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RESEARCH ARTICLE

# The Citrus Flavonoid Nobiletin Inhibits Proliferation and Induces Apoptosis in Human Pancreatic Cancer Cells *In vitro*

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**Abstract** The cellular effects of nobiletin on human pancreatic cancer cells (PANC-1) and the mechanisms by which nobiletin inhibits the proliferation of these cells were investigated. A MTT assay and flow cytometry were used to examine cell proliferation and apoptosis, respectively. A Western blot assay was used to examine expression levels of the apoptotic proteins bax, bcl-2, and p53. NOB induced apoptosis in these cells via up-regulation of the pro-apoptotic protein bax and down-regulation of the anti-apoptotic proteins bcl-2 and p53. The normal cell cycle of PANC-1 cells was arrested by NOB with a significant increase in the proportion of G0/G1 phase cells ($p<0.05$) and a significant decrease in the proportion of S phase cells ($p<0.05$). NOB can inhibit the proliferation of human pancreatic carcinoma cells by inducing apoptosis and arresting the cell cycle progression.

**Keywords:** nobiletin, pancreatic cancer, cell proliferation, apoptosis, cell cycle

## Introduction

Pancreatic cancer is a common malignant tumor with the 4th leading mortality rate among all cancers (1). In 2010, 43,140 new cases of pancreatic cancer were identified in the United States (2). In China, the incidence of pancreatic cancer has been increasing yearly, making it one of the most common causes of mortality in cancer diseases (3). In spite of the high incidence and mortality rate, only 10%-15% of patients suffering from this deadly disease are able to receive medical intervention in China (3). Even when patients do receive treatment, such as excision and postoperative chemotherapy, the 5-year survival rate is no greater than 20% because of cancer metastasis at the time of diagnosis (4). Therefore, developing preventive and/or therapeutic approaches is of particular importance for better control and management of this aggressive malignant tumor disease.

Orange juice constitutes most of the worldwide annual consumption of fruit juice products. The processing of orange juice generates a considerable amount of by-products that contain a great quantity of bioactive substances. Some bioactive substances, such as hesperidin (5), resveratrol (6), apple polyphenols (7), and cordycepin (8) have been shown to possess abilities to delay, suppress, or even reverse carcinogenesis. Nobiletin (NOB) is one of polymethoxylated flavones that have only been found in citrus by-products (9-12). The authors further demonstrated that a high level of NOB is existed in citrus peels, as well as in immature citrus fruits (9-12). NOB is a strong antioxidant and a free radical scavenger (13). In the cell cycle process, the genetic material in the cell is copied and assigned to two daughter cells. When any one stage in the cell cycle is blocked, failure of cell division will occur. Apoptosis plays an important role. The overall cell quantity is retained in a state of dynamic balance by cell apoptosis and proliferation (14). This state of balance is usually destroyed in cancer, and cell proliferation becomes uncontrolled (14), resulting in cancer development. Therefore, inducing cancer cell apoptosis and inhibiting cancer cell proliferation are both important in controlling the development of cancer (14). Previous studies have shown that NOB is able to inhibit the growth of various human tumor cells, including neuroblastoma (15), breast adenocarcinoma (16), gastric adenocarcinoma

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(17), and fibrosarcoma (18). However, the effects of NOB on pancreatic carcinoma cells and the underlying molecular mechanisms are not yet completely understood. Therefore, the objective of this research was to investigate the effect of NOB on human pancreatic carcinoma cells.

## Materials and Methods

**Cell lines and Chemicals reagents** PANC-1 was obtained from the Shanghai Cell Bank (Shanghai, China) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37°C under an atmosphere of normal pressure 5% $CO_2$. NOB was extracted from citrus by-products collected during processing in December, 2010 mainly from *Citrus sinensis* (*Sweet orange*) at Chongqing Sanxia Fruits Group Co., Ltd. (Chongqing, China). The purity of the extracted NOB (>97.6%) was determined using HPLC (Agela Technologies, Co., Ltd., Shanghai, China).

