Effect of liquiritigenin, a flavanone existed from *Radix glycyrrhizae* on pro-apoptotic in SMMC-7721 cells

Shi-ping Zhang, Yi-jing Zhou, Yan Liu, Yun-qing Cai *

Department of Nutrition and Food Hygiene, School of Public Health, Nanjing Medical University, #140 Hanzhong Road, Nanjing 210029, China

**Abstract**

Liquiritigenin is a flavanone existed in *Radix glycyrrhizae*. The objective of this study is to explore the effects of liquiritigenin on SMMC-7721 cells and its possible mechanism. The viability of liquiritigenin treat cells was decreased in a dose-dependent manner assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT), and apoptotic morphological changes also be observed, such as chromatin condensation and nuclear fragmentation. Assessment of apoptotic cells by flow cytometry indicated that cells fell into apoptosis after 0.4 mM liquiritigenin treatment. In addition, a concomitant time-dependent increase in caspase-3 activity was also observed. The level of p53 protein increased and Bcl-2 protein decreased time-dependently. Further studies found the induction of apoptosis by liquiritigenin was accompanied with the production of reactive oxygen species (ROS), disruption of mitochondrial membrane potential and depletion of antioxidant enzymes. The significant ROS generation was firstly found at 3 h and being time-dependent until 9 h. A time-dependent decrease in mitochondrial potential occurred, and significant loss appeared at 9 h and 12 h. Furthermore, pretreatment of N-acetyl-cysteine (NAC), ROS production and apoptosis induced by liquiritigenin were both suppressed. In sum, this paper indicated the cytotoxicity of liquiritigenin on SMMC-7721 cells may via effect on generation of ROS, later lead to cell apoptosis.

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1. Introduction

Hepatocellular carcinoma is one of the most common malignant tumors worldwide, which is responsible for approximately one million deaths annually. The majority of the patients diagnosed with HCC have low recovery rates, and conventional and modified therapies now available are rarely effective, such as surgical resection and chemotherapy (Kern et al., 2002). Therefore, it is essential to seek out other techniques or remedies with efficient and less side effects for hepatocellular carcinoma. Numerous studies suggested that bioactive natural compounds found in medicinal plants could in theory serve as alternatives to chemically designed anticancer agents (Ramirez-Mares et al., 2004). Flavonoids are polyphenolic compounds occurring naturally in the plant kingdom, displaying a wide range of pharmacologic properties, including anti-inflammatory and anticancer effects (Ross and Kasum, 2002).

It had been reported that flavonoids function their potent property of anti-tumor through cell cycle arrest and induction of apoptosis in several cancer cell lines (Hirano et al., 1995; Pan et al., 2002; Wenzel et al., 2000). Recently, the pro-apoptotic effects of flavonoids on tumor cells have received considerable attention in cancer research. Due to the polyphenolic structure, flavonoids have been found to possess antioxidant and prooxidant action, which may be related to cell apoptosis (Najamura et al., 1993; Nakamura et al., 1997; Yodoi and Uchiyama, 1992). There are other emerging studies reported that some flavonoids, regarded as an anti-oxidant, have been found to induce cytotoxicity with prooxidative properties through decrease in mitochondrial membrane potential and production of reactive oxygen species (ROS) (Csokay et al., 1997; Csink et al., 1997; Pan et al., 2005). ROS, a critical role in cancer and apoptosis, could induce the damage to lipids, proteins, and DNA and cell death (Oh and Lim, 2006; Wu et al., 2005).

