The Role of Botanicals and Their AhR-Active Compounds in Estrogen Chemical Carcinogenesis

BY

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DISSERTATION

Submitted as a partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Pharmacognosy
in the Graduate College of the
University of Illinois at Chicago, 2021

Chicago, Illinois

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Acknowledgements

I would first like to thank my first advisor, the late Dr. Judy L. Bolton for her superior mentorship, I would not have applied for or received an F31 grant without her help. She guided my curiosity in pharmacognosy and science, making me a better scientist and more mature person. Her perseverance in life and through cancer was an inspiration to me and everyone who knew her. This journey through my Ph.D. feels somewhat unfulfilled without her being there to see me finish, but her mentorship will never be forgotten by me, and the jade bonsai I have from her will be cherished for the rest of my life. Since the passing of Dr. Judy L. Bolton, Dr. Guido F. Pauli, director of the UIC/NIH Center for Botanical Dietary Supplements Research, generously allowed me to join his lab and be my advisor. His guidance in pharmacognosy and my future was immensely meaningful to me. His passion for natural products has always sparked joy and scientific curiosity in me and I will forever be grateful for his help in finishing my Ph.D.

Dr. Birgit M. Dietz has always been there for me during my Ph.D. studies and has guided my scientific curiosity on a daily basis more so than anyone else. Her expertise in natural products, medicinal chemistry, and pharmacology provided me with inspiration and scientific goals. Her countless hours spent working with me will never be forgotten and I could not have done my work without her expertise and guidance. Dr. Birgit M. Dietz provided me with ideas and new angles of thought and made me a better scientist with every conversation and correspondence. I would like to acknowledge Dr. Tareisha L. Dunlap for her wet lab mentorship and training for most assays and Dr. Caitlin E. Howell for training on LC-MS and EROD assays. I would also like to acknowledge Dr. Dejan Nikolić for his help with LC-MS. All members of Dr. Guido F. Pauli's lab and the former lab members of Dr. Judy L. Bolton's lab deserve

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List of Abbreviations

6-PN   6-prenylnaringenin

8-PN   8-prenylnaringenin

AhR   Aryl hydrocarbon receptor

AHRR   Aryl hydrocarbon receptor repressor

ARE   Antioxidant response element

ARNT   Aryl hydrocarbon receptor nuclear translocator

BA   Biochanin A

BDS   Botanical dietary supplement

BGF   Bioassay guided fractionation

COMT   Catechol-O-methyl transferase

CYP   Cytochrome P450

DESIGNER   Deplete and Enrich Select Ingredients to Generate Normalized Extract Resources

DNMT   DNA methyl transferase

DMSO   Dimethyl sulfoxide

DMX   Desmethylxanthohumol

DNA   Deoxyribonucleic acid

E_1 Estrone

E_2 Estradiol

ER   Estrogen receptor

ERE   Estrogen receptor element

EROD   Ethoxyresorufin O deethylase

ESI   Electrospray ionization

EtOH	Ethanol
FBS	Fetal bovine serum
FDA	Food and drug administration
FN	Formononetin
GG	*Glycyrrhiza glabra*
GI	*Glycyrrhiza inflata*
GU	*Glycyrrhiza uralensis*
GST	Glutathione *S*-transferase
HER2	Human epidermal growth factor receptor 2
HPLC	High performance liquid chromatography
HRT	Hormone replacement therapy
ICI	Fulvestrant, ICI 182 780
ICW	In-Cell Western
IgG	Immunoglobulin G
IRL	Irilone
IX	Isoxanthohumol
KEAP1	Kelch-like ECH-associated protein 1
KO	Knock out
KOE	Knock out extract
Me	Methyl
MeOE$_1$	Methoxyestrone
MeOH	Methanol
mRNA	Messenger ribonucleic acid

MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa B
NMR	Nuclear magnetic resonance
NQO1	NAD(P)H quinone oxidoreductase 1
NRF2	Nuclear factor erythroid 2-related factor 2
OVX	Ovariectomized
P450	Cytochrome P450
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RCE	Red clover extract
RNA	ribonucleic acid
SD	Sprague-Dawley
SEER	Surveillance, epidemiology, and end result
SERD	Selective estrogen receptor degrader
SERM	Selective estrogen receptor modulator
SHBG	Sex hormone binding globulin
SRM	Selective reaction monitoring
TCDD	2,3,7,8-tetrachlorodibenzodioxin
TCM	Traditional Chinese Medicine
TMS	2,2',4,6'-tetramethoxystilbene
UDP	Uridine diphosphate
UGT	Uridine 5'-diphospho-glucuronosyltransferase

UHPLC	Ultra high performance liquid chromatography
XH	Xanthohumol
XRE	Xenobiotic response element

Summary

Dietary supplements are increasingly popular, with sales of botanical dietary supplements being worth nearly 9 billion dollars a year as of 2018. Many women use botanical supplements advertised as containing phytoestrogens as a means of supplementing the natural hormone estrogen during perimenopause and menopause. Estrogen is a crucial sex hormone in pre-menopausal women with three different types utilized physiologically that include estrone (E_1), estradiol (E_2) and estriol (E_3). As women age, the circulation of estrogens fluctuates or reduces during perimenopause (~37.5 years old) and is associated with symptoms including hot flashes and insomnia. In menopausal women estrogen production decreases and ultimately ceases to be produced by the ovaries, thereby increasing the risk of osteoporosis. Although estrogen supplementation with FDA approved products may improve these symptoms, estrogen and progestin supplementation was associated with a 26% increase in breast cancer incidence during the Women's Health Initiative of 2002. Phytoestrogens may be an affordable option and readily available alternative to the historic use of estrogen and progestin hormone therapy and are sold as safe and natural. However, phytoestrogens have shown mixed results in menopausal symptom relief, and may not always be a safe alternative due to their estrogenic nature. Yet some women's health botanicals may exhibit resilience promoting properties, or well-being and beneficial health outcomes, and therefore research into the safety and efficacy of these botanicals which women still use is of great importance.

In this project women's health botanical extracts were analyzed for their ability to influence the estrogen detoxification pathway. The aryl hydrocarbon receptor (AhR) transcribes both P450 1A1 and P450 1B1. Estrogens are metabolized by P450 1B1 and P450 1A1 to their respective 4- or 2-catechol estrogens. The 4-hydroxylated estrogens can form genotoxic quinones

suggesting the CYP1B1 (P450 1B1) pathway may be the genotoxic pathway of estrogen metabolism. Estradiol (E_2) leads to the epigenetic downregulation of the estrogen detoxification pathway through CYP1A1 (P450 1A1), and therefore estrogens increase AhR-ERα (estrogen receptor alpha) crosstalk. Conversely, activated AhR may interact with ERα and promote proteasomal degradation of ERα suggesting an important crosstalk between AhR and ERα.

The amounts of 2- and 4- catechol estrogens have been seen as valuable information when considering estrogen carcinogenesis with the ratio of 2- over 4- catechol estrogens used as a biomarker for estrogen chemical carcinogenesis. Botanical extracts and their bioactive compounds may reverse this AhR-ERα crosstalk and upregulate CYP1A1, leading to increases in the 2-hydroxylation of estrogens and estrogen detoxification. Additionally, the influence of botanical extracts on other targets in estrogen detoxification should be considered, including the enzyme responsible for the reduction of genotoxic estrogen quinones to catechols, NAD(P)H Quinone Dehydrogenase 1 (NQO1). Several popular women's health botanicals were initially evaluated for their effects on the estrogen detoxification and genotoxic pathways. We demonstrated that hops (*H. lupulus*) and *Epimedium* increased both *CYP1A1* and *NQO1* and downregulated ERα, potentially through AhR-activated proteasomal degradation of ERα. By contrast, red clover extract (*T. pratense*) was estrogenic, upregulated *ESR1*, and downregulated *CYP1A1*. Hops and red clover were chosen for further study based on initial evaluation for bioactivity on the estrogen detoxification/genotoxic pathway. These botanicals contain interesting phytochemicals for this study, namely their AhR agonists, 6-prenylnaringenin (6-PN) from hops, and biochanin A and formononetin from red clover. Thus, hops showed resilience potential, while red clover did not and may increase genotoxic pathways of estrogen metabolism (*CYP1B1*). According to the NIH, resilience is defined as a "dynamic property which enables

cells, organs, organisms or individuals to resist or recover from the effects of a physiological or pathological stressor". However, because red clover is popular and thus widely used as a women's health botanical, methods to develop a safer extract which may not promote the estrogen genotoxic pathway of metabolism were also pursued.

DESIGNER, or Deplete and Enrich Select Ingredients to Generate Normalized Extract Resources, methodology allows for fractionation of a botanical extract to provide formulations enriched bioactive compounds. DESIGNER fractions of red clover were analyzed for their ability to influence the estrogen detoxification pathway and AhR. Our botanical center's standardized clinical red clover extract (RCE) did not exhibit resilience activity, so optimization of RCE was attempted using the DESIGNER approach in order to develop a resilience promoting RCE. RCE fractions rich in the AhR agonists increased the transcription of *CYP1A1*, while RCE downregulated *CYP1A1*. Interestingly, the progesterone potentiator irilone potentiated some AhR activity, but downregulated *CYP1A1*. Ultimately, no fraction increased P450 1A1 activity and, therefore, no RCE fraction exhibited this type of resilience (well-being and health promoting) activity on the estrogen detoxification pathway. This may be due to intracellular conversion of the red clover isoflavone AhR agonists, biochanin A and formononetin, to their demethylated and estrogenic counterparts, genistein and daidzein.

The chemically standardized clinical hop extract, used in a Phase 1 clinical trial for safety and drug-drug interactions in Dr. Richard van Breeman's lab, contains the potent phytoestrogen, 8-prenylnarigenin (8-PN), xanthohumol (XH), and a potent AhR agonist, 6-prenylnaringein (6-PN). Hop extract and 6-PN degraded ERα at least in part through an AhR-dependent mechanism. Hop extract and 6-PN also increased expression of P450 1A1. The estrogenic 8-PN downregulated *CYP1A1*, while hop extract and 6-PN preferentially increased the transcription of

CYP1A1 in the presence of E₂, reversing AhR-ERα crosstalk at CYP1A1. Hop extract and 6-PN ultimately reversed the epigenetic inhibition at CYP1A1 caused by estradiol activated DNMT1 and preferentially led to metabolism of estrogens via the estrogen detoxification pathway. Therefore, the standardized clinical hop extract exhibited resilience activity on the estrogen detoxification pathway and was chosen for further preclinical assessment.

The resilience promoting nature of the clinical hop extract can only be realized if the bioactive compounds reach the tissues of interest. Therefore, a distribution study of prenylated hop flavanones and chalcones in a pre- and postmenopausal Sprague-Dawley rat model was undertaken. No estrogenic response was seen for the clinical hop extract in a uterotrophic assay, measuring uterine proliferation, suggesting the clinical hop extract was safe for short term use. All bioactive compounds of interest reached the mammary gland and uterus with XH being the most prevalent. Interestingly, conversion of the chalcone XH to the flavanone IX and IX to the phytoestrogen 8-PN most likely occurred, as there is about 33 times more XH than IX in the extract, but only about 3 times more XH than IX in the serum. 6-PN was always found in the lowest quantities when compared to the other bioactive compounds, even though there is more 6-PN than 8-PN in the clinical hop extract, indicating the likely conversion of IX to 8-PN. The known ability for XH to exhibit antioxidant activities, while 6-PN increases estrogen detoxification, makes the standardized clinical hop extract a potentially resilience promoting women's health botanical.

Many botanical extracts and potential bioactive compounds were initially evaluated for an ability to influence AhR-ERα crosstalk and the estrogen detoxification pathway. Several botanicals following analysis revealed potential to do this or contained AhR agonists and were chosen for further evaluation, but detailed studies into other botanicals, particulary *Epimedium*,

are still needed. Although red clover contains AhR agonists, they are metabolized to estrogenic compounds, which likely led to downregulation of the estrogen detoxification pathway by the clinical RCE. The clinical hop extract showed many bioactivities opposite to those of the red clover extract, namely an ability to preferentially upregulate *CYP1A1* and the estrogen detoxification pathway. Estrogen-induced AhR-ERα crosstalk was shown to preferentially lead to CYP1B1 transcription through epigenetic inhibition of CYP1A1, while the clinical hop extract was shown to reverse this epigenetic inhibition of CYP1A1. Reversing the estrogen-induced epigenetic inhibition of CYP1A1 and upregulation of estrogen metabolism by P450 1A1 is potentially a resilience promoting outcome in the mammary gland. Yet, further study into the safety and efficacy of women's health botanicals standardized to their AhR agonists is needed to determine what role AhR agonists play in resilience, and animal studies to analyze the resilience potential *in vivo* are also needed. Standardization of botanical extracts to multiple bioactive constituents is also a critical step for an understanding of the polypharmacology of a botanical extract. Botanical extracts are complex mixtures of constituents and understanding the bioactivity of a single constituent does not explain the complex bioactivity of a botanical extract. Once standardized to multiple constituents, one can begin to gain an understanding of how these compounds interact and exhibit bioactivity though multiple mechanisms.

Chapter 1: Introduction

       Breast Cancer

          Breast cancer is the most prevalent malignant cancer detected in women, and the second most deadly after lung cancer (American Cancer Society, 2019-2020). Currently the 5-year survival rate for all surveillance, epidemiology, and end result (SEER) stages is 91% (American Cancer Society, 2019-2020). To our current knowledge breast cancer was first described in the Ebers Papyrus and Edwin Smith Papyrus some 3500 years ago in ancient Egypt with no treatment other than possibly cauterization (Lukong, 2017). Written remedies for breast cancer using botanicals began in the year 200 with Galen, suggesting the use of licorice, opium, castor oil, and sulfur (Lukong, 2017). Today breast cancer is generally treated with radiation, targeted chemotherapy, hormonal therapy (antiestrogens), and lumpectomy or mastectomy, but botanical preparations are still commonly used in developing and underdeveloped countries (Laskar et al., 2020; Leo et al., 2020). Breast cancer chemotherapy has drastically improved, with 9 drugs approved in the U.S. from 2009-2018 for breast cancer (Leo et al., 2020). Better chemotherapy has increased 5-year survival with stage I to nearly 100%, but stage IV 5-year survival is 26% (Miller et al., 2019).

       Breast cancer is categorized by the presence or absence of specific receptors. Group 1 is generally referred to as luminal A and is positive for progesterone receptor (PR) and estrogen receptor (ER) (Cancer Genome Atlas, 2012). Luminal B is group 2 and is ER and PR positive as well as positive for receptor tyrosine protein kinase ERBB-2 (HER2) (Cancer Genome Atlas, 2012). Group 3 is HER2 positive only, while group 4 is negative for all 3 receptors and referred to as basal-like or triple negative (Cancer Genome Atlas, 2012). Additionally, breast cancer type 1 and type 2 (BRCA1 and BRCA2) mutations play a major role in hereditary breast cancer

(Mehrgou and Akouchekian, 2016). Based on its site of origin within the breast, breast cancer is classified as either lobular (5-15%) or ductal (50-75%) carcinoma (Waks and Winer, 2019). Breast cancer can also be classified by tumor size, whether it has spread to local lymph nodes, and formed distant metastases, which plays a role in determining the stage of the carcinoma. The level of breast cancer severity is determined in stages 0-4, with 4 being metastatic, the most severe stage. Breast cancer is also histologically graded 1-3, with grade 3 being the most severe and meaning the breast cancer cells have significantly de-differentiated from normal breast tissue and are more likely to grow and spread faster (Johns Hopkins, 2020).

Hormone positive breast cancer of the group 1 and 2 type is initially treated with endocrine therapies. For example, tamoxifen is a selective estrogen receptor modulator (SERM) usually taken for 5 years starting at diagnosis, reducing reoccurrence by about 50% over 5 years (Waks and Winer, 2019). SERMs are a common first line defense in hormone receptor positive breast cancer. Although prone to resistance over time, SERMs work by competitive inhibition of estrogens, including the most potent estrogen, estradiol (E_2), at estrogen receptors, thereby inhibiting its tumor promoting activities. Postmenopausal women with breast cancer may alternatively take an aromatase inhibitor such as anastrozole to inhibit the production of estrogens from androgens within the breast tissue thereby decreasing circulating estrogens and starving the estrogen receptor of its oncogenic ligand (Waks and Winer, 2019). Premenopausal women with hormone positive breast cancer may first be treated with a gonadotropin-releasing hormone agonist to suppress estrogen production in the ovaries before taking an aromatase inhibitor or SERM (Waks and Winer, 2019). Other more specific therapies are also available, such as poly (ADP-ribose) polymerase (PARP) inhibitors. Talazoparib and other PARP inhibitors are used to prevent DNA repair, resulting in cancer cell death for women with BRCA

gene mutations (Waks and Winer, 2019). Women that are HER2 positive take an ERBB2 targeted antibody such as trastuzumab or pertuzumab, sometimes in conjunction with a small molecule tyrosine kinase inhibitor like neratinib (Waks and Winer, 2019). Depending on the stage and grade, cytotoxic chemotherapy agents such as anthracyclines and/or taxanes may also be used to treat all types of breast cancer. In the moment, chemotherapy is the only systemic therapy for triple negative breast cancer (Waks and Winer, 2019). Surgery is also an option that is potentially curative and may be accompanied by additional treatment such as radiation, although radiation may be done without surgery as a part of breast conserving therapy (Waks and Winer, 2019).

Breast Cancer Prevention

In an attempt to thwart breast cancer, some women turn to botanicals for breast cancer prevention, which can be defined as the use of pharmacologic or natural agents that block DNA damage or arrest or reverse the progression of premalignant cells (Cazzaniga and Bonanni, 2012). Although generally unproven, and with some studies showing conflicting results, much evidence suggests the use of botanicals may be beneficial for breast cancer prevention (Dietz et al., 2016; Keiler et al., 2017b; Khan et al., 2012; Yamamoto et al., 2003). Generally regarded as safe, botanicals are added to the diet or taken as supplements for their wellness promoting properties. Botanicals have anti-oxidant, anti-inflammatory, and resilience properties making them popular additions to the lifestyle of postmenopausal women (Hui et al., 2013; Rahman et al., 2006; Rice-Evans, 2001; Rodriguez-Garcia et al., 2019). Resilience can be defined as a "dynamic property which enables cells, organs, organisms or individuals to resist or recover from the effects of a physiological or pathological stressor" (NIA-NIH, 2020). Certain botanicals have been shown to reduce carcinogenic pathways and increase resilience pathways, influencing chemical, hormonal, epigenetic, and inflammatory carcinogenesis in breast cancer cells (Dietz et al., 2016). The bioactive compounds found in these women's health botanicals decrease proliferation, induce apoptosis, induce detoxification enzymes, and reduce estrogen carcinogenic metabolism (Dietz et al., 2016). The influence of botanicals on estrogen carcinogenic metabolism make for interesting targets of study for the reduction of estrogen chemical carcinogenesis and resilience in breast cancer initiation.

Breast Cancer Mechanisms

Estrogen exposure is a well-known risk factor for breast cancer, with the length of exposure correlating to risk, although many additional factors play a role. DNA mutations are the driving force of cancer, some inherited but most accumulated from exposure to environmental and lifestyle factors (Parsa, 2012). Cancer cells depend greatly on sugars as a means of ATP production for the process of glycolysis, and inhibition of this glycolysis results in cell death (Fitzgerald et al., 2018). Cancer cells also do not exhibit senescence and apoptosis like normal cells in part due to sustained telomerase activity (Shay, 2016). Cancer cells also lack contact inhibition due to the distribution of glycoproteins and transmembrane proteins, such as cadherins, resulting in uncontrolled cellular proliferation and tumors (Fitzgerald et al., 2018). Hypoxic and nutrient deprived tumors secrete angiogenic growth factors to promote tumor vascularization, and metastatic spreading of breast cancer is related to the degree of tumor vascularization (Fitzgerald et al., 2018). All cancers exhibit to various degrees glycolysis, angiogenesis, apoptosis evasion, and uncontrolled cell growth, but breast carcinogenesis can also be divided into four general categorical pathways: hormonal, chemical, inflammatory, and epigenetic (Dietz et al., 2016; Fitzgerald et al., 2018; Pfeffer and Singh, 2018).

Hormonal Carcinogenesis

At least 75 % of breast cancer is hormone receptor positive, driven by ERα dependent proliferation of breast tissue (Figure 1) (Santen et al., 2015; Siersbaek et al., 2018). In the classical estrogen binding pathway, estradiol (E_2) and other estrogens bind to ERα to induce conformational change, nuclear translocation, and binding to ERE (estrogen response element) DNA segments to facilitate gene regulation (Dietz et al., 2016). It has been reported that this ERα transcriptional regulation suppresses p53/p21 and activates markers of proliferation events such as proliferating cell nuclear antigen (PCNA) and Ki-67, resulting in cellular proliferation (Liao et al., 2014). Similarly, estrogen and ERα signaling triggers mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3K), and protein kinase B (AKT) pathways resulting in proliferation, cell survival, and metastasis (Cortez et al., 2010). ERα can also interact with transcription factors such as specificity protein 1 (SP1) and activator protein 1 (AP1) among others, leading to increased proliferation and differentiation (Chumsri et al., 2011). Mutations in the ESR1 (ERα) gene are common in breast cancer and can result in ligand independent ERα transcriptional regulation (Bahreini et al., 2017).

The other estrogen receptor, ERβ (Figure 1), plays a significant role in breast cancer prevention and wellness. Often both receptors, ERα and ERβ, play opposite roles in cancer progression: for example, ERα activates the oncogenic transcription factor MYC which induces cell cycle progression leading to proliferation, while ERβ reduces MYC expression (Thomas and Gustafsson, 2011). Normal breast tissue expresses ERβ more than ERα however in invasive ductal breast cancer less than 10% of the cells express ERβ (Huang et al., 2015). Many studies have linked high ERβ expression with improved prognosis (Huang et al., 2015). Activation of ERβ reduces cellular proliferation and is proapoptotic (Thomas and Gustafsson, 2011). Selective

agonists of ERβ have been shown to reduce cellular proliferation of breast cancer cells in preclinical trials (Huang et al., 2015). However, no clinical ERβ agonists have been approved.

Aromatase (CYP19A1) (Figure 1) is the enzyme responsible for the conversion of androgens to estrogens through multiple oxidation steps on the steroid A ring. Specifically, aromatase transforms androstenedione to estrone (E_1) and testosterone to estradiol (E_2). Multiple tissues express aromatase including adipose fibroblasts and malignant epithelial cells (Bulun et al., 2012). Aromatase plays a key role in hormone receptor positive breast cancer being the only source of endogenous estrogen in postmenopausal women and providing ERα with its cognate ligands. Breast cancer of the hormone receptor positive status expresses aromatase at a higher level than non-cancerous cells (Chumsri et al., 2011). Obese post-menopausal women are three times more likely to develop breast cancer than normal weight post-menopausal women, likely due to the increased expression of aromatase due to the higher concentration of adipose fibroblasts associated with obesity (Bulun et al., 2012; Wang et al., 2015b). In an *in vivo* rat model blocking of aromatase with a competitive inhibitor resulted in decreased estrogen concentrations and tumor size regression (Brodie et al., 1977). To this note the development of aromatase inhibitors for clinical use show great benefit, but are also the source of much resistance (Carpenter and Miller, 2005).

Also playing an important role in hormonal carcinogenesis is the progesterone receptor (PR) which can be found in MCF-7 cells, the main cell line used for these studies. Progesterone leads to reactive oxygen species and the proliferation of breast cancer cells *in vitro* (Azeez et al., 2015). The conjugated estrogens plus medroxyprogesterone arm of the women's health initiative led to a 26% increase in the incidence of breast cancer, indicating the importance of both PR and ERα in breast carcinogenesis, yet the conjugated estrogens only arm did not increase breast

cancer risk in women without a uterus, a situation not relevant for most cases of breast cancer or

for these studies (Falk et al., 2019; Ross et al., 2000).

Chemical Carcinogenesis

Estrogens themselves exhibit carcinogenicity in the form of certain metabolites (Figure 1). E_2 is metabolized by P450 enzymes, and some of its metabolites result in catechol estrogens which upon oxidation form genotoxic *o*-quinones (Bolton and Thatcher, 2008). The 3,4 quinone of E_2 (E_2-3,4-Q) forms two types of depurinating DNA adducts, 4-OHE$_2$-1-N3Ade and 4-OHE$_2$-1-N7Gua, and exhibits greater reactivity than the 2,3 quinone of estradiol (E_2-2,3-Q) which forms only 2-OHE$_2$-6-N3Ade adducts (Zahid et al., 2006). Studies have found that 2-hydroxylation of estrogens (2,3-OH-E$_2$) may be associated with a lower risk of breast cancer while conversely more extensive 4-hydroxylation of estrogens (3,4-OH-E$_2$) was associated with higher risk of breast cancer (Falk et al., 2013; Fuhrman et al., 2012; Ziegler et al., 2015). Therefore, the ratio of 4- to 2- hydroxyestradiol has been proposed as a biomarker in the identification of breast cancer risk (Samavat and Kurzer, 2015).

Estrogens are metabolized to their catechol forms by P450 enzymes. In the breast tissue, P450 1A1 (Figure 1) predominantly catalyzes the 2-hydroxylation or non-genotoxic pathway, while conversely P450 1B1 primarily catalyzes the genotoxic 4-hydroxylation pathway producing 3,4-OH-E$_2$, the metabolic precursor of the genotoxic E_2-3,4-Q (Bolton and Thatcher, 2008; Wang et al., 2016). P450 1B1 is translated from *CYP1B1*, its cognate gene and RNA transcript. Expression of *CYP1B1* is of constitutive nature, while *CYP1A1* is expressed at a low basal level yet is highly inducible (Hitzman et al., 2020; Kerzee and Ramos, 2001). The arylhydrocarbon receptor (AhR) is the transcription factor protein responsible for the transcription of *CYP1A1* and *CYP1B1* (Dietz et al., 2016). Xenobiotic compounds like dioxins and benzo[a]pyrene bind to AhR inducing conformational change and transportation into the nucleus, where it binds with xenobiotic response elements (XRE) in DNA and elicits gene

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expression in a potentially overactive manner and carcinogenic manner (Larigot et al., 2018). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a prototypical dioxin and example of a potentially carcinogenic AhR ligand capable of inducing transcription of proliferative factor E2F but also *CYP1A1* (Hitzman et al., 2020; Larigot et al., 2018). Additionally, certain endogenous compounds such as the tryptophan derivatives kynurenine and 6-formyl-indolo[3,2-*b*]carbazole (FICZ) or particular flavonoids such as 6-prenylnaringenin (6-PN) activate AhR, but induce a different conformational change than dioxins and may exhibit resilience properties towards estrogen carcinogenesis through AhR (Dunlap et al., 2017; Giani Tagliabue et al., 2019; Hitzman et al., 2020; Larigot et al., 2018; Safe et al., 2017). Other natural product AhR agonists like biochanin A or isoliquiritigenin directly or indirectly preferentially activate *CYP1B1* and may enhance the 4-hydroxylation of estrogens leading to carcinogenesis (Dunlap et al., 2017; Dunlap et al., 2015).

Catechol estrogens are subject to potential methylation, as part of phase I metabolism, by catechol-*O*-methyltransferase (COMT), resulting in 2- or 4-methoxy estrogens (Figure 1) (Yager, 2015). Once methylated, the oxidative metabolism of a catechol to a quinone is inhibited (Yager, 2015). Interestingly, mutations in COMT are associated with higher rates of breast cancer in Asian women but not in Caucasian women (Peterson et al., 2010; Qiu et al., 2018). Less extensive methylation of catechol estrogens has been shown to be associated with a higher risk of developing breast cancer (Fuhrman et al., 2012). Additionally, inhibitors of COMT lead to increased oxidative damage and depurinating DNA adducts scoring the importance of COMT in reducing the carcinogenic nature of estrogen (Yager, 2015).

Once catechol estrogens have been oxidized to reactive quinones, the capacity to revert them back to their catechol form exists in the form of NAD(P)H-quinone oxidoreductase

(NQO1) (Figure 1) (Chandrasena et al., 2008). Additionally, glutathione *S*-transferase (GST)

conjugates catechol estrogens with glutathione for excretion as a phase II product (Chandrasena

et al., 2008). Both NQO1 and GST are transcribed in the antioxidant response pathway triggered

by nuclear factor erythroid-2-related factor 2 (NRF2) binding to the antioxidant response element

(ARE) segments of DNA in the nucleus (Yao et al., 2010). Many natural products with Michael

acceptor properties are capable of inducing NQO1 activity through binding to sulfhydryl groups

in the Kelch-like ECH-associated protein 1 (KEAP1). In basal settings, NRF2 is sequestered by

KEAP1 in the cytosol. After Michael addition to KEAP1, the concentrations of NRF2 in the

nucleus increase, thus enhancing NQO1 transcription at the ARE (Yao et al., 2010).

Interestingly, AhR increases NQO1 while conversely ERα downregulates NQO1 (Lo and

Matthews, 2013). Ultimately, these targets in the estrogen chemical carcinogenesis pathway may

be influenced by exogenous compounds, such as from botanicals, inducing or inhibiting estrogen

chemical carcinogenesis.

Inflammatory Carcinogenesis

Inflammation fuels carcinogenesis. Tumor associated macrophages are critical for tumor progression, and secrete inflammatory cytokines aiding in breast cancer progression and metastasis (Obeid et al., 2013). One such cytokine, tumor necrosis factor alpha (TNFα), causes CYP1A1 and COMT activity (detoxification pathways) to decrease, and CYP1B1 activity (genotoxic pathway) to increase, a less favorable outcome in terms of estrogen metabolism and cancer (Kamel et al., 2012). Overexpressed inflammatory mediators in breast cancer are of particular relevance for hormone receptor negative breast cancer, correlating with poor prognosis (Dietz et al., 2016; Harris et al., 2014). The transcription factor nuclear factor kappa B (NF-κB) (Figure 1), normally inactivated by inhibitory kappa B proteins, is often constitutively active in breast cancer and is associated with inflammation, proliferation, angiogenesis, and metastasis (Wang et al., 2015a). NF-κB transcribes inflammatory proteins, including cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS), and interleukin-1 (IL-1) among more than 500 others and is, therefore, an important target in breast cancer, particularly hormone receptor negative breast cancer (Wang et al., 2015a).

Epigenetic Carcinogenesis

Increasing evidence points towards epigenetics playing a role in breast cancer initiation (Basse and Arock, 2015; Hervouet et al., 2013). Common epigenetic modifications with potential implications in breast cancer include cytosine methylation and posttranslational histone modifications (Dietz et al., 2016). Histone deacetylase 1 (HDAC1) shows association with *CYP1A1* during its inhibition, and inhibition of *CYP1A1* leads to preferential metabolism of estradiol by P450 1B1 to its 4-hydroxylated genotoxic form and the genotoxic pathway of estrogen metabolism (Schnekenburger et al., 2007). Thymine-DNA glycosylase (TDG) is critical for removing oxidated methylcytosine, a key step in *CYP1A1* initiation (Amenya et al., 2016). Histone variant H2A.Z has been shown to provide physical stability and recruit transcription factors for transcription (Marques et al., 2013). Hypomethylation in CpG regions of DNA are common in many cancers, including breast cancer, yet some genes exhibit hypermethylation leading to inhibition, such as *CYP1A1* (Amenya et al., 2016; Hervouet et al., 2013; Marques et al., 2013). DNA methyltransferase 1 and 3B (DNMT1 and DNMT3B) have both been implicated in the selective inhibition of CYP1A1 through methylation of its promoter region, with involvement of ERα (Hitzman et al., 2020; Marques et al., 2013). It has been suggested that hormone receptor negative breast cancers exhibit epigenetic silencing of ER genes (*ESR1* and *ESR2*) through promoter region methylation by DNMTs (Hervouet et al., 2013). HDAC1 abolishes *ESR1* transcription completely, aiding to a poor breast cancer prognosis (Hervouet et al., 2013). ERα can trigger specific epigenetic patterns leading to repression of tumor suppressor genes like Trefoil factor 1 (TFF1) (Hervouet et al., 2013). While the complex role epigenetics plays in breast cancer is far from fully understood, it is clear that epigenetic carcinogenesis is intertwined with chemical and hormonal estrogen carcinogenesis.

Estrogen Crosstalk

Estrogen carcinogenesis is an intricate, complex, and still unraveling network of different molecular pathways working together, such as the hormonal, chemical, inflammatory, and epigenetic pathways, ultimately potentially leading to breast cancer (Dietz et al., 2016; Hervouet et al., 2013; Hitzman et al., 2020; Marques et al., 2013). Crosstalk in this case is considered between AhR and ERα and the estrogens may play in regulating this crosstalk. The epigenetic pathway of breast carcinogenesis is an important component, as each epigenetic outcome influences chemical and hormonal breast carcinogenesis pathways through estrogen crosstalk (Figure 2). DNMTs inhibit both *CYP1A1* and *ESR1* genes relating to the chemical and hormonal pathways respectively (Hervouet et al., 2013; Hitzman et al., 2020; Marques et al., 2013). Although downregulation of *ESR1* transcription can decrease the extent of estrogen hormonal carcinogenesis, a complete loss of ER receptor can be a form of resistance for cancer, leading to less treatment options and poorer prognosis (Hervouet et al., 2013). The epigenetic methylation of *CYP1A1* by DNMTs causes selective inhibition of AhR mediated *CYP1A1* transcription, and ultimately the 2-hydroxylation of estrogens is reduced, leading to increased genotoxic metabolism of estrogens in the chemical carcinogenesis pathway (Hitzman et al., 2020; Marques et al., 2013). Estrogen hormonal carcinogenesis also influences estrogen chemical and epigenetic carcinogenesis, as estrogen increases DNMT1 inhibition of CYP1A1, with ERα playing a direct role (Hitzman et al., 2020; Marques et al., 2013).

While ERα inhibits AhR-mediated activity, AhR can conversely reduce the extent of ERα and ERα-mediated transcriptional activity (Luecke-Johansson et al., 2017; Tiong et al., 2012). AhR activation can lead to an AhR-ERα complex, in which Cullin-4B (CUL4B) recognizes activated AhR in complex with ERα, labels ERα with ubiquitin, and targets it for proteasomal

degradation (Luecke-Johansson et al., 2017; Wormke et al., 2000). This degradation can have beneficial downstream outcomes for estrogen hormonal carcinogenesis. Growth regulation by estrogen in breast cancer 1 (GREB1), an oncogene transcribed by ERα, is downregulated by AhR activation through decreased ERα expression and overall transcriptional activity (Ahmed et al., 2009; Tiong et al., 2012). The ability for AhR and ERα to influence one another is still under investigation, but overlap between AhR and ERα gene regulation is known to exist (Ahmed et al., 2009). The complex relationship between AhR and ERα may be targeted for oncology research and shows promise for further understanding estrogen carcinogenesis. Botanicals may provide potential for influencing this crosstalk and the outcome of this influence may provide resilience. To this note, a goal of our botanical center is to study the influence of women's health botanical extracts on estrogen crosstalk and resilience promoting mechanisms.
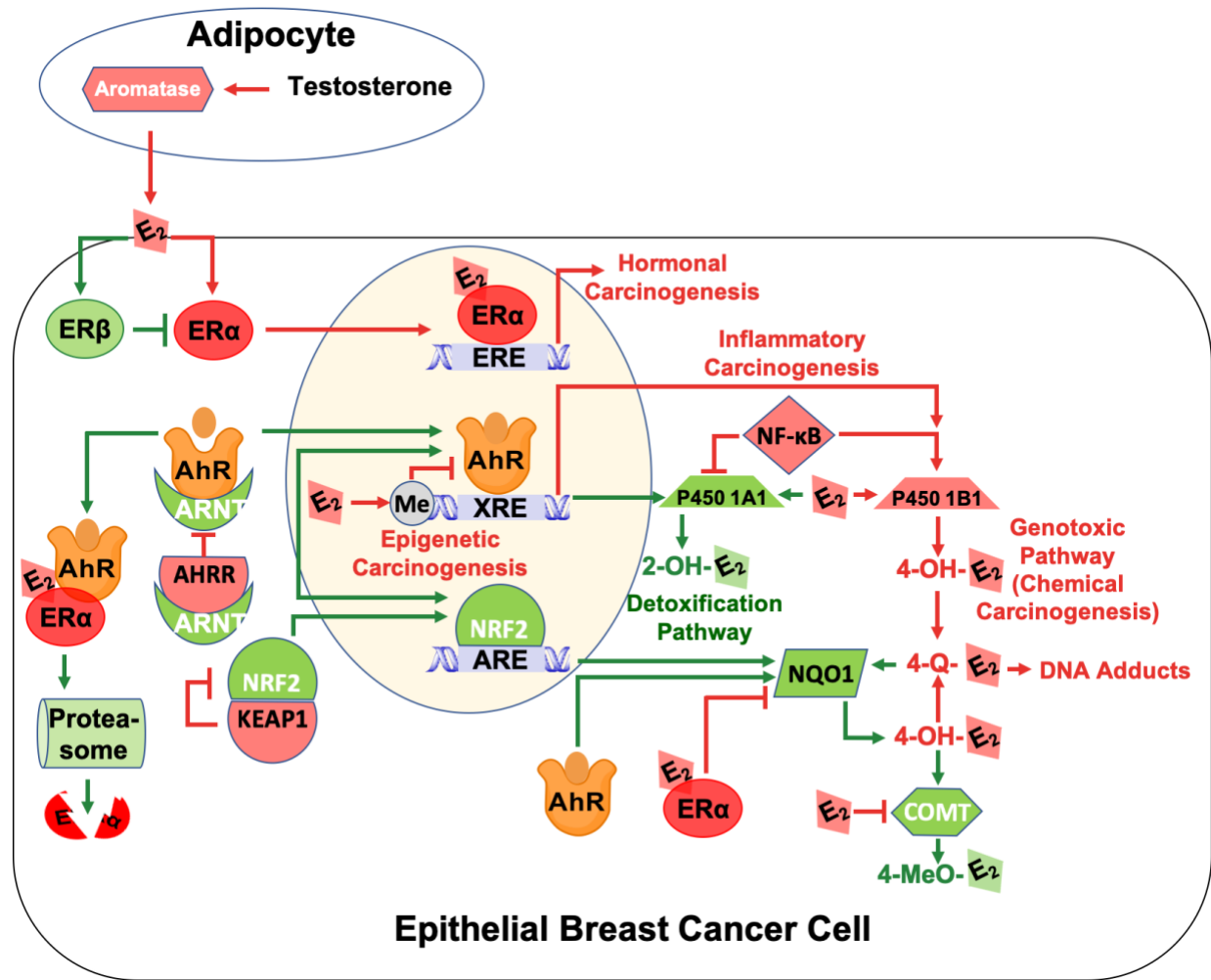
Figure 1. The complex signaling pathways mediating estrogen carcinogenesis. Green symbolizes a resilience promoting pathway, while red symbolizes a potentially carcinogenic pathway or inhibition of a resilience promoting pathway.

Estrogen Crosstalk and Crosstalk Reversal
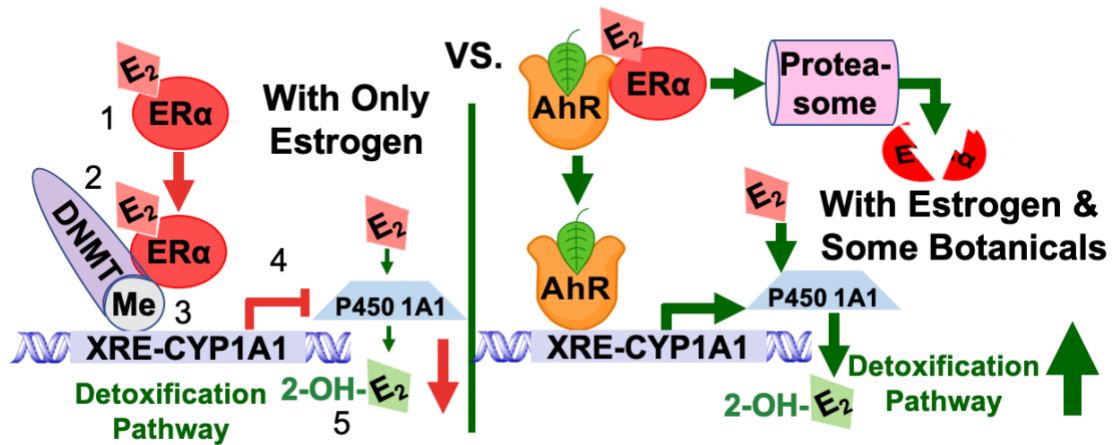
Figure 2. A. Estrogen crosstalk at CYP1A1 occurs through epigenetic recruitment of DNMTs (2) by activated ERα (1) to the promoter of CYP1A1 (3), inhibiting P450 1A1 transcription (4) and the estrogen detoxification pathway (5). B. Some botanicals may reverse estrogen crosstalk by degrading ERα and activating XRE in the CYP1A1 promoter to initiate transcription and promote estrogen detoxification to 2-hydroxyestradiol through P450 1A1.
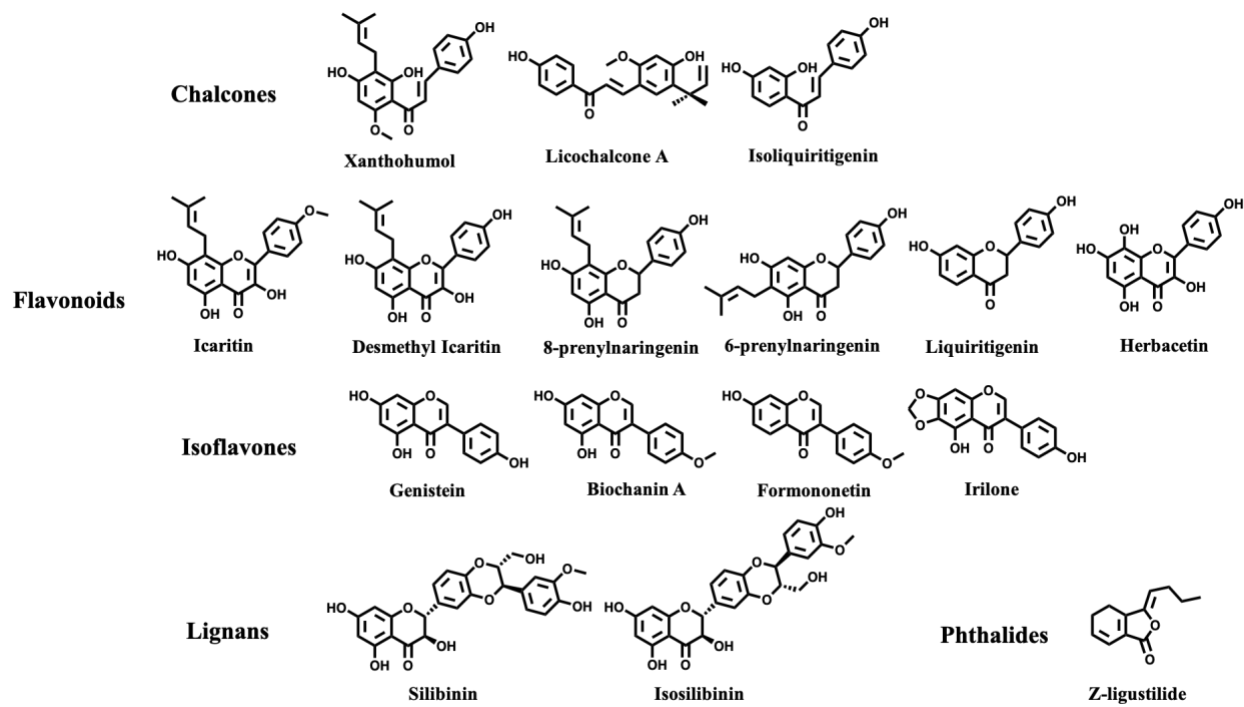
Figure 3. Bioactive compounds of interest found in women's health botanicals.

