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Evaluation of Sinensetin Derived From Flavonoids-Rich Orthosiphon Stamineus as a New Radiosensitizer in MDA-MB-231 and T47D as Breast Cancer Cell Lines

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ABSTRACT

Introduction: Breast cancer is one of the most prevalent diseases around the world. Breast cancer patients treated with radiation may face Side effects as well as cancer recurrence. Some polyphenols exhibit antioxidant effects. Citrus and Orthosiphon Stamineus have source of methylated flavone called Sinensetin (SIN). In this research, we designation of Sinensetin as increasing radiation sensitivity.

Material and Methods: The cytotoxic effect of Sinensetin was examined in MDA-MB-231 and T47D by MTT assay. As well as, the clonogenic ability of cells were Assessmented in the presence of Sinensetin and combination with radiation. To quantify expression alterations of apoptosis related genes, utilized Real-Time PCR method.

Results: In a dose and time-dependent manner**,** Sinensetin decreased the viability of MDA-MB-231 and T47D. The survival fraction was decreased in cells treated with Sinensetin (SIN) prior to X-irradiation compared to cells treated with X-ray only. More ever, expression level of, Bcl-2, STAT3, and increased P53 via treated cells with Sinensetin (SIN) and X-ray.

Conclusion: Due to the results, Sinensetin (SIN) can be mentioned as a novel radiosensitizer and its effects may considered increasing apoptosis following DNA damage induced by irradiation.

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Introduction

Breast cancer is one of the prevalent cancer in women worldwide. The mortality of breast cancer increasing in developed countries (1). During the last decade, radionuclide-based target therapy is one of the most effective ways to treat cancer (2). X-ray and Gamma-ray , can remove electrons from the atoms or produce free radicals to interact with plasma membrane proteins and induction of cell apoptosis (3). Anti-apoptotic genes are overexpressed in radio resistant tumor cells hence, finding a way to restore apoptotic pathways in cancer is one of the most important clinical issues (4). The P53 tumor suppressor is one of the most general mutated genes in various types of human cancer. This gene_plays a main role in the cell cycle as a tumor suppressor (5). Other proteins that have a function as regulators of programmed cell death are BCL-2 and STAT3.

BCL-2activates caspase a member of a family of cysteine proteases, thereby enhancing apoptotic propensity (6). Increasing expression and activating STAT3 by phosphorylation, lead to tumor progression (7). Tumor drug and radiation resistance is one of the main challenges in cancer treatment (8, 9). Polyphenol as plant compound possess antioxidant properties which by inhibiting resistance pathways may lead to radiation sensitization in cancer (10). Sinensetin (SIN) as polyphenol, isolated from leaves of the *Orthosiphon stamineus* and orange oil (11). Sinensetin (SIN) as polyphenol, isolated from leaves of the *Orthosiphon stamineus* and orange oil (11). SIN belongs to the family of polymethyl flavonoid with antioxidant and chemotherapy potentials (12). In the current study, we examined combine therapy (SIN and radiation) in Triple-negative breast cancer (MDA-MB-231) and T47D.

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Materials and Methods

SIN was obtained from (Cayman Chemicals,USA) was dissolved in dimethyl sulfoxide (DMSO) (Merck,Germany). Medium (RPMI-1604) and fetal bovine serum were prepared from (Gibco, UK). Trypsin were provided from (Troge medical Gumby, Germany). Trypan blue, methyl green, penicillin, and 3-[0, 3-Dimethylthiazol-2-yl]-2, 3 diphenyltetrazolium bromide (MTT) were prepared from (Sigma Aldrich, USA).

Cell culture

The breast cancer cell lines MDA-MB-231 and T47D were provided from national cell bank of Iran (Pasteur Institute, Iran). Cells were grown in medium (Gibco,UK), supplemented with penicillin (Sigma, USA) and streptomycin (Jaberebn-Hayan, Tehran, Iran), and 10 % heat-inactivated fetal bovine serum (FBS) (Gibco,UK). Cells were incubated in 5 % CO_2 at 37 ºC and replaced cultured when reaching 80–90% confluence.

Cell viability assay

Cells were seeded in 96-well plates (SPL,Korea) at a density of 6,000 cells/well and incubated overnight. SIN (Cayman Chemicals, USA) was added to medium containing 0.1 % DMSO and cells were treated with various, concentrations of SIN afterward, incubated for 24 h. MTT reagent (Sigma Aldrich,USA) was added to each well and incubate for 3 h.

