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*Original Article*

# Hyperoside enhances the suppressive effects of arsenic trioxide on acute myeloid leukemia cells

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**Abstract:** Hyperoside (Hyp) is the chief component of some Chinese herbs which has anticancer effect and the present study is to identify whether it could enhance the anti leukemic properties of arsenic trioxide ($As_2O_3$) in acute myeloid leukemia (AML). We provide evidence on the concomitant treatment of HL-60 human AML cells with hyperoside potentiates $As_2O_3$-dependent induction of apoptosis. The activation of caspase-9, Bcl-2-associated agonist of cell death (BAD), p-BAD, p27 was assessed by Western blot. Results showed that hyperoside inhibited BAD from phosphorylating, reactivated caspase-9, and increased p27 levels. Importantly, hyperoside demonstrated its induction of autophagy effect by upregulation of LC-II in HL-60 AML cell line. Taken together, hyperoside may serve as a great candidate of concomitant treatment for leukemia; these effects were probably related to induction of autophagy and enhancing apoptosis-inducing action of $As_2O_3$.

**Keywords:** Arsenic trioxide, hyperoside, acute myeloid leukemia, apoptosis, autophagy

## Introduction

Arsenic trioxide ($As_2O_3$) exhibits potent anti-neoplastic activities, including anti-leukemic effects which have led this agent to be introduced to treat patients with acute promyelocytic leukemia (APL). One of the particularly effective strategies for acute myeloid leukemia (AML) is apoptosis-inducing therapy. However, resistance, relapse and toxicity result in the failure of treatment. One vital approach is the combination therapy [1].

Hyperoside (Hyp), which is quercetin-3-O-β-D-galactopyranoside, is a flavonol glycoside mainly contained in plants of the genera *Hypericum and Crataegus* [2]. Recently Hyp has demonstrated various pharmacological activities for chemotherapy of cancer both in vitro and in vivo [3, 4]. We hypothesized that Hyp could demonstrate non toxic effects for human, and it may be a potential candidate of adjuvant agents in combination therapy.

The purpose of the present study is to seek to determine what effects Hyp has on arsenic trioxide-induced anti-leukemic responses. We took great interest in examining whether Hyp is able to enhance arsenic trioxide-induced autophagy or apoptosis in particularly, as latest studies have suggested that besides apoptosis cell death, induction of autophagy also plays a vital role in generating arsenic-induced anti-leukemic effects.

## Materials and methods

### *Cells and reagents*

The HL-60 human myeloid leukemia cell line, cultured in RPMI 1640 medium which is supplemented with 10% fetal bovine serum and antibiotics, were acquired from Anhui academy of medical sciences. Arsenic trioxide ($As_2O_3$) and hyperoside (**Figure 1A**) were gotten from Sigma-Aldrich. Antibodies against PARP, LC3, p27, cleaved caspase-9, BAD, phospho-BAD (Ser136) were purchased from Cell Signaling Technology, Inc (USA). Antibodies against c-Abl and p62/SQSMT1 were acquired from Santa Cruz Biotechnology Inc. (USA), and antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were acquired from Millipore (USA).

### *Cell lysis and immunoblotting*

Cells were treated with the determinate doses of $As_2O_3$ for the designated times and then