Dietary Supplements for Women's Health

Herbal supplements sales totaled nearly 9 billion dollars in 2018 and have steadily increased in the last two decades (Smith et al., 2018). In 2002, the large Women's Health Initiative study found that the common hormone replacement therapy of conjugated estrogens and medroxyprogesterone, usually prescribed for menopausal symptoms, led to an increased risk of breast cancer (Rossouw et al., 2002). These findings have led women to seek alternative options including botanical dietary supplements. Many women's health botanicals are on the top 40 list in sales, including milk thistle (16 million) and horny goat weed (11 million) (Smith et al., 2018). A decline of estrogens during perimenopause has some women choosing to take supplements for hot flashes, insomnia, and night sweats. Menopausal woman may also seek an herbal remedy for their mental health, bone health, and general overall health. Botanicals may achieve these effects through serotonergic or estrogenic mechanisms (Dietz et al., 2016). However, many botanical dietary supplements (BDS) failed to show efficacy in reducing hot flashes in multiple clinical trials, and the health effects can be controversial (Dietz et al., 2016; Hopp, 2015a; b; Roberts, 2010). Some discrepancies in efficacy trials may be resolved with the use of standardized extracts in trials (Dietz et al., 2016). Some clinical trials for hops and isoflavone-rich red clover showed significant reduction in menopausal symptoms compared to placebo (Dietz et al., 2016).

Naturally occurring plant estrogens, or phytoestrogens, are of great interest in women's health. Phytoestrogens consist mainly of flavonoids, isoflavonoids, coumestans, lignans and stilbenes (Desmawati and Sulastri, 2019; Dietz et al., 2016). Isoflavonoids are arguably the most popular of the phytoestrogens, and isoflavone rich diets are associated with lower breast cancer incidence in some Asian countries (Yamamoto et al., 2003). Extensive studies on isoflavones

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have yielded mixed results with some showing inverse correlations between intake and breast cancer risk, while others caution their weak estrogenic nature may lead to breast cancer (Dietz et al., 2016; Roberts, 2010). Popular botanicals containing isoflavones include red clover (*Trifolium Pratense* L.) and soy (*Glycine Max* L.) which contain genistein (Figure 3) and daidzein, with the former also containing coumestrol. Alfalfa (*Medicago sativa* L.) contains isoflavones and coumestans, notably formononetin and coumestrol, respectively. Milk thistle (*Silybum marianum* L.) contains flavonolignans, chiefly silibinin, although its estrogenicity is controversial (Dietz et al., 2016). Horny goat weed (*Epimedium* species) and rose root (*Rhodiola rosea* L.) contain the flavonoid glycosides icariin and rhodionin, respectively. Hops (*Humulus lupulus* L.) and licorice (*Glycyrrhiza* species) contain flavonoids and chalcones. Aside from exerting estrogenic activity, some flavonoids may exhibit other resilience promoting properties. For example, chalcones are effective at inducing anti-oxidant effects (de Freitas et al., 2020; Yao et al., 2015). As such women's health botanicals may be taken throughout life and for a multitude of reasons.

BDS are also taken by premenopausal women as a means for managing PMS symptoms, possibly through opioid, dopaminergic, or estrogenic mechanisms (Dietz et al., 2016). Other premenopausal women may choose to take BDS, such as cranberry extract with proanthrocyanidins, for urinary tract infections which are generally thought to inhibit *E. coli* adhesion to uroepithelial cells (Dietz et al., 2016). Botanicals are also used as galactagogues and women may choose to consume fenugreek or milk thistle during and post pregnancy (Dietz et al., 2016). Increasingly popular are general wellness botanicals which may act as free radial scavengers and induce antioxidant responses. One popular citrus BDS marketed as a bioflavonoid complex contains quercetin and its glycoside counterpart rutin, which had sales over 8 million dollars in 2017 (Smith et al., 2018). Similarly, menopause specific supplements

combining popular women's health botanicals are readily sold in health stores and online. Sales of combinatorial supplements like those mentioned are worth over 3 billion annually and growing twice as fast as single herb supplements (Smith et al., 2018). Although generally considered safe, many of these combinations have yet to be clinically studied for both efficacy and safety.

Botanicals, Breast Cancer, and Breast Cancer Prevention

There is much controversy over the use of BDS by women during breast cancer, and there is little concrete evidence for their benefit or harm. Yet botanicals used for breast cancer prevention have good reasoning and include antioxidant, and/or anti-inflammatory, among other activities targeting hormonal, chemical, inflammatory, and/or epigenetic pathways (Figures 1, 3) (Dietz et al., 2016). Figure 3 shows prominent bioactive compounds found in women's health botanicals. Phytoestrogen rich botanicals are of particular interest in breast cancer due to the estrogen sensitive nature of most breast cancers. Isoflavone rich diets like those typically consumed in Japan have been shown to result in a lower incidence of breast cancer (Yamamoto et al., 2003). Isoflavones, found in soy and red clover among others, have been shown to inhibit aromatase activity in low micromolar ranges *in vitro*, potentially reducing the amount of circulating estrogens *in vivo* (Dietz et al., 2016). Additionally, isoflavones can be more selective towards estrogen receptor beta (ERβ) than estrogen receptor alpha (ERα) and, in turn, reduce cellular proliferation *in vitro*. However, some recent studies have shown that isoflavones stimulate growth of breast cancer cells, but clinical trials with red clover showed safety over 1 year of study (Dietz et al., 2016). The inclusion of long-term use of isoflavones in the diet may point towards breast cancer preventive properties but the fact that they downregulate *CYP1A1* and the 2-hydroxylation of estrogens makes establishing a concentrate answer on the safety and efficacy of isoflavones in breast cancer resilience promoting properties difficult to establish (Dunlap et al., 2017; Kwon, 2014; Yamamoto et al., 2003).

Certain flavonoids may act as phytoestrogens. *Epimedium* contains icariin, which can be biotransformed into the estrogenic 8-prenylated flavonoid icaritin. Icaritin inhibits breast cancer cell proliferation at greater than 1 μM *in vitro* and reduced ERα expression and estrogen induced

breast cancer growth *in vivo* (Indran et al., 2014; Tiong et al., 2012). Hops (*Humulus lupulus*)

contains 8-prenylnaringenin (8-PN), the most potent known phytoestrogen and an agonist of ERα

acting in the nanomolar range (Dietz et al., 2016). 8-PN also acts as an aromatase inhibitor *in*

vitro; however, proliferative activities through ERα may make the intake of 8-PN (Figure 3)

containing hops counterproductive during breast cancer. The polypharmacology of hops is of

great interest, and other bioactive compounds in hops include 6-prenylnaringenin (6-PN) and

xanthohumol (XH), which may in fact counteract the potentially counterproductive proliferative

properties of 8-PN (Dietz et al., 2016). 6-PN induces the estrogen related detoxification enzyme

P450 1A1 *in vitro*, increasing estrogen detoxified metabolites (Hitzman et al., 2020; Wang et al.,

2016). XH is an electrophile and a major hop compound that inhibits cell proliferation *in vitro*

and activates cytoprotective and anti-inflammatory pathways. *In vivo* studies with hops have

shown no uterotrophic activities, or an ability to induce uterine proliferation, and other studies

have shown increased apoptosis and resilience biomarkers in tumors *in vivo*. This suggests that

the phytochemical complexion of a hop extract, a specialized clinical extract, could positively

influence its safety in breast cancer and that hops BDS might have promise as resilience

promoting agents (Dietz et al., 2016).

 Glycyrrhiza (licorice) species exhibit estrogenic properties, although the three

medicinally used species (*G. inflata*, *G. glabra*, *G. uralensis*) have a very distinct phytochemical

composition, and their influence on breast cancer resilience has been shown to vary substantially

(Dietz et al., 2016; Hajirahimkhan et al., 2013). For example, *G. glabra* and *G. uralensis*

stimulate breast cancer cell growth *in vitro* at low concentrations, while inhibiting cell growth at

higher concentrations (Dong et al., 2007; Hu et al., 2009; Jo et al., 2005). The phytoestrogen,

liquiritigenin (Figure 3), which is found in all *Glycyrrhiza* species, is an ERβ selective agonist

and does not enhance proliferation in breast cancer cells or xenografts (Mersereau et al., 2008). *G. glabra* was shown to activate NQO1 enzymes in mammary tissue and glabridin, found in *G. glabra*, exerts resilience effects through anti-inflammatory pathways (Dietz et al., 2016; Hajirahimkhan et al., 2015). *G. inflata* and its characteristic compound, licochalcone A, downregulated estrogen metabolism *in vitro*, while *G. uralensis* and *G. glabra* increased genotoxic estrogen metabolism in the same study (Dunlap et al., 2015).

Silybum marianum (milk thistle) and particularly silymarin, a mixture of flavonolignans found in milk thistle, has been shown to be weakly estrogenic *in vivo* with silibinin B showing ERβ activity *in silico* (El-Shitany et al., 2010). However, silymarin was shown to increase the amount of carcinogen-induced mammary tumors in a rat model (Malewicz et al., 2006). Although *Angelica sinensis* (dong quai) is a popular botanical in botanical mixtures used for breast cancer in Traditional Chinese Medicine (TCM), some studies showed proliferative effects in breast cancer cells *in vitro*, and increased uterine weight *in vivo* (Amato et al., 2002; Circosta et al., 2006). However, the estrogenicity of *A. sinensis* is controversial, and the plant contains unstable compounds in which the extraction process greatly influences the extract bioactivity (Dietz et al., 2016). *Rhodiola rosea* (roseroot) extract contains rosavins and salidrosides, and the latter has been shown to induce apoptosis in breast cancer cell *in vitro* (Gerbarg and Brown, 2016). Salidrosides have also been shown to inhibit reactive oxygen species formation. When roseroot was given *in vivo* to ovariectomized rats, no estrogenicity or change in uterine weight were observed (Gerbarg and Brown, 2016). Although other women's health botanicals, such as soy products, are popular, the focus of this study was generally on the botanicals reviewed in this section.

Botanical Research

Good manufacturing practices are the basis for all of over the counter drugs but the Food and Drug Administration (FDA) in the United States must show adverse effects before acting on BDS, although a manufacturer is responsible for ensuring their product is safe. This is due to the Dietary Supplement Health and Education Act (DSHEA) of 1994, which allowed for botanical dietary supplements to be sold with minimal assurance of identity, quality, or purity (Mahady, 2001). Depending on the quantitative measurement of phytochemicals, the quality of the supplement can vary. For example, over 200 ginseng products of the *Panax* variety were analyzed for ginsenoside content, which ranged from 0.00% to 13.54% (Mahady, 2001). Quality standardization of botanical extracts presents unique problems of intrinsic and extrinsic nature. Botanical composition may vary by species, organ, season, vegetation but also by transportation, storage, adulteration, contamination, processing and manufacturing (Dietz et al., 2016; Mahady, 2001).

In general, starting with a highly characterized plant material aids in providing a better product. For example, the broad leafed *Hypericum perforatum* (St. John's Wort) produces much less of the antidepressant hypericin than the narrow leafed variety (Mahady, 2001). Some compounds exist solely in one species, making species differentiation critical. One such example is licorice: it is comprised of three pharmacopeial and pharmacologically studied species, but glabridin is only found in *G. glabra*, while licochalcone A is only found in *G. inflata* (Dunlap et al., 2015). Identification of a species by its Latin binomial is critical not only for licorice, but for all botanicals, due to chemical variability within a given genus. Collection of the proper plant organ can drastically influence a botanical extract. The roots often store many compounds of interest, such as licorice or in *Echinacea*, where the alkylamides are found. Yet, in *Echinacea*,

the glycoproteins and polysaccharides are also of interest in immunomodulation, but exist almost

solely in the aerial parts and in the plant microbiome (Haron et al., 2019; Mahady, 2001).

Cultivation techniques and locations will also influence a botanical extract. The silymarin

content of *Silybum marianum* is optimized when grown commercially under 60% water/field and

a nitrogen level of 100 (Hammouda, 1993; Mahady, 2001). In this study wild collection of

Rhodiola rosea from both China and Alaska revealed a vast difference in bioactivity.

Adulteration also occurs and can be dangerous for example, when the toxin, digoxin, was found

in plantain supplements (Slifman et al., 1998). More recently samples of *Epimedium* (horny goat

weed), a common botanical for sexual health and stimulation in men and women's health

supplements, were found to contain synthetic PDE-5 inhibitors, used for male sexual

enhancement (Jiru et al., 2019).

Quality assurance and quality control (QA/QC) aims to properly identify botanicals and

supplement composition using taxonomic, chemical, spectroscopic, and microbial protocols

(Mahady, 2001). Genetic fingerprinting involves polymerase chain reaction (PCR) sequencing of

target DNA markers and was a common method of botanical characterization before Sanger

sequencing allowed for quick and lengthy DNA verification of plant material (Nybom et al.,

2014). Once proper botanical identification has been made, additional characterization is

performed such as UV, and high performance liquid chromatography-mass spectrometry (HPLC-

MS) provides mass fragments that can be used for quantification of extract constituents (Choi

and van Breemen, 2008). The immediate molecular peak ion pattern provided by this technique

provides a fingerprint like identification of input material and when done in tandem MS-MS can

be used to quantify secondary metabolites with a high degree of accuracy (Choi and van

Breemen, 2008; Wang et al., 2005). Another potentially more powerful but less practical

technique used to accomplish similar means is nuclear magnetic resonance (NMR), which uses nuclear spin from hydrogen or carbon to solve the molecular puzzle (Pauli et al., 2014; Tang et al., 2020).

Determination of a botanical extract's bioactive compounds is not necessarily revealed through quantification techniques such as LC-MS alone, although the major constituents are known. Sometimes the most prominent extract constituent is not of interest or does not exhibit bioactivity in question. For example, xanthohumol is the most prominent compound in the clinical hop extract used for these studies, yet xanthohumol (XH) does not exhibit estrogenic activity like the extract does because this activity is attributed to the extract's minor constituent, 8-prenylnaringenin (8-PN; 0.3%). When an extract is subjected to fractionation chromatography, the resulting extracts or fractions will contain different phytochemicals and exhibit different bioactivities. These fractions are subjected to bioassays of interest to reveal active fractions. Fractions which show activity in bioassays are then put through spectroscopy techniques to identify and sometimes quantify the compounds of interest. This technique is called bioassay guided fractionation (BGF) and it is a useful tool to determine bioactivity of minor constituents and for formulating optimized extracts and a technique essential to the work of the Botanical Center (Weller, 2012). Fractions may be combined or left out to produce designer fractions and knock out extracts, both powerful tools in extract formulation (Dietz et al., 2017; Malca Garcia et al., 2019; Ramos Alvarenga et al., 2014; Ramos, 2012).

One botanical extract may contain thousands of compounds, exhibiting residual complexity based on their original botanical source. An extract and its residual complexity can be investigated by analyzing separation fractions. Combining active fractions or enriching bioactive compounds in the extract can produce a designer extract tailored to exhibit a certain

bioactivity (Dietz et al., 2017; Ramos Alvarenga et al., 2014). For example, a menopausal

women may benefit from a hop supplement standardized to 8-PN for its estrogenic purposes, but

a premenopausal women taking a XH standardized hop extract containing little to no 8-PN

would still get the cytoprotective properties of XH (Dietz et al., 2017). Fractions may also be

selectively combined to exclude a compound or compounds from an extract to produce a knock-

out extract. This process can aid in bioactivity determination through exclusionary methods but

can also intentionally eliminate bioactivity. For example, an 8-PN knock-out hop extract may

provide resilience properties without exhibiting estrogenicity (Ramos Alvarenga et al., 2014;

Ramos, 2012).

Beside detailed information about chemical composition, the bioassay is a critical step in

determining the efficacy and safety of a botanical extract, providing concurrent chemical and

biological standardization. In the field of breast cancer, bioassays assessing estrogen receptor and

metabolic enzyme activity reveal clues as to whether an extract may exhibit resilience or

carcinogenic properties. Once bioactivity has been established *in vitro*, the extracts may be

subjected to *in vivo* testing to assess safety, pharmacokinetics, and eventually efficacy in

preclinical and clinical settings.

Specific Aims and Hypothesis

The aims of this study involve evaluating botanical extracts used in women's health for their resilience effects on interrelated pathways in estrogen chemical carcinogenesis. Underlying goals of these evaluations include the identification of botanical extracts and their bioactive compounds that modulate targets in the estrogen chemical carcinogenesis pathway *in vitro*. A new resilience pathway is proposed involving ER downregulation by AhR to preferentially increase P450 1A1. This may potentially reduce estrogen chemical carcinogenesis by increasing the ratio of 2-hydroxylated estrogens to 4-hydroxylated estrogens. Additional goals included establishing new epigenetic targets in the estrogen detoxification pathway. Of particular interest were hops for their effects *in vitro* and hops was chosen for *in vivo* analysis with the goal of identifying compound distribution in pre- and postmenopausal rat models. The overall *hypothesis* of this study is that botanicals which beneficially influence the estrogen detoxification pathway may provide resilience by reducing estrogen chemical carcinogenesis. To test this hypothesis, the following aims were proposed:

1. Identification of botanical extracts and bioactive compounds that modulate the estrogen chemical carcinogenesis pathway.

2. Evaluation of mechanisms and epigenetic targets of botanical extracts and bioactive compounds in the estrogen chemical carcinogenesis pathway.

3. Determination of *in vivo* safety and distribution of hop prenylated flavanones and chalcones *in vivo* as an indicator of potential *in vivo* activity.

The present study used the ER+ breast cancer cell line MCF-7 to evaluate botanical extracts and pure compounds. Mechanisms of action were explored through characterization of effects on targets within the estrogen chemical carcinogenesis pathway, with a particular interest in the

estrogen detoxication enzyme CYP1A1 (P450 1A1). Revealing itself as having the most

potential for estrogen detoxication, the clinical hop extract was chosen for an *in vivo* Sprague-

Dawley rat study to analyze tissue distribution of hop compounds. 6-PN from hops was shown to

beneficially influence the estrogen detoxification pathway *in vitro* and accumulate in the

mammary gland of postmenopausal model (ovariectomized and lacking circulating estrogens)

rats. This points to the importance of 6-PN characterization in hop dietary supplements and their

potential for estrogen detoxification and resilience in breast cancer.

Chapter 2: Materials and Methods

In part reprinted (adapted) with permission from Hitzman, *et al.*, 6-Prenylnaringenin from Hops Disrupts ERalpha-Mediated Downregulation of CYP1A1 to Facilitate Estrogen Detoxification. Copyright 2020, American Chemical Society.

Chemicals and Reagents

The chemically standardized clinical spent hop (*Humulus lupulus* L., Cannabaceae) extract, containing 33.20% xanthohumol, 1.22% 6-prenylnaringenin, 1.11% isoxanthohumol, and 0.28% 8-prenylnaringenin, was originally obtained from Hopsteiner (New York, NY, U.S.A. and Mainburg, Germany). This extract was characterized by LC-MS/MS, LC-UV, and ^{1}H NMR (qHNMR) and has been used for previous studies, including a Phase 1 clinical trial in postmenopausal women (Dietz et al., 2017; van Breemen et al., 2014; Wang et al., 2016). Standardized red clover extract (RCE) from *Trifolium pratense* aerial parts was autohydrolyzed using proprietary methods by PureWorld Botanicals, Inc. (South Hackensack, NJ, USA). All presented biological assays also utilized compounds purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) as follows: DMSO, E_2, estradiol benzoate, ethanol, polyethylene glycol (PEG), Tween-20, ICI 182 780 (ICI; Fulvestrant), (±)-6-PN, IX, XH, irilone, formononetin, biochanin A, licochalcone A, liquiritigenin, isoliquiritigenin, silibinin, isosilybinin, herbacectin, Z-liguistilide, icaritin, and desmethylicaritin. TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) was purchased from AccuStandard (New Haven, CT, U.S.A.). 6-PN and 8-PN were previously analyzed and showed a purity of >95%, while XH had a purity of >90% (Bolton et al., 2019; Dietz et al., 2017; Hajirahimkhan et al., 2018). 95.6% pure (+/-)-8-PN was previously obtained by synthesis from Dr. Giovanni Appendino (Overk et al., 2008).

Cell Culture and Conditions

MCF-7 WS8 cells were originally provided by Dr. V. C. Jordan and are generally referred to as MCF-7 cells in this dissertation. These cells were cloned from MCF-7 cells as previously described and were chosen due to increased ER expression and estrogen sensitivity in protein expression and cell growth assays (Jiang et al., 1992; Pink et al., 1995). Otherwise, no significant phenotypic differences have been observed between the parental MCF-7 and the MCF-7 WS8 cell line (Pink et al., 1995). Cells used in experiments were kept within 15 passages and grown in RPMI 1640 media without phenol red containing 10% fetal bovine serum, 1% nonessential amino acids, 1% glutaMAX, 1% AB/AM, and 0.05% insulin (Thermo Fisher, Waltham, MA, U.S.A.). The Ishikawa endometrial carcinoma cell line were originally provided by Dr. R. B. Hochberg and maintained in phenol red free or phenol red containing DMEM/F12 with 10% fetal bovine serum, 1% sodium pyruvate, 1% nonessential amino acids, 1% glutaMAX, and 0.05% insulin. The MCF-7 WS8 cell line used for these studies was authenticated as previously published (Dunlap et al., 2017). The MCF-7 WS8 cells were 93% similar to the MCF-7 cells from ATCC; but contain an allele deletion (D5S818:12), indicating a slight difference between the MCF-7 WS8 subclone and the ATCC MCF-7 cell line. Well defined short tandem repeat profiles of cell lines were used for authentication. Cell treatments consisted of either 10 nM TCDD, 10 nM E_2, 1, 5, or 10 µg/mL extracts, and 1 or 10 µM compounds unless otherwise stated.

Animal Treatments

Ethical approval for this study was granted prior to its initiation, animal care committee approval number 19-218. For the animal study in chapter 6, female intact and ovariectomized (OVX) female Sprague-Dawley rats (56 days of age) were divided in four groups after an

acclimation time of 14 days. Rats were fed a standard diet devoid of phytoestrogens. Each set of animals was divided into a vehicle control group (PEG-300 70%, EtOH 20%, tween 5%, water 5%), estradiol sulfate (E_2) as positive control group (60 µg/kg BW), 8-PN group (4 mg/kg BW), or hop extract (1000 mg/kg BW), a high dose intended to provide enough of hop prenylated compounds for detection *in vivo*, with six animals in each group. All treatments were given orally by gavage for three days and the animals were sacrificed on the 4th day and following sacrifice an uterotrophic assay protocol using excised uterus which involved weighing the wet uterus was performed (Christian et al., 1998). Blood was withdrawn right after sacrifice for serum analysis, and additional tissues (liver, spleen, small intestine, mammary gland, uterus) were collected and frozen in liquid nitrogen then stored at -80°C.

Tissue Preparation for LC-MS/MS

Tissues were weighed then broken down manually. Samples were added to 70% MeOH and 30% Millipore dH_2O and sonicated to produce a homogenate. Tissue and blood were centrifuged at 4°C and 15,000-rpm for 15 minutes. Supernatant and serum were collected for further purification. MeOH was evaporated from samples at 30°C under vacuum centrifugation. 500 µL of 100 mM sodium acetate, 8-isopentylnaringenin (internal calibrant), ^{13}C labeled xanthohumol, and 200 units *Helix pomatia* glucuronidase (Sigma-Aldrich, St. Louis, MO) was added to each sample and obtained from a previous study (Nikolic and van Breemen, 2013). Samples were then incubated at 37°C on a 300-rpm shaker for 1 hour. 2 mL of ether was added to each sample and vortexed for 45 seconds before centrifugation. Supernatant was removed and liquid-liquid extraction was repeated before drying under nitrogen gas. Calibration curve samples were prepared from vehicle treated tissue spiked with standards of xanthohumol, 8-prenylnaringenin, 6-prenylnaringenin, and isoxanthohumol and processed as described above.

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Tissue Sample Analysis for LC-MS/MS

Dried, frozen samples were reconstituted in 200 μL of 70% MeOH 30% Millipore dH$_2$O. Samples and calibration standards were added to a Shimazdu (Kyoto, Japan) Nexera UHPLC system and LCMS-8060 triple quadrupole mass spectrometer instrument. A 3-minute gradient from 40% to 70% acetonitrile/0.1% formic acid with a flow rate of 0.4 mL/min and a column temperature of 40 °C was used to separate analytes on a C18 2.0 x 50 mm 2.5 μm Waters Xbridge column. A negative ion-electrospray LC-MS/MS was performed under selected reaction monitoring (SRM) conditions. Standard calibration curves were produced for XH, IX, and 8-PN. SRM for xanthohumol and isoxanthohumol m/z was 353-233+119; 8-PN and 6-PN m/z was 339-219+119. Standard curves were used to calculate amounts of compounds in LC-MS samples and amounts of compounds in tissue and serum were calculated using mass or volume of original sample. LC-MS/MS analysis was carried out as previously described using LabSolutions (Atlanta, GA, USA) software (Keiler et al., 2017a; Keiler et al., 2017b; Nikolic and van Breemen, 2013).

Instrumentation for DESIGNER Fractions

Preparation of DESIGNER fractions was achieved by Dr. Gonzalo Malca-Garcia in the lab of Dr. Guido F. Pauli, Botanical Center Project 1. The protocol followed was according to previously published studies (Malca Garcia et al., 2019; Malca-Garcia et al., 2021). Briefly, DESIGNER fractions were generated with a SCPE-250 centrifugal partition chromatography (CPC), Armen Instrument Gilson Inc. (Vannes, France). The flow rate was 50 mL/min with a rotation speed of 2500 rpm, and a maximum pressure of 300 bar. Solvents were evaporated on a miVac (Speed Vac, Model: QUC-12060-C00, Genevac LTD. Ipswich, England) centrifugal vacuum concentrator and a Labconco™ Benchtop FreeZone™ freeze-dry system (Kansas City,

MO, USA) lyophilizer. A Waters 2695 (Waters Corp., Milford, MA, USA) solvent delivery system connected to a Waters SYNAPT quadrupole/time-of-flight (Q/TOF) mass spectrometer operated in the negative ion electrospray mode was used for LC-MS/MS quantification. A Shimadzu (Kyoto, Japan) Nexera UHPLC-UV system was used for UHPLC and employed a Kinetex 1.7 μm XB-C18 column (50mm×2.1 mm). Data analysis was done using LabSolutions software. Temperature in the detector cell, column oven, and autosampler temperature remained kept at 40 °C, 40 °C and 4 °C, respectively. NMR spectra were acquired on a JEOL 400 MHz NMR spectrometer at 298 K. JEOL Delta v5.0.4.4 NMR data processing software was used for NMR data as well as Mestrenova 11.0.4 software (Santiago de Compostela, Spain). Before NMR analysis, each sample was dried in a desiccator under vacuum (<1 mbar overnight in order to remove residual solvents. Samples were dissolved in 250 μL DMSO-d_6 for which a 1000 μL analytical syringe (Valco Instruments, Baton Rouge, LA USA) was used to deliver 200 μL into a 3 mm tube (NORELL, Landisville, NJ, USA).

LC-MS of Target Compounds in Red Clover

A Waters YMC AQ C18 column (2×10 mm, 3 μm particle size) was used for LC-MS separations. The mobile phase consisted of methanol and 0.1% formic acid or acetonitrile, with a linear gradient from 10% to 95% B over 30 min. The flow rate was 0.2 mL/min, and the column temperature was set at 30 °C. A mass lock of Leu-enkephalin and a resolving power of 10,000 provided high resolution accurate measurement. Using argon as the collision gas, ion spectra for the products were recorded at 15 or 25 eV. In order to identify compounds, MS/MS and molecular compositions were linked with the typical spectra using botanical center and publicly generated databases, in conjunction with primary literature spectra.

Quantification of Estrogenic/Antiestrogenic/Cytotoxic Activity

This assay was performed according to previous published studies (Hajirahimkhan et al., 2018; Pisha, 1997). Ishikawa cells were grown until about 90% confluency in 96 well plates in estrogen free or 2 nM estradiol for 3 days. Cells were treated with test compounds for 24 hours before lysis with Tris/Triton solution and -80 °C incubation. Thawed cells were subjected to *p*-nitrophenyl phosphate, and alkaline phosphatase activity was measured spectrophotometrically at 405 nm on a BioTek Synergy plate reader. Calculations used the kinetic curve average slope. Estrogenic activity was represented as percentage of alkaline phosphatase induction compared to estradiol treatment, while the percentage of treatment compared to the background control in 2 nM estradiol media was used to represent antiestrogenic activity. Sulforhodamine (SRB) assay was done for cytotoxicity according to published methods (Dietz et al., 2017). Calculations were as follows: estrogenic activity % = [(sample slope – DMSO slope)/(estrogen slope – DMSO slope)]*100; antiestrogenic activity % = [(sample slope – cells slope)/(DMSO slope – cells slope)]*100; cytotoxicity (% growth at 515 nm absorbance) = [(sample OD – day 0 sample OD)/(DMSO OD – day 0 sample OD)]*100.

Quantification of In-Cell Protein Expression

In-Cell Western (ICW) was used as a high-throughput assay for quantification of ERα and β-actin expression, comparable to a traditional Western blot. MCF-7 cells were plated at 50,000 cells/well and incubated at 37 °C in 5% CO_2 for 2 days before treating overnight. Next, 2 h pretreatments were performed with the AhR antagonist, CH-223191 (Sigma-Aldrich, St. Louis, MO, USA), or the proteasome inhibitor, MG-132 (Calbiochem, San Diego, CA, U.S.A.). LI-COR (Lincoln, NE, U.S.A.) protocol for In-Cell Western was performed for fixing, staining, and detection of ERα (F10; Santa Cruz Biotechnology, Dallas, TX, USA) and β-actin (13E5; Cell

Signaling Technology, Danvers, MA, U.S.A.). Wells of equal pixel area were quantified with ImageStudioLite before subtracting background and normalizing to β-actin. The final data was represented relative to the negative control DMSO, which was set at 100%.

Quantification of AhR Activity

A luciferase driven assay was used to obtain AhR activity. MCF-7 cells were plated in 24 well plates and transfected at 70% confluency. Transfection occurred over 5 hours using Lipofectamine 2000 (Thermo Fisher, Waltham, MA, U.S.A.), 1 μg/well of xenobiotic response element (XRE) luciferase plasmid (pGL4.43; Promega, Madison, WI, U.S.A.), and 0.1 μg/well of beta-galactosidase plasmid (pSV-β-Gal; Promega, Madison, WI, U.S.A.). Cells were treated with E_2 and/or compounds for 18 h and lysis occurred with 110 μL of lysis buffer (25 mM glycyl glycine, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT, and 2% Triton X-100). Gen5 microplate reader and imager software were used on a BioTek SYNERGY multimode reader system (BioTek, Winooski, VT, USA) with 50 μL luciferin substrate (Sigma-Aldrich, St. Louis, MO, U.S.A.) per sample. Equal amounts of lysate were mixed with 50 μL of ortho-nitrophenyl-β-galactoside (ONPG) assay buffer (200 mM NaH_2PO_4, 2 mM $MgCl_2$, 7.3% β-mercaptoethanol, and 21 mM ONPG) and incubated for 4 h. Absorbance at 420 nm was measured using the BioTek SYNERGY. Data calculated using mean relative luciferase units (RLU) over the corresponding β-Gal absorbance and normalized to the averaged DMSO for the XRE-Luciferase induction.

Quantification of mRNA

Real-time reverse transcription PCR (qRT-PCR) was used to quantify *CYP1A1*, *CYP1B1*, *ESR1*, *ESR2*, *COMT*, *NQO1*, *AHR*, and *AHRR* in MCF-7 cells. These cells were plated at a density of 40,000 cells/well and incubated at 37 °C in 5% CO_2 for 2 days before 24 h treatment.

Lysis and qRT-PCR was carried out according to manufacturer's protocol for Ambion Cells to

CT 1-Step TaqMan kit from Thermo Fisher Scientific. *HPRT1* (Hs02800695_m1) was used as a

control for the genes of interest, while genes of interest included

CYP1A1 (Hs00153120_m1), *CYP1B1* (Hs00164383_m1), *ESR1* (Hs01046816_m1), *ESR2*

(Hs01100353_m1), *COMT* (Hs00241349_m1), *NQO1* (Hs00168547_m1), *AHR*

(Hs00169233_m1), and *AHRR* (Hs01005078_m1) (Thermo Fisher, Waltham, MA, U.S.A.). CT

(cycle threshold) was obtained with a StepOnePlus fluorescence detection system (Thermo

Fisher, Waltham, MA, U.S.A.) and the comparative CT method ($\Delta\Delta$CT) was used to express

fold induction relative to DMSO.

Quantification of Protein Expression from Lysate

Traditional Western blots employed a previously published protocol with minor changes

(Hemachandra et al., 2012). Briefly, MCF-7 cells were treated with compounds for 24 h before

collection and sonication. Protein concentration was determined using a BCA assay (Pierce BCA

Protein Assay Kit, Thermo Fisher, Waltham, MA, U.S.A.), and 30 µg of protein was added to

each well of a 10% bis-tris gel with LDS sample buffer (Thermo Fisher, Waltham, MA, U.S.A.).

Gel was transferred using the iBlot gel transfer device with PVDF membrane iBlot transfer

stacks (Thermo Fisher, Waltham, MA, U.S.A.). Primary antibodies (1:1000; ERα, PA1-308;

CYP1A1, PA1-340; Thermo Fisher, Waltham, MA, U.S.A.; β-actin, 4967S, Cell Signaling,

Danvers, MA, U.S.A.) were added to blocked membranes and incubated at 4 °C overnight.

Secondary antibody was added (1:1000; Horseradish Peroxidase-linked antirabbit IgG, 7074S,

Cell Signaling, Danvers, MA, U.S.A.) to membranes for 1 h at room temperature. Then,

SuperSignal West Femto (Thermo Fisher, Waltham, MA, U.S.A.) was added and imaged on a

FluorChem system (Protein Simple, Santa Clara, CA, U.S.A.). Quantification used ImageJ

software to determine ratios of protein of interest over β-actin, which were represented as relative to 100% DMSO.

Quantification of *CYP1A1* Associated with a DNMT1 Pulldown

A chromatin immunoprecipitation (ChIP) assay was performed in MCF-7 cells grown in estrogen free media on 10 cm plates until 90% confluency before treating with vs without E_2 and compounds for 12 h. Cross-linking, precipitation, pulldowns, washing, and decross-linking were all done according to previous published protocols, with the exception of antibodies used (Kastrati et al., 2016). DynaBeads Protein A (Thermo Fisher, Waltham, MA, U.S.A.) were rotated in cold for 2 h with either anti-DNMT1 antibody (ab13537, ABCAM, Cambridge, U.K.), anti-DNMT3B antibody (ab2851, ABCAM, Cambridge, U.K.), or normal mouse IgG antibody (sc-2025, Santa Cruz Biotechnology, Dallas, TX, U.S.A.). QIAquick (Qiagen, Hilden, Germany) PCR Purification Kit was used according to manufacturer's protocol to obtain DNA samples. SYBR Green I (Thermo Fisher, Waltham, MA, U.S.A.) was used to perform qPCR. Data was analyzed using the fold induction method, before normalizing to IgG control.

Quantification of Estrogen Metabolites

This protocol was based on a previously published protocol (Wang et al., 2016). E_2 metabolism was studied in MCF-7 cells that were treated with E_2 (10 nM) and hop extract (5 μg/mL), 6-PN (1 μM), or TCDD (10 nM) for 72 h and analyzed by UHPLC-MS/MS. For the last 24 h, cells were treated or cotreated with E_2 (1 μM). The samples were extracted with 4 mL dichloromethane 2x and the organic layer dried under nitrogen gas. Deuterated (d4) 4-methoxyestrone (CDN, Pointe-Claire, Quebec) was used as an internal standard. Derivation occurred with 100 μL 0.1 M $NaHCO_3$ and 100 μL of dansyl chloride in acetone (1 mg/mL) at 60 °C for 10 minutes with gentle shaking before cooling on ice. Standard curves with dansylated 2-

or 4- MeOE$_1$ (methoxyestrone) and samples were analyzed by positive ion electrospray mode on an LC-Agilent 1200 nano flow system (Agilent, Santa Clara, CA) coupled to a Qtrap 5500 (AB Sciex, Framingham, MA). Separation was performed using a C18 100 x 3 mm Waters column filled with 1.7 μm particles (Waters, Milford, CT) and kept at 40 °C. Using Analyst software (AB Sciex, Framingham, MA), the area under the curve of qualifier ions for multiple reaction monitoring conditions (534.4–171.2 for dansylated MeOE$_1$, and 538.3–171.2 for dansylated MeOE$_1$-d4) was used to calculate the amount of 2-MeOE$_1$, and 4-MeOE$_1$ and normalized to E$_2$ control.

Quantification of P450 1A and P450 1B1 Activity

Ethoxyresorufin-*O*-deethylase (EROD) activity assay measuring P450 1A or P450 1A + 1B1 enzyme activity was conducted both in MCF-7 cells as previously described, with small changes described (Wang et al., 2016). Briefly, 1 x 10^4 cells/well were plated in black-walled and rounded-bottom 96-well plates and centrifuged for 5 minutes at 1500 rpm before incubating at 37 °C and 5% CO$_2$. After 3 days, half of the wells were pretreated for one hour with 2 μM of 2,2',4,6'-tetramethoxystilbene (TMS), then the cells were treated with compounds, extracts, or controls for 24 hours in each the TMS and no TMS containing wells. Data gathered from wells containing no TMS corresponded to P450 1A + 1B1 activity, while wells containing TMS provided data for P450 1A activity. Fluorescence was measured on a BioTek SYNERGY (Winooski, VT) multi-mode reader at 530 and 590 nm for 45 minutes. Reaction rate was determined using the slope of the linear regression curves for the last 30 minutes. Data was normalized to cell diameter and then to DMSO. Cytotoxicity in MCF-7 cells was done using MTT assay dose response curves. MTT was dissolved in PBS to 5 mg/mL and 50 μL of MTT

was added to wells for 3 hours before removing and adding 150 μL DMSO, then reading

absorbance at OD = 570 nm.

Statistics

Data from independent experiments, mostly in triplicates, were expressed as mean

plus/minus the standard error of the mean (± SEM) for all assays. Fold induction methodology

was used were appropriate. Using GraphPad Prism version 8 (San Diego, CA, USA),

significance was determined using one or two-way ANOVA with Dunnett's post-test for control

and treatment group comparison. Significance was assigned using a single asterisk (*) for

any $p < 0.05$.

Chapter 3: Evaluation of Botanicals for Effects on Estrogen Resilience Pathways

Rationale and Hypothesis

Breast cancer involves complicated signaling pathway mechanisms, which influence each other through crosstalk. One such molecular pathway results in the genotoxic metabolism of estrogen. This pathway of estrogen chemical carcinogenesis involves the genotoxic metabolism of estrogens by the constitutively active P450 1B1, in the presence of estradiol. Yet P450 1B1 can be transcribed by AhR, but AhR also transcribes P450 1A1, which is normally expressed at a low level, yet is highly inducible. Selective activation and metabolism of estrogens by the detoxification enzyme, P450 1A1, results in less DNA adducts and a reduced carcinogenic potential in breast tissue (Falk et al., 2013; Fuhrman et al., 2012; Samavat and Kurzer, 2015; Ziegler et al., 2015). Evaluating targets along the estrogen metabolism pathway and its targets which exhibit crosstalk amongst each other (Figure 2) can uncover extracts and compounds which may beneficially influence targets of the estrogen detoxification pathway.

The development of most breast cancers is mediated, at least in part, by steroid hormone receptor activity. Although a lack of estrogen and progesterone receptor expression is correlated with poorer prognosis, downregulating ERα expression and activity can reduce the hormonal carcinogenic pathway and may result in reduced tumor progression (Babyshkina et al., 2019; Verma et al., 2020). Meanwhile, highly estrogenic botanical extracts may lead to an induction of hormonal breast cancer pathways. Some botanicals used in women's health downregulate ESR1 or ERα (Figure 4,6) (Hitzman et al., 2020; Szmyd et al., 2018). However, activated ERα upregulates estrogen metabolism through P450 1B1 metabolism to form carcinogenic metabolites.

High throughput assays done in 96 well plates allow for the evaluation of many botanical extracts and are a great tool to provide clues into their bioactivity profiles. The Ishikawa assay (alkaline phosphatase assay) uses the Ishikawa endometrial cancer cell line, which exhibits alkaline phosphatase specific estrogen activity and can be used to show estrogenic or antiestrogenic potential when compared to estradiol or an ability to inhibit estradiol activity, respectively. Estrogenic activity may drive hormonal carcinogenesis through ERα and can downregulate the estrogen detoxification pathway, leading to increased estrogen chemical carcinogenesis (Dietz et al., 2016; Hitzman et al., 2020). Antiestrogenic activity revealed in this assay may, through an ability to inhibit the action of estrogens at the estrogen receptor, reduce hormonal carcinogenesis. Antiestrogen breast cancer treatment involves in part blocking the action of estrogen on the estrogen receptor. Postmenopausal women may use botanical dietary supplements for the relief of menopausal symptoms due to their estrogenic activity or for their resilience properties, which may include antiestrogenic activity.

Estrogenic compounds exhibit their activity through estrogen receptors (ERs). The relative amount of ERα will influence the degree of hormonal carcinogenesis and its crosstalk targets like P450 1A1 (Babyshkina et al., 2019; Hitzman et al., 2020; Verma et al., 2020). Downregulation of ERα can reduce hormonal carcinogenesis and upregulate estrogen detoxification through the 2-hydroxylation of estrogen by P450 1A1 (Figures 1, 2). One mechanism of downregulation for ERα involves proteasomal degradation induced by activated AhR. High throughput screening of relative ERα expression can be determined using the In-Cell Western (ICW) assay. Traditional Western blots from cellular lysates analyze relative protein expression on a gel and are limited in the number of wells for samples, usually a dozen or less. The ICW assay analyzes immunofluorescent labeled protein in cells fixed to wells inside a 96-

well or even 384-well plate, permitting for many more samples to be analyzed in less time than a traditional Western blot.

The changes in mRNA transcription can be associated with similar trends in protein expression and can be used to infer changes in protein expression for key targets of the estrogen chemical carcinogenesis pathway (Koussounadis et al., 2015). The quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay measures the amount of complementary DNA (cDNA) using fluorescent probes associated with primers of interest. Primers are designed to bind to mRNA for proteins of interest, and reverse transcriptase enzymes create cDNA sequences traveling down the 3' end of the primer, creating a sequence complementary to the target mRNA. The amount of cDNA created correlates to the amount of mRNA present. The cDNA is subjected to PCR producing copies of the target. The number of cycles of PCR needed to produce enough copies needed for detection is termed the cycle threshold (CT) and correlates with the amount of starting material. This assay can reveal bioactivity of many targets within the estrogen detoxification pathway including *ESR1* (ERα), *ESR2* (ERβ), *CYP1A1* (P450 1A1), *CYP1B1* (P450 1B1), *NQO1*, and *COMT* among others. Taken together, analysis of these targets through qRT-PCR provides an *in vitro* bioactivity profile for botanical extracts used for women's health.

