Iridoid Glucosides with Insecticidal Activity from *Galium melanantherum*

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The insecticidal activity of the endemic species *Galium melanantherum* was evaluated against *Crematogaster scutellaris* ants and *Kalotermes flavicollis* termites. Iridoid glucosides **1 – 7** were isolated for the first time as metabolites of the investigated plant, along with the coumarin scopolin. The main components of the extract were found to be the non-acetylated iridoids: geniposidic acid (**1**), 10-hydroxyloganin (**2**), deacetyldaphylloside (**3**), monotropein (**4**), deacetylasperulosidic acid (**5**) and scandoside (**6**), while asperulosidic acid (**7**) was present only in minute quantities. All isolated metabolites were identified on the basis of their spectral data. Laboratory bioassays revealed significant levels of toxicity for **1 – 4** against *Kalotermes flavicollis* termites and *Crematogaster scutellaris* ants.

Key words: Galium melanantherum, Iridoids, Insecticidal Activity

Introduction

Galium genus (family Rubiaceae) comprises *ca.* 300 species, 145 of which are present in Europe (Mabberley, 1997). *Galium* species are known from ancient times (Tzakou, 1988) to possess therapeutic properties such as diuretic (BHC, 1992), antispasmodic (Paris and Moyse, 1967), vulnerary, healing cancerous wounds (Harwell, 1982). *G. melanantherum* Boiss. belongs to the section *Leiogalium* and is a member of the *G. incurvum* group. The narrow leaved species of the *G. incurvum* group forms an intriguing, from the point of evolution and taxonomy, polymorphic polyploidy complex (Ehrendorfer and Krendl, 1976; Ehrendorfer and Schönbeck-Temesy, 1982; Krendl, 1987). *G. melanantherum* is endemic in Southeast Greece growing on dry, rocky places (Ehrendorfer and Krendl, 1976). The chemical composition of the petroleum ether extract of *G. melanantherum* as well as its phenolic content have been earlier reported (Tzakou *et al.*, 1988, 1990).

In continuation of our investigations towards the isolation of plant metabolites with insecticidal activity we studied and describe in the present report the iridoidal content of *G. melanantherum* and the activity evaluation of the main compounds against *Kalotermes flavicollis* termites and *Crematogaster scutellaris* ants. Iridoids have exhibited in the past a wide range of activities against insects (Dev and Koul, 1997; Harborne, 1989; Gkinis *et al.*, 2003).

Materials and Methods

General experimental procedures

Optical rotations were measured on a Perkin-Elmer model 341 polarimeter with a 10 cm cell. NMR spectra were recorded on a Bruker AC 200 and a Bruker DRX 400 spectrometer. Chemical shifts are given in δ (ppm) and TMS was used as internal standard (s, singlet; d, doublet; t, triplet; m, multiplet). The 2D experiments (^{1}H-^{1}H COSY, HMQC, HMBC) were performed using standard Bruker microprograms. EIMS data were recorded on a Hewlett Packard 5973 mass selective detector. VLC separations were performed with Kieselgel 60H (Merck), TLC was performed with Kieselgel 60 F_{254} (Merck aluminum support plates) and spots were detected after spraying with a 15% H_2SO_4 solution in MeOH and charing of the plates. HPLC separations were performed using a Waters 2487 pump system with a Dual λ absorbance detector and a Supercosil SPLC-18 (column size 25 cm × 10 mm, 5 μm) column.

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Plant material

Aerial parts of *G. melanantherum* were collected in the flowering stage at Metohi site on Mt Parnes (prefecture of Attica) in July 2000. A voucher specimen (OT-71) has been deposited in the Herbarium of the University of Athens (ATHU).

