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Exploring the anthelmintic properties of Australian native shrubs with respect to their potential role in livestock grazing systems

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SUMMARY

We measured *in vitro* anthelmintic activity in extracts from 85 species of Australian native shrub, with a view to identifying species able to provide a degree of worm control in grazing systems. Approximately 40% of the species showed significant activity in inhibiting development of *Haemonchus contortus* larvae. The most active extracts showed IC_{50} values of 60–300 μg/ml. Pre-incubation with polyvinylpolypyrrolidine removed the activity from some extracts, implicating tannins as the bioactive agent, while in other cases the pre-incubation had no effect, indicating the presence of other anthelmintic compounds. Plant reproductive maturity (onset of flowering or fruiting) was associated with increasing anthelmintic activity in some species. Variability was observed between plants of the same species growing in different environments, while variation between individual plants of the same species within a single field suggests the existence of distinct chemotypes. Significant activity against adult *H. contortus* worms *in vitro* was also demonstrated in a limited number of extracts tested against this life stage. Our study indicates that there is potential for Australian native shrubs to play an anthelmintic role in grazing systems, and highlights some plant biology factors which will need to be considered in order to maximize any anthelmintic effects.

Key words: plant extracts, perennial shrub, tannin, anthelmintic, *Haemonchus contortus*.

INTRODUCTION

Primary producers in the livestock/cropping zone of Southern Australia face significant challenges in maintaining sustainable farming systems in a low/medium rainfall environment (300–650 mm rainfall per annum). In particular, it is often difficult to maintain feed for livestock during the long dry summers using annual crop and pasture systems. A collaborative effort (the 'Enrich' project) was initiated in 2005 to provide new options for sustainable grazing systems in these regions utilizing Australian native perennial shrubs (Revell *et al*. 2008*a*). Perennial shrubs were identified as offering potential to these grazing enterprises due to their persistence under the harsh environmental conditions (e.g. long and hot summers). Forage shrubs could potentially provide feed over the dry summer and autumn to reduce the need for expensive supplementary feeding during this period. Additional benefits from these perennial species included reduced soil erosion, reduced risk of soil acidification and dry land salinity, and shade and wind protection for livestock especially during critical periods such as lambing. The advantages of using Australian native shrubs to provide the grazing and environmental benefits were apparent as they are adapted to the climatic challenges facing this region. The areas targeted by the project are shown in Fig. 1 alongside the distribution of sheep in Australia (in 2001).

An important component of the use of shrubs in grazing systems is their potential to provide bioactives which could act to improve animal health. These potential benefits include improving rumen fermentation profiles (e.g. reducing methane emissions, for example, Soliva *et al*. 2008) and/or providing a degree of control of gastrointestinal nematode parasites (Athanasiadou *et al*. 2007). There is considerable interest worldwide at present in the use of plants with anthelmintic properties, driven by factors such as drug resistance, and a desire to reduce

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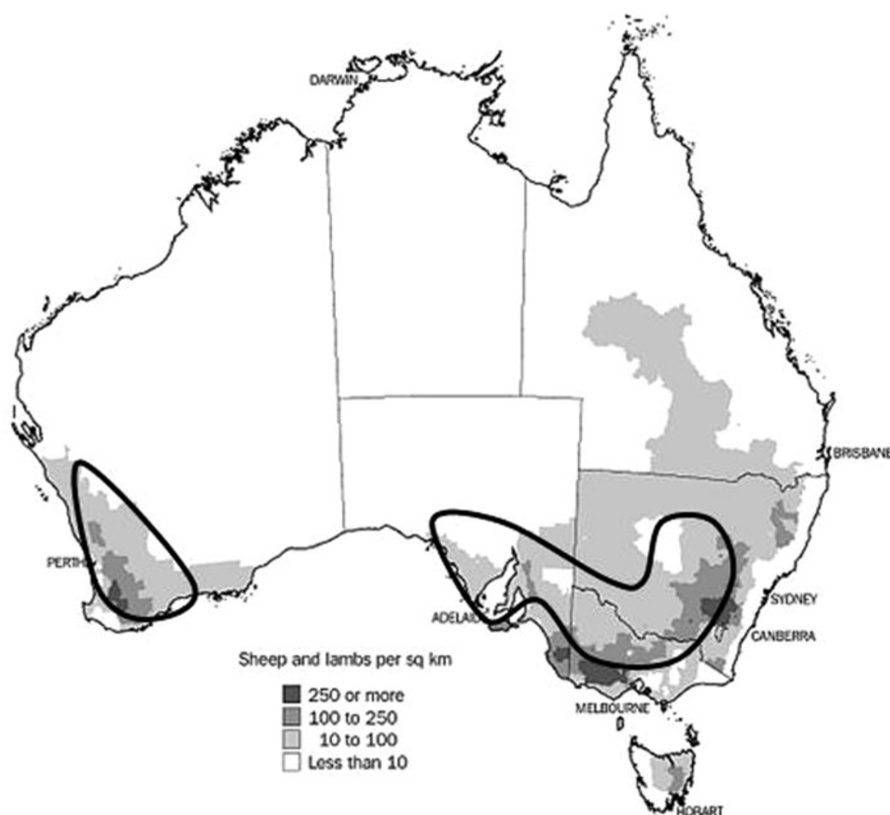

Fig. 1. The regions of southern Australia targeted by the Enrich project (indicated by circled areas) overlaying sheep distribution in Australia in 2001. The sheep distribution map was sourced from the Australian Bureau of Statistics, Year Book Australia, 2006 (http://www.abs.gov.au/ausstats/abs@.nsf/Previousproducts/8345877D2FBA8C4DCA2570DE00167195?opendocument).

the sole reliance on the use of drugs for worm control, as well as concerns about synthetic chemical residues in foodstuffs. The imperative to identify alternatives to drugs for control of parasites is apparent in the areas targeted by the Enrich project which show widespread resistance to existing anthelmintic drugs (Besier and Love, 2003). There is extensive literature on the use of plants to provide an anthelmintic component to the diet of grazing livestock (reviewed by Athanasiadou and Kyriazakis, 2004; Hoste *et al.* 2006; Athanasiadou *et al.* 2007); however, there has been no study in the past of the potential role of Australian native shrub species in this regard. The present study was therefore initiated to use *in vitro* bioassays to identify shrub species that possess significant anthelmintic activity.

We utilized plant material sampled from a field site established at Monarto, in South Australia, for the purpose of propagating and testing under field conditions the forage potential of a number of native shrub species, as well as 3 additional South Australian sources (Murray Bridge field research site, Waite Research Precinct, and Turretfield research station). Plant material was also collected from a field site established at Badgingara, in Western Australia, for the purpose of providing a test grazing site to look at grazing behaviour in shrub/perennial grass mixtures. Plant material was also sourced from a number of roadside collections in South Australia and New South Wales. In looking to optimize the use of plants with anthelmintic properties we compared the activity in samples from particular plant species which differed in their stage of maturity, and which were growing in different locations. The species examined in regard to plant maturity were determined largely by our ability to perform repeat sampling of particular plants at different times of the year. Collections from different roadside locations gave us an ability to examine variations within species due to environmental factors over large distances, while the presence of a large plot of *Rhagodia preissei* at Badginagrra allowed us to examine variations among individual plants in a single paddock in a plant species generating a deal of interest currently as a forage shrub in low rainfall livestock grazing enterprises, particularly in Western Australia. As a preliminary indication of the nature of the anthelmintic compounds in some shrubs we also looked at the effects of the polymer polyvinylpolypyrrolidine (PVPP) on the levels of activity observed with some extracts. The plants examined in this regard included some expected to contain large amounts of tannin (e.g. *Acacia* sp., Dynes and Schlink, 2002; Revell *et al.* 2008*a*), as well as others known to

contain non-tanniferous bioactive compounds (e.g. *Eremophila* sp., Ghisalberti, 1994; Pennacchio *et al.* 1996).

MATERIALS AND METHODS

Parasite egg recovery

The *Haemonchus contortus* used for this study were from the anthelmintic susceptible Kirby 1982 strain, isolated from the field at the University of New England Kirby Research Farm in 1986 (as described by Albers and Burgess, 1988). Infected animals were housed at McMaster Laboratory, CSIRO Livestock Industries, Armidale, New South Wales (NSW). Sheep faeces in air-tight bags were sent regularly by courier to the Queensland Biosciences Precinct laboratory at St Lucia, Brisbane. Nematode eggs were recovered from the faeces by passage through a series of fine sieves (250 μm and 75 μm) followed by centrifugation on a 2-step sucrose gradient (10%, 25% sucrose). The eggs were recovered from the interface between the 10 and 25% sucrose layers, and washed over a 25 μm sieve with water to remove residual sucrose. They were agitated gently in a solution of 8·4 mg/l sodium hypochlorite for 12 min, and then washed again with copious amounts of water. The eggs were diluted in distilled water at a concentration of 50–60 eggs per 30 μl after the addition of amphotericin B (final concentration 25·0 μg/ml) and tylosin tartare (final concentration 800 μg/ml), and used immediately for larval development assays.