To dissolve the crystals (after formation of formazan) was used DMSO (Merck, Germany). After that, incubated for 5 minutes .absorbance was defined at 570 nm with an Elisa (AsysHitech) reader spectrophotometer.

Treatment with SIN and X- irradiation

The Cells were harvested incubated for 24 hours at 37 C and 5 % CO_2. Then pristine medium containing SIN (50 M) was added to each plate and incubated for extra 24 h. Irradiation was performed with Siemens Primus linear accelerator X-ray (Germany) the treatment group exposed 2 to 10 Gy with 6-MV X-ray photons with a dose rate of 2 Gy/min.

Colonogenic survival assay and radiosensitivity

For colony formation assay, the cells were collected after irradiation (7 days for MDA-MB-231) and 14 days (T47D). Afterwards, colonies were stabilized and stained with formaldehyde and crystal violet respectively. The control was untreated-cells kept for 24h. The survival curve was fitted by the linear-quadratic; LQ model to measure cell survival data, as a task of the dose range.

Comet assay

The comet assay was performed for evaluating DNA strand breakages (breakages is better). In brief, SIN-treated, radiation treatment and non-treated irradiated cells were embedded in 1 % low-melting-point agarose in glass microscope slides. The lysed with detergent and high salt (1 % Triton X-100, 100 mM EDTA, 2.5 M NaCl and 10 mM Tris/HCl at pH 10) to form supercoiled loops of DNA attached to the nuclear matrix. Electrophoresis at pH 13 (16 V for 30 min) results DNA double strands to separate. Finally, glass slides neutralized with neutralize buffer, stained with ethidium bromide and observed by fluorescence microscopy. The intensity of the comet tail relative to the total intensity describes the amount of DNA breakage.

RNA extraction, cDNA synthesis and Real-time PCR

The cells were seeded into 6-well plates and incubated for 24 h, then in 2 groups treated with SIN and combinantial SIN (IC50 concentration) and radiation (4GY).

According to manufacturer's instruction, total cellular RNA was extracted by RNX-Plus solution (CinnaGen, Tehran, Iran) .To investigated cDNA synthesis, random hexamer primer (Fermentas, Pittsburgh PA, USA), 1 µg of total RNA, EDTA (CinnaGen, Tehran, Iran), dNTP (CinnaGen ,Tehran, Iran), DEPC Water (CinnaGen, Tehran, Iran) , Reverse Transcriptase (Fermentas, Pittsburgh PA, USA), RNase buffer (Fermentas, Pittsburgh PA, USA) were used. Real-time quantitative PCR was accomplished to quantify the expression levels of p53, Bcl-2 and STAT3 were quantified in MDA-MB-231 and T47D cells using master mix in Real Time Thermo cycler (RotorGene 6000, Corbett Life Science, and USA). QRT-PCR–derived data were analyzed by the $2^{-\Delta\Delta CT}$ methods ($\Delta\Delta$Ct is the difference between the ΔCt values of the treated/experimental sample and the untreated/control sample).

Statistical analysis

Data were analyzed by one-way ANOVA and multiple comparisons by Dunnett's test and reported withmean ± SEM and $P < 0.05$ is considered significant; * represent $P < 0.05$.

Results

Effect of SIN on normal and cancerous cell lines

The viability of treated cells were assessed after 24, 48, and 72 h treatment with various concentrations of SIN (0.1, 1, 10, 50, 100, and 150 µg/ml), the half maximal inhibitory concentration; IC50 for MDA-MB-231 was significantly less than T47D cells. The IC50 values for MDA-MB-231 and T47D cells were acquired to be 0.1 and 1 µM at 48h respectively (Figure1).

IC50 calculation

Cell survival rate (%) = (a - c)/ (b - c) × 100 **(**a = absorbance at each concentration of the 0.1, 1, 10, 50, 100, and 150 µg/ml SIN, b = absorbance at 0 µg/ml, and c = absorbance of the control)

Colony formation ability with SIN

As shown in Figure 2, SIN significantly decreased MDA-MB-231 colony formation ability. In similar way, SIN slightly reduced colony formation of T47D cell lines.

