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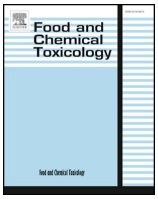

Bioassay-guided isolation of cytotoxic compounds from *Chrysophthalmum montanum* (DC.) Boiss

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ABSTRACT

Bioassay-guided isolation of the 80% methanol extract of the aerial parts of *Chrysophthalmum montanum* (DC.) Boiss. (Asteraceae) led to the isolation of four known guaianolide-type sesquiterpene lactones, 6*α*-acetoxy-4*α*-hydroxy-1*β*H-guaia-9.11(13)-dien-12.8*α*-olide **(1)**, 6*α*-acetoxy-4*α*-hydroxy-9*β*.10*β*-epoxy-1*β*H-guaia-11(13)-en-12.8*α*-olide **(2)**, 4*α*,6*α*-dihydroxy-1*β*,5*α*,7*α*H-guaia-9(10),11(13)-dien-12,8*α*-olide **(3)**, and (4*α*,5*α*,8*β*,10*β*)-4,10-dihydroxy-1,11(13)-guaidien-12,8-olide **(4),** along a steroidal glycoside mixture (**5a** and **5b**). The structures of the compounds were identified on the basis of spectroscopic data. Among them, **2, 4** and a steroidal glycoside mixture were obtained from *C. montanum* for the first time. All isolates were also first time assayed for *in vitro* cytotoxicities against four human cancer cell lines, i.e. breast (MCF-7, MDA-MB 231), colon (HT-29), and lung (PC3). Among the isolates, **1–3** showed significant inhibitory effect on the proliferation of cancer cells with viability ranging from 6.86 to 26.51%, while steroidal glycoside mixture showed no cytotoxicity, except against HT-29 (viability 61.99%). Compound **4** exhibited strong and selective cell growth inhibition against HT-29 with viability 20.99% and was identified as a promising compound with high selectivity between cancer cells and normal human lung cells (BEAS-2B), especially against HT-29 (IC_{50} = 12.2 μg/mL) compared to that of cisplatin. These results suggested that **4** is worthy of further study to determine its cytotoxicity mechanisms.

1. Introduction

The genus *Chrysophthalmum* Schultz. Bip (Asteraceae) comprises of four species in the world, and three of which are distributed in Turkey. Of them, *Chrysophthalmum montanum* (DC.) Boiss. is a herbaceous perennial plant mainly distributed in Eastern Turkey, Syria, and Iraq (Grierson, 1975; Rechinger, 1980; Aytac and Anderberg, 2001). It is locally named as “nezle otu and tutça" in Turkish language. In Turkish folk medicine, the aerial parts of *C. montanum* are used to treat common cold and sinusitis and for wounds healing on the human body and animals (Yeşil and Akalın, 2009; Arasan and Kaya, 2015).

The Asteraceae family is a well-known source of sesquiterpene lactones with different types of skeletons, commonly guaianolides. Sesquiterpene lactones, containing an *α*-methylene-γ-lactone moiety, show good cytotoxic, antimicrobial, anti-inflammatory and phytotoxic activities (Rodriguez et al., 1976; Koukoulitsa et al., 2002; Cheng et al., 2011; Wang et al., 2014). The genus *Chrysophthalmum* is known as the sister genus of *Inula* L. in the family Asteraceae (Eldenäs et al., 1999), and has been reported as a rich source of sesquiterpene lactones with cytotoxicity and antitumor properties (Zhang et al., 2005; Wang et al., 2014). Despite this, only a few studies on morphological characteristics and preliminary evaluation of some biological activities on the genus

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Abbreviations: ANOVA, analysis of variance; CC, column chromatography; DCM, dichloromethane; DMSO, dimethylsulfoxide; MeOH, methanol; BuOH, butanol; R−H_2O, remaining aqueous fraction; SRB, sulforhodamine B

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Chrysophthalmum have been reported (Aytac and Anderberg, 2001; Kirbag et al., 2009; Ozdemir et al., 2013; Selvi et al., 2014). In addition, only one phytochemical study on the isolation of sesquiterpene lactones from *C. montanum* was found in literature (Gürbüz et al., 2016). To the best of our knowledge, no work has been reported on cytotoxic effects of *C. montanum*.

Taking this report into account, in the current study, we have aimed to isolate and identify cytotoxic constituents from the aerial parts of *C. montanum* through bioassay-guided fractionation procedures. It has been found that treatment with *C. montanum* has resulted in promising biological activity on cancer (antigrowth effect).

2. Material and methods

2.1. General experimental procedures

Optical rotations were measured with Rudolph Research Analytical/Autopol IV/6W polarimeter. The UV spectra were recorded on Thermo-Scientific Evolution 300 UV–Visible spectrophotometer. The IR spectra were recorded in KBr pellet or liquid on a Bruker Vector 22 FT-IR spectrophotometer. The ^{1}H-NMR, ^{13}C-NMR, APT, DEPT, COSY, HSQC, HMBC and NOESY spectra were recorded on a Bruker Avance AV-500 NMR, Bruker Avance AV-400 NMR, and Bruker Avance AV-III HD 400 NMR Spectrometers. The chemical shifts are given as δ (ppm). The coupling constants (J) are reported as Hz. The EI-MS was measured on a Jeol the MS route JMS-600H mass spectrometer. The HREI-MS was measured on a Thermo Finnigan Mat 95 XP mass spectrometer.

Column chromatography was performed using silica gel (Kieselgel 60, 0.063–0.200 mm, Merck, Darmstadt, Germany) and Sephadex LH-20 (Sigma-Aldrich, Sweden). Analytical and preparative TLC based separation were carried out on precoated silica gel plates (Kieselgel 60 F_{254}, 0.2 or 0.5 mm thickness, Merck). Spots were visualized under UV light and detected by spraying with anisaldehyde reagent [methanol 76% (Merck), *o*-phosphoric acid 19% (Riedel-De Haën), *p*-anisaldehyde 5% (Merck)] and 20% H_2SO_4 (Merck), followed by heating. Preperative HPLC was conducted on a LC-908W—C60 Recycling Preperative HPLC (Japan Analytical Industry Co., Ltd., Tokyo, Japan), equipped with UV detector 254 and RI detector RI-50. JAIGEL-SIL column (Japan Analytical Industry Co., Ltd., 20 × 250 mm i.d., 10 μm) for normal phase HPLC and JAIGEL-ODS-M-80 C_{18} column (Japan Analytical Industry Co., Ltd., 20 × 250 mm i.d., 10 μm) for reverse phase HPLC were used.

2.2. Plant material

The aerial parts of *Chrysophthalmum montanum* were collected from the valley of Tohma river, Akçadağ, Malatya, Turkey, at flowering stage in July 2014. Plant material was identified by one of the authors (Prof. Hayri Duman). A voucher specimen (H. Duman 10324) was deposited in the Herbarium of GAZI, Gazi University, Ankara, Turkey.

2.3. Plant extraction and purification under bioassay-guided screening

Air-dried and powdered aerial parts of the plant (500 g) were extracted with 80% methanol (MeOH) by stirring at room temperature (v/v, 4 × 3 L, 2 days each time). After filtration, the combined 80% MeOH extracts were evaporated to dryness *in vacuo* to give "MeOH extract" (90.8 g). The crude MeOH extract was suspended in 80% MeOH (250 mL) and successively partitioned with *n*-hexane (11 × 250 mL), chloroform (8 × 250 mL), and *n*-butanol (BuOH) (8 × 250 mL) in a separatory funnel. Each extract, as well as remaining aqueous phase (R—H_2O) after solvent extractions, was evaporated to dryness under reduced pressure to obtain "*n*-hexane fraction" (1.7 g), "$CHCl_3$ fraction" (15.8 g), "*n*-BuOH fraction" (21.4 g) and "R—H_2O fraction" (36.4 g), respectively. Each of these fractions as well as crude MeOH extract was evaluated for their cytotoxic activity against selected cancer lines (MCF-7, MDA-MB-231, PC3, and HT-29) by sulforhodamine B (SRB) assay. The $CHCl_3$ fraction showed potent cytotoxicities against all used cell lines.

