Research Journal of Pharmacognosy (RJP) 10(2), 2023: 1–8
Received: 3 Aug 2022
Final revision: 1 Feb 2023
Accepted: 14 Feb 2023
Published online: 19 Feb 2023
DOI: 10.22127/RJP.2022.354699.1953

Original article

Lichenochemical Analysis and Cytotoxicity of *Diploschistes ocellatus* (Fr.) Norman

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Abstract

Background and objectives: *Diploschistes ocellatus* (Fr.) Norman is a valuable lichen possessing various biological properties which has been traditionally used by indigenous people in southwest of Iran in the treatment of different disorders. The aim of the current study was to evaluate cytotoxicity of different fractions of *D. ocellatus* against breast cancer cell lines through MTT assay, as well as lichenochemical analysis of the most potent fraction. Also, ducking study was performed to investigate the isolated compounds-protein interactions. **Methods:** In this work, aqueous, acetone, chloroform, ethyl acetate, and methanol fractions of *D. ocellatus* were evaluated against three breast cancer cell lines (MCF-7, T-47D, and MDA-MB-231) via MTT assay. Furthermore, docking was performed using the routine method and default parameters of the AutoDock 4.2 software. **Results:** The acetone fraction depicted the most potent cytotoxicity and was candidate for lichenochemical analysis, leading to the isolation and identification of stictic acid and 2-(7'-hydroxy-3,5,6,8-tetramethyl-9-oxooxonan-2-yl) propanoic acid. Docking study of isolated compounds based on the inhibition of survivin, revealed desired interactions with that of amino acid residues. **Conclusion:** Based on the obtained results, *D. ocellatus* can be considered as a natural source of biologically active compounds and complementary studies are in high demand.

Keywords: breast cancer; *Diploschistes ocellatus*; isolation; lichens; molecular docking simulation

Citation: Eftekhari M, Sohrabi M, Balaei Kahnamoei M, Oskou F, Abdshahzadeh H, Mansourian M, Saeedi M, Shams Ardekani MR, Akbarzadeh T, Khanavi M. Lichenochemical analysis and cytotoxicity of *Diploschistes ocellatus* (Fr.) Norman. Res J Pharmacogn. 2023; 10(2): 1–8.

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Introduction

Lichens are a group of composite organisms created from algae or cyanobacteria living among the filaments of the fungi in a symbiotic relation. They grow on tree barks, rocks, walls and soils, distributed in various ecosystems worldwide [1]. Lichens secondary metabolites have shown divers biological properties such as enzyme inhibitory, antioxidant, antifungal, antimicrobial, anticancer, antiviral, and anti-insecticidal activities [2]. Lichens are called "hazaz-al-sakhr" in Persian medicine and their application has also been considered. They have been used in wound healing as well as the treatment of bleeding and skin diseases such as eczema and inflammations [3].

Lichens produce a wide range of active compounds such as amino acid derivatives, sugar alcohols, aliphatic acids, macrolytic lactones, monocyclic aromatic compounds, quinines, chromones, xanthones, dibenzofurans, depsides, depsidones, depsones, terpenoids, steroids, carotenoids, and diphenyl ethers possessing divers biological activities [4]. In this respect, 2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2*H*,9b*H*)-dibenzo-furandione known as usnic acid as well as its semisynthetic derivatives have shown important biological activities and their anti-cancer activity has attracted a special attention [5]. Also, strong cytotoxic effect of depsides and depsidones has been completely proven in the literature [6].

Diploschistes (Thelotremataceae family) is a genus of crustose lichens, containing 43 species, which grow in the arid and semiarid regions of the world. Among different species, *Diploschistes ocellatus* (Fr.) Norman (synonym of *Xalocoa ocellata* (Fr.) Kraichak, Lücking & Lumbsch) has been traditionally used by indigenous people in the southwest of Iran. Antimicrobial and antioxidant activities of *D. ocellatus* have been documented in the literature [7, 8]. Also, Mendili et al. reported the important DPPH scavenging capacity and iron-chelating activity of the *D. ocellats* methanol fraction while the highest ferric-reducing power were obtained with acetone extract [8].