Plant material

A total of 85 species of shrub were sampled for use in this study. Where possible, multiple plants were sampled at every collection. Plant samples were received from the sources described below.

(*1*) *South Australian* (*SA*) *Research Sites*. All plants at these sites were deliberately cultivated for research purposes and samples were collected from 4 sites: (a) Monarto field research site, located approximately 60 km east of Adelaide, (b) Murray Bridge field research site, approximately 70 km east of Adelaide., (c) Waite Research Precinct, located 6 km south-east of Adelaide. (d) Turretfield Research Station, located 55 km north-east of Adelaide.

The samples were taken from plants less than 2 years of age.

(*2*) *Badgingara Research Station, Department of Agriculture and Food Western Australia* (*DAFWA*), *Western Australia* (*WA*). Approximately 180 km north of Perth. *Rhagodia preissii* was sampled from this site.

(*3*) *SA roadside collections*. Samples were taken from natural populations in SA in April 2007. The species collected were *Rhagodia parabolica*, *R. crassifolia*, *R. candolleana*, *Eremophila longifolia* and *E. glabra*. The collection sites were spread over a distance of approximately 230 km in the Yorke Peninsula and mid-north of South Australia, as well as several sites in the lower Murray region.

(*4*) *New South Wales* (*NSW*) *roadside collections*. Samples were collected from natural populations in south west NSW in September 2007. The species collected in this exercise which had not been among the SA Research Site samples were *Alectryon oleifolium*, *Geijera parviflora*, *Santalumacuminatum*, *Exocarpus aphyllus*, *Dodonea viscosa* and *Flindersia maculosa*. Plant growth stage (i.e. vegetative, reproductive) and plant part (i.e. leaf, stem) were recorded for all samples. Soil characteristics were also recorded for the roadside collections in SA.

Plants were received either as ground material which had been either oven (60 °C for 72 h)- or freeze-dried (samples from SA Research Sites, SA roadside collections, and Badgingara), or as fresh specimens (NSW roadside collections). These latter samples were cut into small pieces, placed into a mortar on dry ice until frozen, and then ground within the mortar on dry ice using a pestle.

Plant material was extracted in either water or 70% ethanol. For water extraction, 1 ml of water was added to 100 mg of plant material, and the tube placed onto a roller wheel for 48 h at room temperature. The mixture was then centrifuged at 9000 ***g*** for 30 min and the supernatant recovered, and stored at −20 °C for use in bioassays. For ethanol extraction, 1 ml of 70% ethanol was added to 100 mg of plant material and placed onto a roller for 48 h at room temperature. The mixture was then centrifuged at 9000 ***g*** for 10 min. The supernatant was recovered and dried overnight in a Savant SpeedVac. The pellet was resuspended in 100 μl of DMSO with shaking for 4 h, and stored at −20 °C for use in bioassays.

The weight of dry matter in some of the most toxic extracts was determined in order to allow for the expression of anthelmintic activity on a per mg dry matter basis. For water extracts, an aliquot of the final water extract was dried overnight in a pre-weighed tube, the tube was then reweighed, and the weight of dried material calculated by difference. For ethanol extracts, an aliquot of the initial extract (prior to transfer into DMSO) was dried, and tube weights used to calculate the weight of dry matter as for the water extracts.

A number of plant extracts were treated with polyvinylpolypyrrolidine (PVPP) prior to use in bioassays in order to remove tannins. Extracts (300 μl) in water or ethanol (prior to transfer into DMSO) were added to 30 mg amounts of PVPP (that is, final PVPP concentration 10% (w/v)) and shaken for 4 h. The mixture was centrifuged at 2200 ***g*** for 5 min, and

the supernatant removed and either used directly in assays for water extracts, or dried and resuspended in 30 μl of DMSO for ethanol extracts.

Nematode larval development assay

Assay plates were established in 2 ways according to the type of extract being examined (i) *ethanol extracts*: 0·5 μl aliquots of plant extract (in DMSO) were added to individual wells of 96-well assay plates (DMSO alone was added to control wells). Agar (200 μl of 2% (w/v)) was added to each well, and allowed to solidify. Egg solution (30 μl) was added to each well. (ii) *Water extracts*: plain agar was added to wells of a 96-well plate, and allowed to solidify. Egg solution (30 μl) was added to each well. Aliquots of plant extract (10 μl) were added to each well (control wells received water only).

The plates were placed into bags to prevent drying, and incubated overnight at 26 °C. The next day, 2 μl of a growth medium (consisting of Earle's salt solution (10% v/v), yeast extract (1% w/v), sodium bicarbonate (1 mM) and saline solution (0·9% sodium chloride w/v) (as described by Hubert and Kerboeuf 1984)) was added, along with 2 μl of a solution of *E. coli* cells (XL1-Blue® Stratagene) in LB broth (Maniatis *et al.* 1982), and 6 μl of water. The plates were incubated for a further 5 days, and then killed using Lugol's iodine solution (10 μl per well). The number of fully developed infective-stage larvae (L3) present in each well was counted.

The concentration of the extract used in the screening assay was equivalent to the extracted material from approximately 4 mg of plant material per ml for water extracts, and the extracted material from approximately 2 mg of plant material per ml for the ethanol extracts. Freeze drying of some extracts showed that the recovery of solid material from the extracts amounted to approximately 35 mg solids per 100 mg of original dried plant material for both extraction methods. Hence the screening assay concentrations in terms of extracted plant material solids per ml were approximately 1·4 mg/ml and 0·7 mg/ml for water and ethanol extracts, respectively. A number of plant extracts were also examined in dose response assays in which a series of 2-fold dilutions of the screening extract were prepared and used in larval development assays, alongside 2-fold serially diluted solutions of the anthelmintic drugs ivermectin, thiabendazole and levamisole (technical grade; prepared in DMSO).

Some plants were tested only as oven- or freeze-dried samples, while others were tested after both preparation methods. All plants were tested after extraction with water, while most were also tested after ethanol-extraction. A small number ($n = 6$) were tested as freshly ground samples, without being dried prior to the water or ethanol extraction procedure.

Adult worm motility assays

The effects of 4 plant extracts on adult *H. contortus* worms were assessed by observing the degree of motility shown by worms over a period of exposure to the extracts *in vitro*. Adult worms were recovered from sheep housed at the QDPI animal facility at Yeerongpilly, Brisbane. The adult worm recovery and culture methods were as described by Kotze and McClure (2001). Briefly, worms were recovered from sheep abomasa (approximately 6–10 weeks post-infection) by manual picking from the gut contents. They were placed in culture medium (RPMI-1640, HEPES buffer, glucose, bovine serum, antibiotics and fungicide) for several hours, and then groups of 10 were placed in separate tubes in 0·9 ml of culture medium. The only significant change from the previously described method was the inclusion of 20% newborn bovine serum in the culture medium, and the subsequent maintenance of the worms in an atmosphere of 20% CO_2, 5% O_2 and 75% N_2 (compared to 5% CO_2 in air for the earlier study). Plant extracts (prepared as water extracts as described above) were sterilized by passage through a 0·22 micron filter, and aliquots (100 μl) were added to tubes, which were then kept at 37 °C. Controls received water only. At 24 h intervals, the worms were observed and their degree of motility was scored. The assay tubes were placed onto a warm tray and tubes were held individually near a light for assessment of motility. Each tube was swirled to thoroughly disturb the nematodes and was scored according to the degree of motility shown by the worms using the scoring system described by O'Grady and Kotze (2004). Briefly, the worms were scored as: 3 – most individuals showing significant smooth sinusoidal motion, similar to motion at the start of the culture period; 2 – significant movement shown by a small number of individuals, at least 1 individual able to move in a normal sinusoidal fashion; 1 – only very limited movement in a small number of individuals, no sinusoidal motion; 0 – no movement.

The weight of dry matter in extracts used for adult assays was determined as described above for larval assay extracts. The final concentration of extracted solids per ml of adult assay solution was determined to range between 3 and 7 mg/ml for the 4 plant samples examined.

