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Elucidation of *Leucaena leucocephala* anthelmintic-like phytochemicals and the ultrastructural damage generated to eggs of *Cooperia* spp.

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ABSTRACT

Leucaena leucocephala is a tropical forage legume suggested as an alternative method to control gastrointestinal parasitism in ruminants. This study: (1) performed a bio-guided fractionation of an aqueous extract of *L. leucocephala* using the egg hatch assay (EHA) to identify the anthelmintic (AH)-like phytochemicals present in fresh leaves, and (2) assessed the ultrastructural damage to eggs of *Cooperia* spp. after incubation with the final fraction. Phytochemicals were isolated using silica gel columns and identified using high performance liquid chromatography and standards for comparison. The final fraction was evaluated using EHA at 0.06, 0.125, 0.250, 0.500 and 1.1 mg ml^{-1}. The lethal concentration to inhibit 50% of *Cooperia* spp. egg hatching (LC_{50}) was calculated using a Probit analysis. Scanning and transmission electron microscopy revealed the ultrastructural changes present in *Cooperia* spp. eggs. Bio-guided isolation procedures led to the recognition of an active fraction (LlC1F3) mainly composed of quercetin (82.21%) and caffeic acid (13.42%) which inhibited 90.49 ± 2.8% of *Cooperia* spp. egg hatching ($P < 0.05$), and an LC_{50} of 0.06 ± 0.14 mg ml^{-1}. Scanning electron microscopy (SEM) showed eggs exposed to the active fraction had an irregular external layer with small projections and ruptures of lateral eggshell walls. Transmission electron microscopy (TEM) showed changes to *Cooperia* spp. eggs in electro-density, including the thickness of the eggshell layers and fractures after incubation with the final fraction (LlC1F3).

Changes in bioactivity after purification suggest synergistic interactions between quercetin and caffeic acid.

1. Introduction

Cooperia spp. is an endemic genus of gastrointestinal nematodes (GINs) of grazing cattle in tropical and subtropical environments. These parasites have recently been reported as one of the most prevalent group of GINs in grazing cattle worldwide (Fiel et al., 2012; Stromberg et al., 2012; Vlaminck et al., 2015). The negative impact of *Cooperia* spp. is due to the high costs of control schemes and the decreased productivity of cattle (Stromberg et al., 2012). Broad-spectrum anthelmintics (AHs) are a suitable tool for the control of GINs, which also enhance the productivity and performance of other animals (Sutherland and Leathwick, 2011). However, the increasing presence of *Cooperia* spp. strains resistant to broad-spectrum AHs on cattle farms (Bartley et al., 2012; Becerra-Nava et al., 2014; Njue and Prichard, 2004) supports the development of novel treatments to control GIN populations inside and outside hosts. The use of bioactive plants as a source of secondary metabo-

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lites with AH-like properties represents a viable alternative (Hoste et al., 2006).

The tropical forage *Leucaena leucocephala* (Lam.) de Wit is a member of the family Fabaceae. It is a perennial browse legume native to Central America and southern Mexico, and has been introduced into most tropical and subtropical areas of the world (Nehdi et al., 2014). *L. leucocephala* preparations have been used in traditional medicine mainly for its anti-inflammatory and anti-diabetic activities (Souza Pinto et al., 1995; Syamsudin et al., 2010). Furthermore, *L. leucocephala* has been reported to have AH properties against some of the most important GINs of small ruminants, involving tannins/polyphenols as the phytochemical group responsible for the AH effect (Ademola et al., 2005; Alonso-Diaz et al., 2008). Similar results were found against cattle nematodes where *L. leucocephala* aqueous extracts inhibited both egg hatching and the exsheathment process of *Cooperia* spp. (von Son-de Fernex et al., unpublished data), and flavonoids/polyphenols were identified as the most important compounds involved in the AH effect. Nevertheless, in the latter study, the participation or co-participation of other phytochemical classes in the active extract, especially on the exsheatment process of *Cooperia*, were suggested.

There are limited reports identifying the AH-like phytochemicals present in *L. leucocephala*, but to our knowledge there are no reports targeting cattle nematodes. Therefore, bio-guided fractionation of *L. leucocephala* leaves due to its ovicidal activity against *Cooperia* spp. might help to elucidate and evaluate the phytochemical involved in the AH effect to better understand the possible mechanisms of action on *Cooperia* spp. It has been suggested that the AH effect of phytochemicals such as tannins, might be linked to direct interactions between polyphenols and the structural proteins in the nematode cuticle (Hoste et al., 2006; Brunet et al., 2011). However, there are no studies assessing the ultrastructural damage created by *L. leucocephala* phytochemicals on the free living stages of *Cooperia* spp.

This study: (1) performed a bio-guided fractionation of an aqueous extract from *L. leucocephala* using the EHA to identify the AH-like phytochemicals present in fresh leaves, and (2) assessed the ultrastructural damage to *Cooperia* spp. eggs after incubation with the final fraction.

