Berberine and Evodiamine Act Synergistically Against Human Breast Cancer MCF-7 Cells by Inducing Cell Cycle Arrest and Apoptosis

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Abstract. *Background/Aim: The synergistic combinations of natural products have long been the basis of Traditional Chinese herbal Medicine formulas. In this study, we investigated the synergistic effects of a combination of berberine and evodiamine against human breast cancer MCF-7 cells in vitro and in vivo, and explored its mechanism. Materials and Methods: Cell survival was measured using the MTT assay. Apoptosis-related proteins were observed using western blot analysis. Apoptosis was detected with flow cytometric analysis and by Hoechst 33258 staining. Tumor xenografts were used in vivo. Results: Compared to berberine or evodiamine treatments alone, the combination treatment of berberine (25 μM) and evodiamine (15 μM) synergistically inhibited the proliferation of MCF-7 cells in a time-dependent manner and resulted in the G_0/G_1 phase accumulation of cells that exhibited increased expression levels of the CDK inhibitors p21 and p27 with a concomitant reduction in the expression levels of cell-cycle checkpoint proteins cyclin D1, cyclin E, CDK4, and CDK6. Furthermore, the combination treatment induced apoptosis that was accompanied by increased expression levels of p53 and Bax, reduced expression levels of Bcl-2, activation of caspase-7, and caspase-9, and the cleavage of PARP. The combination of berberine and evodiamine synergistically inhibited tumor growth in vivo in MCF-7 human breast cancer xenografts. Conclusion: Combination of berberine and evodiamine acts synergistically to suppress the proliferation of MCF-7 cells by inducing cell cycle arrest and apoptosis, illustrating the potential synergistic and combinatorial application of bioactive natural products.*

Worldwide, breast cancer is the most common cancer and is the leading cause of cancer deaths among women (1). Although great efforts have been made for the prevention and treatment of breast cancer, much progress remains to be made. It is necessary to identify superior strategies for breast cancer treatment to achieve sustained responses and more favorable outcomes for patients. Natural products are valuable sources of novel anti-cancer drugs due to their diverse biological activities and relatively low toxicities (2). Multi-drug therapy is currently being practiced worldwide for the treatment of numerous types of cancers, that inherently exhibit complex pathologies (3). Thus, the therapeutic application of natural products can synergistically improve the treatment of breast cancer.

Synergistic multi-drug combinations have long been the basis of traditional Chinese herbal medicine formulas, that represent a form of Chinese herbology in which herbs are combined to produce enhanced efficacy compared to the application of individual herbs (4). Recently, berberine and evodiamine, predominant compounds contained in the traditional Chinese herbal medicine formula Zuo-Jin-Wan, were shown to inhibit the proliferation of cancer cells. However, whether berberine and evodiamine elicit synergistic anti-proliferative effects against human breast cancer cells has been unclear. Thus, in this study, we evaluated the potential synergistic effects of berberine and evodiamine against human breast cancer MCF-7 cells.

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Key Words: Breast cancer, MCF-7 cell, berberine, evodiamine, synergistic combination.

Zuo-Jin-Wan (ZJW) consists of two traditional Chinese medicinal herbs, Rhizoma Coptidis and Fructus Evodiae, and was experimentally demonstrated to elicit anti-cancer activity in gastric cancer (5), liver cancer (6), colorectal cancer (7), and multiple cancer cell lines. The anti-cancer activities of ZJW have been suggested to be attributed to the induction of the mitochondria-dependent apoptosis pathway (8). In our previous study, we investigated the effects and safety of Zuo-Jin-Wan (ZJW) using the 4T1 mouse breast cancer model. Our results showed that ZJW inhibits tumor growth and improves kidney and liver functions in the 4T1 breast cancer mice (9).

The compounds berberine and evodiamine, that represent the predominant components of Rhizoma Coptidis and Fructus Evodiae, respectively, have been demonstrated to exhibit significant bioactive functions. Berberine has been widely used in Asian countries for the treatment of diabetes, hypertension, and hypercholesterolemia (10-12). Evodimiane has been shown to exhibit diverse pharmacological activities against obesity and Influenza A Virus, to protect against myocardial ischemia-reperfusion injury, and to regulate testosterone secretion (13-16). Recent studies have shown that both evodiamine and berberine individually exhibit antitumor activities against a broad range of human cancer cells types; they have been shown to inhibit cancer cell proliferation and to induce apoptosis in prostate cells (17-18). Moreover, berberine or evodiamine individually have been shown to decrease levels of Bcl-2, Bcl-xL, and Mcl-1 and to trigger apoptosis (6, 19-22) and have also been reported as potent inhibitors of nuclear factor κB (NF-κB)-mediated transcription (22).

The aim of this study was to determine a scientific rationale for the therapeutic efficacy of Traditional Chinese herbal medicine formula-based combinations of natural products. We investigated the anti-tumor efficacy and mechanisms of action elicited by a combination treatment of berberine and evodiamine in human breast cancer MCF-7 cells through *in vivo* and *in vitro* studies. Our results showed that berberine and evodiamine act synergistically against human breast cancer MCF-7 cells by inducing cell-cycle arrest and apoptosis. Our study demonstrates the potential synergistic therapeutic potential of formula-based combinations of bioactive natural products and might significantly influence the development of new therapeutic strategies in the treatment of cancer.

Materials and Methods

Chemicals. The compound evodiamine was obtained from the Shanghai Research and Development Centre for Standardization of Traditional Chinese Medicine (Shanghai, P.R. China), and berberine hydrochloride was purchased from Shanghai Tauto Biotech Co., Ltd (Shanghai, P.R. China). Stock solutions of evodiamine and berberine were prepared in dimethylsulfoxide (DMSO), stored at 4°C, and

diluted with fresh medium immediately prior to application. An equal volume of DMSO (final concentration ≤0.1%) was added to the controls.