Despite a large amount of effort in the treatment of cancer, some aspects of the therapeutic strategies have still remained ambiguous due to drug resistance, which is usually related to more than one anti-cancer drug, known as multi-drug resistance (MDR). Treatment of cancer using chemotherapy is generally accomplished by inducing apoptosis, the process of programmed cell death, via different pathways. Apoptosis is regulated by different pro-apoptotic and anti-apoptotic proteins such as inhibitors of apoptosis (IAPs) and B-cell lymphoma 2 (Bcl-2) families. Dysregulation of apoptosis has been known as the major reason of MDR[9].

Caspases (cysteinyl aspartate-specific proteases) play critical roles in the process of apoptosis. They are expressed as inactive pro-enzymes and contain an N-terminal pro-domain and a C-terminal catalytic domain. Human caspases can be divided into two categories: initiator caspases (caspase 2, caspase 8, caspase 9 and caspase 10), and effector caspases (caspase 3, caspase 6 and caspase 7). The fate and sequel of each cell ultimately depends on the cross talking of pro-apoptotic and anti-apoptotic signaling pathways. Survivin is the smallest member of the IAPs. It directly inhibits the activity of caspases 3 and 7 through the baculoviral IAP repeat (BIR) domain leading to the inhibition of their protease activity, and ultimately prevents downstream apoptotic events [10, 11]. Survivin is now considered as a cancer specific biomarker and cancer drug target, and related inhibitors have occupied a specific position in the cancer therapy [12].

To the best of our knowledge, there is no report on the investigation of cytotoxicity of *D. ocellatus*. In this regard, various fractions of *D. ocellatus* were evaluated against three breast cancer cell lines including MCF-7, T-47D, and MDA-MB-231. Moreover, lichenochemical analysis was conducted on the most potent fraction and docking study was performed to show that isolated compounds are involved in the inhibition of survivin.

Material and Methods

Ethical considerations

This study was approved at Institute of Pharmaceutical Sciences, Tehran University of Medical Sciences with the ethical code: IR.TUMS.TIPS.REC.1401.107

Chemicals

All solvents and materials were used of analytical grade. Silica gel with different mesh (Merck, Germany), aluminium TLC plates (Si gel 60 F254 and RP_18 F254) (Merck, Germany), Sephadex LH-20 (Fluka, Switzerland), and RP-

18 (Fluka, Switzerland) were used for the isolation of compounds. The medium of RPMI 1640 (PAA, Germany), *N*-hydroxyethylpiperazone-n-2-ethanesulfonic acid (HEPES, Biosera, England), fetal bovine serum (FBS; Gibco, USA), and MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, USA) were used for the cytotoxic evaluation.

Collection of the lichen

The lichen specimen was collected from Kohgiluyeh va Boyer Ahmad province, located in the south-west of Iran in October 2017. It was identified and authenticated by the lichenologist, Dr. Mohammad Sohrabi and deposited in the ICH Lichen Herbarium of Iranian Research Organization for Science and Technology (ICH Number: 16670).

Fractionation

The collected sample was cleaned, dried at ambient temperature, and ground. The powder (700 g) was divided into two parts. One part (350 g) was successively extracted with chloroform, ethyl acetate, and methanol by maceration. Another part (350 g) was similarly extracted using acetone and distillated water, respectively. Each fraction was separately filtered using a Buchner funnel, concentrated under vacuum, and then completely dried. Dry residues were stored in fridge for further experiments [13].

MTT assay

Three different human breast cancer cell lines (MCF-7, MDA-MB-231, and T47D) were obtained from Pasture Institute of Iran, Tehran, Iran. The medium of RPMI 1640 containing sodium bicarbonate and *N*-hydroxyethylpiperazone-n-2-ethanesulfonic acid was used to maintain the cell lines. The medium was enriched with fetal bovine serum and antibiotics including streptomycin (100 µg/mL) and penicillin (100 U/mL) and then, was incubated in air atmosphere enriched with 5% CO_2 at 37 °C. The cytotoxic activity of *D. ocellatus* fractions was evaluated by the MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) assay as described in detail in our previous report [14].

