Trends in Phytochemical Research (TPR)

Journal Homepage: http://tpr.iau-shahrood.ac.ir

Original Research Article

Flavonoids from two Turkish *Centaurea* species and their chemotaxonomic implications

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ABSTRACT

Centaurea asutro-anatolica Hub.-Mor. and *C. kizildaghensis* Uzunh., E. Doğan & H. Duman, two indigenous perennial herbs from the Turkish flora, belong to the medicinally important genus *Centaurea* L. (fam: Asteraceae), which comprises *ca*. 600 species worldwide. While various *Centaurea* species are well-known for producing alkaloids, flavonoids, lignans and terpenoids, there is no report on any thorough phytochemical work on any of these two species available to date. In continuation of our phytochemical and bioactivity studies on the Turkish *Centaurea* species, four flavonoids apigenin (**1**), apigenin 7,4′-dimethyl ether (**2**), genkwanin (**3**) and quercetin (**4**) were isolated from the methanol extracts of the aerial parts of *C. austro-anatolica* and *C. kizildaghensis*, for the very first time. The structures of the flavonoids were elucidated conclusively by spectroscopic means, *i.e.*, UV, MS and 1D and 2D NMR data analyses. The distribution of these flavonoids (**1**-**4**) within the genus *Centaurea* and their possible chemotaxonomic implications within the genus *Centaurea* or the family Asteraceae have been discussed.

ARTICLE HISTORY

Received: 21 October 2017
Revised: 30 October 2017
Accepted: 31 October 2017
ePublished: 11 December 2017

KEYWORDS

Centaurea asutro-anatolica
Centaurea kizildaghensis
Asteraceae
HPLC
Flavonoids
Chemotaxonomy

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1. Introduction

Centaurea austro-anatolica Hub.-Mor. and *Centaurea kizildaghensis* Uzunh., E. Doğan & H. Duman are two Turkish endemic species of the genus *Centaurea* L., which includes about 600 herbaceous thistle-like flowering plants worldwide, and 182 species of which come from Turkey (Sarker et al., 1997; Russo et al., 2016). *C. austro-anatolica* is a 30-60 cm tall woody perennial herb, branched above, leaves are tomentose and hairy, has pink flowers, and prefers macchie, under *Pinus brutia* forest and screes, 460 m above the sea level. This species grows only in Antalya and Muğla Provinces in Turkey (Wagenitz, 1975). On the other hand, *C. kizildaghensis* is a perennial herb with a woody rootstock erect stem up to 52 cm long, slightly striate and scarcely tomentose hairs. It has yellow flowers. This plant prefers serpentine rocky slopes and clearings of a *Pinus nigra* from 1700 m to 1800 m above the sea level. This species only grows in Kızıl Dağ, close to Derebucak District, in Konya Province in Turkey (Uzunhisarcıklı et al., 2007). While several species of the genus *Centaurea* are well-known for their uses in folklore medicines for the treatment of diabetes, diarrhoea, hypertension, malaria, microbial infections, rheumatism and ulcers (Sarker et al., 1997; Baytop, 1999; Ugur et al., 2009), there is no report on any medicinal uses of *C. austro-anatolica* or *C. kizildaghensis* in folklore medicines in Antalya, Muğla or Konya provinces. There is only one report on GC-MS based volatile component analysis and antimicrobial activity assessment of *C. austro-anatolica* (Ugur et al., 2009), and only anthocyanin content was previously detected in *C. kizildaghensis* (Gokbel et al., 2015). As part of our on-going phytochemical and bioactivity

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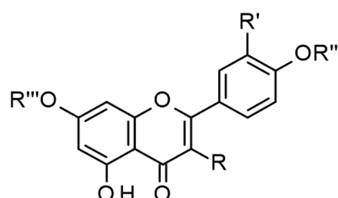
studies on the Turkish *Centaurea* species (Shoeb et al., 2005, 2007a-e; Granger et al., 2009; Sarker et al., 2005, 2007, 2012), we now report, for the very first time, on the isolation and identification of flavonoids, apigenin (**1**), apigenin 7,4′-dimethyl ether (**2**), genkwanin (**3**) and quercetin (**4**) from *C. austro-anatolica* and *C. kizildaghensis* (see Fig. 1), highlighting possible chemotaxonomic implications of these flavonoids within the genus *Centaurea* and the family Asteraceae.



