Original Article:

Evaluation of *Bcl2* gene expression in MCF-7 Human Breast Cancer Cells under treatment of Centaurea Behen extract and Cisplatin

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ABSTRACT

Introduction: Breast cancer is among the most common malignancies of human around the world. Millions of cases of cancer worldwide occur annually, which, if detected in a timely manner, are easier to treat and can be conveniently controlled. In 2008, about 1,384,155 breast cancer cases were detected worldwide, with about 459,000 of them deceased.**Method**:MTT assay was performed to evaluate cell proliferation.The *BCL2* gene is an inhibitor of apoptosis that prevents the release of cytochrome C from mitochondria and leads to inhibition of various apoptosis stimuli. Due to the importance of this gene in the apoptotic process, the level of *BCL2* gene expression under treatment of Cisplatin and Centaurea Behen agents for 24 hours and 48 hours was evaluated in this research,using the Real time-PCR method. It is noteworthy that Cisplatin as a DNA binding agent may be effective in treating breast cancer;moreover, some studies have shown that Centaurea Behenplant has an antioxidant effect that can be a preventive factor in cancer.**Results**: The results obtained related to Cisplatin showed that IC50 for cells treated with Cisplatin for 24 hours was about 2.91 mg/ml while IC50 for cells treated with Cisplatin for 48 hours was about 1.77 mg/ml. Similarly, results obtained related to Centaurea Behenherbal extract showed that IC50 for cells treated with Centaurea Behen for 24 hours was about 9.64 mg/ml while IC50 for cells treated with Centaurea Behen for 48 hours was about 7.85 mg/ml.Results showed that the expression level of gene under treatment of the Cisplatinand Centaurea Behenhas decreased compared to the non-treatment state, so this expression reduction showed a significant difference between samples group and control group .

Keywords: *BCL2* gene; Apoptosis; Cisplatin; Persian herbal medicine; Centaurea Behen herbal extract; Anti-neoplastic agent

INTRODUCTION

Cancer is one of the chronic and non-contagious malignant diseases, which is considered as a serious problem affecting the health of the community. Breast cancer which accounts for 23% of all cancers in women is the most common cancer and malignancy among women[1, 2]. Breast cancer is a major public health concern, with 1,384,155 estimated new cases from population-based cancer registries in 2008 worldwide with nearly 459,000 related deaths[3, 4]. In 2002, there were an estimated 129,000 breast cancer–related deaths among women across Europe. By 2050, the incidence of breast cancer in women is predicted to reach approximately 3.2 million new cases worldwide[5].Breast cancer is caused by very distinct clinical, morphological and molecular cases[6]. Breast cancer is divided into five subtypes: Luminal A, Luminal B, human epidermal growth factor receptor 2 (HER2/ ERBB2)-positive, basal-like, and normal-like breast cancer[7, 8]. Luminal A and Luminal B are estrogen receptor (ER) positive, but luminal B cancer has a weaker outcome[9]. One of the processes involved in cancer is apoptosis.

Apoptosis has been shown to be controlled by many external cellular and intracellular factors, one of which is BCL2 gene (Apoptosis inhibitors)[10]. BCL2 has an anti-apoptotic effect that prevents the release of cytochrome C from mitochondria which leads to inhibition of various apoptosis stimuli. Bcl-2 family members are essential for the conservation of major organ systems and mutations affecting them are implicated in cancer[11].

Pharmacological studies on breast cancer can be cited to Cisplatin[12].Cisplatin is one of the factors used in chemotherapy; and yet, it is not always used to treat breast cancer.Cisplatin as a DNA cross-linking agent may be effective in the treatment of hereditary BRCA1-mutated breast cancer[13, 14].

Throughout time, numerous plants containing alkaloids have been used for treating mental problems.Alkaloids have a strong link with central nervous system (CNS) receptors.Recently, it has been shown that flavonoids also may be involved with enzymes on brain receptor systems and have different effects on the CNS[15]. Flavonoids,in addition to effects on CNS have many biological effects. They have attracted attention as free radical scavengers with antioxidant activity[16].Various studies have shown the presence of flavonoids in Centaurea species[17]. Therefore, the effect of herbal medicine Centaurea Behen with antioxidant properties could be involved in preventing cancer. In this study, the effect of Cisplatin and Centaurea Behen extract was evaluated on BCL-2 gene expression level in breast cancer tissues.

