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Cytotoxic clerodane diterpenes from *Glossocarya calcicola*

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Abstract

Three clerodane diterpenes were isolated and identified from leaf extract of *Glossocarya calcicola*. Compound **1** has been characterised as (*rel*)-10βH-*trans*-12ξ-(2-methylbut-2(*E*)-enoyl)-1β-(isobutanoyl)-6α,13ξ-dihydroxyclerodan-4(20),8(18)-dien-7,15-dione-15,16-oxide, to which we have assigned the trivial name calcicolin-A. The other two compounds had the same skeletal structure and C-12 substituent but in compound **2**, the C-1 esterifying group becomes 2-methylbut-2(*E*)-enoic acid and in **3** it becomes 2-methylbutanoic acid. Although anti-insect activity was not observed for *G. calcicola*, cytotoxicity against insect and human carcinoma cell lines was detected.

Keywords: *Glossocarya calcicola*; Lamiaceae; Cytotoxicity; Insect cell lines; Mammalian cell lines; *Tetranychus urticae*; *Plutella xylostella*; Clerodane diterpenes; Calcicolin

1. Introduction

Approximately 11 species of the genus *Glossocarya* Wallich *ex* Griffith (Lamiaceae) are known to occur in parts of Sri Lanka, Burma, Thailand, Cambodia, Vietnam, West Malaysia and Papua New Guinea (Munir, 1990). A further two species are endemic to Australia; *G. hemiderma* Benth. (syn. *G. coriacea* F.M. Bailey) and *G. calcicola* Domin. The early taxonomic history of *Glossocarya* is contentious. Species were initially placed in *Clerodendrum* L. due to similarities in the leaf and inflorescence type (Munir, 1990). Traditionally *Glossocarya* has been included in the order Lamiales, within the family Verbenaceae and the Caryopterideae subfamily. Recently, *Glossocarya*, together with some other Verbenaceae (*Clerodendrum, Vitex, Premna, Viticipremna, Callicarpa, Gmelina* and *Faradaya* from Australia), have been included in the allied family Lamiaceae (Cantino et al., 1992). These genera were newly assigned within the Lamiaceae to the subfamily Viticoideae, with the exception of *Glossocarya* and *Clerodendrum*, which were placed in the subfamily Ajugoideae (syn. Teucrioideae) (Cantino, 1992). To date, the chemical constituents of *Glossocarya* have not been reported.

As part of our studies on potential insecticidal compounds from Australian Lamiaceae we have noted (Rasikari et al., 2005) that the methanol extract of leaf material of *G. calcicola* was active in inhibiting the growth of two insect cell lines, pupal ovarian tissue from *Spodoptera frugiperda* (Sf9) and embryonic derived *Drosophila melanogaster* (D.mel-II). However, the extract failed to show acaricidal activity against two-spotted spider mite, *Tetranychus urticae* Koch, in whole organism studies. Despite the limited mortality in whole mite bioassay the extract was interesting in that it was among the most highly cytotoxic against insect cell lines from a selection of sixty-seven extracts of Australian Lamiaceae. The extract of related species, *G. hemiderma*, was ineffective against both the more robust cell line D.mel-II and sensitive Sf9 line. In addition, we also observed a lack of activity against mites for this extract. In this paper we report the result of further

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studies on *G. calcicola* designed to isolate and identify the compounds responsible for the cytotoxicity.

2. Results and discussion

2.1. Preliminary insect bioassay data

Preliminary screening of a methanol extract of the leaf of *G. calcicola* revealed IC_{50} values against D.mel-II and Sf9 cell lines of 1.22 (±0.038) and 0.76 (±0.098) μg/mL, respectively (Rasikari et al., 2005). The toxicity of this crude methanol extract (1.0% w/v) against two-spotted spider mite, *T. urticae*, resulted in less than 10% mortality (Rasikari et al., 2005). However, the ethyl acetate soluble fraction of the leaf tested at the same concentration increased mite mortality (38 ± 5.7%) indicating that there was some toxic effect being exhibited by compounds of a lipophilic nature. The antifeeding activity of the ethyl acetate soluble fraction was also assessed against 2nd instar larvae of the diamondback moth, *Plutella xylostella*, in a choice bioassay. Larvae could not discriminate between treated and control leaf discs and were not repelled by crude extract when sprayed at 0.25% (w/v). Observations made at 2, 6, 24 and 48 h gave a low repellency index (RI) value of 10.5%, 20%, 20% and 8.5%, respectively. This suggests low avoidance or similar preference for the control and this product by the caterpillar larvae at this concentration. At 48 h all control and treated leaf discs were consumed (>50%), further demonstrating a low antifeeding effect at this concentration. As a result of the low level of activity of the extract against whole organisms, further study on the *G. calcicola* extract were focused on determining the compounds responsible for the toxicity against insect cell lines.

