Med Chem Res (2013) 22:2946–2953
DOI 10.1007/s00044-012-0297-2

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Antiproliferative activity of pentadeca-(8E, 13Z) dien-11-yn-2-one and (E)-1,8-pentadecadiene from *Echinacea pallida* (Nutt.) Nutt. roots

Ayse Sahin Yaglıoglu · Bayram Akdulum · Ramazan Erenler · Ibrahim Demirtas · Isa Telci · Saban Tekin

Received: 9 July 2012 / Accepted: 20 October 2012 / Published online: 4 November 2012
© Springer Science+Business Media New York 2012

Abstract Several species of *Echinacea*, a perennial plant which belongs to the Asteraceae family, possess medicinal properties and are currently used in phytotherapy. In the present study, antiproliferative activity of methanol extract and isolated structures of pentadeca-(8E, 13Z)-dien-11-yn-2-one **1** and (E)-1,8-pentadecadiene **2** from *Echinacea pallida* roots on C6 cells (Rat Brain tumor cells) and HeLa cells (human uterus carcinoma) was investigated in vitro. Antiproliferative effect of the extract, isolated compounds, and cisplatin were tested at 5, 10, 20, 30, 40, 50, 75, and 100 μg ml^{-1} using BrdU Cell Proliferation ELISA. The methanol extract and Compound **1** significantly inhibited proliferation of HeLa and C6 cancer cell lines.

A. S. Yaglıoglu (✉) · I. Demirtas (✉)
Department of Chemistry, Faculty of Science, Cankiri Karatekin University, 18100 Cankiri, Turkey
e-mail: aysesahin1@gmail.com

I. Demirtas
e-mail: ibdemirtas@gmail.com

B. Akdulum · R. Erenler
Laboratory of Plant Research, Faculty of Science and Art, Gaziosmanpasa University, Taşlıçiftlik, 60240 Tokat, Turkey

I. Telci
Department of Field Crops, Agricultural Faculty of Gaziosmanpasa University, Taşlıçiftlik, 60240 Tokat, Turkey

S. Tekin
Department of Molecular Biology and Genetics, Faculty of Science and Art, Gaziosmanpaşa University, Taşlıçiftlik, 60240 Tokat, Turkey

Keywords *Echinacea pallida* · Antiproliferative activity · C6 cancer cell line · HeLa cancer cell line · Isolation

Introduction

Echinacea is a perennial plant belonging to the Asteraceae family. Three out of nine species, *Echinacea purpurea* (L.) Moench, *E. angustifolia* DC., and *E. pallida* (Nutt.) Nutt. (EP), of the genus are currently used in therapy for their medicinal properties (McGregor, 1968). *Echinacea* has a long history of medicinal use for the treatment of the common cold and respiratory infections (Kindscher, 1989; Li, 1998; Chen *et al.*, 2005; Barnes *et al.,* 2005; Barrett, 2003). A variety of chemical compounds have been isolated and identified from the *Echinacea* genus, including caffeic acid derivatives (Cheminat *et al.,* 1988; Pellati *et al.,* 2004; Pellati *et al.,* 2005), alkamides, acetylenes (polyacetylenes and polyenes) (Bauer *et al.,* 1988a, b; Bauer and Remiger, 1989; Pellati *et al.,* 2007), polysaccharides (Wagner *et al.,* 1988), and glycoproteins (Classen *et al.,* 2000; Thude and Classen, 2005), all of which exhibit diverse pharmacological activities. The in vitro cytotoxic and pro-apoptotic activities of the hexane root extracts from the three medicinally important *Echinacea* species, *E. pallida, E. purpurea*, and *E. angustifolia,* were reported (Bauer *et al.,* 1988a, b; Bauer and Remiger, 1989; Pellati *et al.,* 2007).

Although cytotoxic effects of *E. pallida* root extracted with hexane on several tumor cell lines (the human pancreatic cancer, PaCa-2 and colon cancer, COLO320 cell lines) were observed (Chicca *et al.,* 2007), the antiproliferative effect of *E. pallida* root methanolic extracts (crude extract) and isolated active compounds against HeLa and

C6 were not studied so far. In the present study, the antiproliferative activity of *E. pallida* root methanolic extract and isolated active compounds against HeLa and C6 cells were investigated.

Materials and methods

Collection of *E. pallida*

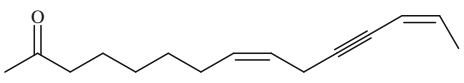
The plant was collected from Tokat province, identified by Dr. Oya Kacar (Uludag University), and cultivated in medicinal plants garden of Field Crops Department in Gaziosmanpasa University, during 2007–2009 vegetation periods. The roots were harvested from cultured *E. pallida* and dried at room condition (at 25 °C) and used for the extraction. All chemicals used were of reagent or higher grade.

Extraction and isolation of compounds

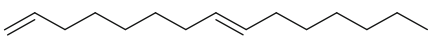
The roots were cut into small pieces and extracted successively with methanol (2.5 L) for 3 times at room temperature. The extracts were filtered through Whatman No: 2 filter paper and vacuum dried. The extract was subjected to silica gel column chromatography, affording 187 fractions. Each fraction was analyzed by TLC and GC–MS, and combined into 20 fractions according to their chromatographic profile. From these fractions, Compounds **1** and **2** (Fig. 1) were isolated by silica gel mesh 60 (0.063–0.200 mm, Merck) column chromatography and preparative TLC. The chemical structure of the compounds was determined on the basis of NMR and MS spectroscopic data.

