Original Article
Shikonin inhibits proliferation and invasion of glioma cells through Wnt/β-catenin signaling pathways

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Abstract: Background: The aim of this study was to observe the effects of shikonin on proliferation inhibition and apoptosis induction in U251 brain glioma cells, examining its roles in Wnt/β-catenin signaling pathways. Methods: U251 glioma cells were treated with either 0, 5, or 20 μmol/L of shikonin. Afterward, cell cycle distribution, apoptosis, and cell invasion capacities, as well as expression of Wnt/β-catenin signaling pathway-related genes and proteins, was analyzed. Results: The survival rate of U251 cells, after 24 hours of incubation, was significantly lower (P < 0.05) and the cell survival rate decreased with increased shikonin concentrations (P < 0.05). The 50% inhibitory concentration (IC50) was 14.29 μmol/L. After 24 hours of treatment with different concentrations of shikonin, the number of apoptotic U251 cells was significantly higher (P < 0.05). With increasing shikonin concentrations, the number of apoptotic cells increased and the proportion of cells in the G2/M phase increased in shikonin 5 μmol/L and 20 μmol/L groups (P < 0.05). The number of invading cells per field in shikonin 5 μmol/L and 20 μmol/L groups was significantly lower (P < 0.05). Expression of apoptosis-related proteins, Cyt c, AIF, and Bax, in shikonin 5 μmol/L and 20 μmol/L groups, was significantly higher (P < 0.05), while expression of apoptosis-suppressing gene Bcl-2 was significantly decreased (P < 0.05). Expression levels of β-catenin and downstream c-Myc genes and proteins in shikonin 5 μmol/L and 20 μmol/L groups were significantly lower (P < 0.05). Fluorescence intensity of β-catenin in the nucleus and cytoplasm of shikonin 5 μmol/L and 20 μmol/L groups was significantly decreased. Conclusion: Shikonin plays important roles in inhibiting proliferation and promoting apoptosis in U251 glioma cell lines. Underlying mechanisms may proceed via inhibition of Wnt/β-catenin signaling pathways.

Keywords: Shikonin, glioma cells, Wnt/β-catenin signaling pathway, inhibit proliferation and invasion

Introduction

Gliomas are one of the most common malignant tumors in neurosurgery, accounting for approximately 40% of brain tumors. The annual incidence of this disease in China is about 6/100,000, with higher occurrence in males than in females. Glioma cells are strongly invasive [1, 2]. They invade and migrate to peripheral tissues through blood vessels and myelinated fiber paths. Therefore, glioma develops rapidly, with high malignancy and high mortality rates [3]. However, at present, the pathogenesis is not clear. In recent years, it has been found that [4] Wnt/β-catenin signaling pathways are abnormally activated in glioma patients, possibly correlating with invasion and metastasis of gliomas.

At present, treatment of gliomas mostly involves routine therapies, such as surgery, radiothera-py and chemotherapy, gene therapy, and immunotherapy [5-7]. However, the clinical effects of these routine therapies have not been satisfactory. Recent studies have demonstrated that a variety of Chinese herbal monomer ingredients, such as quercetin [8], resveratrol [9], and polydatin [10], can strongly inhibit proliferation and induce apoptosis of malignant tumor cells. Puccoon is a common Traditional Chinese Medicine with a cold salty taste and therapeutic functions that include detoxification, fever relief, and blood cooling, as well as rash elimination. Shikonin is the main active ingredient of puccoon. Modern research has illustrated that [11, 12] shikonin has pharmacological effects, including antitumor, anti-inflammation, hepatoprotection, immune regulation, blood sugar lowering, antiviral, and sterilization. A number of studies have demonstrated that [13] shikonin provides significant inhibitory effects on various tumor cells in gastric cancer, bladder can-

cer, breast cancer, and laryngo-carcinomas. However, studies investigating the effects of shikonin on gliomas are rare.

