

Shikonin attenuates H₂O₂-induced oxidative injury in HT29 cells via antioxidant activities and the inhibition of mitochondrial pathway-mediated apoptosis

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Abstract. Shikonin, a natural naphthoquinone extracted from the roots of *Lithospermumery throrhizon*, possesses multiple pharmacological properties, including antioxidant, anti-inflammatory and antitumor effects. It has been hypothesized that the properties of shikonin are associated with its oxygen free radical scavenging abilities. However, the mechanism underlying the antioxidant activity of shikonin is not completely understood. The aim of the present study was to investigate the effect of shikonin against H₂O₂-induced oxidative injury in HT29 cells and to explore the underlying molecular mechanism. The concentration and duration of H₂O₂ treatment to cause maximal damage, and the effects of shikonin (2.5, 5 or 10 μg/ml) on the activity of H₂O₂-induced HT29 cells were determined by MTT assay. The apoptotic rate in HT29 cells was determined by annexin V/propidium iodide staining. HT29 cell cycle alteration was also analyzed by propidium iodide staining. Reactive oxygen species (ROS) production was assessed by monitoring 2',7'-dichlorofluorescein in diacetate fluorescence. Mitochondrial membrane potentials were determined by JC-1 staining. The activities of malondialdehyde, superoxide dismutase, caspase-9 and caspase-3 were measured using spectrophotometric assays. The expression levels of Bcl-2, Bax and cytochrome *c* were determined by western blotting. The results suggested that shikonin increased cell viability, reduced cell apoptosis and increased the proliferation index in H₂O₂-treated HT29 cells. Shikonin also significantly inhibited increases in intracellular reactive oxygen species (ROS), restored the mitochondrial membrane

potential, prevented the release of lactic dehydrogenase and decreased the levels of superoxide dismutase and malondialdehyde in H₂O₂-induced HT29 cells. Furthermore, shikonin significantly decreased caspase-9 and caspase-3 activities, increased Bcl-2 expression and decreased Bax and cytochrome *c* expression levels in H₂O₂-induced HT29 cells. The results indicated that shikonin protected against H₂O₂-induced oxidative injury by removing ROS, ameliorating mitochondrial dysfunction, attenuating DNA oxidative damage and inhibiting mitochondrial pathway-mediated apoptosis.

Introduction

Oxidative stress is caused by an imbalance between the production of reactive oxygen species (ROS) and the antioxidant capacity of cellular antioxidants in biological systems (1). Excessive oxidative stress is related to the pathogenesis of various diseases, including neurodegenerative diseases, tumors and inflammation (2,3). Previous studies have demonstrated that increased levels of ROS production lead to the development of several chronic intestinal inflammatory diseases (4-6). In particular, increased ROS production is typically associated with the pathogenesis of inflammatory bowel disease, which is characterized by chronic inflammation in human gastrointestinal disease (7,8). Therefore, inhibiting oxidative stress-induced injury may serve as an important therapeutic strategy.

Shikonin, a natural naphthoquinone extracted from the roots of the traditional Chinese medicine *Lithospermumery throrhizon*, possesses multiple pharmacological properties, including antioxidant, anti-inflammatory, antiviral, enhanced immunity, antifertility and antitumor effects (9-13). Numerous studies have demonstrated that shikonin displayed efficient antioxidative activities against various types of ROS (14,15). Guo *et al* (2) reported that shikonin attenuated acetaminophen-induced acute liver injury via inhibition of oxidative stress. Several studies indicated that shikonin displayed significant protective effects in brain and hepatic ischemia/reperfusion injury by reducing ROS (16-19). However, the potential antioxidant mechanism underlying shikonin activity is not completely understood.

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The present study aimed to investigate the effects of shikonin against H₂O₂-induced oxidative stress injury in human intestinal epithelial cells and to explore the underlying molecular mechanism. In many studies, human colon cancer cells were used as oxidative damage models (20,21). Therefore, HT29 human colon cancer cells were selected to construct oxidative damage models in the present study.

Materials and methods

Cell culture and treatment. HT29 cells were purchased from American Type Culture Collection. The cell line was established at the Memorial Sloan Kettering Cancer Center and was authenticated using STR profiling. Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere with 5% CO₂ at 37°C. Cells (5x10⁴-10⁵ cells/ml) were harvested and used for subsequent experiments. Cells were divided into six groups for treatment: A control group, cells cultured in medium without H₂O₂ and shikonin; DMSO group, cells cultured in medium with 0.1% DMSO; and H₂O₂ group, cells cultured in medium with 800 μM H₂O₂ (Sigma-Aldrich; Merck KGaA) all of which were cultured for 24 h at 37°C; and 3 shikonin groups, cells pretreated with 2.5, 5 or 10 μg/ml shikonin (Shanghai Yuan Ye Biotechnology Co., Ltd.; purity ≥98%) for 6 h at 37°C, as previously described (22,23), and then co-treated with 800 μM H₂O₂ for 24 h at 37°C.

MTT assay. For the MTT assay, HT29 cells (5x10³) were seeded in 96-well plates and cultured for 24 h at 37°C. cells were treated with H₂O₂ (25-1,600 μM) for 4, 8, 12 or 24 h at 37°C, or treated with shikonin (2.5, 5, 10, 25, 50, 100 and 200 μg/ml) for 24 h at 37°C. Subsequently, cells were incubated with 0.5 mg/ml MTT (Sigma-Aldrich; Merck K Ga A) for an additional 4 h at 37°C. The supernatant was discarded and 100 μl DMSO was added to each well to dissolve the formazan crystals. Absorbance was measured at a wavelength of 490 nm using a microplate reader. The results are presented as a percentage of the control.

Early and late apoptosis detection assay. Early and late apoptosis was measured using a FITC-conjugated annexin V and propidium iodide kit (BD Biosciences). Cells (1x10⁶) were trypsinized, washed with PBS and resuspended in 1X binding buffer. Subsequently, 5 μl FITC-conjugated annexin V and 5 μl propidium iodide were added to 100 μl cell suspension. Following incubation for 15 min at room temperature in the dark, apoptosis was analyzed via flow cytometry (EXPO32 ADC; Epics XL-MCL; Beckman Coulter, Inc.).

Cell cycle assay. Cells (1x10⁶) were trypsinized with 0.25% trypsin-EDTA at room temperature for 24 h and washed three times in PBS. Cell cycle distribution was detected using a Cycle test Plus DNA reagent kit (BD Biosciences). The percentage of cells in each cell cycle phase (G₀/G₁, S and G₂/M) was calculated via flow cytometry (Epics XL-MCL; Beckman Coulter, Inc.). The software used was the inbuilt software provided with

the flow cytometer. Proliferation index [PI;PI (%)=S phase (%) + G₂/M phase (%)].

ROS measurement. Intracellular ROS levels were measured using a ROS assay kit (cat. no. S0033M; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Briefly, cells (1x10⁶) were collected and incubated for 20 min in 500 μl 2',7'-dichlorofluorescein diacetate fluorescence (DCFH-DA) solution (10 μM) at 37°C in the dark. Following washing with PBS, cells were resuspended in 500 μl PBS and analyzed via flow cytometry (EXPO32 ADC, Epics XL-MCL; Beckman Coulter, Inc.).

Levels of malondialdehyde (MDA) and superoxide dismutase (SOD) assays. Cells (1x10⁶) were collected and centrifuged at 10,000 x g for 10 min at 4°C. The levels of SOD and MDA in the supernatant were measured using SOD (cat. no. S0101S) and MDA (cat. no. S0131S) assay kits (both Beyotime Institute of Biotechnology), respectively, according to the manufacturer's protocol.

Lactate dehydrogenase (LDH) activity assay. Cell membrane integrity was determined using an LDH assay. LDH levels in the cell medium from treated cells were determined using an LDH assay kit (cat. no. C0016; Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Absorbance was measured at a wavelength of 490 nm using a microplate reader. LDH levels were calculated according to the following formula: LDH (%)=(sample-blank)/(control-blank) x100.

Mitochondrial membrane potential assay. To assess mitochondrial integrity, the mitochondrial membrane potential assay kit with JC-1 (cat. no. C2006; Beyotime Institute of Biotechnology) was used. Cells (1x10⁶) were resuspended in 500 μl medium, followed by addition of 500 μl JC-1 dye for 20 min at 37°C. Cells were rinsed twice with JC-1 dye buffer. The fluorescent signal in cells was calculated by performing flow cytometry (EXPO32 ADC; Epics XL-MCL; Beckman Coulter, Inc.).

Caspase-3 and caspase-9 activity assays. Cells (1x10⁶) were digested with trypsin and harvested by centrifugation at 1,000 x g for 5 min at 4°C. Caspase-3 and caspase-9 activities were measured using caspase-3 (cat. no. BC3830) and caspase-9 (cat. no. BC3890) activity detection kits (Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's protocol. Caspase-3 and caspase-9 activities are presented as U/mg protein.

Western blotting. Total protein was extracted from cells using RIPA buffer (Beyotime Institute of Biotechnology) containing 1 mM PMSF (Beyotime Institute of Biotechnology) and phosphatase inhibitor for 30 min on ice. Total protein was quantified using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein (20 μg) were separated via 10% SDS-PAGE and transferred to 0.45 μm PVDF membranes. After 5% nonfat milk blocking at room temperature for 2 h, the membranes were incubated with primary antibodies targeted against:

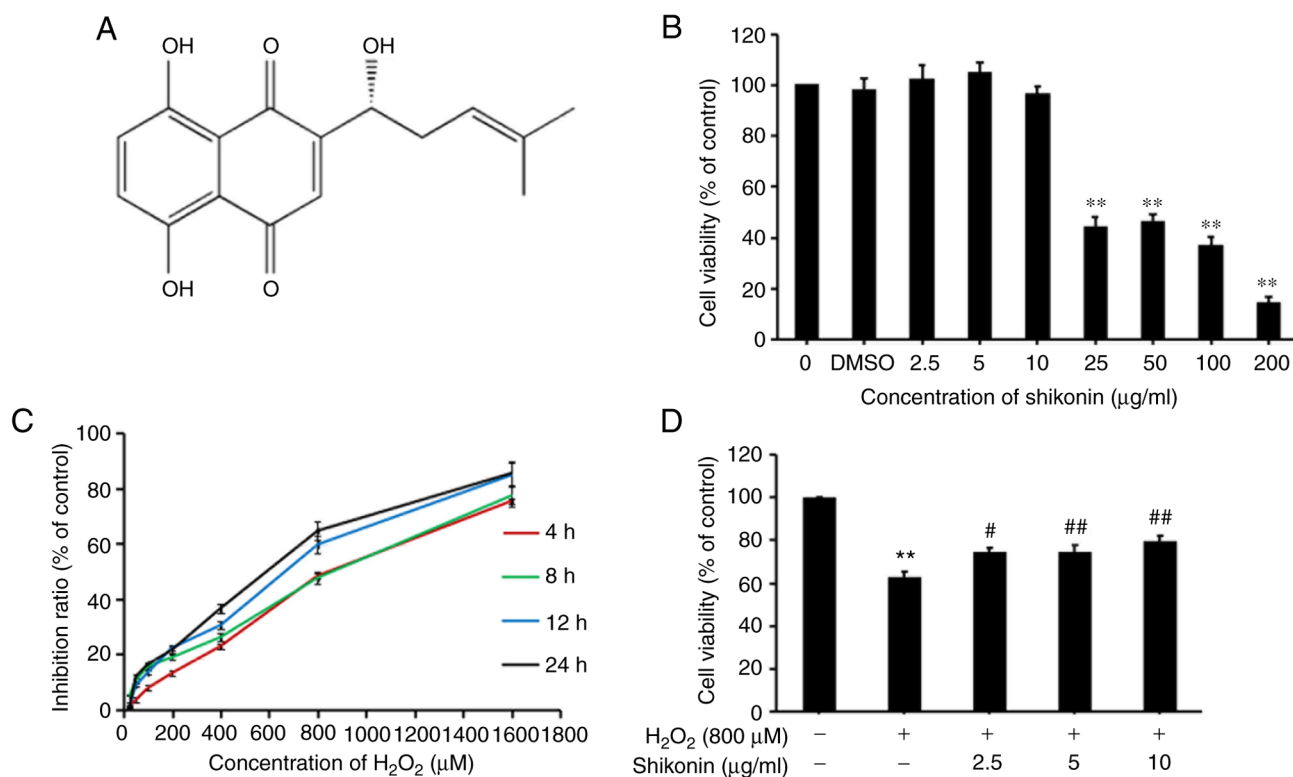


Figure 1. Effects of shikonin and H₂O₂ on cell viability. (A) The chemical structure of shikonin. (B) Cells were treated with different concentrations of shikonin for 24 h. (C) Cells were exposed to different concentrations of H₂O₂ for 4, 8, 12 and 24 h. (D) Cells were pretreated with different concentrations of shikonin for 6 h and then treated with 800 μM H₂O₂ for 24 h. Data are presented as the mean ± SD from at least three independent experiments. **P<0.01 vs. control; #P<0.05 and ##P<0.01 vs. H₂O₂.

Cytochrome *c* (cat. no. 4280; Cell Signaling Technology, Inc.; 1:1,000), Bax (cat. no. 5023; Cell Signaling Technology, Inc.; 1:1,000), Bcl-2 (cat. no. 3498; Cell Signaling Technology, Inc.; 1:1,000) and β-actin (cat. no. 4970; Cell Signaling Technology, Inc.; 1:1,000) at 4°C overnight. Following primary incubation, the membranes were incubated with a horseradish peroxidase-conjugated polymer-tagged secondary antibody (cat. no. 7074; Cell Signaling Technology, Inc.; 1:5,000) for 2 h at room temperature. Protein bands were visualized using ECL reagent (Thermo Fisher Scientific, Inc.) and protein expression was semi-quantified using Image J software (National Institutes of Health, version 1.8.0) with β-actin as the loading control.

Statistical analysis. Data are presented as the mean ± SD. All experiments were performed in triplicate. Statistical analyses were performed using SPSS software (version 19.0; IBM Corp.). Comparisons among multiple groups were analyzed using the one-way analysis of variance (ANOVA) test and Dunnett's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Shikonin attenuates H₂O₂-induced decreases in HT29 cell viability. To identify suitable H₂O₂ and shikonin concentrations and durations of action, an MTT assay was performed to evaluate cell cytotoxicity. The results indicated that H₂O₂ caused marked cytotoxicity in HT29 cells at 800 μM

compared with 0 μM, and when cells treated with 800 μM H₂O₂, the cell inhibition rates at 4, 8, 12 and 24 h were 22.91, 26.12, 30.30 and 36.64%, respectively (Fig. 1C). Therefore, cells treated with 800 μM H₂O₂ for 24 h were selected for subsequent experiments. Subsequently, the cytotoxic effect of shikonin at different concentrations was investigated by performing an MTT assay. Compared with the 0 μg/ml shikonin, no significant cytotoxic effect was observed in cells treated with 2.5, 5 and 10 μg/ml shikonin alone. Therefore, three concentrations of shikonin were used in subsequent experiments (Fig. 1B). Finally, to assess the effects of shikonin against H₂O₂-induced cytotoxicity, HT29 cells were pretreated with different concentrations of shikonin (2.5, 5 and 10 μg/ml) for 6 h, and then co-treated with 800 μM H₂O₂ for 24 h. The results indicated that the cell viability of the shikonin group gradually increased compared with the H₂O₂ group in a dose-dependent manner, suggesting that shikonin reversed H₂O₂-induced decreases in cell viability (Fig. 1D).

Shikonin attenuates H₂O₂-induced HT29 cell apoptosis. To assess the protective effects of shikonin against H₂O₂-induced cell apoptosis, flow cytometry was performed to detect apoptosis following Annexin V and propidium iodide staining (Fig. 2). Compared with the control group, the percentage of total apoptotic cells was significantly increased in the H₂O₂ group. By contrast, the shikonin groups displayed a significantly decreased percentage of apoptotic cells compared with the H₂O₂ group, indicating that shikonin inhibited H₂O₂-induced cell apoptosis.

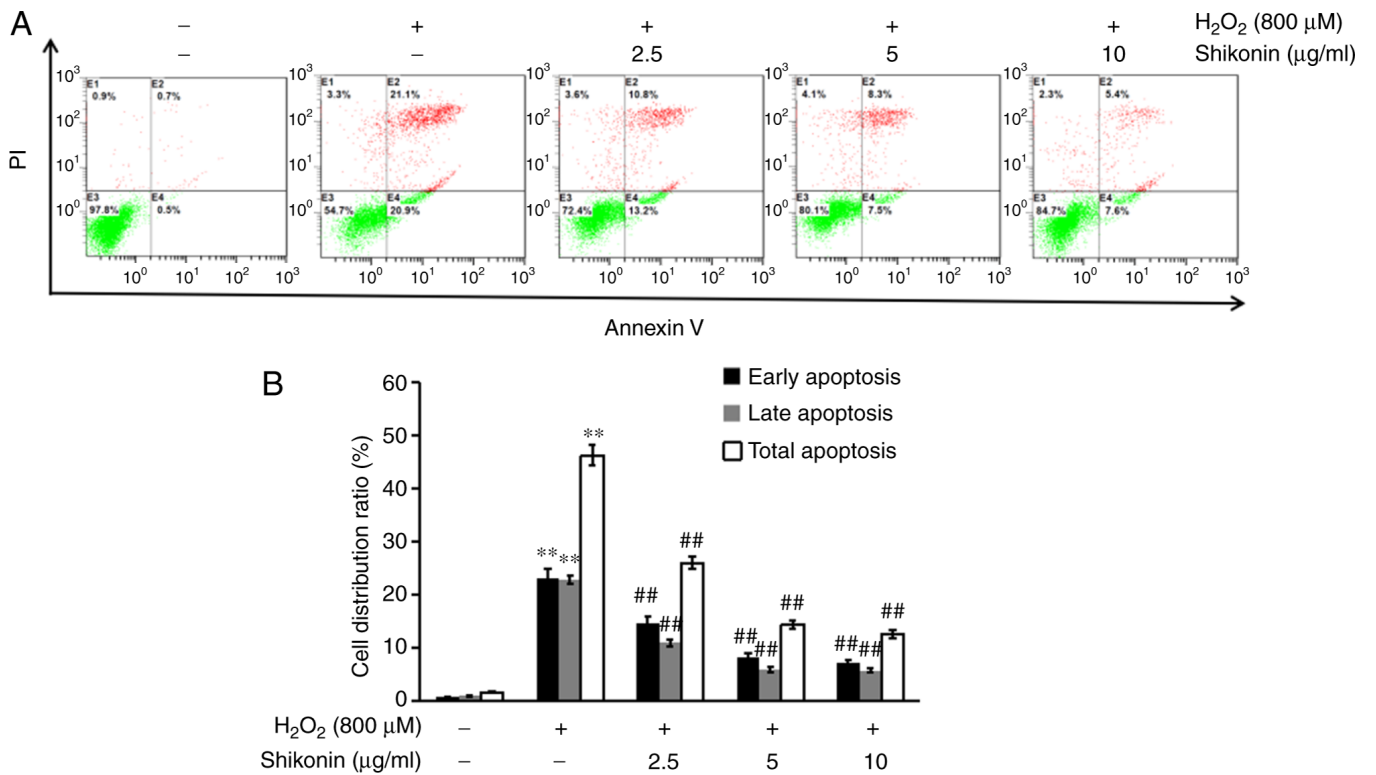


Figure 2. Effect of shikonin (2.5, 5 and 10 μg/ml) on H₂O₂-induced cell apoptosis. HT29 cell apoptosis was (A) assessed via flow cytometry and (B) quantified. Data are presented as the mean ± SD from at least three independent experiments. **P<0.01 vs. control; #P<0.01 vs. H₂O₂.

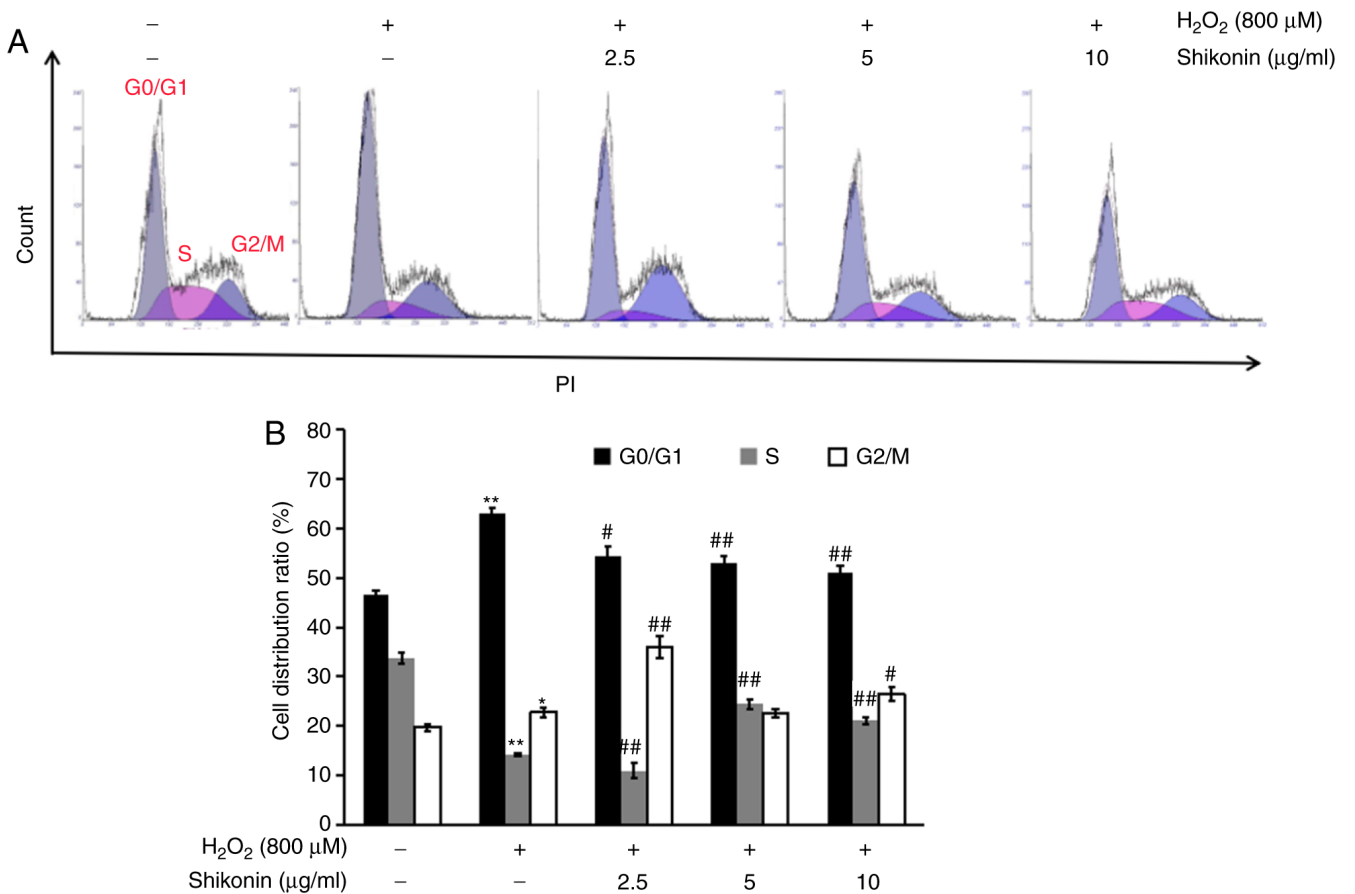


Figure 3. Effects of shikonin (2.5, 5 and 10 μg/ml) on H₂O₂-induced G₀/G₁ cell cycle arrest in HT29 cells. HT29 cell cycle distribution was (A) assessed via flow cytometry and (B) quantified. Data are presented as the mean ± SD from at least three independent experiments. *P<0.05 and **P<0.01 vs. control; #P<0.05 and ##P<0.01 vs. H₂O₂.

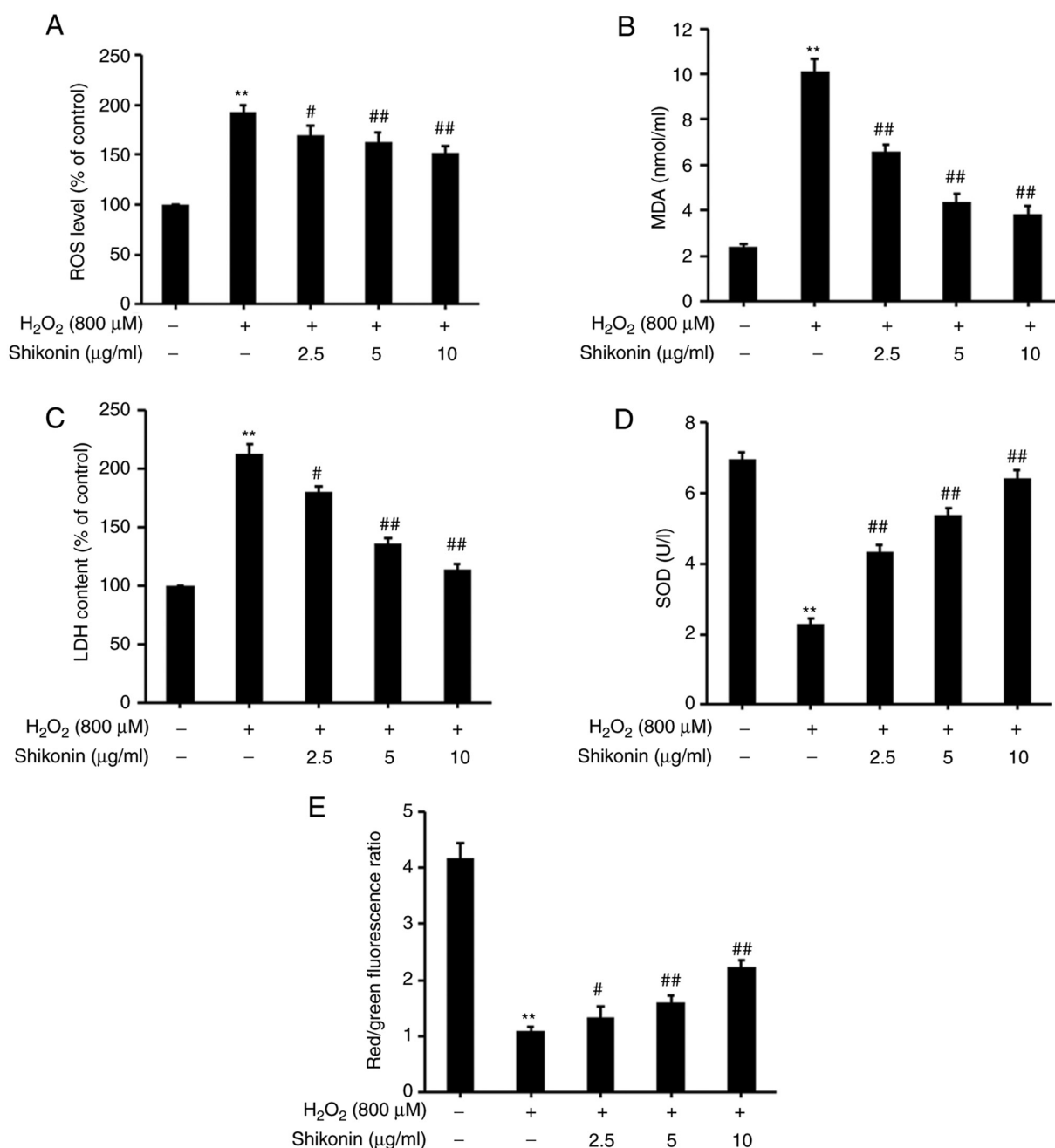


Figure 4. Protective effects of shikonin (2.5, 5 and 10 $\mu\text{g/ml}$) on H_2O_2 -induced oxidative stress injury of HT29 cells. (A) Intracellular ROS levels were assessed via DCFH-DA fluorescence. (B) MDA activity was measured using an MDA assay kit. (C) Extracellular LDH levels were measured using an LDH assay kit. (D) SOD activities were measured using a SOD assay kit. (E) Evaluation of mitochondrial membrane potential was determined by conducting JC-1 staining. Data are presented as the mean \pm SD from at least three independent experiments. ** $P < 0.01$ vs. control; # $P < 0.05$ and ## $P < 0.01$ vs. H_2O_2 . ROS, reactive oxygen species; DCFH-DA, 2',7'-dichlorofluorescein diacetate fluorescence; MDA, malondialdehyde; LDH, lactate dehydrogenase; SOD, superoxide dismutase.

Shikonin suppresses H_2O_2 -induced G_0/G_1 cell cycle arrest in HT29 cells. To investigate the mechanism underlying the effects of shikonin on H_2O_2 -mediated inhibition of cell proliferation, the effects of shikonin on regulating the cell cycle in H_2O_2 -treated HT29 cells were evaluated by conducting flow cytometry. Compared with the control group, an increased percentage of G_0/G_1 cells and a decreased proliferation index (PI) [PI; PI (%) = S phase (%) + G_2/M phase (%)] were observed in the H_2O_2 group (Fig. 3). The PI gradually increased in a dose-dependent manner in the shikonin groups compared with the H_2O_2 group. The results suggested that shikonin suppressed H_2O_2 -induced G_0/G_1 cell cycle arrest.

Shikonin inhibits H_2O_2 -induced increases in ROS and MDA levels in HT29 cells. The levels of intracellular ROS as a major oxidant were assessed by conducting a DCFH-DA assay (Fig. 4A). Following exposure to H_2O_2 , the levels of intracellular ROS were significantly increased compared with the control group. However, significantly lower levels of intracellular ROS were observed in the shikonin groups compared with the H_2O_2 group.

MDA is a biomarker of oxidative stress (24), and MDA activity was measured using an MDA assay kit (Fig. 4B). Upon H_2O_2 exposure, MDA levels were significantly increased compared with the control group. MDA levels in the shikonin

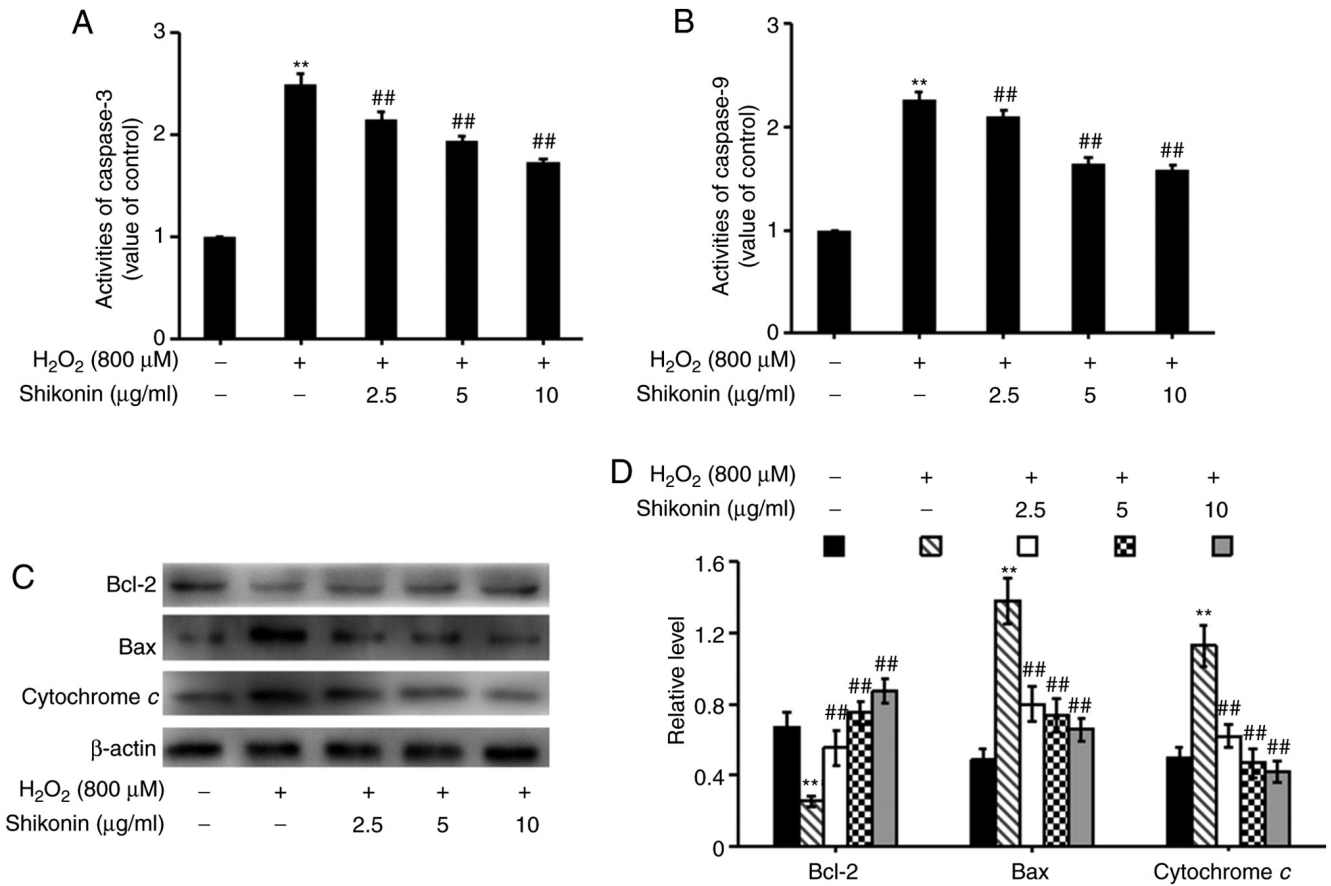


Figure 5. Effects of shikonin (2.5, 5 and 10 μg/ml) on H₂O₂-induced activation of the mitochondria-mediated apoptosis pathway in HT29 cells. (A) Caspase-3 activity was measured using a caspase-3 colorimetric assay kit. (B) Caspase-9 activity was measured using a caspase-9 colorimetric assay kit. (C) Bcl-2, Bax, cytochrome c and β-actin protein expression levels were (C) determined via western blotting and (D) semi-quantified. Data are presented as the mean ± SD from at least three independent experiments. **P<0.01 vs. control; ##P<0.01 vs. H₂O₂.

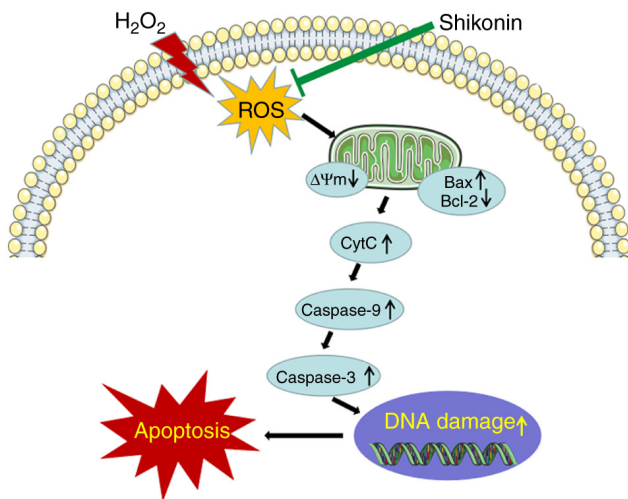


Figure 6. Potential cytoprotective mechanism underlying the effects of shikonin against H₂O₂-induced oxidative injury via elimination of ROS, attenuation of DNA damage and inhibition of mitochondria mediated apoptosis. ROS, reactive oxygen species; CytC, cytochrome c.

groups gradually decreased in a dose-dependent manner compared with the H₂O₂ group. The results suggested that shikonin inhibited H₂O₂-induced intracellular ROS and MDA accumulation.

Shikonin prevents H₂O₂-induced LDH release in HT29 cells. LDH, a glycolytic enzyme, has been suggested to be a key indicator of cell membrane integrity (12). The effects of shikonin on extracellular LDH levels were measured using an LDH assay kit (Fig. 4C). LDH levels were significantly increased in H₂O₂-treated cells compared with the control group. LDH levels in the shikonin groups were significantly lower compared with the H₂O₂ group. The results indicated that shikonin prevented H₂O₂-induced LDH release.

Shikonin inhibits H₂O₂-induced decreases in SOD levels in HT29 cells. SOD is an important antioxidant enzyme in the mitochondria and serves as a defense against oxidative stress (25). SOD activity was measured using a SOD assay kit (Fig. 4D). SOD levels were significantly decreased in the H₂O₂ group compared with the control group. Compared with the H₂O₂ group, SOD levels gradually increased with increasing shikonin concentrations, suggesting that shikonin prevented H₂O₂-mediated decreases in SOD levels.

Shikonin reverses H₂O₂-induced decreases in mitochondrial membrane potential in HT29 cells. Mitochondrial dysfunction has been reported to be associated with H₂O₂-induced cell apoptosis (26). To further investigate whether shikonin was associated with H₂O₂-induced mitochondrial dysfunction, the mitochondrial membrane potential in HT29 cells

was investigated by performing a JC-1 dye assay (Fig. 4E). Mitochondrial membrane potential levels were significantly decreased in HT29 cells exposed to H₂O₂ compared with the control group. However, a gradual increase in mitochondrial membrane potential levels was observed in cells pretreated with 2.5, 5 or 10 µg/ml shikonin compared with the H₂O₂ group. Therefore, the results indicated that shikonin reversed H₂O₂-mediated decreases in mitochondrial membrane potential.

Shikonin protects HT29 cells against oxidative stress via inhibition of the mitochondrial apoptosis pathway. To assess whether the mitochondrial apoptosis pathway was involved in promoting the effects of shikonin on cell apoptosis, caspase-3 and caspase-9 levels were measured by performing caspase assays (Fig. 5A and B), and the expression levels of Bcl-2, Bax and cytochrome *c* were determined via western blotting (Fig. 5C and D). Following exposure to H₂O₂, Bcl-2 expression levels were significantly decreased, whereas the activity levels of caspase-3 and caspase-9, as well as the expression levels of Bax and cytochrome *c* were significantly increased compared with the control group. The opposite effects were observed in H₂O₂-treated cells pretreated with shikonin (2.5, 5 and 10 µg/ml). Therefore, the results indicated that shikonin protected HT29 cells against oxidative stress via inhibiting the mitochondrial apoptosis pathway in a concentration-dependent manner.

The possible mechanism of shikonin. The potential cytoprotective mechanism underlying the effects of shikonin against H₂O₂-induced oxidative injury via elimination of ROS, attenuation of DNA damage and inhibition of mitochondria mediated apoptosis (Fig. 6).

Discussion

The present study investigated the protective effects of shikonin on H₂O₂-induced oxidative stress in HT29 cells and explored the mechanism underlying the antioxidative effects of shikonin. Shikonin protected against H₂O₂-induced injury in HT29 cells. Consistent with previous reports, the results of the MTT assay indicated that H₂O₂ displayed cytotoxicity in HT29 cells in a concentration-dependent manner (23,27). Moreover, the flow cytometry results demonstrated that H₂O₂ decreased the PI and increased apoptosis in HT29 cells compared with the control group. H₂O₂-mediated decreases in HT29 cell viability were significantly reversed following pretreatment with shikonin. In addition, shikonin also increased the PI and attenuated apoptosis in H₂O₂-treated HT29 cells. The results suggested that shikonin exerted a protective effect against H₂O₂-induced oxidative stress in HT29 cells.

ROS is an important antioxidant enzyme in the mitochondria, which has been demonstrated to serve a critical role in DNA oxidative damage and is also a major oxidant *in vivo* (28-32). Under normal physiological conditions, SOD and other antioxidants can scavenge ROS, resulting in a dynamic equilibrium between the generation and removal of ROS (33,34). MDA is a biomarker of oxidative stress that reflects ROS-induced membrane lipid peroxidation (35,36). As cells are damaged, high amounts of ROS accumulate and induce lipid peroxidation on the membrane to produce MDA.

MDA can damage the membrane structure, eventually leading to enhanced membrane permeability, increased generation of intracellular enzymes and the release of LDH (37). The results of the present study indicated that shikonin significantly decreased the levels of intracellular ROS and LDH, decreased MDA levels and restored SOD activity in H₂O₂-treated cells. Collectively, the results indicated that shikonin reduced oxidative stress, at least in part via its antioxidant activity and ROS elimination.

Mitochondria are the major physiological sources of ROS, and H₂O₂ may increase oxidative damage by inducing mitochondrial dysfunction, resulting in increased ROS production and induction of mitochondrial membrane potential loss (38,39). In the present study, shikonin significantly inhibited H₂O₂-induced mitochondrial membrane potential loss, indicating that shikonin may display a protective effect against H₂O₂-induced oxidative damage via the mitochondrial pathway.

The mitochondrial, death receptor and endoplasmic reticulum signaling pathways are three major apoptosis signaling pathways that are dependent on caspases (40). In the mitochondria-mediated apoptosis pathway, oxidative stress induces the opening of mitochondrial permeability transition pores. Mitochondria release the apoptotic promoter and cytochrome *c*, which activates the caspase cascade and induces apoptosis (40). The Bcl-2 family, including antiapoptotic regulator Bcl-2, proapoptotic regulator Bax and death proteins, is a major regulator of the mitochondrial apoptotic pathway (41). The antiapoptotic mechanism underlying Bcl-2 is direct antioxidation, whereas Bax is the primary mediator of the mitochondrial apoptosis pathway (42). Activated Bax leads to the release of cytochrome *c* and mediates apoptosis induced by the mitochondrial pathway (43). It has been reported that Bcl-2 upregulation and Bax downregulation can alleviate the occurrence of apoptosis (44). The results of animal experiments also demonstrated that ischemia-reperfusion injury and heart failure can cause Bcl-2 downregulation and increase apoptosis, whereas Bcl-2 expression in myocardial cells is upregulated following ischemic preconditioning treatment (45). Consistent with previous reports, the present study also suggested that shikonin upregulated Bcl-2 expression and downregulated Bax expression in H₂O₂-induced HT29 cells.

Caspases are a family of proteases that serve important roles in the process of apoptosis (46). Caspase-9 is upstream of the apoptotic signal transduction process and activates caspase-3 (40). Caspase-3-mediated protein cleavage is an important component of the molecular mechanism underlying apoptosis. In addition, caspase-3 serves a key role in nuclear apoptosis, including chromatin condensation and DNA fragmentation (40,47). The present study examined the activities of caspase-3 and caspase-9, and the results indicated that the shikonin group displayed significant downregulation of caspase-3 and caspase-9 activities compared with the H₂O₂ group, suggesting that shikonin protected against H₂O₂-induced oxidative damage of HT29 cells from mitochondrial machinery mediated by the apoptotic pathway.

In conclusion, the present study indicated that the protective effects of shikonin against H₂O₂-induced oxidative stress injury were activated at least in part via removing ROS, ameliorating mitochondrial dysfunction, attenuating DNA oxidative

damage and inhibiting mitochondrial pathway-mediated apoptosis. The results suggested a potential mechanism underlying the antioxidant role of shikonin and a new perspective for the rational use of shikonin for the treatment of oxidation damage-associated diseases in the future.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ and AQ designed the study, acquired the data and drafted the manuscript. QH performed the statistical analysis and drafted the manuscript. JZ and LL performed the statistical analysis. JZ revised the manuscript for intellectual content. KC acquired the data. CC designed the study and drafted the manuscript. All authors have read and approved the final manuscript. CC and YZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Sinha N and Dabla PK: Oxidative stress and antioxidants in hypertension-a current review. *Curr Hypertens Rev* 11: 132-142, 2015.
- Guo H, Sun J, Li D, Hu Y, Yu X, Hua H, Jing X, Chen F, Jia Z and Xu J: Shikonin attenuates acetaminophen-induced acute liver injury via inhibition of oxidative stress and inflammation. *Biomed Pharmacother* 112: 108704, 2019.
- Pistollato F, Iglesias RC, Ruiz R, Aparicio S, Crespo J, Lopez LD, Manna PP, Giampieri F and Battino M: Nutritional patterns associated with the maintenance of neurocognitive functions and the risk of dementia and Alzheimer's disease: A focus on human studies. *Pharmacol Res* 131: 32-43, 2018.
- Yan H, Wang H, Zhang X, Li X and Yu J: Ascorbic acid ameliorates oxidative stress and inflammation in dextran sulfate sodium-induced ulcerative colitis in mice. *Int J Clin Exp Med* 8: 20245-20253, 2015.
- Mrowicka M, Mrowicki J, Mik M, Wojteczak R, Dziki L, Dziki A and Majsterek I: Association between SOD1, CAT, GSHPX1 polymorphisms and the risk of inflammatory bowel disease in the Polish population. *Oncotarget* 8: 109332-109339, 2017.
- Shalkami AS, Hassan M and Bakr AG: Anti-inflammatory, antioxidant and anti-apoptotic activity of diosmin in acetic acid-induced ulcerative colitis. *Hum Exp Toxicol* 37: 78-86, 2018.
- Kruidenier L and Verspaget HW: Review article: Oxidative stress as a pathogenic factor in inflammatory bowel disease-radicals or ridiculous? *Aliment Pharmacol Ther* 16: 1997-2015, 2002.
- Zhu H and Li YR: Oxidative stress and redox signaling mechanisms of inflammatory bowel disease: Updated experimental and clinical evidence. *Exp Biol Med* (Maywood) 237: 474-480, 2012.
- Sakthivel KM and Guruvayoorappan C: Amentoflavone inhibits iNOS, COX-2 expression and modulates cytokine profile, NF- κ B signal transduction pathways in rats with ulcerative colitis. *Int Immunopharmacol* 17: 907-916, 2013.
- Zhong Y, Yu W, Feng J, Fan Z and Li J: Curcumin suppresses tumor necrosis factor-alpha-induced matrix metalloproteinase-2 expression and activity in rat vascular smooth muscle cells via the NF- κ B pathway. *Exp Ther Med* 7: 1653-1658, 2014.
- An S, Park YD, Paik YK, Jeong TS and Lee WS: Human ACAT inhibitory effects of shikonin derivatives from *Lithospermum erythrorhizon*. *Bioorg Med Chem Lett* 17: 1112-1116, 2007.
- Zhang Z, Yang L, Wang B, Zhang L, Zhang Q, Li D, Zhang S, Gao H and Wang X: Protective role of liriiodendrin in mice with dextran sulphate sodium-induced ulcerative colitis. *Int Immunopharmacol* 52: 203-210, 2017.
- Liang W, Cai A, Chen G, Xi H, Wu X, Cui J, Zhang K, Zhao X, Yu J, Wei B and Chen L: Shikonin induces mitochondria-mediated apoptosis and enhances chemotherapeutic sensitivity of gastric cancer through reactive oxygen species. *Sci Rep* 6: 38267, 2016.
- Gao D, Kakuma M, Oka S, Sugino K and Sakurai H: Reaction of beta-alkannin (shikonin) with reactive oxygen species: Detection of beta-alkannin free radicals. *Bioorg Med Chem* 8: 2561-2569, 2000.
- Tong Y, Bai L, Gong R, Chuan J, Duan X and Zhu Y: Shikonin protects PC12 cells against β -amyloid peptide-induced cell injury through antioxidant and antiapoptotic activities. *Sci Rep* 8: 26, 2018.
- Esmailzadeh E, gardaneh M, Gharib E and Sabouni F: Shikonin protects dopaminergic cell line PC12 against 6-hydroxydopamine-mediated neurotoxicity via both glutathione-dependent and independent pathways and by inhibiting apoptosis. *Neurochem Res* 38: 1590-1604, 2013.
- Tong Y, Chuan J, Bai L, Shi J, Zhong L, Duan X and Zhu Y: The protective effect of shikonin on renal tubular epithelial cell injury induced by high glucose. *Biomed Pharmacother* 98: 701-708, 2018.
- Liu T, Zhang Q, Mo W, Yu Q, Xu S, Li J, Li S, Feng J, Wu L, Lu X, *et al*: The protective effects of shikonin on hepatic ischemia/reperfusion injury are mediated by the activation of the PI3K/Akt pathway. *Sci Rep* 7: 44785, 2017.
- Wang Z, Liu T, Gan L, Wang T, Yuan X, Zhang B, Chen H and Zheng Q: Shikonin protects mouse brain against cerebral ischemia/reperfusion injury through its antioxidant activity. *Eur J Pharmacol* 643: 211-217, 2010.
- Moore LD, Le T and Fan G: DNA methylation and its basic function. *Neuropsychopharmacology* 38: 23-38, 2013.
- Sena P, Mancini S, Benincasa M, Mariani F, Palumbo C and Roncucci L: Metformin induces apoptosis and alters cellular responses to oxidative stress in Ht29 colon cancer cells: Preliminary findings. *Int J Mol Sci* 19: 1478, 2018.
- Bai J, Yu J, Wang J, Xue B, He N, Tian Y, Yang L, Wang Y, Wang Y and Tang Q: DNA methylation of miR-122 aggravates oxidative stress in colitis targeting SELENBP1 partially by p53/NF- κ B signaling. *Oxid Med Cell Longev* 2019: 5294105, 2019.
- Zhao X, Fang J, Li S, Gaur U, Xing X, Wang H and Zheng W: Artemisinin attenuated hydrogen peroxide (H₂O₂)-induced oxidative injury in SH-SY5Y and hippocampal neurons via the activation of AMPK pathway. *Int J Mol Sci* 20: 2680, 2019.
- Akbar E, Arbag H, Uyar Y and Ozturk K: Oxidative stress and antioxidant factors in pathophysiology of allergic rhinitis. *Kulak Burun Bogaz Ihtis Derg* 17: 189-1896, 2007 (In Turkish).
- Bresciani G, DA CI and González-Gallego J: Manganese superoxide dismutase and oxidative stress modulation. *Adv Clin Chem* 68: 87-130, 2015.

