Available online at www.sciencedirect.com
SCIENCE DIRECT®
Cancer Letters 230 (2005) 239–247
CANCER Letters
www.elsevier.com/locate/canlet

Chemopreventive properties of the ethanol extract of chinese licorice (*Glycyrrhiza uralensis*) root: induction of apoptosis and G1 cell cycle arrest in MCF-7 human breast cancer cells

Eun-Hye Jo[a], Sung-Hoon Kim[b], Jeong-Chan Ra[c], Sung-Ran Kim[d], Sung-Dae Cho[a], Ji-Won Jung[a], Se-Ran Yang[a], Joon-Suk Park[a], Jae-Woong Hwang[a], Okezie I Aruoma[a,e], Tae-Yung Kim[a,f], Yong-Soon Lee[a], Kyung-Sun Kang[a,*]

[a]*Department of Veterinary Public Health, College of Veterinary Medicine, Seoul National University, San 56-1, Shillim-Dong, Kwanak-gu, Seoul 151-742, South Korea*
[b]*Graduate School of East-West Medical Science, Kyunghee University, South Korea*
[c]*RNL Life Science Ltd, Suwon, Kyunggi-do, South Korea*
[d]*Korea Food Research Institute, Sungnam, South Korea*
[e]*Department of Applied Science, London South Bank University, 103 Borough Road, London SE1 0AA, United Kingdom*
[f]*Animal health division Ministry of Agriculture & Forestry, Kwacheon, South Korea*

Received 22 November 2004; received in revised form 27 December 2004; accepted 29 December 2004

Abstract

Much of the interest on the chemopreventive properties of licorice has been focused on the plant genius *Glycyrrhiza glabra*. In this study the ethanol extract of Chinese licorice root, *Glycyrrhiza uralensis* (*G. uralensis*) was investigated for its estrogenic effect and the ability to inhibit cell proliferation in the MCF-7 human breast cancer cell line. The extract of the root of *G. uralensis* was fractionated in EtOH:H_2O (80:20) (80% ethanol). The extract exhibited estrogenic effects similar to 17β- estradiol (E2) and induced apoptosis at the same dose level (100 μg/ml) in MCF-7 breast cancer cells, results were associated with up-regulation of tumor suppressor gene p53 and pro-apoptotic protein Bax. *G. uralensis* extract caused the up-regulation of $p21^{waf1/cip1}$ and down-regulation of cdk 2 and cyclin E and most significantly, induced G1 cell cycle arrest. This is the first study to show that the ethanolic extract of the root of *G. uralensis* has an estrogen-like activity and anti-cancer effects against MCF-7 human breast cancer cells. Whilst the use of phytoestrogens to protect against hormone-dependent cancers or as a 'natural' alternative to hormone replacement therapy remains controversial, the data in this paper support the suggestion that extracts of root of the Chinese licorice *G. uralensis* might be of importance in this debate.

Keywords: Breast cancer; Chinese licorice root; *Glycyrrhiza uralensis*; Apoptosis; Chemoprevention; Cell cycle arrest

1. Introduction

The incidence of cancers in particular those of the breast and prostate continue to be focus of worldwide

* Corresponding author. Tel.: +82 (0)2 880 1246; fax: +82 (0)2 876 7610.
E-mail address: kangpub@snu.ac.kr (K.-S. Kang).

0304-3835/$ - see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2004.12.038

attention. Breast cancer is the most common form of cancer affecting women with cases in North America and Northern Europe being considerably higher than in Asia, the Far East, Africa and South America. Hulka and Moorman [1] have noted that 'the international differences in breast-cancer incidence and mortality rates indicate important differences in the endogenous hormonal milieu, lifestyle and environmental factors, genetic susceptibility, and mammographic screening activities among countries'. Breast cancer incidence and mortality rates may be explained by differences in the relative risk or prevalence of risk factors including dietary factors but this remain inconclusive. Indeed the extent to which diet is involved in the etiology of breast cancer as opposed to its mediation in part through hormonal mechanisms is being borne out by several studies [2–4] has yet to be fully defined. Although dietary phytoestrogens in edible plants including lignans and isoflavonoids, used in a wide variety of products, can mimic the effects of the main natural estrogen, 17β-estradiol, by binding to the estrogen receptor and influencing the expression of estrogen-dependent genes, the effect of the intake of phytoestrogens in decreasing breast cancer risk remain controversial [3,4]. Nevertheless research in this area is attractive. Most studies on phytoestrogens and breast cancer have addressed the effect of phytoestrogens on the growth of breast tumor cells was biphasic [5–7].

Licorice root is also used as flavoring and sweetening agents for tobaccos, chewing gums, candies, toothpaste and beverages [8]. The genius of licorice widely discussed in the western part of the world is the *Glycyrrhiza glabra* which is indigenous to Turkey, Spain, Iraq, Turkey, Russia and North China. *Glycyrrhiza uralensis* is indigenous to Northern China, Mongolia and Siberia. The content of *G. uralensis* is totally different from that of *G. glabra* in result of HPLC profiles [9]. Most work has been concentrated on the active components and extracts of *G. glabra* in terms of chemoprevention activities [10–13]. Recent research has focused on the activities of extracts of the roots of Chinese licorice *G. uralensis*. The $CHCl_3$, EtOAc, C_6H_{14} and CH_3OH–H_2O (70:30) extracts of *G. uralensis* induced apoptosis in a dose- and time dependent manner by possibly by mediating cleavage of PARP, up-regulation of Bax and cleavage of Bcl-2 in MCF-7 human breast cancer cells [14].

