RESEARCH ARTICLE

Platycodin D Induces Apoptosis, and Inhibits Adhesion, Migration and Invasion in HepG2 Hepatocellular Carcinoma Cells

Ting Li[1&], Wen-Shan Xu[1&], Guo-Sheng Wu[1], Xiu-Ping Chen[1], Yi-Tao Wang[1*], Jin-Jian Lu[1,2*]

Abstract

Background: Platycodin D (PD), a triterpenoid saponin isolated from the Chinese medicinal herb *Platycodonis radix*, possesses anti-cancer effects in several cancer cell lines. The aim of this study was to evaluate its anti-cancer activities in hepatocellular carcinoma cells. Materials and Methods: MTT and colony formation assays were performed to evaluate cell proliferation, along with flow cytometry and Western blotting for apoptosis. Cell adhesion was tested by observing cellular morphology under a microscope, while the transwell assay was employed to investigate the cell migration and invasion. Results: PD concentration-dependently inhibited cell proliferation in both HepG2 and Hep3B cells, and significantly suppressed colony formation and induced apoptosis in HepG2 cells. The protein levels of cleaved poly ADP-ribose polymerase (PARP) and Bax were up-regulated while that of survivin was down-regulated after treatment with PD. Moreover, PD not only obviously suppressed the adhesion of HepG2 cells to Matrigel, but also remarkably depressed their migration and invasion induced by 12-O-tetradecanoylphorbol 13-acetate (TPA) . Conclusions: PD presents anti-cancer potential in hepatocellular carcinoma cells via inducing apoptosis, and inhibiting cell adhesion, migration and invasion, indicating promising features as a lead compound for anti-cancer agent development.

Keywords: Platycodin D - HepG2 - apoptosis - metastasis - cancer

Asian Pac J Cancer Prev, **15 (4)**, 1745-1749

Introduction

Hepatocellular carcinoma is one of the most common and most aggressive cancers worldwide and a leading cause of cancer deaths in China (Chen et al., 2013). Although surgical resection and transplantation have remarkably improved the survival in patients with no metastasis, the prognosis of hepatocellular carcinoma for late stage diseases remains quite poor (El-Serag, 2002). Besides, because of the high rates of recurrence, most patients are not eligible for surgery. What's worse, there was lack of first line chemotherapy drugs for this type of cancer in a long period of time. Recently, sorafenib might be the limited choice for patients with advanced hepatocellular carcinoma (Llovet et al., 2008). However, sorafenib only prolongs patients' median survival time by less than 3 months and many patients have to reduce their dosage or discontinue the drug therapy due to its side effects (Llovet et al., 2008; Cheng et al., 2009). Thus, efficient drugs for treatment of hepatocellular carcinoma are still urgently needed especially for those who are unable to process surgical resection or transplantation.

Natural products, a rich source of compounds with enormous structural diversity, have been extensively explored in the field of drug discovery. A large number of different sources of natural compounds present anti-cancer effects both in vitro and in vivo (Chen et al., 2012; Huang et al., 2012; Lu et al., 2012a; Lu et al., 2012b; Lu et al., 2013). Therefore, it is vital to screen efficient natural compounds for hepatocellular carcinoma treatment. Platycodin D (PD) is a triterpenoid saponin isolated from the Chinese medicinal herb Platycodonis Radix. It possesses important biological activities, including immune stimulation (Xie et al., 2008), anti-inflammation (Kim et al., 2001; Ahn et al., 2005), anti-nociception (Choi et al., 2002), anti-obesity (Han et al., 2002; Lee et al., 2010), anti-atherosclerosis (Wu et al., 2012) and anti-cancer (Kim et al., 2005; Kim et al., 2008a; Kim et al., 2008b; Shin et al., 2009; Choi et al., 2010; Yu and Kim, 2010; Chun et al., 2012) etc. Due to the various pharmacological activities, PD has been listed as the marker for the quality control of Platycodonis Radix in the Pharmacopoeia of the People's Republic of China (Pharmacopoeia Commission of the Ministry of Health of the P.R.China, 2010).

In this study, the anti-proliferative effects and apoptotic induction of PD as well as the anti-adhesion, anti-migration, and anti-invasion potentials of PD were determined which indicates that PD might be a promising lead compound for treatment of hepatocellular carcinoma.

