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Analysis of the Antimicrobial Activity of Flavonoids and Saponins Isolated from the Shoots of Oats (*Avena sativa* L.)

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Abstract

Crude extracts from the shoots of oat cv. Quoll were tested against four species of bacteria and eight species of fungi. Bacterial growth was not inhibited. The mycelia growth of all *Pyrenophora* species tested, except *Pyrenophora avenae* DAR 33699, was inhibited by the crude extract, whereas the mycelia growth of *Fusarium graminearum*, *Mycosphaerella pinodes* and *Rhizoctonia solani* was not affected. Partially purified fractions with high concentrations of flavone-C-glycosides had no inhibitory effect against *P. teres* f. sp. *teres* and *P. teres* f. sp. *maculata*. The saponin 26-desglucoavenacoside B accounted for most of the activity against *P. teres* f. sp. *teres* with a minor contribution from the other saponins, 26-desglucoavenacoside A and avenacosides A and B.

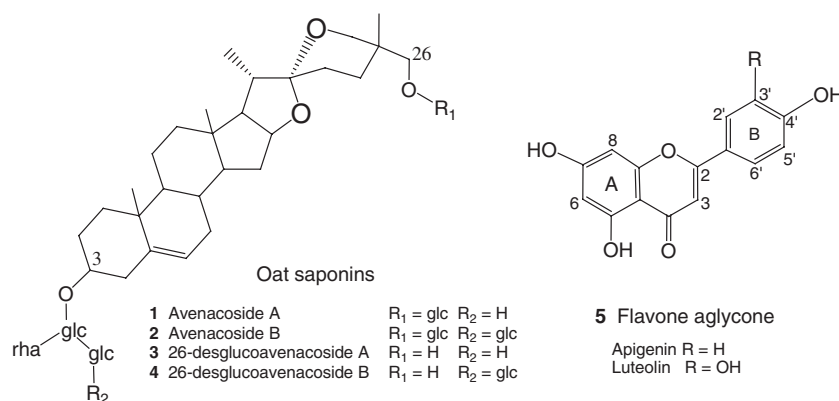
Introduction

Oats (*Avena sativa*) are resistant to a number of important crop diseases, and are grown in crop rotations to limit build up of pathogens (Soriano et al., 2004), such as *Gaeumannomyces graminis* var. *tritici* (Maizel et al., 1964) and *Pyrenophora* sp. (Sebesta

et al., 2001). Growing an oat crop is an effective way to reduce the population density of root lesion nematodes, *Pratylenchus thornei*, (Hollaway, 2002) and alter the soil microflora to suppress pathogens such as *Verticillium* sp. (Konagai et al., 2005).

Two groups of secondary metabolites, saponins and flavonoids, are reported to confer disease resistance in oats (Osbourn, 2003; Osbourn et al., 2003; Soriano et al., 2004). Oat plants contain several saponins including avenacin in the roots, which can inhibit pathogens like *G. graminis* (Crombie and Crombie, 1986; Crombie et al., 1986; Osbourn et al., 1994; Turk, 2005). Oat shoots contain avenacoside A (1) and B (2), which can be converted to the active antifungal compounds, 26-desglucoavenacoside A (26-DGA) (3) and 26-desglucoavenacoside B (26-DGB) (4), when plants are attacked by pathogens (Laudenbach and Kesselmeier, 1982; Wubben et al., 1996).

Flavonoids are a broad group of secondary metabolites with varied and important roles in plant physiology (Martens et al., 2003; Schijlen et al., 2004). Oats contain flavone(5)-C-glycosides that have been reported to have antinematode activity (Soriano et al., 2004). Flavone-C-glycosides are known to help protect



maize (*Zea mays*) against insect damage (Elliger et al., 1980; Lee et al., 1998) and crude extracts from rice leaves with high concentrations of flavone-*C*-glycosides are known to inhibit *Xanthomonas* (Padmavati et al., 1997). Flavone-*C*-glycosides had previously been shown to be present at twofold higher concentrations in oat shoots compared to roots (Soriano et al., 2004).