Spectral analysis

Official methods were used to determine volatile part of the column chromatography by GC–MS on a Perkin Elmer Clarus 500 equipped with BPX 20 capillary column (0.25 μm ID 30 m × 250 μm), filled with 5 % Phenyl polysilphenylene-siloxane, at an ionization voltage of 70 eV. Helium was the carrier gas (1 ml min^{-1}). The injector and detector temperatures were kept at 100 °C for 5 min and then gradually increased to 250 °C at a 5 °C/min rate, and held for 15 min. Diluted samples (1/100, v/v, in *n*-pentane) of 1.0 μl were injected. The hydrocarbons were identified using the molecular formula and fragmentations. The high-resolution NMR spectra (^{1}H and ^{13}C) were run on a Brucker Avence III spectrometer (400 MHz) and *J* values are given in Hz.



1



2

Fig. 1 Structures of pentadeca-(9E, 13Z)-dien-11-yn-2-one (**1**) and (E)-1,8-pentadecadiene (**2**) isolated from *E. pallida* roots

Cell culture and cell proliferation assay

HeLa and C6 cells were cultured in Dulbecco's modified eagle's medium (DMEM, Sigma), supplemented with 10 % (v/v) fetal bovine serum (Sigma, Germany) and PenStrep solution (Sigma, Germany). Cultured cells were detached from the flasks with trypsin–EDTA (Sigma, Germany) at confluency, centrifuged, and pellet resuspended to 3 × 10^5 cells ml^{-1} in DMEM. Cells were plated in 96-well plates (COSTAR, Corning, USA) at a density of 30,000 cells/well and incubated at 37 °C with 5 % CO_2 overnight for attachment. All materials including test and controls were dissolved in sterile DMSO. In each experimental set, cells were plated in triplicates and the experiment was repeated three times ($n = 3$). The cells were treated with crude extract and compounds were isolated at final concentrations of 5, 10, 20, 30, 40, 50, 75, and 100 μg ml^{-1}. Controls, vehicle controls, and positive control wells were treated with culture medium, sterile DMSO, and cisplatin, respectively. Treated cells were incubated at 37 °C with 5 % CO_2 for 24 h.

Cell proliferation was measured using BrdU Cell Proliferation ELISA (Roche, Germany), a colorimetric immunoassay based on BrdU incorporation into the cellular DNA according to manufacturer's procedure. 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 nm with a microplate reader.

Statistical analysis

The results of investigation in vitro are mean ± SD of three separate experiments. Differences between groups were tested by analysis of variance (ANOVA). *P* values of less than 0.05 were considered statistically significant. The mean data which are significant in variance analysis were

grouped with Duncan's multiple range tests (Gomez and Gomez, 1984). All statistical analysis was performed using SPSS (Version 13.5).

Results

Isolation and characterization of active compounds from *E. pallida* root extract

In the present study, the fractionation of the crude methanol extract of *E. pallida* roots by a series of silica gel column chromatographic steps and preparative TLC resulted in the isolation of two bioactive compounds, Compound **1** and **2** (Fig. 1), which are known compounds. Compound **1** has been reported before (Pellati *et al.,* 2006) and Compound **2** has been reported by Voaden and Jacobson (1972) but in the literature its spectral data are missing. The structure of Compound **1** was confirmed by comparison with the literature data (Pellati *et al.,* 2006). The NMR data of the molecules (Table 1) were in perfect agreement with the structures. The compounds isolated were identified from their spectroscopic data, such as GC–MS, NMR, including (^{1}H and ^{13}C) and 2D (COSY, DEPT, TOCSY) techniques. According to ^{1}H-NMR spectra of Compound **1** (Fig. 2a), methyl protons which connected with carbonyl groups were showed as singlet signals at 2.15 ppm. At 2.44 ppm, triplet signals belong to methylene protons which connected with C-3. H-13 protons were determined as doublet signals at 5.47 ppm and the protons interacted with H-14 protons as cis ($J_{13\text{-}14}$ = 10.6 Hz). In DEPT-90, four CH groups which belonged to C-8, C-9, C-13, and C-14 carbons were determined at 131.4, 124.5, 110.3, and 137.2 ppm, respectively (Fig. 2b). The DEPT-45 spectra of Compound **1** supported the presence of CH, CH_2, and CH_3 groups (Fig. 2b). However, at APT spectra of Compound **1** CH_3 groups were exhibited as negative peaks (Fig. 2b). In ^{13}C-NMR spectra of the compounds (Fig. 2c), the signals which belonged to carbonyl groups were obtained at 209.2 ppm. The peak carbons of C-8 and C-9 were observed at 131.4 and 124.5 ppm, respectively. The peaks which belonged to C-12 and C-11 (at 76.7, 92.9 ppm, respectively) concern with the triple bond carbons. In COSY spectra, the corelations of H-13 with H-14 were determined (at 5.47 and 5.91 ppm, respectively) (Fig. 2d). In addition to TOCSY spectra of the compounds, the correlations of H-6 with H-7 (1.40 and 2.09 ppm, respectively) were determined (at 5.47 and 5.91 ppm, respectively) (Fig. 2e).

