

Solanine induced apoptosis and increased chemosensitivity to Adriamycin in T-cell acute lymphoblastic leukemia cells

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Abstract. Solanine is an alkaloid and is the main extract of the traditional Chinese herb, *Solanum nigrum* Linn. It has been reported that Solanine has anti-inflammatory and antitumor properties. The present study aimed to investigate the anti-tumor effect of Solanine in Jurkat cells and demonstrate the molecular mechanism of antitumor activity of Solanine. A Cell Counting Kit-8 assay demonstrated that Solanine inhibited the proliferation of Jurkat cells in a dose- and time-dependent manner. Cell apoptosis was measured by flow cytometry. Flow cytometry revealed that Solanine induced apoptosis in a dose-dependent manner in Jurkat cells. Reverse transcription-quantitative polymerase chain reaction demonstrated that Solanine modulated the mRNA levels of B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax). Additionally, Bcl-2 and Bax expression was measured using western blot analysis. Western blot analysis revealed a significant increase in the expression of Bax and decrease in the expression of Bcl-2. Solanine increased the chemosensitivity of Jurkat cells to Adriamycin. In summary, the present results indicated that the antitumor activity of Solanine was associated with inhibition of cell proliferation, induction of apoptosis and increasing cytotoxicity of Adriamycin. Therefore, Solanine may have potential as a novel agent for the treatment of acute lymphocytic leukemia.

Introduction

Acute lymphoblastic leukemia (ALL) is an aggressive type of blood cancer affecting children and adults, with peak prevalence between 2 and 5 years of age (1). T-cell ALL (T-ALL) is an aggressive hematological cancer that is caused by the malignant transformation of thymocyte progenitors (2).

T-ALL accounts for 10-15% of pediatric and 25% of adult ALL cases (2,3). The age of the patient at diagnosis, leukocyte count, ethnicity, gender and immunophenotype are clinical prognostic parameters that classify ALL patients into different risk groups (4). T-ALL is classified into a high-risk group in ALL (4). The prognosis of T-ALL has improved with the development of high-dose multi-agent chemotherapy, with a cure rate of ~85% in children and ~50% in adults (5). However, the treatment is often accompanied by severe acute toxicities and side effects, such as primary resistance, early relapse and secondary tumors (2,3). The identification of new agents for T-ALL patients is urgently required.

Nightshade, a Chinese herbal medicine, has been used to treat sores, injuries, swelling and fractures (6). Solanine, the main extract of Nightshade, is a trisaccharide glycoalkaloid (7). Solanine has been demonstrated to inhibit the production of cytokine and nitric oxide in stimulated Jurkat cells LPS-stimulated Raw macrophages (8).

High concentrations of Solanine result in cytotoxicity-inducing damage of the plasma membrane, which causes disorder of metabolism, including reduced NAD(P)H productivity and the loss or inactivation of NAD(P)H:menadiene reductase (9). Solanine was demonstrated to have a proliferation-inhibiting and apoptosis-promoting effect on multiple cancer cells, including prostate cancer, pancreatic carcinoma and melanoma cancer cells (6-9). Studies have also shown that Solanine suppresses proliferation and metastasis, and promotes apoptosis, in pancreatic cancer cells (9,10). Solanine induces apoptosis of HepG2 cells by facilitating the opening of the PT channels in the mitochondria and suppressing the expression of Bcl-2 (11,12). Additionally, Solanine has been reported to inhibit human melanoma cells and human prostate cancer cell invasion at non-toxic doses (6,7). However, to the best of our knowledge, the efficacy and the associated molecular mechanisms of Solanine promoting apoptosis in Jurkat cells have not been explored. In the present study, the effects of Solanine on the inhibition of proliferation and induction of apoptosis in Jurkat cells and the underlying molecular mechanism were investigated. Additionally, the effect of Solanine on the chemosensitivity of Jurkat cells to Adriamycin was assessed. The findings indicated the potential of Solanine to improve the therapeutic outcome of T-ALL.

