

## COMBINATION OF BERBERINE AND MATRINE INDUCES INTRINSIC APOPTOTIC PATHWAY AND G1 CELL CYCLE ARREST IN HUMAN CERVICAL CANCER CELLS

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**Abstract:** Cervical cancer is the fourth leading cause of malignancy-related mortality in women worldwide, and effective advanced-stage therapies are urgently required. Berberine is a quaternary ammonium compound extracted from traditional Chinese medicinal herbs, including *Phellodendron* spp., with antibacterial and anti-tumor activities. Matrine, the main active ingredient of *Sophora flavescens* rhizomes, has not only traditionally described health effects but is also widely used for its anti-cancer, anti-inflammatory, immunoregulatory, antiviral, and hepatoprotective effects. We investigated the antitumor activities of berberine and matrine against human cervical cancer HeLa and SiHa cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, along with flow cytometry and western blotting analyses, to assess the effect of the compounds on the cellular status and apoptosis- and cell cycle-related proteins. The 24 h half-maximal inhibitory concentrations (IC<sub>50</sub>) of berberine and matrine were  $123.633 \pm 4.278 \mu\text{mol/L}$  and  $9.625 \pm 0.245 \text{ mmol/L}$  against HeLa cells and  $105.067 \pm 3.745 \mu\text{mol/L}$  and  $8.50 \pm 0.23 \text{ mmol/L}$  against SiHa cells, respectively. Berberine plus matrine inhibited cancer cell growth and caused cell cycle arrest. We observed an increased stimulation of apoptosis, which was likely mediated by enhanced levels of caspase-3, caspase-9, and B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax), as well as decreased Bcl-2 protein expression. Cell cycle arrest in the G1 phase was probably mediated by p21 upregulation and cyclin-dependent kinase (Cdk)-4, Cdk-6, and cyclin D1 suppression. Combination treatment with berberine and matrine effectively inhibited human cervical cancer cell proliferation, most likely by stimulating apoptosis and inducing cell cycle arrest.

**Keywords:** cervical cancer, berberine, matrine, apoptosis

Cervical cancer, as the third most common cancer, is a serious health and safety concern in women. It is a malignant tumor of the vagina and the cervical canal with an incidence rate that ranks fourth among all malignant tumors in women (1). Since the beginning of the last century, the main treatment strategy for cervical cancer has been surgery (2). The current clinical treatment of cervical cancer involves simple uterine radical resection or combined radiotherapy and chemotherapy (3). Although these treatment options offer more choices, most patients are initially diagnosed with advanced cervical cancer, and radiotherapy has become one of the main treatments at this stage. However, the use of surgery alone or combined with radiotherapy and chemotherapy is not effective in patients with advanced cervical cancer (4-6). In

addition, the traditional anti-cancer drugs that inhibit the growth, proliferation, invasion, and metastasis of cervical cancer cells have severe side effects on the surrounding normal tissue cells. Traditional Chinese medicine has significant advantages in the treatment of cervical cancer (7-10). Numerous basic and clinical studies from around the world have confirmed that traditional Chinese medicine formulations, which act on multiple pathways and targets, exert synergistic activities that prevent and treat tumors.

Berberine is a quaternary ammonium compound extracted from traditional Chinese medicinal herbs including *Phellodendron* spp. that has beneficial effects traditionally described as heat-clearing and detoxifying, and some of these effects have been confirmed as antibacterial or antitumor activities (11-14). Matrine, the main active ingredient of

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*Sophora flavescens* rhizomes, has not only the traditionally described health effects, but it also kills insects and relieves itching (15-16). The purified matrine monomer is widely used clinically for its anti-cancer, anti-inflammatory, immunoregulatory, antiviral, and hepatoprotective effects (17-18). In this study, we assessed the combined effects of berberine and matrine on the proliferation and cell cycle of human cervical cancer HeLa and SiHa cells and investigated the underlying mechanisms.

