STUDY ON ANTIOXIDANT ACTIVITY OF PHYTOESTROGEN EXTRACTS FROM SOY GERM

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Abstract. Soy germ is one of the richest phytoestrogen sources and thus has many benefits for health such as improving bone density, cardiovascular health, cancer prevention, and menopausal treatment. In addition, phytoestrogens are reported to act as antioxidants, removing reactive oxygen species and thereby preventing oxidative damage in living tissue. Phytoestrogens in soy germ include isoflavone compounds and their derivatives: daidzein, genistein, glycitein, daidzin, genistin, glycitin, acetyl daidzin, acetyl genistin, acetyl glycitin, malonyl daidzin, malonyl genistin, malonyl glycitin. Isoflavones aglycone forms comprise only about 2 – 5 % of total isoflavones, however, they express more biological effects than the others. The objective of this study was to compare the antioxidant activity among three extracts: purified isoflavone aglycone extract, crude isoflavone aglycone extract and total phytoestrogen extract. The IC₅₀ values of DPPH free radical scavenging capacity of purified isoflavone aglycone extract, crude isoflavone aglycone extract and total phytoestrogen extract were 0.763 ± 0.016; 3.345 ± 0.076; 6.142 ± 0.050 mg/ml, respectively. The IC₅₀ values of reducing power activity of purified isoflavone aglycone extract, crude isoflavone aglycone extract and total phytoestrogen extract were 1.248 ± 0.024; 3.961 ± 0.172; 9.385 ± 0.272 (mg/ml). As our result, the ranking order of the antioxidant activity (from highest to lowest level) was purified isoflavone aglycone extract > crude isoflavone aglycone extract > total phytoestrogens extract.

Keywords: soy germ, phytoestrogen, isoflavone, antioxidant.

Classification numbers: 1.2.1, 1.3.2.

1. INTRODUCTION

Phytoestrogens from soy germ including isoflavones and their derivatives have many benefits for health. The effects of phytoestrogens on improving bone density, cardiovascular health, cancer prevention, cognitive ability and menopausal symptoms have been reported in
Study on antioxidant activity of phytoestrogen extracts from soy germ

many works [1 - 4]. Germ contains the highest level of phytoestrogen among parts of soybean seed. Phytoestrogens in soy germ include: daidzein, genistein, glycitein, daidzin, genistin, glycitin, acetyl daidzin, acetyl genistin, acetyl glycitin, malonyl daidzin, malonyl genistin, and malonyl glycitin. The average concentration of total phytoestrogens was 2887 μg/g in germ (embryo), which was four to five times higher than that of whole seed (575 μg/g) [5]. Phytoestrogen extraction processes mostly are reported from whole soybean seeds or germinated soybean seeds (whole or separated from the sprout). There is a few study on phytoestrogen extraction from soy germ.

Since Wang found 12 phytoestrogens in commercial soybean foods in 1994 [6], there have been a number of studies focused on these natural substances. Among them aglycones have been known to play an important role in all biological mechanisms. Isoflavone aglycones (daidzein, genistein, and glycitein) comprised only about 2 – 5 % of total isoflavones but were rapidly absorbed and showed higher biological activity than other derivatives in digestive [7]. Many works focused on improving isoflavone aglycones in extracts. The commonly used agents which transfer isoflavone glucosides to aglycones were chemicals: acid HCl [8] and biological agents such as enzymes β-glucosidase (cellobiase) [9], galactosidase [10], cellulase [11] and microorganisms capable of producing β-1–4 glucoside binding enzyme [12]. Moreover, in the process of extraction under the effect of temperature, isoflavone derivatives were able to convert one form to another and to the free form of aglycone [13].

The objective of this study was to compare antioxidant activity including the DPPH scavenging and reducing power properties of different extracts (total phytoestrogens extract, crude isoflavone aglycone extract and purified isoflavone aglycone extract) during process of phytoestrogens extraction by cellobiase and purification.

2. MATERIALS AND METHODS

2.1. Materials

Soy germ of size 0.1 mm from Vinanusoy Co. Viet Nam (commercially available) and Cellulase from Trichoderma Reesei (Sigma Aldrich) which contained cellobiase, 28 U/ml were used in this study for isoflavone extraction and purification. DPPH (2,2-diphenyl-1-picrylhydrazyl) (Tokyo Chemical Industry Co. LTD, Japan), Potassium ferricyanide (K3[Fe(CN)6]) BOH Chemicals Ltd, England, and phytoestrogens standards (Wako Pure Chemical Industries Ltd.), ascorbic acid (Guangdong Guanghua Sci-Tech Co., Ltd) were used for analysis.

