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Liquid chromatography–diode array detection–electrospray ionisation mass spectrometry/nuclear magnetic resonance analyses of the anti-hyperglycemic flavonoid extract of *Genista tenera* Structure elucidation of a flavonoid-*C*-glycoside

Amélia P. Rauter[a,*], Alice Martins[a], Carlos Borges[a], Joana Ferreira[a], Jorge Justino[b], Maria-Rosário Bronze[c], Ana V. Coelho[c], Young H. Choi[d], Robert Verpoorte[d]

[a] *Departamento de Química e Bioquímica/Centro de Química e Bioquímica, Faculdade de Ciências da Universidade de Lisboa, Ed. C8, 5° Piso, 1749-016 Lisboa, Portugal*
[b] *Escola Superior Agrária de Santarém, Instituto Politécnico de Santarém, Apartado 279, 2001-904 Santarém, Portugal*
[c] *Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Apartado 127, 2891-901 Oeiras, Portugal*
[d] *Division of Pharmacognosy, Section Metabolomics, Institute of Biology, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands*

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Abstract

The anti-hyperglycemic flavonoid extract obtained from *Genista tenera* was first studied by liquid chromatography (LC)–diode array detection (DAD) which showed the presence of two major compounds. One of them was identified as genistein-7-*O*-glucoside. Luteolin-7-*O*-glucoside was detected as a minor constituent, while luteolin-7,3′-di-*O*-glucoside and rutin were found in trace amounts. LC–DAD–ESI–MS and NMR were used to confirm the structure of these compounds and allowed the elucidation of the structure of the unknown major compound, which is the flavonoid 5,7,4′-trihydroxyisoflavone-8-*C*-glucoside.

Keywords: *Genista tenera*; LC–DAD–ESI–MS; NMR; Flavonoid glycoside; 5,7,4′-Trihydroxyisoflavone-8-*C*-glucoside

1. Introduction

Genista tenera (Jacq. Ex Murr) O. Kuntze is an endemic plant to the Island of Madeira and belongs to the Leguminosae family. The infusion of its aerial parts is used by the local population as an adjuvant for the treatment of diabetes. Previous studies of the secondary metabolites of *Genista* species reported alkaloids and flavonoids as the chemotaxonomic markers of the genus *Genista* [1]. The alkaloids extracted from the aerial parts of the plant studied in this work have recently been described [2]. A previous investigation of the diethyl ether extract of *G. tenera* afforded the flavones apigenin and chrysoeriol, and the isoflavones genistein, 3′-*O*-methylorobol, 5-*O*-methylgenistein and alpinumisoflavone, which were characterized by FAB-MS/MS [3,4]. There is growing evidence that supports a protective role of flavonoids in cardiovascular diseases and various types of cancer [5,6]. Apigenin is a dietary bioflavone with anticarcinogenic properties, which is thought to play a role in cancer chemoprevention and cancer chemotherapy [7,8]. Also the flavone luteolin has been reported as an anticancer agent [9–11]. Some isoflavones, e.g. genistein, possess anticancer activity [12] and estrogen-like activities, which could have a beneficial role in humans against estrogen deficiency [13,14]. Furthermore, flavonoids have been recently reported as aldose reductase inhibitors blocking the sorbitol pathway that is linked to many problems associated with diabetes [15]. A preliminary

* Corresponding author. Tel.: +351 21 7500952; fax: +351 21 7500088.
E-mail address: aprauter@fc.ul.pt (A.P. Rauter).

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study of the anti-hyperglycemic action of the ethyl acetate extract on normal and streptozotocin induced diabetic rats gave promising results [16].

Liquid chromatography (LC) coupled to diode array detection (DAD) and ESI–MS has shown to be a very powerful method for the identification of complex flavonoids [17]. We report herein the study of the flavonoid composition of the anti-hyperglycemic ethyl acetate extract of *G. tenera* by LC–DAD–ESI–MS, which resulted in the identification of the known flavonoid glycosides genistein-7-*O*-glucoside, luteolin-7-*O*-glucoside and trace amounts of luteolin-7,3′-di-*O*-glucoside and rutin. Genistein-8-*C*-glucoside was identified as a major compound of the ethyl acetate extract, which structure was elucidated by LC–DAD–ESI–MS/NMR J-resolved spectra. This paper presents a new methodology, which allows the structure elucidation of unknown isoflavone glucosides present in a complex mixture.

2. Experimental

2.1. Plant material

The plant was identified and collected on the island of Madeira in the beginning of the flowering period. A voucher specimen (MADJ 2508) is deposited in the Herbarium of Jardim Botânico da Madeira, Funchal.

