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Original Research Article

Cytotoxic activity of *lithospermum erythrorhizon* root extract against childhood acute leukemia cells via regulation of PI3K/AKT/mTOR pathway

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Abstract

Purpose: *To investigate the effect and mechanism of action of Lithospermum erythrorhizon root extract (LR) in childhood acute leukemia.*
Methods: *Human leukemic lymphoblast (CCRF-CEM cell line) cells were treated with LR (2, 4, and 8 mg/mL). Cell viability, cell apoptosis and cell cycle were measured using 3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry, respectively. The levels of cell-cycle-related proteins including cyclin D1, cyclin E1, cyclin-dependent kinase 2 (CDK2), CDK4 and CDK6, as well as cleaved caspase 3, cleaved caspase 9 and Bcl-2-associated x (Bax). Also determined were B-cell lymphoma-2 (Bcl-2), E-cadherin, N-cadherin, vimentin, zonula occludens 1 (ZO-1), matrix metalloproteinase-2 (MMP-2) and MMP-9. Cell migration and invasion were assessed using scratch and Transwell assays. Finally, phosphoinositide 3-kinase (PI3K), phosphorylated serine/threonine kinase (p-AKT), total AKT (t-AKT), mammalian target of rapamycin (mTOR), and phosphorylated mTOR (p-mTOR) were measured using western blotting.*
Results: *Lithospermum erythrorhizon root extract not only dose-dependently inhibited cell viability, induced G1 phase accumulation, and downregulated CDK2, CDK4, CDK6, cyclin D1, and cyclin E1, but also elevated apoptosis, cleaved caspase 3, cleaved caspase 9, and Bax and decreased Bcl-2 expression levels. In addition, Lithospermum erythrorhizon root extract suppressed migration and invasion of CCRF-CEM cells, downregulated N-cadherin, vimentin, MMP-2, and MMP-9, and upregulated E-cadherin and ZO-1. Moreover, Lithospermum erythrorhizon root extracts dose-dependently inhibited PI3K, p-AKT (ser473)/t-AKT, p-mTOR (ser2448)/mTOR, and p-mTOR (ser2481)/mTOR.*
Conclusion: *The findings provide a potential therapeutic approach to the treatment of childhood acute leukemia.*

Keywords: *Childhood acute leukemia, PI3K/AKT/mTOR pathway Lithospermum erythrorhizon root extracts*

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INTRODUCTION

Acute leukemia, a hematologic malignancy, which includes acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) [1]. In clinical practice, ALL frequently occurs in children with the peak incidence between 2 and 5 years old, whereas AML is frequently found in adults [2]. Although treatment outcomes have dramatically been improved, acute leukemia remains one of the most common life-threatening diseases in children [3]. Prophylactic cranial irradiation was a standard remedy in children with ALL in clinical practice, which substantially improved 5-year survival rates. Unfortunately, irradiation-induced secondary neoplasms, organ impairment, and psychosocial and cognitive dysfunctions lead to chronic disease occurrence and life expectancy reduction [4-6]. In considering to this problem, chemotherapy was used with an increasing rate, and the satisfactory 5-year disease-free survival (> 80 %) has been achieved[7]. However, pain, obesity, fatigue, cardiomyopathy, osteoporosis and death were therapeutic sequelae in children with ALL after chemotherapy [8]. Therefore, there is a need to develop novel treatment approaches for ALL.

Lithospermum erythrorhizon Sieb.et Zucc. (LR), a Chinese traditional herb for various skin diseases and injuries, is widely used in Asia and Europe [9]. Shikonin, a naphthoquinone, is the main active component of LR which has a variety of pharmacological properties such as anti-inflammation and anti-cancer [10]. A published study demonstrated that shikonin inhibits cell proliferation and induces cell apoptosis partly through modulating MAPKs and c-Myc in acute promyelocytic leukemia [11]. Besides, involvement of ERK/JNK/MAPK and AKT pathways in c-Myc inactivation represents a novel treatment of shikonin and its derivatives for acute leukemia [12]. However, the effects of *Lithospermum erythrorhizon* root extracts on ALL and its molecular mechanism are still poorly elucidated. Therefore, the present study was intended to investigate the role of *Lithospermum erythrorhizon* root extracts on ALL in CCRF-CEM cell line. The underlying mechanism was further investigated.

