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Bioactivity-guided isolation of antiproliferative compounds from *Centaurea jacea* L.

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ABSTRACT

Bioassay-guided fractionation of the chloroform extract of *Centaurea jacea* L. afforded the isolation of cirsiliol, apigenin, hispidulin, eupatorin, isokaempferide, axillarin, centaureidin, 6-methoxykaempferol 3-methyl ether, trachelogenin, cnicin, 4′-acetylcnicin and three aliphatic glucose diesters, including the new natural product 1β-isobutanoyl-2-angeloyl-glucose. The structures of the compounds were established on the basis of spectroscopic analyses (UV, MS and NMR). All compounds were isolated for the first time from this species. The compounds were evaluated for their tumour cell growth inhibitory activities on HeLa, MCF-7 and A431 cells. Different types of secondary metabolites (flavonoids, sesquiterpenes) were found to be responsible for the antitumour effects of the extracts; the highest activity was exerted by centaureidin, in addition to moderately active compounds (cirsiliol, isokaempferide, apigenin, hispidulin, cnicin and 4′-acetylcnicin).

1. Introduction

The genus *Centaurea* (Asteraceae) comprises about 500 species, 221 of which are native to Europe [1]. The species of the genus are not widely applied in European folk medicine, though there are some references to their usage, most frequently for the treatment of ophthalmia (*Centaurea calcitrapa*, *Centaurea cyanus*), fever (*Centaurea cyanus*, *Centaurea jacea*, *Centaurea solstitialis*), gynaecological problems (*C. cyanus*), digestive complaints (*C. calcitrapa*, *C. cyanus*), wounds and dermatological complaints (*C. calcitrapa*, *C. cyanus*, *C. jacea*) [2]. In Hungarian traditional medicine, the most frequently used species is *C. cyanus*, presumably because of its wide-ranging geographical distribution. The majority of the Hungarian *Centaurea* species have been poorly analysed from phytochemical and pharmacological aspects. In a previous investigation the main components of *C. arenaria* were isolated by bioactivity-guided fractionation by our group and the antiproliferative activity of the compounds were evaluated [3].

In our screening programme for antiproliferative activity of Asteraceae species native to Hungary, *Centaurea* species (*C. biebersteinii*, *C. jacea* and *C. spinulosa*) demonstrated high cell proliferation inhibitory activity against cervix adenocarcinoma (HeLa), skin epidermoid carcinoma (A431) and breast epithelial adenocarcinoma (MCF-7) [4,5]. The highest activity was recorded for *C. jacea*, whose chloroform extract, prepared from different plant parts (flowers, leaves and roots), significantly inhibited the growth of HeLa (57–86%), MCF-7 (44–64%) and A431 (43–69%) cells *in vitro*. The antitumour constituents of *C. jacea* were not investigated earlier. The aim of the present work was the isolation and identification of the antiproliferative compounds from the aerial parts of *C. jacea* using bioactivity-guided fractionations.

C. jacea L. (brown knapweed) is a perennial herb occurring widespread in Europe. This species has been used sporadically in traditional medicine (against fever and dermatological

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complaints), but is regarded as bioindicator for monitoring of ozone effect. Previous studies revealed the presence of flavonoids (jaceidine [6], jacein [7], centaurein [8], jaceoside [9], 6-methoxykaempferol 3-methyl ether and its 7-*O*-glucoside [10]), sesquiterpenes (amarine, hydroamarine, melitensin and dihydromelitensin) [11], and cinnamic alcohol glucosides (syringin and coniferin) [12].

In the present study the dried plant materials were extracted with MeOH, and this extract was subjected to solvent–solvent partitioning, yielding *n*-hexane-, $CHCl_3$- and aqueous MeOH-soluble phases. The $CHCl_3$ extract exerted substantial tumour cell growth inhibitory activity at 10 μg/mL concentration primarily against HeLa cell line [4]. Therefore, the $CHCl_3$ extract was selected for a detailed phytochemical-pharmacological analysis in order to identify the antiproliferative constituents.

