

Molecular mechanisms underlying the α -tomatine-directed apoptosis in human malignant glioblastoma cell lines A172 and U-118 MG

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Abstract. In the present study, the molecular mechanisms involved in the α -tomatine-induced apoptosis in human glioblastoma cell lines A172 and U-118 MG were investigated. Wright staining and ApopTag assays were conducted to confirm the apoptosis induced by α -tomatine treatment. Fura-2 assay determined an enhancement in free Ca^{2+} intracellularly, indicating the occurrence of Ca^{2+} -dependent apoptosis induction. Western blot experiments were also performed to predict the apoptosis by measuring the changes in the Bax:Bcl-2 ratio. Increase of calpain activity triggered caspase-12 expression, which in turn further activated caspase-9. In addition, an increase in the ratio of Bax:Bcl-2 accounted for the mitochondrial release of cytochrome *c* into the cytosol for caspase-3 and caspase-9 activation. Elevated activity of calpain and caspase-3 yielded spectrin breakdown products with 145 and 120 kDa, respectively. Caspase-3 activation further cleaved the inhibitor of caspase activated DNase, while the apoptosis-inducing factor detected in the cytosol suggested that apoptosis was independent of caspase. The apoptosis induction was further supported by decreased expression levels of nuclear factor- κ B and increased expression of the inhibitor of nuclear factor, I κ B α . In conclusion, the presented experimental results revealed the stimulation of different molecular mechanisms for α -tomatine-mediated apoptosis in A172 and U-118 MG human glioblastoma cell lines.

Introduction

Glioblastoma is the most widespread and malignant brain tumor, and is often irremediable. This tumor has high mortality rate due to low response to the currently available treatments, resulting in short survival time that may be <1 year between diagnosis and mortality (1). A number of therapies are

available for the treatment of glioblastoma, including surgery, chemotherapy, photodynamic therapy, gene therapy, treatment with traditional Chinese medicine, radiotherapy, heat therapy and immune therapy (2-4). However, these therapies often have insufficient efficacy in curing glioblastoma due to its unusual appearance, powerful invasion and resistance towards chemotherapy. Furthermore, the administration of temozolomide, an alkylating compound, for cancer treatment in patients is limited (met with resistance) due to increased activity of the DNA repair enzyme O⁶-alkylguanine-DNA alkyltransferase enzyme (5). Metastatic cancer cell growth and the high resistance of cells to conventional treatments generate difficulties in complete eradication of the disease. Therefore, novel therapies are essential to defeat these limitations of conventional treatments. Furthermore, the clarification of mechanisms underlying the occurrence and development of gliomas is also essential, since it can contribute towards novel successful treatments.

In the field of cancer research, the evolution of food-derived chemopreventive compounds has been receiving increasing attention. For instance, the use of phytochemicals is an encouraging and contemporary approach for the prevention of cancer and its treatment (6). In addition, traditional herbal therapy may be considered as a favorable substitute for modern medicine (6). Previous studies have been conducted to examine the anti-invasive and antitumor properties of different herbal compounds (7). Researchers identified reduced occurrence of side effects for these herbal components when compared to chemotherapeutic agents. Prior to initiation of expensive clinical trials, *in vitro* evaluation and other studies can be conducted to produce easy, quick and satisfactory data (8).

Plant-based compounds have served important roles in regulating the human health and improving the quality of life for several years (9,10). α -Tomatine, isolated from *Solanum* plants such as *Solanum lycopersicon* L., is a natural steroidal glycoalkaloid found in the leaves, stems and roots of tomatoes. α -Tomatine consists of six-ring steroidal aglycone tomatidine by which two glucose units, xylose and galactose moieties of a tetrasaccharide unit, are linked to the 3-hydroxyl group of aglycone. Different types of tomatine, such as β -tomatine, γ -tomatine and δ -tomatine, are generated by the partial hydrolysis of α -tomatine that leads to the loss of different sugar units (11). α -Tomatine provides protection against pathogenic bacteria, fungi and viruses in plants (12).

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On evaluating the mechanistic effect of α -tomatine, it has been proven that tomatine alters essential signaling pathways involved in the maintenance of cell proliferation, migration and differentiation (13,14). Inhibition in the growth of human liver cancer HepG2 and human colon cancer HT29 cell lines was observed following *in vitro* α -tomatine treatment, and the results were found to be more effective compared with those of standard anticancer drugs, such as camptothecin and doxorubicin (15,16). In addition, the cytotoxicity of α -tomatine towards various other human cancer cells has been investigated, including lung cancer (NCI-H460 and A549) (16,17), prostate cancer (PC3) (18), leukemia (MOLT-4) (19), breast cancer (MCF-7) (20) and EL4 mouse lymphoma cell lines (21). Furthermore, caspase-independent cell death and apoptosis have been identified in leukemia cancer cell lines and PC3 cell lines, respectively, upon treatment with α -tomatine; inactivation of the FAK/PI3K/AKT and ERK signaling pathways with reduced binding potential of nuclear factor- κ B (NF- κ B) occurred upon inhibiting the migration and invasion of cancer cells (20).

Based on the aforementioned facts, the present study aimed to investigate the molecular mechanisms involved in the chemotherapeutic influence of α -tomatine in two human A172 and U-118 MG glioblastoma cell lines through the induction of cell death. The current experiments strongly suggested that several mechanisms are involved in the induction of apoptosis in A172 and U-118 MG cell lines following α -tomatine treatment.

Materials and methods

Materials. α -Tomatine was purchased from Extrasynthese (Genay, France) and its purity was confirmed using high-performance liquid chromatography. Wright staining kit (cat. no. WS16), Fura-2 (cat. no. 47989) and trypan blue (cat. no. T6146) were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Apop Tag assay kit (cat. no. S7100) was obtained from EMD Millipore (Billerica, MA, USA). Anti-calpain IgG antibody (cat. no. 2556; 1:1,000), all the utilized primary antibodies, as well as horseradish peroxidase conjugated goat anti-mouse (cat. no. 7076; 1:2,000) and anti-rabbit IgG (cat. no. 58802; 1:1,000) secondary antibodies, were acquired from Cell Signaling Technology, Inc. (Denver, MA, USA).

