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# Extraction and Purification of Isoflavones from Soybeans and Characterization of Their Estrogenic Activities

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Soybean isoflavones have multiple beneficial health effects especially on estrogen-deficient diseases such as menopausal symptoms. In this study, isoflavones were produced from soybean flour, and the extraction and purification parameters were optimized to give a high yield of total isoflavones, about 0.62 mg of aglycones/g of soybean flour, which is >2 times the initial yield. HPLC analysis and MTT cell proliferation assay using MCF-7 cells revealed that the product thus obtained not only contained a high content of isoflavone aglycones but also had estrogenic activity. MTT data also revealed that both genistein and daidzein exhibited estrogenic effects at lower concentrations and antiproliferative effects at higher concentrations, and 1 $\mu$M genistein and 10 $\mu$M daidzein exerted significant estrogenic activities, which were not more than that of the endogenous level of 17$\beta$-estradiol ($E_2$). The production method developed can be used as a guideline for manufacturing soy isoflavones, and the MTT assay was demonstrated to be suitable for quality control on isoflavone products. The results on the estrogenic properties of isoflavones can be used as reference data for their effective and safe usages in estrogenic therapy.

**KEYWORDS: Isoflavones; soybeans; extraction; purification; optimization; MTT assay; quality control; estrogenic properties; genistein; daidzein**

## INTRODUCTION

Isoflavones are a flavonoid subgroup found in a variety of plants (*1*), with significant amounts in soybeans (*2*). To date, 12 main isoflavones have been characterized in soybeans and soybean products including genistein, daidzein, glycitein, and their respective malonyl, acetyl, and glucosyl forms (*1*, *3*). Interest in soy isoflavones has increased around the world because epidemiologic studies have shown that the consumption of soy isoflavones (*3*) may be associated with low incidence rates of certain types of cancers (*4*−*8*) and reduction in the risk of various diseases including cardiovascular problems (*8*, *9*), osteoporosis (*8*, *10*, *11*) and menopausal symptoms (*8*, *11*, *12*).

Nowadays soy isoflavones are increasingly used instead of traditional hormone replacement therapy (HRT) for estrogen-deficient women in menopause or postmenopause (*13*, *14*). Because isoflavones are a class of phytoestrogens that structurally resemble genuine 17$\beta$-estradiol ($E_2$) and have weak estrogenic activities (*15*), they can mimic or modulate the actions of endogenous estrogens in vertebrates by binding to estrogen receptors (ERs) (*16*). Compared with HRT, they may not only relieve menopausal symptoms and other estrogen-deficient diseases (*8*, *17*, *18*) but also decrease the risk of estrogen-related cancers (*8*, *18*), which are one of the main health risks induced by HRT (*19*, *20*).

Due to the multiple beneficial effects of soy isoflavones on human health, related products have flooded the market, with unsubstantiated claims and few regulations governing their quality or efficacy (*21*). Most products have levels of isoflavones less than their claimed contents, with some containing virtually no detectable isoflavones (*21*). Moreover, many products only contain soy extracts with little isoflavones (i.e., 0.2% for the products of U.S. patents 5,858,449 and 6,818,246) and abundant unknown impurities. More importantly, isoflavones in many products are in the form of glucosides, which have weaker biological activities and are more difficult to be absorbed by the body than the corresponding aglycones (*22*−*24*). In addition, many products are manufactured from soy germ, and consequently their major isoflavones are glycitein (glycitin) and daidzein (daidzin). However, daidzein has a much lower estrogenic potency than genistein (*25*), and glycitein was predicted to be even less estrogenic than daidzein (*21*). Therefore, the quality and efficacy of many isoflavone products are poor, and there is an urgent need to develop novel products with higher purity and efficacy.

The main objective of this study was to develop a highly purified soy isoflavone product that is potentially efficacious for relieving estrogen-deficient diseases (i.e., menopausal symptoms). To maximize the production efficiency and minimize the production cost, simple and low-cost strategies (i.e.,

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antisolvent crystallization) were developed and the extraction and purification parameters were optimized mainly on the basis of the yields of isoflavones. As a result, the products obtained under the optimum conditions were found to have a high content of isoflavone aglycones by high-performance liquid chromatography (HPLC).

To evaluate the biological activities of the purified isoflavone products, a cell proliferation test, the so-called E-SCREEN (*26*), was used. In this experiment, the estrogenic activities of the test samples were measured quantitatively by assaying the proliferation of MCF-7 cells (estrogen-sensitive cell line) (*26*). The results showed that our isoflavone product had a clear estrogenic activity and could be effective for treating menopausal symptoms. In addition, the estrogenic properties of pure isoflavones were explored using the same assay to provide insights into their effective and safe usages in estrogenic therapy.

## MATERIALS AND METHODS

**Chemical Materials.** The isoflavones (genistein, daidzein, and glycitein) with purity >99% were purchased from LC Labs. 17$\beta$-estradiol and 4-hydroxytamoxifen (both with purity >98%) and soybean flour were the products of Sigma Aldrich. Ethanol (96%) was obtained from Riedel de Haën; 37% hydrochloric acid and acetic acid were both from Fisher Scientific. Pure ethanol (Merck) and methanol (Mallinckrodt) used were of HPLC grade. Water was supplied by a Milli-Q water purifier system (Millipore).

**Sample Preparation.** Isoflavone standards were prepared in pure ethanol by serial 2-fold dilutions, and the following concentrations were obtained: from 62.5 to 500 $\mu$g/mL for genistein and daidzein and from 12.5 to 100 $\mu$g/mL for glycitein.

For comparison purposes, four commercial isoflavone supplements were studied. Three solid isoflavone products and a colloidal one were all extracted with pure ethanol by stirring for 2 h at 37 °C. Then the solutions were centrifuged at 4000 *rpm* for 10 min, and the supernatants were used. The above supernatants, our products from soybeans, and the ethanol solutions of isoflavone standards were all stored at 4 °C and filtered through 0.20 $\mu$m syringe filter (Millex) before HPLC analysis.

**HPLC Analysis.** Isoflavone contents in all samples were analyzed using a reversed-phase $C_{18}$ column (Phenomenex, 5 $\mu$m, 250 × 4.6 mm i.d.) on the Agilent 1100 series liquid chromatograph (including the pumping system, vacuum degasser, autosampler, and UV-DAD detector). The sample injection volume was 10 $\mu$L. The mobile phase was water with 0.1% acetic acid (A) and methanol (B). A linear gradient elution was applied from 30 to 50% B starting from 0 to 45 min, at a flow rate of 1.0 mL/min. The temperature of the column was maintained at 40 °C, and the detection wavelength was set at 255 nm, where absorbance peak areas were quantified.

