Evaluation of Estrogenic Activity of Plant Extracts for the Potential Treatment of Menopausal Symptoms

Jianghua Liu,[†] Joanna E. Burdette,[†] Haiyan Xu,[‡] Chungang Gu,[†] Richard B. van Breemen,[†] Krishna P. L. Bhat,[†] Nancy Booth,[†] Andreas I. Constantinou,[‡] John M. Pezzuto,[†] Harry H. S. Fong,[†] Norman R. Farnsworth,[†] and Judy L. Bolton*[,†]

Department of Medicinal Chemistry and Pharmacognosy, Program for Collaborative Research in the Pharmaceutical Sciences, UIC/NIH Center for Botanical Dietary Supplements Research, College of Pharmacy, M/C 781, University of Illinois at Chicago, 833 South Wood Street, Chicago, Illinois 60612, and Department of Surgical Oncology, University of Illinois at Chicago, 840 South Wood Street, Chicago, Illinois 60612

Eight botanical preparations that are commonly used for the treatment of menopausal symptoms were tested for estrogenic activity. Methanol extracts of red clover (*Trifolium pratense* L.), chasteberry (*Vitex agnus-castus* L.), and hops (*Humulus lupulus* L.) showed significant competitive binding to estrogen receptors α (ERα) and *β* (ER*β*). With cultured Ishikawa (endometrial) cells, red clover and hops exhibited estrogenic activity as indicated by induction of alkaline phosphatase (AP) activity and up-regulation of progesterone receptor (*PR*) mRNA. Chasteberry also stimulated *PR* expression, but no induction of AP activity was observed. In S30 breast cancer cells, *pS2* (presenelin-2), another estrogen-inducible gene, was up-regulated in the presence of red clover, hops, and chasteberry. Interestingly, extracts of Asian ginseng (*Panax ginseng* C.A. Meyer) and North American ginseng (*Panax quinquefolius* L.) induced *pS2* mRNA expression in S30 cells, but no significant ER binding affinity, AP induction, or *PR* expression was noted in Ishikawa cells. Dong quai [*Angelica sinensis* (Oliv.) Diels] and licorice (*Glycyrrhiza glabra* L.) showed only weak ER binding and *PR* and *pS2* mRNA induction. Black cohosh [*Cimicifuga racemosa* (L.) Nutt.] showed no activity in any of the above in vitro assays. Bioassay-guided isolation utilizing ER competitive binding as a monitor and screening using ultrafiltration LC-MS revealed that genistein was the most active component of red clover. Consistent with this observation, genistein was found to be the most effective of four red clover isoflavones tested in the above in vitro assays. Therefore, estrogenic components of plant extracts can be identified using assays for estrogenic activity along with screening and identification of the active components using ultrafiltration LC-MS. These data suggest a potential use for some dietary supplements, ingested by human beings, in the treatment of menopausal symptoms.

Keywords: *Estrogen receptor; alkaline phosphatase; progesterone receptor; pS2; dietary supplement; phytoestrogens; isoflavones*

INTRODUCTION

During the period of menopause and postmenopause, many women experience one or more symptoms such as hot flashes, depression, mood swings, sleeping disorders, vaginal dryness, and joint pain, largely due to a lack of estrogens (*1*). Hormone replacement therapy has helped to relieve menopausal symptoms; in addition, the risk of osteoporosis, cardiovascular disease, dementia from Alzheimer's disease, and certain types of cancer are reduced (*2*−*5*). Epidemiological data show that a diet rich in phytoestrogens, such as those found in soy, reduce the number of hot flashes and the incidence of cancer in Oriental women (*6*). Since side-effects of traditional estrogen replacement therapy include a slight but significant increase in the risk of developing breast and endometrial cancer (*3*, *7*−*10*), women are increasingly using herbal remedies as alternative therapy (*11*−*13*).

Estrogen regulates gene expression by binding to intracellular estrogen receptors (ER), which influence the growth, differentiation, and function of many target tissues. When estrogens bind to an ER, receptor dimerization occurs, which in turn binds to an estrogen-responsive element (ERE) in the DNA of estrogen-sensitive cells (*14*). Consequently, the ER−ERE complex modulates the transcription of estrogen-regulated target genes, such as the progesterone receptor (*PR*) and presenelin-2 (*pS2*), and ultimately stimulates cell growth and differentiation (*15*).

The differences between the two estrogen receptors (ERα and ER*β*) include tissue distribution and ligand specificity (*16*, *17*). In the midgestational human fetus, ERα is most abundant in the uterus, and smaller quantities have been detected in the ovaries, testes, skin, and gut by semiquantitative reverse transcriptase−polymerase chain reaction (RT-PCR). In contrast, high amounts of ER*β* mRNA are present in fetal ovaries, testes, adrenals, and spleen (*18*). Both ERα and ER*β* are coexpressed in the human central nervous system, breast, cardiovascular tissue, and bone (*19*).

Black cohosh [*Cimicifuga racemosa* (CR)], red clover [*Trifolium pratense* L. (TP)], hops [*Humulus lupulus* L.

* Author to whom correspondence should be addressed [fax (312) 966-7107; e-mail judy.bolton@uic.edu].

[†] Department of Medicinal Chemistry and Pharmacognosy and Program for Collaborative Research in the Pharmaceutical Sciences.

[‡] Department of Surgical Oncology.

10.1021/jf0014157 CCC: $20.00
Published on Web 03/24/2001

Table 1. ER Binding, AP Induction, *PR* and *pS2* mRNA Expression, and Cytotoxicity of Methanol Extracts

extract	ERα binding IC_{50}, μg/mL	ERβ binding IC_{50}, μg/mL	AP induction, Ishikawa cells IC_{50}, μg/mL	*PR* expression, Ishikawa cells ratio of intensity[a]	toxicity, Ishikawa cells ED_{50}, μg/mL	*pS2* expression, S-30 cells ratio of intensity[b]	toxicity, S-30 cells ED_{50}, μg/mL
dong quai	NA[c]	NA	NA	0.07	>20	0.25	>20
black cohosh	NA	NA	NA		>20		>20
licorice	NA	NA	NA	0.04	>20	0.28	>20
hops	30 ± 0.4	27 ± 2.8	13.1 ± 6.1	1.29	<2.5	0.65	>20
Asian ginseng	NA	NA	NA		>20	0.22	>20
American ginseng	NA	NA	NA		>20	0.75	>20
red clover	5.6 ± 2.1	2.5 ± 0.6	1.0 ± 0.2	1.05	>20	0.35	>20
chasteberry	46 ± 3	64 ± 4	NA	1.23	>20	0.79	>20

[a] Ratio of intensity/net intensity of *PR* band/net intensity of β-actin band. Extracts were tested at a concentration of 20 μg/mL. [b] Ratio of intensity/net intensity of *pS2* band/net intensity of β-actin band. Extracts were tested at a concentration of 20 μg/mL. [c] NA, not active (IC_{50} > 50 μg/mL for ER binding; IC_{50} > 20 μg/mL for AP induction; no *PR* or *pS2* expression at 20 μg/mL).

