Estrogenic And Chemopreventive Properties of Licorice Species Used For Menopausal Symptoms

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DISSERTATION
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To my parents Hadi and Mansoureh whose relentless support and unconditional love has nurtured my enthusiasm for life and knowledge

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-A.H.

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LIST OF ABBREVIATIONS

AhR	Arylhydrocarbon receptor
Akt	Protein Kinase B gene
ANOVA	Analysis of variance
AP	Alkaline phosphatase
AP-1	Activator protein-1
ARE	Antioxidant response element
AUC	Area under the curve
BF	4'-Bromoflavone
BSA	Bovine serum albumin
BW	By weight
C	Chalcone
CD	Concentration doubling the activity
cDNA	Circular deoxyribonucleic acid
CI	Chemopreventive index
COX-2	Cyclooxygenase-2
CYP450	Cytochrome P450
DMSO	Dimethyl sulfoxide
DMX	Desmethylxanthohumol
DNA	Deoxyribonucleic acid
E_2	17β-Estradiol
EC_{50}	Concentration providing 50% of maximal activity
EDTA	Ethylenediaminetetraacetic acid
ER	Estrogen receptor
ERE	Estrogen responsive element
Erk	Extracellular-signal-regulated kinase gene

LIST OF ABBREVIATIONS (continued)

EtOH	Ethanol
F	Flavonone
FBS	Fetal bovine serum
G. glabra (GG)	*Glycyrrhiza glabra*
G. inflata (GI)	*Glycyrrhiza inflata*
Glc	Glycosylated
GPER or GPR30	G-protein coupled estrogen receptor
GREB1	Growth regulation by estrogen in breast cancer 1 gene
GSH	Glutathione
GST	Glutathione-S-transferase
G. uralensis (GU)	*Glycyrrhiza uralensis*
HAPS	Hydroxyapatite Slurry
H. lupulus (HL)	*Humulus lupulus* (hops)
HNMR	Proton nuclear magnetic resonance spectroscopy
HPLC	High performance liquid chromatography
HT	Hormone therapy
5-HT	5-Hydroxy tryptamine
IC_{50}	Concentration inhibiting 50% of maximal activity
iNOS	Inducible nitric oxide synthase
IT-ToF	Ion trap-time of flight
IX	Isoxanthohumol
JNK	c-Jun N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
Ki-67	The antigen associated with cellular proliferation
LC-MS	Liquid chromatography-Mass spectrometry

LIST OF ABBREVIATIONS (continued)

LC-UV	Liquid chromatography-Ultraviolet Spectrometry
LC_{50}	Concentration causing 50% of maximal toxicity
LD_{50}	Dose causing 50% of maximal toxicity
LicA	Licochalcone A
LigC	Isoliquiritigenin
LigF	Liquiritigenin
MAPK	Mitogen-activated protein kinase
MeOH	Methanol
MKK4	dual specificity mitogen activated protein kinase kinase 4
mRNA	messenger ribonucleic acid
MS-MS	Tandem mass spectrometry
MW	Molecular weight
ND	Below the limit of detection
NEAA	Non-essential amino acids
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NQO1	NAD(P)H:quinone oxidoreductase 1
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
p21	Cyclin-dependent kinase inhibitor 1
p53	Tumor protein p53
PBS	Phosphate buffer saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDA	Photo-diode array
pERE	Plasmid containing ERE sequence

LIST OF ABBREVIATIONS (continued)

PEG	Polyethylene glycol
PgR	Progesterone receptor gene
PI3K	Phosphatidylinositol 3-kinase
PK	Pharmacokinetic
6-PN	6-Prenylnaringenin
8-PN	8-Prenylnaringenin
pRL-TK	Plasmid containing renilla luciferase sequence
ProAB	Mutation in proline metabolism (E. Coli)
PUF	Pulsed ultrafiltration
qHNMR	Quantitative proton nuclear magnetic resonance
qRT-PCR	Quantitative real-time polymerase chain reaction
QTOF	Quadrupole time of flight
R^2	Regression
ROS	Reactive oxygen species
R,R-THC	(R,R)-5, 11-Diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol
S.C.	Subcutaneous
SD	Standard deviation
SERM	Selective estrogen receptor modulator
SERT	Serotonin transporters
SFN	Sulforaphane
SRM	Selected reaction monitoring
SSRI	Selective serotonin reuptake inhibitor
STR	Short tandem repeat
TBS	Tris-buffered saline

LIST OF ABBREVIATIONS (continued)

Tff1 (pS2)	Trefoil factor 1 gene
TRAIL	TNF-related apoptosis-inducing ligand
UHPLC	Ultrahigh performance liquid chromatography
WHI	Women's Health Initiative
XH	Xanthohumol
XRE	Xenobiotic response element

SUMMARY

With increased women's life expectancy, menopause and its associated complications are becoming a more prevalent public health issue. Physiological changes due to menopause are mainly related to the lower levels of estrogen in circulation which result in a number of symptoms such as hot flashes, sleep disturbances, mood swings, vaginal dryness, and osteoporosis to name a few. Among several traditional and scientific based approaches, hormone therapy medications have been very successful in relieving menopausal symptoms. However, more recent population studies such as Women's Health Initiative (WHI), Million Women Study, and E3N-EPIC cohort (the French component of the European Prospective Investigation into Cancer and Nutrition) suggested an increased breast cancer risk associated with the use of HT. Therefore, many women have turned to other possible remedies to alleviate menopausal symptoms.

Studies have suggested that Asian women have milder menopausal symptoms likely due to their dietary ingredients such as soy, which is known to have estrogen like constituents. Additionally, there are some traditional approaches such as herbal remedies to manage menopausal discomfort in societies with older histories such as China. Licorice species are among popular herbs for this purpose. Since dietary approaches and traditional medicine are inherently considered as "safe" by general public, many women choose to take them as alternative to HT to relieve menopausal symptoms. However, there are not enough evidence on the safety and efficacy of these remedies.

Another very important public health issue to menopausal women is their higher susceptibility to cancers compared to younger women. In addition to studies on the approaches to combat cancer, chemoprevention through changes in dietary intake and life style could be a great milestone if achieved. Botanical dietary supplements such as licorice that are regularly used by menopausal women as alternatives to HT for long periods of time might have the potential to serve as chemopreventive agents as well.

Licorice species are among the most popular herbal entities in traditional Chinese medicine. They are used alone or in combination with other herbs to treat several conditions such as wound healing, digestive problems, and menopausal symptoms. Licorice has been reported to have more than 30 species among which only three are medicinally approved. *Glycyrrhiza glabra* (GG) is the most popular licorice in western world followed by *Glycyrrhiza uralensis* (GU). *Glycyrrhiza inflata* (GI) is exclusively used in China and most other species are not well known. Different types of licorice originated from or cultivated in various regions of the world are genetically distinct and do not have the same chemical profiles. These differences could potentially influence the biological activity of licorice species.

The major phytoestrogen in all licorice species is liquiritigenin (LigF) which is mostly glycosylated in the plant. Its concentration is different among various types of licorice. All licorice species also contain different amounts of isoliquiritigenin (LigC), a precursor of LigF, which is a Michael acceptor and might have potential chemopreventive effects. Licochalcone A (LicA) is another Michael acceptor which is specific to GI. *The **hypothesis** of this project is that licorice species and their bioactive compounds have estrogenic and chemopreventive properties which could be beneficial to menopausal women.*

The role of the three licorice species GG, GU, and GI in modulating estrogenic and chemopreventive responses was evaluated, using *in vitro* and *in vivo* models. *Humulus lupulus* (hops) was used as a relevant positive control since it has been shown to have estrogenic potential and contains the most potent phytoestrogen, 8-prenylnaringenin (8-PN). Hops also exhibited chemopreventive effects such as the induction of detoxification enzymes which might be attributed to its predominant chalcone, xanthohumol (XH), a Michael acceptor.

Extracts of *Glycyrrhiza* species and spent hops induced estrogen responsive alkaline phosphatase activity in endometrial cancer cells, estrogen responsive element (ERE)-luciferase in MCF-7 cells, and *Tff1* mRNA in T47D cells. The estrogenic activity decreased in the

order hops > GU > GI > GG. Through bioassay guided fractionantion, LigF was found to be the principle phytoestrogen of the licorice extracts; however, it exhibited lower estrogenic effects compared to 8-PN from hops in functional assays. LigC, the precursor chalcone of LigF, also demonstrated significant estrogenic activities which could be the result of its cyclization to LigF under physiological conditions.

In vivo estrogenic studies with female immature Sprague-Dawley rats showed none of the licorice extracts or LigF increased uterine weight; however, LigF did suppress E_2 induced uterine proliferation. This observation might be associated with ERβ activity or partial agonist effects of LigF. Considering the estrogenic potential of licorice extracts especially GU and also LigF *in vitro* it is still quite possible that these treatments exert some estrogenic changes at molecular level that are not translated to increase in uterine weight over a 3 day treatment *in vivo*. Therefore looking into molecular markers such as PCNA and complement C3 as well as ki67 in uterine tissue in the future could better define the estrogenic status of licorice extracts and LigF *in vivo*. Considering the diverse chemical profiles of licorice species, presence of different glycosylated precursors of LigF, and the cyclization of LigC to LigF besides the biological activities of both compounds suggest the importance of precise labeling of botanical supplements.

It was previously shown that hops and its Michael acceptor XH induced the chemoprevention enzyme, NAD(P)H:quinone oxidoreductase 1 (NQO1), *in vitro* and *in vivo*. Licorice species could also induce NQO1, as they contain the Michael acceptors LigC found in GG, GU, and in GI, in addition to LicA which is only found in GI. These licorice species and hops induced NQO1 activity in murine hepatoma (Hepa1c1c7) cells; hops ≫ GI > GG ≅ GU. Similar to the known chemopreventive compounds curcumin (turmeric), sulforaphane (broccoli),

and XH, LigC and LicA were active dose-dependently; sulforaphane ≫ XH > LigC > LicA ≅ curcumin ≫ LigF. Induction of the antioxidant response element luciferase in human hepatoma (HepG2-ARE-C8) cells suggested involvement of the Keap1-Nrf2 pathway. GG, GU, and LigC also induced NQO1 in nontumorigenic breast epithelial MCF-10A cells.

In female Sprague–Dawley rats treated with GG and GU, LigC and LigF were detected in the liver and mammary gland. GG weakly enhanced NQO1 activity in the mammary tissue but not in the liver. Treatment with LigC alone did not induce NQO1 *in vivo* most likely due to its conversion to LigF, extensive metabolism, and its low bioavailability *in vivo*. These data show the chemopreventive potential of licorice species *in vitro* could be due to LigC and LicA and emphasize the importance of chemical and biological standardization of botanicals used as dietary supplements. Although the *in vivo* effects in the rat model after four-day treatment are minimal, it must be emphasized that menopausal women take these supplements for extended periods of time and long-term beneficial effects are quite possible. At the time of our *in vivo* studies well characterized authenticated GI extract was not available to us in enough quantities. Therefore, *in vivo* evaluation of this species will be conducted in the future.

In summary, menopausal complications and the way they are addressed are considerably important public health issues as they influence the quality of life for a significant number of women. The approaches undertaken need to be not only efficacious but also safe. Botanical dietary supplements as one of the alternative options to HT are not regulated by the Food and Drug Administration, therefore, they do not need to go through a rigorous chemical-biological and clinical evaluations prior to marketing. The public perception of these remedies as being “safe” does not urge women to consult with clinicians about using them. Additionally, even health care professionals might not find sufficient body of evidence supporting the efficacy and

safety of botanicals. Our findings in the current research suggest licorice has estrogenic potential primarily by LigF *in vitro*; however, due to the partial agonist effects and ERβ affinity observed with the extracts and LigF, they do not exhibit proliferative effects in the rat uterine tissue. Further, LigF suppresses the proliferative effects of E_2. These observations suggest a safer estrogenic activity with licorice and LigF *in vivo* compared to many other estrogen like agents. Additionally, the minimal but significant NQO1 induction by GG in breast tissue suggests the potential protective effects of this extract. Studies of this kind which closely look into the relevance of the chemical profile and the biological effects of botanicals are needed to clearly define the efficacy and safety of popular menopausal formulations.

1. Introduction

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1.1. Molecular biology of estrogen and estrogen receptors

The importance of estrogen in homeostatic regulation of many cellular and biochemical events is well illustrated by the pathophysiological changes that occur with estrogen deficiency (Ames et al. 2010; Pelekanou and Leclercq 2011; Pastore et al. 2012; Nofer 2012; Boonyaratanakornkit and Pateetin 2015; McGowan et al. 2015; Jia et al. 2015). Endogenous estrogen (estradiol, E_2) is actively involved in the development of the mammary gland and uterus, in maintaining pregnancy and bone density, and in protection from cardiovascular diseases (Krishnan et al. 2000; O'Donnell et al. 2007; Pastore et al. 2012; Nofer 2012). Estrogens mainly exert their biological effects through binding to estrogen receptors (ERs) including ERα and ERβ (Figure 1) (Leitman et al. 2010). These estrogen receptor subtypes have some similar ligands while they are capable of interacting whit quite different compounds. Although ERα and ERβ bind similar binding regions in DNA they can recruit distinct coactivators and generate different responses (Figure 1).

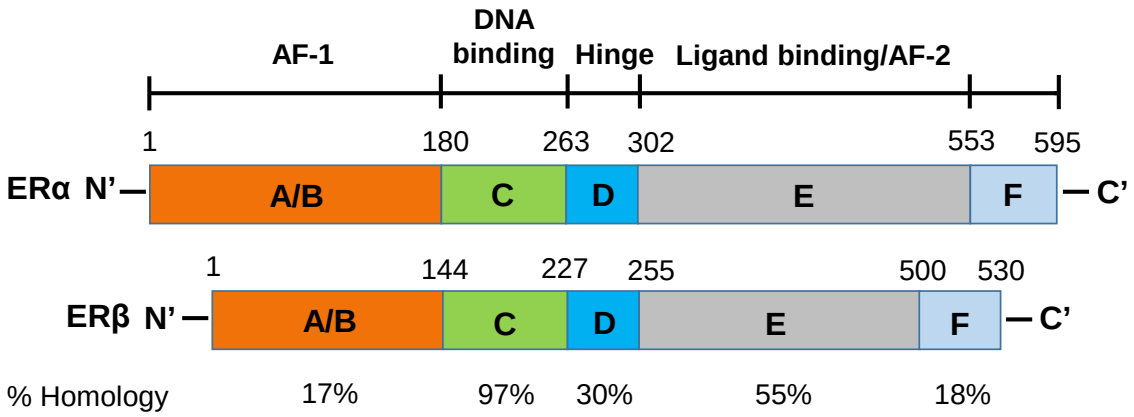


Figure 1: Homology of estrogen receptor (ER) α and ERβ.
While the DNA binding domains of the two receptors are nearly identical, their ligand binding domains containing the activation function (AF-2) and the A/B domain containing AF-1 are the least homologous regions (Leitman et al. 2010).

Upon binding a ligand to ERs, the receptors dimerize and interact with estrogen responsive elements (EREs) at the promoter of the estrogen responsive genes, thus activating transcription and generating estrogenic responses which are crucial for normal physiological functions (Figure 2) (Nilsson et al. 2001; Ellmann et al. 2009; Bjornstrom and Sjoberg 2005; Jia et al. 2015). It is noteworthy that in humans around one third of the genes that are regulated by ERs do not contain ERE-like sequences (Bjornstrom and Sjoberg 2005; O'Lone et al. 2004). ERs can also tether to other transcription factors such as Fos and Jun that are directly bound to DNA through their respective responsive elements such as activator protein-1 (AP1) binding sites to regulate transcription of the related genes (Figure 2) (Bjornstrom and Sjoberg 2005; O'Lone et al. 2004).

Estrogen also activates rapid signaling pathways such as mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase (PI3K) pathways, which, in turn, can modulate transcription and proliferation (Figure 2) (Song and Santen 2006; Bjornstrom and

Sjoberg 2005). Studies have revealed another type of ER, namely G-protein coupled estrogen receptor (GPER or GPR30) that is involved in different signaling pathways and plays a role in several health and disease conditions (Figure 2) (Filardo 2002; Prossnitz et al. 2008; Prossnitz and Hathaway 2015). It is also known that mechanisms of E_2 actions depend on the ligand, the cell type, and the receptor subtype (Bjornstrom and Sjoberg 2005; O'Lone et al. 2004). It is believed that ERα induction is responsible for the proliferative effects of estrogens while ERβ activation balances the ERα-dependent responses (Shanle and Xu 2010; Thomas and Gustafsson 2011; Deroo and Buensuceso 2010; Jia et al. 2015).

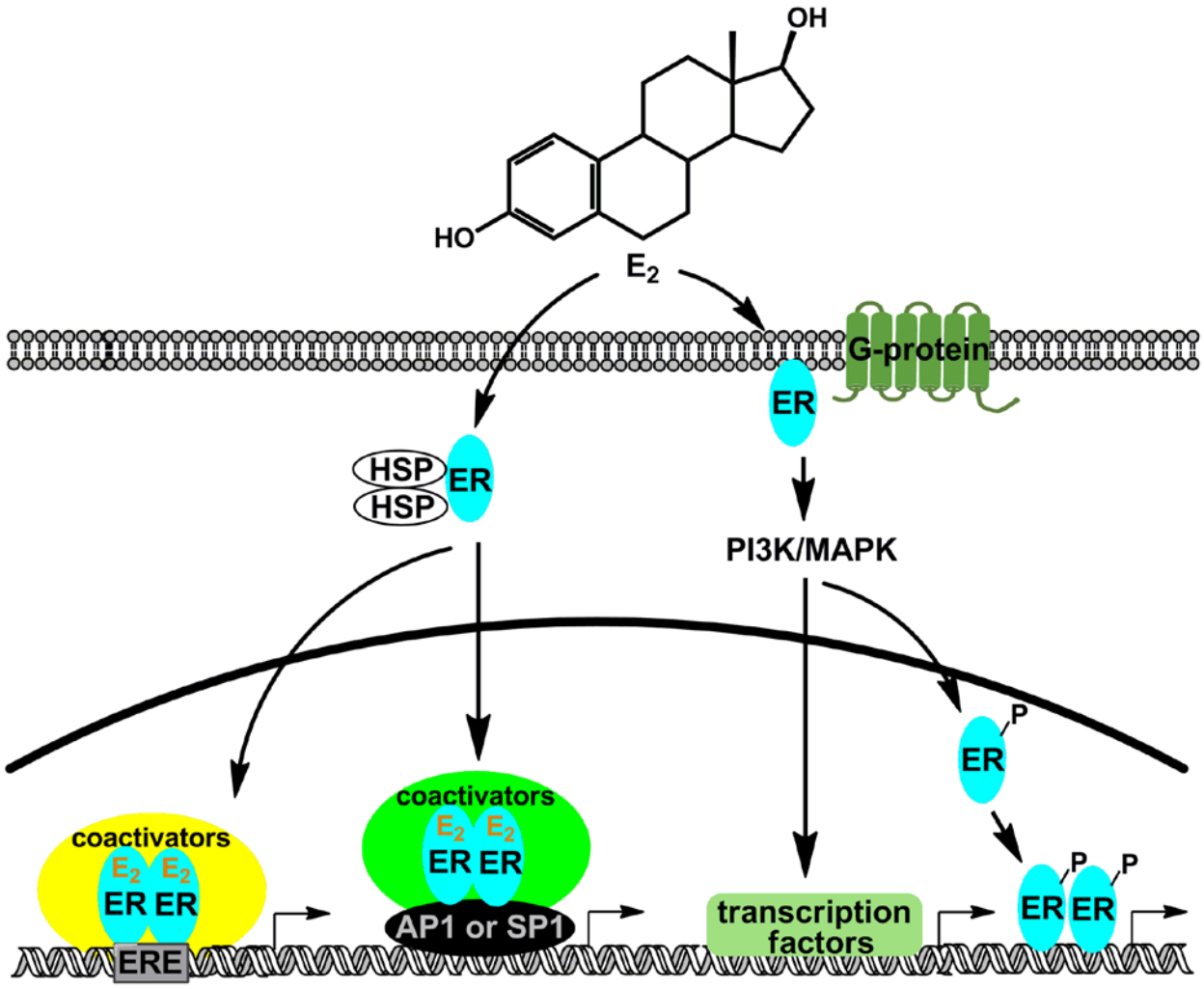


Figure 2: Classical Mechanisms of estrogen receptor activity.
Liganded estrogen receptor (ER) can interact directly with EREs to induce transcription of estrogen dependent genes. It may also tether to some transcription factors such as AP-1 and modulate gene transcription. G-protein coupled ERs or membrane associated ERs can indirectly modulate cell responses through activation of kinase pathways which might result in activation of certain transcription factors or phosphorylation of ERs and the subsequent gene transcriptions.

1.2. Menopause

Women potentially spend the last third of their lives in post menopause, due to their increased life expectancy. Menopause is mainly the state of a female's body when ovaries stop producing estrogen resulting in a significant drop in circulating levels of this hormone (Aidelsburger et al. 2012). While the local production of estrogen in organs such as breast might not be compromised, the drastic decline in circulating estrogen levels and its following events in different organs lead to the rise of a number of symptoms, such as hot flashes, night sweats, mood swings, insomnia, vaginal dryness, in addition to long-term complications such as osteoporosis in menopausal women (Roush 2012; Ortmann and Lattrich 2012).

1.3. Hormone therapy and rising safety concerns

Hormone therapy (HT) (estrogen or estrogen plus progestin) for a long time has been the treatment of choice for the alleviation of menopausal symptoms simply through supplementing the hormones. However, in May 2002 the estrogen plus progestin arm of the Women's Health Initiative (WHI) that was originally planned for 8.5 years to study the role of HT in preventing chronic heart diseases was halted after 5.2 years due to an increased risk of developing invasive breast cancer in healthy menopausal women (Rossouw et al. 2002). The data obtained in this large scale trial which was conducted throughout 40 US clinical centers raised major concerns about the safety of HT and prompted women to seek alternative options to relieve menopausal ailments. However, there were also other crucial information found through this study which were undervalued at the time due to the concerning results of the primary report. It showed a decrease in breast cancer incidence in post-menopausal women in estrogen alone arm with prior hysterectomy (Chlebowski et al. 2015; Anderson et al. 2004). It also suggested the timing of the treatment initiation is a key factor in defining the risk/benefit balance of HT for menopausal women (Rossouw et al. 2007; Lenfant et al. 2011). Women who had 10 years of menopause were found to be at a lower risk or even benefit from protective

effects of HT against coronary heart diseases compared to those with 20 years of menopause (Valera et al. 2015). Nevertheless, the safety of HT is not still quite clear and women are desparately looking for alternative options for the alleviation of menopausal symptoms.

1.4. Alternative treatment options

In order to avoid hormonal approaches, some women choose selective serotonin reuptake inhibitors (SSRIs) to manage menopausal discomforts, particularly vasomotor symptoms (Pearlstein 2012; Mintziori et al. 2015). It is known that estrogen withdrawal during menopause, results in a decline in the release of neurotransmitters, primarily norepinephrine and serotonin (5-hydroxy tryptamine; 5-HT) leading to a change in thermoregulation in the hypothalamus (Shanafelt et al. 2002). This effect ultimately results in frequent sweating and increased peripheral circulation as heat-loss mechanisms generating hot flashes and night sweats. Increase in the amount of serotonin and activating certain 5-HT receptors as well as inhibition of serotonin re-uptake in synapses through the blocking of serotonin transporters (SERTs) are possible approaches in preventing hot flashes (Mintziori et al. 2015). However, there are also a number of undesirable outcomes such as sexual dysfunction, nausea, weight gain, and sleep disturbances associated with these remedies (Nachtigall 2010; Kintscher 2012).

As an alternative to pharmaceutical treatments, a large number of menopausal women turn to natural alternatives such as botanical supplements because of their long history of use in traditional Chinese medicine and the common perception of them as being natural and therefore “safe” (Poluzzi et al. 2014; Geller and Studee 2005; Pitkin 2012; Wang et al. 2011). As of 2014 the sales of botanical dietary supplements including those used to address menopausal discomfort reached $6.4 billion in the United States which marked a 6.8% increase (Smith 2015). The fact that Asian women have less frequent and severe hot flashes suggests that this effect could be associated with their flavonoid-rich diet and that botanicals with high flavonoid

content could be effective in managing menopausal symptoms (Taechakraichana et al. 2002). Flavonoids in botanicals have chemical features of estrogen like compounds, thus might mimic the actions of estrogen; however, the estrogenicity of botanicals and their safety is not fully understood (Mintziori et al. 2015). Moreover, how botanicals might contribute in other wellness parameters such as chemoprevention in their target population is not still clear.

1.5. The role of botanicals in modulating estrogenic responses

Modulation of estrogenic responses has been studied for many popular botanicals such as soy, black cohosh, red clover, hops, and licorice, although their overall efficacy is controversial (Hajirahimkhan, Dietz, et al. 2013; Poluzzi et al. 2014; Mintziori et al. 2015). In this research project we have mainly focused on the medicinally approved licorice species and compared their biological effects to hops.

1.5.1. Humulus lupulus (Hops)

Humulus lupulus L. Cannabaceae is a popular botanical especially in Europe primarily used for its sleep inducing effects (Salter and Brownie 2010; Blumenthal and German Federal Institute for Drugs and Medical Devices. Commission E. 2000; Aidelsburger et al. 2012). It is also present in some dietary supplements for managing menopausal symptoms (Taylor 2012). Previous studies have suggested an estrogenic potential for hops (Hajirahimkhan, Dietz, et al. 2013; Karabin et al. 2015; Poluzzi et al. 2014). A moderate estrogenic activity for hops based on competitive ER binding activity, alkaline phosphatase induction, and *PgR* mRNA induction in Ishikawa cells has been reported (Liu et al. 2001). Overk et al. also showed estrogenic activity for a hops extract in competitive ER binding assays, ERE-luciferase induction in MCF-7 (ERα+) cells, *PgR* mRNA induction in MCF-7 and Ishikawa cells, and induction of alkaline phosphatase enzyme in Ishikawa cells (EC_{50} = 1 µg/mL) (Overk et al. 2005). 8-Prenylnaringenin (8-PN) has been reported as the estrogenic component of hops, equipotent for both ER subtypes, with an activity greater than that of any of the known phytoestrogens (Overk et al. 2005; Milligan et al.

2000; Roelens et al. 2006). Bovee et al.(Bovee et al. 2004) observed estrogenic activity of 8-PN in a yeast based ER-dependent reporter assay. In this study, the potencies of 8-PN for ERα and ERβ were 100 times and 3900 times less than that of estradiol, respectively. Milligan et al (Milligan et al. 2002) reported that 8-PN induced alkaline phosphatase in Ishikawa Var I cells (EC_{50} = 4.41 nM) and was active in a yeast based estrogenic assay.

Milligan et al. showed that administration of 8-PN (15.9 mg/kg/day, equivalent to 100 µg/mL) in the drinking water for 72 h increased vaginal mitosis in ovariectomized Swiss albino mice; however, they did not observe a significant increase in the uterine weight and the uterine mitosis response (Milligan et al. 2002). In contrast, Overk et al. (Overk et al. 2008) observed that 8-PN increased the uterine weight and the height of uterus luminal epithelial cells in Sprague-Dawley rats, significantly; however, an ethanolic extract of hops standardized to its active constituent, 8-PN, and its metabolic precursors, including isoxanthohumol, xanthohumol, and desmethylxanthohumol (Figure 3), did not induce uterotrophy, vaginal cell cornification, and changes in the height of uterus luminal epithelial cells. Similarly, an *in vivo* study by Diel et al. (Diel et al. 2004) in ovariectomized Wistar rats showed that subcutaneous administration of 8-PN (10 mg/kg/day) increased the uterine weight, the height of uterine epithelial, and the height of vaginal epithelial cells. Additionally, ERα and clusterin genes were down-regulated and complement C3 was up-regulated in the uterus, indicating estrogenic activity of 8-PN in this animal model (Diel et al. 2004).