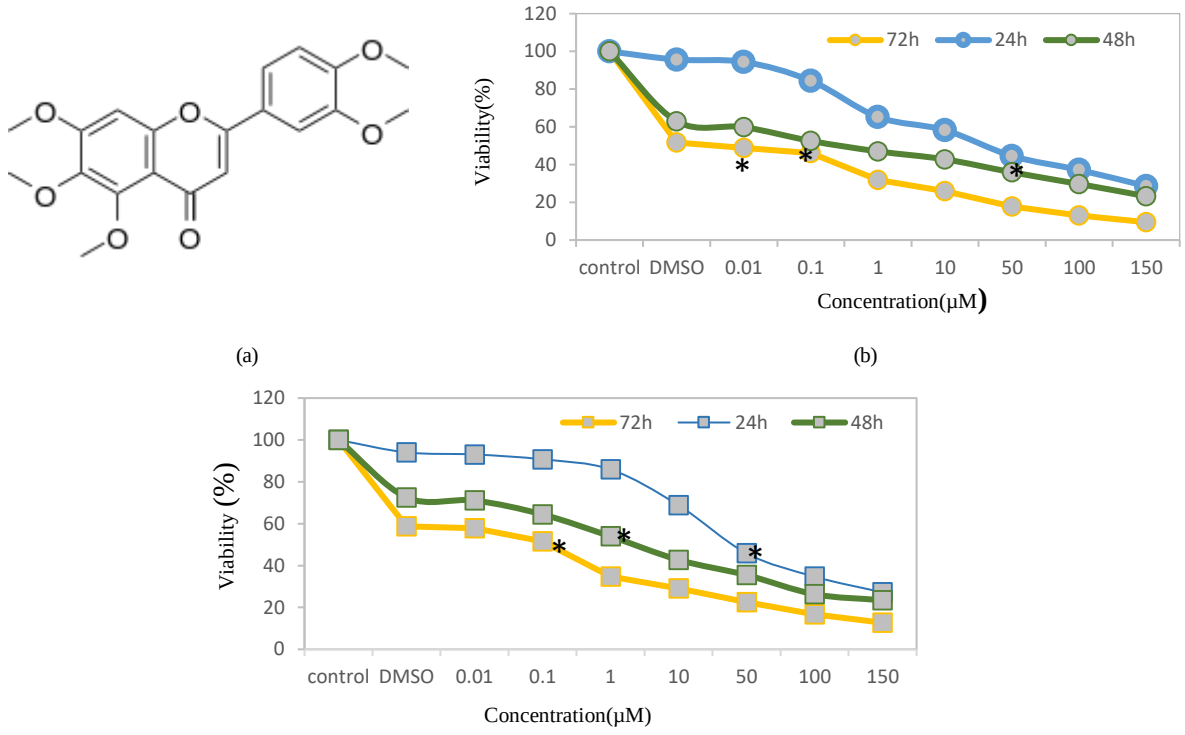


Figure1. a) Chemical structure of SIN. b) The cell viability of MDA-MB-231, c) T47D cells treated with different concentrations of SIN for 24, 48, 72h .* shows the IC_{50}. The results are expressed as mean ± SEM ($P<0.05$). Each data is compared to its representative control group.