Cell culture and treatment. A172 and U-118 MG human glioblastoma cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). These cell lines were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (1%) and streptomycin (100 μ g/ml) in an incubator with a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The medium was replaced every 24 h. In all the experiments, cells were collected using trypsinization after cells were allowed to grow up to passage number 9. Prior to drug addition, cells were starved for 1 day in the same growth medium, followed by preservation using low serum state during addition of α -tomatine. The 100 mM α -tomatine stock solution was prepared using dimethyl sulfoxide (DMSO) and aliquots were diluted serially using growth medium to obtain the desired concentrations for the treatment

of cell lines. In order to determine the suitable α -tomatine dose to induce apoptosis, cell viability was measured by conducting dose-dependent studies (0–50 μ M). It was demonstrated that 25 and 50 μ M α -tomatine were the appropriate doses for apoptosis induction, thus these were used for subsequent experiments. The control group was treated with only DMSO. The morphological and biochemical changes in cells were also analyzed to predict the apoptosis and the mechanistic pathway.

Trypan blue assay for cell viability determination. The cell viability was measured using a trypan blue assay upon treatment with α -tomatine, as previously described (22). The cell viability was determined according to the principle that live cells do not absorb Trypan blue due to membrane integrity, whereas cells with damaged cell membranes absorb the dye and are counted as dead cells. Viable cell counts are presented in terms of the % of the total number of cells.

Wright staining. Wright staining was performed to analyze the changes in morphological characteristics of cells in order to predict the apoptosis. Briefly, cells in the control and α -tomatine groups were washed using phosphate-buffered saline (PBS) solution and then deposited onto slides using a Hettich centrifuge and Cytobuckets (10 min, 150 x g, room temperature; Hettich Lab Technology, Tuttlingen, Germany). Prior to monitoring the morphological changes using Wright staining, cells were fixed in ethanol (95% v/v) (23). Subsequently, various morphological characteristics of the cells were examined, including the chromatin condensation, reduction in cell volume and presence of membrane-bound apoptotic bodies. The percentage of apoptotic cells was measured from three independent experiments.

Peroxidase in situ apoptosis assay. Apoptotic DNA fragmentation upon α -tomatine treatment was evaluated using an ApopTag Peroxidase *In Situ* Apoptosis Detection kit. In brief, cells were treated with α -tomatine as mentioned earlier, washed with PBS, centrifuged (1,000 x g for 3 min at room temperature) and then deposited onto microscopic slides. Cells were fixed in ethanol (95% v/v) and then allowed to dry overnight. A protein digesting enzyme, Proteinase K (cat. no. 21627; EMD Millipore) was used to pretreat the cells for 10 min and the washed using distilled water for ~3 min. This was followed by quenching of the cells using hydrogen peroxide (2% v/v) for 5 min, followed by washing twice in PBS. Next, pre-equilibrated cells were treated with TdT-enzyme and incubated for 1 h at 37°C, followed by addition of stop buffer in the slide, agitation for 10 sec and incubation at room temperature for a further 10 min. After the reaction was stopped, the slides were washed with PBS twice and horseradish peroxidase-conjugated anti-digoxigenin antibody was added and incubated for 30 min. Slides were removed and then washed with PBS, followed by treatment with 3,3'-diaminobenzidine at room temperature for 5 min and finally washing with water (ddH₂O) twice. Methyl green (0.5% v/v) was used to counterstain the slides, which were then washed by water and n-butanol for 10 min. Cells were dehydrated by xylene and fixed in glass coverslips. The percentage of positive apoptotic cells was then determined by counting the brown cells under a microscope (23,24).

Fura-2 assay. Alterations in intracellular calcium ($[Ca^{2+}]_i$) in human glioblastoma cell lines (A172 and U-118 MG) were determined by Fura-2 assay, according to the reported literature (23,24). In brief, cells at a density of 2×10^4 cells/ml were grown in phenol-red medium for 72 h, removed from the culture medium and then centrifuged to obtain pellets for ~5 min at 2,000 rpm. Next, cells were washed twice using PBS buffer and then returned into the suspension, incubated for 2 h and washed twice in Locke's buffer (containing 9 g sodium chloride, 0.4 g potassium chloride, 0.1 g calcium chloride, 0.3 g sodium bicarbonate and 1 g glucose) which was prepared as previously described (25). Cell density was measured with a hemocytometer and then the cells were disseminated in FBS (10%) containing Locke's buffer. Fura-2 stock solution was prepared by dissolving the appropriate amount of Fura-2 in DMSO and then diluting with FBS (10%) containing Locke's buffer to obtain a final concentration of 2 μ M, followed by incubation with cells for 30 min at 37°C. Cells were subsequently diluted to 1×10^5 cells/ml using Locke's buffer solution (Ca^{2+} free), and the $[Ca^{2+}]_i$ was determined using a previously described spectrofluorometric method (26). The fluorescence ratio was measured at wavelengths of 340 and 380 nm using FluoroMax (Horiba Scientific, Kyoto, Japan). Next, 200 μ l digitonin (cat. no. D141; Sigma-Aldrich; Merck KGaA) (250 μ M) and EDTA (500 mM) were used to calculate the maximal and minimal ratios of $[Ca^{2+}]_i$ (R_{max} and R_{min}), respectively. The cell specific dissociation constants for the two cell lines were determined to be 0.378 and 0.477 μ M, respectively, by following the manufacturer's instructions provided in the calcium calibration buffer kit (cat. no. C3008MP; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Western blotting. The adherent and suspended cells from each treatment group were collected and centrifuged at 100 x g for 10 min at room temperature to obtain cell pellets. The cell pellets were washed twice with PBS and resuspended in homogenized buffer solution containing Tris-HCl (50 mM, pH 7.4), EGTA (1 mM), sucrose (325 mM) and phenylmethylsulfonyl fluoride (0.1 mM) for homogenization for ~30 sec at 4°C. Following the homogenization process, the protein concentration in the samples was conducted using a Bradford Protein reagent (Sigma-Aldrich) and measuring spectrophotometrically the absorption at a wavelength of 595 nm (NanoVue spectrophotometer; GE Healthcare Life Sciences, Chicago, IL, USA).

