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Research Article

Bioactive Volatile Content of the Stem and Root of *Centaurea carduiformis* DC. subsp. *carduiformis* var. *carduiformis*

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Several species of *Centaurea*, biennial (or more rarely perennial) plants which belong to the Asteraceae family, possess medicinal properties and are currently used in phytotherapy. In the present study, antiproliferative activity of hexane extract from stems and roots of *Centaurea carduiformis* DC. subsp. *carduiformis* var. *carduiformis* (CCS and CCR) on Vero cells (African green monkey kidney), C6 cells (Rat Brain tumor cells), and HeLa cells (human uterus carcinoma) was investigated *in vitro*. Antiproliferative effect of the extract was tested at 500 μg/mL and 1000 μg/mL using BrDu Cell Proliferation ELISA. The hexane extract was significantly inhibited proliferation of Vero, HeLa, and C6 cancer cell lines with absorbance values. The extract of CCS and CCR showed the highest activity against the Vero, HeLa, and C6 cell lines at 500 μg/mL and 1000 μg/mL.

1. Introduction

The study of lipids has drawn increasing attention in many fields of research and application due to the existence and functioning of living organisms. In many areas reliable qualitative and quantitative fatty acid analyses are required.

The genus *Centaurea* L. belongs to the Cynareae tribe of the Asteraceae family and comprises approximately 500–600 species of annual, biennial, and perennial grassy plants distributed all around the world, particularly in Asia, North Africa, and America [1, 2]. The taxonomy of the genus is still complex; in Turkey *Centaurea* is the third largest genus after Astragalus and Verbascum and is represented by 190 taxa, of which 117 are endemic (endemism ratio: 61.6%) [3]. The high endemism ratio shows that Turkey is one of the gene centers of the genus. The research in herbal medicine has increased in developing countries as a way to rescue ancient traditions and as an alternative solution to health problems in cities. Therefore, with the increasing acceptance of traditional medicine as an alternative form of health care, the screening of plants for active compounds has become very important.

Many species of the genus *Centaurea* L. have traditionally been used for the treatment of various ailments [4–7], and the genus has also been the subject of many antimicrobial activity studies [8–15]. As a continuation of our research on the volatile components of some *Centaurea* species [15–19] in the present study, we report the fatty acids of the stem and root of endemic *Centaurea carduiformis* (CC) plant distributed in the black sea area. CC is perennial, stem 35–70 cm, profusely branched from near base with spreading branches and purple flowers. CC is an endemic species widespread and frequent locally in the Sivas and Tokat provinces. Previous investigations on various extracts allowed the identification of apigenin, scopoletin, β-amyrin, β-sitosterol, and six aromatic compounds in *Centaurea amanicola* [20] and of nine guaiane-type sesquiterpene lactones and two butyrolactone lignans in *Centaurea ptosimopappa* [21]. To the best of our knowledge, no analyses have been previously reported on antiproliferative activity of *Centaurea carduiformis* DC. subsp. *carduiformis* var. *carduiformis*. In the present work, fatty acids of CC on roots (CCR) and stems (CCS) were obtained and tested for antiproliferative activity for Vero, C6, and HeLa cells.

2. Materials and Methods

2.1. Materials. Three human cell lines were used for cytotoxicity screening: Vero cells (African green monkey kidney), C6

cells (rat brain tumor cells), and HeLa cells (human uterus carcinoma) were kindly provided by Assistant Professor Dr. Nazlı Arda (Department of Molecular Biology, Istanbul University, Turkey). *Centaurea carduiformis* (CCS and CCR) was collected from campus area of Tokat, Turkey during June-July 2008 and was identified by Prof. Dr. H. Necati Çelik from Cumhuriyet University, Faculty of Science and Literature, Department of Biology. A voucher specimen has been deposited (CUFH 8935) at the department of Botany, Faculty of Science and Art, Cumhuriyet University. The stem and root parts of the plant was separated and for the preparation of hexane extracts of the collected plant materials was evaporated at low temperature. All chemicals used were of reagent or higher grade.

