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Original Article

Effect of pterostilbene on glioma cells and related mechanisms

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Abstract: Neuroglioma is the most common primary malignant tumor in neurosurgery. Due to unfavorable life quality of patients, the treatment of glioma is a major challenge in clinics. The search for effect treatment drugs thus benefits patient prognosis. As one derivative of resveratrol, pterostilbene has a wide spectrum of pharmaceutical functions, especially with the anti-tumor effects. This study thus investigated the effect of pterostilbene on neuroglioma and related mechanisms. U87 glioma cell line was divided into control, normal culture and different dosages of pterostilbene groups, which received 5 mM or 10 mM pterostilbene for 48 h. MTT assay was used to detect U87 cell proliferation, while invasion assay was employed to test the effect of pterostilbene on cell invasion, followed by flow cytometry assay for analyzing U87 cell apoptosis. Real-time PCR was used to test mRNA expression of Bcl-2 and Bax in glioma cells under the effect of pterostilbene, while Western blotting was used to detect alternation of Bcl-2 and Bax protein levels. Pterostilbene significantly inhibited proliferation and invasion abilities of glioma cells compared to those in control group ($P<0.05$). It can also enhance cell apoptosis, decrease mRNA and protein of Bcl-2 expression, and increase mRNA and protein expressions of Bax ($P<0.05$ compared to control group) in a dose-dependent manner. Pterostilbene can facilitate apoptosis of glioma cells, and inhibit their proliferation and invasion via mediating apoptotic/anti-apoptotic homeostasis.

Keywords: Glioma, pterostilbene, Bcl-2, Bax, cell proliferation, cell invasion

Introduction

Neuroglioma is the most common malignant tumor in neurosurgery, and has a high incidence among all intracranial tumors, with elevated occurrence rate and younger age of onset [1]. Among all primary brain tumors, neuroglioma has high invasiveness and unfavorable prognosis, leading to short lifespan and worse life quality [2, 3]. Neuroglioma has now become the most popular tumor in central nervous system in China [4]. Glioma has a rapid disease progression, and can cause different clinical symptoms due to the size and location of intracranial tumors [5]. Due to the space occupying effect, intracranial pressure (ICP) may be elevated, accompanied with neural symptoms [6]. Although treatment for brain glioma has been developed with major progress, radical surgery is still a major challenge due to complicated mechanism [7, 8]. Residual tumor may again proliferation, leading to its invasive growth, severely compromising treatment efficacy and patient life quality, thus making the treatment of glioma as one major challenge worldwide [9]. Therefore, the development of effective treatment medicine could help to improve patient's prognosis.

Previous studies have found the pluripotent role of resveratrol, including anti-oxidation, anti-bacterial, anti-tumor, modulating vascular dilation, inhibiting platelet coagulation, mediating lipoprotein metabolism and enhancing body immune defense [10, 11]. As one derivative of resveratrol, pterostilbene is one non-flavonoid polyphenol compound that is enriched in grapes, nuts, strawberries, Guangxi Xuexi and propolis.

As one poly-hydroxyl-diphenyl ethylene compound of resveratrol [12, 13], pterostilbene has similar pharmaceutical role as those of resveratrol. In a wide spectrum of functions, pterostilbene could exert certain roles against fungal infection, bacteria, mediating cell proliferation and growth, modulating lipid metabolism and participating in oxidative/reductive reaction or anti-inflammation. Current study has confirmed its effective roles in treating hypoxia-ischemia brain disease, Alzheimer's disease and tumors. With superior biological activity and selectivity over resveratrol, pterostilbene mainly exerts anti-inflammation, anti-oxidation and anti-tumors [14, 15]. This study thus investigated the role of pterostilbene on glioma cells and related mechanisms.

