Modulation of Estrogen Metabolism by Hops Extracts and Bioactive Compounds as Analyzed by LC-MS-MS

BY

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THESIS

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DEDICATION

This work is dedicated to my loving parents and boyfriend for all of the support and encouragement a person could hope for.

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LIST OF ABBREVIATIONS

COMT	Catechol-*o*-methyl transferase
CYP1A1	Cytochrome P450 1A1
CYP1B1	Cytochrome P450 1B1
E_1	Estrone
E_2	Estradiol
ER	Estrogen Receptor
EROD	7-ethoxyresorufin *O*-deethylase
GST	Glutathione-S-transferase
hops	*Humulus lupulus*
2-OH	2-hydoxy
4-OH	4-hydroxy
IXH	Isoxanthohumol
licorice	*Glycyrrhiza glabra* and/or *Glycyrrhiza uralensis*
LC-MS-MS	HPLC tandem mass spectrometry
mRNA	Messenger ribonucleic acid
2-MeO	2-methoxy
4-MeO	4-methoxy
2-$MeOE_1$	2-methoxyestrone
4-$MeOE_1$	4-methoxyestrone
NQO1	NAD(P)H quinone oxidoreductase 1
8PN	8-prenylnaringenin
ROS	Reactive oxygen species

LIST OF ABBREVIATIONS (continued)

RT-PCR	Real time polymerase chain reaction
TCDD	2,3,7,8-Tetrachlorodibenzodioxin
XH	Xanthohumol

SUMMARY

It is estimated that there will be 231,340 new cases of breast cancer diagnosed in 2013 (1). While there have been great strides in early detection leading to decreases in mortality rate, a woman still has a 1 in 36 chance of dying from breast cancer. It is well known that long-term estrogen exposure is linked to an overall increased risk of breast cancer. Although women are exposed to estrogens endogenously, certain events including early menarche, late menopause, nulliparity and hormone replacement use, all increase women's exposure to estrogen, thereby increasing their breast cancer risk.

Estrogen can increase breast cancer risk by two different mechanisms. Estrogens are hormones and enhanced estrogen receptor binding increases cellular proliferation, therefore increasing chances for mutations. Estrogen can also be oxidatively metabolized to generate reactive metabolites that can interact with DNA and proteins creating mutations or aberrations leading to genotoxicity (2-4). Estrogen is converted from testosterone into estradiol by aromatase. Estradiol is then metabolized by CYP1A1, or CYP1B1, which hydroxylate estradiol at the 2- and 4- position respectively. Both 2- and 4- hydroxyestradiol (catechol) can then oxidize into a quinone, or be methylated at the 3-position by catechol-o-methyltransferase (COMT) (5). The quinone can be reduced back to the catechol by NAD(P)H quinone oxidoreductase 1 (NQO1), or conjugated to glutathione by glutathione-s-transferase. The quinone metabolites are capable of alkylating DNA. The 2,3-quinone forms stable adducts while the 3,4-quinone forms depurinating DNA adducts which form apurinic sites that are converted into mutations when there are errors in DNA repair (6). In recent years,

quantification of these oxidative metabolites has become important as we try to understand the complex relationship between estrogen metabolites and breast cancer.

After the Women's Health Initiative trial in 2002 was halted early due to increased risks of many diseases including breast cancer (7, 8), many women have been looking for alternatives to traditional hormone therapy for relief of menopausal symptoms. With the highly publicized dangers of traditional hormone therapy, and the growing popularity of the "green", "natural", and "organic" alternatives some women have turned to dietary supplements for relief. Botanical dietary supplements are perceived to be safe and effective for relief of menopausal symptoms despite any scientific support, leading to an exponential increase in the consuming public's use (9).

Humulus lupulus (hops) has a history of use as a sedative and anti-anxiety agent, and it is used in the beer brewing process (10-13). While hops was not traditionally used for relief of menopausal symptoms, it was used to treat other gynecological issues. After the discovery that extracts contain the most estrogenic compound isolated from a plant, 8-prenylnaringenin, hops became of interest for potential use in menopausal women (10-13).

The hypothesis of this thesis is that botanicals used by women for menopausal symptom relief have beneficial chemopreventive activities by reducing exposure to genotoxic estrogen metabolites and improving overall wellness by reducing the negative effects of estrogen. My approach was to 1) expand the work of our group, which indicated that a specialized extract of *Humulus lupulus* (hops) reduced the genotoxic metabolite marker (14), to include other optimized extracts of *Humulus lupulus* utilizing

a newly optimized LC-MS-MS assay for increased reliability, sensitivity, and throughput, 2) evaluate the ability of three bioactive compounds from hops (8-prenylnaringenin, isoxanthohumol, and xanthohumol) to alter estrogen metabolism, and 3) assess enzyme activity and mRNA expression to interrogate the mechanism of altered metabolite production.

The hypothesis was supported by the observation that five hops extracts altered estrogen metabolism and the one with the highest amount of isoxanthohumol and 8-prenylnaringenin increased the benign metabolite marker 2-methoxyestrone while simultaneously decreasing the genotoxic metabolite marker 4-methoxyestrone. While xanthohumol did not alter estrogen metabolism, 8-prenylnaringenin and isoxanthohumol altered the amount of the genotoxic metabolite 4-methoxyestrone although in opposite manners. 8-prenylnaringenin treatment increased the genotoxic metabolite 4-methoxyestrone by increasing the mRNA for the enzyme responsible for its production (CYP1B1). On the other hand, isoxanthohumol treatment decreased the genotoxic metabolite 4-methoxyestrone by directly inhibiting the enzyme.

This work is the first to show that these constituents in hops have opposing roles on the metabolism of estrogen and it appears to occur through different mechanisms. Modulation of estrogen formation/metabolism has recently become increasingly popular in the treatment of breast cancer through the use of aromatase inhibitors to prevent the synthesis of estrogen in hormone receptor sensitive cancers. The work presented here shows that estrogen metabolism is altered by hops extracts, through 8-prenylnaringenin and isoxanthohumol constituents from the extract. The data suggests

that hops extracts, specifically those with high levels of isoxanthohumol, have beneficial chemopreventive activity by reducing the genotoxic pathway of estrogen metabolism and could potentially improve overall wellness by reducing the negative effects of estrogen. Future experiments will address the mechanism of activity more thoroughly and the implications to malignant transformation and ultimately the ability of the extracts to reduce tumor burden in the ACI rat model for estrogen carcinogenesis.

1. INTRODUCTION

1.1. Breast Cancer and Estrogen

Breast cancer is the leading malignancy among American women, and was estimated to have claimed the lives of more than 39,620 women in 2013 (1). Long-term exposure to estrogen contributes to an increased risk of cancer in hormone sensitive tissues, including breast and endometrium, and occurs from early menarche, late menopause, and/or exogenous estrogens from hormone therapy (formerly hormone replacement therapy). Two mechanisms contributing to the carcinogenic potential of estrogens have been posited.

1.2. Mechanisms of Estrogen Carcinogenesis

The traditional hormonal mechanism of estrogen carcinogenesis involves estrogen binding to the estrogen receptor (ER), which leads to enhanced cell proliferation and increased genomic mutations in DNA (2). It is important to note that there are two estrogen receptors (ERs), ERα and ERβ which have different activity in cells.

ERα was discovered in the late 1950s and is used to assess the clinical status of breast tumors as a prognostic and therapeutic marker (15). ERβ was discovered in 1996 and has been under intense investigation to examine whether ERβ status could be used to help classify breast tumors. The activation of ERβ by estradiol, phytoestrogens, and/or selective estrogen receptor modulators (SERMS) is also being investigated. Stimulation of ERβ is important to systemic estrogen regulation, as ERβ activation appears to reduce the impact of ERα by opposing many of the ERα regulated genes, and down

regulating ERα levels (15). The presence of ERα and ERβ can be significantly different in tissues.

The chemical mechanism of estrogen carcinogenesis involves metabolism of estrogens by cytochrome P450 (CYP) enzymes to reactive, electrophilic, and redox active *ortho*-quinone (*o*-quinone) metabolites (Figure 1.1). Estrogen is converted from testosterone

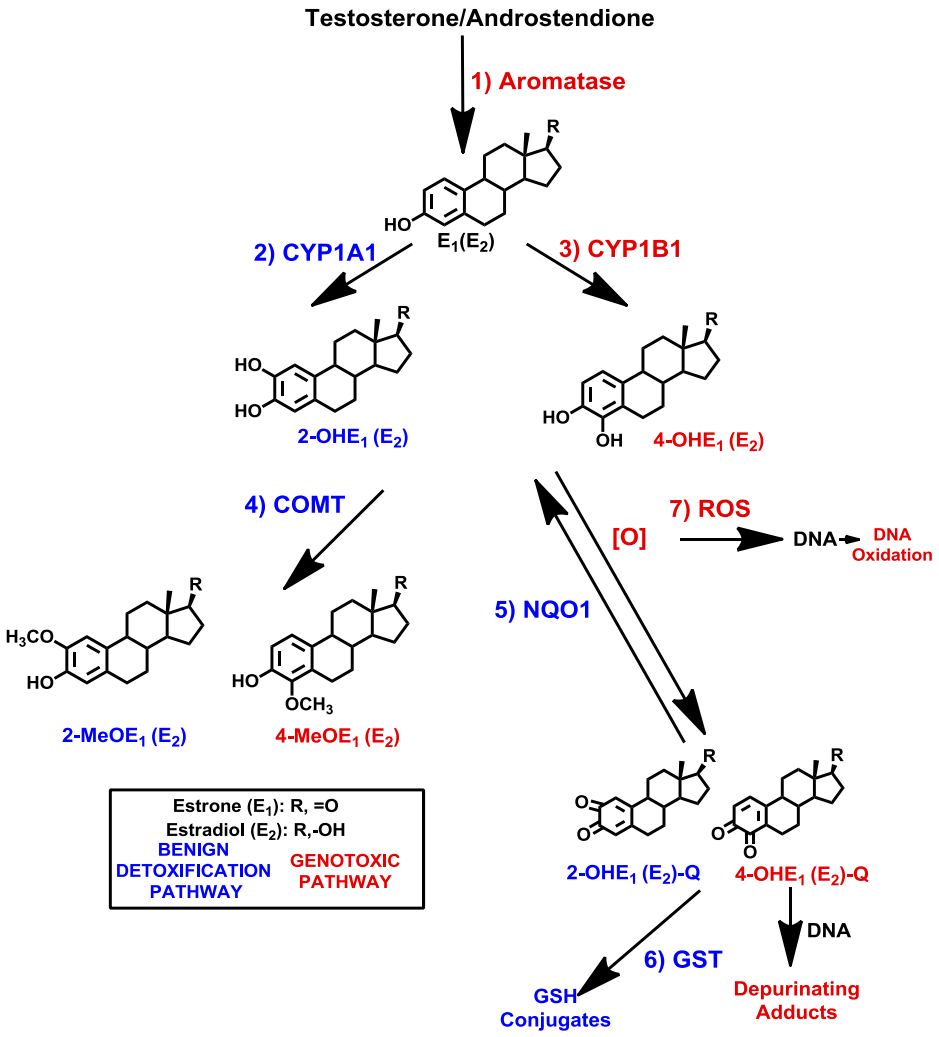

Figure 1.1. Estrogen metabolism leading to carcinogenesis. Estrone (E_1): R,=O, Estradiol (E_2): R,-OH. Blue colors signify benign detoxification pathway and red colors signify the genotoxic pathway.

into estradiol by aromatase. Estradiol is then metabolized by CYP1A1, or CYP1B1 which hydroxlates estradiol at the 2- and 4- position respectively. Both hydroxyestradiol (catechol) metabolites can be oxidized into an *ortho*-quinone (*o*-quinone), or be conjugated at the 3-position by catechol-o-methyltransferase (COMT) (5). The quinone can be reduced back to the catechol by NAD(P)H quinone oxidoreductase 1 (NQO1), or conjugated to glutathione by glutathione-s-transferase. The *o*-quinone metabolites are capable of alkylating DNA. The 2,3-quinone forms stable adducts while the 3,4-quinone forms depurinating DNA adducts which form apurinic sites that are converted into mutations when there are errors in DNA repair (6). The formation of the *o*-quinone generate reactive oxygen species (ROS), which can ultimately increase cancer risk (3, 16). The enzymes involved in estrogen metabolism can be separated into two categories: those responsible for generating estrogens (aromatase) and oxidizing the metabolites into *o*-quinones (CYP1B1, CYP1A1) and those responsible for deactivating the estrogen metabolites into benign metabolites [catechol-*O*-methyltransferase (COMT), NAD(P)H quinone oxidoreductase 1 (NQO1), and glutathione-*S*-transferase (GST)].

1.3. Botanicals as "Safe" Alternative to Hormone Therapy?

In 2002, the highly publicized release of the Women's Health Initiative (WHI) clinical trial results cast serious doubts about the long-term use of hormone therapy(7, 8). Hormone therapy (estrogen plus a progestin for women who have not had a hysterectomy or

estrogen alone for those who have) is the most common treatment for menopausal symptoms and was thought to decrease osteoporosis, risk of stroke, Alzheimer's disease, and coronary heart disease (7). However, the WHI trial was halted early due to an increased incidence of invasive breast cancer, stroke, pulmonary embolism, and coronary heart disease and consequently women began to look for alternatives, especially dietary supplements (17).

Botanical dietary supplements are perceived to be safe and anecdotal claims of efficacy abound; the consuming public, in this case women seeking relief of their menopausal symptoms, often believe the suggested claims despite the lack of convincing scientific evidence in support of such claims (9). The few clinical trials investigating botanical efficacy have shown a very large placebo effect (> 50%) on menopausal symptom relief, further encouraging women to consume these products (18-22). Botanical supplements do contain phytoestrogenic compounds that contribute to its efficacy for menopausal symptom relief and have been shown to have chemopreventive mechanisms of action (23-27). Some phytoestrogens have been shown to have selectivity to either ER (Table 1.2), which has led many investigators to look into phytoestrogens as chemopreventive agents that exhibit selective gene regulation (25-27).