2. Experimental

2.1. General

Optical rotation was determined with a Perkin-Elmer 341 polarimeter. NMR spectra on solutions in $CDCl_3$ were recorded on a Bruker Avance DRX 500 spectrometer at 500 MHz (^1H) or 125 MHz (^{13}C); the signals of the deuterated solvent were taken as reference. Two-dimensional (2D) experiments (^1H,^1H COSY, HSQC, HMBC and NOESY) were set up, performed and processed with the standard Bruker protocol. Polyamide was used for column chromatography (ICN). Silica gel plates were applied for analytical and preparative TLC (Merck 5717 and 5715). ESIMS was performed on an Applied Biosystems 3200 QTrap instrument in ion trap mode. For vacuum liquid chromatography (VLC), silica gel (60G, 15 μm, Merck 11677) was applied. Rotation planar chromatography (RPC) was performed with a Chromatotron instrument (Model 8924, Harrison Research) on manually prepared silica gel (60 GF_{254}, Merck 7730) plates. Preparative layer chromatography (PLC) was carried out on silica gel plates (20 × 20 cm 60GF_{254}, Merck 5715). Separation was monitored at UV 254 nm. HPLC was carried out on a Waters instrument on a LiChrospher RP-18 (5 μm, 250 × 4 mm, Merck) column.

2.2. Plant material

Aerial parts of *C. jacea* were collected in August 2010 in Budaörs, Hungary. Botanical identification of the plant material was performed by Dr. Tamás Rédei (Hungarian Academy of Sciences, Institute of Ecology and Botany, Vácrátót, Hungary) and a voucher specimen (no. 776) has been deposited at the Department of Pharmacognosy, University of Szeged.

2.3. Extraction and isolation

Dried and ground aerial parts of *C. jacea* (1.5 kg) were extracted with 20 L MeOH. After concentration, the extract (250.4 g) was dissolved in 1 L 50% aqueous MeOH, and solvent–solvent partition was performed first with 5 × 500 mL *n*-hexane, and then with 5 × 500 mL $CHCl_3$. After evaporation, the $CHCl_3$ phase (62.5 g) was fractionated by CC on silica gel (940 g), using a gradient system of *n*-hexane–acetone–MeOH (9:1:0, 8:2:0, 7:3:0, 6:4:0, 1:1:0, 50:50:3, 10:10:1, 5:5:1, 5:5:2 and 1:1:1, 500 mL each). Fractions with similar composition were combined according to TLC monitoring, affording fractions F1–F21, which were tested for their antiproliferative effects. The most active compounds were concentrated in F11 (161.6 mg) [cell proliferation inhibition 94.04 ± 0.46% (HeLa); 46.39 ± 9.23% (MCF-7); 86.50 ± 1.62% (A431)], F13 (129.4 mg) [83.41 ± 1.52% (HeLa); 14.91 ± 2.48% (MCF-7); 51.88 ± 2.20%, (A431)] and F15 (198.5 mg) [96.12 ± 0.27% (HeLa); 65.24 ± 4.43% (MCF-7); 81.51 ± 0.59 % (A431)]. These fractions were separated by OCC on polyamide [800 mg (F11), 650 mg (F13) and 1.0 g (F15)], using a solvent system of MeOH–H_2O (1:4, 2:3, 3:2 and 4:1, 50 mL each). Subfractions F11/8 and F11/9, obtained by polyamide CC of F11 with MeOH–H_2O 3:2, were purified by preparative TLC, using cyclohexane–EtOAc–EtOH 50:50:3, which led to the isolation of trachelogenin (**4**) (16.3 mg). Subfractions F11/13 and F11/14, obtained with MeOH–H_2O 3:2, were further fractionated by preparative TLC on silica gel, using toluene–EtOAc–HCOOH 5:4:1 to yield eupatorin (**10**) (8.6 mg). Subfractions F11/15–19, obtained by polyamide CC with MeOH–H_2O 3:2, were separated by RPC with a gradient system of cyclohexane–EtOAc–EtOH (6:2:0, 6:3:0, 60:30:1, 60:30:2, 50:50:3 and 10:10:1). Subfractions 2 and 3, obtained with cyclohexane–EtOAc–EtOH 6:3:0, 60:30:1 and 60:30:2, were rechromatographed by preparative TLC with the solvent system $CHCl_3$–MeOH 19:1, resulting in the isolation of isokaempferid (**11**) (6.1 mg, mp. 261–264 °C), hispidulin (**9**) (13.7 mg), 6-methoxykaempferol 3-methyl ether (**14**) (18.6 mg) and centaureidin (**13**) (20.2 mg). Finally, subfraction F11/20, obtained with MeOH–H_2O 4:1, was purified by preparative TLC, using cyclohexane–EtOAc–EtOH 50:50:3, which led to the isolation of apigenin (**7**) (4.0 mg).