Flavonoids	R	R′	R′′	R′′′
Apigenin (**1**)	H	H	H	H
Apigenin 7,4′-dimethyl ether (**2**)	H	H	Me	Me
Genkwanin (**3**)	H	H	H	Me
Quercetin (**4**)	OH	OH	H	H

Fig. 1. Molecular structures of the characterized flavonoids from *C. austro-anatolica* and *C. kizildaghensis*.

2. Experimental

2.1. Chemicals and regions

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Dorset, UK). All solvents for extraction and chromatography were purchased from Fisher Scientific, (Loughborough, UK). NMR solvents were from GOSS Scientific (Crewe, UK). Mass spectroscopic analyses were performed on a Finnigan MAT 95 spectrometer. The ^{1}H- and ^{13}C-NMR spectra were recorded at 600 MHz and 150 MHz, respectively, on an Ultrashield Bruker AMX 600 NMR spectrometer. Methyl, methylene and methane carbons were distinguished by DEPT experiments. Homonuclear ^{1}H connectivity was determined by using the COSY experiment. ^{1}H-^{13}C one-bond connectivity was established with HSQC gradient pulse factor selection. Two- and three-bond connectivity was confirmed by HMBC experiments. Chemical shifts are reported in δ (ppm) and coupling constants (*J*) were measured in Hz.

2.2. Plant materials

The flowering aerial parts of *C. austro-anatolica* Hub.-Mor. were collected from Antalya, Kumluca District (36° 17′ 18′′N, 030° 24′ 08′′E), about 250 m above the sea level, during 2015 and a voucher specimen (Gokturk 7888) has been deposited in the Herbarium of the Biology Department of Akdeniz University. Similarly, the flowering aerial parts of *C. kizildaghensis* Uzunh., E. Doğan & Duman were collected from Konya, Derebucak District (37° 21′ 01′′N, 031° 40′ 48′′E), 1760 m above the sea level during July 2015, and a voucher specimen (Gokturk 7963) has been deposited in the same herbarium.

2.3. Extraction and isolation

Air-dried and ground flowering aerial parts of *C. austro-anatolica* and *C. kizildaghensis* (400 g each) were separately macerated overnight at room temperature, sequentially, using solvents of increasing polarity, n-hexane, dichloromethane (DCM) and methanol (MeOH) (3×1.5 L each). Extracts were evaporated to dryness using a rotary evaporator (max temp 50 °C). The MeOH extraction afforded the maximum amounts of dried extracts from both species (34 g and 51 g from *C. kizildaghensis* and *C. austro-anatolica*, respectively), and were subjected to subsequent chromatographic separations using a combination of solid-phase extraction (SPE), analytical HPLC and preparative and/or semi-preparative HPLC on reversed-phase C_{18} stationary phase.

2.3.1. Solid-phase extraction

A portion (2.0 g) of the MeOH extract of *C. austro-anatolica* and *C. kizildaghensis*, was individually subjected to SPE fractionation on a Strata (reversed-phase C_{18}, 20 g, manufacturer: Phenomenex) pre-packed cartridge, using eluents of decreasing polarity (water-MeOH mixture: 80:20, 50:50, 20:80 and 0:100, 250 mL each) to obtain four fractions (named as SPE fractions 1-4) from each MeOH extract. All fractions were evaporated to dryness using a rotary evaporator (max temp 50 °C).

2.3.2. Analytical HPLC

All SPE fractions (10 mg/mL) were analysed by analytical HPLC using a Dionex Ultimate 3000 UPLC, coupled with an autosampler, degasser and a photodiode array detector, on a Thermo Scientific™ Hypersil GOLD™ C_{18} column (150 mm×4.6 mm, 5 μm) connected to a guard column, firstly to have an understanding of their chemical profiles (a linear gradient mobile phase: 30-100% MeOH in water in 30 min followed by 100% MeOH for 10 min, 1 mL/min, each solvent contained 0.1% TFA) and also to develop appropriate methods (various gradients of MeOH in water, 1 mL/min) suitable for separation of compounds using a preparative or semi-preparative HPLC. Injection volume was 20 μL. All chromatograms were monitored at four different wavelengths, 220, 250, 280 and 320 nm, and all separated peaks were analysed after each run using the Chromeleon™ 7.2 for UV-Vis data data. Analytical HPLC was also used to check the purity of compounds isolated from the preparative and/or semi-preparative HPLC.