*Radix glycyrrhizae*, the dried root of *Glycyrrhiza uralensis Fisch.*, one of the Chinese herbal medicines for thousands of years, has a broad range of beneficial effects including relieving-cough, anti-inflammatory, anti-anabrosis, immunomodulatory, anti-cancer and anti-virus hepatitis activities (Gao et al., 2004). Liquiritigenin (Fig. 1) is a kind of flavonoids existed in *R. glycyrrhiza*, with the polyphenolic structure. It has been reported that liquiritigenin could possess cytotoxic activity against five human cancer cell
lines in vitro (Falcão et al., 2005). Our previous studies also found liquiritigenin inhibited the cell growth of SGC-7901 and Lovo (data have not shown). In the present study, the aim was to study the effect on human hepatocarcinoma cell line (SMMC-7721) by liquiritigenin as well as its underlying mechanisms. Due to the polyphenolic structure of liquiritigenin, our study would also demonstrate that either prooxidative or antioxidative activity is involved in the effect of liquiritigenin on SMMC-7721 cells.

2. Materials and methods

2.1. Regents and materials

Liquiritigenin (purity $\geq$ 99.68% by high performance liquid chromatogram, HPLC) was provided from Nanjing University of Chinese Medicine. Cell culture reagents were obtained from Gibco Life Technology (Grand Island, NY, USA). Antibodies specific for the bcl-2, p53 and anti-rabbit IgG, HRP-linked antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). The 6-carboxy-2',7' dichlorodihydrofluorescein diacetate (DCFH-DA), Hoechst 33258 staining and caspase-3 activity assay were purchased from Beyotime Institute of Biotechnology (Haimen, China), and Annexin V-FITC Apoptosis Detection kit was purchased from Pharmingen (Becton Dickinson Company, San Jose, CA, USA). Rho-damine123 (Rh123) was purchased from Molecular Probes (Eugene, OR, USA). Reduced glutathione hormone (GSH), glutathione peroxidase (GSH-PX) and superoxide dismutase (SOD) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), N-acetyl-cysteine (NAC), hydrogen peroxide ($\text{H}_2\text{O}_2$), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other reagents in the molecular studies were supplied from Sigma (St. Louis, MO, USA).

2.2. Preparation of Liquiritigenin

Stock solution of liquiritigenin was prepared at a concentration of 1 mmol/mL of DMSO and stored at $-20^\circ$ C until use. For all experiments, final concentrations of the test compound were freshly prepared by diluting the stock with phosphate-buffered saline (PBS, pH 7.2). Carrier solvent (0.1% DMSO) was used as control.

2.3. Cell culture

Human hepatocarcinoma cell line (SMMC-7721) was purchased from Jiangsu Institute of Cancer Research (JICR, Jiangsu, China). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, 100 μg/mL streptomycin in a humidified atmosphere of 5% CO$_2$ at 37°C.

2.4. MTT-colorimetric assay

Inhibition of cell proliferation by liquiritigenin was measured by MTT assay. Briefly, cells attached on culture dishes were collected and planted at a density of $2 \times 10^5$ cells/100 μL in each well of 96-well plates. Then, the cells were treated with liquiritigenin for various concentrations (0.1 mM, 0.2 mM, 0.4 mM, 0.6 mM) for 48 h. Then, 20 μL MTT (5 mg/mL in PBS) was added to each well and continuously incubated at 37°C for 4 h. At the end of this period, the MTT solutions were replaced with 150 μL dimethylsulfoxide and shaken for 10 min. Absorbance was measured by automatic microplate reader (ELR08, Bio-Tek, Winooski, VT, USA) at 570 nm.

2.5. Apoptosis assessment by Hoechst 33258 staining

Apoptotic morphological changes in the nuclear chromatin of cells were detected by Hoechst 33258 staining. SMMC-7721 cells were seeded on sterile cover glasses placed in the 6-well plates. After overnight growth, cells were treated with various concentration liquiritigenin of 0.1 mM, 0.2 mM, 0.4 mM and 0.6 mM for 48 h. Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min, and then incubated with 50 μM Hoechst 33258 staining solution for 10 min. After three washes with PBS, the cells were viewed under a fluorescence microscope (Olympus, IX-70, Japan).

