JBUON 2019; 24(5): 1950-1955
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Psoralidin inhibits the proliferation of human liver cancer cells by triggering cell cycle arrest, apoptosis and autophagy and inhibits tumor growth *in vivo*

Jia Li[1]*, Yongming Fu[2]*, Xingwang Hu[2], Yinghui Xiong[2]

[1]Department of Emergency, Xiangya Hospital Central South University, Changsha, Hunan 410008, China; [2]Department of Infectious Diseases, Xiangya Hospital Central South University, Changsha, Hunan 410008, China.

*These two authors contributed equally to this study.

Summary

Purpose: *Liver cancer is a lethal malignancy with high mortality. Approximately 0.56 million new cases of liver cancer are reported annually. The adverse effects of currently available inefficient chemotherapy remarkably obstruct the treatment of liver cancer. This study was undertaken to investigate the anticancer effects of a natural coumarin, Psoralidin, in vitro and in vivo.*

Methods: *The liver HepG2 cancer cell line was used in this study. The MTT cell viability assay was used for the determination of the proliferation rate. Flow cytometry was used for cell cycle analysis. DAPI was used for detection of apoptosis and transmission electron microscopy (TEM) analysis was performed for the demonstration of autophagy. Protein expression was estimated by western blot analysis.*

Results: *Psoralidin decreased the viability of the liver cancer HepG2 cells and exhibited an IC_{50} of 9 μM. Also, Psoralidin exerted very low toxic effects on the normal AML12 hepatocytes exhibiting an IC_{50} of 100 μM. Flow cytometry showed that Psoralidin triggered G2/M cell cycle arrest of the HepG2 cancer cells. DAPI staining revealed that Psoralidin triggered apoptotic cell death of HepG2 cells which was accompanied with activation of caspases 3 and 9, upregulation of Bax and downregulation of Bcl-2. Additionally, Psoralidin prompted autophagy in the HepG2 cells as revealed by TEM. The Psoralidin-induced autophagy led to upregulation of LC3 II and Beclin-1 expression. Investigation of the in vivo anticancer potential of Psoralidin revealed that this molecule could suppress the growth of xenografted tumors in vivo.*

Conclusion: *Psoralidin may prove essential in the development of systemic therapy for liver cancer.*

Key words: *liver cancer, psoralidin, apoptosis, autophagy, cell cycle arrest*

Introduction

Plant metabolites have shown exceptionally amazing health promoting properties [1]. Across the world, natural products, mainly plants, have been used for the alleviation of human diseases since times immemorial. Even today, a large proportion of the world's population depends on plants for their primary health care [2]. A wide diversity of drugs have been developed from plants and many more await their discovery [3]. Among plant secondary metabolites, coumarins constitute a vast group of bioactive molecules with huge pharmacological potential. Chemically, coumarins are a large group of heterocycles [4]. They have been shown to exhibit anticancer, antimicrobial and several other activities [5]. Psoralidin is prevalently found in *Psoralea corylifolia* belonging to the family Leguminosae [6]. Psoralidin has been shown to suppress the proliferation of gastric cancer cells [7] and

Corresponding author: Jia Li, MD. Department of Emergency, Xiangya Hospital Central South University, Changsha, Hunan 410008, China.
Tel & Fax: +86 13974911157, Email: jasminegarcia67@yahoo.com
Received: 24/01/2019; Accepted: 10/02/2019