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Materials and methods

Chemicals and reagents. Adriamycin (Melone Pharmaceutical Co. Ltd., Dalian, China) was dissolved to a concentration of 2 g/l in dH₂O and divided into 25 aliquots (1.5 ml). Solanine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was dissolved in dimethyl sulfoxide (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) to generate a stock solution (100 µg/ml), and diluted to each designated concentration in RPMI-1640 (HyClone; GE Healthcare Life Sciences). The rabbit polyclonal antibodies against B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax) were obtained from Beijing ZhongShan Golden Bridge Technology Co., Ltd. (Beijing, China). The rabbit polyclonal antibody against GAPDH was obtained from Good here Biotechnology Co., Ltd., Hangzhou, China.

Cell lines and cell culture. The human T-ALL Jurkat cells were obtained from Key Laboratory of Tumour Molecular Biology of Binzhou Medical University (Binzhou, China). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences) at 37°C in a humidified atmosphere containing 5% CO₂.

Cell proliferation assay. Cell Counting Kit 8 (CCK-8; Dojindo Molecular Technologies, Inc., Shanghai, China) was used to determine the cell viability in the presence of Solanine (0, 2, 4, 8 or 16 µg/ml), with/without 0.15 µg/l Adriamycin (4), incubated for 24 h at 37°C in air. Briefly, cells were seeded into 96-well plates at a density of 1x10⁴ cells/well, and, subsequent to treatment with Solanine (0, 2, 4, 6, 8 or 16 µg/ml) with/without 0.15 µg/l Adriamycin, 10 µl CCK-8 solution was added to each well and incubated for 4 h at 37°C in a humidified incubator with 5% CO₂ in air. Cells in control group were supplemented with the equivalent quantity of DMSO. The absorbance was then measured at a wavelength of 490 nm using a fluorescence spectrofluorometer (F-7000; Hitachi Ltd., Tokyo, Japan). A blank well containing only medium and drugs was used as a control.

Flow cytometry analysis. The Jurkat cells (3x10⁵ cells/ml) were seeded into 6 well plates and incubated with various concentrations of Solanine (0, 4 and 16 µg/ml) for 24 h at 37°C in a humidified atmosphere containing 5% CO₂ in air. Cells were collected and washed twice with PBS. Cells were then suspended in binding buffer (KeyGen Biotech Co., Ltd., Nanjing, China) and double-stained with annexin V fluorescein isothiocyanate (FITC)/propidium iodide (PI; KeyGen Biotech Co., Ltd.) for 15 min in the dark at room temperature. The cell-associated mean fluorescence intensity (MFI) was detected by flow cytometer using a FACSCalibur (Beckman Coulter, Brea, CA, USA) to analyze the apoptotic cells.

Determination of gene expression by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from Solanine-treated cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. Reverse transcription was performed to generate first strand cDNA (Takara Biotechnology Co., Ltd., Dalian, China) using 2 µg

of total RNA. The reverse transcription reaction was implemented with PrimeScript™ RT reagent kit with gDNA Eraser (Takara Bio, Shiga, Japan). The primers (Table I) used in this experiment were designed using Primer 5 version 5.6.0 software (PREMIER Biosoft Co., Ltd., CA, USA) and synthesized by Sangon Biotech Co., Ltd., Shanghai, China.

qPCR was performed on an ABI PRISM 7500 qPCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) by using SYBRGreen reaction kit (Takara Bio, Inc., Otsu, Japan). The PCR reaction system consisted of SYBR Green reagent, forward and reverse primers, template cDNA and nuclease-free distilled water. The PCR conditions were 95°C for 30 sec, followed by 45 cycles of 95°C for 5 sec and 60°C for 30 sec. GAPDH was used as an internal control. qPCR for each gene of each cDNA sample was assayed in triplicate. The results were calculated using the 2^{-ΔΔCq} method (4). The following equations were used: ΔCq=Cq (target gene)-Cq (GAPDH); ΔΔCq=ΔCq (Solanine-treated cells)-ΔCq (untreated control).