2.2. Bioassay guided fractionation v D.mel-II

The lipid free extract was separated into 11 fractions by preparative HPLC. The fractions were dried and assessed for cytotoxicity against the D.mel-II insect cell line (Fig. 1). The results for toxicity at 10.0 and 1.0 μg/mL showed the most active fraction as F9 containing **1** (Fig. 2(a)) when tested at both concentrations. Good activity was also observed for F8 (Rt 17–20 min) which contained a complex mixture of compounds including **1**. Fraction F7 contained two minor compounds eluting between 16 and 17 min (Fig. 2(a)) which exhibited toxicity at the lowest screening concentration. However, lower toxicity was observed for F7 at the higher screening concentration (100 μg/mL). Some activity was also observed for fraction F10 (containing **2** and **3**) and F11 (Rt 22–30 min) but only at the higher screening concentration of 100 μg/mL. No cytotoxicity was observed for F1–F4 (Rt < 10 min) at either concentration suggesting that the polar compounds in this extract are inactive, while only minimal activity was observed for fractions F5 and F6 (Rt 10–16 min).

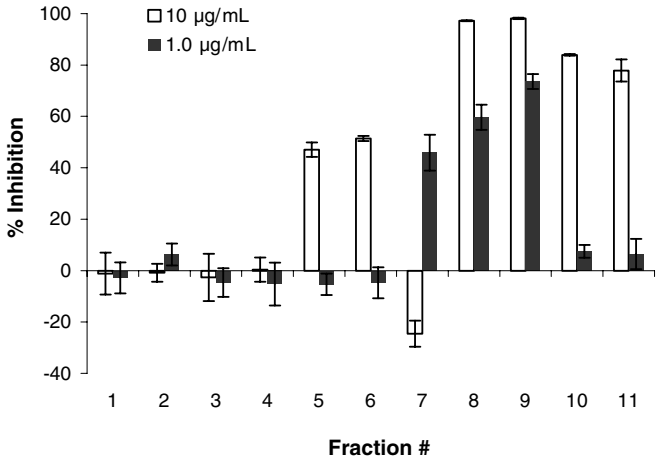

Fig. 1. Cytotoxic effect of fractions of *G. calcicola* (MeOH extract) against D.mel-II at 10.0 and 1.0 μg/mL.

2.3. Cytotoxicity against mammalian cell lines

The cytotoxic activity of most active F9 was further examined against 4 mammalian carcinoma cell lines and one normal cell line (HS27). The results for each cell line were measured and compared against the results for chlorambucil, a standard cytotoxic positive control (Fig. 3). Three concentrations were screened in triplicate (100, 10 and 1.0 μg/mL final concentration) and the results showed the most susceptible cell line to F9 was the human ovary-derived A2780. The IC_{50} value for this cell line was found to be 3.4 ± 0.4 μg/mL and it was equally active with the positive control chlorambucil at a concentration of 100 μg/mL. Fraction F9 was also found to inhibit the growth of liver derived HepG2 cells (similar to chlorambucil) but less activity was observed against prostate-derived PC3 cells (less than chlorambucil). Fraction F9, unlike chlorambucil, showed no cytotoxic effect against breast-derived MCF-7 cells and only minimal effect against the normal cell line HS27 (less than chlorambucil).

Two unidentified compounds from F7 were assessed against HepG2 cells and were significantly less toxic than compounds 1, 2 and 3. The IC_{50} values for compound A (Rt 16.21 min in Fig. 2(a)) and B (Rt 16.69 min in Fig. 2(a)) were 60.82 ± 1.38 and 65.39 ± 0.25 μg/mL, respectively. No further work was carried out on these compounds, however, LC–MS and NMR spectral data suggest that they were flavonoid glycosides.

2.4. Identification of cytotoxic compounds

The defatted methanol extract of *G. calcicola* was fractionated by preparative reverse phase HPLC to yield three new clerodane diterpenes **1**–**3** (Fig. 4). Compound **1**, an amorphous solid, showed $[M + H]^+$ ion peak at 533 *m/z* in positive APCI-MS mode and on HR-EIMS solved for $C_{29}H_{40}O_9$. Examination of the ^1H and ^{13}C NMR 1D spectra (Table 1) and the 2D HSQC direct C–H correlation

