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Metabolic profiling and in vitro assessment of anthelmintic fractions of *Picria fel-terrae* Lour.

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ABSTRACT

Anthelmintic resistance is widespread in gastrointestinal nematode populations, such that there is a consistent need to search for new anthelmintics. However, the cost of screening for new compounds is high and has a very low success rate. Using the knowledge of traditional healers from Borneo Rainforests (Sarawak, Malaysia), we have previously shown that some traditional medicinal plants are a rich source of potential new anthelmintic drug candidates. In this study, *Picria fel-terrae* Lour. plant extract, which has previously shown promising anthelmintic activities, was fractionated via the use of a solid phase extraction cartridge and each isolated fraction was then tested on free-living nematode *Caenorhabditis elegans* and the parasitic nematode *Haemonchus contortus*. We found that a single fraction was enriched for nematocidal activity, killing ≥90% of *C. elegans* adults and inhibiting the motility of exsheathed L3 of *H. contortus*, while having minimal cytotoxic activity in mammalian cell culture. Metabolic profiling and chemometric analysis of the effective fraction indicated medium chained fatty acids and phenolic acids were highly represented.

1. Introduction

Gastrointestinal nematode infections of ruminants remain a major threat to the economic viability of the livestock industry (Nieuwhof and Bishop, 2005; Sackett et al., 2006). The control of these parasitic diseases depends on the use of commercial anthelmintic drugs (Molento et al., 2011), together with management practices (Kahn and Woodgate, 2012; Miller and Waller, 2004; Van wyk et al., 2006, Waller, 1993; Woodgate and Besier, 2010). However, resistance to anthelmintics is widespread in helminth populations (Fleming et al., 2006); therefore, there is a continuous need for the development of novel anthelmintic drugs. The cost of developing new anthelmintic compounds is high, and very few new compounds have been discovered or synthesized in the last decade (Csermely et al., 2013; Mackenzie and Geary, 2013). This is augmented by the fact that nations that suffer most from helminth infections are countries with limited resources to invest in drug discovery research and, as such, are also financially unattractive to the global pharmaceutical industry (Brooker, 2010).

In the last decade, there has been renewed interest in phytomedicine, and many traditional medicinal plants species are being

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tested for pharmacological activities (Agyare et al., 2009; Aremu et al., 2010; Desrivot et al., 2007; Katiki et al., 2011, Ndjonka et al., 2011, Waterman et al., 2010). Although plant-based remedies have been used for centuries in traditional medicine for the treatment of various diseases, including worm infections, there has been limited scientific evaluations of their actual activities, properties and toxicities (Eguale et al., 2011; Kaewintajuk et al., 2010; Ndjonka et al., 2011; Heckendorn et al., 2007; Molan et al., 2003).

Previously, we have shown that traditional medicinal plants are a rich source of anthelmintic drug candidates (Kumarasingha et al., 2014). Plant extracts were selected based on the knowledge of traditional healers from Borneo Rainforests, Sarawak, Malaysia, and screened for anthelmintic compounds in *Caenorhabditis elegans* (Kumarasingha et al., 2014). The present study reports the fractionation of the most active plant extract, derived from the whole plant of *Picria fel-terrae* Lour., and identification of a single fraction (designated Fraction 5) that is enriched for anthelmintic activity. *P. fel-terrae* Lour. is a popular medicinal plant in Asia, and has been studied for its anti-microbial and anti-inflammatory activities (Ahmed and Halaweish, 2014; Huang et al., 1998, 1999; Zou et al., 2006). To the best of our knowledge, apart from our previous work, testing of the anthelmintic activity of this plant has not been reported in the literature.

2. Materials and methods

2.1. Preparation of plant extracts

Plant extracts were prepared at the Sarawak Biodiversity Centre, Kuching, Malaysia. Whole *P. fel-terrae* Lour. Plants were dried, ground into a powder, extracted into 1:1 v/v dichloromethane:methanol and then concentrated using a rotary evaporator. Before use, the powdered plant extracts were dissolved in absolute ethanol (Merck, Australia) and diluted in M9 buffer (85.6 mM NaCl, 4.2 mM Na_2HPO_4, 2.2 mM KH_2PO_4, 1 mM $MgSO_4$) to achieve the desired concentration (Brenner, 1974). The final ethanol concentration of all dilutions was 1% (v/v).

