

## Original Article

**Running Title:** Effect of Dalbergin on T47D and MDA-MB-231 Breast Cancer Cells

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### In Vitro Radiosensitization of T47D and MDA-MB-231 Breast Cancer Cells with the Neoflavonoid Dalbergin

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#### Abstract

**Background:** Radiotherapy is a frequently used therapeutic modality for breast cancer. Dalbergin, a natural antioxidant, inhibits carcinogens and tumor progression. In the present study, we investigated the effect of Dalbergin on the response of T47D and MDA-MB-231 breast cancer cell lines to ionizing radiation.

**Method:** In this experimental in vitro study, doubling time of T47D and MDA-MB-231 were obtained from the growth curve. The cytotoxic effect of Dalbergin on T47D and MDA-MB-231 breast cancer cells were estimated via MTT assay. To determine the clonogenic ability, we treated T47D and MDA-MB-231 with Dalbergin for 48 h prior to irradiation, subsequent to which a colony assay was performed. Real-time polymerase chain reaction was employed to determine the gene expression level.

**Results:** Dalbergin inhibited proliferation of T47D and MDA-MB-231 in a time- and concentration-dependent manner. Additionally, the most appropriate time for the treatment of these types of cancer cells was found to be 48 h and the drug's concentration in both cell lines was different. The IC<sub>50</sub> values of T47D and MDA-MB-231 cells were 0.001 and 0.0001 μM, respectively. Moreover, this drug radiosensitizes both cell lines effectively compared to the radiation only. Finally, the gene expression level of *p53*, *Bcl-2*, and *STAT3* were investigated in cancer cells.

**Conclusion:** Dalbergin showed apoptotic effects probably through the *STAT/p53* signaling pathway. Therefore, Dalbergin could be considered as a radiosensitizer and its effects may be owing to increased cell death.

**Keywords:** Dalbergin, Cells, X-rays, Apoptotic, Cell death

## Introduction

Breast cancer is the major cause of cancer-related death in women worldwide. Pre-clinical and clinical studies have shown extensive differences concerning morphology, histology, genetics, and replies to treatment in breast cancer.<sup>1,2</sup> Radiotherapy is a cancer treatment method aiming to maximize tumor damage while minimizing side effects on normal adjacent tissues.<sup>3</sup> Thus, adjuvant therapy provides more promising approaches to increasing the total effectiveness of radiotherapy in patients with cancer. Combination treatment may also diminish the toxicity caused by a high dose of radiation; therefore, lower doses could be used.<sup>4</sup> In addition, plants polyphenols may increase the tumoricidal effects of chemotherapy and radiotherapy, protect normal cells from therapy-induced damage, and enhance systemic bioavailability of chemotherapeutic drugs.<sup>5</sup> Polyphenols are the main secondary metabolites of plants.<sup>6,7</sup> Polyphenol compounds are found in plants and possess various biological features; they play a critical role in signaling cascades involved in apoptosis and the progression of cancer.<sup>8</sup> Dalbergin is the most common and simple compound of neoflavonoid, which is cultivated in the Indian Subcontinent, Southern Iran, and China.<sup>9</sup> Dalbergin is extracted from *Dalbergia sissoo* and is widely used traditionally as an anti-inflammatory, antipyretic, analgesic, antioxidant, anti-diabetic, antimicrobial, and anti-cancer agent.<sup>10</sup> This polyphenol is also involved in signaling pathways related to radio-resistant cancer cells. Nevertheless, whether the combination of Dalbergin and radiation induces apoptotic cell death still remains unclear. On the other hand, *p53* gene

encodes a transcription factor that contributes to several different cellular activities, including apoptosis, and the ability of cells to express a functional *p53* protein may be an indicator of cellular response to radiation.<sup>11,12</sup> Specifically, signal transducers and activators of transcription (*STAT3*) expression can suppress cell growth and induce cell apoptosis in numerous kinds of cancer cells.<sup>13</sup> *Bcl-2* (B-cell lymphoma 2), encoded in humans by the *Bcl-2* gene, is the founding member of the *Bcl-2* family of regulator proteins; they regulate cell death by inducing apoptosis.<sup>14</sup> DNA is thought to be the original aim of ionizing radiation in cells. Double-strand breaks are the main form of damage that leads to cell death following irradiation.<sup>15</sup> Plant polyphenols, the compounds which resist against radiation-induced DNA damage in cancer cells, have a great potential to be considered into cancer drugs owing to their well-defined efficient effects and insignificant side-effects.

In this study, we evaluated the combined effect of Dalbergin and ionizing radiation on the estrogen receptor-positive T74D and negative MDA-MB-231 breast cancer cell lines by analyzing its proliferation, clonogenic ability, radiation-induced DNA strand breaks, and alteration in cell death-associated genes expression with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay, clonogenic assay, Comet assay, and Real-time polymerase chain reaction (PCR), respectively.

## Materials and Methods

In the present experimental in-vitro research, we studied the effect of Dalbergin on apoptosis

induction and increased cell death on T47D and MDA-MB-231 cells with and without radiotherapy.

#### **Chemicals**

Dalbergin (Nr. AB151655) from abcr GmbH (Germany) was dissolved in DMSO (Merck) before use. Dimethylsulfoxide (DMSO) was supplied from Merck (Germany). Cell culture media (RPMI-1640) and fetal bovine serum were supplied from Gibco (UK).

#### **Cell culture**

T47D and MDA-MB-231 cells were obtained from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). The cells were cultured in RPMI-1640 (Gibco, UK), containing 500 units/ml penicillin and 200 µg/ml streptomycin; they were supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, UK) at 37°C in a 98% humidified atmosphere with 5% CO<sub>2</sub>. Upon reaching the cell density to 90%, the cell culture medium was changed.

#### **Cells growth curve**

To determine the Lag (A), Log (B), Stationary (C), and Decline (D) phases, the cells were plated in 24-well plates (SPL, Korea) at densities of 10,000 cells/well. We then assessed the number of the cells through direct counting on days 0-7 after the culturing.

#### **Cell viability assay**

T47D and MDA-MB-231 cells were counted and appropriate numbers of them were plated in 35-mm Petri dishes followed by incubation at 37°C for 12 and 7 days, respectively. Subsequently, the colonies were fixed, stained, and counted. T47D and MDA-MB-231 cells were respectively plated at densities of 30,000 and 20,000 cells/cm<sup>2</sup> in 24-well plates and incubated for 24 h. For irradiation, the cells in the well plates were exposed to 0, 2, 4, 6, 8, and 10 Gy. Irradiation was performed using a 6 MeV X-ray photon, produced with a linear accelerator (Siemens Primus; Siemens AG, Erlangen, Germany) at a dose rate of 200 cGy/min. Afterwards, the

cells were cultured into 35-mm cell culture plates in RPMI and incubated for 14 days (T47D) and 7 days (MDA-MB231). The colonies were then fixed with formaldehyde (Merck, Germany), stained with crystal violet solution, and counted. We counted the number of colonies to calculate the survival fraction.