Statistical methods

Larval development assays consisted of quadruplicate wells (each initially loaded with approximately 50–60 eggs) for each plant extract. Percentage larval development was calculated by expressing the numbers of larvae in treated wells as a percentage of the mean number in control wells (at least 8 control wells were present on each plate). Experiments were

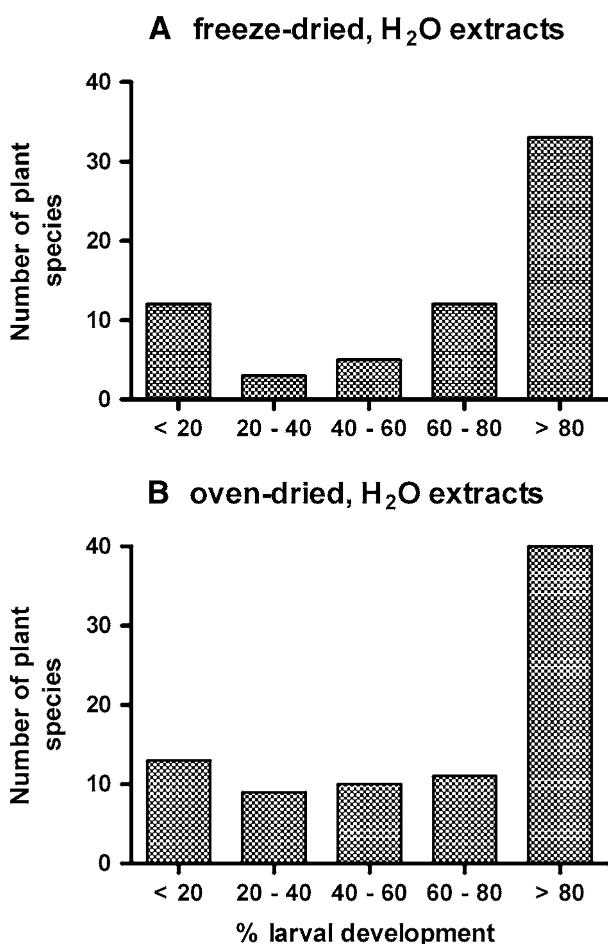

Fig. 2. Frequency distribution of anthelmintic activity in water extracts of freeze-dried (A) and oven-dried (B) samples of various plant species. Total numbers of samples: A = 65; B = 83.

performed at least twice with each extract, on separate nematode egg preparations. Percentage development data for each extract from the separate experiments were pooled to give a mean percentage development for each extract. In some cases, IC_{50} values (concentration of extract which resulted in a 50% inhibition of development) were calculated from percentage development data at a series of 2-fold dilutions of the extract using non-linear regression (sigmoidal dose-response, GraphPad Prism). Two adult worm motility experiments were performed using assay tubes each containing 10 adult worms, with control tubes in triplicate and extracts examined in a single tube. Data from the 2 experiments were combined to give a mean motility score at each time point for each extract ($n = 2$) and a mean score for control tubes ($n = 6$).

RESULTS

The frequency distributions of anthelmintic activity across the water extracts of both freeze- and oven-dried samples are shown in Fig. 2. A large number of samples had no anthelmintic activity, while others showed a spread of activities ranging up to complete inhibition of larval development at the screening concentration of approximately 1·4 mg extracted material/ml for water extracts. For both freeze- and oven-dried samples, there was a substantial number that reduced larval development to less than 40%. This was subsequently used as a cut-off point to identify plants with 'significant' anthelmintic activity. A list of all plants examined in the study, subdivided into those that reduced larval development to less than 40%, and those with less or no effect on development, is shown in Table 1. In cases where activity was only seen in some samples of particular species (see below), a plant is shown as active in Table 1 if anthelmintic activity was detected in at least 1 sample from this species. In cases where multiple plant species from single genera were examined, the prevalence of activity within the genera differed. For example, all 7 *Cullen* spp., and 7 out of 8 *Atriplex* spp. were not active, while 6 out of 7 *Acacia* spp. showed significant activity. *Rhagodia* spp., *Maireana* spp. and *Kennedia* spp. were more evenly spread between those showing a presence or absence of activity.

The sample preparation methods of freeze- or oven-drying, as well as the sample extraction methods using water or 70% ethanol, were compared directly in order to assess the impact of the different methods on subsequent measurement of anthelmintic activity. Only a subset of the plant collections were examined in this regard as some samples were received only in a freeze- or oven-dried form, while ethanol extractions were not performed on all samples. These experiments showed that although there was a deal of overlap in samples identified as anthelmintic by the 2 extraction procedures, there was also a substantial number of samples that were only identified as toxic after one of the extraction methods was used. This was particularly the case for water extractions of freeze-dried material. Additionally, while many samples were identified as anthelmintic after both freeze- and oven-drying, a significant number were only active if prepared by freeze drying.

Several of the most active plant extracts (inhibiting development completely at the screening concentration from Fig. 2) were examined further in dose-response assays, alongside anthelmintic drugs (Fig. 3). The activity of the extracts was at least 300-fold less than levamisole, 4500-fold less than thiabendazole, and 120 000-fold less than ivermectin (in terms of relative IC_{50} values).

The role of tannins in the observed anthelmintic activity was examined by comparing toxicity of some extracts with or without pre-treatment with the tannin-complexing polymer PVPP. The results for some water-extracted samples are presented in Fig. 4, while data from ethanol-extracted samples are

Table 1. Anthelmintic activity in extracts from 85 plant species

(Extracts were considered to have significant anthelmintic activity if they reduced larval development to less than 40% compared to control assays.)

		Species – Inhibition of larval development	
Family	Genus	Yes	No
Myoporaceae	*Eremophila*	*glabra, maculata, longifolia*	*bignoniiflora*
	Myoporum	*platycarpum*	
Chenopodiaceae	*Rhagodia*	*parabolica, crassifolia, preissii*	*candolleana, spinescens*
	Atriplex	*isatidea*	*cinerea, semibaccata, vesicaria, nummularia, amnicola paludosa, rhagodioides*
	Maireana	*brevifolia, planifolia, astrotricha, tomentosa*	*convexa, sedifolia, georgei pyramidata*
	Chenopodium		*auricomum, gaudichaudianum nitrariaceum*
	Enchylaena	*tomentosa*	
Mimosaceae	*Acacia*	*pycnantha, neriifolia, loderi, iteaphylla, saligna myrtifolia*	*ligulata*
Lomandraceae	*Lomandra*	*longifolia*	
Fabaceae	*Medicago*	*citrina*	*sativa, arborea, strasseri*
	Cullen		*palladium, tenax, parvum cinereum, patens, discolor australasicum*
	Viminaria		*juncea*
	Swainsona	*stipularis*	*galegifolia, greyana*
	Indigofera	*australis*	
	Glycine		*canescens, clandestina, tabacina*
	Senna		*planitiicola, barclayana*
	Chameacytisis		*prolifer*
	Kennedia	*nigricans, prorepens, eximia rubicunda*	*prostrata, macrophylla*
	Lotus		*australis*
	Dorycnium	*hirsutum*	
	Colutea	*abyssinica*	
Plantaginaceae	*Plantago*		*lanceolata*
Papilionaceae	*Templetonia*		*retusa*
Nitrariaceae	*Nitraria*		*billardieri*
Asteraceae	*Calotis*		*scapigera*
	Pterocaulon	*sphacelatum*	
	Brachycome	*ciliaris*	
Malvaceae	*Lavatera*		*plebeia*
	Abutilon		*otocarpum*
Rutaceae	*Flindersia*		*maculosa*
	Geijera		*parviflora*
Compositae	*Cichorium*		*intybus*
Santalaceae	*Exocarpus*		*aphyllus*
	Santalum		*acuminatum*
Sapindaceae	*Alectryon*		*oleifolium*
	Dodonaea		*viscosa*
Convolvulaceae	*Convolvulus*	*remotus*	

presented in Fig. 5. The activity of some extracts was unaffected by the PVPP-treatment, indicating no role for tannins in the observed toxicity (e.g. *Eremophila maculata*, *E. glabra*), while in other cases the toxicity was significantly reduced following PVPP treatment (e.g., *Myoporum platycarpum*, *Acacia pycnantha*, *Rhagodia crassifolia*), indicating that tannins contribute at least partly to the observed activity. In general, the impact of PVPP treatment was greater for ethanol extracts than water extracts. This was particularly evident for *R. parabolica* which showed no PVPP effect on water extracts alongside significant effect of the polymer with ethanol extracts, indicating a preferential extraction of tannins using the latter solvent. All cases in which PVPP was assessed with ethanol-extracted samples in Fig. 5

	Drug	IC_{50} (μg/ml)
■	ivermectin	0.00053
▽	Thiabendazole	0.014
○	levamisole	0.21

Plant extract
IC_{50} (μg/ml)
range
64 -- 272

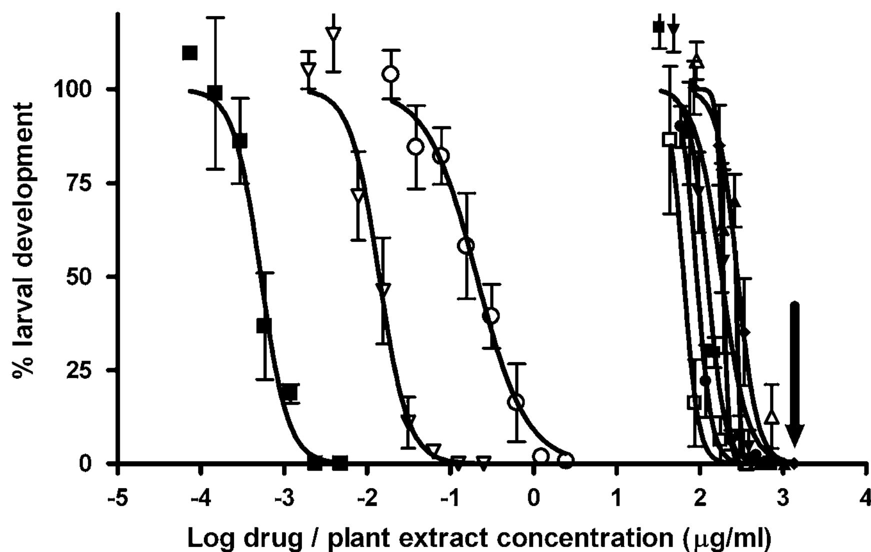

Fig. 3. Activity in larval development assays of commercial anthelmintic drugs and water extracts of several plants. IC_{50} values represent the concentration of material required to reduce the larval development to 50%. The arrow indicates the approximate screening concentration used initially for all water extracts.

showed a significant reduction in toxicity, compared to only selected plants for the water extractions (from Fig. 4).