According to APT, DEPT135, and DEPT90 spectra of Compound **2** (Fig. 3a), four CH groups which belonged to C-1, C,2, C-8, and C-9 carbons were determined at 114.2, 139.1, 129.9, and 129.7 ppm, respectively (Fig. 3a). In APT spectra Compound **2** (Fig. 3a), CH_3 groups (C-15) as positive peak were determined at 15.7 ppm. In addition to the APT spectra, ten CH_2 groups which belonged to C-3, C-4, C-5, C-6, C-7, C-10, C-11, C-12, C-13, and C-14 carbons were determined at 114.2, 139.1, 129.9, and 129.7 ppm as negative peak (Fig. 3a). H-8 and H-9 protons at 5.40 ppm were determined to interact with C-8 and C-9 (129.9 and 129.7 ppm, respectively) in HETCOR spectra (Fig. 3b). C-1 carbons (at 114.2 ppm) with H-1 protons (at 5.04 ppm) and C-15 carbons (at 14.1 ppm) with H-15 protons (at 0.95 ppm) were shown to interacted in HETCOR spectra (Fig. 3b). According to ^{1}H-NMR spectra of Compound **2** (Fig. 3c), methyl protons were showed as triplet at 0.95 ppm. H-1 [15.05 and 4.97 (*dd*) ppm] and H-2 [5.85 (*ddt*)] protons were determined in olefinic region (Fig. 3c).

Table 1 ^{1}H and ^{13}C NMR spectral data of compounds **1** and **2** (400 MHz, $CDCl_3$)

Position	Compound **1**		Compound **2**	
	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)
1	29.9	2.15 *s*	114.2	5.04 *dd* (17.1, 1.7) 4.97 *dd* (10.2, 1.7)
2	209.2	–	139.1	5.85 *ddt* (17.1, 10.2, 6.7)
3	43.7	2.44 *t* (7.4)	33.8	2.10 *m*
4	23.7	1.60 *m*	28.9	1.40 *m*
5	28.8	1.32 *m*	29.0	1.40 *m*
6	29.1	1.40 *m*	29.7	1.40 *m*
7	26.9	2.09 *m*	29.8	2.10 *m*
8	131.4	5.47 *m*	129.9	5.40 *m*
9	124.5	5.47 *m*	129.7	5.40 *m*
10	17.96	3.12 *d* (3.2)	28.8	2.10 *m*
11	92.9	–	27.2	1.40 *m*
12	76.7	–	27.3	1.40 *m*
13	110.3	5.47 *d* (10.6)	31.8	1.40 *m*
14	137.2	5.91 *q* (10.6, 6.6)	22.7	1.40 *m*
15	15.7	1.87 *d* (6.6)	14.1	0.95 *t* (7.0)

Antiproliferative activities of crude extract and Compounds **1** and **2** against C6 and HeLa cells

For the first time, antiproliferative activity of crude extract and Compounds **1** and **2** isolated through bioassay-guided fractionation from *E. pallida* roots were tested on C6 and HeLa cells at 5, 10, 20, 30, 40, 50, 75, and 100 μg ml^{-1} for 24 h. As shown in Fig. 4,