The identification of each isoflavone was made by comparing the retention times with those of pure standards, as well as by UV spectra comparison. Quantitative analysis was done by using calibration curves. Standard solutions were injected in triplicate and detected sequentially. The standard curves were constructed individually by plotting the average peak areas of the standard solutions against the corresponding concentrations. Then the curves characterized by slope (*b*), intercept (*a*), and correlation coefficient ($R^2$) were used to determine the concentration of each isoflavone in the samples and the performance characteristics such as analytical sensitivity (AS), limit of detection (LOD), and limit of quantification (LOQ).

Analytical sensitivity was calculated according to the ALAMIN program (*27*): AS = Ss/*b*, where Ss is the residual standard deviation. The limit of detection ($LOD_{approx}$) was determined by the equation $LOD_{approx} = 3(AS) \times [(n-2)/(n-1)]^{1/2}$ (where $n$ is the number of total measurements for each calibration set; here $n = 3$), and the limit of quantification ($LOQ_{approx}$) was calculated by replacing 3 with 10 in the preceding equation.

The precision and accuracy of the method were evaluated using standard solutions. At each concentration level, the solution was injected consecutively three times, and the relative standard deviation (RSD) was calculated to express the precision. The accuracy was expressed by the mean recovery (percent) of the target compound, the mean percentage of the compound concentration measured in each sample relative to the known amount spiked to the sample.

The stability of the three isoflavones in ethanol and mobile phase was respectively evaluated by assaying the standard solutions and the solutions diluted by 50% methanol. The peak areas were compared at 0 and 12 h. In the meantime, the solutions were kept at room temperature.

**General Extraction and Purification Procedure.** Extraction of isoflavones from dry soybean flour was performed using 40−99.99% ethanol at the material ratio of ethanol to soybean flour in volume (mL)/weight (g) of 1:1 to 10:1. The resulting mixture was heated at the temperatures of 40−90 °C for 2−24 h and stirred constantly. Then the extract was separated from insoluble fractions by vacuum filtration through no. 1 filter paper (Whatman). The extract (filtrate) was hydrolyzed with 37% hydrochloric acid or pure acetic acid, and the pH of resulting mixture was adjusted to 1−5. The mixture was heated at temperatures of 40−90 °C for 1−12 h and stirred constantly. Then the hydrolyzed product was mixed with distilled water at the volume ratio of 1:15 to 1:1 (mL/mL) by stirring constantly at room temperature, and the precipitated isoflavone crystals were separated by vacuum filtration through 0.20 $\mu$m membranes (Millipore). The solids retained on the membrane were dried and dissolved in pure ethanol. This solution was stored at 4 °C until analysis for isoflavone content and estrogenic activity.

**Cell Culture and Conditions.** The MCF-7 cells (ATCC) were maintained in minimum essential medium (MEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 2 mM sodium pyruvate (Invitrogen), and antibiotics (100 units/mL penicillin and 100 $\mu$g/mL streptomycin) (Invitrogen). Cell cultures were grown in T-75 $cm^2$ flasks at 37 °C in a humidified atmosphere with 5% $CO_2$ and subcultured every 2−3 days. At least 4 days before the beginning of the MTT proliferation assay, the cells were switched to phenol red-free Dulbecco's modified Eagle medium (DMEM)/F-12 (Ham) (1:1) (Invitrogen) supplemented with 5% charcoal-stripped FBS (Hyclone) and antibiotics as described previously (*28*).

**MTT Cell Proliferation Assay.** Cell viability was estimated according to the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, and Cell Proliferation Kit I (MTT) (Roche) was used. The number of seeded cells was determined to be (4−5) × $10^3$ cells/well because with this initial cell number there is an almost linear correlation between the numbers of cells and the optical density (OD) values during the incubation period (in our 5-day assays, the OD values did not exceed 0.7) (Supporting Information, Figure 1).

The pure isoflavones were dissolved in ethanol for making stock solutions. The stock solutions, our products, and the samples of four commercial supplements were individually filtered through a 0.20 $\mu$m syringe filter (Millex) for sterilization, serially diluted using phosphate-buffered saline (PBS), and then stored at 4 °C.

After maintenance in standard medium (phenol red-free medium with charcoal-stripped serum) for more than 4 days, MCF-7 cells were seeded into a 96 well plate with 100 $\mu$L of cell solution (about 5 × $10^4$ cells/mL) per well. After 24 h of incubation, the old medium was replaced by the new standard medium containing different test samples when it was $t_0$. MCF-7 cell yields were measured every 24 h until 5 days after $t_0$. At each time point, an aliquot (10 $\mu$L) of MTT labeling solution was added into each well, and the mixture was incubated in the dark for 4 h. Then 100 $\mu$L of solubilization solution was added into each well, and the mixture was incubated for 20 h. Then OD values were measured at the wavelength of 595 nm using an absorbance microplate reader (Bio-Rad). Each test included blank controls containing standard medium (without cells) and triplicate test samples. Net absorbance values were obtained after the average value of blank controls had been subtracted, and the OD value of a test sample is defined as the average of triplicate net absorbance values at day *X*.

The relative induction of cell proliferation (day *X*/day 0) is defined as the ratio of the OD value of a test sample obtained at day *X* to the one at $t_0$. The ratio would reflect the cell yield with this test sample at this time point. The relative induction of cell proliferation (% vehicle or control) is defined as the percentage of the relative induction of cell