**Cell proliferation assessment using an MTT assay** PANC-1 cell proliferation was evaluated using a MTT assay (19). An amount of $1\times10^4$ PANC-1 cells was seeded in 200 μL of RPMI 1640 medium per well in 96-well plates. NOB was added to each well at a 0-250 μM final concentration for dose dependent measurements. After incubation for 24-72 h, 20 μL of MTT (Sigma, Shanghai, China) was added to each well at a concentration of 5 mg/mL, followed by an incubation of the plates at 37°C for 4 h. The medium was then removed and 150 μL of 10% DMSO was added to each well. The plates were centrifuged at 1,000×*g* for 10 min and read at 490 nm to obtain optical density (OD) values using a microplate ELISA reader (Bio-Tek, Burleigh, QLD, Australia). Cell viability was calculated as: cell viability (%)=(corrected treated sample OD/corrected control sample OD)×100%, where corrected OD is the OD value obtained by deducting the mean raw OD of the blank wells from the raw OD of the sample well.

**Flow cytometric analysis of apoptosis** PANC-1 cell apoptosis was analyzed by using an Annexin-V FITC Apoptosis Detection Kit (Beyotime, Shanghai, China) following the manufacturer's instruction. Briefly, PANC-1 cells at a concentration of $3\times10^6$ cells per 2,000 μL in 6-well plates were cultured in RPMI 1640 medium in the presence and absence of 100 μM NOB for 48 h. The medium was removed and the cells were trypsinized, then centrifuged at 1,000×*g* for 5 min to remove trypsin. Next, 5 μL of annexin-V-FITC and 10 μL of propidium iodide from the detection kit were added to each well and the plate was incubated at room temperature for 10 min in the dark, followed immediately by flow cytometric analysis. Flow cytometry was performed on a FACS Vantage SE (Becton Dickinson, Franklin Lakes, NJ, USA). Flow data were analyzed using Cell Quest software (Becton Dickinson).

**Flow cytometric analysis of the cell cycle** Cell cycle analysis was conducted by using a Cell Cycle Analysis Kit (Beyotime) following the manufacturer's instruction. Briefly, after treatment with and without NOB for 48 h, $10^6$ PANC-1 cells per well in 6-well plates were trypsinized and centrifuged at 1,000×*g* for 5 min. The supernatant was removed and the cells were washed with phosphate buffered saline (PBS) and fixed with 70% ethanol at 4°C for 24 h. The cells were then washed and stained with 5 mg/mL of PI and 50 mg/mL of RNase A at 37°C for 30 min. The cellular DNA content and cell cycle phases were analyzed using flow cytometry on a FACS Vantage SE (Becton Dicknson, Shanghai China). Flow data were analyzed using ModFit software (Becton Dickinson).

**Western blot assay** Cells were collected and dissolved using an NE-PER kit (Beyotime) following the manufacturer's instructions. Equal amounts of protein (30 μg) from different samples were loaded, size-fractionated using 12.5% SDS-PAGE, and transferred onto PVDF membranes (Millipore, Billerica, MA, USA) for Western blot analysis. After transfer, the membranes were blocked in 5% skimmed milk and then incubated with the primary antibody at 4°C overnight. After two washes with TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20), the membranes were incubated with horseradish peroxidase-conjugated antibodies and the signals were detected by using an ECL PLUS Chemiluminescence Kit (Millipore) followed by exposure to X-ray film. The intensity of bands on the X-ray film was analyzed using the UVP gel image processing system and Labworks 4.6 software (UVP, Upland, CA, USA). Beta-actin was used as an internal loading control.

**Statistical analysis** All statistical analysis in this study was conducted using Statistical Product and Service Solutions software (SPSS, version 16.0; SPSS Inc., Chicago, IL, USA). Significant differences were considered when *p*-values were less than 0.05. Numeric results are expressed as the mean±standard deviation (SD). All experiments were conducted in triplicate.