has also been shown to stop the growth of colon and breast cancer cells [8]. In prostate cancer cells, Psoralidin has been shown to cause apoptotic cell death [9]. Given this background, the present study was designed to investigate the anticancer effects of Psoralidin against human liver cancer cells and to explore the underlying mechanisms. Liver cancer is the 5th and 8th prevalent type of cancer in males and females, respectively. The risk of liver cancer has been reported to increase with age [10]. Around 0.56 million new cases of liver cancer are reported annually. Additionally, the frequency of liver cancer is comparatively higher in developing countries [11]. The adverse effects of currently available inefficient chemotherapy remarkably obstructs the treatment of liver cancer [12]. This has led to the pursuit for new drugs from plants and herein we, for the first time, examined the anticancer effects of Psoralidin against human liver cancer cells. A previous study indicated that Psoralidin exhibits *in vivo* anticancer effects on xenografted prostate cancer tumors [13]. Therefore, this study also examined the anticancer effects of Psoralidin *in vivo* in xenografted tumors. We believe that this study may serve as basis for establishing Psoralidin as a lead molecule for the development of systemic therapy for liver cancer.

Methods

Cell viability assay

Briefly, the HepG2 cells were treated with 0-200 µM concentrations of Psoralidin for 24 h and then incubated with MTT (500 µg/mL) for 4 h. Dimethyl sulfoxide (10%) was then added to dissolve the blue formazan crystals formed, which are formed as a result of action of cellular oxidoreductase enzymes on MTT dye. Finally, the optical density (OD) was taken at 570 nm and cell viability was taken as the percentage of the control.

Cell cycle analysis

The HepG2 cells were cultured in 96-well plates and then treated with 0, 9, 18 and 36 µM concentrations of Psoralidin for 24 h and then fixed with ethanol (70%). The cells were then suspended in propidium iodide (PI) (0.05 mg/mL) and sodium citrate (0.1%), ribonuclease A (0.015 mg/ml) and NP-40 (0.25%). This was followed by incubation at 37°C for 35 min. Flow cytometry was then used to determine the distribution of the cells in different cell cycle phases.

DAPI staining

In brief, HepG2 cells (0.6×10^6) were grown in 6-well plates. Following incubation of around 12 h, the HepG2 cells were subjected to Psoralidin treatment for 24 h at 37°C. Twenty µl of cell culture were put onto a glass slide and stained with DAPI. The slides were coverslipped and examined under fluorescence microscope.

Transmission electron microscopy

In order to detect autophagy in the Psoralidin-treated HepG2 cells, the cells were subjected to fixation in a solution of 4% glutaraldehyde and 0.05 M sodium cacodylate. The cells were then postfixed in 1.5% OsO4, and dehydrated in alcohol. They were then prepared for flat embedding in Epon 812 and observed using Zeiss CEM 902 electron microscope.

Western blot analysis

Protein expression in Psoralidin-treated HepG2 cells was examined by western blotting. The Psoralidin-treated HepG2 cells were harvested with centrifugation and were then lysed in lysis buffer containing protease inhibitor. The proteins (45 µg) were separated by SDS-PAGE (50 to 60 V for 2 h) and transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). Next, fat-free milk was used to block the membrane at room temperature for 1 h. Thereafter, the membranes were treated with primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with secondary antibodies. Finally, the band signal was detected by Odyssey Infrared Imaging System. Actin was used as control for normalization.

In vivo xenograft study

The NIH guidelines for the care and use of laboratory animals were followed during this study. The mice were injected with 5×10^6 HepG2 cells sub-cutaneously at the flank and in each group they were injected intraperitoneally with Psoralidin dissolved in DMSO (0.1%) and then diluted with 100 µL normal saline at 0, 10, 20 and 40 mg/kg body weight. Psoralidin was given to the mice thrice a week while the control mice were given DMSO (0.1%) in normal saline only. At the end of 6