Materials and methods

Reagent and equipment

Human glioma U87 cell line was purchased from ATCC cell bank (US). Pterostilbene was purchased from Fujistu (Japan). DMEM medium, fetal bovine serum (FBS), and streptomycin-penicillin were purchased from Hyclone (US). DMSO and MTT powders were purchased from Gibco (US). Trypsin-EDTA lysis buffer was purchased from Sigma (US). Caspase 3 activity assay kit and PVDF membrane were purchased from Pall Life Sciences (US). EDTA was purchased from Hyclone (US). Western blotting reagent was purchased from Beyotime (China). ECL reagent was purchased from Amersham Biosciences (US). Rabbit anti-human Bcl-2 monoclonal antibody, rabbit anti-human Bax monoclonal antibody, and mouse anti-rabbit horseradish peroxidase (HRP)-conjugated IgG secondary antibody were all purchased from Cell Signaling (US). Transwell chamber was purchased from Corning (US). RNA extraction kit and reverse transcription kit were purchased from Axygen (US). Annexin V-FITC apoptotic assay kit was purchased from BD (US). FACA Calibur flow cytometry apparatus was purchased from BD (US). Labsystem Version 1.3.1 microplate reader was purchased from Bio-rad (US).

Glioma U87 cell culture and grouping

U87 cells kept in liquid nitrogen were resuscitated in 37°C water-bath until fully thawing. Cells were centrifuged at 1000 rpm for 3 min, and were re-suspended in 1 ml fresh medium and were removed to 5 ml culture flask which contained 3 ml fresh culture medium. Cells were kept in a humidified chamber with 5% CO_2 at 37°C for 24~48 h. U87 cells were seeded in 6-well plate at 1×10^5 per cm^2. Cells at log-phase with 2nd to 8th generation were randomly divided into control, normal culture and high/low dosage of pterostilbene (5 mM or 10 mM) groups in 48 h continuous culture.

MTT assay for cell proliferation

U87 cells at log-phase were seeded into 96-well plate which contained DMEM medium with 10% FBS at 5×10^3 density. After 24 h incubation, the supernatant was removed. Cells were randomly divided into control or high/low pterostilbene groups, which were treated as abovementioned. In brief, 20 μl sterile MTT was added into each test well in triplicates after 48 h cell culture. After 4 h continuous cultivation, the supernatant was removed, with the addition of 150 μl DMSO for 10 min vortex until the complete resolving of crystal violet. Absorbance (A) values was measured at 570 nm in a microplate reader. The proliferation rate was calculated in each group. Each experiment was repeated for more than three times.

Transwell assay for cell invasion

Following the manual instruction, serum-free culture medium was used for 24 h cell culture. Transwell chamber was pre-coated using 1:5 50 mg/L Matrigel dilutions on the bottom and upper layer of the membrane, followed by 4°C air-dry. 500 μl DMEM culture medium containing 10% FBS was then added into inner and outer surface of the chamber, which contained 100 μl tumor cell suspensions prepared by serum-free culture medium in triplicates. The chamber was placed in a 24-well plate. Control cells were cultured in Transwell chamber without Matrigel. After 48 h, PBS was used to rinse Transwell chamber, with the removal of membrane-fixed cells, which were then fixed in cold ethanol and stained by crystal violet. The number of cells at the lower surface of the micropore membrane was then counted in triplicates (N=3).

Flow cytometry measuring U87 cell apoptosis

Tumor cells were digested, counted and inoculated into 50 ml culture flask at 5×10^5/mL concentration, and were randomly divided into

Table 1. Primer sequence

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	ACCAGGTATCTGCTGGTTG	TAACCATGATGTCAGCGTGGT
Bcl-2	ATCATGGTATGATGGACCCCGCAACTTC	ATTCGAAGTCGCACAGCAGCTGGTC
Bax	CTTAGTGGTCTCTGTATGACGT	TCACCCTCTCACAGCTTGAGTCGA