Isolation and purification of compounds

The acetone fraction (3 g) was subjected to silica gel column chromatography (30-70 mesh, 3×45 cm) and eluted with a gradient mixture of chloroform/acetone (10:0 to 0:10) and then, acetone/methanol (10:0 to 0:10) to obtain ten fractions (AC1-AC10). Fraction AC6 (110 mg) was placed on Sephadex LH-20 (2× 60 cm) and eluted with methanol to obtain six fractions (AC6a-AC6f). Compound **A** was afforded from fraction AC6c by recrystallization from n-hexane. Fraction AC6f was subjected to Sephadex LH-20 column chromatography (2× 60 cm) and eluted using methanol to obtain five fractions (AC6f1-AC6f5). AC6f2 was isolated as compound **B** [15].

Docking study

To understand the ligand-protein interactions, docking study was performed using the routine method and default parameters of the AutoDock 4.2 software [16]. It was conducted using the anti-apoptotic protein survivin as one of the targets (receptors) associated with apoptosis. The procedure was performed according to the protocols reported in the literature [17, 18]. The human receptor sequence was received from the NCBI (www.ncbi.nlm.nih.gov). The three-dimensional X-ray structure of the human receptor was used for docking study, downloaded from the RCSB PDB (http://www.rcsb.org).

Survivin is a dimeric protein consisting of A and B chains. The A and B chains have almost the same amino acid sequences, and the chain B only has two additional amino acids at the C-terminus (Met, Asp). The chain A was selected for docking study [19]. The two-dimensional structure of the identified compounds was mapped using ChemOffice 2015 and optimized using HyperChem 7.0 (Hypercube, Inc., Gainesville, FL, USA; http://www.hyper.com). Details of the optimization method have been previously described [17, 18].

Since the binding site of the ligand in the complex is determined through a site-directed mutagenesis, the grid box on survivin (1E31) with a resolution of 2.71 Å [20] was placed at coordinates of X = -24.532, Y = 36.317, and Z = 64.94 [21]. AutoGrid 4 was used to set the network parameter file through the values of axes x, y and z with the grid box dimensions of 60×60×60. The distance of the network point 0.375 Å was considered as the default value. The cluster analysis was performed at the end of the docking test with 200 cycles [18]. Lamarckian genetic algorithm (LGA) program at Autodock was used to calculate different ligand conformers [22].

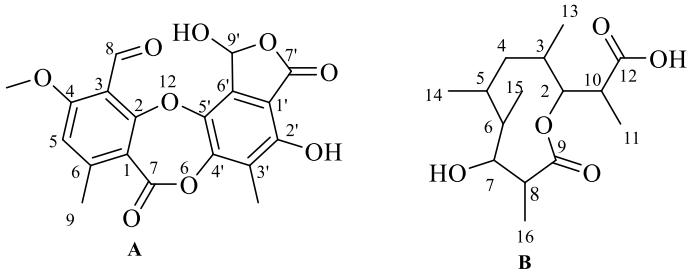

Figure 1. The structure of isolated compounds A (stictic acid) and B **(**2-(7'-hydroxy-3,5,6,8-tetramethyl-9-oxooxonan-2-yl) propanoic acid) from of *Diploschistes ocellatus*

The binding energy was calculated using AutoDock 4.2. Among the various configurations of the interactions between studied compounds and the survivin target, the configuration with the best mode and the lowest binding energy was selected (Table 1). The two-dimensional mode was analyzed visually (graphically) to understand the interaction pattern with the LigPlot software [23].

Statistical analysis

All data were analyzed by Microsoft Excel 2010 and presented as means ± SD of triplicate experiments.

Results and Discussion

The structure of isolated compounds (**A** and **B**) from *D. ocellatus*, was identified based on the NMR spectra as shown in Figure 1.

Compound **A** (10 mg) was obtained as white crystals and identified as stictic acid since the obtained data was in good agreement with those available in the literature [24].

^{1}HNMR (500 MHz, DMSO-d_6): 10.43 (1H, s, CHO-8), 7.09 (1H, s, H-5), 6.86 (1H, s, H-9'), 3.84 (3H, s, MeO-4), 2.46 (3H, s, Me-9), 2.20 (3H, s, Me-8'). ^{13}CNMR (125 MHz, DMSO-d_6): 194.4 (C-8), 166.6 (C-2), 163.0 (C-7'), 162 (C-4), 161.45 (C-7), 155.0 (C-2'), 153.5 (C-6), 143.8 (C-4'), 142.8 (C-5'), 142.0 (C-6'), 117.8 (C-3'), 112.0 (C-3), 111.3 (C-1), 111.0 (C-5), 108.6 (C-1'), 95.8 (C-9'), 56.7 (C-4-OMe), 22.1 (C-9), 10.1 (C-8').

