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Short communication

Anthelmintic effects of forage chicory (*Cichorium intybus*) against free-living and parasitic stages of *Cooperia oncophora*

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ABSTRACT

Chicory shows great promise as an anthelmintic forage for grazing ruminants that can reduce reliance on anti-parasitic drugs. Recently, we reported potent anthelmintic effects of chicory-based diets in infected cattle with significant reductions in worm burdens of the abomasal nematode *Ostertagia ostertagi*, whilst no apparent activity was observed against the small intestinal parasite *Cooperia oncophora*. To explore this discrepancy, we investigated direct anthelmintic effects of forage chicory against *C. oncophora in vitro*. Chicory leaves (cultivar 'Spadona') were extracted with methanol in a Soxhlet apparatus and the resulting extract was purified by solid-phase extraction to concentrate bioactive phytochemicals such as sesquiterpene lactones. *C. oncophora* eggs and adult worms from mono-infected donor calves were exposed to decreasing concentrations of the chicory extract. In an egg hatch assay, the chicory extract induced a marked and dose-dependent inhibition of egg hatching, with 95% inhibition at 2500 µg extract/mL (EC_{50} = 619 [95% CI: 530–722] µg extract/mL). In the adult motility inhibition assays, the chicory extract induced a potent and dose-dependent worm paralysis. At 12 h of incubation, worms exposed to chicory showed a total paralysis at ≥500 µg extract/mL, while after 48 h of incubation a complete inhibition of worm motility was observed at ≥250 µg extract/mL (EC_{50} = 80 [95% CI: 67–95] µg extract/mL). We have demonstrated that forage chicory can induce potent inhibitory effects on the egg hatching and exert direct anthelmintic activity against parasitic stages of *C. oncophora*. These results suggest that the previously reported absence of *in vivo* effects of chicory towards *C. oncophora* in infected animals may be related with host-mediated factors and/or inhibitory digestive conditions, rather than an inherent inactivity of chicory and its bioactive phytochemicals.

1. Introduction

In the search for novel control strategies of livestock nematodes in a context of expanding parasite drug-resistance, a promising and increasingly explored approach is the inclusion of forages with anthelmintic activity in animal diets (Sandoval-Castro et al., 2012; Hoste et al., 2015). One bioactive forage under investigation is chicory (*Cichorium intybus* L., Asteraceae), well known for its content of several biologically active compounds, most notably the sesquiterpene lactones (SL) (Rees and Harborne, 1985; Simonsen et al., 2013). *In vivo*, chicory feeding has been shown to reduce the burdens of abomasal nematodes in sheep (Scales et al., 1995; Tzamaloukas et al., 2005). Recently, we reported a potent anti-parasitic activity of chicory-based diets against the abomasal cattle nematode *Ostertagia ostertagi*, the most pathogenic bovine parasite in temperate regions (Peña-Espinoza et al., 2016). However, in the same study, the small intestinal nematode *Cooperia oncophora* was not affected by dietary chicory. It is unclear whether this lack of effect is due to an inherent inactivity of chicory towards *C. oncophora*, or if other factors such as the host digestive physiology may affect the availability and/or activity of phytochemicals along the gastrointestinal tract, as recently substantiated for condensed tannins in cattle (Desrues et al., 2017). Previously, we reported that forage chicory extracts have direct and dose-dependent activity against *O. ostertagi in vitro* (Peña-Espinoza et al., 2015), but corresponding evidence with *C. oncophora* or other small intestinal nematodes of ruminants is absent. Consequently, the objective of the present study was to investigate

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whether forage chicory can exert direct *in vitro* anthelmintic effects against free-living and parasitic stages of *C. oncophora*.

2. Materials and methods

2.1. Plant material and extraction procedure

Forage chicory extracts were prepared with chicory leaves collected from an organic dairy farm in Årre, Denmark (55°32′50″N, 8°35′49″E) in early December 2012. Chicory leaves were hand-picked from a permanent pasture sown in spring 2010 containing forage chicory (cultivar [cv.] Spadona; < 10% dry matter in the field), ryegrass and white clover. At collection, all chicory plants were at the vegetative stage. Chicory leaves were stored at −20 °C in the dark until extraction. Extracts were prepared following the method described by Foster et al. (2011), with minor modifications. This method enhances the isolation of SL, which are likely the main anthelmintic compounds in chicory (Foster et al., 2011; Peña-Espinoza et al., 2015). Briefly, dried and ground leaf tissue (10 g) was extracted with methanol in a Soxhlet apparatus. The resulting crude extract was evaporated to dryness, dissolved in 48 mL of ethanol/Mili-Q-H_20/methanol (1/1/1; v/v/v) and loaded into 8 × 6 mL solid-phase extraction (SPE) tubes (Empore™ SPE Cartridges, 66873–U Supelco) set in a SPE vacuum manifold. Each SPE tube was further washed with 2 × 6 mL of the ethanol/Mili-Q-H_20/methanol solution and the three resulting eluates were pooled. The obtained extract was concentrated to dryness, resuspended in 48 mL Mili-Q-H_20 and loaded into new 6 × 6 mL SPE tubes. After SPE, all tubes were washed with 2 × 6 mL Mili-Q-H_20 and SL were eluted with 3 × 6 mL methanol. The obtained eluates were pooled and dried under reduced pressure. The resulting purified extract was highly viscous and poorly soluble in Mili-Q-H_20. Therefore, the extract was dissolved in 100% dimethyl sulfoxide (DMSO) at a concentration of 250 mg dry extract/mL (stock solution) and kept at −20 °C until use for *in vitro* assays.

