SUPPLEMENTARY MATERIAL

Secondary metabolites from *Scrophularia canina* L.

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Abstract.

A re-examination of *Scrophularia canina* L. confirmed the presence of iridoid glucosides considered as chemotaxonomic markers for the Scrophulariaceae family, like aucubin, harpagide and 8-*O*-acetylharpagide, besides the further presence of 8-epiloganic acid, which is, indeed, considered the biogenetic precursor of iridoids normally found in Scrophulariaceae, and was recognized here for the first time in the studied species. Also verbascoside and (*E*)-phytol were evidenced for the first time in *S. canina*. The former compound is an almost ubiquitous glycosidic phenyl-ethanoid, which attains systematic importance when in co-occurrence with iridoids, and its taxonomical implications were discussed. The latter compound, even though it is omnipresent, is interestingly endowed with several biological activities, which may give an additional reason for the traditional uses of this plant.

Key words: Secondary Metabolites, Chemotaxonomy, Iridoid glucosides, Phenyl-propanoid Glucosides, Traditional uses.

3 - Experimental.

3.1 - Materials.

NMR spectra were recorded on a Varian (now Agilent Technologies) Mercury 300 MHz instrument using respectively CDCl₃, CD₃OD or D₂O as deuterated solvents. The chemical shifts are expressed in ppm from TMS.

MS spectra were performed on a Q-TOF MICRO spectrometer (Waters, Manchester, UK) equipped with an ESI source operating in negative and/or positive ion mode. The flow rate of the sample infusion was 10 µl per min. with 100 acquisitions per spectrum. Data were analysed by the MassLynx software developed by Waters.

Solvents of RPE grade were purchased from Sigma Aldrich (Milan, Italy) or Carlo Erba Reagenti (Milan, Italy) while silica gel 60 (70-230 mesh ASTM) was from Fluka.

3.2 - Plant material.

A sample of fresh plant material (170 g) was harvested in the Latium slope of the pre-park area of “National Park of Abruzzo, Lazio e Molise” (Central Italy). The botanical identification was performed by one of us (A.V.). The voucher specimen of the studied species is stored in our laboratory for further references and registered under the accession number SC16062013.

3.3 - Extraction and isolation of polar compounds.

The dried plant material (170.0 g) was exhaustively extracted three times using 96% ethanol (0.3 L each, for 48 h). The extracts were collected altogether and the ethanol was eliminated at reduced pressure until a water suspension was obtained. This suspension was frozen and then lyophilized, to preserve also temperature-sensitive compounds, obtaining 20.1 g of crude extract.

Repeated chromatographic separations on silica gel column were conducted on a fraction of the crude extract (6.3 g), using different eluents: i.e. *n*-butanol saturated with water for the first separation and then, on specific fractions derived from that one, mixtures of chloroform/methanol at various percentages, also increasing the polarity during the chromatographic run.

From the first separation procedures were separated five compounds: verbascoside (**2**) (12.4 mg) (Scarpati and Delle Monache, 1963), aucubin (**3**) (45.8 mg) (Birch et al., 1961), 8-*O*-acetylharpagide (**4**) (22.5 mg) (Scarpati et al., 1965), harpagide (**5**) (38.4 mg) (Bobbitt et al., 1969) and 8-epiloganic acid (**6**) (6.7 mg) (Bianco and Passacantilli, 1981). From a further separation of low polarity fractions was isolated another terpenoidic compound called (*E*)-phytol (**1**) (22.4 mg) (Bhattacharya et al., 2013) which was obtained as pure compound.

(E)-phytol (1): $^1\text{H-NMR}$ (CDCl_3 , 300MHz) δ : 4.15 (H-1, d, $J=6.9\text{Hz}$, 2H), 5.41 (H-2, td, $J=6.9/1.0\text{Hz}$, 1H), 1.99 (H-4, t, $J=7.4\text{Hz}$, 2H), 1.39-1.32 (H-5, m, 2H), 1.32-1.20 (H-6 H-8 H-9 H-10 H-12 H-13 H-14, m, 14H), 1.56-1.48 (H-7 H-11 H-15, m, 3H), 0.87 (H-16 H-17, d, $J=6.5\text{Hz}$, 6H), 0.84 (H-18 H-19, d, $J=6.7\text{Hz}$, 6H), 1.67 (H-20, s, 3H).

