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Chemical Defenses of *Trifolium glanduliferum* against Redlegged Earth Mite *Halotydeus destructor*

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Trifolium glanduliferum Boiss. was resistant to redlegged earth mite, *Halotydeus destructor* Tucker (Acari: Penthaleidae), feeding compared with *Trifolium subterraneum* L. Extracts from *T. glanduliferum* leaves, obtained with dichloromethane and methanol, acted as feeding deterrents to the redlegged earth mite. Bioassay-directed fractionation of the extracts led to the isolation of several metabolites, coumarin, α-and β-ionone, linalool, medicarpin, that were shown to be deterrent in laboratory experiments. The compounds contributing to the defense of *T. glanduliferum* were different from those influencing mite feeding on *T. subterraneum* leaves.

KEYWORDS: ***Trifolium glanduliferum*; leaves; *Halotydeus destructor*; plant resistance; deterrent activity; coumarin; α- and β-ionone**

INTRODUCTION

The redlegged earth mite, *Halotydeus destructor* Tucker (Acari: Penthaleidae), is a major pest of pastures and crop legumes in southern Australia. The productivity of subterranean clover (subclover, *Trifolium subterraneum* L.), the most commonly used pasture legume in Australia, is declining in southern Australia due, in part, to the effects of the redlegged earth mite. The adoption of new species resistant to the mite is a priority, and alternative pasture legume species have shown some promise. In particular, mature plants of *Trifolium glanduliferum* Boiss. have been shown to be resistant to mites under laboratory and field conditions (B. Nutt, unpublished data). Mite feeding damage scores from screening young plants show *T. glanduliferum* to be very resistant with breeding line 87182 slightly more resistant (damage 1.0 after 14 days) than 87181 (2.0 after 14 days), compared with *T. subterraneum* (damage 5.0 after 14 days) and *Trifolium nigrescens* (10.0 after 14 days), which is most susceptible (K. Gadja and D. Gillespie, unpublished data; methods in ref *1*). In previous work, we have shown that volatile compounds present in the leaves of *T. glanduliferum* caused deterrence of mites, and bioactivity was observed for coumarin and β-ionone (*2*).

The aim of the present work was to use bioassay-guided fractionation to isolate and identify the relative importance of different metabolites in the trifoliate leaves of *T. glanduliferum* in deterring mite feeding.

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MATERIALS AND METHODS

Screening Plants against Redlegged Earth Mites. Feeding damage by *H. destructor* to mature plants was measured in glasshouse pot experiments. Ten seeds of each species were planted in plastic pots 10 cm in diameter containing soil, and plants thinned down to five per pot. There were three species, *Trifolium glanduliferum* (87182), *T. subterraneum* cv Dalkeith, and *T. vesiculosum* Savi cv Cefalu, and 10 replicate pots of each species. Redlegged earth mites were field-collected, and 100 older mites were added per pot. Seeding was staggered to give plants the same size when mites were added so that Cefalu and variety Prima were 71 days old at the start and Dalkeith 63 days old. There were two experiments, and mites were left to feed for 28 days in 2001 and for 35 days in 2002, and then the mites were removed and feeding damage scores estimated for each pot, where 0 = no damage visible, 1 = 10% damaged, and 10 = 100% damaged. Analyses of variance were carried out using Statistix 8.0 and means compared with a Tukey HSD comparison test. A more detailed description of methods is given by Ridsdill-Smith (*3*).

Redlegged Earth Mite. Mites were collected from pasture near Perth in winter and were cultured in the laboratory on vetch (*Vicia sativa* cv Blanchefleur; Leguminosae) in summer. Mites were separated into developmental stages in the laboratory, and young adults, starved in a humid vial (15 °C) for 2 h each before experiment, were used (*4*).

Plant Material. For extraction studies, mature (3 month old), field grown *T. glanduliferum* (semierect, ~40 cm) of resistant lines (CPI 87812 and CPI 87181) were collected from the University of Western Australia field station at Shenton Park, Perth, in 1997 and 1998.

Assay for Feeding Deterrence. Extracts from leaves and flowers, obtained as described below, and pure samples of the compounds isolated were bioassayed for feeding deterrence toward redlegged earth mites using a membrane sachet technique (*4a*, *4b*). Mites were given a choice of a sachet containing a feeding stimulant (1% aqueous glucose) with or without the test samples. Membrane sachets were made with stretched Parafilm. For each sachet, a membrane was stretched over a 2 cm diameter plastic ring, 35 μL of test solution added, and a

