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Novel Polyphenol Molecule Isolated from Licorice Root (*Glycrrhiza glabra*) Induces Apoptosis, G2/M Cell Cycle Arrest, and Bcl-2 Phosphorylation in Tumor Cell Lines

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Herbal therapies are commonly used by patients with cancer, despite little understanding about biologically active chemical derivatives. We recently demonstrated that the herbal combination PC-SPES, which contains licorice root, had anti-prostate cancer activity attributable to estrogen(s) that produced a chemical castration. A recent study also demonstrated that licorice root alone decreased circulating testosterone in men. Other studies demonstrated antitumor activity of PC-SPES in vitro associated with decreased expression of the anti-apoptotic protein Bcl-2 and in patients independent of chemical castration, suggesting that other mechanisms of antitumor activity exist separate from chemical castration. In the present study, we assessed licorice root extract for effects on Bcl-2 to identify novel cytotoxic derivatives. Licorice root extract induced Bcl-2 phosphorylation as demonstrated by immunoblot and G2/M cell cycle arrest, similarly to clinically used antimicrotubule agents such as paclitaxel. Bioassay-directed fractionations resulted in a biologically active fraction for Bcl-2 phosphorylation. HPLC separation followed by mass spectrometry and NMR identified 6 compounds. Only one molecule was responsible for Bcl-2 phosphorylation; it was identified as 1-(2,4-dihydroxyphenyl)-3-hydroxy-3-(4′-hydroxyphenyl) 1-propanone (β-hydroxy-DHP). The effect on Bcl-2 was structure specific, because α-hydroxy-DHP, 1-(2,4-dihydroxyphenyl)-2-hydroxy-3-(4′-hydroxyphenyl) 1-propanone, in contrast to β-hydroxy-DHP, was not capable of Bcl-2 phosphorylation. Pure β-hydroxy-DHP induced Bcl-2 phosphorylation in breast and prostate tumor cells, G2/M cell cycle arrest, apoptosis demonstrated by Annexin V and TUNEL assay, decreased cell viability demonstrated by a tetrazolium (MTT) assay, and altered microtubule structure. Therefore, these data demonstrate that licorice root contains β-hydroxy-DHP, which induced Bcl-2 phosphorylation, apoptosis, and G2/M cell cycle arrest, in breast and prostate tumor cells, similarly to the action of more complex (MW $>$800) antimicrotubule agents used clinically.

KEYWORDS: Bcl-2; licorice; microtubule; apoptosis; *Glycrrhiza glabra*

INTRODUCTION

Herbs are often used for the treatment of disease (*1*). Although the clinical effect of most herbal products is unknown, many herbs contain derivatives with biological activity (*2, 3*). We recently demonstrated the potent clinical and biological activity of the herbal combination called PC-SPES, a combination of eight herbs including licorice root (*3*). PC-SPES showed anti-prostate cancer activity, which was attributable to estrogen(s) that produced a chemical castration (*3*). A recent study demonstrated that licorice root alone also decreased circulating testosterone in men (*4*). Further studies demonstrated the antitumor activity of PC-SPES in patients already refractory to chemical castration, suggesting that other mechanisms of antitumor activity exist separate from chemical castration (*5*). Preclinical studies found that PC-SPES extracts induced apoptosis in tumor cell lines and decreased the expression of Bcl-2

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(*6*, *7*). Although the mechanism of this effect may be related to estrogenicity, since pharmaceutical estrogens and phytoestrogens can alter Bcl-2, we hypothesized that an estrogenic herb such as licorice root may effect the anti-apoptotic protein Bcl-2 (*8*, *9*). Bcl-2 is a 26-kDa protein that blocks cell death by inhibiting cytochrome *c* release from mitochondria, a critical event in the apoptotic pathway (*8*). Overexpression of Bcl-2 protects cells from death-promoting stimuli, whereas lowering Bcl-2 increases cell death and sensitivity to chemotherapy (*8*). Recent studies suggest that drugs which decrease Bcl-2 expression, or inactivate the molecule through phosphorylation, induce apoptosis (*9*−*13*). In fact, the many active agents used in oncology today, including paclitaxel, docetaxel, vincristine, and vinblastine, alter microtubule structure and induce apoptosis in association with Bcl-2 phosphorylation (*12*−*14*). Therefore, studying herbs such as licorice for effects on Bcl-2 may lead to the identification of novel chemical structures on which newer anti-cancer agents can be derived.

MATERIALS AND METHODS

Materials. Licorice root (*Glycyrrhiza glabra*) was donated by Folexco Corporation (Montgomeryville, PA). T47D (breast cancer), MCF-7 (breast cancer), and HL-60 (leukemia) cells were obtained from the American Type Culture Collection (ATCC). DUPro-1 (prostate cancer) cells were a gift from Dr. S. Ward (*15*). Cells were maintained at 37 °C in an atmosphere of 5% CO_2 and grown in RPMI 1640 supplemented with fetal bovine serum (FBS), 50 units penicillin, and 50 μg/mL streptomycin. Cells were routinely checked and found to be free of contamination by mycoplasma.

General Procedures. ^{1}H NMR and ^{13}C NMR spectra were obtained on a VXR-200 instrument, and mass spectra were obtained using direct probe electron ionization (EI) and atmospheric pressure chemical ionization (APCI) in the negative-ion mode. Direct probe EI-MS was performed on a Finnigan MAT 8230 high-resolution mass spectrometer (San Jose, CA). APCI MS analysis was performed on a Micromass Platform II system (Micromass Co., MA) equipped with a Digital DECPc XL560 computer for analysis of data. The ion source temperature was set at 150 °C, and the probe temperature was set at 450 °C. The sample cone voltage was 10 V and the corona discharge was 3.2 kV. HPLC analysis was performed on a Varian Vista 5500 liquid chromatograph pump coupled to a Varian 9065 Polychrom diode array detector (Sugar Land, TX). Semipreparative fractionation of purified compounds was obtained on a Varian 9012 HPLC pump coupled to a Waters Lambda-Max model 481 LC spectrophotometer (Milford, MA). Column chromatography was performed using octadecyl-functionalized silica gel (60 Å particle size). The column packing was purchased from Aldrich Chemical Co. (Milwaukee, WI), and formic acid (FA), hydrochloric acid (HCl), methanol, ethyl acetate, water, and acetonitrile were purchased from Fisher Scientific (Springfield, NJ). All solvents used for extraction and chromatographic analyses were of HPLC grade. Mixtures of solvents for HPLC were filtered through Whatman nylon membrane filters 0.45 μm, 47 mm (Clifton, NJ) and degassed.