(HL)], and chasteberry [*Vitex agnus-castus* L. (VA)] are four of the most frequently used herbs in Western countries for menopausal symptoms or premenstrual syndrome (PMS) (*6*, *20*−*22*). Dong quai [*Angelica sinensis* (Oliv.) Diels (AS)] is a common Chinese herb used for women's health, and licorice (*Glycyrrhiza glabra* L.) has been reported to have both estrogenic and antiestrogenic activities (*23*, *24*). Several case reports implicate Asian ginseng [*Panax ginseng* C.A. Meyer (PG)] as a possible candidate for the treatment of menopause (*25*, *26*). In addition, the stimulation of the estrogen-sensitive gene, *pS2*, by North American ginseng [*Panax quinquefolius* L. (PQ)] has been shown (*27*, *28*).

In the present study using four in vitro assays, we have systematically evaluated the estrogenic properties of eight botanicals listed above. In addition, we have examined the estrogenic activity of four isoflavones known to be present in red clover (*13*, *29*−*31*). These data suggest that the combination of in vitro bioassays for estrogenic activity along with isolation and characterization of the active components by LC-MS is a valid approach for identification of plant extracts beneficial in the treatment of menopausal symptoms.

MATERIALS AND METHODS

Chemicals and Reagents. All chemicals and reagents were from Fisher (Hanover Park, IL) or Sigma (St. Louis, MO)-unless otherwise indicated. All media for cell culture were purchased from Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was from Atlanta Biologicals (Norcross, GA). Genistein, daidzein, biochanin A, and formononetin were purchased from Indofine Chemical Co. (Belle Mead, NJ). [^{3}H]-Estradiol (83 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA), and Cytoscint was purchased from ICN (Costa Mesa, CA). Human recombinant ERα and ERβ were purchased from Panvera (Madison, WI). Primers of *PR*, *pS2*, and β-actin were obtained from Life Technologies.

Plant Material. *A. sinensis* (dong quai, roots) and *T. pratense* (red clover, flowering aerial parts) were cultivated at the University of Illinois Pharmacognosy Field Station (Downer's Grove, IL). *C. racemosa* (black cohosh, rhizomes and roots) was collected in Rookbridge County, VA. *V. agnus-castus* (chasteberry, berries) and *G. glabra* (licorice, roots) were provided by Pharmavite (San Fernando, CA) and PureWorld Botanicals (South Hackensack, NJ), respectively. *H. lupulus* (hops, strobiles) was purchased from Hops Direct (Mabton, WA). *P. ginseng* (Asian ginseng, roots) was obtained from the Institute of Materia Medica, Chinese Academy of Traditional Chinese Medicine (Beijing, China), and *P. quinquefolius* (North American ginseng, roots) was a gift from Chai-Na-Ta Corp. (Langley, BC, Canada). *V. agnus-castus* (chasteberry, fruits) was a gift from PureWorld Botanicals.

Extraction and Fractionation. Plant materials (100 g) were macerated in MeOH (600 mL) overnight. Following filtration, the marcs were extracted twice with MeOH (600 mL), with gentle heating (<45 °C, 10 min). The extracts were combined, and the solvent was removed in vacuo. Extracts of the eight plants were initially tested in bioassays listed in Table 1. The red clover fraction was redissolved in 30% aqueous MeOH (600 mL) and partitioned against petroleum ether (6 × 250 mL); residual MeOH was removed in vacuo from the aqueous portion, and the latter was partitioned against $CHCl_3$ and BuOH successively (6 × 250 mL). Removal of the solvent yielded the petroleum ether, $CHCl_3$, BuOH, and H_2O soluble fractions. The $CHCl_3$ fraction was chromatographed on a silica gel (70−230 mesh) column and developed successively with solvent mixtures of $CHCl_3$/petroleum ether (4:1, 480 mL), EtOAc (300 mL), $CHCl_3$/MeOH (9:1, 190 mL), and MeOH (80 mL). On the basis of their thin-layer chromatography (TLC) patterns, 14 subfractions were obtained from the original crude chloroform extract.

Cell Culture Conditions. Ishikawa and S30 cell lines were provided by Dr. R. B. Hochberg (Yale University, New Haven, CT) and Dr. V. C. Jordan (Northwestern University, Evanston, IL), respectively. Ishikawa cells were maintained in Dulbecco's Modified Eagle medium (DMEM)/F12 media with 10% heat-inactived FBS, sodium pyruvate (1%), penicillin−streptomycin (1%), and glutamax-1 (1%). One day prior to treating the cells, the medium was replaced with phenol red-free, DMEM/F12 medium containing charcoal/dextran-stripped FBS to remove estrogens. S30 cells were maintained in phenol-free minimum essential medium (MEME) supplemented with 1% penicillin−streptomycin, 6 μg/L insulin, 500 mg/L G418 (geneticin disulfate salt), 1% glutamax, and 5% charcoal/dextran-stripped FBS.

ER Competitive Binding Assays. The procedure of Obourn et al. (*32*) was used with minor modifications. Briefly, 24 h prior to the assay, a 50% v/v hydroxyapatite slurry was prepared using 10 g of hydroxyapatite in 60 mL of TE buffer (50 mM Tris-Cl, pH 7.4, 1 mM EDTA) and stored at 4 °C. The ER binding buffer consisted of 10 mM Tris-Cl (pH 7.5), 10% glycerol, 2 mM dithiothrietol, and 1 mg/mL bovine serum albumin. The ERα and ERβ wash buffers contained 40 mM Tris-Cl (pH 7.5), 100 mM KCl, 1 mM EDTA, and 40 mM Tris-Cl (pH 7.5), respectively. The reaction mixture consisted of 5 μL of test sample in DMSO, 5 μL of pure human recombinant diluted ERα or ERβ (0.5 pmol) in ER binding buffer, 5 μL of "Hot Mix" (400 nM, prepared fresh using 3.2 μL of 25 μM, 83 Ci/mmol [^{3}H] estradiol, 98.4 μL of ethanol, and 98.4 μL of ER binding buffer), and 85 μL of ER binding buffer. The incubations were carried out at room temperature for 2 h, then 100 μL of 50% hydroxyapatite slurry were added, and the tubes were incubated on ice for 15 min with vortexing every 5 min. The appropriate ER wash buffer was added (1 mL), and the tubes were vortexed and then centrifuged at 2000*g* for 5 min. The supernatant was discarded, and this wash step was repeated three-times. The hydroxyapatite pellet containing the ligand−receptor complex was resuspended in 200 μL of ethanol and transferred to scintillation vials. Cytoscint (4 mL/vial) was added, and the tubes were counted using a Beckman (Schaumburg, IL) LS 5801 liquid scintillation counter. The percent inhibition of [^{3}H]estradiol binding to each ER was determined as follows: $[(dpm_{sample} - dpm_{blank})/(dpm_{DMSO} - dpm_{blank}) - 1]$

× 100. The binding capability (percent) of the sample was calculated in comparison to that of estradiol (50 nM, 100%). The data represent the average ± SD of three determinations.