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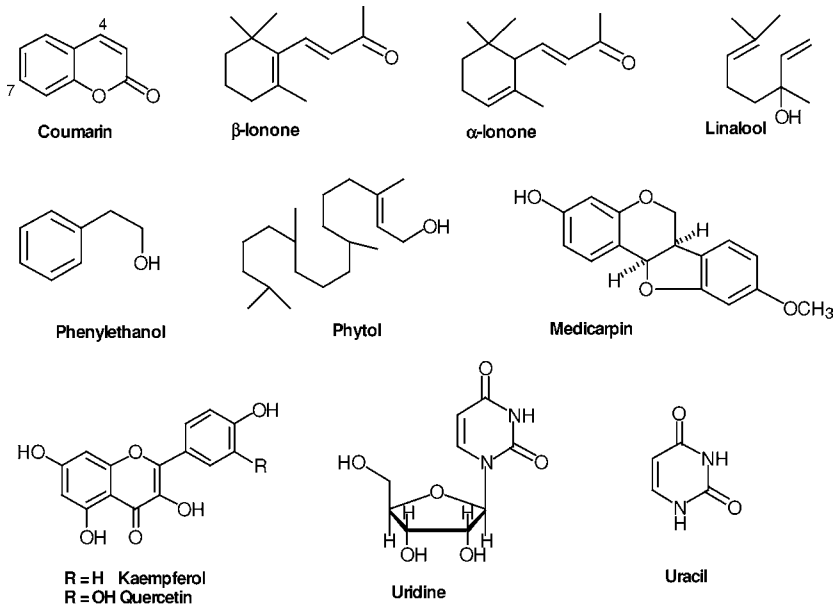

Figure 1. Compounds isolated from *T. glanduliferum.*

second membrane stretched over the top. In choice experiments, two membranes were buried, nearly touching, so as to be just above the surface of the soil (4:1 sand to loam) that was moistened close to field capacity, in a three-quarter filled plastic jar. Twenty mites were released by gently tapping them from a vial onto the soil beside the membrane sachets in the plastic jar, which was then sealed with stretched Parafilm to contain the mites. There were 10 replicates for each experiment. Experiments were carried out at 15 °C under fluorescent light in a room with no natural light. Mites were observed through the Parafilm membrane, and the number of mites on each membrane sachet was counted at 20 or 30 min intervals for 3 h. Not all mites were on sachets. The average number of mites on a sachet gives an index of the amount of mite feeding over 3 h (*4*). Deterrence in the choice test for each experiment was calculated from the average of seven observations as follows: [(mite number on control − mite number on treatment)/total number of mites on sachets] × 100. A positive value implies deterrence. The mean of 10 replicates during 3 h is presented. Differences between means were examined with paired *t* tests.

General Experimental Methods. ^{1}H and ^{13}C NMR spectra were obtained for $CDOD_3$, acetone-d_6, and DMSO-d_6 or D_2O solutions using a Bruker ARX 500 instrument operating at 500.1 and 125.8 MHz. Chemical shifts (δ) were reported in ppm and coupling constants (J) in Hz. Mass spectrometry was carried out using a VG Autospec mass spectrometer (70 eV). Flash column chromatography was performed using 40−60 μm Si gel (BDH) and silica gel 100 C18 reversed phase (Merck) as the stationary phase. For TLC, precoated AL SIL G/UV (Whatman) plates were used and compounds were detected by spraying with a solution of $CeSO_4$ in concentrated H_2SO_4.

Gas Chromatography (GC) and Gas Chromatography−Mass Spectrometry (GC−MS). The presence of coumarin, α-ionone, β-ionone, phenylethanol, and phytol in fractions obtained by chromatography of the extract (see below) was determined by gas chromatography−mass spectrometry (GC−MS) and gas chromatography (GC) using an HP 5790 GC instrument, equipped with an Innowax column (0.24 μm film thickness, 25 m × 0.35 mm i.d.) and helium as the carrier gas at a flow rate of 1.0 mg/min. The injector and FID temperatures were kept at 250 °C, and the oven temperature was programmed from 40 °C (isothermal for 2 min) to 230 °C at a rate of 10 °C/min. Injections of 1 μL were performed. For GC−MS an HP5986 instrument was used. The column and temperatures of oven and injector are the same as those in GC. The auxiliary temperature was 230 °C. The electron impact mass spectra were recorded at 70 eV ionization energy and at a scan mode covering a mass range (m/z) of 50−500 amu. The identifications were made by GC−MS and were confirmed by comparison of the mass spectral data and retention time to those of authentic samples and coinjection of authorized samples and plant sample. Using GC, the amounts of coumarin, β-ionone, and phytol were determined from standard curves of the appropriate compounds at five concentrations using 7-methoxycoumarin as the internal standard.

