Effect of *Polygonatum odoratum* extract on human breast cancer MDA-MB-231 cell proliferation and apoptosis

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Abstract. Traditional Chinese medicine (TCM) is important in the provision of anti-tumor drugs. Recently, studies have shown that certain types of TCM agents are able to control the growth of tumors, enhance the body's immune function and enhance the therapeutic effect of chemotherapeutic drugs. In women, breast carcinoma is the most common tumor type and the second most common cause of death from cancer. *Polygonatum odoratum* (*P. odoratum*) is commonly used in TCM. The aim of the present study was to investigate the effects of *P. odoratum* extract on the proliferation and apoptosis of MDA-MB-231 breast cancer cells. Cell proliferation was assessed using MTT and colony formation assays. In addition, propidium iodide (PI)/Annexin V-FITC staining was used to investigate the apoptosis of MDA-MB-231 cells following treatment with *P. odoratum* extract. The protein expression levels of B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax) were also detected using western blot analysis, while a JC-1 staining assay was used to assess the mitochondrial membrane potential ($\Delta\Psi m$). The results of the MTT assay showed that the proliferation and colony formation of MDA-MB-231 cells were inhibited following treatment with the extract. Furthermore, the PI/Annexin-V staining showed that the apoptosis of MDA-MB-231 cells was enhanced by the extract, in a concentration-dependent manner. The extract also lowered the $\Delta\Psi m$ of MDA-MB-231 cells, upregulated the expression of Bax and inhibited the expression of Bcl-2. In conclusion, these results showed that the *P. odoratum* extract inhibited the proliferation and induced apoptosis of breast cancer MDA-MB-231 cells.

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Introduction

Breast cancer is one of the most common malignant tumors in females, and poses a serious threat to the physical and mental health of women (1). Breast cancer patients may be affected by physical and psychological problems throughout the whole process of disease diagnosis, treatment and rehabilitation (2,3). Psychological problems may increase the side effects of treatment and affect the patient's quality of life (4). Patients with anxiety, depression, happiness and social support situation will affect the patient's emotional state (5). In women, breast cancer is the most common tumor type by incidence and the second cause of cancer-associated mortality (6,7). Adult height has been demonstrated to be positively correlated with breast cancer risk in various epidemiological studies.(8-11). Statistical data revealed that, among all the malignant tumors reported, breast cancer has the highest incidence in major cities in China, as compared with the rest of China (12-15). In comprehensive breast cancer treatment, based on the present risk-benefit ratio optimization scheme, surgery is first line of comprehensive treatment, in addition to chemotherapy, endocrine therapy, radiation therapy, immune therapy, as well as the use of traditional Chinese medicine (TCM) therapy (16-20). Since the majority of tumors develop resistance to certain drugs, the search for novel antitumor agent is essential. Furthermore, adequately sensitive apoptosis cannot be induced by chemotherapy.

TCM occupies an important position in the development of antitumor drugs. Recent studies have demonstrated that TCM agents are able to control the growth of tumors, enhance the body's immune function and improve the therapeutic effect of chemotherapy drugs (21-23). *Polygonatum odoratum* (*P. odoratum*) is a member of the Liliaceae family and is commonly used in TCM (24-26). Previous studies have demonstrated that it increases the level of antibody production, and exerts anti-inflammatory, antiviral and tumor inhibitory effects (27,28). *P. odoratum* has been used to treat various diseases due to its procoagulant activity (29), anti-hyperglycemic effect (30,31), glucose tolerance improvement (32) and anti-herpes simplex virus-II and apoptosis-inducing activities (33). *P. odoratum* has been suggested as a potential therapeutic target for the treatment of breast cancer (34,35).

The aim of the present study was to investigate the effects of *P. odoratum* extract on breast cancer cells. The results of the study may provide novel insights that could assist in the research and development of novel antitumor drugs for the treatment of breast cancer.

Materials and methods

Cell lines. The MDA-MB-231 breast cancer line (American Type Culture Collection, Manassas, VA, USA) was cultured in the laboratory of the Department of Pharmacy, Bengbu Medical College (Bengbu, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, and were maintained at 37˚C with 5% CO_2 in a humidified atmosphere. All the experiments were performed on logarithmically growing cells.

