SUPPLEMENTARY MATERIAL

Unusual molecular pattern in Ajugoideae subfamily: the case of *Ajuga genevensis* L. from Dolomites

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Abstract.

We analysed the ethanolic extract from *Ajuga genevensis* L. (Lamiaceae) growing in Dolomites, part of Italian Alps. Three new compounds for this species were identified: rosmarinic acid (**1**), oleanolic acid (**2**) and maslinic acid (**3**), representative of two different classes of chemical compounds (phenylpropanoids and pentacyclic triterpenes). *A. genevensis* resulted to be a valuable source of these compounds endowed with interesting biological activities (i.e. antioxidant, neuroprotective, anti-inflammatory, antiproliferative). The recognition of compounds (**1**), (**2**) and (**3**) may also confirm the ethnomedicinal uses of this plant. From a chemotaxonomical point of view, is worth to note that iridoids were not evidenced in this accession. Iridoids are considered chemotaxonomic marker in Lamiales, and, in contrast with a previous study on this species, the presence of aucubin was not confirmed. In addition the presence of large amounts of rosmarinic acid (**1**) was unexpected for a species that does not belong to subfamily Nepetoideae.

Key words: *Ajuga genevensis* L., Lamiaceae, phenylpropanoids, pentacyclic triterpenes, ethnopharmacology.

3 - Experimental.

3.1 - Materials.

NMR spectra were recorded on a Varian Mercury 300 MHz instrument using CDCl₃ or D₂O as deuterated solvents. The chemical shifts were 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 the negative and/or positive ion mode. The flow rate of the sample infusion was 10 µl/min. with 100 acquisitions per spectrum. Data were analysed using the MassLynx software developed by Waters.

Solvents of RPE grade were purchased from Sigma Aldrich (Milan, Italy) or Carlo Erba Reagenti (Milan, Italy), silica gel 60 (70-230 mesh ASTM) were from Fluka. Standard compound oleanolic acid (**2**) was purchased from Extrasynthese (Lyon, France), lot number 11012808.

3.2 - Plant material and place of collection.

Plant material was collected in the Dolomitis part of the Italian Alps near the lake of Braies, 1496 m a.s.l., on August 2013, at flowering stage. The environmental conditions of the place of collection correspond to those of a territory that, although particularly popular by tourists, is perfectly preserved as falling in the territory annexed to the Natural Park of Fanes - Senes and Braies. The botanical identification was performed by one of us (A.V.) using available literature (Pignatti, 1982; Conti et al, 2005) and also by correspondence with two authentic samples deposited in the Herbarium of the Department of Environmental Biology of "Sapienza" Università di Roma, which is indexed as: Herb. Anzalone: package number 177; record cards 17316 and 17316. The voucher specimen of the studied plants is stored in our laboratory for further references and registered under the accession number AG08082013.

3.3 - Extraction and isolation of polar compounds.

The dried plant material (9.6 g) was extracted at room temperature four times using 96% ethanol (0.3 L each, for 48 h under continue agitation). The consecutive extracts were collected together and the ethanol was eliminated at reduced pressure until a water suspension was obtained. The suspension was frozen and then lyophilized, to preserve also temperature-sensitive compounds, obtaining 0.64 g of crude dried extract.

Repeated chromatographic separation on silica gel column were conducted on the whole crude extract, using different solvent eluents: i.e. *n*-butanol saturated with water for a first separation and then using mixtures of chloroform/methanol at various percentages and increasing the polarity during the chromatographic run.

From the first separation procedures rosmarinic acid (**1**) (24.7 mg) (Ticli, 2005) was separated as a pure compound, while oleanolic acid (**2**) (7.5 mg) (Seebacher, 2003) and maslinic acid (**3**) (1.5 mg) (Taniguchi, 2002) were obtained by further purification of a low polarity fraction where the presence of triterpenoids was observed in a preliminary NMR spectra. The spectral data of isolated compounds were in accordance with those reported in the literature and the identification was also confirmed by direct comparison with standard compounds

Rosmarinic acid (1): $^1\text{H-NMR}$ (300 MHz, CD_3OD), δ : 7.50 (1H, *d*, $J = 15.9$ Hz, H- β), 7.02 (1H, *d*, $J = 1.8$ Hz, H-2''), 6.91 (1H, *dd*, $J = 8.2, 1.8$ Hz, H-6''), 6.76 (1H, *d*, $J = 1.6$ Hz, H-2'), 6.75 (1H, *d*, $J = 8.0$ Hz, partially overlapped with H-2', H-5''), 6.68 (1H, *d*, $J = 8.0$ Hz, H-5'), 6.62 (1H, *dd*, $J = 8.0, 1.6$ Hz, H-6'), 6.26 (1H, *d*, $J = 15.9$ Hz, H- α), 5.09 (1H, *dd*, $J = 9.4, 3.3$ Hz, H-2), 3.10 (1H, *dd*, $J = 14.1, 3.3$ Hz, H-3a), 2.94 (1H, *dd*, $J = 14.1, 9.4$ Hz, H-3b).

^{13}C NMR (75 MHz, D_2O), δ : 176.9 (C-1), 168.9 (C-4), 147.0 (C-4'), 146.2 (C-3'), 144.2 (C- β), 143.8 (C-4''), 142.7 (C-3''), 130.1 (C-1'), 127.0 (C-1''), 122.7 (C-6'), 121.9 (C6''), 117.3 (C-2'), 116.2 (C2''), 116.1 (C5''), 115.2 (C-5'), 114.2 (C- α), 76.2 (C-2), 36.83 (C-3).

ESI-MS: m/z 383.05 $[\text{M}+\text{Na}]^+$; m/z 358.98 $[\text{M}-\text{H}]^-$.

Oleanolic acid (2): ^1H -NMR (300 MHz, CDCl_3) δ : 5.28 (1H, *br t*, $J = 3.2$ Hz, H-12), 3.22 (1H, *dd*, $J = 9.6, 4.3$ Hz, H-3), 2.82 (1H, *dd*, $J = 13.2, 6.5$ Hz, H-18), 1.19 (3H, *s*, H-27), 0.98 (3H, *s*, H-25), 0.95 (3H, *s*, H-30), 0.93 (3H, *s*, H-29), 0.85 (3H, *s*, H-26), 0.77 (3H, *s*, H-24).

ESI-MS: m/z $[\text{M}+\text{Na}]^+$ 479.61; m/z $[\text{M}-\text{H}]^-$ 455.55.

Maslinic acid (3): ^1H -NMR (400 MHz, CDCl_3) δ : 5.27 (1H, *br t* $J = 3.0$ Hz, H-12), 3.65 (1H, *m*, H-2), 1.10 (3H, *s*, H-27), 1.02 (3H, *s*, H-23), 0.95 (3H, *s*, H-25), 0.88 (3H, *s*, H-30), 0.85 (3H, *s*, H-29), 0.79 (3H, *s*, H-24).

ESI-MS: m/z 495.30 $[\text{M}+\text{Na}]^+$; m/z 471.33 $[\text{M}-\text{H}]^-$.

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