High-Content Screening and Mechanism-Based Evaluation of Estrogenic Botanical Extracts

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Abstract: Symptoms associated with menopause can greatly affect the quality of life for women. Botanical dietary supplements have been viewed by the public as safe and effective despite a lack of evidence indicating a urgent necessity to standardize these supplements chemically and biologically. Seventeen plants were evaluated for estrogenic biological activity using standard assays: competitive estrogen receptor (ER) binding assay for both alpha and beta subtypes, transient transfection of the estrogen response element luciferase plasmid into MCF-7 cells expressing either ER alpha or ER beta, and the Ishikawa alkaline phosphatase induction assay for both estrogenic and antiestrogenic activities. Based on the combination of data pooled from these assays, the following was determined: a) a high rate of false positive activity for the competitive binding assays, b) some extracts had estrogenic activity despite a lack of ability to bind the ER, c) one extract exhibited selective estrogen receptor modulator (SERM) activity, and d) several extracts show additive/synergistic activity. Taken together, these data indicate a need to reprioritize the order in which the bioassays are performed for maximal efficiency of programs involving bioassay-guided fractionation. In addition, possible explanations for the conflicts in the literature over the estrogenicity of *Cimicifuga racemosa* (black cohosh) are suggested.

Keywords: Botanicals, estrogen, estrogen assays, herbal dietary supplements, menopause, selective estrogen receptor modulators.

INTRODUCTION

Symptoms associated with menopause such as insomnia, loss of libido, vaginal atrophy, depression, and hot flashes can greatly affect the quality of life for women. Many women have used hormone replacement therapy (HRT) to alleviate menopausal symptoms; however, with the publication of the Women's Health Initiative in 2002, the number of women using HRT has decreased dramatically [1, 2]. Even before the publication of several large studies of HRT, many women have been turning to herbal remedies for the relief of menopausal symptoms [3-5], perhaps because they are viewed as safe [6]. Among the herbal remedies currently being used, many contain phytochemicals that mimic the effects of human estrogen *in vitro* or *in vivo*. Such phytochemicals are commonly referred to as phytoestrogens. Unfortunately, few botanicals are chemically and biologically standardized to relevant active compounds using an appropriate mechanism of action [7, 8].

There are a number of different target points along the estrogen signaling pathway. Initially the ligand will bind the estrogen receptor (ER) and the dimerized complex will move from the cytosol of the cell through the nuclear pore and into the nucleus. Once inside the nucleus, the ER-dimer will bind to the estrogen response element (ERE) which is located upstream of estrogen-controlled genes. Once bound, coactivators, corepressors, and transcription factors will bind, and transcription will be initiated. During translation, the mRNA will be converted into protein, and posttranslational modifications will then complete the signaling pathway to produce a functional protein.

The ERα and ERβ [^{3}H]-estradiol competitive binding assay [9], ERE-luciferase (ERE-luc) induction [10] in MCF-7 ERα positive and ERβ positive cell lines, and the Ishikawa alkaline phosphatase assay [11] are assays that target key points in the ER signaling pathway. The general paradigm is that a ligand must bind the estrogen receptor in order to have any downstream effects (Scheme **1**); therefore, the competitive binding assay is often utilized as the primary screen [12] to determine if a botanical is further characterized for estrogenic activity. Next, the functional activity, agonist or antagonist, is evaluated using cell-based assays at different points along the pathways. Unfortunately, complicating the matter of characterizing estrogenic activity is the lack of a naturally endogenously expressing ERβ positive cell line [13], which has raised the question of how to show functional activity for ERβ ligands. Cell lines have been stably transfected with ERβ; however, it is clear that the value of an ER stably transfected cell line is not the same as an endogenously expressing cell line [13], since the downstream cellular milieu does not necessarily remain intact. For example, an endogenously expressing ERα cell line will proliferate in the presence of physiological levels of 17β estradiol;

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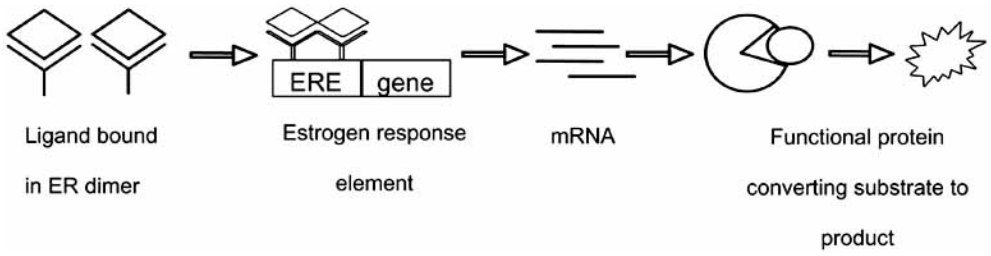

Scheme 1. Simplified depiction of the classical estrogen receptor signaling pathway from ligand binding to functional protein.

whereas, the stably transfected cell line S30 will not grow [14]. However, it is possible to use stable cell lines that are transfected with the estrogen response element-luciferase plasmid [14] since the ERE is the immediate downstream target of the ligand-bound ER.

The purpose of this research was to evaluate seventeen plants (Table **1**) using a variety of established cell-free and cell-based estrogenic assays to biologically characterize the botanicals. Since both estrogens and selective serotonin re-uptake inhibitors (SSRIs) have been known to alleviate symptoms associated with menopause [15], plants were selected based on a literature search of the Natural Products Alert (NAPRALERT) database for the most widely used remedies for menopausal symptoms, menstrual disorders, or reported estrogenic, serotonergic, anti-steroidogenic, and anti-fertility activity. These plants were obtained, extracted, and evaluated for estrogenic activity *in vitro* using the ERα and ERβ [^{3}H]-estradiol competitive binding assay, ERE-luc induction in MCF-7 ERα positive and ERβ positive cell lines, and the Ishikawa alkaline phosphatase assay.

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Itasca, IL) unless stated otherwise.

Plant Material

As indicated in Table **1**, *Angelica sinensis* (roots) was purchased at Yin Wall City, Chicago, IL (2001). *Asclepias tuberosa* was bought from www.blessedherbs.com. *Cimici-*

Table 1. Plants Selected for Estrogenic Screening

Botanical Name	Plant Part	Common Name	Source	Rational
Alisma plantago-aquatica L.	Rhizomes	Water Plantain	[1]PureWorld	Menopausal Symptoms [34]
Angelica sinensis (Oliv.) Diels	Roots	Dang-Gui	[2]Yin Wall City, Inc.	Menopausal Symptoms; [4, 81] serotonergic [35]
Asclepias tuberosa L.	Roots	Butterfly Weed	[2]Blessedherbs	Contains Steroidal Compounds [36]
Beta vulgaris L.	Roots	Beets	[2]Local grocery	Menopausal Symptoms [37]
Cimicifuga americana Michaux	Roots	Yellow Cohosh	[3]Swain County, NC	Chemotaxonomic Relationship [38]
Cimicifuga racemosa (L.) Nutt.	Aerial	Black Cohosh	[3]Sevier County, TN	Related Plant Part
Cimicifuga racemosa (L.) Nutt.	Roots	Black Cohosh	[3]Sevier County, TN	Menopausal Symptoms [39-50]; Serotonergic [22, 51]
Cimicifuga rubifolia (Kearney) Kartesz	Aerial	Appalachian Bugbane	[3]Hancock County; TN	Chemotaxonomic Relationship [38]
Cornus officinalis Sieb. & Zucc.	Fruits	Dogwood	[1]PureWorld	Menopausal Symptoms [34]
Daucus carota L.	Roots	Queen Anne's lace; Carrot	[2]Local grocery store	Anti-Steroidogenic Activity [52]; Estrogenic Activity [53]; Antifertility Activity [54]
Paeonia moutan Sims	Bark	Peony	[1]PureWorld	Menopausal Symptoms [55]
Pueraria lobata (Willd.) Ohwi.	Aerial	Kudzu	[3]Evanston, IL	Estrogenic Activity [98, 99]; Menopausal Symptoms [56]; Serotonergic Activity [57]
Pueraria mirifica Airy, Shaw & Suvatabandhu	Bark	Kwao Keur	[1]PureWorld	Estrogenic Activity [58-61]
Valeriana officinalis L.	Roots	Valerian	[1]PureWorld	Serotonergic Activity [62]
Viburnum opulus L.	Bark	Cramp Bark	[1]PureWorld	Smooth Muscle Antispasmodic [63]
Viburnum prunifolium L.	Bark	Black Haw	[1]PureWorld	Menopausal Symptoms [64]
Vitex agnus-castus L.	Fruits	Chasteberry	[1]PureWorld	Menstrual Disorders [65-70]; Menopausal Symptoms [71]

