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ORIGINAL PAPER

Anti-colon cancer potential of phenolic compounds from the aerial parts of *Centaurea gigantea* (Asteraceae)

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Abstract Reversed-phase HPLC analysis of the methanol extract of the aerial parts of *Centaurea gigantea* afforded chlorogenic acid and five flavonoids, 2″-(4‴-hydroxybenzoyl)-isoorientin, orientin, isoorientin, isoquercetrin and cirsiliol. The structures of the these phenolic compounds were established unequivocally by UV, MS, a series of 1D and 2D NMR analyses and by comparison of their spectroscopic data with literature data. The free radical scavenging properties of these compounds were assessed by the DPPH assay, and their toxicity towards brine shrimps, and cytotoxicity towards cancer cells were evaluated, respectively, by the brine shrimp lethality assay and the MTT assay using CaCo-2 colon cancer cell line. Among the compounds, chlorogenic acid exhibited considerable anti-colon cancer activity (IC_{50}=79.0 μM).

Keywords *Centaurea gigantea* · Asteraceae · CaCo 2 · Anticancer · MTT assay · NMR

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Introduction

Centaurea gigantea Schultz. Bip. ex Boiss. (Family: Asteraceae *alt.* Compositae; Section: *Cynaroides*), is endemic to South East Anatolia, Turkey [1]. This plant is a biennial with erect stems up to 1–1.80 m, densely adpressed-tomentose leaves, and pale purplish to white flowers. To our knowledge, there is no report on any phytochemical or pharmacological study on *C. gigantea* available to date. However, many species of the genus *Centaurea* have long been used in traditional medicine to cure various ailments, e.g. diabetes, diarrhoea, rheumatism, malaria, hypertension, etc., and a variety of secondary metabolites have been reported from different species of this genus [2]. As a part of our continuing phytochemical and bioactivity studies on the species of the genus *Centaurea* [3–8], we now report on the in vitro anti-colon cancer activity of the secondary metabolites: (1) chlorogenic acid, and five flavonoids, (2) 2″-(4‴-hydroxybenzoyl)-isoorientin, (3) orientin, (4) isoorientin, (5) isoquercetrin, and (6) cirsiliolfrom *C. gigantea*. The isolation, unambiguous structure elucidation, antioxidant activity and brine shrimp toxicity of these six compounds are also discussed.

Materials and methods

General procedures

UV spectra were obtained in MeOH using a Hewlett-Packard 8453 UV–Vis spectrometer. MS analyses were performed on a Quattro II triple quadrupole instrument. NMR spectra were recorded in CD_3OD on a Varian Unity INOVA 400 MHz NMR Spectrometer 400 (400 MHz for ^1H and 100 MHz for ^{13}C) using the residual solvent peaks as internal standard. HPLC separation was performed using a Dionex prep-HPLC system coupled with Gynkotek GINA50 autosampler and Dionex UVD340S Photo-Diode-Array detector and/or A JASCO PU-1580 Intelligent HPLC Pump, coupled with JASCO DG-1580-53 Degasser and JASCO LG-1580-02 Ternary Gradient Unit. A Luna C_{18} preparative (10 μm, 250×21.2 mm) and/or a Luna C_{18} semi-preparative HPLC column (5 μm, 250×10 mm) were used. Sep-Pak Vac 35 cc (10 g) C_{18} cartridge (Waters) was used for pre-HPLC fractions. HMBC spectra were optimised for a long range J_{H-C} of 9 Hz and the NOESY experiment was carried out with a mixing time of 0.8 s.

Plant material

The aerial parts of *C. gigantea* were collected in South East Anatolia, Turkey. A voucher specimen PHSH80010 has been retained in the herbarium of the Plant and Soil Science Department, University of Aberdeen, UK.

