# Shikonin induces apoptosis and G0/G1 phase arrest of gallbladder cancer cells via the JNK signaling pathway

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**Abstract.** Shikonin, a natural product isolated from the roots of *Lithospermum erythrorhizon*, is considered to have antitumor effects. Gallbladder cancer (GBC) is a prevalent biliary tract malignancy with few curative therapeutic stragegies and poor prognosis. In the present study, we detected the effects of shikonin on GBC cells as well as the underlying molecular mechanisms. The results demonstrated that GBC cell proliferation was inhibited by shikonin as determined by MTT and colony formation assays. Flow cytometry results demonstrated that shikonin treatment enhanced apoptosis and promoted G0/G1 phase arrest in the GBC cells. Western blot assay showed that shikonin induced mitochondrial-dependent apoptosis via the JNK signaling pathway. Moreover, shikonin suppressed tumor growth in mice bearing GBC-derived xenografts in a dose-dependent manner without side-effects. These results revealed that shikonin exhibits anticancer effects on GBC cells by inducing apoptosis and regulating the cell cycle. Taken together, shikonin may be a novel and safe chemotherapeutic agent for the treatment of GBC.

## Introduction

Gallbladder cancer (GBC) is a prevalent biliary tract malignancy (1,2), and the seventh most common gastrointestinal cancer worldwide (3,4). The prognosis for GBC is extremely poor (5,6), and GBC is usually diagnosed at a late stage due to the lack of specific symptoms (7,8). The only curative therapy for GBC is surgical resection (9). The American College of Surgeons reported in 2010 that the 5-year survival rate for stage IV GBC is ~4% (10), resulting from early infiltration by lymphatic, perineural and hematogenous routes, as well as direct invasion into the liver (11). Thus, the identification of novel and promising agents for the treatment of GBC is urgently needed.

Shikonin (Fig. 1) is a compound isolated from the roots of *Lithospermum erythrorhizon*, a traditional medicinal plant (12). Numerous studies have demonstrated that shikonin exhibits antitumor effects such as inhibition of cancer cell proliferation (13), induction of apoptosis (14), attenuation of invasion and migration (15), and inhibition of proteasome activity (16), angiogenesis (17) and cancer cell glycolysis (18). However, no studies have been conducted concerning the effects of shikonin on GBC cells and the potential mechanisms involved. Therefore, the present study was designed to investigate the effect of shikonin on GBC cells *in vitro* and xenograft tumors *in vivo*, and explore the underlying molecular mechanisms underlying the effects. The present study may offer a promising agent for the treatment of GBC.

## Materials and methods

*Chemicals and reagents.* Shikonin was obtained from the Shanghai Jinsui Biotechnology Co., Ltd. (Shanghai, China). After being dissolved in dimethyl sulfoxide (DMSO) at a stock solution (0.05 mol/l), shikonin was stored at -20°C. Subsequent dilutions were conducted with culture medium. An equal proportion of vehicle was added to the control cells.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) and Hoechst 33342 were obtained from Sigma Chemical Company (St. Louis, MO, USA). An Annexin V/propidium iodide (PI) apoptosis kit was obtained from Invitrogen (Carlsbad, CA, USA). Primary antibodies against Bcl-2, Bax, cleaved caspase-9, cleaved caspase-3, cleaved PARP, cyclin D1, CDK4, JNK, phosphorylated JNK (p-JNK), GAPDH and secondary antibodies (goat anti-rabbit) were obtained from Cell Signaling Technology (Danvers, MA, USA).

*Cell lines and culture.* NOZ and EHGB-1 (human GBC) cell lines were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences and grown in high-glucose

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Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA). The media contained 100 U/ml penicillin (HyClone, Logan, UT, USA), and 10% fetal bovine serum (FBS; Gibco) and 100 $\mu$g/ml streptomycin. The cells were grown at 37˚C in an atmosphere with 5% $CO_2$.

*Cell viability assay.* Cell viability was detected by the MTT assay. NOZ and EHGB-1 cells ($2x10^3$/well) were seeded into 96-well plates and incubated overnight. Then, the cells were treated with shikonin at various concentrations of 0, 0.5, 1, 2, 3 and 4 $\mu$mol/l for 24, 48 and 72 h. After treatment, 10 $\mu$l of MTT solution (5 mg/ml) was added to each well and incubation was carried out at 37˚C for 4 h. The culture medium was then replaced with 100 $\mu$l of DMSO. Absorbance of the solution at 490 nm was measured with a microplate reader (BioTek, Winooski, VT, USA). The results represent the average of three parallel samples.

*Colony formation assay.* NOZ and EHGB-1 cells were liquated as single cell suspensions and 600 cells were seeded into each well of 6-well plates. Cells were treated with shikonin (0, 0.1, 0.2 and 0.3 $\mu$mol/l for NOZ and EHGB-1), and cultured for ~14 days. The cells were then fixed with 4% paraformaldehyde for 15 min, and stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) for 30 min. The total number of colonies (>50 cells/colony) was manually counted.