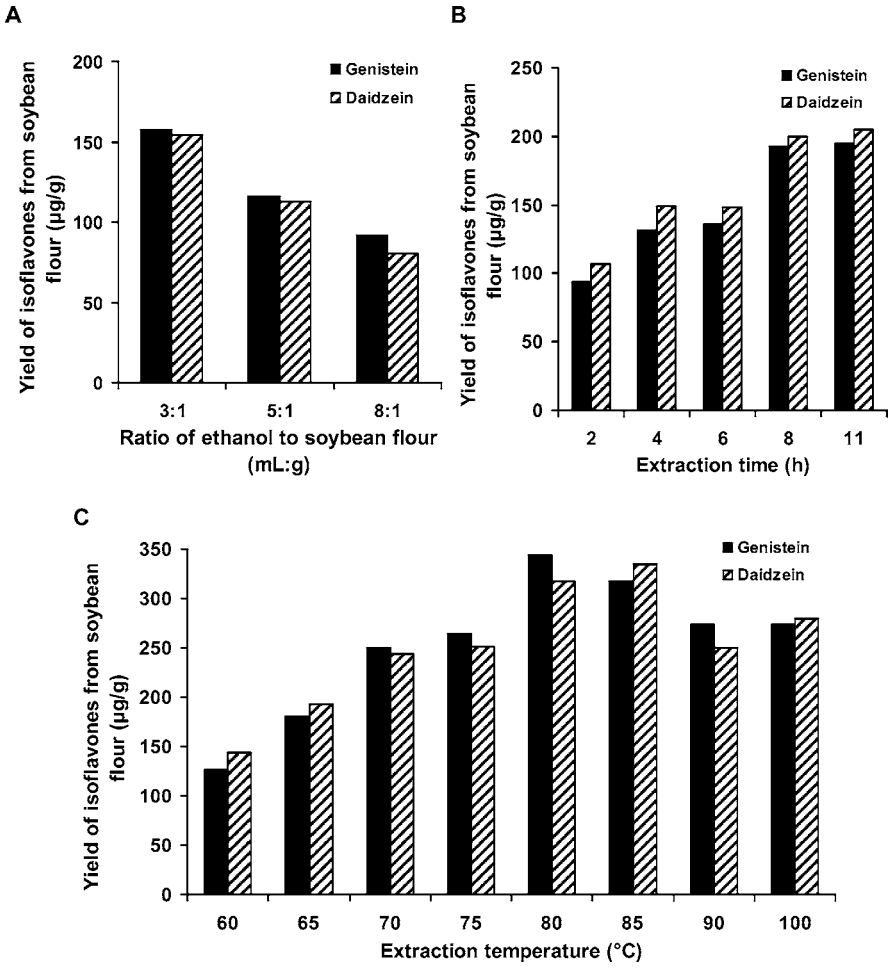


**Figure 1.** Yields of isoflavones (aglycone equivalency) from soybeans ($\mu$g of isoflavones/g of soybean flour) by extraction (**A**) with different material ratios of the volume of ethanol (mL) to the weight of soybean flour (g) at 62 °C for 8 h, (**B**) for different durations with the material ratio of 3:1 at 65 °C, and (**C**) at different temperatures with the material ratio of 3:1 for 8 h. The yield of glycitein was omitted because of its small proportion in the total isoflavones (always <5%).

proliferation (day 4/day 0) of a test sample to that of control (untreated cells) or vehicle (cells treated with solvent only). The cell yield obtained on the fourth day was chosen as the reference because it was reported that significant differences between control and estrogen-treated cultures were apparent after 4 days of treatment (*29*). Under the above experimental conditions, the percentage represented a reliable estimate of the relative proliferative effect achieved by a similar number of seeded cells exposed to different proliferation regulators.

**Statistical Analysis.** Every assay was repeated independently at least three times. All values are expressed as the mean ± standard deviation of three independent experiments. Difference between experimental groups was assessed by analysis of variance and then for significance by applying Student's two-tailed *t* test, taking $P < 0.05$ as significant (*), $P < 0.01$ as highly significant (**), and $P < 0.001$ as very highly significant (***).

## RESULTS

**Extraction and Purification of Isoflavones from Soybeans.** A number of physical and chemical methods have been attempted, and the determined strategy for extracting and purifying isoflavones generally encompasses three steps: (1) extracting isoflavones from soybean flour with an organic solvent (ethanol in this study); (2) hydrolyzing the glucoside fractions of the extract into aglycones using acid (hydrochloric acid in this study); (3) crystallizing the aglycones by the addition of antisolvent (water in this study) to the hydrolyzed product. The resulting isoflavone crystals were then characterized by HPLC.

To maximize the yields of isoflavones and minimize the production cost, the products obtained under different extraction conditions were analyzed by HPLC. Among the solvents used, pure ethanol extracted much higher amounts of total isoflavones (mainly malonyl and glucosyl forms of genistein and daidzein) than aqueous ethanol (i.e., 70% ethanol). To reduce the cost, a relatively cheaper solvent, 96% ethanol, was tested. Because 96% ethanol was found to be able to produce yields of isoflavones similar to those obtained with pure ethanol, it was selected as the solvent for extraction. During extraction, the yield of total isoflavones was also found to be associated with the material ratio of the volume of ethanol to the weight of soybean flour (**Figure 1A**). A lower ratio of 3:1 (mL/g) was preferred for its higher yield and lower solvent requirement than the ratios of 5:1 and 8:1. Moreover, the yield of total isoflavones increased with increasing extraction time and temperature (up to about 80 °C) (**Figure 1B**,**C**), with extraction temperature being more significant. Insufficient extraction duration yielded less isoflavones, and the yield always reached a maximum after 7−8 h of extraction (**Figure 1B**). Furthermore, a higher extraction temperature of 80 °C was preferred for maximizing the ratio of the yield of total isoflavones to the energy consumption (**Figure 1C**). In summary, the optimum extraction conditions were determined to be 80 °C for 8 h with the ethanol over soybean flour ratio of 3:1 to achieve the best extraction of isoflavones [about 0.87 mg of total isoflavones (aglycone equivalency)/g of soybean flour] (the deduction process is not shown).

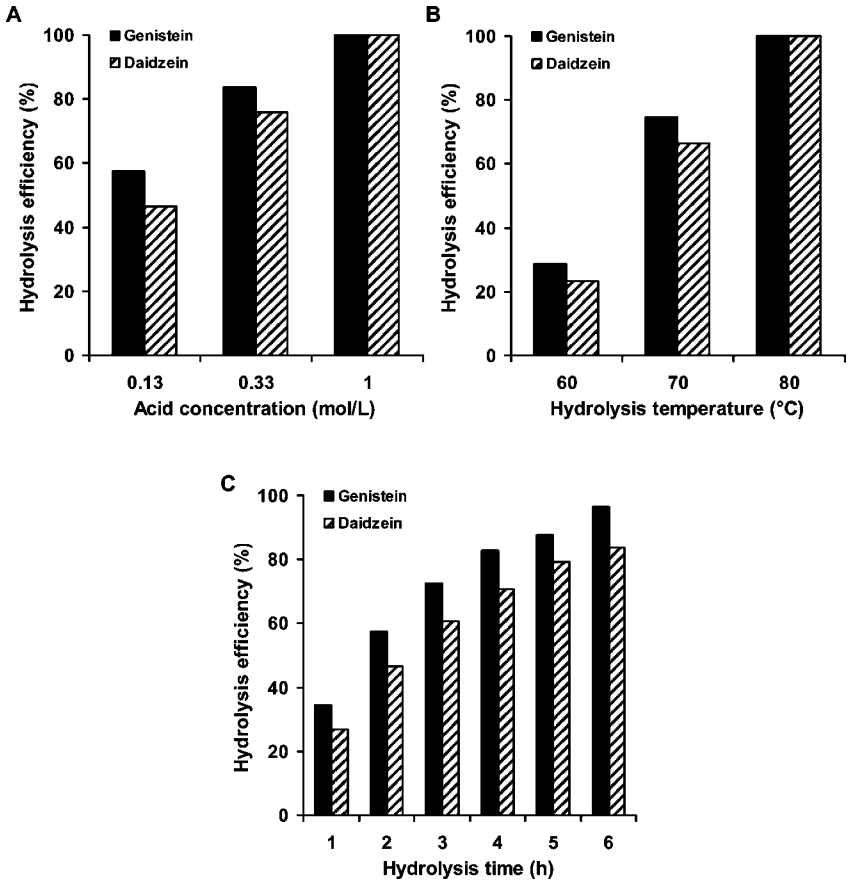


**Figure 2.** Hydrolysis efficiencies (percentages of the glucosides degraded into aglycones) (**A**) with different acid concentrations at 80 °C for 2 h, (**B**) at different temperatures with the acid concentration of 1 mol/L for 2 h, and (**C**) for different durations with the acid concentration of 0.13 mol/L at 80 °C. The efficiency for glycitein was omitted because of its small proportion in the total isoflavones (always <5%).

