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*Original Article*

# Salidroside induces apoptosis via ERK1/2 in human leukemia K562 cells

Ying-Lan Jin, Xiao-Min Zhang, Xi Chen, Juan Liu, Yu-Ying Chang, Xin-Yu Gao, Yan-Ming Xue, Xiu-Shuai Dong, Yao Liu, Yao-Yao Tian, Li-Yan Chen, Ying Wang, Jing-Hua Wang

*Department of Hematology, The Second Affiliated Hospital, Harbin Medical University, 246 Xuefu Road, Nangang District, Harbin 150086, China*

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**Abstract:** Salidroside, a phenylpropanoid glycoside present in all species of *Rhodiola* genus, shows a broad spectrum of pharmacological properties. Here we investigated the effects of salidroside on the viability, cell cycle, apoptosis and its possible molecular mechanisms. Cell viability assay was used to evaluate the cytotoxic effects of salidroside on human leukemia K562 cells, and flow cytometry analyzed the change of cell cycle distribution, cell apoptosis, MMP level and ROS generation induced by salidroside. Western blotting further studied the expression changes of PCNA, CyclinD1, Bax, Bcl-2 and caspase-3, and the activation of ERK1/2 was also detected. We found that salidroside inhibited the growth of K562 cells in dose- and time-dependent manners, caused G2-M phase arrest and induced apoptosis via mitochondrial pathway. Salidroside could result in a decrease of PCNA, CyclinD1 and Bcl-2, and upregulated the levels of Bax and caspase-3 as well as inactivation of ERK1/2. In conclusion, these results suggest that salidroside inhibits cell viability and induces cell cycle arrest and apoptosis via inactivation of ERK1/2 signaling pathway in human leukemia K562 cells and may be a promising candidate for leukemia treatment.

**Keywords:** Leukemia, salidroside, apoptosis, ERK1/2

## Introduction

Salidroside, one of the most potent ingredients extracted from plants of the *Rhodiola* genus, has been reported to possess various pharmacological properties including neuroprotective, cardiovascular protective, anti-inflammatory, and antioxidative effects [1-3]. Recent studies have shown that salidroside inhibits the proliferation of various cancer cells including bladder, hepatocellular, lung and gastric cancer [4, 5]. Salidroside induced the G2-M phase arrest related to the downregulation of CyclinB1 and CDC2 and increases of p21 and p27 in MDA-MB-231 breast cells, and induced apoptosis via upregulating the ration of Bax/Bcl-2 and cleaved caspase-9 in a dose-dependent manner [6]. Moreover, salidroside caused elevation of cell survival and decreased the production of reactive oxygen species (ROS) and osteoclast differentiation inducing factors induced by $H_2O_2$ in bone loss mouse model [7]. However, no report has been issued on the interrelation of salidroside with leukemia progression.

Apoptosis has been described as a highly regulated process, in which caspases are involved in both commitment and execution phases, resulting in the cleavage of specific substrate proteins. The characteristics of apoptosis include DNA fragmentation, chromatin condensation and loss of mitochondrial membrane potential (MMP) [8, 9]. The control of apoptosis is governed by the Bcl-2 family proteins, which include antiapoptotic Bcl-2 and proapoptotic Bax [10]. The activation of caspase-3 is also required for apoptosis, either by the caspase-3 or caspase-9 pathway [11]. Fucoxanthin, a natural biologically active substance isolated from Ishige okamurae, induces apoptosis in human leukemia HL-60 cells through a ROS-mediated Bcl-xL pathway [12]. Annona muricata leaves induces apoptosis in A549 cells through mitochondrial-mediated pathway and involvement of NF-κB [13].

Activation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) has been shown to inhibit apoptosis in response to a wide range of stimuli, such as tumor necrosis factor (TNF), hypoxia, nitric oxide (NO), hydrogen peroxide ($H_2O_2$), and chemotherapeutic agents [14-16]. In the mammalian cell lines, ERK1/2 signaling can block apoptosis at levels upstream, down-

stream, or unrelated to change of mitochondrial transmembrane potential and cytochrome c release [17]. Activation of ERK1/2 by constitutive expression of MEK1 inhibits caspase-8 activation and the loss of MMP induced by activation of TNF [18].