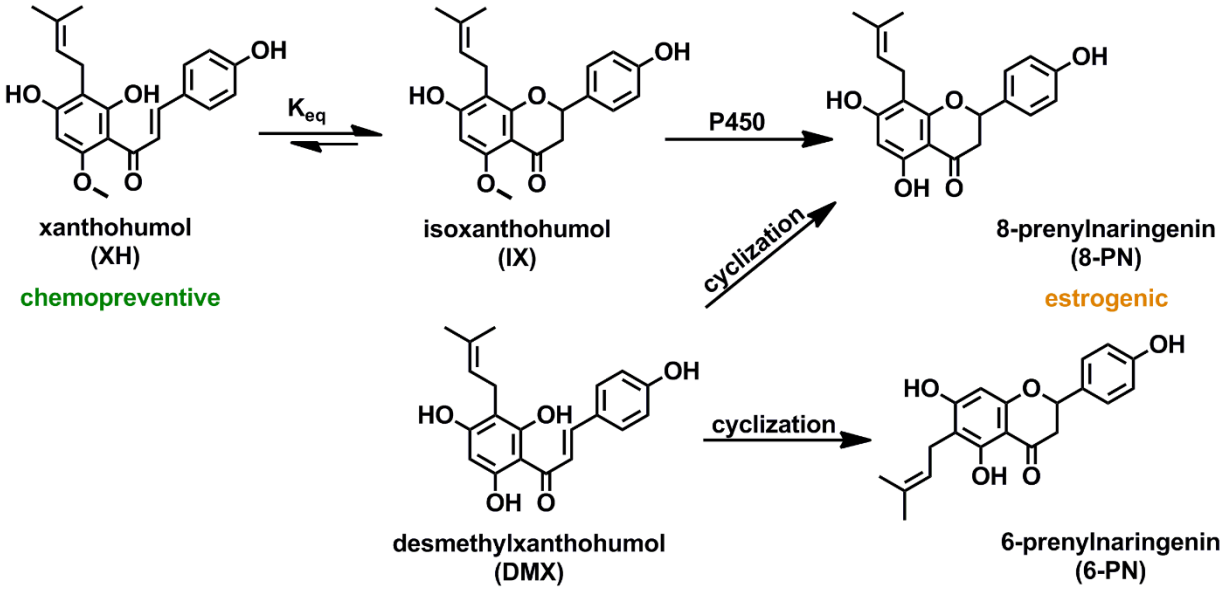


Figure 3: Chemical structures of estrogenic and chemopreventive compounds in hops. Estrogenic compound of hops, 8-PN forms from the chemopreventive compound, XH through metabolism.

Bolca et al. (Bolca et al. 2010) showed that disposition of 8-PN in the women's breast tissue, after hop supplementation for 5 days was associated with the dose and the metabolism of the precursor compounds. It is believed that the formation of the estrogenic compound of hops, 8-PN, is closely related to the metabolism of its precursor, isoxanthohumol (Figure 3), by intestinal microbiota and therefore, subjects with varied microflora could experience different biological outcomes upon hops administration (Bolca et al. 2010; Possemiers et al. 2008; Bolca et al. 2007; Possemiers et al. 2006; Possemiers et al. 2005).

The difference in uterotrophic effects and vaginal histology between hops and its active compound, 8-PN, could be related to the metabolism factor and/or to the other components of hops as an extract. Hops might contain natural progestins which could counteract the estrogenic effects of 8-PN (Toh et al. 2012). Moreover, uterotrophy, and vaginal cell histology are not the

only measures of estrogenicity. Hops as an estrogenic extract might have more pronounced estrogenic effects in other target tissues, such as bone, cardiovascular, and brain which were not evaluated in these studies. In summary, hops flavonoid, 8-PN, is the most potent phytoestrogen known to date and is equipotent for ER subtypes. Since its formation depends on the metabolism of its precursors in hops, the estrogenic activity of hops extract might vary between different subjects depending on their metabolism characteristics. We have used hops and its active constituents in this research for the purpose of comparison.

1.5.2. *Glycyrrhiza* species (licorice)

Glycyrrhiza species is a widely used plant for various purposes. It has more than 30 different species and is a very popular botanical in traditional medicine for different conditions such as digestive problems and wound healing (Shibata 2000; Messier et al. 2012; Asl and Hosseinzadeh 2008; Kao et al. 2014). It is also commonly used as a natural sweetener in the food industry and as a flavoring agent in toothpastes and cigarettes (Liu et al. 2000). Licorice species are also frequently found as components of popular menopausal formulations (Taylor 2012; Nahidi et al. 2012). The United States Pharmacopeia recognizes only two species *Glycyrrhiza glabra* (GG) and *G. uralensis* (GU) as source of licorice botanicals and the European Medicines Agency also considers *Glycyrrhiza inflata* (GI) being a legitimate source plant with medicinal properties (Simmler 2015). Despite the similarities observed in the morphology of the roots of these *Glycyrrhiza* species, they are known to exhibit marked chemical differences, which have been shown to ultimately lead to variation in their biological activities (Farag et al. 2012; Simmler 2015; Hajirahimkhan, Simmler, et al. 2013; Dunlap 2015).

However, such significant differences are often overlooked in botanical research and in the dietary supplement industry (Kondo, Shiba, Yamaji, et al. 2007; Kondo, Shiba, Nakamura, et al. 2007). Besides glycyrrhizin, a triterpene saponin responsible for the sweet taste of licorice, the major constituents found in all three *Glycyrrhiza* species are glycosides of the flavanone

liquiritigenin (LigF, Figure 4) and its chalcone isomer isoliquiritigenin (LigC, Figure 4). Chemical and DNA finger print analysis of various licorice source materials have shown that GU contains the highest levels of LigF amongst the three medicinally used *Glycyrrhiza* species (Table 1) (Kondo, Shiba, Yamaji, et al. 2007; Simmler 2015). It was also shown that the marker compounds of GG, GU, and GI are glabridin (Figure 4), glycocoumarin, and licochalcone A (Figure 4), respectively *Kondo* 2007; Simmler 2015).

The estrogenic activities of different licorice species and extracts are not the same. For example, Liu et al. did not observe any estrogenic effects with the methanolic extract of GG when tested in the competitive ER binding assay, alkaline phosphatase induction in Ishikawa cells, *Tff1* mRNA induction in S30 cells, and *PgR* mRNA induction in Ishikawa cells (Liu et al. 2001). However, Dong et al. showed that the boiling water extract of GG stimulated MCF-7 (ERα+) cell growth at concentrations of 0.1 - 10 µg/mL and enhanced ProAB/luciferase activity in the same cell line at a range of 1 - 10 µg/mL, which was comparable to the estradiol effect at 10 nM (Dong et al. 2007). In this study, the induction of estrogen responsive genes and the activation of rapid signaling pathways trough *Erk1/2* and *Akt* in proliferation of MCF-7 (ERα+) cells was observed at 10 µg/mL of the extract, demonstrating the role of this extract in activating the non-classical mechanism of estrogenic activity (Dong et al. 2007). Simons et al. also observed estrogenic activity for several fractions of an ethyl acetate extract of GG in the yeast based estrogenic assays (Simons et al. 2011). The activity of some fractions was abolished in the presence of either RU58668, selective ERα antagonist, or (*R*,*R*)-5,11-Diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol (*R,R*-THC), selective ERβ antagonist, demonstrating the ER mediated estrogenic effects. The difference between the outcomes of these studies could be associated with using different extracts and concentrations tested as well as the various sources of the plant species.

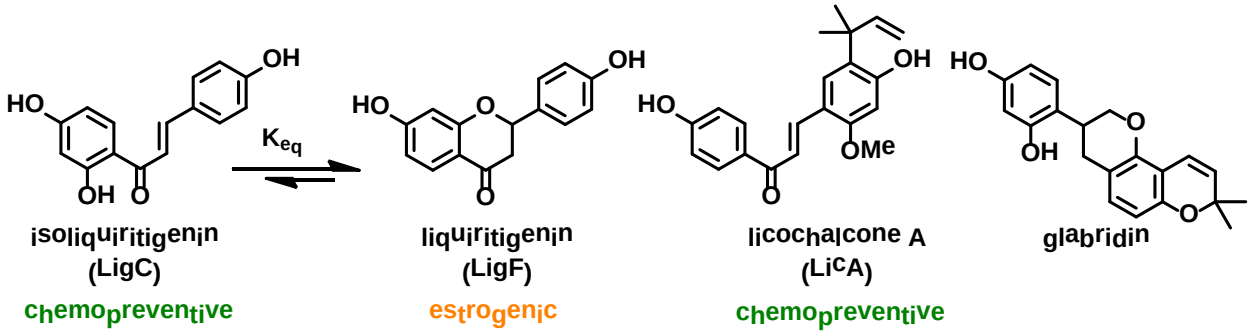


Figure 4: Chemical structures of estrogenic and chemopreventive compounds in licorice species.
LigF, the estrogenic compound of licorice forms through cyclization of the chemopreventive compound, LigC. LicA is the marker compound of GI while glabridin is the marker entity of GG.

Simons et al. also showed that glabrene-rich fractions of GG extract were more estrogenic with higher potency for ERα while glabridin had antiestrogenic properties (Simons et al. 2011). However, Tamir et al. showed that glabridin bound to ER in T47D cell extract (IC_{50}: 5 µM), stimulated ER-dependent cell growth at concentrations lower than 10 µM, and inhibited cell growth at concentrations higher than 15 µM in an ER-independent manner (Tamir et al. 2000). They also observed increased activation of creatine kinase, a marker of estrogenic activity, in female rat uterus, epiphyseal cartilage, diaphyseal bone, and cardiovascular tissues as well as an increased uterine weight effect comparable to that of E_2. Similarly, Somjen et al. showed that glabridin better than glabrene activated creatine kinase, in cultured female human bone cells as well as in female rat skeletal tissues (Somjen, Katzburg, et al. 2004). They also reported the estrogenic activity of glabrene and glabridin in vascular tissues *in vitro* and *in vivo*, with glabrene having selective estrogen receptor modulating (SERM)-like effects (Somjen, Knoll, et al. 2004).

GU was also reported to be estrogenic in yeast-based estrogen receptor activity assays, but the reported activities from different studies were not the same, indicating the lack of a unified standardized extract (Kang et al. 2006; Kim et al. 2008). GU extract was reported to stimulate MCF-7 (ERα+) cell growth at concentrations of 10 - 100 µg/mL with the maximal growth stimulation comparable to that of estradiol at 1 nM (Hu et al. 2009). Cell cycle analysis indicated an increased population of cells in S phase and western blots showed increased PCNA levels in response to proliferative concentrations of the extract, confirming an enhanced cell growth (Hu et al. 2009). They also demonstrated reduced levels of ERα protein as a marker of estrogenicity and a dose-dependent induction of *pS2* (*Tff1*) and *GREB1* mRNA (Hu et al. 2009). These data showed ERα-dependent estrogenic effects by the GU extract. In contrast, an undefined licorice extract was reported to have no proliferative effects in MCF-7 (ERα+) cells and no uterotrophic effects in animal models, but possessed ERβ selectivity in ERE-luciferase induction in transfected HeLa cells (Amato et al. 2002). The contradictory results observed among several studies could be associated with using different extracts and varying source materials which demonstrate the importance of having well-defined standardized licorice extracts.

Among the compounds isolated from licorice, LigF which has a higher availability through GU was reported to be a highly selective ERβ agonist in the ER binding assay and ERβ-ERE-luciferase induction assay in U2OS cells (Mersereau et al. 2008). This flavonoid did not enhance proliferation of MCF-7 (ERα+) xenograft or induction of uterine weight in nude mice, confirming its better potency for ERβ and the corresponding pathways (Mersereau et al. 2008). LigC, the precursor chalcone of LigF (Figure 4), was reported to have estrogenic effects (Maggiolini et al. 2002). However, the observed effects could be associated with the conversion of LigC to LigF under physiological conditions (Simmler et al. 2013). Selective estrogen receptor modulator (SERM)-like activities of LigC and LigF have been also reported in an array of cell

based assays (Boonmuen et al. 2016). This information might lead to the perception that based on the higher levels of LigF in GU, this extract might have higher tendency to activate ERβ dependent signals. However, activation of ERα-dependent responses such as increased proliferation markers could also be observed in some tissues and/or at higher concentrations.(Dietz 2013 ; Kuiper et al. 1997)

Evaluation of the literature has shown that most of the biological activity studies have been performed with GG, while other species of licorice, especially GU and GI (which are medicinally approved) are neglected. Since the genetics and the chemical profiles of these species are different one cannot generalize the observation from one species to others and our findings have clearly shown the significant difference between these medicinal species of licorice. Additionally, it is crucial to have a simultaneous chemical and biological analysis of licorice species, since the global chemical composition of each extract has a significant role in defining the ultimate biological response.

In summary, the most common licorice species in dietary supplements is GG which contains glabridin and glabrene in addition to LigF, while GU contains the highest amount of LigF and GI is less common in dietary supplements. The majority of LigF and LigC are in the form of glycosides in the extracts which may convert to free compounds *in vivo* and enhance the biological effects, although the levels and the types of glycosides are different among the species.(Simmler 2015). The glycosides of LigF in GU have simpler structures and are easier to hydrolyze (Simmler 2015). LigC easily converts to LigF under physiological conditions which in part influences the ultimate response (Simmler et al. 2013). Understanding the relevance of the chemical profiles of licorice species to their estrogenic potential, facilitates the selection and standardization of the more beneficial species for managing menopausal symptoms.

1.6. Chemoprevention

As women age to menopause their chances of developing cancers, especially breast cancer increases (Harman 2006; Rebbeck et al. 2007; Perry et al. 2015). Chemoprevention is an active approach to block, delay, or reverse carcinogenesis processes through diet and life style as well as medicines (Kwon et al. 2007; Penny and Wallace 2015). Botanical dietary supplements are in the daily diet of many menopausal women as a convenient and “safe” option for relieving menopausal symptoms (Hajirahimkhan, Dietz, et al. 2013). Therefore, understanding the chemopreventive potential of these remedies could be important for women’s health.

It is believed that modulation of certain pathways in living cells such as Nrf2 signaling and the subsequent induction of detoxifying phase II metabolism enzymes including NAD(P)H:quinone oxidoreductase 1 (NQO1), modulation of NF-kB and AP-1 signaling, MAPK signaling, and inflammatory mediator (cytokines)-related pathways might contribute to chemoprevention (Kwon et al. 2007; Ko and Moon 2015). One approach to select the biological target for studying the chemopreventive potential of agents is to consider their chemical structures and the relevance to the activation of certain biological pathways.

For example, presence of electrophilic compounds or Michael acceptors in botanicals may suggest the activation of Keap1-Nrf2 pathway through the modification of Keap1 cystein residues by dietary agents (Figure 5) (Eggler et al. 2008). This chemical interaction in part may activate the translocation of Nrf2 to the nucleus and its interaction with antioxidant response elements at the promoter region of the detoxification enzyme genes (Eggler et al. 2008; Yates and Kensler 2007; Wakabayashi et al. 2010; Kwak and Kensler 2010; Chartoumpekis et al. 2015). The subsequent induction of detoxification enzymes, such as NQO1 play a role in preventing diseases such as cancer, cardiovascular problems, and neurological disorders (Ungvari et al. 2011; Zhu et al. 2014). Since licorice and hops contain electrophilic compounds such as LigC and XH, they could serve as interesting candidates among menopausal botanicals

for the evaluation of their chemopreventive potential, particularly for their role in activating detoxification enzymes.

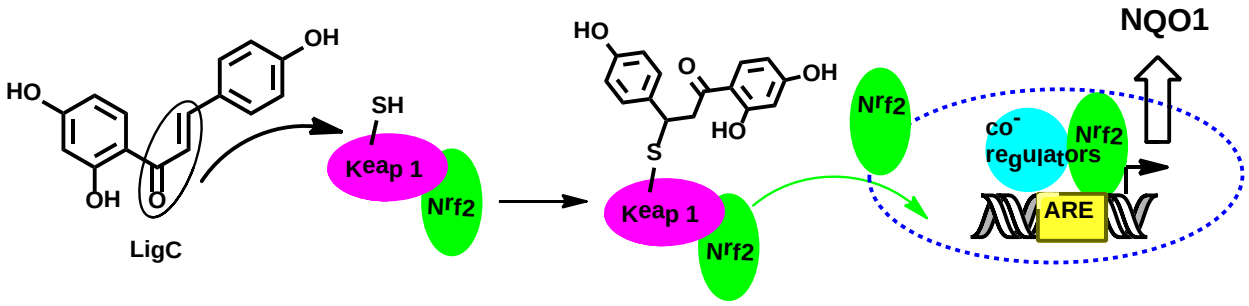


Figure 5: Induction of NQO1 through activation of Keap1-Nrf2 pathway. Electrophilic compounds such as Michael acceptors from botanicals can alkylate cysteine residues of the sensory protein Keap1. This interaction changes the conformation of Keap1 and this protein is no longer able to facilitateNrf2 ubiquitination and degradation. Therefore, Nrf2 levels increase in the nucleus and it interacts with its target sequence ARE at the promoter of certain cytoprotective genes. This interaction could result in the induction of detoxification enzymes such as NQO1, which are responsible for eliminating oxidative metabolites.

1.6.1. Hops

Hops and its constituents have been reported to have chemopreventive effects at different stages of carcinogenesis (Gerhauser et al. 2002; Gerhauser 2005; Dietz et al. 2013). Hops extract and its major Michael acceptor, xanthohumol (XH, Figure 3) induce detoxification enzymes in hepatoma cells and in rat liver likely through modification of cysteine residues of Keap1 and activation of Nrf2 signaling (Figure 5) (Dietz et al. 2005; Dietz et al. 2013). It was also reported that XH increased the expression and activation of Nrf2 in normal hepatocytes and hepatoma cells leading to the activation of glutathione-S-transferase (GST) in both normal and cancer cell lines and NQO1 and p53 in hepatocytes (Krajka-Kuzniak et al. 2013).

Considering other possible mechanisms of chemoprevention, lupulones from hops were reported to have chemopreventive effects against human colon cancer development in a rat model, likely through apoptotic mechanisms (Lamy et al. 2007). Blanquer-Rossello et al. reported that XH from hops at low doses reduced reactive oxygen species production and enhanced mitochondrial oxidative phosphorylation in MCF-7 cells (Blanquer-Rossello et al. 2013). Strathmann et al. demonstrated the pro-apoptotic effect of XH through an interruption in mitochondrial membrane potential and the release of cytochrome C (Strathmann et al. 2010). XH has been reported to block vascular tumor angiogenesis through the suppression of NF-kB and Akt pathways in endothelial cells (Albini et al. 2006).

1.6.2. Licorice

Licorice extracts and bioactive compounds such as LigC (Figure 4) and LicA (Figure 4) have been reported to induce chemopreventive responses through activation of apoptotic pathways, anti-inflammatory effects, and inhibition of oxidative estrogen metabolism (Webb et al. 1992; Takahashi et al. 2004; Jo et al. 2004; Tsai et al. 2014; Dunlap 2015; Wu et al. 2011; Cuendet et al. 2006; Tang et al. 2015; Peng et al. 2015). For example, GU was reported to exert antiproliferative effects in MCF-7 breast cancer cells via the modulation of apoptotic regulators and tumor suppressor pathways (Jo et al. 2004; Jo et al. 2005). It has also been shown that GU induces Nrf2-mediated genes in hepatoma cells and animal tissues (Wu et al. 2011).

LigC, the common chalcone in the three medicinally used licorice (GG GU, GI) extracts was demonstrated to have anti proliferative properties in lung cancer cells via the induction of cell cycle arrest and p21 expression (Ii et al. 2004). Induction of the cell cycle arrest by LigC has been reported in prostate cancer cells as well (Lee et al. 2009). LigC was also shown to modulate COX-2 and iNOS and reduce prostaglandin E-2 and nitric oxide levels and also exert antiproliferative chemopreventive effects *in vitro* and *in vivo* (Takahashi et al. 2004; Lau et al. 2010). LigC has been shown to block colitis-associated tumorigenesis in animal models after 12 weeks of treatment through the suppression of prostaglandin E-2 and interlukine-6 (Zhao et al.

2014). It was also shown that LigC might inhibit breast cancer growth and the associated angiogenesis with an increase in apoptosis (Wang et al. 2013).

Similar to the chalcone XH from hops, LigC is a Michael acceptor with the potential to covalently modify cellular proteins resulting in modulation of biological pathways such as Nrf2 signaling and NQO1 induction (Liu et al. 2005; Eggler et al. 2008; Cuendet et al. 2006). Cuendet et al. reported LigC enhanced NQO1 activity *in vitro* and *in vivo*; however, breast tumor growth was not blocked. This specific observation with LigC could be associated with its conversion to the estrogenic compound LigF as discussed earlier and also to the low bioavailability of LigC *in vivo* (Cuendet et al. 2010; Cuendet et al. 2006; Simmler et al. 2013; Hajirahimkhan, Simmler, et al. 2013).

GI contains a specific marker, LicA which is also a Michael acceptor with an α,β-unsaturated carbonyl (Figure 4) and most likely capable of modulating Keap1-Nrf2 pathway (Kondo, Shiba, Nakamura, et al. 2007; Simmler 2015). LicA has been reported to block estrogen chemical carcinogenesis through inhibition of arylhydrocarbon receptor (AhR) pathways (Dunlap 2015). It was also reported that LicA activated apoptosis in pharyngeal squamous carcinoma cells and reduced the levels of anti-apoptotic mediators, likely though MAPK signaling via ERK1/2 and p38 (Park et al. 2015). It also suppressed the growth of tumor cells xenograft in an animal model through the activation of caspase 3 (Park et al. 2015). LicA also inhibited hepatocellular cancer cell migration and invasion through the modulation of MKK4/JNK and NF-kB pathways, suggesting an anti metastatic effect (Tsai et al. 2014). LicA was reported to sensitize tumor cells to TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-induced apoptosis, which is believed to be highly compromised in many cancers (Szliszka et al. 2010; Szliszka et al. 2012). To our knowledge, despite the frequent reports on the effects of licorice and bioactive compounds on apoptosis related pathways, authenticated medicinally approved licorice species and particularly GI and its marker compound LicA have been rarely studied for modulation of detoxification enzymes. Additionally, the levels of LigC and

the total chalcone contents of different species of licorice is not similar and that could also impact the chemopreventive potential of various species. It is of great importance to emphasize that so far the variety among licorice species has been widely neglected and there is a need to do a simultaneous chemical and biological analysis of different species (Hajirahimkhan, Simmler, et al. 2013; Dunlap 2015). With these types of systematic analyses it would be plausible to standardize botanicals on certain compounds to meet the desired biological outcomes.

In the current research, a parallel analysis of three authenticated licorice species suggests that GG, GU, and GI can activate NQO1 *in vitro* through Keap1-Nrf2 pathway. The active constituents were LigC and LicA, both electrophilic chalcones capable of modulating Keap1 and activating ARE at the promoter region of NQO1. However, LicA might be also antagonizing AhR which could have a suppressing effect on XRE elements close to the AREs at the promoter of NQO1. This dual effect observed with LicA resulted in a less pronounced NQO1 induction by this compound compared to LigC. LigF and LigC were detectable in the liver and mammary gland of mature female rats treated with GG; however, significant NQO1 induction was only observed in the mammary gland. Our findings showed a mechanistic difference between the Michael acceptors in licorice and how that could impact the overall biological response. Additionally it demonstrated the necessity of standardization and chemical/biological characterization of licorice supplements for the desired biological effect.

This project is based on the hypothesis that licorice species and their bioactive compounds have estrogenic and chemopreventive properties as shown in Figure 6. The following aims were pursued:

Aim 1. Licorice species and their bioactive compounds LigC and LigF have varied estrogenic activities *in vitro*. (Chapter 3)

Aim 2. Licorice species and their bioactive constituents LigC and LicA can induce chemopreventive responses through the induction of NAD(P)H:quinone oxidoreductase enzyme *in vitro*. (Chapter 4)

Aim 3. Licorice species can induce estrogenic and chemopreventive responses in the respective preclinical animal models. (Chapter 3&4)

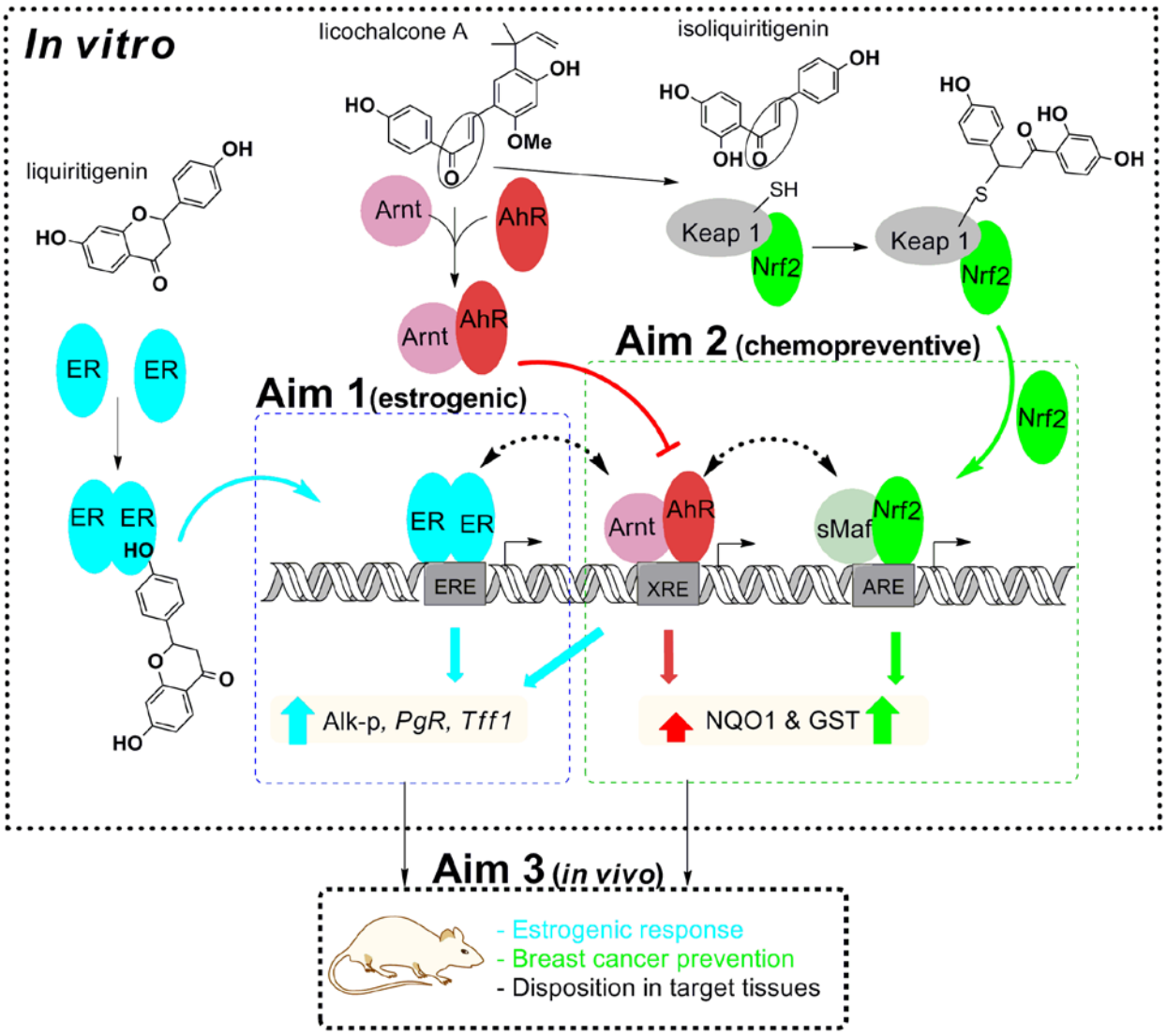


Figure 6. Outline of specific aims.
Licorice contains chemical entities such as liquiritigenin (LigF) and isoliquiritigenin (LigC) which can activate different cellular responses.