Cell culture. MCF-7 cells were obtained from the Chinese Academy of Sciences (Shanghai, China) and were cultured in DMEM medium (Gibco, San Francisco, CA, USA) supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum (ExCell Biology, Shanghai, P.R. China), 0.01 mg/mL insulin (Sigma, St Louis, MO, USA), 2 mmol/L glutamine (Gibco, San Francisco, CA, USA), penicillin (100 U/ml) and streptomycin (100 µg/ml). The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Cell proliferation assay. Inhibition of cell growth was assessed by MTT assay. MCF-7 cells (1×10^4 cells/well) were seeded into 96-well culture plates. After an overnight incubation, the MCF-7 cells were treated with the indicated concentrations of berberine, evodiamine, or berberine plus evodiamine (the concentration of each compound was to controls) Following incubation with the compounds, cell growth was measured at the indicated time points by the addition of 20 µl MTT at 37°C for 4 h. Next, DMSO (150 µl) was added to dissolve the formazan crystals. Optical density (OD) was measured at 490 nm using an ELISA plate reader (BioTek, Winooski, VT, USA).

Isobologram analysis of the efficacy of berberine and evodiamine. Isobologram analysis was used to determine the effects of drug combinations on the MCF-7 cells. The interaction of berberine and evodiamine was quantified by determining the combination index (CI), according to the following classic isobologram: $CI=(D)_1/(Dx)_1 + (D)_2/(Dx)_2$, where Dx is the individual dose of berberine or evodiamine required to produce an effect, and $(D)_1$ and $(D)_2$ are the respective doses of berberine and evodiamine that are necessary to produce the same effect in combination. From this analysis, the combined effects of the two drugs can be summarized as follows: the CI of less than, equal to, or more than 1 indicate synergistic, additive, or antagonistic effects, respectively.

Cellular morphological observation. MCF-7 cells (2×10^5/ml) were seeded onto sterile coverslips that were placed into 6-well plates. After attachment to the coverslips, the MCF-7 cells were treated with 25 µmol/l berberine, 15 µmol/l evodiamine, or 25 µmol/l berberine plus 15 µmol/l evodiamine. After a 48-h incubation, the cells were washed carefully with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min at room temperature, washed with pre-chilled PBS 3 times and stained with 5 µg/ml Hoechst 33258 at room temperature in the dark for 20 min. The samples were visualized using a fluorescent microscope (Olympus, Tokyo, Japan). Cells exhibiting cytoplasmic and nuclear shrinkage, chromatin condensation, or fragmentation were defined as apoptotic cells.

Cell cycle analysis. MCF-7 cells (4×10^5 cells/ml) were cultured in 60-mm dishes to 70-80% confluence. Cells were then treated with 25 µmol/l berberine, 15 µmol/l evodiamine, or 25 µmol/l berberine plus 15 µmol/l evodiamine for 48 h. The cells were harvested and washed twice with PBS, then fixed with 70% ethanol at −20°C for 24 h. The cells were then collected and resuspended in PBS with RNase (25 µg/ml) and propidium iodide (PI, 50 µg/ml) for 30 min at 37°C. The PI-stained cells were analyzed using Mcycle software (Beckman Coulter, Fullerton, CA, USA).
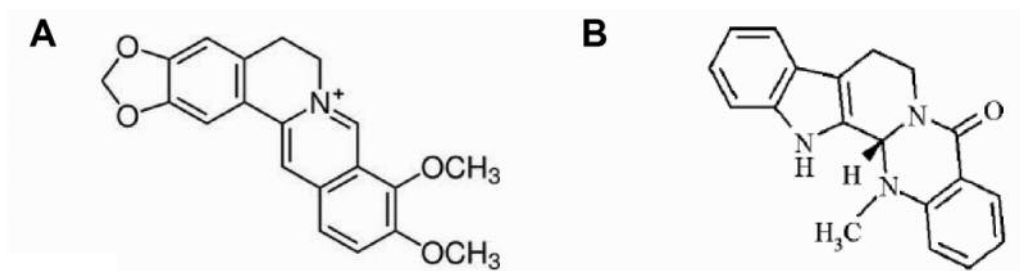
Figure 1. *(A) The chemical structure of berberine. (B) The chemical structure of evodiamine. A: Berberine, $C_{20}H_{18}ClNO_4$, MW:371.81; B: Evodiamine, $C_{19}H_{17}N_3O$, MW:303.36.*

Annexin V-PI staining assay. MCF-7 cells (1×10^6 cells/mL) were cultured in 100-mm dishes to 70-80% confluence. The cells were then treated with 25 µmol/l berberine, 15 µmol/l evodiamine, or 25 µmol/l berberine plus 15 µmol/l evodiamine for 48 h. The cells were collected and evaluated using an Annexin V-FITC/PI Apoptosis Kit (Beckman Coulter, Miami, FL, USA), according to the manufacturer's protocols. The cells were analyzed using a flow cytometer (Beckman Coulter, Fullerton, CA, USA). The percentage of apoptotic cells was determined using Mcycle software.

Western blot analysis. MCF-7 cells (1×10^6 cells/dish in a 100-mm dish) were treated with 25 µmol/l berberine, 15 µmol/l evodiamine, or 25 µmol/l berberine plus 15 µmol/L evodiamine for 48 h. Total cell lysates were generated using RIPA lysate buffer containing 1 mM PMSF and boiled with 5X sample loading buffer. The protein concentration of each lysate was determined by using the BCA method. Lysates (30-50 µg) were subjected to 8-12% SDS-PAGE. The proteins were transferred onto PVDF membranes, and the membranes were incubated with blocking buffer (5% non-fat dry milk in TBS-0.1% Tween 20) for 1 h at room temperature. The membranes were then incubated with the indicated antibodies overnight at 4˚C. The membranes were incubated with corresponding IRDye 800CW or 680CW-conjugated secondary antibodies for 2 h at room temperature. The proteins were detected using an Odyssey infrared imaging system (Li-Cor Bioscience, Lincoln, NE), and the protein bands were subjected to densitometric analysis and shown as the ratio of the intensity of the target protein relative to that of GAPDH.