2.2. Methods

Soy germ was defatted using n-Hexane 95 % at ratio solid/liquid of 1:5 and was shook for 5 hours at 180 rpm. Defatted soy germ with moisture of 5.79 ± 0.09 % was packed in dark glass grinder and stored at – 4 °C until further analysis.

Preparation of extract

Total phytoestrogen extraction was conducted as followed: defatted soy germ flours was added with ethanol 65 %, pH 9 and the solid/liquid ratio was 1:12; extraction time was 90 minutes. The liquid extract was then separated from insoluble fractions by filtration then evaporation [14].
Crude isoflavone aglycone extraction: The extract of total phytoestrogens was adjusted to pH 5 using HCl 0.02 N and kept at room temperature for 1 hour. Then this cloudy suspension was centrifuged at 6000 rpm, at 4 °C for 10 minutes to remove the insoluble. Enzyme reaction was carried out at pH 5, 50 °C with 1.5 U cellobiase/g defatted soy germ flour; reaction time was five hours. The solution was then filtered through to filter paper to remove the insoluble material in order to obtain crude aglycone phytoestrogens [11].

Purification of isoflavone aglycone extract: The crude aglycone phytoestrogens were purified using ethanol with ratio 100 ml of ethanol: 1.0 g crude aglycone for 5 hours at 4 °C. Then ethanol was evaporated and 100 ml ethyl acetate together with 70 ml water were added. After stirring at 500 rpm for 4 hours the solution stayed for next two hours, aglycone phytoestrogens was moved into ethylacetate phase. The ethyl acetate was evaporated to obtain purified aglycone phytoestrogen extract.

Total phytoestrogens extract, crude isoflavone aglycone extract and purified isoflavone aglycone were analysed for soluble dry matter of the extracts. Total phytoestrogens and aglycones were quantified using HPLC.

**HPLC analysis**

Phytoestrogens were analysed by Allicance System, Waters, USA equipped with a Zorbax SB-C18 (5 µm × 4.6 mm × 150 mm). The HPLC conditions were set at 35 °C of column temperature, 260 nm of detective wavelength, mobile phases were A - acetic acid 0.1 %; B - acetic acid/acetonitrile 20/80, flow rate of 1.0 ml /min. The detection was carried out under the linear gradient elution with percentage of mobile phase changing from A 88 %, B 12 % to A 60 %, B 40 % and finish at A 88 %, B 12 %. The quantification of each phytoestrogens was calculated by integrating chromatographic peak areas into calibration curves.

**DPPH radical scavenging activity**

The DPPH radical scavenging activity was determined according to Blois with some improvement [15]. The DPPH scavenging activity was performed using a solution of 0.1 mM in methanol. 1.0 ml sample was added 2.0 ml DPPH 0.1 mM and kept in darkness. After 30 minutes, the absorbance was measured at 517 nm. A blank was prepared without adding the extract. Ascorbic acid at 5, 10, 15, 50, 25 μg/ml was used as standard. Lower the absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation

\[
DPPH \text{ Scavenging} \% = \frac{(R_1 - R_2)}{R_1} \times 100\%
\]

R_1: The absorbance of a control measured at 517 nm; R_2: The absorbance of a test measured at 517 nm.

IC\text{50} value was determined to express antioxidant activity. It is the concentration of fractions that inhibits the formation of DPPH radicals by 50 %.

**Reducing power assay (RPA)**

The reducing activity of the extracts was determined according to the method of Oyaizu [16]. A test sample will transfer ion Fe^{3+} in potassium ferricyanide (K\text{3}[Fe(CN)\text{6}]) to Fe^{2+} in potassium ferrocyanide (K\text{4}[Fe(CN)\text{6}]). By adding FeCl\text{3}, Fe^{3+} would react with ferrocyanide to blue ferrous ferrocyanide (K\text{4}[Fe(CN)\text{6}]). 1.0 ml sample was added with 2.5 ml phosphate buffer
0.2M (pH = 6.6), at 50 °C in 20 minutes. After that, each tube was added with 2.5 ml trichloroacetic acid 10 %. The upper layer (2.5 ml) was mixed with 0.5 ml of 0.1 % ferric chloride and distilled water (2.5 ml). The absorbance was measured at 700 nm, higher the absorbance of the reaction mixture indicated higher reducing antioxidant power assay. A blank was prepared without adding the extract. Ascorbic acid at 20, 40, 60, 80 μg/ml was used as standard.

