Mycotoxins in Root Extracts of American and Asian Ginseng Bind Estrogen Receptors α and β

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The estrogenic activity of ginseng has been the subject of conflicting reports. Cell proliferation, induction of estrogen-responsive genes, and isolated cases of adverse reactions such as postmenopausal vaginal bleeding and gynecomastia have been reported after ginseng treatment. Other studies report antiproliferative effects with no induction of estrogen-responsive genes. We developed estrogen receptor (ER) α and ERβ competitive binding assays using recombinant receptors and [^{3}H]-17β-estradiol to detect phytoestrogens in extracts of Asian ginseng root (*Panax ginseng* C. A. Meyer) and American ginseng root (*Panax quinquefolius L.*). Root extracts contained substances that bound both receptor isoforms. These substances had a two to three times greater affinity for ERβ. Significantly higher binding was found in methanol extracts than in hot water extracts. Subsequent analysis of the extracts revealed significant ER binding attributable to zearalenone, the estrogenic mycotoxin produced by several *Fusarium* species. The ER showed no binding affinity for Rb1 and Rg1, the major ginsenosides found in *P. quinquefolius* and *P. ginseng*, respectively. Thus, ginseng extraction methods, plant species tested, and mycotoxin contaminants may help to explain the disparate literature reports. The prevalence and health significance of fungal contamination in herbal products used for medicinal purposes should be further investigated. Exp Biol Med 229:560-568, 2004

Key words: ginseng; estrogen receptor; zearalenone; phytoestrogen; herbal product

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Herbal remedies are used worldwide to alleviate symptoms, to treat illnesses, and to promote overall wellness. An estimated 60% of the world's population and 40% of Americans use herbal remedies (1). However, use of medicinal herbs may be unsupervised and of unproved efficacy (2–7).

Ginseng is a popular herb with a long history of medicinal use; the Chinese have used ginseng for more than a thousand years to improve energy and longevity (8). In the United States, it ranks among the top five herbal products sold and is an ingredient in energy tonics and used as an immunostimulatory dietary supplement (9). Asian ginseng, *Panax ginseng*, is an official herbal remedy in the *German Pharmacopoeia* and is listed in the *Commission E Monographs* "as a tonic for invigoration and fortification in times of fatigue and debility, for declining capacity for work and concentration, also during convalescence" (10). American ginseng, *Panax quinquefolius*, has been recommended for alleviation of menopausal symptoms (8, 11–15). Ginseng is available in many forms, including capsules, powders, tinctures, teas, and whole roots. Commercial products, such as cosmetics, soaps, toothpaste, chewing gum, beverages, power bars, baby food, cigarettes, and coffee, may list ginseng as an ingredient.

Among the five major species of ginseng, *P. ginseng*, a native plant of Asia, and *P. quinquefolius*, native to North America, are used most frequently in herbal preparations (16). Historically, *P. ginseng* grew abundantly in China, Korea, and parts of Siberia. Today, the wild plant is rare, and most of the ginseng root is cultivated commercially. Similarly, *P. quinquefolius* initially grew wild in North America but is now cultivated for domestic use and for export.

Pharmacological actions of ginseng have been attributed to several active constituents, including the triterpene saponins. More than 30 unique triterpene saponins, named ginsenoside R_x according to their mobility on thin-layer chromatography plates, have been isolated from ginseng.

Similarity of the ginseng molecule to the structure of some steroid hormones may contribute to the plant's pharmacological actions (Fig. 1).

The endocrine activity of ginseng has been investigated. *Panax quinquefolius* may modulate insulin secretion to decrease postprandial glycemia in nondiabetic and type 2 diabetes mellitus patients (17, 18). Ginsenoside Rg1 is a functional ligand of the glucocorticoid receptor, synergistically activating gene transcription by cyclic AMP and downregulating glucocorticoid receptor content of cells (19, 20). *Panax ginseng* root extract bound to progesterone, mineral corticoid, and glucocorticoid receptors but not to androgen or estrogen receptors (ERs) isolated from rat uterine tissue (21). Patients treated with *P. ginseng* extracts had increased spermatozoa concentration and progressive motility, total and free plasma testosterone, dihydrotestosterone, follicle-stimulating hormone, and luteinizing hormone levels but decreased prolactin levels (22). Estradiol (E_2) and *P. quinquefolius* extracts induced RNA expression of presenilin-2 (pS2) equally in ER-positive cells (23). *Panax quinquefolius* extract decreased the proliferation of the ER-positive breast cancer cell line, MCF-7, in one study (23), whereas a second study reported increased proliferation (24). Reports of estrogen-related sequelae from postmenopausal women after ginseng use include mastalgia, vaginal bleeding, and changes in the vaginal and cervical epithelium (25–28). In each case, symptoms ceased when use of the product was discontinued. Male gynecomastia has also been reported after ginseng use (29).