Having identified some plant species with anthelmintic activity, we were interested in examining whether this activity varied among individual plants over time and space. That is, whether individual plants showed variation through the season, as well as whether activities differed between plants from the same species across different geographical zones, ranging from different parts of a single field to different places within a wider region (hundreds of kilometres wide). Figure 6 shows activity in water extracts from 5 *R. preissii* plants collected at the Badgingara Research Station in April 2007 and August 2007. The 2 plants in Fig. 6A showed markedly different activities at the time of the first sampling, with plant 5 being inactive against the worm larvae. These 2 plants both showed little change in activity between the 2 sampling times. On the other hand, plants 2, 3 and 4 showed a significant increase in activity at the second sampling time compared to the first (Fig. 6B). This increase in activity was greatest for plants 3 and 4.

To further examine variation between plants of the same species we sampled from 94 *R. preissii* plants growing in a line over a distance of approximately 400 metres in a field at the Badgingarra Research Station in September 2007. Figure 7A shows that activity varied markedly between the plants. Significant activity was detected in approximately 70% of the plants (larval development $<40\%$) with the remainder showing only low or negligible anthelmintic activity. There were some differences in the physical appearance of the plants; however, it was not possible to correlate this with anthelmintic activity. No obvious pattern of activity occurred with movement along the line of plants (Fig. 7B), indicating the absence of a gradational change in activity that may be expected if soil type changed gradually along the line of plant sampling.

Differences between plants sampled from a number of sites in South Australia were also examined. The plant species sampled had shown activity in our initial screens, except for *R. candolleana* which was collected here to further test the earlier observed presence and absence of activity in different species within the Rhagodia genus. Table 2 shows that significant variation existed between the plants in terms of their anthelmintic activity. The soil texture at the collection sites showed a great deal of variation, with the following types recorded: sand, sandy loam, loam, and clayey sand. Soil pH was acid in some cases, and alkaline in others. The presence or absence

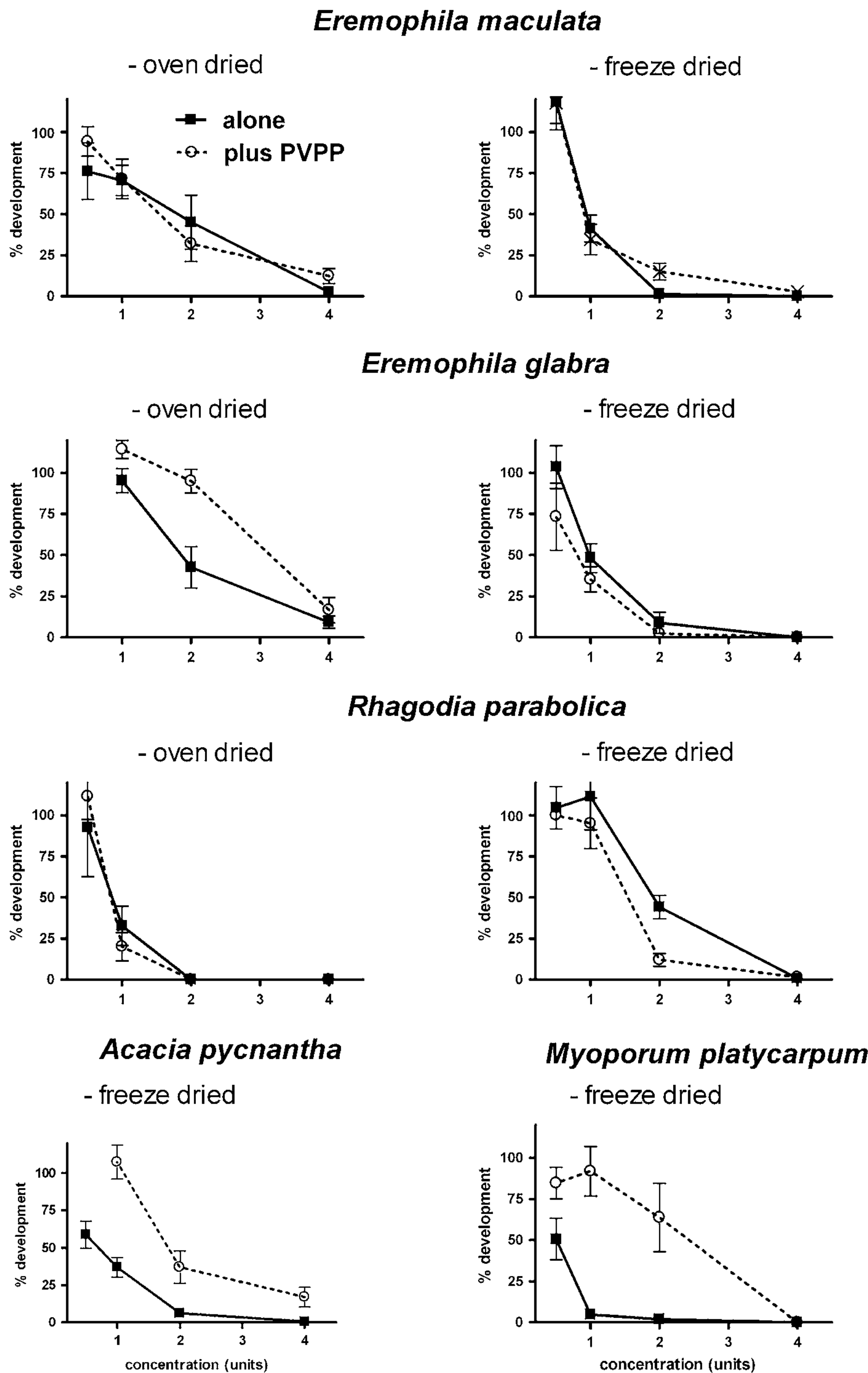

Fig. 4. Effect of pre-treatment with PVPP on the anthelmintic activity in water extracts of oven- or freeze-dried material from various plant species. Extracts not treated with PVPP are shown as solid lines, extracts pre-treated with PVPP are shown as dotted lines. The X-axis represents arbitrary concentration units, with the highest concentration for assays in which 10 μl of plant extract was added to the assay, and lower concentrations representing 2- and 4-fold dilutions of extract before addition to the assay.

of activity did not correlate with soil texture or pH. However, in several of the species there was a clear relationship between the plant growth stage and the presence of anthelmintic activity. The 2 *R. crassifolia* plants that showed toxicity were at the fruiting stage, while the 2 plants that were not active had not fruited, with one having flowered only and the other still vegetative. The 3 *E. longifolia* plants showing activity were at the fruiting stage, while the 2 active plants had not commenced fruiting. There was no obvious discrimination in plant growth stage within the 10 *R. parabolica* plants as all were flowering but had not commenced fruiting. Table 2 also provided some confirmation of the different

