JBUON 2019; 24(5): 2000-2005
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Antiproliferative activity of plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) in human gastric carcinoma cells is facilitated via activation of autophagic pathway, mitochondrial-mediated programmed cell death and inhibition of cell migration and invasion

Xiuli Ma[1], Xiuping Yin[2], Hu Liu[3], Qingming Chen[1], Yuanzhong Feng[1], Xiaoyun Ma[4], Weichang Liu[3]

[1]Department of Pathology, WuWei People's Hospital, WuWei, GanSu, 733000, China; [2]Department of Science and Education Section, WuWei People's Hospital, WuWei, GanSu, 733000, China; [3]Department of General Surgery, WuWei People's Hospital, WuWei, GanSu, 733000, China; [4]Department of Digestive Medicine, WuWei People's Hospital, WuWei, GanSu, 733000, China

Summary

Purpose: *Gastric cancer causes high mortality rates across the globe, mainly due to late diagnosis and the unavailability of effective chemotherapeutic agents. This study evaluated the anticancer potential of plumbagin against gastric cancer cells as well as its effects on autophagic and apoptotic pathways, cell migration and invasion.*

Methods: *MTT assay was used for cell viability assessment. Acridine orange (AO)/ethidium bromide (EB) and annexin V/propidium iodide (PI) staining were used for the detection of apoptosis. Autophagy was demonstrated by electron microscopy. Transwell assay was used for cell migration and invasion. Western blotting was used for the detection of protein expression.*

Results: *The results showed that plumbagin could considerably inhibit the proliferation of AGS gastric cancer cells (IC_{50};8 μM). The anticancer activity of plumbagin against AGS cells was found to be due to the induction of autophagy and apoptosis. Plumbagin-induced apoptosis and autophagy were also associated with alteration in apoptosis (Bax and Bcl-2) and autophagy (LC3I, II, and Beclin 1) - related protein expressions. The effects of plumbagin on the migration and invasion of AGS cells were also investigated by transwell assays and the results showed that plumbagin inhibited both the migration and invasion of AGS cells at IC_{50}.*

Conclusions: *These results indicate that plumbagin significantly inhibits the growth of gastric cancer in vitro and could prove beneficial in the management of gastric cancer and needs further research including in vivo studies.*

Key words: *gastric carcinoma, plumbagin, apoptosis, autophagy, Bax*

Introduction

Gastric cancer accounts for high cancer-related mortality. In 2002 alone, gastric cancer caused 0.65 million deaths and 0.9 million new cases were detected [1,2]. It has been reported that gastric cancer incidence varies with geography; however, developing countries have comparatively higher incidence [3,4]. Although recent reports show considerable improvement in the five-year survival, survival rates are still lower than in other cancers [5]. Lack of effective chemotherapeutic drugs and therapeutic targets hinders gastric cancer treatment [6]. Consequently, there is increasing drive

Corresponding author: Weichang Liu, MD. Department of General Surgery, WuWei People's Hospital, WuWei, GanSu, 733000, China
Tel & Fax: +86 0935-5820132, Email: leekylie618@yahoo.com
Received: 19/01/2019; Accepted: 07/02/2019

to identify new chemotherapeutic agents that could be utilized for the development of gastric cancer systemic therapy. Terrestrial plants have served as a great reservoir for drugs and continue to provide effective lead molecules for the development of anticancer agents via semi-synthetic approaches [7]. Plumbagin is an important chemical scaffold of plant origin. It has been shown to exhibit a wide diversity of bioactivities including anticancer, antibacterial and antifungal activities [8]. Plumbagin has been shown to halt the growth of non-small lung cancer cells via multiple mechanisms [9]. In breast cancer cells, plumbagin inhibits PI-5 kinase to induce its cytotoxic effects [10]. Plumbagin has been shown to cause apoptotic cell death of lung cancer cells [11]. In melanoma cells, plumbagin induces ROS-mediated apoptosis to suppress the proliferation of cancer cells [12]. In yet another study, plumbagin has been reported to induce mitochondrial apoptosis in breast cancer cells [13]. Although the anticancer effects of plumbagin have been studied against gastric cancer cells [14], the molecular mechanisms behind its anticancer effects on gastric cancer cells is yet to be fully explored. This study was therefore conducted in order to investigate the anticancer effects of plumbagin against gastric cancer cells and explore its underlying mechanism. The main aim of the current study was to investigate the antiproliferative potential of plumbagin (which is a naturally occurring napthoquinone) in human gastric cancer cells along with evaluating its effects on cell autophagy, mitochondrial-mediated cell death, and cell migration and invasion.