## Results and Discussion

**Effect of NOB on PANC-1 cell proliferation** The effect of NOB on PANC-1 cell viability and proliferation was investigated. An MTT assay is a common approach for

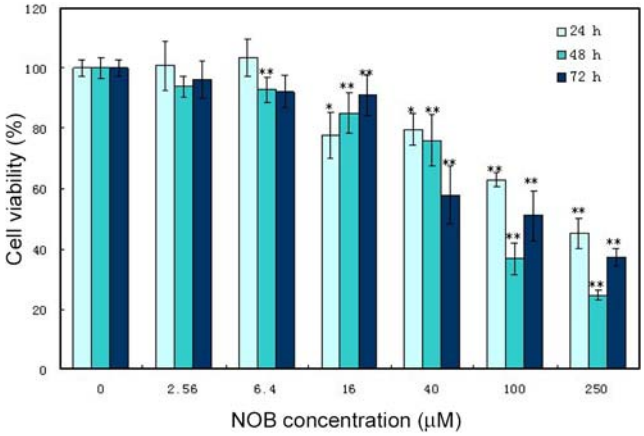


**Fig. 1. Effect of NOB on PANC-1 cell viability.** Cells were treated with different concentrations of NOB for 24-72 h. *$p$<0.05, **$p$<0.01 compared with control. Data are expressed as means±standard deviation ($n$=6).

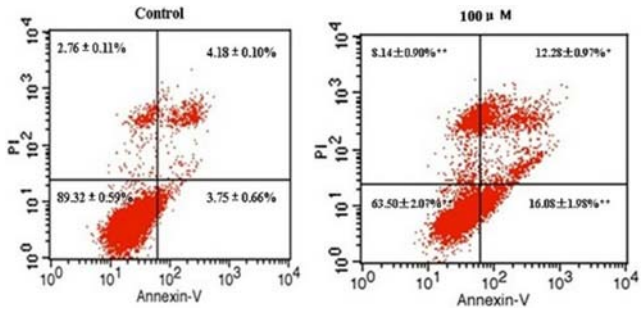


**Fig. 2. Apoptosis analysis of PANC-1 cells.** Apoptosis analysis of PANC-1 cells induced by 100 μM or free of NOB for 48 h, using a flow cytometer with Annexin V-FITC/PI binding assay. *$p$<0.05, **$p$<0.01 compared with control. Data are expressed as means± standard deviation ($n$=6).

evaluation of cell survival and proliferation based on the ability of mitochondria in live cells to reduce MTT to the purple-color compound formazan. A significant dose-dependent inhibitory effect of NOB on PANC-1 cell viability was seen at 24-72 h after treatment (Fig. 1). The minimum effective NOB concentration was 16 μM. At this concentration, the cell viability was decreased to 77.76± 11.71% at 24 h, compared with a control. However, cell viabilities were restored to 84.91±6.68% at 48 h and 91.04±11.71% at 72 h. At a concentration of 40 μM NOB, cell viability decreased to 79.63±10.26% at 24 h, to 76.02 ±8.59% at 48 h, and to 57.77±9.52% at 72 h, indicating NOB inhibition of PANC-1 cell proliferation with time. At concentrations of 100 μM and 250 μM NOB, similar patterns in cell viability over time were observed and the cell survival rate was reduced more. These results indicate that PANC-1 cells are sensitive to NOB, which exerts a dose and time dependent effect on PANC-1 cell proliferation (Fig. 1). To ensure adequate inhibitory effects, 100 μM NOB and 48 h were used for analysis.

