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*Full Length Research Paper*

# Chloroform extract of Tibetan herbal medicine *Dracocephalum tanguticum* Maxim. inhibits proliferation of T98G glioblastomas cells by modulating Caspase-3 cleavage and expression of Bax and p21

**Xinyan Wang, Jinxiu Xu, Mu Yang and Hong Zhou***

School of Life Science and Technology, University of Electronic Science and Technology of China, Chengdu 610054, China.

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**A chloroform extract of *Dracocephalum tanguticum* Maxim. (CEDtM) was obtained and phytochemical analysis showed that this extract contained high content of sapogenin or saponin (53.7%). Subsequently, the anticancer potential of this CEDtM was investigated in T98G glioblastomas cells. Results showed that CEDtM with the dose of 90 μg/ml efficiently induced cytotoxicity in T98G cells but had little cytotoxic effect on mouse primary fibroblast cells. The T98G cells treated with CEDtM showed the features of apoptosis as cell-volume shrinkage, cell blebbing, cell detachment and apoptotic bodies. Moreover, treatment of CEDtM stimulated Caspase-3 cleavage and the expression of proapoptotic protein Bax in T98G cells. Interestingly, CEDtM inhibited the expression of p21 protein, a cell cycle inhibitor, with induction of T98G apoptosis. These findings suggest that CEDtM selectively inhibits T98G glioblastomas cell proliferation by induction of cell apoptosis via Caspase-3 and Bax pathways, along with an inhibition of p21.**

**Key words:** Chloroform extract of *Dracocephalum tanguticum* Maxim., apoptosis, T98G cells.

## INTRODUCTION

Grade IV gliomas, also known as glioblastomas, account for more than 51% of all gliomas which are the most common brain tumors in central nervous system neoplasm (Adamson et al., 2009). Glioblastomas are very aggressive with a highly invasive capacity and often infiltrate critical neurological areas within the brain. Accordingly, in addition to their high resistance to radiation therapy and chemotherapy (Galanis and Buckner, 2000; Hosli et al., 1998), the overall survival of patients with newly diagnosed glioblastomas is 17 to 30% at 1 year, and only 3 to 5% at 2 years (Adamson et al., 2009). So, the new therapeutic strategies and new agents for glioblastomas therapy need to be explored.

Natural products are excellent sources of new drugs for the treatment of human diseases. This was particularly evident in the area of cancer, in which over 60% of drugs for cancer therapy are of natural origin (Newman et al., 2003). Accordingly, a new natural source with anticancer activity and low side effect would be a valuable tool in cancer therapy. Saponins, glycosides in plant, are one kind of nature products which showed the favorable anti-tumorigenic properties. Several saponins inhibit tumor cell growth both *in vivo* and in *vitro* via cellular and systematic mechanisms. Moreover, some combinations of saponins and anti-tumorigenic drugs induce synergistic effects with potentiated tumor growth inhibition (Bachran et al., 2008). On the cellular level, saponins function in tumor therapy via multiple pathways including reduction of invasiveness, induction of cell apoptosis and cell cycle arrest, reduction of inflammatory responses and

*Corresponding author. E-mail: zhouhongzh@uestc.edu.cn. Tel: +86 28 83206437. Fax: +86 28 83208238.

**Abbreviations: CEDtM,** Chloroform extract of *Dracocephalum tanguticum* Maxim.; **Bax,** Bcl2-associated X protein; **WST-8,** 4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H -5-tetrazolio]-1,3-benzene disulfonate sodium salt; **CCK-8**, cell counting kit-8; **DMSO,** dimethylsulfoxide.

induction of detoxifying and antioxidant protein and inhibition of drug efflux (Bachran et al., 2008; Rao and Sung, 1995). However, a number of pathways are observed only for certain cell lines and certain saponins (Bachran et al., 2008).