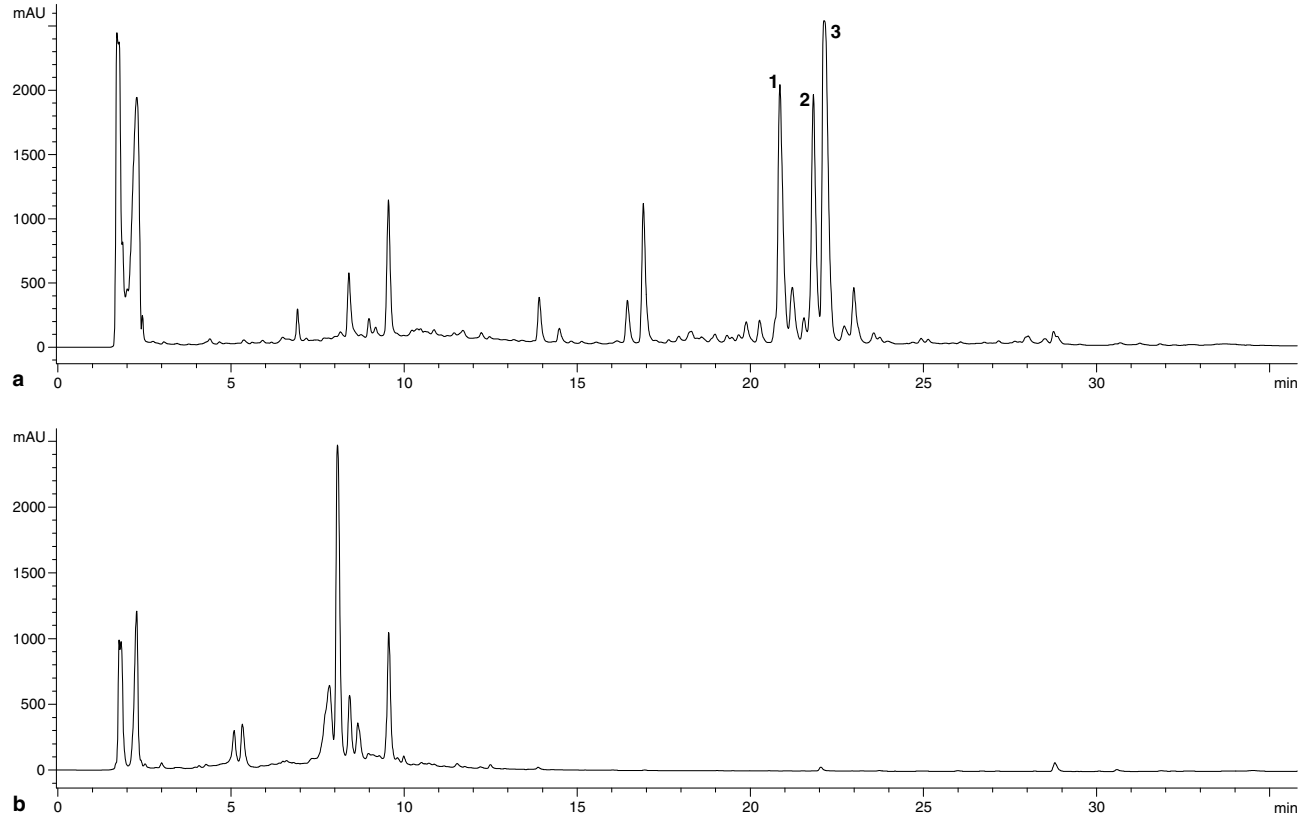

Fig. 2. HPLC (210 nm) profile of (a) *G. calcicola* and (b) *G. hemiderma* MeOH extract.

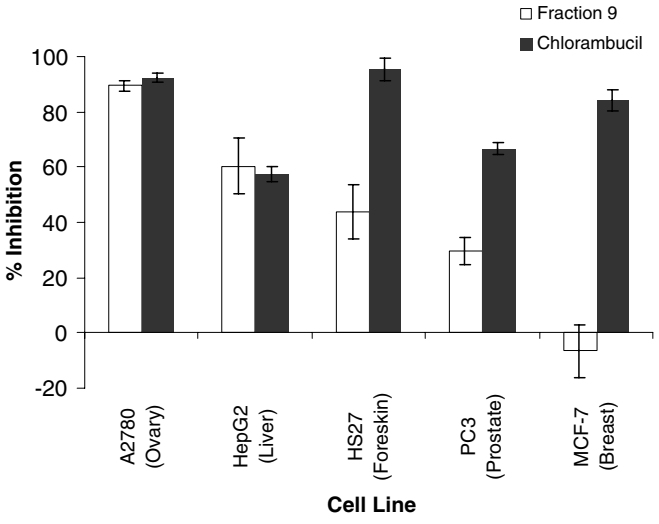

Fig. 3. Cytotoxic effect of F9 and chlorambucil against mammalian cell lines at 100 μg/mL.

