Antiproliferative Constituents of the Roots of *Conyza canadensis*

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Key words
- *Conyza canadensis*
- Asteraceae
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- antiproliferative activity
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Abstract

Bioassay-guided fractionation of the *n*-hexane and $CHCl_3$ phases of the methanol extract of the roots of *Conyza canadensis* (L.) Cronquist led to the isolation of two new dihydropyranones named conyzapyranone A (**1**) and B (**2**), and the known 4*Z*,8*Z*-matricaria-γ-lactone (**3**), 4*E*,8*Z*-matricaria-γ-lactone (**4**), 9,12,13-trihydroxy-10(*E*)-octadecenoic acid (**5**), epifriedelanol (**6**), friedeline (**7**), taraxerol (**8**), simiarenol (**9**), spinasterol (**10**), stigmasterol, β-sitosterol, and apigenin. The structures were determined by means of ESIMS and 1D and 2D NMR spectroscopy, including ^1H-^1H COSY, NOESY, HSQC, and HMBC experiments. The isolated compounds were evaluated for their antiproliferative activities and were demonstrated to exert considerable cell growth-inhibitory activity against human cervix adenocarcinoma (HeLa), skin carcinoma (A431), and breast adenocarcinoma (MCF-7) cells. Some of the active components, including **2, 4**, and **10**, proved to be substantially more potent against these cell lines than against noncancerous human foetal fibroblasts (MRC-5) and can therefore be considered selective antiproliferative natural products.

Introduction

Canadian horseweed [*Conyza canadensis* (L.) Cronquist, syn. *Erigeron canadensis* L., Asteraceae] is a plant species indigenous to America, but is now found globally; it is widely distributed in Hungary. The aerial parts of the plant have been used in different parts of the world to treat several ailments, most commonly diarrhoea and dysentery, and as a diuretic agent. In Chinese folk medicine, horseweed has also been applied for the treatment of wounds, swellings, and pain caused by arthritis [1,2]. Moreover, a decoction of horseweed has traditionally been used to treat cancerous diseases in North America [3].

Previous phytochemical studies revealed the presence of specific C_{10} acetylenes [4,5], sesquiterpene hydrocarbons [6], flavonoids [7], and sterols, triterpenes, and sphingolipids [8,9] in this plant species. Recently, a triterpenoid ester, 3β,16β,20β-trihydroxytaraxastane-3-*O*-palmitoyl ester [5], and phenylpropanoyl 2,7-anhydro-3-deoxy-2-octulosonic acid derivatives were isolated [10]. Analysis of the essential oil of horseweed revealed 25 constituents, including monoterpenes, sesquiterpenes, and acetylenes, among which d-limonene predominated [11].

In the course of the search for antiproliferative compounds from the Asteraceae family, lipophilic and aqueous extracts of different parts of *C. canadensis* have been assayed on human tumour cell lines (HeLa, MCF-7, and A431). The *n*-hexane phase of the methanol extract of the root inhibited markedly the growth of the cells (62.4–70.1%) at 10 µg/mL, and the $CHCl_3$ phase of the methanol extract of the root demonstrated at the same concentration moderate antiproliferative activity (39.3–47.9%) [12]. The present investigation was aimed at the identification of compounds responsible for the anticancer effects of horseweed root. Activity-guided isolation, structure elucidation, and pharmacological analysis of two new C_{10} dihydropyranone derivatives and various known C_{10} acetylenes, triterpenes, sterols, a hydroxy-fatty acid, and apigenin are reported (**Fig. 1**).