IC₅₀ value was determined to express antioxidant activity. It is the concentration of fractions that increase the formation of reducing antioxidant power assay by 0.5.

**Statistical analysis:** All measurements were conducted in triplicates and statistically analyzed by analysis of variance (ANOVA). Duncan’s multiple range test was performed and the relation between using the SPSS software programme version 25 (SPSS Inc., Chicago, IL, USA). Significance of difference was defined at p < 0.05.

The concentration of extract were diluted to a range of concentration that decreased by 50 %. Antioxidant quality is a measure of the effectiveness of total phytoestrogens, crude aglycone phytoestrogens and purified aglycone estrogens. The percentage scavenging and IC₅₀ values were calculated for all models by Microsoft Excel 2010.

The regression models provided by SPSS version 25 are:

- **Linear:** \( Y = b_0 + b_1 * X \)
- **Logarithmic:** \( Y = b_0 + b_1 * \ln(X) \)
- **Inverse:** \( Y = b_0 + (b_1/X) \)
- **Quadratic:** \( Y = b_0 + b_1 * X + b_2 * X^2 \)
- **Cubic:** \( Y = b_0 + b_1 * X + b_2 * X^2 + b_3 * X^3 \)
- **Power:** \( Y = b_0 * X^{b_1} \) or \( \ln(Y) = \ln(b_0) + b_1 * (\ln(X)) \)
- **Compound:** \( Y = b_0 * (b_1^X) \) or \( \ln(Y) = \ln(b_0) + (\ln(b_1) * X) \)
- **S-curve:** \( Y = e^{(b_0+ (b_1/X))} \) or \( \ln(Y) = b_0 + (b_1/X) \)
- **Logistic:** \( Y = 1/(1/u + (b_0 * (b_1^X))) \) or \( \ln (1/Y - 1/u) = \ln(b_0) + (\ln(b_1) * X) \)
- **Growth:** \( Y = e^{(b_0+ (b_1*X)^X)} \) or \( \ln(Y) = b_0 + (b_1*X) \)

\( Y: \) DPPH radical scavenging (%);
\( X: \) phytoestrogen concentration of extract;
\( b_0: \) constant;
\( b_1, b_2, b_3: \) regression coefficient.

The regression model was chosen by following criteria:

- The "R Square" column represents the value (also called the coefficient of determination), which is the proportion of variance in the dependent variable that can be explained by the independent variables. "Adjusted R Square" (adj. R square) to accurately report the data, the adj. R square higher the more accurate the data (describe the varying of DPPH radical scavenging ratio, explained by extraction concentration)

- Sig. value (corresponding P-value) is used to evaluate the suitability (existence) of the model. If the p-value is less than 0.05, we reject the null hypothesis that there's no
difference between the means and conclude that a significant difference does exist. If the p-value is larger than 0.05, we cannot conclude that a significant difference exists.

The correlation coefficient is a statistical measure of the strength of the relationship between the relative movements of two variables.

3. RESULTS AND DISCUSSION

Results of the quantity of total phytoestrogen extract, crude isoflavone aglycone extract and purified isoflavone aglycone extract were 72.20 ± 2.48; 14.50 ± 0.36; 9.40 ± 0.46 (mg/ml), respectively. Then three extracts were subjected to determine the antioxidant activity by DPPH radical scavenging method and reducing power assay with two-fold serial dilution method.