Methods

Cell counting kit-8 (CCK-8) assay

AGS gastric cancer cells in each group were inoculated in a 96-well plate, subjected to treatment with plumbagin at various concentrations and the number of HL-60 cells was measured at each concentration. The procedures were as follows: the Dulbecco's Modified Eagle Medium (DMEM) culture medium was discarded and 100 uL CCK-8 reagents (Beyotime Institute of Biotechnology (Shanghai, China) were added to a fresh DMEM medium. The 96-well plate was incubated in a carbon dioxide incubator for 2 h. The optical density (OD) values were measured by a microplate reader at 450 nm wavelength. The cell proliferation rate (%) was calculated as follows: OD value of experimental well -OD value of control well)/OD value of control well × 100%.

AO/EB staining for apoptosis

AGS gastric cancer cells (0.6×10^6) were grown in 6-well plates. Following an incubation of around 12 h, HL-60 cells were subjected to plumbagin treatment for 24 h at 37°C. Next, 25 µl cell cultures were put onto glass slides and stained with a solution (1 µl) of AO and EB. The slides were cover-slipped and examined under a fluorescent microscope. Annexin V/PI staining was performed as described previously [15].

Cell migration and invasion assay

The migration and invasion abilities of AGS cells were examined by transwell chamber assay. Briefly, 1×10^4 HL-60 cells were kept in the upper chamber with transwells of 8 µM pores. RPIM medium was placed in the lower chamber and incubated for 24 h at 37°C. Extracellular matrix gel was used for cell invasion assay. The non-migrated and non-invaded cells were removed by swabbing. The cells that invaded and migrated to the lower side were fixed and stained by crystal violet and finally observed under microscope.

Transmission electron microscopy (TEM)

For electron microscopy, AGS cells were fixed in the solution of 4% glutaraldehyde 0.05 M sodium cacodylate, postfixed in 1.5% OsO4, and dehydrated in alcohol. They were then prepared for flat embedding in Epon 812 and observed using a Zeiss CEM 902 electron microscope.

Western blot analysis

Western blotting was performed to estimate the expression of the proteins of interest. The plumbagin-treated AGS cells were harvested with centrifugation.

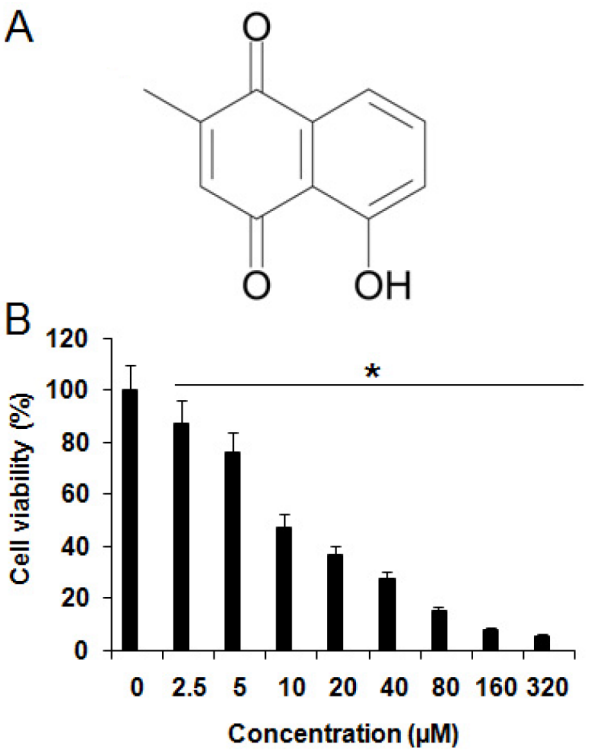

Figure 1. A: Chemical structure of plumbagin. **B:** CCK-8 assay showing the effect of plumbagin on the viability of AGS gastric cancer cells. The experiments were performed in triplicate and shown as mean ± SD (*p<0.01).

