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Modulation of Estrogen Chemical Carcinogenesis by Botanical Supplements used for Postmenopausal Women's Health

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Abstract

Breast cancer risk has been associated with long-term estrogen exposure including traditional hormone therapy (HT, formally hormone replacement therapy). To avoid traditional HT and associated risks, women have been turning to botanical supplements such as black cohosh, red clover, licorice, hops, dong gui, and ginger to relieve menopausal symptoms despite a lack of efficacy evidence. The mechanisms of estrogen carcinogenesis involve both hormonal and chemical pathways. Botanical supplements could protect women from estrogen carcinogenesis by modulating key enzymatic steps [aromatase, P4501B1, P4501A1, catechol-O-methyltransferase (COMT), NAD(P)H quinone oxidoreductase 1 (NQO1), and reactive oxygen species (ROS) scavenging] in estradiol metabolism leading to estrogen carcinogenesis as outlined in Figure 1. This review summarizes the influence of popular botanical supplements used for women's health on these key steps in the estrogen chemical carcinogenesis pathway, and suggests that botanical supplements may have added chemopreventive benefits by modulating estrogen metabolism.

Introduction

Breast cancer is the leading malignancy among American women, and was estimated to have claimed the lives of more than 39,000 women in 2011 [1]. Long-term exposure to estrogens can increase the risk of developing cancer in hormone sensitive tissues, such as the breast or the endometrium. Increased exposures to endogenous estrogens due to early menarche or late menopause, and/or exogenous estrogens from HT are risk factors for breast cancer.

HT (estrogen plus a progestin for women who have not had a hysterectomy and estrogen alone for those who have) is used to relieve menopausal symptoms, and was once believed to decrease osteoporosis, stroke, Alzheimer's disease, and coronary heart disease [2]. The highly publicized release of the Women's Health Initiative (WHI) clinical trial results in 2002 cast serious doubts about the long-term use of HT due to an increased incidence of invasive breast cancer, stroke, pulmonary embolism, and coronary heart disease [2]. After results from the WHI were published in the popular press, women began to look for alternatives to HT to avoid the increased risks associated with the use of HT [3]. Botanical dietary supplements commonly used to relieve menopausal symptoms became an obvious alternative because they are readily available and typically considered to be safer than

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traditional HT. Because 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 [4]. In addition, the few completed botanical clinical trials for efficacy have shown a very large placebo effect (> 50%) on menopausal symptom relief which further persuades women to consume these products [5–9]. Botanical supplements contain phytoestrogenic compounds which may contribute to their efficacy for menopausal symptom relief as well as their potential chemopreventive mechanisms of action [10–14]. This review highlights the key enzymes involved in estrogen chemical carcinogenesis and examines the current literature investigating the potential protective effects of botanicals and bioactive compounds (if known) on these pathways.

Mechanisms of Estrogen Carcinogenesis

Two mechanisms contributing to the carcinogenic potential of estrogens have been posited. 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. This mechanism has been extensively studied and is discussed in detail in other articles in this issue. The second chemical mechanism is the focus of this review discussed below.

It is important to realize that there are two estrogen receptors (ERs), ER α and ER β . ER α was discovered in the late 1950s and is used to assess the clinical status of breast tumors as a prognostic and therapeutic marker [15]. ER β was discovered in 1996 and has been under intense investigation to examine whether ER β status could be used to help classify breast tumors. ER β is also under investigation to determine whether activation of ER β can be stimulated by estradiol, phytoestrogens, or selective estrogen receptor modulators (SERMS). Stimulation of ER β is important to systemic estrogen regulation, as ER β activation appears to reduce the impact of ER α by opposing many of the ER α regulated genes, and down regulating ER α levels [15]. The presence of ER α and ER β can be significantly different in tissues, and are differentially expressed in many of the cell lines commonly used in breast cancer research (Table 1). Some phytoestrogens have been shown to have selectivity to either ER (Table 2), which has led many investigators to look into phytoestrogens as chemopreventive agents that exhibit selective gene regulation [12–14].

