

## Comparison of the in Vitro Estrogenic Activities of Compounds from Hops (*Humulus lupulus*) and Red Clover (*Trifolium pratense*)

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Because the prevailing form of hormone replacement therapy is associated with the development of cancer in breast and endometrial tissues, alternatives are needed for the management of menopausal symptoms. Formulations of *Trifolium pratense* L. (red clover) are being used to alleviate menopause-associated hot flashes but have shown mixed results in clinical trials. The strobiles of *Humulus lupulus* L. (hops) have been reported to contain the prenylflavanone, 8-prenylnaringenin (8-PN), as the most estrogenic constituent, and this was confirmed using an estrogen receptor ligand screening assay utilizing ultrafiltration mass spectrometry. Extracts of hops and red clover and their individual constituents including 8-PN, 6-prenylnaringenin (6-PN), isoxanthohumol (IX), and xanthohumol (XN) from hops and daidzein, formononetin, biochanin A, and genistein from red clover were compared using a variety of in vitro estrogenic assays. The IC<sub>50</sub> values for the estrogen receptor  $\alpha$  and  $\beta$  binding assays were 15 and 27  $\mu\text{g/mL}$ , respectively, for hops and 18.0 and 2.0  $\mu\text{g/mL}$ , respectively, for the red clover extract. Both of the extracts, genistein, and 8-PN activated the estrogen response element (ERE) in Ishikawa cells while the extracts, biochanin A, genistein, and 8-PN, significantly induced ERE-luciferase expression in MCF-7 cells. Hop and red clover extracts as well as 8-PN up-regulated progesterone receptor (PR) mRNA in the Ishikawa cell line. In the MCF-7 cell line, PR mRNA was significantly up-regulated by the extracts, biochanin A, genistein, 8-PN, and IX. The two extracts had EC<sub>50</sub> values of 1.1 and 1.9  $\mu\text{g/mL}$ , respectively, in the alkaline phosphatase induction assay. On the basis of these data, hops and red clover could be attractive for the development as herbal dietary supplements to alleviate menopause-associated symptoms.

**KEYWORDS:** Alkaline phosphatase; estrogen receptor; hops; *Humulus lupulus*; menopause; progesterone receptor; red clover; *Trifolium pratense*

### INTRODUCTION

Many menopausal and postmenopausal women suffer from a variety of complaints including depression, mood swings, sleep disturbances, vaginal dryness, and hot flashes (1–3). Hormone replacement therapy (HRT) has been used as an effective method of treating various menopause-related symptoms (4). Many women have been turning to herbal remedies (5–7) even prior to several large studies of HRT. The effects of HRT on women's

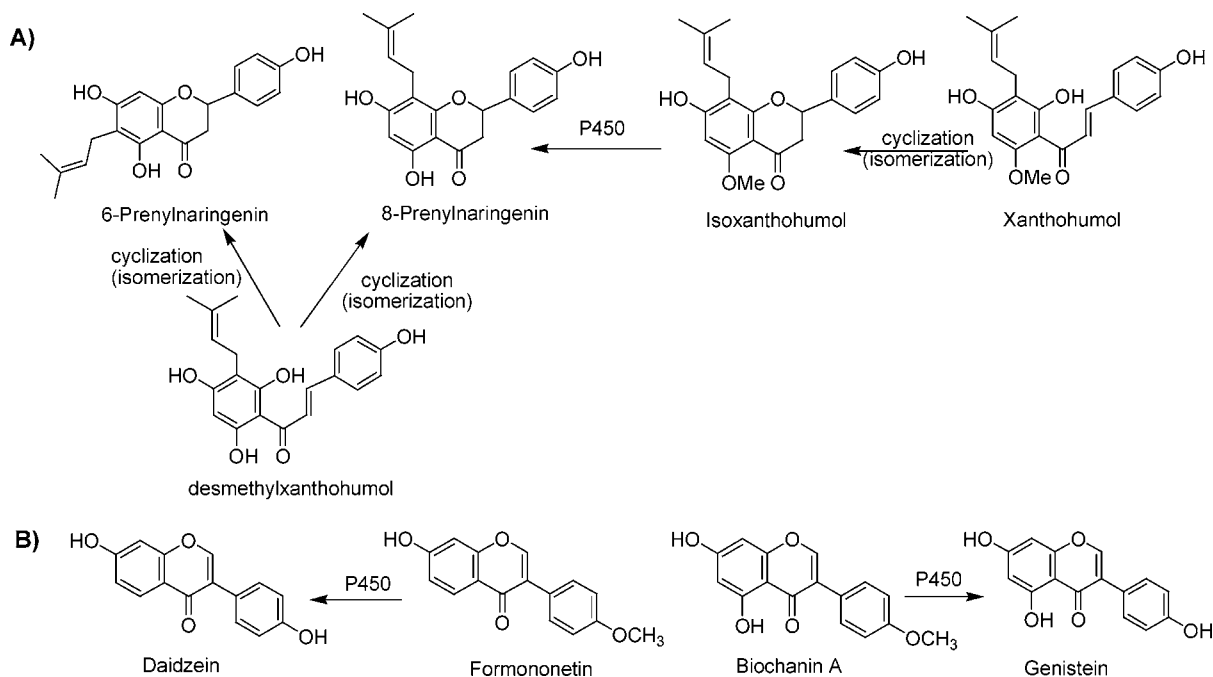
health have been evaluated in the Million Women Study (8), E3N-EPIC (Etude Epidémiologique auprès de femmes de la Mutuelle Générale de l'Education Nationale—European Prospective Investigation into Cancer and Nutrition) (9), and the Women's Health Initiative (WHI) (10–12), all of which showed an increased risk of breast cancer. Considering the growing use of herbal alternatives, it is important to fully understand their mechanism(s) of action to avoid potential adverse effects and to establish their efficacy.