3.1. DPPH radical scavenging activity of extracts

The DPPH radical scavenging activities of the standard and three extracts (total phytoestrogen extract, crude isoflavone aglycone extract, purified isoflavone aglycone extract) were shown in Table 1. The IC₅₀ value of standard was 13.757 ± 0.045 (μg/ml).

| No | Total phytoestrogen extract | Crude isoflavone aglycone extract | Purified isoflavone aglycone extract |
|----|-----------------------------|-----------------------------------|-------------------------------------|
|    | Concentration of extract (mg/ml) | DPPH radical scavenging (%) | Concentration of extract (mg/ml) | DPPH radical scavenging (%) | Concentration of extract (mg/ml) | DPPH radical scavenging (%) |
| 1  | 0.282 | 8.71 ± 0.41 | 0.227 | 17.69 ± 0.39 | 146.875 | 18.50 ± 0.78 |
| 2  | 0.564 | 10.50 ± 0.22 | 0.453 | 20.80 ± 0.20 | 293.750 | 26.37 ± 1.22 |
| 3  | 1.128 | 16.59 ± 0.52 | 0.906 | 23.64 ± 1.69 | 587.500 | 39.62 ± 0.78 |
| 4  | 2.256 | 23.06 ± 0.37 | 1.813 | 33.52 ± 1.88 | 1175.000 | 54.06 ± 0.69 |
| 5  | 4.513 | 40.85 ± 0.25 | 3.625 | 52.60 ± 1.15 | 2350.000 | 68.42 ± 1.33 |
| 6  | 9.025 | 67.18 ± 0.35 | 7.250 | 79.51 ± 1.76 | 4600.000 | 92.82 ± 2.00 |

Different letters are significant at p < 0.05.

Total phytoestrogen extract: The regression model is quadratic,

\[ Y = 6.431 + 7.815*X - 0.118*X^2 \]

(R = 0.997; Adjusted R square = 0.995; Sig Anova = 0.000; Sig (Coefficients) concentration = 0.000; concentration **2 = 0.033; const = 0.000).

Crude aglycone phytoestrogen extract: The regression model is quadratic,

\[ Y = 14.749431 + 11.438*X - 0.343*X^2 \]

(R = 0.995; Adjusted R square = 0.996; Sig Anova = 0.000; Sig (Coefficients) concentration = 0.000; concentration **2 = 0.000; const = 0.000).

Purified aglycone phytoestrogen extract: The regression model is quadratic,

\[ Y = 12.609 + 0.049*X - 1.082E-5*X^2 \]
Study on antioxidant activity of phytoestrogen extracts from soy germ

(R = 0.957; Adjusted R square = 0.993; Sig Anova = 0.000; Sig (Coefficients) concentration = 0.000; concentration **2 = 0.000; const = 0.000).

3.2. Reducing power activity

The results of reducing power activity of the standard and three extracts: total phytoestrogen extract, crude isoflavone aglycone extract, and purified isoflavone aglycone extract were performed in Table 2. The IC₅₀ value of standard was 39.796 ± 0.874 (µg/ml)

Table 2. Reducing power activity.

| No | Total phytoestrogen extract | Crude isoflavone aglycone extract | Purified isoflavone aglycone extract |
|----|----------------------------|----------------------------------|------------------------------------|
|    | Concentration of extract (mg/ml) | Reducing power activity | Concentration of extract (mg/ml) | Reducing power activity | Concentration of extract (µg/ml) | Reducing power activity |
| 1  | 2.256 | 0.162 ± 0.025 | 0.057 | 0.184 ± 0.006 | 36.719 | 0.029 ± 0.004 |
| 2  | 4.513 | 0.271 ± 0.009 | 0.114 | 0.232 ± 0.008 | 73.438 | 0.116 ± 0.006 |
| 3  | 9.025 | 0.510 ± 0.016 | 0.227 | 0.250 ± 0.006 | 146.875 | 0.164 ± 0.007 |
| 4  | 18.050 | 0.888 ± 0.027 | 0.453 | 0.278 ± 0.004 | 293.750 | 0.230 ± 0.005 |
| 5  | 0.906 | 0.346 ± 0.004 | 587.500 | 0.413 ± 0.019 |
| 6  | 1.813 | 0.400 ± 0.021 | 1175.000 | 0.552 ± 0.004 |
| 7  | 3.625 | 0.486 ± 0.008 | 2350.000 | 0.628 ± 0.003 |
| 8  | 7.250 | 0.532 ± 0.004 | 4700.000 | 0.689 ± 0.010 |
| 9  | 14.500 | 0.681 ± 0.009 | 9400.000 | 0.733 ± 0.007 |
| IC₅₀ | 9.385 ± 0.272 (mg/ml) | IC₅₀ | 3.961 ± 0.172 (mg/ml) | IC₅₀ | 1248.500 ± 23.999 (µg/ml) |

Different letters are significant at *p < 0.05.*

Total phytoestrogen extract: The regression model is linear,

\[ Y = 0.069 + 0.046*X \]

(R = 0.997; Adjusted R square = 0.993; Sig Anova = 0.000; Sig (Coefficients) concentration = 0.000; const = 0.000).