2.3.3. Preparative and/or semi-preparative HPLC

Preparative and/or semi-preparative HPLC separation was performed on an Agilent 1200 preparative HPLC system, coupled with an autosampler, online degasser and photo-diode-array detector. For preparative separation a HiChrom ACE5 C_{18} prep-column (150 mm, 21.2 mm, 5 μm; HiChrom; flow rate 10 mL/min) and a Luna semi-prep C_{18} column (150 mm×10 mm, 5 μm; Phenomenex; flow rate: 2 mL/min) were used.

While the preparative HPLC purification (a linear gradient mobile phase: 30-70% MeOH in water in 30 min followed by 100% MeOH for 10 min, 10 mL/min, each solvent contained 0.1% TFA) of the SPE fraction 2 (230 mg) of the MeOH extract of *C. austro-anatolica* afforded two flavonoids, quercetin (**4**, t_R=15.4 min, 10.9 mg) and apigenin (**1**, t_R=19.4 min, 7.8 mg), a semi-preparative HPLC purification (a linear gradient mobile phase: 55-90% MeOH in water in 30 min followed by 100% MeOH for 10 min, 2 mL/min, each solvent contained 0.1% TFA) was carried out for the purification of the SPE fraction 3 (28 mg) to obtain genkwanin (**3**, t_R=17.2 min, 8.3 mg). Similar preparative HPLC purification (a linear gradient mobile phase: 20-60% MeOH in water in 30 min followed by 100% MeOH for 10 min, 2 mL/min, each solvent contained 0.1% TFA) of the SPE fraction 2 (156 mg) of the MeOH extract of *C. kizildaghensis* yielded apigenin (**1**, t_R=20.2 min, 7.6 mg) and semi-preparative HPLC (a linear gradient mobile phase: 30-90% MeOH in water in 30 min followed by 100% MeOH for 10 min, 2 mL/min, each solvent contained 0.1% TFA) of the SPE fraction 3 (37 mg) afforded apigenin 7,4′-dimethyl ether (**2**, t_R=24.6 min, 6.1 mg).

2.3.4. Identification of compounds

All isolated flavonoids were identified conclusively by comprehensive spectroscopic data analysis, *e.g.*, UV, MS and 1D and 2D NMR, and by comparison with respective published data.

Apigenin (**1**): Obtained as yellowish brown amorphous solid. UV λ_{max} (MeOH) nm: 268 and 336. ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD): Table 1. ESIMS (+ve ion mode) *m/z*: 271 $[M+1]^+$ corresponding to $C_{15}H_{11}O_5$ (Mabry et al., 1970; Agrawal, 1989).

Apigenin 7,4′-dimethyl ether (also known as genkwanin 4′-methyl ether, **2**): Obtained as yellowish brown amorphous solid. UV λ_{max} (MeOH) nm: 268 and 334. ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD): Table 1. ESIMS (+ve ion mode) *m/z*: 299 $[M+1]^+$ corresponding to $C_{17}H_{15}O_5$ (Mabry et al., 1970; Agrawal, 1989).

Genkwanin (**3**): Obtained as yellowish brown amorphous solid. UV λ_{max} (MeOH) nm: 267 and 333. ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD): Table 1. ESIMS (-ve ion mode) *m/z*: 283 $[M-1]^-$ corresponding to $C_{16}H_{11}O_5$ and 567 $[2M-1]^-$ (Mabry et al., 1970; Narain, 1976; Agrawal, 1989; Ayatollahi et al., 2009).

Quercetin (**4**): Obtained as yellowish brown amorphous solid. UV λ_{max} (MeOH) nm: 269 and 352. ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD): Table 1. ESIMS (-ve ion mode) *m/z*: 301 $[M-1]^-$ corresponding to $C_{15}H_9O_7$ and 603 $[2M-1]^-$ (Mabry et al., 1970; Agrawal, 1989).

3. Results and Discussion

3.1. Isolation and identification of flavonoids

A combination of SPE, analytical, semi-preparative and preparative reversed-phase HPLC analyses of the MeOH extracts of *C. austro-anatolica* and *C. kizildaghensis* yielded four flavonoids, including three flavones, *i.e.*, apigenin (**1**), apigenin 7,4′-dimethyl ether (**2**) and genkwanin (**3**), and a flavonol, quercetin

Table 1

^{1}H (coupling constant *J* in Hz in parentheses) and ^{13}C NMR data (in CD_3OD) of flavonoids **1**-**4**.

