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Australian plants show anthelmintic activity toward equine cyathostomins *in vitro*

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ABSTRACT

Anthelmintic resistance in gastrointestinal parasites of horses is an increasing problem, particularly in cyathostomins, and there is a need to find alternative means for the control of these parasites. We screened crude extracts from 37 species of Australian native plants for their anthelmintic activity *in vitro* against cyathostomin larvae (development from egg to third larval stage), with the aim of identifying those species that may be suitable for incorporation into sustainable parasite management programs. Water extracts from seven species, namely *Acacia baileyana*, *Acacia melanoxylon*, *Acacia podalyriifolia*, *Alectryon oleifolius*, *Duboisia hopwoodii*, *Eucalyptus gomphocephala* and *Santalum spicatum* completely inhibited larval development (100% inhibition compared to the control), while another 10 species caused 90% inhibition at the initial screening concentration of 1400 μg of extractable solids/mL. The seven most potent extracts produced IC_{50} values (concentration of extract which resulted in a 50% inhibition of development) in the range 30.9–196 μg/mL. Fourteen extracts were incubated with polyvinylpolypyrrolidone (PVPP) before the assays, which removed the anthelmintic activity from 12 of these extracts, indicating that tannins were likely to be the bioactive compound responsible for the effect, while in two species, *i.e. A. melanoxylon* and *D. hopwoodii*, compounds other than tannins were likely to be responsible for their anthelmintic action. Our results suggest that a number of Australian native plants have significant anthelmintic activity against cyathostomin larval development *in vitro*. There is potential for these plants to be used as part of sustainable parasite control programs in horses, although more research is needed to identify the compounds responsible for the anthelmintic effects and confirm their activity *in vivo*.

1. Introduction

Cyathostomins are the most significant parasites of horses worldwide. There are approximately 50 species of cyathostomins, although only about 10 of these species make up the majority of numbers in infested horses (Lyons et al., 1999). The most pathogenic life stage is the larvae. Third stage larvae (L3) encyst in the intestinal mucosa where they develop to fourth stage, L4 (Corning, 2009). These larvae can remain there for up to 2 years and can cause a potentially fatal condition known as "larval cyathostomiasis". This condition is caused by a mass emergence of L4 larvae from the intestinal wall, which can result in acute or chronic diarrhea, weight loss, edema, severe colitis and hypoalbuminemia in the horse (Lyons et al., 2000). Control of these parasites relies largely on the use of anthelmintics, however, this has been severely compromised over recent years by the increasing prevalence of anthelmintic resistance (Brady and Nichols, 2009; Kaplan

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and Nielsen, 2010). Currently, there is widespread resistance in cyathostomins to two major anthelmintic drug classes, the benzimidazoles and tetrahydropyrimidines. In addition, there have been reports of reduced efficacy of macrocyclic lactone anthelmintics (ivermectin, moxidectin) in recent years (Lyons et al., 2008). Molento et al. (2008) found that no anthelmintics tested in a horse yard in Brazil (including benzimidazoles, tetrahydropyrimidines and macrocyclic lactones) adequately controlled cyathostomins up to 28 days after treatment. This was the first report of a multi-resistant cyathostomin population.

The problem of anthelmintic resistance has arisen partly due to the routine use of anthelmintics as a preventative for several decades. This practise, known as the interval treatment program, was proposed in the 1960s by Drudge and Lyons (1966), and introduced mainly to control the very pathogenic large strongyles, particularly the potentially fatal *Strongylus vulgaris*, which were prevalent at that time. It entails dosing horses with an anthelmintic every 6–8 weeks and is still a common method of controlling parasites. While the interval treatment program was successful at controlling the target species, it placed strong selection pressure for anthelmintic resistance on all equine gastrointestinal parasites (Kaplan and Nielsen, 2010).

