Original Article
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The Cytotoxic Effects of Britannin on Acute and Chronic Myeloid Leukemia Cells Through Inducing p21-mediated Apoptotic Cell Death

Britannin'in p21 ile Apoptotik Hücre Ölümü İndüksiyonu Yoluyla Akut ve Kronik Miyeloid Lösemi Hücreleri Üzerindeki Sitotoksik Etkileri

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Abstract

Objective: Following the success of natural compounds in the treatment of solid tumors, interests in applying such agents in the treatment of hematologic malignancies have been fired up more strikingly. Thus far, the anti-leukemic effects of several compounds have been examined in different leukemia cell lines, especially in Acute Lymphoblastic Leukemia (ALL). One of the agents that have recently attracted tremendous attention is Britannin which is derived from *Inula aucheriana*, a plant that grows in Iran (Azerbaijan) and Turkey (Anatolia). In this study, we aimed to evaluate the effects of this compound in myeloid leukemia for the first time.
Materials and Methods: We treated Chronic Myeloid Leukemia (CML)-derived K562 and Acute Myeloid Leukemia (AML)-derived U937 cells with different concentrations of Britannin. We used several assays, including Trypan blue, MTT, BrdU, flow cytometry, and qRT-PCR analysis, to study the anti-leukemic effects of the compound.

Results: Our results showed that while Britannin remarkably reduced the survival of both cell lines in a concentrations-dependent manner, it had cytotoxic effects neither on mouse fibroblast-derived L929 cells nor on normal PBMCs. Moreover, among the tested cell lines, the viability of CML-derived K562 cells was inhibited at higher concentrations of the compound compared to AML-derived U937 cells. We found that Britannin induced apoptotic cell death in both cell lines via altering the expression of anti- and pro-apoptotic genes. Britannin also hampered the proliferative capacity of the cells in a p21/p27-dependent manner.
Conclusion: Overall, we suggest that based on the lack of toxicity on the normal cells and valuable anti-leukemic activities, Britannin could be a promising agent in the treatment strategies of both CML and AML. However, further investigations are required to more precisely study this compound's mechanism of action and evaluate its safety profile.

Keywords: Acute Myeloid Leukemia, Chronic Myeloid Leukemia, Britannin, Cytotoxic effects, Apoptosis

Özet
Amaç: Doğal bileşiklerin solid tümörlerin tedavisinde başarılı bir şekilde kullanılması nedeniyle, bu tür faktörlerin hematolojik malignitelerinin tedavisinde kullanılmasına olan ilgi çarpıcı biçimde artmıştır. Bugüne kadar, farklı lösemi hücre dizilerinde, özellikle akut lenfoblastik lösemide (ALL) çeşitli ekstraktların anti-lösemik etkileri araştırılmıştır. Son zamanlarda çok dikkat çeken şeylerden biri de Britannindir. Brittannin, İran (Azerbaycan) ve Türkiye'de (Anadolu) yetişen Inulaaucheriana bitkisinden elde edilir. Bu çalışmadaki amacımız bu bileşiğin miyeloid lösemi üzerindeki etkilerini ilk kez araştırmaktı.
Malzemeler ve yöntemler: Kronik miyeloid lösemiden (CML) türetilen K562 hücreleri ve akut miyeloid lösemiden (AML) türetilen U937 hücreleri, farklı Britannin konsantrasyonları ile tedavi edildi. Bu ekstraktın anti-lösemik etkilerini incelemek için tripan mavisi, MTT, BrdU, akış sitometrisi ve qRT-PCR analizi dahil olmak üzere birkaç tahlil kullandık.
Sonuçlar: Bulgularımıza göre Britannin, konsantrasyona bağlı bir şekilde her iki hücre hattının hayatta kalmasını önemli ölçüde azaltır. Ayrıca Britannin'in fare fibroblastlarından veya normal PBMC hücrelerinden türetilen L929 hücrelerinin hiçbiri üzerinde sitotoksik etkisinin olmadığı tespit edildi. Üstelik, incelenen hücre hatlarında, bu bileşiğin daha yüksek konsantrasyonlarında CML'den türetilen K562 hücrelerinin hayatta kalma oranı, AML'den türetilen U937 hücrelerine kıyasla azaltılmıştır. Britannin'in, anti-apoptotik ve pro-apoptotik genlerin ekspresyonunu değiştirerek herhangi bir hücrede apoptotik hücre ölümüne neden olduğunu bulmuş olduk. Britannin ayrıca bu hücrelerin çoğalma kapasitesini p21/p27'ye bağlı bir şekilde engelledi.
Sonuç: Genel olarak Britannin, normal hücrelere toksik olmaması ve değerli anti-lösemik aktiviteleri nedeniyle KML ve AML tedavilerinde umut verici bir faktör olarak kabul edilebilir. Bununla birlikte, bu bileşiğin etki mekanizmasını daha kesin bir şekilde incelemek ve güvenlik profilini değerlendirmek için daha fazla araştırma gereklidir.
Anahtar sözcükler: Akut miyeloid lösemi, Kronik miyeloid lösemi, Britannin, Sitotoksik etkiler, Apoptoz

