Hexane/Ethanol Extract of *Glycyrrhiza uralensis* Licorice Suppresses Doxorubicin-Induced Apoptosis in H9c2 Rat Cardiac Myoblasts

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Doxorubicin (DOX) is an anthracycline antibiotic, and has been recognized as one of the most effective anti-neoplastic agents in cancer chemotherapy. However, its usefulness is limited by its profound cardiotoxicity. Licorice is one of the most frequently prescribed agents in traditional herbal medicine, and is also employed as a natural sweetening additive. In traditional Chinese medicine, licorice root is added to a variety of herbal preparations to detoxify the effects of the other herbs in the preparation. In the present study, we explored the possibility that *Glycyrrhiza uralensis* licorice may alleviate DOX-induced cardiotoxicity. The hexane/ethanol extract of *Glycyrrhiza uralensis* (HEGU), which lacks glycyrrhizin, was prepared because glycyrrhizin intake has previously been reported to induce hypertension. In an effort to determine whether HEGU ameliorates DOX-induced cytotoxicity in H9c2 rat cardiac myoblasts, the cells were pretreated with 0–15 mg/L HEGU, then treated with doxorubicin. The pretreatment of cells with HEGU resulted in a significant mitigation of DOX-induced reductions in cell numbers (34 ± 7%) and increases in apoptosis (53 ± 1%). The Western blot analysis of cell lysates showed that HEGU suppressed DOX-induced increases in the levels of p53, phospho-p53 (Ser 15), and Bax. In addition, HEGU induced an increase in the levels of Bcl-xL, regardless of DOX-treatment. HEGU inhibited the DOX-induced cleavage of caspases 9, 3, and 7, as well as DOX-induced poly(ADP-ribose) polymerase cleavage. Furthermore, HEGU caused reductions in the viable cell numbers of HT-29 human colon cancer cells (IC50 = 10.7 ± 0.3 mg/L), MDA-MB-231 human breast cancer cells (IC50 = 7.5 ± 0.1 mg/L), and DU145 human prostate cancer cells (IC50 = 4.7 ± 0.5 mg/L). HEGU augmented DOX-induced reductions in the viability of DU145 cells (15 ± 1%). These results indicate that HEGU may potentially be an effective agent for the alleviation of DOX-induced cardiotoxicity. Exp Biol Med 233:1554–1560, 2008

Key words: licorice; doxorubicin; cardiotoxicity; apoptosis

Introduction

Doxorubicin (DOX) is one of the most effective anti-tumor agents known for the treatment of a variety of cancers, including lymphoma, leukemia, and solid tumors (1, 2). Unfortunately, the clinical use of this drug is somewhat limited by its profound cardiotoxicity (3, 4). Several hypotheses have been suggested to explain this cardiotoxicity, including oxidant stress due to a reduction in myocardial antioxidants (5), perturbation of adrenergic function (6), disturbance of calcium handling (7), release of cardiotoxic cytokines (8), and selective inhibition of the expression of cardiac muscle-specific proteins (9). More recent studies have indicated that apoptosis performs a crucial function in DOX-induced cardiotoxicity (10–12). DOX-induced cardiac apoptosis has been demonstrated to be mediated, at least in part, by the activation of the tumor-suppressor gene product p53 (13–15).

As a transcription factor, p53 upregulates the expression of a variety of genes, some of which are involved in apoptosis induction; these include Fas, death receptor (DR)5 (16), Bax (17), Puma (18), Noxa (19), and Bid (20). It has been firmly established that following the loss of mitochon-

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drial membrane integrity and the release of cytochrome c from the mitochondria to the cytosol, cells are committed to apoptosis. Upon apoptotic stimuli, the proapoptotic Bax translocates from the cytosol to the mitochondrial membrane, where it induces the release of cytochrome c to the cytosol (21, 22), and thereby plays a pivotal role in mitochondria-mediated apoptosis (23). The cytochrome c released into the cytosol can result in the activation of caspase-9, which is responsible for activation of effector caspases, including caspase-3, ultimately resulting in cellular apoptosis (24).

