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Effects of X-irradiation and sinensetin on apoptosis induction in MDA-MB-231 human breast cancer cells

N. Rezakhani[1], B. Goliaei[2*], K. Parivar[1], A.R. Nikoofar[3]

[1]*Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran*
[2]*Department of Biophysics, Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran*
[3]*Department of Radiotherapy, Iran University of Medical Sciences (IUMS), Tehran, Iran*

► ***Original article***

Corresponding authors:
Bahram Goliaei, PhD.,
E-mail: *Goliaei@ut.ac.ir*

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ABSTRACT

Background: Breast cancer is considered as one of the most influential diseases around the world. Radiation is one of many ways that is being use to against cancer. However, patients treated with radiation may face cancer recurrence. Some plant compounds exhibit antioxidant effects. Sinensetin is a methylated flavone present in citrus and *Orthosiphon stamineus*. In this study, we examined the role of sinensetin in increasing the radiation sensitivity. ***Method:*** The cytotoxic effect of sinensetin was evaluated in MDA-MB-231 by MTT assay. Additionally, the clonogenic ability of cells was evaluated in the presence of sinensetin. Real-Time PCR was performed to detect and quantify expression profiles of apoptosis related genes. ***Result:*** Sinensetin decreased the viability of MDA-MB-231 in a concentration and time dependent manner. The survival fraction was decreased in cells treated with sinensetin prior to X-irradiation compared to cells treated with X-ray only. Furthermore, treatment of cells with sinensetin and X-ray could increase expression level of *p53*, *Bcl-2* and *STAT3*. ***Conclusion:*** According to the results, sinensetin combined with X-ray can induce apoptosis in the treated cells.

Keywords: *Radiation, polyphenol, sinensetin, combined therapy, breast cancer.*

INTRODUCTION

Breast cancer is the 5th leading cause of cancer death in Iranian women [1]. It is estimated that various types of cancer incidences will be increased by 2030 [2]. Radiotherapy is one of the techniques used for killing cancer cells. In radiotherapy, the damage caused by high-energy radiation to cells can block the cell division and proliferation processes [3]. However, some normal cells also show apoptosis after radiotherapy. Combined anticancer therapy could be more effective in these cases. For treating cancer cells, combination of chemotherapy and radiotherapy has presented better results compared with that of single therapy [4]. Based on recent investigations, it has been estimated that natural polyphenols can elevate radiation-induced cell killing with minimal toxicity to non-cancer cells [5]. Polyphenols are among phytochemicals [6] found in fruits, vegetables, coffee, chocolates, cereals, and tea. Polyphenols are a group of antioxidants which have been determined to be more prominent in the potential health benefits of dietary [7]. As an example, methoxyflavones in plants have substantial roles such as antioxidant, anti-proliferative, and anti-carcinogenic roles [8].

Citrus flavonoids are generally categorized into two groups: polymethoxylated flavones (such as nobiletin, sinensetin, tangeretin, etc.) and flavanone glycosides (like naringenin, hesperidin, neohesperidin, etc.) [9]. Apoptosis, is one of the most widely-investigated subjects amongst cell biology researchers. Our perception of tumor cell functioning and our ability to utilize this information is hugely implicated by complexity of cellular signaling networks [10].

Irradiation can postpone progression through

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the G1, S, and G2 phases of the cell cycle. The G1 arrest is regulated by the p53 tumor suppressor gene product [11]. *Bcl-2*, an apoptosis gene, can protect cells against radiation [12]. The last gene studied in this work is STAT3. Radiation promoted the phosphorylation of STAT3 [13] and this gene deemed a barrier between tumor cells and immune cells in the tumor microenvironment [14]. This study is aimed at evaluating radiosensitization effect of sinensetin. Furthermore, apoptosis induction as a possible pathway effective in radiosensitization was studied. The radiosensitizing effect of sinensetin in the treatment of breast cancer cell lines is unclear. In this study, we compared the effect of sinensetin treatment alone and in combination with radiation on MDA-MB-231 cell line by analyzing the influence of sinensetin on MDA-MB-231 cytotoxicity and clonogenic ability. We also determined whether sinensetin induces apoptosis in the breast cancer cell line using Real-time PCR.