Protein samples (~50 μ g) were then treated with 2X loading buffer composed of equal amount of Tris-HCl (120 mM, pH 6.8), SDS (6%), glycerol (20%), 1,4-dithio-DL-threitol (200 mM), β -mercaptoethanol (10 mM) and bromophenol blue (0.01%), and boiled for 10 min. Gradient gels (4-20%) were used to load the samples for electrophoresis at 200 V for 30 min using an electrophoretic system (GE Healthcare Life Sciences). The α -spectrin bands were detected using electrophoresis gel (5%) at 100 V for 2 h. Subsequently, the gels were electroblotted onto nylon membranes with an electroblotting apparatus (Bio-Rad Laboratories, Inc., Hercules, CA USA). A blocking buffer solution with Tris-HCl (10 mM, pH 7.4), non-fat powdered milk (5%) and NaCl (120 mM) was used to block the membranes and then washed with a washing buffer composed of Tris-HCl (10 mM, pH 7.4), NaCl (120 mM) and Tween-20

(0.1%). Primary antibodies were then properly diluted, and added to the blots for ~1 h. Primary antibodies against the following proteins were used: Bcl-2 (cat. no. 2872; 1:1,000), cytochrome c (cat. no. 4272; 1:1,000), β -actin (cat. no. 4967; 1:1,000), calpain (cat. no. 2556; 1:1,000), caspase-12 (cat. no. 2202; 1:1,000), caspase-9 (cat. no. 9502; 1:1,000), caspase-3 (cat. no. 9662; 1:1,000), apoptosis-inducing factor (AIF) (cat. no. 4642; 1:1,000), Smac/Diablo (cat. no. 2954; 1:1,000), BIRC-2 (cat. no. 4952; 1:1,000), BIRC-3 (cat. no. 3130; 1:1,000), IkBa (cat. no. 9242; 1:1,000), NFkB (cat. no. 4717; 1:1,000) were purchased from Cell Signaling Technology, Inc. Primary antibody against inhibitor of caspase-activated DNase (ICAD) (cat. no. ab108521; 1:10,000) was purchased from Abcam (Cambridge, MA, USA). Primary antibodies against 145 kDa Spectrin Breakdown Products (cat. no. CSB-EQ028022HU; 1:100) and 120 kDa Spectrin Breakdown Products (cat. no. CSB-EQ028055HU; 1:100) were purchased from Cusabio (Hubei, China). The blots were washed thrice with washing buffer and then secondary antibodies were added for 1 h at a dilution of 1:2,000. Finally, the blots were then visualized with an ECL chemiluminescence system (EMD Millipore) and the optical density of bands was measured using a densitometric technique.

Statistical analysis. The acquired experimental data following α -tomatine treatment of A172 and U-118 MG cells were analyzed using SPSS (ver 21) software (SPSS, Inc., Chicago, IL, USA). Results are presented as the mean \pm standard error of at least three independent experiments ($n \geq 3$). The statistical differences between each group were analyzed using one-way analysis of variance followed by Tukey's honest significant difference post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Cell viability and apoptosis measurements. Two different concentrations of α -tomatine were used to determine whether it reduces cell viability and induces apoptosis in glioblastoma A172 and U-118 MG cell lines (Fig. 1). Cells were treated with different α -tomatine concentrations (25 and 50 μ M) and trypan blue assay was used to determine the cell viability by measuring the absorbance under a light microscope. Treatment with α -tomatine (25 and 50 μ M) significantly reduced the cell viability in A172 cells to 80% ($P < 0.05$) and 65% ($P < 0.01$), respectively, whereas the viability in U-118 MG cells was significantly reduced to 75% ($P < 0.05$) and 70% ($P < 0.05$), respectively, when compared with the control cells (Fig. 1A).

The changes in morphological characteristics due to α -tomatine-induced apoptosis were determined using Wright staining (Fig. 1B). Wright stained cells typically exhibit characteristics including reduction of cell size, membrane blebbing or chromatin condensation as a sign of apoptotic cell death. Treatment with α -tomatine (25 and 50 μ M) in A172 cells resulted in a significant increase in apoptosis of 34% ($P < 0.05$) and 47% ($P < 0.01$), respectively, whereas an increase of 25% ($P < 0.05$) and 30% ($P < 0.05$) was observed in the case of U-118 MG cells, respectively (Fig. 1B). ApopTag assay (Fig. 1C) was further used in order to confirm the Wright staining data. In this assay, brown color stained cells were identified to be

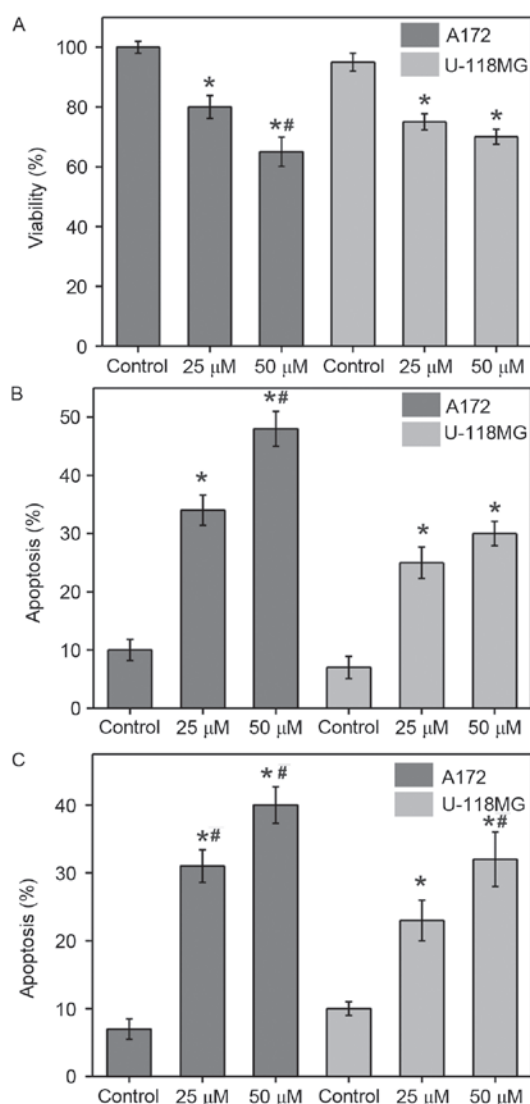


Figure 1. α -Tomatine-treated A172 and U-118 MG demonstrated decreased cell viability and increased apoptotic features. (A) Cell viability tests were conducted using trypan blue test. (B) The apoptotic action following α -tomatine treatment was observed by Wright staining. (C) The biochemical features of the apoptotic reaction were examined by ApopTag assay. * $P < 0.05$, ** $P < 0.01$ vs. control.

apoptotic when presenting one of the morphological changes mentioned earlier. The results revealed that very few control cells exhibited brown staining, indicating a low number of positive ApopTag cells. By contrast, the α -tomatine-treated groups exhibited marked staining and thus apoptosis, which was confirmed by the ApopTag assay and changes in the morphological characteristics. The percentage of apoptotic cells were found to be significantly increased by 33% ($P < 0.01$) and 41% ($P < 0.01$) in the A172 cell line after the treatment with 25 and 50 μ M α -tomatine, respectively, whereas apoptosis of 24% ($P < 0.05$) and 33% ($P < 0.01$) was observed in the U-118 MG cell line, respectively (Fig. 1C).