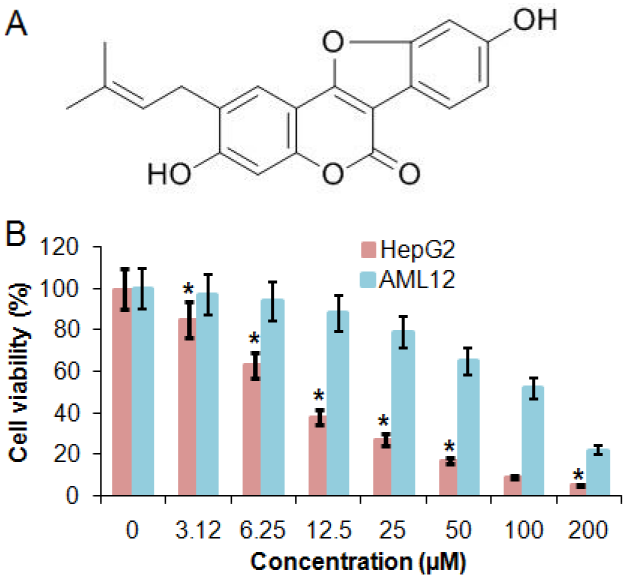

Figure 1. A: Structure of Psoralidin. **B:** MTT assay showing the inhibitory effects of Psoralidin on the viability of liver HepG2 and normal AML12 cells. The experiments were performed in triplicate and shown as mean ± SD (*p< 0.05).

weeks, the mice were euthanized and the tumors were harvested for assessment of tumor growth and other investigations

Results

Psoralidin suppresses the proliferation of liver cancer cells

The results of the MTT cell viability assay showed that Psoralidin (Figure 1A) exerted dose-dependent antiproliferative effects on the HepG2 cells. The viability of HepG2 cells was decreased as the concentration of Psoralidin was increased. The IC_{50} of Psoralidin against the HepG2 cells was found to be 9 μM (Figure 1B). Further, the growth inhibitory effects of Psoralidin were also examined on the normal AML12 hepatocytes and the results showed that Psoralidin exhibited very low growth inhibitory effects and the IC_{50} was 100 μM against the normal AML12 hepatocytes, almost 11-fold higher than that against the HepG2 cells (Figure 1B).

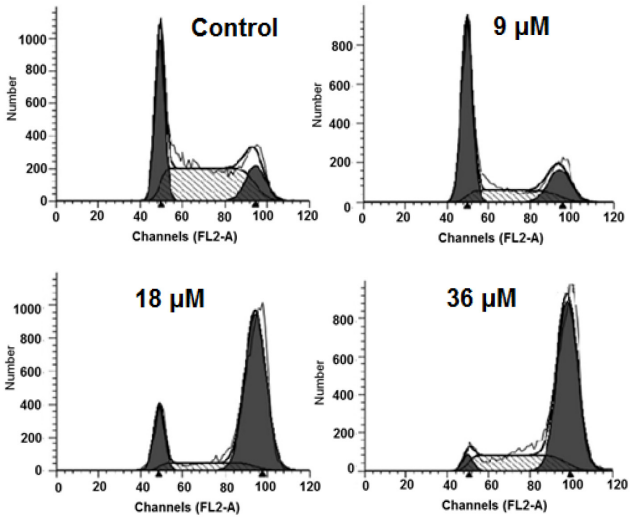

Figure 2. Flow cytometric analysis showing the effect of Psoralidin on the distribution of the HepG2 cells in different phases of the cell cycle. The experiments were performed in triplicate. Psoralidin led to dose-dependent G2/M phase cell cycle arrest in HepG2 human liver cancer cells.

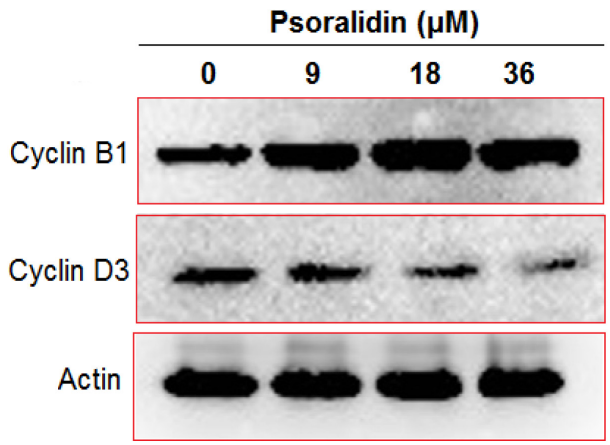

Figure 3. Effect of Psoralidin on the expression of cyclin B1 and Cyclin D3 as determined by western blot analysis. The experiments were performed in triplicate. The expression of cyclin B1 increased while of cyclin D3 decreased with increasing dose of Psoralidin.

