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Original article

Potential Cytotoxic Activity of Galegine on Human Melanoma Cells

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Abstract

Background and objectives: Melanoma, the most lethal type of skin cancer, has a high recurrence rate within one year in melanoma patients following traditional treatment by chemotherapy or immunotherapy. In an effort toward reducing this event, the present study aimed to investigate whether galegine has inhibitory effects on human melanoma cell lines. Galegine is a natural active compound found in the *Galega officinalis* which was known and used in Europe for medicinal purposes for centuries. **Methods:** Cell viability by MTT assay was conducted to measure the 50% inhibitory concentration (IC_{50}) of galegine on DFW and SK-MEL-5 cells. Also, apoptosis level was determined using Annexin V/FITC-propidium iodide (PI) flow cytometry. In addition, quantitative Real-Time PCR (qRT-PCR) for *Bax*, *Bcl2*, and *p53* genes was performed with specific primers to evaluate their expression pattern in each group. **Results:** The experimental results indicated that galegine induced cytotoxicity in a concentration-dependent manner with IC_{50} of 630 µM and 3300 µM in DFW and SK-MEL-5 cells, respectively. Also, apoptosis induction occurred in both melanoma cell lines, in a way that 12.4% of the DFW cells and 41.8% of SK-MEL-5 were detected in the apoptotic phase. Furthermore, it was found that the Bax/Bcl-*2* ratio was upregulated in both melanoma cells. An upregulation in p53 gene expression was observed in SK-MEL-5 cells, as well. **Conclusion:** The results of the present study revealed that galegine induced cytotoxicity and apoptosis in human melanoma cells with the potential toward more research on as a novel therapeutic candidate for melanoma treatment.

Keywords: apoptosis; Bcl-2-associated x protein; biological products; melanoma; tumor suppressor protein p53

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Introduction

Melanoma, the most aggressive skin tumor, is largely resistant or acquires major resistance to

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most of the standard chemo or targeted therapies after a period of therapy [1, 2]. The median survival frequency of malignant melanoma patients remains 6-10 months, despite a large number of clinical studies examining various approaches from surgery to chemo/radiation therapy to improve survival [3, 4]. In fact, based on the American Cancer Society figures, the 5-year-survival of metastatic melanoma patients remains at only 27% [5]. Thus, finding new therapeutic compounds capable of inducing apoptosis or reducing the metastatic capacity of melanoma cells sounds indispensable. Fundamental components of carcinogenesis lead to reduced apoptosis or disruption of apoptosis-associated mechanisms, one of the key hallmarks of malignant cells, as a result of genetic alterations [3]. Apoptosis acts as a critical event in cell differentiation and many other processes by controlling genetically impaired cells or restricting uncontrolled cell proliferation [6]. The antiapoptotic protein Bcl-2 and apoptotic protein Bax are deregulated in a range of human cancers, including prostate carcinoma, melanomas, and gliomas [7-13]. Hence, the induction of cancer cell apoptosis is established as an advantageous method for the management of neoplasia, especially melanoma.

Galegine (isoprenyl guanidine), isolated from Goat's rue (*Galega officinalis* L.), is the major cause of the toxicity of sedge poison and is part of a large group of compounds called biguanides known for various therapeutic applications [14]. *Galega officinalis* belongs to the Fabaceae family and subfamily of Faboideae, native to most of Europe and the west of Asia. It usually grows from 60-150 cm tall and its reproduction happens by seed. Traditionally, it has been used to mitigate polyuria as a consequence of high blood glucose levels. However, other uses for the treatment of plague, tuberculosis, fevers and similar infections have been reported [15, 16]. As a biologically active compound, galegine is known for its anti-diabetic and weight-reducing properties [17]. Two of the most well-known galegine synthetic products, metformin, and phenformin have been also studied for their therapeutic potential in different contexts [18]. For instance, metformin, an oral anti-diabetic agent and a synthetic derivative of galegine is indicated for its antitumor effects in different tumors with a satisfactory safety profile [19-21]. Also, another synthetic derivative of galegine, phenformin, which is estimated to be around fifty times more potent than metformin, has been extensively researched especially for cancer treatment [22]. Nevertheless, its clinical use has been discontinued largely due to its potentially fatal lactic acidosis because of its interference with oxidative phosphorylation [23, 24]. Galegine, phenformin, and metformin are all different members of biguanides with similar chemical structures, and the fact that phenformin causes severe side effects while metformin displays a safe profile, especially in cancer tumor contexts prompted us to investigate the cytotoxic effects of galegine which we speculated might act through a different pathway than phenformin and thus represent milder side effects. In this context, evidence of the underlying mechanisms responsible for galegine antineoplastic activity against melanoma has not been investigated. Therefore, in the present examination, we evaluated whether galegine induces apoptosis in DFW and SK-MEL-2 human melanoma cells and also if there are any changes in the expression ratio of Bax/Bcl-2 during apoptosis induced by galegine.

