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Effect of a Neoflavonoid (Dalbergin) on T47D Breast Cancer Cell Line and mRNA Levels of *p53*, *Bcl-2*, and *STAT3* Genes

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Abstract

Background: Breast cancer is an important cause of death among women. Prevention of cancer through dietary intervention has recently received increasing interest. Lately, dietary polyphenols have gained much attention for their health benefits, including anticancer properties. Dalbergin as a polyphenol is synthesized from a common neoflavene intermediate.
Objectives: This study aimed to examine whether dalbergin can be useful in the chemotherapy of estrogen receptor-positive T47D cell line.
Methods: This experimental study was performed at the Laboratory of Biophysics and Molecular Biology, the Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran, from October 2017 to November 2019. The doubling time of T47D cells was obtained from the growth curve. The cytotoxic effect of dalbergin on T47D breast cancer cells was evaluated. To assess the clonogenic ability, T47D cells were treated with dalbergin for 48 hours and then, the colony assay was performed. A Real-Time PCR was used to determine the transcription levels of *p53*, *Bcl-2*, and *STAT3* genes.
Results: The doubling time of T47D cells was 28.02 $\pm$ 4.22 hours ($P < 0.05$). Dalbergin decreased the viability of the T47D cell line. The half-maximal inhibitory concentration (IC50) values of dalbergin for T47D cells were found to be 1 μM in 24 hours, 0.001 μM in 48 hours, and 0.00001 μM in 72 hours of treatment ($P < 0.05$). In the clonogenic assay, 0.001 μM dalbergin for 48 hours could reduce the surviving fraction of T47D cells ($P < 0.05$). Additionally, dalbergin could change the mRNA levels of *p53*, *Bcl-2*, and *STAT3* genes ($P < 0.05$).
Conclusions: Our results indicated that dalbergin has some anticancer effects probably through inducing apoptosis in cancerous cells by changing mRNA levels of apoptosis-related proteins.

Keywords: Anti-Cancer, Apoptosis, Breast Neoplasms, Cell Line, Dalbergin, Estrogen, Genes, Humans, Receptors, T47D, Tumor Suppressor Protein p53

1. Background

Breast cancer is quite prevalent and is the leading cause of cancer-related death among women worldwide (1, 2). Breast cancer cell lines have been used widely to investigate the pathobiology of cancer and new therapies (3). Chemotherapy uses drugs to kill cancer cells (4). Polyphenols are a group of common substances found in most plants that possess diverse biological features. They play an important role in suppressing carcinogenesis by targeting proteins involved in cell signaling pathways (5). Recent studies have indicated that natural polyphenols can enhance chemotherapy with minimal toxicity to normal cells (6). Neoflavonoids are a class of polyphenolic compounds that have the 4-phenylchromen backbone with no hydroxyl group substitution at position-2, unlike flavonoids that have the 2-phenylchromen-4-one backbone. Several studies have reported neoflavonoids to possess antioxidant, anticancer, nitric oxide reduction, and anti-inflammatory activities (7).

The species of plant Dalbergia have been applied in traditional medicine as a remedy for blood disorders, ischemia, and inflammation. Neoflavonoids, belonging to the neoflavene family, were initially isolated from *Dalbergia sissoo* and *Dalbergia latifolia* (8, 9). This polyphenol has shown to have anticancer effects in different tumor cell lines; however, it shows differential cytotoxic activity against tumor and surrounding normal cells (10). Nevertheless, it has not reported whether dalbergin induces apoptotic cell death. This programmed cell death has been implicated in many diseases including cancer and autoim-

mune diseases. Therefore, it is very important to understand the mode of cell death (11, 12). The *p53* gene is a kind of regulatory factor for tissue apoptosis. In about 50% of cancers, *p53* is subjected to mutations (13). Specifically, improper STAT3 activity has been detected in a wide variety of tumors (14), deriving multiple pro-oncogenic functions. *Bcl-2* is the first apoptotic regulatory protein that has ever been recognized. This oncoprotein is activated via chromosome translocation in human follicular lymphoma. Overexpression of *Bcl-2* and its close relatives has been noticed as significant components of chemo-resistance (15).