EXPERIMENTAL

Cells

Human T lymphoblastic leukemia CCRF-CEM cells were obtained from Shanghai Suer Shengwu Technology Co., LTD (Shanghai, China). Cells were cultured in RPMI-1640 medium (MSKCC Media Facility), supplemented with fetal calf serum (10 %, FCS, Gibco, USA), glutamine (200 mM), and penicillin–streptomycin (1 %, Thermo Fisher Scientific) at 37 °C in 5 % CO_2.

Cell viability

CCRF-CEM cells (1 × 10^4 cells/well) were cultured in 48-well plates overnight. After *Lithospermum erythrorhizon* root extracts (LR) treatment (2, 4 and 8 mg/kg) for the indicated times (24, 48 and 72h) respectively, the 3-(4, 5-dimethylthiazol-2-thiazolyl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma, USA) assay used following the manufacturer's instructions. After 30 min, a microplate reader (BioTek Software, USA) was used to obtain the absorbance at 570 nm.

Cell cycle analysis

After *Lithospermum erythrorhizon* root extracts (LR) treatment (2, 4, 8 mg/kg), samples were rinsed with cold phosphate-buffered saline (PBS) twice. Then, 70 % ethanol was used to fix the cells for 30 min. Samples were resuspended and incubated with propidium iodide (PI)/RNase buffer (BD Bioscience, USA) for 24 h at 4 °C. A FC500 flow cytometer (Beckman Coulter, USA) was used to assess cell cycle fractions.

Cell apoptosis

Samples were harvested and centrifuged for 5 min at 1000 × g (approximately 2000 rpm). After rinsed twice, samples were fixed with ethanol (70 %)for 30 min, resuspended and stained using Annexin V -fluorescein-isothiocyanate (FITC) and phycoerythrin (PE) (Caltag Laboratories, San Francisco, CA). Apoptosis was quantified using flow cytometry.

Scratch assay

Samples were maintained in 24-well tissue culture dishes coated with collagen type I (40 µg/mL) in medium for 2 h at 37 °C until 80 % confluent cell monolayer was reached. Then, a sterile 100 µL plastic pipette was used to scratch a linear wound in the monolayer. The coverslips were washed with PBS to remove cells fragments. Three representative pictures from the scratched area of each coverslip were taken, and relative wound width (24 h/0 h) was calculated using CellC software.

Transwell assay

After transfection with related oligonucleotides according to the protocol, 3 × 10^4 CCRF-CEM cells were cultured for 48 h and samples were

then placed on upper Matrigel-coated chambers (Invitrogen, USA) in serum-free DMEM (10 % FBS), which was also placed in the lower chamber. 24 h later, the invading cells were fixed in 95 % ethanol in duplicate, followed by 0.1 % crystal violet staining. Three representative images were photographed and the number of invading malignant cells was evaluated using a microplate reader at 590 nm.

Western blotting

Cells were washed with cold PBS after *Lithospermum erythrorhizon* root extracts (LR) treatment, and samples were placed on ice in phenylmethanesulfonyl fluoride (PMSF) buffer at 4 °C for 30 min. After sonication and centrifugation, the supernatant was collected for determination of protein concentration. Then, 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins, which were transferred onto polyvinylidene difluoride membranes. The blots were blocked in NaCl/Tris-0.1% (v/v) Tween-20 (NaCl/Tris-T) buffer supplemented with non-fat milk for 1 h. The following primary antibodies were incubated overnight at 4 °C: anti-cyclin D1, anti-cyclin E1, anti-CDK2, anti-CDK4, anti-CDK6, anti-cleaved caspase 9, anti-cleaved caspase 3, anti-Bcl-2, anti-Bax, anti-E-cadherin, anti-N-cadherin, anti-vimentin, anti-ZO-1, anti-PI3K, anti-p-AKT, anti-t-AKT, anti-p-mTOR, and anti-mTOR antibodies (BD Biosciences Franklin Lakes, NJ). Next, secondary goat anti-rabbit IgG (BD Biosciences Franklin Lakes, NJ) antibody was incubated for 1 h. Finally, an enhanced chemiluminescence (ECL) detection system (GE Healthcare, USA) was used to analyze protein expression levels.

