidate the phytochemical involved in the AH effect in the present study; however, this information could be helpful to a better understanding of the possible mechanisms of action on *C. punctata*.

Plant extract yields

Phytochemical extraction showed yield differences among the extraction procedures ($P < 0.05$). The AW extraction provided a yield of 10.04 ± 0.91 % (Mean ± SE). Individual yield percentages are shown in Table 3. In this study, extraction procedures were also compared based on their ovicidal activity. Best inhibitory values were observed using uni-solvent extractions (*G. sepium*-AC and *L. leucocephala*-AQ), leading to the perception that compounds with similar polarity features could enhance bioactivity. Yet the overall performances of each extraction procedure, assessed through LC_{50}, were 16.64 ± 4.27 (Mean ± SE), 12.41 ± 1.19 and 13.49 ± 4.59 mg mL^{-1} for AQ, AW and AC, respectively (Table 3). Furthermore, among the extraction procedures, AW showed the highest yield percentage ($P < 0.05$).

Analyses in this investigation allowed for the determination of both i) flavonoids having ovicidal activity and ii) flavonoids predominant in the AW extractions. The latter is consistent with previous studies that report the acetone:water extraction as the most efficient system for phenol recovery (Chavan *et al.*, 2001; Chavan and Amarowicz, 2013). Previous phytochemical studies have reported a synergistic/antagonistic effect among components from the same extract (Biavatti, 2009),

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Table 3. Phytochemical yield of 4 plant materials using 3 extraction procedures (aqueous, acetone:water, and acetonic). Different letters in the means of each extraction procedure represent statistically significant differences ($P < 0.05$).

Plant material	Extraction yield (%)		
	Aqueous	Acetone:water	Acetonic
L. leucocephala	6.40	7.31	1.33
C. argentea	6.44	9.79	2.67
G. sepium	5.74	9.53	1.01
G. ulmifolia	5.78	10.62	1.00
Mean ± SE	6.09 ± 0.17 [a]	9.31 ± 0.91[b]	1.50 ± 0.31[c]

which could explain how the *G. sepium*-AC extract showed an exceptional ovicidal performance, but when the extractions were performed with acetone:water, the bioactivity was barely noticeable. Thus, such data trends were only observed in one of the four plants analyzed, *G. sepium*, and overall, the AW extraction showed equal or improved AH activity. However, further bio-guided phytochemical fractionation is suggested for the determination of the active molecules present in each plant extract and their interactions.

Conclusions

The present investigation corroborated the ovicidal potential of acetone:water plant extracts against *C. punctata*. The use of PEG indicated that polyphenolic compounds were the main phytochemical class involved in the AH activity. *Leucaena leucocephala* and *Gliricidia sepium* were the forages with the strongest anthelmintic-like phytochemicals, and they could be considered for further *in vivo* evaluations.

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Conflicts of interest

Miguel Ángel Alonso Díaz is the director of Centro de Enseñanza, Investigación y Extensión en Ganadería Tropical from Facultad de Medicina Veterinaria y Zootecnia. The rest of the authors have no financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Author contributions

Elke von Son de Fernex conceived the study, performed all the plant extractions and assays, and wrote the manuscript.

Miguel Ángel Alonso Díaz performed the experimental design and helped with the manuscript redaction and revision.

Pedro Mendoza de Gives helped isolate *C. punctata* and revised the manuscript.

Braulio Valles de la Mora performed the statistical analysis of the experiments and revised the manuscript.

Alejandro Zamilpa designed the plant extraction and phytochemical evaluation methods and revised the manuscript.

Manasés González Cortazar interpreted the results of the phytochemical screening and revised the manuscript.

