

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

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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.

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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

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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 μ L) 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 \times 4 mm, i.d. and particle size 5 μ m) with a guard column with the same stationary phase was used. The ThermoFinnigan pump was operated at 700 μ L/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. MS² 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

¹H NMR, homonuclear J resolved, ¹H–¹H-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 ¹³C. Methanol-*d*₄ was used as the internal lock. ¹H 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 μ s), and relaxation delay (RD) = 1.0 s. FIDs were Fourier transformed with LB = 0.3 Hz and the spectra were zero-filled 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 ¹H 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.

¹H–¹H-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.

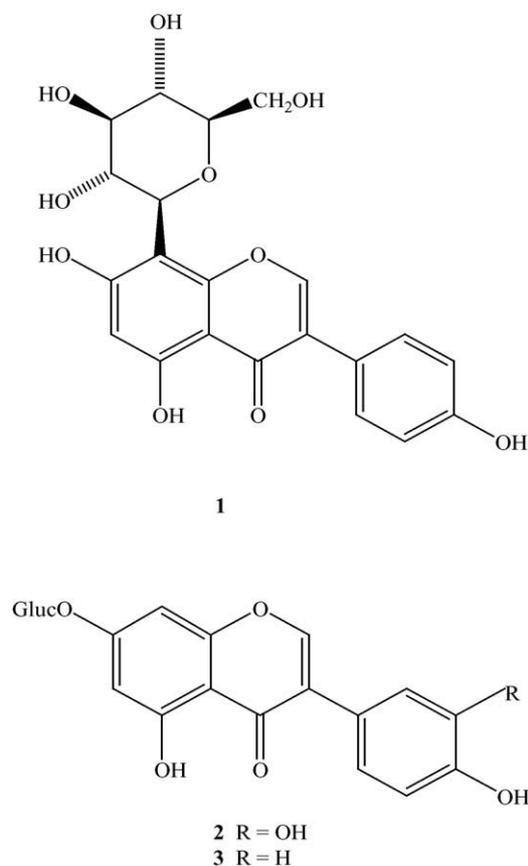


Fig. 1. Structure of the flavonoid glycosides 1–3.

The HMBC spectra were recorded with the same parameters as the HMQC spectrum except for 4252 Hz of spectral width in F2. The optimized coupling constants for HMQC and HMBC were 145 and 8 Hz, respectively.

3. Results and discussion

The ethyl acetate extract of *G. tenera* was analysed by LC–DAD and luteolin-7-*O*-glucoside (**2**) and genistein-7-*O*-glucoside (**3**) (Fig. 1) were detected in the chromatogram of the crude extract (Fig. 2). Peaks were identified by comparing their retention times and UV spectra with chromatograms obtained after analysis of reference compounds in the same conditions. The UV spectra obtained for compounds **2** and **3** showed a similarity index of 0.9975 and 0.998, respectively with luteolin-7-*O*-glucoside and genistein-7-*O*-glucoside standards. The presence of luteolin-7,3'-*O*-di-*O*-glucoside (RT = 51 min) and rutin (RT = 54 min) was confirmed by comparison with retention times of standards analysed in the same conditions, but they are present only in trace amounts. The identity of compounds **2** and **3** was confirmed by HPLC coupled with on-line mass spectrometry using an electrospray ionisation source in positive ion mode (Figs. 3 and 4). The molecular ion species $[M + H]^+$ were detected at m/z 449 and 433 in the linear scan mode (MS). In the daughter scan mode (MS/MS), the spectra (Fig. 4a and b) presented fragments at m/z 283 and at m/z 271, respectively, corresponding to the loss of the glucosyl moiety from the protonated molecule. In previous studies with flavonoids-*O*-glycosides [20], it was observed that at low-energy CID a characteristic loss is obtained due to the fragmentation of the glycosidic bond. In flavonoid-*C*-glycosides the major fragmentation pathways concern cross-ring cleavages of the saccharide residue and the loss of water molecules [19]. The structure of compound **1**, one of the major constituents of the extract is depicted in Fig. 1 and could not be assigned by HPLC since no standard was available. Attempts to hydrolyze this glucoside by treatment of the extract with 2 N HCl–MeOH (1:1) at 100 °C