Induction of Alkaline Phosphatase (AP) with Cultured Ishikawa Cells. The procedure of Pisha et al. (*33*) was used as described previously. Briefly, Ishikawa cells (5×10^4/well) were incubated overnight with estrogen-free media in 96-well plates. Test samples in DMSO were added, and the cells in a total volume of 200 μL media/well were incubated at 37 °C for 4 days. For the determination of antiestrogenic activity, 2×10^{-8} M estradiol was added to the media. Enzyme activity was measured by reading the liberation of *p*-nitrophenol at 340 nm every 15 s for 16−20 readings with an ELISA reader (Power Wave 200 microplate scanning spectrophotometer, Bio-Tek Instrument, Winooski, VT). The maximum slope of the lines generated by the kinetic readings was calculated using a computer program. The percent induction for determination of estrogenic activity was calculated as $[(\text{slope}_{\text{sample}} - \text{slope}_{\text{cells}})/(\text{slope}_{\text{estrogen}} - \text{slope}_{\text{cells}})] \times 100$. For antiestrogenic activity, the percent induction was determined as $[(\text{slope}_{\text{sample}} - \text{slope}_{\text{cells}})/(\text{slope}_{\text{DMSO}} - \text{slope}_{\text{cells}})] \times 100$. The data represent the average ± SD of triplicate determinations.

Cytotoxicity Assays. Ishikawa (15000 cells/well) and S30 cells (4000 cells/well) were preincubated in 96-well plates overnight in estrogen-free media. The Ishikawa cells were incubated with test samples for 4 days, and S30 cells were incubated for 1 day. As an indication of cell viability, absorbance was measured at 515 nm on a microtiter plate reader after the cells were fixed with 20% trichloroacetic acid (TCA) and stained with 0.4% sulforhodamine B (SRB), and the bound dye was liberated with 0.1 M Tris buffer (*33*, *34*). The data represent the average ± SD of triplicate determinations.

RT-PCR Analysis of *PR* and *pS2* mRNA Expression in Ishikawa and S30 Cell Lines. Ishikawa cells (2×10^5/well) were preincubated overnight in estrogen-free media in a six-well plate. Test samples in DMSO were added and incubated at 37 °C for 4 days. S30 cells (4×10^4/well) were preincubated overnight with estrogen-free media in 24-well plates, and then the test samples were added and incubated at 37 °C for 24 h. Total mRNA from both cell lines was extracted with TRIzol reagent (Gibco, Grand Island, NY) following the manufacturer's protocol, and RT-PCR was carried out using the SuperScript one-step RT-PCR system (Gibco) and a DNA thermal cycler 480 (Perkin-Elmer, Foster City, CA). The primers used for *PR* expression were 5′-CCATGTGGCAGATCCCACAGGAGTT-3′ (sense) and 5′-TGGAAATTCAACACTCAGTGCCCGG-3′ (antisense). The primers used for *pS2* expression were 5′-CATGGAGAACAAGGTGATCTG-3′ (sense) and 5′-CAGAAGCGTG-TCTGAGGTGTC-3′ (antisense). The PCR products (5 μL) of *PR* (271 bp) and *pS2* (365 bp) were separated by electrophoresis in 1% agarose gels and visualized by staining with ethidium bromide. The 621 bp sequence for β-actin was used as an internal control for both *PR* and *pS2*. The sense and antisense primers used for β-actin were 5′-ACACTGTGCCCATCTACGAGG-3′ and 5′-AGGGGCCGGACTCGTCATACT-3′, respectively. The net intensity of the bands was measured using Kodak Digital Science 1D software. The ratio of the intensity of the target gene and the internal control of each sample was calculated as shown in Tables 1 and 2.

Detection of ER Ligands in Red Clover Extracts Using Ultrafiltration and LC-MS. Human recombinant ERβ (50 or 100 pmol) was mixed with the test sample in binding buffer containing 50 mM Tris-Cl (pH 7.5), 10% glycerol, 50 mM KCl, and 1 mM EDTA, in a total volume of 150 μL. After a 2 h incubation at room temperature, the reaction mixture was filtered through a Microcon YM-30 centrifugal filter (Millipore) containing a regenerated cellulose ultrafiltration membrane with a 30000 MW cutoff by centrifugation at 10000 rpm for 7 min at 4 °C. Unbound compounds were removed by washing the filter three times by centrifugation with 150 μL aliquots of ammonium acetate buffer at pH 7.5 at 4 °C. To disrupt the ligand−receptor complex and release the bound ligands, 400 μL of MeOH/H_2O (90:10) was added followed by centrifugation at 10000 rpm for 10 min. The solvent in the ultrafiltrate was removed under vacuum, and the ligands were redissolved in

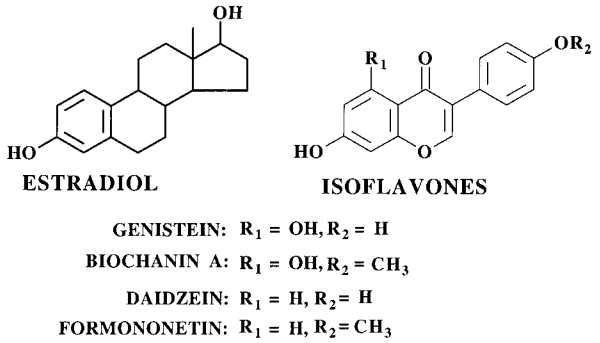

Figure 1. Structures of four major isoflavones in red clover and estradiol.

60 μL of H_2O/MeOH (80:20). Aliquots (10 μL) of this solution were analyzed by using LC-MS, which consisted of a Waters 2690 HPLC system (Waters, Milford, MA) coupled to a Micromass Quattro II electrospray triple quadrupole mass spectrometer (Micromass, Manchester, U.K.). HPLC separations were carried out using a Micra (Northbrook, IL) C_{18} HPLC column, 4.6 × 21 mm, containing 1.5 μm nonporous silica. The mobile phase consisted of H_2O/MeOH (95:5, v/v) containing 0.01% acetic acid (A) and MeOH containing 0.01% acetic acid (v/v) (B), using linear gradients of 5−98% B (v/v) over 20 min. The electrospray source was operated at 155 °C in negative ion mode. Nitrogen was used as both nebulizing and drying gas at flow rates of 20 and 450 L/h, respectively. A control was used to correct for nonspecific binding of the sample, in which ERβ was absent from the incubation solution.