8.3 g of chloroform extract was subjected to silica gel column chromatography (CC) eluted with a solvent system of dichloromethane (DCM): acetone (90:10, 80:20, 70:30, 60:40, 50:50), and followed by DCM: MeOH with a gradient polarity (40:60, 30:70 and 0:100) to obtain eight main fractions by TLC control: Fr. A (1.8 g), Fr. B (455.5 mg), Fr. C (248.6 mg), Fr. D (99.7 mg), Fr. E (314.7 mg), Fr. F (510.0 mg), Fr. G (672.0 mg), and Fr. H (2.9 g). These fractions were evaluated for their cytotoxicity against the selected cancer cell lines.

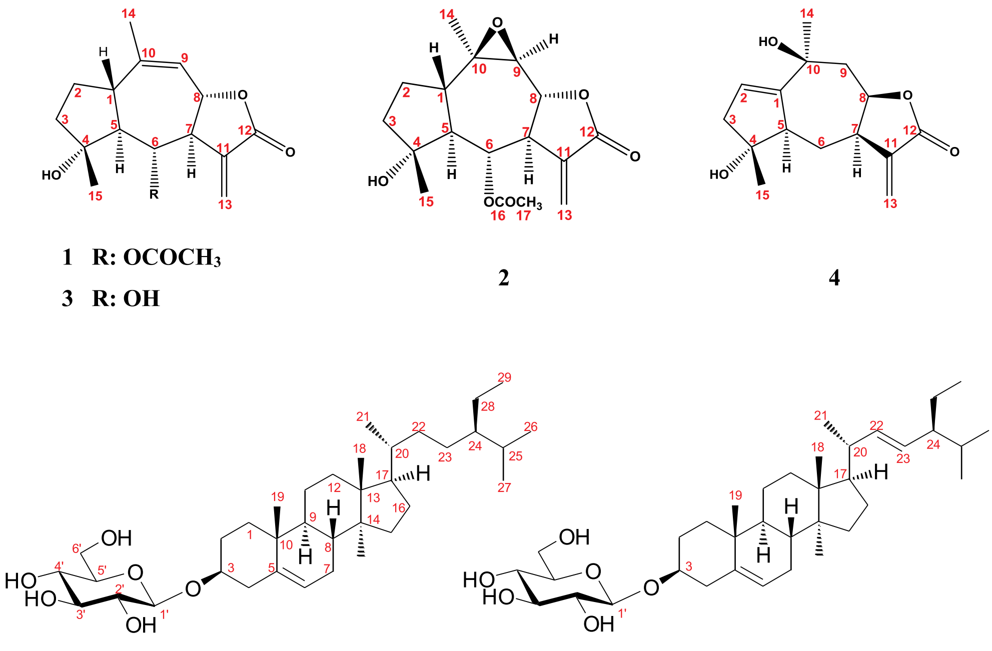
Fr. A (1.8 g) was chromatographed on a silica gel column, eluted with a step gradient of DCM: acetone (100:0 → 70:30) to obtain five subfractions (Fr. $A_{1\text{-}5}$) based on TLC pattern. Fr. A_2 and A_3 afforded compound **1** (513.9 mg) as white crystals by using Sephadex LH 20 CC in MeOH. Fr. A_4 gave compound **2** (13 mg) as a white powder. Fr. B (455.5 mg) was subjected to Sephadex LH 20 column, and eluted with hexane: DCM: MeOH (7:4:1) to MeOH 100% to obtain seven subfractions (Fr. $B_{1\text{-}7}$) based on the TLC profiles. Fr. B_1 was subjected to reverse phase preparative HPLC, eluted with MeOH: H_2O (70:30, 3 mL/min) to afford compound **1** (38.4 mg). Fr. B_3 was applied to preparative TLC using hexane: ethyl acetate (50:50) to afford compounds **2** (18.8 mg) and **3** (61.9 mg) as pure compounds. Fr. C (248.6 mg) was subjected to Sephadex LH 20 column eluting by hexane: DCM: MeOH (7:4:1) to afford three fractions. Fr. C_2 was subjected to preparative TLC using hexane: ethyl acetate (50:50) to afford compound **3** (2.9 mg) as a yellow oil. Fr. C_3 was purified by using crystallization to afford compound **2** (20.0 mg), and then, the rest of Fr. C_3 was subjected to preparative TLC using $CHCl_3$: MeOH (98:2) to afford compound **3** (13.3 mg) and further subjected to normal phase preparative HPLC with $CHCl_3$: MeOH (98:2, 3 mL/min), yielding compound **2** (8.1 mg), as a pure compound. Fr. E (314.7 mg) was chromatographed on a Sephadex LH-20 column, eluted with MeOH to yield four fractions. Fr. E_4 was further purified by preparative TLC with $CHCl_3$: MeOH (97:3) to give compound **4** (10.8 mg) as a white powder. Fr. F (510 mg) was fractionated on Sephadex LH-20 column with DCM: MeOH (1:1) to collect three fractions. Compound **4** (31.2 mg) was obtained as a white compound from Fr. F_3 by reverse phase preparative HPLC using MeOH: water (50:50, 3 mL/min). Fr. G (672 mg) was purified by Sephadex LH-20 in DCM: MeOH (1:1) to yield three fractions. A mixture of compounds **5a** and **5b** (13.4 mg) was obtained from Fr. G_2 as white amorphous powders.

*2.4. Structure elucidation of compounds **1–5***

A bioassay-guided investigation of the aerial parts of *C. montanum* led to the isolation and identification of four known sesquiterpene lactones (**1**–**4**), and a mixture of steroidal glycosides (**5a** and **5b**) (Fig. 1). The structures of the isolated compounds were elucidated on the basis of spectroscopic techniques, UV, IR, 1D and 2D-NMR, and mass spectrometry (EI-MS, and HREI-MS). The signals in the ^{1}H and ^{13}C-NMR spectra of the isolates were unambiguously assigned using 2D-NMR techniques, i.e. COSY, HSQC, HMBC and NOESY. Multiplicities were determined using ^{1}H-NMR and HSQC spectra.

2.4.1. 6α-acetoxy-4α-hydroxy-1βH-guaia-9.11(13)-dien-12.8α-olide (1)

White crystals; $[\alpha]_D^{27}$: 63.7 (*c* 0.315, $CHCl_3$); $C_{17}H_{22}O_5$; UV λ_{max} (MeOH): 214, 222 nm; IR υ_{max} ($CHCl_3$): 3425 (OH), 1765 (*α*-methylene-γ-lactone), 1660 (unsaturation) cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz), APT ($CDCl_3$, 100 MHz), HSQC and HMBC data, see Table 3. EI-MS *m/z* (rel. int.): 246.1 $[M\text{-}CH_3COOH]^+$ (10.3), 228.1 $[246\text{-}H_2O]^+$ (23.7), 213.0 $[228\text{-}CH_3]^+$ (10.3), 188.0 (100); HREI-MS *m/z*: 246.1245 (calculated for $C_{15}H_{18}O_3$, 246.1256), 228.1140 (calculated for $C_{15}H_{16}O_2$, 228.1150).



1 R: OCOCH$_3$

3 R: OH

2

4

5a

5b

Fig. 1. Structures of compounds **1–5b**.

Table 1
Percentage of viable cancer cells treated with extracts of *C. montanum.*

Test samples	% Viability			
	MCF-7	MDA-MB-231	PC3 (p53 mutant)	HT-29
MeOH extract	45.93 ± 1.03	97.20 ± 10.40	46.43 ± 5.82	78.16 ± 5.22
n-Hexane Fr.	66.62 ± 0.17	26.33 ± 0.15	109.29 ± 0.01	44.39 ± 0.39
$CHCl_3$ Fr.	15.59 ± 0.02	22.55 ± 0.12	22.54 ± 0.03	20.80 ± 0.07
n-BuOH Fr.	59.93 ± 0.16	155.62 ± 0.02	151.35 ± 0.15	89.73 ± 0.17
R−H_2O Fr.	56.26 ± 0.14	99.60 ± 0.24	128.87 ± 0.22	87.12 ± 0.32

Data are mean ± SD of three independent experiments carried out in triplicate.