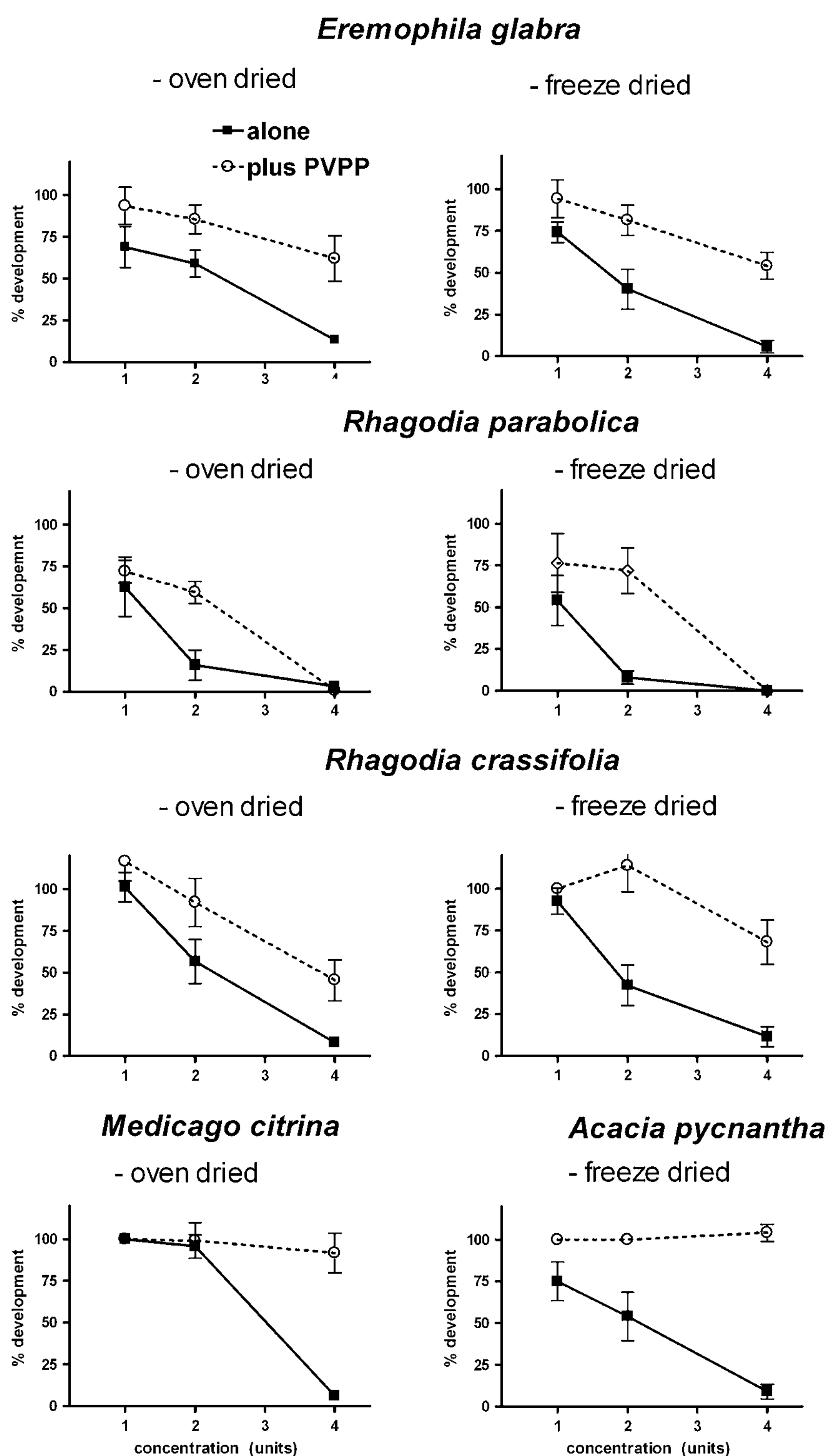

Fig. 5. Effect of pre-treatment with PVPP on the anthelmintic activity in ethanol extracts of oven- or freeze-dried material from various plant species. Extracts not treated with PVPP are shown as solid lines, extracts pre-treated with PVPP are shown as dotted lines. The X-axis represents arbitrary concentration units, with the highest concentration for assays in which 10 μl of plant extract was added to the assay, and lower concentrations representing 2- and 4-fold dilutions of extract before addition to the assay.

solubility patterns noted earlier in comparing water- and ethanol-extracted samples; *R. crassifolia* only showed activity in ethanol-extracted samples, while *E. longifolia* was only active in water-extracted samples.

Given the variation seen in *R. preissii* plants in September 2007 (Fig. 7), and the indications from Table 2 that plant reproductive maturity may be a factor in determining anthelmintic activity in some species, we further investigated this relationship by

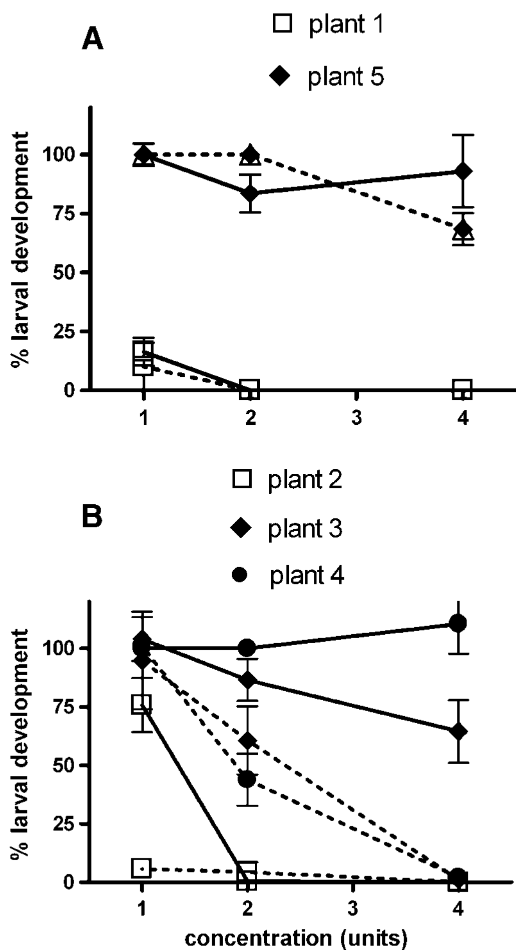

Fig. 6. Anthelmintic activity in water extracts of 5 individual *Rhagodia preissii* plants sampled in April 2007 (solid lines) and again in August 2007 (dotted lines) at the Badgingarra Research Station in WA. For clarity, the plants are shown in separate panels, A and B.

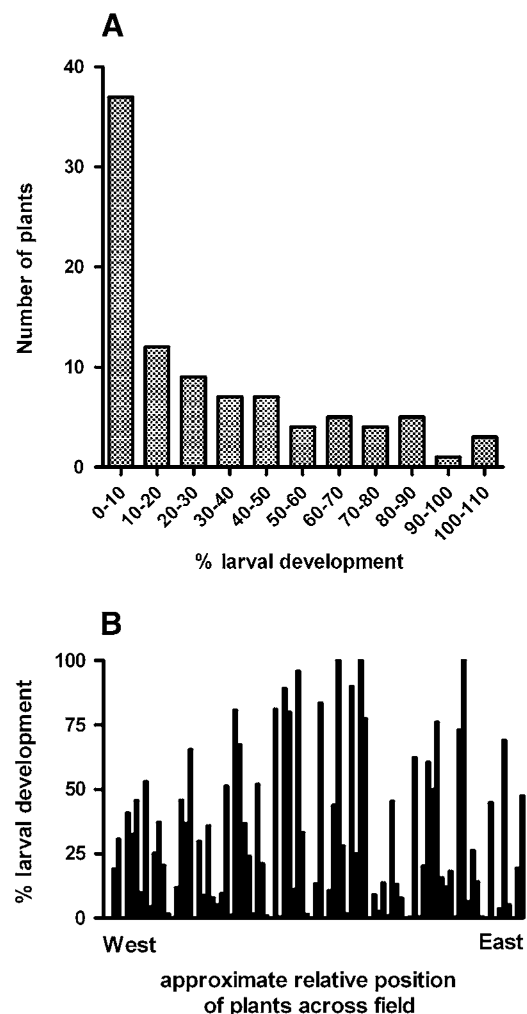

Fig. 7. Anthelmintic activity in 94 *Rhagodia preissii* plants sampled in September 2007 at the Badgingara Research Station. (A) Frequency distribution of activity in individual plants. (B) Activity in plants collected in sequence in a continuous transect from West to East.

sampling a number of individual *R. preissii* plants at the Badgingarra site in February 2008. The samples were taken from both flowering and non-flowering plants, and from plants grouped at the two extremes of the field. While most plants were highly active in the bioassays (median larval development within each grouping was less than 7%), a small number of plants showed only low or negligible activity (Fig. 8). This absence of activity occurred in both flowering and non-flowering plants, and in plants from both areas within the field, suggesting that it was unrelated to plant growth stage or environmental conditions. Of note was the fact that the median larval development for this combined data set was 3·6% ($n = 24$), compared to a median of 18·6% for the 94 plants that had been sampled earlier in the growing season in September 2007 (from Fig. 7), suggesting a greater overall anthelmintic activity at the more reproductive plant stage sampled in early 2008.

We examined the effects of several plant extracts on the motility of adult worms *in vitro*. Figure 9 shows that, while worms in control assays maintained motility throughout the 72 h of the experiment, extracts from 3 plant species (*R. parabolica*, *R. preisii* and *E. longifolia*) significantly reduced the worm motility. Interestingly, despite the *Kennedia exemia* extract showing toxicity to larvae, it did not affect adult worms.

DISCUSSION

This study has demonstrated that there is significant anthelmintic activity in extracts from many Australian native shrub species, raising the possibility that they may show some anthelmintic activity *in vivo*, and hence may be useful in providing a degree of worm control in grazing systems.