Licochalcone-A of licorice root of *G. glabra* induced apoptosis in MCF-7 and HL-60 cell lines, as demonstrated by PARP cleavage, the substrate of ICE-like protease [10]. Isoliquiritigenin of licorice root of *G. glabra* is widely suggested as a candidate agent for the treatment of prostate cancer [11]. The DNA damage response gene GADD153 may play an important role in isoliquiritigenin-induced cell cycle arrest and cell growth inhibition [11]. Tamir et al. [12] studied the effect of glabridin from *G. glabra* on the growth of breast tumor cells and found that there was a biphasic. Glabridin showed an estrogen receptor-dependent, growth-promoting effect at low concentration (10 nM–10 μM) [12]. Low and intermediate concentrations of *G. glabra* derived isoliquiritigenin induced substantial transcriptional activity stimulate the proliferation of MCF-7 cells [13]. In the present study, the unknown properties of Chinese licorice root *G. uralensis* were investigated by comparing their ability to the human ER and their effect on estrogen-responsive human breast cancer MCF-7 cells over a broad range of concentrations. Our results show that the 80% ethanol extract of licorice root of the genius *G. uralensis* has estrogenic activity and anti-proliferative activity on MCF-7 human breast cancer cell.

2. Material and methods

2.1. Materials

Licorice roots were purchased from Kyungdong oriental medicine market, Seoul, Korea. The fresh fruiting bodies of licorice root (*Glycyrrhiza uralensis*) were washed, disintegrated, and extracted with CH_3-CH_2OH (EtOH): H_2O (100:0; 80:20; 60:40; 40:60; 20:80; 0:100) for 3 h. The crude extracts obtained were then subjected into silica gel chromatography and these elutes were evaporated to dry with a rotary evaporator.

2.2. Recombinant yeast cells

The *Saccharomyces cerevisiae* ER+LYS 8127 were gifted from Dr. Donald P. McDonnell (Duke University Medical Center, USA). The yeast cells

were grown in a shaking incubator at 30 °C with 300 rpm in a selective growth medium containing yeast nitrogen base without amino acid (67 mg/ml), 1% dextrose, L-lysine (36 μg/ml), L-histidine (24 μg/ml). Following two days culture, the yeasts were then allowed to grow until OD values at 600 nm reached between 1.0 and 2.0.

2.3. Yeast growth for the estrogenicity assay

The procedures were slightly modified from the method described previously [15]. For the estrogenicity assay, the yeast cells were diluted to an OD_{600nm} value of 0.03 in selective medium plus 50 μM $CuSO_4$ to induce receptor production. The diluted yeasts were aliquoted into 50 ml conical tube (5 ml/tube) and 5 μl of licorice root extracts, ethanol(vehicle control) and E2(positive control) were added. The cultures were incubated for 18 h in a shaking incubator at 30 °C with 300 rpm. After incubation, the yeast culture samples were diluted in the appropriate selective medium to an OD_{600nm} value of 0.25 and 100 μl was added to each well of a 96-well microtiter plate. Each sample was assayed in quadruplicate. β-Galactosidase activity was induced by the addition of 100 μl of a Z buffer (60 mM Na2 HPO4, 40 mM NaH2 PO4, 10 mM KCl, 1 mM MgSO4, pH 7.0) containing 2 mg/ml 0-nitrophenyl-β-D-galactopyranoside (ONPG), 0.1% sodium dodecyl sulfate, 50 mM β-mercaptoethanol, and 200 U/ml oxalyticase (Enzogenetics, Cornavillis, OR). The OD_{420nm} and OD_{590nm} values of each well were measured by using Titertek Multiscan MCC/344 plate reader after allowing the tube to stand for 20 min. The OD420 nm value of each well was corrected by subtracting the OD590 nm value.

2.4. Cell culture and treatment with licorice root

MCF-7 cells (ATCC) were cultured in D-media (EMEM containing 50% increase of all essential amino acids except glutamine, 50% increase of all vitamins and 100 increase of all non-essential amino acids) supplemented with 5% FBS (fetal bovine serum; Gibco Laboratories, USA) and 3 ml/l PSN (penicillin/streptomycin/ neomycin) antibiotic mixture (Gibco Laboratories, USA). Exponentially growing cultures were maintained in humidified atmosphere of 5% CO_2 at 37 °C. The stock solutions of licorice root (100 mg/ml) were dissolved in 80% ethanol, and the experimental concentrations were prepared in the basal medium with a final 80% ethanol concentration of 0.1%.

2.5. Cell proliferation assay

Cell proliferation was determined by the MTT assay as described [16]. In brief, the cells were seeded in 96-well microplates and incubated overnight. Then the cells were treated with different concentrations of the licorice root extracts or its vehicle, ethanol (0.1%) for 24, 48 or 72 h. At the end of periods, 20 μl of MTT stock solution (5 mg/ml, Sigma) were added to each well and the plates were further incubated for 4 h at 37 °C. The supernatant was removed and 100 μl of DMSO were added to each well to solubilize the water insoluble purple formazan crystals. The absorbency at a wavelength of 570 nm was measured with Multiscan MCC 340 microplate reader (Titertek, USA). All the measurements were performed in triplicate. Results were expressed as the percentages proliferation with respect to vehicle-treated cells.

