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In vitro effect of heather (Ericaceae) extracts on different development stages of *Teladorsagia circumcincta* and *Haemonchus contortus*

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ABSTRACT

The aim of the present study was to evaluate the *in vitro* effects of heather (Ericaceae) phenolic extracts on the abomasal nematodes *Teladorsagia circumcincta* and *Haemonchus contortus*. Extracts of three heather species (*Calluna vulgaris*, *Erica cinerea*, *Erica umbellata* and a balanced mixture of all three) were tested *in vitro* on different development stages of *T. circumcincta* (eggs, infective larvae and adult worms) and *H. contortus* (eggs and infective larvae) using an egg hatching assay (EHA), a larval exsheathment inhibition assay (LEIA) and an adult motility inhibition assay (AMIA). The egg hatching rate was measured after incubation with heather extracts for 48 h at 25 °C. Ensheathed infective larvae were incubated for 3 h at 20 °C with heather extracts. Artificial exsheathment was induced *in vitro* by adding hypochloride solution to the larval suspension. The progress of exsheathment over time was measured by repeated observations at 10-min (*T. circumcincta*) and 20-min (*H. contortus*) intervals for 60 min. Adult *T. circumcincta* worms were obtained from two donor goats and incubated with the extracts at 37 °C for 3 days in 48-well multiwell plates. Worm motility was measured at 0, 19, 24, 43, 48, 67 and 72 h. The extracts were tested at concentrations of 75, 150, 300, 600 and 1200 μg/mL. Incubation with *E. cinerea*, *E. umbellata* and mixed heather extracts had a significant ($P < 0.01$) dose-dependent effect on *T. circumcincta* egg hatching. *H. contortus* egg hatching was significantly ($P < 0.01$) inhibited only by the *E. cinerea* extract. All extracts had a significant ($P < 0.01$) dose-dependent effect on the exsheathment of *T. circumcincta* and *H. contortus* infective larvae. The incubation with all heather extracts induced a reduction in adult *T. circumcincta* motility compared to the control, although significant ($P < 0.05$) differences were only found at the highest concentration (1200 μg/mL). The effect of the mixed extract was significant at all concentrations and significant effects were also observed for *C. vulgaris* and *E. umbellata* at 600 μg/mL. These results show anthelmintic properties of heather phenolic extracts against *T. circumcincta* and *H. contort*, thus confirming observations from previous *in vivo* studies.

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1. Introduction

Gastrointestinal nematode parasitism remains a major threat to efficient small ruminant production in pasture-based systems worldwide (Perry and Randolph, 1999). Infections by trichostrongyles are one of the main limitations for efficient sheep and goat production in temperate and tropical areas, especially when the abomasal parasite *Haemonchus contortus* is involved (Hoste et al., 2012). For several decades, the control of this parasitic condition has been achieved mainly through intensive chemoprophylaxis, based on the repeated use of synthetic anthelmintics. However, the development of anthelmintic resistant strains in many nematode species is a worldwide phenomenon, making the control of these parasites increasingly difficult (Kaplan, 2004). Additionally, the increasing consumer demand to reduce the use of chemical substances in the farming industry has prompted active research on alternative or complementary solutions to chemotherapy for the control of gastrointestinal parasitism (Hoste and Torres-Acosta, 2011). The use of nutraceuticals, especially plants rich in tannins, has been suggested as a sustainable alternative for the control of gastrointestinal nematodes (Hoste et al., 2006). The role of other polyphenolic compounds, such as flavonoid glycosides, in anthelmintic activity has also been suggested (Barrau et al., 2005; Ademola et al., 2005).

Browser plants such as shrubs, trees or bushes could provide significant nutritional resources to animals in many small ruminant production systems, especially in goats (Papachristou et al., 2005). The possible anthelmintic activity of these plants (often rich in tannins and other phenolic compounds) included in the vegetation of rangelands is now receiving special attention (Akkari et al., 2008; Landau et al., 2010; Moreno-Gonzalo et al., 2012). The consumption of heather (Ericaceae) by grazing goats naturally infected with gastrointestinal nematodes has shown to be associated with lower fecal egg counts and a greater resilience of animals to infection (Osoro et al., 2007, 2009). This fact was confirmed in controlled trials with goats experimentally infected with *Trichostrongylus colubriformis* (Frutos et al., 2011) and *T. circumcincta* (Moreno-Gonzalo et al., 2013). A significant decrease in the establishment of *T. circumcincta* infective larvae in goats that were fed heather was reported as early as six days post-infection, suggesting that heather could have some effect on *T. circumcincta* larvae exsheathment (Moreno-Gonzalo et al., 2013). Recently, *in vitro* studies showed the anthelmintic effects of heather phenolics on the different development stages of *T. colubriformis* (Moreno-Gonzalo et al., unpublished data). Bahuaud et al. (2006) also found that *Erica erigena* extracts exhibited anthelmintic activity on *H. contortus* but not on *T. colubriformis* larvae.

The objective of the present work was to test the *in vitro* effects of phenolic extracts from the heather species *C. vulgaris*, *E. cinerea*, *Erica umbellata* and a balanced mixture of all three species on different development stages of *T. circumcincta* (eggs, infective larvae and adult worms) and *H. contortus* (eggs and infective larvae) to compare any effects of the heather species, parasite species and/or parasitic stage involved.

2. Materials and methods

2.1. Experimental design

The effects of phenolic extracts from three heather species (*i.e.*, *C. vulgaris*, *E. cinerea*, *E. umbellata*) and a balanced mixture of all three species on the three main stages of the parasite cycle of *T. cirucmcincta* (*i.e.*, egg, infective larvae and adult worms), were measured using an egg hatching assay (EHA), a larval exsheathment inhibition assay (LEIA) and an adult motility inhibition assay (AMIA). For *H. contortus*, only EHA and LEIA were performed.

2.2. Heather extracts

Non-purified extracts of total phenolics were obtained following the procedures described by Makkar et al. (1993). First, pigments and fats were removed from 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.

2.3. Parasites

T. circumcincta eggs, larvae and adults for EHA, LEIA and AMIA were obtained from two donor goats experimentally infected *per os* with 6000 infective larvae (goat strain). Eggs and larvae of *H. contortus* for EHA and LEIA were obtained from one donor goat experimentally infected with 3000 infective larvae (goat strain). For LEIA, batches of 2- to 3-month-old larvae were used in the assays. For AMIA, four weeks after infection, donor goats infected with *T. circumcincta* were euthanised and immediately after death, the abomasa were collected, opened, briefly washed and placed in a Baermann apparatus with saline at 37 °C. After 2 h, worms that had migrated to the saline were collected.

2.4. Egg hatching assay

The method was based on a modification of the egg hatch assay performed to measure anthelmintic resistance (WAAVP recommendations, Coles et al., 1992). Eggs were extracted from fresh feces by repeated centrifugations and washings. Briefly, a water suspension of feces was filtered through a mesh (150 μm pore size) and transferred into 15 mL centrifuge tubes. The suspension was centrifuged (4500 rpm, 5 min, 20 °C), removing the supernatant and adding tap water, three times. After the third centrifugation, the supernatant was removed, replaced with saturated salt solution and centrifuged (4500 rpm, 5 min, 20 °C) two times. The eggs (located on the top of the saline solution, approximately 1 mL) were collected and kept in a plastic tube (50 mL). The tube was filled with Phosphate Buffered Saline (PBS; 0.1 M phosphate, 0.05 M NaCl, pH = 7.2) and the egg solution was centrifuged, the supernatant removed, and the tube filled again with PBS in order to prevent any contact of the eggs with the saline solution.