*Cell apoptosis assay.* Cells were seeded in 6-well plates and treated with shikonin (0, 1, 2 and 3 $\mu$mol/l) for 48 h. After adherence, cells were harvested and washed twice with cold phosphate-buffered saline (PBS), then resuspended at a density of $1x10^6$ cells/ml. Next, 300 $\mu$l of binding buffer containing 5 $\mu$l of Annexin V-FITC and 5 $\mu$l of PI (100 $\mu$g/ml) was added to the cells, followed by incubation in the dark for 30 min. The samples were determined using flow cytometry (BD Biosciences, San Diego, CA, USA).

*Cell cycle analysis.* NOZ and EHGB-1 cells were treated with shikonin in different concentrations (0, 1, 2 and 3 $\mu$mol/l) for 48 h. Cells were harvested, washed with cold PBS, and fixed in 70% ethanol overnight. Then, the cells were washed, added with RNase and PI (Sigma-Aldrich), and incubated in the dark for 30 min. The cells were detected by flow cytometry (BD Biosciences). The percentages of cells in each phase of the cell cycle were analyzed by CellQuest acquisition software (BD Biosciences).

*Hoechst 33342 staining.* After treatment with shikonin (0, 1, 2 and 3 $\mu$mol/l) for 48 h, the NOZ and EHGB-1 cells were washed in cold PBS and added with methanol:acetic acid (3:1) for 10 min. The cells were stained with Hoechst 33342 5 $\mu$g/ml for 10 min at 37˚C and subsequently observed with a fluorescence microscope (Leica Biosystems, Wetzlar, Germany).

*Western blot analysis.* Cells were treated with shikonin (0, 1, 2 and 3 $\mu$mol/l) for 48 h, and then harvested, washed and lysed in RIPA buffer (Beyotime Institute of Biotechnology, Beijing, China) and protease inhibitor (Roche Applied Science, Indianapolis, IN, USA) at 4˚C for 5 min. The lysates were centrifuged at 14,000 x g for 5 min, the supernatant
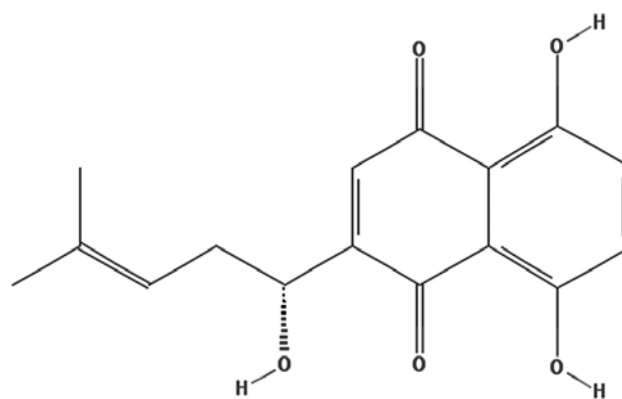


Figure 1. Chemical structure of shikonin.

was collected, and the protein concentration was detected by a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology). The proteins were loaded on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk, and incubated with primary antibodies against Bcl-2, Bax, cleaved caspase-3, cleaved caspase-9, cleaved PARP, cyclin D1, CDK4 and GAPDH overnight. The membrane was washed with Tris-buffered saline with Tween-20 (TBST), and then incubated with HRP-conjugated goat anti-rabbit secondary antibodies (Abcam, Cambridge, UK) for 1.5 h. The bands were detected with Gel Doc 2000 (Bio-Rad, Hercules, CA, USA).

*In vivo tumor xenograft study.* Four-week-old male athymic nude mice were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All procedures were approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University. NOZ cells were resuspended in PBS ($1x10^6$ cells in 0.2 ml), and injected into the right flank of each mouse. Twenty-four hours after inoculation, the mice were randomly divided into three groups (5 mice/group). The control group was injected with vehicle (10% DMSO and 90% PBS) and the others were injected with shikonin (1 or 3 mg/kg) every 2 days for up to 14 days. The body weight was measured every 2 days. On day 15, the tumor tissues were removed and weighed after sacrifice of the mice injected with lethal dose of pentobarbital. The tumor volume (V) = 1/2 x length x width$^2$.

*Statistical analysis.* All assays were performed three dependent times, and data are expressed as means ± SD. The Student's t-test in GraphPad Prism was applied to assess the difference between two groups (GraphPad Software, San Diego, CA, USA). $P<0.05$ ($^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$) was considered to indicate a statistically significant result.

