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Original Article

A new tricyclic abietane diterpenoid from *Salvia chloroleuca* and evaluation of cytotoxic and apoptotic activities

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ABSTRACT

In this study, sahandone (**1**) and a new diterpenoid named sahandol II (**2**) were isolated from the roots of *Salvia chloroleuca* Rech. f. & Aellen, Lamiaceae. The absolute configurations of compounds **1** and **2** were assigned by comparison of experimental electronic circular dichroism spectra and comparing with published data. Cytotoxic and apoptotic evaluation of the isolated compounds and the methanol crude extract and its subfractions including petroleum ether, dichloromethane, ethyl acetate, *n*-butanol and aqueous fraction on two human prostate cancer cell lines and a breast cancer cell lines, showed that non-polar and semi-polar subfractions had the potent cytotoxic effect on PC3 cells with the IC_{50} values of 24.19, 33.59, and 47.15 μg/ml, respectively. Sub-G1 peak in flow cytometry histogram of cells treated with petroleum ether, dichloromethane and ethyl acetate subfractions showed the induction of apoptosis. Change in the Bax/Bcl-2 ratio and cleavage of poly ADP-ribose polymerase were observed.

Introduction

An ideal chemo-therapeutic agent is expected to inhibit or delay in the process of tumor growth through its cytotoxicity and apoptosis-inducing property (Kumar et al., 2012). Many of the current chemotherapeutic agents were originated from natural products or their derivatives (Shakeri et al., 2017). Phytochemicals are a good starting point for discovering new drugs. Many natural compounds isolated from plants were introduced as active cytotoxic and apoptosis-inducing agents (Ghorbani and Hosseini, 2015; Shakeri and Sahebkar, 2015). The genus *Salvia* is represented by over 1000 species which among 61 species in Iran, seventeen are endemic (Maberley, 2008; Jamzad, 2012). A broad spectrum of active natural compounds such as terpenoids and phenolics were found in the *Salvia* species, among them diterpenes have attracted great interest because of their significant biological activities especially cytotoxic and anti-tumor effects. For example salyunnanin D, tanshinone IIA, danshenol A, salyunnanin E, and castanol C as diterpenoids exhibited significant cytotoxic activity ($IC_{50} < 10$ μM) in HeLa cells (Wu et al., 2014). Also, there are many reports on the potent cytotoxic activity of different *Salvia* species. *S. lachnostachys*, *S. pachyphylla* and *S. ballotiflora* are among the cytotoxic species of the genus *Salvia* with strong activity on several human cancers (Guerrero et al., 2006; Campos-Xolalpa et al., 2017). In our previous studies, dichloromethane (DCM) fraction of *S. chorassanica* showed the most cytotoxic activity through induction of apoptosis by increasing caspases 3 and 8 activities in HeLa cells and DNA fragmentation in MCF-7 and DU 145 cell lines as well (Parsaee et al., 2012; Golshan et al., 2016). Taxodione, ferruginol and 6-hydroxysalvinolone three diterpenoids isolated from DCM fraction of *S. chorassanica* increased Bax, cleaved caspase-3 and cleaved PARP in HL-60 and K562 cells (Tayarani-Najaran et al., 2013). *S. chloroleuca* Rech. f. & Allen, Lamiaceae, is an endemic plant of Iran which grows wild in the northeastern and central parts of the country (Salimikia et al., 2016). In the literature the presence of diterpenoids in the root of the plant has reported (Salimikia et al., 2016). According to our knowledge, there is no more report on the isolation of bioactive compounds from the roots of *S. chloroleuca*. Hence, the cytotoxic

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and apoptosis-inducing effect of the MeOH crude extract, its sub-fractions and the two isolated diterpenoids from the roots of *S. chloroleuca* were investigated on PC-3, LNCaP and MCF-7 cancer cells.