They were then lysed in lysis buffer containing the protease inhibitor. Around 45 μg of proteins from each sample were subjected to separation 10% and transferred to polyvinylidene difluoride (PVDF) membrane. Next, fat-free milk was used to block the membrane at room temperature for 1 h. Afterwards, the membranes were treated with primary antibodies at 4°C overnight. Subsequently, the membranes were subjected to incubation with secondary antibodies. Finally, the signal was detected by Odyssey Infrared Imaging System (LI-COR, USA). Actin was used as control for normalization.

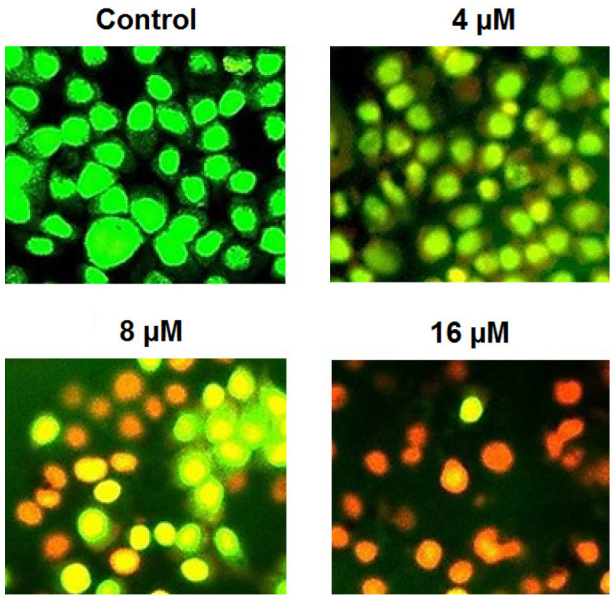

Figure 2. AO/EB staining showing the induction of apoptosis in AGS gastric cancer cells at indicated concentrations of plumbagin. The experiments were performed in triplicate. The apoptotic cells emit yellow and orange fluorescence and the figure shows that the percentage of these apoptotic cells increased with increasing plumbagin dose.

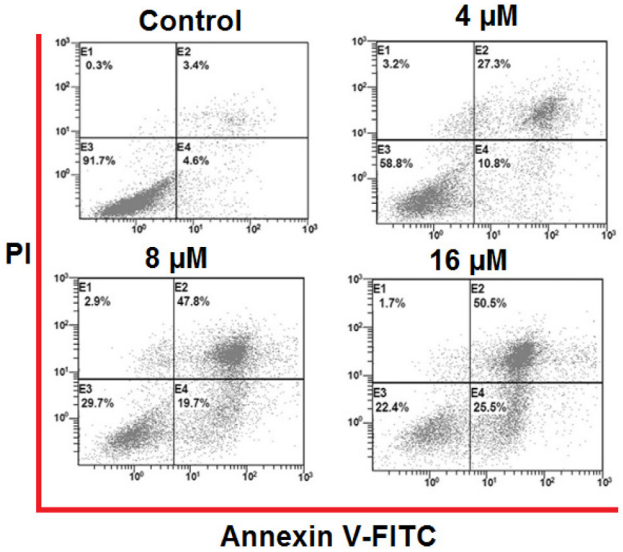

Figure 3. Annexin V/PI staining showing the percentage of apoptotic AGS cells at indicated concentrations of plumbagin. The experiments were performed in triplicate. This assay was used to quantify the percentage of apoptotic cells. The percentage of apoptotic AGS cells increased from 4.6 in control to 25.5% at 16 μM concentration of plumbagin.

