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ARTICLE

Estrogenic and Antiestrogenic Activities of Phytoalexins from Red Kidney Bean (*Phaseolus vulgaris* L.)

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Legumes are the predominant source of isoflavones considered to be phytoestrogens that mimic the hormone 17β-estradiol (E2). Due to the risks associated with hormone replacement therapy, there is a growing need for alternative sources of estrogenic formulations for the treatment of menopausal symptoms. Legume phytoalexins (induced isoflavones) are produced under conditions of stress that include insect damage, wounding, or application of elicitors. The estrogenic and antiestrogenic activities of methanolic extracts obtained from red kidney bean treated with the fungus *Aspergillus sojae* were compared with those of untreated controls using an estrogen responsive element-based (ERE) luciferase reporter assay. *A. sojae*-treated red kidney bean extracts displayed both estrogenic and antiestrogenic activities. Analysis of elicitor-treated red kidney bean extracts showed that *A. sojae* treatments achieved maximal levels of kievitone at 1199 $\pm$ 101 μg/g and phaseollin at 227.8 $\pm$ 44 μg/g. The phytoalexins kievitone and phaseollin were isolated from *A. sojae*-treated red kidney bean extracts and analyzed for estrogenic activity using ERα and ERβ binding, ERE luciferase assays in MCF-7 and HEK 293 cells, and MCF-7 cell proliferation. Kievitone showed the highest relative binding affinity to ERα with kievitone (0.48%) > phaseollin (0.21%), and phaseollin showed the highest relative binding affinity to ERβ with phaseollin (0.53%) > kievitone (0.42%). In an ERE luciferase assay in MCF-7 cells, kievitone displayed high ER transactivation at 10 μM; phaseollin displayed low ER transactivation. Both kievitone and phaseollin stimulated MCF-7 cell proliferation, with kievitone displaying agonist activity between 0.1 and 10 μM. Cotransfection reporter assays performed in HEK 293 demonstrated that phaseollin selectively increased ERE transcriptional activity of ERβ and kievitone selectively increased ERE transcriptional activity of ERα. Although phaseollin displayed attenuation of ER transactivation in the ERE luciferase assay in MCF-7 cells, both phytoalexins attenuated the effects of E2 in an MCF-7 cell colonial survival assay. This work provides evidence that the red kidney bean phytoalexins kievitone and phaseollin possess both estrogenic and antiestrogenic activities.

KEYWORDS: Legume; red kidney bean; isoflavone; phytoalexin; estrogenic; antiestrogenic; kievitone; phaseollin

INTRODUCTION

Legumes are a major source of complex carbohydrates, fibers, proteins, and minerals such as potassium, magnesium, and zinc. Legume consumption has been linked to many health-promoting activities, including reduced risk of various cancers (*1*−*3*) and coronary heart disease (*4*, *5*), and is considered an important part of a healthy diet. Legumes are present in almost every diet throughout the world, and in addition to the seeds many other parts of the plant are also edible. The legume attracting much attention recently is soybean, which contains high concentrations of the isoflavones daidzein and genistein (*3*,*6*), which have several potential health benefits (*3*, *6*, *7*); however, many other isoflavones are also present in legumes, including biochanin A and formononetin (*8*, *9*). Besides isoflavonoids, other flavonoids also exert estrogenic activity, but usually at a much lower level of activity compared to that of isoflavonoids (*10*). Some flavonoids, including kaempferol and quercetin, can exhibit antiestrogenic activity (*10*), and red kidney bean and several other legumes are a source of these flavonoids (*11*−*13*). Coumestrol, a coumestan

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with high estrogenic activity in cell and animal assays, is also present in several legume seeds and sprouts (*8*, *9*). Several other plant components, including the lignans secoisolariciresinol and matairesinol, have estrogenic metabolites (enterodiol and enterolactone); however, high concentrations of lignans are found only in flaxseed (*7*, *8*), with lower amounts occurring in beverages, nuts and seeds, bread, and fruits.

Isoflavonoids and flavonoids in plants are considered phytoestrogens that can interact with estrogen receptors (ER) and exhibit estrogenic/antiestrogenic activities. There are two types of estrogen receptors in humans, hERα and hERβ, with distinct tissue distributions throughout the body. Phytoestrogens exhibit weak estrogenic activity on the order of $10^{-2}-10^{-3}$ that of 17β-estradiol depending on the receptor subtype, but may be present in plasma at concentrations 100-fold higher than endogenous estrogens (*14*−*16*). The antiestrogenic activity of phytoestrogens may be partially explained by their competition with endogenous 17β-estradiol, but several other mechanisms are also involved (*15*−*17*). The ability of phytoestrogens to display both estrogenic and anitestrogenic properties has led to the search for new plant sources and methods to induce phytoestrogens, particularly isoflavones.

Several factors can alter legume isoflavone concentrations, including environment and germination. Changes in isoflavone concentration also occur in response to stress or elicitor treatment. In legumes, isoflavone phytoalexins are produced as plant defensive compounds. Careful examination of over 500 legumes has revealed that compounds belonging to six isoflavonoid classes, including isoflavones, isoflavonones, and coumestans, accumulate in tissues inoculated with fungi or microorganisms (*18*). For example, soybean cotyledons treated with *Phytophthora megasperma* f. sp. *glycinea* wall glucan contained higher concentrations of both isoflavone aglycones and glycosides (*19*). In response to fungal stress, soybean produces the phytoalexins glyceollins I, II, and III that are derived from the isoflavone daidzein (*19*−*22*). Countless other stress factors or physical stimuli induce legume phytoalexin accumulation including wounding, freezing, ultraviolet light exposure, and exposure to microorganisms (*19*−*22*).

Most research utilizing legume phytoalexins has focused on plant defense, and many different elicitors have been identified (*18*−*22*). However, only recently are phytoalexins being explored as nutritional components and a source for development of health-promoting food products. These underutilized plant compounds could hold previously unknown potential for antioxidant activity, anti-inflammation activity, cholesterol-lowering ability, and even anticancer activity. Research in our laboratory has focused on the antiestrogenic activity of the soy glyceollins in vitro (*23*) and in vivo (*24*, *25*). In contrast to the estrogenic effects of the constitutive soy isoflavones daidzein and genistein, the glyceollins displayed a significant antiestrogenic effect on estrogen receptor signaling, which correlated with a comparable suppression of estrogen-induced proliferation of breast cancer cells. These results suggest that legume phytoalexins have unique effects on ER with health-promoting properties, and the potential exists for the development of phytoalexin-enriched functional foods (*26*).

