



Estrogenic activities of extracts of Chinese licorice (*Glycyrrhiza uralensis*) root in MCF-7 breast cancer cells

Chunyan Hu^{a,1}, Huaqing Liu^{b,1}, Juan Du^c, Baoqing Mo^a, Hong Qi^c, Xinru Wang^c, Shengai Ye^d, Zhong Li^{c,*}

^a Department of Nutrition & Food Hygiene, Nanjing Medical University, Jiangsu, Nanjing 210029, China

^b Department of Preventive Medicine, Bengbu Medical College, Anhui, Bengbu 233030, China

^c The Key Laboratory of Reproductive Medicine of Jiangsu Province, Institute of Toxicology, Nanjing Medical University, No. 140 Hanzhong Rd, Jiangsu, Nanjing 210029, China

^d Jiangsu Province Hospital of Traditional Chinese Medicine, Jiangsu, Nanjing 210029, China

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ABSTRACT

Despite the wide use of Chinese licorice root (*Glycyrrhiza uralensis*) for the treatment of menopausal complaints, little is known on its potential estrogenic properties, and available information relative to its effects on cell proliferation is contradictory. In this study, the estrogenic properties of licorice root were evaluated *in vitro* by use of several assays. The effects of increasing concentrations of a DMSO extract of licorice root on the growth of MCF-7 breast cancer cells were biphasic. The extract showed an ER-dependent growth-promoting effect at low concentrations and an ER-independent anti-proliferative activity at high concentrations. In further experiments, licorice root was sequentially extracted to yield four fractions: hexane, EtOAc, methanol and H₂O. Only the EtOAc extract had effects on cell proliferation similar to the DMSO extract. The hexane extract had no effect on cell growth. In contrast, the methanol and water extracts showed an ER-independent, growth-promoting effect. Similar to its effects on cell proliferation, the EtOAc extract had a biphasic effect on S phase cell cycle distribution and the level of PCNA protein. This extract-induced transactivation of endogenous ER α in MCF-7 cells, supported by inducing down-regulation of ER α protein and mRNA levels, and up-regulation of ER α target genes pS2 and GREB1. These results suggest that the activity of licorice root and the balance between increased risk for cancer and prevention of estrogen-dependent breast cancer may depend on the amount of dietary intake.

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1. Introduction

There is growing interest in the use of herbs to aid in the maintenance of health. Licorice root (*Glycyrrhiza*) has been employed in traditional Chinese medicine to treat infectious diseases. It is also useful for detoxification, and it possesses anti-ulcer, anti-inflammation, antiviral, anti-atherogenic, and anticarcinogenic activities [1]. Some components of licorice root demonstrate antimicrobial [2,3] and antioxidant activity *in vitro* [4,5]. In Western countries, licorice root is used as flavoring and sweetening agents for tobacco, chewing gum, candy, toothpaste and beverages [1]. In

the USA, licorice root is classified as Generally Regarded As Safe (GRAS) [6] and is listed by the Council of Europe as a natural source of food flavoring in category no. 2, indicating that small quantities can be added to foodstuffs to limit the amount of an active compound in the final product [7].

American women are increasingly turning to licorice root as a 'more natural' alternative to estrogen replacement therapy in the belief that it has the benefits, without the risks, of estrogen therapy. An important consideration is whether licorice root, as a substitute for hormone replacement therapy, stimulates the progression of estrogen-dependent breast tumors, particularly in hormone-deprived conditions. Previous studies have demonstrated estrogenic effects of individual components or extracts of licorice root [8–13]. These studies have, however, been limited in scope and have not addressed issues of specificity and mechanism of action.

The type of licorice of primary concern to the western world is *Glycyrrhiza glabra*, which is indigenous to Turkey, Spain, Iraq, Turkey, Russia and North China. *Glycyrrhiza uralensis* is indigenous to Northern China, Mongolia and Siberia. As demonstrated by HPLC profiles, the chemical content of *G. uralensis* is totally different from that of *G. glabra* [14]. The purpose of the present study was

Abbreviations: CS-FBS, charcoal-stripped fetal bovine serum; DMSO, dimethylsulfoxide; E2, 17 β -estradiol; ER, estrogen receptor; EtOAc, ethyl acetate; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GREB1, gene regulated by estrogen in breast cancer 1; ICI, ICI 182,780; OHT, 4-hydroxytamoxifen; PBS, phosphate-buffered saline; PCNA, proliferation cell nuclear antigen.

* Corresponding author. Tel.: +86 25 8686 2834; fax: +86 25 8652 7613.

E-mail address: uiucl@126.com (Z. Li).

¹ Note: These authors contribute equally to this work.

to examine the estrogenic effects and mechanisms of action of *G. uralensis* extracts with different polarity on estrogen-dependent human breast cancer cells. Included assays of cell growth and cell cycle progression and determination of licorice extracts their capacity to activate ER α and to modulate ER α target genes. A goal of these studies was to evaluate extracts of different polarity and to assess their modes of action and their specific cellular targets. This study was important in order to assess the potential of licorice extracts for clinical use and their possible adverse side effects.

