A Nematode Larval Motility Inhibition Assay for Screening Plant Extracts and Natural Products

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An *in vitro* nematode larval motility inhibition assay has been developed to screen plant extracts for anthelmintic activity against the sheep parasite *Trichostrongylus colubriformis*. The usefulness of this assay was verified by results for extracts of the liverwort *Plagiochila stephensoniana* and the shrub *Pseudowintera colorata*. The activity of these extracts was due to 4-hydroxy-3′-methoxybibenzyl (**1**) (IC_{50} 0.13 mg/mL) and polygodial (**2**) (IC_{50} 0.07 mg/mL), respectively. Synthetic analogues of **1** displayed enhanced antiparasitic activity. (*Z*)-4-Hydroxy-3′-methoxystilbene (**3**) had an IC_{50} of 0.06 mg/mL. The activity of an extract of the tree *Phyllocladus aspeniifolius* var. *alpinus* was due to the presence of polyphenolics, since treatment of the extract with polyvinylpolypyrrolidone or polyamide removed the activity.

Keywords: *Nematodes; anthelmintics; natural products; assay; plant extracts; Trichostrongylus colubriformis*

INTRODUCTION

Diseases caused by internal parasites of sheep cause significant production losses worldwide and pose a particular threat to the New Zealand sheep and wool industry, a key export sector (Douch, 1990). With nematodes developing resistance to the existing anthelmintics (Prichard, 1990), there is a need for new anthelmintic agents. In the past, natural products have proved a good source of such anthelmintic agents, especially the avermectins (ivermectins), isolated from microorganisms (Campbell et al., 1983). Plants have also provided mankind with many therapeutic agents, including anthelmintics. The anthelmintic activity of plant extracts can arise either from a direct action of a drug on the worms or through induction of gastrointestinal irritation and diarrhea, which causes dislodgement of resident worms. Examples of plant-derived antinematode products are given by Cavier (1973). One plant that has direct anthelmintic activity is *Chenopodium ambrosioides* L. (American wormseed, Jerusalem tea). The active constituent is ascaridole, which *in vitro* causes paralysis and ultimately death of *Ascaris* sp. nematodes (Budavari, 1989). Plants that appear to have indirect anthelmintic action include *Artemisia absinthium* L. (wormwood), which can cause severe gastrointestinal irritation, and *Nicotiana tabacum* L., containing nicotine whose pharmacological action includes promoting evacuation of the bowel, the probable basis of its anthelmintic action (Budavari, 1989).

As part of our program to screen New Zealand plant extracts for natural products with therapeutic potential, we wanted an assay for anthelmintic activity. Several *in vitro* assays have been described for detection of anthelmintic activity (reviewed by Johansen, 1989). Inhibition of nematode larval motility as a method of assessing anthelmintic activity has the advantage of ready availability of nematode larvae (from cultures of feces from infected sheep), extended storage life of the larvae, and relative simplicity of the assay methodology. Some motility assays have the disadvantage that the assessment of motility is subjective (Martin and Le Jambre, 1979; Jurgens et al., 1994). Douch et al. (1983) developed an objective assay for the detection of antiparasitic components in intestinal mucus, based on inhibition of the migration of larvae from agar gels. This assay was subsequently used to ascertain the effects of known anthelmintics such as levamisole, morantel, and ivermectin on larval nematodes (Douch and Morum, 1994). The assay adopted for the present work is based on that described by Rabel et al. (1994).

This paper describes the application of this assay to screening plant extracts for migratory inhibition of infective third stage larvae (L3) of the sheep nematode *Trichostrongylus colubriformis* (Nematoda, Trichostrongylidae), and its use is validated through the successful identification of plant compounds that may serve as lead compounds for future anthelmintic develepement.

MATERIALS AND METHODS

Plant Material. Collection and identification of plant material was made under the supervision of botanists. Voucher samples are available from the Plant Extracts Research Unit collection (codes listed in Table 1). Samples (leaf and stem for higher plants, whole plants for bryophytes) were air-dried (30 °C) and ground.

Preparation of Extract for Assay. Subsamples of ground plant material (5 g) were extracted by shaking with EtOH (95:5 ethanol:H_2O; 50 mL) overnight on an orbital shaker. Extracts were filtered through a plug of cotton wool and stored at −15 °C. For the assay, extracts (1 mL) were dried on a vacuum concentrator (Speedvac Model SC200, Savant Instruments, Farmingdale, NY) and reconstituted in dimethyl sulfoxide (DMSO, 1 mL).