#### **MTT proliferation assay**

T47D and MDA-MB-231 cells were seeded at densities of 6,000 and 3,000 cells/well in 96-well plates and incubated for 48 and 24 h, respectively, to reach the log phase. The cells were then treated with different doses of Dalbergin (0,  $1 \times 10^{-6}$ ,  $1 \times 10^{-5}$ ,  $1 \times 10^{-4}$ ,  $1 \times 10^{-3}$ ,  $1 \times 10^{-2}$ ,  $1 \times 10^{-1}$ , 1, 10, and 30 µM) and incubated for 24, 48, and 72 h. The cells were also treated with DMSO as a control. Subsequently, 100 µL of MTT solution (Sigma-Aldrich, USA), at a concentration of 0.5 mg/ml, was added and the cells were incubated for 3 h followed by the addition of 100 µL DMSO to dissolve the formazan crystals. Absorbance was measured at 570 nm and 630 nm using a microplate spectrophotometer (BioTek, Winooski, USA). The difference in absorbance between the two wavelengths was measured and the percentage of viable cells was calculated as (absorbance of sample/absorbance of control) ×100 for each concentration of Dalbergin.

#### **Colony formation assay and radio sensitivity**

The cells were counted and appropriate numbers of T47D and MDA-MB-231 cells were plated in 24-well plates. Afterwards, the cells were treated with 0.001 and 0.0001 µM Dalbergin and incubated for 48 h. The cells were then exposed to 0, 2, 4, 6, 8, and 10 Gy of X-rays. We analyzed cell survival curve with MATLAB software.

#### **Apoptosis assay**

The cells were counted and appropriate numbers of T47D and MDA-MB-231 cells were cultured in 24-well plates (SPL, Korea) at densities of 15,000 and 10,000 cells/well,

respectively; they were incubated for 48 h at 37 °C in the presence or absence of  $1 \times 10^{-3}$  and  $1 \times 10^{-4}$   $\mu$ M Dalbergin. The cells were irradiated with X-rays (4 Gy) and then stained with AO (100 mg/ml) and EB (100 mg/ml). The viable, apoptotic, and necrotic cells were counted under a fluorescence microscope.

#### **Comet assay**

T47D and MDA-MB-231 cells were cultured in 24-well plates and incubated for 24 h, to reach the log phase. In brief, the cells were suspended in 1% low-melting-point agarose in PBS, with a pH of 7.4, and pipetted onto glass microscope slides percolated with a thin layer of 1% normal-melting-point agarose, which was warmed before use. The agarose was allowed to stay at 4 °C for 10 min. Afterwards, the slides were immersed in lysis buffer (1% Triton X-100, 100 mM EDTA, 2.5 M NaCl, and 10 mM Tris/HCl at pH10) at 4 °C for 1 h in order to remove cellular proteins. The slides were then placed in a horizontal electrophoresis tank containing denaturation buffer (300 mM NaOH and 1m MEDTA, pH 13) at 4 °C for 30 min in order to allow DNA double strands to get separated. Electrophoresis was performed at 16 V (1 V/cm) for 30 min. Finally, we placed the slides in neutralization buffer (0.4 M Tris/HCl) and stained them with ethidium bromide solution (20  $\mu$ g/ml). The prepared Comet assay-stained slides were examined under Axoscope 2 plus fluorescence Spmicroscopy (Zeiss, Germany). For each slide, 60 cells were evaluated and scored using image analysis software. The obtained data represented the average of the three independent experiments.

#### **Quantitative real-time PCR**

Herein, we utilized primer sequences for gene expression analysis via real-time polymerase chain reaction. The primers were designed using Oligo 7. Table 1 depicts the sequences of all the primers used and amplicon lengths. cDNA was synthesized

from the total RNAs. Moreover, *GAPDH*, a housekeeping gene, was used as the control. The fold change of each target gene (*p53*, *Bcl-2*, and *STAT3*), relative to *GAPDH*, was calculated based on relative quantitation using the  $\Delta\Delta CT$  method, according to the  $2^{-\Delta\Delta CT}$  relative expression formula. The primers were synthesized in CinnaGene Company from Iran.

The effect of Dalbergin on the expression level of marker genes *p53*, *Bcl-2*, *STAT3*, and *GAPDH* was analyzed via RT-PCR. In addition, T47D and MDA-MB-231 cells were seeded and incubated for 48 h and 24 h, respectively. Each experiment included four groups, namely control (no treatment), Dalbergin-treated (treated with Dalbergin for 48 h), irradiated (incubated in fresh medium, then exposed to radiation), and Dalbergin plus radiation group (treated with Dalbergin for 48 h, then irradiated) groups. Following incubation, the cells were exposed to radiation (4 Gy). Subsequently, total RNA was extracted by RNX-Plus solution (CinnaGen, Tehran, Iran) according to the manufacturer's instruction. To remove DNA impurity, we employed DNase (Fermentas Pittsburgh, PA, USA) at 37 °C for 30 min. Total RNA was quantified through spectrophotometry and electrophoresed on a 1% agarose. For cDNA synthesis, 1  $\mu$ g of total RNA was used according to the manufacturer's instructions, and 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 also used. The reaction mixture (9  $\mu$ l), containing 1  $\mu$ l primers and 5  $\mu$ l SYBR Green I Master Mix (QuantiFast SYBR Green PCR, Q204054), was added to 1  $\mu$ l cDNA. A Real-time thermal cycler (RotorGene 6000, Corbett Life Science, USA) started. The initial denaturation was

performed at 95 °C for 5 min, which was then at 95 °C for 15 s. The annealing temperature was optimized from 60 to 61 °C for 25 s, and extension at 72 °C for 25 s. We carried out this process for 35 cycles. Furthermore, melting curve analysis was done in each real-time PCR experiment. All the four mentioned steps were applied in different groups, including the controls, *p53*, *Bcl-2*, and *STAT3*.

#### ***Statistical analysis***

We analyzed the differences between the groups with SPSS software using repeated measures analysis of variance (ANOVA). *P-value* < 0.05 was considered to be significant. A fitting curve was obtained using the linear quadratic model. The half maximal inhibitory concentration (IC<sub>50</sub>) was calculated with MATLAB software (MathWorks Inc., Natick, USA). DNA damages were computed via ImageJ. Comparing the expression levels of *p53*, *STAT3*, and *Bcl-2* was performed using Prism7 analysis.