*Dracocephalum tanguticum* Maxim., a member of the Labiatae family, is a traditional Tibetan herbal medicine and distributes widely on the Qinghai-Tibet Plateau of China. It is used to treat hepatitis, gastritis, dizziness, arthritis and ulcer, under the name of “priyangku” in Tibetan (Qupei et al., 2002; Zhao et al., 2006). Phytochemical analysis of this herb showed that its chemical components include multiple triterpenoids (e.g. oleanolic acid (OA) and ursolic acid (UA)), steroids, and flavonoids (Li and Jia, 2006; Zhang et al., 1994). Biological functions of *D. tanguticum* Maxim. extract were also analyzed by several studies, showing that it enhances the activity of superoxide dismutase and has an anti-hypoxia effect in mice (Hai et al., 1997). In the present study, a chloroform extract of *D. tanguticum* Maxim. (CEDtM) was prepared and the content of total saponins in CEDtM was determined by phytochemical analysis. The effects of CEDtM on the proliferation of T98G glioblastomas cells were also investigated. Moreover, the effects of CEDtM on Caspase-3, Bax and p21 protein expression were examined in T98G cells.

## MATERIALS AND METHODS

### Animal and materials

*D. tanguticum* Maxim. was collected from Ganzi, Sichuan province, China and identified by Prof. Yi Zhang (Sichuan Academy of Chinese Medicine Science). Methanol, ethanol and chloroform were obtained from Chengdu Kelong Chemical Reagent Factory (Chengdu, China). BCA reagent Kit and CCK-8 kit were the products of Beyotime Institute Biotechnology (Haimen, China). Antibodies for Bax, Caspase-3, p21 and β-actin were obtained from Boster Biological Technology, Ltd. (Wuhan, China). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was from Beijing ZSGB-BIO Co. (Beijing, China). Lumi-Light Western blotting substrate and Amersham Hyperfilm ECL were purchased from Roche Diagnosis Co. (Mannheim, Germany) and GE Life Science (Piscataway, NJ, USA), respectively. All other chemicals were of analytical grade. Healthy Kunming mice were purchased from the Experimental Animal Center of Sichuan University (Chengdu, China). Animal care and experimental procedures were approved by the Animal Ethical Committee at University of Electronic Science and Technology of China.

### Preparation of CEDtM

The whole dried aerial part of *D. tanguticum* Maxim. (2.50 kg) was pulverized and extracted with 95% ethanol at room temperature for seven days each and total four times. The extract solution was concentrated and evaporated to dryness as crude sample. The crude sample (50.01 g) was suspended in water by the proportion of 1:20 (w/v) and partitioned successively with chloroform. The chloroform fraction was concentrated at 45°C by rotary evaporator and further evaporated the solvent in vacuo to give chloroform-soluble fraction/CEDtM and stored at 4°C for later use.

### Determination of the content of total saponins

The contents of total saponins in the pulverized herb, crude sample and CEDtM were measured using vanillin-acetic acid method (Chen et al., 2007; Wu et al., 2001). Briefly, *D. tanguticum* Maxim. samples were incubated reflux with 2% hydrochloric acid-ethanol at 80°C for 3 h and OA was dissolved in methanol as a standard. The samples were evaporated to dryness and mixed with 5% of vanillin-acetic acid (0.2 ml), followed by 0.8 ml of perchloride acid. Then, the mixtures were incubated at 60°C for 15 min and cooled down by cold water. Finally, 5 ml of absolute acetic acid was added into each tube and the samples were settled down for 30 min at room temperature. Absorbance at 535 nm was measured in 1 h. All experiments were preformed in triplicate. The concentration of total saponins in the pulverized herb, crude sample and CEDtM was determined as milligram of OA equivalents per one gram of each sample.

### Mouse primary fibroblast cell preparation

Primary fibroblast cell was prepared from mouse femurs bone marrow referred to Rahimi’s method (Rahimi et al., 2005). Briefly, mice were killed and the femurs were dissected out. Bone marrow cells in the femurs were collected and maintained in tissue-culture flasks with RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in humidified 5% $CO_2$. The medium was changed to fresh medium after 24, 48 and 72 h, and the debris and nonadherent cells were thereby removed. Cells were replenished with fresh medium every 3 days until they reached confluence. Primary fibroblasts obtained by this method showed the typical morphology of fibroblast cells.