MATERIALS AND METHODS

Materials

Sinensetin, purchased from Cayman Chemicals, was dissolved in Dimethyl sulfoxide (DMSO) Merck (Germany). Cell culture media (RPMI-1640) and fetal bovine serum were from Gibco (UK). Streptomycin and Trypsin were provided from Troge medical Gmbh (Germany) and Merck (Germany), respectively. Trypan blue, methyl green, penicillin, and 3-[4, 5-Dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide (MTT) was bought from Sigma Aldrich (USA).

Cell culture

MDA-MB-231 human breast cancer cell line (estrogen receptor-negative, aggressive, invasion cell and ATCC number HTB-26™) was purchased from the National Cell Bank of Iran (Pastuer Institute, Iran). Cells were cultured in RPMI-1640 culture medium, supplemented with 10 % heat-inactivated fetal bovine serum, 500 units/ml penicillin, and 200 µg/ml streptomycin. Cells were maintained at 37 °C and 5 % CO_2 in a humid atmosphere. Cells were sub cultured using 0.25% trypsin/EDTA in phosphate buffered saline (PBS) solution. MDA-MB231 was sub cultured every three or four days to maintain the exponential growth phase.

Growth curve

MDA-MB-231 cells were seeded at densities of 10000 cells/well in 24-well plates (SPL, Korea). The cells were harvested and counted using the trypan blue dye exclusion method. The number of cells were then assessed by direct counting at days 0-7 after culture. The doubling time (T_d) of each cell line was calculated according to the Patterson formula as equation 1:

$$T_d = T \times \frac{\mathrm{Log}_2}{\mathrm{Log}\left(\frac{N_2}{N_1}\right)} \quad (1)$$

Where, N_1 is cell number on the 1st day of the exponential phase, and N_2 is cell number T h after culture (end of exponential phase); T (h) is the time from N_1 to N_2.

MTT cell viability assay

Viability assay was carried out as follows: first cells were cultured in 96-well plates at a density of 3,000 cells/well and incubated overnight to make sure that the cells are attached. Then, the cells were treated by various concentrations of sinensetin (0.1, 1, 10, 50, 100, and 150 µM) and were incubated for additional times of 24, 48, or 72 h.

After the specific times mentioned above, the medium was removed from each well, with aspiration. Next, 100 µl of 0.5 mg/ml MTT solution was added to each well. Then the cells were incubated for 3 h followed by addition of 100 µl DMSO in order to dissolve the formed formazan crystals. Absorbance at 570 and 630 nm wavelengths was measured using a BioTek (USA) plate reader.

Colony formation assay

Adequate numbers of MDA-MB-231 cells were counted and cultured into 35 petri dishes (SPL, Korea) and were incubated for 7 days. Then, the former colonies were fixed using %2 formaldehyde and stained with %5 crystal violet solution. Afterwards, the colonies were counted and the surviving fraction was calculated as the

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number of cultured cells / number of colonies counted × 100 equals plating effi-ciency.

Furthermore, to evaluate the effect of sinensetin on the clonogenic survival of MDA-MB-231 cells, the cells were treated with 0.1, 1, 10, 50, 100, and 150 µM sinensetin and incubated for 48 h. Then, 500, 750, 1000, and 1500 cells/plate were cultured in 35 mm plates and incubated for 7 days. At the end, the former colonies were fixed, stained, and counted according to the previously mentioned procedure. It is statistically necessary to plate enough cells to obtain about 50 colonies.

Effect of sinensetin combined with radiation on colony forming ability

Moreover, the effect of sinensetin on the radiation response of MDA-MB-231 cells was studied. Briefly, 750, 1500, 8000, 14000, 22000, and 26000 cells/well were cultured in 24-well plates followed by treating with 1 µM sinensetin for 48h. Then, the cells were ex-posed to 2, 4, 6, 8, and 10 Gy irradiation which was performed using 6 MeV X-ray photon, produced by a linear accelerator (Siemens Primus; Siemens AG, Erlangen, Germany) at a dose rate of 200 cGy/ min.

