

## Original Research Article

# Liquiritin (LG), isolated and identified from *Radix Glycyrrhizae* (RG), inhibits the proliferation and induces apoptosis of human gastric cancer cells (MGC-803)

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## ABSTRACT

### Keywords

High Throughput Screening (HTS), *Radix Glycyrrhizae*, Liquiritin (LG), Gastric cancer, Apoptosis

*Radix Glycyrrhizae* (liquorice root) is Chinese liquorice has been used in traditional Chinese medicine for facilitates spleen, stomach functioning, expels phlegm and control coughing, HeLa, Hepatocarcinoma bearing mice, and melanoma cancer studies. In the present study, the initial screening of *Radix Glycyrrhizae* ethanol extract fractions showed strong anti-gastric activity and less toxicity with spleenocytes normal cells. Therefore; we identified a bioactive liquiritin from *Radix Glycyrrhizae* which is responsible for the anti-gastric activity for the first time by using high throughput screening technology (HTS) and used chemical structure analysis techniques (Mass spectrum, <sup>1</sup>H-NMR, <sup>13</sup>C-NMR) to identify liquiritin chemical structure. The anti-gastric activity of liquiritin (LG) was evaluated via morphology cells, MTT assay, and flow cytometry to estimate live, necrosis, and apoptosis ratios and staining with Hoechst 33258. The results showed liquiritin inhibits proliferation of MGC-803 cells followed induced apoptosis and future investigations could develop it action.

## Introduction

Gastric cancer occupied the second rang after lung cancer as one of the most common causes of cancer death due to its death rate >700,000 deaths each year (Max *et al.*, 2005). Gastric cancer exist more in African countries than others (Tan *et al.*, 2006). The incidence and mortality by sex worldwide in men is higher than in women.

The men occupied the second rang while the women the fourth. From the ancient time till nowadays, natural products had shown a great important in the cure of many illnesses. In most herbal preparations in oriental medicine, *Radix Glycyrrhizae* (RG) tree is very widespread (Figure 1). When we take in consideration for example, 120

prescriptions in one Chinese medicine text book, 80 include RG. RG extract is known to have life-enhancing properties, as well as curing and detoxifying effects against injury or swelling. RG products are currently used as flavoring or sweetening agents for tobaccos, chewing gums, candies or beverages in the United States and European countries and are consumed in large quantities at nearly 1.5 kg per person per year (Wang and Nixon, 2001). Carcinogenesis induced by toxicants and hormones are prevented by RG (Zhu *et al.*, 1999). In the uterine corpus, it concealed estradiol induced expression of c-Fos/Jun and prevented endometrial carcinogenesis (Niwa *et al.*, 1999). Up to date study established that diverse solvent extracts from RG specially from its roots (Figure 2) possess wide broad pharmacological actions in vivo models such as effects on central nerve system, cardiovascular system and endocrine system, liver, renal and pancreas functions, anti-ulcer action, anticancer action, anti-allergic and anti-inflammatory effects, anti-virus and antibacterial activities, and effect on immune function (Xiu *et al.*, 2007). The researchers are still continuing having interest in the potential for RG extracts to prevent cancer (Isbrucker and Burdock, 2006; Wang and Nixon, 2001; Chin *et al.*, 2007). It is also known that in vitro, human clinical trials are still having limits (Jung *et al.*, 2006).

The effects of chemical compounds from RG including glabridin, isoliquiritin, glycyrrhizin, glycyrrhizinic acid, carbenoxolone and LG have been studied on mice, rat, and human cancer cell lines. Most studies indicated a dose-dependent action on cell/tumor proliferation and apoptosis. Some cancer cells such as prostate, breast, colon, liver, and lung cancer cell lines have been investigated (Dong *et al.*, 2007; Adams *et al.*, 2006; Lee, 2007; Chintharlapalli *et al.*, 2007). However such effect of LG on human

gastric cancer cells (MGC-803) has not yet been investigated.

Thus, in the present study, to know whether LG could inhibit human gastric cancer (MGC-803) cells proliferation and induced apoptosis were investigated by using cell morphology changes, staining with Hoechst 33258, MTT assay and apoptosis ratio by flow cytometry analysis.

## **Materials and Methods**

### **RG purchasing**

RG was purchased from national institute for food and drug control and Jilin xiancao medical herb limited company by the Key Research Laboratory of Cell Biology, Membrane Channels Research and Anti-Cancer Drug Discovery in the School of Life Science, Northeast Normal University, Changchun, Jilin Province China. Specimen was deposited in this Key Laboratory.

### **Cell culture, reagents and chemicals**

Human gastric cancer cell line MGC-803 was purchased from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies Inc., Grand Island, NY) supplemented with 10% Fetal Bovine Serum (FBS; Life Technologies Inc., Rockville, MD). Cells were cultured at 37°C with 5% CO<sub>2</sub> at 95% humidity. For morphology and density appreciation, the cells were cultured in 10 cm or 24 wells plates and observed after 24 hours under a phase microscope.