Tumor xenograft and treatment. Athymic female BALB/c mice (5 weeks of age) were obtained from the Academia Sinica (Shanghai, China). Five mice were randomly distributed into the normal group. Other mice were assigned to the tumor xenograft groups and treated as follows: prior to inoculation, 17β-estradiol was intraperitoneally injected, and then the MCF-7 cells (1×10^7 cells/ml) were inoculated into the mammary fat pad (m.f.p.) of the mice. Once palpable tumors developed (approximately 2 weeks), the tumor-bearing mice were randomized to 4 groups (n=5 per group). Treatment groups included berberine (10 mg/kg), evodiamine (10 mg/kg), and a combination of berberine (10 mg/kg) and evodiamine (10 mg/kg). All of the compounds were administrated intraperitoneally once every 2 days. Untreated mice were divided into normal (no MCF-7 xenograft) and control groups (xenografted MCF-7 cells) that were injected with physiological saline. Tumor sizes were determined by measuring the largest length/width and perpendicular diameters

every week. Tumor volumes were calculated according to the following formula: $V = A \times B^2 \times 0.52$, where A is the largest superficial diameter and B is the smallest superficial diameter. During the treatment period, the mice of each group were also monitored for body weight, food intake, posture, and movement. After 5 weeks of treatment, the animals were sacrificed. All procedures conformed to Institutional regulations of animal welfare and were approved by the ethical committee of Shanghai University of traditional Chinese medicine.

Statistical analysis. All data are expressed as mean values±SD. Comparisons between groups were performed by Student's *t*-test and one-way analysis of variance (ANOVA). The level of significance was set at $p < 0.05$.

Results

Combination of berberine and evodiamine synergistically inhibit the proliferation of MCF-7 cells. To investigate the anti-proliferative effects of the combination of berberine and evodiamine, MCF-7 cells were treated with or without the indicated concentrations of berberine, evodiamine, or their combination for 48 h. The MTT results showed that both berberine (Figure 1A) and evodiamine (Figure 1B) significantly inhibited cell viability in a concentration-dependent manner. The 50% inhibitory concentrations (IC_{50}) for berberine and evodiamine were 50 and 60 µM, respectively (Figures 2A and B). Next, the effect of berberine in combination with evodiamine was investigated. The dose of berberine was fixed at 25 µM (1/2 IC_{50}) and the dose of evodiamine was fixed at 15 µM (1/4 IC_{50}). We tested both the combination of berberine (25 µM) with a range of concentrations of evodiamine and the combination of evodiamine (15 µM) with a range of concentrations of berberine on MCF-7 cells (Figures 2A and B). After treatment with the combination of 25 µM berberine plus 3.75 µM evodiamine, cell growth was further inhibited by approximately 10% compared to 3.75 µM evodiamine alone ($p < 0.05$). Similarly, after treatment with the combination of 25 µM berberine plus 15, 30, 60 and 120 µM evodiamine respectively,

cell growth was further inhibited by approximately 16, 17, 13, 26% compared to 15, 30, 60, 120 μM evodiamine alone ($p<0.01$). Cell growth inhibition did not significantly change when we combined 25 μM berberine with 7.5 μM evodiamine ($p>0.05$), compared to 7.5 μM evodiamine alone (Figure 2A). Moreover, there were no significant differences between the effects elicited by 25 μM berberine plus 15 μM evodiamine and 25 μM berberine plus 30 μM evodiamine ($p>0.05$).

After a combination treatment of 15 μM evodiamine plus 3.125 μM berberine, 15 μM evodiamine plus 6.25 μM berberine, 15 μM evodiamine plus 25 μM berberine and 15 μM evodiamine plus 100 μM berberine, cell growth was inhibited by approximately 23, 22, 13, and 5%, compared to 3.125, 6.25, 25 or 100 μM berberine alone, respectively ($p<0.05$ or $p<0.01$). However, 15 μM evodiamine plus 12.5 or 50 μM berberine did not change the effect on cell survival ($p>0.05$), as compared to 12.5 or 50 μM berberine alone (Figure 2B). There were no significant differences between 15 μM evodiamine plus 25 μM berberine and 15 μM evodiamine plus 50 μM berberine ($p>0.05$).

Isobologram analysis revealed a CI<1 for 25 μM berberine in combination with 15, 60, or 120 μM evodiamine, respectively, indicating a synergistic relationship between the treatments (Table I). Moreover, the combination treatment of 15 μM evodiamine and 25 or 50 μM berberine also indicated synergistic relationship between the treatments (CI<1) (Table II). The combination of 25 μM berberine and 15 μM evodiamine created a synergistic effect (CI=0.75). Furthermore, the concentration of this combination was lowest compared to the concentrations of all of the other synergistic combinations. Therefore, 25 μM berberine combined with 15 μM evodiamine was selected for the subsequent experiments.

