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Cytotoxic and apoptotic effects of root extract and tanshinones isolated from *Perovskia abrotanoides* Kar.

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ABSTRACT

Objective(s): *Perovskia abrotanoides* Kar., from family Lamiaceae, is a little known medicinal plant growing in various regions of Iran. In the present study, cryptotanshinone (CT), tanshinone 2A (Tan2A), and hydroxycryptotanshinone (HCT) were isolated and purified from the roots of *P. abrotanoides*. In addition, cytotoxic and apoptotic effects of total root extract (TE) and three purified tanshinones were investigated in human cervical carcinoma (HeLa) and human breast cancer (MCF-7) cell lines.

Materials and Methods: Alamar Blue® assay was used to determine cell viability. Cell apoptotic rate was detected using propidium iodide staining of DNA fragmentation by flow cytometry (sub-G1 peak). The PARP cleavage, as the sign of apoptosis, was investigated by Western blotting.

Results: The results revealed that CT, Tan2A, HCT, and TE exhibited significant cytotoxicity in cancer cell lines. All of these compounds caused apoptosis in treated cells and induced sub-G1 peak in the related flow cytometry histograms. HCT was found to have the highest anti-proliferative activity on cancer cells. Western blotting analysis showed cleavage of PARP protein in MCF-7 cells treated with purified tanshinones and TE after 48 hr contact with cells.

Conclusion: The findings suggest that root extract of *P. abrotanoides* and purified tanshinones especially Tan2A and HCT have cytotoxic and apoptotic effects against cancer cell lines. So, they may serve as potential cytotoxic agents for future investigations.

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Introduction

Medicinal plants, as main resources of pharmaceuticals, are the base of traditional medicine for the majority of the world's populations. In recent decades, increasing attention has been attracted to the study of plant secondary metabolites and bioactive compounds, in order to achieve drug discovery and development of novel drugs for the treatment of diverse ailments (1, 2). Screening of plants commonly and historically used in traditional medicines is a logical approach to find new sources of phytochemicals and drugs. So, the role of traditional plant products and their biological activities in finding new potent chemicals is crucial (2).

Perovskia abrotanoides Kar. (Lamiaceae) is one of the medicinal plants used in Iranian traditional medicine to treat leishmaniasis (3). According to the few scientific investigations carried out on this species, the plant also possesses anti-plasmodial, anti-inflammatory, anti - nociceptive, and antibacterial activities (3, 4).

It has been demonstrated that the roots of this little known medicinal plant are a rich source of lipophilic compounds named tanshinones (3, 5).

Tanshinones have attracted attention because of their large variety of biological and pharmacological activities, such as anti-diabetic (6), anti-allergic (7), antioxidative (8, 9), anticancer (10, 11), anti-fibrotic (1), antibacterial (12) and anti-inflammatory properties (13). Recent studies have shown that tanshinones have potent cytotoxic and anti-proliferative effects against various human tumor cell lines including human breast cancer (14, 15), leukemia (11), prostate cancer (16, 17), cervical cancer (10), ovarian cancer (18), and hepatocarcinoma cells (19) by inducing apoptosis and cell death.

Cancer, one of the main public health problems, is a leading cause of death all over the world. The World Health Organization (WHO) has predicted that the number of new cancer cases will reach 15 million every year by 2020 (20).

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The rising occurrence of cancer cases has resulted in a search for additional preventive and therapeutic modalities (15). Because of some serious problems including population growth, drug side effects, high cost of treatment, and increasing drug resistance, natural products in cancer prevention are still of therapeutic interest (21, 22). Plant derived natural products such as terpenoides have received wide attention in recent years due to their diverse pharmacological activities including cytotoxic and cancer chemopreventive effects (23). Since cancer cells usually exhibit active cell division, a helpful way of finding anticancer drugs is to test whether a compound can selectively kill mitotic cells thus blocking the cell cycle progression (24).

Activation of apoptosis pathways is a key mechanism by which cytotoxic drugs kill tumor cells. Thus, induction of apoptosis in cancer cells has now been considered as an indicator of the cancer treatment response and an important manner to evaluate the clinical effectiveness of many anti-tumor drugs (22, 25). Effective cytotoxic treatments with natural products increase apoptosis in cancer cells, but there are some limitations because of the side effects of these agents in normal cells. Scientific studies should be guided by using natural products for better understanding their modes of action and the ways of application.