There are many economically important fungal and bacterial diseases of wheat, barley and pea (e.g. *Pyrenophora* sp., *Fusarium* sp., *Rhizoctonia solani* and *Mycosphaerella pinodes*) and the use of fungicides or bactericides for their control is not always economical and/or desirable. It is not known if flavonoids from oats are effective against any of the major crop pests. This paper investigates the potential of oat compounds to limit the growth of bacteria and fungi *in vitro*. These results suggest a potential mechanism for the beneficial use of oat as a rotation crop.

Materials and Methods

All chemicals unless otherwise stated were obtained from Sigma-Aldrich (St Louis, MO, USA).

Plant material and culture methods

Avena sativa cv. Quoll was obtained from the oat Breeding Group, South Australian Research and Development Institute (SARDI). A few seeds were sown into a pot containing steam pasteurized (65°C for 45 min) University of California (UC) mix. Osmocote (Scotts, Australia), slow release fertilizer, was included at the time of sowing. Plants received Hoagland's solution every other day and tap water on alternate days. Plants were grown in a growth chamber, 85% relative humidity, 22°C light/18°C dark on 12 h cycle, 68 $\mu\text{mol}/\text{m}^2/\text{s}$. Plants were harvested after 3 weeks.

Extraction of bioactive compounds of oat plant

Bioactive compounds were extracted from *A. sativa* cv. Quoll shoots based on the method published by Soriano et al. (2004). Briefly, samples stored at -80°C were freeze-dried and ground to a fine powder. Sixty milligrams of ground sample was extracted with 10 ml methanol [high-performance liquid chromatography (HPLC) grade] by shaking for 48 h at 25°C. Three millilitres of water was added to 7 ml of the methanolic extract and lipids were removed with 10 ml n-hexane shaking for 48 h at 25°C. Eight millilitres of the methanolic phase was evaporated. The residue was dissolved in 5 ml water, 5 ml of 1-butanol (HPLC grade) was added and the mixture was shaken for 24 h at 25°C. A 4 ml aliquot of the resulting butanol phase was dried under vacuum. The final residue was dissolved in 0.4 ml 45% v/v methanol in water immediately prior to HPLC analysis.

Fractionation by high voltage paper electrophoresis

Crude extracts of shoots were fractionated by preparative high voltage paper electrophoresis (HVPE). Shoot extracts (300 μl) were applied to cellulose paper

(115 $\times$ 285 mm; 1 Chr; Whatman, Florham Park, NJ, USA) placed over a glass rod to minimize surface contact between the two wells of the electrophoresis tank (Model 715; Bethesda Research Laboratories, Gaithersburg, MD, USA) containing 0.1 M borate buffer, pH 10 (Smith, 1969). Electrophoresis was performed for 45 min at 300 V. Fractions were identified under UV light at 254 and 366 nm. Relative mobility was estimated using Orange G as an anionic standard and 2'-deoxyadenosine as a neutral standard. Bands were excised and fractions were recovered by eluting with 45% methanol. After drying and re-suspending in water, fractions were passed through a C18 column (SepPak Classic; Waters Corporation, Milford, MA, USA) to remove the borate buffer. The samples were dried again and re-dissolved in 500 μl 45% methanol. Samples were analysed by HPLC and 50 μl of each was assayed against different fungi for biological activity.

Isolation of saponins

The shoots of oat plants were ground and extracted using methanol, hexane, butanol and water as described for flavonoids. The aqueous phase of each extract was loaded onto a 0.5 g RP-C18 preconditioned column (Waters Corporation) and the bound material was sequentially eluted with 20 ml MQ water, then 11 $\times$ 8 ml of 40% methanol and finally with 4 $\times$ 8 ml of 60% methanol. The butanol phase, after drying, was re-dissolved in water and loaded onto a 10 g RP-C18 column (Waters Corporation) and fractionated using 0%, 40% and 60% methanol. The 60% methanol fraction was further purified using a 160 $\times$ 20 mm normal phase column (silica gel 60, Merck KGA, Darmstadt, Germany) and eluted with chloroform : methanol (4 : 1 v/v). Alternatively, the 60% fraction was separated by preparative thin layer chromatography (TLC) using aluminium-backed plates (0.20 mm, silica 60, Merck KGA) with chloroform : methanol : water (140 : 70 : 11 v/v/v) as the solvent. The fractions were analysed by TLC and fractions with similar R_f relative to front, were pooled and analysed by HPLC-mass spectrometry (LC-MS). Purified saponins obtained from column separation and preparative TLC after confirming their purification by TLC were pooled and tested in the bioassay. Three replicates of 400 μg per disc of purified saponins were tested against *P. teres* f. sp. *teres* in the bioassay.