Humulus lupulus (hops) has a history of use as a sedative and anti-anxiety, and it is used in the beer brewing process (10-13). While hops was not traditionally used for relief of menopausal symptoms, it was used to treat other gynecological issues. After the discovery that extracts contain the most estrogenic compound isolated from a plant, 8-prenylnaringenin (8PN), hops became of interest for potential use in menopausal

women (10-13) Hops extracts have over 1000 different compounds (12, 28) and of those compounds 8PN is responsible for estrogenic activity of hops extracts and xanthohumol (XH) has chemopreventive activity. Interestingly, separating out 8PN and XH activity is not straight forward; 8PN can be formed from isoxanthohumol, and isoxanthohumol and xanthohumol cyclize to each other shown in Figure 1.2 (29, 30). These reactions do occur in microsomes and cell culture as well as in the human intestinal microbiota (29-32).

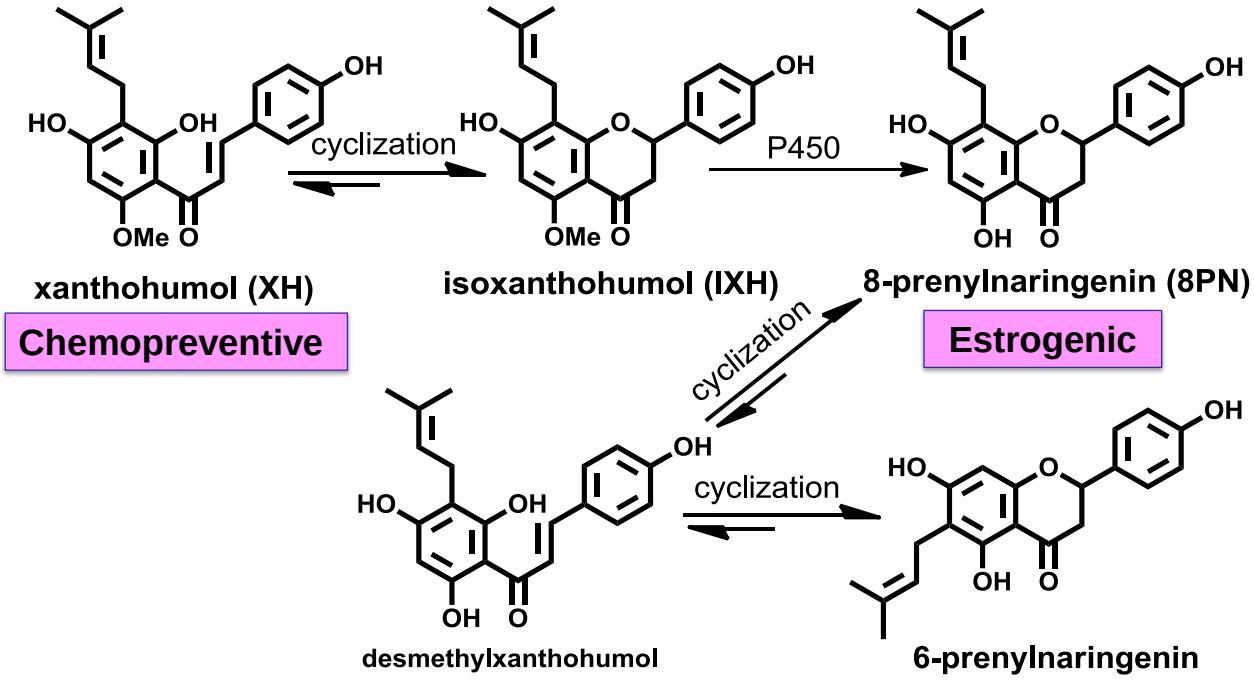

Figure 1.2. Conversion of xanthohumol, isoxanthohumol, and 8-prenylnaringenin.

1.4. Botanical Modulation of Estrogen Carcinogenesis

Figure 1.3 shows potential targets in the estrogen chemical carcinogenesis pathway where the intervention of a botanical could positively modulate estrogen's progression to the *o*-quinone (33). Botanicals could influence the progression of estrogen metabolism by down regulating the formation of estrogen through aromatase or the

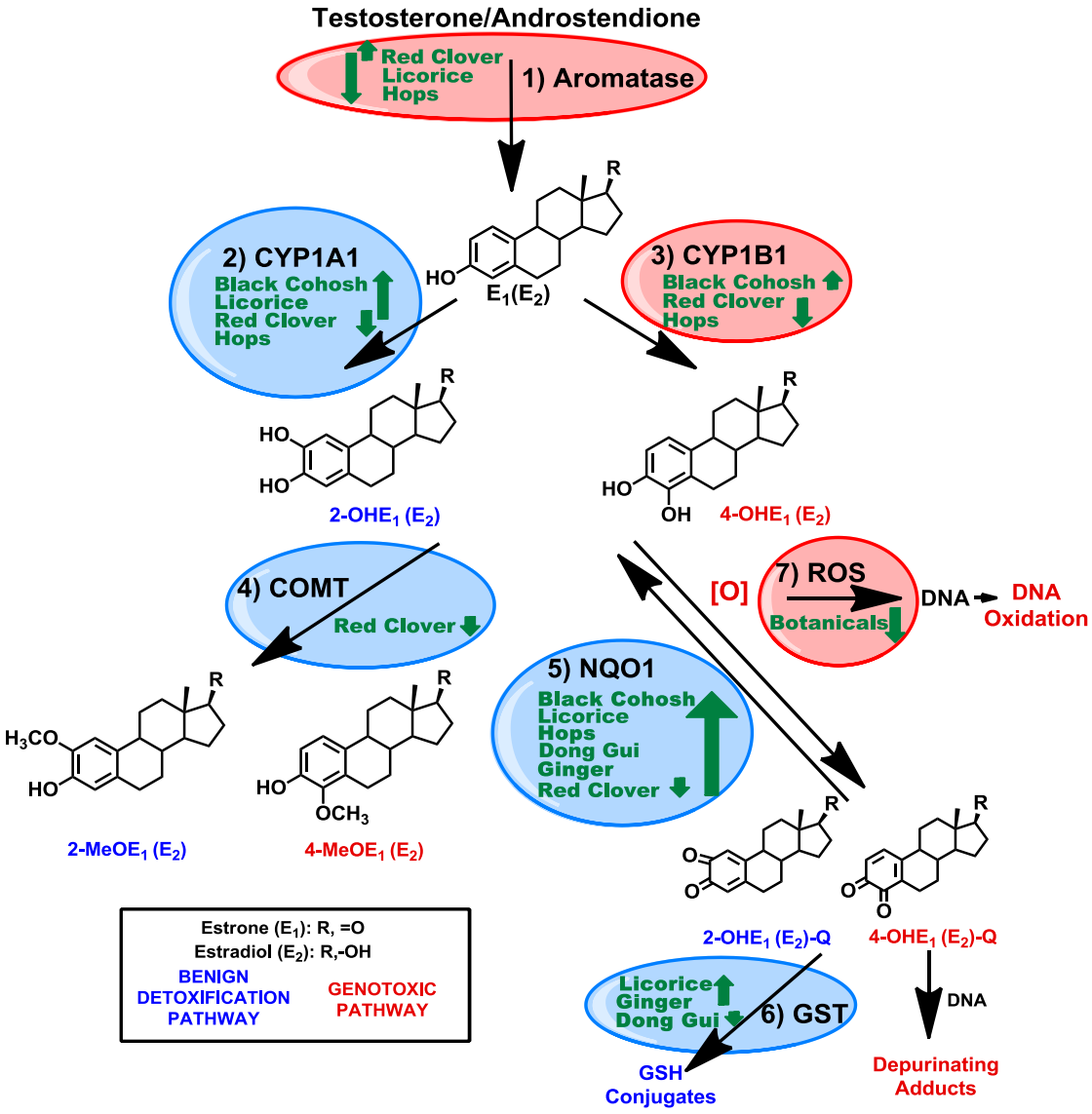

Figure 1.3.Botanicals influence on seven key steps in estradiol metabolism. References found in Table I.

bioactivation of estrogen through CYP1B1, reducing the amount of estrogen or quinone produced (the genotoxic pathway). Alternatively, botanicals could deactivate estrogen into benign metabolites by increasing CYP1A1, COMT, NQO1, or GST, or by scavenging ROS that are produced during estrogen metabolism. Metabolism of estrogen in “healthy” individuals requires a balance of the genotoxic pathway, and the benign pathway and an increased cancer risk is believed to arise from altered regulation of these pathways and typically also effects DNA repair systems (2, 34).The current literature examining the potential botanical modulation of these seven steps is outlined in Table I and discussed below.

1.4.1. Aromatase

Aromatase (Figure 1.3, step 1) catalyzes the conversion of testosterone and androstendione into estradiol (E_2) and estrone (E_1), respectively. Aromatase is expressed in high levels in extrahepatic tissues, which can lead to enhanced levels of estradiol in tissues compared to circulating levels in the blood. For example, aromatase in breast tissue is able to convert circulating androgens to estrogens, and therefore has a crucial function in determining the availability of estrogen in breast tissue, which is

		BIOACTIVATION			DETOXIFICATION			
	ER Selectivity	**Aromatase**	**CYP1B1**	**CYP1A1**	**COMT**	**NQO1**	**GST**	**ANTIOXIDANT**
Black Cohosh (*Cimicifuga racemosa*)	No effect (23)	No Effect (62)	↑ (57, 58)	↑ (57, 58)	n.d.	↑ (50)	n.d.	Yes (35)
Red Clover (*Trifolium pretense*)	β (25)	↓ (63, 64) ↑ (65)	↓ (61)	↑ (59) ↓ (52)	↓ (52, 56)	↑ (43, 51) ↓ (52)	No Effect (43)	Weak (36)
Licorice (*Glycyrrhiza glabara*) (*Glycyrrhiza uralensis*)	β (26, 27)	↓ (66)	n.d.	↑ (60)	n.d.	↑ (45, 46, 53)	↑ (44-46)	Yes (37-39)
Hops (*Humulus lupulus*)	α (25)	↓ (67, 68)	↓ (14)	↓ (14)	No Effect (14)	↑ (40, 54)	n.d.	Weak (40)
Dong Gui (*Angelica sinensis*)	No effect (23)	n.d.	n.d.	n.d.	n.d.	↑ (41)	↓ (47)	Weak (41)
Ginger (*Zingiber officinale*)	n.d.	n.d.	n.d.	n.d.	n.d.	↑ (55)	↑ (48, 49)	Yes (42)

TABLE I: SUMMARY OF BOTANICAL INFLUENCE ON ESTROGEN CARCINOGENESIS.

Literature on the ability of botanicals to influence the seven steps of the estrogen carcinogenesis pathway. n.d. no data

particularly important for post-menopausal women. Modulation of estrogen levels through the inhibition of aromatase has implications especially for postmenopausal women, who no longer experience the circulating estrogens released by the ovaries. Over expression of aromatase in estrogen receptor α negative cells increased their motility, and their anchorage independent growth which could be abolished by treatment with an aromatase inhibitor, suggesting that aromatase is important in cellular transformation (69).

A number of pure compounds isolated from botanicals and one crude botanical extract inhibit aromatase. For example, biochanin A, an active component in *Trifolium pratense* (red clover), inhibited aromatase mRNA expression, aromatase-coupled luciferase activity and repressed transcriptional control of the aromatase promoter in MCF-7 and SK-BR-3 breast cancer cells (63). Additionally, van Meeuwen et al. evaluated aromatase inhibition of biochanin A and its major metabolite genistein in primary mammary fibroblasts. It was shown that both inhibited aromatase at one-third the potency of fadrozole, a known aromatase inhibitor, and genistein was significantly more potent than biochanin A (64). Interestingly, genistein also increased aromatase activity, mRNA, and protein levels in hepatic HepG2 cells (65). Isoliquiritigenin, from *Glycyrrhiza* spp. (licorice), decreased aromatase mRNA in MCF-7 cells (66). Two components of *Humulus lupulus* (hops) extracts, 8-prenylnarnigenin (8PN) and to a lesser extent xanthohumol (XH), inhibited aromatase activity in primary mammary fibroblasts (68) and in JAR choriocarcinoma cells while the mRNA expression levels of aromatase were unaffected (67). An extract of *Cimicifuga racemosa* (black cohosh) was tested in MCF-7 and MDA-MB-231 cell lines and there was no change in the conversion

of androstenedione to estradiol (62). Inhibition of aromatase by the compounds found in red clover, licorice, and hops suggests that these may play a protective role for breast cancer risk in post-menopausal women by decreasing the conversion of androgens into estrogens and lowering estrogen levels in breast tissue.

1.4.2. CYP1B1

The enzyme CYP1B1 hydroxylates estradiol and estrone at the 4-position (Figure 1.3, step 2). The 4-hydroxylated catechol (4-OH) precursor to the *o*-quinone has been shown to be carcinogenic in animal models and mutagenic in cell-based assays, causing cellular transformation and formation of DNA adducts (16, 70-73). CYP1B1 is differentially expressed in normal and tumor tissues and polymorphisms in CYP1B1 have also been identified in some hormone dependent cancers (74).

Isolated compounds from black cohosh, red clover, and an extract of hops have all been shown to affect CYP1B1. Two different extracts of black cohosh increased CYP1B1 mRNA expression in MDA-MB-453 (57) and in MCF-7 cells (58), identified by microarray and confirmed with RT-PCR. However, Hemachandra et al. reported no change in the ratio of 2- to 4-methoxyestrone ($MeOE_1$) metabolites of estrogen in MCF-10A cells co-treated with a black cohosh extract and estradiol (14). The $MeOE_1$ metabolites are a marker for the benign 2- and genotoxic 4- pathways of estrogen metabolism. The contradictory results observed of CYP1B1 inhibition with black cohosh could be explained by differences in the extracts of black cohosh, cell lines, and specific treatments that were used (74).

Genistein and daidzein both inhibited CYP1B1-mediated 7-ethoxyresorufin *O*-deethylase (EROD) activity (61). CYP1B1 inhibition by genistein and daidzein is believed to be due to negative feedback from CYP1B1-mediated conversion of biochanin A and formonetin into genistein and daidzein respectively (61). A hops extract and 8PN reduced the amount of the 4-MeOE_1 metabolite produced from estrogen metabolism (14). The finding was then validated showing a reduction in estradiol induced CYP1B1 protein expression and inhibition of CYP1B1-mediated EROD activity with 8PN and hops, although a cytotoxic dose of hops was needed to observe EROD activity inhibition (14). These results of inhibition and/or reduction of CYP1B1 by genistein, daidzein, and a hops extract demonstrate that these botanicals may be protective against estrogen carcinogenesis by reducing the formation of the 4-OH genotoxic metabolite.

