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ORIGINAL ARTICLE

# Scutellarin exerts anticancer effects on human leukemia cells *via* induction of Sub-G1 cell cycle arrest, apoptosis and also inhibits migration and invasion by targeting Raf/MEK/ERK signalling pathway

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## *Summary*

***Purpose:*** *Leukemia accounts for a significant mortality across the globe every year. The main objective of the present research work was directed towards studying the anticancer effects of scutellarin-a plant flavone, against K562 human leukemia cells, along with examining its effects on cellular apoptosis, cell cycle, cell migration and cell invasion as well as Raf/MEK/ERK signalling pathway.*

***Methods:*** *Cell viability of K562 leukemia cells was evaluated by WTS-1 assay, while apoptotic effects induced by scutellarin in K562 cells were examined by fluorescence microscopy, flow cytometry, and western blot methods. Effects on cell cycle were measured by flow cytometry. Transwell Matrigel assay was performed to evaluate whether scutellarin induces inhibition of cell migration and cell invasion effects in K562 cells*

***Results:*** *Scutellarin was shown to suppress the viability of the K562 cells dose-dependently with an $IC_{50}$ of 6 μM. Further, scutellarin was shown to induce apoptosis which was initially exhibited by DAPI and annexin-V/propidium iodide (PI) staining and then confirmed by western blot in which it was shown to trigger regulation of Bax and downregulation of Bcl-2 in K562 human leukemia cells. Scutellarin also induced G0/G1 cell cycle arrest which was accompanied by suppression of cell migration and invasion. Scutellarin also led to the decline of the expression of p-Raf, p-MEK1/2 and p-ERK1/2 in a concentration-dependent manner.*

***Conclusion:*** *In conclusion, scutellarin could inhibit the growth of K562 human leukemia cells by inducing apoptosis, cell cycle arrest, inhibition of cell migration and invasion and downregulating the expressions of p-Raf, p-MEK1/2 and p-ERK1/2.*

***Key words:*** *scutellarin, apoptosis, cell cycle, flow cytometry, cell migration, cell invasion.*

## Introduction

Leukemia is regarded as one of the lethal human diseases and results in a significant human mortality across the globe. In United States of America alone, more than 0.3 million people are diagnosed with leukemia and out of them 0.2 million people die. Around 3% of all human malignancies constitute leukemia and because of its low survival rates, it is regarded as one of the fatal cancers [1,2]. There are four types of leukemia, namely acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL) and chronic lymphocytic leukemia (CLL). Among them, CLL is very common in middle-aged people or elderly in western countries. However, in China and Japan, CLL is rare. But the published literature supporting this claim is mainly found in China [3,4]. Regarding AML, the median age at diagnosis is 66 years and has a cure rate of less than 10% and a

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median survival less than 1 year. In patients who are less than 60 years of age, around 80% of them can achieve complete remission. However, tumor relapse occurs in most of them and overall survival is only 40-50% at 5 years. AML treatment involves remission induction chemotherapy with a combination of cytarabine/anthracyclines [5]. However, due to the adverse side effects of chemotherapy and its diagnosis at advanced stage, leukemia treatment is seriously affected. Keeping these things in mind, research endeavours are being made to design and develop novel and effective chemotherapeutic drugs which have comparatively lesser side-effects. Plants synthesize a wide array of secondary metabolites and many of these secondary metabolites have been used in treating human disorders including cancer. Many of the plant-derived molecules like taxanes, podophyllotoxins, vincristine, vinblastine, camptothecin etc. have been shown to have significant anticancer activity against a range of human cancer cells [6,7]. In the present study, the anticancer effects of Scutellarin-a plant flavone, mainly found in *Scutellariabarbata* and *Scutellariaateriflora,* were investigated. Its effects on cell cycle phase distribution, cell apoptosis, cell migration and invasion as well as Raf/MEK/ERK signalling pathway were also investigated.

## Methods

*Cell lines, culture conditions, WST-1 cell proliferation assay*

The human leukemia cells K562 were kindly provided by the Department of Biochemistry and Biology, Chinese Academy of Sciences, Shanghai, China. The cells were kept in Dulbecco's modified Eagle's medium. (DMEM) which was also supplemented with 10% fetal bovine serum (FBS), antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), and 2 mM glutamine. The proliferation of these cells was evaluated by WST-1 assay. In brief, K562 cells were cultured in 96-well plates at the density of $1\times10^6$ cells/well and treated with 0, 1.8, 3.6, 7.2, 12.5, 25, 50 and 100 µM concentrations of scutellarin for 24 h at 37°C. This was followed by incubation of the cells with WST-1 at 37°C for 4 h. The absorbance was then measured at 450 nm using a victor 3 microplate reader to determine the proliferation.