One method of ascertaining potential endocrine-modulating activity of a compound is competitive binding to hormone receptors. Although binding of ginsenosides to the glucocorticoid receptor has been examined, no studies have reported the competitive binding of *P. quinquefolius* and *P. ginseng* extracts to human ERα and ERβ (20, 21). Investigating ginseng preparations for bioactive compounds capable of binding ER may explain estrogenic responses observed in individuals using ginseng.

Zearalenone (Fig. 1) is a widely distributed mycoestrogen produced by several species of *Fusarium* fungi. This mycotoxin contaminates food and agricultural commodities infected with the fungus. Zearalenone is rapidly transformed into α- and β-zearalenol by 3α-hydroxysteroid dehydrogenase in the liver (30). These metabolites are more estrogenic than zearalenone, with α-zearalenol approximately three to four times higher than β-zearalenol (31). The potency of zearalenone relative to E_2 in the mouse uterotropic assay was approximately 0.001 (32). A bioassay based on the alkaline phosphatase gene induction by estrogens in the human endometrial Ishikawa cell line showed zearalenone and its metabolites were strongly estrogenic. In this study, α-zearalenol was as potent as ethinyl estradiol and diethylstilbestrol (33). In farm animals, zearalenone ingestion through contaminated feed is associated with decreased reproductive capacity and other hyperestrogenic conditions,

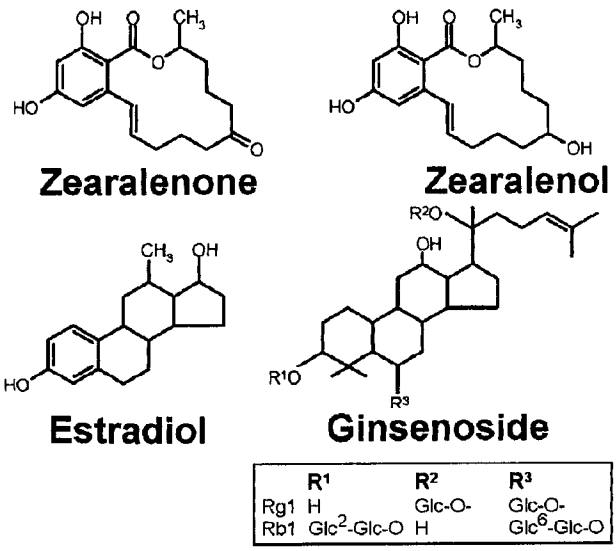

Figure 1. Structures of estradiol-17β, zearalenone, zearalenol, and the ginsenoside backbone. Rg1 and Rb1 are the major ginsenosides in *P. ginseng* and *P. quinquefolius*, respectively.

such as vaginal swelling, enlargement of mammary glands, and testicular atrophy (34).

Herbal preparations, including ginseng extracts, are generally not screened for mycotoxins. The possibility exists that cultivation and storage of herbal products could result in *Fusarium* growth and subsequent zearalenone contamination. The goals of this study were to screen ginseng root extracts using a competitive binding assay for interaction with the ER and to elucidate the source of binding in the extracts using immunoassay and high-pressure liquid chromatography (HPLC).

Methods

Plant Samples and Reagents. Dried roots from 5-year-old plants (identified in these experiments as *P. ginseng* 1, *P. ginseng* 2, and *P. quinquefolius* 1) were obtained from commercial farms in Illinois and Indiana. Additionally, 20 roots of wild *P. quinquefolius* (approximately 5–9 years old and identified as *P. quinquefolius* 2) were collected in an intact, old growth forest at Table Rock Watershed, Oconee County, South Carolina. Wild-crafted plants were stored individually in unsealed plastic bags and allowed to air dry for 8 months at approximately 20°C.

All chemicals were from VWR International, Inc. (Atlanta, GA), Fisher Scientific (Atlanta, GA), or Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Estradiol (2,4,6,7-^{3}H[N]; 71 Ci/m*M*) (^{3}H-E_2) was purchased from NEN Life Sciences Products (Boston, MA). Human recombinant ERα and ERβ were obtained from PanVera (Madison, WI), and ginsenoside Rg1 was purchased from Indofine (Belle Mead, NJ).