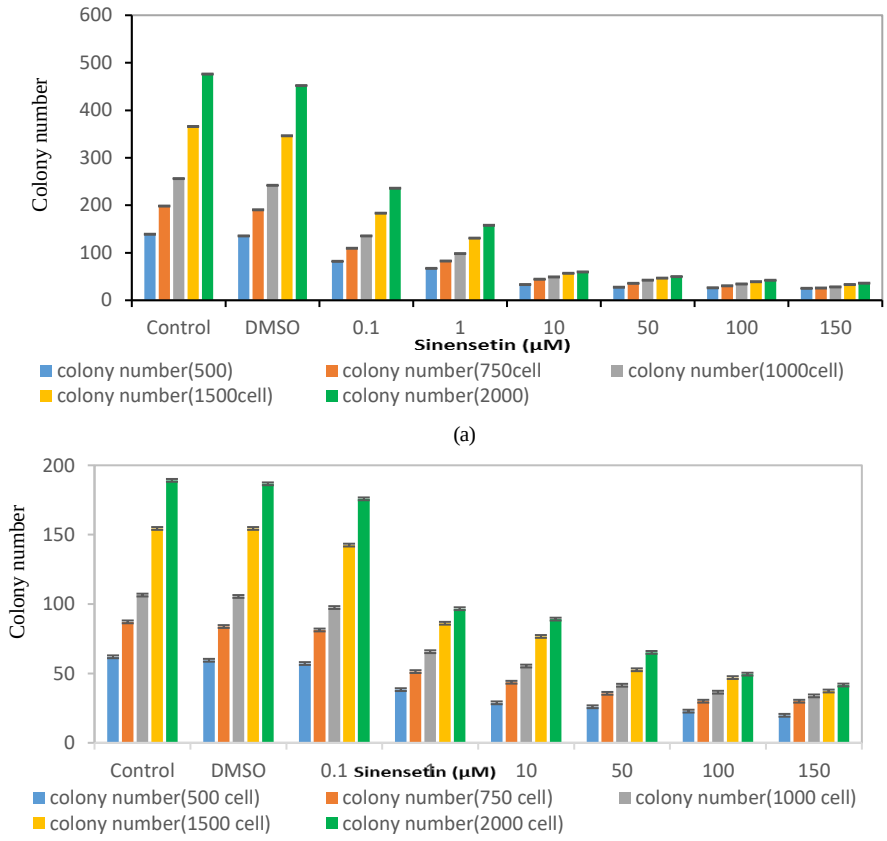
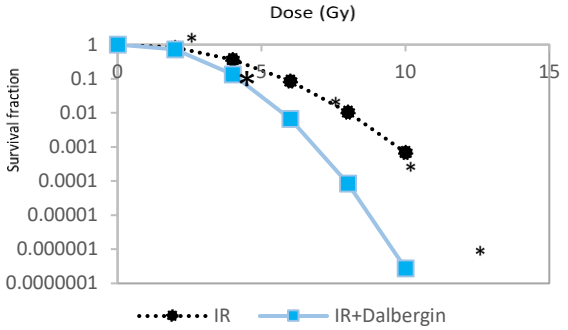


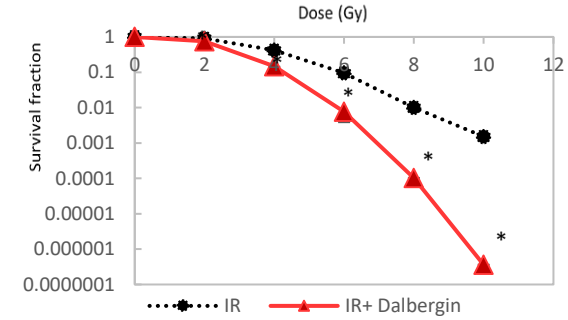
Figure 2. Platting efficiency of MDA-MB-231 (a) and T47D (b) cells. The cells were treated with different concentration of SIN.* $p < 0.05$. Compared with the corresponding control (DMSO=dimethyl sulfoxide).

Colony formation assay with SIN and radiation(combine therapy)

The relationship between SIN and x-ray radiation is shown in Figure 3. At first, MDA-MB-231 and T47D were treated with 0.1 and 1 µg/ml respectively for 48h then compared to the radiation only. Figure 3, shows survival curve of MDA-MB-231 and T47D with radiation alone and both radiation-SIN. Combination treatment remarkably reduced survival fraction compared to the radiation only. The α and β parameters assessed and shown in Table1.



(a)



(b)

Figure 3. Effect of combine therapy of a) MDA-MB-231 and b) T47D. In drug used SIN (0.1 and 1 µg/ml, 48h) were irradiated at 0-10 Gy.The survival curves were fitted by LQ model. * $p < 0.05$. Colony formation SIN and IR compared with the corresponding control.

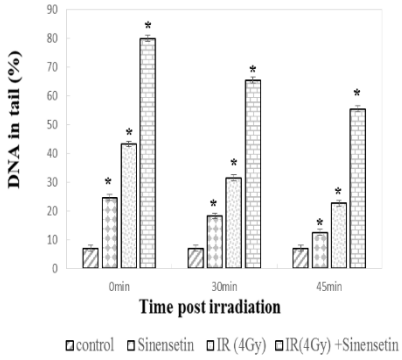
Table1. The α and β parameter calculated from survival curve fitted to linear-quadratic; LQ model.