Fraction F13, which was eluted with *n*-hexane–acetone–MeOH 1:1:0, was separated on polyamide CC. From subfraction F13/1 eluted with MeOH–H_2O 1:4 cirsiliol (**8**) (12.1 mg) was crystallised. The remaining material was further fractionated by preparative TLC on silica gel, using a mobile phase of $CHCl_3$–MeOH 19:1, and finally it was separated by RP-HPLC, using MeOH–H_2O 3:2, to yield 1β-isobutanoyl-2-angeloyl-glucose (**1**) (9.8 mg), 1β,2-diangeloyl-glucose (**2**) (10.0 mg) and 1β-(2-methylbutanoyl)-2-angeloyl-glucose (**3**) (11.0 mg). Subfraction F13/2–5, eluted with MeOH–H_2O 1:4, were separated by RPC with a gradient system of cyclohexane–EtOAc–EtOH 50:50:3 to give 4′-acetyl-cnicin (**6**) (8.2 mg). Moreover, subfraction F13/6, obtained with MeOH–H_2O 3:2, was purified by preparative TLC on silica gel with the mobile phase toluene–EtOAc–HCOOH 5:4:1, which led to the isolation of axillarin (**12**) (21.1 mg).

Fraction F15 was separated on polyamide CC. Subfractions eluted with MeOH–H_2O 1:4 and 2:3 were combined and subjected to RPC, using gradient solvent system of cyclohexane–EtOAc–EtOH (6:3:0, 60:30:1, 60:30:2, 60:30:3, 50:50:3, 10:10:1 and 5:5:1), to give pure cnicin (**5**) (168.2 mg, mp. 147–150 °C).

2.3.1. 1β-Isobutanoyl-2-angeloyl-glucose

(**1**): Colourless oil with $[\alpha]^{27}_D$: + 15 (0.1, $CHCl_3$). ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data, see Table 1. HR-ESIMS: *m/z* 334.2985 (calcd. 334.2984 for $C_{15}H_{26}O_8Na$ $[M+Na]^+$).

2.3.2. 6-Methoxykaempferol 3-methyl ether

(**14**): ^1H NMR δ_H: (CD_3OD): 3.71 (3H, s, 6-OCH_3), 3.81 (3H, s, 3-OCH_3), 6.20 (1H, s, H-8), 6.85 (2H, d, J = 8.8 Hz, H-3′,5′), 7.92

Table 1
NMR data of compounds **1–3** [$CDCl_3$, 500 MHz (^1H), 125 MHz (^{13}C), δ (ppm), (J = Hz)].