1.4.3. CYP1A1

CYP1A1 hydroxylates estradiol and estrone at the 2- position (Figure 1.3, step 3). The 2-hydroxy catechol [2-OHE_1(E_2)] is considered part of the benign pathway of estradiol and estrone because 2-OHE_1(E_2) are not mutagenic or carcinogenic like their 4-OH counterparts (71). In fact, the methyl ether metabolite, 2-methoxyestradiol has reported chemotherapeutic properties and promise as an anticancer drug (75, 76). Although, the 2-OH catechol estrogens can also be oxidized to quinones that can alkylate DNA and form adducts, the 3,4-quinone DNA adducts are present in much higher concentrations than those of the 2,3-quinone in breast cancer patients (77, 78).

CYP1A1 and CYP1B1 isozymes are very similar; therefore, their activity modulation is usually affected in an analogous manner. As with CYP1B1, two extracts of black cohosh increased CYP1A1 mRNA expression identified by microarray and confirmed with RT-PCR (57, 58). The observed difference could be due to the same reasons for the CYP1B1 conflicting results previously discussed.

Biochanin A increased CYP1A1-mediated EROD activity as well as CYP1A1 mRNA in MCF-7 cells (59). Conversely, genistein, and daidzein both inhibited CYP1A1 EROD activity and reduced mRNA levels in MCF-7 BUS cells (52). Additionally, an aqueous extract of licorice, *Glycyrrhiza uralensis,* and glycyrrhetinic acid, a compound from *G. uralensis*, increased *in vivo* CYP1A1 protein expression in livers of rats treated for seven days (60). A hops extract also reduced estradiol induced protein levels of CYP1A1 and reduced the amount of the 2-$MeOE_1$ metabolite produced during estrogen metabolism (14). In summary, genistein, daidzein, a licorice extract, and a hops extract inhibit the CYP1A1 enzyme, which prevents estrogen from forming the therapeutic 2-OH metabolites and possibly increases formation of the genotoxic 4-OH metabolites. Conversely, biochanin A and black cohosh increase CYP1A1 activity and mRNA.

1.4.4. COMT

COMT catalyzes methylation of the 2- and 4-OH metabolites (Figure 1.3, step 4). COMT is part of the detoxification pathway of estrogen, as it lowers the amount of 2- or 4-OH metabolites that could become quinones, which leads to redox cycling and can ultimately form DNA adducts. In an analysis of cancer cells versus normal cells, renal

cancer cell lines consistently showed a lower expression of COMT, suggesting that cancer cells down-regulate the deactivating pathway of estrogen metabolism (79). A COMT polymorphism has also been linked to decreased risk of endometrial cancer in Asian populations and a recent review of COMT and breast cancer describes the association of polymorphisms and breast cancer risk while describing the inconsistent results (5, 80). The 2-MeOE$_2$ metabolite has exhibited non-estrogenic responses on mouse uterine weight and epithelial cell height (81). The 2-MeOE$_2$ metabolite has also exhibited anticancer activities *in vitro* and *in vivo*, including decreased proliferation of cancer cells and has been examined in a few clinical trials. The formulation used in the clinical trials did not allow the best distribution; nevertheless, the results are still promising for an anticancer drug (75, 76).

A botanical's ability to influence COMT activity or expression has been investigated in studies examining red clover and hops. Genistein and daidzein decreased COMT mRNA levels as well as COMT activity in MCF-7 cells (52, 56). This decrease in COMT mRNA levels seems to be ER mediated, because treatments with an ER antagonist caused levels of COMT to return to basal levels. Treatment of MCF-10A cells with either estradiol or a hops extract had no effect on protein COMT levels, which again supports the notion that genistein and daidzein are working through an ER-mediated pathway (14). The ability of genistein and daidzein to down regulate COMT in ER positive breast cancer cells suggests that these compounds may actually increase the carcinogenic metabolites of the estrogen chemical carcinogenesis pathway by reducing the detoxifying role of COMT.

1.4.5. NQO1

NQO1 is responsible for a two-electron reduction of the quinone back to the precursor catechol metabolite (Figure 1.3, step 5). This reduces the reactive quinone without producing ROS and prevents the quinone from binding to DNA. NQO1 deficiency has been shown to increase estrogen dependent tumor formation in MCF-7 xenografts, demonstrating NQO1's role in reducing tumor burden with estrogen exposure (82).

Many of the botanicals have been investigated for effects on NQO1 activity, a well-known detoxifying enzyme. A pure compound from black cohosh, actein, initially decreases NQO1 transcription, but after 24 h, transcription was increased (50). Biochanin A and genistein increased NQO1 mRNA in MDA-MB-231 cells after 24 h (51). Bianco et al. showed that biochanin A, but not genistein, significantly protected against estrogen-induced oxidative DNA damage in these breast cancer cells. Additionally, genistein *in vivo* increased NQO1 activity and mRNA levels in the liver (43). However, it has also been reported that in MCF-7 BUS cells, genistein and daidzein decreased relative NQO1 mRNA after 24 h treatment (52).

An extract of *G. uralensis* (licorice), and dehydroglyasperin C (a compound from *G. uralensis*) increased NQO1 transcription as well as antioxidant response element (ARE) activity in liver cancer cells treated with the extract (46, 53). Isoliquiritigenin, a compound found in licorice, was tested *in vivo* and significantly induced NQO1 activity in the colon and mammary gland; however, the distribution of isoliquirtigenin in the breast was significantly less than in the liver or colon (45). 8PN appears to be responsible for NQO1 induction of hops in the mammary gland (83). Xanthohumol is able to induce NQO1 activity in Hepa 1c1c7 murine hepatoma cells (40). Interestingly,

in mouse microglial BV2 cells xanthohumol increased NQO1 mRNA at 0.5, 1, and 3 h with a decrease at 6 h and no response observed at 12 h; however, an increase in NQO1 protein persisted for 24 h (54). An extract of dong gui was fractionated following NQO1 inducing activity, and five compounds, including lingustilide, with NQO1 inducing activity were identified (41). Topical treatment of zerumbone, from *Zingiber officinale* (ginger), increases mRNA of NQO1on the skin of mice (55). Actein, isoliquiritigenin, xanthohumol, and zerumbone increase NQO1, suggesting that these compounds may be chemopreventative agents. The mixed results from red clover require further investigation and suggest that the effects on NQO1 might be ER mediated.

1.4.6. GST

GST conjugates glutathione (GSH) to electrophiles such as *o*-quinones for detoxification (Figure 1.3, step 7), thereby reducing their potential to cause DNA damage. Many studies examining botanicals' ability to modulate GST levels and activity have initially involved an insult to reduce GSH and GST levels with subsequent analysis of the botanical's ability to reverse the reduction. When genistein was administered *in vivo,* the total GST activity did not change; however, mRNA levels of specific isoforms in the liver were altered (43). An aqueous extract of licorice, administered *in vivo* to rats after CCl_4 reduction of GST protein levels, significantly increased GST levels in the liver (44). Isoliquiritigenin significantly induced GST activity and mRNA in the liver and mammary gland *in vivo* (45). Dehydroglyasperin C, a compound from licorice, was also able to increase GST protein expression in liver hepatoma cells in a dose dependent manner

(46). Raw ginger fed to rats was able to return levels of GST in the kidney to almost normal levels after treatment with doxorubicin (49), and it returned plasma levels of GST to near normal in a 1, 2-dimethylhydrazine-induced colon cancer rat model (48). Two compounds, 11-angeloylsenkyunolide F and tokinolide B, from dong gui both inhibited purified GST (47). While inhibiting GST is not a desirable effect for the estrogen carcinogenesis pathway, Huang et al. speculated that this might be a potentially positive effect with respect to GST's role in the detoxification of chemotherapeutic agents contributing to drug resistance. The ability of these botanicals to induce GST suggests that they may have chemopreventive properties by increasing the detoxification of estrogen quinones, except with dong gui, which inhibits GST.

1.4.7. Antioxidant effects.

The conversion from estrogen semiquinone to the quinone produces ROS (Figure 1.3, step 6). ROS have been implicated in many facets of breast cancer, including increasing genomic instability and altering redox sensitive transcription factors like NF-κB and NRF1, which can ultimately lead to changes in cell growth, survival, transformation, invasion, and apoptosis (84).

It has been established that some botanicals have antioxidant properties, and black cohosh, red clover, licorice, dong gui, hops and ginger are not an exception. Bioactivity-guided fractionation of black cohosh using the 2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH) free radical assay (35), and of licorice using the peroxynitrite assay (37), yield nine different antioxidant compounds from each plant. An extract of

licorice inhibited β-carotene oxidation and scavenged free radicals when tested in the DPPH assay (38, 39). Five compounds from fresh ginger exhibited DPPH radical scavenging activities similar to curcumin and quercetin, which are known botanical antioxidants (42). An extract of red clover exhibited low activity in scavenging free radicals, and three compounds within the extract (fisetin, quercetin, and kaempferol) weakly scavenge DPPH radicals (36). Extracts of dong gui and hops both exhibited weak antioxidant capabilities in the DPPH assay (40, 41). The ability of these botanicals to scavenge free radicals suggests that they would eliminate the ROS produced from the estrogen carcinogenesis pathway, safely preventing them from causing harm in the cell, but it is most likely not the major action of the botanicals.

1.5. Conclusion

Women are taking botanical dietary supplements to ameliorate symptoms of menopause and to avoid traditional hormone therapy due to anxiety and fears related to increased risk of breast cancer associated with hormone therapy use. Nevertheless, some botanicals, although thought to be benign, appear to influence estrogen receptor activity. Genistein for example, has been intensely studied in red clover and soy, and has been shown to act like estradiol binding both ERα and ERβ, leading to increasing cell proliferation *in vitro* and *in vivo* (85). The effect of botanical supplements on estrogen chemical carcinogenesis is less known. My work investigates hops extracts and three isolated compounds for their ability to modulate estrogen chemical carcinogenesis.

Research results that would appear contradictory such as the red clover results, may in fact reflect the variability in tissue milieu in which estrogenic activation might occur, *e.g*. hormone sensitive tissue with varying ratios of ER subtypes. Contributing to this phenomenon of contradictory results is the variability in extracts used among studies. Without standardized extracts, reproducibility is difficult. Therefore, future investigations should be performed utilizing standardized extracts, which will help to investigate the *in vivo* effect of these extracts on estrogen chemical carcinogenesis. The ability of the botanicals to modulate estrogen chemical carcinogenesis seems promising and warrants future investigations into the mechanisms of action.

1.6. Hypothesis and aims

From our previously published work (14) we know that a specialized extract of hops reduced the genotoxic metabolite marker (4-MeOE_1). *The long term hypothesis is that botanicals that women use for menopausal symptom relief have beneficial chemopreventive activities by reducing exposure to genotoxic estrogen metabolites and could improve overall wellness by reducing the negative effects of estrogen.* My approach was to 1) expand the work of our group to include other optimized extracts of *Humulus lupulus* utilizing a newly optimized LC-MS-MS assay for increased reliability, sensitivity, and throughput, 2) evaluate the ability of three bioactive compounds from hops (8-prenylnaringenin, isoxanthohumol, and xanthohumol) to alter estrogen metabolism, and 3) assess enzyme activity and mRNA expression to interrogate the mechanism of altered metabolite production.

2. MATERIALS AND METHODS

2.1. Materials

Estradiol, LC-MS Chromasolv methanol, dansyl chloride, cholera toxin, epidermal growth factor, hydrocortisone, 2,3,7,8-Tetrachlorodibenzodioxin(TCDD), and ascorbic acid were obtained from Sigma Aldrich (St. Louis, MO, USA). Dichloromethane was obtained from Fisher Scientific (Itasca, IL, USA). 2-Methoxyestrone-1,4,16,16-d4 was purchased from CDN isotopes (Quebec, Canada). 2-methoxyestradiol, 2-methoxyestrone, 4-methoxyestradiol, 4-methoxyestrone, were all purchased from Steraloids (Newport, RI, USA). XBridge C18 Column, 3.5 µm, 3.0 x 150 mm and ACQUITY UPLC BEH C18 VanGuard Pre-column, 130Å, 1.7 µm, 2.1 mm X 5 mm, were purchased from Waters (Milford, MA, USA). C18 5uM 20x2.1 mm Guard Column was purchased from Analytical Sales and Service (Pompton Plains, NJ, USA). Xpertek insert 250 µL clear glass tapered bottom spring, and Xpertek Screw Cap 9mm Black with bonded PTFE septa were purchased from Colbert Associates (St. Louis, MO, USA). All cell culture materials were obtained from Fisher Scientific (Itasca, IL, USA) or Invitrogen (Grand Island, NY, USA) unless stated.

API 3000 triple quadrupole mass spectrometer with Analyst software was obtained from Applied Biosystems (Foster City, CA, USA) equipped with Agilent 1200 HPLC from Agilent Technologies (Palo Alto, CA, USA). An AB Sciex QTRAP 5500, tandem quadrupole mass spectrometer (San Jose, CA), was coupled to an Agilent

Technologies 1260 series HPLC system (Santa Clara, CA) consisting of a binary pump, an auto-sampler and a thermo stated column compartment was used with a Waters (Dublin, Ireland) ACQUITY UPLC BEH C18 Column (130Å, 1.7 µm, 3 mm X 100 mm) for chromatographic separation. Data was acquired in the positive ion mode with an electrospray (ESI) source using Analyst software version 1.5.1.

2.2. Botanical Extract Preparations

The hops (*Humulus lupulus*) and licorice (*Glycyrrhiza glabra* and *Glycyrrhiza uralensis*) extracts and pure compounds from hops were prepared by Dr. Guido Pauli's group. The hops (Humulus lupulus) extract previously published on by our group and used for Figure 1, HHEO2, was an ethanol extract of spent hops dispersed in kieselguhr (plant materials were extracted with ethanol after supercritical CO_2 extraction of pelletized strobiles of Humulus lupulus cv. Nugget), which was obtained from Hopsteiner (Mainburg, Germany/New York). The kieselguhr was removed by methanol filtration. Quantitative liquid chromatography/mass spectrometric (LC/MS) analysis using authentic reference compounds as calibrants revealed that this hops extract contained 5.4% XH and 0.084% 8-prenylnaringenin (8PN). XH was isolated and purified (>99.5% purity both by quantitative HNMR and LC/MS) as described previously (40). 8-PN was synthesized and purified (95.0% purity by quantitative HNMR) using the modified literature procedure as previously reported (24).