It was hypothesized that extracts and compounds that are antiestrogenic, degrade ERα, or downregulate *ESR1* may influence estrogen chemical carcinogenesis by reducing ERα-AhR crosstalk. ERα indirectly causes the downregulation of *CYP1A1*, the estrogen detoxification enzyme, while having no effect on CYP1B1; that is achieved through selective epigenetic methylation of *CYP1A1* (Figure 2). An increase in *AhR* or decrease in *AHRR*, the AhR repressor, may enhance AhR activity by providing more protein for AhR agonists or reducing the inhibition

of AhR (Figure 1). Extracts that activate AhR and increase *CYP1A1* may increase the 2-hydroxylation of estrogens (detoxification pathway) and thus reduce estrogen chemical carcinogenesis. Furthermore, extracts that increase the detoxification enzymes, *NQO1* or *COMT*, may further reduce estrogen chemical carcinogenesis by reducing the amount of reactive estrogen quinones and increasing the methylation of hydroxylated estrogens, respectively. The assays of bioactivity of targets with the estrogen detoxification pathway will identify the most promising botanical extracts for further mechanism of action studies.

Results & Discussion

Evaluation of ERα Expression Changes Associated with Women's Health Botanical Extracts

In-Cell Western (ICW) (Figure 4) were performed in ER+ breast cancer cells to quantitate the relative amount of ERα. A decrease in ERα may reduce estrogen induced ERα-AhR crosstalk, which may ultimately lead to an increase in the ratio of 2-hydroxylated estrogens to 4-hydroxylated estrogens. Evaluation of the expression of ERα was done through the high throughput ICW assay using extracts at 10 µg/ml and 1 µg/ml or pure compounds at 10 µM or 1 µM. General trends were observed for most samples tested in that the higher concentrations significantly decreased the expression ERα, although milk thistle showed no activity. Likely more clinically important are samples that significantly decreased ERα at 1 µg/ml or 1 µM such as hop or red clover extracts.

All three pharmacopeial species (*Glycyrrhiza glabra*, *G. uralensis*, and *G. inflata*) of the popular women's health botanical licorice were initially analyzed for changes in ERα expression (Figure 4 A.). Additionally, some of licorice's major bioactive compounds, isoliquiritigenin, and liquiritigenin were analyzed along with the *G. inflata* specific constituent, licochalcone A. No crude licorice extract or compound exhibited a significant decrease in ERα at the lower concentration, while at the higher concentration of extract (10 µg/mL) or compound (10 µM) all samples significantly decreased ERα expression with the greatest decrease seen from *G. inflata*. The present studies reveal moderate activity, but only at higher concentrations and therefore licorice species were not chosen for further mechanistic studies. Past studies have shown that *G. glabra* and *G. uralensis* ultimately increase the 4-hydroxylation of estrogens, aiding in estrogen chemical carcinogenesis, while *G. inflata* and licochalcone A decrease both 2- and 4-

hydroxylation of estrogens which may reduce estrogen chemical carcinogenesis (Dunlap et al., 2015).

Milk thistle is another popular women's health botanical. Milk thistle extract is a mixture of mostly flavonolignans collectively termed silymarin. This was chosen for testing along with potential bioactive compounds silibinin and isosilybinin, the major silymarin constituents (Figure 4 B.). Interestingly no bioactivity was detected for either compound or the silymarin extract at any of the concentrations used in the ICW assay for ERα expression. Therefore, milk thistle was not further explored.

Angelica sinensis and its phthalate bioactive marker, *Z*-ligustilide, were analyzed for ERα expression (Figure 4 C.). The *A. sinensis* extraction was performed using either CO_2, ethanol, or a mixture of ethanol and water to produce three separate extracts. The ethanol and ethanol/water extracts exhibited significant decreases in ERα for both concentrations tested. Interestingly, the *A. sinensis* CO_2 extract and *Z*-ligustilide both increased ERα expression above a basal level at the lower concentration of 1 μg/mL or 1 μM, with the former being significant. These results warranted further investigation, but as these were not resilience promoting results, *A. sinensis* was not prioritized for further mechanistic studies.

Epimedium or horny goat weed is another popular supplement for both men and women. Different *Epimedium* species are often used in Horny Goat Weed supplements, and multiple species of *Epimedium* were used to produce the *Epimedium* extract which was analyzed along with potential bioactive compounds, icaritin and desmethylicaritin, for an ability to decrease ERα expression (Figure 4 D.). The water or methanol *Epimedium* extracts contained mostly icariins, which are glycosides of icaritin. The glycosides are believed to be cleaved *in vivo* to form the bioactive aglycone, icaritin. To analyze the aglycones in the original extract matrix in cell

conditions, the methanol extract was hydrolyzed by incubating it with snailase enzyme.

Chemical analysis showed that the hydrolyzed extract contains icaritin instead of its glycoside.

As anticipated, the hydrolyzed extract exhibited a greater decrease in ERα expression than the

methanolic or water extracts. Icaritin lowered ERα expression more than desmethylicartin,

similar to the hydrolyzed extract at the lower concentration tested. This downregulation of ERα

by icaritin and the hydrolyzed extract may be in part due to the AhR agonist activity associated

with icaritin (Tiong et al., 2012). These promising results warrant further mechanistic studies for

resilience promoting properties associated with *Epimedium* and icaritin.

R. rosea or roseroot was collected from wither Alaska or China and tested for their

influence on ERα expression, along with a potential hydrolyzed bioactive compound, herbacetin

(Figure 4 E.). Interestingly the extracts from different locations exhibited different degrees of

ERα reduction. At the lower concentration of 1 μg/mL, the Alaskan extract exhibited a strong

reduction in ERα expression. Herbacetin showed no activity at either concentration and is not

likely the bioactive compound exhibiting activity in the Alaskan extract. Other potential

bioactive compounds may be rhodiolin, a lignan form of herbacetin, or monoterpene glycosides

also found in *Rhodiola*. The extract constituent makeup warrants further research to explain the

difference in bioactivity between the botanicals collected from different geographic locations.

Further exploration of red clover (*T. pratense*) will be presented in chapter 4, but the

clinical extract and knock out extracts (KOE) were prepared to remove one or both of the red

clover AhR agonists, biochanin A (BA) or formononetin (FN), and analyzed in an attempt to

initially characterize the popular women's health botanical (Figure 4 F.). Pure biochanin A and

formononetin were also analyzed for their influence on ERα expression as well as the

progesterone receptor potentiator irilone (Lee et al., 2018). The clinical and parent red clover

extract exhibited the greatest decrease in ERα expression at 1 μg/mL (Geller et al., 2009). Pure BA, FN, and irilone had similar results, all being less active than the clinical red clover extract to an insignificant extent. KOE of red clover showed less activity than its parent extract at the lower concentration tested, with little difference between each KOE. This indicated the presence of polypharmacology for the parent extract may be stronger than when one or both of the AhR agonists is knocked out. Estrogenic compounds including phytoestrogens tend to reduce ERα similarly to a negative feedback loop and red clover ER agonists, genistein, and daidzein likely contribute to the downregulation of ERα expression seen for red clover extracts.

Although the influence of hop (*H. lupulus*) extract and its bioactive compounds will be explored in further chapters, various hop extracts and bioactive compounds were examined for this chapter (Figure 4 G.). In an attempt to characterize and optimize hop extract's bioactivity, hop KOE were analyzed along with their parent clinical hop extract, which has already been optimized for resilience and menopausal symptom relief, containing 8-prenylnaringenin and high amounts of xanthohumol (Dietz et al., 2017). Interestingly, the prenylated chalcone/flavanone enriched clinical extract was the most potent of the hop extracts. A xanthohumol KOE had the least activity. As this material had low amounts of 6- and 8-prenylnaringenin, in contrast, this underscored the importance of all three compounds for bioactivity, including the degradation of ERα. When hop compounds were tested, xanthohumol did not degrade ERα more than 6- or 8-prenylnaringenin at 1 μM. This showed that each compound tested from hops degrades ERα, indicating all hop bioactive compounds influence the total effect of the clinical hop extract on ERα expression.

Mechanisms involved the degradation of ERα need further exploration for estrogenic ligands, but turnover due to ubiquitin ligase 26S proteasomal degradation has been proposed.

AhR agonists also induce the proteasomal degradation of ERα through AhR recruitment and subsequent ubiquitination (Lonard et al., 2000; Wormke et al., 2003). Estrogen induced degradation of ERα is proposed to be an integral part of estrogen signaling in the continuous turnover of ERα, but more extensive decrease of ERα, without turnover, could result in resilience promoting outcomes, as is the case for antiestrogens such as the SERD, fulvestrant, and the SERM, tamoxifen (Pinzone et al., 2004; Reid et al., 2003).

In summary, many women's health botanicals were evaluated for their ability to influence ERα expression, including hops, red clover, milk thistle, licorice, *A. sinensis*, *Epimedium*, and *R. rosea*. Most investigated extracts downregulated ERα in breast cancer cells. However, milk thistle showed no activity, and the CO_2 extract of *A. sinensis* increased ERα expression at low concentrations. At low test concentrations, some botanical extracts showed ERα expression similar to or lower than the levels seen for treatments with E_2 or TCDD, namely the clinical hop extract, the hydrolyzed *Epimedium* extract, the clinical red clover extract, and the Alaskan *R. rosea* extract. ICW allowed for high throughput screening providing many samples for analysis, making it a suitable tool for initial screening of botanical extracts. Extracts that showed no effect on ERα may have little effect on ERα-AhR crosstalk. Although this type of result does not indicate a lack of bioactivity, it makes an extract a less ideal candidate for further testing for resilience properties towards the estrogen chemical carcinogenesis pathway.

ICW-*Glycyrrhiza*

A.

ICW-Milk Thistle

B.
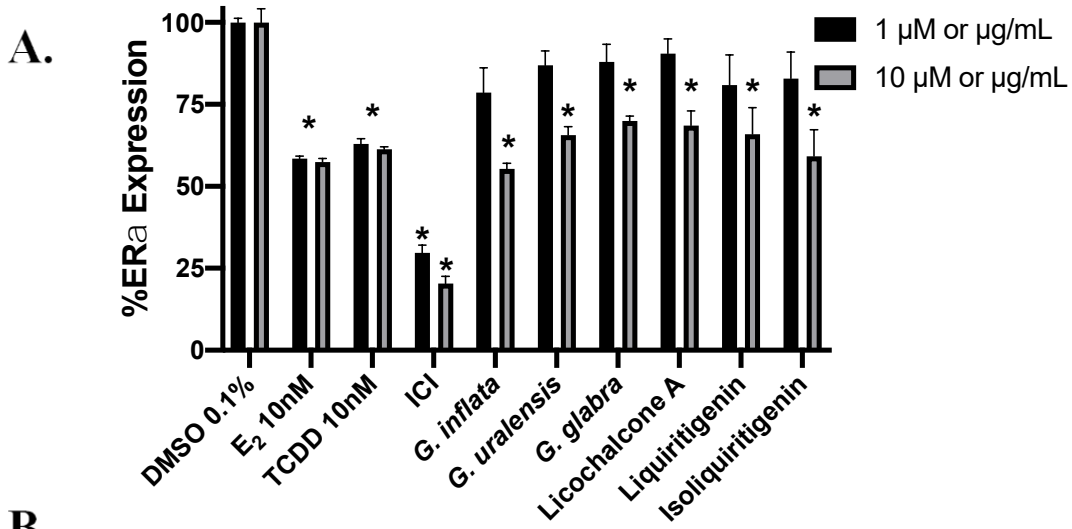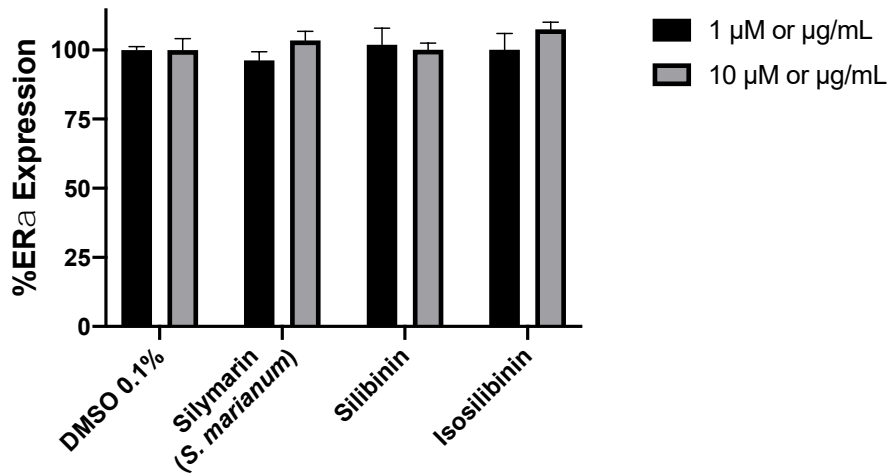
C.

ICW-*A.sinensis*
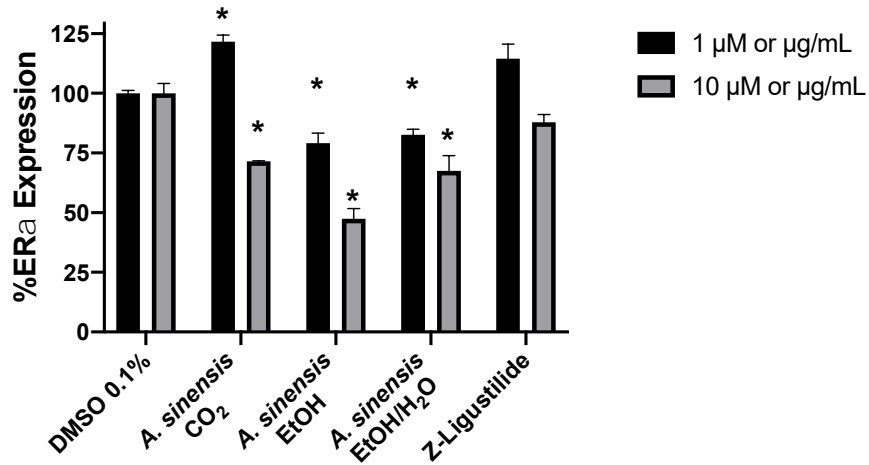
D.

ICW-*Epimedium*
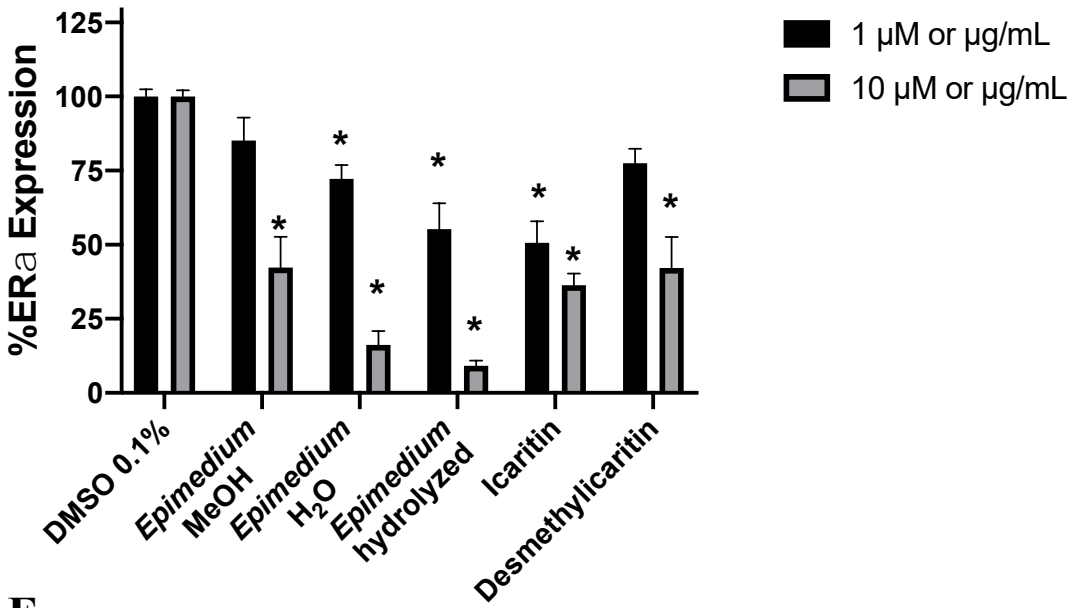
E.

ICW-*Rhodiola*
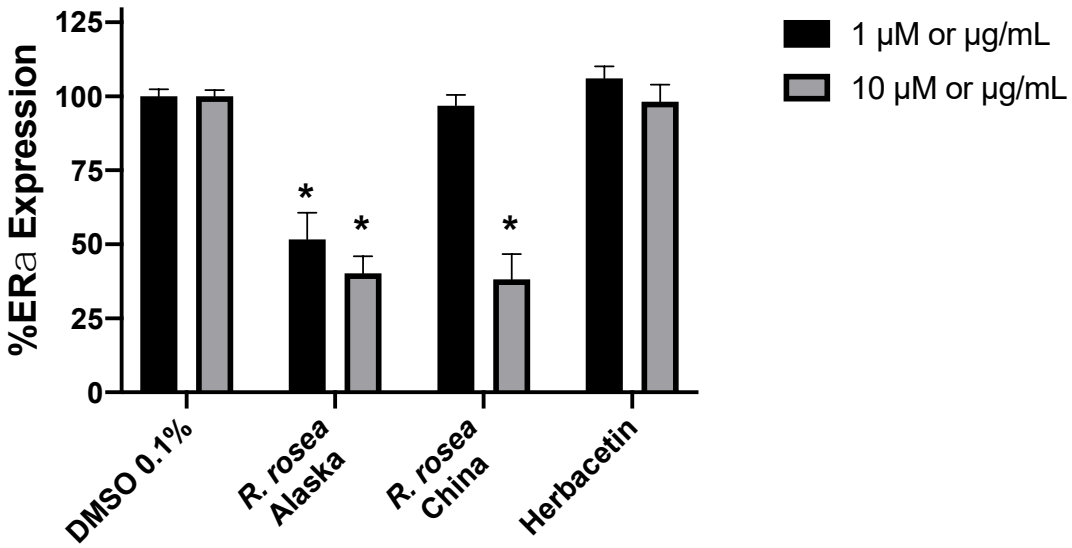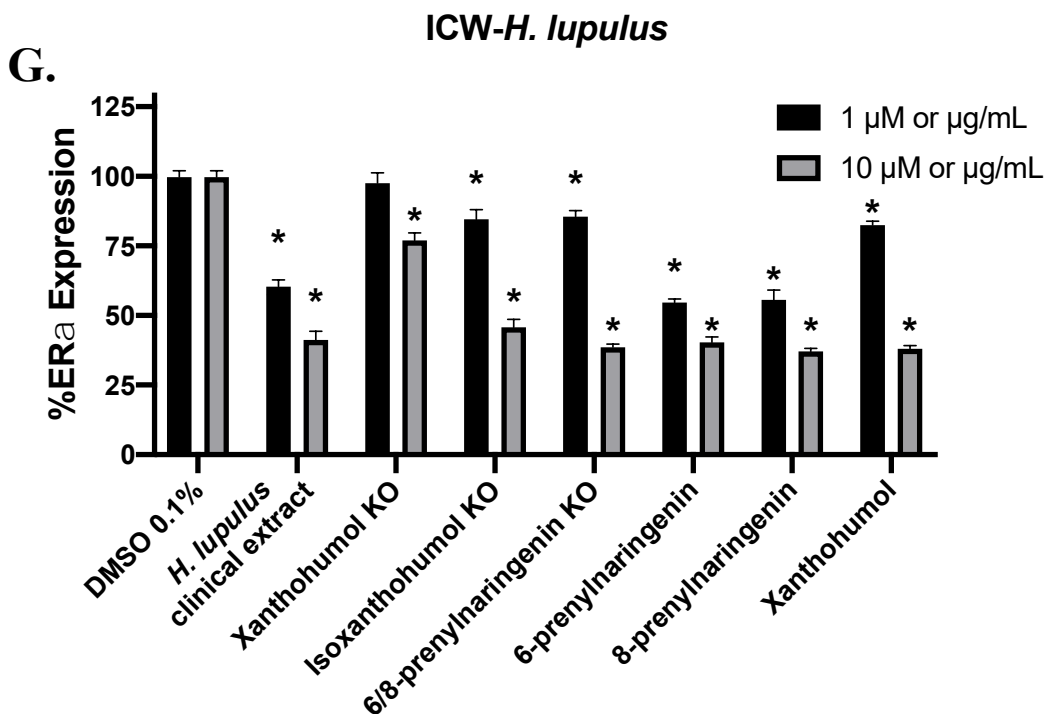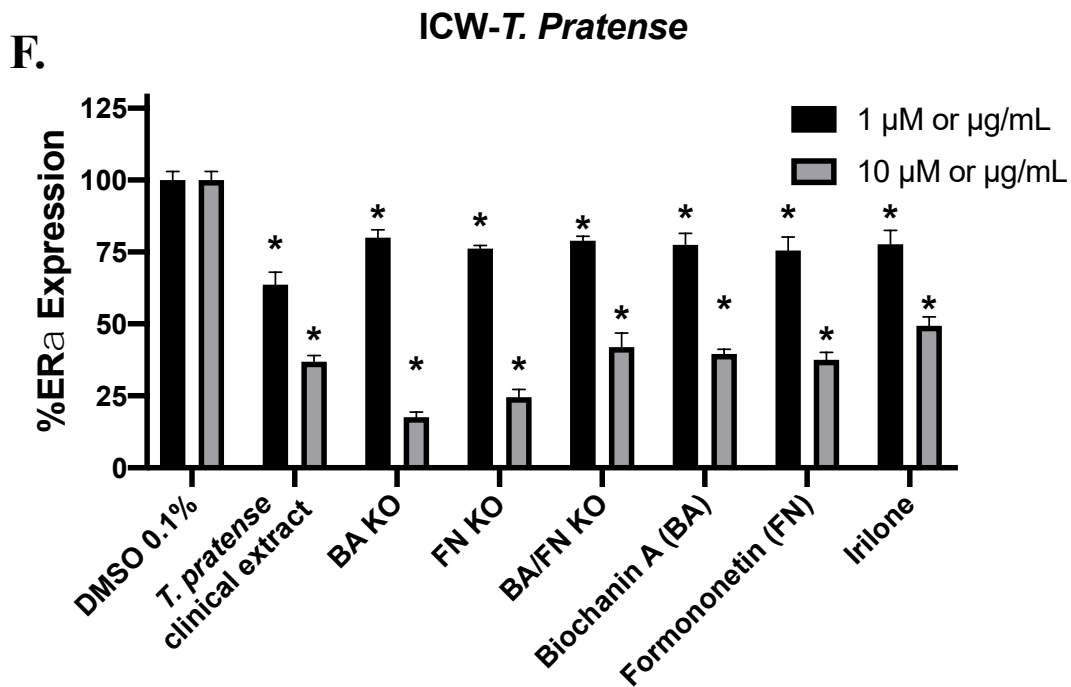
Figure 4. In-Cell Western™ (ICW) assay in MCF-7 breast carcinoma cells for estrogen receptor alpha expression with 24-hour treatments of: 0.1% DMSO; 10 nM TCDD or E_2; 1 or 10 µM compounds, 1 or 10 µg/mL botanical extracts. N ≥ 3; +/- SEM; two-way ANOVA compared to DMSO control, *$p < 0.05$.

Evaluation of Estrogenic/Antiestrogenic Activity Associated with Women's Health Botanical Extracts

The Ishikawa alkaline phosphatase assay uses ERα positive endometrial adenocarcinoma cells (Ishikawa) to test for alkaline phosphatase activity as a proxy for estrogenic activity, as the alkaline phosphatase induction depends on estrogenic compounds (Littlefield et al., 1990). This assay also tests for cytotoxicity concurrently with estrogenic and antiestrogenic activity in order to confirm antiestrogenic results, as cytotoxic doses would provide false antiestrogenic responses. It is important for the safety and efficacy of women's health botanicals to evaluate both estrogenic and antiestrogenic activity. Additionally, activated ERα causes epigenetic inhibition of the estrogen detoxification enzyme *CYP1A1*, resulting in 4-catechol estrogens and their genotoxic quinones. In turn, estrogens cause their own carcinogenic metabolism. Therefore, antiestrogenic activity may aid in reducing ERα-AhR crosstalk and reduce estrogen chemical carcinogenesis. Botanical extracts and their bioactive compounds were tested at a higher concentration (10 µM or 10 µg/mL) for comparative purposes and in order to maximize antiestrogenic activity, while avoiding cytotoxicity. The alkaline phosphatase assay cell line, Ishikawa, differed from assays done for the rest of these studies, MCF-7, yet a decrease in ERα may still allow for antiestrogenic activity as these combined bioactivities may exist. A downregulation of ERα expression may still allow for compounds to reduce the activity of ERα.

Various *H. lupulus* extracts and bioactive hop compounds were subjected to the Ishikawa alkaline phosphatase assay with consistent trends (Figure 5 A, B.). Interestingly, the clinical hop extract exhibited both estrogenic and antiestrogenic activity. An IX knockout extract (KOE) exhibited the greatest estrogenic activity, while a 6- and 8-PN KOE had the least activity. Additionally, 8-PN exhibited the most estrogenic activity of any tested compound, speaking to

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its known estrogenic response as the most potent phytoestrogen (Stulikova et al., 2018). XH and 6-PN both exhibited mixed estrogenic and antiestrogenic activity, indicating they may have importance in reducing estrogenic activity and potentially estradiol induced ERα-AhR crosstalk, although crosstalk experiments were done in MCF-7 cells and estrogenic/antiestrogenic activity in Ishikawa cells. However, at 10 µM XH was slightly cytotoxic in a Sulforhodamine B cytotoxicity assay, as was the clinical hop extract and its knockout extracts except the xanthohumol KOE, similar to published results (data not shown) (Dietz et al., 2017).

Of the licorice species, *G. glabra* and its cognate compound, glabridin, were analyzed and although both exhibited significant estrogenic and antiestrogenic activity this activity can still be considered small when compared to the controls or more potent botanicals such as hops (Figure 5 C, D.). Past studies have also shown ERα antagonistic effects for glabridin (Boonmuen et al., 2016). The red clover clinical extract was highly estrogenic, while exhibiting no antiestrogenic activity at the concentration tested (Figure 5 C, D.), similar to published results (Booth et al., 2006). The hydrolyzed *Epimedium* extract along with its major bioactive compound icaritin both exhibited strong antiestrogenic activity while icaritin also showed estrogenic activity at 10 µM (Figure 5 C, D.), although to a lower extent than previously published results (Mbachu et al., 2020). Estrogenic activity from milk thistle (*S. marianum*) or any of the *A. sinensis* extracts was absent (Figure 5 E.). However, antiestrogenic activity was present for milk thistle and the CO_2 extract of *A. sinensis*, but not the ethanolic or ethanolic/water mixture *A. sinensis* extracts (Figure 5 F.). The CO_2 extract of *A. sinensis* did however exhibit slight cytotoxicity in the Sulforhodamine B assay, possibly due to an increased *Z*-ligustilide and phthalate concentration compared to the other *A. sinensis* extracts (data not shown). The *R. rosea*

extracts exhibited differing bioactivity, with the Chinese extract being more estrogenic and less antiestrogenic than the Alaskan *R. rosea* (Figure 5 E, F.).

The Ishikawa alkaline phosphatase assay revealed how the extrsction solvent can influence extract bioactivity. Mixed agonist/antagonist activities were present for hops, *Epimedium*, and *G. glabra*. Additionally, cytotoxicity was present for xanthohumol (XH) and XH containing KOE at the concentrations tested, but knockout extracts can provide tailored bioactivity, reducing cytotoxicity by removing XH or increasing antiestrogenic activity by removing 8-prenylnaringenin. The antiestrogenic activity seen by hop extracts and hydrolyzed *Epimedium*, although mixed with estrogenic activity, is still of great interest for a potential ability to influence ERα-AhR crosstalk.

A. Ishikawa Estrogenic Activity

10 μg/mL or μM

% of E₂ (estrogenic activity)

DMSO 0.1%, E₂ 2 nM, *H. lupulus* clinical extract, Xanthohumol KO, Isoxanthohumol KO, 6/8-prenylnaringenin KO, 6-prenylnaringenin, 8-prenylnaringenin, Xanthohumol, ICI 1 μM

B. Ishikawa Antiestrogenic Activity

10 μg/mL or μM

% Reduction in Estrogenic Activity

DMSO 0.1%, E₂ 2 nM, *H. lupulus* clinical extract, Xanthohumol KO, Isoxanthohumol KO, 6/8-prenylnaringenin KO, 6-prenylnaringenin, 8-prenylnaringenin, Xanthohumol, ICI 1 μM

C.

Ishikawa Estrogenic Activity

10 μg/mL or μM

y-axis: % of E$_2$ (estrogenic activity)

x-axis: DMSO 0.1%, E$_2$ 2 nM, G. glabra, Glabridin, Epimedium hydrolyzed, Icaritin, T. pratense clinical extract, ICI 1 μM

D.

Ishikawa Antiestrogenic Activity

10 μg/mL or μM

y-axis: % Reduction in Estrogenic Activity

x-axis: DMSO 0.1%, E$_2$ 2 nM, G. glabra, Glabridin, Epimedium hydrolyzed, Icaritin, T. pratense clinical extract, ICI 1 μM

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E.

Ishikawa Estrogenic Activity

10 μg/mL or μM

y-axis: % of E_2 (estrogenic activity)

x-axis: DMSO 0.1%, E_2 2 nM, Silymarin (S. *marianum*), Silibinin, Isosilybin, A. *sinensis* CO$_2$, A. *sinensis* EtOH, A. *sinensis* EtOH/H$_2$O, R. *rosea* Alaska, R. *rosea* China, ICI 1 μM

F.

Ishikawa Antiestrogenic Activity

10 μg/mL or μM

y-axis: % Reduction in Estrogenic Activity

x-axis: DMSO 0.1%, E_2 2 nM, Silymarin (S. *marianum*), Silibinin, Isosilybin, A. *sinensis* CO$_2$, A. *sinensis* EtOH, A. *sinensis* EtOH/H$_2$O, R. *rosea* Alaska, R. *rosea* China, ICI 1 μM

Figure 5. Ishikawa alkaline phosphatase assay in ERα positive endometrial carcinoma cells for estrogenic and antiestrogenic activity in botanical extracts and potentially bioactive compounds. A, C, E: Estrogenic activity compared to 100 % E_2. B, D, F: Anti-estrogenic activity compared to 100 % DMSO with all wells containing 2nM E_2 except DMSO. N = 3; +/- SEM; one-way ANOVA; *p < 0.05.

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Evaluation of Changes in Gene Transcription Associated with the Estrogen

Detoxification Pathway by Women's Health Botanical Extracts

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a powerful tool for the quantitative analysis of messenger ribonucleic acid (mRNA) levels, and in turn predictive of changes in protein expression. qRT-PCR is a high throughput method to analyze multiple mRNA targets. Analysis of gene transcription in this case was done on mRNA targets in the estrogen carcinogenesis pathway in an attempt to further characterize the influence of women's health botanical extracts on estrogen carcinogenesis and their potential for resilience promotion. Although these assays explored genomic effects, non-genomic effects may also exist for women's health BDS, with importance also placed on protein expression and activity.

Aside from inducing estrogen hormonal carcinogenesis in the breast, estrogen induced ERα triggers the epigenetic inhibition of *CYP1A1* (Figure 2). This can indirectly lead to estrogen chemical carcinogenesis through preferential estrogen metabolism by P450 1B1 to genotoxic 4-hydroxylated estradiol. *ESR1* (Figure 6) is the mRNA transcript of ERα and was analyzed to gauge changes in ERα transcription. Reduced *ESR1* transcription may reduce estrogen carcinogenic outcomes, although estrogens can also downregulate *ESR1*, changes in *ESR1* transcription are cyclic for E_2 (Pinzone et al., 2004). Not all estrogenic botanical extracts downregulate *ESR1*: notably, the red clover extract was highly estrogenic (Figure 4C), but upregulated *ESR1* transcription. Hop extract and its bioactive compounds all downregulated *ESR1*. The hydrolyzed *Epimedium* extract exhibited greater downregulation of *ESR1* than the unhydrolyzed water extract, similar to that of silymarin and the Alaskan *R. rosea* extract which interestingly showed opposite results of the Chinese *R. rosea* extract, increasing *ESR1*. Similar to the *R. rosea* extracts, the *A. sinensis* extracts exhibited varying degrees of activity with the water

extract increasing *ESR1* and the ethanolic and CO_2 extracts decreasing *ESR1*. Two of the licorice (*G. glabra*, *G. inflata*) extracts slightly downregulated *ESR1*, while *G. uralensis* had no activity. Although ERα transcription was explored for this assay, targets of ERα-mediated transcription would also be valuable for future studies

ERβ activity reduces the hormonal carcinogenic nature of ERα and upregulation of its mRNA transcript, *ESR2* (Figure 7), may be a sign of resilience promoting properties. Interestingly, the synthetic dioxin and AhR agonist, TCDD, downregulated *ERS2*, a mechanism in need of further study, however evidence suggests TCDD upregulates *ESR2* after 48 hours, and results from this study were done after 24 hours (Dasmahapatra et al., 2001). The hop prenylated flavanone and AhR agonist, 6-prenylnaringenin (6-PN), increased *ERS2* transcription; while the clinical hop extract and the other hop compounds of interest had no activity on *ESR2* transcription. *Epimedium*, red clover, and *R. rosea* extracts had little activity on ERβ mRNA transcription at the concentration tested. All licorice extracts increased *ESR2* transcription along with the water and ethanolic extract of *A. sinensis*, although the CO_2 extract of *A. sinensis* significantly decreased *ESR2*. ERβ transcription was explored for this assay but ERβ expression would be valuable for future experiements.

AHR, the mRNA transcript of the aryl hydrocarbon receptor (AhR), was analyzed to gain insight into botanical extracts' ability to affect AhR transcription and potentially influence estrogen chemical carcinogenesis (Figure 8). Little is known about *AHR* inducibility, but epigenetic regulation is suspected (Englert et al., 2012). Interestingly, hop extract as well as XH and 8-PN had little effect on *AHR*, yet the AhR agonist, 6-PN, did significantly increase *AHR*. *Epimedium* and red clover extracts had no influence on *AHR*. Silymarin and the *R. rosea* extracts significantly decreased *AHR* transcription. *G. inflata* was the only active licorice extract,

significantly upregulating the transcription of *AHR*, and indicating licochalcone A may be worth evaluating for *AHR* transcription. The *A. sinensis* extracts varied in *AHR* transcription with the CO_2 and ethanolic extracts decreasing and the water extract increasing *AHR*. AhR transcription was explored for this study but knockdown of AhR with siRNA or AhR expression may provide valuable information on the role AhR plays in the regulation of AhR-ERα crosstalk by estrogens.

The repressor of AhR, AhRR, is transcribed by *AHRR* (Figure 9) and is normally bound to AhR nuclear translocator (ARNT), preventing translocation of AhR into the nucleus and inhibiting the activity of AhR (Larigot et al., 2018). In a negative feedback loop, AhR agonists also increase *AHRR* transcription, exemplified by the prototypical AhR agonist, TCDD. Interestingly, 8-PN and hop extract decreased *AHRR* transcription significantly, while expectedly 6-PN increased *AHRR*, albeit insignificantly at 1 µM. *Epimedium* had no effect on *AHRR* while red clover, silymarin, and *R. rosea* extracts all significantly downregulated *AHRR*. No licorice extract had activity on *AHRR*. The *A. sinensis* extracts again varied in activity with the CO_2 and ethanolic extracts decreasing and the water extract increasing *AHRR* transcription.

P450 1A1 is an AhR inducible enzyme responsible for the 2-hydroxylation of estrogens (Figure 1) and, therefore, the estrogen detoxification pathway and is transcribed by *CYP1A1* (Figure 10). Hop extract and 6-PN significantly upregulate *CYP1A1*, while 8-PN insignificantly downregulates it. The hydrolyzed *Epimedium* extract and the Alaskan *R. rosea* also significantly increased *CYP1A1*. Silymarin and red clover extract downregulate *CYP1A1*, albeit insignificantly. Genistein from the clinical red clover extract may be responsible for the downregulation of *CYP1A1*. Licorice extracts had little activity, however *G. inflata* insignificantly increased *CYP1A1* at the concentration tested, differing slightly from published results (Wang et al., 2018). The *A. sinensis* CO_2 and ethanolic extract downregulated *CYP1A1*,

while the water extract upregulated it. Both the hop extract and the hydrolyzed *Epimedium* extract contain the known AhR agonists, 6-PN and icaritin, respectively, and these constituents are responsible for much of the *CYP1A1* induction seen for those extracts (Hitzman et al., 2020; Tiong et al., 2012).

P450 1B1 is constitutively active and is transcribed by *CYP1B1* (Figure 11) through AhR, by which AhR agonists may significantly upregulate its transcription. P450 1B1 is responsible for the 4-hydroxylation of estrogens, which form genotoxic quinones and therefore is considered the estrogen genotoxic pathway (Figure 1). Similar to *CYP1A1*, hop extract and 6-PN increased *CYP1B1* transcription, but to a lesser extent than *CYP1A1*. *Epimedium*, red clover, silymarin, and the Chinese *R. rosea* had no activity, but the Alaskan *R. rosea* significantly increased *CYP1B1*, albeit to a lesser extent than *CYP1A1*. Of the licorice species *G. inflata* significantly increased *CYP1B1*. The CO_2 and ethanolic *A. sinensis* extracts significantly decreased *CYP1B1*.

The basis of estrogen chemical carcinogenesis is the reactive 4-quinone estrogens, formed by 4-hydroxylated estrogens. NQO1, transcribed by *NQO1* (Figure 12), reduces quinones to catechols, thereby reducing their carcinogenicity and making it an important enzyme in the estrogen detoxification pathway (Figure 1). Hop extract as well as XH and 6-PN significantly increased transcription of *NQO1*. The hydrolyzed *Epimedium* extract also significantly increased *NQO1*, but not the unhydrolyzed form, speaking to the bioactivity of icaritin. Neither red clover nor *R. rosea* extracts had any activity, while silymarin significantly decreased *NQO1* transcription. Of the licorice species, *G. glabra* and *G. inflata* significantly increased *NQO1* with *G. glabra* showing similar results in MCF-10A cells (Hajirahimkhan et al., 2015). Again, the *A. sinensis* extracts varied in bioactivity with the CO_2 and ethanolic extracts downregulating *NQO1*, while the water extract increased *NQO1* transcription.

Methylated catechol estrogens are not genotoxic and are a sign of estrogen detoxification. COMT (Figure 13) is responsible for the methylation of 2- and 4- hydroxylated estrogens and it is an important enzyme in the estrogen detoxification pathway (Figure 1) (Snelten et al., 2012). Hops and its bioactive compounds had little effect on *COMT* transcription at the tested concentration after 24 hours. *Epimedium*, red clover, and Chinese *R. rosea* had no effect, whereas the Alaskan *R. rosea* and silymarin significantly decreased *COMT*. Licorice extracts had no activity, and the *A. sinensis* extracts varied with CO_2 and ethanolic forms of the extract downregulating *COMT* and the water extract increasing it. Interestingly, little is known about *COMT* inducibility. Yet, estrogen and estrogenic soy isoflavones decrease *COMT* transcription, however, the estrogenic 8-prenylnaringenin did not significantly downregulate *COMT* at the concentration tested in this experiment after 24 hours (Lehmann et al., 2008).

When analyzing the results of all qRT-PCR in the estrogen carcinogenesis pathway, clear trends emerge for botanical extracts. Hop extract downregulated *ESR1* and *AHRR* while upregulating *NQO1* and *CYP1A1*. These results suggest an ability to reduce estrogen chemical carcinogenesis by reducing ERα, AhR inhibition, estrogen quinones, and ultimately increasing the 2-hydroxylation of estrogens in breast cancer cells. Similar to hop extract, hydrolyzed *Epimedium*, but not the unhydrolyzed form, decreased *ESR1*, and increased *NQO1* and *CYP1A1*, suggesting a reduction in estrogen chemical carcinogenesis. Silymarin from *S. marianum* did decrease *ESR1* yet it decreased *CYP1A1*, *NQO1*, *COMT*, and *AHR*, suggesting silymarin may not reduce estrogen chemical carcinogenesis. The red clover extract showed little bioactivity on most qRT-PCR targets analyzed at the concentration tested; however, it did increase *ESR1* and decrease *CYP1A1*, suggesting no reduction in estrogen chemical carcinogenesis in MCF-7 breast cancer cells. The two *R. rosea* extracts varied greatly, with the Alaskan extract decreasing *ESR1*

and preferentially increasing *CYP1A1* over *CYP1B1*, although it did also decrease *COMT*. This suggests that the Alaskan *R. rosea* extract may reduce estrogen chemical carcinogenesis to a greater extent than the Chinese *R. rosea* extract in breast cancer cells. Licorice species showed mixed results with *G. inflata* decreasing *ESR1*, increasing *ESR2*, *AHR*, and *NQO1*, although it also preferentially increased *CYP1B1* over *CYP1A1*. *G. glabra* also decreased *ESR1*, increased *ESR2*, and *NQO1*, while having no bioactivity on the transcription of P450 1A1 or 1B1. The *A. sinensis* extracts varied greatly depending on the solvent used in the extraction process. The water extract of *A. sinensis* increased the transcription of every mRNA tested in the estrogen chemical carcinogenesis pathway except *CYP1B1*. The increase in *ESR1*, and *AHRR* may not be beneficial, but the overall bioactivity of the water extract seemed to have a safer profile than the CO_2 and ethanolic extracts, which decreased every mRNA transcript tested. Testing was done at one concentration for all extracts and compounds (1 µg/mL or 1 µM), respectively, in order to obtain a snapshot of bioactivities at noncytotoxic doses. Dose-response curves to obtain a fuller picture of extract bioactivity would be beneficial in future studies, but at 1 µg/mL no extract was cytotoxic in MCF-7 cells, however the CO_2 *A. sinensis* extract began to show cytotoxicity at a higher dose, and may explain the downregulation of all gene transcription observed for the *A. sinensis* CO_2 extract. Furthermore, changes in mRNA transcription are often time dependent, and may vary drastically by the hour or day. qRT-PCR was shown for one time point of 24 hours for consistency and to maximize *CYP1A1* induction.

A.

PCR-*ESR1*

B.

PCR-*ESR1*

C.