Extraction and isolation

Dried and ground aerial parts of *G. melanantherum* (340 g) were extracted at room temperature with CH_2Cl_2/MeOH (1:1 v/v) to give 39 g of crude extract, which were subjected to vacuum liquid chromatography (VLC) over silica gel (silica gel 60H, Merck Art. 7736) and eluted with solvents of increasing polarity. Fractions were monitored by TLC and similar fractions were combined into thirteen fractions labelled as A1–A13. Fraction A11 was separated by column chromatography (CC) over Sephadex LH-20 using MeOH/H_2O (80:20 v/v) and yielded 40 fractions (B1–B40). Fractions B29–B38 were combined and further separated by CC on silica gel using CH_2Cl_2/MeOH and EtOAc/MeOH mixtures of increasing polarity to yield 100 fractions (C1–C100). Preparative TLC (cellulose, Merck Art. 5716; 10% AcOH) of fractions C83–C100 yielded the coumarin scopolin (**8**, 7.5 mg). Fractions B75–B82 were combined and subjected to CC on silica gel using CH_2Cl_2/MeOH mixtures of increasing polarity to yield geniposidic acid (**1**, 12.2 mg) and asperulosidic acid (**7**, 4.4 mg). Fraction A12 after repetitive CC on silica gel afforded two fractions Z39–Z45 and Z119–Z130. Fraction Z39–Z45 was subjected to CC on silica gel using CH_2Cl_2/MeOH mixtures of increasing polarity and yielded 126 fractions (H1–H126). Fractions H40–H43 were further purified by reversed phase HPLC to yield 10-hydroxyloganin (**2**, 12 mg) and deacetyldaphylloside (**3**, 4.9 mg). Further purification of fraction Z119–Z130 by reversed phase HPLC afforded monotropein (**4**, 5.7 mg), deacetylasperulosidic acid (**5**, 4.1 mg), scandoside (**6**, 4.1 mg) and geniposidic acid (**1**, 11.5 mg).

Geniposidic acid (**1**): α_D = +10.23° (H_2O, *c* = 0.43). – ^{1}H NMR [CD_3OD/D_2O (1:1), 400 MHz]: δ = 5.14 (1H, d, *J* = 6.83 Hz, H-1), 7.15 (1H, s, H-3), 3.15 (1H, m, H-5), 2.75 (1H, m, H-6), 2.05 (1H, dd, *J* = 15.70, 5.46 Hz, H-6), 5.78 (1H, brs, H-7), 2.75 (1H, m, H-9), 4.26, 4.17 (each 1H, d, *J* = 14.34 Hz, H-10), 4.73 (1H, d, *J* = 7.85 Hz, H-1′), 3.14–3.46 (4H, m, H-2′, H-3′, H-4′, H-5′), 3.84 (1H, brd, *J* = 12.12 Hz, H-6$_b$′), 3.66 (1H, dd, *J* = 12.12, 4.78 Hz, H-6$_a$′). – ^{13}C NMR [CD_3OD/D_2O (1:1), 50 MHz]: δ = 97.37 (d, C-1), 148.46 (d, C-3), 118.47 (s, C-4), 36.53 (d, C-5), 39.47 (t, C-6), 129.49 (d, C-7), 143.02 (s, C-8), 47.23 (d, C-9), 60.98 (t, C-10), 171.40 (s, C-11), 99.76 (d, C-1′), 74.14 (d, C-2′), 77.45 (d, C-3′), 70.79 (d, C-4′), 76.97 (d, C-5′), 61.86 (t, C-6′).

10-Hydroxyloganin (**2**): α_D = −0.56° (H_2O, *c* = 1.08). – ^{1}H NMR [CD_3OD/D_2O (1:1), 400 MHz]: δ = 5.15 (1H, d, *J* = 4.78 Hz, H-1), 7.44 (1H, s, H-3), 3.05 (1H, m, H-5), 2.18 (1H, brdd, *J* = 13.67, 7.17 Hz, H-6), 1.54 (1H, ddd, *J* = 13.67, 9.22, 4.78 Hz, H-6), 4.27 (1H, m, H-7), 2.05 (1H, m, H-8), 2.05 (1H, m, H-9), 3.83 (1H, brd, *J* = 11.95 Hz, H-10), 3.62 (1H, m, H-10), 3.67 (3H, s, $-COOCH_3$), 4.68 (1H, d, *J* = 8.2 Hz, H-1′), 3.21–3.43 (4H, m, H-2′, H-3′, H-4′, H-5′), 3.80 (1H, m, H-6$_b$′), 3.65 (1H, m, H-6$_a$′). – ^{13}C NMR [CD_3OD/D_2O (1:1), 50 MHz]: δ = 98.80 (d, C-1), 153.12 (d, C-3), 112.76 (s, C-4), 32.57 (d, C-5), 42.16 (t, C-6), 72.70 (d, C-7), 41.98 (d, C-8), 49.04 (d, C-9), 61.82 (t, C-10), 170.50 (s, C-11), 52.49 (q, $-COO\underline{C}H_3$), 100.05 (d, C-1′), 73.99 (d, C-2′), 77.48 (d, C-3′), 70.75 (d, C-4′), 77.04 (d, C-5′), 61.64 (t, C-6′).