2.2. BrdU Cell Proliferation Assay. Cells were plated in 96-well plates (COSTAR, Corning, NY, USA) at a density of 30.000 cells/well, treated with CCS and CCR, and incubated at 37°C with 5% CO_2 overnight for attachment. In each experimental set, cells were plated in triplicates. Cellular proliferations were measured by colorimetric immunoassay based on BrdU incorporation into the cellular DNA by following the instructions recommended by the vendor (Cell Proliferation ELISA, BrdU Kit; Roche Molecular Biochemical, Germany). Briefly, cells were pulsed with BrdU labeling reagent for 4 h followed by fixation in FixDenat solution for 30 min at room temperature. Thereafter, cells were incubated with 1 : 100 dilution of anti-BrdU-POD for 1.30 h at room temperature. Finally, the immune reaction was detected by adding the substrate solution, and the color developed was read at 450 and 650 nm with a microplate ELISA reader (Rayto, RT-2100C, Germany).

2.3. Cell Culture and Cell Antiproliferation Assay

2.3.1. In Vitro Growth Inhibition Test. Vero cells were grown in tissue culture flasks (TPP) with Eagle's minimum essential medium (MEM; Nutricell), supplemented with 10% (v/v) fetal bovine serum. Cells were detached from the flasks with trypsin-EDTA and resuspended to *c.* 10^5 cells mL^{-1} in MEM. To the 96-well, volumes of 1 mL of the cell suspension were distributed, respectively, and after cell monolayer formation, they were used for the cytotoxic characterization analysis assays.

2.4. Statistical Analysis. Numerical data for treatments of cells were subject to analysis of variance (ANOVA). Significance of differences among treatments in the cells was determined using least significant degree [22]. All statistical analysis was performed using MSTATC software (version 2.1, Michigan State University).

2.5. Chromatographic Analysis

2.5.1. Gas Chromatography (GC) Analysis. Oil obtained from aerial parts of TPS was analysed by gas chromatography (GC) on Perkin Elmer Clarus 500 with BPX-20 capillary column (30 m × 0.25 mm, 0.25 μm film thickness) 5% phenyl polysilphenylene-siloxane at an ionization voltage of 70 eV equipped with an FID detector. Oven temperature was programmed from 50 to 120°C at 5 °C/min and from 120°C to 240°C at 10°C/min and hold for 5 min in the final temperature. Diluted samples of 1.0 μL were injected in the 300 : 1 split mode. Injector and detector temperatures were set at 220°C and 290°C, respectively. Helium was used as carrier gas at a flow rate of 1 mL/min and diluted samples (1/1000).

2.5.2. Gas Chromatography/Mass Spectrometry (GC/MS) Analysis. GC/MS analyses were obtained on Perkin Elmer mass spectrometer with built-in-autosampler using BPX-20 column (30 m × 0.25 mm × 0.25 μm film). For GC/MS detection, an electron ionization system, ionization energy of 70 eV, was used. Helium was the carrier gas, at a flow rate of 1.3 mL/min. The column temperate was operated under the same conditions as described above.

2.6. Fatty Acid Separation. The stem and root parts of *C. carduiformis* (1 kg) were extracted 4 times with 80% EtOH, which yielded the ethanolic extract (110 g CCR and 83 g CCS). The ethanolic extract was evaporated and suspended in H_2O and n-hexane to give lipophilic extract as 3.4 g and 2.0 g for CCR and CCS, respectively.

2.7. Identification of Components. The GC/MS chromatograms of volatile content of *C. carduiformis* root (CCS) and stem (CCR) were given in Figures 1(a) and 1(b), respectively.

Identification of the individual components was based on (a) comparison of their GC retention indices (RI) with those of authentic compounds or literature data and (b) computer matching with a mass spectral library and commercial libraries (WILLEY and NIST database/ChemStation data system).

The composition of the fatty acid fractions from stem (CCS) and root (CCR) of *C. carduiformis* is listed in Table 1 (see Figures 1(a) and 1(b)) according to their retention indices on a PBX-20 column. The yield of oils was 8.25% and 11.09%, for CCS and CCR, respectively. Totally, we identified 23 components: 17 in CCS (96.51% of the oil), 14 in CCR (86.69% of the oil). The compounds may be grouped in four main classes as SFAs, UFAs, alcohols, and hydrocarbons.

C. carduiformis has a higher percentage of fatty acids and their esters (2.00 gr/1 kg, 8.25%, and 3.39 g/1 kg, 11.09%, for CCS and CCR, resp.) compared to the others. Oleic acid and linoleic acid are the main polyunsaturated fatty acids in CCS and CCR, respectively. The palmitic acid is the main polysaturated fatty acid for both CCS and CCR.