Material and Methods

Ethical considerations

Relevant research ethics have been fully considered during this study.

Chemicals and kits

The 4, 5-dimethylthiazole-2-yl, 2, 5-diphenyl tetrazolium (MTT), and trypan blue were obtained from Sigma (St. Louis, USA). Annexin V-FITC apoptosis kit was obtained from Cell Signaling (Cell Signaling, USA). Trypsin-EDTA, High glucose Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, USA). Sodium bicarbonate, dimethyl sulfoxide (DMSO), sodium hydroxide, Triton X-100, and ethylenediaminetetraacetic acid (EDTA) were purchased from Merck (Germany). Galegine powder (3-methyl-2-butenylguanidine) was provided from Pharamaffiliates (India), RNeasy® mini kit was purchased from Qiagen (Qiagen GmbH, Germany), the Prime-Script™ RT reagent kit was obtained from Takara (TaKaRa Bio Inc., Japan). All the oligonucleotides used in this study were ordered from Macrogen (Macrogen Co., South Korea).

Cell lines

DFW (RRID: CVCL_9V18) and SK-MEL-5 (HTB-70) human melanoma cells were purchased from the National Cell Bank of Iran (NCBI, Tehran, Iran).

Cell viability assay

The DFW and SK-MEL-5 cells' cellular viability as an indicator of galegine cytotoxic effect was analyzed using the MTT assay as previously described [23]. Briefly, the cells were seeded at a density of 10^4 cells/well and incubated overnight. Then, the cells were incubated with different concentrations of galegine (0-4 mM) for 24 hours and further exposed to the MTT solution at a final level of 0.05% for 4 hours. DMSO (150 µL) was applied to dissolve the formazan crystals. The absorbance was measured at 570 and 620 nm (background) using a Stat FAX303 plate reader (Awareness Technologies, USA). All tests were performed in triplicate.

Apoptosis flow cytometry assay

Flow cytometry using FITC-labeled annexin V and propidium iodide (PI) was used to determine the apoptosis level. In brief, after 24 h of cell incubation (7×10^5 in a 6-well culture plate) with galegine (IC_{50} and 1/2 IC_{50} [µM]), the cells were harvested and washed twice with ice-cold PBS and re-suspended in 200 µL of 1X binding buffer containing Annexin V. Next, 96 µL of cell suspension was transferred to the flow cytometric tube, then 1 µL of conjugated Annexin V-FITC and PI (12.5 µL) was added to the cells. Later, the cells were incubated for 10 min on ice in the dark. Subsequently, the final volume was set at 250 µL with a 1X binding buffer containing Annexin V. The cell samples were quantified immediately by the BD Facscalibur™ flow cytometer (Becton Dickinson, USA). The flow cytometry data analysis was performed by FlowJo® vX.0.7 (Tree Star, USA). All tests were performed in triplicate.

Quantitative real-time polymerase chain reaction (qRT- PCR)

Total RNA was extracted from the treated melanoma cells (7×10^5 cells/well) according to the RNeasy® mini kit protocol. Next, RNAs were reverse-transcribed utilizing the Prime-Script™ RT reagent kit. Then, qRT-PCR was performed with specific primers for *GAPDH*, p53, Bax, and Bcl-2 (Table 1), which were purchased from the Macrogen. The cDNA amplification was performed utilizing the Light Cycler 96 RT-PCR system (Roche Applied Science, Pleasanton, CA, USA). The $2^{-\Delta\Delta Ct}$ technique was used to analyze the relative expression of the target genes. Gene expression data were normalized to GAPDH. The primer sequences (forward and reverse) are listed in Table 1.