PCR-*ESR1*
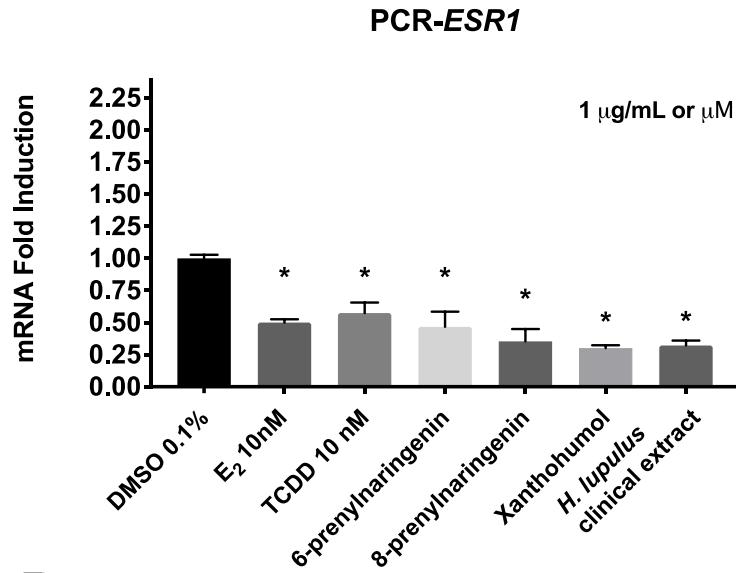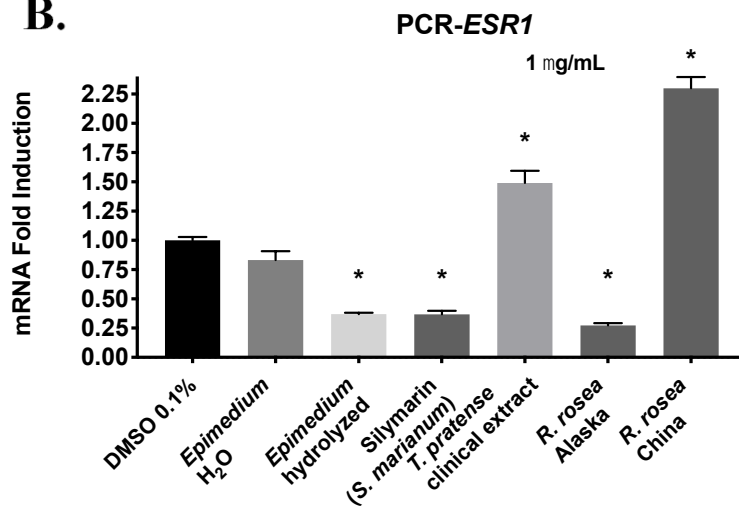
Figure 6. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) in MCF-7 breast carcinoma cells for ESR1 mRNA with 24-hour treatments of botanical extracts and hop compounds. $N \geq 3$; +/- SEM; one-way ANOVA, *$p < 0.05$.

Figure 7. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) in MCF-7 breast carcinoma cells for ESR2 mRNA with 24-hour treatments of botanical extracts and hop compounds. $N \geq 3$; +/- SEM; one-way ANOVA, *$p < 0.05$.

Figure 8. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) in MCF-7 breast carcinoma cells for AHR mRNA with 24-hour treatments of botanical extracts and hop compounds. $N \geq 3$; +/- SEM; one-way ANOVA, *$p < 0.05$.

Figure 9. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) in MCF-7 breast carcinoma cells for AHRR mRNA with 24-hour treatments of botanical extracts and hop compounds. $N \geq 3$; +/- SEM; one-way ANOVA, *$p < 0.05$.
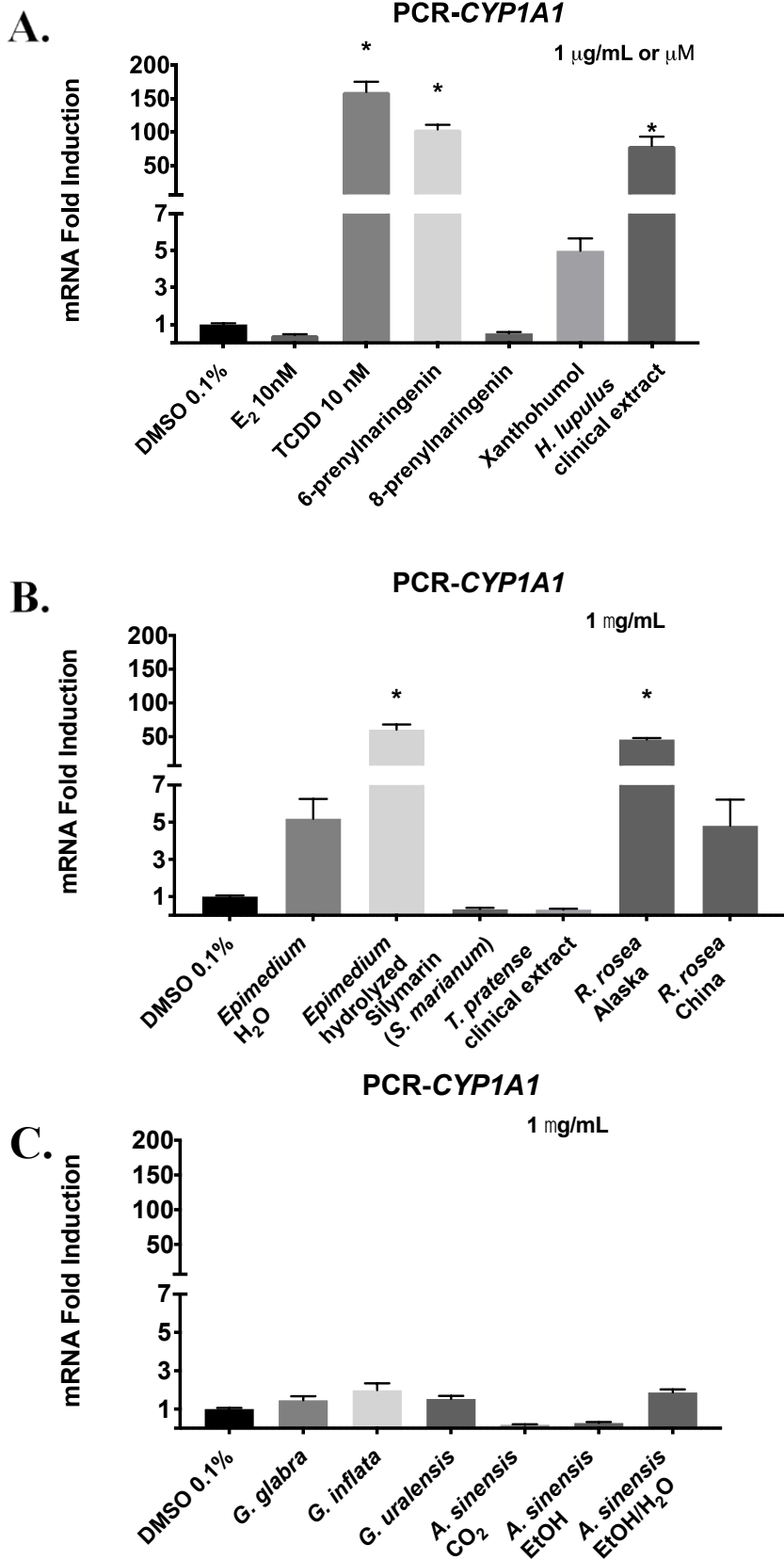
Figure 10. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) in MCF-7 breast carcinoma cells for CYP1A1 mRNA with 24-hour treatments of botanical extracts and hop compounds. $N \geq 3$; +/- SEM; one-way ANOVA, $*p < 0.05$.
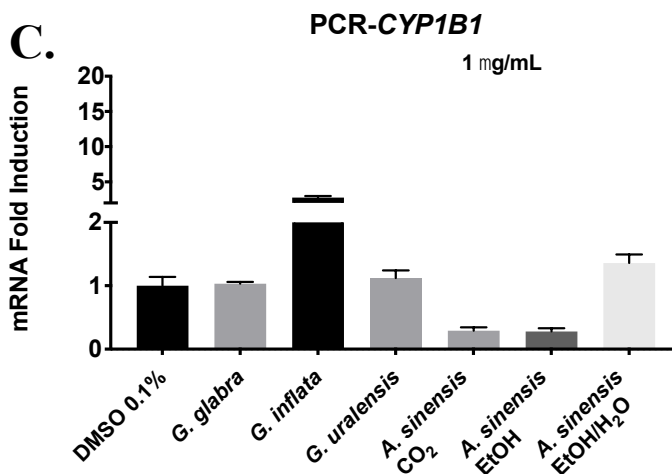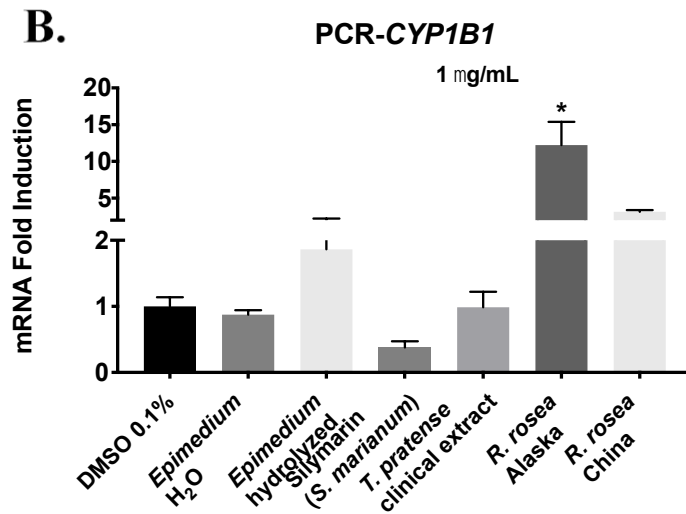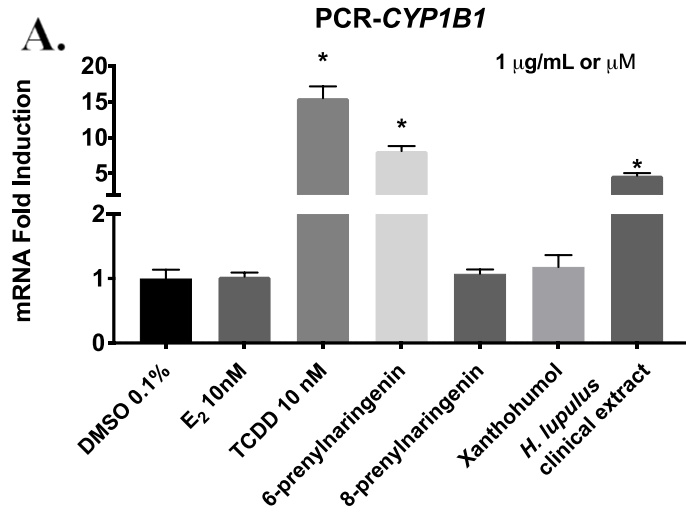
Figure 11. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) in MCF-7 breast carcinoma cells for CYP1B1 mRNA with 24-hour treatments of botanical extracts and hop compounds. $N \geq 3$; +/- SEM; one-way ANOVA, $*p < 0.05$.
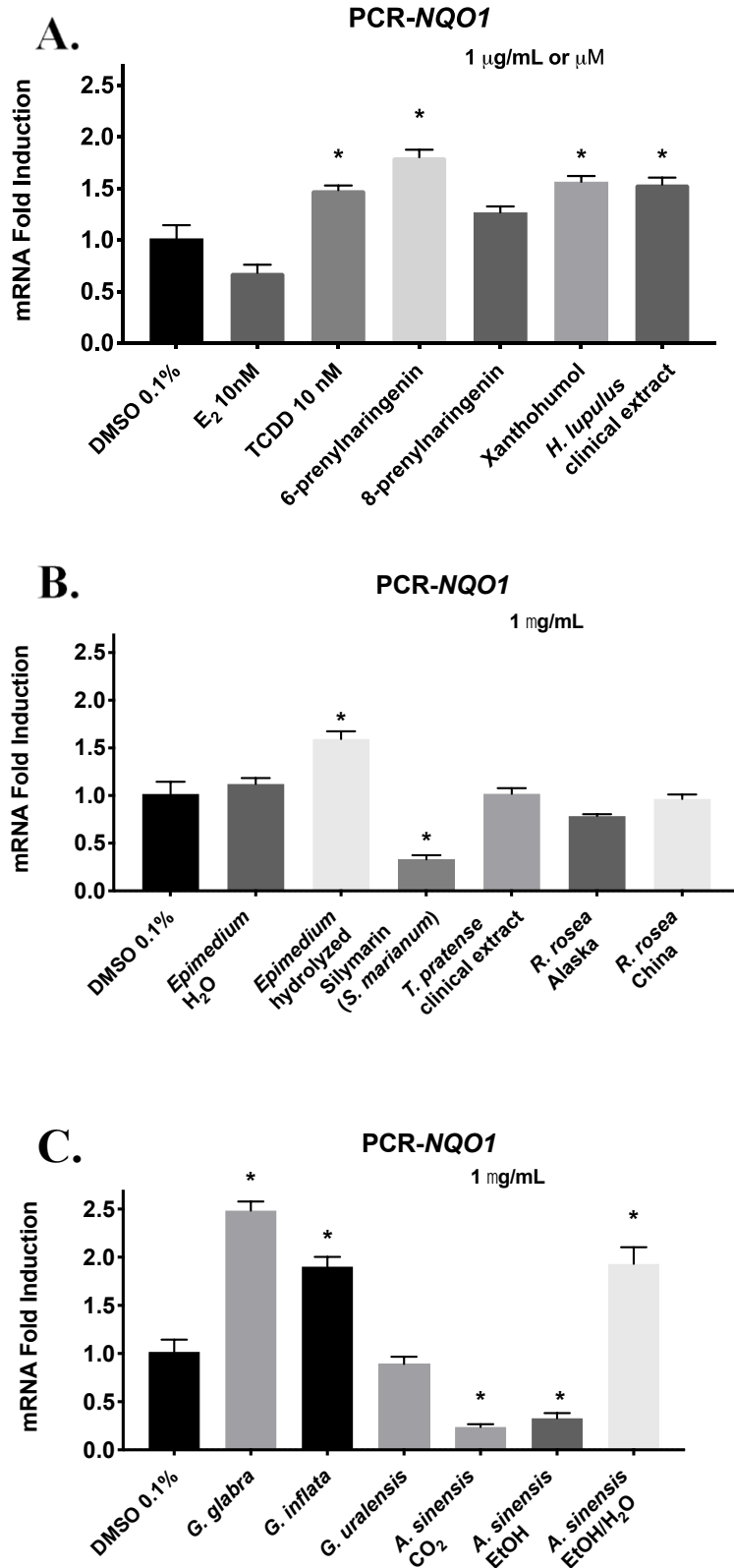
Figure 12. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) in MCF-7 breast carcinoma cells for NQO1 mRNA with 24-hour treatments of botanical extracts and hop compounds. $N \geq 3$; +/- SEM; one-way ANOVA, *$p < 0.05$.
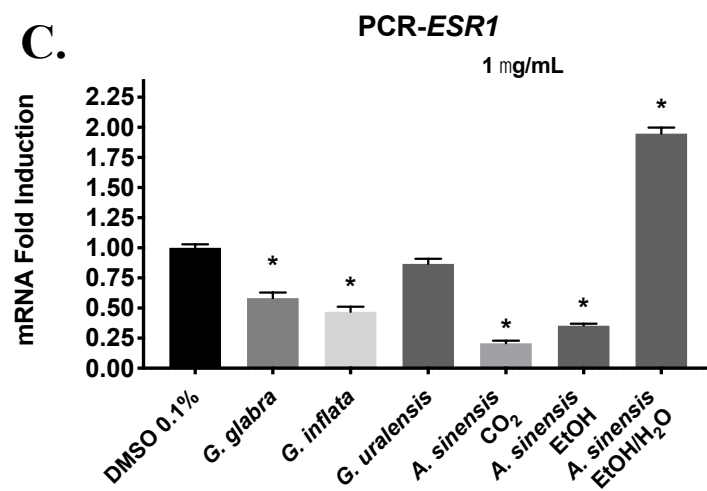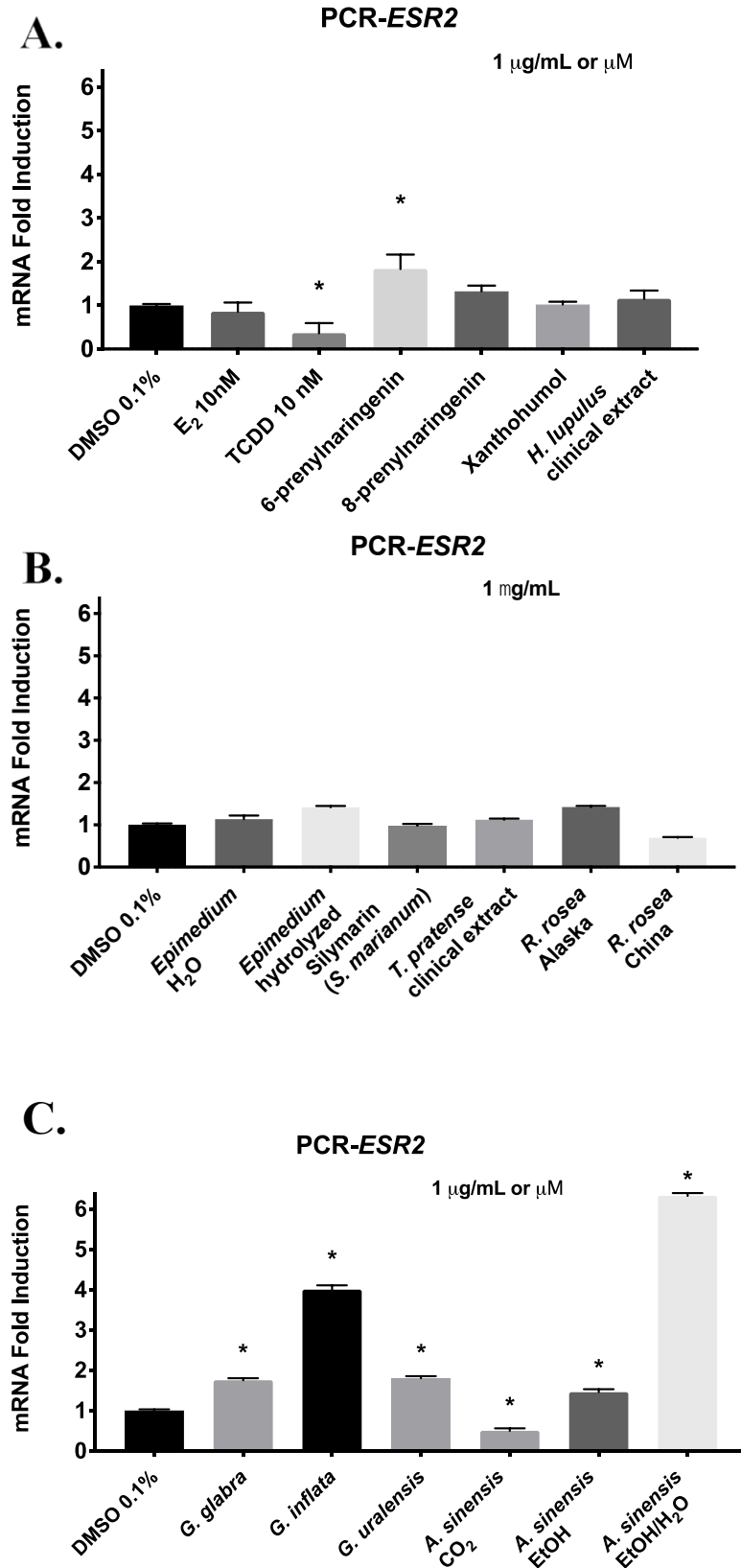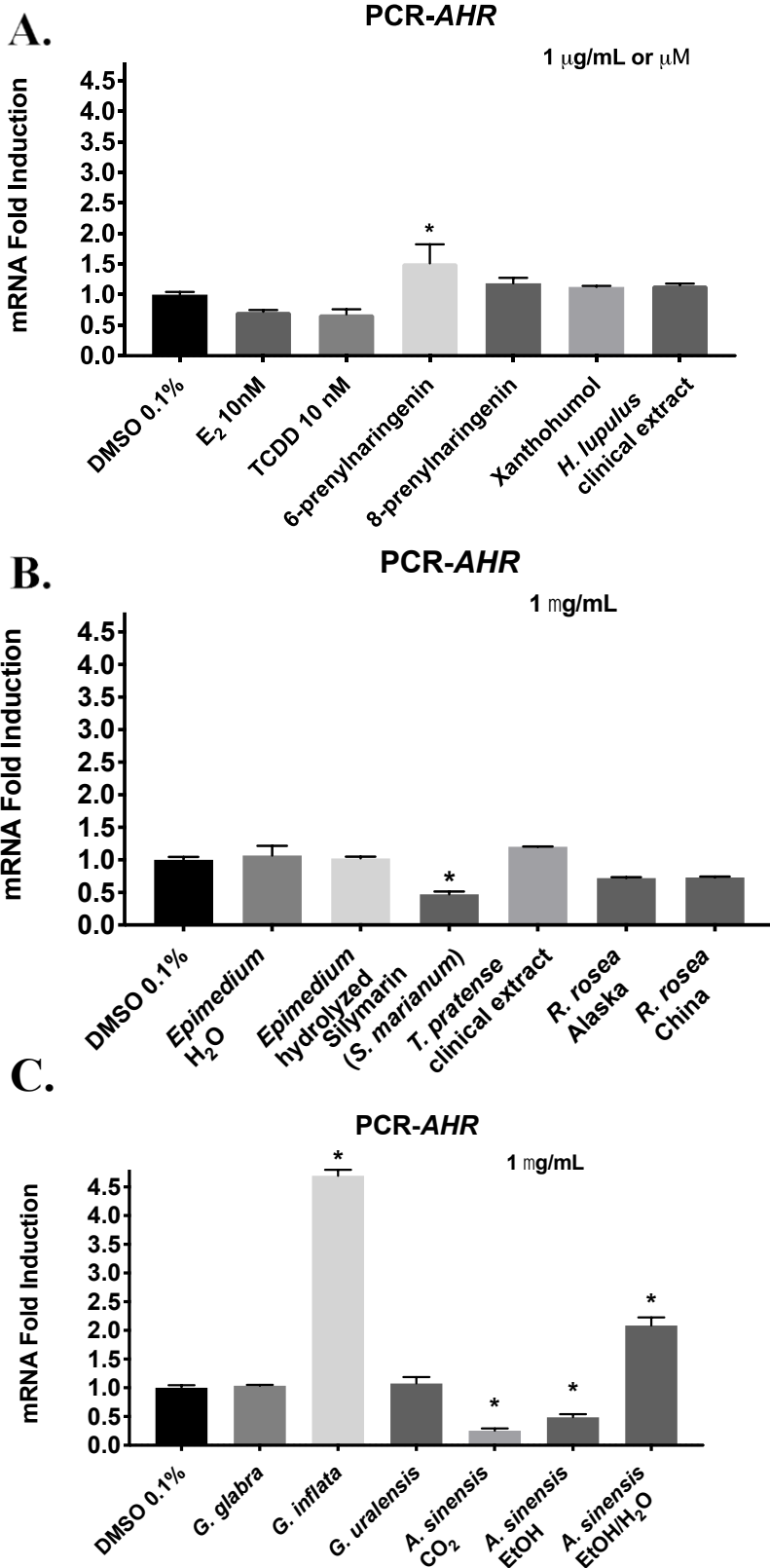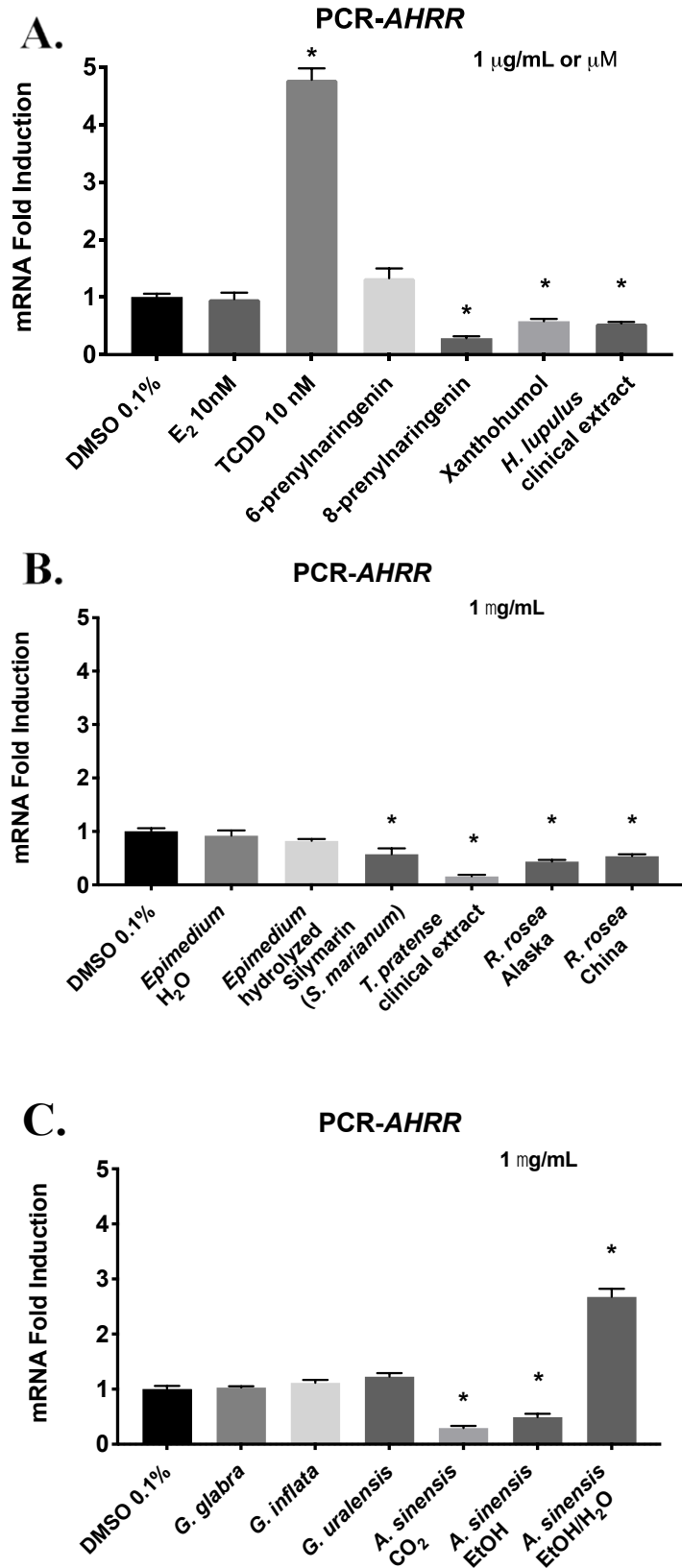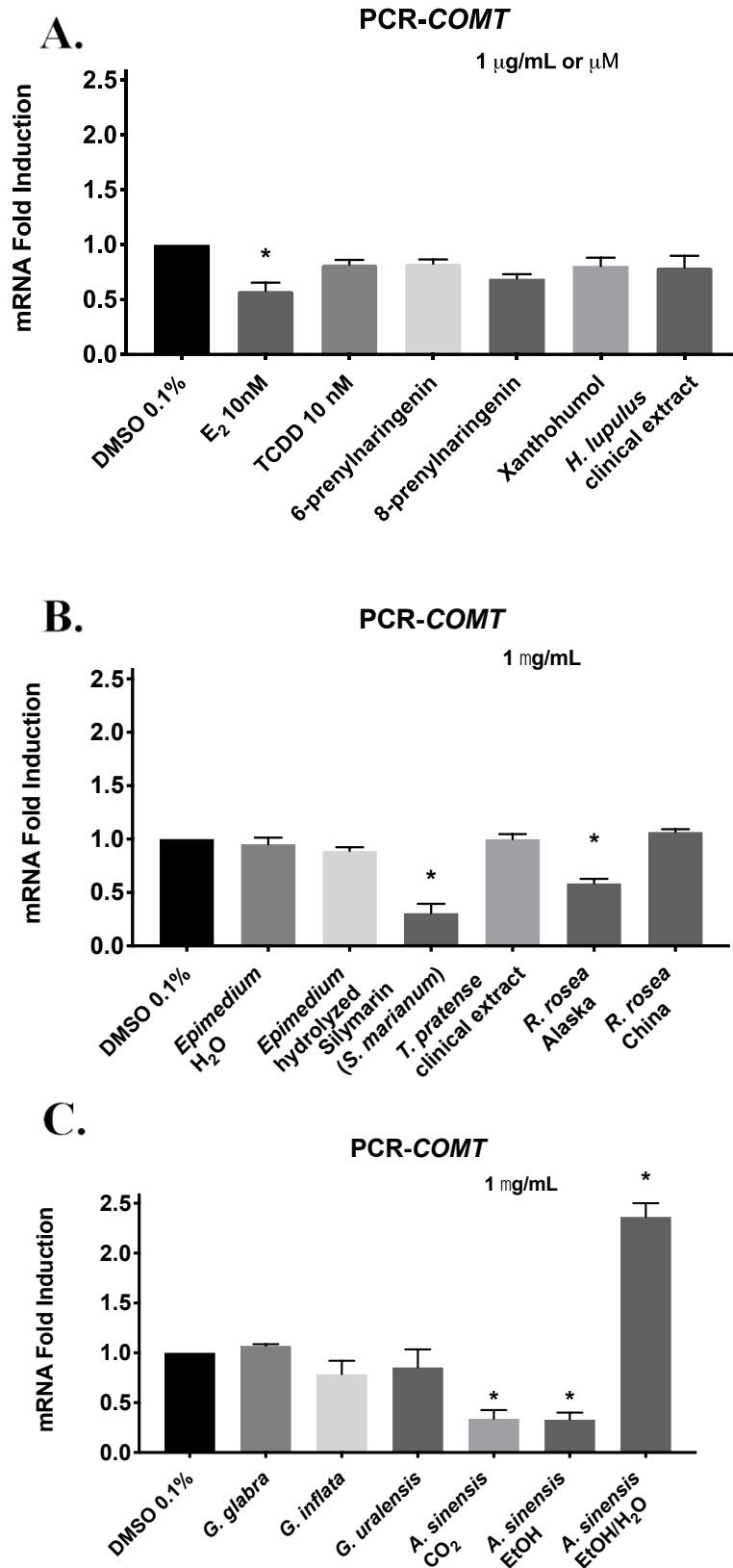
Figure 13. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) in MCF-7 breast carcinoma cells for COMT mRNA with 24-hour treatments of botanical extracts and hop compounds. $N \geq 3$; +/- SEM; one-way ANOVA, *$p < 0.05$.

	ERα	Anti-estrogenic	ESR1	ESR2	AHR	AHRR	CYP1A1	CYP1B1	NQO1	COMT
H. lupulus	↓ (green)	↑ (green)	↓ (green)	—	—	↓ (green)	↑ (green)	↑ (red)	↑ (green)	—
T. pratense	↓ (green)	—	↑ (red)	—	—	↓ (green)	↓ (red)	—	—	—
Epimedium unhydrolyzed	—	—	—	—	—	—	—	—	—	—
Epimedium hydrolyzed	↓ (green)	↑ (green)	↓ (green)	—	—	—	↑ (green)	—	↑ (green)	—
G. glabra	—	↑ (green)	↓ (green)	↑ (green)	—	—	—	—	↑ (green)	—
G. inflata	—	—	↓ (green)	↑ (green)	↑ (green)	—	—	↑ (red)	↑ (green)	—
G. uralensis	—	—	—	↑ (green)	—	—	—	—	—	—
R. rosea Alaska	↓ (green)	↑ (green)	↓ (green)	—	—	↓ (green)	↑ (green)	↑ (red)	—	↓ (red)
R. rosea China	—	—	↑ (red)	—	—	↓ (green)	—	↑ (red)	—	—
S. marianum	—	↑ (green)	↓ (green)	—	↓ (red)	↓ (green)	↓ (red)	↓ (red)	↓ (red)	↓ (red)
A. sinensis CO_2	↑ (red)	↑ (green)	↓ (green)	↓ (red)	↓ (red)	↓ (green)	↓ (red)	↓ (red)	↓ (red)	↓ (red)
A. sinensis H_2O/EtOH	↓ (green)	↓ (red)	↑ (red)	↑ (green)	↑ (green)	↑ (red)	—	—	↑ (green)	↑ (green)

Table I: Summary of results from Aim 1, evaluation of women's health botanical extracts on estrogen chemical carcinogenesis pathways. Green may be seen as potentially beneficial outcomes on the estrogen detoxification pathway while red may be seen as potentially promoting the estrogen genotoxic pathway. An up arrow indicates and upregulation of activity, expression, or transcription while a down arrow indicates a downregulation of activity, expression, or transcription. A blue line indicates a lack of bioactivity for a given bioassay.

Conclusions

The evaluation of women's health botanical extracts on targets in the estrogen chemical carcinogenesis pathway (Table 1) revealed potential resilience promoting mechanisms for these botanical extracts. Hop extract showed activity across all assays, as well as the A-ring prenylated flavanones and chalcones found in the hop extract (Nikolic and van Breemen, 2013). Previous studies have shown resilience activities in breast cancer cells for the AhR agonist, 6-PN from hops, making 6-PN containing hop extract worthy of further study (Hitzman et al., 2020; Wang et al., 2016).

Epimedium also showed activity across all types of assays performed in the estrogen detoxification pathway and warrants further study. The fact that the hydrolyzed extract showed bioactivity but not the unhydrolyzed extract shows that the activity of *Epimedium* is more associated with the aglycone, the prenylated flavonol icaritin, than with the diglycosidic icariins. Icaritin has been shown to be an AhR agonist, and it restricted the growth of breast cancer cells (Tiong et al., 2012). Although the hydrolyzed *Epimedium* extract exhibited bioactivity *in vitro*, an unhydrolyzed version may still exhibit bioactivity, as icariins are metabolized to icaritin by intestinal microflora (Wu et al., 2016).

Strong *Rhodiola* AhR activity was observed in this study for the Alaskan *R. rosea* extract, containing rosavins, salidroside, and monoterpene glycosides as main constituents (Tang et al., 2020). BGF may reveal an AhR agonist with resilience properties and therefore research into how *R. rosea* influences the estrogen detoxification pathway is still needed.

Although similar on many accounts, the three pharmacological licorice species differ in chemical composition and makeup, with licochalcone A specific to *G. inflata* and glabridin specific to *G. glabra* (Dunlap et al., 2015). Similar results regarding *NQO1* induction from this

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study have also been seen for *G. inflata* and *G. glabra* in a past study (Wang et al., 2018). The same study found that although *CYP1A1* levels were elevated in the rat mammary gland, estrogen oxidative metabolism decreased, with *G. inflata* and *G. glabra* indicating potential resilience promoting outcomes (Wang et al., 2018). Past studies using licochalcone A from *G. inflata* showed preferential inhibition of *CYP1B1* and potential resilience properties in breast cancer cells (Dunlap et al., 2015; Hajirahimkhan et al., 2015). Further research into the bioactivity of licochalcone A may explain the induction of *AHR* by *G. inflata* and further *in vivo* estrogen metabolism studies may provide more insight into the resilience potential of licorice species.

The estrogenic standardized red clover extract used contains isoflavones (Booth et al., 2006). Red clover isoflavones have been shown to lead to genotoxic estrogen metabolism (Dunlap et al., 2017). Although containing intriguing phytoconstituents such as the AhR agonists biochanin A and formononetin, red clover extract may not promote resilience, but further research into red clover bioactives is explored in subsequent studies.

Flavonolignan containing silymarin extract from milk thistle may also not promote resilience, as it downregulated the transcription of important enzymes in the estrogen detoxification pathway, although it did have some antiestrogenic activity and further research may explain how silymarin downregulates transcription. Past research has shown silymarin enhances mammary carcinogenesis (Malewicz et al., 2006).

Also enhancing mammary carcinogenesis in past studies was a water extract of *A. sinensis* (Yue et al., 2019). The CO_2 extract of *A. sinensis*, which contained more *Z*-ligustilide and alkylphthalates, downregulated many important enzymes in the estrogen detoxification pathway and may be cytotoxic at lower micromolar concentrations, while the ethanol/water

extract containing little alkylphthalates increased the transcription of detoxification enzymes in MCF-7 cells. Interestingly, in past studies *NQO1* is increased in HepG2 cells by lipophilic extracts, an effect opposite from that seen in breast cancer cells for this study (Dietz et al., 2008). Research into the difference in phytochemistry, bioactivity of the extracts, and bioactivity in different tissues may provide better insight into resilience and carcinogenic potential of *A. sinensis*.

Differences in bioactivity were attributed to extraction solvents and techniques, as well as the location of plant growth. Chemical analysis to characterize the differences in extracts helps determine how plant location and extraction techniques lead to variation in extract bioactivity. Analyzing a botanical extract across multiple bioassays provides a bigger picture of bioactivity than only pursuing one biological endpoint. Many questions regarding the safety and efficacy in estrogen chemical carcinogenesis still exist for the botanical extracts tested, but the results gathered here begin to provide bioactivity profiles in breast cancer cells, hinting towards breast cancer resilience for certain women's health botanicals, especially for hops and *Epimedium*. The bioactivity profiles gathered can be used to design future *in vitro* and *in vivo* studies and point to extracts which are appropriate for further estrogen chemical carcinogenesis studies.

Chapter 4: The Influence of Red Clover DESIGNER Fractions (*Trifolium pratense*) on Estrogen Detoxification Pathways

Introduction and Rationale

Plant extracts are inherently complex mixtures that encompass many different compounds (many inactive compounds, as well as partially and fully active compounds) (Heinrich and Gibbons, 2001). There are often interactions between constituents, and any single constituent may produce many activities. The biological outcomes resulting from this complex chemical matrix is termed network pharmacology or polypharmacology (Hopp, 2015a). An extract may contain a multitude of constituents with various bioactivities, exhibiting polypharmacology, and metabolic conversion of extract constituents may provide various bioactivities depending on the products of extract metabolism.

Red clover contains many compounds of interest, namely formononetin, biochanin A, genistein, daidzein, and irilone, making it a botanical which may exhibit polypharmacology. Traditionally, the various methods for botanical preparation result in differing formulations and were advertised for specific purposes. For example, in 1922 red clover tea was advertised as a treatment for goiter, yet an alcoholic infusion or tincture of the same plant (*Trifolium pratense* L.) was described in the Materia Medica (1927) as a prophylactic against mumps (Boericke, 1927; Meyer, 1922). Nearly a century has passed since the publications. Modern technology and science have afforded us the ability to more thoroughly obtain botanical formulations that are standardized and safe, yet, there is still a lack of understanding for extract polypharmacology.

The high complexity of botanical extracts and bioactive compounds has made discerning polypharmacology difficult, as some compounds have many biological targets, and some compounds will act upon the same targets, enhancing or blocking the effects of other compounds

(Dietz et al., 2017; Dietz et al., 2016; Dunlap et al., 2017). To this extent, this study employed highly specific depletion and enrichment separation techniques to uncover hidden compounds and bioactivities in the plant matrix in order to produce extracts that are safe and optimize bioactivity of red clover. Individual components of this extract exhibit different, and sometimes opposing activities. Therefore, this rationalized the need for DESIGNER (Deplete and Enrich Select Ingredients to Generate Normalized Extract Resources) fractions that are optimized for wellness/resilience promotion in both preclinical research and clinical applications.

T. pratense of the Fabaceae family, also known as red clover, is a medicinal herb used in the treatment of menopausal symptoms, premenstrual syndrome, mastalgia, or high blood cholesterol (Booth et al., 2006). Extracts of the red clover flowers are rich in many phytoestrogens, chiefly isoflavones. Bioactive isoflavones from red clover, particularly biochanin A, formononetin, daidzein, and genistein, have shown promise as candidates for treatment of menopausal symptoms in recent clinical trials (Gartoulla and Han, 2014; Lipovac et al., 2012; Thomas et al., 2014). Isoflavonoids may contribute towards these therapeutic properties due to their affinity for estrogen receptors (Beck et al., 2005; Booth et al., 2006). Yet these same compounds have activities on other receptors, such as aryl hydrocarbon receptor (AhR), complicating their contribution to their overall health outcomes (Dunlap et al., 2017). Enrichment and depletion separation techniques have produced extracts with activities previously masked by other compounds (Dietz et al., 2017). Careful attention should always be exercised when considering the side effects of estrogen supplementation and the dose limitations of isoflavones. Different isoflavones may have significantly divergent bioactivities, therefore, the chemical composition of red clover supplements is critical.

The present study focused on the application of a preparative centrifugal partition chromatography (CPC) method for the phytochemical characterization of isoflavonoids and related metabolites, evaluation of enriched fractions for their estrogenic activity, and generation of valuable information regarding the optimization of red clover extracts for their effect on the estrogen detoxification pathway. The translational nature of these methods to aid in bioactivity analysis cannot be understated, as the complex chemical nature of a botanical extract influences biological analysis. The careful characterization of fraction constituents aided in discerning the polypharmacology of the secondary metabolites present in red clover extract.

The countercurrent separation (CCS) approach of DESIGNER fractions provides a rational and systematic means of high-resolution separation. DESIGNER methodology allows for meaningful investigation of residual complexity and enables the assessment of the therapeutic value of natural products, particularly extracts and other chemically complex mixtures (Dietz et al., 2017; Friesen et al., 2019; Ramos Alvarenga et al., 2014). In the hydrostatic CCS apparatus (centrifugal partition chromatography CPC), the separation potential of the CPC is a result of the rotor design. The rotor comprises hundreds of microcells connected in series (Malca Garcia et al., 2019; Thiebaut, 1992). These microcells emulate liquid-liquid partitioning similar to that performed in separation funnels. The resulting integration and relaxing action allow the compounds to be separated by their respective partition coefficients and relative solubilities in the two phases. Fractionating crude plant extract by using column chromatography may result in sample loss from degradation and stationary phase adsorption (Ito, 1987; Liu et al., 2015; Malca Garcia et al., 2019). However, CCS has a higher sample recovery than conventional liquid solid chromatography, being nearly 100% (Ito, 1987; Malca Garcia et al., 2019).

In order to formulate the ideal DESIGNER extract, initial identification, proper quantification, and extensive biological testing *in vitro* and *in vivo* must take place. Although this is far from a complete process, these DESIGNER fractions allow for initial biological and chemical characterization of major red clover constituents in combination with other extract constituents. This presents the best method to discern the bioactivity of constituents in complex mixtures such as botanical extracts. Ideally this testing resolves questions, but always brings new questions; the latter very true for red clover.

DESIGNER fractions allowed for the unmasking and optimization of extract bioactivities. Safety studies of red clover in women's health have been limited. Studies of red clover isoflavones on chemical and hormonal estrogen carcinogenesis have shown mixed results (Dietz et al., 2016; Dunlap et al., 2017). The biological assays of this study focused on targets within the estrogen chemical carcinogenesis and detoxification pathway. Estrogens are produced locally in postmenopausal mammary tissue where they can be metabolized to 3,4-OH-E$_2$ (3,4-hydroxyestradiol), which upon oxidation can cause DNA mutations, potentially contributing to breast cancer (Bolton and Thatcher, 2008; Fuhrman et al., 2012; Zahid et al., 2006). The 3,4 hydroxylation of estrogens occurs primarily through metabolism by the constitutively active P450 1B1 (Nishida et al., 2013). P450 1A1 primarily catalyzes the 2,3 hydroxylation of estrogens, which are not associated with DNA damage or with breast cancer (Falk et al., 2013; Fuhrman et al., 2012; Nishida et al., 2013; Zahid et al., 2006). Estrogen activated ERα causes epigenetic inhibition of the P450 1A1 pathway, leading to its own carcinogenic metabolism (Marques et al., 2013). Reversing this estrogenic trend by upregulating P450 1A1 activity in breast tissue is associated with reduced cancer risk through increasing the 2-hydroxylation of estrogens (Dietz et al., 2016; Zahid et al., 2006). Therefore, upregulating P450 1A1 activity is a

pharmacodynamic target for women's health botanical dietary supplements to promote resilience and wellness.