Glycyrrhiza uralensis (licorice) has been employed for more than 4,000 years as a flavoring and sweetening agent in foods, candies, toothpastes and tobaccos. In traditional Chinese medicine, licorice root, in amounts of 1–5%, is included in many herbal prescriptions to detoxify, enhance, and/or harmonize the effects of the other herbs in the prescription. However, licorice contains glycyrrhizin (GL) that is converted to glycyrrhetinic acid, which has been reported to induce hypertension, hypokalemia, and posterior encephalopathy with paralysis (25–31). Jo *et al*. (32) reported that licorice extracts prepared with a solvent containing $\geq$ 80% ethanol inhibited proliferation, whereas those prepared with a solvent containing $\leq$ 60% ethanol stimulated the proliferation of MCF-7 human breast cancer cells. These results indicate that the nonpolar components contained in licorice have the ability to inhibit cancer cell growth. In an attempt to prepare a licorice extract which harbors abundant levels of nonpolar components and also contains negligible quantities of GL, we prepared a hexane/ethanol extract of *Glycyrrhiza uralensis* (HEGU). In order to determine whether HEGU alleviates the cardiac toxicity of DOX, the current study evaluated the effects of HEGU on DOX-induced apoptosis in H9c2 rat cardiac myoblasts, which have previously been utilized to study the cellular mechanisms of DOX-induced cardiotoxicity (15, 33, 34).

Materials and Methods

Materials. The following reagents and chemicals were obtained from the respective suppliers: doxorubicin, 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), glycyrrhizin (ammonium salt, $\geq$ 75% purity, Fluka) and 7-amino-actinomycin D (7-AAD) (Sigma, St. Louis, MO); phycoerythrin(PE)-conjugated Annexin V (BD Pharmingen, Franklin Lakes, NJ); antibodies against phospho-p53 (Ser15), Bcl-xL, cleaved caspase-3, cleaved caspase-7, cleaved caspase-9 and cleaved poly(ADP-ribose) polymerase (PARP) (Cell Signaling, Beverly, MA); horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgG (Amersham, Arlington Heights, IL); anti-actin monoclonal antibody (Millipore, Billerica, MA); p53 antibody (Neomarkers, Fremont, CA); and antibodies against Bcl-2 and Bax (Santa Cruz Biotechnology, Santa Cruz, CA).

Preparation of a Deglycyrrhizinated Licorice Extract. Commercially available licorice roots, derived from *Glycyrrhiza uralensis* (GU), were obtained from Daekwang Co. Ltd., Chuncheon, Korea. The dried and ground roots of GU (1 kg) were dip-extracted for 24 h with hexane:ethanol at a ratio of 9:1 (v/v) at room temperature. Afterward, the slurry was filtered through a Whatman No. 2 filter paper and the residue was re-extracted twice. The combined extract was filtered, and the filtrate was then concentrated under reduced pressure at 40°C, yielding HEGU (9 g, 0.9% yield).

Chromatographic System. HPLC analysis was conducted on a Dionex system (Sunnyvale, CA) consisting of a P 580 pump, an ASI 1000 autosampler, and a UVD 170 S detector. A Dionex Chromeleon was utilized for data acquisition and integration. The analytical column was an Agilent prep C18 Scalar, 4.6 $\times$ 250 mm i.d., 5 μm (Agilent, CA) and the column was maintained at room temperature. The mobile phase was a linear gradient of 0.1% formate and acetonitrile (0–5 min 95:5, 5–50 min 10:90, 50–55 min 0:100) at a flow rate of 0.8 mL/min. HEGU and glycyrrhizin were prepared at concentrations of 5 g/L and 0.1 g/L, respectively, and 5 μL were injected into the HLPC system. The column eluent was monitored at UV 254 nm.

Cell Culture. Rat embryonic ventricular myocardial H9c2 cells and DU145 human prostate cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM supplemented with 10% FBS, 100,000 U/L of penicillin, and 100 mg/L of streptomycin. In order to evaluate the effects of DOX on cell viability, we plated the cells in multi-well plates with DMEM containing 10% FBS. 48 h after plating, the cells were treated with various concentrations of DOX. In order to determine whether HEGU mitigates the effects of DOX, the cells were pretreated for 24 h with various concentrations of HEGU. The cells were then incubated for another 24 h in fresh culture medium containing 0 or 0.5 μmol/L of DOX in the absence or presence of HEGU. Viable cell numbers were estimated by the MTT assay, as previously described (35). In brief, the cells were incubated in a fresh medium containing 1 g/L MTT for 3 h at 37°C. After the removal of unconverted MTT, the purple formazan product was dissolved in isopropanol and the absorbance of formazan dye was measured colorimetrically at $\lambda = 570$ nm with background subtraction at $\lambda = 690$ nm.

