Anti-proliferative effect of an extract of the root of *Polygonum multiflorum* Thunb. on MCF-7 human breast cancer cells and the possible mechanisms

HONG-SHENG CHEN[1], YAN LIU[1], LUO-QIANG LIN[1], JIN-LU ZHAO[1], CHUN-PENG ZHANG[1], JUN-CHAO JIN[1], LEI WANG[1], MING-HAN BAI[1], YI-CHONG WANG[1], MING LIU[1] and BAO-ZHONG SHEN[2]

[1]Treatment Center of Oncology; [2]Department of Imaging, The Fourth Affiliated Hospital of Harbin Medical University, Harbin 150001, P.R. China

Abstract. The root of *Polygonum multiflorum* Thunb. (PM) is utilized to treat many diseases associated with aging. Research also indicates that PM inhibits the proliferation of certain types of cancer cells. The aim of the present study was to evaluate the inhibitory effect of PM extract (PME) on the proliferation of MCF-7 cells and to investigate the underlying mechanisms. Inhibition of the proliferation of MCF-7 cells was determined by the MTT assay. Cell cycle distribution and apoptotic rates were evaluated by flow cytometry, and cell cycle and apoptosis-related protein expression was assessed by Western blotting. Apoptotic characteristics of MCF-7 cells were detected by transmission electron microscopy. The present study showed that PME at doses of 100, 150, 200 and 250 µg/ml significantly inhibited proliferation of MCF-7 cells in a time- and dose-dependent manner. Flow cytometry showed that the cell apoptotic rates were 9.1±1.67 and 17.7±2.93% after treatment with 100 and 200 µg/ml PME for 48 h, respectively. The proportions of cells in the G2/M phase were 37.9±1.47 and 42.0±1.71% after treatment with 100 and 200 µg/ml PME for 24 h, respectively. Western blot analysis showed that PME down-regulated the protein expression of Cdc25B and Cdc25C phosphatases accompanied by an increase in phospho-Cdk1, and PME promoted cytochrome c release from mitochondria into the cytosol to activate caspase-9. The present study demonstrated that PME inhibited MCF-7 cell proliferation by inducing cell cycle arrest in the G2/M phase and promoting cell apoptosis. The effects of PME on MCF-7 cells were associated with the modulation of the expression levels of proteins involved in the cell cycle and apoptosis. These data suggest that PME has promise as a treatment against breast cancer by inhibiting the proliferation of cancer cells.

Correspondence to: Dr Ming Liu, Treatment Center of Oncology, The Fourth Affiliated Hospital of Harbin Medical University, NanGang, Harbin 150001, P.R. China
E-mail: mliu35@yahoo.com.cn

Dr Bao-Zhong Shen, Department of Imaging, The Fourth Affiliated Hospital of Harbin Medical University, NanGang, Harbin 150001, P.R. China
E-mail: shenbzh@vip.sina.com

Abbreviations: PME, *Polygonum multiflorum* Thunb. extract; TCM, traditional Chinese medicine; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMSF, phenylmethyl-sulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; TBS, Tris-buffered saline; TEM, transmission electron microscopy; FCM, flow cytometry; Cdks, cyclin-dependent kinases; PP2A, protein phosphatase 2A; PRL-3, phosphatase of regenerating liver-3; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SD, standard deviation

Key words: root of *Polygonum multiflorum* Thunb. extract, anti-proliferation, cell cycle arrest, apoptosis, human breast cancer cells

Introduction

Natural products, which are important sources of medicinal compounds, have been used as drugs to treat many diseases since ancient times. Particularly in the area of cancer, more than 60% of antitumor medicines on the market are based on natural products (1).

The root of *Polygonum multiflorum* Thunb. (PM), a perennial vine-like herb, which is called He-shou-wu, has been widely used in traditional Chinese medicine (TCM) as a tonic and anti-aging agent for centuries. Clinically, it has been utilized in the treatment of poliosis, hemopenia, inflammation, bacterial infections, hyperlipidemia, coronary heart disease, neurosis and other diseases commonly associated with aging (2,3). The medicinal effects of PM in the improvement of health conditions are possibly mediated by the antioxidant capacity of this herb (4). The genus PM is the source of a wide range of phenolic compounds, flavonoids, anthraquinones, stilbenes and tannins (5,6). These constituents which possess biological activities in PM are called phytochemicals. Several studies have demonstrated that these phytochemicals from PM display high pharmacological efficiency, such as antioxidant capacity, anti-proliferative, anti-inflammatory, antiviral activies and improvement in learning and memory ability (7-10). Research indicates that PM inhibits the proliferation of certain types of

carcinoma cells, such as HT29 and SW620 colon cancer cells, and HepG2 human hepatoblastoma cells, but the underlying pharmacological mechanisms are largely unclear (11-13).