*Cell cycle analysis by flow cytometry*

The effects of scutellarin flavone on the progression of cell cycle phases were analysed by flow cytometry using AnnexinV/PI staining. The cultured human leukemia K562 cells were initially treated with 0, 7.5, 15 and 30µM concentration of scutellarin for 24 h at 37oC. The cells were then washed with phosphate buffered saline (PBS). Afterwards, the K562 cells were stained with Annexin V/PI and the distribution of the cells in various cell cycle phases was measured by FACS flow cytometer.

*Apoptosis studies using DAPI (4',6-diamidino-2-phenylindole) and annexin V/propidium iodide (PI) staining assays*

The fact that scutellarin led to induction of apoptosis in K562 human leukemia cells was studied by using fluorescence microscopy along with flow cytometry using DAPI and annexin V/PI staining dyes respectively. The K562 cells were grown in 6-well plates ($2\times10^5$ cells/well) for 12 h. The cells were then treated with 0, 7.5, 15 and 30 µM dose of scutellarin for 24 h at 37°C. Around 25µl cell culture were put onto a glass slide after the cells were sloughed off and stained with a solution of DAPI or separately. The slides were then enclosed with a cover slip and scanned with a fluorescent microscope (Nikon Instruments Inc., NY, USA). Annexin V/PI staining was carried out as previously reported [8].

*Western blot assay*

The apoptotic effects as well as the effects on Raf/MEK/ERK signalling pathway were further examined by western blot assay. The K562 human leukemia cells were washed with ice-cold PBS and then suspended in a lysis buffer at 4°C. Subsequently, Bradford assay was used for evaluating the protein content of each cell extract. About 50 µg of protein were loaded from each sample and separated by SDS-PAGE before being shifted to polyvinylidene fluoride membrane. The membranes were then subjected to tris-buffered saline treatment and then exposed to primary antibodies at 4°C. The cells were treated with appropriate secondary antibodies and the proteins of interest were visualised by enhanced chemiluminescence reagent. Finally, the protein signal was

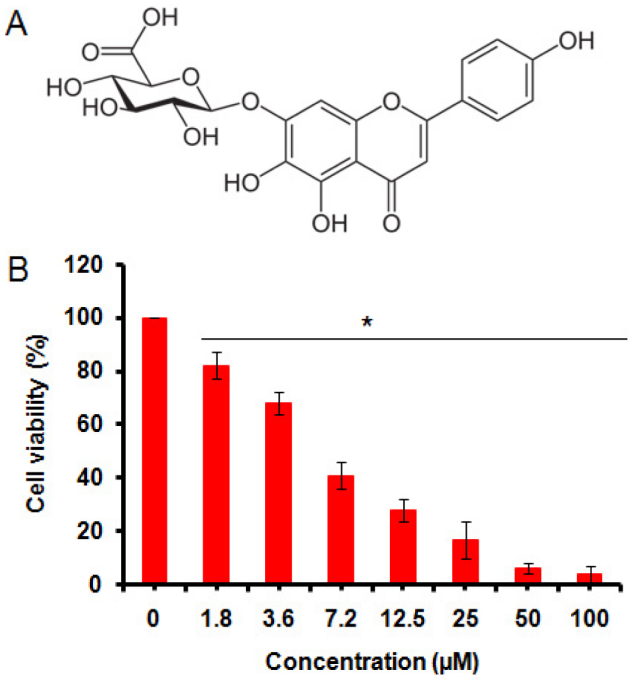


**Figure 1.** Chemical structure of scutellarin **(A)**. **B:** WTS-1 assay showing antiproliferative effects of scutellarin on the viability of K562 human leukemia cells. The experiments were performed in triplicate and shown as mean ± SD (*p<0.05).

detected by Odyssey Infrared Imaging System. Actin and GAPDH were used as control for normalisation.

