Bioactivity-guided isolation of antiproliferative compounds from *Centaurea carduiformis* DC

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**A R T I C L E   I N F O**

Article history:
Available online 24 January 2014

Keywords:
*Centaurea carduiformis* subsp. *carduiformis* Var. *carduiformis*
Flavonoids
Vero
HeLa
*C6*
Antiproliferative activity

**A B S T R A C T**

In this study, antiproliferative activity of methanol extract of *Centaurea carduiformis* DC. subsp. *carduiformis* var. *carduiformis* DC (CC) was examined against Vero, HeLa and C6 cell lines in 1000 and 500 μg/mL concentrations using the BrdU ELISA assay. The CC root (CCR) has the most effective antiproliferative activity. The root extract was fractionated using various solvent and determined antiproliferative activities. Two new and two known flavonoids were isolated from CC roots. The isolated known compound of 7-O-β-D-glucopyranosyl-4′-methylapigenin and new compound of 7-O-β-D-glucopyranosyl-6′-O-β-D-furanosylpinocembrin were isolated from the ethyl acetate fractions. However, wogonin and new compound of N-((pentyloxy)(m-tolyl)methyl)acetamideisowogonin were obtained from chloroform fractions. The chemical structures of pure compounds were elucidated with different chemical and spectroscopic methods (IR, 1H NMR, 13C NMR, HETCOR, COSY, GC-MS, etc.) and their antiproliferative activities were determined against Vero, HeLa and C6 cell lines. The IC₅₀ results showed that the compound 4 has the highest activity against Vero (250 μg/mL) and HeLa (735 μg/mL) cell lines than isolated other compounds from determined values.

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1. Introduction

The *Centaurea* genus is widespread in Turkey with 180 species, 109 of which are endemic (Davis, 1997; Duran and Duman, 2002). The *Centaurea* species have been used in folk medicine because of its anti-diabetic, anti-diarrheic, anti-inflammatory, anti-inflammatory, colagol, choleretic, digestive, stomachic, diuretic, menstrual, astringent, hypotensive, antipyretic, cyotoxic, antibacterial effects (Farrag et al., 1993; Gurkan et al., 1998; Orallo et al., 1998; Kaij-A-Kamb et al., 1992).

This genus has many secondary metabolites in particular flavonoids (Shoeb et al., 2007; Flamini et al., 2001) and sesquiterpene lactones (KouKoulitsa et al., 2002; KouKoulitsa et al., 2005; Saroglou et al., 2005; Yayli et al., 2006).

In present work, the antiproliferative activity and chemical constituents of *Centaurea carduiformis* subsp. *carduiformis* (CC) were investigated by bioassay-guided fractionation procedure. This plant is an endemic species distributed in the central part of Turkey (Wagenitz, 1975). To the best of our knowledge, no report on the isolation of secondary metabolites from *Centaurea carduiformis* or any pharmacological properties of CC is available to date. This paper describes the isolation and structural identification of two new flavonoids of compounds 2 and 4 as well as two known flavonoids (1 and 3) from the CC roots. The fractions and the compounds isolated from bioactive fractions were tested against HeLa, C6 and Vero cell lines in order to evaluate their antiproliferative activity.

2. Results and discussion

2.1. Isolation and structure determination of the compounds

The EtOAc extract of CC roots (CCR) was subjected to silica gel column. Two flavonoids were isolated from the column, 7-O-β-D-glucopyranosyl-4′-methylapigenin (1) and 7-O-β-D-glucopyranosyl-6′-O-β-D-furanosylpinocembrin (2). To our knowledge, compound 2 was obtained for the first time from this plant. The compounds 3 and 4 were isolated from the CHCl₃ extract of CCR. Structure of the isolated compounds was determined using spectral methods, such as ¹H NMR, ¹³C NMR, DEPT, HMBC, and mass spectrometry.