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Bibliography
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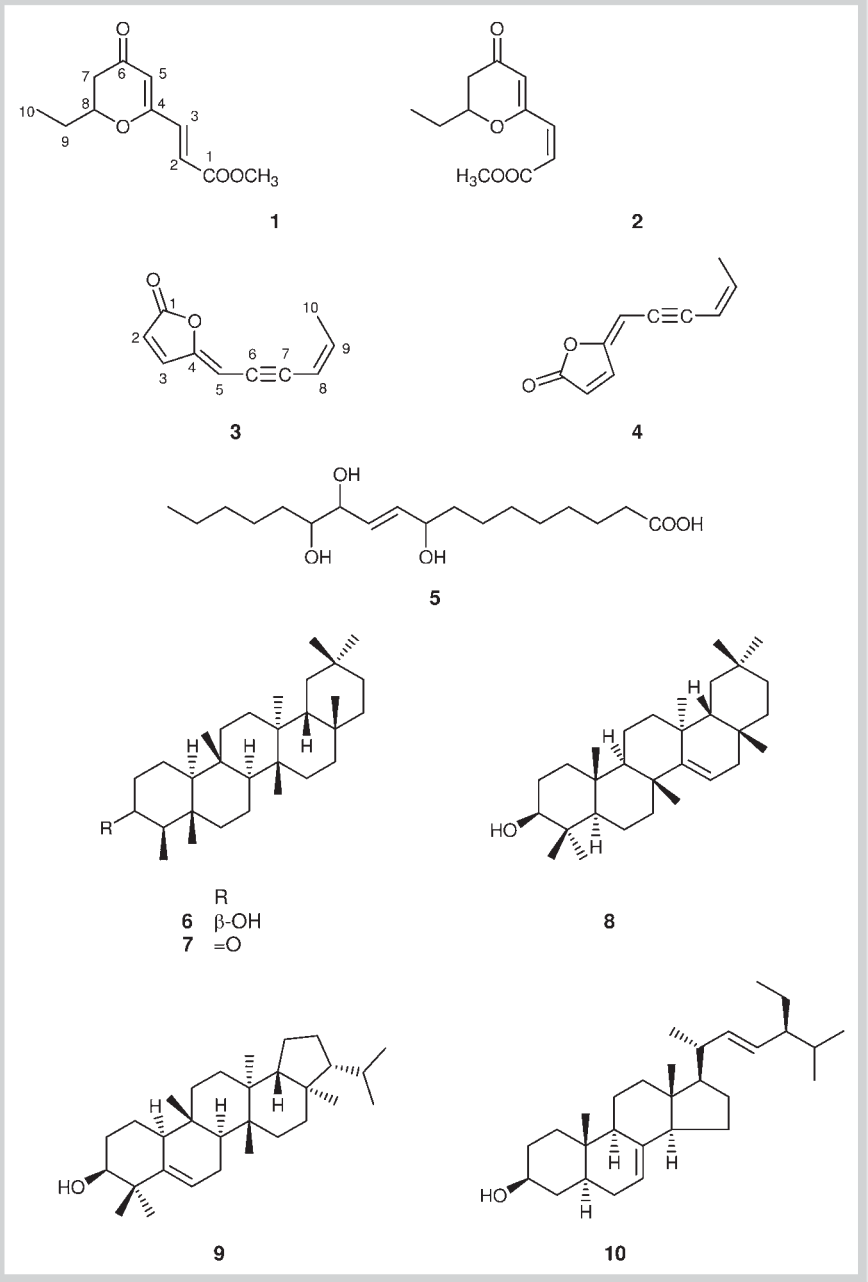

Fig. 1 Chemical structures of compounds **1–10**.

Materials and Methods

General experimental procedures

NMR spectra were recorded in $CDCl_3$ 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 a reference. 2D NMR data were acquired and processed with standard Bruker software. Mass spectrometric measurements were performed on a Finnigan TSQ 7000 tandem mass spectrometer (Finnigan MAT) equipped with a Finnigan electrospray ion source. A voltage of 4.5 kV was used in the ion source. The instrument was scanned in the normal MS mode over the mass range 10–1500, with a scan time of 2 s. UV spectra were obtained from the PDA-HPLC investigations. Silica gel plates were applied for analytical TLC (Merck 5554). The chromatograms were visualised at 254 and 366 nm, and by spraying with concentrated H_2SO_4, followed by heating at 110 °C. For vacuum liquid chromatography (VLC), SiO_2 (silica gel $60GF_{254}$, 15 µm; Merck 11677) was applied. Rotation planar chromatography (RPC) was performed with a Chromatotron instrument (model 8924; Harrison Research) on manually coated SiO_2 (silica gel 60 GF_{254}, Merck 7730; RPC I–VI and RPC VIII–XI) or Al_2O_3 (aluminium oxide G, type E, Merck 1090; RPC VII) plates 1, 2, or 4 mm thick, at a flow rate of 3, 4, or 10 mL/min, respectively. Preparative layer chromatography (PLC) was carried out on SiO_2 plates (20 × 20 cm, silica gel $60F_{254}$; Merck 5715). Separation was monitored by spraying the border of the plates with concentrated H_2SO_4. Reversed-phase HPLC was carried out on a Waters 600 instrument equipped with a LiChrospher 100 RP-18 (10 µm, 250 × 4 mm; Merck) column and a Waters 2998 photodiode array detector. A mobile phase containing MeOH-H_2O 3 : 2 was applied at a flow rate of 0.4 mL/min; the separation was monitored at 220 nm.

Plant material

The roots of *C. canadensis* (L.) Cronq. were collected in the Southern Great Plain (Hungary) in September 2004 and authenticated by Dr. Tamás Rédei (Institute of Ecology and Botany of the Hungarian Academy of Sciences, Vácrátót, Hungary). A voucher specimen (No. 771) has been deposited at the Department of Pharmacognosy, University of Szeged.