Introduction

For a long time, herbal medicines have catered to the basic needs of humans to treat a wide range of diseases such as cancer [1]. The success of *Vinca* alkaloids, a group of drugs isolated from Periwinkle plant *Catharanthus roseus,* in the treatment of hematologic malignancies – indeed– has begun a new era in terms of cancer treatment strategies [2]. Thus far, many compounds that have been provided from natural sources have found their way into the treatment of different human cancers [3]. Curcumin [4], Trabectedin [5], and Resveratrol [6]

are all-natural compounds that recently enjoyed unprecedented success in the treatment of human cancers. Ergolid is another natural compound that has been isolated from *Inula oculus christi*, which showed anti-leukemic effects in Acute Lymphoblastic Leukemia (ALL)-derived cell lines. Also, *Artemisia annua* extract has been reported to not only induce caspase-3-dependent apoptosis in ALL cells but also to increase the sensitivity of the leukemic cells to chemotherapeutic drugs [7]. *Bryonia aspera* extract has also been reported to have anti-proliferative and pro-apoptotic effects in ALL-derived Nalm-6 and REH cells [8]. Amid the burgeoning success of these reports and the astonishing roles of natural compounds in treating hematologic malignancies, there is a consensus that natural compounds could probably change the paradigm of treatment in human leukemia. Recently, promising results have been published about the cytotoxic effects of Britannin, a Sesquiterpene Lactone (SL), which is derived from *Inula aucheriana* DC, a plant that grows in Iran (Azerbaijan) and Turkey (Anatolia) [9-11].

It has been declared that apart from the anti-inflammatory effects, this is the presence of α-methylene-γ-lactone in sesquiterpene lactones that endows their ability to induce cell death in cancer cells [12]. For example, in breast cancer, Hamzeloo-Moghadam et al. have reported that low concentrations of Britannin reduced the viability of MCF-7 cells through suppression of cyclin D1 and CDK4 protein [13]. In another study, this compound has exerted cytotoxic effects in hepatocarcinoma, breast cancer, and lung cancer [9]. Cui YQ et al. also suggested that Britannin induced apoptotic cell death in liver cancer cells [14]. In a most recent study, Mohammadlou et al. reported the anti-leukemic effects of Britannin in acute lymphoblastic leukemia (ALL) cells. The authors suggested that this compound induced G1 cell cycle arrest and increased reactive oxygen species' intracellular levels in ALL cell lines [15, 16]. Although many studies have been focused on the anti-cancer effects of Britannin, as far as we are aware, no study has evaluated the effects of this compound on Myeloid Leukemia cells, and our study aimed to examine the anti-leukemic effect of Britannin in Chronic Myeloid Leukemia (CML)-derived K562 and Acute Myeloid Leukemia (AML)-derivedU937 cells for the first time.

Materials and Methods

Britannin isolation

Inula aucheriana DC was collected, and the scientific name was confirmed at the Herbarium of Traditional Medicine and Materia Medica Research Center (TMRC), Shahid Beheshti University of Medical Science (voucher number: TMRC 3173). To obtain Britannin, 600 g of *I. aucheriana* dried powder was soaked with n-hexane (plant: solvent ratio,1:10) for three days. After filtration, the same process was repeated with chloroform with the residue of the plant. Seven g of the dried chloroform extract was set to vacuum liquid chromatography (VLC) (silica gel 40-63 µm; mobile phase: ethyl acetate and methanol); 400 mg of the fractions eluted with EtOAc-MeOH (2:1) was further processed by solid-phase extraction (SPE) (2.5 ×7.5 cm silica gel 40-63 µm; mobile phase: dichloromethane, ethyl acetate, and methanol) to afford Britannin (70 mg).

Cell culture

To evaluate the effect of the Britannin on Acute Myeloid Leukemia (AML) and Chronic Myeloid Leukemia (CML)-derived cell lines, we cultured U937 and K562 cells in RPMI1640 medium containing 10% fetal bovine serum, 50 mg/ml Streptomycin, and 30 mg/ml Penicillin in a humidified incubator, respectively. We treated the cells with different concentrations of the compound (0-10 µM) for 24 and 48 h. We also cultured L929 cells, a

non-cancerous cell line derived from mouse fibroblasts, and peripheral mononuclear cells (PBMCs) as a control to determine the safety of the compound.

Trypan blue assay

After incubating U937 and K562 cells with different concentrations of Britannin (0, 3, 5, and 10 μM) for 24 and 48 h, we stained the drug-treated cells with Trypan blue dye. After remaining for 2-3 min at room temperature, the number of viable cells was counted manually using a light microscope.

MTT assay

MTT assay was used to evaluate whether Britannin could reduce the metabolic activity of the leukemic cells. Drug-treated leukemic cells were exposed to 100 μL MTT solution (0.5 mg/ml) and were incubated for 4 h. After centrifugation and discarding the medium, 100 μL DMSO was added to each well, and then the absorbance of each sample was measured at 570 nm by an ELISA reader.