2. Materials and methods

2.1. Chemicals and Reagents

All chemicals and reagents were purchased from Fisher (Hanover Park, IL) or Sigma-Aldrich (St. Louis, MO), unless otherwise indicated. All media for cell culture and human recombinant ERα and ERβ were purchased from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). LigF and LigC were purchased from ChromaDex (Irvine, CA). Licochalcone A (LicA), glabridin, 7-hydroxyflavone, and 18*β*-glycyrrhetinic acid were purchased from Sigma-Aldrich (St. Louis, MO). Liquiritin, isoliquiritin, liquiritin apioside, isoliquiritigenin apioside and licuraside were isolated from licorice extract (Simmler, Jones, et al. 2014). 8-PN was synthesized (Overk et al. 2008) and XH was isolated from *H. lupulus* as described previously (Chadwick et al. 2004). Sulforaphane was obtained from Cayman Chemical (Ann Arbor, MI). Curcumin was purchased from Fluka and 4'-bromoflavone (BF) from Santa Cruz Biotechnology (Dallas, TX). LC-MS-grade acetonitrile and methanol were purchased from Thermo Fisher (Fair Lawn, NJ).

2.1.1. Purity control of tested compounds

The purity and identity of all four tested chalcones/flavanones isomers, LigF/LigC and 8-PN/XH, were rigorously determined by orthogonal and complementary techniques using high resolution tandem mass spectrometric analysis (Waters Synapt QToF mass spectrometer) and quantitative ^{1}HNMR (qHNMR; spectra acquired at 298 K, using a 90° pulse experiment, on a Bruker Avance 600.13 MHz, equipped with a 5 mm TXI cryoprobe). The purity of each investigated compound was determined by quantitative 1D ^{1}H NMR using the 100% method (Pauli et al. 2014) and yielded the following purity percentages (in % w/w): LicA 96.1% (ratio *trans/cis* = 93/7), LigF 96.6%, LigC 98.6%, glabridin 99.4%, 8-PN 95.0%, and XH 96.5%.

2.1.2. Plant material, extraction, and characterization

Pelletized strobili of *Humulus lupulus* cv. Nugget were bulk extracted with food-grade ethanol. The fluid extract was dispersed in diatomaceous earth, dried, and bulk extracted with supercritical CO_2 to yield two materials: the bitter acid extract (not used in this study) and the spent hop extract dispersed on the diatomaceous earth was used here. The spent hop extract was free of bitter acids. In preparation of the present experiments, the diatomaceous earth was removed by solubilization in methanol, filtration, and evaporation to dryness en vacuo. Quantitative LC-MS-MS analysis using authentic reference compounds as calibrants revealed that the spent hop extract contained 5.4% XH, 0.084% 8-PN, 0.076% 6-PN, and 0.65% IX (w/w % of the spent hops extract).

Samples of dried root materials of *Glycyrrhiza glabra* L. and *Glycyrrhiza. uralensis* Fisch. ex DC. (Leguminosaea/ Fabaceae) were purchased from Indiana Botanical Gardens and from a local supplier at China Town (Chicago, IL), respectively. *Glycyrrhiza inflata* Batalin, a gift from Dr. Liang Zhao, Lanzhou Institute of Chemical Physics, was collected in Xinjiang province, China in 2008. A Botanical Center number was attributed to each sample which was identified through a series of macroscopic and microscopic analyses as well as DNA authentication compared to authentic voucher samples deposited at the Chicago Field Museum (Kondo, Shiba, Yamaji, et al. 2007; Simmler 2015; Hajirahimkhan, Simmler, et al. 2013; Dunlap 2015).

For the *in vitro* assays, powdered roots from each of the three *Glycyrrhiza* species were exhaustively extracted by percolation with 100% methanol (MeOH, weight powder/volume of solvent: 1/20) at room temperature. Each extract was freeze-dried (mean extraction yield of 25% w/w (weight of extract/weight of root powder), and stored at -20 ˚C prior to any chemical or biological analysis. These crude extracts were compared and characterized through a combination of chromatographic techniques (High Performance Thin Layer Chromatography, High Performance Liquid Chromatography (HPLC) coupled with a photo-diode array (PDA)

detector) and qHNMR analysis in order to obtain their characteristic chemical fingerprint. The marker compounds, LigF and LigC, as well as their glycosylated derivatives, liquiritin and isoliquiritin, were quantified in each *Glycyrrhiza* extract (10 mg/mL in MeOH HPLC grade) by UHPLC on an Acquity BEH C18 column (50 × 2.1 mm, 1.7 *μ*m) with PDA detection at 275 nm for the flavanones (LigF and liquiritin) and 360 nm for the chalcones (LigC and isoliquiritin). Samples (1 *μ*L injected) were eluted at 0.3 mL/min using the following gradient composition (A) H_2O + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid starting from 18% B during 2 min, to 30% B in 8 min and during 2 min, to 57% B at 17 min and during 1 min, and finally to 95% B at 22 min and during 3 min. Under these conditions, the retention time was 5.09 min for liquiritin, 10.56 min for liquiritigenin, 11.12 min for isoliquiritin, and 17.24 min for LigC. Linear regression equations were used to calculate the concentrations of LigC, LigF, Liquiritin and isoliquiritin (in mg/mL) in each extract. The calibration curves were corrected according to the purity of each standard as determined by qHNMR (100% method).

For the *in vivo* studies, powdered roots from *Glycyrrhiza* species were extracted by maceration at room temperature with a solvent mixture composed of ethanol (200 USP proof), isopropranol, and water (90:5:5, v/v) and a plant powder/volume of solvent ratio of 1/15. This extraction procedure optimized the yield concentration of bioactive phenolic constituents, while reducing the extraction of primary metabolites and glycyrrhizin. The chemical equivalence between the *in vivo* and *in vitro* extracts was checked through a combination of Ultra High Pressure Liquid Chromatography (UHPLC) coupled with a photo-diode array (PDA) detector and qHNMR analyses in order to obtain characteristic chemical fingerprints and determine the concentration of bioactive compounds (Figure S4) (Simmler, Nikolic, et al. 2014; Simmler, Jones, et al. 2014; Simmler 2015). The marker compounds glabridin and LicA, as well as LigF, LigC, and their glycosides (liquiritin, liquiritigenin 7-*O*-apiosylglucoside, liquiritin apioside, isoliquiritin, isoliquiritin apioside and licuraside) in addition to glycyrrhizin were quantified in each

Glycyrrhiza extract by UHPLC as described previously (Simmler 2015). The areas under the curve (AUC) were taken at 360 nm for all chalcones, at 275 nm for all flavanones and at 254 nm for glycyrrhizin. Each *Glycyrrhiza* extract was examined in duplicate. Quantitative results obtained for each LigF glycoside were corrected by a factor corresponding to [molecular weight (MW) of LigF]/[MW of LigF glycoside], thereby leading to their concentration as LigF equivalents (Simmler 2015). The same methodology was applied for the quantitative results obtained for each LigC glycoside.

2.2. Cell culture

The Ishikawa cell line was provided by Dr. R. B. Hochberg (Yale University, New Haven, CT) and was maintained in Dulbecco's Modified Eagle Medium (DMEM/F12) containing 1% sodium pyruvate, 1% nonessential amino acids (NEAA), 1% glutamax-1, 0.05% insulin, and 10% heat-inactivated FBS (Littlefield et al. 1990; Hata et al. 1987; Pisha and Pezzuto 1997). The Ishikawa cell line is a well-established ERα (+) endometrial cancer cell line for the evaluation of estrogens and antiestrogens (Pisha and Pezzuto 1997; Littlefield et al. 1990). Two days before treating the cells, the medium was replaced with phenol red-free DMEM/F12 medium containing charcoal/dextran-stripped FBS and supplements. Authentication of this cell line, via determination of the short tandem repeat (STR) profile (Gherezghiher et al. 2012) revealed its similarity with the Ishikawa cells according to the Health Protection Agency Culture Collection in the UK and also with the ECC-1 cells from the American Tissue Culture Collection, ATCC database (Manassas, VA). However, alkaline phosphatase was not inducible in ECC1 cells obtained from ATCC. Despite this controversy, we will keep the conventional name, Ishikawa, for this cell line throughout this paper. The MCF-7 cell line was purchased from ATCC. MCF-7 cells were grown in RPMI 1640 media containing 1% glutamax-1, 1% NEAA, 0.05% insulin, and 5% heat-inactivated FBS. Two days prior to treating the cells, the medium was replaced with phenol red-free RPMI 1640 medium containing charcoal/dextran-stripped FBS with acetone-washed activated charcoal (100mg/mL) at 4 °C for 30 min and centrifuged at

4000 rpm for 15 min at 4 °C. This step was repeated in triplicate. Extracts and compounds were not toxic to cells at the applied concentrations, under these experimental conditions. DMSO concentrations for all cell culture assays were below 0.1%.

Non-tumorigenic breast epithelial MCF-10A cells were obtained from American Type Culture Collection (ATCC) and maintained in DMEM/F12 supplemented with 5% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 0.002% epidermal growth factor (Life Technologies, Grand Island, NY), 0.01% Cholera toxin, 0.005% hydrocortisone, 0.1% insulin, 1% penicillin-streptomycin and incubated in 5% CO_2 at 37 °C. Hepa 1c1c7 murine hepatoma cells were supplied by Dr. J. P. Withlock, Jr. (Stanford University, Stanford, CA). Cells were maintained in α-minimum essential medium (MEME) supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum. HepG2 cells stably transfected with antioxidant response element (ARE) luciferase reporter (HepG2-ARE-C8) were kindly provided by Dr. A. N. Tony Kong (Rutgers University, Piscataway, NJ). Cells were grown in DMEM/F12 supplemented with 10% FBS, 1% penicillin-streptomycin, 1% geneticin, and 0.06% insulin.

2.3. Detection of ER ligands using pulsed ultrafiltration LC-MS

A screening assay based on ultrafiltration mass spectrometry (Sun et al. 2005) was used to identify the ligands of ER present in licorice crude extracts. Briefly, 150 µg/ml of the methanol crude extract was incubated for 1 h at room temperature with 50 pmol of ERα or ERβ in binding buffer consisting of 50 mM Tris-HCl (pH 7.5), 10% glycerol, 50 mM KCl, and 1 mM ethylenediaminetetraacetic acid (EDTA) in a total volume of 50 µL. Identical control incubations in which denatured ER was substituted for active ER was used to correct for nonspecific binding of compounds to the ultrafiltration membrane and holder. After incubation, each mixture was filtered through a Microcon (Millipore, Bedford, MA) YM-30 centrifugal filter containing a regenerated cellulose ultrafiltration membrane with a 30000 MW cutoff and washed three times with 200 µL aliquots of ammonium acetate buffer (pH 7.5) at 4 °C to remove the unbound

compounds. The bound ligands were released by adding 400 µL of methanol/water (90:10; v/v) followed by centrifugation at 10000 × *g* for 10 min. The ultrafiltrates were dried under a stream of nitrogen, and the ligands were reconstituted in 50 µL of methanol/water (50:50; v/v). Aliquots (10 µL) were analyzed using LC-MS, which consisted of a reverse phase separation on a Shimadzu (Kyoto, Japan) Shim-Pack XR-ODS III C_{18} (1.6 µm, 2.0 mm x 50 mm) column and mass spectrometric analysis on a Shimadzu LCMS- IT-TOF mass spectrometer. Both positive ion and negative ion mass spectra were acquired over the range *m/z* 100 to *m/z* 800. The ion source parameters for mass spectrometry included a capillary voltage of 3.5 kV, source block temperature 200 °C, curved desolvation line temperature 200 °C, and nebulizer gas flow of 1.5 L/min. The mobile phase consisted of a 5 min linear gradient from 5 to 100% acetonitrile in water with 0.1% formic acid.

2.4. Estrogen receptor subtype (ERα/ ERβ) competitive binding assay

After identification of ER ligands in licorice crude extracts by mass spectrometry, competitive ERα and ERβ binding assays were used with [^{3}H] estradiol based on the method of Obourn et al. (Obourn et al. 1993) with minor modifications (Liu et al. 2001) to determine in vitro binding affinities of the ligands with the receptors. The reaction mixture consisted of 5 µL of extract in DMSO, 5 µL of purified human recombinant diluted ERα and ERβ (0.5 pmol) in ER binding buffer, 5 µL of “hot mix” [400 nM, prepared fresh using 95 Ci/mmol [^{3}H] estradiol, diluted in 1:1 ethanol:ER binding buffer; obtained from NEN life Science Products (Boston, MA)], and 85 µL of ER binding buffer. To correct for non-specific binding, a control containing all the added components except for the hot mix was considered. The incubation was carried out at room temperature for 2 h before 100 µL of 50% hydroxyapatite slurry (HAPS) was added. The tubes were incubated on ice for 15 min with vortexing every 5 min. The appropriate ER wash buffer was added (1 mL), and the tubes were vortexed before centrifuging at 10000 × g for 5 min. The supernatant was discarded, and this wash step was repeated three times. The HAPS pellet

containing the ligand-receptor complex was re-suspended in 200 µL of ethanol and transferred to scintillation vials. An additional 200 µL of ethanol was used to rinse the centrifuge tube. Cytoscint [4 mL/vial; ICN (Costa Mesa, CA)] was added, and the radioactivity was counted using a Beckman LS 5801 liquid scintillation counter (Schaumburg, IL). The percentage inhibition of [^{3}H] estradiol binding to each ER subtype was determined using eq 1.

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

The binding capability (percentage) of the sample was calculated in comparison with that of estradiol (50 nM).

2.5. Induction of an estrogen-responsive alkaline phosphatase (AP) in Ishikawa cells

The protocol of Pisha et al. was used as described previously (Pisha and Pezzuto 1997). Ishikawa cells (5×10^4 cells/well) were pre-incubated in 96 well plates in estrogen-free medium for 24 h. Test samples dissolved in DMSO, were added at different concentrations and the DMSO concentration was kept lower than 0.1 %. To determine the anti-estrogenic activity, treatments were performed in the presence of 17β-estradiol (2 nM), well above its EC_{50}. Plates were incubated at 37° C for 96 h. Cells were washed with PBS and lysed by adding 50 µL of 0.01 % Triton X-100 in 0.1 M Tris buffer (pH 9.8) followed by a cycle of freeze and thaw at -80° C and 37° C, respectively. *p*-Nitrophenol phosphate (phosphatase substrate) (18 mM) was added to each well and the alkaline phosphatase activity was measured by reading the formation of *p*-nitrophenol at 405 nm every 15 s with a 10 s shake between readings for 16 readings using a Power Wave 200 microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, VT). The maximum slope of the kinetic curve for every experiment well was calculated. The percentage induction of alkaline phosphatase for every treatment, compared to that of the estradiol control was represented as estrogenic activity and calculated

using eq 2. Anti-estrogenic activity was calculated using eq. 3, stating the percent induction of alkaline phosphatase compared to background induction control.

$$[(slope_{sample} - slope_{DMSO})/(slope_{estrogen} - slope_{DMSO})] \times 100 = \text{fold estrogenic induction} \quad (2)$$

$$[1-((slope_{sample} - slope_{cells})/(slope_{DMSO} - slope_{cells}))] \times 100 = \text{fold antiestrogenic induction} \quad (3)$$

2.6. Induction of estrogen responsive element (ERE) in MCF-7 cells

The Dual-Luciferase Reporter Assay System protocol from Promega (Madison, WI) was used to evaluate the activation of ERs through interaction with ERE at the promoter of estrogen responsive genes, resulting in the expression of the fused luciferase reporter. Briefly, MCF-7 cells grown in phenol-red free medium for 48 h, were trypsinized and re-suspended in serum-free medium at 1×10^7 cells/mL followed by a 10 min incubation at room temperature with pERE (3 µg/mL) obtained from Dr. V. C. Jordan, Northwestern University (Catherino and Jordan 1995), and pRL-TK (Promega) (1 µg/mL) in a 4 mm gap cuvette before electroporation at 950 µF and 250 V using the Gene Pulser Xcell (BioRad Laboratories, Hercules, CA). Transfected cells were diluted in serum containing medium and plated in 24-well plates (2×10^5 cells/well). After 24 h, the cells were washed with PBS and treated with two concentrations of the crude extracts (2 µg/mL and 10 µg/mL) and two concentrations of the isolated compounds (0.1 µM and 1 µM), for an additional 24 h. 17-β-Estradiol (E2) (1 nM) was considered as the positive control. Cell lysates (20 µL) were placed in white Costar 96-well plates, before the injection of Luciferase Assay reagent (100 µL) followed by a 12 s read by a FLUOstar OPTIMA (BMG Lab Tech, Offenburg, Germany). To quench the firefly luciferase expression and activation of the renilla expression, Stop & Glo reagent (100 µL) was injected followed by a 12 s read. In order to exclude the errors in transfection efficiency, average read out of the luciferase activity was normalized to the average associated pRL-TK read outs (renilla activity). The results were converted into fold induction by normalizing to DMSO.

2.7. Induction of estrogen-responsive gene mRNA in endometrial and breast cancer cells

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to examine the modulation of the *Tff1* induction following treatment of T47D cells with the extracts and the related compounds. Experiments were performed four independent times in triplicates. T47D cells (4×10^4 cells/mL) were preincubated in estrogen-free media for 72 h. Cells were treated with extracts (10 µg/mL), 8-PN (100 nM), LigF (5 µM), LigC (5 µM) in DMSO for 6 h. Total RNA was isolated using the TRIzol Plus RNA purification kit (Invitrogen) and quantitated by UV analysis at 260 nm. cDNA synthesis was performed using qScript cDNA synthesis kit (Quanta Biosceiences) in a total volume of 15 µL, containing 4 µL qScript reaction mixture (5X), 1 µL qScript RT, 10 µL nuclease-free water, and 5 µL of RNA sample. The reaction was carried out for 5 min at 22 °C, followed by 42 °C for 30 min and a 5 min incubation step at 85 °C. The PCR and subsequent analyses were performed using the ABI StepOne Plus RT-PCR system (Applied Biosystems). Quantitation was performed using the TaqMan technology of Applied Biosystems. *Tff1* was evaluated using a predeveloped gene expression primer/probe set h(Applied Biosystems' Assay on Demand). Briefly, the PCR reaction mixture was prepared in a total volume of 20 µL, containing 1 µL 20X TagMan Gene Expression Assay, 10 µL 2X TaqMan Gene Expression Master Mix, 4 µL cDNA template, and 5 µL RNase-free water. The reaction mixture was incubated at 50 °C for 2 min followed by 10 min at 95 °C. Polymerase chain reactions were performed in triplicate and consisted of 40 cycles with 15 s denaturing step at 95 °C and 1 min annealing/extending step at 60 °C each. The fluorescence signal was measured during the last 30 s of the annealing/extension phase. Following analysis, a fluorescence threshold value was set and threshold cycle (C_t) values were determined. These values were used for further calculations. β-Actin was used as an endogenous control to correct for any differences in the amount of total RNA used for a reaction and to compensate for different levels of transcription during reverse transcription of RNA into cDNA. *Tff1* expression by treatments

and controls were normalized to its respective β-actin expression levels. The final results were expressed as a fold induction, where the levels of *Tff1* observed in the DMSO-treated samples was defined as one.

2.8. Isomerization of isoliquiritigenin to liquiritigenin

As shown in Figure 4, LigC forms a conversion equilibrium with LigF which influences the interpretation of the observations obtained by LigC. To evaluate this conversion and better define the compounds responsible for the observed estrogenic activity, LC-MS monitoring of the isomerization of these active principles in the cell-based assays was performed in parallel to every bioassay. One set of plates was considered for time 0, when there was no treatment and another set was considered for the final harvesting day for every experimental condition. The media exposed to cells were collected and extracted by adding sodium acetate buffer (100 mM, pH 5), followed by liquid-liquid extraction by water-saturated ethyl acetate. After evaporation of the solvent, the residue was dissolved in 50 % methanol and analyzed using LC-MS or LC-UV to detect the conversion of LigC to LigF. When studying the stability of LigC in the competitive binding assay condition, the compound was incubated with the ER binding buffer before analysis.

2.9. NQO1 activity

Murine hepatoma Hepa1c1c7 cells, an established model for the assessment of NQO1 activity, were treated with the licorice extracts, the selected pure constituents ,hops extract, and XH, and the change in NQO1 activity was evaluated as described previously.(Prochaska and Santamaria 1988) Activities were expressed as CD values, referring to the concentration of an agent required to double the activity of NQO1 (Table 2). Chemopreventive indices (CI values) were calculated as the ratio of toxicity (IC_{50}) versus the CD value (Table 2).(Gerhauser et al. 1997)

2.10. ARE luciferase activity

Human hepatoma Hep-G2-ARE-C8 cells stably transfected with ARE were treated with the licorice extracts, the selected pure constituents, the hops extract, XH, curcumin, and sulforaphane. The induction of ARE-luciferase was evaluated after 24 h using the single luciferase kit (Promega, Madison, WI) as described previously (Dietz et al. 2013; Keum et al. 2006). The results were normalized to the protein concentration evaluated using the BCA assay kit.

2.11. Western blotting

Non-tumorigenic breast epithelial MCF-10A cells were treated with the licorice extracts, the selected pure constituents, hops extract, and XH. Protein expression of NQO1 was evaluated using western blot as described previously (Hemachandra et al. 2012). Anti NQO1 and anti β-actin were used as primary antibodies. Antibodies were diluted in blocking solution (5% BSA in TBS with 0.1% tween 20). Blots were incubated with primary antibodies overnight at 4ºC while shaking and upon addition of the appropriate secondary antibody for 1 h at room temperature. Imaging and quantitative densitometric analysis of the blots were performed using luminescence substrate (Thermo Scientific, Waltham, MA) and FluroChem software (Cell Biosciences, Santa Clara, CA). Each protein density band was normalized to its corresponding β-actin band density and reported relative to protein expression. The data represents three independent experiments and is stated as mean ± SD.

2.12. Animal treatment

To assess the estrogenic effects of GG and GU as well as LigF, female immature Sprague-Dawley rats were received at 11 days of age from Harlan (Indianapolis, IN) and while the moms were on phytoestrogen free diet, pups stayed with moms until day 18 when they were whined and were randomized into groups of 6: (i) control diet plus vehicle control (corn oil/50% polyethylene glycol by gavage, s.c. corn oil); (ii) control diet plus GG (2 g/kg BW per day by

gavage); (iii) GU (2 g/kg BW per day by gavage); (iv) control diet plus 17β-estradiol benzoate (2.24 g/kg BW per day, s.c.); (v) control diet plus LigF (50 mg/kg BW per day, s.c.); (vi) control diet plus LigF (150 mg/kg BW per day, s.c.); (vii) control diet plus simultaneous administration of LigF (150 mg/kg BW per day, s.c.) and 17β-estradiol benzoate (2.24 g/kg BW per day, s.c.). The animals were treated for 3 days every 24 h. Animals weight and their food intake were evaluated daily. Animals were sacrificed by CO_2 asphyxiation. Uterus was collected, trimmed of fat and connective tissue, and weighted. One horn was snap frozen and one horn was fixed in 4% paraformaldehyde for 48 h. Then the samples were submerged in 50% EtOH for 1 h and then moved to 70% EtOH before overnight automated processing in a gradient of EtOH and subsequent immersion in xylene. Then the tissue samples were blocked in paraffin for future analysis. The same was applied to mammary tissue, one portion was snap frozen and one portion was fixed and blocked.

For the chemoprevention study, mature female Sprague-Dawley rats were received at 7 weeks of age from Harlan (Indianapolis, IN). All rats consumed powdered Harlan/Teklad purified diet (Indianapolis, IN). After one week of acclimation, animals were divided into six groups based on their weight (n= 5/group) : (i) control diet plus vehicle control (corn oil/50% polyethylene glycol) by gavage; (ii) experimental diet containing 4'-bromoflavone (150 mg/kg BW per day) plus vehicle control by gavage; (iii) control diet plus GU extract (1.3 g/kg BW per day) by gavage; (iv) control diet plus GG extract (1.3 g/kg BW per day) by gavage; (v) control diet plus LigF (80 mg/kg BW per day) by gavage; (vi) control diet plus LigC (40 mg/kg BW per day) by gavage. The recommended human clinical dose of licorice supplements was considered as a basis for calculation of the lower dose for rats and then 20X higher concentration (1.3 g/kg BW per day) was administered to evaluate the potential pharmacological effect. The doses of LigF and LigC were equivalent to their contents (considering all quantified glycosides of LigF and LigC) in the administered GG extracts. The animals were treated for 4 days. All animals and their food were weighted daily. No difference in food intake was observed between treatment

groups. On day 4, animals were sacrificed by CO_2 asphyxiation, and serum and tissues were collected, snap frozen, and stored at - 80 ºC for later analysis. The animal protocol complied with the Guide for the Care and Use of Laboratory Animals and all procedures were approved by UIC's Institutional Animal Care and Use Committee (Protocol No. 08-101).

2.13. UHPLC-MS/MS analysis of LigF and LigC in licorice extracts, rat serum, and tissues

The licorice extracts were dissolved in 50% aqueous methanol at 20 µg/mL. Rat serum (100 µL) was mixed with 400 µL acetonitrile containing 10 ng/mL 7-hydroxyflavone as internal standard. Tissues (300-900 mg) were weighed and homogenized in 3 mL 70% aqueous methanol. A 200 µL aliquot of each homogenate was mixed with 800 µL acetonitrile containing 5 ng/mL 7-hydroxyflavone. Serum/tissue homogenate mixtures were vortexed and then centrifuged for 15 min at 12,000 x *g* at 4 °C. The supernatant was evaporated to dryness and reconstituted with 50 µL of 50% aqueous methanol. A 5 µL aliquot of each serum/tissue extract and a 2 µL aliquot of each licorice extract was analyzed using UHPLC-MS/MS. Calibration curves were prepared by spiking blank serum/tissue homogenate with known concentrations of the reference standards (0.5 - 1000 ng/mL).

UHPLC-MS/MS analyses were carried out using a Shimadzu (Kyoto, Japan) LC/MS-8050 triple quadrupole mass spectrometer equipped with a Shimadzu Nexera UHPLC system. Analytes were separated on a Waters (Milford, MA) Acquity UPLC BEH C_{18} 2.1 x 100 mm column (1.7 µm particle size). For quantitative analysis, the gradient was: 1 - 3.5 min, from 15 - 35% B, 3.5 - 5.5 min, from 35 - 95% B, hold at 95% B for 1 min. For the qualitative analysis of licorice extracts, the gradient was: 0.5 - 14.5 min, from 12 - 80% B. (A: 0.1% formic acid in water, B: acetonitrile) at a flow rate of 0.6 mL/min. The data were acquired using selected reaction monitoring (SRM) in electrospray mode with polarity switching and the following SRM transitions: *m/z* 417 - 255 (liquiritin, isoliquiritin), *m/z* 549 - 255 (liquiritin apioside, isoliquiritin apioside, licuraside), *m/z* 255 - 119 (liquiritigenin, isoliquiritigenin), *m/z* 323 - 201 (glabridin), *m/z*

469 - 425 (glycyrrhetinic acid), *m/z* 339 - 121 (licochalcone A), and *m/z* 237 - 180 (7-hydroxyflavone, internal standard).