### **Identification of small natural molecule from RG extract by High Performance Liquid Chromatography (HPLC)**

To purify the small molecule from RG positive fractions with anti-gastric activity

for first time, we realized ethanol extraction, solvent extraction and HPLC. The roots of RG composed of single pieces stored in a dry and dark place at room temperature with passive ventilation for several times were collected (1kg of crude) and exposed to 95% ethanol extraction. After the ethanol extraction, the extract obtained was rotated on a Rota vapor to eliminate the rest of ethanol found in it. The final material obtained was then dried in an incubator.

Continuous solvent extraction was performed to be able to know the suitable solvent for the isolation of small molecule found in RG positives fractions on MGC-803 cells. A sample of the extract was extracted consecutively with solvents of increasing polarity (Petroleum, Chloroform, ethyl acetate and N-butanol). 250 mL of the extract was mixed with 50 mL of each solvent above and leave at room temperature for one hour or more according to the extraction scheme. After each extraction, followed by filtration, two phases were obtained, the solvent and water phase. From this analysis, chloroform solvent was found to be the best with wave length 277 nm. After several analysis, and purification by using HPLC analysis, HPLC preparative, and HSCCC instruments and every time we test on MGC-803 cells. Finally, we succeed to isolate and purify the small natural molecule which is responsible for the anti-gastric activity for first time from RG herb extract. To identify unknown small molecule with anti-gastric cancer for first time, we used MS, <sup>1</sup>H-NMR, and <sup>13</sup>C-NMR chemical structure analysis (Skoog *et al.*, 1980), and we identified the small natural molecule named Liquiritin (LG).

#### **Liquiritin anti-gastric activity test**

The initial screening of several Chinese herbs paved to observe anti-gastric activity for LG, and we followed the procedure

below to identify the active herb, 100 $\mu$ L of the mixture (DMEM + FBS + Cells) was put in each well of a 96 wells plate and cultured at 37<sup>0</sup>C with 5% CO<sub>2</sub> at 95% humidity and growth for 24hours. After 24 hours, the density of the cells was observed and density reached to 70–80%. Ninety-six wells plate was divided to 3 lines, first line was for untreated cells (negative control), second line was for treated cells with 100  $\mu$ M of LG, and third line was for 100  $\mu$ M of cisplatin (a known drug inhibiting human gastric cancer cells MGC-803) as positive control. The plate was then replaced in an incubator for a second culture. After 24 hours, cells were observed to identify the anti-gastric cancer activity of LG.

#### **Splenocytes cells toxicity test**

To observe the cytotoxic effect of liquiritin on normal splenocytes cells (splenocytes was isolated from CD1 mouse). Briefly, mouse was euthanized by overdose of pentobarbital. Spleen was surgically removed and mashed using the plunger end of the syringe in cold phosphate buffered saline. Cell suspension was centrifuged at 1500 g for 5 minutes and pellet was re-suspended in 1 ml of Dulbecco's Modified Eagle's Medium (DMEM) medium. Red blood cells were lysed with lysis buffer (0.01 M KHCO<sub>3</sub> and 0.15 M NH<sub>4</sub>Cl) for 40 s then 9 ml of medium was added and re-centrifuged. Supernatant was discarded again and the pellet was re-suspended in DMEM medium with 10% FBS. Cells were plated in 96 well plates at 20 cells/well and we followed the same procedure in 2.5 to identify the toxicity of LG on splenocytes cells. The splenocytes cells were stained with 0.4% trypan blue, we observed and photographed under microscopy.

#### **MTT assay**

LG activity on cells growth was estimated

by MTT assay that is based on the cleavage of the yellow tetrazolium salt to purple formazan crystals by intracellular dehydrogenases (Mosmann, 1983; Bounous *et al.*, 1992; Carmichael *et al.*, 1987). Briefly, MGC-803 cells were seeded into sterile 96-wells plate. After 24 hours, LG was added to cell culture medium over a final concentration range of 100µM, 50µM, 25 µM, and 12.5 µM. The cells were incubated at 37°C and 50% CO<sub>2</sub> for a period of 24 hours. After incubation period, MTT was added to each well and incubated for further 4 hours. After 4 hours incubation, the medium was removed, blue crystals of MTT reduced by cells. Cells were dissolved with 100µL DMSO and the cellular metabolism was determined by measuring the absorbance (A) of samples at 540 nm using the software Ascent Software for Multiskan Ascent installed in a computer linked to a measure machine. IC<sub>50</sub> values were estimated following 24 hours incubation. Its formula was:

$$I\% = [A_{540} (\text{control}) - A_{540} (\text{treated})] / A_{540} (\text{control}) * 100$$

For statistics analysis, we used one way analysis of variance (ANOVA) procedures to assess significant differences among the treatment groups and non treated groups. Turkey test was used to compare multiple group means for each significant effect of treatment. The criterion for statistical significance was set at P < 0.05 or P < 0.01.