Observation of $[Ca^{2+}]_i$ changes upon α -tomatine treatment. Fura-2 assay was conducted in order to monitor the $[Ca^{2+}]_i$ changes upon α -tomatine treatment in A172 and U-118 MG cells (Fig. 2). The results revealed that a marked increase in $[Ca^{2+}]_i$ was observed in the two cell lines upon the addition

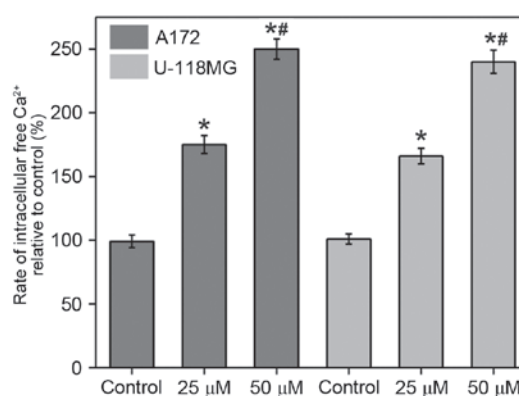


Figure 2. Measurement of intracellular free $[Ca^{2+}]_i$ in A172 and U-118 MG cell lines following α -tomatine treatment. * $P < 0.05$, ** $P < 0.01$ vs. control.

of α -tomatine (25 and 50 μ M). The percentages of change in A172 cells were determined to be 75% ($P < 0.05$) and 150% ($P < 0.01$) following treatment with 25 and 50 μ M of α -tomatine, respectively, compared with the control group. In the case of U-118 MG cells, the percentages of change were found to be 66% ($P < 0.05$) and 138% ($P < 0.01$) for the treatment with 25 and 50 μ M α -tomatine, respectively (Fig. 2). In the two cell lines, treatment of α -tomatine (25 and 50 μ M) was observed to significantly increase the intracellular calcium levels $[Ca^{2+}]_i$ as compared with the control. These $[Ca^{2+}]_i$ enhancements are a strong indication of apoptosis induction through endoplasmic reticulum (ER) stress and of participation of calpain in the degradation of cytosolic substrate.

Identification of α -tomatine-induced apoptosis. It is known that the changes in the expression levels of Bcl-2 proteins are an indicator of cell apoptosis. Western blotting was conducted in the present study to measure the expression levels of Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic), as well as to examine the alterations in Bax:Bcl-2 ratio (Fig. 3A and B). β -actin served as an internal control. It was identified that treatment with α -tomatine resulted in an elevation in Bax expression, whereas Bcl-2 expression was reduced. The calculated ratio of Bax:Bcl-2 was significantly increased in A172 and U-118 MG cells following α -tomatine treatment of 25 ($P < 0.05$) and 50 μ M ($P < 0.01$), respectively (Fig. 3B). These alterations in the ratio of Bax:Bcl-2 upon α -tomatine treatment indicated that the apoptosis induction was mediated through the mitochondrial pathway.

Release of mitochondrial cytochrome c. Apoptosis is initiated by various apoptotic stimuli, which result in the release of cytochrome c from mitochondria. This results in caspase activation through biochemical reactions, leading to cell death (20). Hence, the protein fractions of the cytosolic and mitochondrial parts in all treatment groups were separated to determine the levels of cytochrome c using western blot analysis (Fig. 3A). The results demonstrated that α -tomatine treatment in A172 and U-118 MG cell lines significantly upregulated the cytochrome c protein level in the cytosolic fraction with a consequent reduction in the corresponding mitochondrial portion (Fig. 3C and D). The cytosolic cytochrome c level was increased by 60% ($P < 0.05$) and 90% ($P < 0.01$) in A172 cells

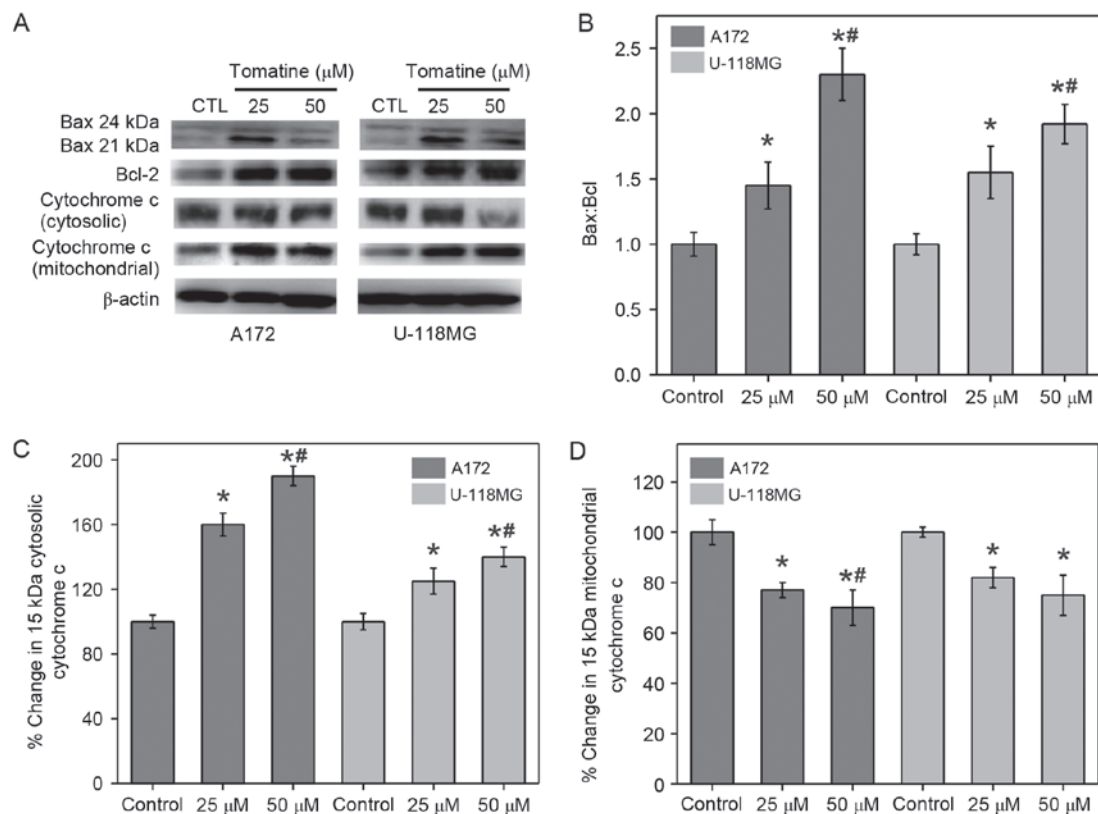


Figure 3. (A) Western blots, and quantified densitometric analysis results demonstrating the expression levels of (B) Bax:Bcl-2, (C) cytosolic cytochrome *c* and (D) mitochondrial cytochrome *c* in α -tomatine-treated A172 and U-118 MG cell lines. * $P < 0.05$, ** $P < 0.01$ vs. control. CTL, control.

and by 25% ($P < 0.05$) and 40% ($P < 0.01$) in U-118 MG cells following 25 and 50 μ M α -tomatine treatment, respectively (Fig. 3C). In the case of mitochondrial cytochrome *c* levels, 25 and 50 μ M α -tomatine treatment caused a significant 22% ($P < 0.05$) and 32% ($P < 0.01$) decrease in A172 cells, respectively, whereas the decrease in U-118 MG cells was observed to be 19% ($P < 0.05$) and 24% ($P < 0.05$), respectively (Fig. 3D). These results revealed that α -tomatine treatment caused cytochrome *c* release from the mitochondria into the cytosol.