2.14. Analysis of NQO1 activity *in vivo*

Frozen samples of liver from the treated rats were homogenized using 0.25 M aqueous sucrose and centrifuged at 15000 x *g* for 1 h at 4 °C (Cuendet et al. 2010). The supernatant was collected and 0.1 M $CaCl_2$ solution added. Samples were incubated for 30 min at 0 °C and centrifuged at 15000 x *g* for 1.5 h at 4 °C. For mammary glands, the frozen tissue was homogenized in ice-cold 0.1 M phosphate buffer (pH 6.5) and centrifuged for 1.5 h at 15,000 x *g* at 4 °C. The supernatant was used for NQO1 analysis. Protein concentrations were measured using BCA assay kit, and the samples were diluted accordingly to give 5 µg liver protein and 30 µg mammary gland protein in 50 µL supernatants, respectively. The samples were then tested for NQO1 activity as described previously (Cuendet et al. 2010).

2.15. Statistical analysis

Linear regression analysis. After obtaining the dose-response curves for the induction of NQO1 activity and ARE-luciferase linear regression analysis was performed with Graph-Pad Prism (version 5.00 for Windows, Graph Pad Software, La Jolla California USA, www.graphpad.com). Linear regression analysis was performed using the concentration ranges not associated with toxicity or saturation of the responses. The slopes of the lines were used as a measure for the inducing potential of the tested materials. *Significance.* Data are reported as means ± SD. Significant differences from control values were determined by one-way ANOVA with a follow-up Dunnett test ($P < 0.05$).

3. Evaluation of the estrogenic properties of glycyrrhiza species used in botanical dietary supplements for managing menopausal symptoms

(Reprinted in part with formatting changes from: Hajirahimkhan, A., Simmler, C., Yuan, Y., Anderson, J. R., Chen, S. N., Nikolic, D., Dietz, B. M., Pauli, G. F., van Breemen, R. B., Bolton, J. L. (2013) Evaluation of the estrogenic activity of licorice species in comparison with hops used for menopausal symptoms. PLoS One 8(7): e67947.)

3.1. Rationale and Hypothesis

Because of an increased life expectancy in recent years, many women spend the last third of their lives in post menopause (Minino 2011). A drastic decline in circulating endogenous estrogen in menopausal women results in a number of symptoms including hot flashes, sleep disturbances, mood swings, vaginal dryness, and osteoporosis (Barlow 1997; Shaver et al. 1991; Kronenberg 1994). Hormone therapy (HT) has been the treatment of choice to alleviate menopausal symptoms. However, in light of the results published from the Women's Health Initiative (WHI), which demonstrated an increased risk of developing hormone dependent cancers, cardiovascular problems, and stroke among women taking HT, many women have turned to alternative therapies such as botanical dietary supplements to alleviate menopausal discomfort (Shumaker et al. 2003; Wassertheil-Smoller et al. 2003; Rossouw et al. 2002). Currently, there is insufficient evidence on the efficacy of botanicals for menopausal symptom relief and their mechanisms of actions are not fully understood (Hajirahimkhan, Dietz, et al. 2013).

Hops (*Humulus lupulus L.*) is a well studied botanical for women's health and a common constituent of dietary supplements, particularly in Europe (Salter and Brownie 2010; Blumenthal and German Federal Institute for Drugs and Medical Devices. Commission E. 2000; Overk et al. 2008; Overk et al. 2005). Hops and its phytoconstituents, including 8-prenylnaringenin (8PN)

and its metabolic precursor chalcone, xanthohumol (XH) (Figure 3) have been studied for their estrogenic and chemopreventive properties (Liu et al. 2001; Overk et al. 2008; Overk et al. 2005; Hemachandra et al. 2012; Dietz et al. 2005; Basly and Lavier 2005). Hops have been shown to exert estrogenic activity in endometrial cancer (Ishikawa) and breast cancer (MCF-7) cells (Overk et al. 2005). One of its bioactive compounds, 8-PN, the most potent phytoestrogen known to date (Roelens et al. 2006), has been shown to be an equipotent ligand of estrogen receptor (ER) subtypes and exhibits estrogenic activity in hormone responsive cell-based assays as well as animal models (Overk et al. 2005; Overk et al. 2008; Diel et al. 2004).

However, XH, a metabolic precursor chalcone of 8-PN does not show estrogenic activity. It has been reported that, while a standardized extract of hops containing 8-PN did not increase uterine weight in ovariectomized Sprague-Dawley rats, 8-PN alone increased uterine weight and the height of luminal epithelial cells in animal models (Overk et al. 2008; Diel et al. 2004). While small amounts of 8-PN are present in most hops preparations, additional 8-PN can be biosynthesized *in vivo* through metabolism of XH (Figure 3). It has been reported that metabolic differences among individuals could impact the formation of 8-PN and likely the ultimate estrogenic responses generated by hops extracts (Bolca et al. 2010). On the other hand, XH, which does not have estrogenic properties, has been reported to possess chemopreventive potential, through the induction of detoxification enzymes (Dietz et al. 2005).

Licorice root is one of the oldest and most frequently used botanicals in traditional Chinese medicine for improving health, curing injury or swelling, detoxification, and for women's health (Wang and Nixon 2001). Today, licorice is mainly used as a flavoring and sweetening agent in tobacco industry, chewing gums, candies, toothpastes and beverages (Wang and Nixon 2001) and is one of the most popular components of menopausal dietary supplements in the United States (Geller and Studee 2005; Taylor 2012; Ju et al. 2008). Licorice has been studied for its estrogenic properties since 1950 (Costello and Lynn 1950), although the findings

about its efficacy have not been conclusive (Hajirahimkhan, Dietz, et al. 2013). There are more than 30 known licorice species in the world which differ genetically and biochemically. The different chemical profiles result in various biological activities and clinical potential among the species. It is ***hypothesized*** that Licorice species and their bioactive compounds LigC and LigF have varied estrogenic activities *in vitro* and *in vivo*.

The licorice species *Glycyrrhiza glabra* (*GG*), *Glycyrrhiza uralensis* (*GU*), *Glycyrrhiza inflata* (*GI*) have been reported to contain various amounts of liquiritin, the glycosylated form of the dihydroflavanone, LigF (Figure 7) and its precursor chalcone, LigC (Figure 7), all of which have been reported to have estrogenic activity *in vitro* (Mersereau et al. 2008; Maggiolini et al. 2002; Kondo, Shiba, Nakamura, et al. 2007). However, a comparative biological evaluation of distinct *Glycyrrhiza* species has not been conducted to date. In addition it is rarely clear which *Glycyrrhiza* species are present in menopausal dietary supplements and what species are better choices for these formulations in terms of estrogenic efficacy and safety. Studies that have reported estrogenic properties of licorice compounds, LigF and LigC, have not addressed the possible interconversion of these compounds which could strongly influence the interpretation of the estrogenic activities depending on the bioassay conditions. Moreover, the potential antiestrogenic effect of LigF in the presence of E_2 in healthy animals has not been reported.

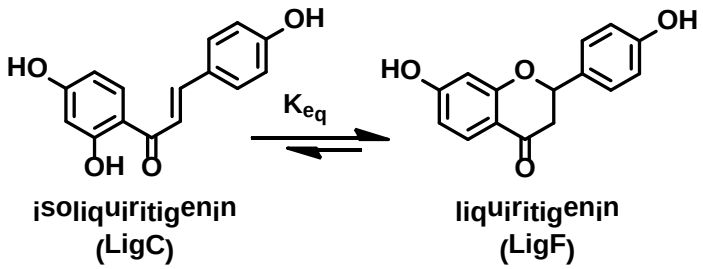


Figure 7. Estrogenic compounds of licorice.

In the present study, crude extracts of three licorice species, GG, GU, and GI, as well as their active compounds, LigF and LigC, were examined for their *in vitro* estrogenic activity and were systematically compared with a spent hops (*Humulus lupulus*) extract in addition to its active constituents, 8-PN and XH. The conversion of LigC to LigF during bioassays was monitored by LC-MS and LC-UV. These results suggest that although licorice species are less estrogenic than hops, they contain an ERβ selective phytoestrogen, LigF and an estrogenic chalcone LigC which in turn can convert to LigF.

The chalcone -> flavanone interconversion (LigC to LigF) in the case of licorice is non-enzymatic and therefore independent of metabolic variations among subjects. In contrast, with hops the two step conversion (XH to 8-PN) depends on CYP450 metabolism as well as gut microbiota (Possemiers et al. 2006; Possemiers et al. 2005) which could differ among individuals with various metabolism characteristics. Additionally, our *in vivo* observations suggested while LigF does not enhance uterine weight, it might inhibit E_2 induced uterine proliferation which could be associated with its ERβ effects as well as partial agonist estrogenic activity. These data suggest that licorice extracts could benefit menopausal women due to moderate estrogenic activity, ERβ selectivity, and a more predictable PK profile.

3.2. Results

3.2.1. Quantification of chalcones/flavanones in *Glycyrrhiza* extracts

The marker compounds, LigF and LigC, as well as their respective glycosylated forms, liquiritin and isoliquiritin, were quantified in the crude MeOH extracts of *Glycyrrhiza* species (Table 1). The analyses showed that the *GU* extract contained the highest amount of LigF (0.16% w/w) compared to *GI* (0.06% w/w) and *GG* (0.05% w/w). Similarly, *GU* had the highest content of LigC (0.06% w/w) compared to *GI* (0.03% w/w) and *GG* (0.02% w/w). However, among the three licorice extracts, *GI* had the highest amount of glycosylated forms of LigF and LigC, liquiritin (5.47% w/w) and isoliquiritin (3.24% w/w), respectively. Nevertheless, the overall ratio of quantified flavanones versus the sum of flavanone and chalcones in *GU* (82% w/w) was considerably higher compared to *GI* (63% w/w) and *GG* (51% w/w). These data are consistent with the estrogenic activities of the various licorice extracts observed in the bioassays described below.

TABLE I: QUANTIFICATION OF CHALCONES AND FLAVANONES IN THE GLYCYRRHIZA EXTRACTS USED FOR ESTROGENIC EVALUATIONS.

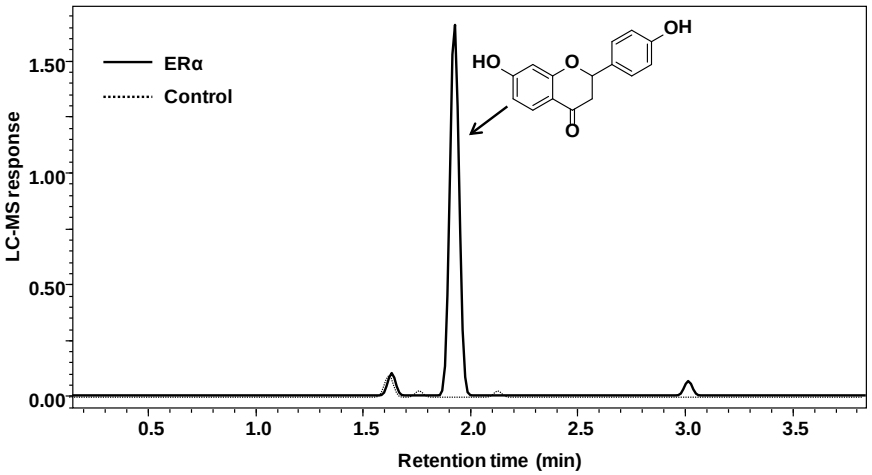
	% in the crude extract (weight/weight dry extract)		
Quantified compounds	**GU**	**GI**	**GG**
Liquiritin (Glc-LigF)	3.23 ± 0.13	5.47 ± 0.18	1.64 ± 0.08
Liquiritigenin (LigF)	0.16 ± 0.05	0.06 ± 0.05	0.05 ± 0.03
Isoliquiritin (Glc-LigC)	0.69 ± 0.15	3.24 ±0.78	1.64 ± 0.16
Isoliquiritigenin (LigC)	0.06 ± 0.03	0.03 ± 0.01	0.02 ± 0.01
Total aglycones : LigC +LigF	0.21 ± 0.04	0.09 ± 0.03	0.07 ± 0.02
Total quantified flavanones (F)	3.39	5.53	1.69
Total quantified chalcones (C)	0.75	3.27	1.66
Total F/Total(F+C)	82%	63%	51%
Total C/Total(F+C)	18%	37%	49%

Values are expressed as mean ± SD of three independent analyses of each crude extract.

3.2.2. Pulsed ultrafiltration mass spectrometric (PUF-MS) screening of the extracts

Pulsed ultrafiltration mass spectrometry is a rapid technique to identify active ligands for receptors in complex mixtures (Johnson et al. 2002). This method was used to find possible hits for ER subtypes in the crude MeOH extracts of the *Glycyrrhiza* species. Figure 8 shows that LigF was the only phytoconstituent which significantly enhanced the peak and bound to ERα and ERβ. Since PUF-MS analysis is a qualitative approach to define ligand-receptor interactions, a competitive binding analysis was also performed to quantitatively determine the affinity of the ligands for ERs.

A)



B)

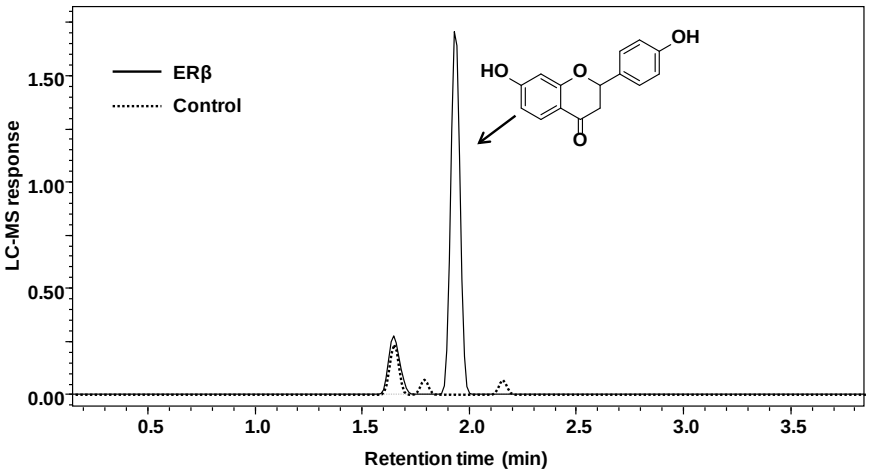
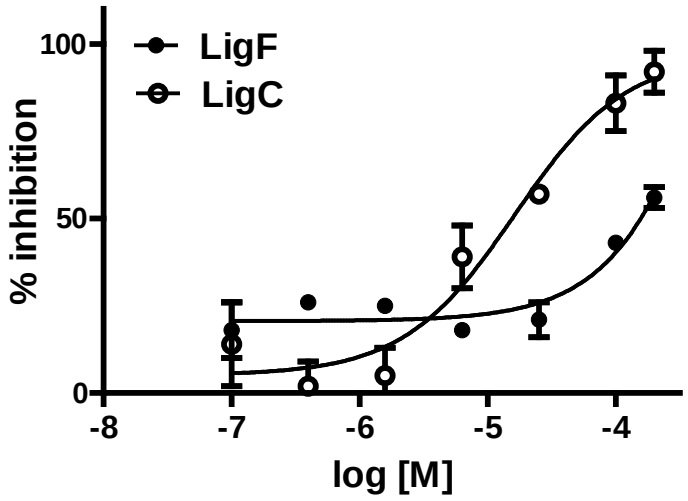


Figure 8. Pulsed ultrafiltration LC-MS of licorice extracts for ER subtypes.
Positive ion electrospray LC-MS chromatograms showing the ultrafiltration mass spectrometric screening of crude extract of *Glycyrrhiza uralensis*; *GU* to A) ERα and B) ERβ. Denatured ER was used as a control for non-specific binding and specific binding is indicated by increases in the chromatographic peak areas. The chromatogram shows that in GU crude extract, LigF bound to both ER subtypes, suggesting the compound is the estrogenic active principle of the extract.

3.2.3. Relative affinity of the *Glycyrrhiza* compounds for ER subtypes

Based on the screening results of PUF-MS analysis as well as literature reports (Maggiolini et al. 2002; Choi et al. 2008), competition of LigF and its precursor chalcone, LigC, with ^{3}[H] estradiol for the ER subtypes was assessed to confirm and quantify the affinity of these compounds for ERs (Table 2, Figure 9). Both LigC and LigF had very similar affinities towards ERβ with IC_{50} values of 7.8 μM and 7.5 μM, respectively. However, LigF had a very weak affinity for ERα while LigC bound to ERα with an IC_{50} value of 16 μM. The selectivity of LigF for ERβ over ERα was 20-fold, which was comparable to the reports by Mersereau et al. (Mersereau et al. 2008) and Kupfer et al. (Kupfer et al. 2008) (ERα IC_{50} = 2.8 μM, ERβ IC_{50} = 0.41 μM). Because different techniques were used the absolute IC_{50} values differed.

A)



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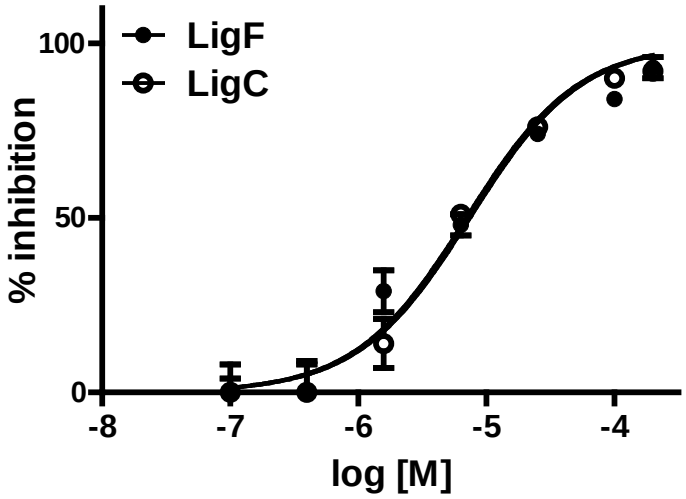


Figure 9. Competitive ER binding.
Competitive ER binding using 3[H] estradiol and human recombinant A) ERα and B) ERβ. Results are the means of three independent experiments and expressed as mean ± SD.

TABLE II. AP INDUCTION, CYTOTOXICITY, ER BINDING, AND ERE LUCIFERASE INDUCTION OF LICORICE, HOPS, AND THEIR ISOLATED COMPOUNDS.[a]

	AP induction Ishikawa cells EC_{50}[b] (n = 9)	Maximum AP fold induction Ishikawa cells	IC_{50}[b] (n = 9)		ERE-luciferase Fold induction MCF-7 cells, (n = 9)
			ERα	ERβ	
17β-estradiol	0.00019 ± 0.00005	137 ± 2.5	0.021 ± 0.03	0.015 ± 0.02	4.8 ± 0.4
GU	8.3 ± 0.8	58.7 ± 2.3	> 200	> 50	2.8 ± 0.4
GG	10.3 ± 0.5	26.9 ± 3.0	> 200	> 50	1.9 ± 0.3
GI	9.8 ± 0.4	61 ± 14.7	> 200	> 25	2.0 ± 0.3
Hops	2.1 ± 0.3	100 ± 15	15 ± 3[i]	27 ± 3[i]	4.2 ± 0.2
LigF	3.4 ± 0.4	83.1 ± 3.4	> 200	7.5 ± 0.5	2.1 ± 0.4
LigC	2.7 ± 0.2	57.9 ± 4.3	16 ± 1	7.8 ± 0.1	3.2 ± 0.9
8-PN	0.00665 ± 0.00140	118 ± 6.0	0.51 ± 0.07[i]	1.7 ± 0.1[i]	4.6 ± 0.9
XH	N/A	N/A	N/A[i]	N/A[i]	N/A

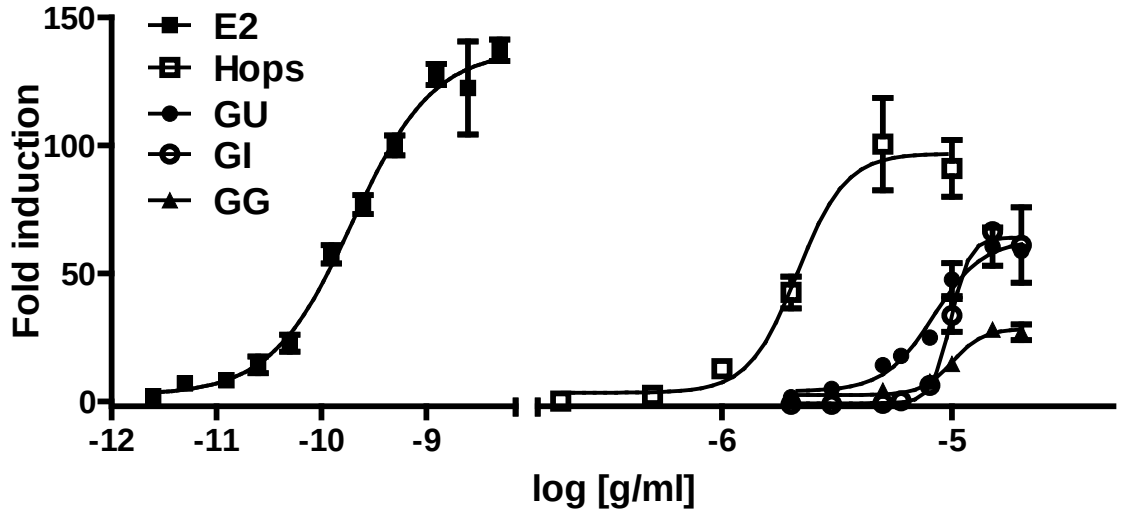
[a] Values are expressed as the mean ± SD of n determinations. Experimental details are described in the Materials and Methods section. [b] Values are expressed in µg/mL for extracts and µM for isolated compounds. [c] Fold inductions tested at 10 µg/mL for extracts (hops at 2 µg/mL) and 100 nM for the isolated compounds where DMSO was set to 1. [d] Ratio of the sum of the firefly and renilla luminescences. [e] N/A, not active. [i] J. Agric. Food Chem. 2005, 53, 6246 - 6253. [j] J. Agric. Food Chem. 2001, 49, 2472 – 2479.

3.2.4. Alkaline phosphatase induction in Ishikawa cells

The Ishikawa cell line is a well-established ERα (+) endometrial cancer cell line for the evaluation of estrogens and antiestrogens (Pisha and Pezzuto 1997). Induction of alkaline phosphatase indicates estrogenic activity, while inhibition of alkaline phosphatase induction in the presence of 17-β-estradiol suggests a possible antiestrogenic effect. The crude MeOH licorice and spent hops extracts showed a dose-dependent induction of alkaline phosphatase (Table 2, Figure 10A). The EC_{50} values of the three *Glycyrrhiza* species were comparable: 7 µg/mL, 9.2 µg/mL, and 9.7 µg/mL for *GU*, *GI*, and *GG*, respectively. However, the maximum efficacy of *GU* and *GI* was around 60 fold of the corresponding E_2 control, while that of *GG* was around 26 fold. On the other hand, the EC_{50} of hops at 2.1 µg/mL was consistent with previous reports (Overk et al. 2005) and was lower than that of the three *Glycyrrhiza* species, whereas its maximum efficacy was 100 fold. The relative EC_{50} ranking of the extracts were hops < *GU* < *GI* ≈ *GG,* while their relative maximum efficacy was hops > *GU* ≈ *GI* > *GG*.

The EC_{50} values for the licorice purified compounds LigC and LigF were 2.7 µM and 3.4 µM, respectively (Table 2, Figure 10B) and their maximum efficacies were 58 fold and 83 fold, respectively. The EC_{50} of 8-PN from hops was 6.6 nM with 118-fold maximum efficacy, and XH was inactive, which was consistent with previous reports (Overk et al. 2005). While the relative EC_{50} ranking of the isolated compounds was 8-PN << LigF ≈ LigC, their relative efficacies ranked: 8-PN > LigF > LigC. None of the extracts and isolated compounds showed antiestrogenic properties (data not shown). LigC showed a reduction in the estrogenic response at concentrations above 7.5 µM, which was associated with its cytotoxic effects at these concentrations. All samples were tested well below their LD_{50} concentrations for the Ishikawa cells (data not shown), unless otherwise stated.

A)



B)

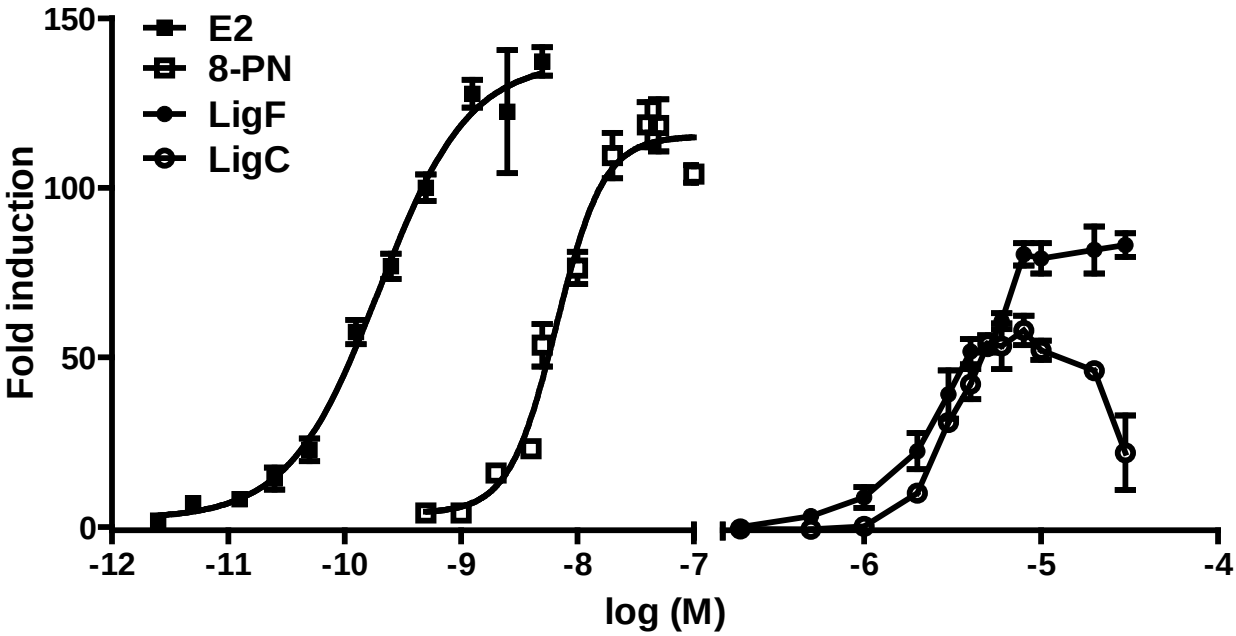
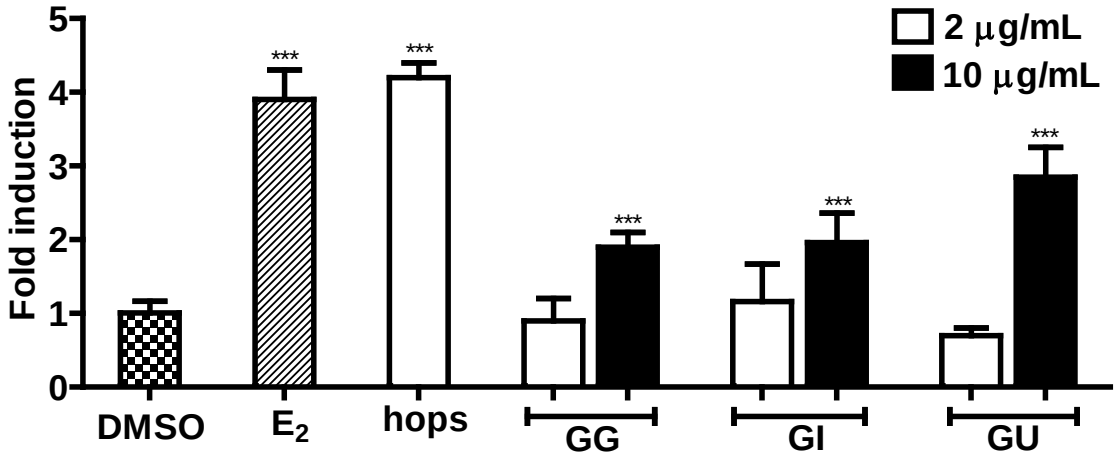


Figure 10. Induction of alkaline phosphatase activity in endometrial carcinoma Ishikawa cells.
A) Crude extracts of *Glycyrrhiza glabra*; *GG*, *Glycyrrhiza uralensis*; *GU*, *Glycyrrhiza Inflata*; *GI*. B) Isolated compounds liquiritigenin and isoliquiritigenin. Results were normalized to DMSO and are shown as the percentage induction relative to 17β-estradiol. Results are the means of three independent determination. Dose-response curves were generated by non-linear regression analysis.