Results

Plumbagin suppressed the growth of gastric cancer cells

The CCK-8 assay was employed to investigate the growth inhibitory effects of plumbagin (Figure 1A) in AGS gastric cancer cells at concentrations ranging from 0 to 320 μM. It was revealed that plumbagin significantly suppressed the viability of AGS gastric cancer cells (Figure 1B). The IC_{50} of plumbagin against AGS gastric cancer cells was found to be 8 μM. Moreover, the growth inhibitory effects of plumbagin on AGS gastric cancer cells were found to be concentration-dependent.

Plumbagin triggered apoptotic cell death of AGS gastric cancer cells

Since plumbagin inhibited the growth of AGS gastric cancer cells and exhibited a very low IC_{50} against them, we sought to ascertain the molecular mechanism behind it. Therefore, plumbagin-treated AGS cells were subjected to staining with AO/EB. The results showed that as the concentration of plumbagin increased, the number of orange colored cells increased, indicative of apoptosis (Figure 2). The annexin V/PI staining further confirmed that plumbagin induces apoptosis in AGS cells. The percentage of apoptotic AGS cells increased from 4.6 in control to 25.5% at 16 μM concentration of plumbagin (Figure 3). The effects of plumbagin on the expression of Bax and Bcl-2 were also examined and the results showed that plumbagin caused considerable increase in the expression of Bax. How-

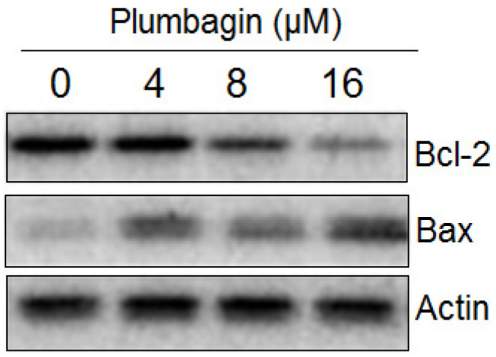

Figure 4. Effect of plumbagin on the expression of Bax and Bcl-2 as depicted by Western blot analysis. The experiments were performed in triplicate. Plumbagin led to increase in the expression of Bax and to decrease in the expression of Bcl-2 indicating that this molecule targets the apoptosis-related proteins within the cancer cells.

ever, plumbagin caused considerable decrease in the expression of Bcl-2 (Figure 4).

Plumbagin caused autophagy in AGS gastric cancer cells

The effects of plumbagin on AGS cells were also examined by electron microscopy. The results showed that plumbagin led to the development of autophagosomes in AGS gastric cancer cells concentration-dependently. These results suggested the induction of autophagic cell death in AGS gastric cancer cells. Autophagy was therefore confirmed by examining the expression of autophagy-related proteins by Western blotting. It was found that plumbagin caused considerable increase in the expression of LC3 II while no apparent effects were observed on the expression of LC3 I. Moreover, plumbagin also caused remarkable