The current study used U251 human glioma cells, similar to gliomas in cellular and molecular features, to observe the effects of shikonin on proliferation inhibition and apoptosis induction in U251 glioma cells, examining its impact on the Wnt/β-catenin signaling pathways.

Materials and methods

Cell line

U251 human glioma cells purchased from Shanghai Cell Bank, Chinese Academy of Sciences.

Reagents

Shikonin was provided by Sigma, USA. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were provided by Gibco, USA. Methyl thiazolyl tetrazolium (MTT) and dimethyl sulfoxide (DMSO) were provided by Cell Signaling, USA. Trypsin was provided by Hyclone, USA. MTT cell proliferation kit was provided by Shanghai Beyotime Biotechnology. Annexin V-FITC/PI apoptosis kit was purchased from BD Company, USA. Real-time PCR kit was purchased from Dalian Takara, China. Total RNA extraction reagent, TRIZOL 20L, was provided by Invitrogen, USA, while the protein assay kit was provided by Shanghai Beyotime Biotechnology. Polyvinylidene difluoride (PVDF) film was provided by Millipore, China. Rabbit anti-human β-catenin polyclonal antibody and Cyclin D1 and C-Myc polyclonal antibodies were provided by SantaCruz, USA. Rabbit anti human β-actin polyclonal antibody and anti-rabbit goat secondary antibody, labeled with horseradish peroxidase (HRP), were purchased from Shanghai Beyotime Biotechnology. This study was approved by the Ethics Committee of Jingzhou Central Hospital, Second Clinical Medical College of Yangtze University.

Cell culture

U251 glioma cells were seeded in DMEM medium containing 10% fetal bovine serum and incubated at 37°C, 5% CO_2 and saturated humidity. The cells were passaged after 0.25% trypsinization. The passage of one generation was completed during the 2nd-3rd day. Cells during the logarithmic growth phase were centrifuged and collected for further experimentation after trypsinization. U251 glioma cells were grouped according to different concentrations of shikonin. These concentrations were 0, 1, 5, and 20 μmol/L.

Quantification of cell proliferation by MTT assay

U251 cells were cultured to the logarithmic growth phase, prepared as single-cell suspensions after trypsin digestion, seeded in 96-well plates with a cell density of 5,000 cells/well, and incubated at 37°C and 5% CO_2. When the cells grew to about 80% confluence, different concentrations of shikonin (1, 5, and 20 μmol/L) were added and set as shikonin 1, 5, and 20 μmol/L groups. Additionally, DMSO, without shikonin, was added for controls. Three replicates were prepared in each group. After the cells had been cultured in the incubator for 24 hours and 48 hours, 20 μL of MTT was added to each well. The medium was discarded after incubation for 4 hours under dark conditions. Next, 150 μL of DMSO was added to each well and stirred for 2 hours on a shaking table. The cells were digested, centrifuged, and collected after incubation for 24 hours at a constant temperature. Absorbance at 490 nm was measured using a TECAN microplate reader.

Detection of cell cycle stage and apoptosis by flow cytometry

U251 glioma cells were cultivated to the logarithmic growth phase and the number of cells was adjusted to 1×10^6 mL^{-1}. Shikonin, at concentrations of 0, 1, 5, and 20 μmol/L, was added to 6-well plates before continuous incubation for 24 hours in a thermostatic incubator. Later, the cells were digested with trypsin to form a monocle suspension, then collected after centrifugation. After centrifugation and collection, 5 μL of Annexin V-FITC was added, followed by incubation at 4°C for 25 minutes in darkness. Next, 10 μL of propidium iodide (PI) was added. This was followed by incubation at 4°C for 25 minutes in the dark. Cell cycle stage and percentages of apoptosis were measured by flow cytometry.

