SUPPLEMENTARY MATERIAL

Phytochemical study of *Bituminaria basaltica* aerial parts, an Italian endemism

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Abstract

The first phytochemical investigation of *Bituminaria basaltica* aerial parts, endemic species of Aeolian Islands, led to the isolation and identification of eight compounds including plicatin B (**3**), two furanocoumarins: angelicin (**1**), psoralen (**2**), three pterocarpans: erybraedin C (**4**), 3,9-dihydroxy-4-isoprenyl-pterocarpan (**5**), bitucarpin A (**8**) and two flavonoids glycosides: isoorientin (**6**), daidzin (**7**). Their structures were elucidated by spectroscospic techniques in comparison with data reported in the literature. Sesquiterpenes characterized the essential oil composition of the titled plant where β-caryophyllene and germacrene D were the main constituents.

Keywords: Fabaceae, essential oil, phytochemicals, CG-MS, NMR, chemotaxonomy

3.1-General procedures.

Chromatographic techniques. The purifications were carried out with following parameters and instruments: column chromatography (CC Silica gel Kiesel gel 60, 70-230 mesh, Merck), flash chromatography using Biotage® instrument Kiesel gel 60 (230-410 μm); gel filtration chromatography, Sephadex LH-20 Pharmacia Chemicals; analytical TLC by Merck Kiesel gel 60 F254. High performance liquid chromatography (HPLC) was performed on a Waters 600 E instrument, photodiode array detector Waters 486 Tunable Absorbance (PDA), Synergic Fusion-RP-18 (250 x 4.60 mm x 5 μm) column (Phenomenex). A Shimadzu instrument with LC-8A pumb, RID-10A diode array detector was used for semi preparative HPLC on a RPC-18 column (Waters, Bondapak, 30 cm x 7.8 mm, flow rate: 1 ml/min.). TLC chromatograms were visualized under UV light at 256 and 366 nm and/or sprayed with Komarowsky reagent, Naturstoff reagents-PEG and cerium sulfate reagents.

Spectroscopic analysis. The nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC-250 (software Topsin) spectrometer using $CDCl_3$, CD_3OD and DMSO-d_6 as solvents.

GC-MS Analysis. GC-MS analysis were carried out with a Varian CP-3800 gas chromatograph equipped with a DB 5 capillary column (30m x 0.25mm; coating thickness: 0.25 µm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions: injector and transfer line temperatures, 220-240 ºC, respectively; carrier gas He (1 ml/min); split ratio, 1:30, injection of 0,2 µl (10% hexane solution). The oven temperature was programmed rising from 60 ºC to 240 ºC at 3º/min. The identification of the components was performed by comparison of their retention time with those of pure authentic samples and by means of their linear retention indices (LRI) relative to the series of *n*-hydrocarbons, and on computer matching against commercial (NIST 98 and ADAMS) and home-made library mass spectra built up from pure substances, components of know oils and MS literature data.

3.2- Plant material.

Aerial parts of *B. basaltica* Miniss., C. Brullo, Brullo, Giusso & Sciandr. were collected in Filicudi (Sicily, Italy) on 3 June 2013 and 14 June 2016. The plant material was identified by one of us (Pietro Minissale) and a voucher specimen (0103062013; 38°33'36.28" N, 14°33'58.80"E) deposited in University of Catania Herbarium (CAT).

3.3- Extraction and isolation of *Bituminaria basaltica*.

Air-dried and powdered *B. basaltica* aerial parts (1220 g) were exhaustively extracted in a Soxhlet apparatus with *n*-hexane (E, 16.8 g), chloroform (C,17.18 g) and methanol (M, 82.6 g) in turn. The *n*-hexane extract was partitioned between MeOH and H_2O (8:2). The methanolic soluble portion (EM, 1.0 g) was purified by flash chromatography column using a gradient elution with mixtures of hexane/ EtOAc from 9:1 to 7:1 (v/v) to obtain seven fractions (EMa-EMg). EMe (0.116g) was purified by preparative TLC on silica gel (hexane: EtOAc, 7:3, v/v) to give compound **1** (10 mg) and **2** (3 mg). EMd was submitted to flash chromatography with the following solvents: hexane/$CHCl_3$ (1:1, 3:7, 2:8; v:v); $CHCl_3$ (100%), $CHCl_3$-MeOH (95:5, 9:1, 8:2, v/v) and MeOH (100%) to afford 14 subfractions (EMd_a-EMd_r). These last fractions were subjected to RP18-HPLC with CH_3CN/ H_2O (7:3) to obtain compounds **3** (6 mg) and **8** (12 mg).