Then the effects of the hydrolysis parameters on hydrolysis efficiency were also analyzed by HPLC. The yields of aglycones (mainly genistein and daidzein) were found to be associated with acid concentration, hydrolysis time, and temperature (**Figure 2**), with acid concentration and hydrolysis temperature being more significant. Hydrolysis was not efficient with the low concentrations of acid used, whereas high concentrations of acid led to certain retention of acid in the crystallization products, which will be harmful to the human body. Hydrochloric acid (37%) was added to the extract to the preferred concentration of about 0.13 mol/L for assuring complete hydrolysis and decreasing acid concentration (**Figure 2A**). On the other hand, hydrolysis was not efficient at low temperatures (**Figure 2B**), and a higher temperature of 80 °C was selected for increasing hydrolysis efficiency. When a concentration of 0.13 mol/L of hydrochloric acid and a temperature of 80 °C were used, 6 h was enough time for almost complete transformation of the glucoside fractions into aglycones (**Figure 2C**).

During crystallization, the ratio of antisolvent to feed mixture greatly affected the yields of crystals (**Figure 3**). In this study, the higher ratio of water to the hydrolyzed product was used, the higher yields of isoflavone crystals were obtained. However, high ratios also led to high loading on the crystallization and filtration steps, which would result in difficulties in manufacturing. The ratio was finally decided to be 4:1 because higher ratios did not give significantly higher yields (**Figure 3**). Here 4:1 could achieve 83% recovery for genistein and 65% for daidzein.

Using all of the above optimum extraction and purification conditions, about 2 times genistein and glycitein and 2.5 times daidzein were produced compared with the initial yields (**Figure 4**). In other words, the yield of total isoflavones was doubled after optimization.

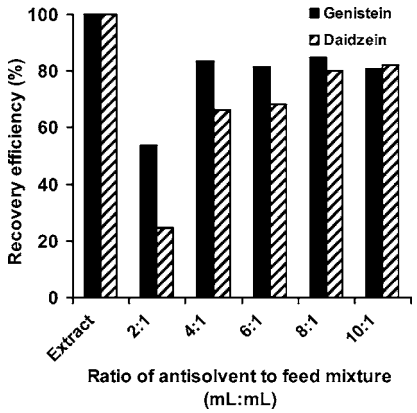


**Figure 3.** Recovery efficiencies of isoflavones with different volume ratios of antisolvent (water) to feed mixture (hydrolyzed product). The efficiency for glycitein was omitted because of its small proportion in the total isoflavones (always <5%).

**Method Validation of HPLC Analysis.** The calibration curves (Supporting Information, Figure 2) of the three isoflavone aglycones have been respectively constructed, and their characteristics are shown in **Table 1**.

The values of correlation coefficient all reached 0.999, confirming the excellent linearity of the three calibration curves. Considering the concentration ranges of isoflavones in all of the samples (genistein >120 $\mu$g/mL, daidzein >100 $\mu$g/mL,

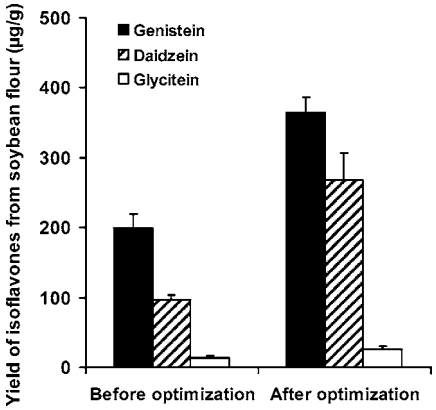


**Figure 4.** Yields of isoflavones from soybeans ($\mu$g of isoflavones/g of soybean flour) before and after optimization. Data are expressed as the mean ± standard deviation of isoflavone products from five independent experiments.

**Table 1.** Linearity and Performance Characteristics for the Analytes

| compd | regression equation[a] | $R^2$ | AS ($\mu$g/mL) | $LOD_{approx}$ ($\mu$g/mL) | $LOQ_{approx}$ ($\mu$g/mL) |
|---|---|---|---|---|---|
| genistein | $y = 75.672x - 33.711$ | 0.99991 | 3.89 | 8.24 | 27.48 |
| daidzein | $y = 51.050x + 82.109$ | 0.99989 | 1.22 | 2.59 | 8.63 |
| glycitein | $y = 45.371x + 70.605$ | 0.99978 | 0.83 | 1.76 | 5.87 |

[a] $y$ = peak area, $x$ = concentration.

glycitein >30 $\mu$g/mL), the values of AS, LOD, and LOQ were low enough, indicating the fairly good sensitivity of the HPLC method.

At each concentration measured, the precision values (RSD) for determining the three isoflavones were all <5%. The mean recovery for each isoflavone varied from 94 to 106%. These results met the acceptance criteria.

In addition, the stability of the three isoflavones in ethanol and mobile phase (aqueous methanol) at room temperature was confirmed at least within 12 h because none of the peak areas of the three isoflavones experienced significant changes (RSD < 5%).

**Measurement of Isoflavone Content of Our Extracts.** The HPLC chromatogram of isoflavones in one of our products obtained under the optimum extraction and purification conditions is shown in **Figure 5B**. The peaks corresponding to three main isoflavone aglycones were eluted from the column in the same order and at similar retention times (33.8 min for daidzein; 37.0 min for glycitein; 43.7 min for genistein) as the authentic standards. UV scans of these peaks also showed that they had the same absorption spectra as the standards (data not shown), which further confirmed the correct identity for each peak.

By applying the corresponding regression parameters (**Table 1**), the level of each aglycone in this optimized product (**Figure 5B**) was deduced: 0.942 mg/mL for genistein, 0.641 mg/mL for daidzein, and 0.071 mg/mL for glycitein. As an example, a total of 60 mL of this product was obtained from the initial 160 g of soybean flour using the optimum conditions. In this example, 56.5 mg of genistein, 38.5 mg of daidzein, and 4.3 mg of glycitein were produced, and the yield of total isoflavones was calculated to be about 0.62 mg/g of soybean flour. According to the HPLC chromatogram (**Figure 5B**), the composition of our product was relatively simple. Genistein and daidzein were the main isoflavone components, with genistein occupying about 57% of total isoflavones and the value for daidzein being 39%. At 255 nm, there were only minor impurities detected in this product.