This study aimed to investigate *in vitro* growth inhibition and apoptosis induction by *P. abrotanoides* Kar. We isolated and purified cryptotanshinone (CT), tanshinone 2A (Tan2A) and hydroxycryptotanshinone (HCT) from the roots of *P. abrotanoides*. Moreover, cytotoxic and apoptotic activities of the purified tanshinones and the total root extract (TE) against human cervix carcinoma (HeLa), human breast cancer (MCF-7) cell lines and human fibroblasts as normal cells were evaluated.

Materials and Methods

Chemicals and reagents

CT (purity ≥ 98%) and Tan2A (purity ≥ 98%) standards were purchased from Sigma Aldrich. AlamarBlue® from BioSource Invitrogen; doxorubicin (10 mg/5 ml) from Ebewe; Roswell Park Memorial Institute-1640 (RPMI-1640), Dulbecco's Modified Eagle medium (DMEM) and FCS from Gibco; β-actin and PARP antibodies, anti-rabbit IgG antibody from Cell Signaling Technology; ECL Western blotting detection reagent from Bio-RaD; the fluorescent probe propidium iodide (PI), protease inhibitor cocktail, phosphatase inhibitor cocktail, sodium citrate and Triton X-100 from Sigma; Silica gel (70– 230 mesh) for column chroma- tography, pre-coated silica gel Gf254 sheets and HPLC grade acetonitrile from Merck; the other solvents as analytical grade from Dr. Mojallali Lab.

Plant material

Roots of *P. abrotanoides* were collected from wild plants in Razavi Khorasan Province, Iran. The plant was identified by Mr Mohammad Reza Joharchi at the Ferdowsi University of Mashhad. A voucher specimen (no. 39299) was deposited at the Herbarium of Research Center for plant sciences, Ferdowsi University of Mashhad, Iran.

Extraction, isolation and purification of tanshinones

Air-dried and powdered roots (500 g) of *P. abrotanoides* were extracted with ethyl acetate (3×3 l, 24 hr each) at room temperature (3). The combined extracts were concentrated in a rotary evaporator below 40°C, followed by removal of residual solvent in a freeze dryer. The yield of extraction was about 3.84%. Three grams of extract was subjected to column chromatography over a silica gel column (100×5 cm, 500 g silica gel 70-230 mesh) eluted with a gradient system of petroleum ether (40 °C–60 °C) :ethyl acetate. Fractions of 250 ml were collected and monitored by TLC as the same mobile phase as column chromato - graphy with different polarity.

The similar obtained fractions were combined according to their TLC profiles.

For further purification, semi-preparative reversed-phase HPLC was performed on a Wellchrom Knauer system (Herbert Knauer GmbH, Germany) that consisted of a Knauer K-1001 pump, operating at 254 nm, using ACE 5 C18 column (250×21.2 mm, 5 µm) eluted with a gradient solvent system 20-100% MeCN - H_2O mixed with 0.05% TFA (v/v) at a flow rate of 10 ml/min. Structure of tanshinones was determined and confirmed by LC-MS, ^{1}H NMR, ^{13}C NMR and DEPT135 methods. LC-DAD-MS analyses were conducted on an Agilent 1200 series HPLC system consisting of an auto-sampler, high-pressure mixing pump, column oven, and DAD detector connected to a Agilent 6410 triple quadruple mass spectrometer. HPLC conditions: Agilent Eclipse XDB -C18 column (4.6 ×100 mm, 3.5 µm), solvent system A: 0.1% formic acid in H_2O, B: MeCN; isocratic 20% B for 6 min, 20-95% B over 18 min, 95-100% B over 6 min, 100% B for 6 min, 100-20% B over 6 min; flow rate: 0.5 ml/min; injection volume: 10 µl; column temperature : 25ºC; DAD condition: 254 nm.

ESI-MS conditions: positive ion mode; drying gas (nitrogen) flow rate: 10 L/min; nebulizing gas pressure: 15 psi; source temperature: 300ºC; ion spray voltage: 4 kv; scan range: 100-1000 amu.

NMR experiments were carried out on a Bruker Avance 400 MHz spectrometer in chloroform-*d*, using TMS as internal standard. The NMR data were processed using MestReC4999 software and compared to those reported in the literature.

TE was obtained by extraction of dried and milled roots of the plant with methanol (1:6 W/V) using the maceration method for 3 days. After every 24 hr, the mixture was filtered and new solvent was added to the plant powder. The combined extracts were concen-trated to dryness under vacuum pressure.