Extraction and isolation

The air-dried and ground roots of the plant (2.6 kg) were percolated with MeOH (50 L) at room temperature. The concentrated extract (300 mL) was diluted with 300 mL H_2O and subjected to solvent-solvent partition, first with *n*-hexane (5 × 2 L) and then with $CHCl_3$ (7 × 2 L). After evaporation, the *n*-hexane-soluble phase (16.0 g) was fractionated by VLC (8.5 × 14 cm), using mixtures of *n*-hexane and EtOAc with increasing polarity [volumetric ratios of 98 : 2, 96 : 4, 94 : 6, 92 : 8, 9 : 1, 8 : 2, 6 : 4, 3 : 7, and 0 : 1 (1120 mL, 800 mL, 640 mL, 2000 mL, 1200 mL, 720 mL, 480 mL, 480 mL, and 800 mL, respectively)]. A total of 103 fractions with a volume of 80 mL each were collected and combined with regard to the results of TLC monitoring, yielding twelve main fractions (A/I–XII). From the marginally active fraction A/III, eluted with *n*-hexane–EtOAc 94 : 6, friedeline (**7**) was crystallised (71.5 mg, white crystals, m. p. 257–260 °C). Fractions A/IV, A/V, A/VI, A/VII, and A/VIII were found to exert pronounced antiproliferative activity and were analysed in detail. Fraction A/IV (eluted with *n*-hexane–EtOAc 92 : 8), which displayed cell growth-inhibitory effects of 37.1% (HeLa), 77.2% (MCF-7), and 63.2% (A-431), was chromatographed by RPC on silica gel in two steps, first with a gradient system of *n*-hexane–acetone (RPC I). Subfractions eluted with *n*-hexane–acetone 19 : 1 from RPC I were next purified by RPC (RPC II) with cyclohexane–EtOAc 9 : 1 as a developing system, affording compound **4** (5.8 mg). Fraction A/IV also contained compound **3**, isolated later from the active fraction A/VII. From fraction A/V [eluent: *n*-hexane–EtOAc 92 : 8; cell proliferation inhibition: 52.9% (HeLa), 38.8% (MCF-7), and 49.7% (A431)], pure epifriedelanol (**6**) was obtained as white crystals (187.0 mg, m. p. 291–292 °C). From the mother liquor of this substance, simiarenol (**9**) (12.0 mg, white crystals, m. p. 203–205 °C) was isolated by means of RPC (RPC III), with a solvent system of *n*-hexane–acetone 9 : 1 as the mobile phase. Fraction A/VI [eluent: *n*-hexane–EtOAc 92 : 8; cell proliferation inhibition: 55.5% (HeLa), 65.7% (MCF-7), and 56.1% (A-431)] was also subjected to RPC (RPC IV), with a solvent system of toluene–CH_2Cl_2 1 : 1, which resulted in the isolation of taraxerol (**8**) (11.1 mg, white crystals, m. p. 283–285 °C). In the prominently active fraction A/VII [eluent: *n*-hexane–EtOAc 9 : 1; cell proliferation inhibition: 87.3% (HeLa), 85.5% (MCF-7), and 84.6% (A-431)], compound **4**, isolated from fraction A/IV, was identified as a minor constituent. To obtain the main component, VLC (6 × 6 cm) was applied, with mixtures of *n*-hexane–acetone of increasing polarity as eluents [96 : 4, 94 : 6, 92 : 8, 9 : 1, and 7 : 3 (280 mL, 370 mL, 440 mL, 390 mL, and 520 mL, respectively); volume of collected fractions: 10 mL each]. The subfractions eluted with *n*-hexane–acetone 92 : 8 in this separation were purified by RPC (RPC V), with petroleum ether–CH_2Cl_2 1 : 1 as a solvent system, which yielded compound **3** (139.2 mg). For the separation of fraction A/VIII [eluent: *n*-hexane–EtOAc 8 : 2; cell proliferation inhibition: 55.9% (HeLa), 55.4% (MCF-7), and 52.4% (A-431)], a subsequent RPC was carried out (RPC VI), with the application of gradient elution (*n*-hexane–EtOAc). From the subfractions eluted with *n*-hexane–EtOAc 7 : 3, a crystalline material was obtained (24.5 mg), which was identified as a mixture of *β*-sitosterol and stigmasterol. From the subfractions eluted with *n*-hexane–EtOAc 6 : 4, another substance was crystallised, which was further purified by preparative TLC on silica gel (developing system: *n*-hexane–EtOAc 13 : 5); this afforded spinasterol (**10**) (9.8 mg, white crystals, m. p. 254–257 °C).

