PERGAMON

Phytochemistry 61 (2002) 433–437

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Flavonoid glycosides from *Centaurea pseudoscabiosa* subsp. *pseudoscabiosa* from Turkey

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Received 4 February 2002; received in revised form 12 June 2002

Abstract

Three flavonoid glycosides were isolated and characterized, together with a further 13 substances belonging to various classes of compounds, in particular two phenolic acids, a coumarin, a sugar and nine flavonoids from the flowered aerial parts of *Centaurea pseudoscabiosa* subsp. *pseudoscabiosa* Boiss. et Buhse (Asteraceae). Some considerations about their evolutionary meaning are provided.

Keywords: *Centaurea pseudoscabiosa* subsp. *pseudoscabiosa*; Asteraceae; Pinocembrin 7-*O*-α-arabinopyranosyl-(1→2)-β-glucopyranoside; Chrysin 7-*O*-β-galactopyranuronoside; Baicalein 6-methylether-7-*O*-β-galactopyranuronoside; Evolution

1. Introduction

Centaurea pseudoscabiosa subsp. *pseudoscabiosa* Boiss. et Buhse [synonyms: *Centaurea pseudoscabiosa* var. *spirokensis* Bornm., *Colymbada pseudoscabiosa* (Boiss. et Buhse) Holub) belongs to Asteraceae family, Sect. Acrocentron]. It is a 40–90 cm herbaceous plant with purple flowers. It usually grows in East Anatolia (Turkey) and in Transcaucasia and northwest Iran in the rest of the World. Although mainly distributed in East Anatolia, the plant has been collected for the first time in Inner Anatolia. This plant prefers the clearings of *Cedrus* and *Abies* forests, rocky slopes and semi-arid, moist-cold climate; it grows on calcareousless Brown Forest soil. This species is not used in the Turkish folk medicine, although other species of the same genus (i.e. *C. cyanus* and *C. scabiosa*) are used against coughs, as liver-strengthening, itch eliminating, and ophthalmic remedies (Poletti, 1978; Baser et al., 1986; Baytop, 1999); for *C. calcitrapa, C. solstitialis* and *C. melitensis* a hypoglycemic effect and for, *C. calcitrapa*, *C. iberica* and *C. jacea* an antipyretic effect have been demonstrated (Masso et al., 1979; Baytop, 1999). The studies on this genus deal mainly with sesquiterpene lactones, while flavonoids are less investigated; the species *C. pseudoscabiosa* has never been studied at all. This paper deals with the isolation and characterization of 16 substances belonging to various classes of compounds, in particular two phenolic acids, a coumarin, a sugar and 12 flavonoids, three of which are new natural compounds.

2. Results and discussion

From the *n*-hexane residue (R_H) two compounds were isolated, namely chrysin and baicalein 6-methyl ether, which were identified by comparison of their physical and spectroscopic properties with literature reports (Shen et al., 1993; Buschi et al., 1981; Agrawal et al., 1989).

Fractionation of the $CHCl_3$–MeOH (9:1) extract (R_{CM}) led to the isolation of nine pure compounds. These were protocatechuic acid, 5-caffeoylquinic acid, chrysin, hispidulin, chrysin 7-*O*-glucuronide, chrysin 7-*O*-glucuronide methyl ester, chrysin 6-*C*-glucoside and chrysin 8-*C*-glucoside, all identified by MS and ^{1}H and

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0031-9422/02/$ - see front matter
PII: S0031-9422(02)00229-7

^{13}C NMR spectroscopy (Collado et al., 1985; Iwahashi et al., 1985; Shen et al., 1993; Agrawal et al., 1989; Yuldashev et al., 1996; Aritomi and Kawasaki, 1984; Miyaichi and Tomimori, 1994; Zhang et al., 1994).