2. Materials and methods

2.1. Chemicals and reagents

17 β -Estradiol (E2), 4-hydroxytamoxifen (OHT) and ICI 182,780 (ICI) were purchased from Sigma (USA). Fetal bovine serum (FBS) was obtained from PAA Laboratories (Austria). Charcoal-stripped FBS (CS-FBS) was from Biological Industries (Israel). The rabbit polyclonal antibody against estrogen receptor α (ER α) was from NeoMarkers (Froment, Canada), the antibody against β -actin, the mouse anti-PCNA and anti-mouse immunoglobulin G, horseradish peroxidase-linked antibody were purchased from Boster (Wuhan, China). Enhanced chemiluminescence detection reagents were obtained from Amersham (USA). The caspase-3 activity kit was obtained from Beyotime Institute of Biotechnology (Haimen, China).

2.2. Preparation of licorice root extracts

Powdered roots of commercial *G. uralensis* were purchased from a local food market (Xian, China). Dimethylsulfoxide (DMSO) was used to extract a range of compounds with differing polarities. In a sequential procedure, hexane was used to extract the non-polar phytochemicals, ethyl acetate (EtOAc) and methanol were

used to extract the compounds with intermediate polarity, and hot water was used to extract polar phytochemicals. The fractionation scheme is outlined in Fig. 1. The hexane, EtOAc, methanol and hot water extracts were redissolved in DMSO at final concentrations of 500 μ g/mL. The stock solutions were serially diluted and added to cell cultures using the same volume of solvent (0.1% DMSO, final concentration). Control cells were exposed only to 0.1% DMSO.

2.3. Cell culture

MCF-7 cells (an estrogen receptor-positive human breast cancer cell line) were obtained from ATCC (Rockville, MD) and routinely maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1 nM E2, 2 mg/mL insulin, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 4 mM glutamine, and 10% fetal bovine serum. Exponentially growing cultures were maintained in a humidified atmosphere of 5% CO₂ at 37 °C. For cell proliferation assays, Western blot analysis, real time-PCR or cell cycle analysis, cells were switched to phenol red-free DMEM (without E2) supplemented with 5% CS-FBS at 5 days before exposure to the extracts.

2.4. Cell growth assay

Experiments were accomplished in 96-well plates containing phenol red-free DMEM supplemented with 5% CS-FBS. MCF-7 cells were seeded at a plating density of 2×10^3 cells/well in 200 μ L of medium and then cultured for 24 h to allow their adhesion to the plate. Two days later, the medium was replaced, and the cells were exposed to E2 or its vehicle, DMSO (0.1%) for 4 days. At appropriate times, MTT stock solution (20 μ L, 5 mg/mL, Sigma) was added to each well, and the plates were further incubated for 4 h at 37 °C. The supernatant was removed, and DMSO (200 μ L) was added to each well to solubilize the formazan crystals. The absorbency at 490 nm was measured with a Multiscan MCC 340 microplate reader

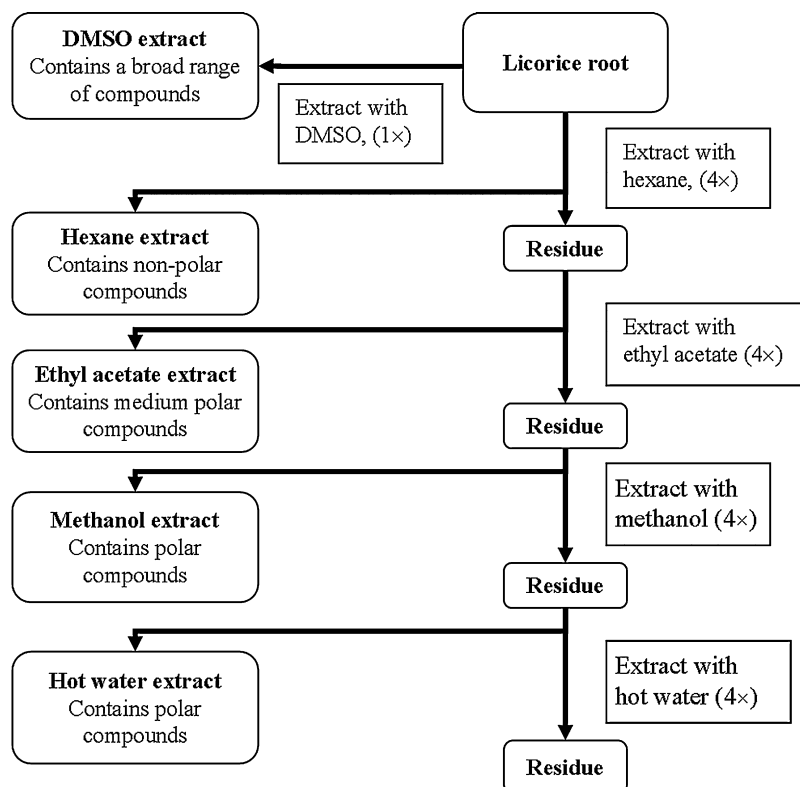


Fig. 1. Procedure used for the fractionation of licorice root.

(Titertek, USA). All measurements were performed in triplicate. Data points represented the means of values for four wells. Results were expressed as the percentages proliferation with respect to vehicle-treated cells.

2.5. Caspase-3 activity assay

For assay of caspase-3 activity, MCF-7 cells were exposed to DMSO (control) or the EtOAc extract for 4 days. Assays were performed on 96-well microtitre plates by incubating 10 μ L protein of cell lysate per sample in 80 μ L reaction buffer (1% NP-40, 20 mM Tris-HCl (pH 7.5), 137 mM NaCl and 10% glycerol) containing 10 μ L caspase-3 substrate (Ac-DEVD-pNA) (2 mM). Lysates were incubated at 37 °C for 4 h. Samples were measured with an ELISA reader at an absorbance of 405 nm.