26. Jablonski RP, Kim SJ, Cheresh P, Williams DB, Morales-Nebreda L, Cheng Y, Yeldandi A, Borade S, Pardo A, Selman M, *et al*: SIRT3 deficiency promotes lung fibrosis by augmenting alveolar epithelial cell mitochondrial DNA damage and apoptosis. *FASEB J* 31: 2520-2532, 2017.
27. Chen XH, Zhou X, Yang XY, Zhou ZB, Lu DH, Tang Y, Ling ZM, Zhou LH and Feng X: Propofol protects against H₂O₂-induced oxidative injury in differentiated PC12 cells via inhibition of Ca(2+)-dependent NADPH oxidase. *Cell Mol Neurobiol* 36: 541-551, 2016.
28. Wagener FA, Dekker D, Berden JH, Scharstuhl A and van der Vlag J: The role of reactive oxygen species in apoptosis of the diabetic kidney. *Apoptosis* 14: 1451-1458, 2009.
29. Yang H, Villani RM, Wang H, Simpson MJ, Roberts MS, Tang M and Liang X: The role of cellular reactive oxygen species in cancer chemotherapy. *J Exp Clin Cancer Res* 37: 266, 2018.
30. Cavallucci V, D'amelio M and Cecconi F: Abeta toxicity in Alzheimer's disease. *Mol Neurobiol* 45: 366-378, 2012.
31. Li C, Jiang W, Liu ZG, Liang PQ and Hu R: Role of reactive oxygen species in GDC-0152-induced apoptosis and autophagy of NB4 cells. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* 27: 1786-1793, 2019 (In Chinese).
32. Prasad S, Gupta SC and Tyagi AK: Reactive oxygen species (ROS) and cancer: Role of antioxidative nutraceuticals. *Cancer Lett* 387: 95-105, 2017.
33. Kehrer JP and Klotz LO: Free radicals and related reactive species as mediators of tissue injury and disease: Implications for Health. *Crit Rev Toxicol* 45: 765-798, 2015.
34. He L, He T, Farrar S, Ji L, Liu T and Ma X: Antioxidants maintain cellular redox homeostasis by elimination of reactive oxygen species. *Cell Physiol Biochem* 44: 532-553, 2017.
35. Tsikas D: Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: Analytical and biological challenges. *Anal Biochem* 524: 13-30, 2017.
36. Del RD, Stewart AJ and Pellegrini N: A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr Metab Cardiovasc Dis* 15: 316-328, 2005.
37. Gallo M, Sapio L, Spina A, Naviglio D, Calogero A and Naviglio S: Lactic dehydrogenase and cancer: An overview. *Front Biosci (Landmark Ed)* 20: 1234-1249, 2015.
38. Chong CM and Zheng W: Artemisinin protects human retinal pigment epithelial cells from hydrogen peroxide-induced oxidative damage through activation of ERK/CREB signaling. *Redox Biol* 9: 50-56, 2016.
39. Li S, Chaudhary SC, Zhao X, Gaur U, Fang J, Yan F and Zheng W: Artemisinin protects human retinal pigmented epithelial cells against hydrogen peroxide-induced oxidative damage by enhancing the activation of AMP-active protein kinase. *Int J Biol Sci* 15: 2016-2028, 2019.
40. Green DR and Lambi F: Cell death signaling. *Cold Spring Harb Perspect Biol* 7: a006080, 2015.
41. Maddika S, Ande SR, Panigrahi S, Paranjothy T, Weglarczyk K, Zuse A, Eshraghi M, Manda KD, Wiechec E and Los M: Cell survival, cell death and cell cycle pathways are interconnected: Implications for cancer therapy. *Drug Resist Updat* 10: 13-29, 2007.
42. Cory S and Adams JM: The Bcl2 family: Regulators of the cellular life-or-death switch. *Nat Rev Cancer* 2: 647-656, 2002.
43. Lin HH, Chen JH, Huang CC and Wang CJ: Apoptotic effect of 3,4-dihydroxybenzoic acid on human gastric carcinoma cells involving JNK/p38 MAPK signaling activation. *Int J Cancer* 120: 2306-2316, 2007.
44. Pistrutto G, Trisciuoglio D, Ceci C, Garufi A and D'orazi G: Apoptosis as anticancer mechanism: Function and dysfunction of its modulators and targeted therapeutic strategies. *Aging (Albany NY)* 8: 603-169, 2016.
45. Maulik N, Goswami S, Galang N and Das DK: Differential regulation of Bcl-2, AP-1 and NF-kappaB on cardiomyocyte apoptosis during myocardial ischemic stress adaptation. *FEBS Lett* 443: 331-336, 1999.
46. Cohen GM: Caspases: The executioners of apoptosis. *Biochem J* 326: 1-16, 1997.
47. Khalilzadeh B, Shadjou N, Kanberoglu GS, Afsharan H, de la Guardia M, Charoudeh HN, Ostadrahimi A and Rashidi MR: Advances in nanomaterial based optical biosensing and bioimaging of apoptosis via caspase-3 activity: A review. *Mikrochim Acta* 185: 434, 2018.



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