Evaluation of Estrogenic Activity of Licorice Species in Comparison with Hops Used in Botanicals for Menopausal Symptoms

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Abstract
The increased cancer risk associated with hormone therapies has encouraged many women to seek non-hormonal alternatives including botanical supplements such as hops (Humulus lupulus) and licorice (Glycyrrhiza spec.) to manage menopausal symptoms. Previous studies have shown estrogenic properties for hops, likely due to the presence of 8-prenylnaringenin, and chemopreventive effects mainly attributed to xanthohumol. Similarly, a combination of estrogenic and chemopreventive properties has been reported for various Glycyrrhiza species. The major goal of the current study was to evaluate the potential estrogenic effects of three licorice species (Glycyrrhiza glabra, G. uralensis, and G. inflata) in comparison with hops. 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 H. lupulus > G. uralensis > G. inflata > G. glabra. Liquiritigenin was found to be the principle phytoestrogen of the licorice extracts; however, it exhibited lower estrogenic effects compared to 8-prenylnaringenin in functional assays. Isoliquiritigenin, the precursor chalcone of liquiritigenin, demonstrated significant estrogenic activities while xanthohumol, a metabolic precursor of 8-prenylnaringenin, was not estrogenic. Liquiritigenin showed ERβ selectivity in competitive binding assay and isoliquiritigenin was equipotent for ER subtypes. The estrogenic activity of isoliquiritigenin could be the result of its cyclization to liquiritigenin under physiological conditions. 8-Prenylnaringenin had nanomolar estrogenic potency without ER selectivity while xanthohumol did not bind ERs. These data demonstrated that Glycyrrhiza species with different contents of liquiritigenin have various levels of estrogenic activities, suggesting the importance of precise labeling of botanical supplements. Although hops shows strong estrogenic properties via ERs, licorice might have different estrogenic activities due to its ERβ selectivity, partial estrogen agonist activity, and non-enzymatic conversion of isoliquiritigenin to liquiritigenin.

Citation: Hajirahimkhan A, Simmler C, Yuan Y, Anderson JR, Chen S-N, et al. (2013) Evaluation of Estrogenic Activity of Licorice Species in Comparison with Hops Used in Botanicals for Menopausal Symptoms. PLoS ONE 8(7): e67947. doi:10.1371/journal.pone.0067947

Editor: Aamir Ahmad, Wayne State University School of Medicine, United States of America

Received March 6, 2013; Accepted May 23, 2013; Published July 12, 2013

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Funding: Support for this work was provided by P50 AT00155 jointly provided to the University of Illinois at Chicago/National Institutes of Health Center for Botanical Dietary Supplements Research by the Office of Dietary Supplements (ods.od.nih.gov/) and the National Center for Complementary and Alternative Medicine (ncim.nih.gov/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction
Because of an increased life expectancy in recent years, many women spend the last third of their lives in post menopause [1]. 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 [2,3,4]. 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 [5,6,7]. Currently, there is insufficient evidence on the efficacy of botanicals for menopausal symptom relief and their mechanisms of actions are not fully understood [8].

Hops (Humulus lupulus L.) is a well studied botanical for women’s health and a common constituent of dietary supplements, particularly in Europe [9,10,11,12]. Hops and its phytoconstituents, including 8-prenylnaringenin (8PN) and its metabolic precursor chalcone, xanthohumol (XH) (Figure 1) have been studied for their estrogenic and chemopreventive properties [11,12,13,14,15,16]. Hops have been shown to exert estrogenic activity in endometrial cancer (Ishikawa) and breast cancer (MCF-7) cells [12]. One of its bioactive compounds, 8-PN, the most potent phytoestrogen known to date [17], 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 [11,12,18]. 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 [11,18]. While small amounts of 8-PN are present in most hops preparations, additional 8-PN can be biosynthesized \textit{in vivo} through metabolism of XH (Figure 1). 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 [19]. On the other hand, XH, which does not have estrogenic properties, has been reported to possess chemopreventive potential, through the induction of detoxification enzymes [15].

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 [20]. Today, licorice is mainly used as a flavoring and sweetening agent in tobacco industry, chewing gums, candies, toothpastes and beverages [20] and is one of the most popular components of menopausal dietary supplements in the United States [21,22,23]. Licorice has been studied for its estrogenic properties since 1950 [24], although the findings about its efficacy have not been conclusive [8]. 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. The licorice species \textit{Glycyrrhiza glabra} (GG), \textit{Glycyrrhiza uralensis} (GU), and \textit{Glycyrrhiza inflata} (GI) have been reported to contain various amounts of liquiritin, the glycosylated form of the dihydroflavanone, liquiritigenin (LigF) (Figure 1) and its precursor chalcone, isoliquiritigenin (LigC) (Figure 1), all of which have been reported to have estrogenic activity \textit{in vitro} [25,26,27]. However, a comparative biological evaluation of distinct \textit{Glycyrrhiza} species has not been conducted to date. In addition it is rarely clear which \textit{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.