Annexin-V/PI staining assay

Annexin V-FITC staining was used to assess the effect of Britannin on the induction of apoptosis in leukemic cells. Both cell lines were treated with desired concentrations of the compound, and after 48 h, 5 μL of Annexin V-FITC dye was added to each sample. Leukemic cells were incubated in the dark for 15 min, and the induction of apoptosis was evaluated using BD FACS Calibur (BD biosciences, SanJose, CA, USA). Flowjo 7.6 software was used for data analysis.

Gene expression analysis

To study the alterations in gene expression, the first RNA was extracted from drug-treated leukemic cells using Trizol reagent. After evaluating the quality of the extracted RNAs by Nanodrop, cDNA was synthesized by TAKARA kit (Japan). Real-time PCR was performed using SYBR Green PCR. ABL was the housekeeping gene in the present study. The primers used to evaluate the expression of genes are shown in Table 1.

Statistical Analysis

One-way ANOVA and post hoc Tukey multiple comparisons were used for data analysis. All analyses were performed using SPSS software version 22. A *P*-value <0.05 was considered to be statistically significant.

Results

The effect of Britannin on the viability and the metabolic activity of U937 and K562 cells

To evaluate whether Britannin could reduce the population of leukemic cells, two myeloid leukemia cell lines, U937 and K562, were treated with increasing concentrations of the compound. The results of the Trypan blue assay showed that while Britannin reduced the number of U937 cells in a concentrations-dependent manner, the anti-leukemic effects of this compound on K562 cells were exerted at the concentration of 10 μM (Fig. 1A). Moreover, to confirm the anti-leukemic effects of Britannin on myeloid leukemia cell lines, we also evaluated the effect of the compound on the metabolic activity of the cells. MTT assay showed that Britannin significantly reduced the metabolic activity of both U937 and K562 cells (Fig. 1B). In agreement with the results of the Trypan blue exclusion, the anti-leukemic effects of Britannin were more potent in U937 cells, as the concentration of 5 μM of this

compound diminished the metabolic activity of U937 cells by almost 50%. However, the significant anti-cancer effect of Britannin on K562 cells was observed at the concentration of 10 μM (Fig. 1B). We also found that U937 cells with an IC_{50} value of 3.9 μM were more sensitive to Britannin as compared to K562 with an IC_{50} value of 8.1 μM (Fig. 1A and B). To check whether the compound at the mentioned concentrations had cytotoxic effects on normal cells, non-cancerous L929 cells and PBMCs were treated with increasing concentrations of the compound (3, 5, and 10 μM). As presented in Fig. 1C, Britannin had minimal effects on the viability of both PBMCs and L929 cells, suggestive of the safety of the compound. To better study the anti-leukemic effects of Britannin on leukemic cell lines, we decided to treated K562 cells with 5, 7, and 9 μM concentrations of the compound and U937 cells with 3, 5, and 7 μM of Britannin for further analysis.

The effects of Britannin on the proliferation of the leukemic cells

Having established that Britannin has anti-leukemic effects on K562 and U937 cells, we aimed to evaluate the effect of the compound on the proliferation of the cells. We treated K562 and U937 cells with different concentrations of the compound (0-9 μM), and then the DNA synthesis rate was determined using BrdU assay. Our results showed that Britannin could reduce the capacity of the leukemic cells to replicate DNA (Fig. 2A). In agreement with this finding, we also found that Britannin could reduce the expression of both p21 and p27 in both cell lines (Fig. 2B).

Britannin induced apoptotic cell death in K562 and U937 cells

To determine whether treatment of cell lines with Britannin could induce apoptotic cell death, we treated both cell lines with different concentrations of the compound (0-9 μM), and then the binding of annexin-V combined with PI was analyzed by flow cytometry. As presented in Fig. 3A and B, we found a significant elevation in the percentage of annexin-V and annexin-V/PI double-positive cells compared to the control group. In agreement with the MTT and Trypan blue assays results, U937 cells were more sensitive to Britannin than K562 cells. Moreover, to confirm these results, we also evaluated the effect of the compound on the expression of both anti- and pro-apoptotic genes. The results of the qRT-PCR analysis showed that while Britannin could remarkably increase the expression levels of pro-apoptotic genes, including Bax, Bad, and PUMA, it could reduce the expression of anti-apoptotic genes such as Bcl2, Bcl-xl, and MCL-1 (Fig. 4).

Discussion

From the first description of human leukemia until now, efforts were made to find a proper treatment for this type of disease. However, the heterogeneity between different sub-types of leukemia, whether they are categorized as chronic leukemia or acute leukemia, did not lead to the development of one single treatment regimen for both diseases [17]. For example, while Chronic Myeloid Leukemia (CML) takes advantage of tyrosine kinase inhibitors such as imatinib for the treatment [18], Acute Myeloid Leukemia (AML) is mainly treated with vincristine, daunorubicin, and other conventional chemotherapeutic drugs [19]. Even targeted therapies also failed to introduce one specific small-molecule inhibitor to treat both acute and chronic myeloid leukemia [17]. Another challenge of the current treatment of human

leukemia is the incidence of severe side effects for the patients, which eventually restrict the clinical dosage of the agents, an event that leads to the disease relapse [20, 21]. The stream of efforts for finding new agents with more potent anti-leukemic effects and lower side effects is on the way. Attention has also been attracted to herbal medicine, as many of the drugs isolated from the plants showed to be safe and well-tolerated in clinical investigations [22]. In this study, we evaluated the effect of Britannin, a compound isolated from *Inula aucheriana,* on two myeloid leukemia cell lines, K562 and U937, which are derived from CML and AML patients, respectively.

