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In vitro effect of heather extracts on *Trichostrongylus colubriformis* eggs, larvae and adults

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ABSTRACT

This study was carried out to evaluate the *in vitro* effects of different heather species on *Trichostrongylus colubriformis* eggs, larvae and adult worms, and obtain scientific evidence to attribute these effects to the action of their phenolic compounds and/or tannins. Total phenolic extracts of three heather species (*Calluna vulgaris*, *Erica cinerea*, and *Erica umbellata*) and an equal mixture of these three extracts were tested *in vitro* in the three development stages of *T. colubriformis* using an egg hatching assay (EHA), larval exsheathment inhibition assay (LEIA), and adult motility inhibition assay (AMIA). The egg hatching rate was measured after incubation with heather extracts for 48 h at 25 °C. Infective third-stage larvae (L3) were incubated for 3 h at 25 °C with heather extracts. The evolution of artificial exsheathment over time was measured with repeated observations at 20-min intervals for 60 min. Adult worms were obtained from one donor goat and incubated with the extracts at 37 °C for 5 days in 48-multiwell plates. Worm motility was measured at 0, 19, 24, 43, 48, 67, 72, 96 and 115 h after the beginning of the experiment. The extracts were tested at concentrations of 75, 150, 300, 600 and 1200 μg/ml. All extracts significantly ($P < 0.001$) inhibited egg hatching and the effect was dose dependent. All extracts inhibited or delayed the exsheathment of *T. colubriformis* L3, and the effect was dose dependent for *C. vulgaris*. Incubation with heather extracts induced a reduction in adult worm motility compared to control, although significant ($P < 0.05$) differences were only found at the highest concentrations. Additional studies showed that purified tannins of the same heather species disturbed *T. colubriformis* larval exsheathment. All these results confirm the anthelmintic properties of heather against *T. colubriformis*, and suggest that not only tannins but also some other phenolic compounds might be involved.

1. Introduction

Gastrointestinal nematode infections are considered an important disease challenge in ruminants at pasture worldwide (Stear et al., 2007). Concerns regarding drug residue in livestock products and the development of resistant nematode populations to the most frequently used

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anthelmintic drugs have stimulated the search for alternative control strategies (Waller, 2006). The use of bioactive plants, mainly those including tannins, has received special attention (Hoste et al., 2006). The anthelmintic effect of these plants is suspected to be related to the ability of tannins to form complexes with parasite proteins. Tannins seem to affect different biological processes of nematodes depending on where and how the tannins bind to various nematode structures (Brunet et al., 2011).

Numerous *in vitro* studies have been carried out to screen the anthelmintic effect of a range of plants or forages, especially in different *Haemonchus contortus* development stages (Manolaraki et al., 2010; Katiki et al., 2011; Kamaraj and Rahuman, 2011; Oliveira et al., 2009, 2011; Cala et al., 2012; Hussain et al., 2011). *In vitro* studies with other nematode species, such as *Trichostrongylus colubriformis* are less numerous (Molan et al., 2000b, 2002, 2003, 2004; Paolini et al., 2004; Brunet et al., 2007). *In vitro* bioassays have the advantage of providing a rapid, simple and low-cost means to test plants or other products for their anthelmintic potential. However, variations in results have been found according to the parasitic stage, particularly depending on whether adult worms, infective larvae, or eggs are submitted to a tannin-rich environment (Paolini et al., 2003).

Although the anthelmintic activity of heather supplementation in goats infected with *Teladorsagia circumcincta* and *T. colubriformis* reported in previous *in vivo* experiments (performed outdoor and indoor) was attributed to its tannin content (Moreno-Gonzalo et al., 2012, 2013a), the effect of heather phenolic extracts was recently showed on eggs, larvae and adult worms of *T. circumcincta* (Moreno-Gonzalo et al., 2013b). Regard to *T. colubriformis*, only Bahuaud et al. (2006) tested the anthelmintic effect of heather on larval exsheathment after incubation with *Erica erigena* extracts, but they found no significant delay in the process of exsheathment. Therefore, the objective of this study was to evaluate the anthelmintic activity of three heather species (*Calluna vulgaris*, *Erica cinerea*, and *Erica umbellata*), which are usually eaten by goats in northern Spain, on the eggs, larvae and adult worms of *T. colubriformis* using different *in vitro* assays, with the final goal of confirming that the anthelmintic effects are due to their phenolic content. Additionally, purified tannin extracts of the same heather species were also tested in *T. colubriformis* third-stage larvae (exsheathment of infective larvae is a key process in the success of ruminant infection). To our knowledge this is the first time that the anthelmintic activity of the extracts of a plant was tested in the three development stages of *T. colubriformis*.

2. Materials and methods

2.1. Experimental design

The effects of four heather (*C. vulgaris*, *E. cinerea*, *E. umbellata* and a equal mixture of all three) total phenolic extracts on the three main stages of the *T. colubriformis* cycle, *i.e.*, eggs, third-stage larvae (L3) and adult worms, were measured using three *in vitro* assays: the egg hatching assay (EHA), larval exsheathment inhibition assay (LEIA), and adult motility inhibition assay (AMIA), respectively. Moreover, LEIA was performed using purified tannin extracts to confirm the role of tannins in the anthelmintic effect of heather.

2.2. Heather extracts

Non-purified extracts of total phenolics were obtained according to the procedures described by Makkar et al. (1993). First, pigments and fats were removed from the dried plant material using diethyl ether containing 1% acetic acid. Total phenols were then extracted with 70% aqueous acetone. This extract contains mainly tannins but also other phenolic compounds.

After freeze-drying these crude extracts, tannin purifications was performed using a slurry of Sephadex LH-20 in 80% ethanol, with sequential washes with 95% ethanol and 50% aqueous acetone, to selectively separate tannins from other compounds, plus extraction with ethyl acetate (Asquith and Butler, 1985; Hagerman, 1991). The resulting extract contained only purified tannins.

2.3. Parasites

Eggs, L3 larvae and adults of *T. colubriformis* for EHA, LEIA and AMIA were obtained from a donor goat experimentally infected *per os* with a pure strain of 6000 *T. colubriformis* L3. For LEIA, batches of 2- to 3-month old L3 larvae were used. For AMIA, four weeks after infection, the goat was euthanized, and immediately after death, the small intestine was collected, opened, briefly washed and placed in a Baermann apparatus with saline at 37 °C. After 2 h, worms that had migrated into the saline were collected. The *T. colubriformis* strain was susceptible to the main anthelmintic drugs.

2.4. Egg hatching assay

This method was based on a modification of the EHA performed to measure anthelmintic efficacy (WAAVP recommendations, Coles et al., 1992). *T. colubriformis* eggs were extracted from feces by repeated centrifugation and washing and distributed in 24-multiwell plates at a density of 100 eggs per well in phosphate buffered saline (PBS; 0.1 M phosphate, 0.05 M NaCl, pH 7.2). Increasing concentrations of plant extracts (75, 150, 300, 600, and 1200 μg dry matter/ml) were obtained from lyophilized extracts dissolved in PBS and added to each well. Each concentration was tested on four replicates. Eggs were incubated for 48 h at 24 °C. Subsequently, the number of larvae per well was counted, and the percentage of eggs hatched was determined as the ratio between the number of larvae and the number of eggs deposited per well. A mean percentage of hatching was calculated for each concentration of the different plant extracts.

2.5. Larval artificial exsheathment assay

First, batches of 1500 ensheathed L3 were incubated for 3 h with each of the four heather extracts at concentrations of 75, 150, 300, 600 and 1200 μg/ml for the raw