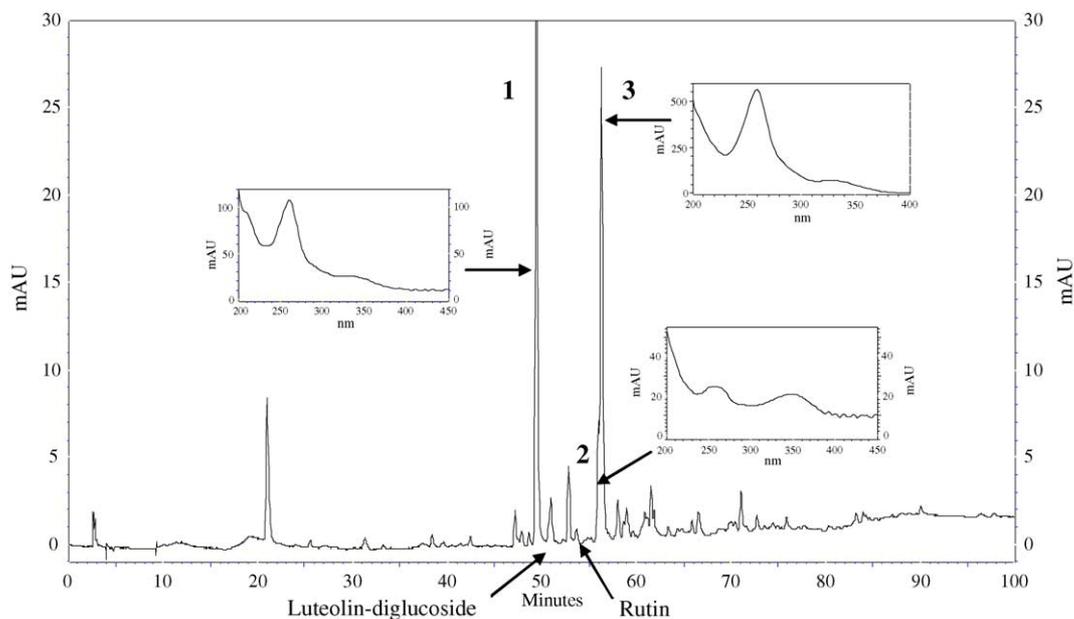


Fig. 2. Chromatogram of the extract detected at 280 nm and UV spectra of the compounds identified as genistein-8-*C*-glucoside (peak 1, RT = 49.5 min), luteolin-7-*O*-glucoside (peak 2, RT = 56 min) and genistein-7-*O*-glucoside (peak 3, RT = 56.4 min).

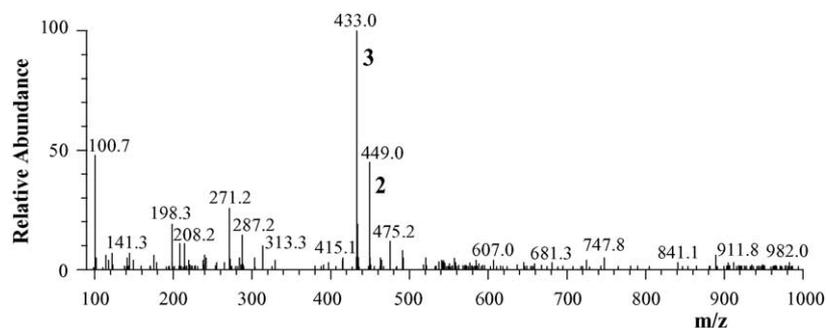


Fig. 3. Mass spectrum at retention time 62 min in the LC-chromatogram of the extract (see Fig. 2). $[M + H]^+$ ions at m/z 449 and 433 correspond to compounds **2** and **3**, respectively.

