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POGOSTONE EFFECT ON DACARBAZINE-INDUCED AUTOPHAGY AND APOPTOSIS IN HUMAN MELANOMA CELLS

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ABSTRACT – ***Objective:*** Chemotherapy is effective for treating malignant melanoma, but drug resistance and occurrence of side effects limited this strategy. The balance between autophagy and apoptosis has an essential role in the chemotherapy of cancers. The present investigation aims to examine the efficacy of pogostone (isolated from *Pogostemon cablin* L.) on the ratio of apoptosis and autophagy caused by dacarbazine in melanoma cells.

Materials and Methods: Human melanoma cells were exposed to different concentrations of dacarbazine and pogostone, and the IC50 values were calculated. The cells were treated with two concentrations higher and lower than IC50 simultaneously, and the dose reduction index and combination index (CI) parameters were calculated. The occurrence of apoptosis and autophagy was evaluated. The expression level of genes related to apoptosis and autophagy pathways was tested.

Results: Pogostone and dacarbazine declined the number of the cells in a dose and time-dependent manner and showed a synergistic effect. There was a significant decrease in autophagy in the co-treatment besides the dacarbazine alone ($p < 0.05$). There was a considerable increment in apoptosis in cultures treated with pogostone and dacarbazine ($p < 0.05$). Also, Real-time PCR data confirmed the obtained results.

Conclusions: Pogostone reduced melanoma cell resistance to dacarbazine via autophagy blockage.

KEYWORDS: Melanoma, Dacarbazine, Pogostone, Apoptosis, Autophagy, Drug resistance.

INTRODUCTION

Melanoma is a type of cancer that shows high invasiveness and metastasis potential[1]. The survival rate of people with metastatic melanoma is about 6 to 10 months, and less than 10% of them can survive up to 5 years[2].

Chemotherapy is one of the prompt treatment trends for cancers that is limited by the occurrence of drug resistance and side effects[3,4]. Combination therapy is effective to dismiss these limitations of anti-cancer drugs.

Dacarbazine is used in the chemotherapy of malignant melanoma, soft tissue sarcomas, and pancreatic carcinoma. This agent is one of the alkylating agents and by adding an alkyl group to nuclear DNA, it inhibits the proliferation of cancer cells. Blood complications, diarrhea, mouth ulcers, vomiting, anorexia, and anemia are seen in some patients treated with dacarbazine. Alkylating chemotherapy drugs such as dacarbazine, has a response rate of less than 5%. O6-methylguanine-DNA methyltransferase, the repair enzyme, represents the most important factor of the chemotherapy resistance related to dacarbazine[5].

The autophagy process, as a type II programmed cell death, can be associated with multiple physiological processes such as cell growth, aging, death, stress response, immune response, and cancer. Although many anticancer agents induce autophagy in cancer cells, evidence indicated that autophagy as a protective mechanism is associated with resistance to chemotherapy. Hence, autophagy inhibition can provide a good approach to enhance the effectiveness of anticancer agents and overcome drug resistance[6].

Indian mint (*Pogostemon cablin* L.), native to the tropical regions of Asia, has many medical benefits[7,8]. Pogostone (a pyranone —a pyran ring containing a ketone functional group) is one of the secondary metabolites obtained from Indian mint and shows diverse therapeutic potentials such as antibacterial, antifungal, and pesticidal properties[9]. Its anti-cancer effects are known in some cancers. Considering the high degree of drug resistance and side effect observed in melanoma chemotherapy by Dacarbazine, the combination of Dacarbazine with other agents should increase the efficacy of this anti-cancer drug. Thus, this study is designed to test if pogostone effectively reduces the number of melanoma cells. Moreover, pogostone may have a synergic effect with Dacarbazine to more removal of melanoma cells, and the disruption in the balance between autophagy and apoptosis could lead to this beneficial potential of pogostone.