[1]PRovided.
[2]Purchased.
[3]Collected.

fuga americana (aerial parts) was collected in Swain County, NC. *Cimicifuga racemosa* (aerial parts) was collected in Sevier County, TN. *Cimicifuga rubifolia* (aerial parts) was collected in Hancock County, TN. *Pueraria lobata* was collected in Evanston, IL. *Alisma plantago-aquatica* (rhizomes and roots), *Cimicifuga racemosa* (rhizomes and roots), *Cornus officinalis* (fruits), *Paeonia moutan* (bark), *Polygonum multiflorum* (roots), *Pueraria mirifica* (bark), *Valeriana officinalis* (roots), *Viburnum opulus* (bark), *Viburnum prunifolium* (bark), and *Vitex agnus-castus* (berries), were provided by PureWorld Botanicals, now NATUREX (South Hackensack, NJ). *Beta vulgaris* (roots) and *Daucus carota* (roots) were purchased from a local grocery store. Voucher specimens have been deposited at the Pharmacognosy Field Station, Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, and verified by botanist Dr. D. D. Soejarto.

Extraction

Using the industry-standard operating procedures for botanical extractions, sequential percolation was used to extract the botanicals as follows: Plant material (200 g each) except *A. sinesis* was minced and macerated in petroleum ether (PE, 600 mL) overnight, and percolated exhaustively with the same solvent (total 8 L). The marc was macerated in dichloromethane (CH_2Cl_2, 600 mL) overnight, and percolated exhaustively with CH_2Cl_2 (8 L). Finally, the marc was macerated in 75% ethanol (EtOH, 600 mL) overnight, and percolated exhaustively with of 75% EtOH (8 L). The PE, CH_2Cl_2, and 75% EtOH percolates were combined separately, and the solvents were removed *in vacuo* to yield respective extracts of different polarity.

The minced sample of *A. sinensis* (2 kg) was macerated in CH_3OH (2 L, 24 h) and percolated exhaustively with CH_3OH (6 L). The percolates were combined and the solvent evaporated *in vacuo* to yield the CH_3OH extract. The CH_3OH extract was dissolved in 15% aqueous CH_3OH and partitioned with petroleum ether (3 × 1 L). The aqueous-methanol partition was evaporated *in vacuo* to remove CH_3OH, and the remaining aqueous partition was successively partitioned with $CHCl_3$ (3 × 1 L) and n-butanol (BuOH, 3 × 1 L). Removal of the solvent yielded the petroleum ether, $CHCl_3$, BuOH, and H_2O soluble partition.

ERα and ERβ Competitive Binding Assays

The competitive ERα and ERβ binding assays were used with tritiated estradiol based on the method of Obourn *et al.* [9] with minor modifications [16], to determine *in vitro* binding affinities of the substrates with the receptors. The reaction mixture consisted of sample in DMSO (5 µL), pure human recombinant diluted ERα and ERβ (0.5 pmol, 5 µL) in ER binding buffer, "Hot Mix" [400 nM, 5 µL prepared fresh using 95 Ci/mmol [^{3}H] estradiol, diluted in 1:1 ethanol:ER binding buffer; obtained from NEN Life Science Products (Boston, MA)], and ER binding buffer (85 µL). The incubation was carried out at room temperature for 2 h before a hydroxyapatite slurry (HAPs, 50%, 100 µL) 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 10,000 × g 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 ethanol (200 µL) and transferred to scintillation vials. An additional volume of ethanol (200 µL) 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 [^{3}H] estradiol binding to each ER was determined using Equation 1. The binding capability (percent) of the sample was calculated in comparison with that of estradiol (50 nM, 90%).

Equation 1. $[1 - (dpm_{sample} - dpm_{blank})/(dpm_{DMSO} - dpm_{blank})] \times 100 = \%$ sample binding

Cell Culture Conditions

The Ishikawa cell line was provided by R. B. Hochberg (Yale University, New Haven, CT) and was maintained in Dulbecco's Modified Eagle medium (DMEM/F12) containing sodium pyruvate (1%), non-essential amino acids (NEAA, 1%), glutamax-1 (1%), insulin (0.05%), and heat-inactivated fetal bovine serum (FBS, 10%). MCF-7 WS8 cells were provided by V. C. Jordan (Fox Chase Cancer Center) and were grown in RPMI 1640 media containing glutamax-1 (1%), NEAA (1%), insulin (0.05%), and heat-inactivated FBS (5%). The MCF-7 C4-12-5 ERβ positive stable cell line ([17] referred to as MCF-7 ERβ) was provided by D. B. Lubahn (University of Missouri) and was grown in MEM (catalogue number 3024) supplemented with stripped calf bovine serum (CBS, 5%), Pen/Strep (2%), insulin (6 ng/mL), sodium carbonate (2.2 g/L), HEPES (1.25 M, 8 mL), glutamax (1%), and G418S (50 mg/mL stock, 6 mL). Stripped serum was prepared by incubating the serum with acetone-washed activated charcoal (100 mg/mL) at 4 °C for 30 min, and centrifuged at 4,000 RPM for 15 min at 4 °C. This step was repeated in triplicate. DMSO concentrations for all cell culture assays were below 0.1%.

Induction of Alkaline Phosphatase in Cultured Ishikawa Cells

The procedure of Pisha *et al.* [11] with minor modifications [16] was used. Ishikawa cells (1.5×10^4 cells/190 µL/well) were preincubated in 96-well plates overnight in estrogen-free medium. Test samples (20 µg/mL final concentration in DMSO) were added to the cells in a total volume (200 µL media/well) were incubated at 37 °C for 4 days. For the determination of antiestrogenic activity, media used to dilute the test samples was supplemented with estradiol (2 nM). The induction plates were processed by washing the plates with PBS and adding Triton x 100 (0.01%, 50 µL) in Tris buffer (pH 9.8, 0.1 M). Plates were subjected to a freeze thaw (-80 ºC for at least 24 h before warming to 37 ºC). An aliquot (150 µL) of *p*-nitrophenylphosphate (phosphatase substrate, 1 mg/mL) in Tris buffer (pH. 9.8, 0.1 M) was added to each well. The enzyme activity was measured by reading the release of *p*-nitrophenol 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. Estrogenic induction was calculated using Equation 2, and for antiestrogenic determination, the percent induction as compared with the background induction control was calculated using Equation 3.