2.2. Parasite material

Parasite material for the *in vitro* assays was collected from two nematode-naïve donor calves (4 months-old) mono-infected with 20,000 third-stage larvae of an anthelmintic-susceptible, laboratory propagated strain of *C. oncophora* (kindly provided by Prof. Dr. Janina Demeler, Freie Universität Berlin, Germany). The experiment was approved by the Animal Experiments Inspectorate of the Danish Ministry of Environment and Food (License j. No. 2013-15-2934-00763). Nematode eggs were detected in faeces from both calves after 14 days post-infection. Unembryonated and clean *C. oncophora* eggs were immediately recovered from fresh faeces following the procedure described by Demeler et al. (2012), and then suspended in Mili-Q-H_20 for use in the egg hatch assay. The two donor calves were euthanised 28 and 29 days post-infection, respectively, to obtain live *C. oncophora* adults for worm motility inhibition assays. Calves were stunned by captive bolt, bled and the digestive tract was immediately removed. The first 5 m of the small intestine were recovered and motile *C. oncophora* adults were isolated from the intestinal content using a modification of the agar-migration method (Christensen et al., 1995) as described in Peña-Espinoza et al. (2015). Isolated nematodes were washed twice in a sterile incubation medium (RPMI 1640 with L-glutamine, Gibco) containing 200 µg/mL streptomycin, 200 U/mL penicillin and 2 µg/mL amphotericin B at 37 °C. After washing, nematodes were kept in Petri dishes with warm incubation medium and used immediately for the *in vitro* assays.

2.3. In vitro assays

First, we investigated the effect of chicory on the hatching of *C. oncophora* eggs by the egg hatch assay (EHA). The assay was conducted according to von Samson-Himmelstjerna et al. (2009), with some modifications. The stock solution of chicory extract was serially diluted in Mili-Q-H_20 and five two-fold decreasing concentrations (in triplicates) were prepared in a 24 well-plate. The final test concentrations were: 2500, 1250, 625, 313 and 156 µg dry chicory extract/mL (final concentration 2% DMSO). Wells containing thiabendazole (Sigma-Aldrich, 1 mg/mL, 2% DMSO) and 2% DMSO diluted in Mili-Q-H_20 were also run in triplicates as positive and negative controls, respectively. Immediately after recovery, ~100 unembryonated *C. oncophora* eggs in Mili-Q-H_20 were added to each well. The 24-well plate was incubated at 25 °C for 48 h, and after incubation 100 µL of Lugol iodine was added to each well. The plate was observed under an inverted microscope and a minimum of 100 eggs and first-stage larvae (L1) were counted per well. Egg hatching percentages were calculated for each well as: % egg hatching = 100 × [(number of L1 per well)/(total number of eggs + L1 per well)].

Next, we investigated the activity of chicory on the motility of *C. oncophora* adults by the adult motility inhibition assay (AMIA). The assays were performed according to Peña-Espinoza et al. (2015), with minor changes. Two independent AMIAs were conducted using decreasing concentrations of the chicory extract with *C. oncophora* adults from each calf (*i.e.* one assay per animal). The chicory extract stock solution was serially diluted in 100% DMSO to obtain six test concentrations. For each assay, 24 well-plates were prepared with the six extract concentrations diluted in the warm sterile incubation media described above. Final tested concentrations in well were: 1000, 500, 250, 125, 60 and 30 µg dry chicory extract/mL media (final concentration 2% DMSO), run in triplicates for each AMIA. Wells containing ivermectin (Sigma-Aldrich, 1 mg/mL, 2% DMSO) and 2% DMSO diluted in the incubation media were run in triplicates as positive and negative controls, respectively. Immediately after recovery, three to four motile *C. oncophora* adults (males and females) were gently added to each well and the plates were incubated at 37 °C. Worm motility was examined at 12, 24 and 48 h after incubation using an inverted microscope and the number of motile and non-motile (no movement detected in 10 s) nematodes per well was recorded. At 24 h of incubation, the incubation media were replaced by fresh media (37 °C) with the same final concentrations run at the start of the assay. At every time point, the number of motile or non-motile worms in the triplicates exposed to the same extract concentration in each AMIA were merged. This resulted in two final replicates per concentration (one replicate per AMIA = per calf) with a larger number of exposed worms to the chicory extract (n = 10–12 worms per replicate and concentration) for analysis of motility inhibition. For each AMIA (*i.e.* replicate) and time point, worm motility percentages per concentration were calculated as: % worm motility = 100 × [(number of motile worms in all wells of the same concentration)/(total number of worms in all wells of the same concentration)].

2.4. Statistical analyses

The tested chicory extract concentrations were log transformed and the hatching/motility percentages were analysed as described by Peña-Espinoza et al. (2015), with bottom values fixed as 0% and top values set as the mean hatching/motility percentages observed in the negative controls. Dose-response curves and the effective extract concentrations needed to inhibit the hatching/motility in 50% of incubated eggs/adults (EC_{50}) were calculated, including the 95% confidence interval (CI) and R squared measure of goodness of fit (R^2) for each dose-response curve. All analyses were performed in GraphPad Prism® version 6.05.

3. Results and discussion

In the EHA, the mean egg hatching rate in negative control wells after 48 h was 90%. In contrast, the chicory extract displayed a marked