In this study, we examined the effect of dalbergin on the estrogen receptor-positive T47D breast cancer cell line by analyzing its clonogenic ability. We also determined whether dalbergin-induced cell death is correlated with the up-regulation of apoptosis-modulating genes, especially *p53*, *Bcl-2*, and *STAT3* genes. The pharmacological effects of dalbergin in T47D have not been reported yet. This study investigated whether dalbergin can mediate its antiproliferative and apoptotic effects in T47D cells through the suppression of *p53*, *Bcl-2*, and *STAT3* pathway.

2. Objectives

Therefore, the current study was designed to determine how dalbergin could affect the T47D breast cancer cell line and disclose its effects on apoptosis and survival of the T47D cell line.

3. Methods

This experimental study was conducted at the Laboratory of Biophysics and Molecular Biology, the Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran, from October 2017 to November 2019. The study was financially supported by the Research Deputy of the Science and Research Branch, Tehran Islamic Azad University, Tehran, Iran.

3.1. Materials

Dalbergin was obtained from ABCR GmbH (Germany). Dimethylsulfoxide (DMSO), formaldehyde, crystal violet, and Trypsin were purchased from Merck (Germany). Trypan blue, methyl green, Penicillin, and (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were provided from Sigma- Aldrich (USA). Cell culture media (RPMI-1640) and fetal bovine serum were purchased from Gibco (UK). Streptomycin was from Troje medical Gmbh (Germany). All the plates and dishes employed in this study were purchased from SPL (Korea). Otherwise, it was mentioned in the manuscript.

3.2. Cell Culture

T47D cells were provided from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). The cells were cultured in RPMI-1640, 500 U/mL of penicillin, and 200 mg/L of streptomycin supplemented with 10% heat-inactivated fetal bovine serum at 37°C in 98% humidity and 5% CO_2 in an incubator (Memmert, Germany). The cells were subcultured when their confluency reached 90%.

3.3. Growth Curve

T47D cells were seeded at densities of 10,000 cells/well in 24-well plates. The number of cells was then assessed by direct counting on days 0 to 7 after culturing. The doubling time (Td) of cells was calculated according to the Patterson formula, as follows:

$$T_d = T \times \frac{Log2}{log\frac{N_2}{N_1}} \quad (1)$$

where N_1 is the number of cells on the first day of the exponential phase, N_2 is the number of cells at time T (hour) after culture (the end of the exponential phase), and T (hour) is the time from N_1 to N_2.

3.4. Clonogenic Assay

Appropriate numbers of T47D cells were seeded in 35-mm Petri dishes. After incubation of cells at 37°C for 11 days, the colonies were fixed, stained, and counted. The surviving fraction was calculated as the number of colonies counted divided by the number of cultured cells multiplied by 100.

3.5. MTT Assay

T47D cells were seeded at densities of 3,000 and 6,000 cells/well for 24 and 48 hours, respectively. Next, the cells were treated by various concentrations of dalbergin (0, 0.000001, 0.00001, 0.0001, 0.001, 0.01, 0.1, 1, 10, and 30 μM) and incubated for 24, 48, and 72 hours. Subsequently, 100 μL of MTT solution at a concentration of 0.5 mg/mL was added and the cells were incubated for 3 hours, followed by the addition of 100 μL of DMSO to dissolve formazan crystals. The absorbance intensities at 570 nm and 630 nm were measured 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 for each concentration of dalbergin as the absorbance of the sample divided by the absorbance of the control multiplied by 100.

3.6. Clonogenic Assay Following Dalbergin Treatment

Sufficient numbers of T47D cells were plated in 24-well plates at densities of 10,000 and 15,000 cells/well. Next, the cells were treated by different concentrations of dalbergin (0, 0.000001, 0.00001, 0.0001, 0.001, 0.01, 0.1, 1, 10, and 30 μM) and incubated for 48 hours. Afterward, appropriate numbers of cells were harvested, seeded in 35-mm Petri dishes, and incubated for 11 days. Finally, the colonies formed were fixed using formaldehyde solution 2% and stained with crystal violet 5%. Then, colonies containing more than 50 cells were counted.