Real time-PCR

The effect of sinensetin and combination of sinensetin and radiation on the expression level of marker genes p53, *Bcl-2*, *STAT3,* and *GAPDH* was analyzed by RT-PCR. Comparative threshold cycle was used for estimation of gene expression. In summary, the C_T (threshold cycle) was obtained from triplicate amplification throughout the exponential stage of the amplification. Next, the C_T value obtained for the reference gene (*GAPDH*) was deducted from the C_T values obtained for the p53, *Bcl-2*, and *STAT3* genes. ΔC_T was calculated for every single gene. Following the calculation of $\Delta\Delta C_T$ for each sample, the relative expression of each gene was evaluated using the ratio formula (ratio=$2^{-\Delta\Delta Ct}$) (table 1).

Table 1. Sequence of primers for (STAT3, Bcl-2, GPPDH, and p53) designed in this Study.

Genes	Primers	Sequence	Amplicon size
STAT3	F Primer	5′CTGAAGCTGACCCAGGTAGC3′	123 bp
	R Primer	5′GCATCAATGAATCTAAAGTGCGGG3′	
BCL2	F Primer	5′GATACTGAGTAAATCCATGCAC3′	356 bp
	R Primer	5′AGTGTTGCAGAATATTCAGCCAC3′	
GAPDH (RT-PCR)	F Primer	5′CCATGAGAAGTATGACAAC3′	115 bp
	R Primer	5′GAGTCCTTCCACGATACC3′	
P53	F Primer	5′GCTCAGATAGCGATGGTCTGGC3′	156 bp
	R Primer	5′AGTGGATGGTGGTACAGTCAGAG3′	
GAPDH (qRT –PCR)	F Primer	5′CCAGCCGAGCCACATCGCTC3′	360 bp
	R Primer	5′ATGAGCCCCAGCCTTCTCCAT3′	

In this case, MDA-MB-231 cells were cultured and treated with IC_{50} concentration of sinensetin for 48 h. Then a group of cells were irradiated after being treated with sinensetin. Next, total RNA was extracted by RNX-Plus solution (CinnaGen, Tehran, Iran) according to manufacturer's instruction. DNase (Fermentas, Pittsburgh PA, USA) was used to remove DNA impurities, at 37 ºC for 30 min and quantified by spectrophotometry and electrophoresis on a %1 agarose (Merck, Germany) gel.

For cDNA synthesis, 1 µg of total RNA, according to the manufacturer's instructions, EDTA (CinnaGen, Tehran, Iran), dNTP (CinnaGen ,Tehran, Iran), random hexamer primer (Fermentas, Pittsburgh PA, USA), Reverse Transcriptase (Fermentas, Pittsburgh PA, USA), RNase buffer (Fermentas, Pittsburgh PA, USA), and DEPC Water (CinnaGen, Tehran, Iran) were used.

In the next step, 9 µl reaction mixture containing 1 µl primers and 8 µl SYBR Green I Master Mix (QuantiFast SYBR Green PCR, Q204054) were added to 1µl cDNA. Then, a Real

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Time Thermo cycler (RotorGene 6000, Corbett Life Science, and USA) was started. Real-time PCR included these stages: the initial denaturation at 95˚C for 5 min, the denaturation at 95˚C for 15 s, the annealing temperature was optimized from 60 to 61˚C for 25 s, and the extension at 72˚C for 25 s. The whole process was repeated for 35 cycles. In addition, melting curve analysis was done for each Real-Time PCR experiment. All the four steps foresaid were applied for different groups including controls, *p53, Bcl-2, and STAT3.*

Data analysis

All data were presented as the mean ± standard deviation 3 independent experiments). Statistical analysis was performed using SPSS 17 software (SPSS Inc., Chicago, USA). For continuous variables, means were compared by one way analysis of variance (ANOVA) and Tukey's posthoc testing. A $p<0.05$ was considered as statistically significant. A fitting curve in cytotoxicity studies was used to calculate the inhibitory concentration 50% (IC_{50}). In the case of X-irradiation studies, a fitting curve was added based on the linear quadratic model to calculate α and β parameters using MATLAB software (Math Works Inc., Natick, USA). Analyses to compare the expression of p53, *STAT9, Bcl-2*, and their active forms were performed using Prism7 software, and results were considered significant if the p-*value* was less than 0.05.