Extraction and Isolation Procedures. The powdered roots of *G. glabra* were extracted with methanol and concentrated under vacuum. The concentrate was then partitioned with acidified ethyl acetate (3% HCl). The dry ethyl acetate extract was reconstituted in methanol and then chromatographed on a reversed-phase octadecyl-functionalized silica gel column (2.5 × 30 cm) such that bio-assay directed fractionation could be performed. The column was packed in methanol and then conditioned with 90:10 water/methanol (500 mL). The dispersed extract was then placed on top of the column, and elution was performed at room temperature. Elution was done using a solvent mixture of water/methanol with an increasing amount of methanol (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100; each 500 mL). Successive fractions were collected and tested for biological activity. The fraction eluted with 70:30 water/methanol was determined to be the most active one. This fraction was then rechromatographed on a semipreparative Zorbax Rx-C18 reversed-phase HPLC column (9.4 mm × 240 mm, 5 μm) purchased from Mac-Mod Analytical (Chadds Ford, PA). Compounds were eluted by a gradient solvent system: A, water and 0.05% FA; B, acetonitrile. The elution program at 3 mL min^{-1} was as follows: 80% A to 40% B (0−45 min). The wavelength monitored was 254 nm. Successive peaks (A−F) were collected and tested for biological activity. Each peak was then reconstituted in methanol and analyzed by APCI LC-MS in the negative ion mode. It was determined that only peak E can phosphorylate bcl-2. Collected peaks were screened for purity using a Discovery C18 reversed-phase HPLC column (250 mm × 4.6 mm, 5 μm) with a column guard purchased from Supelco (Bellefonte, PA). The solvent program was a gradient system: A, water and 0.05% FA; B, acetonitrile. The elution program at 1 mL min^{-1} was as follows: 100% A to 100% B (0−35 min); 100% B (35−55 min). The wavelengths monitored were 220−320 nm with a Varian 9065 diode array detector. It was concluded that peak E was a pure compound and needed no further purification.

Bcl-2 Expression and Phosphorylation. Analysis of Bcl-2 protein by Western blots was determined as previously described (*9*). Protein identification was made using a monoclonal Bcl-2 primary antibody (DAKO Corp) and secondary goat anti-mouse horseradish peroxidase conjugated antibody (Bio-Rad Laboratories, Richmond, CA). The phosphorylation of Bcl-2 was determined by mobility shifts in Western blot as described by Hadlar et al. (*12*−*13*).

Cell Cycle Analysis. Cells were treated with β-DHP for 24 h, and incubated with 10 μM BrdU for 45 min at 37 °C. Cells were then washed with ice-cold PBS, resuspended in 200 μL of PBS, and fixed with cold 70% ethanol. The cells were resuspended, incubated for 30 min in 2 N hydrochloric acid /0.5% Triton X-100 in PBS, and neutralized by rinsing once in 0.1 M sodium tetraborate (pH 8.5). Fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody (Becton-Dickinson) was added (10 μg per sample) in 50 μL of 0.5% Tween 20/1% BSA in PBS and incubated for 30 min. The cells were washed and resuspended in 1 mL of PBS containing 5 μg/mL propidium iodide. Fluorescence intensity was determined by quantitative flow cytometry, and profiles were generated on Becton Dickinson FACS. A minimum of 10,000 cells were analyzed using Modifit LT (Verily Software House, Inc.).

Cell Viability and Apoptosis. The Apoalert Annexin V-EGFP method (CLONTECH, Palo Alto, CA) was used to assess for apoptosis (*16*). Briefly, tumor cells were treated for 2 h with β-DHP, washed with fixing solution, and stained with Annexin V-EGFP and propidium iodide for 15 min in the dark. Cells were viewed using a Nikon Eclipse TE 200 (Nikon Corporation Tokyo, Japan) inverted fluorescent microscope. Photographs were captured using a SPOT digital camera (Diagnostic, Inc., Sterling Heights, MI) in combination with SPOT software, version 2.1. Apoptotic cells were also detected using TdT-mediated dUTP nick end labeling (TUNEL) with an APO-BRDU kit (Pharmingen, San Diego, CA) as previously described (*17*). Cells (1 × 10^6/dish) were treated for 12 h, washed with PBS, and fixed in 1% paraformaldehyde in ice for 30 min. After the cells were fixed, they were washed twice with PBS and fixed in 70% ethanol. The pellets were washed and resuspended in 50 μL of the DNA labeling solution containing Br-dUTP (bromodeoxyuridine triphosphate) and TdT (terminal deoxynucleotidyl transferase) enzyme and incubated for 60 min at 37 °C. After incubation, the pellets were washed, incubated with FITC labeled anti-BrdU antibody in the dark for 30 min at room temperature, and stained with propidium iodide and RNase. The stained cells were analyzed by flow cytometry after 30 min. Cell viability was assessed by the tetrazolium dye method (MTT) as previously described (*18*). Cells were plated in 96-well plates and incubated with various agents for 72 h. Absorbance was measured at 570 nm using a Dynatech microplate reader. Microtubule structure was assessed by indirect immunofluorescence as previously described (*19*).