Thin layer chromatography

Saponins were analysed by TLC, with chloroform : methanol : water (140 : 70 : 11 v/v/v) as the solvent, on aluminium-backed silica gel 60 plates (Merck KGA). The plates were stained with *p*-anisaldehyde solution (Wubben et al., 1996) and baked at 130°C.

High-performance liquid chromatography

Separation was achieved on a C18 column (Platinum EPSC18 100A, 7 $\times$ 53 mm; Alltech Associates Inc., Deerfield, IL, USA) by gradient elution, at a flow rate of 0.5 ml/min at 55°C. Starting with 100% Solvent A

(2.5% formic acid), Solvent B (1% formic acid : 60% acetonitrile : 39% water) was increased from 0% to 40% over 18 min, subsequently from 40% to 100% over 3 min, and finally 100% Solvent B for 1 min. Compounds were monitored at both 254 and 340 nm and quantified at 340 nm using a rutin standard.

Liquid chromatographic mass spectrometry

Mass spectrometry was carried out with an API-300 triple quadrupole mass spectrometer equipped with an electrospray (ES) ion source (MDS-Sciex, Concord, ON, Canada). Compounds in the active fractions of the crude extract were separated using a reversed-phase column (Synergi Hydro-RP Phenomenex, Torrance, CA, USA; 4 μ m, 150 $\times$ 2 mm) with a flow rate of 180 μ l/min for 60 min and an injection volume of 20 μ l in 90% Solvent A (5% formic acid in water) and 10% Solvent B (formic acid : acetonitrile : water; 5 : 80 : 15 v/v/v). Gradient elution was performed by increasing from 10% to 35% Solvent B over 35 min, then 35% to 60% over 15 min, held steady for 6 min and then increasing to 100% Solvent B over 4 min. The mass spectrometer was operated in either positive or negative ion scan mode and scanned from m/z 100 to m/z 2000 with a step size 0.2 and dwell time of 0.25 ms. The ES needle, orifice and ring potentials were set at 5000 V, 30 V and 250 V, respectively. The curtain (nitrogen) and nebulizer (air) gases were set at 8 and 12 units, respectively. The samples were monitored using an UV-vis detector (HP 1100, Agilent, Santa Clara, CA, USA) at 254 and 340 nm. The mass spectral data were processed using BIO-MULTIVIEW (Version 1.2 β 3, PE Sciex, Foster City, CA, USA) and ANALYST (Version 1.4) software.

For improved identification of the purified saponins, the elution gradient for LC-MS was modified as follows, hold at 10% Solvent B for 5 min, increasing to 100% Solvent B from 5 to 35 min, and hold at 100% Solvent B for 30 min. The samples were monitored at 200 and 210 nm.

Microbial culture methods

A 5 mm in diameter plug of each fungus (*Fusarium graminearum*, *Rhizoctonia solani*, *Pyrenophora teres* f. sp. *teres*, *P. teres* f. sp. *maculata* and *P. avenae*) was transferred to potato dextrose agar (PDA) media (DifcoTM; Bacto Laboratories Pty. Ltd., Liverpool, Australia) and incubated at 25°C in the dark until mycelia were approximately 25 mm from the edge of the plate. *Mycosphaerella pinodes* was grown on PDA and incubated at 25°C, with 12 h light/dark cycle. *Pyrenophora tritici-repentis* was grown on V8 medium (Cheong et al., 2004) and incubated at 25°C in dark conditions.