Reagents and antibodies. DMEM, FBS and phosphate-buffered saline (PBS) were purchased from Thermo Fisher Scientific, Inc. (Grand Island, NY, USA). MTT [also known as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was obtained from Sigma-Aldrich (Castle Hill, Australia). Penicillin and streptomycin were purchased from North China Pharmaceutical Group Corp., (Shijiazhuang, China). Propidium iodide (PI)/Annexin V-FITC was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was obtained from Amresco (Solon, OH, USA). Mouse anti-human β-actin (1:10,000; 66009-1-Ig) polyclonal antibody and rabbit anti-B-cell lymphoma-2 (Bcl-2; 1:1,000; 12789-1-AP) and rabbit anti-Bcl-2-associated X protein (Bax; 1:1,000; 23931-1-AP) polyclonal rabbit anti-human antibodies were purchased from Proteintech Group, Inc. (Rosemont, IL, USA). The mitochondrial membrane potential ($\Delta\Psi$m) assay kit (C2006), JC-1, bicinchoninic acid protein concentration kit (P0011) and crystal violet stain were purchased from the Beyotime Institute of Biotechnology (Shanghai, China).

Cell proliferation assay. The effect of the *P. odoratum* extract on cell viability was determined using an MTT assay. *P. odoratum* extract was socked in MeOH for 3 days isolate the filtrate, MeOH extract was obtained using rotary evaporators. MeOH extract was dissolved in a isopyknic mixture of EtOAc and H_2O to obtained the EtOAc layer and the EtOAc extract was isolated using rotary evaporators. Subsequently, EtOAc extract was dissolved in a isopyknic mixture of Hexane and H_2O to obtain the Hexane layer, and *P. odoratum* extract was subsequently obtained using rotary evaporators. Briefly, the MDA-MB-231 cells were seeded at a density of $1x10^4$ cells/well in 96-well plates with 100 ml growth medium. The cells were randomly divided into three groups as follows: Control (no treatment), *P. odoratum* extract (treatment with extract alone) and blank control (no cells, no treatment, just growth medium) groups. Cells in the *P. odoratum* extract group were then incubated for 24 h in the presence of 0, 0.001, 0.01 or 0.1 mg/ml *P. odoratum* extract. Next, 15 μl MTT (5 mg/ml) was added to the cells, which were incubated in the dark at 37˚C for 4 h. Subsequent to removal of the MTT solution, the formazan product (crystals) was dissolved in 150 μl DMSO by shaking the plates for 10 min. Optical density (OD) was then determined at 490 nm using a Synergy HT multi-detection microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The experiments were performed in triplicate, and three parallel samples were measured each time. For calculation of the cell proliferation rate (%), the following formula was used: OD (experimental group) / OD (control group) x 100.

Colony formation assay. MDA-MB-231 cells in the logarithmic growth period, were seeded at a density of $1x10^4$ cells/well in 6-well plates with 2 ml growth medium and incubated for 24 h. Next, the cells were treated with different concentrations of the *P. odoratum* extract (0, 0.02, 0.04 and 0.06 mg/ml) for 5 days and maintained at 37˚C in 5% CO_2 in a humidified atmosphere. The cells were then washed twice with pre-chilled PBS, fixed with paraformaldehyde for 10 min and stained with 2% crystal violet for 10 min. Double-distilled water was used to clean the cells until the color of the sample turned transparent, and the cells were then dried at room temperature. Colonies contained 50-150 cells. Colony numbers were counted under a light microscope at a magnification of x40.