The $CHCl_3$-soluble phase of the extract (20.4 g) was chromatographed via VLC (8.5 × 14.5 cm), using a gradient system of CH_2Cl_2–MeOH [98 : 2, 96 : 4, 94 : 6, 9 : 1, and 8 : 2 (800 mL, 900 mL, 1400 mL, 600 mL, and 500 mL, respectively)]. The combination of the collected fractions (42 × 100 mL) resulted in five main fractions (B/I–V). In fraction B/I (eluted with CH_2Cl_2–MeOH 98 : 2), which exhibited marked cell growth-inhibitory effects [69.4% (HeLa), 80.3% (MCF-7), and 47.6% (A431)], compounds **3** and **4** were identified as main constituents. Fractions B/II, B/III, and B/IV demonstrated moderate antiproliferative activity in the bioassay. Fraction B/II [eluent: CH_2Cl_2–MeOH 96 : 4; cell proliferation inhibition: 36.7% (HeLa), 38.2% (MCF-7), and 35.6% (A431)] was subjected to VLC (6 × 8 cm), with a mixture of toluene–EtOAc–acetone of increasing polarity [6 : 3 : 1, 5 : 5 : 1, and 4 : 5 : 2 (350 mL, 150 mL, and 300 mL, respectively); volume of collected fractions: 25 mL]. The subfractions eluted with toluene–EtOAc–acetone 6 : 3 : 1 were separated by RPC in two steps: RPC VII was carried out on Al_2O_3 as sorbent, with gradient elution (cyclohexane–CH_2Cl_2–MeOH), while the subfractions of RPC VII eluted with cyclohexane–CH_2Cl_2–MeOH 20 : 20 : 1 were purified on silica gel, with *n*-hexane–EtOAc 3 : 2 (RPC VIII), which resulted in a mixture of two compounds. These were separated by RP-HPLC with MeOH–H_2O 3 : 2 as the mobile phase, at a flow rate of 0.4 mL/min, to furnish compounds **1** (7.6 mg) and **2** (15.3 mg). Fraction B/III [eluted with CH_2Cl_2–MeOH 94 : 6; cell proliferation inhibition: 28.4% (HeLa), 26.4% (MCF-7), and 19.1% (A431)] was fractionated by RPC, with a gradient system of *n*-hexane–EtOAc–MeOH (RPC IX). From the subfractions eluted with this system at 5 : 4 : 1, pure apigenin was crystallised (10.6 mg). Fraction B/IV [eluent: CH_2Cl_2–MeOH 9 : 1; cell proliferation inhibition: 29.7% (HeLa), 35.1% (MCF-7), and 43.8% (A431)] was processed by the method used for fraction B/III (RPC X), and the subfractions eluted with EtOAc–MeOH 1 : 1 were then purified by a subsequent RPC (RPC XI), with *n*-hexane–acetone–MeOH 6 : 14 : 5 as eluent, which resulted in the isolation of compound **5** (5.4 mg).

Antiproliferative assay

Antiproliferative effects were measured *in vitro* on four human cell lines (HeLa, MCF-7, A-431, and MRC-5) by means of the MTT ([3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide]) assay [13]. 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 new medium containing the test substances was added. After an incubation period of 72 h, the living cells were assayed by the addition of 20 µL 5 mg/mL MTT solution. 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 formazan was dissolved in 100 µL dimethyl sulfoxide (DMSO) during a 60-min period of shaking. Finally, the reduced MTT was assayed at 545 nm by 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 the cell proliferation. The dose-response curves of the compounds were fitted by means of the computer program GraphPad Prism 4.0 (GraphPad Software), and IC_{50} values were calculated. Doxorubicine and cisplatin were obtained from Ebewe Pharma GmbH as concentrated solutions for intravenous use in human clinical practice. Degree of purity of the tested compounds was determined by HPLC and ^{1}H NMR spectroscopy and found to be over 95% for **1, 3, 5**–**7, 9, 10,** and apigenin, 92% for **2** and **8**, and 90% for **4**.

Conyzapyranone A (**1**): colourless oil; $[\alpha]_D^{29}$ 0 (c = 0.1, $CHCl_3$); UV λ_{max} nm (log ε) 241 (2.56), 306 (2.74); ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$): see ▶ **Table 1**; positive ESIMS: *m/z* 211 $[M+H]^+$

Conyzapyranone B (**2**): colourless oil; $[\alpha]_D^{29}$ 0 (c = 0.1, $CHCl_3$); UV λ_{max} nm (log ε) 228 (2.33), 294 (2.71); ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$): see ▶ **Table 1**; positive ESIMS: *m/z* 211 $[M + H]^+$.

4Z,8Z-Matricaria-γ-lactone (**3**): brownish-yellow oil; ^{1}H NMR ($CDCl_3$, 500 MHz): δ ppm 7.41 (1H, d, J = 5.4 Hz, H-3), 6.25 (1H, d, J = 5.4 Hz, H-2), 6.16 (1H, dq, J = 10.8, 7.0 Hz, H-9), 5.72 (1H, d, J = 10.8 Hz, H-8), 5.49 (1H, d, J = 2.3 Hz, H-5), 1.97 (3H, dd, J = 6.9, 1.3 Hz, H-10). ^{13}C-NMR ($CDCl_3$, 125 MHz): δ ppm 168.8 (C-1), 155.7 (C-4), 142.4 (C-3), 141.8 (C-9), 120.3 (C-2), 109.8 (C-8), 99.3 (C-7), 94.6 (C-5), 87.9 (C-6), 16.4 (C-10).