Preparation of Herbal Extracts. Dried ginseng roots from each source were powdered (Mr. Coffee Grinder; Mr. Coffee, Minneapolis, MN) and stored separately in air-

tight glass containers at 4°C. Crude extracts of 1-g samples used for the competitive binding assays were extracted by using 10 ml of either deionized water (initially at 100°C) or 80% methanol (Mallinkrodt Laboratory Chemicals, Inc., Phillipsburg, NJ). Samples were mixed with solvent for approximately 8 hrs on a lateral shaker at room temperature and centrifuged at 1800 *g* for 15 mins, and the supernatant removed. The pellet was resuspended in 8 ml of solvent and mixed overnight in a lateral shaker. Supernatants from the two extractions were combined, placed in a Thermolyne Dri-Bath (VWR) at 40°C, and evaporated to dryness with filtered air. Samples were resuspended in 1 ml of ethanol and filtered using 0.45 μm of PVDF Acrodisc (Pall Gelman, Ann Arbor, MI). All dried samples were extracted and assayed three times.

Competitive Binding Assay. Estrogen receptor binding studies were performed using the procedure of Obourn *et al.* with modifications (35). The assay binding buffer consisted of 10 m*M* Tris, pH 7.5, 10% glycerol, 2 m*M* dithiothreitol, and 0.1% bovine serum albumin. Varying concentrations of test samples were evaporated to dryness and reconstituted in 50 μl of binding buffer. Competition assay reactions included test samples evaporated to dryness and reconstituted in 50 μl of binding buffer, 50 μl of recombinant receptor α or β, and 100 μl of ^{3}H-E_2 for a total incubation volume of 200 μl. Final concentrations in incubation volume were 4 n*M* ERα or ERβ and 4.9 n*M* ^{3}H-E_2. Each extraction replicate was assayed in triplicate. Assays also included a standard curve using E_2 concentrations at 0.0, 0.1, 0.2, 0.5, 1.0, 2.5, 5.0, 10.0, and 15 ng and nonspecific binding of ^{3}H-E_2. Nonspecific binding counts, determined by adding excess E_2 (1 μg) to a set of assay tubes, were subtracted from total binding to determine specific binding. Standard concentrations (diluted in 100% ethanol) and plant extracts were added to assay tubes, evaporated to dryness under filtered air, and reconstituted in 50 μl of assay buffer. Incubations were conducted for 2 hrs at room temperature followed by 16 hrs at 4°C. Separation of bound and free steroid was performed by the addition of 1 ml of activated charcoal suspension (3%) in binding buffer. Samples were incubated for 5 mins on ice after charcoal addition and centrifuged for 10 mins at 1800 *g*. Supernatant was decanted into 7-ml scintillation vials, 4 ml of Ultima Gold (Packard, Meriden, CT) scintillation fluid was added, and samples were counted using a Beckman LS 1800 liquid scintillation counter (Irvine, CA). A five-parameter logistic standard curve was constructed using StatLIA Analysis software (Brendan Scientific, Carlsbad, CA). Concentration of test samples displacing approximately 50% ^{3}H-E_2 binding from each receptor was determined from the standard curve. The data represent the average ± SD of nine determinations.

Affinities of receptors and estrogenic ligands can be determined by measuring the equilibrium binding of the radioligand in the presence of different concentrations of the unlabeled ligand. These assays are conducted by determining the binding affinity of a test compound for the ER relative to a radiolabeled competitor with a known binding affinity. The plant tissue crude extracts may contain compounds that displace E_2 from its receptor, but concentration of these compounds may be unknown. A convenient way of expressing the estrogenicity of such extracts is by calculation of estrogen binding equivalents (EBEs) in a 1-g sample. Different extract dilutions incubated with the radiolabeled E_2 for receptor binding and EBEs are based on the extract dilution that displaces approximately 50% of the radioligand. Thus, EBEs represent a relative measure of ER binding potential by compounds from a 1-g plant tissue sample.

The ERα and ERβ binding affinities to zearalenone and the metabolites α-zearalenol, β-zearalenol, α-zearalanol, β-zearalanol, and zearalanone were measured by assaying varying concentrations of each compound and determining the concentration required to displace 50% bound ^{3}H-E_2. Similarly, binding affinities for the estrogenic compounds 4-hydroxytamoxifen and diethylstilbestrol (DES) and the ginsenosides Rb1 and Rg1 were determined.

***Fusarium* Culture from Ginseng Roots.** Ginseng root sections were cultured for *Fusarium* according to the method of Nash and Snyder (36). Briefly, using aseptic techniques, 6-mm square sections were cut from root, dipped in 95% ethanol, and flame sterilized. Surface sterilized chips were transferred to 100 × 15-mm, plastic petri plates with potato dextrose agar (Difco Laboratories, Detroit, MI) and incubated for 8 days under normal laboratory lighting and temperature conditions. Fungal identification was made by culture pigmentation and microscopic morphologic analysis, including characteristic length and shape of the macroconidia, presence or absence of polyphialides, and number, shape, and arrangement of formation of microconidia. Three replicate plates were made for each root.