Statistical analysis

SPSS 16.0 statistical software (SPSS Inc, Chicago, IL) was used for statistical analyses. Experimental data was presented as mean ± SD. Analysis of variance (ANOVA) was utilized for comparison between multiple groups, followed by Tukey's multiple comparison tests. The level of statistical significance applied was $p < 0.05$.

RESULTS

Lithospermum erythrorhizon root extract (LR) exerts anti-proliferative effect on CCRF-CEM cell line

To investigate the anti-proliferative effect of *Lithospermum erythrorhizon* root extracts (LR), CCRF-CEM cells were treated with different doses of LR (2, 4, and 8 mg/mL) for 24, 48, and 72 h, respectively. *Lithospermum erythrorhizon* root extracts decreased cell viability both in dose- and time- dependent manners (Figure 1 A). Visible G1 phase accumulation was induced by LR in a dose-dependent manner compared to the negative control (NC) group (Figure 1 B). Western blotting analysis showed that LR dose-dependently decreased levels of CDK2, CDK4, CDK6, cyclin D1, and cyclin E1. The expression levels of CDK4 and cyclin E1after 2 mg/mL of LR treatment were downregulated, but were not significantly different from the NC group (Figure 1 C).

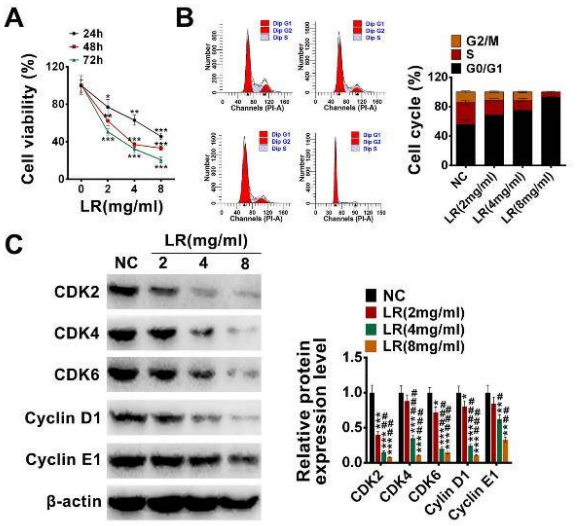

Figure 1: *Lithospermum erythrorhizon* root extracts (LR) inhibits growth of CCRF-CEM cell line; A, MTT assay for measuring viability of CCRF-CEM cells with LR treatment at 24, 48 and 72h; B, Flow cytometry analysis on cell cycle of CCRF-CEM cells after LR treatment. C, Western blotting for detecting CDK2, CDK4, CDK6, cyclin D1, and cyclin E1 levels after LR treatment; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the NC group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ compared with LR (2 mg/mL) group. NC = negative control; LR = *Lithospermum erythrorhizon* root extracts; CDK = cyclin-dependent kinase

Lithospermum erythrorhizon root extracts (LR) induces apoptosis of CCRF-CEM cell line

Compared with the NC group, the proteins levels of cleaved caspase 3, cleaved caspase 9 and Bax were dose-dependently up-regulated, while the level of Bcl-2 was downregulated after *Lithospermum erythrorhizon* root extracts (LR) treatment (Figure 2 A). Apoptosis in CCRF-CEM cells was assayed using flow cytometry. LR dose-dependently enhanced the apoptosis in CCRF-CEM cells as compared with the NC group (Figure 2 B).