### Cell proliferation assay

Cell proliferation was assessed by a CCK-8 assay. Briefly, T98G or mouse primary fibroblast cells (6000 cells/well) in 200 μl of RPMI-1640 medium with 10% FBS were seeded in 96-well plates. After incubation for 24 h, cells were treated with different concentrations of CEDtM or vehicle, for 24 to 72 h. The morphology of T98G cells was observed and the photos were taken by using the phase-contrast microscopy during drug incubation. After drug administration, 20 μl of WST-8 solution (Beyotime) was added to each well and the plates were further incubated for 3 h at 37°C. WST-8 reagent can be reduced by dehydrogenases to give a yellow-colored formazan and the amount of the formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells. Since the peak absorbance of WST-8 formazan is at about 450 nm, the cell viability was evaluated by measuring the absorbance at 450 nm with the reference at 603 nm by a microreader (Bio-Rad, San Diego, CA, USA). All the measurements were performed in quadruplicate.

### Western blot analysis

Whole cell lysates of T98G cells were prepared for Western blot analysis. Cell culture media were removed and the cells were washed with 1 ml of ice cold phosphate-buffered saline and lysed by radio immunoprecipitation assay (RIPA) buffer supplemented with proteinase inhibitors. Cells were scraped into centrifuge tubes and sonicated on ice. Cell lysates were centrifuged at 12,000 g for 10 min at 4°C and the supernatant was collected. Seventy microgram of total protein was separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 120 V for about 1.5 h. Protein samples were transferred to PVDF membrane (Millipore, Billerica, MA, USA) using a semi-dry transfer apparatus (Bio-Rad).

**Table 1.** Content of total saponins in the herb, crude sample and CEDtM.

| | Total saponins (mg/g) |
|---|---|
| Herb | 35.9±0.1 |
| Crude sample | 274.9±2.3 |
| CEDtM | 537.0±2.8 |

Total saponins content was determined using colorimetric method and expressed as milligram of OA equivalents per one gram of each samples. The experiments were performed in triplicates (n=3).

After incubation with 5% (w/v) non-fat milk in TBS (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.4) for 2 h at room temperature, the membrane was incubated with rabbit anti-Bax, anti-Caspase-3 (p12) and anti-p21 (Boster) polyclonal antibody at 1:400 dilution at 4°C, separately. After a brief washing, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (ZSGB-BIO) at 1:10,000 dilution with gentle agitation for 1 h at room temperature. Signals were detected using Lumi-Light Western blotting substrate kit (Roche) and visualized after exposure to an Amersham Hyperfilm ECL film (GE). After that, the films were scanned and the relative protein levels of activated capspase-3, Bax, p21 and β-actin were quantified using Image J software (http://rsb.info.nih.gov/ij/index.html).

#### Statistical analysis

In cell proliferation assay, cell viability was expressed as the percentage of the mean value of control group (as "% Ctrl"). Data presented (Mean±SEM) were analyzed by one-way or two-way ANOVA followed by a Student's t-test for comparison between two groups. Differences were considered statistically significant at $P < 0.05$. In Western blotting assay, data were analyzed by one-way ANOVA followed by a Student's t-test for comparison between two groups. The results were considered significant if the P value was less than 0.05.

## RESULTS

### Determination of the content of total saponins

About 2.5 kg of whole dried aerial part of *D. tanguticum* Maxim. was pulverized and extracted by 95% ethanol and 50.01 g of crude sample were obtained. With the subsequent extraction with chloroform, total 26.74 g of CEDtM was obtained for the following experiments.

The content of total saponins in the pulverized herb, crude sample and CEDtM was analyzed and expressed as milligram per gram of samples. The content of total saponins was significantly increased from 35.9±0.1 mg/g in the herb to 274.9±2.3 mg/g in the crude sample and to 537.0±2.8 mg/g in CEDtM (Table 1).