## MATERIALS AND METHODS

### Materials and chemicals

Matrine was purchased from China Food and Drug Control Institute (article number: S31035, CAS number: 519-02-8, purity = 98%), and berberine was purchased from Shanghai Yuanye Biotechnology Co., Ltd. (China, article number: B21379, CAS number: 2086-83-1, purity = 98%). Roswell Park Memorial Institute (RPMI)-1640 medium and fetal bovine serum (FBS) were purchased from Corning Inc. (Corning, NY, USA). Rabbit polyclonal antibodies against p21, caspase-3, caspase-8, caspase-9, and  $\beta$ -actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X protein (Bax), cyclin-dependent kinase (Cdk)-4, Cdk-6, and cyclin D1 were purchased from Bioworld Technology, Inc. (Bioworld, MN, USA). Materials and chemicals for electrophoresis from obtained from Bioworld Technology, Inc. and Bio-Rad Laboratories (Hercules, CA, USA).

### Cell culture conditions

Human cervical cancer HeLa (ATCC® HTB-35) and SiHa (ATCC® CCL-2™) cell lines (American Type Culture Collection, ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and 100 U/mL cyan and streptomycin. All cells were cultured in a cell incubator at 5% CO<sub>2</sub> and maintained at 37°C (19-21).

### Cell culture assays

*3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to determine the half-maximal inhibitory concentration (IC<sub>50</sub>)*

The effects of the test agents were investigated in logarithmic growth phase HeLa and SiHa cells (22), which first were detached using 0.25% trypsin, followed by the addition of serum-containing medium to terminate the detach. The single cells were

collected and resuspended in fresh medium at a density of 10<sup>4</sup> cells/mL. Then, 200  $\mu$ L aliquots of the single-cell suspensions were seeded into 96-well plates and cultured in an incubator at 5% CO<sub>2</sub> and 37°C for 24 h. The experiment included blank controls, experimental controls, and samples treated at different concentrations (23). After the cells had attached, the medium in all wells was replaced with fresh culture medium for the controls or medium containing the respective compound concentrations. Four replicates of each concentration were tested to investigate the effect of berberine (3, 10, 30, 100, and 300  $\mu$ mol/L) and matrine (1, 3, 10, 30, and 100 mmol/L) against HeLa and SiHa cells. After incubating for 24 h, 20  $\mu$ L of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well, followed by incubation for 4 h in the incubator, after which the supernatant in each well was carefully discarded (24-25). An aliquot of 150  $\mu$ L dimethyl sulfoxide (DMSO) was added to each well with shaking for 10 min to fully dissolve the formazan crystals. The absorbance (A) of each well was measured at 570 nm using a plate reader. The survival and inhibition rates were calculated according to the following formulas: survival rate (%) = 100  $\times$  (A compound sample - A blank sample)/(A control sample - A blank sample) and inhibition rate (%) = 100 - survival rate (%).

### Flow cytometry analysis to determine apoptosis

HeLa and SiHa cells were seeded in six-well plates at a density of 5  $\times$  10<sup>5</sup> cells/well. After allowing the cells to adhere for 24 h, the incubation was continued in the presence of the test agents at their respective IC<sub>50</sub> except for the normal control wells. The HeLa and SiHa cells were incubated with berberine (30  $\mu$ mol/L), matrine (1.0 and 2.0 mmol/L) or two combinations of berberine plus matrine (30  $\mu$ mol/L + 1.0 mmol/L; 30  $\mu$ mol/L + 2.0 mmol/L). After 24 h, the adherent and suspended cells were collected and washed twice with pre-chilled phosphate-buffered saline (PBS); each sample was resuspended with 195  $\mu$ L Annexin V-fluorescein isothiocyanate (FITC) binding buffer (10 mM HEPES/sodium hydroxide [NaOH], 140 mM sodium chloride [NaCl], and 2.5 mM calcium chloride [CaCl<sub>2</sub>]). Then, 5  $\mu$ L Annexin-FITC solution (25  $\mu$ g/mL) was added, followed by gentle mixing and incubation at room temperature (21.0-23.0°C) for 10 min in the dark (26). Then, the mixture was centrifuged at 111  $\times$  g for 5 min, and the supernatant was discarded. The cells were resuspended in 190  $\mu$ L Annexin V-FITC binding solution and 10  $\mu$ L

propidium iodide (PI) solution (250  $\mu\text{g}/\text{mL}$ ) was added and mixed gently. The rate of apoptosis was analyzed using flow cytometry (27).