The strobiles of *Humulus lupulus* L. (hops) are primarily used to flavor beer, although they have been studied since 1953 for a potential estrogenic mechanism of action (13–15). In the past, the flavonoids, 8-prenylnaringenin (8-PN) and 6-prenylnarin-

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**Figure 1.** Formation of (A) 8-PN from precursors from hops and (B) genistein and daidzein from precursors from red clover.

genin (6-PN), and the chalcone, isoxanthohumol (IX), have been isolated and reported to be estrogenic *in vitro* and/or *in vivo* (16, 17). The most potent estrogen in hops is 8-PN, which is an artifact formed through isomerization of the precursor chalcone, desmethylxanthohumol (18, 19). There are several reports on 8-PN in various biological models (20–22). On the other hand, *Trifolium pratense* L. (red clover) contains the estrogenic isoflavones, daidzein, formononetin, biochanin A, and genistein (23, 24). Metabolism and clinical pharmacokinetics studies (25) have shown that formononetin and biochanin A, the same isoflavones found in *Glycine max* Merrill (soy) (26), can be converted by cytochrome P450 to their more active estrogenic metabolites, daidzein and genistein, respectively (Figure 1B). Red clover has been tested in clinical trials as a menopause therapy (27) with formulations based on the active metabolites and their metabolic precursors (13). In the present study, we confirmed the presence of 8-PN as the most potent estrogen in hops using a mass spectrometry-based screening assay developed in our laboratory (28). *In vivo*, additional 8-PN can be formed as a metabolite of the more abundant compounds IX and xanthohumol (XN) (29). This is similar to red clover in that both plants have weak or inactive precursors that can be converted to more potent estrogens.

While estrogenic activities have been reported in the literature for both hops and red clover, few studies have directly compared the estrogenicities of the extracts (23, 30, 31), and to our knowledge, none of the previous reports have compared the isolated compounds responsible for estrogenic activity. Extracts of hops and red clover and their individual constituents including daidzein, formononetin, biochanin A, and genistein from red clover and 8-PN, 6-PN, IX, and XN from hops (Figure 1) were compared using a variety of *in vitro* estrogenic assays. These data suggest that hops and red clover have the potential for development as herbal dietary supplements to alleviate symptoms associated with menopause.

## MATERIALS AND METHODS

**Chemicals and Reagents.** All chemicals and reagents were purchased from Fisher (Hanover Park, IL) or Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. All media for cell culture and human

recombinant estrogen receptor (ER)  $\alpha$  and ER $\beta$  were purchased from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). Genistein, daidzein, biochanin A, and formononetin were purchased from Indofine Chemical Co. (Belle Mead, NJ). 8-PN, 6-PN, IX, and XN were isolated from *H. lupulus* L. cv. Nugget as described previously (19).

**Plant Material, Extraction, and Fractionation.** The chloroform partition of a methanol extract from a previously CO<sub>2</sub>-extracted Nugget cultivar of *H. lupulus* L. (Cannabaceae) (provided by Yakima Chief, Inc., Sunnyside, WA) was used as previously described (19). The *T. pratense* L. (Fabaceae) extract (provided by PureWorld, Hackensack, NJ) was an enzymatically treated ethanolic extract, in which glycosidic isoflavones were converted to aglycones such that it contained 30% isoflavones as described previously (32).

**Cell Culture Conditions.** The Ishikawa cell line was provided by Dr. R. B. Hochberg (Yale University, New Haven, CT) and was maintained in Dulbecco's modified Eagle medium (DMEM/F12) containing 1% sodium pyruvate, 1% nonessential amino acids (NEAA), 1% glutamax-1, 0.05% insulin, and 10% heat-inactivated FBS. A day prior to treating the cells, the medium was replaced with phenol red-free DMEM/F12 medium containing charcoal/dextran-stripped FBS and supplements. The MCF-7 cell line was purchased from American Tissue Culture Collection (Manassas, VA). MCF-7 cells were grown in RPMI 1640 media containing 1% glutamax-1, 1% NEAA, 0.05% insulin, and 5% heat-inactivated FBS. A day prior to treating the cells, the medium was replaced with phenol red-free RPMI 1640 medium containing charcoal/dextran-stripped FBS and supplements. Stripped FBS was prepared by incubating FBS with acetone-washed activated charcoal (100 mg/mL) at 4 °C for 30 min and centrifuged at 4000 rpm for 15 min at 4 °C. This step was repeated in triplicate. The concentration of extracts and compounds did not cause any significant cell death under these experimental conditions. DMSO concentrations for all cell culture assays were below 0.1%.

**ER $\alpha$  and ER $\beta$  Competitive Binding Assays.** A screening assay based on ultrafiltration mass spectrometry (28) was used to identify the estrogenic compounds in the hops and red clover extracts. Briefly, 13.3  $\mu$ g/mL of a DMSO stock extract was incubated for 2 h at room temperature with 100 pmol of ER $\alpha$  or ER $\beta$  in binding buffer consisting of 50 mM Tris-HCl (pH 7.5), 10% glycerol, 50 mM KCl, and 1 mM ethylenediaminetetraacetic acid (EDTA), in a total volume of 150  $\mu$ L. Identical control incubations in which denatured ER was substituted for active ER was used to correct for nonspecific binding of estrogens to the ultrafiltration membrane and holder. After incubation, each mixture was filtered through a Microcon (Millipore, Bedford, MA) YM-