Deacetyldaphylloside (**3**): α_D = +10.30° (H_2O, *c* = 0.40). – ^{1}H NMR (CD_3OD, 400 MHz): δ = 5.08 (1H, d, *J* = 9.22 Hz, H-1), 7.68 (1H, d, *J* = 1.03 Hz, H-3), 3.04 (1H, ddd, *J* = 7.51, 5.80, 1.03 Hz, H-5), 4.82 (1H, dd, *J* = 5.80, 2.42 Hz, H-6), 6.04 (1H, d, *J* = 2.05 Hz, H-7), 2.56 (1H, dd, *J* = 8.54, 8.54 Hz, H-9), 4.48, 4.23 (each, 1H, d, *J* = 15.70 Hz, H-10), 3.76 (3H, s, $-COOCH_3$), 4.74 (1H, d, *J* = 7.85 Hz, H-1′), 3.24–3.43 (4H, m, H-2′, H-3′, H-4′, H-5′), 3.87 (1H, brd, *J* = 12.63 Hz, H-6$_b$′), 3.64 (1H, dd, *J* = 12.63, 5.80 Hz, H-6$_a$′). – ^{13}C NMR (CD_3OD, 50 MHz): δ = 101.59 (d, C-1), 155.91 (d, C-3), 107.80 (s, C-4), 41.86 (d, C-5), 75.02 (d, C-6), 129.67, (d, C-7), 150.65 (s, C-8), 45.39 (d, C-9), 61.20 (t, C-10), 171.31 (s, C-11), 52.60 (q, $-COO\underline{C}H_3$), 100.05 (d, C-1′), 74.17 (d, C-2′), 77.55 (d, C-3′), 70.83 (d, C-4′), 77.00 (d, C-5′), 61.93 (t, C-6′).

Monotropein (**4**): α_D = −74° (H_2O, *c* = 0.05). – ^{1}H NMR [CD_3OD/D_2O (1:1), 400 MHz]: δ = 5.56 (1H, d, *J* = 1.95 Hz, H-1), 7.08 (1H, s, H-3), 3.59 (1H, m, H-5), 6.23 (1H, dd, *J* = 5.62, 2.44 Hz, H-

6), 5.68 (1H, dd, J = 5.62, 1.46 Hz, H-7), 2.69 (1H, dd, J = 8.80, 1.95 Hz, H-9), 3.67 (2H, m, H-10), 4.79 (1H, d, J = 8.07 Hz, H-1′), 3.22–3.93 (4H, m, H-2′, H-3′, H-4′, H-5′), 3.70 (2H, m, H-6$_a$′, H-6$_b$′). – ^{13}C NMR [CD_3OD/D_2O (1:1), 50 MHz]: δ = 95.23 (d, C-1), 151.83 (d, C-3), 112.03 (s, C-4), 39.26 (d, C-5), 138.19 (d, C-6), 133.27 (d, C-7), 86.19 (s, C-8), 45.47 (d, C-9), 68.07 (t, C-10), 171.79 (s, C-11), 99.71 (d, C-1′), 74.28 (d, C-2′), 77.41 (d, C-3′), 71.05 (d, C-4′), 77.85 (d, C-5′), 62.15 (t, C-6′).

