

## STUDIES ON THE IDENTIFICATION OF CONSTITUENTS IN ETHANOL EXTRACT OF RADIX GLYCYRRHIZAE AND THEIR ANTICANCER ACTIVITY

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**Abstract**

**Background:** The main functions of Radix Glycyrrhizae include regulating middle warmer, moistening lung, relieving toxicity, harmonizing property of drugs which is a traditional Chinese medicine widely used in clinical settings. The objective of the paper is to isolate and identify the constituents in ethanol extract of Radix Glycyrrhizae, and to study their anticancer activity.

**Materials and Methods:** Column chromatography, ODS column chromatography, preparative thin layer chromatography and NMR spectroscopy techniques were used to isolate compounds from ethanol extract of Radix Glycyrrhizae; optical microscopy and flow cytometry were used to determine the anticancer effect of Radix Glycyrrhizae extract.

**Results:** Four compounds were isolated from the ethanol extract of Radix Glycyrrhizae, namely oleanolic acid, isoliquiritin, glycyrrhetic acid and licochalcone A. Optical microscopic observation showed that the growth of gastric cancer cell line SGC-7901 was inhibited in the experimental groups, and apoptotic morphological changes were observed in adherent cells; flow cytometry with PI staining showed that Radix Glycyrrhizae extract could induce SGC-7901 cell apoptosis within a concentration range of 0.5-1.5 mg/mL, compared with the control group, the apoptosis was positively correlated with the drug concentration, which exhibited an apparent dose-dependence.

**Conclusion:** We conclude Ethanol extract of Radix Glycyrrhizae has an anti-proliferative activity on SGC-7901 cells.

**Keywords:** Radix Glycyrrhizae; oleanolic acid; isoliquiritin; glycyrrhetic acid; licochalcone A; SGC-7901 cell.

**Introduction**

Radix Glycyrrhizae is the root and rhizome of *Glycyrrhiza uralensis* Fisch., *G. inflata* Bat. or *G. glabra* L. of family Leguminosae. Its main functions include regulating middle warmer, moistening lung, relieving toxicity, harmonizing property of drugs, etc. which is a traditional Chinese medicine widely used in clinical settings (Suzuk et al., 2004; Torkin et al., 2005). The main active fraction of Radix Glycyrrhizae is flavonoids, domestic and foreign scholars have conducted a number of studies on them (Surget et al., 2011; Paizanis et al., 2007). In this paper, the ethanol extract of Radix Glycyrrhizae is studied, and its inhibitory effect on SGC-7901 cells is explored.

**Materials****Instruments and reagents**

DRX-500 NMR spectrometer (Bruker, USA); rotary evaporator (Shanghai Yarong Biochemical Instrument Factory); CO-150 CO<sub>2</sub> incubator (NBS, USA); flow cytometer (BD Biosciences); RPMI 1640 medium, purchased from Gibco, USA; propidium iodide (PI), purchased from Sigma, USA.

**Cells and drugs**

Radix Glycyrrhizae, purchased from the pharmacy, which was identified as *Glycyrrhiza uralensis* Fisch. of family Leguminosae by Pro. Diaoyanping from Dalian Medical University, stored in herbarium. Radix Glycyrrhizae extract, self-prepared. SGC-7901 cell lines, purchased from China Medical University.

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## Methods

### Extraction and isolation of compounds from Radix Glycyrrhizae

5 kg of Radix Glycyrrhizae was taken, ground into a coarse powder, and then extracted by heat reflux three times with 95% ethanol, each extraction lasted 2 h. The extracts were combined, and solvent was removed to give 1.6 kg of ethanol extract. The ethanol extract was added with appropriate amount of water, and extracted with ethyl acetate, then solvent was removed under reduced pressure to give 125 g of ethyl acetate extraction fraction. The extract was then isolated by column chromatography, and gradient-eluted with dichloromethane and methanol as solvents, identical fractions were combined, and four fractions were obtained. The first and second fractions were subjected to polyamide chromatography, ODS column chromatography and preparative thin layer chromatography to obtain four compounds.