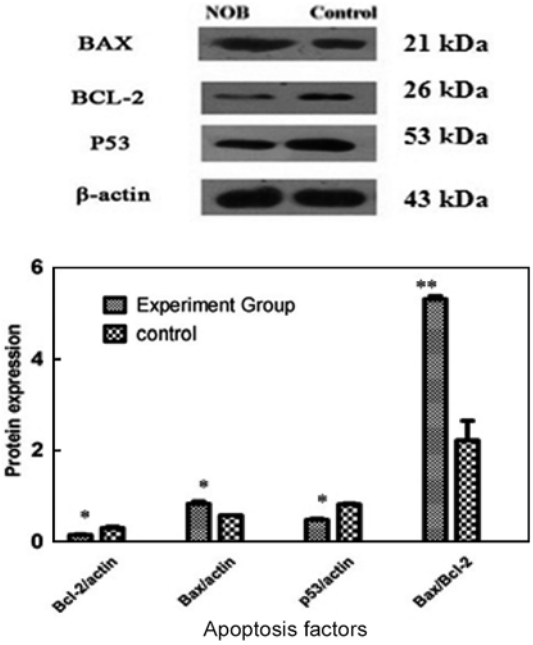


**Fig. 3. Effect of NOB on the expression of apoptosis-related proteins in PANC-1 cells.** *$p$<0.05, **$p$<0.01 compared with control. Data are expressed as mean±SME ($n$=6).

**NOB induced apoptosis in PANC-1 cells** Inhibition of PANC-1 cell viability and proliferation associated with the NOB-induced apoptosis was investigated. Apoptosis was analyzed using annexin-V-FITC and PI double staining, followed by flow cytometry. After 48 h of treatment with and without 100 μM NOB, there was a significant increase in the proportion of annexin-FITC single positive cells in NOB-treated samples (16.08±1.98%), compared with control samples (3.75±0.66%) ($p$<0.05) (Fig. 2) . There was also a significant increase in the proportion of annexin-FITC and PI double positive cells in NOB-treated (12.28± 0.97%) cells, compared with the control (4.18±0.10%) ($p$<0.05). In the presence of NOB, both early (annexin-V single positive) and late apoptotic (annexin-V and PI double positive) cell proportions were increased, compared to an absence of NOB.

**Effects of NOB on expression of apoptotic proteins** Western blot analysis was used to analyze expressions of the bax, bcl-2, and p53 proteins to further characterize the mechanism of NOB-induced apoptosis in PANC-1 cells. Bcl-2 is one of the best known apoptosis-suppressing gene products (20,21) and bax is a prototype apoptosis-promoting gene product (22). The Bax/bcl-2 ratio, to some extent, determines the fate of cells (23). There are both wild-type and mutation-type p53 cells. Wild-type p53 has an anti-tumor effect, but mutation-type p53 can promote tumor development (24). More than half of p53 cells in human cancer are mutation-type p53 (24). The expression level of