RESULTS

Licorice Root Extract Induced Bcl-2 Phosphorylation and G2/M Cell Cycle Arrest. To determine the effect of licorice root on mitosis we measured the effect of licorice root on Bcl-2 and cell cycle. To determine the effect of licorice extract on Bcl-2, various fractions of whole licorice root were assessed

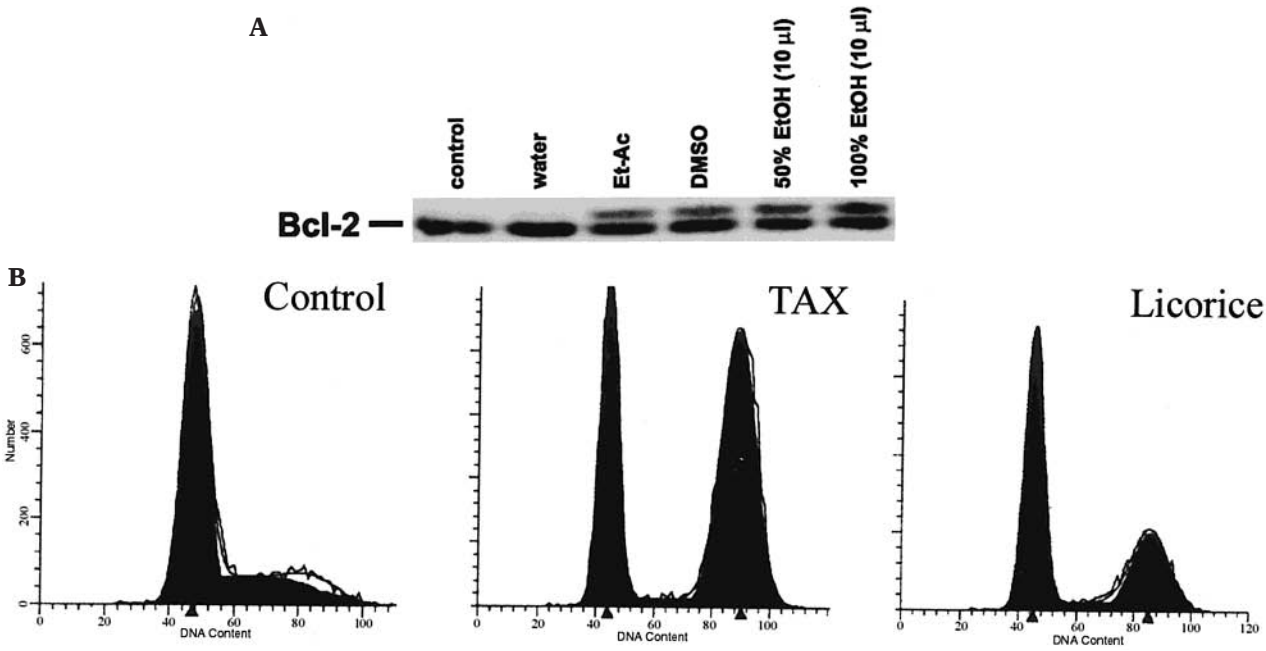

Figure 1. The effect of licorice root extract on Bcl-2 and cell cycle. 1A, Immunoblot of MCF-7 cells treated with licorice root extracted with ethanol vehicle control, water, ethyl acetate (Et-Ac), DMSO, and ethanol (EtoH) for 12 h. Cells were lysed and assayed with a monoclonal Bcl-2 antibody. 1B, Cell cycle analysis of T47D cells treated with licorice root extracted in 100% ethanol, 10 μM paclitaxel (TAX) in ethanol, or ethanol vehicle control for 12 h.

by immunoblotting. Licorice root extracted with ethyl acetate, DMSO, or ethanol induced Bcl-2 phosphorylation, as demonstrated by a slower migrating band, in contrast to the vehicle control (ethanol alone) or a water extraction (**Figure 1A**). Because previous studies have shown an association between Bcl-2 phosphorylation and cell-cycle arrest at G2/M, we also assessed the effect of licorice extract on the cell cycle (*19*−*22*). As shown in **Figure 1B**, licorice extract induced G2/M cell cycle arrest similar to paclitaxel, as represented by a second peak in the flow cytometric analysis.

Extraction and Identification of Active Compound Capable of Bcl-2 Phosphorylation. To identify the active molecule from licorice root capable of Bcl-2 phosphorylation, an ethyl acetate extract of *Glycrrhiza glabra* was subjected to bioassay directed fractionation. The most active fraction 70:30 water/methanol was analyzed by reversed-phase HPLC at 254 nm. To identify the active molecule with biological activity, the 70:30 water/methanol fraction was then rechromatographed on a semipreparative reversed-phase C18 HPLC column such that successive peaks (A−F) could be collected and subjected to bioassays (**Figure 2**). Peak **A** had a mass of 418 $[M-(H_2O)]^-$ and a fragment at *m*/*z* 417 $[M-(H_2O-H)]^-$ and was tentatively identified as a hydroxydihydrochalcone glucoside. Its corresponding aglycon was observed at mass 255 $[A-(H_2O-H)]^-$. The *m*/*z* 463 is a formic acid adduct from the mobile phase and the *m*/*z* 486 = 463 + Na. Structure **B** also had a mass of 418 $[M-(H_2O)]^-$. Its corresponding aglycon was also observed at *m*/*z* 255 $[A-(H_2O-H)]^-$. As shown in **Figure 2**, licorice root extracts 70:30 water/methanol fraction contained multiple derivatives and we are able to identify only 6 compounds (Peaks **A**−**F**) by NMR and EI-MS (**Figure 3**). The hydroxydihydrochalcone isolated from peak **E** was the only active molecule that induced Bcl-2 phosphorylation, and was determined to be 1-(2,4-dihydroxyphenyl)-3-hydroxy-3-(4′-hydroxyphenyl)1-propanone. It is estimated that 1 kg of licorice root contains 80−250 mg of β-hydroxy DHP in four separate batches obtained from different sources. The two hydroxydihydrochalcone glucosides from Peaks **A** and **B** were determined to be 1-(2,4-dihydroxyphenyl)-3-hydroxy-3-(4′-hydroxyphenyl-4′-*O*-β-D-glucopyranoside)-1-propanone, and 1-(2,4-dihydroxyphenyl-4-*O*-β-D-glucopyranoside)-3-hydroxy-3-(4′-hydroxyphenyl)-1-propanone. Structures **C** (2,3,4-trihydroxy-4′-methoxychalcone), **D** (1-(2,4-dihydroxyphenyl)-2-hydroxy-3-(4′-hydroxyphenyl)-1-propanone), and **F** (2,4,4′-trihydroxychalcone) were found to be known structures. Because the structures of **C**, **D**, and **F** were known, the NMR data were not included.