The EtOAc and BuOH residues (R_{Ac} and R_{Bu}) gave sucrose, chrysin 7-glucuronide, luteolin 7-glucoside, and the new compounds **1**, **2** and **3**. Known isolates were identified by comparison with literature data or authentic samples (Lu and Foo, 2000; Agrawal et al., 1989).

Compound **1** was obtained as a pale yellowish powder that appears on TLC as a yellow spot after treatment with Naturstoffreagenz A-PEG. The negative FABMS spectrum gave a quasimolecular peak $[M-H]^-$ at 549 m/z, corresponding to the molecular formula $C_{26}H_{30}O_{13}$, supported also by elemental analysis (see Section 3). The ^{13}C NMR spectrum showed 22 signals, sorted by DEPT experiments into 13 CH, 3 CH_2 and 6 quaternary C. In the ^{1}H NMR spectrum two coupled one-proton doublets (J = 2.0 Hz) at δ 6.13 and δ 6.17 indicated two *meta*-related hydrogens on ring A of a flavonoid. Furthermore a three-proton multiplet coupled with a two-proton one were visible at δ 7.43 and δ 7.53, respectively. This situation is typical of a flavonoid nucleus with an unsubstituted B ring. Three one-proton coupled double doublets (δ 5.64, J = 12.7 and 2.9 Hz; δ 3.33, J = 16.6 and 12.7 Hz; δ 2.82, J = 16.6 and 2.9 Hz) suggested that ring C was saturated; this splitting pattern was due to the coupling between the H-2 axial proton and the H-3 geminal protons. The flavanonic nature of **3** was confirmed by the very deshielded signal of C-4 (δ 196.9). Furthermore, the resonances of a sugar moiety were present. By means of ^{13}C NMR it was possible to identify the presence of a terminal α-arabinopyranoside residue linked to the C-2 of a β-glucopyranose (downfield shift of C-2 and upfield shift of C-1). This situation was confirmed by COLOC experiments. The linkage site of the sugar moiety was determined on the 7-OH. Therefore **1** was identified as pinocembrin 7-*O*-α-arabinopyranosyl-(1→2)-β-glucopyranoside (Fig. 1).

The ^{1}H NMR spectrum of **2** showed the typical pattern of chrysin: two one-proton *meta*-related doublets (J = 1.9 Hz) at δ 6.88 and δ 6.46, a singlet at δ 7.04 and the two multiplets at δ 7.58 and δ 8.08 due to the five protons of ring B. Furthermore the signals of a glycosidic moiety were visible, in particular the doublet (J = 6.8 Hz) of the anomeric hydrogen at δ 5.08. The ^{13}C NMR spectrum confirmed the identification of chrysin and permitted to recognize the sugar as β-galacturonic acid. It was possible to distinguish between the galacturonic and the glucuronic acid derivatives (both isolated from the plant), besides from the different ^{13}C NMR chemical shifts, also because in the former was absent the *trans*-diaxial doublet (J = 9.3 Hz) at δ 4.05 (H-5″) since the different C-4 stereochemistry. Infact, in the latter compound, H-5″ showed a doublet with a J value of 7.1 Hz due to the axial/equatorial coupling. Furthermore the hydrolysis products were compared with reference compounds. Placement of the sugar moiety on 7-OH was determined on the basis of the typical glycosilation shifts that occurred with respect to chrysin, particularly the C-7 upfield shift (1.2 ppm) and the downfield shifts of the *ortho* (ca. 0.8 ppm) and *para* (1.1 ppm) carbons. This structure is also supported by the observed COLOC correlations (Fig. 1) and by its negative FABMS spectrum where, besides the quasimolecular peak at m/z 429, $[M-H]^-$, the peak due to the loss of galacturonic acid $[M-H\text{-}176]^-$ at 255 m/z was evident. Therefore, **2** was identified as chrysin 7-*O*-β-galactopyranuronoside (Fig. 1).