Calpain expression and activation of caspases. α -Tomatine mediated ER stress can increase $[Ca^{2+}]_i$ and trigger Ca^{2+} -dependent calpain expression for activating caspase. Western blot experiments were performed to study the induction of calpain expression and caspase activation in A172 and U-118 MG cells upon treatment with α -tomatine (Fig. 4A). The results indicated that treatment with 25 and 50 μ M α -tomatine caused an increase in calpain protein expression of 7% (non-significant) and 36% ($P < 0.01$) in A172 respectively, and of 15% ($P < 0.05$) and 26% ($P < 0.01$) in U-118 MG cell lines, respectively, as compared with the control (Fig. 4B). In addition, α -tomatine (25 and 50 μ M) significantly increased the protein levels of caspase-12 (40 kDa) in A172 cell lines, by 24% ($P < 0.05$) and 46% ($P < 0.01$) respectively and by 28% ($P < 0.05$) and 56% ($P < 0.01$) respectively in U-118 MG cell lines (Fig. 4C). Similarly, the protein levels of caspase-9 (35 kDa) were significantly upregulated upon administration with α -tomatine (25 μ M, $P < 0.05$; 50 μ M, $P < 0.01$) in A172 and U-118 MG cell lines (Fig. 4D). These results revealed the participation of caspase activation in the apoptosis induction.

Calpain and caspase-3 activities. The enhancement in calpain and caspase-3 activities from the generation of α II-spectrin breakdown products (SBDP) (145 and 120 kDa, respectively) were analyzed (Fig. 5A). The results indicated that the protein levels of SBDP (145 and 120 kDa subunits) were significantly upregulated upon addition of α -tomatine (25 μ M, $P < 0.05$; 50 μ M, $P < 0.01$) in A172 and U-118 MG cell lines (Fig. 5B and C). The above results are in concordance with the results of caspase-3 in A172 and U-118 MG cell lines.

Caspase-3 activation and inhibition of ICAD cleavage. The current study also investigated the activation of caspase-3 and ICAD cleavage, which is capable of translocating DNase activated by caspase to the nucleus for DNA fragmentation (Fig. 6). In A172 and U-118 MG cell lines, the protein expression of caspase-3 fragments (20 kDa) and ICAD (40 kDa) was significantly enhanced after treatment with α -tomatine (25 and 50 μ M). Treatment with 25 and 50 μ M α -tomatine resulted in an increase in the protein expression of caspase-3 fragments (20 kDa) by 43% ($P < 0.05$) and 71% ($P < 0.01$) in A172 respectively, and by 10% (non-significant) and 38% ($P < 0.01$) in U-118 MG cell lines, respectively, as compared with the control (Fig. 6B). Treatment with 25 and 50 μ M α -tomatine increased the protein expression of ICAD (40 kDa) by 13% (non-significant) and 21% ($P < 0.05$) in A172 respectively, and by 40% ($P < 0.05$) and 48% ($P < 0.01$) in U-118 MG cell lines, respectively, as compared with the control (Fig. 6C).

Enhancement in AIF cytosolic levels. Elevation of the permeability of the mitochondrial membrane can cause AIF release

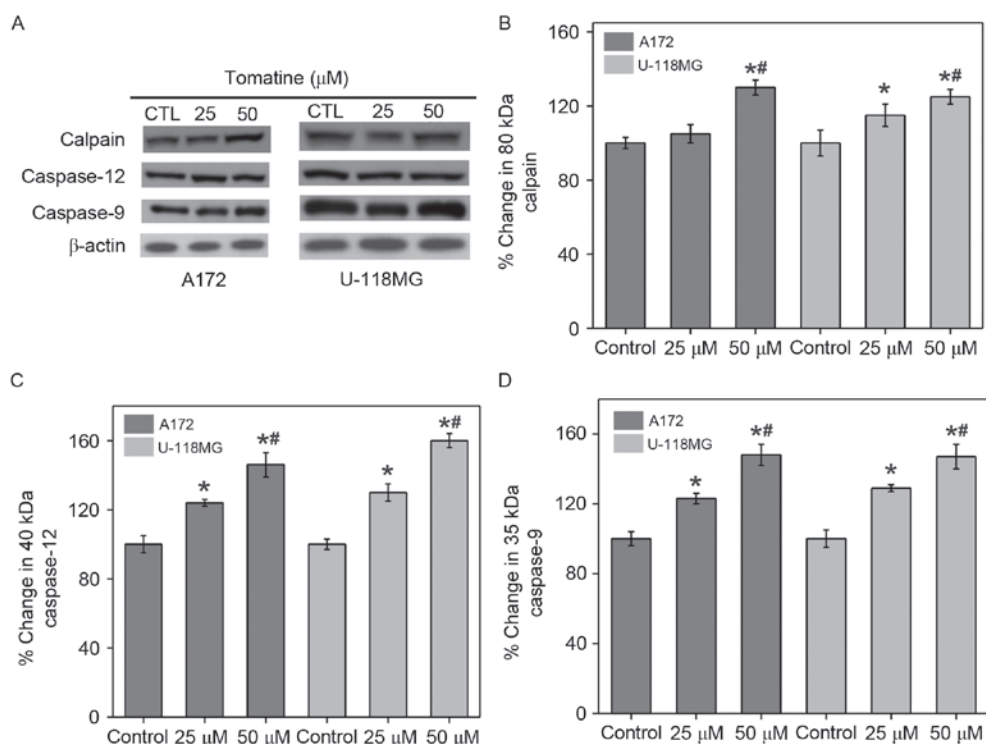


Figure 4. (A) Western blots, and quantified densitometric analysis results demonstrating the expression levels of (B) calpain, (C) caspase-12 and (D) caspase-9 in α -tomatine-treated A172 and U-118 MG cell lines. * $P < 0.05$, ** $P < 0.01$ vs. control.