MATERIALS AND METHODS

Materials and cells

The melanoma cell line (A-375) was purchased from the Pasteur Institute of Iran (Tehran, Iran). Ethanol, dimethyl sulfoxide (DMSO), trypsin/EDTA solution, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), pogostone, and dacarbazine were prepared from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). The study was approved by the Ethical Committee of Kermanshah University of Medical Sciences (IR.KUMS.MED.REC.1400.138).

Culturing the cells

The cells were harvested in DMEM, containing 10% FBS, and 1% penicillin/streptomycin, and incubated at 37°C with 5% CO_2. They were cultured in cell culture flasks and after reaching the appropriate density, they were detached from the bottom of the flask using trypsin/EDTA solution.

Viability assay

The cells were grown in 96-well plates (15×10^3 cells/each well). Following 24 hr, the cells were exposed to pogostone (1, 2, 4, 8, 16, 32, 64, and 128 µg/ml) or dacarbazine (7.81, 15.62, 31.25, 62.5, 125, 250, 500, and 1000 µg/ml), respectively and incubated. After 24, 48, 72, and 96 hr, the medium was discarded and 10 µl of MTT solution (5 mg/ml) was added to wells and incubated. After 4 hr DMSO was added to wells (150 µl). The absorbance of the wells was read at 570 nm by an ELISA reader. The control cells received a serum-free medium. The cell viability was analyzed from the following equation: Cell viability (%) = (Abs test cells/Abs control cells) × 100. The half-maximal inhibitory concentration (IC50) values, defined as the drug concentration that produced 50% cytotoxicity, were determined by nonlinear regression from the sigmoidal dose-response curve using GraphPad Prism software (La Jolla, CA, USA) version 9.3.

Median effect analysis

The treatment was done by combining two agents simultaneously in two higher and two lower concentrations than IC50 values and the MTT assay was repeated. 5 co-treatment groups were used, including: Group 1: 17.5 µg/ml of pogostone + 412.75 µg/ml dacarbazine; Group 2: 35 µg/ml of pogostone + 825.5 µg/ml dacarbazine; Group 3: 70 µg/ml of pogostone + 1651 µg/ml dacarbazine; Group 4: 140 µg/ml of pogostone + 3302 µg/ml dacarbazine; and Group 5: 280 µg/ml of pogostone + 6604 µg/ml dacarbazine (Table 1).

Table 1. Fraction affected (Fa), Combination index (CI) and dose reduction index (DRI) values for pogostone and dacarbazine combination.

Co-treatments groups	Fa	CI	DRI pogostone	DRI dacarbazine
1	0.45 ± 0.09	0.69	5.12	2.72
2	0.6 ± 0.07	0.68	3.37	2.61
3	0.78 ± 0.08	0.5	5.12	3.28
4	0.89 ± 0.07	0.38	7.44	3.98
5	0.95 ± 0.09	0.28	11.22	4.98

Group 1: 17.5 µg/ml of pogostone + 412.75 µg/ml dacarbazine; Group 2: 35 µg/ml of pogostone + 825.5 µg/ml dacarbazine; Group 3: 70 µg/ml of pogostone + 1651 µg/ml dacarbazine; Group 4: 140 µg/ml of pogostone + 3302 µg/ml dacarbazine; and Group 5: 280 µg/ml of pogostone + 6604 µg/ml dacarbazine.

Determination of combination indexes (CI) and dose reduction indexes (DRI) were performed by CompuSyn software (NJ, USA). CI was determined based on the equation CI = (D)1 / (Dx)1 + (D)2 / (Dx)2, where (Dx)1 and (Dx)2 are the dose of pogostone and dacarbazine alone that inhibits x% and (D)1 and (D)2 are the amounts of pogostone and dacarbazine in the combination that inhibit the experimentally observed x. A CI < 1 indicates synergy, a CI = 1 indicates additively and a CI > 1 indicates antagonism. In addition, DRI was calculated according to the equation (DRI)1 = (Dx)1/(D)1 and (DRI)2 = (Dx)2/(D)2. DRI value defines the amount to which the dose of a drug is decreased when combined to sustain an equal efficacy, and Fa is the fraction of cell death and varies from 0 (no death) to 1 (total death).