Enzyme-Linked Immunoabsorbent Assay (ELISA) Determination of Zearalenone. Ginseng root samples were extracted and assayed using the Veratox Quantitative Zearalenone Test CD-ELISA (Neogen Corporation, Lansing, MI), a kit used commercially to detect food and feed possibly contaminated with zearalenone. Five grams of root sample was ground into a fine powder and extracted by vigorously shaking for 3–5 mins in 25 ml of 70% methanol and water. Aliquots of 100 μl from each sample were spin-filtered to remove particulate matter (0.45 μm of GHP Nanosep centrifugal device; Pall Gelman) and added to the microassay plates. Free zearalenone in the samples and controls competed for the antibody binding sites with enzyme-labeled zearalenone. Microassay plates were washed and substrate was added, which binds with the conjugate to produce blue color. Plates were read in a microwell reader using a 650-nm filter. Concentrations were read on a log-logit curve generated by the reader from a set of standards provided in the kit. Range of quantitation was

100–1000 ppb. Root samples were extracted and assayed in two separate experiments.

The HPLC Determination of Zearalenone. The sample extraction method for HPLC analysis was adapted from the modified Association of Official Analytical Chemists (AOAC) official method for zearalenone (37). Briefly, 1-g, dry, powdered ginseng root samples were extracted in duplicate as previously described for the binding assay, except solvent was 70% methanol as recommended in the AOAC zearalenone extraction method. Supernatant was evaporated to dryness and resuspended in 20% methanol (8 ml). Samples were further purified using octadecyl (C_{18}), disposable, 3-ml extraction columns (BAKERBOND SPE; J. T. Baker, Phillipsburg, NJ) with a vacuum manifold. The C_{18} columns were preconditioned by sequentially passing through 2 ml of methanol and 2 ml of water. Extracted test samples were aspirated through the conditioned column under vacuum (<0.8 cm [2 in.] Hg). The column was washed with 2 ml of 30% methanol and then dried under a vacuum for 3 mins. Zearalenone was eluted from the column by gently aspirating two 0.5-ml portions of 70% methanol. Eluates from the C_{18} column were evaporated to dryness, reconstituted in 1 ml of mobile phase buffer to a volume equivalent to 1 g/ml, and used for zearalenone determination. The HPLC method was adapted from Visconti and Pascale (38). A Hewlett Packard 1090 HPLC (Palo Alto, CA) with a 3-μm, 10cm × 4.6-mm C_{18} reverse phase column (Regis Technologies, Inc., Morton Grove, IL) was used for the analysis. The mobile phase consisted of acetonitrile-water-methanol (46:46:8) with a flow rate of 0.5 ml/min. The sample volume size injected into the HPLC for zearalenone determination depended on zearalenone concentration and varied from 10–100 μl. Standard curves were prepared by injecting standards (31, 62, 125, 250, 500, 1000, and 5000 ng/ml) at volumes equivalent to the test sample injection volume. Before injection, test samples and standards were filtered using 0.45 μm of Acrodisc LC13 PVDF (Pall Gelman). A diode array detector (λ = 236 nm) was used for detection. Identification of zearalenone was determined by comparing retention times of test samples with those of the standard and comparison of spectra (170–400 nm) of test samples to those of standards. Concentrations of zearalenone were calculated by comparing test sample peak area with areas from the appropriate standard curve. Data represent the mean of two HPLC determinations using two different extractions.

Extracts of *P. ginseng* and *P. quinquefolius* were fractionated to determine the source of estrogenic activity. Approximately 0.25-ml fractions of eluant were collected during an HPLC gradient run beginning with 34:6:60 acetonitrile-methanol-water and ending with 60:10:30 acetonitrile-methanol-water. Samples were collected over 15 mins with a flow rate of 0.5 ml/min. Twenty-microliter aliquots of *P. ginseng* or *P. quinquefolius* (equivalent to 40 mg of dried root) were injected for each run. Fractions collected from 7 individual runs of *P. ginseng* runs were pooled for each collection time and each fraction assayed for estrogenic activity using the binding assay. The procedure was repeated using *P. quinquefolius* root. To determine elution patterns for the mycotoxin standards, zearalenone, α-zearalenol, and β-zearalenol were added to a ginseng extract previously determined to have no ER binding for a final concentration of 20 μg/ml. Twenty-microliter aliquots of the spiked samples were run using the same HPLC and collection conditions. Individual fractions from samples and spikes were analyzed for ERβ binding. Fractions were also collected from blanks for analysis.