*Effects on cell invasion and cell migration*

The cell invasion and migration effects of scutellarin on K562 human leukemia cells were evaluated by transwell assay (8 µM pore size polycarbonate filters) with Matrigel. Around 100 ml cell culture were positioned onto the upper chambers and only medium was put in the bottom chambers. However, for invasion assay, the inserts were coated with extracellular matrix gel (30 µl) (ECM, Sigma, USA). Swabbing was performed to remove the non-migrated and non-invaded cells from the upper surface. However, the migrated and the invaded cells on the lower surface were fixed with methanol for about 40 min followed by crystal violet (0.5%) staining for about 50 min, and washed with PBS. Finally, inverted microscope was used to count the number of invaded and migrated cells at 200x magnification.

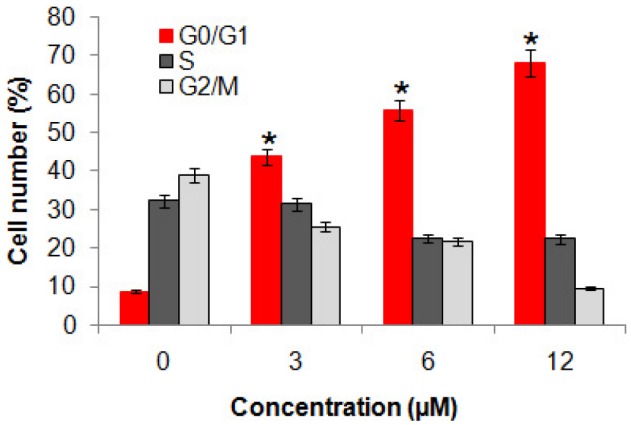


**Figure 2.** Scutellarin induces G0/G1 cell cycle arrest of the K562 human leukemia cells as evidenced by flow cytometry. The experiments were performed in triplicate and shown as mean ± SD (*p<0.05).

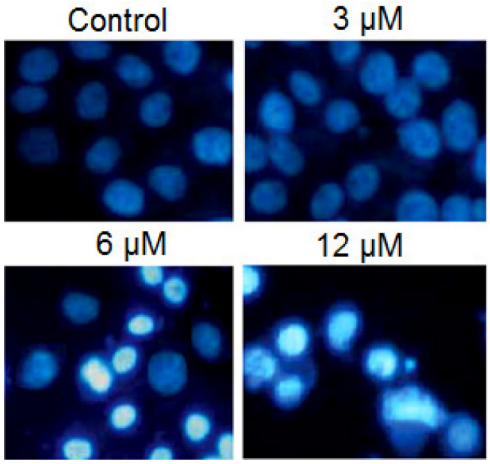


**Figure 3.** Fluorescence microscopy using DAPI (4',6-diamidino-2-phenylindole) staining, showing that scutellarin induces apoptosis in K562 human leukemia cells. The experiments were performed in triplicate.

*Statistics*

The experiments were done in triplicate. The values presented are the mean of three repeats ±SD. *p<0.05, **p<0.01 and **p<0.001 were considered statistically significant. Student's t-test using GraphPad prism 7 software was employed for statistical analyses.

## Results

*Scutellarin inhibits K562 human leukemia cell growth*

The growth inhibitory effects of scutellarin (Figure 1A) were seen on the K562 leukemia cells by WTS-1 assay at concentrations ranging from 0 to 100 µM. Scutellarin was shown to suppress the viability of the K562 cells dose-dependently (Figure 1B). The $IC_{50}$ of scutellarin against the K562 cells was found to be 6 µM. The cell viability was much more pronounced at 100 µM concentration, killing more than 95% of the cells.

*Scutellarin induces G0/G1 cell cycle arrest in K562 human leukemia cells*

The growth inhibitory effects of scutellarin were further observed to be mediated via inhibiting cell cycle phase distribution. Results obtained from flow cytometric measurements indicated that scutellarin led to G0/G1 cell cycle arrest in a dose-dependent manner. With increase in the scutellarin dose, the K562 cells in G0/G1 phase were also seen to increase and at 12 µM dose around 67.5% of the cells were observed to lie in G0/G1 phase of the cell cycle (Figure 2).