To ascertain whether HEGU has any effects on the viability of cancer cells, DU145 cells, HT-29 human colon cancer cells and MDA-MB-231 human breast cancer cells (ATCC) were plated in DMEM/F12 containing 10% FBS, and the cell monolayers were serum-deprived in DMEM/F12 containing 1% FBS for 24 h. After serum-deprivation, the cells were treated with different concentrations of HEGU for 48 h and the viable cell numbers were estimated via an MTT assay.

Quantification of Apoptotic Cell Numbers. The cells were pretreated for 24 h with 0 or 15 mg/L HEGU and then treated with 0.5 μmol/L DOX in the absence or

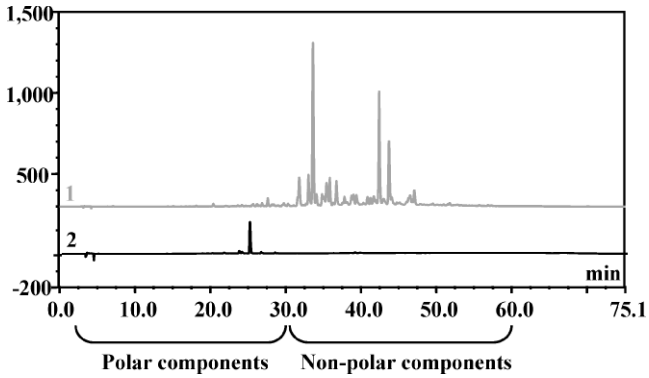

Figure 1. HPLC-UV chromatograms of the hexane/ethanol extract of *Glycyrrhiza uralensis* (HEGU) and glycyrrhizin (GL). The mobile phase was a linear gradient of 0.1% formate and acetonitrile (0–5 min, 95:5; 5–50 min, 10:90; 50–55 min, 0:100) and the flow rate was 0.8 mL/min. 1, HEGU; 2, GL.

presence of 15 mg/L HEGU for 12 h, and the early apoptotic cells were stained with Annexin V as described previously (36). The number of apoptotic cells was quantified via flow cytometry using the FACScan system (Becton Dickinson, Franklin Lakes, NJ). The data were analyzed using ModFit V.1.2. software.

Western Blot Analysis. The cells were lysed as previously described (37) and the protein concentrations of lysates were determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). For Western blot analysis, the proteins (50 μg) were separated via electrophoresis in 4–20% or 10–20% SDS-PAGE and then transferred to a polyvinylidene fluoride membrane (Millipore). The blots were blocked for 1 h with 5% skim milk in 20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% Tween 20 (TBST) and incubated for 1 h with anti-phospho-p53 (Ser 15) (1:1000), anti-p53 (1:1000), anti-Bax (1:2000), anti-Bcl-xL (1:1000), anti-Bcl-2 (1:1000), anti-cleaved caspase-9 (1:500), anti-cleaved caspase-3 (1:500), anti-cleaved caspase-7 (1:500), anti-cleaved PARP (1:500) or anti-actin (1:2000) antibody. The membranes were washed with TBST and incubated with anti-mouse or anti-rabbit HRP-conjugated antibody. The signal was then detected with a chemiluminescence detection system (Millipore).

Statistical Analysis. The results were expressed as the means ± SEM for the indicated number of separate experiments. Statistical data analysis was conducted with ANOVA. Differences between the treatment groups were analyzed via Duncan's multiple range test. Differences were considered significant at $P < 0.05$. Statistical analyses were conducted using the SAS system for Windows, version 8.12.

Results

Preparation of a Deglycyrrhizinated Licorice Extract. It has been previously noted that the optimal extraction condition for GL from licorice was water or ethanol/water (30:70, 50:50 v/v) as an extraction solvent with a 60 min dipping time (38). In order to obtain the deglycyrrhizinated extract, we used a mixture of hexane and ethanol (90:10, v/v) as an extract solvent, because GL is a water-soluble triterpenoid glycoside. Figure 1 shows the HPLC spectrum of the analysis of GL in HEGU. It is clear that GL is well separated from the rest of the sample constituents, which contained mostly nonpolar compounds. The quantification of GL was based on the peak area at UV 254 nm. The calibration curves of GL evidenced good linearity and the regression equation of GL was $y = 9.238x - 0.239$ with $r^2 = 0.998$. However, the GL content in HEGU was too low to be quantified, thereby indicating that the present extraction method using hexane/ethanol (9/1) is efficient with regard to the removal of GL present in GU (Fig. 1).