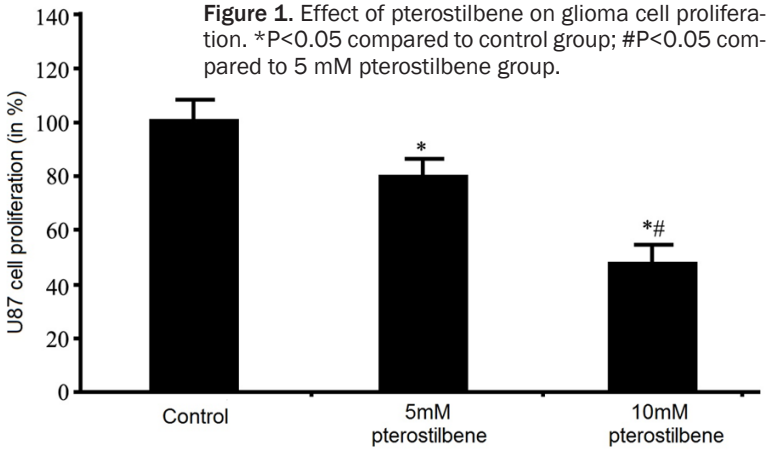

Figure 1. Effect of pterostilbene on glioma cell proliferation. *P<0.05 compared to control group; #P<0.05 compared to 5 mM pterostilbene group.

three groups as abovementioned (N=3 each). After 48 h transfection, cells were counted, and collected at 2×10^6 per ml. After centrifugation in 1× PBS at 1000 rpm for 5 min, cells were rinsed and fixed in 75% cold ethanol for 4°C overnight incubation. After discarding 75% ethanol, cells were-centrifuged in 1× PBS at 1000 rpm for 1 min, followed by rinsing and re-suspension in 800 μl 1× PBS containing 1% BSA, with the addition of 100 μg/ml PI dye (in 3.8% Na-citrate, pH 7) and 100 RNAase A (10 mg/ml) for 37°C dark incubation (30 min). Flow cytometry apparatus was used to test cell apoptosis, with the data analysis by FCSExpress3.0 software.

Real-time PCR for measuring Bcl-2 and Bax mRNA expression

Trizol reagent was used to extract RNA from all groups for cells. Reverse transcription was performed according to the manual instruction, using primers designed by Primer6.0 and synthesized by Invitrogen, Shanghai (China) as shown in **Table 1**. Real-time PCR was performed on target genes under the following conditions: 55°C for 1 min, followed by 35 cycles each containing 92°C for 30 s, 58°C for 45 s, and 72°C for 35 s. Data were collected and calculated for CT values of all samples and standards based on fluorescent quantification using GAPDH as the baseline. Standard curve was firstly plotted using CT values of standards, followed by semi-quantitative analysis by 2-ΔCt method.

Western blotting

U87 cell proteins were firstly extracted. In brief, RIPA lysis buffer containing proteinase inhibitor was used to lyse cells on ice for 15~30 min, followed by ultrasound rupture (5 s×4) and centrifugation (4°C, 10000 g, 15 min). Supernatants were saved and quantified for protein contents, and were stored at -20°C for further Western blotting. Proteins were then separated using 10% SDS-PAGE gel, and were transferred to PVDF membrane using semi-dry method. Non-specific background was removed by 5% defatted milk powder at room temperature for 2 h, followed by the addition of anti-Bcl-2 monoclonal antibody (1:1000 dilution) in 4°C overnight incubation. On the next day, the membrane was rinsed in PBST, and incubated with 1:2000 goat anti-rabbit secondary antibody for 30 min incubation. After PBST rinsing, ECL reagent was used to develop the membrane, which was exposed under X-ray for observing results. Protein imaging analysis system and Quantity One software were used to scan X-ray films for observing band density. Each experiment was repeated for four times (N=4) for further analysis.

Statistical analysis

All data were presented as mean ± standard deviation (SD). Student t-test was used to compare means between two groups. SPSS11.5 software was used in statistical analysis. Analysis of variance (ANOVA) was used for between-group analysis. A statistical significance was identified when P<0.05.