Extraction and isolation of compounds

Dried and ground aerial parts of *C. gigantea* (100 g) were Soxhlet-extracted, successively, with *n*-hexane, dichloromethane and methanol (MeOH) (1 l each). The MeOH extract was fractionated by solid-phase extraction method using a Sep-Pak C_{18} (10 g) cartridge eluting with a step gradient: 30, 60, 80 and 100% MeOH in water (200 ml each). Preparative-HPLC (eluted with a linear gradient-water:MeCN=90:10 to 60:40 over 50 min followed by 40% MeCN for 10 min, 20 ml/min) of the Sep-Pak fraction, which was eluted with 30% MeOH, yielded (1) 15.5 mg, t_R=7.3 min, (2) 34.9 mg, t_R=13.3 min, (3) 33.5 mg, t_R=14.3 min, (4) 7.9 mg, t_R=15.3 min and (5) 3.5 mg, t_R=18.0 min. Prep-HPLC (eluted with a linear gradient-water:-MeOH=75:25 to 30:70 over 50 min followed by 70% MeOH for 10 min, 15 ml/min) of the Sep-Pak fraction, which was eluted with 60% MeOH, afforded (6) 12.1 mg, t_R=25.9 min.

1. Chlorogenic acid

Gum; UV λ_{max} (MeOH): 332, 220 nm; IR ν_{max} (neat): 3,459, 1,765, 1,591, 1,514, 1,460 and 1,266 cm^{-1}; ESIMS *m/z* 353 $[M-H]^-$; ^1H NMR (400 MHz, CD_3OD): δ 7.38 (d, *J*=15.6 Hz, H-7′), 7.00 (d, *J*=2.0 Hz, H-2′), 6.91 (dd, *J*=8.0, 2.0 Hz, H-6′), 6.70 (d, *J*=8.0 Hz, H-5′), 6.16 (d, *J*=15.6 Hz, H-8′), 5.08 (m, H-3), 4.11 (m, H-5), 3.68 (dd, *J*=9.6, 3.2 Hz, H-4), 2.42 (dd, *J*=14.8, 3.2 Hz, H-2a), 2.02 (m, H-6a), 1.98 (m, H-6b), 1.92 (m, H-2b); ^{13}C NMR (100 MHz, CD_3OD): δ 176.8 (C-7), 166.9 (C-9′), 149.1 (C-4′), 146.3 (C-3′), 145.3 (C-7′), 126.2 (C-1′), 121.9 (C-6′), 116.5 (C-5′), 115.4 (C-2′), 115.2 (C-8′), 74.0 (C-4), 73.3 (C-3), 71.9 (C-5), 69.2 (C-1), 39.2 (C-2), 38.3 (C-6) [9].

2. 2″-(4‴-Hydroxybenzoyl)-isoorientin

Gum; UV λ_{max} (MeOH): 233, 252, 295, 314; IR ν_{max} (neat): 3,459, 1,679 and 1,205 cm^{-1}; ESIMS *m/z* 591 $[M+Na]^+$; ^1H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD): Tables 1 and 2; [10].

3. Orientin

Gum; UV λ_{max} (MeOH): 254, 272, 334; IR ν_{max} (neat): 3,459, 1,679 and 1,205 cm^{-1}; ESIMS *m/z* 471 $[M+H]^+$; ^1H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD): Tables 1 and 2; [11–13].

4. Isoorientin

Gum; UV λ_{max} (MeOH): 214, 250, 280, 314; IR ν_{max} (neat): 3,459, 1,679 and 1,205 cm^{-1}; ESIMS *m/z* 471 $[M+H]^+$; ^1H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD): Tables 1 and 2; [14, 15].

5. Isoquercetrin

Gum; UV λ_{max} (MeOH): 213, 248, 285, 313; IR ν_{max} (neat): 3,459, 1,679 and 1,205 cm^{-1}; ESIMS *m/z* 487 $[M+Na]^+$; ^1H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD): Tables 1 and 2; [16].