Sample Preparation

Stock solutions of TE and purified tanshinones (CT, Tan2A, and HCT) were prepared by dissolving them in dimethylsulfoxide (DMSO). Different concentrations of TE (1, 5, 25, 50, 100, and 200 µg/ml) and purified tanshinones (1, 5, 10, 25, and 50 µM) were then obtained by diluting stock solutions with media so that the final concentrations of DMSO did not exceed 0.05%. All dilutions were sterilized by filtering through a microfilter (0.2 µm) and prepared fresh before addition to the cells.

Cell Culture

HeLa and MCF-7 cell lines were obtained from Pasteure institute (Tehran, Iran) and respectively cultured in DMEM and RPMI-1640 media with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a humidified atmosphere of 90% air and 5% CO_2. Periodontal fibroblast cells were obtained according to Tayarani-Najaran *et al.* (2014) and cultured in DMEM medium in the conditions mentioned above (21).

Cell viability and cytotoxicity assay

The effects of purified tanshinones and TE on HeLa and MCF-7 cells proliferation were determined by AlamarBlue® assay (26). Briefly, cells were cultured overnight at a density of approximately 10^4 cells/well and then treated with various concentrations of CT, Tan2A, HCT (0-50 µM), and TE (0-200 µg/mL) for 48 hr. AlamarBlue® reagent was added to each well up to 10% of tissue culture medium and incubated for an additional 4 hr at 37 °C. The cell viability was determined by measuring the absorbance at 600 nm using Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, USA). Cells without tanshinone treatment which received an equal volume of the solvent, served as control. The cytotoxicity of root extract and purified tanshinones was determined as IC_{50}, which was calculated by Graph Pad prism version 5 software. Doxorubicin was used as a reference drug and positive control for the cytotoxicity evaluation of tanshinones.

PI staining

For the apoptosis assay, hypo-diploid DNA content was assessed by PI staining and flow cytometry. The procedure of Tayarani-Najaran *et al.* (2010) was employed (27). In brief, HeLa and MCF-7 cells were seeded overnight at a density of approximately 10^4 cells/well and then treated with different concentrations of CT, Tan2A, HCT, and TE for 48 hr. Floating and adherent cells were 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). The fluorescence of cells was analyzed using a FACScan flow cytometer (Becton Dickinson, USA). Sub-G1 peaks and apoptosis percentage were analyzed using WinMDI software (ver. 2.8).

Western blot analysis

MCF-7 cells were treated with TE (25, 50, and 100 µg/mL), CT, Tan2A, and HCT (each at 10 and 50 µM) for 48 hr. The cells were harvested and rinsed with cool PBS. The cell pellet was homogenized 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 on ice for 30 min. After centrifugation at 14000 rpm for 15 min at 4 °C, the cell supernatants were collected and protein concentration was determined according to Bradford assay and equal amounts of proteins were subjected to 10% SDS-PAGE (W/V). The proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% nonfat milk, the blots were subjected to immunoblotting using β-actin and poly (ADP ribose) polymerase (PARP) antibodies as primary antibodies and anti-rabbit IgG, HRP-linked antibody as secondary antibody. PARP cleavage was detected by enhanced chemiluminescence using the ECL Western blotting detection reagent (21).

Statistical analysis

All experiments were carried out in a completely randomized design. Data were subjected to Duncan's Multiple Range Test. All results were reported as means ± standard error of means (SEM) and $P \leq 0.05$ was considered as significant.

Results

Purification and identification of tanshinones from the roots of P. abrotanoides

Ethyl acetate extract of *P. abrotanoides* roots was fractionated by column chromatography. Semi-preparative HPLC of the active fractions led to purification of three tanshinones including CT, Tan2A, and HCT (purity > 95%) (Figure 1). The structure of HCT was determined by the NMR method. Spectral data of HCT are presented in Table 1. Two other compounds were identified as CT and Tan2A by comparison of their TLC profiles and LC-MS spectra with related standards.