### **Results**

#### ***Growth curves and doubling times***

Figure 1 illustrates the growth curves of these cells. According to the results, doubling time of MDA-MB-231 and T47D cells were respectively 22.87 h and 28.02h.

#### ***Clonogenic survival of T47D and MDA-MB-231 after irradiation***

We analyzed the effect of irradiation (exposure to 0, 2, 4, 6, 8, and 10 Gy). Figure 2 exhibits a comparison between T47D and MDA-MB-231 cell survival exposed to graded doses of radiation.

#### ***Cytotoxic effect of Dalbergin on the toxicity of T47D and MDA-MB-231***

The cytotoxic effect of Dalbergin on T47D and MDA-MB-231 was studied with MTT assay. The cells were treated with Dalbergin at concentrations ranging from  $1 \times 10^{-6}$  to 30  $\mu\text{M}$  for 24, 48, and 72h, following which the percentage of viable cells were determined. The toxicity of Dalbergin was

time- and concentration-dependent in the two cell lines. As shown in Figures 3A and B, the viability of T47D and MDA-MB-231 cells strikingly decreased at higher concentrations of Dalbergin ( $1 \times 10^{-6}$ -30  $\mu\text{M}$ ) at the three different incubation times. Nonetheless, a remarkable reduction in the number of viable T47D and MDA-MB231 cells occurred at 48 h. The IC<sub>50</sub> values for T47D and MDA-MB231 cells were found to be  $1 \times 10^{-3}$  and  $1 \times 10^{-4}$   $\mu\text{M}$  at 48 h, respectively.

#### ***Radiosensitizing effect of Dalbergin***

T47D and MDA-MB-231 were treated with  $1 \times 10^{-3}$  and  $1 \times 10^{-4}$   $\mu\text{M}$  Dalbergin, respectively, for 48 h and irradiated with various doses of ionizing radiation. Figure 4 shows the survival curves of T47D and MDA-MB231 cells treated with Dalbergin and X-ray, respectively. The survival of T47D and MDA-MB-231 cells decreased at 4 Gy after the combination treatment in comparison with irradiation only (Figures 5A and B).

#### ***Effect of Dalbergin on radiation-induced apoptosis***

Figures 5A and B demonstrate the percentage of apoptotic cells after X-irradiation with and without Dalbergin treatment. For the T47D and MDA-MB-231 irradiated without Dalbergin treatment, the percentages of apoptosis were approximately 23.25 and 26.28, respectively. For those irradiated and treated with Dalbergin, the percentage of apoptotic cells notably increased. These data indicated that the apoptosis induced by Dalbergin and X-ray was significantly more than that induced by Dalbergin or radiation alone. Additionally, the percentage of necrosis in T47D saw a slight increase with and without Dalbergin and radiation; however, the rate of necrosis in MDA-MB-231 remained unchanged with IR and Dalbergin compared to the control. Meanwhile, necrosis in MDA-MB-231 with Dalbergin + IR considerably increased.

### ***Single-cell gel electrophoresis (Comet) assay***

In the Comet assay, DNA damage was assessed by measuring the fraction of DNA in the tail moment (Figure 6A and 6B) in the four groups of the controls, Dalbergin alone, radiation alone, and combination of Dalbergin and radiation at various time periods after irradiation. T47D and MDA-MB231 cells were treated with  $1 \times 10^{-3}$  and  $1 \times 10^{-4}$   $\mu$ M Dalbergin alone, respectively, which slightly changed the level of DNA damage as compared to that in the control ( $P > 0.05$ ). Radiation alone caused DNA damage in T47D and MDA-MB-231 cells directly after exposure. In irradiated T47D and MDA-MB-231 cells, the damage was rapidly repaired in nearly 30 min after exposure. Combined treatment of the cells with Dalbergin and radiation significantly postponed the repair process for up to 45 min following their exposure to radiation. A significant difference was observed in the fraction of DNA in the tail moment for 30 and 45 min.

### ***qRT-PCR for STAT3, Bcl-2, and p53***

qRT-PCR for *STAT3*, *Bcl-2*, and *p53* was done with of the control, Dalbergin, IR, and combination of Dalbergin and IR as described using RT-PCR. T47D and MDA-MB-231 cells were treated with Dalbergin at  $1 \times 10^{-3}$  and  $1 \times 10^{-4}$   $\mu$ M, respectively, for 48 h (Figures 7A and B). T47D presented decreased levels of *STAT3*, *Bcl-2*, and *p53* genes increased. MDA-MB-231 cells also presented increased levels of *STAT3*. *Bcl-2* witnessed a significant decreased while *p53* grew significantly in MDA-MB-231. The expression of *STAT3*, *Bcl-2*, and *p53* genes was quantified after the treatment with ionizing radiation alone (IR) and combination of Dalbergin and radiation treatment in T47D and MDA-MB-231 cells (Figures 8A and B).

### **Discussion**

In the current study, we observed that Dalbergin acts as a radiosensitizer whose effects may be attributed to increased cell death. Moreover, Dalbergin showed apoptotic effects probably through the STAT/P53 signaling pathway. Nowadays, breast cancer is the most prevalent malignant cancer among women and remains the second leading cause of cancer-related deaths among females. Ionizing radiation has been extensively used in the treatment of breast cancer.<sup>16</sup> Tumor resistance against radiotherapy is the main obstacle to an effective treatment. A number of factors, such as genetic or epigenetic alterations, along with some environmental factors, have been reported to aggravate radio resistance of cancer cells. Therefore, finding new factors that can sensitize cancer cells to ionizing radiation is believed to be imperative.<sup>17</sup> A broad range of evidence has shown that fruits and vegetables are rich in various bioactive phytochemicals, such as polyphenols, which have been identified to preferentially sensitize cancer cells to radiation. Moreover, dietary polyphenols have presented prominent effects on anti-tumorigenesis properties.<sup>18</sup> Neoflavonoids are a class of polyphenolic compounds with anti-oxidant, anti-cancer, nitric oxide-reducing, and anti-inflammatory activities.<sup>19</sup> T47D cell line is epithelial, adherent, hypo triploid, and ER-positive.<sup>21</sup> On the other hand, MDA-MB-231 cells are highly aggressive and invasive, and poorly differentiated triple-negative breast cancer (TNBC) cell lines.<sup>22</sup> In this study, we investigated whether Dalbergin can sensitize these two human breast cancer cells to X-ray irradiation. Initially, the doubling times were calculated to be 28.02 and 22.87h for T47D and MDA-MB-231, respectively; the doubling time represents the growth pattern of the cells. The clonogenic assay is frequently used in cancer research to