When the chemical properties of the fatty oils for CCS and CCR are compared to each other, SFA more closely resembles (35.65% for CCS and 37.19% for CCR), while UFA appears somewhat different (51.09% for CCS and 40.64% for CCR), see Table 1 (Figure 1).

Among the fatty acids, UFA-containing compounds were present in a concentration a little higher in comparison to those of SFA (lipids), and in all analyzed oils, palmitic acid, linoleic acid, and oleic acid were present in appreciable amounts (20.82–28.30%). The oil of CCS was characterized by

Table 1: Volatile constituents of the stem and root of *C. carduiformis* DC. subsp. *carduiformis* var. *carduiformis* (CCS and CCR).

RI	RT	Component	CCS	CCR
		Polysaturated fatty acids (SFAs)		
1174	14.109	C_{12}: 0, lauric acid	0.14	
1285	18.947	C_{14}: 0, myristic acid	1.03	
1478	25.995	C_{20}: 0, arachidic acid ethyl ester		0.23
1492	27.798	C_{16}: 0, palmitic acid	26.74	20.82
1679	30.406	C_{16}: 0, palmitic acid ethyl ester	0.78	9.64
1693	34.520	C_{18}: 0, stearic acid	5.82	6.50
1790	36.173	C_{11}: 0, undecanoic acid	0.14	
1882	39.104	C_{20}: 0, arachidic acid	1.00	
		Total	**35.65**	**37.19**
		Polyunsaturated fatty acids (UFAs)		
1376	21.970	C_{12}: 1,9-dodecenoic acid, (E)-	0.15	
1483	26.614	C_{16}: 1, palmitoleic acid	2.03	
1682	33.740	C_{18}: 2, linoleic acid	20.61	26.57
1683	33.88	C_{18}: 3, linolenic acid		13.53
1685	33.961	C_{18}: 1, Oleic acid	28.30	0.54
		Total	**51.09**	**40.64**
		Total fatty acids	**86.74**	**77.83**
		Alcohols and hydrocarbons		
1267	17.411	9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-		4.02
1358	23.482	Hexahydrofarnesyl acetone	0.55	0.77
1478	25.364	Phytol	7.76	2.42
1681	33.343	1-Hexadecanol	0.11	
1790	38.472	Eicosane	0.52	2.63
1816	40.462	Farnesane	0.11	
1982	43.74	Tetratriacontane		0.86
	47.836	Cembrane		0.22
	48.703	11-n-Decyldocosane		0.94
	48.731	Tetratetracontane	0.72	
		Total	**9.77**	**11.86**
		General total	**96.51**	**86.69**

the highest contents of oleic acid (28.30%) and palmitic acid (26.74%) while in CCR linoleic acid (26.57%) and palmitic acid (20.82%) represented the most abundant fatty acids. Although we did not find any high concentration of omega-3 fatty acid family in CCS (only 0.15% of (E)-9-Dodecenoic acid, C_{12}:1), the CCR sample show higher omega-3 fatty acid (13.53% of linolenic acid, C_{18}:3; Table 1).

3. Results and Discussion

3.1. The Effect of Fatty Acids and Hydrocarbons on Cancer Cell Lines. In the present study to determine whether the reduction in cell number mediated by UFAs and SFAs was a result of a cytotoxic effect from fatty acids, we monitored the viability of cells during the course of incubation. After 48 hours of incubation with fatty acids and hydrocarbons, the viability of cancer cells was less than 25%. Thus the growth arrest observed in C6, Vero, and HeLa cells during the incubation with UFAs, SFAs, and hydrocarbons isolated from CCS and CCR was either lipids, or alcohols and hydrocarbons, due to the effect of toxicity.

3.2. Effect of CCS and CCR on Proliferation of Vero Cells. We tested the antiproliferative activity of CCS and CCR against Vero cells at 1000 and 500 μg/mL for 48 hrs. As shown in Figure 2, both CCS and CCR significantly inhibited the proliferation of Vero cells during incubation for 48 h.

CCS inhibited the proliferation of Vero cells in a dose-dependent manner. After incubation, the absorbance of Vero cells was approximately 0.24 at 1000 μg/mL, and 0.38 at 500 μg/mL for CCS. The CCR almost completely suppressed the proliferation of Vero cells at two concentrations of 1000 and 500 μg/mL as 0.22 and 0.21 μg/mL, respectively (Table 2).

When C6, Vero, and HeLa cells were treated with CCS or CCR in combination with DMSO, it was found that CCS and CCR increased the antiproliferative effect compared to treatment with DMSO alone (Table 2).