To culture bacteria, soft buffer agar (SBA; 0.2 M NaH_2PO_4 , 0.2 M Na_2HPO_4 and 7 g/l agar adjusted to pH 7) held at approximately 60°C was inoculated and well mixed with 300 μ l of suspension from a 5-day-old broth culture of bacteria. The concentration of bacteria was adjusted to 5×10^8 cells/ml using a haemocytometer and the optical density at 600 nm (OD600)

was recorded. Suspensions of *Pseudomonas* in SBA (5 ml) were overlaid on Kings B medium (20 g Bacto Proteose Peptone No. 3, 1.5 g $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 g glycerol and 20 g Bacto agar per litre H_2O adjusted to pH 7.2) and suspensions of *Rathyibacter* and 'Corynebacterium' on M523 medium (10 g sucrose, 2 g casamino acids, yeast extracts, 2 g K_2HPO_4 , 0.3 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 15 g agar per litre H_2O) to give a uniform overlay.

Bioassays

Fungal bioassays were performed using autoclaved filter paper discs (6 mm in diameter). Each disc was loaded with 5×10 μ l of the crude shoot extract. The discs were dried between each application. Loaded paper discs were placed on the medium approximately 15 mm from the margin of the growing mycelia and 10 mm from the edge of the plate. Plates were incubated at 25°C and the radius zone of inhibition (distance between the centre of the paper disc and margin of the inhibited mycelium) was recorded.

Bacteria were analysed using a similar method. A loaded paper disc was prepared as described above and placed on SBA inoculated with the respective bacteria. The plate was inverted and incubated at 25°C for approximately 3 days and the diameter of the inhibition zone was recorded.

Results and Discussion

Of the seven genera of pathogens tested (Table 1) only *Pyrenophora* species were inhibited by methanolic crude extract of oats containing flavone-C-glycosides (5). *Pyrenophora avenae* DAR 33699, was the only *Phyrenphora* species in this study whose growth was not affected by the crude shoot extract. Generally, *P. avenae* is regarded as a compatible pathogen of oats. However, another isolate of *P. avenae*, DAR32137, was inhibited by the crude shoot extract. Our results showing that *P. avenae* has different responses to oat shoot extracts is consistent with other work showing that selected oat cultivars are resistant to *P. avenae* (Sebesta et al., 2001). Pigmentation and conidia formation were induced in *Mycosphaerella pinodes* by the oat extracts. The pigmentation was first visible at 24 h after addition of discs and by day 3 a strong black colour was observed (data not shown).

The crude methanol extract of oat shoots was separated into 10 fractions according to relative mobility by HVPE and monitored at 254 nm (UV) and fluorescence at 366 nm. The λ_{max} of each HVPE fraction helped distinguish between different fractions (Table 2) and the concentration of flavone(5)-C-glycosides was estimated by HPLC (Fig. 1). Fractions 6 and 8 had the highest concentration of flavone-C-glycosides and other fractions contained negligible flavone-C-glycosides (Table 2). Fraction 6 contained 0.713 mg (rutin eq.)/g dry weight mainly as *O*-methyl-apigenin-C-deoxyhexoside while fraction 8 contained 1.344 mg (rutin eq.)/g dry weight, principally as apigenin-C-hexoside-*O*-pentoside.

Organism	Isolate/strain	Source	Origin	Crude extract inhibition
<i>Pyrenophora avenae</i>	DAR 33699	<i>Avena sativa</i>	OAI	No
<i>P. avenae</i>	DAR 32137	<i>Avena sativa</i>	OAI	Yes
<i>P. teres</i> f. sp. <i>teres</i>	43961	<i>Hordeum sativum</i>	SARDI	Yes
<i>P. teres</i> f. sp. <i>maculata</i>	1998	<i>Hordeum sativum</i>	SARDI	Yes
<i>P. tritici-repentis</i>	Mar04	<i>Triticum aestivum</i>	SARDI	Yes
<i>Fusarium graminearum</i>		<i>Triticum aestivum</i>	SARDI	No
<i>Rhizoctonia solani</i>	317		SARDI	No
<i>Mycosphaerella pinodes</i>	1201	<i>Pisum sativum</i>	SARDI	Minor
<i>Pseudomonas syringae</i> pv. <i>syringae</i>	CS201	<i>Pisum sativum</i>	SARDI	No
<i>P. syringae</i> pv. <i>pisi</i>	CS200	<i>Pisum sativum</i>	SARDI	No
<i>Rathayibacter tritici</i>	CS21	<i>Triticum aestivum</i>	SARDI	No
' <i>Corynebacterium agropyri</i> '	CS35	<i>Agropyron smithii</i>	SARDI	No

OAI, Orange Agricultural Institute, NSW, Australia; SARDI, South Australian Research and Development Institute, SA, Australia.