Psoralidin induces G2/M arrest of HepG2 cells

The distribution of the Psoralidin-treated HepG2 cells was determined by flow cytometry. The results showed that Psoralidin caused remarkable increase in the percentage of the HepG2 in G2/M phase cells. The percentage of the HepG2 cells increased from 12.88% in the control to around 76.52% at 36 μM concentration (Figure 2). Western blot analysis was then performed to determine the effect of Psoralidin on the expression of Cyclin B1 and Cyclin D3 in HepG2 cells (Figure 3) which revealed that this molecule caused increase in Cyclin B1 expression while the expression of Cyclin D3 was decreased concentration-dependently.

Psoralidin induces apoptosis of HepG2 cells

DAPI staining of the Psoralidin-treated cells was performed to investigate if this agent induces apoptosis in the HepG2 cells. The results showed that Psoralidin caused nuclear fragmentation of the HepG2 cells concentration-dependently, which is hallmark of apoptosis (Figure 4). The western blot analysis of Psoralidin-treated HepG2 cells showed that this molecule caused concentration-

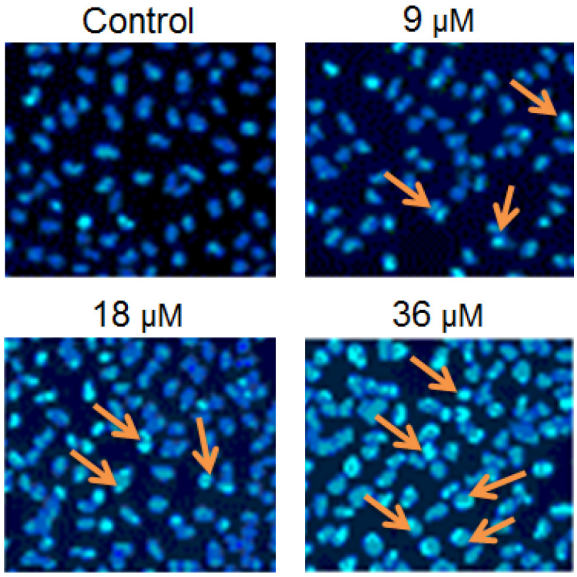

Figure 4. DAPI staining showing that Psoralidin induces apoptosis in the HepG2 cells at the indicated concentrations (arrows depict apoptotic cells). The experiments were performed in triplicate.

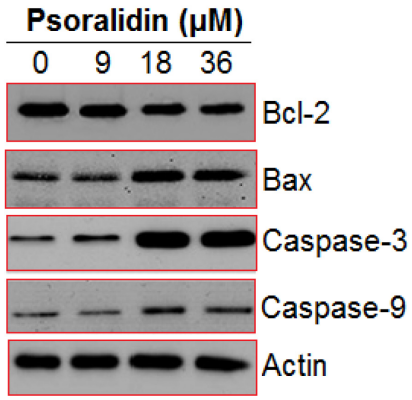

Figure 5. Western blot analysis showing the effects of Psoralidin on the expression of Bcl-2, Bax, Caspase 3 and caspase-9. The experiments were performed in triplicate and shown as mean ± SD. Psoralidin led to a dose-dependent increase in the expression of Bax, caspase 3 and caspase 9 and a decrease in the expression of Bcl-2.