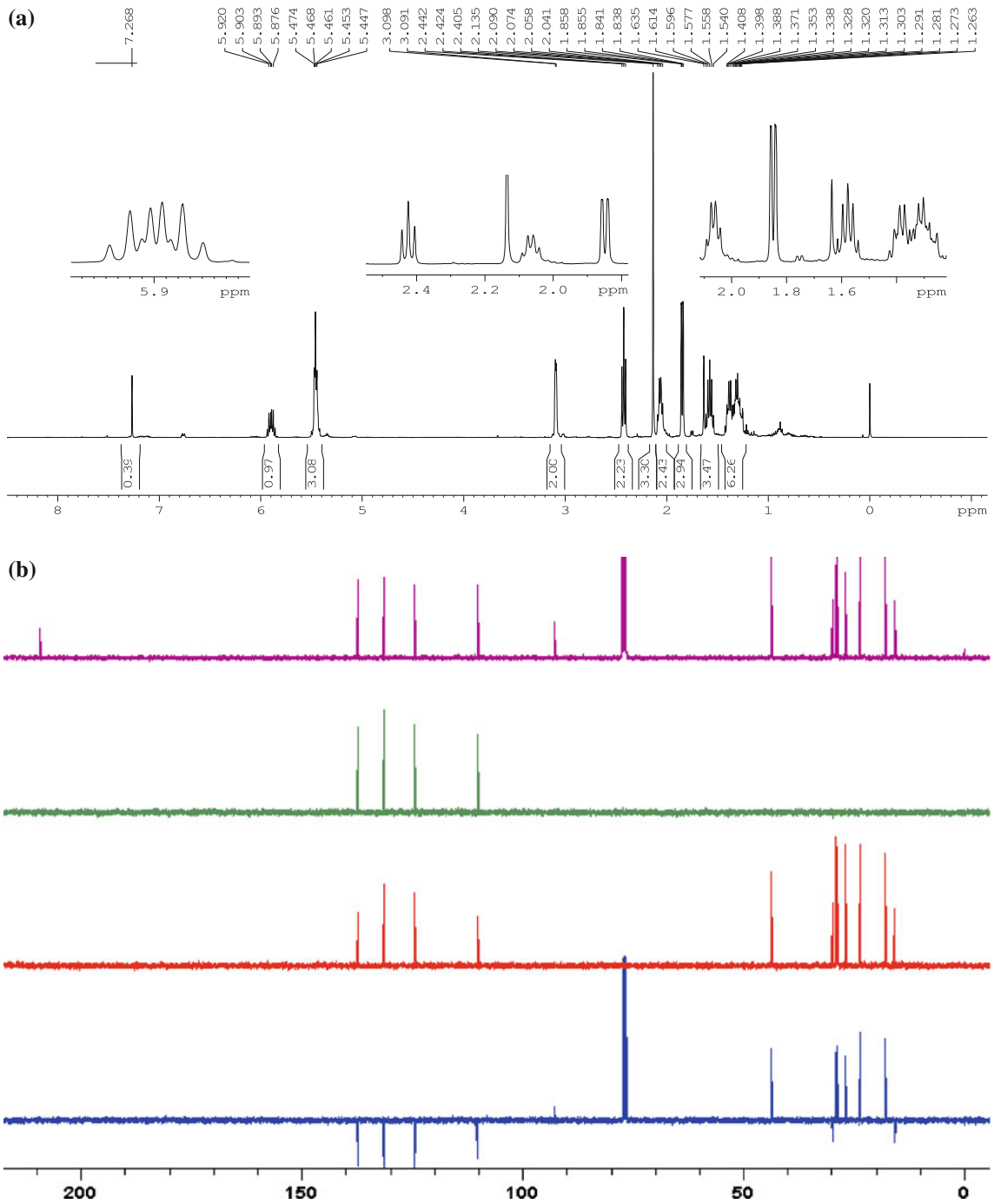


Fig. 2 **a** ^{1}H-NMR spectra for pentadeca-(8E,13Z)-dien-11-in-2-on, **b** ^{13}C, DEPT90, DEPT45, and APT spectra for pentadeca-(8E,13Z)-dien-11-in-2-on, **c** ^{13}C-NMR spectra for pentadeca-(8E,13Z)-dien-11-in-2-on, **d** cosy spectra for pentadeca-(8E,13Z)-dien-11-in-2-on, **e** Tcosy spectra for pentadeca-(8E,13Z)-dien-11-in-2-on

E. pallida extract and isolated Compound **1** significantly ($p < 0.01$) inhibited the proliferation of C6 cells compared to anticancer agent, Cisplatin. The antiproliferative activities of crude extract and Compound **1** at concentrations of 75–5 μg ml^{-1} were high. In addition, crude extract and Compound **1** showed a significant antiproliferative activity against HeLa cells at concentrations of 5, 10, 20, 30, and 40 μg ml^{-1} only (Fig. 5). As observed on C6 cells, the crude extract and Compound **1** were more effective at low concentrations against HeLa cells. However, Compound **2** had no antiproliferative activity against both cell lines at all concentrations tested.