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Table 1. Larval Motility Inhibition Assay Screening Results for Selected Plant Extracts

plant name	collection code	% LMI	SD (% of mean; $n = 3$)
Artemisia absinthium	920504-01	9	20
Nicotiana tabacum	911001-01	3	13
Phyllocladus aspleniifolius var. *alpinus*	910814-39	53	27
Plagiochila stephensoniana	911203-06	62	6
Pseudowintera colorata	920121-12	29	23
Effect of Polyphenolic Screening on Plant Extracts			
Phyllocladus aspleniifolius var. *alpinus*			
nontreated	910814-39	44	20
PVPP-treated	910814-39	9	12
polyamide-treated	910814-39	9	7
Plagiochila stephensoniana			
nontreated	921029-05	34	8
PVPP-treated	921029-05	23	10

Assay Method. The assay procedure was essentially that of Rabel et al. (1994). Basically, infective larvae (L3) of *T. colubriformis*, obtained from fecal cultures of monospecifically infected sheep, were exsheathed in sodium hypochlorite solution (0.025% available chlorine), washed with tap water, and concentrated to 1500 L3/mL in tap water. For assay, reconstituted test samples (10 μL) were diluted with water (90 μL) and larval suspensions (100 μL), made up to 500 μL with phosphate buffered saline (PBS), and then incubated together at 37 °C in 48-well tissue culture plates (Costar, Cambridge, MA). Triplicate samples were run for each extract, and usually 12 extracts were tested simultaneously in one assay. A solvent control and positive controls (6 replicates per control) were included on each plate. Solvent controls contained DMSO at an equivalent concentration to the sample wells (2%), and positive controls contained levamisole at 1 μg/mL. After initial incubation (2 h), samples were quantitatively transferred to test sieves (7 mm i.d. translucent acrylic tube with 20 μm nylon mesh glued to one end) for overnight incubation (16 h) at ambient temperature. After this incubation, test sieves were removed, and Lugol's iodine was added to the wells to stain and kill larvae. The number of L3 that had moved through the sieve was counted under a microscope. The numbers migrating were expressed as percent inhibition of larval migration (LMI) was compared with control samples. Plant extracts were regarded as inhibitory or activating if LMI was outside the 95% confidence interval for the control samples (mean $\pm$ standard deviation $\times$ Z value for 5% significance) or alternatively $> \pm 20\%$ LMI.

Dose−response curves using a dilution series (run in the range of 0.005−1.0 mg/well) were determined for inhibitory extracts and for pure compounds. The dilution series comprised 5−10 steps, depending on the availability of test material. Four replicate samples were run at each concentration beside the control.

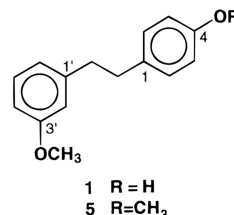
A graphical approach was used for preliminary estimation of the concentration of the sample in the well, which gives 50% inhibition (IC_{50}) as recommended by Finney (1971). SAS Probit analysis (SAS version 6) was undertaken to determine the intervals and fiducial limits for the IC_{50}s. The data for all substances showed heterogeneity that was adjusted for in calculating fiducial limits and is reflected in their wide spread. Fiducial limits could not be calculated for some substances due to large heterogeneity factors coupled with the small number of dose levels tested.

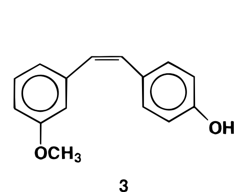
Polyphenolic Screening. *Polyvinylpolypyrrolidone (PVPP).* Extract (2 mL) was added to a test tube containing PVPP (40 mg, Sigma) and shaken on an orbital shaker overnight. After allowing the sample to settle, the supernatant was filtered and assayed along with the original extract.

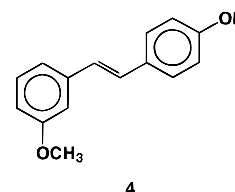
Polyamide. A short polyamide column was prepared using polyamide resin (1 g, Macherey Nagel SC6), pre-swollen overnight in H_2O. Extract (1 mL) was added to the column, and the column was washed with EtOH, MeOH, and MeOH/H_2O (3 mL of each). The combined eluents were evaporated to dryness and reconstituted with EtOH (1 mL) and assayed along with the original extract.

