Antiproliferative Constituents of the Roots of Conyza canadensis

Abstract

Bioassay-guided fractionation of the n-hexane and CHCl₃ 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 4Z,8Z-matricaria-γ-lactone (3), 4E,8Z-matricaria-γ-lactone (4), 9,12,13-trihydroxy-10(E)-octadecenoic acid (5), epifriedelanol (6), friedelone (7), taraxerol (8), simiareol (9), spinasterol (10), stigmasterol, β-sitosterol, and apigenin. The structures were determined by means of ESIMS and 1D and 2D NMR spectroscopy, including "H-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 diarrohea 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 C₁₀ 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-trihydroxyaraxastane-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 monoterpens, sesquiterpenes, and acetylens, 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 CHCl₃ 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 C₁₀ dihydropyranone derivatives and various known C₁₀ acetylenes, triterpenes, sterols, a hydroxy-fatty acid, and apigenin are reported (Fig. 1).
Materials and Methods

General experimental procedures

NMR spectra were recorded in CDCl₃ on a Bruker Avance DRX 500 spectrometer at 500 MHz (¹H) or 125 MHz (¹³C); 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 H₂SO₄, followed by heating at 110 °C. For vacuum liquid chromatography (VLC), SiO₂ (silica gel 60GF₂₅₄, 15 µm; Merck 11677) was applied. Rotation planar chromatography (RPC) was performed with a Chromatotron instrument (model 8924; Harrison Research) on manually coated SiO₂ (silica gel 60 GF₂₅₄, Merck 7730; RPC I–VI and RPC VIII–XI) or Al₂O₃ (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 SiO₂ plates (20 × 20 cm, silica gel 60F₂₅₄; Merck 5715). Separation was monitored by spraying the border of the plates with concentrated H₂SO₄. 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–H₂O 3:2 was applied at a flow rate of 0.4 mL/min; the separation was monitored at 220 nm.

Fig. 1 Chemical structures of compounds 1–10.
Plant material
The roots of *C. canadensis* (L.) Cronq. were collected in the South ern Great Plain (Hungary) in September 2004 and authenticated by Dr. Tamás Rédei (Institute of Ecology and Botany of the Hun garian Academy of Sciences, Vácfrátó, Hungary). A voucher spec imen (No. 771) has been deposited at the Department of Pharma cognosy, University of Szeged.

Extraction and isolation
The air-dried and ground roots of the plant (2.6 kg) were per colated with MeOH (501) at room temperature. The concentrat ed extract (300 mL) was diluted with 300 mL H2O and subjected to solvent-solvent partition, first with n-hexane (5 × 2 L) and then with CHCl3 (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, 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/II–XII). From the marginally active fraction A/III, eluted with n-hexane–EtOAc 96:6, 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 sys tem, 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% (A-431), pure epifriedelanol (6) was obtained as white crystals (187.0 mg, m.p. 291–292°C). From the mother liquor of this substance, sim larenol (9) (12.0 mg, white crystals, m.p. 203–205°C) was iso lated 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–CH2Cl2 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/VI 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–CH2Cl2 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 stigmastanol. 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 CHCl3-soluble phase of the extract (20.4 g) was chromat ographed via VLC (8.5 × 14.5 cm), using a gradient system of CH2Cl2–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 CH2Cl2–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/I, B/II, and B/IV demonstrated moderate antiproliferative activity in the bioassay. Fraction B/I [eluent: CH2Cl2–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 an Al2O3 as sorbent, with gradient elution (cyclohexane–CH2Cl2–MeOH), while the subfractions of RPC VII eluted with cyclohexane–CH2Cl2–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–H2O 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 CH2Cl2–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: CH2Cl2–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 bro midle)] 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 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. Doxorubicin and cisplatin were obtained from Ebewe Pharma GmbH as concentrated solutions and 13C NMR (125 MHz, CDCl3) was obtained from Ebewe Pharma GmbH as concentrated solutions.

Results and Discussion

The n-hexane and CHCl3 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 base of the peak at m/z 211 [M + H]^+ in the ESIMS spectrum, its UV absorptions at 241 (2.56), 306 (2.74); 1H NMR (500 MHz, CDCl3) proved a CH=CH-COOCH3 structural moiety (B) in the molecule. Fragments A and B were connected with the aid of the HMBC correlations of C-5 with H-3, resulting in the overall structure. 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-COOCH3 structural moiety (B) in the molecule. 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 stereocentre, C-8, but the optical rotation data [α]_D^20~0 (c = 0.1, CHCl3) are indicative of a racemic mixture.