RESULTS

Growth curve

The growth curve of MDA-MB-231 human breast cancer cells is shown in Fig1. According to the figure, lag, log, stationary, and decline phases are observed in 0-2, 2-5, 5-6, and 6-7 days, respectively. Doubling time is defined as the time needed for each doubling of cell population during log phase. In this study, the doubling time for MDA-MB-231 cells was 21.6±0.8 hours (figure 1).

Cytotoxic effect of sinensetin

MTT assay was performed to determine the cytotoxic effects of sinensetin (0.1, 1, 10, 50, 100, and 150 µM) on MDA-MB-231 cells at 24, 48, and 72 hours. The IC_{50} val-ues for these cells were found to be 50 µM in 24 hours, 0.1 µM in 48 hours, and 0.01 µM in 72 hours. As shown in figure 2, the viability of MDA-MB-231 cells was markedly decreased at high concentrations of sinensetin 0.1-150 µM. The number of viable cells was then calculated as following:

Number of viable cells (%) = (Abs of sample ÷ Abs of control) ×100.

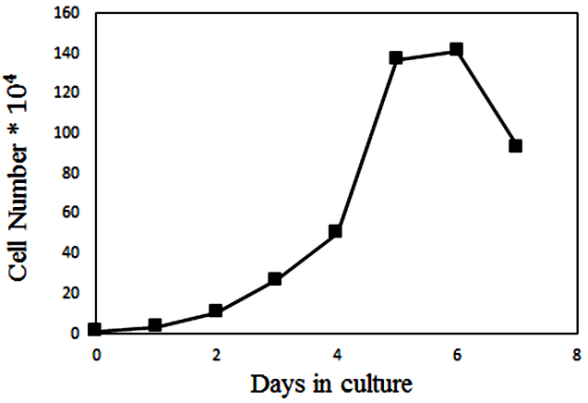

Figure 1. MDA-MB-231 (p-value<0.05) growth curve. The number of cells was counted during 7 days.

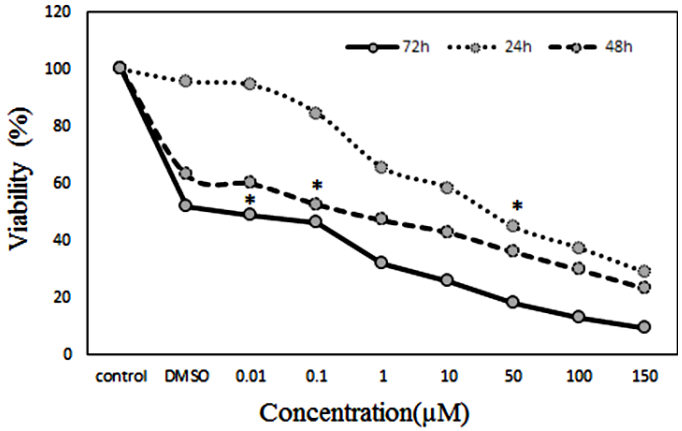

Figure 2. Effect of sinensetin on cell viability. MDA-MB-231 cells were treated for 24, 48, and 72 h by sinensetin (0.1-150 µM). The cell viability was determined by MTT assay after the indicated times.

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Colony formation assay

The number of cells that we seeded in 35 mm petri dishes were 500, 750, 1000, 1500, and 2000. The number of colonies formed increased with the number of cells seeded in each plate. This is a particular parameter for cell proliferation rate (figure 3).

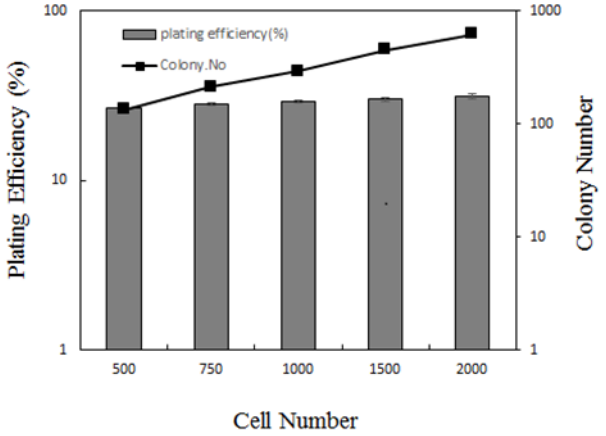

Figure 3. Plating efficiency of MDA-MB-231 cells. The cells were incubated for 7 days to allow colony formation ($p<0.05$).