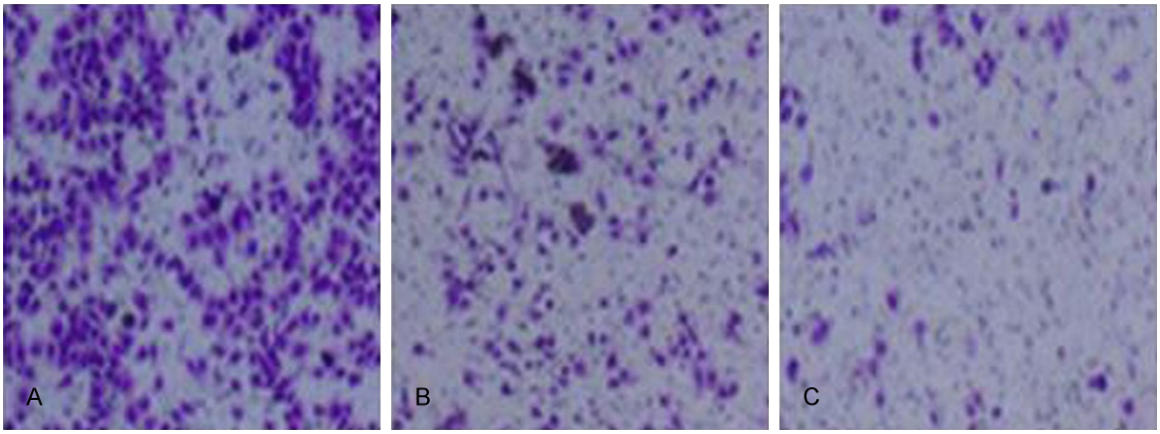

Figure 2. Effect of pterostilbene on glioma cell invasion. A. Control group; B. 5 mM pterostilbene group; C. 10 mM pterostilbene group.

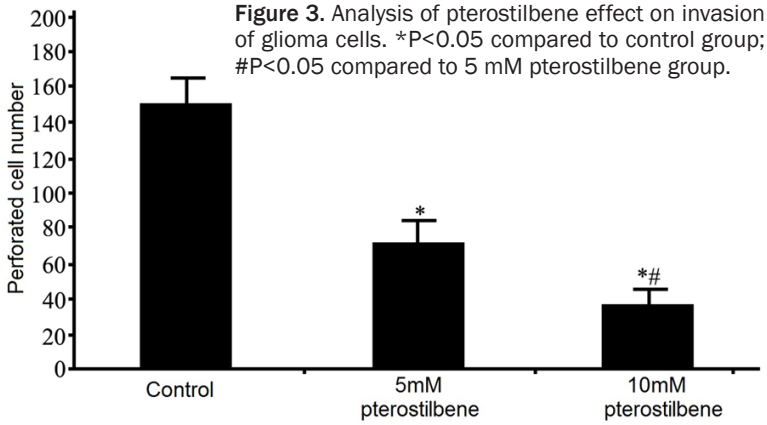

Figure 3. Analysis of pterostilbene effect on invasion of glioma cells. *P<0.05 compared to control group; #P<0.05 compared to 5 mM pterostilbene group.

Results

Effect of pterostilbene on glioma cell proliferation

We employed MTT assay to check the mediation of pterostilbene on glioma U87 cell proliferation. Results showed the significant inhibition of pterostilbene on cell proliferation (P< 0.05) in a positive dose-dependent manner, as higher pterostilbene enhanced the inhibitory effect on tumor cell proliferation (**Figure 1**). These results suggested that pterostilbene could inhibit proliferation and survival of glioma cells.

Effects of pterostilbene on glioma cell invasion

We performed cell invasion assay using Transwell chamber under the influence of pterostilbene. Results showed that, after 48 h of pterostilbene treatment on glioma U87 cells, the invasion potency of tumor cells was remarkably suppressed (P<0.05 compared to control group). With elevated pterostilbene dosage, such inhibition was further potentiated (**Figures 2** and **3**), suggesting that pterostilbene could inhibit tumor growth via suppressing glioma cell invasion.

Effect of pterostilbene on glioma cell apoptosis

Flow cytometry was used to analyze the effect of pterostilbene on apoptosis of U87 cells. Results showed that after 48 h of pterostilbene treatment, the apoptosis of glioma cells was significantly inhibited (P<0.05 compared to control group). With elevated pterostilbene concentration, such potentiation effect on tumor cell apoptosis was further enhanced (**Figures 4** and **5**). These results thus indicated the induction of cell apoptosis by pterostilbene.