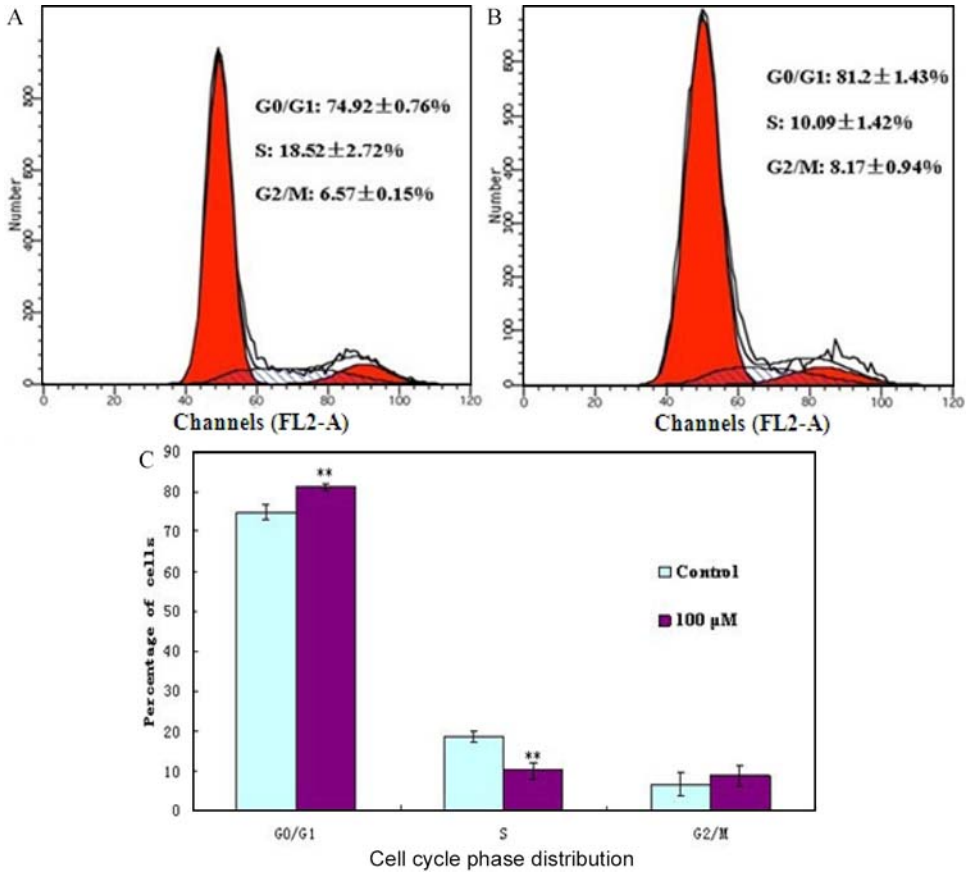


**Fig. 4. Flow cytometry analysis of cell cycle phase distribution in PANC-1 cells.** A, control; B, PANC-1 cells were treated with 100 μM NOB; C, the cell cycle phase distribution of control and sample. *$p$<0.05, **$p$<0.01 compared with control. Data are expressed as mean±SME ($n$=6).

the pro-apoptotic bax protein was significantly increased in PANC-1 cells treated with NOB, compared with the control ($p$<0.05) (Fig. 3). The expression level of the anti-apoptotic protein bcl-2 decreased markedly in NOB-treated cells, compared with control samples ($p$<0.05). The expression level of p53 (mutation-type) also decreased in NOB-treated samples ($p$<0.05). These results are consistent with apoptosis detected using annexin-V and PI double staining. Up-regulation of bax and down-regulation of bcl-2 unbalances the pro-apoptotic/anti-apoptotic ratio and causes apoptosis, and is at least partially responsible for the inhibitory effect of NOB on PANC-1 cell proliferation.

**NOB induced G0/G1 phase arrest in PANC-1 Cells**

Many antitumor compounds execute their anti-tumor effects by inducing apoptosis or by arresting the cell cycle at a particular checkpoint, or both (25,26). Cell cycle analysis using PI staining and flow cytometry was performed to investigate the potential anti-mitogenic effect of NOB. In a control group (without NOB) the frequencies of PANC-1 cells in the G0/G1 phase, S phase, and G2/M phase were 74.92±0.76%, 18.52±2.72%, and 6.57±0.15%, respectively (Fig. 4). After treatment with 100 μM NOB for 48 h, the frequencies of PANC-1 cells in the G0/G1 phase, S phase, and G2/M phase shifted to 81.2±1.43%, 10.09±1.42%, and 8.17±0.94%, respectively (Fig. 4A, 4B). The significant increase in the frequency of cells in the G0/G1 phase ($p$<0.05) and the significant decrease in the frequency of cells in the S phase ($p$<0.05) upon NOB treatment indicate that NOB affects PANC-1 cell cycle progression by imposing a G0/G1 arrest (Fig. 4C). Thus, PANC-1 cell proliferation was inhibited by NOB. These results indicate a mechanism that is different from other anti-tumor compounds by which NOB suppresses PANC-1 cell proliferation. The effects of casticin, oridonin nano-suspension, and cantharidin on cell cycle arrest take place in the G2/M phase (27-29). There was a small, non-significant increase in the proportion of G2/M phase cells in NOB-treated cells, compared with non-treated cells ($p$>0.05). p53 has an influence on DNA replication and, thus, influences the cell cycle, so p53 expression is closely related to the cell cycle (24). Also, p53 expression in PANC-1 cells in the presence and absence of NOB was examined because p53 has been seen to cause stagnation of the cell cycle at the G0/G1 phase in reaction to stress-induced cellular changes, and to induce apoptosis (30). The