	MDA-MB-231		T47D	
	α	β	α	β
Radiation	0.079	0.076	0.16	0.13
SIN + Radiation	0.16	0.12	0.19	0.15

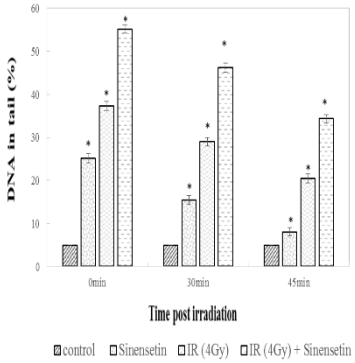
Radiation-induced DNA damage assay

This test could show DNA damages induced in 3 groups following by treatment with SIN, radiation (4GY) and combination therapy. All groups demonstrated that DNA could be damaged but in the combine treatment this result shown obviously. These observations were more notable in MDA-MB-231. The levels of DNA breakage (breakage is more suitable) were quantified by measuring the percentage of DNA in tail, tail moment (Figure 4).

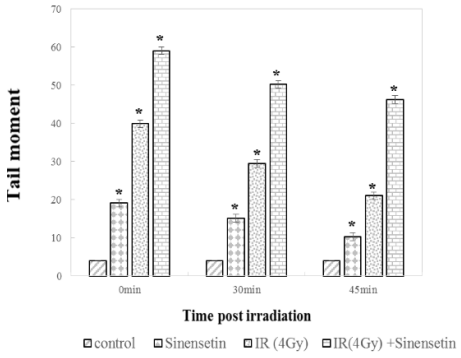
With 0.1 µM SIN were administered for 4 hours in MDA-MB-231 and T47D cell lines. Images were analyzed by Image J software (%.)



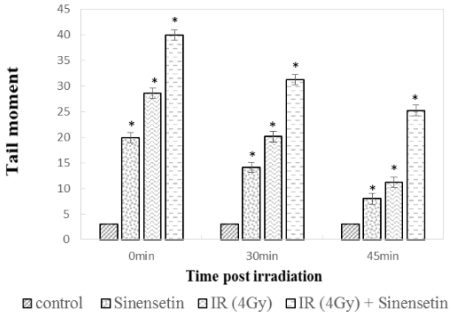
(a)



(b)



(c)



(d)

Figure 4. SIN could retain processes of radiation-induced DNA damages in a) MDA-MB-231 and b) T47D. The cells MDA-MB-231 and T47Dcell were treated with 0.1 and 1 µg/ml, 48h and irradiation 4 G respectively. The images were achieved using a fluorescence microscope. c) Tail moment in MDAMB-231 and d) T47D. Data illustrated mean ± SEM of three independent experiments. Each category is compared to its envoy control group (* $p < 0.05$).

Evaluation of Gene Expression (BCL-2, STAT3, and P53)

Expression of, Bcl-2, p53, and STAT3,3 genes in MDA-MB-231 and T47D cells that were survived. The cells were treated with SIN (0.1 and 1µg/ml) for MDA-MB-231 and T47D) 48 h is shown in Figure 5. In combine therapy (SIN and 4GY radiation) in both BC cells, expression levels of the apoptosis-related genes BCL-2, P53, STAT3 were significantly decreased (Figure6).

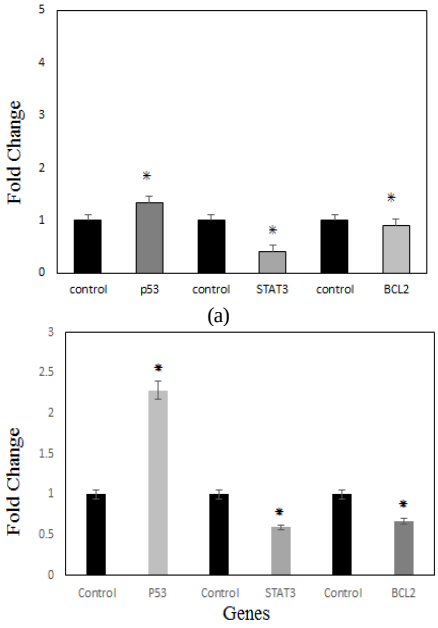


Figure 5. Relative gene expression of a) MDA-MB-231 and b) T47D (treated with IC50 concentration) were analyzed using the three genes for qPCR. * $p < 0.05$. Genes compared with the control group.