### Cell culture

Gastric cancer SGC-7901 cell lines were cultured in RPMI 1640 medium containing 10% fetal bovine serum, and routinely subcultured in a 37°C, 5% CO<sub>2</sub> incubator, cells in logarithmic growth phase were taken and set aside.

### Optical microscopic observation

Logarithmic growth phase SGC-7901 cells were taken, and treated with different concentrations of Radix Glycyrrhizae extracts (final concentrations of 0.5, 1.0 and 1.5 mg/ml) for 24 h, control group was added with an equal volume of PBS. Cell morphology was observed under an optical microscope.

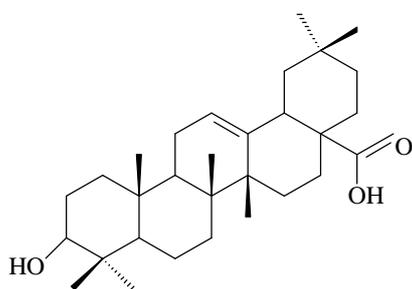
### Detection of cell cycle change

The SGC-7901 cells were seeded in culture flasks at  $5 \times 10^4$  cells/mL, and incubated at 37°C under 5% CO<sub>2</sub> for 24 h, then the medium was replaced and the flasks were added with Radix Glycyrrhizae extracts with final concentrations of 0.5, 1.0 and 1.5 mg/ml, and incubated for 48, 72 h. After digestion with 0.25% trypsin, the cells were collected, centrifuged for 5 min, washed twice with PBS, added with 75% ethanol, and allowed to stand overnight at 4°C. After removal of ethanol by centrifugation, the cells were washed twice with PBS, PI stained, and incubated at 37°C under dark conditions for 30 min, followed by determination of cell cycle distribution by flow cytometry.

## Results

### Structural identification of compounds

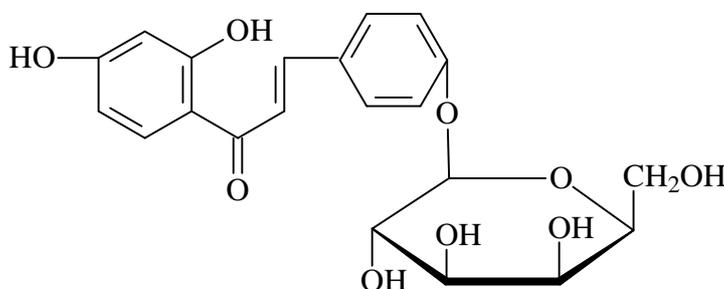
Compound 1: white powder. <sup>1</sup>H-NMR (300MHz, CDCl<sub>3</sub>) δ: 0.79 (3H, s, H-23), 0.78 (3H, s, H-24), 0.92 (3H, s, H-25), 0.91 (3H, s, H-26), 0.95 (3H, s, H-29), 0.98 (3H, s, H-30), 1.15 (3H, s, H-27), 5.24 (1H, t, J = 2.4 Hz, H-12), 3.24 (1H, dd, J = 4.4, 11.6 Hz, H-3α), 2.85 (1H, dd, H-18); <sup>13</sup>C-NMR (300 MHz, CDCl<sub>3</sub>) δ: 38.6 (C-1), 27.3 (C-2), 79.2 (C-3), 38.6 (C-4), 55.4 (C-5), 18.5 (C-6), 32.8 (C-7), 39.3 (C-8), 47.4 (C-9), 37.3 (C-10), 23.6 (C-11), 122.7(C-12), 143.8 (C-13), 41.6 (C-14), 27.5 (C-15), 23.7 (C-16), 46.4 (C-17), 41.2 (C-18), 45.7 (C-19), 30.5 (C-20), 33.6 (C-21), 32.6 (C-22), 28.3 (C-23), 15.7 (C-24), 15.4 (C-25), 17.3 (C-26), 25.7 (C-27), 183.4 (C-28), 33.2 (C-29), 22.8 (C-30). The above data were consistent with the reported literature (Ding et al., 2010), so the compound was identified as oleanolic acid.



