ARTICLE

CYTOTOXIC EFFECT OF THE ROOT EXTRACT OF BERBERIS ORTHOBOTRYS ON HELA CELL LINE

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ABSTRACT

Background: *Berberis orthobotrys have long been used traditionally as an herbal remedy for treatment of a variety of Diseases in north of Iran.* ***Objectives:*** *The aim of this study was to evaluate the cytotoxic effect of the root extract of Berberis orthobotrys on HeLa cell line.* ***Materials and Methods:*** *Different concentrations of the ethanolic extracts of the roots of B. orthobotrys (10, 7.5, 5, 2.5, 1.25,0.625 , 0.312, 0.156 mg/ml) were subjected to cytotoxic study against HeLa cervical cancer cell lines by employing MTT assay, and apoptosis of the cancer cells was confirmed by acridine orange-propidium iodide(AO/PI) staining.* ***Results:*** *The ethanolic extract of the roots of B. orthobotrys showed a significant cytotoxicity in (2.5, 1.25, 0.625, 0.312, 0.156 mg/ml) concentrations. The maximum reducing power of the Berberis orthobotrys extract to HeLa cell line at 492 nm was found to be 0.0506±0.01125 (P≤0.01) at 0.156 mg/ml versus control group. The highest inhibition percentage with regard to cytotoxicity was found to be 83.2 7% at 0.156 mg/ml with IC_{50} value of 2.68 mg/ml. This extract did not have any cytotoxic effect on normal cells.* ***Conclusion:*** *Our study shows that ethanolic extract of the roots of B. orthobotrys have cytotoxic effects on HeLa cell line. It seems that B. orthobotrys could be considered as a promising chemotherapeutic agent in cancer treatment. But further study is needed to fractionation for isolation and identification of biologically active substance from this extracts.*

KEY WORDS
Cytotoxicity, Berberis orthobotrys, HeLa cell line, MTT assay.

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INTRODUCTION

Berberis L. includes about 500 species of spiny or unarmed, evergreen or deciduous shrubs which grow in Asia and Europe, especially in India and Iran [1]. Some Berberis spp. including *B. crataeging, B. integerrima, B. khorasanic, B. orthobotrys, B. vulgaris, B. thunbergii* mainly grow in Iran. Their roots, barks, leaves and fruits are often used as a folk medicine [2,3]. *Berberis orthobotrys* have long been used traditionally as a herbal remedy in North of Iran. The fruit or freshly pressed juice, is used in treatment of liver and gall bladder problems, kidney stones, menstrual pains etc. [4,5]

It is shown that the amount of seven alkaloids (oxyacanthine, berbamine, isotetrandrine, columbamine, jatrorrhizine, palmatine, and berberine) in 17 species of *Berberis* Such as: *B.tabiensis, B.coletioides, B.microphyll, B.koroeana, B.vulgris*, and etc. were the highest in root barks followed by stem barks, root wood, and stem wood. Among these 17 species, berberine was the richest alkaloid while berbamine, jatrorrhizine, and palmatine were irregular in content [6,7]. Mazumder and et al, reported that the roots in some species of Berberis such as: *B.asiatica, B.lycium, B.vulgaris, B.aristata, B.nepalensis* contain alkaloids , taxilamine protoberberine and berberine ,that berberine is a yellow alkaloid, a bitter substance, which dissolves in acids and forms salts of the alkaloid [8] . Yang and et al found that berberine and berbamine are two major pharmaceutical compositions in the roots of different species such as *B.aristata, B.sanguine, B.taronesis, B.vulgaris* that have potential bioactivities such as antitumor, antidiabetic, antihyperlipidemic, anti-arrhythmic, and neuro-protective activities[1]. And also Gan in 2012 in his latest studies on berberine has shown more bioactivities, such as antioxidant, anti -microbial, anti-cancer, cardiovascular protective, anti-obesity, hepatoprotective, gastrointestinal protective, anti-rheumatic, anti-angiogenic and anti-clastogenic effects [9]. Recently, the effect of berberine was investigated on cancer cell and berberine showed potential anti-cancer activity against cancer cells. Its anti-cancer effect was mainly attributed to its actions on inducing cancer cell death, suppressing cancer cell growth and inhibiting cancer cell metastasis [10].

We briefly introduced phytochemical and pharmaceutical bioactive components of the Berberis spp. in medicinal use. Although a large number of studies on the pharmacological effects of berberies spp. and its components, in particular berberin, has been reported [1, 9, 10], no research has been done on cytotoxic effects of *B. orthobotrys*. So in this study we evaluated the cytotoxic effect of ethanolic extract of the roots of *B.orthobotrys* on HeLa cell line.