*[1]State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macao, [2]College of life Sciences, Zhejiang Chinese Medical University, Hangzhou, Zhejiang, China [&]Equal contributors *For correspondence: ytwang@umac.mo; jinjian.lu@163.com*

Materials and Methods

Materials

PD was purchased from Best-Reagent (Chengdu, China). It was diluted using DMSO and the stock concentration was 20 mM. 3-[4, 5-Dimethyl-2-thiazolyl]-2, 5-diphenyltetrazolium bromide (MTT) and 12-O-tetradecanoylphorbol 13-acetate (TPA) were obtained from Sigma (Saint Louis, Missouri, USA) and Beyotime (Nantong, China), respectively. Propidium iodide (PI) was purchased from Invitrogen (Carlsbad, CA, USA).

Cell culture

The hepatocellular carcinoma cell lines HepG2 and Hep3B (ATCC, Rockville, MD, USA) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS, GIBCO BRL, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin, and were grown in an incubator with 5% CO_2 at 37°C.

MTT assay

Exponentially growing HepG2 and Hep3B cells were planted into 96-well plates, and then treated with serial concentrations of PD for 48 h after adhesion. Cell proliferation was determined by addition of 1 mg/mL MTT-containing medium for 4 h, addition of 100 μL DMSO to solubilize the formazan, and shaking for 10 min in the dark. The absorbance at 570 nm was recorded using the SpectraMax M5 Microplate Reader (Molecular Devices).

Colony formation assay

HepG2 cells were treated with serial concentrations of PD for 24 h and then suspended and re-seeded into 6-well plates at a density of 2×10^4 cells per well after treatment. Cells were subsequently fixed using 4% paraformaldehyde and stained with crystal violet staining solution (Beyotime, Nantong, China) after 10 days-incubation. Typical images were captured using an ordinary NIKON camera.

Flow cytometry analysis of DNA content

HepG2 cells seeded into 6-well plates were treated with series concentrations of PD for 24 or 48 h. Cells were harvested and fixed in 70% ethanol and then stored at 4°C overnight. Cells were stained in PBS containing 5 μg/mL RNase and 20 μg/mL PI in the dark at room temperature for 30 min and analyzed using a flow cytometry (Becton Dickinson FACS Canto™ , Franklin Lakes, NJ). At least 10,000 events were counted for each sample.

Cell adhesion assay

Matrigel was diluted in sterile conditions and applied to 96-well plates overnight at 4°C. Nonspecific binding sites were blocked with 1% bovine serum albumin for 1 h at 37°C. Cells were harvested after serum starved for 45 min and then plated in triplicate onto wells in low-serum medium (0.5% FBS) with series concentrations of PD. Following 1 h-incubation, the cellular morphology was photographed using a CCD camera (AxioCam HC, Carl Zeiss, Thornwood, NY) attached to a microscope. Non-adherent cells were washed away and adherent cells were fixed with 4% paraformaldehyde and stained with crystal violet. After extensive washing with PBS, the dye was extracted with 10% acetic acid and the absorbance at 595 nm was recorded using the SpectraMax M5 Microplate Reader (Molecular Devices).

Cell migration assay

A cell suspension containing 0.5% FBS DMEM medium and different concentrations of PD was deposited into the upper chamber of each well. The lower chambers were loaded with 10% FBS DMEM medium containing 100 nM TPA as well as different concentrations of PD. After 24-h incubation, the non-invasive cells were removed via gentle scraping. The invasive cells attached to the lower surface of the membrane were fixed in 4% paraformaldehyde at room temperature for 30 min and stained with crystal violet for 10 min. Three randomly selected views of the upper chamber were photographed using a CCD camera (AxioCam HC, Carl Zeiss, Thornwood, NY) attached to a microscope.

Cell invasion assay

Cell invasion assay was performed as described in migration with a slight modification in that both sides of the insert were pre-coated with Matrigel.

Western blot analysis

Cells were lysed in the lysis buffer, and the proteins of the lysates were quantified using a BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA). About 50 μg of total proteins was subjected to SDS-PAGE, transferred onto PVDF membranes, and then blocked with 5% nonfat milk in TBST (20 mM Tris, 500 mM NaCl, and 0.1% Tween-20) at room temperature for 2 h with rocking. The membranes were probed with specific primary antibodies against poly ADP-ribose polymerase (PARP), Bax, and survivin (Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C. After washing with TBST three times for 15 min each, the membranes were incubated with horseradish-peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc.) in TBST at room temperature for 1 h, and specific protein bands were visualized using an ECL advanced western blot detection kit. Equal protein loading was verified through re-hybridization of the membranes and re-probing with anti-β-actin antibody.