30 centrifugal filter containing a regenerated cellulose ultrafiltration membrane with a 30000 MW cutoff and washed three times with 150  $\mu\text{L}$  aliquots of ammonium acetate buffer (pH 7.5) at 4 °C to remove the unbound compounds. The bound ligands were released by adding 400  $\mu\text{L}$  of methanol/water (90:10; v/v) followed by centrifugation at 10000g for 10 min. The ultrafiltrates were dried in vacuo, and the ligands were reconstituted in 60  $\mu\text{L}$  of methanol/water (50:50; v/v). Aliquots (30  $\mu\text{L}$ ) were analyzed using LC-MS, which consisted of a reverse phase separation on a Waters (Milford, MA) Xterra MS C<sub>18</sub> (3.5  $\mu\text{m}$ , 2.1 mm  $\times$  100 mm) high-performance liquid chromatography (HPLC) column and mass spectrometric analysis on a Micromass (Manchester, United Kingdom) Quattro II electrospray triple quadrupole mass spectrometer. The mobile phase consisted of a 15 min linear gradient from 15 to 50% acetonitrile in water, and negative ion electrospray was used for mass spectrometric detection of the ligands.

After ER ligands were identified in the hops extract using mass spectrometry, competitive ER $\alpha$  and ER $\beta$  binding assays were used with tritiated estradiol based on the method of Obourn et al. (33) with minor modifications (23) to determine in vitro binding affinities of the substrates with the receptors. The reaction mixture consisted of 5  $\mu\text{L}$  of extract in DMSO, 5  $\mu\text{L}$  of pure human recombinant diluted ER $\alpha$  and ER $\beta$  (0.5 pmol) in ER binding buffer, 5  $\mu\text{L}$  of "Hot Mix" [400 nM, prepared fresh using 95 Ci/mmol [<sup>3</sup>H]estradiol, diluted in 1:1 ethanol:ER binding buffer; obtained from NEN Life Science Products (Boston, MA)], and 85  $\mu\text{L}$  of ER binding buffer. The incubation was carried out at room temperature for 2 h before 100  $\mu\text{L}$  of 50% hydroxyapatite slurry (HAPS) was added. 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 before centrifuging at 10000g for 1 min. The supernatant was discarded, and this wash step was repeated three times. The HAPS pellet containing the ligand-receptor complex was resuspended in 200  $\mu\text{L}$  of ethanol and transferred to scintillation vials. An additional 200  $\mu\text{L}$  of ethanol was used to rinse the centrifuge tube. Cytoscint [4 mL/vial; ICN (Costa Mesa, CA)] was added, and the radioactivity was counted using a Beckman LS 5801 liquid scintillation counter (Schaumburg, IL). The percent inhibition of [<sup>3</sup>H]estradiol binding to each ER was determined using eq 1.

$$\left[ \frac{(\text{dpm}_{\text{sample}} - \text{dpm}_{\text{blank}})}{(\text{dpm}_{\text{DMSO}} - \text{dpm}_{\text{blank}})} - 1 \right] \times 100 = \text{\% sample binding (1)}$$

The binding capability (percent) of the sample was calculated in comparison with that of estradiol (50 nM, 90%).

**Measure of Estrogen Responsive Element (ERE) Activation.** The Dual-Luciferase Reporter Assay System from Promega (Madison, WI) was used to evaluate the functional formation of the ER-ERE complex and luciferase protein expression. Briefly, Ishikawa cells, grown overnight in phenol-free medium, were plated (1.0  $\times$  10<sup>5</sup> cells/well) in six well dishes. The following day, the cells were cotransfected using Effectene (Qiagen, Valencia, CA) according to the company's protocol with ERE plasmid (0.4  $\mu\text{g}/\text{mL}$ ), pRL-TK plasmid (0.2  $\mu\text{g}/\text{mL}$ ), and the respective ratios of reagents. MCF-7 cells, grown overnight in phenol-free medium, were trypsinized and resuspended at 1  $\times$  10<sup>7</sup> cells/mL of serum-free medium. Cells (5  $\times$  10<sup>6</sup>) were incubated with the ERE plasmid (3  $\mu\text{g}/\text{mL}$ ) obtained from Dr. V. C. Jordan, Northwestern University (34), and pRL-TK plasmid (Promega) (1  $\mu\text{g}/\text{mL}$ ) in a 4 mm gap cuvette for 5 min at room temperature before electroporation at 950  $\mu\text{F}$  and 250 V using the GenePulser Xcell (BioRad Laboratories, Hercules, CA). Transfected cells were diluted in serum containing medium and plated in six well dishes (8.0  $\times$  10<sup>5</sup> cells/well). Following a 24 h recovery period, the cells were washed with PBS and treated with 2  $\mu\text{g}/\text{mL}$  of extract or 100 nM compounds, including the 17 $\beta$ -estradiol (E2) positive control, for an additional 24 h. Cell lysates (20  $\mu\text{L}$ ) were placed in 96 well plates. Luciferase Assay Reagent II (100  $\mu\text{L}$ ) was injected followed by a 12 s read by a FLUOstar OPTIMA (BMG LabTech, Offenburg, Germany). Stop & Glo (100  $\mu\text{L}$ ) was added followed by a 12 s read, thus allowing the termination of the firefly luciferase expression and the activation of the renilla expression. The sample results were normalized to pRL-TK, to account for transfection efficiency, by dividing the sum of the luciferase activity by the sum of

the renilla activity. Samples were converted into fold induction such that the DMSO fold induction was 1.