3.2.5. ERE-luciferase induction in MCF-7 cells

MCF-7 cells cotransfected with pERE-luciferase reporter and pRL-TK control were used to evaluate the ERE transcriptional activity of ERs in response to the applied treatments. The reporter response was evaluated relative to the control transfection and was presented as the fold induction after normalizing to the response of the DMSO treated cells (Table 2, Figure 11A). Induction of ERE-luciferase for hops (2 µg/mL) was 4-fold higher than that of DMSO. The *Glycyrrhiza* species were inactive at 2 µg/mL, but did demonstrate significant induction at 10 µg/mL. Both LigF and 8-PN showed induction of ERE-luciferase in MCF-7 cells (Figure 11B). On the other hand, while XH, the closely related chalcone of 8-PN, did not show any induction of ERE-luciferase in MCF-7 cells, LigC had a 7-fold induction at 1 µM, which was comparable to the induction levels by 8-PN (1 µM) and LigF (1 µM).

A)



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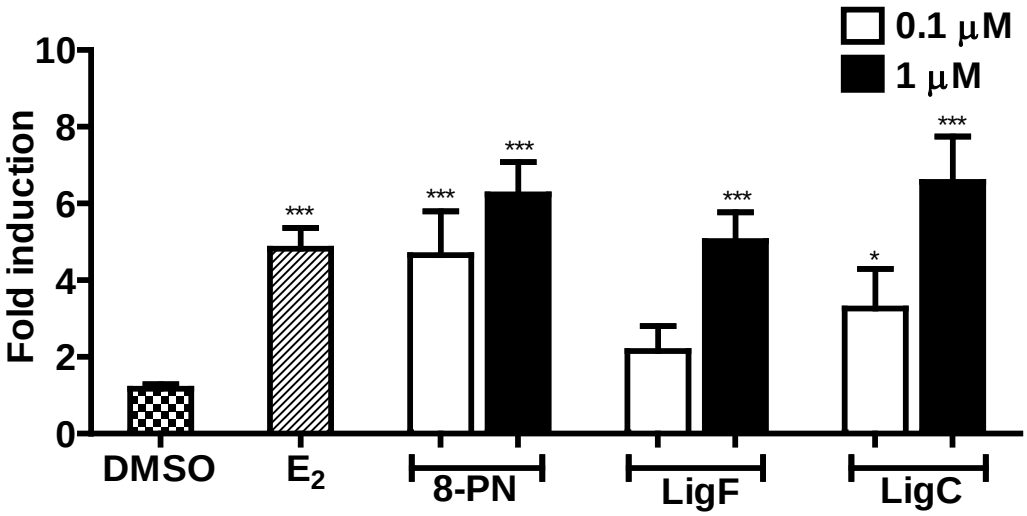
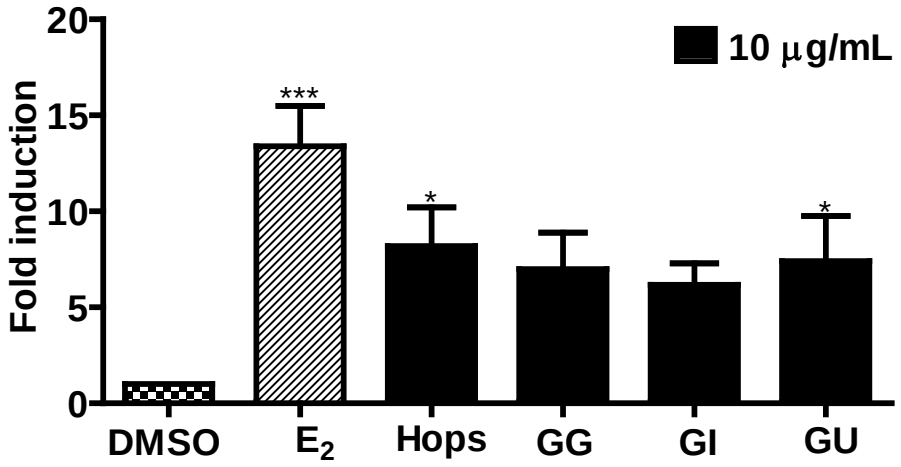


Figure 11. Induction of ERE-luciferase in breast cancer MCF-7 cells.
ERE-luciferase induction in ERα (+) MCF-7 cells by A) licorice and hops extracts and B) their respective compounds. Cells were cotransfected with pERE and pRL-TK 24 h before being treated with either extracts (2 μg/mL, open bars and 10 μg/mL, closed bars) or pure compounds (0.1 μM, open bars and 1 μM, closed bars). 17β-Estradiol (1 nM) was used as positive control. Since hops extract showed a considerable estrogenic activity at 2 μg/mL, higher concentrations were not tested. Chemiluminescence analysis was performed after 24 h. Results were normalized for transfection efficiency, and they are shown as a fold induction relative to the level observed in cells treated with vehicle only. Results are the means of three independent determinations in duplicates ± SD.

3.2.6. Induction of estrogen responsive gene, *Tff1*, in T47D cells

Induction of trefoil factor 1(*Tff1)*, in ER(+) breast cancer T47D cells is a well-established tool to evaluate estrogenic activity of xenobiotics. Upon treating T47D cells with the extracts and the purified compounds, the total RNA was extracted and subjected to cDNA synthesis and qRT-PCR. The response was normalized to the corresponding effect of every treatment on β-actin gene induction and stated relative to the response of DMSO treated cells when DMSO response was considered as one. The results (Figure 12A) showed that *GU*, *GI*, and *GG* at 10 µg/mL induced *Tff1* in T47D cells, and induction of *Tff1* for the three *Glycyrrhiza* species and hops (10 µg/mL) were similar. Induction of *Tff1* by LigF (5 µM) was lower than that of 8-PN (0.1 µM), but the difference was not significant (Figure 12B). On the other hand, despite no *Tff1* activity by XH from hops, LigC from licorice induced *Tff1*, significantly.

A)



B)

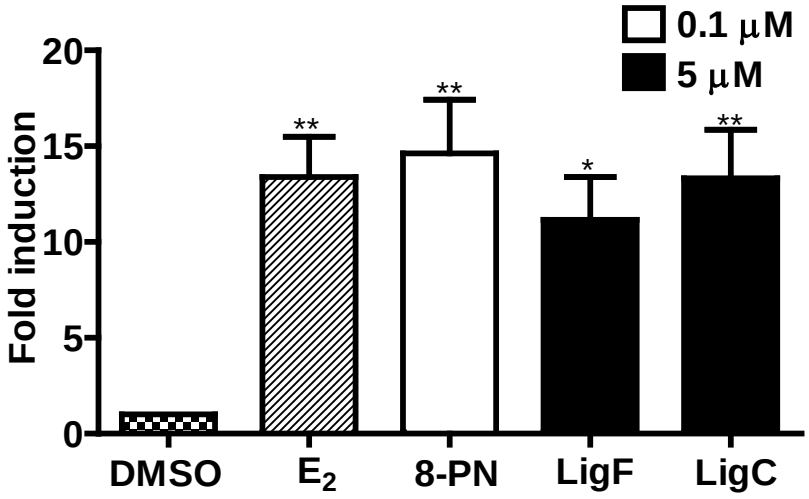
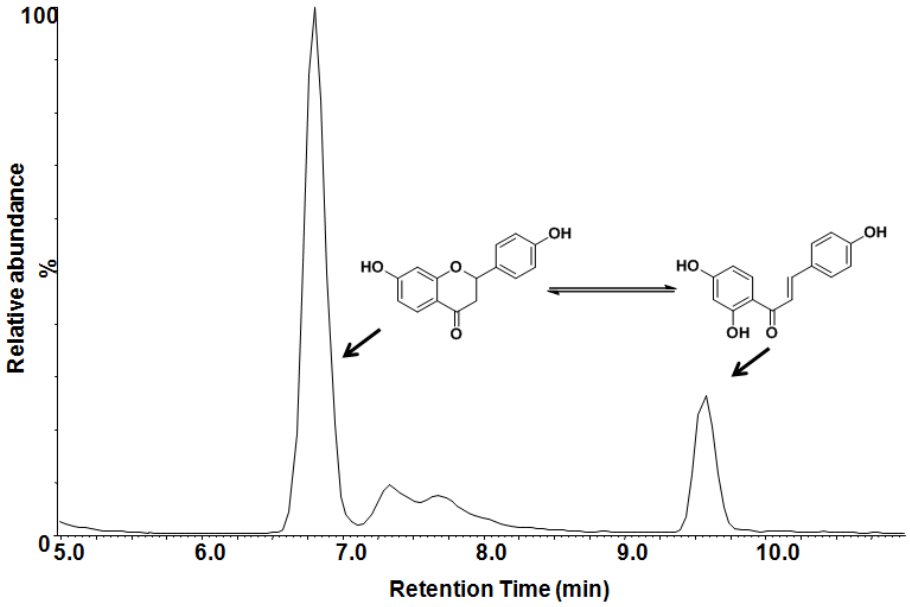


Figure 12. Induction of estrogen responsive genes in breast cancer T47D cells. Estrogen responsive gene (*Tff1*) induction in T47D cells by A) licorice and hops extracts (10 μg/mL) and B) the related compounds 8-PN (100 nM), LigF (5 μM), and Lig C (5 μM). 17β-Estradiol (100 nM) was used as positive control. Results are the means of four independent determinations in duplicates ± SD.

3.2.7. Analysis of the isoliquiritigenin-liquiritigenin isomerization *in vitro*

Results from the alkaline phosphatase induction, ERE-luciferase induction, competitive binding to ERs, and estrogen responsive gene induction showed that LigC has estrogenic activity. The in situ isomerization of LigC to LigF (Figure 7) could potentially be involved in generating estrogenic responses observed with LigC. This hypothesis was confirmed by LC-MS analysis of the cell media of the alkaline phosphatase induction assay after 96 h which showed a significant reduction in the LigC content and a corresponding increase in LigF formation (Figure 13A). However, LC-MS analysis of the cell media of the mRNA induction assay after 6 h did not show a significant formation of LigF from LigC (Figure 13B) which indicates the role of incubation time in this 37°C conversion reaction. LC-UV analysis of the ER binding buffer in the competitive binding assay after 2 h incubation at room temperature did not show any isomerization of LigC to LigF (data not shown), which emphasizes the effect of incubation time in addition to temperature on the stability of LigC (Simmler et al. 2013). These data suggest that LigC could activate estrogenic responses on its own, although formation of the isomerization product, LigF, likely also contributes to observed estrogenic properties of LigC.

A)



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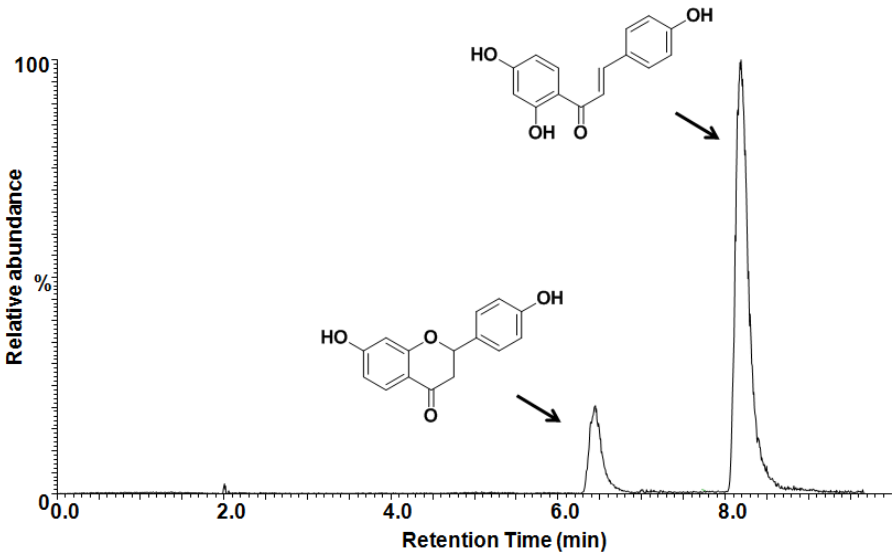
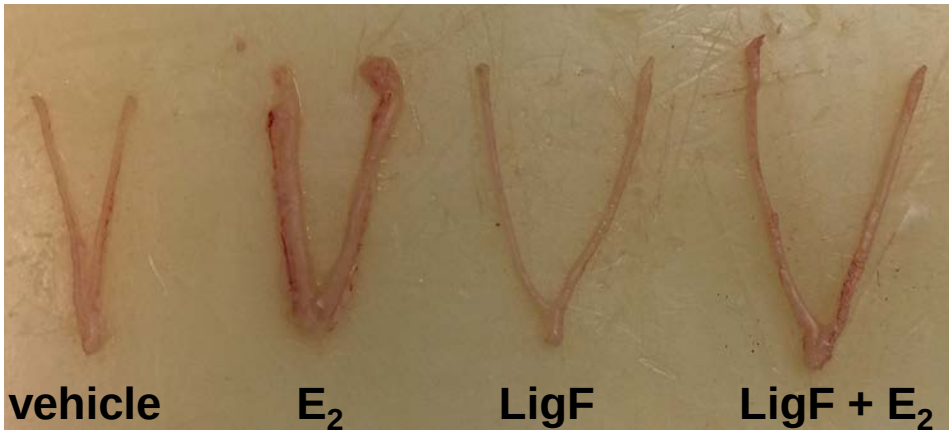


Figure 13. Stability of LigC in alkaline phosphatase assay in Ishikawa cells.
LC-MS analysis of the isomerization of LigC to LigF: A) in the alkaline phosphatase induction assay conditions, 96 h incubation with cultured Ishikawa cells at 37°C. B) in the mRNA induction assay conditions, 6 h incubation with cultured T47D cells at 37°C.

3.2.8. Analysis of the estrogenic/antiestrogenic properties *in vivo*

Uterotrophy in immature female rats is a classic tool to evaluate the estrogenic activity of xenobiotics. Immature female Sprague-Dawley rats start to produce estrogen from 23rd day postnatal. Therefore in order to detect a meaningful estrogenic effect (uterotrophy) by exogenous estrogens the treatment window needs to be limited to days 18-22. We observed a significant increase in uterine weight in animals treated with 17β-estradiol benzoate (2.24 mg/kg BW per day). Since GG and GU are considered the only medicinally approved licorice source materials to be used in botanical dietary supplements in the US and because the fully characterized and authenticated GI extract was not available in enough quantities, in this study only GG and GU were evaluated *in vivo*. A change in uterine weight was not observed in the animals treated with either GG (2 g/kg BW per day) or GU (2 g/kg BW per day) (Figure 14A, Figure 14B). Similarly, LigF at the two applied doses (50 mg/kg BW per day and 150 mg/kg BW per day) did not enhance uterine weight, significantly (Figure 14A, Figure 14B). However, when LigF (150 mg/kg BW per day) was applied as a co-treatment with 17β-estradiol benzoate (2.24 mg/kg BW per day), it blocked the E_2 induced increased uterine weight, significantly (Figure 14A, Figure 14B).

A)



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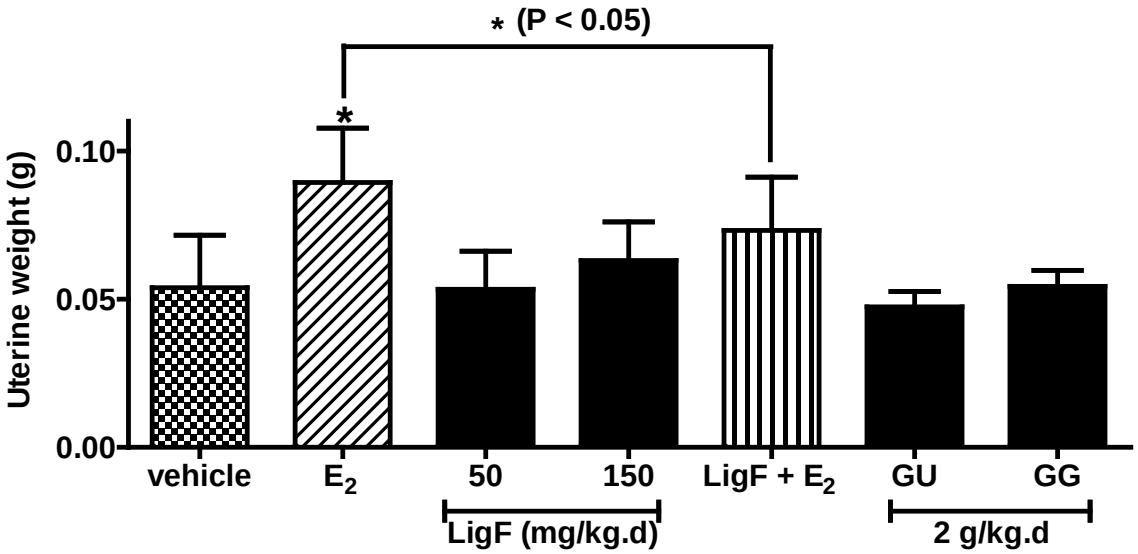

Figure 14. The effect of licorice extracts and LigF on uterine weight.
Immature female Sprague-Dawley rats were treated every 24 h with control diet plus vehicle control (corn oil/50% polyethylene glycol by gavage, s.c. corn oil), control diet plus GG (2 g/kg BW per day by gavage), GU (2 g/kg BW per day by gavage), control diet plus 17β-estradiol benzoate (2.24 g/kg BW per day, s.c.); control diet plus LigF (50 mg/kg BW per day, s.c.), control diet plus LigF (150 mg/kg BW per day, s.c.), control diet plus simultaneous administration of LigF (150 mg/kg BW per day, s.c.), and 17β-estradiol benzoate (2.24 g/kg BW per day, s.c.). After 3 days animals were sacrificed and the weight of dry uterine tissue was recorded.

3.3 Discussion

Previous studies on licorice extracts have primarily focused on the characterization of estrogenic properties of *GG,* because it is the most widely used licorice source material and most likely to be found in botanical supplements for women's health in North America (Dong et al. 2007; Liu et al. 2001; Lutomski 1983; Simons et al. 2011). Both previous findings (Liu et al. 2001) and the present work have demonstrated that *GG* does not have a strong estrogenic activity and, in fact, *GU* and *GI* are more estrogenic (Table 2).

Nevertheless, similar potencies were observed for the three *Glycyrrhiza* species in the alkaline phosphatase induction assay in ERα (+) endometrial cancer cells (Table 2, EC_{50}'s, Figure 10A). This suggests that the estrogenic active principle is likely the same among the species. This observation was consistent with the findings of the pulsed ultrafiltration LC-MS study that showed LigF as the ER ligand in all *Glycyrrhiza* crude extracts (Figure 8). However, the pronounced difference in the efficacy of the estrogenic responses (Table 2, maximum AP) might be attributed to the varied amounts of LigF and its precursor chalcone (LigC) in the three investigated species (Table 1).

Previously, Kondo et al. (Kondo, Shiba, Nakamura, et al. 2007) had shown that *Glycyrrhiza uralensis* has the highest content of LigF (0.11%) and its glycosylated form, liquiritin (1.68%), among the three investigated licorice species. Similarly, quantitation of the chalcone/flavanone ratio in each *Glycyrrhiza* extract (Table 1) revealed that *GU* contained the highest amount of LigF (0.16% w/w) compared to the other extracts (0.05% w/w for GG and 0.06% w/w for *GI*). Total LigF and LigC content of *GU* (0.21% w/w) was more than twice as high as that of *GI* (0.09% w/w) and *GG* (0.07% w/w). Moreover, the total flavanone content of *GU* extract (82% w/w) was significantly higher than that of *GI* (63% w/w) and *GG* (51% w/w) extracts, all explaining the higher estrogenic activity of *GU* extract.

GG has a higher ratio of chalcone content over total flavanones and chalcones (49% w/w) in comparison to *GI* (37% w/w) and *GU* (18% w/w). Chalcones with an accessible Michael acceptor/electrophilic moiety, such as LigC and XH, can interact with cysteine residues of cellular proteins and activate protective responses such as apoptosis (Kensler and Wakabayashi 2010). XH can switch on some cytoprotective mechanisms including activation of detoxification enzymes and can lead to cytotoxicity at higher concentrations (Dietz et al. 2005). Recent studies have demonstrated that LigC induces apoptosis in different cell lines (Yuan et al. 2012; Zhou et al. 2013). Therefore, LigC might be responsible for the observed cytotoxicity of GG at concentrations > 10 µg/mL (data not shown). LigC as a Michael acceptor can activate cellular chemopreventive processes (Dietz and Bolton 2011; Cuendet et al. 2006; Eggler et al. 2005; Calliste et al. 2001; Jung et al. 2006; Kinghorn et al. 2004; Maggiolini et al. 2002). However, in one animal study it has been reported that LigC enhanced breast tumor growth (Cuendet et al. 2010), which might be associated with its estrogenic properties or conversion to the estrogenic compound LigF.

The estrogenic activity of the *Glycyrrhiza* species has been confirmed in the ERE-luciferase assay in MCF-7 cells (Figure 11), as well as in the gene induction assay in T47D cells (Figure 12) suggesting that licorice has estrogenic properties in different estrogen sensitive tissues. The overall results showed that *GU* and *GI* have higher estrogenic activity than *GG*, *in vitro*. However, the *in vivo* observations using GG and GU showed that these extracts as well as LigF did not enhance uterine weight as an ERα related estrogenic response (Figure 14). Interestingly, LigF inhibited E_2 induced uterine proliferation suggesting an anti esrogenic effect with this compound *in vivo* (Figure 14). These findings indicate that although *GG* is widely used in dietary supplements for menopausal symptoms, it is not the most estrogenic *Glycyrrhiza* species and might not be the most suitable for such a purpose. Further *in vivo* evaluations are needed to define the estrogenicity of the licorice extracts.

Isomerization of chalcones to flavanones is a well-established chemical phenomenon (Figure 7) (Cisak and Mielczarek 1992; Nudelman and Furlong 1991; Andujar et al. 2003; Yamin et al. 1997). However, this conversion has often been neglected when studying the biological properties of chalcone/flavanone pairs such as LigC/LigF in bioassays. LC-MS analysis of this isomerization under bioassay conditions (Figure 13) in addition to a report by Simmler et al. (Simmler et al. 2013) led to the conclusion that the cyclization is dependent on the pH, temperature, and incubation time. After 96 hours incubation at 37°C with Ishikawa cells, LigC was mostly converted to LigF (Figure 13A); however, after 6 h incubation with T47D cells, the conversion was limited (Figure 13B). In case of the competitive ER binding assays, the analysis after 2h incubation at room temperature did not show a conversion of LigC to LigF, suggesting the ability of LigC to bind to ER subtypes (Figure 9) without converting to LigF (data not shown). Therefore, the active estrogenic compound(s) in licorice preparations could be both LigF and LigC, depending on the bioassay conditions. The conversion of LigC to LigF could be more pronounced under physiological conditions and needs to be evaluated *in vivo*.

LigF and LigC from licorice were compared with 8-PN and XH from hops in a variety of estrogenic assays. While LigF selectively bound to ERβ either in the current study as well as in previous reports (Kupfer et al. 2008; Mersereau et al. 2008), Overk et al. (Overk et al. 2005) had reported that 8-PN bound ER subtypes with almost equal affinities, thus presents a considerable difference between LigF and 8-PN in terms of the estrogenic pathways the two compounds can activate. LigF has also been reported to selectively activate ERβ in functional assays (Mersereau et al. 2008; Kang et al. 2011) which shows not only its selective binding towards ERβ, but also an activating role for ERβ dependent pathways in the cells. Selective activation of ERβ by estrogenic ligands has been reported to be correlated with down-regulation of ERα activities, including proliferation (Gustafsson 1999). Therefore, selective activation of ERβ by

LigF might present a safer mode of estrogenic activity of licorice, while hops and its constituents do not possess this preferential ERβ modulatory effect.

In the present study, LigF showed estrogenic activity in alkaline phosphatase induction assay in Ishikawa cells at micro molar concentrations and in a partial agonistic manner, while 8-PN was active in nano molar ranges with an efficacy close to the full agonist, E_2. However, the activities of LigF in ERE-Luciferase induction in MCF-7 cells and *Tff1* mRNA induction in T47D cells were similar to the activities observed with 8-PN, which might be related to cell type/signaling pathway specific activities of these compounds. LigC, the precursor chalcone of LigF was an equipotent ligand of ER subtypes in competitive binding assays, exhibited partial agonistic estrogenic activity in alkaline phosphatase induction assay in Ishikawa cells at concentrations < 7.5 µM, and induced ERE-luciferase in MCF-7 cells as well as *Tff1* mRNA in T47D cells. However, XH, the precursor chalcone of 8-PN from hops, neither bound to ERs (Overk et al. 2005) nor exhibited any estrogenic effects in corresponding bioassays. These data are consistent with previous studies which demonstrated that LigC is able to bind to the ER binding pocket and activate estrogenic responses (Calliste et al. 2001; Choi et al. 2008). XH does not have this property and requires metabolism to convert to an estrogenic entity, 8-PN (Figure 3).

Hops extract exhibited higher estrogenic potency and efficacy in the alkaline phosphatase induction assay and higher activity in the ERE-luciferase induction assay compared to the licorice species. The stronger estrogenic activities of hops could be associated with the presence of 8-PN and its precursors in hops. Moreover, the licorice extract showed partial agonistic behavior, which could be a therapeutic advantage as partial agonists are better tunable agents for their respective receptor signaling pathway (MacGregor and Jordan 1998). Partial agonists can play the role of antagonists when a full agonist is present and can also work

more selectively. These properties make licorice and its active constituents an attractive target for further characterizations of their estrogenic activity.

