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PAPER

In vitro activity of *Pithecellobium dulce* and *Lysiloma acapulcensis* on exogenous development stages of sheep gastrointestinal strongyles

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Abstract

An experiment was conducted to evaluate the effects of two lyophilised aqueous extracts of *Lysiloma acapulcensis* (LAE) and *Phitecellobium dulce* (PDE) tree leaves on *in vitro* assessment of hatching of eggs, larval development and migration of gastrointestinal nematodes of sheep using a general linear model. Treatments contained extracts from both species at concentrations of 0, 125, 250 and 500 µg/mL. Both albendazole and levamisole were used at a level of 1% as positive control. The extract of LAE, compared to PDE, showed better inhibition ($P<0.05$) of egg hatching. Different doses of both the LAE and PDE extracts showed a larvicidal effect ($P<0.05$) on all larvae exposed to different doses of the extracts. In the larval migration assay, a similar effect with levamisole at doses of 250 and 500 µg/mL occurred with the LAE extract. The extract of *P. dulce* had a lower larvicidal effect ($P<0.05$) than levamisole and *L. acapulcensis* extracts. Using aqueous extracts of both species of *L. acapulcensis* and *P. dulce* could be a promising alternative to synthetic anthelmintics as treatments of gastrointestinal nematodes of sheep in organic and conventional production systems under subtropical conditions.

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Introduction

Gastrointestinal parasitism in small ruminants in tropical regions has been classified as a major health and welfare problem. Helminthic infections are a major cause for reduced productivity in livestock, particularly those owned by poor farmers (Hounzangbe-Adote *et al*., 2005). Development of resistant strains of nematodes to synthetic anthelmintics (Jackson and Coop, 2000) and the move to organic farming systems over the past few years have increased the demand for alternatives to chemoprophylaxis as a means of reducing the use of anthelmintic drugs to control parasites (Athanasiadou *et al*., 2000; Waller and Thamsborg, 2004).

Use of plants containing high levels of condensed tannins (CT) or CT extracts (CTE) are potential alternative methods to reduce the worm burden, nematode female fecundity and egg hatchability (Athanasiadou *et al*., 2001; Nguyen *et al*., 2005; Githiori *et al*., 2006; Meja-Hernandez *et al.* 2014). These plants, with anthelmintic properties, are promising due to their beneficial effects on health, rather than for their direct nutritional value. Both *L. acapulcensis* and *P. dulce* are native tree species widely distributed in the semi-arid regions of Mexico, which have high condensed tannins concentrations (Camacho *et al*., 2010), and constitute a substantial part of the diet consumed by grazing goats and sheep.

The objective of the present study was to evaluate the anthelmintic effects of *Lysiloma acapulcensis* (LAE) and *Phitecellobium dulce* (PDE) on egg hatching, *P. dulce* larval development and migration of gastrointestinal nematodes of sheep.

Materials and methods

Plant materials

Fresh leaves of *L. acapulcensis* and *P. dulce* were harvested in July 2011 from an area located at the Centro Universitario Universidad Autónoma del Estado de México, Temascaltepec, Mexico. Geographically, this is located at 19°02'04" west longitude at an elevation of 1720 m asl. The climate is moderately humid with an average temperature of 15 to 18°C an annual rainfall of 950 to 1000 mm (García, 1987).

Preparation of plant extracts

Plant extracts were prepared according to Salem *et al*. (2006). Briefly, 100 g of each plant species leaves were chopped and extracted with distilled water (100 mL of water: 10 g of leaves). This solution was incubated for 48 h at room temperature, and then filtered through three layers of cheesecloth. Finally, the remaining fractions were lyophilised and kept refrigerated at 4°C in air-tight containers until use for biological assays.