Defective cell cycling and apoptotic mechanism are considered to play a role in resistance to therapeutic drugs. In this study, we investigate the effects of salidroside on cell viability, cycle and apoptosis in human leukemia K562 cells. The results demonstrated that salidroside inhibits viability via blocking cell cycle progression at G2-M phase and subsequently progression to apoptosis. The underlying events relevant to mitochondria were studied in detail, including the alteration of MMP and the generation of ROS. The expression of cell cycle- and apoptosis-related molecules as well as the ERK1/2 activity was also investigated.

## Materials and methods

### *Cell culture*

Human leukemia K562 cell line was obtained from Shanghai Institute of Cell Biology (Shanghai, China). Cells were cultured in RPMI-1640 medium with 10% fetal calf serum (FBS, Gibco BRL, Rockville, MD, USA), 100 U/ml penicillin G and 100 μg/ml streptomycin in an incubator (37°C, 100% humidity and 5% $CO_2$).

### *Cell viability assay*

Cell viability was measured by a Cell Titer 96 AQueous One Solution Cell Proliferation Assay (Promega, Beijing, China). Briefly, K562 cells were treated with or without salidroside (5, 10, 20, 30 and 40 mg/ml) at a density of $5\times10^3$ cells per well into a 6-well plate. Cell viability was examined at 0, 6, 12, 24, 48 and 72 h after seeding. Culture medium, 100 ml, was added together with 20 ml of Cell Titer 96 AQueous One Solution and then incubated at 37°C for 2 h. Optical density was measured at 490 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

### *Cell cycle assay*

Cell cycle was measured by propidium iodide (PI; Sigma-Aldrich, St. Louis, MO, USA) staining and flow cytometer (BD Biosciences, San Diego, CA, USA). Briefly, K562 cells were treated with or without salidroside (10, 20 and 30 mg/ml) for 24 h and were washed once with PBS, resuspended in 500 ml hypotonic staining buffer (sodium citrate 250 mg, Triton X 0.75 ml, PI 25 mg, ribonuclease A 5 mg, and 250 ml water), and analyzed by flow cytometry.

### *Cell apoptosis assay*

Cell apoptosis was measured by annexin V-FITC/PI staining and flow cytometer (BD Biosciences, San Diego, CA, USA). Briefly, K562 cells were treated with or without salidroside (10, 20 and 30 mg/ml) for 24 h and were collected and re-suspended in 195 μl annexin V-FITC and 5 μl PI, followed by incubation for 5 min at 4°C in the dark. Analysis was immediately performed using a flow cytometer.

### *Measurement of ROS and MMP*

ROS detection was performed by flow cytometry analysis as described previously. Briefly, K562 cells were treated with or without salidroside (10, 20 and 30 mg/ml) for 24 h and were washed with PBS and resuspended in 50 μM Dihydroethidium (DHE), followed by incubation for 5 min at 4°C in the dark. ROS fluorescence intensity was determined by cytometry with excitation at 490 nm and emission at 590 nm. Tetramethyl rhodamine methyl ester (TMRM) dye (Sigma) was used to detect the changes in MMP. Briefly, K562 cells were treated with or without salidroside (10, 20 and 30 mg/ml) for 24 h and were washed with PBS and resuspended in 100 nM TMRM, followed by incubation for 15 min at 37°C in the dark.