Extract #	Extract name	IXH	8PN	XH	6PN
HL 05-03-32	HHEO2	0.65	0.084	5.4	
HL 05-04-26	Clinical	0.99 ± 0.03	0.36 ± 0.007	32 ± 0.76	1.18 ± 0.03
HL 07-50-20	IsoXantho	12 ± 0.4	0.51 ± 0.01	0.70 ± 0.005	0.53 ± 0.01
HL 07-50-21	IsoXanthoFlav	62 ± 0.67	0.83 ± 0.01	2.22 ± 0.02	0.82 ± 0.02
HL 07-50-22	Xantho	1.1 ± 0.02	0.31 ± 0.004	8.8 ± 0.17	0.74 ± 0.02
HL 07-51-22	XHKO	0.58 ± 0.003	0.01 ± 0.0003	0.07 ± 0.0005	0.15 ± 0.002
HL 07-51-24	IX KO	0.07 ± 0.003	0.1 ± 0.001	16.33 ± 0.29	0.74 ± 0.01
HL 07-51-26	6PN KO	0.83 ± 0.012	0.2 ± 0.0007	15 ± 0.35	0.37 ± 0.006
HL 07-51-28	3 KO	0.08 ± 0.005	0.04 ± 0.002	0.10 ± 0.005	0.1 ± 0.005

TABLE II TABLE OF HOPS EXTRACTS USED. w/w% of compounds ± standard deviation as analyzed by UHPLC with PDA (provided by Rene Ramos and Shao-Nong Chen)

The “designer” extracts (HL-07-50-20/21/22) were obtained from Hopsteiner and were enhanced for specific compounds with known biological activity. Extract HL 07-50-22, Xantho was made to have in increased level of xanthohumol and isoxanthohumol. Extract HL 07-50-20, IsoXantho, is the isomerization of the Xantho extract. The extract HL 07-50-21, IsoXanthoFlav extract is the IsoXantho extract but enriched for isoxanthohumol.

Deprived (Knock-Out) Extracts (HL-07-51-22/24/26/28) were derived from the hops clinical extract. The deprived extracts were prepared by subtracting prenylated phenols xanthohumol [XH], isoxanthohumol [IX], 8-prenylnaringenin [8-PN], and 6-prenylnaringenin [6-PN] by a two-step HSCCC method, using HEMWat 0 as initial solvent system, and then HEMWat-3 and the orthogonal HterAcWat+3 for removal of XH/8-PN/6-PN, and IX, respectively. Four deprived extracts were obtained with lessened content of XH/8-PN (HL-07-51-22), IXH (HL-07-51-24), 6-PN (HL-07-51-26), and XH/IX/8-PN/6-PN (HL-07-51-28). It should be noted that it is very difficult to remove 8PN completely from hops extracts since it is already in low amounts in the extract.

w/w% of constituents in the extracts were obtained by UHPLC with PDA detection shown in Table II. All extracts were prepared at 0.4 to 10 mg/ mL solutions in MeOH. Samples were analyzed on a Shimadzu Nexera UHPLC equipped with a Acquity UPLC® BEH C18 column (1.7µm x 2.1 x 50 mm) and using PDA and fluorescence as detection modes. Gradient: A = H_2O 0.1 % FA ; B = ACN 0.1 % FA; 5 % to 57 % B in

18 min, 57% to 98 % B in 7 min, isocratic mode at 98 % B during 3 min; flow rate: 0.6 mL/ min. The method is in validation process.

Xanthohumol standard was obtained from Hopsteiner (95%) and further purified by HSCCC using HEMWat 0 as solvent system. A 98.68% pure XH was obtained and measured by qHNMR. Isoxanthohumol standard was obtained by chemical isomerization of xanthohumol (Intramolecular Michael addition) under alkaline conditions. The compound thus obtained was then purified by HSCCC using the same conditions as for XH. A 99.75% pure IX was produced and assessed by qHNMR (100% method).

2.3. Cell Culture

MCF-10A cells were obtained from American Type Culture Collection were maintained in Dulbecco's Modified Eagle's Medium and F12 medium (DMEM/F12) supplemented with 1% penicillin-streptomycin, 5% fetal bovine serum (FBS), cholera toxin (0.1 µg/mL), epidermal growth factor (20 ng/mL), hydrocortisone (0.5 µg/mL), and insulin (10 µg/mL), in an incubator held at 5% CO_2 at 37°C. Estrogen-free medium for treating the MCF-10A cells, was prepared supplementing charcoal-dextran treated FBS to phenol red-free DMEM/F12, whereas other components remained the same.

2.4. Analysis of Estrogen Metabolites

2.4.1. Initial Screening Cell Culture Conditions

MCF-10A cells were seeded into 6-well plates at 20,000 cell/well in estrogen free medium. 24 hours later, cells were incubated with 1 µM estradiol in the presence or absence of test extracts for 6 days. Treatments were renewed every 3 days: cell media was collected (5 mL/well) and stored at -20°C with the addition of 2 mM ascorbic acid in water which was freshly prepared for each experiment. At the end of the 6^{th} day cell media was collected and pooled with the third day cell media (10 mL total volume) and a second amount of ascorbic acid (2 mM final concentration) and 15ng of 2-$MeOE_1$-d_4 internal standard was added to each pooled sample. Samples were then stored in -20°C until sample preparation.

2.4.2. Pure Compound/Dose Response Cell Culture Condition

The assay was changed to a 3 day assay to increase throughput, decrease variability, and reduce the amount of extract utilized. MCF-10A cells were seeded into 6-well plates at 80,000 cell/well in estrogen free medium. 24 hours later, cells were incubated with 1 µM estradiol in the presence or absence of test extracts for 3 days. After 3 days, cell media was collected (3 mL/well) and stored at -20°C with the addition of 2 mM freshly prepared ascorbic acid in water and 1.2ng of 2-$MeOE_1$-d_4 internal standard. Samples were then stored in -20°C until sample preparation, up to 2 months.

2.4.3. Sample Preparation

A schematic of the different extraction methods tested is diagramed in Figure 2.1.

Method 1: Samples were lyophilized to approximately 2 mL aqueous solution, and the

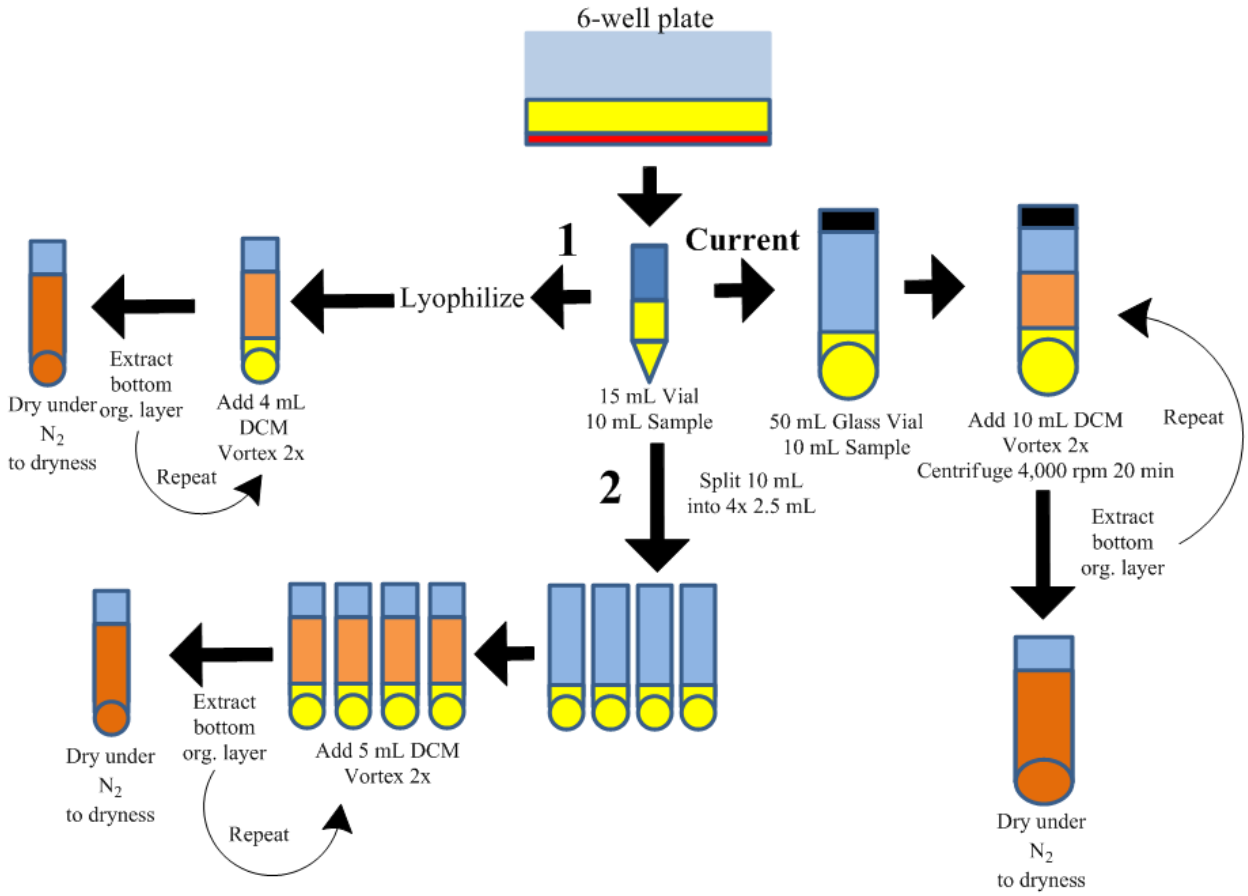

Figure 2.1 Diagram of different extraction methods utilized

estrogen metabolites were extracted twice by adding 5 mL dichloromethane to each sample quickly vortexing and extracting the bottom organic layer. The dichloromethane layer was evaporated under a stream of nitrogen gas and reconstituted with 200µL of 0.1 M sodium bicarbonate buffer (pH 9) and 200 µL of freshly prepared dansyl chloride (1mg/mL in acetone), Figure 2.2. The reaction mixture was incubated at 60°C for 10

minutes to complete the derivatization. Samples (50 µL) were analyzed by liquid chromatography/tandem mass spectrometry (LC-MS-MS) as described later.

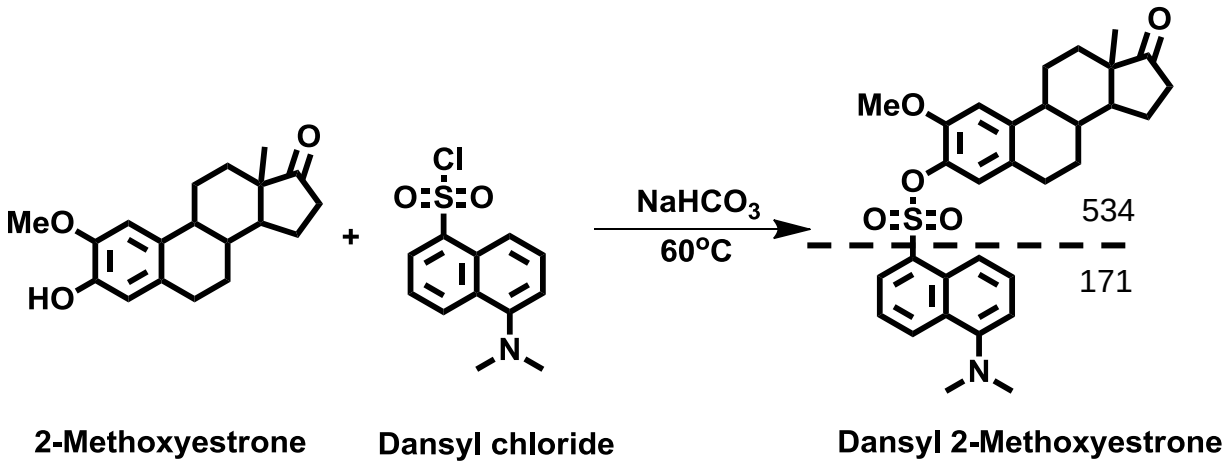

Figure 2.2 Derivatization of methoxyestrone by dansyl chloride.

Method 2: Samples were split into 4 test tubes with approximately 2.5 mL aqueous solution, and the estrogen metabolites were extracted twice by adding 5 mL dichloromethane quickly vortexing and extracting the bottom organic layer. The extracted layers of the same sample were pooled together. The pooled organic layer was evaporated under a stream of nitrogen gas and reconstituted with 200 µL of 0.1 M sodium bicarbonate buffer (pH 9) and 200 µL of freshly prepared dansyl chloride (1mg/mL in acetone). The reaction mixture was incubated at 60°C for 10 minutes to complete the derivatization. Samples (50 µL) were analyzed by LC-MS-MS as described later

Current Method: Samples were transferred to 50 mL glass centrifuge tubes. The estrogen metabolites were extracted twice by adding an equal amount of

dichloromethane quickly vortexing and then centrifuging at 4,000 rpm for 20 minutes at 20°C. The bottom organic layer was transferred to a test tube. The extraction was repeated and organic layers of the same sample were pooled together. The pooled organic layer was evaporated under a stream of nitrogen gas, reconstituted in 700 µL of methanol, transferred to an eppendorf tube and dried down. Samples were then placed in -20°C until ready for analysis. Before analysis the samples were reconstituted with 100 µL of 0.1 M sodium bicarbonate buffer (pH 9) and 100 µL of freshly prepared dansyl chloride (1 mg/mL in acetone). The reaction mixture was incubated at 60°C for 10 minutes to complete the derivatization. Samples (50 µL) were analyzed by LC-MS-MS as described later.

2.5. LC-MS-MS Method

2.5.1. Initial Method Using API 3000

Initial metabolism experiments used positive ion electrospray tandem mass spectrometry on an API 3000. Liquid chromatography was carried out on an XBridge C-18 column. The mobile phase, operating at the flow rate of 300 µL per minute consisted of water with 0.1% (v/v) formic acid and 10% methanol as solvent A and 0.1% (v/v) formic acid in methanol as solvent B. Initial conditions for the 30-minute run were set at 80% solvent B. The gradient was held at the initial conditions for 5 minutes followed by a linear gradient of B from 80% to 95% over 20 minutes and held at 95% solvent B for 5 minutes. The mass spectrometer parameters were optimized to the values found in Table IV.