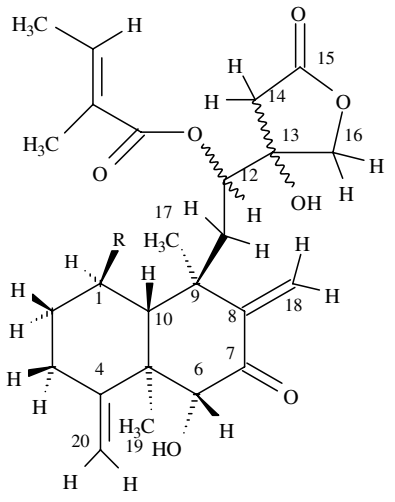

Fig. 4. Structure of isolated clerodane compounds **1**–**3** (**1**, calcicolin-A, R = isobutanoyl; **2**, calcicolin-B, R = 2-methylbut-2(*E*)-enoyl; **3**, calcicolin-C, R = 2-methylbutanoyl).

spectrum showed 29 carbon signals that were identified as 10 quaternary carbons [including one ketone (200.5 ppm), three ester carbonyls (167.7, 174.9, 176.0 ppm), three sp2 and three sp3 carbons with one of the latter oxygen bearing (128.1, 149.0, 150.7, 79.3, 46.8, 42.4 ppm)], six methine carbons [one sp2 (140.6 ppm), three oxygen-bearing sp3 (72.7, 73.7, 78.2 ppm) and two other sp3 (47.8, 34.9 ppm)], seven methylene carbons [two are sp2 (109.6, 128.6 ppm), one oxygen-bearing sp3 (75.8 ppm) and four other sp3 (44.0, 38.8, 34.7, 30.6 ppm)] and six methyl carbons resonating at 29.8, 19.4, 18.8, 14.8, 14.4 and 12.3 ppm.

Full 2-dimensional analysis, including COSY, HMBC, HSQC and NOESY revealed the presence of two esterifying groups that could be identified as isobutanoic acid and 2-methylbut-2-enoic acid with proton and carbon resonances assigned (Fig. 5). Subtracting these leaves a

Table 1
^{1}H and ^{13}C chemical shift values for diterpenes **1**–**3**

C/H	^{1}H			^{13}C		
	1	**2**	**3**	**1**	**2**	**3**
1	5.23 *td* (10.9, 4.9)	5.24 *td* (10.9, 4.8)	5.34 *td* (10.9, 5.0)	72.7	72.7	72.6
2	2.21 *m*	2.35 *m*	2.25 *m*	34.7	34.9	34.9
	1.16 *m*	1.13 *m*	1.21 *m*			
3	2.32 *td* (13.4, 4.3)	2.31 *td* (13.2, 4.2)	2.34 *td* (13.0, 4.2)	30.6	30.6	30.7
	2.09 *m*	2.11 *m*	2.12 *m*			
4				150.7	150.7	150.8
5				46.8	46.8	46.8
6	4.30 *br.s*	4.30 *br.s*	4.31 *br.s*	78.2	78.2	78.2
7				200.5	200.5	200.5
8				149.0	149.0	149.2
9				42.4	42.5	42.4
10	2.09 (10.9)	2.11 *d* (10.9)	2.15 *d* (10.9)	47.8	47.8	48.0
11	2.82 *dd* (15.2, 9.1)	2.81 *dd* (15.4, 9.2)	2.84 *dd* (15.6, 9.3)	44.0	43.9	43.9
	1.75 *d* (15.2)	1.81 (15.4)	1.76 *d* (15.6)			
12	5.09 *d* (9.1)	5.09 *d* (9.2)	5.13 *d* (9.3)	73.7	73.7	74.0
13				79.3	79.3	79.5
14	2.74 *d* (17.8)	2.73 *d* (17.5)	2.71 *d* (17.5)	38.8	38.8	39.1
	2.42 *d* (17.8)	2.43 *d* (17.5)	2.42 *d* (17.5)			
15				174.9	175.1	174.4
16	4.12 *d* (10.0)	4.12 *d* (10.1)	4.11 *d* (10.0)	75.8	75.8	75.6
	4.02 *d* (10.0)	4.02 *d* (10.1)	4.03 *d* (10.0)			
17	1.25 *s*	1.23 *s*	1.23 *s*	29.8	29.8	29.9
18	6.71 *s*	6.71 *s*	6.72 *s*	128.6	128.6	128.2
	5.64 *s*	5.64 *s*	5.61 *s*			
19	0.98 *s*	0.98 *s*	1.00 *s*	14.4	14.4	14.4
20	5.10 *s*	5.10 *s*	5.12 *s*	109.6	109.6	109.6
	4.83 *s*	4.83 *s*	4.84 *s*			
1 – C-1 ester	C=O 176.0; CH 2.57 hept (7.3), 34.9; Me-1 1.25 *d* (7.3), 19.4; 1.24 *d* (7.3), 18.8.					
2 – C-1 ester	C=O 166.8; C 129.3; Me 1.92 *br.s*, 12.5; CH 6.99 *q* (7.1), 138.5; Me 1.87 *d* (7.1), 14.8					
3 – C-1 ester	C=O 175.9; CH 2.38 *m*, 42.0; Me 1.20 *d* (6.9) 16.5; $CH_2$1.73 *m* + 1.57 *m*, 27.2; Me 0.95 *t* (6.9), 11.8					
1 – C-12 ester	C=O 167.7; C 128.1; Me 1.83 *br.s*, 12.3; CH 6.96 *q* (7.1), 140.6; Me 1.83 *d* (7.1), 14.8					
2 – C-12 ester	C=O 167.8; C 128.1; Me 1.83 *br.s*, 12.3; CH 6.96 *q* (7.1), 140.6; Me 1.81 *d* (7.1), 14.8					
3 – C-12 ester	C=O 167.9; C 128.1; Me 1.84 *br.s*, 12.3; CH 6.94 *q* (7.1), 140.6; 1.83 *d* (7.1), 14.8					

