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Short communication

Comparing the sensitivity of two *in vitro* assays to evaluate the anthelmintic activity of tropical tannin rich plant extracts against *Haemonchus contortus*

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ABSTRACT

The present trial aimed at comparing the sensitivity of two *in vitro* methods, i.e. the larval migration inhibition assay (LMIA) and the larval exsheathment inhibition assay (LEIA), to evaluate the anthelmintic (AH) properties of tannin-rich plant extracts against *Haemonchus contortus* infective larvae. The two assays were applied on the same batch of *H. contortus* infective larvae exposed to water/acetonic extracts obtained from four tropical plants with different tannin contents: *Acacia gaumeri*, *Brosimum alicastrum*, *Havardia albicans* and *Leucaena leucocephala*. Increasing concentrations (0, 75, 150, 300, 600, 1200 μg/ml PBS) of lyophilized extracts were used in both *in vitro* assays. A general lineal model test was used to determine the dose-effect in the LMIA or the difference in the percentage of exsheathed larvae between the respective control and treated groups. The LMIA showed a dose-dependent AH effect for *H. albicans* ($P < 0.001$) and *A. gaumeri* ($P < 0.05$), but not for *L. leucocephala* and *B. alicastrum*. In contrast, the exsheathment process was significantly affected by all doses of *H. albicans* and *A. gaumeri* extracts and a significant dose-dependent effect was found for *B. alicastrum* and *L. leucocephala*. Calculation of lethal dose (LD) was possible with LEIA using *B. alicastrum* and *L. leucocephala* but not with *H. albicans* and *A. gaumeri* as the lowest tested concentration was achieving more than 50% inhibition. Calculation of LD with the LMIA results was not feasible. These results suggest that tannin-rich plant extracts are more potent inhibitors of the exsheathment of *H. contortus* L_3 larvae than their motility. This information underlines the difference of sensitivity between methodological procedures to evaluate the AH properties of plant extracts on the same nematode stage.

1. Introduction

Some tannin-rich (TR) plants can have direct anthelmintic (AH) effects against the main nematode species of sheep and goats (Athanasiadou et al., 2001; Paolini et al., 2004). The AH effect has been related to the tannins and their ability to form complexes with parasite proteins. Tannins seem to affect the biological processes of nematodes depending on where and how the tannins bind with various nematode structures (e.g. cuticle, digestive or reproductive tract) (Hoste et al., 2006). Previous *in vitro* studies reported discrepancies between the conclusions drawn from results obtained with the larval migration inhibition assay (LMIA) and the larval exsheathment inhibition assay (LEIA), using the same TR source (Alonso-Díaz et al., 2008a,b). In particular, *Piscidia piscipula* extracts (a plant with low quantity of tannins and

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low biological activity) at the concentration of 1200 μg/ml PBS did not show any AH effect based on LMIA results but it significantly inhibited the exsheathment process of both *Haemonchus contortus* and *Trichostrongylus colubriformis* (Alonso-Díaz et al., 2008a,b). It was hypothesized that tropical TR plant extracts were more potent inhibitors of the exsheathment process than the motility of the infective larvae. Thus, the LMIA could be less sensitive than LEIA to investigate the *in vitro* AH effect of TR plant extracts against infective larvae. However, because only a single high dose level (1200 μg/ml) was used in the LEIA, it was difficult to verify this assumption based on those results. To assess differences in sensitivity between assays, the same tannin source and a dose–response design for both tests must be used. Some authors have pointed out the necessity to compare the methodology used for the *in vitro* evaluation of plant AH effects (Ketzis et al., 2006; Athanasiadou et al., 2007). The present study aimed at comparing the sensitivity of two *in vitro* methods (LMIA and LEIA) for the evaluation of the AH activity of TR plant extracts against *H. contortus* infective larvae.