**Measurement of Progesterone Receptor (PR) mRNA Expression Levels.** Quantitative real-time polymerase chain reaction (RT-PCR) was used to examine the modulation of the PR following treatment of Ishikawa and MCF-7 cells with the herbal extracts and test compounds. Experiments were performed three independent times in triplicate. Using the method described previously (35), Ishikawa and MCF-7 cells (2  $\times$  10<sup>6</sup>) were preincubated overnight in estrogen-free medium. Cells were treated with extracts (2  $\mu\text{g}/\text{mL}$ ) and test compounds (100 nM) in DMSO for 4 days. mRNA was isolated using the RNAqueous kit (Ambion Inc., Austin, Texas). Following isolation, mRNA was quantitated by UV analysis at 260 nm. cDNA synthesis was performed in a total volume of 10  $\mu\text{L}$ , containing 1  $\times$  TaqMan RT buffer, 5.5  $\mu\text{M}$  MgCl<sub>2</sub>, 2 mM dNTPs mixture, 2.5  $\mu\text{M}$  random hexamers, 4 U RNase inhibitor, 12.5 U MultiScribe RT (Perkin-Elmer/Applied Biosystems, Foster City, CA), and 0.2  $\mu\text{g}$  of RNA. The reaction was carried out for 10 min at 25 °C, followed by 48 °C for 30 min and a 5 min incubation step at 95 °C. The PCR and subsequent analyses were performed using the GeneAmp 5700 Sequence Detection System (Applied Biosystems). Quantitation was performed using the TaqMan technology of Applied Biosystems. PR was evaluated using a predeveloped gene expression primer/probe set (Applied Biosystems' Assay on Demand). The reaction mixtures were first incubated at 50 °C for 2 min, followed by 10 min at 95 °C. PCR reactions were performed in triplicate and consisted of 40 cycles with 15 s at 95 °C and 1 min at 60 °C each. The fluorescence signal was measured during the last 30 s of the annealing/extension phase. Following analysis, a fluorescence threshold value was set and threshold cycle (C<sub>t</sub>) values were determined, i.e., the fractional cycle at which the fluorescence signal reached this threshold. These values were used for further calculations. 18S (TaqMan PDAR control, Applied Biosystems) was used as an endogenous reference to correct for any differences in the amount of total RNA used for a reaction and to compensate for different levels of transcription during reverse transcription of RNA into cDNA. PR expression was related to a standard curve derived from a serial dilution of their estradiol-treated cDNA. Subsequently, normalization was achieved by dividing the expression level of PR by its respective 18S expression level. Finally, results were expressed as a fold induction, where the levels of PR observed in the DMSO-treated samples were defined as 1.

**Induction of Alkaline Phosphatase (AP) in Cultured Ishikawa Cells.** The procedure of Pisha et al. was used as described previously (36). Ishikawa cells (1.5  $\times$  10<sup>4</sup> cells/190  $\mu\text{L}$ /well) were preincubated in 96 well plates overnight in estrogen-free medium. Test samples (10  $\mu\text{L}$  at varying concentrations in DMSO) were added to determine EC<sub>50</sub> values, and the cells in a total volume of 200  $\mu\text{L}$  media/well were incubated at 37 °C for 4 days. For the determination of antiestrogenic activity, 2  $\times$  10<sup>-8</sup> M estradiol was added to the media. The induction plates were processed by washing the plates with PBS and adding 50  $\mu\text{L}$  of 0.01% Triton  $\times$  100 in 0.1 M Tris buffer (pH 9.8). An aliquot (150  $\mu\text{L}$ ) of 24 mM *p*-nitrophenyl phosphate (phosphatase substrate) was added to each well. The enzyme activity was measured by reading the release of *p*-nitrophenyl phosphate at 405 nm every 15 s with a 10 s shake between readings for 16 readings using a Power Wave 200 microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, VT). The maximal slopes of the lines generated by the kinetic readings were calculated. For estrogenic determination, the percent induction as compared with the estradiol control was calculated using eq 2. For antiestrogenic determination, the percent induction as compared with the background induction control was calculated using eq 3.

$$\left[ \frac{(\text{slope}_{\text{sample}} - \text{slope}_{\text{DMSO}})}{(\text{slope}_{\text{estrogen}} - \text{slope}_{\text{DMSO}})} \right] \times 100 = \text{\% estrogenic induction (2)}$$

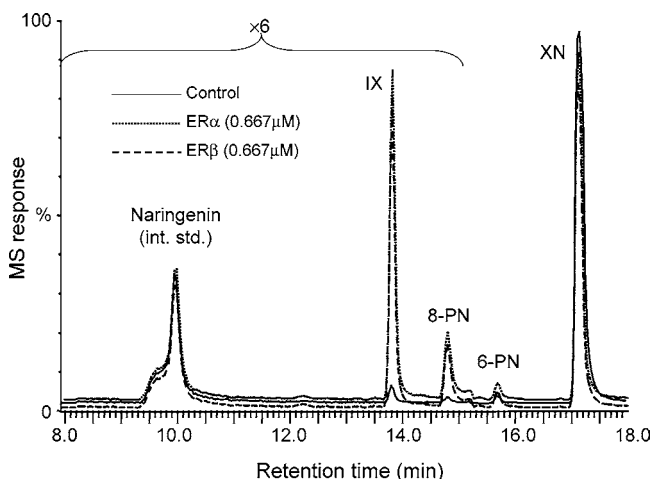
$$\left\{ 1 - \left[ \frac{(\text{slope}_{\text{sample}} - \text{slope}_{\text{cells}})}{(\text{slope}_{\text{estrogen}} - \text{slope}_{\text{cells}})} \right] \right\} \times 100 = \text{\% antiestrogenic induction (3)}$$

**Statistics.** Statistical analyses were performed using the SAS statistical package (SAS Institute, Cary, NC). One-way analysis of variance (ANOVA) was used to analyze the ERE-luciferase data,

**Table 1.** ER Binding, ERE-Luciferase, PR mRNA Expression, and AP Induction of Hops, Red Clover, and Their Respective Compounds<sup>a</sup>