Spectra obtained at 500 MHz (^{13}C at 125 MHz) in $CDCl_3$.

20-carbon moiety containing a ketone, an ester carbonyl, five further quaternary carbons, four methine carbons, seven methylene carbons and two methyls (both quaternary according to the ^{1}H spectrum). The quaternary carbons at 150.7 and 149.0 ppm together with the sp2 methylene carbons at 109.6 and 128.6 ppm could be assigned to two exocyclic methylene groups. Starting from these, using HMBC correlation data it was possible to establish a bicyclic decalin system (Table 2) substituted at C-4 and C-8 with exocyclic methylene groups, at C-5 and C-9 with methyl groups, at C-7 with a carbonyl, at C-6 with an oxygen, a methylene at C-3, a methane at C-10 and the remainder of the structure linked to the decalin through C-9. The remaining two positions of the decalin, C-1 and C-2, were made up of a second methylene and a second oxymethine which could not be unambiguously assigned from HMBC data. Fortunately the COSY spectrum revealed

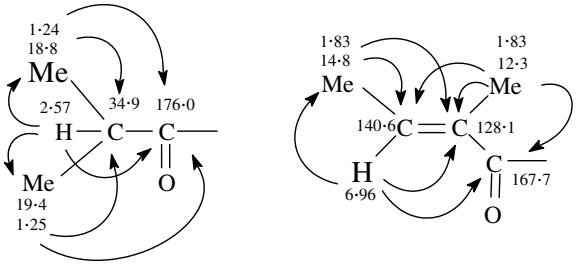

Fig. 5. HMBC correlations of the two esterifying groups in compound **1**.

Table 2
Unambiguous correlations observed in the HMBC spectrum of **1**

Proton	Observed correlation to carbon	
	J_2	J_3
H-6	C-5, C-7	C-4, C-19
H-10	C-1, C-5, C-9	C-4, C-6
H_2-11	C-9, C-12	C-8
H_2-14	C-13, C-15	C-12w, C-16
H_2-16	C-13	C-12w, C-14w, C-15
H_2-17	C-9	C-8, C-10, C-11
H_2-18	C-8	C-7, C-9
H_3-19	C-5	C-4, C-6, C-10
H_2-20	C-4	C-3, C-5

w, weak signal.

direct coupling of the oxymethine PROTON to H-10 requiring it to be placed at C-1.

The remaining six carbons that made up the C-9 side chain consisted of three methylene groups, two of which where remote from other H-bearing carbons (one of these was oxygen bearing) while the third formed an ABX spin system with the proton of an oxymethine. This leaves an ester carbonyl and a quaternary oxygen-bearing sp3 carbon. HMBC correlations established the ester carbonyl at C-15 linked through an oxygen bridge to C-16 with further oxygenation on C-12 and C-13. This allows the structure of the diterpene to be assigned as in **1**, onto which the 2-methylbut-2-enoic acid group is esterified at C-12 (HMBC correlation from H-12 to esterifying carbonyl) and the isobutanoic acid group esterified to C-1 (no correlation seen but deshielded resonance of H-1 requires esterification).

As a starting point in resolving relative stereochemistry, H-1 and H-10 exhibit a coupling constant of >10 Hz which requires these two protons to be *trans* diaxial. H-1 exhibits strong nOe interactions with the methyl groups C-17 and C-19, requiring that these methyls are both axial and on the same face of the decalin nucleus as H-1. By contrast the isolated H-6 proton exhibits a strong nOe to H-10, so requiring that H-6 is axial and on the same face of the molecule as H-10. All strong nOe's observed are shown in Fig. 6. In addition the olefinic proton of the 2-methylbut-2-enoic acid ester showed an nOe to only one methyl so requiring that the two methyls are *cis* across the double bond.