Deacetylasperulosidic acid (**5**): α_D = +10.60° (MeOH, c = 0.40). – ^{1}H NMR (CD_3OD, 400 MHz): δ = 5.06 (1H, d, J = 8.87 Hz, H-1), 7.63 (1H, s, H-3), 3.03 (1H, dd, J = 6.45, 6.45 Hz, H-5), 4.90 (1H, m, H-6), 6.03 (1H, s, H-7), 2.57 (1H, dd, J = 8.06, 8.06 Hz, H-9), 4.48, 4.23 (each, 1H, d, J = 15.58 Hz, H-10), 4.74 (1H, d, J = 7.79 Hz, H-1′), 3.23–3.43 (4H, m, H-2′, H-3′, H-4′, H-5′), 3.87 (1H, dd, J = 12.09, 1.62 Hz, H-6$_b$′), 3.64 (1H, dd, J = 12.09, 5.91 Hz, H-6$_a$′). – ^{13}C NMR (CD_3OD, 50 MHz): δ = 101.30 (d, C-1), 154.66 (d, C-3), 109.53 (s, C-4), 42.97 (d, C-5), 75.49 (d, C-6), 129.78 (d, C-7), 151.54 (s, C-8), 45.91 (d, C-9), 61.75 (t, C-10), 171.86 (s, C-11), 100.34 (d, C-1′), 74.98 (d, C-2′), 78.51 (d, C-3′), 71.64 (d, C-4′), 77.81 (d, C-5′), 62.81 (t, C-6′).

Scandoside (**6**): α_D = −5.60° (H_2O, c = 0.18). – ^{1}H NMR (CD_3OD, 400 MHz): δ = 4.86 (1H, d, J = 8.53 Hz, H-1), 7.32 (1H, s, H-3), 2.92 (1H, dd, J = 7.51, 7.51 Hz, H-5), 4.57 (1H, brd, J = 5.80 Hz, H-6), 5.84 (1H, s, H-7), 2.79 (1H, dd, J = 8.20, 8.20 Hz, H-9), 4.39, 4.20 (each, 1H, d, J = 15.70 Hz, H-10), 4.74 (1H, d, J = 7.85 Hz, H-1′), 3.21–3.42 (4H, m, H-2′, H-3′, H-4′, H-5′), 3.85 (1H, dd, J = 12.02, 2.05 Hz, H-6$_b$′), 3.65 (1H, dd, J = 12.02, 5.12 Hz, H-6$_a$′). – ^{13}C NMR (CD_3OD, 50 MHz): δ = 99.09 (d, C-1), 151.98 (d, C-3), 113.85 (s, C-4), 47.19* (d, C-5), 82.84 (d, C-6), 129.89 (d, C-7), 147.42 (s, C-8), 47.38* (d, C-9), 61.42 (t, C-10), 169.98 (s, C-11), 100.26 (d, C-1′), 74.87 (d, C-2′), 78.36 (d, C-3′), 71.49 (d, C-4′), 77.81 (d, C-5′), 62.59 (t, C-6′). * Values could be interchangeable.

Asperulosidic acid (**7**): α_D = +13.80° (MeOH, c = 0.44). – ^{1}H NMR (CD_3OD, 400 MHz): δ = 5.04 (1H, d, J = 8.99 Hz, H-1), 7.58 (1H, s, H-3), 3.05 (1H, m, H-5), 4.87 (1H, m, H-6), 6.02 (1H, s, H-7), 2.63 (1H, dd, J = 7.83, 7.83 Hz, H-9), 4.95 (1H, m, H-10), 4.83 (1H, m, H-10), 2.11 (3H, s, CH_3CO–), 4.74 (1H, d, J = 7.82 Hz, H-1′), 3.23–3.42 (4H, m, H-2′, H-3′, H-4′, H5′), 3.87 (1H, brd, J = 11.70 Hz, H-6$_b$′), 3.64 (1H, dd, J = 11.70, 5.48 Hz, H-6$_a$′). – ^{13}C NMR (CD_3OD, 50 MHz): δ = 100.97 (d, C-1), 153.90 (d, C-3), 109.18 (s, C-4), 43.00 (d, C-5), 75.61 (d, C-6), 131.77 (d, C-7), 146.00 (s, C-8), 46.46 (d, C-9), 63.88 (t, C-10), 170.18 (s, C-11), 172.57 (s, $CH_3\underline{CO}$–), 20.73 (q, $\underline{CH_3}CO$–), 100.46 (d, C-1′), 74.95 (d, C-2′), 78.55 (d, C-3′), 71.56 (d, C-4′), 77.85 (d, C-5′), 62.96 (t, C-6′).