Table 1 . The sequence of primers in the current study

Gene symbol	Gene name	Primers (5′ → 3′)
Bax	Bcl-2-associated X protein	Forward: GGAGCTGCAGAGGATGATTG Reverse: CCAGTTGAAGTTGCCGTCAC
Bcl-2	B-cell lymphoma 2	Forward: CTGAGGAGCTTTGTTTCAACCA Reverse: TCAAGAAACAAGGTCAAAGGGA
p53	Tumor suppressor protein	Forward: ACCCTTGCTTGCAATAGGTG Reverse: AACAAAACACCAGTGCAGGC
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Forward: ACAACTTTGGTATCGTGGAAGG Reverse: GCCATCACGCCACAGTTTC

Statistical analysis

Data were expressed as the mean ± standard error of mean (SEM). All statistical analyses were performed using GraphPad Prism® 7.01 software (GraphPad Software, USA) and the values were compared using one-way analysis of variance (ANOVA) followed by Dunnett test. The p-value<0.05 was regarded as statistically significant.

Results and Discussion

Melanoma cells were treated with varying concentrations of galegine (0-4 mM) for 24 h. As depicted in Figure 1, galegine reduced the viability of melanoma cells in a dose-dependent manner. The obtained half-maximal inhibitory concentration (IC_{50}) values for galegine in DFW and SK-MEL-5 cells were 630 µM and 3300 µM after 24 h, respectively.

To determine if galegine induces apoptosis in melanoma cells, we studied the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. As shown in Figures 2 and 3, the majority of the control cells were healthy and unstained neither by FITC-labelled annexin V or PI in the two treatment periods.

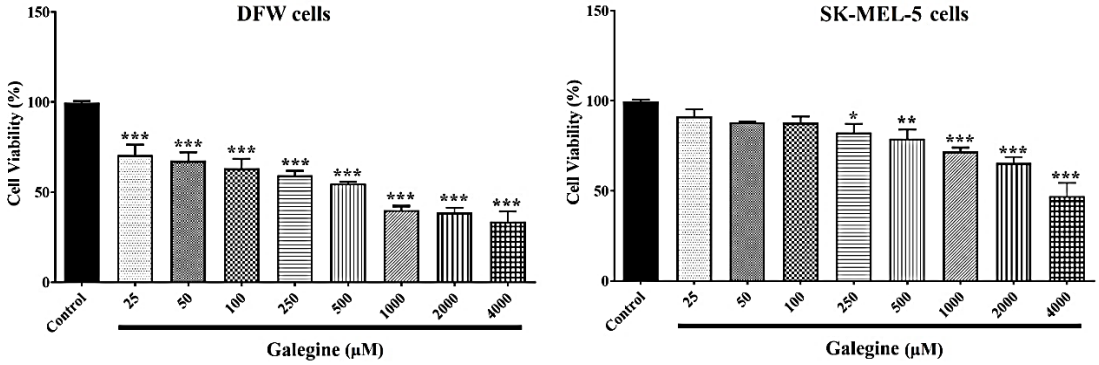

Figure 1. Melanoma cells proliferation (MTT assay) after 24 h of treatment with galegine. The IC_{50} of galegine in DFW and SK-MEL-5 cells was determined at 630 µM and 3.3 mM for 24 h, respectively. Each column represents the cell viability (in percent) mean±SEM for each concentration. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ show significant differences compared to the control group (n=8).

Galegine treatment gave rise to the translocation of PS molecules, as evidenced by the binding of FITC-labelled annexin V. Following treatment of DFW cells with 315 and 630 µM of galegine for 24 h, approximately 4.28% and 4.09% of cells population were in the early stages of apoptosis (annexin V+/PI−), respectively. Additionally, late apoptotic (annexin V+/PI+) and necrotic DFW cells at galegine concentrations of 315 and 630 µM were reported to be 10.08 and 12.9%, respectively, as shown in Figure 2A. The percentage of apoptotic cells in each phase and altogether is summarized in Figure 2B. The results revealed that galegine markedly induced apoptosis in DFW cells after 24 h of treatment (Figure 2C).

Also, 38 and 40.4% of the SK-MEL-5 cells at concentrations of 1.65 and 3300 µM, were in apoptotic and necrotic phases, respectively (Figure 3A). Figure 3B shows the percentage of apoptosis in each phase and altogether. The results revealed that galegine induced apoptosis in SK-MEL-5 cells after 24 h of treatment in a statistically significant manner (Figure 3C).