for 2 h and 2 N HCl at 100 °C for 2 h failed, suggesting that this compound might be a C-glycoside. The ESI-MS spectrum showed $[M + H]^+$ at m/z 433 (Fig. 5a), while its MS/MS spectrum (Fig. 5b) presented the fragments E_1^+ , E_2^+ , E_3^+ at m/z 415, 397, 379 due to the loss of one, two, and three water molecules, respectively, from the protonated molecule. The fragment ion $^{2,3}X^+_{-2w}$ at m/z 367 (45%) and those due to fragmentations involving the glycosidic bond $^{0,4}X^+_{-2w}$ at m/z 337 (14%), $^{0,2}X^+$ at m/z 313 (27%), $^{0,2}X^+_{-w}$ at m/z 295 (8%) and $^{0,1}X^+$ at m/z 283 (4%) (see Fig. 6), are in agreement with the structure of a C-glucoside. Comparing these results with those reported for 6-C- and 8-C-flavone glucosides using LSIMS [19], the high abundance of some fragment ions would suggest that compound **1** might be a 6-C-glucoside. The final assignment of the structure of compound **1** was possible after examination of its 1H NMR and ^{13}C NMR data (Table 1), using several 2D-NMR spectra, COSY, HMQC,

HMBC. In particular J-resolved spectra were largely applied in order to evaluate the signal purity and to obtain exact coupling constants. The HMBC correlations observed (Fig. 7) were also compared with the corresponding data obtained for compound **3**. The singlet at δ 8.10 and the doublet at δ 4.91 for compound **1** were assigned to H-2 and H-1'', respectively, being these chemical shifts characteristic of an isoflavone- β -C-glucoside, presenting H-1'' $J_{1'',2''} = 10.1$ Hz. These two protons show an HMBC correlation with C-8a at δ 158.1, clearly indicating that this compound is an 8-C-glucoside. The singlet at δ 8.09 also confirms that compound **3** is an isoflavone. HMBC correlations of H-2 of compounds **1** and **3** were detected with C-3 (δ 125.0, 124.4, assigned either to compound **1** or **3**), with C-4 (δ 182.4) and, for compound **3**, also with C-8a (δ 159.1). HMBC correlations of the anomeric proton of compound **1** with C-7 at δ 164.7 and C-8 at δ 104.1 indicate that the C-glucosyl moiety is bonded to position 8,

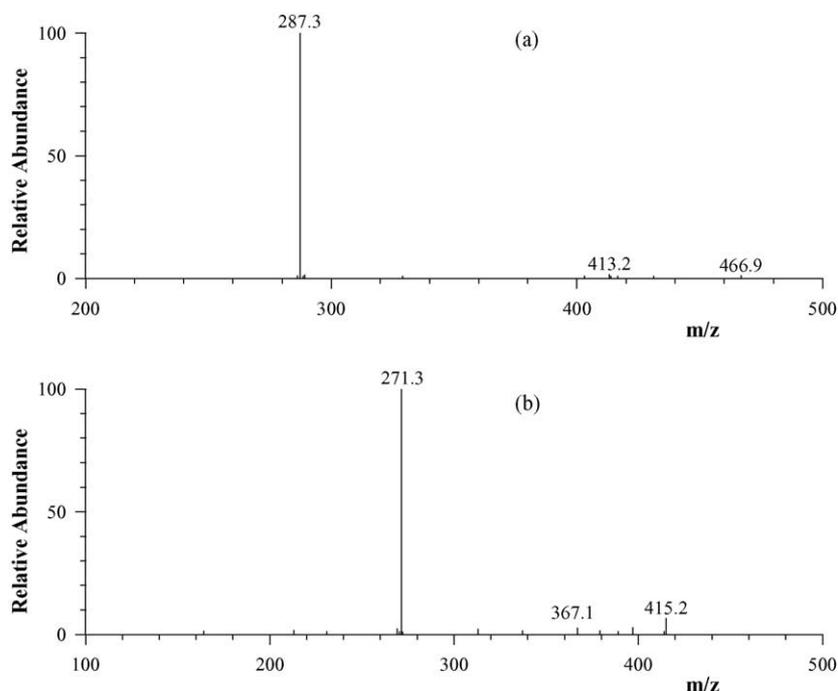


Fig. 4. MS/MS for the $[M + H]^+$ ions of (a) compound **2** and (b) compound **3**.