Position	^{1}H NMR chemical shift --in ppm				^{13}C NMR chemical shift δ in ppm			
	1	2	3	4	1	2	3	4
2	-	-	-	-	163.4	163.7	163.8	158.7
3	6.67 (1H, s)	6.40 (1H, s)	6.54 (1H, s)	-	103.5	103.9	103.6	136.1
4	-	-	-	-	182.5	184.9	183.9	180.1
5	-	-	-	-	157.4	157.5	157.7	163.1
6	6.51 (1H, d, *J*=2.2)	6.28 (1H, d, *J*=2.3)	6.31 (1H, d, *J*=2.3)	6.18 (1H, d, *J*=2.1)	101.4	101.4	99.2	100.6
7				-	164.1	167.1	167.2	168.3
8	6.84 (1H, d, *J*=2.2)	6.45 (1H, d, *J*=2.3)	6.62 (1H, d, *J*=2.3)	6.38 (1H, d, *J*=2.1)	94.2	94.2	93.3	95.3
9	-	-	-	-	161.8	160.1	160.0	159.9
10	-	-	-	-	105.7	105.9	105.9	105.3
1′	-	-	-	-	121.4	119.0	118.9	123.0
2′	-	-	-	7.36 (1H, d, *J*=1.9)	-	-	-	116.2
2′ and 6′	7.91 (2H, d, *J*=8.9)	7.56 (2H, d, *J*=8.8)	7.79 (2H, d, *J*=8.8)	-	129.4	129.4	129.4	-
3′	-	-	-	-	-	-	-	146.5
3′ and 5′	6.95 (2H, d, *J*=8.9)	6.98 (2H, d, *J*=8.8)	6.78 (2H, d, *J*=8.8)	-	117.2	117.2	116.9	-
4′	-	-	-	-	161.6	164.0	161.7	150.0
5′	-	-	-	6.97 (1H, d, *J*=8.2)	-	-	-	116.4
6′	-	-	-	7.32 (1H, d, *J*=8.2, 1.9)	-	-	-	122.5
7-OMe	-	3.98 (3H, s)	3.90 (3H, s)	-	-	55.9	55.8	-
4′-OMe	-	3.86 (3H, s)	-	-	-	56.4	-	-

(**4**) in reasonable yields (6-11 mg). While apigenin (**1**) was identified from both species, *C. austro-anatolica* also afforded genkwanin (**3**) and quercetin (**4**), and *C. kizildaghensis* provided apigenin 7,4′-dimethyl ether (**2**), which is also known as genkwanin 4′-methyl ether. The UV spectra obtained from the HPLC-PDA analysis indicated that these compounds were flavonoids (Mabry et al., 1970). ^{1}H and ^{13}C NMR analyses (Table 1) together with 2D NMR experiments, *i.e.*, COSY, HMBC and HSQC, unequivocally established the structures of these flavonoids. ESI-MS analysis of these flavonoids (**1**-**4**) showed respective pseudomolecular ions, either $[M+1]^+$ or $[M-1]^-$ ions and thus further confirmed the identity of these flavonoids.

All spectroscopic data were comparable with respective literature data. Although all these flavonoids are known natural products, they have not been previously reported from any of these species. Besides, the occurrence of apigenin 7,4′-dimethyl ether (**2**) and genkwanin (**3**) is rather limited within the genus *Centaurea*.

3.2. Distribution and chemotaxonomic implications

The genus *Centaurea* is well-known for producing various types of secondary metabolites, mainly, alkaloids, flavonoids, lignans and terpenoids (Sarker et al., 1997). However, all these compounds do not occur in all species of this genus; there are significant variations in the distribution of these compounds within this genus. Flavonoids and their glycosides occur in certain *Centaurea* species, and can be used as chemotaxonomic markers (De Oliveira et al., 2017). Flavonoids have also been successfully employed as chemotaxonomic markers within the Asteraceae (Emerenciano et al., 2001), at the tribe and sub-tribe levels, because flavonoids possess a wide structural diversity and have been isolated from a number of species of the Asteraceae, and it was previously shown that flavonoids could be used as taxonomic markers at lower hierarchical levels (Crawford, 1978).