Compound **B** was isolated as a white and amorphous solid and appeared as dark blue spots when sprayed with *p*-anisaldehyde-sulfuric acid and heated. The molecular formula of $C_{15}H_{26}O_5$ was obtained from EIMS (m/z 286.6 $[M]^+$; calcd. 286.18), accounting for three degrees of unsaturation. The ^{13}C-NMR spectrum revealed 15 carbon signals, which were identified with the aid of HSQC and DEPT spectra as five methyl, a methylene, seven methane, and two carbonyl groups. The ^{1}H-NMR spectrum displayed the characteristic signals of four methyl doublets (δ_H 0.86, 0.89, 1.08, 1.16), one methyl broad singlet (brs) (δ_H 0.84), an oxygenated methine (δ_H 3.72, dd, J = 9.5 Hz, J = 2.1 Hz). Unambiguous assignment of all protons and their connectivity was achieved with the aid of ^{1}H-^{1}H COSY, HSQC and HMBC experiments (Fig. 2).

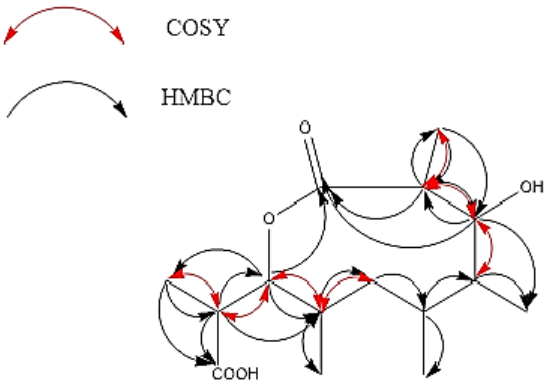

Figure 2. ^{1}H-^{1}H COSY (red arrows) and ^{1}H-^{13}C HMBC (black arrows) correlations observed in compound B

In this respect, the structure of compound **B** was elucidated to be 2-(7'-hydroxy-3,5,6,8-tetramethyl-9-oxooxonan-2-yl) propanoic acid.

^{1}H-NMR ($CDCl_3$, 400 MHz): δ 0.84 (3H, bs, H-15), 0.86 (3H, d, J = 2.1 Hz, H-14), 0.89 (3H, d, J = 3.3 Hz, H-13), 0.9 (1H, bs, H-4α), 1.08 (3H, d, J = 7.1 Hz, H-16), 1.16 (3H, d, J = 7.2 Hz, H-11), 1.25 (1H, bs, H-4β), 1.45 (1H, m, H-5), 1.71 (1H, m, H-6), 1.90 (1H, m, H-3), 2.60 (1H, m, H-8), 2.77 (1H, m, H-10), 3.72 (1H, dd, J = 2.1 Hz, 9.5 Hz, H-7), 5.17 (1H, dd, J = 2.1 Hz, 9.7 Hz, H-2).

^{13}C-NMR ($CDCl_3$, 100 MHz): δ 11.1 (C-14), 12.5 (C-15), 13.7 (C-11), 13.8 (C-13), 14.5 (C-16), 30.7 (C-3), 30.8 (C-6), 31.2 (C-5), 41.1 (C-4),

42.2 (C-10), 44.2 (C-8), 74.7 (C-7), 76.4 (C-2), 175.8 (C-9), 178.6 (C-12).

According to the phylogenetic investigation of *Diploschistes* genus conducted by Fernández-Brime et al., *D. ocellatus* is a monotypic subgenus classified in *Diploschistes* subg. *thorstenia*. The criterion that distinguishes this species from others is the presence of β-orcinol depsidones instead of orcinol depsides. In the present study, stictic acid belonging to the β-orcinol depsidones was isolated from the acetone fraction of *D. ocellatus* [25, 26].