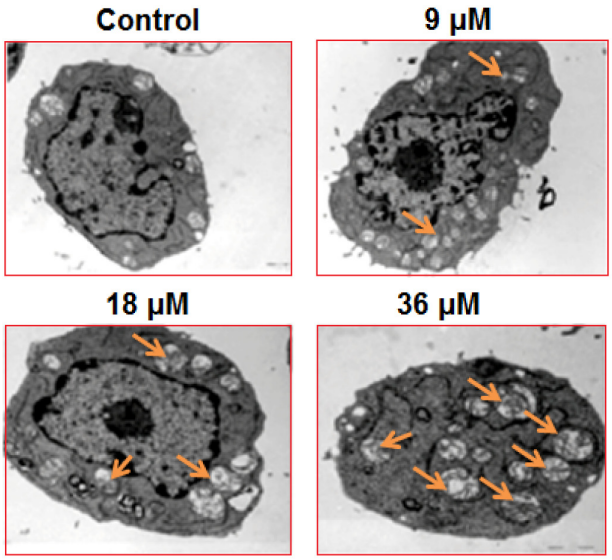

Figure 6. Transmission electron microscopy analysis showing the effects of Psoralidin on the induction of autophagy in the HepG2 cells at indicated concentrations (arrows depict autophagosomes). The experiments were performed in triplicate.

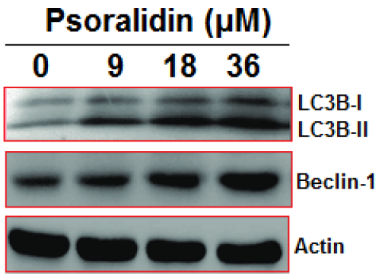

Figure 7. Effect of Psoralidin on the expression of LC3B-1, LC3B-II and Beclin 1 in HepG2 cells as depicted by western blotting. The experiments were performed in triplicate. Psoralidin led to increased expression of LC3B-II and Beclin-1, while the expression of the LC3B-I remained apparently constant.

dependently activation of Caspase 3 and 9 (Figure 5). Moreover, the expression of Bax was increased and that of Bcl-2 was decreased upon treatment of the HepG2 cells with Psoralidin.

Psoralidin induces autophagy in HepG2 cells

Electron microscopic analysis of the Psoralidin-treated HepG2 cells showed that this molecule caused development of autophagosomes in the HepG2 cells (Figure 6). The incidence of autophagy in HepG2 cells was increased with increase in the concentration of Psoralidin. Moreover, Psoralidin led to increased expression of LC3B-II and Beclin-1, while the expression of the LC3B-I remained apparently constant in the HepG2 cells (Figure 7).

Psoralidin inhibits tumor growth in vivo

The *in vivo* anticancer effects of Psoralidin were evaluated in xenografted mice model. The results showed that Psoralidin could suppress the tumor volume and tumor weight of the xenografted tumors (Figure 8A and B). These effects of Psoralidin were found to be concentration-dependent.

Discussion

Liver cancer is a lethal malignancy ranking second major cause of cancer-related mortality around the globe [15]. The incidence of liver cancer has significantly increased over the last few decades and varies geographically [16]. Naturally occurring coumarins have shown impressive anticancer properties and a number of plant-derived coumarins has been evaluated for their anticancer properties [17]. Herein, the anticancer effects of Psoralidin were examined against the HepG2 liver cancer cells and showed remarkable anticancer effects against them. One of the interesting features of Psoralidin was that it exhibited comparatively lower anticancer effects against the normal AML12 hepatocytes. These results suggest that this molecule exhibits cancer cell specific growth inhibitory effects. Previous studies have also proved the anticancer properties of Psoralidin, further complementing our results. For instance, Psoralidin suppresses the proliferation of human esophageal cancer cells by activating apoptotic cell death and deactivation of PI3K/AKT signalling pathway [18]. In yet another study Psoralidin has been shown to cause inhibition of cell proliferation of prostate cancer cells [13]. Several of the natural coumarins have been shown to cause cell cycle arrest of cancer cells. In the present study similar results were observed as Psoralidin could arrest the cells at the G2/M check point and this was also accompanied