Chemical composition and condensed tannins determination

Lyophilised samples of LAE and PDE were analysed for dry matter (DM) (method 934.01), organic matter (OM) (method 942.05), crude protein (CP) (method 954.01) according at AOAC (1990). Neutral (NDF) and acid detergent fiber (ADF) were determined by the method of Van Soest *et al*. (1991). Total condensed tannins (TCT) were analysed using the butanol-HCL method (Terrill *et al*., 1992), as modified by López *et al.* (2004), as internal standards using *L. acapulcensis*. Analyses of the free (FCT1), protein- (PCT) and fibre-(FCT2) bound CT were conducted following Porter *et al.* (1986). Purification was with Sephadex LH-20 as described by Asquith and Butler (1985), with the modifications of Hedqvist *et al*. (2000).

Gastrointestinal nematodes eggs recovery

A mixture of gastrointestinal nematodes (95% *Haemonchus contortus*, 2% *Trichostrogylus colubriformis* and 3% *Oesophagostomum columbianum*) was recovered as described by Hubert and Kerboeuf (1992). Faeces (~1000 g) were obtained from two sheep, maintained as experimental donors, by hanging a faecal collection bag on the sheep overnight. The fresh faeces were suspended in water and cleared of organic debris by filtration through 250, 150, 100 and 60 µm sieves. Eggs were collected on a 32 µm sieve and the organic debris was further cleared by centrifugation (3500 rpm for 5 min) in saline solution. The concentration in the suspension was estimated by counting the number of eggs in 20 µL aliquots, where the eggs suspension was adjusted to 400 eggs/mL.

Treatments

Nine treatments were evaluated, being: *P. dulce* (PD-125, PD-250 and PD-500 µg/mL), *L. acapulcensis* (LA-125, LA-250 and LA-500 µg/mL), phosphate buffer solution (PBS, was used as negative control and solvent of extracts), albendazole and levamisole both at 1% (ABZ and LV, were used as positive control) and dimethyl sulphoxide (DMSO, was used as negative control and solvent of ABZ).

Eggs hatch assay

The *in vitro* egg hatch assay was based on the method of Marie-Magdeleine *et al.* (2010). One mL of egg suspension was distributed in each well of 48 well plates containing ~400 eggs/well, and mixed with the same volume of plant extracts (*i.e.*, 125, 250, 500 µg/mL). Albendazole at a 1% concentration was used as the positive control. The plates were incubated at 25°C for 48 h, when one drop of Lugol's iodine solution was added to stop egg hatching. All the eggs and first stage larvae (L_1), in each plate were counted with three replicates for each concentration and control.

Larval development assay

The procedure used was described by Hubert and Kerboeuf (1992) with modification of Assis *et al*. (2003). One thousand larvae (L_1 and L_2) were distributed into the wells of the 24 weel plates. Larvae were recovered by the procedures described for eggs hatch assay and feed for 48 h in a nutritive solution of fecal juice and Amphotericin B to avoid fungal development. At this time, the same volume of plants extracts (*i.e.*, 125, 250, 500 µg/mL) were added and albendazole was used as the positive control as previously. There were three replicates for each treatment. Third- stage larvae (L_3) were obtained 7 d later. At this time, the parasites were counted under an inverted microscope by separation of the larvae into third stage larvae (L_3), and larvae of other development stages (L_1 and L_2).

Larval migration assay

The procedure used was that of Marie-Magdeleine *et al* (2010). One thousand live L_3 were added to centrifuge tubes (total of 30 tubes) containing negative control (PBS), a positive control (Levamisole at 1% concentration) and each extract to be tested (*i.e.*, 125, 250, 500 µg/mL). Use of PBS aimed to avoid interference with any non-specific effect due to pH change. All incubations were for 3 h at 25°C. Thereafter, the L_3 from each tube was washed with PBS and centrifuged (5000 rpm for 5 min) three times. Larvae were then transferred to sieves (inserts equipped with a 20 µm mesh positioned a conical tube). After 3 h at room temperature, the numbers of L_3 larvae which migrated through the mesh were counted in an optical microscope at 40 x in a 10% aliquot.

Statistical analyses

Data were transformed into Arcoseno √x and analysed with a completely randomised design using SAS (2006), where mean comparisons used Tukey's test at a confidence level of 95% (Steel and Torrie, 1980).

