Patrinia scabiosaefolia inhibits the proliferation of colorectal cancer *in vitro* and *in vivo* via G_1/S cell cycle arrest

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Abstract. *Patrinia scabiosaefolia* (PS) has long been used as an important component in traditional Chinese medicine formulas to treat gastrointestinal malignancies including colorectal cancer (CRC). We recently reported that PS can inhibit CRC growth through induction of apoptosis and inhibition of tumor angiogenesis. To further elucidate the mode of action of PS, in the present study, we used a CRC mouse xenograft model and a human CRC cell line HT-29 to evaluate the effect of the ethanol extract of PS (EEPS) on cancer cell proliferation and investigated the underlying molecular mechanisms. We found that EEPS inhibited CRC growth both *in vivo* and *in vitro*, which was associated with the inhibitory effects of EEPS on cancer cell proliferation. In addition, EEPS treatment significantly blocked G_1 to S phase cell cycle progression in HT-29 cells. Moreover, EEPS treatment decreased the expression of pro-proliferative CyclinD1 and CDK4, at both the mRNA and protein levels. Thus, inhibition of cell proliferation via G_1/S cell cycle arrest might be a potential mechanism whereby PS effectively treats cancers.

Introduction

Colorectal cancer (CRC) is one of the most common malignancies with high incidence in Western countries (1). At present, 5-fluorouracil-based regimens have been performed as international standard chemotherapy for CRC treatment (2). However, 5-FU-based regimens are often coupled with serious toxicity and side effects such as anemia, leucopenia, thrombocytopenia and peripheral neuropathy (3-5). Thus, the development of novel therapeutic drugs is necessary. Natural products, including traditional Chinese medicines (TCM), have been considered as alternative cancer remedies for many years. Numerous plants and their constituents have been shown to possess beneficial therapeutic effects for various diseases including cancer (6,7). *Patrinia scabiosaefolia* (PS), a perennial plant natively distributed in Eastern Asia, has long been used in China to clinically treat edema, appendicitis, endometritis and other inflammatory diseases (8-10). More importantly, PS has also been used as a major component in several TCM formulas for the treatment of gastrointestinal cancers (11,12). We recently reported that PS can inhibit CRC growth through induction of apoptosis and inhibition of tumor angiogenesis (12-14). To further elucidate the mode of action of PS, in the present study we used a CRC mouse xenograft model and a human CRC cell line HT-29 to evaluate the effect of the ethanol extract of PS (EEPS) on cancer cell proliferation and investigated the underlying molecular mechanisms.

Materials and methods

Materials and reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA, and TRIzol reagent were purchased from Life Technologies (Carlsbad, CA, USA). SuperScript II Reverse Transcriptase was obtained from Promega Corporation (Madison, WI, USA). PCNA assay kit was purchased from R&D Systems (Minneapolis, MN, USA). CDK4 and CyclinD1, β-actin antibodies, and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). A cell cycle kit was purchased from BD Biosciences (San Jose, CA, USA). All other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of ethanol extract from PS. Ethanol extract from *Patrinia scabiosaefolia* (EEPS) was prepared as described previously (12). For animal experiments, EEPS powder was dissolved in saline to a working concentration of 250 mg/ml. In cell-based experiments, EEPS powder was dissolved in 50% DMSO to a stock concentration of 250 mg/ml, and the

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Abbreviations: CRC, colorectal cancer; TCM, traditional Chinese medicine; EEPS, ethanol extract of *Patrinia scabiosaefolia*; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IHS, immunohistochemical staining

Key words: Patrinia scabiosaefolia, herbal medicine, anticancer treatment, proliferation, cell cycle

EEPS working concentrations were obtained by diluting the stock solution in the culture medium. The final concentration of DMSO in the medium for all cell experiments was <0.5%.

Cell culture. Human CRC HT-29 cells were obtained from the cell bank of the Chinese Academy of Science (Shanghai, China). The cells were grown in DMEM containing 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured at 37˚C in a humidified incubator with 5% CO_2.