**Figure 1:** Structure of oleanolic acid

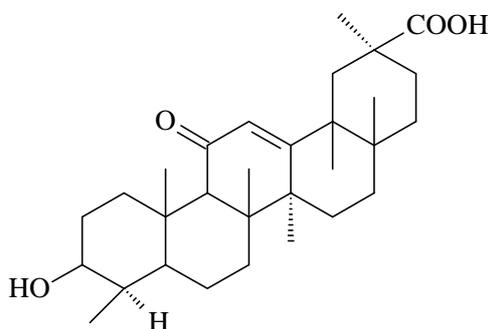
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Compound 2: yellow crystals, mp: 148–150°C, easily soluble in methanol and ethyl acetate. <sup>1</sup>H-NMR (300MHz, CDCl<sub>3</sub>) δ: 6.33 (1H, d, J=2.3Hz), 6.54 (1H, dd, J=9.0Hz, 2.3Hz), 8.03 (1H, d, J=9.0Hz), 7.45 (1H, d, J=18Hz), 7.76 (2H, d, J=18Hz), 7.69 (2H, d, J=8.6Hz), 7.43 (2H, d, J=8.6Hz); <sup>13</sup>C-NMR (300MHz, CDCl<sub>3</sub>) δ: 145.4 (C-1), 120.5 (C-2), 193.3 (C-3), 114.7 (C-4), 167.6 (C-5), 104.5 (C-6), 166.5 (C-7), 109.4 (C-8), 133.4 (C-9), 130.6 (C-10), 131.7 (C-11), 118.5 (C-12), 161.8 (C-13), 118.7 (C-14), 131.6 (C-15), 102.4 (C-16), 78.3 (C-17), 78.4 (C-18), 75.4 (C-19), 71.6 (C-20), 62.4 (C-21). The above data were consistent with the reported literature (Wang et al., 2004), so the compound was identified as isoliquiritin



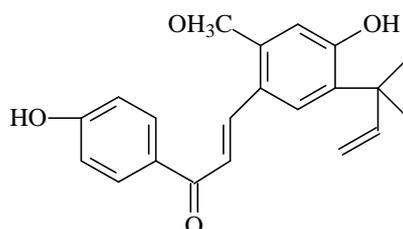
**Figure 2:** Structure of isoliquiritin

Compound 3: white powder, <sup>1</sup>H-NMR (300MHz, CDCl<sub>3</sub>) δ: 12.23(1H, s, 29-COOH), 3.42 (1H, dd, H-3), 3.15 (1H, dd, H-5), 2.76 (2H, d, H-2), 2.32 (1H, m, H-9), 0.7-1.5 (21H, 7×CH<sub>3</sub>); <sup>13</sup>C-NMR (300 MHz, CDCl<sub>3</sub>) δ: 38.3 (C-1), 26.6 (C-2), 76.1 (C-3), 7.2 (C-4), 54.5 (C-5), 6.5 (C-6), 30.2 (C-7), 42.6 (C-8), 48.5 (C-9), 36.8 (C-10), 177.8 (C-11), 127.5 (C-12), 169.3 (C-13), 44.3 (C-14), 25.5 (C-15), 28.5 (C-16), 32.6 (C-17), 61.6 (C-18), 39.3 (C-19), 31.3(C-20), 38.5 (C-21), 43.4 (C-22), 27.4 (C-23), 17.6 (C-24), 16.5 (C-25), 16.3 (C-26), 18.6 (C-27), 28.3 (C-28), 199.3 (C-29), 23.5 (C-30). The above data were consistent with the reported literature (Chim, 2001), so the compound was identified as glycyrrhetic acid.