### *Western blot assay*

Total protein was extracted from K562 cells treated with or without salidroside (10, 20 and 30 mg/ml) for 3 h and 24 h using radioimmunoprecipitation buffer (JRDUN Biotechnology Co., Ltd. Shanghai, China). The protein concentration was assessed using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific). 40 μg protein lysates was separated by 10-15% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Sigma-Aldrich), followed by incubated with primary antibodies against PCNA (1:1000), CyclinB1 (1:1000), Bax (1:300), Bcl-2 (1:400), caspase-3 (1:500), ERK1/2 (1:1000), p-ERK1/2 (1:1000) and GAPDH (1:1500), respectively, and incubated with secondary antibody labeled with horseradish peroxidase (1:1000). The blots were developed using enhanced chemiluminescence (GE Hea-

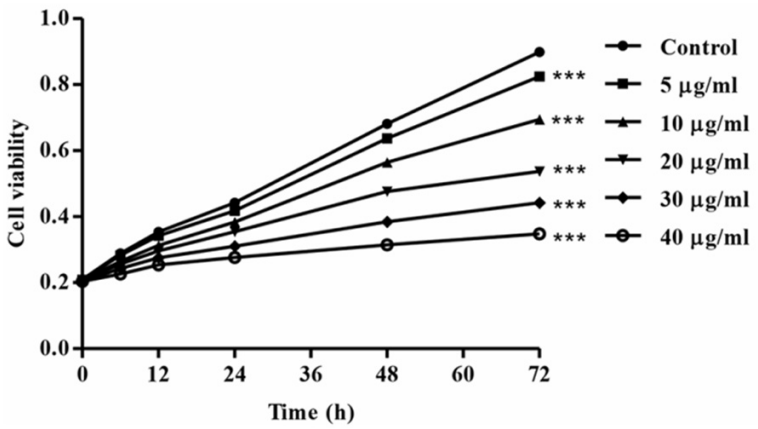


**Figure 1.** Effects of salidroside on the viability of K562 cells. Cell viability was measured by the Cell Titer 96 AQueous One Solution Cell Proliferation Assay. Salidroside (5, 10, 20, 30 and 40 μg/ml) significantly inhibited K562 cells viability in a time- and dose-dependent manner when compared with the control group. $^{***}P < 0.001$ compared with control.

lthcare, Chalfont St. Giles, Buckinghamshire, UK) and determined using Image J software (National Institutes of Health, Bethesda, MD, USA).

*Statistics*

All experiments were performed repeatedly in three times. The data are expressed as the mean ± SD, and samples were evaluated by the unpaired two-tailed Student's t-test using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). The limit of significance for all analyses was defined as a *P* value of 0.05.

## Results

*Salidroside inhibits cell viability of K562 cells*

Cell Titer 96 AQueous One Solution Cell Proliferation Assay was performed to determine the cell viability of K562 cells under the influence of salidroside. Salidroside (5, 10, 20, 30 and 40 μg/ml) were added to the culture medium for 0, 6, 12, 24, 48 and 72 h. The result showed that salidroside inhibited K562 cells viability in a time- and dose-dependent manner (**Figure 1**, n = 3). The doses of 10, 20 and 30 μg/ml were determined to carry out further investigations.

*Salidroside induces cell cycle arrest of K562 cells*

Propidium iodide (PI) stain assay and flow cytometry analysis were carried out to substantiate cell cycle induced by salidroside treatment under various concentrations. As shown in **Figure 2A** and **2B**, the percentage of G0-G1 phase cells in the cell line was decreased in a dose-dependent manner from 42.27% ± 1.19% to 32.15% ± 1.89% (n = 3) compared with control cells at 57.52% ± 2.18%. The percentage of K562 cells in S phase was also decreased particularly when treated with 20 and 30 μg/ml salidroside for 24 h. However the percentage of G2-M phase cells in the cell line increased after treatment with 10, 20 and 30 μg/ml salidroside, respectively, from 31.82% ± 2.78% to 46.93% ± 3.69% (n = 3) compared with control cells at 14.79% ± 1.54%. These results suggest that salidroside inhibits cell viability via inducing G2-M phase cell cycle arrest in K562 cells.