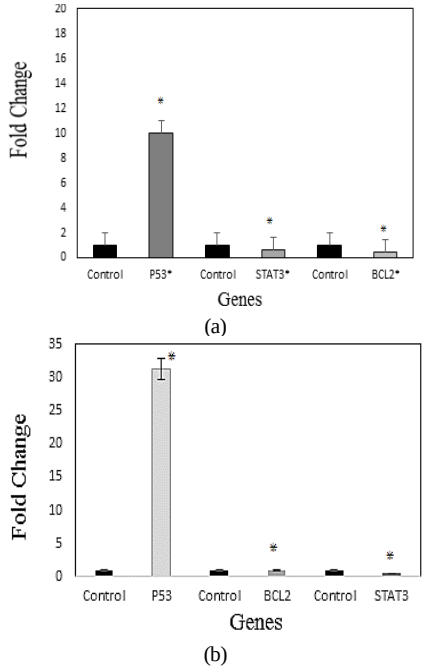


Figure 6. Gene expression level e of STAT3, Bcl-2, p53 genes after combine therapy (SIN) and radiation. A) MDA-MB-231 and B) T47D**.** * $p < 0.05$. Genes compared with the corresponding control.

Discussion

Radiotherapy is one of the most common and effective treatments against breast cancer. On the other hand, radioresistance that mainly because of the presence of cancer stem cells is a common limitation of this modality(12). Applying radiosensitizer agents that make tumor cells more sensitive to ionizing radiation, is a promising way leading to improved survival (13)SIN is an isoflavone derived from citrus and Orthosiphon Stamineus plant used as an anticancer drug and can suppress signaling pathways in radioresistant cells. The plant polyphenols can selectively radiosensitize tumor cells compared to normal cells, however, the molecular mechanism behind this radiation response is unclear.

ER positive breast cancer usually have a good prognosis and are often responsive to anti-estrogen drugs, whereas ER-negative breast cancer are poor prognosis and insensitive to anti-estrogen modalities (14).

In this investigation, we used MDA-MB-231 cell lines as ER-negative (negative means do not need estrogen to grow cells) and T47D cell lines as ER-positive, to study whether SIN can enhance the radiation response in these cells(15).

Our results showed the dose-dependent inhibition of cell growth and clonogenic ability in both ER-positive and ER-negative cancer cells with SIN (16,17). Previous researches have also demonstrated this similar inhibition effect against different cancer cells (18,19) Additionally, surviving fraction of cells treated with SIN and exposed to cells irradiated only decreased significantly compared to cells irradiated only. Based on our results, higher radiosensitivity of MDA-MB-231 compared to T47D related to increasing α and β parameters. Tumor cells with a higher value of α and β are more susceptible to radiation.

The ability of tumor cells to repair radiation induced DNA damages is a main cause of radio resistance. Thus, disruption and delay in DNA repair signaling pathway constrains tumor cells (20). Based on the comet assay (Figure 4) results SIN could retain DNA lesions post irradiation in both cell lines.

SIN was also able to increase the expression of main apoptotic pathway genes like P53. On the other hand BCL-2 and STAT-3 decreased following irradiation in MDA-MB-231 and T47 cells (Figure 7).

Apoptosis induced by SIN in X-irradiated cells may be mediated through inhibition of the NF-κB pathway, which regulates the DNA breaks. Figure 7 provides an insight at a possible action mechanism for SIN in radiation induced DNA damage lesions.In this work, for the first time we presented proof that SIN displays radiosensitivity properties on both MDA-MB-231 and T47D cells as shown by its capacity to hinder DNA repair pathway and induction of apoptosis(21). Based on these results, it may be concluded that MDA-MB-231are more susceptible to radiation by treatment with

SIN. The variation of radiation response between two cells could be associated to the expression level of estrogen receptor, however further studies are needed to elucidate this issue.

Conclusion

In this study the prevalent techniques and new approaches in development to treat breast cancer cells is described. The various concentrations of sinensetin is an unprecedented radio sensitizing factor for MDA-MB-231 and T47D cell lines. This new way can regulate cell apoptosis via intrinsic and extrinsic mechanisms of action in vitro conditions. Based on the data presented in this research, treatment with radiation and sinensetin increase apoptotic cell death in accord with DNA and real time PCR curve. In addition, studies are needed to gain the molecular mechanism.

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