2.6. Determination of cell apoptosis by annexin V and PI double staining

Measurement of phosphatidylserine redistribution in a plasma membrane was conducted according to the protocol outlined by the manufacturer of the Annexin V-FITC Apoptosis Detection kit (Becton Dickinson Company, San Jose, CA, USA). Cells ($1 \times 10^5$) were treated with vehicle alone (0.1% DMSO), various concentration of liquiritigenin and 0.1 mM H$_2$O$_2$ for designed time, or pre-treatment with 0.5 mM NAC for 30 min, followed by 0.4 mM liquiritigenin for 48 h. Cells were harvested and suspended in a binding buffer (1×). An aliquot of 100 μl was incubated with 5 μl of Annexin V-FITC and 5 μl of PI for 15 min in dark, and 400 μl binding buffer (1×) was added to each sample. The stained cells were analyzed directly by flow cytometry using the Cell Quest program (Becton Dickinson, Franklin, NJ).

2.7. Detection of intracellular reactive oxygen species (ROS)

Cells were treated with 0.4 mM liquiritigenin and 0.1 mM H$_2$O$_2$ for designed time, or pre-treatment with 0.5 mM NAC for 30 min, followed 0.4 mM liquiritigenin for 9 h. Cells then were washed twice in phosphate-buffered saline (PBS pH = 7.2), and incubated with 10 μM DCFH-DA at 37°C for 20 min according to the manufacturer’s instructions. A FACScan flow cytometer (FL-1, 530 nm) was used to detect the fluorescence.

2.8. Measurement of mitochondrial membrane potential ($\Delta \psi_m$)

Cells treated with liquiritigenin (0.4 mM) were incubated with Rh 123 (5 mg/ml) for 30 min at 37°C, harvested and suspended in PBS (pH = 7.2). Mitochondria membrane potential ($\Delta \psi_m$) was measured by the fluorescence intensity (FL-1, 530 nm) of 10,000 cells.

2.9. Intracellular reduced glutathione hormone (GSH), glutathione peroxidase (GSH-PX) and superoxide dismutase (SOD) activity assay

GSH activity was measured by reacting on DTNB (5,5'-DITHIOBIS-(2-NITROBENZOIC ACID)) to form a yellow substance, which can be determined by colorimetry. The GSH-PX activity was detected by the oxidizing speed of GSH, which can be expressed by the GSH reduction in a certain time. One unit of GSH-PX activity was defined as 1 μM GSH oxidized to glutathione disulphide (GSSG) per milligram of protein per minute. SOD activity was determined by hydroxylamine assay-developed from xanthine oxidase assay. Cells were treated with liquiritigenin 0.4 mM for designed time, then washed twice and resuspended in PBS, sonicated for 30 s on ice, and centrifuged at 1000g for 15 min according to the manufacturer’s instructions. Supernatants were subjected to intracellular GSH, GSH-PX and SOD activity assays, which were determined with commercial kits from Janccheng Bioengineering Institute.

2.10. Caspase-3 activity assay

Activity of caspase-3 was determined using the caspase-3 activity kit (Beyotime Institute of Biotechnology, Haimen, China). Cell lysates were prepared after their respective treatments. Assays were performed on 96-well microtitre plates by incubating 10 μl protein of cell lysate per sample in 80 μl reaction buffer (1% NP-40, 20 mM Tris–HCl (pH = 7.5), 137 mM NaCl and 10% glycerol) containing 10 μl caspase-3 substrate (Ac-DEVD-pNA) (2 mM). Samples were incubated at 37°C for 4 h and measured with an ELISA reader at an absorbance of 405 nm. The detailed analysis procedure was described in the manufacturer's protocol.