Cell cycle is the mechanism through which cells divide, and it is an orderly and tightly regulated phenomenon, including four phases (G1, S, G2 and M). Cancer cells have deregulated cell cycle progression with overexpression of positive regulators and inhibition of negative regulators, giving them unlimited replication potential. Therefore, development of agents targeting the deregulated cell cycle has been considered as an ideal strategy for cancer therapy. Cdc25 phosphatase inhibitors, which are cell cycle-based agents, inhibit the proliferation of many types of tumor cells by causing cell cycle arrest. Many of the most potent Cdc25 phosphatase inhibitors reported to date are quinonoid compounds. PM contains copious anthraquinone compounds, thus we assumed that PME may inhibit Cdc25 phosphatases to cause anti-proliferation activity in cancer cells.

The aim of this study was to investigate the roles of PME in cell cycle distribution and cell apoptosis in MCF-7 human breast cancer cells and its underlying mechanisms. We revealed that PME inhibits the proliferation of MCF-7 cells by arresting the cell cycle in the G2/M phase and by promoting cell apoptosis. The effects are mainly mediated by inhibition of Cdc25 phosphatases and modulation of the bax-dependent apoptotic pathway.

Materials and methods

Materials. PM was obtained from the Harbin branch of Peking Tong Ren Tang TCM Co., Ltd. (Harbin, China). Antibodies against Cdc25B, Cdc25C, cyclin B1, Cdk1, phospho-Cdk1 (p-Cdk1, Tyr15), bcl-2-associated X protein (bax), bcl-2, cytochrome c, cleaved caspase-9 (Asp315, 35 kDa) and β-actin were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Annexin V-FITC kit, propidium iodide (PI) and RNase A were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The BCA protein assay kit was purchased from Pierce Biotechnology, Inc. (Rockford, IL, USA) and the BCIP/NBT Alkaline Phosphatase Color Development kit was purchased from Sangong Biotech Co., Ltd. (Shanghai, China). All of the other reagents were of analytical grade.

Preparation of PME. The dried crude root of PM (10 g) was cut into small pieces and ground into coarse powder that passed through a 20-mesh (0.9-mm) sieve and extracted with 50% ethanol (200 ml) and agitated continuously on a rocker platform at 30°C for 20 h. After centrifugation at 8,000 rpm for 15 min, the supernatant was concentrated to obtain a brown powder (182 mg) under reduced pressure by a rotary evaporator and stored at -20°C. PME was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 2 mg/ml prior to the experiments.

Cell lines. The MCF-7 human mammary epithelial cell line was purchased from the cell bank of the Shanghai Institute of Biological Sciences, Chinese Academy of Sciences. MCF-7 cells were routinely cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin solution at 37°C in an atmosphere of 95% air and 5% CO_2 in a

humidified incubator. Cell cycle was synchronized at G0 phase by incubation in FBS-free RPMI-1640 for 16 h, followed by washing with phosphate-buffered saline (PBS) and fresh medium replacement when the experiments concerning cell cycle were performed.

Proliferation assay. MCF-7 cells were seeded in 96-well microtiter plates at a density of 1×10^4 cells/well in 200 µl medium; the medium was replaced the following morning with 200 µl of fresh complete medium containing PME of different final concentrations. Cells were treated with PME for different times according to the experimental requirement, using DMSO as vehicle at a maximum concentration of 0.1%. After PME treatment, MTT solution (5 mg/ml, 20 µl, final concentration 0.5 mg/ml) in RPMI-1640 without FBS was added to every well for 4 h at 37°C. Dark blue formazan crystals forming in the intact cells were dissolved with DMSO for 10 min on a rocker platform, and the absorbance was measured at 570 nm using an ELISA reader (Tecan Sunrise, Switzerland). The results were then expressed as percentages of MTT reduction, with the absorbance exhibited by the control cells being arbitrarily set as 100%. The inhibitory rate was calculated according to: (OD control - OD sample)/OD control x 100%.