Of great interest are the isoflavones, biochanin A and formononetin, that act as AhR agonists, the estrogenic genistein and daidzein, and irilone, which can function as a potentiator of progesterone signaling (Dunlap et al., 2017; Lee et al., 2018). Although irilone is not known to have activity on the progesterone receptor alone, it's potentiation of progesterone signaling in the presence of progesterone may be relevant for cell models containing both ERs and PR as well as *in vivo* for individuals with ER and PR positive breast cancer. It has been known that biochanin A and formononetin are metabolized to genistein and daidzein respectively, but the extent and ultimate significance of this biotransformation has been called into question, with metabolism data not known in cells (Tolleson et al., 2002). AhR agonists, such as biochanin A and formononetin, may aid estrogen detoxification in breast cancer cells through P450 1A1 transcription, yet this depends on a compound's dose and extract polypharmacology, as AhR also induces the transcription of P450 1B1 and potentially estrogen carcinogenic metabolism (Dietz et al., 2017; Dunlap et al., 2017; Tolleson et al., 2002). The estrogenic nature of red clover may mostly be attributed to daidzein, genistein, and potentially irilone; however, daidzein and genistein are ERβ preferential ligands, which may show positive correlation with breast cancer prevention and resilience (Dietz et al., 2016; Mbachu et al., 2020). Previous studies have shown biochanin A and formononetin to preferentially upregulate P450 1B1 metabolism (Dunlap et al., 2017). The last decade has revealed that irilone is highly resistant to metabolism and degradation, likely providing some estrogenic activity seen for red clover, as well as progesterone activity, yet much can still be learned regarding its bioactivities or effects on red clover extract bioactivities (Braune et al., 2010; Lee et al., 2018; Lutter et al., 2014). The

DESIGNER fraction methodology allows for various combinations of red clover extract to be

analyzed and is an attempt to optimize the botanical extract for biological purposes and to

provide a safer extract.

Results and Discussion

A specialized isoflavonoid-enriched red clover extract (RCE) was used as the starting material in this investigation (Phansalkar et al., 2017). This RCE has been studied extensively and a clinical trial over a period of a year showed RCE to be safe in menopausal women (Geller et al., 2009). DESIGNER methodology further allows for evaluation of complex red clover mixtures. DESIGNER fractions (DFs) were generated by centrifugal partition chromatography (CPC), combining fractions according to CPC UV chromatogram that showed separation of major components such as daidzein/genistein (DF 01: 8-17), formononetin (DF 02: 18-33), irilone (DF 03: 34-45), biochanin A (DF 04: 46-77), hydrophilic portion (DF 05: 78-150), and a whole extract similar to RCE (DF 06: 8-150) after extrusion. Major and minor ingredients were quantified using orthogonal absolute qHNMR method and UHPLC UV by Dr. Gonzalo Malca-Garcia (Table 2). LC-MS analysis was used for aiding the structural characterization of low-abundance compounds (residual complexity) through each DF.

Although the methylated biochanin A has been shown to exhibit AhR activity, the demethylated genistein is known for binding the estrogen receptor (Dietz et al., 2016; Dunlap et al., 2017). Therefore, conversion from biochanin A to genistein (Figure 14) in breast cancer cells would result in different biological activities associated with the difference between AhR agonists and estrogenic compounds, and could explain genotoxic estrogen metabolism seen with the red clover extract in previous studies and the role isoflavones may play in AhR-ERα crosstalk, yet this does not consider isoflavone selectivity for ERβ and the role ERβ may play in reducing breast cancer risk(Dunlap et al., 2017). However, it has not been shown whether breast cancer cells can metabolize biochanin A to genistein. Commercially available biochanin A was obtained in order to evaluate the *in vitro* conversion of genistein from biochanin A in the

presence of MCF-7 cells, an estrogen sensitive cell line commonly used in breast cancer research. LC-MS/MS analysis of genistein from MCF-7 cells treated with biochanin A showed a dose dependent conversion of biochanin A to genistein at a rate of about 7.5% over 24 hours, likely due to an intracellular P450 metabolism, a known metabolic pathway for biochanin A and genistein (Figure 14) (Dunlap et al., 2017). Interestingly, genistein has potency on ERα and ERβ in the nanomolar range (Hajirahimkhan et al., 2018). This suggests that AhR active concentrations of biochanin A would result in concentrations of genistein which may inhibit P450 1A1 metabolism, and potentially result in genotoxic estrogen metabolism, particularly at higher concentrations. Due to similar chemical structure with biochanin A, formononetin may exhibit a similar metabolism resulting in potentially bioactive doses of daidzein.

The DESIGNER fractions DF 01 through DF 06 as well as purified irilone were subjected to biological assays for estrogenic activity, as well as their ability to modulate breast cancer cellular estrogen chemical carcinogenesis *in vitro*. The selective separation process can ultimately be used to formulate DESIGNER extracts enriched in resilience promoting compounds and depleted of potentially harmful compounds (Dietz et al., 2017; Malca Garcia et al., 2019). For the case of red clover, this may involve maximizing AhR activity while preventing ERα mediated inhibition of P450 1A1. At a basal level, and with no AhR activation, the result of the red clover phytoestrogens is preferential activation and metabolism of estrogens by P450 1B1 over 1A1 (Dunlap et al., 2017; Marques et al., 2013; Tiong et al., 2012). Activation of AhR can result in preferential transcription of, and metabolism by P450 1A1, a generally favorable outcome for estrogen metabolism, and opposing results from estrogenic compounds which tend to downregulate CYP1A1 (Fuhrman et al., 2012; Marques et al., 2013; Tiong et al., 2012).

The ability to inhibit estrogen-mediated activity by botanicals can be important for reducing estrogen-mediated carcinogenesis (Dietz et al., 2016). While estrogenic activity may alleviate menopausal symptoms, RCE has been shown to be no more effective than a placebo for reducing menopausal symptoms in a clinical trial (Geller et al., 2009). Alkaline phosphatase induction in the endometrial cancer cell line, Ishikawa, has been used to assess estrogen activity, with RCE used as a standard for estrogen-induced activity (Bolton et al., 2019). Interestingly, DF 02, 03, 04, and irilone all showed estrogenic activity (Figure 15). Irilone is highly resistant to degradation and has been shown to exhibit estrogenic activity, and may contribute to the overall bioactivity of RCE or red clover DESIGNER fractions, exhibiting some dose dependent bioactivity and biphasic activity (Braune et al., 2010; Lutter et al., 2014). Due to irilone's relative high bioavailability compared to other red clover isoflavones, activities exhibited by irilone on AhR-inducible P450 enzymes are also of great interest (Braune et al., 2010).

Additionally, degradation of ERα is associated with positive outcomes in ER+ breast cancer (Tiong et al., 2012; Wormke et al., 2003). The ER can cause many transcriptional and regulatory actions, one such being the inhibition of *CYP1A1* and ultimately P450 1A1 mediated non-genotoxic metabolism of estrogens (Dietz et al., 2016). E_2 has been shown to exhibit this activity, preferentially downregulating *CYP1A1* and the estrogen detoxification pathway (Hitzman et al., 2020). Degradation of ERα reverses this effect and can be induced by AhR agonists through a specific AhR-ERα degradation pathway (Tiong et al., 2012; Wormke et al., 2003). Interestingly, all fractions and irilone exhibited an ability to reduce expression of ERα in an In-Cell Western (Figure 16). DF 06, which has similar phytochemical makeup to RCE, had activity similar to RCE. While the hydrophilic fraction containing small quantities of biochanin A and formononetin, DF 05, exhibited the greatest decrease in ERα expression.

Gene transcription of the P450 enzymes, *CYP1A1* and *CYP1B1*, was analyzed using quantitative reverse transcription polymerase chain reaction, and relative luciferase activity in an XRE-luciferase assay was assessed for RCE, IRL, TCDD, and DF 01 – DF 06. DF 02 and 04, rich in formononetin and biochanin A, exhibited significant XRE-luciferase activity (Figure 17) and preferential upregulation of *CYP1A1* over *CYP1B1* (Figure 18). In the presence of ERα degrader, Fulvestrant, biochanin A, and formononetin showed similar results to this study, preferentially upregulating *CYP1A1* over *CYP1B1* (Dunlap et al., 2017). RCE data also correlated with previous studies, preferentially downregulating *CYP1A1*, yet RCE upregulated AhR-dependent XRE-luciferase activity in this study (Dunlap et al., 2017). DF 03, rich in irilone, although slightly inducing *CYP1A1* over *CYP1B1*, was not significant, nor was the XRE-luciferase activity for DF 03. This correlated with the irilone data exhibiting no AhR activity in either assay at 10 µM. Although this study showed irilone alone had no effect on upregulating *CYP1A1* or *CYP1B1*, it did downregulate *AHRR* (AhR repressor) transcription, it also revealed interesting effects when in combination with TCDD (Figure 20). An XRE-luciferase assay revealed irilone may potentiate the ability of TCDD to induce translocation of AhR to the xenobiotic response element (XRE)-motif (Figure 21). This potentiation of AhR mediated activity by irilone seemed to be of a biphasic nature, with the middle dose tested exhibiting the greatest response and the low (0.5 µM) and high (10 µM) doses having a lower response than the maximum seen for 3 µM (Figure 21).

The XRE-luciferase assay is usually used to represent AhR-mediated activity including initiating the AhR signaling cascade, but XRE activation is just one step in the initiation of transcription. Many proteins are ultimately involved in the transcription of *CYP1A1*, and although TCDD alone may significantly activate XRE and upregulate mRNA transcription of

CYP1A1, trends seen for a combination of TCDD plus irilone did not correlate between XRE-luciferase (Figure 21) and qRT-PCR (Figure 19). Irilone + TCDD seemed to potentiate AhR-dependent XRE activity yet irilone reduced the transcriptional potential of TCDD on *CYP1A1* in qRT-PCR. A similar outcome has been described for resveratrol, with no or potentiating effects of TCDD on XRE, yet a reduction of maximum TCDD induction of *CYP1A1* (Beedanagari et al., 2009). This was attributed to an ability for resveratrol to inhibit RNA polymerase II from binding to the *CYP1A1* promoter, a necessary step for mRNA gene transcription (Beedanagari et al., 2009). This provides one potential explanation for the qRT-PCR and XRE-luciferase data gathered here, and future studies would need to analyze this mechanism further. Additionally, combinations of irilone plus formononetin (red clover AhR agonist) exhibited similar potentiating effects for XRE-luciferase (Figure 22), while tending to reduce transcription of *CYP1A1* at a low irilone concentration (Figure 23). Formononetin did not induce CYP1A1 or CYP1B1 significantly as other AhR agonists like TCDD or 6-prenylnaringenin. Interestingly, the standardized red clover extract (RCE) also significantly activated XRE-luciferase but inhibited *CYP1A1* transcription (Figures 16, 17). Although at 10 µg/mL, RCE downregulated *CYP1A1* transcription in this study, past studies have shown a dose dependent regulation of *CYP1A1* transcription, slightly upregulating it at 10 µg/mL and downregulating it at 1 µg/mL (Dunlap et al., 2017). The freshness of the extract may play a role in RCE discrepancies between studies and between DF 06. Interestingly, DF 06 and RCE have a very similar phytochemical makeup, yet DF 06 exhibits the characteristics of biochanin A and formononetin rich extracts, upregulating *CYP1A1* transcription at 10 µg/mL while RCE downregulated transcription (Figure 18). This may be due to dynamic residual complexity associated with the freshness of the extract, as the

RCE used in bioassays was less fresh than the DESIGNER fractions and was exposed to DMSO for longer.

The present study further explored influence on estrogen metabolism through an activity assay, analyzing P450 1A and P450 1B1 activity as a proxy for detoxification and genotoxic estrogen metabolism, respectively. In an ethoxyresorufin-O-deethylase (EROD) assay, P450 1A activity can be discerned by adding the selective P450 1B inhibitor, 2,2',4,6'-tetramethoxystilbene (TMS). P450 1A was only upregulated by the AhR agonist, TCDD, while formononetin and irilone rich fractions, DF 02 and DF 03 respectively, exhibited an ability to downregulate P450 1A activity (Figure 24). Pure irilone exhibited characteristics similar to the irilone rich fraction, DF 03, and to E_2, downregulating P450 1A activity in breast cancer cells *in vitro*. When P450 1A + P450 1B1 activity was analyzed in combination, biochanin A and formononetin rich fractions slightly upregulated activity while irilone rich DF 03 slightly downregulated activity (Figure 25).

Previous studies in our Botanical Center have shown red clover extract (RCE) preferentially upregulates *CYP1B1* over *CYP1A1*, and were confirmed in these studies (Dunlap et al., 2017). Additionally, 4-MeOE$_1$ was preferentially upregulated over 2-MeOE$_1$ for RCE, indicative of genotoxic estrogen metabolism (Dunlap et al., 2017). Compounds found in red clover, such as irilone, genistein, and daidzein, have been shown to inhibit P450 1A or P450 1B enzyme activity, and this may affect P450 1A1 or P450 1B1 activity differently, possibly adding to the mixed activity and dose dependent nature of most fractions tested for these studies (Dunlap et al., 2017; Roberts et al., 2004; Wollenweber et al., 2003). These results suggest that the non-genotoxic P450 1A1 estrogen metabolism pathway may be reduced by some red clover DESIGNER fractions, potentially leading to genotoxic estrogen metabolism.

Ultimately, an extract which preferentially induces the non-genotoxic metabolism of estrogens is most promising. Although some compounds or fractions may preferentially upregulate *CYP1A1* transcription, this may not necessarily lead to preferential metabolism of estrogens through P450 1A1. Previous studies have shown that pure compounds biochanin A, formononetin, daidzein, and genistein significantly upregulated 4-MeOE$_1$, a biomarker for P450 1B1 metabolism of estrogens and the estrogen genotoxic pathway (Dunlap et al., 2017). Additionally, 2-MeOE$_1$, a biomarker for P450 1A1 estrogen metabolism, consistently was seen to a lower extent than 4-MeOE$_1$ for biochanin A, formononetin, daidzein, and genistein, indicating that the pure compounds act to enhance genotoxic estrogen metabolism in breast cancer cells *in vitro* (Dunlap et al., 2017). The polypharmacology of these compounds in red clover needs to be considered when evaluating these results, as some may be weak AhR ligands, or ER ligands, and exhibit dose dependent potency and competing bioactivities, such as P450 inhibition.

The present studies have revealed that irilone may act as an AhR modulator and inhibitor of P450 1A, with an irilone enriched DESIGNER extract drastically reducing the ability of the AhR agonist to activate the estrogen detoxification pathway in breast cancer cells (Figures 24, 25). The similarities in pharmacological profiles for red clover extract (RCE) and in irilone and the irilone rich fraction vs the stark difference for biochanin A and formononetin rich extracts in PCR data is intriguing (Figure 18). Consistent standardization of red clover extracts is key to understanding bioactivity; as extract composition varies, so will results. Future preclinical and clinical trials of red clover supplements and/or extracts should include analysis of irilone content for additional safety studies, as it may reduce estrogen detoxification in breast cancer cells among other cell and tissue types. This study also presents methodology to provide irilone

enriched and irilone depleted extracts, as well as formononetin and biochanin A enriched or depleted fractions. Irilone enriched fractions may potentiate some AhR activity. While formononetin or biochanin A enriched fractions may induce the estrogen detoxification pathway more than other fractions, and ultimately this methodology can be used to discriminate red clover fraction bioactivities.

The methodology used for the chemical separations provided distinct DESIGNER fractions, which aided in bioactivity studies. Ultimately, these studies revealed bioactivities for DESIGNER fractions of red clover extract within the estrogen metabolism pathway. Irilone showed an ability to reduce AhR-mediated transcription and along with an irilone rich fraction, exhibited P450 1A and P450 1B1 inhibition. Although biochanin A and formononetin rich fractions exhibited AhR activity, they exhibited no activity or weak activity of P450 1A, a marker for estrogen detoxification. Overall, differences in bioactivity between fractions was stark for many assays, yet P450 1A inhibition occurred for every fraction. A reduction of P450 1A activity could reduce the amount of estrogen metabolized to the non-genotoxic 2-hydroxylated estrogens and result in increased estrogen-induced genotoxicity. The resulting, and potentially genotoxic inhibition of P450 1A may be due to the presence of irilone, genistein, and daidzein, all of which have been shown to inhibit P450 activity. Although further *in vivo* studies are needed for safety, the present outcomes indicate that red clover may have adverse effects on estrogen detoxification for some women. Yet dose-dependent activities are difficult to say, as most assays were done with one or two concentrations, with the exception of some irilone studies, and dose-dependent studies would be valuable for future studies.

Compounds (%w/v)	(H-2) δ_n (ppm) Chemical shift	DF 01	DF 02	DF 03	DF 04	DF 05	DF 06	RCE
quercetin		3.75±0.06	-	-	-	-	0.71±0.10	1.47±0.01
formononetin	8.331	0.41±0.01	70.3±0.56	18.6±0.30	1.62±0.76	2.62±0.04	17.1±0.27	14.6±0.14
calycosin	8.283	1.42±0.02	-	-	-	-	0.41±0.01	0.49±0.01
daidzein	8.281	2.25±0.04	<LOQ	-	-	-	0.54±0.01	0.40±0.01
pratensein	8.319	0.22±0.01	4.89±0.08	-	-	-	0.64±0.01	1.20±0.02
kaempferol		0.14±0.01	1.75±0.03	-	-	-	0.37±0.01	1.08±0.01
genistein	8.315	<LOQ	2.29±0.05	0.70±0.01	-	-	0.56±0.01	0.42±0.01
pseudobaptigenin	8.328	-	2.81±0.05	0.70±0.01	-	-	0.89±0.01	0.93±0.01
biochanin A	8.364	-	-	2.82±0.04	82.6±0.99	2.46±0.32	15.7±0.25	15.1±0.16
prunetin	8.410	-	>LOQ	1.84±0.03	3.01±0.04	-	0.27±0.01	0.83±0.01
irilone	8.434	-	0.73±0.01	41.6±0.66	-	-	1.22±0.02	2.81±0.02
Maackiain		-	-	0.78±0.04	>LOQ	-	-	-
isoflavonoid 01	8.315	0.29±0.01	-	-	-	-	-	-
isoflavonoid 02	8.331	0.46±0.01	-	-	-	-	-	-
isoflavonoid 03	8.396	0.28±0.01	-	-	-	-	-	-
ononin	8.439	0.63±0.01	-	-	-	-	-	-
sissotrin	8.475	0.68±0.01	-	-	-	-	-	-
isoflavonoid 04	8.494	0.08±0.01	-	-	-	-	-	-
Maackiain type		-	-	0.78±0.02				
Weight (mg)		66.6	35.9	6.44	24.1	19.3	8.50	-

Table II. Percentage of isoflavonoids and related compounds calculated by using EC qHNMR method in each of the DESIGNER fractions (DF), structures for some bioactive isoflavones can be seen in Figure 3. Courtesy of Dr. Gonzalo Malca-Garcia (manuscript in preparation).

LC-MS/MS Quantification of Genistein from Biochanin A
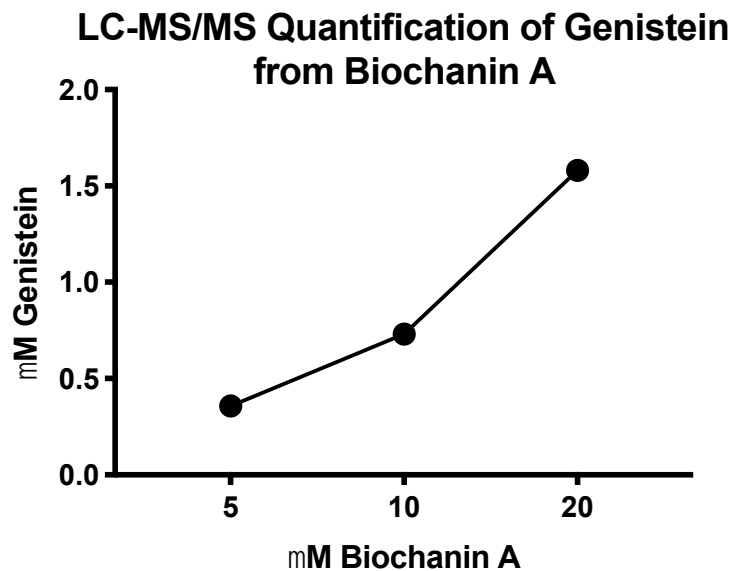
Figure 14A. LC-MS/MS quantification for the formation of genistein from *in vitro* cellular treatment with biochanin A. MCF-7 cells were treated with biochanin A at listed concentrations for 24 hours before media was collected for LC-MS/MS.

≈ 7.5% conversion

P450

Biochanin A (BA)
AhR agonist

Genistein (GN)
ER agonist/
P450 1A1/1B1 direct inhibitor
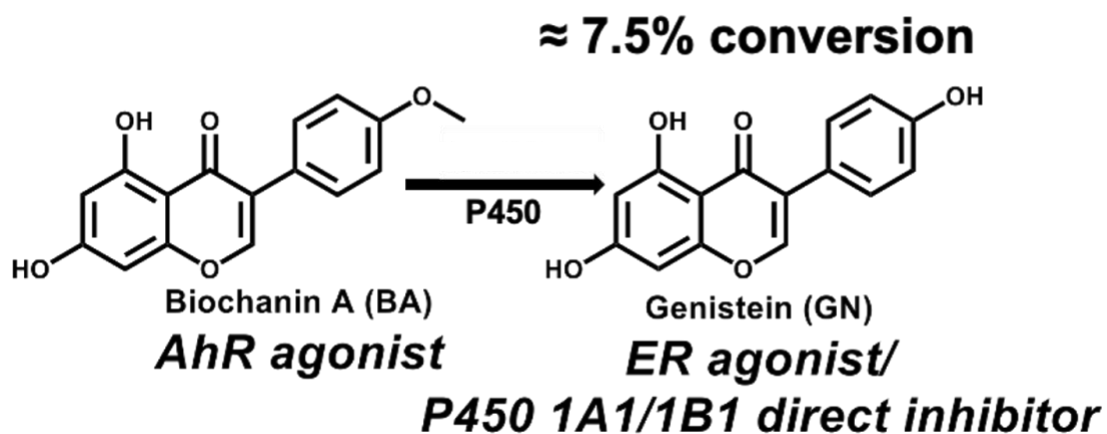
Figure 14B. Schematic representation of intracellular metabolism of biochanin A to genistein.
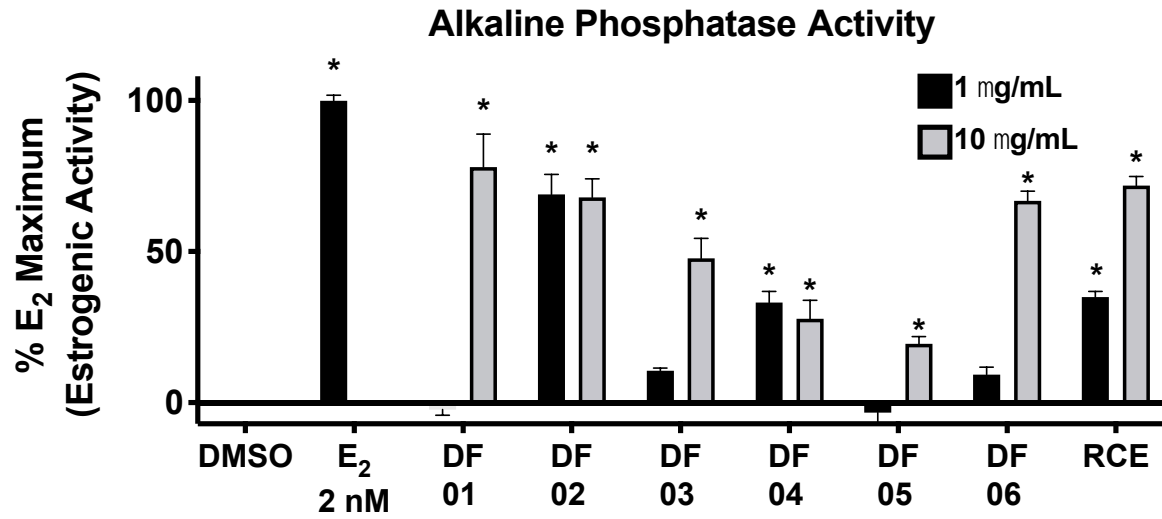
Figure 15A. Alkaline phosphatase assay in endometrial carcinoma cells (Ishikawa) for estrogenic activity of red clover extract DESIGNER fractions 01-06 and clinical red clover extract (RCE). N = 3; +/- SEM; two-way ANOVA; *p < 0.05.

Alkaline Phosphatase Activity (mM)
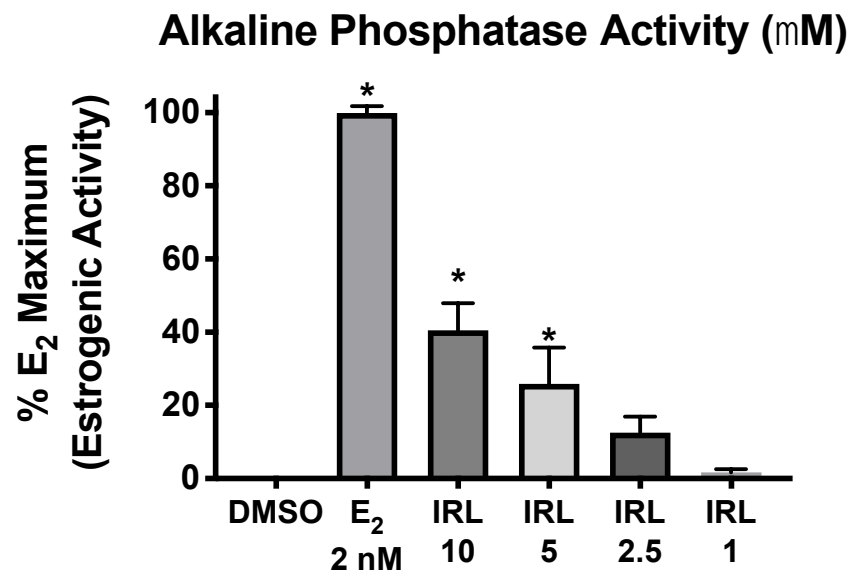
Figure 15B. Ishikawa assay in endometrial carcinoma cells for cytotoxicity and estrogenic and antiestrogenic activity for treatments with varying doses of irilone. N = 3; +/- SEM; one-way ANOVA; *p < 0.05.
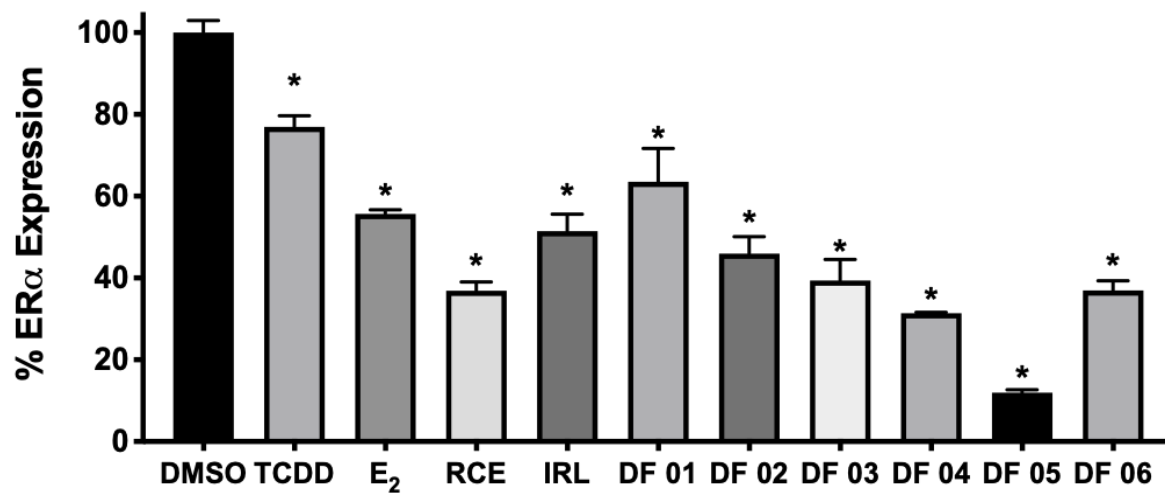
Figure 16. In-Cell Western™ assay in MCF-7 breast carcinoma cells for estrogen receptor alpha (ERα) expression with 24-hour treatments of: 0.1% DMSO; 10 nM TCDD or E_2 (positive controls); 10 µM irilone; and 10 µg/mL RCE or DF 01-06. $N \geq 3$; +/- SEM; one-way ANOVA, $*p < 0.05$.
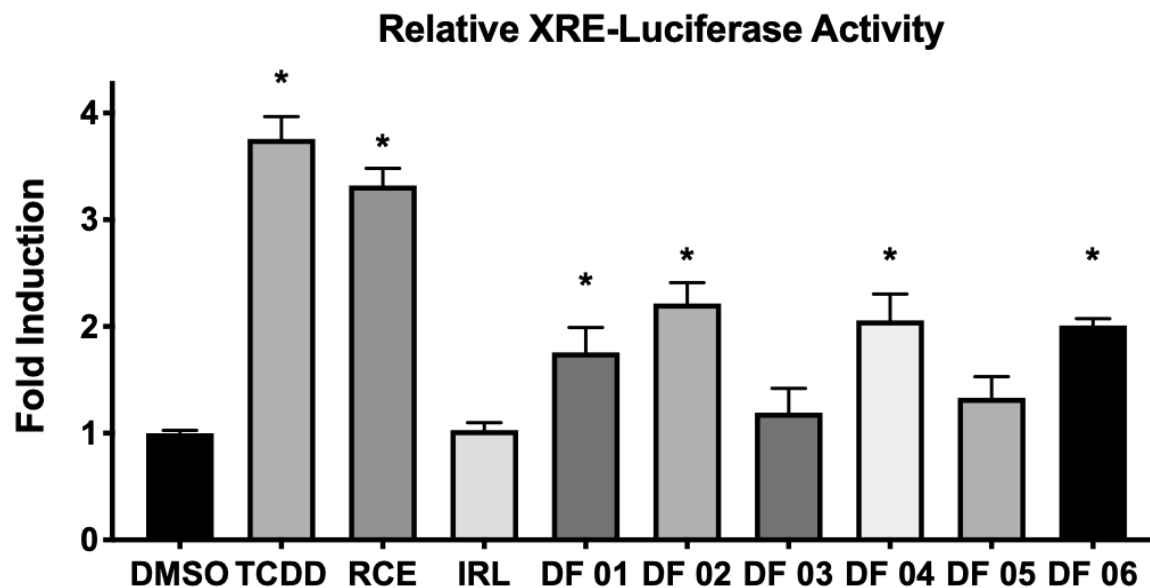
Figure 17. Xenobiotic response element (XRE)-luciferase assay in transiently transfected MCF-7 breast carcinoma cells for AhR activity with 24-hour treatments of: 0.1% DMSO; 10 nM TCDD; 10 µM irilone; and 10 µg/mL RCE or DF 01-06. N ≥ 3; +/- SEM; one-way ANOVA, *p < 0.05.
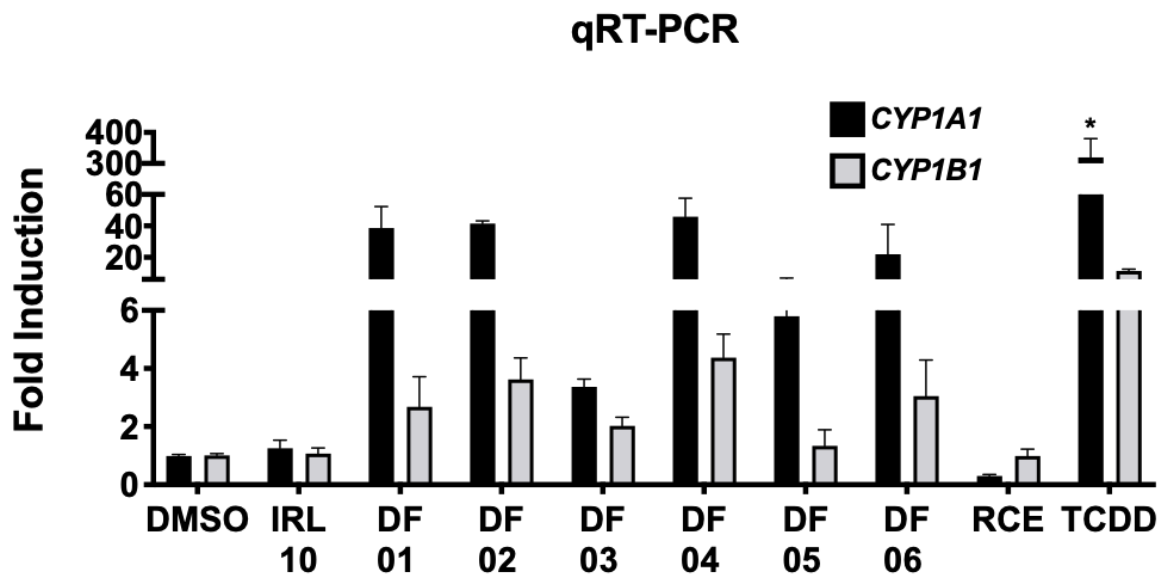
qRT-PCR

Figure 18. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) in MCF-7 breast carcinoma cells for AhR dependent transcription of CYP1A1 and CYP1B1 with 24-hour treatments of: 0.1% DMSO; 10 nM TCDD; 10 µM irilone; and 10 µg/mL RCE or DF 01-06. N ≥ 3; +/- SEM; two-way ANOVA, *p < 0.05.
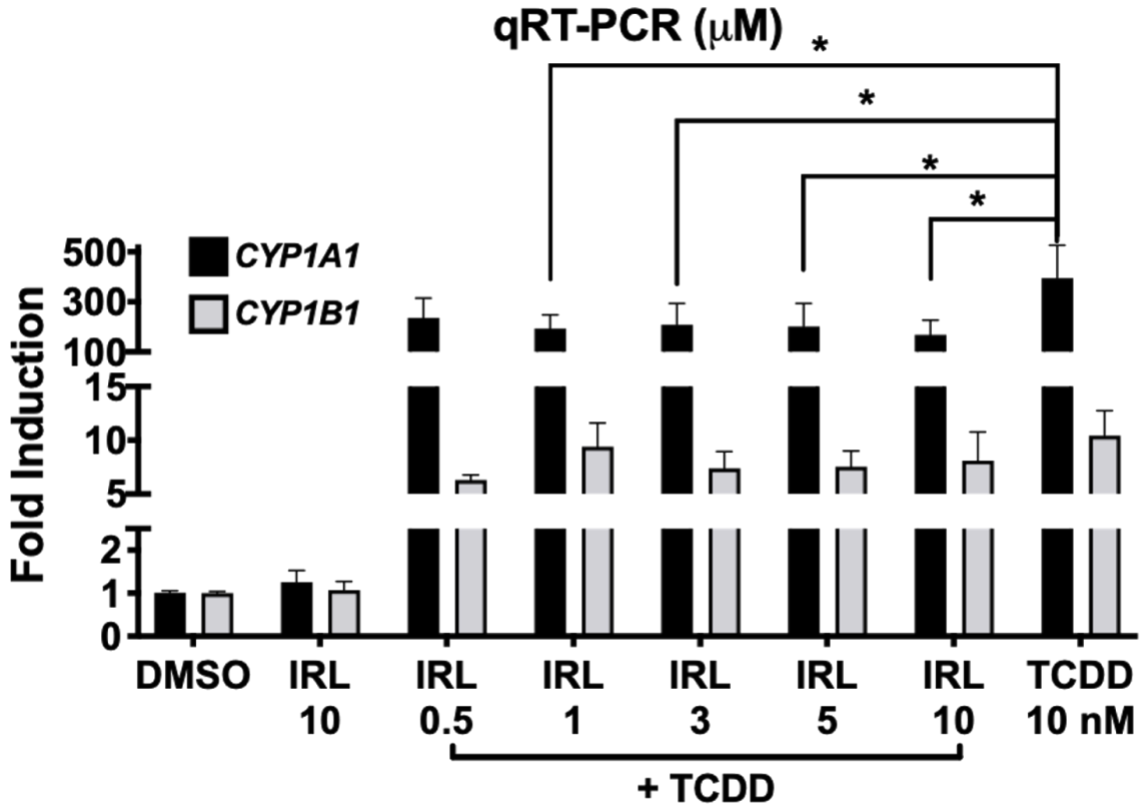
Figure 19. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) in MCF-7 breast carcinoma cells for AhR dependent transcription of CYP1A1 and CYP1B1 with 24-hour treatments of: 0.1% DMSO; 10 nM TCDD; or doses of irilone (µM) irilone with or without TCDD. N ≥ 3; +/- SEM; two-way ANOVA, *p < 0.05.

Figure 20A. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) in MCF-7 breast carcinoma cells for transcription of *AHRR* with 24-hour treatments of: 0.1% DMSO; 10 nM TCDD; or irilone. Normalized to DMSO. N = 3; +/- SEM, one-way ANOVA, *p < 0.05.