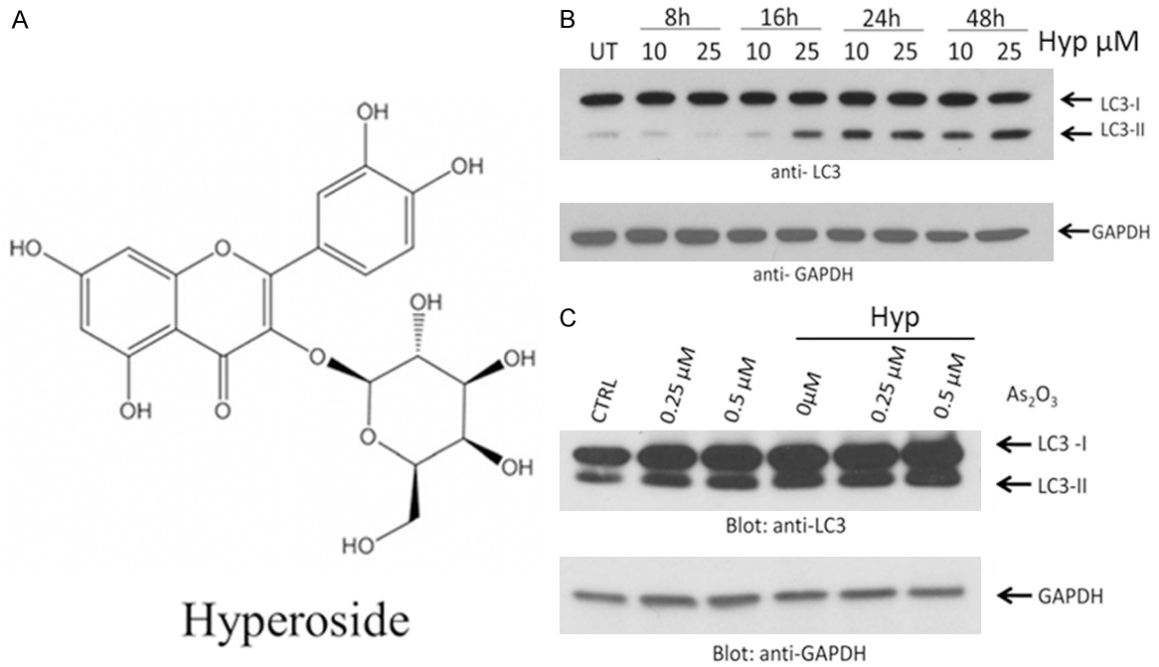


**Figure 1.** Structure of hyperoside and the induction of autophagy effect of hyperoside or $As_2O_3$. A. The chemical structure of hyperoside. B. HL-60 cells were treated with the indicated concentrations of hyperoside for the indicated times. Total cell lysates were resolved by SDS-PAGE and immunoblotted with anti-LC3 or anti-GAPDH antibodies, as indicated. C. HL-60 cells treated with $As_2O_3$ at the indicated concentrations for 24 h, in the presence or absence of hyperoside (25 μM), as indicated. Total cell lysates were resolved by SDS-PAGE and immunoblotted with anti-LC3 or anti-GAPDH antibodies, as indicated.

lysed in the phosphorylation lysis buffer as depicted earlier [5]. Immunoblotting was conducted using an enhanced chemiluminescence (ECL) method as depicted earlier [6].

*Cell proliferation assays*

Cells were treated with the determinate doses of $As_2O_3$, with or without hyperoside for the designated times. Cell proliferation assays were performed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) method as previously described [7].

*Analysis of apoptosis*

Cells were treated for as many as 48 hours with hyperoside (10, 20, 50 μmol/L), $As_2O_3$ (1-5 μmol/L), or combinations of hyperoside and $As_2O_3$. Cells ($1.0\times10^5$) which were harvested and cleaned twice using ice-cold PBS, and then resuspended in 100 μL binding buffer. Afterwards, 5 μL annexin V-FITC and 10 μL PI were added to the resuspended cells. 400 μL PBS was added and analyzed by flow cytometric analysis after incubating for 15 minutes at room temperature away from light.

*Western blot analysis*

Western blot analysis was applied to assess apoptosis-related proteins expression. Briefly, HL-60 cells ($1\times10^7$) were seeded in 6-well plates overnight, then treated with TBMS1 with four different concentrations of 0, 5, 10 and 15 μmol/L. The treatment should last for 24 hours. The solubilization and extraction of total proteins were achieved through lysis buffer (0.5 mM EDTA, 20 mM HEPES, 200 mM KCl, pH 7.9, 20% glycerol, 0.5% NP-40 and 1% protease inhibitor cocktail) after the treatment was finished. The determination of protein concentration was done through the usage of bicinchoninic acid (BCA) protein assay. In order to identify the expression levels of p27, cleaved caspase-9, β-actin proteins, BAD and phospho-BAD (Ser136), SDS-PAGE was used to separate all samples. It is through an ECL kit that blots were developed.