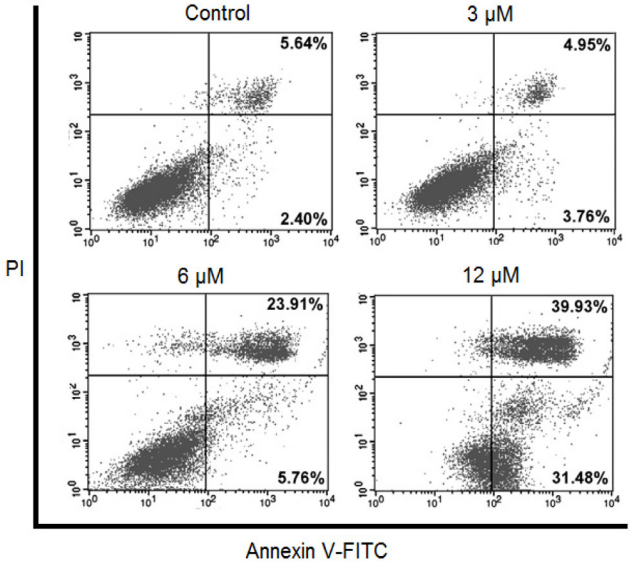


**Figure 4.** Annexin V/PI assay showing the percentage of K562 human leukemia apoptotic cells at the indicated concentrations of scutellarin. The experiments were performed in triplicate.

*Scutellarin induced programmed cell death in K562 human leukemia cells*

The results also showed that the growth inhibitory effects of scutellarin were mediated via inducing apoptosis in these cells. Fluorescence microscopy using DAPI staining indicated that scutellarin induced changes in K562 cells resembling apoptosis, and these changes included nuclear fragmentation, chromatin condensation, and splitting of the nucleus. These effects were upregulated with increasing scutellarin dose (Figure 3). The annexin V/PI staining showed that K562 apoptotic cell percentage enhanced in a concentration-dependent manner. The apoptotic cell percentage increased from 8.04% at 0 µM drug dose to 8.71%, 29.67% and 71.41% at 3, 6 and 12µM respectively (Figure 4). Scutellarin induced both early and late apoptosis, indicating its potential as an apoptosis-inducing agent. Further, for the authentication of apoptosis, the expression of apoptosis-associated proteins (Bax and Bcl-2) was observed and it was shown that scutellarin triggered upregulation of Bax and downregulation of Bcl-2 in K562 human leukemia cells (Figure 5). Taken together, all three assays confirmed the fact that scutellarin inhibits the growth of K562 leukemia cells by triggering programmed cell death.

*Scutellarin led to suppression of cell migration and invasion*

In addition, the effects of scutellarin on cancer cell migration and invasion were evaluated by transwell assay at varying doses of the molecule. It was seen that scutellarin at $IC_{50}$ dose exerted potent inhibitory effects on cancer cell migration and invasion (Figures 6 and 7). These results sug-

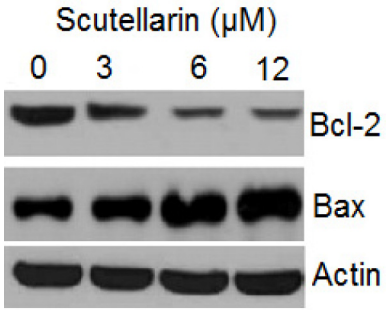


**Figure 5.** Effect of tested concentrations of scutellarin on the expression of apoptosis related proteins (Bcl-2 and Bax) by western blot analysis. The experiments were performed in triplicate.

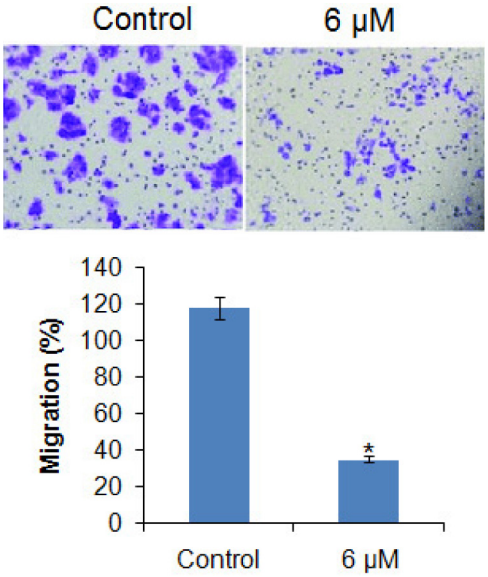


**Figure 6.** Scutellarin induced dose-dependent inhibition of cell migration in K562 human leukemia cells. The experiments were performed in triplicate and shown as mean ± SD (*p<0.05).