MATERIAL AND METHODS

Preparation of Centaurea BehenExtract

*Centaurea Behen*was purchased from Biological Resource Center, Tehran, Iran. The ethics approval number is 15930553952022. The extraction method was maceration. In this case, 20 grams of each plant sample was immersed in a 300 ml of 80% ethanol. The plant was soaked in solvent for 6 days, then incubated at 37 ° C until complete evaporation. The final extracts were stored in aluminum-coated containers until use.

Cell Culture

MCF-7 (human breast cancer cell line) was purchased from Biological Resource Center, Tehran, Iran. The cells were cultured in RPMI-1640 with 500 µl of *Pen/Strep (*Penicillin 100 u/ml – Streptomycin 100 µg/ml) and 5 ml of FBS; then,*Cell Suspension*cultures wereincubated in 5% CO2 at 37°C.

Preparation of Extracts with Different Concentrations

The MCF-7 cells were incubated with different concentrations of *Centaurea Behen*extract (61, 63 and 64 mg in 1 ml of RPMI 1640, respectively) for 24 and 48 hrs.The cells were treated with different concentrations of Cisplatinfor positive control.

Cell Proliferation Assay

MTT assay was performed to evaluate cell proliferation where 10^4 cells were seeded in 96 well plates and incubated with different concentrations of *Centaurea Behen*extractor different concentrations of Cisplatin for 24 and 48 hrs. Then, 20 µl MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well, and the plates were incubatedin 5% CO2 at 37°C for 4 hrs. Then, the medium was replaced with 150 µl of 2-Propanoland the absorbance was recorded at 570 nm.

Treatment of MCF-7 cells with Cisplatin and Centaurea Behen herbal extract, RNA extraction and cDNA Synthesis

The MCF-7 cells were treated with different concentrations of *Centaurea Behen*herbalextract and Cisplatin for 24 and 48 hrs. Total RNA was isolated from each cellthrough using RNX-Plus (Cinnagene ® Cat. no.: RN7713C, Iran) according to the manufacturer's specifications. The concentration of total RNA was determined by spectrophotometry (ND-1000, Wilmington, DE). cDNA synthesis was performed using the Prime Script RT reagent kit (Perfect Real Time) RR037A (Takara, Japan) according to the manufacturer's specifications. The obtained cDNAs were stored in -80℃ until use.

Real-time Quantitative PCR

Real-time PCR was performed in a 20-µl reaction, containing 10-µl of RealQ Plus 2x Master Mix Green High ROX™ (*Ampliqon, Denmark*), 2-µl of cDNA (20 ng/ µl), 6-µl of H2O, 1-µl of the forward primer and 1-µl of reverse primers (10 Pmol/µl concentration). Real-time PCR amplifications were done as follows: PCR amplification was set to an initial 95°C for 15 min and then for BCL2 gene, a

total of 35 cycles, 94°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds. For internal control, we used Beta-actin and all samples were analyzed in duplicate. The primers used for real-time PCR are listed in Table 1.

Table 1. Primer sequences used for real-time PCR.

Target genes		Sequences (5`→ 3`)	Product length
BCL2	Forward	ATTGGGAAGTTTCAAATCAGC	105bp
	Reverse	TCCTCTGTCAAGTTTCCTT	
Beta-actin	Forward	TCCTCCTGAGCGCAAGTAC	89bp
	Reverse	CCTGCTTGCTGATCCACATCT	

Statistical Analysis

Gene expression was calculated using the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method using REST© (Relative Expression Software Tool, Germany). A *P* value <0.05 was considered statistically significant for all tests.

RESULTS

Assessment of cell viability

The MCF-7 cells were treated with different concentrations of Centaurea Behen herbal extract and Cisplatin for 24 and 48 hrs. Results obtained related to Cisplatin showed that IC50 for cells treated with Cisplatin for 24 hours was about 2.91 mg/ml while IC50 for cells treated with Cisplatin for 48 hours was about 1.77 mg/ml. Likewise, results obtained related to Centaurea Behen herbal extract showed that IC50 for cells treated with Centaurea Behen for 24 hours was about 9.64 mg/ml while IC50 for cells treated with Centaurea Behen for 48 hours was about 7.85 mg/ml. (Figure.1 Shows the results of cell viability with different concentration).