6. Cirsiliol

Gum; UV λ_{max} (MeOH): 213, 255, 276, 343; IR ν_{max} (neat): 3,459, 1,679 and 1,205 cm^{-1}; EIMS *m/z* 330 $[M]^{+\cdot}$ 315, 75 (100); ^1H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD): Tables 1 and 2 [17, 18]

Free radical scavenging activity: the DPPH assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH), molecular formula $C_{18}H_{12}N_5O_6$, was obtained from Fluka Chemie,

Table 1 ^{1}H NMR data (coupling constant *J* in Hz in parentheses, CD_3OD, 400 MHz) of flavones 2–6[a]

Position	Chemical shifts δ in ppm				
	2	3	4	5	6
3	6.45 s	6.58 s	6.45 s	–	6.68 s
6	–	6.21 s	–	6.17 d (2.0)	
8	6.41 s	–	6.38 s	6.36 d (2.0)	6.83 s
2′	7.27 d (2.0)	7.42 d (2.0)	7.27 d (2.0)	7.66 d (2.0)	7.39 d (2.0)
5′	6.83 d (8.4)	6.80 d (8.4)	6.80 d (8.4)	6.83 d (8.4)	6.85 d (8.0)
6′	7.28 dd (2.0, 8.4)	7.48 dd (2.0, 8.4)	7.28 dd (2.0, 8.4)	7.55 dd (2.0, 8.4)	7.40 dd (2.0, 8.0)
6-OMe	–	–	–		3.67 s
7-OMe	–	–	–		3.87 s
Sugar moiety					
1″	4.98 d (7.7)	4.62 d (7.8)	4.82 d (7.8)	5.21 d (7.6)	–
2″	4.15 m[b]	3.79 m[b]	4.13 t (8.8)	3.36 m[b]	–
3″	3.50 m[b]	3.26 m[b]	3.52 m[b]	3.48 m[b]	–
4″	4.05 m[b]	3.29 m[b]	3.50 m[b]	3.24 m[b]	–
5″	3.49 m[b]	3.10 m[b]	3.40 m[b]	3.33 m[b]	–
6″	3.90 dd (5.4, 11.2) 3.75 m[b]	3.75 dd (5.2, 11.2) 3.50 m[b]	3.84 dd (5.6, 11.2) 3.71 m[b]	3.79 dd (5.2, 11.0) 3.54 m[b]	–
Acyl moiety					
2‴	7.90 d (8.8)	–	–	–	–
3‴	6.87 d (8.8)	–	–	–	–
5‴	7.90 d (8.8)	–	–	–	–
6‴	6.87 d (8.8)	–	–	–	–

[a] *2* 2″-(4‴-hydroxybenzoyl)-isoorientin, *3* orientin, *4* isoorientin, *5* isoquercetrin, *6* cirsiliol

[b] Overlapped peaks

Bucks. Quercetin was obtained from Avocado Research Chemicals, Shore road, Heysham, Lancs. The method used by Takao et al. [19] was adopted with appropriate modifications [20, 21]. DPPH (4 mg) was dissolved in MeOH (50 ml) to obtain a concentration of 80 μg/ml.

Qualitative assay

Test compounds (1–6) were applied on a TLC plate and sprayed with DPPH solution using an atomiser. It was allowed to develop for 30 min. The colour change (purple on white) was noted.

Quantitative assay

Test compounds (1–6) were dissolved in MeOH to obtain a concentration of 0.5 mg/ml each. Dilutions were made to obtain concentrations of 5×10^{-2}, 5×10^{-3}, 5×10^{-4}, 5×10^{-5}, 5×10^{-6}, 5×10^{-7}, 5×10^{-8}, 5×10^{-9}, 5×10^{-10} mg/ml. Diluted solutions (1.00 ml each) were mixed with DPPH (1.00 ml) and allowed to stand for 30 min for any reaction to occur. The UV absorbance was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. The same procedure was followed for the positive control, quercetin, a well-known natural antioxidant.