Table 2
Characteristics of fractions from oat cv. Quoll shoots fractionated by HVPE

HVPE fraction	Relative mobility	$\lambda_{\max}$	Flavone-C-glycoside mg (rutin eq.)ml ^a
1	0.00	257.8	nd ^b
2	0.10	204.8, 272.6	0.02
3	0.16	206.2	0.025
4	0.22	207.8	nd
5	0.26	206.1	nd
6	0.36	197.7, 256.7, 331	0.713
7	0.56	194.9, 261.6	0.013
8	0.67	204.6, 267, 350.6	1.344
9	0.81	195.2, 267.4	0.011
10	1.00	nd	nd
Crude			2.505

^aFlavone-C-glycosides were quantified at 340 nm.
nd, not detected; HVPE, high voltage paper electrophoresis.

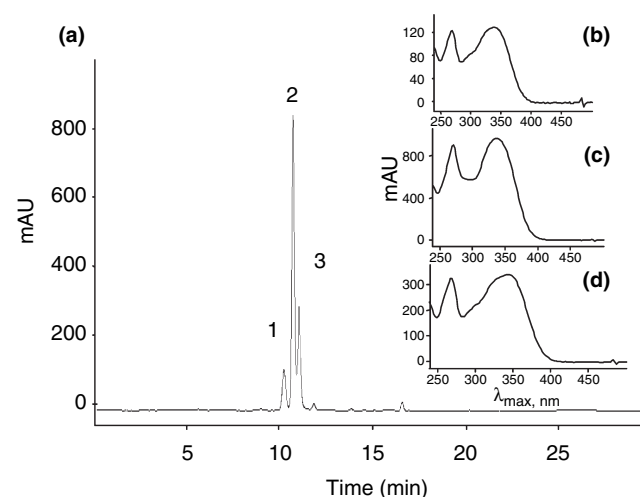


Fig. 1 High-performance liquid chromatography chromatogram and absorbance spectra of the flavonoids in oat (*Avena sativa*) shoots. (a) Flavonoids can be separated into three major peaks using gradient elution with formic acid as Solvent A and formic acid : acetonitrile : water as Solvent B; peaks 1, 2, 3 contain luteolin-C-hexoside-O-pentoside, apigenin-C-hexoside-O-pentoside and O-methyl-apigenin-C-deoxyhexoside-O-hexoside, respectively, based on the absorbance spectra (b, c and d) that are consistent with these previously characterized flavone-C-glycosides (Soriano et al., 2004)

Table 1
List of fungi and bacteria and effect of oat shoots crude extract

High voltage paper electrophoresis fractions 3, 4 and 5 had an inhibitory effect on the mycelia growth of *P. teres* f. sp. *teres* (Fig. 2). These fractions contained low or negligible flavone-C-glycosides (Table 2). Fractions 6 and 8, with the highest concentrations of flavone-C-glycosides, had only a minor inhibitory effect suggesting that they were not responsible for the inhibition observed in the crude extract.

Two of the three active fractions (fractions 3 and 4) that were separated and partially purified by HVPE (Table 2) were further analysed by LC-MS. HPLC chromatograms showed that most of the material eluted as a series of peaks between retention times (R_t) 47 and 50 min. Based on the mass and ionization patterns, these compounds were identified as saponins (Wubben et al., 1996).