Western blot analysis. The Jurkat cells were incubated with Solanine (0, 4 or 16 µg/ml) for 24 h at 37°C. The cells were then harvested. Lysis buffer (100 µl; Beyotime Institute of Biotechnology, Shanghai, China) was added and the protein concentration of the lysate was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). The lysed samples containing 50 µg total protein were separated on 10-12% SDS-PAGE (Beyotime Institute of Biotechnology), with a constant voltage of 80 V for 0.5 h, and then 100 V for another 1.5 h. The resolved proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Merck KGaA) and blocked with 5% skimmed milk for 2 h at room temperature. Subsequently, the membranes were incubated overnight at 4°C with specific antibodies. The primary antibodies used were rabbit polyclonal antibodies against Bcl-2 (dilution, 1:500), Bax (dilution, 1:500) and GAPDH (dilution, 1:1,000). The following day, the membranes were incubated in horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (dilution, 1:5,000; catalog no., ZB-5301; Beijing ZhongShan Golden Bridge Technology Co., Ltd.) for 2 h at room temperature. Finally, images were captured using a FluorChem FC2 gel imaging system (ProteinSimple; Bio-Techne, Minneapolis, MN, USA). The intensity of each band was normalized by GAPDH for their respective lanes.

Data analysis. Statistical analyses were performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation. Differences between ≥3 groups were evaluated by one-way analysis of variance followed by Student-Newman-Keuls test. Independent two sample t-tests were used to compare the differences between 2 groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Solanine decreased the viability of Jurkat cells. The ability of Solanine to inhibit Jurkat cell proliferation was assessed. Jurkat cells were treated with various concentrations of Solanine (0, 2, 4, 8 or 16 µg/ml) for 24, 48 and 72 h. Cell viability was

determined by CCK-8 assay. The results demonstrated that Jurkat cells were sensitive to Solanine, and Solanine inhibited Jurkat cell proliferation in a dose- and time-dependent manner (Fig. 1; $P < 0.05$).

Solanine induced Jurkat cell apoptosis. Subsequent to treatment of Jurkat cells with various concentrations of Solanine (0, 4 and 16 $\mu\text{g/ml}$) for 24 h, the apoptotic rate of Jurkat cells was detected by flow cytometry to confirm that Solanine induces Jurkat cell apoptosis. The results revealed that Solanine promotes Jurkat cell apoptosis in a dose-dependent manner (Fig. 2; $P < 0.05$).

Effect of Solanine on mRNA levels of Bcl-2 and Bax. RT-qPCR analysis was performed to assess whether Solanine modulates the expression of Bcl-2 and Bax genes. The results showed that Bcl-2 mRNA level decreased and Bax mRNA level increased subsequent to treatment with various concentrations of Solanine (Fig. 3; $P < 0.05$).

Effect of Solanine on the expression of Bcl-2 and Bax protein in Jurkat cells. Bcl-2 and Bax are involved in cell apoptosis. The expression of Bcl-2 and Bax was measured to explore the molecular mechanism underlying Solanine-induced apoptosis. The western blot assay showed that the expression of Bcl-2 decreased significantly while the expression of Bax increased subsequent to Jurkat cell incubation with various concentrations of Solanine for 24 h (Fig. 4; $P < 0.05$).

Solanine enhanced chemosensitivity of Jurkat cells to Adriamycin. Based on the aforementioned findings, the present study explored whether Solanine could increase the chemosensitivity of Jurkat cells to Adriamycin. The cells were incubated with various concentrations of Solanine (0, 4 and 16 $\mu\text{g/ml}$) in the presence of Adriamycin for 24 h, and inhibition of cell proliferation was measured by CCK-8 assay. As shown in Fig. 5, Solanine significantly increased the Adriamycin-induced inhibitory rate of Jurkat cell proliferation, which indicated that Solanine enhanced sensitivity to Adriamycin compared to the controls [Cells treated with Adriamycin (0.15 $\mu\text{g/l}$) in the absence of Solanine].

Discussion

Glycoalkaloids are secondary plant metabolites that contain nitrogen and are found in Solanaceous plants and possess anticarcinogenic activity (13). Solanine is a steroidal glycoalkaloid (9). Studies have shown that Solanine has antitumor potency (6-9), and inhibited the proliferation of U937 cells (14). In addition, data has shown that Solanine has antitumor activity in other types of cancer. Solanine has been shown to induce apoptosis of mice breast cancer cells by inducing Bax expression and decreasing Bcl-2 expression (14). Solanine also inhibited the activity of matrix metalloproteinase (MMP)-2 and MMP-9 by suppressing the phosphoinositide 3-kinase/Akt and c-Jun N-terminal kinase signaling pathways at non-toxic doses, causing inhibition of migration and invasion in melanoma cells (7). Additionally, Solanine suppressed pancreatic cancer cell migration and invasion by inhibiting MMP-2 and MMP-9 expression (9,10). Solanine downregulated the Bcl-2/Bax ratio and processed the capase-3 zymogen into an active form, thereby

Table I. Primers used in reverse transcription-quantitative polymerase chain reaction.