4E,8Z-Matricaria-γ-lactone (**4**): brownish-yellow oil; ^{1}H NMR ($CDCl_3$, 500 MHz): δ ppm 7.76 (1H, d, J = 5.4 Hz, H-3), 6.30 (1H, dd, J = 5.4, 1.6 Hz, H-2), 6.14 (1H, dq, J = 10.8, 6.9 Hz, H-9), 5.92 (1H, s, H-5), 5.79 (1H, dd, J = 10.8, 1.5 Hz, H-8), 1.94 (3H, dd, J = 6.9, 1.3 Hz, H-10). ^{13}C-NMR ($CDCl_3$, 125 MHz): δ ppm 168.8 (C-1), 157.3 (C-4), 140.8 (C-3), 140.2 (C-9), 121.3 (C-2), 109.7 (C-8), 95.7 (C-5), 16.4 (C-10).

12,13-Trihydroxy-10(E)-*octadecenoic acid* (**5**): white solid; M.p. 144–147 °C; ^{1}H NMR data were in good agreement with published data (Oueslati et al., 2006); ^{13}C NMR ($CDCl_3$, 125 MHz): δ ppm 175.0 (C-1), 38.0 (C-2), 26.6 (C.3), 30.5 (C-4), 30.5 (C-5), 30.6 (C-6), 27.1 (C-7), 38.4 (C-8), 73.1 (C-9), 131.1 (C-10), 136.6 (C-11), 76.6 (C-12), 75.8 (C-13), 33.6 (C-14), 26.5 (C-15), 33.1 (C-16), 23.7 (C-17), 14.2 (C-18); positive ESIMS: *m/z* 348 $[M + NH_4]^+$, 353 $[M + Na]^+$; negative ESIMS: *m/z* 329 $[M - H]^-$.

Results and Discussion

The *n*-hexane and $CHCl_3$ phases of the methanol extract of the root of *C. canadensis*, which exerted noteworthy tumour cell growth-inhibitory effects in a preliminary screening experiment, were subjected to multiple chromatographic separations with the guidance of an antiproliferative assay. Thirteen compounds were isolated from the active fractions and tested for their antiproliferative action.

Compound **1** was obtained as colourless oil. Its UV absorptions at 241 and 306 nm indicated a conjugated enone system. On the basis of the base peak at *m/z* 211 $[M+H]^+$ in the ESIMS spectrum, its molecular mass was established as 210. The ^{1}H-NMR spectrum of **1** contained fourteen proton signals, and the ^{13}C-NMR spectrum eleven carbon resonances (▶ **Table 1**), indicating the molecular composition $C_{11}H_{14}O_4$. The presence of one methoxy group was easily recognized from the signals at δ_H 3.81 and δ_C 52.1. Analysis of the ^{1}H-^{1}H COSY and HSQC spectra provided information allowing the identification of one primary methyl, two methylene and four methine groups, and three quaternary carbons, including one keto (δ_C 193.3) and one carbonyl group (δ_C 165.9). The proton-proton connectivities detected in the ^{1}H-^{1}H COSY spectrum revealed the existence of two sequences of correlated protons: CH_3-CH_2-CH-CH_2- [fragment A, δ_H 1.07 (3H), 1.79 (1H), 1.90 (1H), 4.38 (1H), 2.50 (2H)] and a disubstituted olefin group with *trans* geometry (δ_H 7.05 d, 6.59 d, J = 15.6 Hz). Moreover, one isolated *sp*^{2} methine was detected at δ_H 5.59 and δ_C 109.7. The overall structure was assembled by analysis of the long-range C–H correlations gained from an HMBC experiment. Two- and three-bond correlations of the quaternary carbon at δ_C 193.3 (C-6) with protons at δ_H 5.59 (H-5), 2.50 (H-7), and 4.38 (H-8) demonstrated that the isolated methine and fragment A are connected through the keto group. This was corroborated by the HMBC correlations between C-5 and H-7, and C-7 and H-5. The long-range couplings of the carbonyl carbon at δ_C 165.9 (C-1) with the olefin protons at δ_H 7.05 (H-3) and 6.59 (H-2) and the methyl group at δ_H 3.81 proved a –CH=CH-$COOCH_3$ structural moiety (B) in the molecule. Fragments A and B were connected with the aid of the HMBC cross-peaks between C-4 (δ_C 165.2) and H-2, H-3 and H-5, and the correlations of C-5 with H-3, resulting in the overall structure **1**. The stereochemistry of **1** was determined in a NOESY experiment. The *Overhauser* effect between H-5 and H-3 revealed the steric proximity of these protons. Compound **1** has one stereogenic centre, C-8, but the optical rotation data $[\alpha]_D^{29}$ 0 (c = 0.1, $CHCl_3$) are indicative of a racemic mixture.