The unoptimized products showed similar chromatograms (i.e., **Figure 5A**) because optimization did not change the composition of our products on the whole. According to the results from five independent experiments (**Figure 4**), before optimization genistein and daidzein were still the main isoflavone components (sum >95%), and the proportion changes of genistein and daidzein induced by optimization were both <10%. For example, in one unoptimized product (**Figure 5A**) obtained using the same amount of soybean flour for extracting isoflavones and the same volume of ethanol for dissolving the crystals, genistein accounted for 66% of total isoflavones and the value for daidzein was 30%, and only minor impurities were detected.

The most significant change induced by optimization was the improvement of the yields of isoflavones. For example, on the basis of the low levels of isoflavones in the unoptimized product (**Figure 5A**) (0.606 mg/mL for genistein, 0.276 mg/mL for daidzein, and 0.037 mg/mL for glycitein), the yield of total isoflavones was calculated to be about 0.34 mg/g of soybean flour, which was only about half of the value (0.62 mg/g) of the optimized product (**Figure 5B**). Therefore, the main function of optimization is not to change the product composition but to improve the yield of total isoflavones. More examples (**Figure 4**) have demonstrated this point.

**Characterization of Estrogenic Properties of Pure Isoflavones.** To evaluate the estrogenic activities of the test samples, E-SCREEN was applied. The test has been suggested to be a reliable tool to assess the ER-mediated estrogenic activity from many chemical compounds (*26*). The proliferative potency of the test compounds measured by E-SCREEN was demonstrated to be correlated well with their relative binding affinities to ER and with their in vivo biological effects determined by uterotropic assays (*26*, *30*).

In terms of $E_2$, it showed a proliferative effect at concentrations of 100 pM−1 $\mu$M according to our MTT results (data not shown). Considering that 100 pM is the endogenous level of $E_2$ (*31*, *32*) and $E_2$ could induce maximal MCF-7 cell yields at the concentration range of 10−100 pM, especially 100 pM (*26*, *33*), the estrogenic activity of 100 pM $E_2$ was used as a positive control in this study. As shown in **Figure 6A**, $E_2$ stimulated cell proliferation significantly, with a highly significant ($P < 0.01$) difference between the treatment with $E_2$ and no-hormone control. When ER antagonist 4-hydroxytamoxifen (OHT) was added together with $E_2$, the induced cell proliferation was blocked effectively ($P < 0.001$), indicating the ER-dependence of the proliferative effect.

Then the estrogenic properties of genistein and daidzein, the two most important soy isoflavones, were analyzed. Genistein showed a biphasic effect: it reduced the proliferation of MCF-7 cells significantly ($P < 0.001$) at higher concentrations (25−100 $\mu$M) (**Figure 7A**), and evident apoptotic morphology was observed in a portion of cells at 50−100 $\mu$M (pictures not shown), whereas it had an evident stimulatory effect on cell proliferation at lower concentrations, with a highly significant ($P < 0.01$) effect at concentrations of 1−10 $\mu$M (**Figure 7C**). Similarly, higher concentrations (50−100 $\mu$M) of daidzein could evidently ($P < 0.05$ or <0.001) inhibit cell proliferation (**Figure 7B**), but the inhibition effect was weaker than that of genistein at the same concentration. The lower concentrations of daidzein also stimulated cell proliferation, but the proliferative effect was much weaker than that of genistein; that is, the effect of 10 $\mu$M daidzein was significantly ($P < 0.05$) less than that of 1 $\mu$M genistein (**Figure 7C**). In a word, daidzein has a similar but weaker biphasic effect.

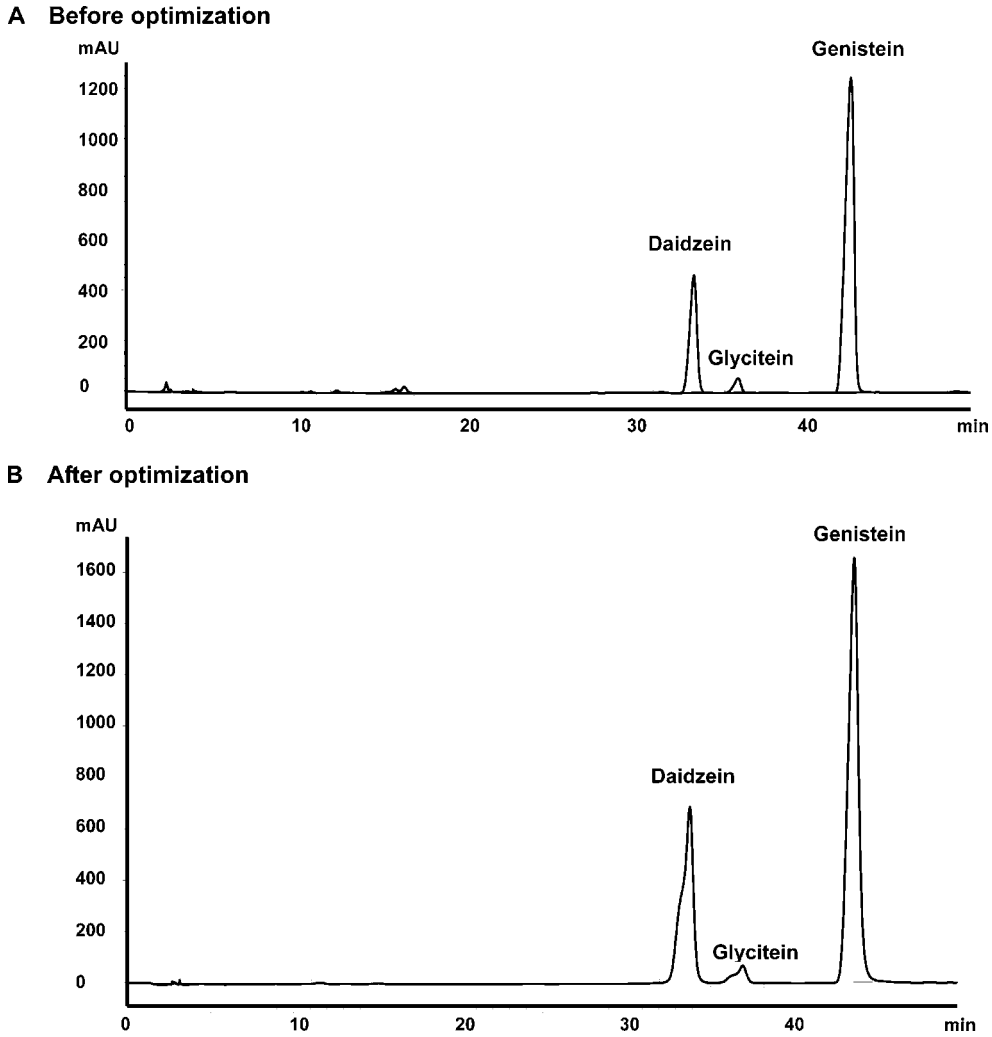


**Figure 5.** HPLC analysis of genistein, daidzein, and glycitein in our isoflavone products before optimization (**A**) and after optimization (**B**).