Testing of samples from polyphenolic screening experiments used a slight modification of the above assay method. Samples (extract, compound, or control) were dissolved in DMSO and added directly to the well containing the larvae in PBS. The level of DMSO was maintained at 4% (v/v) per well, and PBS was added to bring the well volume to 500 μL.

Sources of Pure Compounds. 4-Hydroxy-3′-methoxybibenzyl (**1**) was isolated from *Plagiochila stephensoniana* Mitt. (University of Otago Herbarium voucher code OTA 046557), and compounds **3**−**5** were synthesised as previously described (Lorimer et al., 1993).







Polygodial (**2**) was isolated from *Pseudowintera colorata* (Raoul) Dandy (Plant Extracts Research Unit collection 901200-01) as previously described (Gerard et al., 1993).

RESULTS AND DISCUSSION

The first stage of the screening program is the extraction of plant material with ethanol (containing 5% H_2O). This solvent was chosen because it will extract compounds with a wide range of polarities, is volatile, and has relatively low toxicity (Farnsworth, 1990). Rabel et al. (1994) showed that organic solvents inhibited *T. colubriformis* larval motility. Maximum solvent concentrations (v/v) tolerated by larvae in this assay (causing $<10\%$ inhibition) were dimethyl sulfoxide (DMSO) 10%; ethanol 5%; methanol 5%; and propanol $<1\%$. Preliminary tests in which ethanolic plant extracts were dried and then redissolved in saline to avoid any solvent effect on larvae showed substantially lower larval motility inhibition (LMI) than did the same extracts in ethanol or DMSO. This indicated that the active components were not particularly water soluble. For example, the LMI activities of the *Phyllocladus aspleniifolius* var. *alpinus* extract in the different solvents were H_2O 14%, EtOH 56%, and DMSO 53%. DMSO was chosen for the assay due to its greater solvating ability. This is particularly important when using the assay for bioactivity-directed isolations, when fractions with wide ranges of polarities will need to be tested.

An important consideration in selecting an assay for use in a screening program is the amount of sample consumed by the assay process. In this assay, only small amounts of sample (100 μL) are required for the initial screening. The dose−response curves required approximately 15 mg of sample, depending on the type of sample and its activity range.

Results for the screening of selected plant extracts are given in Table 1. Neither wormwood (*Artemisia absinthium*) nor tobacco (*Nicotiana tabacum*) foliage extracts

Table 2. IC_{50} Data for Extracts, Purified Compounds, Analogues, Levamisole, and Piperazine

extract	n^a	IC_{50}^b (mg/mL)	FL_{95}^c
Pl. stephensonia	4	1.9	(0.88, 20.6)
4-hydroxy-3′-methoxybibenzyl (**1**)	4	0.13	(0.10, 0.15)
(*Z*)-4-hydroxy-3′-methoxystilbene (**3**)	4	0.06	(0.02, 0.08)
(*E*)-4-hydroxy-3′-methoxystilbene (**4**)	3	0.07	ND[d]
Ps. colorata	4	3.6	ND
polygodial (**2**)	4	0.07	(0.019, 0.121)
levamisole	3	1.5×10^{-3}	ND
piperazine	6	3.7×10^{-4}	$(1.4 \times 10^{-4}, 9.6 \times 10^{-4})$

[a] *n* is the number of replicates used in the LMI assay itself. [b] Values derived using MiniTab statistical routine. In all cases the slope of the regression line was significant ($P < 0.05$). [c] Fiducial limits to 95% confidence. [d] ND, not able to be determined using this statistical routine.

showed significant LMI (Table 1), as expected from the reported indirect mechanism of anthelmintic action (see above). Some plant extracts including those of *Ph. aspleniifolius* var. *alpinus* and *Ps. colorata* gave very large standard deviations for numbers of larvae migrating in the assay (Table 1). Negative and positive control experiments, which omitted plant extracts, showed coefficients of variation of <10%, suggesting that the large variations between sieves were due to the plant extracts. One possible cause was that some plant extracts, when incubated in the saline environment, threw down precipitates in the sieves. It is likely that such precipitates, by causing blockage of the entire sieve surface, could have inhibited larval migration (Rabel et al., 1994). In other cases, precipitation caused minor sieve blockage but did not result in larval inhibition. Extracts that produced precipitates were re-assayed using a modified procedure where a centrifugation step was added after addition of the extract to PBS, thus removing the precipitate.