The ^{1}H NMR spectrum of **3** was very similar to that of **2**, both for the aglycone and the sugar moieties. The main differences consisted in the absence of the two doublets of ring A, replaced by a one-proton singlet at δ 7.00 and a new three-proton singlet of an aromatic methoxyl group at δ 3.77. The high resonance value of the latter (δ 60.3) in the ^{13}C NMR spectrum suggested that both the *ortho* positions should be substituted, so it must be placed only at the 6 or 8 position. The unsubstituted methine resonated at δ 94.4, so the methoxyl must be linked to C-6 (Agrawal et al., 1989; Markham and Chari, 1982). The sugar moiety was identified as β-galacturonic acid and placed on the 7-OH following the same reasoning described for **2**. These circumstances

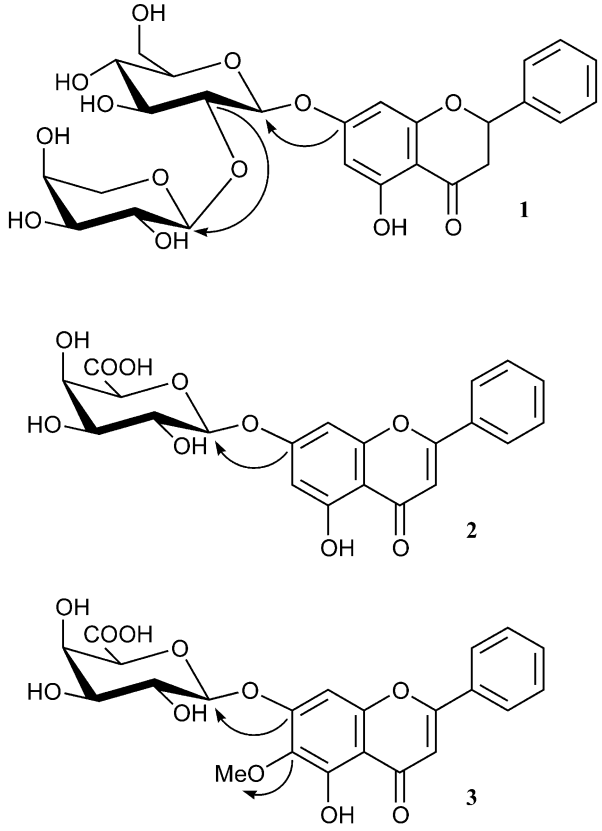

Fig. 1. Significant COLOC correlations observed for **1–3**. Arrows point from C to H.

were supported by COLOC and FABMS spectrum (see Section 3). Therefore, **3** was characterized as baicalein 6-methylether-7-*O*-β-galactopyranuronoside.

One of the known compounds, chrysin 7-glucuronide methyl ester, was previously identified only by mean of ^{1}H NMR data (Yuldashev et al., 1996), so we report here its ^{13}C NMR values for the first time.

Summarizing the aerial parts of *C. pseudoscabiosa* subsp. *pseudoscabiosa* yielded 16 substances, among which 12 were flavonoids, two phenolic acids, a coumarin, and a sugar. The usual pattern for this genus is the polymethoxylation of the flavonoids, but in this species, with the exception of baicalein 6-methyl ether and its glycoside, all the flavonoids were not methoxylated. Also in the case of baicalein derivatives only one methoxyl is present in the molecules. It seems that polymethoxylation of the flavonoids represent an advanced character in many plant families; furthermore, the presence of uronic acids seems to be an ancient character (Gottlieb, 1990; De P. Emerenciano et al., 1987; Giannasi, 1988; Richardson and Young, 1982). Flavonoid *C*-glycosides are quite rare in the genus *Centaurea*, having been isolated only from *C. solstitialis*, *C. virgata* (Kaïji-a-Kamb et al., 1992), *C. montana* (Gonnet, 1992), *C. triunfetti* (Gonnet, 1993) and *C. horrida* (Flamini et al., 2002). From a literature survey, sesquiterpene lactones are the main class of substances isolated from the genus *Centaurea,* but no sesquiterpene lactones were present in this species. The complete absence of these chemicals, the lack of polymethoxylation on the flavonoid nucleus and the presence of many glucuronides and galacturonides exhibited by *C. pseudoscabiosa* subsp. *pseudoscabiosa* could be due either to the antiquity of this species, which may not have developed biosynthetic pathways for these substances or to mutations that have blocked these pathways at relatively early levels. Section Acrocentron, in which *C. pseudoscabiosa* subsp. *pseudoscabiosa* was included, contains primitive species of this genus (Davis, 1975), so the former hypothesis should be the more acceptable one. Furthermore, a similar chemistry was found also for *C. horrida*, a wellknown very ancient species (Pignatti, 1982), in which polymethoxylated flavonoids and sesquiterpene lactones were absent, while *C*-glycosides and flavonoid glucuronides were detected (Flamini et al., 2002).