2. Materials and methods

2.1. Plant material

Fresh leaves of *L. leucocephala* (1865 g) were harvested during February 2014 from an experimental area located at the Centro de Enseñanza, Investigación y Extensión en Ganadería Tropical, de la Facultad de Medicina Veterinaria y Zootecnia, de la Universidad Nacional Autónoma de México (CEIEGT-FMVZ-UNAM) (Centre for Research, Teaching and Extension in Tropical Livestock, of the Faculty of Veterinary Medicine, of the National Autonomous University of Mexico), located in Martinez de la Torre (20°03′ N and 93°03′ W; 151 masl), Veracruz, Mexico.

2.2. Extraction procedure

Fresh leaves were air-dried at 60 °C for 72 h and placed in a grinding mill to obtain smaller particles (1 mm). Air-dried powdered material (404 g) was placed in a glass beaker with distilled water (previously heated to 58 °C) and a magnetic stir-bar, and was continuously sonicated for 4 h in a water bath (Branson Sonicator 2510®; 40 KHz). The extract was separated from the solid material using filter paper (Whatman® qualitative filter paper, Grade 1), and the solvent was evaporated from the extracts at 58 °C using low pressure distillation in a rotovapor machine (Rotovapor® R-3). Extracts were washed four times with 500 ml of *n*-hexane to remove chlorophyll and lipids, and a separation funnel was used for discarding the *n*-hexane fraction. Finally, extracts were frozen and lyophilized to obtain the dry ground extracts.

2.3. Chemical fractionation of the aqueous extract using the egg hatch assay

The aqueous extract (2.77 g) was fractioned through a bipartition process using ethyl acetate and water, obtaining a low-polarity fraction (LlC'-AcoET, organic) and a polar fraction (LlA'-Aq, aqueous). The fraction LlC'-AcoET (1.6 g) was subjected to further fractionation using a silica gel column, eluted with dichloromethane and methanol with an ascending polarity of 5% resulting in five main fractions (LlC1F1, 35.3 mg; LlC1F2, 48.9 mg; LlC1F3, 500.0 mg; LlC1F4, 409.9 mg and, LlC1F5, 42.0 mg). The best AH value was obtained with LlC1F3, which was further analyzed using high performance liquid chromatography (HPLC) (Section 2.4.2). Due to the identification of a compound mixture, LlC1F3 was suspended in dichloromethane resulting in two final fractions (soluble fraction LlC1F3A and precipitate fraction LlC1F3B) for which AH evaluations also were performed and were analyzed using HPLC. Prior to all AH evaluations performed during the experiment, fractions were concentrated to dryness using low-pressure distillation in a rotovapor machine (Rotovapor® R-3), and then were lyophilized. The EHA was used to perform the bio-guided fractionation of *L. leucocephala* because in previous studies the exsheathment process showed higher sensitivity to plant extracts.

2.4. Phytochemical identification

2.4.1. Thin layer chromatography analysis of fractions obtained through isolation procedures

Qualitative phytochemical screening of *L. leucocephala* fractions was performed in silica gel 60 F_{254}-pre-coated thin layer chromatography (TLC) plates using capillary tubes, and developed in a TLC chamber using suitable mobile phases for either terpenoids or flavonoids. The developed TLC plates were air-dried and observed under ultraviolet light at both 254 nm and 365 nm. They were later sprayed with natural products-polyethylene glycol reagent and anisaldehyde–sulphuric acid reagent, for flavonoids and terpenoids, respectively (Wagner and Bladt, 1996). Finally, the plates were placed in a hot air oven for 1 min for the development of color in separated bands. Qualitative determination of compounds was performed according to the colorimetric standards established by Wagner and Bladt (1996). Retention factors (Rf) of phytochemicals were obtained using the formula: Rf = MDP/MDS, where MDP and MDS represent the migration distance of the phytochemical and of the solvent, respectively. Qualitative TLC analysis was employed starting with the aqueous crude extract, leading up to the final fractions in order to perform an accurate fractionation procedure.

2.4.2. High performance liquid chromatography analysis of fractions obtained through isolation procedures

High performance liquid chromatography (HPLC) was used for the final fractions analysis, using a Waters® 2695 separation-module HPLC system equipped with a Waters® 996 photodiode array detector and Empower Pro software (Waters Corporation®, USA). Compounds were separated on a supersphere 100 RP-18 column (4 × 125 mm, 5 μm) (Merck, Darmstadt, Germany). The mobile phase consisted of water (5% TFA, solvent A) and acetonitrile (solvent B). The gradient system was: 0–1 min, 0% B; 2–4 min, 10% B; 5–7 min, 20% B; 8–14 min, 30% B; 15–18 min, 40% B; 19–22 min, 80% B; 23–26 min, 100% B, and 27–28 min, 0% B. Flow rate was maintained at 1 ml min^{-1} and the injection volume was 20 μL. Absorbance was measured at 360 nm. Flavonoid peaks