*Salidroside induces cell apoptosis of K562 cells*

An annexin-V fluorescein isothiocyanate (FITC)/propidium iodide (PI) double stain assay and flow cytometry analysis were carried out to substantiate cell apoptosis induced by salidroside treatment under various concentrations. The number of apoptotic cells was counted as late apoptotic cells shown in the upper right quadrant and early apoptotic cells as shown in lower right quadrant of the histograms. As shown in **Figure 3A** and **3B**, treatment of salidroside at the dose of 10, 20 and 30 μg/ml for 24 h significantly increased the number of early apoptotic cells, respectively, from 12.33% ± 1.30% to 35.30% ± 2.78% (n = 3) in a dose-dependent manner compared with control cells with that of 2.30% ± 0.89%. The significant induction of apoptosis indicated the anticancer effect of salidroside against K562 cells.

*Salidroside induces apoptosis in the mitochondrial pathway*

Loss of MMP is related to the mitochondrial apoptotic pathway. To assess the effect of salidroside on the changes of MMP in K562 cells, flow cytometry analysis was carried out to detect the fluorescence intensity of TMRM dye.

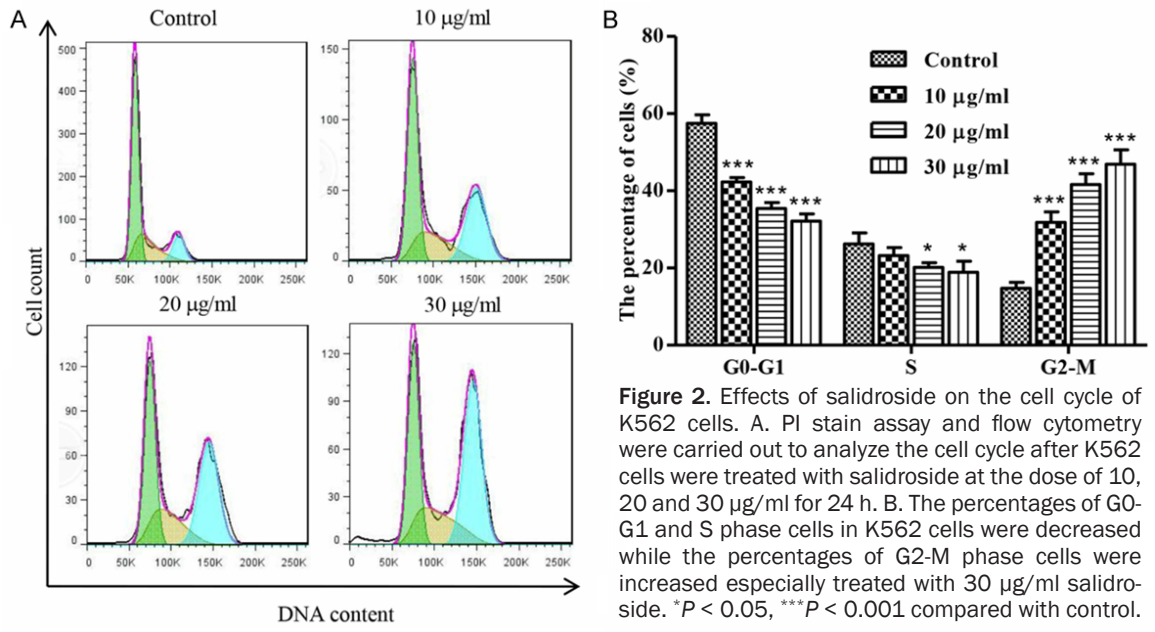


**Figure 2.** Effects of salidroside on the cell cycle of K562 cells. A. PI stain assay and flow cytometry were carried out to analyze the cell cycle after K562 cells were treated with salidroside at the dose of 10, 20 and 30 μg/ml for 24 h. B. The percentages of G0-G1 and S phase cells in K562 cells were decreased while the percentages of G2-M phase cells were increased especially treated with 30 μg/ml salidroside. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with control.