#### Flow cytometry analysis to determine the cell cycle status of human cervical cancer cells

HeLa and SiHa cells in the logarithmic growth phase were seeded into six-well plates at a density of  $5 \times 10^5$  cells/well. After adherence for 24 h, the cells were incubated in the presence of the test agents at their respective  $\text{IC}_{50}$  except for the normal control samples. HeLa and SiHa cells were incubated with berberine (30  $\mu\text{mol}/\text{L}$ ), matrine (1.0 and 2.0  $\text{mmol}/\text{L}$ ) or two combinations of berberine plus matrine (30  $\mu\text{mol}/\text{L}$  + 1.0  $\text{mmol}/\text{L}$ ; 30  $\mu\text{mol}/\text{L}$  + 2.0  $\text{mmol}/\text{L}$ ). After 24 h, the cells were harvested by digestion, washed twice with pre-cooled PBS, resuspended in 75% ethanol (EtOH) solution, and fixed for 2 h. The cells were centrifuged at  $111 \times g$  for 5 min, the supernatant was discarded, and 0.1% Triton X-100 was added, followed by incubation for 5 min at room temperature (21.0–23.0°C). Then, the cells were stained with PI (containing 0.2  $\text{mg}/\text{mL}$  RNase A) for 30 min. The cell cycle distribution was measured using flow cytometry (28–29).

#### Western blot analysis of apoptosis and expression of cycle-associated proteins

In order to study the synergistic effect of berberine and Matrine, according to the MTT experiment, we selected the dose of berberine and Matrine to inhibit the proliferation of cervical cancer cells by less than 15% as the follow-up experi-

ment. The dose of berberine is set as a single dose, through the interaction of different concentrations of Matrine, which can fully reflect the synergistic effect of the two and whether there is a dose-dependence. After allowing HeLa and SiHa cells to adhere for 24 h, the cells were incubated with berberine (30  $\mu\text{mol}/\text{L}$ ), matrine (1.0 and 2.0  $\text{mmol}/\text{L}$ ) or two combinations of berberine plus matrine (30  $\mu\text{mol}/\text{L}$  + 1.0  $\text{mmol}/\text{L}$ ; 30  $\mu\text{mol}/\text{L}$  + 2.0  $\text{mmol}/\text{L}$ ) for 24 h. Then, the cells were collected, and the protein was extracted and quantified using the Bradford method. After adding the loading buffer, the protein lysates were boiled for 5 min for denaturation. Then, 30  $\mu\text{g}$  of each protein lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% polyacrylamide for the separating gel and 6% for the stacking gel. Subsequently, the separated proteins were transferred onto the nitrocellulose (NC) membrane and incubated in a blocking buffer with 10% skim milk powder for 2 h. The primary antibodies were added (dropwise) at normal temperature (21.0–23.0°C), followed by incubation at 4°C overnight. Then, the membranes were equilibrated at room temperature (21.0–23.0°C), washed three times with Tris-buffered saline plus Tween (TBST) for 5 min each time, incubated with the corresponding horseradish peroxidase (HRP) secondary antibody for 2 h at room temperature, washed three times again with TBST, developed using a chemiluminometer (Clinux Science Instruments Co., Ltd., Shanghai, China), and scanned for quantification.  $\beta$ -Actin was used as an internal reference (30).

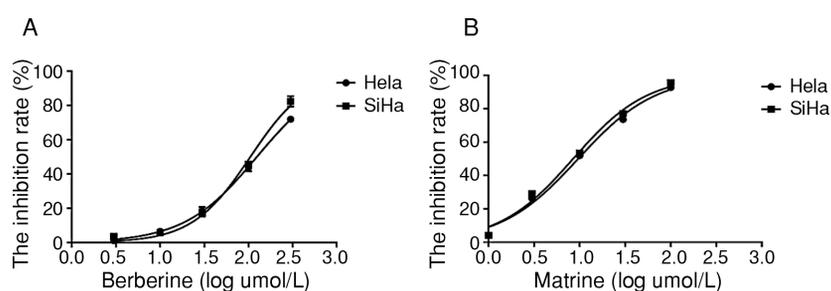


Figure 1. Effects of berberine and matrine on the viability of cervical cancer HeLa and SiHa cells. (A) Berberine treatment (3, 10, 30, 100, and 300  $\mu\text{mol}/\text{L}$ ) and (B) matrine treatment (1, 3, 10, 30, and 100  $\text{mmol}/\text{L}$ ) were performed on HeLa and SiHa cells

Table 1 Half-maximal inhibitory concentration ( $\text{IC}_{50}$ ) values of test compounds against HeLa and SiHa cells.