Results and discussion

Chemical composition and condensed tannins

The leaves of *Lysiloma acapulsencis* had higher concentrations of OM, NDF, ADF, FCT1, PCT and TCT than leaves of *P. dulce* which had a higher content of CP and FCT2 (Table 1).

Eggs hatch assay

Both aqueous extracts of LAE and PDE, in addition to the negative and positive controls (*i.e.*, PBS, ABZ, and DMSO), affected gastrointestinal nematodes egg hatching. Phosphate buffer solution and DMSO had the highest ($P<0.05$) egg hatching (96.7 and 68.1%, respectively) followed by PDE and LAE ($P<0.05$), with ABZ having the lowest ($P<0.05$) hatching percentage, *i.e.* 30.5% (Figure 1). Within PDE extract doses, there was increased hatching as the dose of extract increased (62.7, 59.6, and 56.3% for PD-125, PD-250, and PD-500, respectively) while, in LAE, the highest hatching (47.4%) was with the highest extract dose (*i.e.*, 500 µg/mL) compared to the intermediate dose of 250 µg/mL which had the lowest value (32.6%; Figure 1).

Larval development and migration assay

Both the LAE and PDE extracts at different doses, and ABZ, resulted in almost no larval development ($P<0.05$) compared to 100 and 86.4% larval development for DMSO and PBS, respectively (Figure 2). The highest ($P<0.05$) larval migration was with PBS (79.3%) compared to other treatments which had values ($P<0.05$) of 2.9% for LV-500 and 16.1% with PD-500. The lowest ($P<0.05$) larval migration was with LV at all doses (4.6, 6.7, and 2.9% for LV-125, LV-250, and LV-500, respectively; Figure 3).

Eggs hatch and larval development assay

This study examined the *in vitro* bioactive properties of aqueous extracts from *L. acapulcensis* and *P. dulce* on common gastrointestinal nematodes of sheep. Results showed that LAE has an ovicidal effect with doses of 250 and 500 µg/mL, which may be due to the condensed tannins in *L. acapulcensis* (116.3 g/kg DM; Table 1). Ademola and Eloff (2011), in their study with *Vernonia amygadalina* acetone extracts, inhibited egg hatching and larval

Table 1. Chemical composition and concentration of condensed tannins (g/kg DM) in leaves of *L. acapulcensis* and *P. dulce*.

Species	OM	CP	NDF	ADF	FCT1	PCT	FCT2	TCT
L. acapulcensis	945.9	177.0	607.3	500.8	116.3	67.8	3.7	187.8
P. dulce	909.6	261.5	495.8	365.7	36.6	21.8	4.1	62.8

OM, organic matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; FCT1, free condensed tannins; PCT, protein-bound condensed tannins; FCT2, fibre-bound condensed tannins; TCT, total condensed tannins.

development of *H. contortus* larvae at a concentration of 957 µg/mL. It is well known that CT of tanniniferous plants varies in their molecular weight and composition (Mueller-Harvey, 2006; Gea *et al*., 2011). Thus possible anthelmintic effects of tanniniferous plants may depend on their CT contents as well as on other CT parameters such as the monomeric composition and degree of polymerisation (*i.e*., average size of tannin molecules). Molan *et al*. (2003) and Brunet and Hoste (2006) suggested that prodelphidin-rich tannins and their monomers have a stronger anthelmintic activity against sheep nematodes than do procyanidin-rich tannins and their monomers.