### **Hoechst 33258 staining**

To detect the apoptotic morphological changes in the nuclear chromatin of cells we performed Hoechst 33258 staining. MGC-803 cells were cultured in the 6-wells plate. After overnight growth, they were treated with different concentrations LG of 100 µM, 50 µM, 25 µM, and 12.5 µM for 48 hours. After 48 hours, cells were washed with

phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 minutes. Using Hoechst 33258 staining solution, we incubated the cells for 10 minutes. After incubation, cells were washed three times with PBS then viewed under a fluorescence microscope. Cells exposed for 48 hours exhibited morphological changes typical of apoptosis from 100 µM, 50 µM, 25 µM and 12.5 µM.

### **Flow cytometry**

MGC-803 cells were grown in 6 wells Plate culture flasks at 37°C under 5% CO<sub>2</sub> using DMEM + FBS. At 80–90% confluence, half of the amount of DMSO that inhibited the cells was added in the first well. In the second well, we added 50 µM of cisplatin as a positive control. In the third, we added 50µM of LG. The plate was then incubated to 24 hours. After incubation, cultured media was discarded; the cells were detached under the pates using PBS (1mL of PBS for each well) and distributed to 6 tubes of 1mL each, mixed then centrifuge at the speed of 5000 rmp, time: 5 minutes, and 4°C. After centrifugation, the supernatant is discarded. The same amount of PBS is then added in each well as here above and the cells were centrifuge as above. After this second centrifugation, the supernatant is discarded as above. The different kits of flow cytometry were added as indicated in the experiment respecting the conditions imposed. After this period of time, the cells were taken to the general laboratory to read the results.

### **Results and Discussion**

The initial screening of Chinese medicine herbs on MGC-803 cells was to identify many small natural molecules with anti-gastric cancer activity for the first time which will accelerate to discover new anti-gastric agent from natural herbs in the near

future. We observed a strong anti-gastric toxicity of *Radix Glycyrrhizae* (RG) paved us to identify the small natural molecule via the procedure as shown in Figure 3. Cisplatin was serving as positive control. MGC-803 cells were treated with 100 $\mu$ M of RG and Cisplatin for a period of 24 hours in 96 wells plate.

After 24 hours of treatment, fractions and Cisplatin were found to be positives on MGC-803 cells, which are inhibiting MGC-803 cells. RG extract fractions showed a strong anti-gastric toxicity (F<sub>4</sub>-F<sub>7</sub>, F<sub>11</sub>, G<sub>2</sub>-G<sub>9</sub> of RG from 96 well/ plate) and the rest of all the other fractions of RG were negatives. We analyzed positive RG fractions by using HPLC analysis and preparative to identify the positive small natural molecule which is responsible for anti-gastric cancer for first time.

To identify the small natural molecule with anti-gastric activity for first time from RG herb extract, we used analysis chemical structure instruments which are MS, 1H-NMR, and 13C-NMR and the literature characterization of small natural molecular from RG herb and the small molecular identified as Liquiritin, the physical property of LG is white powder with purity > 98%, the molecular formula is C<sub>21</sub>H<sub>22</sub>O<sub>9</sub>, and the molecular weight is 418g/mole (Bolledula J et al, 2009).

