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Phenolic constituents of *Gnaphalium uliginosum* L.

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ABSTRACT

The herb *Gnaphalium uliginosum* L. is an annual plant widely used in Russian and Bulgarian phytotherapy in the treatment of hypertension, thrombophlebitis, phlebothrombosis and ulcers. Decoction and infusion of *G. uliginosum* are known to possess anti-inflammatory, astringent, and antiseptic properties. Oil extracts are used in the treatment of laryngitis, upper respiratory catarrh and tonsillitis. However, there is still lack of information about the active compounds.

Ten phenolic compounds have been identified from the aerial parts of *G. uliginosum* including seven flavonoid glucosides and three phenylpropanoids. Their chemical structures were elucidated on the basis of 1D and 2D NMR and HRESIMS. Among the identified compounds the first full assignments of the ^{1}H and ^{13}C NMR spectra of, 5,7,4′-trihydroxy-6,3′-dimethoxyflavone-7-*O*-β-D-(6″-*O*-caffeoyl)-glucopyranoside are firstly reported in this paper.

1. Introduction

The herb of *Gnaphalium uliginosum* L. syn *Filaginella uliginosa* L. or Marsh Cudweed is an annual plant which is widely used in Russian phytotherapy (Yakovlev and Blinova, 1999). Decoction and infusion of *G. uliginosum* are known to possess anti-inflammatory, astringent, and antiseptic properties (Sokolov, 2000). Oil extracts are used both internally and externally in the treatment of laryngitis, upper respiratory catarrh and tonsillitis (Gammerman et al., 1984). In Russian and Bulgarian phytotherapy it is used in the treatment of hypertension and ulcer (Shchepotin et al., 1984; Ivancheva and Stantcheva, 2000). There is some information about the usage of the herb *G. uliginosum* for the treatment of the thrombophlebitis and phlebothrombosis (Turova and Sapozhnikova, 1989). Some of these activities are attributed to phenolics compounds present in the plant (Barnaulov et al., 1984; Yakovlev and Blinova, 1999).

The phenolics that have been reported to occur in *G. uliginosum* include the flavonoids: gnaphaloside A [5,7,4′-trihydroxy-6,3′-dimethoxyflavone-7-*O*-β-D-(6″-*O*-caffeoyl)-glucopyranoside], 6-methoxyluteolin, 6-hydroxyluteolin 7-*O*-β-D-glucopyranoside, and scutellarein 7-*O*-β-D-glucopyranoside (Konopleva et al., 1979, 1980) together with coumarins, and tannins (Gammerman et al., 1984). In the lipid complex of the herb of *G. uliginosum* α-, β-, and γ-carotenes and lycopene were identified (Bogdanova et al., 1983). However, there is still lack of detailed chemical data such as NMR assignments for the compounds. The present paper reports the structure elucidation of the phenolic constituents of *G. uliginosum* using 1D and 2D NMR, and HRESIMS spectra. The data presented should make a further contribution to the current knowledge regarding the chemistry of *G. uliginosum* and help to better understand the functional properties of this interesting, yet little investigated, herb.

2. Results and discussion

Compound **1** (Fig. 1) was isolated from the EtOH extract of the aerial parts of *G. uliginosum*. The positive HRESIMS of **1** displayed a molecular ion peak $[M+Na]^+$ at *m*/*z* 677.1506, suggesting the molecular formula $C_{32}H_{30}O_{15}Na$. The ^{1}H NMR spectrum suggested

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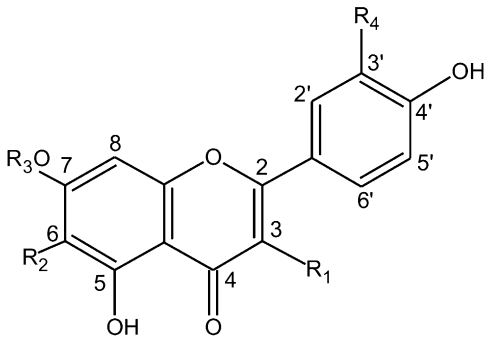