Despite the ineffectiveness of the current treatments, it is not expected that any new synthetic anthelmintics for use in horses will become available in the near future (Stratford et al., 2011). Subsequently, there is a need to implement new sustainable approaches to parasite control in horses, with treatments based on the biology of the target parasite, effectiveness of the drug, and the needs of the individual horses, rather than using regular preventative treatments (Kaplan and Nielsen, 2010). Interest has grown in the use of some novel methods for nematode control, in particular those that have the potential to maintain low egg numbers, which reduce the frequency that anthelmintic drugs need to be administered and slow the spread of resistance.

One such novel method for nematode control that has potential in the equine industry is the use of anthelmintic plants. Medicinal plants have been used to treat parasitism in animals for hundreds of years, although the majority of evidence on the anthelmintic activity of these plants had been largely anecdotal. More recently, scientific reports have emerged, describing the anthelmintic properties of plants in variety of animal species, both *in vitro* and *in vivo* (reviewed by Athanasiadou and Kyriazakis, 2004; Githiori et al., 2006; Hoste et al., 2006; Athanasiadou et al., 2007). In Australia, selected plants and their secondary compounds have been found to have antiviral and antibacterial activity (Semple et al., 1998; Palombo and Semple, 2001; Ndi et al., 2007). Recently, Kotze et al. (2009) reported anthelmintic properties against sheep worms *in vitro* amongst some Australian plants. The study revealed that plant species such as *Eremophila maculata* and *Acacia pycnantha* completely inhibited larval development of *Haemonchus contortus* *in vitro* at 1400 μg extractable solids per mL. Additionally, some tropical Australian plants have been found to have anthelmintic activity against *H. contortus* and *Trichostrongylus colubriformis* in goats, significantly reducing the egg output for both species (Moreno et al., 2012). Many of the plants examined in the study are known to be rich in secondary compounds, due to the harsh environment where they grow and which allow them to tolerate water, soil and climatic stress and also protect against herbivores (Dynes and Schlink, 2002). To date, the activity of bioactive Australian plants against horse helminths have not been investigated. The present study aimed to screen a range of Australian plants for anthelmintic activity against cyathostomins *in vitro*, in order to identify plants with potential to be incorporated into parasite control programs for horses. Firstly, this involved testing 37 species of plants in larval development assays at the concentration of 1400 μg extractable solids per mL, and secondly, the determination of the IC_{50} values for the most active species. Thirdly, the polymer PVPP was used to verify if tannin compounds were responsible for any anthelmintic activity in fourteen of the plant species.

2. Materials and methods

2.1. Experimental design

We examined the anthelmintic activity of 37 plant species using crude plant extracts and *in vitro* assays with cyathostomin larvae. All samples were screened at one concentration of 1400 μg/mL and following this, the most potent extracts ($n = 25$) were examined in a dose–response manner. Fourteen plant extracts were also tested in the presence of polymer polyvinylpolypyrrolidone (PVPP) as a means to determine whether anthelmintic activity observed with some extracts was due to the presence of tannins (Makkar, 2003). The fourteen plants were chosen as they showed the highest anthelmintic activity in the initial screening assays ($n = 12$), or because there was anecdotal evidence that they were used as fodder and/or were palatable to livestock ($n = 2$). Each treatment was run in quadruplicate wells and assays were replicated on two separate occasions, using separate nematode egg preparations.

2.2. Plant material

Samples from 37 species of Australian plants held in a collection at the University of Western Australia were used for this study (Table 1). Plants were selected on the basis of previous reports indicating that they contained bioactive substances (anthelmintic, antibacterial or other medicinal properties), or have potential as a fodder shrub (Ghisalberti, 1994; Dynes and Schlink, 2002; Durmic et al., 2008). The samples were collected by the Department of Conservation and Land Management throughout Spring 2005, identified botanically and voucher specimens were deposited. The plant material was then stored at −20 °C until processed. The samples were freeze-dried, reweighed and milled to coarse powder using a cyclone grinder (CYCLOTECH 1093 Sample Mill; Tecator, Hoganas, Sweden) fitted with a 4 mm screen followed by a 1 mm screen. A control of oaten chaff (not containing any known bioactive secondary compounds) was also prepared in the same manner. Ground material was stored at room temperature in sealed containers until use. The samples consisted of a mixture of leaves