RESULTS

Relative Affinity of Plant Extracts and Isoflavones for ERα and ERβ. Among the eight methanol extracts tested, red clover, hops, and chasteberry showed significant binding affinities with both ERα and ERβ on the basis of their 50% inhibitory (IC_{50}) values (Table 1). The order of binding potency was red clover ≫ hops > chasteberry, and their affinities for ERα and ERβ were not significantly different. Dong quai and licorice showed weak binding affinity (IC_{50} >50 μg/mL), whereas Asian ginseng, North American ginseng, and black cohosh displayed no binding (<20% at a concentration of 200 μg/mL). Standards of isoflavones, known to be present in red clover (see structures in Figure 1), were tested with both ER receptor subtypes and exhibited competitive binding potency following the order genistein > daidzein > biochanin A > formononetin, based on their IC_{50} values (Table 2). These four isoflavones displayed higher affinity with ERβ compared to ERα, consistent with previous studies (*35*).

Detection of ER Ligands in Red Clover by a Combination of Ultrafiltration and LC-MS. Since red clover showed the highest ER binding affinity among the crude extracts tested, it was fractionated with different solvents to help identify the active compound(s). As shown in Table 3, the chloroform fraction of red clover displayed the greatest potency compared to other fractions. As a result, the chloroform extract was analyzed using affinity ultrafiltration LC-MS, which is a variation of pulsed ultrafiltation LC-MS (*36*) developed by Wieboldt et al. (*37*). Unlike other ultrafiltration LC-MS applications, this affinity method was applied in the present study for the rapid screening of botanical extracts for ligands to ERβ. Using ultrafiltration and LC-MS, daidzein (9.9 min retention time), genistein (11.3 min), and biochanin A (14.9 min) were identified as ERβ ligands in the chloroform extracts of

Table 2. ER Binding, AP Induction, *PR* and *pS2* mRNA Expression, and Cytotoxicity of Phytoestrogens in Red Clover

compound	ERα binding IC_{50}, μM	ERβ binding IC_{50}, μM	AP induction Ishikawa cells IC_{50}, μM	*PR* expression Ishikawa cells, ratio of intensity[a]	toxicity Ishikawa cells ED_{50} μM	*pS2* expression S-30 cells, ratio of intensity[b]
genistein	0.3 ± 0.01	0.018 ± 0.002	0.51 ± 0.1	0.88	>5[c]	0.70
daidzein	17 ± 2.5	1.2 ± 0.0	1.2 ± 0.6	1.15	>5[c]	0.62
biochanin A	35 ± 1.4	4.1 ± 0.8	5.1 ± 0.4	1.36	47 ± 6.0	0.21
formononetin	104 ± 8.2	60 ± 7.1	12 ± 3.0	0.97	>100	0.16
estradiol	0.0065 ± 0.00058	0.0024 ± 0.00014	0.00014 ± 0.000014	1.40	>0.005	0.93

[a] Ratio of intensity/net intensity of *PR* band/net intensity of β-actin band. Compounds were tested at a concentration of 5 nM. [b] Ratio of intensity/net intensity of *pS2* band/net intensity of β-actin band. Compounds were tested at a concentration of 0.1 μM. [c] Milli.

Table 3. ER Binding, AP Induction, and Cytotoxicity of Red Clover Fractions and Subfractions

fraction/subfraction	ERα binding[a]	ERβ binding[a]	AP induction[b] Ishikawa cells	toxicity[c] Ishikawa cells	bound ligands detected by LC-MS
MeOH	78	72	30	70[d]	genistein, daidzein, biochanin A
PE	61	77	<20	<80	ND[e]
$CHCl_3$	83	93	33	50[f]	genistein, daidzein, biochanin A
BuOH	28	34	77	<80	ND
H_2O	7	0	<20	<80	ND
$CHCl_3$ fraction 2	92	90	toxic	7	biochanin A
$CHCl_3$ fraction 6	82	97	44	59	genistein
$CHCl_3$ fraction 7	90	98	68	<80	genistein
$CHCl_3$ fraction 8	57	93	76	<80	genistein, daidzein

[a] Percent inhibition at 200 μg/mL. [b] Percent induction at 20 μg/mL. [c] Percent cell survival at 20 μg/mL. [d] $IC_{50} = 1 \pm 0.2$ μg/mL [e] ND, none detected. [f] $IC_{50} = 2.6 \pm 0.1$ μg/mL.

red clover on the basis of molecular weight, tandem mass spectra, and HPLC retention time in comparison with authentic standard compounds. The affinity of genistein for ERβ was confirmed by the large enhancement of the LC-MS peak following affinity ultrafiltration (solid line in Figure 2A) compared with that of a control sample which did not contain ERβ (dashed line, Figure 2A). Of the 14 subfractions of the most potent chloroform extract of red clover separated by column chromatography, the four fractions showing the highest competitive binding ability to ERβ were subjected to the ultrafiltration and LC-MS ERβ binding assay. Genistein was detected as the most ERβ-active component in subfractions 6−8 (Table 3; Figure 2B). In addition to genistein, daidzein was detected in fraction 8. Biochanin A was the ERβ ligand detected in chloroform subfraction 2 (Table 3); however, this fraction (Table 3) and the pure isoflavone (Table 2) were found to be cytotoxic with Ishikawa cells.

AP Induction in Ishikawa Cells. Ishikawa is an ER positive endomentrial adenocarcinoma cell line derived from a glandular epithelial cell line. This cell responds to estrogens and antiestrogens at concentrations approximating physiological levels (*38*). Induction of AP activity in Ishikawa cells indicates an estrogenic response, whereas inhibition represents an antiestrogenic effect (*33*). This cell line was used to investigate the estrogenic or antiestrogenic effects of the test samples, and *PR* expression was carried out to confirm the results of AP induction. These two assays give consistent results in comparison with ER binding data in terms of the estrogenic activity of the test samples. In Ishikawa cells, the red clover extract showed the strongest AP induction ability with an IC_{50} value of 1.0 μg/mL (Table 1). Chloroform subfractions 6−8, which showed the strongest ER binding affinity, also displayed high AP induction in the Ishikawa cells, whereas subfraction 2 appeared to be cytotoxic with these cells (Table 3). Although the hops extract exhibited strong cytotoxicity (Table 1), its estrogenic activity was still detected with an IC_{50} value of 13.1 μg/mL. Chasteberry displayed weak estrogenic activity (40%) at a concentration of 20 μg/mL, whereas the other plant extracts were not active (Table 1). Genistein, daidzein, biochanin A, and formononetin, which are all present in red clover, exhibited AP induction activity with potency that correlated with the ER binding assay, based on IC_{50} values (Table 2). None of the extracts or isoflavone standards exhibited antiestrogenic activity (data not shown).