3. Experimental

3.1. General

Melting points (uncorrected) were determined with a Kofler apparatus; optical rotations were measured on a Perkin-Elmer 241 polarimeter; FABMS were recorded, in the negative mode, with a VG ZAB instrument; ^{1}H- and ^{13}C NMR spectra were obtained with a Brüker AC200 spectrometer in CD_3OD, DMSO-d_6, and $CDCl_3$, using TMS as internal standard. All 1D and 2D NMR experiments were performed using the standard Bruker library of microprograms; known compounds were identified by comparison of their spectral data with those of literature and, when available, with authentic samples.

The following adsorbents were used for purification: flash chromatography, Merck Kieselgel 60 (230–410 mesh); low-pressure chromatography, Merck Lobar Lichroprep RP-8 and RP-18 (31×2.5 cm); size-exclusion chromatography, Pharmacia Fine Chemicals Sephadex LH-20; analytical TLC, Merck Kieselgel 60 F_{254} precoated plates; chromatograms were visualized under UV light at 254 and 366 nm and/or sprayed with Komarowsky or cerium sulphate or Naturstoffreagenz A-PEG reagents.

3.2. Plant material

The flowered aerial parts of *Centaurea pseudoscabiosa* subsp. *pseudoscabiosa* Boiss. et Buhse were collected in Turkey, C4 Konya, Hadim, Gevne Vadisi, in open *Cedrus* and *Abies* forest, 1500 m above the sea level, at the end of June 2000 (36°52′07″N, 32°20′47″E). Voucher specimens are deposited at GAZI (Gazi University, Science and Art Faculty, The Herbarium of Biology Department) as Ertugrul 2267.

3.3. Extraction and isolation

The dried and ground aerial parts (1200 g) were extracted, successively, in a Soxhlet apparatus with *n*-hexane, $CHCl_3$, $CHCl_3$–MeOH (9:1) (4.5 1×30 h) and, at room temperature, with MeOH (3.5 1×7 days×3 times). After removal of solvents, in vacuo up to 40 °C, the following residues were obtained: R_H (5.8 g), R_C (10.1 g), R_{CM} (40.2 g), and R_M (16.8 g).

R_M was suspended in MeOH–H_2O (6:4) and extracted, in turn, with EtOAc and *n*-BuOH obtaining, after removal of the solvents, the residues R_{MAc} and R_{MBu} (2.5 and 3.2 g, respectively).

The R_H residue, after repeated SiO_2 gravity and flash column chromatographies and preparative TLCs, gave chrysin (12 mg) and baicalein 6-methyl ether (8 mg).