p53 expression level in NOB-treated PANC-1 cells was lower, compared with non-treated cells, suggesting either that p53 is not a major contributor to the NOB related G0/G1 arrest in these cells, or that down-regulation of the mutated abnormally functional p53. This is the first mention of mutated abnormally functional p53. NOB has a negative regulatory role in development of human pancreatic cancer.

In summary, NOB effectively inhibits the proliferation of PANC-1 cells by inducing apoptosis and arresting the cell cycle at the G0/G1 phase. Unbalanced expressions of pro-apoptotic versus anti-apoptotic proteins are a key factor that contributes to the inhibitory effects of NOB. More detailed studies of the role of NOB in pancreatic cancer in the future will help with development of NOB as a new drug for cancer treatment or prevention.

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## References

1. Jemal A, Siegel R, Ward E, Hao YP, Xu JQ, Michael J. Cancer statistics. CA-Cancer J. Clin. 59: 225-249 (2009)
2. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics. CA-Cancer J. Clin. 60: 277-300 (2010)
3. Guo X, Cui Z. Current diagnosis and treatment of pancreatic cancer in China. Pancreas 31: 13-22 (2005)
4. Ardeesy N, DePinho RA. Pancreatic cancer biology and genetics. Nat. Rev. Cancer 2: 897-909 (2002)
5. Inoue T, Tsubaki S, Ogawa K, Onishi K, Azuma J. Isolation of hesperidin from peels of thinned *Citrus unshiu* fruits by microwave-assisted extraction. Food Chem. 123: 542-547 (2010)
6. He L, Gongke L, Zhanxia Z. Effects of operating parameters on the extraction of resveratrol in Huzhang (*Polygonum cuspidatum*) using microwave-assisted extraction. Chinese J. Anal. Chem. 31: 1341-1344 (2003)
7. Zhilu A, Juan G, Yuhong W, Yanxia L, Qiuyan Z. Microwave-assisted extraction technique of apple polyphenols in apple pomace. T. Chinese Soc. Agr. Eng. 6: 188-191 (2006)
8. Min X, Lu W. Study on extraction of cordycepin with microwave. Food Sci. 10: 248-251 (2006)
9. Mouly PP, Gaydou EM, Aazouyan C. Separation and quantitation of orange juices using liquid chromatography of polymethoxylated flavones. Analusis 27: 284-288 (1999)
10. Delaney B, Phillps K, Vasquez C, Wilson A, Cox D, Wang HB, Manthey J. Genetic toxieity of astandardized mixture of citrus polymethoxylated flavones. Food Chem Toxicol. 40: 617-624 (2002)
11. Delaney B, Phillip K, Buswell D, Mowry B, Nickels D, Cox D, Wang HB, Manthey J. Immunotoxicity of a standardized citrus polymethoxylated flavone extract. Food Chem. Toxicol. 39: 1087-1094 (2001)
12. Manthey JA, Grohmann K. Phenols in citrus peel by products concentrations of hydroxycinnamates and polylmethoxylated flavones in citrus peel molasses. J. Agr. Food Chem. 49: 3268-3273 (2001)
13. Londono-Londono J, De Lima V, Lara O, Gil A, Cresynski Pasa TB, Arango GJ, Pineda JR. Clean recovery of antioxidant flavonoids from citrus peel: Optimizing an aqueous ultrasound-assisted extraction method. Food Chem. 119: 81-87 (2010)
14. Waldman T, Zhang Y, Dillehay L, Yu J, Kinzler K, Vogelstein B, Williams J. Cell-cycle arrest versus cell death in cancer therapy. Nat. Med. 3: 1034-1036 (1997)
