Veterinary Parasitology 249 (2018) 63–69

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Procyanidin A2 in the Australian plant *Alectryon oleifolius* has anthelmintic activity against equine cyathostomins *in vitro*

S.E. Payne[a,b,*], G.R. Flematti[c], A. Reeder[d], A.C. Kotze[e], Z. Durmic[a,b], P.E. Vercoe[a,b]

[a] School of Agriculture and Environment, The University of Western Australia, Crawley, Australia
[b] Institute of Agriculture, The University of Western Australia, Crawley, Australia
[c] School of Molecular Sciences, The University of Western Australia, Crawley, Australia
[d] Centre for Microscopy, Characterisation and Analysis, The University of Western Australia, Crawley, Australia
[e] CSIRO Agriculture and Food, Queensland Bioscience Precinct, University of Queensland, Brisbane, Australia

ARTICLE INFO

Keywords:
Cyathostomin
Anthelmintic
Tannins
Horse
HPLC
Procyanidin A2

ABSTRACT

There is a need to investigate new methods of controlling cyathostomins in horses due to increasing anthelmintic resistance amongst these parasites. In a previous study we identified the Australian plant *Alectryon oleifolius* as having anthelmintic activity towards cyathostomins. This study aimed to isolate and identify the bioactive compound(s) responsible for all or part of this anthelmintic activity and quantify its activity *in vitro*. The condensed tannin procyanidin A2 was isolated from the plant through a process of bioassay guided fractionation and identified using 1D and 2D nuclear magnetic resonance spectroscopy and high performance liquid chromatography with mass spectrometry. Procyanidin A2 demonstrated significant anthelmintic activity in larval development assays, completely inhibiting development from egg to third larval stage at concentrations as low as 50 μg/mL and having an IC_{50} value of 12.6 μg/mL. Procyanidin A2 also significantly inhibited larval migration at concentrations of 25 μg/mL. This study indicates that procyanidin A2 is the principal anthelmintic compound in extracts from *A. oleifolius*, and further highlights the potential for the use of this plant as a component of cyathostomin control programs in the future.

1. Introduction

Anthelmintic resistance in cyathostomin parasites of horses is increasing, and hence, investigation into new methods of controlling these parasites is needed. In an earlier study (Payne et al., 2013), several Australian plants were found to have significant anthelmintic activity against cyathostomin parasites *in vitro,* which indicated potential for them to be useful as a component of parasite control programs. *Alectryon oleifolius* (Sapindaceae, syn. *Heterodendum oleifoium,* "Bullock bush"), was one of the most potent plants tested, completely inhibiting larval development when tested as a crude aqueous extract (Payne et al., 2013). This plant is known to be palatable to herbivores, and is used as fodder for livestock when pasture is scarce (Cunningham et al., 2011).

There is limited information about *A. oleifolius* in the literature, especially regarding its chemical composition. In our earlier examination of this species (Payne et al., 2013), we found that the anthelmintic activity of *A. oleifolius* was removed when the plant extract was first exposed to the tannin-binding compound polyvinylpolypyrrolidone (PVPP), suggesting that the anthelmintic activity was likely to be due to tannin compounds in this plant. Tannins are polyphenolic compounds that are common plant secondary compounds, and have been widely reported to have anthelmintic properties, particularly condensed tannins (Hoste et al., 2012). The direct inhibitory effects of tannins on nematodes *in vitro*, most likely caused by interactions with proteins (Brunet and Hoste, 2006; Williams et al., 2014), have been demonstrated with several important species of parasitic nematodes infecting livestock, including the sheep parasites *Haemonchus contortus* and *Trichostrongylus colubriformis* (Molan et al., 2004; Alonso-Díaz et al., 2008a; Alonso-Díaz et al., 2008b). The aim of the current study was to isolate and identify the main anthelmintic compound(s) in *A. oleifolius* through bioassay-guided fractionation, and to quantify its anthelmintic activity using *in vitro* larval development and larval migration inhibition assays with equine cyathostomins.

2. Materials and methods

2.1. Plant material and chemicals

A. oleifolius used in this study was part of a collection of plant

* Corresponding author at: School of Agriculture and Environment, The University of Western Australia, 35 Stirling Hwy, Crawley, WA 6009, Australia.
E-mail address: stephanie.payne@uwa.edu.au (S.E. Payne).

https://doi.org/10.1016/j.vetpar.2017.11.008
Received 25 April 2017; Received in revised form 16 November 2017; Accepted 18 November 2017

samples held at The University of Western Australia which were collected and processed as described by Payne et al. (2013).

The amphotericin B solution 'Fungizone', tylosin solution, and anthelmintic compounds ivermectin, levamisole and thiabendazole were purchased from Sigma-Aldrich (St Louis, MO, USA). The anthelmintic compounds were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO, USA) to create stock solutions of 10 mg/mL for use in assays. A synthetic standard of procyanidin A2 was purchased from Extrasynthese (Genay, France). The compound was dissolved in 10% DMSO to make stock solutions of 5 mg/mL for use in the larval development assays (LDA) and 4 mg/mL for the larval migration inhibition assays (LMIA).

2.2. Cyathostomin parasites

The cyathostomin eggs used for this study were collected from the faeces of naturally-infected horses located in Perth metropolitan area, Western Australia, as described by Payne et al. (2013). No worm species other than cyathostomins were observed in any of the samples. This was determined after larval culture (Zajac et al., 2006) by the use of an identification key (Soulsby, 1965).