Increase in the weight of the estrogen responsive tissue, uterus is a classic experiment to define the estrogenic activity of xenobiotics. Since estrogen is not produced in the ovaries of immature rats before the 23rd day of their lives, and the circulating estrogen levels are lower than detectable, this animal model can clearly show any uterine proliferation associated with the potential estrogenic agents over three days of treatment. Treating immature female Sprague-Dawley rats with GG and GU for three consecutive days showed that despite the estrogenic effects observed *in vitro*, GG and GU did not enhance uterine weight in animals (Figure 14). The same results were obtained before when mature female rats were treated with hops extract. This observation might be associated with the metabolism and low bioavailability of the active compounds especially when the treatment rout is oral. Additionally, licorice extracts might have natural progestins that oppose the proliferative estrogenic effects *in vivo*. Unlike the reported proliferative effects of 8-PN, the potent ERα ligand from hops, on uterus, subcutaneous injection of LigF from licorice did not enhance uterine weight (Figure 14) which might be associated with its low concentration *in vivo* or its tendency to interact with ERβ (Figure 7B). However, LigF was able to suppress the E_2-induced uterine proliferation, significantly (Figure 14). This effect could be explained by the ERβ selectivity of LigF having an opposing influence on ERα dependent responses such as proliferation. More importantly LigF is a partial agonist estrogenic compound which could in part inhibit the effects of E_2 as a full agonist and act as an antiestrogen in the presence of E_2. The animal tissues will be further examined in the future to obtain more insights based on the molecular markers of estrogenicity.

In conclusion, these data show that *Glycyrrhiza* species have different estrogenic activities with *GU* showing the highest estrogenic properties. This further emphasizes the importance of precise labeling and definition of plant species in botanical supplements. Licorice

and its compounds have partial agonistic estrogenic activities, LigF is an ERβ selective ligand and LigC shows dual estrogenic/chemopreventive activities. LigC and LigF are easily interconvertible without enzymatic metabolism (Simmler et al. 2013). While GG, GU, and LigF did not enhance uterine weight in rats, LigF exhibited antiestrogenic effects in the presence of E_2. All these properties suggest that licorice might have more moderate, potentially safer, and more predictable estrogenic activities than hops. Therefore, standardized licorice preparations could be considered as an option for menopausal women. Nevertheless, before estrogenic herbal supplements such as licorice are recommended, *in vivo* safety studies are necessary, since estrogenic compounds have the potential to increase the risk of endometrial cancer in women with intact uterus or the risk of breast cancer. Further evaluations of the animal tissues using molecular markers will provide more information on the estrogenic status of GG and GU as well as LigF in the uterine and mammary tissues of the treated animals. Moreover, GI extract needs to be examined *in vivo* for its estrogenic properties.

4. Evaluation of breast cancer prevention potential of *Glycyrrhiza* species used in botanical dietary supplements for managing menopausal symptoms

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4.1. Rationale and Hypothesis

Due to the cancer risk associated with hormone therapy (HT), menopausal women often use botanical dietary supplements such as hops strobiles (*Humulus lupulus*, Cannabaceae) and licorice roots (*Glycyrrhiza* species, Fabaceae) as "natural alternatives" primarily for the alleviation of menopausal symptoms (Rees 2009; Rossouw et al. 2002). Despite the controversy on the efficacy of these botanicals for menopausal discomfort,(Geller and Studee 2005; Hajirahimkhan, Dietz, et al. 2013) they remain popular since they are natural, have a long history in traditional medicine, and are therefore perceived as safe (Hajirahimkhan, Dietz, et al. 2013). Menopausal women are more susceptible than younger women to the development of cancers including breast cancer (Harman 2006; Rebbeck et al. 2007). They are also frequent and longtime consumers of botanicals. Therefore, understanding the chemopreventive potential of women's health botanicals such as licorice would be particularly beneficial for menopausal women.

One strategy for chemoprevention involves induction of detoxification enzymes, such as NAD(P)H:quinone oxidoreductase 1 (NQO1), which play a role in preventing diseases such as cancer, cardiovascular problems, and neurological disorders (Ungvari et al. 2011; Zhu et al. 2014). We have previously shown that a specialized exract from the strobiles of hops has

chemopreventive effects (Dietz et al. 2013). The hops extract and its major Michael acceptor, xanthohumol (XH, Figure 15) induce detoxification enzymes in hepatoma cells and in rat liver likely through modification of cysteine residues of Keap1 and activation of Nrf2 signaling (Dietz et al. 2005; Dietz et al. 2013). This effect has been previously reported with other naturally occurring electrophilic compounds such as curcumin from turmeric and sulforaphane from broccoli (Figure 15) (Balogun et al. 2003; Gafner et al. 2004; Shen et al. 2006; Francy-Guilford and Pezzuto 2008; Kong et al. 2001; Juge et al. 2007; Cheung and Kong 2010; Liu et al. 2005; Eggler et al. 2008).

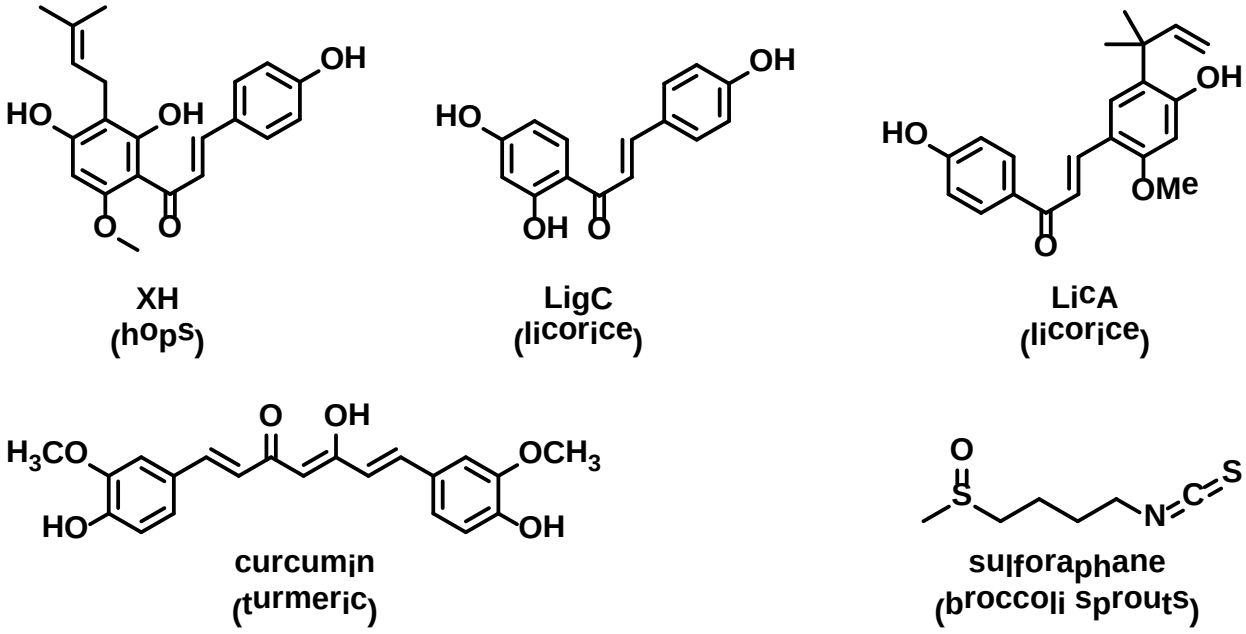

Figure 8: Electrophilic compounds from botanicals.
Electrophilic compounds such as the ones found in plants are capable of interacting with cellular proteins and activate biological responses.

Licorice has more than 30 different species and is a very popular botanical in traditional medicine for various conditions such as digestive problems and wound healing (Shibata 2000; Messier et al. 2012). It is also commonly used as a natural sweetener in the food industry and as a flavoring agent in toothpastes and cigarettes (Liu et al. 2000). Licorice species are also frequently found as components of popular menopausal formulations. The United States Pharmacopeia recognizes only two species *Glycyrrhiza glabra* (GG) and *G. uralensis* (GU) as source of licorice botanicals and the European Medicines Agency also considers *Glycyrrhiza inflata* (GI) being a legitimate source plant with medicinal properties (Simmler 2015). Despite the similarities observed in the morphology of the roots of these *Glycyrrhiza* species, they are known to exhibit marked chemical differences, which have been shown to ultimately lead to variation in their biological activities (Farag et al. 2012; Simmler 2015; Hajirahimkhan, Simmler, et al. 2013; Dunlap 2015). However, these significant differences have mostly been overlooked in botanical research and in the dietary supplement marketplace (Kondo, Shiba, Yamaji, et al. 2007; Kondo, Shiba, Nakamura, et al. 2007). It is ***hypothesized*** that Licorice species and their bioactive constituents LigC and Licochalcone A (LicA) (Figure 15) can induce chemopreventive responses through the induction of NAD(P)H:quinone oxidoreductase enzyme *in vitro* and *in vivo*.

Besides glycyrrhizin, a triterpene saponin responsible for the sweet taste of licorice, the major constituents found in all three *Glycyrrhiza* species are glycosides of the flavanone liquiritigenin (LigF) and its chalcone isomer isoliquiritigenin (LigC, Figure 15) (Hajirahimkhan, Simmler, et al. 2013; Simmler, Nikolic, et al. 2014). We have recently shown that LigC and LigF (Figure 7) are interconvertible under physiological conditions influencing their biological responses profoundly (Simmler et al. 2013; Hajirahimkhan, Simmler, et al. 2013). Similar to the chalcone XH from hops, LigC is a Michael acceptor with the potential to covalently modify cellular proteins resulting in modulation of biological pathways (Liu et al. 2005; Eggler et al. 2008; Cuendet et al. 2006). LigF is an estrogenic compound with a higher tendency to interact

with estrogen receptor beta (ERβ) than with ERα (Hajirahimkhan, Simmler, et al. 2013; Mersereau et al. 2008; Matthews and Gustafsson 2003).

LicA which is specific to GI also contains an α,β-unsaturated carbonyl and is an electrophilic Michael acceptor (Figure 15) (Kondo, Shiba, Nakamura, et al. 2007; Simmler 2015). Different chemopreventive properties have been described for LicA such as the inhibition of carcinogenic oxidative estrogen metabolism through inhibition of arylhydrocarbon receptor (AhR) pathways (Dunlap 2015).

The aim of the present study was to systematically analyze the *in vitro* and *in vivo* NQO1 induction potential of the three medicinally used *Glycyrrhiza* species that have been DNA authenticated and chemically well characterized and to highlight any differences between extracts and the selected bioactive constituents (Simmler 2015). Due to the similarities between the classes of compounds in licorice and hops, a comparative analysis was performed. Our findings emphasize on the importance of the simultaneous chemical/biological characterization of licorice botanicals used for women's health.

4.2. Results

4.2.1. UHPLC-UV metabolite profiling and characterization of licorice extracts

Licorice extracts were analyzed by UHPLC-UV to quantify the bioactive aglycones, LigF and LigC, as well as their major glycosides, glabridin and LicA (Table 1) (Dunlap 2015). The UHPLC-UV profiles revealed that both *in vivo* and *in vitro* extracts displayed similar chemical profiles (Figure S1, Table 1). LigF and LigC glycosides can be considered as bio-precursors or pro-drugs of their corresponding aglycones since they are likely deglycosylated *in viv*o as will be discussed later in the tissue analysis results and discussion sections. Deglycosylation can occur throughout the digestive tract, and there are controversies on whether this process can start in the stomach (Kamei et al. 2005). Therefore, all quantified glycosides of LigF and LigC were expressed as LigF and LigC equivalents (Table 1) (Dunlap 2015). The results presented in Table 1 demonstrated that the extracts from the three *Glycyrrhiza* species showed characteristic differences in terms of flavanone and chalcone composition. The GI extract had the highest total concentration of chalcones (LicA and LigC), whereas the GG and GI extracts had similar amounts of LigC equivalents. GU had the lowest levels of chalcones and therefore the rank order for the chalcone contents of the three *Glycyrrhiza* species would be GI> GG > GU. As a result, we hypothesized that the relative NQO1 induction could follow the same order and would be GI > GG > GU.

TABLE III. CONCENTRATION OF THE BIOACTIVE COMPOUNDS IN THREE LICORICE EXTRACTS DETERMINED BY *UHPLC-UV*, USED FOR CHEMOPREVENTIVE EVALUATIONS.

Species	Glabridin	LicA[b,c]	LigC[c]	LigC equivalents[b,c]	LigF[c]	LigF equivalents[b,c]
			% w/w crude extract ± SD			
GG	0.96 ± 0.03	ND[a]	0.06 ± 0.00	2.97 ± 0.01[d]	0.24 ± 0.01	5.61 ± 0.02[d]
GU	ND[a]	ND[a]	0.09 ± 0.01	0.81 ± 0.03	0.41 ± 0.01	2.96 ± 0.02
GI	ND[a]	5.42 ± 0.34	0.13 ± 0.01	2.47 ± 0.02[d]	0.12 ± 0.04	0.82 ± 0.06[d]

[a]ND: below the Limit of Detection

[b,c]The term LigC equivalents is used to represent the total amount of LigC aglycone plus LigC glycosides (isoliquiritin, isoliquiritin apioside and licuraside) in each crude extract. LigF equivalents is used to represent the total amount of LigF aglycone plus LigF glycosides (liquiritin, liquiritin apioside and liquiritigenin-7-*O*-apiosylglucoside) in each crude extract. The values are expressed as mean ± SD of independent measures.(Dunlap 2015)

[d]More than 70% of all LigF equivalents correspond to liquiritin apioside.

4.2.2. Licorice species as well as LigC and LicA induce NQO1 activity in murine hepatoma cells

Murine Hepa1c1c7 cells are a well-established model for evaluating the NQO1 inducing potential of xenobiotics (Prochaska and Santamaria 1988). The licorice and hops extracts showed a dose-dependent induction of NQO1 in Hepa1c1c7 cells (Figure 16A, Table 4). The induction potency of GI was comparable to that of GG and higher than that of GU (Table 2). The efficacy of GI was better than either GG or GU (Figure 16A). Hops extract was more active than any of the three licorice extracts with a more pronounced toxicity compared to GG and GU (Figure 17A,Table 4). The rank order for concentration to double the activity (CD) values is Hops << GI ≅ GG < GU (Table 4). The different NQO1 inducing properties of the extracts can also be illustrated by the slope of the linear regression analysis of the linear part of the dose-response curves revealing a similar rank order of activity as the CD values: Hops << GI < GG < GU (Table 4). The line fit was set to include the concentrations in the linear part of the dose-response curves, in which toxicity and saturation effects do not play a role (Table 4).

LigC, the common Michael acceptor chalcone in all three licorice species, showed a dose-dependent induction of NQO1 with a significant efficacy (Figure 16B, Table 4). Similarly, the other Michael acceptor, LicA, which is the marker compound of GI, induced NQO1 dose-dependently but with lower efficacy (Figure 16B, Table 4). LigF, the estrogenic flavonone in *Glycyrrhiza* species and the cyclization product of LigC under physiological conditions, was a weak NQO1 inducer (Figure 16B, Table 4). This observed activity was probably due to conversion to LigC (Simmler et al. 2013). Glabridin (Figure 4), the species-specific marker compound of GG did not show induction of NQO1 activity (Figure 16B). XH, the electrophilic chalcone of hops, induced NQO1 at lower concentrations than LigC and LicA (Figure 16B) and was more toxic than LigC (Figure 17B, Table 4).

The known chemopreventive compounds, curcumin from turmeric and sulforaphane (SFN) from broccoli (Figure 15), were also tested for comparison (Figure 16C, Table 4) (Gullett et al. 2010; Aggarwal and Shishodia 2006). Curcumin induced NQO1 with activity similar to LicA, while sulforaphane exhibited a higher NQO1 induction over a broad concentration range demonstrating the highest activity of all compounds tested (Figure 16C, Table 4). Linear regression analysis (Figure S2 Table 4) confirmed these observations showing a similar rank order of NQO1 activity projected in CD values. Relative to its high NQO1 inducing activity, the toxicity of sulforaphane was low resulting in the highest chemopreventive index [CI] (Kang and Pezzuto 2004) (Figure 17B, Table 4). The rank order of CI values was sulforaphane >> XH > LigC > LicA = curcumin (Figure 17C, Table 4).

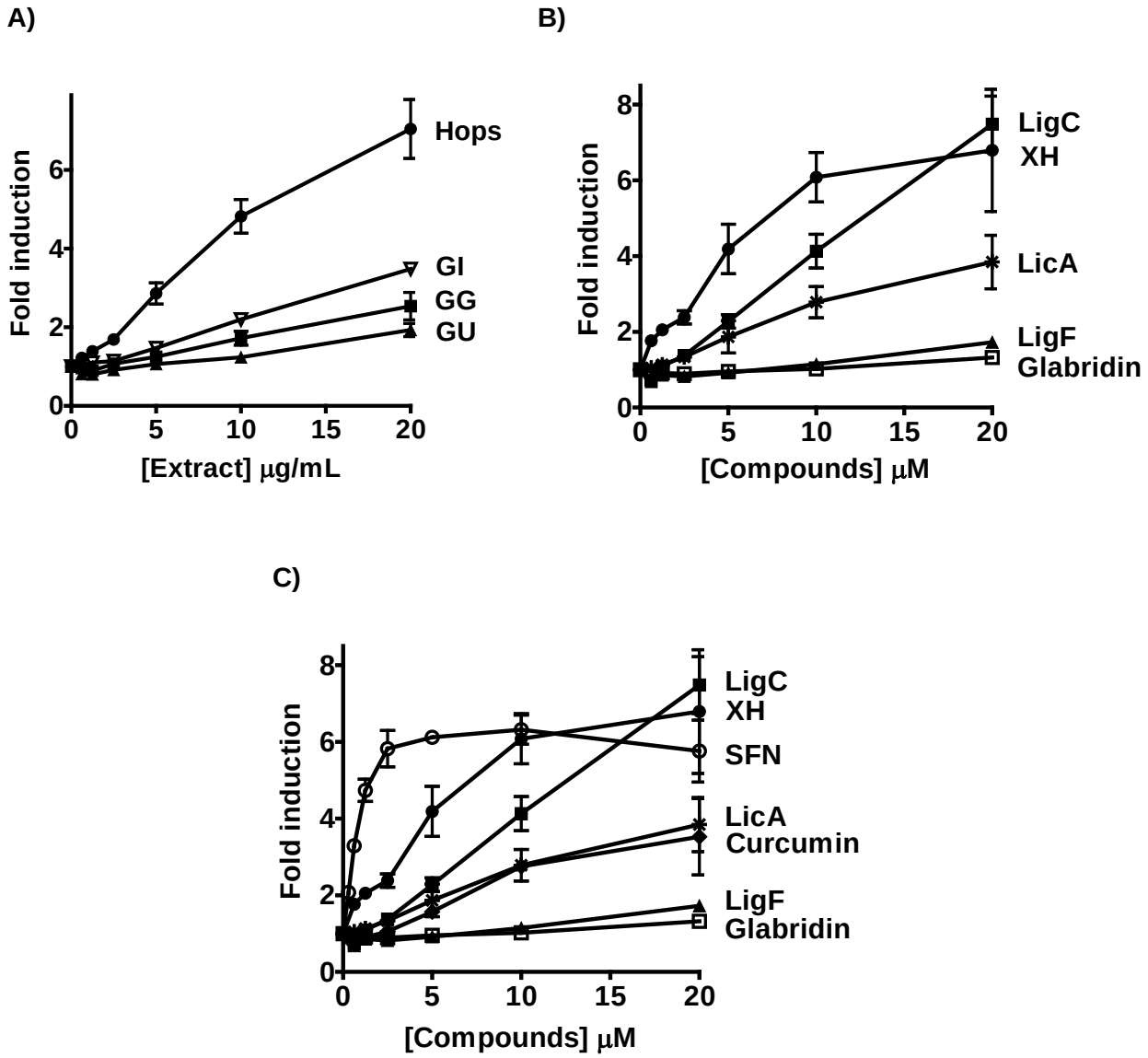

Figure 16. Induction of NQO1 in hepa1c1c7 cells.
NQO1 activity was measured after 48 h treatment with A) licorice extracts from the three major species, GG, GU, and GI in comparison to hops extract. B) LigC/LigF, and LicA from licorice in comparison to XH from hops. C) Licorice bioactive constituents relative to XH, curcumin from turmeric, and sulforaphane from broccoli sprouts. Results are shown as fold induction and are the means of three independent determinations.

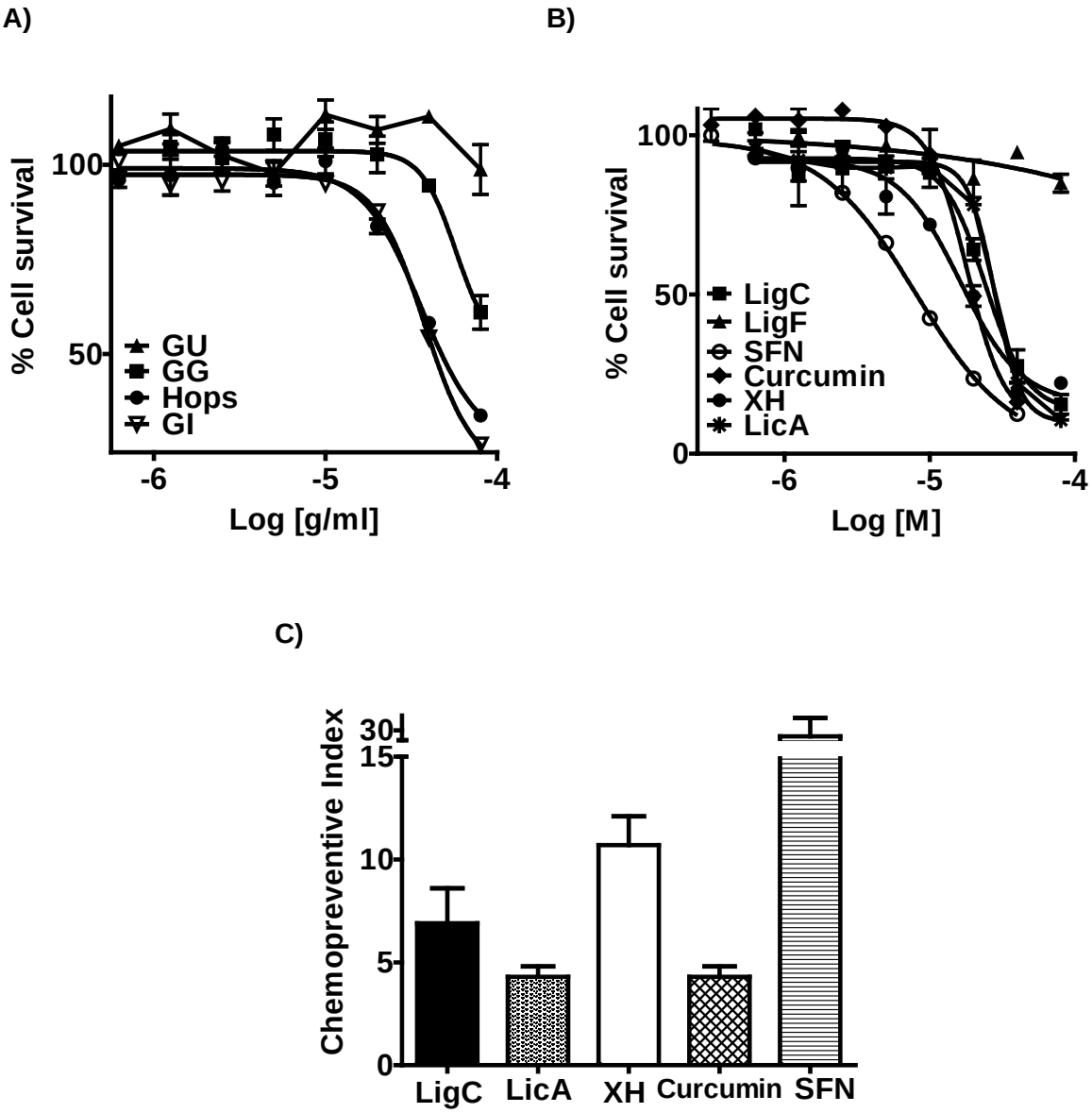

Figure 17. Cytotoxicity and chemoprevention index.
Cell viability of murine hepatoma hepa1c1c7 cells was assessed by crystal violet staining after 48 h of treatment with A) GG, GU, GI, hops, and B) LigC, LigF, LicA from licorice, XH from hops, curcumin from turmeric, and sulforaphane from broccoli. C) Chemopreventive index (CI) was calculated as a ratio of the LC_{50} values obtained from the curves in A and B to the CD values obtained from Figure 23. Results are the means of three independent determinations and expressed as mean ± SD.

TABLE IV. NQO1 ACTIVITY AND ARE INDUCTION OF LICORICE AND COMPOUNDS.

Extracts	NQO1 induction CD[a] ± SD	NQO1 induction Slope[b]	NQO1 induction R^{2b}	LC_{50}[c]	CI[d]	ARE induction Slope[b]	ARE induction R^{2b}
GG	8.7 ± 0.62	0.08	0.93	58 ± 3.2	6.6 ± 0.83	0.11	0.59
GU	12 ± 1.9	0.05	0.89	> 60	N/A	0.07	0.83
GI	8.3 ± 0.33	0.13	0.99	37 ± 4.2	4.4 ± 0.66	0.57	0.97
Hops	2.9 ± 0.15	0.31	0.96	36 ± 5.1	12 ± 7.9	0.21	0.98
Compounds							
LigC	3.5 ± 0.7	0.34	0.98	24 ± 2.0	6.9 ± 1.7	0.37	0.64
LigF	11 ± 0.61	0.05	0.93	> 100	N/A	0.00	0.07
LicA	5.5 ± 0.36	0.18	0.93	24 ± 1.1	4.3 ± 0.50	1.9	0.97
XH	1.5 ± 0.10	0.49	0.97	16 ± 1.0	10.7 ± 1.4	2.0	0.93
Curcumin	5.4 ± 0.11	0.19	0.97	18 ± 1.2	3.3 ± 0.29[e]	0.28	0.91
sulforaphane	0.29 ± 0.06	2.9	0.98	8.1 ± 0.5	28 ± 7.5[f]	2.6	0.99
4'-bromoflavone	0.03 ± 0.002	20	0.93	> 100	> 3000[g]	N/A[g]	N/A[g]

[a]CD, concentration (μM) doubling the NQO1 activity
[b]Slope, the slope of the linear regression analysis line fit
[c]LC_{50}, the concentration (μM) leading to 50% of the maximal toxicity
[d]CI, chemopreventive index calculated as $\frac{LC50}{CD}$
[e]Consistent with literature(Gerhauser et al. 2003), [f]Consistent with literature,
[g]N/A, not analyzed

4.2.3. Licorice species as well as LigC and LicA induce ARE-luciferase in human hepatoma Hep-G2-ARE-C8 cells

The luciferase gene linked to the ARE element in Hep-G2-ARE-C8 cells is a unique tool to evaluate the capacity of xenobiotics to activate antioxidant responses and to test for the involvement of the Keap1-Nrf2-ARE pathway (Chen and Kong 2004). Similar to the NQO1 induction results, all licorice extracts GG, GU, and GI induced ARE luciferase activity dose dependently (Figure 18A). Unlike the NQO1 induction activity assay, the data showed that the activity of GI was significantly higher than hops, GG, and GU (Figure 18A). This observation was reflected in the linear regression analysis of the data (Table 4).