Summary of Mass Spectrometer Parameters		
Ion Spray Voltage	4.5 kV	
Source Temperature	350°C	
Nebulizer Gas	12 instrument units	
Curtain Gas	8 units	
Collision Gas	5 units	
Focusing Potential	370 V	
Declustering Potential	81 V	
Collision Energy	2-$MeOE_1$, 4-$MeOE_1$, 2$MeOE_1$-d_4 59 V	E_1, E_2 57 V
MRM Channels	E_1	504/171
	E_2	506/171
	2-$MeOE_1$	534/171
	4-$MeOE_1$	534/171
	2-$MeOE_1$-d_4	538/171

TABLE III MASS SPECTROMETER PARAMETERS FOR API 3000.

The MRM channels used correspond to positive ion mode with a loss of the dansyl group (171). The estrogen metabolites were quantified by analyzing peak areas. 2-$MeOE_1$ and 4-$MeOE_1$ were normalized against 2-$MeOE_1$-d_4 internal standard to account for extraction efficiencies and represented as peak area ratios. 2-$MeOE_1$ and 4-$MeOE_1$ peak area ratios in E_2-treated samples were considered as 100%, and all the other samples were normalized against that and represented as relative peak area ratio in graphs.

2.5.2. Final Method Using AB Sciex 5500

Current metabolism experiments use positive ion electrospray tandem mass spectrometry on an AB Sciex 5500. Liquid chromatography was carried out on an Acuity UPLC C-18 column. The mobile phase, operating at the flow rate of 600 µL per minute

consisted of water with 0.1% (v/v) formic acid and 30% methanol as solvent A and 0.1% (v/v) formic acid in methanol as solvent B. Initial conditions for the 5-minute run were set at 82% solvent B. The gradient was held at the initial conditions for 1 minute followed by a linear gradient of B from 82% to 86% over 3.7 minutes and held at 86% solvent B for 0.3 minutes before returning back to 82% B for 1 minutes. The mass spectrometer parameters were optimized to the values found in Table V.

Summary of Mass Spectrometer Parameters		
Ion Spray Voltage	5.5 kV	
Source Temperature	500°C	
Nebulizer Gas	12 instrument units	
Curtain Gas	30 units	
Collision Gas	Medium	
Entrance Potential	10 V	
Declustering Potential	50 V	
Collision Energy	50 V	
MRM Channels	E_1	504/171
	E_2	506/171
	2-$MeOE_1$	534/171
	4-$MeOE_1$	534/171
	2-$MeOE_1$-d_4	538/171

TABLE IV MASS SPECTROMETER PARAMETERS FOR AB SCIEX 5500.

The MRM channels used correspond to positive ion mode with a loss of the dansyl group (171). The estrogen metabolites were quantified by analyzing peak areas. A standard curve of six concentrations of 2-$MeOE_1$, 4-$MeOE_1$, and 2-$MeOE_1$-d_4 were run each time samples were analyzed to account for extraction efficiencies, machine variability, and to calculate the concentration of each metabolite in the sample. The peak area for 2-$MeOE_1$ and 4-$MeOE_1$ were normalized against 2-$MeOE_1$-d_4 internal

standard to account for extraction efficiencies and represented as peak area ratios. These peak area ratios were then converted to concentration of metabolite using a standard curve.

2.6. Ethoxyresorufin-O-deethylase

This assay was performed by Shuai Wang (86). Inhibition of CYP450 1B1 activity was assessed by the ethoxyresorufin-O-deethylase (EROD) assay. Human recombinant CYP450 1B1 isozymes with CYP450 reductase were purchased from BD bioScience. Briefly, recombinant CYP450 1B1 was incubated with NADPH(1 mmol/L) in potassium phosphate buffer (50 mmol/L, pH 7.4) in the presence and absence of 8PN, IXH, and XH (1 µM). Fluorescence was measured at 530/590 for 20 min every minute by the BioTek Synergy H4 plate reader. The slope of each line was determined and used.

2.7. RT-PCR

This assay was performed by Shuai Wang (86). MCF-10A cells were treated with 1 µM E_2, 10 nM TCDD, or XH, IXH, 8PN (1 µM). After 24 h treatment cells were lysed using QiaShredder Kit (Qiagen). mRNA was purified using RNA mini kit (Qiagen). mRNA was reverse transcribed using Superscript III RT (Invitrogen). Real-time PCR evaluation of the expression of CYP1A1 (Hs00153120_m1), CYP1B1 (Hs00164383_m1), COMT (Hs00241349_m1) was performed using the TaqMan Gene Expression Assay on the Step One Plus (Applied Biosystems). The relative expression level of CYP1A1,

CYP1B1, and COMT mRNA was calculated using the Delta Delta Ct method by comparing it with the relative mRNA levels of beta actin (Hs 99999903_m1) and then vehicle treated samples.

2.8. Statistical Analysis

All statistics were performed in GraphPad Prism 5 using a 1-way ANOVA with Dunnett's post-hoc test.

3. METHOD OPTIMIZATION FOR ANALYSIS OF BOTANICALS ABILITY TO MODULATE ESTROGEN CHEMICAL CARCINOGENESIS

The oxidative metabolism of estrogen is known to play a role in breast cancer risk. In recent years, absolute quantification of these oxidative metabolites has become increasingly popular as we try to understand the complex relationship between estrogen metabolism and the metabolites it produces, with breast cancer. The most efficient way to detect and quantify estrogen metabolites is to utilize LC-MS-MS techniques. My goal was to optimize the method established in our lab to increase sensitivity, reduce variation, and make the overall assay shorter. The increase in sensitivity was necessary as we are planning on analyzing animal samples where the level of metabolites would be significantly less than what we observe in our cell culture experiments, and to increase the assay throughput.

We measure the amount of methylated catechol as a surrogate marker for catechol due to efficient methylation by catechol-*o*-methyltransferase (87) and the methylated catechols are much more stable in cell media. MCF-10A cells express a high level of 17β-hydroxysteroid dehydrogenase (17β-HSD) which converts estradiol into estrone (88, 89). 17β-HSD converts a large majority of the estradiol into estrone and the most abundant metabolites in MCF-10A media are estrone metabolites. Therefore, the methylated catechol of estrone ($MeOE_1$) is used as a marker for the estradiol catechol (OHE_2).

Building upon the work of our group (14) which indicated that a specialized extract of hops reduced the carcinogenic metabolite marker, we were interested in investigating other optimized hops extracts, and the major compounds found in hops

(8PN, XH, and IXH) to determine if these compounds were responsible for the activity observed with the initial extract.

3.1. Method Optimization for Robust Quantification of Estrogen Metabolites

The procedure previously utilized in our lab used lyophilization of the cell media to reduce the total volume to be extracted (Figure 2.1; Method 1). The lyophilization procedure would sometimes not proceed fully, thawing half way through and/or sample would be lost due to bubbling out of the top of the containers therefore introducing a large source of variation to the experiment. To eliminate the lyophilization, the samples were split up evenly into 4 different test tubes and extracted (Figure 2.1: Method 2). While this method eliminated the lyophilization, it increased the amount of work and by splitting the samples up and it increased the chance of losing more of the sample. These methods of extraction were also inefficient because the samples were not being centrifuged to make sure that there was a good separation between the organic and aqueous layers. The current method used (Figure 2.1: Current Method), extracts the cell media without splitting it up, centrifugation for a cleaner separation of layers, and yields an extraction efficiency ~85%.

In order to assess that we are capturing the majority of the metabolites, the amount of metabolites inside the cell were quantified. After the media was removed from the cells, cells were washed with PBS, trypsinized, pelleted, re-suspended in PBS, sonicated, and then extracted. There was a low level of metabolites present in the cell

lysates, however the amount was negligible compare to the amount located in the cell media (Figure 3.1).

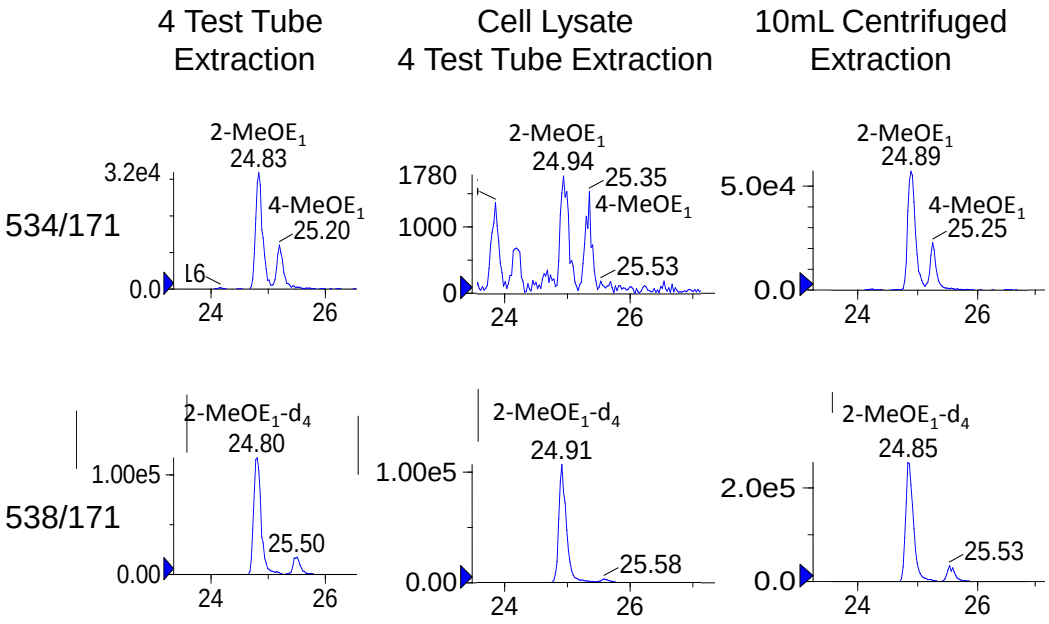

Figure 3.1 MRM Ion Chromatograms comparing different extraction methods. Testing two extraction methods and determination of metabolites in cell lysate. Top row m/z 534/171. Bottom row m/z 538/171.

The API-3000 was able to measure estrogen metabolites using 30 minute runs. Utilizing the AB Sciex 5500 LC-MS-MS system that the University of Illinois at Chicago Research Resource Center recently acquired, with gradient optimization the run was reduced to 5 minutes. The AB Sciex increased sensitivity, in both peak resolution and intensity can be directly compared after the results obtained from the API 3000 when analyzing a 0.1 nM mixture of 2-MeOE$_1$ and 4-MeOE$_1$ shown in Figure 3.2.

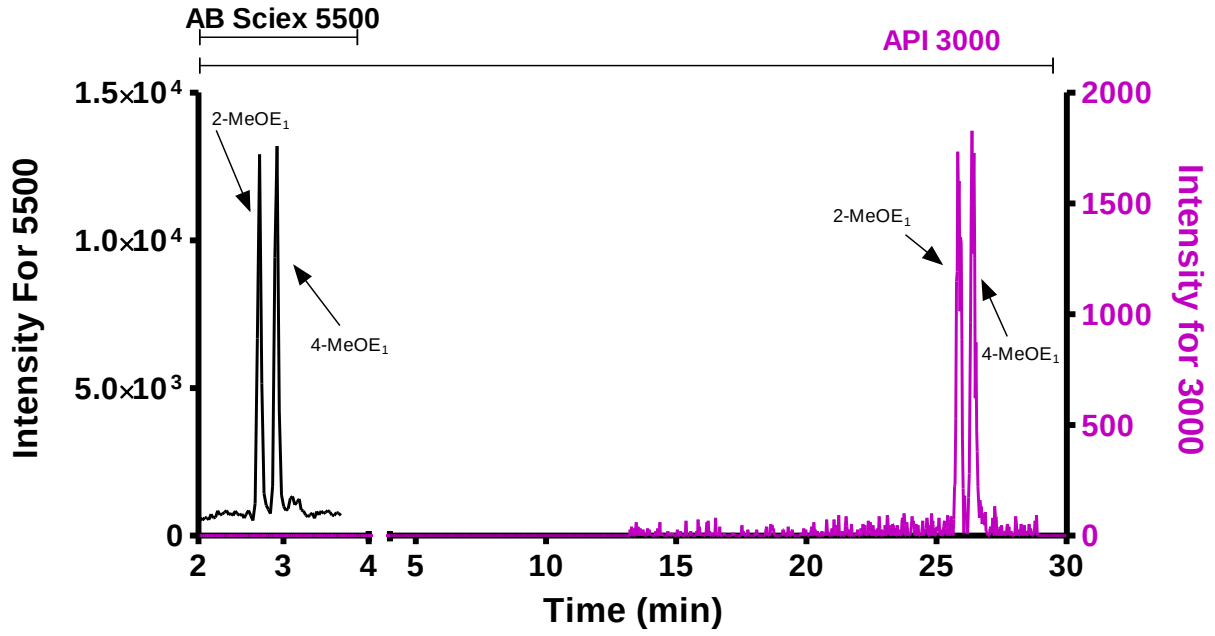

Figure 3.2. Comparison API-3000 and AB Sciex 5500. (0.1 nM mixture of 2-MeOE$_1$ and 4-MeOE$_1$). m/z 534/171

With each LC-MS-MS run, a standard curve was prepared by spiking 20 µL of stock solutions of 2-MeOE$_1$, and 4-MeOE$_1$ at 0.001 µM, 0.005 µM, 0.01 µM, 0.05 µM, 0.1 µM, 0.2 µM, and 1.2 ng of 2-MeOE$_1$-d$_4$ into stripped phenol red free media. The standards in media were extracted, and prepared for analysis like the samples. The standard curves were plotted peak area ratio vs. [analyte] and used to determine the concentration of 2-MeOE$_1$, and 4-MeOE$_1$ (Figure 3.3).