### Inhibitory effects of CEDtM on T98G cell proliferation

The cell viability of T98G cells was examined by CCK-8 assay. CEDtM reduced cell viability in a time- and dose-dependent manner (Figure 1). After incubation for 48 h, CEDtM at the concentrations of 90 and 270 μg/ml decreased cell viability to 63.18±2.40% and 30.25±0.69%, respectively (Figure 1A). However, administration of CEDtM with the doses of 10, 30 and 90 μg/ml separately for 48 h had no significant effect on the viability of mouse primary fibroblast cell (Small inset in Figure 1A), which is used here as normal cell model. In the presence of 90 μg/ml of CEDtM, cell viability was reduced to 60.76±2.31% and 31.62±1.49% at 48 h and 72 h of incubation, respectively (Figure 1B).

### CEDtM induced cell morphological change in T98G cells

The morphology of T98G cells treated with CEDtM was observed by phase-contrast microscopy. The cells treated by 30 μg/ml of CEDtM for 72 h and treated by 90 μg/ml of CEDtM for 48 h showed the characteristic features of apoptosis as cell-volume shrinkage, cell blebbing, cell detachment and apoptotic bodies compared with the adherent and spindle-shaped control cells (Figure 2). These cells were considered apoptotic since their morphological changes are the characteristic features of apoptotic T98G cells (Karmakar et al., 2006; Karmakar et al., 2007).

### Effects of CEDtM on Bax, Caspase-3 and p21 expression in T98G cells

Since CEDtM significantly decreased the viability of T98G cells, it was of interest to examine some cellular proteins that are related to cell apoptosis or cell cycle. Caspase-3, a key factor in cell apoptosis, is one of important functional enzymes in the pathway of cellular apoptotic signal which can be activated by enzymatic cleavage on procaspase-3. Procaspase-3 is expressed in cells as a 32-kDa protein from which a 17-kDa (p17) and a 12-kDa (p12) subunits of the mature Caspase-3 are generated by enzymatic cleavage during apoptosis (Cohen, 1997). Examination of the expression of Caspase-3 protein showed that 90 μg/ml of CEDtM increased the active form of Caspase-3 (12 kDa) after incubation for 48 h (Figure 3). Bax, a proapoptotic protein of Bcl-2 family, is essential for the completion of apoptosis (Lalier et al., 2007). In the present study, CEDtM stimulated Bax protein expression at the concentration of 90 μg/ml after