Materials and methods

Chemicals and instruments

AlamarBlue® (resazurin) from Sigma (Saint Louis, MO, USA); β-actin, Bcl-2, Bax and PARP antibodies, anti-rabbit IgG and HRP linked antibody from Cell Signaling Technology (Boston, USA); ECL Western blotting detection reagent from Bio-RaD (USA); RPMI-1640 and FCS from Gibco; the fluorescent probe propidium iodide (PI), protease inhibitor cocktail, phosphatase inhibitor cocktail, sodium citrate, Triton X-100, phenylmethylsulfonyl fluoride and QuantiPro BCA Assay Kit from Sigma (Steinheim, Germany); all solvents as analytical grade were purchased from Dr. Mojallali Lab. (Tehran, Iran). Electronic circular dichroism (ECD) spectra were recorded in MeOH (120 μg/ml) on a Chirascan™ CD spectrometer. NMR spectra were recorded at a target temperature of 18 °C on a Bruker Avance III 500 MHz spectrometer operating at 500.13 MHz for ^{1}H, and 125.77 MHz for ^{13}C. A 1 mm TXI-microprobe with a z-gradient was used for ^{1}H-detected experiments. ^{13}C-NMR spectra were recorded with a 5 mm BBO-probe head with z-gradient. Spectra were analyzed using Bruker TopSpin 3.0 software. HRESI-MS spectra in positive and negative ion modes were recorded on a Bruker microTOF ESI MS system

Plant materials

The roots of *Salvia chloroleuca* Rech. f. & Aellen, Lamiaceae, were collected from Mount Binalud, Pivehzhan, Khorasan Razavi province in June 2016. Voucher specimen (No: 21617) was deposited in the herbarium of School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.

Extraction, fractionation and isolation

Dried roots of *S. chloroleuca* (100 g) were ground into powder and then were percolated with pure MeOH. Obtained extract was filtered and the solvent eliminated under reduced pressure at 45 °C. After drying the extract, the obtained powder was suspended in water and then partitioned with petroleum ether (Pet), DCM, ethyl acetate (EtOAc) and *n*-butanol (n-BuOH), successively. After that the solvents were removed under reduced pressure at 45 °C to afford different extracts. By the cytotoxic activity, DCM was chosen as the extraction solvent to finding bioactive compounds. The air-dried powdered material (350 g) was extracted with DCM (3 × 2 l × 24 h) at room temperature and the combined extracts were concentrated to give a crude extract (12 g). The latter was subjected to column chromatography over silica gel, and a step gradient system of Pet–EtOAc–MeOH was used with increasing of EtOAc and then MeOH contents from the ratio of 1:0:0 to 0:1:0 and then 0:0:1. The obtained sub-fractions were repeatedly subjected to reversed-phase semi-preparative HPLC using a C18 Eurospher column (Thermo Finnigan, Dreieich, Germany) (5 μM, 250 mm × 16 mm) with a gradient elution of MeOH and H_2O as an eluent including 0.1% TFA at a flow rate 9 ml/min within 30 min. Compounds **1** (12 mg) and **2** (6.8 mg) were obtained from fractions 8 and 6. This yielded sahandone (**1**) and sahandol II (**2**), respectively.

*Sahandol II (**2**)*

Yellow gumy material; UV (MeOH): λ_{max} (log ε) 198 (3.48), 244 (3.11), 339 (2.75); CD (MeOH, c = 0.05 mM, 1.0 cm): $[\theta]_{211}$ = −82648, $[\theta]_{244}$ = +21736, $[\theta]_{339}$ = +23700, $[\theta]_{386}$ = −20634; ^{1}H-NMR ($CDCl_3$, 500 MHz) and ^{13}C-NMR ($CDCl_3$,125 MHz), see Table 2. HRESIMS: m/z 335.1619 $[M+Na]^+$ (calcd. for $C_{20}H_{24}O_3Na$, 335.1623).

Bioassay

MCF-7, LNCaP and PC-3 cell lines were purchased from the Pasteur Institute (Tehran, Iran) and maintained at 37 °C in a 5% CO_2 atmosphere. All cell lines were cultured in RPMI-1640 with 10% (v/v) fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin.