Apoptosis assay

Acridine orange/ethidium bromide double staining was used to evaluate morphological changes in apoptotic cells. After exposure of cells to pogostone and dacarbazin for 24 hr, the cells were stained with a mixture of acridine dye (100 µg/ml) and ethidium bromide (100 µg/ml). After 5 min, the cells were washed with PBS and were observed with a fluorescence microscope. In this method, cells were divided into four different categories: living cells with yellow nuclei, apoptotic cells in the early stage with shiny green nuclei with compact and fragmented chromatin, apoptotic cells in the late stage with orange-colored nuclei with compressed and fragmented chromatin and necrotic cells with the unusual orange colored cell nucleus.

Autophagy assay

To analyze the induction of autophagy, AVOs, which are mainly composed of autophagosomes and autolysosomes, were stained using acridine orange. Acridine orange accumulates protonates, is trapped inside the acidic organelles in cells, and emits red fluorescence. At neutral pH, it emits green fluorescence. After treatment, the cells were stained with acridine orange (1 µM). After 20 min, they were washed with PBS and observed by a fluorescence microscope. The percentage of autophagic cells is calculated as the number of cells containing red dots/total number of cells × 100.

Molecular analyses

The gene expression analysis was done by real-time PCR. After treatment with IC50 concentration for 24 hr, RNA was isolated using 1 × 106 cells control and treated group with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). The purity and integrity of RNA were evaluated using a NanoDrop spectrophotometer and gel electrophoresis. Complementary DNA (cDNA) was synthesized according to the cDNA synthesis kit (Vivantis Technologies kit, Selangor DE, Malaysia) procedure. Real-time PCR was conducted by SYBR Premix Ex Taq Technology (TaKaRa Bio Inc., Otsu, Shiga, Japan) according to the manufacturer's protocol. The fold changes were obtained based on the comparative Ct (2e-ΔΔct) method. GAPDH was used as an internal control. All the primer sequences were planned by GeneRunner software (Hastings Software, Hastings, NY, USA) version 3.05 and listed in Table 2.

Table 2. The primer sequences of studied genes.

Genes	Sequences
Bax	F: 5′-CCTGTGCACCAAGGTGCCGGAACT-3′
	R: 5′-CCACCCTGGTCTTGGATCCAGCCC-3′
Bcl-2	F: 5′-TTGTGGCCTTCTTTGAGTTCGGTG-3′
	R: 5′-GGTGCCGGTTCAGGTACTCAGTCA-3′
Beclin-1	F: 5′-GCCGAAGACTGAAGGTCA-3′
	R: 5′-GTCTGGGCATAACGCATC-3′
LC-3	F: 5′-GATGTCCGACTTATTCGAGAGC-3′
	R: 5′-TTGAGCTGTAAGCGCCTTCTA-3′
GAPDH	F: 5′-TCCCTGAGCTGAACGGGAAG-3′
	R: 5′-GGAGGAGTGGGTGTCGCTGT-3′

STATISTICAL ANALYSIS

The tests were done in triplicate and the results were expressed as means ± Standard error of the mean (S.E.M). One-way analysis of variance with Tukey's test correction was used to determine significant differences between groups, with $p < 0.05$ considered statistically significant.

RESULTS

Viability assay

Figures 1 A and B show the impact of pogostone and dacarbazine on cell survival. After 24, 48, 72, and 96 hr, both pogostone and dacarbazine reduced cell viability in a concentration- and time-dependent manner. IC50 values for pogostone were 70.83, 40.01, 32.29, and 10.09 µg/ml and for dacarbazine were 1651.85, 1238.83, 473.67, and 188.78 µg/ml for 24, 48, 72, and 96 hr, respectively. The CI values were presented in Table 1. The obtained values were less than 1 (synergistic effect). The DRI values were greater than 1 demonstrating a dose decline for a certain therapeutic effect in both of them.