Gene	Primer sequence, 5'-3'	Product length, bp
<i>Bax</i>		117
Forward	CCGAGAGGTCTTTTCCGAG	
Reverse	GTGCACAGGGCCTTGAGC	
<i>Bcl-2</i>		152
Forward	GGATTGTGGCCTTCTTTGAG	
Reverse	TACCCAGCCTCCGTTATCCT	
<i>GAPDH</i>		121
Forward	TGACTTCAACAGCGACACCCA	
Reverse	CACCCTGTTGCTGTAGCCAAA	

Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein.

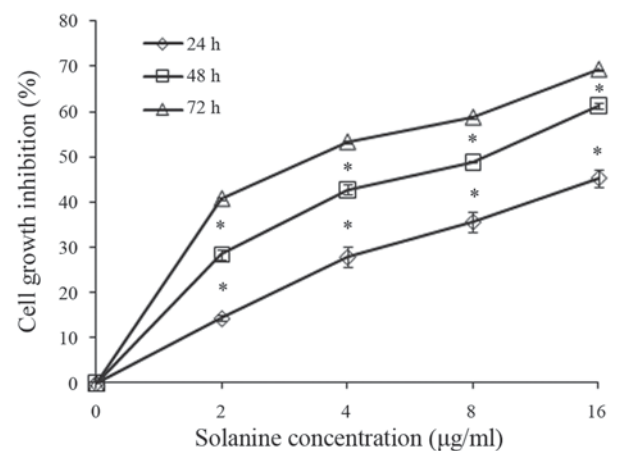


Figure 1. Solanine inhibited the proliferation of T-cell acute lymphoblastic leukemia Jurkat cells. Jurkat cells were treated with different concentrations of Solanine (0, 2, 4, 8 and 16 $\mu\text{g/ml}$) for 24, 48 and 72 h. Growth curves were based on data from Cell Counting Kit-8 assays. Data are expressed as the mean \pm standard deviation of triplicate experiments. * $P < 0.05$ vs. 24 h.

promoting pancreatic cancer cell apoptosis (10). However, to the best of our knowledge, the effect of Solanine on T-ALL cells remains unknown. The present study aimed to investigate the cellular functions of Solanine to elucidate the mechanism by which it contributes to promote apoptosis and inhibit proliferation in T-ALL Jurkat cells *in vitro*.

The CCK-8 assay showed that Solanine significantly inhibited Jurkat cell proliferation in a time- and dose-dependent manner (Fig. 1). Therefore, the results of the present study demonstrated that Solanine treatment inhibited T-ALL Jurkat cell proliferation *in vitro*.

Apoptosis, also termed programmed cell death, is characterized by morphological changes, including cell shrinkage, chromatin condensation and membrane blebbing without disruption of the plasma membrane (15). Apoptosis plays an important role in homeostasis (16). Therefore, apoptosis plays a crucial role in cancer treatment (17). In the present study, Solanine induced apoptosis of Jurkat cells, which was demonstrated by flow cytometry (Fig. 2).