3.7. Quantification of Gene Transcription by Real-Time PCR

The effect of dalbergin on the mRNA levels of marker genes *p53*, *Bcl2*, *STAT3*, and GAPDH was determined by RT-PCR. T47D cells were seeded and incubated for 48 hours. Afterward, the cells were treated with dalbergin for 48 hours. Next, total RNA was extracted by RNX-Plus solution (CinnaGen, Tehran, Iran) according to the manufacturer's instruction. To remove DNA impurity, DNase (Fermentas Pittsburgh PA, USA) was applied at 37°C for 30 minutes, quantified by spectrophotometry, and electrophoresed on a 1% agarose gel. For cDNA synthesis, 1 μg of total RNA was used according to the manufacturer's instructions. Moreover, we used 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). Then, 9 μL of the reaction mixture containing 1 μL of primers and 5 μL of SYBR Green I Master Mix (QuantiFast SYBR Green PCR, Q204054) were added to 1 μL of cDNA and the Real-Time Thermocycler (RotorGene 6000, Corbett Life Science, USA) started. The thermocycler program was as follows: initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 15 seconds, optimized annealing temperature from 60 to 61°C for 25 seconds, and extension at 72°C for 25 seconds. The whole process was repeated for 35 cycles. The melt curve analysis was done in each real-time PCR experiment. All the four mentioned steps were applied in different groups including controls, *p53*, *Bcl-2*, and *STAT3*.

3.8. Statistical Analysis

The data were analyzed by one-way analysis of variance (ANOVA). All experiments were performed in triplicate and P values of < 0.05 were considered significant. Comparing the mRNA levels of *p53*, *STAT3*, *Bcl-2*, and their active forms were made using the GraphPad Prism 7 (GraphPad Prism Software Inc., San Diego, CA, USA).

4. Results

4.1. Doubling Time

Figure 1 shows the growth curve of T47D cells. The number of cells was calculated for seven days. T47D cells showed the lag, log, stationary, and decline phases in 0 - 1, 1 - 5, 5 - 6, and 6 - 7 days, respectively. The doubling time is the time required for cells to double in the exponential phase. Thus, the baseline doubling time of T47D cells was calculated as 28.02 $\pm$ 4.22 hours ($P < 0.05$; Table 1).

Table 1. Results of T47D Doubling Time After Seven-Day Culture

T47D Samples	Doubling Time, h	Mean $\pm$ SD
1	23.19	28.02 $\pm$ 4.22
2	29.85	
3	31.02	

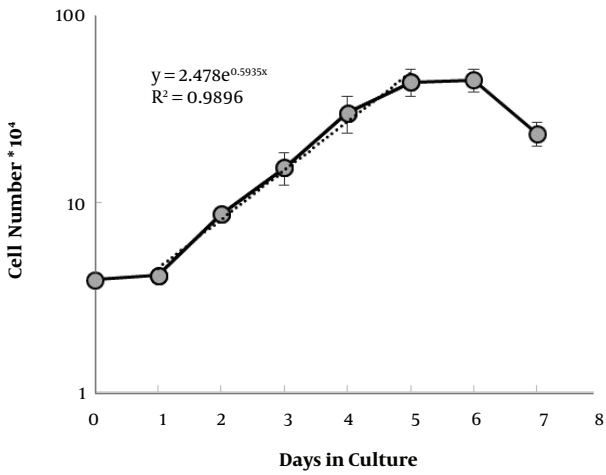

Figure 1. The T47D growth curve showing the number of cells in seven days ($P < 0.05$). Values are expressed as mean $\pm$ SD of three independent experiments.

4.2. Plating Efficiency Assay

The plating efficiency of T47D cells was determined. The number of colonies formed increased with increasing the number of cells seeded in each plate. This step was necessary to determine the suitable number of cells for the clonogenic assay (Figure 2 and Table 2).

4.3. MTT Cell Viability Assay

The effect of dalbergin on the proliferation of T47D cells was examined by the MTT assay. Figure 3 presents the cytotoxicity of dalbergin to T47D cells. The induced toxicity depended on the incubation time and dalbergin concentration. The IC50 values for T47D cells were found to be

Table 2. The Results of the Clonogenic Ability of T47D Cells in the Presence of Different Concentrations of Dalbergin[a]

Cell Number	Colony Number	SEM	Plating Efficiency, %	SEM
500	61	2.67	12.2	0.53
750	98.33	6.33	13.11	0.84
1000	142	6.33	14.2	0.63
1500	227.83	8.83	15.19	0.59
2000	322	6.33	16.1	0.32

[a]Values are expressed as mean ± SD of three independent experiments.