All statistical analyses were conducted using SAS statistical software (SAS Institute Inc., Cary, NC). Analysis of variance was used to determine significant differences, and Pearson's coefficient was used for correlation.

Results

Affinity of Crude Plant Extracts and Compounds for ERα and ERβ. Extracts of *P. ginseng* and *P. quinquefolius* from the four different root sources bound to ERα and ERβ in a concentration-dependent manner (Table 1). Overall, ERβ bound methanol and water extracts with greater affinity (P = 0.05) than ERα. Methanol extracts of ginseng roots from one source, *P. ginseng* 2, bound ERα and ERβ with significantly greater affinity than roots from the other sources. Water extracts of *P. quinquefolius* 1 had significantly higher binding to ERα than the other three sources yet had significantly lower binding to ERβ when compared with *P. ginseng* 1 and *P. ginseng* 2.

ERα and ERβ binding affinity for zearalenone and some metabolites was examined. Based on an arbitrary relative binding affinity of 100 for ERα and ERβ to E_2, both ERα and ERβ had less affinity for zearalenone, 4.2 and 6.6, respectively, than for E_2 (Table 2). Data from the competitive binding tests showed the ERα affinities for zearalenone and its metabolites relative to E_2 and some other estrogenic compounds were as follows: DES > α-zearalenol > E_2 > 4-hydroxytamoxifen > α-zearalanol > β-zearalanol > zearalanone > β- zearalenol > zearalenone. ERβ affinities of these same compounds were as follows: DES > α-zearalenol > E_2 > 4-hydroxytamoxifen > α-zearalanol > β-zearalanol > zearalanone > zearalenone. Affinity of the ER for the metabolite α-zearalenol is 15 times greater than affinity for zearalenone. The purified ginsenosides Rg1 and Rb1, the primary ginsenosides in *P. ginseng* and *P. quinquefolius*, respectively, had no detectable binding to either receptor in the binding assay.

***Fusarium* Culture from Ginseng Root.** *Panax ginseng* 1, *P. ginseng* 2, and *P. quinquefolius* 2, but not *P. quinquefolius* 1, were confirmed positive for *Fusarium* by identification of characteristic microconidia, macroconidia, and polyphialides (data not shown). Cultures were characteristically white with tinges of pink and yellow.

Zearalenone Concentrations in Ginseng Root Extracts Using ELISA. Crude extracts of dried ginseng root from the four sources were screened for zearalenone content using CD-ELISA. All four ginseng root samples tested positive for zearalenone in the extracts. Zearalenone concentrations were highest in *P. quinquefolius* 2, the wild-crafted ginseng sample, at 680.1 ppb. *Panax ginseng* 1, *P. ginseng* 2, and *P. quinquefolius* 1 had zearalenone concentrations of 183.2, 386.1, and 177.4 ppb, respectively. Ginsenosides Rb1 and Rg1 did not bind to the antibody.

Zearalenone Concentrations in Ginseng Root Extracts. Crude extracts of roots from each of the four sources were evaluated for zearalenone concentration using HPLC. The highest concentrations of zearalenone were found in extracts of *P. ginseng* 2 at 11.7 μg/g followed by *P. ginseng* 1 at 6.13 μg/g. Extracts of *P. quinquefolius* 2 had a concentration of 2.60 μg/g and *P. quinquefolius* 1 had the lowest concentration at 0.25 μg/g. Pearson's correlation coefficient (r^2) between binding affinities (EBEs) and zearalenone concentration in the root extracts was 0.62 for ERα (α = 0.0035) and 0.59 for ERβ (α = 0.004) (Fig. 2).

Estrogen Receptor Binding in HPLC Fractionated Ginseng Samples. Spiked ginseng samples had HPLC retention peaks at 10.08, 7.75, and 6.70 mins for zearalenone, α-zearalenol, and β-zearalenol, respectively (Fig. 3). The ERβ binding assay detected peak binding activity from aliquots collected at times 10.6, 8.6, and 7.1 mins, indicating approximately a 0.5-min delay in collection time of eluted standards. The *P. ginseng* 2 extract had ERβ binding peaks in fractions collected at 10.6, 8.6, and 6.6 mins (Fig. 4). Unexpectedly, binding peaks were also detected at 2.6, 4.1, and 5.6 mins. No binding was detected in fractionated samples of *P. quinquefolius* 1 or from the solvent blanks collected between runs.