On the basis of the above evidence compound **1** has been characterised as (*rel*)-10βH-*trans*-12ξ-(2-methylbut-2(*E*)-enoyl)-1β-(isobutanoyl)-6α,13ξ-dihydroxyclerodan-4(20),8(18)-dien-7,15-dione-15,16-oxide, and assigned the trivial name calcicolin-A.

Compounds **2** and **3** were obtained as amorphous solids. The molecular formulae $C_{30}H_{40}O_9$ and $C_{30}H_{42}O_9$ attributed to **2** and **3** were deduced from LC–MS analyses showing $[M+H]^+$ ion peaks at 545 and 547 *m/z*, respectively. The ^1H and ^{13}C NMR spectra (Table 1) of the decalin units for **2** and **3** show closely similar signals to those of **1**. Differences between the compounds and **1** resided only in the structure of the C-1 esterifying group. In **2**, the C-1 esterifying group becomes 2-methylbut-2(*E*)-enoic acid and in **3** it becomes 2-methylbutanoic acid. In both **2** and **3** the C-12 esterifying group remains as 2-methyl-but-2(*E*)-enoic acid and were assigned the trivial names calcicolin-B and calcicolin-C, respectively.

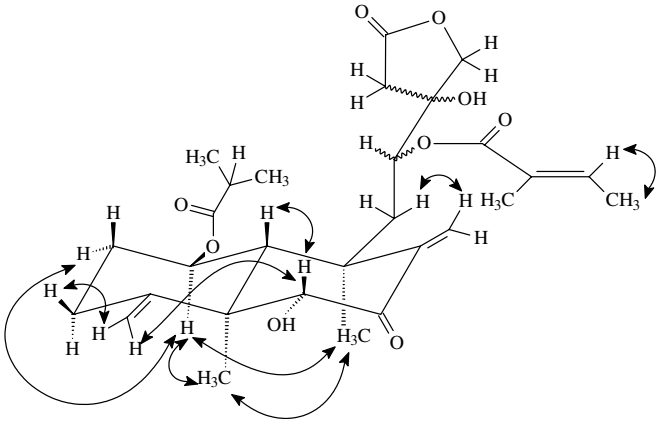

Fig. 6. Key nOe correlations observed in compound **1**.

Table 3
Cytotoxicity of diterpenes **1**–**3** against one insect and mammalian cell line

Compound	IC_{50}, μg/mL (±SD)	
	D.mel-II	HepG2
1	2.06 ± 0.20	9.04 ± 0.13
2	3.09 ± 0.24	16.16 ± 0.27
3	2.10 ± 0.26	8.30 ± 0.12

D.mel-II, embryonic tissue of ferment fly, *D. melanogaster*; HepG2, Caucasian hepatocyte adenocarcinoma.

2.5. Cytotoxicity of diterpenes *1*–*3*

To illustrate the structure/activity relationship of the isolated compounds, a cytotoxic assessment was made against one insect cell line and one human cell line. Each compound was tested in triplicate at five concentrations between 100 and 1.0 μg/mL. Although the HepG2 cells were less susceptible to the toxic effects exhibited by compounds **1**, **2** and **3**, the results show that most active diterpenes were **1** and **3** against both the D.mel-II and HepG2 cell line (Table 3). Compound **2** also showed good toxicity against D.mel-II cells but was not as effective against HepG2 cells ($IC_{50} = 16.16 \pm 0.27$).

3. Discussion

Previous work has highlighted that the occurrence of diterpenes in Lamiaceae could be used to support subfamilial classification (Cole, 1992). Over 90 diterpene structural skeletons have been identified from Lamiaceae (Alvarenga et al., 2001). Clerodane diterpenes have occurred in at least 13 genera most predominantly in *Ajuga*, *Teucrium* (Ajugoideae), and *Scutellaria* (Scutellarioideae), and to a lesser extent in subfamilies Viticoideae (Hu et al., 2002), Chloanthoideae (Fletcher et al., 2000) and to some extent the Nepetoideae (*Salvia* spp.) (Alvarenga et al., 2001). This is the first report on the chemistry of *G. calcicola* and the finding of clerodane diterpenes supports its inclusion in the Ajugoideae syn. Teucrioideae subfamily, as proposed by Cantino (1992). The inspection of HPLC data showed that compounds 1, 2 and 3 were not present in *G. hemiderma* (Fig. 2(b)). However, the polar compounds present in this species were similar to those found in *G. calcicola*.