	1		2	3
Atom	^1H	^{13}C	^{13}C	^{13}C
1	5.72 d (8.2)	91.6	92.0	91.8
2	5.02 t (8.1)	72.5	72.5	72.2
3	3.74 m	75.2	75.4	75.0
4	3.74 m	70.1	70.2	69.8
5	3.52 m	76.3	76.3	76.2
6	3.91 brd (12.6), 3.88 brd (12.6)	61.5	61.5	61.3
1-*O*-acyl	Isobutanoyl		Angeloyl	Isovaleroyl
1′	–	175.4	165.8	172.0
2′	2.56 sept (7.0)	33.8	126.6	43.0
3′	1.11 d (7.0)	18.8	141.4	25.3
4′	1.13 d (6.9)	18.3	15.9	22.0
			20.2	22.0
2-*O*-Acyl	Angeloyl		Angeloyl	Angeloyl
1″	–	167.4	167.5	167.0
2″	–	127.0	127.1	127.0
3″	6.13 q (6.8)	140.0	139.5	139.7
4″	1.95 d (6.8)	15.9	15.8	16.0
5″	1.85 s	20.4	20.4	20.3
OH	4.25 brs	–	–	–
OH	4.1 brs	–	–	–
OH	3.10 brs	–	–	–

(2H, d, J = 8.8 Hz, H-2′,6′). ^{13}C NMR δ_C: 60.4 (3-OCH_3), 60.5 (6-OCH_3), 98.0 (C-8), 102.2 (C-10), 117.4 (C-3′,5′), 121.6 (C-1′), 131.0 (C-2′,6′), 135.9 (C-6), 138.2 (C-3), 152.8 (C-9), 155.4 (C-5), 156.7 (C-2), 164.1 (C-7), 171.1 (C-4′), 178.7 (C-4).

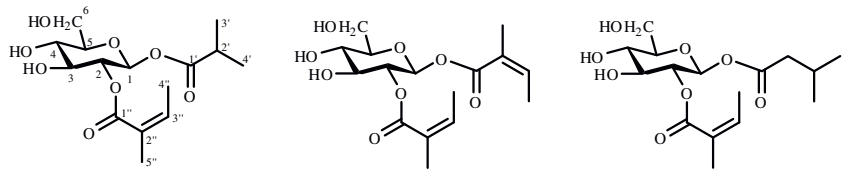
2.4. Antiproliferative assay

Antiproliferative effects were measured *in vitro* on three human cell lines (HeLa, MCF-7 and A431) with the MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay (Mosmann, 1983). Briefly, a limited number of human cancer cells (5000/well) were seeded onto a 96-well microplate and became attached to the bottom of the well overnight. On the second day of the procedure, the original medium was removed and 200 μL MTT solution (5 mg/mL) was added. MTT was converted by intact mitochondrial reductase and precipitated as blue formazan crystals during a 4 h contact period. The medium was then removed, and the precipitated crystals were dissolved in 100 μL DMSO during 60 min period of shaking. Finally, the reduced MTT was assayed at 545 nm using a microplate reader, wells with untreated cells being taken as the control. All *in vitro* experiments were carried out on two microplates with at least five parallel wells. Doxorubicin and cisplatin were used as positive controls. Stock solutions of 10 mg/mL of the tested compounds and extracts were prepared with DMSO. The highest DMSO concentration (0.3%) of the medium did not have any significant effect on cell proliferation. The dose–response curves of the compounds were fitted by means of the computer programme GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA), and IC_{50} values (the concentration at which the cell proliferation is 50% of the untreated control) were calculated.