	HeLa cells	SiHa cells
	24 h	
Berberine ( $\mu\text{mol}/\text{L}$ )	123.633 $\pm$ 4.278	105.067 $\pm$ 3.745
Matrine ( $\text{mmol}/\text{L}$ )	9.625 $\pm$ 0.245	8.50 $\pm$ 0.23

### Statistical analysis

The statistical analysis was carried out by SPSS 17.0 statistical software, and the data were expressed as  $\bar{x} \pm s$ . One-way ANOVA and minimum significant difference (LSD) t test were used to evaluate the difference between groups.

## RESULTS

### Effect of berberine and matrine on cell viability

The MTT assay (31) was used to test the inhibitory activity of berberine and matrine on the growth of HeLa and SiHa cells. The 24 h cell survival rates and  $IC_{50}$  values were calculated using the Statistical Package for the Social Sciences (SPSS) software. The 24 h  $IC_{50}$  values of berberine and matrine were  $123.633 \pm 4.278 \mu\text{mol/L}$  and  $9.625 \pm 0.245 \mu\text{mol/L}$  against HeLa cells and  $105.067 \pm 3.745 \mu\text{mol/L}$  and  $8.50 \pm 0.23 \text{ mmol/L}$  against SiHa cells, respectively (Table 1 and Fig. 1).

### Berberine and matrine promote apoptosis

The results of the analysis of berberine- and matrine-induced apoptosis are shown in Figures 2 and 3. Co-incubation with berberine and matrine significantly increased apoptosis in HeLa and SiHa cells with apparent synergism (32-33). The apoptosis rates (early plus late apoptosis) of HeLa and SiHa cells in the normal control samples were  $2.0 \pm 0.4\%$  and  $1.9 \pm 0.4\%$ , and those of berberine-treated HeLa and SiHa cells were  $16.2 \pm 1.5\%$  and  $18.9 \pm 1.4\%$ , respectively. Matrine at 1 mmol/L induced apoptosis in HeLa and SiHa cells at rates of  $5.7 \pm 1.2\%$  and  $6.7 \pm 1.5\%$ , respectively, whereas the corresponding rates at 2 mmol/L were  $13.2 \pm 1.5\%$  and  $15.6 \pm 1.4\%$ , respectively. Furthermore, berberine plus matrine cotreatment caused enhanced apoptosis in HeLa and SiHa cells. Berberine plus 1 mmol/L matrine showed apoptosis rates of  $24.0 \pm 1.3\%$  and  $28.4 \pm 1.5\%$  in HeLa and SiHa cells, respectively, and those of berberine plus 2 mmol/L matrine were

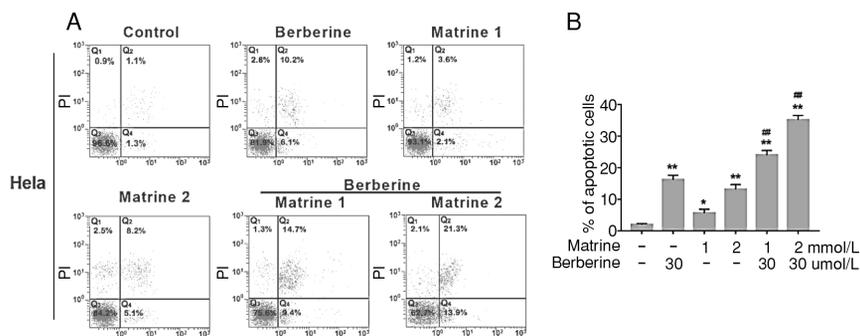


Figure 2. Flow cytometry analysis of the effect of berberine and matrine on apoptosis in HeLa cells. (A) In the flow histogram of diagram A: (Q<sub>1</sub> area) necrotic cells; (Q<sub>2</sub> area) early apoptotic cells; (Q<sub>3</sub> area) viable cells; (Q<sub>4</sub> area) late apoptotic cells. The doses of berberine, matrine 1 and matrine 2 were explained. (B) Statistical analysis: \*\**p* = 0.01 and ##*p* = 0.01 vs control and single drug groups, respectively.