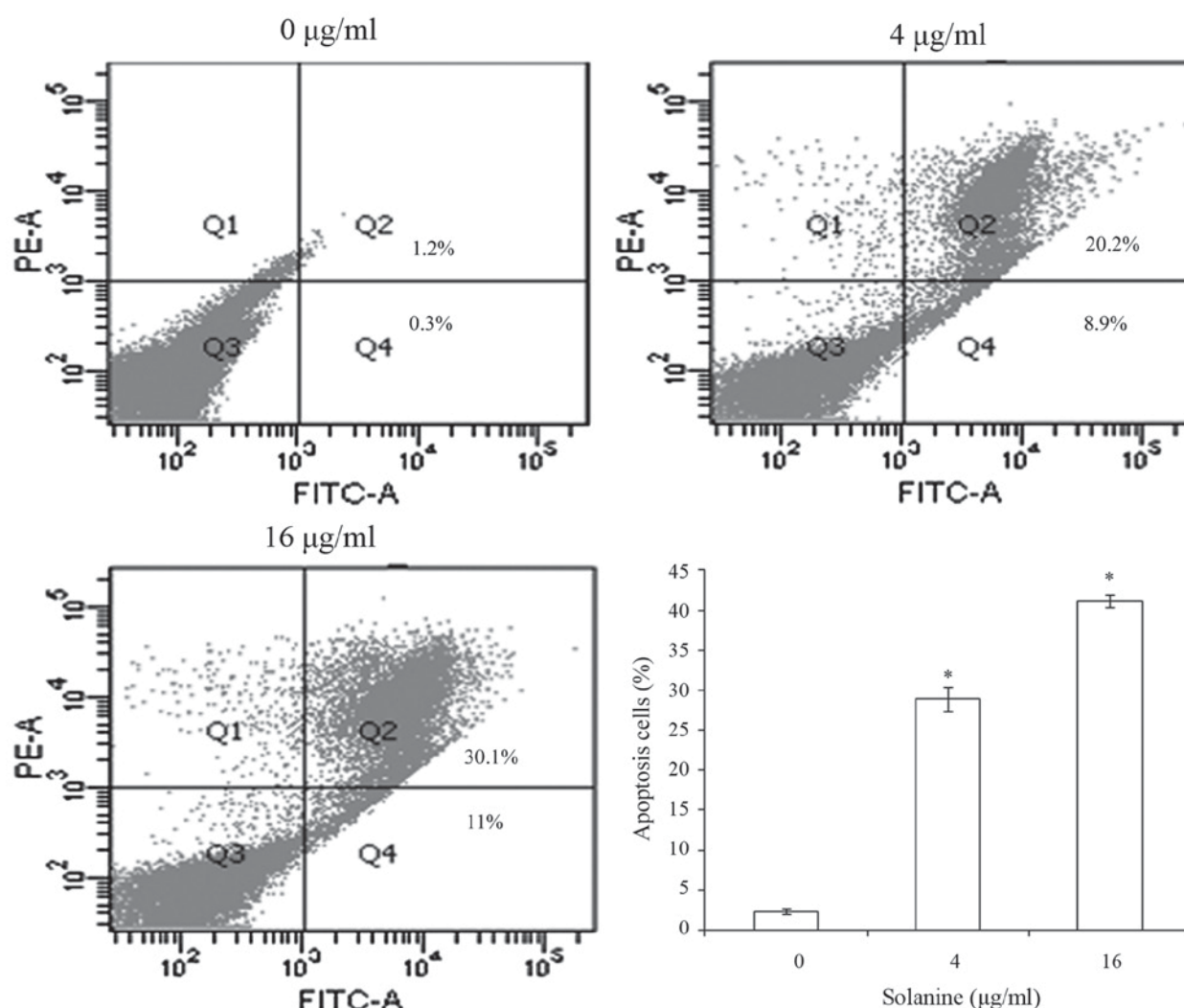


Figure 2. Solanine induced apoptosis in T-cell acute lymphoblastic leukemia Jurkat cells. Jurkat cells were treated with different concentrations of Solanine (0, 4 and 16 µg/ml) for 24 h. Annexin VFITC/phycoerythrin staining was conducted in order to measure cell apoptosis. Data are expressed as the mean \pm standard deviation of triplicate experiments. * $P < 0.05$ vs. 0 µg/ml Solanine. FITC, fluorescein isothiocyanate; PE, phycoerythrin.