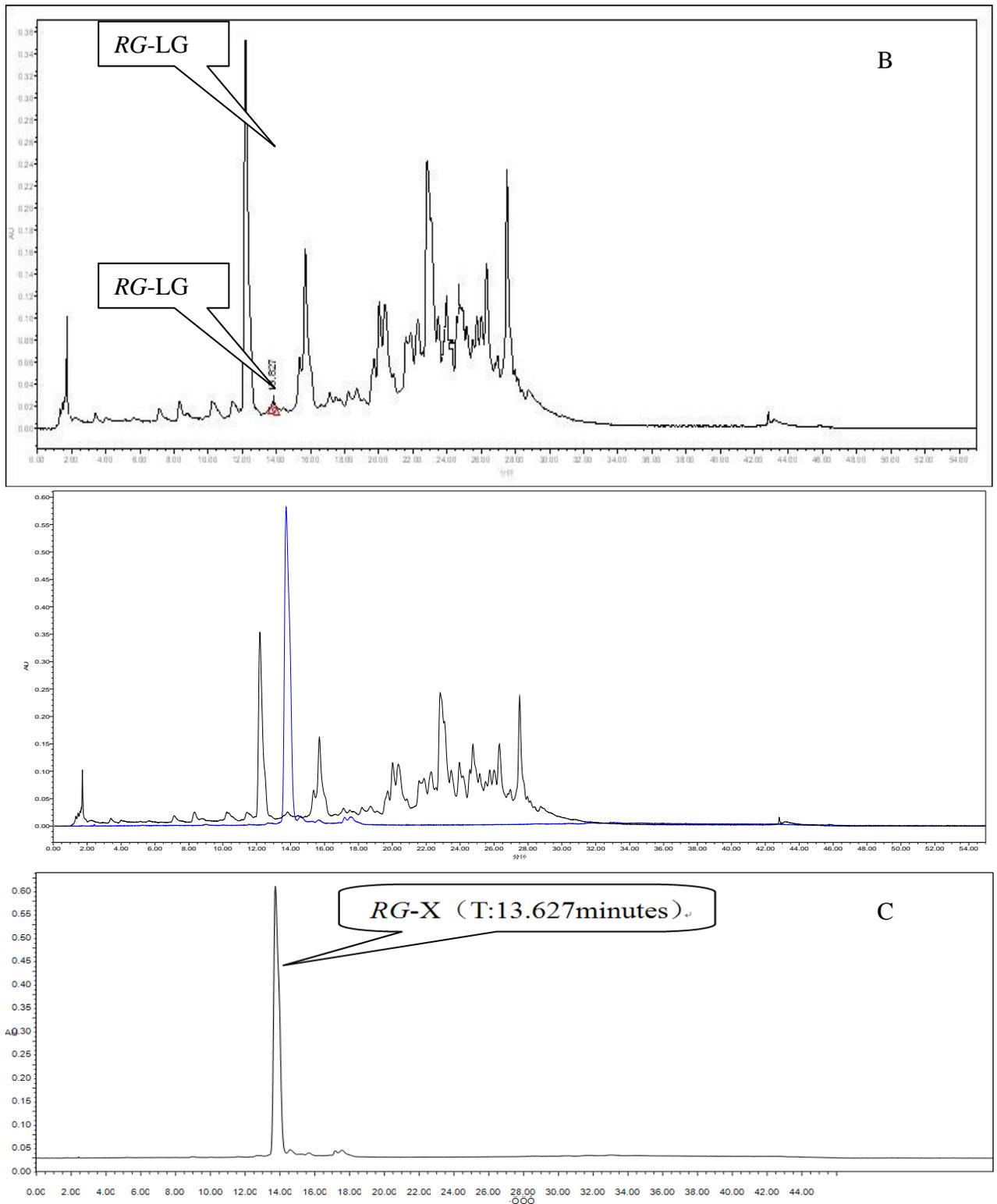
The effects of LG on MGC-803 cell proliferation were examined by treating MGC-803 cells with various concentrations of LG for 24 hours. As shown in Figure 4, an increase in cells death was observed from 12.5  $\mu$ M to 100 $\mu$ M. These cells death confirmed the present of an inhibition of the proliferation of MGC-803 cells by LG. After the treatment of MGC-803 cells with LG at 100  $\mu$ M, 50  $\mu$ M, 25  $\mu$ M, and 12.5  $\mu$ M, the rates of growth inhibition were respectively: 12.68% (12.5  $\mu$ M), 40.57% (25  $\mu$ M),

70.98% (50 $\mu$ M) and 75.57 % (100 $\mu$ M) for 24 hours.

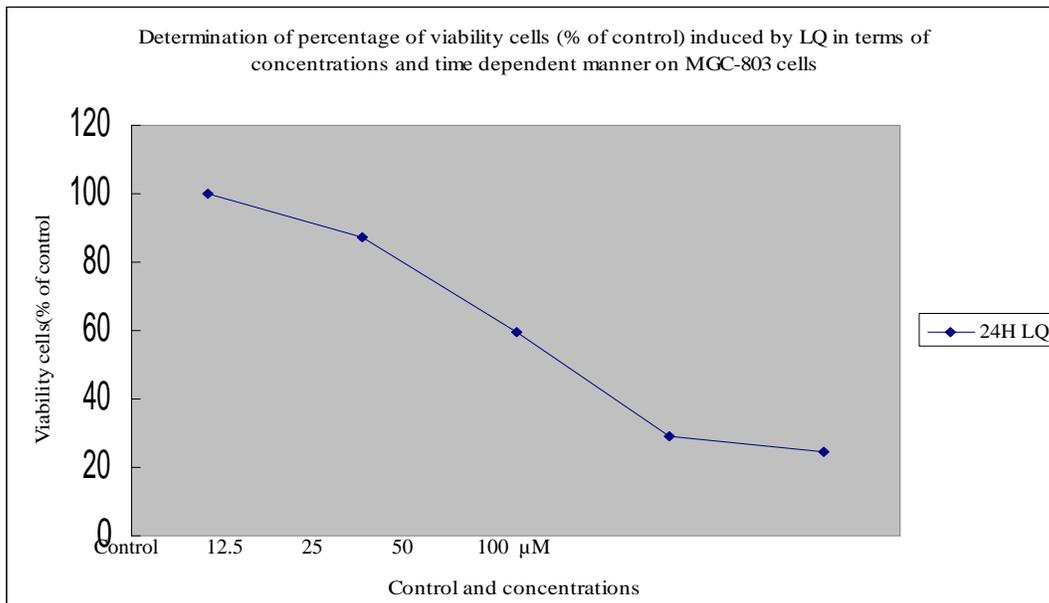
Our main objective here was to verify whether LG has toxicity on MGC-803 cells were of no action on splenocytes via morphology cells test. Splenocytes cells were cultured and treated for a period of 24 hours with LG on MGC-803 cells. The results we obtained showed that LG has toxicity on MGC-803 gastric cells and no action on splenocytes cells as showing in Figure 5 and 6.