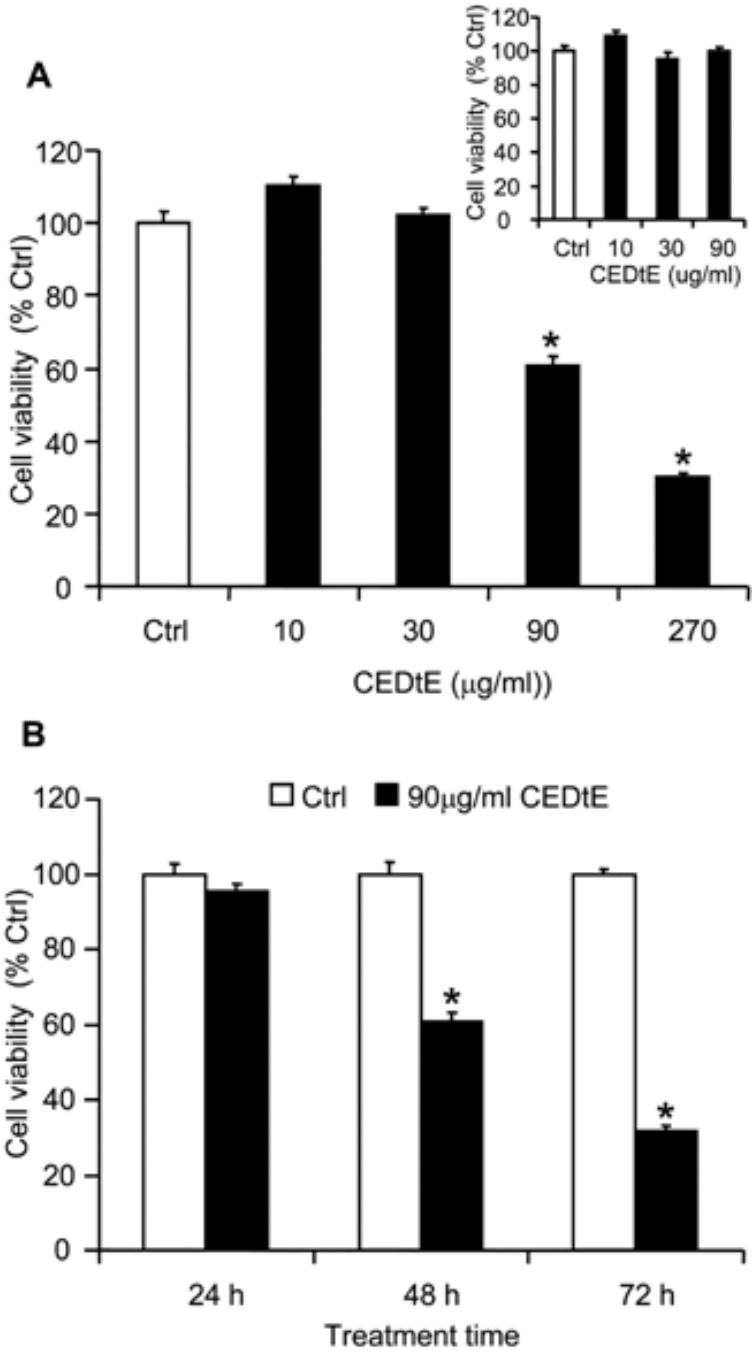


**Figure 1.** Effects of CEDtM on the proliferation of T98G glioblastomas cells. Cells were incubated for 48 h with different concentrations (A, 0, 10, 30, 90 and 270 μg/ml) of CEDtM or with 90 μg/ml of CEDtM for different times (B, 24, 48 and 72 h). Small inset in A showed the effects of CEDtM on mouse primary fibroblast cells. Cell viability was examined by CCK-8 assay. Data presented are expressed as Mean±SEM (n=4) and groups denoted by stars represent a significant difference at $P<0.05$ versus the control group.

treatment for 48 h (Figure 3). Expression of p21 protein, a universal inhibitor of cyclin-dependent kinases (Xiong et al., 1993), was also examined by Western blot. In this case, p21 expression in T98G cells was inhibited by CEDtM with the concentration of 90 μg/ml after treatment for 48 h (Figure 3).