2.11. Western blot analysis

Cells were grown at 5 × 10^4 cells/well in 6-well microplates and treated with liquiritigenin for designed time. Following treatment, cells were washed with PBS, and total cells were prepared by scraping in 200 μl of lysis buffer (20 mM Tris–HCl (pH = 8.0), 1 mM sodium orthovanadate, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 mM ethylenediaminetetraacetate (EDTA), 1% Triton X-100, 50 mM β-glycerolphosphate, and 10 mg/ml each of aprotinin, leupeptin, and pepstatin). Fifty micrograms of proteins determined by Bradford assay were electrophoretically separated using a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene fluoride (PVDF) membrane and immuno blotted with the corresponding antibodies. Immunodetection was performed with enhanced chemiluminescence (ECL) detection kit (Cell Signaling Technology, Beverly, MA). The protein bands were followed by densitometry scanning using a Chemilumager™ 5500 Fluorescence system equipped with the analysis software AlphaEase FC™ (Alpha Innotech Corporation, San Leandro, CA 94577, USA).
2.12. Statistical analysis

Data were expressed as means ± SD of three independent experiments. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences (*p < 0.05 or **p < 0.01) between the means of control and treated cells were analyzed by Dunnett’s test.

3. Results

3.1. Effects of Liquiritigenin on SMMC-7721 cells viability and Morphological changes

The effect of liquiritigenin on viability of SMMC-7721 cells was examined using the MTT assay. As shown in Fig. 2A, exposure of SMMC-7721 cells to various concentrations of liquiritigenin for 48 h resulted in a concentration-dependent decrease in cell viability. After treatment with liquiritigenin at 0.4 mM and 0.6 mM, the cell viability decrease became significant (p < 0.01). To further investigate whether the liquiritigenin mediated cell death in SMMC-7721 cells due to an apoptotic mechanism, the morphological changes that occurred during liquiritigenin treatment were observed by Hoechst 33258 staining. Fig. 2B shows that liquiritigenin treated cells exhibited characteristic features of apoptosis including chromatin condensation and nuclear fragmentation, while almost no apoptotic nuclei were observed in control cells.

3.2. Flow cytometric analysis of liquiritigenin-induced SMMC-7721 cells apoptosis

Above results showed that liquiritigenin could inhibit cell growth of SMMC-7721 cells in a dose-dependent manner, and 0.4 mM liquiritigenin was chosen for next experiments. To further explore whether liquiritigenin-induced apoptosis, SMMC-7721 cells were stained with annexin V-FITC and propidium iodide, and subsequently analyzed by flow cytometry. The annexin V assay measures phospholipid turnover from the inner to the outer lipid layer of the plasma membrane, an event typically associated with apoptosis. H2O2 was used as a positive control, which was reported to be a cell apoptosis inducer (Mariko et al., 2004). As indicated in Fig. 3, the numbers of apoptotic cells induced by 0.4 mM liquiritigenin for indicated time were 11.08%, 14.35%, 18.20% and 28.17%, respectively, which were incremental time-dependently. The results also showed that the apoptotic cells caused by 0.4 mM liquiritigenin were slightly fewer than that caused by 0.1 mM H2O2 at 12 h, and more after 12 h.
3.3. Effect of liquiritigenin on the activation of caspase-3

Caspase-3 is one of the principal caspases found in apoptotic cells (Zhang et al., 1999), and plays a central role in mediating various apoptotic responses. In this study, caspase-3 protease activity significantly increased in a time-dependent manner after liquiritigenin treatment. As shown in Fig. 4, 0.4 mM liquiritigenin strongly induced activation of caspase-3 protease as early as 24 h, and the maximum activity at 72 h was 773.94 ± 31.04%, which was about 7-fold increase compared with control.

3.4. Effects of liquiritigenin on levels of p53 and bcl-2 proteins in SMMC-7721 cells

The effect of liquiritigenin on the expression of p53 and Bcl-2 in SMMC-7721 cells is shown in Fig. 5. As shown in Fig. 5A, 0.4 mM liquiritigenin induced a significant increase in the expression of p53. The level of p53 increased significantly when SMMC-7721 cells were treated with 0.4 mM liquiritigenin for 12 h, and remained elevated until 72 h. In contrast, the level of Bcl-2 protein significantly decreased in a time-dependent manner after liquiritigenin treatment, which gradually disappeared at 72 h (Fig. 5B). Densitometric analysis showed the increases in the ratio of p53/β-actin band densities reached statistical significance at 48 h (2.0-fold, p < 0.05) and 72 h (2.4-fold, p < 0.01) (Fig. 5A). And the ratio of Bcl-2/β-actin band densities was observed in a time-dependent decrease significantly (Fig. 5B).