In our experiments, there was no significant difference ($P > 0.05$) between the proliferative activity of genistein at 1 $\mu$M and that at 10 $\mu$M (**Figure 7C**). Previous papers suggested that the maximal effect of genistein was at 1.56 $\mu$M in the yeast estrogen screen assay (*34*) and at 1 $\mu$M in a 6-day E-Screen assay (*35*). Therefore, genistein may have the best estrogenic efficiency at about 1 $\mu$M, and so the estrogenic activity of genistein was measured at 1 $\mu$M (**Figure 6B**): the significant proliferative effect was identified to be ER-dependent.

Because daidzein demonstrated an evident ($P < 0.05$) proliferative activity at only 10 $\mu$M among the tested concentrations (0.1, 1, 10, 20, 50, 100 $\mu$M) in our experiments (**Figure 7B**,**C**, 0.1 $\mu$M not shown) and was reported to have the maximal proliferative activity at 10 $\mu$M in the 6-day E-Screen assay (*35*), 10 $\mu$M daidzein was examined for its estrogenic activity (**Figure 6C**): the significant proliferative effect was also ER-dependent. In addition, the estrogenic activity comparison showed that the activity of 1 $\mu$M genistein was close to that of 100 pM (the endogenous level) $E_2$, while the activity of 10 $\mu$M daidzein was weaker than that of 100 pM $E_2$ (**Figure 7C**). The stronger estrogenic activity of genistein than daidzein could be explained by the fact that the estrogenic potency of genistein is about 40-fold higher than that of daidzein for both ER subtypes (*25*).

The antiproliferative activity of isoflavones at higher concentrations was supported by other researchers and found not only in estrogen-sensitive cells (i.e., MCF-7 cell line) but also in estrogen-independent cells (i.e., MDA-MB-231 cell line) (*7*, *36*−*39*), suggesting its ER independence. The activity might be due to the regulation of isoflavones on cell cycle (*7*, *37*, *38*).

When MCF-7 cells were treated with 10 $\mu$M daidzein and 100 pM $E_2$ together, a significant ($P < 0.01$) inhibition on cell proliferation was observed compared to the treatment with $E_2$ alone (**Figure 7D**), which could be explained by the strong competitive binding of daidzein with $E_2$ to ERs at this high concentration (10 $\mu$M, 100000-fold excess compared with $E_2$) (*32*, *33*, *40*) and the much lower estrogenic potency of daidzein than $E_2$ for both ER subtypes (daidzein has only $^1/_{1000}$ the potency of $E_2$ for ER$\alpha$ and $^1/_{200}$ the potency of $E_2$ for ER$\beta$) (*25*). However, no evident reduction effect was observed by the combined addition of 1 $\mu$M genistein and 100 pM $E_2$ ($P > 0.05$) (**Figure 7D**), maybe due to the much higher estrogenic potency of genistein than daidzein (*25*).

**Quality Evaluation of Our Isoflavone Extract.** To evaluate the quality of our isoflavone products, their estrogenic activities were assessed by E-SCREEN assay. The result of one optimized product (**Figure 5B**) is shown in **Figure 6D**. The product demonstrated a significant ($P < 0.01$) stimulation activity toward cell proliferation even at the concentration of 0.4 $\mu$g of total isoflavones/mL [the concentration of genistein was about 0.89 $\mu$M (0.24 $\mu$g/mL), and the value for daidzein was about 0.64 $\mu$M (0.16 $\mu$g/mL)], and the induced cell proliferation could be effectively blocked by ER antagonist 4-OHT, identifying its ER-dependent estrogenic activity.

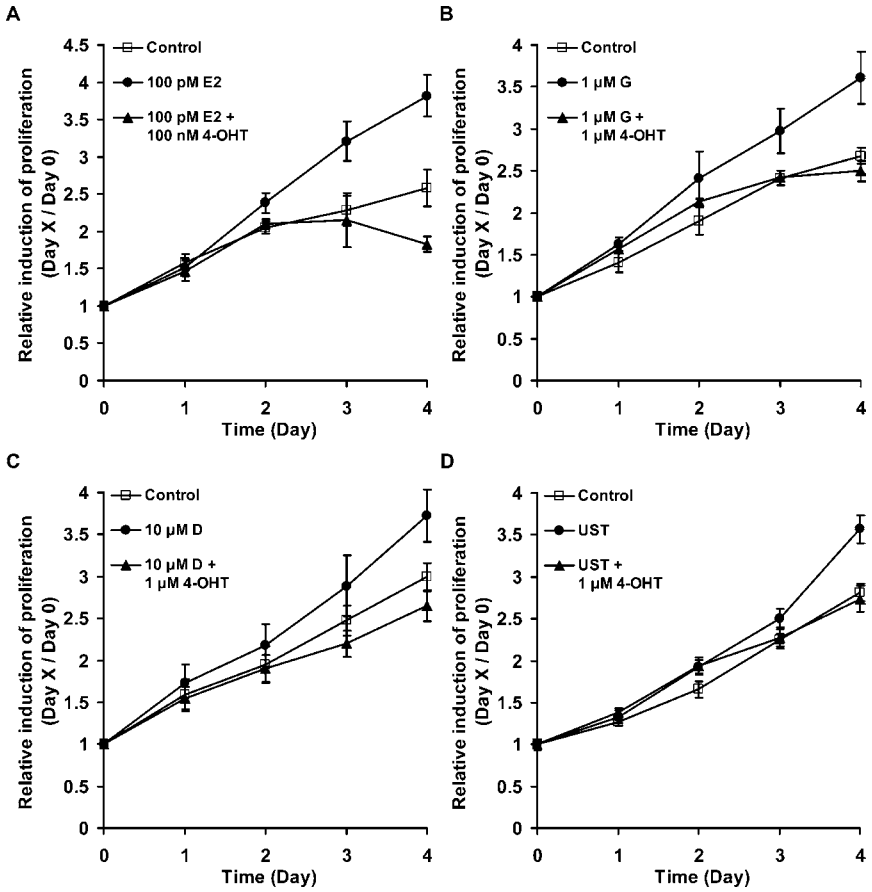


**Figure 6.** Cell proliferation assay in the estrogen-sensitive cell line MCF-7: estrogenic activity of $E_2$ (**A**), genistein (**B**), daidzein (**C**), and our isoflavone product (**D**). OD, optical density value obtained from MTT assay. Control, untreated cells; $E_2$, 17$\beta$-estradiol; G, genistein; D, daidzein; UST, our isoflavone product (concentration = 0.4 $\mu$g of total isoflavones/mL); 4-OHT, 4-hydroxytamoxifen. Data are expressed as the mean ± standard deviation of the OD values obtained from three independent experiments.