High Performance Liquid Chromatography Quantification. The concentrations of medicarpin were determined by high performance liquid chromatography (HPLC) using a Waters 600E HPLC instrument, equipped with an Econosil C18 Micron column (5 mm, 250 × 4.6 mm i.d.). The photodiode assay detector was set at 220, 260, 320 nm. The mobile phase used (1.0 mL/min) was 25 mM KH_2PO_4 (pH 3.25) (A) and acetonitrile (B) in the following gradient: 15% B in A increasing linearly to 25% B over 40 min, and then linearly increasing to 65% B over 20 min, being maintained for 12 min at 65% B. The concentrations of analytes in the extracts were determined from standard curves of the appropriate compounds at five concentrations using flavone as internal standard.

Extraction and Isolation of Metabolites. The bioassay technique described above was used to guide the extraction and fractionation of the extracts. Leaves of *T. glanduliferum* have prominent glands, and it might be expected that bioactive metabolites are present on the leaf surface. To obtain the surface components, leaves or flowers of lines 87182 and 87181 were dipped for 10 s in hexane, dichloromethane, or methanol. Evaporation of the solvent afforded extracts, which were used for the bioassay. Total extracts were obtained as follows. Fresh leaves (180 g fresh wt) or flowers (210 g fresh wt) were powdered in liquid nitrogen and extracted sequentially with CH_2Cl_2 (×3) and MeOH (×3). The combined CH_2Cl_2 or MeOH solutions were concentrated under reduced pressure to afford the extracts. Typical amounts obtained were as follows: CH_2Cl_2 extract from leaves, 18 mg/g fresh wt; from flowers, 5.7 mg/g fresh wt; MeOH extract from leaves, 50 mg/g fresh wt; from flowers, 49 mg/g fresh wt. The CH_2Cl_2 extract (2.8 g) combined with CH_2Cl_2 fractions from methanol extract from the leaves was subjected to vacuum liquid chromatography (VLC) on silica G using hexanes−ethyl acetate (9:1) to ethyl acetate as eluent to give eight fractions (A1−A8). Samples of each of these fractions were tested for antifeedant activity test. The activities of fractions were analyzed by GC−MS (FA2) or subjected to column chromatography to isolate active compounds. Analysis of the active fraction FA2 by GC and GC−MS showed the presence of α-ionone, β-ionone, phytol, and phenylethanol (**Figure 1**) from comparison of retention times and mass spectral parameters with those of authentic samples. Fraction A4 was further purified by flash column chromatography with hexane:ethyl acetate (9:1 to 8:2) to yield pure coumarin (20 mg) and β-sitosterol (16 mg). Fraction A5 was separated by flash column chromatography on silica G with hexanes−ethyl acetate to yield eight fractions (B1−B8), of which B3 consisted of coumarin. Fraction B4 was purified by C18 Sep-Pak cartridge using MeOH−H_2O to give pure medicarpin (9 mg),

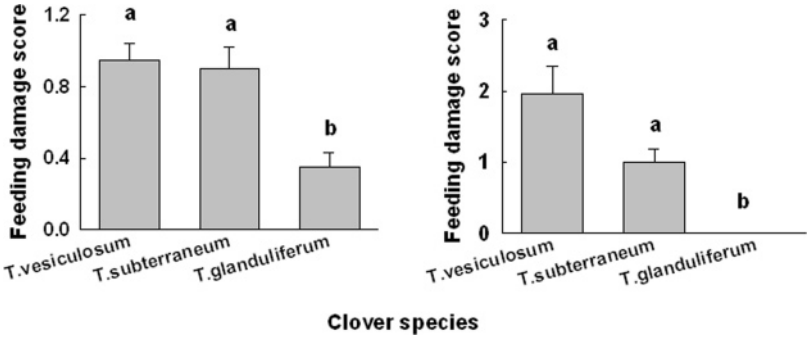

Figure 2. Damage scores for *H. destructor* feeding on mature plants of three *Trifolium* species. Feeding damage after 100 mites fed on 10 week-old plants for four weeks (mean ± SE). In each experiment means for feeding on *T. vesiculatum* and *T. subterraneum* have the same letter, "a", denoting that they were not significantly different from each other, while the mean for *T. glanduliferum* has a different letter, "b", denoting that it was significantly lower ($P < 0.05$).

whose proton and carbon NMR parameters were identical to those published (*5, 6*).

Methanolic extract (32.6 g) was separated by solvent partition to yield four fractions including dichloromethane (4.4 g), ethyl acetate (4.6 g), *n*-butanol (7.6 g), and aqueous fractions (16.0 g). Four fractions were tested for antifeedant activity. The dichloromethane fraction was combined with the dichloromethane extract. The ethyl acetate fraction (4.3 g) was subjected to column chromatography on Sephadex LH-20 on elution with methanol to yield eight fractions (C1−C8). Fraction C2 was subjected to VLC with CH_2Cl_2:MeOH (90:10 to 0:100) to yield pure uracil (28 mg) and uridine (30 mg). Fraction C7 was further purified using flash column chromatography with CH_2Cl_2:MeOH (95:5 to 90:10) to yield kaempferol (5.1 mg) and quercetin (6.1 mg). From fraction C4, a pure 4-hydroxybenzoic acid (30 mg) was obtained after VLC on reverse phase C18 with H_2O to MeOH (**Figure 1**). These compounds were identified by comparison of TLC and ^1H and ^{13}C NMR data with those of authentic samples.

