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

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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 (ERβ). 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 genestin was the most active component of red clover. Consistent with this observation, genestin 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 ERβ) 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 ERβ mRNA are present in fetal ovaries, testes, adrenals, and spleen (18). Both ERα and ERβ 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.]

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

<table>
<thead>
<tr>
<th>Extract</th>
<th>ERα binding IC50, µg/mL</th>
<th>15β binding IC50, µg/mL</th>
<th>AP induction, Ishikawa cells IC50, µg/mL</th>
<th>PR expression, Ishikawa cells ratio of intensity</th>
<th>Toxicity, Ishikawa cells ED50, µg/mL</th>
<th>pS2 expression, S-30 cells ratio of intensity</th>
<th>Toxicity, S-30 cells ED50, µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dong quai</td>
<td>NA</td>
<td>NA</td>
<td>0.07</td>
<td>&gt;20</td>
<td>0.25</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Black cohosh</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Licorice</td>
<td>NA</td>
<td>NA</td>
<td>0.04</td>
<td>&gt;20</td>
<td>0.28</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Hops</td>
<td>30 ± 0.4</td>
<td>27 ± 2.8</td>
<td>13.1 ± 6.1</td>
<td>1.29</td>
<td>&lt;2.5</td>
<td>0.65</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Asian ginseng</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>&gt;20</td>
<td>0.22</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>American ginseng</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>&gt;20</td>
<td>0.75</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Red clover</td>
<td>5.6 ± 2.1</td>
<td>5 ± 0.6</td>
<td>1.0 ± 0.2</td>
<td>1.05</td>
<td>&gt;20</td>
<td>0.35</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Chasteberry</td>
<td>46 ± 3</td>
<td>64 ± 4</td>
<td>NA</td>
<td>1.23</td>
<td>&gt;20</td>
<td>0.79</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>

* Ratio of intensity/net intensity of PR band/net intensity of β-actin band. Extractions were tested at a concentration of 20 µg/mL. 
* Ratio of intensity/net intensity of pS2 band/net intensity of β-actin band. Extractions were tested at a concentration of 20 µg/mL. 
* NA, not active (IC50 > 50 µg/mL for ER binding; IC50 > 20 µg/mL for AP induction; no PR or pS2 expression at 20 µg/mL).

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). Estradiol (83 C1/mmol) was obtained from NEN Life Science Products (Boston, MA), and Cytosprint was purchased from ICM (Costa Mesa, CA). Human recombinant ERα and ERβ were purchased from Panvera (Madison, WI). Primes 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. glagos-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 (Malton, WA). P. ginseng (Asian ginseng, roots) was obtained from the Institute of Materia Medica, Chinese Academy of Traditional Chinese Medicine (Beijing, China), and K. quenchea (North American ginseng, roots) was a gift from Chai-Na-Ta Corp. (Langley, BC, Canada). V. glagos-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 marc 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 CHCl3 and BuOH successively (6 × 250 mL). Removal of the solvent yielded the petroleum ether, CHCl3, BuOH, and H2O soluble fractions. The CHCl3 fraction was chromatographed on a silica gel (70–230 mesh) column and developed successively with solvent mixtures of CHCl3/petroleum ether (4:1, 480 mL), EtOAc (300 mL), CHCl3/MeOH (9:1, 190 mL), and MeOH (80 mL). On the basis of their thin-layer chromatography (TLC) pattern, 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. Jordon (Northwestern University, Evanston, IL), respectively. Ishikawa cells were maintained in Dulbecco's Modified Eagle medium (DMEM)/F12 media with 10% heat-inactivated FBS, sodium pyruvate (1%), penicillin-streptomycin (1%), and gentamicin (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/mL 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 dithiothreitol, 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 C1/mmol [3H] 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 2000g 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. Cytosprint (4 mL/A) was added, and the tubes were counted using a Beckman (Schaumburg, IL) LS 5801 liquid scintillation counter. The percent inhibition of [3H]estradiol binding to each ER was determined as follows: [(dpm sample – dpm blank)/(dpm blank – 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 GraphPad Prism software. The percent induction for the determination of estrogenic activity was calculated as [slope_{sample} − slope_{estrogen} − slope_{cells}] × 100. For antiestrogenic activity, the percent induction was determined as [(slope_{sample} − slope_{DMSO} − slope_{estrogen})] × 100. The data represent the average ± SD of triplicate determinations.