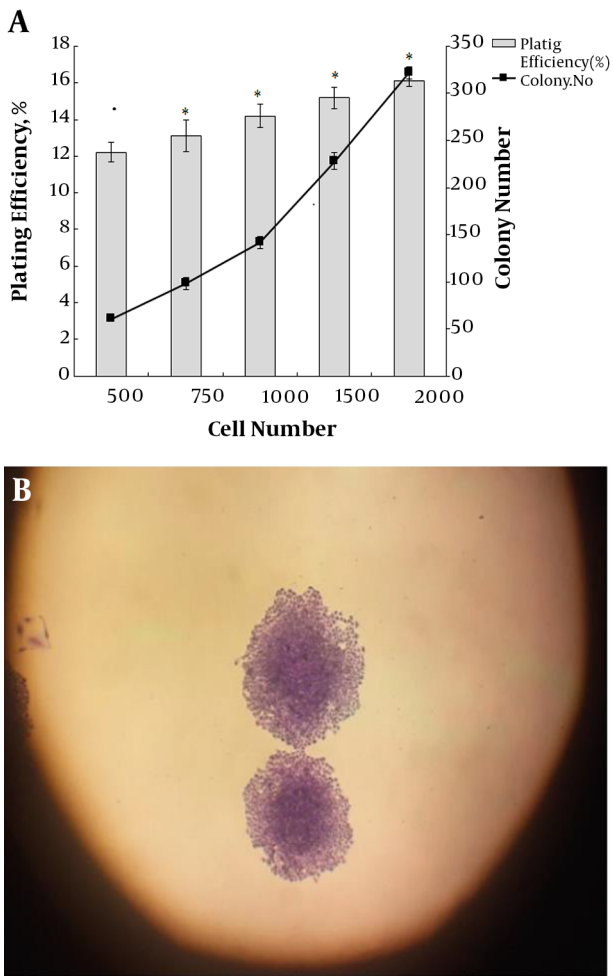

Figure 2. The plating efficiency of T47D cells; A, the values are the mean ± SEM of three experiments ($P < 0.05$); B, the photomicrograph of colonies formed by T47D cells (with cell numbers of higher than 50) observed under a microscope (×400).

1 μM in 24 hours, 0.001 μM in 48 hours, and 0.00001 μM in 72 hours of incubation. The cytotoxicity of 0 - 30 μM dalbergin to T47D cells gradually decreased from 24 hours to 72 hours ($P < 0.05$; Table 3).

Table 3. The IC50 Values for This Cells After 24, 48, 72 Hours[a]

Dalbergin, μM	Incubation Time, h	Mean ± SD
1	24	54.62 ± 3.58
0.001	48	51.45 ± 2.12
0.00001	72	50.58 ± 1.02

[a]Values are expressed as mean ± SD of three independent experiments.

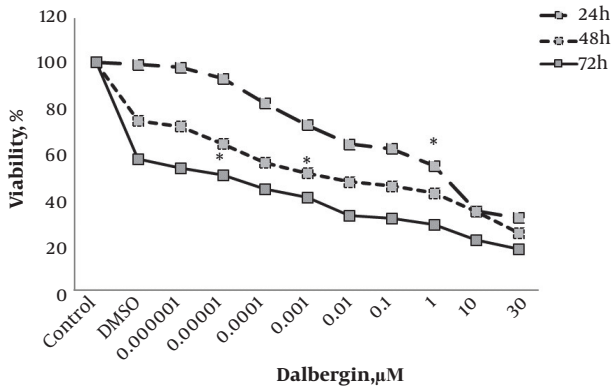

Figure 3. The effect of dalbergin on the viability of the T47D breast cancer cell line. The cells were treated with 0, 0.000001, 0.00001, 0.0001, 0.001, 0.01, 0.1, 1, 10, and 30 μM dalbergin for 24, 48, and 72 hours. The cell viability was evaluated by MTT assay. Dalbergin reduced cell viability.

4.4. Colony Assay in the Presence of Dalbergin

The plating efficiency of T47D cells was examined following for 48 hours of treatment with various concentrations of dalbergin (0, 0.000001, 0.00001, 0.0001, 0.001, 0.01, 0.1, 1, 10, and 30 μM). Different cell numbers (500, 750, 1000, 1500, and 2000) were tested. Based on the results presented in Figure 4A - E, dalbergin could considerably decrease the colony formation ability of T47D cells. As can be seen in Figure 5, a concentration of 0.001 μM dalbergin decreased the number of colonies to half of the control's count after 48 hours. Data represent the mean ± standard deviation (SD) of three independent experiments ($P < 0.05$; Table 4).

