In vitro activity of *Phitecellobium 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.

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; Mejia-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 (Garcia, 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 990.01) according to 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 (1983), with the modifications of Hedqvist et al. (2000).
Gastrointestinal nematodes eggs recovery

A mixture of gastrointestinal nematodes (95% *Haemonchus contortus*, 2% *Trichostrongylus 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-Magdaleine 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 (*L1*), 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 (*L1* and *L2*) were distributed into the wells of the 24 well 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 (*L3*) were obtained 7 d later. At this time, the parasites were counted under an inverted microscope by separation of the larvae into the parasites were counted under an inverted microscope by separation of the larvae into development stages (*L1* and *L2*).

Larval migration assay

The procedure was used that of Marie-Magdaleine et al. (2010). One thousand live *L3* 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 *L3* 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 *L3* 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 acapulcensis* 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). The leaves of *P. dulce* had a higher content of OM, NDF, ADF, FCT1, PCT and TCT than leaves of *P. dulce* which had a higher content of CP and FCT2 (Table 1).

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 | 485.8 | 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 latisiliquum*, *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. acapulcensis* 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 L3 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 L3 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. acapulcensis*, 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...
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 subropical conditions.

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