	IC <sub>50</sub> <sup>b</sup> (n = 6)		ERE-luciferase fold induction <sup>c,d</sup> (n = 2)		PR mRNA fold induction <sup>c</sup> (n = 9)		AP induction EC <sub>50</sub> <sup>b</sup> (n = 9)
	ER $\alpha$	ER $\beta$	Ishikawa	MCF-7	Ishikawa	MCF-7	
E2	0.021 ± 0.003	0.015 ± 0.002	3.5 ± 0.7 <sup>g</sup>	4.3 ± 0.2 <sup>g</sup>	39 ± 12 <sup>g</sup>	10 ± 6 <sup>g</sup>	0.00014 ± 0.00003
hops	15 ± 3	27 ± 3	3 ± 0.3 <sup>g</sup>	4.2 ± 0.2 <sup>g</sup>	19 ± 2 <sup>g,e</sup>	5.6 ± 2.4 <sup>g</sup>	1.1 ± 0.8
red clover	18 ± 5	2.0 ± 0.8	7.2 ± 2.8 <sup>g</sup>	6.3 ± 0.3 <sup>g</sup>	22 ± 11 <sup>g</sup>	5.9 ± 2.4 <sup>g</sup>	1.9 ± 0.5
daidzein	17 ± 3	1.20 ± 0.01	1.1 ± 0.1	1.6 ± 0.3	2.0 ± 1 <sup>e</sup>	1.5 ± 1.3	0.5 ± 0.1
formononetin	104 ± 8	60 ± 7	1.1 ± 0.3	1.2 ± 0.3	1.9 ± 0.3	1.5 ± 0.8	N/A
biochanin A	35 ± 1	4.1 ± 0.8	0.8 ± 0.2	4.0 ± 0.4 <sup>g</sup>	1.4 ± 0.3 <sup>g</sup>	3.4 ± 2.0 <sup>g</sup>	4.6 ± 0.8
genistein	0.30 ± 0.01	0.020 ± 0.002	3.5 ± 1.2 <sup>g</sup>	4.0 ± 0.5 <sup>g</sup>	2.8 ± 0.3	4.0 ± 2.2 <sup>g</sup>	0.3 ± 0.1
8-PN	0.51 ± 0.07	1.7 ± 0.1	3.5 ± 0.1 <sup>g</sup>	4.2 ± 0.3 <sup>g</sup>	46 ± 15 <sup>g</sup>	8.6 ± 2.9 <sup>g</sup>	0.013 ± 0.002
6-PN	N/A <sup>f</sup>	N/A	0.9 ± 0.4	0.7 ± 0.1	1.3 ± 0.3	1.1 ± 0.6	N/A
IX	266 ± 56	56 ± 13	0.8 ± 0.1	1.8 ± 0.3 <sup>h</sup>	1.8 ± 0.7	2.5 ± 2.2 <sup>g</sup>	1.1 ± 0.8
XN	N/A	N/A	0.7 ± 0.3	0.7 ± 0.2	2.0 ± 0.7	0.8 ± 0.4 <sup>e</sup>	N/A

<sup>a</sup> Values are expressed as the mean ± SD of *n* determinations. Experimental details are described in the Materials and Methods. Values were significantly different from the control as determined by one-way ANOVA with a follow-up Dunnett test ( $p < 0.05$ ). <sup>b</sup> Values are expressed in  $\mu\text{g}/\text{mL}$  for extracts and  $\mu\text{M}$  for pure compounds. <sup>c</sup> Fold inductions tested at 2  $\mu\text{g}/\text{mL}$  for extracts and 100 nM concentrations for compounds where DMSO is equal to 1. Results were normalized relative to 18S. <sup>d</sup> Ratio of the sum of the firefly and renilla luminescences. <sup>e</sup>  $n = 6$ . <sup>f</sup> N/A, not active. <sup>g</sup> Statistically significant fold induction as compared with DMSO ( $p < 0.05$ ). <sup>h</sup> Marginally significant fold induction as compared with DMSO ( $0.1 > p > 0.05$ ).



**Figure 2.** Negative ion electrospray LC-MS chromatograms showing the ultrafiltration mass spectrometric screening of a hop extract for ligands to ER $\alpha$  and ER $\beta$ . Denatured ER was used as a control for nonspecific binding, and specific binding is indicated by increases in the chromatographic peak areas. IX and 8-PN were detected as the highest affinity ligands to both ER $\alpha$  and ER $\beta$ .

followed by a Dunnett test for pairwise comparison between the DMSO control and each of the other compounds. A general linear model with two main effects and their interaction was used to analyze the RT-PCR data, followed by posthoc comparison between the control vs each of the other compounds. All of the tests were two-sided, and results were considered significant when  $p < 0.05$ .

## RESULTS

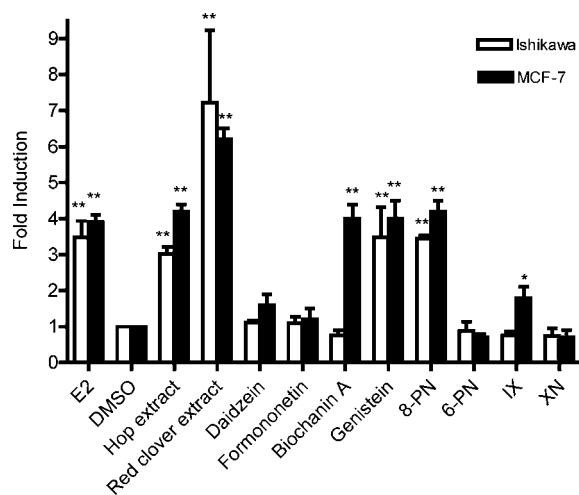
**Relative Affinity of Extracts and Compounds for ER $\alpha$  and ER $\beta$ .** Ultrafiltration LC-MS screening was used to identify ligands for ER $\alpha$  and ER $\beta$  in the complex botanical extracts. In addition, the competition of botanical ligands with tritiated estradiol was used to confirm that binding occurred at the estradiol site of ER and to measure the relative affinities of these compounds based on their IC<sub>50</sub> values. As shown in the ultrafiltration LC-MS chromatograms (Figure 2), two ligands for ER were detected in the hop extract. One of the ligands was identified as the known hop estrogen, 8-PN, by comparison of its HPLC retention time and mass spectrum with a standard that had previously been isolated over the course of several months using traditional ER binding assay-directed fractionation.