In our study, LAE showed anthelmintic properties with reduced egg hatching (32.6%), although the dose of 250 µg/mL was higher than that of PDE at 59.6%. Alonso-Díaz *et al.* (2008) showed that extracts of some tropical tanniniferous plants (*i.e*., *Acacia pennulata*, *Lysiloma latisiliqum*, *Leucanena leucocephala*), had *in vitro* anthelmintic effects against gastrointestinal nematodes using egg hatching and larval development assays. The hatching of nematode eggs is initiated by environmental stimuli which cause release of enzymes, such as proteases, lipases and chitinases, by the larvae which function to degrade the egg membrane (Mansfield *et al*., 1992). The tanniniferous compounds which are in extracts of *L. acapusencis* may act to inhibit activity of these enzymes. Both LAE and PDE are more potent inhibitors of larval development than of egg hatching. Indeed results were similar to those of Molan *et al*. (2002), who found that extracts of *Lotus pendunculatus*, *Lotus corniculatus*, *Hedychium coronarium* and *Onobrychis viciifolia*, were more potent in inhibiting larval development (91%) than of egg hatching (34%). Unfortunately the design of this study did not include confirmation of the role of tannins in the anthelmintic effect observed using polivynil-pirrodilone or polyethylene glycol as tannin blocking agents. However, it is possible to speculate that the more specific and strong actions of the tannin-rich extracts on larval development is related to the presence of proline and hydroxiproline rich proteins in the nematode larval sheath and cuticle, and the high affinity of tannins to those proteins (Page, 2001).

Larval migration assay

The LAE (250 and 500 µg/mL) was more consistent on larval migration. Purified condensed tannins from several plant species were used *in vitro* against *T. colubriformis* and *T. circumcincta* (Molan *et al*., 2000). The viability, motility and migration ability of the L_3 larvae of these nematodes were severely affected by the presence of CT in their environment. Lorimer *et al*. (1996) found that CT extracts reduced migration of L_3 larvae of *T. colubriformis*. Other *in vitro* studies have shown that both purified condensed tannins and terpenoids from several legumes reduced the mobility, and consequent migration ability, of ovine nematode larvae (Molan *et al*., 2000, 2003). In our study *L. acapulsencis*, a legumes with a high CT content, was probably responsible for its anthelmintic properties.

Tannins are biochemical structures with a nature consistent with its constitutive monomer

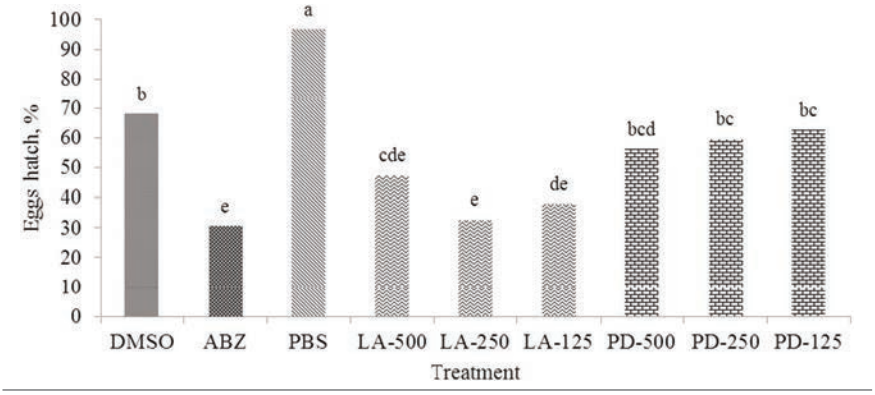

Figure 1. Effects of aqueous extracts of *L. acapulcensis* and *P. dulce* on egg hatching of gastrointestinal nematodes of sheep. Averages with different letters differ at P<0.05.

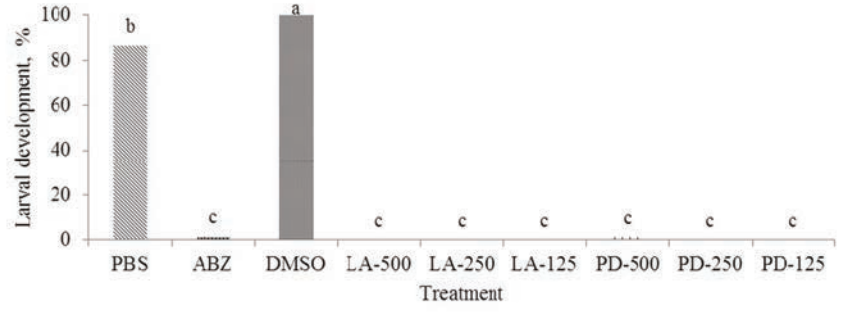

Figure 2. Effects of aqueous extracts of *L. acapulcensis* and *P. dulce* on larval development of gastrointestinal nematodes of sheep. Averages with different letters differ at P<0.05.