*Statistical analysis*

Statistical analysis was conducted through the SPSS 13.0 package (SPSS Inc., Chicago, IL, USA). All experiments were carried out more

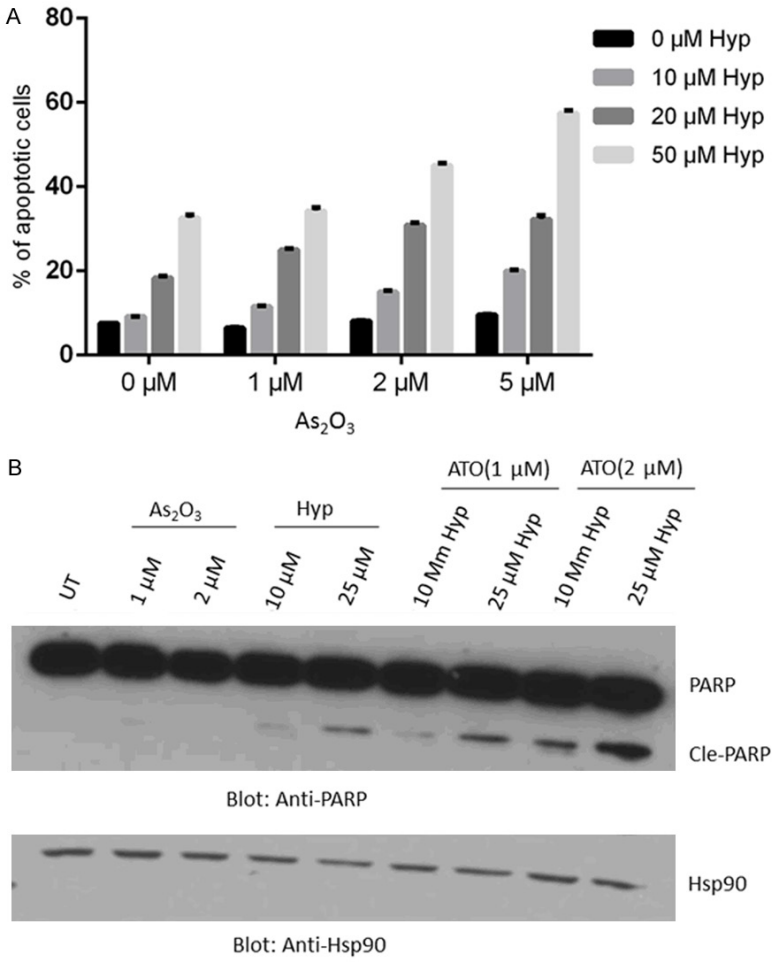


**Figure 2.** Combination treatment $As_2O_3$ and hyperoside induces apoptosis in a dose-dependent manner. A. cells were treated with the indicated concentrations of $As_2O_3$ and hyperoside for 48 h and subjected to flow cytometric analysis for annexin V staining. Means ± SE of 2 independent experiments are shown. B. HL-60 cells treated with $As_2O_3$ and hyperoside for 48 h at the indicated concentrations. Total cell lysates were resolved by SDS-PAGE and immunoblotted with anti-PARP or anti-HS P90 antibodies, as indicated.

than three times. All data were expressed as the mean ± SD. The Student's t-test and ANOVA were both used to test the significance of the data's statistical correlations. $P<0.05$ and $P<0.01$ were two criterions to suggest that there are statistically significant differences.