HEGU Inhibits DOX-Induced Decreases in Viability of H9c2 Cells. In order to determine the effects of DOX on the viability of H9c2 cells, the cells were treated with various concentrations (0.1, 0.3 and 0.5 μmol/L) of DOX and the viable cell numbers were estimated via MTT assay. DOX reduced the number of viable H9c2 cells in a concentration-dependent manner (Fig. 2A). The pretreatment of cells with increasing concentrations of HEGU dose-dependently suppressed DOX-induced reductions in cell viability. Treatment with 10 mg/L HEGU completely prevented DOX-induced reductions in cell viability (Fig. 2B). The treatment of cells with HEGU in the absence of DOX also resulted in an increase in the number of viable cells.

HEGU Protects Against DOX-Induced Apoptosis of H9c2 Cells. In order to estimate the number of early apoptotic cells, the cells were stained with annexin V and 7-AAD, then analyzed via flow cytometry. We noted a significant increase in apoptotic cell numbers in the DOX-treated H9c2 cells, and the pretreatment of cells with HEGU mitigated this effect. HEGU alone exerted no effect on apoptosis in H9c2 cells (Fig. 2C).

HEGU Inhibits DOX-Induced Increases in the Levels of Proapoptotic Proteins in H9c2 Cells. Because HEGU suppressed DOX-induced apoptosis (Fig. 2C) and the activation of the tumor suppressor protein can result in apoptosis (39), we assessed the effects of DOX on the levels of p53 protein in H9c2 cells. 12 hours of treatment of the cells with DOX resulted in significant increases in both phospho-p53 and p53 protein levels, and HEGU treatment prevented these increases (Fig. 3A). A similar trend was also observed with regard to Bax protein levels. The addition of DOX induced a nearly 3.3-fold increase in the levels of Bax protein. The treatment of cells with HEGU resulted in a significant inhibition of the DOX-induced increase in Bax protein levels (Fig. 3B). However, DOX had no effect on Bcl-xL levels, whereas HEGU increased the Bcl-xL levels regardless of DOX treatment. Neither DOX nor HEGU had any effect on the levels of Bcl-2.