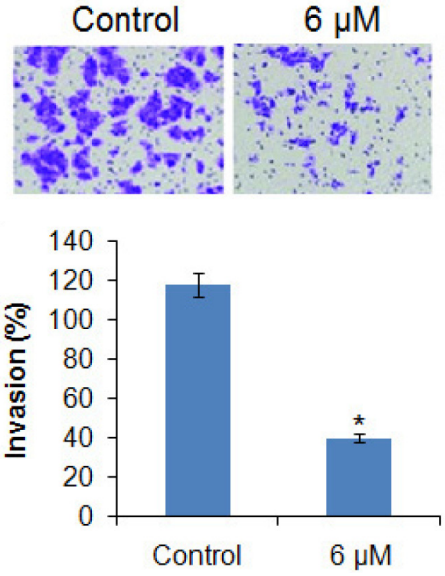


**Figure 7.** Scutellarin induced dose-dependent inhibition of cell invasion in K562 human leukemia cells. The experiments were performed in triplicate and shown as mean ± SD (*p<0.05).

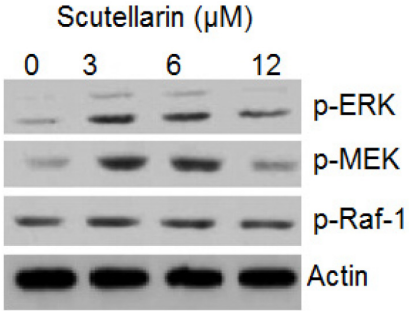


**Figure 8.** Scutellarin targets Raf/MEK/ERK signalling pathway by decreasing the expression of p-Raf, p-MEK1/2 and p-ERK1/2 in a concentration-dependent manner. The experiments were performed in triplicate.

gested that scutellarin could inhibit cell migration and invasion and as a result it could be quite useful in stopping the cancer cell metastases, which is a challenging problem in cancer treatment.

*Scutellarin inhibits the Raf/MEK/ERK signalling pathway in K562 leukemia cells*

One of the final experiments that we did was to assess the effects of scutellarin on Raf/MEK/ERK signalling pathway and this was done by western blot assay. The results convincingly indicated that scutellarin led to the decline of the expression of p-Raf, p-MEK1/2 and p-ERK1/2 in a concentration-dependent manner (Figure 8).

## Discussion

Naturally occurring molecules have always played key roles in treating human diseases, especially infections and cancers. Numerous clinically valuable anticancer drugs have been isolated from plants which have been used in treating diverse cancers in the last 50-60 years. Plant-derived natural products play a major role in drug discovery keeping in view the increasing number of new and novel drugs which are currently in different phases of clinical development [9]. Scutellarin is a plant flavone that belongs to phenolic class of molecules with wide range of pharmacological effects. These effects include antioxidant, anti-inflammatory, antiplatelet, anti-coagulation, and anti-myocardial infarction. Scutellarin has been reported to enhance antitumor effects of bleomycin- a broad spectrum antitumor drug- in H22 Ascites Tumor-bearing mice [10]. This molecule has also been reported to suppress colorectal cancer cell growth and colony formation along with inhibiting tumor growth in *in vivo* mouse xenografts. It has also been reported to inhibit angiogenesis along with inhibiting human umbilical vascular endothelial cells (HUVECs) migration [11]. This molecule has been reported to exert anticancer effects against a wide range of cancer cells including hepatocellular cancer cells, colorectal cancer cells, kidney cancer cells, lung, prostate etc. The mechanism of anticancer action of scutellarin has been shown to be mediated by its ability to induce programmed cell death, inducing cell cycle arrest as well as targeting various biochemical signaling pathways [12-15]. In one published study it is reported to inhibit cancer cell growth by inducing apoptosis along with downregulation of Bcl-2, Bax, and caspase-3 [16]. It is also reported that scutellarin also led to reduction of reactive oxygen species (ROS) along with activating STAT-3 transcription signalling pathway, and targeting Bcl-XL, and Mcl-1. It is also reported in a much recent study of 2017 that this molecule suppresses cell invasion and migration of liver cancer cells by suppressing STAT-3 and AKT protein expressions [17,18].