3.5. Effect of liquiritigenin on intracellular reactive oxygen species (ROS) in SMMC-7721 cells

ROS producing is an important marker of prooxidant activity in polyphenolic compounds-induced apoptosis. As liquiritigenin is a polyphenolic compound, this study examined whether liquiritigenin-induced apoptosis mediated by its prooxidant activity. In order to demonstrate the role that ROS play in liquiritigenin-induced apoptosis, production of ROS was examined by staining with DCFH-DA and subsequently analyzed by flow cytometry. The results showed that a significant ROS production was generated in both 0.4 mM liquiritigenin and 0.1 mM H₂O₂ treated cells (Fig. 6A). A significant ROS production was firstly observed at 3 h, the fluorescence of which was about 229.7 ± 14.5%. Highest fluorescence at 9 h indicated approximately a 15-fold increase of ROS.
This result is similar to that in cells treated with 0.1 mM H$_2$O$_2$, as evident in Fig. 6A. The formations of ROS induced by H$_2$O$_2$ were greater than that induced by liquiritigenin before 6 h and lower after 6 h.

### 3.6. Effects of liquiritigenin on mitochondrial membrane potential loss ($\Delta \psi / m$) in SMMC-7721 cells

Since mitochondria membrane potential is an important mitochondrial parameter controlling key cellular processes of prooxidantive flavonoids, we examined the effect of liquiritigenin on mitochondria membrane potential. The result suggested that liquiritigenin treatment caused disruption of mitochondrial membrane potential in a time-dependent manner (Fig. 7). Being an early marker of apoptosis, a decrease in mitochondrial membrane potential occurred as early as 3 h (74.53 ± 7.7%), and the loss in mitochondrial membrane potential became significant at 9 h and 12 h ($p < 0.05$, $p < 0.01$).

### 3.7. Effects of liquiritigenin on intracellular superoxide dismutase (SOD), reduced glutathione hormone (GSH) and glutathione peroxidase (GSH-PX) activities in SMMC-7721 cells

To further investigate whether the production of ROS is an important factor in liquiritigenin-induced SMMC-7721 cells apoptosis, the antioxidant enzymes namely reduced glutathione hormone (GSH), glutathione peroxidase (GSH-PX) and superoxide dismutase (SOD), were examined by commercial kits. In this study, the changes of these antioxidant enzymes activities were given in Table 1. The data show that all the activities of GSH, GSH-PX and SOD decreased in time-dependent manner after cells treated with 0.4 mM liquiritigenin, and the GSH-PX activities significantly decreased at 24, 48 and 72 h ($p < 0.05$), which decreased to 15.4 ± 4.7 U (72 h) from 37.7 ± 2.9 U (0 h).

### 3.8. Protective effect of N-acetyl-cysteine (NAC) against liquiritigenin-induced reactive oxygen species (ROS) production and apoptosis in SMMC-7721 cells

N-acetyl-cysteine (NAC) (0.5 mM) was shown to be capable of suppressing the ROS production induced by 0.4 mM liquiritigenin, as reflected by the decrease of DCF relative fluorescence staining from 1545 ± 90.8% to 213 ± 18.9% (Fig. 8A). Interestingly, another experiment demonstrated NAC also could suppress the apoptosis induced by liquiritigenin. As shown in Fig. 8B, 0.4 mM liquiritigenin for 48 h caused the apoptosis cells increase to almost 17.18% as compared to 9.03% in the control population. When cells were pretreated with 0.5 mM NAC for 30 min, then treated with 0.4 mM liquiritigenin for 48 h, the proportion of apoptosis cells decreased to 9.94%. These results suggest that ROS generation may be a curial mediator in the process of liquiritigenin-induced apoptosis.