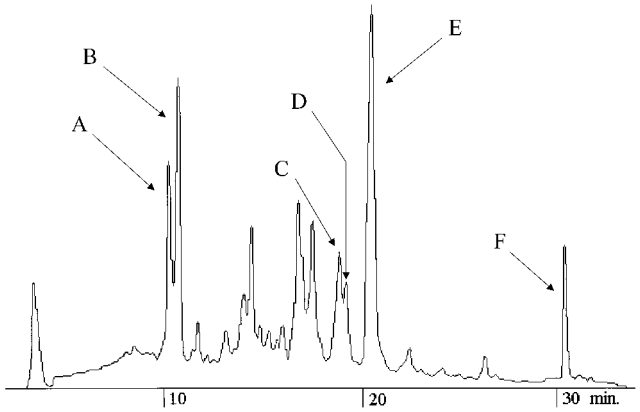

Figure 2. Reversed-phase HPLC chromatogram of licorice root extract 70:30 water/methanol fraction at 254 nm. Semipreparative Zorbax Rx-C18 reversed-phase HPLC column (9.4 mm × 240 mm, 5 μm) eluted by a gradient solvent system: A, water 0.05% FA; B, acetonitrile; and elution program at 3 mL min^{-1} was 80% A to 40% B (0–45 min).

NMR Data for New Isolated Compounds. *Structures A and B*. Structure confirmation of peaks **A** and **B** were determined using the following ^{1}H NMR and ^{13}C NMR data. 1-(2,4-dihydroxyphenyl)-3-hydroxy-3-(4′-hydroxyphenyl-4′-*O*-β-D-glucopyranoside)-1-propanone (peak **A**). ^{1}H NMR: δ 2.70 (1H, dd, J = 16.4, 3.2 Hz, H-α′), δ 3.04 (1H, dd, J = 16.4, 12.8 Hz, H-α″), δ 3.30−3.54 (4H, m, H-2″, 3″, 4″, 5″), δ 3.72 (1H, dd, J = 12.0 Hz, 4.1 Hz, H-6′), δ 3.90 (1H, brd, J = 12.0 Hz, H-6″), δ 5.44 (1H, dd, J = 12.8, 3.0 Hz, H-β), δ 6.38 (1H, d,

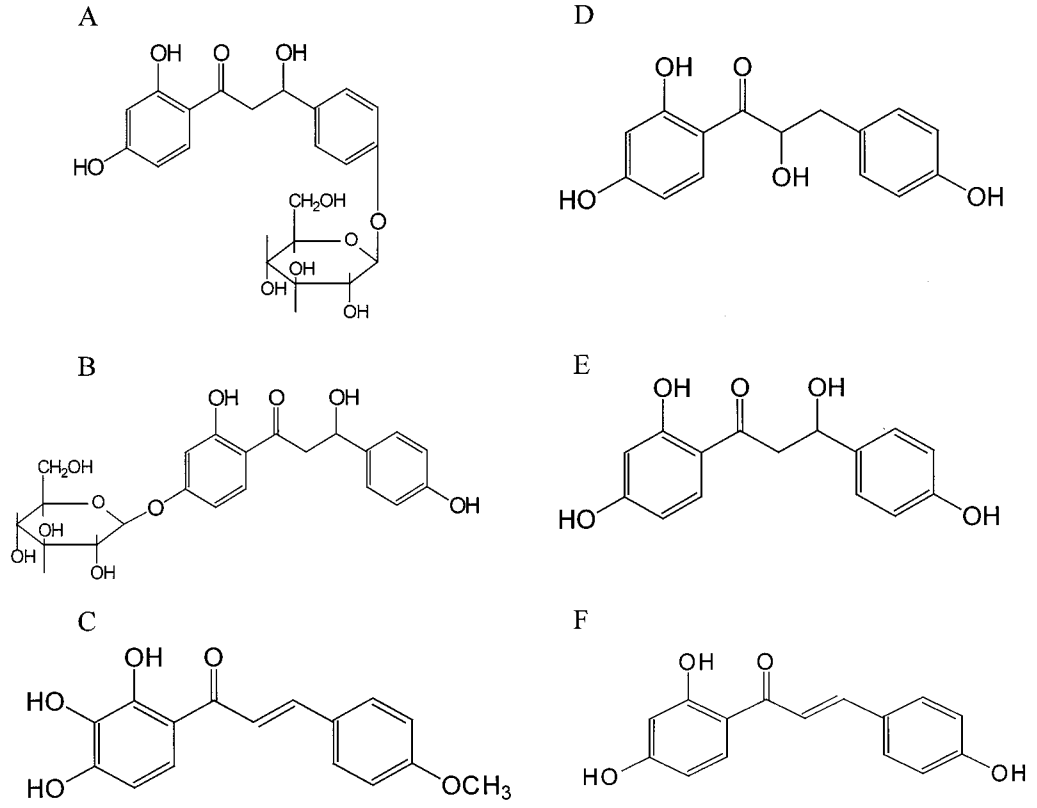

Figure 3. Structures of 6 compounds (peaks A–F) identified by mass spectrometry and NMR: 3A, 1-(2,4-dihydroxyphenyl)-3-hydroxy-3-(4′-hydroxyphenyl-4′-*O*-*β*-D-glucopyranoside)-1-propanone; 3B, 1-(2,4-dihydroxyphenyl-4-*O*-*β*-D-glucopyranoside)-3-hydroxy-3-(4′-hydroxyphenyl)-1-propanone; 3C, 2,3,4-trihydroxy-4′-methoxychalcone; 3D 1-(2,4 dihydroxyphenyl)-2-hydroxy-3-(4′-hydroxyphenyl)1-propanone; 3E 1-(2,4-dihydroxyphenyl)-3-hydroxy-3-(4′-hydroxyphenyl) 1-propanone; and 3F 2,4,4′-trihydroxychalcone.