Several phytoalexins are produced in red kidney bean plants and seeds during wounding, insect damage, or elicitor treatment. Phaseollin and kievitone are the two primary phytoalexins produced in both red kidney beans and green beans during abiotic (*18*) and biotic elicitor treatment (*18*, *27*−*30*). These phytoalexins have been well-characterized for antifungal activities (*18*, *27*, *31*), and kievitone has been shown to inhibit the growth of breast cancer cells (*32*), but further characterization of these inducible compounds is necessary. Although elicitor treatment is critical for production of high concentrations of phytoalexins in legume seeds or plants, several studies have shown that damaged or infected legumes can contain low levels of these compounds. Postharvest damage frequently occurs with more delicate legumes such as the snap or French bean, *Phaseolus vulgaris*, which produces phaseollin and kievitone (*18*). Phaseollin levels increased (from 10 to 40 μg/g) in injured pod tissue within 48 h of injury, and accumulation occurred in healthy tissue adjacent to injured sites (*33*). This production of phytoalexins (glyceollins) has been observed in our laboratory in soybean using mechanical wounding (*34*). Injury, mechanical wounding, and infection of legumes are all methods of creating legume phytoalexins postharvest and can introduce low to moderate levels of these compounds into the food supply.

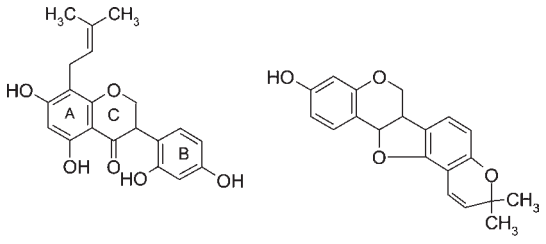
The possibility for legumes to contain low levels of phytoalexins during injury necessitates further characterization of the biological effects of these compounds, including their estrogenic activity. Also, the potential exists for the production of newer functional foods containing legume phytoalexins; however, toxicity and biological characterization would be critical before human consumption could be promoted. This area of research is growing; however, few data are available on the estrogenic and antiestrogenic activities of extracts obtained from elicitor-treated legume extracts. In the present study, we evaluated the estrogenic and antiestrogenic activities of red kidney bean extracts using an estrogen responsive element-based (ERE) luciferase assay in MCF-7 cells. The ERE luciferase assay in MCF-7 cells has been widely used to evaluate the estrogenic activity of isoflavones and plant extracts (*10*, *15*, *23*). The estrogenic and antiestogenic activities of untreated red kidney bean extracts were compared with those of extracts containing phytoalexins obtained from seeds exposed to *Aspergillus sojae*. Additionally, the isolated phytoalexins kievitone and phaseollin obtained from *A. sojae*-treated red kidney bean were examined for estrogenic activities in ERE luciferase in MCF-7 and HEK 293 cells, MCF-7 cell proliferation, and ER binding affinity assays. The antiestrogenic activity of each phytoalexin was analyzed in ERE luciferase in MCF-7 cell and MCF-7 cell colonial survival assays. HPLC with both UV and mass spectrometry was utilized to provide both qualitative and quantitative analysis of *A. sojae*-treated red kidney bean extracts.

MATERIALS AND METHODS

Materials. Red kidney bean (*P. vulgaris* L.) seeds were obtained from Homegrown Harvest (Kennesaw, GA). HPLC grade methanol, acetonitrile, and water were used as solvents for the study and were purchased from Fisher Scientific (Pittsburgh, PA). Genistein and coumestrol were obtained from Indofine Chemical Co. (Hillsborough, NJ). E2 and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO).

Elicitors. *A. sojae* (SRRC 1125) cultures were grown at 25 °C in the dark on potato dextrose agar. After 5 days, inoculum was prepared by harvesting conidia (3.4×10^7/mL) in 15 mL of sterile, distilled water. Yeast extract (Becton Dickson 212750) was prepared in distilled water at a concentration of 0.1 g/mL.

Red Kidney Bean Treatments. Red kidney bean seeds were surface-sterilized for 3 min in 70% ethanol followed by a quick deionized H_2O rinse and two 2 min rinses in deionized H_2O. Seeds were soaked in sterile deionized H_2O for 4 h prior to placement into Petri dishes. Each Petri dish (100 × 15 mm) was lined with one autoclaved filter paper (Whatman) moistened with 0.5 mL of distilled H_2O. Seeds were wounded by cutting with a sterile razor longitudinally along the length of the seeds. *A. sojae* spore suspension (10 μL) or yeast extract (10 μL) was applied to the cut surface of each seed for elicitor treatments. All chambers were stored at 25 °C in the dark for 3 days and then transferred to −70 °C. Controls were whole dry seeds.



KIEVITONE PHASEOLLIN

Figure 1. Chemical structures of the phytoalexins phaseollin and kievitone isolated from red kidney bean (*Phaseolus vulgaris* L.).

Preparation of Red Kidney Bean Extracts. Treated seeds were lyophilized and ground. Approximately 1 g of ground seed material from each legume was extracted with 5 mL of methanol for 1 h using ultrasonic treatment. The extracted solvent was filtered through 0.45 μm filters. The filtrate was used for analytical HPLC. For analysis of estrogenic activity separate extracts using the same lyophilized source material were prepared in a similar manner. The resulting extracts were centrifuged at 10000*g* for 20 min, decanted, filtered, concentrated, and dissolved in DMSO at a concentration of 100 mg/mL. The concentration of each legume extract in DMSO is based on dry extract mass. For semipreparative isolation of phytoalexins, 0.5 kg of legume seeds was used and prepared as described with *A. sojae* elicitor. The extraction solvent was 2 L of methanol. Rotary evaporation for 2 h (temperatures between 35 and 45 °C) was used to concentrate the methanolic legume extract almost to dryness before semipreparative HPLC.