AHRR mRNA qRT-PCR (μM)
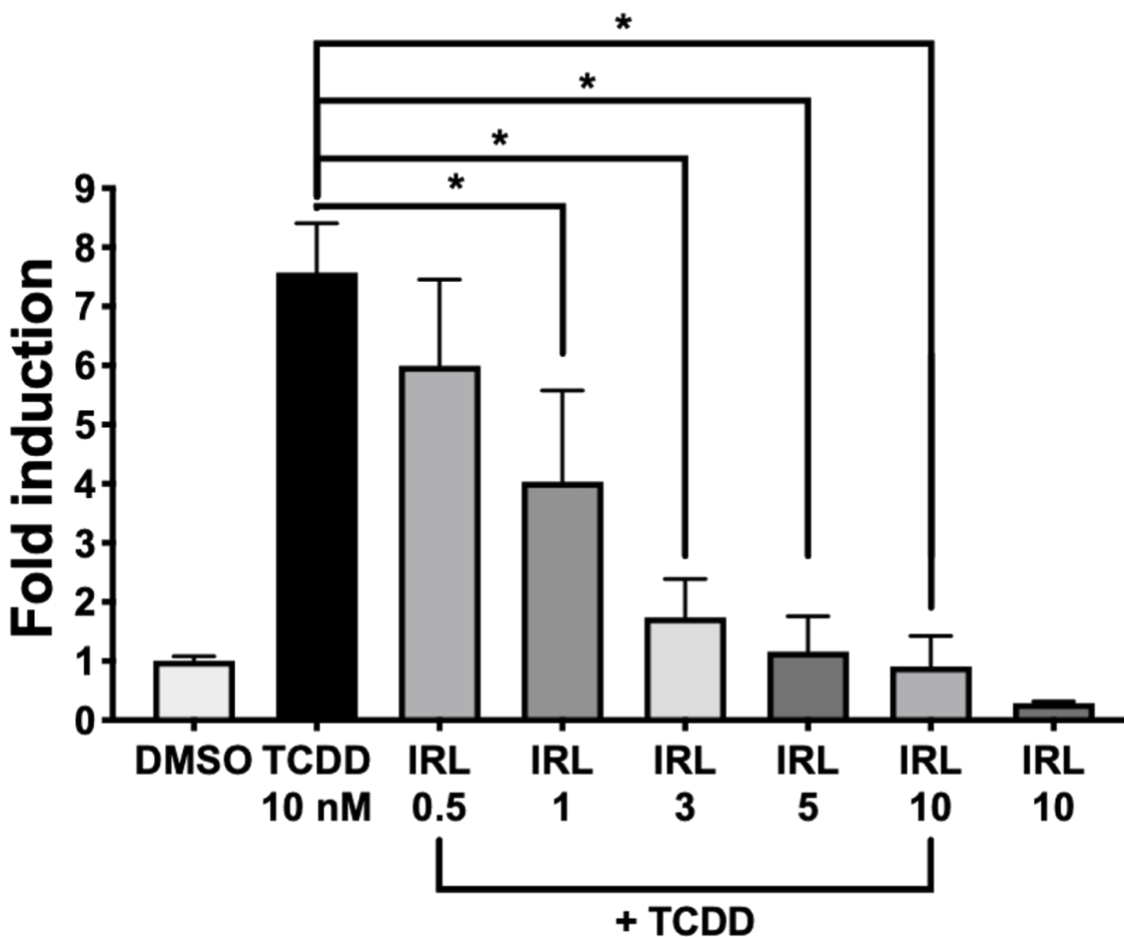
Figure 20B. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) in MCF-7 breast carcinoma cells for transcription of *AHRR* with 24-hour treatments of: 0.1% DMSO (not shown); 10 nM TCDD; or irilone (IRL) plus 10 nM TCDD. Normalized to DMSO. N = 3; +/- SEM, one-way ANOVA, *p < 0.05 .
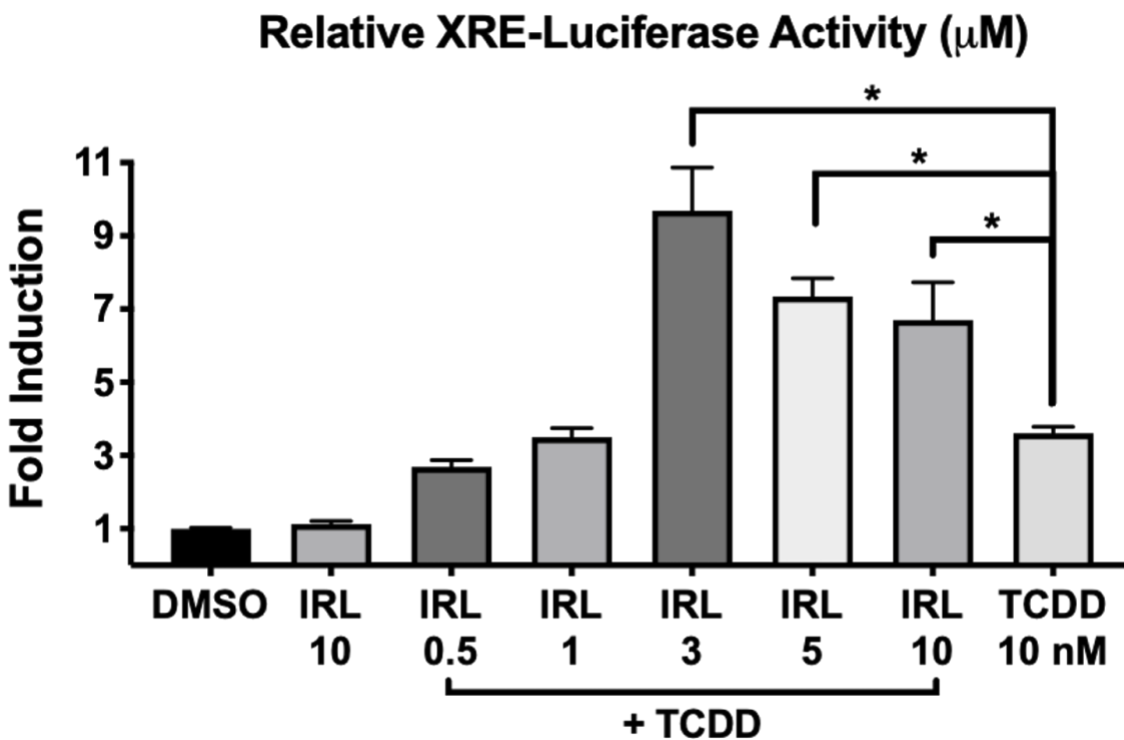
Figure 21. Xenobiotic response element (XRE)-luciferase assay in transiently transfected MCF-7 breast carcinoma cells for AhR activity with 24-hour treatments of: 0.1% DMSO; 10 nM TCDD; or irilone (IRL) plus 10 nM TCDD. Normalized to DMSO. N = 3; +/- SEM; one-way ANOVA, *p ≤ 0.05.
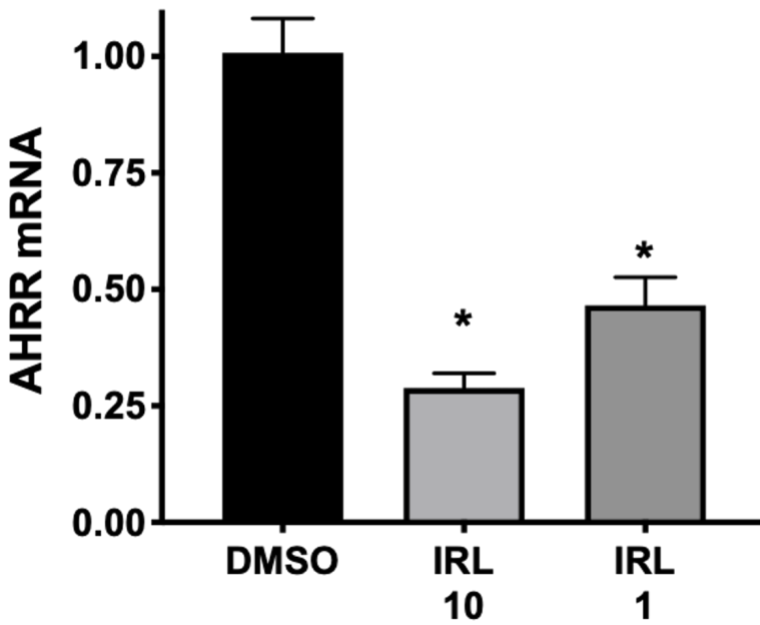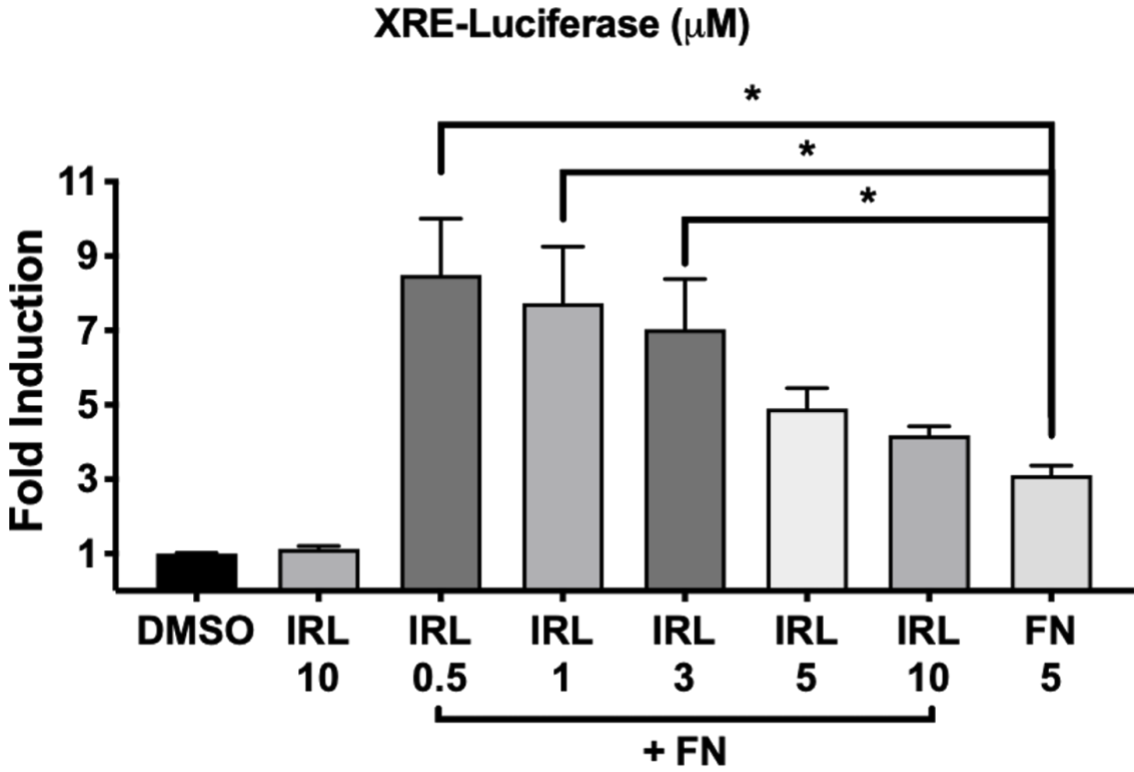
Figure 22. Xenobiotic response element (XRE)-luciferase assay in transiently transfected MCF-7 breast carcinoma cells for AhR activity with 24-hour treatments of: 0.1% DMSO; irilone (μM); formononetin (μM); or formononetin (FN) plus irilone (IRL). Normalized to DMSO. N = 3; +/- SEM; one-way ANOVA, *$p \leq 0.05$.
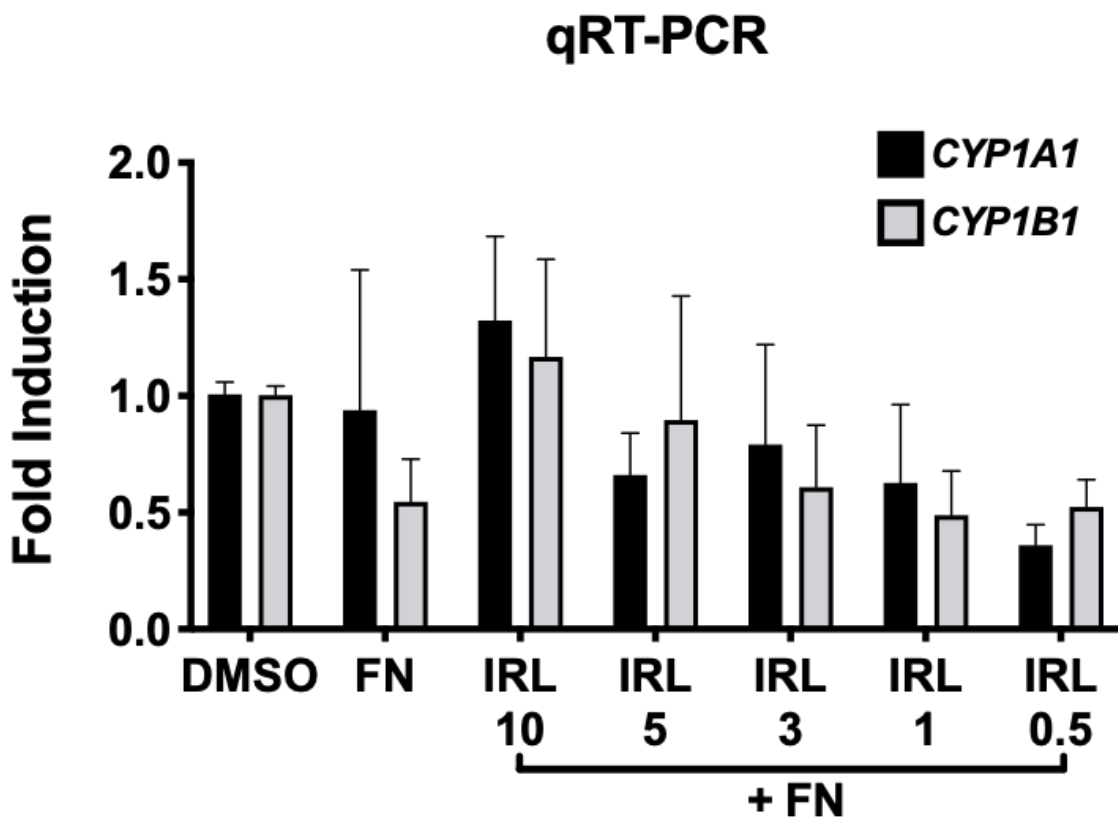
Figure 23. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) in MCF-7 breast carcinoma cells for transcription of *CYP1A1* or *CYP1B1* with 24-hour treatments of: 0.1% DMSO; irilone (µM); formononetin (5 µM); or formononetin (FN) plus irilone (IRL). Normalized to DMSO. N = 3; +/- SEM.
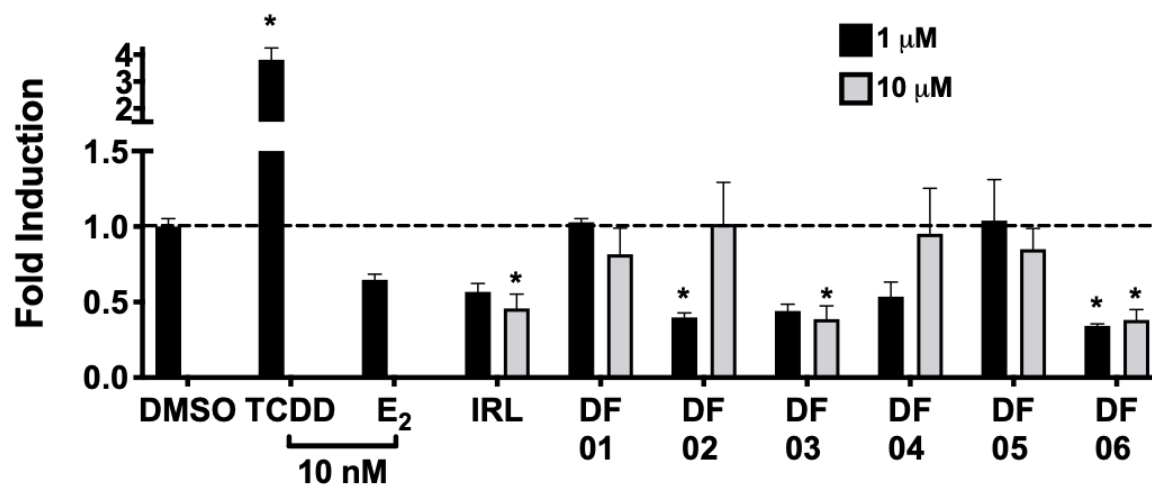
Figure 24. Ethoxyresorufin-*O*-deethylase (EROD) assay in MCF-7 breast carcinoma cells for activity of P450 1A, a biomarker for non-genotoxic estrogen metabolism, with 24-hour treatments described in graph and 1 hour of 2 µM 2,2',4,6' tetramethoxystilbene (TMS) pretreatment. N ≥ 3; +/- SEM; two-way ANOVA, *p < 0.05.
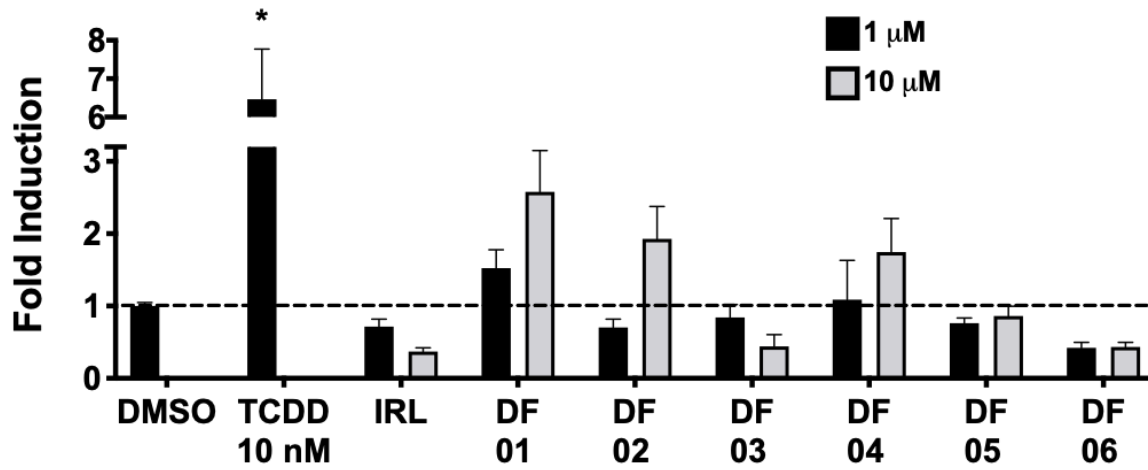
Figure 25. Ethoxyresorufin-*O*-deethylase (EROD) assay in MCF-7 breast carcinoma cells for activity of P450 1A + 1B1, with 24-hour treatments described in graph. Normalized to DMSO. N ≥ 3; +/- SEM; two-way ANOVA, p ≤ 0.05.

Chapter 5: 6-Prenylnaringenin from Hops Disrupts ERα-mediated Downregulation of

CYP1A1 **to Facilitate Estrogen Detoxification**

INTRODUCTION

The use of botanical dietary supplements (BDS), such as hops (*Humulus lupulus* L., Cannabaceae), have steadily increased in part due to the findings of the Women's Health Initiative study (Smith et al., 2018). This study found that traditional hormone therapy, involving conjugated estrogens and medroxyprogesterone, led to a 26% increase in the incidence of breast cancer (Rossouw et al., 2002). Women taking hop supplements tend to use them as an alternative to hormone therapy, as a sleep inducing remedy, and for their cytoprotective properties (Chadwick et al., 2006; van Breemen et al., 2014). Hop extract contains prenylated chalcones and flavanones as bioactive compounds, which according to the long-term research in our Botanical Center can elicit multiple women's health resilience promoting properties: cytoprotection attributed to the chalcone xanthohumol (XH), estrogenicity and aromatase (P450 19A1) inhibition effected by 8-prenylnaringenin (8-PN), and aryl hydrocarbon receptor (AhR) activation by 6-prenylnaringenin (6-PN) (Figure 26) (Dietz et al., 2016; Helle et al., 2014; Milligan et al., 2000; Monteiro et al., 2007; Rad et al., 2006; Smith et al., 2018; Wang et al., 2016; Yao et al., 2015).

Increased local estrogen levels in the mammary gland of postmenopausal women are associated with a greater risk of breast cancer (Key et al., 2002; Travis and Key, 2003; Yaghjyan and Colditz, 2011). Estrogen carcinogenesis, or the role of estrogen in carcinogenesis, can be

divided mainly into two pathways, hormonal and chemical (Dietz et al., 2016; Hemachandra et al., 2012). Chemical estrogen carcinogenesis involves the metabolism of estrogens to reactive quinones that can lead to DNA damage and, subsequently, genotoxicity and mutagenesis, comprising processes involved in tumor initiation (Dietz et al., 2016). This can occur in the breast, as estrogens (estradiol/estrone, E_2/E_1) are produced locally in the mammary gland (Dietz et al., 2016; Wang et al., 2016). Steroidal estrogens can be metabolized by P450 1A1 into their non-genotoxic 2-hydroxylated form termed the 2-OHE$_2$/E$_1$ estrogen detoxification pathway (Figure 27). However, the hormonal pathway involves steroidal estrogens that can also be metabolized into the genotoxic 4-hydroxylated form (4-OHE$_2$/E$_1$) by P450 1B1, also located in the breast tissue (Murray et al., 2014; Spink et al., 1992; Wang et al., 2016; Wang et al., 2015b; Yaghjyan and Colditz, 2011; Zahid et al., 2006).

Both P450 1A1 (*CYP1A1*-mediated) and P450 1B1 (*CYP1B1*-mediated) are transcribed by the transcription factor aryl hydrocarbon receptor (AhR) and are important enzymes responsible for estrogen metabolism in mammary tissue (Dietz et al., 2016). However, P450 1B1 estrogen metabolism is considered particularly genotoxic due to the formation of the reactive 4-OHE-*O*-quinone that can form DNA adducts (Bolton and Thatcher, 2008; Fuhrman et al., 2012; Samavat and Kurzer, 2015; Zahid et al., 2006). In accordance, epidemiological studies indicated a reduced risk for breast cancer with enhanced levels of 2-hydroxylated estrogens in postmenopausal women (Falk et al., 2013; Fuhrman et al., 2012; Samavat and Kurzer, 2015; Zahid et al., 2006). Targeting a reduction in estrogen chemical carcinogenesis has clinical relevance for breast cancer prevention (Falk et al., 2013; Fuhrman et al., 2012; Samavat and Kurzer, 2015; Zahid et al., 2006). Identifying plant-derived extracts and compounds which preferentially activate the benign, P450 1A1 pathway, has plausible health benefits, as they may

reduce factors related to estrogen chemical carcinogenesis. To this note, we have previously

shown that the unique hop flavanone, 6-PN (Figure 26), acts as an agonist of AhR to

preferentially induce *CYP1A1* over *CYP1B1* (Wang et al., 2016).

AhR has also been shown to be important in the attenuation of estrogen receptor alpha

(ERα) responses by inducing degradation of ERα (Tiong et al., 2012; Wormke et al., 2003). In

regard to estrogen carcinogenesis, the crosstalk interactions of ERα and AhR may be of

particular relevance (Murray et al., 2014; Safe et al., 1991; Wihlen et al., 2009). One example of

ERα-AhR crosstalk involves E_2-activated ERα epigenetic downregulation of the benign *CYP1A1*

(P450 1A1) estrogen detoxification pathway (Dunlap et al., 2017; Luecke-Johansson et al.,

2017). Past studies revealed that agonist-activated AhR induces the proteasomal degradation of

ERα (Dunlap et al., 2017; Luecke-Johansson et al., 2017). This degradation of ERα has been

shown to preferentially upregulate *CYP1A1* transcription, potentially reversing ERα-mediated

downregulation of *CYP1A1* (Dunlap et al., 2017; Luecke-Johansson et al., 2017). Reversing this

crosstalk could result in a preferential increase in 2-hydroxylated estrogens and possibly

contribute to a reduction in estrogen chemical carcinogenesis.

Epigenetic regulation of proteins have been an evolving target for understanding

crosstalk mechanisms, including those involved in this ERα-AhR crosstalk (Amenya et al., 2016;

Beedanagari et al., 2010; Guo et al., 2015; Marques et al., 2013). These epigenetic ERα-

mediated regulations likely contribute to selective transcription of AhR-mediated genes, such as

CYP1A1, but not *CYP1B1* (Amenya et al., 2016; Marques et al., 2013). Among others, DNA

methyltransferases (DNMTs) have been implicated in selective epigenetic silencing of *CYP1A1*

by ERα (Marques et al., 2013). Therefore, reducing ERα-mediated recruitment of DNMTs to

CYP1A1 may be important for preferential AhR-activation of the P450 1A1 estrogen detoxification pathway.

Recent studies have shown that 6-prenylnaringenin (6-PN), the A-ring prenyl regioisomer of the phytoestrogen, 8-PN, contained in hop extract acts as an AhR agonist, upregulating *CYP1A1* transcription, and thereby is a suitable bioactive marker of standardized hop extracts (Wang et al., 2016). However, little else is known about how hops and 6-PN in particular may alter ERα-AhR crosstalk under estrogenic conditions (Bolton et al., 2019; Wang et al., 2016). Considering our prior research on hops, we hypothesize that 6-PN in a standardized hop extract contributes to a large degree in the ability of a hop extract to promote the estrogen detoxification pathway *in vitro* through activation of AhR under estrogenic conditions in a model with ER+ MCF-7 cells (Bolton et al., 2019; Chadwick et al., 2006; Wang et al., 2016). The present chapter presents evidence substantiating this hypothesis by elucidation of a new mechanism. This mechanism involves the ability of both hop extract and 6-PN to activate AhR, degrade ERα, and reverse the E_2-activated and ERα-mediated downregulation of *CYP1A1* in ER+ breast cancer cells, collectively resulting in upregulation of the estrogen detoxification pathway. An understanding of how 6-PN contributes to the overall bioactivities of the hop extract, and how hop extracts may be optimized to reduce carcinogenic metabolism of estrogens, represents significant progress in the understanding of BDS that are widely used to promote resilience in women's health.

RESULTS

Hop extract and 6-PN induce ERα degradation through an AhR-dependent mechanism.

Quantification of ERα protein was analyzed using In-Cell Western^{TM} in MCF-7 cells with the hop extract and the prenylated hop compounds all demonstrating significant increased degradation of ERα (Figure 28A). E_2 significantly decreased ERα protein, confirming previous results (Wormke et al., 2003). This is an example of E_2 acting as a protective modulator through compound-induced ER protein-downregulation in breast cancer cells (Pinzone et al., 2004). This degradation in the presence of E_2 was potentiated for treatment with hop extract, 6-PN, and XH, as well as with the positive control, the AhR agonist, TCDD (Figure 28A). 8-PN did not potentiate ERα degradation in the presence of E_2, nor did the selective ER degrader/downregulator (SERD), ICI (Figure 28A). This suggests that the phytoestrogen, 8-PN, and ICI use a mechanism similar to that of E_2 and likely compete with E_2 at the ERα binding site. However, the data also indicate that 6-PN, XH, and hop extract at least in part partake in a different mechanism of action than E_2 or the phytoestrogen, 8-PN, suggesting multiple pathways of ERα degradation by hop constituents. To analyze whether the compounds/extracts reduce ER through a proteasome degrading mechanism, the cells were pretreated with the proteasome inhibitor, MG-132 (Figure 28B). In the presence of the proteasome inhibitor known to inhibit ERα degradation, the ERα protein-reducing effects seen by the test compounds, extract, and controls were significantly attenuated (Figure 28B) (Wormke et al., 2003). This indicates that hop extract and its bioactive compounds facilitate ERα degradation through a proteasomal pathway. AhR activation is known to lead to proteasomal degradation of ERα (Wormke et al., 2003). To analyze the involvement of AhR in hops-mediated ERα downregulation, the cells

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were pretreated with the AhR antagonist, CH-223191. After pretreatment with the AhR

antagonist, sustained and significantly higher levels of ERα expression were observed for

treatments with TCDD, 6-PN, and hop extract (Figure 28C), thereby indicating involvement of

AhR. TCDD-induced ERα expression was fully restored by the AhR antagonist; however,

cotreatment with the AhR antagonist did not fully restore 6-PN and hop extract-induced ERα

degradation. The specificity of the AhR antagonist for dioxins over flavonoids may be a cause

for the lack of complete restoration of ERα expression for 6-PN when compared to TCDD

treatment (Figure 28C) (Giani Tagliabue et al., 2019; Zhao et al., 2010).

Hop extract and 6-PN act on AhR to activate XRE-motifs

Upon AhR binding to its cognate ligand, the complex is translocated into the nucleus and

ultimately associates with the xenobiotic response element (XRE), initiating, among others, gene

transcription of the P450 1 family (Lusska et al., 1993; Wang et al., 2016). To test whether hop

extract or its bioactive compounds activate AhR in ER+ breast cancer cells, transiently

transfected MCF-7 cells with XRE-luciferase were analyzed for luciferase activity. E$_2$ slightly

lowered XRE activation, albeit insignificantly (Figure 29A). In the presence of E$_2$, both 6-PN

and hop extract, exhibited significant luciferin output, reversing the reducing effect of E$_2$ on the

ability for AhR to bind and to activate its cognate XRE-motifs (Figure 29A). Similarly, hop

extract and 6-PN significantly activated AhR-dependent XRE-motifs without E$_2$, although to a

slightly greater extent (Figure 29B).

Hop extract and 6-PN induce preferential transcription of *CYP1A1* over *CYP1B1*

Reverse transcription PCR (qRT-PCR) was used to analyze changes in relative

transcription for P450 1A1 and P450 1B1 genes in the presence of E$_2$ and hop extract or hop

constituents of interest. The current data confirms that E$_2$ alone significantly downregulates

CYP1A1 without affecting *CYP1B1* transcription (Figure 30A) (Dunlap et al., 2017). Similarly, treatment with the potent phytoestrogen, 8-PN alone, significantly downregulated *CYP1A1* (Figure 30A). In contrast, the present studies also confirmed that the hop extract and 6-PN significantly induce *CYP1A1* (Figure 30C) (Wang et al., 2016). Additionally, E_2-mediated downregulation of *CYP1A1* mRNA levels was reversed by hop extract, 6-PN, and the positive control, TCDD, leading to a significant induction of *CYP1A1* mRNA (Figure 30B). 8-PN or XH cotreated with E_2 showed no significant change in *CYP1A1* or *CYP1B1* gene expression (Figure 30B).

Hop extract and 6-PN decrease ERα expression and increase P450 1A1 expression

Quantification of relative protein expression from MCF-7 cells utilized traditional Western blot (Figure 31). Similar to the In-Cell Western^{TM} blot data (Figure 28), ERα expression was reduced for all treatments when compared to DMSO, while this decrease in ERα expression was significant for treatments with TCDD, 6-PN, 8-PN, and hop extract (Figure 31A). Confirming the gene expression analysis of *CYP1A1* (Figure 30C), P450 1A1 (CYP1A1) protein expression was significantly increased for treatments with TCDD, 6-PN, and hop extract when compared to DMSO (Figure 31B). Interestingly, hop extract exhibited the greatest decrease in ERα and increase in P450 1A1 protein, respectively.

Hop extract and 6-PN reverse E_2 associated DNMT1 recruitment to *CYP1A1*

Because only the hop extract and 6-PN degraded ERα through AhR activation, showed enhanced XRE-reporter activity, and increased *CYP1A1* and P450 1A1 levels (Figures 28-31), a potential epigenetic mechanistic link controlling *CYP1A1* was analyzed for 6-PN and hop extract treatment. A Chromatin Immunoprecipitation (ChIP) pulldown with an anti-DNMT1 or anti-DNMT3B antibody was performed for treatment groups with E_2 alone and in combination with

hop extract, 6-PN, or TCDD. An anti-DNMT1 ChIP was also done without E_2. Using qPCR, purified DNA was amplified with *CYP1A1* promoter specific primers (Marques et al., 2013). An anti-DNMT1 pulldown for E_2 showed a significant increase in gene amplification compared to DMSO suggesting involvement of activated ERα in recruitment of DNMT1 to *CYP1A1*, (Figure 32A). Anti-DNMT1 cotreatment of E_2 with hop extract or 6-PN, when compared to E_2 alone, showed a significant decrease in the amount of DNMT-associated *CYP1A1* that was precipitated similar to the positive control, TCDD (Figure 32A). This trend of DNMT1 downregulation at *CYP1A1* continued for hop extract and 6-PN without E_2 (Figure 32B). This shows that methylation and downregulation of *CYP1A1* by DNMT1 and ERα is reversed through AhR activating 6-PN and hop extract. This effect was not observed using an anti-DNMT3B antibody for precipitation (Figure 32C).

Hop extract and 6-PN preferentially induce non-genotoxic estrogen metabolism

The UHPLC-MS/MS approach previously used for the analysis of oxidative estrogen metabolism modulation by botanicals was applied to quantify the amount of 2- and 4-methoxyestrones (Wang et al., 2016). In the presence of 1 µM E_2, only TCDD increased the amount of 4-methoxyestrone significantly when compared to E_2 alone (Figure 33C). However, the non-genotoxic 2-methoxyestrone was significantly increased for E_2 cotreatments with TCDD, 6-PN, and hop extract (Figure 33B). Moreover, the ratio of 2- to 4-methoxyestrone was significantly increased for TCDD, 6-PN, and hop extract (Figure 33A). These results were in accordance with previous results for 6-PN in MCF-7 and MCF-10A cells (Wang et al., 2016).
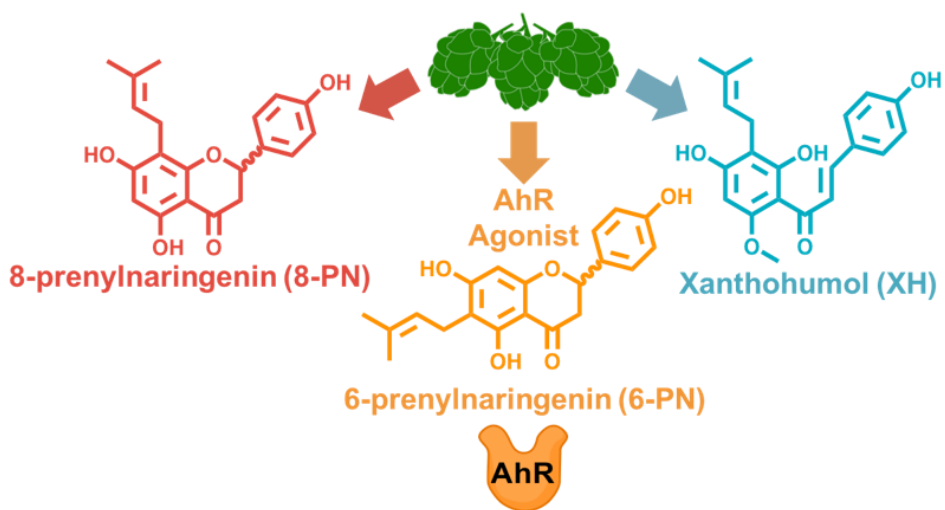
Figure 26: Hop bioactive compounds of interest.(Bolton et al., 2019)
From left to right: 8-PN, an ER agonist and aromatase inhibitor; 6-PN, an AhR agonist; and XH, a cytoprotective compound activating NRF2/ARE pathways.
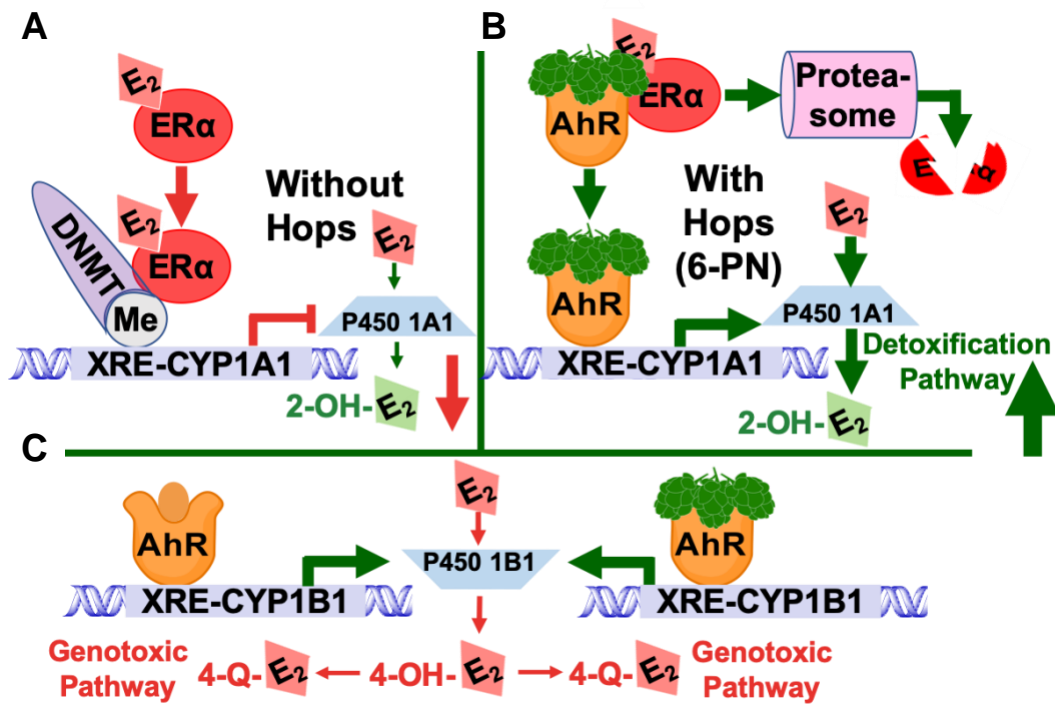
Figure 27: The influence of the standardized hop extract on oxidative estrogen metabolism: estrogen detoxification- and genotoxic metabolism pathways. **A**. Epigenetic regulation decreases the estrogen detoxification pathway in the presence of E_2. **B.** Hop extract standardized to 6-PN inhibit estrogen-mediated epigenetic downregulation of *CYP1A1* in MCF-7 cells. **C** The constitutive activity of AhR on *CYP1B1* exhibits little change by hop extract. (Bolton and Thatcher, 2008; Zahid et al., 2006)
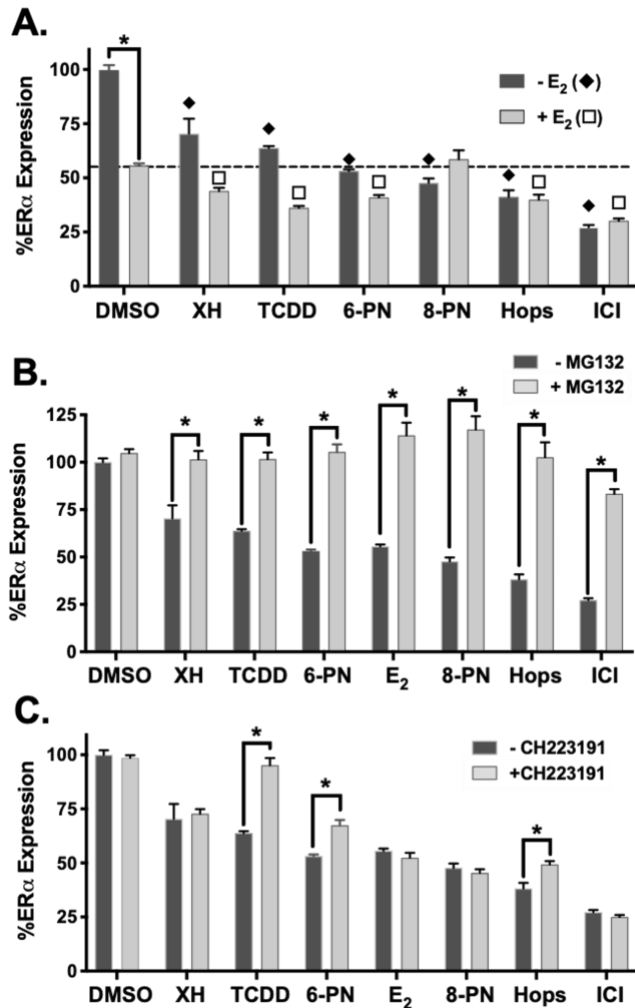
Figure 28. Percent degradation of ERα by hop extract and 6-PN is partially mediated by AhR. MCF-7 cells were treated with either 1 µM of 6-PN, 8-PN, XH, or ICI, 10 nM TCDD, or 5 µg/mL hops; **A**. with and without 10 nM E_2. **B**. with and without 2-hour pretreatment with the specific proteasome inhibitor, MG-132 (10 µM). **C**. with and without 2-hour pretreatment with AhR antagonist, CH-223191 (10 µM). After 24 hours ICW was performed, normalized to DMSO, and analyzed by two-way ANOVA with Dunnett's multiple comparison post-test. N ≥ 3, mean +/- SEM. Test groups were compared to DMSO (◆) or E_2 (◆) controls (**A**), or within treatment groups for E_2 and DMSO (**A**), +/- MG-132 (**B**) and +/- CH-223191 (**C**) */◆/◆ $p < 0.05$.
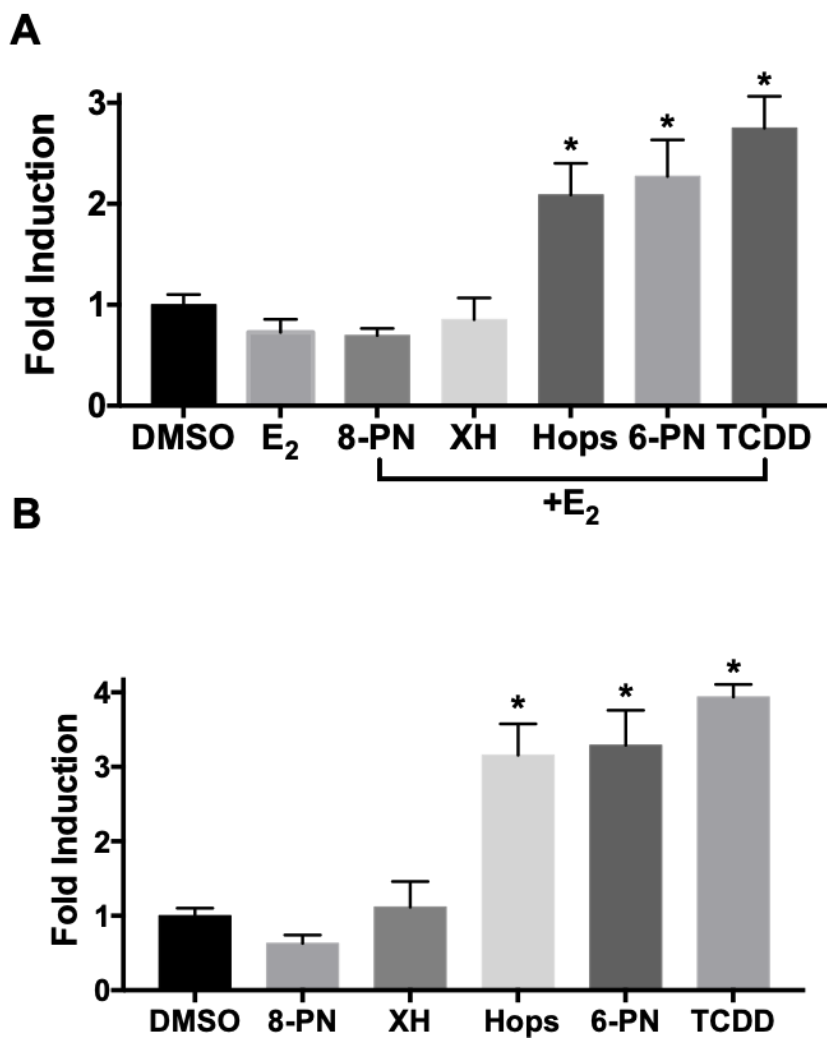
Figure 29. Hop extract and 6-PN induce XRE luciferase activity. MCF-7 cells were treated with 1 µM 6-PN, 8-PN, or XH, 5 µg/mL hops, or 10 nM TCDD together with 10 nM E_2 (**A**) and without E_2 (**B**) for 18 hours. Luciferase output normalized for cell activity then to DMSO. One-way ANOVA for comparisons to E_2 (**A**) or to DMSO alone (**B**). n ≥ 3, mean +/- SEM. *p < 0.05.
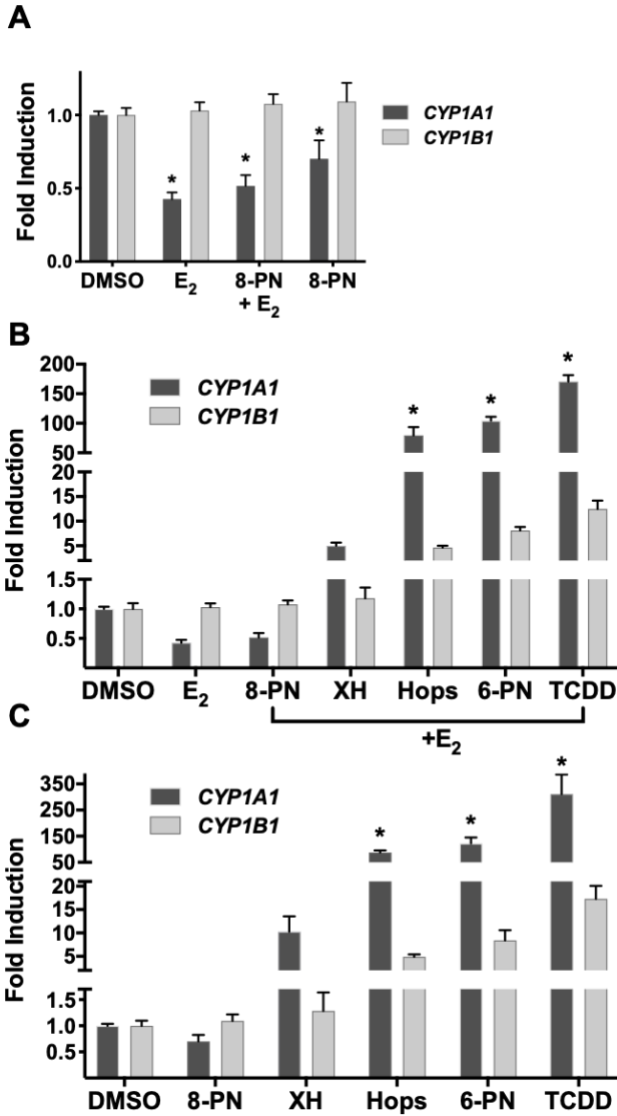
Figure 30. In the presence of E$_2$, hop extract, and 6-PN preferentially upregulate *CYP1A1*. MCF-7 cells were treated with E$_2$ (10 nM), 8-PN (1 µM), or a combination of the two (**A**), or 1 µM 6-PN, 8-PN, or XH, 5 µg/mL hop extract, or 10 nM TCDD together with 10 nM E$_2$ (**B**) and without E$_2$ (**C**) for 24 hours. ΔΔCT method for qRT-PCR of *CYP1A1* and *CYP1B1* mRNA was done using HPRT1 as a control gene. Data was normalized to DMSO before two-way ANOVA with Dunnett's multiple comparison test. N ≥ 3, mean +/- SEM. *$p < 0.05$. Comparisons made to DMSO (**A,C**) or to E$_2$ (**B**).
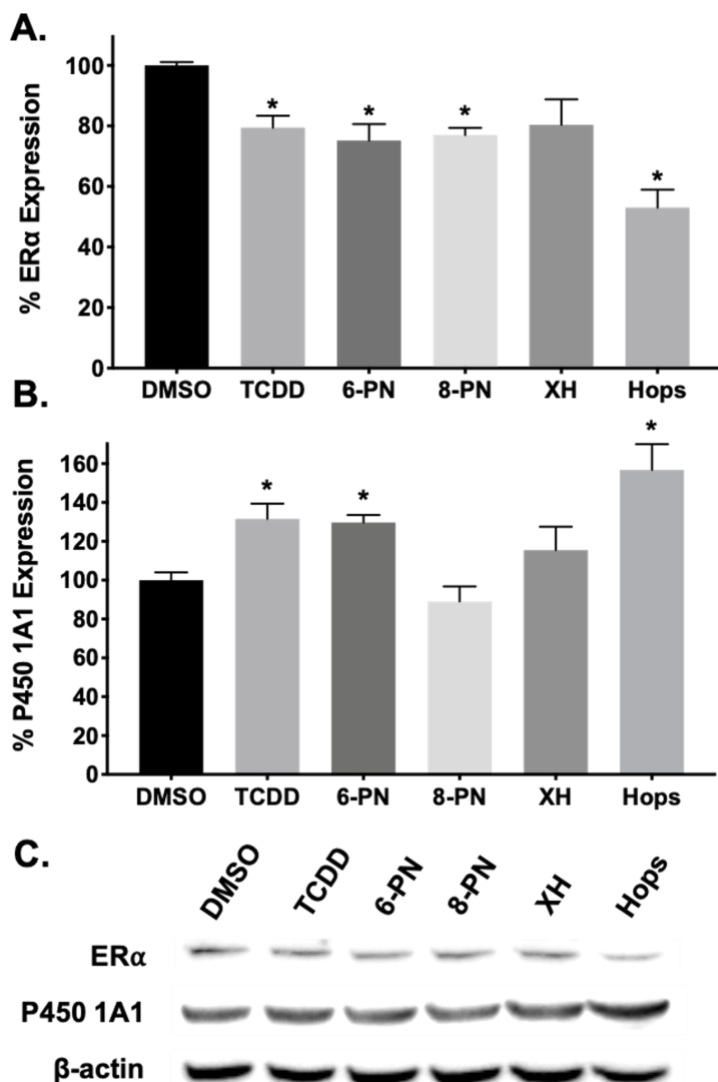
Figure 31. Traditional Western blots confirm ERα downregulation and P450 1A1 upregulation by hop extract and 6-PN. Western blot quantification of relative ERα (**A**) and P450 1A1 (CYP1A1) (**B**) in MCF-7 cells treated 24 hours with 0.1% DMSO, 10 nM TCDD, 1 μM 6-PN, 8-PN, or XH, or 5 μg/mL hop extract. One-way ANOVA was used to discern significance, n ≥ 3, mean +/- SEM. *$p < 0.05$. (**C**) Representative traditional Western blot image of an experiment for P450 1A1 (CYP1A1), ERα, and β-actin.
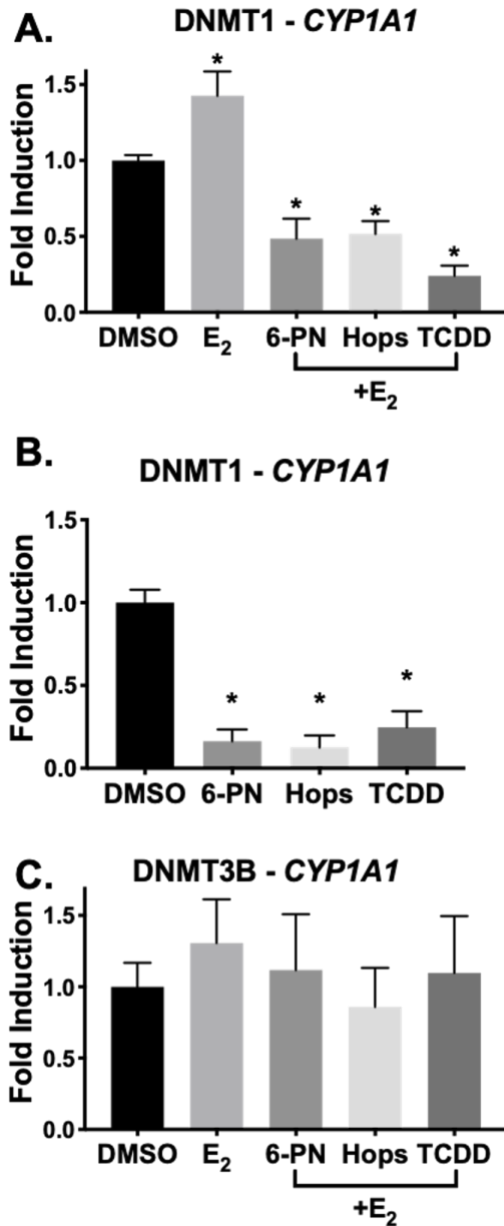
Figure 32: Treatments with hop extract or 6-PN attenuate E_2-induced DNMT1 at *CYP1A1*. MCF-7 cells were treated with 1 µM 6-PN, 5 µg/mL hops, 10 nM TCDD and/or 10 nM E_2 for 12 hours. Cells were analyzed using a ChIP assay for DNMT1 with E_2 (**A**), without E_2 (**B**), or for DNMT3B with E_2 (**C**) pulldowns followed by qPCR of the *CYP1A1* promoter. Data were first normalized to IgG (1) and fold induction method was used with a one-way ANOVA compared to DMSO. $N \geq 3$, mean +/- SEM. *$p < 0.05$.
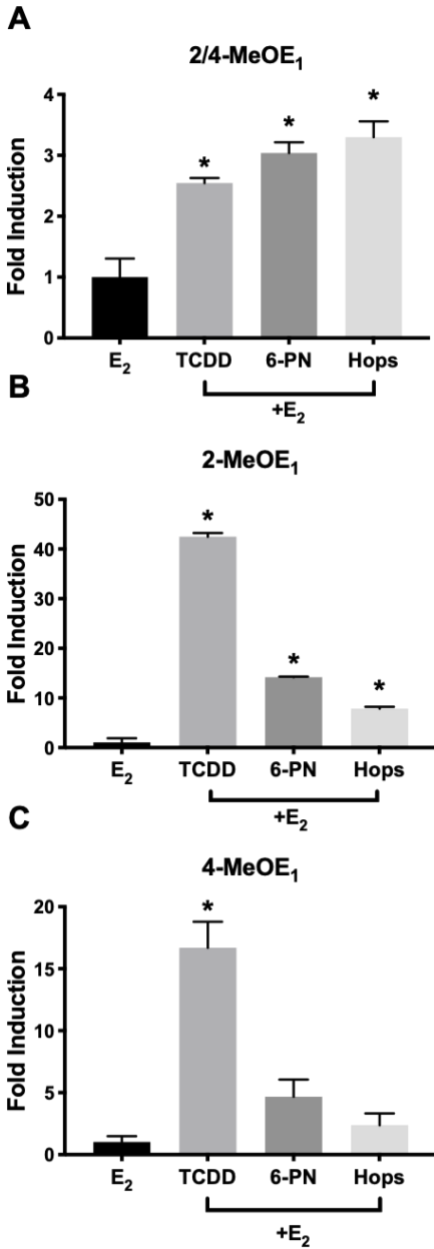
Figure 33: LC-MS/MS analysis of 2- or 4-methoxyestrone (2/4-MeOE$_1$) metabolites from MCF-7 cells treated with 1 µM E$_2$ alone or in combination with 10 nM TCDD, 1 µM 6-PN, or 5 µg/mL hop extract for 24 hours. Data were normalized to E$_2$ for fold induction of 2-MeOE$_1$ (**B**), 4-MeOE$_1$ (**C**), and the ratio of these values for 2- over 4-MeOE$_1$ shown in (**A**). One-way ANOVA was used to discern significance, n = 3, mean +/- SEM. *p < 0.05.