2.6. Cell cycle analysis

5×10^5 cells were incubated at 37 °C overnight in triplicate 10-cm plastic dishes in phenol red-free DMEM supplemented with 5% CS-FBS and then for 2 days with various concentrations of the EtOAc extract of licorice root. Cells were trypsinized, washed in cold phosphate-buffered saline (PBS, pH 7.4), fixed in 70% ethanol/30% PBS and stored at 4 °C until processing. A portion (1 mL) of the fixed cell suspension containing 1×10^6 cells was washed twice in cold PBS. The fixed cells were treated for 30 min at 4 °C in the dark with fluorochrome DNA staining solution (1 mL) containing 40 μ g of propidium iodide and 0.1 mg of RNase A; the stained cells were analyzed by flow cytometry.

2.7. Western blotting

The MCF-7 cells were plated on culture dishes and allowed to attach for 24 h, followed by the addition of 0, 1, 10, 100, 250, or 500 μ g/mL EtOAc extracts of licorice root. Control cells were incubated in the medium with DMSO. After incubation, the cells were washed twice with ice-cold PBS and then scraped off in 0.2 mL of buffer (20 mM HEPES, pH 6.8; 5 mM EDTA; 10 mM EGTA; 5 mM NaF; 0.1 μ g/mL okadaic acid; 1 mM DTT; 0.4 M KCl; 0.4% Triton X-100; 10% glycerol; 5 μ g/mL leupeptin; 50 μ g/mL PMSF; 1 mM benzamide; 5 mg/mL aprotinin; 1 mM Na orthovanadate) and incubated on ice for 30 min, followed by centrifugation at 12,000 rpm for 20 min. The supernatant was stored at -70 °C. Protein concentrations were measured with the BCA protein assay (Pierce, Rockford, IL). Afterwards, proteins were diluted to equal concentrations, boiled for 5 min and separated by 12% SDS-PAGE. Proteins were transferred to nitrocellulose membranes, which were probed with ER α primary antibodies (NeoMarkers, CA) overnight at 4 °C. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature before enhanced chemiluminescence (Amersham Biosciences, USA) and exposure to film. β -Actin was used to normalize for protein loading. All the experiments were performed at least twice with similar results.

2.8. Real-time PCR

Cells were grown in 6-cm plastic dishes (1×10^6 cells/dish in 5 mL of estrogen-free medium). At 24 h after plating, the test compounds (dissolved in DMSO) were added. The medium was changed every 24 h after plating; the various test compounds were added fresh with each change. After 48 h, total RNA was isolated by TRIzol (Invitrogen), and 5 μ g of each sample was reverse transcribed by use of the M-MLV first-stand synthesis system (Promega). cDNAs were analyzed, in triplicate, with the MJ Real-Time PCR System (Bio-Rad). For ER α , pS2, GREB1

and the internal control gene GAPDH, the primers were: 5'-CCACCAACCAGTGACCAATT-3' (ER α forward) and 5'-GTCTTCCGT-ATCCACCTTTC-3' (ER α reverse), 5'-GATTGGTGGACCGATTGC-3' (GREB1 forward) and 5'-CTCCGACAGCCCGAAGTA-3' (GREB1 reverse), 5'-TTCTATCCTAATACCATCGACG-3' (pS2 forward) and 5'-TTTGAGTAGTCAAAGTCAGAGC-3' (pS2 reverse), 5'-GAAGGTGAAG-GTCGGAGTC-3' (GAPDH forward) and 5'-GAAGATGGTGATGGGA-TTTC-3' (GAPDH reverse). Normalization was achieved by dividing the expression level of mRNA by its respective GAPDH expression level. The results were expressed as fold inductions.

2.9. Statistical analyses

Data were expressed as means \pm S.D. Statistical differences were analyzed by one-way ANOVA followed by Tukey's post hoc test. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Effects of extracts of licorice root on the growth of MCF-7 human breast cancer cells

To determine whether licorice root contains estrogenic compounds, DMSO, a solvent that dissolves nearly all compounds, was used to extract chemicals from licorice root. The effects of the DMSO extract on cell growth are shown in Fig. 2A. Cell growth was biphasic. At concentrations of 10–100 μ g/mL, the DMSO extract stimulated growth, reaching a maximum effect at about 100 μ g/mL. Maximal growth stimulation by the DMSO extract was equal to that of E2 at 1 nM. Growth stimulation by the DMSO extract (100 μ g/mL) was inhibited by the anti-estrogen OHT (1 μ M) or by ICI (100 nM) (Fig. 2B), confirming that an ER α -mediated mechanism is involved in cell proliferation. In contrast to its growth-promoting effects at lower concentrations, the DMSO extract at concentrations >100 μ g/mL, inhibited cell growth. When combined with 1 nM E2, the DMSO extract dose-dependently inhibited the growth of MCF-7 cells within the concentration range used (Fig. 2C).

To determine which polar fractions of licorice root have estrogenic activity, licorice root was fractionated by use of a series of extractions with solvents of different polarity. The effects of these extracts on growth of MCF-7 cells are shown in Fig. 3A. The hexane extract of licorice root had no effect on cell growth. The effect of the EtOAc extract on the growth of cells was biphasic. It stimulated cell growth at concentrations of 1–100 μ g/mL and inhibited cell growth above 100 μ g/mL. Growth stimulation by 100 μ g/mL of the EtOAc extract was inhibited by the anti-estrogens OHT (1 μ M) or ICI (100 nM) (Fig. 3B). Methanol and water extracts dose-dependently stimulated the growth of MCF-7 cells within the concentration range used (Fig. 3A); the anti-estrogens did not block the growth stimulation by these extracts (Fig. 3C and D). These results indicated that the EtOAc extract contained the estrogenic components of licorice root. Growth stimulations by the methanol and water extracts were both ER-independent.