Saponins were purified from 3-week-old oat cv. Quoll plants from either aqueous phase (Fig. 3a) or butanol phase (Fig. 3b). The aqueous phase of the extract was fractionated by C18 column using elution with 40% and 60% methanol to give two fractions. These fractions were analysed by TLC and the saponins visualized with the *p*-anisaldehyde stain (Hostettmann and Marston, 1995). The 60% methanol fraction contained two *p*-anisaldehyde-positive bands, whereas the 40% methanol fraction contained only one band (Fig. 3a). The single band of the 40% methanol had a neutral mass of 1225.3. The positive ion mass spectrum ionization pattern of this compound was: m/z 1226.2 ($M + H^+$), 1063.8 ($M + H^+ - \text{hexose}$), 901.8 ($M + H^+ - 2 \times \text{hexose}$), 739.6 ($M + H^+ - 3 \times \text{hexose}$), 593.8 ($M + H^+ - 3 \times \text{hexose-deoxyhexose}$), 431.0 ($M + H^+ - 4 \times \text{hexose-deoxyhexose}$) and 413.2 ($M + H^+ - 4 \times \text{hexose-deoxyhexose-water}$) (Fig. 3c). Based on these LC-MS data and TLC data this compound was identified as avenacoside B (2) ($C_{57}H_{92}O_{28}$). The minor band eluted by 60% methanol was not of sufficient purity for identification. This band, however, was later isolated and fractionated from the butanol phase.

The fractions obtained from the butanol phase were separated and partially identified by TLC as four *p*-anisaldehyde-positive saponins; compound 1 (R_f 0.50),

Fig. 2 Inhibitory effect of high voltage paper electrophoresis derived fractions on the growth of *Pyrenophora teres* f. sp. *teres*. Forty-five percentage of methanol was used as a control. Each mean was calculated from three replicates. Bars represent the standard error

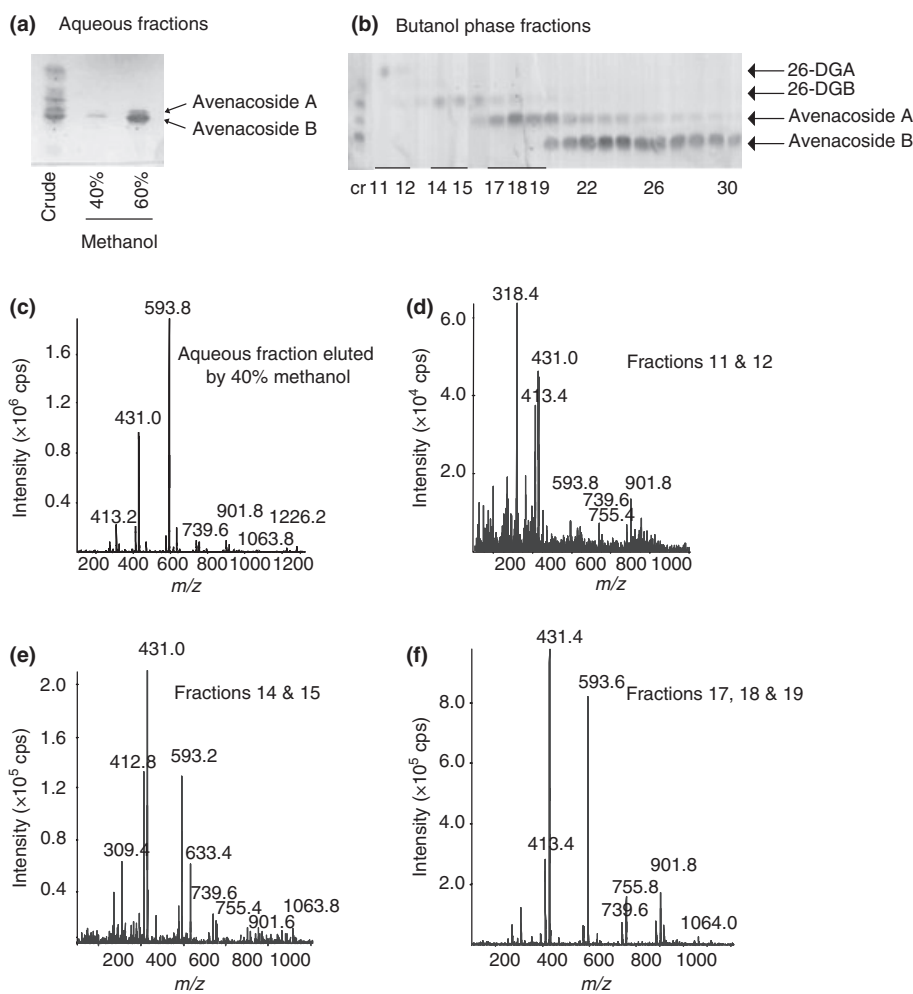
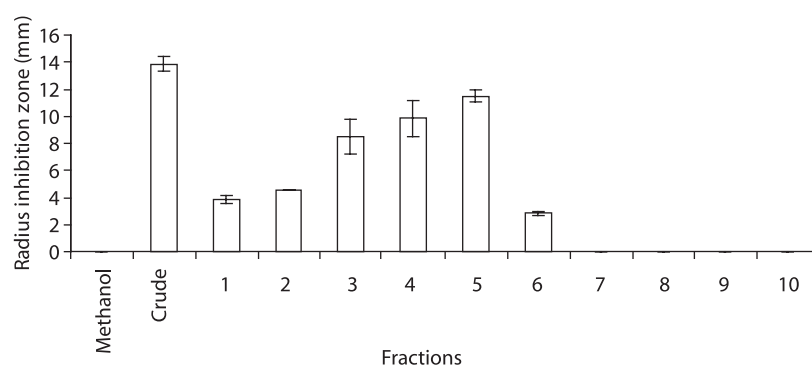