Combination of berberine and evodiamine enhances apoptosis of MCF-7 cells. We investigated the rate of apoptosis of MCF-7 cells by morphological evaluation after combination treatment with berberine and evodiamine for 48 h. The images showed that MCF-7 cells underwent apoptosis when treated with the combination of berberine with evodiamine. Hoechst 33258 staining showed that MCF-7 cells in the control group exhibited uniform chromatin and regular nuclear morphology. After treatment with the combination of berberine and evodiamine, typical features of apoptosis including cytoplasmic and nuclear shrinkage, chromatin condensation, formation of apoptotic bodies and smaller cellular bodies, cell volume shrinkage, intracellular vacuole formation, chromatic agglutination, and chromatorrhexis were observed at the indicated time points (Figure 3A). To determine the apoptotic ratio induced by the compound combinations, apoptosis was analyzed by flow cytometry after MCF-7 cells were exposed to 25 μM berberine, 15 μM evodiamine or 25 μM berberine plus 15 μM evodiamine for 48 h. The combination treatments
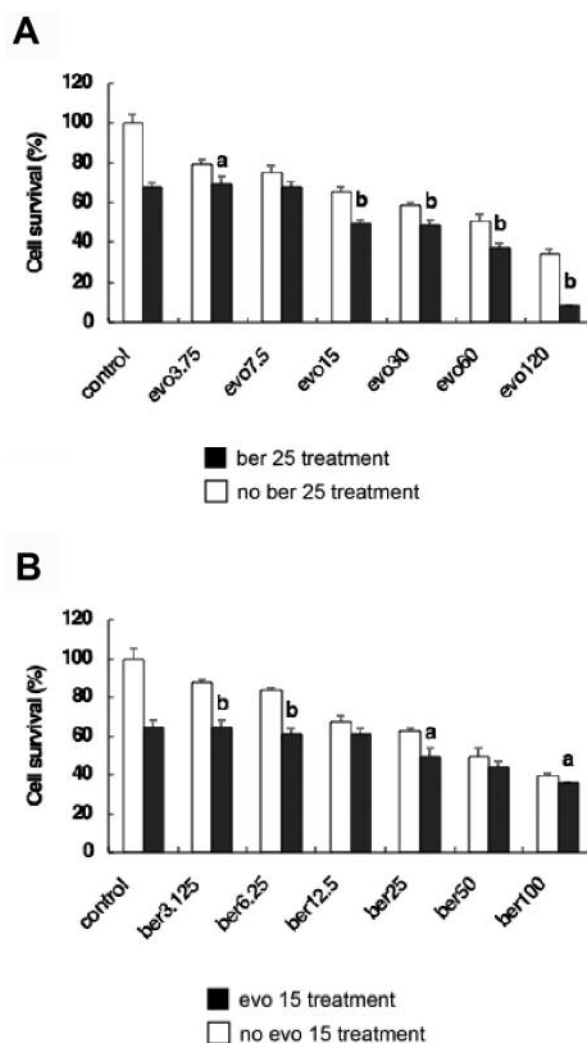

Figure 2. *The effect of berberine (ber), evodiamine (evo), or their combination on the viability of MCF-7 cells. (A) MCF-7 cells were treated with 3.75, 7.5, 15, 30, 60, 120 μM evodiamine with (black columns) or without (white columns) 25 μM berberine for 48 h. a<0.05, b<0.01 (B) MCF-7 cells were treated with 3.125, 6.25, 12.5, 25, 50, 100 μM berberine with (black columns) or without (white columns) 15 μM evodiamine for 48 h. a<0.05, b<0.01. Values are expressed mean values±SD. Data shown represent experiments performed in biological triplicates.*

significantly increased apoptosis ($p<0.01$) (by 20 and 32%, respectively) compared to either drug alone; when berberine and evodiamine were applied individually, only 29.1 and 17.1% of the cells exhibited apoptotic features at 48 h, respectively (Figures 3B and C).

Combined berberine and evodiamine induces cell-cycle arrest of MCF-7 cells. The combination of berberine and evodiamine not only enhanced apoptosis, but also induced
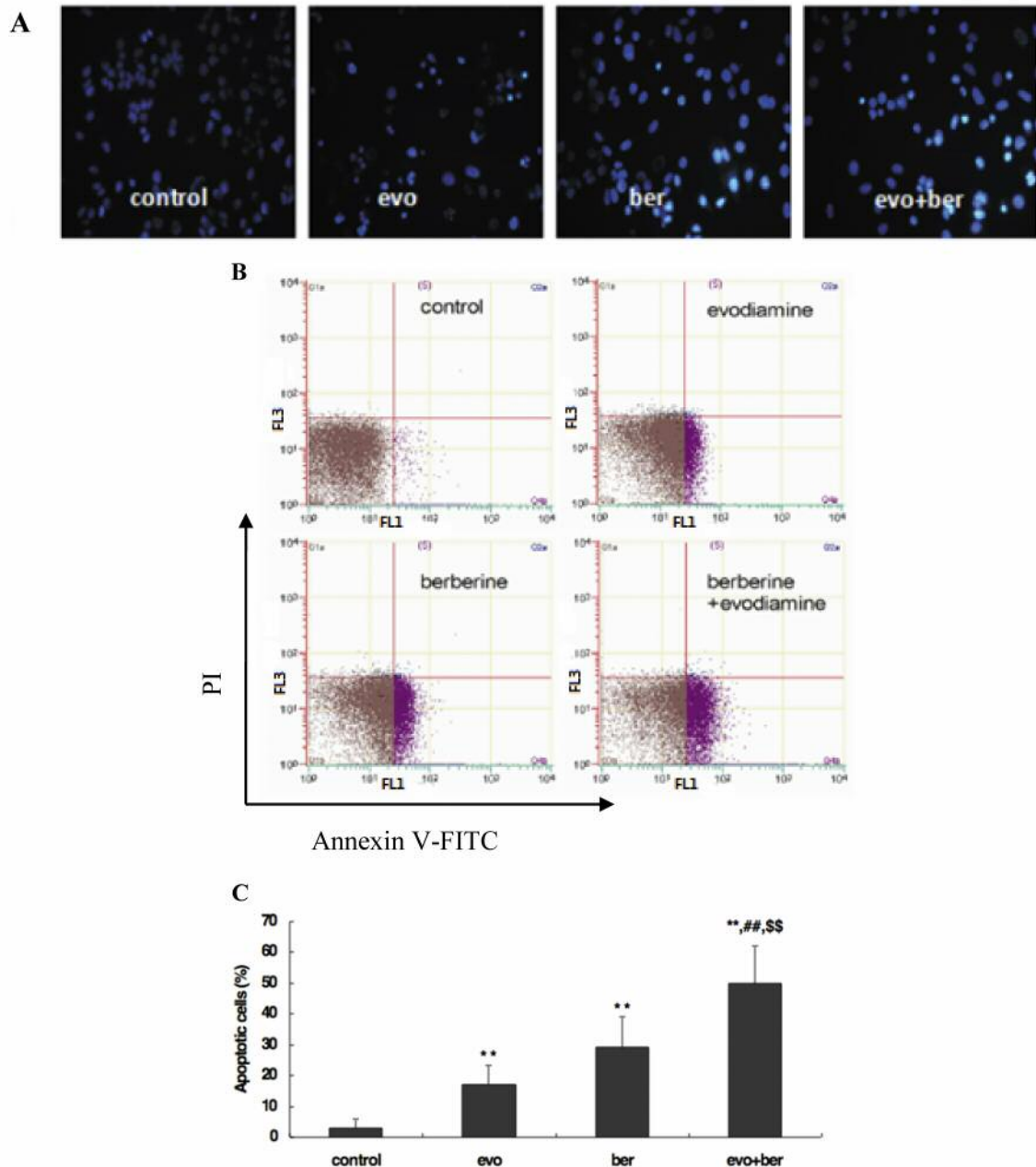
Figure 3. *The effect of berberine (ber), evodiamine (evo), or their combination on apoptosis. (A) MCF-7 cells were treated with 25 μM ber, 15 μM evo or their combination for 48 h. The cells were stained with Hoescht 33258 and visualized using a fluorescence microscope (×400). (B) MCF-7 cells were treated with 25 μM berberine, 15 μM evodiamine, or their combination for 48 h and analyzed by FACS. Representative flow cytometric profiles are shown. (C) The quantity of apoptotic cells was calculated. The data shown represent experiments performed in biological triplicates.* **$p<0.01$ evo, ber, evo+ber vs. con; ##$p<0.01$ evo+ber vs. evo; $$P <0.01 evo+ber vs. ber.*