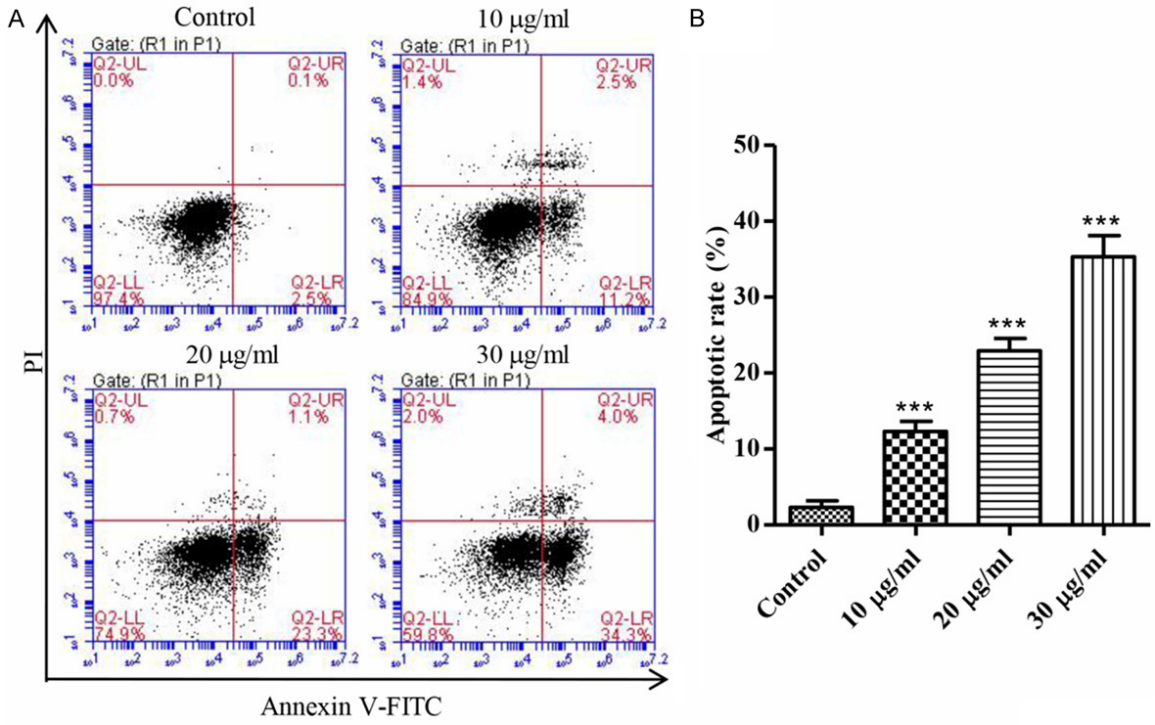


**Figure 3.** Effects of salidroside on apoptosis of K562 cells. A. Annexin-V/PI double stain assay and flow cytometry analysis were carried out to substantiate cell apoptosis. B. Treatment of salidroside at doses of 10, 20 and 30 μg/ml for 24 h dose-dependently increased the apoptotic population of K562 cells. The lower left quadrant shows vital cells. The lower right quadrant indicates early apoptotic cells (Annexin-V positive but PI negative). The upper right quadrant represents late apoptotic cells or necrotic cells (double positive). $^{***}P < 0.001$ compared with control.

As shown in **Figure 4A** and **4B**, treatment of salidroside at doses of 10, 20 and 30 μg/ml for 24 h significantly decreased the fluorescence intensity of the K562 cells, respectively, from 41989.03% ± 1687.49% to 13057.59% ± 484.85% (n = 3) in a dose-dependent manner