Aided by the knowledge of the HPLC retention time and mass spectrometric data gained through ultrafiltration mass spectrometry, the second ligand was isolated from a crude extract and identified as IX by its mass and NMR spectra in only 5 days. Both compounds showed affinity for ER $\alpha$  as well as ER $\beta$ . Although isomeric with 8-PN, 6-PN did not show any specific binding to ER (Figure 2). Also, the abundant prenylated hops constituent XN did not show affinity for ER during ultrafiltration LC-MS. Screening of the red clover extract using ultrafiltration LC-MS has been reported previously (23) and resulted in the identification of daidzein, formononetin, biochanin A, and genistein as the most estrogenic constituents.

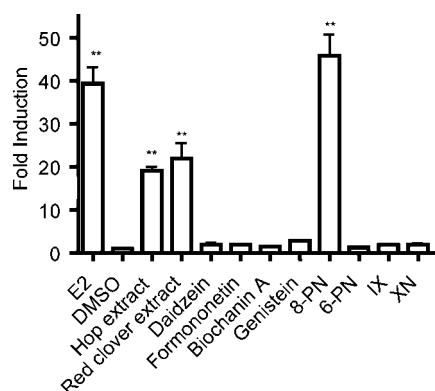
Tested as complex botanical extracts, hops and red clover showed similar ER $\alpha$  activity with IC<sub>50</sub> values of 15 and 18  $\mu\text{g}/\text{mL}$ , respectively (Table 1). However, these extracts had different ER $\beta$  IC<sub>50</sub> values of 27 and 2  $\mu\text{g}/\text{mL}$ , respectively. For the isolated compounds, the relative binding affinities for ER $\alpha$  were as follows: genistein  $\approx$  8-PN > daidzein > biochanin A > formononetin > IX, while the relative binding affinities for ER $\beta$  were genistein > 8-PN  $\approx$  daidzein > biochanin A > formononetin  $\approx$  IX (Table 1). Genistein and 8-PN had similar affinities for ER $\alpha$  with IC<sub>50</sub> values of 0.51 and 0.3  $\mu\text{M}$ , respectively, whereas genistein was more selective for ER $\beta$  as indicated by the IC<sub>50</sub> values of 0.020 and 1.7  $\mu\text{M}$ , respectively.

**ERE Luciferase Activity.** Ishikawa and MCF-7 cells that had been transiently cotransfected with the ERE-luciferase and the pRL-TK control plasmid were used to measure the formation of the functional ER-ERE complex in response to treatment with the botanical extracts or individual compounds. Samples were normalized to the control transfection and then expressed as a fold induction as compared with DMSO-treated cells (Table 1). In Ishikawa cells (Figure 3), the hop and red clover extracts had significant fold inductions, 3 and 7.2, respectively. Genistein and 8-PN had significant fold inductions each of 3.5. None of the other compounds had significant activity. In MCF-7 cells (Table 1 and Figure 3), hop and red clover extracts both activated ERE similarly (4.2 and 6.3, respectively). Consistent with Jung et al. (37), biochanin A and genistein had significant activity (4.0 and 4.0, respectively), 8-PN was statistically active (4.2), while IX had a 1.8-fold induction and was considered marginally active ( $p$  value = 0.0978).

**PR mRNA Expression.** The estrogen inducible PR mRNA expression was studied by using RT-PCR in the Ishikawa and MCF-7 cell lines (Table 1 and Figures 4 and 5). In the Ishikawa



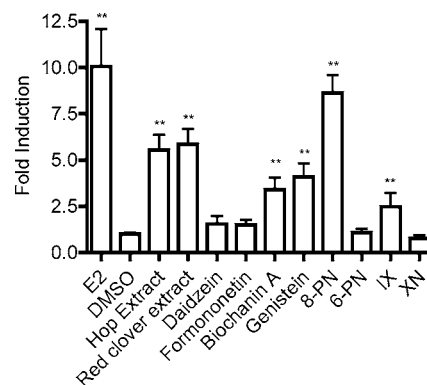
**Figure 3.** ERE-luciferase induction in Ishikawa and MCF-7 cells by hop and red clover extracts and their respective compounds. Cells were treated with either extracts (2  $\mu\text{g/mL}$ ) or pure compounds (100 nM) for 24 h and then analyzed for chemiluminescence. Results were normalized for transfection efficiency, and they are shown as a fold induction relative to the level observed in cells treated with solvent only. Results are the means of two determinations  $\pm$  SD. Significant differences from the control value were determined by one-way ANOVA with follow-up Dunnett test: \*0.1 >  $p$  > 0.05 and \*\* $p$  < 0.05.



**Figure 4.** PR mRNA expression levels in the Ishikawa cell line by hops, red clover, and their respective compounds. Ishikawa cells were treated with either extracts (2  $\mu\text{g/mL}$ ) or pure compounds (100 nM) for 96 h and then analyzed for PR mRNA. Results are shown as a fold induction relative to the level observed in cells treated with solvent only. Results are the means of nine determinations  $\pm$  SD with the exception of the hop extract, daidzein, and biochanin A where  $n = 6$ ; \*\* $p$  < 0.05.

cell line, PR mRNA was similarly and significantly up-regulated in both the hop and the red clover extracts (19 and 22, respectively). This was consistent with previous published results obtained in our laboratory using semiquantitative RT-PCR (23). The up-regulation induced by 8-PN was equivalent to E2 (46 and 39, respectively). The remaining compounds did not up-regulate the PR mRNA. In the MCF-7 cell line, PR mRNA was significantly up-regulated in both the hop and the red clover extracts (5.9 and 5.6). Biochanin A, genistein, 8-PN, and IX all significantly up-regulated the PR gene (3.4, 4.0, 8.6, and 2.5, respectively).