Chloroformic extract was purified by Sephadex LH-20 ($CHCl_3$-MeOH, 1:1) to give nine fractions (C*a*-C*g*). Fraction C*g* was fractionated on silica gel column, eluted with $CHCl_3$: EtOAc in mixtures of increasing polarity (95:5, 9:1, 8:2, 1:1, v/v) to obtain 27 fractions (X1-X27). These subfractions were further purified by silica gel chromatography ($CHCl_3$: EtOAc, 9:1, v/v) afforded to compounds **4** (46 mg), **5** (17 mg) and **3** (6 mg).

Compounds **6** (4 mg) and **7** (6 mg) were isolated from the methanolic extract after purification by Sephadex LH-20 (MeOH-H_2O, 8:2, v/v) and RP18-HPLC (Bondapack column) using MeOH-H_2O (1:1, v/v) as eluent.

Angelicin (**1**)

^{1}H NMR (CD_3OD) δ: 6.40 (1H, d, 9.50 Hz, H-3); 7.14 (1H, d, 2.1 Hz, H-3'); 7.38 (1H, d, 8.4 Hz, H-5); 7.45 (1H, d, 8.5 Hz, H-6); 7.70 (1H, d, 2.2 Hz, H-2'); 7.82 (1H, d, 9.60 Hz, H4).

^{13}C NMR ($CDCl_3$) δ: 104.8 (C-3'); 109.5 (C-6); 114.2 (C-10); 114.8 (C-3); 117.6 (C-8); 124.5 (C-5); 145.2 (C-4); 146.5 (C-2'); 149.2 (C-9); 158.0 (C-7); 161.57 (C-2).

Psoralen (**2**)

^{1}H NMR ($CDCl_3$) δ: 6.39 (1H, *d*, J= 9.6Hz, H-3); 6.84 (1H, *d*, 2.5 Hz, H-3'); 7.49 (1H, brs); 7.70 (1H, s, H-5); 7.71 (1H, d, 2.5Hz, H-2').

^{13}C NMR ($CDCl_3$) δ: 100.6 (C-8); 107.0 (C-3'); 115.4 (C-2); 120.5 (C-5); 126.0 (C-6); 144.7 (C-3); 147.6 (C-2').

Plicatin B (**3**)

^{1}H NMR ($CDCl_3$) δ: 1.77 (3H, *s*, H-5'); 1.78 (3H, *s*, H-4'); 3.36 (2H, *d*, 7.2 Hz); 3.79 (3H, *s*, OCH_3); 5.31 (1H, *t*, 7.3 Hz, H-2'); 6.28 (1H, *d*, 16 Hz, H-8); 7.30 (2H, *m*, H-2 and H-6); 7.63 (1H, *d*, 16 Hz, H-7).

^{13}C NMR ($CDCl^3$) δ: 18.2 (C-5'); 26.2 (C-4'); 28.7 (C-1'); 52.0 (OCH_3); 114.3 (C-8); 115.7 (C-5); 122.2 (C-2'); 126.5 (C-3); 128.0 (C-2); 129.1 (C-1); 130.2 (C-6); 133.9 (C-3'); 146.2 (C-7); 157.4 (C-4); 169.0 (CO).