Results

PD inhibited cell proliferation and colony formation in hepatocellular carcinoma cells

Herein, the hepatocellular carcinoma cell lines HepG2 and Hep3B were used to detect the anti-proliferative potential of PD. Cells were treated with various concentrations of PD for 48 h and the inhibition rate was tested by MTT assay. As shown in Figure 1A, B, PD dose-dependently inhibited the cell proliferation in both HepG2 and Hep3B hepatocellular carcinoma cell lines. To further investigate the anti-cancer effect of PD, the colony formation assay was used. The number of colony foci was obviously decreased after treatment with 10 μM

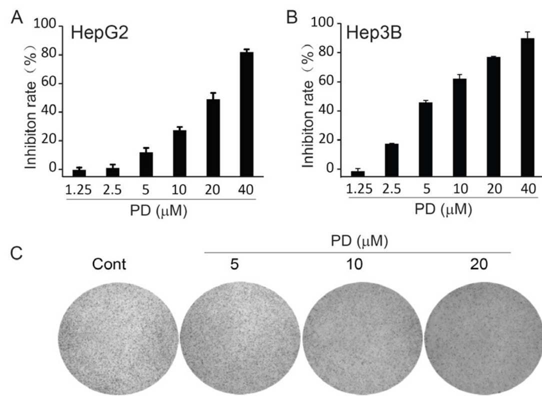

Figure 1. PD Inhibited Proliferation and Colony Formation in Hepatocellular Carcinoma Cells. (A, B) HepG2 (A) and Hep3B (B) cells were treated with indicated concentrations of PD for 48 h and the cell proliferative inhibition was tested by MTT assay. (C) HepG2 cells were treated with various concentrations of PD for 24 h. After treatment, cells were suspended and re-seeded into 6-well plates at a density of 2×10^4 cells per well and then fixed and stained with 4% paraformaldehyde and crystal violet after 10 days. The representative images of colony formation assay were obtained

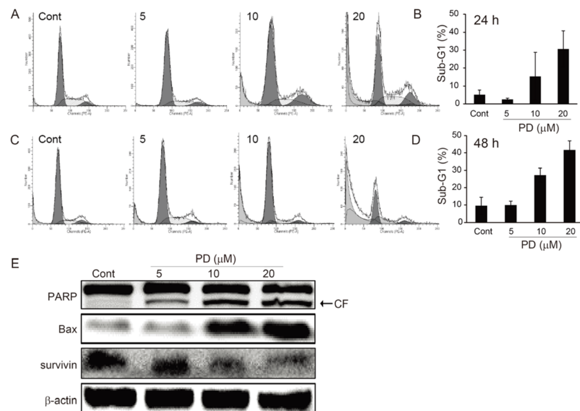

Figure 2. PD Induced Apoptosis in HepG2 Cells. (A-D) Cells were treated with indicated concentrations of PD for 24 h (A, B) or 48 h (C, D), and cells in the sub-G1 cells were tested using flow cytometry assay. (E) Cells were treated with indicated concentrations of PD for 24 h and the protein levels of PARP, Bax and survivin were determined by western blot analysis. CF: cleavage fragment

or 20 μM PD in human HepG2 cells (Figure 1C), which is consistent with the results of MTT assay, indicating that PD effectively presents anti-proliferative activity in hepatocellular carcinoma cells.

PD induced apoptosis in HepG2 cells

As induction of apoptosis is one of the major contents that mediate cell proliferative inhibition, we tested cells in sub-G1 phase, which is a characteristic of apoptosis, after PD treatment using flow cytometry. Upon treatment with PD for 24 h, PD induced sub-G1 peek (Figure 2A, B) in a dose-dependent manner and the apoptotic cells appeared more obvious accompanied with the increased incubation time (Figure 2C, D). We further detected the levels of several proteins which are related with apoptosis. After PD treatment, a cleavage fragment (CF) of PARP and pro-apoptotic protein Bax were obviously up-regulated