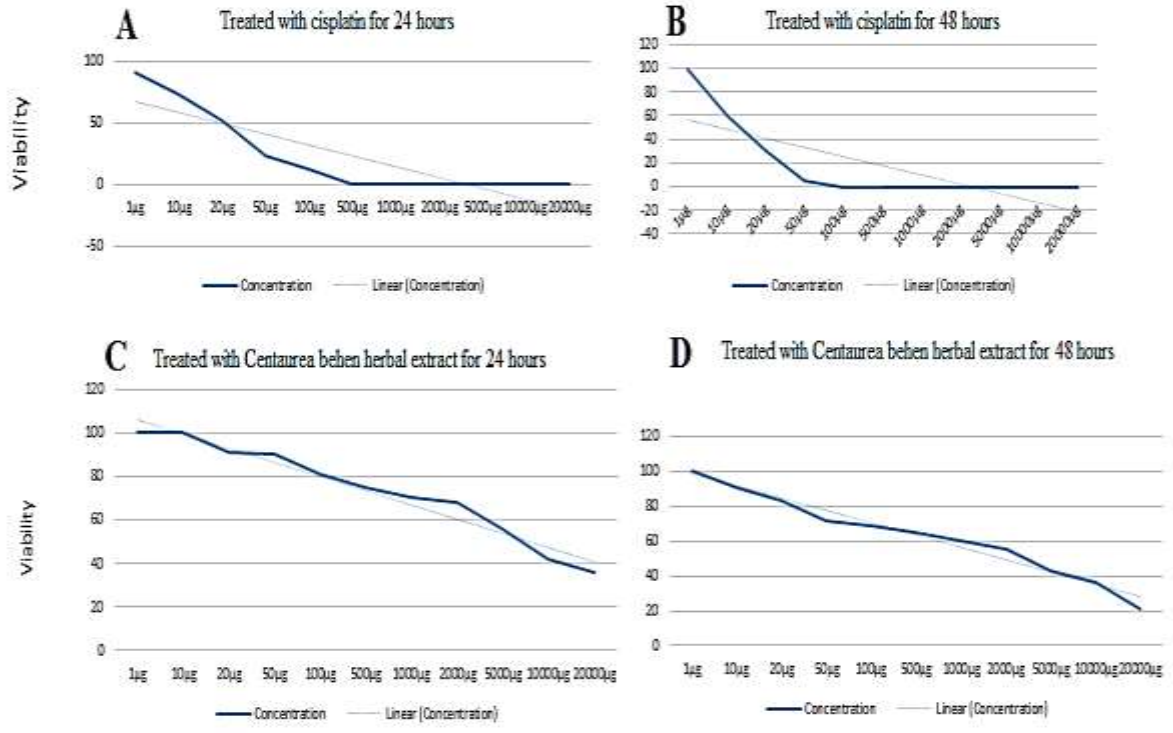

Figure1. The graph (A) related to treatment with Cisplatin for 24 hours, (B) related to treatment with Cisplatin for 48 hours, (C) related to treatment Centaurea Behen herbal extract for 24 hours and (D) related to treatment Centaurea Behen herbal extract for 48 hours.

Checking Level Expression of BCL2 Gene Under Treatment by Cisplatin for 24 and 48 Hours

Results obtained in non-treatment status showed that there was no significant difference between the level of BCL2 gene expression in sample group (no treatment) compared to control group (P=0.91). Results of under treatment for 24 hours by Cisplatin showed that the expression level of BCL2 gene has decreased so that there was significant difference between the level of BCL2 gene expression in sample group compared to control group (P=0.013). Also, the results of 48 hours treatment with Cisplatin showed that BCL2 gene expression level has decreased compared with level expression of this gene in 24 hours status, so there was a significant difference between the level of expression of BCL2 gene expression in sample group compared to control group (P=0.001). (Fig.2 and Fig.4 show the results of BCL2 gene expression level by treatment with Cisplatin).

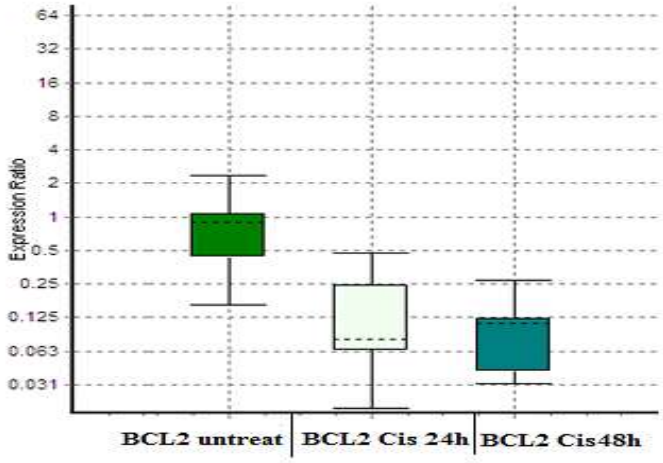

Figure2. Difference in the level of gene expression BCL2 under the treatment with Cisplatin