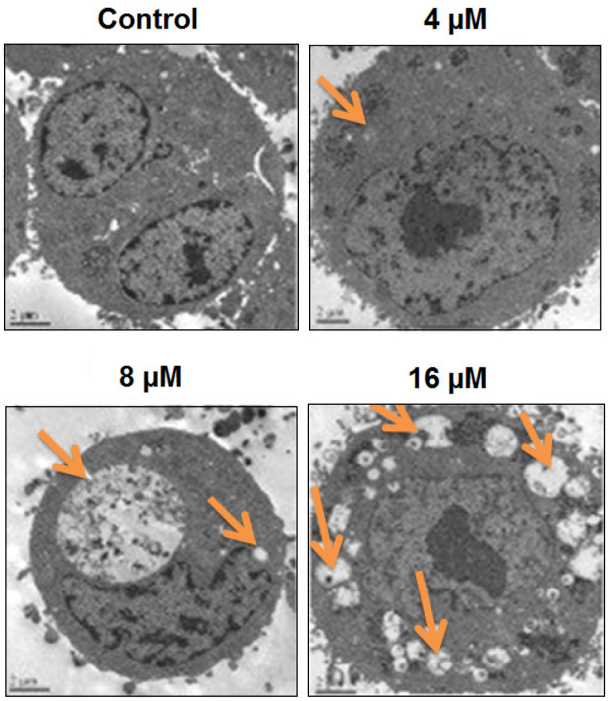

Figure 5. Electron microscopy analysis showed that plumbagin induces autophagy in AGS gastric cancer cells (Arrows depict autophagosomes). The experiments were performed in triplicate. Plumbagin treatment at increasing doses led to dose-dependent formation of autophagosomes indicating onset of autophagic cell death.

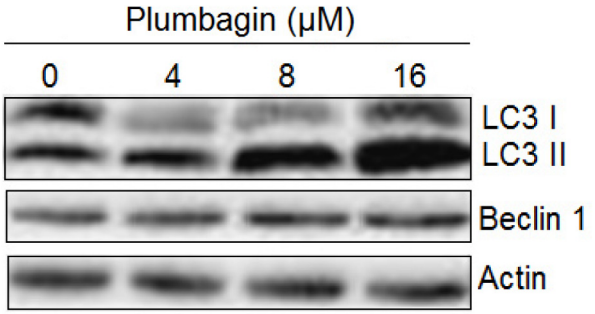

Figure 6. Effect of plumbagin on the expression of autophagy-related proteins. The experiments were performed in triplicate. Plumbagin treatment led to increase in the expression of LC3-I, LC3-II and Beclin-1 indicating that this molecule targets autophagy-related proteins.

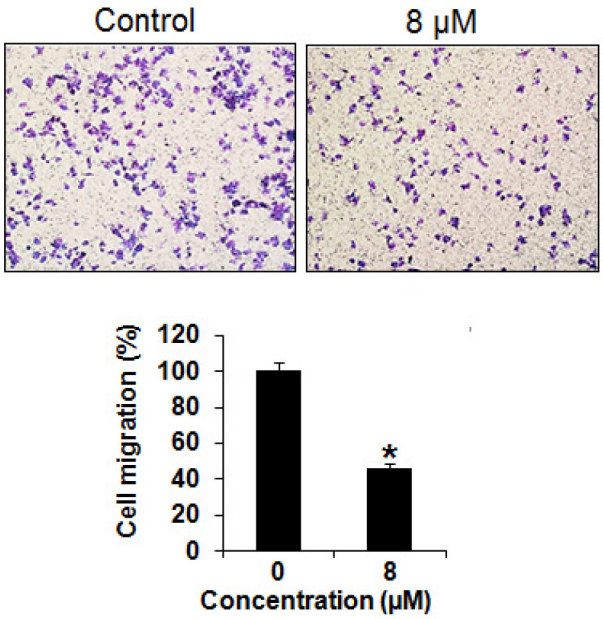

Figure 7. Effect of plumbagin on the migration of AGS gastric cancer cells as depicted by transwell assay. The experiments were performed in triplicate and shown as mean ± SD (*p<0.01). The figure shows that 8 μM dose of plumbagin led to significant inhibition of cell migration.