cell-cycle arrest. As shown in Figure 4, the combination treatment increased the fraction of cells in the G_0/G_1 phase by 15%, as compared to the control treatment at 48 h, suggesting that the combination of berberine and evodiamine induced a G_0/G_1 arrest. There were no significant differences between 15 μM evodiamine plus 25 μM berberine, and 15 μM evodiamine or 25 μM berberine alone ($p>0.05$). However, sub-G_1 cells, which represent the apoptotic cells, increased significantly by 27 and 33% with the combination treatments, compared to either drug alone.
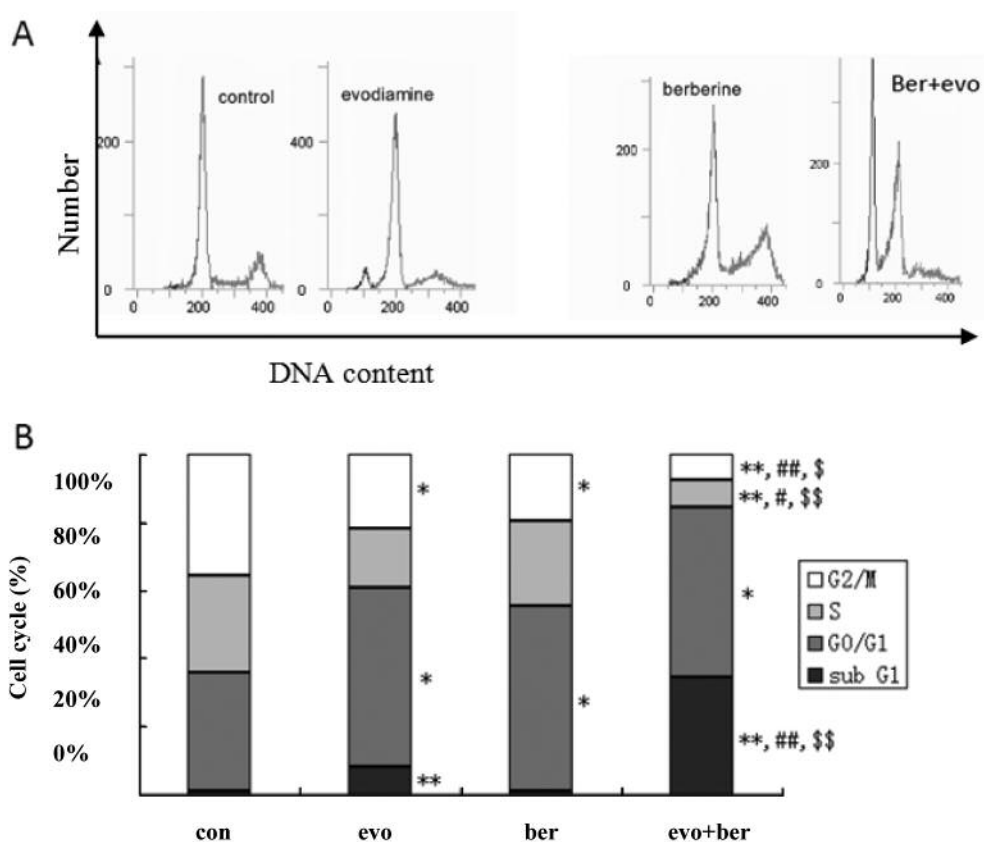
Figure 4. *The effects of berberine (ber), evodiamine (evo), or combination on the cell-cycle progression of MCF-7 cells. MCF-7 cells were treated with 25 µM berberine, 15 µM evodiamine, or their combination for 48 h. The MCF-7 cells were stained with PI and analyzed by flow cytometry. (A) Representative flow cytometric profiles. (B) The quantity of cells in different cell-cycle phase. Data shown represent experiments performed in biological triplicates. *$p<0.05$, **$p<0.01$ evo, ber, evo+ber vs. con; #$p<0.05$, ##$p<0.01$ evo+ber vs. evo, $$p<0.05$, $$$$p<0.01$ evo+ber vs. ber.*

Table I. *The CI of Evo 15 combination with different concentrations of Ber (µM).*

Group	Evo+Ber 3.125	Evo+Ber 6.25	Evo+Ber 12.5	Evo+Ber 25	Evo+Ber 50	Evo+Ber 100
CI	1.208	1.178	1.418	0.75	0.834	1.079

Table II. *The CI of Ber 25 combination with different concentrations of Evo (µM).*

Group	Ber+Evo 3.75	Ber+Evo 7.5	Ber+Evo 15	Ber+Evo 30	Ber+Evo 60	Ber+Evo 120
CI	2.586	1.536	0.75	0.966	0.75	0.525