Detection of cell migration by scratch assay

U251 glioma cells in the logarithmic growth phase were collected to prepare a single-cell suspension, inoculated in a 6-well plate with 400,000 cells per well. The cells were continu-

Table 1. Effects of shikonin on the survival rate of U251 cells

Groups (Concentration of shikonin)	Cell viability (%)
0 µmol/L	98.55±11.46
1 µmol/L	82.68±9.35
5 µmol/L	65.21±9.51
20 µmol/L	36.42±8.09

ously cultured until adherent growth. A scratch was made at the bottom of the well using 200 µL tips. Wells were rinsed with PBS three times, followed by the addition of a serum-free culture medium containing shikonin at concentrations of 0, 1, 5, and 20 µmol/L. Cells were imaged using an inverted microscope. Cells were then cultured for 24 hours and 48 hours in the thermostatic incubator. Afterward, parts of the scratched area were observed under a microscope at tenfold magnification and at different time points. Relative area ratios were then measured using ImageJ software to compute cell migration capacities.

Detection of cell invasion capacity by Transwell chambers

The inner surface of each well with the polycarbonate membrane was coated with 30 µg of Matrigel. The chambers possess an upper layer and a lower layer. U251 cells at the logarithmic growth phase were suspended in serum-free DMEM medium to achieve a cell density of 1×10^6 cells/mL. Next, 100 µL of the obtained suspension was added to the upper chamber, while 600 µL of serum-free DMEM medium was added to the lower chamber of the coated well. After 48 hours of incubation in the thermostatic incubator, non-adherent cells were removed from the filtration membrane. The filtration membrane was fixed for 5 minutes, using paraformaldehyde, and stained with hematoxylin and eosin (HE). Later, five fields were randomly selected under the 400 folds microscope. The number of cells that penetrated the filtration membrane (invading cells) in the view fields was noted, evaluating the invasion capacity of cells.

Analysis of expression of Wnt pathway-related genes by real-time fluorescence quantitative PCR

TRIzol Reagent was used to extract total RNA from each group of cells. After determination of
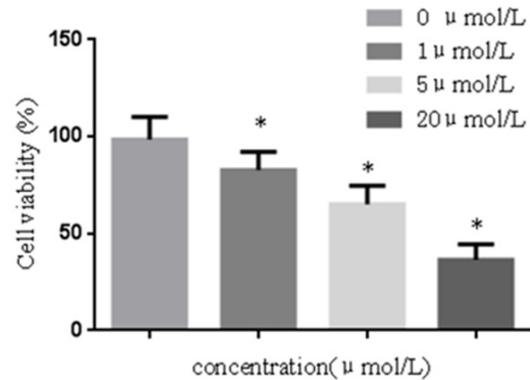

Figure 1. Effects of shikonin on survival rates of U251 cells. Comparison of the concentration of shikonin on the experimental group with the control group (*P < 0.05).

RNA purity, reverse transcription was performed to synthesize cDNA. The cDNA was then PCR-amplified using following primers and reaction conditions: β-catenin: Positive-sense 5'-CGTTTCGCCTTCATTATGGACTACCT-3', Antisense 5'-GCCGCTGGGTGTCCTGATGT-3', amplicon size 197 bp; C-Myc: Positive-sense 5'-TTCGG-GTAGTGGAAAACCAG-3', Antisense 5'-CAGCAG-CTCGAATTTCTCC-3', amplicon size 203 bp; β-actin: Positive sense 5'-GGTGCTGAGTATGTCG-TGGAG-3', Antisense 5'-ATGCAGGGATGATGTTC-TAGG-3', amplicon size 390 bp; Reaction conditions: pre-denaturation 95°C for 30 seconds, for 1 cycle; PCR reaction conditions: 95°C for 5 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, for 40 cycles. The purity of RNA was measured by the absorbance of β-catenin, c-Myc, and β-actin RT-PCR products. RNA quantification was then performed.

Detection of Wnt pathway-related protein expression by Western blotting

Different concentrations of shikonin were used to treat U251 cells for 48 hours. This was followed by collection of the cells and extraction of total protein. Protein content was analyzed using the BCA method and SDS-PAGE electrophoresis was conducted for protein separation. Proteins were transferred onto the membranes and incubated with 5% skim milk powder for 1 hour. This was followed by the addition of 1:500 rabbit anti-human β-catenin and c-Myc polyclonal primary antibodies and rabbit anti-human β-actin polyclonal antibody (1:1000).