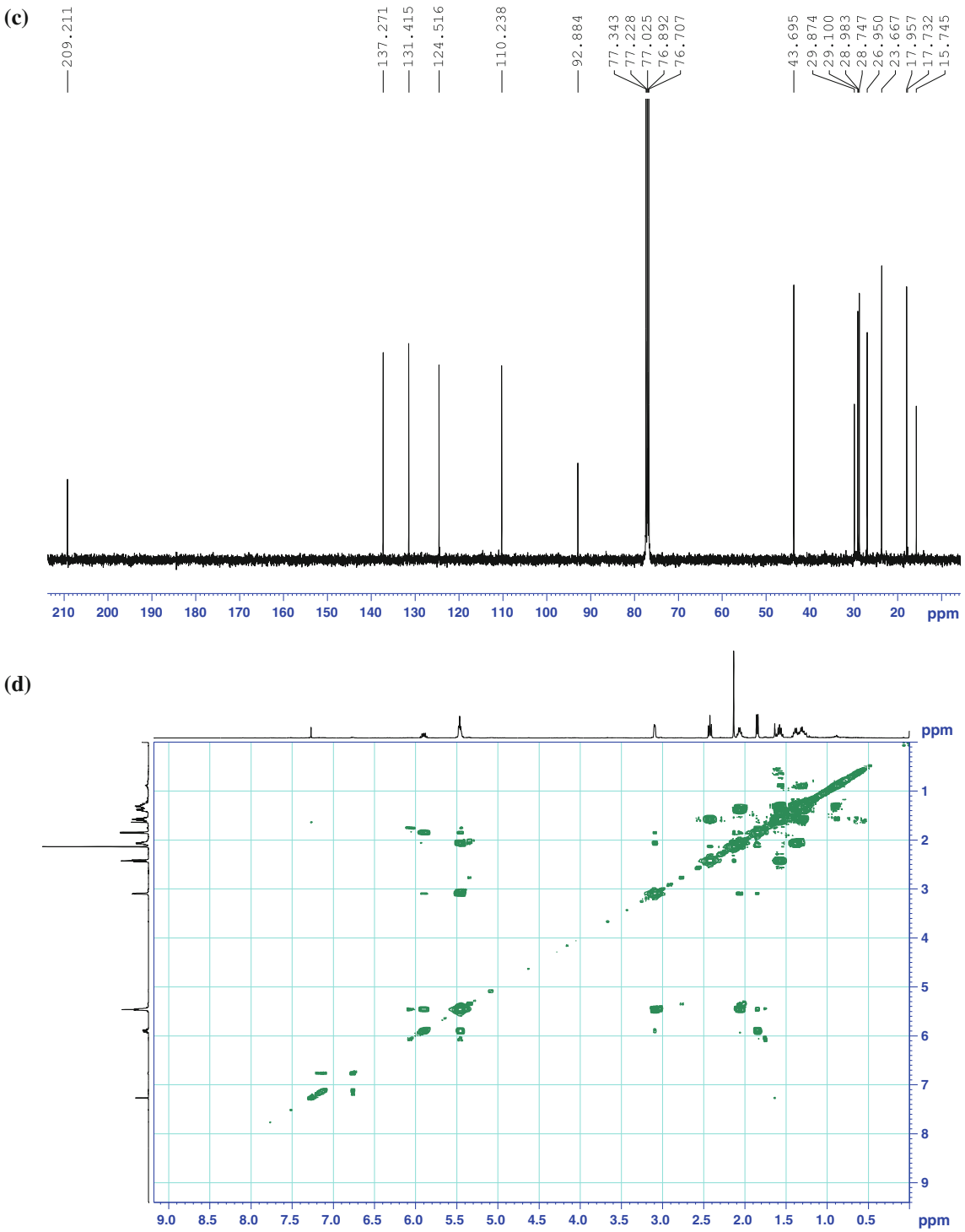


Fig. 2 continued

Discussion

Echinacea has a long history of medicinal use for the treatment of the various diseases (Kindscher, 1989; Li, 1998; Chen *et al.,* 2005; Barnes *et al.,* 2005; Barrett, 2003). A numerous of compounds have been isolated and identified from the *Echinacea* genus, including caffeic acid derivatives (Cheminat *et al.,* 1988; Pellati *et al.,* 2004; Pellati *et al.,* 2005), alkamides, acetylenes (polyacetylenes and polyenes) (Bauer *et al.,* 1988a, b; Bauer and Remiger, 1989; Pellati *et al.,* 2007), polysaccharides (Wagner *et al.,* 1988), and glycoproteins (Classen *et al.,* 2000; Thude and