### 4. Discussion

Recently, flavonoids compounds have been shown to act many beneficial activities such as preventing cancer, cardiovascular disease and neurodegenerative diseases (Engelhart et al., 2002; Ikeda et al., 2005; Sun et al., 2002). The cytotoxic effects of flavonoids on cancer cells have become an area of extensive investigation by emerging evidence (Chen et al., 2004; Chiang et al., 2006). In the present study, we demonstrate that liquiritigenin, a kind of flavonoids existed in Radix glycyrrhizae, has an anticancer activity on SMMC-7721 cells, the results of which showed that liquiritigenin revealed significant growth inhibitory effects on cells screened by MTT, and that the cytotoxic mechanism involves the induction of apoptosis. The latter was characterized by typical characteristic features of apoptosis and time-dependent increase in apoptosis rate. Meanwhile, the activity of caspase-3 was found to be elevated.
in the progress of liquiritigenin induce-apoptosis. It is known that caspases, a family of cysteine proteases, have been recognized as a critical role during apoptosis. Their genes encode proteins that are translated as inactive proenzymes, and these precursors are activated by cleavage at specific aspartate residues and assembly into heterotetramers. Caspase-3 is one of the principle caspases found in apoptotic cells, which is required for many of the nuclear changes associated with apoptosis (Lee et al., 2002). Further studies showed a time-dependent increase of p53 protein and decrease of Bcl-2 protein were also regulated by liquiritigenin. Numerous studies demonstrated that p53, a tumor suppressor gene, played an important role in tumor growth inhibition and induction of apoptosis, and exerted its control on apoptosis by interacting with other important apoptotic molecules, such as members of the Bcl-2 family (Agarwal et al., 1998; Pucci et al., 2000; Vidal and Koff, 2000). Bcl-2 is known as an upstream effecter molecule in the apoptotic pathway and is identified as a potent suppressor of apoptosis (Hockenbery et al., 1993). In addition, it was observed that Bcl-2 promotes cell survival by preserving the integrity of the external mitochondrial membrane, which prevents the release of cytochrome c from mitochondria and hence induce cell death (Scorrano and Koreshmeyer, 2003). Taken together, the changes of these proteins-correlated apoptosis expressions suggested that liquiritigenin induce-apoptosis may be involved with the participation of p53 and Bcl-2. From above facts, it is reasonable to presume that liquiritigenin functions its cytotoxic effects on SMMC-7721 cells via induction of apoptosis.

Several studies reported that flavonoids have been shown to exhibit antioxidant and pro-oxidant activities, which often been linked to their beneficial effects in cancer therapy (Birt et al., 2001; Matsuzaki et al., 1996; Richter et al., 1999). It is well accepted that flavonoids function there antioxidant property with their ability to scavenge free radicals and ROS (Bors et al., 1994; Bors and Saran, 1987; Jovanovic et al., 1994) and to form complexes with metal ions, thus preventing oxidation of the metals with oxygen yielding ROS (Haber–Weiss and Fenton reactions) (Mira et al., 2002; Morel et al., 1994; Potapovich and Kostyk, 2003). However, there is also a number of works demonstrating the prooxidant activity of some flavonoids (Ueda et al., 2002; Wang et al., 1999). The prooxidant activity of flavonoids are possibly due to: (1) its structure, such as myricetin which exerts the strong cytotoxic effects by undergoing autoxidation and producing hydrogen peroxide and the effects are associated with its relatively unstable pyrogallol structure (Kyungmi et al., 2008); (2) the presence of Fe²⁺ due to the reaction between Fe²⁺ and H₂O₂ yielding the highly reactive hydroxyl radical (Schmalhausen et al., 2007); (3) another possible presumption mechanism may be related to their lipophilicity (Nemeikaite-Ceniene et al., 2005). Actually, the prooxidant action of flavonoids rather than their antioxidant action may play an important role in their anticancer and apoptosis-inducing properties (Sergediene et al., 1999). Firstly, ROS was predominantly produced in the mitochondria, then leading to the free radical attack of membrane phospholipids and loss of mitochondrial membrane potential. As a consequence of oxidative membrane damage, the impaired transmembrane potential and permeability-barrier function would lead to further mitochondrial damage. Recently, studies have shown that the oxidative damage to the mitochondrial membrane potential caused by the increased generation of ROS play a role in apoptosis (Peled-Kamar et al., 1995).