**Figure 3:** Structure of glycyrrhetic acid

Compound 4: orange crystals, <sup>1</sup>H-NMR (300MHz, CDCl<sub>3</sub>) δ: 7.32 (1H, d, A-H), 7.89 (1H, d, B-H), 6.76 (2H, d, 3c, 5c-H), 7.86 (2H, d, 2c, 6c-H), 6.34 (1H, s, 3-H), 7.42 (1H, s, 6-H), 3.69 (3H, s, -OCH<sub>3</sub>), 1.52[6H, s, Ar-C(CH<sub>3</sub>)<sub>2</sub>], 6.18 (1H, dd, -CH=), 5.43 (1H, dd, cis-HC=CH<sub>2</sub>), 5.33 (1H, dd, trans-CH-CH<sub>2</sub>); <sup>13</sup>C-NMR (300MHz, CDCl<sub>3</sub>) δ: 115.5 (C-1), 158.6 (C-2), 99.7 (C-3), 161.5 (C-4), 126.3 (C-5), 127.5 (C-6), 117.3 (C-A), 138.4 (C-B), 129.2 (C-1c), 130.3 (C-2c), 115.6 (C-3c), 159.5 (C-4c), 115.6 (C-5c), 130.6 (C-6c), 39.7 (C-1d), 147.3 (C-2d), 110.6 (C-3d), 55.3 (-OCH<sub>3</sub>), 187.6 (C=O), 26.3 (C-4d), 26.4 (C-5d). By comparison, the data of compound 4 was basically consistent with the literature (Zou, 1993), so it was identified as licochalcone A.



**Figure 4:** Structure of licochalcone A

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### Observation of cell morphology

Optical microscopic observation showed that the growth of gastric cancer cell line SGC-7901 was inhibited in the experimental groups, and apoptotic morphological changes were observed in adherent cells, that is, cell shape gradually changed from spindle to round, and transparency and adhesion of cells were reduced; cell growth in the control group was normal.

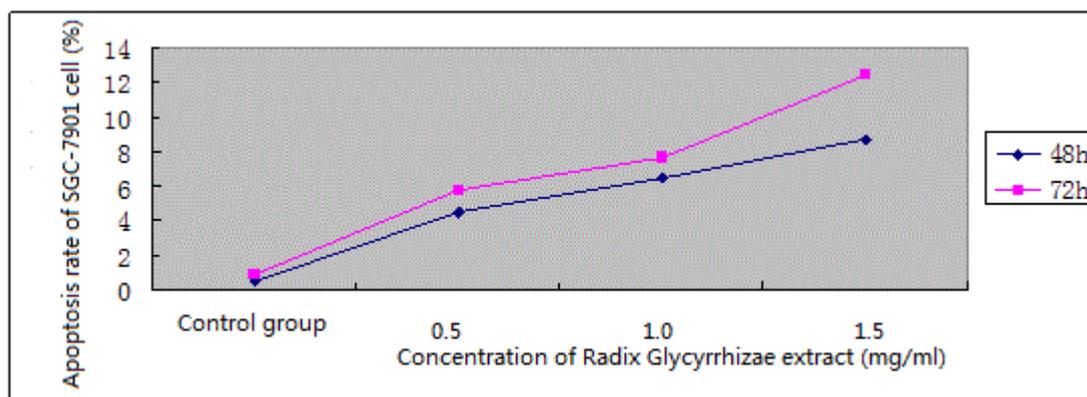
### Change in cell cycle

Flow cytometry with PI staining showed that Radix Glycyrrhizae extract could induce SGC-7901 cell apoptosis within 0.5-1.5 mg/mL, compared with the control group, the apoptosis was positively correlated with the drug concentration, which exhibited an apparent dose-dependence. The results in Tab. 1 show that after 72 h of action, the number of G<sub>0</sub>/G<sub>1</sub> phase cells increased apparently in various Radix Glycyrrhizae extract groups compared with the control group, indicating that different concentrations of Radix Glycyrrhizae extracts can effectively induce SGC-7901 cell cycle arrest in the G<sub>0</sub>/G<sub>1</sub> phase in a dose-dependent manner.