Equation 2. $[(slope_{sample} - slope_{cells})/(slope_{estrogen} - slope_{cells})] \times 100 = \%$ estrogenic induction

Equation 3. $[1-((slope_{sample} - slope_{cells})/(slope_{estrogen} - slope_{cells}))] \times 100 = \%$ antiestrogenic induction

Cytotoxicity Assay

Cytotoxicity can be a false negative in the agonism assay and a false positive in the antagonism assay. To identify false responses, a 96-hour assay is run in parallel with the induction assay. Cytotoxicity was determined for the Ishikawa alkaline phosphatase induction assays using ~1,000 cells per well in a 96-well plate (5 x 106 cells/mL). Cells were treated with the same samples used in the inductions assays for the respective cell lines. The plates were harvested after 96 h. A day zero plate was prepared by plating at least half of a 96-well plate and allowing the cells to settle overnight. To process the plates 50 µL of cold 50% trichloroacetic acid (TCA) was added to the media (final concentration 20%) and stored at 4 ºC for 30 min. The plates were washed with tap water and dried overnight. The following day the plates were stained with 100 µL of sulphohodamine B (SRB) at room temperature and washed with 1% acetic acid and dried in the dark overnight. The dye was suspended in 200 µL Tris buffer (0.1 mM) and mixed using a plate shaker until the dye was completely solubilized. The plates were read using the endpoint mode at 515 nm. Calculation of the percent cytotoxicity was determined using Equation 4. Generally cytotoxicity greater that 20% will interfere with cell-based alkaline phosphatase assays.

Equation 4. $[1-((OD_{sample} - OD_{day0})/(OD_{DMSO} - OD_{day0}))] \times 100 = \%$ cytotoxic

Measure of 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. Both MCF-7 WS8 and MCF-7 ERβ cell lines were cultured in estrogen-free media 96 h before transfection. The cells were transfected with the pERE-luciferase plasmid (2 µg), which contains three copies of the Xenopus laevis vitellogenin A2 ERE upstream of fire fly luciferase (a gift from Dr. V.C. Jordan). To normalize transfection efficiency, pRL-TK plasmid (1 µg, Promega) was co-transfected. Cells (5 x 10^6) in serum-free media were transfected by electroporation in a 0.4 cm cuvette (Bio-Rad Laboratories) at a voltage (0.320 kV) and a high capacitance (950 µF) in a GenePulser X-cell (Bio-Rad Laboratories). The cells were resuspended in estrogen-free media, transferred to 6-well plates immediately after electroporation, and incubated overnight. The cells were treated with the extracts for 24 h. The luciferase activities in the cell lysates were measured using the Dual-Luciferase Reporter Assay System from Promega (Madison, WI) with a FLUOstar OPTIMA (BMG LABTECH, Durham, NC).

RESULTS

ER Alpha and ER Beta Competitive Assay

The plant extracts were screened in the ERα and ERβ competitive assay at 200 µg/mL (n = 5, two independent measurements). Extracts where the mean percent inhibitory activity was within one standard deviation of 50% or greater were considered active (Table **2**). Six plants were active in the ERα assay (Fig. **1**): the petroleum ether extracts of *A. sinensis* (81%), *A. tuberosa* (69%), *C. racemosa* (aerial parts; 55%), *V. officinalis* (43%), the petroleum ether and the dichloromethane extracts of *C. rubifolia* (56% and 51%, respectively), and the dichloromethane extract of *P. lobata* (63%). There were thirteen plant extracts that bound the ERβ (Fig. **1**): the 75% ethanol and the dichloromethane extracts of *P. lobata* (63 and 45%, respectively), the petroleum ether extract and chloroform partition of *A. sinensis* (99 and 46%, respectively), the petroleum ether extracts of *A. plantago-aquatica* (51%), *A. tuberosa* (85%), *B. vulgaris* (44%), *C. americana* (47%), *C. officinalis* (48%), *C. racemosa* (aerial parts; 64%), *C. rubifolia* (66%), *P. moutan* (60%), *P. multiflorum* (BC 268; 50%), *V. officinalis* (81%), and *V. agnus-castus* (68%).

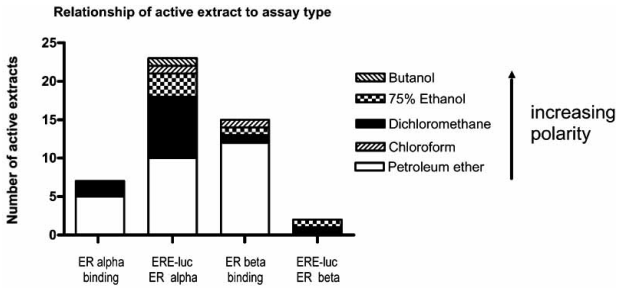

Fig. (1). Bar chart representation of the frequency of extracts with activity in either ER alpha binding or ER beta binding assays and ERE-luc ER alpha or ERE-luc ER beta positive cell lines.

Estrogenic Alkaline Phosphatase Induction in the Ishikawa Cell Line

The ALP assay was used to distinguish agonist activity from antagonist activity [11]. At the tested concentration of 20 µg/mL (n = 3 independent times in triplicate), the petroleum ether extract of *A. sinensis* (49%), and the dichloromethane extracts of *B. vulgaris* (44%), *C. americana* (79%) had antiestrogenic activity that did not appear to be caused by cytotoxicity (Fig. **2**). Cytotoxicity can have a negative impact on the results of the alkaline phosphatase assays when it is above 20%, as well as on the ERE-luc assay. In the estrogenic ALP assay, cytotoxicity was apparent at 20 µg/mL for the dichloromethane extracts of *A. tuberosa* (106%), *C. officinalis* (31%), *P. multiflorum* (BC 286; 31%), *P. mirifica* (115%), *V. prunifolium* (42%), and *V. agnus-castus* (112%), the petroleum ether extracts of *V. agnus-castus* (39%), *P. mirifica* (100%), and *C. officinalis* (31%), the 75% ethanol extracts of *A. tuberosa* (99%), *P. mirifica* (60%), and the chloroform partition of *A. sinensis* (31%). In the ERE-luc assay ERα positive cell line, cytotoxicity was apparent in all of the *A. tuberosa* treated samples as indicated by the high standard deviation and low value for the transfection control vector, pRL-TK.

Interestingly, some extracts when combined with estradiol appear to have a greater estrogenic response than the extract alone (Fig. **3**). The 75% ethanol extract of *C. racemosa*, aerial parts had a -48% antiestrogenic response indicating that more alkaline phosphatase enzyme was induced when compared to the extract without 17β estradiol (4%).

Table 2. Screening Results in ER Binding, ERE-Luciferase, and Alkaline Phosphatase Assays