Checking level expression of BCL2 gene under treatment by Centaurea Behen herbal extract for 24 and 48 hours

Results of under treatment for 24 hours by Centaurea Behen herbal extractdemonstrated that the expression level of BCL2 gene has decreased. Therefore, there was a significant difference between the level of BCL2 gene expression in sample group comparedwith control group (P=0.02). Also, the level of BCL2 gene expression in the 48-hour treatment period with Centaurea Behen herbal extract has decreased with cpmpared to the 24-hour status (P=0.001). (Fig.3 and Fig.4 show the results of BCL2 gene expression level by treatment with Centaurea Behen).

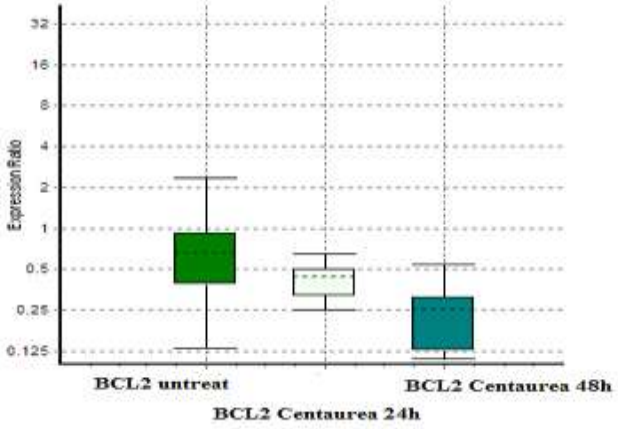

Figure3. Difference in the level of gene expression BCL2 under the treatment with Centaurea Behen

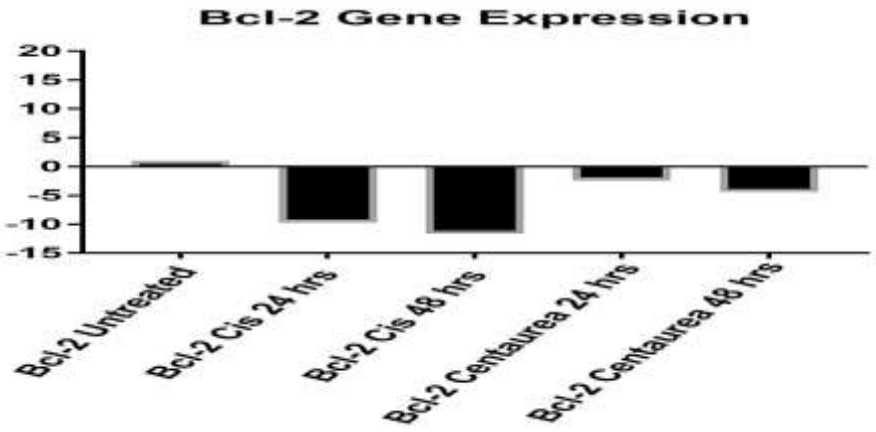

Figure4. The difference in the level of BCL2 gene expression under treatment with Cisplatin and Centaurea Behen agents

DISCUSSION

Herbal extracts usually exert their effects on the cancer cells through direct action on tumor cells or with increased immune responses. Using herbal extracts may cause beneficial effects, such as reduction of the cytotoxic effects of chemotherapy in patients with cancer. In the onset and progression of breast cancer, many factors and genes are involved. Among them, the genes which are involved in apoptosis play a significant role in the treatment and survival of patients. It has been shown that the Bcl-2 gene plays an important regulatory role in apoptosis. This gene is a cellular protein that inhibits apoptosis in normal tissues, including breast tissues. In the present study, the extract of Centaurea Behen and Cisplatin were assessed on Bcl-2 gene expression in MCF-7 cell line;it has been manifested that Bcl-2 gene expression down regulated 2.42 and 4.4 fold, when treated with Centaurea Behen, and 9.7 and 11.62 fold, when treated with Cisplatin during 24h and 48h, respectively. Considering the results of this study, the Centaurea Behen extract and Cisplatin could induce apoptosis in MCF-7 cell line.

CONCLUSION

The present study shows that the Centaurea Behen extract and Cisplatin could induce apoptosis in MCF-7 cell line through reducing expressionof Bcl-2 gene. Due to the natural properties of Centaurea Behen extract compared to chemical Cisplatin, the use of this herbal product along with other anti-cancer drugs could be more effective.

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