Pterostilbene and Bcl-2 mRNA expression in glioma cells

Real-time PCR was further replenished to test the effect of pterostilbene on mRNA expression of Bcl-2 in U87 glioma cells. Results showed that after 48 h of pterostilbene treatment, mRNA expression of Bcl-2 gene was remarkably depressed (P<0.05 compared to control group). With further higher concentration, the inhibitory effect on Bcl-2 mRNA level in tumor cells was potentiated (**Figure 6**).

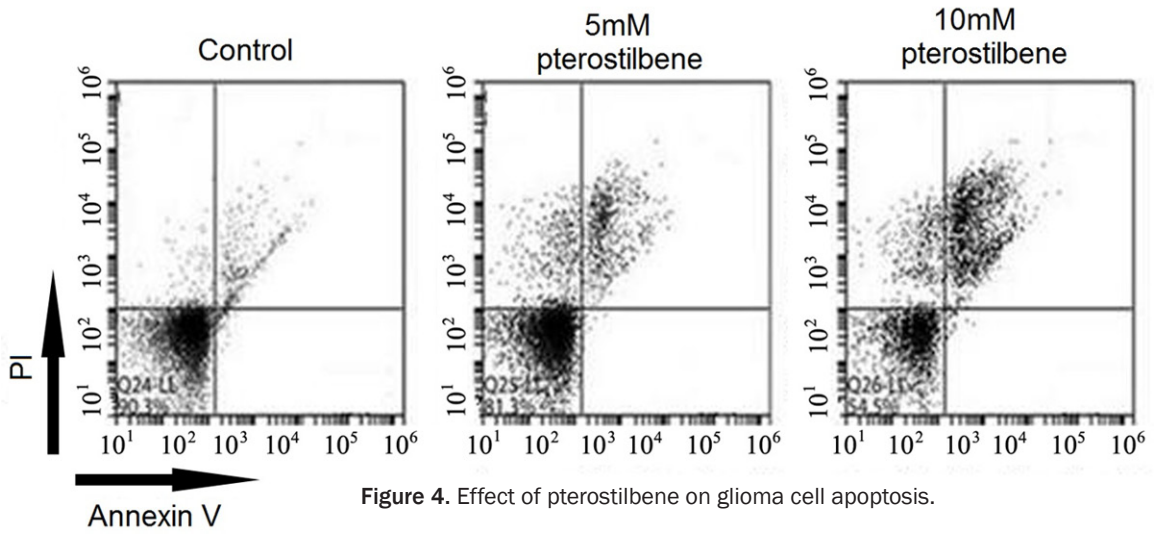

Figure 4. Effect of pterostilbene on glioma cell apoptosis.

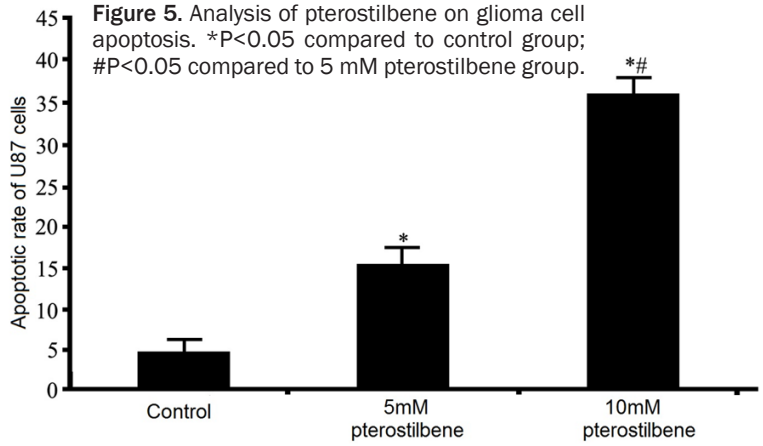

Figure 5. Analysis of pterostilbene on glioma cell apoptosis. *P<0.05 compared to control group; #P<0.05 compared to 5 mM pterostilbene group.