7-O-β-D-glucopyranosyl-4′-methylapigenin (1) is an amorphous light yellow powder with melting point 223–224 °C and UV max.: 272 and 311 nm. The strong band of carbonyl stretching of the flavone was observed at 1656 cm⁻¹, the hydroxyl stretching and aromatic stretching bonds were observed at 3394 and 3072 cm⁻¹, respectively, in the IR spectrum. The molecular formula C₁₉H₂₂O₁₀ was determined by LC-MS/MS showing
molecular ion peak [M+Na]+ at m/z 469.11 (calcd. for C_{22}H_{22}O_{10}Na, 469.11). The DEPT spectrum displayed seven quarternary carbons, five methine carbons, a methane carbon and a methoxy carbon. The carbonyl carbon was observed at δC 182.8 (C-4) in the 13C NMR spectrum. The 1H NMR spectrum of 1 (Table 1) displayed the signals at δH 7.63 (2H, H-3' and H-5'), δH 8.07 (2H, H-2' and H-6'), δH 7.44 (2H, H-2' and H-6'), d, J = 7.44 Hz, δH 7.08 (2H, H-2' and H-6'), m, J = 6.44 Hz, and δH 7.25 (2H, H-2' and H-6'), m, J = 7.25 Hz, and two doublets, δH 7.68 and δH 7.75 Hz, were observed at δH 5.67 (H-6), δH 5.12 (H-5) and δH 6.16 (H-7, d, J = 1.89 Hz), δH 7.53 (H-2' and H-6', d, J = 7.25 Hz) and δH 7.45 (H-3' and H-5', dd, J = 6.44, 7.25 Hz) and δH 7.40 (H-4', t, J = 6.44 Hz).

The flavonoid skeleton was investigated by a flavone anthocyanin, a glucose and a furoxone ring. The flavonoid signals were observed at δH 5.67 (H-2, dd, J = 12.66 Hz, J = 2.87 Hz), δH 2.85 and 3.38 (2H, d, J = 15.4 Hz), δH 6.12 (H-6, d, J = 1.89 Hz), δH 6.16 (H-7, d, J = 1.89 Hz), δH 7.53 (H-2' and H-6', d, J = 7.25 Hz) and δH 7.45 (H-3' and H-5', dd, J = 6.44, 7.25 Hz) and δH 7.40 (H-4', t, J = 6.44 Hz).

Compound 1 | Compound 2 | Compound 3 | Compound 4
<table>
<thead>
<tr>
<th>H</th>
<th>C</th>
<th>H</th>
<th>C</th>
<th>H</th>
<th>C</th>
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<th>C</th>
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<tbody>
<tr>
<td>2</td>
<td>163.8</td>
<td>5.67 dd (12.7, 2.9)</td>
<td>79.2</td>
<td>163.6</td>
<td>6.98 s</td>
<td>104.5</td>
<td>6.96 s</td>
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<tr>
<td>3a</td>
<td>7.05 s</td>
<td>197.2</td>
<td>182.7</td>
<td>182.5</td>
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<tr>
<td>3b</td>
<td>152.3</td>
<td>163.4</td>
<td>153.2</td>
<td>152.9</td>
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</tr>
<tr>
<td>6</td>
<td>7.08 d (3.6)</td>
<td>95.0</td>
<td>6.16 d (1.9)</td>
<td>96.5</td>
<td>6.59 s</td>
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</tr>
<tr>
<td>8</td>
<td>7.64 d (3.6)</td>
<td>132.2</td>
<td>6.12 d (1.9)</td>
<td>97.09</td>
<td>131.0</td>
<td>132.6</td>
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<td></td>
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<tr>
<td>2'</td>
<td>8.07 dd (7.4)</td>
<td>126.4</td>
<td>7.53 d (7.3)</td>
<td>128.9</td>
<td>8.05 t (6.7)</td>
<td>126.3</td>
<td>7.68 m</td>
</tr>
<tr>
<td>3'</td>
<td>7.63 d (7)</td>
<td>129.1</td>
<td>7.45 dd (7.3, 6.4)</td>
<td>127.1</td>
<td>7.54 d (6.9)</td>
<td>129.4</td>
<td>8.06 d (6.7)</td>
</tr>
<tr>
<td>4'</td>
<td>132.9</td>
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<td>7.54 d (6.9)</td>
<td>129.4</td>
<td>8.06 d</td>
<td>126.9</td>
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<tr>
<td>5'</td>
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<td>129.1</td>
<td>7.45 dd (7.3, 6.4)</td>
<td>127.1</td>
<td>7.54 d (6.9)</td>
<td>129.4</td>
<td>8.06 d</td>
</tr>
<tr>
<td>6'</td>
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<td>128.9</td>
<td>8.05 t (6.7)</td>
<td>126.3</td>
<td>7.68 m</td>
</tr>
<tr>
<td>7'</td>
<td>(C=O)</td>
<td>166.9</td>
<td>166.9</td>
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</tbody>
</table>

NH | 3.20 m | 3.81 |
9' | 1.61 m | 38.1 |
11' | 4.13 m | 67.4 |
12' | 1.29 m | 23.2 |
13' | 1.27 m | 29.8 |
14' | 1.24 m | 22.4 |
15' | 0.88 m | 10.9 |