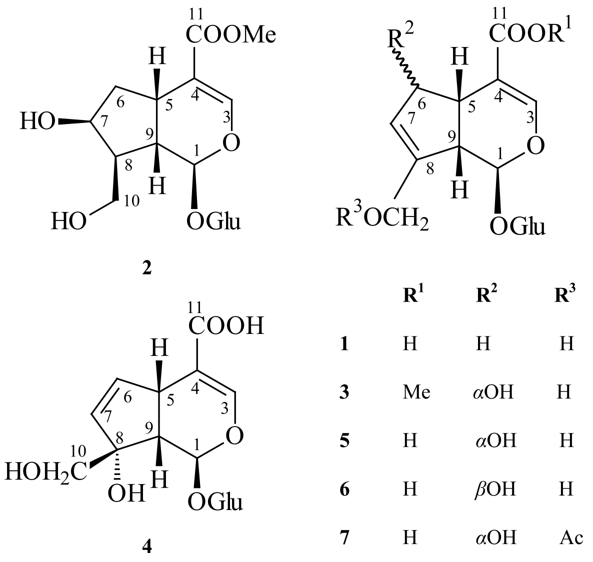
Insect collection and bioassays

Assays were performed against the arboricolous ant *C. scutellaris* (Olivier) (Hymenoptera, Formicidae) and the termite *K. flavicollis* Fabricius (Isoptera, Kalotermitidae). Both insects were collected from a pine forest at the foot of Mt Hemitus (prefecture of Attica). The ants were collected from the bole of *Pinus halepensis* where they attended the pine scale *Marchalina hellenica* and the termites from a colony in a stump of the same pine species. Both insects were transferred to laboratory Plexiglas rearing cages and supplied the necessary populations for the bioassays. The queenless ant colony was fed with artificial food made from insecticide-free corn flour and the ingredients suggested by Castane and Zapata (2005) except beef. Live pine scales were offered to ants since they regularly use them for their honeydew and as food on pine branches. Dichloromethane (200 μl) was used to dissolve 100 μg of each assayed metabolite. The solution was introduced in a flat-bottomed Haggerton insect rearing bottle. After the evaporation of the solvent the bottle was sealed with Whatman No 4 filter paper in sets of *ca.* 20 repetitions for each insect and compound. 5–10 insects were placed in each bottle. The observations were made in specific time intervals and insects were considered dead if found irreversibly motionless.

The data from the bioassays were processed by using the binary regression algorithm proposed by Throne *et al.* (1995) and the binary model was the probit one (Dobson, 1990; Cochran and Snedecor, 1980) in a quantal mode.

Results and Discussion

Seven pure compounds were isolated and identified as the iridoid glucosides (Fig. 1): geniposidic acid (**1**) (Akdemir and Calis, 1991; Chaudhuri *et al.*, 1980; El-Naggar and Beal, 1980; Takeda *et al.*,



	R^1	R^2	R^3
1	H	H	H
3	Me	αOH	H
5	H	αOH	H
6	H	βOH	H
7	H	αOH	Ac

Fig. 1. Iridoid glucosides from the studied *Galium melanantherum.*

1975), 10-hydroxyloganin (**2**) (Kamiya *et al.*, 2002; Boros and Stermitz, 1990), deacetyldaphylloside (**3**) (Kamiya *et al.*, 2002; Boros and Stermitz, 1990; Ishiguro *et al.*, 1983), monotropein (**4**) (Boros and Stermitz, 1990; Davini *et al.*, 1981; El-Naggar and Beal, 1980), deacetylasperulosidic acid (**5**) (Kamiya *et al.*, 2002; Deliorman *et al.,* 2001; Heyden, 1983; El-Naggar and Beal, 1980), scandoside (**6**) (Kamiya *et al.*, 2002; Mitova *et al.*, 1999; Heyden, 1983; El-Naggar and Beal, 1980) and asperulosidic acid (**7**) (Kamiya *et al.*, 2002; Deliorman *et al.*, 2001; Mitova *et al.*, 1999; El-Naggar and Beal, 1980; Chaudhuri *et al.*, 1980) in addition to the coumarin scopolin (Fliniaux *et al.*, 1997; Harborne, 1973). The spectral data of the isolated compounds were identical with those reported in the literature.