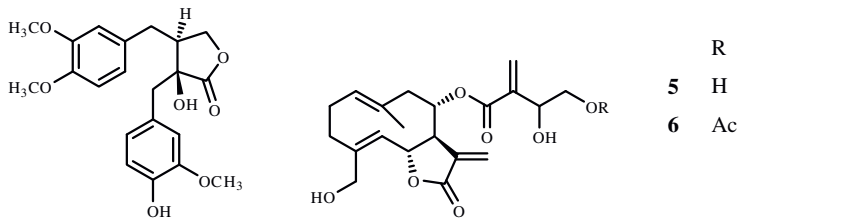
3. Results and discussion

Dried and ground aerial parts of *C. jacea* were extracted with MeOH. After concentration, the extract was dissolved in 50% aqueous MeOH, and solvent–solvent partition was performed first with *n*-hexane and then with $CHCl_3$. After evaporation, the $CHCl_3$ phase was fractionated by OCC on silica gel, using a gradient system of *n*-hexane–acetone–MeOH. Fourteen compounds (**1**–**14**) were isolated from the highly and moderate active fractions through the use of VLC, CPC, preparative TLC and HPLC techniques. The compounds were identified by means of UV, ESIMS and NMR spectroscopy (^1H-NMR, JMOD, ^1H,^1H COSY, HSQC, HMBC and NOESY) and comparison of the spectral data with literature data.

Compound **1** was isolated as colourless oil with $[\alpha]^{27}_D + 15$ ($CHCl_3$, *c* 0.1). It was shown by HRESIMS to have the molecular formula $C_{15}H_{26}O_8$ (M = 332) through the presence of quasi-molecular ion peak at *m/z* 355.1363 $[M+Na]^+$ (calcd for $C_{15}H_{26}O_8Na$ 355.1369). The ^1H and ^{13}C NMR spectra of **1** revealed the presence of one isobutanoate group (δ_H 2.56 sept, 1.13 d and 1.11 d; δ_C 175.4, 33.8, 18.8 and 18.3) and one angelate group (δ_H 6.13 q, 1.95 d and 1.85 s; δ_C 167.4, 127.0, 140.0, 20.4 and 15.9) (Table 1). The remaining signals indicated the presence of a diacylated glucose in the molecule. The positions of the ester groups were established via the HMBC experiment. The correlations of the carbonyl signal at δ_C 175.4 (isobutanoyl CO) with the proton signals at δ_H 5.72 (H-1) indicated the presence of the isobutanoyl group on C-1. Similarly, the HMBC cross-peak of the carbonyl carbon signal at δ_C 167.4 with the proton signal at δ_H 5.02 (H-2) demonstrated the presence of the angeloyl group on C-2. All of the above evidence confirmed the structure of this compound as 1β-isobutanoyloxy-2-angeloyloxyglucose (**1**).

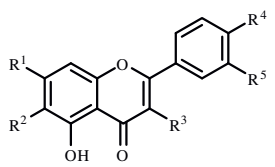


1 **2** **3**



4

	R
5	H
6	Ac



	R^1	R^2	R^3	R^4	R^5
7	OH	H	H	OH	H
8	OCH_3	OCH_3	H	OH	OH
9	OH	OCH_3	H	OH	H
10	OCH_3	OCH_3	H	OCH_3	OH
11	OH	H	OCH_3	OH	H
12	OH	OCH_3	OCH_3	OH	OH
13	OH	OCH_3	OCH_3	OCH_3	OH
14	OH	OCH_3	OCH_3	OH	H

Fig. 1. Structures of compounds isolated from *Centaurea jacea*.

Compounds **2** and **3** were identified as 1β,2-diangeloyl-glucose (**2**) and 1β-(2-methylbutanoyl)-2-angeloyl-glucose (**3**), which were isolated previously only from *Centaurea napifolia* and *Centaurea diffusa* [13,14]. Their ^{13}C NMR data are published for the first time in this article (Table 1).

Compounds **4**–**14** were found to be identical in all of its characteristics, including NMR data, with trachelogenin (**4**) [15], cnicin (**5**) [3], 4′-acetylcnicin (**6**) [16], apigenin (**7**) [17], cirsiliol (**8**) [18], hispidulin (**9**) [19], eupatorin (**10**) [20], isokaempferide (**11**) [3,21], axillarin (**12**) [22], centaureidin (**13**) [23] and 6-methoxykaempferol 3-methyl ether (**14**) [24] (Fig. 1). Interestingly our bioassay directed isolation procedure led to the identification of other compounds than isolated earlier from this species with the only exception of 6-methoxykaempferol 3-methyl ether (**14**). Methoxylated flavones, such as cirsiliol (**8**), hispidulin (**9**), and the flavonol isokaempferid (**11**) were described previously from different *Centaurea* species [25]. Similarly, the germacranolide cnicin (**5**) was detected in some *Centaurea* species [26]. 4′-Acetyl-cnicin (**6**) is a structurally related analogue of amarine, isolated previously from the plant [27].