As HEGU inhibited DOX-induced changes in Bax

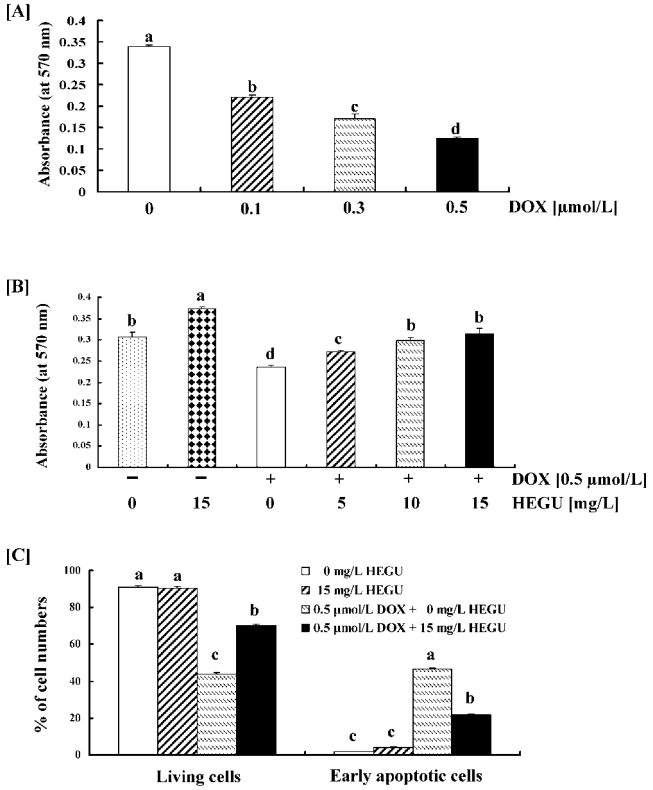

Figure 2. Effects of the hexane/ethanol extract of *Glycyrrhiza uralensis* (HEGU) on doxorubicin (DOX)-induced cell death in H9c2 cells. [A] The cells were treated for 48 h with DOX at the indicated concentrations. [B] The cells were incubated for 24 h with various concentrations (0–15 mg/L) of HEGU, and then treated with 0 or 0.5 μmol/L DOX for another 24 h in the absence or presence of HEGU. Viable cell numbers were estimated via MTT assay. [C] Cells were pretreated for 24 h with 0 or 15 mg/L HEGU and then treated with 0.5 μmol/L DOX for another 12 h in the absence or presence of 15 mg/L HEGU. The cells were trypsinized, loaded with 7-aminoactinomycin D (7-AAD) and annexin V, and then analyzed via flow cytometry. The number of living cells (that exclude 7-AAD and are negative for annexin V) and apoptotic cells (that are positive for annexin V but negative for 7-AAD) is expressed as a percentage of the total cell number. Each bar represents the mean ± SEM ($n = 6$). Means without a common letter differ, $P < 0.05$.

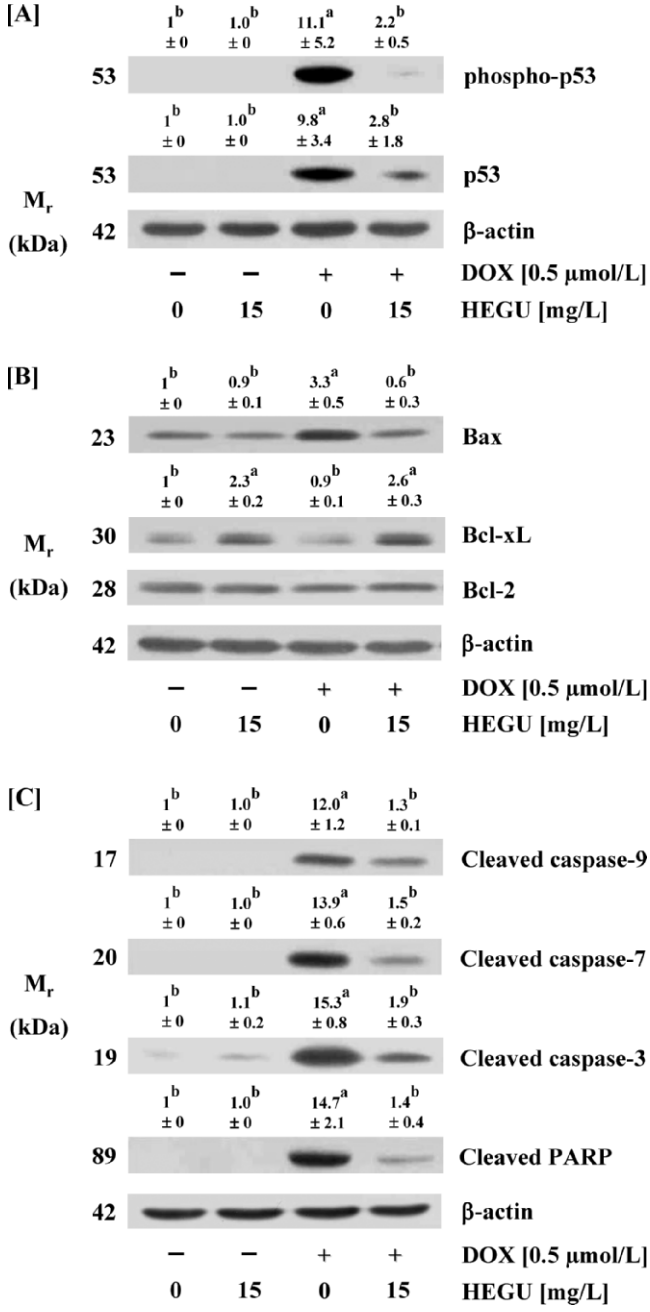

Figure 3. Effects of HEGU on the levels of apoptosis regulating proteins in DOX-treated H9c2 cells. Cells were treated with DOX and/or HEGU as described in Figure 2C. The cell lysates were analyzed via Western blotting with the indicated antibodies. Photographs of the chemiluminescent detection of the blots, which were representative of three independent experiments, are shown. The relative abundance of each band to its own actin was quantified and the control levels (0 μmol/L DOX, 0 mg/L HEGU) were set at 1. The adjusted mean ± SEM ($n = 3$) of each band is shown above each blot. Means without a common letter differ, $P < 0.05$.

levels and increased the levels of Bcl-xL, we then attempted to determine whether HEGU suppresses the DOX-induced cleavage of caspases and PARP. Treatment of the cells with DOX resulted in a marked increase in the levels of cleaved caspase-9, –7, and –3, and HEGU inhibited these effects. Cleaved PARP levels were increased dramatically by DOX treatment, and this effect was significantly inhibited by HEGU treatment (Fig. 3C).