To know whether MGC-803 cells death is a result of apoptosis, Hoechst 33258 staining was performed. The cells treated at 100 $\mu$ M, 50 $\mu$ M, 25 $\mu$ M, and 12.5 $\mu$ M with LG for 48 hours exhibited nuclear morphology changes with Hoechst 33258 staining at 50 $\mu$ M-100 $\mu$ M while almost no apoptosis nuclei were observed in controls cells. Figure 7 here below at 100 $\mu$ M shows nuclear condensation, blabbing of membrane, nuclear fragmentation, and apoptotic bodies were also detected by Hoechst staining microscopically.

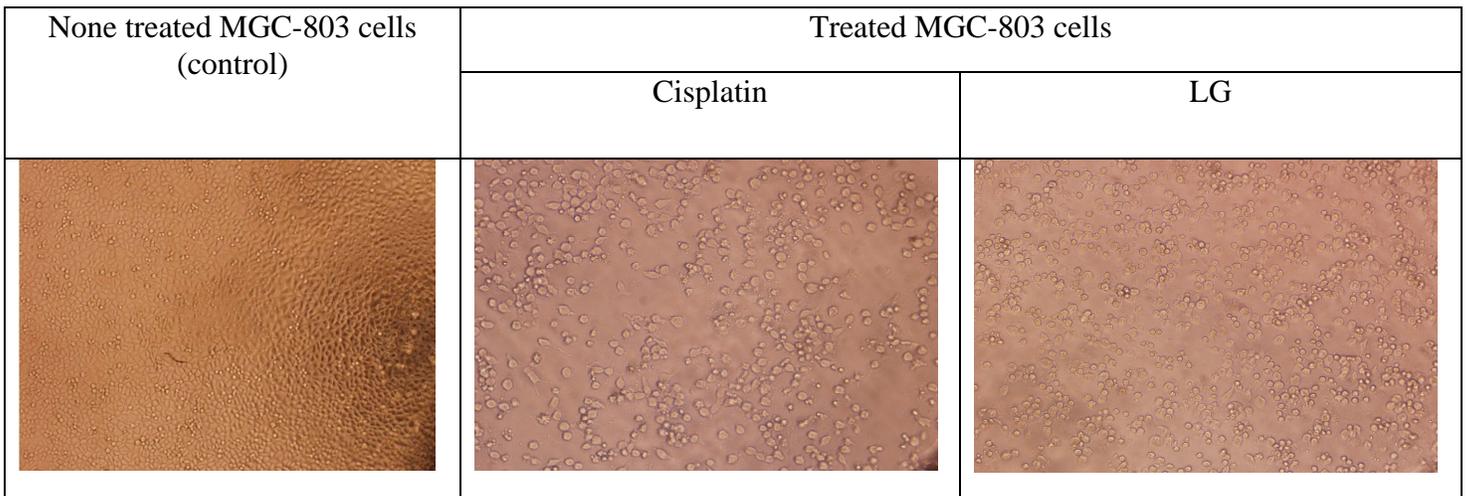
In addition, to examine the apoptosis induced by Cisplatin, a known and commercial drug treating cancer (e.g. gastric cancer) and LG on MGC-803 cells, we realized Flow cytometry. MGC-803 cells were treated with Cisplatin and LG at 50 $\mu$ M for 24 hours. The population was separated into three groups: late apoptosis and necrotic cells (B<sub>2</sub>) showing late apoptosis and necrotic cell death, live cells (B<sub>3</sub>) showing the present of living cells, apoptotic cells (B<sub>4</sub>) were showing the present of apoptosis cells. The percentages of cells in each group in control and treated cells were determined by fluorescence-activated cell sorting (FACS), an aspect of flow cytometry and the results were showed in Figure 8.