MATERIALS AND METHODS

Plant material

The plant *B. orthobotrys* was collected from around Veresk and was identified by Dr. Bahman Eslami, (Assistant Prof. of plant system, Islamic Azad University of Qaemshahr, Iran). Voucher specimens were deposited with the faculty of biology herbarium (as NO 720-654).Then, about 2 kilos of its roots were collected, washed with cold water, cut into small pieces, dried in the shade, and mechanically ground to produce a fine powder. Dry powder was extracted with ethanol for about 2 days at room temperature. Dry

ethanolic extracts were obtained after removing the solvent by evaporation. The dried ethanolic extract was used for evaluation of cytotoxicity activities.

Cell lines

The HeLa cell line used for assay was purchased from the cell bank, Pasture Institute (Tehran, Iran). Cells were grown in RPMI-1640(PAA) [each 500 ml of RPMI-1640 was supplemented with 10% of fetal calf serum (FBS) (PAA), penicillin (100 IU/ml) streptomycin (100µg/ml) (PAA)] in a humidified atmosphere of 5% Co_2 at 37ºC. The cells were dissociated with 0.2 % trypsin (Gibco) in phosphatebuffer saline solution. The stock cultures were grown in 25 cm^2 tissue culture flasks and all cytotoxicity experiments were carried out in 96 well plates.

Isolation of peripheral blood mononuclear cells

Peripheral blood was immediately drawn into heparinized sterile 15 ml conical tube, layered on to an equal volume of Ficoll and centrifuged at 1500×g for 20 min. Cells were then harvested from the Ficoll-plasma interface and washed three times in RPMI1640 medium. Cells were suspended at 1×10^4/ml in RPMI1640 supplemented as above. Cell viability was always >95٪, as estimated by trypan blue exclusion.

MTT Assay for cytotoxic activity

Cytotoxic effect of the extracts against HeLa cells was measured by MTT assay (MTT, Sigma) [11]. 100 µl of cell suspension (1×10^4 cell/ml) were cultured in 96 well microplate and were incubated for 24 h (37ºC, 5% CO_2 air humidified). Then 100 µl of different concentrations of extract including 0.156, 0.312, 0.625, 1.25, 2.5, 5, 7.5 and 10 mg/ml, were added and the microplates were further incubated for 72 h at the same qualification. Dilution of stock solutions was made in culture medium yielding final extracts concentrations with a final DMSO concentration of 0.1%. This concentration of DMSO did not affect cell viability. Control cells were incubated in culture medium only. All concentrations of plants extracts were in triplicates on the same cell group. For examination of the effect of the extract on normal cells, we used peripheral blood mononuclear cells at 1×10^4/ml in RPMI1640. To consider the cell survival, each well was then incubated with 20 µl of MTT solution (5 mg/ml in phosphate buffer solution) for 3-4 h. Then, the media in each well was gradually replaced with 100 µl DMSO and gently shaken to dissolve the formazan crystals. The absorbance of each well was determined at 492 nm using the ELISA reader (Awareness, USA).

The percentage of growth inhibition was calculated using following formula,
%cell inhibition = 100- {(At-Ab)/ (Ac-Ab)} × 100

Where,
At=Absorbance value of test compound
Ab=Absorbance value of blank
Ac=Absorbance value of control
The effects of extracts were expressed by IC_{50} values (the drug concentration reducing the absorbance of treated cells by 50% with respect to untreated cells).

Observation of morphological changes

The HeLa cells were cultured in 96 well culture plates (1×10^4 cells per well) in RPMI-1640 supplemented with 10% FBS for 72 h. The cells were treated with or without the root extract of B. *orthobotrys* at various concentrations. After 72 h, cellular morphology was observed under the inverted microscope and pictures were taken.

Detection of apoptotic cells

Morphological changes of apoptosis were assessed using acridine orange-propidium iodide (AO/PI) staining. Cell suspensions (1×10^4 cell/ml) were seeded in 96-well plate and incubated for 24 h . In the next day, the Cells were treated with 1.25 mg/ml concentration of extract for 4, 24, 48,72 h . Cells were then fixed with 70% ethanol for 20 min and stained with a solution composed of Ao/PI (50 µl of 10 µg/ml AO and 50 µl of 50 µg/ml PI) for 30 min, after being washed with PBS, cells were examined using fluorescence microscopy Motic AE 31 (Australia) and photographed.

Statistical Analysis

Results were presented as means ± SEM. Differences between control and test values were determined by Student's *t* test and were accepted as significant when $p<0.05$. The IC_{50} values were calculated from linear regression analysis.