## Results

*Induction of autophagy by hyperoside*

Initially we examined the autophagy-induction effects of Hyp in leukemia cells. As we expected, treatment with Hyp led to upregulation of LC3-II in the HL-60 AML cell line (**Figure 1B**), and similarly, treatment with $As_2O_3$ led to upregulation of LC3-II as well (**Figure 1C**). However, no synergy was generated by the two agents in inducing the LC3-II expression, which is the marker of autophagy (**Figure 1C**).

*Hyperoside inhibited HL-60 cell proliferation*

Treatment of leukemic cells with $As_2O_3$ led to apoptosis induction, as shown by annexin V staining (**Figure 2A**). Such apoptosis induction was obviously increased through combinations of $As_2O_3$ with Hyp (**Figure 2A**). Immunoblotting experiments which were done by probing for the expression of the late apoptosis marker, poly (ADP-ribose) polymerase (PARP) and its cleaved isoform (**Figure 2B**) also achieved the similar results.

*Hyperoside inhibited HL-60 cell viability*

To make researches on Hyp's apoptosis-inducing effect, different concentrations of Hyp & $As_2O_3$ were used to the treatment of the HL-60 cells. Just like we expected, as doses of Hyp & $As_2O_3$ increased, cell viability of HL-60 cells was decreased with a record of remarkable effects even at low Hyp concentrations at either 48 h or 120 h (**Figure 3A** and **3B**).

*Effect of hyperoside on expression levels of the caspase-9*

To illustrate the mechanism of Hyp induced apoptosis, we assessed the involvement of caspase-9. Western blot analysis, as depicted in **Figure 4**, compared with the control, the protein level of cleaved caspase-9 was slightly increased with Hyp, while considerable increase was shown in the HL-60 cells treated with the Hyp-$As_2O_3$ combinations.

*Effect of hyperoside on expression levels of the Bcl-2 gene family*

To gain more information about the Hyp-induced apoptotic mechanism, we next evaluated the protein expression of the pro-apoptot-

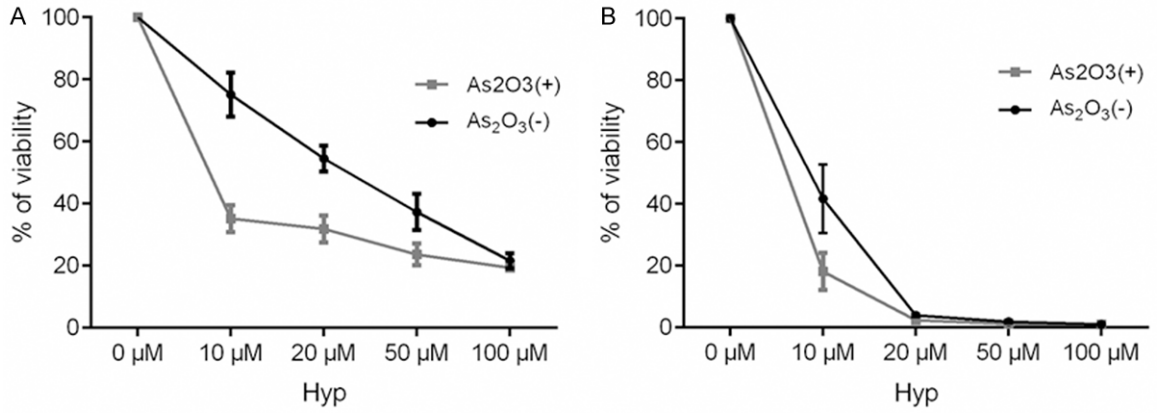


**Figure 3.** Combination treatment of $As_2O_3$ and hyperoside reduces cell viability in a dose-dependent manner. HL-60 cells were incubated in the presence or absence of $As_2O_3$ (2 μM) and the indicated concentrations of hyperoside for either 48 h (A) or 120 h (B). Cell viability was determined by an MTT assay. Data are expressed as a percentage of viability of control untreated samples. Means ± SE of 2 independent experiments are shown.