3.2. Effects of the EtOAc extract of licorice root on the cell cycle

The capacity of an extract or purified compound to affect specific phases of the cell cycle may provide clues to its mechanism of action. To determine the effects of the EtOAc extract on the cell cycle, MCF-7 cells were exposed to increasing concentrations of this extract for 48 h. The effects on cell cycle distribution were assessed by flow cytometry (Fig. 4). After exposure to 1, 10, or 100 μ g/mL of the EtOAc extract, there was an increase of cells in the S phase, compared to the control ($P < 0.05$, $n = 3$). After exposure to 500 μ g/mL of the EtOAc extract, there was a decrease of cells in S phase, compared

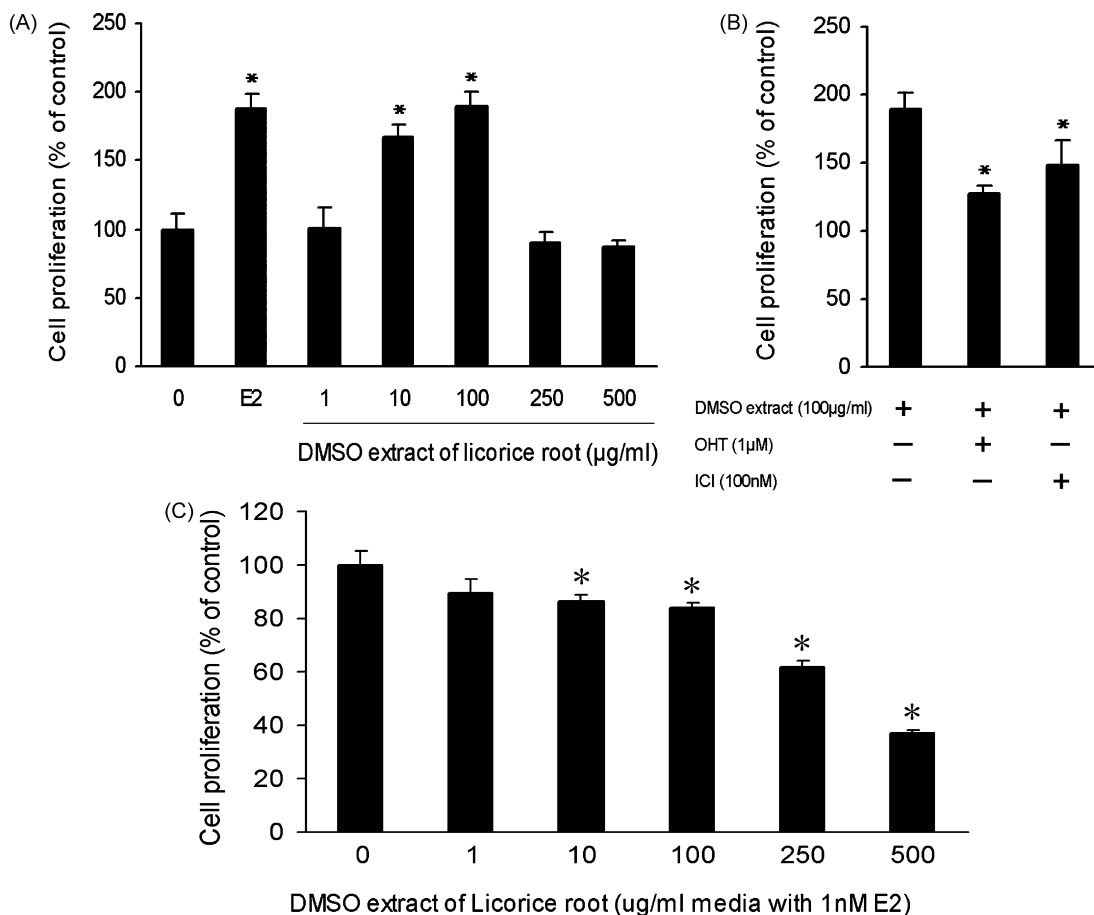


Fig. 2. Effects of the DMSO extract of licorice root on the growth of MCF-7 cells. (A) Dose–response of DMSO extract on cell growth in estrogen-depleted medium, with 1 nM E2 as a positive control. (B) OHT and ICI block DMSO extract-induced cell growth. (C) Dose–response of DMSO extract on cell growth in estrogen medium (1 nM E2, 0.00027 µg/mL). Cells were plated in phenol red-free DMEM medium plus 5% CS-FBS in the absence (0.1% DMSO only, control cells) or in the presence of the indicated concentrations of licorice root extracts, E2, OHT and ICI for 96 h, and cell viability was determined by the MTT assay. Results are expressed as percentages of proliferation relative to the untreated control (mean ± S.D., $n = 4$). *Significantly different from control, $P < 0.05$.

to the control ($P < 0.05$, $n = 3$). These results indicate that the EtOAc extract stimulates cell growth through induction of DNA synthesis.