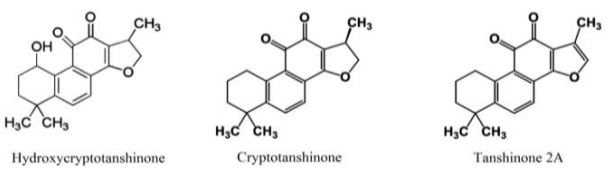

Figure 1. Molecular structures of three tanshinone compounds in *Perovskia abrotanoides* roots

Table 1. 400 MHz ^{1}H-NMR and 100 MHz ^{13}C-NMR spectral data for hydroxycryptotanshinone ($CDCl_3$)

Carbon number	^{13}C-NMR	Reference*	^{1}H-NMR	Reference*
1	63.4	(63.38)	5.06 (m)	5.04 (br,q), $J_{1,2}\approx J_{1,OH}\approx$3.5 Hz
2	26.9	(26.91)	2.11 (m), 1.91 (m)	2.11 (m), 1.88(m)
3	31. 9	(31.92)	1.57(m), 2.16 (m)	1.55 (m), 2.16 (m)
4	35.1	(35.13)	-	-
5	152.1	(152.11)	-	-
6	134.1	(134.09)	7.74 (d), $J_{6,7}$=8.0 Hz	7.72 (d), $J_{6,7}$=8.1 Hz
7	124.5	(124.54)	7.62 (d), $J_{6,7}$=8.0 Hz	7.61 (d), $J_{6,7}$=8.1 Hz
8	126. 9	(126.93)	-	-
9	129.8	(129.83)	-	-
10	143.0	(143.43)	-	-
11	186.3	(186.31)	-	-
12	175.4	(175.37)	-	-
13	118.5	(118.49)	-	-
14	170.8	(170.71)	-	-
15	34.5	(34.59)	3.62 (m)	3.61 (m)
16	81.8	(81.81)	4.42 (dd), 4.94 t	4.40 (dd), 4.92(t)
17	19.1	(19.13)	1.39 (d), $J_{15,17}$=7.2 Hz	1.37 (d), $J_{15,17}$=6.8 Hz
18	31.1	(31.19)	1.42 (s)	1.40 (s)
19	31.5	(31.57)	1.27 (s)	1.26 (s)

Multiplicity of signals is given in parentheses: s, singlet; d, doublet; t, triplet; q quartet; sp, septet; m, multiplet, br, broad; coupling constants (numeric values) are specified only once at the first occurrence of the coupled resonance
*Sairafianpour *et al.* 2001 (3)

Cytotoxicity of purified tanshinones and total extract isolated from the roots of P. abrotanoides

Results of the cytotoxicity assay showed that TE, CT, Tan2A, and HCT decreased cell viability in HeLa and MCF-7 cells in a concentration dependent manner after 48 hr. As indicated in Figure 2, TE severely inhibited cancer cells proliferation. HeLa cells were more sensitive to TE than MCF-7 cells.

Among the three purified tanshinones, HCT showed the most potent cytotoxicity against HeLa and MCF-7 cell lines and its IC_{50} values (17.55 and 16.97 µM for HeLa and MCF-7 cells, respectively) were considerably less than the values obtained for Tan2A and CT (Table 2).

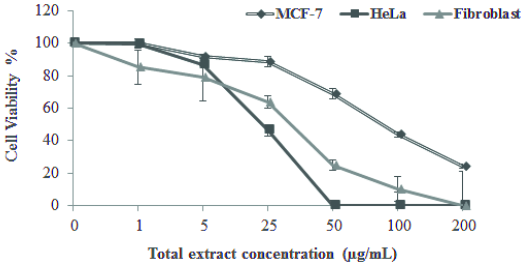

Figure 2. Growth inhibition of cancer cell lines (MCF-7 and HeLa) and normal cells (human fibroblasts) by different concentrations of total root extract of *Perovskia abrotanoides*. Cells exposed to increasing levels of root extract showed cytotoxicity. Data are mean ± SEM of three independent experiments, each in triplicates

Table 2. IC_{50} values for total extract (TE), cryptotanshinone (CT), tanshinone 2A (Tan2A), and hydroxycryptotanshinone (HCT) isolated from the roots of *Perovskia abrotanoides* in cancer cell lines and normal fibroblast cell.

Cell line	TE (µg/ml)	CT (µM)	Tan2A (µM)	HCT (µM)
	IC50			
Fibroblast	28.14	68.7	> 1000	84.79
HeLa	24.83	73.18	59.53	17.55
MCF-7	87.69	80.00	36.27	16.97