Flow cytometry (FCM). The effect of PME on cell cycle distribution was determined by FCM after staining the cells with PI. Briefly, 5×10^5 cells were seeded in 25-cm² culture flasks and allowed to attach for 6 h of incubation and FBS-free medium replacement overnight. The medium was replaced with fresh complete medium containing PME at the desired concentrations. DMSO (final concentration <0.1%) was used as a negative control. After treatment for 24 h at 37°C, floating and adherent cells were collected, washed with ice-cold PBS and fixed with 70% ethanol for at least 12 h at 4°C. The cells were then treated with 80 mg/ml RNase A and 50 µg/ml PI at a density of 1×10^6 cells/ml for 30 min, and the stained cells were analyzed using a BD FACS Calibur. Data acquisition (10,000 events for each sample) was performed using Cell Quest software (Becton Dickinson, USA).

The apoptotic rate was measured by FCM according to the instructions provided by the Annexin V-FITC kit. In brief, following treatment with PME, cells were harvested after digestion with 0.25% trypsin, washed three times with ice-cold PBS containing calcium and resuspended in binding buffer (500 µl) at a concentration of 1×10^6 cells/ml, in which 500 µl of cell suspension was added to a 5-ml FCM tube. Annexin V-FITC (50 µg/ml, 5 µl) and PI (50 µg/ml, 5 µl) were added, and incubation was carried out for 30 min at room temperature in the dark. Quantitative analysis of the apoptotic level was performed using FCM. The apoptotic percentage of 10,000 cells was determined.

Transmission electron microscopy (TEM). MCF-7 cells were seeded at a density of 1×10^6 cells/flask and treated with DMSO or PME-supplemented medium for 48 h. At the end of the incubation period, cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 24 h at 4°C. Then, the cells were washed in the same buffer three times, fixed with 1% osmium tetroxide and dehydrated in graded ethanol. The 100% ethanol solution was then replaced by propylene oxide

and embedded in epoxy resin, which was polymerized at 70°C for 8 h. Sections were stained with uranyl acetate and lead citrate, and then observed with a Hitachi H-7650 TEM.

Western blotting. The expression levels of Cdc25B, Cdc25C, cyclin B1, Cdk1, p-Cdk1, bax, bcl-2, cytochrome c, cleaved caspase-9 and β-actin were detected by Western blotting, with specific antibodies against these proteins.

MCF-7 cells ($5x10^5$) were seeded in 25-cm^2 culture flask for 6 h to be adherent, using the same procedure for cell synchronizing. After PME treatment, cells were washed three times in ice-cold PBS and lysed with 200 μl lysis buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.5 mg/ml leupetin, 1 mM EDTA, 1 mg/ml pepstatin and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). After the cells had been incubated on ice for 30 min and scraped from the flasks, the lysates were centrifuged at 5,000 rpm for 15 min to discard the precipitate. Protein concentrations were determined using the BCA protein assay kit. Total cellular proteins (20 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% nonfat dry milk and 0.05% Tween-20 in Tris-buffered saline (TBS) for 45 min, and subsequently incubated with the primary antibody in TBS for 1 h at room temperature. The antibody-antigen complexes were then detected using alkaline phosphatase-conjugated anti-rabbit or anti-mouse IgG secondary antibodies, followed by the use of a BCIP/NBT Alkaline Phosphatase Color Development kit. The band was recorded by a digital camera and analyzed using ImageJ software (NIH, USA), normalized with the gray value of β-actin.

For cytochrome c determination, $5x10^7$ cells were suspended in five volumes of 20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF and 10 mM leupeptin containing 20 mM sucrose. The cells were disrupted by stroking in a glass homogenizer. The nuclei were centrifuged at 1,000 x g for 10 min at 4°C. The resulting supernatant was centrifuged at 10,000 x g for 30 min to pellet mitochondria. A final centrifugation at 100,000 x g for 1 h at 4°C generated the cytoplasmic fraction.

Statistical analysis. All of the experiments were performed three times and data were expressed as the means ± standard deviation (SD) and analyzed by SPSS using the Student's t-test. In all cases, a value of $P<0.05$ was considered statistically significant.