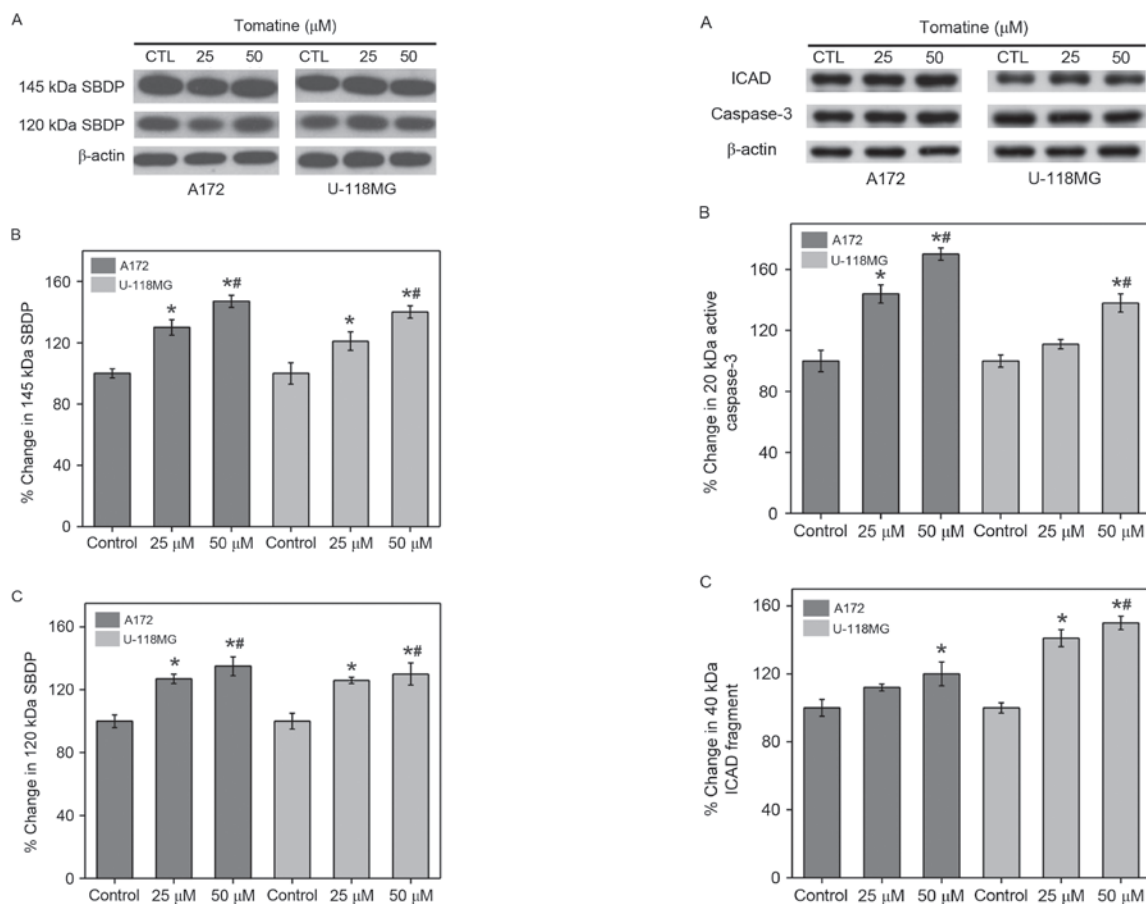


Figure 5. (A) Western blot analysis of the expression levels of SBDP in α -tomatine-treated A172 and U-118 MG cell lines. Densitometric analysis showed the quantified expression of (B) 145 kDa SBDP and (C) 120 kDa SBDP. * $P < 0.05$, ** $P < 0.01$ vs. control. SBDP, α II-spectrin breakdown products.

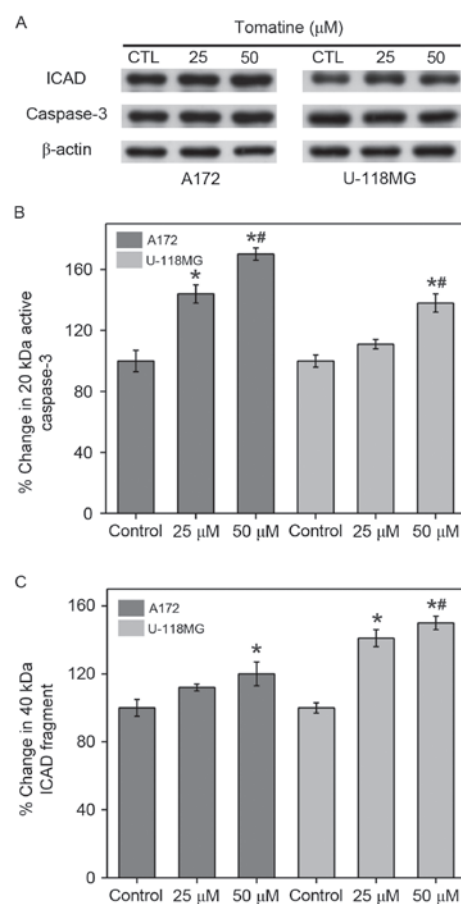


Figure 6. (A) Western blots, and quantified densitometric analysis results demonstrating the expression levels of (B) caspase-3 and (C) ICAD fragment in α -tomatine-treated A172 and U-118 MG cell lines. ICAD, inhibitor of caspase-activated DNase. * $P < 0.05$, ** $P < 0.01$ vs. control.

into the cytoplasm. Thus, AIF levels in cytosolic fractions were determined in α -tomatine-treated A172 and U-118 MG cells (Fig. 7). The protein expression level of AIF in the two cell lines was found to be increased by α -tomatine treatment. Treatment with 25 μ M α -tomatine was observed to significantly increase AIF expression in A172 cells compared with control cells ($P<0.05$), whereas this increase was non-significant in U-118 MG cells. Treatment with 50 μ M α -tomatine in the A172 and U-118 MG cell lines produced a significant increase in the cytosolic AIF levels of 60% ($P<0.01$) and 17% ($P<0.05$), respectively. These results suggested the involvement of a caspase-independent pathway of apoptosis.