Apoptosis is triggered by two pathways; one is the death receptor-mediated extrinsic pathway, and the other is the mitochondrial-dependent intrinsic pathway (17,18). Bcl-2 family members are key components of the mitochondrial-dependent intrinsic apoptosis pathway (19). Bcl-2 family members are classified into three subgroups: The pro-apoptotic proteins, including Bax and Bak; the anti-apoptotic proteins, including Bcl-2, myeloid cell leukemia-1 and Bcl-extra large; and the BH3-only proteins, including BH3 interacting domain death agonist, p53 upregulated modulator of apoptosis and Noxa (20). Bax and Bcl-2 are the most characterized apoptosis regulators in mitochondrial-associated apoptosis (21). Bax was the first identified pro-apoptotic protein member of the Bcl-2 protein family, which is able to promote apoptosis (21). In the presence of apoptotic stimuli, Bax translocates to the mitochondria, promoting the release of cytochrome c into the cytosol, leading ultimately to apoptotic cell death (22). Bcl-2, a major anti-apoptotic protein of the Bcl-2 family inhibits cells apoptosis by protecting mitochondrial membrane integrity and blocking the release of cytochrome c (22). The Bax/Bcl-2 ratio determines whether

a cell will survive or undergo apoptosis (18). It has been reported that a reduced level of Bax and increased level of Bcl-2 affect the relapse rate of patients with ALL (21). In the present study, RT-qPCR revealed that Solanine inhibited Bcl-2 and promoted Bax mRNA expression (Fig. 3). In addition, the results of the present study indicated that Solanine increased Bax protein expression and decreased Bcl-2 protein expression in a dose-dependent manner, promoting the apoptosis of Jurkat cells (Fig. 4). Therefore, the results of the present study confirmed that Solanine regulates the expression of apoptosis-associated genes and proteins in the T-ALL Jurkat cell line.

Adriamycin is a highly effective anthracycline that is widely used in chemotherapy treatment of a wide range of cancer, including leukemia. In the present study, it was demonstrated that Solanine significantly enhanced the cytotoxicity of Adriamycin in T-ALL cells. The results indicated that Solanine may sensitize T-ALL cells to Adriamycin. The results from the present study indicated the potential of Solanine as an attractive therapeutic strategy for T-ALL.

In conclusion, the present results indicated that Solanine possesses antitumor activity in Jurkat cells. Additionally,

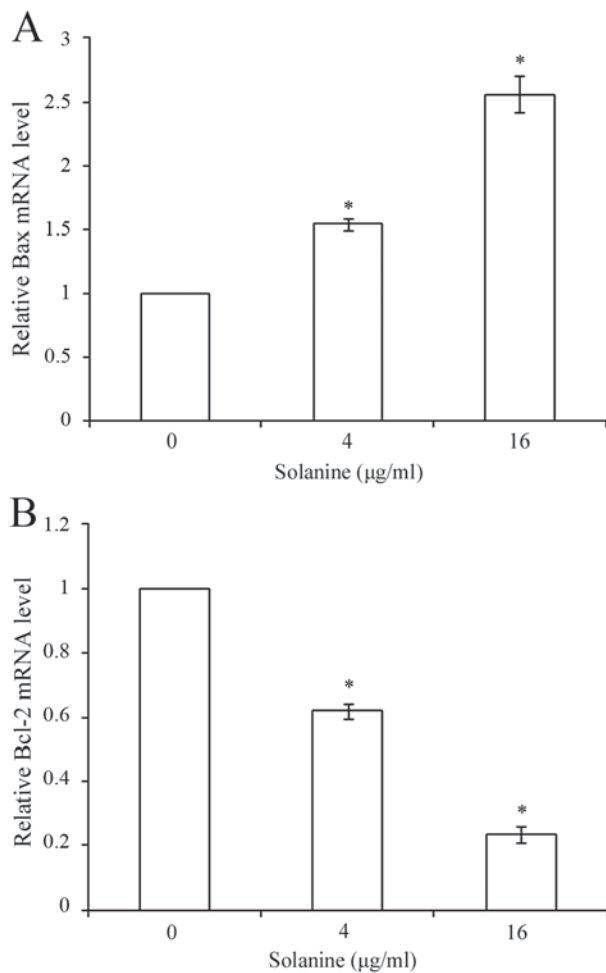


Figure 3. Effect of Solanine on (A) Bax and (B) Bcl-2 mRNA expression in Jurkat cells. The Jurkat cells were treated with different concentrations of Solanine (0, 4 and 16 µg/ml) for 24 h before Bcl-2 and Bax mRNA levels were determined by reverse transcription-quantitative polymerase chain reaction. Data are expressed as the mean \pm standard deviation of triplicate experiments. * $P < 0.05$ vs. 0 µg/ml Solanine. Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein.

the results showed that the anticancer activity of Solanine in Jurkat cells may be associated with inhibition of proliferation and induction of apoptosis by regulating the expression of apoptosis-associated genes and proteins. Solanine may significantly increase the chemosensitivity of Jurkat cells to Adriamycin. Therefore, these findings may provide a novel approach for the development of T-ALL therapy using Solanine.