2.6. Apoptosis assay

The apoptotic effect of the licorice root extracts on MCF-7 cells were analyzed by nuclear DNA staining and DNA fragmentation assay. For nuclear DNA staining, control and compound-treated cells were fixed in 4% paraformaldehyde in PBS for 20 min, washed with PBS, then stained with Hoechst 33258 at 1 μg/ml in PBS for 15 min. Stained cells were washed twice with PBS. The changes in nuclei were observed with a fluorescent microscope (Olympus, USA) through UV-filter.

2.7. Cell cycle analysis

MCF-7 cells in exponential phase of growth were treated with the extract of licorice root (50 μg/ml) for 24 and 48 h, then harvested by trypsinization, and washed twice with ice-cold PBS and fixed by 70% ethanol at −20 °C for at least 30 min. The fixed cells were then washed twice with ice-cold PBS and stained with 50 μg/ml of propidium iodide in the presence of

100 μg/ml RNase A for 30 min. Cell cycle distribution was analyzed by using FACSCalibur (Becton&Dickinson, USA). Data from 10,000 cells per sample were collected and analyzed by using the Cell Fit Cell analysis program.

2.8. Western blot analysis

MCF-7 cells were grown in a 10 cm dish, and when cell density reached 80–90% confluence, cells were treated with the extract of licorice root (100 μg/ml) for the indicated times. The cells were then washed once with ice-cold PBS and lysed with lysis buffer (20% SDS containing 2 mM phenymethylsulgonyl fluoride, 10 mM iodoacetoamide, 1 mM leupeptin, 1 mM antipain, 0.1 mM sodium orthovanadate and 5 mM sodium fluoride). The lysates were sonicated three times at 10 s intervals, aliquoted and stored at −20 °C. The protein concentration was determined by the Bio-Rad DC protein assay (Bio-Rad Laboratories, USA). Equal amounts of protein (20 μg/lane) were subjected to 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were subsequently incubated with the corresponding primary antibodies, as indicated: a anti-PARP monoclonal antibody (Santa Cruz, CA), a anti-Bcl-2 monoclonal antibody (Santa Cruz, CA); a anti-Bax antibody (Sigma), a anti-caspase-7 antibody (Santa Cruz, CA), a anti-p53 antibody (Santa Cruz, CA), a anti-cdk2 antibody (Santa Cruz, CA), a anti-cyclin E antibody (Santa Cruz, CA), a anti-E2F-1 antibody (Santa Cruz, CA). Antibody recognition was detected with the respective secondary antibody, ether anti-mouse IgG, or anti-rabbit IgG antibodies linked to horseradish peroxidase (Zymed Laboratories Inc, CA). Antibody-bound proteins were detected by the ECL western blotting analysis system (Amersham Pharmacia Biotech UK Limited).

2.9. Statistical analysis

The data were expressed as the mean ± SE. Analyses were performed using SPSS statistical software. When ANOVA revealed $P<0.05$, the data were further analyzed by Sheffe's multiple range tests. Differences were considered statistically significant at $P<0.05$.

3. Results

3.1. Estrogenic activity of the ethanol extracts of licorice root in recombinant yeast assays

A two-plasmid system consisting of human estrogen receptor (hER)-expression plasmid containing estrogen response element (ERE) was employed to study the estrogenic property of licorice root extracts. The reporter gene, β-galactosidase was expressed depending on the ligand-dependent transactivation. Treatment with increasing concentrations of the solvent, ethanol with licorice root induced a concentration-dependent increase in β-galactosidase activity in the yeast (Fig. 1). The activity of 80% ethanol extract of licorice root was maximum and similar to E2 (10^{-8} μM). These 100%, 80%, 60%, 40% and 20% ethanol extracts of licorice root had strong estrogenic activities while 0% ethanol extract of licorice root did not induce estrogenic activity compared with the control.

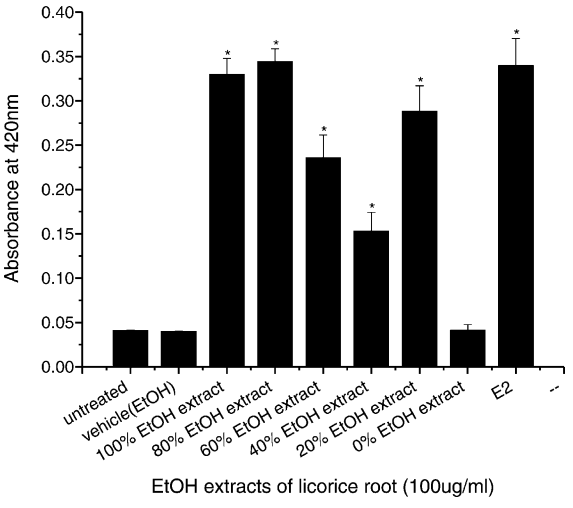

Fig. 1. Estrogenicity of the ethanol extracts of licorice root in YER assay. Test materials were added to yeast cultures at concentration 100 μg/ml. The induction of β-galactosidase activity was determined by OD_{420nm}. Values represent the mean ± SD of three separate experiments for each material. Significant differences from vehicle (0.1% EtOH) are indicated; $^{*}P<0.05$ by ANOVA.