Fig. 1. Chemical structures of **1**–**7**. **1**: R_1 = H, R_2 = OCH_3, R_3 = β-D-(6″-*O*-caffeoyl)-glucose, R_4 = OCH_3. **2**: R_1 = H, R_2 = OCH_3, R_3 = β-D-glucose, R_4 = OH. **3**: R_1 = O-β-D-glucose, R_2 = OCH_3, R_3 = H, R_4 = OH. **4**: R_1 = O-β-D-glucose, R_2 = H, R_3 = H, R_4 = OH. **5**: R_1 = O-β-D-glucose, R_2 = H, R_3 = H, R_4 = OCH_3. **6**: R_1 = H, R_2 = OCH_3, R_3 = O-β-D-glucose, R_4 = OCH_3. **7**: R_1 = H, R_2 = H, R_3 = O-β-D-glucose, R_4 = H.

1 to be a 5,6,7,3′,4′-pentasubstituted flavone derivative, showing the signals due to two aromatic singlets (1H each, *s* at δ 6.51, H-3 and δ 6.85, H-8) and B-ring protons at δ 7.31 (H-2′, 1H, *d*, *J* = 2.0), δ 6.84 (H-5′, 1H, *d*, *J* = 8.3), and δ 7.38 (H-6′, 1H, *dd*, *J* = 8.3, 2.0). Two methoxy groups at δ 3.90 (3H, *s*) and δ 3.94 (3H, *s*), and an anomeric proton of β-D-glucose at d 5.15 (1H, *d*, *J* = 7.8) were detected in ^{1}H NMR spectrum of the compound **1**. In addition, a caffeoyl moiety was confirmed by the detection of the resonances at δ 6.58 (H-2‴, 1H, *d*, *J* = 1.4), δ 6.46 (H-5‴, 1H, *d*, *J* = 8.2), δ 6.44 (H-6‴, 1H, *dd*, *J* = 8.2, 1.4), δ 7.35 (H-7‴, 1H, *d*, *J* = 15.8), and δ 6.08 (H-8‴, 1H, *d*, *J* = 15.8).

The glucose signals of H-1″, H-2″, H-3″, H-4″, H-5″, and H-6″ in the ^{1}H NMR spectrum of **1** were assigned by analysis ^{1}H–^{1}H COSY spectrum (Table 1). The large downfield shift of H-6″ compared with that of free glucose (1.0–1.7 ppm) and separation of two protons at δ 4.20 (1H, *dd*, *J* = 11.8, 8.4) and δ 4.74 (*dd*, *J* = 11.8, 2.1) show the attachment of caffeic acid at C-6″ position. The conjugation of glucose and caffeic acid was confirmed by HMBC correlation between H-6″ and C=O of caffeic acid. The glucose attachment at C-7, and methoxyl groups at C-6 and C-3′ to flavone moiety were also confirmed by the HMBC correlation (H-1″ and C-7, OCH_3 and C-6, and OCH_3 and C-3′). The remaining signals were assigned by comparison of ^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H-J-resolved, COSY, HSQC, and HMBC spectra (Table 1).

On the basis of these NMR data the chemical structure of **1** was elucidated to be 5,7,4′-trihydroxy-6,3′-dimethoxyflavone-7-*O*-β-D-(6″-*O*-caffeoyl)-glucopyranoside (gnaphaloside A). This compound has been reported as a constituent of *G. uliginosum* before (Konopleva et al., 1979, 1980) but the NMR assignments were ambiguous. In this paper, the full assignments of the ^{1}H and ^{13}C NMR spectra are reported for the first time.