Animals. Male BALB/c athymic (nude) mice (with an initial body weight of 20-22 g) were obtained from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and housed under pathogen-free conditions with controlled temperature (22˚C), humidity, and a 12-h light/dark cycle. Food and water were given *ad libitum* throughout the experiment. All animal treatments were strictly in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. The experiments were approved by the Institutional Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine.

In vivo tumor xenograft study. HT-29 cells ($1.5x10^6$) mixed with Matrigel (1:1) were subcutaneously injected into the right flank of the mice to initiate tumor growth. After 5 days of xenograft implantation, mice were randomly divided into 2 groups (n=6) and given intragastric administration with 1.93 g/kg/day of EEPS or saline daily, 5 days per week for 3 weeks. Tumor size was determined by measuring the major (L) and minor (W) diameter with a caliper. The tumor volume was calculated according to the following formula: Tumor volume = $\pi/6$ x L x W^2.

Immunohistochemical staining (IHS). After being fixed with 10% formaldehyde for 12 h, the tumor samples were processed conventionally for paraffin-embedded 4-μm-thick tumor slides. The slides were subjected to antigen retrieval, and the endogenous peroxidase activity was quenched with hydrogen peroxide. After blocking non-specific proteins with normal serum in phosphate-buffered saline (PBS) (0.1% Tween-20), the slides were incubated with rabbit polyclonal antibodies against PCNA (1:200 dilution) for 1 h. After washing with PBS, slides were incubated with a biotinylated secondary antibody followed by conjugated HRP-labeled streptavidin (Dako), and then washed with PBS. The slides were then incubated with diaminobenzidine (Sigma) as the chromogen, followed by counterstaining with diluted Harris' hematoxylin (Sigma). After staining, five high-power fields (x400 magnification) were randomly selected in each slide, and the average proportion of positive cells in each field was counted using a true color multi-functional cell image analysis management system (Image-Pro Plus; Media Cybernetics, Rockville, MD, USA). To rule out any nonspecific staining, PBS was used to replace the primary antibody as a negative control.

Evaluation of cell viability by MTT assay. The HT-29 cell viability was assessed by MTT colorimetric assay. HT-29 cells were seeded into 96-well plates at a density of $1x10^4$ cells/well in 0.1-ml medium. The cells were treated with various EEPS concentrations for 24 h. At the end of the treatment, 100 μl MTT (0.5 mg/ml in PBS) was added to each well, and the samples were incubated for an additional 4 h at 37˚C. The purple-blue MTT formazan precipitate was dissolved in 100 μl DMSO. The absorbance was measured at 570 nm using an ELISA reader (BioTek, Model ELX800, BioTek Instruments, Inc., Winooski, VT, USA).

Colony formation assay. HT-29 cells were seeded in 6-well plates at a density of $2x10^5$ cells/well in 2 ml medium. After treatment with various EEPS concentrations for 24 h, the cells were collected and diluted in fresh medium in the absence of EEPS and then reseeded in 6-well plates at a density of $1x10^3$ cells/well. Following incubation for 7 to 8 days in a 37˚C humidified incubator with 5% CO_2, the formed colonies were fixed with 4% paraformaldehyde for 15 min, stained with 0.01% crystal violet, and counted. The numbers of colonies were observed, and the data were normalized to the number of control cells (100%).

Cell cycle analysis. Cell cycle was analyzed by flow cytometry using fluorescence-activated cell sorting (FACSCalibur; Becton-Dickinson, San Jose, CA, USA) and propidium iodide (PI) staining. Subsequent to treatment with various EEPS concentrations (0, 0.5, 1 and 2 mg/ml) for 24 h, HT-29 cells were collected and adjusted to a concentration of $5x10^5$ cells/ml, and fixed in 70% ethanol at 4˚C overnight. The fixed cells were washed twice with cold PBS and then incubated for 30 min with RNase (8 μg/ml) and PI (10 μg/ml). The fluorescent signal was detected through the FL2 channel, and the DNA proportion in different phases was analyzed using ModfitLT ver. 3.0 (Verity Software House, Topsham, ME, USA).