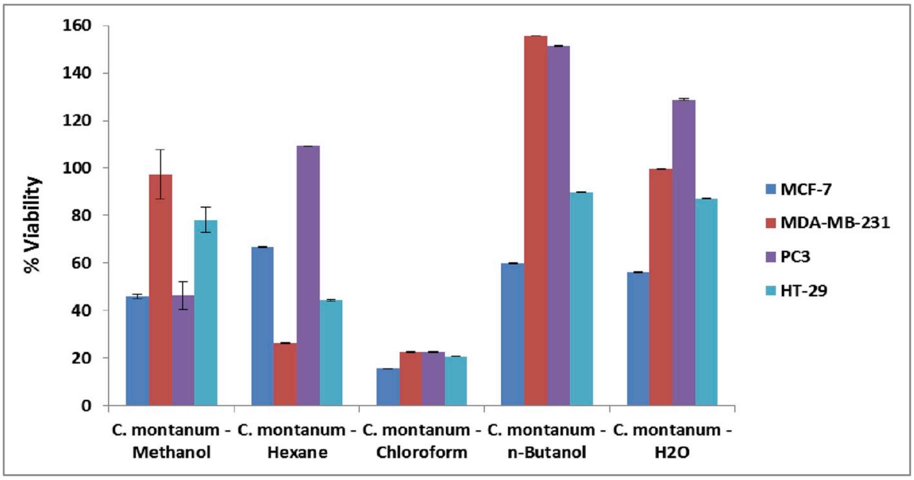


Fig. 2. Effects of the *C. montanum* extracts (20 μg/mL) on the viability of different cancer cell lines during 48 h by SRB assay (Data represent the mean ± SD of three independent experiments carried out in triplicate).

2.4.2. 6α-acetoxy-4α-hydroxy-9β.10β-epoxy-1βH-guaia-11(13)-en-12.8α-olide (2)

White crystals; $[\alpha]_D^{27.6}$: 54.7 (*c* 0.1, $CHCl_3$); $C_{17}H_{22}O_6$; UV λ_{max} (MeOH): 214, 222 nm; IR υ_{max} (KBr): 3441 (OH), 1769 (α-methylene-γ-lactone), 1730 (OAc) cm^{-1}; ^1H-NMR ($CDCl_3$, 400 MHz), APT ($CDCl_3$, 100 MHz), HSQC and HMBC data, see Table 4. EI-MS *m/z* (rel. int.): 262.2 [M- $CH_3COOH]^+$ (8.95), 244.1 $[262\text{-}H_2O]^+$ (12.24), 219.1 $[262\text{-}CH_3CO]^+$ (24.03), 186.1 (100); HREI-MS *m/z*: 262.1214 (calculated for $C_{15}H_{18}O_4$, 262.1205), 244.1113 (calculated for $C_{15}H_{16}O_3$, 244.1099).

Table 2
In vitro cytotoxic activity of fractions from the chloroform extract of *C. montanum* after 48 h incubation.

Test samples	% Viability			
	MCF-7	MDA-MB-231	PC3 (p53 mutant)	HT-29
Fr. A	30.13 ± 9.42	30.31 ± 1.51	25.45 ± 16.00	19.47 ± 5.03
Fr. B	25.80 ± 5.56	30.21 ± 1.61	27.81 ± 14.13	20.09 ± 3.41
Fr. C	56.20 ± 19.95	47.21 ± 0.87	35.47 ± 15.77	36.76 ± 5.67
Fr. D	64.02 ± 18.45	51.84 ± 3.13	43.50 ± 12.88	37.34 ± 1.55
Fr. E	47.51 ± 6.96	42.02 ± 1.48	38.67 ± 14.12	33.62 ± 5.10
Fr. F	73.52 ± 1.05	75.94 ± 0.56	63.86 ± 1.01	54.56 ± 2.76
Fr. G	71.88 ± 1.42	76.22 ± 4.08	61.20 ± 7.62	62.45 ± 1.97
Fr. H	79.70 ± 16.25	100.82 ± 9.71	99.35 ± 5.91	88.24 ± 5.72

All results are presented as mean ± SD of three independent experiments carried out in triplicate.

2.4.3. *4α,6α-dihydroxy-1β,5α,7αH-guaia-9(10),11(13)-dien-12,8α-olide (3)*

Yellow oil; $[\alpha]_D^{28}$: 63.2 (*c* 0.085, CH_3OH); $C_{15}H_{20}O_4$; UV (MeOH) λ_{max}: 229 nm; IR (KBr) υ_{max} 3432 (OH), 1763 (α-methylene-γ-lactone), 1646 (unsaturation) cm^{-1}; ^1H-NMR ($CDCl_3$, 400 MHz), APT ($CDCl_3$, 100 MHz), HSQC and HMBC data, see Table 5; EI-MS *m/z* (rel. int.): 246.1 $[M-H_2O]^+$ (9.56), 228.2 $[246-H_2O]^+$ (8.86), 213.1 $[228-CH_3]^+$ (7.98), 55.1 (100); HREI-MS *m/z*: 264.1358 (calculated for $C_{15}H_{20}O_4$, 264.1362), 246.1307 (calculated for $C_{15}H_{18}O_3$, 246.1256).

2.4.4. *(4α,5α,8β,10β)-4,10-dihydroxy-1,11(13)-guaidien-12,8-olide (4)*

White crystals; $[\alpha]_D^{28}$: +20.3 (*c* 0.08, $CHCl_3$); $C_{15}H_{20}O_4$; UV (MeOH) λ_{max}: 214, 222, 228 nm; IR (KBr) υ_{max}: 3343 (OH), 1756 (α-methylene-γ-lactone), 1653 (unsaturation) cm^{-1}; ^1H-NMR (CD_3OD and $CDCl_3$, 400 MHz), ^{13}C-NMR (CD_3OD, 125 MHz), HSQC (CD_3OD) and HMBC (CD_3OD), see Table 6; EI-MS *m/z* (rel. int.): 246.1 $[M-H_2O]^+$ (32.3), 231.0 $[246-CH_3]^+$ (37.7), 213.1 $[231-H_2O]^+$ (7.6), 119.0 (100); HREI-MS *m/z*: 246.1252 (calculated for $C_{15}H_{18}O_3$, 246.1256), 231.1027 (calculated for $C_{14}H_{15}O_3$, 231.1021).

2.4.5. *A mixture of β-sitosterol-3-O-β-glucopyranoside (daucosterol) (5a) and stigmasterol 3-O-β-glucopyranoside (5b) with ratio 1:0.5*

White powders; $C_{35}H_{60}O_6$ and $C_{35}H_{58}O_6$, respectively; UV (MeOH) λ_{max}: 208, 229 (sh) nm; IR (KBr) υ_{max}: 3412 (OH), 2934 (CH), 1068 and 1024 (—C—O—C—) cm^{-1}; ^1H-NMR ($CDCl_3$ + CD_3OD, 600 MHz), ^{13}C-NMR ($CDCl_3$ + CD_3OD, 125 MHz), HSQC and HMBC data, see Table 7. EI-MS *m/z* (rel. int.): 414.1 [M-glucose + H]$^+$ (26.3), 396.2 $[414-H_2O]^+$ (100); 412.2 [M-glucose + H]$^+$ (25.5), 394.2 $[412-H_2O]^+$ (48.5); HREI-MS *m/z*: 414.3871 (calculated for $C_{29}H_{50}O_1$, 414.3862), 396.3767 (calculated for $C_{29}H_{48}$, 396.3756); 412.3719 (calculated for $C_{29}H_{48}O_1$, 412.3705), 394.3615 (calculated for $C_{29}H_{46}$, 394.3600).

Table 3
Spectral data of compound **1** (^1H-NMR and APT data in $CDCl_3$, δ_H 400 MHz, δ_C 100 MHz).