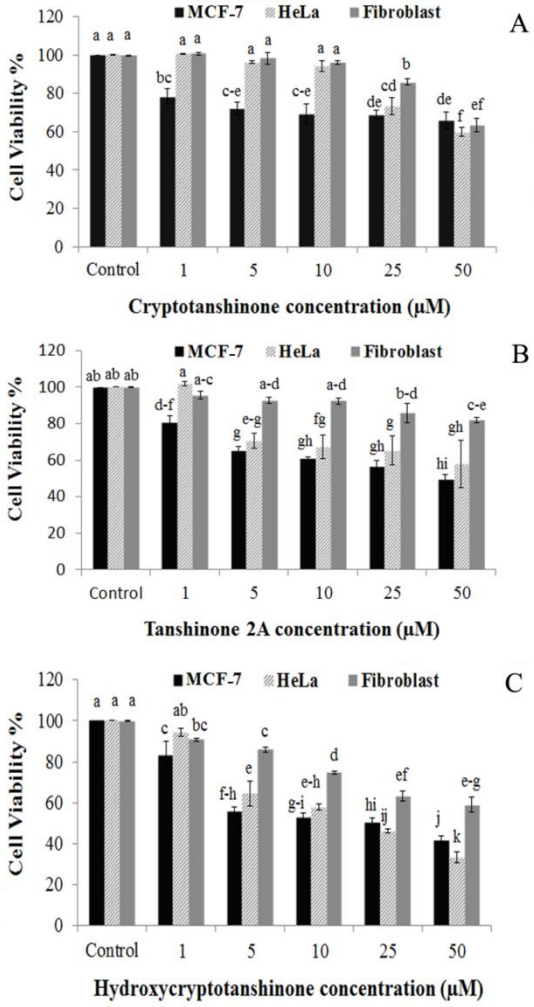

Figure 3. Inhibitory effects of different concentrations of crypto - tanshinone (A), tanshinone 2A (B), and hydroxycry- ptotanshinone (C) on HeLa, MCF-7 and fibroblast cell viability after 48 hr. Cancer cell lines were more sensitive to tanshinones treatment than normal fibroblast cells. Data are mean±SEM of three independent experiments, each in triplicates. Bars with different letters indicate significant difference (P≤0.05)

Besides, Tan2A decreased cancer cell viability more than CT (Figure 3). The alamarBlue® results demons - trated that treatment of HCT and Tan2A in the same

concentrations had a less cytotoxic effect on human fibroblast cells than cancer cells. In other words, the inhibitory effects of tanshinones, particularly Tan2A, on the normal cells were lower than their effects on the cancer cells. It seemed that MCF-7 cells were more sensitive to Tan2A and HCT treatments than HeLa cells (Figure 3). CT and Tan2A did not show significant cytotoxicity on normal fibroblast cells at the concentrations below 25 µM. The IC_{50} values of TE, CT, Tan2A, and HCT have been presented in Table 2. Doxorubicin at concentration of 0.92 µM was used as positive control. At this concentration, doxorubicin decreased HeLa and MCF-7 cell viability to 35.3% ± 2.71 and 41.5%±1.89 compared to untreated control cells, respectively (data not shown).

In general, the results suggest that tanshinones may have potent anti-proliferative and cytotoxic activity against cancer cells but limited unfavorable effects on normal cells.

Apoptosis induction by purified tanshinones and total extract isolated from the roots of Perovskia abrotanoides

The effects of TE and tanshinones on apoptosis induction in HeLa and MCF-7 cells were investigated by PI staining to detect sub-G1 peak resulted from DNA fragmentation. Incubation of cancer cells with various concentrations of TE (5, 25, 50, and 100 µg/ml), CT, Tan2A, and HCT (each at 5, 25, and 50 µM) for 48 hr induced apoptosis and led to accumulation of the sub-G1 region. Untreated control cells did not exhibit sub-G1 peak (Figure 4A). This indicates the role of the apoptosis process in cell death induced by tanshinone compounds.

TE induced apoptosis more effectively in HeLa cells than MCF-7 ones. At the 50 µg/mL concentration, 62.7% and 98.95% apoptosis occurred in MCF-7 and HeLa cells, respectively (Figures 4B and 4D). After treatment of HeLa and MCF-7 cells with 25 µg/mL TE, the sub-G1 cell populations were respectively 57.2% and 36.7% while the percentage of sub-G1 phase populations in untreated HeLa (4.5%) and MCF-7 cells (1.9%) were crucially decreased.

The presence of tanshinones also led to the significantly higher apoptosis percentage in MCF-7 and HeLa cells (Figures 4C and 4E). Among the three tested tanshinones, HCT was the most powerful compound to induce apoptosis in both cancer cells especially at a dose of 50 µM. In most cases, the rate of apoptosis in Tan2A-treated cells was also significantly higher than those obtained of CT treatments (Figures 4C and 4E). The crude root extract and isolated tanshinones enhanced the level of apoptotic cells in all treatments in a dose-dependent manner.