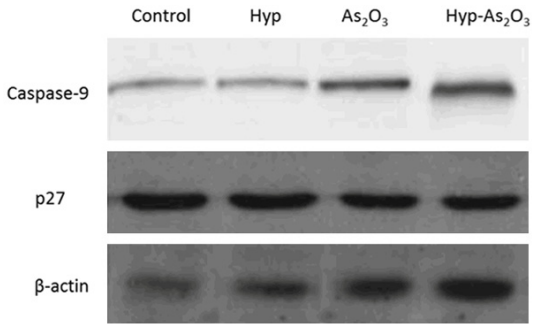


**Figure 4.** Analysis of Caspase-9 and p27 expression by western blot in HL-60 cell line.

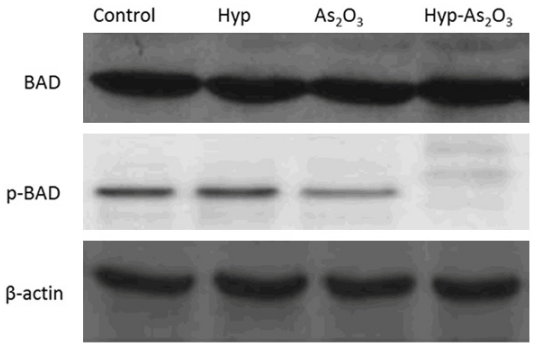


**Figure 5.** Analysis of BAD and p-BAD expression by western blot in HL-60 cell line.

ic protein BAD and phosphorylated BAD by western blot analysis. As can be seen in **Figure 5**, regardless of constant levels of the BAD protein, phosphorylated BAD level was decreased after single drug & combination treatment.

## Discussion

$As_2O_3$ has clinical potentials for treatment of different types of leukemia, however, its clinical potentials are greatly restricted due to the mechanisms of leukemic cell resistance and simultaneous activation of negative feedback regulatory pathways which impose restrictions on induction of arsenic biologic responses. More and more attention has been paid to the use of natural products isolated from Chinese medicinal herbs for leukemia therapy [8-10]. Hyperoside was proved to have anti-tumor activity towards various human cancer cell lines and xenograft systems of human tumors, making it an excellent candidate for new anti-cancer agents [11-13].

Previous research has indicated that signaling pathway of AKT/mTOR is a negative feedback regulators activated in the process of responding to treatment of leukemia cells with arsenic trioxide underscoring the complication of the mechanisms of leukemic cell resistance [14]. The simultaneous involvement of diversified feedback loops gives challenges for treatment and causes selective targeting of leukemic cells to be difficult and limited. Thus it is of great importance to make efforts to research and develop combinations of arsenic trioxide with other agents which boost anti-leukemic effects through unique action mechanisms, particularly when the demonstrated arsenic capacity to eliminate leukemic cells works

effectively even under low dosage is taken into account [15].

The present study proved that t hyperoside sensitizes cell lines of AML leukemia to the effects of $As_2O_3$ in vitro and revealed that hyperoside enhances $As_2O_3$'s inhibitory effects on primary AML cells in vitro. Our researches show that such effects of hyperoside are likely to reflect that pro-apoptotic properties of arsenic trioxide was potentiated and arsenic-dependent autophagic cell death do not seem to be modulated. It should be noted that previous study has proven that both apoptosis induction and induction of autophagy are essential for $As_2O_3$ to generate inhibitory effects on primitive leukemic precursors [16-18]. This was revealed by experiments indicating that pharmacological inhibitors of autophagy or apoptosis is capable of reversing arsenic's suppressive effects on leukemic clonogenic assays in methylcellulose to some degree. Although current study is unable to demonstrate the strengthening effect of hyperoside on arsenic-dependent induction of an autophagic state, which can be owed to the mutual effect between the various pathways of cell death occurring simultaneously. Future studies need to illuminate the issue and determine the specific components of pro-apoptotic or autophagic mechanisms that are modified and probably be responsible for the strengthening effects of hyperoside. However, our research findings proved that there is great potential for combination therapy to enhance the effects of arsenic on primitive AML cells and further efforts in that direction are warranted.