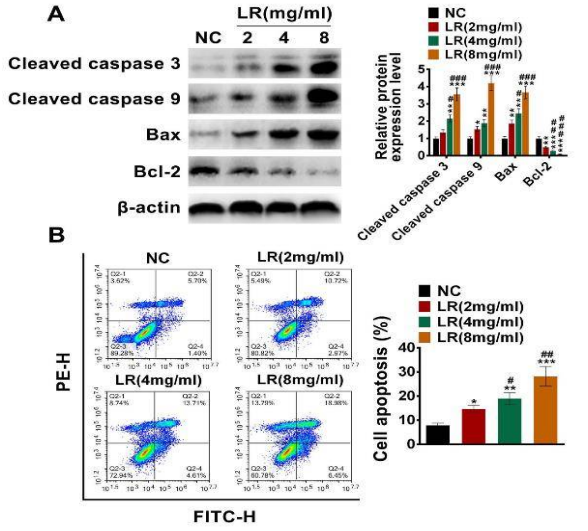

Figure 2: *Lithospermum erythrorhizon* root extracts (LR) induces apoptosis of CCRF-CEM cell line. A, Western blotting was used to examine protein levels; B, Flow cytometry was applied to quantify apoptosis of CCRF-CEM cell line; CCRF-CEM cells were treated with 2, 4 and 8 mg/ml of LP . $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ *vs.* NC group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ *vs.* LR (2 mg/mL) group. NC = negative control; LR = *Lithospermum erythrorhizon* root extracts; Bcl-2 = B-cell lymphoma-2; Bax = Bcl-2-associated x

Lithospermum erythrorhizon root extracts (LR) inhibits migration and invasion of CCRF-CEM cell line

An *in vitro* scratch assay was carried out to ascertain the migration of CCRF-CEM cells. Compared with NC group, *Lithospermum erythrorhizon* root extracts (LR) significantly inhibited the migration of CCRF-CEM cells in a dose-dependent manner (Figure 3 A). The transwell assay showed that LR remarkably inhibited the invasion of CCRF-CEM cells in a dose-dependent manner (Figure 3 B). Compared to the NC group, the levels of N-cadherin, vimentin, MMP-2, and MMP-9 were decreased, and the levels of E-cadherin and ZO-1 were increased after LR treatment. (Figure3C). Totally, LR effectively inhibited migration and invasion of CCRF-CEM cells.

Lithospermum erythrorhizon root extracts (LR) inhibits CCRF-CEM cells via PI3K/AKT/mTOR pathway

As shown in Figure 4**,** the protein levels of PI3K, p-AKT (ser473)/t-AKT, p-mTOR (ser2448)/mTOR, and p-mTOR (ser2481)/mTOR were significantly decreased in the LR treated group when compared to the NC group. These results indicated that LR dose-dependently inhibited the PI3K/AKT/mTOR pathway.

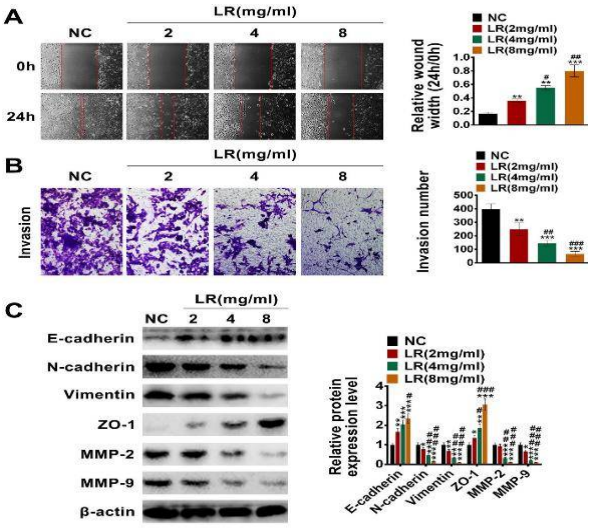

Figure 3: *Lithospermum erythrorhizon* root extracts (LR) inhibits migration and invasion of CCRF-CEM cell line. A:, Scratch assay for analyzing the migration of CCRF-CEM cells; B:, Transwell assay for investigating invasion of CCRF-CEM cells; C:, Western blotting for detecting E-cadherin, N-cadherin, vimentin, ZO-1, MMP-2, and MMP-9 levels. CCRF-CEM cells were treated with 2, 4 and 8 mg/ml of LP at 0h and 24 h. $^{*}P < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* NC group. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ *vs.* LR (2 mg/mL) group. NC = negative control; LR = *Lithospermum erythrorhizon* root extracts; MMP = matrix metalloproteinase; ZO-1 = zonula occludens 1