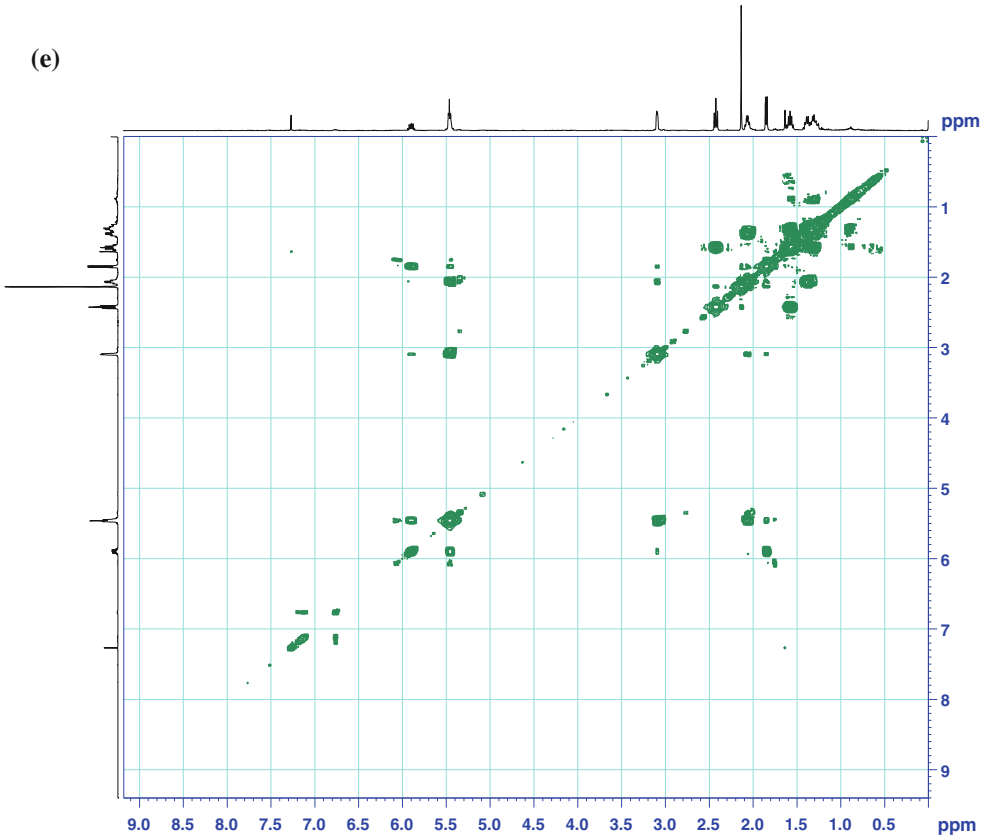


Fig. 2 continued

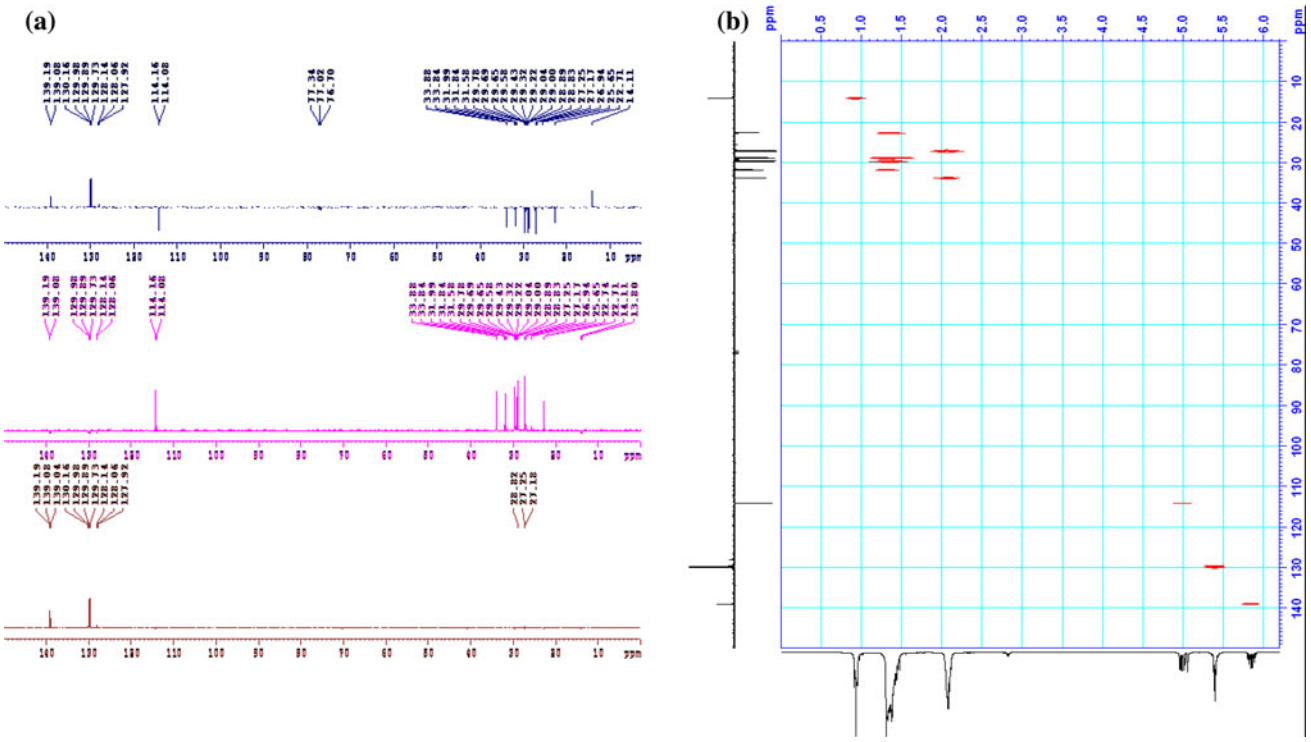


Fig. 3 **a** APT, DEPT135, and DEPT90 spectra for E-1.8-pentadecadien, **b** HETCOR spectra for E-1.8-pentadecadien, **c** ^{1}H-NMR spectra for E-1.8-pentadecadien, **d** Hetcor spectra for E-1.8-pentadecadien