Phytoalexin Isolation and Identification. Phaseollin and kievitone were isolated using techniques developed at the Southern Regional Research Center (ARS, USDA, New Orleans, LA) (*23*). For the isolation of the phytoalexins semipreparative HPLC-UV (UV detection) was utilized. The column was a Whatman ODS-2, 10 mm × 500 mm, using a flow rate of 3.0 mL/min with the following solvent system: A, acetonitrile; B, water; 5% A for 15 min, then 5−90% A in 40 min followed by holding at 90% A for 20 min. Acetonitrile was removed from fractions containing phytoalexins and lyophilized to remove water. Phytoalexin purities of ≥98% were achieved as revealed by HPLC-UV analyses. Kievitone and phaseollin shown in **Figure 1** were confirmed by UV−vis spectrophotometry, APCI mass spectrometry, and ^{1}H analysis. ^{1}H NMR spectra were recorded in deuterated acetone with a Bruker DMX-500 spectrometer (Billerica, MA).

HPLC and Mass Spectrometric Analysis of Red Kidney Bean Extracts. To identify phytoalexins, each legume extract was analyzed using HPLC-UV combined with mass spectrometry. HPLC analyses were performed on a Waters 2695 combined with a Waters UV−vis 2996 detector. Separations were carried out using a Phenomenex Luna C_{18} (4.6 × 250 mm; 5 μm) reverse-phase column. A guard column containing the same packing was used to protect the analytical column. The injection volume of sample was 20 μL with a flow rate of 1.0 mL/min with the following solvent system: A, aqueous acetic acid (pH 3.0); B, acetonitrile; 15% B for 8 min, then to 58% B in 50 min, then to 90% B in 10 min followed by holding at 90% B for 10 min. The system was equilibrated with 15% B for 10 min at the end of each run. The mass spectrometer utilized was a Finnigan MAT LCQ ion trap (San Jose, CA) equipped with a heated nebulizer APCI. HPLC effluent at 1 mL/min was introduced directly into the interface without splitting using a source temperature of 500 °C. Positive ion mode was used with a corona needle voltage of 4 kV. The capillary temperature was 210 °C. The full scan spectra of the isoflavones from m/z 100 to 1000 were measured using 500 ms for collection time, and three microscans were summed. Peak areas of phaseollin and kievitone were quantified at 280 and 294 nm, respectively. Linear calibration curves ($R^2 > 0.99$) for phaseollin and kievitone were prepared over a range of 4−200 μg/mL using purified compounds. The limits of detection (LOD) and quantification (LOQ) were established as the amounts for which the signal-to-noise ratios (S/N) were 3 and 10, respectively. The LOD for phaseollin and kievitone were 0.25 and 0.31 μg/mL, respectively; the LOQ for phaseollin and kievitone were 0.51 and 0.62 μg/mL, respectively. Kievitone and phaseollin concentrations were reported as micrograms per gram of red kidney bean on a dry weight basis.

ERα and ERβ Binding Assays. ERα and ERβ receptor binding determinations were achieved using the method of Bolger (*35*). In this method, recombinant estrogen receptor (ERα and ERβ) is in equilibrium with fluorescent-labeled E2 (ES2; Panvera, Madison, WI) and a concentration of the competitor (phytoalexin). The relative displacement of ES2 is measured as a change in polarization anisotropy. Specifically, a 200 μM working stock solution of kievitone and phaseollin was prepared from the original 10 mM DMSO stock solution and was serially diluted in triplicate in screening buffer to the desired concentrations. hrER and ES2 were added to final concentrations of 2 and 3 nM, respectively. Negative (ER + ES2, equivalent to 0% inhibition) and positive (free ES2, equivalent to 100% inhibition) controls in the absence of competitor were measured in duplicate. After 2 h at room temperature, the anisotropy values in each tube were measured using the Beacon 2000 system CO. The anisotropy values were converted to percent inhibition using the following formula: $I(\%) = (A_0 - A)/(A_0 - A_{100}) \times 100$, where $I(\%)$ is the percent inhibition, A_0 is 0% inhibition, A_{100} is 100% inhibition, and A represents the observed value. Polarization values were converted to percent inhibition to make the data more intuitive to the reader and to normalize the day-to-day differences in the starting 0% inhibition polarization values. The percent inhibition versus competitor concentration curves were analyzed by nonlinear least-squares curve fitting and yielded an IC_{50} value (the concentration of competitor needed to displace half of the bound ligand). To compare binding affinities of the test compounds to those reported in the literature, IC_{50} values were converted to relative binding affinities (RBA) using E2 as a standard (*16*). The E2 RBA was set equal to 100 [RBA = (IC_{50} of E2/IC_{50} of phytoalexin) × 100].

Cell Culture. MCF-7 breast cancer cells (expressing both ERα and ERβ) (*36*) and HEK 293 human embryonic kidney were cultured in 150 cm^2 culture flasks in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Co.) supplemented with 10% FBS (Life Technologies, Inc., Gaithersburg, MD), basic minimum MEM essential (50×, Invitrogen Co.) and MEM nonessential (100×, Invitrogen, Co.) amino acids, sodium pyruvate (100×, Invitrogen Co.), antimycotic−antibiotic (10000 U/mL penicillin G sodium; 10000 μg/mL streptomycin sulfate; 25 μg/mL amphotericin B as Fungizone), and human recombinant insulin (4 mg/mL, Invitrogen Co). The culture flasks were maintained in 5% CO_2 at 37 °C.

Determination of Estrogenic Activity Using ERE-Luciferase Assay. The isolated phytoalexins phaseollin and kievitone were each prepared in DMSO at a concentration of 10 mM (stock solution). The final concentration of DMSO was adjusted to below 0.1% (v/v). MCF-7 cells were placed in phenol red-free DMEM supplemented with 5% dextran-coated charcoal-treated (DCC) FBS (5% CS−FBS) for 48 h prior to plating. The cells were plated in 24-well plates at 5 × 10^5 cells/well in the same media and allowed to attach overnight. The next day the cells were transfected with ER(2)-luc plasmid (Panomics) using jetPEI transfection reagent (Polyplus tranfection). After 6 h, cells were treated with DMSO, estradiol (0.1 nM), phytoalexin (0.1, 1, and 10 μM), legume extract (1, 10, and 100 μg/mL), or estradiol (0.1 nM) plus legume extract and incubated at 37 °C. After 18 h, the media were removed, and 200 μL of 1× lysis buffer (Promega) was added per well and incubated for 15 min at room temperature. The cell debris was then pelleted by centrifugation at 15000*g* for 5 min. The cell extracts were normalized for protein concentration using Bio-Rad reagent following the supplied protocol (Bio-Rad Laboratories, Hercules, CA). Luciferase activity for the cell extracts was determined using 1× luciferase assay substrate (Promega, Madison, WI) in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). Reported data are the mean (± SEM) of three independent experiments.