In the present study, crude extracts of three licorice species, GG, GU, GI, as well as their active compounds, LigF and LigC, were examined for their \textit{in vitro} estrogenic activity and were systematically compared with a spent hops (\textit{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 ER\textsubscript{B} selective phytoestrogen, LigF and an estrogenic chalcone LigC which in turn can convert to LigF. The chalcone \texttt{-} 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 [28,29] which could differ among individuals with various metabolism characteristics. These data suggest that licorice extracts could benefit menopausal women due to moderate estrogenic activity, ER\textsubscript{B} selectivity, and potentially a more predictable PK profile.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Estrogenic compounds in licorice and hops are formed from chalcones. Metabolism of bioactive compounds from A) hops and B) licorice. \textit{doi:10.1371/journal.pone.0067947.g001}}
\end{figure}
### Table 1. Quantification of chalcones and flavanones in the *Glycyrrhiza* extracts.

| Quantified compounds | % in the crude extract (weight/weight dry extract) |
|----------------------|--------------------------------------------------|
|                      | GU                  | GI        | GQ        |
| Liquiritin (Glc-LigF) | 3.23±0.13          | 5.47±0.18 | 1.64±0.08 |
| Isoliquiritin (LigF)  | 0.16±0.05          | 0.06±0.05 | 0.05±0.03 |
| Isoliquiritigenin (LigC) | 0.69±0.15      | 3.24±0.78 | 1.64±0.16 |
| Isoliquiritin (Glc-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% 43% 51%      | 63% 43% 51% |
| Total C/Total(F+C) | 18% 23% 49%      | 37% 23% 49% |

Values are expressed as mean ± SD of three independent analyses of each crude extract.
doi:10.1371/journal.pone.0067947.t001

### Materials and Methods

#### 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). 8-PN was synthesized [11] and XH was isolated from *H. lupulus* cv. Nugget as described previously [30].

#### 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 ¹H NMR (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 100% qHNMR method [31] was applied to determine the purity of each compound and gave the following results: LigF (lot 12290-620) 96.9% w/w (calculated weight/ measured weight), LigC (lot 9265–720) 92.6% w/w, 8-PN 95.0% w/w, XH 96.5% w/w.

#### Plant material, extraction, and characterization

Pelletized strobili of *Humulus lupulus* cv. Nugget were bulk extracted with food-grade ethanol. The fluid extract was dispersed on the diatomaceous earth, dried, and bulk extracted with supercritical CO₂ 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 earthen 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. (Leguminosae/ 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 Xining province, China in 2008. A Botanical Center number was attributed to each sample which was identified through a series of macroscopic and microscopic analyses compared to authentic voucher samples deposited at the Chicago Field Museum.

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 μm) with PDA detection at 275 nm for the flavanones (LigF and liquiritin) and 360 nm for the chalcones (LigC and isoliquiritin). Samples (1 μL injected) were eluted at 0.3 mL/min using the following gradient composition (A) H₂O+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).
Cell culture conditions

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 [32,33,34]. The Ishikawa cell line is a well-established ERα (+) endometrial cancer cell line for the evaluation of estrogens and antiestrogens [32,34]. 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 [35] 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%.

Detection of ER ligands using pulsed ultrafiltration LC-MS

A screening assay based on ultrafiltration mass spectrometry [36] 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 xg 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 C18 (1.6 µm, 2.0 mm ×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

**Table 2.** AP induction, cytotoxicity, ER binding, and ERE-luciferase induction of licorice, hops and their isolated compounds. 