Autophagy assay

The control group and pogostone treated group showed negligible AVOs (Figures 2 A and B), but the dacarbazine treated group exhibited abundant AVOs, which is a marker of autophagy (Figure 2 C). There was a significant reduction in AVOs in the combination group compared to dacarbazine-treated group.

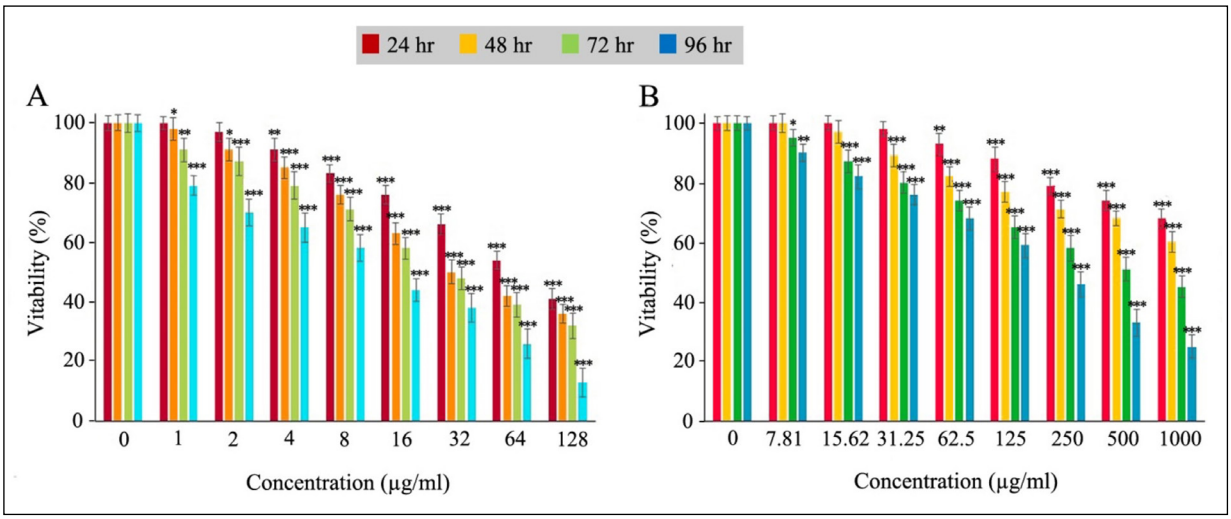

Figure 1. The effects of (A) pogostone and (B) dacarbazine on melanoma cells viability. *$p < 0.05$, ** $p < 0.01$, and *** $p < 0.01$ compared with control.