Table 1 ^{1}H and ^{13}C NMR data on compounds **1** and **2** [500 MHz (^{1}H), 125 MHz (^{13}C), $CDCl_3$, δ ppm (J = Hz)].

Position	1		2	
	^{1}H	^{13}C	^{1}H	^{13}C
1	–	165.9	–	166.7
2	6.59 d (15.6)	125.2	6.13 d (12.4)	127.0
3	7.05 d (15.6)	137.4	6.17 d (12.4)	129.2
4	–	165.2	–	166.0
5	5.59 s	109.7	5.49 s	107.8
6	–	193.3	–	193.4
7a	2.50 m (2H)	41.2	2.51 dd (16.8, 12.6)	40.7
7b			2.42 dd (16.8, 3.4)	
8	4.38 m	80.6	4.37 m	80.9
9a	1.90 m	27.5	1.81 m	27.4
9b	1.79 m		1.75 m	
10	1.07 t (7.4)	9.3	1.01 t (7.5)	9.0
OMe	3.81 s	52.1	3.79 s	51.9

The ESIMS and ^{1}H-NMR and JMOD spectra of compound **2** indicated the same molecular mass and composition as in the case of **1** (▶ **Table 1**). The HSQC and ^{1}H-^{1}H COSY experiments on **2** allowed identification of the same scalarly coupled spin systems CH_3-CH_2-CH-CH_2- (A) and –CH=CH-$COOCH_3$ (B), one isolated methine, and two quaternary carbons. Via the HMBC correlations, the same assignments of the subunits were elucidated. The only significant difference was observed in the coupling constant and ^{13}C chemical shifts of the disubstituted olefin. The coupling constant value J = 12.4 Hz demonstrated *cis* geometry of the C-2-C-3 double bond. For **2**, NOE effect was detected between H-3 and H-5; accordingly, its structure can be formulated as **2**. For this compound too, the presence of two enantiomers (in a ratio of 1 : 1) was indicated by the optical rotation data.

The bioactivity-guided chromatographic purification led to the isolation of eleven known compounds, including the C_{10} acetylene 4*Z*,8*Z*-matricaria-γ-lactone (**3**), 4*E*,8*Z*-matricaria-γ-lactone

Table 2 Antiproliferative effects of the isolated compounds on different human tumour and non-tumour cell lines.

Compound	Calculated IC_{50} values (μM) ± SEM			
	HeLa	MCF-7	A431	MRC-5
Conyzapyranone A (**1**)	61.40 ± 5.71	48.20 ± 1.89	35.32 ± 3.36	61.12 ± 4.03
Conyzapyranone B (**2**)	31.83 ± 1.96	46.00 ± 4.54	37.13 ± 3.92	79.63 ± 5.58
4*Z*,8*Z*-Matricaria-γ-lactone (**3**)	27.03 ± 3.61	6.90 ± 2.95	32.45 ± 3.54	28.10 ± 3.43
4*E*,8*Z*-Matricaria-γ-lactone (**4**)	24.46 ± 1.39	18.74 ± 2.19	22.81 ± 2.47	73.75 ± 5.12
9,12,13-Trihydroxy-10(*E*)-octadecenoic acid (**5**)	inactive	inactive	inactive	not tested
Epifriedelanol (**6**)	16.39 ± 1.87	61.43 ± 4.37	5.40 ± 1.02	inactive
Friedeline (**7**)	inactive	inactive	inactive	not tested
Taraxerol (**8**)	inactive	inactive	2.65 ± 0.71	inactive
Simiarenol (**9**)	inactive	inactive	inactive	not tested
Spinasterol (**10**)	13.93 ± 2.11	26.50 ± 3.58	13.66 ± 2.61	71.14 ± 11.42
Apigenin	10.64 ± 1.42	13.88 ± 2.38	12.34 ± 2.65	> 100.0
β-Sitosterol + stigmasterol	inactive	inactive	2.62 ± 0.14*	11.31 ± 0.89*
Doxorubicin	0.15 ± 0.03	0.28 ± 0.01	0.15 ± 0.04	0.50 ± 0.03
Cisplatin	12.43 ± 1.05	9.63 ± 0.75	2.84 ± 0.61	4.11 ± 1.05

* In μg/mL. „Inactive" indicates that the compound elicited less than 50 % inhibition of cell proliferation at 30 μg/mL, and no higher concentration was tested