J = 2.1 Hz, H-3), δ 6.53 (1H, dd, J = 8.4, 2.1 Hz, H-5), δ 7.14 (2H, d, J = 8.4 Hz, H-3′, 5′), δ 7.44 (2H, d, J = 8.4 Hz, H-2′, 6′), δ 7.73 (1H, d, J = 8.4 Hz, H-6). ^{13}C NMR: δ 45.25 (C-α), δ 80.98 (C-β), δ 104.14 (C-3), δ 112.12 (C-5), δ 115.31 (C-1), δ 118.11 (C-3′, 5′), δ 129.07 (C-2′, 6′), δ 130.16 (C-6), δ 134.73 (C-1′), δ 159.52 (C-4′), δ 165.69 (C-4), δ 167.10 (C-2), δ 193.49 (C═O), Glucose 102.47 (C-1″), 75.19 (C-2″), 78.27 (C-3″), 71.66 (C-4″), 78.46 (C-5″), 62.80 (C-6″).

1-(2,4-dihydroxyphenyl-4-*O*-β-D-glucopyranoside)-3-hydroxy-3-(4′-hydroxyphenyl)-1-propanone (peak **B**). ^{1}H NMR: δ 2.74 (1H, dd, J = 16.4, 3.0 Hz, H-α′), δ 3.12 (1H, dd, J = 16.4, 13.0 Hz, H-α″), δ 3.00−3.52 (4H, m, H-2″, 3″, 4″, 5″), δ 3.69 (1H, dd, J = 12.0 Hz, 4.0 Hz, H-6′), δ 3.90 (1H, brd, J = 12.0 Hz, H-6″), δ 5.43 (1H, dd, J = 13.0, 3.0 Hz, H-β), δ 6.70−6.88 (4H, m, H-3, 5, 3′, 5′), δ 7.34 (2H, d, J = 8.4 Hz, H-2′, 6′), δ 7.82 (1H, d, J = 8.6 Hz, H-6). ^{13}C NMR: δ 45.27 (C-α), δ 81.60 (C-β), δ 105.52 (C-3), δ 112.50 (C-5), δ 116.59 (C-3′, 5′), δ 117.27 (C-1), δ 129.38 (C-2′, 6′), δ 129.75 (C-6), δ 131.43 (C-1′), δ 159.35 (C-4′), δ 165.38 (C-4)*, δ 165.83 (C-2)*, δ 193.88 (C═O), Glucose 101.76 (C-1″), 74.99 (C-2″), 78.12 (C-3″), 71.49 (C-4″), 78.56 (C-5″), 62.66 (C-6″).

*Structure **E***. Structure confirmation of licorice peak **E** (DHP-β) was determined using the following ^{1}H NMR and ^{13}C NMR data. 1-(2,4-dihydroxyphenyl)-3-hydroxy-3-(4′-hydroxyphenyl)-1-propanone. ^{1}H NMR: δ 2.69 (1H, dd, J = 16.3, 3.1 Hz, H-α′), δ 3.06 (1H, dd, J = 16.3, 13.0 Hz, H-α″), δ 5.38 (1H, dd, J = 13.0, 3.0 Hz, H-β), δ 6.36 (1H, d, J = 2.2 Hz, H-3), δ 6.51 (1H, dd, J = 8.6, 2.2 Hz, H-5), δ 6.82 (2H, d, J = 8.6 Hz, H-3′, 5′), δ 7.33 (2H, d, J = 8.6 Hz, H-2′, 6′), δ 7.74 (1H, d, J = 8.6 Hz, H-6). ^{13}C NMR: δ 45.24 (C-α), δ 81.33 (C-β), δ 104.11 (C-3), δ 112.05 (C-5), δ 115.41 (C-1), δ 116.60 (C-3′, 5′), δ 129.31 (C-2′, 6′), δ 130.15 (C-6), δ 131.64 (C-1′), δ 159.10 (C-4′), δ 166.00 (C-4), δ 167.21 (C-2), δ 193.84 (C═O). No effort was made to determine the sterochemistry of compound **E**.

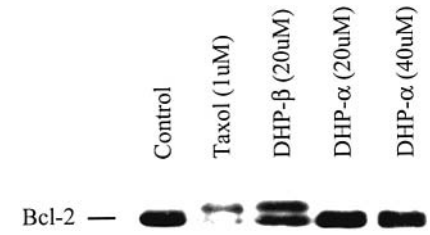

Figure 4. Effect of α-hydroxy-DHP (Figure 3D) compared to β-hydroxy-DHP (Figure 3E) on Bcl-2 protein. Immunoblot of MCF-7 cells treated using taxol, α-hydroxy-DHP, or β-hydroxy-DHP for 12 h. Cells were lysed and assayed with a monoclonal Bcl-2 antibody.

Novel Polyphenol Identified as the Active Component of Licorice Root. To determine active compound(s) from licorice root extract capable of Bcl-2 phosphorylation, fractions were initially collected and assessed by HPLC. As shown in **Figure 2**, licorice roots extract 70:30 water/methanol fraction contained multiple derivatives, and we are able to identify only 6 compounds, as described above. Analysis by NMR and mass spectroscopy identified an active compound 1-(2,4-dihydroxyphenyl)-3-hydroxy-3-(4′-hydroxyphenyl) 1-propanone, (β-hydroxy-DHP) (MW 264) in peak **E**. The effect of β-hydroxy-DHP (**Figure 3E**) on Bcl-2 phosphorylation was structure specific, because α-hydroxy-DHP (**Figure 3D**), in contrast to β-hydroxy-DHP, was not capable of Bcl-2 phosphorylation, as shown in **Figure 4**. As shown in **Figure 4**, β-hydroxy-DHP induced Bcl-2 phosphorylation similar to paclitaxel (taxol) in contrast to α-hydroxy-DHP. Peaks **A**, **B**, **C**, **D**, and **F** (**Figure**

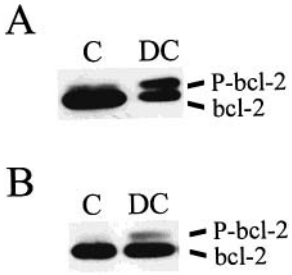

Figure 5. The effect of β-hydroxy-DHP on Bcl-2 protein. Immunoblot of MCF-7 (5A) and DuPro-1 (5B) cells treated using 10 μM β-hydroxy-DHP (DC) or vehicle control for 12 h. Cells were lysed and assayed with a monoclonal Bcl-2 antibody.