There are many different types of plant secondary compounds that could potentially act against nematodes, for example, tannins, saponins, polyphenols, alkaloids, glycosides (Athanasiadou and Kyriazakis, 2004; Hoste *et al.* 2006). Plant cysteine proteases

Table 2. Presence or absence of anthelmintic activity in extracts from 23 plant populations sampled in April 2007 in South Australia

	Toxicity expected based on initial screening		H_2O extracts		EtOH extracts	
Species	H_2O extract	EtOH extract	Number toxic	Number non-toxic	Number toxic	Number non-toxic
R. parabolica	Yes	Yes	5	5	3	7
R. crassifolia	No	Yes	—	4	2	2
R. candolleana	No	No	—	1	—	1
E. longifolia	Yes	No	3	2	—	5
E. glabra	Yes	Yes	—	3	—	3

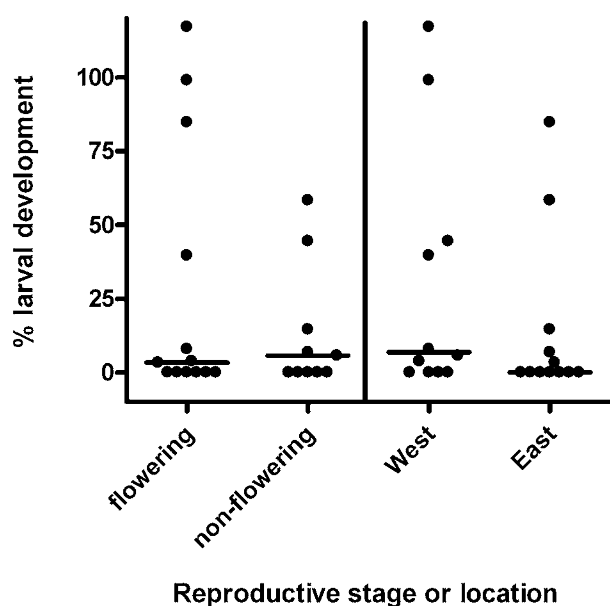

Fig. 8. Anthelmintic activity in 24 *Rhagodia preissii* plants sampled in February 2008 at the Badgingara Research Station. Plants are grouped either as flowering or non-flowering (left panel), or as growing at Western or Eastern end of the field (right panel). Horizontal lines represent the median value for percentage larval development.

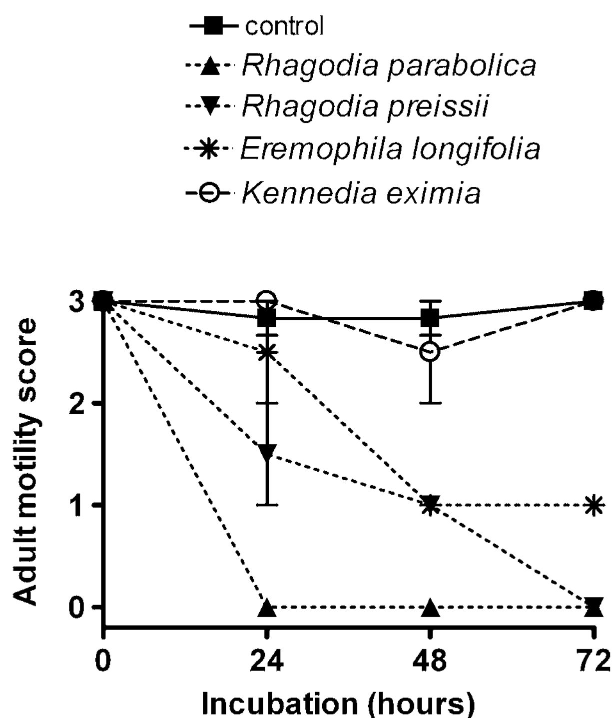

Fig. 9. Activity of plant extracts against adult *Haemonchus contortus* worms *in vitro*. The degree of motility shown by worms in the presence or absence of extracts was scored at various time points. Motility scoring system is described in the Materials and Methods section of the text. Pooled data from 2 experiments, each with 3 control assays, and single plant extract assays (data are shown as mean ± S.E.).

have also been shown to have significant *in vitro* anthelmintic activity (Stepek *et al.* 2004). We used the tannin-absorbing compound PVPP to determine whether tannins contributed significantly to the observed anthelmintic activities. It was clear that the activity in some species was largely due to tannins, while activity in others did not involve these compounds. While low doses of tannins are beneficial to ruminants by protecting fermentable protein from degradation in the rumen, high doses can be detrimental by reducing voluntary feed intake and plant digestibility, thereby reducing rates of body and wool growth (Barry and McNabb, 1999; Min *et al.* 2003). Hence, the identification of tannins as being the anthelmintic agent in some extracts in the present study needs to be examined closely to determine whether the anthelmintic potential can be realised at a level of feed intake that does not result in anti-nutritional effects. In addition, many other plant secondary compounds can also have anti-nutritional properties when ingested at sufficiently high concentrations; for example, saponins have been associated with reduced feed intake (Milgate and Roberts, 1995). The nature of the non-tannin components of the plants studied here is unknown. Australian native shrubs have been shown to contain bioactives with antiviral, antibiotic and medicinal properties (Ghisalberti, 1994; Semple *et al.* 1998;

Ndi *et al.* 2007*a*, Revell *et al.* 2008*a*), with the bioactive compounds in some cases being identified as saponins, diterpenes, essential oils, or glucosides (Ghisalberti 1994; Pennachio *et al.* 1996; Ndi *et al.* 2007*b*; Revell *et al.* 2008*a*). Further studies to identify and characterize the active agents in the anthelmintic extracts identified in the present study will require fractionation guided by *in vitro* larval development assays.

A number of *Acacia* spp. showed significant anthelmintic activity. This is not surprising given the prevalence of tannins among the secondary compounds in Australian acacias (Dynes and Schlink, 2002), and previous studies demonstrating the anthelmintic properties of various *Acacia* spp. due to the presence of condensed tannins (Kahiya *et al.* 2003; Cenci *et al.* 2007; Akkari *et al.* 2008). However, the potential for the use of Australian *Acacia* spp. in grazing systems is considered to be limited by their generally low palatability and nutritive value (Dynes and Schlink, 2002; Revell *et al.* 2008*a*). Consequently, plants such as these are likely to be eaten in small amounts and represent only a component of the diet yet, importantly, this can still be beneficial in some circumstances (Howard *et al.* 2002). For example, Ben Salem *et al.* (2005) showed that, with lambs offered an oaten hay-based diet, feeding a small amount of foliage from the tannin-containing *Acacia saligna* (also known as *Acacia cyanophylla*) before the main protein source (soyabean meal) was offered, reduced degradation in the rumen and increased live weight gain of the lambs.

Various *in vitro* assays have been used in studies of the anthelmintic properties of plants (Hoste *et al.* 2006). We chose larval development assays as our bioassay method for screening of plant extracts. Larvae were allowed to develop in an aqueous solution on an agar base (as commonly used for drug bioassays, for example Gill *et al.* 1995) rather than in an aqueous environment alone, as *H. contortus* L1 stage larvae that hatch onto agar appear 'fitter' (more motile) than if hatching in water in a plastic assay well (A. Kotze, unpublished observation). This may be due to access to soluble nutrients released from the agar. Addition of water extracts to the assay wells was straightforward. On the other hand, due to the toxicity of 70% ethanol to the larvae, to allow the addition of a similar amount of ethanol extract to the assays as utilized for the water extracts, we dried the samples and then redissolved them at a higher concentration in DMSO (the solvent used routinely for larval development assays, see for example, Gill *et al.* 1995).

Our comparison of sample preparation and extraction techniques indicated the presence of bioactive compounds with quite different physical properties in the various plant extracts. Some plants contained actives that were soluble in both water and ethanol, while some were soluble in only one solvent. Some plants contained bioactive agents that were stable to oven drying, while the active agents in others were only detected after freeze drying, indicating a range of susceptibilities of the plant bioactive agents to the extremes of oven-drying. These data suggest that sample preparation and extraction techniques will need to be optimized for further focused study of particular plant species. The influence of the extraction technique on the nature of the bioactives recovered from extracts was illustrated in the larval assays with PVPP; for *E. glabra*, *A. pycnantha*, and *R. parabolica* the role of tannins in the observed anthelmintic activity was greater for the 70% ethanol extracts than the water extracts. Tannins are most commonly extracted with aqueous organic solvents, and our data suggest that aqueous ethanol is superior to water as an extraction solvent for the specific study of anthelmintic tannins, while in other cases anthelmintic agents were only detected after extraction in water.