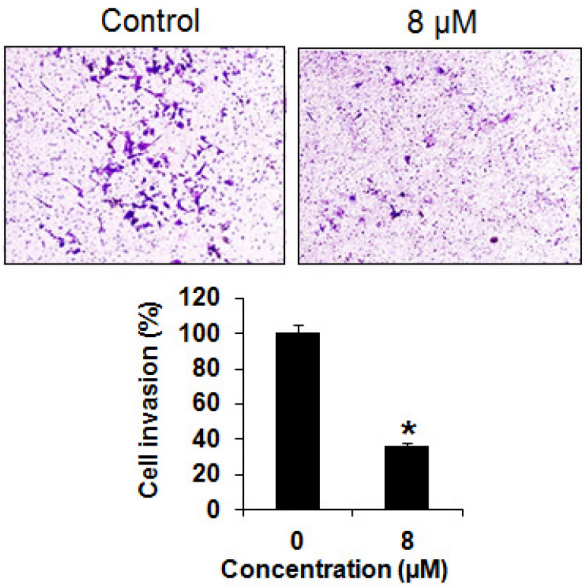

Figure 8. Effect of plumbagin on the invasion of AGS gastric cancer cells as depicted by transwell assay. The figure shows that 8 μM dose of plumbagin led to significant inhibition of cell invasion. The experiments were performed in triplicate and shown as mean ± SD (*p<0.01).

and dose-dependent increase in the expression of Beclin-1.

Plumbagin inhibited the migration and invasion of AGS cells

The effects of plumbagin on the migration of AGS gastric cancer cells were monitored by transwell chamber assays. It was found that plumbagin treatment could considerably inhibit the migration of cancer cells in a dose-dependent manner (Figure 7).

Plumbagin suppressed the invasion of AGS cells

Transwell assay was also used to examine the effects of plumbagin on the invasion ability of AGS gastric cancer cells. The results showed that plumbagin treatment could significantly decrease the invasion ability of AGS gastric cancer cells (Figure 8).

Discussion

Although there have been recent improvements in the overall survival rate of gastric cancer, it continues to cause very high mortality rates all over the world [16]. For instance, half of the cancer cases detected in East Asian countries are gastric cancers [17]. The currently used chemotherapy for gastric cancer has negative effects on patient health and effective therapeutic targets are lacking [17]. In this study, the anticancer effects of plumbagin were investigated against AGS gastric cancer cells and the results showed that plumbagin inhibited their growth and exhibited an IC_{50} of 8 μm. The analysis of the underlying mechanism revealed that plumbagin induced apoptotic cell death of AGS gastric cancer cells, which was also accompanied by an increase in Bax and a decrease in Bcl-2 expression. Apoptosis is one of the important mechanisms by which anticancer agents stop the growth of cancer cells and it also prevents the development of drug resistance [18]. Plumbagin has also been previously shown to induce apoptosis in cancer cells. For example, plumbagin induced apoptosis of HER2-overexpressing breast cancer cells [13]. Moreover, it has also been shown to induce apoptosis in promyelocytic leukemia cells [19]. Plumbagin also caused significant changes in the nuclear morphology of AGS cancer cells, as indicated by electron microscopy, and led to the development of autophagosomes, which are the hallmarks of autophagy [20]. The fact that plumbagin induced autophagy in AGS gastric cancer cells was further confirmed by Western blot analysis and the results, which showed that plumbagin increased the expression of LC3 II and Beclin 1. Nonetheless, the expression of LC3 I remained almost unaltered. Previous studies support our results as plumbagin has been reported to induce autophagy in breast cancer cells by targeting the AKT signaling pathway [20]. Moreover, the migration and invasion of cancer cells is considered imperative for cancer cell metastasis. Therefore, the anti-metastatic potential of plumbagin was examined by transwell and we observed that this molecule inhibited both the migration and invasion of cancer cells.

Conclusion

The findings of the present study showed that plumbagin inhibits the growth of gastric cancer cells via induction of apoptosis and autophagy. Moreover, plumbagin could also inhibit the migration and invasion of gastric cancer cells, indicative of its significant anticancer potential.

Conflict of interests

The authors declare no conflict of interests.