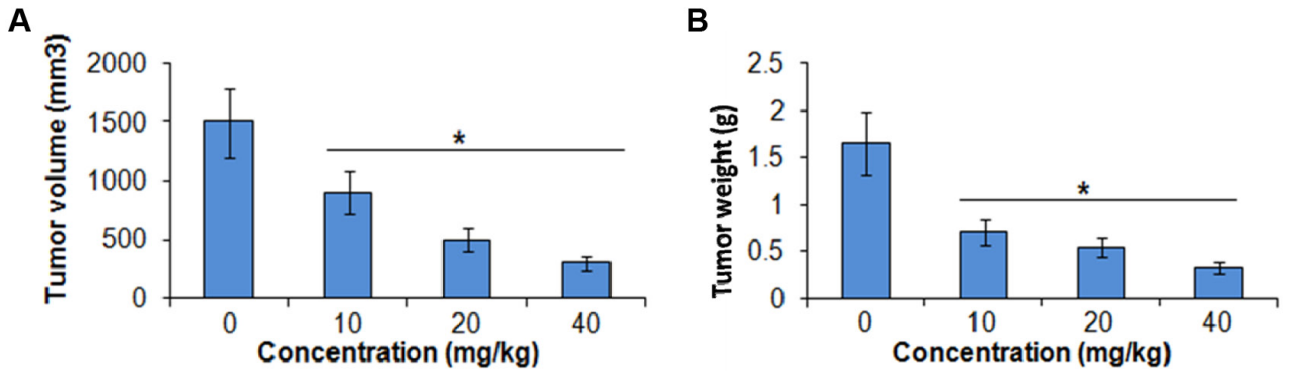

Figure 8. Effect of Psoralidin on tumor volume **(A)** and tumor weight **(B)** of the xenografted tumors in mice. The experiments were performed in triplicate and shown as mean ± SD (*p< 0.05). Psoralidin treatment led to reduction in the tumor volume as well as in the tumor weight in a dose-dependent manner.

with suppression of the Cyclin D3 expression and upregulation of Cyclin B1.

The DAPI and annexin V/PI staining also revealed that this compound prompted apoptosis in the HepG2 cells via activation of Caspases 3 and 9, upregulation of Bax and suppression of Bcl-2. Several studies that have been carried out on Psoralidin have also proved the apoptosis-inducing properties of this molecule. For instance, Psoralidin caused apoptosis of colon cancer cells via activation of ROS triggered apoptotic cell death [19]. There are many studies which have revealed that Psoralidin triggers autophagy in cancer cells, for instance Psoralidin has been shown to induce autophagy in breast cancer cells [20]. Similarly, in lung cancer cells, this molecule has been shown to induce autophagy via generation of ROS [21]. Given this background, electron microscopic analysis of HepG2 cells was also performed and the results showed that Psoralidin triggered the development of autophagosomes in HepG2 cells. The Psoralidin-induced autophagy was also concomitant with upregulation of LC3B-II and Beclin-1 expression. The evaluation of the anticancer activity of Psoralidin in xenografted mice models revealed that it suppressed the volume and weight of the xenografted tumors concentration-dependently, indicative of its anticancer potential.

Conclusion

The findings of the present study suggest that Psoralidin inhibits the proliferation of human liver cancer cells via multiple mechanisms which include apoptosis, autophagy and cell cycle arrest. Moreover, Psoralidin could suppress the growth of xenografted tumors *in vivo*, indicating that it may prove a lead molecule for the development of systemic therapy in liver cancer.

Acknowledgements

This study was supported by National Natural Sciences Foundation of China (No.81402623).

Conflict of interests

The authors declare no conflict of interests.

