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Research Article

Studies on the Identification of Constituents in Ethanol Extract of Radix Glycyrrhizae and Their Anti-Primary Hepatoma Cell Susceptibility

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The objective of this paper is to study the chemical constituents of Radix Glycyrrhizae and to apply the resulting natural products in the study of drug susceptibility of hepatoma cells so as to provide a scientific basis for quality standards and clinical application of medicinal Radix Glycyrrhizae. Chromatographic materials were used for isolation and purification; structural identification was performed based on physicochemical properties and spectral data. MTT colorimetry was used to detect the proliferation inhibition rate against primary hepatoma cells by natural products, and flow cytometry was used to detect the changes in cell cycle progression. Five compounds were isolated and identified, namely, liquiritigenin (1), liquiritin (2), isoliquiritigenin (3), betulinic acid (4), and oleanolic acid (5). In the study, 5-FU (5-fluorouracil) is used as a positive control to the hepatoma cells. Primary hepatoma cells were highly susceptible to 5-FU and liquiritigenin, both of which markedly inhibited the proliferation of hepatoma cells; flow cytometry results showed an increase in G0/G1 phase cells, a decrease in S phase cells, and a relative increase in G2/M phase cells. Primary hepatoma cells are highly susceptible to liquiritigenin, a natural product; the testing of tumor cell susceptibility is of important significance to the improvement of therapeutic effect of cancer.

1. Introduction

Radix Glycyrrhizae is a legume, which is recorded in the Chinese Pharmacopoeia 2010 Edition Vol. I as the root and rhizome of *Glycyrrhiza uralensis* Fisch., *G. inflata* Bat., or *G. glabra* L. [1]. Radix Glycyrrhizae has a complex chemical composition, whose main constituents are flavonoids and saponins [2–4]. Despite extensive studies on chemical constituents of Radix Glycyrrhizae, pharmacological studies on monomeric compounds, especially the studies on anticancer activities and drug susceptibility, are still few [5]. To further improve the quality evaluation of Radix Glycyrrhizae and to better guide clinical medication, a systematic study of chemical constituents in Radix Glycyrrhizae is necessary, in order to obtain monomeric compounds and to study their antihepatoma activities.

Primary liver cancer is a common malignancy. Incidence of liver cancer is relatively high in many countries, and the majority of patients are not diagnosed until they are in the advanced stages. Liver cancer is relatively less sensitive to chemotherapy and is prone to drug resistance, which forms major reasons affecting chemotherapeutic effect on liver cancer. Among them, the multidrug resistance (MDR) of liver cancer not only limits the chemotherapeutic effect on liver cancer, but is also an important cause of recurrence and metastasis of liver cancer. Human hepatoma Bel-7402 cell line has become one of the commonly used in vitro biological models in the screening of novel anticancer drug candidates. Domestic scholars have used this model to screen out baicalin, cordycepin, melittin, ampelopsin, ligustrazine, and other natural active constituents with relatively high inhibitory activities on hepatoma Bel-7402 cells from natural medicines and explored their application values in adjuvant therapy, susceptibility, and other aspects of liver cancer [6, 7]. In this study, hepatoma tissues obtained from surgical resection were used to prepare and culture primary hepatoma

cells, which were used to perform anticancer drug sensitivity test, with the aim of improving drug efficacy and reducing side effects.

2. Methods

2.1. Instruments. NMR spectra were determined using a Bruker AV-500 NMR Spectrometer. TLC silica gel GF-254 and column chromatography silica gel (200-mesh) were both manufactured by Xindi Chemical Plant in Yantai; RPTLC plates were products of Merck, Germany; Sephadex LH-20 was a product of Pharmacia; RP-18 reversed phase silica gel was a product of Great Door, Germany; MCI GEL CHP-20P was a product of Mitsubishi Chemical; and D101 macroporous resin is a product of Dashi Technology Development Co., Ltd. in Nanjing.