3.3. Effect of CCS and CCR on Proliferation of C6 Cells. We tested the antiproliferative activity of CCS and CCR against C6 cells at 1000 and 500 μg/mL for 48 h. As shown in Figure 3, CCS significantly inhibited the proliferation of C6 cells in a dose-dependent manner during incubation for 48 h.

Table 2: The antiproliferative effect of hexane extract of *C. carduiformis* in C6, Vero, and HeLa cells*.

	C6		Vero		HeLa	
	1000 μg/mL	500 μg/mL	1000 μg/mL	500 μg/mL	1000 μg/mL	500 μg/mL
Control	2.01 ± 0.34^{a}	2.18 ± 0.21^{a}	2.06 ± 0.25^{a}	2.28 ± 0.25^{a}	1.58 ± 0.41^{a}	1.51 ± 0.52^{a}
DMSO	1.41 ± 0.49^{b}	1.75 ± 0.35^{a}	1.06 ± 0.16^{b}	2.06 ± 0.13^{a}	0.74 ± 0.47^{b}	1.05 ± 0.43^{a}
CCS	0.19 ± 0.06^{c}	0.51 ± 0.25^{b}	0.24 ± 0.07^{c}	0.38 ± 0.03^{b}	0.20 ± 0.05^{b}	0.28 ± 0.02^{b}
CCR	0.21 ± 0.07^{c}	0.21 ± 0.05^{b}	0.22 ± 0.02^{c}	0.21 ± 0.04^{b}	0.17 ± 0.08^{b}	0.17 ± 0.05^{b}

*Cells were treated with CCS and CCR (1000–500 μg/mL) in combination with BrdU for 48 hrs, $P < 0.05$.

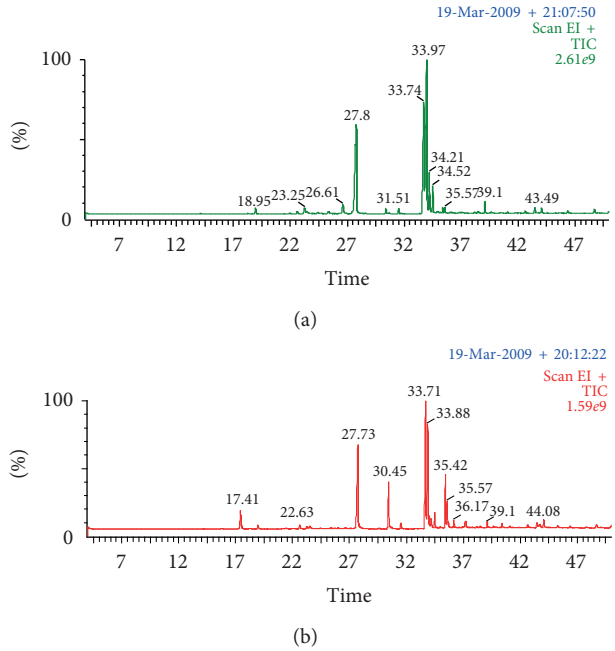

Figure 1: The chromatograms of CCS (a) and CCR (b).

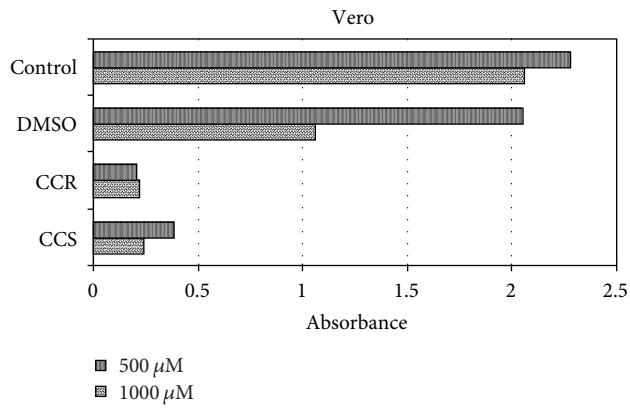

Figure 2: Antiproliferative effect of CCS and CCR on the Vero cells.

Although CCR significantly inhibited the proliferation of C6 cells, it is not in dose-dependent manner. After incubation, the absorbance of CCS was approximately 0.19 at 1000 μg/mL and 0.51 at 500 μg/mL (Table 2). The absorbance of CCR was 0.21 μg/mL for the concentrations of 1000 and 500 μg/mL.