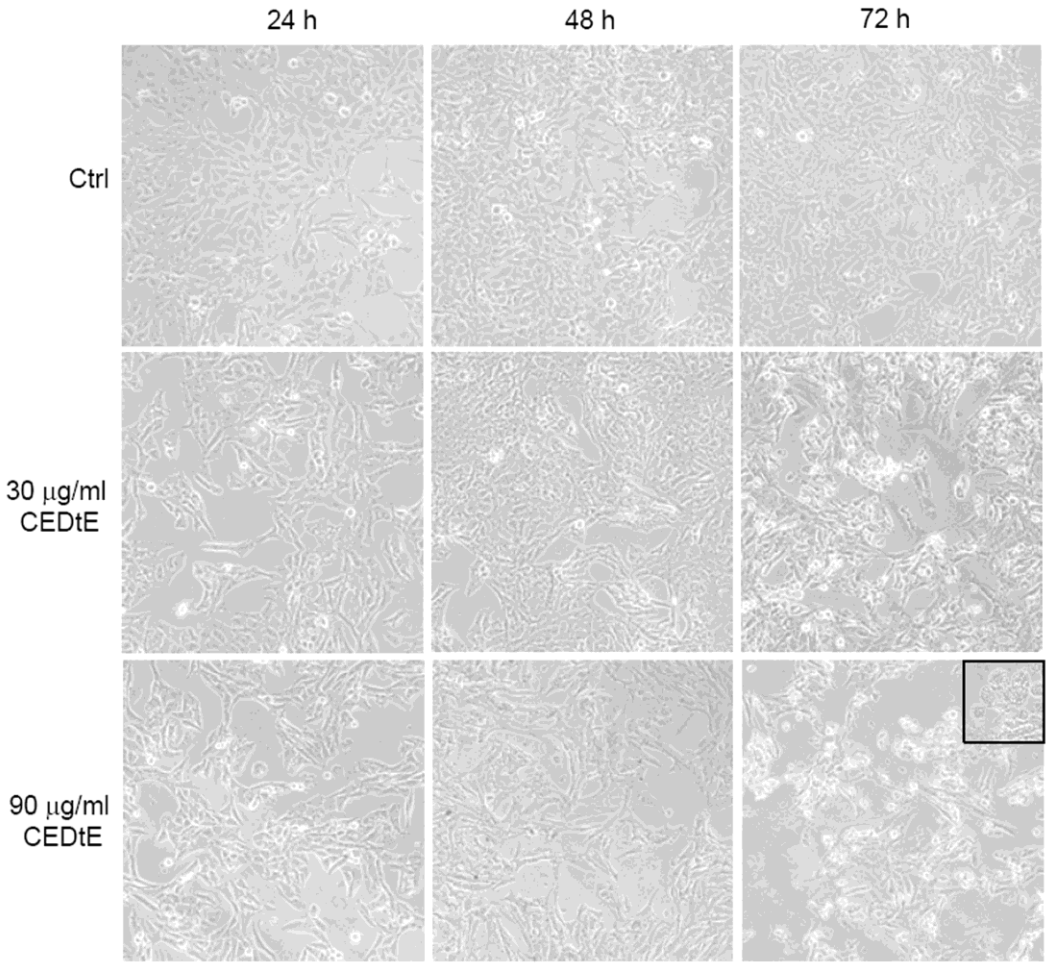


**Figure 2.** CEDtM induced morphological change in T98G cells. The morphology of T98G cells was observed by phase-contrast microscopy. Cells were cultured in 96-well plate and treated with different doses of CEDtM (0, 30 and 90 μg/ml) for different times (24, 48 and 72 h). Photomicrographs were taken in 96-well plate under phase-contrast microscopy. All photos were taken at ×100 magnification except the small inset at ×200 magnification.

## DISCUSSION

*D. tanguticum* Maxim. is a Tibetan herbal medicine which is a member of the Labiatae family. In this family, saponins and triterpenoid sapogenin were isolated and identified from various plants (Fan et al., 2002; Lee et al., 2008; Zhang et al., 2008). Among these studies, consistent with the cytotoxic effect of CEDtM, cytotoxic activities of saponins and triterpenoic acids from *Craniotome furcata* and *Prunella vulgaris var. lilacina*, respectively, were revealed (Fan et al., 2002; Lee et al., 2008). It has also been reported that chloroform fractions were more effective to inhibit cancer cell proliferation and lower cytotoxic to a normal liver cell line than other fractions among different fractions of some herbs which belong to the Labiatae family (Cha et al., 2004; Chan et al., 2006; Wang et al., 2010; Yu et al., 2007). Thus, we used organic solvent, chloroform, to enrich the active components in *D. tanguticum* Maxim. during the extraction. The phytochemical analysis showed that the content of total saponins in chloroform-soluble fraction of *D. tanguticum* Maxim. was 537.0±2.8 mg/g (Table 1). As we described previously, saponins has anti-tumor effects through various pathways dependent on the type of the cells and saponins (Bachran et al., 2008; Rao and Sung, 1995), so it is likely that saponins contributed at least partially to the growth inhibitory effect of CEDtM on T98G glioblastomas cells.