**AP Induction in Ishikawa Cells.** The Ishikawa cell line is a well-differentiated ER $\alpha$  positive endometrial adenocarcinoma cell line that responds to estrogens and antiestrogens at near physiological levels (38). Induction of AP is indicative of an estrogenic response, while competition with estrogen-supplemented media and prevention of AP expression indicate an



**Figure 5.** PR mRNA expression levels in the MCF-7 cell line by hop and red clover extracts and their respective compounds. MCF-7 cells were treated with either extracts (20  $\mu\text{g/mL}$ ) or pure compounds (100 nM) for 24 h and then analyzed for PR mRNA. Results are shown as a fold induction relative to the level observed in cells treated with solvent only. Results are the means of nine determinations  $\pm$  SD with the exception of XN where  $n = 6$ ; \*\* $p$  < 0.05.

antiestrogenic response (38). In this assay, the EC<sub>50</sub> values for the hop and red clover extracts were comparable, 1.1 and 1.9  $\mu\text{g/mL}$ , respectively. The EC<sub>50</sub> values for the compounds found in the red clover extract, namely, genistein, daidzein, formononetin, and biochanin A, were consistent with previous results obtained in our laboratory (23). Furthermore, EC<sub>50</sub> values for genistein and daidzein, as well as compounds isolated from the hop extract, namely 8-PN, IX, and XN (Table 1), were consistent with values reported by Milligan et al. (16). The isolated compound 6-PN was not active in this assay even when tested at 20  $\mu\text{g/mL}$ . The relative EC<sub>50</sub> rankings for all eight compounds were as follows: 8-PN  $\gg$  genistein  $\approx$  daidzein > IX > biochanin A (Table 1). None of the samples had antiestrogenic activity (data not shown). All of the samples were tested well below their LC<sub>50</sub> concentrations for the Ishikawa cell line (data not shown).

## DISCUSSION

In this study, the estrogenicity of hops and red clover extracts and eight isolated compounds, identified as ligands for ER $\alpha$  and ER $\beta$  based on the ultrafiltration LC-MS prescreening or as a precursor of an active compound, was evaluated using several in vitro estrogenic assays. We investigated different mechanisms to evaluate the estrogenic response of the sample since one end point in one tissue of interest may not predict clinical pharmacology (39). The competitive binding of the isolated ER, induction of a transiently transfected ERE-luciferase plasmid, and the up-regulation of the mRNA transcripts of the estrogen sensitive gene, PR, in both MCF-7 and Ishikawa cell lines, and the induction of the endogenous AP enzyme in the Ishikawa cell line were used. On the basis of these data, the activities of the two extracts and eight compounds were directly compared and were found to be consistent with the ultrafiltration LC-MS screening results and previous reports of activity for specific samples.

The competitive binding of ER $\alpha$  and ER $\beta$  provides direct information on ligand-receptor binding in a cell-free environment. On the basis of the ER distribution, ER $\beta$  binding is generally considered beneficial based on the relative tissue distributions of the two receptors (40, 41). The red clover extract preferentially bound to the ER $\beta$  receptor nine times greater than to ER $\alpha$ . The hop extract had nearly a 2-fold preference for ER $\alpha$  as compared with ER $\beta$ . Because all of these studies were carried

out using cell-based assays with ER $\alpha$  positive cell lines, it is important to note that the hop and red clover extracts had equivalent ER $\alpha$  activity.

Daidzein, formononetin, biochanin A, and genistein from red clover and 8-PN and IX from hops bound to ER $\alpha$ . Genistein and 8-PN had equivalent IC<sub>50</sub> values for ER $\alpha$ , 0.3 and 0.51  $\mu$ M, respectively, followed by daidzein and biochanin A, which had weaker IC<sub>50</sub> values of 17 and 35  $\mu$ M, respectively. Formononetin and IX had much weaker IC<sub>50</sub> values of 104 and 266  $\mu$ M, respectively. Daidzein, formononetin, biochanin A, and genistein, as well as 8-PN and IX, bound ER $\beta$ . Genistein bound the strongest with an IC<sub>50</sub> of 0.02  $\mu$ M. Daidzein, biochanin A, and 8-PN had similar activities, 4.1, 1.2, and 1.7  $\mu$ M, respectively. Formononetin and IX also had similarly weak activities, 60 and 56  $\mu$ M, respectively. The relative binding affinities observed for the compounds are in agreement with the ultra-filtration LC-MS screening and previously published data (42, 43).

The activation of a transiently transfected ERE-luciferase construct was designed to test the binding of ER $\alpha$  with the ERE using both the Ishikawa endometrial cell line and the MCF-7 breast cancer cell line. Studies of the DNA response element by Paech et al. (44) provide evidence of a potential transcription control mechanism for estrogen sensitive genes. In this assay, both the hop and the red clover extracts were significantly active. Genistein and 8-PN had significant activity, but the weaker estrogens biochanin A, genistein, and IX did not have activity in the Ishikawa cell line. When ERE-luciferase activity was measured in MCF-7 cells, the hop and red clover extracts had significant induction as compared with DMSO. There was a marked difference in the compounds that responded. Genistein and 8-PN were active, as were compounds that had less affinity for ER $\alpha$ , biochanin A, and IX. Joung et al. used a stably transfected ERE-luciferase MCF-7 cell line treated with genistein, daidzein, or biochanin A for a similar time period and found that genistein and biochanin A gave a dose-dependent response (37). Our results for 8-PN are in agreement with Zierau et al. who used the MVLN bioluminescent MCF-7-derived cell line to verify the modulation of estrogenic activity (20).