## Results

*Shikonin suppresses proliferation and colony forming of GBC cells.* The cell proliferation was assessed by MTT assay. We discovered an obvious reduction in the viability of shikonin-treated cells (Fig. 2A). The inhibitory concentration
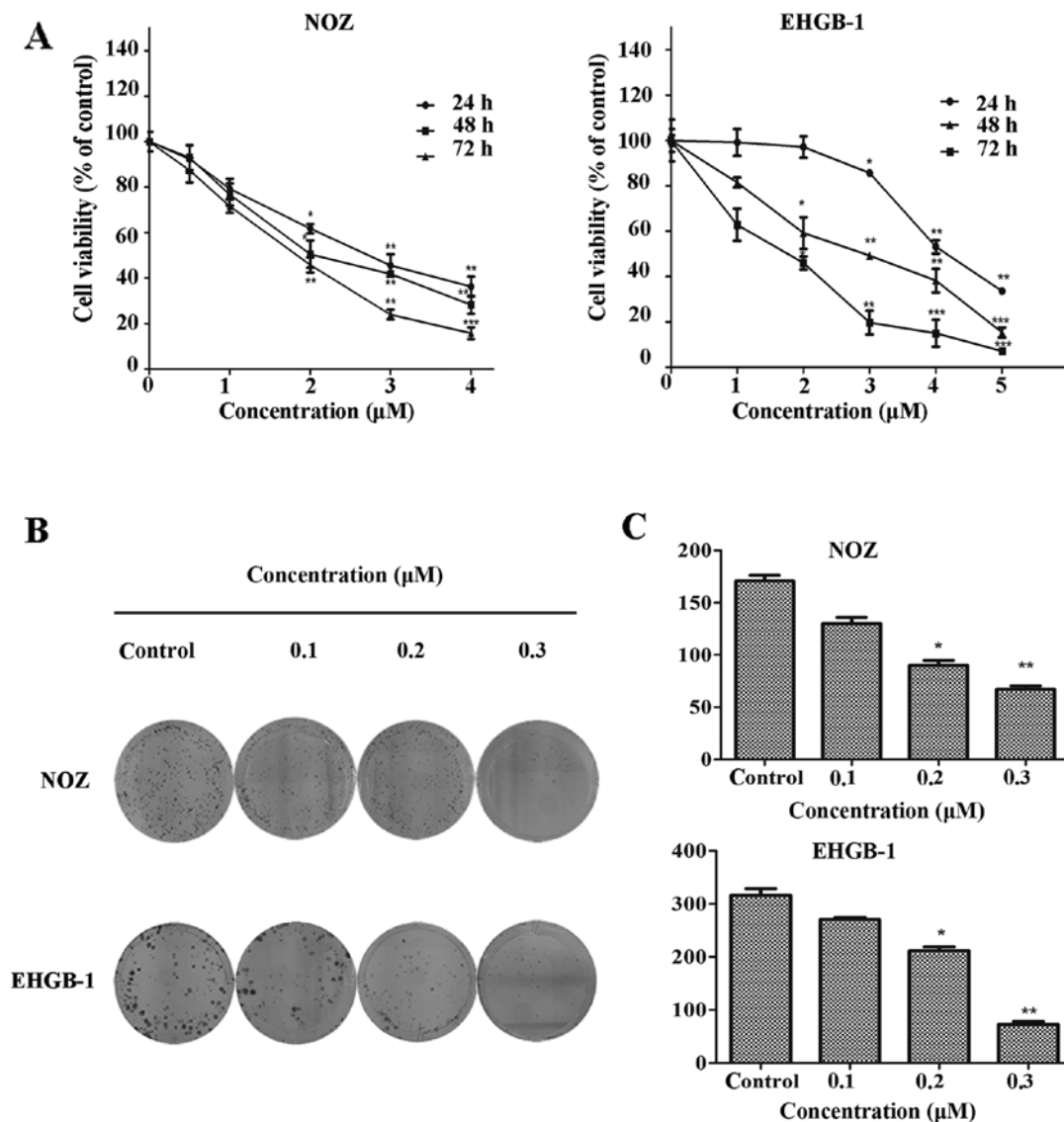
Figure 2. (A) Shikonin suppresses proliferation and colony forming of GBC cells. Cell viability was determined separately by MTT assay following treatment with shikonin for 24, 48 and 72 h. (B) NOZ and EHGB-1 cells were treated with different concentrations of shikonin (0, 0.1, 0.2 and 0.3 $\mu$mol/l) for 14 days. (C) The colony counts are shown. All data are expressed as means ± SD, obtained from three independent experiments; *P<0.05, **P<0.01, ***P<0.001.

$(IC)_{50}$ for NOZ and EHGB-1 cells was ~2 $\mu$mol/l at 48 h treatment. The ability of NOZ and EHGB-1 cells to form colonies when treated with shikonin was demonstrated by the colony formation assay (Fig. 2B). The result suggested that shikonin exerted a negative influence on colony formation ability. Furthermore, we found less amount of clone formations in shikonin-treated groups than those in the control groups (Fig. 2C). These results indicated that shikonin obviously suppressed the proliferation and colony forming of NOZ and EHGB-1 cells.

*Shikonin induces mitochondrial-dependent apoptosis via the JNK signaling pathway in GBC cells*. The impacts of shikonin on apoptosis in the NOZ and EHGB-1 cells were detected using Annexin V/PI staining and flow cytometry. As shown in Fig. 3A, the percentage of surviving cells was reduced, whereas the percentage of apoptotic cells was obviously increased (Fig. 3B).