2) were unable to induce Bcl-2 phosphorylation (data not shown). Given these data, β-hydroxy-DHP (**Figure 3E**) was the subject of further biological assessment.

***β*-Hydroxy-DHP Induced Bcl-2 Phosphorylation and G2/M Cell Cycle Arrest.** To confirm the activity of pure compound, β-hydroxy-DHP was studied further. As shown in **Figure 5**, β-hydroxy-DHP induced phosphorylation of Bcl-2 in both breast (**Figure 5A**) and prostate (**Figure 5B**) tumor cells. Prior studies demonstrated that anti-microtubule agents such as paclitaxel and vinca alkaloids induce Bcl-2 phosphorylation, in association with G2/M cell cycle arrest (*12*−*13*, *20*−*21*). To confirm that pure β-hydroxy-DHP induced G2/M cell cycle arrest, as did whole licorice root, T47D tumor cells were treated with β-hydroxy-DHP and assessed by flow cytometry. As shown in **Figure 6**, β-hydroxy-DHP (**Figure 6C**) induced a G2/M cell cycle arrest similar to that of the known antimicrotubule agent paclitaxel (**Figure 6B**). To determine the effect of β-hydroxy-DHP on microtubules, indirect immunofluorescence was used to assess microtubule. As shown in **Figure 7D** and **E**, β-hydroxy-DHP altered microtubule structure similar to that of cells treated with vinblastine, which is known to destabilize microtubules (**Figure 7C**) (*19*). In contrast, paclitaxel is known to induce microtubule stabilization, as is shown in **Figure 7B**.

***β*-Hydroxy-DHP Induced Apoptosis.** To assess the cytotoxicity of β-hydroxy-DHP, tumor cells were treated with pure β-hydroxy-DHP and assessed for cell viability and apoptosis. β-hydroxy-DHP induced apoptosis in MCF-7 cells, as demonstrated by the detection of extracellular phosphatidylserine, which redistributes to the outer layer of the cell membrane during apoptosis (**Figure 8**). Apoptotic cells were indicated by green fluorescence under microscopy after treatment with 1 to 5 μM β-hydroxy-DHP (**Figure 8D** and **F**) and yellow-red intracellular staining after treatment with 10 μM β-hydroxy-DHP (**Figure 8H**). β-Hydroxy-DHP also induced DNA strand breaks in HL-60 cells as detected by a TUNEL assay (**Figure 9, top**) and decreased cell viability in MCF-7 cells in a dose-dependent manner (IC_{50} of approximately 13 μM) (**Figure 9, bottom**).

DISCUSSION

Anti-microtubule agents are widely used for the treatment of malignancy (*10*, *12*−*14*). Most of the known drugs such as paclitaxel, docetaxel, vincristine, and vinblastine are large complex structures with molecular weights of over 800. In our study, we demonstrated for the first time that licorice root has biological activity similar to that of known anti-microtubule drugs, and we identified the active compound as β-hydroxy-DHP (peak **E**, molecular weight 264), which alters microtubule structure, induces G2/M cell cycle arrest, induces Bcl-2 phosphorylation, and causes apoptosis similar to known anti-microtubule agents.

Previous studies have demonstrated that licorice root extract has other potent biological effects (*23*−*24*). For example, a water extract of licorice root was found to inhibit angiogenesis (*23*). In our study, isolation of active compounds in licorice root with biological activity on Bcl-2 protein and the cell cycle required more hydrophobic solvents. Prior studies also identified isoflavonoids from licorice with anti-microbial activity and anti-parasitic activity (*24*, *26*). For example, Shibata et al. (*27*) demonstrated antitumor activity of Licochalcone-A (LA) in vitro and in vivo in a mouse skin papilloma model, but the mechanism

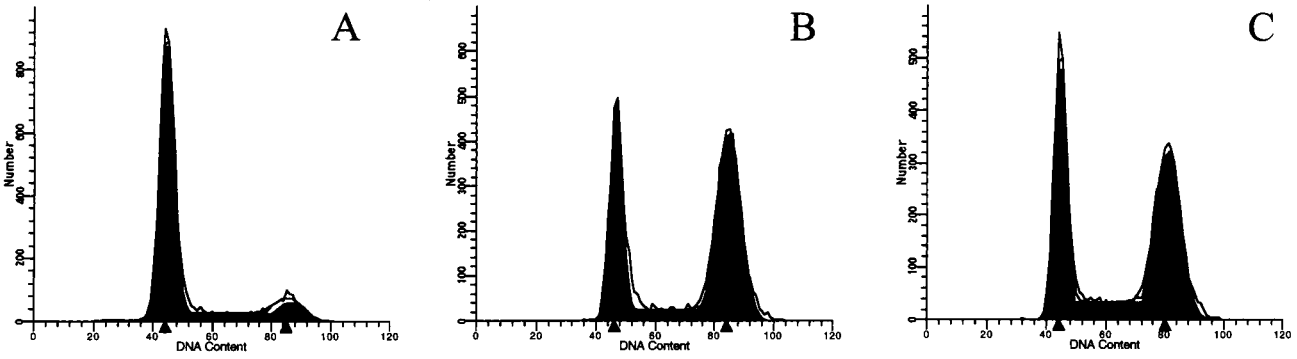

Figure 6. Effect of β-hydroxy-DHP on tumor cell cycle. Cell cycle analysis of T47D cells treated using 10 μM β-hydroxy-DHP (6C), 10 μM paclitaxel (6B), or vehicle control (6A) for 12 h.