OH | 3.66 m | 3.66 m |

OCH3 | 3.60 m | 3.60 m |

OCH3 | 3.77 s | 3.77 s |

a At 400 MHz in DMSO-d6.

b At 100 MHz in DMSO-d6, s: singlet, d: doublet, t: triplet, m: multiplet, dd: doublet of doublet.
Anomeric protons were the characteristic signals of flavone glycoside determined at δH 5.16 (d, J = 6.84 Hz) and δH 4.52 (d, J = 5.16 Hz). In addition, eight methine and two methylene signals were showed between δH 3.7–4.4, appertaining to a glucose and a furonose ring. In 1H NMR spectrum of 2 with D2O was investigated to disappear of hydroxyl groups. In the HMBC spectrum C-7 (165.5 ppm) was correlated with H-1" (5.18 ppm) anomeric proton belonging to a glucose. The correlation between H-1" anomeric proton at C-6" and C-5" carbons were established HMBC correlations belonging to a furonose ring. The COSY coupling was determined as H-3-α (dd, J = 15.4, J = 2.9 Hz) and H-3-β (dd, J = 15.4, J = 12.66 Hz) with δH 5.67 H-2 (dd, J = 12.66, J = 2.87 Hz).

Wogonin (3) is an amorphous yellow powder. This compound was named as wogonin in the literature and determined by using spectral data, i.e. 1H NMR, 13C NMR, DEPT, HMBC, mass spectrometry. These data were compared with literature (Enomoto et al., 2007; Jang et al., 2005). The compound 3 was also isolated from Scutellaria baicalensis GEORG (Sohn et al., 2006; Li-Weber, 2009). Its melting point is 205–207 ºC and UV max: 274 and 320 nm. The strong band of carbonyl stretching of the flavone was observed at 1643 cm⁻¹, hydroxyl stretching was observed at 3432 cm⁻¹ in the IR spectrum. The molecular formula C16H12O5 was determined by LC-MS/MS showing molecular ion peak [M⁺] at m/z 284.93 (calcd. for C16H12O5, 284.07). The 1H NMR spectrum was observed a flavone skeleton. The signals at δH 8.05 (H-2' and H-6', J = 6.86 Hz), δH 7.54 (H-3' and H-5'), m, J = 6.84 Hz, δH 7.61 (H4', t, J = 6.86 Hz) and δH 3.80 (methoxy groups), δH 6.98 (H-3), δH 6.59 (H-6) indicated the correct structure as seen in Table 1. In the 13C NMR spectrum, there were sixteen carbon signals. The carbons were determined as eight quaternary carbons, seven methine carbons which was a positive signal in DEPT-90 spectrum and a methoxy carbon (δC 60.0). The signal which was δH 8.05 (H-2' ve H-6) was correlated with δH7.54 (H-3' and H-5') and δH 7.61 (H-4' in the COSY spectrum. C-2 carbon correlated with H-2', H-6' and H-3 protons in the HMBC spectrum. In addition, C-5 with H-6 and 5-OH, C-10 with H-3, H-6 and 5-OH, C-1 with H-2' and H-3, C-8 with methoxy protons showed correlation. 1H–13C HETCOR correlations between C-3 (δc 104.5), δH 6.98 (H-3) and C-6 (δc 94.7) δH 6.59 (H-6) were investigated. C-2' and C-6' (δc 126.3) δH 7.54 (H-2' and H-6'), C-3' and C-5' (δc 129.4), δH 8.05 (H-3' and H-5') and methoxy carbon (δc 60.0) δH 3.73 were interacted in the HETCOR spectrum. 

N-(pentyloxy(m-tolyl)methyl)acetamidesisowogonin (4) is a yellow crystalline compound with melting point 194–196 ºC and UV max 257 and 321 nm. The strong bands of two carbonyl stretching were observed at 1627 and 1740 cm⁻¹, the hydroxyl stretching was observed at 3428 cm⁻¹ in the IR spectrum. The elemental analysis resulted as C 69.82%, H 6.09%, N 2.99%, O 21.09%. The molecular formula C30H31NO6 was determined by LC-MS/MS showing molecular ion peak [M–216]⁺ at m/z 285.94 (C29H27NO5, 285.07) and [M–334]⁺ at m/z 166.72 (calcd. for C24H22NO3, 166.03).