We have indicated that approximately 40% of the plant species examined in this study had 'significant' anthelmintic activity. Such a percentage of positive results is high for a natural product discovery exercise; however, this value is arbitrary in the sense that we have used a figure of 60% inhibition of larval development at our screening assay concentration as the criteria for being 'active'. However, the nature of the plants examined in this study may provide some basis for expecting a higher rate of positive (anthelmintic) samples than may be observed from other such plant screening exercises. Dynes and Schlink (2002), examining a number of Australian *Acacia* species, noted that 'plants that grow in inhospitable environments commonly contain secondary compounds which play a role in plant survival'. One suggested function for such compounds was as a grazing deterrent. Coley *et al.* (1985) proposed that resource availability was the major factor influencing the amount of plant defensive metabolites, with slow-growing species in resource-limited environments showing higher levels of defensive secondary metabolites (for example, tannins). They suggested that plants in hostile environments had a greater need to deter grazing animals due to the greater relative costs of replacing plant parts compared to fast-growing plants in rich environments. The harsh environmental conditions to which Australian perennial shrubs have adapted may contribute to our demonstration of some degree of *in vitro* anthelmintic activity in a large number of species. Given that the Enrich project aims to identify shrubs for incorporation into grazing systems, it will be important to determine whether the anthelmintic agents present in some plants will also act as grazing deterrents.

Anthelmintic activity of some plant species showed variability between samples. Some cases of

variability were most likely related to plant growth stage, for example, (i) the increase in anthelmintic activity in *R. preissii* plants 2, 3 and 4 between the two time-points may be associated with the increasing reproductive maturity of the plants as they approach flowering in October – January, and (ii) activity in samples of *R. crassifolia* and *E. longifolia* limited to more advanced fruiting specimens. It is known that levels of some plant secondary compounds may increase as plants mature (Barry, 1989; Kozukue and Friedman, 2003; Nair *et al.* 2006; Siskos *et al.* 2007), and hence it may not be surprising that the compounds toxic to nematode larvae are present at increased levels at flowering and fruiting times. Seasonal variation in plant secondary compounds has also been shown to be common in some species investigated in this study, such as *Atriplex semibaccata* (Mathams and Sutherland, 1952), *Acacia saligna* (Salem, 2005) and *Chamaecytisus prolifer* (Assefa *et al.* 2008). However, in these cases it has been correlated with increasing temperature or water stress, and not plant growth stage.

Variation within *R. preissii* plants at single time-points within a field was also observed. This may relate to the presence of chemotypes, that is, *R. preissii* plants within the population at the field site with differences in the composition of their secondary metabolites. The *R. preissii* plants at the Badgingara site were not clonal, being derived from seed collections from wild populations over a wide geographical range (I. Pullbrook, personal communication). It was noted by the plant collector at the time of sampling for anthelmintic assays that there were some differences in the appearance of the plants, particularly leaf colour and, to a lesser extent, leaf size and shape. The individual plants from which extracts showed anthelmintic activity tended to be bigger, more upright in structure, with larger, thicker and darker green leaves, but none of these traits on their own were significantly different between toxic and non-toxic plants (Revell *et al.* 2008*b*). It is well known that chemotypes exist within plant species. Chemotypes that show differing toxicities to insects have been described in a number of reports (Lattanzio *et al.* 2000; Cheng *et al.* 2004; van Leur *et al.* 2008). Further work is needed to determine whether chemotypes exist in the *R. preissii* examined in this study, as well as in other species showing variable levels of activity (for example, *R. parabolica* from Table 2). Until such work is undertaken, it is not possible to apply general 'anthelmintic' labels to plant species identified in the present study as showing activity in our assays of extracts from particular plant collections.

Plant variability may also relate to other factors, for example, soil variability, soil pH, recent insect damage, or recent grazing. These factors are known to affect the types and levels of secondary compounds produced by plants (Barry and Manley, 1986; Barry, 1989; Olsen and Roseland, 1991; Edwards, 1999; Nair *et al.* 2006; Staudt and Lhoutellier, 2007). A controlled study in which all such data are recorded alongside anthelmintic activity may be required to provide explanations for the type of plant variability observed in the present study.

An ability to quantify the bioactive agents would be a more accurate means of monitoring anthelmintic activity than reliance solely on the worm bioassay. An understanding of the nature of the variability will enable plants to be better utilized to maximize their anthelmintic effects; for example, a defined pattern of activity associated with flowering and fruiting stages would enable effective use of the anthelmintic activity that those shrubs would provide at these times in the season. Given the likely existence of chemotypes within the promising plant species identified in this study, the identification of the most active chemotypes within particular species could allow for the specific use of these to generate seed or cutting populations for distribution to graziers.

The plant extracts were significantly less active than commercial anthelmintic drugs in the larval assay. However, while the anthelmintic drugs used in the assays were pure compounds, the anthelmintic agents in the plant extracts may represent only a small proportion of the total material in the extract, and hence, in terms of toxicity on the basis of weight of the bioactive compound(s) alone, the activity against worms could be much greater than indicated by the IC_{50} of the whole extract. Direct comparison with anthelmintic drugs will need to wait until the bioactives are identified and purified from plant extracts. However, while commercial anthelmintics are designed to remove 100% of a worm population within an animal with a single dose, the aims of the Enrich project are quite different. We are looking for bioactives that will provide a degree of anthelmintic effect when ingested over a period by a host animal, not expecting that they will remove all worms or prevent establishment of all worms. Such a need could be satisfied by bioactives showing significantly less intrinsic potency than compounds suitable for use as commercial anthelmintics.

A potential issue with respect to the adult worm assays in the present study is the possibility that the decrease in motility seen in worms treated with some extracts was due to a lack of protein available as a food source in the culture medium as a result of protein absorption by tannins present in the extract. If this was the case then the observed toxicity may have been associated with a general lack of nutrition in the worms rather than any direct toxic effect of bioactives within the extracts. However, while the medium used in the present study contained 20% newborn bovine serum, inclusion of this agent is not a requirement for worm survival in the *in vitro* assay. Kotze and McClure (2001) maintained active

adult *H. contortus* worms for up to 2·5 days using a medium identical to that described in the present study except with no bovine serum added, indicating that adult worms given no serum proteins can maintain their motility for a period considerably longer than the 24 and 48 h time-points at which toxic effects were apparent in some assays in the present study. Hence, if the tannin in the extracts acted solely by removing the serum proteins from the adult *in vitro* assays in the present study, worm motility would be expected to be unaffected. In contrast, clear decreases in motility were seen in Fig. 9.

The bioassay used for the screening aspect of this study examined the free-living life stage of the parasite during development from the egg to the L3 stage. On the other hand, effective control of existing parasite populations within a sheep requires that the bioactive is toxic towards adult parasites. The limited number of *in vitro* adult worm assays in the present study showed that extracts from several of the plants were indeed toxic to both larval and adult life stages. In another case (*K. eximia*) though, larval activity did not translate into significant adult activity. This translation from larval to adult stages will clearly need to be tested experimentally *in vivo* for the most promising plant species. The use of free-living larvae in the early stages of a bioactive or drug discovery project such as the present one is necessary due to the difficulty and expense associated with obtaining fresh adult worms for regular bioassays. Promising leads may then be examined in assays with adult worms *in vitro*, as done here with several species; however, confirmation of usefulness as an anthelmintic agent only comes with *in vivo* trials. The record of translation of promising *in vitro* anthelmintic activity of plant extracts into useful *in vivo* activity is poor (Athanasiadou and Kyriazakis, 2004; Athanasiadou *et al*. 2007). Host pharmacokinetics can greatly limit the amount of active material that actually reaches the parasite in the intestinal tract. Components of this include absorption into host and excretion via urine, absorption onto digesta material within the gut, host metabolism in the liver, microbial metabolism in the rumen, and pH effects. Hence, while the demonstrated *in vitro* activity against *H. contortus* larval stages and, in a limited number of cases, against adult stages, in the present study is encouraging, *in vivo* experiments are required to judge the real potential of these plants.

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REFERENCES

Akkari, H., Ben Salem, H., Gharbi, M., Abidi, S. and Darghouth, M. A. (2008). Feeding *Acacia cyanophylla* Lindl. foliage to Barbarine lambs with or without PEG: Effect on the excretion of gastro-intestinal nematode eggs. *Animal Feed Science and Technology* **147**, 182–192.

Albers, G. A. A. and Burgess, S. K. (1988). Serial passage of *Haemonchus contortus* in resistant and susceptible sheep. *Veterinary Parasitology* **28**, 303–306.

Assefa, G., Sonder, K., Wink, M., Kijora, C., Steinmueller, N. and Peters, M. J. (2008). Effect of variety and harvesting management on the concentration of tannins and alkaloids in tagasaste (*Chamaecytisus palmensis*). *Animal Feed Science and Technology* **144**, 242–256.

Athanasiadou, S. and Kyriazakis, I. (2004). Plant secondary metabolites: antiparasitic effects and their role in ruminant production systems. *Proceedings of the Nutrition Society* **63**, 631–639.

Athanasiadou, S., Githiori, J. and Kyriazakis, I. (2007). Medicinal plants for helminth parasite control: facts and fiction. *Animal* **1**, 1392–1400.

Barry, T. N. (1989). Condensed tannins: their role in ruminant protein and carbohydrate digestion and possible effects upon the rumen ecosystem. In *The Roles of Protozoa and Fungi in Ruminant Digestion* (ed. Nolan, J. V., Leng, R. A. and Demeyer, D. I.), pp. 153–169. Penambul Books, Armidale, Australia.