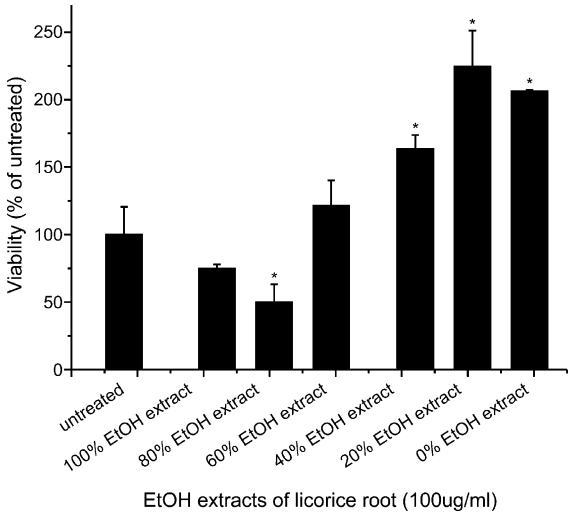

Fig. 2. Inhibition of proliferation by various ethanol extracts of licorice root. Cells were treated with 100 μg/ml concentration of test materials for 72 h, and cell viability was determined by the MTT assay. Results are expressed as percentages proliferation compared with untreated control (mean ± SE, $n = 3$). Significant differences from untreated control are indicated; $^*P < 0.05$ by ANOVA.

3.2. Ethanol extracts of licorice root inhibited proliferation of MCF-7 cells

To evaluate the effects of licorice root extracts on the growth of MCF-7 cells, the cells were treated with licorice root extracts (100 μg/ml) of various solvent concentrations for 72 h. Fig. 2 shows that 100% and 80% ethanol extracts of *G. uralensis* licorice root inhibited the proliferation, while the others induced the proliferation of MCF-7 cells. After 72 h of treatment, 80% ethanol extracts caused nearly a 60% inhibition of cell growth compared with control. The potential biphasic responses of the extracts were evaluated using a concentration range 6.25 μg/ml-400 μg/ml (Fig. 3(A)) on MCF-7 cells. At concentrations 6.25–25 μg/ml, the 80% ethanol extract of *G. uralensis* licorice root had no effect on the growth-promoting activity, but interestingly a dose-dependent effect was observed at concentrations above 50 μg/ml. The anti-proliferation activity of the 80% ethanol extract of *G. uralensis* licorice root assessed at 100 μg/ml concentration, was indeed time-dependent manner (Fig. 3(B)).

3.3. Ethanol extract of licorice root induced apoptosis in MCF-7 cells

The ability of the 80% ethanol extract of *G. uralensis* licorice root to induce apoptosis in MCF-7 human breast cancer cells was assessed using Hoechst 33258 staining (Fig. 4). The cells shrank, turned around, and had a relatively smaller volume than control cells. It was clear that apoptosis was induced in MCF-7 cells within 48 h.

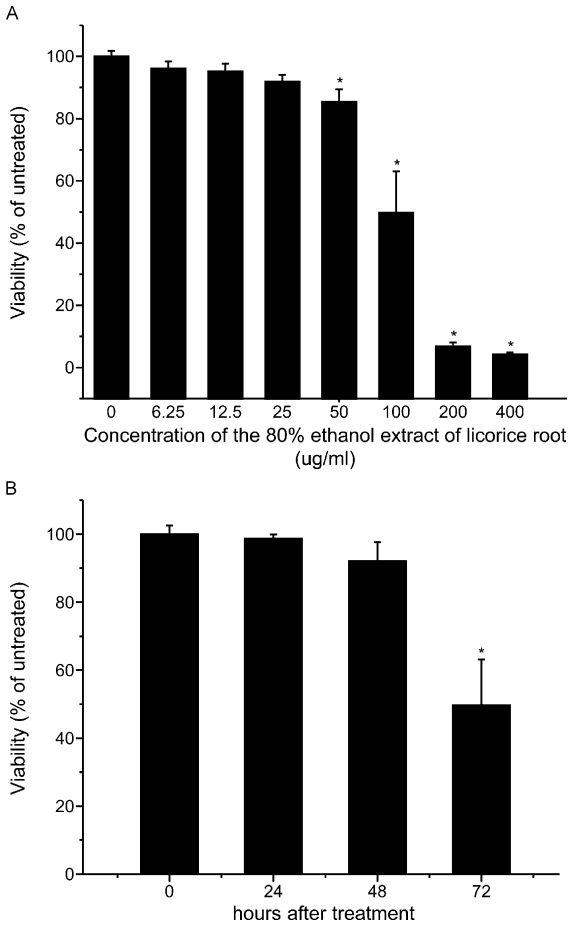

Fig. 3. Inhibition of proliferation by the 80% ethanol extract of licorice root. Cells were treated with various concentrations of test materials for 72 h (A) and 100 μg/ml concentration of test materials for various exposure time (B). Cell viability was determined by the MTT assay. Results are expressed as percentages proliferation compared with untreated control (mean ± SE, $n = 3$). Significant differences from untreated control are indicated; $^*P < 0.05$ by ANOVA.