Interestingly, our results showed that Britannin remarkably reduced the number of viable cells, cell growth, and the metabolic activity of U937 cells in a concentrations-dependent manner. However, for K562 cells, the significant anti-leukemic effect of the compound was obtained a higher concentration (10 μM). This finding suggested that as compared to U937 cells, CML-derived K562 cells were more resistant to the lower concentrations of Britannin. Our results also showed that this single compound at the same concentrations had neither cytotoxic effects on L929 cells nor normal PBMCs.

It should be noted that among the tested cell lines, we found that the viability of CML-derived K562 cells was inhibited at higher concentrations of the drug, as compared to AML-derived U937 cells. This difference in cell sensitivity could probably be due to the excessive activation of the PI3K/Akt signaling pathway in K562 cells as a result of the presence of BCR-ABL [23]. It has been well-established that the PI3K/Akt activity could induce the drug-resistance in CML cells [24, 25]. However, Britannin reduced the survival of K562 cells at concentrations that had no toxic effects on the normal cells.

Having established the anti-leukemic activity of Britannin on both leukemic cell lines, we also examined the mechanism through which this compound might induce its effects. Our results showed that Britannin reduced the capability of the leukemic cells to replicate DNA. The results of the BrdU assay revealed that in the presence of Britannin, there was a significant decrease in the DNA synthesis rate in both K562 and U937 cells, which in turn hampered the ability of the cells to proliferate. Likewise, Britannin reduced the number of viable cells in a concentration-dependent manner in both cell lines. In agreement with our finding, Zhang et al. also indicated that Britannin could reduce the proliferation of cancer cells [26]. It has been indicated that Britannin interacts with c-Myc and thereby hamper the proliferative activity of PDL-1 positive T cells in the tumor site, an event which in turn prevents the growth and metastasis of cancer cells [26]. The proliferation of leukemic cells could be regulated via the alteration in the genes controlling the progression of the cell cycle. Thus far, several cell-cycle regulatory genes such as p21 and p27 have been identified [27]. Moreover, the results of a previous study indicated that Britannin could reduce the expression of cyclin D1 and CDK4, two other cell-cycle regulatory genes in breast cancer cells [13]. In line with these results, we also found that Britannin increased the expression levels of both p21 and p27 in leukemic cell lines, suggesting that the anti-proliferative capacity of Britannin in K562 and U937 cells are mediated, at least in part, through altering the expression of the genes controlling the distribution of the cells in the cell cycle. Apart from regulating the progression of the cells from the G1 phase of the cell cycle to the S phase, p21 could also increase the expression of pro-apoptotic genes such as Bax in the cells [28]. It has also been declared that elevation in the expression of p21 could increase the sensitivity of cancer cells to apoptosis via increasing the expression of p53-related genes [29]. In another study, Hastak et al. reported that ablation of p21 in prostate cancer cells prevented epigallocatechin-3-gallate, a polyphenolic compound of green tea, induced apoptotic cell death [30]. In agreement with the up-regulation of p21, we found that not only Britannin increased the expression of p53-dependent pro-apoptotic genes but also diminished the expression of anti-apoptotic genes such as Bcl2, MCL-1, and BCL-xl. Moreover, this compound significantly

increased the population of apoptotic cells in both K562 and U937 cells. Cui et al. also showed that this compound hampered the viability of liver cancer cells via elevating the intracellular concentrations of Reactive Oxygen Specious (ROS), which eventually led to the induction of apoptotic cell death [14]. As the most straightforward interpretation of our results, we suggested that probably Britannin reduced the survival and proliferative capacity of myeloid leukemia cells via p21-mediated suppression of DNA synthesis and induction of apoptotic cell death.

Conclusion

Taken together, the results of the present study indicated that based on minimal toxicity on the normal cells and valuable anti-leukemic activities, Britannin could be introduced in the treatment strategies of leukemia, whether Chronic Myeloid Leukemia or Acute Myeloid Leukemia. However, further investigations are required to more precisely study the mechanism of action of the compound and also to evaluate its safety profile in a xenograft model.