HEK 293 cells were cultured in 5% FBS−DMEM and seeded into 24-well plates at a density of 50000 cells/well and allowed to attach overnight in 5% CS−FBS. Cells were transfected with 0.2 μg of ER(2)-luc plasmid (Panomics, Santa Clara, CA), 0.2 μg of pcDNA3.1B-ERβ, or 0.2 μg of pcDNA3.1B-ERα plasmids the next day using Effectene transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol. After a 6 h transfection, cells were treated with compounds or DMSO overnight. The following day, the cells were lysed with 150 μL of M-Per mammalian extraction reagent (Pierce, Rockford, IL). One hundred microliters of cell extract was assayed using the Bright-glo luciferase assay substrate (Promega, San Luis Obispo, CA) and determined

in a Berthold AutoLumat Plus luminometer. Reported data are the mean (± SEM) of three independent experiments.

Determination of Estrogenic Activity Using MCF-7 Cell Proliferation Assay. The proliferation of MCF-7 cells used is a modified version of published methods (*37*−*39*). MCF-7 cells were placed in phenol red-free DMEM supplemented with 10% DCC FBS (5% CS−FBS) for 7 days prior to plating. The cells were plated in 96-well plates at 4.5×10^3 cells/well in the same media and allowed to attach overnight. After 24 h, the cells were dosed with treatment media at 100 μL/well. Treatment media consisted of 10% DCC FBS into which isolated phytoalexins and controls in DMSO were added. The experimental cells were redosed with isolated phytoalexins on day 4. E2 carrier used as a positive control (0.1 nM) increased cell proliferation 2.5-fold over negative controls (DMSO carrier only). Cell proliferation was measured on day 7 when positive control wells reached 90−100% confluence. Alamar Blue dye was added to the medium (10 μL/well), and the plates were incubated for 3 h at 37 °C with 5% CO_2. Fluorescence was monitored at 560 nm excitation and 595 nm emission using an HTS7000 series bioassay reader (Perkin-Elmer, Boston, MA). Within proliferation assays, each dose was run in four wells. Reported data are the mean (± SEM) of three independent experiments.

Determination of Antiestrogenic Activity Using MCF-7 Cell Colonial Survival Assay. Similar to our previous studies (*40*, *41*), cells were cultured in 5% FBS−DMEM and medium was changed to phenol red-free 5% CS−DMEM for 48 h prior to assay. MCF-7 cells were seeded at a density of 1×10^3 cells/well in a 6-well plate containing phenol red-free 5% CS−DMEM. The cells were allowed to attach overnight and treated on the following day with DMSO vehicle, 0.1 nM E2, and 10 μM phytoalexin + 0.1 nM E2. Cells were incubated at 37 °C and 5% CO_2 in a humidified incubator. Media were replaced every 7 days and treated with appropriate drug for 3 weeks. After 3 weeks, the media were removed and the cells fixed with formaldehyde and dried overnight. The cells were then washed and stained with crystal violet and dried. The colonies were counted.

Statistical Analyses. All experimental data were analyzed using XLSTAT (version 2007.6; Addinsoft, Inc., New York, NY). Statistical evaluation of the results was performed by Dunnett's multiple-comparison test. The Dunnett procedure compares the means as measured for the treatment groups to the control mean. Differences are considered to be significant at $p < 0.05$. Each value is presented as the mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Effect of *A. sojae* on Red Kidney Bean Extracts. One method to produce red kidney bean phytoalexins is to treat cut seeds with the fungus *A. sojae* (*23*). Changes in legume seeds inoculated with *A. sojae* were analyzed utilizing HPLC-UV. A representative HPLC-UV profile comparison between noninoculated legumes and inoculated legumes with *A. sojae* is displayed in **Figure 2**. **Figure 2A** displays the HPLC-UV chromatogram obtained from noninoculated red kidney beans. **Figure 2B** displays the HPLC chromatogram obtained from *A. sojae*-treated red kidney beans. Significant changes in HPLC-UV chromatograms are observed including the presence of the phytoalexins kievitone and phaseollin (structures in **Figure 1**).

Identification of Red Kidney Bean Phytoalexins. Phaseollin and kievitone were successfully identified using HPLC combined with atmospheric pressure chemical ionization (APCI). The positive ion mass spectra for each phytoalexin $(M + H)^+$ were phaseollin at m/z 323 and kievitone at m/z 357. Evaluation of the UV spectra for the peaks corresponding to phytoalexins revealed identical spectra compared with those in earlier papers with UV_{max} for phaseollin at 280 nm and for kievitone at 294 nm (*18*, *27*, *30*, *42*−*44*). ^{1}H NMR results for each phytoalexin matched earlier results (*30*, *42*−*44*).

Quantitation of Legume Phytoalexins. Phytoalexin contents in elicitor treatments of red kidney bean extracts are presented in **Table 1**. The two different elicitors used for preparation of treated legume extracts were a yeast extract and an *A. sojae* spore suspension. The use of a yeast extract as an elicitor has been reported by Hahn and Albersheim (*45*). In our study, treatment with *A. sojae* produced the highest levels of phaseollin (227.8 μg/g) and kievitone (1199 μg/g) in red kidney bean extracts. Lower levels of phaseollin (91.2 μg/g) and kievitone (479.1 μg/g) were observed in seed treatments using the yeast extract. The phytoalexins were not detected in untreated red kidney bean extracts.