Brine shrimp lethality assay

Shrimp eggs were purchased from The Pet Shop, Kittybrewster Shopping Complex, Aberdeen, UK. The bioassay was conducted following the procedure described by Meyer et al. [22]. The eggs were hatched in a conical flask containing 300 ml artificial seawater. The flasks were well aerated with the aid of an air pump, and kept in a water bath at 29–30°C. A bright light source was left on and the nauplii hatched within 48 h. The compounds (1–6) were dissolved in 20% aq. DMSO to obtain a concentration of 1 mg/ml. These were serially diluted twice and seven different concentrations were obtained. A solution of each concentration (1 ml) was transferred into clean sterile universal vials with pipette, and aerated sea-water (9 ml) was added. About ten nauplii were transferred into each vial with pipette. A check count was performed and the number alive after 24 h was noted. LD_{50}s were determined using the Probit analysis method [23].

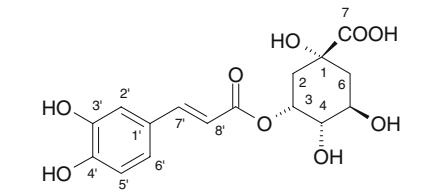
MTT cytotoxicity assay

CaCo-2 cells were maintained in Earle's minimum essential medium (Sigma), supplemented with 10% (v/v) foetal calf serum (Labtech Int.), 2 mM L-glutamine (Sigma), 1% (v/v) non-essential amino acids

Table 2 ^{13}C NMR data (CD_3OD, 100 MHz) of flavones 2–6[a]

Position	Chemical shifts δ in ppm				
	2	3	4	5	6
2	165.0	164.7	164.5	157.3	164.9
3	102.6	103.0	103.0	134.4	103.3
4	182.7	182.7	182.7	180.1	182.8
5	160.8	161.0	161.0	161.9	152.7
6	107.9	98.8	107.2	98.6	132.5
7	163.6	163.2	163.6	165.0	159.2
8	94.0	105.2	94.0	93.5	92.1
9	157.4	156.6	156.2	153.0	153.2
10	104.0	104.7	104.6	104.1	105.7
1′	122.3	122.7	122.8	122.0	122.0
2′	113.0	114.7	114.7	114.2	114.1
3′	145.8	146.4	146.4	144.7	146.5
4′	149.8	150.2	150.2	150.1	150.6
5′	115.7	116.3	116.3	116.3	116.6
6′	119.1	120.0	122.7	120.0	119.7
6-OMe	–	–	–	–	60.7
7-OMe	–	–	–	–	57.1
Sugar moiety					
1″	74.1	74.0	74.0	101.6	–
2″	75.3	71.4	71.4	70.0	–
3″	78.9	79.4	79.4	77.1	–
4″	71.2	71.3	71.4	74.5	–
5″	81.4	82.6	82.5	77.2	–
6″	61.7	62.3	62.4	61.3	–
Acyl moiety					
1‴	122.1	–	–	–	–
2‴	128.9	–	–	–	–
3‴	115.8	–	–	–	–
4‴	161.6	–	–	–	–
5‴	115.8	–	–	–	–
6‴	128.9	–	–	–	–
7‴	165.4	–	–	–	–

[a] *2* 2″-(4‴-hydroxybenzoyl)-isoorientin, *3* orientin, *4* isoorientin, *5* isoquercetrin, *6* cirsiliol



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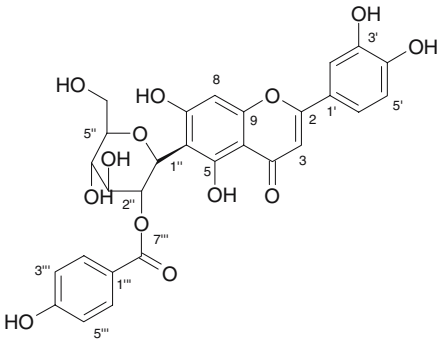

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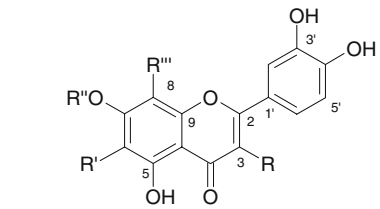

	R	R’	R’’	R’’’
3	H	C-Glucosyl	H	H
4	H	H	H	C-Glucosyl
5	*O*-Glucosyl	H	H	H
6	H	OMe	Me	H

Fig. 1 Structures of compounds (1–6)

(Sigma), 100 IU/ml penicillin and 100 μg/ml streptomycine (Sigma). Exponentially growing cells were plated at 2×10^4 cells cm^{-2} into 96-well plates and incubated for 72 h before the addition of drugs. Stock solution of compounds was initially in DMSO or H_2O and further diluted with fresh complete medium (Fig. 1).