RNA extraction and RT-PCR analysis. Total RNA was isolated from the tumor tissues or HT-29 cells with TRIzol reagent. Oligo(dT)-primed RNA (1 μg) was reverse-transcribed with SuperScript II reverse transcriptase according to the manufacturer's instructions. The obtained cDNA was used to determine the mRNA amount of CDK4 and CyclinD1 by PCR. GAPDH was used as an internal control. The sequences of the primers used for amplification of CyclinD1 and CDK4 were as follows: Cyclin D1 forward, 5'-GGA GCA GAA GTG CGA AGA-3' and reverse, 5'-GGG TGG GTT GGA AAT GAA-3' (Tm = 57˚C, 394 bp); CDK 4 forward, 5-CTT CCC GTC AGC ACA GTTC-3 and reverse, 5'-GGT CAG CAT TTC CAG TAGC-3' (Tm = 55˚C, 687 bp); GAPDH forward, 5'-CGA CCA CTT TGT CAA GCTCA-3' and reverse, 5'-AGG GGT CTA CAT GGC AACTG-3' (Tm = 58˚C, 240 bp). Samples were analyzed by gel electrophoresis (1.5% agarose). The DNA bands were examined using a Gel Documentation system (Model Gel Doc 2000; Bio-Rad, Hercules, CA, USA).

Western blot analysis. Tumor tissues were homogenized in non-denaturing lysis buffer and centrifuged at 14,000 x g for 15 min. Protein concentrations of the clarified supernatants were determined by BCA protein assay. HT-29 ($1x10^6$) cells were seeded into culture flasks in 5 ml medium and treated with various concentrations of EEPS for 24 h. Treated cells were lysed in mammalian cell lysis buffer (M-PER, Thermo