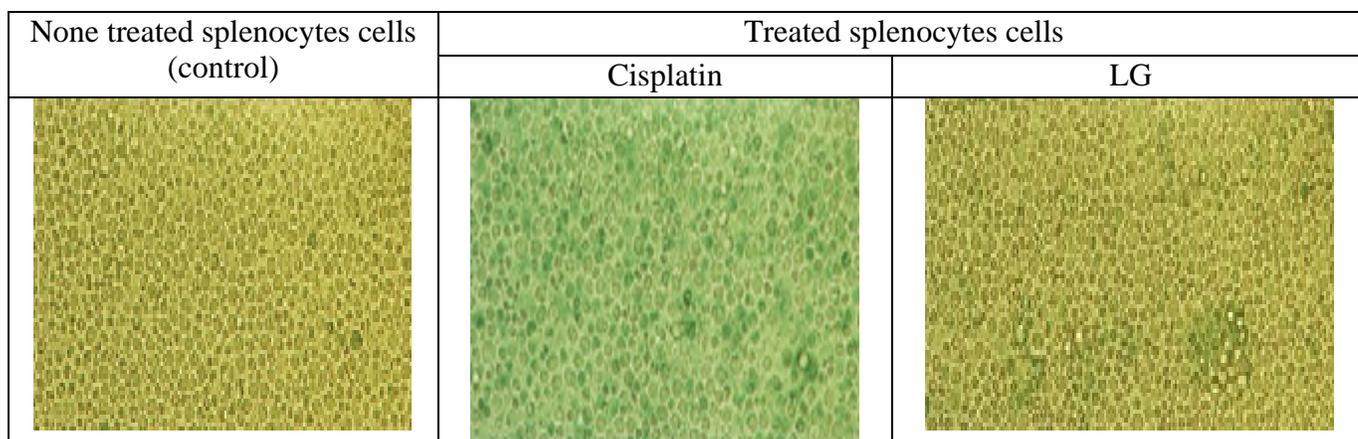
**Figure.3** A- HPLC chromatogram of RG curve showing the peak of RG-LG, the estimated compound inhibiting MGC-803 cells. RG curve showed three compounds estimated to inhibit MGC-803 cells. But our study was based only on RG-LG. B- HPLC chromatograms of RG (positive and negative fractions) curves put together to identify the correspondence of RG-LG compound. C- HPLC chromatogram of LG, the isolated compound from RG-X (X= LG).



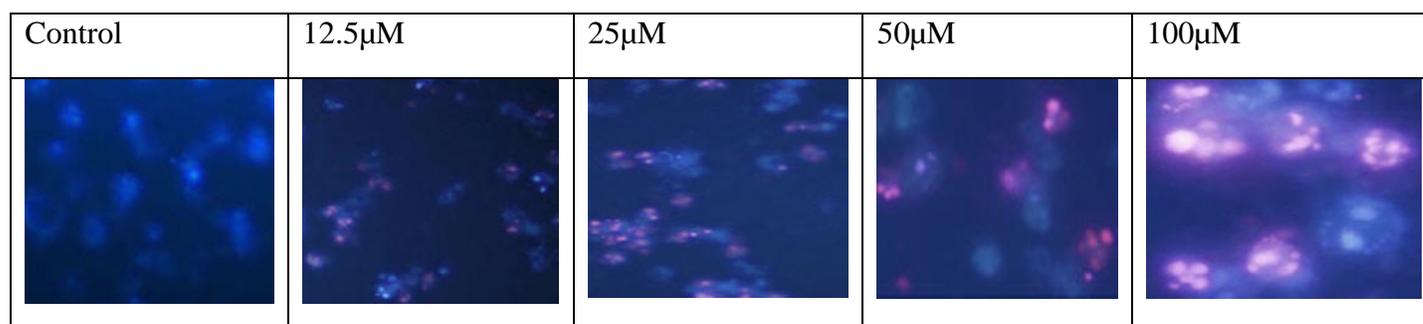
**Figure.4** Growth inhibitory effects of LG on MGC-803 cells. Cells incubated with varying concentrations of LG for 24 hours, and cell survival was determined by MTT assay.