The chemical pathway of estrogen carcinogenesis involves metabolism of estrogens by cytochrome P450 (CYP) enzymes to reactive, electrophilic, and redox active α -quinone metabolites (Figure 1). These α -quinones are capable of alkylating DNA and forming DNA adducts which can lead to DNA mutations, as well as generating reactive oxygen species (ROS) ultimately increasing cancer risk [16]. The enzymes involved in estrogen metabolism can be separated into two categories; those enzymes responsible for generating estrogens and oxidizing the metabolites into α -quinones [aromatase, cytochrome P4501B1 (CYP1B1), cytochrome P4501A1 (CYP1A1)], and those responsible for deactivating the estrogen metabolites into benign metabolites [catechol- α -methyltransferase (COMT), NAD(P)H quinone oxidoreductase 1 (NQO1), and glutathione-S-transferase (GST)].

Botanical Modulation of Estrogen Carcinogenesis

There are seven targets in estrogen carcinogenesis (Figure 1; 1–7) where the intervention of a botanical could positively modulate estrogen's progression to the α -quinone. Botanicals could influence the progression of estrogen metabolism by down regulating the formation of estrogen through aromatase or the bioactivation of estrogen through CYP1B1 reducing the amount of estrogen or quinone produced. Alternatively, botanicals could deactivate estrogen into benign metabolites by increasing CYP1A1, COMT, NQO1, GST, or by scavenging

ROS that are produced during estrogen metabolism. The current literature examining the potential botanical modulation of these seven steps is outlined in Table 1 and discussed below.

Aromatase

Aromatase (Figure 1, 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 extra hepatic 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 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 monthly surge and ebb of circulating estrogens released by the ovaries.

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 [17]. 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 than fadrozole, a known aromatase inhibitor, and genistein was significantly more potent than biochanin A [18]. Interestingly, genistein also increased aromatase activity, mRNA, and protein levels in hepatic HepG2 cells [19].

Isoliquiritigenin, from *Glycyrrhiza* spp. (licorice), decreased aromatase mRNA in MCF-7 cells [20]. Two components of *Humulus lupulus* (hops) extracts, 8-prenylnarigenin (8-PN) and to a lesser extent xanthohumol (XH), inhibited aromatase activity in primary mammary fibroblasts [21] and in JAR choriocarcinoma cells while the mRNA expression levels of aromatase were unaffected [22]. 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 [23]. 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.

CYP1B1

The enzyme CYP1B1 hydroxylates E_2 and E_1 at the 4-position (Figure 1, step 2). The 4-hydroxylated catechol (4-OH) precursor to the α -quinone has shown to be carcinogenic in animal models and mutagenic in cell-based assays causing cellular transformation and formation of DNA adducts [16,24–26].

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 [27] and in MCF-7 cells [28] identified by microarray and confirmed with RT-PCR. However, Hemachandra et al. reported no change in the ratio of 2- to 4- MeOE₁ metabolites of estrogen in MCF-10A cells co-treated with a black cohosh extract and estradiol [29]. 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.

Genistein and daidzein both inhibited CYP1B1 mediated 7-ethoxyresorufin *O*-deethylase (EROD) activity [30]. CYP1B1 inhibition by genistein and daidzein is believed to be due to negative feedback from CYP1B1 mediated catalysis of the conversion of biochanin A and formononetin into genistein and daidzein [30]. A hops extract and 8-PN reduced the amount of the 4-MeOE₁ metabolite produced from estrogen metabolism [29]. While the decrease in the 4-MeOE₁ metabolite does not directly demonstrate a reduction in CYP1B1, it does infer that the conversion to the 4- metabolite is decreased. The finding was then validated showing a reduction in estradiol induced CYP1B1 protein expression and inhibition of CYP1B1 mediated EROD activity with 8-PN and hops, although a cytotoxic dose of hops was needed to observe EROD activity inhibition [29]. These results of inhibition and/or reduction of CYP1B1 by genistein, daidzein, and a hops extract demonstrates that these botanicals may be protective against estrogen carcinogenesis by reducing the formation of the 4-OHE₂ carcinogenic metabolite.