|                     | 17β-estradiol | GU | GG | GI | hops | LigF | LigC | 8-PN | XH |
|---------------------|---------------|----|----|----|------|------|------|------|----|
| ERE-luciferase fold induction (n = 9) | 4.8±0.4       | 2.8±0.4 | 1.9±0.3 | 2.0±0.3 | 4.2±0.2 | 2.1±0.4 | 3.2±0.9 | 4.6±0.9 | N/A |
| IC₅₀ (n = 9) ERβ    | 0.015±0.02    | >50 | >50 | >25 | 27±3¹ | 7.5±0.5 | 7.8±0.1 | 1.7±0.1¹ | N/A² |
| IC₅₀ (n = 9) ERα    | 0.021±0.03    | >200 | >200 | >200 | 15±3¹ | >200 | 16±1 | 0.51±0.07³ | N/A² |
| Maximum AP fold induction Ishikawa cells | 137±2.5 | 58.7±2.3 | 26.9±3.0 | 61±14.7 | 100±15 | 83.1±3.4 | 57.9±4.3 | 118±6.0 | N/A |
| AP induction Ishikawa cells | 0.00019±0.00005 | 8.3±0.8 | 10.3±0.5 | 9.8±0.4 | 2.1±0.3 | 3.4±0.4 | 2.7±0.2 | 0.00665±0.00140 | N/A |

*Values are expressed as the mean ± SD of n determinations. Experimental details are described in the Materials and Methods section. † Values are expressed in µg/mL for extracts and µM for isolated compounds. § 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. ¶ Ratio of the sum of the firefly and renilla luminescences. N/A, not active. J. Agric. Food Chem. 2005, 53, 6246–6253. J. Agric. Food Chem. 2001, 49, 2472–2479.

doi:10.1371/journal.pone.0067947.t002

Figure 3. Liquiritigenin selectively binds to ERβ. Competitive ER binding using human recombinant A) ERα and B) ERβ. doi:10.1371/journal.pone.0067947.g003

Figure 4. Different *Glycyrrhiza* species and their bioactive compounds are partial ER agonists with varied estrogenic potency and efficacy in Ishikawa cells. Induction of alkaline phosphatase in Ishikawa cells by A) crude extracts of *Glycyrrhiza glabra*; *GG*; *G. uralensis*; *GU*; *G. inflata*; *GI* in comparison to hops and estradiol and B) isolated compounds liquiritigenin and isoliquiritigenin in comparison to 8-PN and estradiol. Results were normalized to DMSO and are shown as fold induction. Results are the means of three independent determinations. Dose-response curves were generated by non-linear regression analysis. doi:10.1371/journal.pone.0067947.g004
determinations in duplicates treated with vehicle only. Results are the means of three independent experiments shown as a fold induction relative to the level observed in cells control. Results were normalized for transfection efficiency, and they showed a considerable estrogenic activity at 2 µM. Estradiol (1 nM) was used as positive control. Since hops extract data was obtained from NEN Life Science Products (Boston, MA), and 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. doi:10.1371/journal.pone.0067947.g006

Figure 5. Different *Glycyrrhiza* species and their bioactive compounds induce ER dependent estrogenic response in 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. doi:10.1371/journal.pone.0067947.g006

Figure 6. *Glycyrrhiza* species and their bioactive compounds induce the estrogenic marker, TFF1 mRNA, in 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. doi:10.1371/journal.pone.0067947.g006

**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 [3H] estradiol based on the method of Obourn et al. [37] with minor modifications [13] 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 [3H] 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 10,000 x g for 3 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 nL/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 [3H] estradiol binding to each ER subtype and the subsequent analysis were determined as were described previously [12].

**Induction of an Estrogen-Responsive Alkaline Phosphatase (AP) in Ishikawa Cells**

The protocol of Pisha et al. was used as described previously [34]. Ishikawa cells (5 x 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 EC50. 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 was calculated. The fold induction of alkaline phosphatase for every treatment, compared to that of the estradiol control was represented as estrogenic activity and calculated as described previously [34]. Anti-estrogenic activity was stated as the fold induction of alkaline phosphatase compared to background induction control [34].
Luciferase Assay reagent (100 μL) were placed in white Costar 96-well plates, before the injection of
(1 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 Biosciences) in a total volume of 15 μL, containing 4 μL qScript reaction mixture (5X), 1 μL qScript RT, 10 μL nuclelease-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 (Applied Biosystems’ Assay on Demand). Briefly, the PCR reaction mixture was prepared in a total volume of 20 μL, containing 1 μL 2X TaqMan 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/extension 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 (Ct) 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.

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 Biosciences) in a total volume of 15 μL, containing 4 μL qScript reaction mixture (5X), 1 μL qScript RT, 10 μL nuclelease-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 (Applied Biosystems’ Assay on Demand). Briefly, the PCR reaction mixture was prepared in a total volume of 20 μL, containing 1 μL 2X TaqMan 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/extension 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 (Ct) 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.