Position	C/H	δ_C ppm	δ_H ppm, *J* (Hz)	HMBC (C→H)
1	CH	39.61	2.57 brdd (10.0/10.4)	H_3-14, H-9, H_2-2, H_2-3, H-5
2	CH_2	22.45	1.94 m, 1.75–1.66 m	H-1, H-5, H_2-3, H_3-14
3	CH_2	42.00	1.75–1.66 m	H_3-15, H_2-2, H-1, H-5
4	C	79.98	–	H_2-2, H_2-3, H-5, H_3-15, H-6
5	CH	55.09	1.94 m	H_3-15, H_2-3, H-6, H-1
6	CH	76.15	5.17 brdd (10.0/10.4)	H-5, H-7, H-8, CO***CH₃***, H-1
7	CH	49.14	3.04 ddt (3.2/10.0/10.0)	H_2-13, H-6, H-8, H_3-14, H-5
8	CH	76.43	4.72 brdd (4.0/9.6)	H-7, H_2-13, H-6
9	CH	125.78	5.80 dq (2.0/4.0)	H_3-14, H-8, H-1, H-7
10	C	140.84	–	H_3-14, H_2-2, H-5, H-1, H-8
11	C	136.51	–	H_2-13, H-6, H-7
12	C	169.65	–	H_2-13, H-6
13	CH_2	122.56	6.20 d (3.6), 5.50 d (3.2)	H-7
14	CH_3	20.87	1.81 s	H-5,H-9
15	CH_3	22.25	1.28 s	H_2-3
COCH_3	C	170.52	–	CO***CH₃***
CO***CH₃***	CH_3	21.65	2.1 s	

2.5. *Cancer cell lines and cell culture*

Breast cancer cell lines MCF-7 was kindly provided by Dr. Bahadir Ozturk (School of Medicine, Selcuk University) while MDA-MB 231 was provided by Dr. Ceyda Acılan (TUBITAK MARMARA Research Center). These cell lines were cultured in RPMI 1640 Medium (Lonza, Verviers, Belgium) supplemented with 100 U/mL penicillin +100 μg/L streptomycin (Gibco, Grand Island, NY, USA), 2 mM L-glutamine (Gibco, Grand Island, NY, USA), and 10% fetal bovine serum (Lonza, Verviers,

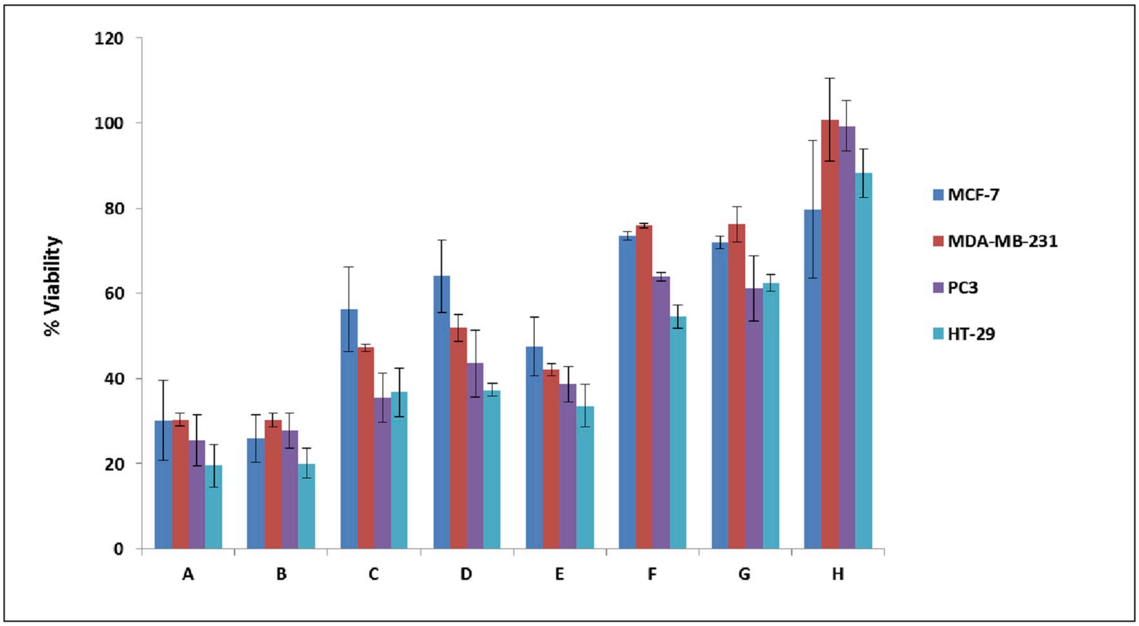


Fig. 3. Viability screening of the cancer cells treated with fractions from the chloroform extract of *C. montanum* A-H (20 μg/mL) for 48 h by SRB assay (Data represent the mean ± SD of three independent experiments carried out in triplicate).

Table 4
Spectral data of compound **2** (^{1}H-NMR and APT data in $CDCl_3$, δ_H 400 MHz, δ_C 100 MHz).

Position	C/H	δ_C ppm	δ_H ppm, *J* (Hz)	HMBC (C→H)
1	CH	42.39	1.70 m	H_2-2, H_3-14, H-5
2	CH_2	20.95	1.91 m, 1.70–1.63 m	H-1, H_2-3
3	CH_2	41.89	1.70–1.63 m	H_3-15
4	C	79.97	–	H_3-15, H_2-2, H-1, H-5,
5	CH	53.75	2.27 brdd (10.0/10.4)	H_3-15, H_2-2, H-1
6	CH	75.26	4.98 brdd (10.0/10.0)	H-8, CO$\boldsymbol{CH_3}$
7	CH	46.07	3.39 m	H_2-13, H-8
8	CH	78.49	4.01 dd (6.4/10.4)	H-9, H_3-14
9	CH	63.36	3.12 d (6.4)	H_3-14, H-8
10	C	59.20	–	H_3-14, H-1, H_2-2, H-9, H-5
11	C	135.06	–	H_2-13
12	C	168.97	–	H_2-13
13	CH_2	123.70	6.27 d (3.2), 5.63 d (2.8)	–
14	CH_3	16.66	1.41 s	H-9
15	CH_3	21.75	1.23 s	H-1
COCH_3	C	170.56	–	CO$\boldsymbol{CH_3}$
CO$\boldsymbol{CH_3}$	CH_3	21.59	2.1 s	–

Table 5
Spectral data of compound **3** (^{1}H-NMR and APT data in $CDCl_3$, δ_H 400 MHz, δ_C 100 MHz).

Position	C/H	δ_C ppm	δ_H ppm, *J* (Hz)	HMBC (C→H)
1	CH	38.69	2.51–2.53 m	H_3-14, H-5, H-9, H_2-3, H_2-2, H-6
2	CH_2	22.82	1.98–2.02 m, 1.77–1.68 m	H-1, H-5
3	CH_2	40.97	1.77–1.68 m	H_3-15, H_2-2
4	C	81.13	–	H-6, H_3-15, H-5, H_2-2, H_2-3
5	CH	56.69	1.58 dd (10.4/10.8)	H_3-15, H_2-3, H-6, H-1
6	CH	74.95	4.02 dd "t" (10.0)	H-5, H-7, H-1, H-8
7	CH	51.58	2.62 dddd (3.2/3.6/9.6/9.6)	H_2-13, H-6
8	CH	76.41	4.62 brdd (4.4/10.0)	H-7, H-6
9	CH	125.82	5.80 m	H_3-14, H-1, H-7
10	C	140.41	–	H_3-14, H-5, H_2-2, H-1
11	C	137.61	–	H-6, H_2-13, H-7
12	C	170.47	–	H_2-13
13	CH_2	123.95	6.26 m, 6.25 m	H-7
14	CH_3	21.14	1.80 s	H-9
15	CH_3	22.30	1.36 s	H_2-3

Belgium). On the other hand, colon cancer cell line HT-29 was provided by Dr. Konstantinos Dimas (School of Medicine, Thessaly University), and lung cancer cell line PC3 (p53 mutant) and non-tumorigenic lung cells BEAS-2B were generously donated by Dr. Hakan Akca (School of Medicine, Pamukkale University) were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin + 100 μg/mL streptomycin and 1% L-glutamine and non-essential amino acid (Lonza, Verviers, Belgium). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.6. *Cytotoxicity*

The SRB assay was employed to evaluate the cytotoxicity of the extract/fractions and the isolated compounds from *C. montanum* against selected cancer cell lines, based on the measurement of the cellular protein content. The cells were seeded in 96-well culture plates at a density of 5×10^3 cells, and subsequently treated with either extracts/fractions (20 μg/mL) or isolated compounds (20 μM) for 48 h. In addition, the growth inhibitory effect of compound **4** on the same cancer cell lines, and a normal human cell line (BEAS-2B) were performed in a dose dependent-manner, with concentration range varying 100–1.56 μg/mL. All samples were dissolved in dimethyl sulfoxide (DMSO), with the final concentration of 0.2% (v/v) in the cell culture. Cisplatin was used as a positive control. Each experiment was performed in triplicates.