3.3. Effects of the EtOAc extract of licorice root on the expression of PCNA protein

Expression of proliferating cell nuclear antigen (PCNA) by cells during the S and G2 phases of the cell cycle makes the protein a good cell proliferation marker [15,16]. Western blot analyses performed with antibodies against PCNA were performed to elucidate the mechanism of the EtOAc extract-induced cell proliferation. Representative Western blots for expression of PCNA in lysates from control and the EtOAc extract-treated MCF-7 cells are shown in Fig. 5A. Treatment of MCF-7 cells for 4 days with increasing concentrations of the EtOAc extract caused a biphasic expression in the level of the PCNA protein. At concentrations of 10–100 µg/mL, the EtOAc extract increased the level of PCNA protein, reaching a maximum effect at about 100 µg/mL. In contrast to its promoting effects at lower concentrations, the EtOAc extract at concentrations >100 µg/mL, decreased the level of PCNA protein.

3.4. Effects of the EtOAc extract of licorice root on the caspase-3 activity

Caspases are aspartate-specific, cysteine proteases that mediate apoptosis. To determine whether the EtOAc extract of licorice root

inhibited cell proliferation at higher concentrations through cell apoptosis. Apoptosis induction by the EtOAc extract was established by determining its effect on caspase-3 activity in MCF-7 cells. As shown in Fig. 5B, the EtOAc extract of licorice root had no effect on caspase-3 activity within the concentration range used.

3.5. Effects of the EtOAc extract of licorice root on the expression of ERα in MCF-7 cells

17β-Estradiol down-regulates the levels of ERα in breast cancer cell lines through both an increased turnover of the E2-activated ERα protein and a reduced transcription rate of its own gene [23]. Therefore, the repression of ERα protein levels might be considered a characteristic of receptor activation by an agonist. This prompted us to investigate whether the levels of ERα in MCF-7 cells are also sensitive to the EtOAc extract. A 48-h exposure to increasing concentrations of EtOAc extracts dose-dependently down-regulated the content of ERα protein (Fig. 6A) and mRNA (Fig. 6B).

3.6. Effects of the EtOAc extract of licorice root on the mRNA levels of pS2 and GREB1 in MCF-7 cells

ERα is a ligand-dependent transcription factor that regulates gene expression through interaction with DNA sequences termed estrogen response elements located within the regulatory regions of target genes such as pS2 and GREB1. In this way, we determined

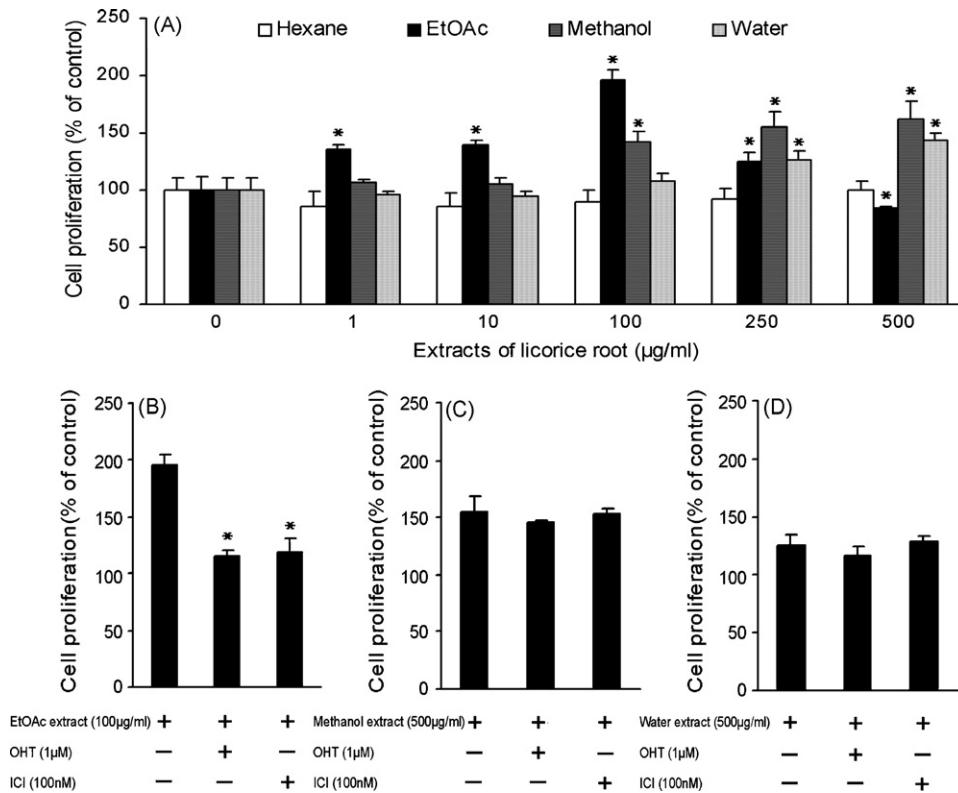


Fig. 3. Effects of the fractionation of licorice root on the growth of MCF-7 cells in estrogen-depleted medium. (A) Dose–response of hexane, EtOAc, methanol and water extracts on cell growth. (B) OHT and ICI block EtOAc extract-induced cell growth. (C) OHT and ICI have no effect on methanol extract-induced cell growth. (D) OHT and ICI have no effect on water extract-induced cell growth. Cells were plated in phenol red-free DMEM medium plus 5% CS-FBS in the absence (0.1% DMSO only, control cells) or in the presence of the indicated concentrations of licorice root extracts, E2, OHT and ICI for 96 h, and cell viability was determined by the MTT assay. Results are expressed as percentages of proliferation relative to the untreated control (mean ± S.D., n = 4). *Significantly different from control, P < 0.05.