Table 2. Effects of shikonin on apoptosis of U251 cells

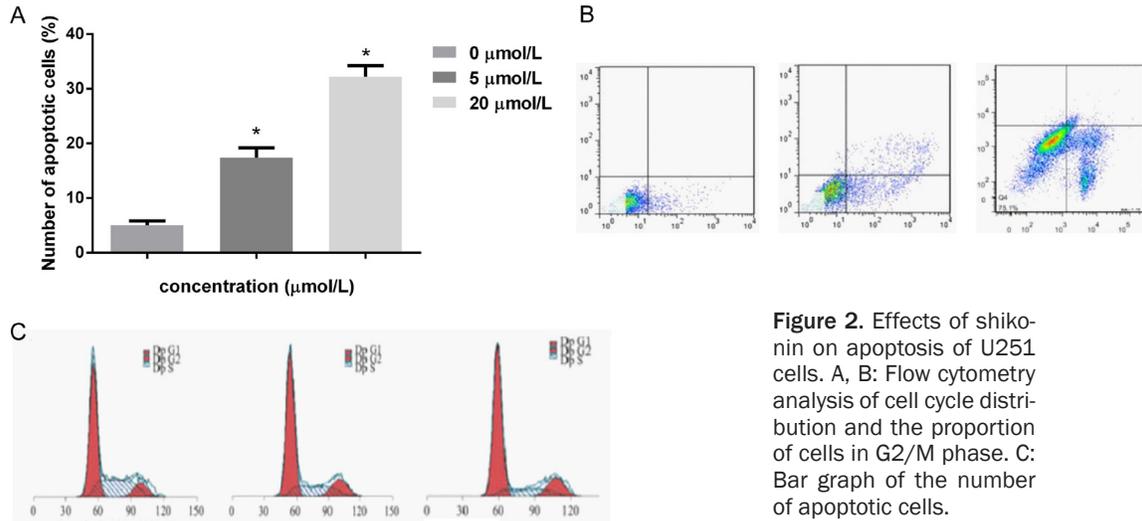| Groups (Concentration of shikonin)	Number of apoptosis	Cell cycle		
		G0/G1	S	G2/M
0 μmol/L	4.91±0.86	52.32±2.74	39.21±3.55	8.47±3.75
5 μmol/L	17.24±1.87	50.61±5.63	30.45±3.09	18.94±2.69
20 μmol/L	32.05±1.94	42.09±3.94	29.06±4.31	28.85±4.75

Figure 2. Effects of shikonin on apoptosis of U251 cells. A, B: Flow cytometry analysis of cell cycle distribution and the proportion of cells in G2/M phase. C: Bar graph of the number of apoptotic cells.

Membranes were incubated overnight at 4°C, then washed 3 times with PBST. Next, the HRP-labeled secondary antibody was added to the membranes. This was followed by incubation for 1 hour at 24°C. Detection of proteins on the membranes was carried out using the enhanced chemiluminescence method, followed by imaging.

Statistical analysis

SPSS19.0 statistics software was employed to analyze data. Data are presented as mean ± standard deviation (x ± s). Differences between groups were analyzed using univariate analysis of variance. The significance level is set at P < 0.05.

Results

Shikonin inhibits proliferation of U251 glioma cells

MTT experiments demonstrated that U251 cells, cultured under different concentrations of shikonin for 24 hours, had a significantly lower survival rate, compared with the control group (P < 0.05). With increases in shikonin concentrations, cell survival rates gradually decreased (P < 0.05), illustrating that the inhibitory effects of shikonin on glioma cells were concentration-dependent. Higher concentrations of shikonin led to stronger inhibitory effects on cell proliferation.

The 50% inhibitory concentration (IC50), obtained by the above experiment, was 14.29 μmol/L. Therefore, 5 and 20 μmol/L of shikonin were selected for subsequent experiments (**Table 1** and **Figure 1**).