## DISCUSSION

In the present study, isoflavones were extracted and purified from soybean flour using the following strategies. Ethanol was chosen as the solvent of extraction mainly because both the solubilities and the steadiness of isoflavones are high in ethanol. In hydrolysis, its main function is to convert most of the isoflavone glucosides (mainly malonyl and glucosyl forms) of the extract into aglycones. In this study, >85% of genistin and daidzin was degraded by hydrolysis, whereas the concentrations of genistein and daidzein were increased about 40-fold (details not shown). The predominant isoflavone forms in natural soybeans and nonfermented soybean products are glucosides (*41*), but aglycones have stronger biological activities and can be more easily absorbed by the body when compared with corresponding glucosides (*26*−*28*), so hydrolysis is required to improve the efficacy and absorbability of the isoflavone products derived from natural soybeans. The crystallization step is based on the fact that the solubilities of isoflavone aglycones in water are much lower than those in ethanol (*42*). Thus, water can be used as an antisolvent to promote isoflavone aglycones to crystallize from the solution. In addition, hydrolysis also can promote the crystallization of isoflavones because the aqueous solubilities of aglycones are lower than those of their corresponding glucosides (*42*). Therefore, isoflavone aglycones can be effectively produced using the procedure described in this paper. As a result, the yield of total isoflavones reached about 0.62 mg of aglycones/g of soybean flour after optimization. Considering that natural soybeans contain 1−3 mg/g of total isoflavones (*43*), the yield of this procedure is fairly good.

The main advantages of our method are as follows. First of all, simple and low-cost steps such as crystallization and filtration were used instead of expensive and complicated ones such as repeated extraction, chromatography, and centrifugation, which are used in most of the present manufacturing processes. Therefore, this novel method is appropriate for large-scale manufacturing. Second, there is no doubt of the high yield of our procedure (0.62 mg of isoflavone aglycones/g of soybeans). Extraction is the key step of the manufacturing process, and the yield of our exaction step is 0.87 mg of total isoflavones (aglycone equivalency)/g of soybeans. It was reported that the extraction yields (genistein + daidzein) of six different methods were all <0.08 mg/g of soybeans (*44*). The extraction yield reached 0.78 mg of total isoflavones (aglycone equivalency)/g of soybeans by another fully optimized method using a high temperature of 100 °C and a high pressure of 100 atm (*3*). Then considering the high recovery of our crystallization step (>80% for genistein and >60% for daidzein after optimization), the isoflavone yield of our procedure should be fairly high. Third, edible alcohol was used as the solvent of extraction, which ensures the products will not endanger human health even if a trace amount of the solvent remains in the products.

The products were identified to have a high content of isoflavones through HPLC analysis. More importantly, isoflavones in our products were all in the form of aglycones. It has been reported that the physiological effects of soy products are mainly contributed by their aglycones (*45*). Moreover, the major isoflavone component in our products was genistein, which has the highest biological activity among the 12 soy isoflavones

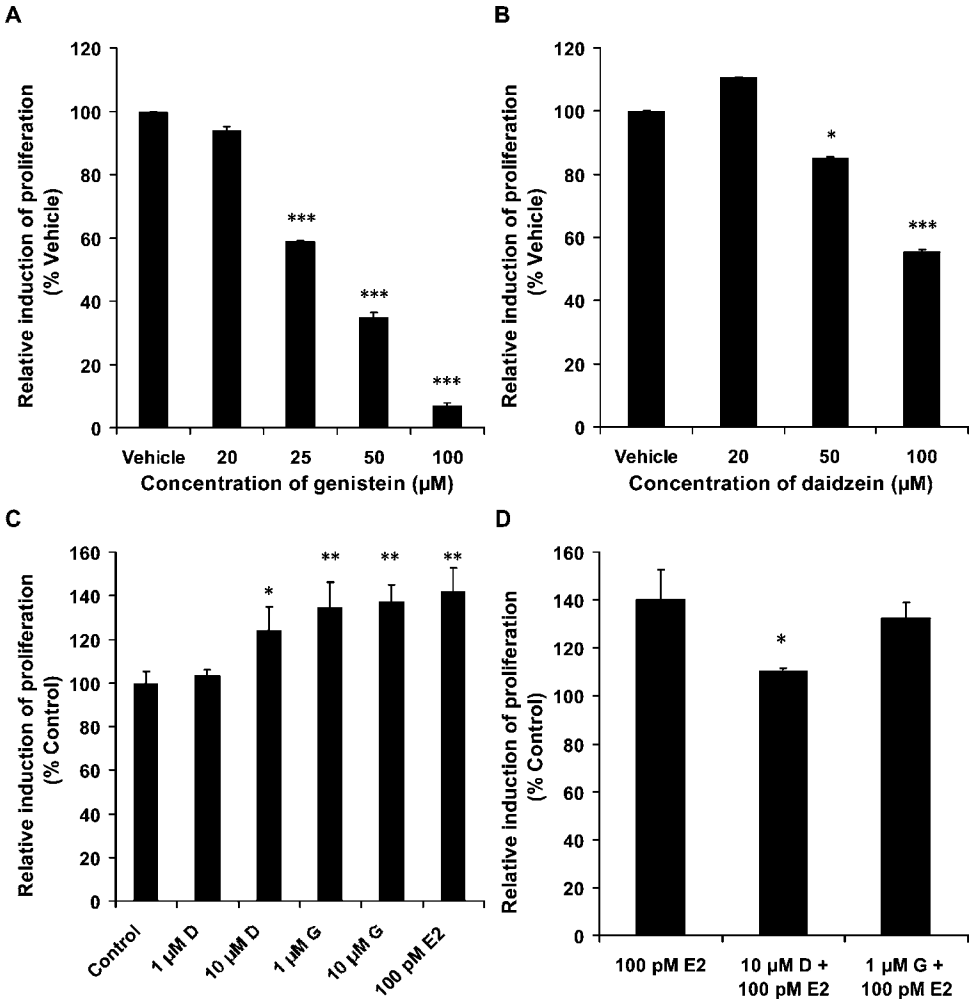


**Figure 7.** Cell proliferation assay in MCF-7 cell line: relative induction of cell proliferation over vehicle (**A**, **B**) or control treatment (**C**, **D**) by various concentrations of isoflavones. Control, untreated cells; vehicle, 0.5% ethanol. Data are expressed as the mean ± standard deviation of the OD values obtained from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, when compared with vehicle (**A**, **B**) or control (**C**) or the treatment with 100 pM $E_2$ alone (**D**) using Student's two-tailed *t* test.

(*46*, *47*). Therefore, our extracts should have strong biological activities especially directed to specific health problems.

E-SCREEN was applied to illuminate the estrogenic activities of the test samples. According to the MTT results, our product exerted strong ER-dependent estrogenic activity (**Figure 6D**). Our isoflavone product not only contained a high level of isoflavone aglycones but also had identified estrogenic activity. Therefore, it would be efficacious for relieving menopausal symptoms and other estrogen-deficient diseases. In addition, the above result accords with the estrogenic properties of isoflavones. According to some literature (*34*, *35*), genistein exerts maximal activity at about 1 $\mu$M and the value is 10 $\mu$M for daidzein. Our MTT results also supported that genistein had significant estrogenic activity at about 1 $\mu$M (**Figure 6B**). Our product indeed had strong estrogenic activity at the concentration where genistein was 0.89 $\mu$M.