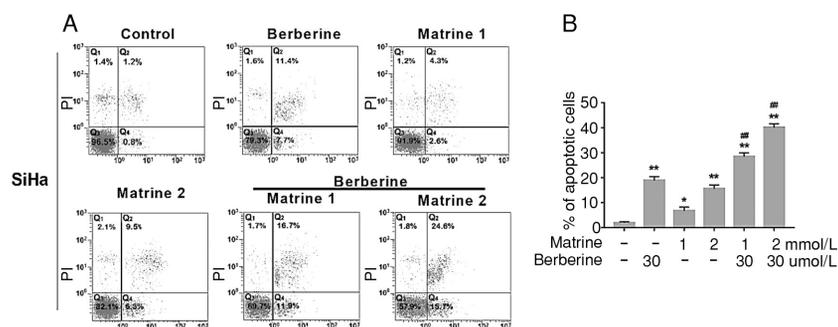


Figure 3. Flow cytometry analysis of the effects of berberine and matrine on apoptosis in SiHa cells. (A) In the flow histogram of diagram A: (Q<sub>1</sub> area) necrotic cells; (Q<sub>2</sub> area) early apoptotic cells; (Q<sub>3</sub> area) viable cells; (Q<sub>4</sub> area) late apoptotic cells. The doses of berberine, matrine 1 and matrine 2 were explained. (B) Statistical analysis: \*\**p* = 0.01 and ##*p* = 0.01 vs control and single drug groups, respectively.

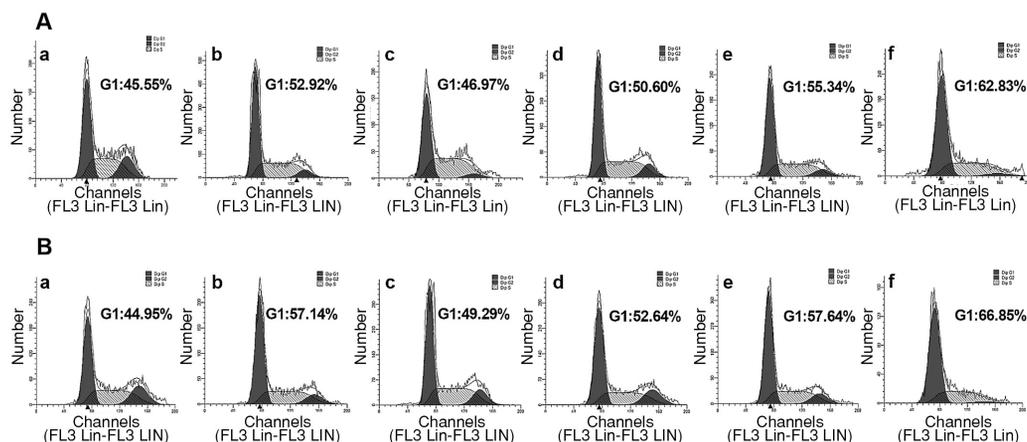


Figure 4. Flow cytometry analysis of the effects of berberine and matrine on the cell cycle phase in cervical cancer cells. (A) HeLa and (B) SiHa cell cycle flow pattern diagrams: (a) control; (b) berberine, 30  $\mu\text{mol/L}$ ; (c) matrine, 1.0  $\text{mmol/L}$ ; (d) matrine, 2.0  $\text{mmol/L}$ ; (e) berberine plus matrine, 30  $\mu\text{mol/L}$  + 1.0  $\text{mmol/L}$ ; and (f) berberine plus matrine, 30  $\mu\text{mol/L}$  + 2.0  $\text{mmol/L}$ .

35.1  $\pm$  1.2% and 40.1  $\pm$  1.5% in HeLa and SiHa cells, respectively. The apoptosis rate was significantly enhanced in the cancer cells treated with each compound alone for 24 h compared to that in untreated control cells ( $P < 0.01$ ). Therefore, the effect of berberine and Matrine on apoptosis rate was significantly higher than that of a single drug, which indirectly indicated that there was a synergistic effect between berberine and Matrine.