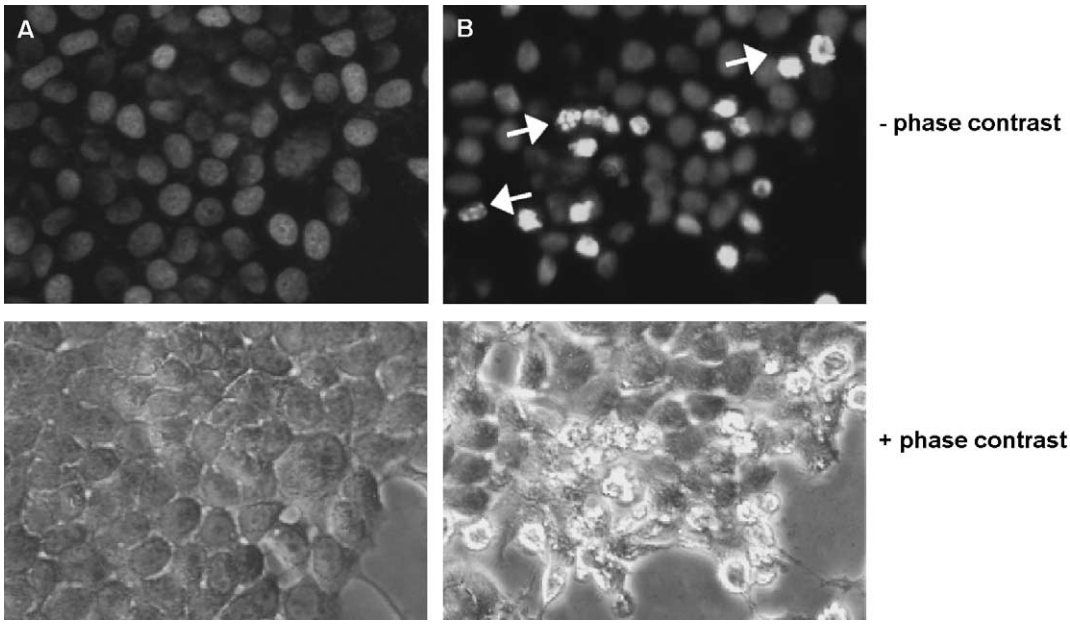

Fig. 4. Detection of apoptotic morphological changes in MCF-7 cells treated with the 80% ethanol extract of licorice root at concentration 100 μg/ml. Nuclei were stained with Hoechst 33258 and examined by fluorescence microscopy. (A) Control; (B) treated with the 80% ethanol extract of licorice root for 48 h. Arrows indicate chromatin condensation and nuclear fragmentation.

3.4. Effects of the ethanol extract of licorice root on the levels of Bax and Bcl-2 in MCF-7 cells

In order to explore the potential signaling pathways with which the extract induced apoptosis, Western blotting analysis was used to evaluate the expression of the genes for the d transcription factor p53 and the Bcl-2 family of proteins. The Bax protein level was increased up to 48 h after treatment and remained elevated up to 72 h. No change was observed in protein expression of Bcl-2 (Fig. 5).

3.5. Effects of the ethanol extract of licorice root on the PARP cleavage and caspase-7 dependency in MCF-7 cells

The expression of caspase-7 was examined by western blot analysis. As shown in Fig. 5, the 35 Kd proenzyme caspase-7 was cleaved to its active 20 Kd form after treatment with the 80% ethanol extract of *G. uralensis* licorice root in a time-dependent manner. The 116 Kd PARP protein was cleaved to 85 Kd fragment in a time-dependent manner and up to a maximum at 72 h. But, it just appeared PARP degradation fragments which did not lead to a persistent fragment.

3.6. Ethanol extract of licorice root induced cell cycle arrest at G1 phase in MCF-7 cells

Given that the licorice root extract decreased cell proliferation and induced cell death, its effect on cell

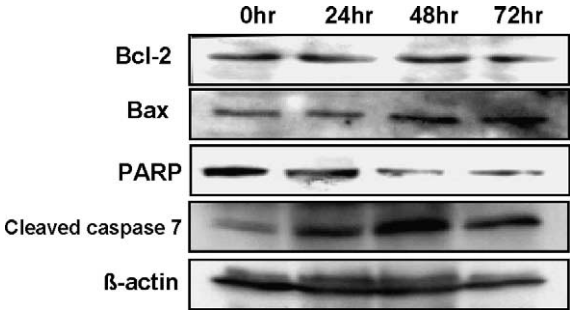

Fig. 5. Effects of the 80% ethanol extract of licorice root on expression of Bcl-2, Bax, PARP and caspase-7 proteins. Cells were treated with 80% ethanol extract of licorice root for indicated times. Total cellular proteins were prepared and western blot were performed with an antibody specific for corresponding proteins.

Table 1
Cell viability and DNA profiles of MCF-7 cells after exposure to 50 μg/ml for 24 and 48 h

	24 h untreated	24 h licorice root	48 h untreated	48 h licorice root
% Apoptosis	1.47±0.083	0.99±0.025[a]	2.20±0.081	9.75±0.144[a]
% G1/S phase	38.90±0.517	52.29±0.470[a]	39.05±0.350	41.38±1.650[a]
% G2/M phase	33.67±0.523	34.85±0.419	36.16±0.292	40.58±0.400

[a] Significant difference with untreated group ($P<0.05$).

cycle distribution was assessed by flow cytometry. As shown in Table 1, cells accumulated in the G1 phase with the number of cells increasing in 24 and 48 h compared to untreated cells after treatment with the 80% ethanol extract of *G. uralensis* licorice root extracts.