The crude extract of *Plagiochila stephensoniana* Mitt. (family Plagiochilaceae) was subjected to dose−response testing, which confirmed its LMI activity and allowed the calculation of an IC_{50} of 1.9 mg/mL (Table 2). Previous work on *Pl. stephensoniana* has shown **1** to be a major component of this extract (about 10%) and to be responsible for its antifungal activity (Lorimer et al., 1993). **1** was active in the LMI assay, with an IC_{50} of 0.13 mg/mL (Table 2). This level of activity could account for the activity of the crude extract. As part of our study of the antifungal activity of **1**, we prepared a number of analogues. These were also tested in the LMI assay, and it was found that (*Z*)-4-hydroxy-3′-methoxystilbene (**3**) and (*E*)-4-hydroxy-3′-methoxystilbene (**4**), were more active than **1** (Table 2). 4,3′-Dimethoxybibenzyl (**5**) showed reduced activity (30% inhibition at 0.6 mg/mL) as compared to **1** (85% inhibition at 0.6 mg/mL). These LMI activities mirror the relative antifungal activities of these compounds (Lorimer et al., 1993). We could find no previous reports of anthelmintic activity for these compounds.

The crude extract of foliage of the shrub *Pseudowintera colorata* (Raoul) Dandy (family Winteraceae) was also subjected to dose−response testing, which confirmed its activity and allowed the calculation of an IC_{50} of 3.6 mg/mL (Table 2). The large heterogeneity that precluded calculation of fiducial limits for the crude extract of *Ps. colorata* may be due to partial removal of the active component at higher concentration by coprecipitation with other compounds in the extract (see above).

Previous work on *Ps. colorata* has shown polygodial (**2**) to be a major component of this extract (about 5%) and to be responsible for its insect antifeedant and antifungal activity (Gerard et al., 1993; McCallion et al., 1982). **2** was active in the LMI assay with an IC_{50} of 0.07 mg/mL (Table 2), suggesting that it is the main active component in the *Ps. colorata* extract. We could find no previous reports of anthelmintic activity associated with **2**.

Extracts from the foliage of the New Zealand tree *Ph. aspleniifolius* var. *alpinus* (Hook. f.) H. Keng (family Podocarpaceae) also showed LMI activity (Table 1). An initial fractionation of an extract from this species did not lead to concentration of the biological activity with either this assay or with antimicrobial assays. Recent reports of the isolation of polyphenolics from this species (Foo et al., 1985; Vilain et al., 1986) led to speculation that these were the source of the activity in the LMI assay. Polyphenolics are often antimicrobial (Scalbert, 1991) and are not easily chromatographed on silica-based stationary phases (Porter, 1989). Recently, two methods of removing polyphenolics from plant extracts have been reported, one using PVPP (Kellam et al., 1992) and the other using polyamide (Cardellina et al., 1993). Both were developed in response to the number of hits (positive responses) caused by polyphenolics in plant extract screening programs. Both methods of polyphenolic removal gave *Ph. aspleniifolius* var. *alpinus* extracts with much reduced LMI activity (Table 1). Of the two methods, the treatment with PVPP is preferred due to its simplicity. The selectivity of the method in removing polyphenolic compounds and not simple phenolics was tested by treating a sample of *Pl. stephensoniana* extract. The activity of this extract has been found to be due to the phenolic bibenzyl (**1**) (see above). Analysis of the treated extract by ^1H NMR showed no loss of bibenzyl, and the treated extract remained active (Table 1). Thus, the proposed PVPP (or polyamide) screening protocol would enable detection of extracts where the activity is due to polyphenolics. The anthelmintic activity of polyphenolics does not appear to have been extensively investigated, although in one report polyphenolics and tannins were associated with activity against plant parasitic nematodes (Taylor and Murant, 1966). These results indicate that further study of the anthelmintic activity of polyphenolics may be useful. However, the main emphasis of this screening program is the discovery of classes of anthelmintic compounds with previously unknown structural features, which we are continuing to pursue.

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