References

1) Alonso-Diaz MA, Torres-Acosta JF, Sandoval-Castro, CA, Aguilar-Caballero, A.J., Hoste, H. 2008a. In vitro larval migration and kinetics of exsheathment of Haemonchus contortus larvae exposed to four tropical tanniniferous plant extracts. *Veterinary Parasitology*, 153:313-319.
2) Alonso-Diaz MA, Torres-Acosta JF, Sandoval-Castro CA, Capetillo-Leal C, Brunet S, Hoste H. 2008b. Effects of four tropical tanniniferous plant extracts on the inhibition of larval migration and the exsheathment process of *Trichostrongylus colubriformis* infective stage. *Veterinary Parasitology*, 153:187-192.
3) Alonso-Diaz MA, Torres-Acosta JF, Sandoval-Castro CA, Hoste H. 2010. Tannins in tropical tree fodders fed to small ruminants: A friendly foe? *Small Ruminant Research*, 89:164-173.
4) Arnaud-Ochoa R, Alonso Díaz MA. 2012. Unidades de producción bovina con nematodos gastrointestinales resistentes al albendazol (benzimidazoles) en México. *Revista Cientifica*, XXII (4):315-320.
5) Athanasiadou S, Kyriazakis I, Jackson F, Coop RL. 2001. Direct anthelmintic effects of condensed tannins towards different gastrointestinal nematodes of sheep *in vitro* and *in vivo* studies. *Veterinary Parasitology*, 99:205-219.
6) Barrau E, Fabre N, Fouraste I, Hoste H. 2005. Effect of bioactive compounds from Sainfoin (*Onobrychis viciifolia* Scop.) on the *in vitro* larval migration of *Haemonchus contortus*: Role of tannins and flavonol glycosides. *Parasitology*, 131:531-538.
7) Bartley DJ, McArthur CL, Devin LM, Sutra JF, Morrison AA, Lespine A, Matthews JB. 2012. Characterisation of macrocyclic lactone resistance in two field-derived isolates of *Cooperia oncophora*. *Veterinary Parasitology*, 190:454-460.
8) Becerra-Nava R, Alonso-Diaz MA, Fernandez-Salas A, Quiroz RH. 2014. First report of cattle farms with gastrointestinal nematodes resistant to levamisole in Mexico. *Veterinary Parasitology*, 204:285-290.
9) Biavatti MW. 2009. Synergy: An old wisdom, a new paradigm for pharmacotherapy. *Brazilian Journal of Pharmaceutical Sciences*, 45:371-378.
10) Bizimenyera ES, Githiori JB, Eloff JN, Swan GE. 2006. *In vitro* activity of *Peltophorum africanum* Sond. (*Fabaceae*) extracts on the egg hatching and larval development of the parasitic nematode *Trichostrongylus colubriformis*. *Veterinary Parasitology*, 142:336-343.

DOI: http://dx.doi.org/10.21753/vmoa.3.2.365
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11) Botura MB, dos Santos JD, da Silva GD, de Lima HG, de Oliveira JV, de Almeida MA, Batatinha MJ, Branco A. 2013. In vitro ovicidal and larvicidal activity of *Agave sisalana* Perr. (sisal) on gastrointestinal nematodes of goats. *Veterinary Parasitology*, 192:211-217.
12) Charlier J, Hoglund J, von Samson-Himmelstjerna G, Dorny P, Vercruysse J. 2009. Gastrointestinal nematode infections in adult dairy cattle: Impact on production, diagnosis and control. *Veterinary Parasitology*, 164:70-79.
13) Chavan UD, Amarowicz R. 2013. Effect of various solvent systems on extraction of phenolics, tannins and sugars from beach pea (Lathyrus maritimus L.). *International Food Research Journal*, 20(3):1139-44.
14) Chavan UD, Shahidia F, Naczkb M. 2001. Extraction of condensed tannins from beach pea (*Lathyrus maritimus L.*) as affected by different solvents. *Food Chemistry*, 75:509–512.
15) Coles GC, Bauer C, Borgsteede FHM, Geerts S, Klei TR, Taylor MA, Waller PJ. 1992. World Association for the Advancement of Veterinary Parasitology (WAAVP) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. *Veterinary Parasitology*, 44:35-44.
16) Demeler J, Krucken J, AlGusbi S, Ramunke S, De Graef J, Kerboeuf D, Geldhof P, Pomroy WE, von Samson-Himmelstjerna G. 2013. Potential contribution of P-glycoproteins to macrocyclic lactone resistance in the cattle parasitic nematode *Cooperia oncophora*. *Molecular & Biochemical Parasitology*, 188:10-19.
17) Dobson RJ, Donald AD, Waller PJ, Snowdown KL. 1986. An egg-hatch assay for resistance to levamisole in trichostrongyloid nematode parasites. *Veterinary Parasitology*, 19:77-84.
18) Fitzpatrick JL. 2013. Global food security: The impact of veterinary parasites and parasitologists. *Veterinary Parasitology*, 195:233-248.
19) Flores-Guido JS. 2001. Leguminosae. *Florística, Etnobotánica y Ecología*. Etnoflora Yucatense. Universidad Autónoma de Yucatán; Mérida, Yucatán, México.
20) Gasbarre LC. 2014. Anthelmintic resistance in cattle nematodes in the US. *Veterinary Parasitology*, 204:3-11.
21) Gibbons LM. 1981. Revision of the african species of the genus *Cooperia Ransom*, 1907 (*Nematoda*, *trichostrongylidae*). *Systematic Parasitology*, 2:219-252
22) Hoste H, Martinez-Ortiz-De-Montellano C, Manolaraki F, Brunet S, Ojeda-Robertos N, Fourquaux I, Torres-Acosta JF, Sandoval-Castro CA. 2012. Direct and indirect effects of bioactive tannin-rich tropical and temperate legumes against nematode infections. *Veterinary Parasitology*, 186:18-27.
23) Katiki LM, Chagas AC, Bizzo HR, Ferreira JF, Amarante AF. 2011. Anthelmintic activity of *Cymbopogon martinii*, *Cymbopogon schoenanthus* and *Mentha piperita* essential oils evaluated in four different *in vitro* tests. *Veterinary Parasitology*, 183:103-108.
24) Kenyon F, Jackson F. 2012. Targeted flock/herd and individual ruminant treatment approaches. *Veterinary Parasitology*, 186:10-17.
25) Kozan E, Anul SA, Tatli II. 2013. *In vitro* anthelmintic effect of *Vicia pannonica* var. *purpurascens* on trichostrongylosis in sheep. *Experimental Parasitology*, 134:299-303.
26) Makkar HPS. 2003. Quantification of tannins in tree and shrub foliage. *A laboratory manual*. Dordrecht, Netherlands: Kluwer Academic Publishers.