RESULTS

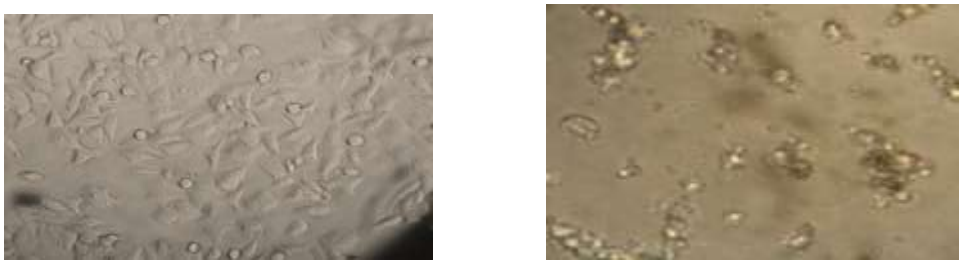
The results of cytotoxic assays of different concentrations of the root extracts of the B. *orthobotrys* on HeLa cells are shown in [Fig. 1, 2, 3] and [Table 1]. The ethanolic extracts of the roots of B. *orthobotrys* showed significant cytotoxicity in (2.5, 1.25, 0.625, 0.312, 0.156 mg/ml) concentrations. The maximum reducing power of the *B. orthobotrys* extract to HeLa cell line at 492 nm was found to be 0.0506±0.011 (P≤0.01) at 0.156 mg/ml. The highest inhibition percentage with regard to cytotoxicity was found to be 83.27% at 0.156 mg/ml with IC_{50} value of 2.68 mg/ml . The results showed that the extract had no effect on normal (lymphocyte and monocyte) cells.

Table 1: Cytotoxic Activity of Ethanolic Extract of the roots of *B. orthobotrys* on HeLa Cell Line at Different Concentrations by MTT Assay

Concentration of *Berberisorthobotrys*(mg/ml)	Absorbance, Mean ± SEM	%Inhibition	IC50(mg/mlL)
0.156	0.05 ± 0.011**	83.27	
0.312	0.072 ± 0.005**	72.08	
0.625	0.071 ± 0.002**	72.2	
1.25	0.055 ± 0.008*	79.09	
2.5	0.083 ± 0.007*	65.7	
5	0.22 ± 0.006	5.8	
7.5	0.33 ± 0.034	-50.7	
10	0.43 ± 0.017	-100.2	
Lymph	0.235 ± 0.013	-67.6	
DMSO	0.473 ± 0.034	-7.2	
Control	0.227 ± 0.021		

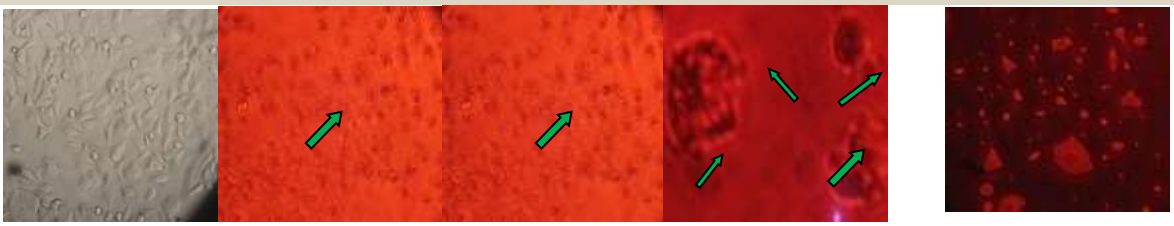
Morphology of untreated and treated cells was observed under inverted microscope, to evaluate apoptotic features of the cells. From [Fig. 1], it can be seen that untreated control cells showed normal appearance, which were elongated and flattened outgrowths, while the treated cells showed obvious morphological changes at concentration of 1.25mg/ml of extract. The transformed cells were shrunken, rounded, shiny, and condensed and showed surface blebs.

In addition, morphological changes in nuclei were observed in all cell line. It indicated that the extract caused marked apoptotic changes in a time- dependent manner, characterized by nuclear shrinkage and chromatin condensation [Fig. 2].



(A) (a)

Fig.1: Morphological changes of cancer cell line after being treated with extract under inverted microscope (A) HeLa cell - Control, (a) Treated HeLa cell with 0.156 mg/ml of extract (Magnification 200×)



(b) (c) (d) (e) (f)

Fig. 2: Induction of morphological changes in shape and in nuclear (a) Control HeLa cells (b) HeLa cells treated with 1.25 mg/ml concentration of root extract of *B. orthobotrys* for 4 (b),24 (c)(Magnification 200×), 48 (d), 72 (f) (Magnification 400×) hours when observed under the fluorescence microscope