Hyperoside is also called quercetin-3-O-arabinoglucoside, and previous studies showed that glycosylation site the C-3 position own well-documented antiviral, anti-inflammatory as well as immunosuppressive activities [19-21]. Given our results, hyperoside seems to be effective in terms of against acute leukemia cells. Noteworthy, Literatures reported that hyperoside could reduce cardiotoxicity and nephrotoxicity caused by $As_2O_3$ treatment [22], evidences also showed that hyperoside is able to enhance the anti-leukemic effects of imatinib mesylate on CML cells [23]. Taken together, hyperoside induced cell arrest and cell apoptosis in HL-60 cells and enhanced the effects induced by low concentrations of $As_2O_3$, which was related with upregulation of p27, activation of caspace-9, and inhibition of BAD phosphorylation. Whether the effects were directly related with the modulation of autophagy, further study is required. However our data provide evidence that hyperoside may have potential to serve as a promising adjuvant in the combination therapy for acute myeloid leukemia.

## Disclosure of conflict of interest

None.

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## References

[1] Alimoghaddam K. A review of arsenic trioxide and acute promyelocytic leukemia. Int J Hematol Oncol Stem Cell Res 2014; 8: 44-54.

[2] Kim SJ, Um JY, Lee JY. Anti-inflammatory activity of hyperoside through the suppression of nuclear factor-κB activation in mouse peritoneal macrophages. Am J Chin Med 2011; 39: 171-81.

[3] Park SH, Kim HJ, Yim Sheet, Kim AR, Tyagi N, Shen H, Kim KK, Shin BA, Jung DW, Williams DR. Delineation of the role of glycosylation in the cytotoxic properties of quercetin using novel assays in living vertebrates. J Nat Prod 2014; 77: 2389-96.

[4] Ku SK, Zhou W, Lee W, Han MS, Na M, Bae JS. Anti-inflammatory effects of hyperoside in human endothelial cells and in mice. Inflammation 2015; 38: 784-99.

[5] Giafis N, Katsoulidis E, Sassano A, Tallman MS, Higgins LS, Nebreda AR, Davis RJ, Platanias LC. Role of the p38 mitogen-activated protein kinase pathway in the generation of arsenic trioxide-dependent cellular responses. Cancer Res 2006; 66: 6763-71.

[6] Uddin S, Sassano A, Deb DK, Verma A, Majchrzak B, Rahman A, Malik AB, Fish EN, Platanias LC. Protein kinase C-δ (PKC-δ) is activated by type I interferons and mediates phosphorylation of Stat1 on serine 727. J Biol Chem 2002; 277: 14408-16.

[7] Galvin JP, Altman JK, Szilard A, Goussetis DJ, Vakana E, Sassano A, Platanias LC. Regulation of the kinase RSK1 by arsenic trioxide and generation of antileukemic responses. Cancer Biol Ther 2013; 14: 411-6.

[8] Nakazato T, Sagawa M, Kizaki M. Triptolide induces apoptotic cell death of multiple myeloma cells via transcriptional repression of Mcl-1. Int J Oncol 2014; 44: 1131-8.

[9] Hui H, Chen Y, Yang H, Zhao K, Wang Q, Zhao L, Wang X, Li Z, Lu N, Guo Q. Oroxylin A has thera-