Effects of shikonin on apoptosis and cell cycle distribution of U251 cells

Results of flow cytometry demonstrated that the apoptosis of U251 cells was significantly higher in the 5 and 20 μmol/L groups than in the 0 μmol/L group (P < 0.05) after 24 hours of treatment with different concentrations of shikonin. With increases in shikonin concentrations, the number of apoptotic cells gradually increased (**Table 2** and **Figure 2**). Additionally, flow cytometry analysis of cell cycle distribution indicated that the proportion of cells in the G2/M phase increased significantly after shikonin 5 μmol/L and 20 μmol/L treatment of U251

Table 3. Effects of shikonin on invasiveness of U251 cells

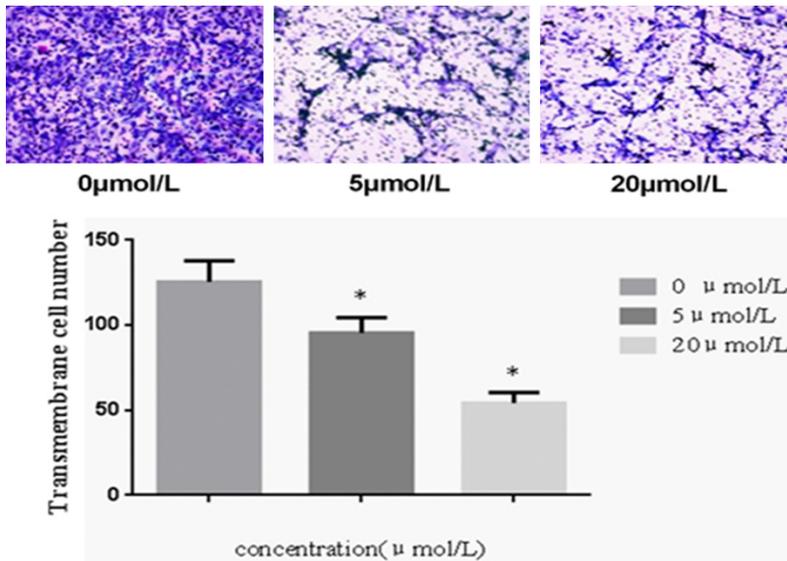| Groups (Concentration of shikonin)	Transmembrane cell number
0 µmol/L	125.57±12.33
5 µmol/L	95.52±8.99
20 µmol/L	54.26±6.14

Figure 3. Effects of shikonin on invasiveness of U251 cells. Comparison of the concentration of shikonin on the experimental group with the control group (*P < 0.05).

cells (P < 0.05). Data suggests that shikonin effectively prevented cells from arresting in the G2/M phase and significantly inhibited cell proliferation (**Table 2**).

Effects of shikonin on invasiveness of U251 cells

Results of Transwell invasion assays showed that the number of invading cells in each field in shikonin 5 µmol/L and 20 µmol/L groups was significantly lower than that of the shikonin 0 µmol/L group (P < 0.05), suggesting that shikonin effectively inhibits the invasive ability of cells (**Table 3** and **Figure 3**).

Effects of shikonin on expression of apoptosis-related proteins in U251 cells

Western blot analysis showed that expression levels of apoptosis-related proteins, Cyt c, AIF, and Bax, in shikonin 5 µmol/L and 20 µmol/L groups were significantly higher than those in the 0 µmol/L shikonin group (P < 0.05). Expression of apoptosis-inhibiting gene Bcl-2 was

significantly decreased (P < 0.05) (**Table 4** and **Figure 4**).

Effects of shikonin on expression of Wnt/β-catenin signaling pathway proteins and mRNA in U251 cells

Expression levels of β-catenin, downstream c-Myc genes, and proteins in shikonin 5 µmol/L and 20 µmol/L groups were significantly lower than in the shikonin 0 µmol/L group (P < 0.05) (**Table 5**, **Figures 5** and **6**), suggesting that shikonin may repress the core components of Wnt/β-catenin signaling pathways.