Table 4. The Mean Colony Count of T47D Cells After Treatment with Various Concentrations of Dalbergin for 48 Hours[a]

Colony Count	500 Cells	750 Cells	1000 Cells	1500 Cells	2000 Cells
Control	100.68	147.83	183.17	264.83	339.33
DMSO	97.83	141.67	173.67	248.5	318
0.000001 μM	91.5	132.17	165.33	233.67	292.33
0.00001 μM	89	121.5	153.83	217.17	273.33
0.0001 μM	79	116	145.67	201.83	248.5
0.001 μM	41.67	58.33	67.67	81.17	98.17
0.01 μM	38	47.67	58.17	77.67	94.33
0.1 μM	38	46	54.67	76.83	88.83
1 μM	31.33	43.17	49.67	62.67	79.5
10 μM	23.5	27.17	33	45.67	57
30 μM	11.5	13.17	14	15	16.33
SDEV	33.26	50.41	63.93	93.66	120.13
SEM	23.52	35.64	45.21	66.23	84.95

[a]The colonies were counted after 11 days. The experiment was performed in triplicate.

4.5. Primers

Primer sequences were used in this study for gene transcription analysis by real-time PCR. The sequences of all primers used and amplicon lengths are listed in Table 5. GAPDH, a housekeeping gene, was used as a control. The fold change of each target gene (*p53*, *Bcl-2*, and *STAT3*) relative to GAPDH was calculated based on the relative quantitation using the $\Delta\Delta C_T$ method, calculated by the $2^{-\Delta\Delta CT}$ relative transcription formula.

Table 5. Primer Sequences for Each Target Gene

Genes/Primers	Sequence	Amplicon, bp
STAT3		123
F	5'CTGAAGCTGACCCAGGTAGC3'	
R	5'GCATCAATGAATCTAAAGTGCGGG3'	
Bcl-2		356
F	5'GATACTGAGTAAATCCATGCAC3'	
R	5'AGTGTTGCAGAATATTCAGCCAC3'	
GAPDH (RT-PCR)		115
F	5'CCATGAGAAGTATGACAAC3'	
R	5'GAGTCCTTCCACGATACC3'	
p53		156
F	5'GCTCAGATAGCGATGGTCTGGC3'	
R	5'AGTGGATGGTGGTACAGTCAGAG3'	
GAPDH (qRT-PCR)		360
F	5'CCAGCCGAGCCACATCGCTC3'	
R	5'ATGAGCCCCAGCCTTCTCCAT3'	

4.6. Real Time-PCR

Because the *p53*, *Bcl-2*, and *STAT3* genes have been reported to play a role in positive estrogen receptor breast cancer cells, it was of interest to compare the mRNA levels in the highly invasive breast cancer cell line. Therefore, gene transcriptions were compared in the absence and presence of 0.001 μM dalbergin. The results showed that *p53*, *Bcl-2*, and *STAT3* mRNA levels were significantly higher in the T47D cells treated with dalbergin than in the untreated cells. All measurements were done twice in a duplicate analysis (Figure 6 and Table 6).

Table 6. The mRNA Levels of *p53*, *STAT3*, and *Bcl-2* Genes in T47D Cells After 48-Hours Treatment with 0.001 μM Dalbergin

Samples	mRNA Level	SD
T47D (*p53*)	0.063	0.347
T47D + Dalbergin (*p53*)	0.111	0.337
T47D (*STAT3*)	0.055	0.397
T47D + Dalbergin (*STAT3*)	0.095	0.316
T47D (*Bcl-2*)	0.093	0.284
T47D + Dalbergin (*Bcl-2*)	0.427	0.011

5. Discussion

Breast cancer is the most common female cancer, accounting for 23% of all cancer cases among women around the globe (16). The use of cytotoxic chemotherapy has been effective in both advanced and early-stage breast cancer