In vitro *cell proliferation*

About 10^4 LNCaP, MCF-7 and PC3 cell lines were placed in each well of a 96-microwell plate and different concentrations of pure compounds (100, 50, 25, 2.5 μg/ml) and each extracts (200, 100, 50, 25, 2.5 μg/ml) of *S. chloroleuca* were treated. After 48 h incubation, 1% of total well content AlamarBlue® was added to each well and the absorbance was measured at 570 nm and 600 nm in a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, USA) after 4 h of incubation. The cytotoxic potential of isolated compounds and extracts were defined as IC_{50}. Doxorubicin (DOX) was used as positive control.

Propidium iodide staining

Apoptotic cells were determined by propidium iodide (PI) staining of treated cells followed by flow cytometry to detect the so-called sub-G1 peak (Mousavi et al., 2014). Briefly, 10^5 PC3 cell lines were seeded in each well of a 24-well plate and treated with various concentrations (200, 100, 50 μg/ml) of the PDE extracts of *S. chloroleuca* for 48 h. Floating and sticking cells were then collected and incubated at 4 °C overnight in the dark with 400 μl of a hypotonic buffer (50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100) before flow cytometric analysis using a FACScan flow cytometer (Partec GmbH, Münster, Germany) was used.

Western blotting analysis

10^6 PC-3 cell lines were treated with Pet and DCM extracts of *S. chloroleuca* at 50 and 100 μg/ml for 48 h. The cells were collected and washed with ice-cold PBS. Proteins were obtained after the cell pellet was resuspended in a lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.2% SDS, 1% protease inhibitor cocktail, 1% phosphatase inhibitor cocktail and 1 mM phenylmethylsulfonyl fluoride at 0 °C for 30 min. The protein content was equalized after centrifugation at 18,620 *g* for 20 min at 4 °C and subjected to 12.5% SDS-PAGE (w/v). The polyvinylidene fluoride (PVDF) membrane was subjected to β-actin, PARP, Bcl-2 and Bax as the primary antibodies and anti-rabbit IgG as secondary antibody and detected by enhanced chemiluminescence using the ECL Western blotting detection reagent. Images were quantified using the Gel-Pro Analyzer v.6.0 Gel Analysis Software.

Results and discussion

In this study, the cytotoxicity of different extracts (MeOH crude extract, PDE, BuOH and aqueous (H_2O) sub-fractions) of *S. chloroleuca* was evaluated *in vitro* against three cancer cell lines; LNCaP and PC-3 and MCF-7. Other authors have reported the cytotoxic activity of several *Salvia* species such as *S. miltiorrhiza*, *S. sahendica* and *S. menthaefolia*, on several types of tumor cells (Fiore et al., 2006; Fronza et al., 2011). As shown in

Table 1
In vitro antiproliferative activity of *Salvia chloroleuca* sub-fractions and compounds **1** and **2** against cancer cell lines (IC_{50}, μg/ml).

Cell lines	MeOH	PE	DCM	EtOAc	*n*-BuOH	H_2O	Sahandol (**1**)	Sahandol II (**2**)	DOX
MCF7	>200	33.39	39.55	32.00	>200	>200	>100	>100	2.78
PC3	143	24.19	33.59	47.15	>200	>200	>100	>100	2.49
LNCaP	166.6	62.22	38.53	49.48	59.38	23.33	>100	>100	1.55