Discussion

Studies have shown that [14-16] shikonin significantly inhibits the growth and metastasis of gastric cancer cell SGC-7901, bladder cancer cell T24, breast cancer, and many other malignant tumor cells. However, there are few reports concerning its inhibitory effects on glioma cells and the mechanisms of inhibition. In this study, glioma U251 cells were used and shikonin was administered for intervention. Present results demonstrated that shikonin promotes the apoptosis of U251 cells and inhibits invasion and metastasis. This may be achieved by inhibiting Wnt/β-catenin signaling pathways, upregulating apoptosis-related proteins, and inducing apoptosis of glial cells.

The continuous division and proliferation of cancer cells is the basis for progression of malignant tumors. Therefore, detection of proliferation of cancer cells enables researchers to monitor the effects of drug treatment [17]. In this study, the proliferation of U251 cells was detected by MTT assay. Survival rates of U251 cells gradually decreased with increased shikonin concentrations, suggesting that the inhibitory effects of shikonin on glioma cells were concentration-dependent. Apoptosis or the pr-

Table 4. Effects of shikonin on expression of apoptosis-related proteins in U251 cells

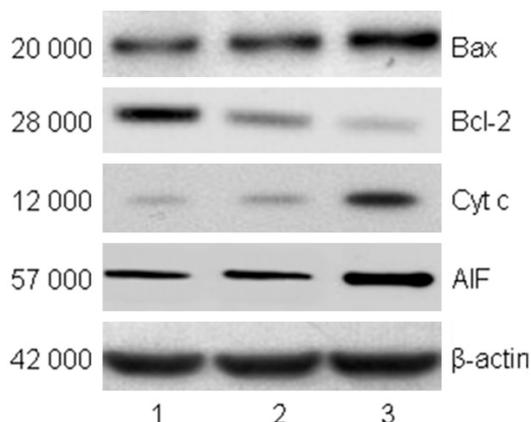| Groups (Concentration of shikonin)	Bax	Bcl-2	Cyt-c	AIF
0 μmol/L	0.586±0.025	0.892±0.021	0.187±0.019	0.315±0.022
5 μmol/L	0.791±0.031	0.614±0.018	0.608±0.023	0.514±0.036
20 μmol/L	0.873±0.035	0.422±0.015	1.099±0.032	0.899±0.039

Figure 4. Effects of shikonin on apoptosis-related protein expression in U251 cells.

ogrammed cell death of tumor cells upon a drug treatment indicates that the anti-tumor drug played a therapeutic role. In the current study, flow cytometry was used to detect the apoptosis of U251 cells. Results showed that, with increased shikonin concentrations, the number of apoptotic cells gradually increased. Analysis of cell cycle distribution revealed that the proportion of cells in the G2/M phase increased significantly when shikonin 5 μmol/L and 20 μmol/L was applied to U251 cells. Results suggest that shikonin effectively prevented cells from arresting in the G2/M phase and significantly inhibited cell proliferation. Studies have shown that [18, 19] shikonin significantly inhibits cell proliferation and promotes apoptosis of U251 human glioma cells, consistent with present findings.

Cyt c, AIF, Bax, and Bcl-2 proteins are currently recognized as crucial proteins in apoptosis signaling pathways [20]. Bcl-2 and Bax belong to the BCL-2 family. Both increase the permeability of mitochondria in cells and promote the release of apoptotic activators, such as Cyt c and AIF, thereby promoting apoptosis. The current study showed that shikonin significantly decreased levels of anti-apoptotic protein Bcl-2 and upregulated expression of pro-apoptotic

proteins Cyt c, AIF, and Bax, in a concentration-dependent manner. Results suggest that shikonin plays a role in promoting apoptosis of tumor cells.