To further confirm the cell apoptosis, we applied Hoechst 33342 staining to examine the change in nuclear morphology. The untreated cells were round with uniformity in chromatin distribution, while vivid chromatin condensation and lobulated nuclear fragmentation were revealed in cells treated with shikonin (Fig. 3C). Furthermore, the proportion of apoptotic nuclei that was obviously increased agreed with the gradually increased shikonin concentration.

Proteins of the Bcl-2 and caspase family play major roles in initiation and maintenance of mitochondrial apoptosis (19). To further explore the molecular mechanism underlying shikonin-mediated apoptosis, we evaluated the quantitative levels of apoptosis-related proteins via western blot analysis, including Bax, bcl-2, cleaved caspase-9, cleaved caspase-3 and cleaved PARP. As shown in Fig. 4A, we observed increased expression of Bax, cleaved caspase-9, cleaved caspase-3 and cleaved PARP and downregulation of Bcl-2. Moreover, the Bcl-2/Bax ratio represents apoptotic activity (20), as cell apoptosis is promoted when the ratio decreases. In the present study, we found a significant decrease in the ratio of Bcl-2/Bax in the shikonin-treated cells (Fig. 4B). We also observed
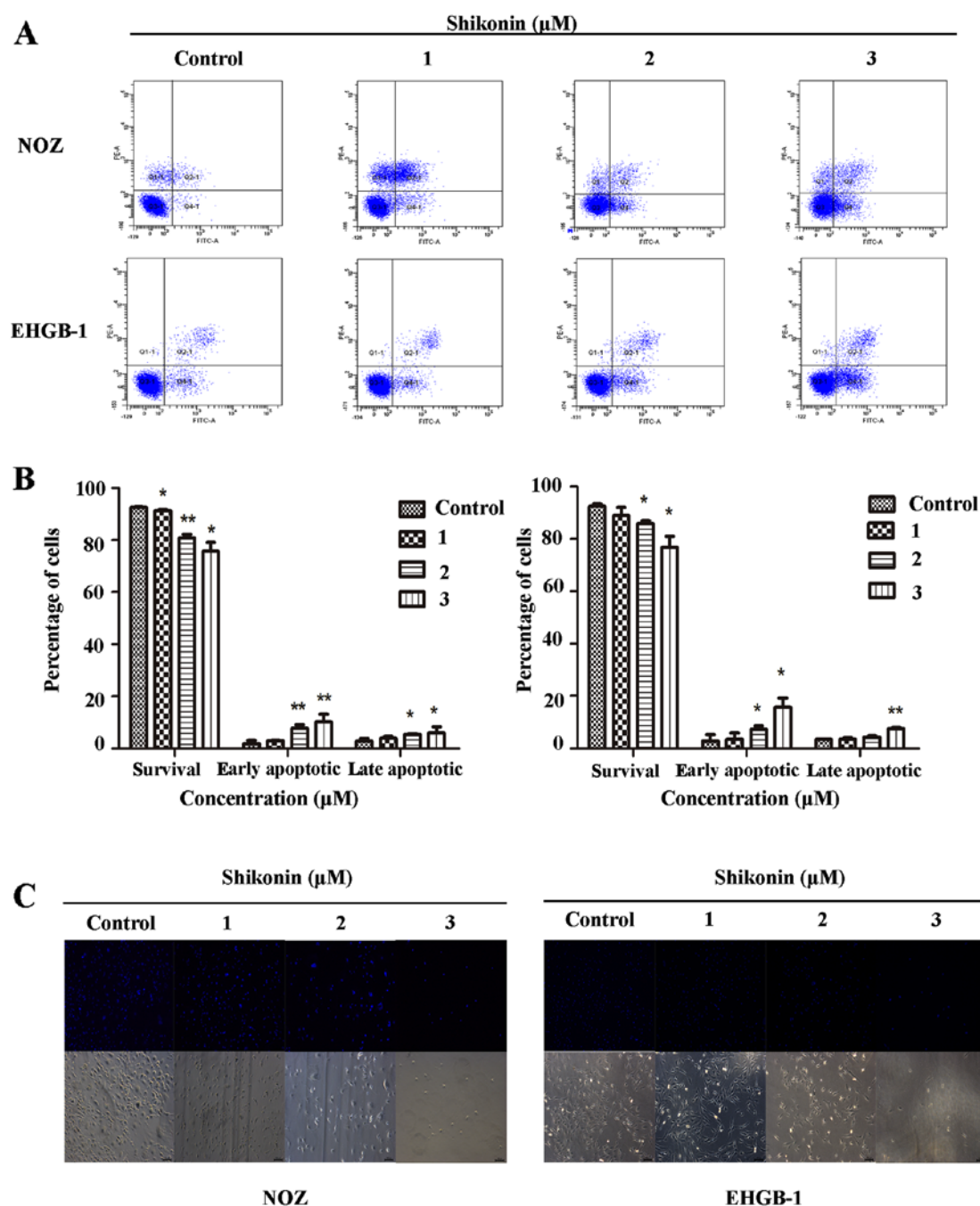
Figure 3. Shikonin induces apoptosis in GBC cells. (A) NOZ and EHGB-1 cells were stained with Annexin V-FITC/PI and analyzed by flow cytometry following treatment with shikonin (0, 1, 2 and 3 $\mu$mol/l) for 48 h. The right lower quadrant and right upper quadrant represent the percentage of early and late apoptotic cells, respectively. (B) The proportion of apoptotic cells is shown (mean ± SD). (C) Differences in nuclear morphology were determined by Hoechst 33342 staining and observed under fluorescence microscopy. Representative images from three independent experiments are shown; *P<0.05, **P<0.01 vs. the control group.