if the expressions of pS2 and GREB1 in MCF-7 cells were sensitive to the EtOAc extract of licorice root. The mRNA levels were determined by real-time PCR and standardized using the mRNA levels of the house-keeping gene, GAPDH. After 48 h, the EtOAc extract up-regulated the mRNA levels of pS2 (Fig. 7A) and GREB1 (Fig. 7B)

in a concentration-dependent manner. Next, we exposed MCF-7 cells to a combination of a receptor antagonist, OHT or ICI, and the EtOAc extract. The EtOAc extract-induced up-regulation of the mRNAs of pS2 and GREB1 was inhibited by OHT or ICI (Fig. 7A and B), consistent with the receptor dependence of the observed effects.

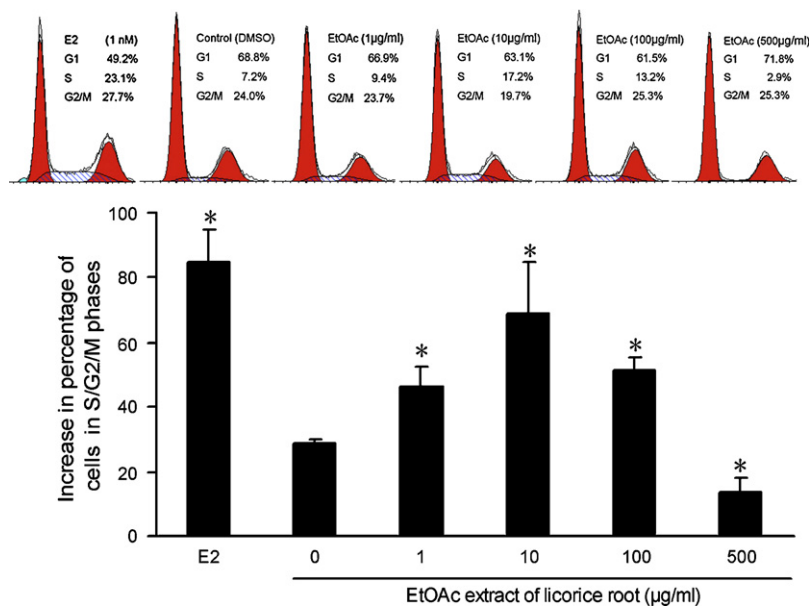


Fig. 4. Dose–response of the cell cycle distribution in MCF-7 cells exposed to different concentrations of the EtOAc extract of licorice root. Cells were incubated with the EtOAc extract of licorice root at the concentration of 0, 1, 10, 100, and 500 µg/mL for 48 h, and the percentage of cells in each cell cycle phase (G1, S and G2/M) was determined by flow cytometry. *Significantly different from control, P < 0.05 (n = 3).

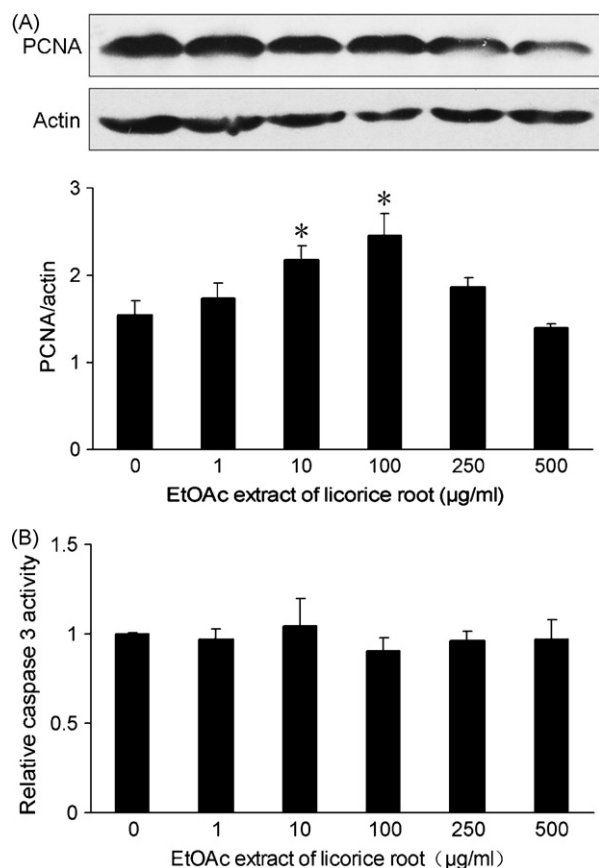


Fig. 5. (A) Effects of the EtOAc extract on the expression of PCNA protein. Cells were incubated with the EtOAc extract at concentrations of 0, 1, 10, 100, 250 and 500 µg/mL for 4 days. The representative Western blots for the expression of PCNA was presented. Actin protein was blotted as a control. Each experiment was repeated twice with similar results. Histograms represent densitometric measurement of specific bands using actin level as control. *Significantly different from control, $P < 0.05$. (B) Effects of the EtOAc extract on caspase-3 activity. Caspase-3 activity in lysates of MCF-7 cells exposed to DMSO (control) or different concentrations of the EtOAc extract for 4 days was determined. *Significantly different from control, $P < 0.05$ ($n = 3$).