Stimulation of *PR* mRNA Expression in Ishikawa Cells. Estradiol-mediated *PR* expression was not observed in S30 cells, so experiments were conducted using the Ishikawa cell line. *PR* expression, as measured by RT-PCR, was significantly up-regulated by red clover, hops, and chasteberry extracts at concentrations of 20 μg/mL (Table 1; Figure 3A). Dong quai and licorice exhibited weak stimulation of *PR* expression at this concentration; however, extracts of black cohosh and the two ginseng species did not show activity. The four isoflavones induced *PR* expression at concentrations of 5 nM (Table 2; Figure 3B). These results are consistent with the ER binding data.

The chloroform subfractions of red clover demonstrated strong *PR* induction in comparison to the petroleum ether, butanol, and H_2O fractions at a concentration of 20 μg/mL (data not shown). Chloroform subfractions 6−8, which displayed high ER binding, also displayed significant *PR* up-regulatory activity (data not shown). Subfraction 2 was not tested in this assay due to its toxicity with Ishikawa cells.

Stimulation of *pS2* mRNA Expression in S30 Cells. The stimulation of *pS2* expression in the estrogen receptor-positive breast cancer cell line MCF-7 has been reported previously (*39*−*41*). However, the expression of the *pS2* mRNA in MCF-7 was constitutive under our experimental conditions, despite a change to estrogen-free media for 4 days (data not shown). In contrast, Ishikawa cells did not show *pS2* expression in incubations with estradiol (data not shown). S30 is a subclone of the ER-negative MDA-MB-231 breast cancer cell line that is stably transfected with ERα. We utilized this cell line for *pS2* expression because it was responsive to estradiol; results were consistent with the other

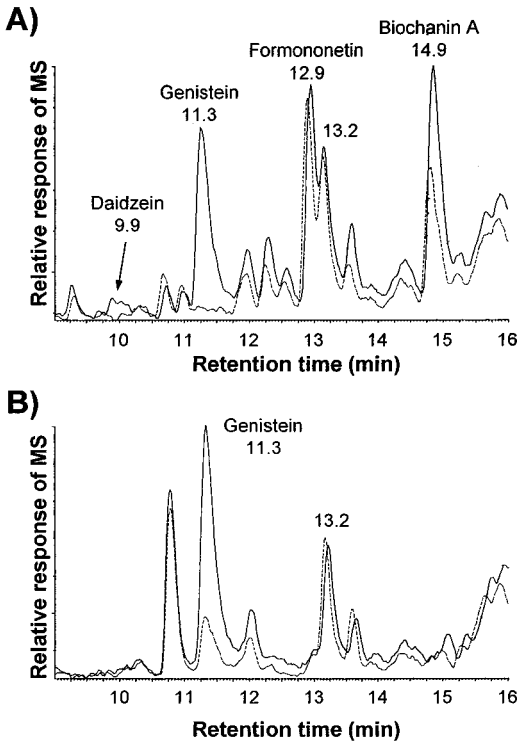

Figure 2. Overlaid total ion chromatograms showing affinity ultrafiltration and LC-MS screening results of (A) red clover chloroform extract (10 μg/mL) and (B) one of its bioactive subfractions (fraction 7, 20 μg/mL). The solid line (—) represents the experiment with ERβ (0.667 μM), and the dashed line (- - -) indicates the control experiment without the receptor. By a combination of ultrafiltration and LC-MS, the enhanced peaks of genistein (11.3 min), daidzein (9.9 min), and biochanin A (14.9 min) were identified and confirmed as active ligands in the chloroform extract. Genistein was the active ligand in subfraction 7. See Table 3 for active ligands identified in other bioactive subfractions of the red clover chloroform fraction. The peak at 13.2 min is due to an impurity in the water mobile phase collected during the equilibration of the LC column. It serves as an internal standard for normalizing the experiment with the control, because the equilibration time of the LC column was the same in the LC sequence before sample injection for both samples.

assays with the exception of data obtained with ginseng. In S30 cells, all extracts except that of black cohosh induced *pS2* expression (Figure 4A) at a concentration of 20 μg/mL. Interestingly, Asian ginseng and North American ginseng did not show activity in the three assays described above.

Similar to its *PR* induction in Ishikawa cells, the chloroform fraction of red clover showed stronger *pS2* expression than the petroleum ether, BuOH, and H_2O extracts at 20 μg/mL (data not shown). The purified isoflavones from red clover also induced *pS2* expression. Expression induced by genistein and daidzein was significantly stronger than that of biochanin A and formononetin at a concentration of 0.1 μM (Figure 4B).

DISCUSSION

Zava et al. (*42*) previously reported the estrogenic and progestin bioactivities of over 150 herbs including the 8 plants studied in this investigation. In their radioreceptor assay, red clover, licorice, and hops extracts were reported to bind to the ER of MCF-7 cells. Red clover also bound to the *PR* of the T47D cell line. Using cell proliferation as an indicator of possible estrogenic

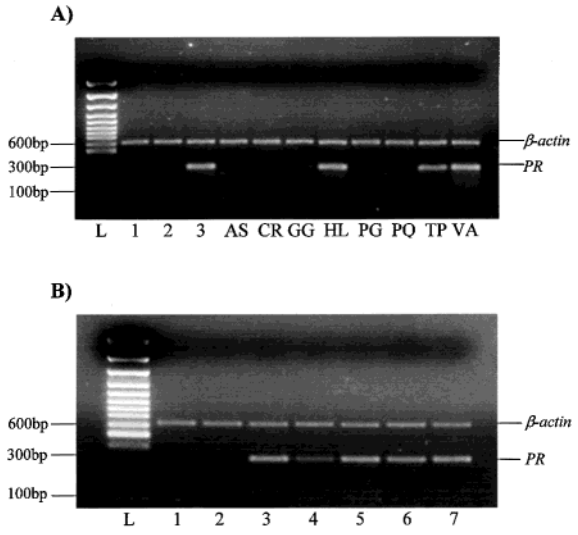

Figure 3. Induction of *PR* mRNA expression in Ishikawa cells: (A) methanol extracts (20 μg/mL) [1, control; 2, DMSO; 3, estradiol; AS, *A. sinensis* (dong quai); CR, *C. racemosa* (black cohosh); GG, *G. glabra* (licorice); HL, *H. lupulus* (hops); PG, *P. ginseng* (Asian ginseng); PQ, *P. quinquefolius* (North American ginseng); TP, *T. pratense* (red clover); VA, *V. agnus-castus* (chasteberry)]; (B) phytoestrogens (5 nM) (1, control; 2, DMSO; 3, estradiol; 4, genistein; 5, daidzein; 6, biochanin A; 7, formononetin).