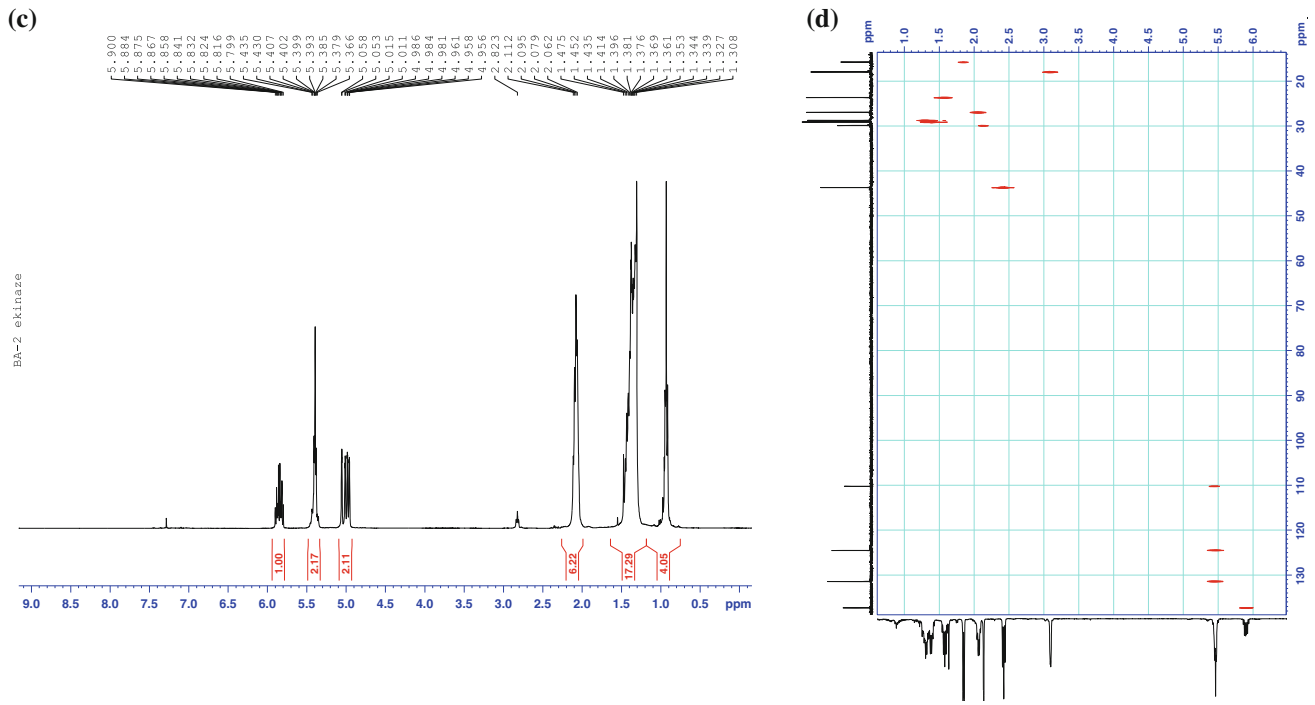


Fig. 3 continued

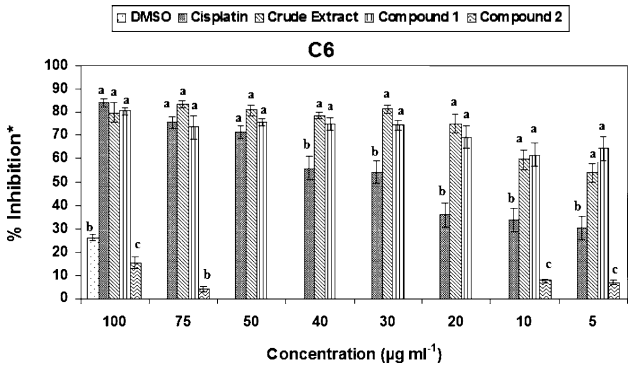


Fig. 4 Antiproliferative activities of *E. pallida* crude extract and isolated compounds against C6 cells, $*p < 0.01$

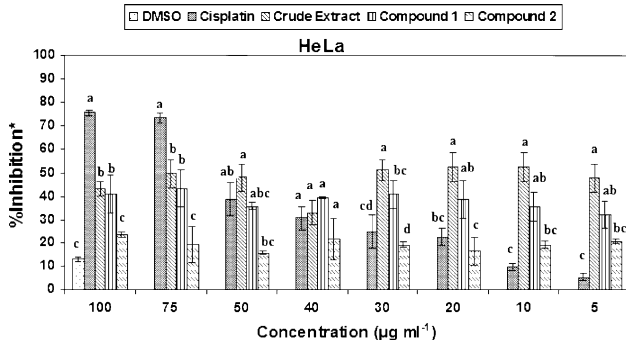


Fig. 5 Antiproliferative activities of *E. pallida* crude extract and isolated compounds against HeLa cells, $*p < 0.01$

Classen, 2005), all of which exhibit diverse pharmacological activities. The previously isolated Compound 1 was obtained for the first time from the *E. pallida* roots in this study. Beside the contribution to literature, it may also be used medicinally like the previously described compounds isolated from *E. pallida*.

Species from *Echinacea* genus are currently used in therapy for their medicinal properties (McGregor, 1968). In the present study, antiproliferative activity of crude extract of *E. pallida* and two isolated compounds, Compound **1** and **2** were investigated. Results showed that at least, crude extract and Compound **1** both have antiproliferative potential against C6 cells at concentrations of 75-5 μg ml^{-1} and HeLa cells at concentrations of 50-5 μg ml^{-1}. These findings were similar to previous reports such as hexane root extracts from three medicinally important *Echinacea* species, *E. pallida, E. purpurea*, and *E. angustifolia*, which showed cytotoxic and pro-apoptotic activities (Bauer *et al.,* 1988a, b; Bauer and Remiger, 1989; Pellati *et al.,* 2007). The crude extract and compound 1 were more antiproliferative against C6 cells than HeLa cells, indicating a cell specific activity.

The antiproliferative activity of the crude extract was generally slightly higher than isolated Compound **1**, indicating contribution from isolated Compound **1** and unknown compounds in the extract. Compound **1** was potent inhibitor of proliferation which is related to its chemical structure. As Compound **1**, many polyphenols

and flavonoids have been reported to inhibit proliferation and angiogenesis of tumor cells in vitro (Fotsis *et al*., 1997; Demirtas *et al.,* 2009; Demirtas and Sahin 2013) and inhibit carcinogenesis and tumorigenesis in animal experiments (Hertog *et al.,* 1993; Elangovan *et al.,* 1994). Since both crude extract and Compound **1** have antiproliferative potential, it is suggested that they are likely to show in vivo activity.