Clerodane diterpenes are often associated with insect antifeeding activity notably those from species of *Ajuga* (Belles et al., 1985; Ben Jannet et al., 2000; Bondi et al., 2000; Bremner et al., 1998; Camps and Coll, 1993; Zhi-da et al., 1989) and *Teucrium* (Bruno et al., 1992; Camps

et al., 1987; Lopez-Olguin et al., 1999; Simmonds et al., 1989). It is accepted that the presence of a spiroepoxide substituent at C-4, acetate groups at C-6 and C-19 and a hexahydrofurofuran moiety at the C-9 position is responsible for such activity (Camps and Coll, 1993). The absence of these components in the clerodanes isolated from *Glossocarya* may account for the observed lack of activity against the larva of *P. xylostella* and adult *T. urticae* mites. However, the clerodanes isolated from *Glossocarya* were highly cytotoxic to the insect cell line and some of the human carcinoma cell lines tested here. Greatest cytotoxicity was exhibited by compounds **1** and **3**, particularly against the HepG2 cell line which proved to be less susceptible than the D.mel-II cell line. The presence of the additional 2–3 double bond in the C-1 esterifying group in compound **2**, must be responsible for the observed reduction in activity in comparison to **1** and **3** against both lines.

4. Experimental

4.1. General detection of metabolites

The optical rotations were measured with a Perkin–Elmer polarimeter. The ^{1}H NMR (500 MHz) and ^{13}C NMR (125.7 MHz) were recorded on a Bruker Avance DRX-500 spectrometer. Pure compounds were dissolved in $CDCl_3$ and chemical shifts were reported in parts per million with respect to the solvent signal while the coupling constants (*J*) are given in hertz.

APCI-MS spectra were obtained on an Agilent (series 1100) HPLC equipped with a vacuum degasser, binary pump, auto-injector, diode array detector and coupled to an Agilent mass selective detector. Positive fragmentor voltage was set at 150 V and absorbance was monitored between 190 and 900 nm. The HPLC/MS system operation was controlled using ChemStation software, and was used to determine the mass and retention times of UV/visible components separated on a Phenomonex aqua C_{18} column (5μ: 150 × 4.6 mm ID) using a 10–95% acetonitrile (0.0005% TFA) and water gradient at 1 mL/min over 30 min.

4.2. Plant material

Field collected leaf material of *G. calcicola* was collected by Paul I. Forster in February 2002, voucher # PIF-28260, and *G. hemiderma* in July 2003, # PIF-29177 and deposited at the Queensland Herbarium (BRI), Brisbane Botanic Gardens, Mt Coot-tha Road, Toowong, Qld, Australia.

4.3. Extraction and isolation

Dried and ground leaf material (52 g) was extracted with MeOH (1:4 w/v with 15 min sonication × 3) at room temperature. Extracts were pooled, filtered through a scintered glass funnel and evaporated in vacuo (<40 °C) to afford 4.9 g of crude extract. Part of this extract (3.4 g) was defatted with hexane, taken to dryness and partitioned with ethyl acetate and water to yield 1.6 g of crude ethyl acetate soluble fraction for insect bioassay.

For preparative bioassay guided fractionation, the remaining crude extract (1.5 g) was similarly defatted with hexane, vacuum concentrated by rotary evaporation (1.2 g), dissolved in 50:50 acetonitrile:water (100 mg/mL) and fractionated by preparative RP-HPLC (Gilson; 10–95% ACN/H_20 with 0.005% TFA), on an Altima C_{18} column (5 μm packing, 50 × 22 mm ID), run over a 30-min period at 20 mL/min and operating at wavelengths of 210 and 280 nm. Fractions were collected at 30 s intervals and were combined accordingly to yield 11 fractions with the most cytotoxic fraction F9 enriched with compound **1** (190 mg). Fraction F10 was a combination of compounds **2** (270 mg) and **3** (210 mg), which were separated on the same preparative RP-HPLC system using a 40–85% solvent gradient.

4.3.1. Calcicolin-A. (rel)-10βH-trans-12ξ-(2,methylbut-2(E)-enoyl)-1β-(isobutanoyl)-6α,13ξ-dihydroxyclerodan-4(20),8(18)-dien-7,15-dione-15,16-oxide (**1**)

Amorphous solid, $[\alpha]_D^{24}$ +4.3° (MeOH, *c*. 1.00/100 mL); UV ACN:H_20 (0.0005% TFA) λ_{max} 226. ^{1}H and ^{13}C NMR see Table 1. Found: 555.2571; $C_{29}H_{40}O_9Na$ requires 555.2559.