determine the effect of drugs or radiation on proliferating tumor cells.<sup>23, 24</sup> Hence, we studied the ability of T47D and MDA-MB-231 breast cancer cell lines to IR with colony forming assays. The number of colonies of MDA-MB-231 cells saw a slight decrease compared to T47D cells.<sup>25, 26</sup> Considering the shoulder region of the curve as a measure of the repair capacity of the cell for radiation damage after exposure to low doses, especially 4 Gy,<sup>27</sup> it could be proposed that Dalbergin sensitizes T47D and MDA-MB-231 in 4Gy doses of radiation via inhibition of repair pathway. Our data revealed that the surviving fraction significantly decreased in the cells treated with Dalbergin and radiation simultaneously compared to that of the cells subjected to radiation only. This indicates that the radiosensitization of Dalbergin is related with an increase in  $\beta$  parameter values in MDA-MB-231 cells. On the contrary, the increase in the radiosensitizing effect in T47D cells by Dalbergin may be related to the great disturbance at lower doses of radiation, which then acts as a  $\alpha$ -type sensitizer. Based on a previous study, an increase in  $\alpha$  parameter was ascribed to the DNA damage caused by a single-hit effect of radiation interaction. This damage included double-strand breaks, which can be mortal. The changes in the  $\beta$  parameter are caused by two radiation interactions.<sup>28, 29</sup> Thus, compared with MDA-MB-231 cells, T47D cells are more sensitive to damage by combinational treatment with Dalbergin. The clonogenic radiosensitization was noticeable for T47D and MDA-MB-231. According to our results, the most pronounced effect of Dalbergin was on the induction of apoptosis. As expected, the apoptotic shock was more severe in MDA-MB-231 cultures as compared to that in T47D. Our findings indicated that Dalbergin can sensitize T47D and MDA-MB-231 cells to radiation; meanwhile, under similar conditions, Dalbergin and 4Gy of radiation are more

effective on MDA-MB-231 than on T47D. There is a difference concerning the efficacy of Dalbergin on the cell death between MDA-MB-231 and T47D cells, which is due to the fact that the drug can reach all the cells, especially those located in the core of the cellular assemblies.<sup>30</sup> Kabala-Dzik and colleagues, in 2017, compared CA and CAPE activity on triple-negative human breast adenocarcinoma line cells. They reported that polyphenols induced apoptosis whereas CAPE induced a higher apoptotic effect. CAPE also induced cell cycle arrest in S phase. CA did it only for 50 and 100  $\mu$ M. Dalbergin is also a polyphenol that could induce apoptosis in breast adenocarcinoma line cells (MDA-MB-231 and T47D).<sup>31</sup> In the Comet assay, the amount of DNA damage rapidly decreased in the irradiated cells. Dalbergin could maintain DNA damage during the combined treatment with radiation compared to the irradiated cells which were not treated with Dalbergin. Our data supported that Dalbergin postponed the repair mechanism by up to 45 min in T47D and MDA-MB-231 cells and could disrupt DNA repair by up to 45 min after radiation. In the MDA-MB-231 and T47D, we observed an additive and synergistic interaction following the combinational treatment. In 2005, Piero Dolaro and colleagues indicated wine polyphenols, administered to rats not treated with carcinogens, which resulted in a significant decrease in the basal level of DNA oxidative damage of the colon mucosa as measured with the Comet assay.<sup>32</sup> In addition, Alrena V Lightboun and colleagues reported that treatment with *Ficus carica* leaf extract inhibits spontaneous DNA damage and reverses non-steroidal estrogen (DES)-induced DNA strand breaks in individuals, similar to human epithelial breast tumor cells. *Ficus carica* differentially promotes DNA repair and ameliorates Comet formation owing to the irreversible

interaction of oxidative quinine metabolites of DES with the nuclear apparatus.<sup>33</sup> Therefore, combination of Dalbergin and radiation is able to treat breast adenocarcinoma cell lines (MDA-MB-231 and T47D). Quantitative RT-PCR analysis demonstrated that treatment with this drug can increase the expression of mRNA levels of *p53*. However, the expression of mRNA levels of *Bcl-2* and *STAT3* saw a significant decrease. This result showed that Dalbergin presents antioxidant activities and can induce apoptosis in T47D cells. Once exposed to radiation and Dalbergin, there was an increase in *p53* whereas *Bcl-2* and *STAT3* in the T47D and MDA-MB-231 cells were reduced. Previous research has demonstrated that this polyphenol acts more efficiently than equol and caffeic acid for treatment of T47D cells and MDA-MB-231. T47D and MDA-MB-231 treatment with Dalbergin and IR that the least concentration of Dalbergin and dose radiation effect showed radiosensitizer in breast cancer cells.<sup>34</sup> Dalbergin inhibited clonogenicity and maintained radiation-induced DNA damages in the two cell lines. Given the similarity in structures between Dalbergin and estrogen, Dalbergin may be more effective in T47D cells than in MDA-MB-231 cells. In accordance with the results of the Comet assay, there was a synergistic interaction between Dalbergin and radiation. Altogether, the Dalbergin/ *P53/Bcl-2/STAT3* signaling axis may have potential therapeutic values in the treatment of breast cancer cells with radiation (Figure 9). N. In 2020, Rezakhani and colleagues showed that polyphenol group (sinensetin) induced apoptosis with *Bcl-2*, *STAT3*, *p53* in human breast cancer, and sinensetin induced the overexpression of *STAT3*, *Bcl-2* and *p53* genes. This phenomenon leads to apoptosis induction in breast adenocarcinoma line cells treated with X-ray or X-ray combined with sinensetin.<sup>35</sup> Hence, we investigated the combination of

Dalbergin and radiation in breast cancer cells treatment through expressed *p53*, *Bcl-2*, *STAT3* signaling pathway.

In the study, we obtain many strengths point which T47D and MDA-MB-231 cells can be a treatment with the lowest concentration of Dalbergin and dose radiation. Beside, this manuscript needs to artificial intelligence and machine learning for clinical trial, which are able to develop new drug delivery systems and also, radiosensitization efficacy in the treatment breast cancer.

### Conclusion

The obtained results demonstrated that Dalbergin can act as a radiosensitizer in breast cancer cells. Several studies have proposed the potential capability of some nutraceuticals to target important hallmarks of cancer cells, such as cell cycle, apoptosis, inflammation and angiogenesis, underlining the complexity of the molecular mechanisms involved in the anticancer properties by these compounds. Evaluating these polyphenols in combination with radio-therapeutic regimens could enhance the efficacy as well as the tolerability of conventional anticancer therapies, improving the clinical outcome and survival of breast cancer patients. In conclusion, this prospective study could suggest that several classes of polyphenols with radio-therapeutic could be potentially conducive to breast cancer treatment.