The cytotoxicity of the different fractions of *D. ocellats* was evaluated on three breast cancer cell lines (MCF-7, T47D, and MDA-MB-231) comparing with etoposide as the reference drug (Table 2). It was found that the aqueous fraction was completely inactive toward all cell lines (IC_{50}> 500 µg/mL), however, the acetone and ethyl acetate fractions showed the highest activity against the three cell lines. They depicted potent activity against MCF-7 with IC_{50} values of 80.87 and 81.23 µg/mL, respectively. The corresponding IC_{50} values against T-47D were calculated as 81.20 and 102.88 µg/mL, respectively indicating the higher potency of the acetone fraction against T-47D. It should be noted that the acetone and ethyl acetate fractions showed lower cytotoxicity against MDA-MB-231 since calculated IC_{50} values were obtained as 144.36 and 114.41 µg/mL, respectively. The chloroform and methanol fractions showed moderate activity. With these results in hand, the acetone fraction seemed to be more cytotoxic against breast cancer cell lines which was candidate for the lichenochemical analysis.

Stictic acid, which was isolated from the acetone fraction, has shown anti-cancer activity [27]. It has depicted moderate anticancer activity against HT-29 with IC_{50} value of 29.29 µg/mL and a low growth inhibition on nonmalignant MRC-5 cells (IC_{50} = 2478.40 µg/mL) [28]. However, the compound **B** needs to be comprehensively investigated for its anticancer activity.

As shown in Figure 3, compounds **A** and **B** constructed desired interactions with survivin residues including Cys60, Phe61, Lys62, Glu63, His80, Ser81, Gly83, Asn111, Lys115, Asn118, Asn119, and Lys122.

As shown in Table 1, stictic acid (compound **A**) formed hydrogen bonding interactions with Ser81 and Lys115 residues at a binding distance of 2.95 and 2.67 Å. These H-bonding interactions are known as the ligand-protein interaction stabilizers [22, 23]. Moreover, hydrophobic interactions were constructed with Cys60, Phe61, Lys62, His80, Gly83, Asn111, and Ala114 residues. Compound **B** also established a bonding interaction with Lys115 as well as hydrophobic interactions with Cys60, Phe61, Lys62, His80, Ser81, Gly83, and Asn111 residues.

Table 1. Energy-based interactions detail of the identified structures as the survivin inhibitors

Ligand	Docking score (kcal/mol)	Electrostatic energy (kcal/mol)	Estimated inhibition constant (K_i)	H-bond interactions details			Distance (Å)	Hydrophobic interactions
				Ligand	Receptor			
Comp A*	-5.69	-0.16	67.32 µM	OAN* OAS*	Ser81 Lys115	OG** NZ**	2.95 2.67	Cys60, Phe61, Lys62, His80, Gly83, Asn111, Ala114
Comp B*	-6.72	-2.73	11.90 µM	OAS	Lys115	NZ	2.52	Cys60, Phe61, Lys62, His80, Ser81, Gly83, Asn111

*The isolated compounds: **A** (stictic acid) and **B** (2-(7'-hydroxy-3,5,6,8-tetramethyl-9-oxooxonan-2-yl) propanoic acid) from *Diploschistes ocellatus*; types are the names of atoms

Table 2. Cytotoxic activity of different fractions of *Diploschistes ocellatus* on breast cancer cell lines

Fraction	IC_{50} (µg/mL)		
	MCF-7	T-47D	MDA-MB-231
Acetone	80.87 ±0.593	81.20 ± 0.064	144.36 ± 0.011
Aqueous	>500	>500	>500
Chloroform	164.10 ± 0.210	206.77 ± 0.038	166.46 ± 0.056
Ethyl acetate	81.23 ±0.518	102.88 ± 0.015	114.41 ± 0.016
Methanol	213.38 ±0.462	161.63 ± 0.018	175.37 ± 0.006
Etoposide	18.53 ± 0.239	23.34 ± 0.096	19.94 ± 0.006