Cell apoptosis assay. A single cell suspension was prepared from MDA-MB-231 cells in the logarithmic growth period and counted using flow cytometry (Accuri C6; BD Biosciences, Franklin Lakes, NJ, USA). Annexin-V FITC/PI staining was performed according to the manufacturer's instructions to assess cellular apoptosis. Cells were seeded in 6-well culture plates ($1x10^4$ cells/well) for 24 h prior to the cell apoptosis assay. Next, the cells were incubated with the *P. odoratum* extract at increasing concentrations (0, 0.02, 0.04 and 0.06 mg/ml) for 24 h and then washed twice with pre-chilled PBS. The cells were resuspended in 500 μl binding buffer, following centrifugation at 13,223 x g for 10 min. Subsequently, 100 μl cell suspension was transferred to 5-ml Eppendorf tubes, and 5 μl PI/Annexin V-FITC solution was added to each tube and mixed for 15 min (4˚C; dark). Following incubation for 15 min at 20˚C, the apoptosis rate was determined using an BD Accuri C6 flow cytometer, and the results were analyzed using the flow cytometer software.

Western blot analysis. Stable expression cells were washed three times with cold PBS (1 ml/well) and digested with trypsin in lysis buffer for 30 min on ice. The protein concentrations of the cell lysates were detected using a bicinchoninic acid protein concentration kit. The protein sample was mixed with the sample buffer (2:1) and boiled at 100˚C with the denaturation buffer for 5 min, and steps were taken to prevent protein degradation. Protein samples from each test group (30 μg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A condensation protein electrophoresis gel was run at 70 V for 30 min and 150 V for 90 min, and the proteins were transferred onto polyvinylidene fluoride membranes at 50 V in an ice bath for 90 min. The membrane was then blocked in 5% skim milk, followed by an overnight incubation at 4˚C with the primary antibodies against β-actin. Bcl-2 and Bax (1:1000).

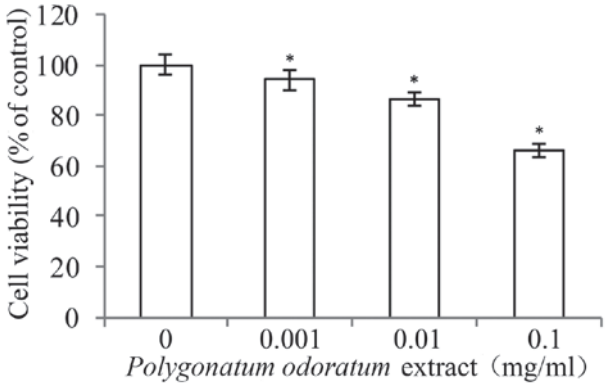

Figure 1. Viability and cell proliferation of MDA-MB-231 breast cancer cells treated with the *Polygonatum odoratum* extract. The cell viability was inhibited in a concentration-dependent manner. *P<0.05 vs. 0 mg/ml.

Subsequently, the membrane was washed three times with Tris PBS (TPBS) and once with PBS, for 10 min each time. The sample was then labeled with the peroxidase-conjugated rabbit (109525) or mouse (117228) secondary antibodies (1:5,000; ZSGB Bio, Beijing, China) for 2 h at room temperature on a shaker. Next, the membrane was washed with TPBS, and the bands were visualized using western lightning with enhanced chemiluminescence (EMD Millipore, Billerica, MA, USA). β-actin was used as the protein control, and the gray values of the protein bands in each group were analyzed using Quantity One 4.6.8.27 gel image analysis software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The experiments were repeated at least twice to confirm the results.

Measurement of ΔΨm. The mitochondrial stability was assessed using a ΔΨm assay kit with JC-1. MDA-MB-231 cells in the logarithmic growth period were seeded at a density of $2x10^5$ cells/well in 12-well plates with 1 ml growth medium and incubated for 24 h. Next, the cells were treated with 0.1 mg/ml of the drug. After treatment for 24 h, the cells were washed once with PBS and then incubated with 1 ml JC-1 fluorescent dye for 20 min in the dark at 37˚C. Subsequently, the cells were washed twice with pre-chilled buffer solution. The ΔΨm was imaged using a fluorescence microscope (Olympus Corp., Tokyo, Japan) at 550 nm excitation and 570 nm emission.

Statistical analysis. All experiments were performed at least in triplicate. Data are presented as the mean ± standard error of the mean. A double-sided Dunnett's test was used for between-group comparisons. All statistical analyses were performed using the SPSS version 13.0 software (SPSS, Inc., Chicago, IL, USA) and statistically significant differences were indicated by P<0.05.

Results

Inhibitory effects of P. odoratum extract on the viability and proliferation of MDA-MB-231 cells. An MTT assay was performed to determine the effect of *P. odoratum* extract on the proliferation of MDA-MB-231 cells. As shown in Fig. 1, the viability of MDA-MB-231 cells was altered following 24-h treatment with various concentrations of the *P. odoratum* extract up to 0.1 mg/ml. The extract inhibited MDA-MB-231 cell proliferation in a dose-dependent manner (P<0.05).

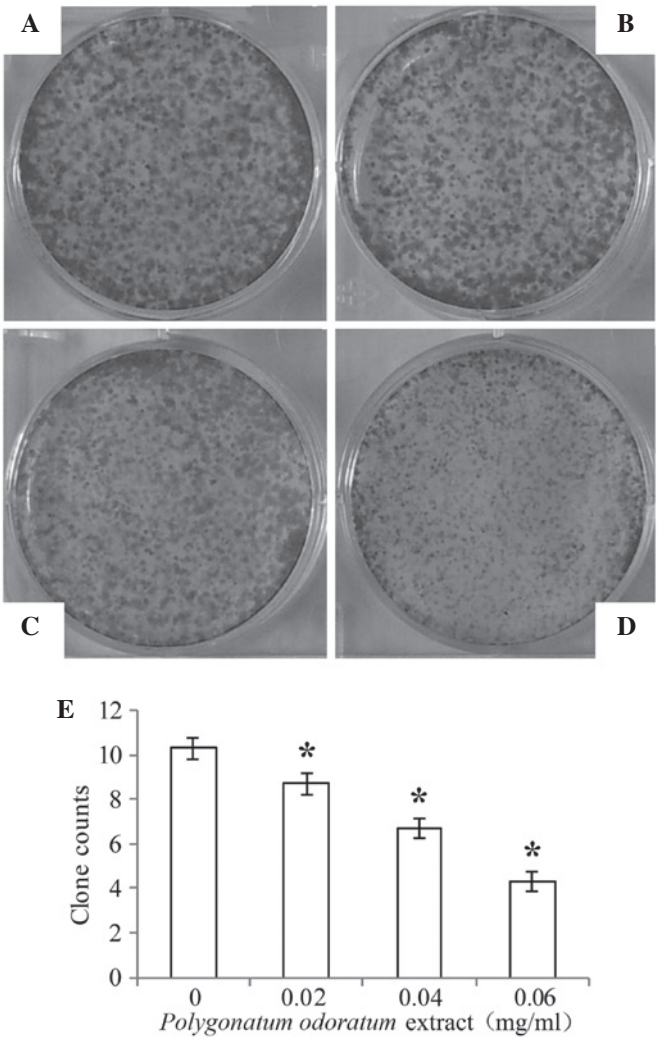

Figure 2. Representative images of MDA-MB-231 breast cancer cell colonies treated with (A) 0 mg/ml (control), (B) 0.02 mg/ml, (C) 0.04 mg/ml and (D) 0.06 mg/ml *Polygonatum odoratum* extract for 5 days. (E) Quantitifaction of the clone counts demonstrated that treatment with the extract had a significantly inhibitory effect on colony formation in the MDA-MB-231 cells. *P<0.05 vs. 0 mg/ml.

Furthermore, the colony forming ability of the MDA-MB-231 cells was analyzed using a colony formation assay. As shown in Fig. 2 (P<0.05), a significant inhibitory effect was observed in cells treated with increasing concentrations of the *P. odoratum* extract (0, 0.02, 0.04 and 0.06 mg/ml) for 5 days, while low concentration significantly inhibited the cell colony formation. These results suggest that the *P. odoratum* extract has inhibitory effects on breast cancer cell proliferation.

Effect of P. odoratum extract on the apoptosis of MDA-MB-231 cells. To determine whether the reduced cell viability was due to apoptosis, PI/Annexin V FITC double staining using the Accuri C6 flow cytometry was conducted. As indicated in Fig. 3 (P<0.05), MDA-MB-231 cells treated

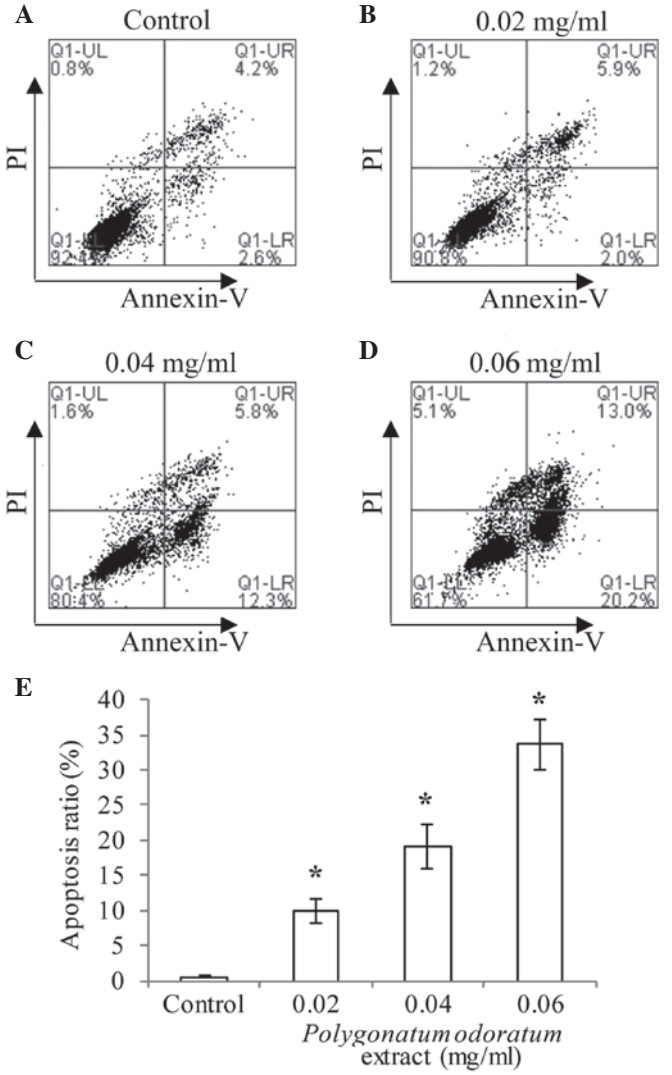

Figure 3. Effect of *Polygonatum odoratum* extract on apoptosis of MDA-MB-231 breast cancer cells. The cells were (A) untreated (control), or treated *Polygonatum odoratum* extract at (B) 0.02, (C) 0.04 and (D) 0.06 mg/ml for 24 h and compared with untreated cells. (E) Quantitative analysis demonstrated that the extract significantly induced apoptosis of human breast cancer cells in a concentration manner. *P<0.05 vs. control.

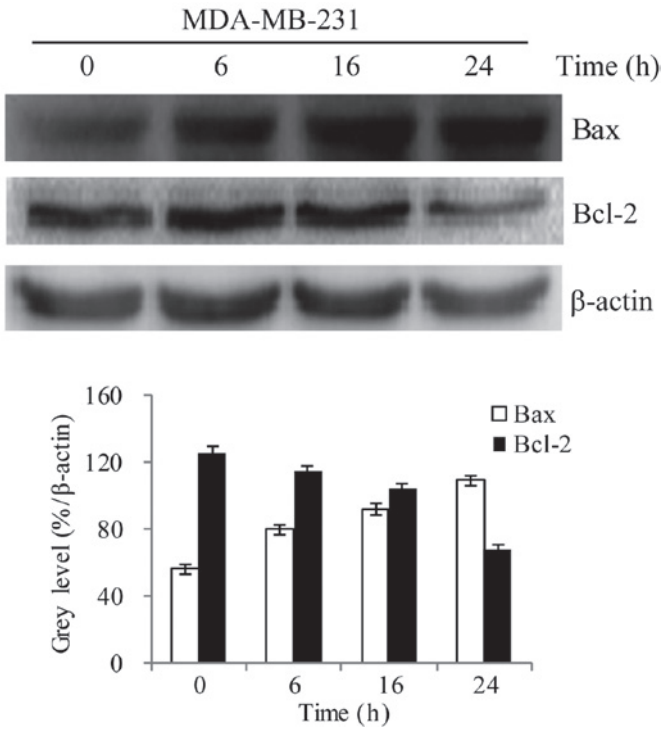

Figure 4. Effects of 0.1 mg/ml *Polygonatum odoratum* extract on the expression levels of Bcl-2 and Bax in MDA-MB-231 breast cancer cells after incubation for 0, 6, 16 and 24 h. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