CYP1A1

CYP1A1 hydroxylates E₂ and E₁ at the 2- position (Figure 1, step 3). The 2-hydroxy catechol [2- OHE₁(E₂)] is considered part of the deactivating pathway of estradiol or estrone because 2- OHE₁(E₂) are not mutagenic or carcinogenic like their 4-OH counterparts [25]. In fact, the methyl ether metabolite, 2-methoxyestradiol has reported chemotherapeutic properties and promise as an anticancer drug [31,32]. Although, the 2-hydroxy catechol estrogens can also be oxidized to quinones that can alkylate DNA and form adducts, in breast cancer patients, the 3,4-quinone DNA adducts are present in much higher concentrations than those of the 2,3-quinone [33]. Also, the amount of the 3,4-quinone adducts in serum albumin of breast cancer patients compared to healthy controls was significantly increased while there was no increase in the concentration of the 2,3-quinone adducts [33].

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 [27,28]. 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 [34]. Conversely, genistein, and daidzein both inhibited CYP1A1 EROD activity and reduced mRNA levels in MCF-7 BUS cells [35]. Additionally, an aqueous extract of licorice, *Glycyrrhiza uralensis*, and glycyrrhetic acid, a compound from *G. uralensis*, increased *in vivo* CYP1A1 protein expression in liver of rats treated for seven days [36]. Also, a hops extract reduced estradiol induced protein levels of CYP1A1 and reduced the amount of the 2-MeOE₁ metabolite produced during estrogen metabolism [29]. In summary, genistein, daidzein, a licorice extract, and a hops extract inhibit the CYP1A1 enzyme which prevents estrogen from forming the therapeutic 2-OHE₁(E₂) metabolites and possibly increasing formation of the genotoxic 4-OH metabolites. Conversely, biochanin A and black cohosh increase CYP1A1 activity and mRNA.

COMT

COMT catalyzes the methylation of the 2- and 4-OH metabolites (Figure 1, step 4). COMT is part of the detoxification pathway of estrogen as it lowers the amount of 2- or 4-OH metabolites that 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 [37]. The 2-methoxyestradiol

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 but the formulation did not allow for the best distribution nevertheless the results are still promising for an anticancer drug [31,32].

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 [35,38]. 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 [29]. 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.

NQO1

NQO1 is responsible for a two-electron reduction of the quinone back to the precursor catechol metabolite (Figure 1, step 5). This reduces the reactive quinone without producing ROS and prevents the quinone from binding 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 [39].

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 [40]. Biochanin A and genistein increased NQO1 mRNA in MDA-MB-231 cells after 24 h [41]. 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 [42]. However, it has also been reported that in MCF-7 BUS cells, genistein and daidzein decreased relative NQO1 mRNA after 24 h treatment [35].

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 [43,44]. 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 isoliquiritigenin in the breast was significantly less than in the liver or colon [45]. Xanthohumol is able to induce NQO1 activity in Hepa 1c1c7 murine hepatoma cells [46]. 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 [47]. An extract of dong gui was fractionated following NQO1 inducing activity, and five compounds including lingustilide with NQO1 inducing activity were identified [48]. Topical treatment of zerumbone, from *Zingiber officinale* (ginger), increases mRNA of NQO1 on the skin of mice [49]. 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.

GST

GST conjugates glutathione (GSH) to electrophiles like *o*-quinones for detoxification (Figure 1, 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 [42]. An aqueous extract of licorice, administered *in vivo* to rats after CCl₄ reduction of GST protein levels, significantly increased GST levels in the liver [50]. 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 [44]. Raw ginger fed to rats was able to return levels of GST in the kidney to almost normal levels after treatment with doxorubicin [51], and it returned plasma levels of GST to near normal in a 1, 2-dimethylhydrazine-induced colon cancer rat model [52]. Two compounds, 11-angeloylsenkyunolide F and tokinolide B, from dong gui both inhibited purified GST [53]. 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 in 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.

Antioxidant—The conversion from the semiquinone to the quinone produces ROS (Figure 1, step 6). ROS have been implicated in many facets of breast cancer, including increasing genomic instability and altering redox sensitive transcription factors like NF- κ B and NRF1, which can ultimately lead to changes in cell growth, survival, transformation, invasion, and apoptosis [54].