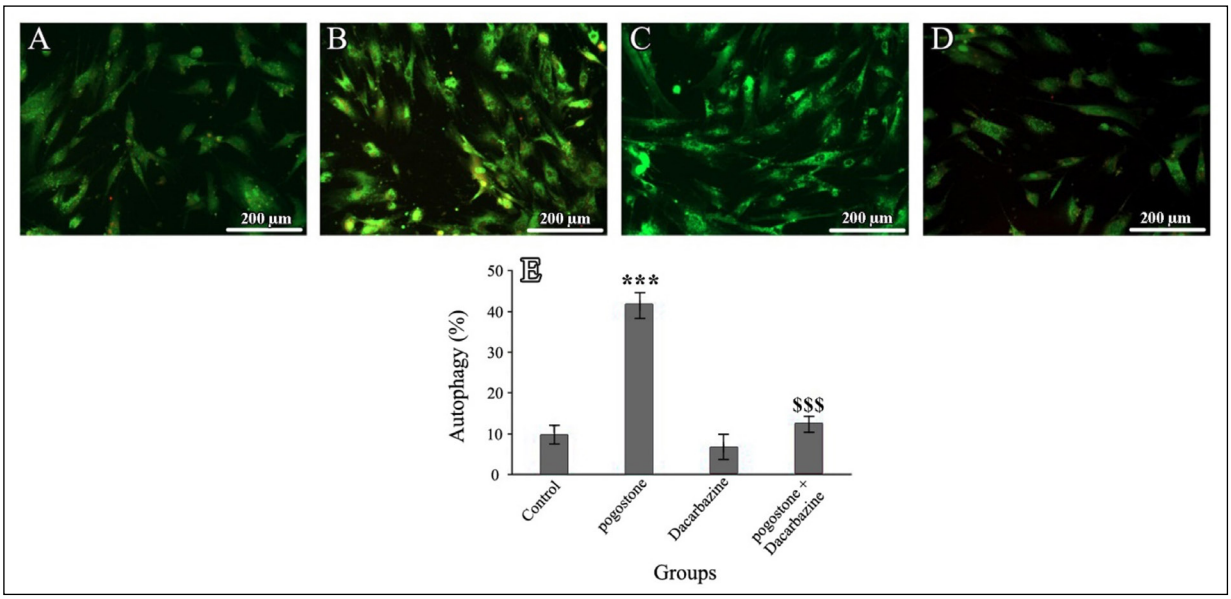

Figure 2. The effects of pogostone and/or dacarbazine on autophagy in melanoma cells. A) Control cells, B) in the presence of 70 µg/ml of pogostone, C) in the presence of 1650 µg/ml of dacarbazine, D) in the presence of 70 µg/ml pogostone and 1650 µg/ml of dacarbazine, and E) Columns mean percentage of autophagic cells from three independent experiments. *** $p < 0.001$ than control cells and $$$ $p < 0.001$ against dacarbazine-treated cells.

Apoptosis assay

The results of acridine orange/ethidium bromide staining showed that a vast number of living cells and normal morphology were seen in the control group, whereas early and late apoptosis occurred in cells treated with pogostone and dacarbazine. There was a significant increase in apoptosis in the combination group compared with the dacarbazine treated group (Figure 3).