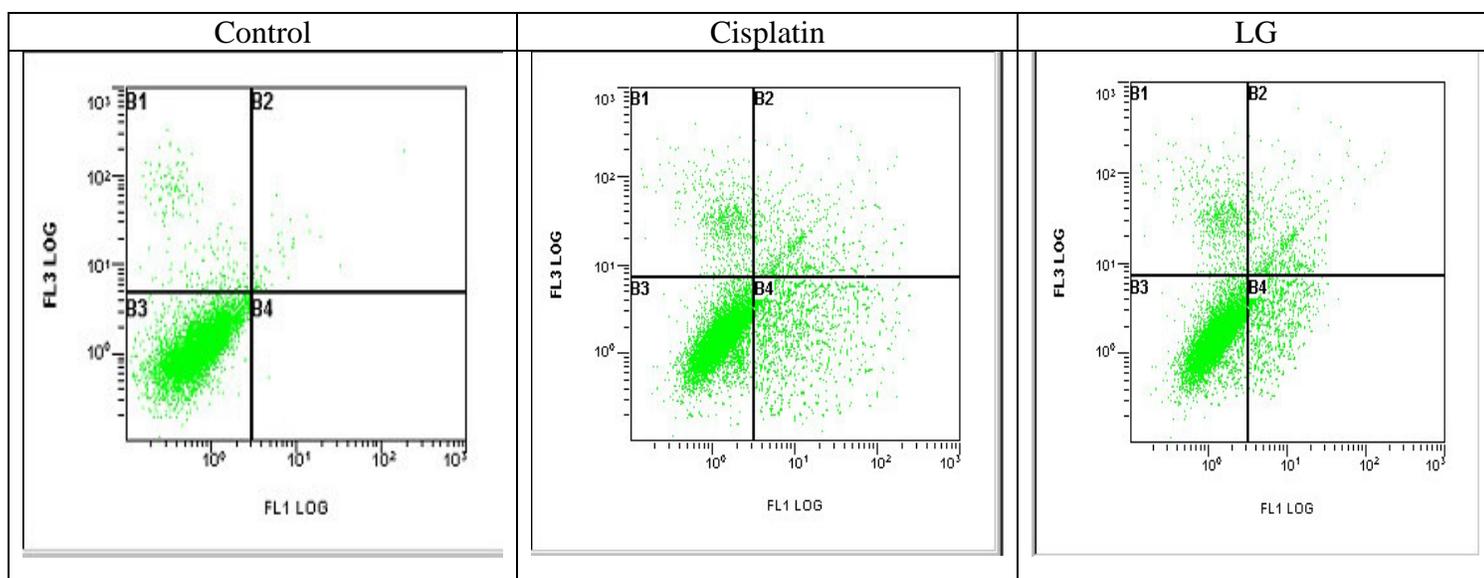
**Figure.5** Morphological changes of MGC-803 cells treated with cisplatin were observed positive control. The same morphological changes were found in LG and absence in none treated MGC-803 cells as negative control with concentration of 50μM.



**Figure.6** Morphological changes of splenocytes cells treated with cisplatin were observed positive control. The same morphological changes were found in LG and absence in none treated splenocytes cells as negative control with concentration of 50 $\mu$ M.



**Figure.7** Effects of LG on MGC-803 cells apoptosis determined by Hoechst 33258 staining. In controls, the majority of cells had uniformly stained nuclei. Cells exposed for 48 hours to (12.5-100 $\mu$ M exhibited morphological changes typical of apoptosis.



**Figure.8** Flow cytometric analysis of MGC-803 cells following exposure to Cisplatin and LG at the concentration of 50  $\mu$ M for 24 hours

After the determination, we noticed that the number of live cells (group B<sub>3</sub>) of the control, Cisplatin and LG was (24729.802, 6532.302 and 8681.42, respectively) with a % gated of (98.96%, 26.138% and 34.740%, respectively). We also observed that the number of apoptotic cells (group B<sub>4</sub>) of the control, Cisplatin and LG was (49.979, 18197.496 and 16048.382, respectively) with a % gated of (0.2%, 72.82% and 64.22%, respectively). Synchronously, taking in consideration group B<sub>4</sub> which indicated apoptotic-associated chromatin degradation, when we compared the control with the effect of Cisplatin and LG, significantly we noticed an increase of % gated after cells cultured with Cisplatin and LG (72.62% and 64.02%, respectively) at the concentration of 50µM for 24 hours. These results suggested that as Cisplatin, LG may induce apoptosis in MGC-803 cells with a dose-dependent manner.

The consumption of Flavonoids found in numerous plants is associated with the reduction of the rates of cancer. The scientific and medical study of the causes and transmission of disease within a population known as epidemiology have constantly suggested that these phenolic compounds may play an important roles in cancer prevention (Birt and Hendrich, 2001). Current anti-cancer agents' research on natural diet has focused on the activity capable of selective or preferential elimination of cancer cells by inhibiting cell cycle progression and/or causing apoptosis.

Apoptosis is a form of cell death necessary to make way for new cells and to remove cells who's DNA has been damaged to the point at which cancerous change is liable to occur. Knowing that apoptosis inhibition lies at the heart of all tumor

development, in all cancers, it remains an obvious target for preventive and therapeutic intervention (Evan *et al.*, 2001). The anti-cancer activities of certain chemotherapeutic agents are known and confirmed to be mediated through the inhibition of apoptosis considered as a favored way to handle cancer. This has been demonstrated by emerging evidence (Hen, 2000; Brown and Wouters, 1999). Liquiritin reduces cell viability in a concentration and time dependent manner, MGC-803 cells were maintained in medium for 24 hours, and then incubated with various concentrations (100µM, 50µM, 25µM, 12.5µM). A significant increase in cell death was observed due to exposure to LG at 50µM and 100µM concentrations and time dependent manner of control value in 24 hours. This is why the cells viability decreased much at 50µM-100µM and even a little at 25µM. The effects of LG on MGC-803 cells apoptosis determined by Hoechst 33258 staining and examined by fluorescence microscopy showed in control the majority of cells having uniformly stained nuclei and the cells exposed for 24 hours exhibited morphological changes typical of apoptosis from 50µM-100µM. MTT assay and Hoechst 33258 staining results compared to previous study for example Isoliquiritigenin induces apoptosis by depolarizing mitochondrial membranes in prostate cancer cells (Jung *et al.*, 2006), on how to examine the inhibition of cells proliferation