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 [55], and of licorice using peroxynitrite assay [56], 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 [57,58]. Five compounds from fresh ginger exhibited DPPH radical scavenging activities similar to curcumin and quercetin, which are known botanical antioxidants [59]. 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 [60]. Extracts of dong gui and hops both exhibited weak antioxidant capabilities in the DPPH assay [46,48]. 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.

Conclusions

Women are taking botanical dietary supplements to ameliorate symptoms of menopause and to avoid traditional HT due to anxiety and fears related to increased risk of breast cancer associated with HT 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 ER α and ER β , leading to increasing cell proliferation *in vitro* and *in vivo* [61]. Notwithstanding this

body of knowledge, the effect of botanical supplements on estrogen chemical carcinogenesis is less known. Studies reviewed here have been performed with botanical extracts and their isolated compounds; results suggest that botanicals may have the potential to modify estrogen metabolism and impact estrogen carcinogenesis. The data examined thus far suggest that botanical extracts from hops and licorice may have the highest potential to reduce chemical estrogen carcinogenesis. The results for black cohosh, dong gui and ginger are less clear so far. Literature on red clover reveals that extracts might have the potential to both induce or reduce estrogen metabolism and carcinogenesis.

Research results that would appear contradictory as the red clover results reveal, 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.

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Figure 1.

Seven key steps in estradiol metabolism leading to increased cancer risk and how botanical compounds and/or extracts can influence the enzymes. 1) Inhibition of aromatase with licorice [20], hops [21,22], and mixed results with red clover [17,18]. 2) CYP1B1 inhibition with hops [29], red clover [30], and induction with black cohosh [27,28]. 3) Inhibition of CYP1A1 with hops [29] and an induction with black cohosh [27,28], licorice [36], while red clover has been shown to increase CYP1A1 [34] and decrease CYP1A1 [35]. 4) COMT has been inhibited by red clover [35,38]. 5) NQO1 was induced by black cohosh [40], licorice [43–45], hops [46,47], dong gui [48], ginger [49] with mixed results from red clover [35,41,42]. 6) GST was increased by licorice [44,45,50] and ginger [51,52] and inhibited by dong gui [53]. 7) ROS were scavenged by all the botanicals in this review; black cohosh [55], licorice [56–58], and ginger [59] with weak antioxidant activity from red clover [60], hops [46], and dong gui [48].

Table 1

	ER	ER	Reference
MCF-10A	Low	Low	[62]
MCF-7	+	Low	[62,63]
MDA-MB-453	–	–	[63,64]
MDA-MB-231	–	–	[63,65]
SK-BR-3	–	–	[63,66]

Table 2

Estrogen receptor (ER) status of the most commonly used cell lines in breast cancer research. Summary of literature on the six botanicals ability to influence the seven steps of the estrogen carcinogenesis pathway. Descriptions of experiments and conclusions can be found in the botanical modulation of estrogen carcinogenesis section.

	ER Selectivity	BIOACTIVATION		CYP1A1	DETOXIFICATION			GST	ANTIOXIDANT
		Aromatase	CYP1B1		COMT	NQO1			
Black Cohosh (<i>Cimicifuga racemosa</i>)	No effect [10]	No Effect [23]	[27,28]	[27,28]	?	[40]	?	?	Yes [55]
Red Clover (<i>Trifolium pretense</i>)	[12]	[17,18] [19]	[30]	[34] [35]	[35,38]	[41,42] [35]	No Effect [42]	Weak [60]	
Licorice (<i>Glycyrrhiza glabra</i>) (<i>Glycyrrhiza uralensis</i>)	[13,14]	[20]	?	[36]	?	[43-45]	[44,45,50]	Yes [56-58]	
Hops (<i>Humulus lupulus</i>)	[12]	[21,22]	[29]	[29]	No Effect [29]	[46,47]	?	Weak [46]	
Dong Gui (<i>Angelica sinensis</i>)	No effect [10]	?	?	?	?	[48]	[53]	Weak [48]	
Ginger (<i>Zingiber officinale</i>)	?	?	?	?	?	[49]	[51,52]	Yes [59]	