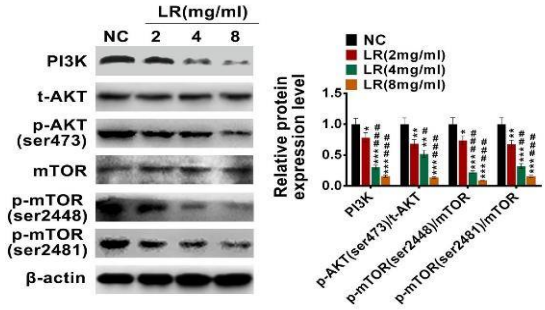

Figure 4: *Lithospermum erythrorhizon* root extracts (LR) exerts inhibitory effect on CCRF-CEM cells via PI3K/AKT/mTOR pathway. $^{*}P < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ *vs.* NC group. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ *vs.* LR (2 mg/ml) group. NC, negative control; LR = *Lithospermum erythrorhizon* root extracts; PI3K = phosphoinositide 3-kinases; t-AKT = total serine/threonine kinase; p-AKT = phosphorylated serine/threonine kinase; mTOR = mammalian target of rapamycin

DISCUSSION

Acute lymphoblastic leukemia (ALL) is a major cause of pediatric cancer-associated death, which is responsible for 30 % of all malignancies

in children [2]. There is an urgent need to develop a novel strategy for effective treatment of children with ALL. As previously reported, shikonin and its derivatives could induce apoptosis in AML through the ROS/JNK pathway [13] and promote AML cell differentiation by regulating the Nrf2/ARE pathway [14]. However, there are few studies considering on the protective role of *Lithospermum erythrorhizon* root extracts (LR) in childhood ALL. Thus, this study was to investigate the protective role of LR on childhood acute leukemia cells and reveal the underlying mechanisms.

In the present study, LR exerted anti-proliferative effect on CCRF-CEM cells and induced G1 phase cell cycle arrest. *Lithospermum erythrorhizon* root extracts caused apoptosis and inhibited migration and invasion of CCRF-CEM cells. In addition, LR dose-dependently inhibited expression of PI3K, t-AKT, p-AKT, mTOR, and p-mTOR. These findings indicate that LR attenuates childhood acute leukemia through regulation of the PI3K/AKT/mTOR pathway.

Activated PI3K/AKT/mTOR is crucial to many aspects of cellular functions, including transcription, cell cycle progression, apoptosis, differentiation, and growth [15-17]. LY294002 and rapamycin longitudinally inhibited the PI3K/AKT/mTOR pathway, and adult T-cell leukemia growth inhibition and G1 phase accumulation were both observed [18]. Bergenin inhibited cell viability of human colorectal cancer cells and induced G1 phase accumulation through inhibiting the PI3K/AKT/mTOR pathway [17]. Furthermore, decreased levels of p-AKT in line with inactivation of the PI3K/AKT/mTOR pathway led to visibly increased cell apoptosis in ETV6/RUNX1 (E/R)-positive leukemia cells [19]. Besides, reduction in proliferation and cell survival occurred after suppressing the endogenous E/R fusion protein. The findings suggest that LR effectively inhibited cell viability while inducing G1 phase accumulation and apoptosis in CCRF-CEM cells.

It is reported that AKT is a PI3K lipid product activated by phosphorylation, and migrates to the nucleus. The nuclear p-AKT plays pivotal roles in promoting tumorigenesis [20]. In the current study, the ratio of p-AKT/t-AKT was dose-dependently downregulated by LR, implying that LR could target PI3K/AKT signaling via reduction of phosphorylation of AKT to exert anti-tumor effects.