**Table 1:** Effect of Radix Glycyrrhizae extract on cell cycle of SGC-7901 cells (72 h)

Group	Concentration (mg/mL)	G <sub>0</sub> /G <sub>1</sub> phase	S phase	G <sub>2</sub> /M phase
Control group		47.43 ± 6.54	19.45 ± 4.97	33.13 ± 5.75
Radix Glycyrrhizae extract	0.5	54.35 ± 4.65	18.47 ± 5.73	27.18 ± 4.34
	1.0	63.57 ± 5.85*	17.54 ± 6.64	18.89 ± 4.48
	1.5	71.24 ± 7.37*	16.54 ± 5.73	12.22 ± 3.23

Note: comparison with the blank control group, \* P<0.05



**Figure 5:** Effect of Radix Glycyrrhizae extract on apoptosis of gastric cancer SGC-7901 cells

## Discussion

Radix Glycyrrhizae flavonoids are a class of constituents with relatively strong physiological activity contained in Radix Glycyrrhizae. There have been relatively many studies on the pharmacological activity of Radix Glycyrrhizae flavonoids, which are mainly focused on anti-depressant and anti-cancer aspects. Fan Zi-zhou et al. established chronic stress depression model by administering nine different stimuli to rats, and continuously intervened the established model using different doses of Radix Glycyrrhizae flavonoids, whose anti-depressant effects were determined by open field test, forced swimming test and tail suspension test. Their results suggested that the total flavonoid fraction of Radix Glycyrrhizae has a good antidepressant pharmacological activity on chronic unpredictable stress-induced depressive behaviors in rats, as well as a protective effect on stress-induced hippocampal neurogenesis impairment at larger doses (Li et al., 2010; Wang et al., 2004; Fan et al., 2012). Radix Glycyrrhizae flavonoids can induce typical apoptotic changes in tumor cells, including reduced nucleus volume, nuclear chromatin condensation into clumps and formation of apoptotic bodies. Electron microscopy and agarose gel electrophoresis found that Radix Glycyrrhizae

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flavonoids can cause relatively obvious apoptotic morphological changes and DNA fragmentation in tumor cells (Ji et al., 2005; Zhao et al., 2005; Ji et al., 2004; Ji et al., 2002).

In this paper, four compounds, namely oleanolic acid, isoliquiritin, glycyrrhetic acid and licochalcone A, were isolated from the ethanol extract of *Radix Glycyrrhizae* by column chromatography, ODS column chromatography, preparative thin layer chromatography and NMR spectroscopy techniques.

In the experiment on the growth inhibitory effect of *Radix Glycyrrhizae* extract on gastric cancer cell line SGC-7901, optical microscopic observation showed that the growth of gastric cancer cell line SGC-7901 was inhibited in the experimental groups, and apoptotic morphological changes were observed in adherent cells, that is, cell shape gradually changed from spindle to round, and transparency and adhesion of cells were reduced; cell growth in the control group was normal. Flow cytometry with PI staining showed that *Radix Glycyrrhizae* extract could induce SGC-7901 cell apoptosis within 0.5-1.5 mg/mL, compared with the control group, the apoptosis was positively correlated with the drug concentration, which exhibited an apparent dose-dependence. The results in Tab. 1 show that after 72 h of action, the number of G<sub>0</sub>/G<sub>1</sub> phase cells increased apparently in various *Radix Glycyrrhizae* extract groups compared with the control group, indicating that different concentrations of *Radix Glycyrrhizae* extracts can effectively induce SGC-7901 cell cycle arrest in the G<sub>0</sub>/G<sub>1</sub> phase in a dose-dependent manner.

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