At the end of the treatment period, cells were fixed with 50% (w/v) ice-cold trichloroacetic acid (50 μL) for 1 h at 4 °C. The plates were then washed slowly with deionized water, and allowed to dry. After then, 50 μL of SRB dye (4% SRB in 1% acetic acid) was added at room temperature to each well for 30 min in the dark. At the end of the staining period, unbound SRB was removed by washing four times with 1% acetic acid, and air dried. 150 μL of Tris base solution (10 mM, pH:10) was added into each well to dissolve the cell-bound SRB dye. After 10 min, the absorbance was measured at the wavelength of 564 nm using a microplate reader (FLASH Scan S12, Analytik Jena AG, Jena, Germany). Viability of treated cells was calculated in reference to the untreated control cells by using the following equation:

$$\text{Viability (\%)} = [100 \times (\text{Sample Abs}) / (\text{Control Abs})]$$

Table 6
Spectral data of compound **4** (^{1}H and ^{13}C-NMR data in CD_3OD, δ_H 400 MHz, δ_C 125 MHz; ^{1}H-NMR data in $CDCl_3$, δ_H 400 MHz).

Position	C/H	CD_3OD			$CDCl_3$
		δ_C ppm	δ_H ppm, *J* (Hz)	HMBC (C→H)	δ_H ppm, *J* (Hz)
1	C	153.19	–	H_3-14, H_2-6, H_2-3, H_2-9 ve H-5	–
2	CH	121.34	5.65 m	H_2-3, H-5 ve H_2-9	5.69 m
3	CH_2	47.15	2.38 brd (16.4) 2.30 brd (16.4)	H_3-15, H-2	2.40 m
4	C	82.57	–	H_3-15, H_2-3, H-5, H_2-2	–
5	CH	56.73	2.49 brd (12.0)	H_3-15, H_2-6, H-2	2.45 brd (12.4)
6	CH_2	32.96	1.97[a]m, 1.46 ddd (12.4/12.8/13.2)	H-8, H_3-15	1.93–1.99[a]m 1.43–1.47 m
7	CH	43.09	3.30 m	H_2-6, H_2-9, H_2-13	3.23 m
8	CH	79.17	4.87 m	H_2-9, H_2-6, H_3-14	4.75 ddd (4.4/8.0/12.0)
9	CH_2	44.53	2.12 dd (4.4/12.8) 1.97[a] dd (12.4/12.8)	H_3-14	2.24 dd (4.4/13.2) 1.93–1.99[a] dd (12.4/12.8)
10	C	71.66	–	H_3-14, H_2-9	–
11	C	142.29	–	H_2-13, H_2-6, H-7, H-8	–
12	C	171.82	–	H_2-13, H-8	–
13	CH_2	123.27	6.20 d (2.4) 5.74 d (2.4)	–	6.28 d (2.8) 5.62 d (2.4)
14	CH_3	27.24	1.33 s	H_2-9	1.37 s
15	CH_3	24.67	1.25 s	H_2-3	1.29 s

[a] Overlapped protons.

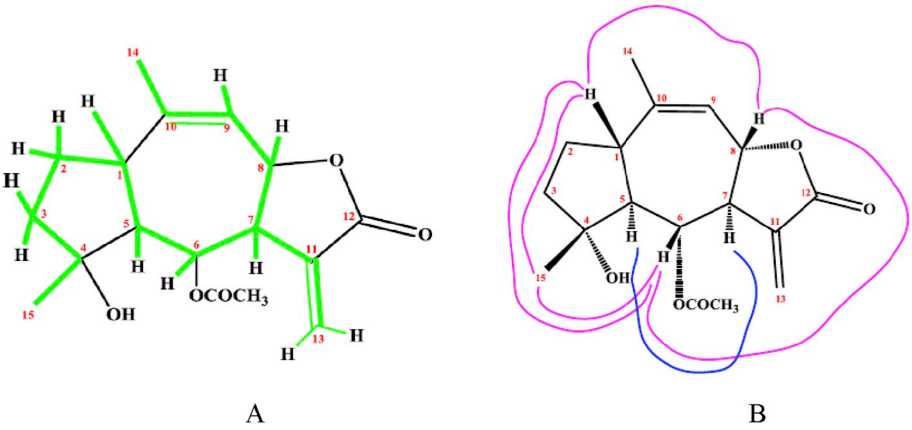


Fig. 4. Key COSY (A), and NOESY (B) correlations for **1**.

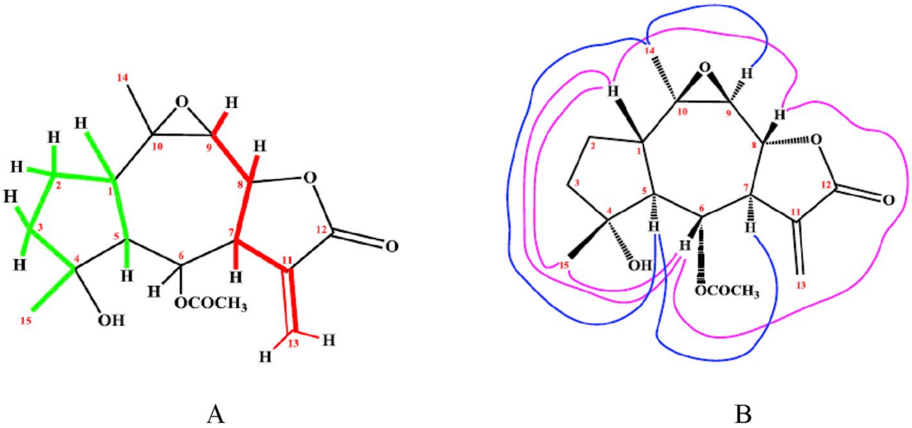


Fig. 5. Key COSY (A), and NOESY (B) correlations for **2**.

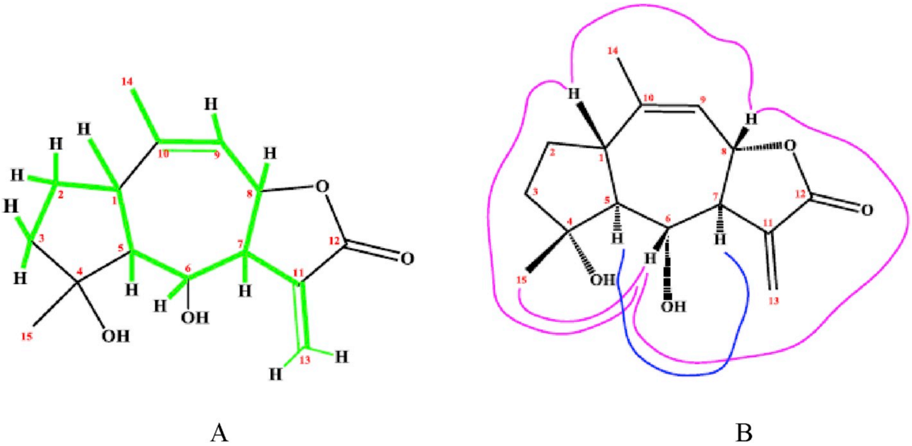


Fig. 6. Key COSY (A), and NOESY (B) correlations for **3**.

2.7. Statistical analysis

All of the statistical analyses were performed by using the Graphpad Prism 6.0 statistical Software for Windows. The significance was calculated using two-way analysis of variance (ANOVA), followed by multiple Tukey's comparison test. All results were expressed as mean ± SD (standard deviation).