Cytotoxicity Assays. Ishikawa (15,000 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 × 10^4/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 plate, 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′-CCATGTGGCAGATCCCACAG-3′ (sense) and 5′-TGGAAATCTAACAACCTGTCG-5′ (antisense). The primers used for pS2 expression were 5′-CATGGAACAACTGATCCTG-3′ (sense) and 5′-CAGAAGGCCTGCTGACTGCTC-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 21β receptor was used as an internal control for both PR and pS2. The sense and antisense primers used for β-actin were 5′-ACACTGTGGAACAAGGCTGC-3′ (sense) and 5′-ACTGGGCTAGACTGTTACT-3′ (antisense). The PCR products (10 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 21β receptor was used as an internal control for both PR and pS2. The sense and antisense primers used for β-actin were 5′-ACACTGTGGAACAAGGCTGC-3′ (sense) and 5′-ACTGGGCTAGACTGTTACT-3′ (antisense). The PCR products of PR (271 bp) and pS2 (365 bp) were separated by electrophoresis in 1% agarose gels and visualized by staining with ethidium bromide. The 21β receptor was used as an internal control for both PR and pS2. The sense and antisense primers used for β-actin were 5′-ACACTGTGGAACAAGGCTGC-3′ (sense) and 5′-ACTGGGCTAGACTGTTACT-3′ (antisense). 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 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 ultrafiltration 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 red clover.
samples, and the estrogenic or antiestrogenic effects of the test
tations approximating physiological levels (responds to estrogens and antiestrogens at concentra-
derived from a glandular epithelial cell line. This cell
ER positive endometrial adenocarcinoma cell line
isoflavone (Table 2) were found to be cytotoxic with
A was the ER
strongest AP induction ability with an IC50 value of 1.0
In Ishikawa cells, the red clover extract showed the
consistent results in comparison with ER binding data
the results of AP induction. These two assays give
response, whereas inhibition represents an antiestro-
genic effect (of AP activity in Ishikawa cells indicates an estrogenic

- 
- 
- 

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

<table>
<thead>
<tr>
<th>compound</th>
<th>ERα binding IC50, μM</th>
<th>ERβ binding IC50, μM</th>
<th>AP induction Ishikawa cells</th>
<th>PR expression Ishikawa cells, ratio of intensity</th>
<th>toxicity Ishikawa cells ED50, μM</th>
<th>pS2 expression S-30 cells, ratio of intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>genistein</td>
<td>0.3 ± 0.01</td>
<td>0.018 ± 0.002</td>
<td>0.51 ± 0.1</td>
<td>0.88</td>
<td>&gt;5 g/mL</td>
<td>0.70</td>
</tr>
<tr>
<td>daidzein</td>
<td>17 ± 2.5</td>
<td>1.2 ± 0.0</td>
<td>1.2 ± 0.6</td>
<td>1.15</td>
<td>&gt;5 g/mL</td>
<td>0.62</td>
</tr>
<tr>
<td>biochanin A</td>
<td>35 ± 1.4</td>
<td>4.1 ± 0.8</td>
<td>5.1 ± 0.4</td>
<td>1.36</td>
<td>47 ± 6.0</td>
<td>0.21</td>
</tr>
<tr>
<td>formononetin</td>
<td>104 ± 8.2</td>
<td>60 ± 7.1</td>
<td>12 ± 3.0</td>
<td>0.97</td>
<td>&gt;100</td>
<td>0.16</td>
</tr>
<tr>
<td>estradiol</td>
<td>0.0056 ± 0.00058</td>
<td>0.0024 ± 0.00014</td>
<td>0.00014 ± 0.000014</td>
<td>1.40</td>
<td>&gt;0.005</td>
<td>0.93</td>
</tr>
</tbody>
</table>