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### Conflict of Interest

None declared.

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Table 1. Primers used in the present study (*STAT3*, *Bcl-2*, *p53*, *GAPDH*)

Genes	Primers	Sequence	Amplicon
<i>STAT3</i>	F Primer	5'CTGAAGCTGACCCAGGTAGC3'	123 bp
	R Primer	5'GCATCAATGAATCTAAAGTGC GGG3'	
<i>Bcl-2</i>	F Primer	5'GATACTGAGTAAATCCATGCAC3'	356 bp
	R Primer	5'AGTGTTGCAGAATATTCAGCCAC3'	
<i>p53</i>	F Primer	5'GCTCAGATAGCGATGGTCTGGC3'	156 bp
	R Primer	5'AGTGGATGGTGGTACAGTCAGAG3'	
<i>GAPDH</i> ( <i>qRT-PCR</i> )	F Primer	5'CCAGCCGAGCCACATCGCTC3'	360 bp
	R Primer	5'ATGAGCCCCAGCCTTCTCCAT3'	

*Bcl-2*: B-cell lymphoma 2; *STAT3*: signal transducer and activator of transcription 3; *p53*: tumor-suppressor protein; *qRT-PCR*: Quantitative reverse transcription-polymerase chain reaction

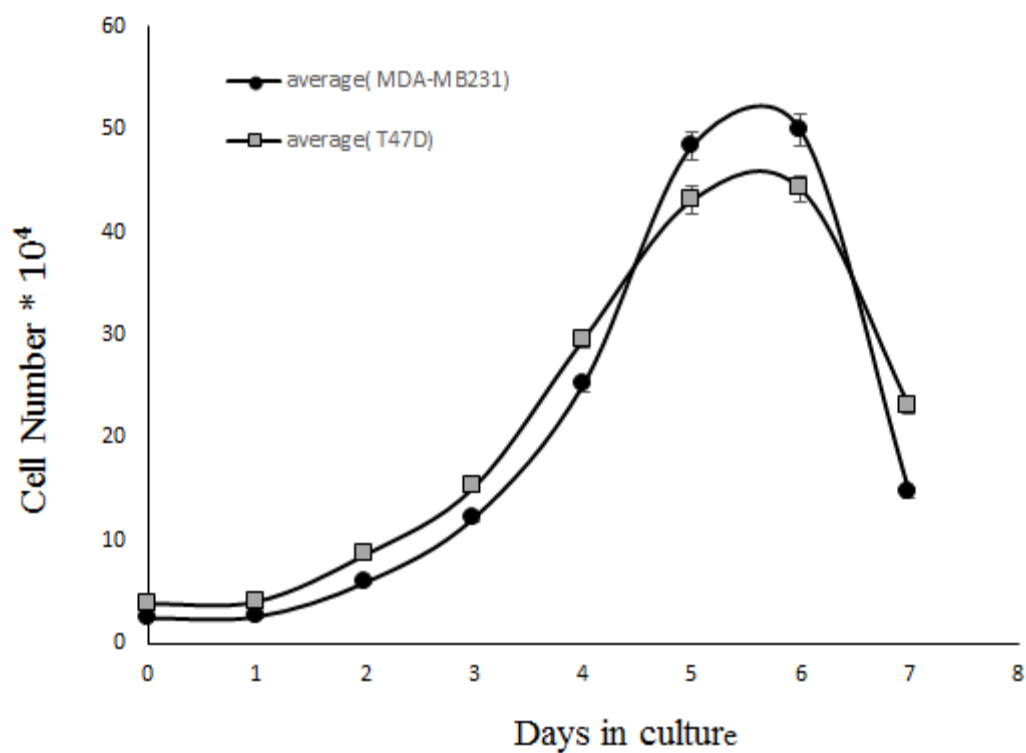


Figure 1. Reproducibility of the doubling time determined by the crystal violet assay. T47D and MDA-MB-231 were plated at an approximate density of 10,000 cells. The doubling times were calculated from an average of three experiments.

MDA-MB-231: Isolated at M D Anderson from a pleural effusion of a patient with invasive ductal carcinoma; T47D: Differentiated epithelial substrain

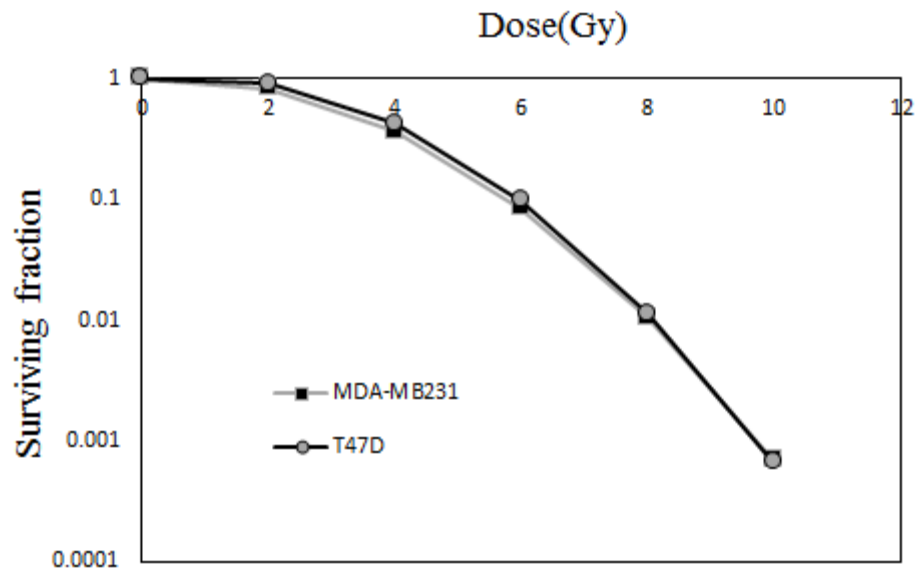
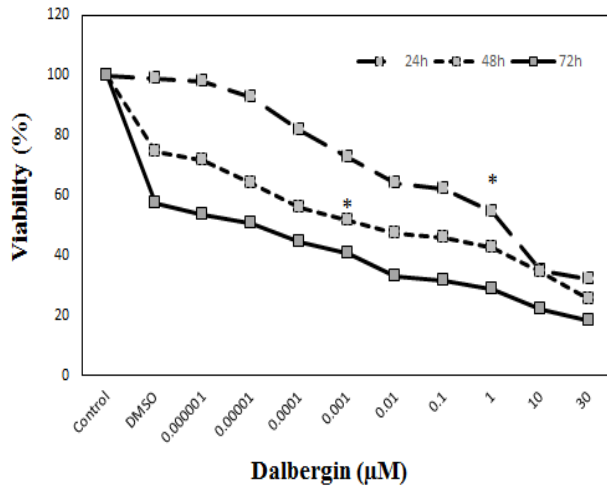


Figure 2. Colony-forming efficiency of irradiated (●) T47D and (■) MDA-MB-231 cells. Mean  $\pm$  SEM values of triplicate determinations are shown.