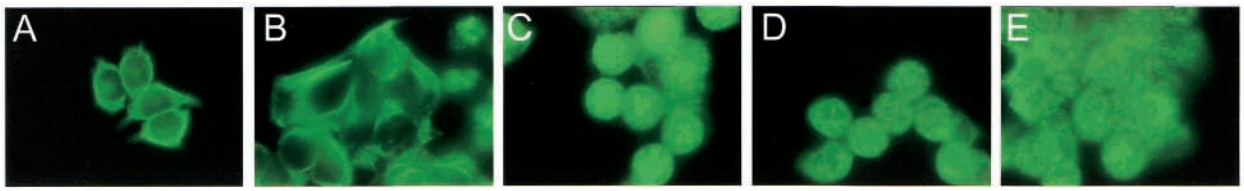

Figure 7. Effect of β-hydroxy-DHP on microtubules. Indirect immunofluorescence of MCF-7 cells using antibody to tubulin after 12 h of treatment. (A) vehicle control, (B) cells treated with 10 μM paclitaxel, (C) cells treated with 10 μM vinblastine, (D) cells treated with 20 μM β-hydroxy-DHP, and (E) cells treated with 40 μM β-hydroxy-DHP.

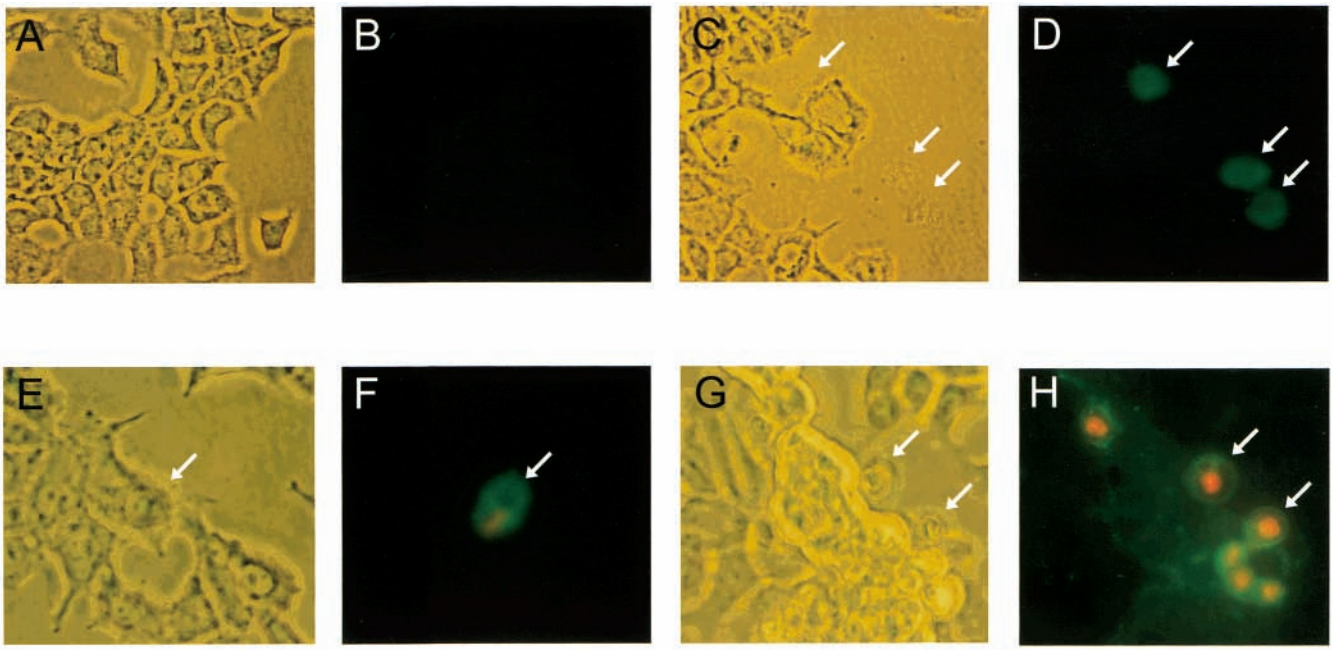

Figure 8. Effect of β-hydroxy-DHP on early apoptosis as demonstrated by the detection of extracellular phosphatidylserine. Fluorescent microscopy of MCF-7 cells stained with Annexin V-EGFP and propidium iodide: brightfield (A) and fluorescent (B) view of cells treated with vehicle; brightfield (C) and fluorescent (D) view of cells treated with 1 μM β-hydroxy-DHP for 2 h; brightfield (E) and fluorescent (F) view of cells treated with 5 μM β-hydroxy-DHP over 2 h; brightfield (G) and fluorescent (H) view of cells treated with 10 μM β-hydroxy-DHP over 2 h. Arrows indicate the same apoptotic cells viewed in the fluorescent and brightfield views.