Table 1. ERα and ERβ EBEs from Methanol and Water Extracts of Ginseng Root Using a Competitive Binding Assay

	EBE* (ng/g ± SD)[b]	
	ERα	ERβ
Methanol extract		
Panax ginseng 1	62.4[b] ± 39.8	122.5[b] ± 106.4
P. ginseng 2	232.4[a] ± 145.5	860.6[a] ± 733.4
Panax quinquefolius 1	39.4[b] ± 16.9	131.4[b] ± 119.0
P. quinquefolius 2	67.2[b] ± 19.5	144.8[b] ± 74.9
Mean	85.6[c] ± 91.7	338.6[d] ± 505.4
Water extract		
P. ginseng 1	45.6[b] ± 52.6	206.6[a] ± 244.2
P. ginseng 2	55.3[b] ± 32.9	210.1[a] ± 125.5
P. quinquefolius 1	129.5[a] ± 110.1	68.3[b] ± 40.0
P. quinquefolius 2	15.3[b] ± 6.6	68.4[b] ± 39.7
Mean	60.8[c] ± 69.5	138.4[d] ± 151.6

Note. EBEs are calculated by adjusting the ER binding concentration of *P. ginseng* or *P. quinquefolius* as determined from the standard curve to express binding concentration in a 1-g plant tissue sample. ER, estrogen receptor; EBEs, estrogen binding equivalents.
[a,b] EBEs that have different small capital letters are significantly different (P = 0.05) within extract type (methanol or water) and receptor type (ERα or ERβ).
[c,d] EBEs for mean values for ERα and ERβ which have different superscripts are significantly different (P = 0.05) within extract type (methanol or water).

Discussion

Increasingly, more patients are seeking alternative therapies (39–42). Women seeking treatments for relief of menopausal symptoms often use herbal products, and among these women ginseng is frequently used. Traditionally, ginseng is considered a safe herb with few adverse effects. However, estrogen-like effects after use of ginseng products have been reported (26–29). Our experiments revealed an estrogenic mycotoxin, zearalenone, in ginseng samples during ER assays. The discussion will center on interpreting the current results with respect to the relevant ginseng and mycotoxin literature.

Results from studies that investigated the estrogenic action of ginseng crude extracts and purified ginsenosides are conflicting. Although no significant binding of ginseng extracts to the ER have been previously demonstrated, studies have shown induction of the estrogen responsive protein pS2 in MCF-7 cancer cells and pS2 messenger RNA in S30 breast cancer cells, suggesting an ER-mediated response (9, 23, 43, 44). However, these studies reported either no change or decreased proliferation of ER-positive cells, suggesting the action of the extract is not singularly estrogenic. In contrast, a ginseng extract that failed to transactivate either ERα or ERβ in a transient gene expression assay system and had no effect on uterine weight *in vivo* caused a 27-fold increase in MCF-7 cell proliferation (24). Effects of ginsenosides on estrogen-related cancer cell proliferation are similarly contradictory. Using various cancer cell lines, ginsenosides have been

Table 2. The RBAs of ERα and ERβ for Some Potentially Estrogenic Compounds Using a Competitive Binding Assay

	RBAs*	
	ERα	ERβ
17β-estradiol (E_2)	100	100
Zearalenone	4.2	6.6
Zearalanone	10.8	6.7
α-zearalenol	111	125
β-zearalenol	7.3	5.6
α-zearalanol	24.4	43.5
β-zearalanol	13.0	13.2
Diethylstilbestrol	218	237
4-hydroxytamoxifen	43	46
Ginsenoside Rb1	<0.01	<0.01
Ginsenoside Rg1	<0.01	<0.01

* The RBA was the ratio of E_2 and estrogenic compound at a concentration sufficient to displace radioligand binding by 50%. The RBA value for E_2 is arbitrarily set at 100. RBA, relative binding affinity; ER, estrogen receptor.

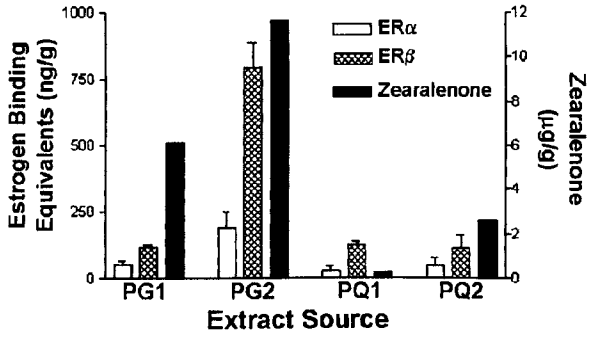

Figure 2. Estrogen binding equivalents in methanol extracts of *P. ginseng* (PG) and *P. quinquefolius* (PQ) from ERα and ERβ competative binding assays and zearalenone concentrations determined from HPLC. Correlation (r^2) with zearalenone concentration in corresponding extracts was 0.62 for ERα and ERβ.