2.2. Extraction

The powdered aerial parts (2200 g) were exhaustively extracted in a Soxhlet apparatus with EtOH. The crude extract was concentrated to dryness under vacuum and the residue (238 g) was dissolved in warm water, filtered and partitioned against diethyl ether and ethyl acetate. The ethyl acetate extract was concentrated to dryness (15.5 g).

2.3. LC–DAD–ESI–MS analyses

The ethyl acetate extract was dissolved in methanol (HPLC grade) (1%, w/v). Samples (20 μL) were analysed using a HPLC system coupled with a photodiode array detector (DAD) (Surveyor ThermoFinnigan) and an autosampler (Surveyor ThermoFinnigan). The equipment was controlled by Chromquest software. A reversed-phase C18 (Lichrospher 100, Merck) column (250 mm × 4 mm, i.d. and particle size 5 μm) with a guard column with the same stationary phase was used. The ThermoFinnigan pump was operated at 700 μL/min using the following eluents: 99.9% water–phosphoric acid (99.1:0.1) (eluent A) and water–acetonitrile–phosphoric acid (59.5:40:0.1) (eluent B). Solvents were HPLC grade. The following elution program was used: from 0 until 20% eluent B in 15 min, isocratic 10 min, until 70% eluent B in 45 min, isocratic 5 min, until 100% B in 10 min, isocratic 15 min, equilibrium time 15 min. DAD detector was operated between 220 and 800 nm.

LC–DAD–MS analyses were carried out in a LCQ Advantage ThermoFinnigan mass spectrometer equipped with an electrospray ionisation source and using an ion trap mass analyser. It was controlled by Xcalibur software (ThermoFinnigan). The chromatographic separation was performed with the same type of equipment and conditions described before. In order to use an eluent system compatible with MS analysis, 0.1% phosphoric acid was replaced by 0.5% formic acid.

The ionisation conditions were adjusted at 350 °C and 13 V for capillary temperature and voltage, respectively and at 4 kV for spray voltage. Nitrogen was used as sheath and auxiliary gas. The full scan mass covered the range from *m*/*z* 50 up to 1000. Collision-induced fragmentation experiments were performed in the ion trap by increasing the amplitude of the supplementary potential applied to the end cap electrodes (i.e., 2 V) using helium as the collision gas. Mass spectrometry data were acquired in the positive ionisation mode. MS^2 was carried out in the data dependent mode on the more abundant parent ions. The ion nomenclature followed is according to that suggested by Domon and Costello [18] for glycoconjugates and adapted by Li et al. [19] for flavonoid glycosides.

2.4. NMR spectroscopy

^{1}H NMR, homonuclear J resolved, ^{1}H-^{1}H-COSY, HMQC, and HMBC spectra were recorded at 25 °C on a 400 MHz Bruker AV-400 spectrometer operating at a proton NMR frequency of 400.13 MHz and 100.16 MHz for ^{13}C. Methanol-d_4 was used as the internal lock. ^{1}H NMR spectrum consisted of 128 scans requiring 10 min acquisition time with the following parameters: 0.25 Hz/point, pulse width (PW) = 90° (6.6 μs), and relaxation delay (RD) = 1.0 s. FIDs were Fourier transformed with LB = 0.3 Hz and the spectra were zerofilled to 32 K points. The resulting spectra were manually phased and baseline corrected, and calibrated to residual solvent of methanol-d_4 at 3.30 ppm, using XWIN NMR (version 3.5, Bruker). Two dimensional J-resolved ^{1}H NMR spectra were acquired using 8 scans per 32 increments that were collected into 16 K data points, using spectral widths of 5208 Hz in F2 (chemical shift axis) and 50 Hz in F1 (spin-spin coupling constant axis). A 1.0 s relaxation delay was employed, giving a total acquisition time of 14.52 min. Datasets were zero-filled to 512 points in F1 and both dimensions were multiplied by sine-bell functions (SSB = 0) prior to double complex FT. J-resolved spectra tilted by 45°, symmetrized about F1, and then calibrated, using XWIN NMR (version 3.5, Bruker). Data were exported as the 1D projection (F2 axis) of the 2D J-resolved spectra.

^{1}H-^{1}H-COSY spectrum was acquired with 1.0 s relaxation delay, 4194 Hz spectral width in both dimensions. Window function for COSY spectra was sine-bell (SSB = 0). The HMQC spectrum is obtained with 1.3 s relaxation delay, 4401 Hz spectral width in F2 and 19996 Hz in F1. Qsine (SSB = 2.2) was used for the window function of HMQC.