3.7. Effects of the ethanol extract of licorice root on the expressions of p21 cyclin E, cdk2 and E2F-1 in MCF-7 cells

The p53 protein expression was increased 24 h after treatment and remained elevated up to 72 h (Fig. 6). The p53-target gene, $p21^{waf1/cip1}$, is the key player in G1 arrest. The $p21^{waf1/cip1}$ was also increased 24 h after treatment and remained elevated up to 72 h (Fig. 6). The effect of the 80% ethanol extract of *G. uralensis* licorice root on G1 arrest in MCF-7 cells was evaluated by Western blotting and using cyclin E, cyclin-dependent protein kinase 2 (cdk2) and transcription factor E2F-1. Indeed the levels of cyclin E, cdk2 and E2F-1 were decreased in a time-dependent manner (Fig. 6).

4. Discussion

This study investigated the antiproliferative activity of the ethanolic extract of *G. uralensis* licorice root in MCF-7 human breast cancer cells. We have chosen hormone-dependent cell line, MCF-7, because this cell line is ER-positive. In previous study, $CHCl_3$, EtOAc, C_6H_{14} and CH_3OH–H_2O (70:30) extracts of *G. uralensis* licorice root was found to exert anti-proliferative activity in this cell line [14].

Although the estrogenic activity of the licorice root of the genius *G. glabra* has been the subject many investigations [10–13], little is known about estrogenic activity and their mechanisms of the Chinese licorice root (*G. uralensis*). The chalcone isoliquiritigenin is a component of *G. glabra* extract exhibiting estrogen receptor (ER) -dependent growth promoting effects on breast cancer cells [13]. Glabrene and isoliquiritigenin in the *G. glabra* extract can bind to the human ER with higher affinity (IC_{50}, 1 and 0.5 μM) than glabridin (IC_{50}, 5 μM). The stimulatory effects of glabrene in vivo were tissue specific and similar to those of estradiol [17]. So, the effect of increasing concentrations of glabrene and isoliquiritigenin on the growth of breast tumor cell was biphasic.

However, the effect of ethanol extract of Chinese licorice root (from 6.25 to 400 μg/ml) was not concentration-dependent biphasic on the proliferation of MCF-7 breast cancer cell. One explanation is that there was cell growth arrest through up-regulation of p53 and $p21^{waf1/cip1}$, down-regulation of cdk2, cyclin E and E2F-1, and inducing apoptosis through increasing Bax protein expression and activation of caspase-7. Indeed the $p21^{waf1/cip1}$ plays a direct role in

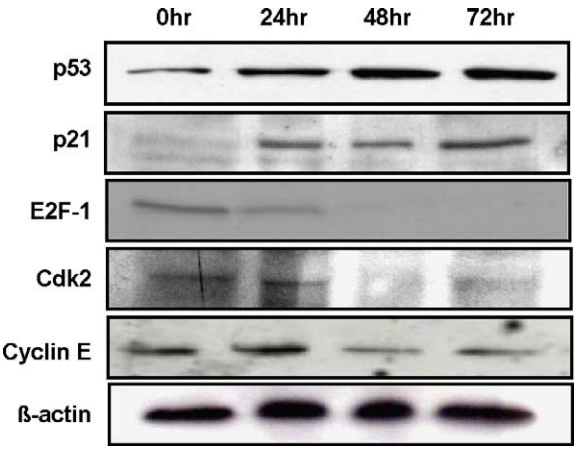

Fig. 6. Effects of the 80% ethanol extract of licorice root on expression of p53, p21, E2F-1, cdk2 and cyclin E in MCF-7 cells. Cells were treated with 80% ethanol extract of licorice root for indicated times. Total cellular proteins were prepared and western blot were performed with an antibody specific for corresponding proteins.

mediating p53-induced G1 arrest and p53 is its transcription factor. Whereas p53 is mutated in 20–40% of human breast cancers, $p21^{waf1/cip1}$ is mutated in relatively few [18–20]. Therefore, relative phenotypic expression of $p21^{waf1/cip1}$ and p53 appears to be important in breast cancer where tumors expressing both genes were less aggressive than those expressing only one [21]. The cdks exist predominantly in quaternary complexes consisting of a cdk, a cyclin, a proliferating cell nuclear antigen (PCNA), and the $p21^{waf1/cip1}$ [22]. Moreover, estrogen activates cyclin E-cdk2 complexes and cyclin D1-cdk4 complexes, substantially preceding entry into S phase [23]. The cell cycle phase-specific of the effects of estrogens on proliferation and differentiation has focused attention on the role of estrogens and their receptors in regulating processes controlling the en try into, progression through, and exit from the G1 phase of the cell cycle [24]. In contrast, the cyclin E-cdk2 has a particularly important role in estrogen-induced cell cycle progression. Recent studies have shown that licorice root (*G. glabra*) can inhibit cell growth by G2/M cell cycle arrest in breast and prostate tumor cells [25]. Results reported here are consistent these previous reports. Thus the ethanol extract of Chinese licorice root *G. uralensis* was also capable of inducing cell cycle arrest at G1 phase in MCF-7 cells. Further mechanistic study suggested that this process might be mediated through up-regulation of $p21^{waf1/cip1}$ and down-regulation cyclin E, which inhibited or inactivated cdk2 and may be led to the dephosphorylation of Rb. This is turn resulted in the inactivation of the transcription factors E2F involved in the expression of genes required for DNA synthesis and cell cycle progression. The estrogenic effect of the *G. uralensis* licorice ethanolic extract could be serving as an estrogen receptor agonist in MCF-7 breast cancer cells. The possible molecular interaction are currently being evaluated, a process that will be facilitated by use of isolated compounds from the Chinese licorice.