Furthermore, 500, 750, 1000, and 1500 cells were seeded in 24 wells plates and treated with 0.1, 1, 10, 50, 100, and 150 µM sinensetin for 48 h and allowed to form colonies. According to the results presented in figure 4 A-E, sinensetin decreased the colony forming ability of MDA-MB231 cells.

Effect of sinensetin combined with radiation on colony forming ability

Human breast cancer cell lines MDA-MB-231 were seeded in 24 well petri dishes. The number of cells, exposure unit were different. Then, MDA -MB-231 cells were treated with 0.1 µM of sinensetin for 48 h followed by exposure to various doses of X-irradiation (2, 4, 6, 8, and 10Gy).

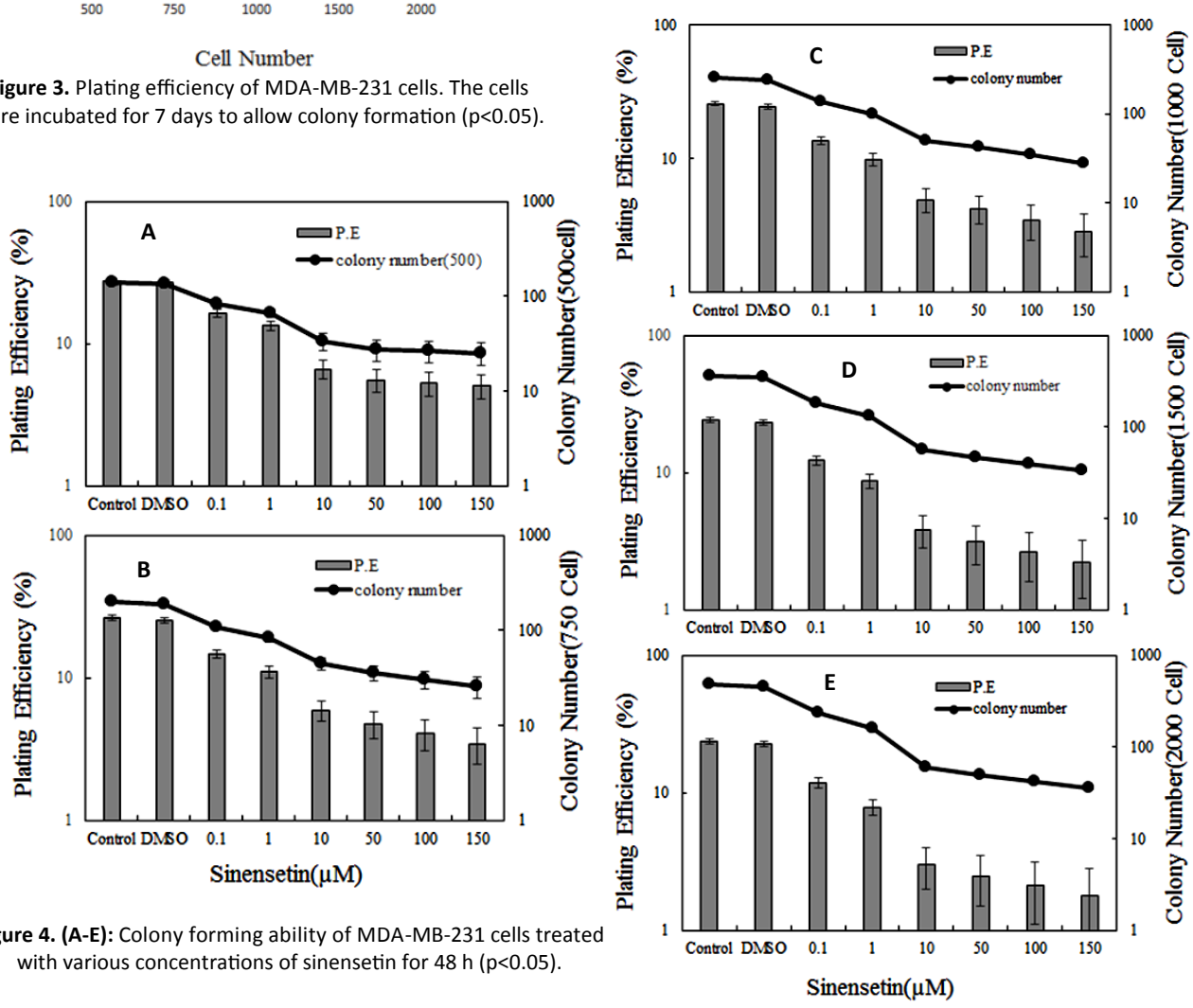

Figure 4. (A-E): Colony forming ability of MDA-MB-231 cells treated with various concentrations of sinensetin for 48 h ($p<0.05$).

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In this study, we combined the methylated flavone (sinensetin) and X-rays. The α and β parameters calculated based on the survival curve are shown in table 2. α parameter was related to the DNA damage caused by a single hit effect of radiation interaction. This damage included double-strand breaks, which can be lethal. The changes in the β parameter are caused by two radiation interactions. The survival of MDA-MB-231 cells decreased at 2 and 4Gy (p <0.05) (figure 5).

p53, Bcl-2, and *STAT3* genes in MDA-MB-231 cells