Plant or Sample Name	Extract Type	[1]Binding	[2,3]ERE-Luc	[3,4]ALP	[3,4]ALP	[3,4]SRB	[1]Binding	[2,3]ERE-Luc
		ER Alpha	ER Alpha	Estrogenic	Antiestrogenic	Cytotoxicity	ER Beta	ER Beta
17 β estradiol	Control	**95 ± 1**	**21 ± 7**	**100 ± 10**	-10 ± 8	4 ± 2	**90 ± 1**	**7 ± 3**
4-hydroxytamoxifen	Control	**96 ± 1**	1 ± 0.1	0 ± 5	**94 ± 1**	2 ± 1	**94 ± 1**	1 ± 0.2
DMSO	Control	0	1 ± 0.2	1 ± 2	0 ± 5	1 ± 2	0	1 ± 0.2
Alisma plantago-aquatica	Petroleum ether	31 ± 5	0.9 ± 0.4	-7.3 ± 4.2	-1.5 ± 13.3	10.7 ± 16.2	**51 ± 7**	2.3 ± 1.9
Angelica sinensis	Petroleum ether	[5]**81 ± 10**	**9.7 ± 3.5**	-4.7 ± 6.2	**48.7 ± 9.5**	16.4 ± 13.6	**99 ± 9**	1.1 ± 0.4
	Chloroform	19 ± 9	**5.9 ± 3**	12.3 ± 4.7	19.4 ± 9.8	30.6 ± 8	**46 ± 6**	0.8 ± 0.5
	Butanol	4 ± 3	**9.5 ± 2.2**	12.4 ± 8.2	-16.3 ± 4.5	3.3 ± 15.2	0 ± 2	1.2 ± 0.2
Asclepias tuberosa	Petroleum ether	**69 ± 7**	1.1 ± 0.4	-17.9 ± 15.5	-9.1 ± 21	-19.9 ± 9.8	**85 ± 5**	1.1 ± 0.8
	Dichloromethane	16 ± 9	4.1 ± 3.1	-26.5 ± 4.5	114.6 ± 6.7	**106.1 ± 5.3**	32 ± 10	**10.7 ± 0.8**
	75% ethanol	0 ± 2	3.9 ± 4.3	-10.3 ± 8.9	110.1 ± 8.5	**99.1 ± 3.6**	5 ± 8	1.3 ± 1.1
Beta vulgaris	Petroleum ether	35 ± 10	**4.7 ± 1.9**	-2.4 ± 17.4	4.4 ± 14.8	-15.9 ± 29.4	**44 ± 7**	1.1 ± 0.3
	Dichloromethane	33 ± 11	3.2 ± 2	-2.3 ± 5.5	**43.7 ± 8.6**	-20.2 ± 30.9	30 ± 5	1.1 ± 0.1
Cimicifuga americana	Petroleum ether	37 ± 8	1.6 ± 0.7	-5.8 ± 14.4	27.1 ± 27.5	-3.7 ± 37	**47 ± 10**	1.4 ± 0.3
	Dichloromethane	31 ± 5	2.8 ± 1.1	-16.2 ± 14.9	**79.2 ± 19.5**	5.8 ± 32.1	21 ± 9	1.4 ± 0.6
Cimicifuga racemosa	DCM extract	34 ± 7	**6.6 ± 3.2**	-2.5 ± 1.3	3.5 ± 8.7	8.3 ± 5.2	45 ± 3	1.1 ± 0.4
	75% EtOH	21 ± 5	**5.8 ± 1.8**	4.3 ± 5.3	**-48.4 ± 4.9**	0.1 ± 5.7	23 ± 3	1 ± 0.2
	PE extract	**55 ± 8**	3.2 ± 1.5	-4.5 ± 4.8	21.3 ± 7.2	4.8 ± 10.9	**64 ± 8**	1 ± 0.5
Cimicifuga rubifolia	Petroleum ether	**56 ± 2**	2.5 ± 2	-13.1 ± 12.4	33.2 ± 17	-23.2 ± 25	**66 ± 6**	1.1 ± 0.5
	Dichloromethane	**51 ± 5**	**7.4 ± 1.1**	0.3 ± 15.9	21 ± 14.2	4.4 ± 19.8	40 ± 8	0.8 ± 0.3
Cornus officinalis	Petroleum ether	26 ± 16	**24.3 ± 6.6**	2.6 ± 5.9	41.8 ± 4.1	11.5 ± 3.4	**48 ± 4**	1.5 ± 0.5
	Dichloromethane	0 ± 4	2.5 ± 0.7	-2.2 ± 4.8	24.9 ± 5.2	**30.6 ± 19.1**	0 ± 2	1.3 ± 0.3
Daucus carota	Petroleum ether	21 ± 6	**12.3 ± 5.1**	-6.8 ± 11.2	-26.6 ± 14.6	-18.7 ± 19.1	36 ± 6	0.9 ± 0.5
	Dichloromethane	22 ± 5	**5.4 ± 1.8**	-5.7 ± 8	-11.5 ± 16.1	13.7 ± 14.1	26 ± 6	0.7 ± 0.2
Paeonia moutan	Petroleum ether	21 ± 5	2.8 ± 1	-9.7 ± 11.4	8.7 ± 16.7	7.7 ± 19.7	**60 ± 6**	2 ± 1.7
Polygonum multiflorum	Petroleum ether	15 ± 8	**21.7 ± 8.5**	6.5 ± 1.8	1.2 ± 3.3	7.6 ± 14.3	**50 ± 9**	1.4 ± 0.7
	Dichloromethane	30 ± 7	**25.8 ± 11.2**	12.3 ± 3.4	0.4 ± 12.3	12.7 ± 17.8	36 ± 13	3 ± 2.5
Pueraria lobata	Petroleum ether	32 ± 2	**10.3 ± 2.8**	-10.8 ± 12.6	**46.9 ± 18.9**	12.7 ± 30	36 ± 2	1.5 ± 1.4
	Dichloromethane	**63 ± 6**	**24.5 ± 9.9**	-0.3 ± 25.6	36 ± 32.6	-3.3 ± 5.5	**45 ± 19**	1.2 ± 0.5
	75% ethanol	17 ± 16	**26.9 ± 12.7**	-1.3 ± 13.8	28.9 ± 40.7	7.6 ± 50.1	**63 ± 3**	**3.5 ± 1.3**
Pueraria mirifica	Petroleum ether	12 ± 8	[6]N.T.	-2.7 ± 2.6	58.5 ± 37	**100.6 ± 38.8**	18 ± 12	N.T.
	Dichloromethane	12 ± 5	**25.7 ± 9.5**	-4.2 ± 1.1	101.1 ± 8.6	**115 ± 2.2**	19 ± 11	3.2 ± 1.6
	75% ethanol	18 ± 7	**28 ± 8.6**	-1.4 ± 4.8	52.2 ± 61	**60.4 ± 62.2**	31 ± 8	2.4 ± 1.3
Valeriana officinalis	Petroleum ether	**43 ± 11**	**7.2 ± 3.1**	-7.8 ± 7.6	33.7 ± 16.8	12.5 ± 19.3	**81 ± 4**	1.3 ± 0.8
	Dichloromethane	13 ± 12	**5.9 ± 1.6**	-8.5 ± 7.7	19.4 ± 8.2	-7.6 ± 21	13 ± 6	0.7 ± 0.1
Viburnum prunifolium	Dichloromethane	3 ± 6	0.7 ± 0.5	-3.5 ± 3	68.5 ± 6.3	**42 ± 4.4**	3 ± 4	1 ± 0
Vitex agnus-castus	Petroleum ether	37 ± 6	**9.5 ± 2.5**	-1.1 ± 1.6	43.5 ± 3.3	**39.1 ± 8.3**	**68 ± 9**	1.1 ± 0.7
	Dichloromethane	13 ± 3	**9.6 ± 6.6**	-2.3 ± 1.5	94.5 ± 7.2	**112.5 ± 8.8**	20 ± 19	0.8 ± 0.3

[1]Percent binding at 200 μg/mL.
[2]Fold induction where DMSO is 1.
[3]Tested at 20 μg/mL.
[4]Percent induction (estrogenic), inhibition (antiestrogenic), or cytotoxic.
[5]Bold face-type indicates extract with assay activity.
[6]Not tested due to the limited quantities available.

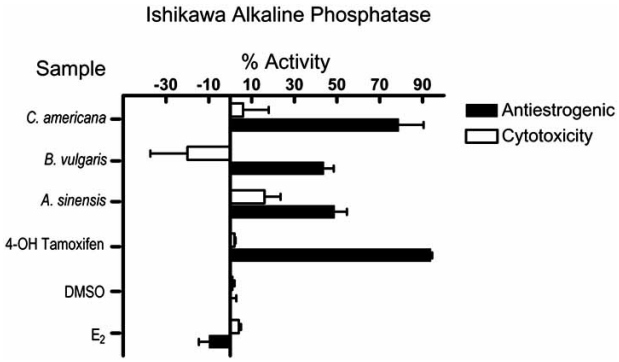

Fig. (2). Antiestrogenic and cytotoxic graph of active samples and controls. The petroleum ether extract of *A. sinensis*, and the dichloromethane extracts of *B. vulgaris* and *C. americana* had antiestrogenic activity without apparent cytotoxicity in the Ishikawa alkaline phosphatase inhibition assay when tested at 20 μg/mL. Samples were tested at least three independent times in triplicate. Samples were considered active if they were within one standard deviation or greater of 50% for the antiestrogenic assay. Samples with greater than 20% cytotoxicity are know to interfere with the accuracy of the antiestrogenic assay by causing a false positive result.