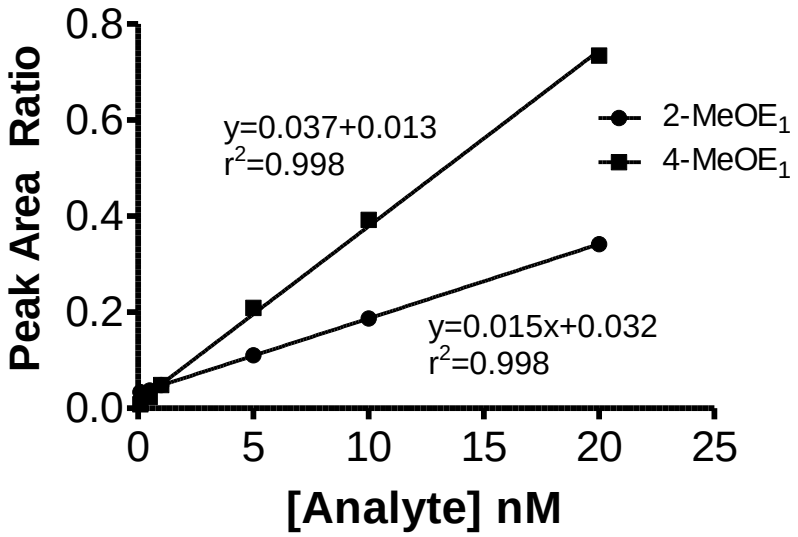

Figure 3.3. Representative standard curves for 2-$MeOE_1$, and 4-$MeOE_1$.

Due to the increased peak resolution and decreased assay time, the newer extraction method and 5500 LC/MS-MS method will be utilized for future experiments. This method change allows for 24 samples (with appropriate washes) to be run in 9 hours, 24 samples with the older method would have taken just under 48 hours to acquire. This increase in throughput of the assay along with the increase in sensitivity should be enough to analyze data from the planned animal experiments looking at metabolism of estrogen in different tissues.

3.2. Reproduction of Hops Extract Reduction of Estrogen Metabolism

Utilizing the optimized extraction method and newer LC-MS-MS method, we confirmed our method by replicating the results published by our lab (14) using the same hops extract, as well as testing extracts from two licorice species which were under

investigation in the botanical center for promising estrogenic activity, and wide use in menopausal women's supplements, Figure 5.

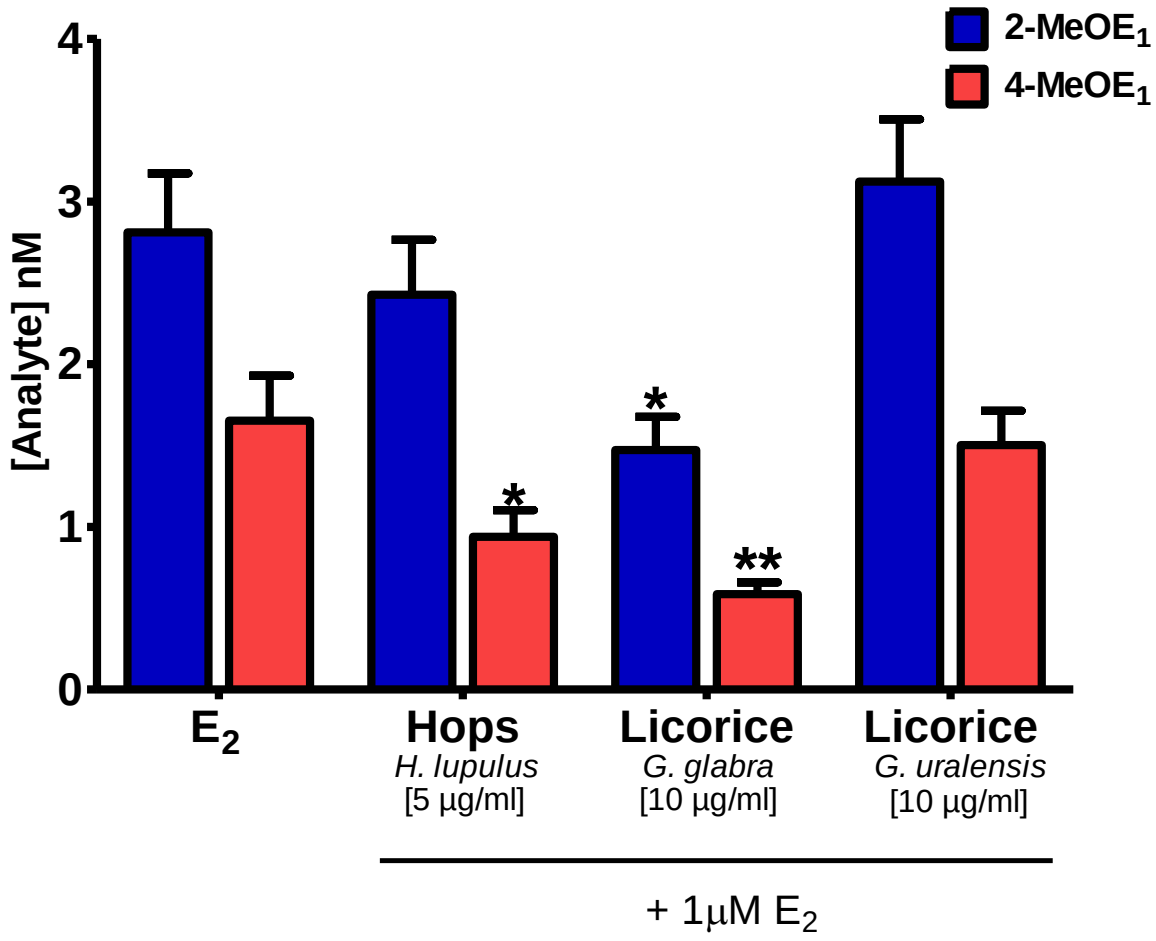

Figure 3.4 Verification of hops reducing genotoxic marker metabolite and one species of Licorice reduces both benign and genotoxic marker metabolites. 6 day metabolism experiment. Each data point represents an average of 3 independent experiments done in duplicate ± SEM and the statistic was calculated with a 1-way ANOVA with Dunnett post-test. * $p<0.05$ and ** $p<0.01$.

Our previous report found that there was ~50% reduction in the 4-$MeOE_1$ metabolite formation and the data here shows a 43% reduction confirming that the new method

gives comparable data to the older method. Interestingly, Licorice's activity appears to be species specific. *G.glabra* reduced the genotoxic marker, 4-MeOE$_1$, 65% and the benign, 2-MeOE$_1$, was reduced 48% while *G.uralensis* had no effect. The interesting licorice activity is not discussed further, and hops will be the focus of the remainder of this thesis.

3.3. Test of optimized Hops extracts

Utilizing the improved method a variety of hops extracts that have been optimized for different ratios of flavonoids (Table VI) were tested in the assay to get a better idea

Extract name	IXH	8PN	XH	6PN	"Major Compound"
3 KO	0.08%	0.04%	0.10%	0.10%	**Equal**
XHKO	0.58%	0.01%	0.07%	0.15%	**Equal**
IXH KO	0.07%	0.10%	16.3%	0.74%	**XH**
6PN KO	0.83%	0.20%	15%	0.37%	**XH**
Xantho	1.11%	0.31%	8.81%	0.74%	**XH**
Clinical	0.99%	0.36%	32.26%	1.18%	**XH**
IsoXantho	12.2%	0.51%	0.70%	0.53%	**IXH**
IsoXanthoFlav	62.4%	0.83%	2.22%	0.82%	**IXH**

TABLE V W/W% CONSTITUENTS OF OPTIMIZED HOPS EXTRACTS BY "MAJOR COMPOUND" (provided by Rene Ramos). Those extracts with the major compound labeled as equal had relatively the same amount of all 4 of the measured compounds so not one single compound dominates the extract.

how the compounds within the constructs of the extract might be responsible for the reduction in metabolism Figure 3.5. Those extracts with the major compound labeled as

equal had relatively the same amount of all 4 of the measured compounds so not one single compound dominates the extract. Four of the extracts increased the benign

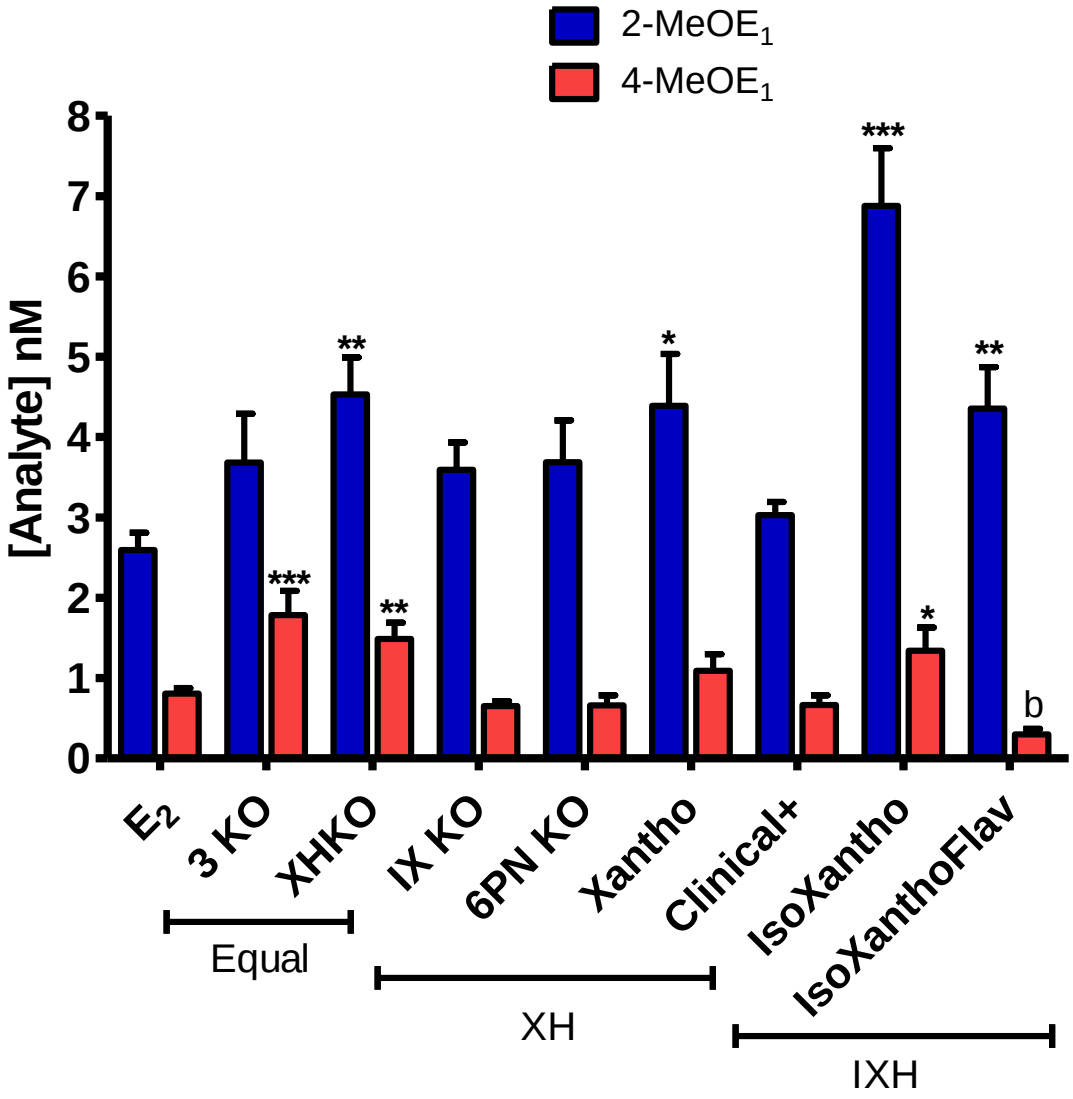

Figure 3.5 Optimized hops extracts (2.5 µg/ml or 1 µg/ml for clinical extract) suggest that 8PN and IXH account for activity. 6 day metabolism experiment. Each data point represents an average of 3 independent experiments done in duplicate ± SEM. Statistically significant increases * $p<0.05$, ** $p<0.01$, and *** $p<0.001$. Statically significant decrease in metabolite production b $p<0.05$.

metabolite (XHKO, Xantho, IsoXantho, and IsoXanthoFlav; $p<0.01$, $p<0.05$, $p<0.001$, and $p<0.01$ respectively), three extracts significantly increased the genotoxic metabolite marker (3KO, XHKO, and IsoXantho; $p<0.001$, $p<0.01$, and $p<0.05$ respectively), while only one extract (IsoXanthoFlav) simultaneously increased the benign metabolite ($p<0.01$) and decreased the genotoxic metabolite ($p<0.05$).

The extracts with the highest 8PN and IXH had the most significant effect on the benign metabolite marker, and both IsoXantho and IsoXanthoFlav effected the genotoxic metabolite marker but in opposite manners. Since the IsoXanthoFlav extract exhibited the most desirable effect of increasing the benign pathway while also decreasing the genotoxic pathway of estrogen metabolism, this extract's activity was investigated further in a dose response (Figure 3.6).