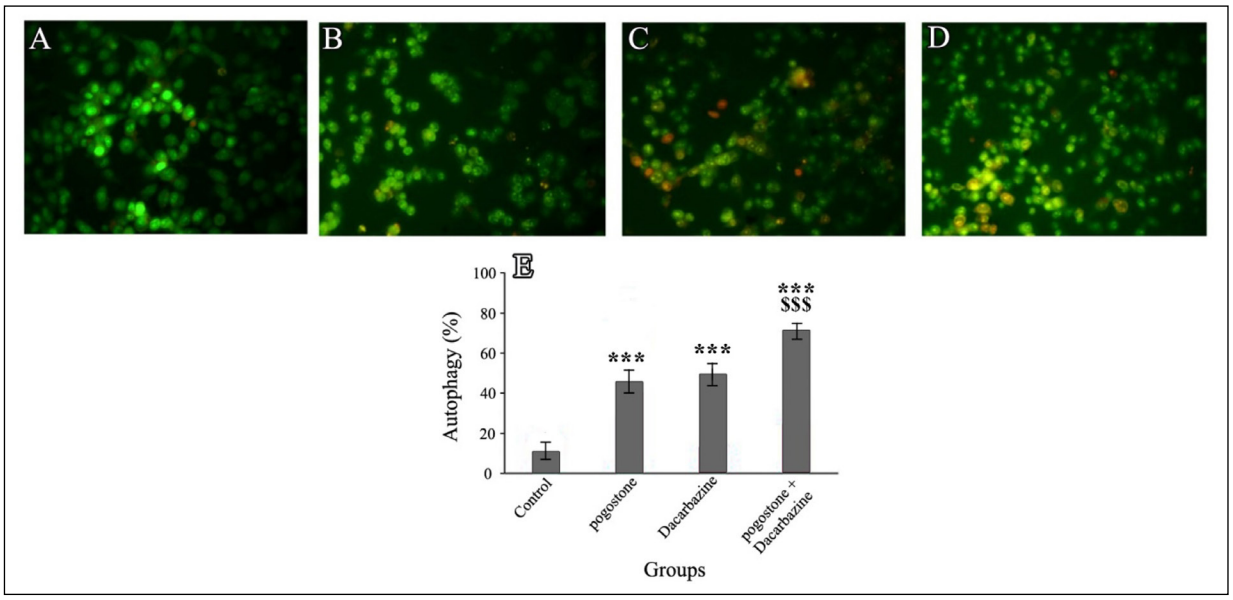

Figure 3. The effects of pogostone and/or dacarbazine on apoptosis in melanoma cells. A) Control cells, B) in the presence of 70 µg/ml of pogostone, C) in the presence of 1650 µg/ml of dacarbazine, D) in the presence of 70 µg/ml pogostone and 1650 µg/ml of dacarbazine, and E) Columns mean percentage of apoptotic cells from three independent experiments. $^{***}p < 0.001$ in comparison with control cells and $^{\$\$\$}p < 0.001$ compared with dacarbazine-treated cells.

Real-time pcr assay

The expression of some genes involved in apoptosis and autophagy pathways was tested by real-time PCR. After 24 hr exposure to pogostone and/or dacarbazine, there was a down-regulation of Bcl-2 and up-regulation of Bax mRNA expression. Also, dacarbazine increased the LC-3, Beclin-1 mRNA expression. But pogostone decreases these levels. There was a significant decline in autophagy-related genes in the combination group compared with the dacarbazine treated group (Figure 4).

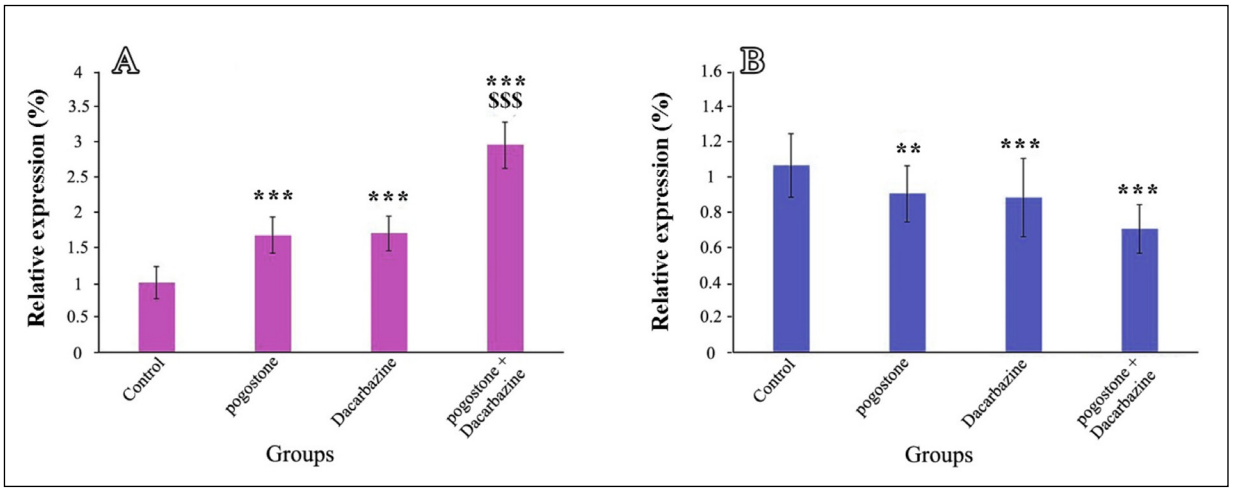

Figure 4. The effects of pogostone and/or dacarbazine on (A) Bax and (B) Bcl-2 expression in melanoma cells. $^{**}p < 0.005$ and $^{***}p < 0.001$ compared to control cells and $^{\$\$\$}p < 0.001$ compared with dacarbazine-treated cells.