4. Discussion

Recent surveys estimate that between 12% and 17% of Americans have used herbal remedies and that women often use such medicine as hormone replacement therapy [17]. Despite the widespread use of these herbs, little is known about their safety and efficacy.

Although the estrogenic activity of the licorice root of the genus *G. glabra* has been the subject of many investigations [8,10,18,19], little is known about estrogenic activity and the mechanisms of action of the Chinese licorice root (*G. uralensis*). Several components (glabrene, glabridin and isoliquiritigenin) have been isolated from this root, and their estrogenic activities have been confirmed [9,11,12]. Nevertheless, the potential estrogenic effect of the whole extract is controversial [13,20].

To evaluate whether extracts of *G. uralensis* contain estrogenic compounds, DMSO was first used to extract components of licorice root. The DMSO extract promoted the growth of ER-dependent MCF-7 breast cancer cells, and this stimulation was blocked by the anti-estrogens, OHT and ICI. The results demonstrated that the growth-promoting effect of the DMSO extract was mediated by ER [21,22]. Nevertheless, the proliferative pattern of cells exposed to the DMSO extract was biphasic. The DMSO extract at low concentrations stimulated the proliferation of MCF-7 breast cancer cells whereas it became cytotoxic at relatively high levels. A similar

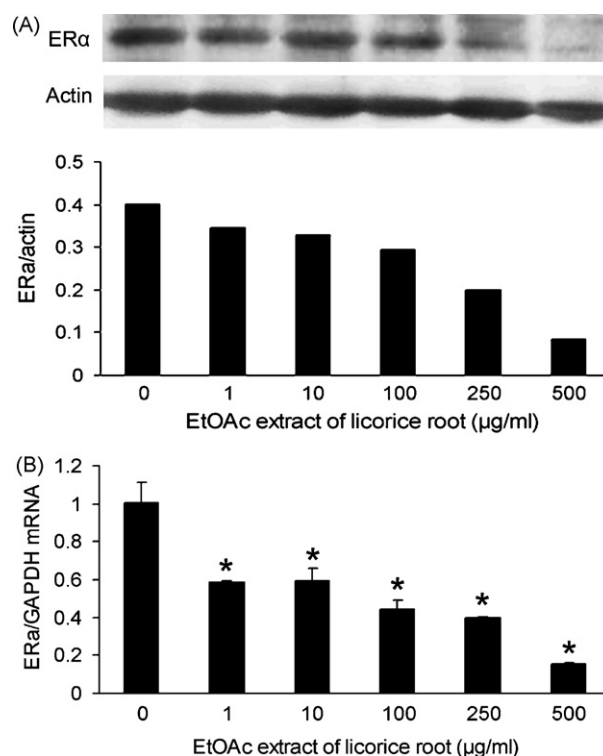


Fig. 6. Effects of the EtOAc extract on the expression of ERα protein and mRNA. Cells were incubated with the EtOAc extract at concentrations of 0, 1, 10, 100, 250 and 500 µg/mL for 48 h. Fig. 4A is a representative Western blot for the expression of ERα, with β-actin as a control. This experiment was repeated twice with similar results. Fig. 4B, after 48 h exposure to the EtOAc extract, total RNA was isolated and subjected to cDNA synthesis followed by real-time PCR analysis using specific primer pairs. The signals were normalized to a GAPDH internal control, and the results were expressed as fold induction in comparison to control (mean ± S.D., $n = 3$). *Significantly different from control, $P < 0.05$.

biphasic effect has been noted for genistein [21]. When combined with 1 nM E2, the DMSO extract dose-dependently inhibited the growth of MCF-7 cells. Our data support the hypothesis that the actions of phytoestrogens are mediated not only via the ER as estrogen agonists, but also, at higher concentrations, they interact with other ER-independent cellular mechanisms to inhibit cell proliferation [23,24].

To determine which polar fractions of licorice root have estrogenic activity, licorice root was extracted sequentially to yield four additional fractions: hexane, EtOAc, methanol and H₂O. Of the four, only the EtOAc extract had a biphasic effect on cell proliferation similar to that of the DMSO extract. This stimulation was blocked by the anti-estrogens, OHT and ICI. Methanol and water extracts dose-dependently stimulated the growth of MCF-7 cells, but the anti-estrogen did not block the stimulation. These results indicated that the EtOAc extract contained the estrogenic component(s) in licorice root. Growth stimulations by methanol and water extracts both were ER-independent.

A recent study demonstrated that extracts of licorice root do not induce cell cycle arrest in MCF-7 cells [11]. To confirm this result, flow cytometric analysis was performed. In MCF-7 cell lines, the EtOAc extract stimulated cell growth and induced cell cycle arrest at the transition S phase. Consistent with the effects on cell proliferation, the EtOAc extract had a biphasic effect on DNA synthesis. These results indicate that the EtOAc extract stimulates cell growth through promotion of DNA synthesis.