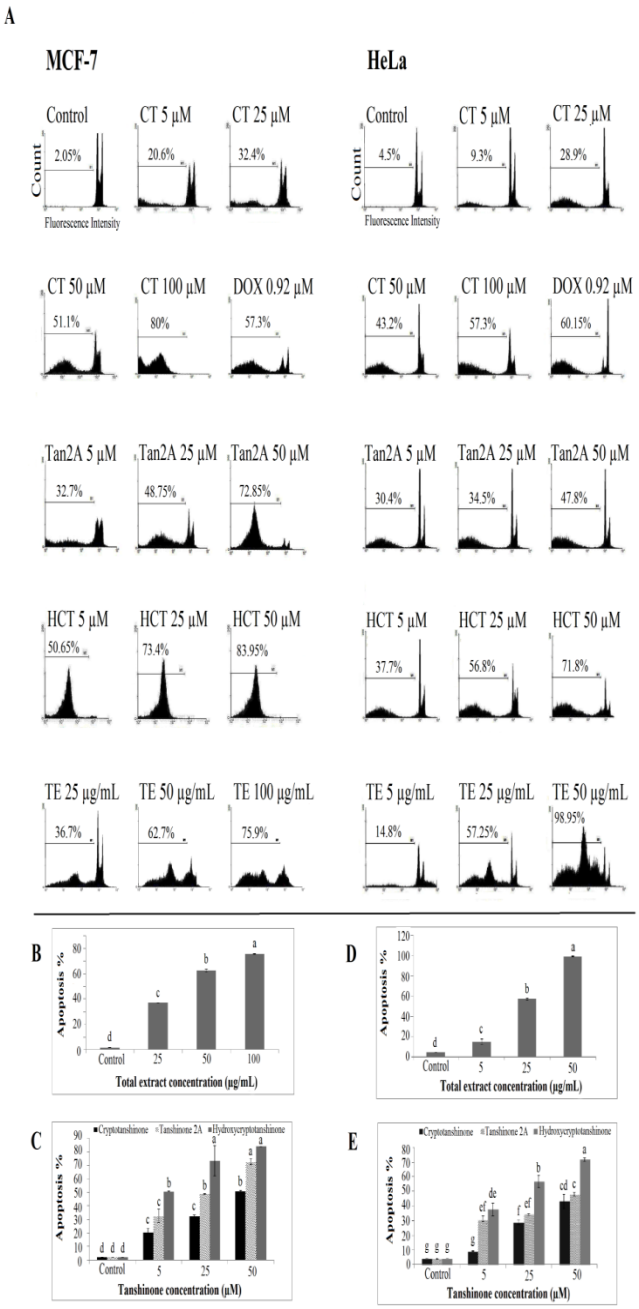

Figure 4. Flow cytometry histogram of apoptosis assay by PI staining method in MCF-7 and HeLa cells treated with different concentrations of isolated tanshinone and total root extract of *Perovskia abrotanoides* after 48 hr. Sub-G1 peak as an indicative of apoptotic cells induced in the cells treated with total root extract and tanshinones but not in control cells (A). Effects of different concentrations of total root extract and tanshinones isolated from the roots of *P. abrotanoides* on apoptosis percentage in MCF-7 (B and C) and HeLa (D and E) cells after 48 hr. Increasing levels of total root extract and tanshinones, increased the percentage of apoptosis. Data are representative of at least three independent experiments. Bars with different letters indicate significant difference ($P \leq 0.05$). CT: Cryptotanshinone; Tan2A: Tanshinone 2A; HCT: hydroxycryptotanshinone; TE: Total extract of roots; DOX: Doxorubicin

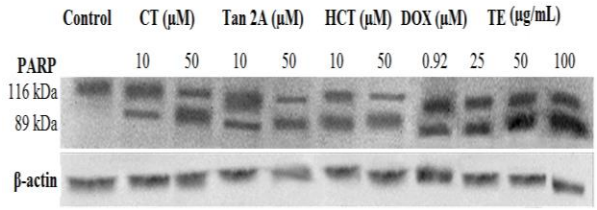

Figure 5. Western blot analysis of PARP of the MCF-7 cells after 48 hr exposure to different concentrations of total extract and tanshinones isolated from the roots of *Perovskia abrotanoides*. β-actine was used as loading control. All Western blots were representative of three independent experiments. CT: Cryptotanshinone; Tan2A: Tanshinone 2A; HCT: Hydroxycryptotanshinone; TE: Total extract of roots; DOX: Doxorubicin

Effect of purified tanshinones and total extract isolated from the roots of Perovskia abrotanoides on poly (ADP ribose) polymerase cleavage

Proteolytic cleavage of 116 kDa PARP to 89 kDa fragment is considered as a biochemical hallmark of apoptosis. Western blot analysis showed that PARP protein was cleaved after exposure of MCF-7 cells to different concentrations of TE (25, 50, and 100 µg/ml), CT, Tan2A, and HCT (10 and 50 µM) for 48 hr (Figure 5).