2.3. Larval development assay (LDA)

The assay was carried out as described by Kotze et al. (2009) and Payne et al. (2013). All plant extracts and fractions generated through the bioassay guided fractionation process were tested in this assay, the preparations of which are detailed in Section 2.5. Following bioassay guided fractionation, the pure compound procyanidin A2 was tested at 200 µg/mL and five 2-fold serial dilutions (100, 50, 25, 12.5, and 6.25 µg/mL). Parallel assays were also run with anthelmintic compounds as positive controls: ivermectin (2.47×10^{-3} ng/mL – 650 ng/mL), levamisole (0.0016 µg/mL – 10.4 µg/mL) and thiabendazole (3.98×10^{-5} µg/mL – 2.6 µg/mL).

2.4. Larval migration inhibition assay (LMIA)

The LMIA was used to test the anthelmintic activity of procyanidin A2 once it had been identified through the bioassay guided fractionation process. Faecal larval cultures for the cultivation of L3 larvae were established according to the methods described by Zajac et al. (2006). Fresh faeces was broken up and mixed with vermiculite in a beaker to form a moist, crumbly mix. The beaker was covered with foil and kept at room temperature for approximately 14 days. Water was added when needed to keep the mixture moist, and the faeces were stirred daily to prevent the formation of mould on the surface. After day 14, a modified Baermann apparatus was used to remove the larvae from the culture. Briefly, the faecal sample was removed from the beaker and wrapped in double layer cheesecloth. The cheesecloth was suspended in a closed funnel filled with lukewarm water. This was left overnight to let the larvae migrate out of the sample and settle at the bottom of the funnel. The faecal sample was removed and a few millilitres containing the larvae from the bottom of the funnel were removed by pasteur pipette. The larvae were then again left overnight to migrate through the double layers of cheesecloth into clean water.

The larvae were used in the LMIA on the same day if possible, otherwise they were stored at 4 °C for no longer than one week before use. For use in the LMIAs, the larval solution was diluted to contain approximately 20–25 larvae per 10 µL. Amphotericin B solution 'Fungizone' was added at a rate of 100 µL per mL of larval solution (final concentration of 25 µg/mL) to prevent fungal growth (Kotze et al., 2006).

The migration inhibition assay procedure was based on the methods described by Kotze et al. (2006). First, larvae were exposed to the plant compound during an incubation period in 96-well assay plates. A stock solution of 4 mg plant compound per mL of 10% DMSO was prepared, followed by five 2-fold serial dilutions. Each well received 10 µL of plant compound solution, 60 µL phosphate buffered saline solution (PBS) and 30 µL of larval solution, giving a total volume of 100 µL, except for the 800 µg/mL concentration that received 20 µL of the plant compound stock solution, 30 µL larval solution and 50 µL PBS. The dilution of the plant compound solutions into this total well volume gave eight final concentrations: 12.5, 25, 50, 100, 200, 400, and 800 µg/mL. Negative controls included water or 10% DMSO in place of the plant compound solution. Anthelmintic compounds were also tested on a separate plate and used as positive controls. Stock solutions (10 mg/mL in 100% DMSO) of levamisole and ivermectin were serially diluted by 2-fold. One micro liter of solution was then added to each well with 70 µL PBS and 30 µL larval solution to give final concentrations ranging from 6×10^{-6}–25 µg/mL. Each treatment was run in triplicate, and then repeated with a different larval preparation at a later time. Plates were placed in a zip lock bag (to prevent evaporation) and incubated for 48 h at room temperature.

During the incubation period, a 'receiver' plate (96-well assay plate), in which the filter plate was to be suspended, was prepared to contain the same concentration of plant compound as the incubation plates. The volume of the wells was to be 300 µL, so the amount of plant compound and control solutions was three times the volumes that had been used previously for the incubation plates (described above). The volume was then made up to 300 µL by the addition of PBS.

After the 48 h incubation period, the mesh filter plate was placed on top of the receiver plate. Using a multi-tip pipette set at 120 µL, the contents of the incubation plate wells were transferred to their corresponding places in the filter plate. Care was taken to mix the incubation wells and to ensure that all larvae were collected by taking the liquid up and down five times with the pipette before transfer. Therefore, there was 300 µL of plant compound solution 'below' the filter in the receiver plate wells, and 100 µL of plant compound solution and larvae 'above' the mesh in the filter plate wells. The plates were placed in zip lock bags on a black lined tray. They were incubated overnight at room temperature in a well-lit room. The next day, the filter plate was removed and 10 µL of Lugol's iodine solution was added to each receiver plate well. The number of larvae in each receiver well was counted under the compound microscope.

2.5. Bioassay guided fractionation

2.5.1. Solvent extracts

One gram of ground dried plant material was extracted with 20 mL of the following solvent systems: 1/1 (v/v) methanol/chloroform, 4/1 (v/v) methanol/water, and acetone. These were stirred for approximately 1 h at room temperature. Once filtered, the three extractions were concentrated under reduced pressure in a water bath not exceeding 40 °C. Approximately 10% (v/v) of the extract was removed as a bioassay sample, therefore representing an equivalent to the extract derived from 0.1 g of original plant material. Both the extracts and bioassay samples were dried under nitrogen and stored at 4 °C until needed.

For LDA testing, the bioassay samples of each solvent extract were resuspended in 1 mL of a 10% DMSO solution for use in LDAs (10 µL per assay well). Thus, the amount of plant material per assay well was equivalent to the extracted material from 1 mg dried plant. Dilution of this material into a total assay volume of 250 µL of agar, egg suspension, plant extract and nutrient medium, resulted in a concentration equivalent to the extractable material from 4 mg of original plant material per mL. Thus, the extracts were tested at 4 mg/mL, plus three 2-fold dilutions of 2, 1 and 0.5 mg/mL. To determine IC_{50} values further dilutions of 250, 125, 62.5 and 31.3 µg/mL were tested. The extracts were tested in LDAs and the 4/1 (v/v) methanol/water extract was found to be the most active, so it was selected for further analysis.