Also, the 75% ethanol extract of *V. prunifolium* also had a similar effect when combined with 17β estradiol (-33%) compared to the extract alone (0%). This may also be true for the 75% ethanol extract of *C. rubifolia* (-31%) and the petroleum ether extract of *D. carota* (-27%), but the standard deviations for both samples were large (22 and 15, respectively).

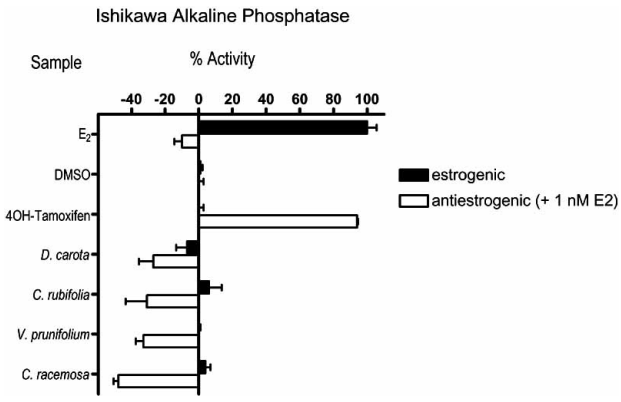

Fig. (3). Samples with possible synergistic activity when combined with 17β estradiol in the Ishikawa alkaline phosphatase assay. Samples were treated alone to determine the inherent estrogenic activity of the sample, or in combination with 1 nM E_2 to determine if there was an antiestrogenic effect of the samples. 17β Estradiol shows the stereotypical estrogenic response alone and is therefore estrogenic, while 4-hydroxytamoxifen shows a stereotypical antiestrogenic response when combined with 1 nM E_2, but it is not estrogenic alone. The 75% ethanol extract of *C. racemosa* (aerial parts) was not estrogenic alone, but when combined with 1 nM E_2 was actually more estrogenic than when E_2 is tested in the presence of 1 nM E_2. The 75% ethanol extract of *V. prunifolium* and *C. rubifolia*, and the petroleum ether extract of *D. carota* are also displayed. Samples were tested in triplicate at least three independent times, and are represented as averages ± standard deviation.

ERE-Luciferase Induction in ER Alpha and ER Beta Positive Cell Lines

Following the ERα and ERβ competitive assay, the transient transfection of MCF-7 WS8 or MCF-7 C4-12-5 ERβ+ cell lines with the ERE-luciferase plasmid was used to help confirm functional activity of the extracts that had activity in the isolated ER competitive assay. The cells were treated with extracts (20 μg/mL) for 24 h, and the activity was first normalized for transfection efficiency using the pRL-TK vector, and then normalizing to the DMSO control (DMSO = 1). Extracts with > two–fold induction were considered active (Table **1**; $n \geq 3$).

In the MCF-7 WS8 (ERα positive) cell line, the petroleum ether, chloroform, and butanol partitions of *A. sinensis* (9.7, 5.9, and 9.5, respectively), the petroleum ether, dichloromethane, and 75% ethanol extracts of *P. lobata* (10.3, 24.5, and 26.9, respectively), the dichloromethane and 75% ethanol extracts of *C. racemosa* (aerial parts; 6.6 and 5.8, respectively) and *P. mirifica* (25.7 and 28.0, respectively), the petroleum ether and dichloromethane extracts of *D. carota* (12.3 and 5.4, respectively), *P. multiflorum* (BC 268: 21.7 and 25.8; BC 286: 15.1 and 16, respectively), *V. officinalis* (7.2 and 5.9), and *V. agnus-castus* (9.5 and 9.6, respectively), the petroleum ether extracts of *B. vulgaris* (4.7), *C. rubifolia* (7.4), *C. officinalis* (24.3) were active (Fig. **1**). All extracts of *A. tuberosa* were cytotoxic at 20 μg/mL, which caused some false positive activity. In the MCF-7 ERβ cell line, none of the petroleum ether extracts were active. The 75% ethanol extract of *P. lobata* (3.5) and the dichloromethane extracts of *A. tuberosa* (10.7) and *P. mirifica* (3.2) had activity (Fig. **1**).

DISCUSSION

The UIC/NIH Center for Botanical Dietary Supplements Research (the Center) was established in the fall of 1999 to address issues of standardization, quality, safety, and efficacy of botanical dietary supplements. Using a multidisciplinary strategy to achieve its basic and clinical research objectives, the Center has focused on botanicals with potential benefits for women's health. Based on these data, over time a paradigm shift has occurred within the Center in utilizing the assays to provide the most meaningful data. For example, during the initial years when testing botanicals in the Center, the competitive estrogen receptor binding assay was primarily performed, and then the induction of alkaline phosphatase in Ishikawa cells was used to determine if the botanicals were acting as antagonists or agonists. The logical thought was that botanicals that were not active in the competitive binding assay would not be active in the alkaline phosphatase assay. However, recently increased evidence of nonclassical estrogen receptor signaling has been more fully understood [18-20]. Additionally, the Center has identified botanicals that do not bind to the isolated receptor, but exhibited activity in the cell-based assays. Furthermore, *C. racemosa* (roots) is an example of a botanical that questioned this paradigm since it was not estrogenic either *in vitro* or *in vivo* [21, 22]*;* however, it is one of the most popular botanicals used by menopausal women. Alternative mechanisms such as the serotonergic [22] and opiod [23] pathways have since been implicated.

While the selected assays are not new, and the throughput for these assays has not been improved, the summation of data from these assays has improved the total information content and enabled possible new combinations of activity for plant extracts. The ER binding assays provide information on binding to the receptor in a cell-free environment. While this information is important, it does not indicate if the activity is functional, or what kind of function it has, agonist or antagonist activity [9]. The Ishikawa ALP assay is used to distinguish agonist activity from antagonist activity, but it is only ERα positive, and is affected by cytotoxicity [11] whereas the cell-free ER-binding assays are not affected by cytotoxicity. Finally, the ERE-luc assay provides functional agonist activity in ERα or ERβ cell lines and is capable of some metabolism [24]. While not incorporated into the panel of assays to identify antagonist activity, it is fully capable of distinguishing agonist from antagonists and compounds without functional activity [24]. It is also affected by cytotoxicity, and it is significantly slower to process agonist and antagonist assays in parallel to the same extent as the ALP assays. Therefore each assay provides some information about mechanism of action individually. However, due to the inherent limitations of each assay, the characterization of a sample is significantly improved by incorporating these assays into a panel.

In order to identify plants that might have a beneficial effect on symptoms associated with menopause, a NAPRALERT search was conducted to select plants with previously reported hormonal or neurotransmitter activity. Among the plants selected, nine plants had previously been reported to be useful for the relief of menopause symptoms, and four plants were specified as having estrogenic activity. In general, to cover the whole range of polarity for phytoconstituents three extracts of each plant, petroleum ether, dichloromethane, and 75% ethanol, were prepared and tested in the isolated ERα and ERβ competitive assay, ERE-luc fold-induction in both ERα and ERβ positive breast cancer cell lines, and the induction of alkaline phosphatase in the Ishikawa endometrial cell line. The extracts were also tested in a secondary assay for cytotoxicity. Due to the nature of *in vitro* assays we have had to define for the purpose of evaluating these data the terms "false positive" and "false negative". False positive activity is attributed to a mechanism assumed to be independent of the tested mechanism such as non-specific binding of lipophilic material to an isolated receptor which blocks the tritiated-estradiol from specifically binding to the binding pocket. A false negative result has been defined to mean a lack of activity which is attributed to a mechanism assumed to be independent of the tested mechanism such as a sample that is cytotoxic rather than specifically inhibits a specific enzymatic reaction in the cell.