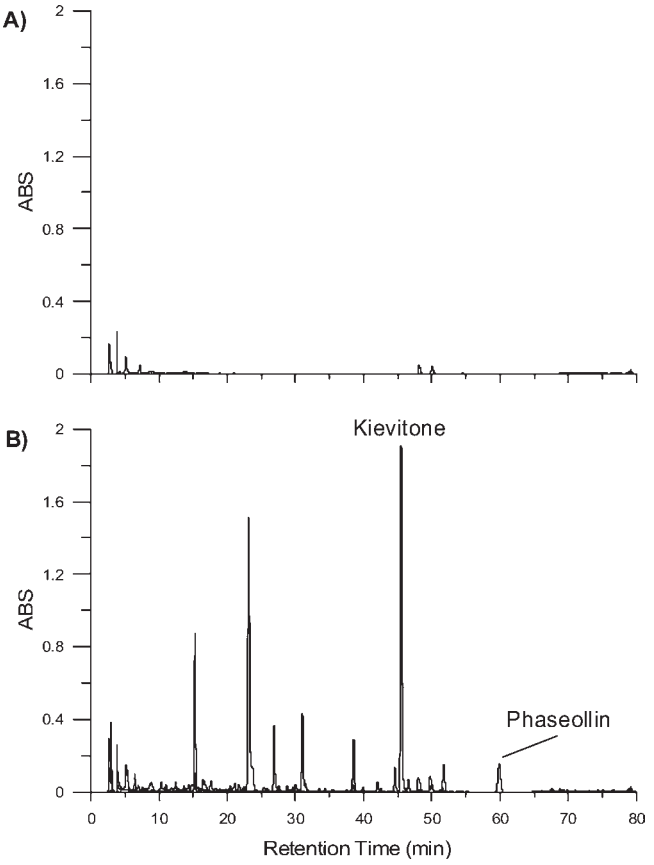


Figure 2. HPLC-UV chromatograms (λ = 285 nm) of the methanol extracts of red kidney beans: (**A**) untreated; (**B**) *Aspergillus sojae*-treated. The phytoalexins phaseollin and kievitone are identified.

Table 1. Phytoalexin Composition of Untreated and Elicitor-Treated Red Kidney Beans

phytoalexin	untreated (μg/g dry wt)	yeast extract (μg/g dry wt)	*Aspergillus sojae* (μg/g dry wt)
phaseollin	ND[a]	91.2 ± 22	227.8 ± 44
kievitone	ND	479.1 ± 17	1199 ± 101

[a] ND, not detected.

Transcriptional Activation and Inhibition of ER by Legume Extracts in MCF-7 Cells. The estrogenic activities of the red kidney bean extract at various concentrations (1−100 μg/mL) were analyzed by measuring the transcriptional activation using an MCF-7 reporter gene assay (**Figure 3**). To determine if extracts were antiestrogenic, MCF-7 cells were transfected with ERE-luciferase and treated with E2 (0.1 nM) in addition to increasing concentrations of extracts (1−100 μg/mL). Low ER transcriptional activation (8−9%) was observed with the untreated red kidney bean extract (**Figure 3**); however, higher transcriptional activation (40%) was detected at the highest concentration of the *A. sojae*-treated red kidney bean extract. Antiestrogenic activity (67−59%) was observed in the untreated red kidney bean extract (10−100 μg/mL); however, treatment with *A. sojae* produced higher antagonist activity (40%) at the highest concentration (100 μg/mL).

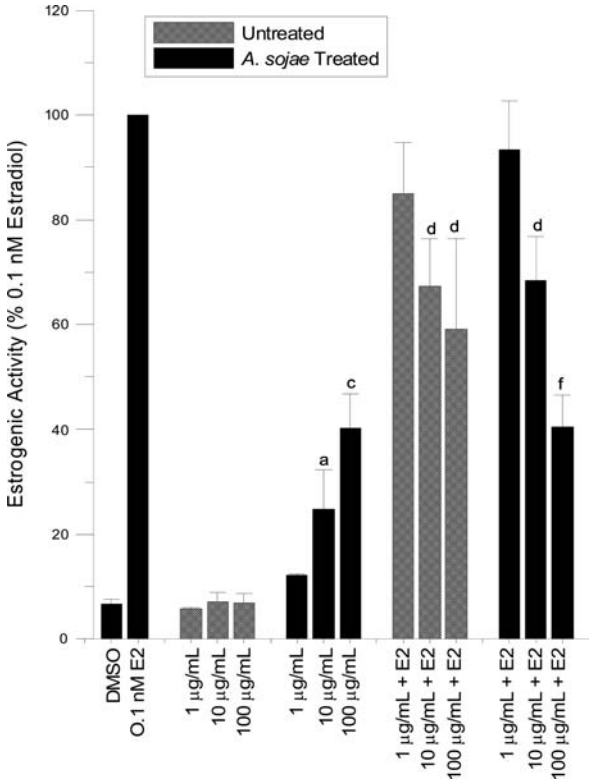


Figure 3. Effects of red kidney bean extracts on ERE transcriptional activity in MCF-7 cells. MCF-7 cells were transiently transfected with pGL2-ERE2x-TK-luciferase plasmid. After a 6 h transfection, cells were treated with untreated and *Aspergillus sojae*-treated red kidney bean extracts (alone and combined with E2). Estrogenic activity was set to 100% using 0.1 nM estradiol. Data represent the mean ± SEM of three independent experiments. Letters above bars: a, significant difference from DMSO control, $p < 0.05$; c, significant difference from DMSO control, $p < 0.001$; d, significant difference from E2 (100%), $p < 0.05$; f, significant difference from E2 (100%), $p < 0.001$.

The transcriptional activation of the estrogen receptor by legume extracts is a well-characterized phenomenon (*46*). The ability of *A. sojae*-treated red kidney bean extract to increase the expression of an ERE−LUC reporter gene in MCF-7 cells suggests that they may exert their estrogenic activity, at least in part, via an ER-dependent mechanism. Several components of red kidney bean, including coumestrol (*42*), biochanin A (*8*, *9*), kaempferol (*11*), other isoflavones (*47*), kievitone, and phaseollin, may be responsible for this observed estrogenic activity.

The ability of the untreated red kidney bean extract to attentuate or down-regulate ER gene transcription suggests a constitutive component is necessary for the observed activity. The weak to moderate ER antagonist activity detected may be due to constitutive isoflavone components. However, the moderate antagonist activity observed after treatment with *A. sojae* suggests enhanced antiestrogenic activity when compared to the untreated extract. The further down-regulation of ER that is observed using the *A. sojae*-treated red kidney bean extract suggests that an inducible component or phytoalexin contributes to this antagonistic activity. The ability of legume extracts to antagonize ER gene transcription has been observed in *A. sojae*-treated soybean extracts, and phytoalexin components glyceollins were identified as the antiestrogens (*23*). A similar phytoalexin component of red kidney bean may contribute to the antagonist activity observed.

Transcriptional Activation and Inhibition of ER by Phytoalexins in MCF-7 Cells. To further elucidate the components responsible for the observed estrogenic and antiestrogenic activities observed with legume extracts, the induced phytoalexins kievitone and phaseollin were isolated and tested in further assays. The estrogenic activities of the isolated legume phytoalexins at various concentrations (0.1−10 μM) were analyzed by measuring the transcriptional activation using an ERE luciferase assay in MCF-7 cells (**Figure 4**). The phytoalexin phaseollin (**Figure 4A**) displayed only low ER transactivation of 15.8% at the highest dose (10 μM) tested. However, kievitone displayed higher ER transactivation of 52% at 1.0 μM and 86.4% at 10 μM (**Figure 4B**). Kievitone, which is induced during *A. sojae* treatment, may be a component responsible for the enhanced estrogenic activity observed with the *A. sojae*-treated red kidney bean extract. When combined with E2 in this assay, phaseollin displayed no significant antiestrogenic activity. Attenuation of ER transactivation to 80% was observed with kievitone at 1 μM, but no antiestrogenic activity was displayed at 0.1 and 10 μM.

Estrogenic Activities of Phytoalexins in MCF-7 Cells. The proliferation of MCF-7 cells is a well-established biological response to 17β-estradiol and a useful screening tool for compounds that may function as estrogen agonists (*37*, *48*). The estrogenic activity of the legume phytoalexins was analyzed by measuring the MCF-7 cell proliferation in response to various concentrations (0.01−10 μM) shown in **Figure 5**. Both phytoalexins tested displayed estrogenic activity in this assay. Of the two phytoalexins, kievitone displayed the highest level of cell proliferation of 107% at 10 μM and 84.6% at 1.0 μM. Estrogenic activity was observed with phaseollin at 10 μM (79.8%) and much lower agonist activity at 1.0 μM (13.8%). The ability of kievitone to induce MCF-7 cell proliferation correlates well with the activation of ER transcriptional activity, and this correlation points to kievitone acting through an ER-dependent mechanism. However, the ability of phaseollin to induce MCF-7 cell growth does not correlate with the low ER transcriptional activity observed in the reporter gene assay. Phaseollin may enhance cell proliferation through a non-ER-mediated pathway. The isoflavones coumestrol (97%) and genistein (67.9%) were agonists at lower concentrations (0.1 μM) in the MCF-7 proliferation assay when compared to kievitone and phaseollin.

Relative Affinity of Phytoalexins for ERα and ERβ. Several studies have demonstrated that phytoestrogens exert their stimulatory effect on the ER by binding to the same site as E2 with some differences in ligand binding specificity and transactivation between ERα and ERβ (*15*, *16*, *49*, *50*). Of particular interest was the observation that certain isoflavones may bind with higher affinity and possess higher agonistic activity toward ERβ (*15*, *16*, *49*, *50*). To assess the ability of phytoalexins to bind to ERα and ERβ, a competitive binding assay with fluorescent detection was utilized. **Table 2** details the results for the competitive binding assay using ERα and ERβ. For ERα, kievitone had the highest relative binding affinity at 0.485% of the two phytoalexins tested. Phaseollin displayed the highest relative binding affinity (0.530%) for ERβ. Kievitone displayed only slightly higher binding for ERα (0.485%) compared to ERβ (0.418%). However, overall the two phytoalexins tested displayed low binding to ERα and ERβ when compared to other more estrogenic constitutive isoflavones such as genistein and daidzein from soybean (*51*).

Transcriptional Activation of ERα and ERβ. A reporter gene assay using HEK 293 cells transfected with either ERα (**Figure 6A**) or ERβ (**Figure 6B**) expression plasmids was used to determine whether estrogenic activity of kievitone and phaseollin was mediated via ERα and/or ERβ. Many isoflavones, particularly the soy isoflavones daidzein and genistein, have demonstrated preferential binding affinity toward ERβ (*15*, *16*). Also, genistein has demonstrated an ERβ-selective affinity in reporter gene assays,