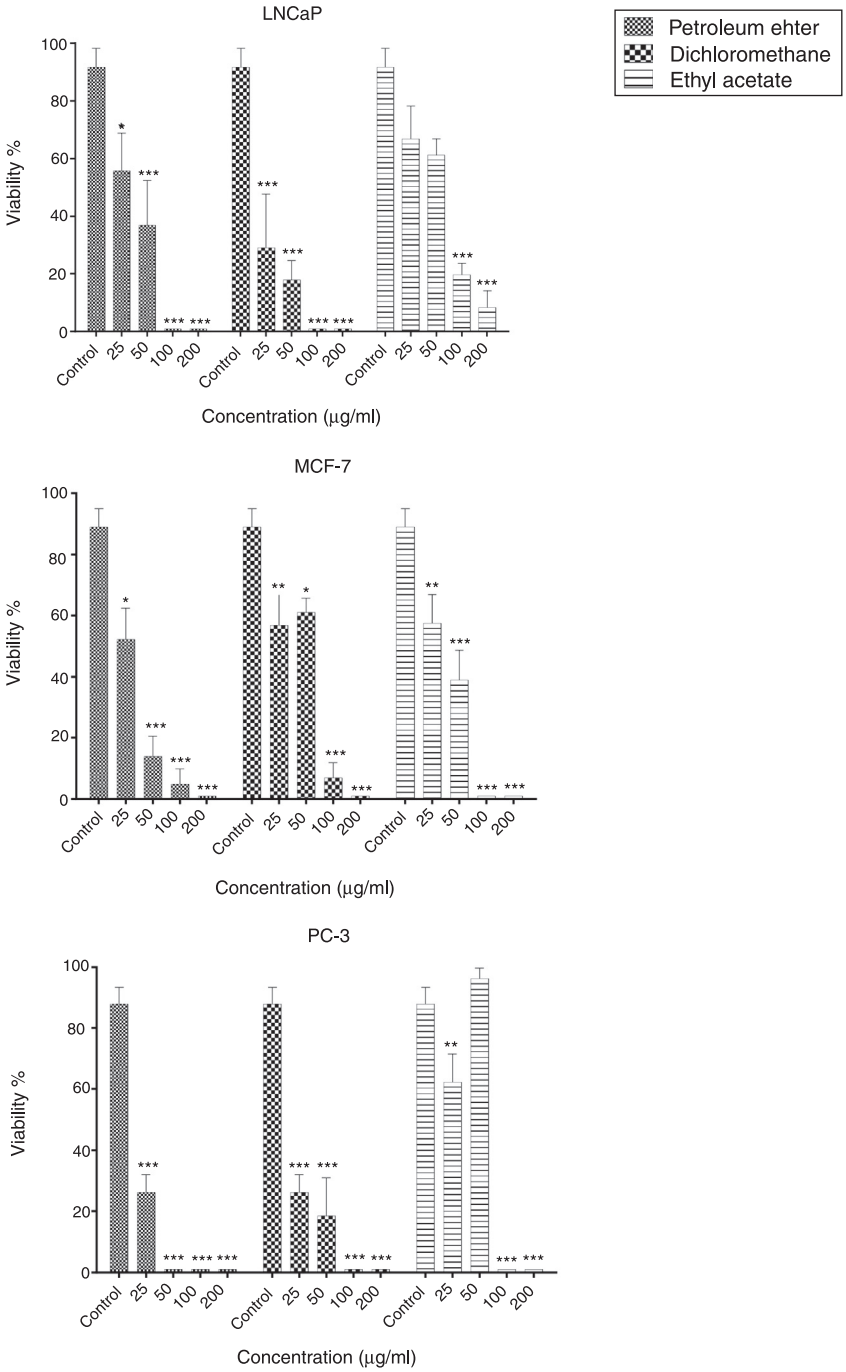


Fig. 1. The cytotoxic effects of the PDE fractions on three different cancer cells (PC-3, LNCaP and MCF-7). Cells were treated with different concentrations (25, 50, 100 and 200 μg/ml) for 24 h and cell viability was quantified by resazurin assay (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control). Results are mean ± SD. The test was done in triplicate ($n = 3$).

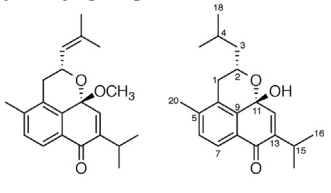
Table 1, our results indicated that the polar fractions of the plant including MeOH, *n*-BuOH and H_2O extracts had not or had lower cytotoxic activity compared to those of non-polar (Pet) and intermediate polar fractions (DCM and EtOAc) on MCF7 and PC3 cell lines. In this case, LNCaP, as an exception, showed odd behavior and was more susceptible toward polar fractions (*n*-BuOH and H_2O) with the IC_{50} values of 59.38 and 23.33 μg/ml, respectively. Generally, among all tested fractions, the PDE subfractions were shown to be more effective than the other ones ($IC_{50} < 100$ μg/ml) (Fig. 1).