2.2. Drugs and Reagents. Radix Glycyrrhizae was purchased from Hebei Ecological Technology Co., Ltd., which was identified by Professor Deng Fei from the Beijing University of Chinese Medicine as the root and rhizome of *Glycyrrhiza uralensis* Fisch. All the reagents used were of analytical grade. Five cases of hepatoma tissues were provided by the Department of Laboratory, General Hospital of Beijing Military Region of PLA, which had been pathologically diagnosed; RPMI-1640 medium and MTT reagents were purchased from Sigma; 5-FU (5-fluorouracil) was purchased from the Department of Pharmacy, General Hospital of Beijing Military Region of PLA; fetal bovine serum was purchased from Beijing Lixiapu Biology Co., Ltd.

2.3. Preparation of Compounds

2.3.1. Extraction and Isolation. Radix Glycyrrhizae medicinal material was ground, passed through a 60-mesh sieve, soaked overnight in a 6-fold volume of 80% aqueous solution of ethanol, and then extracted under reflux three times, each time lasted 2 h, followed by filtration. Solvent was then removed under reduced pressure to give the extract.

The 80% ethanol solution extract was dried to give the powder (0.5 kg), which was then fully dissolved in water, loaded on the D101 macroporous resin column, and eluted sequentially with water and 30%, 60%, and 90% aqueous solutions of ethanol. The fraction eluted with 90% ethanol solution was isolated through silica gel column chromatography and gradient-eluted with cyclohexane-acetone (10 : 1; 9 : 1; 8 : 1; 7 : 1; 6 : 1; 5 : 1; 4 : 1; 3 : 1; 2 : 1; 1 : 1) to give compound 4 (100 mg) and compound 5 (50 mg). The fraction eluted with 60% ethanol solution was purified repeatedly through silica gel column chromatography and eluted with petroleum ether-acetone (20 : 1) to give compound 2 (50 mg) and compound 3 (100 mg). The fraction eluted with 30% aqueous solutions of ethanol was subjected to column chromatography on MCI gel, gradient-eluted with aqueous solutions of ethanol (40%, 50%, 60%, 70%, and 80%), and recrystallized repeatedly with methanol to give compound 1 (5 g).

2.3.2. Structural Identification (Figure 1). Compound 1: white powder, mp. 259~261°C. ^{1}H-NMR (DMSO-d_6) δ: 7.74 (1H, d, J = 8.6 Hz, H-10), 7.16 (1H, s, H-13), 6.90 (1H, d, J = 8.4 Hz, H-11), 6.72 (1H, s, H-8), 5.23 (1H, t, J = 7.0 Hz, H-2′), 3.96 (3H, OCH_3), 3.36 (2H, d, J = 7.0 Hz, H-1′), 1.78 (s, CH_3), 1.70 (s, CH_3). The above H-NMR spectral data were consistent with the reported liquiritigenin data [8], so the compound was identified as liquiritigenin.

Compound 2: white powder, mp. 207~209°C. ^{1}H-NMR (DMSO-d_6) δ: 10.66 (s, OH-7), 7.49 (2H, d, J = 8.4 Hz, H′-2′, 6), 7.69 (1H, d, J = 8.6 Hz, H-5), 7.10 (2H, d, J = 7.2 Hz, H-3′, 5′), 6.50 (1H, d, J = 8.6 Hz, H-6), 6.34 (1H, s, H-8), 5.55 (1H, brd, J = 12.4 Hz, H-2), 2.76 (2H, m, H-3), 4.92 (1H, d, J = 6.2 Hz, H-1″). The above H-NMR spectral data were consistent with the reported liquiritin data [9], so the compound was identified as liquiritin.

Compound 3: yellow powder. ^{1}H-NMR (DMSO-d_6) δ: 13.66 (2′-OH), 8.19 (1H, d, J = 8.8 Hz, 6′-H), 7.79~7.70 (4H, α, β, 2, 6-H), 6.88 (2H, d, J = 8.4 Hz, 3, 5-H), 6.38 (1H, dd, J = 8.8, 2.4 Hz, 5′-H), 6.29 (1H, d, J = 2.4 Hz, 3′-H). The above data were basically consistent with the isoliquiritigenin reported in the literatures [10, 11], so the compound was identified as isoliquiritigenin.