MDA-MB-231: Isolated at M D Anderson from a pleural effusion of a patient with invasive ductal carcinoma; T47D: Differentiated epithelial substrain; SEM: Standard error of mean

A



B

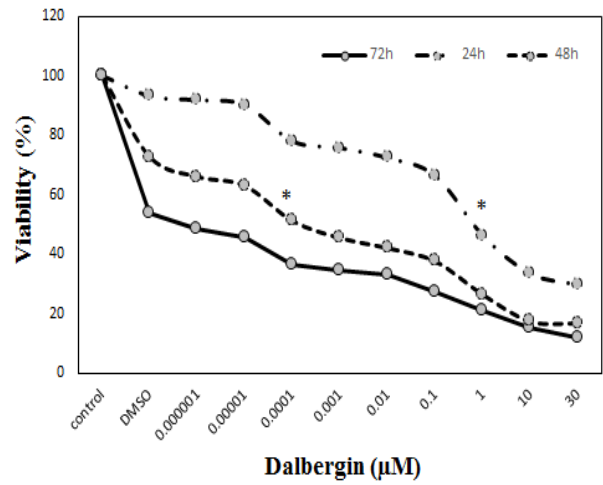


Figure 3. Effect of Dalbergin on the proliferation of T47D and MDA-MB-231 cells. The cells were treated with  $1 \times 10^{-6}$ – $30 \mu\text{M}$  Dalbergin for 24, 48, and 72 h. The percentage of cell viability was measured via MTT assay. The data represent the means  $\pm$  SEM. (A) T47D; (B) MDA-MB231. \* $P < 0.05$ . At the end,  $\text{IC}_{50}$  48 and 72 samples were compared with the control groups  $\text{IC}_{50}$  in 24h. MDA-MB-23: isolated at M D Anderson from a pleural effusion of a patient with invasive ductal carcinoma; T47D:

differentiated epithelial substrain; SEM: Standard error of mean

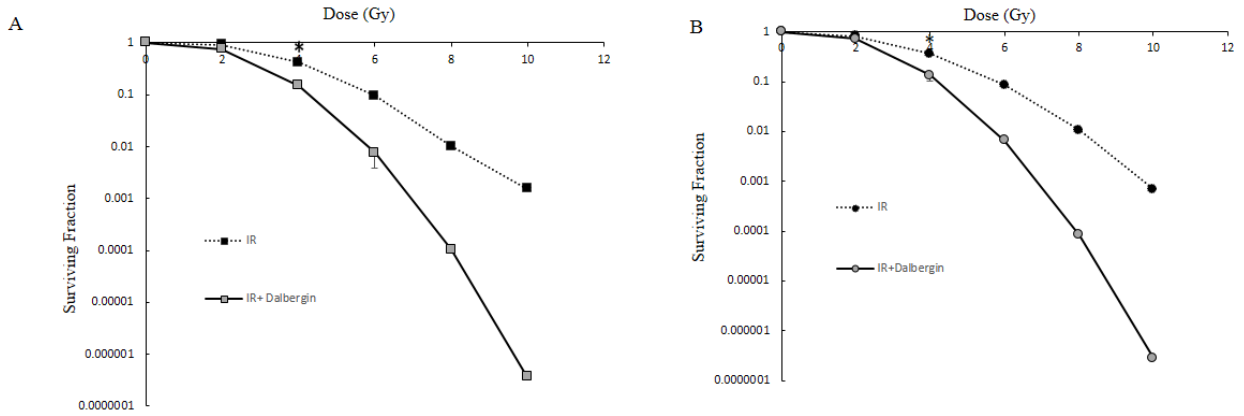


Figure 4. Dalbergin effects on T47D and MDA-MB-231 cells radiosensitivity; (A) Survival curve of T47D; (B) MDA-MB-231 cells. \* $P < 0.05$ . Colony formation of Dalbergin and IR compared with the corresponding control.

IR: Irradiation; MDA-MB-231: Isolated at M D Anderson from a pleural effusion of a patient with invasive ductal carcinoma; T47D: Differentiated epithelial substrain



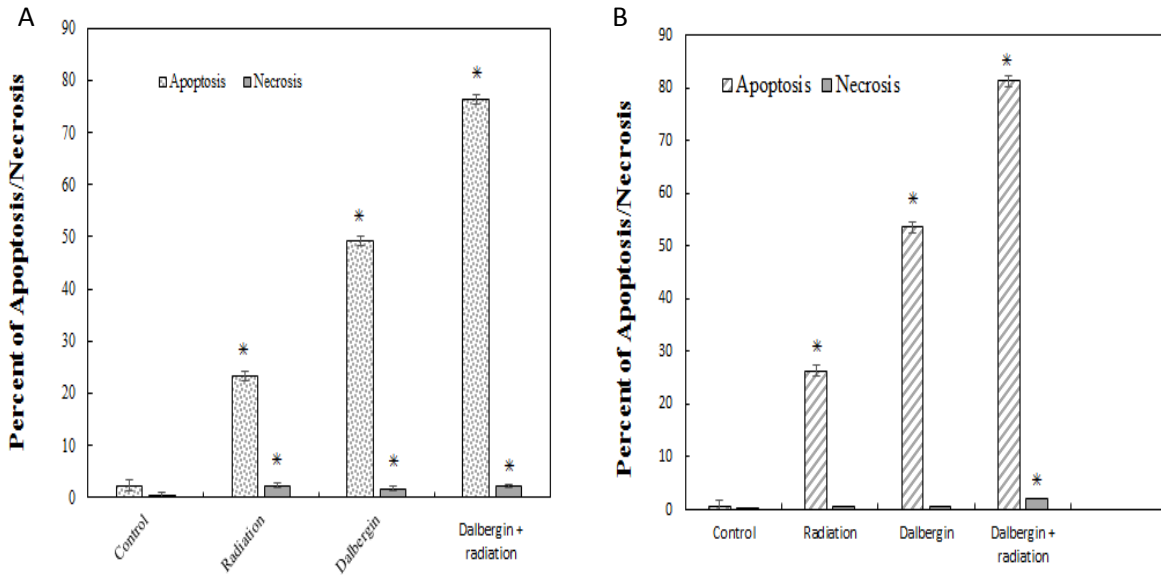


Figure 5. A) Extent of apoptosis in T47D cells irradiated with X-rays, with or without Dalbergin treatment; B) Extent of apoptosis in MDA-MB-231 cells irradiated with X-rays, with or without Dalbergin treatment. Values are the means  $\pm$  SEM of the three independent experiments. \* $P < 0.05$ .