Changes in Smac/Diablo and inhibitor of apoptosis proteins (IAPs). The release of mitochondrial Smac/Diablo proteins into the cytoplasm and the quantity of IAPs in the cytosol serve major functions in apoptosis. Therefore, the present study determined these levels with the help of western blotting experiments using α -tomatine-treated A172 and U-118 MG cells. Treatment with 25 μ M α -tomatine resulted in a 35% ($P<0.05$) and 47% ($P<0.01$) increase in Smac/Diablo protein expression in A172 and U-118 MG cells, respectively (Fig. 8A and B). Also, treatment with 50 μ M α -tomatine significantly increased the Smac/Diablo cytosolic levels in both A172 (54%; $P<0.01$) and U-118 MG (48%; $P<0.01$) cells.

The current study also monitored the expression levels of two IAPs, namely c-IAP1 and c-IAP2 that are also known as Baculoviral IAP repeat-containing-2 (BIRC-2) and BIRC-3, respectively, using western blot experiments. Treatment with 25 μ M α -tomatine did not show any significant difference on BIRC-2 expression in A172 cells (7%), while it caused a significant reduction in U-118 MG cells (27%; $P<0.05$) as compared with the control. However, 50 μ M α -tomatine treatment significantly reduced the protein expression of BIRC-2 in A172 (20%; $P<0.01$) and U-118 MG (29%; $P<0.01$) cells as compared with control (Fig. 8C). Furthermore, treatment with 25 μ M α -tomatine significantly downregulated the protein expression of BIRC-3 in A172 (17%; $P<0.05$) and U-118 MG (20%; $P<0.05$) cell lines as compared with control. Treatment with 50 μ M α -tomatine significantly reduced the expression of BIRC-3 in A172 (37%; $P<0.01$) and U-118 MG (23%; $P<0.05$) cells as compared with control (Fig. 8D). These reduced expression levels of BIRC-2 and BIRC-3 are favorable for the apoptosis process.

Effect on NF- κ B expression. Cancer cells generally increase NF- κ B and reduce the inhibitor of NF- κ B (I κ B α) expression levels in order to promote IAP expression for cell survival. Therefore, western blotting experiments were conducted in the present study to assess the NF- κ B and I κ B α levels in A172 and U-118 MG cells (Fig. 9). Treatment with 25 μ M α -tomatine generated a significant ~16% ($P<0.05$) decrease in NF- κ B expression in A172 cells, and a significant decrease of ~25% ($P<0.05$) in U-118 MG cells as compared with the control. Treatment with 50 μ M α -tomatine significantly decreased NF- κ B expression in A172 (~34%; $P<0.01$) and U-118 MG (~48%; $P<0.01$) cells (Fig. 9B). Furthermore, treatment with 25 μ M α -tomatine significantly increased the protein expression of I κ B α in A172 (26%; $P<0.05$), whereas no significant increase was observed in U-118 MG cells. However, 50 μ M

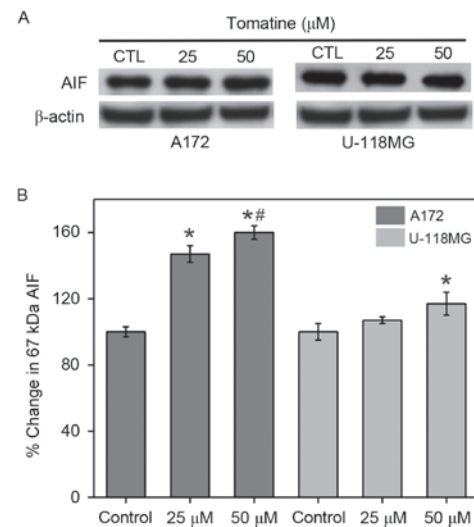


Figure 7. (A) Western blots and (B) densitometric analysis results demonstrating the change in cytosolic AIF levels following α -tomatine treatment in A172 and U-118 MG cell lines. * $P<0.05$, ** $P<0.01$ vs. control. AIF, apoptosis-inducing factor.

α -tomatine treatment significantly increased I κ B α expression in both A172 (35%; $P<0.01$) and U-118 MG (28%; $P<0.05$) cell lines (Fig. 9C). These results suggest that the alterations in the expression levels of NF- κ B and I κ B α were due to α -tomatine-induced apoptosis.

Discussion

In the present study, α -tomatine-induced cell death in A172 and U-118 MG human glioblastoma cell lines was reported for the first time. Based on the obtained results from these experiments, it was demonstrated that apoptosis was induced by α -tomatine treatment and the occurrence of multiple molecular mechanisms was observed. Apoptosis was investigated by observing the morphological and biochemical characteristics of A172 and U-118 MG cells. For instance, α -tomatine triggered an enhancement in $[Ca^{2+}]_i$ that can induce cell death. The obtained results revealed a connection between $[Ca^{2+}]_i$ elevation and calpain activity in the induction of cell death. Bax and Bcl-2 levels were also examined in the cell lines, and the ratio of Bax:Bcl-2 was found to be raised following α -tomatine treatment, which may assist in the induction of apoptosis. Overexpression of Bax is known to alter the mitochondrial membrane permeability to release cytochrome *c*, and thus the current study further investigated the changes in the cytochrome *c* levels in both cytosolic and mitochondrial fragments. The results indicated that the cytosolic fraction levels were raised and mitochondrial levels were decreased upon α -tomatine addition, strongly indicating the participation of mitochondrial cytochrome *c* release in apoptosis induction.

The present study also examined the α -tomatine-induced cell death along with caspase-9, caspase-12 and calpain activation in A172 and U-118 MG cells. The results indicated that ER stress was caused by α -tomatine treatment for apoptosis induction in human glioblastoma cell lines. The current study results are comparable with similar apoptosis models shown by other researchers (24,27,28). Earlier studies demonstrated