The classical Wnt/β-catenin signaling pathway, of which β-catenin is the core protein, plays a key role in the generation and progression of tumors [21]. Under normal circumstances, β-catenin in the cytoplasm combines with Axin, GSK-3p, and APC and maintains a low activity state. When the Wnt signal is activated, β-catenin protein accumulates in the cytoplasm and migrates to the nucleus, thereby initiating the transcription of c-Myc and other deuterium genes downstream of the pathway. β-catenin stimulates cell proliferation to induce the loss of control of cell proliferation and failure of apoptosis, inducing the occurrence of tumors [22]. Studies have shown [23] that abnormal activation of Wnt/β-catenin signaling pathways is involved in the occurrence and progression of gliomas. Some scholars found that [4] there was β-catenin expression in glioma cells. This expression increased with increases in the levels of gliomas.

Results of the current study showed that expression levels of β-catenin and downstream c-Myc genes and proteins in shikonin 5 μmol/L and 20 μmol/L groups were significantly reduced, suggesting that shikonin represses the core components of Wnt/β-catenin signaling pathways. Immunofluorescence experiments suggested that fluorescence intensities of β-catenin in the nucleus and cytoplasm of shikonin 5 μmol/L and 20 μmol/L groups were significantly reduced, indicating that shikonin can strongly inhibit the activity and expression of β-catenin in the nucleus and cytoplasm. Results suggest that shikonin inhibits the proliferation of glioma cells by repressing the activity of Wnt/β-catenin signaling pathways, because of a decrease in the free cytosolic β-catenin proteins into the nucleus. The current study, in accord with conclusions of other studies, sug-

Table 5. Effects of shikonin on β-catenin and c-myc protein and gene expression in U251 cells

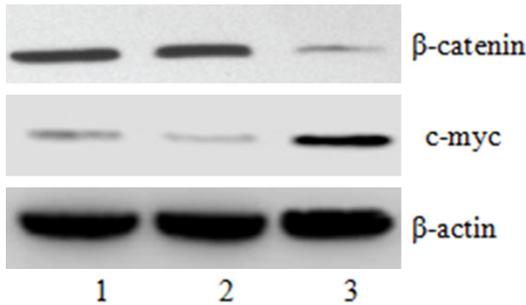| Groups (Concentration of shikonin)	Protein		Gene	
	β-catenin	c-myc	β-catenin	c-myc
0 µmol/L	0.924±0.031	0.957±0.028	0.382±0.015	0.279±0.018
5 µmol/L	0.609±0.024	0.688±0.023	0.209±0.013	0.115±0.013
20 µmol/L	0.372±0.014	0.406±0.016	0.165±0.012	0.096±0.009

Figure 5. Effects of shikonin on apoptosis-related protein expression in U251 cells. 1. shikonin 0 µmol/L; 2. shikonin 5 µmol/L; 3. shikonin 20 µmol/L.
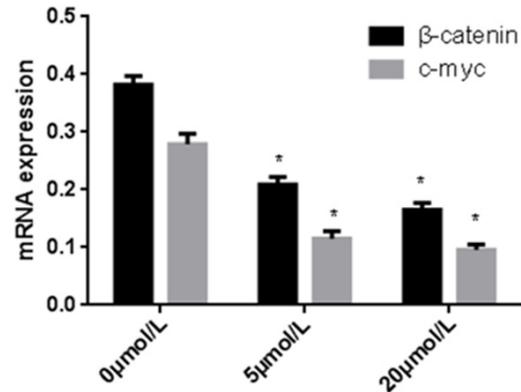
gests the inhibitory effects of shikonin on glioma cells [24].

Conclusion

In summary, shikonin significantly inhibits proliferation and promotes apoptosis of U251 glioma cells. The mechanisms may proceed through the inhibition of Wnt/β-catenin signaling pathways. Therefore, shikonin may be applied as a therapeutic TCM monomer component for treatment of gliomas. However, the current study is a preliminary investigation into the *in vitro* anti-tumor activity of shikonin. Future studies should be carried out using animal model systems to further characterize the inhibitory effects of shikonin on proliferation of glioma cells.

Disclosure of conflict of interest

None.

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Figure 6. Effects of shikonin on apoptosis-related mRNA expression in U251 cells.

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