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Short communication

Anti-angiogenic activity of iridoids from *Galium tunetanum*

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ABSTRACT

The phytochemical study of *Galium tunetanum* Lam., Rubiaceae, leaves led to the isolation of 13 compounds from the chloroform–methanol and the methanol extracts, including six iridoid glycosides, one non-glycoside iridoid, two *p*-coumaroyl iridoid glycosides, two phenolic acids, and two flavonoid glycosides. The structural determination of the isolated compounds was performed by mono- and bidimensional NMR spectroscopic data, as well as ESI-MS experiments. All compounds were isolated from this species for the first time. The anti-angiogenic effects of the isolated iridoids were also reported on new blood vessels formation using the chick embryo chorioallantoic membrane as *in vivo* model. Results showed that among the isolated iridoids tested at the dose of 2 μg/egg, asperuloside (**1**), geniposidic acid (**2**), and iridoid V1 (**3**) reduced microvessel formation of the chorioallantoic membrane on morphological observations using a stereomicroscope. The anti-angiogenic effects of the active compounds, expressed as percentages of inhibition *versus* control, were 67% (**1**), 59% (**2**), and 54% (**3**), respectively. In addition, the active compounds were able to inhibit angiogenesis in the chorioallantoic membrane assay, in a dose-dependent manner (0.5–2 μg/egg) as compared to the standard retinoic acid.

Introduction

Plants belonging to *Galium* genus, Rubiaceae, comprising approximately 1300 species, are known in ethnobotanical field for the treatment of a variety of pathological conditions, such as psoriasis, skin infections (Oumeish, 1999), hepatitis (Bolivar et al., 2011), kidney disorders, and as sedative, diuretic, and to treat the epilepsy and hysteria (Shah et al., 2006). *G. tunetanum* Lam. is a perennial herb, native to Tunisia, Algeria, Marocco, Spain, and Sicily (Casimiro et al., 2012). To the best of our knowledge, in the literature there is only one report about the antioxidant activity of the methanol extract of its leaves (Gaamoune et al., 2014) but no phytochemical studies have been carried out so far.

Galium genus is well-known for producing several classes of secondary metabolites such as iridoid glycosides, saponins, triterpenes, anthraquinones, and flavonoid glycosides (Mocan et al., 2016). Iridoids are a large class of natural products, exhibiting a wide range of pharmacological activities such as anti-inflammatory, anticancer, cardioprotective, and neuroprotective. Interestingly, the iridoid glycoside geniposide was found to have a potent anti-angiogenic activity in the chick embryo chorioallantoic membrane (CAM) assay (Koo et al., 2004). Angiogenesis is the growth of new blood vessels to ensure wound healing, reproduction, and developments of cells. This physiological process plays an important role in the expansion of veins and blood capillaries and in the nutrition of tumor cells. Thus, angiogenesis inhibition might be a promising approach for anticancer therapies.

In the course of our investigation on plants belonging to the African flora (Beladjila et al., 2017), the chemical study of *G. tunetanum* leaves was performed, and the isolation and structural characterization of 13 compounds, including nine iridoid glycosides (**1**–**9**), two phenolic acids (**10**–**11**), and two flavonoid glycosides (**12**–**13**) was herein reported. The anti-angiogenic effect of iridoids **1**–**8** on new blood vessels formation, using the CAM assay as *in vivo* model, was also explored.

Materials and methods

One and two-dimensional NMR experiments were performed on a Bruker DRX-600 spectrometer at 300 K (Bruker BioSpin, Rheinstetten, Germany) equipped with a Bruker 5 mm TCI CryoProbe, acquiring the spectra in methanol-d_4. Pulse sequences and phase cycling were used for DQF-COSY, TOCSY, HSQC, and HMBC, experiments. NMR data were processed using XWinNMR

