In Vitro Comparison of Estrogenic Activities of Popular Women's Health Botanicals

BY

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THESIS

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<u>DEDICATION</u>

This work is dedicated to my three biggest supporters, my mother Elizabeth, my father William, and my fiancé Christopher. Their encouragement, love, and support means the world to me.

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First, I would like to thank my Lord and Savior, Jesus Christ, without whom I'd be nothing. I could never praise or thank Him enough for the blessings that he continues to bestow upon me. This thesis is proof that I can indeed do all things through Christ who strengthens me.

So many thanks go to my advisor, Dr. Judy Bolton, who gave me an amazing opportunity to work in her lab within the UIC/NIH Botanical Center. Her guidance, support, and leadership have helped me grow into a great researcher and an even better person. Though she wears many hats and has a host of responsibilities, she has always made time in her busy schedule to sit and talk with me about any problem or issue that I'm facing. My decision to pursue naturopathic medical school was a tough one but, needless to say, she was so supportive and encouraging. Thank you so much, Dr. Bolton! I would also like to thank the other members of my thesis committee, Dr. Birgit Dietz and Dr. Joanna Burdette. I honestly could not have finished any of this without either of these beautiful and smart women. Not only did they help me with my thesis preparation but they were available to answer my countless questions about experiments, classes, etc. I truly grateful for their help and encouragement.

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LIST OF ABBREVIATIONS

8-PN	8-Prenylnaringenin
black cohosh	*Cimicifuga racemosa* L.
DPN	Diarylpropionitirile
E_2	Estradiol
ER	Estrogen receptor
ERE	Estrogen response element
GG	*Glycyrrhiza glabra*
GU	*Glycyrrhiza uralensis*
GI	*Glycyrrhiza inflata*
hops	*Humulus lupulus*
IC_{50}	Half maximal inhibitory concentration
ICI	7-alkylsulfinyl analogue of estradiol
IX	Isoxanthohumol
LicA	Licochalcone A
LigC	Isoliquiritigenin
LigF	Liquritigenin
licorice	*Glycyrrhiza glabra, Glycyrrhiza uralensis*, and/or *Glycyrrhiza inflata*
mRNA	Messenger ribonucleic acid
OHT	4-OH Tamoxifen
PPT	Propylpyrazole triol
red clover	*Trifolium pretense*
RT-PCR	Real time polymerase chain reaction

LIST OF ABBREVIATIONS (continued)

UHPLC   Ultra high performance liquid chromatography

XH   Xanthohumol

SUMMARY

By the year 2030 there will be approximately 1.2 billion menopausal and postmenopausal women in the world (1). With 47 million new menopausal women every year, there is an increased demand for safe and efficacious treatment options for menopausal symptoms. Traditional hormone therapy (HT) was once thought to be the gold standard in the treatment of menopausal symptoms; however, the use of HT has been associated with a variety of conditions including but not limited to cardiovascular disease, osteoporosis, and an increased incidence of hormone dependent cancers (2). As an alternative, many women have turned to botanical dietary supplements for menopausal symptom relief since they contain potent phytoestrogens that possess estrogenic activity. Nevertheless, the evidence to substantiate the safety and efficacy of these botanicals is scarce.

Previous studies have indicated that hops (*Humulus lupulus*), red clover (*Trifolium pratense*), and three medicinal licorice extracts (*Glycyrrhiza glabra*, *Glycyrrhiza uralensis*, *Glycyrrhiza inflata*) might differentially activate estrogen receptor ERα or ERβ signaling pathways. ERα agonists potentiate the negative effects associated with estrogens such as excessive cell proliferation in hormone sensitive cells (breast, uterus). In contrast to ERα agonists, it is believed that ERβ agonists do not initiate proliferative effects. In fact, ERβ agonists have been shown to inhibit the cell proliferation caused by ERα activation. The **hypothesis** is that botanicals that preferentially bind to and activate ERβ instead of ERα may have a better safety profile. The primary goal of this study is to compare the effects of hops, red clover, licorice, and black cohosh along with their active compounds, in modulating ERα and ERβ signaling.

SUMMARY (continued)

To measure the extracts and compounds' ability to modulate ERα signaling, an alkaline phosphatase assay was performed in an ERα endometrial carcinoma cell line (Ishikawa). The results indicated that the hops extract, red clover, and the licorice extract GI were equally potent; however, red clover and hops acted as full ERα agonists whereas GI had 50% less efficacy. The other licorice species (GG, GU) were significantly less active and also behaved as partial ERα agonists. As seen in previous studies, black cohosh, displayed no estrogenic activity. When assessing the ERα activity of the most estrogenic compounds in the botanical extracts, 8-prenylnaringenin (hops) had the highest agonist activity, followed by genistein (red clover), and liquiritigenin (licorice species). 8-PN was a full ERα agonist whereas genistein and liquiritigenin were partial ERα agonists. Propylpyrazole triol (PPT), a positive control, behaved as a full ERα agonist; however, this synthetic ERα selective ligand was considerably less potent compared to 8-PN.

The modulation of ERβ activity by these botanicals was analyzed using an ERE luciferase assay in the stably transfected ERβ expressing, malignant breast carcinoma cell line (MDA-MB-231/β41). To validate the accuracy of the assay, the potent, synthetic agonists Diarylpropionitirile (DPN) (ERβ) and PPT (ERα) were also tested. DPN, the ERβ agonist, has an EC_{50} of 1.7 μM, which is 100 times less than the most potent phytoestrogen, genistein, which has an EC_{50} of 7.62 ηM. PPT, the ERα agonist, has little estrogenic activity and its EC_{50} was undetermined due to its low activity. The results from the ERE luciferase assay in ERβ expressing cells show a different order of activity than the alkaline phosphatase assay. In this ERβ assay, red clover was the most potent

SUMMARY (continued)

botanical instead of hops, as seen in the ERα assay. Among the licorice species, *G. inflata* was the most potent and most effective, followed by GG and GU. The order of activity among the botanicals was similar to results gathered in the ERα assay, where GI was found to be ten times more potent in ERβ cells than ERα. In regards to the compounds, genistein was the most potent and was over one hundred times more potent in ERβ cells than ERα. Liquritigenin was more effective than genistein (its efficacy was quite similar to DPN) but less potent. Licochalcone A, a compound solely found in GI, showed a similarly limited level of potency followed by 8-PN and PPT. Based on these data, red clover appears to be the safest option for women seeking botanical supplements for menopausal symptom relief. This is due to the extract's ability to provoke ERβ signaling at lower concentrations than what is needed to stimulate ERα signaling. Genistein, a pure compound present in the red clover extract, has over 100-fold higher ERβ selectivity over ERα.

Our hypothesis is that popular botanicals for menopausal symptom relief that display ERβ selectivity, potency, and efficacy will have a better safety profile than botanicals that preferentially engage ERα. The aims of this project involve A) establishing and optimizing a functional assay that examined the estrogenic activities of botanicals via estrogen receptor beta, B) evaluating the estrogenic activity of 5 botanicals (*Trifolium pratense, Humulus lupulus, Glycyrrhiza glabra/uralensis/inflata, and Cimicifuga racemosa* (L.) Nutt.) and their corresponding pure compounds in ERβ positive cells, and lastly C) measuring mRNA expression of an ERβ target gene to determine the depth and breadth of ERβ activation initiated by the potent phytoestrogens genistein and 8-

PN. This hypothesis was supported by the observation that the activation of ERβ

actually mitigates the onset of increased proliferation associated by the activation of

ERα by estradiol. Future experiments will address the discovery of the selectivity of GI,

which warrants further studies to determine the presence of an unknown ERβ agonist.
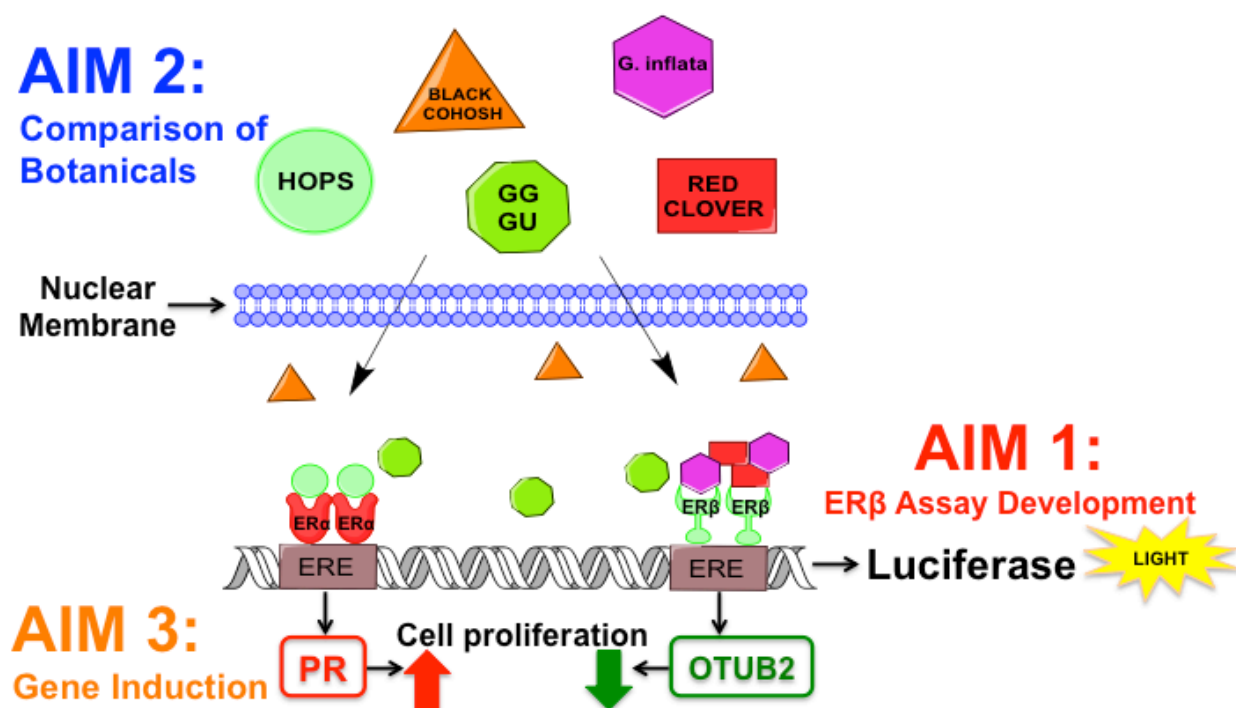
Figure 1: Botanicals, like red clover, that preferentially activate ERβ initiate the anti-proliferative effects associated with ERβ. The activation of ERα increases cell proliferation whereas the activation of ERβ reduces cell proliferation and hinders the negative effects of ERα activation. PE denotes phytoestrog

1. INTRODUCTION

1.1   Menopause

Menopause is a natural and inevitable phase of life for every woman. Characterized by at least 12 months of amenorrhea, menopause marks the end of a woman's fertile years due to the decrease in activity of the ovaries and the subsequent decline in estrogen and other hormones. Menopause is also associated with a variety of physical symptoms including but not limited to: hot flashes, insomnia, fatigue, anxiety, depression, mood changes, and vaginal atrophy (1). The treatment options vary for each woman but generally include hormone therapy, vaginal estrogen, or low-dose antidepressants (3).