In conclusion, our results demonstrated the antiproliferative effect of isolated bioactive compound from *E. pallida* root extract against different cancer cell lines. The crude extract itself and Compound **1** showed potent antiproliferative activity. The results suggest an anticancer property and support the ethnomedical claims for the *E. pallida*. In vivo studies are needed to confirm the pharmacological efficacy and safety of *Echinacea pallida* extract and active compound, Compound **1**.

Acknowledgments The authors wish to thank Gaziosmanpasa University (BAP 2009-18) for financial support and Dr. Oya Kacar (Uludag University) for identifying the plant used in this study.

References

Barnes J, Anderson LA, Gibbons S, Phillipson JD (2005) *Echinacea* species (*Echinacea angustifolia* (DC.) Hell., *Echinacea pallida* (Nutt.) Nutt., *Echinacea purpurea* (L.) Moench): a review of their chemistry, pharmacology and clinical properties. JPP 57:929–954

Barrett B (2003) Medicinal properties of *Echinacea*: a critical review. Phytomedicine 10:66–86

Bauer R, Remiger P (1989) TLC and HPLC analysis of alkamides in *Echinacea* drugs. Planta Med 55:367–371

Bauer R, Khan IA, Wagner H (1988a) TLC and HPLC analysis of *Echinacea pallida* and *E. angustifolia* roots. Planta Med 54:426–430

Bauer R, Remiger P, Wagner H (1988b) Alkamides from the roots of *Echinacea purpurea*. Phytochemistry 27:2339–2342

Cheminat A, Zawatzky R, Becker H, Brouillard R (1988) Caffeoyl conjugates from *Echinacea* species: structure and biological activity. Phytochemistry 27:2787–2794

Chen Y, Fu T, Tao T, Yang J, Chang Y, Wang M, Kim L, Qu L, Cassady J, Scalzo R, Wang X (2005) Macrophage activating effects of new alkamides from the roots of *Echinacea* species. J Nat Prod 68(5):773–776

Chicca A, Adinolfi B, Martinotti E, Fogli S, Breschi MC, Pellati F (2007) Cytotoxic effects of *Echinacea* root hexanic extracts on human cancer cell lines. J Ethnopharmacol 110:148–153

Classen B, Witthohn K, Blaschek W (2000) Characterization of an arabinogalactan-protein isolated from pressed juice of *Echinacea purpurea* by precipitation with the β-glucosyl Yariv reagent. Carbohydr Res 327:497–504

Demirtas I, Sahin A (2013) Bioactive volatile content of the stem and root *Centaurea carduiformis* DC. subsp carduiformis var. carduiformis. J Chem. doi:10.1155/2013/125286

Demirtas I, Sahin A, Ayhan B, Tekin S, Telci I (2009) Antiproliferative effects of the methanolic extracts of sideritis. Rec Nat Prod 3(2):104–109

Elangovan V, Sekar N, Govindasamy S (1994) Chemopreventive potential of dietary bioflavonoids against 20-methylcholanthrene-induced tumorigenesis. Cancer Lett 87(1):107–113

Fotsis T, Pepper MS, Aktas E, Breit S, Rasku S, Adlercreutz H, Wahala K, Montesano R, Schweigerer L (1997) Flavonoids, dietary-derived inhibitors of cell proliferation and in vitro angiogenesis. Cancer Res 57(14):2916–2921

Gomez KA, Gomez AA (1984) Statistical procedures for agricultural research. Wiley, New York 680

Hertog MG, Hollman PC, Katan MB, Kromhout D (1993) Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. Nutr Cancer 20:21–29

Kindscher K (1989) Ethnobotany of purple coneflower (*Echinacea angustifolia*, Asteraceae) and other *Echinacea* Species. Econ Bot 43:498–507

Li TSC (1998) *Echinacea*: cultivation and medicinal value. Hort-technology 8:122–129

McGregor R (1968) The taxonomy of the genus *Echinacea* (Compositae). Univ Kans Sci Bull 48:113–142

Pellati F, Benvenuti S, Magro L, Melegari M, Soragni F (2004) Analysis of phenolic compounds and radical. J Pharm Biomed Anal 35(2):289–301

Pellati F, Benvenuti S, Melegari M, Lasseigne T (2005) Variability in the composition of anti-oxidant compounds in *Echinacea* species by HPLC. Phytochem Anal 16(2):77–85

Pellati F, Calo S, Benvenuti S, Adinolfi B, Nieri P, Melegari M (2006) Isolation and structure elucidation of cytotoxic polyacetylenes and polyenes from *Echinacea pallida*. Phytochemistry 67:1359–1364

Pellati F, Calò S, Benvenuti S (2007) High-performance liquid chromatography analysis of polyacetylenes and polyenes in *Echinacea pallida* by using a monolithic reversed-phase silica column. J Chromatogr A 114:56–65

Thude S, Classen B (2005) High molecular weight constituents from roots of *Echinacea pallida*: an arabinogalactan-protein and an arabinan. Phytochemistry 66:1026–1032

Voaden DJ, Jacobson M (1972) Tumor Inhibitors. 3. Identification and synthesis of an oncolytic hydrocarbon from American coneflower roots. J Med Chem 15:619–623

Wagner H, Stuppner H, Schafer W, Zenk M (1988) Immunologically active polysaccharides of *Echinacea purpurea* cell cultures. Phytochemistry 27:119–126