Effects of berberine and evodiamine on the levels of p21, p27, cyclin D1, cyclin E, and CDK 2, 4, 6 in MCF-7 cells. In mammalian cells, replication is regulated in an orderly fashion from G_1 to S to mitosis by phase-specific oscillations in the level of Cyclins, Cyclin-dependent protein kinases (CDKs), and CDK inhibitors. Cell-cycle progression is accelerated by cyclins and CDKs, and decelerated by CDK inhibitors (such as p16, p21 and p27), p53, retinoblastoma tumor suppressor protein (RB), and ARF. The D-type cyclins (cyclin D1, D2 and D3) are involved in regulation of the
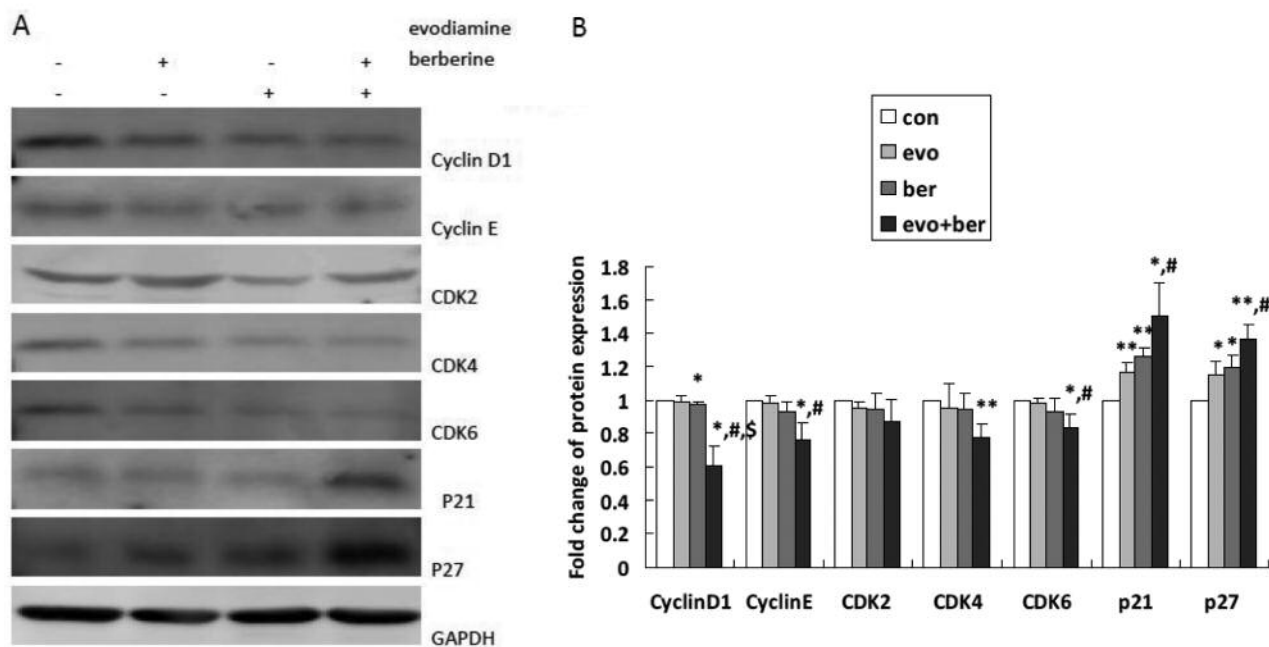
Figure 5. *The expression levels of p21, p27, Cyclin D1, Cyclin E, CDK2, CDK4, and CDK6 as affected by 25 μM berberine (ber), 15 μM evodiamine (evo), or their combination on MCF-7 cells. (A) MCF-7 cells were treated with ber, evo, or ber plus evo for 48 h. The densitometric ratios of the indicated protein bands to GAPDH are shown as relative expression. The values are expressed as mean values±SD, n=3. *p<0.05, **p<0.01, evo, ber, evo+ber vs. con. #p<0.05, evo+ber vs. evo; $p<0.05, evo+ber vs. ber.*

G1/S transition. Their crucial function is to activate CDK4 and CDK6. (9-11) To elucidate the mechanisms underlying the cell-cycle arrest induced by the combination berberine and evodiamine in MCF-7 cells, we performed immunoblot analysis to evaluate p21/Waf1, p27- cell cycle inhibitors, cyclin D1, cyclin E, cyclin-dependent protein kinase 2 (CDK2), CDK4 and CDK6. The p21 and p27 levels increased after combination treatment. In contrast, the levels of cyclin D1, cyclin E, CDK4 and CDK6 decreased, whereas the combination treatment did not affect CDK2 expression ($p>0.05$) (Figure 5).

Effects of berberine and evodiamine on the levels of p53, Bcl-2, Bax, caspase-7, -8, -9 and PARP in MCF-7 cells. To determine which proteins are regulated by the compound combination, we analyzed the protein levels of caspase-9, caspase-8, caspase-7, PARP, the Bcl-2 family including Bax and Bcl-2. As shown in Figure 6, caspase-9, caspase-7 and PARP cleavage products were observed upon treatment with the drug combination at 48 h in MCF-7 cells. The level of Bcl-2 decreased by the combined drug treatment at 48 h. In contrast, the levels of Bax and p53 in MCF-7 cells increased by the combined drug treatment at 48 h, as compared to the control treatment. However, the combination treatment did not affect caspase-8 expression ($p>0.05$). Apoptosis occurs either through the extrinsic pathway or the intrinsic pathway.

Efficacy of berberine and evodiamine on human breast cancer MCF-7 xenografts. To evaluate the anti-tumor effects of combined berberine and evodiamine *in vivo*, the experimental MCF-7 human breast cancer xenografts in nude mice were established to evaluate treatments with berberine (10 mg/kg), evodiamine (10 mg/kg), and their combined application. Mice with visible subcutaneous tumors were divided randomly into 4 groups. The tumor volume and body weight were measured every week. The average body weights of the control, evodiamine-, berberine- and combination-treated mice did not differ significantly throughout the study period (Figure 7A). Movement, indigestion, and swelling appeared normal among the 3 groups of treated mice. When the experiment was terminated after 6 weeks, the tumor volumes and weights were significantly reduced by the combination treatment, compared with berberine or evodiamine treatments alone (Figures 7B and D).