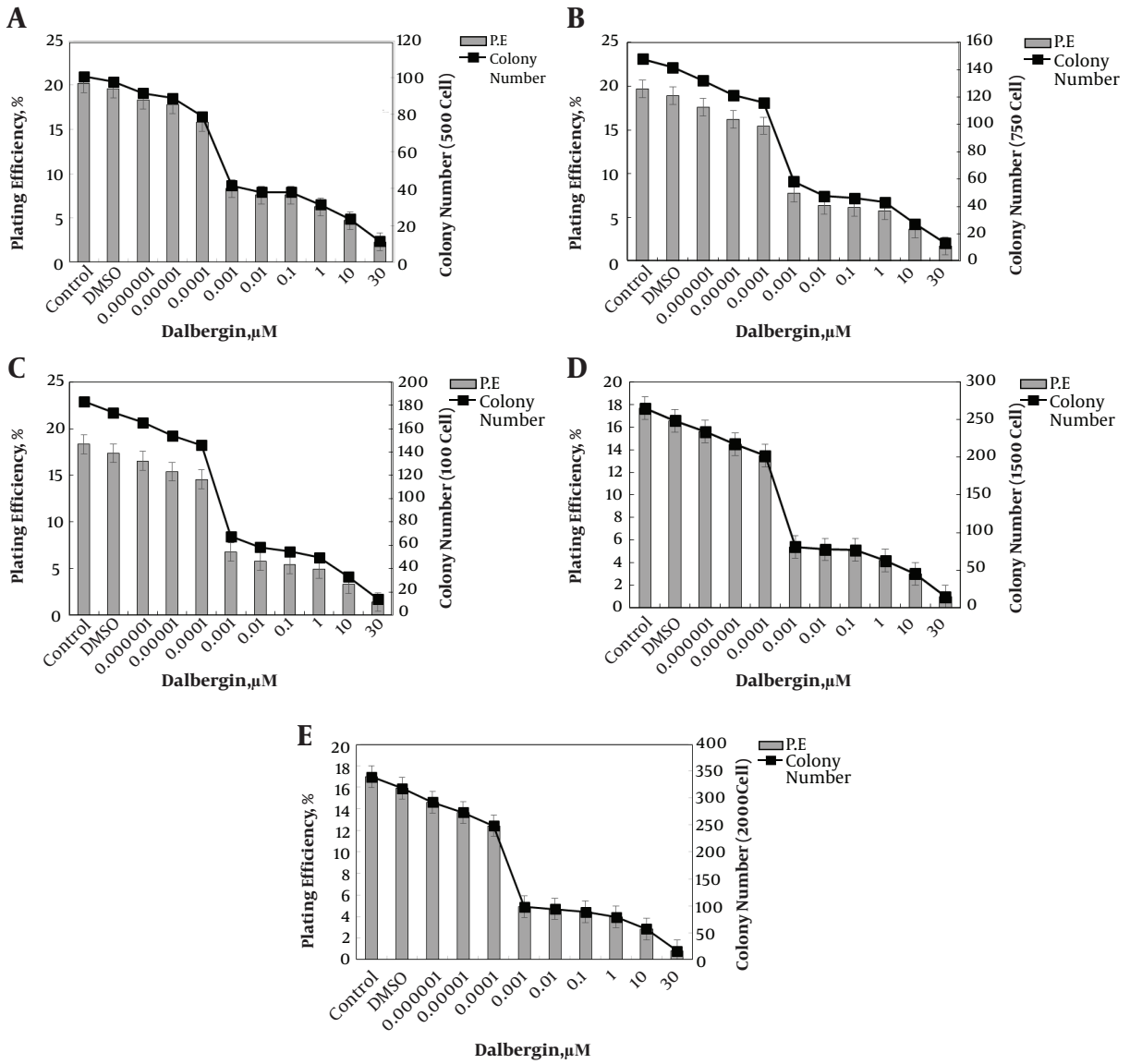

Figure 4. The effect of various concentrations of dalbergin on different T47D cell numbers (500, 750, 1000, 1500, and 2000) after 48-hours incubation. Values are expressed as mean ± SD of three independent experiments ($P < 0.05$).

(17). Polyphenols are found in plants, fruits, and vegetables. They are specific components of a healthy diet (18). Anti-inflammatory and antioxidant properties have been reported for polyphenols (19). Natural polyphenols are among the plants' secondary metabolites (20). Dalbergin as a polyphenolic compound is the most prevalent neoflavene in the plant kingdom (21). It has been used traditionally to cure many diseases (22). Dalbergin is characterized by antioxidant and free radical scavenging properties. Its antioxidant activity has been shown by in vitro studies (23). We examined the effect of dalbergin on T47D cells viability using four complementary tests. In this experiment, we assessed T47D cell growth (24, 25). The doubling time was 28.02 ± 4.22 hours, which a suitable time in growth curves.