To determine mRNA expression levels of genes involved in apoptosis (Bax, Bcl-2, p53), we performed a qRT-PCR. The results revealed upregulation in the Bax/Bcl-2 ratio with galegine at concentrations of 315 and 630 µM in DFW cells (Figure 4). A marked upregulation in Bax/Bcl-2 ratio and *p53* mRNA expression in SK-MEL-5 cells was observed as well after treatment by galegine (1650 µM) (Figure 5). No significant change in *p53* gene expression was found in DFW cells ($p>0.05$).

Melanoma is the most severe type of skin cancer, and its frequency in all Caucasians is on the rise [25]. The number of cases of melanoma and its related mortality has risen more than all other cancers worldwide over the previous ten years; there ,foreskin cancers could become a major public health issue in the upcoming years. The current treatment modalities include surgery, radiation therapy, chemotherapy, or a combination of these treatments. However, these methods are often accompanied by adverse effects [26, 27]. It is therefore essential to study skin cancer in vitro, especially melanoma cell lines, to expand our understanding of this tumor which in turn would hopefully translate into lower morbidity or mortality rates.

In the present study, melanoma cell proliferation was found to be hindered by galegine. Apoptosis induction appears to be responsible for a large part of the reduced growth. Interestingly, it was found that the DFW cells were more susceptible than the melanotic line SK-MEL-5 to galegine-induced cytotoxicity. In this study, we observed that the IC_{50} of galegine in DFW cells was lower than in SK-MEL-5 cells, implying that galegine was more cytotoxic to DFW cells. Only one study on the impact of galegine on melanoma appears to be published. Lee et al. [20] found that galegine suppressed the growth of the mouse melanoma B16F1 but did not decrease its metastatic potential. However, the cytotoxic effects of galegine in specific melanoma cells have yet to be elucidated. Hence, we evaluated the cytotoxic and apoptogenic impacts of galegine on DFW and SK-MEL-5 melanoma cells.

Our results demonstrated that galegine represents medium cytotoxicity to human melanoma cells concentration-dependently where the IC_{50} was 630 µM and 3300 µM in DFW and SK-MEL-5 cells for 24 h of treatment, respectively. In line with this finding, it has been found that 1000 µM galegine showed a significant anti-melanogenic effect in mouse melanoma B16F1 cells [18].

Modulating the activity of apoptotic proteins, such as Bax and Bcl-2, is an appealing approach for investigating their function in rising the apoptosis of tumor cells and thus identifying future therapy strategies [28]. Our study has shown that galegine significantly increases the apoptotic and necrotic phases 24 h after treatment, suggesting that galegine-induced cytotoxicity is through apoptosis induction. Induction of apoptosis is one of the most critical strategies for the treatment of cancer, and in this case melanoma [29-32]. Studies have shown that galegine is a potent inducer of apoptosis through truncated Parp-1, a typical apoptotic event [20].