The bioassays did not focus on the effect of concentrations but on the range of duration. The duration between two observations was considered as dose, and since the iridoid metabolite and the concentration were constant throughout the experiments, the measurements constituted serial time-mortality data (Krager and Hartmann, 2001). The rational behind this consideration lies in the fact that the tested compounds are continuously dissolved in the lipid layer lining the ectodermis of the insect cuticle. In this way they are transferred in the sensitive tracheal openings and the buccal cavity through the external mouthparts.

Probit regression was preferred over logit or the complementary log-log (CLL) regression because of its simplicity in estimating lethal time 50% (LT$_{50}$); that is the time required to kill 50% of the tested insects. In addition probit had a smaller log-likelihood ratio D statistic except in one case where it was inferior to CLL (Krager and Hartmann, 2001; Dobson, 1990).

Between the two insects *K. flavicollis* termites were found to be more sensitive to the tested iridoids (Table I). Most probably this is due to their lightly chitinized cuticle compared to the cuticle of *C. scutellaris* ants. In addition the wandering of the ants on the vertical walls of the Haggerton bottle minimized the compound particles delivered to the insect. Geniposidic acid (**1**) was found to be

Table I. Insecticidal activity of iridoids **1**–**6** against *Kalotermes flavicollis* termites and *Crematogaster scutellaris* ants.

Compound	r^2	Binary model	LT$_{50}$ (sd) [100 μg·min]
Kalotermes flavicollis			
Geniposidic acid (**1**)	0.993***	Probit	744.3 (281.0)
10-Hydroxyloganin (**2**)	ns	–	$>4\cdot10^3$
Deacetyldaphylloside (**3**)	0.990**	Probit	1060.5 (334.2)
Monotropein (**4**)	0.991**	Probit	833.0 (368.6)
Deacetylasperulosidic acid (**5**)	0.997***	Probit	1293.3 (312.6)
Scandoside (**6**)	0.982**	Probit	861.1 (436.5)
Crematogaster scutellaris			
Geniposidic acid (**1**)	0.988**	Probit	3093.4 (1181.6)
10-Hydroxyloganin (**2**)	0.989**	Probit	1481.6 (665.1)
Deacetyldaphylloside (**3**)	0.999***	Probit	828.8 (127.6)
Monotropein (**4**)	0.877*	Probit	9361.1 (5358.5)
Deacetylasperulosidic acid (**5**)	0.962**	CLL	3931.9 (1143.1)
Scandoside (**6**)	0.997***	Probit	9185.1 (6846.9)

* $P < 0.05$; ** $P < 0.01$;
*** $P < 0.001$;
ns, not significant.
Performance parameters shown are the coefficients of determination (r^2) and the LT$_{50}$ (sd).

the more efficient killing agent for termites and scandoside (**6**) to be second in rank almost equally effective to monotropein (**4**). These three compounds are the least effective killing agents for ants that are affected by deacetyldaphylloside (**3**) and 10-hydroxyloganin (**2**).

In the taxa of Rubioideae the iridoids asperuloside and/or deacetylasperulosidic acid are uniformly distributed (Inouye *et al.*, 1988). Iridoids among other compounds have been used in attempts to clarify the classification problems in *Galium* and closely related genera (Corrigan *et al.*, 1978). It is known that in the genus *Galium* iridoids are biosynthesized through iridodial and deoxyloganic acid to deacetylasperulosidic acid and asperuloside (Inouye *et al.*, 1988; Inouye and Uesato, 1986). It seems that *G. melanantherum* contains mostly primitive iridoids, the non-acetylated iridoid acids, with geniposidic acid prevailing. Also two members of the *G. incurvum* group, *G. mirum* and *G. rhodopeum*, have been reported to contain primitive iridoids, with most abundant the deacetylasperulosidic acid, whereas geniposidic acid was found only in traces in *G. mirum* (Mitova *et al.*, 1999).

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