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software (De Leo et al., 2017). ESI-MS were obtained from an LCQ Advantage ThermoFinnigan spectrometer (ThermoFinnigan, USA), equipped with Xcalibur software. Column chromatographies (CC) were performed over Sephadex LH-20 (40–70 μm, Amersham Pharmacia Biotech AB, Uppsala, Sweden) and Isolera® Biotage® purification system (flash Silica gel 60 SNAP 340 g cartridge, flow rate 90 ml/min) (Milella et al., 2016). Reverse phase – high performance liquid chromatography (RP-HPLC) separations were conducted on a Shimadzu LC-8A series pumping system equipped with a Shimadzu RID-10A refractive index detector and Shimadzu injector on a C_{18} μ-Bondapak column (30 cm × 7.8 mm, 10 μm Waters, flow rate 2 ml/min, Milford, MA, USA). Thin Layer Chromatography (TLC) analyses were carried out using precoated Kieselgel 60 F_{254} (0.20 mm thickness) plates (Merck, Darmstadt, Germany); compounds were detected by cerium disulfate/sulfuric acid (Sigma–Aldrich, Milan, Italy). All the solvents used for the extraction and separation processes and retinoic acid used for the CAM assay as antiangiogenic reference compound were purchased from Sigma-Aldrich (Milan, Italy).

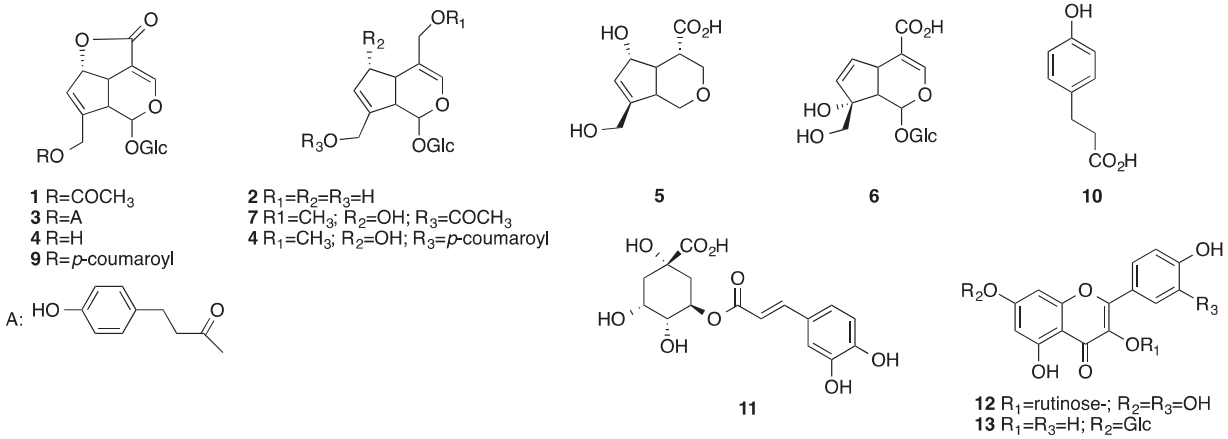
Galium tunetanum Lam., Rubiaceae, leaves were collected and identified by authors Smain Amira and Fatima Benchikh in Djilma, 45 km away from Jijel, Northeast Algeria, in June 2013. A voucher specimen has been deposited at the Herbarium Horti Botanici Pisani, Pisa, Italy (n. 8486 *Galium tunetanum*/1, Nuove Acquisizioni).