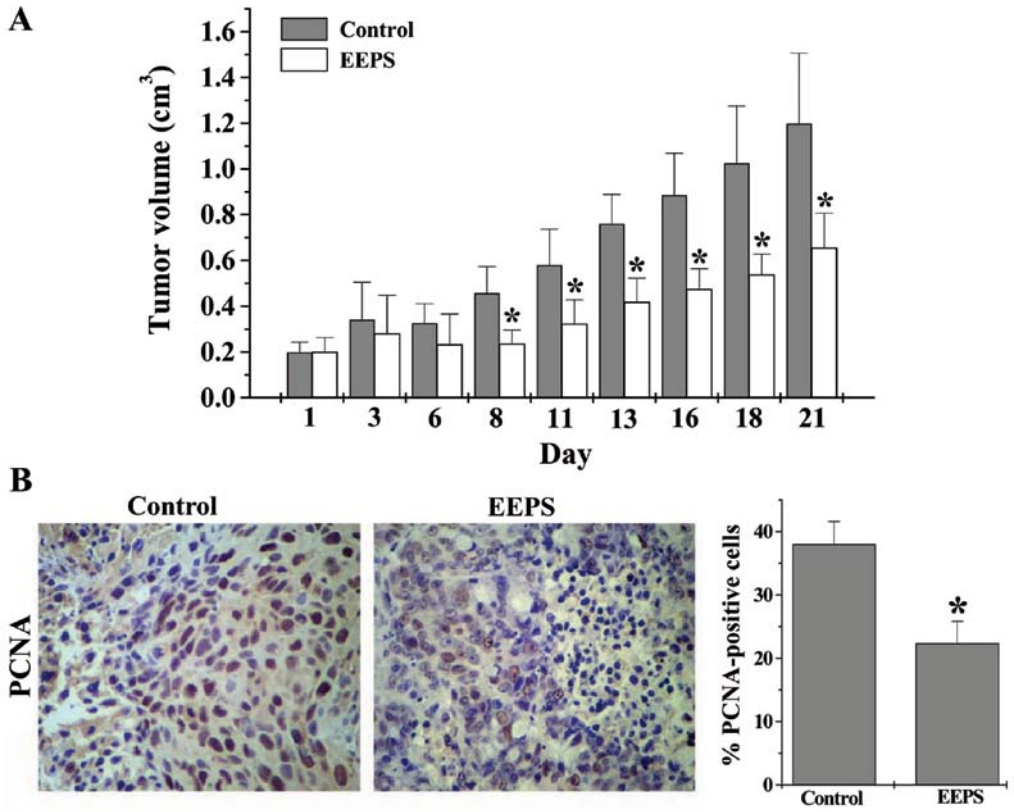

Figure 1. Effect of EEPS on cancer cell proliferation in CRC xenograft mice. (A) After tumor development, the mice received an intragastric administration of a 1.93 g/kg/day of EEPS or saline daily, 5 days a week for 3 weeks. Tumor volume was measured during the course of study. Data shown are averages with SD (error bars) from 6 individual mice in each group. *P<0.05, vs. the controls. (B) At the end of the experiment, tumor tissues were processed for immunohistochemical staining (IHS) for PCNA. The photographs are representative images captured at a magnification, x400. Quantification of IHS assay was represented as the percentage of positively stained cells. Data shown are averages with SD (error bars) from 6 individual mice in each group. *P<0.05 vs. controls.

Scientific, Rockford, IL, USA) containing protease (EMD Biosciences) and phosphatase inhibitor (Sigma-Aldrich) cocktail and centrifuged at 14,000 x g for 15 min. Protein concentrations in the cell lysate supernatants were determined by BCA protein assay and resolved on 12% Tris-glycine gels. Equal amounts of protein from each tumor or cell lysate were separated by 10% SDS-PAGE gels and transferred onto PVDF membranes. Membranes were blocked for 2 h with blocking solution at room temperature, washed in TBS with 0.25% Tween-20 (TBS-T), and exposed to primary antibodies against CDK4, CyclinD1 and β-actin (all at 1:1,000 dilutions) overnight at 4˚C. After the membranes were washed in TBS-T, secondary HRP-conjugated antibodies (anti-rabbit or anti-mouse) were added at a 1:2,000 dilution for 1 h at room temperature, and the membranes were washed again in TBS-T followed by enhanced chemiluminescence detection.

Statistical analysis. Data are presented as means ± SD for the indicated number of independently performed experiments and analyzed using the SPSS package for Windows (ver. 18.0). Statistical analysis of the data was performed with the Student's t-test and ANOVA. Differences with P<0.05 were considered statistically significant.

Results and Discussion

EEPS inhibits cancer cell proliferation in CRC xenograft mice. Cancer cells are characterized by an uncontrolled increase in cell proliferation (15); therefore inhibiting excessive proliferation of tumor cells is one of the key approaches for development of anticancer drugs. We first evaluated the efficacy of EEPS against tumor growth *in vivo* in the CRC mouse xenograft model. As shown in Fig. 1A, EEPS treatment significantly reduced the tumor volume in CRC mice (P<0.05, vs. controls), demonstrating its *in vivo* anticancer activity. We next determined the cell proliferation in CRC tumor tissues using IHS to examine the expression of proliferating cell nuclear antigen (PCNA), a processivity factor for DNA polymerase that has been recognized as a histologic marker for cell proliferation (16). As shown in Fig. 1B, the percentages of PCNA-positive cells in the control and the EEPS-treated mouse group were 38.0 and 22.3%, respectively (P<0.05), suggesting that EEPS significantly suppressed CRC cell proliferation *in vivo*.

EEPS inhibits cell proliferation in HT-29 cells. The *in vitro* effect of EEPS on CRC cell proliferation was determined by MTT assay to compare the viability of HT-29 cells in EEPS-treated monolayers to untreated controls. As shown in Fig. 2A, treatment with 0.5 to 2 mg/ml of EEPS for 24 h dose-dependently reduced cell viability by 21-62% compared with the untreated control cells (P<0.05). These results were verified using a colony formation assay. EEPS treatment reduced the survival rate of HT-29 cells in a dose-dependent manner (P<0.05, Fig. 2B), suggesting that EEPS can inhibit CRC cell proliferation *in vitro*.