3. Results and discussion

In this study, a bioassay-guided fractionation and isolation, based on the cytotoxic activity of *C. montanum* against four cancer cell lines, MCF-7 and MDA-MB-231 breast cancer, PC3 (p53 mutant) lung cancer, and HT-29 colon cancer cell lines, was performed. As the initial step, the crude MeOH extract of *C. montanum* was successively subjected to solvent-solvent extractions to afford *n*-hexane, chloroform, *n*-butanol and remaining aqueous fractions. The crude MeOH extract and its fractions were tested for their cytotoxic activities against the cancer cell lines. Since the chloroform extract exhibited the highest cytotoxic activity with viability values ranging from 15.59 to 22.55% on all tested cancer cell lines (Table 1 and Fig. 2), it was selected for the isolation of active compounds.

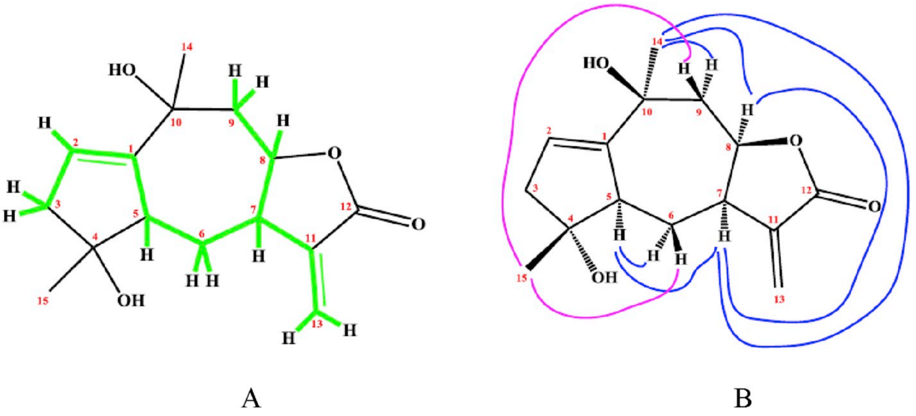


Fig. 7. Key COSY (A), and NOESY (B) correlations for **4**.

Table 7
Spectral data of a mixture of compounds **5a** and **5b** (^{1}H and ^{13}C-NMR data in $CDCl_3$ + CD_3OD, δ_H 600 MHz, δ_C 125 MHz)[a].

Sitosterol-3-*O*-β-glucopyranoside (Daucosterol) (5a)					Stigmasterol 3-*O*-β -glucopyranoside[b] (5b)		
Position	C/H	δ_C ppm	δ_H ppm, *J* (Hz)	HMBC (C→H)	C/H	δ_C ppm	δ_H ppm, *J* (Hz)
1	CH_2	36.99	1.74[c]/0.94[c]		CH_2		
2	CH_2	31.67	1.85[c]/1.32[c]		CH_2		
3	CH	78.91	3.46 m	H-1′	CH		
4	CH_2	38.41	2.28 dd (2.4/13.2) 2.15 dd (11.4/13.2)		CH_2		
5	C	140.01	–	Me-19	C		
6	CH	121.97	5.25 brd		CH		
7	CH_2	29.33	1.78[c]/1.48[c]		CH_2		
8	CH	31.60	1.85[c]		CH		
9	CH	49.89	0.79[c]	Me-19	CH		
10	C	36.46	–	Me-19, Me-21	C		
11	CH_2	20.80	1.36[c]		CH_2		
12	CH_2	39.48	1.87[c]/1.05[c]		CH_2		
13	C	42.06	–		C		
14	CH	56.48	0.88[c]		CH	56.50	
15	CH_2	24.05	1.45[c]/0.96[c]		CH_2		
16	CH_2	28.02	1.73[c]/1.14[c]		CH_2		
17	CH	55.76	1.0[c]	Me-18, Me-21	CH	55.70	
18	CH_3	11.58	0.57 s		CH_3	11.77	
19	CH_3	18.70	0.88 s		CH_3		
20	CH	35.91	1.22[c]	Me-21	CH		
21	CH_3	18.49	0.78 d (6.6)		CH_3		
22	CH_2	33.65	1.20[c]/0.88[c]		CH	138.15	5.03 dd (9.0/15.0)
23	CH_2	25.69	1.04[c]		CH	129.00	4.90 dd (9.0/15.0)
24	CH	45.55	0.80[c]		CH		
25	CH	28.81	1.13[c]	Me-26, Me-27	CH		
26	CH_3	19.54	0.72 d (9.0)		CH_3		
27	CH_3	19.06	0.70 d (9.0)		CH_3		
28	CH_2	22.77	1.14[c]		CH_2		
29	CH_3	11.67	0.73[c]		CH_3	11.99	
1′	CH	100.8	4.29 d (7.5)		CH	101.76	
2′	CH	73.26	3.12 dd (7.5/9.0)		CH		
3′	CH	76.12	3.32 dd “t” (9.0)		CH		
4′	CH	69.77	3.33 dd “t” (9.0)		CH		
5′	CH	75.15	3.17[c]		CH		
6′	CH_2	61.50	3.73 dd (3.0/12.0) 3.64 dd (4.8/12.0)		CH_2		

[a] All data were detected by 2D-NMR (COSY, HSQC ve HMBC) analysis.

[b] Minor compound, it was shown only different signals.

[c] J values could not be evaluated due to overlapping.

The $CHCl_3$ extract was further fractionated through column chromatography over silica gel to obtain eight main fractions (Fr. A-H). Each fraction was tested on the cancer cell lines. Among the all fractions, both Fr. A and B had significant cytotoxic effects on HT-29 with viability of 19.47 and 20.09%, respectively. In addition, the Fr. A and B displayed a considerable cell growth inhibition against PC3 and MCF-7 with viability of 25.45 and 25.80%, respectively (Table 2 and Fig. 3).

Column chromatographic fractionation of the chloroform extract, followed by silica gel CC, Sephadex CC, preparative TLC and HPLC, afforded of four known guaianolide-type sesquiterpene lactones (**1–4**), and a mixture of steroidal glycosides (**5a** and **5b**) from Fr. A-G (Fig. 1). The structures of all the isolated compounds were identified through spectroscopic analysis, including 1D and 2D-NMR and, EI-MS and HREI-MS. In this study, we report detailed spectral data of sesquiterpene lactones (**1–4**) for the first time (Tables 3–6). The chemical shift assignments were confirmed by HMBC correlations as well as COSY analysis as shown in Figs. 4–7. The relative configurations of isolates were determined by NOESY experiments (Figs. 4–7, see the spectra in the Supporting information).

The structure of compound **1**, obtained as a major constituent of the chloroform extract of *C. montanum*, was elucidated to be "6*α*-acetoxy-4*α*-hydroxy-1*β*H-guaia-9.11(13)-dien-12.8*α*-olide" by comparing with the literature data, including ^{1}H-NMR (Rustaiyan et al., 1989) and ^{13}C-NMR (Chang et al., 2012) experiments. Compound **1** was "6*α*-acetoxy-isoinuviscolide" in a study conducted by Chang et al. (2012). ^{1}H-NMR spectrum of **1** indicated three singlets methyl [δ_H 1.81 (H_3-14), 1.28 (H_3-15), and 2.1 (CO***CH₃***)], one olefinic methine [δ_H 5.80 (dq, H-9)] and typical signals for one "*α*-methylene γ-lactone" moiety [δ_H 3.04 (ddt, H-7), δ_H 4.72 (brdd, H-8) and δ_H 6.20 d, 5.50 d (H_2-13)] (Table 3). These observed structural features and COSY correlations (especially H-1/H-5) were identical with those of a guaianolide-type sesquiterpene lactones. The majority of sesquiterpene lactones from higher plants contain *α*-methylene-γ-lactone moiety in which H-7 is generally *α*-oriented (Simonsen et al., 2013). Additionally, the large coupling constants of H-6 (δ_H 5.17 brdd, $J = 10.0/10.4$ Hz) indicated that the acetoxyl group was in equatorial *β*-orientation. According to NOESY spectrum of **1**, H-7/H-5 showed correlation in *α*-orientation; and H-6/H_3-15, H_3-15/H-1 and H-1/H-8 showed correlation in *β*-orientation (Fig. 4). Compound **1** was previously isolated from different plants of Inuleae tribe-Asteraceae, such as *Postia bombycina* (Rustaiyan et al., 1989), *Inula helianthus-aquatica* (Chang et al., 2012), *I. sericophylla* (Cheng et al., 2012), *I. hupehensis* (Qin et al., 2011), and *C. montanum* (Gürbüz et al., 2016) up to now.