#### Compound-induced cell cycle arrest in cervical cancer cells

Compound-treated HeLa and SiHa cells were stained with PI, and the effect of the treatment on the cell cycle was assessed using flow cytometry. The results are shown in Figure 4. The proportion of cells in the G1 phase was altered in the cells co-treated with berberine and matrine compared to that in the untreated control cells, i.e., after alkali treatment, the percentage of G1 cells significantly increased, suggesting that the compound combination induced G1 arrest in HeLa cells. In addition, the decrease in the proportion of S phase cells suggested that cell proliferation was inhibited by the treatments. The data also showed that the berberine–matrine combination had a more significant effect than either drug alone (34–35).

#### Effect of the compounds on the expression of apoptosis-related proteins

Western blot analysis showed that the levels of activated caspase-3 and caspase-9 but not caspase-8 increased in cells after treatment with berberine and matrine compared to those in cells without treatment.

The expression of Bax protein significantly increased in HeLa cells, and that of the anti-apoptotic protein Bcl-2 significantly decreased in a concentration- and time-dependent manner. There was a significant difference between the compound-treated and the untreated control cells ( $p < 0.01$ ), and the effect of the berberine–matrine combination was significantly enhanced, apparently indicating a synergism (Fig. 5).

#### Effect of the compounds on the expression of proteins related to cell cycle regulation

We found that berberine and matrine induced G1 arrest in cervical cancer HeLa and SiHa cells using flow cytometry (36). Cyclin D1 binds to Cdk4 and Cdk6 to regulate the G1-to-S-phase transition in cells. This experiment was performed to determine the effect of the test compounds on the expression of these three proteins. We found that the levels of cyclin D1, Cdk4, and Cdk6 significantly decreased after treatment with berberine and matrine. The p21 factor is an inhibitory protein of Cdk, which prevents the passage of damaged DNA and inhibits the replication and accumulation of damaged DNA, thereby exerting a tumor-suppressing effect. We found that the expression level of the p21 protein increased in HeLa and SiHa cells after treatment with various concentrations of the test compounds. These results suggested that berberine and matrine inhibited the activity of the Cdk-4/Cdk-6-cyclin D1 complex by upregulating the p21 protein expression and arresting the cell cycle in the G1 phase, while berberine combined with matrine showed a significantly synergistic, dose-dependent G1 phase arrest in HeLa and SiHa cells (Fig. 6).

**DISCUSSION**

Apoptosis plays an important role in maintaining the normal physiological function of the body (37). Cells with signs of aging or abnormal division can be eliminated by apoptotic mechanisms. Conversely, when apoptosis regulation is disrupted, abnormal cells cannot be cleared and accumulate over time in the body, which can potentially induce malignant tumors. In this study, we first used the Annexin V-FITC/PI double dye method to monitor the effects of berberine and matrine on apoptosis in cervical cancer HeLa and SiHa cells using flow cytometry. Both berberine and matrine increased the apoptosis rate in cervical cancer cells to different degrees. Further analysis showed that berberine combined with matrine increased the apoptosis rate in HeLa and SiHa cells more significantly than that by treatment with either agent alone. This finding indicated that berberine and matrine had synergistic effects on apoptosis in cervical cancer HeLa and SiHa cells.

Non-activated caspase is found in the cytoplasm of normal cells. Among caspases, caspase-9 promotes apoptosis; the enzyme is activated via cleavage by the mitochondrial programmed death signal. In our study, we investigated the test compound treatment effect on activated caspase-3, caspase-8, and caspase-9. The level of activated caspase-9 significantly increased after berberine and matrine treatment, indicating that the compounds may induce tumor cell apoptosis via a caspase-dependent programmed cell death pathway (38).

The Bcl-2 protein family has critical regulatory roles. The regulation of apoptosis by the Bcl-2 family depends on the interactions between its family members. In tumor cells, the expression level of anti-apoptotic protein Bcl-2 is increased, and the expression of pro-apoptotic protein Bax is decreased, resulting in decreased responsiveness of tumor cells to pro-apoptotic stimuli (39). In this experiment, after treatment with berberine and matrine, the expression of the anti-apoptotic protein Bcl-2 decreased in the cancer cells, but that of the