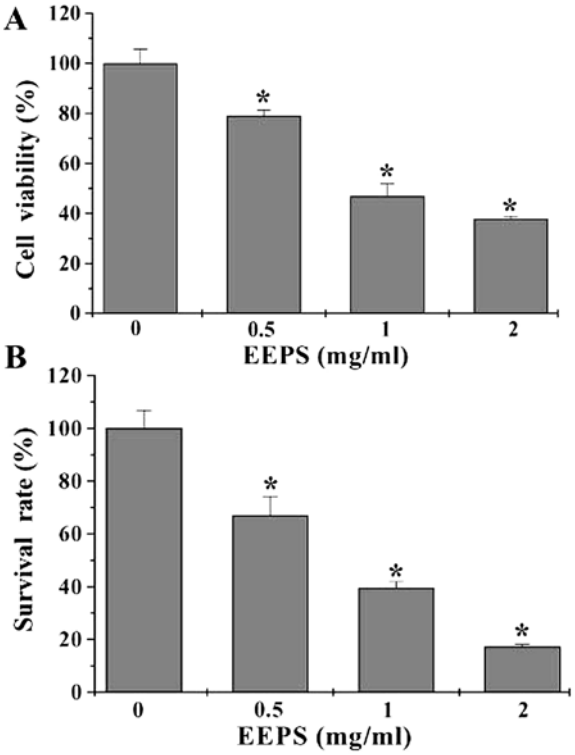

Figure 2. Effect of EEPS on the proliferation of HT-29 cells. After treatment with the indicated concentrations of EEPS for 24 h, (A) the viability of HT-29 cells was determined by the MTT assay, and (B) the cell survival rate was evaluated by colony formation assay. The data were normalized to the viability or survival of the untreated control cells (100%). Data are averages with SD (error bars) from 3 independent experiments. *P<0.05 vs. controls.

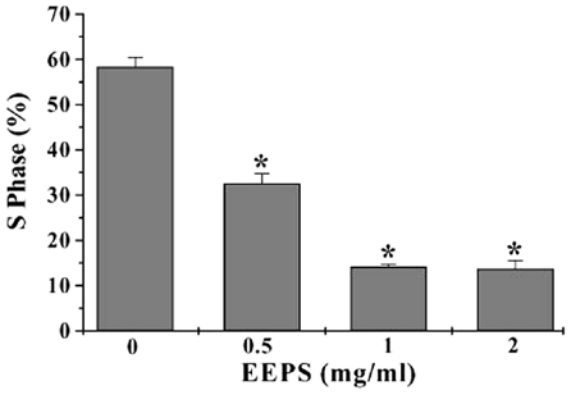

Figure 3. Effect of EEPS on the cell cycle progression in HT-29 cells. Cells were treated with the indicated concentrations of EEPS for 24 h, stained with PI, and analyzed by FACS. The proportion of DNA in the S-phase was calculated using ModfitLT ver. 3.0 software. Data shown are averages with SD (error bars) from 3 independent experiments. *P<0.05, vs. control cells.

EEPS blocks cell cycle G_1/S progression in HT-29 cells. Eukaryotic cell proliferation is strongly regulated by the cell cycle, which consists of S, M, G_1 and G_2 phases. G_1 to S transition is one of the two main checkpoints of the cell cycle that is responsible for initiation and completion of DNA replication (17). We, therefore, investigated the effect of EEPS on the G_1 to S phase progression. Using PI staining followed by FACS analysis we found that the percentage of HT-29 cells in the S-phase following treatment with 0, 0.5, 1 or 2 mg/ml of EEPS was 58.3, 32.6, 14.1 or 13.7%, respectively (P<0.05, vs. untreated control cells) (Fig. 3), indicating that the inhibitory effect of EEPS on CRC cell proliferation was mediated by G_1/S cell cycle arrest.