(**4**) [14], the fatty acid 9,12,13-trihydroxy-10(*E*)-octadecenoic acid (**5**) [15], the triterpenes epifriedelanol (**6**) [16], friedeline (**7**) [17], taraxerol (**8**) [18], and simiarenol (**9**) [19], the sterols spinasterol (**10**) [20] and a mixture of β-sitosterol and stigmasterol, and the flavone apigenin. Taraxerol (**8**), simiarenol (**9**), and 9,12,13-trihydroxy-10(*E*)-octadecenoic acid (**5**) are reported for the first time from this plant species. The structures of the compounds were determined by means of ESIMS and ^{1}H and ^{13}C NMR spectroscopy, and in some cases with the aid of ^{1}H-^{1}H COSY, NOESY, HSQC, and HMBC experiments. The 2D NMR investigations permitted the complete ^{1}H and ^{13}C chemical shift assignments for matricaria-γ-lactones **3** and **4**, and yielded ^{13}C NMR data on 9,12,13-trihydroxy-10(*E*)-octadecenoic acid (**5**) in $CDCl_3$ for the first time, as listed in the experimental section. After the isolation of 4*Z*,8*Z*-matricaria-γ-lactone (**3**) and 4*E*,8*Z*-matricaria-γ-lactone (**4**) in pure form, the formation of the isomeric compound was observed in both cases, suggesting an *E*/*Z* isomerization process. In contrast with this, it was reported earlier that **4** is produced when **3** is irradiated with UV light, yielding an equilibrium mixture, and **4** was therefore regarded as an artefact [14].

The isolated compounds were evaluated for their inhibitory activities on HeLa, MCF-7, A431, and MRC-5 cells (**Table 2**), and it was found that different types of secondary metabolites are responsible for the cell growth-inhibitory action of the plant extracts. To different extents, flavone, sterol, triterpene, C_{10} acetylene, and dihydropyranone derivatives exhibited concentration-dependent antiproliferative effects. In some cases [4*Z*,8*Z*-matricaria-γ-lactone (**3**) (MCF-7 IC_{50} 6.90 μM), taraxerol (**8**) (A431 IC_{50} 2.65 μM), and apigenin (HeLa IC_{50} 10.64 μM)], the measured activities were comparable to that of the reference compound cisplatin.

Taraxerol (**8**), epifriedelanol (**6**), conyzapyranone A (**1**), and the mixture of stigmasterol and β-sitosterol displayed the highest activity against A431 cells, while matricaria lactones **3** and **4** demonstrated the highest efficacy against MCF-7 cells, with calculated IC_{50} values of 6.90 and 18.74 μM, respectively.

Interestingly, friedeline (**7**), a close analogue of epifriedelanol (**6**), was found to be inactive, indicating that the hydroxy group at C-3 is an important structural requirement for the antitumour activity of triterpenes. The antitumour activity of epifriedelanol (**6**) was demonstrated *in vitro* in a potato disc bioassay study [16], but no cytotoxicity was observed against P-388, A-549, MCF-7, HT-29, or KB cells at 10 μg/mL [21]. In our antiproliferative assay, marked activity was detected for apigenin against all three human tumour cell lines, while 9,12,13-trihydroxy-10(*E*)-octadecenoic acid (**5**) proved to be inactive.

The selective cytotoxic activity of new anticancer drug candidates, natural or synthetic, is one of their most critical pharmacological features. An additional set of MTT assays was therefore performed on noncancerous human foetal lung fibroblast cells (MRC-5) in order to evaluate the selectivity of the currently presented antiproliferative action. Although an ideal anticancer agent is expected not to suppress the proliferation of intact cells, most of the currently used agents, including doxorubicin and cisplatin, are clearly toxic for noncancerous cells. The potency of the two new isolated compounds is not outstanding: conyzapyranone B (**2**) exhibits substantially lower activity against MRC-5 cells than against malignant cell lines. Similarly, the calculated IC_{50} values of epifriedelanol (**6**), spinasterol (**10**), and apigenin indicate more pronounced toxicity on the utilized cancer cells than on MRC-5.

In conclusion, this bioactivity-guided phytochemical study of *Conyza canadensis* resulted in the isolation of two new unusual γ-dihydropyranone derivatives, named conyzapyranone A (**1**) and B (**2**), and eleven known compounds, and led to the conclusion that the significant inhibitory effect of the *n*-hexane and $CHCl_3$ extracts on the proliferation of cultured human tumour cell lines (HeLa, MCF-7, and A431) may be attributed mainly to flavone, C_{10} acetylene, triterpene, and sterol-type compounds.

Although 3-hydroxy-γ-pyranone and its derivatives have been reported to be abundant constituents of *Conyza* species [22], the structure of conyzapyranones, based on C_{10} unsaturated carbon skeleton and having a carboxymethyl functionality, suggests a closer relationship to C_{10} acetylenic compounds than to 3-hydroxy-γ-pyranone derivatives. C_{10} acetylenes, including diyn-ene (e.g., lachnophyllum ester) or ene-diyn-ene (e.g., matricaria ester) chromophore-containing compounds and C_{10}-lactones (e.g., lachnophyllum lactone, matricaria lactone), are typical constituents of the genus *Conyza*. Incorporation studies have revealed that these compounds are biosynthesized from C_{18} acetylenes by multistep β-oxidation or by direct oxidation. It has been supposed that C_{10} lactones can be originated from C_{10} acetylene acids, and other O-

heterocyclic compounds may also be biosynthetised by a similar way [23]. In case of conyzapyranone A (**1**) and B (**2**), the cyclisation of the lachnophyllum methylester [$CH_3CH_2CH_2(C{\equiv}C)_2$-$CH{=}CH$-$COOCH_3$] precursor can be supposed; in this cyclisation the C-4-C-8 part of the molecule may be involved.