In addition to compound **1**, other flavonoid glucosides were identified as mixtures from HPLC fractions. The positive HRESIMS of the 3rd HPLC fraction show a molecular ion peak $[M+Na]^+$ at *m*/*z* 501.1008, 517.0955 and 487.0849 suggesting the molecular formula $C_{22}H_{22}O_{12}Na$ (**2**), $C_{22}H_{22}O_{13}Na$ (**3**) and $C_{21}H_{20}O_{12}Na$ (**4**), respectively. The ^{1}H NMR spectrum of **2** is similar as of **1** but the resonance of the caffeoyl and a methoxy ^{1}H NMR are absent. The attached positions of glucose and methoxy group were confirmed by HMBC spectrum in which H-1″ at δ 5.12 (*d*, *J* = 7.8 Hz) and 6-methoxy at δ 3.89 (*s*) correlated with C-7 at δ 157.6 and C-6 at δ 132.9, respectively. In the case of compound **3**, glucose was found to be attached at C-3 as the HMBC spectrum of compound **3** shows a correlation between H-1″ at δ 5.25 (*d*, *J* = 7.9 Hz) and C-3 at δ 135.2. Compared with the singlet of H-8 at δ 6.50 of compound **3**, compound **4** shows clear doublets of H-6 at δ 6.20 (*d*, *J* = 2.1 Hz) and H-8 at δ 6.39 (*d*, *J* = 2.1 Hz) which are correlated in the COSY spectrum. Based on the NMR and HRESIMS data, compounds **2**, **3** and **4** were thus elucidated as 5,7,3′,4′-tetrahydroxy-6-methoxy-flavone-7-*O*-β-D-glucoside, 3,5,7,3′,4′-pentahydroxy-6-methoxy-flavone-3-*O*-β-D-glucoside and 3,5,7,3′,4′-pentahydroxy-flavone-3-*O*-β-D-glucoside, respectively (Fig. 1). ^{1}H NMR assignments of the phenolic moieties and the anomeric protons (H-1″) of compounds **2**, **3** and **4** are listed in Table 2.

Table 1
^{1}H NMR and ^{13}C NMR data of **1** in CH_3OH-d_4 [a].

Position	^{1}H	^{13}C
2	–	165.7
3	6.51 (*s*)	103.6
4	–	182.9
5	–	156.2
6	–	132.9
7	–	156.4
8	6.85 (*s*)	95.5
9	–	153.5
10	–	105.7
6-OCH_3	3.90 (*s*)	61.7
1′	–	120.7
2′	7.31 (*d*, *J* = 2.0)	110.2
3′	–	147.7
4′	–	150.8
5′	6.84 (*d*, *J* = 8.3)	116.4
6′	7.38 (*dd*, *J* = 8.3, 2.0)	121.7
1″	5.15 (*d*, *J* = 7.8)	101.7
2″	3.61 (*dd*, *J* = 9.1, 7.8)	74.5
3″	3.54 (*t*, *J* = 9.1)	77.5
4″	3.40 (*t*, *J* = 9.3)	72.6
5″	3.88 (*m*)	75.0
6″	4.74 (*dd*, *J* = 11.8, 2.1), 4.20 (*dd*, *J* = 11.8, 8.4)	64.4
3′-OCH_3	3.94 (*s*)	56.0
1‴	–	125.8
2‴	6.58 (*d*, *J* = 1.4)	114.9
3‴	–	145.4
4‴	–	147.7
5‴	6.46 (*d*, *J* = 8.2)	115.9
6‴	6.44 (*dd*, *J* = 8.2, 1.4)	122.6
7‴	7.35 (*d*, *J* = 15.8)	147.5
8‴	6.08 (*d*, *J* = 15.8)	114.6
COO–	–	166.9

[a] Chemical shift in ppm and coupling constant in Hz. ^{1}H- and ^{13}C-chemical shift were calibrated to δ 3.30 for ^{1}H and δ 49.0 for ^{13}C of the residual CH_3OH-d_4.