peutic potential in acute myelogenous leukemia by dual effects targeting PPARγ and RXRα. Int J Cancer 2014; 134: 1195-206.
[10] Cheng X, Xiao Y, Wang X, Wang P, Li H, Yan H, Liu Q. Anti-tumor and pro-apoptotic activity of ethanolic extract and its various fractions from Polytrichum commune L.ex Hedw in L1210 cells. J Ethnopharmacol 2012; 143: 49-56.
[11] Yang FQ, Liu M, Li W, Che JP, Wang GC, Zheng JH. Combination of quercetin and hyperoside inhibits prostate cancer cell growth and metastasis via regulation of microRNA-21. Mol Med Rep 2015; 11: 1085-92.
[12] Li W, Liu M, Xu YF, Feng Y, Che JP, Wang GC, Zheng JH. Combination of quercetin and hyperoside has anticancer effects on renal cancer cells through inhibition of oncogenic microRNA-27a. Oncol Rep 2014; 31: 117-24.
[13] Kim HI, Quan FS, Kim JE, Lee NR, Kim HJ, Jo SJ, Lee CM, Jang DS, Inn KS. Inhibition of estrogen signaling through depletion of estrogen receptor alpha by ursolic acid and betulinic acid from Prunella vulgaris var. lilacina. Biochem Biophys Res Commun 2014; 451: 282-7.
[14] Altman JK, Yoon P, Katsoulidis E, Kroczynska B, Sassano A, Redig AJ, Glaser H, Jordan A, Tallman MS, Hay N, Platanias LC. Regulatory effects of mammalian target of rapamycin-mediated signals in the generation of arsenic trioxide responses. J Biol Chem 2008; 283: 1992-2001.
[15] Beauchamp EM, Kosciuczuk EM, Serrano R, Nanavati D, Swindell EP, Viollet B, O'Halloran TV, Altman JK, Platanias LC. Direct binding of arsenic trioxide to AMPK and generation of inhibitory effects on acute myeloid leukemia precursors. Mol Cancer Ther 2015; 14: 202-12.
[16] Duan XF, Wu YL, Xu HZ, Zhao M, Zhuang HY, Wang XD, Yan H, Chen GQ. Synergistic mitosis-arresting effects of arsenic trioxide and paclitaxel on human malignant lymphocytes. Chem Biol Interact 2010; 183: 222-30.
[17] Seo T, Urasaki Y, Takemura H, Ueda T. Arsenic trioxide circumvents multidrug resistance based on different mechanisms in human leukemia cell lines. Anticancer Res 2005; 25: 991-8.
[18] Szymanska B, Wilczynska-Kalak U, Kang MH, Liem NL, Carol H, Boehm I, Groepper D, Reynolds CP, Stewart CF, Lock RB. Pharmacokinetic modeling of an induction regimen for in vivo combined testing of novel drugs against pediatric acute lymphoblastic leukemia xenografts. PLoS One 2012; 7: e33894.
[19] Choi JH, Kim DW, Yun N, Choi JS, Islam MN, Kim YS, Lee SM. Protective effects of hyperoside against carbon tetrachloride-induced liver damage in mice. J Nat Prod 2011; 74: 1055-60.
[20] Lee S, Park HS, Notsu Y, Ban HS, Kim YP, Ishihara K, Hirasawa N, Jung SH, Lee YS, Lim SS, Park EH, Shin KH, Seyama T, Hong J, Ohuchi K. Effects of hyperin, isoquercitrin and quercetin on lipopolysaccharide-induced nitrite production in rat peritoneal macrophages. Phytother Res 2008; 22: 1552-6.
[21] Huo Y, Yi B, Chen M, Wang N, Chen P, Guo C, Sun J. Induction of Nur77 by hyperoside inhibits vascular smooth muscle cell proliferation and neointimal formation. Biochem Pharmacol 2014; 92: 590-8.
[22] Zhou L, An XF, Teng SC, Liu JS, Shang WB, Zhang AH, Yuan YG, Yu JY. Pretreatment with the total flavone glycosides of Flos Abelmoschus manihot and hyperoside prevents glomerular podocyte apoptosis in streptozotocin-induced diabetic nephropathy. J Med Food 2012; 15: 461-8.
[23] Hosseinimehr SJ, Azadbakht M, Tanha M, Mahmodzadeh A, Mohammadifar S. Protective effect of hawthorn extract against genotoxicity induced by methyl methanesulfonate in human lymphocytes. Toxicol Ind Health 2011; 27: 363-9.