Results

Effect of PME on MCF-7 cell proliferation. In order to evaluate the effect of PME on the proliferation of MCF-7 cells, the cells were treated with increasing concentrations of PME for different times. PME inhibited the growth of MCF-7 cells in a dose- and time-dependent manner (Fig. 1). IC$_{50}$ values of PME (24 h, 200.3±12.3 μg/ml; 48 h, 146.8±4.4 μg/ml; 72 h, 113.2±6.6 μg/ml) were obtained by extrapolation from the linear regression analysis.

Effect of PME on cell cycle arrest. We designed experiments to determine whether PME-mediated suppression of MCF-7
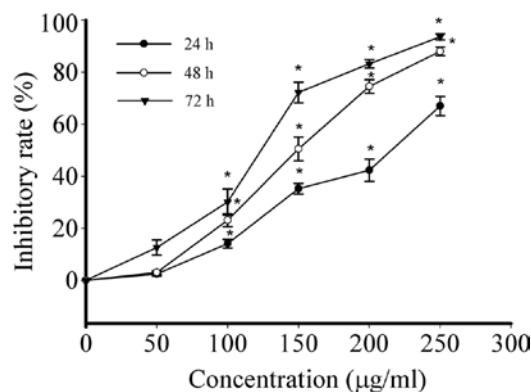

Figure 1. Effect of PME on the proliferation of MCF-7 cells. MCF-7 cells were exposed to increasing dosages of PME (50, 100, 150, 200 and 250 μg/ml) with fresh medium for a total of 24, 48 or 72 h. Values are expressed as the percentage of control group (DMSO). Values are the means ± SD; n=6. Data are representative of three independent experiments. *Different from the vehicle control, P<0.05.
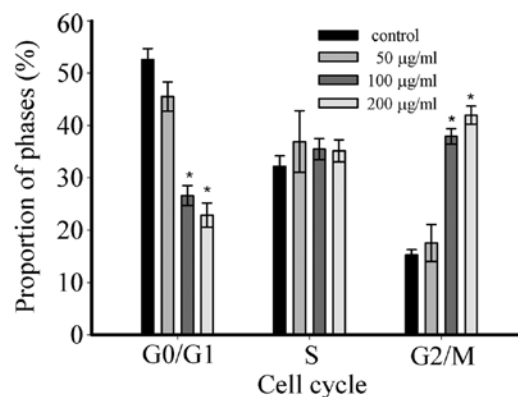

Figure 2. Effect of PME on MCF-7 cell-cycle progression. Synchronized MCF-7 cells were subjected to PME of increasing concentrations (50, 100 and 200 μg/ml) for 24 h. The cells were stained with PI and analyzed for DNA content by FCM to determine the percentages of cells in the G0/G1, S and G2/M phases of the cell cycle. Data are the means ± SD from three independent experiments. Columns, mean (n=3); bars, SD. *P<0.05 compared to the control group treated with DMSO.
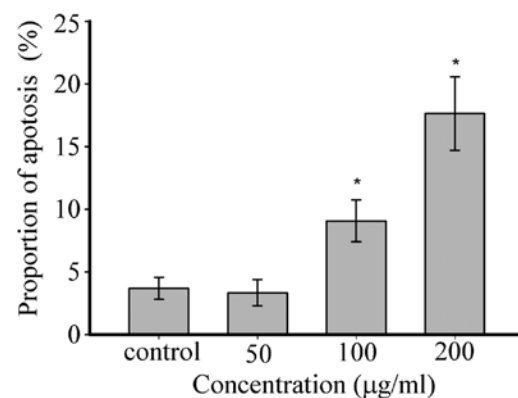

Figure 3. Effect of PME on apoptosis in MCF-7 cells. MCF-7 cells were subjected to PME of increasing concentrations (50, 100 and 200 μg/ml) for 48 h. Analysis of the presence of phosphatidylserine on the outer leaflet of the cell membrane was performed using a double-labeling experiment with Annexin V-FITC and PI to discriminate apoptotic from necrotic cells. Approximately 9 and 18% of apoptotic cells were detected in MCF-7 cell lines treated with 100 and 200 μg/ml PME, respectively. Data are the means ± SD from three independent experiments. Columns, mean (n=3); bars, SD. *P<0.05 compared to the control group treated with DMSO.
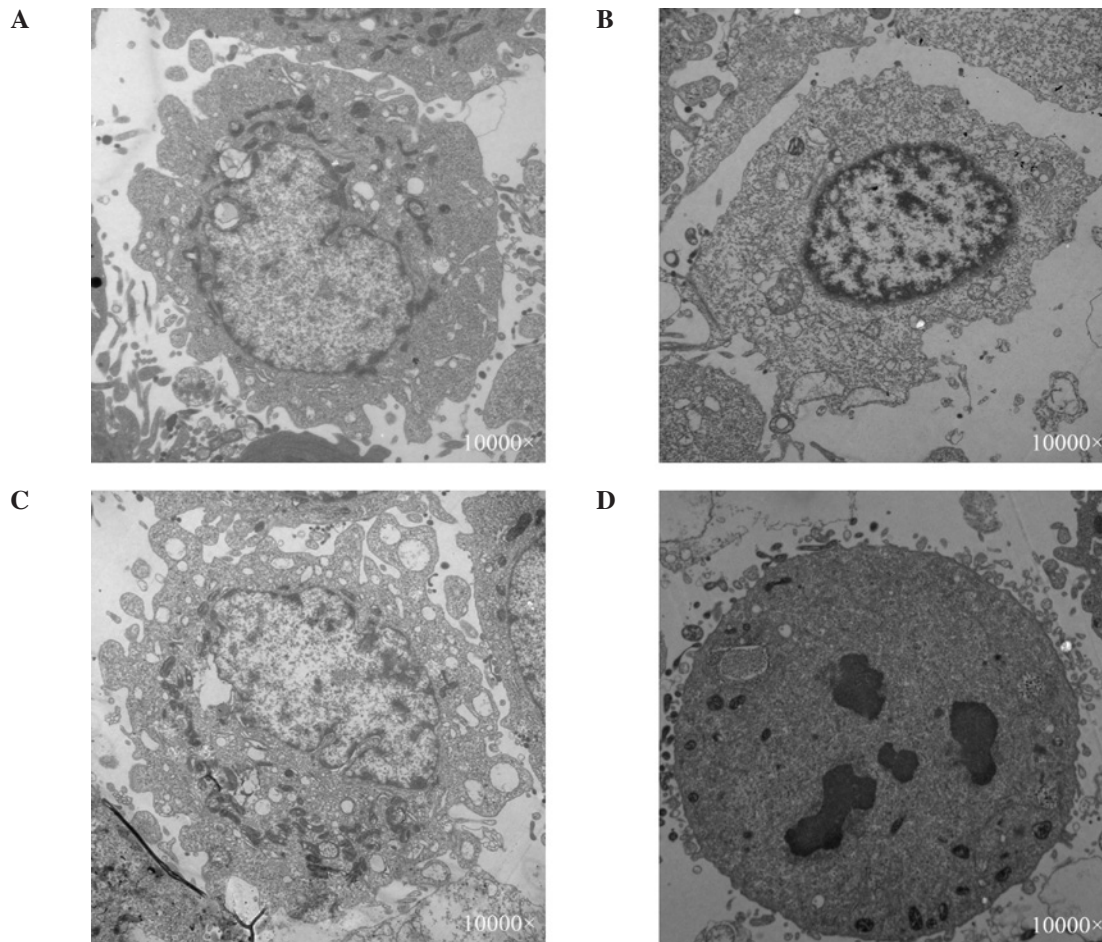
A

B

C

D

Figure 4. Morphology of MCF-7 cells analyzed by TEM. MCF-7 cells were treated with 150 μg/ml PME for 48 h and then examined by TEM at magnification x10,000. (A) In the control cells, the structure of the nucleus, abundance of cytoplasm, as well as the size and shape of the mitochondria, were normal. (B) In the treated cells, the compaction and margination of nuclear chromatin were quite obvious. (C) Cell detachment, cell shrinkage, folded nuclear membrane, membrane blebbing, increased nuclear heterochromatin, swelling of the endoplasmic reticulum cisternae and vesicle formation (abundant vacuoles with multivesicular bodies) were observed. (D) Nuclear pyknosis and fragmentation occurred.

cell proliferation correlated with cell cycle arrest. As shown in Fig. 2, after a 24-h treatment of MCF-7 cells with increasing dosages of PME, FCM assay showed that the proportions of cells in the G2/M phase were 15.3±1.00, 17.6±3.55, 37.9±1.47 and 42.0±1.71% after 0, 50, 100 and 200 μg/ml of PME, respectively. PME resulted in a significant increase in the cells in the G2/M phase and a decrease in the G0/G1 phase in a dose-dependent manner.

Effect of PME on cell apoptosis. In order to determine whether the addition of PME induced cell apoptosis, FCM and TEM were conducted in PME-treated MCF-7 cells and the control. FCM showed that the cell apoptotic rates were 9.1±1.67 and 17.7±2.93 after treatment with 100 and 200 μg/ml PME for 48 h, respectively. MCF-7 cells treated with increasing concentrations of PME exhibited a statistically significant increase in the apoptotic cell fraction, indicating apoptosis induction (Fig. 3).

Morphological and ultrastructural changes in MCF-7 cells exposed to PME for 48 h were examined by TEM (Fig. 4). In the treated cells, characteristics of apoptosis were noted.

Effect of PME on the cell cycle and apoptosis regulatory protein expression. Western blots of the effect of PME treatment on the levels of proteins involved in the regulation of G2-M transition are shown in Fig. 5A. The levels of Cdc25B, Cdc25C and Cdk1 proteins were reduced markedly in the treated cells compared to the control. The decreased level of Cdc25B was not significant compared to that of Cdc25C. PME treatment caused a statistically significant increase in the protein level of p-Cdk1. A change in cyclin B1 was not detected in the MCF-7 cells.

To determine which type of apoptotic pathway was induced by PME, lysates from the MCF-7 cells after PME treatment for 48 h were examined by Western blotting using bax-, bcl-2-, cytochrome c- and cleaved caspase-9-specific antibodies (Fig. 5B). Our results clearly demonstrated an increase in the bax protein content in the MCF-7 cells treated with different concentrations of PME, suggesting that PME induces apoptosis in MCF-7 cells likely via a bax-dependent pathway. A change in the anti-apoptotic protein bcl-2 was never detected under any conditions. We then examined whether the expression of bax promoted the release of mitochondrial cytochrome c into the cytosol leading to activation of the caspase cascade that is essential for the death program. Fig. 5B shows that as the level of bax increased, cytochrome c was detected in the cytoplasm, whereas its level in the mitochondria decreased. Finally, the caspase cascade was also induced by PME, as shown by the cleavage of caspase-9.
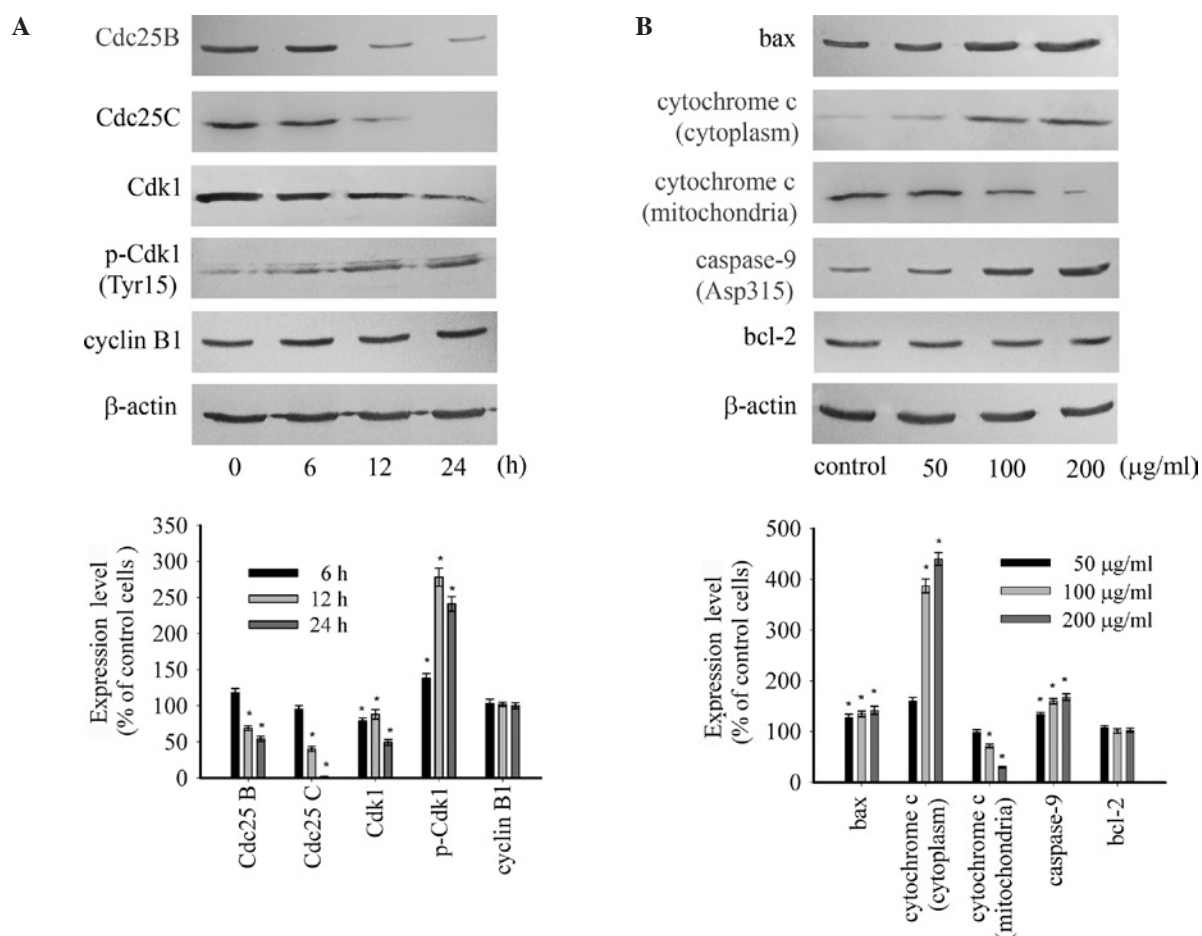
Figure 5. Change in protein expression levels in the MCF-7 cells treated by PME. (A) Western blotting for Cdc25B, Cdc25C, Cdk1, p-Cdk1 and cyclin B1 using lysates from MCF-7 cells treated with 150 µg/ml PME for 0, 6, 12 and 24 h. Proteins (20 µg) were separated by SDS-PAGE and probed with the indicated antibodies. (B) Western blotting for bax, bcl-2, cytochrome c and cleaved caspase-9 using lysates from MCF-7 cells treated with PME at various concentrations (50, 100 and 200 µg/ml) for 48 h. The gray values were determined and analyzed by the ImageJ software package, normalized with the gray value of β-actin. Data are presented as the means ± SD from three repeated tests. $^{*}P<0.05$ compared to the control group treated with DMSO.