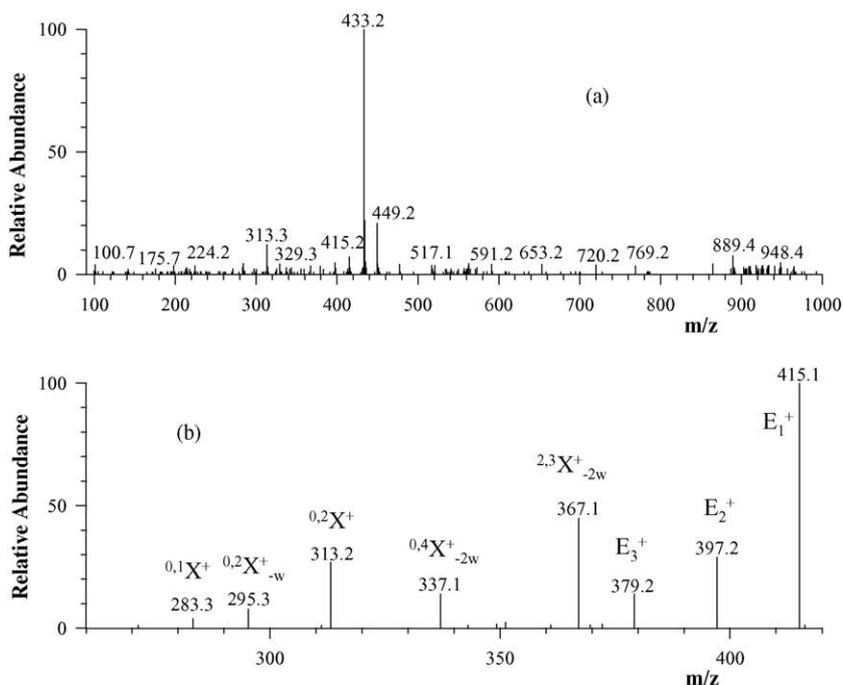


Fig. 5. (a) Mass spectrum at retention time 56 min in the LC-chromatogram of the extract (see Fig. 2). $[M + H]^+$ ion at m/z 433 corresponds to compound **1**. (b) MS/MS spectrum for the parent ion m/z 433.

Table 1
 ^1H and ^{13}C NMR chemical shifts of compound **1** and **3** in methanol- d_4

Atom number	Chemical shifts δ (ppm) ^a			
	1		3	
	^1H NMR	^{13}C NMR	^1H NMR	^{13}C NMR
2	8.10 (s)	155.2 ^b	8.09 (s)	154.6 ^b
3	–	125.0 ^c	–	124.4 ^c
4	–	182.4	–	182.4
5	–	163.4	–	163.4
6	6.25 (s)	101.1	6.48 (<i>d</i> , $J=2.2$ Hz)	101.6
7	–	164.7	–	164.7
8	–	104.1	6.66 (<i>d</i> , $J=2.2$ Hz)	95.9
8a	–	158.1	–	159.1
4a	–	106.8	–	107.4
1'	–	123.2 ^d	–	123.1 ^d
2'	7.36 (<i>d</i> , $J=8.4$ Hz)	131.3	7.36 (<i>d</i> , $J=8.4$ Hz)	131.3
3'	6.83 (<i>d</i> , $J=8.4$ Hz)	116.8	6.83 (<i>d</i> , $J=8.4$ Hz)	116.8
4'	–	158.9	–	158.9
5'	6.83 (<i>d</i> , $J=8.4$ Hz)	116.8	6.83 (<i>d</i> , $J=8.4$ Hz)	116.8
6'	7.36 (<i>d</i> , $J=8.4$ Hz)	131.3	7.36 (<i>d</i> , $J=8.4$ Hz)	131.3
1''	4.91 (<i>d</i> , $J=10.1$ Hz)	75.2	5.03 (<i>d</i> , $J=7.5$ Hz)	101.8
2''	3.2–4.2 (m)	74.7	3.2–4.2 (m)	72.9
3''	3.2–4.2 (m)	80.1	3.2–4.2 (m)	77.8
4''	3.2–4.2 (m)	71.2	3.2–4.2 (m)	71.2
5''	3.2–4.2 (m)	82.6	3.2–4.2 (m)	78.3
6''	3.2–4.2 (m)	62.9 ^e	3.2–4.2 (m)	62.4 ^e

^a Reference to residual solvent of methanol- d_4 at δ 3.30.