2.2. Fractionation of P. fel-terrae Lour. whole plant extract

A solid phase extraction (SPE) strata C18-E cartridge (Silica-based sorbent; Phenomenex, USA) was used for sample fractionation. Plant extract (100 mg) was dissolved in 1 ml absolute ethanol and diluted with 19 ml MilliQ water. The column was washed with 20 ml of 100% Acetonitrile (ACN; Sigma, Australia) and then 20 ml of MilliQ water was sent through to condition the column. The dissolved plant extract was loaded on the column and eluted by passing 20 ml of 10%, 25%, 40%, 55%, 70%, 85% and 100% ACN dilution series. The fractions obtained were dried by rotary evaporation. Individual fractions were solubilised in 1 ml of absolute ethanol and 10 µl aliquots were added to 990 µl of M9 to make working solutions.

2.3. C. elegans strains and maintenance

C. elegans "Bristol N2" wild type strain was used for all the experiments. All the strains were grown on Nematode Growth Medium (NGM) in petri dishes containing a lawn of *Escherichia coli* OP50 (Brenner, 1974). Strains were grown at 20 °C and synchronised populations were obtained by a modified alkaline bleaching method (Lenaerts et al., 2008). Briefly, worm culture plates with eggs and egg-laying adults were washed in M9 and then incubated in a freshly prepared bleaching solution (4 ml commercial bleach, 1 ml 1 M NaOH and 9 ml H_2O) for 3.5 min, followed by washing 3 times with M9. Eggs in M9 were kept on a rotaty shaker at 20 °C overnight to hatch. First stage larvae (L1s) were put on to NGM plates the next morning and incubated at 20 °C. Young adults were isolated 48 h later. These worms were washed 4 times in M9 before being used in experiments.

2.4. Efficacy of fractions of P. fel-terrae Lour. Whole-plant extracts on C. elegans adults

The 9 fractions collected following SPE fractionation were tested on *C. elegans* young adults. Assays were performed in 96-well microtitre plates using 150 µl media per well, with 3 replicates per condition (≥10 worms per well) as described previously (Kumarasingha et al., 2014). The plates were covered and kept in a humid chamber on a shaker at 20 °C, and survivors were counted at specific time points for up to 72 h. Worms were considered dead if they were immobile, even after provocation with a platinum wire, and if no pharyngeal pumping was detected. Two conventional anthelmintic drugs, doramectin (Pfizer, Australia) and levamisole (Sigma, Australia) were used as positive controls, and M9 with 1% ethanol was used as a negative control. Each condition was performed in triplicate, and three biological repeats were performed for each experiment.

2.5. Production and storage of H. contortus third-stage larvae (L3s)

Haemonchus contortus (Haecon-5 strain; cf. Schwarz et al., 2013) was maintained in experimental sheep as described previously (Preston et al., 2015) and in accordance with the institutional animal ethics guidelines (permit no. 1111938; The University of Melbourne). To produce L3s, faecal samples were incubated at 27 °C for 7–10 days before larvae were harvested (cf. Schwarz et al., 2013). L3 were stored at 10 °C for up to three months prior to use.

2.6. Exsheathment of L3s

L3s were exsheathed and sterilised by incubation in 0.15% (v/v) sodium hypochlorite (NaClO) at 37 °C for 20 min as described by Preston et al. (2015). Following exsheathment, L3s (designated xL3s) were suspended at a density of 300 xL3 in 50 µl of Luria Bertani medium (LB) supplemented with 100 IU/ml of penicillin, 100 µg/ml of streptomycin and 2.5 µg/ml of amphotericin (Fungizone®,Thermo Fisher Scientific, Australia; designated LB*).

2.7. H. contortus exsheathed third stage larvae (xL3) motility assay

To test for activity, the test fractions (0.01 mg/ml) and the solvent control in LB* with 1% ethanol were arrayed in triplicate in 96 well flat-bottomed plates (Corning, 3650, Life Sciences, USA). Six wells were used for the negative control (LB* + 1% ethanol); 300 L3s were dispensed (in 50 µl) into the wells using a multi-channel pipette and a mini-air pump (Air-pump-S100; Aquatrade, Australia) to keep the parasites suspended, as described by Preston et al. (2015). The perimeter wells were filled with 200 µl of sterile water. Plates were incubated at 38 °C and 10% CO_2 (v/v). Following a 48 h incubation period, the motility of the xL3s was assessed as described previously (Preston et al., 2015). In brief, the plates were agitated on an orbital shaker for 30 min at 38 °C prior to recording a 10 s video of each well using an eyepiece camera (Dino-eye, ANMO Electronic Corporation, Taiwan) attached to a stereo dissecting microscope (Olympus, Japan). Each video was then processed for motility by calculating the changes in pixel intensity using a custom macro in the program ImageJ (1.47v, imagei.nih.gov/ij), as described in detail by Preston et al. (2015).