Apoptosis is regulated by members of the Bcl-2 family [26], which acts upstream of a family of cysteine proteases known as the interleukin-1 converting enzyme (ICE) protease family, which are often called caspases [27]. The result presented clearly demonstrated an up-regulation of Bax, a member of Bcl-2 family. The dysregulation of apoptosis contributes to the pathogenesis of breast cancer, at least in part, due to an imbalance between Bcl-2/Bcl-x and Bax [28]. The apoptosis-promoting Bax protein led to mitochondrial dysfunction and the release of cytochrome c from the mitochondria. The released cytochrome c then interacts with specific adapter, such as Apaf-1, which in turn prototypically converts procaspases to active caspases [16]. The key of next step was the members of the ICE family of cysteine proteases, which cleaves several substrates including the PARP, a nuclear enzyme involved in DNA repair and maintenance of genome integrity and post-translational ribosylation of proteins, whereby apoptosis occurs [29]. Indeed caspase-3, caspase-6 and caspase-7, have been implicated in the execution phase of apoptosis. Caspase-3 did not express in the MCF-7 cell lines however. Our results demonstrated that the licorice root ethanol extract induced the activation of caspase-7 in MCF-7 human breast cancer cell. An inactive caspase-7 precursor was cleaved to form the active protease 20 Kd during apoptosis. This occurred PARP degradation fragments. It could be hypothesized that PARP degradation continues after its first cleavage.

In conclusion, ethanol extract of Chinese licorice root *G. uralensis* inhibits cell proliferation through up-regulation of p53 and $p21^{waf1/cip1}$ and down-regulation cyclin E and cdk2, and induces apoptosis through overexpression of Bax in MCF-7 human breast cancer cells. Thus extracts of the root of the Chinese licorice *G. uralensis* has anti-tumor effect which is mediated through G1 arrest and apoptosis in MCF-7 human breast cancer in vitro system. Furthermore, these results suggested that ethanol extract of licorice root of *G. uralensis* might have a beneficial effect on the treatment of estrogen-related disease such as heart and blood vessel disease, osteoporosis, bone loss and cognitive decline without breast cancer risk. The clinical implications of these observations needs to be established through trials to assess the bioefficacy of the phytoestrogens and to understand the potential pharmacokinetics of the use of the Chinese licorice *G. uralensis*.

Acknowledgements

This study was supported by technology Development Program for Agriculture and Forestry, Ministry

of Agriculture and Forestry (200001-03-3-CG000, 203004-03-HD110), Republic of Korea. And Research Institute for Veterinary Science, Seoul National University also supported this work. Professor Aruoma acknowledges the 'Brain Pool Award' from the Korean Ministry of Science and Technology (2004-2005) and its hosting by the Seoul National University, College of Veterinary Medicine.