References

1. Venerito M, Vasapolli R, Rokkas T, Malfertheiner P. Gastric cancer: epidemiology, prevention, and therapy. Helicobacter 2018;23:e12518.
2. Sitarz R, Skierucha M, Mielko J, Offerhaus GJ, Maciejewski R, Polkowski WP. Gastric cancer: epidemiology, prevention, classification, and treatment. Cancer Manage Res 2018;10:239.
3. Crew KC, Neugut AI. Epidemiology of gastric cancer. World J Gastroenterol 2006;12:354-62.
4. Karimi P, Islami F, Anandasabapathy S, Freedman ND, Kamangar F. Gastric cancer: descriptive epidemiology, risk factors, screening, and prevention. Cancer Epidemiol Prevent Biomarkers 2014;23:700-13.
5. Fock KM. The epidemiology and prevention of gastric cancer. Aliment Pharmacol Therap 2014;40:250-60.
6. Smyth EC, Verheij M, Allum W, Cunningham D, Cervantes A, Arnold D. Gastric cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2016;27(Suppl_5):v38-49.
7. Mann J. Natural products in cancer chemotherapy: past, present and future. Nat Rev Cancer 2002;2:143.
8. Krishnaswamy M, Purushothaman KK. Plumbagin: a

study of its anticancer, antibacterial and antifungal properties. Indian J Experim Biol 1980;18:876-7.

9. Gomathinayagam R, Sowmyalakshmi S, Mardhatillah F, Kumar RA, Akbarsha MA, Damodaran C. Anticancer mechanism of plumbagin, a natural compound, on non-small cell lung cancer cells. Anticancer Res 2008;28:785-92.
10. Lee JH, Yeon JH, Kim H et al. The natural anticancer agent plumbagin induces potent cytotoxicity in MCF-7 human breast cancer cells by inhibiting a PI-5 kinase for ROS generation. PloS One 2012;7:e45023.
11. Hsu YL, Cho CY, Kuo PL, Huang YT, Lin CC. Plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone) induces apoptosis and cell cycle arrest in A549 cells through p53 accumulation via c-Jun NH2-terminal kinase-mediated phosphorylation at serine 15 in vitro and in vivo. J Pharmacol Experim Therap 2006;318:484-94.
12. Wang CC, Chiang YM, Sung SC, Hsu YL, Chang JK, Kuo PL. Plumbagin induces cell cycle arrest and apoptosis through reactive oxygen species/c-Jun N-terminal kinase pathways in human melanoma A375. S2 cells. Cancer Lett 2008;259:82-98.
13. Kawiak A, Zawacka-Pankau J, Lojkowska E. Plumbagin induces apoptosis in Her2-overexpressing breast cancer cells through the mitochondrial-mediated pathway. J Nat Products 2012;75:747-51.
14. Li J, Shen L, Lu FR et al. Plumbagin inhibits cell growth and potentiates apoptosis in human gastric cancer cells in vitro through the NF-κB signaling pathway. Acta Pharmacol Sin 2012;33:242.
15. Hua F, Li CH, Chen XG, Liu XP. Daidzein exerts anticancer activity towards SKOV3 human ovarian cancer cells by inducing apoptosis and cell cycle arrest, and inhibiting the Raf/MEK/ERK cascade. Int J Molec Med 2018;41:3485-92.
16. Ang TL, Fock KM. Clinical epidemiology of gastric cancer. Singapore Med J 2014;55:621.
17. Correa P (Ed). Diet and gastric cancer. In: Diet, Nutrition and Cancer: A Critical Evaluation. CRC Press, 2018, pp 1-10.
18. Fisher DE. Apoptosis in cancer therapy: crossing the threshold. Cell 1994;78:539-42.
19. Xu KH, Lu DP. Plumbagin induces ROS-mediated apoptosis in human promyelocytic leukemia cells in vivo. Leukemia Res 2010;34:658-65.
20. Kuo PL, Hsu YL, Cho CY. Plumbagin induces G2-M arrest and autophagy by inhibiting the AKT/mammalian target of rapamycin pathway in breast cancer cells. Mol Cancer Ther 2006;5:3209-21.
21. Lin C, Hu Z, Yuan G et al. MicroRNA-1179 inhibits the proliferation, migration and invasion of human pancreatic cancer cells by targeting E2F5. Chem Biol Interact 2018;291:65-71.