Apigenin (**1**) is a well-known flavone widely distributed in the genus *Centaurea*, *e.g.*, *C. arenaria*, *C. chilensis*, *C. cyanus*, *C. davidovii*, *C. galicicae*, *C. jacea*, *C. macrocephala*, *C. mircanthos*, *C. montana*, *C. nervosa*, *C. nicaeensis*, *C. parilica*, *C. phrygia*, *C. repens*, *C. rupestotilis*, *C. sadleriana*, *C. scabiosa*, *C. scoparia*, *C. schischkinii*, *C. soskae*, *C. stenolepis*, *C. suaveolens*, *C. tomorosii*, *C. triumfetti*, *C. urvillei*, and *C. virgata* (Negrete et al., 1988; Christensen, 1991; Gonnet, 1993; Youssef and Frahm, 1995; Peter and Dosa, 2002; Zheng et al., 2004; Shoeb et al., 2005; Csapi et al., 2010; Gulcemal et al., 2010; Forgo et al., 2012; Hammoud et al., 2012; Pirvu et al., 2012; Csupor et al., 2013; Nikolova and Bancheva, 2013; Tesevic et al., 2014; Mishio et al., 2015; Tuzun et al., 2017), Similarly, quercetin (**4**) is also quite common in the genus *Centaurea*, *e.g.*, *C. aksoyi*, *C. amaena*, *C. bracteata*, *C. chilensis*, *C. collina*, *C. cyanus*, *C. floccosa*, *C. horrida*, *C. isaurica*, *C. kotschyri*, *C. macrocephala*, *C. mircanthos*, *C. omphalotricha*, *C. rupestotilis*, *C. rupestris*, *C. scabiosa*, *C. suaveolens* (Kamanzi et al., 1982; Negrete et al., 1987, 1988; Oksuz and Putun, 1987; Peter and Dosa, 2002; Flamini et al., 2000, 2001, 2004; Mouffok et al., 2012; Pirvu et al., 2012; Curkovic-Perica et al., 2014; Mishio et al., 2015; Albayrak et al., 2017). Both apigenin (**1**) and quercetin (**4**) are also found in other genera of the family Asteraceae.

The methylated derivatives of apigenin, *i.e.*, apigenin 7,4′-dimethyl ether (**2**) and genkwanin (apigenin 7-methyl ether, **3**) are rather rare flavones. To the best of our knowledge, genkwanin (**3**) was previously reported from only one *Centaurea* species, *i.e.*, *C. urvillei* (Ulubelen and Oksuz, 1982), but the flavone, apigenin 7,4′-dimethyl ether (**2**), has never been reported from the genus *Centaurea*. However, genkwanin (**3**) has been reported from other genera of the family *Asteraceae* *e.g.*, *Artemisia*, *Baccharis*, *Chromolaena*, *Gueldenstaedtia* and *Vernonia*, and its chemotaxonomic value is well-documented (Nakasugi and Komai, 1998; Kraft et al., 2003; Li et al., 2008; Avula et al., 2009; Piao et al., 2012; De Oliveira et al., 2017). Similarly, apigenin 7,4′-dimethyl ether (**2**) was reported from the genus *Calea* (*C. tenuifolia*) of the family Asteraceae (Koehler et al., 2002). Within the genus *Centaurea*, apigenin-based flavones including their glycosides and glucuronides are quite common, but methylation of the hydroxyls of the apigenin skeleton, as in **2** and **3**, may create a sub-chemical group indicating the presence of a slightly more advanced biosynthetic pathway in certain *Centaurea* species.

4. Concluding remarks

Isolation of the flavones (**1**-**3**) and the flavonol (**4**) from these two relatively less investigated *Centaurea* species from the Turkish flora, contributes to the understanding of the chemistry of the genus *Centaurea*, and also identifies new sources for relatively less common flavones, apigenin 7,4′-dimethyl ether (**2**) and genkwanin (**3**). The co-occurrence of these flavonoids within the genus *Centaurea* or the family Asteraceae may be chemotaxonomically important, especially when controversies about phylogeny and affinity of infra-familial groups as well as uncertain positioning of some genera of the family Asteraceae still exist.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

EPSRC National Mass Spectroscopy Service (NMSS), Swansea, is thanked for conducting the MS experiments on the isolated compounds.

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