PCNA is a nuclear protein and DNA polymerase auxiliary protein that is necessary for DNA synthesis in eukaryotic cells. It plays an important role for DNA replication, and its expression reflects the level of cell proliferation. PCNA expression and synthesis are

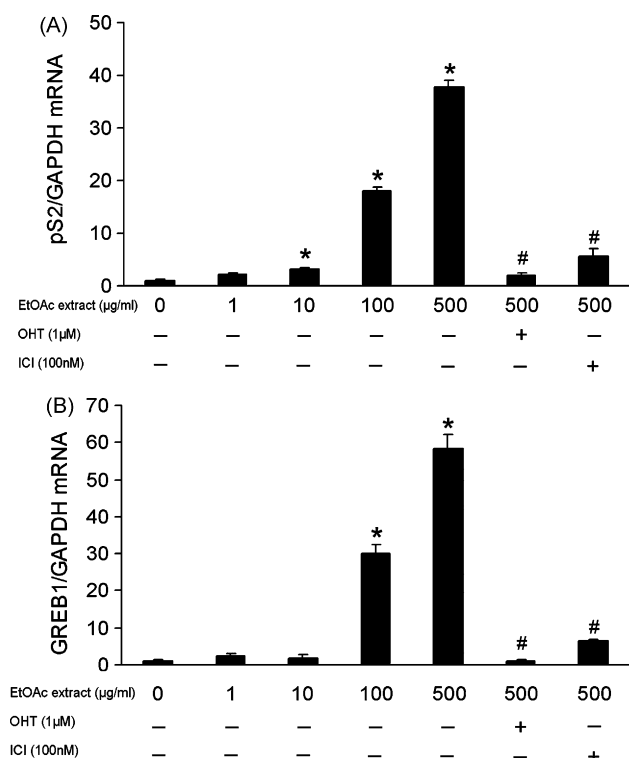


Fig. 7. Effects of the EtOAc extract on the mRNA expressions of pS2 and GREB1 in MCF-7 cells. Cells were plated in phenol red-free DMEM medium plus 5% CS-FBS in the absence (0.1% DMSO only, control cells) or in the presence of indicated concentrations of the EtOAc extract and OHT or ICI. Total RNAs were isolated at 48 h and examined for the expressions of pS2 (A) and GREB1 (B) mRNA by real-time PCR. The signals were normalized to a GAPDH internal control, and the results were expressed as fold induction in comparison to control (mean \pm S.D., $n = 3$). *Significantly different from control ($P < 0.05$). #Significantly different from 500 μ g/mL EtOAc extract ($P < 0.05$).

closely related to the cell proliferation cycle. The expression of PCNA increases gradually in G1 phase, peaks in S phase, and reduces in G2 and M phase [25]. Our study found that, consistent with the effects on cell proliferation and cell cycle, the EtOAc extract had a biphasic effect on the expression level of PCNA protein. These results suggested that the EtOAc extract induces DNA synthesis through promotion of the expression of PCNA protein.

Caspases are cysteine proteases involved in the process of apoptosis. Caspase-3, a cell-death protease, is activated in response to various apoptotic stimuli [26]. In the present study, the EtOAc extract of licorice root had no effect on caspase-3 activity, even at highest concentration. We also did not observe the sub-G0/G1 peak after treatment with the EtOAc extract, suggesting an apoptosis mechanism is not involved in the EtOAc extract-inhibited cell proliferation at higher concentrations.

Results from our study indicate that exposure of MCF-7 cells to the EtOAc extract of licorice root resulted in a down-regulation of ER mRNA and protein. The ligand-receptor activation in MCF-7 cells was confirmed by the ER α down-regulation, which may reduce agonistic activity through a proteasomal degradation process [27]. Control of ER protein levels has been studied for various cell types [28,29], and it is now generally accepted that this protein is targeted for rapid degradation via the ubiquitin-proteasome pathway in response to E2 in breast cancer cells [30]. The activity of the proteasome pathway in controlling ER degradation in MCF-7 cells is directly linked to activation of transcription through the ERE. Degradation of E2-liganded ER requires transcriptional activity [31]. Thus, the EtOAc extract-mediated loss of ER is dependent on proteasome activity and is necessary for ER-mediated transcription.

To confirm that the EtOAc extract contains estrogenic components acting via an ER mechanism, we evaluated the expression of estrogen-responsive genes (pS2 and GREB1) in MCF-7 cells. The EtOAc extract-induced the expression of pS2 mRNA in a concentration-dependent manner. The pS2 gene, which is regulated by estrogen *in vitro* [32] and *in vivo* [33], is not required for estrogen-induced cell proliferation. This may be the underlying reason why pS2 expression in primary breast cancers does not provide better prediction of hormonal therapy than estrogen receptor status [34]. GREB1, discovered in the human brain [35], is an estrogen-regulated gene. Its expression is associated with ER expression in breast cancer cell lines. It is an early-response gene directly regulated by ER and may function in hormone-responsive tissue and cancer [36]. siRNA-mediated gene 'knockdown' in MCF-7 cells shows that GREB1 is involved in estrogen-stimulated growth [37]. The present data demonstrate that the EtOAc extract of licorice root up-regulates the mRNA of GREB1 through the ER-mediated signaling pathway. Need to note, the EtOAc extract-inhibited cell growth above 100 μ g/mL, however, up-regulation of pS2 and GREB1 mRNA was observed at this concentration range. This estrogen-independent activity is probably due to non-specific inhibitory effects such as those caused by flavonoid compounds [38,39].

In summary, our results provide evidence for the estrogenic activity of extracts from Chinese licorice root on ER α . In MCF-7 human breast cancer cells, an EtOAc extract of licorice root induced cell proliferation through ligand-receptor activation. Cell growth inhibition by licorice root extract was ER-independent. These observations indicate that licorice root contains a variety of phytoestrogen compounds that could be considered as potential estrogen and chemopreventive agents. Thus, the amount of licorice root in normal/supplemented food or pills should be considered in the treatment of postmenopausal women affected by hormone-sensitive breast cancer.

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