The results of Western blot assay together with the results obtained from PI staining and flow cytometry indicated that the crude root extract and isolated tanshinones of *P. abrotanoides*, at least in part, inhibited the proliferation of cancer cells by inducing apoptosis.

Discussion

The application of medicinal plants to make useful drugs for various diseases and also as a source of effective anticancer agents has attracted recent attention. Due to an increasing rate of mortality associated with cancer and adverse or unwanted side effects of cancer chemotherapy and radiation therapy, discovery of new anticancer natural products and the screening of medicinal plants for this purpose have become the focus of attention (28). It has been proven that biologically active compounds derived from medicinal plants play a key role in integrative cancer treatment and healing (11, 23, 27). Several drugs are used to treat and prevent the development of the tumor, but they are not always effective and usually are accompanied with side effects. Alternative treatments based on plant phytochemicals might be a potential safe candidate to treat cancer (29). Recent studies have shown that tanshinones, which are widely applied to treat cardiovascular diseases, inhibit the proliferation of different cancer cell lines (8, 11, 15, 25).

In this study, the fractionation of root extract of *P. abrotanoides* by column chromatography and then HPLC analysis of the fractions led to purification of three diterpenoids named cryptotanshinone, tanshinone 2A, and hydroxycryptotanshinon. It should be noted that four tanshinones including cryptotanshinone, hydroxycryptotanshinone, oxocryptotanshinone, and oxomiltrione were isolated from this source by Sairafianpour *et al* (3). To the best of our knowledge, there have been no published reports on the existence of Tan2A in the roots of *P. abrotanoides*.

The results demonstrated that TE had cytotoxic effect against HeLa and MCF-7 cells and significantly decreased cell viability. Regarding the presence of CT, Tan2A, and HCT in the roots of *P. abrotanoides*, some of the biological activities of the plant might be related to these compounds. The cytotoxicity of *P. abrotanoides* root extract on MCF-7 and WEHI cells has also been reported (30). Other studies have indicated that total extract of the roots of *Salvia miltiorrhiza*, another rich source of tanshinons, prevented the proliferation of human hepatoma HepG2 (31) and P388 lymphocytic leukemia cells (32). Crude natural extract is usually a complex mixture of various substances which have different physicochemical and pharmacological properties (27). The cytotoxic property of root extract of *P. abrotanoides* against cancer cells could be attributed to the synergistic effects of tanshinones. The use of total plant extracts in biological assays provides an assessment for the synergistic and/or antagonistic interactions between different components in the crude extract. To better understand the biological activity and decrease possible side effects attributed to the chemical constituents of plant materials, further investigations on the individual pure substances are necessary (33).

The findings of the present study indicate that treatment of HeLa and MCF-7 cells with CT, Tan2A, and HCT significantly inhibited cell proliferation in a dose dependent manner. The effect of tanshinones especially Tan2A and HCT on malignant and normal cells showed a degree of specificity for malignant cells. It has been shown that CT inhibited the proliferation of epidermoid cancer (KB-3-1) (3), urinary bladder cancer (5637), Lung cancer (A-427), and breast cancer (MCF-7) cell lines (34). Similarly, the cytotoxicity, growth inhibition, and apoptosis induction of CT have been demonstrated on DU145 prostate cancer cells (17) and P 388 lymphocytic leukemia cells (32). On PC3 prostate cancer and HepG2 cells, CT caused cell cycle arrest at the S phase and thus inhibited cell proliferation (16, 19). Our results showed the potent cytotoxic activity of Tan2A against malignant cells (IC_{50} values for HeLa and MCF-7 cell lines were 59.53 and 36.27 µM, respectively). Interestingly, normal fibroblast cells were significantly resistant to cytotoxicity induced by this secondary metabolite (IC_{50}>1000 µM). Tan2A effectively inhibited the proliferation of different cancer cell lines including PC3 prostate cancer (16), HeLa (10), leukemia THP-1 (11), and human breast cancer COC1/DDP (14). Studies carried out on HeLa, PC3, and HepG2 cancer cells revealed that Tan2A affects cell cycle progress and arrests the cells at the G2/M phase (10, 16, 19).