Table 2
^{1}H and ^{13}C NMR spectral data of sahandol II (**2**) (500 MHz, δ in ppm, J in Hz, $CDCl_3$).

No.	^{1}H (J, Hz)	^{13}C	COSY	HMBC
1α	2.55, dd (16.9, 10.5)	30.0	H-2, H-1β	10, 3, 2
1β	2.77, dd (16.9, 4.4)		H-1α, H-2	5, 10
2	5.37, ddd (10.5, 8.5, 4.4)	65.6	H-1α, H-1β	3, 4, 11
3	5.42, dt (8.5, 1.2)	125.3	H-2, H-18, H-19	18, 19
4	–	136.7	–	–
5	–	136.9	–	–
6	7.11, d (7.6)	130.4	H-20, H-7	20, 8, 10, 14
7	6.99, d (7.6)	126.2	H-6	9, 5
8	–	127.8	–	–
9	–	132.4	–	–
10	–	133.3	–	–
11	–	90.1	–	–
12	6.95, br.s	135.6	–	14, 9, 15
13	–	139.8	–	–
14	–	195.4	–	–
15	2.97, sep (6.7)	27.2	H-12, H-17	17, 13
16	1.15, d (6.7)	21.5	H-15	13, 15, 17
17	1.16, d (6.7)	21.4	H-15	13, 15
18	1.79, d (1.0)	25.7	–	4, 3, 18
19	1.79, d (1.0)	18.7	–	
20	2.22, s	18.7	–	10, 5

Similarly, in a previous study, the hexane and DCM fractions of the extract from *S. chloroleuca*, showed significant cytotoxicity against the C32 cell lines with the IC_{50} values of 11.2 and 13.6 μg/ml, respectively (Tundis et al., 2011). Therefore, PDE subfractions were selected for subsequent studies. Since the IC_{50} values of DCM fraction were below the 50 μg/ml against all tested cell lines, we chose DCM extract to isolate the active compounds, which may be responsible for the cytotoxicity. Compounds **1** and **2** were isolated from the DCM extract. The structure elucidation of isolated compounds was carried out by spectroscopic analysis, using one- and two-dimensional NMR (^{1}H–^{1}H COSY, HSQC, HMBC, and NOESY) spectroscopic and HR-ESIMS experiments (Table 2). The molecular formula of compound **2** ($C_{20}H_{24}O_3$) was determined by ^{13}C-NMR and HR-ESIMS spectra (*m/z* 335.1619 $[M+Na]^+$, calcd. 335.1623). The ^{1}H-NMR spectrum showed resonances of five methyl groups at δ H1.15 (H-16), 1.16 (H-17), 1.79 (H-18), 1.79 (H-18), and 2.22 (H-20), three aromatic and one olefinic proton resonating at δ H 7.11 (H-6), 6.99 (H-7), 6.95 (H-12), and 5.42 (H-3), two methylene protons at δ H 2.77 (H-1β), 2.55 (H-1α), one methine proton at δ H 2.97, (H-15), and a signal of a deshielded proton at δ H 5.37, (H-2). HMBC correlations between H-16 and C-13 (139.8), C-15 (27.2), and C-17 (21.4) confirmed the attachment of the isopropyl moiety at C-13. The key HMBC correlations of H-12 with C-9 (132.4) and C-14 (195.4) and C-15 (27.2) were observed. HMBC correlations of H-1α with C-2 (65.5), C-3 (125.2) and H-2 with C-3 (125.2) and C-4 (136.6) confirmed the position of a vinyl group at C-2. In the NOESY spectrum, the correlations of H-1β and H-2 indicated that they were on the same side of the structure. Compound **2** was a new compound and named as sahandol II. The structure of the known compound, sahandone (**1**) was identified by comparisons of its observed and reported NMR data (Salimikia et al., 2016).

The comparison of spectral data of compounds **1** and **2** revealed as both belong to diterpenoids. A detailed analysis of NMR experiments showed that the structure of compound **1** was almost comparable with those of compound **2** except for the absence of a hydroxyl group which substituted by a methoxy group.