1.2.   Dangers of Hormone Therapy

On May 31st, 2002 the National Institutes of Health elected to end the Women's Health Initiative (WHI) clinical trial after breast cancer test statistics surpassed the maximum boundary for adverse effects (2). This groundbreaking study was designed to measure the long-term effects of estrogen and progestin combination hormone therapy used for the amelioration of menopausal symptoms. Hormone therapy was originally thought to decrease the risk of stroke, Alzheimer's disease, coronary heart disease, and osteoporosis. Initially scheduled to last 8.5 years, the study was abruptly ended due to the increased risk in a number of dangerous adverse effects. According to the study, the primary outcome associated with hormone therapy was coronary heart disease and the primary adverse outcome was invasive breast cancer. The study concluded that the overall health risks associated with hormone therapy significantly exceeded the benefits over a 5 year time period (3). During the trial, women who were taking estrogen plus

progestin experienced coronary heart disease at a rate that was 29% higher than the placebo group. In addition, the 26% increase in invasive breast cancer rates observed in the estrogen plus placebo group concerned the investigators enough to recommend discontinuing the trial (3). Additionally, researchers have determined that estradiol and Premarin® (conjugated estrogens) treatments activate ERα which increases cell proliferation and a woman's cancer risk. One of the many benefits of this study was that physicians now understand that hormone therapy may not work for every woman and it is imperative that they identify patients who are not good candidates for hormone therapy to avoid the incidence of adverse effects.

1.3. Estrogen receptor alpha vs. estrogen receptor beta

In the human body, there are two subtypes of estrogen receptors, alpha and beta that belong to the nuclear receptor gene family (4). Though they are encoded by unique genes, the two receptors express a degree of homology in the functional domains of the DNA-binding and ligand-binding domains.
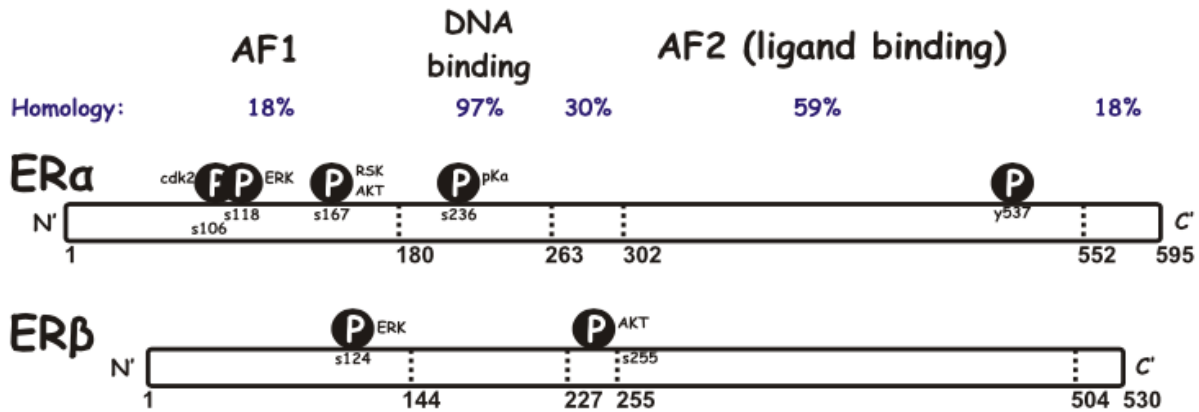
Figure 2: Structural domains of estrogen receptors alpha and beta (5).

As expected, the two receptors have a tendency to interact with analogous DNA response elements and have similar binding affinities for an array of ligands (6). The differences between the two receptors appear in their tissue distribution and expression patterns. ERα is commonly localized and expressed in the breast, uterus, cervix, vagina, liver, kidney and heart, whereas ERβ is localized and expressed in the ovary, prostate, testis, spleen, lung, bladder hypothalamus, and thymus (6) (7).

There are areas of the body where both receptors are co-expressed such as the mammary gland, epididymis, thyroid, adrenal gland, bond, and particular regions of the central nervous system (5). According to Saji et al. ERβ is expressed ubiquitously during all stages of development, whereas ERα expression fluctuates regularly with an increase during puberty and lactation then a down-regulation of its expression during pregnancy and post lactation (4).

1.4. **Effects of estrogen receptor beta on hormone sensitive tissues**

Studies have shown that in breast carcinoma cell lines T47D, over expression of ERβ inhibited ERα- mediated cell proliferation driven by estradiol (4). Patients that express ERβ in their tumors often experience better survival with adjuvant treatment of tamoxifen. Additional studies have shown that ERβ expression decreases as breast tissue and ovarian tissue become increasingly tumorigenic, suggesting possible protective effects of ERβ (8).

Previous studies have investigated the importance of ERβ in the uterus by using ERβ knockout mice and assessing any changes in embryo implantation (9). Though no changes were found, investigators wondered if ERβ had any other roles in the endometrium. Estrogen receptor beta can be found in both pre-pubertal and post-menopausal endometrial tissue and has been detected in all endometrial cell types. ERβ abnormalities have been reported in most benign and malignant endometrial proliferative disease. This suggests that ERβ not only plays a role in normal endometrial function but may also be involved in malignant endometrial disease when its expression is compromised (9).

Additionally, ERβ agonists have been reported to be able to sensitize mesothelioma cells to cisplatin in vitro and in vivo (10). According to Pinton et al, co-treatment of ERβ agonist, KB9520, with cisplatin and pemetrexed significantly improved the cytotoxic effects of in mesothelioma cells and in CD1 nude male mice. The same effects were seen with pre-treatment with KB9520 before treatment with cisplatin (10).

1.5.   **Botanicals as safe alternatives to Hormone Replacement Therapy**

The results of the WHI study incited an interest in alternative remedies for menopausal symptoms that would not increase a woman's risk of cancer in hormone sensitive tissues (2) (11). Botanicals have been used for thousands of years as a part of traditional Chinese medicine to aid in the relief of symptoms of menopause and other women's health conditions (12). Due to their natural origins, botanical dietary supplements are generally perceived as safe. Despite the lack of convincing scientific evidence regarding the efficacy of botanicals used to relieve menopausal symptoms, research suggests that the relief that these women experience are indeed attributed to the bioactive compounds present in the botanical supplements.

Although in vitro studies have shown that the botanicals do possess estrogenic activity, previous clinical trials evaluating the efficacy of estrogenic botanical have shown an effect similar to placebo (50% reduction in vasomotor symptoms) [(13)- 17)] questioning the validity of estrogenic botanical dietary supplements in human subjects versus cell based assays. To further analyze the mechanisms of action of these phytoestrogens, previous studies have determined that these botanicals display a degree of binding selectivity for estrogen receptor alpha or beta subtypes (18). It should be noted that Burdette, et al determined that black cohosh displayed poor estrogenic activity for either receptor (19).

As previously discussed, some of the botanicals and pure compounds examined in this study have estrogenic activity, however, they also have selective binding affinities for certain estrogen receptor subtypes; as seen in Table I.

Table I: COMEPETITIVE ER BINDING ANALYSIS OF RED CLOVER, HOPS, AND LICORICE EXTRACTS AND COMPOUNDS

Binding affinities of extracts/compounds		
Extract/Compound	IC_{50}	
	ERα	**ERβ**
Hops[a]	15 ± 3	27 ± 3
8-PN[b]	0.5 ± 0.1	1.7 ± 0.1
Red Clover[a]	18 ± 5	2.0 ± 0.8
Genistein[b]	0.3 ± 0.01	0.02 ± 0.002
GG[a]	> 200	> 50
GU[a]	> 200	> 50
LigF[b]	> 200	7.5 ± 0.5
Black Cohosh[a]	-	-

Superscript 'a' denotes IC_{50} in units of g/L. Superscript 'b' denotes IC_{50} in units of uM.

1.5.1. *Cimicifuga racemosa* (L.) Nutt. (black cohosh)

Black cohosh is undoubtedly the most popular and extensively studied botanical used for menopausal symptom relief. Traditionally, black cohosh was used for pain during childbirth, dysmenorrhea, and a host of other women's health conditions and other complaints (20). Though several studies, including this one, have reported no estrogenic activity associated with black cohosh, clinical trials have reported an increase in luteinizing hormone (LH) in women treated with black cohosh (21), (22), (23). The rhizome of this plant contains triterpene glycosides 23-epi-26-deoxyactein and 26-deoxyactein, neither of which is considered a phytoestrogen (24), (20).

1.5.2. *Trifolium pratense* (red clover)

Trifolium pratense, Fabaceae is one of the most popular botanicals used for the relief of menopausal symptoms (25). It contains the phytoestrogens, genistein and daidzein, which are also present in soy. Unlike soy, the predominant isoflavones present in red clover are the methoxy ethers biochanin A and formononetin. These compounds require

cytochrome p450 metabolism to produce the active phytoestrogens, genistein and daidzein (26). Quantitative analysis reported in Booth et al. (27) revealed that the standardized extract of red clover contained 14.47% biochanin A, 14.26% formononetin, 0.23% daidzein, and 0.41% genistein (Table II, Figure 3). The abundance of biochanin A and formononetin, in comparison to the phytoestrogens, is an indication that the cytochrome P450 metabolism is the primary method through which red clover displays its estrogenic activity (27).

Red clover's estrogenic activity has been studied in both in vitro and in vivo assays. Data reported in Overk et al. showed that the standardized red clover extract was able to not only stimulate estrogen-inducible alkaline phosphatase activity but also induced progesterone receptor mRNA expression in Ishikawa cells (26). In Burdette et al. the standardized extract was able to increase the uterine weight of ovarectomized Sprague-Dawley rats when compared to estradiol. Though the increase was not large, it was statistically significant and was seen at concentrations as high as 750 mg/kg (28). In summary, red clover is predominantly composed of the methoxy esters, biochanin A and formononetin, the precursors of the phytoestrogens genistein and daidzein through P450 metabolism. The robust estrogenic activity of this extract is due to these potent phytoestrogens and is potentially mediated through ERβ (29),(28),(30).

Table II: QUANTITATION (W/W%) OF BIOACTIVE COMPOUNDS PRESENT IN RED CLOVER EXTRACT (31).

Compounds in red clover (% w/w)			
Species	**Biochanin A**	**Formononetin**	**Genistein**	**Daidzein**
Red Clover	14.47	14.26	0.41	0.23
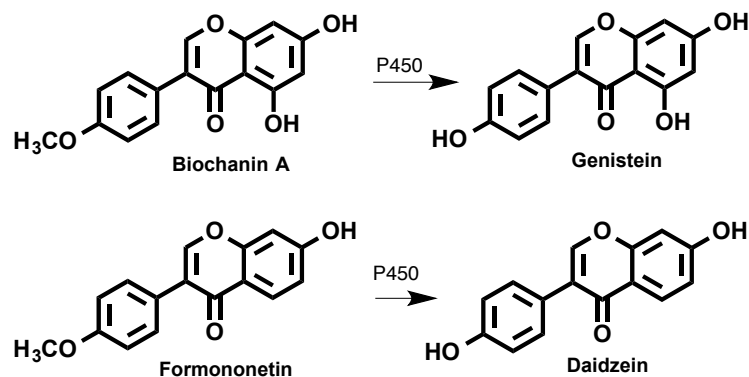
Figure 3: Structures of bioactive compounds present in *Trifolium pratense.*

1.5.3. *Humulus lupulus* (hops)

Hops is native to Europe and is one of the most well-known botanicals used for medicinal purposes. Hops is best known for its sedative and estrogenic effects (32). The extract contains prenylated chalcones and flavonones xanthohumol, isoxanthohumol, and 8-prenylnaringenin. Quantitative analysis of the standardized extract revealed that it is primarily composed of xanthohumol (33.5%), followed by isoxanthohumol at 1.1% and 8-prenylnaringenin at 0.33%. Similar to the red clover extract, the most estrogenic compound present in the hops extract, 8-prenylnaringenin (8-PN) is the least abundant in the hops extract (Table III, Figure 4).

While there is numerous data confirming hops' estrogenic activity it is noted that its activity was recorded in both in vitro and in vivo studies. In vivo studies of 8-PN showed significant increase in the uterine weight of ovarectomized Sprague-Dawley rats, while the hops extract, xanthohumol and isoxanthohumol did not induce uterotrophy or changes in the height of uterine epithelial cells(18).

Table III: QUANTITATION (W/W%) OF BIOACTIVE COMPOUNDS PRESENT IN STANDARDIZED *HUMULUS LUPULUS* (32).

Compounds in hops (% w/w)			
Species	**Xanthohumol**	**Isoxanthohumol**	**8-prenylnaringenin**
Clinical Hops	33.5	1.1	0.33
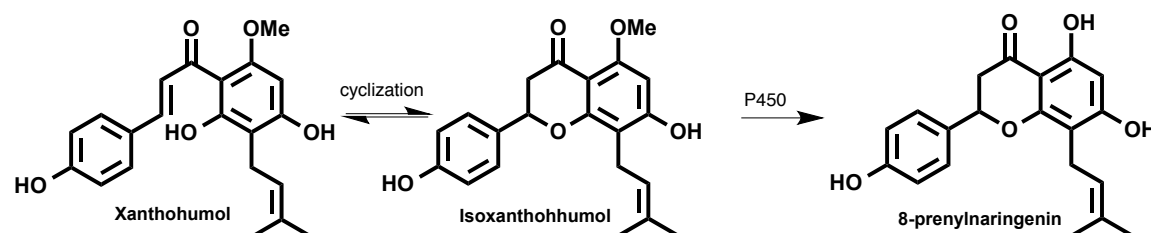
Figure 4: Structures of bioactive compounds present in *Humulus lupulus*.

1.5.4. *Glycyrrhiza glabra, uralensis, and inflata* (Licorice)

Licorice species are widely used, mostly as sweetening agents in the food/beverage industry, and in toothpaste (21). Asl et al. notes that there are more than 30 *Glycyrrhiza* species, all with diverse traditional uses such as treating peptic ulcers, pulmonary conditions, women's health conditions, and skin diseases due to the species' antiviral,

antimicrobial, estrogenic, and anticancer properties (21). In the United States, the species most commonly present in botanical dietary supplements designed for menopausal symptom relief is *Glycyrrhiza glabra (European licorice).* In this project, three species of licorice were studied: *Glycyrrhiza glabra (*GG)*, Glycyrrhiza uralensis* GU)*, and Glycyrrhiza inflata* (GI). These three licorice species have varying levels of estrogenic activity across a number of studies. Liu et al. reported not seeing any estrogenic activity in the methanolic extract of Glycyrrhiza glabra when tested in Ishikawa cells (19). Contradictory to that report, Hajirahimkhan et. al. and Simons et. al reported estrogenic activity in both the methanolic extract and the ethyl acetate extract of GG (33), (34). In this study, the methanolic extract of GG showed similar potency across both estrogen receptor subtypes. The primary bioactive compounds that are present in GG are liquiritigenin (LigF) and isoliquiritigenin (LigC) at 5.51% and 2.97%, respectively (Table IV).

The GU species (Chinese licorice) also shows estrogenic activity in both ERα and ERβ cells with similar potency across subtypes. According to studies by Hu et al. the DMSO extract of GU displayed estrogenic activity in ERα positive MCF-7 cells via induction of cell-proliferation. Anti-estrogens, 4-hydroxytamoxifen and ICI subsequently blocked the initiation of cell proliferation by GU, which is indicative of GU's estrogenic mechanism of action (35). Like GG, the primary phytoestrogens present in GG are liquiritigenin (LigF) and isoliquiritigenin (LigC) at 2.96% and 0.81% respectively (Table IV, Figure 5). Hajirahimkhan et. al. showed that LigC exists in equilibrium with LigF and is converted, non-enzymatically, to LigF during bioassay incubation (33).

The third estrogenic licorice species, GI, contains LigF, LigC, and a unique compound, licochalcone A, which has been shown to have antioxidant and anti-inflammatory properties (Table IV) (36). This extract has not been studied as extensively as the other two, and often exists as a hybrid with GU.

Table IV: QUANTITATION (W/W%) OF BIOACTIVE COMPOUNDS PRESENT IN STANDARDIZED *Glycyrrhiza glabra, uralensis, and inflata EXTRACT (36)*.

Species	Compounds in licorice (%w/w)				
	LicA	LigF	LigF equivalents	LigC	LigC equivalents
GG	-	0.24 ± 0.01	5.61 ± 0.02	0.06 ± 0.00	2.97 ± 0.01
GU	-	0.41 ± 0.01	2.96 ± 0.02	0.09 ± 0.01	0.81 ± 0.03
GI	5.42 ± 0.34	0.12 ± 0.04	0.82 ± 0.06	0.12 ± 0.01	2.72 ± 0.02

LicA was only detected in GI and was below the limit of detection in GG and GU. The LigF equivalents represent the amount of LigF that is present in the crude extract in the form of LigF glycosides (Liquiritin, liquiritin apioside, liquiritigenin -7-O-apiosylglucoside). Similarly, LigC equivalents represent the total amount of LigC in the crude extract that is in the form of LigC glycosides (isoliquiritin, isoliquiritin apioside, licuraside). The values (% weight compound/ weight crude extract) are expressed as mean ± SD from three independent measures.

Liquiritigenin (LigF) — cyclization — Isoliquiritigenin (LigC)
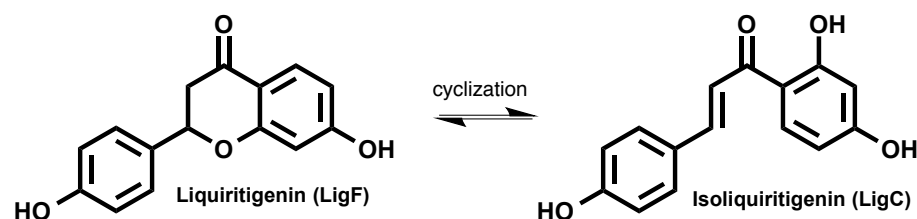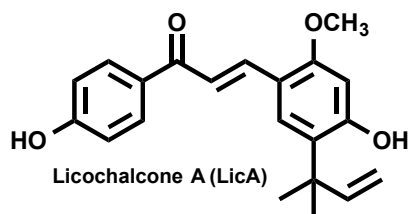
Figure 5: Structures of bioactive compounds present in *Glycyrrhiza glabra, uralensis, and inflata.*

1.6. Hypothesis and Aims

Our previously published work revealed that popular botanical extracts are not only estrogenic in ERα positive cells but they also exhibit preferential binding for a specific receptor (30). *Our hypothesis is that popular botanicals for menopausal symptom relief that display ERβ selectivity, potency, and efficacy will have a better safety profile than botanicals that preferentially engage ERα.* My methodology began with A) establishing and optimizing a functional assay that examined the estrogenic activities of botanicals via estrogen receptor beta, B) evaluating the estrogenic activity of 5 botanicals (*Trifolium pratense, Humulus lupulus, Glycyrrhiza glabra/uralensis/inflata, and Cimicifuga racemosa* (L.) Nutt.) and their corresponding pure compounds in ERβ positive cells, and C) measure mRNA expression of an ERβ target gene to determine the depth and breadth of ERβ activation initiated by potent phytoestrogens genistein and 8-PN. The results will allow standardization of botanicals to ERβ ligands, which should improve safety and efficacy profiles for these women's health botanicals

2. MATERIALS AND METHODS

2.1   Materials

Estradiol, diarylpropionitrile (DPN), propylpyrazole triol (PPT), genistein, S-equol, diadzein, isoxanthohumol, and xanthohumol were obtained from Sigma Aldrich (St. Louis, MO, USA). *Trifolium pratense* was obtained from PureWorld Botanicals, Inc. (South Hackensack, NJ)*, Humulus lupulus* was provided by Yakima Chief Inc. (Sunnyside, WA; lot #PE-MANU004). *Glycyrrhiza glabra* was purchased from Mountain Rose Herbs*, Glycyrrhiza uralensis* was purchased from a supplier in China Town (Chicago, IL)*, Glycyrrhiza inflata* was a gift from Dr. Liang Zhao at Lanzhou Institute of Chemical Physics, CAS*, and Cimicifuga racemosa* was a gift from Dr. G. Ramsey (Lynchburg College, Lynchburg, VA).

The pure compounds 8-prenylnaringenin, licochalcone A, liquiritigenin, and isoliquiritigenin were isolated from their respective crude extracts by the UIC/NIH Center for Botanical Dietary Supplement Research (Chicago, IL, USA). All cell culture materials were obtained from Fisher Scientific (Itasca, IL, USA), Sigma Aldrich (St. Louis, MO, USA), and Invitrogen (Grand Island, NY, USA) unless stated.

Power Wave 200 microplate scanning spectrophotometer was obtained from Bio-Tek Instruments (Winooski, VT). The Dual-Luciferase Reporter Assay System protocol was obtained from Promega (Madison, WI) and was analyzed by the FLUOstar Optima luminometer (BMG Lab Tech, Offenburg, Germany).

2.2   Botanical Extract Preparations

The red clover (*Trifolium pratense)*, hops (*Humulus lupulus*), licorice (*Glycyrrhiza glabra, Glycyrrhiza uralensis,* and *Glycyrrhiza inflata),* and black cohosh (*Cimicifuga racemosa*) extracts were prepared by Dr. Guido Pauli's group; as were the respective pure compounds. The red clover clinical trial extract was standardized to contain a minimum of 30% isoflavones of which consisted of 0.41% genistein, 0.23% daidzein, 14.47% biochanin A, and 14.26% formononetin as previously discussed (31)**.**

The hops clinical extract was extracted with ethanol after supercritical CO_2 extraction of pelletized strobiles of Humulus lupulus L. cv. Nugget (37).   Quantitative liquid chromatography/mass spectrometric (LC/MS) analysis, using authentic reference compounds as calibrants, revealed that this clinical hops extract contained 33% Xanthohumol (XH), 3.0% Isoxanthohumol (IX), and 0.35% 8-prenylnaringenin (8-PN) (37)**.** XH was isolated and purified (>99.5% purity both by quantitative HNMR and LC/MS) as described previously (37). Using the modified literature procedure, as previously reported in Overk CR. et. al, 8-PN was synthesized and purified (95.0% purity by quantitative HNMR) (18).

Dried root samples of Glycyrrhiza glabra L. and Glycyrrhiza uralensis Fisch. (Leguminosaea/ Fabaceae) were purchased from Mountain Rose Herbs (Eugene, OR, USA) and a local supplier in Chicago, IL, respectively. The Glycyrrhiza inflata sample was a gift from Dr. Liang Zhao at Lanzhou Institute of Chemical Physics, CAS and was collected in Kuga county, Xinjiang province in China. The three Glycyrrhiza species were authenticated by DNA barcoding and then the roots were powdered and

exhaustively extracted by percolation with re-distilled methanol at room temperature, as previously described (33). The ratio of sample to solvent was 1/40 w/v. Before evaporation, in vacuo, each extract solution was divided into three separate vials with varying amounts: ~4 mg, ~30 mg, and ~500 mg. After evaporation the samples were kept overnight in a vacuum desiccator under Drierite with indicator. Lastly, the vials were covered and stored at -20°C before biological analysis (33).

The pure compounds liquiritigenin (LigF), isoliquiritigenin (LigC), and licochalcone A (LicA) were quantified in each Glycyrrhiza extract through ultra high performance liquid chromatography (UHPLC) analysis. The Glycyrrhiza glabra extract contained 5.61% LigF and 2.97% LigC; no LicA was identified. The Glycyrrhiza uralensis extract contained 2.96% LigF and 0.81% LigC; no LicA was identified. Lastly, the Glycyrrhiza inflata species contained 0.83% LigF, 2.72% LigC, and 5.42% LicA (33).

Black cohosh, *Cimicifuga racemosa* (L.) Nutt, extract was collected in Rookbridge County, VA by Dr. G. Ramsey (Lynchburg College, Lynchburg, VA) and deposited into the Field Museum of Natural History Herbarium (Chicago, IL) (38). The dried roots/rhizomes of C. racemosa were exhaustively extracted with MeOH and evaporated to yield a syrup-like residue (38).

2.3. Cell Culture

The estrogen receptor alpha positive, Ishikawa endometrial carcinoma cells were provided by Dr. R. B. Hochberg (Yale University, New Haven, CT) and were maintained in Dulbecco's Modified Eagle Medium (DMEM/F12) containing 1% sodium pyruvate, 1% non-essential amino acids (NEAA), 1% glutamax-1, 0.05% insulin, and 10% heat-

inactivated FBS. Estrogen-free media was prepared by adding 10% charcoal-stripped FBS to phenol-free DMEM media, instead of heat-inactivated FBS. Other components remained unchanged.

The estrogen receptor alpha positive, MCF-7 breast carcinoma cell line was purchased from ATCC and were grown in RPMI 1640 media containing 1% glutamax-1, 1% NEAA, 0.05% insulin, and 5% heat-inactivated FBS. Estrogen-free media was prepared by adding 5% charcoal-stripped FBS to phenol-free RPMI media, instead of heat-inactivated FBS. Other components remained unchanged.

The MDA-MB-231/β41 breast carcinoma cell line, stably transfected with estrogen receptor beta was a gift from Dr. Debra Tonetti (University of Illinois at Chicago, Chicago, IL) and were maintained in Modified Eagle Medium (MEM) containing 1% non-essential amino acids (NEAA), 1% glutamax, 1% anti-biotic/anti-mitotic, 5% charcoal stripped calf serum, and 0.05% insulin (38).

2.4. Animals

All procedures followed the guidelines established by the institutional Animal Care and Use Committee along with state and federal regulations. The protocol complied with the Guide for the Care and Use of Laboratory Animals, and the facilities are Association for the Assessment and Accreditation of Laboratory Animals Care approved. Immature, female, Sprague–Dawley rats weighing ~50 g were obtained from Harlan (Indianapolis, IN). Animals were arrived at 12 days and were allowed 6 days to acclimate.

All rats consumed a diet that was certified phytoestrogen-free (Indianapolis, IN) in order to minimize the potential for abnormal experimental results due to phytoestrogens

commonly found in rodent feed. Access to food and water was unrestricted. Genistein, Genistein + 17β-Estradiol co-treatment, the positive control (17β-Estradiol; Sigma-Aldrich), and the vehicle (sesame oil), were administered by gavage.

The dosing of genistein was calculated by determining the concentration of genistein that activates ERβ but not ERα. This concentration was scaled to an equivalent rat dose of 2.5 mg/kg/day. The rats were treated with 10 μg/kg/day dose of 17β-Estradiol. The co-treatment of genistein and estradiol contained a 1:1 ratio of 2.5 mg/kg genistein and 2.24 μg/kg 17β-Estradiol. All rats were weighed to monitor body weight fluctuations and toxicity.

The animals began treatment on day 18 and were treated daily until day 21 when they were euthanized via CO_2 inhalation. Mammary, liver, and uterine tissue were collected along with blood and urine then placed on ice and ultimately stored in -80°C. The uteri were excised, trimmed of fat, and weighed before being placed on ice then stored in -80°C. Half of the tissue samples were stored in 4% paraformaldehyde in phosphate buffer to process for paraffin wax embedding.

2.5   Analysis of estrogenic activity

2.5.1. Estrogen Responsive Alkaline Phosphatase induction in Ishikawa Cells

The protocol used for the Ishikawa assay in Pisha et al. was used as described (39). Endometrial carcinoma Ishikawa cells were plated at 5×10^4 cells/well and in 96 well plates in estrogen-free medium for 24 h. Extracts and/or compounds were dissolved in DMSO and added at varying concentrations while ensuring that the DMSO concentration was less than 0.1%. To determine anti-estrogenic activity, the cells were

co-treated with 2nM 17β-estradiol, suitably above its EC_{50}. After treatment the plates were incubated at 37°C for 72 h then washed with PBS and lysed by adding 50μL of 0.01% Triton X-100 in 0.1 M Tris buffer at pH 9.8; followed by a freeze and thaw cycle at -80°C and 37°C, respectively. The phosphatase substrate, p-nitrophenol phosphate was added to each well and the alkaline phosphatase activity was measured by assessing the presence of p-nitrophenol at 405nm using a Power Wave 200 microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, VT). The fold induction of alkaline phosphatase for each individual treatment, in comparison to the estradiol control, denoted estrogenic activity and was calculated as previously described (39). Anti-estrogenic activity compared the induction of alkaline phosphatase by each treatment to the negative control, 4-OH tamoxifen.

2.5.2. Estrogen Response Element (ERE) Induction in MDA-MB-231/β41 cells

The protocol for the Dual-Luciferase Reporter Assay System from Promega (Madison, WI) was used to evaluate the activation of estrogen receptor beta through interaction with the ERE at the promoter of estrogen responsive genes. This presents as the expression of the fused luciferase reporter. MDA-MB-231/β41 cells are grown in phenol-red free medium and plated at 4×10^5 cells/mL in a 12 well plate. Following a 24 h incubation at 37°C, the cells were washed with PBS and Opti-MEM media was added for transfection. The cells were transfected with pERE at 3 μg/mL and pRL-tK at 1μg/mL for 6 h then washed twice with PBS and the phenol-free MEM media was added before treatment with extracts or compounds for 18 h. E2 and Diarylpropionitirile (DPN), a selective ERβ agonist, were used as positive controls. After the 18 h incubation at 37°C,

the cells were lysed with 1X cell lysis buffer and frozen at -80°C for 10 minutes to 24 h.

Once thawed, the cell lysates were collected in eppendorf tubes, centrifuged at 14,000

RPM at 4°C for 10 minutes and then 20 µL of the supernatant were placed in white

Costar 96-well plates. The plates were placed into the FLUOstar OPTIMA luminometer

(BMG Lab Tech, Offenburg, Germany) where 100 µL of the Luciferase Reagent was

injected into the wells followed by 100 µL of the Stop and Glo reagent to quench the

firefly luciferase expression and activation of the *Renilla* vector. To account for

transfection efficiency, the average read-out for the luciferase activity was normalized to

the average of the *Renilla* (pRL-tK) activity. To convert the data to fold induction the

results were normalized to the DMSO control.

2.5.3. <u>Advantages and disadvantages of comparing Ishikawa and β41 assays</u>

One of the advantages of using the MDA-MB-231/β41 cell line is that the cells are

breast carcinoma cells which is a hormone-sensitive model, compared to the traditional

ERβ model carried out in osteosarcoma U2OS cells. In addition, this luciferase assay is

relatively short, taking 3 days from start to finish, compared to the ERα assay that takes

7 days to complete.

A disadvantage would be the fact that comparing the Ishikawa assay to this β41

luciferase assay is much like comparing apples and oranges. For one, the Ishikawa cell

line is an endometrial carcinoma cell line and the β41 cells are breast carcinoma cells.

Also, the Ishikawa assay measures enzymatic activity, whereas, the β41 assay

measures chemiluminescence. In order to combat these challenges, an ERα-luciferase

assay, in MCF-7 breast carcinoma cells, was developed to establish a comparable ERα assay to the β41 assay.

2.5.4. Induction of mRNA expression of estrogen receptor beta target gene by Genistein and 8-PN.

Real-time polymerase chain reaction (RT-PCR) was used to determine the modulation of Otubain 2 (OTUB2), an estrogen receptor beta target gene, following treatment of MDA-MB-231/β41 cells with potent ERβ agonist genistein and potent ERα agonist 8-PN. The experiments were performed three different times in triplicate. The MDA-MB-231/β41 cells were plated in phenol-free media at 6×10^5 cells/mL in 6 well plates. After a 24 h incubation at 37°C, the cells were treated with genistein and 8-PN (both at 1 and 0.1uM concentrations) for 24 h. Cell lysis and RNA extraction were performed using the QiaShredder kit and RNEasy kit, respectively (Qiagen). The cells were lysed with RLT buffer and the RNA was extracted according to the RNeasy protocol. The cDNA synthesis was performed using Superscript III RT (Invitrogen). The PCR and associated analyses were conducted with the ABI StepOne Plus RT-PCR system (Applied Biosystems). The relative expression level of OTUB2 (Hs01027047_m1) was calculated using the delta-delta C_T method by comparing it with the relative mRNA expression levels of the endogenous gene beta actin (Hs 99999903_m1) and then the DMSO treated samples.

3. RESULTS

3.1. Alkaline Phosphatase induction in Ishikawa Cells

The Ishikawa is a reliable ERα positive endometrial cancer cell line that is primarily used for the evaluation of estrogens and anti-estrogens (39). Estrogenic activity is determined by a sample's ability to induce alkaline phosphatase activity, given that the enzyme's activity in this cell line is estrogen inducible. Anti-estrogenic activity is determined by the inhibition of alkaline phosphatase activity in the presence of 17β-estradiol.

The red clover clinical trial extract, spent hops extract, and the crude MeOH licorice extracts all showed the ability to induce alkaline phosphatase in a dose-dependent manner (Figure 6A). The hops, red clover, and GI extracts had similar EC_{50}s: 2.14 μM, 1.83 μM, and 0.8 μM respectively. Among the licorice species, *GI* was the most active with an EC_{50} of 0.8 μM. The EC_{50} values of *GG* and *GU* were similar: 8 and 10 μM, respectively. Although GI was the most potent licorice species, GU was the most efficacious with a maximum efficacy of around 60 fold. The weakest extract was black cohosh with relatively no estrogenic activity. The relative EC_{50} ranking of the extracts is: hops ≈ red clover ≈ GI > GU > GG >> black cohosh, while the ranking of the relative maximum efficacy is: hops > red clover > GU > GI > GG >> black cohosh.

The pure compounds were also able to induce alkaline phosphatase in a dose-dependent manner (Figure 6B). The most potent pure compound, 8-PN, had an EC_{50} of 7.23 ηM followed by genistein with an EC_{50} of 0.36 μM. The EC_{50} values for the licorice compounds LigF, LigC, and LicA are: 3.45 μM, 2.82 μM, and 1.35 μM respectively. The most efficacious phytoestrogen was also 8-PN, which had a maximum efficacy of 123 fold (Figure 6B). Genistein's maximum efficacy was 79 fold, whereas the maximum

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efficacy for the licorice compounds (LigF, LigC, and LicA) were as follows: 85, 54, and 26, respectively (Figure 6B). The ranking of EC_{50} values of the pure compounds is 8-PN >> genistein > LicA > LigC ≈ LigF. The ranking of maximum efficacy of the pure compounds is 8-PN >> genistein > LigF > LigC > LicA. There was no anti-estrogenic activity associated with any of the extracts or pure compounds.
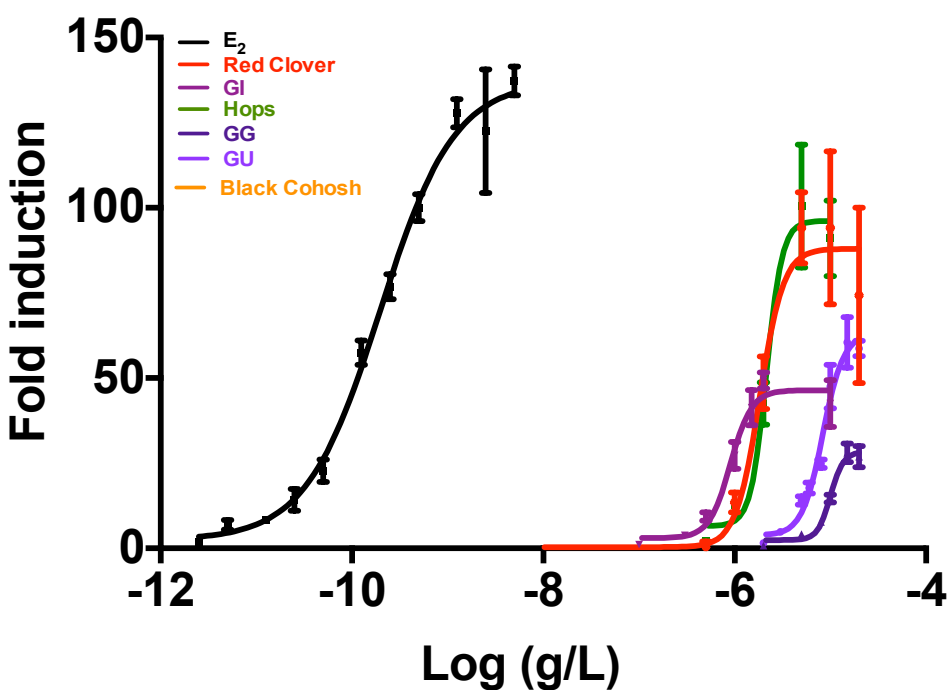
Figure 6A: Popular botanicals red clover and hops are full ERα agonists, whereas the licorice species are partial ER agonists with various estrogenic potency and efficacy in Ishikawa cells.

ERα expressing, malignant, endometrial carcinoma cells (Ishikawa) were treated with

botanical extracts and incubated for 72 hours. Cells were analyzed for estrogen

inducible alkaline phosphatase enzyme activity.
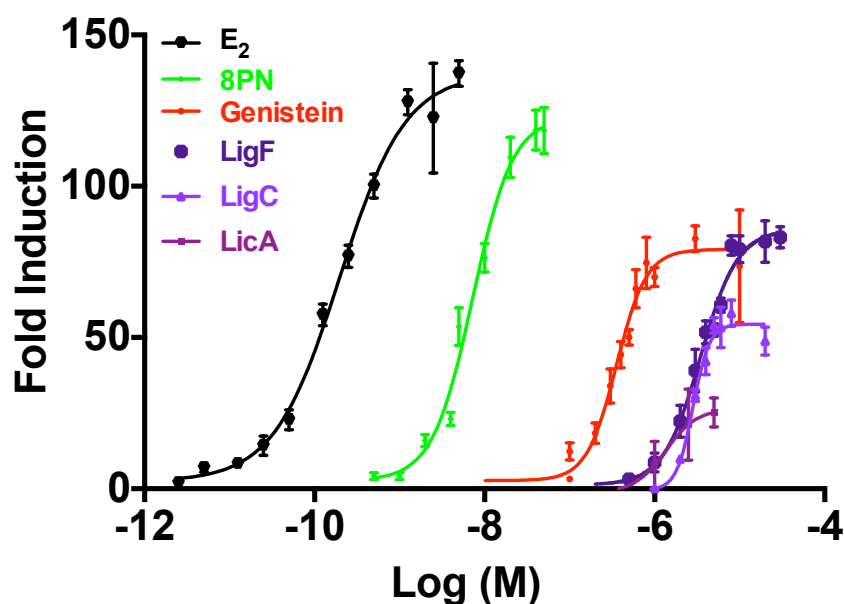
Figure 6B: The potent phytoestrogen 8-PN is the most potent ERα agonist in Ishikawa
cells, followed by genistein and the licorice compounds.

ERα expressing, malignant, endometrial carcinoma cells (Ishikawa) were treated with

pure compounds and incubated for 72 hours. Cells were analyzed for estrogen inducible

alkaline phosphatase enzyme activity.

3.2.   Induction of ERE-luciferase in MDA-MB-231/β41 cells.

The MDA-MB-231/β41 cells were stably transfected with estrogen receptor beta in order to establish an estrogen receptor beta positive cell culture model (38). The β41 cells were co-transfected with pERE-luciferase reporter and the pRL-tK control vector in order to evaluate the ERE transcriptional activity of ERβ in response to treatment with the botanical extracts and pure compounds. The extracts were able to induce ERE-luciferase activity in a dose- dependent manner (Figure 7A). The red clover extract was the most potent ERβ agonist, followed by GI with EC_{50}s of 0.228 μM and 0.233 μM, respectively. Both red clover and GI are 10 times more potent in the ERβ cells than the ERα cells. The hops, GG, and GU extracts had EC_{50}s of 0.69 μM, 2.12 μM, and 8.81 μM, respectively. As seen with the Ishikawa assay, the black cohosh extract showed no estrogenic activity. Red clover was the most efficacious extract as well with a maximum efficacy of 124 fold. The maximum efficacy of hops, GI, GU, and GG are as follows: 72, 92, 83, and 51 fold, respectively. The EC_{50} rankings are: red clover ≈ GI > hops > GU > GG. The maximum efficacy rankings are: red clover > GI > GU > hops >GG.

Overall, the pure compounds mirrored the activity of their corresponding extracts (Figure 7B). Genistein was the most potent phytoestrogen with an EC_{50} of 7.624 ηM and a maximum efficacy of 108 fold. 8-PN, DPN, PPT, LigF, and LicA have EC_{50}s of 29.220 μM, 1.705 μM, 2.486μM, and 2.071 μM fold, respectively. Genistein was shown to be 100 times more potent in ERβ cells, whereas, 8-PN was more than 1000 times more selective for ERα. The maximum efficacy of genistein was 108 fold while the maximum efficacy of 8-PN was 212 fold. Though 8-PN has a higher maximum efficacy, it was obtained at a relatively high concentration, 80 μM. The maximum efficacies of the

licorice compounds LigF and LicA were 122 and 64 fold, respectively. The highly

selective ERβ agonist, DPN, had a maximum efficacy of 137 fold while the highly

selective ERα agonist, PPT, had a maximum efficacy of 9 fold. The EC50 ranking is:

Genistein >>DPN > LigF > LicA > LigC > PPT >> 8-PN. The maximum efficacy ranking

is: 8-PN > DPN > LigF > Genistein > LicA >> PPT. There was no anti-estrogenic activity

associated with any of the extracts or pure compounds.
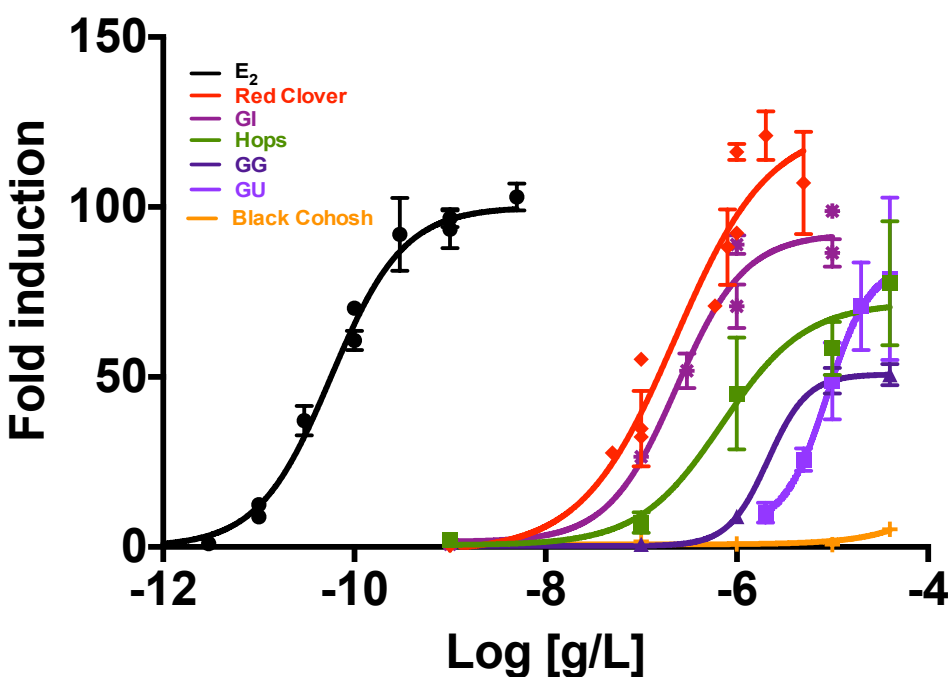
Figure 7A: Popular botanicals red clover and GI are full ERβ agonists, whereas the hops, GG, and GU are partial ERβ agonists with various estrogenic potency and efficacy in MDA-MB-231/β41 cells.

ERβ expressing, malignant breast carcinoma cells (MDA-MB-231/β41) were transfected

with estrogen response element (ERE) for 6 hours then treated with the botanical

extracts for 18 hours. After treatment, the cells were analyzed for chemiluminescence in

a standard luciferase assay.
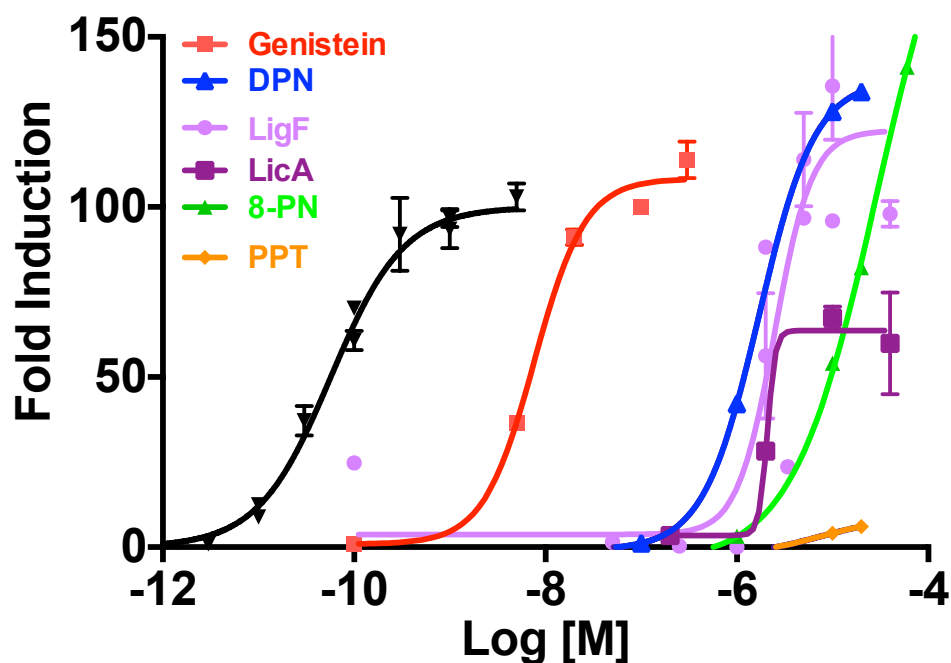
Figure 7B: Genistein is the most potent ERβ agonist in MDA-MB-231/β41 cells.
Selective ERβ agonist DPN and LigF have similar potency and efficacy, highlighting
LigF's potent estrogenic activity in ERβ cells.

ERβ expressing, malignant breast carcinoma cells (MDA-MB-231/β41) were transfected

with estrogen response element (ERE) for 6 hours then treated with the compounds for

18 hours. After treatment, the cells were analyzed for chemiluminescence in a standard luciferase assay.

3.3. <u>Induction of ERE-luciferase in MCF-7 cells.</u>

The ERα expressing breast carcinoma cell line, MCF-7, was co-transfected with pERE-luciferase reporter and the pRL-tK control vector in order to evaluate the ERE transcriptional activity of ERα in response to treatment with the botanical extracts and pure compounds. This assay was used to determine if the findings in the Ishikawa assay, that used ERα expressing endometrial carcinoma cells, were similar in an ERα expressing breast carcinoma cell line. Genistein and 8-PN were the only compounds tested and were found to be able to induce ERE-luciferase activity in a dose- dependent manner (Figure 8). In contrast to the Ishikawa data, this preliminary data suggests that genistein was the most potent compound with an EC_{50} of 0.71 µM followed by 8-PN with an EC_{50} of 2.7 µM. Both compounds are full ERα agonists.
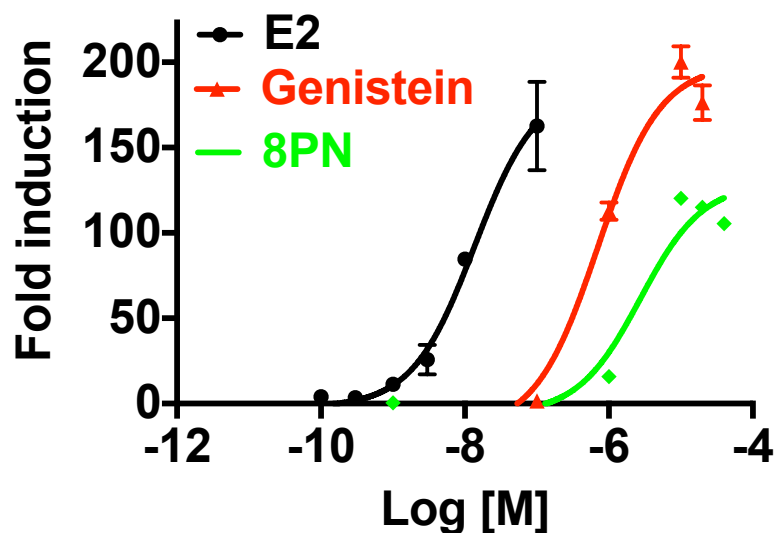
<u>Figure 8</u>**:** Preliminarily data showing that the potent phytoestrogen genistein is the most potent ERα agonist in MCF-7 cells, followed by 8-PN.

ERα expressing, malignant breast carcinoma cells (MCF-7) were transfected with estrogen response element (ERE) for 6 hours then treated with the compounds for 18 hours. After treatment, the cells were analyzed for chemiluminescence in a standard luciferase assay.

3.4.   Induction of OTUB2 mRNA expression in MDA-MB-231 cells

The induction of otubain2 (OTUB2) mRNA expression in ERβ positive breast cancer MDA-MB-231/β41 cells is used to confirm the selectivity of 8-PN, an ERα selective phytoestrogen, and genistein, ERβ selective phytoestrogens. Upon treating the β41 cells with the pure compounds and completing the RNA extraction, cDNA synthesis,

and PCR experiments the gene induction results were calculated. The full ERβ agonist genistein was able to induce OTUB2 mRNA expression in a dose dependent manner (Figure 9A). To confirm that 8-PN does not activate ERβ, its ability to induce mRNA expression of OTUB2 was also measured (Figure 9B).
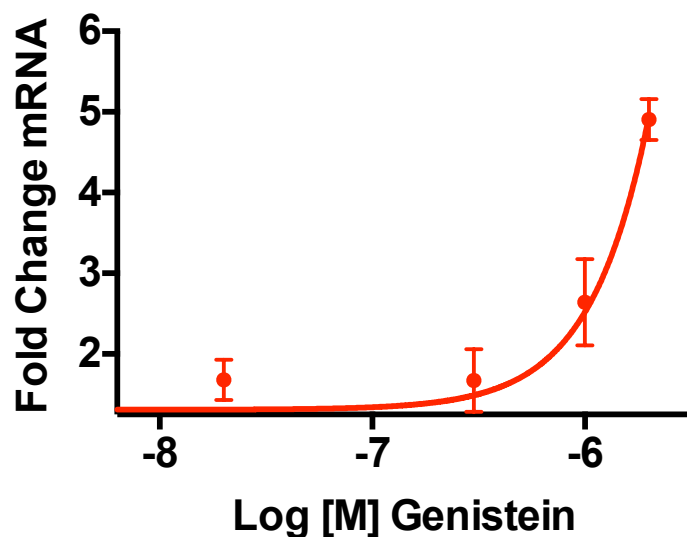
Figure 9A: Genistein, a full ERβ agonist, induces OTUB2 mRNA expression in a dose dependent manner
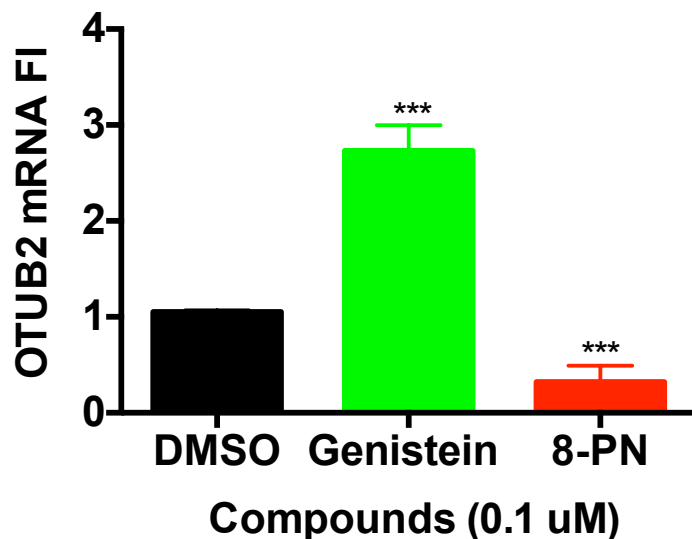
Figure 9B: The full ERβ agonist, Genistein, was able to induce OTUB2 mRNA expression, contrary to the ERα agonist 8-PN. ***P < 0.01.

MDA-MB-231/β41 cells were treated with genistein and 8-PN for 24 h and then

analyzed for ERβ selective gene mRNA expression. Results are shown as fold induction

relative to the level observed in cells treated with solvent only. Results are the means of

nine determinations ± SD;***p< 0.01.

3.5.   Comparison of extracts and compounds across ER subtypes

In this study, extracts from *Trifolium pratense, Humulus lupulus, Glycyrrhiza glabra,*

Glycyrrhiza inflata, Glycyrrhiza uralensis, and *Cimicifuga racemosa* (L.) Nutt, were

assessed for their estrogenic activity and their selectivity for a particular subtype of

estrogen receptor. The hops extract had the strongest binding affinity for ERα of all of

the extracts tested. Ironically, the most potent phytoestrogen in hops, 8-PN, is the least

abundant in the extract. Xanthohumol is the most abundant compound and yet has no

estrogenic activity (32). It is noted that although xanthohumol is the most abundant compound, it is readily cyclized to isoxanthohumol, which is then metabolized by cytochrome P450 to 8-prenylnaringenin. In red clover, the methoxy ethers biochanin A and formononetin are the most abundant flavonones in the extract. Both of the methoxy esters are metabolized by cytochrome P450 to genistein and daidzein, respectively. GG and GU are primarily composed of liquiritigenin (LigF) and isoliquiritenin (LigC) with LigF being the most abundant in both extracts. GI also contains LigF and LigC but its most abundant compound is the chalcone licochalcone A.

Extracts from *Trifolium pratense* and *Glycyrrhiza inflata* were showed to preferentially engage estrogen receptor β as full ERβ agonists. Both red clover and GI were ten times more potent in ERβ cells than in ERα (Figure 10A). In contrast, *Humulus lupulus* was shown to preferentially engage estrogen receptor α as a full agonist (Figure 10A), as noted in previous studies (30). The remaining licorice species *Glycyrrhiza glabra* and *Glycyrrhiza uralensis* showed no significant difference in activity between the two subtypes signifying their roles as partial agonists (Figure 10A). *Cimicifuga racemosa* (L.) Nutt. did not display any estrogenic activity, which is confirmatory of previous studies (33).
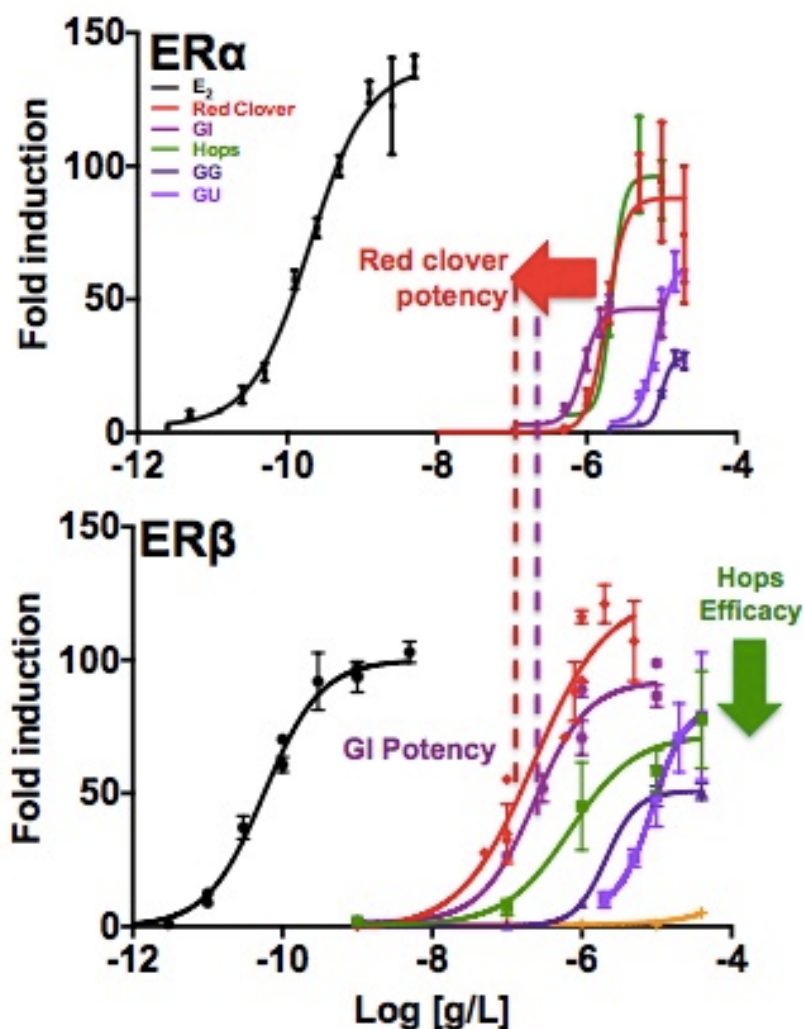
Figure 10A: Red Clover & G. Inflata are 10x more potent in ERβ cells than ERα.

Genistein, the phytoestrogen in Trifolium pratense, was over one hundred times more selective for ERβ than ERα and was a full ERβ agonist (Figure 10B), echoing the activity of the extract. Genistein was the most potent phytoestrogen tested in ERβ cells. 8-prenylnaringenin, in Humulus lupulus, is a full ERα agonist and was greater than one thousand fold more selective for ERα; PPT, a known ERα agonist is 400 fold more

selective for alpha (Figure 10B). The ERβ selective ligand DPN and LigF have similar potency and efficacy, which is indicative of LigF's potential as an ERβ agonist. Surprisingly, the predominant phytoestrogen in Glycyrrhiza inflata, LicA, showed very weak activity in both cell lines, despite the extract's full agonistic activity in ERβ cells (Figure 10B). This finding suggests the presence of an unknown ERβ selective ligand in the GI extract. The potencies of both the extracts and compounds in ERα and ERβ cells are listed in Table V.
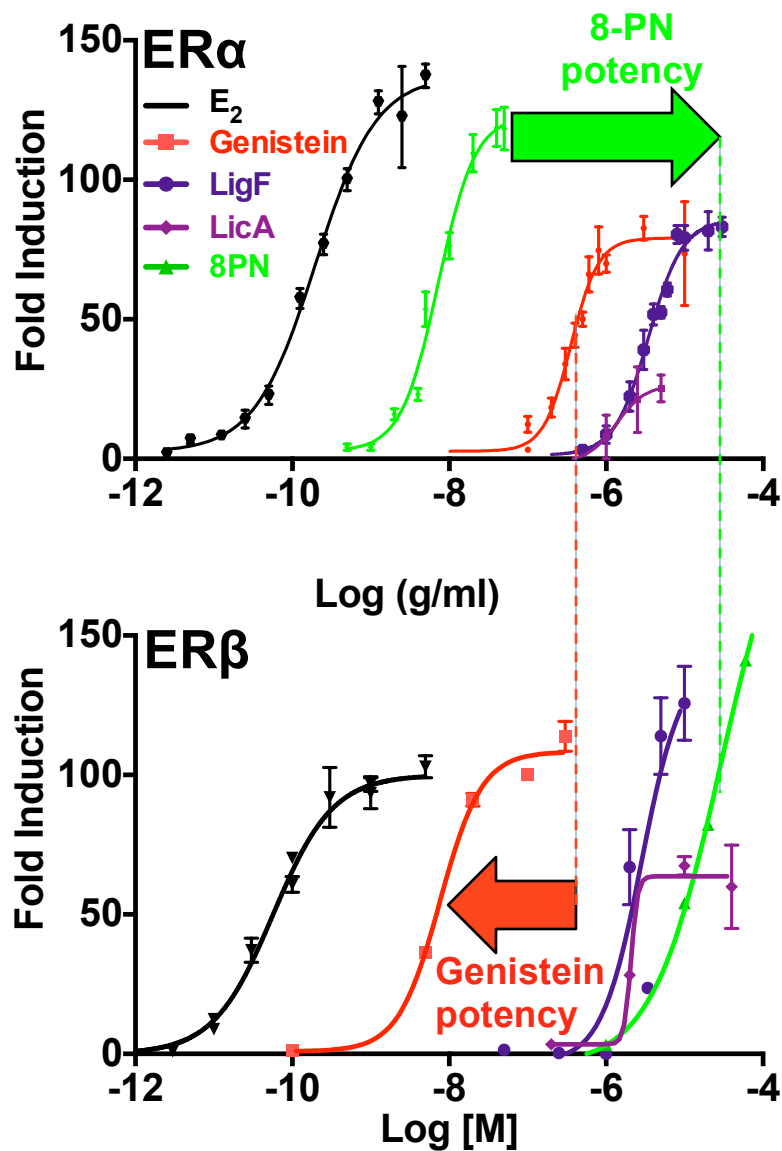
<u>Figure 10B:</u> Genistein is 100x more potent in ERβ cells than ERα. 8-PN is over 1000x more selective for ERα.

Table V: COMPARISON OF POTENCIES OF BOTANICAL EXTRACTS AND COMPOUNDS IN ERα AND ERβ

Extracts	$EC_{50}{}^a$	
	ERα	**ERβ**
Hops	2.15 ± 0.04	1.62 ± 0.49
Red Clover	1.70 ± 0.4	0.204 ± 0.10
GG	10.0 ± 0.02	2.12 ± 0.12
GU	8.34 ± 0.02	8.82 ± 0.18
GI	0.910 ± 0.1	0.233 ± 0.08
Compounds	$EC_{50}{}^b$	
8-PN	0.007 ± 0.03	2.92 ± 0.09
Genistein	0.47 ± 0.2	7.62 ± 0.06
LigF	3.46 ± 0.03	2.84 ± 0.2
LicA	3.61 ± 2.6	2.07 ± 0.01
DPN	0.55 ± 0.83	1.71 ± 0.01
PPT	2.63 ± 2.69	NA

[a] EC_{50} extracts was measured in g/L. [b] EC_{50} of compounds was measured in uM.

3.6. Additive effects of Genistein when co-treated with estradiol in-vivo

This uterotrophic assay was performed to determine if low-dose genistein, which does not induce uterotrophic activity alone, inhibits estradiol induced uterotrophic activity in immature rats As previously mentioned, the animals were treated with a low dose of estradiol, high dose of estradiol, genistein at 2.5 mg/kg/BW, and a co-treatment of genistein and low dose estradiol. After collection, the uterus from each animal was weighed and entered into an electronic spreadsheet for further calculations. Though previous in vivo studies reported mixed results, we hypothesized that the low concentration of genistein would preferentially activate ERβ and subsequently prevent uterine growth. As seen in Tinwell et al, the low dose of genistein, did not display any

additive estrogenic effects (40). However, when co-treated with estradiol, genistein did display synergistic uterotrophic effects.

Figure 11: Genistein alone did not display uterotrophic activity but did display additive estrogenic effects on immature rat uterine weight. P < 0.0002.

3.7.   Discussion and Conclusions

In the last decade there has been a paradigm shift within the healthcare industry that trends towards "natural" and "holistic" treatments for a variety of conditions, including
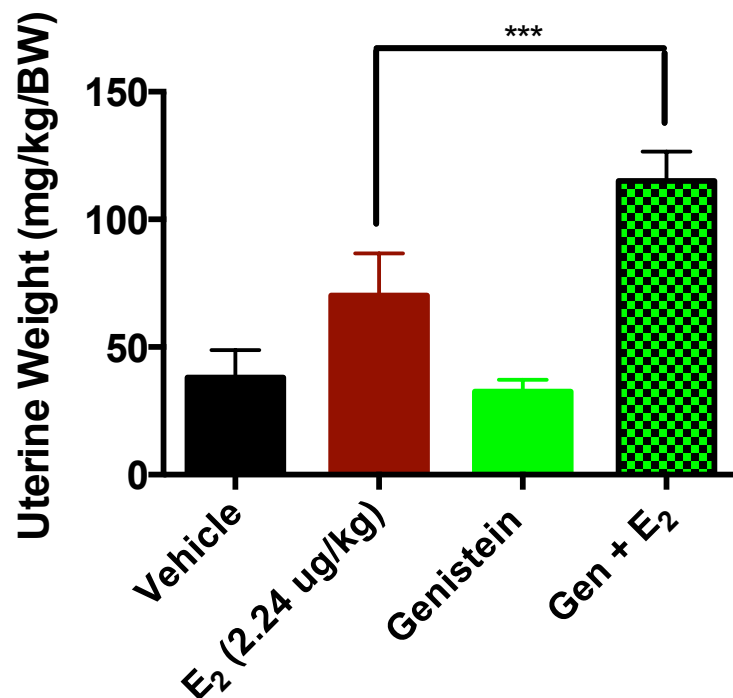
menopause (41), (42), (43). The botanical dietary supplement industry is a multi-billion dollar industry with a growing sector dedicated to women's health, indicating it's growing popularity. It has been well established that although the phytoestrogens present in these botanicals are weak compared to the natural ligand, 17β-estradiol, they do possess estrogenic activity. However, a comprehensive evaluation of the selectivity of these popular botanicals in functional assays has not been well documented. (31), (18), (33).

The selectivity of these botanicals is an important aspect of this project because the activation of ERα has been associated with an increased risk of cellular proliferation in hormone sensitive tissues, while the activation of ERβ has been shown to inhibit cellular proliferation and tumor formation (44). Thus, ERβ agonists have been thought to be ideal, novel targets for breast cancer treatment (45). In addition, ERβ agonists are considered to be new targets for menopausal symptom relief. Bionovo, Inc. has developed an estrogen receptor beta modulator, Menerba (MF-101), which contains 22 botanicals known to be used in traditional Chinese medicine for the treatment of menopausal symptoms (46). During pre-clinical studies Menerba, whose active estrogenic compound is liquiritigenin (LigF) from GU, did not promote the growth of breast cancer nor did it stimulate uterine growth. Phase II clinical studies revealed that this compilation of ERβ selective botanicals were safe and showed to be more efficacious in relieving hot flashes than the placebo (46). Although studies like these have shown the beneficial effects of modulating ERβ over ERα, few have done side-by-side analyses of the activities of a variety of botanicals in both receptor subtypes.

In this study, five of the six extracts evaluated for their estrogen receptor activity and selectivity had actually displayed estrogenic activity. Hops was the only extract to preferentially bind to and activate ERα. As expected and previously determined in Hajirahimkhan et al, 8-PN, also displayed selectivity for ERα and like its parent extract, Hops, it was a full ERα agonist (33). The extracts red clover and GI were the only botanicals to preferentially engage estrogen receptor β. Genistein, found in red clover, also preferred to bind to and activate ERβ and was also a full ERβ agonist. Estrogen receptor β activity is preferred due ERβ's well-known anti-proliferative activity. This is not the first study to show genistein's selectivity for ERβ. Jiang et al showed that genistein was not only able to selectively bind to ERβ but was also able to elicit an estrogenic response in adenovirus infected, ERβ-expressing MCF-7 cells (47).

To confirm the accuracy of the ERE-luciferase assay in MDA-MB-231 cells, the potent ERβ agonist DPN and the potent ERα agonist PPT were evaluated. As expected, DPN showed estrogenic activity in the ERβ cells, whereas PPT showed little to no activity. This data confirms the results in Vijaykumar et al, which showed that PPT was synthesized to be an estrogen receptor α selective ligand and has a four hundred fold preference for ERα (48). Much like PPT, 8-PN was not very active in ERβ cells, however the data indicates that it was over one thousand times more selective for ERα than ERβ.

Gene induction assays were performed to further highlight the differences in selectivity and potency between genistein and 8-PN. Similar to data found in Jiang et al, genistein was able to induce the mRNA expression of Otubain 2 (OTUB2), an ERβ target gene at concentrations as low as 0.1 μM. As predicted, 8-PN was not able to

induce the mRNA expression of OTUB2 at 0.1 µM. This serves, as confirmation that 8-PN is a potent ERα selective ligand and down-regulates the expression of an ERβ gene.

The in vivo study results suggest that genistein, when co-treated with estradiol, initiates additive estrogenic effects on immature rat uterus. The treatment with genistein alone did not increase the uterine weight compared to the vehicle. Similar results were found in Tinwell et al with immature rats treated with 1 mg/kg genistein. This occurrence could be attributed to genistein's preference for ERβ, which, upon activation, would not induce endometrial cell proliferation.

Interestingly, licochalcone A, the most abundant compound present in the ERβ agonist GI, showed very little estrogenic activity in both ERα and ERβ cells. Though there is very little data available regarding licochalcone A's estrogenic activity, the strong ERβ activity present with the GI extract suggests the presence of an unknown ERβ agonist. It can be deduced that the ERβ-mediated estrogenic activity of GI is not associated with the phytoestrogens LigF and LigC because if so, the remaining licorice extracts, GU and GG would also display ERβ selectivity and potency. In fact, LicA, LigF and LigC are all partial ERβ agonists. LigF, specifically, is partially selective for ERβ according to ligand assays and literature (33).

This project serves as a great addition to the body of knowledge concerning estrogenic botanicals by highlighting the differences in selectivity of a wide range of popular women's health botanicals, specifically the pure compounds genistein and 8-prenylnaringenin. The activation of ERβ has been suggested to be beneficial in the reduction and prevention of cell proliferation and this work provides comprehensive data

on the botanicals that selectively bind and engage ERβ. Based on our findings, the ERβ selectivity and the potency of genistein suggest that red clover might be a relatively safe menopausal remedy.

3.8.   Future Directions

Based on the data gathered in this study, it is imperative that future experiments investigate the source of GI's ERβ selectivity. To further analyze the selective activity of GI, future experiments include bioassay-guided fractionation of the Glycyrrhiza inflata extract to identify the unknown ERβ selective ligand responsible for the increased ERβ activity of the extract. In addition, binding data for the Glycyrrhiza inflata extract along with licochalcone A could also provide clarity.

The ERα assay will be redesigned with MDA-MB-231/S30 cells that have been stably transfected with ERα, which will be a better model for comparison. Lastly, an ERβ animal model will be designed to further investigate how genistein interacts with ERβ in vivo.

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EDUCATION

MS in Pharmacognosy, University of Illinois at Chicago, 2015
Thesis title: "Investigating the modulation of estrogen receptor signaling by popular botanical dietary supplements used by menopausal women."
Thesis Advisor: Judy L. Bolton, PhD.

BS in Biology & Chemistry, Georgia State University, 2012
Undergraduate Thesis: "Analysis of Cleavage of Phosphatidylcholine by Cerium IV metal complexes"
Thesis Advisor: Kathyrn B Grant, PhD

RESEARCH EXPERIENCE

Graduate Research Assistant, UIC/NIH Center for Botanical Dietary Supplements Research, University of Illinois at Chicago, August 2012- July 2014

Investigated the estrogenic effects of popular dietary supplements used by women for menopausal symptom relief in malignant endometrial and malignant breast cancer cell lines. Also investigated the modulation of estrogen receptor signaling by popular botanical dietary supplements used by menopausal women. Results indicated that botanical dietary supplements from the Licorice and Red Clover species demonstrate partial estrogen receptor alpha agonistic effects and stimulate the estrogen receptor element promoter in breast cancer cells.

- Maintained all cell lines
- Prepared all reagents, buffers, and experimental instruments
- Conducted lab experiments using tissue culture, high-throughput cell based assays, real-time polymerase chain reaction (RT-PCR), microscopy, gel electrophoresis, transfection, luciferase assays and western blotting.
- Documented detailed accounts of experiment protocols, results, and data in laboratory notebook for research publication.
- Analyzed data for statistical significance using Graph Pad Prism
- Presented data in project meetings

- Investigated the use of botanical dietary supplements in current literature
- Followed safety procedures required for laboratory use
- Maintained functionality of common lab instruments

Graduate Summer Research Fellow, National Institutes of Health/ National Human Genome
Research Institute, Cancer Genetics Branch, Metastasis Genetics Section, Bethesda, MD
Summer 2013

Investigated the functional analysis of inherited germline polymorphisms in Necdin, a modifier of mammary tumor metastasis in malignant mouse breast cancer cell line. Results indicated that Necdin serves as metastasis modifier by binding to the c-Myc locus, possibly regulating its oncogenic activity.

- Maintained all cell lines
- Performed all laboratory experiments using chromatin-immunoprecipitation sequencing and qPCR (ChIP-seq/ChIP-qPCR), RT-PCR, Western blotting, soft agar migration/invasion assays, and tissue culture.
- Documented detailed accounts of experiment protocols, results, and data in laboratory notebook.
- Conducted a review of relevant literature and supporting documentation
- Developed and organized abstracts and posters for conference presentation
- Presented data in project meetings
- Adhered to laboratory safety regulations at all times.
- Attended professional development seminars
- Participated in genome-wide sequencing journal club

Study Abroad Research, Current Topics in Environmental Health and Diseases in Italy, Georgia State University, Ca'Foscari University, Summer 2012

This study abroad program was an interdisciplinary curriculum that combined the disciplines of immunology and environmental sciences. The course covered topics related to ecotoxicology and environmental cleanup, the immune system, environmental pollutants, endocrine disruptors, heavy metal toxicity, global warming, modulation of immune responses by environmental pollutants, causal links between exposure and immunomodulation.

- Attended lectures and seminars by Ca'Foscari immunologists and environmental scientists
- Studied the importance of environmental regulation
- Examined the correlation between environmental hazards and chronic diseases such as cancer, asthma, and respiratory diseases.

Undergraduate Research Assistant, Department of Chemistry, Georgia State University, Fall 2010
Summer 2012

Analyzed the hydrolysis of the ligand phosphatidylcholine with Cerium IV metal complexes to identify a possible therapeutic agent for this disease. Lysosomal Storage Disease is characterized by the accumulation of macromolecules in the lysosome, which can cause cellular dysfunction. Also conducted spectroscopic titrations to identify the types of DNA interactions used by 9-Aminomethyl anthracene dye to photocleave pUC19 plasmid DNA in the presence of salt.

- Performed all laboratory experiments including UV-vis, gel electrophoresis, sonication, and
- Presented data at project meetings
- Organized abstracts and posters for annual Georgia State Undergraduate Research Conference
- Documented detailed accounts of experiment protocols, results and data in laboratory notebook for research publication.
- Prepared all reagents, buffers, and instruments

Study Abroad Research, Medical Virology in Argentina, Georgia State University, National University of Cordoba, Dr. JM Vanella Institute of Virology, Summer 2010

Focused on viruses of public health concern in Argentina and current research on these viruses at the Institute of Virology.
- Toured the Centers of Disease Control
- Performed laboratory research in a wet lab using microscopy, gel electrophoresis, and basic microbiology techniques
- Attended medical virology seminars and lectures
- Toured the Malbrann Institute in Buenos Aires, Argentina
- Compared public health virology initiatives between the United States and Argentina.

TEACHING EXPERIENCE

Graduate Teaching Assistant, Department of Medicinal Chemistry & Pharmacognosy, University of Illinois at Chicago, fall 2012- spring 2015
- Assigned course grades
- Maintained regular office hours for student discussions and tutoring
- Monitored student attendance and class participation
- Managed lecture materials, assignments, and grades using the online learning system *BlackBoard*
- Proctored and graded exams
- Created supplemental course materials

Molecular Biology Teaching Intern, Department of Biology, Georgia State University, 2012 (summer)
- Supervised laboratory experiments
- Prepared laboratory reagents and calibrated instruments needed for experiments
- Managed assignments and grades using the online learning system *ULearn*
- Provided technical assistance for the laboratory exercises and projects
- Graded labs and projects
- Proctored lab and course exams

Teaching Assistant, Department of Chemistry, Georgia State University, fall 2010-spring 2012
- Prepared laboratory reagents and calibrated instruments needed for experiments
- Provided instruction and assistance for laboratory experiments
- Graded lab exercises and assignments
- Proctored lab and course exams
- Evaluated lab assignments for completeness and comprehension

PUBLICATIONS Non-Peer Reviewed

2015
Green SE. In vitro comparison of estrogenic activities of popular women's health botanicals used for menopausal symptom relief. MS Thesis. University of Illinois at Chicago, Chicago, IL.

CONFERENCE PARTICIPATION

Spring 2015
University of Illinois at Chicago College of Pharmacy Research Day, Chicago, IL "Red clover and Glycyrrhiza inflata display ERbeta selectivity, suggesting better safety profile for women's health", **Poster Presentation**

Fall 2014
National Organization for the Professional Advancement of Black Chemists and Chemical Engineers (NOBCChE), New Orleans, LA "In vitro comparison of estrogenic activities of popular women's health botanicals used for menopausal symptom relief", **Poster Presentation**

Summer 2013
National Human Genome Research Institute Summer Research Conference, Bethesda, Maryland " Functional Analysis of Germline Polymorphisms in Necdin, a Modifier of

Mammary Tumor Metastasis", **Poster Presentation**

National Institute of Health Summer Research Conference, Bethesda, Maryland " Functional Analysis of Germline Polymorphisms in Necdin, a Modifier of Mammary Tumor Metastasis", **Poster Presentation**

Spring 2012
5th Annual Georgia State Undergraduate Research Conference, Atlanta, Georgia, "Analysis of Cleavage of Phosphatidylcholine by Cerium IV metal complexes", **Poster Presentation**

PROFESSIONAL MEMBERSHIPS

Who's Who Among Students in American Colleges and Universities

Beta Beta Beta Biological Honor Society

MIKI Medicinal Chemistry Conference Planning Committee