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Condensed tannins act against cattle nematodes

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ABSTRACT

The use of natural plant anthelmintics was suggested as a possible alternative control of gastrointestinal nematodes (GIN) in ruminants. Direct anthelmintic effects of tannin-containing plants have already been shown in sheep and goat GIN. These anthelmintic properties are mainly associated with condensed tannins. In the present study, we evaluated possible *in vitro* effects of three tannin-containing plants against bovine GIN. Effects of *Onobrychis viciifolia*, *Lotus pedunculatus* and *Lotus corniculatus* condensed tannin (CT) extracts on *Cooperia oncophora* and *Ostertagia ostertagi* were determined by a larval feeding inhibition assay (LFIA) and a larval exsheathment assay (LEA). In the LFIA, all three plant extracts significantly inhibited larval feeding behaviour of both *C. oncophora* and *O. ostertagi* first stage larvae in a dose-dependent manner. The *L. pedunculatus* extract, based on EC_{50} (effective concentration for 50% inhibition), was the most effective against both nematodes, followed by *O. viciifolia* and *L. corniculatus*. The effect of CT extracts upon larval feeding behaviour correlates with CT content and procyanidin/prodelphidin ratio. Larval exsheathment of *C. oncophora* and *O. ostertagi* L3 larvae (third stage larvae) was also affected by CT extracts from all three plants. In both *in vitro* assays, extracts with added polyvinylpolypyrrolidone, an inhibitor of tannins, generated almost the same values as the negative control; this confirms the role of CT in the anthelmintic effect of these plant extracts. Our results, therefore, indicated that tannin-containing plants could act against cattle nematodes.

1. Introduction

Plants or crops with natural anti-parasitic properties may play a role in future grazing systems for livestock (Waller and Thamsborg, 2004). Several tannin-containing forages, in particular those with condensed tannins (CT), have shown anthelmintic activity against gastrointestinal nematodes (GIN) of sheep and goats (Hoste et al., 2006). Although some effects of CT on GIN may be indirect due to nutritional benefits (Niezen et al., 1995), substantial evidence points to direct anthelmintic effects of CT on nematodes in both species (Athanasiadou et al., 2000, 2001; Paolini et al., 2003). Several *in vitro* studies have demonstrated the influence of extracted CT on small ruminant nematodes, e.g. reduced egg hatching and larval development in *Trichostrongylus colubriformis* (Molan et al., 2002), inhibited migration of infective larvae (L3) of *Haemonchus contortus* and *T. colubriformis* (Molan et al., 2000; Barrau et al., 2005) and a delay or complete inhibition of the

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exsheathment of L3 of *H. contortus* and *T. colubriformis* (Brunet et al., 2007; Alonso-Diaz et al., 2008). However, studies on the effects of CT or tanniniferous plants against bovine GIN are lacking.

Most of the *in vitro* assays for testing plant products have been developed to assess the efficacy of synthetic anthelmintic compounds, e.g. detection of anthelmintic resistance in nematodes (Athanasiadou and Kyriazakis, 2004). The main advantages of using *in vitro* assays are their ease of performance, high reproducibility, rapid results and low costs. They may also target different stages of the nematode life cycle (Hoste et al., 2008). In addition, purified compounds isolated from plants can be tested without interference from other plant components or nutrients. It is, however, important, that *in vitro* results are always evaluated *in vivo* before making general conclusions about anthelmintic properties (Athanasiadou and Kyriazakis, 2004). The larval feeding inhibition assay (LFIA) was originally developed for detecting anthelmintic resistance in sheep (Alvarez-Sanchez et al., 2005) and was recently used for the screening of plants with putative anthelmintic effects in sheep nematodes (Bartley et al., 2009). The principle of the assay is based on the reduction of food ingestion by first stage larvae (L1), where fluorescein-labeled *Escherichia coli* serve as feed in the presence of plant extracts. Fed larvae are characterized by the presence of fluorescent *E. coli* in their gut. Thus, LFIA measures whether plant compounds can affect the function of pharyngeal muscles of nematode larvae.