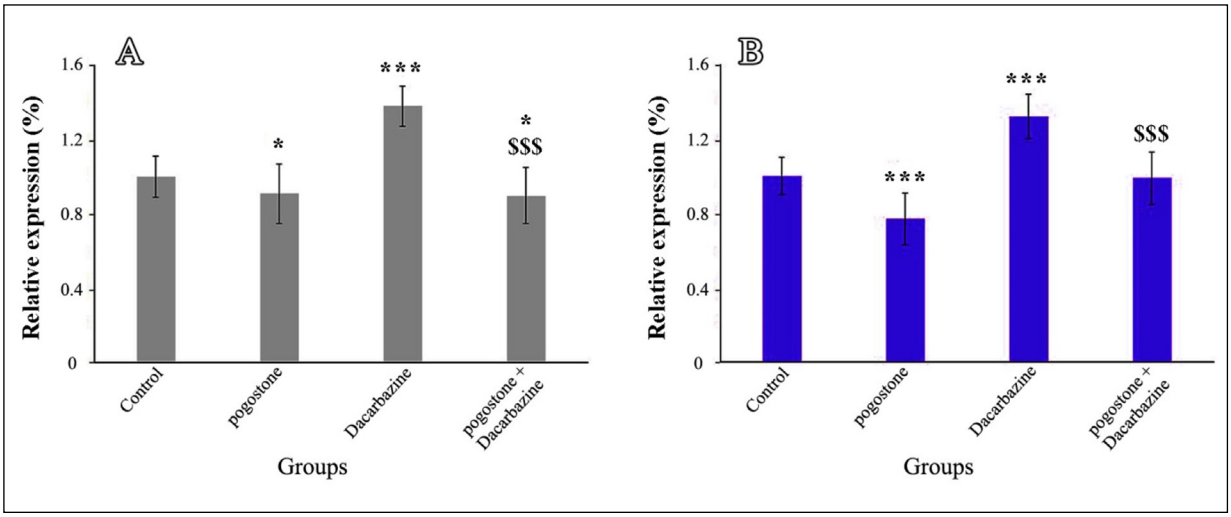

Figure 5. The effects of pogostone and/or dacarbazine on (A) Beclin-1 and (B) LC-3 expression in melanoma cells. Data are means ± S.E.M. of values from three independent experiments and are expressed as a percentage relative to control values, $^{*}p < 0.05$ and $^{***}p < 0.001$ than control cells and $^{\$\$\$}$ $p < 0.001$ compared with dacarbazine-treated cells.

DISCUSSION

In this study, the cytotoxicity of pogostone and/or dacarbazine was investigated on melanoma cancer cells. The data indicated that the effect of pogostone on reducing viability was significant. Pogostone displays a considerable cytotoxic effect against some human cancer cell lines. It induces autophagy and apoptosis via the regulation of LC3-dependent, caspase-7, and caspase-3 expression and lowering of AKT/mTOR phosphorylation[10].

First, the cells were treated with pogostone (1, 2, 4, 8, 16, 32, 64, and 128 µg/ml) or dacarbazine (7.81, 15.62, 31.25, 62.5, 125, 250, 500, and 1000 µg/ml) alone. After 24, 48, 72, and 96 hr exposure, the viability gradually decreased with the increasing concentration of dacarbazine than the control cells. 24 hr later of dacarbazine exposure, the reduction in viability was seen significantly at 62.5, 8, 125, 250, 500, and 1000 µg/ml doses beside the control group. After 48 hr, the viability was decreased significantly at concentrations of 31.25, 62.5, 8, 125, 250, 500, and 1000 µg/ml compared with the control cells. After 72, and 96 hr exposure, the effect of dacarbazine on reducing viability was significant in all groups.

Despite the current progresses in cancer treatment, drug resistance is yet one of the main obstacles to the successful outcome of treatment. Therefore, the development of new strategies to overcome this issue is necessary. Newly, the drug combination is the most widely therapeutic strategy used in lethal disorders such as cancer and AIDS. The benefits of reducing toxicity and minimizing resistance can be the result of synergistic interactions. Newly, the use of anti-cancer agents' combination is used in the treatment of all types of cancers.