HEGU Decreases the Viability of HT-29, MDA-MB-231, and DU145 Cells. In order to determine whether HEGU increases the viability of cancer cells, HT-29 human cancer cells, MDA-MB-231 human breast cancer cells, and DU145 human prostate cancer cells were treated with increasing concentrations of HEGU. HEGU significantly reduced the viable cell numbers of these three cancer cells (Fig. 4A–C). In addition, the pretreatment of DU145 cells with 5 mg/L HEGU prior to DOX treatment augmented DOX-induced reductions in cell viability. However, increasing HEGU concentrations beyond 5 mg/L did not further decrease the viability of DOX-treated DU145 cells (Fig. 4D).

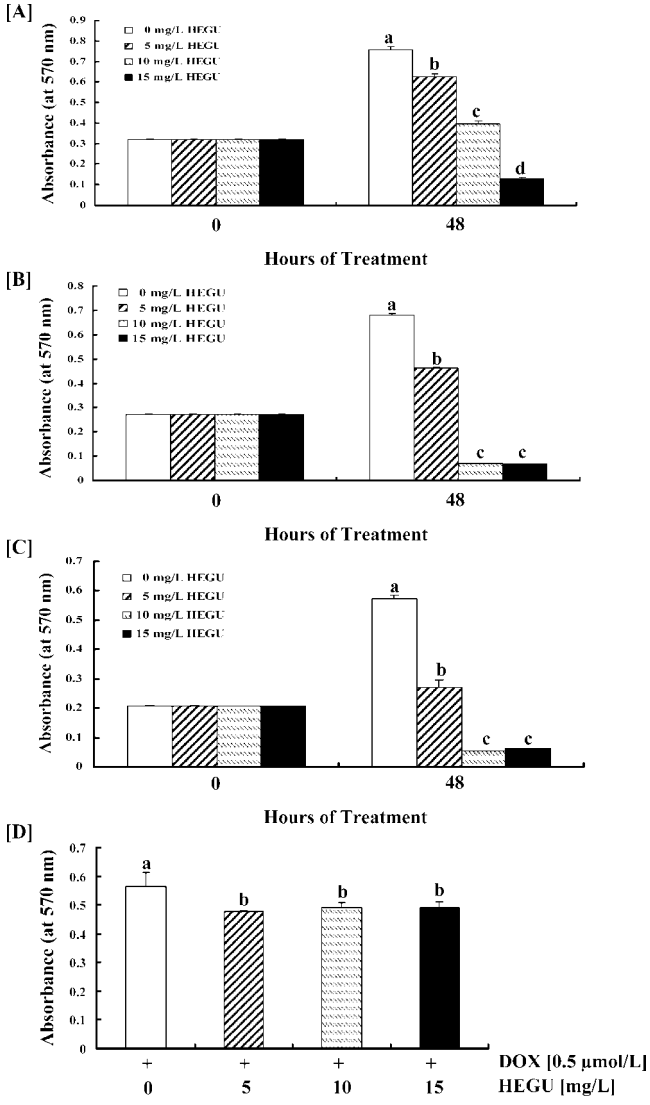

Figure 4. Effects of HEGU on the viability of cancer cells. HT-29 [A], MDA-MB-231 [B] and DU145 [C] cells were serum-deprived and treated with various concentrations of HEGU for 48 h. Cell numbers were estimated via MTT assay. Each bar represents the mean ± SEM ($n = 6$). Means without a common letter differ, $P < 0.05$. [D] DU145 cells were treated with 0–15 mg/L of HEGU and 0.5 μmol/L DOX as described in Figure 2B. Cell numbers were estimated by the MTT assay. Each bar represents the mean ± SEM ($n = 6$). Means without a common letter differ, $P < 0.05$.