References

1. Cragg GM, Newman DJ. Plants as a source of anti-cancer agents. J Ethnopharmacol 2005;100:72-9.
2. Gordaliza M. Natural products as leads to anticancer drugs. Clin Transl Oncol 2007;9:767-76.
3. Cragg GM, Newman DJ, Snader KM. Natural products in drug discovery and development. J Nat Prod 1997;60:52-60.
4. Wu L, Wang X, Xu W, Farzaneh F, Xu R. The structure and pharmacological functions of coumarins and their derivatives. Curr Medicinal Chem 2009;16:4236-60.
5. Jain PK, Joshi H. Coumarin: chemical and pharmacological profile. J Applied Pharmaceut Sci 2012;2:236-40.
6. Xiao G, Li G, Chen L et al. Isolation of antioxidants from Psoralea corylifolia fruits using high-speed counter-current chromatography guided by thin layer chromatography-antioxidant autographic assay. J Chromatogr A 2010;1217:5470-6.
7. Yang YM, Hyun JW, Sung MS et al. The cytotoxicity of psoralidin from Psoralea corylifolia. Planta Medica 1996;62:353-4.
8. Mar W, Je KH, Seo EK. Cytotoxic constituents of Psoralea corylifolia. Arch Pharmacol Res 2001;24:211.

9. Srinivasan S, Kumar R, Koduru S, Chandramouli A, Damodaran C. Inhibiting TNF-mediated signaling: a novel therapeutic paradigm for androgen independent prostate cancer. Apoptosis 2010;15:153-61.
10. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 2007;132:2557-76.
11. Gomaa AI, Khan SA, Toledano MB, Waked I, Taylor-Robinson SD. Hepatocellular carcinoma: epidemiology, risk factors and pathogenesis. World J Gastroenterol 2008;14:4300.
12. Bruix J, Sherman M. Management of hepatocellular carcinoma: an update. Hepatology 2011;53:1020-2.
13. Kumar R, Srinivasan S, Koduru S et al. Psoralidin, an Herbal Molecule, Inhibits Phosphatidylinositol 3-Kinase-Mediated Akt Signaling in Androgen-Independent Prostate Cancer Cells. Cancer Prev Res 2009;2:234-43.
14. Okuda K, Ohtsuki T, Obata H et al. Natural history of hepatocellular carcinoma and prognosis in relation to treatment study of 850 patients. Cancer 1985;56:918-28.
15. El-Serag HB, Marrero JA, Rudolph L, Reddy KR. Diagnosis and treatment of hepatocellular carcinoma. Gastroenterology 2008;134:1752-63.
16. Shah U, Shah R, Acharya S, Acharya N. Novel anticancer agents from plant sources. Chin J Nat Medicines 2013;11:16-23.
17. Emami S, Dadashpour S. Current developments of coumarin-based anti-cancer agents in medicinal chemistry. Eur J Medicinal Chem 2015;102:611-30.
18. Jin Z, Yan W, Jin H, Ge C, Xu Y. Psoralidin inhibits proliferation and enhances apoptosis of human esophageal carcinoma cells via NF κB and PI3K/Akt signaling pathways. Oncol Lett 2016;12:971-6.
19. Jin Z, Yan W, Jin H, Ge C, Xu Y. Differential effect of psoralidin in enhancing apoptosis of colon cancer cells via nuclear factor κB and B cell lymphoma 2/B cell lymphoma 2 associated X protein signaling pathways. Oncol Lett 2016;11:267-72.
20. Ren G, Luo W, Sun W et al. Psoralidin induced reactive oxygen species (ROS)-dependent DNA damage and protective autophagy mediated by NOX4 in breast cancer cells. Phytomedicine 2016;23:939-47.
21. Hao W, Zhang X, Zhao W, Chen X. Psoralidin induces autophagy through ROS generation which inhibits the proliferation of human lung cancer A549 cells. Peer J 2014;2:e555.
22. Kumar R, Srinivasan S, Koduru S et al. Psoralidin, an herbal molecule inhibits PI3K mediated Akt signaling in androgen independent prostate cancer (AIPC) cells. Cancer Prev Res 2009;2:234.