## References

- Adams LS, Seeram NP, Hardy ML, Carpenter C, Heber D 2006. Analysis of the interactions of botanical extracts combinations against the viability of prostate cancer cell lines. *ECAM*, 3: 117-124.
- AI-Haroni, M., Skaug, N. 2007. Incidence of

- antibiotic prescribing in dental practice in Norway and its contribution to national consumption. *J. Antimicrob. Chemother.*, 59: 1161–1166.
- Birt, D.F., Hendrich, S., Wang W 2001. Dietary agents in cancer prevention: flavonoids and isoflavonoids. *Pharmacol. Ther.*, 90: 157–177.
- Bounous, P., Campagnoli, R.P., Brown, J. 1992. Comparison of MTT colorimetric assay and tritiated thymidine uptake for lymphocyte proliferation assays using chicken splenocyte. *Avian Dis.*, 36: 1022–1027.
- Brown, J.M., Wouters, B.G. 1999. Apoptosis, p53, and tumor cell sensitivity to anticancer agents. *Cancer Res.*, 59: 1391–1399.
- Carmichael, J., Collins, J.M., Minna, J.D., Gazdar, A.F. 1987. Evaluation of tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res.*, 47: 936–942.
- Chin, Y.W., Jung, H.A., Liu, Y., Su, B.N., Castoro, J.A., Keller, W.J. 2007. Antioxidant constituents of the roots and stolons of licorice (*G. glabra*). *J Agric Food Chem.*, 55: 4691–4697.
- Chintharlapalli, S., Papineni, S., Jutooru, I., Indira, J., Alan, M., Stephen, S. 2007. Structure dependent activity of glycyrrhetic acid derivatives as peroxisome proliferator-activated receptor (gamma) agonists in colon cancer cells. *Mol Cancer Ther.*, 6: 1588–1598.
- Dong, S., Inoue, A., Zhu, Y., Tanji, M., Kiyama, R. 2007. Activation of rapid signaling pathways and the subsequent transcriptional regulation for the proliferation of breast cancer MCF-7 cells by the treatment with an extract of *Glycyrrhiza glabra* root. *Food Chem. Tox.*, 45: 2470–2478.
- Evan, C.A., Whetton, A.D. 2001. Proliferation, cell cycle and apoptosis in cancer. *Nature*, 411: 342–348.
- Hen, G. 2000. The biochemistry of apoptosis. *Nature*, 407: 770–776.
- Isbrucker, R.A., Burdock, G.A. 2006. Risk and safety assessment on the consumption of Licorice root (*Glycyrrhiza sp.*), its extract and powder as a food ingredient, with emphasis on the pharmacology and toxicology of glycyrrhizin. *Regul. Toxicol. Pharmacol.*, 46: 167–192.
- Jung, J.I., Lim, S.S., Choi, H.J., Cho, H.J., Shin, H.K., Kim, E.J. 2006. Isoliquiritigenin induces apoptosis by depolarizing mitochondrial membranes in prostate cancer cells. *J. Nat. Biochem.*, 17: 689–696.
- Lee, C.K. 2007. Effects of the licorice extract against tumor growth and cisplatin-induced toxicity in a mouse xenograft model of colon cancer. *Biol. Pharm. Bull.*, 30: 2191–2195.
- Max, P., Freddie, B., Ferlay, J., Paola, P. 2005. Global cancer statistics. *CA Cancer J. Clin.*, 55: 74–108.
- Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Meth.*, 65: 55–63.
- Niwa, K., Hashimoto, M., Morishita, S. 1999. Preventive effects of *Radix Glycyrrhizae* extract on estrogen-related endometrial carcinogenesis in mice. *Jpn. J. Cancer Res.*, 90:726–32.
- Skoog, D.A., West, D.M., Saunders, C., Pheladelphia, P.A. 1980. Principles of instrumental analysis. 2<sup>nd</sup> edn., Research library.
- Tan, G., Gyllenhaal, C., Soejarto, D.D. 2006. Biodiversity as a source of anticancer drug. *Curr. Drug Targets*, 7: 265–277.
- Wang, Z.Y., Nixon, D.W. 2001. Licorice and cancer. *Nutr. Cancer*, 39: 1–11.
- Xiu, P.S., Pei, G.X., Chang, X.L. 2007. Research and application of *Radix Glycyrrhizae*. *Asian J. Pharmacodyn. Pharmacokinet.*, 7: 181–200.
- Zhu, X.H., Shen, Y.L., Jing, Y.K., Cai, X., Jia, P.M., Huang, Y. 1999. Apoptosis and growth inhibition in malignant lymphocytes after treatment with arsenic trioxide at clinically achievable concentrations. *J. Nat. Cancer Inst.*, 91: 772–778.