^b Changeable.

^c Changeable.

^d Changeable.

^e Changeable.

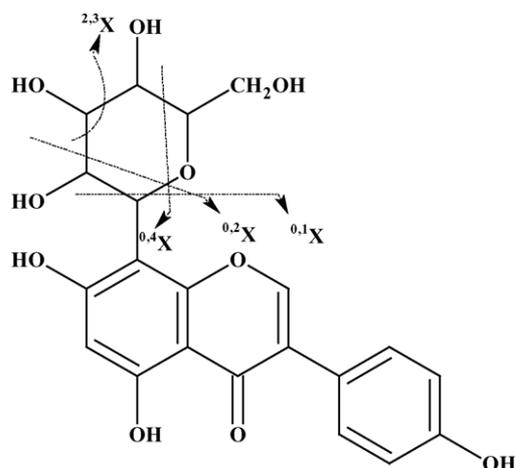


Fig. 6. Proposed structure for compound **1** indicating some fragmentations occurred by ESI–MS/MS.

being detected H-6 as a singlet at δ 6.25, presenting HMBC correlations with C-8, C-4a at δ 106.8, C-5 at δ 163.4 and C-7, as expected. The signal of C-1'' appeared at δ 75.2 and is in full agreement with the structure of a C-glucoside, while the resonance of C-1'' of compound **3** appeared at δ 101.8, thus confirming that this compound is a β -O-glucoside. The

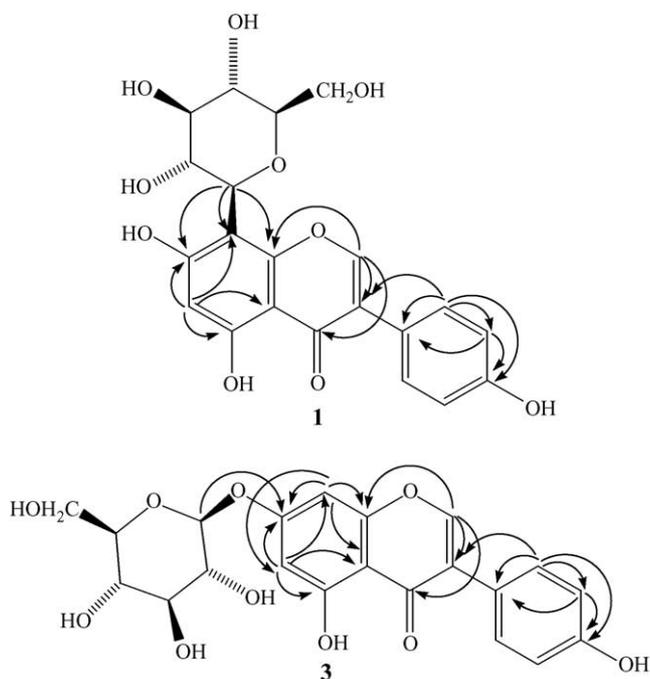


Fig. 7. Important HMBC correlations for the structure elucidation of compounds **1** and **3**.

resonance of H-1'' at δ 5.03 as a doublet with $J_{1,2} = 7.5$ Hz for **3** also indicates that this compound is the glucoside β -anomer. The substitution pattern at ring A was confirmed by the presence of H-6 at δ 6.48 as a doublet with $J_{6,8} = 2.2$ Hz, which shows HMBC correlations with C-5 at δ 163.4, C-7 at δ 164.7, C-4a at δ 107.4 and C-8 at δ 95.9. The resonance of H-8 appeared at δ 6.66 and presented the expected HMBC correlations indicated in Fig. 7. The chemical shifts of C-2'', C-3'', C-4'', C-5'' and C-6'' for compounds **1** and **3** were in agreement with those expected for β -glucosides.

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