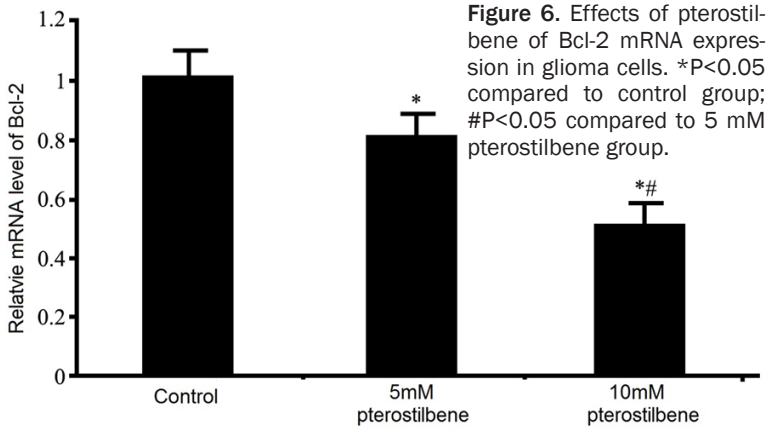

Figure 6. Effects of pterostilbene of Bcl-2 mRNA expression in glioma cells. *P<0.05 compared to control group; #P<0.05 compared to 5 mM pterostilbene group.

The effect of pterostilbene on Bax mRNA expression in glioma cells

Further real-time PCR assay was employed to test the effect of pterostilbene on Bax mRNA expression of glioma U87 cells under the treatment of pterostilbene. Results showed that after 48 h of pterostilbene treatment, Bax mRNA level in U87 cells was significantly elevated (P<0.05 compared to control group). With higher concentration of pterostilbene, such enhancing effect was further potentiated (**Figure 7**).

Regulatory effects of pterostilbene on protein levels of Bcl-2 and Bax in glioma cells

Western blotting was used to test the effect of pterostilbene treatment on Bcl-2 and Bax protein expression in U87 glioma cells. Results were consistent with those in mRNA analysis by real-time PCR, as 48 h pterostilbene treatment significantly suppressed anti-apoptotic Bcl-2 protein expression and facilitated pro-apoptotic Bax protein expression. The ratio of Bcl-2/Bax protein was significantly lowered (P<0.05 compared to that in control group). With higher dosage of pterostilbene, such Bcl-2 inhibition and Bax potentiation effects were further enhanced (**Figures 8** and **9**).

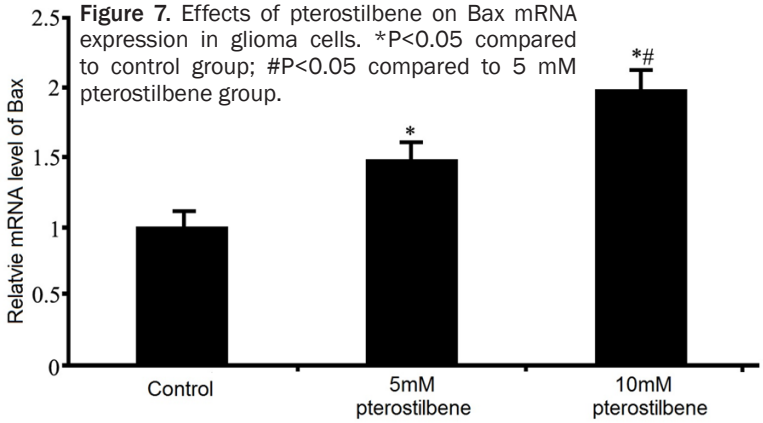

Figure 7. Effects of pterostilbene on Bax mRNA expression in glioma cells. *P<0.05 compared to control group; #P<0.05 compared to 5 mM pterostilbene group.

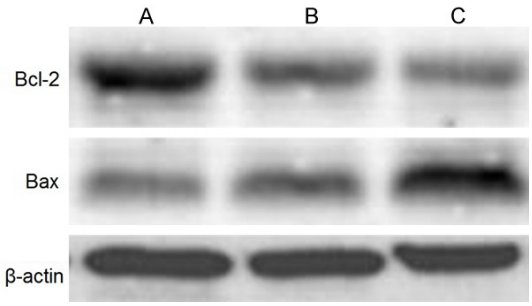

Figure 8. Western blotting bands showing Bcl-2 and Bax proteins in glioma cells. A. Control group; B. 5 mM pterostilbene group; C. 10 mM pterostilbene group.