Compound 4: white powder (chloroform). ^{1}H-NMR ($CDCl_3$) δ: 0.66, 0.74, 0.82, 0.88, 0.92, 1.66 (each 3H, 6×CH_3), 4.08 (1H, H-3), 4.62, 4.75 (each 1H, C=CH_2); ^{13}C-NMR ($CDCl_3$) δ: 38.6 (C-1), 27.6 (C-2), 76.9 (C-3), 38.7 (C-4), 55.3 (C-5), 19.0 (C-6), 34.3 (C-7), 40.6 (C-8), 51.0 (C-9), 36.9 (C-10), 20.8 (C-11), 26.4 (C-12), 37.2 (C-13), 43.4 (C-14), 31.5 (C-15), 32.1 (C-16), 56.3 (C-17), 49.5 (C-18), 46.8 (C-19), 150.4 (C-20), 29.7 (C-21), 38.2 (C-22), 28.0 (C-23), 15.1 (C-24), 15.9 (C-25), 16.1 (C-26), 14.9 (C-27), 179.9 (C-28), 109.9 (C-29), 19.4 (C-30). The above data were basically consistent with the reported literature [12], so compound 4 was identified as betulinic acid.

Compound 5: white powder (chloroform). ^{1}H-NMR ($CDCl_3$) δ: 0.76 (3H, s, H-23), 0.81 (3H, s, H-24), 0.90 (3H, s, H-25), 0.92 (3H, s, H-26), 0.93 (3H, s, H-29), 0.99 (3H, s, H-30), 1.13 (3H, s, H-27), 5.29 (1H, t, J = 2.4 Hz, H-12), 3. 22 (1H, dd, J = 4.2, 11.4 Hz, H-3α), 2.84 (1H, dd, J = 13.8, 3.6 Hz, H-18); ^{13}C-NMR ($CDCl_3$) δ: 39.2 (C-1), 26.9 (C-2), 79.1 (C-3), 38.7 (C-4), 55.5 (C-5), 18.3 (C-6), 32.6 (C-7), 40.1 (C-8), 47.7 (C-9), 37.1 (C-10), 23.5 (C-11), 122.6 (C-12), 143.6 (C-13), 41.6 (C-14), 27.7 (C-15), 23.7 (C-16), 46.5 (C-17), 41.9 (C-18), 45.9 (C-19), 30.9 (C-20), 34.8 (C-21), 33.4 (C-22), 29.1 (C-23), 15.7 (C-24), 16.3 (C-25), 18.1 (C-26), 26.2 (C-27), 183.2 (C-28), 33.1 (C-29), 23.2 (C-30). The above data were consistent with the reported literature [13], and Rf value and spot color on TLC were exactly identical with those of oleanolic acid reference substance, so compound 5 was identified as oleanolic acid.

2.4. Anticancer Pharmacological Activity Test

2.4.1. Preparation of Primary Hepatoma Cells. Surgically resected hepatoma tissues were taken and soaked in sterile Hanks' solution; small amounts of nonnecrotic carcinoma tissues were selected, repeatedly washed in RPMI-1640 medium containing 1% double-antibody, cut into 1 mm^3 pieces, then digested by addition of a 10-fold amount of 0.25% trypsin solution and 0.02% EDTA at 37°C for 35 min, and shaken every 5 min; digestion solutions were discarded, and the

