SUPPLEMENTARY MATERIALS

Iridoids and phenylethanoid glycosides from the aerial parts of *Ajuga tenorei*, an endemic Italian species

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Abstract.

We report the first analysis in absolute, and in particular concerning its phytochemical pattern, about an endemic Italian species, *Ajuga tenorei* C. Presl. The analysis, performed by means of techniques such as Column Chromatography and NMR spectroscopy and Mass spectrometry, led to the isolation and the identification of five compounds namely **verbascoside (1)**, **echinacoside (2)**, **ajugoside (3)**, **harpagide (4)** and **8-O-acetylharpagide (5)**. The presence of these compounds is important from both chemotaxonomic and ethno-pharmacological point of view., For what concerns the first point is confirmed the correct botanical classification of the species. The isolated compounds are also known to exert peculiar pharmacological activities and their presence may give a rationale to the historical medicinal properties associated to the *Ajuga* genus in general since these plants have a long traditional use in many parts of the world. Such fact might suggest the use of also this species in this sense.

Keywords: *Ajuga tenorei* C. Presl, phenyl-ethanoid glycosides, iridoids, chemotaxonomy, ethno-pharmacology.

3. Experimental.

3.1. General.

NMR spectra were recorded on a Varian (now Agilent Technologies) Mercury 300 MHz instrument and/or on a Bruker Avance II 400 MHz instrument using D₂O or CD₃OD as deuterated solvents; the chemical shifts were expressed in ppm using the HDO signal to set the ¹H-spectra for those in D₂O while the internal solvent signal (m5) at 3.31 ppm was used as reference for the spectra in CD₃OD.

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

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

3.2. Plant Materials.

The studied sample was collected in the town of Rivisondoli in the province of L'Aquila (Abruzzo Region, Central Italy) (geographic coordinates: 41°86'13" N; 13°98'99" E) in June 2015 at an altitude of about 1750 m a.s.l.. To conciliate the need to have a representative sample and the impossibility to collect a large amount of plant material from plants growing in a protected area, and considering the rarity of this species, we have taken the minimal amount of plant materials meaning 50.0 g.

The botanical identification of the species was performed by the botanists of the Park (Dr. Mirella Di Cecco and Dr. Giampiero Ciaschetti) using the Italian and the European analytic floras (Pignatti, 1982; Tutin et al., 1972). The voucher specimen of the studied plants is stored in our laboratory for further references and registered under the accession number AT08082015.

3.3. Extraction and isolation of polar compounds.

The dried plant material 50.0 g (leaves) was extracted **at room temperature** with ethanol 96% three times (150 mL for each extraction, **48 h of infusion**). The extracts were collected altogether in the same flask and the ethanol was eliminated at reduced pressure until a water suspension was obtained.

The suspension was frozen and then lyophilized, to preserve temperature-sensitive compounds possibly present, obtaining 3.5 g of dried crude extract.

A portion of this (2.6 g) was then subjected to a chromatographic fractionation on silica gel column using *n*-butanol saturated with water (**82:18 v/v**) as eluting system.

From this separation step were directly eluted and identified: verbascoside (1) (fr. 15-16) (8.7 mg) (Scarpati & Delle Monache, 1963; Venditti et al., 2016a), and a mixture (fr. 49-67) of **harpagide (4)** (9.4 mg) (Bobbitt et al., 1969) and **8-O-acetyl-harpagide (5)** (12.1 mg) (Scarpati et al., 1965).

A second chromatographic separation was performed a collection of fractions (fr. 10-19) deriving from the first chromatographic run for the weight of 120.1 mg **on silica gel CC (3.1 g)** and using a mixture of chloroform/methanol as eluting system **(starting from 9:1 and gradually increasing the polarity to 8:2 and to 7:3 during the chromatographic run)**. **This second separation step allowed to the identification of ajugoside (3)** (fr. 14-19) (4.3 mg) (Guiso et al, 1974; Venditti et al., 2016b) and **echinacoside (2)** (fr. 54) (2.7 mg) (Kobayashi et al., 1984; Venditti et al., 2016). The experimental spectroscopic data obtained from these isolates were in accordance with literature and/or with experimental properties of original standard available in our laboratory.

References.

Bobbitt JM, Segerbarth KP. in Taylor WI, Battersby R. 1969. Cyclopentanoid Terpene Derivatives. Marcel Dekker Inc., New York, N.Y., Chapter 1.

Guiso M, Marini-Bettolo R, Agostini A. 1974. Iridoids. XIII. Ajugoside and ajugol. Structure and configuration. Gazz. Chim. Ital. 104(1-2): 25-33.

Kobayashi H, Karasawa H, Miyase T, Fukushima S. 1984. Studies on the constituents of *cistanchis herba*. III. Isolation and structures of new phenylpropanoid glycosides, cistanosides A and B. Chem. Pharm. Bull. 32:3009–3014.

Pignatti S, 1982. Flora d'Italia, vol. 2. Edagricole, Bologna.

Scarpati ML, Delle Monache F. 1963. Isolation from *Verbascum sinuatum* of two new glucosides, verbascoside and isoverbascoside. Ann. Chim-Rome. 53:356–367.

Scarpati ML, Guiso M, Panizzi L. 1965. Iridoids. I. Harpagide acetate from *Melittis melissophyllum*, Tetrahedron Lett. 39: 3439-3443.

Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA, eds, 1972. Flora Europaea. Vol. 3. Diapensiaceae to Myoporaceae. Cambridge University Press., Cambridge, UK.

Venditti A, Frezza C, Serafini M, Bianco A. 2016a. Iridoids and phenylethanoid from *Pedicularis kernerii* Dalla Torre growing in Dolomites, Italy. Nat. Prod. Res. 30(3): 327-331.

Venditti A, Frezza C, Guarcini L, Maggi F, Bianco A, Serafini M. 2016b. Reassessment of *Melittis melissophyllum* L. subsp. *melissophyllum* iridoidic fraction. Nat. Prod. Res. 30(2): 218-222.