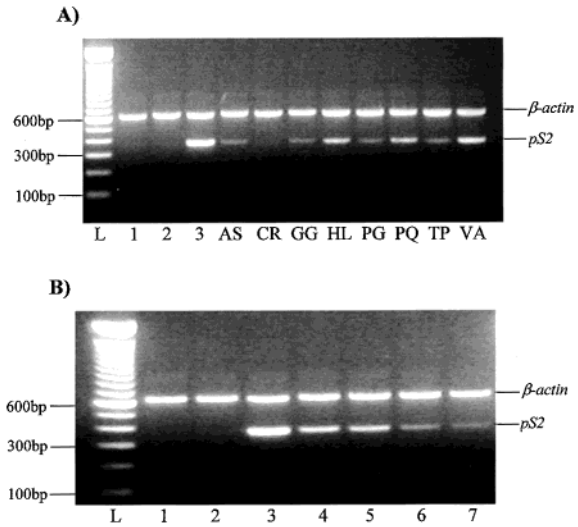

Figure 4. Induction of *pS2* mRNA expression in S-30 cells: (A) methanol extracts (20 μg/mL) [1, control; 2, DMSO; 3, estradiol; AS, *A. sinensis* (dong quai); CR, *C. racemosa* (black cohosh); GG, *G. glabra* (licorice); HL, *H. lupulus* (hops); PG, *P. ginseng* (Asian ginseng); PQ, *P. quinquefolius* (North American ginseng); TP, *T. pratense* (red clover); VA, *V. agnus-castus* (chasteberry)]; (B) phytoestrogens (0.1 μM) (1, control; 2, DMSO; 3, estradiol; 4, genistein; 5, daidzein; 6, biochanin A; 7, formononetin).

activity, red clover, hops, and licorice extracts demonstrated growth that was significantly higher than that of controls. Our results are consistent with these conclusions based on the estrogenic activities of red clover and hops. However, in our tests, the licorice extract displayed only weak binding affinity to ER, weak stimulation of *PR* expression in Ishikawa cells, weak *pS2* expression in the S30 cell line, and a lack of AP induction in Ishikawa cells. Although different assays and cell lines were used, further investigation of licorice appears to be necessary to evaluate its potential estrogenicity.

Genistein, daidzein, biochanin A, and formononetin have been implicated as causative for the estrogenic activity of red clover (*13*, *29*−*31*). Although many flavonoids in red clover have been identified by using LC-MS (*43*, *44*), whether substances other than isoflavones contribute to its estrogenicity remains unclear. The utilization of mass spectrometric characterization combined with affinity enrichment of receptor ligands from compound libraries or metabolite mixtures by ultrafiltration assays has been reported previously (*36*, *37*, *45*−*47*). In this study, we applied this technique in screening botanical extracts and successfully demonstrated that genistein likely plays the most important role in terms of the estrogenic activity of red clover followed by daidzein and biochanin A. Although abundant in red clover, formononetin had insufficient affinity for ERβ to be detected in our affinity ultrafiltration LC-MS assay.

The North American Menopause Society (NAMS) recently published the results of a study concerned with the therapeutic role of isoflavones in menopausal women (*48*). As noted by NAMS, it is not clear whether the observed health effects in humans are attributable to isoflavones alone or to isoflavones plus other components in whole foods. Whereas a reduction in low-density lipoproteins and triglycerides and an increase in high-density lipoproteins was associated with isoflavone ingestion, no differences in the incidence and severity of hot flashes were observed between the isoflavone recipients and the controls. Inadequate data exist to establish the potential of isoflavones to modulate breast and other hormone-dependent cancers, bone mass, and vaginal dryness. Therefore, further work is necessary to characterize the in vivo estrogenic activity of isoflavones.

As currently reported, extracts of black cohosh displayed no estrogenic activity in the assays presented here, which is consistent with previous results (*21*, *49*). Clinical trials with black cohosh have demonstrated a significant reduction in serum lutenizing hormone (LH) levels with women demonstrating climacteric symptoms; however, the extract had no effect on follicle stimulating hormone (FSH) (*50*). These data indicate that black cohosh may alleviate menopausal symptoms by actions discrete from estrogen receptor regulation.

Chasteberry extract exhibited significant ER binding and induced *PR* and *pS2* mRNA expression, but no AP induction activity was noted in Ishikawa cells. In addition to the different sensitivities of the assays and various targets detected in these assays, these results may be due to the use of cell lines derived from different tissues. Although hops extract was strongly cytotoxic with Ishikawa cells, the estrogenic activity was still detectable in the AP induction and *PR* expression assays.

Extracts of Asian ginseng and North American ginseng mediated *pS2* expression in S30 cells, but no ER binding, AP induction, or *PR* expression in Ishikawa cells. Stimulation of *pS2* expression by American ginseng has been reported in MCF-7 cells, and this was presumed to be partially mediated through the ER (*27*, *28*). However, our data with S30 cells suggest that the stimulation of *pS2* expression by ginseng might not occur through ER modulation. It is possible that the constituents of ginseng modulate one or more elements involved in ER function rather than directly through the ER. In a similar fashion, 3,3′-diindolylmethane (DIM), a metabolite of indole-3-carbinol (I3C), was shown to increase *pS2* gene transcription in MCF-7 cells without binding to the ER (*51*). This suggests a promoter-specific, ligand-independent activation of ER signaling is a possible mechanism for natural modulation of ER function. Several papers have suggested that the specific down-regulation of *pS2* expression is an early event in sporadic late-onset Alzheimer's disease (*52*) and may be involved in the pathology of some cases of Alzheimer's (*53*). Therefore, extracts that can stimulate *pS2* expression, such as ginseng, might benefit patients suffering from Alzheimer's disease.

Further studies are needed to fully understand the mechanisms of dong quai, licorice, hops, chasteberry, ginseng, and black cohosh. Alternative mechanisms might involve receptors specific to other hormones or neurotransmitters, such as luteinizing hormone release hormone (LHRH), luteinizing hormone (LH), follicle stimulating hormone (FSH), serotonin, and γ-aminobutyrate (GABA).

CONCLUSIONS

Of the eight plants tested, red clover extracts showed the most consistent estrogenic effects in four different in vitro assays. Hops extracts also displayed consistent estrogenic potency, but it was found to be cytotoxic with Ishikawa cells. Combined utilization of ultrafiltration and LC-MS confirmed genistein was the most active ERβ ligand in red clover, and this compound might be responsible for AP induction, as well as *PR* and *pS2* expression. Future studies utilizing affinity ultrafiltration LC-MS for ER binding as well as the four in vitro bioassays will identify the estrogenic compounds in other botanicals used for women's health.

ABBREVIATIONS USED

AP, alkaline phosphatase; DMEM, Dulbecco's Modified Eagle Medium; E2, estradiol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether) tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; ERE, estrogen-responsive element; FBS, fetal bovine serum; LC-MS, liquid chromatography−mass spectrometry; MEME, minimum essential medium; PE, petroleum ether; PMS, premenstrual syndrome; *PR*, progesterone receptor; *pS2*, presenelin-2; RT-PCR, reverse transcriptase−polymerase chain reaction; SRB, sulforhodamine B; TCA, trichloroacetic acid; TLC, thin layer chromatography.