The MTT assay is a widely used test to measure cell proliferation, cell viability/survival, and drug toxicity. Recently, MTT has been described as one of the main techniques for testing tumor cell cytotoxicity of polyphenols. MTT assay disclosed the cytotoxic activity of dalbergin (26). Dalbergin reduced cell viability measured by MTT assay to approximately 50% after 24, 48, and 72-hours treatment of

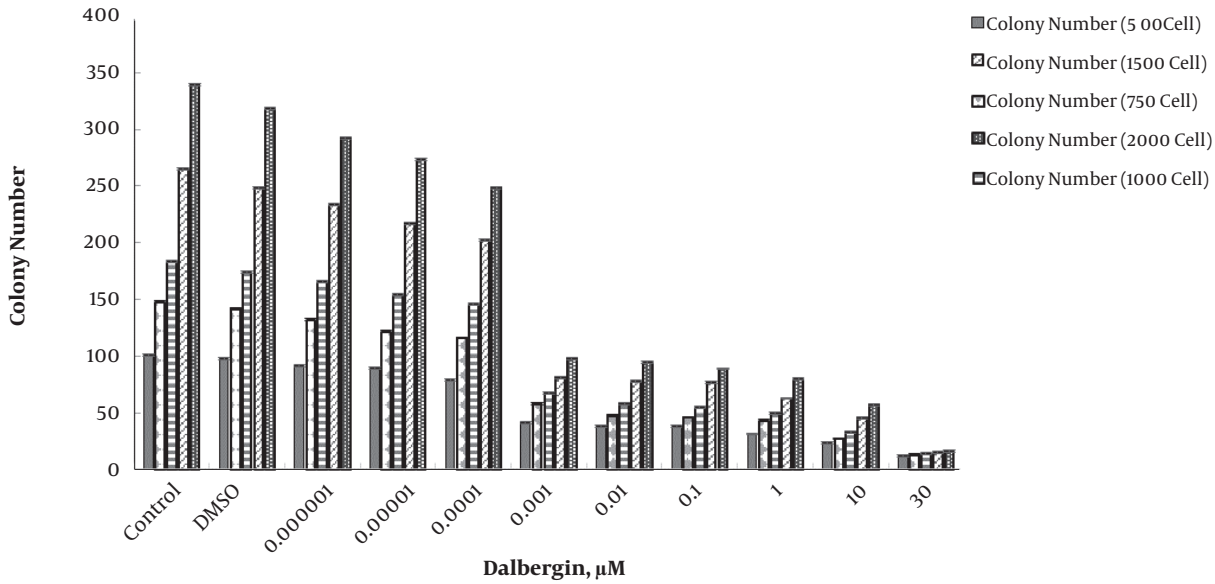

Figure 5. The plating efficiency for various cell numbers in the presence of different dalbergin concentrations ($P < 0.05$). Values are expressed as mean ± SD of three independent experiments.

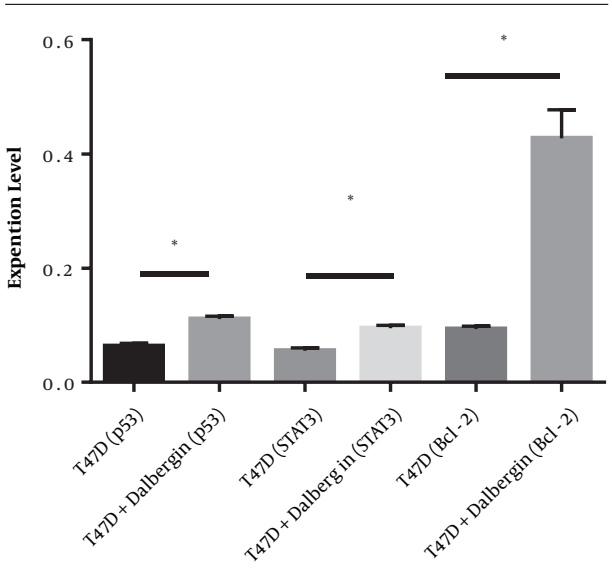

Figure 6. Relative mRNA levels of *p53*, *Bcl-2*, and *STAT3* in T47D cells in the presence or absence of 0.001 μM dalbergin. Values are expressed as mean ± SD of two independent experiments ($P < 0.05$).