shown to exhibit antiproliferative activity (44–49). In contrast, it was recently reported that ginsenoside Rg1 extracted from *Panax notoginseng* stimulated MCF-7 breast cancer cell proliferation in a dose-dependent manner (12). Although Rg1 stimulated MCF-7 cell proliferation, it failed to displace ^3H-E_2 in a cell lysate, leading the authors to suggest stimulation occurred without direct interaction with ER. Rg1, a major active molecule from *P. ginseng*, was shown to be a functional ligand of glucocorticoid receptor, but there has been no evidence that the ginsenoside binds the ER (20). In another study, Rb1, the principal ginsenoside in *P. quinquefolius*, activated transcription of the estrogen-responsive luciferase reporter gene in MCF-7 cells and induced the estrogen-responsive gene c-fos, suggesting interaction of this ginsenoside with the ER (50).

Data from our study showed crude extracts of ginseng roots bound to ERα or ERβ, but neither receptor type interacted with the ginsenoside compounds Rb1 or Rg1. This contradiction to previous studies that reported negative binding to ER may be related to the method used to obtain the extract, the concentration of extract tested, or receptor type used. In our study, both hot water and methanol solvents were used to extract compounds with different solubilities (Fig. 1). Zearalenone and its metabolites are more soluble in methanol; therefore, methanolic extracts of samples containing these potent compounds had greater estrogen binding than the hot water extracts. Although Lui *et al.* (44) reported no binding to ERα or ERβ using an extract dilution equivalent to 1 μg of plant material, we detected receptor binding when using a more concentrated extract, equivalent to 1 mg of plant material. Furthermore, in our study, ERβ had three to four times higher binding affinity than ERα for most ginseng extracts, suggesting that cell lines that express a high percentage of ERβ may be more responsive than cells that express primarily ERα.

These experiments identified zearalenone or zearalenone-like compounds from *P. ginseng* and *P. quinquefolius* roots. Roots from three of the four sources cultured positive for *Fusarium* fungus, the only known natural source of

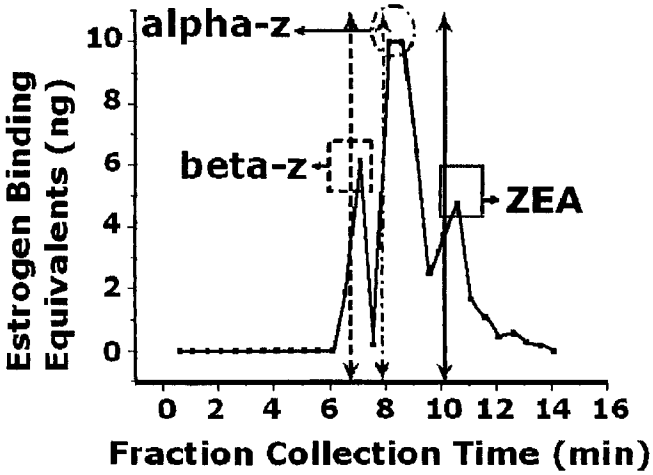

Figure 3. Estrogen binding equivalents (ng) in ERβ binding assay from HPLC fractionation of a ginseng sample spiked with zearalenone (ZEA), α-zearalenol (alpha-z), and β-zearalenol (beta-z). The ginseng sample used for the spiking matrix was previously determined to have no detectable ER binding or zearalenone as determined by HPLC. Vertical lines indicate HPLC retention times for respective compounds.

zearalenone. Zearalenone in the extracts was detected using ELISA and HPLC. The root sample that cultured negative for *Fusarium* was the same sample showing the lowest zearalenone concentration using HPLC. Explanations as to why zearalenone was identified in a sample that cultured negative for *Fusarium* are as follows: (i) spores were nonviable before sterilization and the zearalenone persisted in the sample; and (ii) the sample was weakly infested, with the primarily surface-localized spores being killed by flame sterilization. Concentrations of zearalenone in some *P. ginseng* roots in the parts per million range (10 μg/g or 10 mg/kg) were measured using HPLC. In comparison, lower zearalenone concentrations detected by ELISA may result

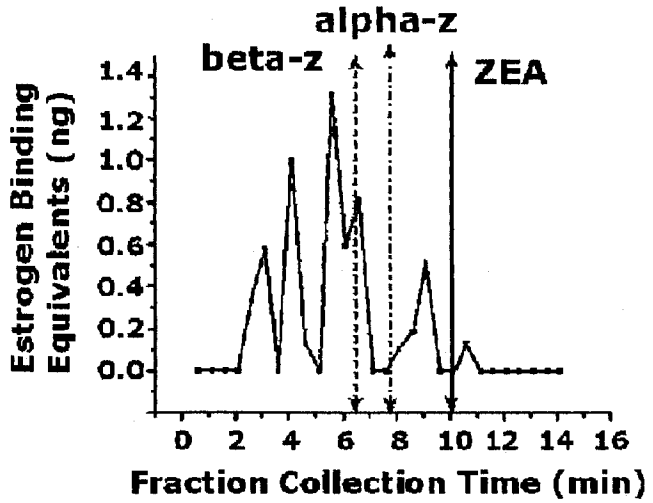

Figure 4. Estrogen binding equivalents (ng) in ERβ binding assey from HPLC fractionation of *P. ginseng* 2 extract. Vertical lines represent HPLC retention times for standards.