The R_{CM} residue was submitted to Sephadex LH-20 size-exclusion chromatography with MeOH–$CHCl_3$ (4:1) as eluent (fractions A–L) obtaining, after crystallization from fraction C, pure chrysin 7-*O*-glucopyranouronide (66 mg). Fraction E, after SiO_2 gravity column chromatography, eluting with $CHCl_3$–MeOH (gradient from 9:1 to 7:3) followed by flash chromatography ($CHCl_3$–MeOH, gradient from 9:1 to 8:2) yielded protocatechuic acid (18 mg), 5-caffeoylquinic acid (7 mg), scopoletin (8 mg), and again

chrysin 7-*O*-glucopyranouronide (48 mg). Fraction H was chromatographed on a SiO_2 column eluting with $CHCl_3$–MeOH–H_2O (gradient from 9:1:0 to 6:4:1) obtaining, directly or after preparative TLC, chrysin 7-*O*-glucopyranouronide methyl ester (17 mg), chrysin 6-*C*-glucopyranoside (22 mg), and chrysin 8-*C*-glucopyranoside (20 mg). Fraction J, after SiO_2 column chromatography, gave hispidulin (4 mg) and chrysin again (7 mg).

R_{MAc} was submitted to Sephadex LH-20 size-exclusion chromatography with MeOH as eluent (fractions A′–G′) obtaining from fraction B' sucrose as precipitate (100 mg). Another precipitate, recovered from fraction D', was identified as pinocembrin

Table 1
^{1}H NMR data of compounds **1**–**3** and chrysin 7-glucuronide and baicalein 6-methylether 7-glucuronide (200 MHz, DMSO-d_6); *J* (Hz) in parentheses

H	**1**	**2**	Chrysin 7-glucuronide	**3**	Baicalein 6-OMe 7-glucuronide
2	5.64, *dd*, (12.7 e 2.9)	–	–	–	–
3	2.82 eq, *dd* (16.6 e 2.9) 3.33 ax, *dd*, (16.6 e 12.7)	7.04, *s*	7.07, *s*	7.03, *s*	7.10, *s*
6	6.13, *d*, (2.0)	6.46, *d*, (1.9)	6.49, *d*, (2.0)	–	–
8	6.17, *d*, (2.0)	6.88, *d*, (1.9)	6.91, *d*, (2.0)	7.00, *s*	7.03, *s*
2′–6′	7.53, *m*	8.08, *m*	8.09, *m*	8.06, *m*	8.07, *m*
3′–4′–5′	7.43, *m*	7.58, *m*	7.59, *m*	7.58, *m*	7.59, *m*
1″	5.13, *d* (7.3)	5.08, *d* (6.8)	5.29, *d* (6.9)	5.13, *d*, (6.9)	5.30, *d*, (6.7)
2″	3.39, *m*	3.65, *m*	n.d.[a]	3.65, *m*	n.d.
3″	3.45, *m*	3.61, *m*	n.d.	3.63, *m*	n.d.
4″	3.17, *m*	3.74, *m*	n.d.	3.80, *m*	n.d.
5″	3.13, *m*	3.82, *d*, (7.1)	4.05, *d*, (9.3)	3.83, *d*, (7.2)	4.02, *d*, (9.1)
6″	3.65, *m* 3.42, *m*	–	–	–	–
1‴	4.50, *d* (7.8)	–	–	–	–
2‴	3.44, *m*	–	–	–	–
3‴	3.50, *m*	–	–	–	–
4‴	3.62, *m*	–	–	–	–
5‴	3.63, *m* 3.36, *m*	–	–	–	–
6-OCH3	–	–	–	3.77, *s*	3.77, *s*

[a] n.d., not determined.

Table 2
^{13}C NMR data of compounds **1–3**, chrysin 7-glucuronide and baicalein 6-methylether 7-glucuronide, and chrysin 7-glucuronide methyl ester (50 MHz, DMSO-d_6)