The initial biological characterization of the extracts indicated that many of the petroleum ether extracts had activity in the ER binding assays, particularly in the ERβ assay, where twelve of the fifteen active extracts were petroleum ether extracts (Fig. **1**). Petroleum ether extracts are known to contain fatty acids, which have been reported in the literature to non-specifically bind isolated receptors and/or may only have weak estrogenic activity [25]. This hypothesis was supported by the observation that the 75% ethanol extract of *P. lobata*, which had activity in the ERβ competitive assay, also had activity in the ERE-luc assay in the ERβ positive cell line (Fig. **1**), an observation consistent with other reported literature [26, 27]. In the ERα assays, there was a correlation between ERα competitive binding activity and ERE-luc assay for the petroleum ether extract of *A. sinensis* (81% binding affinity and 9.7 fold induction) and the dichloromethane extracts of *C. rubifolia*, and *P. lobata*. For the latter two extracts, there was relatively weak activity (51% and 63%, respectively) in the ERα competitive binding assay, but relatively strong fold-induction (7.4 and 25, respectively) at one-tenth the concentration in the ERE-luc assay. There appeared to be a pattern of activity where the first solvent used in the sequential extraction has more activity (Fig. **1**). This may be partially related to the sequential extraction method, which is industry-standard. Since compounds can dissolve in more than one solvent to varying degrees, it is possible that the first extracting solvent, petroleum ether, extracted more of the active constituents compared to the following solvents. Based on in-house evaluation of specific plants subject to individual extraction and sequential extraction, the order, parallel or sequential did not show as cause for concern. In general, the relative proportions of activity would be maintained if the plant material had been extracted in parallel. At best a change in extraction method may change the degree to which an extract is active, but it would not cause an inactive extract to become active or *vice versa*.

However, what was unexpected was the number of extracts that did not bind the receptor, but did have activity in the ERE-luc assay (Fig. **1**). In the ERβ assays, *A. tuberosa* and *P. lobata* were the only plants that did not have activity in the competitive ERβ binding assay while having activity in the ERE-luc assay in ERβ cells. In the ERα assays, this was also the case for the tested chloroform and butanol partitions of *A. sinensis* and certain extracts of *C. racemosa*, aerial parts, *C. officinalis*, *D. carota*, *P. multiflorum*, *P. lobata*, *P. mirifica*, *V. officinalis*, and *V. agnus-castus*. One important example is *A. sinensis*, which did not have ERα isolated receptor competitive binding activity for any of the partitions except for petroleum ether, but showed potent activity in the ERE-luc assay for both the chloroform and butanol partitions. While the petroleum ether extracts generally did not have functional activity, *A. sinensis* did have functional activity in the ERE-luc assay and antiestrogenic activity in the Ishikawa alkaline phosphatase assay. *Angelica sinensis* is one of the most commonly used herbs in China for relief of PMS and menopause; however, the mechanism of action has not clearly been identified. One reason might be the interesting pattern of activity where it can have receptor binding activity, ERE-luc and antiestrogenic activity as in the case of the petroleum ether extract, but it also might have components that are not ligands for the receptor, but still have activity in the ERE-luc assay.

Classically, extracts that do not bind the estrogen receptor would not be thought to be active in the ERE-luc assay, which is generally used to identify ligand-ER complexes that bind the ERE causing gene transcription. The disconnection between the binding to the estrogen receptor and the activation of the ERE has raised many hypotheses. One is that these plant extracts do not bind to the ER, but instead use a non-classical estrogen pathway that phosphorylates the ER [28]. The non-liganded, phosphorylated ER has been demonstrated to bind the ERE and recruit transcription factors. This could explain why it has been challenging to explain the mechanism

of action for plants that have been traditionally used by women, but when the extracts were scientifically tested, they do not contain ER ligands. Another hypothesis is that the extract contains ligands that do not competitively bind to the ER, but instead bind to a second binding site [29]. The full implication of this situation with different structures has yet to be explored, but does raise some interesting possibilities. These hypotheses raise the concern that women taking extracts that are considered to be "non-estrogenic" due to health concerns of hormone-dependent cancers might still be at risk. A third explanation might be metabolism of botanical compounds in the cells to estrogenic products. It has previously been shown that biochanin A, a compound found in *Trifolium pratense*, does not inherently have estrogenic activity, but can be metabolically converted in the MCF-7 WS8 cell line to the estrogenic compound, genistein [16]. Similarly, isoxanthohumol, a compound found in *Humulus lupulus*, can also be metabolized to 8-prenylnaringenin, a potent phytoestrogen [16, 30].

In the Ishikawa assay, none of the extracts had agonist activity, but some had antiestrogenic activity that appeared to be unrelated to cytotoxicity or direct inhibition of alkaline phosphatase. At 20 μg/mL the petroleum ether partition of *A. sinensis* was the only sample that had ERα activity in all three assays. In fact, *A. sinensis* might be considered a true SERM since it was an agonist in the ERE-luc assay, but had antagonistic activity in the Ishikawa assay. *Angelica sinensis* may also have selective tissue effects since the antiestrogenic Ishikawa assay was in an endometrial cell line, while the ERE-luc was in a breast cancer cell line. Other extracts that appear to have antiestrogenic activity in the ALP assay were the dichloromethane extracts of *B. vulgaris* and *C. americana*. It is noteworthy that the ethnobotanical use of *B. vulgaris* has generally been attributed to its vitamin content rather than the presence of antiestrogenic compound(s). *Cimicifuga americana* has not been reported in the literature as being used to alleviate any hormone-related symptoms; however, it is taxonomically closely related to *C. racemosa*.

There were some unexpected results in the alkaline phosphatase assay. Some extracts, had large negative values in the antagonist ALP assay. This initially was overlooked, but after carefully review of the data, and multiple independent repetitions of the assay, the values were determined to be reproducible. The antagonist assay was designed to identify compounds that blocked the effect of estrogen. When this occurs, the ALP enzyme is not produced, and substrate is not converted to product. Therefore, a score of 100% in this assay would indicate complete blockage of the estrogenic activity, and 0% would indicate that the estrogenic activity led to the ALP enzyme converting substrate to product. When the data go into the negative range, it might indicate a synergistic effect with estrogen where even more ALP is produced causing faster conversion of substrate to product (Fig. **3**).

Four plants had negative values in the antagonist ALP assay indicating a possible synergistic activity: the 75% ethanol extracts of *C. racemosa* (aerial parts), *C. rubifolia*, and *V. prunifolium*, and the petroleum ether extracts of *D. carota*. The activity was most prevalent in the 75% ethanol extract of *C. racemosa* (aerial parts). The extract alone at 20 μg/mL was not estrogenic in the Ishikawa assay (4.3%); however, when combined with estrogen it had a -48% activity in the antiestrogenic ALP assay. These data might be interpreted as the extract combined with estrogen induced enough ALP enzyme to convert substrate to product twice as fast as estrogen alone. This may have occurred by the extract working through a non-estrogen pathway that upregulates the ER, such as the progesterone pathway. A striking observation was that this extract also had activity in the ERE-luc assay (5.8 fold-induction) in the ERα positive cell line, but did not bind to the estrogen receptor (24%). Investigation of the contribution of aerial parts to the biological profile of adulterated *C. racemosa* preparations may also contribute to the resolution of the conflict in the literature revolving around the estrogenic activity of C. racemosa [31, 32]. While completely unexplored in the literature, the biological activities of the aerial parts of *C. racemosa* clearly deserve further investigation.