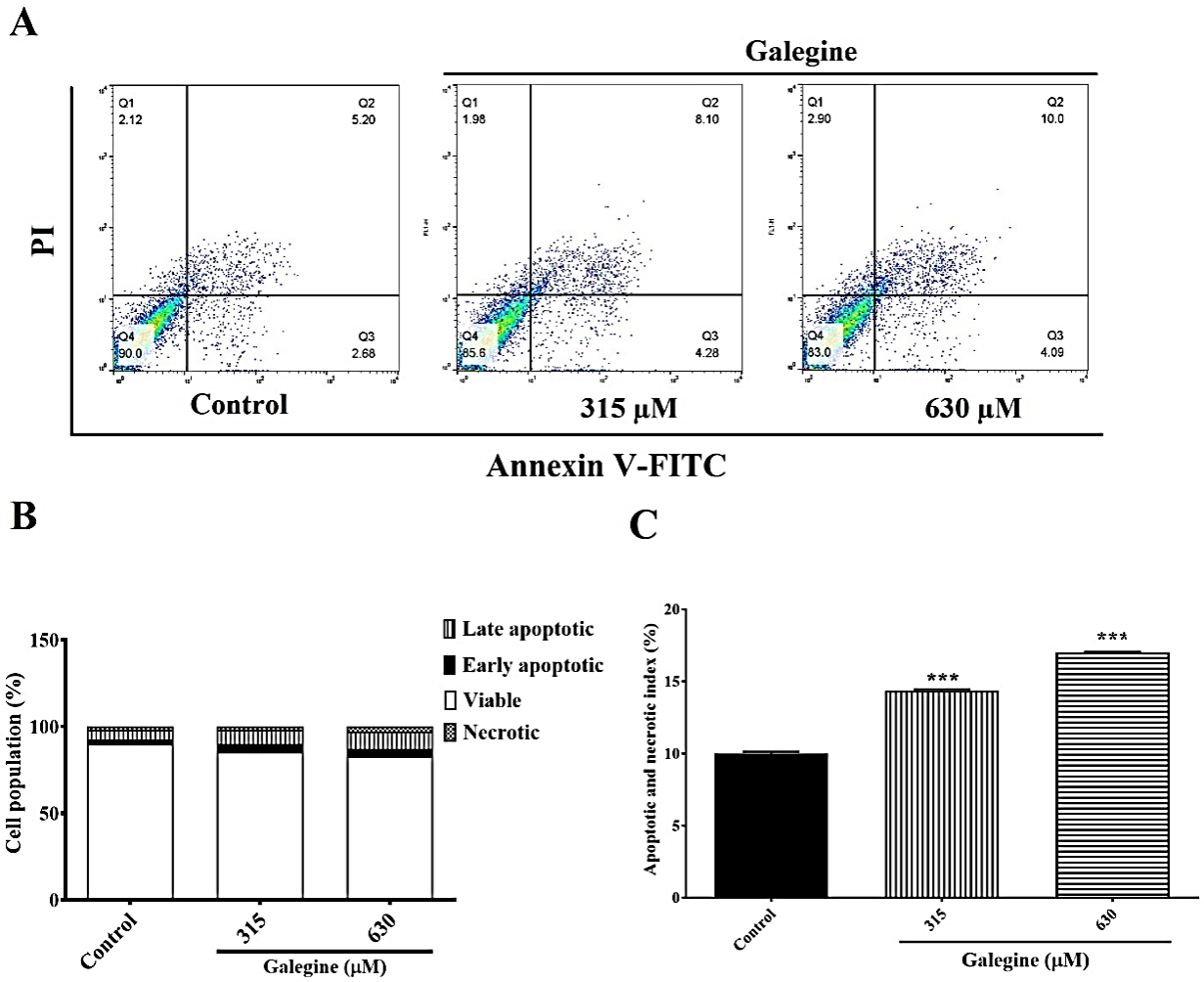

Figure 2. A: Galegine-induced apoptosis in DFW melanoma cells. These cells were collected and stained as directed by the producers with annexin V-FITC and propidium iodide (PI). The viable cells are in the lower-left quadrant (negative for both annexin V-FITC and PI). Early apoptotic cells are located at the lower right quadrant (annexin V-FITC positive). Late apoptotic cells showing the progressive cellular membrane and nuclear membrane damage are in the upper right quadrant (double-positive). Necrotic cells (lacking a cell membrane structure) are located in the upper left quadrant (PI positive). B and C: The percentages of viable, early/late apoptotic, and necrotic cells in treated samples compared to the control group, 24 h after treatment by galegine. Each column represents the mean±SEM in the samples; ***$p<0.001$ as compared with the control group (n=5)