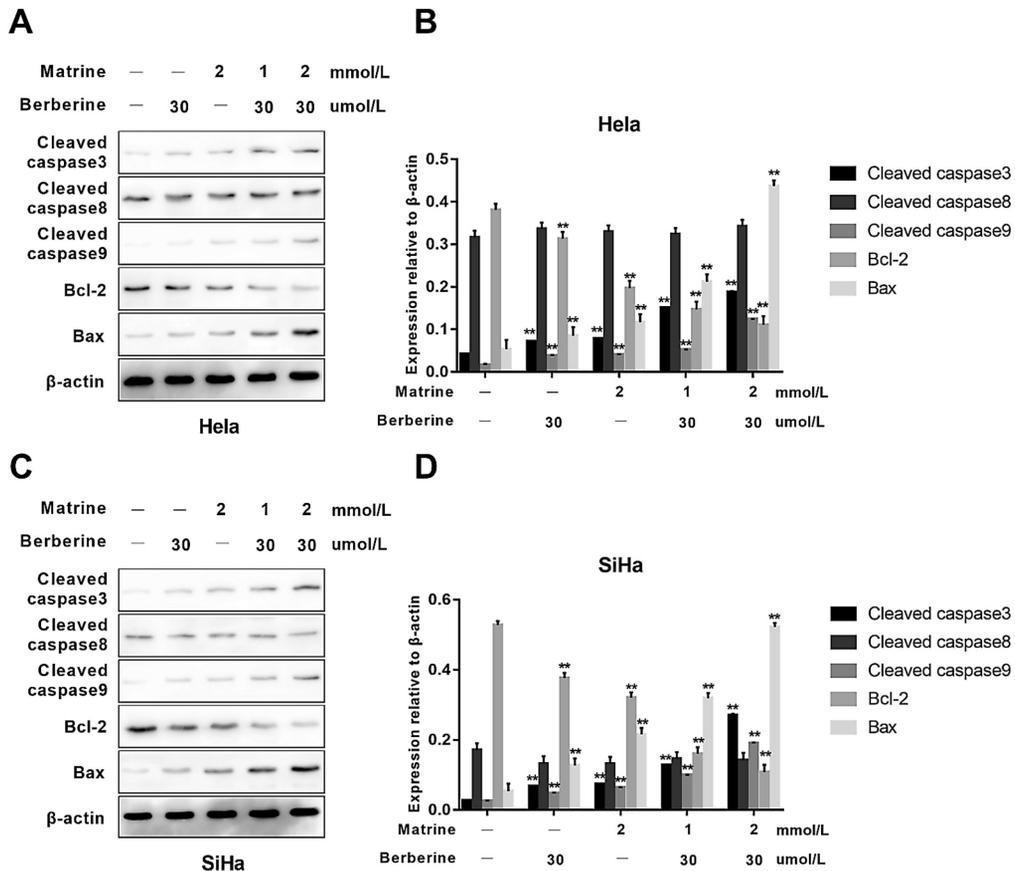


Figure 5. Effect of berberine and matrine on the expression of apoptosis-related proteins in HeLa and SiHa cells.