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Competing interests

The authors declare that they have no competing interests.

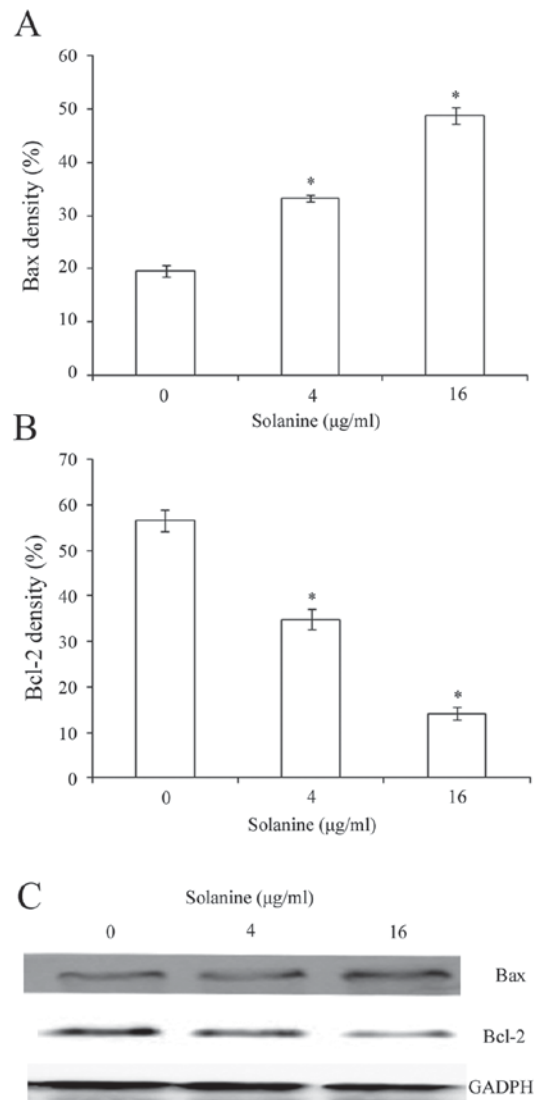


Figure 4. Effect of Solanine treatment on (A) Bax and (B) Bcl-2 expression in T-cell acute lymphoblastic leukemia Jurkat cells. Jurkat cells were treated with Solanine (0, 4 and 16 µg/ml) for 24 h. (C) Protein expression levels of Bcl-2 and Bax were measured by western blot analysis. GAPDH was used as a positive control. Data are expressed as the mean \pm standard deviation of triplicate experiments. * $P < 0.05$ vs. 0 µg/ml Solanine. Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein.

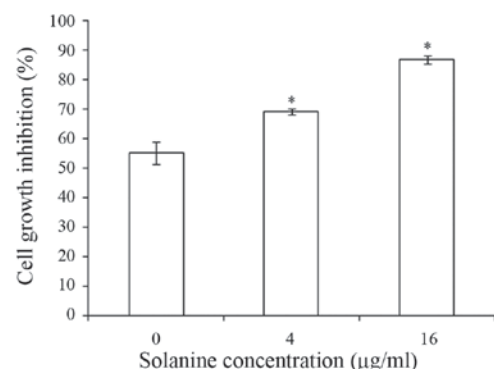


Figure 5. Effect of Solanine on the chemosensitivity of Jurkat cells to Adriamycin. Cell viability was measured by Cell Counting Kit-8 assay subsequent to treatment with Solanine in the presence of Adriamycin (2 µg/ml for 24 h). Data are expressed as the mean \pm standard deviation of triplicate experiments. * $P < 0.05$ vs. 0 µg/ml Solanine in the presence of 2 µg/ml Adriamycin.

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