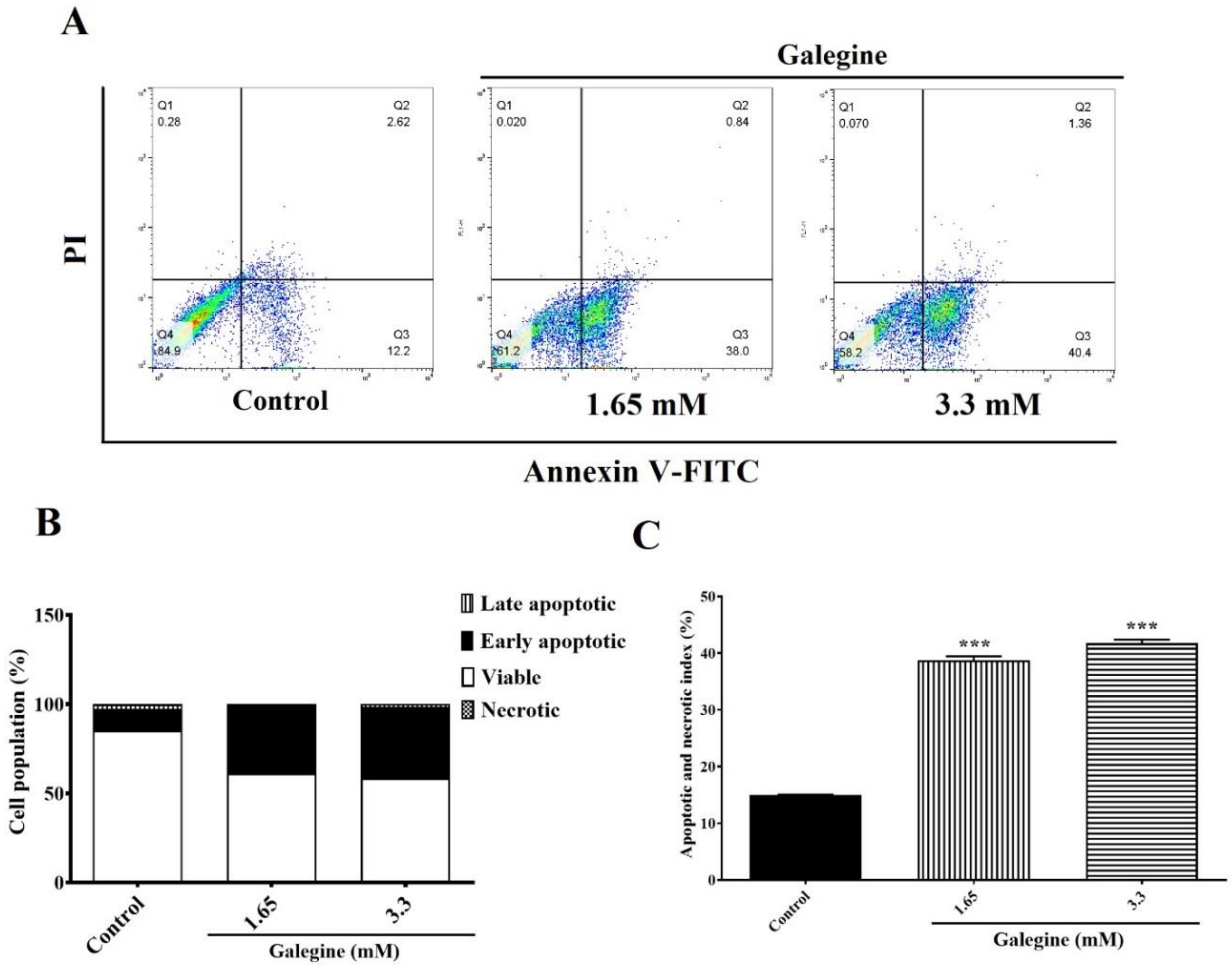

Figure 3. A: Galegine-induced apoptosis in SK-MEL-5 melanoma cells. These cells were collected and stained as directed by the producers with annexin V-FITC and propidium iodide (PI). The viable cells are in the lower-left quadrant (negative for both annexin V-FITC and PI). Early apoptotic cells are located in the lower right quadrant (annexin V-FITC positive). Late apoptotic cells showing the progressive cellular membrane and nuclear membrane damage are in the upper right quadrant (double-positive). Necrotic cells (lacking a cell membrane structure) are located in the upper left quadrant (PI positive). B and C: The percentage of viable, early/late apoptotic, and necrotic cells in treated samples compared to the control group, 24 h after treatment by galegine. Each column represents the mean±SEM in the samples; ***p<0.001 as compared with the control group (n=5)