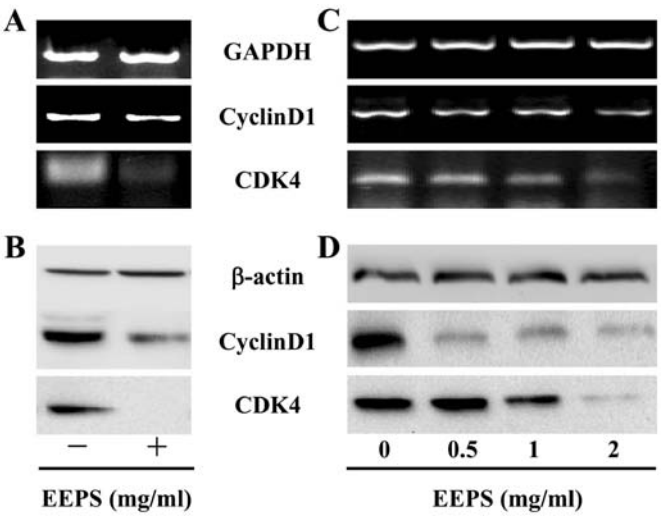

Figure 4. Effect of EEPS on the expression of CyclinD1 and CDK4 in CRC xenograft mouse tissues and HT-29 cells. (A and C) The mRNA levels of CyclinD1 and CDK4 in CRC xenograft mouse tissues and in HT-29 cells were determined by RT-PCR. (B and D) The protein expression levels of CyclinD1 and CDK4 were determined by western blot analysis. GAPDH and β-actin were used as the internal controls for the RT-PCR or western blotting, respectively. Images are representative from 6 individual mice in each group or from 3 independent cell-based experiments.

EEPS downregulates the expression of CyclinD1 and CDK4 in vivo and in vitro. G_1/S progression is highly mediated by CyclinD1, which forms an active complex with its CDK major catalytic partners (CDK4/6) (18-20). Overexpression of CyclinD1 and CDK4 is commonly found in many types of human cancer (21-24). To further explore the mechanism of the anti-proliferative activity of EEPS, we performed RT-PCR and western blot analyses to examine the mRNA and protein levels of CyclinD1 and CDK4. The results of the RT-PCR assay showed that EEPS treatment significantly reduced the mRNA expression of pro-proliferative CyclinD1 and CDK4 both in CRC tumor tissues and in HT-29 cells (Fig. 4A and C); and the pattern of protein expression of CyclinD1 and CDK4 was similar to their respective mRNA level (Fig. 4B and D).

In conclusion, in the present study, we report that *Patrinia scabiosaefolia* inhibits colorectal cancer cell proliferation through blockade of G_1/S progression and the modulation of cell cycle-regulatory gene expression, which may be one of the mechanisms through which *Patrinia scabiosaefolia* exerts its antitumor function.

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References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.

