Modulation of Estrogenic Activity in Estrogen Receptor α and β by Flavonoids in Women's Health Botanicals

BY

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THESIS

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Dedicated to my God the King for His glory, and to my love-my wife Liz.

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CONTRIBUTION OF AUTHORS

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LIST OF ABBREVIATIONS

aa	Amino acids
AF-1	Activation function 1
AF-2	Activation function 2
AhR	Aryl hydrocarbon receptor
AI	Aromatase Inhibitors
AP-1	Activator protein-1
ATF	Activating transcription function
AhR	Aryl hydrocarbon receptor
cAMP	Cyclic adenosine monophosphate
CBP	Cyclic-AMP response element binding protein
COMT	Catechol-O-methyltransferases
DBD	DNA binding domain
DHEA	Dehydroepiandrosterone
DPN	2,3-bis(4-hydroxyphenyl)-propionitrile
E_1	Estrone
E_2	Estradiol
E_3	Estriol
E_4	Estretrol
EGFR	Epidermal growth factor receptor
ER	Estrogen Receptor
ERE	Estrogen Response Element
FSH	Follicle Stimulating hormone

LIST OF ABBREVIATIONS (continued)

GPER1	G-protein coupled receptor-1
HDAC	Histone deacetylase
HR (+)	Hormone Receptor positive
3β-HSD	3β-hydroxysteroid dehydrogenase/Δ5-4 isomerase
17β-HSD	17β-hydroxysteroid dehydrogenase
IGFR1	Insulin-like growth factor-1 receptor
LBD	Ligand binding domain
LDL	low density lipoprotein
LH	Luteinizing hormone
NCOR	Nuclear receptor coactivator repressor
PKC	Protein kinase C
PPT	4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol
RR	Relative risk
ROS	Reactive oxygen species
SERM	Selective estrogen receptor modulator
SMRT	Silencing mediator for retinoid and thyroid hormone
Sp-1	Stimulating protein-1
SRC	Steroid receptor coactivator
StAR	Steroidogenic Acute Regulatory protein

SUMMARY

Estrogens are endogenous hormones that are integral to proper physiological development and function in the body. These include development and proper function of reproductive system as well as maintenance of metabolism and the homeostatic function of organ systems. Although estrogens chemically comprise a number of steroid metabolites, the term is mostly used to refer to estradiol (E_2), the most biologically active hormone among them. E_2 induces its biological activity through a number of estrogenic pathways. In the classical pathway, E_2 interacts primarily with two intracellular receptors estrogen receptors (ER) α and β. In the presence of E_2, ERα drives a number of cellular functions including cell proliferation, while ERβ is associated with cellular differentiation and attenuating ERα driven proliferation in tissues.

Breast cancer, the most frequently occurring type of cancer and the second leading cause of death in women is associated with estrogen exposure. In estrogen carcinogenesis, there are two notable pathways: chemical pathway and hormonal pathway. In the chemical pathway, E_2 is metabolized to 2-hydroxyestradiol or 4-hydroxyestradiol by P450 1A1 and P450 1B1 enzymes, respectively. P450 1B1 is known to be expressed in the breast. The 4-hydroxyestradiol metabolite can be oxidized to a genotoxic quinone. Through redox cycling of the quinone with its semiquinone radical, reactive oxygen species are formed. Ultimately, this leads to alkylation and oxidation of DNA causing DNA damage and genotoxoicity. A second pathway is the hormonal pathway. In this pathway, a ligand-bound ER translocates into the nucleus, recruits' transcription factors such as the steroid receptor coactivator

(SRC), and binds to specific sites on DNA, initiating transcription. These recruited transcription factors regulate genomic transcription. However, studies report that under certain conditions, when tissue such as mammary gland is exposed to prolonged E_2 stimulation, proliferation rates increase and the risk of genetic mutation is elevated. These events can lead to mammary carcinogenesis which is driven by some pathways including ERα activation. A considerable loss in mammary ERβ expression during early breast carcinogenesis has also been reported in women, suggesting a possible chemoprotective role for ERβ. The putative antiproliferative and chemoprotective functions of activated ERβ, has driven an interest in identifying ligands that favor regulation of the ERβ pathway.

There have been reports of increased risk of breast cancer in postmenopausal women using combined hormone replacement therapy to manage postmenopausal symptoms. These hormone treatments received by women have comprised estrogens and medroxyprogesterone. Consequently, many women have turned to natural alternatives such as botanicals to manage their postmenopausal symptoms. These relatively poorly characterized botanicals when consumed modulate the function both ER subtypes in a manner not fully understood. Thus, there is still significant need to characterize the pharmacology and safety of dietary supplements derived from botanicals.

We hypothesized that the selected women's health botanicals contain ERβ–preferential constituents. These ERβ–preferential (iso)flavonoids and botanicals may

provide chemoprotection against breast cancer, due to their antiproliferative activity that counterbalances ERα activity. In this work, several (iso)flavonoids and botanicals where studied using cell-based *in vitro* models originating from endometrial and breast carcinomas cells. The three aims of this project were: (1) to optimize assay methods for the analysis of estrogenic activity of ERα and ERβ (2) to study selected (iso)flavonoids and botanical extracts for these estrogenic activities, and (3) to determine chemical structures in botanicals that demonstrate ERβ-preferential activity.

In Aim 1, an assay method was optimized to confirm that its estrogenic activity results of the tested bioactive compounds and botanical extracts reflected their estrogenic activity *in vitro*. For the ERα activity analysis assay, endometrial carcinoma Ishikawa ERα (+) cells were used and E_2–dependent alkaline phosphatase activity was employed as the endpoint. For the activity analysis assay for ERβ, stably transfected ERβ (+) MDA-MB-231/β41 cells where used in an ERE-luciferase assay; luciferase activity was the colorimetric readout. The ERα selective ligand 4,4',4''- (4-propyl-[1*H*]-pyrazole-1,3,5-triyl) *tris*phenol (PPT) showed low nanomolar EC_{50} values in the alkaline phosphatase assay and evoked no response in the ERβ assay. The ligand 2,3-*bis* (4-hydroxyphenyl)-propionitrile (DPN), an ERβ-selective synthetic ligand, showed 30-fold selectivity for ERβ over ERα and low nonmolar EC_{50} in ERβ. Cotreatment with E_2 and fulvestrant (an ER antagonist and ER degrader) in the ER(+) assays and using MDA-MD-231 ER (-) and E_2 in the ERE-luciferase assay showed significantly low activity in both assays. These results confirmed the direct modulation of ER by the compounds and validated our functional estrogenic assay.

In an effort to identify ERβ-preferential compounds in botanical dietary supplements (Aim 2), the estrogenic activity of all three medicinal species of *Glycyrrhiza* (*G. inflata*, *G. glabra*, and *G. uralensis)*, was analyzed using the optimized and validated assays. *G. inflata* had some ERβ-preferential activity and had the most overall estrogenic potency of the three species. *G. inflata* (or *inflata* licorice) extract were used for bioassay guided fractionation. Using this technique, we obtained several estrogenic extract fractions, of which fraction 8 was selected due to its likely chemical composition and high estrogenic activity. Upon further analysis of this fraction, 8-prenylapigenin (8-PA) was identified and found to exhibit ERβ-preferential activity with a low nanomolar EC_{50} value that is comparable to the known ERβ-preferential isoflavonoid genistein.

The third aim was to determine (iso)flavonoid structures that exhibit ERβ-preferential activity. Several structurally similar (iso)flavonoids, including the identified 8-PA from our second aim, and selected botanical extracts were tested in a structure activity relationship (SAR) study using our optimized method assay from Aim 1. The data demonstrated that C-8 prenylation in the A-ring of flavonoids, as in 8-PA and 8-PN, resulted in significantly higher overall estrogenic activity than their non-prenylated congeners apigenin (Api) and naringenin (Nrg), respectively. Moreover, site-specific prenylation with C-ring unsaturation at C2-C3 increased ERβ-preferential potency over ERα as was observed with 8-PA. In flavonols, C3 hydroxylation on the C-ring and 4'-O-methylation on the B-ring, as in desmethylicaritin and icaritin, reduced overall estrogenic activity compared to 8-PA and 8-PN. In contrast, for isoflavonoids the absence of C-8 prenylation on A-ring increased estrogenic activity compared to the

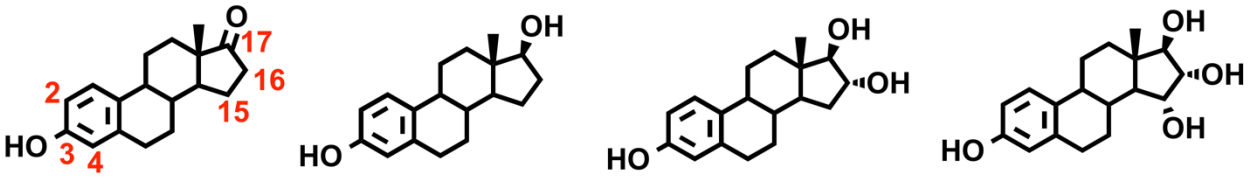
prenylated compounds [(8-prenylgenistein, (8-PG)]. This non-prenylation with the presence of C-ring unsaturation at C2-C3 favored ERβ activity over ERα activity, as was observed with genistein. These general trends were observed in the SAR results from the ER binding analysis, SRC recruitment, and functional assay analysis in this study. Computational models employed in this work showed that the prenyl group at the C-8 of the A-ring interacts with a hydrophobic region in the ER active site, resulting in higher estrogenic activity compared to unprenylated flavonoids. Conversely, prenylation at the identical site in isoflavonoids results in unfavorable interactions with a hydrophilic region in the binding site, thereby reducing estrogenic activity. In addition, the SAR work suggested that C-ring unsaturation (at C2-C3) created a more 'planar' structure, leading to a better fit into the ERβ active site, as is observed with 8-PA and genistein.

In conclusion, the work of this dissertation demonstrates that 8-PA, a compound found in licorice may be able to contribute ERβ chemoprotective benefits by reducing risk of mammary carcinogenesis when extracts such as *inflata* licorice are consumed for health purposes. Significant *in vivo* studies have already been conducted using genistein. The results reported here suggest that estrogenic *in vivo* studies with 8-PA and *inflata* licorice are warranted. The animal studies should determine if 8-PA activation of ERβ by botanicals is translatable from *in vitro* to *in vivo,* and results in a demonstrable reduction in breast carcinogenesis.

1. INTRODUCTION

1.1. Estrogens

Estrogens are a group of endogenous hormones that provide support to numerous beneficial biological functions (1, 2). The pharmacophores are fused tetracyclic carbon structures consisting of 17 carbons (2). The endogenous estrogens comprise four C18 carbon tetracycles which feature an aromatic A-ring with a C3 hydroxy group (2). Further substitution differentiates the members of the estrogen family: a ketone at C17 yields estrone (E_1), a hydroxyl group at the C17 gives 17β-estradiol (E_2), a hydroxyl group at C16 and C17 gives estriol (E_3), and hydroxyl group at C15, C16, and C17 yields estretrol (E_4) (Figure 1). E_2, commonly referred to as estrogen, is produced mainly in the granulosa cells of the ovary in women of child bearing years, and in smaller amounts adipose tissue, breast, bone, brain, and other extra-gonadal tissues during the postmenopausal period (1, 2). E_2 is also produced in the testicular leydig cells in males (1, 3).



Estrone (E_1) **17β–Estradiol (E_2)** **Estriol (E_3)** **Estretrol (E_4)**

Figure 1. Structure of Endogenous Estrogens

Each of the estrogens interacts with estrogen receptors (ER) at different potencies, and induce biological responses of varying degrees (4). E_4 and E_3 are most prevalent during pregnancy; E_4 is produced in fetal hepatic tissue, and E_3 is synthesized by the placenta (5). E_1, produced in peripheral tissue from androstenedione by aromatase (CYP 19A1), has relatively higher serum levels during menopause and can subsequently be converted to back E_2 (5-7). Among all the estrogens, E_2, is most biologically dominant and active, especially during women's reproductive years, in comparison to its metabolites E_1 and E_3 (2, 3).

1.1.1. Estrogen Biosynthesis

The synthesis of estrogens occurs mainly in the granulosa cells of the ovary through the process of steroidogenesis (2). Steroidogenesis uses cholesterol derived from low density lipoprotein (LDL) as the starting substrate (2, 8). In the initial phase of this pathway, the Luteinizing hormone (LH) interacts with its native G-protein coupled receptor, which is expressed by the theca cells of the ovary (2). This molecular recognition initiates a signaling cascade that ends with the transcription and expression of the Steroidogenic Acute Regulatory Protein (StAR) (2). Free cholesterol from LDL cholesterol, is then translocated to the inner membrane of the mitochondria by StAR where it becomes the substrate for CYP 450css (side-chain cleaving) enzymes and converted to pregnenolone (9). This 21 carbon steroid, a precursor for the steroid hormones, is converted to dehydroepiandrosterone (DHEA) after 17α-hydroxylation and consecutive side chain cleavage by CYP 17A1, and then to androstenedione by 3β-hydroxysteroid dehydrogenase/Δ5-4 isomerase (3β-HSD) (2). Androstenedione is

metabolized by two different pathways. It can be converted to testosterone by 17β-hydroxysteroid dehydrogenase (17β-HSD), followed by oxidation to E_2 by aromatase (2). Androstenedione can also be converted to E_1 by aromatase, and then to E_2 in the granulosa cells of the ovary by 17β-HSD (2). Figure 2 shows a putative scheme for estrogen biosynthesis. The Follicle Stimulating Hormone (FSH) regulates aromatase and 17β-HSD expression in the granulosa cells and, as a result, FSH also has influence on E_2 production by aromatase and 17β-HSD in these cells (2, 5). In the bone and adipocytes, testosterone is converted to E_2 by aromatase (10). However, aromatase is the rate-limiting enzyme in both the conversion of androstenedione to E_1 and the conversion of testosterone to E_2, making it an important pharmacological target in the cancer treatment (5). Estrogens are also synthesized in the placenta during pregnancy, largely by the action of aromatase and importation of the 19-carbon precursor steroids for estrogen synthesis (11, 12).

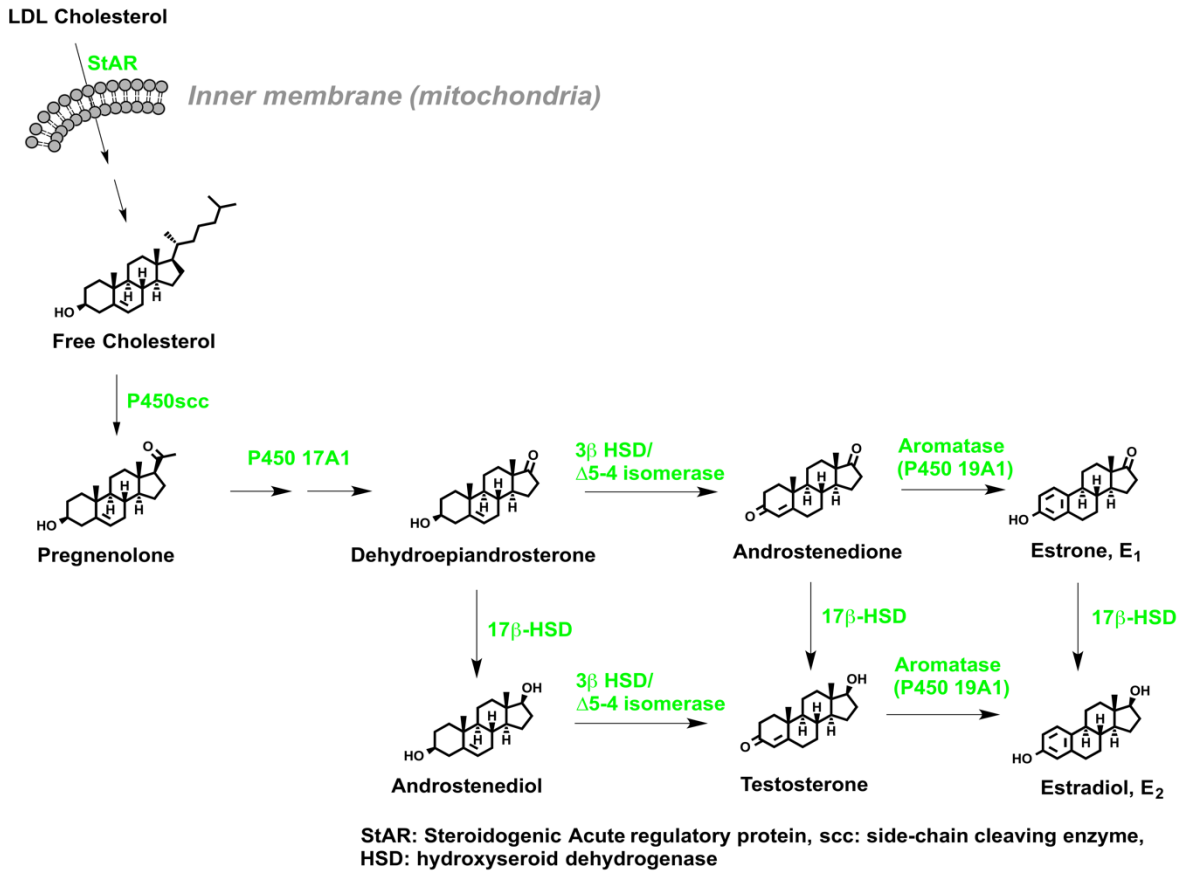


Figure 2. Putative Scheme of Estrogen Biosynthesis

1.1.2. Estrogen Metabolism

Estrogens are metabolized and excreted from the body via the gastrointestinal tract in bile or feces, or in urine (2). E_3 is the most abundant estrogen in female urine (2, 5). E_1 and E_2 are metabolized following the P450 oxidative pathways in the liver; the principle enzymes involved are P450 3A4 and P450 1A2 (13-15). These are part of the cytochrome P450 family of enzymes and are responsible for 2-hydroxylation and 16-

hydroxylation of E_1 and E_2 in target cells (2, 14, 15). In the mammary gland, P450 1B1 metabolizes estradiol to the 4-hydroxylated metabolite and P450 1A1 to the 2-hydroxylated metabolite (16). The P450 1B1 enzyme is also found in the renal gland and parts of the central nervous system (2). The 2-hydroxyestrone/estradiol and 4-hydroxyestrone/estradiol are sometimes referred to as catechol estrogens (2). They can undergo further *O*-methylation by catechol-O-methyltransferases (COMT) to methoxy-estrogens (5). Additionally, estrogens are also conjugated in order to make them more soluble for efficient excretion (17). These conjugating enzymes include sulfotranferases and UDP-glucoronosyltransferases (18, 19).

1.1.3. Biological Functions

Free estrogens are able to reach target tissue and exert a variety of physiological effects. Central to estrogen signaling pathways are the proper development and function of reproductive organs (1, 2). Additionally, during puberty in females, E_2 is responsible for the growth and proliferation of the epithelial cells in breast and uterine endometrial tissue; E_2 is also important for lactation in pregnant women (20-23). It also regulates inflammation and maintains various metabolic activity and proper functioning of the skeletal, cardiovascular and central nervous systems (1, 2). These myriad functions arise from estrogen interactions with the endogenous estrogen receptors *in vivo*.

1.2. Estrogen Receptors

Estrogen receptors belong to the global class of transcription regulating nuclear hormone receptors. There are two known ER subtypes expressed in tissue; ERα and ERβ that can be located intracellularly, including in the nucleus (1, 2). ERα, a 67 kDa, protein consisting of 595 amino acids is encoded by ESR-1 on chromosome 6 (1, 2). In contrast, ERβ, a 59 kDa protein comprising 530 aa, is encoded by ESR2 on chromosome 14 (1, 2).

Estrogen receptors are expressed on the cell membrane as G-protein coupled estrogen receptor-1 (GPER1). This membrane-bound GPER-1 is encoded on chromosome 7 and consists of 7 transmembrane helical subunits. ERα is found in the mammary gland, bone, and uterus, ovary (thecal cells) and adipose tissue, while ERβ is also expressed in mammary tissue and prostate (epithelial cells) and ovary (granulosa cells), CNS, colon, lungs, and cardiovascular system (1, 24-27). ERα was first identified in the mid 1950's (1, 2). ERα activity includes the induction of cellular proliferation in the presence of E_2 (1, 28). ERβ, identified in 1996, is known for functions including that attenuation of tissue proliferation and maintenance of proper central nervous system function (1, 2). Both receptors share a generally similar structure. Important differences can be found in their various functional domains, which are notated as regions A through F. The A/B region contains the N-terminal domain and activation function 1 (AF-1), which regulates ligand independent gene transcription. The C region contains the DNA binding domain (DBD) that interacts with specific sites on the DNA prior to transcription; these DNA binding sites are known as estrogen response elements (ERE) prior to transcription. This C region consists of two 'zinc fingers' with regions named P

box and D box, respectively (29). The P box is necessary for DNA site recognition during ER-DNA binding, while the D box participates in ER dimerization (29). The D region contains the 'hinge' portion of the receptor, and E/F region is the carboxy-terminal containing the ligand binding domain (LBD) and AF-2. This last region comprises 12 α-helices (H1 – H12). ER homo-or heterodimerization is governed by this region, as is ligand-dependent estrogenic transcription. Region E/F also features coactivator and corepressor interaction (1, 2, 29-31). H12 is of particular importance in ligand binding. When an ER agonist is bound to the receptor, H12 becomes positioned next to H3 and H11 (32). When H12 is in this agonist conformation, a hydrophobic pocket is formed for coactivator binding (32-34). However, when an antagonists such as tamoxifen binds the ER, it stabilizes the H12 in the hydrophobic pocket, blocking coactivator interaction (32, 35). When H12 is in agonist mode and the hydrophobic pocket is formed on the ligand bound ER, coactivators interact with this pocket through a Leu-X-X-Leu-Leu (LXXLL where X can be any amino acid) amino acid sequence in a region called the NR box (34). This NR box consists of amphipathic α-helices (34). This amphipathic property of the helices keeps the hydrophilic aa residues separated from hydrophobic aa (36) (such as leucine in the LXXLL motif) residues, which are necessary for interaction for interaction with the AF-2 of the ER. Steroid receptor coactivators (SRC) are examples of these coactivators that interact with the ligand-bound ER complex in order to enhance transcription (37). In turn, the carboxyl end of these SRC's has regions that bind other necessary transcription cofactors such as cyclic AMP response element binding protein (CBP), and p300, that are necessary for SRC-mediated transcription (37). In other instances, nuclear receptor corepressors

(NCoR) or silencing mediator for retinoid and thyroid hormone (SMRT) can also be recruited to the ER. These cofactors suppress the transcriptional activity of ER in a ligand-independent mode or in the presence of an antagonist (38). Their repression of transcriptional activity is due to the activity of histone deacetylase (HDAC) (38, 39). It is suggested that these HDAC's are recruited, and they inhibit the acetyltransferase functions of the coactivator complex, thereby repressing transcription (40).

ERβ is reported to have a smaller binding site compared to ERα, and this influences the ligand selectivity and, potentially, biological activity of some exogenous ligands (1, 2). In comparison to ERα and ERβ, GPER-1 responsible for fast cellular responses of estrogen-mediated cell signaling (2, 41-43). The different cellular pathways that are induced upon activation of these receptors are summarized in the next section.

1.3. Estrogenic Signaling Pathways

1.3.1. Direct Genomic Signaling

According to the classic estrogenic pathway (Figure 3) of transcription activation, E_2 passes through the cell membrane and binds with cytoplasmic ERα and/or ERβ. This is binding event is followed by a change in receptor conformation, resulting in a sequestering of chaperone proteins such as heat shock proteins (hsp), dimerization, and eventual translocation of the ligand-ER complex to the nucleus (44-48). In the nucleus, the complex recruits important transcription cofactors (CF) such as steroid receptor coactivators (SRC), cyclic AMP response element binding protein (CBP), and

p300, after binding to the estrogen Response Element (ERE) (1, 37). Among a number of functions, this protein complex performs methyltransferase and acetyltransferase activity for chromatin remodeling in preparation for other transcription factors at the promotor to initiate transcriptional activity (37).

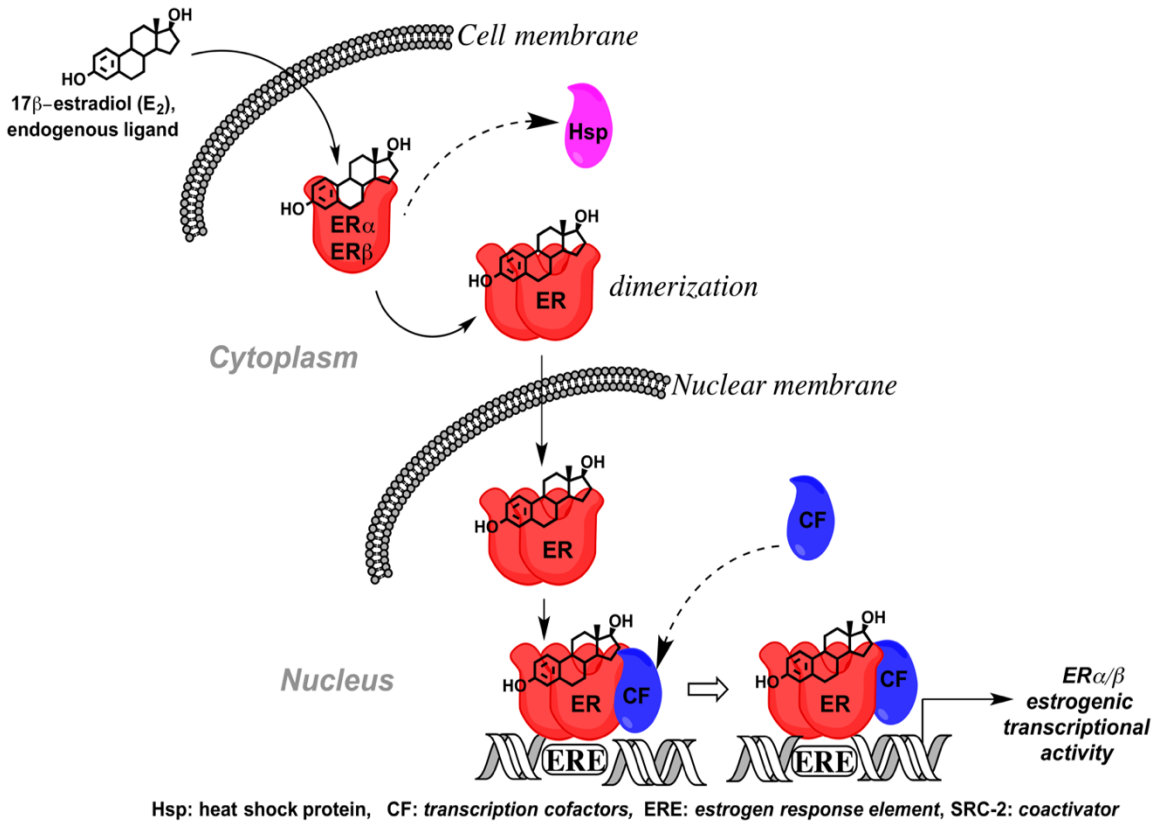


Figure 3. Direct Genomic Signaling

1.3.2. Indirect Genomic Signaling

The indirect estrogenic pathway is so named because function is achieved occurs without direct contact of the ER-ligand complex with DNA. Instead, the ER-

ligand complex interacts with specific transcription factors at specific sites on DNA through protein-protein interactions (2, 29, 49). These transcription factors include stimulating protein 1 (Sp-1) which interacts with promoters in proximity to CG rich regions of DNA and activator protein-1 (AP-1) (50, 51). Genes induced through Sp-1 interaction with ER include those for low density lipoprotein (LDL) receptor, progesterone receptor B, and endothelial nitric oxide synthase (eNOS) (52-54). AP-1 is a complex consisting of different proteins including c-Fos, c-Jun, and activating transcription factor (ATF) and its functions include modulation of cellular proliferation, stimulating differentiation and triggering apoptotic cell death (55).

1.3.3. Indirect Non-genomic Signaling

This estrogenic pathway transduces signaling pathways that involve intracellular second messengers and kinases, producing signaling cascades that ultimately modulate gene expression (56). This mode of signaling follows a variety of pathways. Some of the main categorized pathways include the phospholipase C/protein kinase C (PKC), phosphatidyl inositol 3 kinase/Akt kinase, Ras/Raf/MAPK, and cyclic adenosine monophosphate (cAMP)/protein kinase A (57-61). When stimulated, the kinases involved in these pathways ultimately phosphorylate intracellular transcription factors, including ER (2). Phosphorylation alters their function and ability to interact with the cellular genome, and this event ultimately influences gene expression (2). Studies also indicate that E_2 binding to GPER-1 also stimulates second messengers and results in transcriptional activity (2, 58). A cartoon representation of this putative pathway is shown on figure 4.

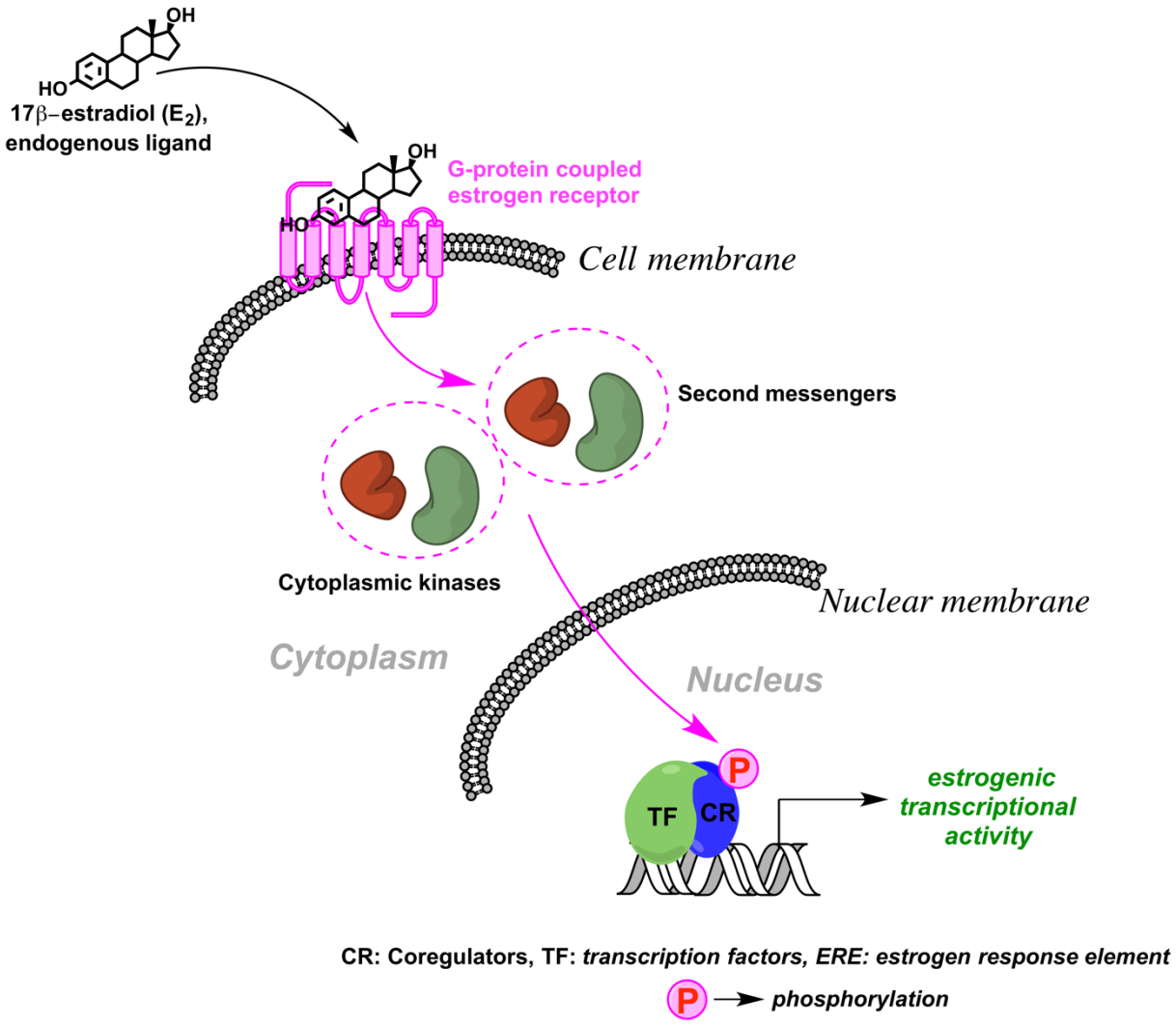


Figure 4. Putative scheme of indirect non-genomic signaling

1.4. Breast Cancer

In 2019, it is estimated that there will be over 260,000 new breast cancer cases in women in the United States alone, and more than 40,000 women will die from the disease (62). These alarming statistics correlate to 30% and 15% of all cancer cases and cancer deaths in women, respectively (62). Breast cancers can be categorized into groups based on immunohistochemistry (IHC) classification (63). IHC classification provides information for treatment and disease outcomes (63). IHC categories are usually grouped based on the presence or absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2): ER/PR(+), HER2(+); ER/PR(+), HER2(**-**); ER/PR(**-**),HER2(**-**),or ER/PR(**-**), HER2(+) (63). Breast cancers are also categorized into different classes based upon tissue of origin, tumor size, histological status, and metastasis to lymph node or other tissue (63). Factors such as alcohol consumption, age, family history, early menarche, late menopause, age, race, lifestyle, hormone replacement therapy, and obesity are reported to increase the risk of breast cancer incidents (64-68). Other conditions such as inheritable mutations in breast cancer susceptible genes BRCA-1 and BRCA-2, are also known to increase the risk of breast cancer (69).

1.4.1. Estrogen and Breast Cancer

There is an established correlation between the presence of estrogen and breast cancers (70, 71). Evidence of this connection was first observed by Dr George Beatson a over century ago when breast cancer remission in premenopausal women occurred after removal of their ovaries (72). A number of studies point to the connection of

prolonged estrogen exposure to the increase in risk for breast cancer (73). Additionally, hormone-associated risk factors are associated with an elevated relative risk of this disease. For example, the correlation of obesity with elevated risk is thought to be due to increased estrogen levels resulting from additional aromatase activity in the breast adipose tissue in postmenopausal women (74). Elevated levels of estrogens in the serum is considered a biomarker for higher relative risk of breast cancer (75). In a large study conducted by the Endogenous Hormones and Breast Cancer Collaborative group (JNCI 2002), data on serum analysis was collected from 663 women that had breast cancer and 1765 women that were breast cancer free. The subjects did not use exogenous reproductive hormones during the study and serum was obtained to determine the relative risk (RR) of breast cancer in correlation to varying serum hormone levels. Hormones analyzed included free E_2, E_2 (conjugated), albumin bound E_2, E_1, E_1 sulfate, androstenedione, testosterone, and dehydroepiandrosterone. The study concluded that there is a strong correlation between sex-related hormone levels and increased risk of breast cancer in postmenopausal women (75). The association between estrogen and increase in breast cancer risk was further supported by Fisher and Constantino, et al. showing the reduction in invasive and non-invasive breast cancer risk by the antiestrogen tamoxifen in pre- and postmenopausal women, and women with a history of breast cancer (76). Results from Fisher and Constantino, et al also showed that the risk of recurrence of ER (+) tumors was reduced significantly with daily long term treatment of tamoxifen (76, 77). Tamoxifen is also an established therapy for breast cancer prevention.

1.4.2. Estrogen Carcinogenesis

Estrogen chemical carcinogenesis and estrogen hormonal carcinogenesis are two generally accepted mechanisms for the onset of breast cancer. Estrogen chemical carcinogenesis involves the hydroxylation of the C4 position on E_2 forming a catechol estrogen that is associated with genotoxic effects. Estrogen hormonal carcinogenesis begins when genetic mutations or errors occur during increased cell division and proliferation through E_2-dependent activation of ER (68, 78).