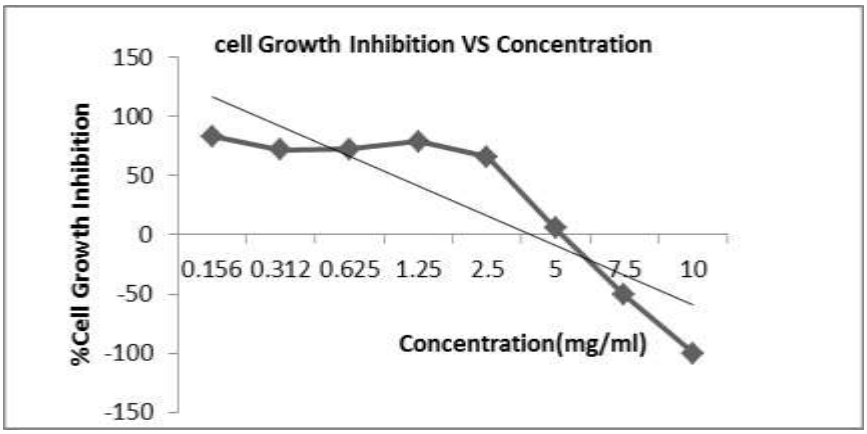


Fig. 3: Growth Inhibition of *B. orthobotrys* Roots Extract Against HeLa cell Line by MTT Assay

DISCUSSION

Natural herb extracts have biologically active compounds and have been used for a long time as a resource of new drugs. Anticancer activity is one of those effect among most of all effects that has been reported in vitro research. *B.orthobotrys* is a traditional remedy that is used in North of Iran. The major component of the roots of berberis plants, alkaloid berberine, has shown some bioactivities, such as antioxidant, anti -microbial and anti-cancer [9]. In this research, the cytotoxic effect of *B.orthobotrys* in different concentrations(10, 7.5, 5, 2.5, 1.25, 0.625, 0.312, 0.156 mg/ml) was studied.

The results of cytotoxic assays of different concentrations of the roots extract of *B. orthobotrys* on HeLa showed significant cytotoxicity in 2.5, 1.25 ($P \leq 0.05$), 0.625. 0.312 and 0.156 mg/ml($P \leq 0.01$) concentration [Table 1]. The highest cytotoxicity of this extract against HeLa cell was found in 0.156 mg/ml concentration with percent of cell growth inhibition [Fig. 3]. And this extract did not have any cytotoxic effect on normal (lymphocyte and monocyte) cells. The approximate concentrations of the extracts to reduce viability of the cells to about 50% (IC50) showed for HeLa, 2.68 (mg/ml).

This result indicates that, the cytotoxic effects of this extract may be mostly due to the presence of alkaloids berberine, while the other ingredients of the crude extract may also contribute to these activities.

It was reported that berberine could suppress the growth and proliferation of different cancer cells. Cell cycle arrest was the main mechanism involved in this process. Berberine could promote cell cycle arrest at G0/G1 checkpoint via inhibiting expression of cyclin D_1[12,13] and also induce G_1-phase cell cycle arrest [14] by increasing the expression of Cdk inhibitory proteins and inhibiting the expression of cyclin-dependent kinase (Cdk)2, Cdk4, Cdk6 and cyclins D1,D2, and E [15]. In addition, G1/S,G2/M and s-phase cell cycle arrests were involved in berberine-induce cell cycle arrest [16].

In this study, we found that *B.orthobotrys* exhibited a significant effect on the morphological features of the cancer cells tested, indicating that *B.orthobotrys* induces apoptosis in time-dependent manner. We observed that treatment of cells with 1.25mg/ml of extract, reduced the cell viability, inhibited the cell growth, changed cell adhesion to the substrate, created a star-shaped morphology, pigmented the cells and formed apoptotic bodies [Fig. 1, 2].

Choi and et al had also shown that apoptosis is the most common way involved in berberin-induced cancer cell death in many cell lines [10]. Berberine could activate mitochondria and caspase-dependent

apoptotic pathway in vitro [17]. In cultured cancer cell lines, it could induce the disruption of the mitochondrial Trans membrane potential, release of cytochrome c and apoptosis-inducing factor from the mitochondria to the cytosol [18].

By our analyses through MTT assay, morphological observation with the help of fluorescence microscopy and inverted microscopy, we could confirm that ethanolic extract of *B.orthobotrys* have cytotoxic effect on HeLa cells. Our study for the first time proves the anticancer and cytotoxic potentials of *B.orthobotrys* on HeLa cells by inducing apoptosis in these cancer cells.

These findings conform the folklore medicinal uses and suggest that *B.orthobotrys* has great potential as a source for new drug development.

CONCLUSION

Our study shows that ethanolic extract of the roots of *B. orthobotrys* has cytotoxic effects on tumor cells, but it does not have any cytotoxic effect on normal cells. It seems that berberine in *B. orthobotrys* could be considered as a promising chemotherapeutics agent in cancer treatment.

CONFLICT OF INTEREST
There is no conflict of interest

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FINANCIAL DISCLOSURE
None

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