ACKNOWLEDGMENT

We thank Dr. R. B. Hochberg of Yale University for the Ishikawa cell line and Dr. V. C. Jordan of Northwestern University for the S30 cell line. We are grateful to S. Schlecht, L. Chadwick, and D. Fabricant for the extraction of the plants and to Steven Totura for cultivation of red clover. Finally, we thank Mrs. Bambi Teague at Blue Ridge Parkway National Park for National Park Service Permits BLRI-00-030, BLRI-99-028, Keith Langdon at Great Smoky Mountains National Park for National Park Service Permits GRSM-00-096, GRSM-99-097, and Dr. Gwynn Ramsey and Aubrey Neas for collection of plant material.

LITERATURE CITED

(1) Brosage, P. Hormone therapy: The woman's decision. *Contemp. Nurse Pract.* **1995**, *1* (S), 3.

(2) Harris, R. B.; Laws, A.; Reddy, F. M.; King, A.; Haskell, W. L. Are women using postmenopausal estrogens? A community survey. *Am. J. Public Health* **1990**, *80*, 1266−1268.

(3) Colditz, G. A.; Hankinson, S. E.; Hunter, D. J.; Willett, W. C.; Manson, J. E.; Stampfer, M. J.; Hennekens, C.; Rosner, B.; Speizer, F. E. The use of estrogens and progestins and the risk of breast cancer in postmenopausal women. *New Engl. J. Med.* **1995**, *332*, 1589−1593.

(4) Grodstein, F.; Stamfer, M. J.; Colditz, G. A.; Willett, W. C.; Manson, J. E.; Joffe, M.; Rosner, B.; Fuchs, C.; Hankinson, S. E.; Hunter, D. J.; Hennekens, C. H.; Speizer, F. E. Postmenopausal hormone therapy and mortality. *New Engl. J. Med.* **1997**, *336*, 1769−1775.

(5) Wickelgren, I. Estrogen: A new weapon against Alzheimer's. *Science* **1997**, *276*, 676−677.

(6) Kurzer, M. S.; Xu, X. Dietary phytoestrogens. *Annu. Rev. Nutr.* **1997**, *17*, 353−381.

(7) Henderson, B. E.; Ross, R.; Bernstein, L. Estrogen as a cause of human cancer. The Richard and Hinda Rosenthal Foundation Award Lecture. *Cancer Res.* **1988**, *48*, 246−253.

(8) Liehr, J. G. Genotoxic effects of estrogens. *Mutat. Res.* **1990**, *238*, 269−276.

(9) Stampfer, M. J.; Willett, W. C.; Hunter, D. J.; Manson, J. E. Type of postmenopausal hormone use and risk of breast cancer: 12-year follow-up from the Nurses' Health Study. *Cancer Causes Control* **1992**, *3*, 33−39.

(10) Bolton, J. L.; Pisha, E.; Zhang, F.; Qiu, S. Role of quinoids in estrogen carcinogenesis. *Chem. Res. Toxicol.* **1998**, *11*, 1113−1127.

(11) Setchell, K. D. R. Phytoestrogens: The biochemistry, physiology, and implications for human health of soy isoflavones. *Am. J. Clin. Nutr.* **1998**, *68*, 1333S−1346S.

(12) Setchell, K. D. R.; Cassidy, A. Dietary isoflavones: Biological effects and relevance to human health. *J. Nutr.* **1999**, *129*, 758S−767S.

(13) Murkies, A. L.; Wilcox, G.; Davis, S. R. Clinical review 92: Phytoestrogens. *J. Clin. Endocrinol. Metab.* **1998**, *83*, 297−303.

(14) Gaido, K. W.; Maness, S. C.; Waters, K. M. Exploring the biology and toxicology of estrogen receptor β. *AIIT Activities* **1999**, *19*, 1−5.

(15) Saegusa, M.; Okayasu, I. Change in expression of estrogen receptor α and β in relation to progesterone receptor and *pS2* status in normal and malignant endometrium. *Jpn. J. Cancer Res.* **2000**, *91*, 510−518.

(16) Kuiper, G. G.; Enmark, E.; Pelto-Huikko M.; Nilsson, S.; Gustafsson, J.-A. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 5925−5930.

(17) Mosselman, S.; Polman, J.; Dijkema, R. ERβ: Identification and characterization of a novel human estrogen receptor. *FEBS Lett.* **1996**, *392*, 49−53.

(18) Brandenberger, A. W.; Tee, M. K.; Lee, J. Y.; Chao, V.; Jaffe, R. B. Tissue distribution of estrogen receptors α (ER-α) and β (ER-β) mRNA in the midgestational human fetus. *J. Clin. Endocrinol. Metab.* **1997**, *82*, 3509−3512.

(19) Gustafsson, J.-A. Estrogen receptor β—a new dimension in estrogen mechanism of action. *J. Endocrinol.* **1999**, *163*, 379−383.

(20) Stolze, H. The other way to treat symptoms of menopause. *Gyne* **1982**, *1*, 14.

(21) Lieberman, S. A review of the effectiveness of *Cimicifuga racemosa* (black cohosh) for the symptoms of menopause. *J. Womens Health* **1998**, *7*, 525−529.

(22) Tham, D. M.; Gardner, C. D.; Haskell, W. L. Clinical review 97: Potential health benefits of dietary phytoestrogens: a review of the clinical, epidemiological, and mechanistic evidence. *J. Clin. Endocrinol. Metab.* **1998**, *83*, 2223−2235.

(23) Kumagi, A.; Nishino, K.; Shimomura, A. Effect of glycyrrhizin on estrogen action. *Endocrinol. Jpn.* **1967**, *14*, 34−38.

(24) Sakamoto, K.; Murabe, K.; Watanabe, M. Inhibitory effect of glycyrrhetinic acid on testosterone production. *Jpn. J. Pharmacol.* **1985**, *39* (S), Abstr. 02E1600.

(25) Punnonen, R.; Lukola, A. Oestrogen-like effect of ginseng. *Br. Med. J.* **1980**, *281*, 1110−1118.

(26) Palmer, B. V.; Montgomery, A. C. V.; Monteiro, J. C. M. P. Ginseng and mastalgia. *Br. Med. J.* **1978**, *279*, 1284−1289.

(27) Duda, R. B.; Taback, B.; Kessel, B.; Dooley, D. D.; Yang, H.; Marchiori, J.; Slomovic, B. M.; Alvarez, J. G. *pS2* expression induced by American ginseng in MCF-7 breast cancer cells. *Ann. Surg. Oncol.* **1996**, *3*, 515−520.

(28) Duda, R. B.; Zhong, Y.; Navas, V.; Li, M. Z.; Toy, B. R.; Alavarez, J. G. American ginseng and breast cancer therapeutic agents synergistically inhibited MCF-7 breast cancer cell growth. *J. Surg. Oncol.* **1999**, *72*, 230−239.