Medicarpin:[5,6] solid. ^1H NMR (CD_3OD, 500.1 MHz): δ 7.38 (d, J = 8.4 Hz, H-1), 6.55 (dd, J = 2.5, 8.4 Hz, H-2), 6.42 (d, J = 2.5 Hz, H-4), 4.23 (ddd, J = 10.8, 5.0, 0.6 Hz, H-6), 3.62 (t, J = 11.0, H-6′), 3.53 (ddd, J = 11.1, 6.7, 5.0 Hz, H-6a), 7.13 (dd, J = 8.8, 0.4 Hz, H-7), 6.45 (dd, J = 8.8, 2.5 Hz, H-8), 6.46 (d, J = 2.5 Hz, H-10), 5.49 (d, J = 6.8 Hz, H-11a), 3.7 (s, OMe). ^{13}C NMR (CD_3OD, 125.8 MHz): 132.2 (C-1), 112.6 (C-1a), 109.8 (C-2), 157.1 (C-3), 103.6 (C-4), 156.4 (C-4a), 66.5 (C-6), 39.5 (C-6a), 124.7 (C-7), 119.1 (C-7a), 96.3 (C-8), 161.6 (C-9), 106.4 (C-10), 160.6 (C-10a), 78.5 (C-11a), 55.5 (OMe).

Bioassays of Metabolites and Related Compounds for Feeding Deterrence. The bioassay technique described above was used to test the activity of samples of the metabolites isolated at concentrations ranging from 0.005% to 0.5%. Samples of β-ionone, α-ionone, linalool, phytol, and phenylethanol were obtained from Sigma-Aldrich, Castle Hill, NSW, Australia. The samples of medicarpin, β-sitosterol, coumarin, quercetin, kaempferol, 4-hydroxybenzoic acid, uracil, and uridine used were those isolated from plant material.

RESULTS

Mite Feeding Damage on Plants. There was a significant difference in feeding damage between the *Trifolium* species in experiment 1 ($F = 14.8$, $P > 0.001$, 2.26 df) and experiment 2 ($F = 11.3$, $P < 0.001$, 2.27 df). In both experiments damage was not significantly different betweem *T. vesiculosum* and *T. subterraneum*, but was lower in *T. glanduliferum* (**Figure 2**). Thus *T. glanduliferum* was consistently highly resistant, with no or very few mites surviving over the 4−5 week experiments. Actual levels of feeding damage varied between experiments.

Bioassay of Surface Components and Total Extracts from Leaves and Flowers of *T. glanduliferum* 87181 and 87182. Extracts from the leaf surface prepared by rinsing of leaves or flowers of 87181 and 87182 with a variety of solvents, hexane, dichloromethane, and methanol, were tested for feeding deterrence (**Table 1**). For the leaf surface components, the dichloromethane extracts showed significant deterrent activity toward mites, with 13% and 23% deterrence, respectively. The methanol leaf extract from 87181 was more attractive than the control. The flower surface components recovered with dichloromethane from variety 87182 were deterrent to mites.

Table 1. Mean Number of Mites on Control and Treatment Membrane Sachets Containing 1% Surface Components Obtained with Various Solvents from Leaves (L) and Flowers (F) of *T. glanduliferum* Varieties over 3 h

variety	solvent	control (mean ± SE)	treatment (mean ± SE)	deterrence[a] (%)
87181L	hexane	1.80 ± 0.280	1.74 ± 0.319	2.0
87182L	hexane	1.30 ± 0.181	1.00 ± 0.324	13.0
87181L	CH_2Cl_2	3.57 ± 0.312	2.78 ± 0.345	12.6*
87182L	CH_2Cl_2	2.78 ± 0.391	1.74 ± 0.178	23.0*
87181L	MeOH	2.21 ± 0.317	4.10 ± 0.360	−30.0**
87182L	MeOH	2.61 ± 0.301	3.50 ± 0.509	−14.6
87182F	CH_2Cl_2	3.73 ± 0.383	1.88 ± 0.321	33.0**
87182F	MeOH	2.16 ± 0.394	2.86 ± 0.384	−13.9

[a] (*) $P < 0.05$; (**) $P < 0.005$.

Table 2. Mean Number of Mites on Control and Treatment Membrane Sachets Containing CH_2Cl_2 and MeOH Total Extract from Leaves or Flowers (0.5 g) of *T. glanduliferum* Varieties over 3 h

variety	tissue	solvent	control	treatment	deterrence[a] (%)
87181	leaves	CH_2Cl_2	6.08 ± 0.725	1.62 ± 0.174	57.9**
87182	leaves	CH_2Cl_2	7.63 ± 0.543	0.78 ± 0.173	81.5***
87181	flower	CH_2Cl_2	5.83 ± 0.690	0.15 ± 0.039	95.0***
87182	flower	CH_2Cl_2	5.44 ± 0.766	0.03 ± 0.019	98.9***
87181	leaves	MeOH	7.26 ± 0.776	0.90 ± 0.188	77.9***
87182	leaves	MeOH	6.76 ± 0.672	2.35 ± 0.450	48.4**
87181	flower	MeOH	4.00 ± 0.794	3.95 ± 0.541	0.6
87182	flower	MeOH	4.63 ± 0.619	4.70 ± 0.559	−0.7

[a] (**) $P < 0.001$; (***) $P < 0.0001$.

The dichloromethane and methanol total extracts from leaves and flowers of the two lines of *T. glanduliferum* were tested for feeding deterrence (**Table 2**). The dichloromethane extracts from leaves and flowers showed significant activity toward mites (58% to 99% deterrence). The methanol total extracts from leaves of both varieties showed feeding deterrence to mites, but the extract from 87182 exhibited stronger activity than that from 87181. The methanol total extracts from the flowers did not display significant deterrence.

Isolation and Identification of Compounds from the Total Extract from Leaves of Variety 87182. Separation of the

Table 3. Mean Number of Mites on Control and Treatment Membrane Sachets Containing Coumarin, β-Ionone, and α-Ionone over 3 h

compd and concn (%)	control (mean ± SE)	treatment (mean ± SE)	deterrence[a] (%)
coumarin			
0.5	6.28 ± 1.01	0	100***
0.1	2.95 ± 0.468	0.42 ± 0.193	75.5**
0.05	2.36 ± 0.367	0.44 ± 0.064	80***
0.01	3.28 ± 0.524	1.14 ± 0.177	48.4**
0.007	2.40 ± 0.391	1.58 ± 0.367	20.6*
0.005	2.16 ± 0.255	1.70 ± 0.250	11.9
β-ionone			
0.5	2.33 ± 0.512	0.93 ± 0.573	42.9*
0.1	5.71 ± 0.866	0.14 ± 0.042	94.2***
0.05	3.44 ± 0.612	0.23 ± 0.115	87.5**
0.01	3.54 ± 0.805	0.73 ± 0.222	65.5*
0.005	2.46 ± 0.674	1.28 ± 0.330	31.7
α-ionone			
0.5	2.13 ± 0.717	0.14 ± 0.127	87.7*
0.05	6.97 ± 0.655	0.342 ± 0.071	90.6***
0.01	7.97 ± 0.923	1.96 ± 0.180	60.5***
0.005	3.97 ± 0.691	6.51 ± 0.507	–24.2*

[a] (***) $P < 0.001$; (**) $P < 0.005$; (*) $P < 0.05$.

components of CH_2Cl_2 total extract and methanol total extract was conducted by bioassay-guided fractionation. Separation of the compounds present in the CH_2Cl_2 total extract and CH_2Cl_2 fraction from methanol extract from leaves of 87182 yielded the terpenes β-ionone, α-ionone, and phytol, the pterocarpan medicarpin, and coumarin, phenylethanol, and β-sitosterol. The methanol total extract afforded the pyrimidine derivatives uracil and uridine, 4-hydroxybenzoic acid, and the flavones quercetin and kaempferol. The structures of all the compounds isolated were verified from their NMR and MS parameters.

Bioassay of Compounds Isolated from the Total Extracts. Medicarpin, coumarin, uracil, uridine, the flavones quercetin and kaempferol, β-sitosterol, and 4-hydroxybenzoic acid isolated from the extracts and authentic samples of α-ionone, β-ionone, linalool, phytol, and phenylethanol were tested for feeding deterrence to mites. Of these, coumarin, β-ionone, and α-ionone (**Table 3**) were most deterrent to mites, showing strong activity at 0.01%. Medicarpin (55%), linalool (76%), phenylethanol (91%), uracil (36%), and quercetin (28%) showed significant deterrent activity at 0.5% concentration (**Table 4**). At lower concentrations, medicarpin (0.1%), phytol (0.1%), and quercetin (0.05%) exhibited attractant properties and uracil was not significant. Phytol (0.1%) and β-sitosterol (0.5%) appeared to be attractant to the mites. Kaempferol was not active in the concentration range 0.1% to 0.05%, whereas quercetin was deterrent at 0.5% and attractant at 0.05%. The activities of 4-hydroxybenzoic acid and uridine were not significant at 0.5% (data not shown).

There was little difference in feeding deterrence to mites between β-ionone and α-ionone (**Table 3**). Both compounds showed significant feeding deterrence at a concentration of 0.01%.

Coumarin exhibited the highest deterrent activity to mites with 100% deterrence at 0.5% and retained significant deterrence (21%) at 0.007%.

Quantitation of Levels of Some Constituents in Dichloromethane Extracts. The levels of coumarin, β-ionone, and phytol in the CH_2Cl_2 total extracts from leaves and flowers of the two *T. glanduliferum* lines 87182 and 87181 were determined by GC and the levels of medicarpin by HPLC (**Table 5**). Leaves contained higher amounts of all compounds than flowers. Coumarin was higher in 87181, and phytol was in 87182. β-Ionone was a bit higher in 87182. The ratio of β- and α-ionone in plants was about 2:1. The two varieties 87181 and 87182 contained similar amounts of medicarpin, but no medicarpin was detected in the flowers.

Table 4. Mean Number of Mites on Control and Treatment Membrane Sachets Containing Compounds Present in Leaves of *T. glanduliferum* 87182 over 3 h

compd and concn (%)	control	treatment (mean ± SE)	deterrence[a] (%)
medicarpin			
0.5	5.45 ± 0.572	1.58 ± 0.190	55.0***
0.1	1.84 ± 0.332	3.03 ± 0.370	–24.4***
linalool			
0.5	1.04 ± 0.335	0.156 ± 0.096	76.2*
0.1	1.34 ± 0.396	0.713 ± 0.171	30.7
phenylethanol			
0.5	3.46 ± 0.362	0.17 ± 0.055	90.6***
0.1	2.08 ± 0.257	1.74 ± 0.166	8.9
phytol			
0.5	2.46 ± 0.331	2.6 ± 0.29	–2.8
0.1	2.54 ± 0.421	4.00 ± 0.303	–22.3*
β-sitosterol			
0.5	1.13 ± 0.168	2.00 ± 0.294	–27.8**
0.1	1.50 ± 0.215	2.27 ± 0.294	–20.4
uridine			
0.5	5.14 ± 0.789	3.34 ± 0.581	21.0
0.1	6.05 ± 0.597	6.58 ± 0.718	–4.1
uracil			
0.5	7.26 ± 0.776	3.40 ± 0.364	36.2*
0.1	6.55 ± 0.823	5.01 ± 0.422	12.5*
quercetin			
0.5	2.40 ± 0.289	4.23 ± 0.320	27.6**
0.05	3.08 ± 0.320	6.45 ± 0.614	–35.3***
kaempferol			
0.1	3.80 ± 0.81	3.80 ± 0.54	0
0.05	3.40 ± 0.55	3.90± 0.43	–6.8

[a] (*) $P < 0.05$; (**) $P < 0.005$; (***) $P < 0.001$.

Table 5. Minimum Concentrations for Deterrent Activity of Some Constituents to Mites, and Concentrations of Compounds in the CH_2Cl_2 Extract from Leaves and Flowers of *T. glanduliferum* Varieties (% Fresh Wt)

		leaf		flower	
compound	MPC (%)	87181 (%)	87182 (%)	87181(%)	87182 (%)
medicarpin[a]	0.5	0.015	0.017	nd[b]	nd
coumarin	0.007	0.0072	0.0057	0.0004	0.00059
β-ionone	0.01	0.0024	0.0030	0.00048	0.00088
phytol	0.5	0.0026	0.0074	nd	nd

[a] The amount of medicarpin was determined by HPLC, and the other amounts were determined by GC. [b] Not detected.

DISCUSSION

T. glanduliferum was strongly resistant to *H. destructor*, as measured by the feeding damage test, compared with the main annual clover species used in Australia, *T. subterraneum*. *T. glanduliferum* Boiss. is so-called because it has glands on the edges of its leaves. Two distinct varieties have been recognized (*7*). Variety CPI 87182 is *T. glanduliferum* Boiss. var. *glanduliferum* Zoh. and has been released in Australia as cv Prima. CPI 87181 is *T. glanduliferum* Boiss. var. *nervulosum* (Boiss. & Heldr.) Zoh. In the description, var. *glanduliferum* is consistently glandular, whereas var. *nervulosum* is not. While both of the breeding lines 87181 and 87182 were highly resistant to the mites, there is some evidence from feeding damage that 87181, the less glandular line, is slightly less resistant than