MDA-MB-231: Isolated at M D Anderson from a pleural effusion of a patient with invasive ductal carcinoma; T47D: Differentiated epithelial substrain; SEM: Standard error of mean

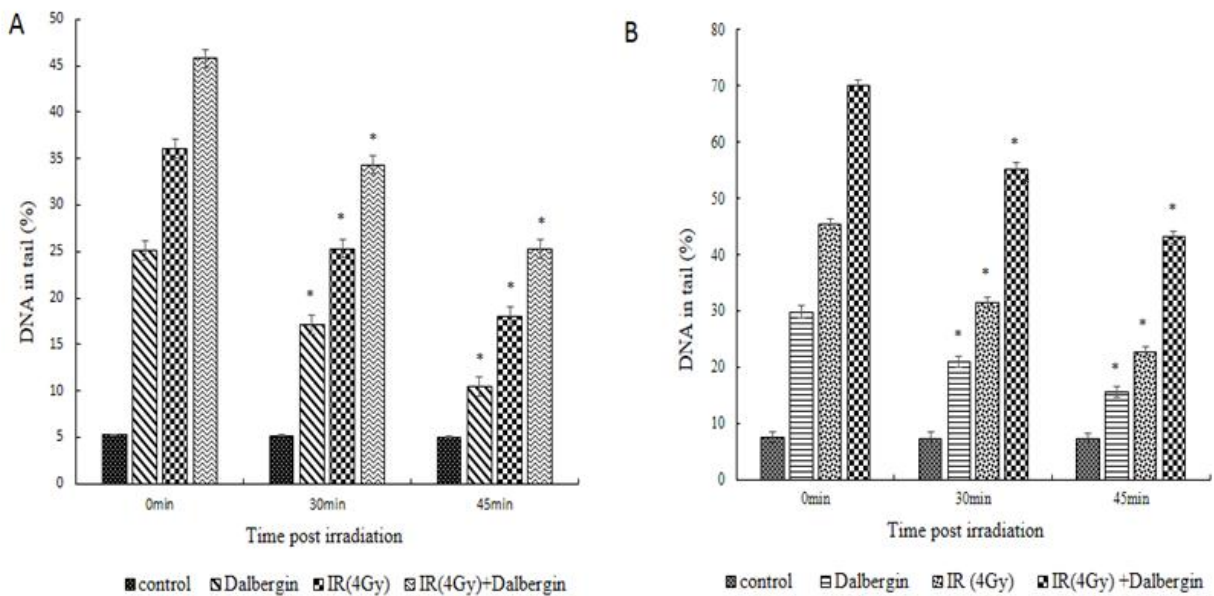


Figure 6. Level of radiation-induced breaks in the presence of Dalbergin; (A) %DNA in tail in T47D; (B) %DNA in tail in MDA-MB-231. The mean  $\pm$  SEM value of each parameter was measured through the three independent experiments. \* $P < 0.05$ . % DNA in tail in T47D and MDA-MB-231 to treatment with Dalbergin and IR were compared with the corresponding control, which these graphs resulted of the three independent experiments.

IR: Irradiation; MDA-MB-231: Isolated at M D Anderson from a pleural effusion of a patient with invasive ductal carcinoma; T47D: Differentiated epithelial substrain; SEM: Standard error of mean

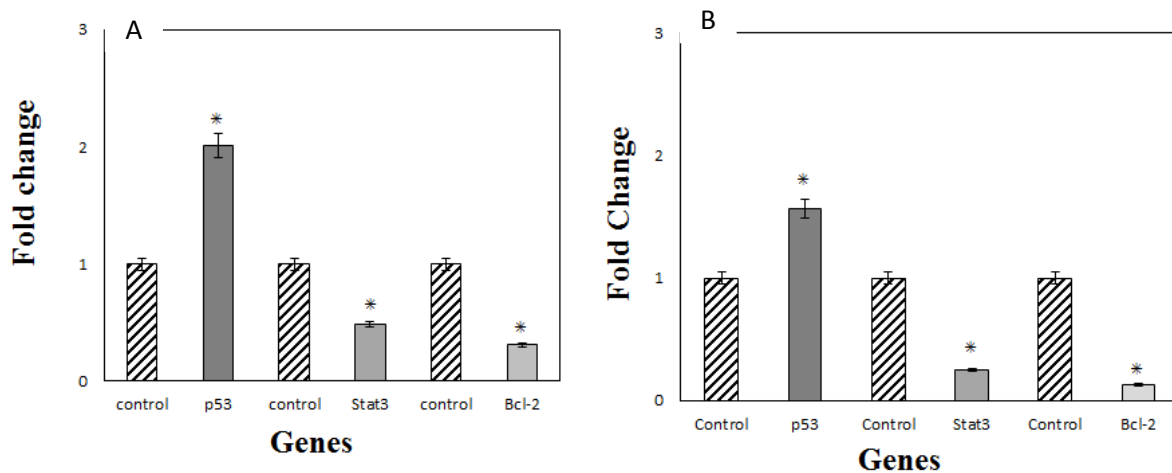


Figure 7. Effect of Dalbergin on the levels of *STAT3*, *Bcl-2*, and *p53* in human breast cancer cell lines. T47D and MDA-MB-231 were treated with Dalbergin for 48 h and then RT-PCR analysis was performed for the transcription level the genes interest of (A) T47D and (B) MDA-MB-23. \* $P < 0.05$ . Fold change of Dalbergin on the levels of *STAT3*, *Bcl-2*, and *p53* was compared with the corresponding control, which these graphs resulted the three independent experiments.