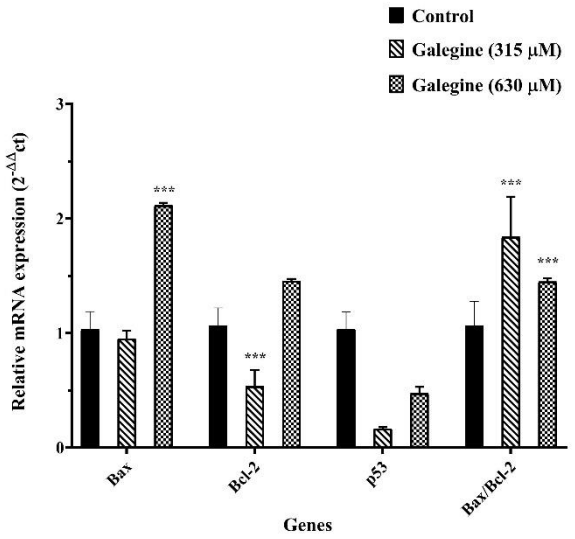

Figure 4. The DFW melanoma cells were treated with the indicated concentrations of galegine for 24 h. Total RNA was isolated, and mRNA expression was analyzed by qRT-PCR. The relative mRNA expression levels of Bax, Bcl-2, p53, and Bax/Bcl-2 ratio were determined; *** $p<0.001$ compared to the control group

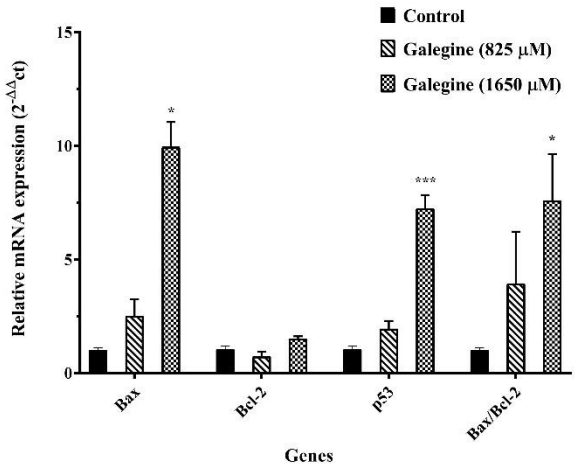

Figure 5. The SK-MEL-5 melanoma cells were treated with galegine for 24 h. Total RNA was isolated, and mRNA expression was analyzed by qRT-PCR. The relative mRNA expression levels of Bax, Bcl-2, p53, and Bax/Bcl-2 ratio were determined; *$p<0.05$ and ***$p < 0.001$ compared to the control group

Since galegine was able to induce apoptosis, the impact of galegine on the apoptotic-related genes involved in the melanoma cells was investigated. Studies have shown that the apoptosis pathway is controlled by the Bcl-2 (antiapoptotic) and Bax (apoptotic) group of proteins, regulating the integrity of the mitochondrial external layer in cancer [33,34]. In our study, it was concluded that galegine is an apoptosis inducer agent by upregulating Bax/Bcl-2 gene expression ratio. Besides, p53, as one of the most critical genes in cell cycle progression [35,36], was significantly increased at mRNA levels in SK-MEL-5 melanoma cells. This disparity in *p53* expression could be due to different cellular gene expression profiles between the two cell lines and also the dosage of galegine which can potentially activate or deactivate alternative apoptosis-related pathways as evidenced in other studies [37].

Finally, future studies focus on the combined treatment of melanoma cell lines or models with the currently established chemoradiotherapeutic modalities and novel potentially effective compounds can lead to new therapeutic regimens. This in turn would reduce the side effects of the current therapies while maintaining the overall survival of the patients. The other strategy would be to test the toxicity of this compound in mammalian melanoma models such as rats or mice to investigate the in vivo effects of galegine.

Conclusion

Galegine exerts cytotoxic effect on melanoma cells that involves the induction of apoptosis, possibly through the regulation of apoptosis-related gene expression. Based on this preliminary in vitro data it could happen dependent or independent of the P53 tumor suppressor as a major apoptosis regulator. Galegine, as a potential natural product, could be useful in melanoma management. However, further research on the underlying mechanisms of this promising agent is needed.

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Author contributions

Mohammad-Hassan Arjmand designed and supervised the project; Hamed Sabri and Abolfazl Maghrouni performed the experiments; Elmira Zarei provided galegine for cellular treatment; Massoumeh Hotelchi was involved in writing the manuscript; Amir Reza Afshari performed the statistical analyses and provided fund for the project.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

IC_{50}: 50% inhibitory concentration; qRT-PCR: quantitative real-time polymerase chain reaction; Bax: Bcl-2-associated x protein; p53: tumor protein; FITC: fluorescein isothiocyanate; DMEM: Dulbecco's modified Eagle's medium; EDTA: ethylenediaminetetraacetic acid; DMSO: dimethyl sulfoxide; PI: propidium iodide