The exsheathment of L3 larvae occurs in parts of the gastrointestinal tract immediately anterior to the habitat of the adult nematodes and it features the transition from free-living to parasitic stage (Hertzberg et al., 2002). Since exsheathment of L3 larvae plays an important role in the life cycle of trichostrongylid nematodes, interference with the exsheathment process by plant compounds can contribute to anti-parasitic properties of plants. To test for effects of plant compounds on larval exsheathment, an artificial exsheathment assay (LEA) has been developed (Bahuaud et al., 2006). It has shown that *in vitro* exsheathment of *T. colubriformis* and *H. contortus* is delayed/inhibited by tannin-containing extracts of sainfoin (*Onobrychis viciifolia*) and this has been confirmed *in vivo* in rumen-cannulated sheep (Brunet et al., 2007).

Sainfoin, greater trefoil (*Lotus pedunculatus*) and birdsfoot trefoil (*Lotus corniculatus*) are tannin-containing legume forages, which are widely grown in Europe, Asia and Australia. Low concentrations of *L. corniculatus* CT in diet cause increased amino acid uptake in the small intestine and have positive effects on ruminant digestion (Waghorn et al., 1987). In addition, tannin-containing plants reduce the incidence of pasture bloat in cattle (Majak et al., 1995; McMahon et al., 2000). All together, anti-parasitic properties in small ruminants and nutritional benefits suggest that tannin-containing plants could be useful also for cattle. Thus, the objectives of the present study were to assess the anti-parasitic effects of three tannin-containing plants against bovine GIN, namely *Ostertagia ostertagi* and *Cooperia oncophora* using two different larval assays, LFIA and LEA. Finally, the role of CT was verified by using polyvinylpolypyrrolidone (PVPP) (Alonso-Diaz et al., 2008). Bis-alkyl substituted amide nitrogen promotes strong binding to tannins (Hagerman, 1992). A large number of such amide nitrogen groups occur in PVPP and PVP and generate a high specificity of PVPP and PVP for tannins. As a result, highly specific interactions cause preferential binding to the tannin-binding agents rather than proteins (Hagerman and Butler, 1981), which are found on larval surfaces and membranes (Brunet, 2008).

2. Materials and methods

2.1. Preparation of plant extracts

Plants were grown at the National Institute of Agricultural Botany (Cambridge, UK). Freeze-dried plant material of *O. viciifolia* (variety Cotswold Common), *L. pedunculatus* (variety Maku) and *L. corniculatus* (variety Goldie) were harvested at ~70% flowering and used for preparation of extracts according to Barrau et al. (2005) with modifications. Samples (10 g) consisting of freeze-dried leaves and stems (<1 mm) were extracted for 60 min with 100 ml of acetone/water (7:3, v/v) containing ascorbic acid (1 g/l) to avoid oxidation. The suspension was filtered through two layers of cheesecloth and centrifuged at 3000 × *g* for 2 min to remove plant material. Acetone was evaporated using a rotary evaporator at 35 °C. Following evaporation, the extract was washed 4 times in a separating funnel with 70 ml of dichloromethane to remove chlorophyll and lipids. Residual dichloromethane was removed by evaporation in a rotary evaporator at 35 °C. These CT-containing plant extracts were freeze-dried and stored at −20 °C until use.

2.2. Tannin analysis

Plants were extracted as above. Freeze-dried extract (4 mg) was weighed into a glass tube containing a magnetic stirrer; methanol (1.5 ml) was added, followed by acidified methanol (3.3 ml concentrated HCl in 100 ml methanol; 0.5 ml) and benzyl mercaptan (50 μl). The reaction mixture was stirred at 40 °C for 1 h. The tube was cooled in ice water, water (2.5 ml) was added and then dihydroquercetin as the internal standard (0.047 mg/ml; 0.5 ml). Samples were analysed by HPLC as described Gea et al. (2011). Total condensed tannin (CT) content of all three extracts was determined by HPLC, which also provided information on mean degree of polymerization (mDP), procyanidin/prodelphidin (PC/PD) and *cis*/*trans* ratios.

2.3. Preparation of nematode larvae

Four calves (6 month old) infected with either *O. ostertagi* or *C. oncophora* (anthelmintic susceptible strains) were used as source of nematode eggs for the studies. L1 for LFIA were produced as follows. Eggs were recovered from fresh, rectal faeces by differential sieving (212 and 90 μm) and collected on a 20 μm sieve followed by 2 times flotation in sugar–saline solution. Washed eggs were transferred into Petri dishes and incubated in tap water at 26 °C for 16–22 h. Hatched L1 larvae were then baermannized through a 20 μm nylon mesh and concentrated to 1000 larvae/100 μl. L3 were produced by incubating fresh faeces