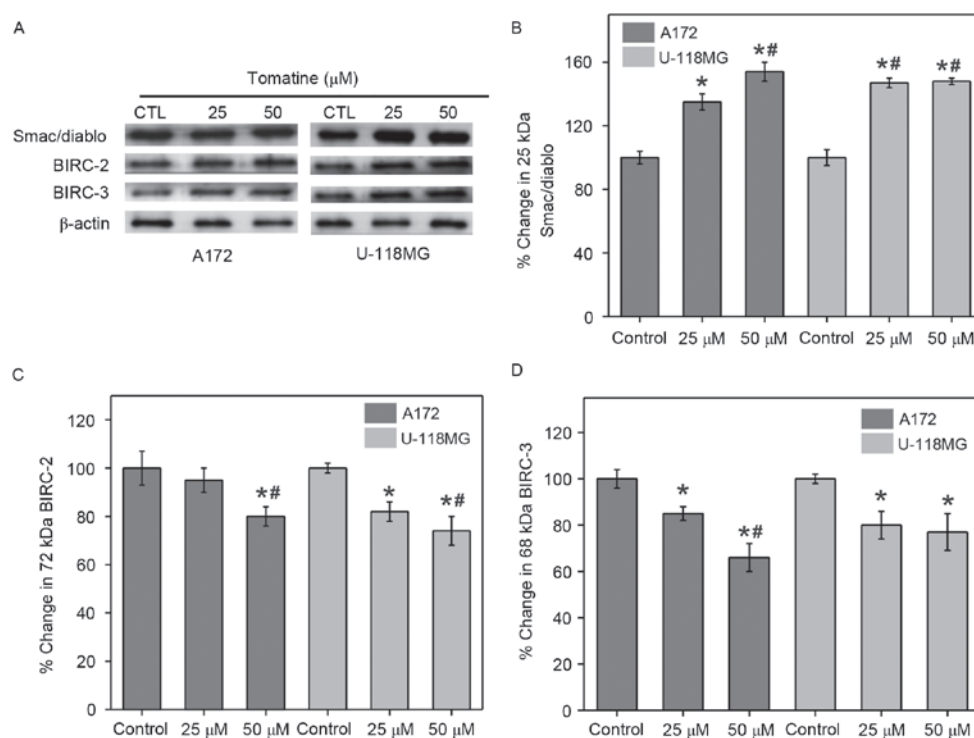


Figure 8. (A) Western blots, and quantified densitometric analysis results demonstrating the expression levels of (B) Smac/Diablo, (C) BIRC-2 and (D) BIRC-3 in α -tomatine treated A172 and U-118 MG cell lines. * P <0.05, *# P <0.01 vs. control. BIRC, Baculoviral IAP repeat-containing protein.

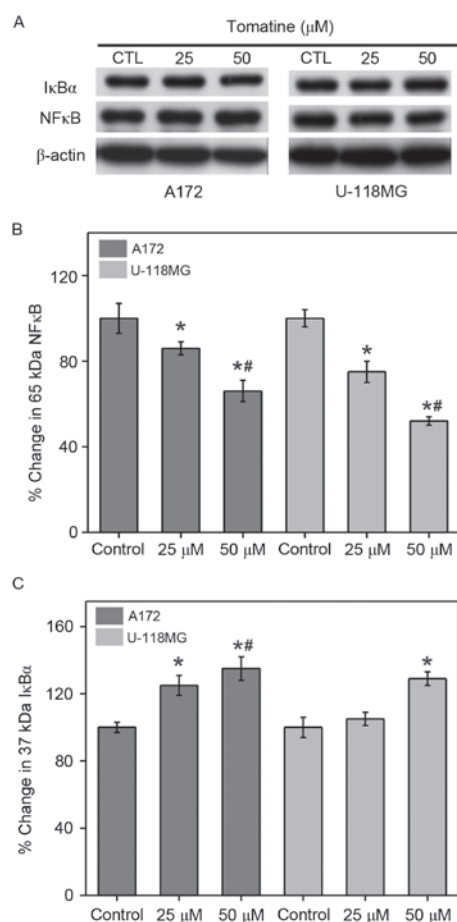


Figure 9. (A) Western blots, and quantified densitometric analysis results demonstrating the expression levels of (B) NF- κ B and (C) I κ B α in α -tomatine-treated A172 and U-118 MG cells. * P <0.05, *# P <0.01 vs. control. NF- κ B, nuclear factor- κ B; I κ B α , inhibitor of NF- κ B.

that ER stress can induce caspase-12 activation, which is directly associated with caspase-9 activation (29,30). Furthermore, the present study measured the activities of calpain and caspase-3 activation, according to previous findings stating that the degradation of α -spectrin to 145 and 120 kDa SBDP is responsible for calpain and caspase-3 activation, respectively (31,32). From the results obtained, it was proven that elevated calpain and caspase-3 levels served a crucial function in α -tomatine-triggered apoptosis in A172 and U-118 MG cell lines. A previous relevant report also supported the association of calpain and caspase-3 activation with apoptosis (33).

Furthermore, α -tomatine was observed to trigger the activation of caspase-3 as identified from the 20 kDa fragment of caspase-3 and ICAD cleavage in the present study. These results demonstrated that α -tomatine induced caspase-3 activation, which is able to cleave ICAD to permit translocation of CAD into the nucleus for caspase-dependent DNA nuclear fragmentation, and this finding is consistent with the observations of a previous study (34).

Caspase-independent apoptosis is known to also be induced by mitochondria upon the release of AIF (35); thus, the present study examined the changes in AIF cytosolic levels to determine the feasibility of caspase-independent apoptosis following α -tomatine treatment in the glioblastoma cell lines. The data strongly indicated the enhancement of AIF cytosolic levels after α -tomatine treatment, proving that α -tomatine induced AIF mitochondrial efflux into the cytosol. This translocation may produce caspase-independent apoptosis and DNA nuclear fragmentation (36). According to the aforementioned results, it can be demonstrated that both caspase-dependent and independent pathways participated

in the α -tomatine-directed apoptosis in A172 and U-118 MG glioblastoma cell lines.

The Smac/Diablo release from mitochondria upon α -tomatine treatment was also investigated. Since IAPs, such as BIRC-2 and BIRC-3, can be downregulated by Smac/Diablo pro-apoptotic molecules in the cytosol, the BIRC-2 and BIRC-3 cytosolic levels were investigated. α -Tomatine treatment was observed to cause reduction in the expression levels of these two genes, which are considered as feasible oncogenes (37). Thus, this suggests that the apoptosis of α -tomatine may be due to the oncoprotein downregulation. In addition, collective results indicated that α -tomatine treatment induced apoptosis by elevating Smac/Diablo and reducing IAP levels, as well as by caspase activation.

In cell survival, NF- κ B activation serves a crucial role (38), and thus, the downregulation of NF- κ B by compounds extracted from plant may be a promising approach for preventing cancer development (39). In the experiments of the present study, α -tomatine treatment triggered I κ B α elevation and IAP downregulation in the two cell lines, suggesting that other than the apoptosis induction, the mechanisms of α -tomatine action include IAP and NF- κ B inhibition for the prevention of cell survival signals. The α -tomatine-induced inhibition of NF- κ B was due to its decreased binding with DNA. Furthermore, the apoptosis induction in A172 and U-118 MG by α -tomatine treatment was shown to be p53 independent (40).

In conclusion, the present study explored the multiple molecular mechanisms involved in the apoptosis induction following α -tomatine treatment in A172 and U-118 MG cell lines. α -Tomatine exhibited its proapoptotic potentials at 25 and 50 μ M levels within the physiological dosage. Xenografted/allografted animal models will be used in our future studies for further confirmation of these findings.

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