4.3.2. Calcicolin-B. (rel)-10βH-trans-1β,12ξ-di(2-methylbut-2(E)-enoyl)-6α,13ξ-dihydroxyclerodan-4(20),8(18)-dien-7,15-dione-15,16-oxide (**2**)

Amorphous solid, $[\alpha]_D^{24}$ −8.98 °C (MeOH, *c* 1.00/100 mL); UV ACN:H_20 (0.0005% TFA) λ_{max} 220. ^{1}H and ^{13}C NMR see Table 1. Found: 567.2044; $C_{30}H_{40}O_9Na$ requires 567.2034.

4.3.3. Calcicolin-C. (rel)-10βH-trans-12ξ-(2-methylbut-2(E)-enoyl)-1β-(2-methylbutanoyl)-6α,13ξ-dihydroxyclerodan-4(20),8(18)-dien-7,15-dione-15,16-oxide (**3**)

Amorphous solid, $[\alpha]_D^{24}$ +2.31 °C (MeOH, *c* 1.00/100 mL); UV ACN:H_20 (0.0005% TFA) λ_{max} 226. ^{1}H and ^{13}C NMR see Table 1. Found: 569.2720; $C_{30}H_{42}O_9Na$ requires 569.2715.

4.4. Cytotoxicity

The embryonic *D. melanogaster* (Schneiders D.mel-II) insect cell line was sourced from the American Type Culture Collection (ATCC). In addition, four human tumourous cell lines, A2780 (ovarian carcinoma), HepG2 (Caucasian hepatocyte carcinoma), PC3 (Caucasian prostate adenocarcinoma), MCF-7 (mammary gland adenocarcinoma), and one healthy cell line HS27 (normal foreskin) were used to assess F9. The insect lines were housed on the laboratory bench in a glass incubator at 27 °C whilst the mammalian lines were kept separate and maintained at 37 °C with 5% CO_2 supplied.

All extracts were dissolved in DMSO (Sigma) at 10.0 mg/mL and diluted to working concentrations. Each concentration was screened in triplicate using the ATP-Lite™-M (Perkin–Elmer) luminescence kit assay (Cree and Androtti, 1997). Cells were seeded at approximately 2×10^5 cells/mL (99 μL) into a 96-well plate (Nunc) prior to the addition (1 μL) of a test agent or control blank. The inhibition of cell viability was calculated by comparing the number of viable treated cells with the cells exposed to blank DMSO.

4.5. Whole organism bioassays

Extract residue was dissolved in MeOH or EtOAc and added to a pre-prepared solution (200 ppm) of surfactant Triton X-100™ (octyphenol ethylene oxide condensate; Union Carbide, Sigma Chemicals, St. Louis, MO, USA) to the desired concentration (1.0% and 0.5% (w/v)). Insecticidal sprays were carried out by a Potter precision spray tower (Burkard, Rickmansworth, Herts, UK), at a pressure of 18.5 psi.

Young female adult *T. urticae* were collected from a mass culture of an organophosphate susceptible strain maintained at the University of Western Sydney's Hawkesbury campus (Beneficial Bug Company Pty. Ltd., Richmond, NSW, Australia 2753) and reared on insecticide free potted French bean (*Phaseolus vulgaris* L.) seedlings in a glasshouse under conditions of 25 ± 5 °C, $65 \pm 5\%$ RH and photoperiod 14:10 h L:D. Mites ($n = 20$ mites, 4 replicates) were randomly selected then transferred to French bean leaf discs (25 mm diameter) mounted on moistened cotton wool in Petri dishes (90 mm × 15 mm). Mites were sprayed (5 mL aliquot) at 1.0% (w/v) and after spraying, were maintained at 23 ± 5 °C; 14:10 h L:D. Mites were considered dead if appendages did not move after a gentle prod 24 h post treatment.

In a separate experiment, early second instar of *P. xylostella* were used to assess repellency and antifeeding activity of the ethyl acetate soluble fraction in a choice bioassay. Both sides of cabbage leaf discs, *Brassica oleracea* var. Sugarloaf (15 mm diameter), were treated with 5 mL of the test product at the desired concentration via the Potter tower. Three treated and three control discs were alternately arranged in a Petri dish (90 mm diameter) lined with moistened filter paper ($n = 4$ replicates). Larvae were deprived of food 3 h before use in bioassay and exactly ten larvae were placed into the middle of the test arena where they could choose to settle and feed on a treated or a control leaf disc. Observations on the number of larvae on each disc were made at 2, 6, 24 and 48 h and a repellency index (RI) was calculated where RI = # Control − # Treated/(Control + # Treated) × 100 (Cole, 1994). A visual assessment of the extent of feeding was also recorded after 48 h at which time the experiment was terminated as 50% of leaf discs were consumed, making the bioassay no-choice.

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