15. Akao Y, Ohguchi K, Iinuma M, Nozawa Y. Interactive effects of polymethoxy flavones from citrus on cell growth inhibition in human neuroblastoma SH-SY5Y cells. Bioorgan. Med. Chem. 16: 2803-2810 (2008)
16. Androutsopoulos VP, Surichan S, Arroo RRJ, Boarder MR. Citrus flavonoid nobiletin induces CVP1-enzyme activity and CYP1-mediated oxidative metabolism in MCF-7 breast adenocarcinoma cells. Int. J. Mol. Med. 26: S52-S52 (2010)
17. Lee YC, Cheng TH, Lee JS, Chen JH, Liao YC, Fong Y, Wu CH, Shih YW. Nobiletin, a citrus flavonoid, suppresses invasion and migration involving FAK/PI3K/Akt and small GTPase signals in human gastric adenocarcinoma AGS cells. Mol. Cell Biochem. 347: 103-115 (2011)
18. Miyata Y, Sato T, Imada K, Dobashi A, Yano M, Ito A. A citirus polymethoxyflavonoid, nobiletin, is a novel MEK inhibitor that exhibits antitumor metastasis in human fibrosarcoma HT-1080 cells. Biochem. Bioph. Res. Co. 366: 168-173 (2008)
19. Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchel JB. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer. Res. 47: 943-946 (1987)
20. Burlacu A. Regulation of apoptosis by bcl-2 family proteins. J. Cell Mol. Med. 7: 249-257 (2003)
21. Cory S, Adams JM. The bcl-2 family: Regulators of the cellular life-or-death switch. Nat. Rev. Cancer. 2: 647-656 (2002)
22. Kuwana T, Mackey MR, Perkins G. Ellisman MH, Latterich M, Schneiter R, Green DR, Newmeyer DD. Bid, bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell 111: 331-342 (2002)
23. Del Poeta G, Venditti A, Del Principe MI, Maurillo L, Buccisano F, Tamburini A, Cox MC, Franchi A, Bruno A, Mazzone C, Panetta P, Suppo G, Masi M, Amadori S. Amount of spontaneous apoptosis detected by bax/bcl-2 ratio predicts outcome in acute myeloid leukemia (amL). Blood 101: 2125-2131 (2003)
24. Pietenpol JA, Stewart ZA. Cell cycle checkpoint signaling: cell cycle arrest versus apoptosis. Toxicology 12: 475- 481(2002)
25. Chan KT, Meng FY, Li Q, Ho CY, Lam TS, To Y, Lee WH, Li M, Chu KH, Toh M. Cucurbitacin B induces apoptosis and S phase cell cycle arrest in BEL-7402 human hepatocellular carcinoma cells and is effective via oral administration. Cancer Lett. 294: 118-124 (2010)
26. Shen JK, Du HP, Yang M, Wang YG, Jin J. Casticin induces leukemic cell death through apoptosis and mitotic catastrophe. Ann. Hematol. 88: 743-752 (2009)
27. Ding C, Khan M, Zheng B, Yang JB, Zhong LL, Ma TH. Casticin induces apoptosis and mitotic arrest in pancreatic carcinoma PANC-1 cells. Afr. J. Pharm. Pharmaco. 6 : 412-418 (2012)
28. Qi X, Zhang DR, Xu X, Feng FF, Ren GJ, Chu QQ, Zhang Q, Tian K. Oridonin nanosuspension was more effective than free oridonin on G2/M cell cycle arrest and apoptosis in the human pancreatic cancer PANC-1 cell line. Int. J. Nanomed. 7: 1793-1804 (2012)
29. Li W, Xie L, Chen Z, Zhu Y, Sun YJ, Miao Y, Xu ZK, Han X. Cantharidin, a potent and selective PP2A inhibitor, induces an oxidative stressindependent growth inhibition of pancreatic cancer cells through G2/M cellcycle arrest and apoptosis. Cancer Sci. 101 : 1226-1233 (2010)
30. El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. Cell 75: 817-825 (1993)