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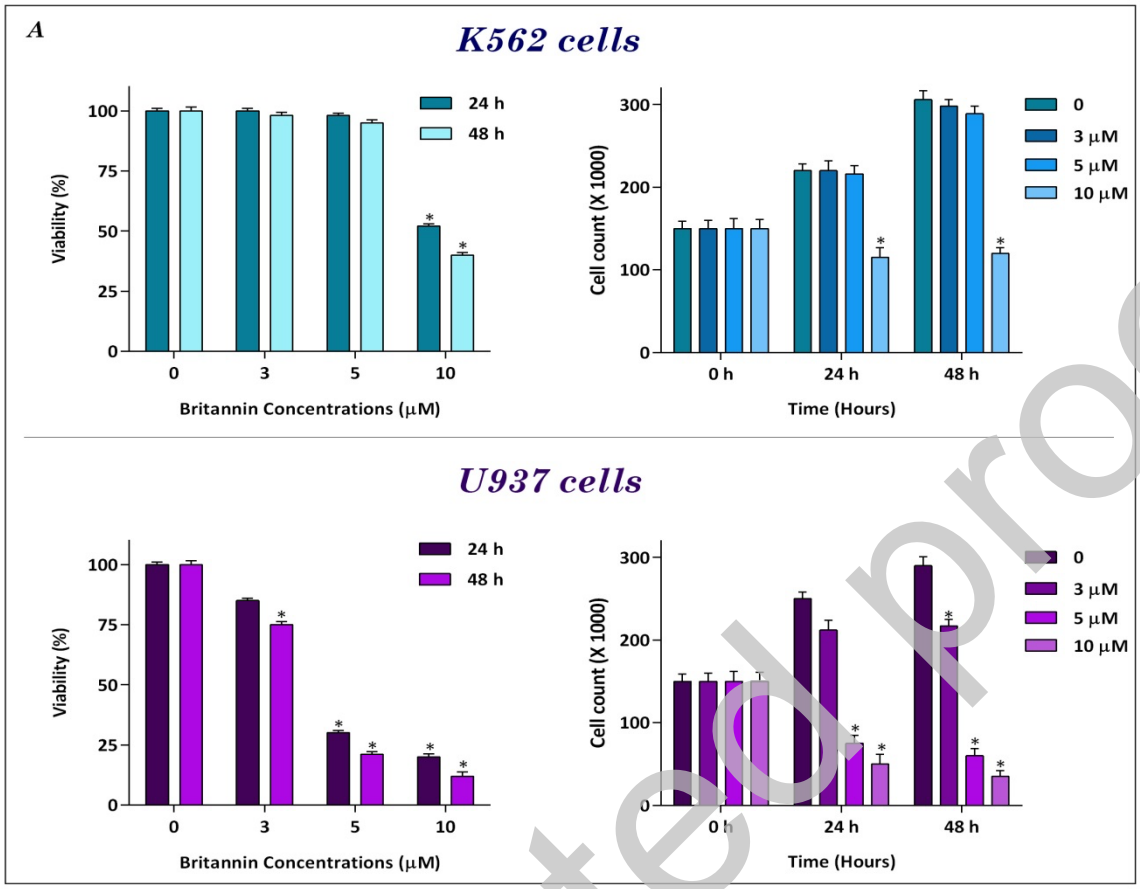
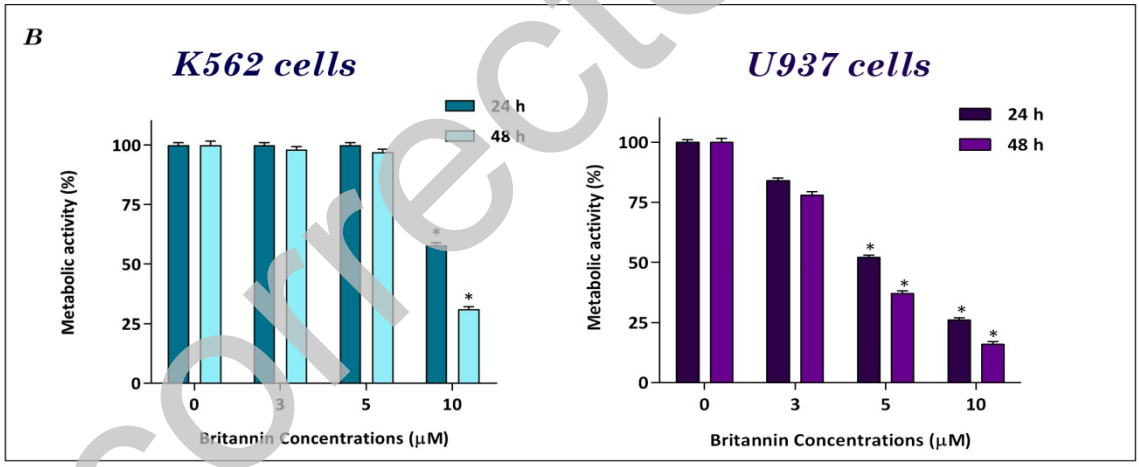
Table 1. Nucleotide sequences of primers used for real-time RT-PCR.

Gene	Accession number	Forward primer (5′-3′)	Reverse primer (5′-3′)	Size (bp)
ABL	NM_005157	AGTCTCAGGATGCAGGTGCT	TAGGCTGGGGCTTTTTGTAA	290
p21	NM_000389	CCTGTCACTGTCTTGTACCCT	GCGTTTGGAGTGGTAGAAATCT	130
p27	NM_004064	AACGTGCGAGTGTCTAACGG	CCCTCTAGGGGTTTGTGATTCT	139
Bax	NM_138761	CGAGAGGTCTTTTTCCGAGTG	GTGGGCGTCCCAAAGTAGG	242
PUMA	NM_014417	GACCTCAACGCACAGTACGAG	AGGAGTCCCATGATGAGATTGT	98
Bad	NM_004322	CCCAGAGTTTGAGCCGAGTG	CCCATCCCTTCGTCGTCCT	249
Bcl-2	NM_000633	CGGTGGGGTCATGTGTGTG	CGGTTCAGGTACTCAGTCATCC	90
MCL-1	NM_021960	AGAAAGCTGCATCGAACCAT	CCAGCTCCTACTCCAGCAAC	183
BCL-xl	NM_138578	GAGCTGGTGGTTGACTTTCTC	TCCATCTCCGATTCAGTCCCT	119