Treatment with CEDtM induced the morphological features of apoptosis in T98G cell, similar to that in other studies characterized as the small retracted cell bodies and thin elongated cytoplasmic processes (Khalid et al., 1999) or shrinkage of the cells, rounding up of the cell body and detachment from the bottom (Zupanska et al., 2005). Cells treated with 90 μg/ml of CEDtM for 48 h showed small retracted cell bodies and thin cytoplasm

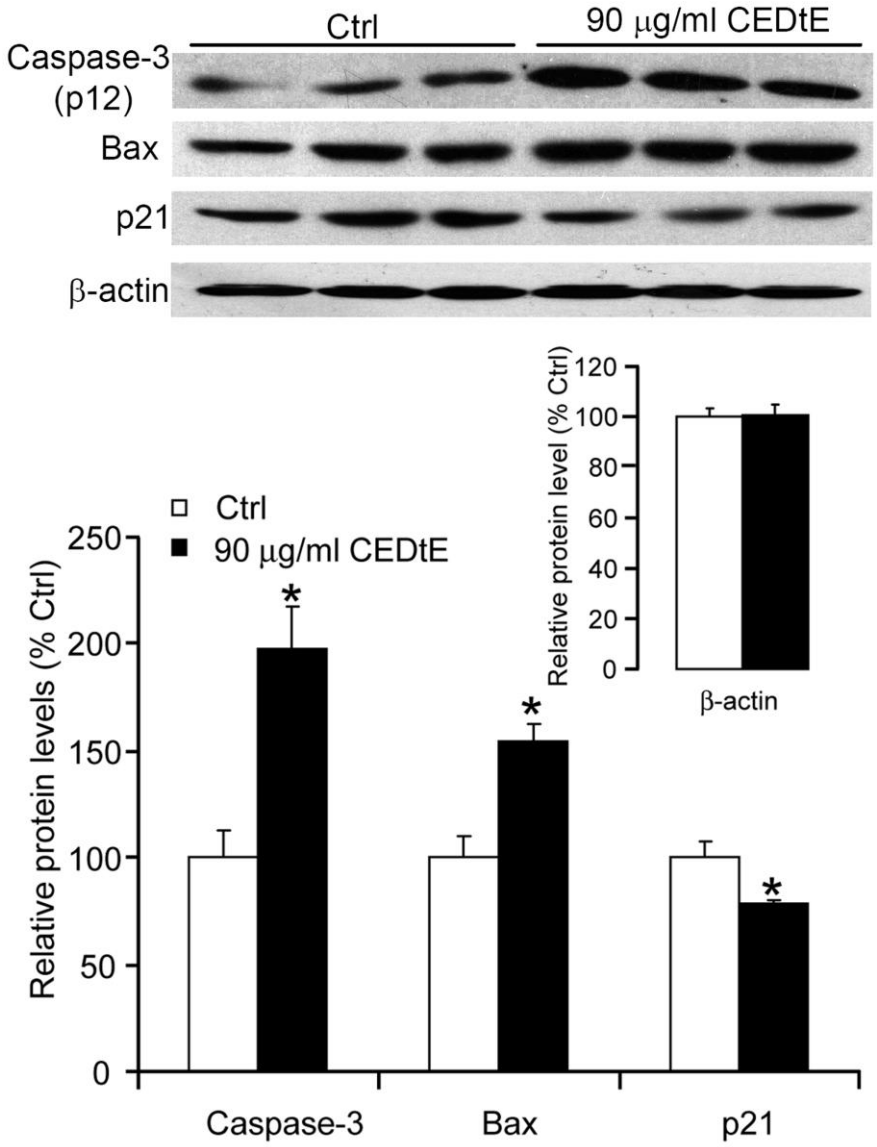


**Figure 3.** Effects of CEDtM on the activation of Caspase-3 and expression of Bax and p21 protein in T98G glioblastomas cells. Upper panel: Western blotting of Caspase-3, Bax and p21 protein. β-actin was conducted to serve as an internal control. Lower panel: Statistic analysis of Caspase-3, Bax, p21 and β-actin protein levels. The raw data for the immunoreactivity of the target proteins were quantified in terms of "arbitrary density unit" by using Image J software and transformed to a ratio of control samples with no treatment. Groups denoted by stars showed a significant difference at $P<0.05$ versus control group.

compared the cells in control group. In cells treated with 30 and 90 μg/ml of CEDtM for 72 h, cell detachment and apoptotic bodies were observed (Figure 2), indicating that CEDtM was effective to induce T98G cell apoptosis.