Discussion

The treatment of cancer requires treatments with similar levels of complexity as its complex etiology (23). TCM formula-based combinations of natural products provide this type of complexity as their constituents elicit therapeutic effects *via* multi-targeted mechanisms to achieve synergistic efficacy (24). In this study, the combination treatment
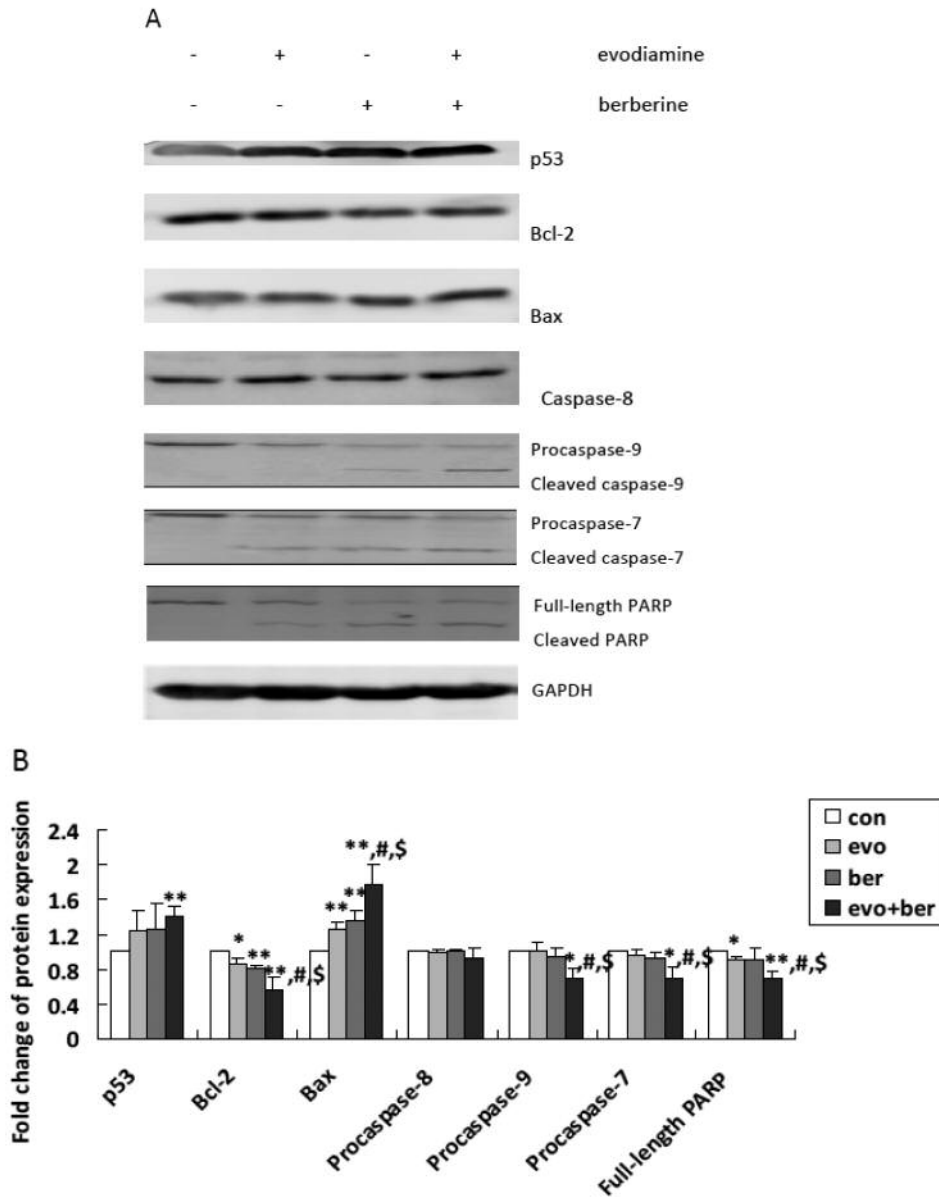
Figure 6. *The expression levels of p53, Bcl-2, Bax, caspase-8, caspase-9, Caspase-7, and PARP as affected by 25 μM berberine (ber), evodiamine (evo) 15 μM, and their combinatorial application. MCF-7 cells were treated with ber, evo, or ber plus evo for 48 h. The densitometric ratio of proteins relative to GAPDH is shown as relative expression values. Values are expressed as mean values±SD, n=3. *p<0.05, **p<0.01 evo, ber, evo+ber vs. administration; #p<0.05, evo+ber vs. evo; \$p<0.05, evo+ber vs. ber.*

investigated was synergistic and significantly inhibited cell growth of human breast cancer MCF-7 cells by inducing cell-cycle arrest and inducing apoptosis. This synergistic method showed particular effectiveness in targeting different cell death-inducing processes.

As shown in Figure 8, the combination of berberine and evodiamine induced cell-cycle arrest at G_1 with multiple-target actions through up-regulation of p21, p27, and p53 and