Cytotoxicity can play a large role in the false positive and false negative interpretation of cell based assays. In the ALP assays, cytotoxicity could cause a false negative in the agonist assay, and similarly, cytotoxicity can cause a false positive in the antagonist ALP assay where a lack of enzyme is really caused by a lack of cell viability. At 20 μg/mL, the dichloromethane and the 75% ethanol extracts of *A. tuberosa* were completely cytotoxic in the Ishikawa cell line, and was the likely cause of the large standard deviations in the ERE-luc assay. The vector control for *A. tuberosa* in the ERE-luc assay was low for a few of the replicates compared with other samples tested in parallel. This indicates that the cells were not able to utilize the control vector, because the cells were not viable rather than due to poor transfection efficiency, which is what the control vector is supposed to indicate. This resulted in dividing by a small denominator, which resulted in a large product with a large standard deviation. The dichloromethane and the 75% ethanol extracts had activity in the ERE-luc in ERα positive cells, and the dichloromethane extract had activity in the ERβ positive cells. When the values for the transfection control vector were evaluated, data were consistent with other plant extracts that did not have cytotoxicity. Another case where cytotoxicity was a factor was for *P. mirifica*, which was completely cytotoxic for the petroleum ether and dichloromethane extracts, and 60% cytotoxic for the 75% ethanol extract when tested in the Ishikawa cell line. While *P. mirifica* has been reported to be estrogenic in the literature [33], a different and more gentle extraction procedure was used in the present study and the lack of estrogenicity may be a result of the cytotoxicity, extraction procedure, or a combination of both. Of interest was that, while the extracts were toxic in the four-day assays, they did not appear to have a significant effect on the 24-hour assays.

In summary, based on these findings, the Center has moved toward cell-based assays for evaluating the hormonal activities of botanicals. Employing a diverse panel of bioassays, several extracts have been identified which do not conform to the classical estrogen receptor signaling pathway as they do not bind the estrogen receptor, but appear to have cell-based activity in the ERE-luc assay. Extracts that are not inherently estrogenic, but appear to increase the estrogenic activity in the presence of estrogen have also been identified. Finally, the synergistic activity opens avenues that need to be explored concerning the overall biological activity of the plant extracts and the value of multiple bioassays in characterizing the plant activities.