(29) Collins, B. M.; McLachlan, J. A.; Arnold, S. F. The estrogenic and antiestrogenic activities of phytochemicals with the human estrogen receptor expressed in yeast. *Steroids* **1997**, *62*, 365−372.

(30) Saloniemi, H. S.; Wahala, K.; Nykanen-Kurli, P.; Kallela, K.; Saastamoinen, I. Phytoestrogen content and estrogenic effect of legume fodder. *Proc. Soc. Exp. Biol. Med.* **1995**, *208*, 13−17.

(31) Kallela, K.; Saastamoinen, I.; Huokuna, E. Variations in the content of plant estrogens in red clover-timothy-grass during the growing season. *Acta Vet. Scand.* **1987**, *28*, 255−262.

(32) Obourn, J. D.; Koszewski, N. J.; Nortides, A. C. Hormone-binding and DNA-binding mechanisms of the recombinant human estrogen-receptor. *Biochemistry* **1993**, *32*, 6229−6236.

(33) Pisha, E.; Pezzuto, J. M. Cell-based assay for the determination of estrogenic and anti-estrogenic activities. *Methods Cell Sci.* **1997**, *19*, 37−43.

(34) Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. New colormetric cytotoxicity assay for anti-cancer-drug screening. *J. Natl. Cancer Inst.* **1990**, *82*,1107−1112.

(35) Kuiper, G. G.; Lemmen, J. G.; Carlsson, B.; Corton, J. C.; Safe, S. H.; ver der Saag, P. T.; van der Burg, B.; Gustafsson, J. A. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinology* **1998**, *139*, 4252−4263.

(36) van Breemen, R. B.; Huang, C. R.; Nikolic, D.; Woodbury, C. P.; Zhao, Y. Z.; Venton, D. L. Pulsed ultrafiltration mass spectrometry: A new method for screening combinatorial libraries. *Anal. Chem.* **1997**, *69*, 2159−2167.

(37) Wieboldt, R.; Zeigenbaum, J.; Henion, J. Immunoaffinity ultrafiltration with ion spray HPLC/MS for screening small-molecule libraries. *Anal. Chem.* **1997**, *69*, 1683−1691.

(38) Holinka, C. F.; Hata, H.; Kuramoto, H.; Gurpide, E. Effects of steroid hormones and antisteroids on alkaline phosphatase activity in human endometrial cancer cells (Ishikawa). *Cancer Res.* **1986**, *46*, 2771−1774.

(39) Hirota, M.; Furukawa, Y.; Hayashi, K. Expression of *pS2* gene in human breast cancer cell line MCF-7 is controlled by retinoic acid. *Biochem. Int.* **1992**, *26*, 1073−1078.

(40) Sathyamoorthy, N.; Wang, T. T.; Phang, J. M. Stimulation of *pS2* expression by diet-derived compounds. *Cancer Res.* **1994**, *54*, 957−961.

(41) Knowlden, J. M.; Gee, J. M.; Bryant, S.; McClelland, R. A.; Manning, D. L.; Mansel, R.; Ellis, I. O.; Blanmey, R. W.; Roberston, J. F.; Nicholson, R. I. Use of reverse transcription-polymerase chain reaction methodology to detect estrogen-regulated gene expression in breast cancer specimens. *Clin. Cancer Res.* **1997**, *3*, 2165−2172.
(42) Zava, D. T.; Dollbaum, C. M.; Blen, M. Estrogen and progestin bioactivity of foods, herbs, and spices. *Proc. Soc. Exp. Biol. Med.* **1998**, *217*, 369−378.
(43) He, X. G.; Lin, L. Z.; Lian, L. Z. Analysis of flavonoids from red clover by liquid chromatography-electrospray mass spectrometry. *J. Chromatogr.* **1996**, *755*, 127−132.
(44) Lin, L. Z.; He, X. G.; Lindenmaier, M.; Yang, J.; Cleary, M.; Qiu, S. X.; Cordell, G. A. LC-ESI-MS study of the flavonoids glycoside melonates of red clover (*Trifolium pretense*). *J. Med. Chem.* **2000**, *43*, 354−365.
(45) Lim, H. K.; Stellingweif, S.; Sisenwine, S.; Chan, K. W. Rapid drug metabolite profiling using fast liquid chromatography, automated multiple-stage mass spectrometry and receptor-binding. *J. Chromatogr.* **1999**, *831*, 227−241.
(46) Nikolic, D.; Fan, P. W.; Bolton, J. L.; van Breemen, R. B. Screening for xenobiotic electrophilic metabolites using pulsed ultrafiltration-mass spectrometry. *Comb. Chem. High Throughput Screening* **1999**, *2*, 165−175.
(47) Nikolic, D.; Habibi-Goudarzi, S.; Corley, D. G.; Gafner, S.; Pezzuto, J. M.; van Breemen, R. B. Evaluation of cyclooxygenase-2 inhibitors using pulsed ultrafiltration mass spectrometry. *Anal. Chem.* **2000**, *72*, 3853−3859.
(48) The North American Menopause Society. The role of isoflavones in menopausal health: Consensus opinion of the North American Menopause Society. *Menopause* **2000**, *7*, 215−229.
(49) Pepping, J. Black cohosh: *Cimicifuga racemosa*. *Am. J. Health Syst. Pharm.* **1999**, *56*, 1400−1402.
(50) Ducker, E. M.; Kopanski, H.; Jarry, H.; Wuttke, W. G. Effects of extracts from *Cimicifuga racemosa* on gonadotropin release in menopausal women and ovariectomized rats. *Planta Med.* **1991**, *57*, 420−424.
(51) Riby, J. E.; Chang, G. H.; Firestone, G. L.; Bjeldances, L. F. Ligand-independent activation of estrogen receptor function by 3,3′-diindolymethane in human breast cancer cells. *Biochem. Pharmacol.* **2000**, *60*, 167−177.
(52) McMillan, P. J.; Leverenz, J. B.; Dorsa, D. M. Specific downregulation of presenilin 2 gene expression is prominent during early stages of sporadic late-onset Alzheimer's disease. *Mol. Brain Res.* **2000**, *78*, 138−145.
(53) Huynh, D. P.; Vinters, H. V.; Ho, D. H. D.; Ho, V. V.; Pulst, S. M. Neuronal expression and intracellular localization of presenilins in normal and Alzheimer disease brains. *J. Neuropathol. Exp. Neurol.* **1997**, *56*, 1009−1017.

Received for review November 28, 2000. Revised manuscript received February 12, 2001. Accepted February 12, 2001. This research was supported by NIH Grant P50 AT00155, the Office of Dietary Supplements (ODS), the National Institute of General Medicine (NIGMS), the Office for Research on Women's Health (ORWH), and the National Center for Complementary and Alternative Medicine (NCCAM).

JF0014157