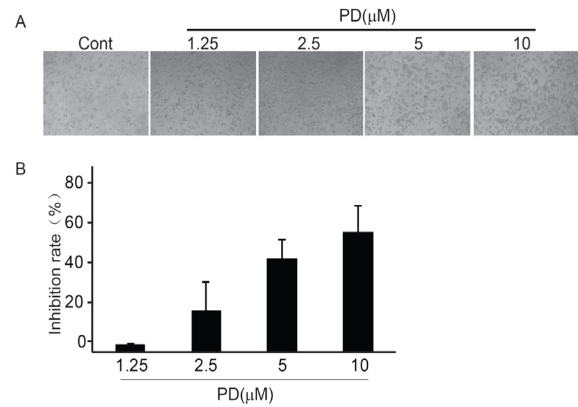

Figure 3. PD Inhibited Adhesion in HepG2 Cells. (A) Cells were plated onto Matrigel-coated 96-well plates in low-serum medium (0.5% FBS) with various concentrations of PD for 1 h. The representative images were obtained. (B) Adherent cells were stained with crystal violet and the OD value was detected by a microplate reader

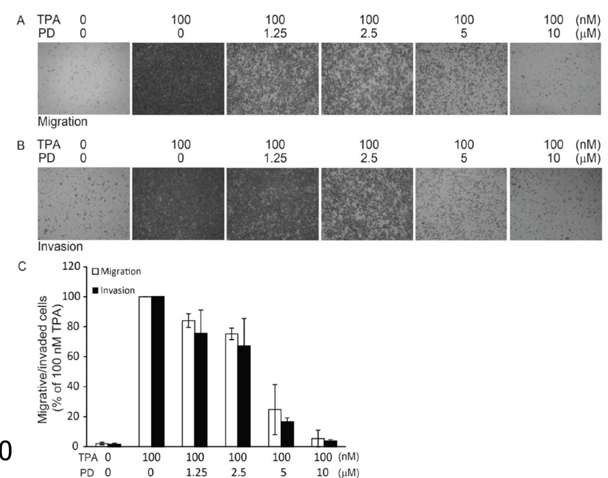

Figure 4. PD Inhibited Metastasis and Invasion Induced by TPA in HepG2 Cells. (A, B) Cells were co-treated with TPA and PD for 24 h. The migrated (A) or invasive (B) cells attached to the lower surface of the membrane were fixed and stained with crystal violet. The representative images were shown. (C) Semi-quantitation of the results obtained from (A) and (B)

while the anti-apoptotic protein survivin was remarkably down-regulated (Figure 2E), indicating PD indeed induces apoptosis in HepG2 cells.

PD inhibited adhesion in HepG2 cells

In order to systematically evaluate the anti-hepatocellular carcinoma activities of PD, the potential of cancer cells adhering to Matrigel was tested. Cells were harvested and plated into wells with series concentrations of PD for 1 h and the cellular morphology was photographed. As shown in Figure 3 A, the cells without PD treatment showed spreading state while those cells treated with PD presented much more round phenotype in a concentration-dependent manner. Data obtained from the microplate reader showed the similar results as phenotype observation (Figure 3B), indicating PD effectively suppresses HepG2 cells adhering to Matrigel.

PD inhibited migration and invasion induced by TPA in HepG2 cells

We further investigated the anti-migration and anti-invasion abilities of PD in HepG2 cells. TPA is a well-known tumor promoter, and it may act as a potential inducer of tumor migration and invasion in cancer cells (Shimao et al., 1999). The results of the transwell assay (without Matrigel pre-coated) showed that after 24 h treatment, the amounts of cells translocated into the lower surface of the membrane were significantly increased in the group with 100 nM TPA treatment (Figure 4A). PD concentration-dependently inhibited TPA-induced migration in HepG2 cells (Figure 4A). Similarly, the amounts of cells degraded the Matrigel and invaded into the lower surface of the membrane were obviously increased after induced by TPA and PD also significantly reduced the invasive cells in the invasive model (Figure 4B). Figure 4C showed the semi-quantitative results obtained from (A) and (B).

Discussion

Herein, we found that PD obviously inhibited hepatocellular carcinoma cells proliferation and induced apoptosis in a concentration-dependent manner (Figure 1 and 2). PARP cleaved by caspases is a hallmark of apoptosis (Boulares et al., 1999) and Bax is a pro-apoptotic member of the Bcl-2 family which regulate apoptosis (Brady and Gil-Gomez, 1998). Survivin has multiple functions including cytoprotection and inhibition of cell death among others which favor cancer survival (Yamamoto et al., 2008). Together with the data obtained from flow cytometry with PI staining and western blot assay, PD indeed induced apoptosis in hepatocellular carcinoma cells and the up-regulation of Bax and down-regulation of survivin, at least in part, may contribute to the initial of apoptosis.