DISCUSSION

The scope of this current research involves elucidating the mechanism responsible for the preferential transcription of *CYP1A1* over *CYP1B1* by the standardized hop extract and 6-PN (Figure 30). Although it has been shown previously that 6-PN acts as an agonist of AhR, it is unknown in how far the involvement of ERα-AhR crosstalk plays a role in this preferential *CYP1A1* transcription (Figure 27) (Wang et al., 2016). Thus, it was hypothesized that the standardized hop extract and its constituent, 6-PN, degrade ERα through AhR activation to facilitate preferential transcription of *CYP1A1*. The fact that AhR-mediated degradation of ERα was confirmed for some constituents in these studies, but not all compounds is indicative that hop compounds, including 6-PN, promote ERα degradation through multiple pathways (Figure 28). This degradation would likely have a favorable effect on estrogen metabolism, as ERα degradation leads to an increase in P450 1A1 transcription (Figure 27), seen for ICI in previous studies (Dunlap et al., 2017). Interestingly, hop extract and 6-PN both significantly decrease ERα and increase P450 1A1 expression (Figure 28, 30, and 31).

Induction of AhR pathways is dependent upon XRE recognition on genes such as *CYP1A1* and *CYP1B1*, yet the extent of individual *CYP1A1* or *CYP1B1* activation varies and can depend upon specific ligands, cofactors, epigenetic influences, or cell types (Amenya et al., 2016; Dunlap et al., 2017; Wang et al., 2016; Wen and Walle, 2005). AhR activation can also vary based on its ligands, due to the different confirmations of the AhR receptor (Giani Tagliabue et al., 2019; Zhao et al., 2010). This variability was confirmed by the observed full restoration of ERα for treatments with an AhR antagonist and TCDD (Figure 28C). However, the AhR antagonist only exhibited partial ERα restoration of 6-PN and hop extract induced ERα degradation (Figure 28C). This is likely due to different AhR binding conformations for dioxins

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and flavonoids and the specificity of the antagonist for the dioxin binding site (Giani Tagliabue et al., 2019; Zhao et al., 2010). The genotoxic *CYP1B1* estrogen metabolism pathway, for example, is dominant for E_2 or estrogenic ligands, such as genistein, from red clover or soy (Dunlap et al., 2017; Wen and Walle, 2005). However, preferential transcription of *CYP1A1*, and consequently metabolism by the estrogen detoxification pathway, occurs with endogenous AhR ligands, such as 6-formylindolo[3,2-b]carbazole (FICZ), exogenous agonists, such as TCDD or the SERD, ICI (Larsen et al., 1998; Mullen Grey and Riddick, 2011; Wincent et al., 2009). The results seen in the XRE-luciferase assay in MCF-7 cells (Figure 29) indicate significant AhR activity for 6-PN and hops, which is in accordance with the *CYP1A1* induction data (Figure 30). However, the stark increase seen in the qRT-PCR data is less apparent in the XRE-luciferase assay due to decreased specificity, as XRE motifs are associated with multiple genes, and each gene has specific cofactors that influence its expression (Lusska et al., 1993). The same can be concluded for the effect of E_2 on the change in XRE activity, as the downregulation of *CYP1A1* by E_2 is more likely dependent on epigenetic factors such as DNMT1 in the direct promoter region of *CYP1A1*, than XRE in general (Dunlap et al., 2017). Additionally, results from these investigations are supported by previous studies showing 6-PN exhibits significant XRE-activation in MCF-7 cells (Wang et al., 2016).

Prior studies have been shown that DNMT-mediated methylation of *CYP1A1* occurs through recruitment by and association with ERα (Marques et al., 2013). For this study, the decrease in DNMT1 recruitment by 6-PN, hop extract, and TCDD with or without E_2 (Figure 32) correlates with the ability to activate *CYP1A1* (Figure 30, 31) and reverse E_2-mediated activity (Figure 30, 32) (Amenya et al., 2016; Marques et al., 2013). DNMTs are associated with the inhibition of P450 1A1 transcription, and E_2 increases the recruitment of both ERα and DNMTs

to the *CYP1A1* promoter (Marques et al., 2013). Previous studies have also shown increased

XRE methylation in the *CYP1A1* promotor region with combinatorial E_2/TCDD treatment

compared to TCDD treatment alone (Marques et al., 2013). Additionally, combinations of 10 nM

TCDD/E_2 have shown sustained degradation of ERα starting after 3 hours of treatment and

maintained at least for 24 hours (Wormke et al., 2003). All these results suggest the involvement

of DNMTs in E_2-mediated repression of AhR-induced *CYP1A1* expression, although responses

can vary for cell types or ligands and doses used (Amenya et al., 2016; Beedanagari et al., 2010;

Marques et al., 2013).

Crosstalk between ERα and AhR is complex, with this study revealing a role for DNMT1

in E_2-mediated inhibition of *CYP1A1*. DNMT proteins can catalyze the 5-methylation of cytosine

in a promoter, inhibiting gene transcription (Marques et al., 2013). Isoforms of DNMTs may be

highly important in discerning epigenetic influence on crosstalk. DNMT1 and DNMT3 have

shown relevance in epigenetic crosstalk between AhR and ERα, but DNMT2 to our knowledge

has not been reported to be involved (Marques et al., 2013; Ward et al., 2018). DNMT3B has

been implicated to drive an ERα-dependent methylation pattern at *CYP1A1* (Marques et al.,

2013). In past studies, the ERα cofactor SRC-1 has been associated with increased expression of

DNMT1 and DNMT3A (Ward et al., 2018). Histone variant H2A.Z has shown importance in

TCDD-induced transcription of *CYP1A1* by providing a stable physical environment and

recruiting RNA polymerase II (Marques et al., 2013). Thymine-DNA glycosylase (TDG)

removes oxidated 5-methylcytosine, a key role in reversing epigenetic *CYP1A1* inhibition, and

critical for TCDD-activated AhR transcription of *CYP1A1* (Amenya et al., 2016). Histone

deacetylase 1 (HDAC1) is associated with the promoter region of *CYP1A1* during inhibition, and

its removal is needed for AhR-mediated transcription of *CYP1A1* (Schnekenburger et al., 2007).

Recently HDAC inhibitors have been implicated in activation of AhR responsive gene induction

(Jin et al., 2017). Additionally, trimethylation of lysine 4 on histone 3 (H3K4me3) in the

promoter of *CYP1A1* is enriched by TCDD, common for active gene transcription (Amenya et

al., 2016). ERα recruits DNMT to methylate the promoter of *CYP1A1*, and AhR agonists can

cause the demethylation of affected DNA through complex epigenetic influences not yet fully

elucidated. As epigenetics is an evolving field it is likely that additional epigenetic effects for

AhR agonists remain to be discovered, and that the influence of hop extract on these and other

epigenetic factors has yet to be studied.

The present studies suggest ERα-AhR crosstalk is in part regulated by DNMT1, whose

key role in E_2-mediated *CYP1A1* inhibition is reversed by AhR-active 6-PN and the standardized

hop extract. Although the dose of 6-PN (1 µM) used for analysis was approximately 5 times the

amount of 6-PN found in the hop extract (179 nM in 5 µg/mL), the hop extract exhibited an

activity similar to that of 6-PN in assays tested. This apparent discrepancy is a ubiquitous

phenomenon for botanical extracts and herbal medicines, and may be interpreted as a

prototypical example of chemical potentiation relating to over-additive effects of

phytoconstituents and, specifically, chemical potentiation between 6-PN and congeneric prenyl-

phenolic hop constituents (Caesar and Cech, 2019; Schmidt et al., 2008). Although multiple

compounds may act on the same enzyme, other hop compounds may indirectly cause chemical

potentiation, influencing the pharmacodynamic profile of 6-PN through activity on additional

targets, an example of polypharmacology (Dietz et al., 2017). For example, the prenylated

chalcone and Michael acceptor, XH (4.68 µM in 5 µg/mL hop extract), is the most abundant and

therefore most prominent constituent of the hop extract. Interestingly, the polypharmacological

properties of hops depend greatly on XH, which exhibits resilience promoting properties *in vitro*

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through activation of the NRF2 antioxidant cascade, inhibition of inflammatory NF-κB signaling, and reduction of cell viability (Bolton et al., 2019; Dorn et al., 2012). The nature of XH as a Michael acceptor permits for KEAP1 alkylation, enhancing nuclear NRF2 concentration, and allowing for potential increase in AHR gene transcription (Shin et al., 2007). This is one example of a mechanism by which XH may potentiate the ability of hops and its preparations to promote estrogen detoxification.

In contrast, the polypharmacology of the investigated clinical hop extract on estrogen metabolism likely also includes antagonistic effects of 6-PN-mediated transcription of *CYP1A1*, for example, by the downregulating effect of the phytoestrogen, 8-PN (41.1 nM in 5 µg/mL hop extract). Yet, 8-PN concentrations may increase *in vivo* due to metabolic reactions mediated by intestinal gut microbiota or via phase I metabolism of isoxanthohumol to 8-PN (Dietz et al., 2016). At the same time, the isoxanthohumol concentration can increase through cyclization of XH, providing more isoxanthohumol for subsequent 8-PN formation (Dietz et al., 2013; Miranda et al., 2018). Additionally, 8-PN has been shown to be roughly five times more bioavailable than 6-PN when purely administered *in vivo* (Calvo-Castro et al., 2018). However, 8-PN may play a valuable role by inhibiting aromatase, the enzyme responsible for conversion of certain androgens to estrogens (Monteiro et al., 2007; Simpson, 2003). Additional prenylated phenols found in hops, such as isoxanthohumol and desmethylxanthohumol, may also contribute to the overall polypharmacology of hop extracts (Bolton et al., 2019). In summary, optimizing the bioactivity of a hop product for estrogen chemical detoxification seems to depend on 6-PN, but still involves many other compounds, their chemical transformations and reactions, as well as their pharmacodynamic interactions – as could be expected for a botanical agent.

Aiming for a reduction in estrogen chemical carcinogenesis through an induction of the beneficial 2-hydroxylation pathway has clinical importance for breast cancer prevention and resilience in women (Fuhrman et al., 2012). Therefore, the induction of the 2-hydroxylation estrogen detoxification pathway by hop extract and 6-PN and the increased ratio of 2- over 4-methoxyestrone (Figure 33) indicates a protective and resilient effect against breast cancer (Wang et al., 2016). However, *in vivo* studies are warranted to substantiate these *in vitro* results. Although the favorable 2-hydroxylation of estrogens is mainly catalyzed by P450 enzymes in the breast tissue, P450 1B1 can also catalyze the 2-hydroxylation in the breast tissue, and P450 1A2 is the main liver enzyme conducting the 2-hydroxylation of estrogens (Cribb et al., 2006; Dietz et al., 2016; Samavat and Kurzer, 2015). Additionally, while P450 1B1 is the predominant enzyme in the breast tissue catalyzing the genotoxic metabolism of estrogen to its 4-hydroxy catechol, this metabolism pathway can also occur through P450 1A1 in breast tissue and through P450 3A4 and P450 1A2 in the liver (Cribb et al., 2006; Samavat and Kurzer, 2015). However, P450 1A1 and P450 1B1 are primarily responsible for the initiation of estrogen detoxification and the carcinogenic metabolism of catechol estrogens in the local mammary tissue, respectively (Falk et al., 2013; Fuhrman et al., 2012; Samavat and Kurzer, 2015; Zahid et al., 2006).

The Phase I enzyme, NAD(P)H-quinone oxidoreductase 1 (NQO1) plays an important role in estrogen detoxification (Dietz et al., 2016; Samavat and Kurzer, 2015). NQO1 can chemically reduce the genotoxic 4-OHE-*O*-quinone to the catechol (Bolton et al., 2019; Dietz et al., 2016). Also, Phase II enzymes play an important role in detoxification of estrogens (Dietz et al., 2016; Samavat and Kurzer, 2015). Sulfotransferases and glucuronosyltransferases aid in estrogen detoxification through increasing water solubility for eventual excretion (Dietz et al., 2016; Samavat and Kurzer, 2015). The reactivity of catechol estrogens is reduced through

methylation by catechol-*O*-methyltransferase (COMT), and is associated with a reduction in breast cancer risk (Bolton et al., 2019; Dietz et al., 2016; Samavat and Kurzer, 2015; Wang et al., 2016). The 2- and 4-methoxyestrone metabolites formed by COMT are stable and can be assessed as biomarkers of their respective catechol estrones, indicative of estrogen detoxification or genotoxic estrogen metabolism, respectively (Wang et al., 2016). Ultimately, the estrogen metabolite profiles are important for the clinical influence of hops and its botanical preparations.

Chemical and biological analysis and multi-constituent standardization of extract polypharmacology is especially important for optimal safety and bioactivity of hop supplements, as they are used by women worldwide. Botanicals are complex agents and may interact differently in various populations depending on the constituent ratio in the extract. As shown by the present study, a hop formulation optimizing the activation of estrogen detoxification pathways with 6-PN, in particular, may enhance resiliency in women. To begin this process, an understanding of ERα-AhR crosstalk and the pharmacology of individual hop compounds was necessary. The present study revealed that 6-PN and a standardized hop extract decreased ERα-mediated epigenetic inhibition of *CYP1A1*, and preferentially upregulated the AhR-dependent estrogen detoxification pathway through attenuation of DNMT1-mediated repression of *CYP1A1* transcription. This suggests that 6-PN drives the ability of the hop extract to influence ERα-AhR crosstalk and increases activity of the estrogen detoxification pathway. Additional hop constituents provide chemical potentiation of estrogen detoxification for 6-PN and support the concept of polypharmacology in hops. Supplementing the present *in vitro* outcomes, *in vivo* studies are needed for further mechanistic insight and analysis of reasonable expectation for translational outcomes. Based on our investigations, it can be hypothesized that a 6-PN-rich hop extract may enhance resilience against breast cancer in preclinical outcomes, through the

attenuation of ERα-mediated epigenetic inhibition of AhR-dependent *CYP1A1* transcription, accompanied with preferential 2-hydroxylation of estrogens by P450 1A1 in breast tissue. This hypothesis should be suitable for subsequent translational studies of hops BDS.

Chapter 6: The Distribution of Prenylated Hop Compounds in ER Positive Tissues in a Premenopausal and Postmenopausal Rat Model.

Introduction

Hops products are popular BDS used by women to promote wellness or as an alternative to traditional hormone therapy for menopausal symptoms. According to a clinical trial of hop extract done in our Botanical Center, hops is safe for short term use and daily use may be appropriate (van Breemen et al., 2014). Hops contains many bioactive compounds of interest, particularly a spent hop extract contains prenylated flavanones and chalcones (Figure 34) (Karabin et al., 2016). Research over the last two decades in our Botanical Center and other labs has shown cytoprotection and antidiabetic properties attributed to xanthohumol (XH), aromatase inhibition and estrogenicity from 8-prenylnaringenin (8-PN), and AhR activation and an increase in estrogen detoxification by 6-prenylnaringenin (6-PN) (Chadwick et al., 2006; Dietz et al., 2017; Dietz et al., 2016; Helle et al., 2014; Hitzman et al., 2020; Milligan et al., 2000; Miranda et al., 2016; Monteiro et al., 2007; Rad et al., 2006; van Breemen et al., 2014; Wang et al., 2016; Yao et al., 2015).

A postmenopausal woman does not produce estrogen from the ovaries like a premenopausal woman. However, postmenopausal women still produce estrogen locally for example, in the mammary gland putting them at risk for breast cancer. Breast cancer is the most commonly diagnosed malignant cancer in women (American Cancer Society, 2019-2020). Hormone receptor positive breast cancers are regulated by estrogens and Estrogen Receptor Alpha (ERα) and leads to proliferation, and metastasis (Chumsri et al., 2011; Cortez et al., 2010; Santen et al., 2015; Siersbaek et al., 2018). Phytoestrogens found in botanical products may relieve menopausal symptoms but can also influence estrogen hormonal and chemical

carcinogenesis (Dunlap et al., 2017; Dunlap et al., 2015; Hitzman et al., 2020; Snelten et al., 2012).

Estrogen chemical carcinogenesis occurs through the metabolism of estrogens to their 4-catechols which upon oxidation form reactive quinones (Bolton and Thatcher, 2008; Snelten et al., 2012). These quinones form DNA adducts and lead to mutations and cancer (Falk et al., 2013; Fuhrman et al., 2012; Ziegler et al., 2015). The 4-hydroxylation of estrogens is mediated by the constitutively active but also AhR-inducible P450 1B1, while the 2-hydroxylation of estrogens occurs through the AhR-inducible P450 1A1, although basal level expression of P450 1A1 does exist (Kerzee and Ramos, 2001). Estrogens which are 2-hydroxylated are generally benign and excreted; therefore, this pathway is termed the estrogen detoxification pathway (Falk et al., 2013; Fuhrman et al., 2012; Hitzman et al., 2020). 6-PN from hops has been shown to preferentially activate the estrogen detoxification pathway *in vitro* (Hitzman et al., 2020; Wang et al., 2016). A higher amount of 6-PN is likely important for a wellness/resilience targeted hop extract. Yet the distribution of 6-PN in tissues is also important to consider, as 6-PN must reach hormone responsive tissues like the mammary gland or uterus in considerable quantities for any potential estrogen detoxification.

The influence of phytoestrogens as ligands of ERs is well documented (Dietz et al., 2017; Dietz et al., 2016; Hajirahimkhan et al., 2018; Helle et al., 2014). Scientific consensus on the resilience potential of phytoestrogens is mixed (Bilal et al., 2014; Ziegler, 2004). Phytoestrogens which are selective for ER beta (ERβ) may have resilience properties, reducing proliferation (Thomas and Gustafsson, 2011). Phytoestrogens which act as selective ERα agonists may have similar effects as estrogens, resulting in proliferation and inhibition of estrogen detoxification through ERα-mediated downregulation of *CYP1A1*, both carcinogenic outcomes (Dietz et al.,

2016; Hitzman et al., 2020; Sakamoto et al., 2010). 8-PN from hops is a potent and selective agonist of ERα but its carcinogenicity is debated (Keiler et al., 2013). Some publications show antiproliferative effects at high concentrations which are not clinically relevant, with others showing weak proliferation stimulation at lower concentrations (Brunelli et al., 2007; Helle et al., 2014; Stulikova et al., 2018). Therefore, caution must be taken when considering 8-PN in a hop extract, with a lower concentration in the extract likely being safer, particularly in postmenopausal women. More importantly, the distribution of 8-PN to hormone responsive tissues like the mammary gland and uterus must be considered.

Resilience properties are well documented for the main constituent of spent hop extract, the prenylated chalcone xanthohumol (Dietz et al., 2017; Dietz et al., 2013; Plazar et al., 2007; Suh et al., 2018). The importance of xanthohumol in the total bioactivity of hop extract cannot be understated. Xanthohumol, being a Michael acceptor, activates the antioxidant NRF2 cascade and inhibits the inflammatory NF-κB pathway (Bolton et al., 2019; Rucker et al., 2015). It has been theorized that the cytoprotective effects of xanthohumol balances potential carcinogenic properties of 8-PN (Dietz et al., 2017). A wellness/resilience promoting hop extract should contain a high percentage of xanthohumol which is ideally distributed to all tissues with high efficacy.

Safety evaluations of hop BDS for long-term use have yet to be established but it is suggested they are safe for daily use (van Breemen et al., 2014). However, the pharmacodynamics of a hop extract can vary and depends on the phytochemical makeup of the extract (Dietz et al., 2017). The pharmacokinetics of a hop extract is also an important factor to consider when evaluating safety and resilience properties. That is to say compounds with resilience properties, such as XH and 6-PN, must be distributed well to the relevant tissues. A

clinical trial to assess tissue distribution of hop prenylated compounds is infeasible; however, a similar *in vivo* assessment can be made with a rat model. Models for menopausal status can also be achieved using intact and ovariectomized (OVX) Sprague-Dawley rats. OVX rats no longer produce estrogen from the ovaries and lack circulating estrogens similar to a postmenopausal woman after a 14-week wash out phase, while the female rats with intact ovaries can be used as a model for women of premenopausal status.

A clinical study of 8-PN and 6-PN in humans showed that serum contains roughly 5 times more 8-PN than 6-PN, but this study was done only with blood samples and only in young men and women (Calvo-Castro et al., 2018). To our knowledge no *in vivo* studies have been conducted to compare hop compound tissue distribution in a pre- and postmenopausal model. We hypothesized that the distribution of the prenylated hop compounds occurs to all tissues, but biotransformation and ovarian status will influence this distribution. The process of digestion, as well as metabolism, may influence the concentration of prenylated hop compounds in hormone sensitive tissues. Sex hormone transporters may influence the distribution of phytoestrogens like 8-PN. For example, if sex hormone transporter levels are low or bound to estradiol, 8-PN levels may be reduced in hormone sensitive tissues and vice-versa. The present study sheds light on the distribution of prenylated hop compounds in a pre- and postmenopausal model and provides some evidence to substantiate the hypothesis. These studies of hop compound distribution *in vivo* provide valuable safety information and moves toward a better understanding of hop BDS used for women's health.

Results

Hop extract and 8-PN do not increase uterine weight.

Stimulation of uterine wet weight is an important factor to assess risk of endometrial cancer. Estrogen supplementation is known to increase uterine weight (Medlock et al., 1994; Overk et al., 2008). This was the case for the estrogen deprived OVX rats supplemented with estrogen in this study, but not in the rats with intact ovaries (Figure 35). 8-PN (4 mg/kg BW, orally) or hop extract (1000 mg/kg BW, orally) did not increase uterine weight in either OVX or intact ovary rats in this three-day study. This three-day time frame was chosen in order to assess estrogenic uterotrophic results, but outcomes may vary in a long-term study with daily hop extract administration. However, a 21-day study using a similar hop extract (400 mg/kg/day) and a 56-day bone study (60 mg/kg/day) found no change in uterine weight either (Keiler et al., 2017a; Overk et al., 2008). Additionally, hops are generally recognized as safe by the FDA.

Hop compound distribution in serum.

Blood is a common tissue used in distribution studies; therefore, the blood serum was analyzed for prenylated flavonoids and xanthohumol after hydrolysis (Figure 34). Free, bound, and conjugated compounds found in serum are transported throughout the body for tissue distribution or excretion. XH was present in the greatest quantities of the compounds tested, followed by IX, 8-PN, and 6-PN, all in the low ng/mL range (Figure 37). There was no significant difference due to the ovarian status. There was a difference in the ratios of each compound in the serum when compared to the ratios found in the botanical extract; in the extract XH is by far the highest, followed by 6-PN, IX, then 8-PN, while in the serum there is more 8-PN and IX than 6-PN and XH concentrations are much closer to the other prenylated hop constituents (Figures 34, 37).

Hop compound distribution in mammary gland.

Mammary gland is an important tissue for hormonal compounds like 8-PN, but also resilience promoting compounds like XH and 6-PN. Concentrations of 6-PN and XH are important when considering effects on estrogen chemical and hormonal carcinogenesis for their ability to regulate the estrogen detoxification pathway and antioxidant response pathways, respectively. XH was the most prominent compound averaging between 150-200 ng per g of tissue. XH was followed by 8-PN, IX, and 6-PN in females with intact ovaries. In OVX mammary gland, XH was followed by IX, 8-PN, and 6-PN, with 6-PN and 8-PN being very similar. (Figure 38).

Hop compound distribution in uterus.

Similar to the mammary gland, the uterus is an important estrogen sensitive tissue when considering a resilience promoting and estrogenic botanical extract such as hops. Again, of the four compounds analyzed XH was present in the highest concentration. XH and IX concentrations increased in OVX uteri compared to rats with intact ovaries, with XH significantly increasing to nearly 1 mg per g of tissue. XH concentrations were followed by 8-PN, IX, and 6-PN in the premenopausal model, while in the postmenopausal model XH concentrations were followed by IX, 8-PN, then 6-PN (Figure 39).

Ratio of 8-PN to 6-PN.

An interesting approach to analyzing the distribution and resilience potential of the clinical hop extract is the ratio of prenylated hop flavanones. Serum and mammary glands in both intact and OVX Sprague-Dawley rats was analyzed for the amount of 8-PN and 6-PN. The ratio of 8-PN to 6-PN was about the same in the serum, around 3:1. The ratio of 8-PN to 6-PN in

the premenopausal model roughly 6:1 and it dropped in the mammary gland for OVX

postmenopausal model to 2:1.

Discussion

The hop extract used in this study has been established to be safe for short term use and may be safe for daily use (van Breemen et al., 2014). That study involved serum and urine analysis of the four prenylated hop compounds of interest. Serum distribution of 8-PN and 6-PN had little difference in postmenopausal women, occurring in the low nanogram per milliliter range (van Breemen et al., 2014). A previous clinical study administered 500 mg of 6-PN or 8-PN to premenopausal women and found serum levels of 8-PN to be four to five times higher than 6-PN (Calvo-Castro et al., 2018). Although these studies provided valuable pharmacokinetic data on hop constituents in humans, they were limited in the scope of tissue analysis. Animal studies using Sprague-Dawley rats allowed for compound distribution analysis in estrogen sensitive tissues. The current study used intact females and ovariectomized females as a way to distinguish distribution between the ovarian status which represent a model of the menopausal status. This work can provide valuable hop pharmacokinetics for further clinical trials in pre- versus postmenopausal women.

The current research involved evaluating the distribution of hop prenylated bioactive compounds (Figure 34) *in vivo*. Compounds of interest included xanthohumol (XH), isoxanthohumol (IX), 8-prenylnaringenin (8-PN), and 6-prenylnaringenin (6-PN). The clinical hop extract prepared by Hopsteiner contains 33.20% of the resilience promoting XH (Bolton et al., 2019; Dietz et al., 2017; Ramos Alvarenga et al., 2014). The content of the related flavonoid IX was 1.1%. The content of the AhR agonist 6-PN was 1.2%, while the concentration of estrogenic 8-PN was 0.3% in the extract (Bolton et al., 2019; Dietz et al., 2017; Hitzman et al., 2020; Ramos Alvarenga et al., 2014; Wang et al., 2016). The chemical makeup of an extract can change once ingested. Concentrations of these four compounds, along with

desmethylxanthohumol (DMX) can fluctuate *in vivo* (Figure 34). Cyclization of xanthohumol to isoxanthohumol occurs in acid-catalyzed environments such as stomach acid, reducing XH concentrations and increasing IX concentrations (Figure 34) (Nikolic et al., 2005). Xanthohumol can theoretically be demethylated by gut microbiota or P450 1A2 to produce DMX (Legette et al., 2014; Wang et al., 2016). Either 6-PN or 8-PN can theoretically be spontaneously formed from DMX (Legette et al., 2014; Wang et al., 2016). Isoxanthohumol can also be demethylated by gut microbiota or P450 1A2 to produce 8-PN, a major pathway for metabolism (Legette et al., 2014; Wang et al., 2016). Together the pharmacokinetic biotransformation of hop prenylated bioactive compounds may produce tissue concentrations different from the original compound concentrations found in the clinical hop extract. Specifically, XH may be lower and IX, 6-PN, and 8-PN levels may be elevated. Indeed, when analyzing the serum XH concentration in this study, XH was still the major compound, but was only roughly present at a ratio of 12:6:3:1 for XH:IX:8-PN:6-PN, respectively. This is drastically different than the ratios of these compounds when compared to XH in the extract and speaks to the poor bioavailability of XH and the level of conversion to hop prenylated flavanones. The higher level of 8-PN also suggests that IX is converted to 8-PN, as 8-PN exists at only about 0.3% of the extract compared to 6-PN at 1.2%, yet there is roughly 3x more 8-PN than 6-PN in serum. These results indicate that there is more conversion to 8-PN than to 6-PN, and speaks to the lack of DMX biotransformation, instead it points to the likely prevalence of XH to IX and IX to 8-PN biotransformation. High variation in *in vivo* results speak to the individual variability in absorption, metabolism, and distribution of hop prenylated compounds in such a controlled animal model.

The scope of this study is limited in that the doses of hops was only administered for three consecutive days and at one dose. Pharmacokinetics of prenylated hop compounds may be

altered with long term use and may have dose dependent kinetics. The half-life of the phytoestrogen may differ from its regioisomer 6-PN, and half-life may be influenced by long-term use. Further studies are needed to address long term use of hop extract and to address questions regarding dose dependent kinetics of prenylated hop compounds but a past study using three doses of clinical hop extract in postmenopausal women found dose dependent kinetics, enterohepatic recirculation led to long half-lives, and 8-PN concentration increased after decreasing, suggesting *in vivo* conversion of IX or DXM to 8-PN (van Breemen et al., 2014). Another limit to these studies is that it measured only aglycones following hydrolysis and did not discriminate between conjugated metabolites. The influence of estrogen cycle should also be considered, as any three-day time frame may produce great variability due to the fluctuation of hormones in the premenopausal model. The methods of administration may also influence pharmacokinetics. In this study hop extract was administered via gavage so as to imitate an oral administration in humans.

Flavonoids undergo phase I and phase II first pass metabolism in the intestines and liver (Manach and Donovan, 2004; Thilakarathna and Rupasinghe, 2013). Glucuronides and some free prenylated hop flavanones reach serum and are transported throughout the body. Free compounds, but not glucuronides or other phase II metabolites, are able to freely cross the cell membrane and elicit biological responses, although transporters may aid in glucuronide uptake. Interestingly, certain flavanols have been shown to reduce beta-glucuronidase activity and phytoestrogens have been shown to increase UDP-glucuronosyltransferase family 2 member B15 enzyme (UGT2B15) (Harrington et al., 2006; Revesz et al., 2007). Activity of phase II metabolism enzymes can influence the transport and distribution of compounds and it could be that hop extract increases glucuronides and detoxification. This study utilized the addition of

beta-glucuronidase to samples so as to analyze the vast majority of all glucuronides and aglycone compounds present. This provides a higher amount of aglycone sample for analysis and better represents the distribution of compounds yet is limited in determining the actual bioavailable bioactive compound.

Transport proteins play a pivotal role in distribution of xenobiotics such as hop prenylated compounds. Albumin is the most common transporter and likely plays a role in serum transport of hop compounds, but sex hormone specific transporters such as sex hormone binding globulin (SHBG) aside from transporting estrogens also bind phytoestrogens and may transport the phytoestrogen, 8-PN, and to a lesser extent 6-PN (Dechaud et al., 1999). SHBG transports estrogens through the serum to hormone sensitive tissues expressing the SHBG receptor (SHBG-R). Interestingly, phytoestrogens such as genistein and naringenin, but not their glycoside counterparts, bind to SHBG and may transported like estrogens (Dechaud et al., 1999). It has also been shown that phytoestrogens like genistein increase the level of SHBG in cell cultures and in postmenopausal women (Loukovaara et al., 1995; Pino et al., 2000). It can be theorized that 8-PN may also increase SHBG, and in an estrogen deprived state like an ovariectomized rat may bind and transport 8-PN to tissues which express the SHBG-R such as hormone positive tissues. In a rat with intact ovaries and higher circulating estrogen, SHBG is bound to estrogen and may not bind to 8-PN with the same affinity. This may produce a disparity between premenopausal and postmenopausal models in the amount of phytoestrogen reaching hormone sensitive tissues, although no significance was seen between models for 8-PN in this study or for the ratio of 8-PN to 6-PN.

XH, 6-PN, and 8-PN elicit important bioactivities when considering estrogenic and resilience activities in the mammary gland. An extract containing 8-PN may influence estrogenic

influences due to its estrogenicity (Dietz et al., 2017; Heyerick et al., 2006). A 21-day study using ovariectomized rats showed a significant increase in uterine wet weight for 8-PN with intraperitoneal injection (4 mg/kg/day) (Overk et al., 2008). Interestingly, this study showed no change in uterine weight for 8-PN (4 mg/kg/day) or hop extract for 3 days with oral gavage administration and could speak to the high level of first pass effect and low bioavailability of oral administration. In a 3-day study 8-PN (15 mg/kg/day, subcutaneous) increased terminal end buds and proliferative markers in the mammary gland, yet in an 8-week study hop extract (56 mg/kg/day, food) showed no tumorigenic or proliferative response (Helle et al., 2014; Keiler et al., 2017b). This may speak to the balancing nature of the extract, and XH and 6-PN may play a role in reducing the potential carcinogenic nature of 8-PN. 6-PN (1 µM) and hop extract (5 µg/mL) have been shown to preferentially increase the 2-hydroxylation over 4-hydroxylation metabolism pathway of estrogens in breast cancer cells, reducing estrogen chemical carcinogenesis *in vitro* (Hitzman et al., 2020; Wang et al., 2016).

This study explored the distribution of prenylated flavanones and chalcones in hormone sensitive tissues and serum. All four prenylated compounds analyzed were available in the target tissues, the mammary gland and uterus. Although XH made up the greatest extent of the extract, the ratio of XH to the other compounds in these tissues was markedly different than in the extract, with XH ratios decreasing in tissue suggesting metabolism of XH to IX and IX to 8-PN. The results of this study showed 8-PN is more concentrated than 6-PN in serum and hormone sensitive tissues. There was no significant difference in prenylated flavanones between models dependent on the ovarian status with some exceptions. The prenylated chalcone XH was significantly increased in the OVX uteri when compared to a female with intact ovaries, as was IX, although insignificantly. Also, of interest was the decrease in the ratio of 8-PN to 6-PN seen

for the mammary gland in the ovariectomized (postmenopausal) model when compared to the

intact (premenopausal) model, although these results may be due to the small sample size used.

These results indicate the role of XH and 6-PN in resilience promoting properties seen for

previous chapters may be relevant in hormone sensitive tissues where these compounds can be

found upon hop extract oral administration. The fact that 6-PN reaches tissues, albeit to a lesser

extent than other hop compounds, indicates that 6-PN may have low bioavailability, although it

may still have the ability to increase estrogen detoxification in hormone sensitive tissues,

although the clinical relevance regarding the concentration of 6-PN which reaches tissues as well

as the clinical importance of 6-PN are unknown. Results also indicate that resilience promoting

XH was the dominant prenylated hop constituent in all tissues analyzed, but XH concentrations

may increase in the postmenopausal uterus, although these results need further research and

based on a small sample size, a larger study may resolve these discrepancies. The mechanisms

which underlie these differences associated with menopausal status need to be further explored.

Better biotransformation studies could be done through administering pure amounts of XH and

analyzing IX, 8-PN, and 6-PN metabolites. This would provide more definitive answers

regarding *in vivo* conversion of prenylated hop chalcones and flavanones. Furthermore, *in vivo*

immunohistochemistry of P450 1A1 expression, and ultimately LC-MS analysis of 2- and 4-

catechol estrogens in hop treated mammals would provide valuable data regarding the resilience

potential of hop BDS. Additionally, the current results speak to the safety and resilience

promoting nature of short-term hop extract supplementation in pre- and postmenopausal women.