Fig. 3 Separation and identification of compounds isolated from the aqueous and butanol phases of shoot extracts of *Avena sativa* cv. Quoll. (a) Thin layer chromatography (TLC) separation of two fractions obtained from RP-C18 separation of the aqueous phase from the methanolic extract. The two fractions were eluted by 40% and 60% methanol, respectively. (b) TLC analysis of saponins from the purification of the compounds of butanol phase. (c) Positive ion mass spectrometry (MS) of avenacoside B (2) purified from the aqueous phase. (d) MS (positive mode) of 26-DGA (3) isolated from fractions 11 and 12 of the butanol phase, (e) MS (positive mode) of 26-DGB (4) isolated from fractions 14 and 15 of the butanol phase, (f) MS (positive mode) of avenacoside A (1) isolated from fractions 17, 18 and 19 of the butanol phase

compound 2 (R_f 0.40), compound 3 (R_f 0.31) and compound 4 (R_f 0.22) (Fig. 3b). The identity of each of the purified saponins was established according to LC-MS data and published TLC profiles of saponins (Wubben et al., 1996). Compound 1 was identified as 26-DGA (3) ($C_{45}H_{72}O_{18}$), with positive ion masses at m/z 901.8 ($M + H^+$), m/z 739.6 ($M + H^+$ -hexose), 593.8 ($M + H^+$ -hexose-deoxyhexose), 431.6 ($M + H^+$ -2 $\times$ hexose-deoxyhexose), 413.4 ($M + H^+$ -2 $\times$ hexose-deoxyhexose-water) and 318.4 (Fig. 3d). Compound 2 was recognized as 26-DGB (4) ($C_{51}H_{82}O_{23}$), with ions at m/z 1063.8 ($M + H^+$), 901.6 ($M + H^+$ -hexose),

739.6 ($M + H^+$ -2 $\times$ hexose), 633.0, 593.2 ($M + H^+$ -2 $\times$ hexose-deoxyhexose), 431.0 ($M + H^+$ -3 $\times$ hexose-deoxyhexose) and 412.8 ($M + H^+$ -3 $\times$ hexose-deoxyhexose-water) (Fig. 3e). Compound 3 was identified as avenacoside A (1) ($C_{51}H_{82}O_{23}$), with ions at m/z 1064.0 ($M + H^+$), 901.8 ($M + H^+$ -hexose), 739.6 ($M + H^+$ -2 $\times$ hexose), 593.6 ($M + H^+$ -2 $\times$ hexose-deoxyhexose), 431.4 ($M + H^+$ -3 $\times$ hexose-deoxyhexose), 413.4 ($M + H^+$ -3 $\times$ hexose-deoxyhexose-water) (Fig. 3f). Compound 4 (R_f 0.22) was previously identified in the aqueous phase as avenacoside B (2). The mass spectra of compounds 1, 2, 3 and 4 in negat-