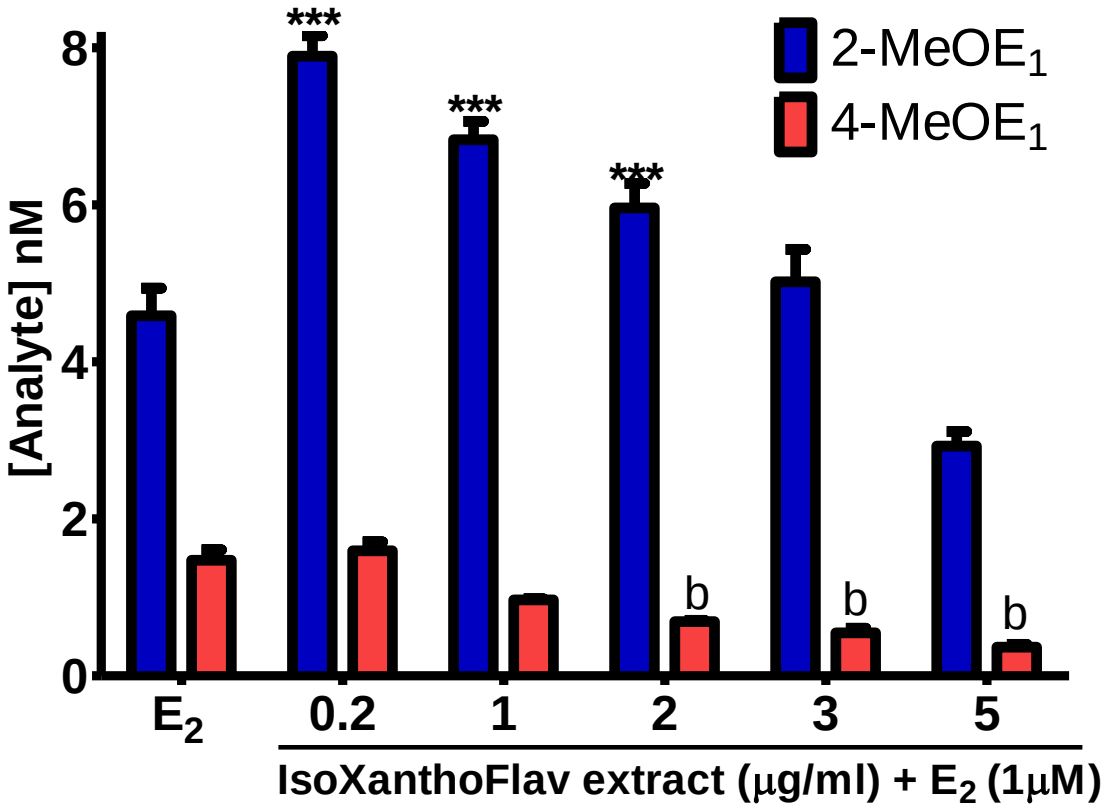

Figure 3.6 IsoXanthoFlav extract significantly increases 2-$MeOE_1$ at low doses, and at higher doses, 4-$MeOE_1$ is reduced, while there is no change in 2-$MeOE_1$. 3 day metabolism experiment. Each data point represents an average of 2 independent experiments done in duplicate ± SEM. Statistically significant increase *** $p<0.001$. Statically significant decrease in metabolite production b $p<0.05$.

3.4. Bioactive compounds from Hops alter metabolism differently

Because the IsoXanthoFlav extract is enriched for IX and contains relatively high levels of 8PN as well we investigated IXH, 8PN and XH ability to modulate estrogen metabolism, Figure 3.7. 8PN and IXH appears to increase the production of the benign

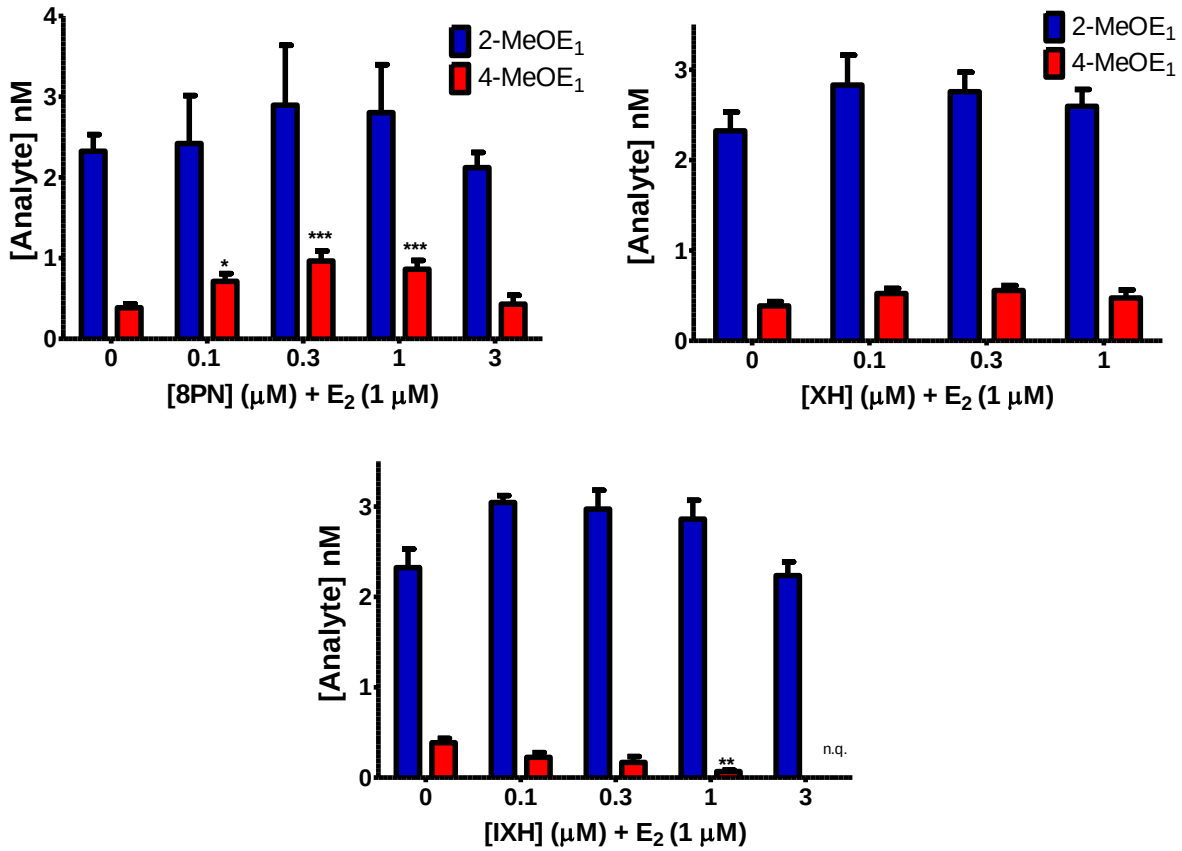

Figure 3.7 Analysis of the most commonly investigated constituents in hops; 8-prenylnaringenin and isoxanthohumol significantly affects 4-$MeOE_1$ levels but in opposite ways. 3 day metabolism experiment. Each data point represents an average of 3 independent experiments (only 2 experiments for IXH) done in duplicate ± SEM. Statistically significant increases * $p<0.05$, and *** $p<0.001$. Statically significant decrease in metabolite production b $p<0.05$ and n.q. not quantifiable under limit of quantitation.

metabolite however it is not statistically significant. 8PN and IXH have the opposite effect on the amount of 4-$MeOE_1$ which can be seen in Figure 3.8. XH on the other hand does not affect the production of either metabolite.

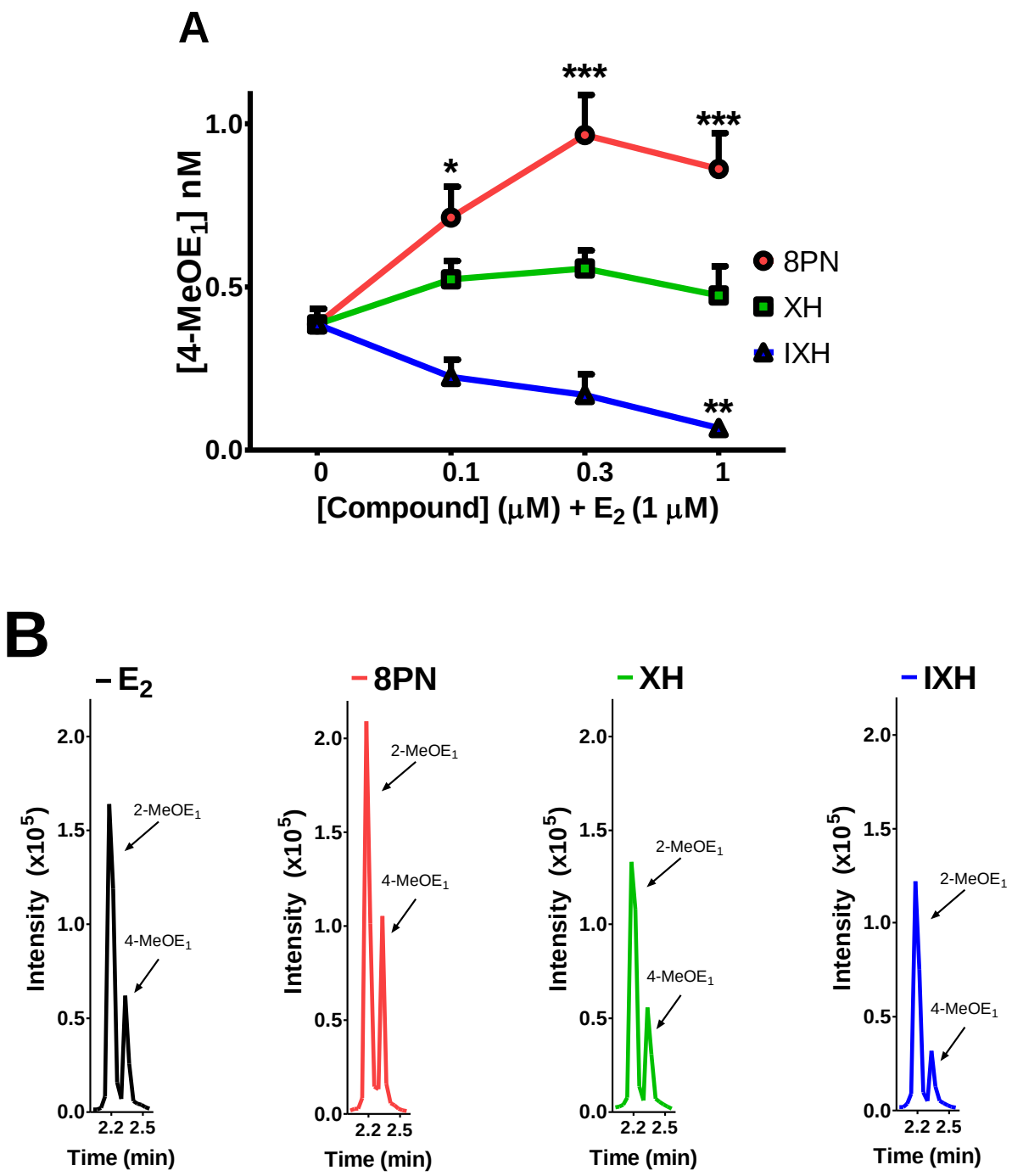

Figure 3.8 Comparison of 8PN, IXH, XH ability to alter genotoxic metabolite marker. A) 4 doses of 8PN, IXH, and XH ability to alter estrogen the level of the genotoxic 4-MeOE$_1$. 3 day metabolism experiment. Each data point represents an average of 3 independent experiments (only 2 experiments for IXH) done in duplicate ± SEM. *

p<0.05, ** p<0.01, and *** p<0.001. B) Overlaid representative MRM chromatograms of the formation of 2-MeOE$_1$ and 4-MeOE$_1$ after treatment with E$_2$ (1 µM) and E$_2$ + 8PN, XH, or IXH (1 µM).

3.5. Mechanism of 8PN and IXH modulation genotoxic metabolite marker level

In order to assess the mechanism behind the increase of 4-MeOE$_1$ by 8PN or the mechanism of reduction by IXH, the EROD assay was run with purified CYP1B1 and CYP1A1 enzymes to determine if the compounds are directly inhibiting the enzymes (Figure 3.9; (86)). While 8PN, XH, and IXH all inhibit CYP1B1 with about the same intensity, IXH preferentially inhibits CYP1B1, not really inhibiting CYP1A1. XH and to a lesser extent 8PN inhibit CYP1A1 activity.

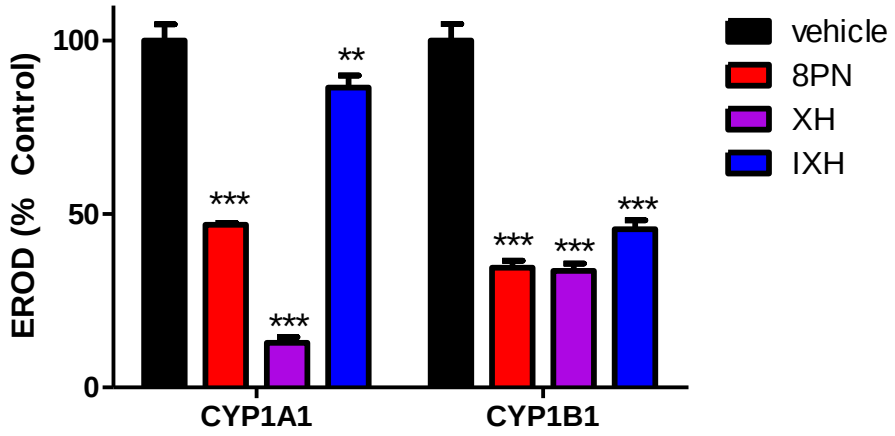

Figure 3.9 Purified enzyme inhibition by 8PN, XH, and IXH. 0.15 pmole of CYP1A1 or 0.8 pmole of CYP1B1 were pre-incubated with 1 µM of test compound or 0.1% DMSO for 5 minutes then the fluorescence read out from EROD conversion was measured.

Each data point represents an average of 3 independent experiments done in triplicate ± SEM. ** p<0.01 and *** p<0.001. unpublished data from Shuai Wang (86).

The ability of the three compounds to alter the expression of the enzymes were assessed by 24 h treatment and then RT-PCR for CYP1A1, CYP1B1, and COMT (Figure 3.10; (86)). TCDD is a known inducer of both CYP1A1 and CYP1B1.

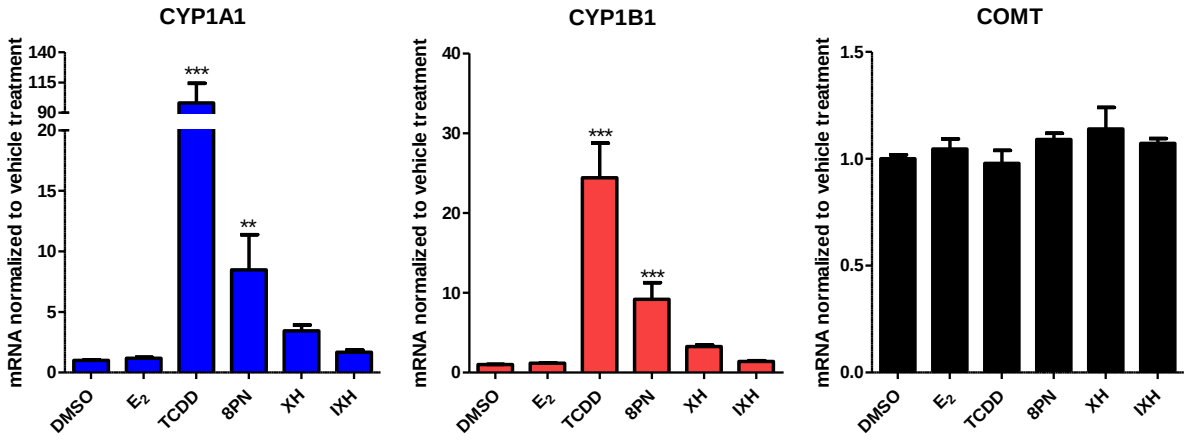

Figure 3.10 8PN increased mRNA Expression of CYP1A1 and CYP1B1. No compound reduced COMT expression. MCF-10A cells were treated with E_2, 8PN, XH, XH, or IXH at 1 µM or TCDD at 10 nM for 24 h. mRNA isolated and analyzed after by RT-PCR. Each data point represents an average of 3 independent experiments done in duplicate ± SEM. ** p<0.01 and *** p<0.001. unpublished data from Shuai Wang (86).