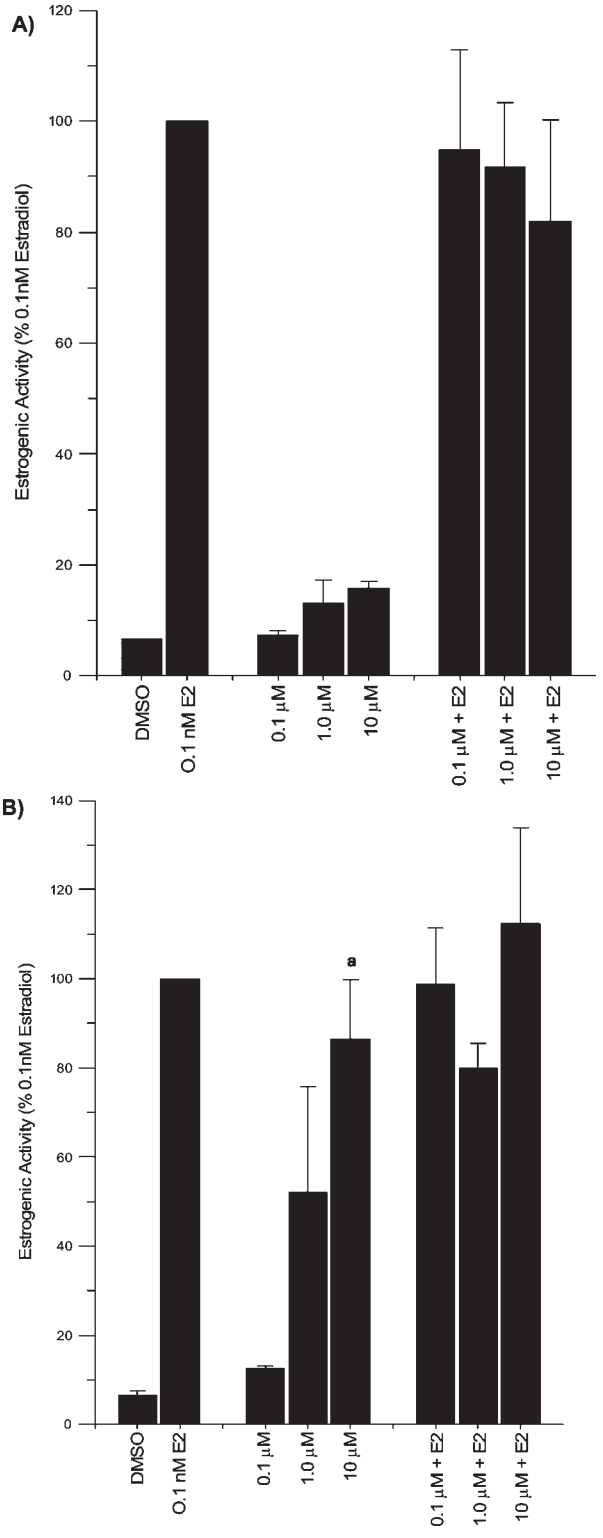


Figure 4. Effects of phaseollin and kievitone on ERE transcriptional activity in MCF-7 cells. MCF-7 cells were transiently transfected with pGL2-ERE2x-TK-luciferase plasmid. After a 6 h transfection, cells were treated with (**A**) phaseollin and (**B**) kievitone (alone and combined with E2). Estrogenic activity was set to 100% using 0.1 nM estradiol. Data represent the mean ± SEM of three independent experiments. Letters above bars: a, significant difference from DMSO control, $p < 0.05$.

but an ERα-selective efficacy (*49*). In our assay, phaseollin displays no ERα transactivation but displays higher ERβ transactivation at 1−10 μM with a maximal effect observed at 10 μM of 31.6% relative to E2. These data point to phaseollin preferentially

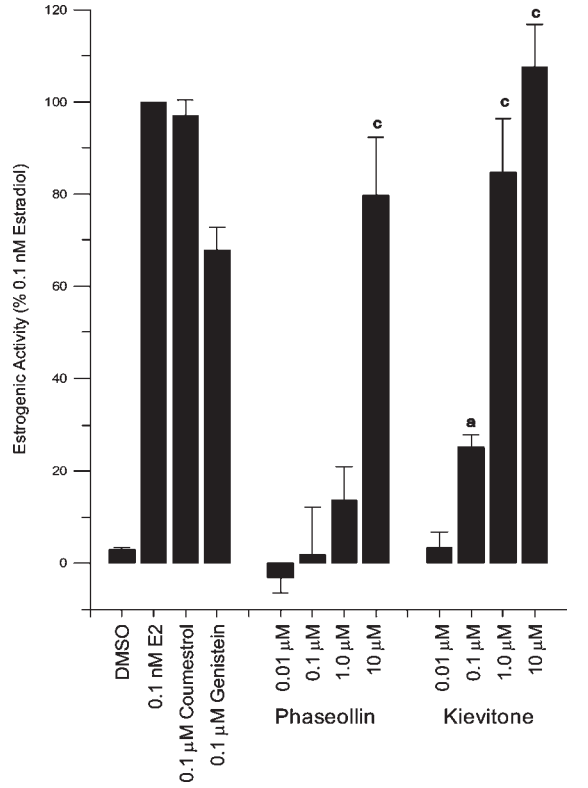


Figure 5. Estrogenic activity of phaseollin and kievitone using the MCF-7 cell proliferation assay. Estrogenic activity was set to 100% using 0.1 nM estradiol. Data represent the mean ± SEM of three independent experiments. Letters above bars: a, significant difference from DMSO control, $p < 0.05$; c, significant difference from DMSO control, $p < 0.001$.

Table 2. Relative Binding Affinities of Phytoalexins with ERα and ERβ[a]

	ERα		ERβ	
phytoalexin	IC_{50}	RBA (%)	IC_{50}	RBA (%)
17β-estradiol	7.00×10^{-10}	100	7.23×10^{-10}	100
phaseollin	3.28×10^{-7}	0.213	1.34×10^{-7}	0.530
kievitone	1.45×10^{-7}	0.485	1.73×10^{-7}	0.418

[a] The E2 relative binding affinity (RBA) was set equal to 100 [RBA = (IC_{50} of E2/IC_{50} of phytoalexin) × 100].

activating ERβ in agreement with earlier ER binding results (**Table 2**). This activity also correlates well with the low ER transactivation observed using the MCF-7 cell reporter gene assay. Kievitone displayed ERβ transactivation of 52.8% only at the highest concentration tested (10 μM). However, kievitone displayed maximal ERα transactivation (55.2%) at 1 μM and slightly lower ERα transactivation (45.2%) at 10 μM. These data point to kievitone as activating ERα preferentially in agreement with ER binding results (**Table 2**). The isoflavones daidzein and genistein are potent agonists of both ERα and ERβ (data shown for 0.1 μM for each) in this assay, and both kievitone and phaseollin are weaker agonists by comparison.

Antiestrogenic Activity of Phaseollin and Kievitone. To further evaluate the antiestrogenic activities of phaseollin and kievitone, the phytoalexins were tested by a colonogenic assay for their ability to inhibit the estradiol-induced proliferation of MCF-7 cells. In the colony assay MCF-7 cells were treated with each phytoalexin at 10 μM in the presence of 0.1 nM E2. Results are illustrated in **Figure 7**, where estrogen-dependent cell growth was normalized to 100 for E2. Both phytoalexins attenuated cell clonogenicity at 10 μM. Phaseollin attenuated clonogenicity to