To reduce the metastasis could be a promising stratagem for treatment of hepatocellular carcinoma, as the poor prognosis of hepatocellular carcinoma is largely due to a high rate of metastasis (Genda et al., 1999; Ye et al., 2003). Metastasis of cancer cells includes several steps while change of cell-cell and cell-extracellular matrix (ECM) adhesion capacity is the initial step in this process. Matrigel mainly contains the structural proteins such as laminin and entactin (Hughes et al., 2010) which has been widely used to mimic the ECM in cancer. Herein, we found that PD significantly inhibited HepG2 cell adhesion to Matrigel in a concentration-dependent manner only after one hour treatment (Figure 3), indicating the potential anti-adhesion activity of PD. We further detected the anti-migration and anti-invasion potentials of PD employing transwell assays and the data showed that PD remarkably reduced TPA-induced HepG2 cell migration and invasion (Figure 4). It is noteworthy that combinational treatment with 5 μM PD and 100 nM TPA reduced the cell proliferation about 30% in HepG2 cells (data not shown), indicating the proliferative inhibition could not be excluded when the concentration of PD is high. Because cancer cells need to degrade and traverse the Matrigel-coated membrane to successfully penetrate the transwell chamber, PD might also affect the basement membrane hydrolysis which deserves further study.

In conclusion, PD inhibited HepG2 cell proliferation, and induced apoptosis by regulating the protein expression of Bax and survivin. Moreover, PD effectively inhibited HepG2 cell adhesion, migration and invasion in hepatocellular carcinoma cells. Our results support PD might be a novel candidate chemotherapeutic agent for treatment of hepatocellular carcinoma.

Acknowledgements

This study was supported by the Research Fund of University of Macau (SRG026-ICMS13-LJJ, MRG012/WYT/2013/ICMS and MRG013/WYT/2013/ICMS), Administration of Traditional Chinese Medicine of Zhejiang Province (2012ZA028) and Science and Technology Development Fund of Macau Special Administrative Region (070/2013/A, 074/2012/A3 and 077/2011/A3).

References

Ahn KS, Noh EJ, Zhao HL, et al (2005). Inhibition of inducible nitric oxide synthase and cyclooxygenase II by Platycodon grandiflorum saponins via suppression of nuclear factor-kappaB activation in RAW 264.7 cells. *Life Sci*, **76**, 2315-28.

Boulares AH, Yakovlev AG, Ivanova V, et al (1999). Role of poly(ADP-ribose) polymerase (PARP) cleavage in apoptosis. Caspase 3-resistant PARP mutant increases rates of apoptosis in transfected cells. *J Biol Chem*, **274**, 22932-40.

Brady HJ, Gil-Gomez G (1998). Bax. The pro-apoptotic Bcl-2 family member, Bax. Int J Biochem *Cell Biol*, **30**, 647-50.

Chen WQ, Zheng RS, Zhang SW (2013). Liver cancer incidence and mortality in China, 2009. *Chin J Cancer*, **32**, 162-9.

Chen X, Bao J, Guo J, et al (2012). Biological activities and potential molecular targets of cucurbitacins: a focus on cancer. *Anticancer Drugs*, **23**, 777-87.

Cheng AL, Kang YK, Chen Z, et al (2009). Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol*, **10**, 25-34.

Choi SS, Han EJ, Lee TH, et al (2002). Antinociceptive mechanisms of platycodin D administered intracerebroventricularly in the mouse. *Planta Medica*, **68**, 794-8.

Choi YH, Yoo DS, Cha MR, et al (2010). Antiproliferative effects of saponins from the roots of Platycodon grandiflorum on cultured human tumor cells. *J Nat Prod*, **73**, 1863-7.

Chun J, Joo EJ, Kang M, et al (2012). Platycodin D induces anoikis and caspase-mediated apoptosis via p38 MAPK in AGS human gastric cancer cells. *J Cell Biochem*. ????

El-Serag HB (2002). Hepatocellular carcinoma: an epidemiologic view. *J Clin Gastroenterol*, **35**, S72-8.