Three additional flavonoids were identified from the 4th HPLC fraction. The positive HRESIMS of the 3rd HPLC fraction showed a molecular ion peak $[M+Na]^+$ at *m*/*z* 501.1013, 515.1158 and 471.0910 suggesting the molecular formula $C_{22}H_{22}O_{12}Na$ (**5**), $C_{23}H_{24}O_{12}Na$ (**6**) and $C_{21}H_{24}O_{12}Na$ (**7**), respectively. ^{1}H NMR spectrum of compound **5** is similar to that of compound **4**, except for a downfield shift of H-2′ from δ 7.60 (*d*, *J* = 2.2 Hz) to δ 7.92 (*d*, *J* = 2.1 Hz). The shift is caused by the attachment of a methoxy group at C-3′ which was confirmed by the correlation between 3-methoxy at δ 3.94 (*s*) and C-3′ at δ 147.7 in HMBC spectrum. The position of β-D-glucose was also confirmed by the correlation between H-1 at δ 5.40 (*d*, *J* = 7.7) and C-3 at δ 135.2. The compound **6** has a similar pattern of the ^{1}H NMR spectrum as compound **1**, but 7-*O*-β-D-glusoside is without caffeoyl moiety. The presence of two methoxy groups at C-6 and C-3′, and an *O*-β-D-glucosyl moiety at C-7 were confirmed by the HMBC spectrum. Using the 1D and 2D NMR spectra and HRESIMS the structures of compounds **5** and **6** were elucidated as 3,5,7,4′-tetrahydroxy-3′-methoxyflavone-3-*O*-β-D-glucoside and 5,7,4′-trihydroxy-3′-methoxyflavone-7-*O*-β-D-glucoside, respectively (Fig. 1). The compound **7** was elucidated as 5,7,4′-trihydroxyflavone-7-*O*-β-D-glucoside (Fig. 1) by comparison with authentic compound. ^{1}H NMR assignments of the phenolic moieties and the anomeric protons (H-1″) of compounds **5**, **6** and **7** are listed in Table 2.

Table 2
^{1}H NMR data of **1**–**6** in CH_3OH-d_4 [a].

Position	2	3	4	5	6	7
3	6.51 (*s*)	–	–	–	6.70 (s)	6.69 (s)
6	–	–	6.20 (d, 2.1)	6.20 (d, 2.1)	–	6.50 (d, 2.1)
8	6.95 (s)	6.50 (s)	6.39 (d, 2.1)	6.40 (d, 2.1)	7.01 (s)	6.85 (d, 2.1)
2′	7.41 (d, 2.3)	7.59 (d, 2.2)	7.60 (d, 2.2)	7.92 (d, 2.1)	7.52 (d, 2.1)	7.90 (d, 8.8)
3′	–	–	–	–	–	6.92 (d, 8.8)
5′	6.90 (d, 8.3)	6.86 (d, 8.4)	6.86 (d, 8.4)	6.90 (d, 8.4)	6.93 (d, 8.6)	7.90 (d, 8.8)
6′	7.43 (dd, 8.3, 2.3)	7.58 (dd, 8.4, 2.2)	7.58 (dd, 8.4, 2.2)	7.58 (dd, 8.4, 2.1)	7.56 (dd, 8.6, 2.1)	6.92 (d, 8.8)
6-OCH_3	3.89 (s)	3.87 (s)	–	–	3.89 (s)	–
3′-OCH_3	–	–	–	3.94 (s)	3.96 (s)	–
1″	5.12 (d, 7.8)	5.25 (d, 7.9)	5.25 (d, 7.9)	5.40 (d, 7.7)	5.12 (d, 7.7)	5.07 (d, 7.5)

[a] Chemical shift in ppm and coupling constant in Hz. ^{1}H-chemical shift were calibrated to δ 3.30 for ^{1}H of the residual CH_3OH-d_4.

In addition to these flavonoids the three phenylpropanoids, *trans*-caffeic acid (**8**), chlorogenic acid (**9**) and cynarin (**10**) were isolated and identified by comparison with authentic samples.

3. Experimental

3.1. General experimental procedures

^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H-J-resolved, COSY, HSQC, and HMBC (600 MHz for ^{1}H and 150 MHz for ^{13}C) were recorded at 25 °C on a Bruker AV-600 spectrometer equipped with cryoprobe (Bruker, Karlsruhe, Germany). ^{1}H- and ^{13}C-resonances were calibrated to the residual solvent signal of CH_3OH-d_4 at δ 3.30 for ^{1}H and δ 49.0 for ^{13}C.

Mass spectra were measured by an ESITOFMS. The operating conditions of the ESI ion source (Jeol, Tokyo, Japan) coupled to a JMS-T100TD (AccuTOF-TLC) in the positive ion modes were a discharge needle voltage of 2000 V, nebulizing nitrogen gas flow at 1 L/min. The first orifice lens was set to 100 V and Ring lens voltage was set to 13 V. The TOFMS was set with a peak voltage of 2500 V, a bias voltage of 29 V, a pusher bias voltage of −0.76 V, and a detector voltage of 2300 V.