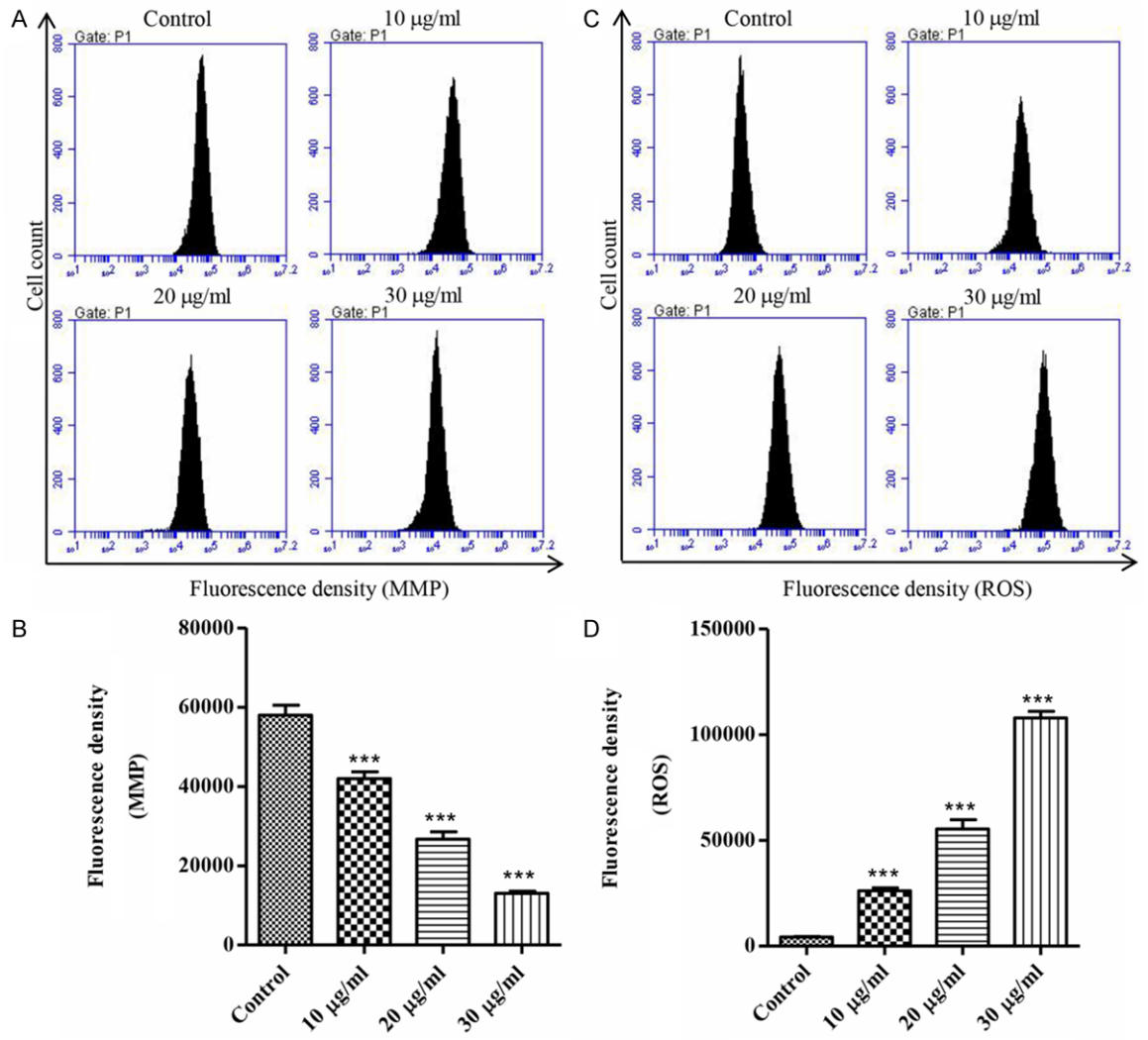


**Figure 4.** Effects of salidroside on MMP level and ROS generation in K562 cells. A. After treatment with salidroside at doses of 10, 20 and 30 μg/ml for 24 h, cells were resuspended with TMRM dye and analyzed by flow cytometry. B. Salidroside treatment dose-dependently decreased MMP levels of K562 cells. C. After treatment with salidroside at doses of 10, 20 and 30 μg/ml for 24 h, cells were resuspended with DHE and analyzed by flow cytometry. D. Salidroside treatment dose-dependently increased ROS generation of K562 cells. $^{***}P < 0.001$ compared with control.

compared with control cells with 57963.68% ± 2505.17%.

On the other hand, ROS generation is also linked to mitochondria. Dihydroethidium (DHE) was used to determine the levels of ROS production in K562 cells. As shown in **Figure 4C** and **4D**, treatment of salidroside at doses of 10, 20 and 30 μg/ml for 24 h significantly increased the fluorescence intensity of the K562 cells, respectively, from 26187.33% ± 1423.77% to 107918.60% ± 3145.55% (n = 3) in a dose-dependent manner compared with control cells with 4291.42% ± 217.71%.