Discussion

Glioma has complicated pathogenesis mechanisms among malignant tumors of nervous system, causing its unfavorable prognosis and the major challenge for neurosurgeon. As its increasing incidence worldwide, glioma has become a major concern threating the public health and leads to heavy economic burden and mental stress [16]. Although the advancement of medical sciences has improved the treatment of neuroglioma, the composited application of various treatment strategies did improve patient's life quality to certain extents. The clinical treatment efficacy is still unfavorable, repressing the overall 5-year survival rate. In addition, higher recurrence rate also existed for glioma even after surgery, making it one refractory tumor [17, 18]. The invasion and migration property of glioma has a close relationship with tumor recurrence and refractory nature. Glioma cells has intrinsic features of migration and invasion, both of which are correlated with anti-apoptosis and enhanced cell adhesion of tumor cells, composing on complicated pathological process [19]. Therefore, the establishment of effective anti-glioma drugs can improve the treatment efficacy of glioma.

Pterostilbene can effectively clear reactive oxygen, and regulate oxidation/reduction homeostasis via anti-oxidation pathway, and may exert functions for various diseases including ischemia-reperfusion injury, inflammation and tumors. As one of the most effective natural compound for anti-tumor treatment currently used in clinics [20], pterostilbene can induce the apoptosis of human acute promyelocytic leukemia cells, and inhibit dimethylbenzanthracene-induced skin tumor cells, with chemical anti-cancer effects. Pterostilbene has more potent pharmaceutical functions over resveratrol, due to its advantage of medicine effects and highly selective specificity [21, 22]. However, the role of pterostilbene has not been illustrated yet. The overexpression of Bcl-2 and inhibition of Bax expression are closely correlated with anti-apoptosis/apoptosis imbalance of glioma cells. As one regulatory mechanism for body homeostasis, apoptosis can retard the occurrence of tumors via inhibiting its over-growth. When Bcl-2 is over-expressed, those injured cells resist apoptosis, induce the interaction between downstream proliferation or growth related genes, developing into tumors. When apoptotic protein Bax is over-expressed, the apoptosis signal is initiated for facilitating cell apoptosis, which may antagonize or inhibit Bcl-2 protein expression [23]. This study demonstrated that after pterostilbene treatment, the proliferation or invasion of glioma cells was remarkably inhibited, enhancing tumor cell apoptosis, decreasing mRNA/protein expression of Bcl-2 while increasing Bax mRNA or protein expression.

In summary, pterostilbene could facilitate the apoptosis of glioma cells via regulating apoptotic/anti-apoptotic balance for inhibiting glioma cell proliferation or invasion. This study provided evidence for analyzing the pathogenesis mechanism of glioma in clinics, and suggested novel strategies for treatment.

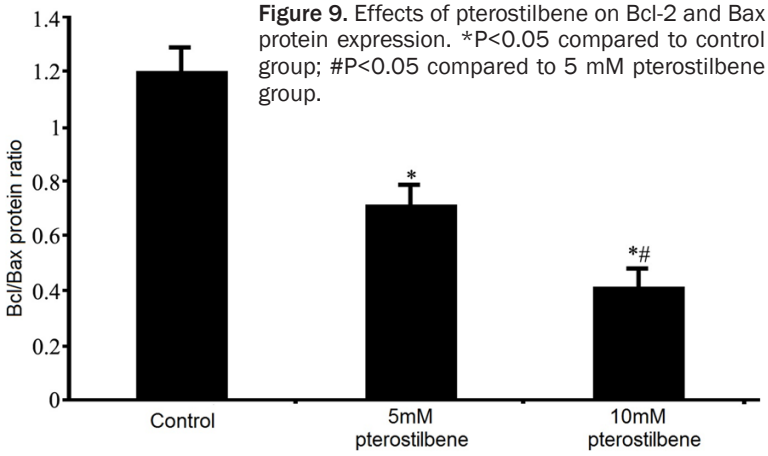

Figure 9. Effects of pterostilbene on Bcl-2 and Bax protein expression. *P<0.05 compared to control group; #P<0.05 compared to 5 mM pterostilbene group.

Disclosure of conflict of interest

None.

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