2. Materials and methods

2.1. Plant materials and extraction procedure

Fresh leaves of *Acacia gaumeri*, *H. albicans*, *Brosimum alicastrum* and *Leucaena leucocephala* were used. *A. gaumeri* was chosen for its high content of tannins. On the other hand, *B. alicastrum* is a plant that has normally negligible levels of condensed tannins (Alonso-Díaz et al., 2008c) and was included as a negative control for the bioassays. The *H. albicans* and *L. leucocephala* plant extracts were included as a positive control because previous studies showed AH effect against *H. contortus* (Alonso-Díaz et al., 2008a; Hernández-Orduño et al., 2008) or *T. colubriformis* (Alonso-Díaz et al., 2008b).

Five hundred grams of fresh leaves of each plant species were chopped to obtain the extracts. The chopped material was extracted with acetone:water (70:30) containing ascorbic acid. Then, the acetone was evaporated and the extract was washed four times with methylene chloride in order to eliminate pigments. Finally, each plant extract was lyophilized.

2.2. Quantification of condensed tannins

The condensed tannin (CT) content of the extracts was quantified using the Butanol–HCl assay (Makkar, 2003) reading with a spectophotometer at 550 nm. The CT contents were expressed as leucocyanidin equivalent.

2.3. Infective larvae

The third stage larvae (L_3) were obtained from a donor goat with a monospecific infection of *H. contortus* susceptible to anthelmintics (INRA strain, France). The larvae were stored at 4 °C for two months before use.

2.4. Larval migration inhibition assay (LMIA)

The mobility of ensheathed *H. contortus* L_3 larvae was performed as described by Wagland et al. (1992), modified by Rabel et al. (1994). Live ensheated L_3 (*c.* 1000) were added to centrifuge tubes containing either the negative control (PBS; pH 7.2) (BioMerieux®) or a commercial anthelmintic control (levamisole at 1% concentration) or each solution to be tested (75, 150, 300, 600 and 1200 μg of extract/ml). All incubations were carried out for 3 h at 20 °C. Thereafter, the L_3 from each tube were washed with PBS and centrifuged (3500 rpm) three times. The larvae were then transferred to sieves (inserts equipped with a 20 μm mesh positioned in a conical tube). After 3 h at room temperature, the number of larvae that migrated through the mesh was counted at a 40× magnification using a 15% aliquot technique. The percentage of migration was calculated as $M/T \times 100$ (where T is the total number of L_3 deposited on the sieve and M the number of L_3 that had successfully migrated through the sieve). Four replicates were run for each plant extract and for the negative and the positive (levamisol) controls.

2.5. Larval exsheathment inhibition assay (LEIA)

Ensheathed *H. contortus* L_3 larvae (*c.* 1000), from the same batch used for the LMIA, were incubated for 3 h with each plant extract at concentrations of 75, 150, 300, 600 and 1200 μg/ml in PBS before being re-suspended. After incubation, the larvae were washed and centrifuged (1000 rpm) three times in PBS (pH 7.2). The larvae were then submitted to an artificial exsheathment process by contact with a solution of sodium hypochloride (2%, w/v) and sodium chloride (16.5%, w/v) diluted in 1 to 300 in PBS (pH 7.2) as described by Bahuaud et al. (2006). The kinetics of larval exsheathment in the different experimental treatments was then monitored by microscopic observation (40×). The percentages of exsheathed larvae were identified at 0, 10, 20, 30, 40, 50 and 60 min intervals. Four replicates were run for each plant extract to examine the changes in proportion of exsheated larvae with time.

2.6. Statistical analyses

A General Lineal Model (GLM) test was used to determine the dose effect of each plant extract in the LMIA and LEIA (SAS, 1991). Calculation of the lethal dose 50 (LD50), 90 (LD90 and LD99) were performed using a probit analysis (LeOra, 2003).

3. Results

3.1. Condensed tannins in plant extracts

The highest levels of CT were found in *H. albicans* followed by *A. gaumeri* and *L. leucocephala* (16.45, 12.2 and 7.75 g/kg respectively). *B. alicastrum* extract had low levels of CT (0.81 g/kg).