with increasing concentrations (0.02, 0.04 and 0.06 mg/ml) of the extract showed an increase in the ratio of apoptotic cells (7.9, 18.1, and 33.2%, respectively) for 24 h. These data showed that the *P. odoratum* extract induced dose-dependent apoptosis in the cells.

Effect of P. odoratum extract on the protein expression levels of Bcl-2 and Bax. MDA-MB-231 cells were treated with the 0.1 mg/ml *P. odoratum* extract for 0, 6, 16, and 24 h, and western blot analysis was used to investigate the effect on the protein expression levels of Bcl-2 and Bax. The results showed that, compared with the control group, there was a time-dependent decrease and increase in the protein expression levels of Bcl-2 and Bax, respectively (Fig. 4; P<0.05). These findings indicate that the extract treatment resulted in downregulation of Bcl-2 and upregulation of Bax. β-actin expression was used as an internal control, and its level was not changed.

Determination of ΔΨm using JC-1. The ΔΨm following JC-1 staining was also examined using fluorescence analysis. The results indicated that there was a significant change in the ΔΨm loss in MDA-MB-231 cells following treatment with the *P. odoratum* extract (0.1 mg/ml, 24 h). As shown in Fig. 5, fluorescence conversion from red to green was observed in response to the extract treatment. This illustrates the early apoptosis that occurred in the cells subsequent to treatment. The intensity of the JC-1 staining was measured using fluorescence microscopy. JC-1 passed across the membrane into the living cells and aggregated in the mitochondrial membrane, and the concentration of JC-1 increased as mitochondrial membrane potential increased. When the ΔΨm is high, JC-1 accumulates in the mitochondrial matrix forming J aggregates and red fluorescence is produced. By contrast, when the ΔΨm is low, JC-1 cannot accumulate in the mitochondrial matrix and the monomer with the green fluorescence is produced. Therefore, the ΔΨm can be easily determined based on the fluorescent color change, and a decrease in ΔΨm is a sign of early apoptosis. Change in fluorescence from red to green induced by JC-1 can easily be detected with the decline of the membrane potential. Therefore, JC-1 staining can be used as an index for the detection of early apoptosis.

Discussion

Recent research has been focusing on the search for novel antitumor drugs (36). The present study demonstrated that the *P. odoratum* extract inhibited the proliferation of the breast cancer MDA-MB-231 cell line, enhanced the expression of