The ESIMS and 1H-NMR and 13C NMR data of compound 1 are given in Table 1 (Table 1). The HSQC and 1H-13C COSY experiments on 1 allowed identification of the same scalarly coupled spin systems CH_3-CH=CH-CH_2- (A) and -CH=CH-COOCH_3 (B), one isolated methine, and two quaternary carbons. Via the HMBC correlations detected in the 1H-1H COSY spectrum the proton-proton connectivities detected in the 1H-1H COSY spectrum revealed the existence of two sequences of correlated protons: CH_3-CH=CH-CH_2- [fragment A, δ_H 1.07 (3H), 1.97 (1H), 1.94 (3H, dd, J = 6.9, 1.3 Hz, H-10) - fragment B, 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.

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The ESIMS and 1H-NMR and 13C NMR data of compound 2 are given in Table 1 (Table 1). The HSQC and 1H-13C COSY experiments on 2 allowed identification of the same scalarly coupled spin systems CH_3-CH=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 13C 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 C10 acetylene 4Z,8Z-matricaria-γ-lactone (3). 4E,8Z-matricaria-γ-lactone...
Table 2 Antiproliferative effects of the isolated compounds on different human tumour and non-tumour cell lines.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Calculated IC50 values (µM) ± SEM</th>
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<tbody>
<tr>
<td></td>
<td>Hela</td>
</tr>
<tr>
<td>Conyza pyranone A (1)</td>
<td>61.40 ± 5.71</td>
</tr>
<tr>
<td>Conyza pyranone B (2)</td>
<td>31.83 ± 1.96</td>
</tr>
<tr>
<td>4E,Z/8-Matricaria-γ-lactone (3)</td>
<td>27.03 ± 3.61</td>
</tr>
<tr>
<td>4E,8-Z/Matricaria-γ-lactone (4)</td>
<td>24.46 ± 1.39</td>
</tr>
<tr>
<td>9,12,13-Trihydroxy-10(E)-octadecenoic acid (5)</td>
<td>inactive</td>
</tr>
<tr>
<td>Epifriedelanol (6)</td>
<td>16.39 ± 1.87</td>
</tr>
<tr>
<td>Friedelanol (7)</td>
<td>inactive</td>
</tr>
<tr>
<td>Taraxerol (8)</td>
<td>inactive</td>
</tr>
<tr>
<td>Simiareolen (9)</td>
<td>inactive</td>
</tr>
<tr>
<td>Spinasterol (10)</td>
<td>13.93 ± 2.11</td>
</tr>
<tr>
<td>Apigenin</td>
<td>10.64 ± 1.42</td>
</tr>
<tr>
<td>β-Sitosterol + stigmasterol</td>
<td>inactive</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>12.43 ± 1.05</td>
</tr>
</tbody>
</table>

* 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.

In conclusion, this bioactivity-guided phytochemical study of Conyza canadensis resulted in the isolation of two new unusual γ-dihydropyranone derivatives, named conyzapyanone A (1) and B (2), and eleven known compounds, and led to the conclusion that the significant inhibitory effect of the n-hexane and CHCl₃ extracts on the proliferation of cultured human tumour cell lines (HeLa, MCF-7, and A431) may be attributed mainly to flavone, C₁₀ 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 C₁₀ unsaturated carbon skeleton and having a carbonyl functionality, suggests a closer relationship to C₁₀ acetylenic compounds than to 3-hydroxy-γ-pyranone derivatives. C₁₀ acetylenes, including diyn-ene (e.g., lachnophyllum ester) or ene-diyne-ene (e.g., matricaria ester) chromophore-containing compounds and C₁₀-lactones (e.g., lachnophyllum lactone, matricaria lactone), are typical constituents of the genus Conyza. Incorporation studies have revealed that these compounds are biosynthesized from C₁₈ acetylenes by multistep β-oxidation or by direct oxidation. It has been supposed that C₁₀ lactones can be originated from C₁₀ acetylene acids, and other O-
heterocyclic compounds may also be biosynthesised by a similar way [23]. In case of conyzapyranone A (1) and B (2), the cyclisation of the lachnophyllum methylester [CH$_3$CH$_2$CH$_2$(C≡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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