However, a long-term study to assess pharmacokinetics and pharmacodynamics in hormone

receptor positive tissues may further answer lingering questions regarding the safety and efficacy

for the daily use of hop extract in postmenopausal women, as well as provide relevance for future clinical studies.
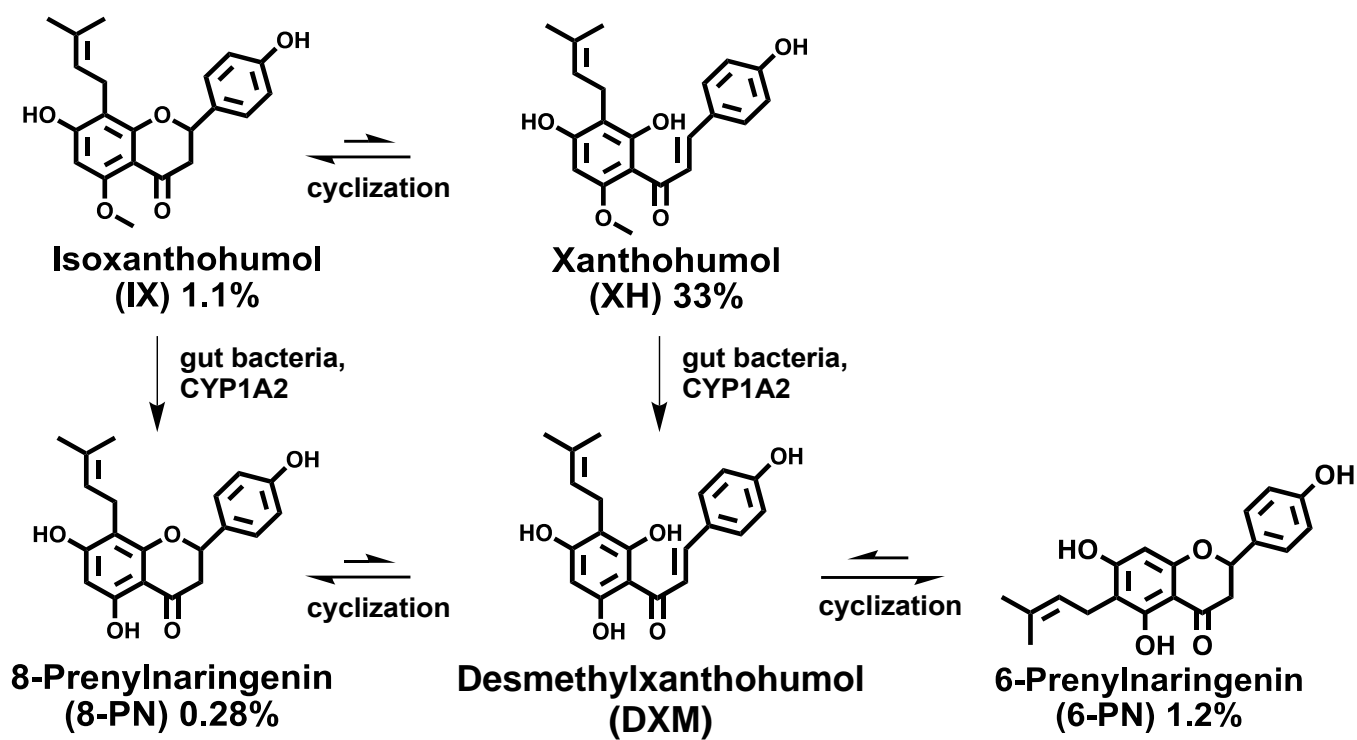
Figure 34: Hop prenylated flavanones and chalcones and their biotransformations with concentrations listed for the hop clinical extract.
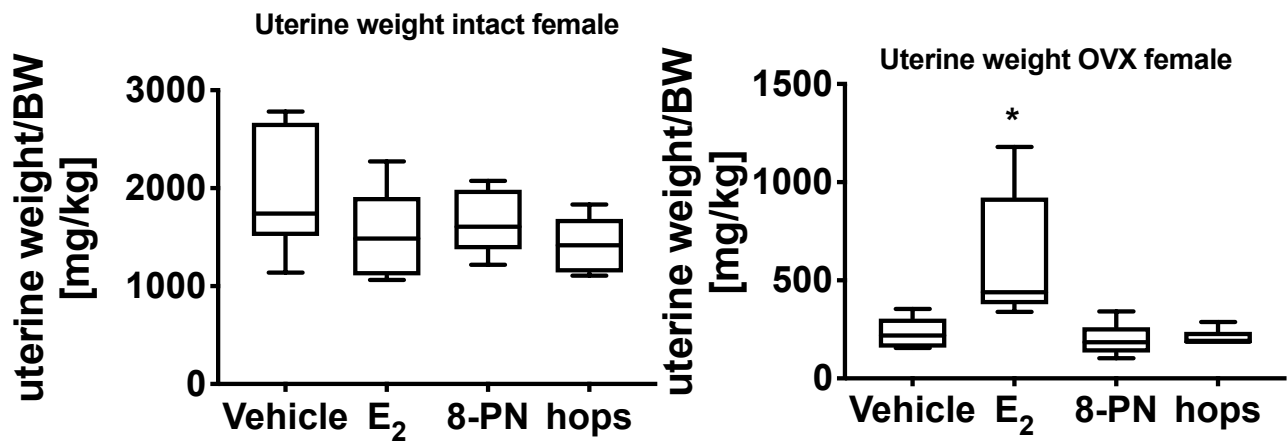
Figure 35: Uterine wet weight for Sprague-Dawley females with (intact) and without (OVX) ovaries treated with vehicle, E_2, 8-PN, or hop extract. Box indicates upper and lower quartiles with a line for the average. N=6, +/- SEM, one-way ANOVA *p < 0.05.

A. Intact Female Serum
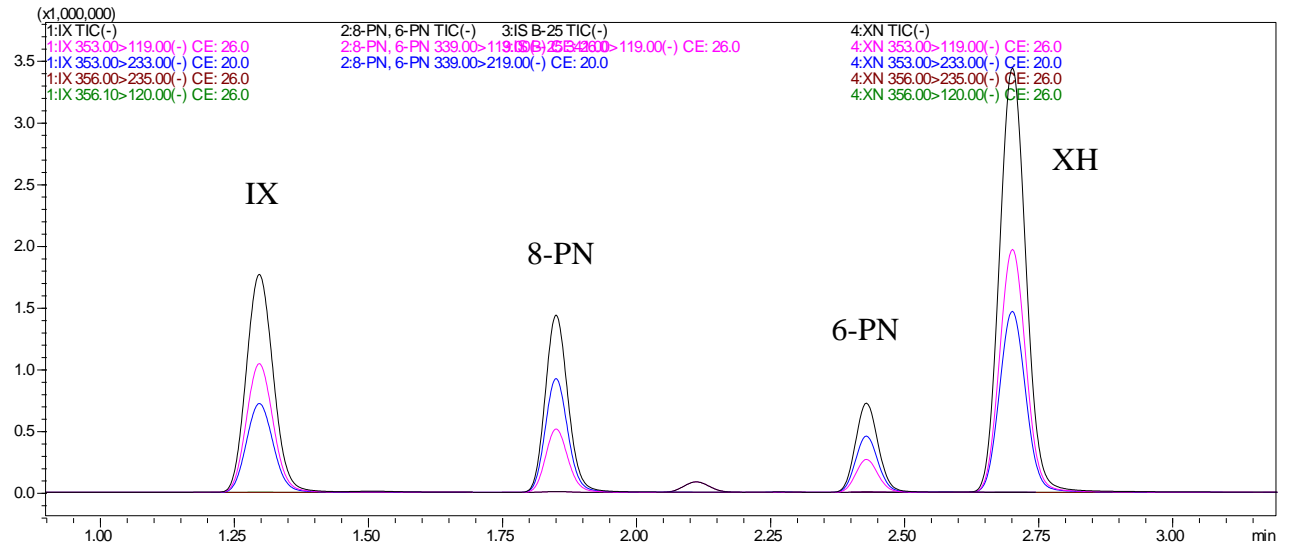
B. OVX Female Serum
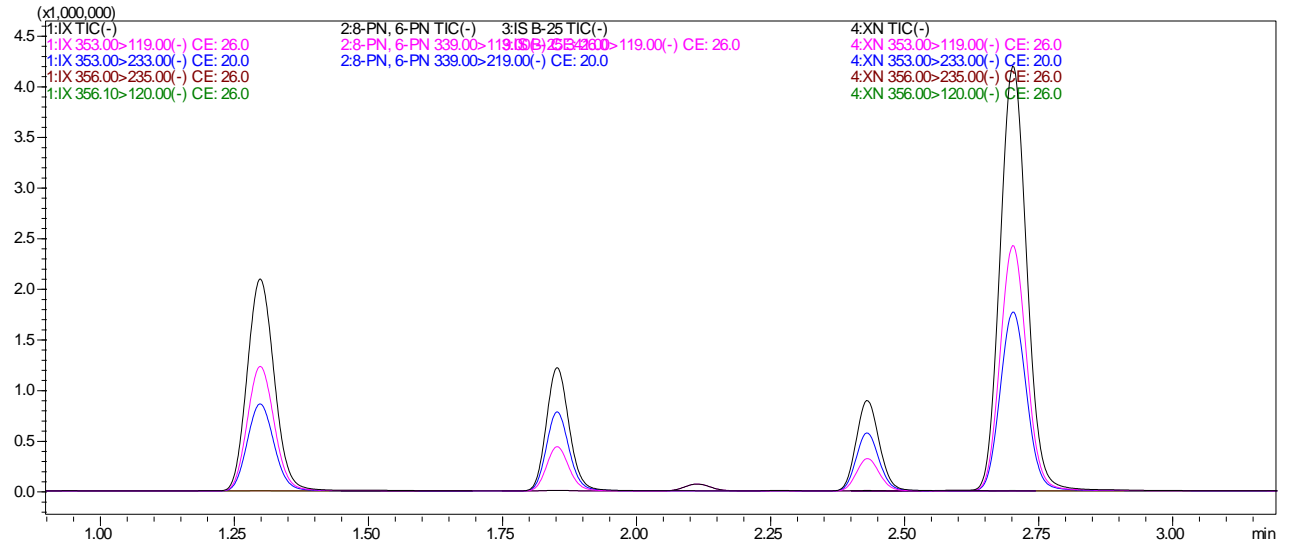
C. Intact Female Mammary Gland
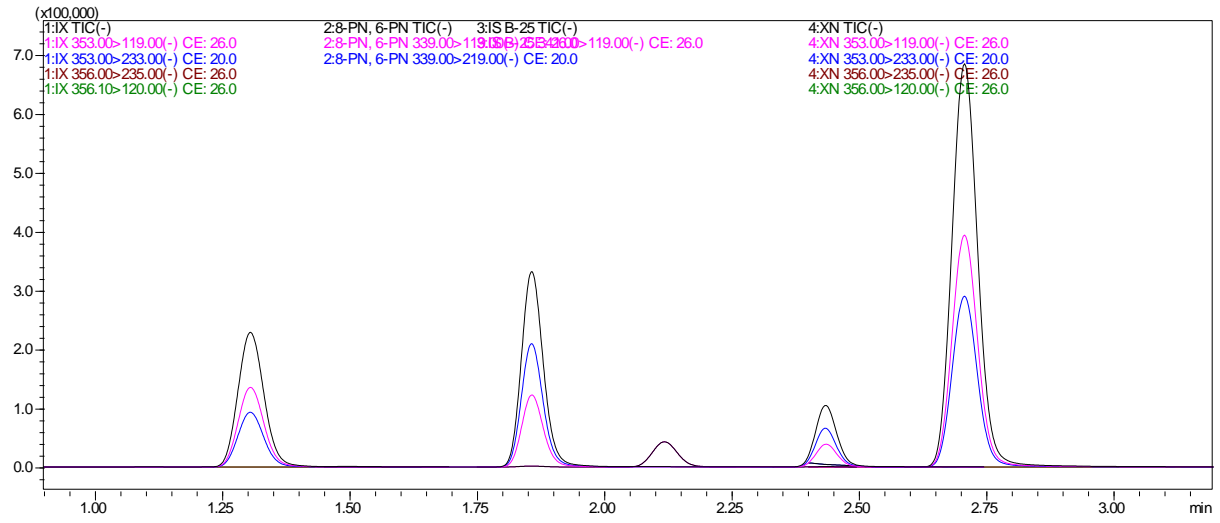
D. OVX Female Mammary Gland
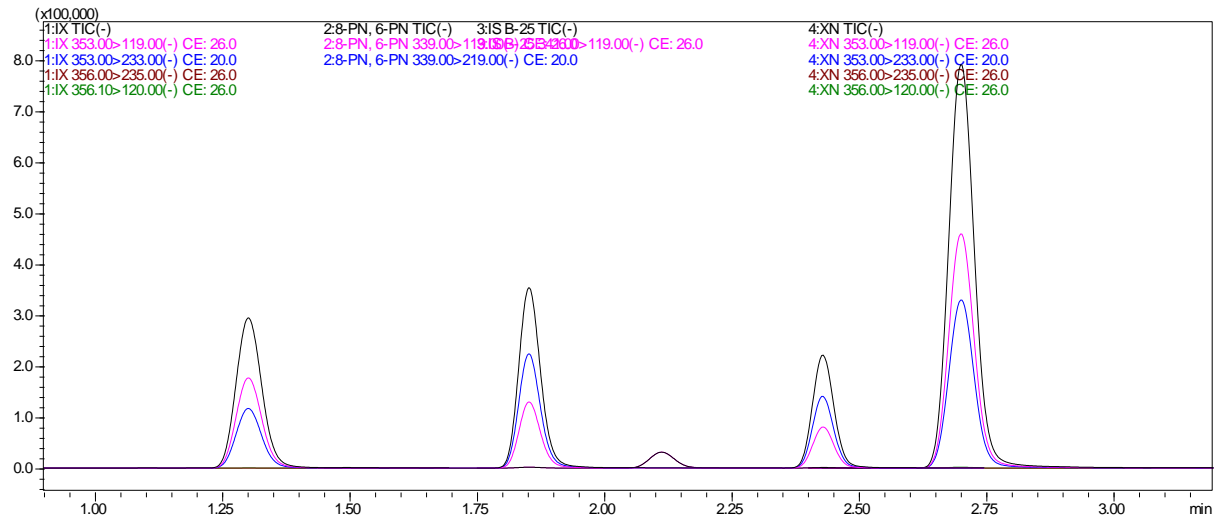
E. Intact Female Uterus
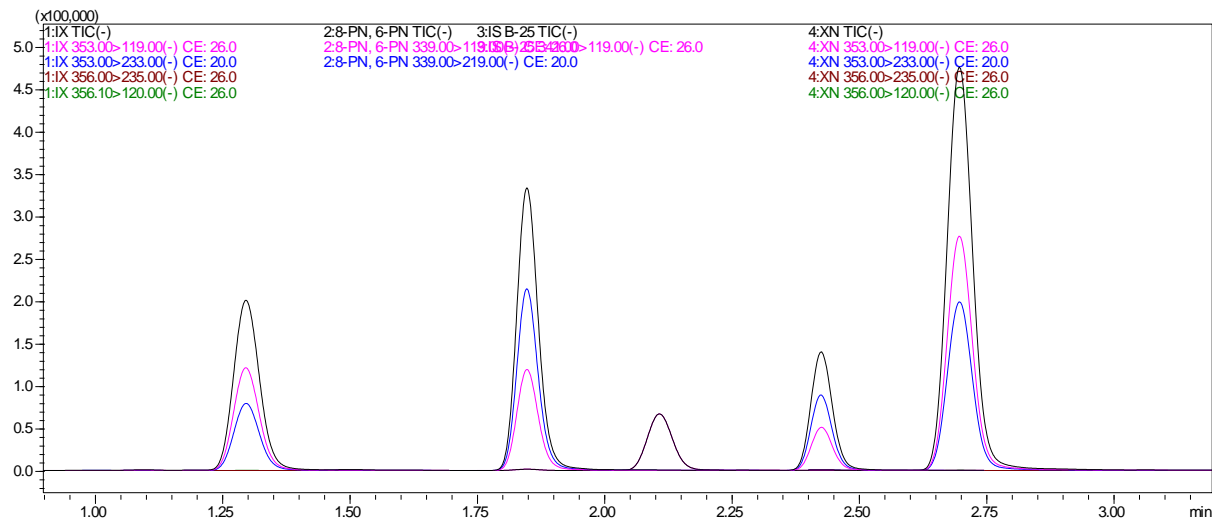
F. OVX Female Uterus
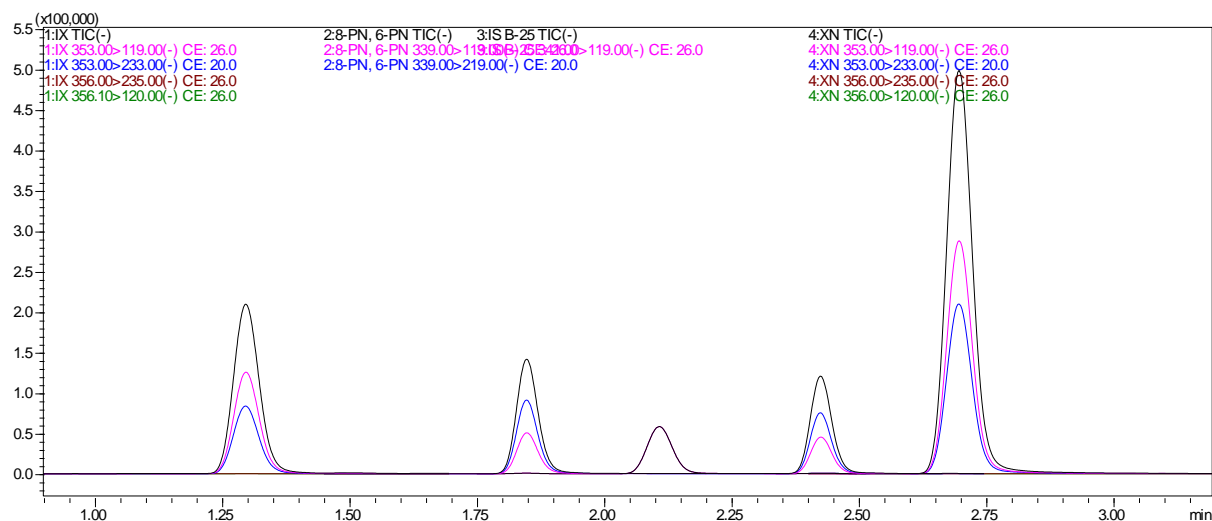
Figure 36: Example of LC-MS/MS chromatograms for the serum, mammary gland, and uterus of a hop extract-treated female Sprague-Dawley rat with intact ovaries (top) or from an ovariectomized Sprague-Dawley rat (bottom). From left to right, isoxanthohumol (IX), 8-prenylnaringenin (8-PN), 8-isopentylnaringenin, 6-prenylnaringenin (6-PN), xanthohumol (XH). Serum: A, B. Mammary gland: C, D. Uterus: E, F.
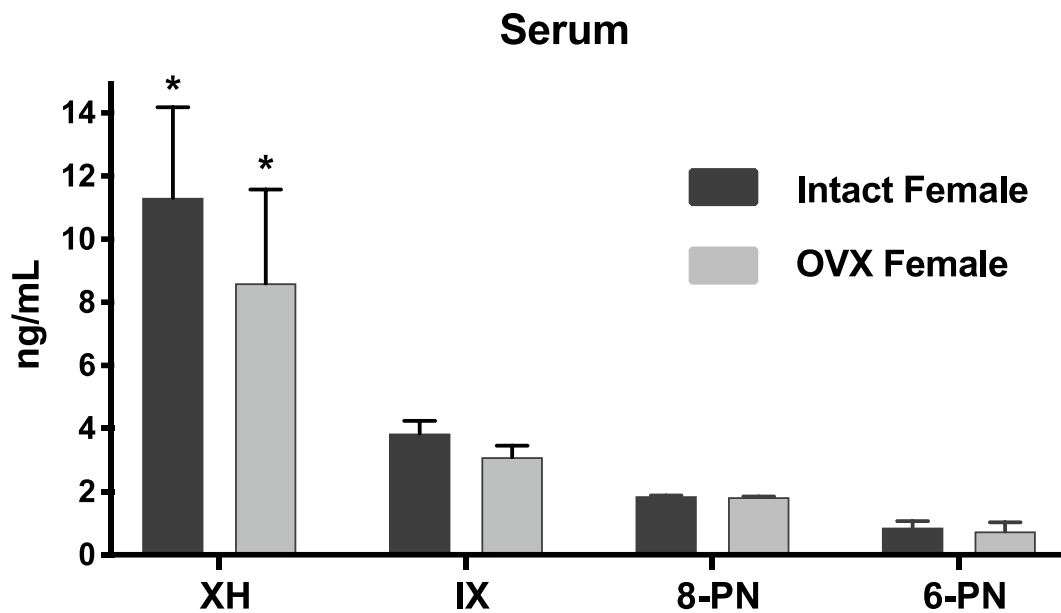
Figure 37: Quantification of hop prenylated compounds in the serum of Sprague-Dawley rats treated for three days with hop extract or vehicle. Significance associated against vehicle which was at zero for all hop compounds. N=3-6, +/- SEM, two-way ANOVA, *p < 0.05.
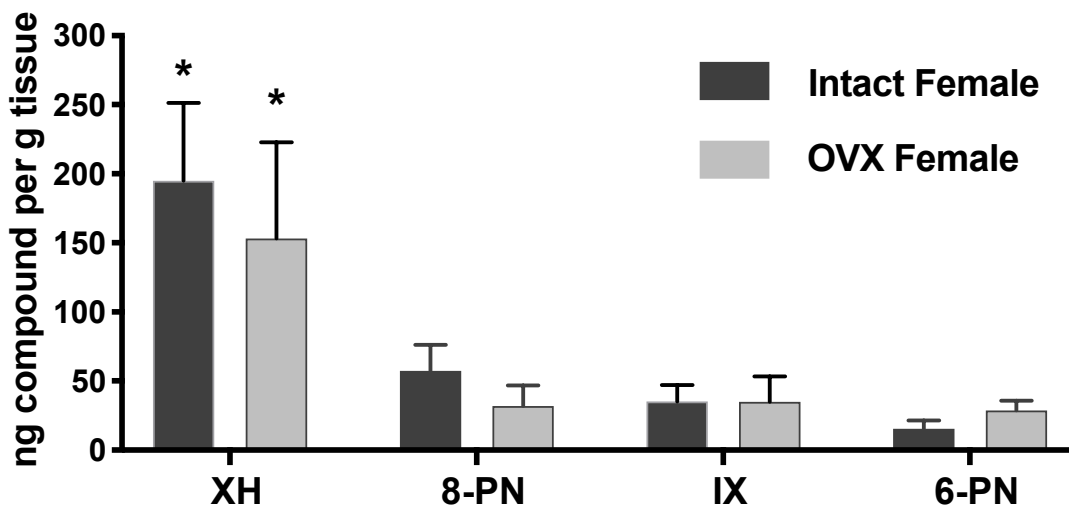
Figure 38: Quantification of hop prenylated compounds in the mammary gland of Sprague-Dawley rats treated with hop extract or vehicle. Significance against vehicle which as zero. 6-PN was excluded from analysis due to low sample number. N=2-4, +/-SEM, two-way ANOVA, *p < 0.05.
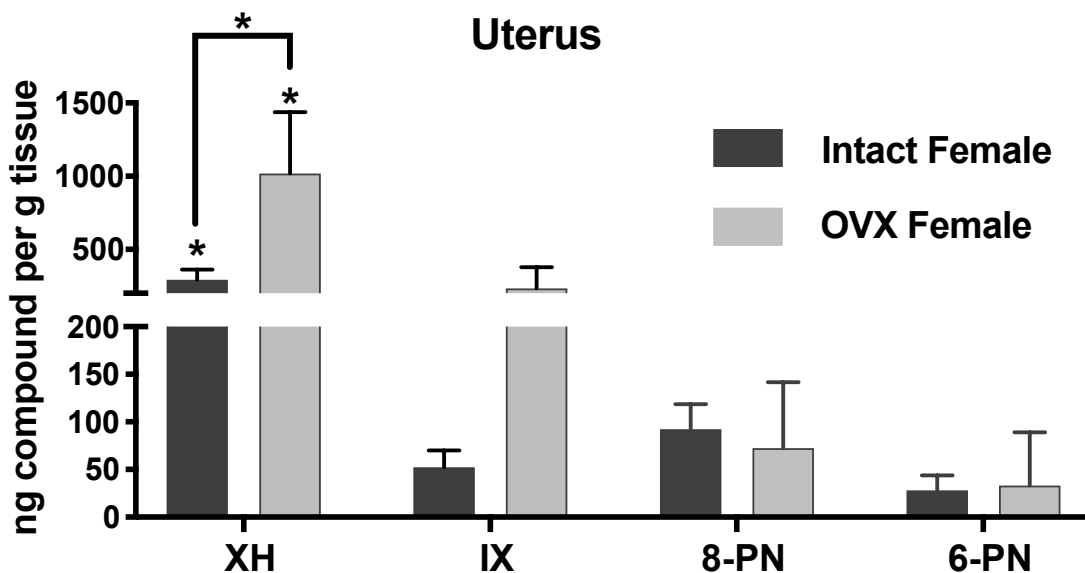
Figure 39: Quantification of hop prenylated compounds in the uterus of Sprague-Dawley rats treated for three days with hop extract or vehicle. Significance against vehicle, which was zero, or between ovariectomized and intact ovary rats. 6-PN and 8-PN were excluded due to low sample size. N=2-4, +/-SEM, two-way ANOVA, *p < 0.05.
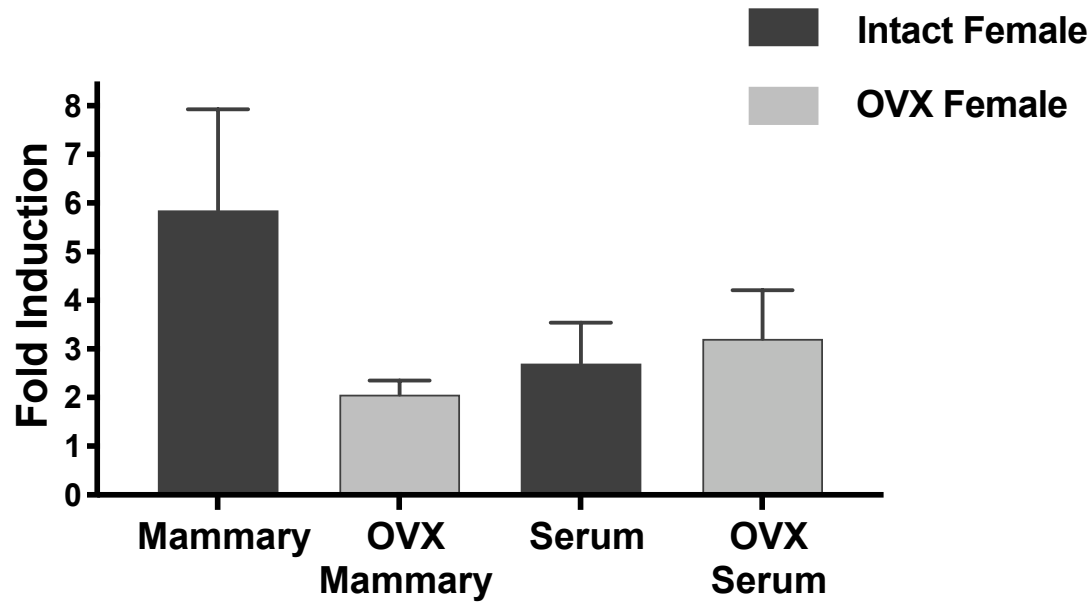
Figure 40: Ratio of 8-PN to 6-PN in serum and mammary gland of Sprague-Dawley rats treated for three days with hop extract. N=2-4, +/-SEM, two-way ANOVA, *p < 0.05.

Chapter 7. Conclusions and Future Directions

Women's health BDS are increasingly popular options for the relief of menopausal symptoms and to promote general wellness; however, safety, mechanisms of actions, and efficacy are still relatively poorly understood. This series of studies involved evaluating women's health botanical extracts and their designated bioactive compounds for an ability to influence the estrogen chemical carcinogenesis pathway *in vitro*, discerning potential mechanisms of action, and evaluating the distribution of bioactive prenylated hop chalcones and flavanones *in vivo*. It was hypothesized that botanical extracts which beneficially influence the estrogen detoxification pathway may reduce estrogen chemical carcinogenesis. Part of this hypothesis was based on the theory that estrogen crosstalk occurs on AhR pathways, or the ability for activated ER to selectively suppress transcription of *CYP1A1* but not *CYP1B1*. This would then lead to preferential metabolism of estrogen through P450 1B1 to its genotoxic 4-hydroxylated metabolite, and estrogen chemical carcinogenesis. As a result, botanical extracts that reverse estrogen crosstalk may reduce estrogen chemical carcinogenesis.

Many well-established extracts from women's health botanicals were evaluated for an ability to degrade ERα, exhibit estrogenic and antiestrogenic activity, and modulate transcription of enzymes involved in the estrogen detoxification pathway. The bioactivities exhibited varied based on the botanical source and species, as well as extraction solvents and extraction technique. Generally, hops and *Epimedium* or their bioactive compounds decreased ERα expression in breast cancer cells and exhibited mixed estrogenic/antiestrogenic activity in Ishikawa endometrial cancer cells. An extract may be designed to exhibit this antiestrogenic activity by removing estrogenic compounds such as 8-prenylnaringein. Knock-out DESIGNER extracts of hops proved the importance of xanthohumol in these bioactivities. The importance of

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hydrolysis for *Epimedium* extracts was also evident from the observation that the hydrolyzed extract exhibited far greater activity than the unhydrolyzed extract, containing glycosides of icaritin. Bioactivity of *Epimedium* extracts can be associated with icaritin, which is likely formed from the unhydrolyzed icariin through the microbiome and metabolic enzymes.

Various women's health botanical extracts investigated in our Botanical Center were also analyzed for abilities to modulate transcription of enzymes involved in the estrogen detoxification pathway. Notably, it is hypothesized that both the clinical hop extract and the hydrolyzed *Epimedium* extract generally had beneficial impact on the transcription of enzymes involved in the estrogen detoxification pathway. Other extracts had mixed results, for example the Alaskan *Rhodiola rosea* extract significantly increased *CYP1A1*, but decreased *COMT*. Interestingly the *Angelica sinensis* extracts varied greatly based on the solvents used in the extraction process, the ethanolic/water extract generally increased the transcription of every enzyme tested, notably *COMT* and *NQO1*. The licorice species showed similar results with *G. inflata* being most active on *ESR2* and *AHR*, while *G. inflata* and *G. glabra* both upregulated *NQO1*. The clinical red clover extract preferentially increased mRNA for *ESR1* over *ESR2* and preferentially decreased *CYP1A1* but not *CYP1B1*, indicating red clover extract may not promote resilience, which is a dynamic property enabling cells, organs, organisms, or individuals to resist or recover from the effects of a physiological or pathological stressor according to the NIH (NIA-NIH, 2020). Similarly, silymarin from milk thistle decreased the transcription of most enzymes tested, including *CYP1A1*, *NQO1*, and *COMT* and may not promote resilience either. Based on the results gathered when analyzing targets in the estrogen detoxification pathway, hop extract and hydrolyzed *Epimedium* exhibited the best resilience properties *in vitro*. Both hops

and *Epimedium* make promising extracts for further study and hops was further analyzed for mechanisms of resilience and *in vivo* distribution analysis.

The clinical red clover extract was highly estrogenic and led to preferential metabolism of estrogen through the genotoxic P450 1B1 pathway (Dunlap et al., 2017). Yet red clover contains the AhR agonists formononetin and biochanin A, as well as irilone, which reduced transcription of the AhR repressor. When subjected to the DESIGNER (Deplete and Enrich Select Ingredients to Generate Normalized Extract Resources) fraction methodology, red clover fractions rich in each of those compounds were produced. Fractions rich with AhR agonists preferentially activated CYP1A1, yet the irilone rich fraction had no such activity. Interestingly, irilone potentiated AhR activity, but reduced AhR-dependent transcription. All red clover fractions, including those rich with AhR agonists, reduced P450 1A activity, and, therefore, a DESIGNER fraction which reduced estrogen chemical carcinogenesis could not be produced. This can be explained by the intracellular conversion of the AhR agonists formononetin and biochanin A to their demethylated and estrogenic forms, daidzein and genistein. Both daidzein and genistein are estrogenic ligands and known to downregulate *CYP1A1* yet act as preferential ERβ ligands, although these are likely unrelated. A red clover KOE also upregulated *CYP1A1*, but based on its bioactive compounds, either formononetin or biochanin A, such extracts too will likely cause metabolic conversion of those AhR active compounds to their demethylated estrogenic counterparts. These results indicate that red clover represents the case of a botanical extract that is difficult to optimize for certain resilience promoting properties and also may not be the safest choice as a female menopausal botanical, although the European Food Safety Authority has deemed isoflavones safe in peri- and postmenopausal women. The safety of red

clover extracts makes for interesting discussion and further analysis of red clover should be included in future safety studies of women's health BDS.

Based on Chapter 3 initial screening of women's health botanical extracts on the estrogen detoxification pathway, the clinical hop extract was chosen for additional *in vitro* mechanism studies in MCF-7 breast cancer cells. E_2-activated ERα causes the selective downregulation of *CYP1A1*, thereby initiating estrogen crosstalk on AhR-ERα and leading to its own carcinogenic metabolism. Clinical hop extract and its 6-prenylated flavanone constituent, 6-PN, preferentially upregulated *CYP1A1* in the presence of estradiol, reversing estrogen crosstalk on AhR-ERα. The present study showed that both hop extract and 6-PN caused degradation of ERα, in part through AhR-induced proteasomal degradation. The outcome of 6-PN exposure may be reduced or fewer ERα-mediated effects in general. Indeed, it was shown that E_2 leads to inhibitory epigenetic effects on CYP1A1 by DNMT1, an outcome of estrogen crosstalk on AhR-ERα, and that 6-PN and hop extract reverse DNMT1-mediated epigenetic inhibition of CYP1A1. The ultimate outcome of reversing this estrogen crosstalk on AhR-ERα and preferentially increasing CYP1A1 transcription is that it led to preferential metabolism of estradiol to its detoxified 2-catechol, ultimately forming the benign methoxy catechol, 2-methoxyestrone by COMT, and leading to an increase in estrogen detoxification. This study also showed the importance of 6-PN standardization for hop extract bioactivity, revealed a novel mechanism of action for the action of hop extract involving 6-PN to reverse estrogen crosstalk on AhR-ERα, and showed that both hop extract and 6-PN increase the estrogen detoxification pathway as well as detoxified estrogen metabolites. Thus, this study confirmed the resilience promoting nature of hop extract *in vitro*.

Due to its resilience promoting *in vitro* profile, the hop extract was chosen for more detailed *in vivo* studies. It was particularly important to analyze whether hop bioactive

compounds reach the tissues of interest for resilience promoting properties. Accordingly, a three-day study analyzed the safety and estrogenicity of hop extract, as well as the distribution of hop prenylated compounds in serum and hormone sensitive tissues of intact and ovariectomized Sprague-Dawley rats in a pre- and postmenopausal model, respectively. Notably, no uterotrophic, or uterine proliferation effects were observed for hop extract at 1000 mg/kg, thereby indicating safety of the extract in this short-term rat study. Interestingly, XH, the most prominent compound in the extract, present at about 30-fold the concentration of that of IX, and about 100 times more than 8-PN, was also the most prominent compound in tissue and serum. However, cyclization through Michael addition can produce IX from XH, metabolic processes of the intestinal microbiome and intracellular metabolism enzymes can lead to 8-PN from IX. This could explain why the resulting distribution of these hop prenylated compounds was much closer than expected with serum and tissue levels of XH being only 3-4 times higher than IX and only 4-5 times higher than 8-PN. 6-PN was consistently found in all tissues and serum, but to a lesser extent than 8-PN, though 6-PN is 4 times more concentrated than 8-PN in the administered hop extract. However, the fact that 6-PN was found in hormone sensitive tissues indicates that the resilience enhancing nature of the hop extract due to 6-PN may be exhibited in the mammary gland as an important target tissue. Interestingly, the ratio of 8-PN to 6-PN was noticeably yet insignificantly lower in the ovariectomized postmenopausal model compared to the intact premenopausal model. This observation was made in the mammary gland, but not in the serum. An explanation for the mechanisms responsible for this the difference in pre- and postmenopausal models requires further research.

From a more general perspective the work involved in these studies confirmed that certain women's health botanical extracts studied in our Botanical Center can indeed exhibit

activity on the estrogen detoxification pathway by engaging mechanisms involved in reversing estrogen crosstalk on AhR-ERα and increasing estrogen detoxification. This work also highlighted the importance of biological standardization of botanical extracts in combination with chemical standardization, as a prerequisite to eliciting specific biological responses: specifically, the importance of 6-PN standardization of a hop extract capable of reducing estrogen chemical carcinogenesis. Additionally, the present studies exemplified the importance of standardization to multiple bioactive markers in order to optimize biological responses and cover the full pharmacology of a botanical extract more comprehensively. Further mechanistic, animal, and clinical studies are needed to address the biological standardization of botanical extracts, as these extracts may elicit different pharmacokinetics and pharmacodynamics based on age, menopause, cancer status, duration of intake, and other factors.

In vitro epigenetic regulation changes induced by hop extract are of great interest in elucidating mechanisms of AhR-ERα crosstalk. Crosstalk mechanisms can be further explored using immunohistochemistry and PCR of P450 1A1, ERα, AhR, and other targets in the estrogen detoxification pathway in animal models. Translational studies in a clinical setting could analyze estrogen metabolites and the ratio of 2- to 4- catechol estrogens in serum and urine to assess the ability for hop extract to influence the estrogen detoxification pathway and exhibit resilience properties *in vivo*. In order to maximize efficacy and safety, the polypharmacology and dosage of a botanical extract should be established in preclinical and clinical settings. The ability for bioactive compounds to biotransform, exhibit dose dependent pharmacology, and ultimately influence estrogen detoxification makes further clinical evaluation of hop extract and other women's health botanicals essential for safety and efficacy establishment. Further epigenetic mechanisms studies, as well as preclinical and clinical evaluations of hop extract effects on

estrogen detoxification pathway targets and estrogen metabolites needs to be done to fully

substantiate the clinical use of hops and other women's health botanicals for their resilience

potential. However, the current marketing status of BDS makes hop extract and other women's

health BDS continuously available for over the counter purchase. While hop extracts and other

BDS are generally regarded as safe, the still booming dietary supplement industry would gain

more scientific validity from incorporating biological standardization of BDS for their

polypharmacology, as a demonstrated factor of enhanced safety, and likelihood of efficacy.

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Chapter 9. Appendices

6-Prenylnaringenin from Hops Disrupts ERα-Mediated Downregulation of CYP1A1 to Facilitate Estrogen Detoxification

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Publication:

Chemical Research in Toxicology

Publisher:

American Chemical Society

Date:

Nov 1, 2020

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Chapter 10. VITA

Ryan T. Hitzman

<u>Education</u>

2016-2021   Ph.D., Pharmacognosy, University of Illinois at Chicago, Chicago, IL

                 Advisors: Dr. Guido F. Pauli, Dr. Judy L. Bolton

2009-2015   B.S., Biology, University of Illinois at Chicago, Chicago, IL

<u>Research Experience</u>

University of Illinois at Chicago College of Pharmacy, Chicago, IL (2016-present)

Description: At the UIC/NIH Center for Botanical Dietary Supplements Research I investigated botanical modulation of estrogen carcinogenesis

Advisor: Dr. Guido F. Pauli

- Utilized biological extractions and LC-MS to investigate the distribution and metabolism of botanical compounds in an *in vivo* model.

- Evaluated derivatives of natural products for estrogen activity and cytotoxicity.

Advisor: Dr. Judy L. Bolton

- Wrote a successful NIH F31 grant to research the role of botanicals on AhR-ERα crosstalk.

- Utilized cell culture, animal studies, luminescence, fluorescence, PCR, LC-MS, and other assays to investigate the role of botanicals on AhR-ERα crosstalk.

- Developed assay methodology to investigate AhR-ERα crosstalk.

- Discovered a role for DNMT1 in the epigenetic inhibition of CYP1A1.

- Mentored five students (2 Ph.D., 1 master's, 1 PharmD, and 1 undergraduate) in scientific topics relating to cell culture, experimental design, assay methods, and scientific reasoning.

University of Illinois at Chicago College of Medicine, Chicago, IL (2015-2016)

Advisor: Dr. Kishore K. Wary

- Led animal handling and assisted in genetic engineering for a myocardial infarction mouse model.

- Developed a role for NANOG and Wnt in the regeneration of cardiac micro vessels.

University of Illinois at Chicago College of Dentistry, Chicago, IL (2013)

Advisor: Dr. Srilata Bagchi

- Performed cell culture and assisted in analyzing DDB and E6/E7 function in cervical cancer cells.

Genome Institute of Singapore, Singapore (2012)

Advisors: Dr. Shyam Prabhakar and Dr. Jeremie Poschmann

- Learned cell culture, blotting, ELISA, and genetic analysis in DNA damage models.

- Developed dot blot techniques and analyzed DNA damage in colon cancer cells.

Teaching Experience

2018-present   Graduate Research Fellow, University of Illinois at Chicago

2016-2018   Graduate Research Assistant, University of Illinois at Chicago

2016-2017   Teaching Assistant, University of Illinois at Chicago

2013-2015   STEM Tutor, Smart Kids Digital Tutoring

Publications

1. **Hitzman R.**, Dunlap T., Howell C., Chen S.N., Vollmer G., Pauli G., Bolton J., and Dietz B. 6-Prenylnaringenin from Hops Disrupts ERα-Mediated Downregulation of *CYP1A1* to Facilitate Estrogen Detoxification. *Chem. Res. Tox.* **2020** 33(11) 2793-2803.

2. Mbachu O., Howell C., Simmler C., Malca-Garcia G., Skowron K., Dong H., Ellis S., **Hitzman R.**, Hajirahimkhan A., Chen S.N., Nikolic D., Moore T., Vollmer G., Pauli G., Bolton J., and Dietz B. SAR Study on Estrogen Receptor α/β Activity of (Iso)flavonoids: Importance of Prenylation, C-Ring (Un)Saturation, and Hydroxyl Substituents. *J. of Agric. Food Chem.* **2020** 68(39) 10651-10663.

3. Baruah J., Chaudhuri S., Mastej V., Axen C., **Hitzman R.**, Ribeiro I., and Wary K. Low-Level Nanog Expression in the Regulation of Quiescent Endothelium. *Arteriosc. Throm. and Vasc. Bio.* **2020** 40(9) 2244-2264.

4. Baruah J., **Hitzman R.**, Zhang J., Chaudhuri S., Mastej V., and Wary K. The allosteric glycogen synthase kinase-3 inhibitor NP12 limits myocardial remodeling and promotes angiogenesis in an acute myocardial infarction model. *J. Biol. Chem.* **2017** 292(50) 20785-20798.

Poster Presentations

1. **ASP Conference**: Hitzman R., Dunlap T., Chen S.N., Pauli G., Dietz B., Bolton J. *Epimedium* sp. and Icaritin Promote Non-Genotoxic Estrogen Metabolism. Madison, WI. July 2019.

2. **NIH CARBON Conference**: Hitzman R., Dunlap T., Chen S.N., Pauli G., Dietz B., Bolton J. *Epimedium* sp. and Icaritin Promote Non-Genotoxic Estrogen Metabolism. Bethesda, MD. May 2019.

3. **UIC Research and Impact Day**: Hitzman R., Dunlap T., Chen S.N., Pauli G., Dietz B., Bolton J. *Humulus lupulus* and 6-PN Activate AhR to Promote Breast Cancer Chemoprevention through Epigenetic Regulation of CYP1A1. Chicago, IL. April 2019.

4. **UIC College of Pharmacy Research Day**: Hitzman R., Dunlap T., Chen S.N., Pauli G., Dietz B., Bolton J. *Humulus lupulus* and 6-PN Activate AhR to Promote Breast Cancer Chemoprevention through Epigenetic Regulation of CYP1A1. Chicago, IL. February 2019.

5. **NIH CARBON Conference**: Hitzman R., Dunlap T., Chen S.N., Pauli G., Dietz B., Bolton J. *Humulus lupulus* (Hops) Activation of AhR Induces Estrogen Detoxification Pathways. Bethesda, MD. May 2018.

6. **MIKI Medicinal Chemistry Conference**: Hitzman R., Dunlap T., Chen S.N., Pauli G., Dietz B., Bolton J. *Humulus lupulus* (Hops) Activation of AhR Induces Estrogen Detoxification Pathways. Chicago, IL. April 2018.

Awards and Honors

2019   Edward Benes Scholarship, UIC College of Pharmacy

2018   F31 Grant: AT010090, NCCIH/NIH

Affiliations and Memberships

2018-present   American Society of Pharmacognosy

2017-present   American Chemical Society

2017-present   International Society for the Study of Xenobiotics

2017-present   UIC/NIH Center for Botanical Dietary Supplements Research

2016-present   UIC Center for Biomolecular Sciences

2015-present   American Society of Biochemistry and Molecular Biology