High performance liquid chromatography (HPLC) was performed on an Agilent 1100 (Waldbronn, Germany) using a reversed phase column (Phenomenex Luna C18, 250 mm × 10 mm, 5 μm, Phenomenex, Torrence, CA, USA). Separation of the compounds was performed with a gradient of water–MeOH–formic acid (70:30:0.1) to water–MeOH–formic acid (20:80:0.1) in 26 min at a flow rate of 2.0 mL/min.

Thin layer chromatography (TLC) was performed on silica gel $60F_{254}$ plate (Merck, Darmstadt, Germany) using EtOAC–formic acid–acetic acid–water (100:11:11:27). The fractions obtained from Sephadex LH-20 column chromatography were pooled by the UV profiling (254 nm).

3.2. Plant material

Aerial parts of *G. uliginosum* L. were collected by Mr. Bertalan Galambosi from plantation of MTT Agrifood Research Finland, Mikkeli (61°44′N, 27°18′E) in August 2007. Voucher specimens have been deposited in the herbarium of the St-Petersburg Institute of Pharmacy (St. Petersburg, Russia). The plant samples were dried at 40 °C in an air-forced dryer, ground to a powder in an excelsior mill and stored in closed vessels.

3.3. Extraction and isolation of compounds

Five grams of dry plant material was extracted using a Soxhlet apparatus for 5 h (20–25 cycles) with EtOH and obtained extract was evaporated *in vacuo*. Residue was dissolved in 10.0 mL of 10% NaCl water solution on boiling water bath, filtered and loaded onto column (10 mm × 150 mm) with polyamide sorbent (ICN Biomedicals GmbH, Eschwege, Germany). The column was washed by deionized water (30 mL), and eluted by EtOH. The solvent was removed under vacuum.

The dried ethanol extract (250 mg) was redissolved in MeOH and subjected to column chromatography over Sephadex LH-20 (60 g, Pharmacia, Amsterdam, The Netherlands) using MeOH as eluents. A total of 64 fractions (10 mL each) were collected. Fractions showing similar TLC profiles were pooled to afford seven combined fractions. Chlorogenic acid (5-caffeoyl quinic acid, 1.5 mg, **9**) and cynarin (1,5-dicaffeoyl quinic acid, 2.0 mg, **10**) were isolated from the 3rd and the 7th fraction, respectively. Those chemical structures were confirmed by the comparison with ^{1}H NMR spectra of authentic compounds. The 6th fraction obtained from Sephadex LH-20 was further separated by high performance liquid chromatography. Fractions showing similar HPLC-UV profiles at 254 nm were pooled to afford six combined fractions. The 2nd fraction yielded 3.5 mg of caffeic acid (**8**) and was confirmed by the comparison with the ^{1}H NMR spectrum of the reference compound. A caffeoyl flavone glycoside, 5,3′,4′-trihydroxy-6,3′-dimethoxyflavone-7-*O*-β-D-(6″-*O*-caffeoyl)-glucopyranoside (4.5 mg, **1**) was isolated from the 5th fraction. The chemical structure was elucidated using ^{1}H NMR, ^{1}H–^{1}H-J-resolved, COSY, HSQC, and HMBC spectra together with HRESITOFMS spectrum. The 3rd and 4th fractions of HPLC separation showed the mixture of flavonoid glucosides. Using 1D and 2D NMR spectra as well as HRESIMS spectrum the 3rd fraction was confirmed to contain 5,7,3′,4′-tetrahydroxy-6-methoxyflavone-7-*O*-β-D-glucoside (**2**), 3,5,7,3′,4′-pentahydroxy-6-methoxyflavone-3-*O*-β-D-glucoside (**3**) and 3,5,7,3′,4′-pentahydroxy-flavone-3-*O*-β-D-glucoside (**4**). In the 4th fraction 3,5,7,4′-tetrahydroxy-3′-methoxyflavone-3-*O*-β-D-glucoside (**5**), 5,7,4′-trihydroxy-3′-methoxyflavone-7-*O*-β-D-glucoside (**6**) and 5,7,4′-trihydroxyflavone-7-*O*-β-D-glucoside (**7**).

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