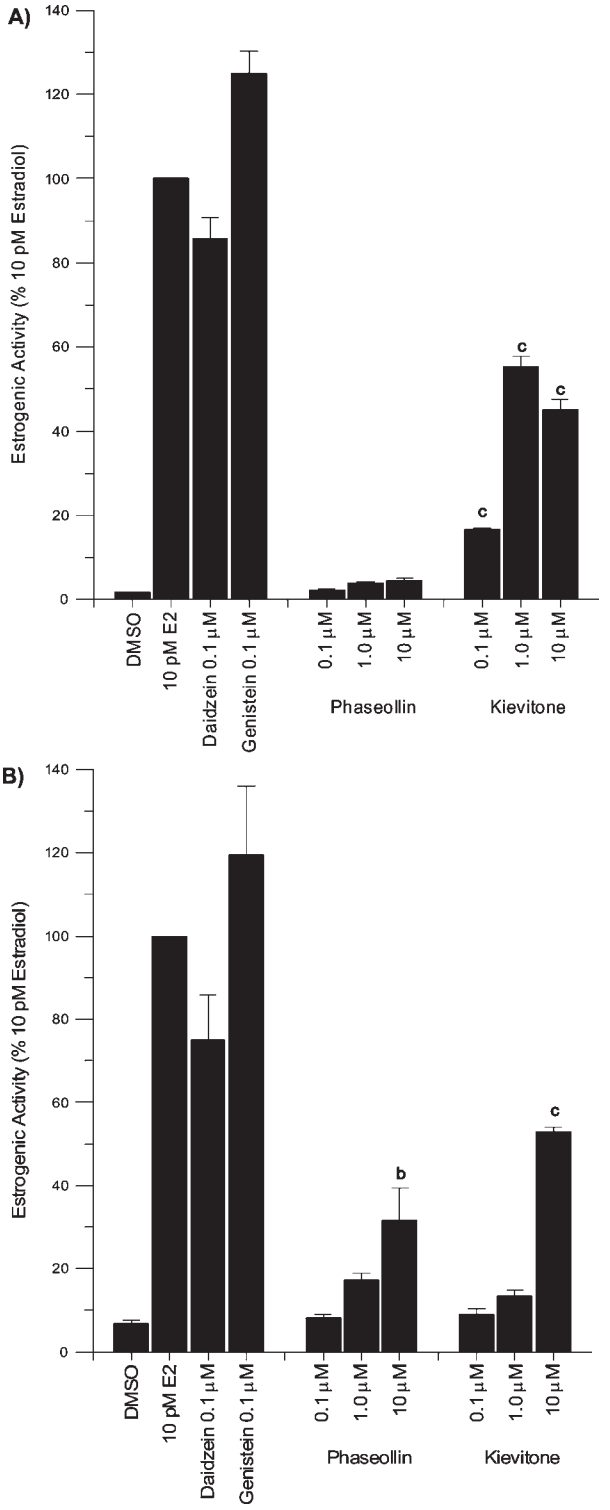


Figure 6. Transcriptional activation of ERα (**A**) and ERβ (**B**) in response to phaseollin and kievitone. Transfection assays were conducted in HEK 293 cells using the pG12-ERE2X-TK-luciferase reporter. Estrogenic activity was set to 100% using 10 pM estradiol. Data represent the mean ± SEM of three independent experiments. Letters above bars: b, significant difference from DMSO control, $p < 0.01$; c, significant difference from DMSO control, $p < 0.001$.

65.4% versus E2. Kievitone was the most effective antiestrogen, decreasing clonogenicity to 49.6% versus E2.

The ability of kievitone to attenuate the growth of breast cancer cells has been reported by other researchers (*32*). Kievitone was effective in attenuating the proliferation of ER-positive

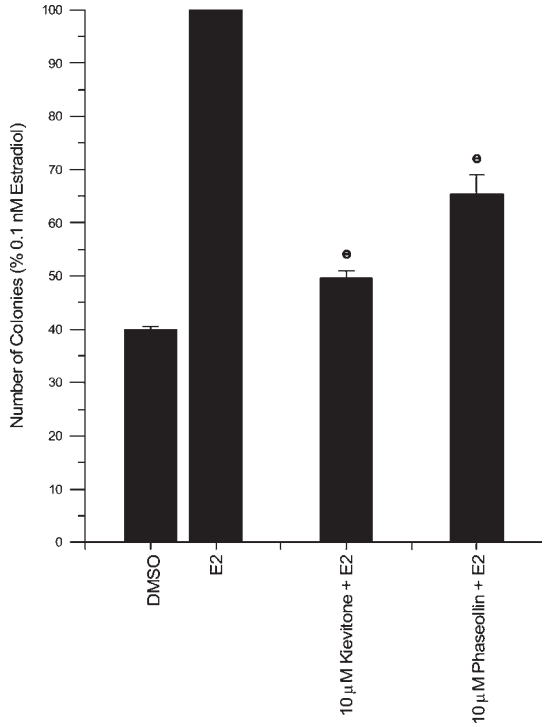


Figure 7. Antiestrogenic activity of phaseollin and kievitone at 10 μM using the MCF-7 colony formation assay. Estrogenic activity was set to 100% using 0.1 nM estradiol. Data represent the mean ± SEM of three independent experiments. Letters above bars: e, significant difference from E2 (100%), $p < 0.01$.

breast cancer cell lines MCF-7 and T47D and the ER-negative breast cancer cell line SKBR3 (*32*). Compared with kievitone, genistein was 3−9-fold weaker as an inhibitor of the proliferation of the breast cancer cell lines and of growth factor-stimulated DNA synthesis (*32*). Kievitone is structurally similar to genistein but contains a prenyl group at the C8 of ring A (**Figure 1**), a chromanone ring (ring C), and an additional OH group at position 6′ of ring B. Several studies have shown that prenylation can enhance estrogenic activity of isoflavones, particularly naringenin; however, prenylation at the C8 position of genistein weakened estrogenic activity in several in vitro assays (*52*). This weakening of agonist activity suggests that the prenyl group of kievitone may be partially responsible for the attenuation of breast cancer cell growth. The addition of the prenyl group may also lead to enhanced antiestrogenic activity. Antiestrogenic activity was enhanced in several prenylated isoflavones from *Melletia pachycarpa* when prenylation occurred at positions 6 and 8 on the A-ring (*53*).

In conclusion, phytoalexins induced by elicitor treatment in red kidney bean extracts possess estrogenic and antiestrogenic activities. *A. sojae*-treated extracts from red kidney bean exhibited enhanced estrogenic activity and also displayed antiestrogenic activity at the highest doses tested. Kievitone displayed estrogenic activity in both MCF-7 proliferation and reporter gene assays with selective ERα transactivation. Phaseollin exhibited estrogenic activity in MCF-7 proliferation, but lower activity in a reporter gene assay with selective ERβ transactivation. Both phaseollin and kievitone exhibited antiestrogenic activity in an MCF-7 colonial survival assay. This work provides evidence that the red kidney bean phytoalexins kievitone and phaseollin possess both estrogenic and antiestrogenic activities. This study also points to the potential for phytoalexin-enriched functional foods using elicitor-treated red kidney beans; however, further research is warranted.

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