2. Gustin DM and Brenner DE: Chemoprevention of colon cancer: current status and future prospects. Cancer Metastasis Rev 21: 323-348, 2002.
3. Longley DB, Allen WL and Johnston PG: Drug resistance, predictive markers and pharmacogenomics in colorectal cancer. Biochim Biophys Acta 1766: 184-196, 2006.
4. Sun Y, Zhao H, Guo Y, Lin F, Tang L and Yao Y: Clinical study of combining chemotherapy of oxaliplatin or 5-fluorouracil/leucovorin with hydroxycamptothecine for advanced colorectal cancer. Clin Oncol Cancer Res 6: 117-123, 2009.
5. Boose G and Stopper H: Genotoxicity of several clinically used topoisomerase II inhibitors. Toxicol Lett 116: 7-16, 2000.
6. Newman DJ, Cragg GM and Snader KM: The influence of natural products upon drug discovery. Nat Prod Rep 17: 215-234, 2000.
7. Gordaliza M: Natural products as leads to anticancer drugs. Clin Transl Oncol 9: 767-776, 2007.
8. Cho EJ, Shin JS, Noh YS, Cho YW, Hong SJ, Park JH, Lee JY, Lee JY and Lee KT: Anti-inflammatory effects of methanol extract of *Patrinia scabiosaefolia* in mice with ulcerative colitis. J Ethnopharmacol 136: 428-435, 2011.
9. Gao L, Zhang L, Li N, Liu JY, Cai PL and Yang SL: New triterpenoid saponins from *Patrinia scabiosaefolia*. Carbohydr Res 346: 2881-2885, 2011.
10. Ju HK, Baek SH, An RB, Bae K, Son KH, Kim HP, Kang SS, Lee SH, Son JK and Chang HW: Inhibitory effects of nardostachin on nitric oxide, prostaglandin E2, and tumor necrosis factor-alpha production in lipopolysaccharide activated macrophages. Biol Pharm Bull 26: 1375-1378, 2003.
11. Chiu LC, Ho TS, Wong EY and Ooi VE: Ethyl acetate extract of *Patrinia scabiosaefolia* downregulates anti-apoptotic Bcl-2/Bcl-XL expression, and induces apoptosis in human breast carcinoma MCF-7 cells independent of caspase-9 activation. J Ethnopharmacol 105: 263-268, 2006.
12. Peng J, Chen YQ, Lin JM, Zhuang QC, Xu W, Hong ZF and Sferra TJ: *Patrinia scabiosaefolia* extract suppresses proliferation and promotes apoptosis by inhibiting the STAT3 pathway in human multiple myeloma cells. Mol Med Rep 4: 313-318, 2011.
13. Liu LY, Shen AL, Chen YQ, Wei LH, Lin JM, Sferra TJ, Hong ZF and Peng J: *Patrinia scabiosaefolia* induces mitochondrial-dependent apoptosis in a mouse model of colorectal cancer. Oncol Rep 30: 897-903, 2013.
14. Chen LW, Liu LY, Ye L, Shen AL, Chen YQ, Sferra TJ and Peng J: *Patrinia scabiosaefolia* inhibits colorectal cancer growth through suppression of tumor angiogenesis. Oncol Rep 30: 1439-1443, 2013.
15. Evan GI and Vousden KH: Proliferation, cell cycle and apoptosis in cancer. Nature 411: 342-348, 2001.
16. Zhong W, Peng J, He H, Wu D, Han Z, Bi X and Dai Q: Ki-67 and PCNA expression in prostate cancer and benign prostatic hyperplasia. Clin Invest Med 31: E8-E15, 2008.
17. Nurse P: Ordering S phase and M phase in the cell cycle. Cell 79: 547-550, 1994.
18. Chen Y, Robles AI, Martinez LA, Liu F, Gimenez-Conti IB and Conti CJ: Expression of G_1 cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors in androgen-induced prostate proliferation in castrated rats. Cell Growth Differ 7: 1571-1578, 1996.
19. Grana X and Redy EP: Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). Oncogene 11: 211-219, 1995.
20. Morgan DO: Principles of CDK regulation. Nature 374: 131-134, 1995.
21. Harakeh S, Abu-El-Ardat K, Diab-Assaf M, Niedzwiecki A, El-Sabban M and Rath M: Epigallocatechin-3-gallate induces apoptosis and cell cycle arrest in HTLV-1-positive and -negative leukemia cells. Med Oncol 25: 30-39, 2008.
22. Kessel D and Luo Y: Cells in cryptophycin-induced cell-cycle arrest are susceptible to apoptosis. Cancer Lett 151: 25-29, 2000.
23. Purohit A, Hejaz HAM, Walden L, MacCarthy-Morrogh L, Packham G, Potter BVL and Reed MJ: The effect of 2-methoxyoestrone-3-*O*-sulphamate on the growth of breast cancer cells and induced mammary tumours. Int J Cancer 85: 584-589, 2000.
24. Zafonte BT, Hulit J, Amanatullah DF, Albanese C, Wang C, Rosen E, Reutens A, Sparano JA, Lisanti MP and Pestell RG: Cell-cycle dysregulation in breast cancer: breast cancer therapies targeting the cell cycle. Front Biosci 5: D938-D961, 2000.