The structure of compound **2** possess an epoxy moiety between C-9 and C-10. On the basis of its spectral data, **2** was identified as "6*α*-acetoxy-4*α*-hydroxy-9*β*.10*β*-epoxy-1*β*H-guaia-11(13)-en-12.8*α*-olide" by comparing with the reported ^{1}H-NMR data (Rustaiyan et al., 1989). ^{13}C-NMR data of **2** showed that C-10 signal (δ_C 59.20 ppm) was shifted upfield as a quaternary carbon of an epoxy moiety. Signal of oxymethine, H-9 (δ_H 3.12 d $J = 6.4$ Hz), attached the epoxy group was identified (Table 4, Fig. 5). Our study is the first report of the isolation of **2** from *C. montanum*. The presence of **2** has been previously reported in *Postia bombycina* (Rustaiyan et al., 1989).

On the basis of the spectral data, compound **3** was identified to be "4*α*,6*α*-dihydroxy-1*β*,5*α*,7*α*H-guaia-9(10),11(13)-dien-12,8*α*-olide", which is distinctly similar to **1**, except for an acetoxyl moiety at C6 that was hydroxylated in **3**. Compound **3** was also named as "6α-hydroxy-isoinuviscolide" by us for the first time. In ^{1}H-NMR spectrum of **3**, characteristic signals of exocyclic methylene were shifted downfield (δ_H 6.26 m, 6.25 m) due to a hydroxyl group in *α*-orientation at C-6 (Table 5, Fig. 6). The structure of **3** was identified by comparing its spectral data with those of literature (Cheng et al., 2011). According to the literature, compound **3** was previously isolated from *Inula falconeri* (Cheng et al., 2011), and *C. montanum* (Gürbüz et al., 2016) until now.

The structure of compound **4** was identified to be "(4*α*,5*α*,8*β*,10*β*)-4,10-dihydroxy-1,11(13)-guaiadien-12,8-olide", by comparing its ^{1}H-NMR spectral data to those of reported data in $CDCl_3$ (Ahmed et al., 1993). In ^{1}H-NMR spectrum of **4**, the olefinic proton (δ_H 5.65 m) indicated unsaturation in the guaianolide skeleton. The ^{13}C-NMR spectrum of **4** indicated that the typical downfield shift of δ_C 153.19 was due to unsaturation between C-1 and C-2. The specific coupling constants of H-8 (δ_H 4.75 ddd J = 4.4/8.0/12.0 Hz) indicated that H-8 was in *α*-orientation, including the presence of *cis*-fused lactone ring (Table 6). According to NOESY spectrum of **4**, H-5/H-7, H-7/H-8, H-8/H_3-14 showed correlations in *α*-orientation (Fig. 7). This is the first report of the isolation of **4** from *C. montanum*. Compound **4** was previously isolated from *Jasonia candicans* (Ahmed et al., 1993), *Carpesium faberi* (Li et al., 2011) and *Leucophyta brownie* (Hyldgaard et al., 2015).

A mixture of steroidal glycoside "sitosterol-3-*O*-*β*-glucopyranoside (daucosterol) (**5a**)" and "stigmasterol 3-*O*-*β*-glucopyranoside (**5b)**" was obtained in a ratio (1:0.5). Their structures were identified by comparing their spectroscopic data with the literature (Kojima et al., 1990; Rashed et al., 2014). Our spectral data of mixture of **5a** and **5b** is presented in Table 7. The structure of major compound, daucosterol (**5a**), and minor compound, stigmasterol 3-*O*-β-glucopyranoside (**5b**) were deduced. In our study, the isolation of a mixture of sitosterol and stigmasterol glycosides from *C. montanum* was firstly reported.

The cytotoxicity of all the isolates was evaluated against four human cancer cell lines, MDA-MB-231, MCF-7, PC3, and HT-29, using cisplatin as a standard drug. Compounds **1**–**3** exhibited a significant growth inhibition with viability values ranging from 6.86 to 26.51%. Compound **4** showed superior and selective inhibitory effects on HT-29 and PC3 cancer cell lines with viability values of 20.99 and 32.61%, respectively. In addition, mixture of compounds **5a** and **5b** showed no cytotoxicity against the cell lines used, except for HT-29 (Table 8 and Fig. 8). Moreover, the cytotoxicity of compound **4** against MCF-7, MDA-MB 231, HT-29 and PC3, as well as normal human lung cell line (BEAS-2B), was tested by the SRB assay, and compared with that of cisplatin (Figs. 9 and 10). Noticeably, compound **4** was found to be the most promising compound against HT-29 with an IC_{50} value of 12.2 μg/mL. It exhibited a strong inhibitory effect and showed specific selectivity towards HT-29 cancer cells. On the other hand, it is worth mentioning that **4** induced significantly less toxicity on normal cells. In addition, **4** showed a moderate activity against PC3 cell lines with the IC_{50} value of 21.7 μg/mL (Table 9 and Fig. 9).

Asteraceae species, such as *Inula, Tanacetum, Centaurea, Cichorium, Artemisia, Anthemis, Achillea, Erigeron* and *Xanthium* have been extensively studied for cytotoxic activity (Koukolulitsa et al., 2002; Stavri et al., 2005; Wang et al., 2014). A number of phytochemical studies on *Inula* have been reported including sesquiterpene lactones and their anticancer, anti-inflammatory and antibacterial activities (Wang et al., 2014; Seca et al., 2015). The active sesquiterpene lactones containing *α*-methylene γ-lactone obtained from several Asteraceae plants exhibit cytotoxic activity, and some of them shave been found to have potential to enter into clinical cancer trials (Zhang et al., 2005; Ghantous et al., 2010). Certain sesquiterpene lactones cause apoptosis in the cells by forming a covalent bond with the α-methylene, γ-lactone group. This structural group reacts with nucleophiles, especially thiol groups of cysteine in proteins, by a Michael-type addition. The other important contributors towards the bioactivity of sesquiterpene lactones are their lipophilicity and molecular geometry. High lipophilicity increases activity as it facilitates the penetration of the compound through the cell membrane. Conformational flexibilty is also found to be important on the bioactivity of sesquiterpene lactones (Ghantous et al., 2010).

Table 8
Cytotoxic activity of the isolated compounds (20 μM) from *C. montanum.*

Compounds	% Viability				
	1	2	3	4	A mixture of 5a and 5b
MDA MB-231	17.16 ± 5.87	26.51 ± 6.83	16.43 ± 4.93	84.82 ± 10.09	112.78 ± 7.15
HT-29	6.86 ± 0.37	9.39 ± 4.25	7.73 ± 0.77	20.99 ± 1.01	61.99 ± 7.44
MCF-7	14.91 ± 2.17	15.76 ± 6.46	13.54 ± 0.45	104.85 ± 0.78	116.37 ± 3.59
PC3 (p53 mutant)	10.03 ± 2.84	7.95 ± 3.57	15.11 ± 3.08	32.61 ± 7.17	91.20 ± 2.75

Results are presented as mean ± SD of three independent experiments carried out in triplicate.

In the present study, a bioassay-guided investigation of *C. montanum* led to the isolation of four known guaianolide-type sesquiterpene lactones (**1–4**) and a mixture of steroidal glycosides (**5a** and **5b**). In the literature, there are only a few studies on biological activities of the isolated sesquiterpene lactones (Cheng et al., 2011; Li et al., 2011; Qin et al., 2011; Hyldgaard et al., 2015). As for the cytotoxic activity, only one report described that compound **4** had cytotoxic effect against MCF-7 breast cancer cell line ($IC_{50} = 26.5 \pm 0.6\,\mu g/mL$) (Li et al., 2011). In our study, we found that compound **4** has a low cytotoxicity against MCF-7 with IC_{50} value of > 100 µg/mL by SRB assay.