1.4.3. Estrogen Chemical Carcinogenesis

The enzymes P450 1A1 and 1B1 are part of the P450 1 class of enzymes. Estrogen in the body undergoes phase 1 metabolism by 2-hydroxylation by P450 1A1, and 4-hydroxylation catalyzed by P450 1B1 in the mammary tissue (79). The human isoforms of these enzymes have been reported to convert polycyclic aromatic organic compounds into toxic intermediates that caused cancer in rat mammary glands (80, 81). These P450 enzymes and their mRNA are present in normal human breast tissue and in tumors (81-85). P450 1B1 is reported to be more active than P450 1A1 during E_2 metabolism (86, 87). Human Aromatic hydrocarbon Receptor (AhR) acts as a transcription factor and modulates the expression of P450 1A1 and P450 1B1 (79, 88). The 4-hydroxylation of E_2 by P450 1B1 associated with oxidative metabolism promotes estrogen chemical carcinogenesis (89). This metabolic pathway leads to formation of reactive oxygen species (ROS) and formation of the genotoxic E-3,4-quinone species that can form DNA-adducts leading to DNA depurination *in vivo* (Figure 5) (90-92). Consequently, this leads to DNA damage, genotoxicity, and potential carcinogenesis.

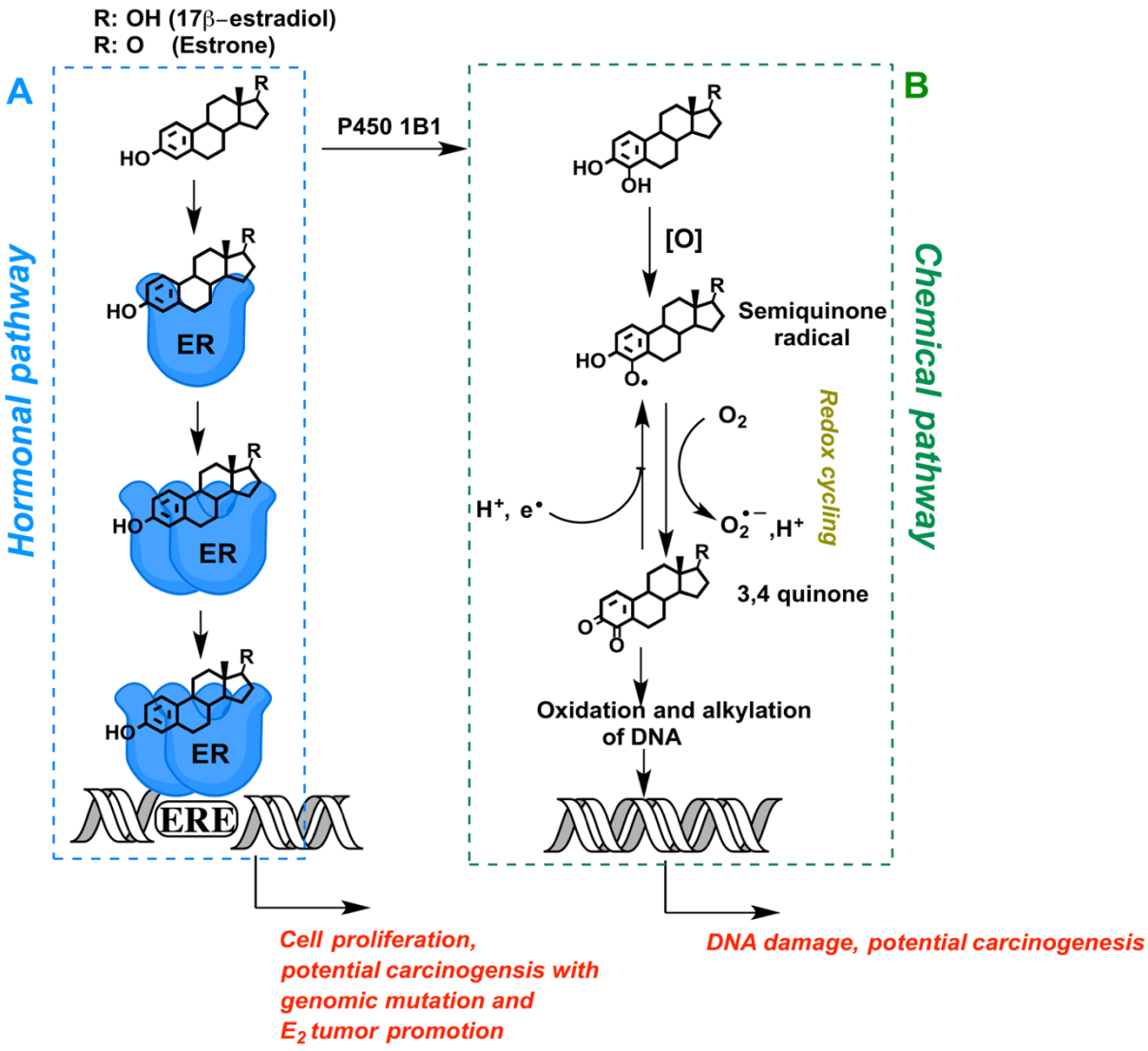


Figure 5. Schematic representation of estrogenic hormonal pathway and chemical pathway in carcinogenesis.

A. The hormonal pathway is induced by binding of E_2/E_1 to the native receptor, followed by dimerization and interaction with ERE site on DNA, activating transcription and ultimately proliferation. Genomic mutation and hormone stimulation can drive carcinogenesis. B. In the chemical pathway E_2/E_1 is hydroxylated at the 4 position by P450 1B1, followed by oxidation to the semiquinone radical, then 1 e^- to the 3,4 quinone. This quinone through the redox cycle is reduced back to the semiquinone radical. The reactive quinone can cause genomic damage (93, 94). (Figure modified from a figure generated from Dr Shuai Wang and Dr Tareisha Dunlap)

1.4.4. Estrogen Hormonal Carcinogenesis

It has been suggested for years that excessive exposure of estrogens to a target organ that is estrogen sensitive can promote the risk of carcinogenesis (78). Increased exposure to hormones such as estrogens results in cell division and proliferation *in vivo*; over time this can increase the chances of genomic replication errors, genetic mutations and tissue carcinogenesis (68, 78). This carcinogenic pathway can continue to progress with the presence of exogenous or endogenous hormone stimulation (68). Normal tissue can consequently progress to a state of hyperplasia then neoplasia (Figure 6) (68, 78). The carcinogenesis and progression of hormone-induced malignancy is also dependent on the types genomic mutations that occur. Studies from Henderson and Feigelson (2000), Ross et al (1998), Sager (1989) and Stanbridge (1990) suggest that mutation of tumor suppressor and DNA repair genes, as well as genes involved in the estrogenic pathway are among these responsible for carcinogenesis (68, 95-98). Specifically, mutation of tumor suppressor genes BRCA-1 and BRCA-2, are included and germline mutation of the TP53 gene can increase breast cancer risk (68, 99, 100). The over-expression of the HER2 gene is also observed in advanced breast cancer (101).

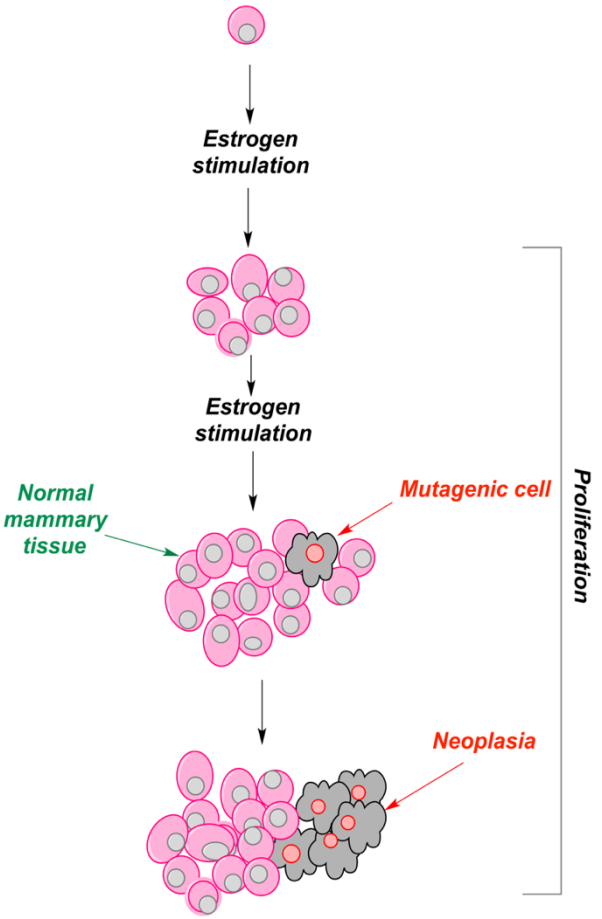


Figure 6. Estrogen Hormonal Carcinogenesis

1.5. Breast Cancer Chemoprevention

Cancer incidence is increased by environmental and lifestyle risk such as smoking, obesity, alcohol use, and diet (102, 103). Among women, breast cancer ranks as first among cancer types that occur and the second cause of death (62). Therefore, chemoprevention is of significant importance and could have a dramatic effect upon the number of women succumbing to this disease.

Selective estrogen receptor modulators (SERMs) have been extensively studied as potential breast cancer therapeutics. These include tamoxifen and raloxifene. These compounds act as ERα-antagonists, opposing ERα-driven cellular activity in the presence of E_2. Although, these drugs exhibit toxic side effects such as cardiovascular complications and an increased rate of endometrial cancers (104-106), they have been a mainstay in treatment of breast cancer in women and can be used to prevent recurrence or a second breast cancer after a first breast malignancy and to prevent breast cancer in women that are at high risk.

Aromatase inhibitors (AI) that reduce the generation of estradiol in breast tissue have been shown to be an effective adjuvant treatment in prevention of breast cancer recurrence in postmenopausal women (107-109). Exemestane and anastrazole have been proven effective in postmenopausal women with medium and high respective risk of breast cancer carcinogenesis (108, 110). A five year phase 3 double blind study demonstrated that letrozole decreased the risk of recurrent breast cancer in postmenopausal women (111). However, long term use of AI's is correlated with severe bone loss and cardiovascular side effects.

When tissue is stimulated to proliferation through the classical estrogenic pathway, the ERα subtype is stimulated by estrogens resulting in cell division, while ERβ is reported to attenuate ERα hyperproliferative activity (1, 112-114). In normal breast tissue, ERα and ERβ are coexpressed, but there is a significant loss of ERβ expression in the early stages of breast carcinogenesis (115, 116). Therefore a chemoprotective role in ERβ activity suggested (116). Helguero et al (2005) reported that when ERβ expression was suppressed in mouse mammary cells that express both

isoforms, an ERα-driven cell proliferation in response to E_2, was observed (113). This *in vitro* cell growth in soft agar indicates potential for *in vivo* tumorigenesis (113). However, when ERα expression was silenced, apoptosis in the presence of E_2 occurred (113). Helguero et al (2005), who used ERα and ERβ-selective ligands, also demonstrated the distinct *in vivo* activity of these ER subtypes. When the normal murine mammary epithelial HC11 cells were treated with 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT), an ERα-selective ligand, cell proliferation was induced (113, 117). In contrast, treatment of the same cells with 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN), a selective ERβ ligand, resulted in inhibition of cell growth and cell death (113, 118). This beneficial effect of ERβ expression is also suggested to be observed in humans; analysis of breast cancer tissue from women who received adjuvant therapy with tamoxifen seem to show that ERβ expression is associated with notably better patient survival (119-122).

Phytoestrogens, plant-sourced compounds that function like estrogens, may play an important role in breast cancer prevention (120, 123). Phytoestrogens are present in various botanicals, fruits and vegetables that are used for food or for medicinal purposes. Epidemiological studies show that a diet rich in phytoestrogens is correlated with a decreased risk of breast cancer (124, 125). Upon consumption, phytoestrogens modulate ERα and ERβ *in vivo*, resulting in pharmacological effects that are complex and still poorly understood. All phytoestrogens so far identified have a lower affinity for ER than E_2, but, intriguingly, a number of them have higher affinity for ERβ than ERα as opposed to E_2 that exhibits a similar affinity to both receptor subtypes (120, 123). Some reported ERβ-preferential phytoestrogens, include genistein, daidzein, liquiritigenin, and

8-prenylapigenin (123, 126, 127). Genistein and liquiritigenin, both of which are classified as (iso)flavonoids, have been reported to suppress carcinogenesis in prepubescent and adult rodent models (126, 128, 129). Moreover, these ERβ-selective flavonoids showed little toxicity, even in the long term administration in the rodent model (128, 129). Overall, the potential benefits of enhancing ERβ activity has piqued interest into identifying ERβ-preferential ligands (1). ERβ-preferential phytoestrogens present in botanicals may be able to provide chemoprotective benefits against breast carcinogenesis.

1.6. Menopause and Hormone Therapy

Most women enter the stage of menopause between 49 and 52 years of age (130). During this stage, there is a drastic decline in estrogen levels. This leads to symptoms such as hot flashes, night sweats, insomnia, vaginal atrophy, fatigue, weight gain and menstrual irregularities (131). Consequently, women for decades have turned to hormone replacement therapy to manage their symptoms. These are synthetic hormones usually consisting of equine estrogens and medroxyprogesterone (132). In 2002, studies from the Womens' Health Initiative (WHI) reported an increase in risk of breast cancer as a result of hormone replacement treatments consisting of estrogens and medroxyprogesterone (132). These findings by the WHI have prompted postmenopausal women to seek alternative therapies, like botanicals, to alleviate their symptoms (132, 133).

1.7. Botanicals for Women's Health Research

1.7.1. Natural Product Research in the NIH/UIC Botanical Center

The NIH/UIC Botanical Center for Detary Supplements Research (the botanical center) is nationally renowned in the study of natural products, including botanicals for women's health (134). Established in 1999, it has been responsible for numerous high impact publications detailing the *in vivo* and *in vitro* activities of plant-based natural products. Botanicals studied include hops, red clover, licorice, and epimedium. The center has also conducted human clinical trials using some of these popular botanicals. For example, isoflavone-enriched red clover was analyzed in phase 1 and phase 2 trials (135, 136). The center is a leader in isolating, identifying, and assessing the biological activity of plant based natural products.

1.8. Women's Health Botanicals and Phytoestrogens

Natural products have been used for various health purposes through human history. Some notable botanicals of historical importance include red clover, hops, licorice, and epimedium (137).

1.8.1. Hops

Hops (*Humulus lupulus*) is part of the Cannabaceae family. Historically it was used in beverage, as a preservative, as a mild sedative, and to manage infections (138, 139). Hops and hops extracts have been used as a diuretic, an anxiolytic, and is recently used to manage gynecological problems such as menstrual irregularities, and to help

with sleep disorders (138, 140). Hops has been used as a flavoring agent and a fragrance in select cosmetics (138). It contains the most potent ERα phytoestrogen known to date, 8-prenylnaringenin (8-PN), as well as 6-prenylnaringenin (6-PN), xanthohumol (XH), and isoxantohumol (IX) (139, 141-143).

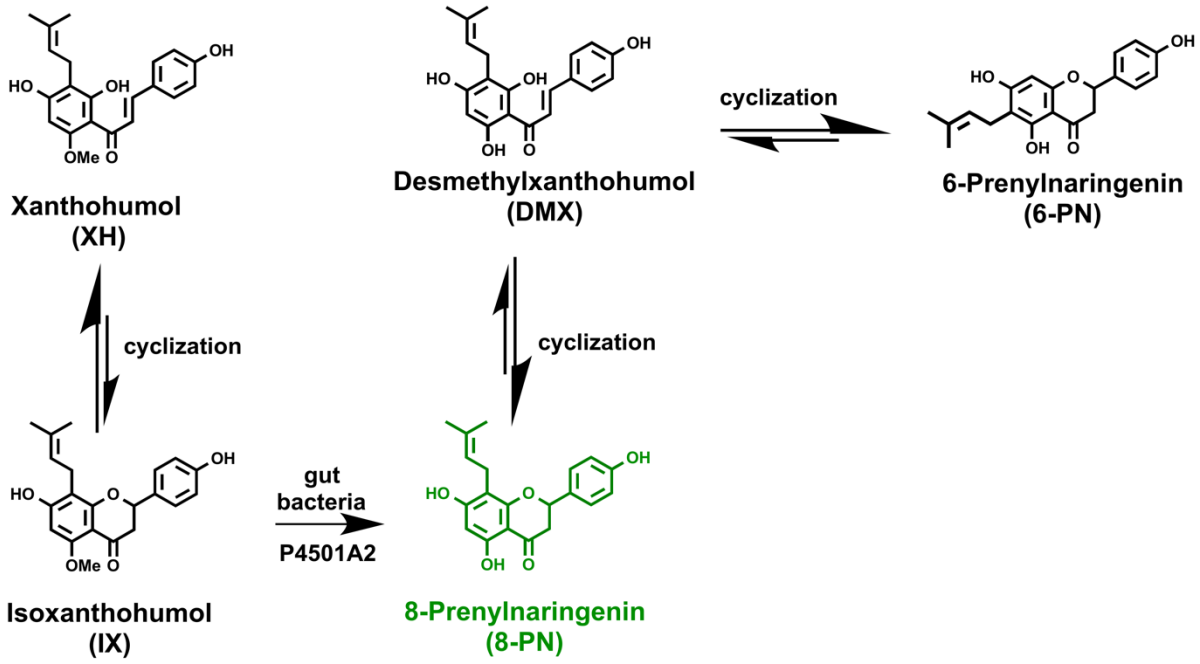


Figure 7. Bioactive Compounds in Hops

1.8.2. Red clover

Red clover (*Trifolium pratense*) is a part of the Fabeceae plant family.

Red clover has been traditionally used to treat cough and gout (144). It was usually used as an additive to ointments or as tea to treat ulcers (145). Moreover, red clover has been used to treat burns, bruises, fungal infections and even ocular diseases.

(145). Some Native Americans used red clover to treat fevers and kidney inflammation. When prepared as a tea it was used to treat pulmonary infections (145). Lately, it is used in some circles as a sedative, for decongestion, and various joint pains. However, it is not regularly used to enhance fertility (145). This botanical contains a number of isoflavonoids including genistein, a potent ERβ preferential agonist, biochanin A, daidzein, and formononetin (145).

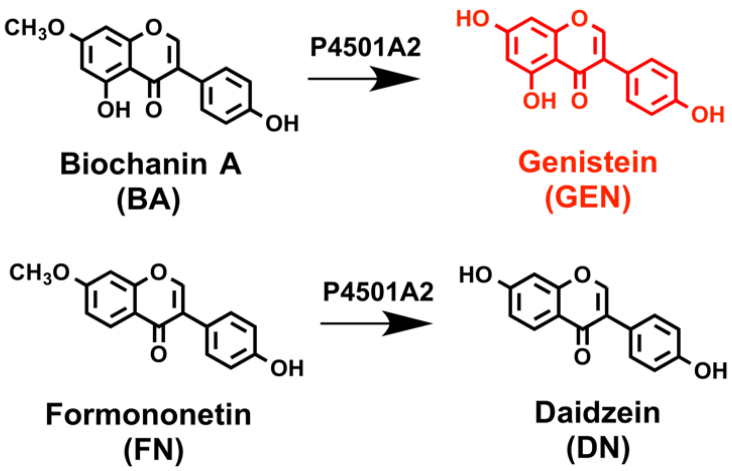
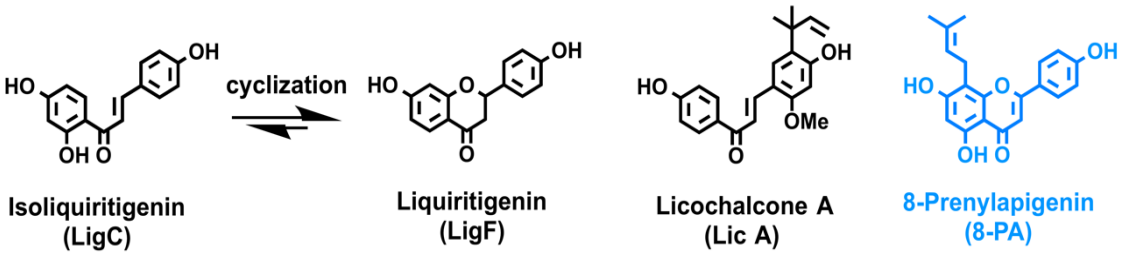


Figure 8. Bioactive Isoflavonoids in Red clover

1.8.3. Licorice

Licorice (*Glycyrrhiza sp.*) is a botanical that belongs to the Fabaceae plant family and is mainly found in various provinces in Asia and Europe (146, 147). Three licorice species are used medicinally and approved in international pharmacopeias; these include:

Glycyrrhiza uralensis, *Glycyrrhiza glabra*, and *Glycyrrhiza inflata* (127, 146). Licorice has been used for pain relief, as well as stomach inflammation, respiratory problems, and for the management of postmenopausal symptoms (147, 137, 146). Lately, it has been marketed to treat stomach ulcers, hepatic dysfunction and even diseases caused by inflammation such as Addison's disease (146). Studies report various pharmacologic properties of this botanical, including antiviral, anti-inflammatory, anti-tumorigenic activity. These differing pharmacological properties are thought to arise from the various bioactive compounds, found in licorice (146). Some notable bioactive licorice compounds include liquiritigenin (LigF), isoliquiritigenin (LigC), licochalchone A (LicA) and 8-prenylapigenin (8-PA), a potent ERβ preferential agonist (127).



Isoliquiritigenin (LigC) | Liquiritigenin (LigF) | Licochalcone A (Lic A) | 8-Prenylapigenin (8-PA)

Figure 9. Bioactive Compounds present in Licorice

1.8.4. Epimedium

Horny goat weed is the common name used for 50 different known species of Epimedium, which include *E. sagittatum*, *E. grandiflorum*, and *E. koreanum* which have been widely studied (148-150). As part of the Berberaceae plant family, these

Epimedium species are well known in Traditional Chinese Medicine (TCM) for natural hormone therapy, including the treatment of menstrual irregularities and osteoporosis (148, 150). Icaritin (Ict) is regarded as the major bioactive flavonoid present in this botanical.

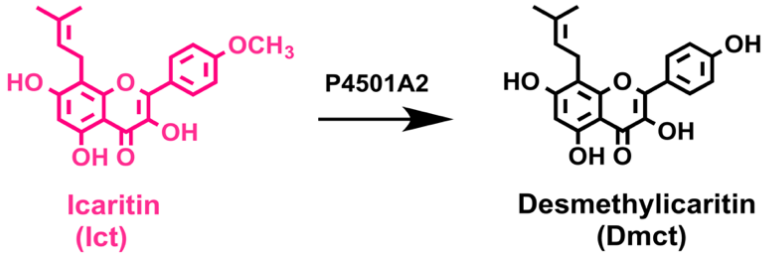


Figure 10. Bioactive compound, Icaritin, present in Epimedium and its *in vivo* Metabolite

1.9. Specific Aims for Study

The goal of this study was to identify and characterize ERβ-preferential (iso)flavonoids present in select botanical extracts used in women's health botanicals. It is ***hypothesized*** that ERβ-preferential (iso)flavonoids in women's health botanicals may be able to contribute to chemoprevention while maintaining an improved safety profile by reducing risk of breast carcinogenesis by counteracting potential ERα-induced hyperplasia. Since hops, red clover, licorice, and epimedium are among the most frequently used botanicals for menopausal symptoms containing estrogenic compounds, and are used by menopausal women, this study focused on identifying and

studying select estrogenic flavonoids, flavonols, and isoflavonoids that they contain. Three aims were proposed for these studies:

i. Optimization of estrogenic assays to assess ERα and ERβ potency and efficacy of select botanical extracts and respective bioactive compounds
ii. Identification of any ERβ-preferential compounds in the estrogenic licorice extracts.
iii. Determination of structural elements that favor ERβ-preferential activity by a structure activity relationship analysis of various (iso)flavonoids.

In this study, authenticated botanical extracts and select pure (iso)flavonoids (compound purity of > 95%) where studied using *in vitro* models of ERα+ endometrial carcinoma-(Ishikawa) cells and ERβ+ MDA-MB-231/β41 breast cancer cells. The ER receptor binding affinity and transcription factor recruitment of select (iso)flavonoids was determined in ERα and ERβ using recombinant protein models. The potency and efficacy of the (iso)flavonoids and select extracts was quantified in ERα and ERβ activity assays to assess functional estrogenic activity. Data from ERα and ERβ were analyzed comparatively to determine ER subtype selectivity. Furthermore, this study identified an ERβ-preferential flavonoid and provided data on flavonoid structural characteristics that favor ERβ estrogenic activity.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) and 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) were purchased from Cayman Chemical (Ann Arbor, MI, 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 otherwise stated. 8-Prenylnaringenin (8-PN), naringenin (Nrg), apigenin (Api), genistein (Gen), kaempferol (Kfl), icaritin (Ict) and licochalcone A were acquired from Sigma-Aldrich (St. Louis, MO, USA). Desmethylicaritin (Dmct) was obtained from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). 8-Prenylapigenin was initially purchased from ChemFaces (Wuhan, Hubei, People's Republic of China) which was misidentified, and then obtained from Ryan Scientific Inc. (Mount Pleasant, SC, USA) along with 8-Prenylgenistein (8-PG). Dehydrogenistein (Dgn) was obtained from TRC Canada (Toronto Research Chemicals Inc., Toronto, Canada). The purity of each purchased compound was determined as described previously using either the relative 100% or the absolute qHNMR methods (Figure S1, Appendices) (151). All commercial compounds (Api, Ict, Kfl, Dmct, Gen, Dgn, 8-PG) with a purity below 95% w/w or containing structurally related impurities were re-purified by semi-preparative HPLC-UV prior to any biological assays.

2.2. Botanical Extract Preparations

All extract preparation and verification for this study was performed by Core B or Project 1 of the NIH/UIC botanical center.

Hops (*Humulus lupulus* L., Cannabaceae) extract was prepared with ethanol at Hopsteiner (New York, NY, USA) from spent hops (152). The contents (% w/w) of four markers, xanthohumol (XN), isoxanthohumol (IX), 6-prenylnaringenin (6-PN), and 8-PN, in freshly prepared extracts were determined by HPLC-MS/MS to be 0.28 ± 0.03 for 8-PN (see supporting information), 1.22 ± 0.09 for 6-PN, 1.11 ± 0.07 for IX, 33.2 ± 2.8 for XN. Desmethylxanthohumol (DMX) content was determined in the freshly prepared sample to be 0.63 ± 0.06 % w/w. This compound isomerize slowly to 6PN/8PN (5.7:1) (153).

Red clover (*Trifolium pratense* L., Fabaceae) standardized extract was prepared by autohydrolysis using proprietary methods and manufactured by Pure World Botanicals (Purchased by Naturex, Inc., South Hackensack, NJ). Biochanin A and genistein content quantified by QM-qHNMR were 15.4 ± 0.6 and 0.64 ± 0.04 %w/w respectively (154).

Leaves from *Epimedium* botanicals sold as horny goat weed (*Epimedium sp*., Berberidaceae) were purchased from Starwest Botanicals (part # 209365-51, lot# 60901). The powder was analyzed by means of microscopic analyses, chemical profiling and DNA barcoding (manuscript in preparation, supporting information).

Enzymatic hydrolysis of Epimedium botanicals: An auto-hydrolyzed extract (70.0 mg) was dissolved with 171.60 mg snailase in 2 mL of Milli-Q water and stirred at 40°C. After four days, enzymatic activity was stopped by adding 2.0 mL of methanol.

Aglycones were extracted with 40 mL of organic solvents (hexanes, chloroform, and ethyl acetate). A total of 18.2 mg of snailase-hydrolyzed extract, enriched in icaritin, was thus obtained (155, 156, 157). Icaritin concentration in this extract was determined to be 3.40 % w/w by HPLC-UV (manuscript in preparation, supporting information). Licorice (G. *glabra*, G. *uralensis*, and G. *inflata*) extracts were prepared as described previously (158). Dried root samples of *G. glabra* and *G. uralensis* were purchased from a local supplier in Chicago, IL. and the Indiana Botanical Garden, respectively. The licorice species for these studies was *Glycyrrhiza inflata* Batalin (Fabaceae), collected in China (Kuga County, Xinjiang Province, People's Republic of China) and generously provided by Dr. Liang Zhao at Lanzhou Institute of Chemical Physics, CAS. *G. inflata* was identified using macroscopic/microscopic analyses, chemical profiling, and DNA barcoding as previously described (159). The three *Glycyrrhiza* species were identified by means of macroscopic/microscopic analyses as well as DNA barcoding and compared to voucher specimens from the Field Museum of Natural History (Chicago, IL) (160). The powdered roots were extracted by maceration and percolation at room temperature with a solvent mixture composed of ethanol (200 USP proof), isopropranol, and water (90:5:5, v/v) and a plant powder/volume of solvent ratio of 1/15. After concentration, the produced extract was freeze dried leading to an extraction yield of ~10% (w/w) of the initial powdered roots (158, 160, 161). The powdered roots of *G. inflata* (licorice) were extracted by hydro-alcoholic maceration and 8-PA content was determined by LC-MS to be 0.168 ± 0.014 % w/w as previously described (127).

2.2. Fractionation of *G. inflata* Crude Extract

Fractionation of the *G. inflata* extract for this study was by the analytical core of the NIH/UIC botanical center (Project 1). Fractionation of the crude *G. inflata* extract was performed by high-speed countercurrent separation (HSCCC) with the solvent system composed of hexanes-ethyl acetate-methanol-water (5:5:5:5 v/v) in an isocratic and descending mode (reverse phase mode). A HSCCC Tauto TBE-300B (Shanghai Tauto Biotech Co., Ltd., Shanghai, People's Republic of China) integrated with the Cherry-One automated CCS system (Cherry Instruments, Chicago, IL., USA) was filled with the organic upper phase (UP) at a flow rate of 3 mL/min at 200 rpm. The system was then equilibrated at a flow rate of 1.5 mL/min leading to a Sf of 88% (VS =256 mL and Vm = 34 mL) with a rotation speed adjusted to 800 rpm. *G. inflata* crude extract (521.48 mg), diluted in 2 mL of UP and 2 mL of lower phase (LP) was injected into the column. Fraction collection was set up for 7.5 mL/fraction. The extrusion was performed after 2.6 column volumes. A total of 100 tubes was collected. The fractions were pooled according to their TLC profiles, leading to a total of 17 final fractions, defined as follows: fraction 1: vials 25-29, fraction 2: vials 30-31, fraction 3: vials 32-33, fraction 4: vials 34-36, fraction 5: vials 37-39, fraction 6: vials 40-42, fraction 7: vials 43-45, fraction 8: vials 46-53, fraction 9: vials 54-56, fraction 10: vials 58-65, fraction 11: vials 66-72, fraction 12:vials 66-72, fraction 13: vials 76-78, fraction 14: vials 79-81, fraction 15: vials 82-83, fraction 16:vials 84-85, fraction 17: vials 85-100. All TLC were performed on Alugram silica gel plates (SiO_2 F254, Macherey-Nagel), eluted with $CHCl_3$-MeOH (90:10, v/v) and visualized with 5% H_2SO4/vanillin reagent. All the fractions were dried in order to calculate the weight recovery as % weight fraction/ weight crude extract. Because of

their very low final amounts, fractions 9 and 10 were pooled together yielding fraction 10, likewise fractions 11, 12 and 13 were pooled to give fraction 12.

Isolation and Dereplication of Compounds from Fraction 8. Abyssinone II, 8-prenylapigenin, licochalcone C, and licochalcone A were isolated from fraction 8 by semi-preparative HPLC performed on a Waters 600 instruments using a photodiode array detector (Waters 2996) (Figure S1). The separation was performed on a YMC-Pack ODS AQ column (250 × 10 mm, 5 µm, part no. 102500531) utilizing an isocratic elution mode with 58% acetonitrile in water and a flow rate of 1.8 mL/min. Under these conditions, **4** (Abyssinone II) was eluted at 28 min, **3** (8-prenylapigenin, 8-PA) at 31.6 min, **5** (licochalcone C, LicC) at 36 min, and **6** (licochalcone, LicA) at 38 min. The fraction was prepared at 28 mg/mL and 100 µL of solution were injected at each semi-preparative run. The identity of all compounds was confirmed by means of MS/MS, (1D and 2D) NMR analyses (see Supporting Information and freely available NMR dataset at Harvard Dataverse (doi:10.7910/DVN/JZOL2U), and comparison with published data (162-164).

2.3. Confirmation of the Identity and Purity of Commercial Standards

The authentication of commercial standards (verification of identity and purity) was performed by the analytical core of the NIH/UIC botanical center (Project 1) by both LC-MS/MS and qHNMR analyses. For NMR analysis, approximately, 1 mg of each sample was precisely weighed, whenever possible, with a Mettler Toledo XS105 Dual Range analytical balance and diluted in 200 µL of DMSO-d6 (D 99.9%, Cambridge Isotope

Laboratories Inc., Andover, MA, USA). The solution was then transferred with calibrated glass pipets into 3 mm standard NMR tubes (Norell part no. S-3-HT-7, Norell Inc., Landisville, NJ, USA). The 1D ^{1}H NMR spectra were acquired at 298 ºK under quantitative conditions (qHNMR) using a 90º excitation pulse experiment (Bruker pulprog: zg), on a Bruker AVANCE 900 MHz equipped with a 5 mm CPTCI probe, and/or on a Bruker AVANCE 600.13 MHz spectrometer equipped with a 5 mm TXI cryoprobe. The 90° pulse width for each sample was determined by prorating the measured 360° pulse width (p90 = 1/4 × p360). The probe was frequency tuned and impedance matched before each acquisition. For each sample, 32 scans (ns) and 4 dummy scans (ds) were recorded with the following parameters: pulse width (P1) of typically 10.65 µ sec. (90° at 900 MHz) and 9.20 µ sec., spectral width of 30 ppm, relaxation delay (D1) of 30-60 sec. Off-line data processing was performed using the Mnova NMR software package (v.6.0.2, MestreLab Research S.L., A Coruña, Spain). ^{1}H and ^{13}C chemical shifts (δ) were expressed in ppm with reference to the residual solvent signal (DMSO-d_5: ^{1}H spectrum: 2.500 ppm). The following processing scheme was used: a mild Lorentzian-to-Gaussian window function (line broadening = −0.3 Hz, Gaussian factor = 0.01) was applied, followed by zero filling to 256 K acquired data points before Fourier transformation. After manual phasing, a fifth order polynomial baseline correction was applied.

LC-MS/MS analysis was carried out using Waters 2695 solvent delivery system connected to Waters SYNAPT quadrupole/time-of-flight (q/TOF) mass spectrometer operated in the positive ion electrospray mode. Separations were carried out using YMC AQ C18 column (2 x 100 mm, 3 µm particle size), eluted with a mobile phase

consisting of 0.1% formic acid (solvent A) and acetonitrile (solvent B) with a linear gradient from 10-95% B over 30 min. The flow rate was 0.2 mL/min and the column was thermostated at 30 ºC. Mass spectrometric measurements were carried out at 10,000 resolving power (FWHM) using leu-enkelphalin as the lock mass. For identification, molecular compositions and tandem mass spectra were compared with the standard spectra from public (MassBank, MoNA) and in-house generated databases as well as with spectra published in the primary literature (162-164).

The purity determination of each commercial compound was performed as described previously using the 100% qHNMR method (165). Hence the (commercial phytochemical) purity was calculated to be 95.90% w/w for 8-prenylnaringenin (+/-) (**2**) (Sigma), 95.49% w/w for licochalcone A (**6**) (Sigma), 99.51% w/w for genistein (**1**) (Sigma), 98.84% w/w for 8-prenylapigenin (**3**) (Ryan Scientific Inc, WuxinPO1673) (see Supporting Information for 8-prenylapigenin (**3**) and freely available NMR dataset at doi:10.7910/DVN/JZOL2U).

Quantitative Analysis of Tested Licorice Extracts. Quantitative UHPLC-UV analyses were performed on licorice extracts in order to determine the level (in % w/w) of liquiritigenin (**7**) equivalents, isoliquiritigenin (**8**) equivalents as well as the amount of G. inflata species-specific licochalcone A (**6**), as previously described (Table III) (166). In addition, 8-prenylapigenin (**3**) was quantified using HPLC-MS/MS with CID and selected reaction monitoring (SRM). The analyses were carried out on a Shimadzu (Kyoto, Japan) LC-MS-8050 triple quadrupole mass spectrometer equipped with a Shimadzu Nexera UHPLC system and Waters Xbridge C18 column (2.5 x 50 mm, 3 µm). The mobile phase consisted of a 10-min linear gradient from 35% to 70%

acetonitrile in water containing 0.1% formic acid. The flow rate was 0.3 mL/min, and the column oven temperature was 45 °C. The negative ion electrospray SRM transitions for 8-prenylapigenin (**3**) were m/z 337 to 281 and 337 to 293 (quantifier and qualifier, respectively) and 353 to 119 for internal standard xanthohumol. The collision energy was 28 eV and the SRM dwell time was 20 m sec. per transition.

For the preparation of calibration curves, authenticated commercial 8-prenylapigenin (**3**) (Ryan Scientific Inc.) was diluted with 50% CH_3CN/water to produce a calibration curve from 10 to 500 nM. Licorice crude extracts were prepared at 0.1 mg/mL in 70% acetonitrile and 3 µL was injected for analysis.

2.4. Cell Culture Condition

The ERα (+), endometrial carcinoma cells (Ishikawa) 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 fetal bovine serum (FBS), as described previously (158). An estrogen-free medium was prepared similarly but with phenol red-free medium and 10% charcoal-stripped FBS replacing heat-inactivated FBS.

The MDA-MB-231/β41 breast carcinoma cell line, stably transfected with ERβ was a gift from Dr. Debra Tonetti (University of Illinois at Chicago, Chicago, IL.) and was maintained in phenol red-free Modified Eagle Medium (MEM) containing 1% non-essential amino acids (NEAA), 1% glutamax, 1% anti-biotic/anti-mycotic, 5% charcoal stripped calf serum, and 0.05% insulin (167).

Treatment with the extracts and compounds did not result in significant cell death under the concentrations used in this study. All DMSO concentrations for the cell culture assays were below 0.1%. All cell lines were authenticated and had well-defined STR profiles.

2.5. Estrogen Responsive Alkaline Phosphatase Induction in Ishikawa cells

The protocol from Pisha and Pezzuto (168) for the Alkaline Phosphatase assay was used as previously described (158). 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 compounds were dissolved in DMSO and added in serial concentrations while ensuring that the DMSO concentration was less than 0.1%. After treatment, the plates were incubated at 37 °C for 96 h, washed with PBS and lysed by adding 50 µL of 0.01% Triton X-100 in 0.1 M Tris buffer at pH 9.8; followed by a freeze -80 °C till assay was conducted. Plates were thawed at 37 °C. and 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 405 nm using a Power Wave 200 microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA). The fold induction of alkaline phosphatase for each individual treatment, in comparison to the estradiol control (1 nM), denoted estrogenic activity and was calculated as previously described (158). In parallel, the cytotoxicity of the treatments was evaluated using sodium rhodamine B (SRB) reagent, as described previously (158).

2.6. ERβ-ERE-luciferase Induction in MDA-MB-231/β41 Cells

Briefly, ERβ stably transfected MDA-MB-231/β41 cells were 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 medium was added for transfection. The cells were transfected with pERE-luciferase at 3 µg/mL and pRL-tK at 1 µg/mL for 6 h then washed twice with PBS and the phenol-free MEM medium was added before treatment with extracts or compounds for 18 h. E_2 (1 nM) and Diarylpropionitirile (DPN), a selective ERβ agonist (1 µM), 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 min to 24 h. Once thawed, the cell lysates were collected in Eppendorf tubes, centrifuged at 14,000 X g at 4 °C for 10 min, 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 were 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.

The data obtained were the mean of three biological replicates and are stated as means ± SD.

2.7. ERα and ERβ Competitive Binding assay

The protocol used and validated for this analysis is as previously described with minor modifications (169-172). Briefly, the reaction mixture consisting of 5 µL of compound in DMSO, 5 µL of purified full length human recombinant ERα or ERβ (Invitrogen Carlsbad, CA) diluted in ER binding buffer (3 nM), and 5 µL of "Hot Mix" (400 nM) prepared fresh with 95 Ci/mmol [^{3}H] estradiol (E_2) was diluted in 1:1 ethanol:ER binding buffer (NEN Life Science Products, Boston, MA, USA). [^{3}H]-E2 concentration of 20 nM was used as tracer in the reaction mixture in accordance with previously described methods (169-171). Then 85 µL ER binding buffer was added and the mixture was incubated at room temperature for 2 h. Hydroxyapatite (BioRad, Herculus, CA) slurry (HAPS) was added (100 µL) to the reaction mixture, which was on ice for 15 min, being vortexed every 5 min. A wash step, repeated three times, was by performed adding 900 µL of appropriate wash buffer for the respective receptor subtype to reaction mixture, which was vortexed, centrifuged at 10,000 g at 4 °C for 1 min and decanted. The HAPS pellet with receptor-ligand complex was re-suspended in 200 µL of ethanol (200 proof) and added to scintillation tubes. Reaction tubes were further rinsed with 200 µL of ethanol and the rinse was added to the scintillation tubes. Cytoscint [(4 ml; ICN (Costa Mesa, CA)] was added to the tube and a Beckman LS 6500 liquid scintillation counter (Schaumburg, IL) was used to count radioactivity. The % inhibition of [^{3}H]-E2 was calculated using equation (1):

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

The % binding of the sample was calculated by comparison with that of estradiol (50 nM). The data obtained were the mean of three analytical replicates in triplicates. % Relative Binding Affinity (RBA) values were calculated for each compound using equation (2) to quantitate binding activity relative to 17β-E_2 (173).

% relative binding affinity (RBA)= [$IC_{50\ E2}$/ $IC_{50\ (iso)flavonoid)}$] X 100 (2)

RBA β/α values *[(%RBA β of compound (A)] / [%RBA* α *of compound (A)]* were used to express the measure of preferential interaction of each specific (iso)flavonoid for each ER subtype (α or β) (174).

2.8. Time Resolved Fluorescence Energy Transfer (TR-FRET) assay in ERα and ERβ

The following agonist mode TR-FRET assay protocol was adapted from established procedures (175, 176). For ERα-ligand recruitment assessment, (iso)flavonoids at varied concentrations were incubated with 4 nM ERα-417 (amino acids 304–554; C381,530S; site-specifically labeled at C417 with biotinmaleimide), 1 nM streptavidin terbium chelate (LanthaScreen® Tb-Streptavidin; ThermoFisher Scientific catalog number: PV3965), and 100 nM steroid receptor coactivator 2 (residues 627–829; labeled nonspecifically with 5-iodoacetamidofluorescein) in 80 µL of TR FRET buffer. For ERβ-ligand recruitment assessment, (iso)flavonoids at varied concentrations were incubated with 7 nM ERβ-LBD (labeled with GST), 5 nM terbium-anti-GST antibody and

250 nM steroid receptor coactivator 2 (residues 627–829; labeled nonspecifically with 5-iodoacetamidofluorescein) in 80 µL of TR FRET buffer. TR FRET buffer contained 20 mM Tris-HCl, 10% glycerol, 50 mM NaCl, 0.02% Nonidet® P 40 substitute at pH 7.5 with a DMSO concentration of 3%. After a 1 hour incubation at room temperature for each respective ER subtype-ligand recruitment measurement, TR-FRET readings were taken on a Biotek Neo2 reader (Gen5 v. 3.02 software package) using 360/40 nm excitation with a 100 µs delay and a 500 µs collection time. Emission was measured at 495/5 nm and 520/25 nm. Experiments were performed in triplicate using Corning black, polystyrene, flat bottom, nonbinding surface area, 96-well half area assay plates. Graphpad Prism v. 7.02 was used to generate bestfit curves of the data (ratio of emission at 520 nm / emission at 495 nm) to sigmoidal, 4PL, where X is log (concentration of inhibitor).

2.9. Computational docking model

In silico docking analysis was performed to investigate the interactions of the flavonoids and isoflavanoids with ERα and ERβ. Binding sites of ERα and ERβ bound to genistein were obtained from Protein Data Bank (PDB ID: 1x7r and 1x7j, respectively) and uploaded to Molecular Operating Environment (MOE; Chemical Computing Group, version 2016.0208). All unnecessary water molecules were removed, and the structure was prepared using the quick prep option in MOE. Compounds were docked with triangle matcher placement with London dG scoring and induced fit refinement with GBVI/WSA dG scoring. Figures are shown with the docked compound in molecular surface showing hydrophobicity and lipophilicity of the binding site. *In silico* data was generated by Caitlin Howell.

3. METHOD OPTIMIZATION FOR DETERMINATION OF ERα AND ERβ ACTIVITY OF *(ISO)*FLAVONOIDS

(Reprinted in part from: Atieh Hajirahimkhan, Obinna Mbachu, Charlotte Simmler, Sarah Green, Huali Dong, Dejan Nikolic, David C. Lankin, Richard B. van Breemen, Shao-Nong Chen, Guido F. Pauli, Birgit M. Dietz, and Judy L. Bolton "Estrogen Receptor (ER) Subtype Selectivity Identifies 8-Prenylapigenin as an ERβ Agonist from Glycyrrhiza inflata and Highlights the Importance of Chemical and Biological Authentication" Journal of Natural Products 81(4), 966-975 (2018))

3.1. Rationale and Hypothesis

The ER subtype selectivity of phytoestrogens has been investigated in numerous studies (123, 174). Since the identification of ERα decades ago and ERβ in the mid 90's, there has been a concerted effort to identify or develop ligands that can modulate them (1, 117, 177). These two ER subtypes have distinct, as well as complementary activities *in vivo* as it relates to mammary gland proliferative activity. ERα activity has been shown to be associated with cell proliferation, while ERβ activity is reported to be antiproliferative and attenuates ERα activity in breast cells (3, 113, 120, 178). E_2 stimulation of ERα drives the proliferation of some breast cancers cell lines (179, 180). In this aim, it is hypothesized that phytoestrogens and botanicals that exhibit ERβ-preferential activity may be a safer option for women's health BDS, as they won't lead to proliferation in mammary tissue, presenting a better safety profile and potentially reducing the risk of mammary gland carcinogenesis. ERβ activity may include a protective effect *in vivo* (116). This is due to findings that ERβ mRNA levels and relative

expression is decreased in a number of cancers, compared to normal tissue (115, 181-183). It has also been suggested that in some clinical cases, targeting a specific ER subtype over its counterpart may offer some biological benefits such as fewer unwanted side effects (1). These side effects can occur through the increased risk of hyperproliferation due to ERα stimulation by E_2. An *in vitro* cell model was optimized to assess ER subtype activities of the respective (iso)flavonoids and women's health botanicals.

3.1.1. Validation of the Bioassays for Differentiating ERα versus ERβ Effects

A cell-based estrogenic assay protocol was used consisting of an induction of alkaline phosphatase activity assay in Ishikawa (ERα+) cells and an ERβ-ERE-luciferase assay in MDA-MB-231/β41 cells was developed to compare ERα and ERβ activity. Alkaline phosphatase activity in Ishikawa cells is mainly induced by activators of ERα dependent pathways (184, 185). MDA-MB-231/β41 cells (ER negative cells transfected with ERβ), on the other hand, specifically indicate the estrogenic effects associated with ERβ through the activation of ERβ-ERE-luciferase (167). The known selective ERα ligand 4,4',4''- (4-propyl-[1*H*]-pyrazole-1,3,5-triyl) *tris*phenol (PPT) and the selective ERβ ligand 2,3-*bis* (4-hydroxyphenyl)-propionitrile (DPN) developed by the Katzenellenbogen laboratory, were used as positive controls in these assays (186-188). In addition, the potent ERα/ERβ phytoestrogen 8-prenylnaringenin (8-PN), the potent ERβ-preferential isoflavonoid genistein, and the endogenous ER ligand 17β-Estradiol (E_2) were used to further validate the method development assay (123, 139, 141, 189, 190). A validated assay was expected to show ERα-selectivity for PPT, nanomolar EC_{50} for 8-PN in ERα,

and for ERβ, nanomolar EC_{50}'s with ERβ preferential activity over ERα for DPN and genistein.

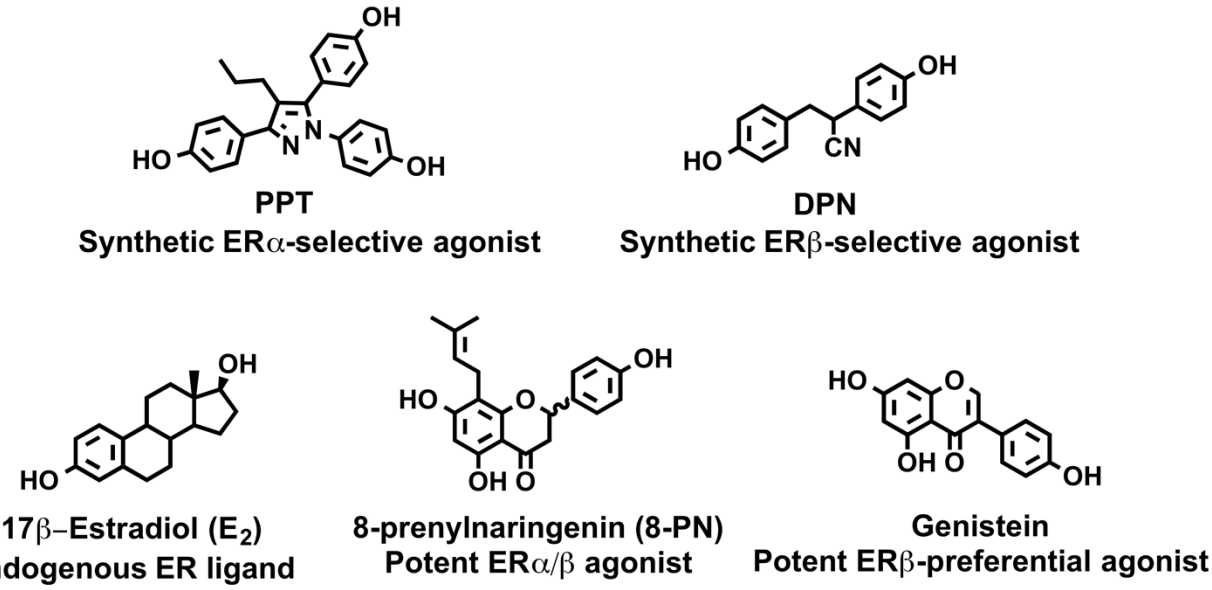


Figure 11. ER Subtype Selective Ligands

3.1.2. Results and Discussion

The ERα ligand PPT induced alkaline phosphatase activity in ERα (+) Ishikawa cells with a low nanomolar potency (Table I, Figure 12A and 13A), but it did not exhibit any estrogenic response in MDA-MB-231/β41 ERβ (+) cells (Table I, Figure 12B and 13B). These data were consistent with previous studies and confirmed the ERα selectivity of PPT and validated the use of the optimized ERα assay (117, 191). The ERβ ligand DPN showed estrogenic sub-micromolar potency in the alkaline phosphatase activity assay in Ishikawa cells (Table I, Figure 12A and 13A) and low nanomolar potency and

ERβ preferential activity in the ERβ-ERE-luciferase assay in MDA-MB-231/β41 cells (Table I, Figure 12B and 13B) with a considerable (30-fold) selectivity for ERβ. Other *in vitro* studies report DPN as having a 12 to 30-fold selectivity for ERβ over ERα (191,192, 193). Finally, the known phytoestrogens 8-PN also had low nanomolar ERα and ERβ potency but no selectivity for either of the two ER subtypes, and genistein with a 100-fold ERβ selectivity exhibited the expected effects in these estrogenic assays (Figure 12A and B).

Further steps were taken to further confirm that results from the developed method were reflective of the ER specific activity. Dose-response assays using DPN in MDA-MB-231 ER (-) cells were used in the ERβ-ERE-luciferase assay (Figure 13B). As expected, the absence of ERβ expression resulted in significantly lower luciferase activity at every DPN concentration (Figure 13B). Additionally, an alkaline phosphatase activity assay in Ishikawa (ERα+) cells was conducted comparing cells cotreated with E_2 and fulvestrant (ICI 182.780, an ER antagonist) with cells treated with E_2 alone (Figure 14A). Results showed that cells cotreated with E_2 and fulvestrant had significantly lower activity compared to those the cells that were treated with E_2 only (Figure 14A). This further confirmed that ERα activity is modulated in the developed assay. This same assay verification was conducted using MDA-MB-231/β41 cells in the ERβ-ERE-luciferase assay (Figure 14B). The findings showed that cotreatment of these cells with E_2 and fulvestrant significantly reduced ERβ activity compared to those treated with E_2 alone (Figure 14B), confirming the modulation of ERβ activity in this assay. The use of different cell lines and assay types to analyze the respective ER subtype activities in this study were recognized limitations for our method. This may also explain the

difference in our ERα EC_{50} value for genistein of 0.4 μM from that of other studies of 0.038 μM (192). However, the results in our stated assays were generally consistent with previous publications (117,191,192). Taken together, our data suggests that the developed bioassays functioned effectively and were suitable for the purpose of evaluating plant extracts and their phytoconstituents for differential ERα versus ERβ effects (158, 186, 187, 194, 195). Our validated biological assays enabled the identification of ERα and ERβ-preferential extracts and bioactive compounds which were necessary for our next aim (Aim 2). The goal of this next project was to identify ERα and ERβ biological activity of extracts, especially licorice, used in women's health and their various phytoestrogens.

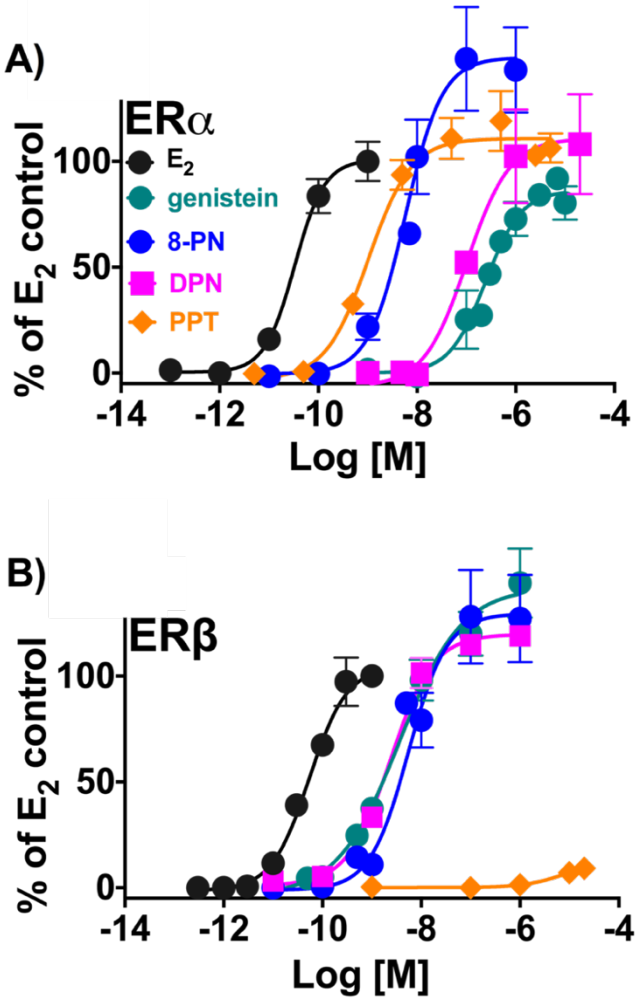


Figure 12. Method Validation for Defining ER Selectivity by Select Compounds. A) ERα-dependent alkaline phosphatase induction. B) ERβ-ERE-luciferase induction using E2 (black, filled circle), PPT (tangerine, filled diamond), and 8-PN (blue, filled circle) and DPN (pink, filled square) and genistein (ERβ selective ligands) (teal, filled circle). The methods for the Ishikawa and ERE-luciferase assays are described in the methods section. The data represent the averages +/- SEM of three independent determinations.

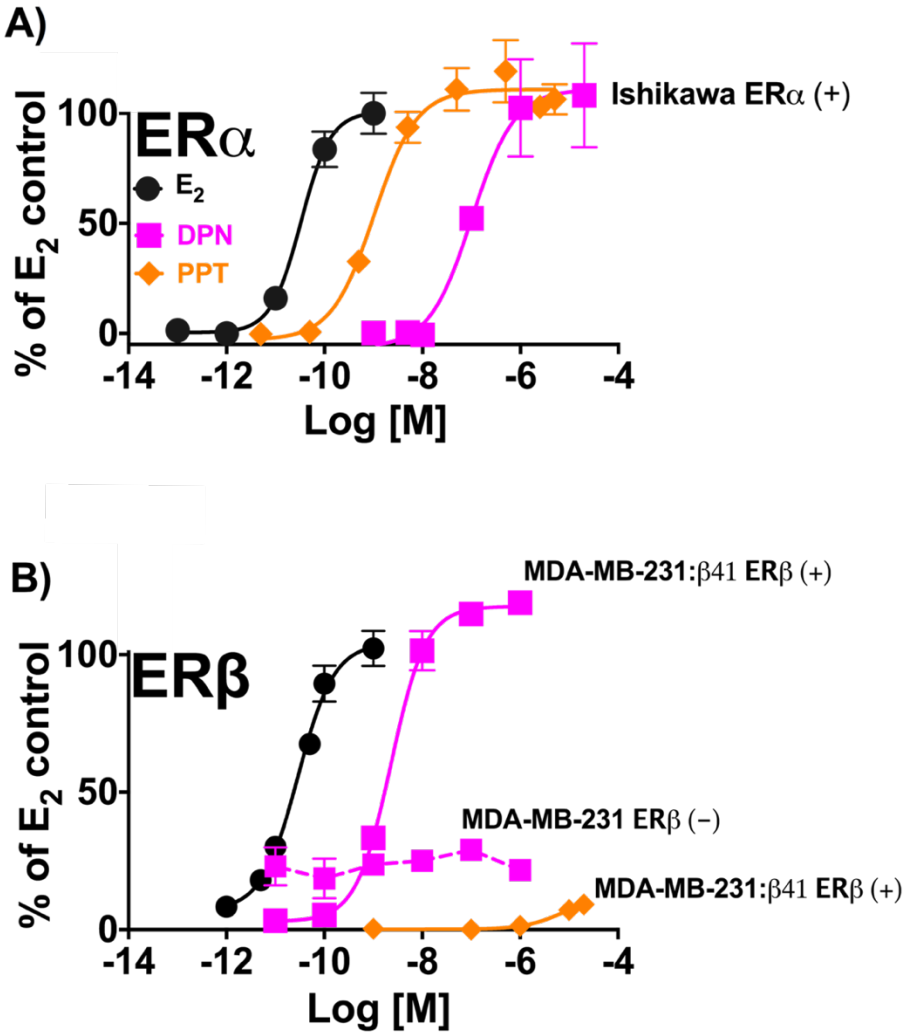


Figure 13. Method Validation Assay Confirming ERβ Modulation for Estrogenic Analysis. A) ERα-dependent alkaline phosphatase induction. B) ERβ-ERE-luciferase induction using E_2 (black, open circle), PPT (tangerine, filled diamond) and DPN (pink, closed square). The methods for the Ishikawa and ERE-luciferase assays are described in methods section. The dashed lines in magenta represent ERE-luciferase activity of DPN in ERβ(-) MDA-MB-231 cells. The data represent the averages +/- SEM of three independent determinations.

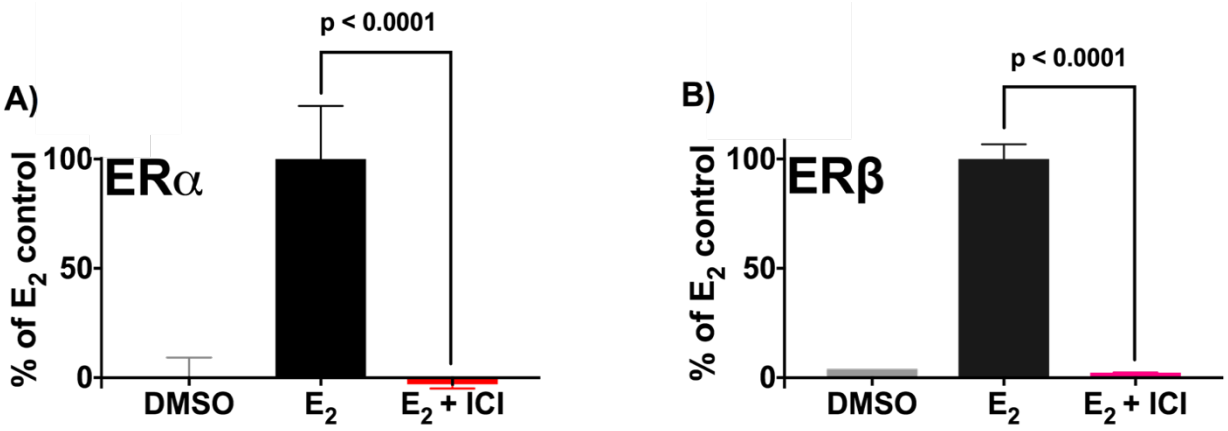


Figure 14. Method Validation Confirming assay ER Modulation for Estrogenic Analysis. A) ERα-dependent alkaline phosphatase induction. B) ERβ-ERE-luciferase induction using DMSO, E_2 (1nM), E_2 (1nM) + ICI (10 μM). The methods for the Ishikawa and ERE-luciferase assays are described in Experimental Section. The data represent the averages +/- SEM of three independent determinations defines receptor route. Data analyzed with by two-tailed unpaired T-test.

	EC_{50} [b]	Maximum efficacy	EC_{50} [b]	Maximum efficacy
17β-estradiol	0.03 ± 0.00[c]	100 ± 10	0.03 ± 0.00c	100 ± 4
PPT	1.0 ± 0.2[c]	119 ± 14	N/A	N/A
DPN	0.08 ± 0.02	90 ± 7.0	0.0024 ± 0003	119 ± 3
Genistein	0.24 ± 0.10	92 ± 4.0	0.0024 ± 0.0002	121 ± 11
8-PN	0.0050 ± 0.001	108 ± 18	0.0050 ± 0.0005	87 ± 9.0

Table I. ERα and ERβ Method Optimization Values. [a]Values are expressed as the mean ± SEM of at least 3 independent determinations in triplicate/duplicate. Experimental details are described in the methods section. [b] μM compounds in general. [c]nM. N/A, not active.

4. IDENTIFICATION OF ESTROGEN RECEPTOR BETA PREFERENTIAL COMPOUNDS IN LICORICE SPECIES

(Reprinted in part from: Atieh Hajirahimkhan, Obinna Mbachu, Charlotte Simmler, Sarah Green, Huali Dong, Dejan Nikolic, David C. Lankin, Richard B. van Breemen, Shao-Nong Chen, Guido F. Pauli, Birgit M. Dietz, and Judy L. Bolton "Estrogen Receptor (ER) Subtype Selectivity Identifies 8-Prenylapigenin as an ERβ Agonist from Glycyrrhiza inflata and Highlights the Importance of Chemical and Biological Authentication" *Journal of Natural Products* 81(4), 966-975 (2018))

4.1. Rationale and Hypothesis

Menopause is a phase of life for women that is marked by a drastic decline in the levels of estrogen in the circulation. This hormonal change causes a number of symptoms such as hot flashes, insomnia, mood changes, and vaginal atrophy, and bone loss, which could have a dramatic negative influence on the quality of life of women (196). It is well known that E_2 (Figure 15) plays a crucial role in human physiology (197). In its classical pathway, E_2 binds to two estrogen receptors (ERs), ERα and ERβ, followed by the interaction of the ERs with estrogen responsive elements (EREs) at the promoter region of the estrogen dependent genes, which ultimately results in the transcription of these genes and the final biological responses (Figure 15) (197). With the onset of menopause, these events are significantly decreased due to the scarcity of estrogens. While hormone therapy (HT) can ameliorate this situation by supplementing estrogens, the 2002 Women's Health Initiative (WHI) showed that an increased breast cancer risk

is associated with HT comprising of estrogen and medroxyprogesterone (198-201). Because of the elevated risk associated with HT, there have been rigorous investigations to find safer options for treating menopausal symptoms (198). It has been reported that activators of ERβ pathways may balance the proliferative effects associated with ERα and might have a better safety profile than typical hormonal therapy (Figure 15) (197, 202-205).

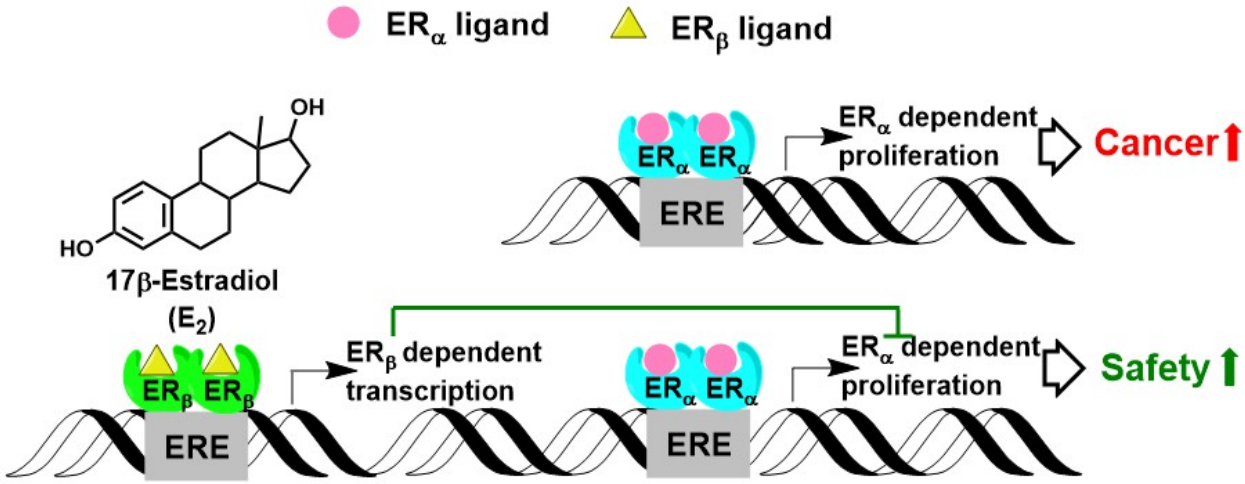


Figure 15. The Effect of ERβ Dependent Pathways on ERα Dependent Proliferation.

Increasing evidence suggests that ERβ can play a potential protective role in various disease conditions including mammary tissue (206-208). Some constituents of botanical dietary supplements used in women's health that have become popular after the WHI report have shown preferential activity for ERβ pathways (203, 209, 210). For

example, soy, red clover, and their isoflavone, genistein [(1) (Figure 19)] have exhibited ERβ effects in various models, although the *in vivo* results have not been conclusive (195, 196, 211-215). Studies have suggested that genistein plays a protective role against various cancers (203). However, other studies report that genistein increases estrogen dependent breast tumors cells *in vivo* (216). These findings, along with the fact that Asian women consuming a phytoestrogen-rich diet have a lower breast cancer incidence and less frequent and/or less severe hot flashes, warrant a more in-depth evaluation of the estrogenic effects of botanicals used for women's health (196, 217, 218). Licorice, which is among the popular botanicals in traditional medicine, is being used for various women's health indications, and is marketed in the U.S. as a dietary supplement ingredient targeting menopausal women (218, 219). Among its 30 different reported species, *Glycyrrhiza glabra* L. (G. glabra), *G. inflata* Batal., and G. uralensis Fisch ex DC. are the three species approved in international pharmacopeias (127). At the same time, it has been shown that these three *Glycyrrhiza* species in the family Fabaceae have distinctly different chemical profiles and, consequently, demonstrate varying levels and various types of estrogenic activity (158, 160, 194, 196, 220). Additionally, studies have suggested the lack of proliferative effects for licorice species in reproductive and mammary tissues of rodents (221). It is suggested that these observations could be associated with the ERβ preferential activity of select licorice extracts (221). Liquiritigenin (LigF) is common to all licorice species and exhibits weak estrogenic effects with a moderate selectivity for ERβ (126, 158, 160, 161, 220, 222, 223). Other studies have also evaluated the estrogenic properties of various components of licorice and have suggested selective ER modulator (SERM) like effects

with some of these phytochemicals (222, 224, 225). However, the different species of licorice have not been explored systematically for comparative ER subtype selectivity, and previously unidentified, preferential and potent ERβ ligands might yet to be found in licorice dietary supplements. The current study compared the ERα and ERβ activities of the three medicinal licorice species. The outcomes showed that *G. inflata* is the most estrogenic of the licorice species that were investigated, and it comprises components that have nanomolar potency for ERβ. Collectively, this suggests that this species might be a safe and effective botanical for postmenopausal women's health. Consequently, this study's aim was determined to identify the presence of ERβ-preferential compound(s) in *G. inflata* that may be contributing to this observed ERβ activity.

4.2. Results and Discussion

4.2.1. Comparison of ERα versus ERβ Activity of Botanicals

When tested in the alkaline phosphatase activity assay in Ishikawa cells (ERα), all three extracts (*G. glabra*, *G. inflata, and G. uralensis*) showed dose-dependent activity (Figure 17A). The relative EC_{50} and the maximum efficacy rankings of the extracts in Ishikawa cells were: *G. inflata* > *G. uralensis* > *G. glabra* (Table II, Figure 16A). The results with *G. uralensis* and *G. glabra* were consistent with previous publications while there are very few reports on the estrogenic activity of authenticated *G. inflata* (158, 224, 226). When the extracts were studied in the ERβ-ERE-luciferase induction assay in MDA-MB-231/β41 cells, the rank order for the potency of these extracts was: *G. inflata* > *G. glabra* > *G. uralensis* (Table II, Figure 16B). Interestingly, a 2-fold increase in potency

was observed in ERβ-ERE-luciferase signal for *G. inflata* in MDA-MB-231/β41 cells compared to the estrogenic activity of this extracts in Ishikawa cells (ERα) (Table II, Figure 16A, Figure 16B). While the increase in ERβ potency of *G. uralensis* and *G. glabra* was 2.3-fold and 3.4-fold, respectively, *G. inflata* had the highest ERβ potency. The rank order for the maximum efficacy of these extracts in ERβ-ERE-luciferase induction was: *G. uralensis* > *G. inflata* > *G. glabra* (Table II, Figure 16B). Additionally, when compared with the alkaline phosphatase data, all the three extracts exhibited increased maximum efficacy in ERβ-ERE-luciferase signal (Table II, Figure 16A and B). These data suggested that overall *G. inflata* has ERβ preferential activity at lower concentrations, and that it might have a better safety profile because its ERβ activity could protect hormone responsive tissues against ERα dependent proliferation. While the ERβ selectivity of the licorice extracts have not been fully investigated, previous studies have suggested that liquiritigenin (LigF) is responsible for the ERβ-selectivity for licorice extracts in the competitive ER binding assays [(**7**) (Figure 19)]. This compound was previously identified as a selective ERβ ligand (126, 158). Moreover, the most potent of the tested licorice extracts (*G. inflata*) contained relatively little of the putative bioactive component liquiritigenin (**7**) (Table III). Interestingly, liquiritigenin (**7**) was present in relatively higher concentrations in the *G. glabra* extract, and yet this extract was not the most ERβ potent of the tested licorice extracts (Table II and III). Therefore, it was evident that another compound in *G. inflata* was likely responsible for the observed ERβ potency.

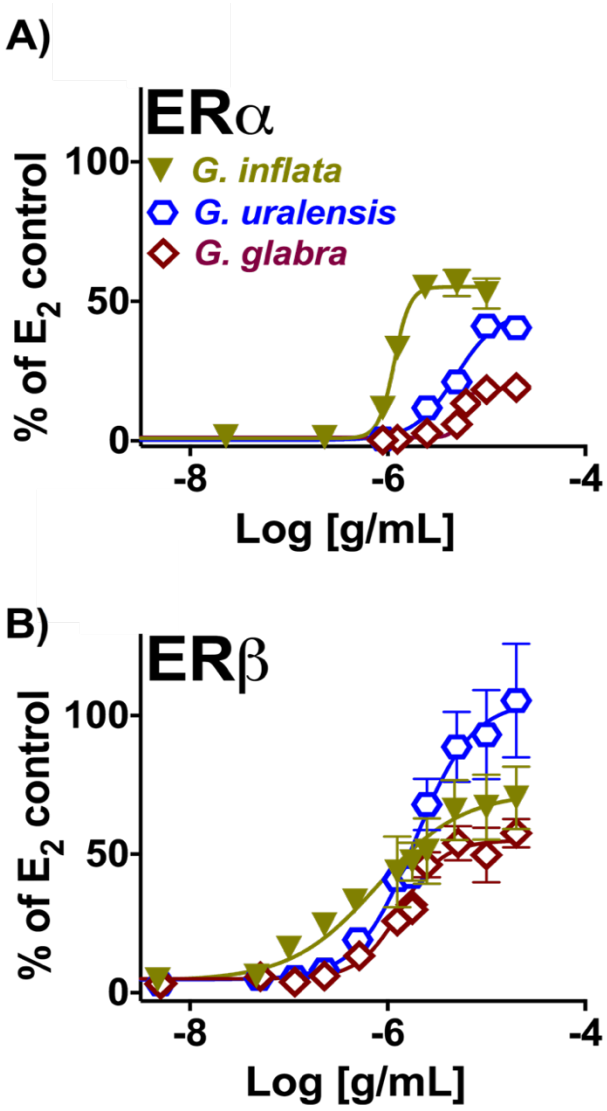


Figure 16. *G. inflata* has the most overall Estrogenic Activity and is moderately ERβ-preferential. Induction of estrogenic activity with the three medicinal licorice extracts (*G. inflata*, green, filled triangle; *G. glabra*, brown, open diamond; *G. uralensis*, blue, open hexagon). A) alkaline phosphatase activity induction in Ishikawa cells and B). ERβ-ERE-luciferase assay in MDA-MB-231/β41 cells. The data represent the averages + SEM of three independent determinations.

4.2.2. Bioassay-Guided Fractionation of *G. inflata*

The observed higher ERβ potency of *G. inflata* (Table II, Figure 16), suggested the presence of unidentified potent ERβ ligands [other than the weak estrogenic compound, liquiritigenin (**7**)] in this extract. Therefore, bioassay-guided fractionation of *G. inflata* extract (Figure 17 and 20) was performed and eventually led to the selection of fractions 8 and 10, both of which displayed estrogenic activities in both the alkaline phosphatase induction assay in Ishikawa cells (ERα+) and the ERβ-ERE-luciferase assay in MDA-MB-231/β41 cells (ERβ+) (Figure 17 and 20), as a potential source of new ERβ ligands.

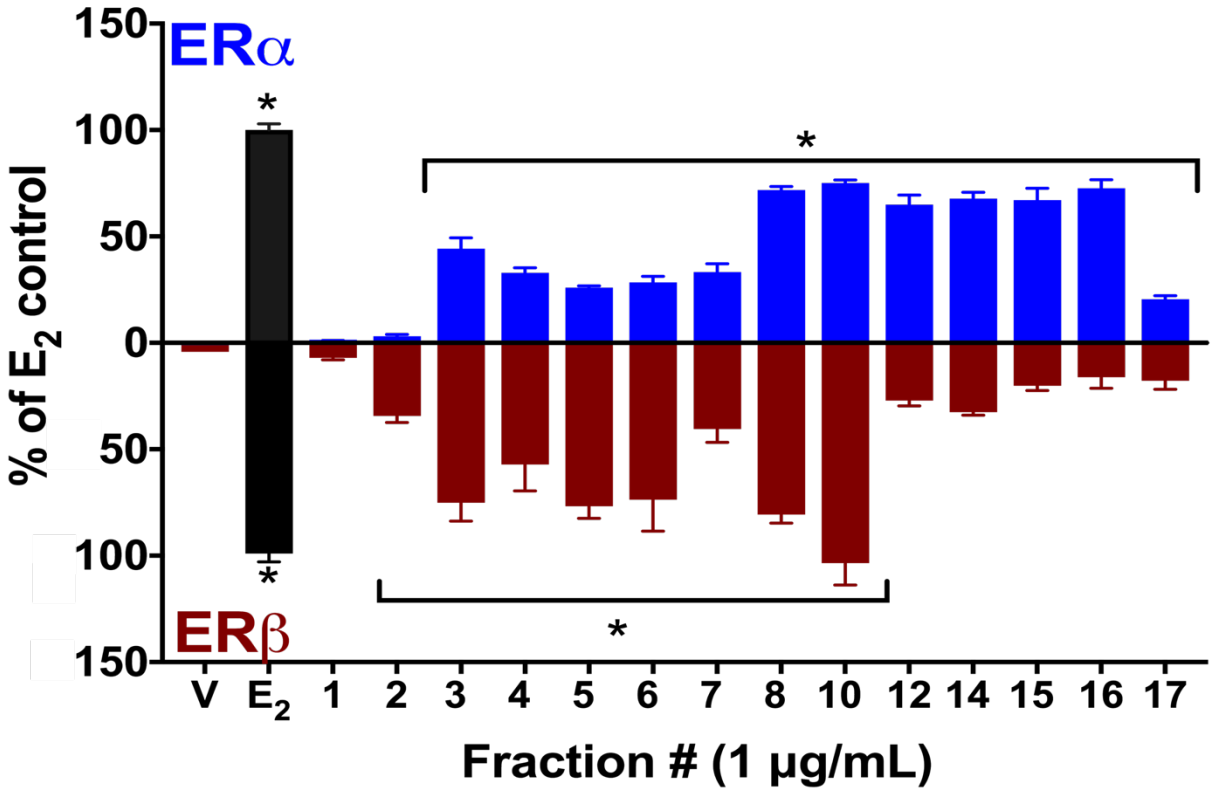


Figure 17. Estrogenic Fractions present in *G. inflata.* Bioassay guided fractionation of *G. inflata* extract yielding fractions (1-17). Fractions assessed for ERα activity by alkaline phosphatase induction assay in Ishikawa cells (ERα+) and ERβ-ERE-luciferase assay in MDA-MB-231/β41 cells for ERβ activity. Fraction 8 was selected for isolation of pure compound(s) to be used for further determination of estrogenicity. The data represent the averages +/- SEM of three independent determinations. Data analyzed with one way ANOVA and Dunnett's multiple comparison post test. (*p < 0.05)

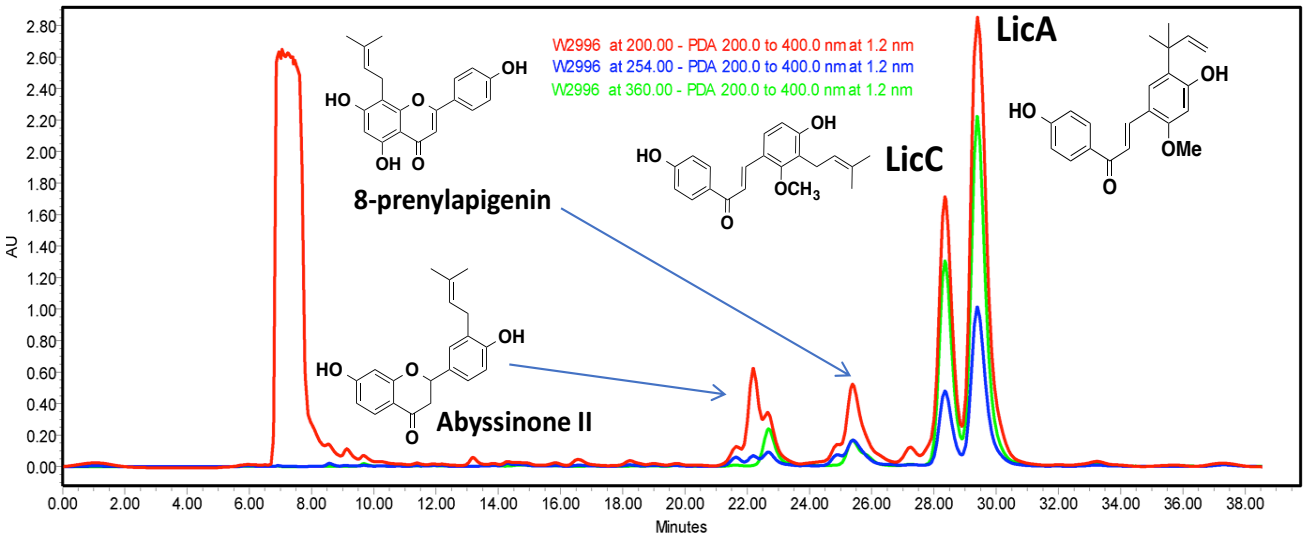


Figure 18. HPLC Chromatogram of Identified Compounds from Fraction 8

Fraction 10, which represented only 0.8% w/w of the crude extract, had a rather complex phytochemical profile (see Appendices), indicating that isolation of pure compounds in amounts sufficient for structure elucidation and further bioassay assessment would be challenging. Interestingly, fraction 8, representing 2.2% w/w of the crude extract was characterized by four major compounds. After purification by semi-preparative HPLC (Figure 18), these four compounds were obtained and identified as, 8-prenylapigenin (**3**, 8-PA, also called licoflavone C), abyssinone II (**4**), licochalcone C (**5**), and licochalcone A (**6**), by means of NMR (1D/2D) MS analyses, and comparison to published data (Figure 19) (162-164, 227-230). Quantitative ^{1}H NMR analysis was performed to estimate the relative abundance of each of the four compounds within fraction 8. Relative percentages of each major component in the extract were as follows: (**4**) 4.5% w/w, (**3**) 5.1%w/w, (**5**) 25.5% w/w, (**6**) 64.8% w/w. Interestingly, (**6**), which is usually regarded as a the most relevant and species-specific bioactive marker of *G. inflata*, showed no estrogenic activity in either assay (Figure 20B). The estrogenic activities of **3** - **6** were evaluated in estrogenic activity assays. Only 8-PA (**3**) exhibited significant activity in both the alkaline phosphatase induction and the ERβ-ERE-luciferase assays. 8-PA (**3**) had been previously reported to have estrogenic activity in ER(+) MCF-7/BOS cells (162). However, the preference of 8-PA (**3**) for ERβ had not been recognized. This ERβ activity provides additional evidence suggesting an enhancement of botanical safety profile of licorice preparations.

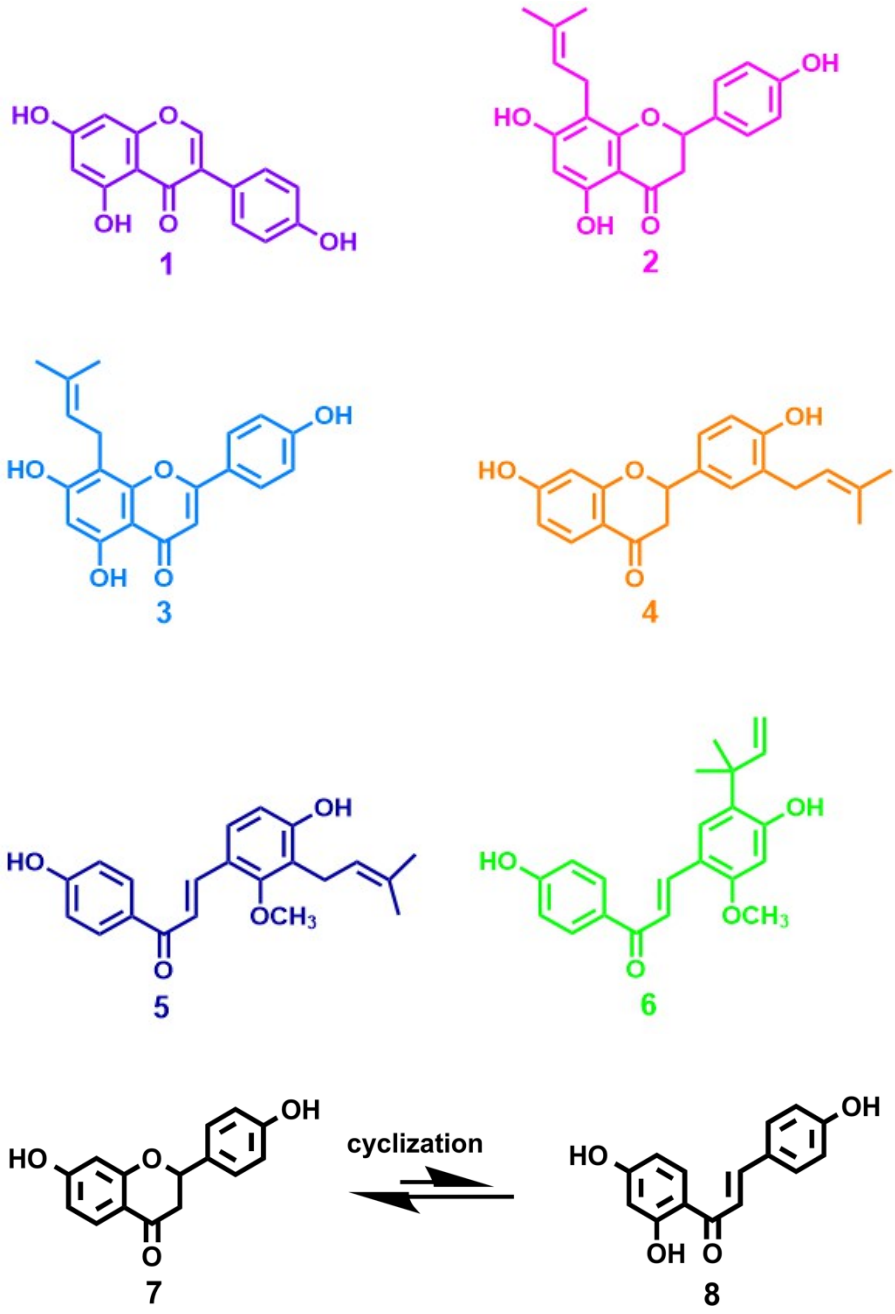


Figure 19. Natural products from red clover, soy, hops, and licorice. Genistein (**1**) is an ERβ preferential compound from red clover and soy. 8-prenylnaringenin (**2**) is a potent estrogenic compound from hops. 8-prenylapigenin (**3**) is an ERβ preferential compound isolated from licorice (*G. inflata*). Abyssinone II (**4**), licochalcone C (**5**), and licochalcone A (LicA) (**6**) are isolated compounds from licorice (*G. inflata*) with no estrogenic activity. Liquiritigenin (**7**) and isoliquiritigenin (**8**) are the estrogenic pair isolated from various licorice extracts.

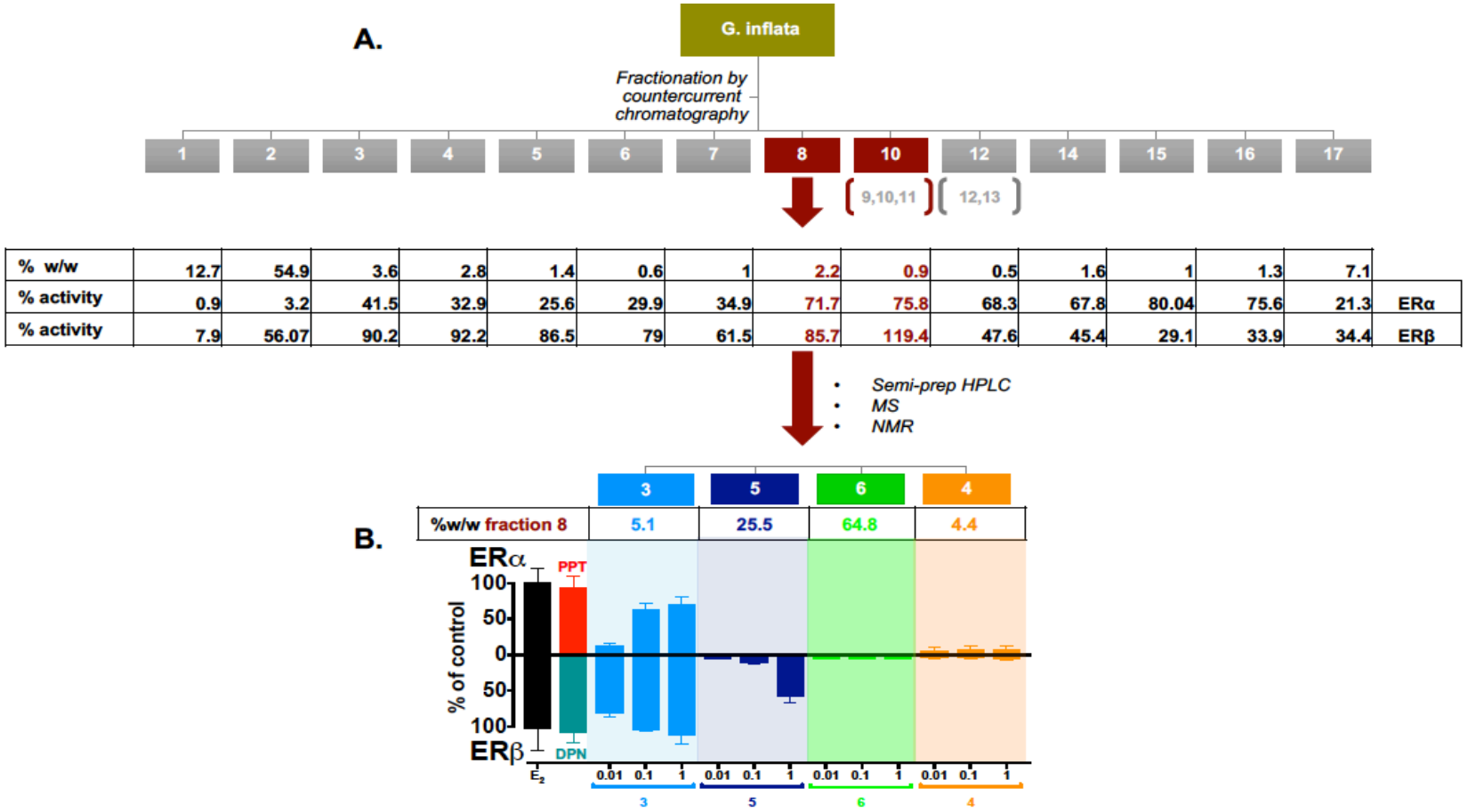


Figure 20. Scheme of Bioassay-guided Fractionation of *G.inflata* Extract.
The crude extract of *G. inflata* was fractionated by countercurrent chromatography (CCC). The different *G. inflata* fractions were tested for their estrogenic properties on both ERα and ERβ models. Fractions 8 and 10 displayed significant activity on both the ERα and ERβ models. Fraction 8, with the highest mass yield at 2.2 % w/w crude extract was further processed by semi-preparative HPLC to isolate and identify four major compounds, namely, 8-prenylapigenin (3), licochalcone C (5), licochalcone A (6), and abyssinone II (4). B). Induction of differential estrogenic activity with the isolated compounds from the active bioassay-guided fraction 8 (Figure 18) in alkaline phosphatase activity induction assay in Ishikawa cells (ERα) and in ERβ-ERE-luciferase assay in MDA-MB-231/β41 cells. The methods for the Ishikawa and ERE-luciferase assays are described in Materials and Methods. The data represent the averages +/- SEM of three independent determinations.

While liquiritigenin and its (pro-drug) bio-equivalent glycosylated derivatives (158, 166) are present in all licorice species, and contribute to the estrogenic activity observed with these extracts, the presence of 8-PA (**3**) could fully explain the higher ERβ potency and observed with *G. inflata* compared to *G. glabra* and *G. uralensis*. In order to evaluate this hypothesis, quantitation of 8-PA (**3**) in all three *Glycyrrhiza* extracts was carried out by LC-MS/MS (Table III). The quantitative data revealed that 8-PA (**3**) was 33 times more concentrated in *G. inflata* compared to the other two *Glycyrrhiza* extracts. These data suggest that **3** plays a fundamental role in the high ERβ potency observed with *G. inflata*.

treatment	alkaline phosphatase induction		ERβ-ERE-luciferase	
	EC_{50} [b]	Maximum efficacy	EC_{50} [b]	Maximum efficacy
17β-estradiol	0.03 ± 0.00[c]	100 ± 10	0.03 ± 0.00[c]	100 ± 4
PPT	1.0 ± 0.2[c]	119 ± 14	N/A	N/A
DPN	0.08 ± 0.02	90 ± 7.0	0.020 ± 0.005	117 ± 10
8-PA**, 3**	0.050 ± 0.006	93 ± 7.0	0.0035 ± 0.0004	104 ± 6.0
8-PN**, 2**	0.005 ± 0.001	108 ± 18	0.0050 ± 0.0005	87 ± 9.0
Genistein**, 1**	0.24 ± 0.10	92 ± 4.0	0.0024 ± 0.0002	121 ± 11
G. glabra	5.4 ± 0.5	19 ± 2.0	1.6 ± 0.4	58 ± 9.0
G. uralensis	4.7 ± 0.2	41 ± 3.0	2.1 ± 0.3	101 ± 17
G. inflata	1.10 ± 0.2	57 ± 6.0	0.6 ± 0.2	80 ± 10

Table II. ERα- and ERβ-dependent Estrogenic Effects of Licorice Species and Isolated Compounds. [a]Values are expressed as the mean ± SEM of at least 3 independent determinations in triplicate/duplicate. Experimental details are described in the method section. [b]Values are expressed in μg/mL for extracts and μM for isolated compounds. N/A, not active. [c]nM.

Species	**6**	**8** equivalents[b]	**7** equivalents[c]	**3**[d]
		% w/w crude extract		
G. glabra	ND[a]	3.61 ± 0.06	8.55 ± 0.06[c]	<LOQ
G. uralensis	ND[a]	0.59 ± 0.01	3.86 ± 0.16	0.005 (52 ± 2.86 ppm)
G. inflata	7.07 ± 0.61	2.32 ± 0.04	3.67 ± 0.31[c]	0.168 ± 0.045

Table III. Comparative Concentrations of Bioactive Compounds in the Licorice Extracts. [a]ND: below the Limit of Detection, [b]The term LigC equivalents is used to represent the total amount of LigC aglycone plus LigC glycosides (isoliquiritin, isoliquiritin apioside and licuraside) in each crude extract. [c]LigF equivalents is used to represent the total amount of LigF aglycone plus LigF glycosides (liquiritin, liquiritin apioside and liquiritigenin-7-O-apiosylglucoside) in each crude extract. [d]8-PA was quantified by LC-MS analysis. The values are expressed as means ± SD of independent measures.

4.2.3. A Cautionary Tale: The Importance of Characterizing Purchased Standards.

As the steps of the bioassay-guided fractionation were underway and small quantities of the compounds were isolated and characterized, the acquisition of reference standards became necessary to enable a thorough study of the pharmacological activities of isolated 8-PA (**3**). The first reference materials for 8-PA (**3**) were acquired commercially from a recognized vendor. This material was immediately subject to biological testing for its estrogenic properties. However, to our surprise, no estrogenic activity was observed, leading to initial questioning of the bioassay-guided fractionation results altogether (Figure 21A). However, in-depth NMR and MS analyses of this commercial reference material demonstrated that the material consisted of a mixture of the chalcone, 4'-*O*-methylbroussochalcone B (**10**), and its flavanone isomer, bavachinin (**9**) (Figure 21A) (162, 228-230).

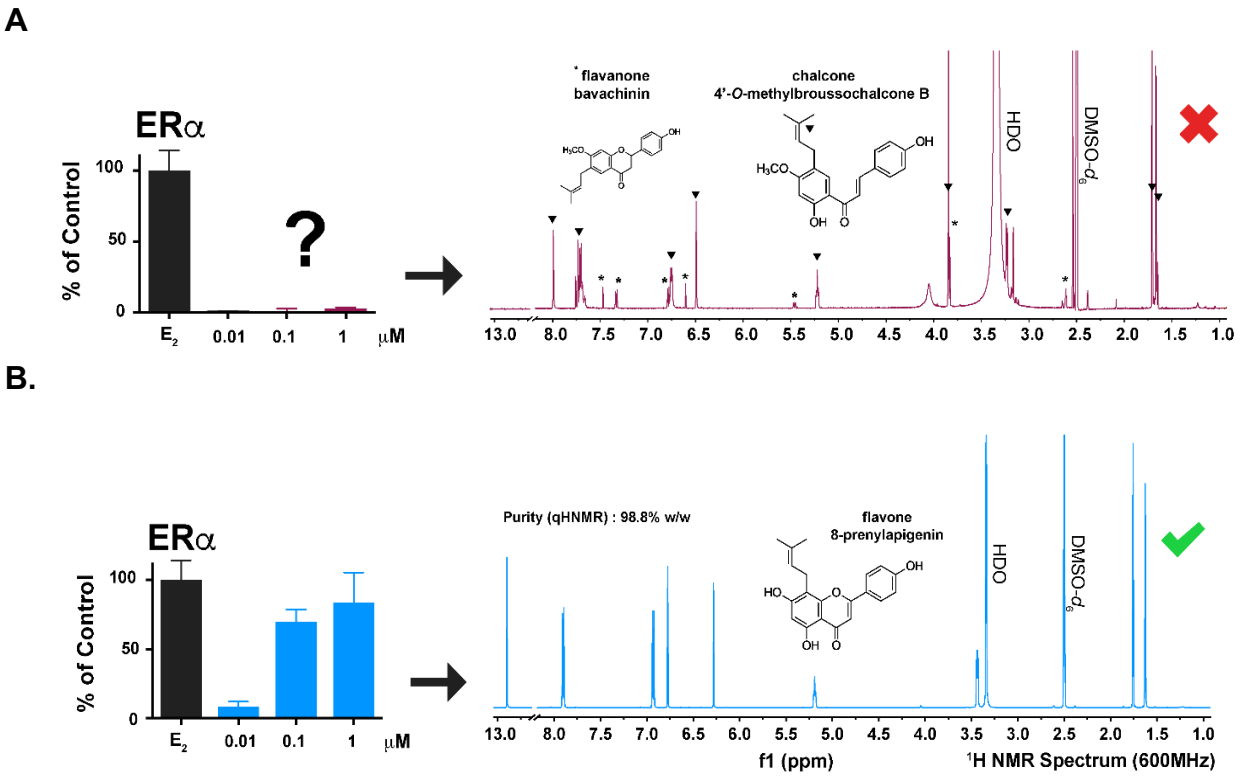


Figure 21. Comparative Estrogenic Activity and qHNMR analysis of Commercial 8-PA Samples. A) The first commercial compound. Identified by NMR (and MS/MS) analyses as being 4'-O-methylbroussochalcone B (10) (triangle highlight within the NMR spectrum). The ^{1}H NMR spectrum of the adulterated commercial compound also displays proton resonances belonging to its flavanone isomer, bavachinin (*) and B) The second commercial compound. Identified by NMR (and MS) analyses and had expected estrogenic activity. The purity of 3 was found to be 98.8% using the qHNMR 100% method.

Interestingly, both 8-PA (**3**) and 4'-O-methylbroussochalcone B (**10**) have the same molecular mass but a different molecular formula. Hence performance of HR-MS analysis was an integral part of the quality control measures, aimed at assuring the

botanical integrity for the overall study. A second batch of "pure" 8-PA (**3**) was purchased from a second company, and in-house quality control combining NMR and HR-MS analysis (see Appendices) was performed to verify the identity and determine the purity of the material. This successfully authenticated batch of 8-PA (**3**) (purity 98.8% w/w, determined by the 100% method) displayed the expected estrogenic activity in the alkaline phosphatase induction assay (Figure 21B). Collectively, these results emphasized once more the fundamental need for the implementation of a rigorous quality control element for chemical authentication (verification of identity and purity determination) of commercial standards intended to be tested in bioassays (231). Such measures are prerequisites to ensure that *in vitro* and *in vivo* evaluations of pure and more complex plant natural products and their formulations can be performed with botanical integrity (https://nccih.nih.gov/research/policies/naturalproduct.htm).

4.2.4. Differential Activation of the ER Subtypes by 8-Prenylapigenin and Known Phytoestrogens.

In order to define the ER subtype selectivity of 8-PA (**3**) in comparison to known phytoestrogens including 8-PN (**2**) from *Humulus lupulus* (hops) and genistein (**1**) from *Trifolium pratense* (red clover) and *Glycine max* (soy), the compounds were studied in the alkaline phosphatase induction assay in Ishikawa cells (ERα+), and in the ERβ-ERE-luciferase assay in MDA-MB-231/β41 cells (ERβ+). 8-PN (**2**) exhibited the highest potency in the alkaline phosphatase induction assay, followed by 8-PA (**3**) and genistein (**1**) (Table II, Figure 22A). When studied in the ERβ-ERE-luciferase assay, **1** exhibited the highest potency, followed by 8-PA (**3**) and 8-PN (**2**) (Table II, Figure 22B). The

comparison of the potencies shown in Figure 22A and Figure 22B suggested a 14-fold ERβ preferential activity over ERα for 8-PA (**3**) and a 100-fold preference with genistein (**1**). 8-PA showed considerably more ERβ potency than liquiritigenin (Figure 34). Additionally, the difference in the abundance of liquiritigenin in the three licorice extracts is not as large as the abundance of 8-PA (**3**), which is 33 times more concentrated in *G. infata*. Based upon our resulting observations, 8-PA (**3**) is suggested to be responsible for the high ERβ potency of *G. inflata.* Moreover, this greater ERβ preferential activity might enhance the safety profile of this extract compared to *G. glabra* and *G. uralensis*. While genistein (**1**) has ERβ preferential activity in various *in vitro* studies, *in vivo* experiments have yielded conflicting results; uterine proliferation effects have been reported in certain concentration ranges (232, 233). To establish the safety of *G. inflata* extracts and the role of its ERβ preferential ligand, 8-PA (**3**), *in vivo* studies are warranted.

To conclude, botanical dietary supplements have become increasingly popular among menopausal women for the alleviation of menopausal symptoms, and establishing their potential efficacy as well as their safety profiles are important areas of research. While estrogenic effects are essential for relieving menopausal discomfort, especially hot flashes and night sweats, studies have suggested that ERα-dependent estrogenic activity could be associated with enhanced tissue proliferation and hormonal carcinogenesis. In contrast, ERβ-dependent estrogenic effects may oppose ERα-dependent proliferation and enhance the safety profile. Therefore, botanical supplements with preferential ERβ effects could be beneficial for menopausal women. *G. inflata* and its active compound 8-prenylapigenin (**3**) which display enhanced ERβ

effects, along with ERα activities could be considered a safer licorice species for menopausal symptom relief. Future *in vivo* studies are needed to define the clinical relevance of the *in vitro* findings. This study also presents an important cautionary note with regard to the pitfalls of natural products with compromised integrity, specifically with misidentified and/or sufficiently impure compounds. Adulteration of crude botanicals affects good research practices in the field of botanicals. Recognition of these supply chain challenges is particularly important for researchers and trainees in natural product chemistry, who must be ever vigilant when they acquire chemical standards for their research. While many vendors provide quality products with reliable certificates of analysis, it is still crucial to perform full authentication (i.e., structure verification and purity determination) of commercially available compounds prior to their application in expensive biological and clinical studies.

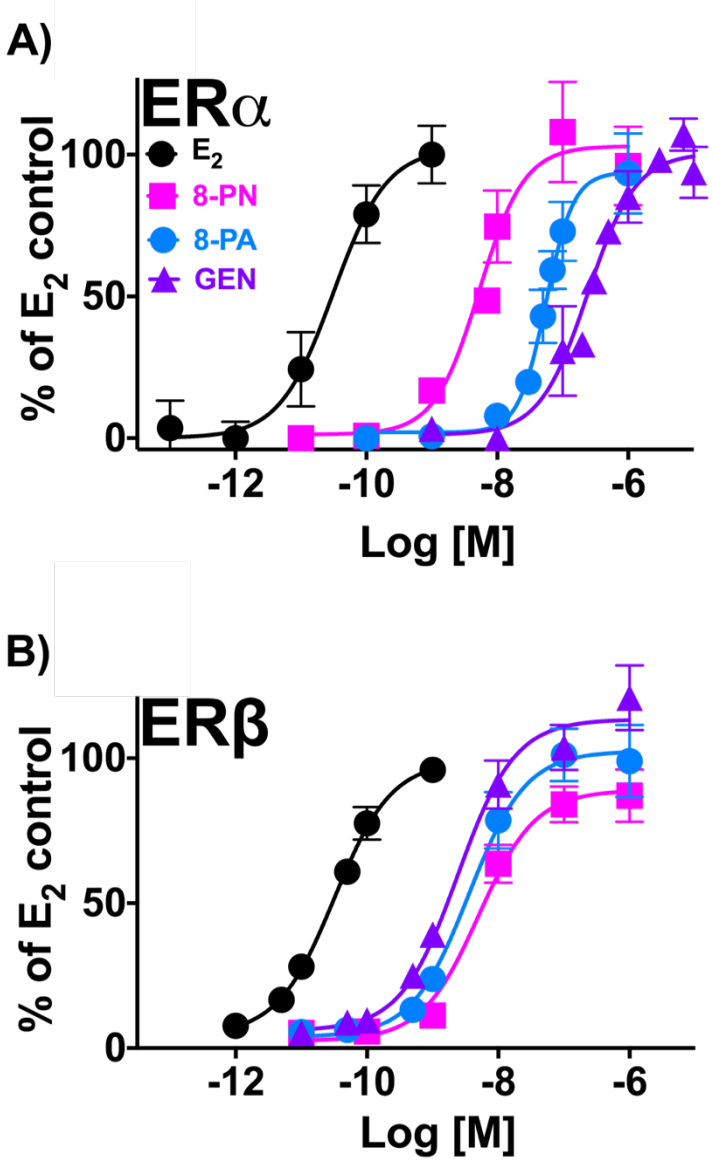


Figure 22. 8-PA and Genistein show ERβ-preferential activity, 8-PN is Equipotent in both ER subtypes. Induction of differential estrogenic activity with 8-PA (3) (blue, filled circle) compared to E_2 (black, closed circle) and the known phytoestrogens, genistein (purple, filled triangle) and 8-PN (pink, filled square) in A) alkaline phosphatase activity induction assay in Ishikawa cells and B) in ERβ-ERE-luciferase assay in MDA-MB-231/β41 cells. The methods for the Ishikawa and ERE-luciferase assays are described in the methods section. The data represent the averages +/- SEM of three independent determinations.

5. MODULATION OF ESTROGENIC ACTIVITY BY ESTROGEN RECEPTOR BIOACTIVE (ISO)FLAVONOID STRUCTURES IN WOMEN'S HEALTH BOTANICALS

(Reprinted in part from: Obinna C. Mbachu, Caitlin Howell, Charlotte Simmler, Gonzalo R. Malca Garcia, Kornelia J. Skowron, Huali Dong, Sarah G. Ellis, Atieh Hajirahimkhan, Shao-Nong Chen, Dejan Nikolic, Terry W. Moore, Günter Vollmer, Guido F. Pauli, Judy L. Bolton, Birgit M. Dietz "SAR study on Estrogen Receptor α/β activity of (iso)flavonoids: importance of prenylation, C-ring (un)saturation, and hydroxyl substituents" *Journal of Agriculture and Food Chemistry* (Manuscript in preparation for submission, 2019)

5.1. Rationale and Hypothesis

Botanicals have been used for many years because of the perceived health benefits they provide (148, 234, 235). The Women's Health Initiative study (WHI) report which showed an increased risk of breast cancer and cardiovascular complications associated with conventional hormone therapy of conjugated equine estrogens and medroxyprogesterone acetate (132), stimulated an uptick in botanical use as an alternate choice in women's health, including usage for the alleviation of menopausal symptoms (234). This increased interest arises out of the perception that botanicals are generally safer option than conventional drugs (132, 234, 236, 237). Frequently used botanicals for women's health include (1) red clover leaves (*Trifolium pratense L.*), (2) hops strobili (*Humulus lupulus L.*), (3) licorice roots (*Glycyrrhiza sp.*), and (4) horny goat weed leaves (*Epimedium sp.*) (234). Historically, red clover was used to treat respiratory ailments in traditional medicine (145). Today, red clover constituents are contained in many botanical dietary supplements aimed at relief of menopausal

symptoms (145). Similarly, hops were traditionally utilized as a sedative before their use in women's health (140, 144). Licorice roots have been widely used for stomach inflammation, respiratory problems, and for the management of postmenopausal symptoms (147, 137, 146). Three licorice species, which include *Glycyrrhiza inflata*, to be referred to as "*inflata* licorice", are used medicinally and approved in international pharmacopeias (127). Horny goat weed is the common name used for 50 different known species of Epimedium, which include *E. sagittatum*, *E. grandiflorum*, and *E. koreanum* to cite a few widely studied species (148-150). These Epimedium species are staples of Traditional Chinese Medicine (TCM) for natural hormone therapy, including the treatment of menstrual irregularities and osteoporosis (148). In the present manuscript, the term "*Epimedium* botanical" is used to describe plant material of the Epimedium genus sold as horny goat weed.

All four described botanicals contain phytoestrogenic (iso)flavonoids, plant-sourced compounds that function like endogenous estrogen (234, 238, 239). Spent hops contains the most potent ERα agonistic phytoestrogen known to date, 8-prenylnaringenin (8-PN) and red clover is known to contain the potent ERβ agonist genistein (Gen) and its precursor biochanin A (141, 189, 123, 139, 190). Alcoholic extracts from *inflata* licorice showed preferential activity for ERβ over ERα and contain the highest concentration of the ERβ-preferential flavone 8-prenylapigenin (8-PA), in comparison to the remaining two approved medicinal species of licorice (127). Lastly, *Epimedium* botanicals contain glycosides of the major bioactive flavonol, icaritin (Ict), which is metabolized to desmethylicaritin (Dmct) *in vivo* (Figure 10) (240, 241). Both Ict and Dmct are reported to be estrogenic (240).

When consumed, the bioavailable flavonoids in these botanicals can interact with the two known estrogen receptors (ER), ERα and ERβ (1, 112). ERα expression is predominant in organs like the mammary gland, uterus, ovary (thecal cells), and adipose tissue, while ERβ, is present in the breast (mainly epithelial cells), ovary (granulosa cells), brain, as well as colon (1, 27, 25, 26, 24). ERα activity drives cellular proliferation in estrogen responsive tissues such as breast and uterus, and studies point to potential increased risk of hormone–dependent cancers when ERα is selectively stimulated (1, 113, 242). For example, purified 8-PN, which is equipotent in both ER subtypes, has been shown to increase proliferative activity in breast and uterine tissues in murine models (127, 173, 243). In contrast, ERβ activity is reported to be antiproliferative, suppressing ERα-driven cellular proliferation (Figure 23), potentially reducing the risk of carcinogenesis (1, 112, 174, 244, 245). There has been an increased interest in ERβ-selective ligands due to their potential antiproliferative properties and their additional health benefits (1, 112).

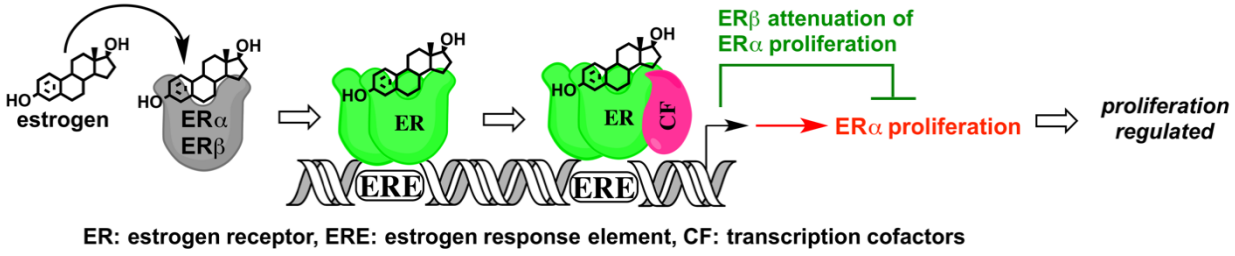


Figure 23. Putative Scheme of Transactivation of ERα and ERβ

In this study, a Structure Activity Relationship (SAR) analysis of respective structurally similar bioactive (iso)flavonoids present in select botanicals was conducted. The compounds examined were: 8-PN, 8-PA, naringenin (Nrg), apigenin (Api), Ict, Dmct, kaempferol (Kfl), Gen, dehydrogenistein (Dgn), and 8-prenylgenistein (8-PG) (Figure 24). 8-PN, 8-PA, Dmct, Ict, and 8-PG have site-specific prenylation, and all the tested isoflavonoids have other subtle structural differences that could give rise to an interesting SAR study (Figure 24). The primary aim was to determine how structural variations to the conserved (iso)flavonoid pharmacophore modulate ER-subtype selectivity—specifically ERβ-preferential activity—and overall ER activity. It was hypothesized that site-specific prenylation, (un)saturation, hydroxylation, and methylation can modulate ER-subtype affinity and activity of (iso)flavonoids.

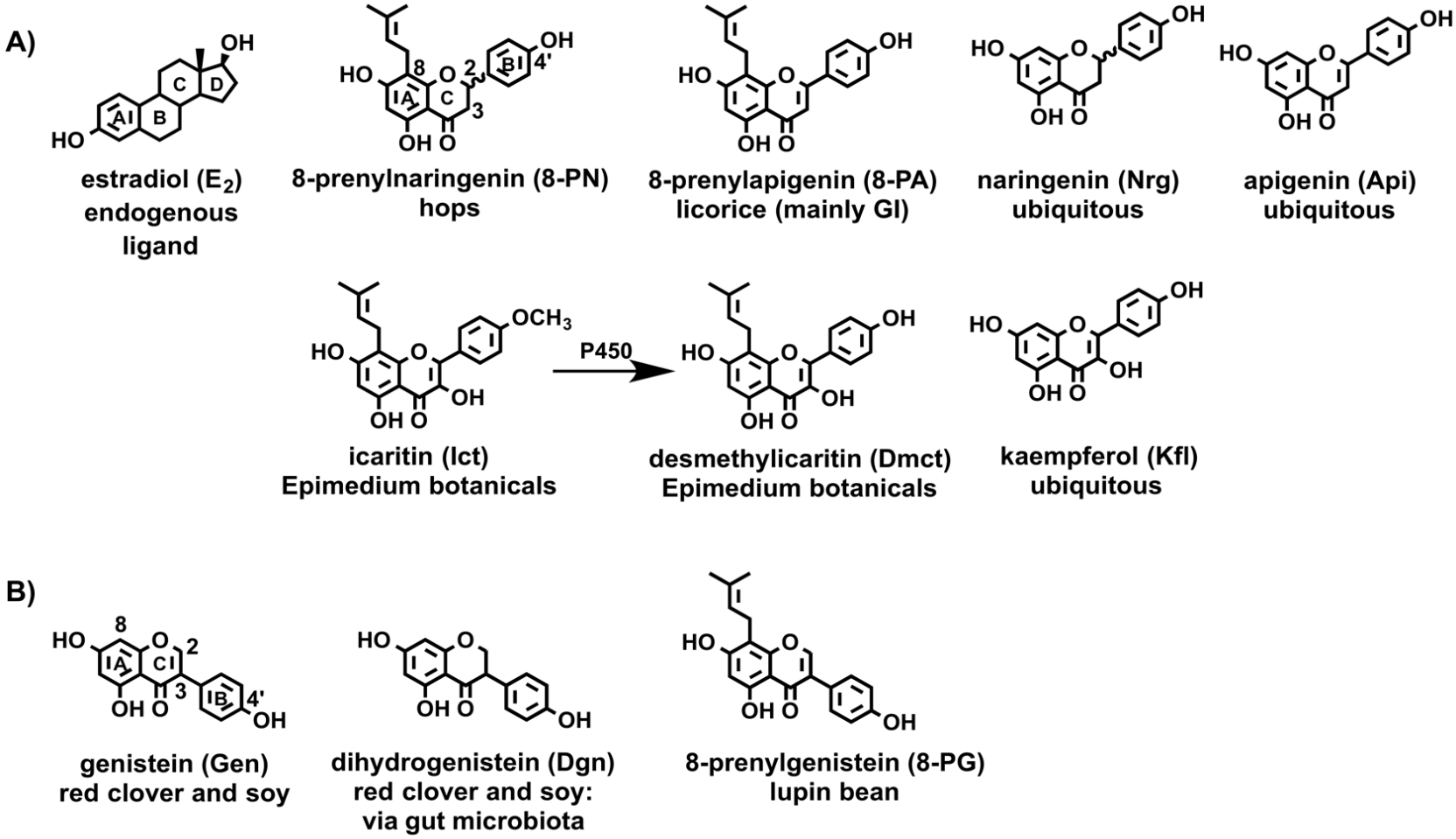


Figure 24. Select (Iso)flavonoids and their Respective Botanical Sources.

A) Estradiol, endogenous ligand; identified bioactive flavonoids present in hops, licorice (mainly Glycyrrhiza inflata : GI), Epimedium botanical sold as horny goat weed, and flavonoids present in ubiquitous plant sources as indicated. B) Identified bioactive isoflavonoids present in red clover/soy and lupin bean, as well as dihydrogenistein, a biotransformed metabolite of genistein.

Additionally, the estrogenic activity of selected extracts containing some of these (iso)flavonoids — hops (8-PN), red clover (Gen), *inflata* licorice (8-PA), and *Epimedium* botanical (Ict)—were also assessed. Another aim was to determine whether the estrogenic activity of the studied extracts correlates with the observed estrogenic behavior of their respective identified bioactive (iso)flavonoids. This approach may show the extent of influence these bioactive compounds have on the overall estrogenicity of their respective botanical extracts. Botanicals containing potent ERβ-preferential (iso)flavonoids may be able to provide the proposed ERβ-related protective benefits by counterbalancing ERα proliferative activity *in vivo*.

5.2. Results

5.2.1. Competitive binding assays of (iso)flavonoids in ERα and ERβ

This assay was used to assess the comparative binding affinity of the tested isoflavonoids for the respective ER subtypes. Among the flavanones and flavones, 8-PN had the highest affinity (% RBA) for ERα, followed by 8-PA (Figure 25). 8-PN showed greater affinity for ERα over ERβ, while 8-PA demonstrated the strongest binding affinity for ERβ with preferential binding for ERβ over ERα (Table IV, Figure 25). Api and Nrg had significantly weaker affinity for ERα and ERβ than their prenylated congeners. The decreasing order of affinity for flavanones/flavones in ERα was 8-PN > 8-PA>> Nrg ≈ Api, while for ERβ the order was 8-PA >> 8-PN >> Api > Nrg (Table IV). The flavonols (Dmct, Kfl, Ict) showed significantly less overall ER affinity than the corresponding flavone, 8-PA (Table IV, Figure 25). The low affinity for ERα and ERβ by

Dmct and Kfl was similar to the unprenylated flavonoids, Api and Nrg (Table IV, Figure 25). However, all four flavonoids, Api, Nrg, Dmct, and Kfl, demonstrated low, but preferential ERβ affinity. The 4' methoxylated Ict had negligible binding activity in ERα and ERβ. All three isoflavonoids (Gen, Dgn, 8-PG) had significantly weaker affinity for ERα than for ERβ. Gen had the strongest affinity for ERβ among the isoflavonoids (Table IV). The overall decreasing affinity ranking order in ERα was Gen >> Dgn ≈ 8-PG and in ERβ Gen > Dgn > 8-PG, demonstrating a clear decrease in affinity with isoflavonoid prenylation as in 8-PG (Table IV). 8-PN showed preferential affinity for ERα at RBA β/α of 0.3 (Table IV). In contrast, 8-PA and Gen had ERβ-preferential affinity with RBA β/α of 2.7 and 3.5, respectively (Table IV). The 8-PN and Gen RBA β/α values were consistent with some previous publications (172, 173). Among all the (iso)flavonoids tested, 8-PA showed the strongest ERβ binding affinity, while Gen showed the best overall ERβ-preferential affinity (Table IV).

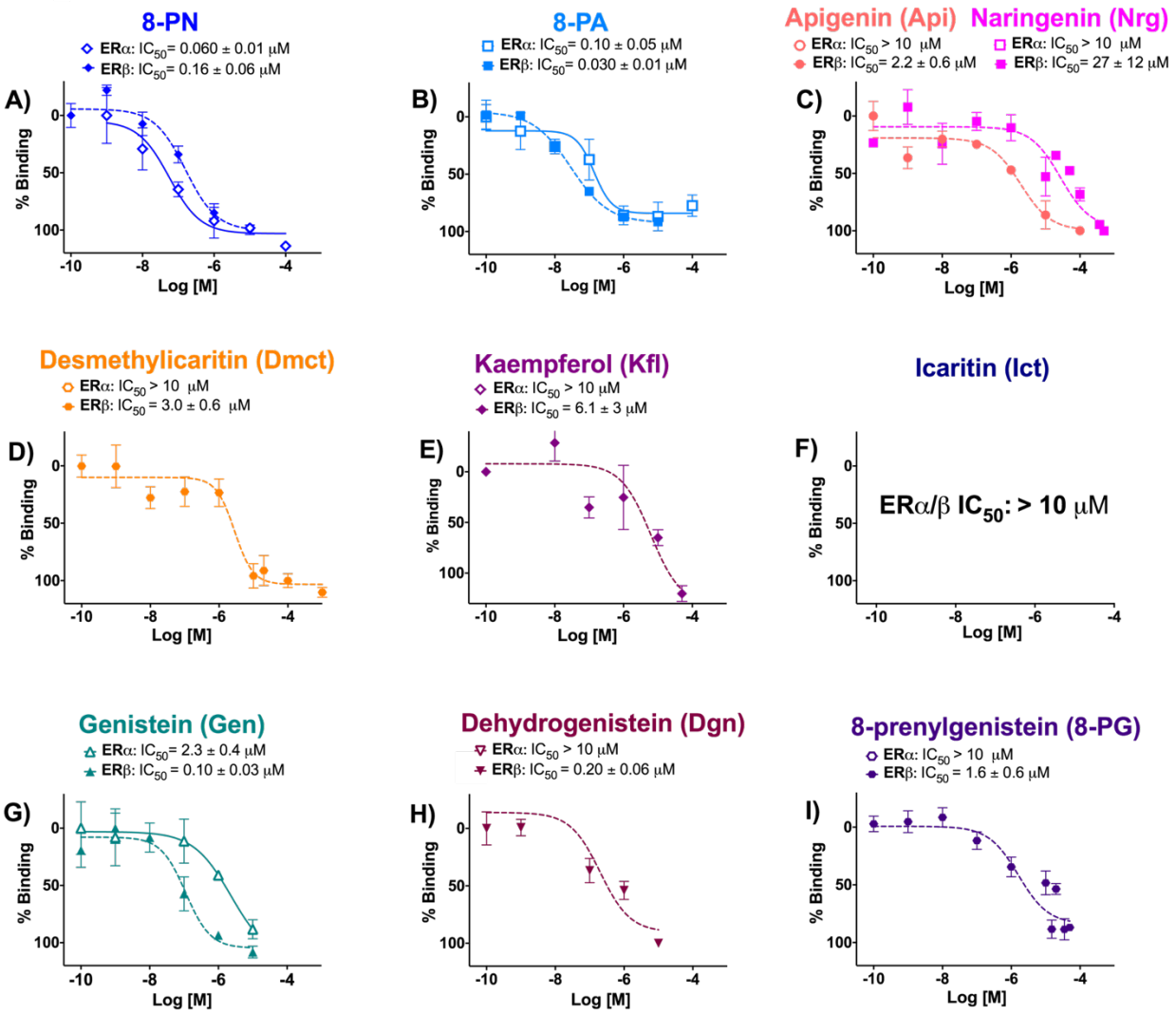


Figure 25. Competitive Radioligand Binding to ERα and ERβ of (Iso)flavonoids. Flavanones, flavones (A - C), flavonols (D - F) and isoflavonoids (G - I) as dose-response evaluations to assess affinity for ERα and ERβ. Data represents average ± SEM from three independent determinations plus IC_{50} (μM) as determined by Graph Pad Prism® 7.0 (Graph Pad software, San Diego, CA).

5.2.2. In Silico Computational Models

Our in vitro findings are further explained by our *in silico* docking results (Figure 26A and B), in which the C-8-prenyl group on 8-PN interacts with lipophilic regions in the ERα binding pocket (Figure 26A). This interaction is positioned to strengthen 8-PN binding to ERα and helps explain the potent IC_{50} values that were determined for 8-PN in comparison to its unprenylated counterpart, Nrg (Table IV, Figure 25A and C). In contrast, the prenyl group of the isoflavonoid, 8-PG, interacts with hydrophilic regions in the ERβ binding pocket (Figure 26B), leading to an unfavorable binding interaction, as opposed to the unprenylated counterpart, Gen, that exhibits strong potency in this ER subtype (Table IV, Figure 25G and I)

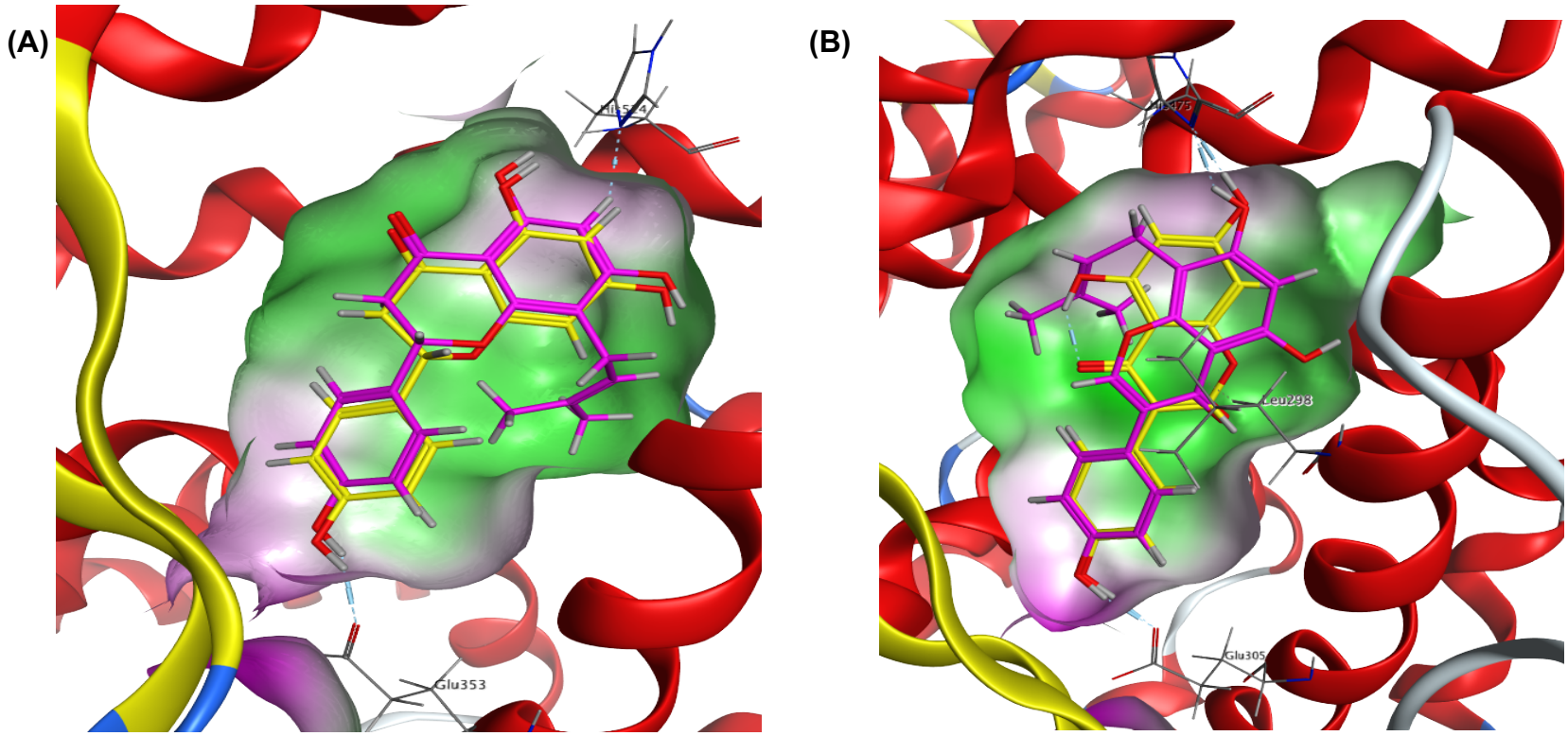


Figure 26. In silico computational modelling of ERα and ERβ binding pocket. The green regions of the binding pocket are lipophilic and contain non-polar residues, which interact favorably with a prenyl group; the purple regions are hydrophilic with polar amino acid residues that interact unfavorably with a prenyl group. A) 8-PN (magenta) and Nrg (yellow) are docked in the binding pocket of ERα. The prenyl group of 8-PN interacts with the lipophilic region of the ERα pocket creating favorable binding conditions. B) Gen (yellow) and 8-PG (magenta) are docked in the ERβ binding pocket. Gen binds well in the ERβ pocket, while 8-PG places its prenyl group in a hydrophilic region creating unfavorable binding conditions

Compounds	ERα		ERβ		RBA β/α
	% RBA[b]	% RRE[c]	% RBA[b]	% RRE[c]	
17β-Estradiol	100	100	100	100	---
Flavanones, Flavones					
8-PN	30	94.7	9	51.2	0.3
8-PA	18	43.2	48	47.2	2.7
Nrg	<0.002	12.6	0.05	23.0	---
Api	<0.002	15.4	0.6	32.8	---
Flavonols					
Dmct	<0.002	9.1	0.5	16.6	---
Ict	<0.002	10.2	<0.002	11.9	---
Kfl	<0.002	31.1	0.2	18.8	---
Isoflavonoids					
Gen	5.6	62.7	19.4	50.2	3.5
Dgn	<0.002	25.1	14	38.7	---
8-PG	<0.002	33.5	0.9	17.2	---

Table IV. ERα and ERβ (Iso)flavonoid ER subtype and Coactivator Affinity[a]

[a]Values are expressed as the mean of at least 3 independent analytical determinations in triplicate. Method details are described in the Experimental Section. [b]% relative binding affinity (RBA)= (IC_{50} E2/ IC_{50} (iso)flavonoid))*100, [c]% relative recruitment efficacy (RRE) = (max FRET efficacy (iso)flavonoid) / max FRET efficacy E_2)*100, E_2 arbitrarily set at 100%.

5.2.3. TR-FRET assay of (iso)flavonoids in ERα and ERβ

The % Relative Recruitment Efficacy (RRE) (246) reflects a measure of the efficacy of the transcription factor, steroid receptor coactivator-2 (SRC-2), recruitment by each ER-ligand complex. This assay comparatively assesses the affinity of each respective (iso)flavonoid-ER complex for the SRC-2, relative to the affinity of the E_2-ER complex reference. In ERα, 8-PN had the strongest % RRE, followed by 8-PA. The order of % RRE readings in ERα for flavanones and flavones were: 8-PN > 8-PA >> Api ≈ Nrg (Table IV, Figure 27). In ERβ, 8-PN and 8-PA showed comparable activity, while Api showed more activity than Nrg (Table IV, Figure 27). Both 8-PN and 8-PA had greater activity than Api and Nrg, giving an ERβ % RRE order of 8-PN ≈ 8-PA > Api > Nrg (Table IV, Figure 27). As observed in the ER-binding assay, the flavonols, Dmct, Ict, and Kfl, exhibited weak overall results, but Kfl showed comparatively the highest activity in both ER subtypes. However, in comparison to 8-PA the flavonols had significantly lower overall % RRE values (Table IV). Lastly, Gen exhibited the highest % RRE in ERα and ERβ among the isoflavonoids, followed by 8-PG and Dgn resulting in a ranking order of ERα: Gen > 8-PG > Dgn and ERβ: Gen > Dgn >> 8-PG (Table IV).

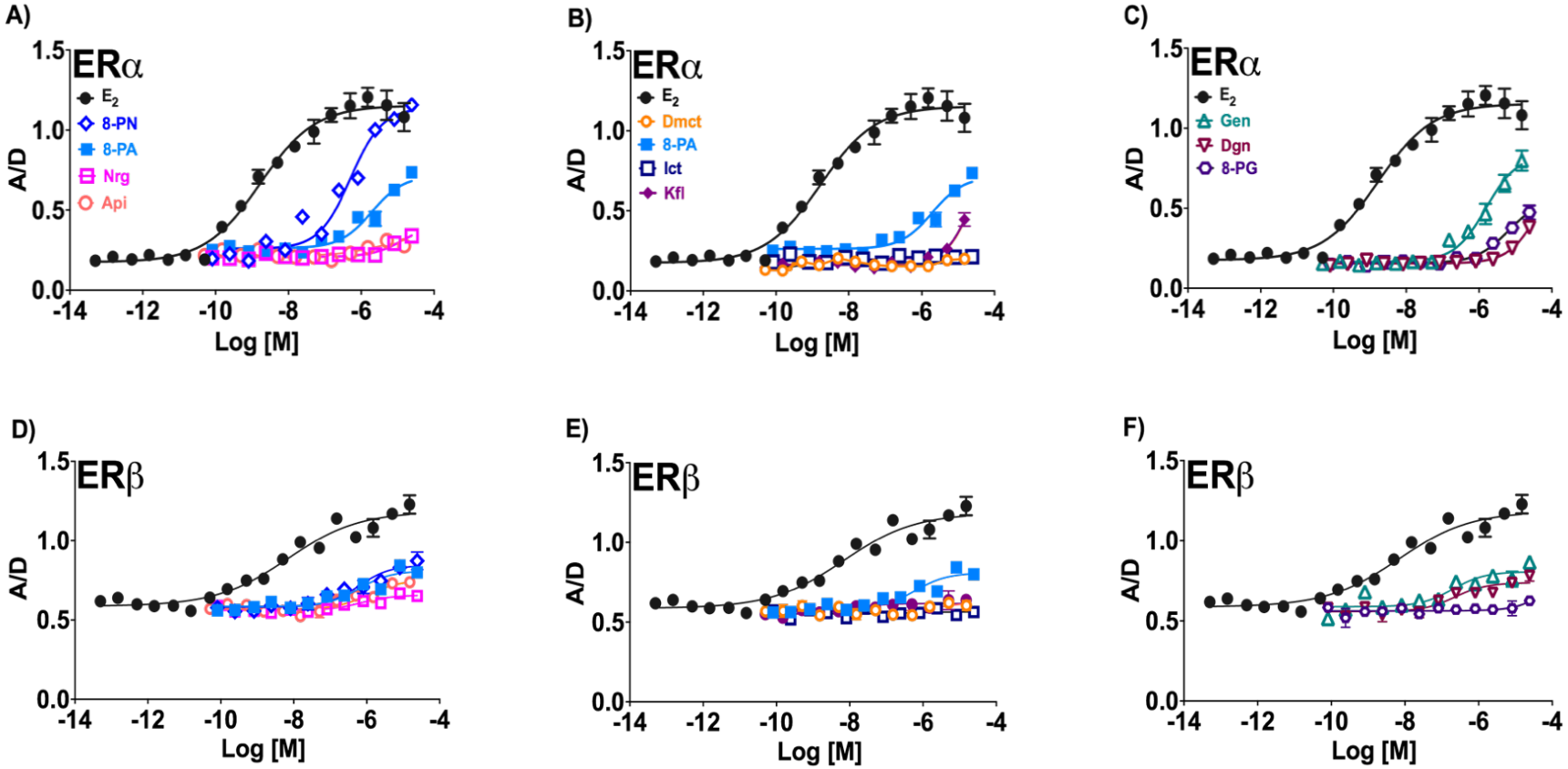


Figure 27. TR-FRET Assessing Coactivator Transcription Factor Recruitment.

SRC-2 recruitment is increased by prenylation on the flavonoid in the ligand-ER complex, but recruitment is decreased when isoflavonoids in the complex are prenylated. Flavanones and flavones (A and D) flavanols (B and E), isoflavonoids (C and F) assessed in ERα and ERβ. The measure of respective ligand-ER recruitment efficacy is represented by A/D: the normalized acceptor molecule emission signals (A) /normalized donor molecule emission signal (D). Data represents average from two or three independent determinations as calculated with Graph Pad Prism® 7.0 (GraphPad software, San Diego, CA).

5.2.4. Comparative functional estrogenic activity of (iso)flavonoids

The functional activity of the (iso)flavonoids and select botanical extracts enriched in these targeted compounds was determined in an alkaline phosphatase activity assay in ERα (+) endometrial carcinoma-(Ishikawa) cells and in ERβ-Luciferase assay in ERβ (+) MDA-MB-231/β41 breast cancer cells with validated methods (117, 118). Prenylation at C-8 on the A-ring of flavonoids showed higher ERα and ERβ functional activity as was observed in 8-PN, 8-PA, and Dmct in comparison to their unprenylated congeners, Nrg, Api, and Kfl, with the exception of the 4'-O-methylated, Ict (Figure 24A, Table V, Figure 28A - D). However, prenylation at the same site on isoflavonoids, as in 8-PG, showed significantly lower functional activity compared to Gen and Dgn (Figure 24B, Table V, Figure 29).

a) Flavanones and flavones. The prenylated flavanone, 8-PN which has saturation at the C2-C3 position on the C-ring showed the highest ERα potency, followed by the prenylated flavone 8-PA with C2-C3 unsaturation at the same position. The order of potency observed in the ERα assay is as follows: 8-PN > 8-PA >> Nrg ≈ Api (Table V, Figure 28A). However, 8-PA demonstrated considerably higher ERβ potency and was the most ERβ-preferential over ERα among the flavonoids in this assay, while 8-PN was equipotent in both subtypes. The resulting ERβ potency order was 8-PA ≈ 8-PN >> Nrg ≈ Api (Table V, Figure 28B). While Nrg and Api were the least estrogenic flavanone and flavone, respectively, both show some preferential ERβ potency (Table V, Figure 28B).

b) Flavonols. Similar to results from the ER-binding and FRET assay, C-ring hydroxylation at 3-position, as seen in Dmct, Ict, and Kfl, reduced the overall estrogenic activity of these compounds compared to 8-PA (Table V, Figure 28C-D). Additional 4′ O-methylation, as observed in Ict, further reduced the estrogenic potency compared to the non-methylated congener, Dmct (Table V, Figure 28C-D). In ERα the order of estrogenic activity was 8-PA >> Dmct > Ict > Kfl (Table V, Figure 28C). However, in ERβ, O-methylation at the 4′ position seemed to obscure the potentiating effect of C-8 prenylation of Ict, making unprenylated Kfl more active than Ict for this ER subtype: 8-PA > Dmct > Kfl >> Ict (Table V, Figure 28D). In comparison to the tested flavonols, 8-PA also showed the most ERβ-preferential activity.

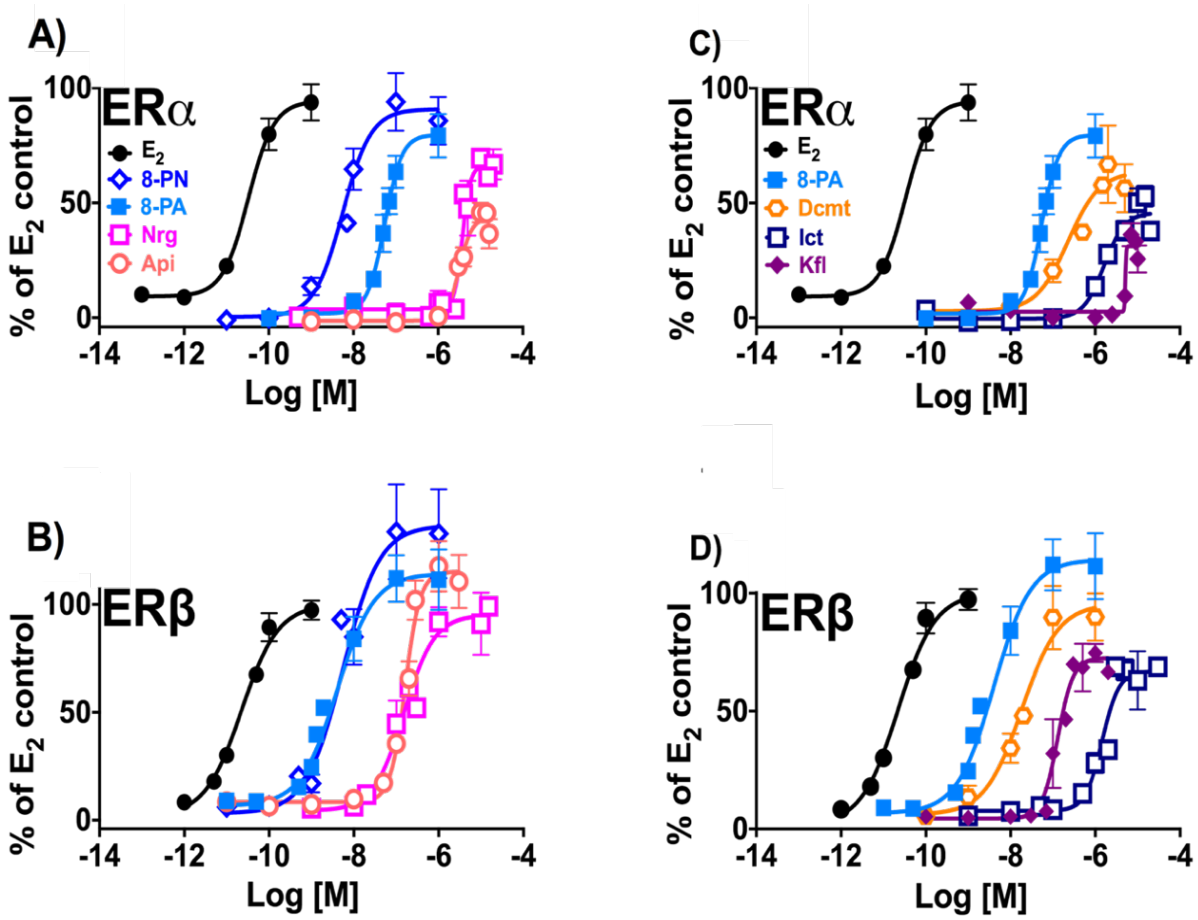


Figure 28. Prenylation increases induction of functional estrogenic effects *in vitro.* Flavanones/flavones (A and B) and flavonols (C and D): (A, C) ERα, estrogen-dependent induction of alkaline phosphatase activity in Ishikawa cells. ERβ, estrogen-dependent induction of ERE-luciferase activity in MDA-MB-231/β41 cells. Data represents average ± SEM from three independent determinations.

Extracts[b]	ERα		ERβ	
	EC_{50}	Maximum efficacy	EC_{50}	Maximum efficacy
Hops	0.030 ± 0.010	72 ± 4.0	0.50 ± 0.050	107 ± 8.0
Red clover	1.8 ± 0.20	94 ± 12	0.45 ± 0.10	139 ± 7.0
inflata licorice	1.1 ± 0.20[127]	57 ± 6.0[127]	0.60 ± 0.20[127]	80 ± 10[127]
Epimedium botanical	3.2 ± 0.20	53 ± 5.0	2.5 ± 0.090	53 ± 0.60
Compounds[c]	**EC_{50}**	**Maximum efficacy**	**EC_{50}**	**Maximum efficacy**
17β-Estradiol	0.030 ± 0.00[d,127]	100 ± 10[127]	0.030 ± 0.00[d,127]	100 ± 4.0[127]
		Flavanones, Flavones		
8-PN	0.0050 ± 0.0010[127]	108 ± 18[127]	0.0050 ± 0.00050[127]	87 ± 9.0[127]
8-PA	0.050 ± 0.0060[127]	93 ± 7.0[127]	0.0035 ± 0.00040[127]	104 ± 6.0[127]
Nrg	3.7 ± 0.50	69 ± 6.0	0.10 ± 0.040	99 ± 4.0
Api	3.2 ± 0.030	45 ± 3.0	0.16 ± 0.020	118 ± 5.0
		Flavonols		
Dmct	0.20 ± 0.020	67 ± 12	0.010 ± 0.0070	90 ± 11
Ict	1.6 ± 0.40	53 ± 8.0	1.7 ± 0.70	69 ± 4.0
Kfl	5.1 ± 4.0	36 ± 5.0	0.20 ± 0.050	75 ± 4.0
		Isoflavonoids		
Gen	0.40 ± 0.050	117 ± 25	0.0022 ± 0.0004	131 ± 3.0
Dgn	0.80 ± 0.10	84 ± 4.0	0.020 ± 0.010	114 ± 12
8-PG	0.50 ± 0.20	49 ± 10	0.30 ± 0.0030	74 ± 7.0

Table V. ERα- and ERβ-dependent Estrogenic Effects of Extracts and (Iso)flavonoids. [a]Values are expressed as the mean ± SEM of at least 3 independent determinations in triplicate. Method details are described in the Method Section. [b]Values are expressed in µg/mL for extracts. [c]Values are expressed in µM for compounds. [d]nM

c) Isoflavonoids. In contrast to what was observed with flavonoids, prenylation of isoflavonoids resulted in a significant decrease of overall estrogenic efficacy and potency as was observed in 8-prenylgenistein (Figure 24B, Table V, Figure 29A and B). Saturation at the C2-C3 position in ring C also led to an overall reduction in estrogenic functional activity as was observed for Dgn in comparison to Gen. The isoflavonoids were significantly less potent ERα ligands than ERβ ligands; the order of ERα potency was Gen ≈ Dgn ≈ 8-PG. However, these compounds showed a more defined decreasing order of efficacy in ERα: Gen > Dgn > 8-PG (Table V, Figure 29A). This was determined by the measure their comparative alkaline phosphatase activity relative to % of E_2 control (Figure 29). In ERβ, Gen had the strongest potency as expected followed by Dgn, with 8-PG showing the lowest potency and efficacy relative to the former compounds: Gen >> Dgn >> 8-PG (Table V, Figure 29B).

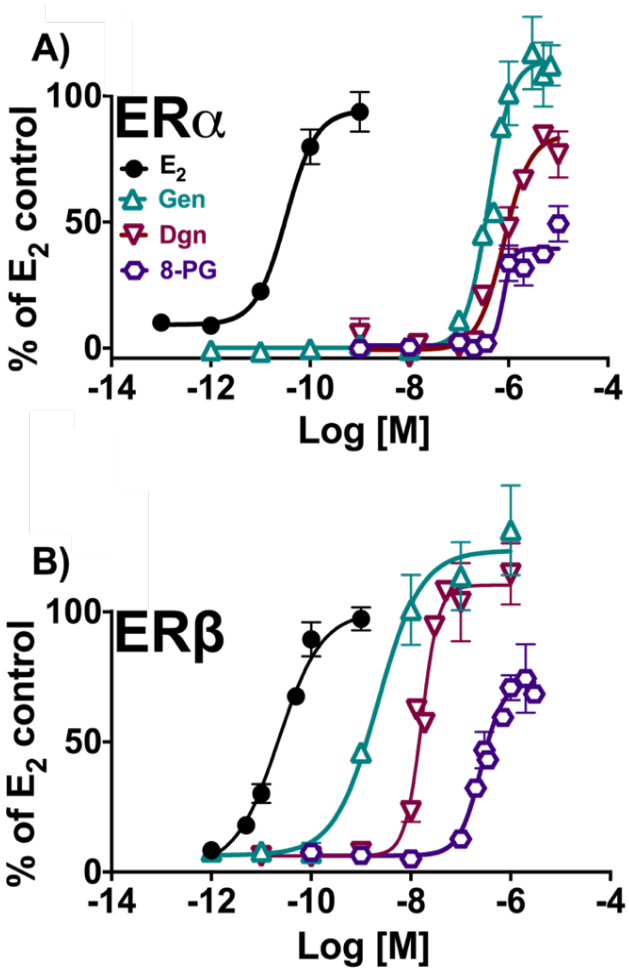


Figure 29. Prenylation on isoflavonoids decreases induction of functional estrogenic effects *in vitro*. Gen, Dgn, 8-PG. (A) Estrogen-dependent induction of alkaline phosphatase activity in Ishikawa cells. (B) Estrogen-dependent induction of ERE-luciferase activity in MDA-MB-231/β41 cells. Data represents average ± SEM from three independent determinations.

5.2.5. Functional estrogenic activity of extracts

The botanical extracts, each containing one of the studied flavonoids/flavonols (8-PN, 8-PA, Ict) and isoflavonoids (Gen) were analyzed for their ERα and ERβ potency. The tested hops extract was generally equipotent in both ERα-(Ishikawa) and ERβ (MDA-MB-231/β41) cell-based assays. In addition, hops extract displayed the highest potency in ERα assay compared to the other tested botanical extracts (Table V, Figure 30). Hops, red clover, and *inflata* licorice extracts did show similar overall activity in ERβ. Red clover and *inflata* licorice extracts both demonstrated preferential potency for ERβ over ERα. While red clover behaved as a full agonist in ERα and ERβ inflata licorice mainly demonstrated partial agonistic activity in ERα. However, *inflata* licorice extract had greater functional efficacy in ERβ compared to ERα (Table V, Figure 30). Epimedium botanical showed the least overall activity, behaving as a partial agonist in ERα and ERβ. The order of functional activity of botanical extracts for ERα was: hops > inflata licorice > red clover >> Epimedium botanical (Table V, Figure 30). In the ERβ assay the rank order was red clover ≈ hops ≈ inflata licorice >> Epimedium botanical (Table V, Figure 30)

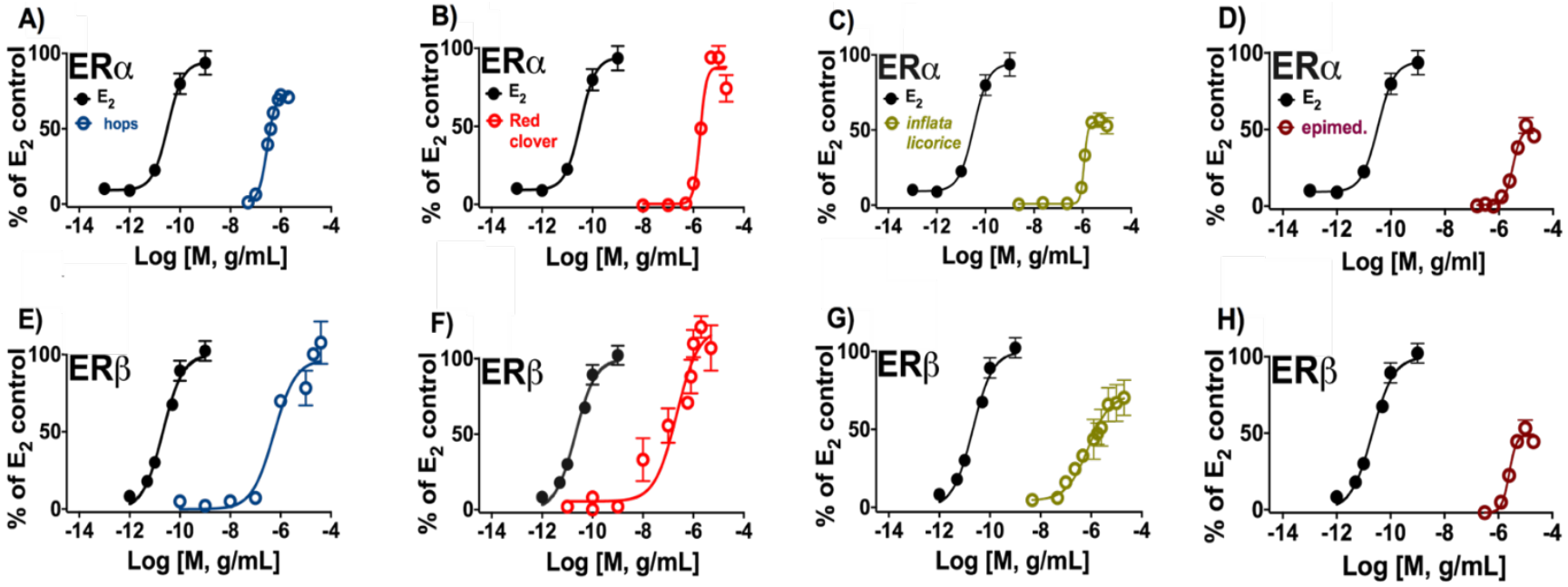


Figure 30. Hops, red clover, and inflata licorice show greater overall functional estrogenic activity than epimedium botanicals.

Induction of estrogenic effects *in vitro* by botanical extracts enriched in target (iso)flavonoids: (A, E) hops, (B, F) red clover, (C, G) inflata licorice, and (D, H) Epimedium botanical. The panels A to D represent the ERα-dependent induction of alkaline phosphatase activity in Ishikawa cells. The panels E to H represent the ERβ-dependent induction of ERE-luciferase activity in MDA-MB-231/β41 cells. Data represent average ± SEM from three independent determinations.

5.2.6. Discussion

ERα and ERβ are co-expressed in a number of estrogen-sensitive tissues, such as the mammary gland, but the relative ratio of these ER subtypes varies depending on the tissue (247, 248). For example, the uterus is reported to have a higher ERα to ERβ expression ratio, while ERβ is reported to be more prominently expressed than ERα in normal mammary tissue (27, 247, 249). Therefore, the relative ratio of ERβ and ERα in a given tissue can influence the level of ERβ-driven protection from ERα-mediated proliferation and potential estrogen carcinogenesis (245, 247, 248). Studies suggest a potential protective role of ERβ from carcinogenesis in normal cells. This is indicated by loss of ERβ expression in early stages of breast carcinogenesis such as ductal carcinomas, as well as its inverse relationship in expression with Ki-67, a biomarker of cell proliferation (115, 249-254). Studies also show that in the presence of E_2, ERβ activity also increased *in vitro* expression of p21 WAF1/cip1, a tumor suppressor cellular protein that inhibits cell cycle progression (179), and inhibited the expression of the pro-proliferative proteins such as c-Myc and cyclin D1 (255). In cells, this p21WAF1/cip1 is reported to bind to, and inhibit the activity of the proliferating cell nuclear antigen (PCNA), responsible for DNA replication during cell proliferation (256, 257). In *in vivo* studies, tissue exposure to ERβ-preferential (iso)flavonoids did not induce endometrial tissue growth and suppressed risk of uterine and mammary carcinogenesis (128, 126, 129, 258, 259).

The initial steps in induction of estrogenic activity involve the binding of the compound to the ER. Both ER receptor subtypes contain polar binding residues in the

ligand binding site, and these residues interact with the A- and D-ring of the endogenous ligand E_2 (Figure 31) (1). These specific regions contain polar amino acid residues crucial for ligand binding including arginine and glutamic acid (A-ring pocket) and histidine (D-ring pocket), which interact with select hydroxyl moieties on ligands, promoting receptor binding (Figure 31, B.1 - B.3) (1). One of the basic requirements for binding of a ligand to the ER is the presence of two optimally positioned hydroxyl groups (or bio-isosteres in some cases), approximately 11Å apart, separated by a hydrophobic core (1). This helps to form crucial hydrogen bonds with the respective amino acids for adequate ligand-binding affinity for both ER subtypes as in the case of E_2 (Figure 31) (1). However, binding affinity is ligand-dependent (246). A majority of (iso)flavonoids used in our study generally harbor these structural characteristics and consequently displayed estrogenic activity (Tables IV and V, Figure 31). Planarity generally tends to favor ERβ selectivity, as is observed in the ERβ agonist Gen, although this feature has some limitations (1, 260). The ERβ active site is narrower than its counterpart, ERα (1). As a result planar flavonoids, such as Gen, are able to fit into the active site with closer proximity to non-polar residues resulting in stronger hydrophobic interactions with amino acids such as methionine (Met 336) (1). This is likely one explanation for the increased preferential binding activity for ERβ over ERα of planar flavonoids (Table IV, Figure 25). However, some non-planar compounds like 8β-vinyl estradiol also display very strong ERβ selectivity due to ligand rigidity and optimally positioned substituents (1).

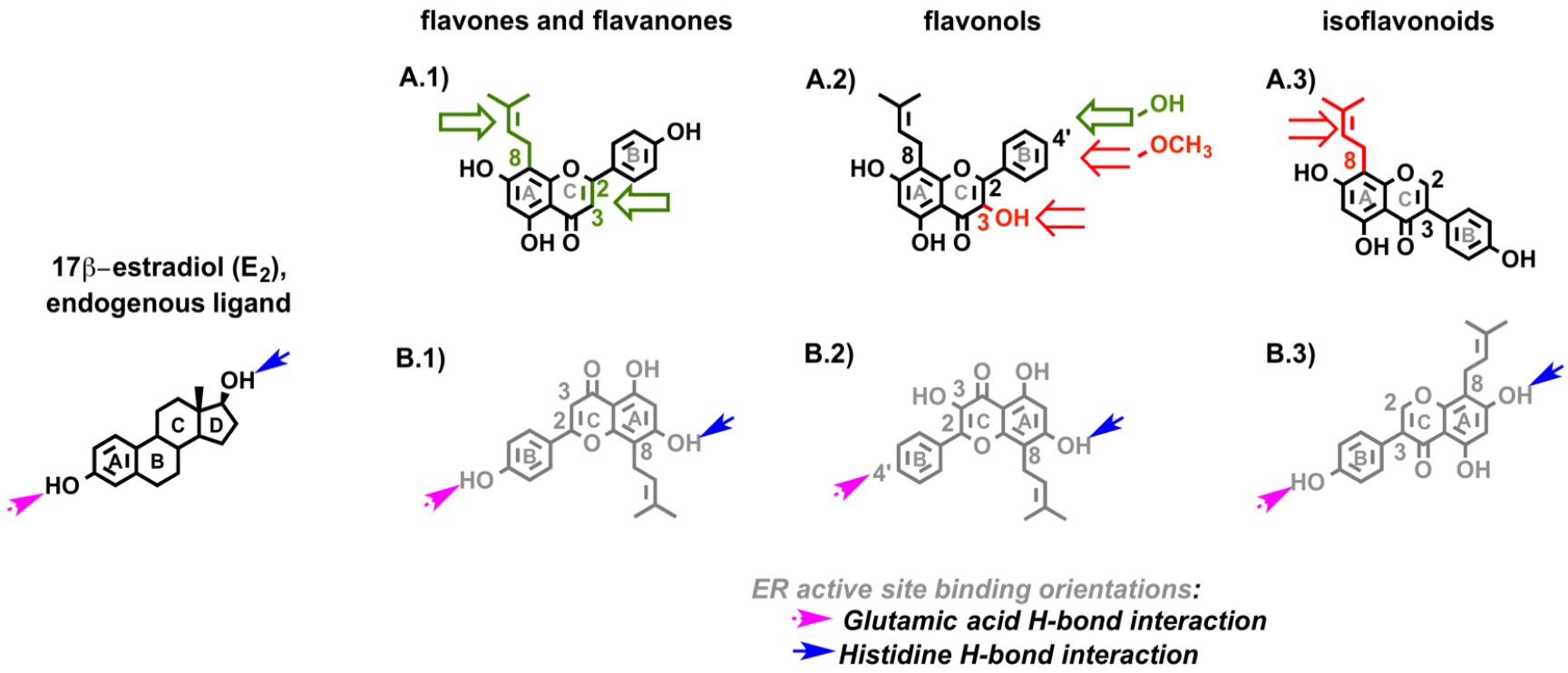


Figure 31. Putative flavonoid structures with ER binding orientations.

(A.1) Flavanone and flavone structure with A-ring prenylation at 8 position (C-8) and (un)saturation at C2-C3. (A.2) Flavonol structure with C-3 hydroxyl group and B-4' position for hydroxyl or methoxy group (A.3) Isoflavonoid structure with C-8 prenylation. The open green arrow represents an increased activity when the considered substitution is present on the core flavonoid structure, whereas the open red arrow indicated a decreased activity. (B.1) ER binding orientations of flavanones and flavones, (B.2) of flavonols, (B.3) of isoflavonoids in relation to E2. Blue full-tailed arrows represent moieties with hydrogen bond (H-bond) interactions with the histidine residue in the ER binding site; magenta dash-tailed arrows represent moieties with H-bond interactions with glutamic acid residues in ER binding site

After the ligand is bound to the ER, coregulators, for example SRC-2, are recruited to the ligand-ER complex (246). When complexed with the ER, the ligand structure also determines the resulting conformation of the bound ER, which in turn uniquely determines the respective strength of coactivator recruitment activity (246). Consequently, two different ligands with similar binding affinities can reflect different recruitment abilities (246), resulting in their different functional biological activities. Binding affinity of a compound and subsequent coactivator recruitment are closely associated to the resulting biological response of the cell; however, there are some exceptions to this correlation (246, 261). The SRC-2 coactivator has a moderately stronger affinity for ERα over ERβ (262). This likely explains the stronger TR-FRET assay readouts observed in our ERα data compared to ERβ (Table IV, Figure 27). In addition to the SRC-2; there are two other isoforms, SRC-1 and SRC-3. Studies report they have stronger affinities for ERα over ERβ (37, 262).

In general, the current SAR results from the binding assays mirror observations from the TR-FRET and functional estrogenic results in both ER subtypes (Table IV and V, Figure 25 - 29). Flavanone and flavone prenylation at C-8 showed increased ligand binding, elevated SRC-2 recruitment to the ligand-ER complex and higher functional activity compared to the unprenylated congeners, while prenylation on the same site in isoflavonoids decreased these estrogenic activities (Table IV and V). The computational studies provide a clearer explanation for the observed estrogenic effects of prenylation. A hydrophobic groove is formed in the binding pocket of ER when bound by prenylated flavonoids similar to those tested in this study (260). This hydrophobic

groove interacts with the prenyl group on the A-ring of flavonoids like 8-PN, as shown in the computational docking analysis (Figure 26A) (260). This groove likely envelops the prenyl group present on the 8-position of the A-ring of similar flavonoids such as 8-PA, stabilizing the compound in the pocket (Figure 26) (260). This may explain the greater affinity of the 8-prenylated flavonoids for the ER and subsequent SRC-2 recruitment, as well as higher overall functional activity compared to their unprenylated counterparts, Nrg and Api (Table IV and V, Figure 25, 27 and 28) (260). These observations are consistent with those reported in prior publications (263, 260, 264). In contrast, prenylation on C-6, as in 6-PN found in hops, leads to significantly lower functional estrogenic activity compared to that observed with 8-PN (152).

The C-3 hydroxylation on flavonols resulted in low estrogenic activity, (Table IV and V, Figure 24A, 25, 27, and 28). The prenylated Dmct had the highest overall estrogenic functional activity compared to the B-ring 4'O-methylated Ict and unprenylated Kfl (Table V, Figure 28C and D). Additionally, the functional potency of the unprenylated Kfl with a 4'-OH group was significantly stronger than the prenylated and 4'O-methylated Ict in ERβ (Figure 24A, Table V, Figure 28D). These results highlight the important role of the B-ring 4′-OH group in these flavonoid-receptor interactions as observed with Kfl and Ict (Table V, Figure 28D). This B-ring 4′-OH group mirrors the activity of the C3-hydroxyl group on the endogenous ligand, E_2, because both moieties form hydrogen bonds with the same polar amino acid residues in the ER active site during ligand binding (Figure 31) (1, 239). Interestingly, unprenylated Kfl showed the highest overall SRC-2 recruitment activity compared to the prenylated congeners Dmct and Ict, especially in ERα in the coactivator affinity studies (Table IV,

Figure 27). Additional comparative studies of cofactor recruitment, including the impact of binding upon ER conformational change, by Kfl and other similar structures may be beneficial to further explain these observations.

The isoflavonoid, 8-PG, and the flavone, 8-PA, are constitutional isomers. However, the B-ring in 8-PG is attached to the C-3-position instead of the 2-position, as it is the case with 8-PA (Figure 24A and B). As a result, the B-ring in 8-PG will be positioned differently from that of flavonoids, such as 8-PA, in the ligand binding site (Figure 26B). This can lead to differences in binding affinity and activity (Figure 25 - 29). Interestingly, for isoflavonoids, the functional effects of prenylation are mainly observed in differences in the efficacy in ERα assays, while in ERβ these effects are reflected in both potency and efficacy (Table V, Figure 29). The reduction in estrogenic efficacy and potency that was observed for 8-PG as compared to unprenylated Gen correlated with results from past studies. The origin of these effects was further clarified here using computational docking studies (Figure 26B) (264).

Api and Gen, which are constitutional isomers of each other (Figure 24), should also bind differentially to the ER subtypes resulting in varying estrogenic activity (Table V, Figure 28 and 29). While both compounds showed preferential ERβ activity, the isoflavone, Gen, was significantly more potent in ERβ (Figure 25 and 29). Similarly, in the ERβ assay, 8-PA also showed much stronger potency than the unprenylated congener Api, and comparable ERβ potency and ERβ-preferential activity to Gen (Table V, Figure 28 and 29) (127). In keeping with the SAR study, a comparative estrogenic analysis of 8-PN (racemic), 8-PN (R) isomer and 8-PN (S) isomer was conducted (Figure 32). Our results did not show any ERβ preferential activity by any of the 8-PN

isomers, and all three generally equipotent in ERα and ERβ, while 8-PN (racemic) showed the most efficacy in ERα (Figure 32).

Studies report that ERα tends to be more transcriptionally dominant than ERβ (265), and thus the potential for ERα dependent proliferation leading to an increased risk of mammary carcinogenesis is a significant concern. The results in this study showed that 8-PA and Gen have stronger binding affinity to ERβ than to ERα and higher ERβ-preferential functional activity over ERα (Table IV and V, Figure 25, 27 and 28). Therefore, these compounds may be of physiological benefit *in vivo* since sustained leverage towards ERβ cellular activity by these ERβ-preferential (iso)flavonoids could provide a counterweight to ERα pro-proliferative gene expression, thereby maintaining normal overall activity in mammary tissue. Further animal studies are warranted to substantiate this *in vitro* observation.

Our results indicate that the estrogenic activity of the tested extracts mimicked the potent/abundant bioactive (iso)flavonoids they contain. Studies suggest that 8-PN was shown to be mainly responsible for the estrogenic activity observed in hops (169, 152). Like 8-PN, the tested hops extract was equipotent in both ER subtypes in functional assays (Table V, Figure 30). The tested red clover extract contains the potent isoflavonoid Gen and its precursor biochanin A, present at 0.64% w/w and 15.2% w/w, respectively (154, 190). Studies report that these two compounds contribute considerably to the observed estrogenic activity of red clover compounds (154, 171). Although some ERβ-preferential binding activity has been reported for biochanin A in competitive ER ligand binding assays (171), much of its reported in cell ERβ-preferential estrogenic activity is likely due to its metabolism to Gen (123, 266-268). Similarly, in the

current results, the tested red clover extract showed ERβ-preferential activity over ERα (Table V, Figure 30). Likewise, 8-PA, present in an *inflata* licorice extract at 0.17 % w/w, was also an ERβ-preferential flavonoid (Table V, Figure 28) (127). In addition, the flavanone, liquiritigenin (LigF) present at 0.12 % w/w in this inflata licorice extract has also been demonstrated to have estrogenic activity (238, 127, 159). However, 8-PA exhibited greater overall estrogenicity and a stronger ERβ-potency than LigF with an ERα EC_{50} for 8-PA of 0.050 μM (Table V) and for LigF of 3.4 μM, as well as an ERβ EC_{50} of 8-PA 0.0035 μM (Table V) and for LigF of 0.037 μM for LigF (127, 238, 126). The *inflata* licorice extract, like 8-PA and LigF, displayed overall preferential activity for ERβ over ERα (Table V, Figure 30). Due to 8-PA's higher estrogenic activity, 8-PA most likely had a greater contribution to the estrogenic activity of inflata licorice than LigF.

During menopause, there is a considerable decrease in E_2 serum levels, which may lead to undesired symptoms (171, 269). In clinical studies, isoflavones from soy botanicals have been reported to alleviate menopausal symptoms without evidence of mammary tissue hyperproliferation, although these studies have limitations (270). Women that use soy, red clover, and *inflata* licorice for menopausal symptoms may benefit from their ERβ-protective properties against increased risk of mammary gland carcinogenesis provided by their respective bioactive phytoestrogens, Gen and 8-PA. The botanical extract obtained from *Epimedium* botanical had the lowest ERα and ERβ activity of all the extracts studied (Table V, Figure 30). *Epimedium* botanicals contain different glycosides of Ict (icariins) (271). To mimic the hydrolysis of the glycosides in the intestine *in vivo*, the extract from *Epimedium* botanical used in this study was

hydrolyzed prior to biological analysis yielding an extract enriched in aglycones including the weakly estrogenic Ict (Figure 28). While Dmct was only detected in traces in the hydrolyzed extract, Ict is metabolized by P450 enzymes to the estrogenic metabolite, Dmct, (Figure 24, Figure 28C and D) *in vivo* (272). It is likely that in cells similar to biochanin A and Gen, Ict is also in part metabolized to the more estrogenic Dmct. The estrogenic results with Epimedium botanical may correlate with the effects observed with its weakly estrogenic major flavonol, Ict, and its metabolite, Dmct. Our findings suggest that the estrogenic effects of the tested extracts could correlate with the order of activity of their corresponding bioactive (iso)flavonoids; in ERα, 8-PN / hops > 8-PA / inflata licorice ≈ Gen / red clover > Dmct / Epimedium botanical > Ict (Table V), in ERα assays. Likewise, in ERβ studies, the observed activity ranking was Gen / red clover ≥ 8-PA / *inflata* licorice ≈ 8-PN / spent hops >> Dmct / Epimedium botanical >> Ict / Epimedium botanical (Table V).

The analyzed phytoestrogens can activate both ER receptors (ERα and ERβ), therefore, inducing various cellular pathways (e.g., ERα relief of hot flashes, proliferation; ERβ: influence on memory, antiproliferation) and leading to an overall "polypharmacological" effect (273, 274). Other constituents in the extract can also influence the specific phytoestrogen's activity. In line with this, the concentration of the selected bioactive (iso)flavonoid in the extract, timing of supplementation (275), *in vivo* pharmacokinetics, estrogenic potency, and influence by the activity of other phytoconstituents in the extract, could influence how much a specific bioactive flavonoid modulates the activity of botanical extracts (276). For example, studies report that hops neither increased or decreased uterine tissue weight, while its bioactive flavonoid 8-PN,

in dietary concentrations, increased uterine weight in rats (277), likely indicating ERα activity (243).

As previously mentioned, the *Epimedium* botanical was hydrolyzed prior to biological analysis. The functional estrogenic activity of this extract was conducted in comparison to its unhydrolyzed form. The data from this study showed that hydrolysis of our tested epimedium botanical extract increased its overall functional estrogenic activity in both ER subtypes compared to its unhydrolyzed form (Figure 34A and B). Similarly, the flavonol aglycone Ict showed significantly greater estrogenic activity in both ER subtypes compared to its glycosylated congener icariin (Figure 34C and D).

Overall, it needs to be mentioned that there may be other molecular pathways, other than ERβ activation, that may be induced by the tested compounds and parent extracts that can confound any biological benefits and breast cancer risk reduction in women.

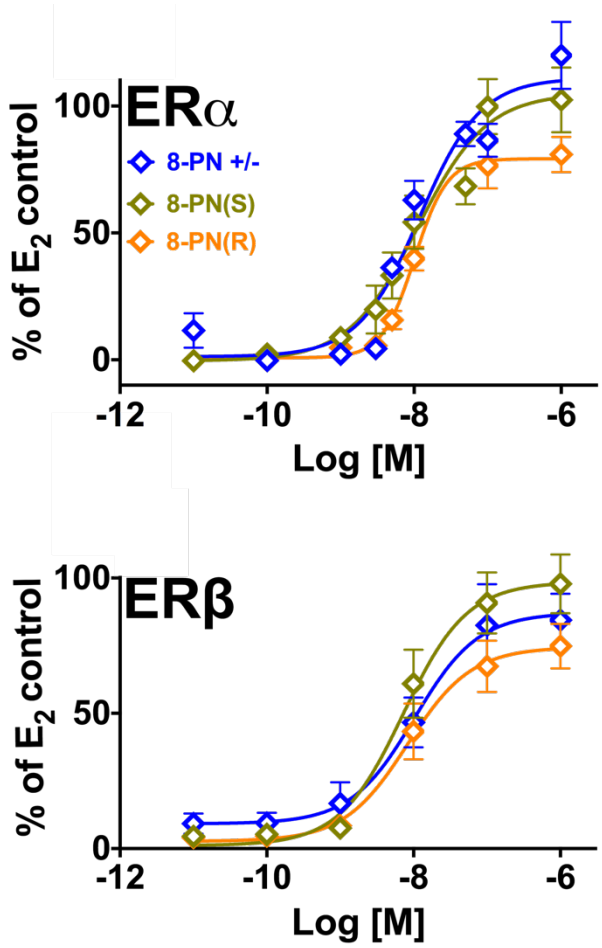


Figure 32. Comparative Functional Estrogenic Activity of 8-PN and Isomers R and S. 8-PN, 8-PN isomers R and S induction of estrogenic effects *in vitro*. 8-PN, R isomer, and S isomer, were generally equipotent in both ER subtypes. A) Estrogen-dependent induction of alkaline phosphatase activity in Ishikawa cells. B) Estrogen-dependent induction of ERE-luciferase activity in MDA-MB-231/β41 cells. Data represents average ± SEM from three independent determinations.

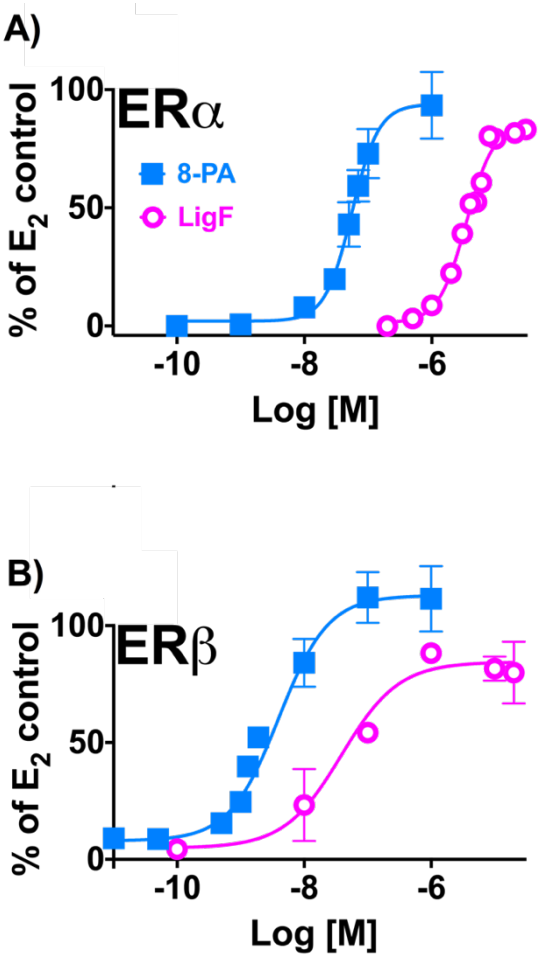


Figure 33. Comparative Functional Estrogenic Activity of 8-PA and LigF. Induction of estrogenic effects *in vitro* by 8-PA and LigF. 8-PA demonstrated the most overall estrogenic potency followed by LigF. A) Estrogen-dependent induction of alkaline phosphatase activity in Ishikawa cells. B) Estrogen-dependent induction of ERE-luciferase activity in MDA-MB-231/β41 cells. Data represents average ± SEM from three independent determinations.

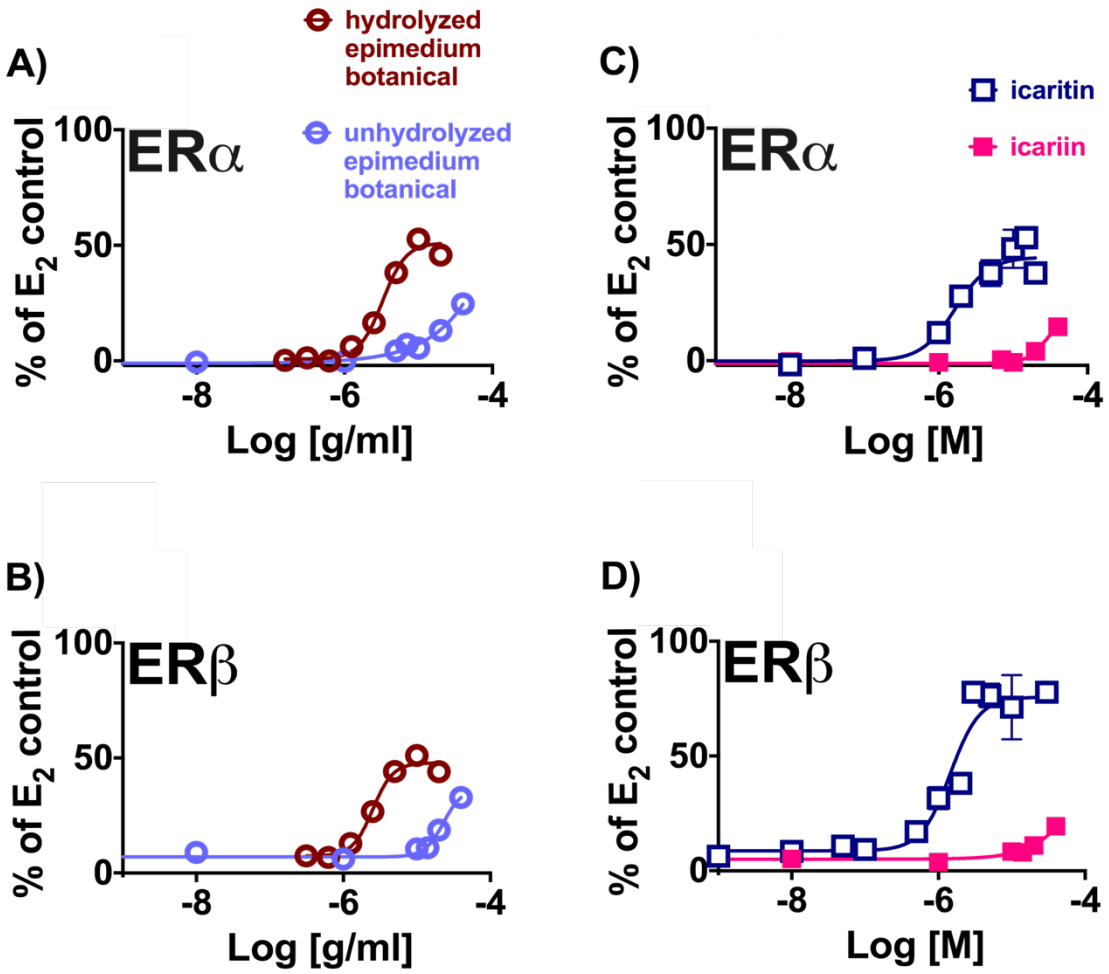


Figure 34. Comparative Functional Estrogenic Activity of Hydrolyzed and Unhydrolyzed Epimedium Botanical Extracts, Icaritin and Icariin. Induction of estrogenic effects *in vitro* by flavonols and epimedium botanicals. The hydrolyzed epimedium botanical extract was more estrogenic than the unhydrolyzed epimedium extract in both ER subtypes; the aglycone icaritin showed more overall estrogenic activity than the glycoside, icariin. A) Estrogen-dependent induction of alkaline phosphatase activity in Ishikawa cells. B) Estrogen-dependent induction of ERE-luciferase activity in MDA-MB-231/β41 cells. Data represents average ± SEM from three independent determinations.

In conclusion, we have shown that 8-PA and Gen possess ERβ-preferential activity. Both compounds can contribute to the properties of *inflata* licorice and red clover, respectively, to regulate ERα pathways by preferentially stimulating ERβ activity. This preferential activation may increase the safety of these dietary supplements when consumed by women for health purposes. The ERα activities of these extracts may offer favorable health benefits including assistance in management of postmenopausal symptoms. *In vivo* studies on estrogenic effects of Gen and red clover preparations have already been reported; however, *in vivo* estrogenic studies evaluating the effects of 8-PA and inflata licorice are warranted. From the described SAR observations, we may be able to predict the potential estrogenic activity and related health benefits of similar iso(flavonoids) and of botanicals containing these types of (iso)flavonoid phytoestrogens, in the absence of any known or suspected genotoxicity.

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6. CONCLUSIONS AND FUTURE DIRECTIONS

Botanicals have been used throughout human history by various cultures to manage or treat various disorders and maintain health (234, 278, 279). Lately, the increased use of botanicals for health purposes has been driven, in part, by the desire to prevent illness and the belief that they are safer alternatives to conventional medication (280, 132, 234). Additionally, there has been growing skepticism about hormone therapy for postmenopausal women due to the reported increased risk of breast cancer associated with estrogen and medroxyprogesterone hormone treatment. Continued study on the pharmacological activity of these natural products and bioactive compounds is still needed to provide evidence for their efficacy, ensure their safety, and understand their pharmacodynamics (280). Our focus on the safety on the safety of these botanicals led us to seek to identify and study ERβ preferential (iso)flavonoids present in botanical extracts in keeping with the hypothesis that enhanced ERβ activity is correlated with antiproliferative effects. In order to conduct this study, estrogenic analysis of (iso)flavonoids and select parent extracts was conducted to determine their comparative ERα and ERβ activity.

This research project addressed three aims. The first aim was to optimize an *in vitro* cell model to assess ER subtype activities of the respective (iso)flavonoids and botanicals tested. Results from the optimized functional assays used in this study have been verified as able to reflect the ER subtype functional activity of the tested (iso)flavonoids in *in vitro* mammalian cell models. The results obtained using the ERα and ERβ selective ligands, PPT and DPN, respectively, generally correlate with other studies reported in literature as previously stated. Using the optimized assay method,

PPT behaved as a full agonist in ERα but showed no estrogenic activity on ERβ, while DPN showed ERβ-preferential activity. Using this valuable assay, we verified that the observations for the tested samples (EC_{50} and efficacy) truly reflect ER modulating activity. Confirmation was also evident in the lower estrogenic readouts obtained in the optimized method ER assay with inhibition of ER with ICI (fulvestrant), or absence of ERβ in the *in vitro* cell models. It should be noted that an optimal *in vitro* cell model for this assay would have employed stably transfected epithelial mammary cells expressing either ERα or ERβ, or expressing both ERα and ERβ, and would have used the same assay readout for analysis. Here we used ERα (+) uterine endometrial cells instead of ERβ (+) mammary epithelial cells and an alkaline phosphatase assay instead of ERE-luciferase assay. However, the estrogenic values obtained in this aim were in general agreement with those reported in literature.

In the second aim, the optimized assays from the first aim (Aim 1) were used to determine the comparative estrogenicity of three medicinal licorice species, *G. inflata*, *G. glabra*, and *G. uralensis* and to identifiy ERβ preferential ligand(s) present in licorice. Our findings showed that *G. inflata* was the most ERβ potent licorice species compared to the latter two species. With the goal of finding the ERβ compound(s) in *G. inflata* that may be responsible for this observation, we used bioassay guided fractionation with *G. inflata* to isolate and elucidate the structures of bioactive constituents in *G. inflata.* As a selected fraction (fraction 8) showed considerable estrogenic activity, compounds were isolated from this compound. Among the isolated compounds, LicA had the highest concentration in fraction 8 at 64% w/w, but showed no estrogenic activity. However, 8-PA which was present at a lower concentration in the same fraction at 5.1% w/w, had

the highest overall estrogenicity compared to the other compounds. Importantly, 8-PA showed ERβ-preferential activity and a comparable ERβ EC_{50} value to genistein, the known ERβ preferential phytoestrogen (3.5 nM vs 2.2 nM, respectively). The estrogenic activity for the flavonoid in *G. inflata*, LigF, was also conducted in this study (Figure 33). Although our findings show that LigF was ERβ-preferential (Figure 34), which correlates with past findings (126), 8-PA is the most potent ERβ ligand identified in the extract; our ERβ EC_{50} value for 8-PA was 3.5 nM, and one for LigF was 38 nM (Figure 33). The bioassay guided fractionation performed with *G. inflata* yielded other estrogenic fractions. Besides the selected fraction 8 used to identify 8-PA, a number of them also showed ERβ/ERα activity. For example, the tested *G. inflata* fractions 2 – 6 had higher ERβ activity than ERα activity (Figure 17). Further analysis of these fractions may possibly lead identification of other potent ERβ-preferential agonists. However, 8-PA identified as a potent ERβ-preferential agonist in licorice, was most concentrated in the tested *G. inflata* extract at 0.168% w/w (Table III).

In the third aim, structurally similar flavans, flavones, flavonols, and isoflavonoids from various botanical sources were used in a structure activity relationship (SAR) study using the optimized assays from the first aim (Aim 1). The objective of this aim was to identify structures that favor ERβ-preferential activity. The binding affinity, transcription coactivator recruitment affinity (SRC-2), and functional estrogenic activity of select compounds were analyzed. According to the findings, C-8 prenylation on the A-ring in flavonoids increases overall estrogenic activity (Table IV and V, Figure 24 and 28). The *in silico* data showed the importance of the positioned prenyl group as it interacts with a hydrophobic pocket in the ER, improving binding. This C-8 prenylation on the A-ring,

with C2 - C3 unsaturation on the C-ring significantly increases ERβ functional potency, and increases ERβ preferential activity as observed in 8-PA compared to 8-PN (Table V, Figure 24, 28A and B, 35A). However, C2 - C3 saturation at C-ring results in loss of this ERβ-preferential functional activity as observed in 8-PN. In contrast, for isoflavonoids, C-8 prenylation on the A-ring, and C2 - C3 unsaturation on the C-ring significantly reduced the overall functional estrogenic activity (8-PG). Absence of this prenylation on isoflavonoids, with C2 - C3 unsaturation on the C-ring, resulted in increased ERβ-preferential functional activity over ERα as observed in Gen (Figure 24, 29 and 35B).

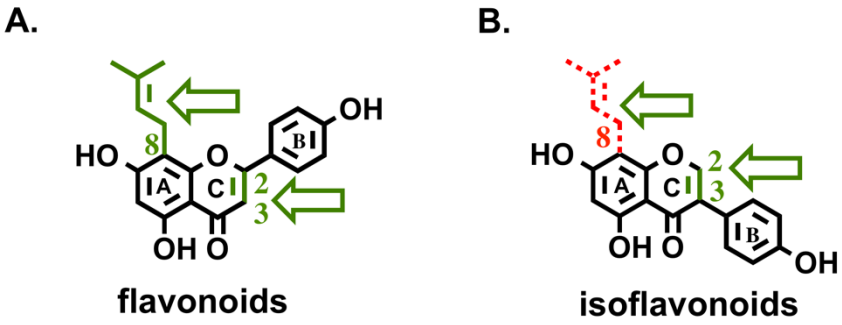

Figure 35. (Iso)flavonoid Substituents Enhancing ERβ potency. Presence of green open arrows indicate structures contributing to ERβ potency. Red dashed structures on isoflavonoids convey absence of indicated structure.

Additionally, the C3 hydroxylation on the C-ring of the tested prenylated flavonols reduced overall functional estrogenic activity compared to the prenylated flavone 8-PA, as it is observed in Dmct and Ict (Figure 24 and 28). The presence of the methoxy group at the 4' position on the prenylated icaritin also resulted in significantly lower ERβ functional activity compared to the unprenylated kaempferol with a 4' hydroxyl group (Figure 24 and 28). This highlights the importance of the 4' positioned hydroxyl group in the tested flavonols as vital for robust estrogenic binding interactions and functional activity. In this aim, the SAR study was limited and it would be interesting to conduct a more comprehensive study with more structurally diverse flavonoids and different compound classes. Additionally, ER subtype specific gene and protein expression assays of the identified ERβ-preferential agonists would be needed to better understand the biological endpoints of the select compounds.

The functional estrogenic activity of hops, red clover, *inflata* licorice, and epimedium, were assessed. Their trend of estrogenic activity seems to mimic the activity of the most prominent bioactive compounds they contain; 8-PN, genistein, 8-PA, and icaritin, respectively. It is suggested in this study that these potent bioactive compounds contribute to the estrogenic behavior of their parent extracts. From the described SAR observations, we may be able to predict the potential estrogenic activity and related health benefits of other botanicals containing these types of (iso)flavonoid phytoestrogens.

Different *in vivo* studies on estrogenic effects of Gen and red clover preparations have already been reported. For the future, *in vivo* estrogenic studies evaluating the chemoprotective and estrogenic properties of 8-PA and *G. inflata* are still needed to

provide a better picture of the potential biological benefits of ingesting these botanical dietary supplements. Such studies are feasible since they can employ rodent models to study ERβ-specific genes, (anti)proliferative biomarkers, and relevant phenotypes such as terminal end buds (TEB) in mammary gland tissue. An example of the *in vivo* models may include use of adult ovariectomized rats, in an attempt to represent the low serum E_2 status that occurs in postmenopausal women (269).

The development of 8-PA-enriched designer extracts of *G. inflata* for *in vivo* and clinical studies may be advantageous for a better understanding of the mentioned safety and health benefits in postmenopausal women. As previously mentioned, the concentration of 8-PA in our tested *G. inflata* extract was only 0.168 % (127). Although the concentration may vary from batch to batch, enrichment of the botanical extracts may ensure that 8-PA activity through ERβ as well as ERα at tissue target site.

Women that consume various botanicals may be able to benefit from a reduction in risk of breast cancers when they consume botanical extracts that contain the ERβ-preferential (iso)flavonoids. The health benefit of some of these botanicals may also include management of menopausal symptoms, due to the retained ERα activities of some of these natural products in the absence of genotoxic properties. This needs to be shown in further *in vivo* and clinical studies. However, the ERβ-preferential (iso)flavonoids present in these products used by menopausal women may contribute a chemoprotective and safer profile by attenuating the tissue proliferation may result from ERα activation. We may also be able to predict in the future the potential estrogenic activity and related health benefits of other (iso)flavonoids and women's health botanicals containing these types of ERβ-preferential phytoestrogens.

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APPENDICES

Appendices 1

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Appendices 1 (continued)

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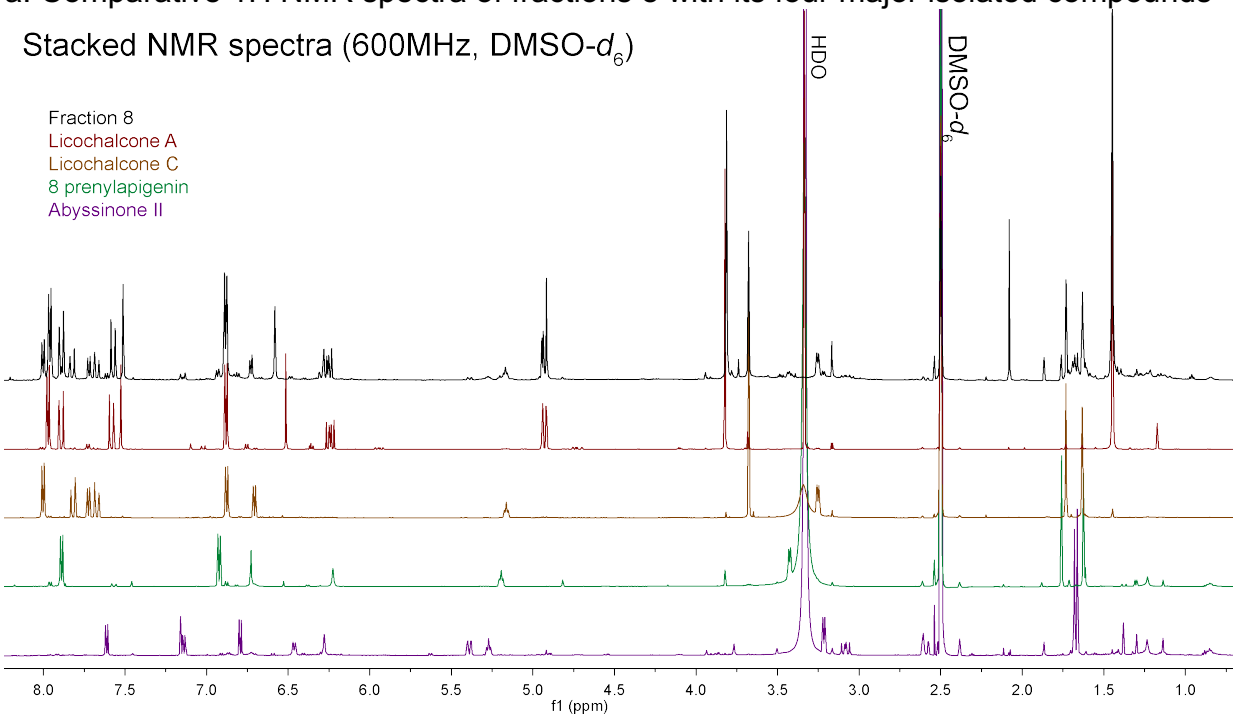
Appendices 2

Note: The raw and annotated NMR data, as well as the ^{1}H iterative full spin analysis (HiFSA) of 8-prenylapigenin, 4'O-methylbavachalcone and its isomer, abyssinone II, and licochalcone C are made freely available at Harvard Dataverse doi:10.7910/DVN/JZOL2U

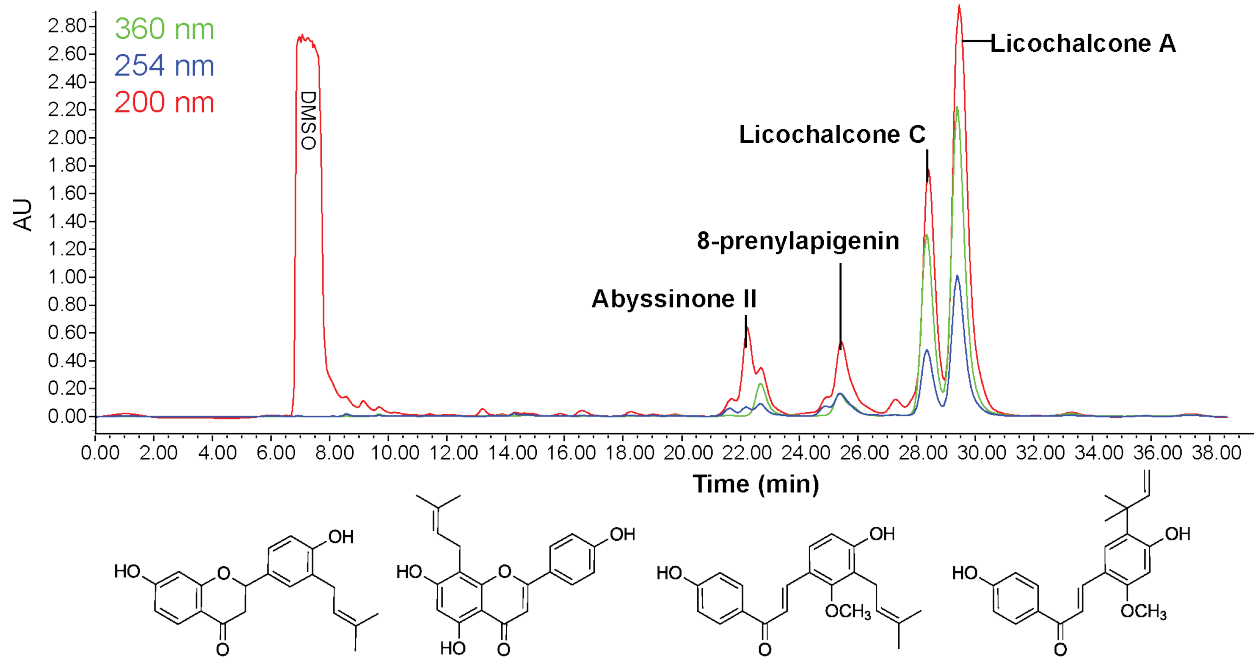
S1. ^{1}H NMR and chromatographic analyses of fraction 8 (2.1% w/w crude GI extract)

a. Comparative 1H NMR spectra of fractions 8 with its four major isolated compounds



Appendices 2 (continued)

b. Semi-preparative chromatogram of fraction 8 after NMR analysis



	Abyssinone II	8-prenylapigenin	Licochalcone C	Licochalcone A
% w/w fr.8	4.4	5.1	25.5	64.8

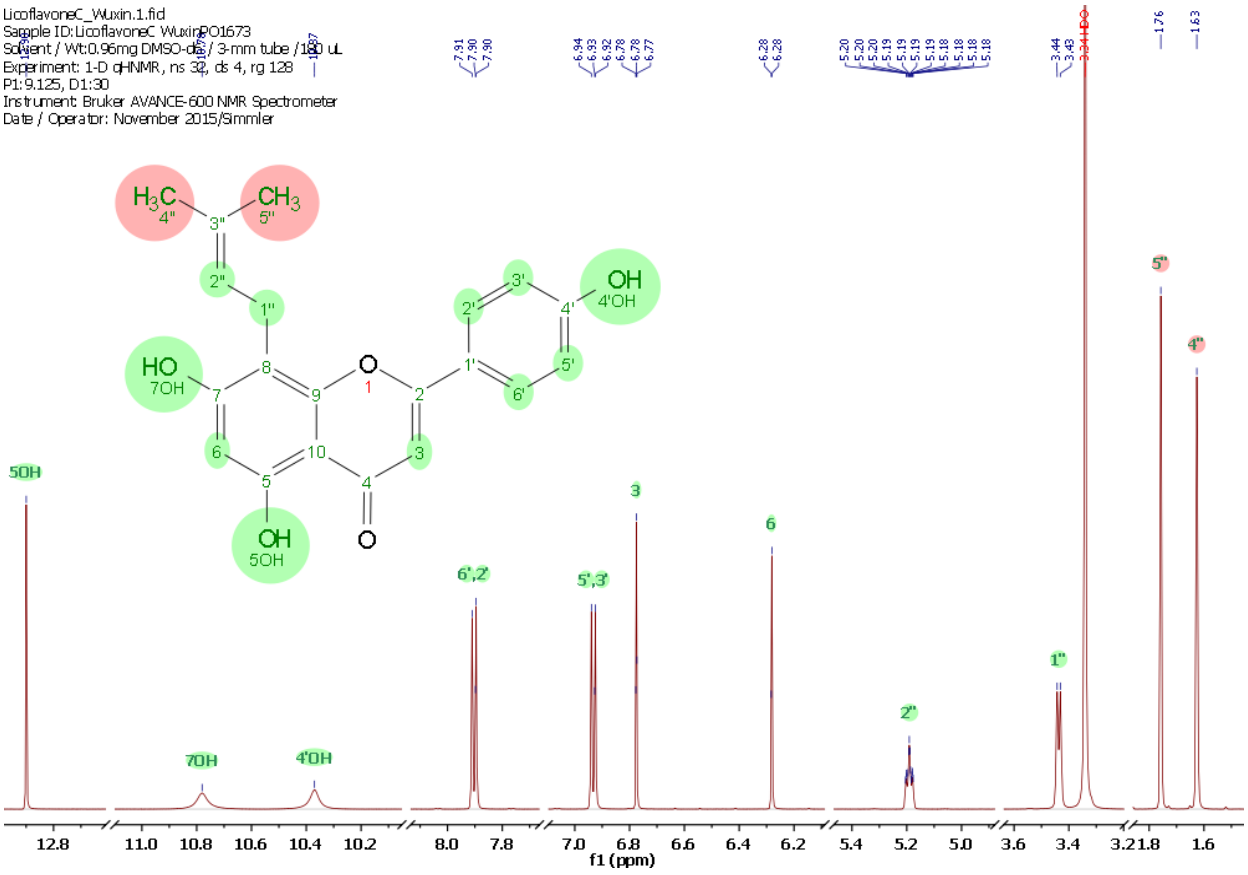
Column: YMC- Pack ODS AQ (1250 x10 mm ID, S-10 µm, 12 mm AQ12S11-251 OWT)
Gradient: (B: Acetonitrile, A: Water) 58% B for 40 minutes in isocratic mode. Flow rate at 1.8 ml/min

Both the semi-preparative LC-UV chromatogram and the ^{1}H NMR spectrum indicate that licochalcone A and licochalcone C are the most abundant compounds in fraction 8, whereas 8-prenylapigenin (8-PA) is a minor metabolite. Knowing the fraction yield (7.13 mg = 2.1% w/w extract), the proportion of 8-PA in fraction 8 has been estimated at 5.1%w/w fraction, and thus the proportion of 8 PA in the crude extract is estimated at~ 0.11% w/w.

Appendices 2 (continued)

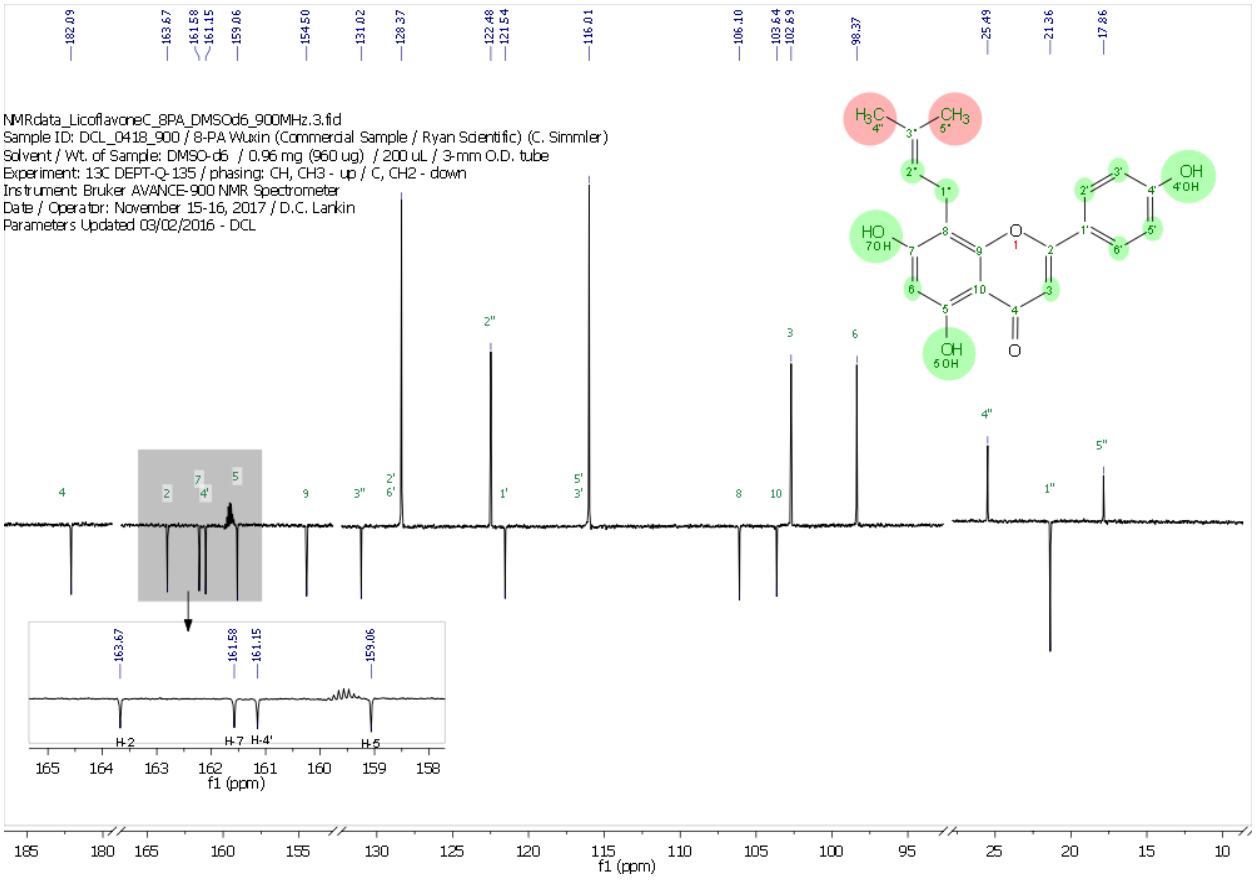
S2. Annotated $^{1}H/^{13}C$ NMR and MS/MS spectra of 8-PA (DMSO-d_6, 600 and 145MHz)

a. Annotated ^{1}H NMR spectrum of verified 8-PA



Appendices 2 (continued)

b. Annotated ^{13}C NMR spectrum of verified 8-PA



Appendices 2 (continued)

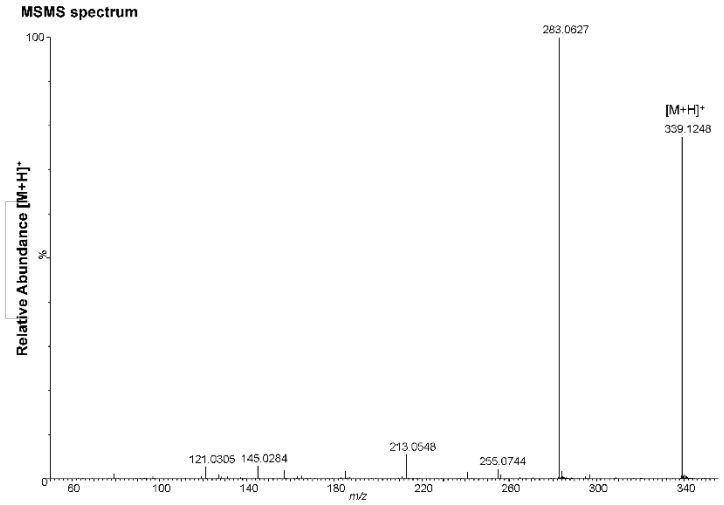
c. ^{1}H and ^{13}C NMR data of 8-prenylapigenin

		8-prenylapigenin (Licoflavone C)	
Position	**mult.**	**δ_H (*J* in Hz)**	**δ_C**
C=O			181.74
2			163.73
3	1H	6.78, *s*	102.72
5 (OH)		12.85, *s*	159.10
6		6.28, *s*	98.38
7			161.64
8			106.10
9			154.49
10			103.66
1’	1H		121.54
2’/6’	2H	7.89 *AA’ type* ($J_{H2'/6'-H3'/5'}$:8.63/0.24, $J_{H2'/6'-H2'/6'}$:2.88)*	127.61
3’/5’	2H	*6.92 XX’ type* ($J_{H2'/6'-H3'/5'}$:8.63/0.24, $J_{H3'/5'-H3'/5'}$:2.88)*	115.17
4’	1H		161.15
1”	2H	*3.42 d* ($J_{H2"-1"}$:7.039, $J_{H1"-4"}$:0.96, $J_{H1"-5"}$:0.73)*	22.06
2”	1H	5.21 *ddqq* type ($J_{H2"-1"}$:7.04, $J_{H2"-4"}$: -1.57, $J_{H2"-5"}$: -1.37)*	122.56
3’’			131.03
4” CH3 5” CH3	3H 3H	1.62 *brs* ($J_{H1"-4"}$:0.96, $J_{H2"-4"}$: -1.57)* 1.75 *brs* ($J_{H1"-5"}$:0.74, $J_{H2"-5"}$: -1.37)*	25.47 17.85
7’-OH		10.78 *brs*	
4’-OH		10.37 *brs*	

*Calculated values obtained through full spin analysis using Perch NMR software, in order to obtain the exact coupling constants.

Appendices 2 (continued)

d. MS/MS spectrum of verified 8-prenylapigenin



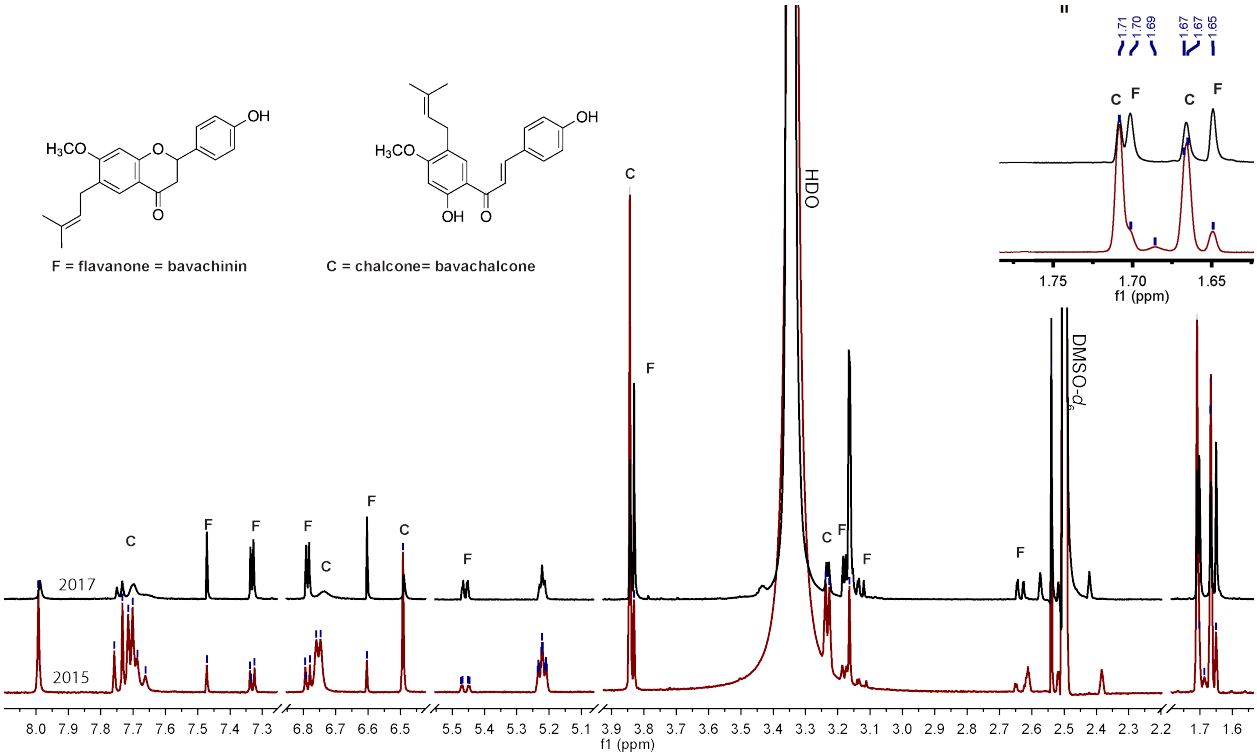
MS/MS spectrum of verified 8-prenylapigenin. The MS/MS spectrum was taken with a CE ramp between 6-50 eV in positive ionization mode.

Synonym: 8-prenylapigenin, licoflavone C
CAS Registry Number: 72357-31-4 Molecular Formula: C20H18O5
Pubchem CID: 10246505 Chemspider ID: 8421992

Appendices 2 (continued)

S3. Annotated ^{1}H/ ^{13}C NMR and MS/MS spectra of 4'-O-methylbavachalcone claimed to be 8-PA

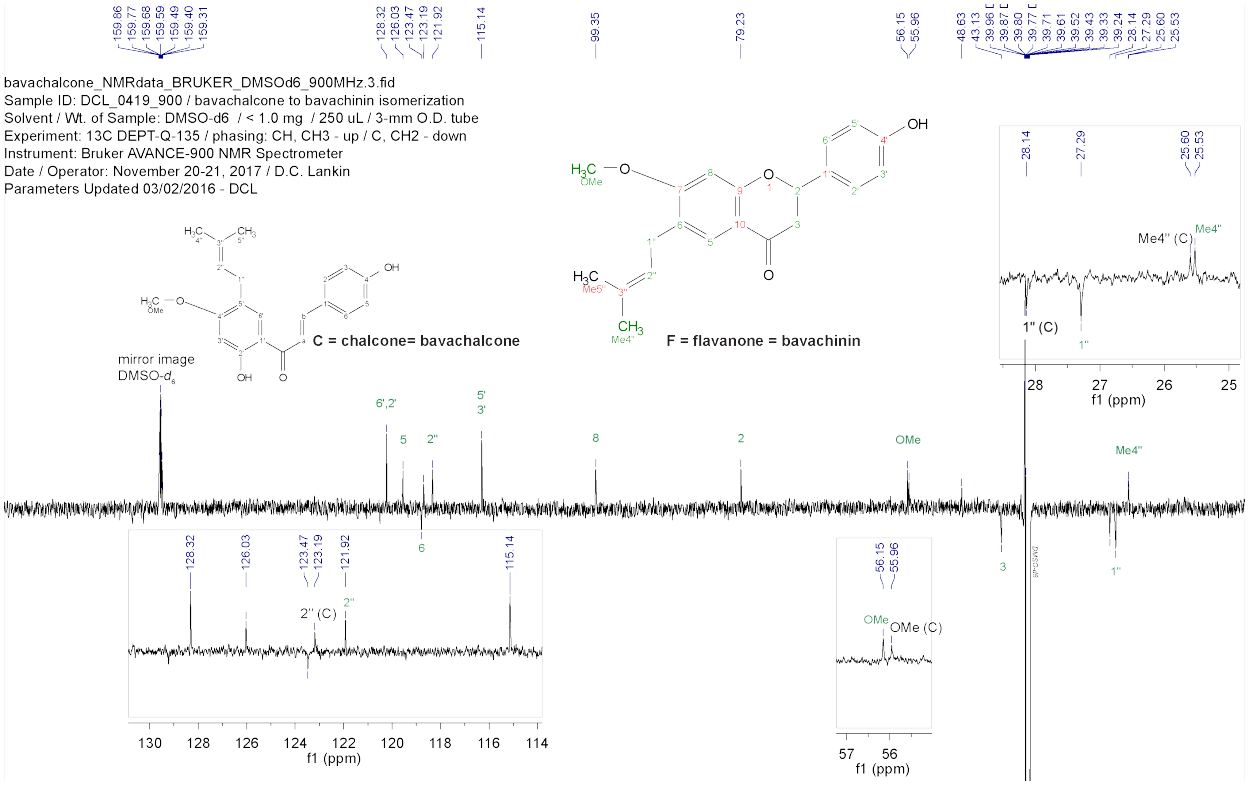
a. ^{1}H NMR spectra of sample acquired at 2-year interval (DMSO-d_6, 600-900MHz)



The results presented here clearly demonstrated the isomerization of the chalcone into its flavanone isomer, in the NMR tube. The ^{13}C data was acquired on the 2 year-sample and thus reflects only the ^{13}C resonances of the flavanone isomer.

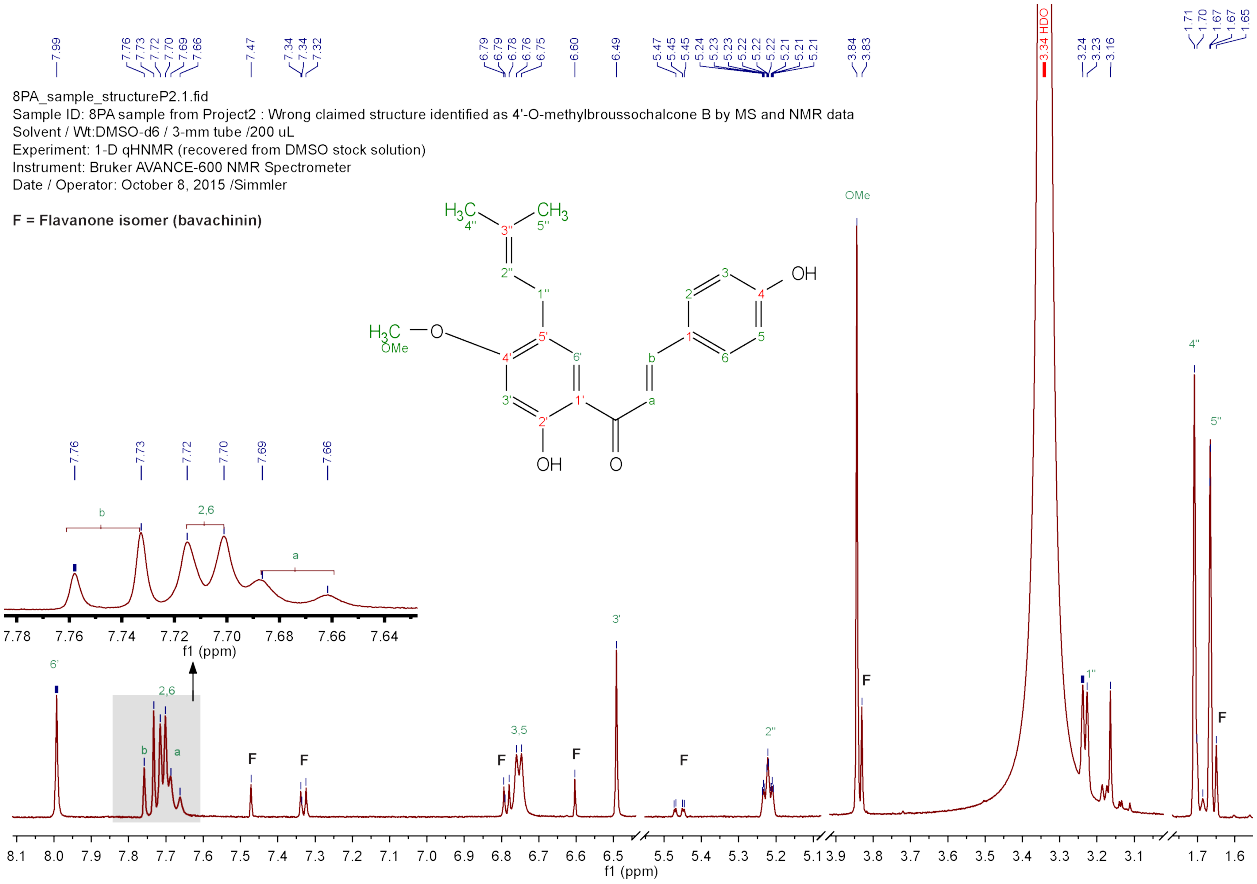
Appendices 2 (continued)

b. Annotated ^{13}C NMR spectrum of 2-year sample containing mainly bavachinin (syn. Bavachinin A, 7-O-methylbavachinin)



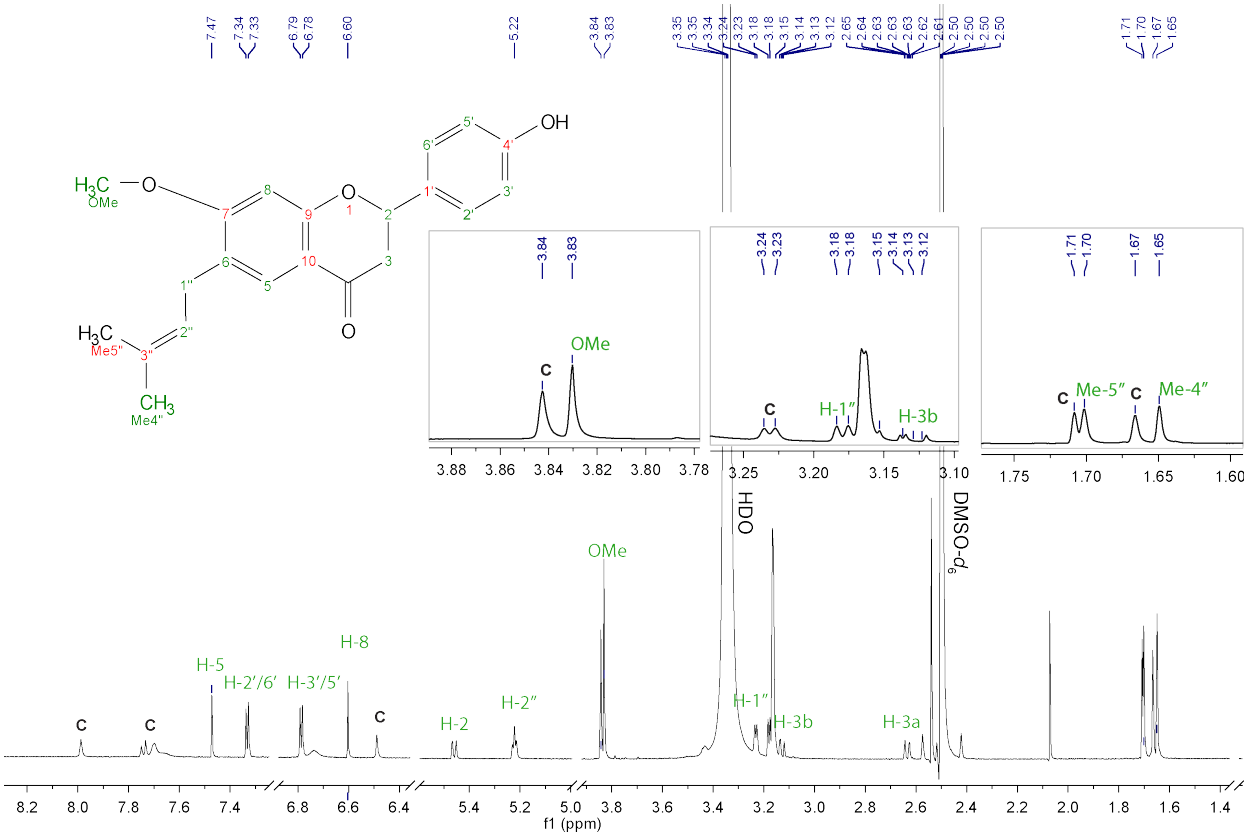
Appendices 2 (continued)

c. Annotated ^{1}H NMR spectrum of 4'O-methylbavachalcone (DMSO-d_6, 600 MHz)



Appendices 2 (continued)

d. Annotated ^{1}H NMR spectrum of bavachinin (DMSO-d_6, 900 MHz)



Appendices 2 (continued)

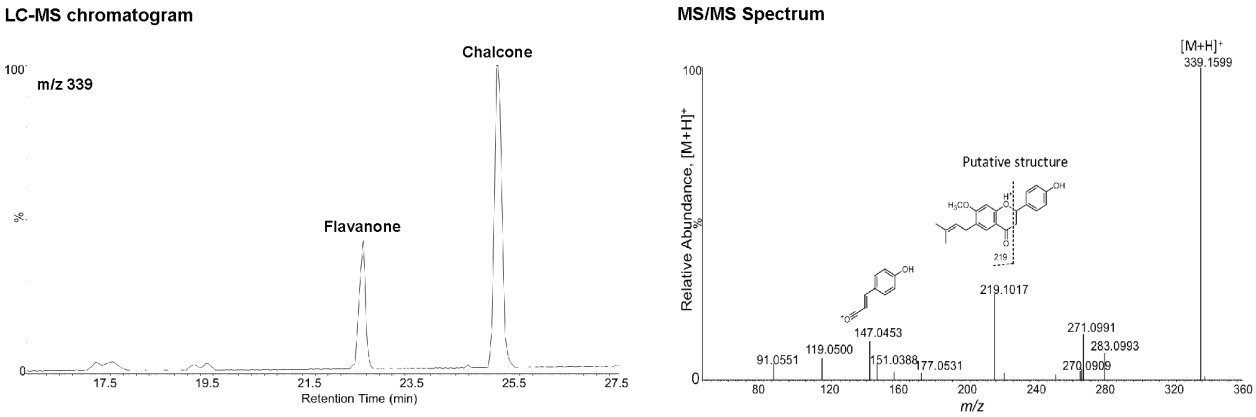
e. ^{1}H and ^{13}C NMR data of 4'O-methylbavachalcone and its isomer (DMSO-d_6, 600 MH, 145 MHz)

		4'*O*-methylbavachalcone		**Bavachinin (flavanone isomer)**	
Pos.	**mult.**	**δ_H (*J* in Hz)**	**δ_C[a]**	**δ_H (*J* in Hz)**	**δ_C[d]**
C=O			191.90		nd
2		*Not present*		5.45 *dd* (J_{3a-2}:13.13; J_{3b-2}:2.84)*	79.19
3b 3a	1H 1H			3.14, *dd* (J_{3a-3b} :-16.95; J_{3b-2}:13.13)* 2.63, *dd* (J_{3a-3b} :-16.95; J_{3a-2}:2.84)*	43.13
3' (8)	1H	6.49, *s* ($J_{H3'-6'}$:0.92)	99.14	6.61, *s* (J_{8-5} :0.006)	99.14
4 (4')			nd		nd
5' (6)			121.01		123.47
6' (5)		7.99, *s* ($J_{H3'-6'}$:0.92)	130.04	7.48, *s* (J_{H5-8} :0.006)	125.95
α	1H	7.67, *d* ($J_{H\alpha-\beta}$:15.13)		*Not present*	
β	1H	7.74, *d* ($J_{H\alpha-\beta}$:15.13)		*Not present*	
1' (10)	1H		113.52		114.17
2' (9)			164.56		162.29
2/6 (2'/6')	2H	7.71 *AA'*($J_{H2/6-H3/5}$:8.29/0.80, $J_{H2/6-H2/6}$:1.69)*	131.16	7.34 *AA'* ($J_{H2'/6'-H3'/5'}$:8.15/0.62, $J_{H2'/6'-H2'/6'}$:1.71)*	128.29
3/5 (3'/5')	2H	6.75 *XX* ($J_{H2/6-H3/5}$:8.63/0.24, $J_{H3/5-H3/5}$:1.87)*	nd	*6.79 XX'*($J_{H2'/6'-H3'/5'}$:8.15/0.62, $J_{H3'/5'-H3'/5'}$:1.71)*	114.95
4' (7)	1H		163.42		164.13
O-CH3	3H	3.84, *s*	56.03	3.83, *s*	56.03
1"	2H	3.23, *d* ($J_{H2"-1"}$:7.64, $J_{H1"-4"}$:-0.80, $J_{H1"-5"}$:0.64)*	28.56	3.18, *d* ($J_{H2"-1"}$:7.45, $J_{H1"-4"}$:-0.34, $J_{H1"-5"}$:0.79)*	27.29
2"	1H	5.22, *ddqq* ($J_{H2"-1"}$:7.64, $J_{H2"-4"}$:-1.57, $J_{H2"-5"}$: -1.34)*	123.19	5.22, *ddqq* ($J_{H2"-1"}$:7.45, $J_{H2"-4"}$: -1.39, $J_{H2"-5"}$: -1.24)*	121.92
3''			131.13		132.0
4" CH3 5"CH3	3H 3H	1.67, *brs* ($J_{H1"-4"}$:-0.80, $J_{H2"-4"}$: -1.57)* 1.71, *brs* ($J_{H1"-5"}$:0.64, $J_{H2"-5"}$: -1.37)*	26.01 17.25	1.65, *brs* ($J_{H1"-4"}$: -0.34, $J_{H2"-4"}$: -1.39)* 1.70, *brs* ($J_{H1"-5"}$:0.79, $J_{H2"-5"}$: -1.23)*	26.08 17.86

The numbers between brackets are related to bavachinin *Calculated values obtained through full spin analysis using Perch NMR software, in order to obtain the exact coupling constants. nd=- not detected [d] determined from HMBC/HSQC data and ^{13}C data for bavachinin.

Appendices 2 (continued)

f. LC-MS chromatogram of the sample received and MS/MS spectrum of 4'-O-methylbavachalcone



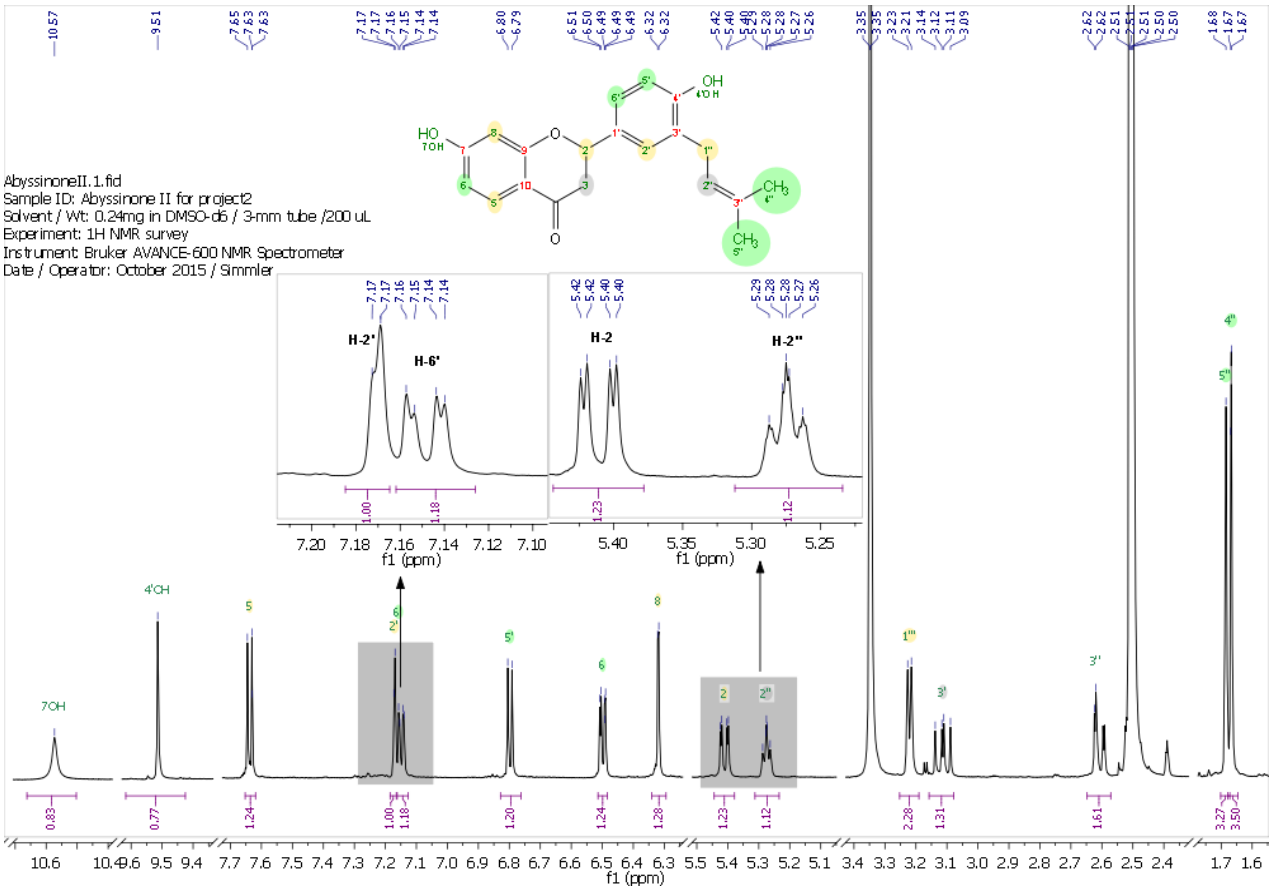
LC-MS Chromatogram (left side) and MS/MS spectrum (right side) obtained for the sample claimed to be 8-prenylapigenin. The MS/MS spectrum was taken with a CE ramp between 6-50 eV in positive ionization mode. The extracted ion chromatogram (*m/z* 339) dysplayed two peaks with the exact same MS /MS spectra, thereby suggesting the presence of two isomeric forms

Pubchem CID: 5321765 **Chemspider ID:** 4479431
CAS Registry Number: 20784-60-5 **Molecular Formula**: $C_{21}H_{22}O$

Appendices 2 (continued)

S4. Annotated ^{1}H NMR spectrum of Abyssinone II (DMSO-d_6, 600 MHz)

a. Annotated ^{1}H NMR spectra



Appendices 2 (continued)

b. ^{1}H and ^{13}C NMR data (DMSO-d_6, 600MHz)

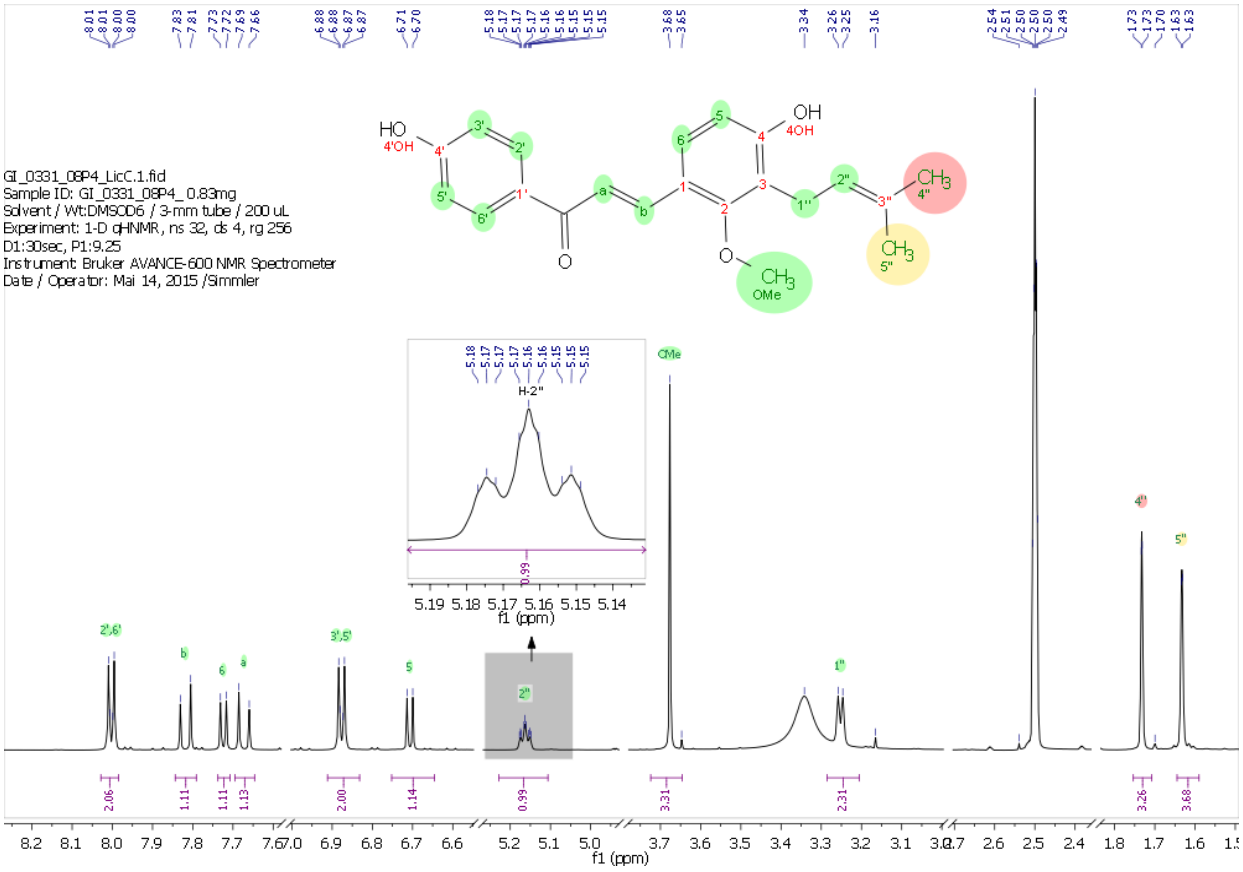
Position	mult.	δ_H (*J* in Hz) in ppm	δ_C in ppm
C=O			191.97
2	1H	5.41 *dd* ($J_{H2\text{-}3b}$:12.78, $J_{H2\text{-}3a}$:2.92)*	79.31
3a 3b	1H 1H	2.62 *dd* ($J_{H3a\text{-}3b}$:-16.76, $J_{H2\text{-}3a}$:2.92)* 3.11 *dd* ($J_{H3a\text{-}3b}$:-16.76, $J_{H2\text{-}3b}$:12.78)*	43.56
5	1H	7.64,*d* ($J_{H6\text{-}5}$: 8.22, $J_{H5\text{-}8}$:0.55)*	128.79
6	1H	6.50, *dd* ($J_{H6\text{-}5}$:8.22, $J_{H8\text{-}6}$ 2.25)*	110.60
7 (OH)			164.80
8	1H	6.32, *d* ($J_{H8\text{-}6}$: 2.25, $J_{H5\text{-}8}$:0.55)*	102.85
9			162.29
10			114.33
1’			nd
2’	1H	7.17, *d* ($J_{H2'\text{-}6'}$: 2.4, $J_{H5'\text{-}2'}$: 0.21, $J_{H2'\text{-}1''ab}$: 0.05)*	128.75
3’		w	nd
4’ (OH)			156.58
5’	1H	6.80, *d* ($J_{H5'\text{-}6'}$8.66, $J_{H5'\text{-}2}$: 0.21)*	nd
6’	1H	7.15, *dd* ($J_{H5'\text{-}6'}$8.66, $J_{H2'\text{-}6'}$: 2.41)*	**125.68**
1”a 1”b	 1H 1H	3.22 *d* ($J_{H2''\text{-}1''a}$:7.40, $J_{H1''a\text{-}4''}$:0.70, $J_{H1''a\text{-}5''}$:0.93 , $J_{H1''a\text{-}b}$:-18, $J_{H2'\text{-}1''a}$: 0.05)* 3.22 *d* ($J_{H2''\text{-}1''b}$:7.16, $J_{H1''b\text{-}4''}$:1.02, $J_{H1''b\text{-}5''}$:0.61 , $J_{H1''a\text{-}b}$:-18 $J_{H2'\text{-}1''b}$: 0.05)*	27.50
2”	1H	5.27 *ddqq* ($J_{H2''\text{-}1''a}$:7.40, $J_{H2''\text{-}1''b}$:7.16, $J_{H2''\text{-}4''}$:-1.53, $J_{H2''\text{-}5''}$: -1.28)*	123.06
3’’			132.02
4” CH3 5” CH3	3H 3H	1.68, *brs* ($J_{H1''a\text{-}4''}$:0.70, $J_{H1''b\text{-}4''}$:1.02, $J_{H2''\text{-}4''}$:-1.53) * 1.67, *brs* ($J_{H1''a\text{-}5''}$:0.93, $J_{H1''b\text{-}5''}$:0.61, $J_{H2''\text{-}5''}$:-1.28) *	26.15 18.27

*Calculated values obtained through full spin analysis using Perch NMR software, in order to obtain the exact coupling constants. Nd=- not detected [d] Determined from HMBC/HSQC data.

Appendices 2 (continued)

S5. Annotated ^{1}H NMR spectrum of licochalcone C (DMSO-d_6, 600 MHz)

a. Annotated ^{1}H NMR spectra



Appendices 2 (continued)

b. ^{1}H and ^{13}C NMR data (DMSO-d_6, 600 MHz)

Position	mult.	δ_H in ppm (*J* in Hz)	δ_C in ppm
C=O			nd
1			113.47
2 (-OMe)			158.31
3			nd
4 (-OH)			159.89
5		6.71, *d* (J_{H5-6}: 8.58)	112.32
6	1H	7.72, *d* (J_{H5-6} 8.58)	127.35
2', 6'	2H	8.00, AA' type ($J_{H2'/6'-H3'/5'}$:8.55/0.37, $J_{H2'/6'-H2'/6'}$:2.19)*	131.45
5', 3'	2H	6.88, XX' type ($J_{H2'/6'-H3'/5'}$:8.55/0.37, $J_{H3'/5'-H3'/5'}$:2.26)*	115.80
α	1H	7.67, *d* ($J_{Hα-β}$ 15.56)*	119.71
β	1H	7.82, *d* ($J_{Hα-β}$ 15.56)*	138.64
1"	2H	3.25, *d* ($J_{H2"-1"}$:7.04, $J_{H1"-4"}$: 1.36, $J_{H1"-5"}$: -1.36)*	22.88
2"	1H	5.16 *ddqq* ($J_{H2"-1"}$:7.04, $J_{H2"-4"}$: 0.84, $J_{H2"-5"}$: -1.34)*	123.53
-CH3 (4")	3H	1.63, *brs* ($J_{H1"-4"}$: 1.37, $J_{H2"-4"}$: - 0.84) *	26.11
-CH3 (5")	3H	1.73, *brs* ($J_{H1"-5"}$: -1.36, $J_{H2"-5"}$: -1.34) *	18.06
O-CH3	3H	3.68 s	63.50

*Calculated values obtained through full spin analysis using Perch NMR software, in order to obtain the exact coupling constants. Nd=- not detected [d] Determined from HMBC/HSQC data.

VITA

EDUCATION:

University of Illinois at Chicago Graduate College, 2014 to 2019
Chicago, IL.

Ph.D., Medicinal Chemistry, Department of Pharmaceutical Sciences

- Research advisor: Dr. Judy L. Bolton

Midwestern University College of Pharmacy, 2010 to 2014
Downers Grove, IL.

- ***PharmD*** *conferred May 22, 2014.*

RESEARCH EXPERIENCE:

(NIH Botanical Dietary Supplements Research Center)

Work on 3 projects to study differential modulation of estrogenic activity in estrogen receptor (ER)α and β by flavonoids in women's health botanicals with chemopreventive properties.

- **Project 1:** Identification of ERβ-preferential small molecules, (iso)flavonoids, present in health botanicals with potential chemoprotective properties *in vitro*.
  - In collaboration with UIC Botanical Center/analytical core, prepped botanical extracts, isolated and characterized respective bioactive compounds by UHPLC and NMR.
  - Performed colorimetric cell-based (alkaline phosphatase and ERE-Luciferase) assays using uterine and breast carcinoma cell lines to determine and compare ERα and ERβ activity of select botanical extracts/bioactive compounds.

- **Project 2:** Pharmacological study to determine optimal (ER)β-preferential (iso)flavonoid structures present in select botanicals *in vitro*
  - Performed SAR on various related (iso)flavonoids to determine lead compound structures that favor ERβ activity.
  - Performed alkaline phosphatase and ERE-Luciferase assays using uterine and breast carcinoma cell lines to determine ERβ-preferential compound structures.

- **Project 3:** *In vivo* assessment of chemoprevention through antiproliferative activity by ERβ-preferential flavonoid 8-prenylapigenin (licoflavone C), isolated from licorice botanical extract.
  - Performed *in vivo* and histological study to assess (anti)proliferative activity of 8-prenylapigenin (licoflavone C) in animal tissue.
  - In collaboration with UIC Department of Pathology performed whole-mount tissue prep to study rodent mammary terminal end buds (TEB) as phenotypic biological endpoints and tissue biomarkers.

INTERNSHIPS:

Adello Biologics, Chicago, IL. Summer 2018

Summer Intern, *Manufacturing Science and Technology/Technical Services*

- Developed and optimized of CEX and HIC methods for recombinant *granulocyte colony-stimulating growth factor* (GCSF) protein purification.
- Used protein solubilizing and refolding methods to perform upstream and downstream production.

Quality Control Department

- Prepared samples using desalting columns for cation exchange HPLC and Empower® and Apex® software.
- Tabulated and verified recombinant GCSF protein sample stability data.

Takeda Pharmaceuticals, Deerfield, IL Summer 2011

Summer Intern, *Department of Regulatory Affairs, Promotion, and Advertising*

- Applied FDA regulation to ensure *fair balance* on drug label prior to new drug product launch.
- Assessed FDA drug-labelling compliance by top pharmaceutical companies in US.

GRANTS AND AWARDS:

- Scientist Mentoring and Diversity Program Biotech Scholar Award 2019
- Oscar Robert Oldberg Award in Pharmaceutical Chemistry 2019
  - *Awarded at: 21st Annual Graduate Students Awards Ceremony, UIC Research Day.*
- Victor A. Drill Award, *Midwest Regional Chapter, Society of Toxicology* 2018
- W. E. van Doren Scholar Research Award 2018
  - *Annual Graduate Student Awards Ceremony, UIC Research Day.*
- Ruth L. Kirschstein National Research Service Award (NRSA) 2015
  Institutional Research Training Grant (T32)
  - *Awarded by*: NIH/National Center for Complementary & Integrative Health.
  - *Project Title:* Research Training in Natural Products, Complementary and Alternative Medicine.
- Midwestern University ***Kenneth A. Suarez Research Award*** 2012

SELECTED POSTER PRESENTATIONS (out of 12):

- **Mbachu**, **O**., Howell, C., Dong, H., Simmler, C., Chen, S.N., Malca-Garcia, G.R., Nikolic, D., Pauli, G.F., Dietz, B.M., Bolton, J. L.***** "Differential modulation of estrogenic activity in estrogen receptor (ER) α and β by flavonoids in women's health botanicals." *Great Lakes Drug Metabolism and Disposition Group Meeting, Ann Arbor, MI.* (May 2019)

- **Mbachu**, **O**., Howell, C., Hajirahimkhan, A., Simmler, C., Dong, H., Chen, S.N., Pauli, G.F., Dietz, B.M., Bolton, J. L.***** "SAR study of flavonoids/isoflavonoids on estrogen receptor α and β selectivity: prenylation enhances flavonoid estrogenicity but decreases isoflavonoid activity." *Society of Toxicology, Midwest Regional Chapter Meeting, Mundelein, IL.* (May 2018)

- **Mbachu**, **O**., Simmler, C., Green, S., Dong, H., Nikolic, D., Chen, S.N., Pauli G.F., van Breemen, R.B., Dietz, B.M., Bolton, J.L.*8-Prenylapigenin (8-PA) contributes to the estrogenic activity of Glycyrrhiza inflata. *NIH Centers for Advancing Research on Botanicals and other Natural Products (CARBON) meeting/NIH Botanical Center Directors meeting, Bethesda, MD.* (May 2016)

- Puppala, B., Awan, I., Briyal, S., **Mbachu**, **O**., Leonard, M. and Gulati, A.* "Ontogeny of Endothelin Receptors in brain, heart, and kidney of neonatal rats." *Midwestern University Kenneth A. Suarez Research Day, Downers Grove, IL.* (May 2012)

SELECTED ORAL PRESENTATIONS (out of 6):

- "Is the Verification of Identity & Purity of Commercial Standards Necessary?" *NIH Centers for Advancing Research on Botanicals and other Natural Products (CARBON) meeting/NIH Botanical Center Directors meeting, Chicago, IL.* (May 2017)

- "Identification of Estrogen Receptor 🚲Selective Agonist 8-Prenylapigenin from Licorice and SAR of Similar Phytoestrogens Found in Women's Health Botanicals" 55th *Annual MIKI conference, Minneapolis, MN.* (April 2017)

- "Estrogenic activities of flavonoids in women's health botanicals; importance of prenylation, C-ring unsaturation, and hydroxyl substituents." *NIH Botanical Center meeting, Chicago, IL.* (January and February, 2017)

- Presentation at Takeda Pharmaceuticals "*Clinical Studies by the Top 12 U.S. Pharmaceutical Companies from May 2011 to May 2012*", Deerfield, IL. (Summer 2012)

MANUSCRIPTS (*indicates corresponding author, ** co-authorship):

- **Mbachu, O**., Howell, C., Dong, H., Simmler, C., Gonzalo, R. M. G., Chen, S.N., Pauli, G.F., Dietz, B.M., Bolton, J. L.* "Comparative SAR study on estrogen receptor α/β activity of *(iso)*flavonoids: importance of prenylation, C-ring saturation, and hydroxyl substituents." *Journal of Agriculture and Food Chemistry* (2019) *(manuscript in preparation)*

- Hajirahimkhan, A **., **Mbachu, O.****, Simmler, C., Green, S., Dong, H., Nikolic, D., Lankin, D., van Breemen, R., Chen, S., Pauli, G., Dietz, B., Bolton, J. L.* "Estrogen Receptor (ER) Subtype Selectivity Identifies 8-Prenylapigenin as ERβ Agonist from G. inflata and Highlights the Importance of Chemical and Biological Authentication." *J Nat Prod.* 81 (4), 966 - 975 (2018)

- Bolton, J., Dunlap, T., Hajirahimkhan, Dr. A,. **Mbachu, O.,** Chen, S., Chadwick, L., Nikolic, D., van Breemen, R., Pauli, G., Dietz, B. "The Multiple Biological Targets of Hops and Bioactive Compounds" *Chem Res Toxicol.* 32 (2), 222 - 233 (2018)

- Wang, S., Dunlap, T.L., Howell, C.E., **Mbachu, O.,** Rue, E.A., Phansalkar, R.S., Chen, S.N., Pauli, G.F., Dietz, B.M. and Bolton, J.L.* "Hop (Humulus lupulus L.) Extract and 6-Prenylnaringenin Induce P450 1A1 Catalyzed Estrogen 2-Hydroxylation.*" Chem. Res. Toxicol*. *29* (7), 1142 -1150 (2016). American Chemical Society Press Release, June 22, 2016**.** https://www.acs.org/content/acs/en/pressroom/presspacs/2016/acs-presspac-june-22-2016/hops-and-breas-cancer.html**.** PMC4951797

- Puppala, B., Awan, I., Briyal, S., **Mbachu, O**., Leonard, M. and Gulati, A.* "Ontogeny of Endothelin Receptors in brain, heart, and kidney of Neonatal Rats." *Brain Dev.* 37.2: 206-215 (2015)

References

Supplied on request.