3.4. Effect of CCS and CCR on Proliferation of HeLa Cells. The antiproliferative activity of CCS and CCR was tested on HeLa

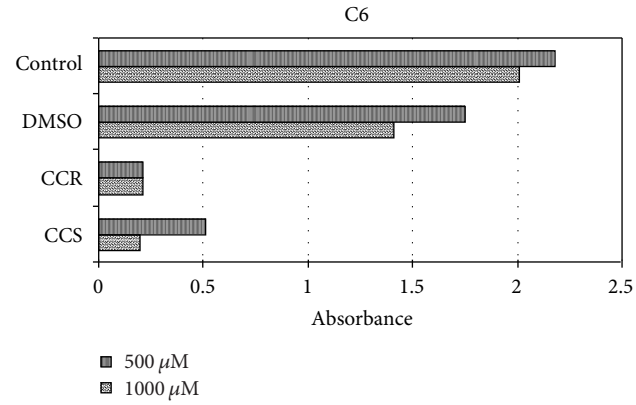

Figure 3: Antiproliferative effect of CCS and CCR to the C6 cells.

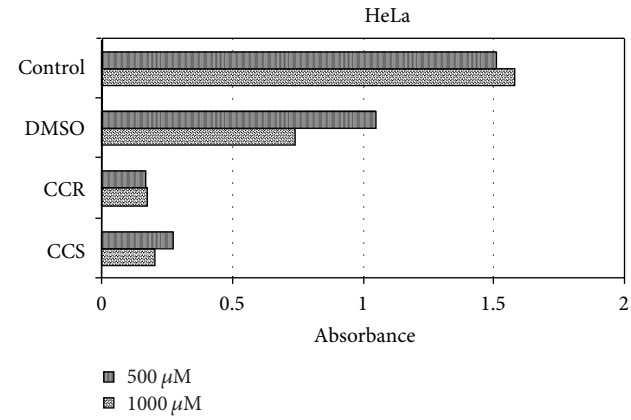

Figure 4: Antiproliferative effect of CCS and CCR on the HeLa cells.

cells at 1000 and 500 μg/mL for 48 h. As shown in Figure 4, CCR significantly inhibited the proliferation of HeLa cells as 0.17 at 1000 μg/mL and 500 μg/mL and CCS inhibited the proliferation as 0.20 at 1000 and 0.28 at 500 μg/mL in a dose-dependent manner (Table 2). CCR extract almost completely suppressed the proliferation of HeLa cells at concentrations of 1000 and 500 μg/mL.

4. Discussion

Many polyphenols and flavonoids have been shown to inhibit proliferation and angiogenesis of tumor cells *in vitro* [16] and inhibit carcinogenesis and tumorigenesis in animal experiments [17, 18]. Flavonoids and the related compounds are found to be widely distributed in *Sideritis libanotica* in

our ongoing study [19]. However, the lipophilic extracts prepared withs n-hexane were not analysed in terms of their antiproliferative activities. The lipophilic extracts of CCS and CCR were analysed by capillary gas chromatography-mass spectrometry (GC-MS), and their fatty acid compositions were characterized.

The results of the study demonstrated that the fatty acids inhibited the proliferation of C6, Vero, and HeLa cells. However, no reports or information are available on the effects of UFAs, SFAs, alcohols, and hydrocarbons

The antiproliferative activity of the stem and root of *C. carduiformis* was tested in terms of capacity to inhibit proliferation of cells at different concentrations. The crude extract of the apolar solvent fraction (n-hexane) showed significant antiproliferative activity against Vero, C6, and HeLa cells especially in lower doses as shown in Figures 2, 3, and 4. The hexane-soluble portions of the plant (CCS and CCR) were antiproliferative; however, the CCS extract showed significant dose-dependent antiproliferative activity against all tumour cell lines but not for CCR. The CCR was the most effective on HeLa cells than the others (Table 1).

Cell proliferation involves complex combinations of many biochemical processes, and different chemicals might influence different biochemical processes or stages in different manners. The apolar omega-3 and omega-6 such as linolenic acid and linoleic acids might inhibit cell growth by mechanisms different from those of the polysaturated fatty acids.

The results suggest strong antiproliferative properties and support the ethnomedical claims for the *C. carduiformis* plant. *In vivo* studies are needed to confirm the pharmacological efficacy and safety of *C. carduiformis*.

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