The Michael acceptors from licorice, LigC and LicA, also induced ARE-luciferase activity dose dependently; however, LicA exhibited a higher ARE-luciferase induction level than LigC in contrast to their NQO1 activities (Figure 16B, Figure 18B). This observation was also reflected in the enhanced slope of LicA compared to that of LigC, suggesting a higher tendency of LicA to activate ARE (Figure S3, Table 4). LicA showed toxic effects at concentrations of ~10 µM in HepG2-ARE-C8 cells, leading to a decline in ARE-luciferase signal. LigF is not a Michael acceptor and was not active in the ARE-luciferase assay consistent with its low NQO1 induction activity (Figure 16B, Figure 18B). The ARE-luciferase induction of XH was comparable to that of LicA, with both compounds sharing very similar slopes (Figure 18B, Table 4). The ARE-luciferase activities of XH and curcumin were comparable to their NQO1 inducing activities (Figure 18B). However, while sulforaphane exhibited a higher slope in inducing NQO1 compared to other tested compounds (Figure S2, Table 4), its slope in inducing ARE-luciferase was comparable to that of XH and LicA (Figure S3, Table 4).

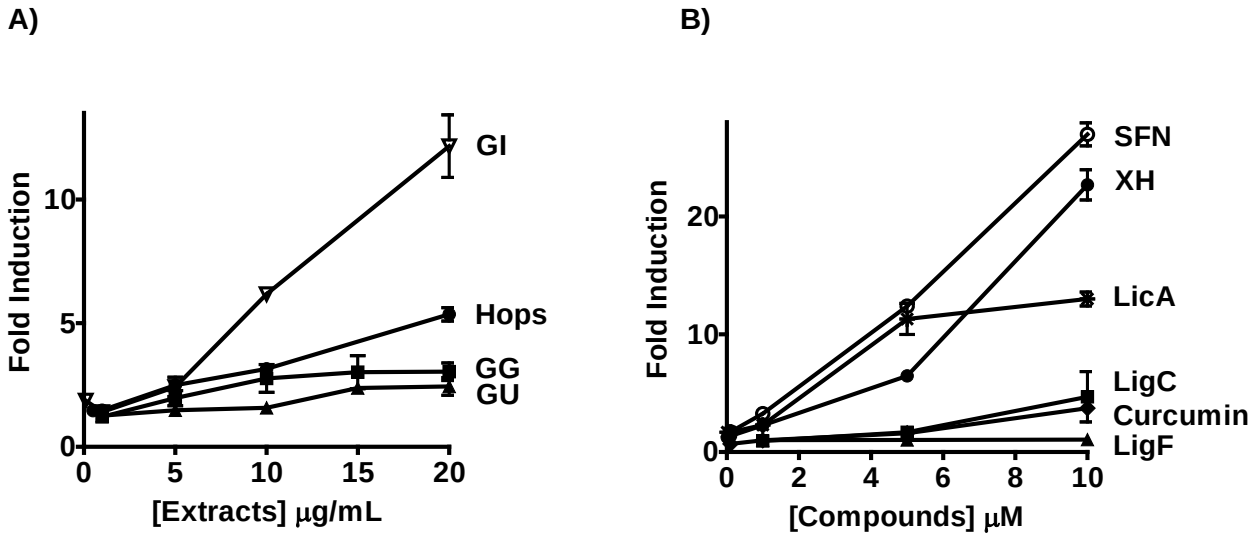

Figure 18. Induction of ARE-luciferase in stably transfected human hepatoma Hep-G2-ARE-C8 cells.
ARE-luciferase induction by A) licorice extracts from the three major species, GG, GU, and GI in comparison to hops extract, B) by LigC/LigF, and LicA from licorice, XH from hops, curcumin from turmeric, sulforaphane from broccoli. Results are normalized to the corresponding protein concentrations and the DMSO control. Results are shown as fold induction and are the means of three independent determinations.

4.2.4. Licorice species and LigC induce NQO1 protein expression in non-tumorigenic breast epithelial MCF-10A cells

To determine whether the hops and licorice extracts also induce NQO1 in non-tumorigenic breast cells, western blot analysis of NQO1 protein was performed in non-tumorigenic ER(-) breast epithelial MCF-10A cells. The western blot analysis showed that NQO1 is induced significantly by GG and GU extracts at 10 µg/mL in MCF-10A cells after 24 h (Figure 19A). Hops extract exhibited a higher induction level at 5 µg/mL (Figure 19A). The Michael acceptors from licorice, LigC and LicA, also induced NQO1 at 5 µM; however, the effect caused by LicA was not statistically significant (Figure 19B). XH from hops had much stronger activity at the same concentration (Figure 19B). These data suggested that licorice extracts and the tested constituents might increase NQO1 protein levels in normal breast cells.

In order to confirm the *in vitro* effects described above, animal models were employed subsequently.

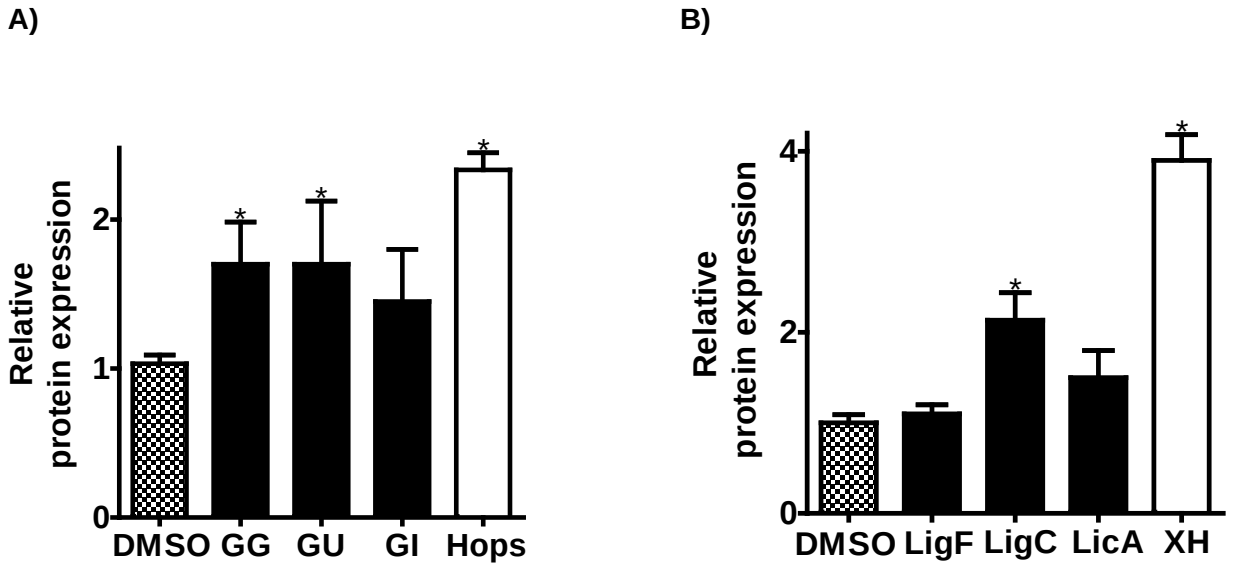

Figure 19. Induction of NQO1 in non-tumorigenic ER (-) human mammary epithelial cells (MCF-10A).
Quantitation of western blot analysis of the Induction of NQO1 in MCF-10A cells by A) crude extracts of *G. glabra*; *GG* (10 µg/mL), *G. uralensis*; *GU* (10 µg/mL, open bar), *G. Inflata*; *GI* (10 µg/mL) in comparison to hops extract (5 µg/mL) and B) characteristic compounds LigC (5 µM), LigF (10 µM), and LicA (5 µM) in comparison to XH (5 µM). Results are shown as fold induction and are the means of three independent determinations.

4.2.5. LigC and LigF are better absorbed when administered through the licorice extracts, and reach the target tissue mammary gland

As described in the Materials and Methods section, female mature Sprague-Dawley rats were randomly distributed in groups of 5 and treated orally every 24 h for four days. The LC-MS/MS analysis of GG and GU extracts, used in the *in vivo* study, showed the presence of free

LigC and LigF as well as their different glycosylated forms in the extracts (Figure 20A, Figure 20B). It also showed the presence of glabridin (a marker compound of GG) in GG extract (Figure 20A). In the animals treated with GG or GU extracts, only trace amounts of unconjugated LigC and LigF were detected in the serum (below limit of quantitation, < 0.5 ng/mL) (Figure 20A, Figure 20B). However, tissue analysis of the animals treated with the extracts showed quantifiable amounts of free LigF (2-10 ng/g tissue) in both liver and mammary tissue homogenates (Figure 20A, Figure 20B). For LigC (Figure 20C) and LigF (data not shown) administered alone to animals, free LigC and LigF were detected in the serum (<0.5 ng/mL). Free LigF (10-20 ng/g tissue) was measured in the tissues of LigF-treated animals, but less than 1 ng/g tissue of LigF and LigC were detected in LigC-treated animals. An enrichment of estrogenic LigF was observed in mammary tissue in all treatment groups including the LigC treated animals. In all groups of animals, LigC and LigF were observed as aglycones and glucuronides (data not shown). After enzymatic deconjugation of serum samples obtained 24 h after the last dose, quantitation of LigC in the hydrolyzed serum showed the highest levels of LigC in animals treated with GG (10.28 ng/mL) compared with those treated with equivalent amount of LigC (< 0.5 ng/mL, data not shown).

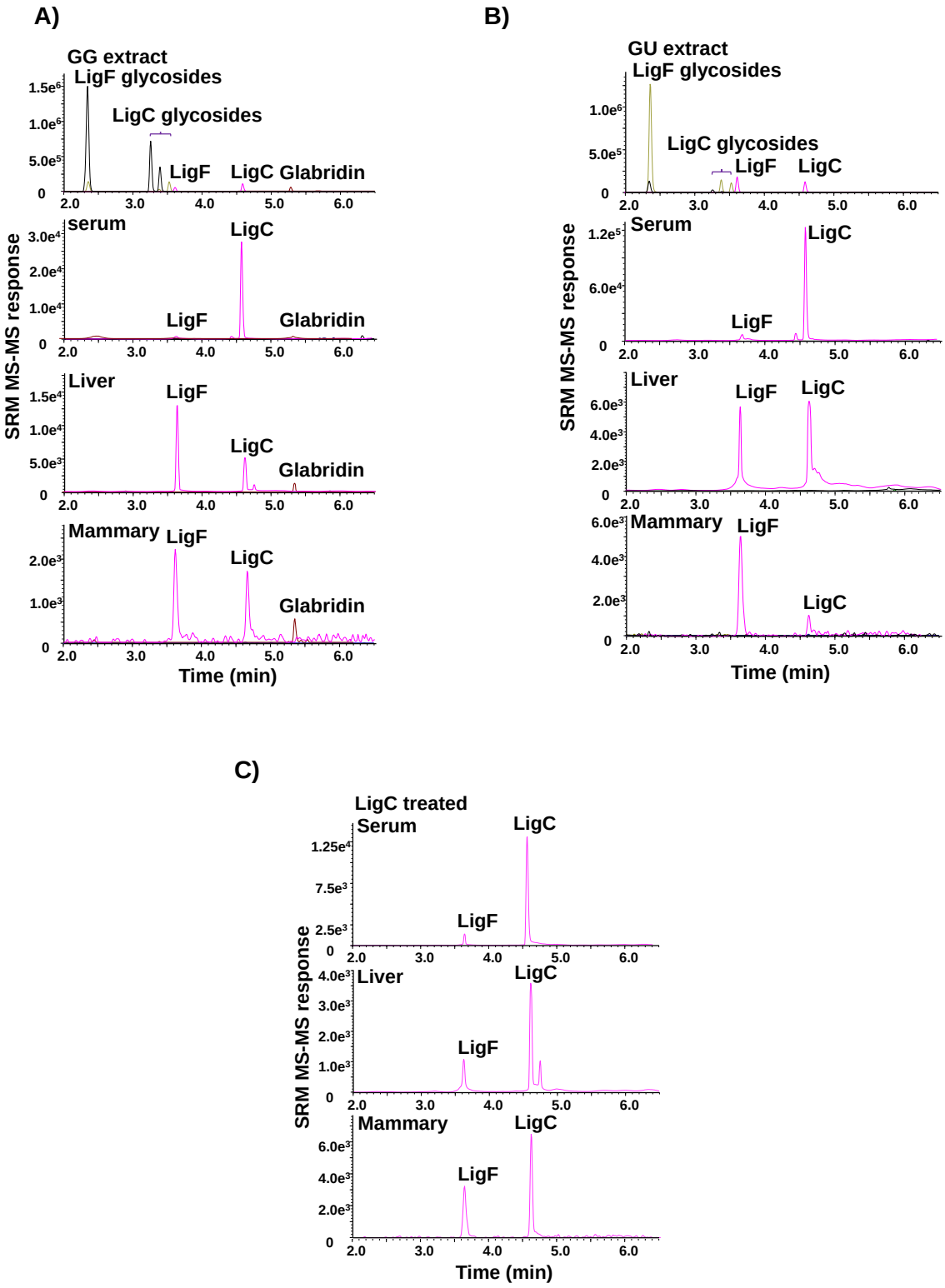

Figure 20. Tissue distribution of LigF and LigC *in vivo.*
Mature female Sprague-Dawley rats were treated with A) GG (1.3 g/kg BW per day), B) GU (1.3 g/kg BW per day), and C) LigC (40 mg/kg BW per day) for 4 consecutive days by gavage.

4.2.6. NQO1 activity increased in the mammary glands of the animals treated with GG extract

Induction of NQO1 in the rat liver homogenates after treatment with the positive control, 4'-bromoflavone, was significant relative to vehicle treatment, which was consistent with previous reports (Dietz et al. 2013; Song et al. 1999). While GG extract did not change the NQO1 activity in the liver (Figure 21A), it induced NQO1 in mammary glands significantly (Figure 21B). GU extract and pure LigC did not change NQO1 activity in either of the tissues (Figure 21A, Figure 21B). LigF reduced NQO1 activity in the liver significantly (Figure 21A), but did not change NQO1 levels in mammary tissue (Figure 21B).

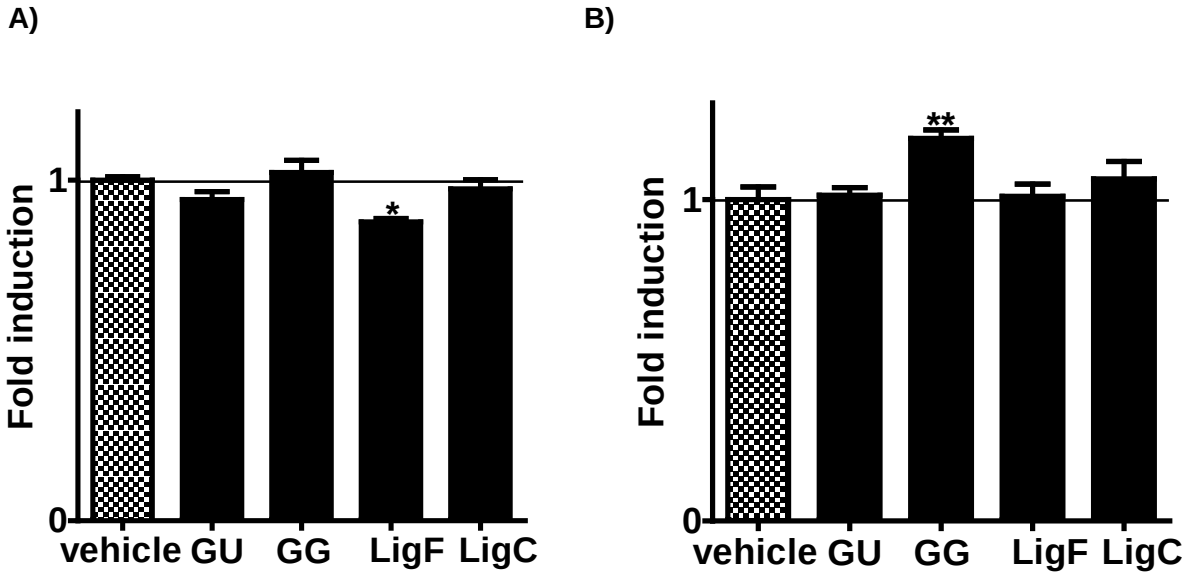

Figure 21. Induction of NQO1 by licorice extracts and bioactive compounds *in vivo*. Induction of NQO1 was assessed in A) Liver homogenate and B) mammary tissue homogenate of mature female Spargue-Dawley rats treated with GG (1.3 g/kg BW per day), GU (1.3 g/kg BW per day), LigF (80 mg/kg BW per day), and LigC (40 mg/kg BW per day) for 4 consecutive days by gavage.

4.3. Discussion

Previous studies have reported cytoprotective effects by licorice and by some of its constituents such as LigC and LicA (Jo et al. 2004; Wu et al. 2011; Cuendet et al. 2006; Tsai et al. 2014; Dunlap 2015). For example, the extracts and bioactive compounds have been reported to induce chemopreventive responses through activation of apoptotic pathways, anti-inflammatory effects, and inhibition of oxidative estrogen metabolism (Webb et al. 1992; Takahashi et al. 2004; Jo et al. 2004; Tsai et al. 2014; Dunlap 2015). It has also been shown that GU induces Nrf2-mediated genes in hepatoma cells and animal tissues (Wu et al. 2011). LigC was also reported to activate NQO1 *in vitro* and *in vivo*; however, it failed to retard breast tumor growth in animal models which might be associated with its conversion to the estrogenic compound LigF under physiological conditions as well as its poor bioavailability when administered alone, as suggested by the results presented herein (Cuendet et al. 2010; Cuendet et al. 2006; Simmler et al. 2013; Hajirahimkhan, Simmler, et al. 2013).

The fact that licorice has more than 30 different species with various chemical profiles and thus distinct biological activities has often been neglected (Asl and Hosseinzadeh 2008; Kondo, Shiba, Yamaji, et al. 2007; Dunlap 2015). The majority of previous biological evaluations of licorice have been performed with GG, which is the most commercialized species in the western world, and with GU, which is more popular in Asian countries. Comparatively, the biological properties of GI extracts have not been extensively investigated. More importantly, up to now, studies simultaneously comparing the chemical profiles and biological properties of these three medicinal *Glycyrrhiza* species are rare (Hajirahimkhan, Simmler, et al. 2013; Dunlap 2015). Nevertheless, these comparative analyses are fundamental for the meaningful interpretation of the biological results and adequate chemical standardization of licorice botanical dietary supplements.

The dose-dependent induction of NQO1 activity in Hepa1c1c7 cells by extracts from the three different *Glycyrrhiza* species (Figure 16A, Table 4) was consistent with the differences in

their chemical profiles (Table 3). GI contains more of the electrophilic chalcones that could potentially lead to better induction of NQO1 (Figure 16A). However, its CD value and slope of the NQO1 induction dose-response curve were only slightly better compared to GG. Both licorice chalcones, LigC and LicA, induced NQO1; however, LigC was more effective than LicA (Figure 16B). The intensity of NQO1 activity observed with GG and GI as well as LigC and LicA might be explained by the mechanistic differences between the active chalcones as will be further discussed. The higher NQO1 activity and the enhanced slope of hops extract in comparison to the three licorice extracts (Figure 16A, Table 4) could be due to the increased stability of XH in hops as compared to LigC in licorice which easily converts to LigF under physiological conditions (Figure 7, Figure 16A) (Simmler et al. 2013).

The mechanism of induction of detoxification enzymes such as NQO1 is believed to proceed through interaction of the transcription factor Nrf2 with the antioxidant response elements (AREs) in the promoter of their genes (Figure 22) (Jaramillo and Zhang 2013; Wakabayashi et al. 2010; Schnekenburger et al. 2014; Li and Zhou 2014). Under normal conditions, Nrf2 is sequestered in the cytosol by a cysteine-rich sensory protein, Keap1, which targets Nrf2 for ubiquitination and degradation. However, electrophilic compounds such as the chalcones in this study as well as reactive oxygen species (ROS) can interact with sulfhydryl groups in Keap1 and change the conformation of the protein (Figure 22) (Eggler et al. 2009; Eggler et al. 2005). This will result in the inability of Keap1 to target Nrf2 for degradation resulting in its accumulation in the nucleus and its enhanced interaction with ARE. This will ultimately lead to higher levels of detoxification enzymes such as NQO1, which contribute to the inactivation and excretion of reactive oxidative metabolites and therefore to cytoprotection (Figure 22) (Zhang 2006; Dinkova-Kostova and Talalay 2010). It has also been shown that xenobiotic response elements (XRE) that are regulated by the AhR are present in the promoter region of NQO1 and other Nrf2 genes in close proximity to ARE elements (Nioi and Hayes 2004; Wakabayashi et al. 2010). The licorice Michael acceptors in this study, have been shown

to modulate the AhR pathway (Dunlap 2015) and might ultimately influence the induction of NQO1 (Figure 22); however, the detailed mechanistic events leading to the cross talk between AhR and Nrf2 in influencing NQO1 induction are not well understood (Kohle and Bock 2007; Nioi and Hayes 2004; Kalthoff et al. 2010).

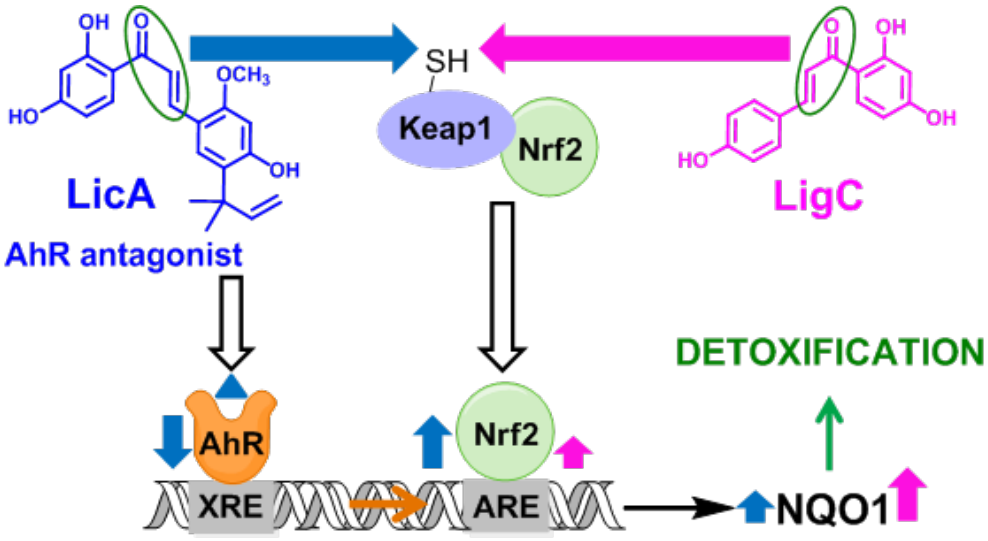

Figure 9: Differential mechanisms of NQO1 induction by Michael acceptors in licorice.
Michael acceptors LigC and LigF modulate NQO1 through different mechanisms based on the *in vitro* observations. The left side clear arrow with black borders shows the inhibitory effect of LicA on AhR pathway. The blue arrow shows the effect of LicA on Keap1-Nrf2 pathway and the induction of ARE and NQO1 by this compound. Pink arrow shows the effect of LigC on Keap1-Nrf2 pathway and the induction of ARE and NQO1 by this compound.
LigC interacts with Keap1 and the consequent translocation of Nrf2 to the nucleus and its interaction with ARE might result in NQO1 induction. LicA could influence the induction of NQO1 through two parallel, yet opposing molecular interactions at the promoter of NQO1. It can increase ARE induction through interacting with Keap1 and decrease XRE induction through inhibiting AhR, which might result in a lower NQO1 induction compared to that of LigC.

All three Michael acceptors, XH, LigC, and LicA significantly induced ARE-reporter activity suggesting activation of the Keap1-Nrf2 pathway (Figure 18B, Table 4). Interestingly, the level of induction by LicA was much higher than that of LigC and comparable to sulforaphane and XH in the ARE-luciferase induction assay; however, the induction of NQO1 by LicA was

significantly lower (Figure 18B, Figure 16B, Table 4). Recently, it was shown that LicA is an antagonist of AhR, inhibits XRE activity, and blocks carcinogenic oxidative estrogen metabolism (Dunlap 2015). Therefore, LicA might activate AREs at the promoter of NQO1 while simultaneously inhibiting the proximal XREs at this region, resulting in a lower NQO1 induction (Figure 16B, Figure 22). LigC, on the other hand, was not shown to inhibit XRE, and it promoted carcinogenic oxidative estrogen metabolism (Dunlap 2015). Other studies demonstrated that LigC and XH mainly induce NQO1 through ARE, but not through XRE (Cuendet et al. 2006; Miranda et al. 2000). These data suggested that similar to hops and XH, the licorice extracts, LigC, and LicA induced NQO1 through Keap1-Nrf2 pathway; however, LicA and thus GI might modulate Nrf2-mediated detoxification genes through XRE and ARE, simultaneously (Figure 22) (Nioi and Hayes 2004; Eggler et al. 2005). The weak NQO1 induction by LigF could be related to its conversion to LigC after 48 h (Figure 4) (Simmler et al. 2013).

To compare the NQO1 inducing properties of licorice compounds with other well-known chemopreventive compounds, curcumin (Figure 15) and sulforaphane (Figure 15) were analyzed in parallel. While sulforaphane exhibited very good NQO1 inducing properties (Figure 16C) and the best CI value (Figure 17C, Table 4) compared to the other tested compounds, its ARE-luciferase inducing activity was comparable to XH and LicA (Figure 18B, Table 4). This observation suggests that sulforaphane-induced NQO1 through ARE and other parallel mechanisms as previously reported (Kang and Pezzuto 2004; Juge et al. 2007). Nevertheless, apart from the mechanistic diversity between the tested compounds, the varied responsiveness of different cell lines (Hepa1c1c7 versus HepG2-ARE-C8) to certain compounds might contribute to the differences observed between NQO1 induction and ARE-luciferase data. Additionally, varied reactivities of the Michael acceptors towards certain proteins might contribute to the observed differences. While sulforaphane is effective for chemoprevention, it is not associated with menopausal symptom relief. In contrast, licorice and its active compounds are directly relevant to the relief of menopausal symptoms (Menati et al. 2014; Hajirahimkhan,

Dietz, et al. 2013; Nahidi et al. 2012) and the *in vitro* observed detoxification enzyme inducing effects as well as literature reports might suggest further benefit (Cuendet et al. 2010; Wu et al. 2011). As the *in vivo* detoxification enzyme inducing effects of licorice in this short-term rat model are minimal, long-term efficacy studies are necessary to analyze whether licorice has chemopreventive effects *in vivo.*

As the major goal of the study was to understand the chemopreventive potential of licorice in breast tissue, MCF-10A cells were employed as a model to evaluate the NQO1 induction by the three major *Glycyrrhiza* species and their bioactive constituents in non-tumorigenic breast epithelial cells. The significant induction of NQO1 by GG, GU, and LigC in MCF-10A cells (Figure 19) further confirmed the chemopreventive potential of these licorice species and LigC. It has been shown that sulforaphane reduced estrogen DNA adducts in MCF-10A cells through up-regulating enzymes that detoxify genotoxic estrogen quinones, such as NQO1 (Yang et al. 2013). Similarly, it was recently shown that LicA significantly inhibits estrogen metabolism to genotoxic catechols in MCF-10A cells (Dunlap 2015).