$^{13}\text{C-NMR}$ (CDCl_3 , 75 MHz) δ : 59.5 (C-1), δ 123.2 (C-2), δ 140.4 (C-3), δ 40.0 (C-4), δ 25.2 (C-5), δ 36.8 (C-6), δ 32.8 (C-7), δ 37.5 (C-8), δ 24.6 (C-9), δ 37.5 (C-10), δ 32.9 (C-11), δ 37.4 (C-12), δ 24.9 (C-13), δ 39.5 (C-14), δ 28.1 (C-15), δ 22.8 (C-16), δ 22.7 (C-17), δ 19.9 (C-18), δ 19.8 (C-19), δ 16.3 (C-20).

ESI-MS: m/z 319.15 $[\text{M}+\text{Na}]^+$; m/z 295.26 $[\text{M}-\text{H}]^-$.

Verbascoside (2): $^1\text{H-NMR}$ (CD_3OD , 400MHz), δ : 4.00 (H- α a Tir, dd, $J=16.0/6.4\text{Hz}$, 1H), 3.74-3.65 (H- α b Tir, m, 1H), 2.78-2.73 (H- β Tir, m, 2H), 6.66 (H-2, d, $J=2.0\text{Hz}$, 1H), 6.63 (H-5, d, $J=8.0\text{Hz}$, 1H), 6.52 (H-6, dd, $J=8.0/2.0\text{Hz}$, 1H), 6.23 (H- α Caff, d, $J=15.8\text{Hz}$, 1H), 7.55 (H- β Caff, d, $J=15.9\text{Hz}$, 1H), 7.12 (H-2'', d, $J=1.6\text{Hz}$, 1H), 6.74 (H-5'', d, $J=8.2\text{Hz}$, 1H), 6.91 (H-6'', dd, $J=8.3/2.0\text{Hz}$, 1H), 4.33 (H-1', d, $J=8.0\text{Hz}$, 1H), 5.15 (H-1'', d, $J=1.5\text{Hz}$, 1H), 1.05 (H-6'', d, $J=6.2\text{ Hz}$, 3H).

$^{13}\text{C-NMR}$ (CD_3OD , 100 MHz) δ : 69.8 (C- α Tir), 34.4 (C- β Tir), 131.4 (C-1), 116.2 (C-2), 143.8 (C-3), 142.3 (C-4), δ 116.7 (C-5), 121.2 (C-6), 168.2 (CO), 113.7 (C- α Caff), 147.4 (C- β Caff), 126.7 (C-1'''), 115.2 (C-2'''), 144.3 (C-3'''), 147.5 (C-4'''), 116.2 (C-5'''), 122.9 (C-6'''), 102.0 (C-1'), 73.9 (C-2'), 80.7 (C-3'), 69.4 (C-4'), 73.6 (C-5'), 60.1 (C-6'), 101.5 (C-1''), 71.0 (C-2''), 70.2 (C-3''), 71.7 (C-4''), 68.7 (C-5''), 17.0 (C-6'').

ESI-MS: m/z 647.23 $[\text{M}+\text{Na}]^+$; m/z 623.17 $[\text{M}-\text{H}]^-$.

Aucubin (3): $^1\text{H-NMR}$ (CD_3OD , 300MHz), δ : 4.85 (H-1, d, $J=7.2\text{Hz}$, 1H), 6.21 (H-3, dd, $J=5.9/1.6\text{Hz}$, 1H), 4.99 (H-4, dd, $J=5.9/3.9\text{Hz}$, 1H), 2.73-2.65 (H-5, m, 1H), 4.39 (H-6, dd, $J=2.5/1.9\text{Hz}$, 1H), 5.67 (H-7, bs, 1H), 2.79 (H-9, t, $J=7.6\text{Hz}$, 1H), 4.25 (H-10a, d, $J=15.5\text{Hz}$, 1H), 4.12 (H-10b, d, $J=17.7\text{Hz}$, 1H), 4.58 (H-1', d, $J=7.9\text{Hz}$, 1H).

$^{13}\text{C-NMR}$ (CD_3OD , 75 MHz) δ : 98.5 (C-1), 139.5 (C-3), 105.2 (C-4), 46.3 (C-5), 80.5 (C-6), 128.5 (C-7), 146.7 (C-8), 48.9 (C-9), 60.7 (C-10), 98.3 (C-1'), 75.6 (C-2'), 76.1 (C-3'), 70.8 (C-4'), 76.3 (C-5'), 60.6 (C-6').