In the aggregate, there were 30 carbons in the 13C NMR spectrum. These carbons were investigated as twelve quaternary carbons, four methylene carbons which was negative signal in the DEPT 135 spectrum, eleven methine carbons which was shown as positive signal in the DEPT 90 spectrum, three methyl carbons, one of them belonging to methoxy group at δc 60.5. In the 1H NMR spectrum of compound 4 (Table 1) showed a flavone skeleton. δH 6.65 (H-6, s), δH 6.96 (H-3, s), δH 7.68 (H-2' and H-6', m) and δH 8.06 (H-3' and H-5', d, J = 6.8 Hz) signals were determined in the 1H NMR. In addition, methoxy group belonging to flavonoid was observed at δH 3.76 as singlet. δH 7.62 (H-2, H-3 and H-4, d,
$J = 6.8\text{ Hz}$ signals were investigated concerning with phenyl ring. $^{1}H-^{13}C$ HETCOR coupling between C-3 ($\delta_{C} 104.8, \delta_{H} 6.96$) and C-6 ($\delta_{C} 95.0, \delta_{H} 6.65$), C-3’ and C-5’ carbons ($\delta_{C} 126.9, \delta_{H} 8.06$), C-2’ and C-6’ carbon ($\delta_{C} 129.1$ and $131.6$) $\delta_{H} 7.7$ was determined. In the molecules, other phenyl carbons ($\delta_{C} 132.0, 132.0, 132.0, 131.7$) were correlated at $\delta_{H} 7.62, 7.62, 7.62$ and 7.7, respectively. The methylene carbons ($\delta_{C} 22.37, 23.21, 28.33, 29.76, 64.4$) with $\delta_{H} 1.24, 1.29, 1.21, 1.27$ and 4.13 sequentially, methyl carbons ($\delta_{C} 10.9$ ve 14.4) with $\delta_{H} 0.88$ ve 0.85, methoxy carbon (59.6) with $\delta_{H} 3.77$, methine carbons ($\delta_{C} 38.1$) with $\delta_{H} 1.61$ were established in the HETCOR spectrum. C-7 carbon which belongs to flavone skeleton was correlated with methoxy proton in the HMBC spectrum. In addition, C-2 carbon was shown to interact with H-2’ ve H-6’ protons. In the HMQC spectrum, H-3’ and H-5’ protons ($\delta_{H} 8.06$) with $\delta_{C} 126.9$, H-6 proton ($\delta_{H} 6.65$) with $\delta_{C} 95.0$ and H-3 protons ($\delta_{H} 6.96$) with $\delta_{C} 104.8$ were correlated. The COSY coupling between $\delta_{H} 8.06$ (H-3’), $\delta_{H} 7.68$ (H-2’) was determined. NH ($\delta_{H} 3.20$) protons and H-9’ ($\delta_{H} 1.61$) protons were correlated in the COSY spectrum.

2.2. Antiproliferative activities of extracts and isolated compounds

Antiproliferative activities were determined for the methanol extracts of flower, steam and root of Centaurea carduiformis subsp. carduiformis var. carduiformis (CC) (Fig. 1). CC roots have the highest activity against Vero, HeLa and C6 cell lines (at 1000 and 500 $\mu$g/mL dose). As results in Fig. 1, especially CC root showed higher activity than cisplatin and 5-Fluorouracil (5-FU) against Vero cell line.

According to this data (Fig. 2), we prepared the ethanol (80%) extract of CCR. The extract was subjected to fractionation through solvent–solvent extraction and obtained hexane extract, CHCl3 extract, n-ButOH extract, H2O extract. All extracts were investigated for antiproliferative activity against the three cell lines. The results show that EtOAc extracts have the strongest activity (Fig. 2). The second higher activity was obtained from the chloroform extract.

In this study, we purposed to isolate the extract which showed strong activity, through bioassay-guided fractionation procedures. Therefore, firstly the EtOAc extract was subjected to silica gel column, after the CHCl3 extract. Compounds 1 and 2 were obtained from the EtOAc extract. Compounds 3 and 4 were purified from the CHCl3 extract. Structure of the isolated compounds determined by using spectral data, i.e. $^{1}H$ NMR, $^{13}C$ NMR, DEPT, HMBC, mass spectrometry. All compounds were tested against Vero, HeLa and C6 cell lines for antiproliferative activity. To our knowledge, there is no report on either the isolation of secondary metabolites or determination of biological activities for the endemic plant of CC. The isolated compounds 2 and 4 were obtained first time from this plant. Antiproliferative activities of isolated compounds were compared with the extracts’ activities (Fig. 3). The results showed that the extracts have the highest activity than isolated compounds. Therefore, we said that this extract’s activity may be from synergic effects. However, IC50 and IC75 values of the isolated compounds are given in Table 2.