Expression of the p53, *Bcl-2,* and *STAT3* genes in MDA-MB-231 cells treated by IC_{50} concentration of sinensetin and exposed to 4Gy radiation was analyzed by qPCR. Firstly, the expression level of *GAPDH* was checked out. Results did not show any effect by sinensetin treatment. This gene is an appropriate housekeeping gene for transcription analysis (table 1). According to the results, *Bcl-2, p53,* and STAT3 gene were up-regulated in the both group of cells. However, the increase in expression level was higher in the cells treated with sinensetin combined with X-ray compared to the cells receiving sinensetin only (figures 6-7).

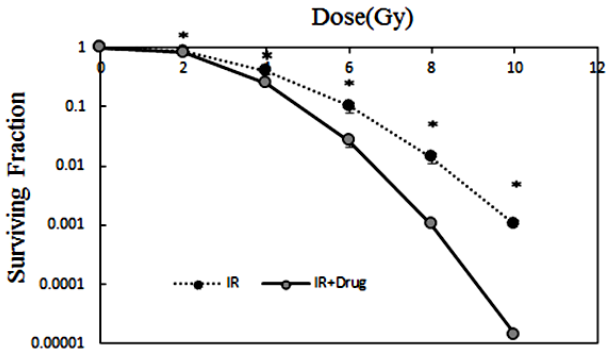

Figure 5. The surviving fraction of group treated with sinensetin plus radiation was compared to that of the irradiated group. This data represent average of at least three independent experiments. IR=irradiation; Drug= Sinensetin.

Table 2. The α and β parameters to compare MDA-MB-231 cells treated with radiation or radiation along with drug. The parameters were calculated based on the survival curve fitted to linear-quadratic model.

	MDA-MB-231	
	α-value	β-value
IR	0.07972	0.076
IR+Sinensetin	0.1644	0.1281

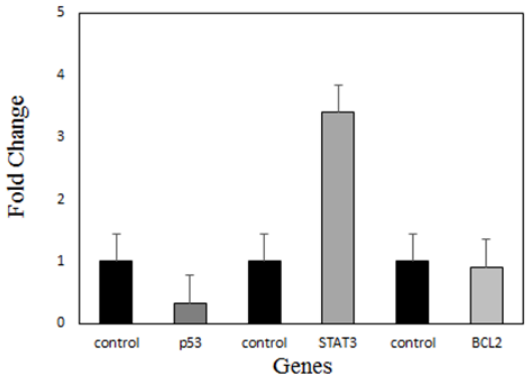

Figure 6. Expression levels of p53, Bcl-2, and STAT3 genes in MDA-MB-231 cells treated with sinensetin.

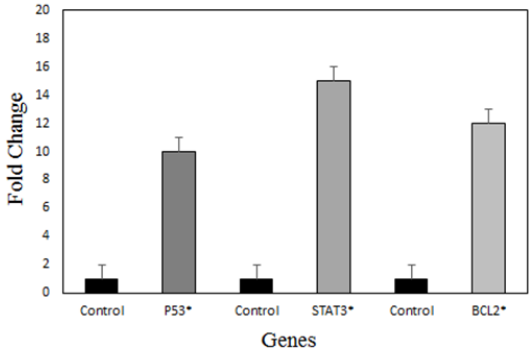

Figure 7. Expression levels of p53, Bcl-2, and STAT3 genes in MDA-MB-231 cells treated with sinensetin combined with X-ray.