T47D cells. The IC50 values for T47D cells were found to be 1 μM at 24 hours, 0.001 μM at 48 hours, and 0.00001 μM at 72 hours of treatment.

Colony forming or clonogenic assay is an in vitro quantitative technique to examine the capability of a single cell to grow into a large colony through clonal expansion. Many polyphenols can decrease colony counts in estrogen receptor-positive human breast cancer cell lines (27). T47D cell colonies comprising more than 50 cells were detected after day 10 of growth (28). According to our results, treatment of various counts of T47D cells (500, 750, 1000, 1500, and 2000 cells) with various concentrations of dalbergin for 48 hours could decrease colony counts. According to colony formation assays, we found that dalbergin could inhibit the colony forming ability of T47D cells.

Furthermore, we evaluated the apoptotic effect of dalbergin by measuring the transcription level of apoptotic-related genes in T47D cells in the presence or absence of dalbergin. Quantitative real-time PCR demonstrated that treatment with this drug could increase the mRNA levels of *Bcl-2*, *p53*, and *STAT3* genes (29). This result showed that dalbergin presents anticancer activities and can induce apoptosis in T47D cells. We suggest that *p53*, *Bcl-2*, and *STAT3* are novel, valuable therapeutic targets for T47D treatment because they are implicated at many stages of tumor progression, including cell growth, survival, and invasion. This signaling pathway may have potential therapeutic values in the treatment of breast cancer cells.

In this study, we used a polyphenol to treat cancer cells because this plants' secondary metabolite has some features such as antioxidant and anti-inflammatory properties and can modulate multiple molecular events involved in carcinogenesis (30). Another reason for its use as an anticancer is its oxygen scavenging property (31). This group

of polyphenols, specifically dalbergin, is superior to other groups due to their fewer side effects and their capability to kill cancer cells. Studies have shown that anthocyanidin treatment induced apoptosis and cell cycle arrest in several types of cancer. However, this polyphenol can do this function by the suppression of the NF-κB pathway (32) whereas dalbergin can induce apoptosis via *p53*, *Bcl-2*, and *STAT3*. Jahanafrooz et al. used neoflavonoids as chemopreventive agents and showed they could block TNF-α-induced NF-κB activation. The IC50 values were in the range of 0.11 - 3.2 μM. However, in this investigation, we used low concentrations compared to the mentioned range. Silibinin, a polyphenol, could cause G1 cell cycle arrest in MCF-7 and MCF-10A cells but had no effect on the T47D cell cycle (33, 34). This is while in this study, dalbergin could induce apoptosis. Like dalbergin, another polyphenol could decrease the viability of T47D cells in the concentration and time-dependent manners (35). Thus, polyphenols can arrest the cell cycle in men and women-specific cancer. Further research is required to confirm if dalbergin can inhibit breast cancer in vivo. The authors can use mouse models in future studies. Although the data are still limited, the results appear promising. Moreover, using dalbergin as a potential modulator of chemotherapy and radiotherapy deserves further investigation.

5.1. Weak Points and Limitations

We did not use more molecular and cellular tests, such as comet assay, western blot, etc. because of a budget limitation. Moreover, we can use mouse models in future studies.

5.2. Strong Points

Breast cancer is a highly important issue in medicine. This study reports that dalbergin can activate apoptotic-inducing pathways in the T47D cell line. The researchers hope that the findings of this preliminary study are promising.

5.3. Conclusions

The study focused on the analysis of the anticancer effect of dalbergin. In conclusion, the results demonstrate the possibility of using dalbergin as a drug for cancer therapy. Based on our findings, it is necessary to conduct more in vitro studies and clinical trials to identify the anticancer effects and mechanism of dalbergin action inside the human body.

Acknowledgments

This work was performed at the Institute of Biochemistry and Biophysics, University of Tehran, and the Science and Research Branch, Tehran Islamic Azad University, Tehran, Iran.

Footnotes

Authors' Contribution: Bahram Goliaei, Kazem Parivar and Alireza Nikoofar developed the original idea and the protocol. Fereshte Mahdizade abstracted and analyzed data, wrote the manuscript, also contributed to the development of the protocol, abstracted data, and prepared the manuscript. All the authors had an effective contribution to the manuscript preparation.

Conflict of Interests: All authors declare no conflict of interest.

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