C	**1**	**2**	Chrysin 7-glucuronide	**3**	Baicalein 6-OMe 7-glucuronide	Chrysin 7-glucuronide methyl ester
2	78.7	163.7	163.8	163.7	163.7	163.8
3	42.3	105.5	105.5	104.9	104.9	105.6
4	196.9	182.2	182.2	182.5	182.5	182.3
5	163.0	161.1	161.2	152.3	152.2	161.3
6	96.7	99.6	99.1	133.7	132.8	99.4
7	165.1	163.3	162.7	156.8	156.3	162.7
8	95.6	94.9	94.8	94.4	94.2	94.8
9	162.6	157.1	157.1	152.4	152.4	157.2
10	103.4	105.6	105.7	106.0	106.1	105.1
1′	138.5	130.6	130.6	130.6	130.6	130.6
2′–6′	126.8	126.5	126.5	126.4	126.3	126.6
3′–5′	128.7	129.2	129.3	129.2	129.1	129.2
4′	132.1	132.3	132.2	132.1	132.1	132.3
1″	98.0	99.9	99.7	100.0	99.5	99.6
2″	81.2	74.0	72.9	74.1	72.8	72.7
3″	77.0	73.0	75.5	73.0	75.3	75.2
4″	69.2	69.8	71.4	71.8	71.3	71.6
5″	76.1	76.5	75.8	76.7	76.0	75.5
6″	60.5	172.3	170.1	171.9	170.4	169.3
1‴	104.0	–	–	–	–	–
2‴	71.2	–	–	–	–	–
3‴	69.2	–	–	–	–	–
4‴	66.7	–	–	–	–	–
5‴	64.1	–	–	–	–	–
OCH3	–	–	–	60.3	60.3	52.0

7-*O*-α-arabinopyranosyl-(1→2)-β-glucopyranoside (**1**) (51 mg). Fraction E' was constituted by pure chrysin 7-*O*-glucopyranouronide (23 mg). Fraction F' after chromatography on a Lobar RP-18 column eluting with MeOH–H_2O–formic acid (7:3:0.1) yielded chrysin 7-*O*-β-galactopyranuronoside (**2**) (18 mg). From fraction G' luteolin (9 mg) was purified by preparative TLC.

R_{MBu} was filtered on a Sephadex LH-20 column eluting with MeOH (fractions A″–H″). After crystallization fraction C″ gave baicalein 6-methylether-7-*O*-β-galactopyranuronoside (**3**) (40 mg). Fraction E″ was submitted to lobar RP-8 chromatography (MeOH–H_2O–formic acid 7:3:0.1 as eluent) obtaining chrysin 7-*O*-glucopyranouronide (26 mg) again. From fraction F″, after preparative TLC, further 8 mg of chrysin 7-*O*-β-galactopyranuronoside (**2**) were purified. Fraction H″ was constituted by pure luteolin 7-*O*-glucopyranoside (12 mg).

3.4. New compounds

Pinocembrin 7-O-α-arabinopyranosyl-(1→2)-β-glucopyranoside (**1**): pale yellowish powder; ^1H and ^{13}C NMR spectral data see Tables 1 and 2; FABMS (negative mode) *m*/*z* 572 $[M+Na-H]^-$, 549 $[M-H]^-$, 417 $[M-H-132]^-$, 255 $[M^-H-294]^-$; Elemental analysis: found: C 56.45% H 5.66%, requires: C 56.73% H 5.49%.

Chrysin 7-O-β-galactopyranuronoside (**2**): pale yellowish amorphous powder; ^1H and ^{13}C NMR spectral data see Tables 1 and 2; FABMS (negative mode) *m*/*z* 452 $[M+Na-H]^-$, 429 $[M-H]^-$, 253 $[M-H-176]^-$; Elemental analysis: found: C 58.33% H 4.36%, requires: C 58.61% H 4.22%.

Baicalein 6-methylether-7-O-β-galactopyranuronoside (**3**): yellowish amorphous powder; ^1H and ^{13}C NMR spectral data see Tables 1 and 2; FABMS (negative mode) *m*/*z* 482 $[M+Na-H]^-$, 459 $[M-H]^-$, 283 $[M-H-176]^-$; Elemental analysis: found: C 57.12% H 4.51%, requires: C 57.39% H 4.38%.

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