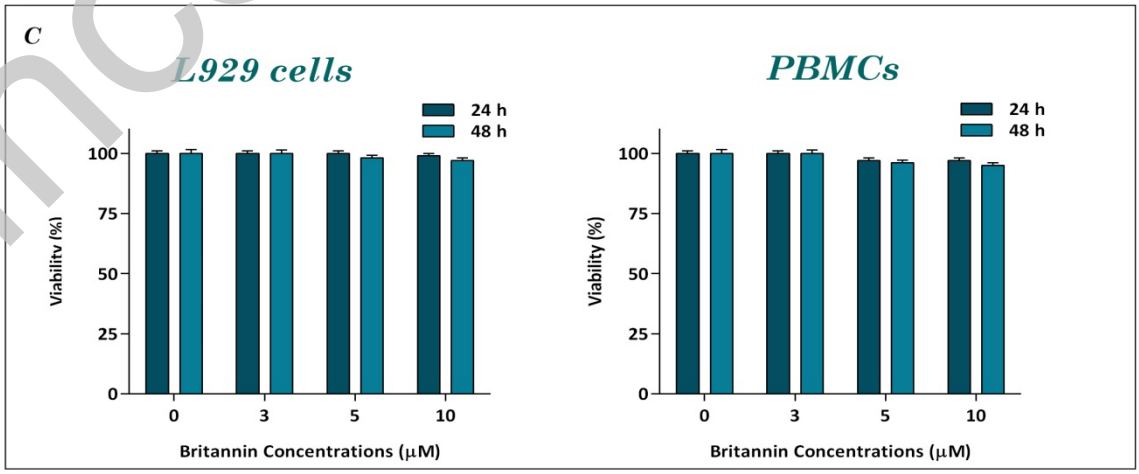


Fig.1 The effect of Britannin on the viability and the metabolic activity of K562 and U937 cells. A) Britannin concentrations of 3, 5, and 10 µM significantly reduced the survival and the number of viable cells in both leukemic cell lines. **B)** The results of the MTT assay revealed that Britannin could hamper the metabolic activity of the cells in a concentrations-dependent manner. **C)** Britannin at the concentrations of 3, 5, and 10 µM had toxic effects neither on L929 cells nor on PBMCs. Values are given as mean± S.D. of three independent experiments. *, $P \leq 0.05$ represents significant changes from untreated control.