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References

1 *Grünwald J.* Canadian fleabane. PDR for herbal medicines. Oxford: Wiley; 2000: 144–145
2 *Li TSC.* Chinese and related North American herbs – phytopharmacology and therapeutic values. Boca Raton, London, New York, Washington: CRC Press; 2002: 179–180
3 *Duke JA, Bogenschutz-Godwin MJ, Ottesen AR.* Canada fleabane. Duke's handbook of medicinal plants of Latin America. Boca Raton, London, New York: CRC Press; 2009: 225–227
4 *Bohlmann F, Jakupovic J.* 8-Oxo-α-Selinen und neue Scopoletin-Derivate aus *Conyza*-Arten. Phytochemistry 1979; 18: 1367–1370
5 *Xie WD, Gao X, Jia ZJ.* A new acetylene and a new triterpenoid from *Conyza canadensis*. Arch Pharm Res 2007; 30: 547–551
6 *Lenfeld J, Motl O, Trka A.* Anti-inflammatory activity of extracts from *Conyza canadensis*. Pharmazie 1986; 41: 268–269
7 *Czeczot H, Tudek B, Kusztelak J, Szymczyk T, Dobrowolska B, Glinkowska G, Malinowski J, Strzelecka H.* Isolation and studies of the mutagenic activity in the Ames test of flavonoids naturally occurring in medical herbs. Mutat Res 1990; 240: 209–216
8 *Mukhtar N, Iqbal K, Anis I, Malik A.* Sphingolipids from *Conyza canadensis*. Phytochemistry 2002; 61: 1005–1008
9 *Mukhtar N, Iqbal K, Malik A.* Novel sphingolipids from *Conyza canadensis*. Chem Pharm Bull 2002; 50: 1558–1560
10 *Ding X, Su Y, Guo H, Yang F, Mao H, Gao X, Zhu Z, Tu G.* Phenylpropanoid esters from horseweed (*Conyza canadensis*) and their inhibitory effects on catecholamine secretion. J Nat Prod 2010; 73: 270–274
11 *Hrutfiord BF, Hatheway WH, Smith DB.* Essential oil of *Conyza canadensis*. Phytochemistry 1988; 27: 1858–1860
12 *Réthy B, Csupor-Löffler B, Zupkó I, Hajdú Z, Máthé I, Hohmann J, Rédei T, Falkay G.* Antiproliferative activity of Hungarian Asteraceae species against human cancer cell lines. Part I. Phytother Res 2007; 21: 1200–1208
13 *Mossmann T.* Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65: 55–63
14 *Lam J.* Polyacetylenes of *Solidago virgaurea*: their seasonal variation and NMR long-range spin coupling constants. Phytochemistry 1971; 10: 647–653
15 *Oueslati MH, Jannet HB, Mighri Z, Chriaa J, Abreu PM.* Phytochemical constituents from *Salsola tetrandra*. J Nat Prod 2006; 69: 1366–1369
16 *Kundu JK, Rouf ASS, Nazmul Hossain M, Hasan CM, Rashid MA.* Antitumor activity of epifriedelanol from *Vitis trifolia*. Fitoterapia 2000; 71: 577–579
17 *Mahato SB, Kundu AP.* ^{13}C NMR spectra of pentacyclic triterpenoids – a compilation and some salient features. Phytochemistry 1994; 37: 1517–1575
18 *Jutiviboonsuk A, Zhang HJ, Kondratyuk TP, Herunsalee A, Chaukul W, Pezzuto JM, Fong HHS, Bunyapraphatsara N.* Isolation and characterization of cancer chemopreventive compounds from *Barringtonia maunwongythiae*. Pharm Biol 2007; 45: 185–194
19 *Yoo NH, Jang DS, Kim JS.* Phytochemical constituents of the roots of *Erigeron annuus*. J Korean Soc Appl Biol Chem 2008; 51: 305–308
20 *Kojima H, Sato N, Hatano A, Ogura H.* Sterol glycosides from *Prunella vulgaris*. Phytochemistry 1990; 29: 2351–2355
21 *Zheng GQ.* Cytotoxic terpenoids and flavonoids from *Artemisia annua*. Planta Med 1994; 60: 54–57
22 *Li X, Pan J, Gao K.* γ-Pyranone derivatives and other constituents from *Erigeron annuus*. Pharmazie 2006; 61: 474–477
23 *Christensen LP, Lam J.* Acetylenes and related compounds in Asteraceae. Phytochemistry 1991; 30: 2453–2476