ESI-MS: m/z 369.30 $[\text{M}+\text{Na}]^+$; m/z 345.12 $[\text{M}-\text{H}]^-$.

8-O-acetylharpagide (4): $^1\text{H-NMR}$ (D_2O , 300MHz) δ : 6.09 (H-1, s, 1H), 6.46 (H-3, d, $J=6.4\text{Hz}$, 1H), 5.06 (H-4, dd, $J=6.6/1.4\text{Hz}$, 1H), 3.78 (H-6, d, $J=3.0\text{Hz}$, 1H), 2.02 (H-7a, d, $J=14.9\text{Hz}$, 1H), 2.17 (H-7b, d, $J=15.0\text{Hz}$, 1H), 2.87 (H-9, s, 1H), 1.45 (H-10, s, 3H), 2.06 (OAc, s, 3H), 4.64 (H-1', d, $J=7.9\text{Hz}$, 1H).

$^{13}\text{C-NMR}$ (D_2O , 75 MHz) δ : 93.2 (C-1), 142.4 (C-3), 105.6 (C-4), 71.9 (C-5), 76.8 (C-6), 44.7 (C-7), 87.2 (C-8), 54.1 (C-9), 21.1 (C-10), 171.9 (OAc), 20.8 (OAc).

ESI-MS: m/z 429.14 $[\text{M}+\text{Na}]^+$; m/z 405.25 $[\text{M}-\text{H}]^-$.

Harpagide (5): $^1\text{H-NMR}$ (D_2O , 300Hz) δ : 5.73 (H-1, s, 1H), 6.36 (H-3, d, $J=6.4\text{Hz}$, 1H), 5.03 (H-4, d, $J=6.6\text{Hz}$, 1H), 3.78-3.71 (H-6, m, 1H), 1.81 (H-7a, dd, $J=14.0/4.0\text{Hz}$, 1H), 1.99 (H-7b, dd, $J=14.0/4.9\text{Hz}$, 1H), 2.55 (H-9, s, 1H), 1.24 (H-10, s, 3H), 4.73 (H-1', d, $J=8.0\text{Hz}$, 1H).

$^{13}\text{C-NMR}$ (D_2O , 75 MHz) δ : 94.8 (C-1), δ 143.0 (C-3), 105.2 (C-4), 73.7 (C-5), 77.4 (C-6), 46.9 (C-7), δ 86.0 (C-8), 55.2 (C-9), 23.4 (C-10), 102.0 (C-1'), 74.5 (C-2'), 77.8 (C-3'), 72.0 (C-4'), 78.4 (C-5'), 63.0 (C-6').

ESI-MS: m/z 387.28 $[\text{M}+\text{Na}]^+$; m/z 363.41 $[\text{M}-\text{H}]^-$.

8-epiloganic acid (6): $^1\text{H-NMR}$ (CD_3OD , 400MHz) δ : 5.67 (H-1, d, $J=0.9\text{Hz}$, 1H), 7.34 (H-3, s, 1H), 3.07 (H-5, m, 1H), 2.06-2.04 (H-6a, m, 1H), 1.96-1.94 (H-6b, m, 1H), 4.03-3.95 (H-7, m, 1H), 2.27-2.23 (H-8, m, 1H), 2.74 (H-9, t, $J=6.3\text{Hz}$, 1H), 1.11 (H-10, d, $J=6.4\text{Hz}$, 3H), 5.12 (H-1', d, $J=7.5\text{Hz}$, 1H).

$^{13}\text{C-NMR}$ (CD_3OD , 100MHz) δ : 93.9 (C-1), 108.3 (C-3), 120.4 (C-4), 30.6 (C-5), δ 39.9 (C-6), δ 77.9 (C-7), 39.8 (C-8), δ 47.3 (C-9), 14.4 (C-10), 173.9 (C-11), 98.1 (C-1'), 74.8 (C-2'), 78.1 (C-3'), 71.2 (C-4'), 78.0 (C-5'), 62.7 (C-6').

ESI-MS: m/z 399.29 $[\text{M}+\text{Na}]^+$; m/z 375.20 $[\text{M}-\text{H}]^-$.

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