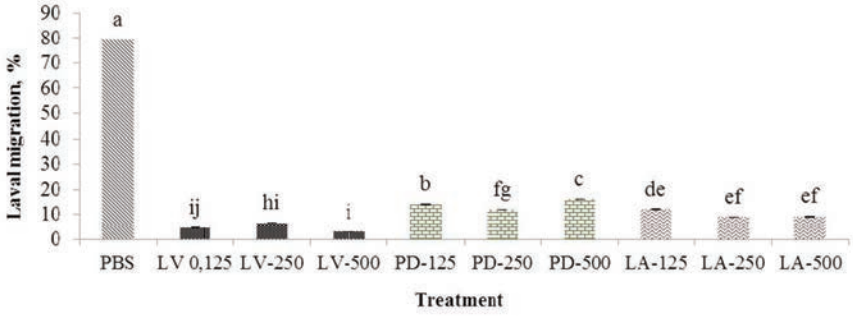

Figure 3. Effects of aqueous extracts of *L. acapulcensis* and *P. dulce* on larval migration of gastrointestinal nematodes of sheep. Averages with different letters differ at P<0.05.

(flavan-3-ol). Condensed tannins can be distinguished into four classes: i) prodelphinidins, ii) procyanidins, iii) prorobetinidins, and iv) profisetinidins. In legume forages, both prodelphinidins and procyanidins are commonly present. However their ratios strongly differ among plant species and/or varieties (Mueller Harvey *et al*., 2006). By experimentally measuring effects of the different flavan-3-ols (monomers) which constitutute either prodelphinidins or procyanidins, some dta suggests that prodelphinidins have more potent anthelmintic activity than do procyanidins (Molan *et al*., 2003; Brunet *et al*., 2006, 2008). This hypothesis is also supported by the reality that legume fodders with a high content of prodelphinidin and/or procyanidins (*e.g.* sainfoin, sericea lespedeza, *Lotus pedunculatus*) have shown more consistent anthelmintic activity than species with a low prodelphinidin and/or procyanidins ratio (*Lotus corniculatus*; Molan *et al*., 2003).

In vitro and *in vivo* assays indicate that tannins disturb the two early steps of nematode establishment, being larval exsheathment (Brunet and Hoste, 2006; Brunet *et al*., 2007) and penetration of the exsheated larvae within the digestive mucosae (Brunet *et al*., 2008). These functional modifications have been associated with major changes in larval ultrastructure. Similarly, observations of transmission and scanning electron microscopy on adult *H. contortus*, after *in vitro* and/or *in vivo* contact with tree rich plants. have shown modifications to the cuticle, the digestive tract and the female reproductive tract, which may explain their negative consequences on adult worm populations, particularly those affecting egg excretion (Brunet *et al*., 2011).

Ovicidal and larvicidal activity against gastrointestinal strongyles in our study showed evidence that extracts of the two trees have anthelmintic activity. This is of vital importance in southern Mexico State, and other areas of the world, where the production systems of sheep and goats during the dry period depend mainly on feeding animals trees and shrubs, among which the two species which we studied dominate. That is why use of extracts of both species tested may represent an alternative to control of gastrointestinal nematodes in sheep, reducing indiscriminate use of synthetic wormers. However *in vivo* studies are required to support the promising *in vitro* assay data of our study.

Conclusions

Aqueous extracts of *Lysiloma acapulcensis* and *Phitecellobium dulce* species could be beneficial as phytogenic anthelmintics in sheep, and could be used to control gastrointestinal nematodes in sheep under subtropical conditions.

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