87182. The bioassay of 1% surface components was twice as deterrent with the CH_2Cl_2 leaf extract from 87182 (23%) than 87181(13%), and the MeOH leaf surface extract from 87181 was twice as attractive (30% to 15%). Variety CPI 87182 has more glands than CPI 87181, and was slightly more resistant, as measured by mite feeding damage. However, bioassays of total CH_2Cl_2 leaf extracts of CPI 87182 also exhibited greater deterrent activity to mites than CPI 87181, indicating that factors in the leaf play an important role, as well as the surface glands. In *T. subterraneaum* there was a better correlation between resistance to *H. destructor* and levels of isoflavones on the leaf surface than between resistance and total amount of extractable isoflavones in the mature leaves (*8*). The membrane assay makes use of a behavioral response of *H. destructor* that they probe all possible feeding sites, and settle at sites where food is acceptable but move away from sites where food contain a deterrent. However, the choice assays do provide an index of the influence of plant compounds on mite feeding. The deterrence caused by 1% catechin in a membrane assay (72%) is very similar to the reduction in weight gain by mites on membranes in a no-choice situation (74%) (*4a*). The membrane assay is also able to detect dose-dependent deterrent effects on the mites, as demonstrated with a solvent extract from *Eucalyptus* leaves (*4a*).

The dose responses for 12 compounds extracted from *T. glanduliferum* were determined in mite bioassays. Of these, α-ionone, β-ionone, and coumarin showed good deterrence at the lowest concentrations (0.01%). Coumarin, and α- and β-ionone, which co-occur in a 1:2 ratio in line 87182, had been detected previously as components of the volatile compounds produced by leaves of *T. glanduliferum* with bioactivity against redlegged earth mite (*2*). A more detailed examination now reveals that coumarin retains significant activity at a concentration as low as 0.007%. The level of coumarin in the leaves (57−73 μg/g fresh wt) represents a concentration of 0.006−0.007%, within the range at which this compound is active. Coumarin has been reported to be a feeding deterrent to a range of insects (*9*−*13*).

The isomeric modified terpenes α- and β-ionone retain activity at concentrations of 0.01%, which are much higher than the levels of these compounds found in the leaves. The concentrations of β- and α-ionone in plants are about 24.2 to 29.6 (0.002% to 3%) and 12 to 25 (0.0012% to 0.0025%) μg/g fresh wt, respectively. Interestingly, the ionones have been found to be repellent toward the livestock tick, *Rhipicephalus appendiculatus* Neumann (Acari: Ixodidae), but α-ionone was significantly more active than β-ionone (*14*). In the same assay, linalool was as active as α-ionone, whereas against *H. destructor* it was found to be less active. Linalool is repellent to aphids, mosquitoes, cockroaches, and ants (*15*) and has been found to be a component of a volatile *Tetranychus urticae* (spider mite) dispersing pheromone (*16*).

The pterocarpan medicarpin is the most common phytoalexin in *Trifolium*. It is produced by a number of *Trifolium* species, including *T. glanduliferum*, after inoculation with *Helminthosporium carbonum* (*17*). In the present study, medicarpin was shown to occur at levels of 0.017%. In the bioassay, medicarpin was deterrent at a concentration of 0.5%, but became weakly attractant at 0.1%, suggesting that it plays no part in deterrence. Surprisingly, no evidence for the presence of the likely isoflavone precursors of medicarpin was obtained. Of the other compounds isolated, phenylethanol, linalool, and uracil showed activity at 0.5%, but showed no activity at 0.1%, at which concentration the levels of compounds phenylethanol and linalool are much higher than those found in the plant (data not shown). Interestingly, quercetin was deterrent at concentrations of 0.5%, but became attractant at 0.05%, a concentration which is much higher than that found (0.01%) in the plant. Attractant activity was also observed for phytol and β-sitosterol, but at concentrations (0.1%) higher than that found (0.01%) for these two compounds in the plant.

In contrast to *T. subterraneum*, in which deterrence is conferred largely by constitutive isoflavones on the leaf surface, *T. glanduliferum* relies on a greater variety of endogenous compounds both on the leaf surface and in the leaf. In *T. glanduliferum*, it appears that coumarin dominates the activity. It is worthwhile noting that coumarin, the ionones, and linalool are regarded as volatile metabolites. Other compounds with in vitro deterrent activity are also present in *T. glanduliferum*, although not in high enough concentrations to mediate deterrence individually. It is likely that these compounds act synergistically or, at least, that the palatability of *T. glanduliferum* to redlegged earth mite depends on the overall stimulant−deterrent balance.

The production of coumarin in *T. glanduliferum* may appear problematic since coumarin can be converted to dicoumarol if overheating or spoilage takes place during hay or silage making. However, analyses of extracts from several lines grown under different stress conditions have shown that levels of coumarin range from 130 to 500 ppm (dry wt) (unpublished data), which places *T. glanduliferum* 87812 cv Prima in the low-coumarin group of clover cultivars (*18*).