Erybraedin C (**4**)

^{1}H NMR ($CDCl_3$) δ*:* 1.70 (6H, *s*, H-4' and H-4"); 1.76 (6H, *s*, H-4' and H"4); 3.28 (2H, *d*, 7.3 Hz, H-1'); 3.38 (2H, *d*, 7.3 Hz, H-1"); 3.38 (1H, *dt*, H-6a); 3.55 (1H, *d*, 11.2 Hz, H-6); 4.12 (1H, *dd*, 10.7 and 4.8 Hz, H-6); 5.21 (1H, *d*, 6.8 Hz, H-11a); 6.36 (1H, *s*, H-7); 6.53 (1H, *d*, 8.8 Hz, H-2); 6.94 (1H, *s*, H-10); 7.23 (1H, *d*, 8.8 Hz, H-1).

^{13}C NMR ($CDCl_3$) δ: 18.4(C-4'and C-4"); 23.0 (C-1'); 26.4 (C-5' and C-5"); 40.2 (C-6a); 67.5 (C-6); 79.7 (C-11a), 99.0 (C-10); 110.3 (C-2); 113.0 (C-1a); 115.8 (C-4);

119.6 (C-7a); 119.8 (C-8); 122.5 (C-2"); 123.0 (C-2'); 125.8 (C-7); 129.8 (C-1); 134.6 (C-3"); 134.7 (C-3'); 154.6 (C-4a); 155.7 (C-9); 156.2 (C-3); 159.4 (C-10a).

3,9-dihydroxy-4-isoprenyl-pterocarpan (**5**)

^{1}H NMR ($CDCl_3$) δ: 1.63 (3H, *s*, H-4'); 1.74 (3H, *s*, H-5'); 3.25 (2H, *m*, H-1'); 3.46 (2H, *t*, H-6); 5.44 (1H, *d*, 5.6 Hz, H-2'); 6.23 (1H, *d*, 2.2 Hz, H-10); 6.32 (1H,*dd*, 2.2 Hz, H-8); 6.49 (1H, *d*, 8.3 Hz, H-2); 7.09 (1H, *d*, 8.1 Hz, H-7); 7.13 (1H, *d*, 8.2 Hz, H-1).

^{13}C NMR ($CDCl_3$) δ: 17.1 (C-4'); 22.4 (C-1'); 25.2 (C-5'); 40.1 (C-6a); 67.1 (C-6); 79.9 (C-11a); 97.9 (C-10); 107.8 (C-8); 109.2 (C-2); 112.1 (C-1a); 116.4 (C-4); 119.0 (C-7a); 123.5 (C-2'); 125.3 (C-7); 129.1 (C-1); 130.7 (C-3'); 154.9 (C-4a); 156.6 (C-3); 159.0 (C-9).

Isoorientin (**6**)

^{1}H NMR (DMSO) δ: 3-4.1 (glucosyl moiety); 4.56 (1H, 9.6 Hz, G-1); 6.41 (1H, *s*, H-8); 6.60 (1H, *s*, H-3); 6.82 (1H, *m*, H-5'); 7.36 (1H, *d*, 2.2, H-2'); 7.40 (1H, broad *s*, H-6').

^{13}C NMR (DMSO) δ: G6 (63.2); G2 (72.0); G4 (72.4); G1 (75.0); G3 (80.4); G5 (83.3); 95.6 (C-8); 103.0 (C-3); 104.0 (C-10); 110.8 (C-6); 114.0 (C-2'); 117.0 (C-5'); 120.8 (C-1'); 127.8 (C-6'); 147.0 (C-3'); 148.0 (C-4'); 162.5 (C-2 and C-7); 183.4 (C-4).

Daidzin (**7**)

^{1}H NMR (CD_3OD) δ: 5.10 (1H, d, 7.6 Hz, H-6'); 6.86 (2H, d, 2.3 Hz, H-3'/5'); 7.22 (1H, dd, 8.8 Hz and 2.3 Hz, H-6); 7.26 (1H, d, 2.3 Hz, H-8); 7.38 (2H, *d*, 8.7 Hz, H-2'); 8.15 (1H, *d*, 8,8 Hz, H-5); 8.21 (1H, s, H-2).