Briefly, dried leaves of the plant (1 kg) were extracted with solvents of increasing polarity: *n*-hexane, chloroform, chloroform–methanol (9:1), and methanol by exhaustive maceration to give 4.0, 13.3, 11.9, and 48 g of the respective residues. The methanol extract was partitioned between *n*-butanol and water to afford a *n*-butanol residue (10.8 g), that was submitted to Sephadex LH-20 column chromatography (5 × 75 cm, flow rate 1 ml/min) using methanol as eluent and collecting nine major fractions (A–I) grouped by TLC. Part of the fraction B (1.5 g) was subjected to RP-HPLC with methanol–water (3:7) as eluent, to give compounds **2** (0.7 mg, t_R 7 min) and **7** (1.4 mg, t_R 14 min). Fractions E (273.3 mg), F (707.3 mg), G (724.0 mg), and I (818.2 mg) were submitted to RP-HPLC using methanol–water (35:65) as eluent, to give compounds **3** (5.0 mg, t_R 14 min) and **8** (1.7 mg, t_R 55 min) from fraction E; compounds **10** (1.5 mg, t_R 9 min) and **9** (0.5 mg, t_R 22 min) from fraction F; compounds **11** (6.0 mg, t_R 6 min) and **12** (1.3 mg, t_R 32 min) from fraction G; compound **13** (2.6 mg, t_R 39 min) from fraction I, respectively. The remaining fractions B (874.2 mg) and C (922.3 mg) were subjected to RP-HPLC with methanol–water (1:4) as eluent, to give compound **6** (1.3 mg, t_R 5 min) from fraction B and compound **2** (1.3 mg, t_R 8 min) from fraction C, respectively. Part of the chloroform–methanol residue (5.6 g) was subjected to Isolera Biotage column chromatography (340 g silica SNAP cartridge, flow rate 90 ml/min), eluting with chloroform followed by increasing concentrations of methanol in chloroform (between 1% and 100%). Fractions of 27 ml were collected, analyzed by TLC and grouped into five major fractions (A–E). Fractions B (331.4 mg) and C (1481.8 mg) were subjected to RP-HPLC with methanol–water (3:7) as eluent, to give compounds **5** (1.3 mg, t_R 6 min) and **7** (3 mg, t_R 15 min) from fraction B; compound **1** (23.6 mg, t_R 8 min) from fraction C, respectively. Fraction E (509.7 mg) was submitted to RP-HPLC with methanol–water (1:4) as eluent, to give compound **4** (6.6 mg, t_R 7 min).

The CAM assay was performed following the method of Germanò et al. (2015) modified (Certo et al., 2017). Fertilized eggs of *Gallus gallus* were previously maintained in a humidified incubator at 37 °C and, after four days of incubation, a small window was created on the broad side of the eggs to apply different doses of pure compounds (0.5–2 μg/egg) directly on the CAM surface, previously suspended in albumen. Retinoic acid (2 μg/egg) was used as antiangiogenic reference compound. After treatment, the eggs were reincubated for 24 h, then they were observed by means of a steromicroscope (Zeiss Stemi 2000-c) equipped with a digital camera (Axiocam MRc 5 Zeiss) and photographed. The antiangiogenic effects on the CAM were quantified by counting the number of blood vessel branch points in a standardized area using a Zeiss software for micromorphometric analysis and expressed as % of inhibition respect to control. Each experiment was repeated three times. The significance of the differences was assessed on the basis of the *t*-test, considering the differences for $p < 0.05$, and finally calculated with respect to the lot of control eggs treated only with albumen.

Results and discussion

The phytochemical study of chloroform–methanol and methanol extracts of *G. tunetanum* leaves afforded the isolation of thirteen compounds **1**–**13**. Their structural determination was performed by 1D and 2D NMR spectroscopic techniques, mass spectrometry analyses, and comparison of these data with those reported in the literature. Isolated compounds included six iridoid glycosides identified as asperuloside (**1**) (Otsuka et al., 1991), geniposidic acid (**2**) (Güvenalp et al., 2006), iridoid V1 (**3**) (Mitova et al., 1999), deacetylasperuloside (**4**) (Otsuka et al., 1991), monotropein (**6**) (Tzakou et al., 2007), and daphylloside (**7**) (Demirezer et al., 2006); one non-glycoside iridoid macedonine (**5**) (Mitova et al., 1996); two *p*-coumaroyl iridoid derivatives, 10-*O*-*p*-coumaroyl-10-deacetyldaphylloside (**8**) (Ahn and Kim, 2012) and 10-*O*-*p*-coumaroyl-10-deacetylasperuloside (**9**) (Bai and Hu, 2006); two phenolic acids characterized as *p*-hydroxyhydrocinnamic acid (**10**) and chlorogenic acid (**11**) (Owen et al., 2003); and two flavonoid



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