References

[1] B.S. Hulka, P.G. Moorman, Breast cancer: hormones and other risk factors, Maturitas 38 (2001) 103–113.
[2] P.L. Horn-Ross, E.M. John, M. Lee, S.L. Stewart, J. Koo, L.C. Sakoda, et al., Phytoestrogen consumption and breast cancer risk in a multiethnic population: the bay area breast cancer study, Am. J. Epidemiol. 154 (2001) 434–441.
[3] H. Adlercreutz, Phyto-oestrogens and cancer, Lancet Oncol. 3 (2002) 364–373.
[4] L. Keinan-Boker, Y.T. van Der Schouw, D.E. Grobbee, P.H. Peters, Dietary phyto-estrogens and breast cancer risk, Am. J. Clin. Nutr. 79 (2004) 282–288.
[5] P.J. Magee, I.R. Rowland, Phyto-oestrogens, their mechanism of action: current evidence for a role in breast and prostate cancer, Br. J. Nutr. 91 (2004) 513–531.
[6] P.J. Magee, H. McGlynn, I.R. Rowland, Differential effects of isoflavones and lignans on invasiveness of MDA-MB-231 breast cancer cells in vitro, Cancer Lett. 208 (2004) 35–41.
[7] J.C. Le Bail, F. Varnat, J.C. Nicolas, G. Habrioux, Estrogenic and antiproliferative activities on MCF-7 human breast cancer cells by flavonoids, Cancer Lett. 130 (1998) 209–216.
[8] Z.Y. Wang, D.W. Nixon, Licorice and cancer, Nutr. Cancer 39 (2001) 1–11.
[9] H. Hayashi, S. Hattori, K. Inoue, K. Sarsenbaev, M. Ito, G. Honda, Field survey of glycyrrhiza plants in Central Asia (1) characterization of G. uralensis, G. glabra and the putative intermediate collected in Kazakhstan, Biol. Pharm. Bull. 26 (2003) 867–871.
[10] M.M. Rafi, R.T. Rosen, A. Vassil, C.T. Ho, H. Zhang, G. Ghai, et al., Modulation of bcl-2 and cytotoxicity by licochalcone-A, a novel estrogenic flavonoid, Anticancer Res. 20 (2000) 2653–2658.
[11] M. Kanazawa, Y. Satomi, Y. Mizutani, O. Ukimura, A. Kawauchi, T. Sakai, et al., Isoliquiritigenin inhibits the growth of prostate cancer, Eur. Urol. 43 (2003) 580–586.
[12] S. Tamir, M. Eizenberg, D. Somjen, N. Stern, R. Shelach, A. Kaye, et al., Estrogenic and antiproliferative properties of glabridin from licorice in human breast cancer cells, Cancer Res. 60 (2000) 5704–5709.
[13] M. Maggiolini, G. Statti, A. Vivacqua, S. Gabriele, V. Rago, M. Loizzo, et al., Estrogenic and antiproliferative activities of isoliquiritigenin in MCF7 breast cancer cells, J. Steroid Biochem. Mol. Biol. 82 (2002) 315–322.
[14] E.H. Jo, H.D. Hong, N.C. Ahn, J.W. Jung, S.R. Yang, J.S. Park, et al., Modulations of the Bcl-2/Bax family were involved in the chemopreventive effects of licorice Root (*Glycyrrhiza uralensis* Fisch) in MCF-7 Human Breast Cancer Cell, J. Agric. Food Chem. 52 (2004) 1715–1719.
[15] K.S. Kang, S.D. Cho, Y.S. Lee, Additive estrogenic activities of the binary mixtures of four estrogenic chemicals in recombinant yeast expressing human estrogen receptor, J. Vet. Sci. 3 (2002) 1–5.
[16] H. Hu, N.S. Ahn, X. Yang, Y.S. Lee, K.S. Kang, *Ganoderma lucidum* extract induces cell cycle arrest and apoptosis in MCF-7 human breast cancer cell, Int. J. Cancer. 102 (2002) 250–253.
[17] S. Tamir, M. Eizenberg, D. Somjen, S. Izrael, J. Vaya, Estrogen-like activity of glabrene and other constituents isolated from licorice root, J. Steroid Biochem. Mol. Biol. 78 (2001) 291–298.
[18] S.P. Hussain, C.C. Harris, Molecular epidemiology and carcinogenesis: endogeneous and exogenous carcinogens, Mutat. Res. 462 (2002) 311–322.
[19] J. Lukas, S. Groshen, B. Saffari, N. Niu, A. Reles, W.H. Wen, et al., WAF1/Cip1 gene polymorphism and expression in carcinomas of the breast, ovary, and endometrium, Am. J. Pathol. 150 (1997) 167–175.
[20] K.E. McKenzie, A. Siva, S. Maier, I.B. Runnebaum, R. Seshadri, S. Sukumar, Altered WAF1 genes do not play a role in abnormal cell cycle regulation in breast cancers lacking p53 mutations, Clin. Cancer Res. 3 (1997) 1669–1673.
[21] C. Keshava, B.L. Frye, M.S. Wolff, E.C. McCanlies, A. Weston, Waf-1 (p21) and p53 polymorphisms in breast cancer, Cancer Epidemiol. Biomarkers Prev. 11 (2002) 127–130.
[22] Y. Xiong, H. Zhang, D. Beach, D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA, Cell 71 (1992) 505–514.
[23] J.S. Foster, J. Wimalasena, Estrogen regulates activity of cyclin-dependent kinases and retinoblastoma protein phosphorylation in breast cancer cells, Mol. Endocrinol. 10 (1996) 488–498.
[24] S.F. Doisneau-Sixou, C.M. Sergio, J.S. Carroll, R. Hui, E.A. Musgrove, R.L. Sutherland, Estrogen and antiestrogen regulation of cell cycle progression in breast cancer cells, Endocrinol. Relat. Cancer 10 (2003) 179–186.
[25] M.M. Rafi, B.C. Vastano, N. Zhu, C.T. Ho, G. Ghai, R.T. Rosen, et al., Novel polyphenol molecule isolated from licorice root (*Glycrrhiza glabra*) induces apoptosis, G2/M cell cycle arrest, and Bcl-2 phosphorylation in tumor cell lines, J. Agric. Food Chem. 50 (2002) 677–684.
[26] S.J. Korsmeyer, Regulators of cell death, Trends Genet. 11 (1995) 101–105.
[27] E.S. Alnemri, D.J. Livingston, D.W. Nicholson, G. Salvesen, N.A. Thornberry, W.W. Wong, Human ICE/CED-3 protease nomenclature, Cell 8 (1996) 71.
[28] R.C. Bargou, P.T. Daniel, M.Y. Mapara, K. Bommert, C. Wagener, B. Kallinich, et al., Expression of the bcl-2 gene family in normal and malignant breast tissue: low bax-alpha expression in tumor cells correlates with resistance towards apoptosis, Int. J. Cancer 60 (1995) 854–859.
[29] J.Y. Yuan, S. Shaham, S. Ledoux, H.M. Ellis, H.R. Horvitz, The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme, Cell 75 (1993) 641–652.