*Effect of salidroside on protein expression in K562 cells*

To clarify the mechanism of K562 cells apoptosis induced by salidroside, cell cycle- and apoptosis-related proteins and the phosphorylation of kinases were detected by western blot. As shown in **Figure 5A** and **5B**, treatment with salidroside (20 and 30 μg/ml) for 6 h decreased ERK1/2 phosphorylation in a dose-dependent manner, while K562 cells treated with salidroside had no effect on the protein levels of t-ERK1/2. Treatment with salidroside (30 μg/ml) for 24 h also evidently decreased the pro-

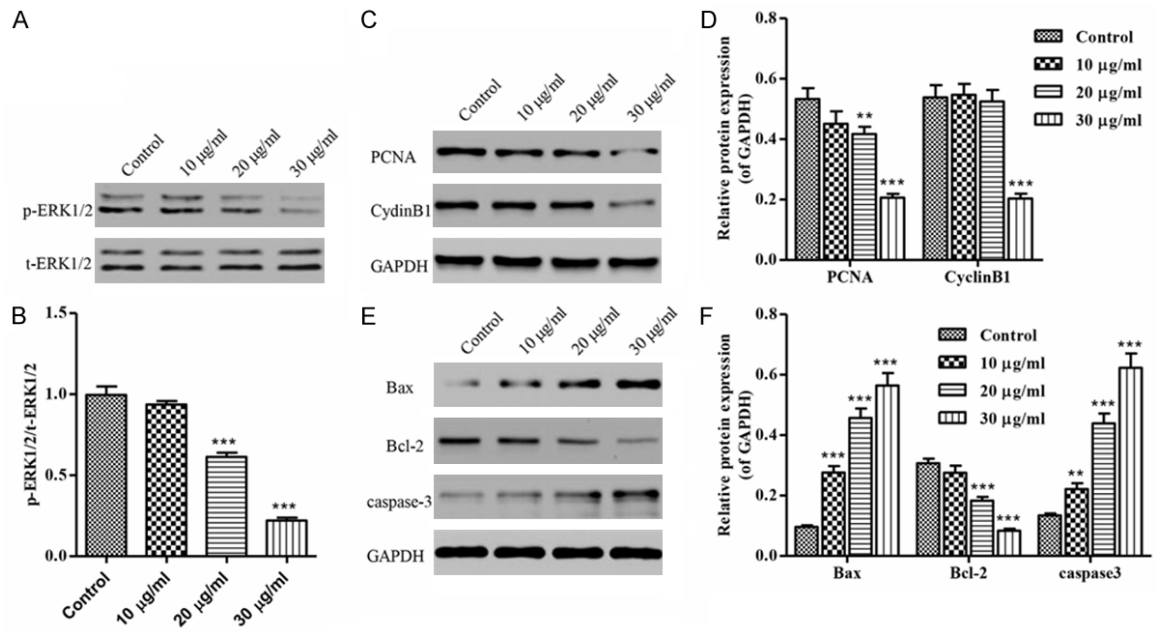


**Figure 5.** Effects of salidroside on the expression of key proteins involved in cell cycle and cell apoptosis. A, B. Treatment with salidroside for 6 h significantly decreased ERK1/2 phosphorylation, measured by Western blot. C, D. Treatment with salidroside for 24 h significantly decreased PCNA and CyclinB1 expression, measured by Western blot. E, F. Treatment with alidroside for 24 h significantly increased the ratio of Bax/Bcl-2 and the active form of caspase-3, measured by Western blot. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control.

tein levels of PCNA and CyclinD1 compared to the control group (**Figure 5C** and **5D**), but increased levels of Bax/Bcl-2 ratio and the active form of caspase-3 (**Figure 5E** and **5F**).