1 **2**

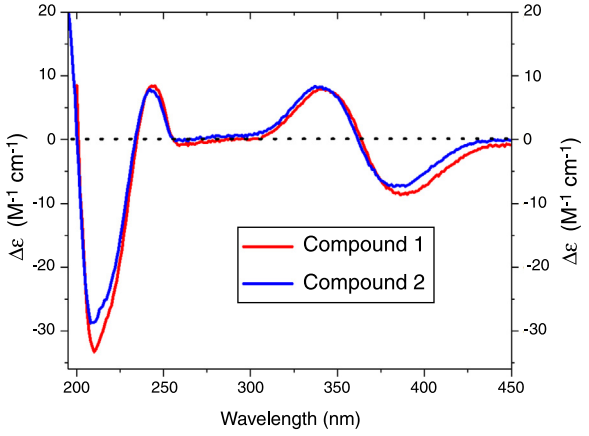


Fig. 2. ECD spectra of compounds sahandone (**1**) and sahandol II (**2**) in MeOH.

The absolute configuration of sahandol II (**2**) has been established by ECD measurement and comparing with published data (Ebrahimi et al., 2013). In the experimental compound **2** showed two negative Cotton effects (CE) on 390 and 211 nm along with two sequential positive CE at 340 and 243 nm (Fig. 2). Comparing data of **1** with those of **2** showed identical ECD spectra with two negative and positive CE, therefore the (2*R*,11*S*)-configuration established for **2**.

To assess whether the isolated compounds could decrease the viability, cancer cells were treated with 2.5–100 μg/ml of purified compounds for 48 h. However, they showed no significant cytotoxic effect (>100 μg/ml), which could be due to the required synergy of different compounds or maybe there are other compounds responsible for the cytotoxic activity in the extract. One of the essential mechanisms for the development and maintenance of tissue homeostasis is apoptosis (Li et al., 2016). Induction of apoptosis is therefore considered as one of the main strategies in the discovery of new anticancer therapeutics (Russo et al., 2006). In the present study, to find the role of apoptosis in the cytotoxicity of sub-fractions of *S. chloroleuca*, PC3 cells were incubated with various concentrations of PDE (50, 100 and 200 μg/ml) for 48 h and the sub-G1 peak in the flow cytometry histogram of cells were compared as an indicator for DNA fragmentation in apoptotic cells (Fig. 3).

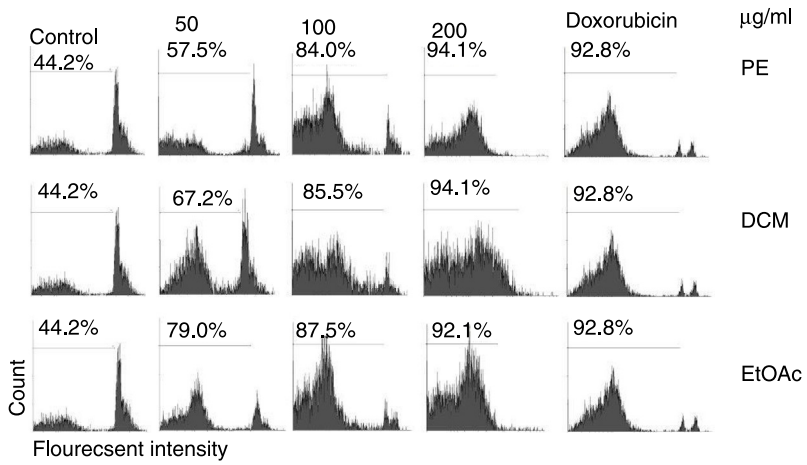


Fig. 3. Apoptotic activity of PE and DCM fractions in PC3 cells. Cells were treated with different concentrations (50, 100 and 200 µg/ml) for 24 h. Apoptosis was quantified by flow cytometry after PI staining of PC3 cells. The test was done in triplicate ($n = 3$).