Intact, mature, female Sprague-Dawley rats were employed to study the role of licorice extracts and their characteristic aglycones LigC and LigF in modulating NQO1 activity *in vivo.* As GI is not recognized by the United States Pharmacopoeia and obtaining pure plant material of this single species in the marketplace is a challenge, thus genetically and chemically authenticated GI was not available in sufficient quantities for *in vivo* evaluations. Therefore, only authenticated and fully characterized GG and GU extracts were prepared for the *in vivo* study. A high dose of 1.3 g extract/kg BW per day was employed, as the clinically relevant dose (65 mg/kg BW per day) did not enhance NQO1 activity *in vivo* (data not shown). The doses of LigC and LigF were calculated to mimic the contents of the high dose of GG extract, which is characterized by a higher amount of LigC equivalents than GU (Table 3). The administered GG and GU extracts contained mainly the glycosylated forms of LigC and LigF and only a small portion of the extracts were aglycones (Figure 20A and Figure 20B, Table 3). However, *in vivo*,

only LigC and LigF aglycones (Figure 20A, Figure 20B) and their metabolites (data not shown) were detectable suggesting the hydrolysis of LigC/LigF glycosides *in vivo*. Deglycosylation can occur in digestive tract starting from stomach with a low pH, although the current literature reports are controversial (Kamei et al. 2005). There are also limited information on the absorption and pharmacokinetics of various glycosides in comparison to their unconjugated counterparts in licorice extracts (Zuo et al. 2002).

Interestingly, LigC and LigF, which also have estrogenic properties (Hajirahimkhan, Simmler, et al. 2013) were distributed to mammary glands (Figure 20). Recent pharmacokinetic studies have shown that the bioavailability of LigC is relatively low due to its conversion to LigF as well as its fast and extensive metabolism (Choi et al. 2015; Hajirahimkhan, Simmler, et al. 2013; Simmler et al. 2013). Our data also showed that in LigC treated animals, LigC is mainly converted to LigF (Figure 20C). Extensive rapid metabolism, cyclization, and the interaction with glutathione could contribute to the negligible induction of NQO1 with free LigC (Figure 20C, Figure 21). However, in animals treated with extracts, in which glycosylated forms of LigC and LigF are abundant, LigC might be protected from rapid conversion to LigF. This may lead to a longer and sustained availability of LigC. Hence, GG extract, containing more LigC glycosides compared to GU, was able to induce NQO1 *in vivo* (Table 3, Figure 20A, Figure 20B, Figure 21). For example, in serum higher total LigC concentrations were observed after application of GG extract (10.28 µg/mL), while the levels of LigC in the samples treated with the equivalent purified LigC was below the limit of quantification. In addition, matrix effects of the extract might play a role in providing higher LigC serum concentrations after GG administration compared to purified LigC. Also, it is noteworthy that there might be some other active components in the GG extract, different from LigC, that may contribute to the higher NQO1 induction.

LigC as a Michael acceptor, has been shown to form glutathione conjugates in rat liver (Cuendet et al. 2010). GSH conjugates of Michael acceptors are often reversible which could suggest that LigC GSH conjugates could regenerate LigC in tissues where GSH concentrations

are low (i.e., mammary gland) (Chandrasena et al. 2008; Randall et al. 2013). The higher NQO1 induction by GG in the mammary gland compared to the liver might be associated with the lower levels of glutathione leading to more available free LigC in mammary tissue to induce NQO1. In addition, the different electrophilicities of the chalcones (LigC, LicA) towards glutathione as well as other biological targets need to be considered in future studies.

The minimal NQO1 induction observed *in vivo*, could be due to several factors. Oral administration of the extracts and LigC results in extensive degradation and Phase I and Phase II metabolism throughout the gastrointestinal tract (Choi et al. 2015; Cuendet et al. 2010). In addition, LigC is prone to rapid cyclization to LigF (Figure 7) (Simmler et al. 2013). All of these transformations result in low free LigC concentration (< limit of quantitation). Glycosylated LigC in the extracts might be hydrolyzed to the aglycone throughout the digestive tract and liver; however, hydroxylation and extensive glucoronidation of the LigC aglycone leads to low bioavailability of LigC (Choi et al. 2015; Lee et al. 2013). It was previously shown that dietary administration of much higher doses of LigC (10g/kg) in a long-term animal study (85 days) caused a small but significant NQO1 induction in the colon and mammary gland of female Sprague-Dawley rats (Cuendet et al. 2010). However, this study also described extensive metabolism of LigC, which might have ultimately impacted the chemopreventive outcomes (Cuendet et al. 2010). It should be noted that the effects observed with licorice and its bioactive compounds in a four day rat study could be very different from the outcomes that might be observed in menopausal women taking them over a long period of time. Considering the *in vitro* data and the minimal induction of NQO1 by GG in the mammary tissue, the chemopreventive potential of licorice and its bioactive compounds needs further evaluations before recommending licorice supplements for chemoprevention in women. This recommendation will depend on having fully characterized extracts that are precisely standardized to their well studied bioactive compounds.

In conclusion, our *in vitro* comparison of three authenticated *Glycyrrhiza* species suggests that the electrophilic compounds such as LigC and LicA could contribute to the activation of detoxification enzymes through the Keap1-Nrf2 pathway. However, LicA might employ an additional mechanism as an AhR antagonist which could modulate NQO1 through parallel yet opposing effects on ARE and XRE elements. In addition, comparison with known chemopreventive compounds, such as sulforaphane, curcumin, and XH showed the following rank order of NQO1 induction; sulforaphane >> XH > LigC > LicA ≈ curcumin >> LigF. The bioactive compounds LigF and LigC were detectable in the liver and mammary gland of rats treated with GG; however, the amount of the aglycones are very low and significant NQO1 induction was only observed in the mammary gland. This study clearly demonstrates the differential roles of Michael acceptors, LigC and LicA, in exerting chemopreventive effects by licorice extracts *in vitro* and it shows the weak NQO1 induction by GG *in vivo*. Further experiments are planned to test the NQO1 *in vivo* induction potential of GI and LicA especially since the chemical profile and biological activities are likely quite different.

Although the *in vivo* effects of GG and GU in the rat model are minimal, it must be emphasized that menopausal women take these supplements for extended periods of time and long-term efficacy is currently unknown. Finally, this study further emphasizes the importance of standardization and chemical/biological characterization of botanical supplements to their specific bioactive compounds.

5. Conclusions and future directions

A growing number of menopausal women use dietary supplements to alleviate menopausal symptoms. Data regarding the safety and efficacy of these remedies are scarce. Licorice as one of the popular botanicals for management of menopausal symptoms has been found to consist of more than 30 different species. The current study is a novel example of the simultaneous chemical and biological characterization of licorice species *in vitro* and *in vivo*. While licorice species and LigF exhibit estrogenic activity in multiple cell based assays, they did not increase uterine weight in a rat model. However, subcutaneous LigF suppresses the proliferative effects of E_2. This observation could be associated with the partial agonist effects of LigF and licorice extracts as well as the higher affinity for ERβ. Moreover, apart from the phenotypic results such as uterine weight, there might be certain estrogenic effects induced by the licorice species and LigF at the molecular level. Therefore, the next step would be the immunohistochemistry analysis of the animal tissues to study changes in estrogenic markers such as Ki67, PCNA, and complement C3. Also it would be interesting to study licorice and its constituents for the activation of non-classical estrogenic effects including their role in modulating GPERs and the associated pathways.

Licorice extracts, LigC, and LicA exhibited a considerable NQO1 induction *in vitro* via activation of Keap1-Nrf2 pathway. The effects were minimal *in vivo* which could be associated with the low bioavailability of the active constituent LigC when administered orally in addition to its rapid metabolism. However, long term use of licorice botanicals might show different outcomes. GI and its active compound LicA which will be evaluated in the future could provide a more pronounced chemopreventive effects in animals as LicA is a more stable chalcone than LigC.

Our findings in this study demonstrates that licorice does not promote unsafe proliferative responses in estrogen responsive tissues, uterus and LigF can inhibit proliferative

effects of estradiol. It also suggests licorice could exert cytoprotective effects such as the induction of NQO1 in mammary glands. Therefore, licorice might be a beneficial plant for menopausal women. The study also further emphasize the necessity of simultaneous chemical and biological evaluations of botanicals such as licorice. It also shows the importance of authentication and precise labeling of botanical dietary supplements.

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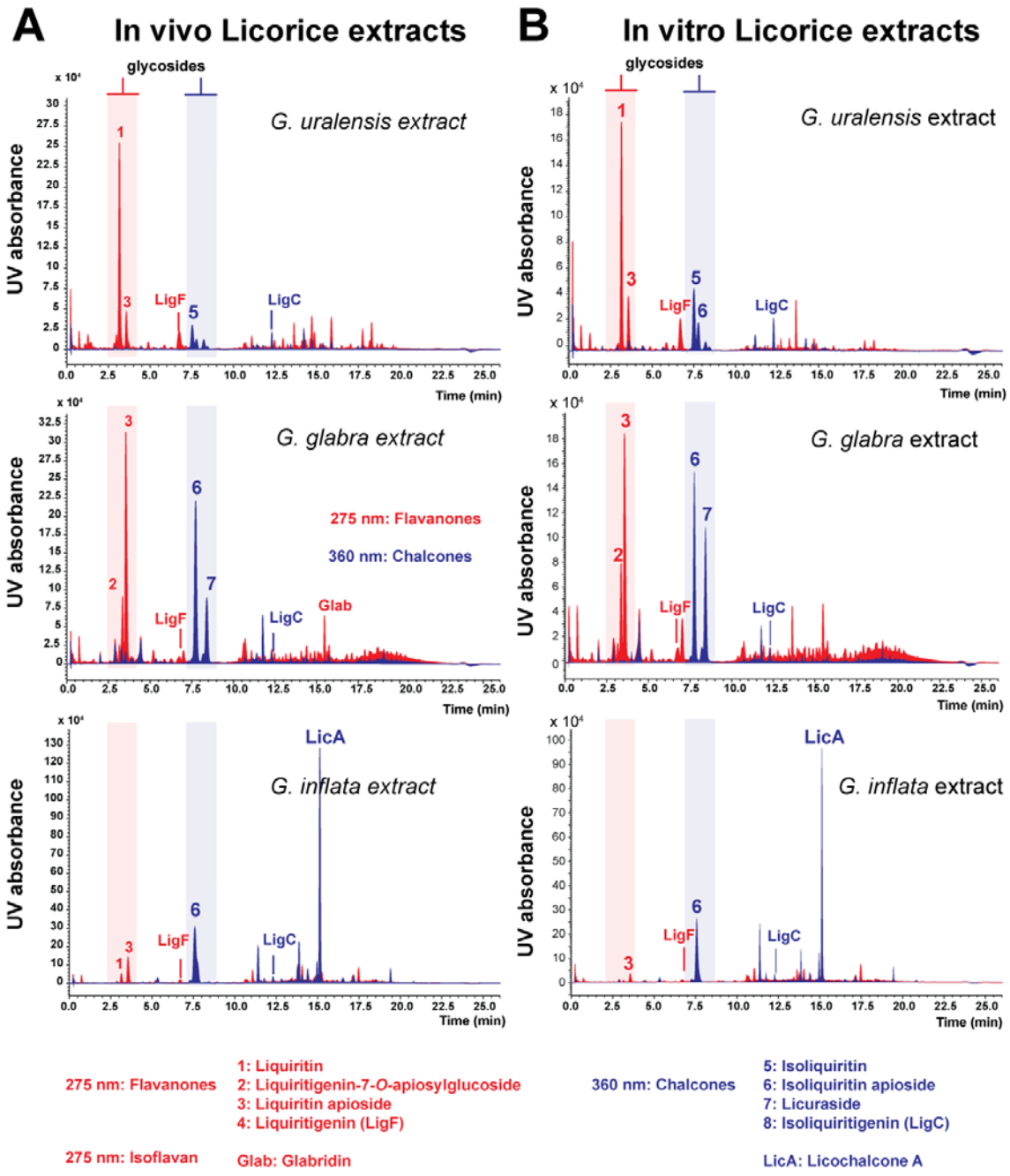
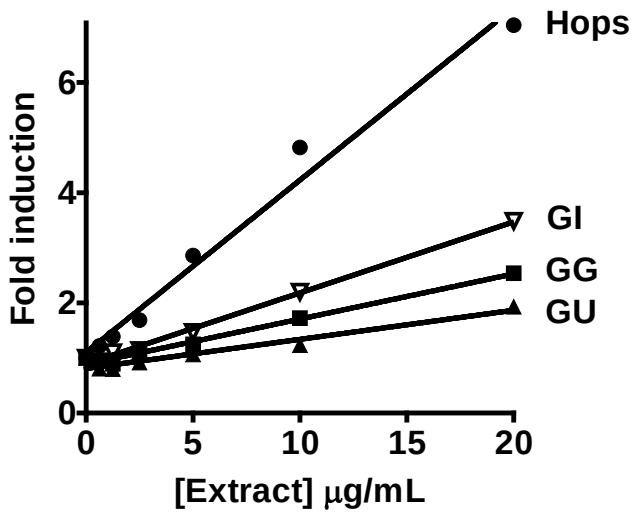
Appendices

Appendix A



Appendix A (continued)

A)



B)

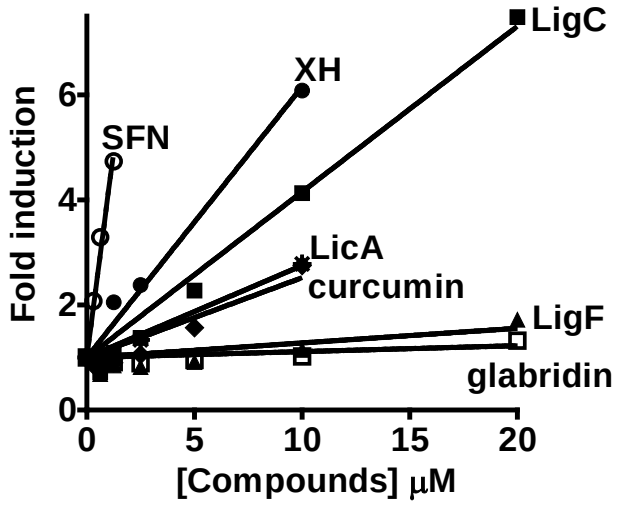
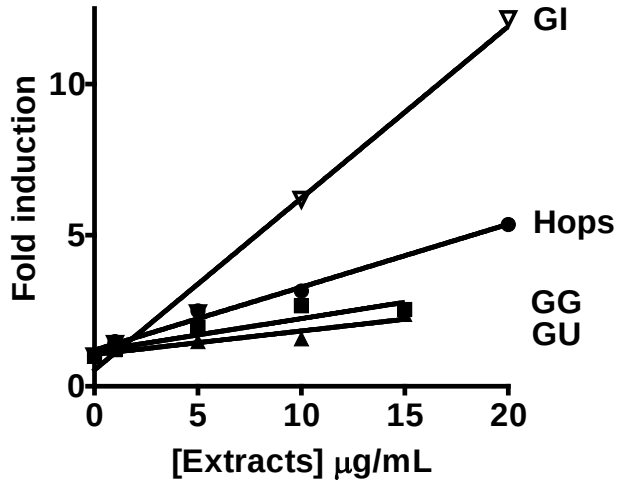


Figure S 1. Chemical profile comparison of the licorice extracts.

Figure S 2. Linear regression analysis of NQO1 induction in hepac1c1c7 cells. The analysis was performed on the NQO1 data obtained by A) GG, GU, GI, and hops extracts, B) LigC, LigF, and LicA from licorice, XH from hops, sulforaphane from broccoli, curcumin from turmeric, and glabridin.

Appendix A (continued)

A)



B)

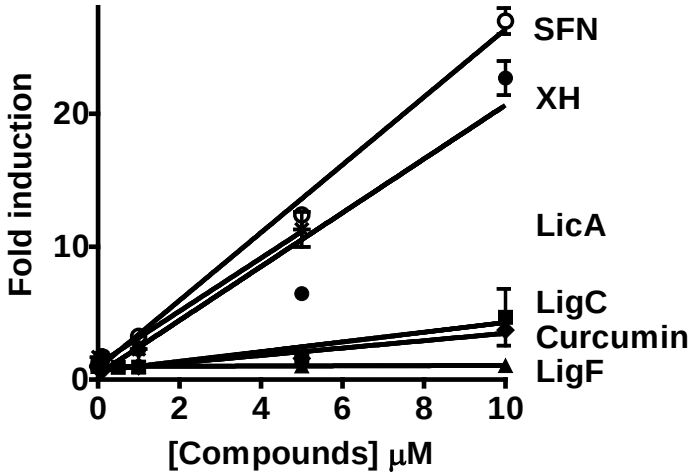


Figure S 3. Linear regression analysis of ARE-luciferase induction in HepG2-ARE-C8 cells.
The analysis was performed on ARE-luciferase data obtained by A) GG, GU, GI, and hops extracts B) LigC, LigF, and LicA from licorice, XH from hops, sulforaphane from broccoli, and curcumin from turmeric.

Appendix B

Permission letters

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Appendix B (continued)

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Via E-Mail

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VITA

Atieh Hajirahimkhan

Education

2009-2016	PhD, Medicinal Chemistry, University of Illinois at Chicago, Chicago, IL.
2003-2007	MSc, Clinical Biochemistry, Urmia University of Medical Sciences, Urmia, Iran
1995-2000	BSc, Chemistry, Isfahan University of Technology, Isfahan, Iran

Experiences

2010-present	Research assistant, University of Illinois at Chicago
2009-2010	Teaching assistant, University of Illinois at Chicago
2013-2014	Certification of the Program for Academic Instruction, University of Illinois at Chicago
2005-2007	Teaching assistant, Urmia University of Medical Sciences, Urmia, Iran

Publications

1. Hajirahimkhan, A., Simmler, C., Dong, H., Lantvit, D., Li, G., Chen, S., Nikolic, D., Pauli, G. F., van Breemen, R. B., Dietz, B. M., Bolton, J. L. (2015) Induction of NAD(P)H:quinone oxidoreductase 1 (NQO1) by *Glycyrrhiza* species used for women's health: differential effects of the Michael acceptors isoliquiritigenin and licochalcone A. *Chem Res Toxicol.* 28: 2130-2141.

2. Krause, E., Yuan, Y., Hajirahimkhan, A., Dong, H., Dietz, B. M., Nikolic, D., Pauli, G. F., Bolton, J. L., van Breemen, R. B. (2014) Biological and Chemical Standardization of a Hop (*Humulus lupulus*) Botanical Dietary Supplement. *Biomed Chromatogr.* 28: 729-734.

3. Hajirahimkhan, A., Simmler, C., Jeffery R. Anderson, Yuan, Y., Nikolic, D., Chen, S., Dietz, B. M., Pauli, G. F., van Breemen, R. B., and Bolton, J. L. (2013) Evaluation of the estrogenic

activity of licorice species in comparison with hops for menopausal symptoms. *PLoS One*. 8: e67947.

4. Simmler, C., Hajirahimkhan, A., Lankin, D., Bolton, J. L., Jones, T., Soejarto, D. D., Chen, S., Pauli, G., F. (2013) Dynamic residual complexity of the isoliquiritigenin-liquiritigenin interconversion during bioassays. *J Agric Food Chem.* 61: 2146-2157.

5. Hajirahimkhan, A., Dietz, B. M., Bolton, J. L. (2013). Botanical Modulation of menopausal symptoms: Mechanisms of action. *Planta Med.* 79: 538-553.

6. Eftekhar, E., Hajirahimkhan, A., Taghizadeh Afashari, A., Nourooz-Zadeh, J. (2012) Plasma glutathione peroxidase activity in kidney recipients with and without adverse outcomes. *Renal Fail*. 34: 628-633.

Poster presentations

1. "*In vitro* and *in vivo* evaluation of NAD(P)H:quinone oxidoreductase 1 (NQO1) induction by licorice species used in botanical dietary supplements for women's health.", Hajirahimkhan, A., Simmler, C., Dong, H., Lantvit, D., Li, G., Chen, S., Nikolic, D., Pauli, G. F., van Breemen, R. B., Dietz, B. M., Bolton, J. L. Minnesota, Illinois, Kansas, Iowa Medicinal Chemistry meeting, Lawrence, KS. (April 2015).

2. "Chemopreventive effects of licorice species used in botanical dietary supplements for women's health: *in vitro* and *in vivo* evaluation of NAD(P)H:quinone oxidoreductase 1(NQO1) induction.", Hajirahimkhan, A., Simmler, C., Dong, H., Lantvit, D., Li, G., Chen, S., Nikolic, D., Pauli, G. F., van Breemen, R. B., Dietz, B. M., Bolton, J. L. UIC College of Pharmacy Research Day, Chicago, IL. (March 2015).

3. "Induction of detoxification enzyme NAD(P)H:quinone oxidoreductase 1 by licorice species used in botanical supplements for women's health.", Hajirahimkhan, A., Dietz, B. M., Dong, H., Simmler, C., Chen, S., Nikolic, D., Pauli, G. F., van Breemen, R. B., Bolton, J. L., 248th American Chemical Society National meeting, San Francisco, CA. (August 2014).

4. "Induction of detoxification enzyme NAD(P)H:quinone oxidoreductase 1 by licorice species used in botanical supplements for women's health.", Hajirahimkhan, A., Dietz, B. M., Dong, H., Simmler, C., Chen, S., Nikolic, D., Pauli, G. F., van Breemen, R. B., Bolton, J. L., UIC Women's health research day, Chicago, IL. (April 2014).

5. "Induction of detoxification enzyme NAD(P)H:quinone oxidoreductase 1 by licorice species used in botanical supplements for women's health.", Hajirahimkhan, A., Dietz, B. M., Dong, H., Simmler, C., Chen, S., Nikolic, D., Pauli, G. F., van Breemen, R. B., Bolton, J. L., UIC Cancer research forum, Chicago, IL. (April 2014).

6. "Induction of detoxification enzyme NAD(P)H:quinone oxidoreductase 1 by licorice species used in botanical supplements for women's health.", Hajirahimkhan, A., Dietz, B. M., Dong, H., Simmler, C., Chen, S., Nikolic, D., Pauli, G. F., van Breemen, R. B., Bolton, J. L., UIC Student Research Forum, Chicago, IL. (April 2014).

7. "Induction of detoxification enzyme NAD(P)H:quinone oxidoreductase 1 by licorice species used in botanical supplements for women's health.", Hajirahimkhan, A., Dietz, B. M., Dong, H., Simmler, C., Chen, S., Nikolic, D., Pauli, G. F., van Breemen, R. B., Bolton, J. L., UIC College of Pharmacy Research Day, Chicago, IL. (March 2014).

8. "Comparison of the chemopreventive potential of two licorice species with hops.", Hajirahimkhan, A., Dietz, B. M., Dong, H., Simmler, C., Chen, S., Nikolic, D., Pauli, G. F., van Breemen, R. B., Bolton, J. L., Gordon Conference in Molecular and Cellular Mechanisms of Toxicity, Andover, NH. (August 2013).

9. "Comparison of the estrogenic activity of licorice species with hops in botanical dietary supplement formulations for women's health." Hajirahimkhan, A., Simmler, C.; Yuan, Y., Nikolic, D., Chen, S., Dietz, B. M., Pauli, G. F., van Breemen, R. B., Bolton, J. L., 52th SOT annual meeting, San Antonio, TX. (March 2013).

10. "Comparison of the chemopreventive potential of two licorice species with hops." Hajirahimkhan, A., Dietz, B. M., Dong, H., Simmler, C., Chen, S., Nikolic, D., Pauli, G. F.,

van Breemen, R. B., Bolton, J. L., Great Lakes Nuclear Receptor Conference, IL. (October 2012).

11. "Comparison of the chemopreventive potential of two licorice species with hops.",Hajirahimkhan, A., Dietz, B. M., Dong, H., Simmler, C., Chen, S., Nikolic, D., Pauli, G. F., van Breemen, R. B., Bolton, J. L., Reproductive Sciences Symposium, Chicago, IL. (October 2012).

12. "Evaluation of estrogenic activity of licorice species and hops for menopausal symptoms", Hajirahimkhan, A., Simmler, C., Yuan, Y., Nikolic, D., Chen, S., Dietz, B. M., Pauli, G. F., van Breemen, R. B., and Bolton, J. L., Minnesota, Illinois, Kansas, Iowa Medicinal Chemistry meeting, Iowa City, IO. (April 2012).

13. "Evaluation of estrogenic activity of licorice species and hops for menopausal symptoms", Hajirahimkhan, A., Simmler, C., Yuan, Y., Nikolic, D., Chen, S., Dietz, B. M., Pauli, G. F., van Breemen, R. B., and Bolton, J. L., UIC Cancer Forum, Chicago, IL. (March 2012).

14. "Evaluation of estrogenic activity of licorice species and hops for menopausal symptoms", Hajirahimkhan, A., Simmler, C., Yuan, Y., Nikolic, D., Chen, S., Dietz, B. M., Pauli, G. F., van Breemen, R. B., and Bolton, J. L., UIC College of Pharmacy Research Day, Chicago, IL. (March 2012).

15. "L-Citrulline as a surrogate biomarker of kidney transplantation outcome, a follow-up study", Hajirahimkhan, A., Eftekhar, E., Ahmadpour, P., Nourooz-Zadeh, J. 8th Iranian International Congress of Biochemistry and Molecular Biology, Tehran, Iran (September 2006).

Oral presentations

1. "Induction of detoxification enzyme NAD(P)H:quinone oxidoreductase 1 by licorice species used in botanical supplements for women's health.", Hajirahimkhan, A., Dietz, B. M., Dong, H., Simmler, C., Chen, S., Nikolic, D., Pauli, G. F., van Breemen, R. B., Bolton, J. L., Illinois Symposium on Reproductive Sciences, Chicago, IL. (October 2014).

2. "Evaluation of the estrogenic and chemopreventive properties of licorice species used in botanical dietary supplements for menopausal women.", Hajirahimkhan, A., Simmler, C., Dong, H., Lantvit, D., Huang, K., Chen, S., Nikolic, D., Dietz, B. M., Pauli, G. F., van Breemen, R. B., Bolton, J. L., Minnesota, Illinois, Kansas, Iowa Medicinal Chemistry meeting, Chicago, IL. (April 2014).

Awards and honors

2014 Van Doren Scholar award, UIC College of Pharmacy, Chicago, IL.

2014 Best oral presentation, Minnesota, Illinois, Kansas, Iowa regional Medicinal Chemistry meeting, Chicago IL.

2014 UIC Graduate College travel award.

2014 UIC Graduate Student Council travel award.

2013 Van Doren Scholar award, UIC College of Pharmacy, Chicago, IL.

2013 Society of Toxicology (SOT) travel award, San Antonio, TX.

2013 Gordon Conference travel award, Andover, NH.

2013 UIC Graduate College travel award.

2013 UIC Graduate Student Council travel award.

2012 Best poster presentation award in Great Lakes Nuclear Receptor Conference, Chicago, IL., 2012.

2012 Honorary membership of the Endocrine Society, Chicago, IL.

2007 Best translational research thesis in biomedical research, Urmia university of Medical Sciences, Iran.

Affiliations/memberships

2014-present	Member of the organizing committee of “Expanding Your Horizons-Chicago” annual symposium
2014-present	Member of the graduate student council of the UIC Department of Medicinal Chemistry
2009-present	Member of the UIC Graduate Student Council
2013-present	Member of the American Association for the Advancement of Science
2012-present	Member of the American Society of Pharmacognosy
2012-2013	Honorary membership of the Endocrine Society
2012-present	Member of the Society of Toxicology
2012-present	Member of the American Chemical Society