3. Experimental

3.1. General experimental procedures

NMR spectra were measured on Bruker Avance III Spectrometers at 400 MHz. Mass spectra were run on Micromass quattro LC-MS/MS spectrometer. IR spectra determined on KBr plates on Jasco-430 FT/IR Spectrometers. UV spectra were recorded in MeOH on JASCO V 530. Silica gel 60 F254 (Merck) were used for TLC analyses. Spots on TLC were visualized by UV irradiation (254 and 366 nm), and by spraying with Ce(SO4)2 (1% in 10% aqueous H2SO4) followed by heating (105 °C, 5 min).

![Fig. 2. Antiproliferative activity of the fractions from CCR. The values represent the mean ±SEM (n = 3). *P < 0.01 when compared to control groups (one-way ANOVA following the Duncan’s multiple comparison test).](image-url)
3.2. Plant material

*Centaurea carduiformis* subsp. *carduiformis* var. *carduiformis* were collected from Tokat in July 2008. Identification of the voucher specimens was done at the Faculty of Pharmacy, Istanbul University by Prof. Dr. Nerman Ozhata. Voucher specimens are deposited in the herbarium of Faculty of Pharmacy, Istanbul University (ISTE 85426).

3.3. Root extraction of Centaurea carduiformis

The dried root (1 kg) were macerated with 80% EtOH/H₂O (6 L × 2 L) in a desicator at room temperature for 2 weeks and then filtered. This solution was evaporated to dryness. The yield of ethanolic extract of *Centaurea carduiformis* roots (CCR) was 110.9 g (11.09%).

3.4. Fractionation of CCR through solvent–solvent extraction

CCR was dissolved in 1 L MeOH/H₂O (9:1) and further extracted with n-hexane (5 L × 2 L). After evaporation of MeOH, the remaining extract was diluted with H₂O (1 L) and extracted with CHCl₃ (5 L × 1.5 L), EtOAc (4 L × 1.5 L), and n-BuOH, respectively. Each extract and the remaining aqueous part were then evaporated to give n-hexane extract (3.39 g), CHCl₃ extract (5.28 g), EtOAc extract (8.73 g), n-BuOH extract (18.96 g) and remaining aqueous extract (57.51 g) (Fig. 4).

3.5. Isolation of the compounds from EtOAc extract

EtOAc extract (6.11 g) was subjected to silica gel column (364 g, Kieselgel 60, 70–230 mesh, Merck) using MeOH–CHCl₃ (1:6) solvent systems on fraction 1–220; MeOH–CHCl₃ (1:5) on fraction 221–817; MeOH–CHCl₃ (1:1) solvent systems on fraction 818–895; MeOH–CHCl₃ (1:1) solvent systems on fraction 896. A total of 896 fractions were collected. The fractions volumes were 20 mL. Fractions were combined due to their TLC profiles. Compound 1 (84 mg; fractions 73–79) and 2 (66 mg; fractions 236–259) (Fig. 5).

3.6. Isolation of the compounds from CHCl₃ extract

The chloroform extract (1 g) was chromatographed on silica gel column (364 g, Kieselgel 60, 70–230 mesh, Merck) using EtOAc:CHCl₃ (1:1) solvent systems. A total of 250 fractions were collected. The fractions volumes were 20 mL. Fractions were combined due to their TLC profiles. Compound 3 (19 mg) was obtained from fractions 15–18 (Fig. 5).

3.7. Separation of the CHCl₃ extract with flash chromatography

The chloroform extract (500 mg) was dissolved in MeOH. This solution is subject to flash chromatography column (max. 45 PSI, 3.1 Bar. Redi Sep) using CH₃Cl₂ and EtOAc:CH₃Cl₂ (1:9). A total of 300 fractions were collected. The fraction volumes were 10 mL. Fractions were combined due to their TLC profiles. Compound 4 (27 mg) was obtained from fractions 1–10 (Fig. 5).

3.8. Isolation of compound 2 by different methods

*Centaurea carduiformis* roots were crushed with liquid nitrogen. The obtained powder (550 g) was dissolved in water (1 L) and refluxed for 4 h. After that, the mixture was filtered using filter paper and extracted with EtOAc (3 mL × 300 mL). Organic phase was evaporated to dryness (Demirias et al., 2013). Compound 2 (211 mg) was obtained as a pure compound.

Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC₅₀ (Vero)</th>
<th>IC₇₀ (Vero)</th>
<th>IC₅₀ (HeLa)</th>
<th>IC₇₀ (HeLa)</th>
<th>IC₅₀ (C6)</th>
<th>IC₇₀ (C6)</th>
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<tr>
<td>Compound 1</td>
<td>*</td>
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<td>875</td>
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<td>805</td>
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<tr>
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<td>*</td>
<td>750</td>
<td>875</td>
<td>*</td>
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<tr>
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<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Compound 4</td>
<td>250</td>
<td>625</td>
<td>735</td>
<td>867</td>
<td>750</td>
<td>875</td>
</tr>
</tbody>
</table>

*IC₅₀* and *IC₇₀* values of compounds 1, 2, 3 and 4.

*“* <0, **“** >1000 μg/mL.
3.9. Acid hydrolysis of compound 2

Compound 2 (134 mg) was refluxed 2 M HCl/MeOH (20 mL) for 3 h, concentrated under reduced pressure and diluted with H2O (10 mL). The aqueous phase was extracted with EtOAc (4 mL × 10 mL). Pinocembrin (69.8 mg) were obtained after evaporation of the organic phase to dryness, (Fig. 5, compound 5). Optical rotation of the aglycone was measured as +7. This value was compared with the literature (Yuan et al., 2008). The result showed that the structure of compound 2 was having the R-pinocembrin form.

3.10. Bioassays

Three cancer cell lines were used for antiproliferative activities: Vero cells (African green monkey kidney), C6 cells (Rat Brain tumor cells) and HeLa cells (human uterus carcinoma) were kindly provided by Prof. Dr. Nazli Arda and Associate Prof. Ali Karagoz (Department of Molecular Biology, Istanbul University, Turkey). All other chemicals and solvents used in this study were of analytical grade. Cisplatin and 5-FU were used as positive control.

3.10.1. Preparation methanolic extracts of CC for bioassays

CC was into three parts as flowers, steams and roots. Each organ (approximate 50 g) was extracted with methanol (500 mL) three times at room temperature. The extracts were filtered through Whatman No. 2 filter paper, and then concentrated in vacuum until dryness. Stock solutions of C. carduiformis steams, flowers and roots were prepared in DMSO and diluted with Dulbecco’s modified eagle medium (DMEM). DMSO final concentration is below 1% in all tests.

Fig. 4. Extraction and fractionation chart of CCR by solvent extraction.

Fig. 5. Structure of active compounds isolated from Centaurea carduiformis DC. subsp. carduiformis var. carduiformis DC. and R-Pinocembrin.
3.11. Cell lines and cell culture

HeLa, Vero and C6 cancer cell lines were grown in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2% penicillin streptomycin. The medium was changed twice a week.

3.11.1. Cell proliferation assay

Antiproliferative effects of the plants were investigated on HeLa, Vero and C6 cell lines using proliferation BrdU ELISA assay (Demirtas et al., 2009; Demirtas and Sahin, 2013). Cultured cells were grown in 96-well plates (COSTAR, Corning, USA) at a density of 3 × 10^4 cells/well. In each experimental set, cells were plated in triplicates and replicated twice. The cell lines were exposed to two concentrations of methanolic extracts of different organs (flower, stem and root) of CC, for 24 h at 37 °C in a humidified atmosphere of 5% CO₂. 5-Fluorouracil, cisplatin were used as standard compounds. Cells were then incubated for overnight before applying the BrdU Cell Proliferation ELISA assay reagent (Roche, Germany) according to manufacturer’s procedure. The amount of cell proliferation was assessed by determining the A450 nm of the culture media after addition of the substrate solution by using a microplate reader (Ryto, China). Results were reported as percentage of the inhibition of cell proliferation, where the optical density measured from vehicle-treated cells was considered to be 100% of proliferation. All assays were repeated at least twice using HeLa and C6 cells. Percentage of inhibition of cell proliferation was calculated as follows:

\[ \frac{[\text{A} - \text{Asample/AControl}] \times 100}{1} \]

3.12. Statistical analysis

The results of investigation in vitro are means ±SD of nine measurement. Differences between groups were tested by using ANOVA. P values of <0.01 were considered as significant.

Acknowledgments

We thank Gaziosmanpasa University (BAP 2008/12) and State Planning Organization, Turkey (DPT 2003K120510) and TUBITAK (TBAG-109T056) for the financial support and Prof. Dr. Saban Tekin for antiproliferative activity tests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phyto.2014.01.003.

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