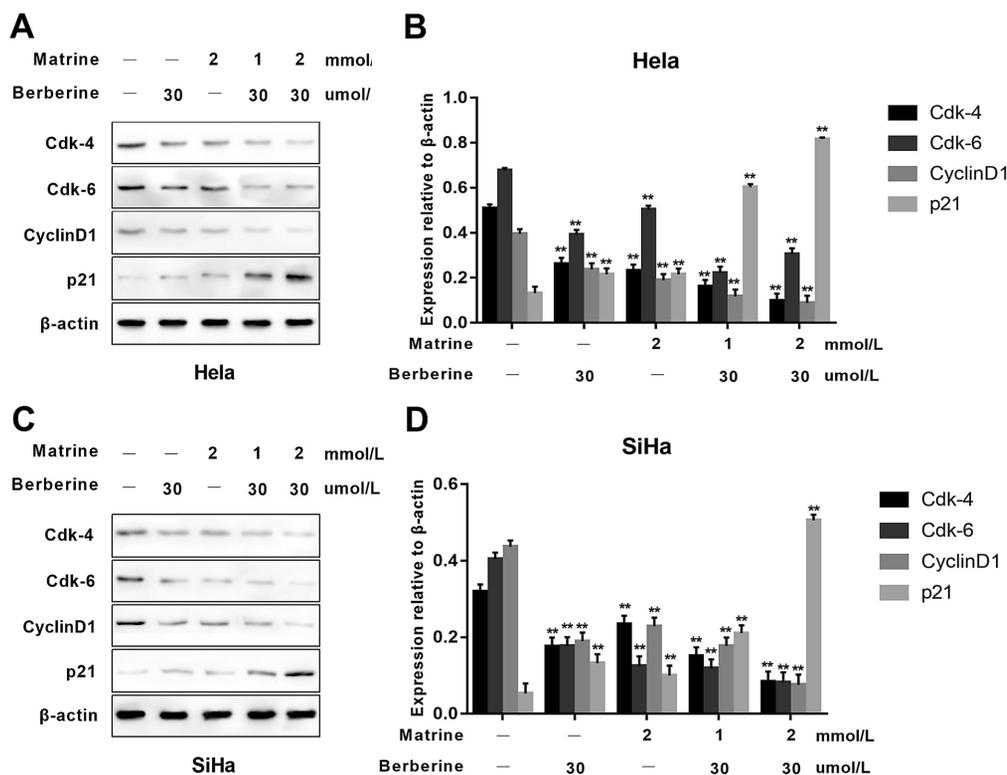


Figure 6. Effect of berberine and matrine on the expression of key cell cycle regulatory proteins in HeLa and SiHa cells.

pro-apoptotic Bax increased, indicating that berberine and matrine induced apoptosis in cancer cells by mechanisms related to the ratio of anti-apoptotic and pro-apoptotic proteins. Further studies showed that berberine combined with matrine showed significantly enhanced and concentration-dependent effects in the cancer cell lines.

The cycle phase transitions of normal cells are tightly regulated. When cells appear to be dysfunctional before entering the next phase, specific regulatory mechanisms often block the cell cycle at a specific stage, i.e., cell cycle arrest (40-41). To determine the effect of drugs on the cell cycle, we first analyzed the cell cycle distribution in HeLa cells after drug treatment using flow cytometry combined with PI staining. We found that berberine and matrine induced G1 arrest in cervical cancer cells. G1 phase regulatory proteins include cyclin D1, D2, and D3, along with cyclin C and cyclin E. To further investigate the underlying mechanism of the test compound effect on cervical cancer cells, we examined the expression of proteins involved in cell cycle regulation, i.e., Cdk-4, Cdk-6, and cyclin D1. We

found that the levels of these cell cycle-related proteins were downregulated after 24 h treatment with berberine and matrine.

The p21 protein can prevent the passage of damaged DNA, reduce abnormal DNA replication rate, and prevent the occurrence of tumors. We found that berberine and matrine upregulated the expression of p21 in cervical cancer cells, indicating that berberine and matrine blocked the cancer cell cycle in the G1 phase by upregulating p21 and inhibiting the expression of Cdk-4, Cdk-6, and cyclin D1. Furthermore, the berberine-matrine combination had a significantly increased effect on the G1 arrest, and the levels of the regulatory proteins Cdk-4, Cdk-6, and cyclin D1 decreased significantly (42). In addition, the protein expression of p21 significantly increased, and the effect was more pronounced with increasing matrine concentration. However, despite accumulating evidence on the effects of berberine and matrine on apoptosis in cervical cancer HeLa and SiHa cell lines, additional research will be needed to fully comprehend the mechanism of action of those compounds in cancer

cell apoptosis (43-44). Most studies on the anti-cancer properties of berberine have only been conducted over the past 10 years, and numerous details of its clinical potential, such as berberine-susceptible cancer types, molecular targets, dosage, and indications in different individuals, remain to be addressed (45).

Therefore, further studies are still warranted to enhance the knowledge of the antineoplastic actions of berberine and matrine and discover their potential clinical applications by using advanced approaches. Future studies to elucidate the effects of berberine and matrine on cancer and normal cells will facilitate the development of effective treatment strategies with possible clinic applications (46).

## CONCLUSIONS

Our results indicate that berberine and matrine inhibited cervical cancer cell growth and induced both apoptosis and cycle arrest, and their combination exerted enhanced effects on these critical cellular events. The underlying mechanism was likely mediated by effects on the G1 phase of the cell cycle, which affected the intrinsic caspase activity of the cells. This study provides critical data for future in-depth research on the mechanism of action and the development of new treatment options for cervical cancer by using this drug combination.

## Acknowledgments

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## Conflict of interest

The authors report no conflicts of interest.

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