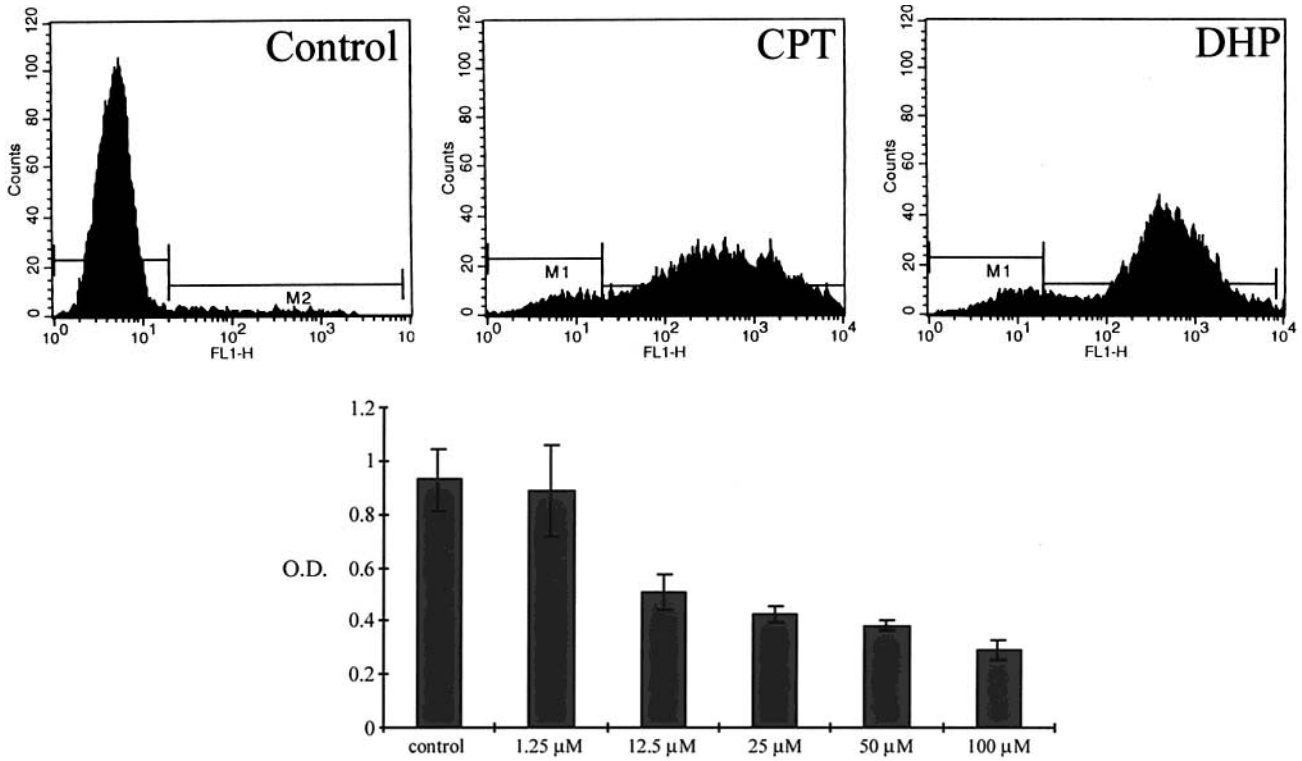

Figure 9. Effect of β-hydroxy-DHP on late apoptosis as demonstrated by TUNEL assay and cell viability. (Top) Flow cytometry analysis of HL-60 cell lines using APO-BRDU. Apoptosis was detected by incorporation of Br-dUTP using a fluorescein labeled anti-BrdU monoclonal antibody after treatment with vehicle control, 1 μM camptothecin (CPT), or 50 μM β-hydroxy-DHP for 12 h. (Bottom) Cell viability of MCF-7 cells assessed by MTT assay after treatment with various concentrations of β-hydroxy-DHP or vehicle control for 72 h. Optical density (O. D.) is represented ± SEM.

of activity is unknown. LA, in contrast to β-hydroxy-DHP, did not induce Bcl-2 phosphorylation or G2/M cell cycle arrest (data not shown). In addition to in vitro biological effects, licorice root extract is also biologically potent in humans, as studies have demonstrated alterations in blood pressure and serum testosterone levels (*4*, *28*). β-Hydroxy-DHP induced Bcl-2 phosphorylation in tumor cell lines (**Figure 5**) similar to that of the known clinically active anti-microtubule chemotherapy agents (*12*−*14*). Halder et al. demonstrated that paclitaxel, vincristine, docetaxel, and vinblastine induced Bcl-2 phospho-

rylation, which inactivated Bcl-2 function (*12*−*14*). Although phosphorylation of Bcl-2 is associated with anti-microtubule activity, the relationship of Bcl-2 phosphorylation and apoptosis remains unclear (*21*, *22*). For example, some investigators have demonstrated that paclitaxel induced apoptosis requires the phosphorylation of Bcl-2 (*22*). In contrast, other investigators have reported that phosphorylation of Bcl-2 is a marker of G2/M Phase events and not a determinant of apoptosis (*21*). In agreement with this latter study, β-hydroxy-DHP induced G2/M cell cycle arrest (**Figure 6**). β-hydroxy DHP also induced apoptosis as demonstrated in **Figures 8** and **9**, but further studies would be needed to determine if Bcl-2 phosphorylation was required for this effect. Further studies would also be warranted to determine whether β-hydroxy DHP enhanced the effect of known anti-microtubule agents capable of Bcl-2 phosphorylation.

Given the structural complexity of known agents capable of Bcl-2 phosphorylation, such as paclitaxel and vinblastine, the study of structure and activity of this simple polyphenolic compound may improve our ability to develop novel clinically active agents (*29*−*33*). Yoshida et al. demonstrated the synthesis of β-hydroxy-DHP from a polyphenol glycoside derived from *Rosa cymosa*, but did not study biological activity (*30*). Edwards et al. synthesized similar chalcone structures, many of which are isolated from plants, with multiple methoxy groups and found that 1-(2,5-dimethoxyphenyl)-3-[4-dimethylamino)phenyl]-2-methyl-2-propen-1-one had potent anti-microtubule activity (*32*). In contrast to these chalcone structures, β-hydroxy-DHP lacks methoxy groups (**Figure 3E**); this finding may be important in understanding the structure needed to affect microtubules, as prior studies have demonstrated that methoxy groups of the colchicine A ring and carbonyl group of the C ring are important for its binding to tubulin (*33*). In addition, the effect by β-hydroxy-DHP was structure specific, because an isomer, α-hydroxy-DHP, was not capable of phosphorylating Bcl-2 at the same dose and timing tested (**Figure 4**). Given these data, further studies including the methylation of β-hydroxy-DHP and α-hydroxy-DHP would be warranted. These analogues will hopefully serve as lead compounds in the synthesis of more potent analogues that may ultimately lead to the development of improved chemotherapy drugs with specific biological targets.

In conclusion, these data demonstrate for the first time that licorice root has biological activity capable of Bcl-2 phosphorylation and G2/M cell cycle arrest. The molecule causing this activity was identified as the polyphenol β-hydroxy-DHP, which was purified, and induced Bcl-2 phosphorylation, apoptosis, and G2/M cell cycle arrest, and altered microtubule structure in a manner similar to that of the more complex known anti-microtubule agents. These effects were structurally specific because α-hydroxy-DHP, in contrast to β-hydroxy-DHP, was incapable of Bcl-2 phosphorylation. Further studies of the structure−activity relationship of β-hydroxy-DHP as a parent compound are warranted.

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