In the present study, treatment of HeLa and MCF-7 cells with HCT induced strong cytotoxicity. The inhibitory effect of HCT on KB-3-1 cancer cells growth

has been shown by other researches (3). Mothana *et al.* (2009) have also reported the potent cytotoxic effect of HCT, isolated from the roots of *Meriandera benghalensis*, on urinary bladder cancer (5637), lung cancer (A-427) and MCF-7 cell lines (34). According to our results, among three tanshinones studied, HCT was the most powerful cytotoxic compound and its IC_{50} values were less than 18 µM in both investigated cancer cell lines. Besides, Tan2A had stronger anti-proliferative activity than CT. Consistent with this finding, higher cytotoxicity of Tan2A than CT has also been reported in PC3 (16), HepG2 (19), and P388 cancer cells (32). The lower IC_{50} value for HCT could suggest this substance as a promising therapeutic candidate to treat human cervix and breast cancers.

Because of apoptosis induction by many anticancer drugs in cancer cells, the apoptosis inducing activity of TE of *P. abrotanoides* and purified tanshinones were also investigated. The qualitative and quantitative analysis of apoptosis events are considerably important in order to determine and confirm the mechanism of action played by the extract (35). Root extract and three purified tanshinones induced apoptosis in HeLa and MCF-7 cells significantly and increased cell population in the sub-G1 phase. This indicated that apoptosis was involved in the cell death and cytotoxicity of root extract and tanshinones in HeLa and MCF-7 cells. So far no study has been conducted on apoptosis induction by the root extract of *P. abrotanoides* on cancer cells. The roots of *P. abrotanoides* contain tanshinone compounds causing apoptosis, thereby revealing its potential cytotoxicity. This cytotoxic activity can be exploited in cancer treatment, leading cancer cells to apoptosis. So, this little known medicinal plant can frequently serves as a source of new drugs with little or no side effects.

The compound HCT was more efficacious in apoptosis induction than Tan2A or CT while the apoptotic activity of CT was less than the other compounds. The results were consistent with the results of alamarBlue® assay. Taken together, according to the results of PI staining and alamarBlue® assays, HCT was the strongest apoptosis inducing agent while the weakest activity belonged to CT. Thus HCT can act as a powerful antiproliferative substance on HeLa and MCF-7 cells. To the best of our knowledge, there is no other report in the literature showing the possible effect of HCT on apoptosis induction in cancer cell lines. The higher apoptosis induction of Tan2A than CT has also been reported in the PC3 cell line (16). Apoptosis is a well-known biological response expressed by cells after suffering DNA damage and is a useful marker for screening compounds for subsequent development as possible anticancer agents (23). Apoptotic cell death is known to be induced by many chemotherapeutic substances routinely used to treat cancer. Normal development of organs is controlled by a balance between cell proliferation and apoptosis (36, 37). On the other hand, apoptosis is a key regulatory of tissue homeostasis, and imbalances between cell death and proliferation may result in tumor formation. The objective of using anticancer agents is to induce apoptosis related signaling pathways and disrupt cell proliferation (23). Thus, induction of apoptosis in cancer cells is considered as a valuable manner to treat cancer. In addition, a wide variety of natural secondary metabolites have been identified to have the potential of apoptosis induction in different cancer cell lines. So, screening of apoptosis inducers from the plants, either as crude extract or as pure compounds isolated from them, is important (36, 37).

PARP is a nuclear enzyme which is involved in DNA repair process and this 113 KDa protein is cleaved to 89 KDa and 24 KDa fragments by Caspase 3 protease (38). Treatment of MCF-7 cells with CT, Tan2A, HCT, and TE caused apoptotic cell death, as indicated by PARP cleavage.

Conclusion

In summary, it can be concluded that root extract of *P. abrotanoides* and isolated tanshinones (CT, Tan2A, and HCT) can decrease cell viability in MCF-7 and HeLa cell lines in which induction of apoptosis partly could be involved. To our knowledge, induction of apoptosis by the roots of *P. abrotanoides* and HCT are reported for the first time. So root extract of this species and the secondary metabolites purified from the roots could be considered as promising candidates for further investigations to develop natural anticancer drugs.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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