The mammalian target of rapamycin (mTOR) is well-known to regulate cell proliferation and survival [15]. mTOR exists as mammalian target of rapamycin complex 1 (mTORC1) and mammalian target of rapamycin complex 2 (mTORC2). The former plays an important role in regulating cell growth [21], whereas the later exerts an oncogenic effect [22]. A selective mTORC1 inhibitor, RAD001, was demonstrated to decrease the levels of p-mTOR on the Ser 2448 and Ser 2481 phosphorylation sites in B-precursor ALL [23]. Consistently, the present study showed that the protein levels of ser2448 p-mTOR (indicative of mTORC1 effect) and ser2481 p-mTOR (indicative of mTORC2 activity) were both remarkably decreased after LR treatment, indicating that LR regulated ALL cell growth via inhibiting mTOR signaling.

This is the first evidence investigating the role of LR in childhood ALL *in vitro* and the results show the protective effect of LR through regulating the PI3K/AKT/mTOR signaling pathway. There are limitations in current study. *In vivo* experiments to depict the inhibitory effect of LR are still unknown. In addition, the accurate interaction between LR and the PI3K/AKT/mTOR pathway remains further study.

CONCLUSION

Lithospermum erythrorhizon root extract alleviates childhood acute leukemia by regulating PI3K/AKT/mTOR signaling pathway, suggesting that LR provides a potential therapeutic strategy for children with ALL.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors listed in this article. All liabilities related with the content of this article will be borne by the authors. Zhongxia Fu and Li Wang designed all the experiments and revised the paper. Wei Zhang and Jingjing Wang formed the experiments. Shengdong Zhu and Weideng wrote the manuscript.