Barry, T. N. and Manley, T. R. (1986). Interrelationships between the concentrations of total condensed tannin, free condensed tannin and lignin in *Lotus* sp. and their possible consequences in ruminant nutrition. *Journal of the Science of Food and Agriculture* **37**, 248–254.

Barry, T. N. and McNabb, W. C. (1999). The implications of condensed tannins on the nutritive value of temperate forages fed to ruminants. *British Journal of Nutrition* **81**, 263–272.

Ben Salem, H., Makkar, H. P. S., Nefzauoi, A., Hassayoun, L. and Abidi, S. (2005). Benefit from the association of small amounts of tannin-rich shrub foliage (*Acacia cyanophylla* Lindl.) with soya bean meal given as supplements to Barbarine sheep fed on oaten hay. *Animal Feed Science and Technology* **122**, 173–186.

Besier, R. B. and Love, S. C. J. (2003). Anthelmintic resistance in sheep nematodes in Australia: the need for new approaches. *Australian Journal of Experimental Agriculture* **43**, 1383–1391.

Cenci, F. B., Louvandini, H., McManus, C. M., Dell'Porto, A., Costa, D. M., Araújo, S. C., Minho, A. P. and Abdalla, A. L. (2007). Effects of condensed tannin from *Acacia mearnsii* on sheep infected naturally with gastrointestinal helminthes. *Veterinary Parasitology* **144**, 132–137.

Cheng, S. S., Liu, J. Y., Tsai, K. H., Chen, W. J. and Chang, S. T. (2004). Chemical composition and mosquito larvicidal activity of essential oils from leaves of different *Cinnamomum osmophloeum* provenances. *Journal of Agriculture and Food Chemistry* **52**, 4395–4400.

Coley, P. D., Bryant, J. P. and Chapin, F. S. 3rd. (1985). Resource availability and plant antiherbivore defense. *Science* **230**, 895–899.

Dynes, R. A. and Schlink, A. C. (2002). Livestock potential of Australian species of *Acacia*. *Conservation Science of Western Australia* **4**, 117–124.

Edwards, N. J. (1999). A review of tannins and other secondary metabolites in the fodder shrub tagasaste (*Chamaecytisus proliferus*). In *Tannins in Livestock and Human Nutrition* (ed. Brooker, J. D.), pp. 160–164. Australian Centre for International Agricultural Research, Canberra, Australia.

Ghisalberti, E. L. (1994). The ethnopharmacology and phytochemistry of *Eremophila* species (Myoporaceae). *Journal of Ethnopharmacology* **44**, 1–9.

Gill, J. H., Redwin, J. M., van Wyk, J. A. and Lacey, E. (1995). Avermectin inhibition of larval development in *Haemonchus contortus*–effects of ivermectin resistance. *International Journal for Parasitology* **25**, 463–470.

Hoste, H., Jackson, F., Athanasiadou, S., Thamsborg, S. M. and Hoskin, S. O. (2006). The effects of tannin-rich plants on parasitic nematodes in ruminants. *Trends in Parasitology* **22**, 253–261.

Howard, D., Krebs, G. L. and Van Houtert, M. (2002). The value of *Acacia saligna* as a source of feed for sheep. *Conservation Science of Western Australia* **4**, 135–138.

Hubert, J. and Kerboeuf, D. (1984). A new method for culture of larvae used in diagnosis of ruminant gastrointestinal strongylosis: comparison with fecal cultures. *Canadian Journal of Comparative Medicine* **48**, 63–71.

Kahiya, C., Mukaratirwa, S. and Thamsborg, S. M. (2003). Effects of *Acacia nilotica* and *Acacia karoo* diets on *Haemonchus contortus* infection in goats. *Veterinary Parasitology* **115**, 265–274.

Kotze, A. C. and McClure, S. J. (2001). *Haemonchus contortus* utilises catalase in defence against exogenous hydrogen peroxide *in vitro*. *International Journal for Parasitology* **31**, 1563–1571.

Kozukue, N. and Friedman, M. (2003). Tomatine, chlorophyll, beta-carotene and lycpene content in tomatoes during growth and maturation. *Journal of the Science of Food and Agriculture* **83**, 195–200.

Lattanzio, V., Arpaia, S., Cardinali, A., Di Venere, D. and Linsalata, V. (2000). Role of endogenous flavonoids in resistance mechanism of Vigna to aphids. *Journal of Agriculture and Food Chemistry* **48**, 5316–5320.

van Leur, H., Vet, L. E., van der Putten, W. H. and van Dam, N. M. (2008). *Barbarea vulgaris* glucosinolate phenotypes differentially affect performance and preference of two different species of lepidopteran herbivores. *Journal of Chemical Ecology* **34**, 121–131.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982). *Molecular Cloning; a Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA.

Mathams, R. H. and Sutherland, A. K. (1952). The oxalate content of some Queensland pasture plants. *Queensland Journal of Agricultural Science* **9**, 317–334.

Milgate, J. and Roberts, D. C. K. (1995) The nutritional and biological significance of saponins. *Nutrition Research* **15**, 1223–1249.

Min, B. R., Barry, T. N., Attwood, G. T. and McNabb, W. C. (2003). The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. *Animal Feed Science and Technology* **106**, 3–19.

Nair, R. M., Whittall, A., Revell, D. K., Dowling, K., Hughes, S. J., Craig, A. D. and Auricht, G. C. (2006). Effect of defoliation stress on 2-hydroxy cinnamic acid content at different growth stages in *Melilotus albus* (Medik.). *Australian Journal of Experimental Agriculture* **46**, 1601–1603.

Ndi, C. P., Semple, S. J., Griesser, H. J. and Barton, M. D. (2007*a*). Antimicrobial activity of some Australian plant species from the genus *Eremophila*. *Journal of Basic Microbiology* **47**, 158–164.

Ndi, C. P., Semple, S. J., Griesser, H. J., Pyke, S. M. and Barton, M. D. (2007*b*). Antimicrobial compounds from *Eremophila serrulata*. *Phytochemistry* **68**, 2684–2690.

O'Grady, J. and Kotze, A. C. (2004). *Haemonchus contortus: in vitro* drug screening assays with the adult life stage. *Experimental Parasitology* **106**, 164–172.

Olsen, M. M. and Roseland, C. R. (1991). Induction of the coumarins scopoletin and ayapin in sunflower by insect feeding stress and effects of coumarins on the feeding of sunflower beetle (*Coleoptera, Chrysomelidae*). *Environmental Entomology* **20**, 1166–1172.

Pennacchio, M., Syah, Y. M., Ghisalberti, E. L. and Alexander, E. (1996). Cardioactive compounds from *Eremophila* species. *Journal of Ethnopharmacology* **53**, 21–27.

Revell, D. K., Durmic, Z., Bennell, M., Sweeney, G. C. and Vercoe, P. E. (2008*a*). The *in situ* use of plant mixtures including native shrubs in Australian grazing systems: the potential to capitalise on plant diversity for livestock health and productivity. In *Harvesting Knowledge, Pharming Opportunities* (ed. Skaife, J. F. and Vercoe, P. E.), pp. 36–49. Cambridge University Press, Cambridge, UK.

Revell, D. K., Kotze, A. and Thomas, D. T. (2008*b*). Opportunities to use secondary plant compounds to manage diet selection and gut health of grazing herbivores. *Proceedings of the International Grasslands Congress/International Rangelands Congress Hohhot, China, July 2008*.

Salem, A. Z. M. (2005). Impact of season of harvest on *in vitro* gas production and dry matter degradability of *Acacia saligna* leaves with inoculum from three ruminant species. *Animal Feed Science and Technology* **123/124**, 67–79.

Semple, S. J., Reynolds, G. D., O'Leary, M. C. and Flower, R. L. (1998). Screening of Australian medicinal plants for antiviral activity. *Journal of Ethnopharmacology* **60**, 163–172.

Siskos, E. P., Konstantopoulou, M. A., Masomenos, B. E. and Jervis, M. (2007). Insecticidal activity of *Citrus aurantium* fruit, leaf, and shoot extracts against adult olive fruit flies (Diptera: Tephritidae). *Journal of Economic Entomology* **100**, 1215–1220.

Soliva, C. R., Zeleke, A. B., Clement, C., Hess, H. D., Fievez, V. and Kreuzer, M. (2008). *In vitro*

screening of various tropical foliages, seeds, fruits and medicinal plants for low methane and high ammonia generating potentials in the rumen. *Animal Feed Science and Technology* **147**, 53–71.

Staudt, M. and Lhoutellier, L. (2007). Volatile organic compound emission from hohn oak infested by gypsy moth larvae: evidence for distinct responses in damaged and undamaged leaves. *Tree Physiology* **27**, 1433–1440.

Stepek, G., Behnke, J. M., Buttle, D. J. and Duce, I. R. (2004). Natural plant cysteine proteinases as anthelmintics? *Trends in Parasitology* **20**, 322–327.