DISCUSSION

Cancer is considered as a crucial cause of death worldwide, and its fatality is rising every year [15]. In average, Iranian patients with breast cancer are one decade younger compared to western patients [16]. Although radiotherapy is the most well-known cancer treatment, a large portion of cancer patients experience radioresistance [17]. Combination of radiation

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and chemical compounds are particularly low-toxicity agents [18]. Numerous studies have shown that plant secondary products play an important role in the cellular level as regulatory modulators of gene expression in plant growth [19]. In this study, we examined MDA-MB-231 growth curve to study the growth process of these invasive cells. Doubling time is the time required for doubling of cell growth/count [20]. This quantity can help us to have a better choice of the time required for treatment. This data was compared with that of ATCC [21] (American Type Culture Collection). The doubling time reported in ATCC for MDA-MB-231 was 38 h which is around 16 h higher than the doubling time obtained here.

Several reports have shown that sinensetin cytotoxicity is time-dependent [22]. Bigdeli *et al.*, found that increasing the concentration of entrolacton decreases the viability of cancer cells in a time- and concentration-dependent manner. IC_{50} was 100 µM during 48 h [23] but here, IC_{50} for sinensetin was 0.1 µM for 48 h. The concentrations of sinensetin tested here did not promote the proliferation of MDA-MB-231 cells at 24, 48, and 72 h. We found that increasing of sinensetin concentration leads to a decrease in the cell viability. Therefore, this flavones (Sinensetin) may have a cytotoxic effect on estrogen receptor-negative breast cancer cells.

In 2016, Patties *et al.*, tested clonogenic survival of human medulloblastoma cells by multimodal treatment with ionizing irradiation. Their results indicates that we can use clonogenic assay to inquire radio synergistic action [24]. In another work, Equol as a polyphenol was investigated using clonogenic assays and it was shown that Equol can enhance radio sensitivity [25]. Our data also illustrated that the surviving fraction significantly decreased after combined therapy. Based on our results, increasing concentrations of sinensetin and X-ray doses reduced the viability and colony -forming ability of MDA-MB-231. Combined therapy may exhibit anticancer effects by inhibiting cell growth and decreasing viability in a time and dose dependent manner. In 2016, Masoudi Khoram *et al* investigated radio sensitivity of Cafeic Acid (polyphenol). Their results were comparable with that of Zhang G *et al* and Patties *et al.* which means that Cafeic acid increased radio sensitivity in human breast cancer [26]. In that article, some reports showed that polyphenols can change expression level of genes. In 2018 Wang J *et a*l, demonstrated the same result [27]. In one study, it was shown that one of the polyphenol groups (flavones), naringenin, can induce apoptosis via reducing activation of NF-κB/COX-2-caspase-1 pathway in HeLa [28], Here, another member of the same polyphenol group (Sinensetin) induced apoptosis with *Bcl-2,STAT3,P53* in human breast cancer. Some researchers in 2018 used nobiletin to study cancer inhibition. This polyphenol is a member of the flavones group and can induce apoptosis with STAT3 [29] similar to our polyphenol (data was shown). In 2016, Nallanthighal S *et al* evaluated Pomegranate polyphenol which can protects against genomic instability induced by medical X-rays [30] whereas, sinenensetin, induces the overexpression of STAT3, *Bcl-2* and *p53* genes. This phenomenon leads to apoptosis induction in MDA-MB-231 cells treated with X-ray or X-ray combined with sinensetin.

CONCLUSION

Our results demonstrated that combined therapy via radiation and polyphenol (sinensetin) can change the viability of human breast cancer cells. Sinenensetin is recognized as a type of drug capable of inducing apoptosis in cancer cells. While, many researches have been focused on the *Bcl-2* mRNA expression, we found that mRNA transcription of *Bcl-2 may be* promoted in MDA-MB-231 cells treated with this drug.

***Conflicts of interest*:** *Declared none.*

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