The growth-inhibitory effects of the compounds (1–6) were measured using standard tetrazolium MTT assay [24]. After 72 h of incubation at 37°C, the medium was removed, and 100 μl of MTT reagent (1 mg/ml) in serum free medium was added to each well. The plates were incubated at 37°C for 4 h. At the end of the incubation period, the medium was removed and pure DMSO (200 μl) was added to each well. The metabolised MTT product dissolved in DMSO was quantified by reading the absorbance at 560 nm on a micro plate reader (Dynex Technologies, USA). The IC_{50} values were calculated from the equation of the logarithmic line determined by fitting the best line (Microsoft Excel) to the curve formed from the data. The IC_{50} value was obtained from the equation y=50 (50% value).

Results and discussion

Reversed phase preparative HPLC analysis of the methanol extract of the aerial parts of *C. gigantea* led to the isolation of (1) chlorogenic acid, five flavonoids, (2) 2″-(4‴-hydroxybenzoyl)-isoorientin, (3) orientin, (4) isoorientin, (5) isoquercetrin and (6) 6-hydroxyluteolin 6,7-dimethylether (or cirsiliol). The structures of the compounds were established unequivocally by UV, MS, a series of 1D and 2D NMR analyses and also by comparison of their spectroscopic data with respective literature data.

The ESIMS spectrum of compound (1) revealed the *pseudo*molecular ion peak at m/z 353 $[M\text{-}H]^+$,

suggesting *Mr*=354 and the molecular formula $C_{16}H_{18}O_9$. The ^{1}H NMR signals at δ 7.38 (d, *J*=15.6 Hz), 7.00 (d, *J*=2.0 Hz), 6.91 (dd, *J*=8.0, 2.0 Hz), 6.70 (d, *J*=8.0 Hz) and 6.16 (d, *J*=15.6 Hz) suggested the presence of a caffeoyl moiety. The ^{1}H NMR signals at δ 5.08, 4.11, 3.68, 2.42, 2.02, 1.98 and 1.92, together with the ^{13}C NMR signals at δ 176.8, 74.0, 73.3, 71.9, 69.2, 39.2 and 38.3, could be assigned to a quinic acid moiety. A ^3J long-range HMBC correlation between δ_H 5.08 (H-3) to δ_C 166.9 (C-9′) indicated that the caffeoyl moiety was attached to quinic acid at C-3. Thus, compound (1) was identified as chlorogenic acid. All spectroscopic data of compound (1) were in good agreement with the published data for chlorogenic acid [9].

Compounds (2–6) could be identified as flavones from their characteristic UV absorption maxima [25]. The ^{1}H NMR and ^{13}C NMR data (Tables 1 and 2) for these compounds also confirmed the presence of flavone nucleus in these molecules. Flavones (3–6) were unambiguously identified as orientin, isoorientin, isoquercetrin and cirsiliol, respectively, by direct comparison of their spectroscopic data with respective published data (Tables 1 and 2) [11–18]. The ESIMS spectrum of compound (2) showed the pseudomolecular ion peak at *m/z* 591 $[M+Na]^+$, suggesting *Mr*=568, and was calculated for the molecular formula $C_{28}H_{24}O_{13}$. The UV, ^{1}H and ^{13}C NMR data (Tables 1 and 2) revealed the striking similarity between (2) and isoorientin (4), with the exceptions that the ^{1}H NMR spectrum of (2) showed additional resonances at δ_H 7.90 (d, *J*=8.8 Hz) and 6.87 (d, *J*=8.8 Hz) which could be assigned to a 4-hydroxybenzoyl moiety. The ^{13}C NMR spectrum of **2** (Table 2), in addition to the signals associated with isoorientin skeleton, exhibited seven more signals at δ_C 165.4, 161.6, 128.9, 128.9, 122.1, 115.8 and 115.8 corresponding to this 4-hydroxybenzoyl group. The attachment of this moiety at C-2″ of the glucose unit was confirmed by a ^3J ^{1}H-^{13}C correlation from H-2″ (δ_H 4.15) to the carbonyl carbon C-7‴ (δ_C 165.4) observed in the HMBC spectrum. Thus, the structure of (2) was determined as 2″-(4‴-hydroxybenzoyl)-isoorientin. This compound was previously isolated from *Gentiana asclepiadea* [10]. However, this is a new report on the occurrence of this compound in *C. gigantea* and even in the genus *Centaurea*.