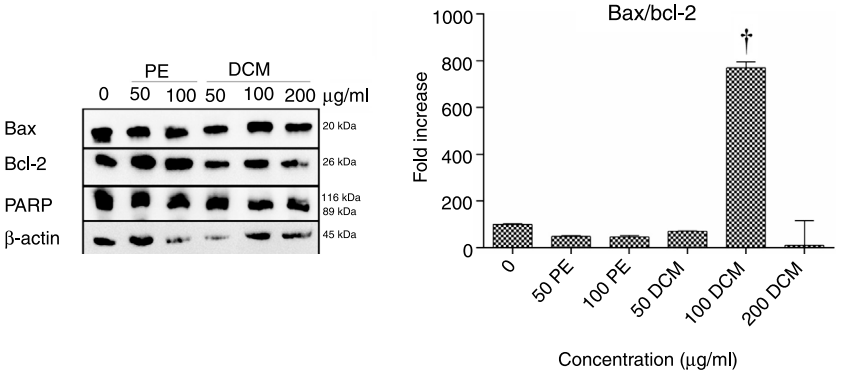


Fig. 4. The cleavage of PARP and protein level of Bcl-2, Bax in PC3 cells after western blot analysis. Cells were exposed to 50, 100 and 200 µg/ml for 24 h. β-Actin was used as a protein-loading control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control). The test was done in triplicate ($n = 3$).

As shown in Fig. 3, sub-G1 peak was increased in flow cytometry histograms of cells treated with PDE subfractions of *S. chloroleuca*, but not in untreated control cells. Apoptotic cell populations (sub-G1 peak) of treated cells with PDE were 13.3–49.9% compared to control. The results of flow cytometry histograms confirmed that the mechanism of cell death was mainly due to apoptosis, while necrosis has minimal or no effect on cells. Our results are in accordance with our previous study that the DCM fraction of *S. chorassanica* showed the strongest cytotoxic activity compared to the other polar fractions (Parsaee et al., 2012). The more cytotoxic activity of the non-polar and moderate polar fractions could be due to a higher concentration of cytotoxic agents found in the fractions. To further investigate the potential mechanism for PC3 cells to undergo apoptosis by *S. chloroleuca*, the western blot was used to detect apoptosis-related protein poly ADP-ribose polymerase (PARP), Bcl-2/Bax before and after the treatment with Pet and DCM subfractions of *S. chloroleuca*. Balance between pro-survival and antiapoptotic Bcl-2 proteins is an essential factor that determines cells undergo apoptosis or not. In fact, increase in the level of Bax protein can induce apoptosis, while up cells undergo apoptosis show decreased or unchanged level of Bcl-2 protein (Simsek and Uysal, 2013). In addition, to detect the cleavage of PARP, PC3 cells were treated with 50, 100 and 200 µg/ml of Pet and DCM subfractions for 48 h. A significant change in the ration of Bax/Bcl-2 and cleavage of PARP after 48 h was seen when cells were treated with 100 µg/ml of DCM fraction. There was also cleavage of PARP in cells treated with 100 and 200 µg/ml of DCM fraction. As shown in Fig. 4 change in the Bax/Bcl-2 ratio and cleavage of PARP were considered as a clue of apoptosis induction.

Previously our research group has shown that DCM fraction of the same plant had cytotoxic effect through the induction of apoptosis, which is associated with DNA fragmentation and ROS increasement in MCF-7 cells (20). A diversity of biochemical events are considered as markers of apoptosis which indicates the high complexity of the mechanisms of apoptosis. It has been reported that sibiriquinone A, sibiriquinone B, cryptotanshinone, and dihydrotanshinone I, cytotoxic diterpenoid compounds isolated from *S. miltiorrhiza*, induce apoptosis by inhibiting hypoxia-inducible factor-1 (HIF-1) expression (Wu et al., 2012). In another study in the same plant it was shown that tanshinon II, as a diterpenoid, induces apoptosis in HeLa cells through mitotic arrest (Zhou et al., 2008). However in our study isolated diterpenoids had no cytotoxic effect and further studies are needed to find the main compounds responsible for cytotoxic activity.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2018.09.007.

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