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REFERENCES

1. *Rowley J. Molecular genetics in acute leukemia. Leukemia 2000; 14(3): 513.*
2. *Pui CH, Robison LL, Look AT. Acute lymphoblastic leukaemia. The Lancet 2008; 371(9617): 1030-1043.*
3. *Pui CH, Gajjar AJ, Kane JR, Qaddoumi IA, Pappo AS. Challenging issues in pediatric oncology. Nat Rev Clin Oncol 2011; 8(9): 540.*
4. *Marta GN, Murphy E, Chao S, Yu JS, Suh JH. The incidence of second brain tumors related to cranial irradiation. Expert Rev Anticancer Ther 2015; 15(3): 295-304.*
5. *Cheung YT, Eskind A, Inaba H, Hudson MM, Pui CH, Krull KR, Wolf J. Association of Bacteremic Sepsis With Long-term Neurocognitive Dysfunction in Pediatric Patients With Acute Lymphoblastic Leukemia. JAMA Pediatr 2018; 172(11): 1092-1095.*
6. *Boittin FX, Denis J, Mayol JF, Martigne P, Raffin F, Coulon D, Grenier N, Drouet M, Hérodin F. The extent of irradiation-induced long-term visceral organ damage depends on cranial/brain exposure. PloS one 2015; 10(4): e0122900.*
7. *Pui CH, Campana D, Pei D, Bowman WP, Sandlund JT, Kaste SC, Ribeiro RC, Rubnitz JE, Raimondi SC, Onciu M. Treating childhood acute lymphoblastic leukemia without cranial irradiation. New Eng Med 2009; 360(26): 2730-2741.*
8. *Cox CL, Montgomery M, Oeffinger KC, Leisenring W, Zeltzer L, Whitton JA, Mertens AC, Hudson MM, Robison LL. Promoting physical activity in childhood cancer survivors: results from the Childhood Cancer Survivor Study. Cancer 2009; 115(3): 642-654.*
9. *Kawata J, Kameda M, Miyazawa M. Cyclooxygenase-2 inhibitory effects and composition of the volatile oil from the dried roots of Lithospermum erythrorhizon. J Nat Med 2008; 62(2): 239-243.*
10. *Staniforth V, Wang SY, Shyur LF, Yang NS. Shikonins, phytocompounds from Lithospermum erythrorhizon, inhibit the transcriptional activation of human tumor necrosis factor α promoter in vivo. J Biol Chem 2004; 279(7): 5877-5885.*
11. *Shan ZL, Zhong L, Xiao CL, Gan LG, Xu T, Song H, Yang R, Li L, Liu BZ. Shikonin suppresses proliferation and induces apoptosis in human leukemia NB4 cells through modulation of MAPKs and c□Myc. Mol Med Rep 2017; 16(3): 3055-3060.*
12. *Zhao Q, Assimopoulou AN, Klauck SM, Damianakos H, Chinou I, Kretschmer N, Rios JL, Papageorgiou VP, Bauer R, Efferth T. Inhibition of c-MYC with involvement of ERK/JNK/MAPK and AKT pathways as a novel mechanism for shikonin and its derivatives in killing leukemia cells. Oncotarget 2015; 6(36): 38934.*
13. *Mao X, Yu CR, Li WH, Li WX. Induction of apoptosis by shikonin through a ROS/JNK-mediated process in Bcr/Abl-positive chronic myelogenous leukemia (CML) cells. Cell Res 2008; 18(8): 879.*
14. *Zhang B, Chen N, Chen H, Wang Z, Zheng Q. The critical role of redox homeostasis in shikonin-induced HL-60 cell differentiation via unique modulation of the Nrf2/ARE pathway. Oxid Med Cell Longev 2012; 2012: 781516.*
15. *Barrett D, Brown VI, Grupp SA, Teachey DT. Targeting the PI3K/AKT/mTOR signaling axis in children with hematologic malignancies. Pediatr Drugs 2012; 14(5): 299-316.*
16. *Wang Z, Zheng Py. Apigenin inhibits proliferation and migratory properties of Barrett's esophageal adenocarcinoma cells by targeting PI3K/Akt/mTOR pathway. Trop Pharm Res 2016; 15(11): 2365-2370.*
17. *Gao X, Wang Y, Zhang J, Lin L, Yao Q, Xiang G. Bergenin suppresses the growth of colorectal cancer cells by inhibiting PI3K/AKT/mTOR signaling pathway. Trop Pharm Res 2017; 16(10): 2307-2313.*
18. *Ikezoe T, Nishioka C, Bandobashi K, Yang Y, Kuwayama Y, Adachi Y, Takeuchi T, Koeffler HP, Taguchi H. Longitudinal inhibition of PI3K/Akt/mTOR signaling by LY294002 and rapamycin induces growth arrest of adult T-cell leukemia cells. Leukemia Res 2007; 31(5): 673-682.*
19. *Fuka G, Kantner H, Grausenburger R, Inthal A, Bauer E, Krapf G, Kaindl U, Kauer M, Dworzak M, Stoiber D. Silencing of ETV6/RUNX1 abrogates PI3K/AKT/mTOR signaling and impairs reconstitution of leukemia in xenografts. Leukemia 2012; 26(5): 927.*
20. *Martelli AM, Faenza I, Billi AM, Manzoli L, Evangelisti C, FalàF, Cocco L. Intranuclear 3'-phosphoinositide metabolism and Akt signaling: new mechanisms for tumorigenesis and protection against apoptosis? Cell Signal 2006; 18(8): 1101-1107.*
21. *Tamburini J, Green AS, Chapuis N, Bardet V, Lacombe C, Mayeux P, Bouscary D. Targeting translation in acute myeloid leukemia: a new paradigm for therapy? Cell Cycle 2009; 8(23): 3893-3899.*
22. *Guertin DA, Stevens DM, Saitoh M, Kinkel S, Crosby K, Sheen JH, Mullholland DJ, Magnuson MA, Wu H, Sabatini DM. mTOR complex 2 is required for the development of prostate cancer induced by Pten loss in mice. Cancer cell 2009; 15(2): 148-159.*
23. *Neri L, Cani A, Martelli A, Simioni C, Junghanss C, Tabellini G, Ricci F, Tazzari P, Pagliaro P, McCubrey J. Targeting the PI3K/Akt/mTOR signaling pathway in B-precursor acute lymphoblastic leukemia and its therapeutic potential. Leukemia 2014; 28(4): 739.*