down-regulation of cyclin D1, cyclin E, CDK4, and CDK6. Our results suggest that caspase-9, caspase-7, and PARP are activated by combination treatment of berberine and evodiamine. This study showed that the down-regulation of Bcl-2 and up-regulation of Bax were triggered by the combination of berberine and evodiamine but not to treatment with berberine or evodiamine alone. The levels of p53 were up-regulated by the combined drug treatment,
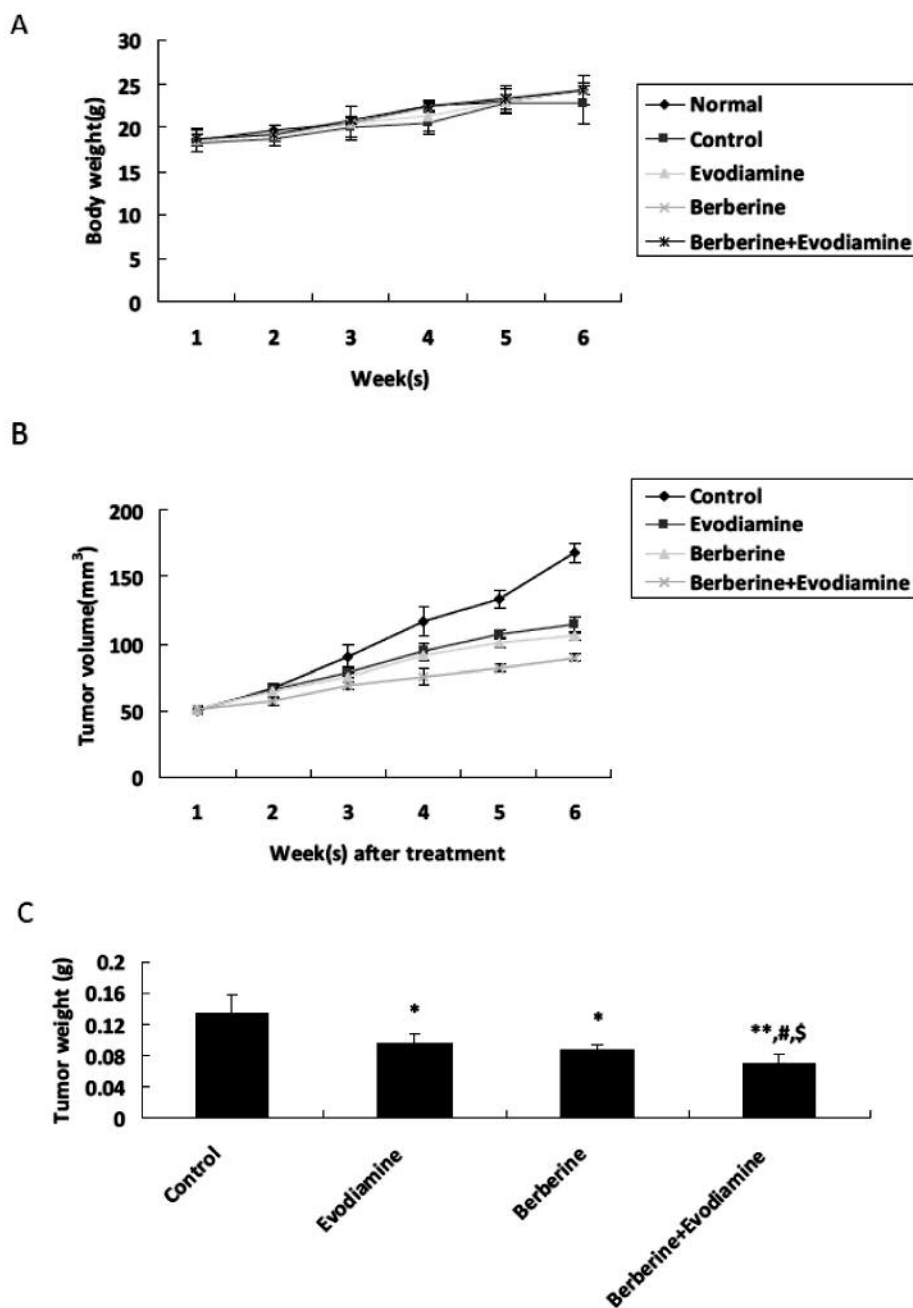
Figure 7. *The body weight (A) and tumor growth curves (B) of 4 treatment and normal groups during 6 weeks (n=5). (C) Tumor weights of the 4 treatment groups are shown. Values are expressed mean values±SD. *p<0.05, **p<0.01, evo, ber, evo+ber vs. Model; #p<0.05, ##p<0.01, evo+ber vs. evo; $p<0.05, evo+ber vs. ber.*

suggesting that p53 is involved in the compound combination-induced apoptotic process.

Synergistic drug combinations have been explored to achieve one or more favorable outcomes, such as enhanced efficacy, decreased dosage at an equal or increased level of efficacy (25). Previously, berberine was evaluated for its ability to sensitize cancer cells to the anti-cancer effects of doxorubicin (26). In another study, evodiamine augmented the therapeutic efficacy of gemcitabine (27). In this study, 25 μmol/l berberine or 15 μmol/l evodiamine alone resulted in
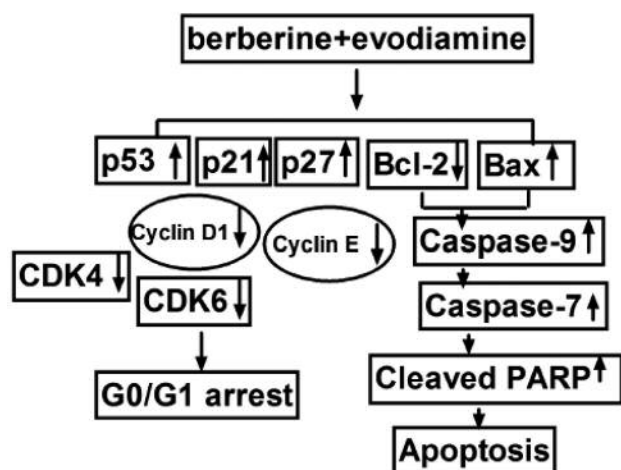
Figure 8. *A schematic diagram illustrating the molecular mechanism underlying how berberine (ber) and evodiamine (evo) inhibit MCF-7 breast cancer cell proliferation. The mechanisms of ber and evo might involve the expression of cyclins, CDKs, Bcl-2 and the activation of the caspase-9 apoptotic pathway, leading to reduced cell proliferation.*

moderate or no changes in the expression of proteins related to cell-cycle arrest or induction of apoptosis. However, the same doses of the combination treatment elicited synergistic effects on the up-regulation or down-regulation of these proteins, effectively inhibiting cell growth.

Conclusion

Through this study, the combination of berberine and evodiamine, which are found in the TCM formula Zuo-Jin-Wan, was used to treat MCF-7 cells *in vitro* and *in vivo*. This combination treatment acts synergistically against the human breast cancer MCF-7 cells by inducing cel-cycle arrest and apoptosis. These promising results suggest that the TCM formula-based combination of berberine and evodiamine exhibits significant anti-breast cancer activity and demonstrated that TCM formulas might represent valuable resources for the future study of synergistic natural product treatments for cancer.

Conflicts of Interest

The Author (s) declare that they have no competing interests.

Acknowledgements

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