DOI: http://dx.doi.org/10.21753/vmoa.3.2.365
Vol. 3 | No. 2 | April-June | 2016

27) Makkar HPS, Blummel M, Becker K. 1995. Formation of complexes between polyvinyl pyrrolidones or polyethylene glycols and tannins, and their implication in gas production and true digestibility *in vitro* techniques. *British Journal of Nutrition*, 73:897-913.
28) Mansfield LS, Gamble HR, Fetterer RH. 1992. Characterization of the eggshell of haemonchus contortus-i. Structural components. *Comparative Biochememistry and Physiology*, 103B(3):681-686.
29) Martinez-Ortiz-de-Montellano C, Vargas-Magana JJ, Canul-Ku HL, Miranda-Soberanis R, Capetillo-Leal C, Sandoval-Castro CA, Hoste H, Torres-Acosta JF. 2010. Effect of a tropical tannin-rich plant *Lysiloma latisiliquum* on adult populations of *Haemonchus contortus* in sheep. *Veterinary Parasitology*, 172:283-290.
30) Molan AL, Duncan AJ, Barry TN, McNabb WC. 2003. Effects of condensed tannins and crude sesquiterpene lactones extracted from chicory on the motility of larvae of deer lungworm and gastrointestinal nematodes. *Parasitology International*, 52:209-218.
31) Molan AL, Waghorn GC, Min BR, McNabb WC. 2000. The effect of condensed tannins from seven herbages on *Trichostrongylus colubriformis* larval migration in vitro. *Folia Parasitologica*, 47:39-44.
32) Novobilsky A, Mueller-Harvey I, Thamsborg SM. 2011. Condensed tannins act against cattle nematodes. *Veterinary Parasitology*, 182:213-220.
33) Perri AF, Mejia ME, Licoff N, Lazaro L, Miglierina M, Ornstein A, Becu-Villalobos D, Lacau-Mengido IM. 2011. Gastrointestinal parasites presence during the peripartum decreases total milk production in grazing dairy Holstein cows. *Veterinary Parasitology*, 178:311-318.
34) Rafi M, Rohaeti E, Miftahudin A, Darusman LK. 2011. Differentiation of *Curcuma longa*, *Curcuma xanthorrhiza* and *Zingiber cassumunar* by thin layer chromatography fingerprint analysis. *Indonesian Journal of Chemistry*, 11(1):71-74.
35) Stromberg BE, Gasbarre LC, Waite A, Bechtol DT, Brown MS, Robinson NA, Olson EJ, Newcomb H. 2012. *Cooperia punctata*: Effect on cattle productivity? *Veterinary Parasitology*, 183:284-291.
36) Sutherland IA, Leathwick DM. 2011. Anthelmintic resistance in nematode parasites of cattle: A global issue? *Trends in Parasitology*, 27:176-181.
37) Von Son-de Fernex E, Alonso-Diaz MA, Valles-de la Mora B, Capetillo-Leal CM. 2012. *In vitro* anthelmintic activity of five tropical legumes on the exsheathment and motility of *Haemonchus contortus* infective larvae. *Experimental Parasitology*, 131:413-418.
38) Wabo Poné J, Kenne Tameli F, Mpoame M, Pamo Tedonkeng E, Bilong Bilong C. 2011. *In vitro* activities of acetonic extracts from leaves of three forage legumes (*Calliandra calotyrsus*, *Gliricidia sepium* and *Leucaena diversifolia*) on *Haemonchus contortus*. *Asian Pacific Journal of Tropical Medicine*, 4:125-1 28.