## Discussion

Natural products provide one of the most important sources of promising leads for the development of novel chemotherapeutics. Although a few research results indicated that salidroside could inhibit the growth of cancer cells, but lacking for the enough information to further verify the anticancer action of salidroside on cancer cells. The effects on human leukemia K562 cells of the plant ingredients, salidroside, were investigates in this study. The present study demonstrated for the first time that salidroside treatment effectively initiated a series of events leading to viability inhibition and cell cycle arrest and apoptosis, such as the mitochondrial dysfunction, ROS generation, caspase activation and ERK1/2 inactivation of K562 cells. Hu et al. reported that salidroside inhibited proliferation and induced apoptosis in cancer cells [5, 6], whereas other studies showed that salidroside has protective effects against apoptosis in PC12 cells, erythrocyte and cardiomyocytes [19-21].

Our data indicate that salidroside induce cell cycle arrest in K562 cells in a dose-dependent manner. Lower concentration salidroside (2 μg/ml) inhibited the growth of MDA-MB-231 and A549 cells by G1-phase arrest markedly, but inhibitory action of higher concentration salidroside (4 μg/ml) on two cancer cells resulted from G2-phase arrest [5], which similar to our findings that high concentration salidroside (10-30 μg/ml) induced G2-M phase cell cycle arrest in K562 cells. As we known, cell cycle progression is regulated by proliferating cell nuclear antigen (PCNA) [22] and several different cyclin-dependent kinases, which are activated through binding with different types of cyclins [23]. G2-M transition is positively regulated by CyclinB1. Since salidroside treatment resulted in strong G2-M phase arrest in K562 cells, we assessed the effects of salidroside on PCNA and CyclinB1 in the cells, and the results showed that G2-M phase arrest could be relative with the down-regulation of PCNA and CyclinB1.

Apoptosis is a programmed cell death via the expression and translocation of the Bcl-2 family proteins, changes of the MMP and the release of cytochrome c from mitochondria and activation of caspases to cause DNA fragmentation

[24]. We found that salidroside upregulated Bax expression and caspase-3 activation and inhibited the levels of Bcl-2 in K562 cells. Moreover, the increased expression of caspase-3 activity in association with the decrease of the levels of MMP and Bcl-2 and increase of ROS generation suggests that salidroside-induced apoptosis is mitochondria-dependent and signaling pathways may be involved in regulating caspase-3 activity. Depolarization of the mitochondrial inner membrane during the apoptosis has been reported [25], as observed in the present study. ROS are known as intracellular second messengers and may act as a mediator in apoptotic pathways and in turn act on MMP to influence mitochondrial function.

The MAPK signaling cascade, including ERK1/2, c-Jun N-terminal kinase (JNK), and P38, has also been implicated in the apoptosis of leukemia [26, 27]. ROS generation has been shown to be coupled with the sustained activation of the ERK signaling pathway for a variety of cellular effects, including apoptosis [28]. Our results showed that salidroside treatment decreased the phosphorylation of ERK1/2 in K562 cells. Sun et al. found that salidroside inhibits migration and invasion of human fibrosarcoma HT1080 cells via inhibiting the activation of ERK1/2 [29]. Zhao et al. reported that salidroside inhibited the tumor growth of human breast cancer *in vivo* via inhibiting the ROS formation and ERK1/2 pathway activation [30]. Our work showed that salidroside treatment increased the intracellular ROS level in K562 cells might be associated with the inhibitory effect of salidroside on ERK1/2 activation.

In conclusion, salidroside caused viability inhibition, cell cycle G2-M arrest and apoptosis in human leukemia K562 cells. The results revealed the associations between mitochondrial dysfunction, ROS generation, caspase activation and ERK1/2 inactivation. This study may provide a mechanistic background for the introduction of this new type of promising therapeutic agent in the study of cancer chemotherapy.

## Disclosure of conflict of interest

None.

**Address correspondence to:** Jing-Hua Wang, Department of Hematology, The Second Affiliated Hospital, Harbin Medical University, 246 Xuefu Road, Nangang District, Harbin 150086, China. Tel: +86-451-86605442; Fax: +86-451-86605442; E-mail: wjhharbin@126.com

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