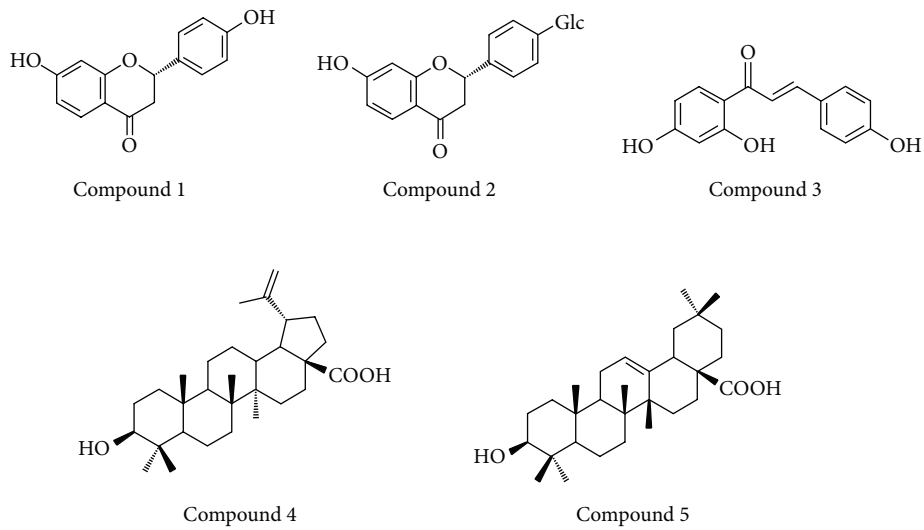

Figure 1: Structural formulas of compounds 1–5.

remaining was washed by addition of blank culture medium, then added with a small amount of culture solution, and repeatedly blown with a pipette into single cells, which were then passed through a 40-mesh sieve, and stained with 1% trypan blue, followed by counting of the number of viable cells.

2.4.2. MTT Colorimetry. 2×10^5 hepatoma cell suspension was taken and seeded in 96-well plastic culture plates. Wells contained different drugs at 100 μL per well, with 90 μL added medium and 10 μL drug; the concentration of all drugs is 1 mg/mL. 10 replicate wells were set up for each drug; the plates were statically cultured in a CO_2 incubator set at 37°C for 24 h. After incubation, MTT reagent was added at 20 μL/well, and the cultivation was continued for an additional 6 h, then the supernatant was aspirated, and each well was added with dimethyl sulfoxide at 150 μL/well and shaken for 15 min, so that the MTT reduction product was completely dissolved. Absorbance (A value) of each well was measured at 570 nm using a microplate reader, and the proliferation inhibition rates against hepatoma cells by different drugs were calculated according to the following formula. Hepatoma cell proliferation inhibition rate = (1 − A value of cells in treated wells ÷ A value of cells in control wells) × 100%.

2.4.3. Flow Cytometry. Changes in each cell cycle phase of primary hepatoma cells by different drugs were detected using flow cytometry. Cell culture and grouping methods were the same as "MTT colorimetry," with the exception that 96-well plastic plates were replaced by 25 mL culture flasks; after cultivation, cells were collected and washed three times with PBS; supernatant was discarded and the remaining was fixed in 70% cold ethanol and stored within 4°C; before being subjected to flow cytometry, the cells were passed through a 40-mesh sieve, concentration adjusted to 1×10^6 cells/mL, and PI stained, followed by detection with flow cytometry; cell cycle results were analyzed using Modiffi 2.0 software.

2.5. Statistical Processing. Data were analyzed using SPSS 11.0 statistical software, comparison of cell proliferation inhibition rate was performed by Student's t-test, and comparison of cell cycle between groups by F test. All data are expressed as mean ± standard deviation ($\overline{x} \pm s$).

3. Results

Positive control 5-FU is effective for inhibiting primary hepatoma cells. Comparing with 5-FU, liquiritigenin also had apparent proliferation inhibitory effects on primary hepatoma cells, while liquiritin and isoliquiritigenin had relatively low sensitivities to hepatoma cells, and betulinic acid and oleanolic acid had no clear effect on these cells. 5-FU and liquiritigenin could significantly inhibit hepatoma cell progression from G1 phase to S phase, where G0/G1 phase cells increased, S phase cells decreased, and G2/M phase cells relatively increased. See Tables 1 and 2.