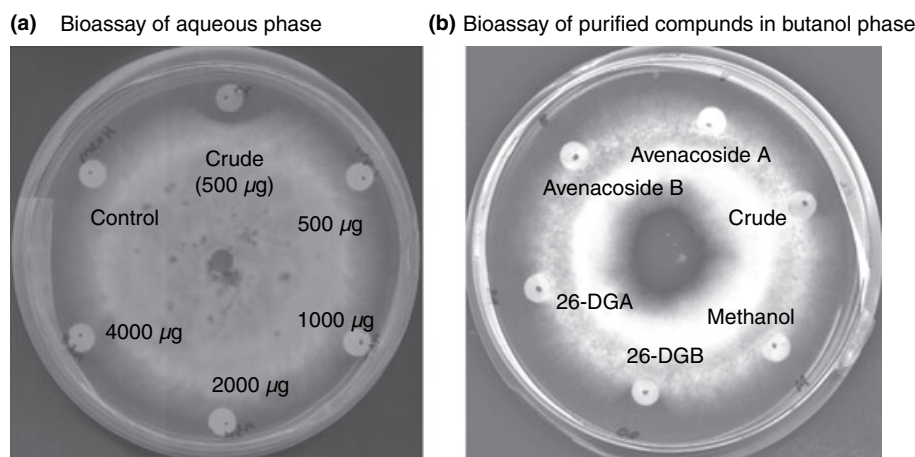


Fig. 4 Bioassay of saponins from oat cv. Quoll shoot extracts against *Pyrenophora teres* f. sp. *teres*. (a) Bioassay with 500, 1000, 2000 and 4000 μg (50 μl of 10, 20, 40 and 80 $\mu\text{g}/\mu\text{l}$) of the aqueous phase extract from shoot and 45% methanol as control. (b) Bioassay of purified compounds from the butanol phase of shoot extract that were obtained from silica gel column followed by preparative thin layer chromatography; 40 μl avenacoside A, avenacoside B, 26-DGA and 26-DGB (each 10 $\mu\text{g}/\mu\text{l}$ in 45% methanol) to give total 400 μg . Crude extract (400 μg) was used as a positive control and 45% methanol as a negative control

ive mode produced parent ions at m/z 900.0, 1062.0, 1062.2, 1224.2 ($\text{M} - \text{H}^+$), respectively, and no fragmentation was observed.

When the crude aqueous phase extracts, containing only two of the four saponins [avenacoside A (1) and avenacoside B (2)], were used against *P. teres* f. sp. *teres*, no inhibition was observed (Fig. 4a). However, when each of the four purified saponins (from the butanol phase) was tested separately, 26-DGB (4) exhibited greatest inhibition of *Pyrenophora* mycelia (Fig. 4b). 26-DGA (3) and avenacosides A (1) and B (2) showed minor inhibition of the mycelia growth. The activity of 26-DGB (4) was consistent with previous reports showing that 26-DGA (3) and 26-DGB (4) are active antifungal compounds released by deglycosylation of avenacoside A (1) and B (2) in infested leaves (Wubben et al., 1996).

The specificity of the flavone-*C*-glycoside, *O*-methylapigenin-*C*-deoxyhexoside-*O*-hexoside (Soriano et al., 2004) as an antinematode compound and the saponin, 26-DGB (4) as an anti-*Pyrenophora* fungicide, suggests these compounds can be used to target particular organisms. However, this specificity also presents challenges for the oat breeder who is interested in controlling a broad range of pathogens. Oats also contains other biologically important compounds such as the phytoalexins, avenanthramides (hydroxycinnamic acid amide) which are produced in oat shoots in response to fungal attack (Mayama et al., 1981; Miyagawa et al., 1996; Okazaki et al., 2004). It is not known whether these compounds have a broad range of activity or are highly specific like the saponins and flavone-*C*-glycosides.

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