*STAT3*: Signal transducer and activator of transcription 3; *p53*: Tumor-suppressor protein; *Bcl-2*: B-cell lymphoma 2; MDA-MB-231: Isolated at M D Anderson from a pleural effusion of a patient with invasive ductal carcinoma; T47D: Differentiated epithelial substrain

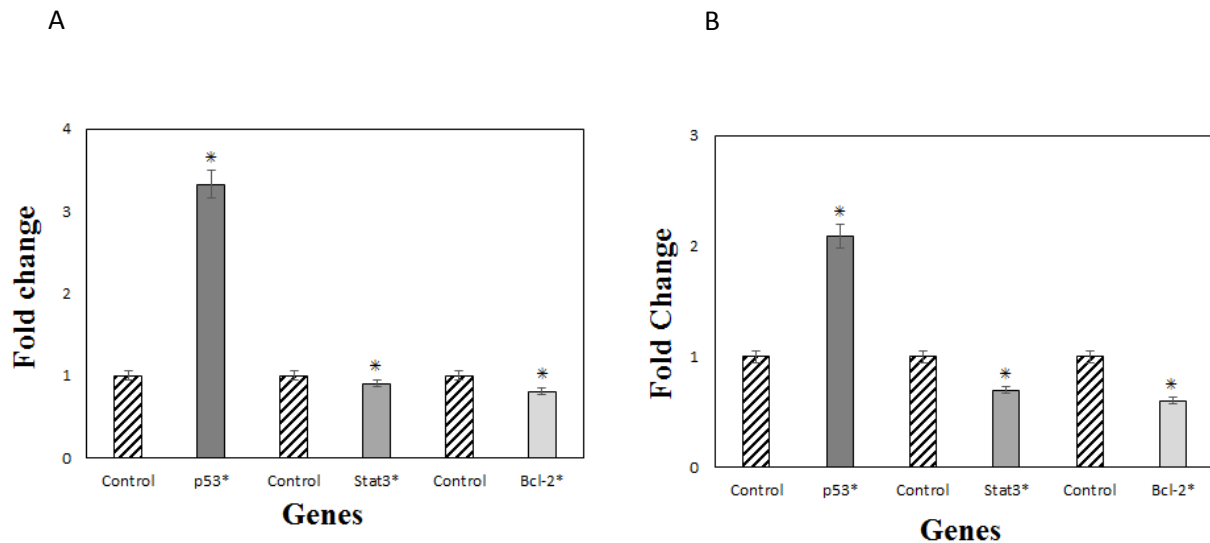


Figure 8. Ionizing radiation- and Dalbergin-induced mRNA expression in (A) T47D and (B) MDA-MB231. The expression levels of various *STAT3*, *Bcl-2*, and *p53* were determined by RT-PCR and quantified by the comparative  $2^{-\Delta\Delta CT}$  method. \* $P < 0.05$ . Fold change of ionizing radiation and Dalbergin on the levels of *STAT3*, *Bcl-2*, and *p53* was compared with the corresponding control, which these graphs resulted the three independent experiments.

*STAT3*: Signal transducer and activator of transcription 3; *p53*: Tumor-suppressor protein; *Bcl-2*: B-cell lymphoma 2; MDA-MB-231: Isolated at M D Anderson from a pleural effusion of a patient with invasive ductal carcinoma; T47D: Differentiated epithelial substrain

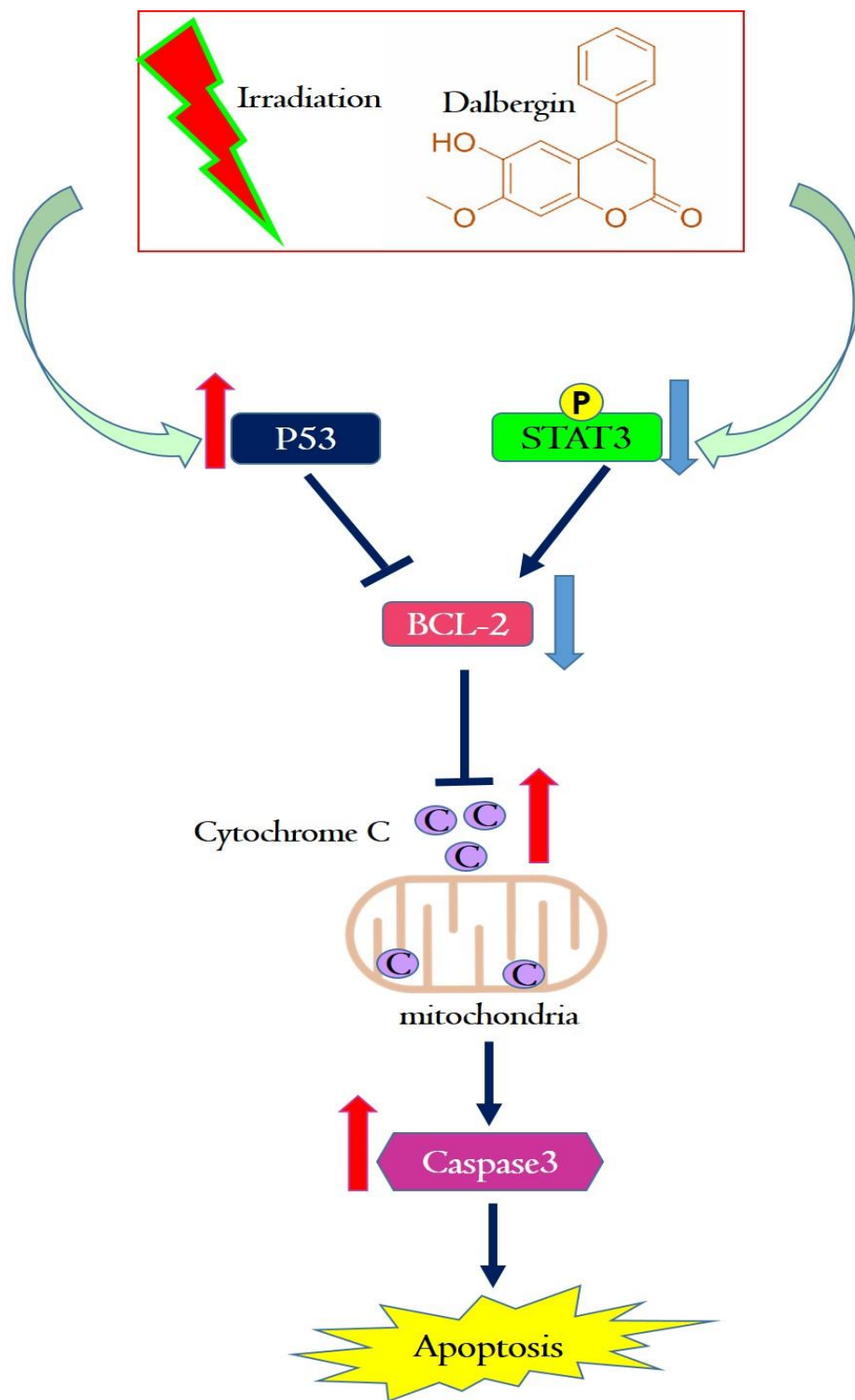


Figure 9. The possible molecular interaction of Dalbergin and signaling pathways involved the radiation-induced DNA damage.  
 STAT3: Signal transducer and activator of transcription 3; p53: Tumor-suppressor protein, Bcl-2: B-cell lymphoma 2