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LITERATURE CITED

(1) Gillespie, D. J. Redlegged earth mite (*Halotydeus destructor*) resistance in annual legumes. In *Pests of Pastures*; Delfosse, E., Ed.; CSIRO Aust.: Melbourne, 1993; pp 211−213.

(2) Wang, S. F.; Ghisalberti, E. L.; Ridsdill-Smith, T. J. Volatiles from *Trifolium* as feeding deterrents of redlegged earth mites. *Phytochemistry* **1999**, *52*, 601−605.

(3) Ridsdill-Smith, T. J. Responses and feeding damage of redlegged earth mite (Acari: Penthaleidae) to seedlings of resistant and susceptible subterranean clover varieties. *Aust. J. Agric. Res.* **1995**, *46*, 1091−1099.

(4) (a) Ridsdill-Smith, T. J.; Jiang, Y.; Ghisalberti, E. L. A method to test compounds for feeding deterrence towards redlegged earth mite (Acarina: Penthaleidae). *Ann. Appl. Biol.* **1995** *127*, 593−600. (b) Ridsdill-Smith, T. J. Biology and control of *Halotydeus destructor* (Tucker) (Acarina: Penthaleidae): a review. *Exp. Appl. Acarol.* **1997**, *21*, 195−224.

(5) Pachler, K. G. R.; Underwood, W. G. E. A proton magneic resonance study of some pterocarpan derivatives. *Tetrahedron* **1967**, *23*, 1817−1826.

(6) Chalmers, A. A.; Rall, G. J. H.; Oberholzer, M. E. ^{13}C NMR of pterocarpans. *Tetrahedron* **1977**, *33*, 1735−1737.

(7) Zohary, M.; Heller, D. *The genus Trifolium*; The Israel Academy of Sciences and the Humanities: Jerusalem, 1984.

(8) Wang, S. F.; Ridsdill-Smith, T. J.; Ghisalberti, E. L. Levels of isoflavonoids as indicators of resistance of subterranean clover trifoliates to redlegged earth mite *Halotydeus destructor*. *J. Chem. Ecol.* **1999**, *25*, 795−803.

(9) Roseland, C. R.; Grosz, T. J. Induced responses of common annual sunflower *Helianthus annuus* L. from geographically diverse populations and deterrence to feeding by sunflower beetle. *J. Chem. Ecol.* **1997**, *23*, 517−542.

(10) Bernays, E. A. Relationship between deterrence and toxicity of plant secondary compounds for the grasshopper *Schistocerca americana*. *J. Chem. Ecol.* **1991**, *17*, 2519−2526.
(11) Mansour, M. H.; Dimetry, N. Z.; Rofaeel, I. S. The role of coumarin as secondary plant substance in the food specificity of the cowpea aphid *Aphis craccivora* Koch. *Z. Angew. Entomol.* **1982**, *93*, 151−157.
(12) Manglitz, G. R.; Gorz, H. J.; Haskins, F. A.; Akeson, W. R.; Beland, G. L. Interactions between insects and chemical components of sweet clover. *J. Environ. Qual.* **1976**, *5*, 417−435.
(13) Yatagai, M.; Ohira, T.; Nakashima, K. Composition, miticidal activity and growth regulation effect on radish seeds of extracts from *Melaleuca* species. *Biochem. Syst. Ecol.* **1998**, *26*, 713−722.
(14) Lwande, W.; Ndakala, A. J.; Hassanali, A.; Moreka, L.; Nyandat, E.; Ndungu, M.; Amiani, H.; Gitu, P. M.; Malonza, M. M.; Punuya, D. K. *Gynandropsis gynandra* essential oil and its constituents as tick (*Rhipicephalus appendiculatus*) repellents. *Phytochemistry* **1999**, *50*, 401−405.
(15) Bowers, W. S.; Ortego, F.; You, X.; Evans, P. H. Insect repellents from the Chinese prickly ash *Zanthoxylum bungeanum*. *J. Nat. Prod.* **1993**, *56*, 935−938.
(16) Dicke, M.; Van Beek, T. A.; Posthumus, M. A.; Ben Dom, N.; Van Bokhoven, H.; De Groot, A. E. Isolation and identification of volatile kairomone that affects acarine predator-prey interactions. Involvement of host plant in its production. *J. Chem. Ecol.* **1990**, *16*, 381−396.
(17) Ingham, J. L. A further investigation of phytoalexin formation in the genus *Trifolium*. *Z. Naturforsch.* **1990**, *45c*, 829−834.
(18) Sanderson, M. A.; Meyer, D. W.; Casper, H. H. Dicoumarol concentrations in sweetclover hay treated with preservatives and in spoiled hay of high- and low-coumarin cultivars of sweetclover. *Anim. Feed Sci. Technol.* **1986**, *14*, 221−230.

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