Discussion

Licorice has been added to many prescriptions in traditional Chinese medicine for the detoxification of other plant compounds in the prescription. However, the consumption of large amounts of the GL present in licorice extract can result in hypokalemia, hypertension, and a syndrome known as apparent mineralocorticoid excess (29). GL is a water-soluble triterpenoid glycoside (38). Jo *et al.* previously reported that 100% ethanol and ethanol:H_2O (80:20) extracts of GU inhibit the proliferation of MCF-7 human breast cancer cells, whereas the proliferation was increased in MCF-7 cells treated with GU extracted with ethanol:H_2O (60:40; 40:60; 20:80; 0:100) (32). HEGU did not harbor measurable quantities of GL and contained mostly nonpolar compounds (Fig. 1). The extraction technique utilized in the present study was simple and rapid, and the yield of the extract was 0.9%. In the current study, HEGU reduced the viability of prostate, colon and breast cancer cells (Fig. 4), which is consistent with the results reported by Jo *et al.* These results suggest that HEGU would not interfere with DOX chemotherapy regimens and, rather, would augment the effects of the therapy. The mechanisms by which HEGU decreases the viability of cancer cells remain to be clearly elucidated.

Even though DOX is one of the most effective anticancer drugs, it induces a cardiomyopathy that, in turn, initiates heart failure. A variety of attempts have been made to mitigate and/or prevent DOX-induced cardiotoxicity, but the results have been relatively disappointing thus far. Using H9c2 rat cardiac myoblasts as an experimental model, we attempted to assess the possibility that HEGU lacking GL exerts protective effects against DOX-induced cardiotoxicity. The findings that HEGU treatments prevented the DOX-induced reduction of H9c2 cell viability but augmented that of DU145 cell viability suggest that HEGU may be employed to prevent DOX-induced cardiotoxicity in patients receiving DOX chemotherapy treatment. The mechanisms by which HEGU selectively protects cardiomyocytes from DOX treatment, but further decreases the viability of cancer cells, remain to be clearly elucidated.

Apoptosis has been suggested as one of the most important contributors to DOX-induced cardiomyopathy. In the current study, we observed that DOX induces apoptosis, upregulates p53, phosphorylated p53, and Bax, and increases the cleavage of caspases 9, 3, and 7 in H9c2 rat cardiac myoblasts. These results are consistent with the results reported by other investigators (15). In addition, we have demonstrated for the first time that HEGU inhibits DOX-induced apoptosis, DOX-induced accumulation of p53, DOX-induced increases in the levels of Bax, and DOX-induced caspase activation of H9c2 cells. In addition, HEGU increased the protein levels of the antiapoptotic Bcl-xL.

In normal healthy cells, the level of p53 is kept low due to its short half-life, but increases in response to cellular stress. Some evidence suggests that p53 becomes phosphorylated on Ser15 after cell damage; this phosphorylation impairs the ability of p53 to associate with its negative regulator, mdm2, which in turn prevents the degradation of the p53 protein and allows it to be translocated to the nucleus (40, 41). Within the nucleus, p53 stimulates the transcription of a variety of proapoptotic genes, including Bax. HEGU was shown to markedly block the accumulation of p53 and phospho-p53 in DOX-treated cells (Fig. 3A). The data uncovered in the present study suggest that the downregulation of p53 contributes to the reduction in Bax levels in HEGU-treated cells.

Bax is a proapoptotic member of the Bcl-2 family,

which, in response to apoptotic stimuli, translocates to the outer mitochondrial membrane, influences its permeability, and induces the loss of cytochrome c from the intermembrane space of the mitochondria, and subsequent release into the cytosol. The antiapoptotic Bcl-xL is associated with the outer mitochondrial membrane, where it stabilizes membrane permeability, thus preserving mitochondrial integrity and suppressing the release of cytochrome c [reviewed in (42, 43)]. In this study, HEGU suppressed DOX-induced increases in Bax levels and increased Bcl-xL levels, thereby suggesting that the reduction of Bax and/or increased levels of Bcl-xL contributes to the alleviation of apoptosis in cells treated with HEGU.

The cytosolic cytochrome c, together with ATP and Apaf1, activates caspase-9, which in turn activates caspase-3 and caspase-7, which execute the death program (44). In the present study, HEGU suppressed the DOX-induced cleavage of caspases 9, 7, and 3 and PARP (Fig. 3C). The inhibition of the cleavage of these proteins thereby results in the suppression of DOX-induced apoptosis in HEGU-treated H9c2 cells.

In conclusion, we have demonstrated that the licorice extract lacking GL protects cardiac myoblasts against DOX-induced apoptosis. The data uncovered in the current study indicate that HEGU attenuates DOX-induced apoptosis via the inhibition of p53 activation, thereby preventing the activation of p53 downstream events including Bax expression and caspase activation in H9c2 cells. HEGU deserves further evaluation of its potential as a protective agent against DOX-induced cardiac injury in animal studies.

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