The DPPH assay [19] is an easy and straightforward method for determining the free radical scavenging property of a compound. DPPH is a molecule containing a stable free radical. In the presence of an antioxidant that can donate an electron to DPPH, the purple colour, which is typical of the free DPPH radical, decays, and the change in absorbance at 517 nm is monitored spectrophotometrically. All compounds (1–6) showed significant free radical scavenging activity (IC_{50}=2.9×10^{-3} to 4.6×10^{-2} mg/ml) (Table 3). This finding also proved that the presence of the highest number of phenolic hydroxyl groups in the molecule will increase antioxidant properties and the sesquiterpene lactones are not common to be used as antioxidants.

The brine shrimp lethality assay [22], which has been proven to be an effective and rapid assay method to screen compounds for potential general toxicity and cytotoxic activity, was used to determine the general toxicity of compounds 1–6. Circiliol (6) was found to be the most toxic of all test compounds towards brine shrimp (LD_{50}=6.4×10^{-3} mg/ml, respectively,) and displayed toxicity comparable to that of the positive control podophyllotoxin (LD_{50}=2.8×10^{-3} mg/ml), a well-known cytotoxic lignan.

The in vitro cytotoxicities (IC_{50} μM) of all the compounds isolated and characterised in this work were determined by the MTT assay against colon cancer cell line, CaCo-2 (Table 3). Among the compounds, chlorogenic acid (1) and cirsiliol (6) showed moderate levels of cytotoxic properties (IC_{50}=79.0 and 96.0 μM, respectively). The activity of chlorogenic acid (1) could be due to the fact that this compound possesses an α,β-unsaturated carbonyl moiety, which can be considered as Michael acceptor, an active moiety often employed in the design of anticancer drug [26]. The degree of general toxicity displayed by the test compounds in the brine shrimp lethality assay corresponded well with the cytotoxic potentials of these compounds observed in the MTT assay using colon cancer cell line.

Table 3 Antioxidant (DPPH assay) and cytotoxic (MTT assay) activities, and brine shrimp toxicity (Brine shrimp lethality assay) of compounds **1–6**

Compounds	Antioxidant activity IC_{50} (mg/ml)	Cytotoxicity IC_{50} (μM)	Brine shrimp toxicity LD_{50} (mg/ml)
1	2.3×10^{-2}	79.0	2.5×10^{-2}
2	4.6×10^{-2}	285.7	>1,000 μg/ml
3	3.5×10^{-2}	290.3	>1,000 μg/ml
4	3.9×10^{-3}	>300.0	>1,000 μg/ml
5	2.9×10^{-3}	>300.0	>1,000 μg/ml
6	2.8×10^{-2}	96.0	6.4×10^{-3}
Methanol extract of *C. gigantea*	7.2×10^{-2}	43.2	69.2×10^{-2}
Quercetin	2.8×10^{-5}	–	–
Podophyllotoxin	–	0.06	2.8×10^{-3}

Conclusion

This is the first report on phytochemical and bioactivity screening of the aerial parts of *C. gigantea*. While all compounds showed considerable levels of antioxidant properties due to their phenolic nature, chlorogenic acid (1) and cirsiliol (6) were found to be promising candidates for their use as templates for anti-colon cancer drug development.

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