SUPPLEMENTARY MATERIAL

Screening Results in ER Binding, ERE-Luciferase, and Alkaline Phosphatase Assays

Plant or Sample Name and Plant Part	Extract Type	[1]Binding ER Alpha	[2,3]ERE-luc ER Alpha	[3,4]ALP Estrogenic	[3,4]ALP Antiestrogenic	[3,4]SRB Cytotoxicity	[1]Binding ER Beta	[2,3]ERE-Luc ER Beta
17 β estradiol	Control	**95 ± 1**	**21 ± 7**	**100 ± 10**	-10 ± 8	4 ± 2	**90 ± 1**	**7 ± 3**
4-hydroxy tamoxifen	Control	**96 ± 1**	1 ± 0.1	0 ± 5	**94 ± 1**	2 ± 1	**94 ± 1**	1 ± 0.2
DMSO	Control	0	1 ± 0.2	1 ± 2	0 ± 5	1 ± 2	0	1 ± 0.2
Alisma plantago-aquatica	Petroleum ether	31 ± 5	0.9 ± 0.4	-7.3 ± 4.2	-1.5 ± 13.3	10.7 ± 16.2	**51 ± 7**	2.3 ± 1.9
(Water Plantain)	Dichloromethane	25 ± 13	2.4 ± 1.5	-9.8 ± 11	3.3 ± 14	-5.7 ± 7.8	26 ± 3	2.1 ± 1.3
rhizomes	75% ethanol	2 ± 2	1.2 ± 0.4	-10.4 ± 7.8	-2.1 ± 10.4	-17.8 ± 11.8	8 ± 6	0.8 ± 0.4
Angelica sinensis	[5]Methanol	8 ± 7	1.8 ± 0.5	-2.3 ± 5.5	15.3 ± 8.8	3.1 ± 17.1	34 ± 5	1.3 ± 0.6
(Dang-Gui)	[5]Petroleum ether	[6]**81 ± 10**	**9.7 ± 3.5**	-4.7 ± 6.2	**48.7 ± 9.5**	16.4 ± 13.6	**99 ± 9**	1.1 ± 0.4
roots	[5]Chloroform	19 ± 9	**5.9 ± 3**	12.3 ± 4.7	19.4 ± 9.8	30.6 ± 8	**46 ± 6**	0.8 ± 0.5
	[5]Butanol	4 ± 3	**9.5 ± 2.2**	12.4 ± 8.2	-16.3 ± 4.5	3.3 ± 15.2	0 ± 2	1.2 ± 0.2
	[5]Water	7 ± 11	1.3 ± 0.8	-0.3 ± 1.2	-13.5 ± 2.5	-9.4 ± 6.1	0 ± 4	1 ± 0.1
Asclepias tuberosa	Petroleum ether	**69 ± 7**	1.1 ± 0.4	-17.9 ± 15.5	-9.1 ± 21	-19.9 ± 9.8	**85 ± 5**	1.1 ± 0.8
(Butterfly weed)	Dichloromethane	16 ± 9	4.1 ± 3.1	-26.5 ± 4.5	114.6 ± 6.7	**106.1 ± 5.3**	32 ± 10	**10.7 ± 0.8**
roots	75% ethanol	0 ± 2	3.9 ± 4.3	-10.3 ± 8.9	110.1 ± 8.5	**99.1 ± 3.6**	5 ± 8	1.3 ± 1.1
Beta vulgaris	Petroleum ether	35 ± 10	**4.7 ± 1.9**	-2.4 ± 17.4	4.4 ± 14.8	-15.9 ± 29.4	**44 ± 7**	1.1 ± 0.3
(Beets)	Dichloromethane	33 ± 11	3.2 ± 2	-2.3 ± 5.5	**43.7 ± 8.6**	-20.2 ± 30.9	30 ± 5	1.1 ± 0.1
roots	75% ethanol	6 ± 5	1 ± 0.2	-7.4 ± 10.4	-8.7 ± 17.7	-14 ± 33.7	8 ± 13	1.1 ± 0.4
Cimicifuga americana	Petroleum ether	37 ± 8	1.6 ± 0.7	-5.8 ± 14.4	27.1 ± 27.5	-3.7 ± 37	**47 ± 10**	1.4 ± 0.3
(Yellow cohosh)	Dichloromethane	31 ± 5	2.8 ± 1.1	-16.2 ± 14.9	**79.2 ± 19.5**	5.8 ± 32.1	21 ± 9	1.4 ± 0.6
roots	75% ethanol	9 ± 8	1.1 ± 0.2	-4.4 ± 14.3	-10 ± 12.4	-22.4 ± 25	7 ± 6	1 ± 0.1
Cimicifuga racemosa	DCM extract	34 ± 7	**6.6 ± 3.2**	-2.5 ± 1.3	3.5 ± 8.7	8.3 ± 5.2	45 ± 3	1.1 ± 0.4
(Black cohosh)	75% EtOH	21 ± 5	**5.8 ± 1.8**	4.3 ± 5.3	**-48.4 ± 4.9**	0.1 ± 5.7	23 ± 3	1 ± 0.2
aerial parts	PE extract	**55 ± 8**	3.2 ± 1.5	-4.5 ± 4.8	21.3 ± 7.2	4.8 ± 10.9	**64 ± 8**	1 ± 0.5
Cimicifuga rubifolia	Petroleum ether	**56 ± 2**	2.5 ± 2	-13.1 ± 12.4	33.2 ± 17	-23.2 ± 25	**66 ± 6**	1.1 ± 0.5
(Appalachian bugbane)	Dichloromethane	**51 ± 5**	**7.4 ± 1.1**	0.3 ± 15.9	21 ± 14.2	4.4 ± 19.8	40 ± 8	0.8 ± 0.3
aerial parts	75% ethanol	4 ± 3	2.3 ± 1.4	6 ± 13	-30.5 ± 22.1	6.8 ± 11.7	4 ± 7	1.1 ± 0.6
Cornus officinalis	Petroleum ether	26 ± 16	**24.3 ± 6.6**	2.6 ± 5.9	41.8 ± 4.1	11.5 ± 3.4	**48 ± 4**	1.5 ± 0.5
(Dogwood)	Dichloromethane	0 ± 4	2.5 ± 0.7	-2.2 ± 4.8	24.9 ± 5.2	**30.6 ± 19.1**	0 ± 2	1.3 ± 0.3
fruits	75% ethanol	17 ± 2	0.5 ± 0.7	-0.2 ± 7.9	-10.8 ± 6.3	-8.9 ± 6.1	5 ± 3	1 ± 0.5
Daucus carota	Petroleum ether	21 ± 6	**12.3 ± 5.1**	-6.8 ± 11.2	-26.6 ± 14.6	-18.7 ± 19.1	36 ± 6	0.9 ± 0.5
(Carrots)	Dichloromethane	22 ± 5	**5.4 ± 1.8**	-5.7 ± 8	-11.5 ± 16.1	13.7 ± 14.1	26 ± 6	0.7 ± 0.2
roots	75% ethanol	8 ± 8	0.8 ± 0.6	-7.7 ± 8.7	-19.5 ± 14.9	-6.3 ± 9.5	19 ± 10	1 ± 0.5
Paeonia moutan	Petroleum ether	21 ± 5	2.8 ± 1	-9.7 ± 11.4	8.7 ± 16.7	7.7 ± 19.7	**60 ± 6**	2 ± 1.7
(Peony)	Dichloromethane	19 ± 6	3.4 ± 2.8	-10.7 ± 13	24.4 ± 14.7	11.5 ± 16.1	19 ± 5	1.8 ± 0.2
bark	75% ethanol	14 ± 13	0.8 ± 0.3	-10 ± 8.9	-30.2 ± 22.7	-10.1 ± 18.3	3 ± 5	1 ± 0.4
[7]*Polygonum multiflorum*	Petroleum ether	15 ± 8	**21.7 ± 8.5**	6.5 ± 1.8	1.2 ± 3.3	7.6 ± 14.3	**50 ± 9**	1.4 ± 0.7
(Fo-Ti)	Dichloromethane	30 ± 7	**25.8 ± 11.2**	12.3 ± 3.4	0.4 ± 12.3	12.7 ± 17.8	36 ± 13	3 ± 2.5
roots	75% ethanol	4 ± 3	1.5 ± 0.2	0.7 ± 3.3	3.9 ± 18.2	2.7 ± 7.5	0 ± 3	1.2 ± 1.1
[8]*Polygonum multiflorum*	Petroleum ether	22 ± 16	**15.1 ± 9.1**	-1.5 ± 0.6	36.1 ± 48.6	18.4 ± 22.6	26 ± 9	1.2 ± 0.9
(Fo-Ti)	Dichloromethane	16 ± 5	**16 ± 3.4**	-0.2 ± 1.6	68.3 ± 11.5	**31.1 ± 19.1**	16 ± 7	0.9 ± 0.7
roots	75% ethanol	5 ± 3	0.7 ± 0.2	-0.4 ± 2.5	16.5 ± 26.2	18 ± 20.5	0 ± 3	1 ± 0.2
Pueraria lobata	Petroleum ether	32 ± 2	**10.3 ± 2.8**	-10.8 ± 12.6	**46.9 ± 18.9**	12.7 ± 30	36 ± 2	1.5 ± 1.4
(Kudzu)	Dichloromethane	**63 ± 6**	**24.5 ± 9.9**	-0.3 ± 25.6	36 ± 32.6	-3.3 ± 5.5	**45 ± 19**	1.2 ± 0.5
aerial parts	75% ethanol	17 ± 16	**26.9 ± 12.7**	-1.3 ± 13.8	28.9 ± 40.7	7.6 ± 50.1	**63 ± 3**	**3.5 ± 1.3**
Pueraria mirifica	Petroleum ether	12 ± 8	[9]N.T.	-2.7 ± 2.6	58.5 ± 37	**100.6 ± 38.8**	18 ± 12	N.T.
(Kwao Keur)	Dichloromethane	12 ± 5	**25.7 ± 9.5**	-4.2 ± 1.1	101.1 ± 8.6	**115 ± 2.2**	19 ± 11	3.2 ± 1.6
bark	75% ethanol	18 ± 7	**28 ± 8.6**	-1.4 ± 4.8	52.2 ± 61	**60.4 ± 62.2**	31 ± 8	2.4 ± 1.3
Valeriana officinalis	Petroleum ether	**43 ± 11**	**7.2 ± 3.1**	-7.8 ± 7.6	33.7 ± 16.8	12.5 ± 19.3	**81 ± 4**	1.3 ± 0.8
(Valerian)	Dichloromethane	13 ± 12	**5.9 ± 1.6**	-8.5 ± 7.7	19.4 ± 8.2	-7.6 ± 21	13 ± 6	0.7 ± 0.1
roots	75% ethanol	12 ± 6	1.7 ± 1.1	-10.4 ± 9.5	-0.8 ± 5.4	-18.9 ± 15.6	7 ± 11	1.1 ± 0.5
Viburnum opulus	Petroleum ether	17 ± 17	1.3 ± 0.4	-0.3 ± 0.9	-2.1 ± 5.8	7.1 ± 12.6	11 ± 8	1.2 ± 0.6
(Guelder Rose)	Dichloromethane	11 ± 4	1.4 ± 0.2	1.6 ± 1.4	14 ± 7.4	22.5 ± 4	6 ± 2	0.8 ± 0.2
bark	75% ethanol	22 ± 10	1.4 ± 0.3	-1.2 ± 0.7	-17.5 ± 11.4	11.4 ± 6.9	24 ± 16	0.7 ± 0.6
Viburnum prunifolium	Petroleum ether	12 ± 6	0.8 ± 0.3	-3.7 ± 0.7	-9.8 ± 0.8	7.7 ± 5.7	6 ± 3	1 ± 0.5
(Black Haw)	Dichloromethane	3 ± 6	0.7 ± 0.5	-3.5 ± 3	68.5 ± 6.3	**42 ± 4.4**	3 ± 4	1 ± 0
bark	75% ethanol	10 ± 10	4.9 ± 3.7	-0.5 ± 2.2	-33 ± 7.9	-2.7 ± 11.2	3 ± 9	0.9 ± 0.6
Vitex agnus-castus	Petroleum ether	37 ± 6	**9.5 ± 2.5**	-1.1 ± 1.6	43.5 ± 3.3	**39.1 ± 8.3**	**68 ± 9**	1.1 ± 0.7
(Chasteberry)	Dichloromethane	13 ± 3	**9.6 ± 6.6**	-2.3 ± 1.5	94.5 ± 7.2	**112.5 ± 8.8**	20 ± 19	0.8 ± 0.3
fruits	75% ethanol	10 ± 8	2.3 ± 0.9	-1.5 ± 2.7	-14.3 ± 7.6	2.3 ± 7.9	7 ± 7	1.3 ± 0.7

[1]Percent binding at 200 µg/mL.
[2]Fold Induction where DMSO is 1.
[3]Tested at 20 µg/mL.
[4]Percent induction (estrogenic), inhibition (antiestrogenic),
[5]Fraction instead of partition.
[6]Bold face-type indicates assay activity.
[7]BC number 268.
[8]BC number 286.
[9]Not tested due to the limited quantities available.

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ABBREVIATIONS

ER	=	Estrogen receptor
HRT	=	Hormone replacement therapy
ERE	=	Estrogen response element
ERE-luc	=	ERE-luciferase
SSRIs	=	Selective serotonin reuptake inhibitors
NAPRALERT	=	Natural Products Alert
SERMs	=	Selective estrogen receptor modulators
PE	=	Petroleum ether
DMEM/F12	=	Dublecco's Modified Eagle medium
NEAAs	=	Non-essential amino acids
FBS	=	Fetal bovine serum
CBS	=	Calf bovine serum
HAPs	=	Hydroxyapatite slurry

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