4. Discussion

Ideal goal of adjuvant cancer chemotherapy is to pick out the most sensitive and most effective adjuvant chemotherapy regimens based on clinical manifestations, pathological type, and genetic characteristics of patients, thus ensuring maximum benefit for patients and achieving truly individualized treatment [14]. In this study, hepatoma tissues obtained from surgical resection were used to prepare single cell suspension and for primary cell culture, and drug sensitivity in cells was observed; MTT results showed that 5-FU and liquiritigenin had apparent inhibitory effects on primary hepatoma cells, with the inhibition rates of 42.9% and 35.7%, respectively, while the inhibition rates of drugs in other groups were not high. Difference in the sensitivity of various drugs was larger between the groups. Therefore, sensitivity of chemotherapeutic drugs should be tested before application to facilitate the individualized treatment for the same patient, in order to

Table 1: Primary hepatoma cell proliferation inhibition rates of various drugs ($\overline{x} \pm s$, %).

Group	Concentration (μg/mL)	A value	Inhibition rate (%)	P value
Control group	0	1.4 ± 0.1	—	—
5-FU group	10	0.8 ± 0.2	42.9	<0.01
Liquiritigenin group	100	0.9 ± 0.2	35.7	<0.01
Liquiritin group	100	1.0 ± 0.0	28.6	<0.05
Isoliquiritigenin group	100	1.0 ± 0.2	28.6	<0.05
Betulinic acid group	100	1.3 ± 0.2	7.1	>0.05
Oleanolic acid group	100	1.3 ± 0.1	7.1	>0.05
Extract group	100	1.2 ± 0.3	14.3	>0.05

Table 2: Effects of various drugs on cell cycle phases of primary hepatoma cells ($\overline{x} \pm s$, %).

Group	Concentration (μg/mL)	G0/G1	P value	S	P value	G2/M	P value
Control group	0	26.7 ± 4.3		68.5 ± 3.2		5.8 ± 1.3	
5-FU group	10	58.7 ± 3.6	<0.01	26.7 ± 3.2	<0.01	14.3 ± 3.2	<0.05
Liquiritigenin group	100	55.9 ± 4.1	<0.01	28.7 ± 1.2	<0.01	14.8 ± 0.3	<0.05
Liquiritin group	100	39.8 ± 2.3	<0.05	49.2 ± 1.1	<0.05	11.2 ± 0.6	<0.05
Isoliquiritigenin group	100	38.5 ± 3.1	<0.05	49.3 ± 1.5	<0.05	12.3 ± 0.4	<0.05
Betulinic acid group	100	30.7 ± 3.2	—	63.5 ± 2.0	—	6.5 ± 0.6	—
Oleanolic acid group	100	28.5 ± 3.1	—	64.6 ± 1.6	—	6.8 ± 0.2	—

reduce blind medication, thereby improving the quality of treatment and enhancing survival rate. MTT assay results can objectively reflect the activation state of tumor cells, because the MTT reagent can be reduced to blue formazan particles by dehydrogenases in the mitochondria of living cells of mammals, and the amount of formazan production is linearly correlated with the number of living cells and cell activation state; this experiment is widely used in the screening of anticancer drugs and the experimental studies of cytotoxicity.

Flow cytometry results showed that different drugs all had effects on each cell cycle phase of primary hepatoma cells, but the sensitivities of 5-FU and liquiritigenin were higher. 5-FU converts into fluorouracil deoxynucleotide in the body and specifically binds with thymidylate synthase, thus affecting cell DNA synthesis; so the results showed an increase in G1 phase cells, resulting in accumulation of G1 phase cells and failure to enter S phase; S phase cells decreased, so that the G2/M phase cells were relatively increased, while the increase in G2/M phase cells is a common reaction of cell damage [15]. Numerous studies have shown that [16] the tumor cell killing effects of many chemotherapeutic drugs are achieved by arresting cell cycle progression. Therefore, drug susceptibility testing prior to chemotherapy can not only achieve specific killing of tumor cells, but also help avoid damage to normal tissues and organs. The present study not only found that liquiritigenin has a potential antihepatoma activity, but also stressed the importance of susceptibility in the clinical treatment of cancer.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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