upregulation of p-JNK while no significant change in the expression of JNK was noted.

Together, these results revealed that shikonin may initiate mitochondrial-dependent apoptosis via the JNK signaling pathway in GBC cells.

*Shikonin triggers G0/G1 phase arrest by regulating cell cycle-related proteins in GBC cells.* The percentage of cells in each phase of the cell cycle was determined via flow cytometry. We found an obvious increase in the proportion of G0/G1 cells (Fig. 5B), indicating that shikonin suppressed cell

cycle progression in the NOZ and EHGB-1 cells (Fig. 5A). We detected the expression of cell cycle-related proteins cyclin D1 and CDK4, and observed an obvious decrease in the NOZ and EHGB-1 cells (Fig. 5C). Therefore, shikonin may inhibit GBC cell proliferation by triggering G0/G1 phase arrest.

*Shikonin exhibits anticancer effects in vivo.* To further detect whether shikonin suppresses tumor growth *in vivo*, nude mice with palpable tumor xenografts were injected with vehicle (10% DMSO and 90% PBS) or shikonin (1 and 3 mg/kg) every other day. This dose was determined to be effective (21,22) and
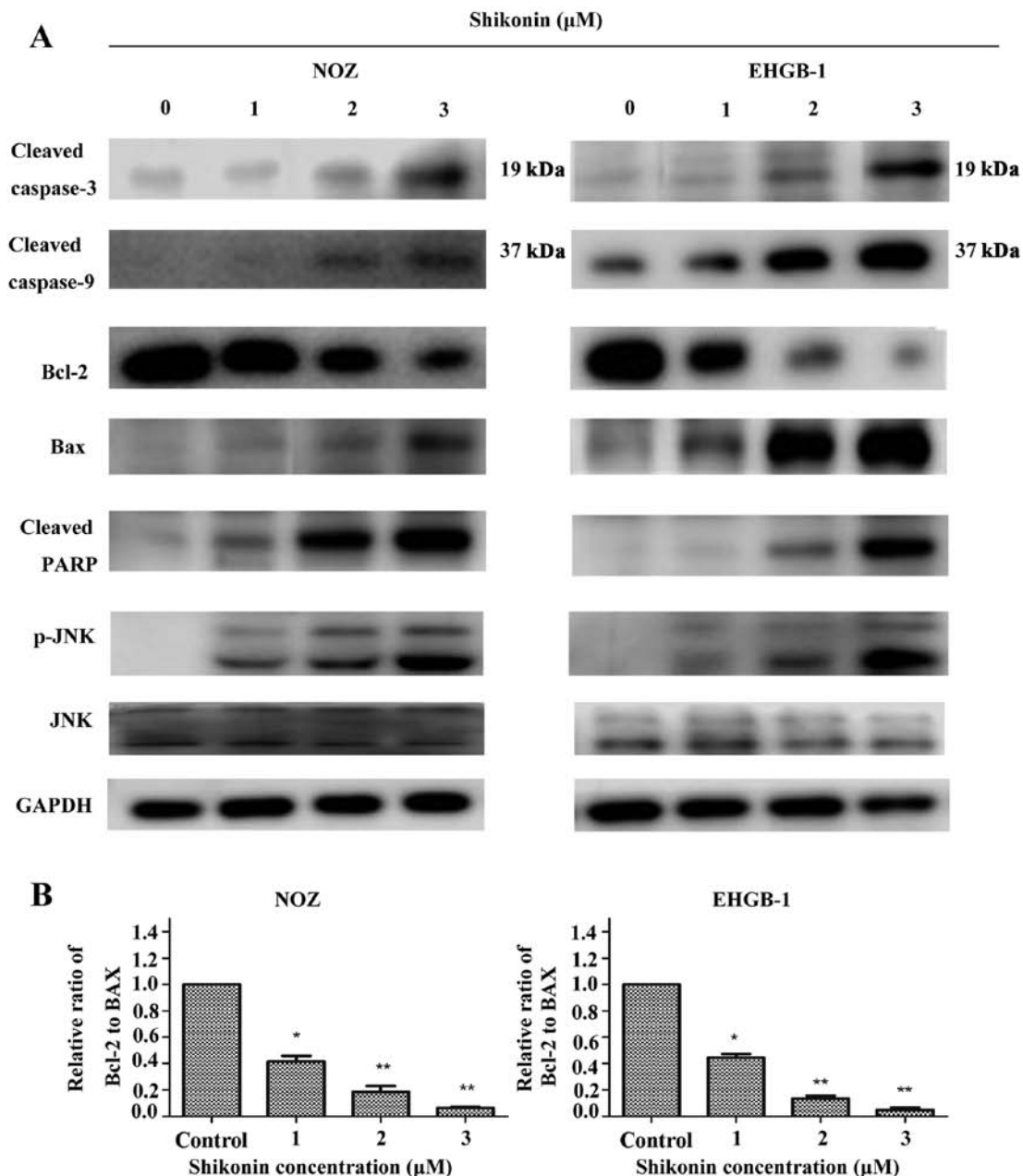
Figure 4. Shikonin induces apoptosis via the JNK signaling pathway in GBC cells. (A) NOZ and EHGB-1 cells were treated with shikonin (0, 1, 2, and 3 $\mu$mol/l) for 48 h. The expression levels of Bcl-2, Bax, cleaved caspase-9, cleaved caspase-3, cleaved PARP, p-JNK and JNK were determined by western blot analysis. (B) Relative ratio of Bcl-2/Bax are shown; *P<0.05, **P<0.01 vs. the control group.

a non-toxic level; LD$_{50}$ of shikonin in the mouse is 20 mg/kg when injected in an intraperitoneal manner (23). After injection, the bile and liver were previously found to contain the highest levels of shikonin, and most of the excreted metabolite was transformed (24). As shown in Fig. 6A-D, the shikonin group produced the smaller tumors when compared with the tumor volume of the vehicle group. The results demonstrated that tumor growth was obviously suppressed in the shikonin-treated mice in a dose-dependent manner. Moreover, we found no significant difference in regards to body weight between the vehicle and shikonin-treated groups (Fig. 6E), suggesting that shikonin had no side-effects in nude mice. As shown in Fig. 6F, we detected the upregulation of Bax, cleaved caspase-9, cleaved caspase-3 and cleaved PARP and downregulation of Bcl-2 in the tumor tissues by western blot assay.

**Discussion**

Recently, various studies have shown that shikonin acts as an anticancer agent. However, the effects of shikonin on gall-bladder cancer (GBC) cells required elucidation. In the present study, we demonstrated that shikonin induced apoptosis and G0/G1 phase arrest in human GBC cells.

The cytotoxic effects of shikonin were assessed by MTT and colony formation assays. We found that shikonin significantly inhibited cell growth in a time- and dose-dependent manner. To better understand the apoptotic effect of shikonin in GBC cells, we performed flow cytometric analysis and Hoechst 33342 staining. The results showed that shikonin induced apoptosis in GBC cells. To date, two pathways play crucial roles in cell apoptosis, including the mitochondrial intrinsic pathway
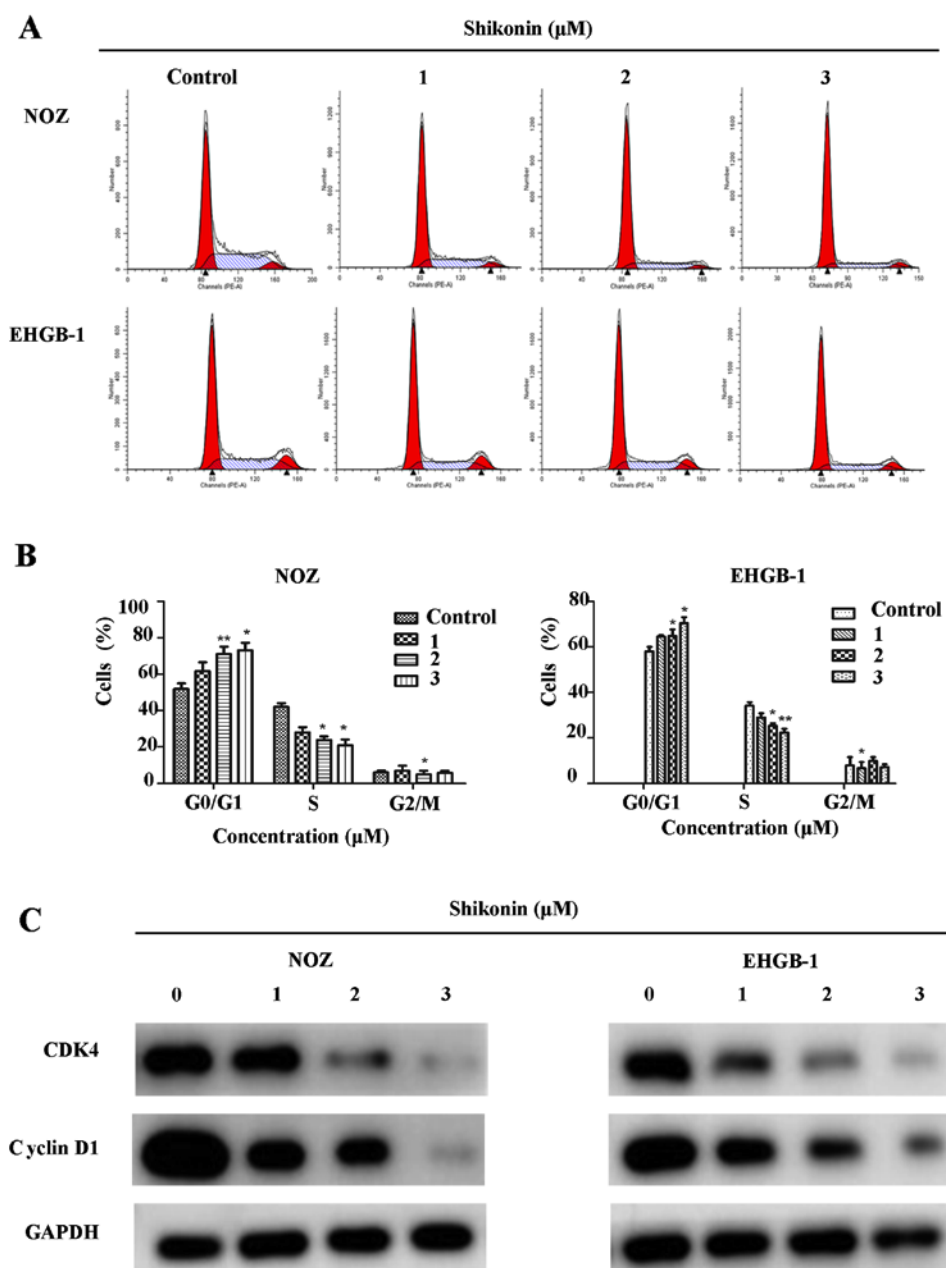
Figure 5. Shikonin triggers G0/G1 phase arrest via regulating cell cycle-related proteins in GBC cells. (A) The percentages of cells in each cell cycle were determined by flow cytometry. (B) Data are presented as the mean ± SD (n=3). (C) Western blot analysis of expression levels of cyclin D1 and CDK4. Representative blots from three independent experiments are shown; *P<0.05, **P<0.01 vs. the control group.

and the death receptor-induced extrinsic pathway (25). In the present study, we demonstrated the importance of the intrinsic pathway in shikonin-induced apoptosis. Proteins in the Bcl-2 family are crucial regulators of the mitochondrial-dependent apoptotic pathway (26). Increased expression levels of Bax and reduced expression of Bcl-2, the dominant inhibitor of Bax, were observed in the present study. Moreover, the ratio of Bcl-2/Bax is a critical factor which determines the apoptosis threshold (27). In the present study, we detected that the Bcl-2/Bax ratio was significantly decreased following treatment with shikonin. These results suggest that shikonin induced apoptosis in the GBC cells.

We also detected the expression level of caspase and cleaved PARP. Caspase-9 is activated in a mitochondrial-dependent intrinsic pathway, which then mediates the activation of

caspase-3 (28). Caspase-3 is an important molecule to trigger the cleavage of downstream proteins, such as PARP, in the caspase-dependent apoptosis pathway, eventually leading to apoptosis (29). PARP can be triggered in cells undergoing stress stimulus, resulting in chromatin lysis and finally triggering apoptosis (30). In the present study, we observed an obvious upregulation of cleaved caspase-3, and -9, and PARP.

Shikonin has been shown to induce cell apoptosis and inhibit proliferation in dozens of cancers through various signaling pathways, such as Erk (31), PI3K/AKT (32) and NF-κB signaling pathways (33). In the present study, we detected the expression levels of JNK and p-JNK. JNK plays an essential role in the intrinsic apoptotic pathway (34). After initially activated by extracellular stimuli, JNK activates caspase-9 and inhibits the anti-apoptotic protein Bcl-2 (35).
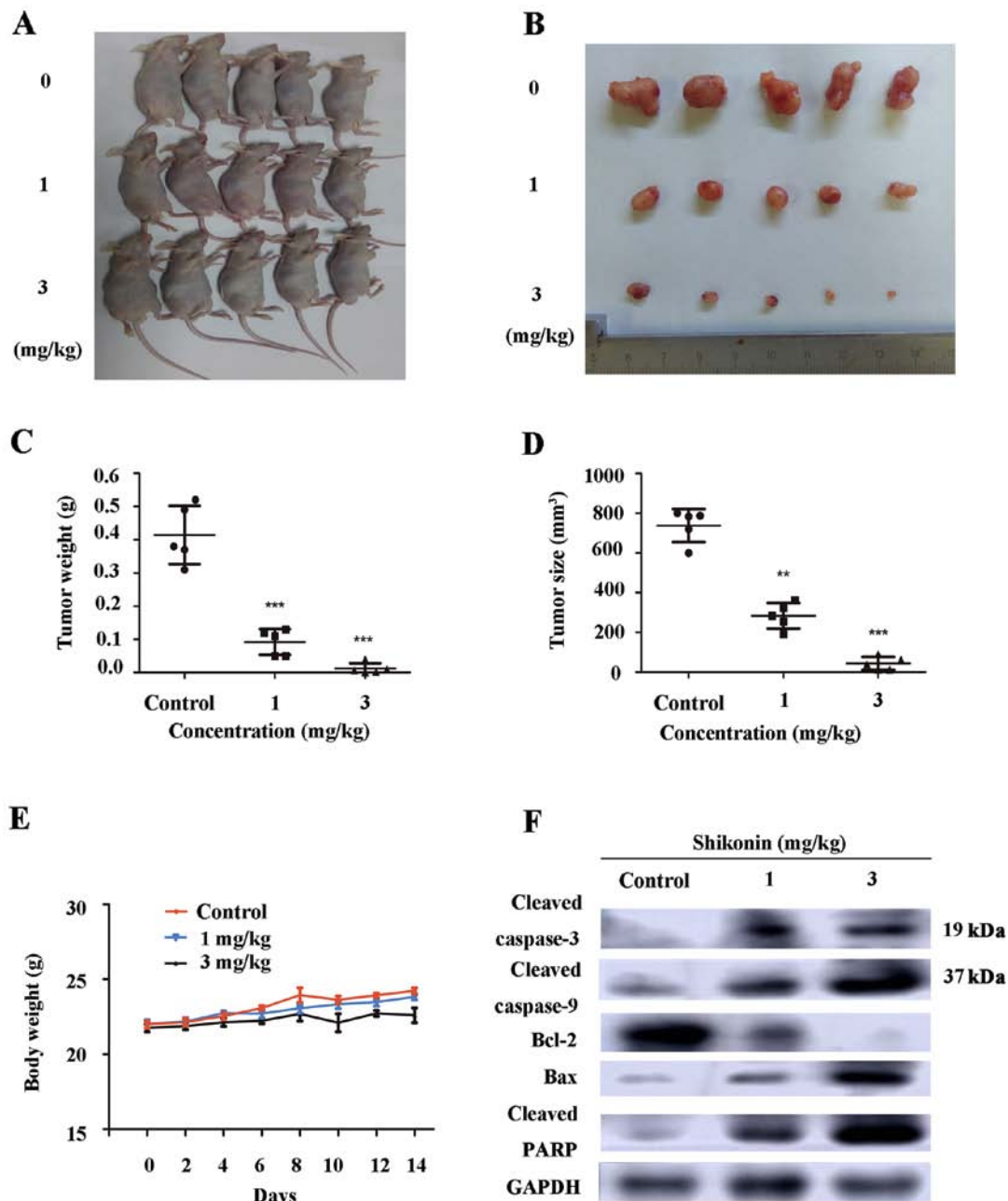
Figure 6. Shikonin exhibits anticancer effect *in vivo*. (A) Nude mice were injected with NOZ cells to establish tumor xenografts. Subsequently, the mice were injected with a vehicle (10% dimethyl sulfoxide and 90% phosphate-buffered saline) or shikonin (1 and 3 mg/kg) for up to 14 days. The sizes of tumors of the mice are shown in the images. (B-D) Tumors were collected after sacrificing the animals and subjected to weighing on day 14. (E) The body weight of the nude mice was measured during the administration period (n=5); [**]P<0.01, [***]P<0.001 vs. the control group. (F) The expression levels of apoptosis-related molecules in the tumors were detected by western blot analysis.

The data revealed upregulation of p-JNK and no obvious change in JNK, suggesting that JNK was activated in the shikonin-treated GBC cells. All in all, shikonin may trigger the apoptosis of GBC cells via the JNK signaling pathway, subsequently enhancing cell death.

Blockage of cell cycle progression has been considered as an effective strategy for the treatment of human malignancies (33). In the present study, cell cycle analysis showed that the G0/G1 phase arrest of GBC cells was induced by shikonin. The G0/ G1 phase of the cell cycle is regulated by cell cycle checkpoint proteins, such as cycling D1 and CDK4 (36), which was verified by the downregulation of CDK4 and cyclin D1 by western blot analysis.

To further confirm the apoptotic effects of shikonin, we established xenograft tumors in nude mice, and then treated them with shikonin. According to the difference in tumor volume, weight and apoptosis-related molecules in the tumors removed from the sacrificed mice, the antitumor effect of shikonin *in vivo* was verified. The insignificant change in body weight among the three groups indicated the safety of shikonin in the treatment of GBC.

In conclusion, the results demonstrated that shikonin exhibited marked anticancer effects by inducing apoptosis via the JNK signaling pathway and G0/G1 phase arrest. Moreover, tumor growth was also inhibited *in vivo* with no side-effects. Therefore, shikonin may be a novel and safe chemotherapeutic agent for the treatment of GBC.

## Acknowledgements

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