The induction of the estrogen sensitive gene PR in the ER $\alpha$  positive Ishikawa endometrial cell line was designed to evaluate the up-regulation of mRNA using quantitative RT-PCR. Following the formation of the ER-ERE complex, transcription of the target gene begins, in this case PR. As seen in the ERE-luciferase assay using the Ishikawa cell line, hop and red clover extracts significantly up-regulated PR, as did 8-PN. Results for biochanin A and daidzein were consistent with the results from the ERE-luciferase assay performed in the Ishikawa cell line. It was surprising that genistein did not have a statistically significant response; however, there was a trend toward estrogenic activity. Interestingly, 8-PN had greater than 10-fold induction as compared with genistein and had a similar fold induction for PR with that of estradiol when all compounds, including estradiol, were tested at the same concentration (100 nM). This may be a key point since 8-PN and genistein had essentially the same affinity for ER $\alpha$ , but they had considerable differences in ERE-dependent transcriptional activity, although not to the extent that was seen in the target gene induction. It appears, at least in the Ishikawa cell line, that 8-PN has a 10-fold higher activity as compared with genistein. This is consistent with 8-PN being considered one of the most potent phytoestrogens (16), and it acts as a pure estrogen with activity similar to estrone (45).

Both the hops and the red clover extracts were evaluated for PR gene induction in the ER $\alpha$  positive MCF-7 breast cancer cell line and showed significant activity. Furthermore, genistein, 8-PN, and the weaker estrogens, biochanin A and IX, also demonstrated significant induction as compared with DMSO. Biochanin A was also found to be estrogenic even though poor binding affinity to both receptors was observed in the competitive binding assay. This was attributed to the conversion of biochanin A to genistein and would also explain the estrogenic activity of biochanin A that was also seen with the ERE-luciferase assay. We confirmed (unpublished results) that the estrogenic activity of biochanin A in the MCF-7 cell line is due to the metabolism of biochanin A to genistein as previously reported (46). The conversion of biochanin A to genistein was not observed in the Ishikawa cell line (unpublished results) explaining why no significant induction of the PR gene was observed.

The AP induction assay was designed to evaluate the estrogen-induced production of the AP enzyme. As expected, on the basis of the ER $\alpha$  competitive binding assay, both the hop and the red clover extracts compared similarly in this assay with EC<sub>50</sub> values of 1.9 and 1.1  $\mu$ g/mL, respectively. Genistein and 8-PN were the most active compounds; however, 8-PN was 10 times more active than genistein. As was seen with the ERE-luciferase assay, there appears to be some ERE affinity and transcriptional regulation that effectively caused 8-PN, which had similar ER $\alpha$  binding, to have a 10-fold increase in PR gene up-regulation, which was also seen in the production of AP.

In the AP assay, formononetin and XN gave results that were slightly different from previous reports. Formononetin was previously reported to have an EC<sub>50</sub> value around 12  $\mu$ M (23). We demonstrated 50% activity at approximately the same concentration as previously reported; however, concentrations at 20 and 40  $\mu$ M consistently gave between 50 and 60% activity. We therefore conclude that formononetin has a maximum induction of 50% in the AP enzyme at concentrations of 12  $\mu$ M and above. It should also be noted that Gerhäuser et al. (47) reported antiestrogenic activity for XN of  $6.6 \pm 0.2$   $\mu$ M. We did not find any antiestrogenic activity for XN or any of the other pure compounds reported in this paper. One caveat of the antiestrogenic assay is that false positives can occur due to cell death, and a cytotoxicity assay is run concurrent with the AP induction assay. During this study, we found that XN had a cytotoxic effect as determined using the sulforhodamine B (SRB) assay described in ref 36 with an LC<sub>50</sub> of  $7.1 \pm 2.3$   $\mu$ M. This is essentially the same concentration that was reported for the antiestrogenic IC<sub>50</sub> of XN. While Gerhäuser et al. did perform a cytotoxicity assay, there were methodological differences between their investigation (47) and the current study, which might explain the differences in antiestrogenic activity for XN.

Overall, the hop and red clover extracts evaluated in the present study (a chloroform partition of a methanol extract from a previously CO<sub>2</sub>-extracted Nugget hops cultivar and a red clover ethanol extract containing 30% isoflavones prepared for a phase II clinical trial) gave results that are similar to previously reported data from our laboratory, which were assessed using a methanol extract of a Galena hops cultivar and a 15% isoflavone methanol extract of red clover (23). Both extracts demonstrated significant activities in the ER competitive binding, activation of transiently transfected ERE-luciferase, quantitative RT-PCR of an estrogen inducible gene, and AP enzyme induction assays. The equivalent estrogenic potency of the hop extract as compared with that of red clover suggests that future in vivo

studies are warranted to further validate the estrogenic nature of a standardized hop extract in comparison with a red clover extract already in clinical trials.

#### ABBREVIATIONS USED

6-PN, 6-prenylnaringenin; 8-PN, 8-prenylnaringenin; AP, alkaline phosphatase; DMEM/F12, Dulbecco's modified Eagle/F12 medium; E2, 17 $\beta$ -estradiol; EDTA, ethylenediaminetetraacetic acid; ER, estrogen receptor; ERE, estrogen responsive element; FBS, fetal bovine serum; HAPS, hydroxyapatite slurry; IX, isoxanthohumol; NEAA, nonessential amino acids; PR, progesterone receptor; SRB, sulforhodamine B; WHI, Women's Health Initiative; XN, xanthohumol.

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