SOD, GSH and GSH-PX are the important antioxidant enzymes of a cells defense against free radical damage, since SOD has the activity to convert superoxide into hydrogen peroxide, while GSH-PX could reduce the hydrogen peroxide to water at the expense of GSH. Our results found that the earliest observable response in liquiritigenin treatment was ROS generation, which was produced at 3 h and decreased after 9 h, and then a significant decrease in mitochondrial membrane potential was detected at 9 h, later than ROS production. The time-dependent decrease of the activities of SOD, GSH and GSH-PX are also found in our studies. Comparing that the balance between antioxidant enzymes

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**Fig. 6.** Effects of liquiritigenin on reactive oxygen species generation (ROS) in SMMC-7721 cells. Cells were treated with 0.4 mM liquiritigenin and 0.1 mM H₂O₂ for 0, 3, 6, 9 and 12 h at 37 °C. A. DCF fluorescence measured using a flow cytometer with FL-1 filter.

B: Each value was expressed the ratio of ROS fluorescence level, the value of control was set to 100 (n = 3). Each column and bar represents means ± SD. Indicates significant difference compared to control group at p < 0.05 analyzed using Dunnett’s test.
and ROS with the general agreement, the data suggested that the descent of antioxidant enzymes may be related to an increase in ROS generation in cells which is formed during the autoxidation of liquiritigenin. All of these above results suggest that liquiritigenin-induce apoptosis in SMMC-7721 cells is accompanied with the ROS production. However, the relationship between the ROS generation and apoptosis induction are needed further studies.

So in the next study, we used NAC, a free radical scavenger, to explore the role of ROS in liquiritigenin-induced apoptosis. The results showed that NAC could suppress both liquiritigenin-induced apoptosis and ROS production. Give the above facts, we could conclude that the intracellular redox was involved in liquiritigenin-induced apoptosis and liquiritigenin plays a pro-oxidant role in this apoptotic pathway.

Finally, we have to confess that the concentration liquiritigenin used in this study was rather higher. However, the purpose of this study is to successfully establish a vitro model to evaluate the possible mechanism of liquiritigenin in its cytotoxic effects on human hepatocarcinoma cell line. In addition, the effect of liquiritigenin, at least in this study has been proven to be specific because liquiritigenin at 0.4 mM did not cause significant human hepatic cells

![Image](image_url)

**Table 1** Effects of liquiritigenin on intracellular superoxide dismutase (SOD), reduced glutathione hormone (GSH) and glutathione peroxidase (GSH-PX) activities in SMMC-7721 cells.

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<th>Anti-oxidant enzymes</th>
<th>Time (h)</th>
<th>0</th>
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<th>6</th>
<th>9</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>72</th>
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<tr>
<td>GSH (mg/g pr)</td>
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<td>149.9 ± 45.8</td>
<td>120.1 ± 25.7</td>
<td>108.8 ± 18.3</td>
<td>98.7 ± 10.4</td>
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<td>GSH-PX(U)</td>
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<td>27.5 ± 6.7</td>
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<tr>
<td>SOD (U/mg pr)</td>
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Data are the means ± SD of three independent experiments. The asterisk indicates a significant difference from 0 h treated cells by Dunnett’s test; *p < 0.05.
L-02 cell death (data have not shown). The most parameters measured in this experiment were in a time-dependent manner, which would make it easier to understand the kinetic process of liquiritigenin-induced apoptosis.

In conclusion, our experiments suggested that liquiritigenin could inhibit the growth of SMMC-7721 cells via apoptosis, which was probably mediated by reactive oxygen species generation. Present results may provide an information on some underlying...
mechanisms involved in liquiritigenin-induced cytotoxic effects on SMMC-7721 cells.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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