^{13}C NMR (DMSO) δ: G-6 (60.7); G-4 (69.6); G-2 (73.1); G-5 (76.5); G-3 (77.2); G-1 (100.0); 103.4 (C-8); 115.6 (C-3' and C-5'); 115.9 (C-6); 118.4 (C-10); 122.3 (C-3); 124.8 (C-1'); 126.9 (C-5); 130.1 (C-2' and C-6'); 153.3 (C-2); 157.0 (C-4'); 157.3 (C-9); 161.7 (C-7); 178.0 (C-4).

3.4- Essential oils extraction.

Dried aerial parts from *B. basaltica* were subjected to hydrodistillation by Clevenger apparatus (3h). The essential oils (EOs) were collected with *n*-hexane (HPLC grade) due to very low yield (≤ 0.01%) and stored at -4ºC in the dark until use and analyzed by GC-MS.

Table S1. Chemical composition of the essential oil from *Bituminaria basaltica* aerial parts.

Entry	Class	Component	LRI	Content (%)
1	NT	Heptanal	903	0.2
2	MH	Tricyclene	938	1.4
3	MH	α-pinene	940	5.0
4	MH	Camphene	955	0.8
5	MH	Sabinene	978	0.2
6	MH	Myrcene	993	1.5
7	MH	Limonene	1032	0.4
8	OM	1,8-cineole	1036	tr
9	MH	β-ocimene	1042	0.1
10	NT	*n*-nonanal	1104	0.2
11	OM	Menthol	1178	0.1
12	OM	4-terpinenol	1180	0.1
13	NT	*n*-decanal	1206	0.1
14	SH	α-cubebene	1351	0.4
15	SH	cyclosativene	1371	0.2
16	SH	α-ylangene	1372	0.1
17	SH	α-copaene	1376	3.2
18	SH	β-bourborene	1383	1.6
19	SH	β-cubebene	1390	0.2
20	SH	β-elemene	1392	0.2
21	SH	α-cubebene	1396	0.1
22	SH	β-caryophyllene	1418	31.0
23	SH	β-copaene	1429	1.3
24	SH	Aromadendrene	1445	1.4
25	SH	*cis*-muurola-3,5-diene	1448	0.1
26	SH	α-humulene	1456	4.3
27	SH	Alloaromadendrene	1461	4.9
28	SH	γ-muurolene	1477	2.4
29	SH	Germacrene D	1481	15.8
30	AC	(*E*)-β-ionone	1485	0.1
31	SH	Bicyclogermacrene	1495	2.3
32	SH	α-muurolene	1499	0.9
33	SH	Germacrene A	1503	0.3
34	SH	δ-amorphene	1505	0.2
35	SH	*Trans*-γ-cadinene	1513	0.8
36	OS	Cubebol	1518	1.7
37	SH	δ-cadiene	1523	3.9
38	SH	*Trans*-cadina- 1(2), 4-diene	1533	0.2
39	OS	Germacrene D-4-ol	1576	0.2
40	OS	*Trans*-sesquisabinene hydrate	1547	0.6
41	OS	Caryophyllene oxide	1582	4.4
42	OS	Isoaromadendrene epoxide	1595	0.2

43	OS	Humulene epoxide II	1607	0.3
44	OS	1,10-di-epicubenol	1614	0.3
45	OS	1-epi-cubenol	1630	0.3
46	OS	Eremoligenol	1632	0.8
47	OS	*Cis*-cadin-4-en-7-ol	1637	0.2
48	OS	*Epi*-α-cadinol	1642	0.9
49	OS	α-muurolol	1651	0.3
50	OS	Ledene oxide	1680	2.2
		Total identified		***98.4***
		Non terpene derivatives		*0.5*
		Sesquiterpene hydrocarbons		*75.8*
		Oxygenated sesquiterpenes		*12.4*
		Monoterpene hydrocarbons		*9.4*
		Oxygenated monoterpenes		*0.2*
		Apocarotenoids		*0.1*

[a]LRI, Linear retention index. Compounds with relative percentages lower or equal to 0.1% are considered traces (tr.). The components are listed in order of their elution on the DB-5 column. Chemical compounds showed relatives percentages smaller than 0.01% were excluded from the table and the analysis.