- 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

<table>
<thead>
<tr>
<th>fraction/subfraction</th>
<th>ERα binding a</th>
<th>ERβ binding a</th>
<th>AP induction b Ishikawa cells</th>
<th>toxicity c Ishikawa cells</th>
<th>bound ligands detected by LC-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH</td>
<td>78</td>
<td>72</td>
<td>30</td>
<td>70 g/mL</td>
<td>genistein, daidzein, biochanin A</td>
</tr>
<tr>
<td>PE</td>
<td>61</td>
<td>77</td>
<td>&lt;20</td>
<td>&lt;80 ND</td>
<td>genistein, daidzein, biochanin A</td>
</tr>
<tr>
<td>CHCl3</td>
<td>83</td>
<td>93</td>
<td>33</td>
<td>50 g/mL</td>
<td>genistein, daidzein, biochanin A</td>
</tr>
<tr>
<td>BuOH</td>
<td>28</td>
<td>34</td>
<td>77</td>
<td>&lt;80 ND</td>
<td>ND</td>
</tr>
<tr>
<td>H2O</td>
<td>7</td>
<td>0</td>
<td>&lt;20</td>
<td>&lt;80 ND</td>
<td>ND</td>
</tr>
<tr>
<td>CHCl3 fraction 2</td>
<td>92</td>
<td>90</td>
<td>toxic</td>
<td>7</td>
<td>biochanin A</td>
</tr>
<tr>
<td>CHCl3 fraction 6</td>
<td>82</td>
<td>97</td>
<td>44</td>
<td>59 genistein</td>
<td>genistein A</td>
</tr>
<tr>
<td>CHCl3 fraction 7</td>
<td>90</td>
<td>98</td>
<td>68</td>
<td>&lt;80 genistein</td>
<td>genistein A</td>
</tr>
<tr>
<td>CHCl3 fraction 8</td>
<td>57</td>
<td>93</td>
<td>76</td>
<td>&lt;80 genistein</td>
<td>genistein, daidzein</td>
</tr>
</tbody>
</table>

- a Percent inhibition at 200 μg/mL. b Percent induction at 20 μg/mL. c Percent cell survival at 20 μg/mL. d IC50 = 1 ± 0.2 μg/mL. e ND, none detected. f IC50 = 2.6 ± 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 ERα was confirmed by the large enhance-
ment of the LC-MS peak following affinity ultrafiltration
(solid line in Figure 2A) compared with that of a control
sample which did not contain ERα (dashed line, Figure
2A). Of the 14 subfractions of the most potent chloroform
e xtract of red clover separated by column chromatog-
raphy, the four fractions showing the highest competi-
tive binding affinity to ERα were subjected to the
ultrafiltration and LC-MS ERα binding assay. Genistein
was detected as the most ERα-active component in
subfractions 6–8 (Table 3; Figure 2B). In addition to
genistein, daidzein was detected in fraction 8. Biochanin
A was the ERα 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 endometrial adenocarcinoma cell line
derived from a glandular epithelial cell line. This cell
responds to estrogens and antiestrogens at concentra-
tions approximating physiological levels (38). Induction
of AP activity in Ishikawa cells indicates an estrogenic
response, whereas inhibition represents an antiestro-
genic 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 IC50 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 IC50 value of 13.1 μg/mL. Chasteberry
displayed weak estrogenic activity (40%) at a concentra-
tion 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 IC50
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 H2O 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 transfect ed with ERα. We utilized this
cell line for pS2 expression because it was responsive
to estradiol; results were consistent with the other
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 H2O 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 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 ER/β 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 luteinizing hormone (LH) levels with women demonstrating dianamic 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, 3,5-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 as 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 ER/β 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-aminoethylether) tetraacetic acid; ELISA, enzymelinked 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.

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