To quantify the ability of CEDtM to induce T98G cell apoptosis, the cell viability was determined by WST-8 assay. In the present study, 90 μg/ml of CEDtM efficiently inhibited T98G cell proliferation and had little effect on mouse primary fibroblast cells. CEDtM (90 μg/ml) showed similar efficiency in cell proliferation inhibition by some crude extracts from other members of the Labiatae family (Russo et al., 2009; Tayarani-Najaran et al., 2009; Yin et al., 2004). Since CEDtM with the concentration of 90 μg/ml had no cytotoxic effect on the primary fibroblast cells, suggesting that this extract has cancer cell-specific selectivity.

The cysteine proteases, called caspase, are important mediators of apoptosis in which Caspase-3 is the main executioner caspase in apoptotic process since Caspase-3 is thought to be tightly linked to the final events in the

execution cell death program via specific and extensive apoptotic DNA fragmentation (Porter and Janicke, 1999). Caspase-3 is synthesized as pro-caspase-3 protein with the size of 30 KDa and activated by cleavage to p12 subunit and a 20-kDa (p20) peptide (Han et al., 1997). Thus, CEDtM enhanced the level of Caspase-3 (p12) (Figure 3), suggesting this drug could stimulate Caspase-3 activation.

Bax belongs to the Bcl-2 family which functions as a "life/death switch" that integrates diverse inter- and intracellular cues to determine whether or not the stress apoptosis pathway should be activated (Adams and Cory, 2007). Bax mediates pore formation in the outer mitochondrial membrane for the release of cytochrome-*c* into cytosol from the mitochondria. This event is considered the key regulatory step irreversibly committing cells to apoptosis in most cases of intrinsic apoptosis (Kumarswamy and Chandna, 2009). In the present study, CEDtM stimulated Bax protein expression, indicating that CEDtM induced T98G cell apoptosis via intrinsic pathway.

p21 is the founding member of the Cip/Kip family of Cyclin-dependent Kinase Inhibitors, which inhibit a broad range of cyclin/Cdk complexes and negatively regulate cell cycle progression. It can synergize with other tumor suppressors to protect against tumor progression in mice. CEDtM inhibited p21 expression in T98G cells and we can deduce that inhibition of p21 expression attenuates its inhibition on growth arrest or cell proliferation. It seems that this deduction is controversial to the previous conclusion that CEDtM can induce T98G cell apoptosis. In fact, in addition to being an inhibitor of cell proliferation, p21 also acts as an inhibitor of apoptosis in a number of systems (Gartel and Tyner, 2002). In multiple gliomas cells, p21 facilitates active cyclin-Cdk complex formation and induces cell proliferation (Besson and Yong, 2000) and blockage of p21 protein expression by antisense oligonucleotides results in prominent sensitization to CD95-mediated mitochondrial cytochrome-*c* release and apoptosis. These are consistent with the results that CEDtM inhibited p21 expression, and simultaneously stimulated Bax (the mediator of pore formation in the outer mitochondrial membrane) and induced T98G cell apoptosis. This phenomenon was also revealed in HT1080 cells, in which triptolide (20 ng/ml), an natural product from the Chinese herb *Tripterygium wilfordii*, inhibited p21 expression and induced this cell apoptosis (Chang et al., 2001). However, the mechanisms in these responses are still undetermined (Gartel and Tyner, 2002).

## Conclusion

We obtained a saponin-rich fraction by 95% ethanol and chloroform extraction from *D. tanguticum* Maxim. This saponin-rich extract displayed significant anti-proliferation properties in T98G glioblastomas cells. This cytotoxic activity was associated with the Caspase-3 activation and increase of Bax expression. Interestingly, inhibition of p21, a cyclin-dependent kinase inhibitor, by saponin-rich fraction from *D. tanguticum* Maxim. was also revealed in T98G glioblastomas cells.

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