The estrogenic activity comparison by MTT assay (results not shown) further suggested that our product had better estrogenic efficacy than three of the four tested commercial supplements. When all of the samples were diluted to the same level of total isoflavones according to their claimed values, one commercial supplement did not show estrogenic activity at all ($P > 0.05$ when compared with control) and another demonstrated only weak activity, in accord with the actual low isoflavone contents of these two supplements, whereas our product and the other two supplements with actual high contents of total isoflavones exerted significant activities ($P < 0.001$ when compared with control). More interestingly, although the main isoflavone components of the three effective products (our product and two commercial supplements) were all genistein and daidzein, differences were seen among their estrogenic activities when they were used actually at the same concentration of total isoflavones (0.4 $\mu$g/mL), which could be explained by their different proportions of genistein and the much higher activity of genistein than daidzein. The above relatively good correlation between the results of estrogenic activity comparison and HPLC analysis indicated that this bioassay might be suitable for quality control on isoflavone products. This bioassay could reflect the combined biological activities of total isoflavones, which is related to both the level of total isoflavones and the isoflavone composition. Isoflavone composition also plays a key role because different isoflavones have different biological activities (i.e., the estrogenic potency: genistein ≫ daidzein ≫ glycitein) and their function mechanism may be complex (i.e., daidzein demonstrates competitive effect when combined with $E_2$).

The estrogenic properties of genistein and daidzein were then explored. Both of them were identified as having authentic estrogenic activities (**Figure 6B**,**C**) and could mimic the stimulation effect of $E_2$ on cell proliferation, indicating they

could be used for relieving estrogen-deficient diseases. Genistein was found to have a much stronger estrogenic activity than daidzein (**Figure 7C**), so it should be much more efficacious than daidzein as an estrogenic agent in therapy. Because of the antiproliferative effects of genistein and daidzein (**Figure 7A**,**B**), their concentrations used in estrogenic therapy should not be too high; otherwise, they could exert side effects such as inhibiting normal cell proliferation.

In estrogenic therapy, one of the good choices with regard to concentration might be about 1 $\mu$M for genistein and about 10 $\mu$M for daidzein. The choice is based on at least three findings from this study. First, genistein and daidzein had evident estrogenic activities at the above concentrations (**Figure 6B**,**C**). Second, the estrogenic activities of 1 $\mu$M genistein and 10 $\mu$M daidzein were close to or less than the activity of 100 pM $E_2$ (**Figure 7C**), indicating the effects of isoflavones used at the above concentrations will not exceed the original effect of the endogenous level of $E_2$. In other words, the above concentrations should be safe for seriously estrogen-deficient patients (with very low levels of $E_2$) such as postmenopausal women. Third, when isoflavones were added together with the endogenous level of $E_2$ to MCF-7 cells, neither 10 $\mu$M daidzein nor 1 $\mu$M genistein further increased the cell proliferative effect of $E_2$ (**Figure 7D**), indicating the safety of using genistein and daidzein at the above concentrations for patients with a certain level of $E_2$. Because these effective and safe doses of genistein and daidzein are based on only MCF-7 cells, further investigation by animal experiments and clinical trials should be conducted for determining the appropriate therapeutic levels of isoflavones in treating estrogen-deficient patients.

Recently isoflavones from natural soybeans have been regarded as a promising alternative to HRT due to their estrogenic activities, but their proliferative effects on some cancer cells at certain concentrations (i.e., **Figure 6B**,**C**) have caused the concern that they may increase the risk of estrogen-related cancers such as breast cancer. Actually, this concern may not be necessary because more and more studies have shown that isoflavones can inhibit or delay mammary tumor formation and development (*18*, *48*, *49*). The different function mechanisms of the two isoforms of ER, ER$\alpha$ and ER$\beta$, may underlie such an emerging view. ER$\alpha$ and ER$\beta$ mediate different physiological effects of $E_2$; that is, ER$\alpha$ promotes the proliferation of many cells (i.e., breast epithelial cells), whereas ER$\beta$ has a restraining influence (*49*). Considering the much higher estrogenic potency of some isoflavones for ER$\beta$ than for ER$\alpha$ [i.e., the potency of genistein for ER$\beta$ is about 30-fold higher than for ER$\alpha$ (*25*)], it is not surprising that soy isoflavones do not increase breast cancer risk, and possibly may diminish it (*49*). Our results (**Figure 7C**,**D**) also demonstrated that the proliferative effects of genistein and daidzein at the estrogenic concentrations did not exceed the effect of endogenous level of $E_2$. Therefore, soy isoflavones will have safe and moderate effects on estrogen-deficient diseases and thus can be used as a better alternative to HRT. In addition, because ER$\beta$ is also associated with bone metabolism and many other physiological pathways (*49*), soy isoflavones with a high potency for ER$\beta$ [i.e., genistein has 87% potency of $E_2$ for ER$\beta$ (*25*)] have multiple beneficial health effects.

At present there are still many problems to be solved for isoflavone products. First of all, the quality of certain isoflavone products on the market is questionable. For example, in this study two of the tested supplements were found with very low levels of isoflavones, much lower than their claimed values, and one supplement contained almost no isoflavones. Therefore, to ensure the quality of isoflavone products, specific content standards should be established and administered. Moreover, it should be noted that most products are labeled with the content of only total isoflavones (*21*). However, even with the same level of total isoflavones, one supplement had lower estrogenic activity than our product, indicating that not only the content of total isoflavones but also isoflavone composition, especially the level of the most effective component, genistein, should be used as a marker for quality control of isoflavone products. The second important problem is that there is little information about the efficacy and bioavailability of isoflavone products on the market. Bioassays are able to provide more assurance for isoflavone products, and this study suggested that the cell proliferation test could be a good choice for quality and efficacy control. It is worth mentioning that the isoflavone product prepared using our protocol not only contained a high level of isoflavone aglycones, especially genistein, but also exerted strong estrogenic activity (comparable with or better than the commercial products measured). In other words, our products have solved the above two problems. A third notable problem is about the therapeutic doses of isoflavones. Although soy isoflavones are totally natural products, higher concentrations of isoflavones were found with evident antiproliferative effects and might affect the normal proliferation of cells. On the other hand, to achieve the expected therapeutic effects, isoflavones should reach certain concentrations. Therefore, appropriate dosages should be determined. The daily consumption of isoflavones is mostly estimated at 15−50 mg (*50*−*52*), but the dangers of overdosing and low efficacy due to underdosing have not received much attention. This study has provided some reference data for the effective and safe usages of genistein and daidzein in estrogenic therapy.

**Supporting Information Available:** Standard curve of MCF-7 cell absorbance and calibration curves of the three isoflavone aglycones. This material is available free of charge via the Internet at http://pubs.acs.org.

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