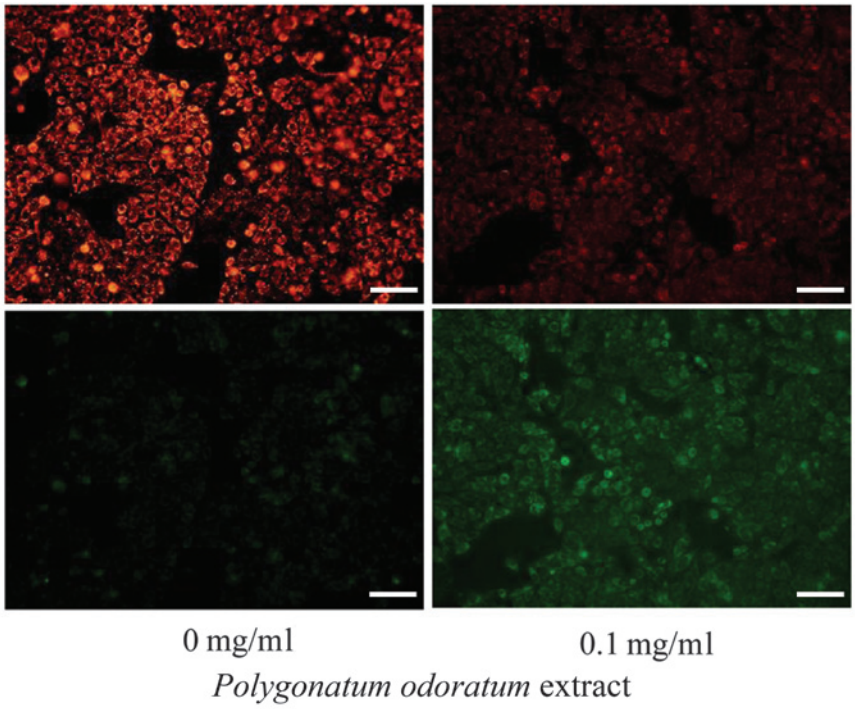

Figure 5. Treatment with 0.1 mg/ml *Polygonatum odoratum* extract decreased the mitochondrial membrane potential in the MDA-MB-231 breast cancer cell line. Induction of apoptosis was observed in MDA-MB-231 breast cancer cells after 24 h of treatment (as observed by change in the fluorescence from red to green). Scale bar, 100 μm.

Bax, inhibited Bcl-2 and reduced the ΔΨm, thereby resulting in MDA-MB-231 cell apoptosis.

Under physiological and pathological stimuli, normal cells undergo a spontaneous death process known as apoptosis. This process is active, highly ordered, signal-dependent, and controlled by genes and a series of enzymes. The initiation of the apoptotic process directly determines the 'fate' of the cell (37,38). A high expression of the Bcl-2 gene maintains cell survival and has no effect on cell proliferation, as first demonstrated by Vaux *et al* in 1988 (39). The main physiological function of the Bcl-2 protein is inhibition of apoptosis, thereby prolonging the life of cells without any effect on cell differentiation (40). Furthermore, the expression of other genes of the Bcl-2 family serves an important role in the process of apoptosis, which includes mitochondrial regulation (41,42). Numerous apoptotic cells exhibit enhanced expression of Bax, which is the most widely studied pro-apoptotic protein in the Bcl-2 family (43). Alsabeh *et al* (44) examined the expression of Bcl-2 in 371 cases of breast cancer, and identified a positive expression as high as 79.3%, while a generally strong intensity of staining was observed, indicating that the Bcl-2 test for the clinical diagnosis of breast cancer has a reference value. In the present study, expression levels of Bcl-2 increased and Bax decreased, as compared with the original tissues. Bax/Bcl-2 downregulation exhibited significance. The present findings that the expression levels of Bax and Bcl-2 increased and decreased, respectively, may indicate that a mechanism that promotes various factors that induce tumor cell apoptosis.

JC-1 is an ideal fluorescent probe and is widely used in the detection of ΔΨm in purified cells or tissues (45). When the ΔΨm is high, JC-1 accumulates in the mitochondrial matrix forming J-aggregates and red fluorescence is produced (46). By contrast, when the ΔΨm is low, JC-1 cannot accumulate in the mitochondrial matrix and the monomer with the green fluorescence is produced (47-49). Therefore, the ΔΨm can be easily determined based on the fluorescent color change, and a decrease in ΔΨm is a sign of early apoptosis (50). Change in fluorescence from red to green induced by JC-1 can easily be detected with the decline of the membrane potential. Thus, JC-1 staining can be used as an index for the detection of early apoptosis. In the present study, red fluorescence intensity was attenuated, whereas the intensity of green fluorescence enhanced. These findings indicated that the mitochondrial membrane potential declined and apoptosis was activated.

In conclusion, the present study revealed that the *P. odoratum* extract inhibited the proliferation and induces the apoptosis of human breast cancer MDA-MB-231 cells. This treatment was also found to affect the expression of apoptosis-associated proteins. These results suggest that the *P. odoratum* extract may have certain value in the treatment of breast cancer. However, the molecular mechanism involved in the extracts' effects requires further elucidation. Furthermore, toxicological and *in vivo* experiments are also necessary to confirm the findings of the present study.

Acknowledgements

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