8PN significantly increased the amount of both CYP1A1 and CYP1B1 mRNA levels, while IXH had a negligible effect on both. XH did increase the mRNA level of both CYP1B1 and CYP1A1 but not significantly. All three compounds did not affect COMT mRNA levels.

3.6. Discussion and Conclusion

There have been recent advancements in breast cancer treatment and risk factors, however, it is still responsible for 14% of all cancer deaths among women -second only to lung and bronchus cancers (1). It has been well established that estrogen contributes to breast cancer risk through a hormonal pathway by estrogen binding to the estrogen receptor and increasing cellular proliferation, thereby increasing chances of mutations. Since 1999, a lot of research has been investigating the role of estrogen's oxidative metabolism in the initiation and progression of cancer (2). The 4-pathway of metabolism has been associated with more genotoxicity, increases in 3,4,-quinone DNA adducts, and have been linked to higher incidence of breast cancer (77, 90), 4-OH metabolites are carcinogenic in animal models and mutagenic in cell-based assays, causing cellular transformation and formation of DNA adducts (16, 70-72), while the 2-pathway is more benign and may even have a beneficial effect (71, 75, 76).

Women are currently taking supplements that contain botanicals for a multitude of reasons including overall health improvement, menopausal symptom relief, and bust enhancement. Many botanicals used in those supplements have been investigated for their estrogenic and chemopreventive properties; however this is one of the few investigations into the ability of botanicals to modulate the chemical mechanism of estrogen carcinogenesis involving estrogen metabolism. *The hypothesis that botanicals women use for menopausal symptom relief have beneficial chemopreventive activities by reducing exposure to genotoxic estrogen metabolites and improving overall wellness by reducing the negative effects of estrogen* was supported by five extracts altering estrogen metabolism, and the observation that 8PN increases the genotoxic pathway

(4-) and IXH decreases the same pathway in MCF-10A cells. A summary of the observations can be seen in Table VII.

	Metabolism		**Purified Enzyme Activity**			**mRNA**		
	2-MeOE$_1$	4-MeOE$_1$	1A1	1B1	COMT	1A1	1B1	COMT
8PN	~↑	↑	↓	↓		↑	↑	-
XH	-	-	↓	↓		~↑	↑	-
IXH	-	↓	-	↓		Low↑	-	-

TABLE VI SUMMARY OF 8PN, XH, IXH RESPONSES

In the present study, a variety of extracts of *Humulus lupulus* were assessed for their ability to alter the production of two estrogen metabolites as markers for the genotoxic and benign pathways of estrogen metabolism in order to better understand the complex relationship between the constituents in any given extract of this plant. Five of the eight tested extracts had some significant effect on metabolism, however one extract (IsoXanthoFlav) simultaneously increased the benign metabolite ($p<0.01$) and decreased the genotoxic metabolite ($p<0.05$; Figure 3.5). This effect is the most desired because the estrogen metabolites that are known to be more genotoxic are reduced, while increasing the benign metabolites with suggested beneficial properties.

Because the IsoXanthoFlav extract had the most desirable effect on metabolism and it had the highest amount of 8PN and IXH, and XH is known to have

chemopreventive properties, the ability of each of these individual compounds to modulate estrogen metabolism were evaluated (Figure 3.7). When MCF-10A cells were treated with 8PN, there was a significant increase in the 4-methoxyether metabolite. Conversely, IXH dose dependently decreased the 4- genotoxic metabolite marker. The LC-MS-MS analysis of the methoxyether metabolites of estrogen as a marker for the 2- and 4- pathways depends on the extracts and compounds not affecting the expression or activity of the COMT enzyme which is responsible for the methylation. We have already shown that 8PN, XH, and IXH do not affect the mRNA expression of COMT, and the activity assay for COMT is currently in process. Our data (summarized in Table 3.2) suggests that IXH reduction of 4-$MeOE_1$ may be due to enzyme inhibition ($p<0.001$) and not altered gene regulation (Figure 3.9 and Figure 3.10). While it appears that 8PN increases 4-$MeOE_1$ at the gene transcription level by increasing CYP1B1 mRNA ($p<0.001$).

This is not the first report of the contradictory activities of IXH and 8PN. While 8PN is the most potent phytoestrogen to date; IXH does not have estrogenic activity. IXH does not increase uterine weight *in vivo* like 8PN or estradiol (24). 8PN and IXH had opposite effects on angiogenesis and inflammation; 8PN stimulated endothelial and smooth muscle cell growth, motility and invasion and capillary like structure formation in umbilical vein endothelial cells grown in matrigel whereas IXH inhibited those effects (91). IXH has also been shown to decrease capillary like tubules in MDA-MB-231 cells and block TGF-β signaling cascades leading to decreased pro-inflammatory gene expression (92).

While there are some noted differences in the activities between 8PN and IXH, they do have some similar effects including inhibition of prostate cancer cell proliferation and viability of colon cancer cells (93, 94). Interestingly, 8PN and IXH altered the colon cancer cells cell cycles differently. A follow up study to the prostate cancer proliferation showed that IXH and 8PN induced cell death in absence of caspase 3 and the cells had an increased number of vacuoles suggesting autophagy, however the doses used in this study were 100-200 µM which is very high (95). 8PN also inhibits aromatase, the enzyme responsible for the synthesis of estradiol (IC_{50}=0.08 µM), and to a lesser extent XH (3.2 µM) and IXH (25.4 µM) inhibit as well (96). 8PN and IXH inhibited androgen production in Leydig cells (97). 8PN and IXH also inhibited colorectal carcinoma cell invasion around 50% in a matrigel assay (94). 8PN and IXH both appear to be able to affect hormone dependent cancers by altering steroidogenesis and altering cancer cell viability and invasiveness.

Our results add to the body of knowledge on the different activities of 8PN and IXH, by showing that they have opposite effects on modulating estrogen metabolism. 8PN treatment increased the genotoxic metabolite by increasing the mRNA for the enzyme responsible for its production while treatment with IXH decreased the genotoxic metabolite by directly inhibiting the enzyme.

3.7. Future Directions and Conclusions

Based on our results short-term follow up experiments include confirming enzyme inhibition in a cellular system, and measuring the amounts of 8PN, IXH, and XH

in media after assay times to assess conversion rates. The inhibition of CYP1B1 will be tested by pretreating MCF-10A cells with the compounds, washing the cells, and then incubating with just estradiol for 24 hours and analyzing the media for metabolites. We suspect that there would be no reduction in metabolites if IXH is in fact only altering the metabolism by directly inhibiting CYP1B1 activity. Due to inability to obtain good CYP1B1 antibodies in order to analyze functional protein levels, we will assess MCF-10A cells' ability to convert 7-ethoxy-o-resorufin after treatment with compounds in order to give a more accurate picture of what is happening in the cells. The specific action of CYP1A1 vs. CYP1B1 will be determined by using specific inhibitors of those enzymes.

A critical point to consider is that 8PN, IXH, and XH can be converted to one another and a necessary follow up to this study is to measure the actual amounts of IXH, XH, and 8PN present in the cell media to understand their rate of conversion in the assays used. While 8PN can be formed from IXH, and IXH and XH cyclize to each other (29, 30), our data shows each compound has different activity on the metabolism of estrogen. The conversion of 8PN, IXH, and XH could have the ability to alter the results of experiments however both 8PN and IXH are the cyclized forms which are more stable and less likely to be converted. XH is the one compound most likely to be affected by cyclization to XH since this conversion is favored.

Longer term follow up studies include testing the pure compounds and the IsoXanthoFlav extract for inhibition of estradiol induced malignant transformation in MCF-10A cells. Those compounds and extract (if possible) that show a reduction in transformed foci would then be tested if they could reduce the tumor burden in the ACI rat model for estrogen carcinogenesis.

This work is the first to show that, the different constituents in hops have opposing roles on estrogen metabolism and it appears to occur through different mechanisms. The use of hops and some of the isolated compounds have been suggested as potential cancer preventive or therapeutic agents, and this work suggests that 8PN increases the genotoxic pathway of estrogen metabolism, and that IXH is more desirable because it reduces the genotoxic pathway of estrogen metabolism. The data suggests that hops extracts, specifically those with high levels of isoxanthohumol, have beneficial chemopreventive activities by reducing the genotoxic pathway of estrogen metabolism and could potentially improve overall wellness by reducing the negative effects of estrogen.

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5. CURRICULUM VITAE

Courtney Snelten

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EDUCATION

2013 UNIVERSITY OF ILLINOIS AT CHICAGO CHICAGO, IL
MS Pharmacognosy GPA: 3.7/4.0
Project Description: Understand the role of botanical supplements women commonly use in their ability to modulate estrogen carcinogenesis.

2013 REGULATORY AFFAIRS PROFESSIONAL SOCIETY
Online Regulatory Affairs Basics Bundle of Classes
Pharmaceuticals: Definitions & Lifecycle, Medical Devices: Definition & Lifecycle, Role of the Regulatory Professional, and Introduction to Global Healthcare Product Regulations

2010 CARTHAGE COLLEGE KENOSHA, WI
Bachelor of Arts GPA: 3.4/4.0
Majors: Biology and Neuroscience Minor: Chemistry
Senior Thesis: Proposal to investigate how the route of administration and time affect *Ginkgo biloba* extract's ability to reduce symptoms of Parkinson's disease after the disease is induced in rats.

ACADEMIC AWARDS/FELLOWSHIPS

2012 American Foundation for Pharmaceutical Education Pre-Doctoral Fellow

RESEARCH EXPERIENCE

2010-Current JUDY BOLTON LAB
UNIVERSITY OF ILLINOIS AT CHICAGO CHICAGO, IL
Department of Medicinal Chemistry and Pharmacognosy
UIC/NIH Center for Botanical Supplements Research
Graduate Research Assistant

- Use LC/MS-MS to detect changes in estradiol metabolism with the addition of different botanical extracts
- Serve as the go-to problem solver for the lab group, often dealing in crisis mode and looking for the most effective solution
- Coordinate and planned weekly group meetings, organized lab space, and delegated joint responsibilities for a group of 25 members

PUBLICATIONS

2013 **Snelten, C.S.**, Dietz, B., Bolton, J.L. Modulation of Estrogen Chemical Carcinogenesis by Botanical Supplements used for Postmenopausal Women's Health*. Drug Discovery Today: Disease Mechanisms.* 2013

POSTERS

2013 "Hops and Licorice Reduce Carcinogenic Estrogen Metabolites" **Snelten, C.S.**, Wang, S., Ramos, R., Chen, S.N., Dietz, B., Pauli, G.F., Bolton, J.L. MIKI Meeting in Miniature Minneapolis, MN and Great Lakes Drug Metabolism Meeting Toledo, OH.

PRESENTATIONS

2013 "Investigating the Modulation of Estrogen Metabolism by *Humulus lupulus*, Hops." **Snelten, C.S.** UIC Masters Departmental Seminar. Chicago IL

2012 "Highlights from participation in the ODS-sponsored *Mary Frances Picciano Dietary Supplement Research Practicum*" **Snelten, C.S.** and Martinez, L. UIC Botanical Center Plant Center Meeting Journal Club. Chicago IL

"Soy Isoflavone Supplementation for Breast Cancer Risk Reduction: A Randomized Phase II Trial" Snelten, C.S. UIC Botanical Center Plant Center Meeting Journal Club. Chicago IL

2010 Proposal for the investigation into the role of *Ginkgo biloba* in Parkinson's disease.*"* Snelten, C.S.. Carthage College Biology Research Day. Kenosha, WI

2009 *"*Proposal for the investigation into the role of *Ginkgo biloba* in Parkinson's disease.*"* Snelten, C.S.. Carthage College Undergraduate Thesis Defense. Kenosha, WI

PROFESSIONAL WORKSHOPS

July 2013 Accepted to Participate in 2013 ACS Publications Graduate Student/Postdoc Summer Institute

June 2012 Participated in Mary Frances Picciano Dietary Supplement Research Practicum

PROFESSIONAL AFFILIATIONS

2013 Regulatory Affairs Professional Society
2013 National Association of Scientific Writers
2013 American Medical Writers Association
2012-Current American Chemical Society
2011-Current American Society of Pharmacognosy

COMPUTER SKILLS

- Microsoft Office
- SPSS
- GraphPad Prism
- ChemOffice

RESEARCH TECHNIQUES

- Cell Culture
- Liquid Chromatography/Mass Spectrometry
- Luciferase Assay
- Cytotoxicity Assay

VOLUNTEER EXPERIENCE

2011-Current METRO ACHIEVEMENT CENTER CHICAGO, IL

Academic Tutor

- Tutored female inter-city high school students in coursework, and monitored their work weekly throughout the school year.
- Instructed girls on proper writing techniques, essay layouts, and critical thinking in preparation to succeed in college.
- Mentored girls in various difficult life situations that arise during high school

GRADUATE COURSEWORK

- Pathobiology of Cancer
- Cancer Biology & Therapeutics
- Drug Discovery
- Pharmacognosy Research Techniques
- Biochemistry
- Essentials for Animal Research

TEACHING EXPERIENCE

2011-2012 GRADUATE STUDENT TEACHING CERTIFICATE PROGRAM CHICAGO, IL

Engaged in a year-long program designed to help graduate students become familiar with teaching at a university level from policies, and contracts to how to design effective lectures and discussions. The program also helped in the development of teaching philosophies and start my teaching portfolio.