Isomerization of isoliquiritigenin to liquiritigenin

As shown in Figure 1, 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.
Statistics
The data are reported as the mean ± SD. Significant differences from control values were determined by one-way ANOVA with follow-up Dunnett test (P<0.05) using Graph-Pad Prism, version 5.00 for Windows, GraphPad Software.

Results
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, GI 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 GI (92% 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.

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 [39]. This method was used to find possible hits for ER subtypes in the crude MeOH extracts of the Glycyrrhiza species. Figure 2 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.

Relative affinity of the Glycyrrhiza compounds for ER subtypes
Based on the screening results of PUF-MS analysis as well as literature reports [26,40], competition of LigF and its precursor chalcone, LigC, with [3H] estradiol for the ER subtypes was assessed to confirm and quantify the affinity of these compounds for ERs (Table 2, Figure 3). Both LigC and LigF had very similar affinities towards ERβ with IC50 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 IC50 value of 16 μM. The selectivity of LigF for ERβ over ERα was 20-fold, which was comparable to the reports by Mersecou et al. [25] and Kupfer et al. (ERα IC50 = 2.8 μM, ERβ IC50 = 0.41 μM) [41]. Because different techniques were used the absolute IC50 values differed.

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 [34]. Induction of alkaline phosphatase indicates estrogenic activity, while inhibition of alkaline phosphatase induction in the presence of 17β-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 4A). The EC50 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, while that of GG was around 26 fold. On the other hand, the EC50 of hops at 2.1 μg/mL was consistent with previous reports [12] and was lower than that of the three Glycyrrhiza species, whereas its maximum efficacy was 100 fold. The relative EC50 ranking of the extracts was hops < GU < GI < GG, while their relative maximum efficacy was hops > GU > GI > GG. The EC50 values for the licorice purified compounds LigC and LigF were 2.7 μM and 3.4 μM, respectively (Table 2, Figure 4B) and their maximum efficacies were 56 fold and 83 fold, respectively. The EC50 of 8-PN from hops was 6.6 nM with 118-fold maximum efficacy, and XH was inactive, which was consistent with previous reports [12]. While the relative EC50 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 LD50 concentrations for the Ishikawa cells (data not shown), unless otherwise stated.

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 5A). 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 show some limited ERE-luciferase induction at 10 μg/mL. Both LigC isolated 8-PN showed induction of ERE-luciferase in MCF-7 cells (Figure 5B). 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).

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 6A) 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 LigC (5 μM) was lower than that of 8-PN (0.1 μM), but the difference was not significant (Figure 6B). On the other hand, despite no Tff1 activity by XH from hops, LigC from licorice induced Tff1, significantly.

Analysis of the liquiritigenin-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 1) 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 7A). 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 7B) indicating the rate 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 [42]. 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.

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 [13,43,44,45]. Previous findings [13] 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 estrogenic potencies were observed for the three Glycyrrhiza species in the alkaline phosphatase induction assay in ERα (+) endometrial cancer cells (Table 2, EC50s, Figure 4A). The estrogenic activity of the Glycyrrhiza species was confirmed in the ERE-luciferase assay in MCF-7 cells (Figure 5), as well as in the gene induction assay in T47D cells (Figure 6) suggesting that licorice has estrogenic properties in different estrogen sensitive tissues. These data show 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 2).

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. [27] had shown that GU 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 (31%/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 [46]. XH can switch on some cytoprotective mechanisms including activation of detoxification enzymes and can lead to cytotoxicity at higher concentrations [15]. Recent studies have demonstrated that LigC induces apoptosis in different cell lines [47,48]. Therefore, LigC might be responsible for the observed cytotoxicity of GG at concentra-
a therapeutic advantage as partial agonists are better tunable agents for their respective receptor signaling pathway [60]. 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 characterization of their estrogenic activity.

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 [42]. 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. Future animal studies are also warranted to better define the estrogenic efficacy of *Glycyrrhiza* species *in vivo*.

**Acknowledgments**

The authors thank Ping Yao for her technical support. The authors also thank Harald Schwarz and Dr. Martin Biendl from Hopsteiner (S. S. Steiner, New York, NY and Steiner Hopfen GmbH, Mainburg, Germany) for providing the hops extracts, and Dr. Liang Zhao from Lanzhou Institute of Chemical Physics, CAS, for providing *Glycyrrhiza inflata* Batalin sample as a generous gift.

**Author Contributions**

Conceived and designed the experiments: AH BMD JLB. Performed the experiments: AH JRA YY. Analyzed the data: AH BMD DR B JB. Wrote the paper: AH BMD JLB. Prepared the extracts, isolated compounds, and analyzed phytochemistry: CS SC GF.

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