The isolated compounds were evaluated for their growth inhibitory activity on HeLa, MCF-7 and A431 cells (Table 2), and it emerged that flavones and sesquiterpenes exerted pronounced concentration-dependent effects. The highest activity was demonstrated by the flavonol centaureidin (**13**), with IC_{50} values of 0.08 μM (HeLa), 0.13 μM (MCF-7) and 0.35 μM (A431). The extremely high cytotoxicity of centaureidin (**13**) was detected earlier by Beutler et al. in an *in vitro* screening in the NCI 60-cell line panel [28]. Interestingly, isokaempferide (**11**) and axillarin (**12**), the close analogues of centaureidin (**13**) were found to be less active, and only on HeLa cell line. Moreover, apigenin (**7**), cirsiliol (**8**), hispidulin (**9**), cnicin (**5**) and 4′-acetyl-cnicin (**6**) inhibited the proliferation of all three tumour cells significantly, while sugar esters (**1**–**3**) and trachelogenin (**4**) were found to be inactive against all the tested cell lines.

In summary, our bioactivity-guided isolations led to the conclusion that the strong inhibitory effect of the $CHCl_3$ extract of *C. jacea* on the proliferation of cultured human tumour cell lines (HeLa, MCF7 and A431) may be attributed mainly to flavones and sesquiterpenoids. Predominantly, the content of the most active compound, centaureidin (**13**), determines the antitumour activity of the extract, with the other compounds (**5**–**12**, **14**) playing an additional role in this effect. Moreover, a favourable interaction between chemically unrelated compounds (phenolics and sesquiterpenoids) could be suggested which is responsible for the overall antiproliferative action of the extract.

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Table 2
Antiproliferative effects (IC_{50}) of the isolated compounds on different human tumour cell lines.

Compound	IC_{50} (μM)		
	HeLa	MCF-7	A431
1β-Isobutanoyl-2-angeloyl-glucose (**1**)	Inactive	Inactive	Inactive
1β,2-Diangeloyl-glucose (**2**)	Inactive	Inactive	Inactive
1β-(2-Methylbutanoyl)-2-angeloyl-glucose (**3**)	Inactive	Inactive	Inactive
Trachelogenin (**4**)	Inactive	Inactive	Inactive
Cnicin (**5**)	34.50 ± 3.61	16.84 ± 1.99	17.86 ± 1.30
4′-Acetyl-cnicin (**6**)	6.76 ± 0.62	37.88 ± 1.52	31.28 ± 3.12
Apigenin (**7**)	10.64 ± 1.42	13.88 ± 2.38	12.34 ± 2.65
Cirsiliol (**8**)	10.96 ± 0.31	10.12 ± 1.14	12.30 ± 0.64
Hispidulin (**9**)	5.68 ± 0.31	37.97 ± 2.04	80.97 ± 3.72
Eupatorin (**10**)	29.79 ± 0.83	Inactive	52.50 ± 1.53
Isokaempferid (**11**)	15.05 ± 1.97	Negative	Negative
Axillarin (**12**)	26.91 ± 2.70	Inactive	Inactive
Centaureidin (**13**)	0.08 ± 0.004	0.13 ± 0.03	0.35 ± 0.05
6-Methoxykaempferol 3-methyl ether (**14**)	22.63 ± 2.42	40.76 ± 4.77	55.25 ± 4.59
Doxorubicin	0.15 ± 0.03	0.28 ± 0.01	0.15 ± 0.04
Cisplatin	12.34 ± 1.05	9.63 ± 0.75	2.84 ± 0.61

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2012.04.006.

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