Genda T, Sakamoto M, Ichida T, et al (1999). Cell motility mediated by rho and Rho-associated protein kinase plays a critical role in intrahepatic metastasis of human hepatocellular carcinoma. *Hepatology*, **30**, 1027-36.

Han LK, Zheng YN, Xu BJ, et al (2002). Saponins from platycodi radix ameliorate high fat diet-induced obesity in mice. *Journal of Nutrition*, **132**, 2241-5.

Huang M, Lu JJ, Huang MQ, et al (2012). Terpenoids: natural products for cancer therapy. *Expert Opin Investig Drugs*, **21**, 1801-18.

Hughes CS, Postovit LM, Lajoie GA (2010). Matrigel: a complex protein mixture required for optimal growth of cell culture.

Proteomics, **10**, 1886-90.

Kim MO, Moon DO, Choi YH, et al (2008a). Platycodin D induces mitotic arrest in vitro, leading to endoreduplication, inhibition of proliferation and apoptosis in leukemia cells. *Int J Cancer*, **122**, 2674-81.

Kim MO, Moon DO, Choi YH, et al (2008b). Platycodin D induces apoptosis and decreases telomerase activity in human leukemia cells. *Cancer Lett*, **261**, 98-107.

Kim YP, Lee EB, Kim SY, et al (2001). Inhibition of prostaglandin E2 production by platycodin D isolated from the root of Platycodon grandiflorum. *Planta Med*, **67**, 362-4.

Kim YS, Kim JS, Choi SU, et al (2005). Isolation of a new saponin and cytotoxic effect of saponins from the root of Platycodon grandiflorum on human tumor cell lines. *Planta Med*, **71**, 566-8.

Lee H, Kang R, Kim YS, et al (2010). Platycodin D inhibits adipogenesis of 3T3-L1 cells by modulating Kruppel-like factor 2 and peroxisome proliferator-activated receptor gamma. *Phytother Res*, **24** Suppl 2, S161-7.

Llovet JM, Ricci S, Mazzaferro V, et al (2008). Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med*, **359**, 378-90.

Lu JJ, Bao JL, Chen XP, et al (2012a). Alkaloids isolated from natural herbs as the anticancer agents. *Evid Based Complement Alternat Med*, **2012**, 485042.

Lu JJ, Bao JL, Wu GS, et al (2013). Quinones derived from plant secondary metabolites as anti-cancer agents. *Anticancer Agents Med Chem*, **13**, 456-63.

Lu JJ, Dang YY, Huang M, et al (2012b). Anti-cancer properties of terpenoids isolated from *Rhizoma curcumae*--a review. *J Ethnopharmacol*, **143**, 406-11.

Pharmacopoeia Commission of the Ministry of Health of the P.R.China (2010). Pharmacopoeia of the People's Republic of China. *China Medical Science Press*.

Shimao Y, Nabeshima K, Inoue T, et al (1999). TPA-enhanced motility and invasion in a highly metastatic variant (L-10) of human rectal adenocarcinoma cell line RCM-1: selective role of PKC-alpha and its inhibition by a combination of PDBu-induced PKC downregulation and antisense oligonucleotides treatment. *Clin Exp Metastasis*, **17**, 351-60.

Shin DY, Kim GY, Li W, et al (2009). Implication of intracellular ROS formation, caspase-3 activation and Egr-1 induction in platycodon D-induced apoptosis of U937 human leukemia cells. *Biomed Pharmacother*, **63**, 86-94.

Wu JT, Yang GW, Zhu WX, et al (2012). Anti-atherosclerotic activity of platycodin d derived from roots of *Platycodon grandiflorum* in human endothelial cells. *Biological Pharmaceutical Bulletin*, **35**, 1216-21.

Xie Y, Deng W, Sun H, et al (2008). Platycodin D2 is a potential less hemolytic saponin adjuvant eliciting Th1 and Th2 immune responses. *International Immunopharmacology*, **8**, 1143-50.

Yamamoto H, Ngan CY, Monden M (2008). Cancer cells survive with survivin. *Cancer Sci*, **99**, 1709-14.

Ye QH, Qin LX, Forgues M, et al (2003). Predicting hepatitis B virus-positive metastatic hepatocellular carcinomas using gene expression profiling and supervised machine learning. *Nat Med*, **9**, 416-23.

Yu JS, Kim AK (2010). Platycodin D induces apoptosis in MCF-7 human breast cancer cells. *J Med Food*, **13**, 298-305.