The mechanisms for dacarbazine resistance to melanoma are unclear. A study showed that dacarbazine increased IL-8 and VEGF secretion, which can cause resistance to the cytotoxic effects of this drug in melanoma cells[11].

The co-treatment was done by combining two agents simultaneously in two higher and two lower concentrations than IC50 values (Table **1**). Pogostone intensified dacarbazine cytotoxicity with a CI between 0.28 and 0.69, which indicates a synergistic effect in all the combined concentrations used in this study. The average of CI values was 0.50, which indicates the overall synergistic effect of pogostone and dacarbazine combination on the melanoma cells. This combination decreased the dose of dacarbazine and pogostone. Therefore, the IC50 values were reduced for both agents. Decreasing the dose of dacarbazine to produce a certain effect is very valuable from a clinical point of view because this dose reduction decreases the general side effects of chemotherapy. The synergistic effect of the combination of dacarbazine and pogostone on the viability of melanoma cells was reported for the first time and the best-chosen doses were 280 of pogostone and 6604 of dacarbazine (Group 5) because this combination showed the lowest CI (0.28).

The autophagy test indicated that the number of cells with AVO significantly increased by the treatment with dacarbazine, which indicates the increase in the autophagy process in these cells, and it showed a significant decrease by the treatment with pogostone indicates inhibition of autophagy, and finally, in the simultaneous group, AVO positive cells were significantly lower than the dacarbazine treated group, but there was no significant difference with the control group.

Molecular investigations showed that autophagy-promoting genes expression (beclin-1 and LC-3) was significantly enhanced by dacarbazine and decreased by pogostone. The results of the molecular test are in complete agreement with the results of the fluorescence staining of the cells. Dacarbazine-induced autophagy can be a reason for melanoma cell resistance to this drug, and its inhibition at the transcription level by pogostone increases the sensitivity of cells to drug toxicity and increases its effectiveness.

Examining the morphological changes created in the nucleus during cell death after separate and simultaneous treatment with dacarbazine and pogostone by acridine orange/ethidium bromide staining method indicated that most of the cell death induced by both agents was done through apoptosis and the necrosis caused by these compounds was very low and can be ignored.

Apoptosis and autophagy have complex interactions and the balance between these two pathways determines the death or survival of the cell. Autophagy is a homeostatic cellular mechanism that inhibits apoptosis and stimulates aging. In addition, autophagy has an important role in cancer cell survival because inhibition of this pathway can decrease cancer cell proliferation[12]. Therefore, inhibiting autophagy in cancer cells treated with chemotherapy can improve the cell-killing effect of a drug and improve the therapeutic response. Our study showed the aggravation of apoptosis induced by dacarbazine in co-treatment. This phenomenon is probably due to the inhibition of autophagy by pogostone.

One of the main causes of tumor formation is a defect in the processes of apoptosis, which causes the production of immortal clones of cells. Most chemotherapy drugs increase apoptosis in tumor cells. Failure to induce apoptosis is one reason for drug resistance in cancer.

CONCLUSIONS

Data from this study indicated that combinations of pogostone and dacarbazine had a synergistic anti-tumor potential in melanoma cells. We proposed that pogostone may reduce cell resistance to dacarbazine through autophagy blockage.

Ethics approval and informed consent:

The study was approved by Kermanshah University of Medical Sciences (Number: IR.KUMS.MED.REC.1400.138). The study was carried out in accordance with the Declaration of Helsinki.

Availability of data and material:

The data and material are available by corresponding author upon request.

Conflict of interests:

Authors report no conflict of interest.

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Author's contribution:

All authors contributed equally to this work. Ali Ghanbari takes responsibility for the integrity of the work as a whole from inception to published article and should be designated as 'guarantor'.

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