from using a nylon spin-filter to remove particulate matter. Visconti and Pascale (38) reported that up to 40% zearalenone concentration in corn extracts was lost due to filtration with a nylon spin filter. Additionally, the CD-ELISA, unlike HPLC, is an indirect method and can be subject to cross-reactivity and matrix effects and should be used only qualitatively as a preliminary screen.

A correlation greater than 50% between ER binding data and zearalenone concentration was found in the test samples, suggesting a substantial relationship between these factors. Also, α-zearalenol, a highly estrogenic metabolite of zearalenone, was identified in the root extract using HPLC. Results from fractionation of *P. ginseng* 2, the sample with highest EBE and zearalenone concentrations, confirm zearalenone, α-zearalenol, and β-zearalenol in the extract. In contrast, no ER binding was detected in *P. quinquefolius* 1 fractions collected from HPLC. This sample had almost seven times less EBE concentration in the ER binding assay when compared with *P. ginseng* 2 and more than 10 times less zearalenone concentration as determined by HPLC. Perhaps fractionation of the sample diluted the estrogenic components of the extract beyond the range of the binding assay. In addition to the zearalenone and zearalenol binding peaks demonstrated in the fractionated *P. ginseng* 2, there were three unidentified binding peaks. Absorbance data on these peaks did not resemble zearalenone or the metabolites. Although ERβ had three to four times higher affinity than ERα for most ginseng extracts, affinity of the two receptors for zearalenone and the metabolites was only slightly higher for ERβ. Perhaps the xenoestrogen in the three unidentified peaks could account for the three to four times greater ERβ binding affinity detected in the assays. The source of the unidentified xenoestrogen may be fungal or plant derived, and the possibility exists that this compound is the result of plant-fungal interaction (i.e., metabolic conversion).

The health consequences of zearalenone exposure in humans are uncertain. However, mice, rats, swine, and monkeys are susceptible to the estrogenic effects of zearalenone; hyperestrogenicity and severe reproductive and infertility problems can occur, especially in swine (32, 51–57). Zearalenone at concentrations of 0.3–30 μg/ml induced dose-dependent decrease in bovine oocyte maturation rate and chromatin abnormalities (58). Zearalenone possesses tumor-promoting activity similar to estrogen and may be associated with carcinogenesis (59–61). This mycotoxin has been detected in human endometrial adenocarcinoma tissue (62) and has been suggested as a contributing factor to outbreaks of precocious puberty in Puerto Rico (63). Zearalenone contamination of grains used for animal and human consumption is considered a health concern at the parts per billion level (37). Toxicity problems are seen in domestic animals, particularly swine and poultry, when ingesting feed contaminated with zearalenone at the parts per billion level (64, 65).

Ginseng is used medicinally at 0.5–4 g/d, a much lower consumption than would be expected from grains. However, as shown in the binding data and by others, the zearalenone metabolite α-zearalenol is as potent an estrogenic compound as E_2. Certainly, levels of zearalenone in ginseng root at a parts per million concentration could present a potential health risk, particularly in consumers in whom estrogen exposure should be minimized.

There appears to be no uniform explanation for the contradictions in ginseng effects with respect to estrogen-related biological activity in individuals. Although commercial extracts of ginseng are usually standardized to a certain level of the saponin ginsenosides, this standardization does not reflect the concentration of ginseng root and other potentially biologically active components in the preparation, including zearalenone. Estrogenic responses reported after ginseng use may be attributed to biologically active compounds present in the root other than the ginsenosides. Herbal manufacturers often have proprietary methods for preparing their ginseng extracts, resulting in the inclusion or exclusion of potentially bioactive compounds.

In conclusion, the unexpected findings of zearalenone-like compounds in *P. ginseng* and *P. quinquefolius* extracts could explain the sporadic reports of estrogen toxicity after ginseng use. The presence of this mycotoxin in an herbal product should be investigated further to determine the source and prevalence of *Fusarium* fungi and zearalenone in ginseng root. Additionally, this finding raises the issue of mycotoxin testing for all herbal products. The methods described herein provide a valid approach for screening herbal products for ER binding and identification of zearalenone contamination.

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