In the present study, scutellarin was investigated for its anticancer activity against K562 human leukemia cells. Furthermore, its effects on cellular apoptosis, cell cycle, cell migration and cell invasion along with Raf/MEK/ERK signalling pathway were also examined. The molecule was shown to suppress the viability of the K562 cells dose-dependently with an $IC_{50}$ of 6 µM. In addition, scutellarin was shown to induce apoptosis which was initially exhibited by DAPI and annexin-V/PI staining and then confirmed by western blot in which it was shown to trigger upregulation of Bax and downregulation of Bcl-2 in K562 human leukemia cells. Scutellarin also induced G0/G1 cell cycle arrest which was accompanied by suppression of cell migration and invasion. This molecule also led to the decline of the expression of p-Raf, p-MEK1/2 and p-ERK1/2 in a concentration-dependent manner.

## Conclusion

Combining all the experimental pieces together, it can be safely suggested that scutellarin exerts potent anticancer effects in K562 human leukemia cells by inducing apoptosis, G0/G1 cell cycle arrest, inhibition of cell migration, cell invasion as well as suppression of Raf/MEK/ERK signalling pathway.

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## Conflict of interests

The authors declare no conflict of interests.

## References

1. Deschler B, Lübbert M. Acute myeloid leukemia: epidemiology and etiology. Cancer 2006;107:2099-107.
2. Zhang L, Freeman LE, Nakamura J et al. Formaldehyde and leukemia: epidemiology, potential mechanisms,

and implications for risk assessment. Environm Molec Mutagenesis 2010;51:181-91.

3. Boggs DR, Chen SC, Zhang ZN, Zhang A. Chronic lymphocytic leukemia in China. Am J Hematol1987;25:349-54.
4. Chen B, Wang YY, Shen Y et al. Newly diagnosed acute lymphoblastic leukemia in China (I): abnormal genetic patterns in 1346 childhood and adult cases and their comparison with the reports from Western countries. Leukemia 2012;26:1608-16.
5. Roboz GJ. Current treatment of acute myeloid leukemia. Curr Opin Oncol 2012;24:711-9.
6. Newman DJ, Cragg GM. Natural products as sources of new drugs from 1981 to 2014. J Nat Products 2016;79:629-61.
7. Harvey AL, Edrada-Ebel R, Quinn RJ. The re-emergence of natural products for drug discovery in the genomics era. Nat Rev Drug Discov 2015;14:111.
8. Hammill AK, Uhr JW, Scheuermann RH. Annexin V staining due to loss of membrane asymmetry can be reversible and precede commitment to apoptotic death. Experim Cell Res 1999;251:16-21.
9. Rayan A, Raiyn J, Falah M. Nature is the best source of anticancer drugs: Indexing natural products for their anticancer bioactivity. PLoS One 2017;12: e0187925.
10. Nie J, Yang HM, Sun CY et al. Scutellarin Enhances Antitumor Effects and Attenuates the Toxicity of Bleomycin in H22 Ascites Tumor-Bearing Mice. Front Pharmacol 2018;9:615.
11. Zhu PT, Mao M, Liu ZG, Tao L, Yan BC. Scutellarin suppresses human colorectal cancer metastasis and angiogenesis by targeting ephrinb2. Am J Transl Res 2017;9:5094-104.
12. Chledzik S, Strawa J, Matuszek K, Nazaruk J. Pharmacological effects of scutellarin, an active component of genus Scutellaria and Erigeron: a systematic review. Am J Chin Med2018;46:319-37.
13. Deng W, Han W, Fan T et al. Scutellarin inhibits human renal cancer cell proliferation and migration via up-regulation of PTEN. Biomed Pharmacother 2018;107:1505-13.
14. Chan JY, Tan BKH, Lee SC. Scutellarin sensitizes drug-evoked colon cancer cell apoptosis through enhanced caspase-6 activation. Anticancer Res 2009;29:3043-8.
15. Feng Y, Zhang S, Tu J et al. Novel function of scutellarin in inhibiting cell proliferation and inducing cell apoptosis of human Burkitt lymphoma Namalwa cells. Leuk Lymphoma 2012;53:2456-64.
16. Wu Y, Fan Q, Lu N et al. Breviscapine induced apoptosis of human hepatocellular carcinoma cell line HepG2 was involved in its anti-tumour activity. Phytother Res 2010;24:1188-94.
17. Xu H, Zhang S. Scutellarin-induced apoptosis in HepG2 hepatocellular carcinoma cells via a STAT3 pathway. Phytother Res 2013;27:1524-8.
18. Ke Y, Bao T, Wu X et al. Scutellarin suppresses migration and invasion of human hepatocellular carcinoma by inhibiting the STAT3/Girdin/Akt activity. Biochem Biophys Res Commun 2017;483:509-15.