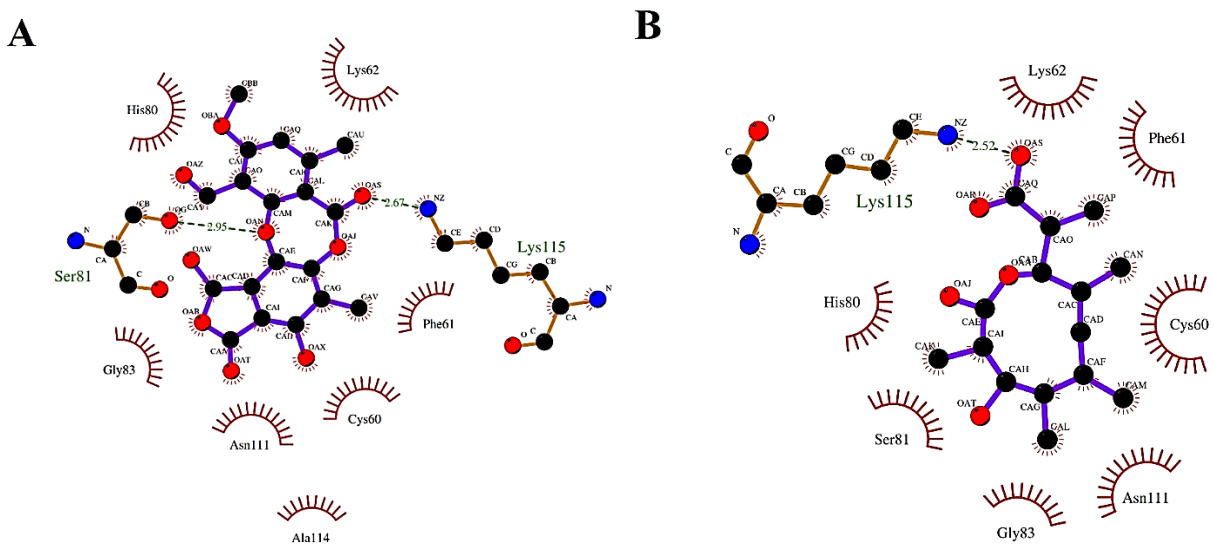

Figure 3. Schematic interactions of the best docking resulting from AutoDock software provided by LigPlot software for survivin and A (stictic acid) and B **(**2-(7'-hydroxy-3,5,6,8-tetramethyl-9-oxooxonan-2-yl) propanoic acid) from *Diploschistes ocellatus*. The hydrogen bonding interaction is shown in green, and hydrophobic interactions are shown with brown circles

The electrostatic force of compound **B** was recorded as -2.73 kcal/mol, indicating a good protein-drug interaction. Based on the interactions of compound **B** and survivin, the interaction with binding free energy of -6.72 kcal/mol is desirable. Both identified compounds formed a hydrogen bonding interaction with Lys115 (Figures 3 A and B).

Conclusion

We studied cytotoxicity of different fractions of *Diploschistes ocellatus* against three human breast cancer cell lines (MCF-7, T-47D, and MDA-MBA-231). The acetone fraction was found to be the most potent cytotoxic fraction; however, the aqueous fraction showed no activity. Lichenochemical analysis of the acetone fraction confirmed the presence of stictic acid and 2-(7'-hydroxy-3',5',6',8'-tetramethyl-9'-oxooxonan-2'-yl) propanoic acid which were probably responsible for the cytotoxic activity. In this respect, docking study based on the survivin inhibition confirmed the formation of desired interactions between those compounds and survivin amino acid residues. It seems that these compounds can regulate apoptosis to be well considered in anticancer therapeutic approaches.

Acknowledgements

Research reported in this publication was supported by Elite Researcher Grant Committee under award number [958762] from the National Institutes for Medical Research Development (NIMAD), Tehran, Iran.

Author contributions

The study was conceptualized, supervised by Mahnaz Khanavi, Mahdieh Eftekhari and Mohammad Reza Shams Ardekani; Mohammad Sohrabi identified the lichen; Lichnochemical investigation was done by Mahdieh Eftekhari, Fatemeh Oskou and Helia Abdshahzadeh; Cytotoxic activity was obtained by Tahmineh Akbarzadeh and Mina Saeedi; Docking analysis was obtained by Mahboubeh Mansourian; Interpretation of data was prepared by Mahdieh Eftekhari and Majid Balaei Kahnamoei; Writing and revising the manuscript was obtained by Mahdieh Eftekhari, Mina Saeedi, Mahboubeh Mansourian and Mahnaz Khanavi. All authors approved the final manuscript.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

BIR: baculoviral IAP repeat; IAPs: inhibitors of apoptosis; Bcl-2: B-cell lymphoma 2; DPPH: 2,2-diphenyl-1-picrylhydrazyl; MDR: multi-drug resistance; MTT: 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide