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Full Length Research Paper

Anti-cancer activity of flavonoids from Xinjiang *Glycyrrhiza inflata* Licorice on proliferation, cytotoxicity and apoptosis in cervical carcinoma cells

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Glycyrrhiza inflata **Licorice, as a traditional Chinese medicine, has long been used as therapeutic ingredients as anti-inflammatory, and anti-viral in many chronic diseases. Anti-cancer activity of licorice total flavonoids (LTFs) from Xinjiang *G. inflata*, on cervical carcinoma cells was investigated. LTFs were prepared from *G. inflate*. After determination of content by high-performance liquid chromatographic (HPLC), the finger print map was characterized by Electro Spray Mass Spectrometry. SiHa cervical carcinoma cells were treated with LTFs, and cell viability and cellular apoptosis were determined by both MTT method and flow cytometry. In the dose range of 0 to 500 μgml^{-1} LTFs, the cell viability dropped gradually with the LTFs treatment, the lowest level by MTT detection was 12%; in the dose range of 0 to 1000 μgml^{-1} LTFs, the ratio of cellular apoptosis induced by LTFs increased gradually, the highest level by flow cytometry detection was up to 78%. It can be concluded that LTFs had relatively high anti-cancer activity on cervical carcinoma cells including the inhibition of cell growth and viability by induction of cellular apoptosis. This work may provide important evidence for further screening and isolation of active ingredients of flavonoids as well as the development of new natural anti-cancer drugs.**

Key words: Xinjiang *Glycyrrhiza inflata*, licorice total flavonoids (LTFs), anti-cancer activity.

INTRODUCTION

Glycyrrhiza inflata is one of the main botanical sources of licorice. It is distributed throughout central Asia: Kyrgyzstan, Kazakhstan, Tajikistan, Turkmenistan, Uzbekistan, Mongolia, and west China (Jun et al., 2010). Xinjiang Uyghur autonomous region of China has an abundant *Glycyrrhiza* plant resource. *G. inflata* is called *Ququk buya* or *Bihsus* in Uyghur; its root, *G. inflata* licorice, has long been used as a main ingredient in many drug prescriptions of traditional Uyghur medicine (TUM), especially in the preparation of drugs to regulate the four abnormal body fluids by munziq of abnormal savda - Compound Munziq Particles [State medical permitment No: Z65020166] which contains considerable proportion of *Glycyrrhiza* root composition (Liu, 1999).

The main chemical composition of *Glycyrrhiza* licorice includes three terpenes, flavonoids, and polysaccharides. Its flavonoids composition has up to 150 species which exhibit multiple biological activities including antiallergic, anti-infammatory, antiviral, anxiolytic, and anticarcinogenic activities (Manthey et al., 2001; Teillet et al., 2008; Boumendjel et al., 2009). Especially, flavonoids such as isolicorice element, licorice element and licorice chalcone A have significant antioxidant, anti-inflammatory and estrogenic functions (Le Bail et al., 1998; Hsu et al., 2005). *In vitro* experiments indicate that these flavonoids

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Abbreviations: MTT, 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide; **SiHa,** cervical carcinoma cell lines; **HPLC,** high-performance liquid chromatographic; **LTFs,** licorice total flavonoids; **RSD,** relative standard deviation.

could control the cell growth of prostate, breast, colon and liver cancer (Le Bail et al., 1998; Hsu et al., 2005; Erhart et al., 2005; Kilani-Jaziri et al., 2011), but the studies about the cytotoxic effects of these flavonoids on human cervical carcinoma cell lines were not reported yet. It was also speculated that *glycyrrhiza* licorice flavonoids might have a strong anti-cancer activity in estrogen-dependent tumors.

Cervical cancer is the second malignant tumor morbidity of women worldwide which ranks just behind breast cancer. It takes away nearly 27 million women's lives every year and has been severely threatening the health and life of women (Yang et al., 2004; Ferlay et al., 2007; Cohen, 2005).

The prevalence rate of cervical cancer in south Xinjiang Uyghur women (590/10 million) is four times greater than women's average prevalence (138/10 million) in China, therefore cervical cancer was listed as one of high-incidence endemic diseases in Xinjiang (Lalai et al., 2006; Guzalnur et al., 2004). Thus, it is important to make clear the anti-tumor activity of Xinjiang glycyrrhiza licorice flavonoids from Xinjiang on cervical carcinoma cells in order to improve the treatment by using of local resources.

In this study, the preparation of general flavonoids (LTFs) of Xinjiang *G. inflata* licorice and its mass fingerprint analysis were reported. Anti-cancer cytotoxic drug activity of *Glycyrrhiza* LTFs was evaluated through the drug intervention, cell viability and cell apoptosis test of SiHa cervical carcinoma cell. Hence, this work will provide a foundation and evidence for the development of anti-cancer drugs form Xinjiang regional peculiar plants such as *G. inflata* licorice.

MATERIALS AND METHODS

Instruments

LCQ-ESI-MS (Thermo Fishers), CO_2 Incubation box (Hera Cell-150, Thermo), Biosafety cabinet (Thermo KS-18, Thermo), ELISA Reader (Beckmann Coulter), Flow cytometer (LSR II, BD), Annex in V-FITC kit (Calbiochem). SiHa cells were provided by the cell laboratory of Shanghai Branch of Chinese Academy of Science. RPM I1640 culture medium, fetal bovine serum (FBS), antibiotics, trypsin, 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide (MTT) and dimethylsulfoxide (DMSO) were purchased from Sigma or Invitrogen.

Preparation and content determination of LTFs of *Glycyrrhiza inflata* Licorice

Xinjiang *G. inflata* licorice was obtained from Xinjiang Licorice Base, Maralbexi (Bachu), Kashgar. *G. inflata* licorice LTFs were extracted from *Glycyrrhiza* root with ultrasonic wave using macroreticular polymeric absorbents in purification. The LTFs content of *G. inflata* licorice was determined by high-performance liquid chromatographic (HPLC) analysis. The method of preparation, purification and identification was reported in detail in our previously published work (Ma et al., 2008).

Ion trap electron spray mass spectrometry (ESI-MS) analysis

The ESI-MS spectrum of LTFs from Xinjiang glycyrrhiza inflate was taken with LCQ-ESI-MS (Thermo Fishers). The optimal parameters of MS analysis: ionization source: electrospray (ESI), capillary temperature: 150°C, cone gas (N_2) flow: 350 L/h, desolvation gas (N_2) flow: 50 L/h, capillary voltage: 3.5 kV, scan mass range: 200 to 1000/50 to 350, cone voltage: 30 V/60 V, collision gas: Ar and collision energy: 30 V.

Preparation of sample solution

After dissolving the LTFs with pure DMSO, dilutions at different concentrations with RPM I1640 cell culture medium were made, the DMSO concentration of each sample was fixed to 0.5%, and all samples were demerged by 0.22 μm filter. The drug concentration was determined as the ratio of LTFs weight to solution volume.

Cell culture

SiHa cells were cultured and maintained with RPM I1640 cell culture medium supplemented with10% of FBS, 100 Uml^{-1} of toximycin, and 100 $Ugml^{-1}$ of streptomycin in an atmosphere of 5% CO_2 at 37°C. Culture medium was changed every 2 to 3 days, when the cell confluence reached the volume of 80% the cells were passaged to the ratio of 1:3 or 1:4 by trypsin (0. 25%) digest method.

MTT assay

Cell viability was determined using MTT assay. During logarithmic growth phase, SiHa cells were seeded in 96-well microplates (5000 cells/well, 200 μl/well in a humidified incubator for 24 h). Pre-experiments proved that when drug intervention time was controlled between 16 to 48 h, MTT assay determination results remained almost stable, and that the drug affection time has little effect on the results. So, we chose 20 to 24 h as the best drug intervention time. The next day, 20 μl of MTT (5 $mgml^{-1}$) were added to each well in order to keep the original culture medium volume constant. After cultivating the cells for 4 h in 5% CO_2 cell culture incubator at 37°C, the culture medium was discarded, and the cells were dissolved with 150 μl DMSO. When the crystal violet in cells was dissolved completely, its absorbance value (OD) was measured at 570 nm with a microplate reader, then cell survival rate was calculated and cell viability was determined. Independent experiments were repeated for six times, the average value was calculated, and the t-test method in statistical analysis was sued by SPSS.17.0 software.

Apoptosis rate by flow cytometry

The chosen logarithmic phase SiHa cells were planted in 6-well with cell density of 1×10^6 cells / well, cultivated for 20 to 24 h. When the cell confluence reached the volume of 70 to 80%, a predetermined concentration of drug was added and cultivated further. After the complements of drug intervention, the cells were collected and processed with reference to annex in V-FITC kit manual, and cell apoptosis rate including cell suspension, combined with the annex in V-FITC (1.25 μl) and propidium iodide (PI, 10 μl) was tested by flow cytometry. Analysis conditions for flow cytometry: excitation wavelength, Ex = 488 nm, emission wavelength, Em = 530 nm. Independent experiments were repeated three times, the average value was calculated, and the t-test method in statistical analysis was sued by SPSS.17.0 software.

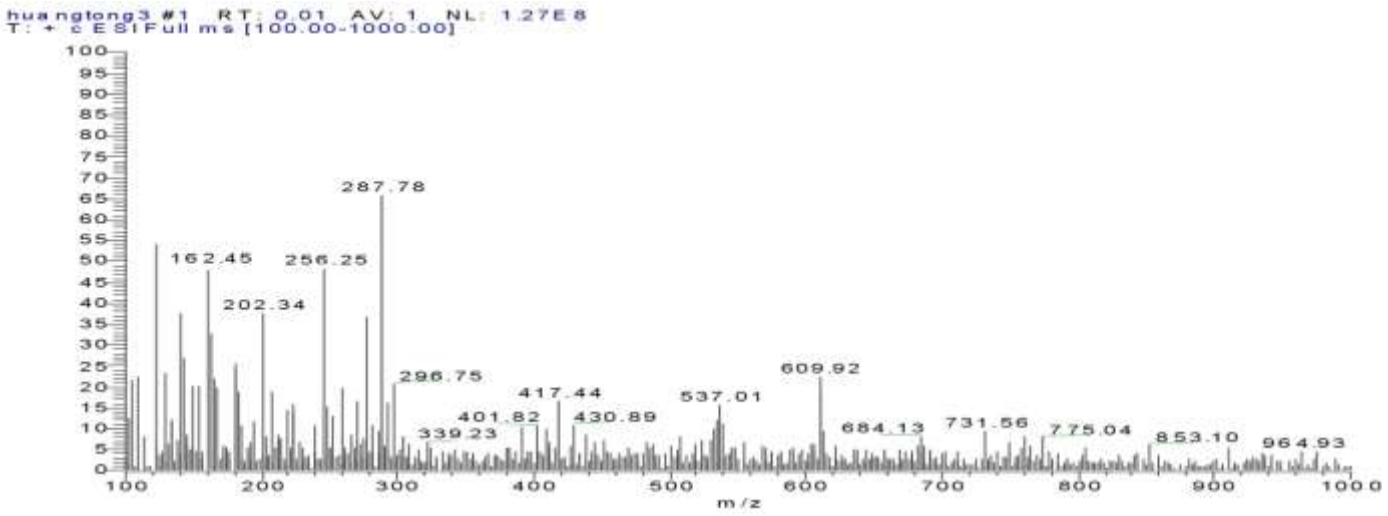

Figure 1. ESI-MS characteristics of LTFs from Xinjiang *Glycyrrhiza inflata.*

Table 1. Effects of LTFs from Xinjiang *Glycyrrhiza inflata* on SiHa growth and viability by MTT Assay.

Group	DMSO (0.5 - 1.0%)	LTFs dosage (□g/ml)	OD_{570} Value[a]	Relative cell viability[b] (%)
Blank control group	0	0	0.995 ± 0.171	91
Normal control group	+	0	1.092 ± 0.156	100
Drug intervention group	+	10	0.840 ± 0.098	77
	+	50	0.175 ± 0.016*	16
	+	100	0.136 ±0.011*	12
	+	150	0.163 ± 0.011*	15[†]
	+	200	0.180 ± 0.033*	16[†]
	+	250	0.289 ± 0.028	27[†]
		500	0.499 ± 0.058	46[†]

[a] Mean value, repeated for 6 times; [b] Percentage of the mean OD_{570} value of each group to normal control group; [+] The group with significant discrepancy ($\overline{x} \pm s$, $\rho < 0.05$) when compared its OD_{570} value with the control group; [†] Light absorbance of high concentration LTFs at 570 nm.

RESULTS

Determination of LTFs content

The HPLC method provides a repeatability of the quantitative analysis of LTFs below 1% relative standard deviation (RSD), which makes the method suitable when the legal authorities require the content to be within ±5% of the declaration. We found that Xinjiang *G. inflata* licorice contains about 35.4% of LTFs content. LTFs are a light yellow powder that displays brown color when reacted with $FeCl_3$.