The most common plant sterol β-sitosterol, is a 24-ethyl analogues of cholesterol. Whereas, stigmasterol contains additional unsaturation in the side-chain, a *trans*-Δ^{22} double bond (Kojima et al., 1990). β-Sitosterol is a constituent of many herbal drugs used for the treatment of benign prostatic hyperplasia, and prostate cancer (Holtz et al., 1998; Wilt et al., 1999). In the literature, β-sitosterol and daucosterol were tested against K-562 (leukemia), NIH-3T3 (normal), T47D (breast ductal carcinoma) and HT-29 (colon carcinoma) cell lines by MTT assay. It was found that β-sitosterol inhibited the HT-29, while daucosterol was more active against K-562. Based on the selectivity index

Table 9
Cytotoxic activities of compound **4** from *C. montanum* and cisplatin with IC_{50} (µg/mL) values.

Test Samples	IC_{50} (µg/mL)				
	MCF-7	MDA-MB 231	PC3 (p53 mutant)	HT-29	BEAS-2B
4	> 100	93.8 ± 0.8	21.7 ± 0.2	12.2 ± 0.3	92.2 ± 0.5
Cisplatin	3.0 ± 0.1	7.8 ± 0.2	6.2 ± 0.6	2.5 ± 0.4	> 7.6

The IC_{50} values (the concentration of sample for 50% inhibition) are mean ± SD calculated from three independent experiments carried out triplicate.

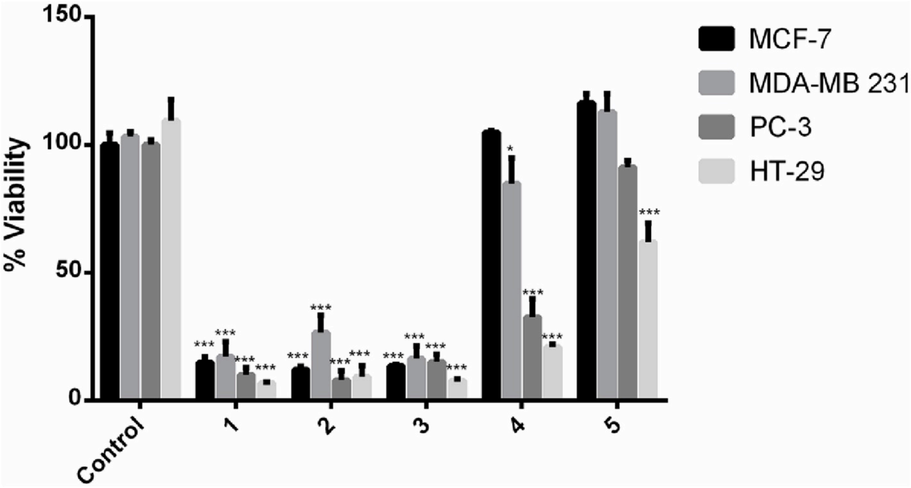


Fig. 8. *In vitro* cytotoxic activity of the isolated compounds (20 µM) from *C. montanum* after 48 h incubation by using SRB assay (**5**: a mixture of **5a** and **5b**) (Data represent triplicate determinations carried out three times. Analysis of variance was used to demonstrate statistical significance between different doses with a Tukey multiple comparison post test) (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

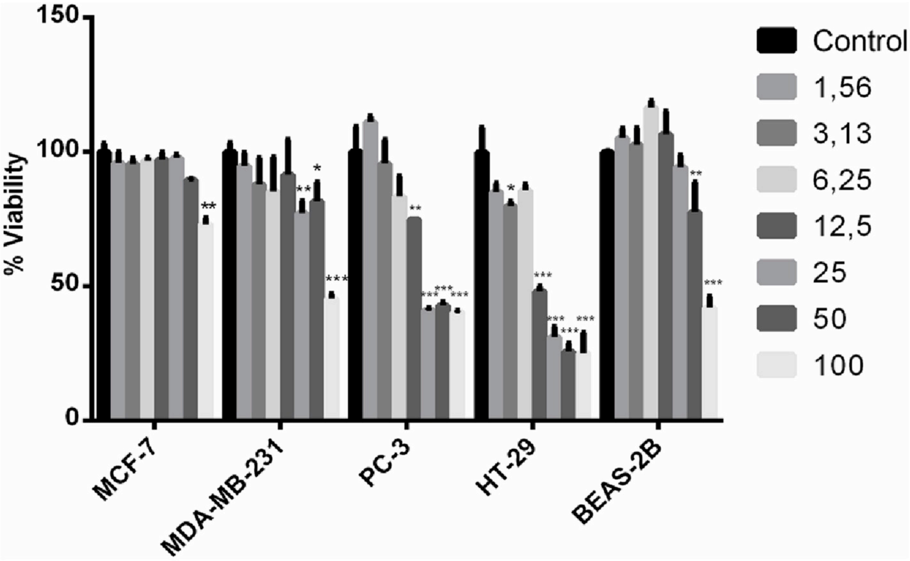


Fig. 9. *In vitro* cytotoxic activity of compound **4** at various concentrations after 48 h incubation (Data represent triplicate determinations carried out three times. The asterisk denotes statistically significant differences in comparison with the control (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

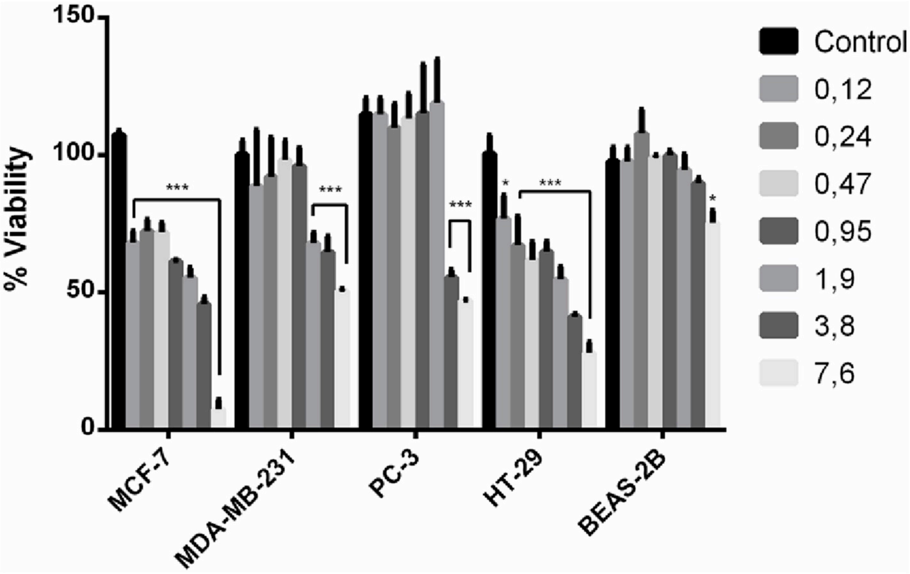


Fig. 10. *In vitro* cytotoxic activity of cisplatin at various concentrations after 48 h incubation (Results are presented as viability ratio compared with the control group. Data are expressed as the mean of three independent experiments significantly different from the control). (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

values, daucosterol was identified as more selective to HT-29, than positive control methotrexate (Manayi et al., 2013). According to our results, mixture of compounds **5a** and **5b** exhibited a moderate cytotoxicity against only HT-29 cell line with viability valueof 61.99%.

4. Conclusions

In the present study, *C. montanum* was found to be a novel source of natural anticancer agents, against particularly colon cancer. We identified the compounds responsible for the growth inhibitory activity of *C. montanum* through bioactivity-guided fractionation for the first time. Among the isolated compounds, **2**, **4**, and a mixture of **5a** and **5b** were obtained from *C. montanum* for the first time. The cytotoxic activities of all the isolated compounds were first time screened against various human cancer cell lines. Compound **4** exhibited high selectivity between cancer and normal cells, especially superior cytotoxicity against HT-29 cell lines. Further studies are warranted in order to determine the mechanism of cytotoxicity of compound **4**. Our results are likely to contribute the future studies of anticancer constituents from medicinal plants.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2018.12.029.

Transparency document

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