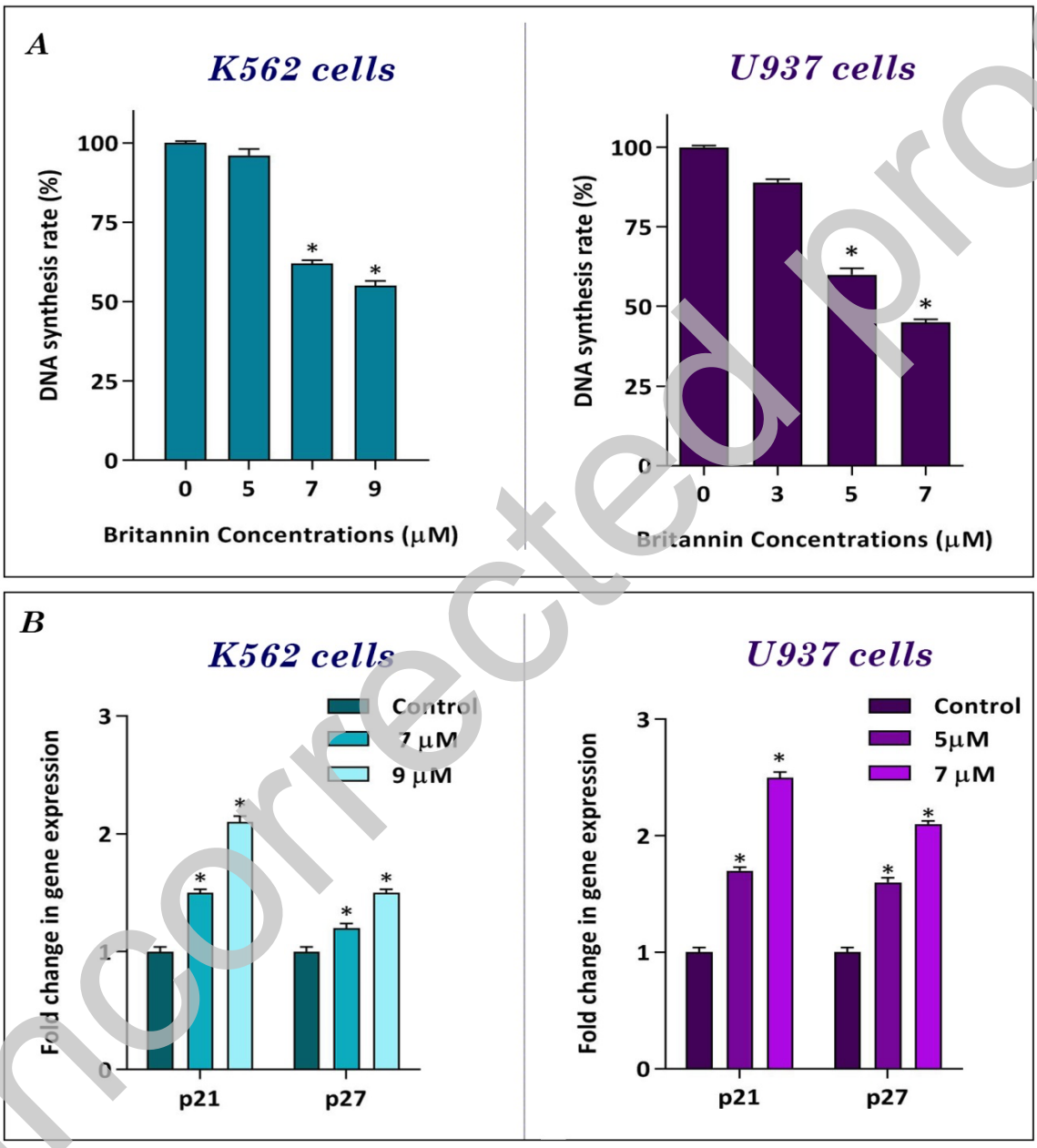


Fig. 2. The anti-proliferative effects of Britannin on K562 and U937 cells. A) Treatment of K562 and U937 cells with increasing concentrations of Britannin led to the decrease in the DNA synthesis rate of the cells. **B)** Britannin significantly increased the expression of p21 and p27 in both cell lines. Values are given as mean± S.D. of three independent experiments. *, $P \leq 0.05$ represents significant changes from untreated control.