ES I-MS analysis of *Glycyrrhiza* LTFs

By ESI-MS full scan analysis, fingerprint of chemical composition of glycyrrhiza LTFs was obtained (Figure 1). LTFs components of *Glycyrrhiza* contain isoliquiritigenin and licorice chalcone A, according to the mass-to-charge ratio with the corresponding peaks at 256.25 and 339.23, respectively.

Affects of *Glycyrrhiza* LTFs on SiHa cell growth and cell activity

LTFs mainly contain liophilic compounds, soluble in conventional cell culture reagents such as DMSO. Via the SiHa cell cytotoxicity test the final concentration range of DMSO used in drug intervention was determined as 0.5±1.0%. In previously published papers, 490 nm was chosen for the MTT assay. However, we repeatedly measured and found that LTFs self absorbance at 570 nm is lower.

It was found that in the LTFs concentration range between 0 and 500 $\mu g ml^{-1}$, as LTFs concentration increases, SiHa cells activity decreases (Table 1). After

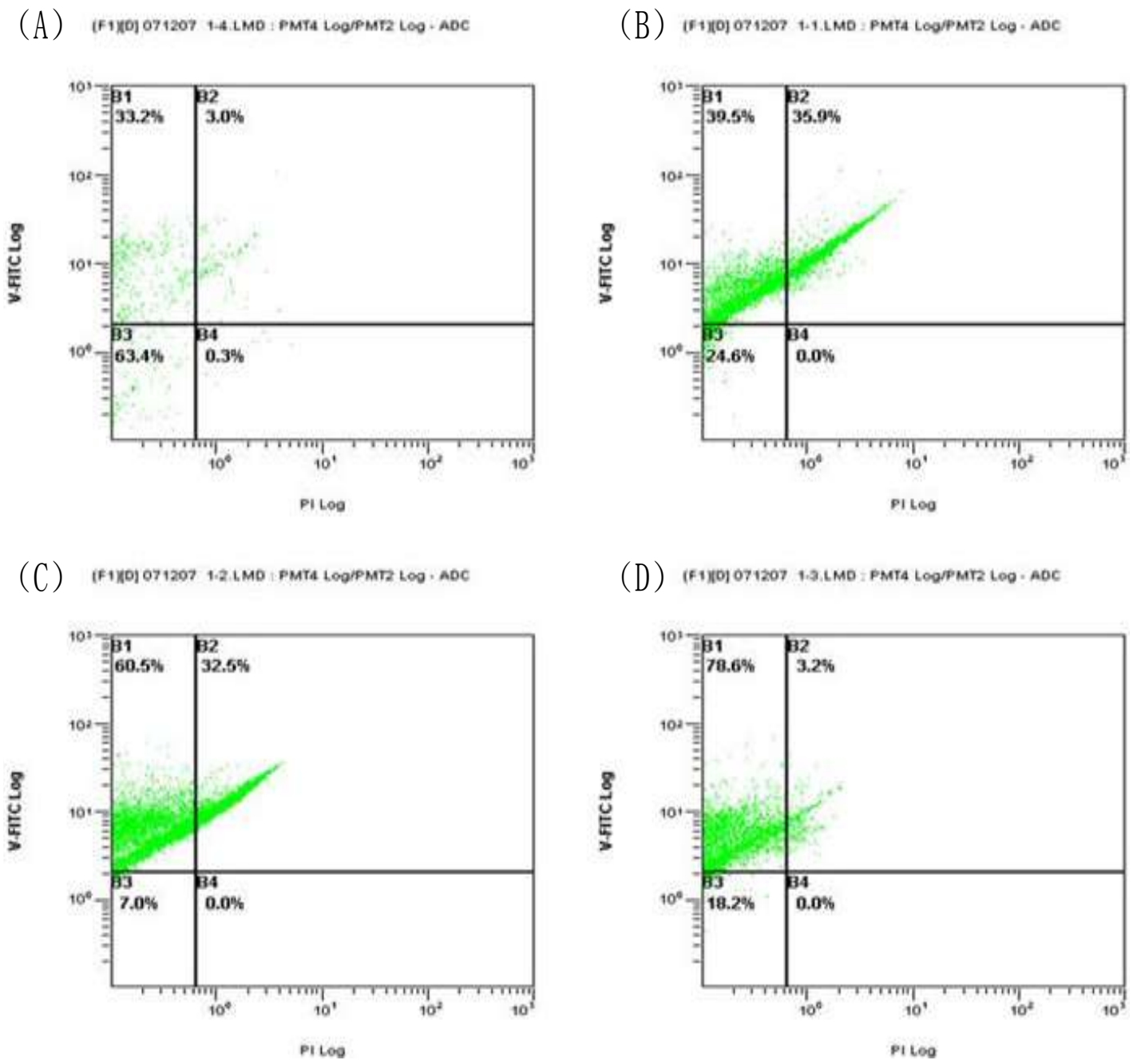

Figure 2. FACS detection of SiHa cell apoptosis induced by LTFs treatment. SiHa cells after treated with LTFs remarked with Annexin-V-FITC and PI to test their apoptosis rate by flow cytometry at Ex = 488 nm, Em = 530 nm. (A) 0 μgml^{-1}GFs; (B) 100 μgml^{-1}GFs; (C) 500 μgml^{-1}GFs; (D) 100 μgml^{-1}GFs.

repeated experiments, we found that at LTFs concentration below 150 μgml^{-1}, there is a dose-response relationship (Figure 3). The best sensitivity is achieved at 50 μgml^{-1}. When LTFs concentration is more than 150 μgml^{-1}, LTFs self absorption causes an interference on the detection signal.

MTT assay results reflect that LTFs have combined effects on cell growth, reproduction, activity and cell apoptosis, and it was proven that LTFs have the potential anti-cancer activity in cervical cancer.

Glycyrrhiza LTFs-induced SiHa cell apoptosis by flow cytometry

Fluorescent labeling and flow cytometry are helpful to determine the level of apoptosis, and to eliminate the

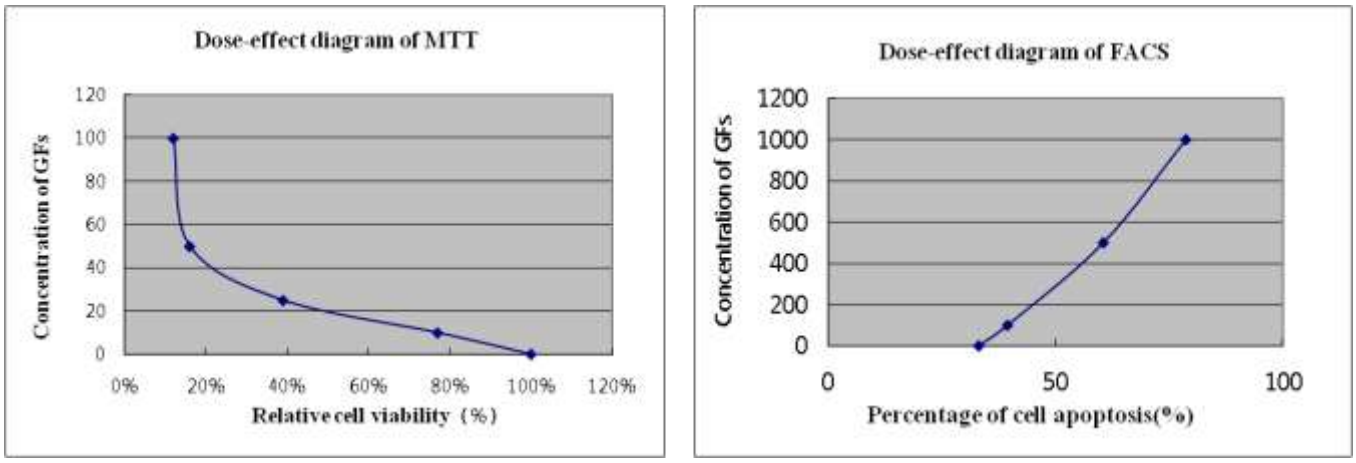

Figure 3. Dose-effect diagrams of MTT Assay and FACS.

Table 2. Effects of LTFs from Xinjiang *Glycyrrhiza inflata* on SiHa apoptosis by FACS method.

Group	LTFs dosage (μg/ml)	Apoptotic cell percent [a]
Normal control group	0	33.3667 ± 0.86217
Drug intervention group	100	40.2667 ± 1.35769*□
	500	62.2667 ± 1.66233*
	1000	80.7667 ± 1.95533*

[a] Mean value, repeated for 3 times; *The group with significant discrepancy ($\bar{x} \pm s$, ρ < 0.05) when compared its cell apoptosis percentage with other groups.

flavonoids self absorption of light. The drug intervention of SiHa cell with 0 + 1000 μgml^{-1} of LTFs, induces apoptosis, and apoptosis rate increases proportionally to drug concentration. There is a dose-effect relationship; the minimal effective concentration is 100 μgml^{-1} and the maximum apoptosis rate is 78% (Figure 3 and Table 2). The effects of LTFs in cell apoptosis induction are in accordance with the cell vitality determined by MTT method, and this is also a further evidence for the strong anti-cancer activity of *Glycyrrhiza* LTFs.

DISCUSSION

Research on the anti-cancer activity of plant constituents from both domestic and foreign sources has been in existence for decades. The possible mechanism of anti-tumor activity of some plant drugs has also been successfully explained to some extent. Flavonoids (LTFs) are a class of low molecular weight natural plant components, which have a common nucleus of C_6+C_3+C_6, which mainly contain flavonoids, flavonols, isoflavones, chalcones, flavanones, dihydro-flavonoids, dihydro-chalcones etc.

Recent *in vitro* and *in vivo* studies have demonstrated that flavonoids have the effect of suppressing carcinogenesis as a promising candidate for cancer prevention (Yang et al., 2001; Le Marchand, 2002; Kawabata et al., 1999; Wietrzyk et al., 2000; Pollard and Suckow 2006). Several mechanisms of action have been identified for flavonoids chemoprevention functions such as, estrogenic/antiestrogenic activity, antiproliferation, induction of cell-cycle arrest or apoptosis, prevention of oxidation, induction of detoxification enzymes, regulation of the host immune system, anti-inflammatory activity, and changes in cellular signaling (García-Lafuente et al., 2009; Birt et al., 2001). Furthermore, an extensive panel of flavonoids has reversal impact upon BCRP-, P-gp-, and MRP1-mediated drug resistance (Katayama et al., 2007). In our study, LTFs of Xinjiang *G. inflata* licorice inhibit cell growth by inducing cell apoptosis, therefore it could be one of the most possible mechanisms of LTFs' anti-cancer activity.

In the search for high-efficient and low toxic natural drugs, licorice has always been an interesting plant species. Approximately 150 kinds of flavonoids have been found in licorice species. Xinjiang has rich resources of wild and cultivated licorice plant species. A number of studies have also been reported about the extraction process, composition and pharmacological

identification of licorice flavonoids (Ma et al., 2008; Mourboul et al., 2008). They have provided significative guidance for the screening and identification of single active components of licorice LTFs by revealing its anti-cancer mechanism.

In this study, Xinjiang *Glycyrrhiza* LTFs contains 35.4% of LTFs content. By ESI-MS analysis, the chemical composition fingerprint of Xinjiang *Glycyrrhiza* LTFs was identified. It was inferred that Xinjiang *Glycyrrhiza* LTFs contain anti-cancer active components such as, isoliquiritigenin and licorice chalcones A. Cell drug intervention and MTT assay results indicated that when the concentration range of LTFs was between 25 to 100 μgml^{-1}, it produced inhibitory effect on SiHa cervical carcinoma cell growth and cell activity, and the cell activity decreased to 12%. In order to further identify the function of the drug on cervical carcinoma cell apoptosis alone, we applied the Annexin V fluorescent labeling and flow cytometry. Results indicated that the drug induced cell apoptosis and the apoptosis rate reached up to 78% within the LTFs concentration range of 100 to 1000 μgml^{-1}. By cell viability and apoptosis analysis, we believed that Xinjiang *Glycyrrhiza* LTFs have active components with anti-cancer ability which could inhibit growth, reproduction and vitality of cervical carcinoma cells by inducing cell apoptosis. Through this research, we preliminary evaluated the anti-tumor biological activity of *Glycyrrhiza* LTFs in cervical cancer. This finding made an important basis for further studies on screening and separation of active components of Xinjiang *Glycyrrhiza* LTFs and to determine the mechanism of the exact apoptotic pathway both at the *in vitro* and *in vivo* level.

In summary, our studies suggest that, general flavonoids (LTFs) isolated from Xinjiang *G. inflata* licorice may be developed as chemotherapeutic agents for cervical carcinoma treatment due to LTFs' function in inhibition of cancer cell growth and induction of apoptosis with the presence of its antitumor components such as isoliquiritigenin and licorice chalcones A.

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