Discussion

Phosphorylation and dephosphorylation of proteins mediated by protein kinases and phosphatases are a core component of cellular regulation and signal transduction (14). Currently, multiple phosphatases have been determined to be involved in tumorigenesis and development: protein phosphatase 2A (PP2A), Cdc25 phosphatases (Cdc25A, Cdc25B and Cdc25C), phosphatase of regenerating liver-3 (PRL-3) and phosphatase and tensin homologue deleted on chromosome 10 (PTEN) (15-18). Eukaryotic cell cycle progression involves sequential activation of Cdks, whose activity is dependent on their dephosphorylation status mediated by Cdc25 phosphatases. The G2-M transition and entry into mitosis is mainly regulated by cyclin B1/Cdk1 complex activity, which is negatively regulated by reversible phosphorylations at Thr14 and Tyr15 of Cdk1 during the G2/M phase (19). Cdc25 phosphatases stimulate cell cycle progression by activating cyclin B1/Cdk complexes by catalyzing the removal of phosphate from the Cdk proteins, and the reaction is believed to be a rate-limiting step for entry into mitosis (20,21). Most previous studies indicate that each Cdc25 homolog controls distinct aspects of cell cycle progression, namely non-overlapping roles for each

isoform during a specific cell cycle phase. Briefly, Cdc25A is important for entry into S-phase, whereas Cdc25B is essential for pre-initiating G2-M transition and progression of S-phase. Cdc25C dephosphorylates and activates cyclin B1/Cdk1 (22). However, recent evidence suggests that multiple Cdc25 isoforms cooperate to control each cell cycle transition. All three Cdc25 isoforms play essential roles during the G1-S and G2-M transitions, and in mitosis, where they show specific activity towards Cdk1/cyclin B1 (23,24). Certain quinone-derived compounds, which were identified as Cdc25 inhibitors, may cause cell cycle arrest and inhibit tumor cell proliferation markedly. Therefore, development of Cdc25 inhibitors has been considered as a promising strategy for cancer therapy. Cdc25 inhibitor BN-82685 is active *in vivo* and inhibits the growth of human pancreatic tumor Mia PaCa-2 cells xenografted into athymic nude mice (25). Another Cdc25 inhibitor, ARQ-501, has undergone phase I clinical trials in patients with advanced and chemotherapy-unresponsive solid tumors, and is undergoing a phase II trial in patients with leimyosarcoma and head and neck cancer (26). These Cdc25 inhibitors have demonstrated pre-clinical efficacy, but their efficacy in the clinic has been modest and has been hampered by various side effects (26). Accordingly, preparation and evaluation of

a new Cdc25 inhibitor with potent efficacy and minor side effects would have great interest in the therapeutics of human malignant tumors. Choi *et al* reported that anthraquinones isolated from PM have potent inhibitory activities on Cdc25B and anti-proliferation effects on human colon cancer SW620 cells, while the inhibitory activities on Cdc25C have not been detected (12).

In the present study, we initially discovered that PME inhibited the proliferation of MCF-7 cells by arresting the cell cycle in the G2/M phase, so we assumed that the effectiveness may be mediated by inhibition of Cdc25B and/or Cdc25C phosphatases. The present study showed that the inhibitory activity on Cdc25C by PME was noticeable compared to Cdc25B. We also found that PME caused a sustained decrease in Cdk1, Cdc25B and Cdc25C expression and an increase in p-Cdk1 expression at 24 h. These results suggest that PME treatment may cause inactivation of Cdk1 possibly due to a decrease in the level of Cdk1 protein; an increase in p-Cdk1 resulted from attenuation of dephosphorylation of Cdk1 which is catalyzed by Cdc25 phosphatases. These PME-induced modulations of G2/M regulatory protein expression may contribute to the PME-induced G2/M arrest in MCF-7 cells.

Our result also showed that PME-induced MCF-7 cell apoptosis was accompanied by an overexpression of bax. Bax acting as a promoter of cell apoptosis is a protein present predominantly in the cytoplasm, which binds with the outer membrane of the mitochondrion and is capable of forming a selective ion channel (mitochondrial permeability transition pore: PT pore) in membranes, a critical event in the loss of cell viability that mediates the release of cytochrome c, finally activating the caspase cascade (27-29). Previous studies have shown that the activation of the caspase cascade is involved in apoptosis (30). Caspases which exist as inactive pro-caspases are proteolytically activated via multiple cleavages in cells undergoing apoptosis (31). Then, the activated caspases cleave specific substrates, including poly (ADP-ribose) polymerase, lamin and focal adhesion kinase, resulting in the apoptosis of cells (32). In our study, overexpression of the bax protein was correlated with cytochrome c release, suggesting it triggers caspase-dependent apoptosis. This was evidenced by the presence of active caspase-9, and the cleavage of it was up-regulated by increasing dosages of PME in the treated cells. In contrast, the PM extract was found to protect human umbilical vein endothelial cells from the inflammatory factor TNF-α which causes apoptotic damage, accompanied by down-regulation of caspase-3 gene expression (33). Tetrahydroxystilbene glucoside extracted from PM has protective effects against MPP(+)-induced apoptosis in PC12 cells mediated via the inhibition of reactive oxygen species generation and modulation of JNK activation (34). Taken together with these studies, our results indicated that the responses of tumor cells and non-tumor cells to PME may be different.

In summary, the present study demonstrated that PME inhibited MCF-7 cell proliferation by inducing cell cycle arrest in the G2/M phase and promoting cell apoptosis. These PME-induced responses are associated with changes in expression and activity of cell cycle regulatory proteins and apoptosis proteins. It will now be of substantial interest to determine the nature of PME interaction with cellular targets responsible for triggering mitotic cell cycle arrest to develop antitumor drugs from PM.

A major concern of scientists investigating herbal treatments is that the chemical compositions contained in the herbs contributing to their biological effects are largely unknown. PME is an admixture comprising many compounds, while the constituent contributing to the main effect remains unknown. Further studies are therefore required to assess the efficacies of the components of this herb on antitumor and possible adverse side effects. The precise mechanisms of cell-cycle arrest and apoptosis caused by PME warrant subsequent investigation.

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