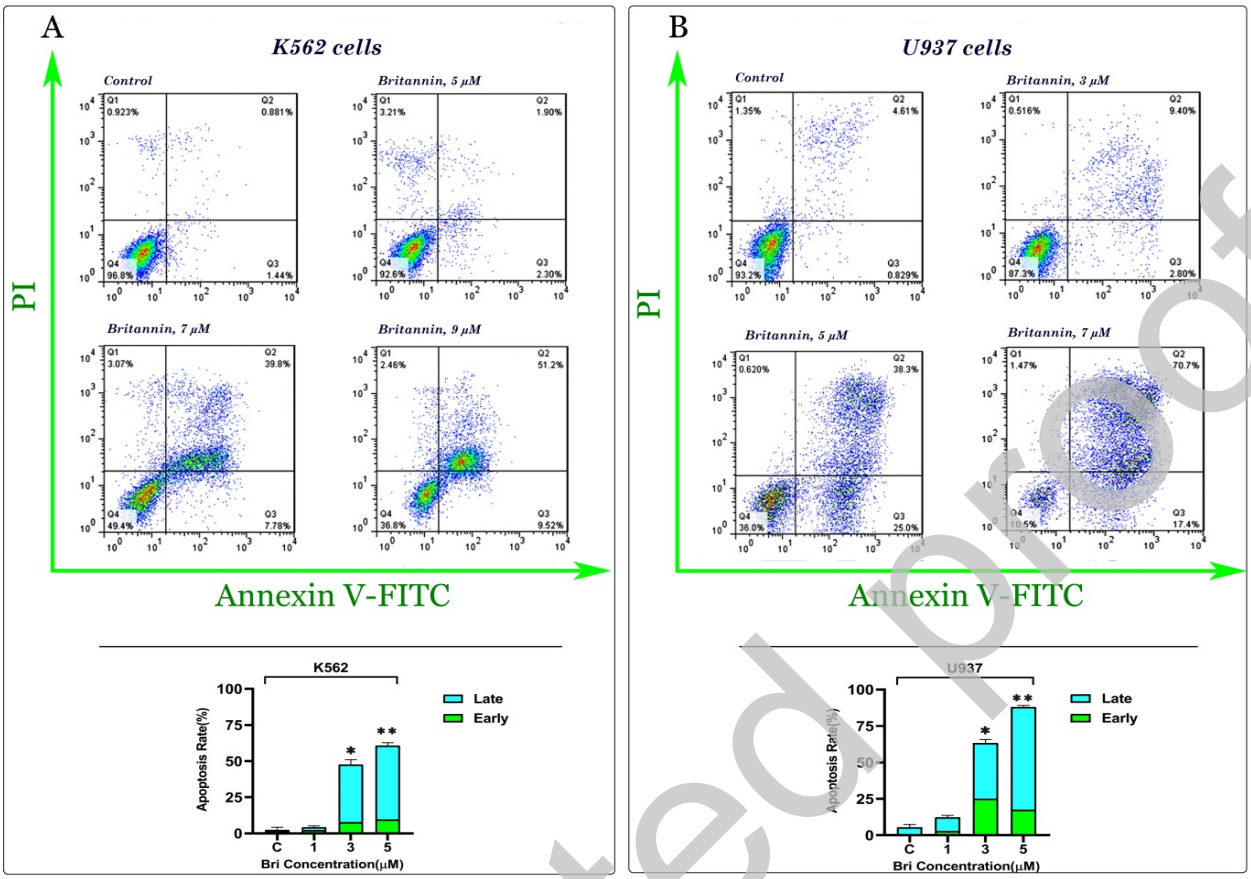


Fig. 3 Britannin induced apoptotic cell death in leukemic cells. A and B) Britannin elevated the percentage of both annexin-V and annexin-V/PI double-positive cells in K562 and U937 cells. Values are given as mean± S.D. of three independent experiments. *, P ≤ 0.05 represents significant changes from untreated control.

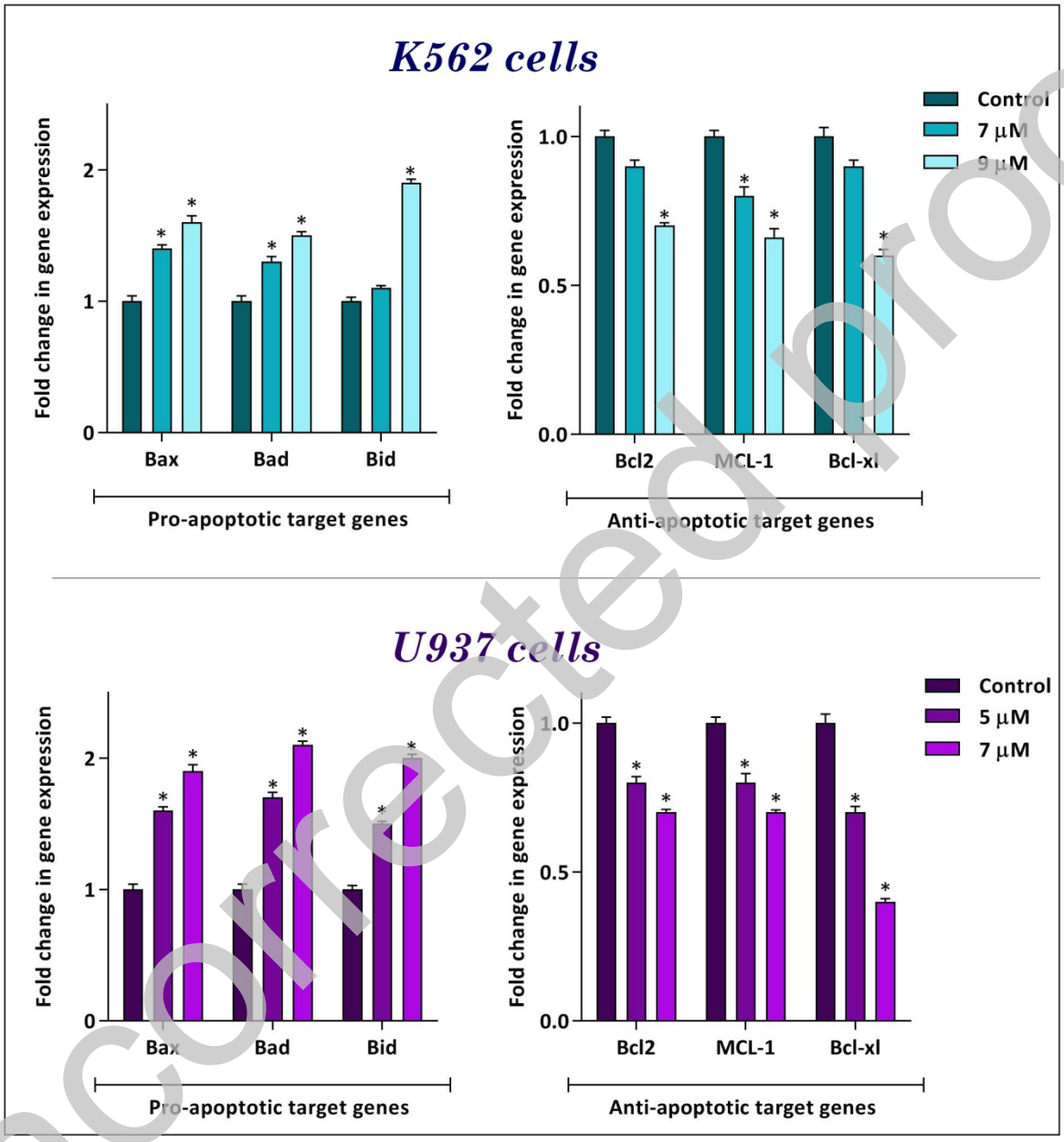


Fig. 4 Britannin altered the expression of pro-and anti-apoptotic genes in K562 and U937 cells. The results of the qRT-PCR analysis showed that while Britannin elevated the expression of pro-apoptotic target genes, this compound diminished the expression of anti-apoptotic target genes in both cell lines. Values are given as mean± S.D. of three independent experiments. *, P ≤ 0.05 represents significant changes from untreated control.