Crude aglycone phytoestrogen extract: The regression model is logarithmic,

\[ Y = 0.385 + 0.084*\ln(X) \]

(R = 0.923; Adjusted R square = 0.940; Sig Anova = 0.000; Sig (Coefficients) concentration = 0.000; const = 0.000).

Purified aglycone phytoestrogen extract: The regression model is logarithmic,

\[ Y = -0.492 + 0.139*\ln(X) \]

(R = 0.773; Adjusted R square = 0.973; Sig Anova = 0.000; Sig (Coefficients) concentration = 0.000; const = 0.000),
3.3. Discussion

Phytoestrogens are isoflavones which occur naturally in a wide range of food and plants. The isoflavones are best studied group among polyphenols. A number of phytoestrogens are either being actively developed or already currently sold as dietary supplements and herbal, derived medicines because their antioxidant properties.

Since 1997, Ruiz-Larrea et al. determined antioxidant activity of phytoestrogenic isoflavone [17], the health benefits of phytoestrogens have been attributed to the antioxidant capacity. Extracts issued from soy germ, normally a waste product from soybean processing due to its off-flavour, could offer an interesting alternative as starting material for extract phytoestrogens. According to Kim et al., the concentration of phytoestrogens in germ was higher (2887 μg/g) than that of whole seed or other parts of seed such as cotyledon, seed coat [5]. Moreover, the radical scavenging capacity of soy germ extracts was reported at much higher level than those of cotyledon extracts [18].

In this paper, the antioxidant activity of three extracts from soy germ were evaluated using two different assays. The first model of scavenging the stable radical DPPH was sensitive enough to show that soy germ extracts contain particular hydrogen–donor substances which may convert free radicals into harmless substances. Secondly, the reducing power of the soy germ extract was strong. Applied SPSS in statistical analysis helped to establish the relation between phytoestrogen concentrations and antioxidant activities (DPPH radical scavenging or reducing power). The regression models of phytoestrogens concentrations and antioxidant activities were not linear, they might be quadratic or logarithmic.

Result of DPPH radical scavenging method showed that the IC_{50} value of total phytoestrogen extract was higher than that of crude isoflavone aglycone extract; the last one was higher than purified isoflavone aglycone extract. It meant the DPPH radical scavenging activity of purified isoflavone aglycone extract was strongest with IC_{50} = 0.763 ± 0.016 mg/ml, subsequently crude isoflavone aglycone extract and the weakest was total phytoestrogen extract (Table 1).

Result of RPA indicated that the IC_{50} value of total phytoestrogen extract was higher than crude isoflavone aglycone extract, and higher than purified isoflavone aglycone extract. It meant the reducing power activity of purified isoflavone aglycone extract is highest then crude isoflavone aglycone and reducing power activity of total phytoestrogen extract is the weakest. (Table 2).

As our result, the antioxidant activity showed the highest in purified isoflavone aglycone extract, subsequently lower in crude isoflavone aglycone extract, and the lowest was total phytoestrogens extract. All these results could be explained by the HPLC analysis of three extracts showed in Table 3.

When the concentration of total isoflavones in the extract increased the antioxidant activity of this extract increased, too. Morever, these results indicated that the antioxidant activity not only depended on the concentration of total isoflavones but also depended on the concentration of total isoflavone aglycones in the extract. The higher value of concentration of total isoflavone aglycone in the extract was the higher of antioxidant activity of this extract was. (The value of concentration of isoflavone aglycones/total isoflavones in total phytoestrogens extract, crude isoflavone aglycones extract, purified isoflavone aglycones extract was 8.43 %; 73.88 % and 87.09 %, respectively). The intensity of the isoflavones antioxidant was reported to be strongly dependent on chemical structure, especially influenced by the number and position of hydroxyl
groups that linked with two aromatic rings [19]. Thus after hydrolysis reaction and purification process, the conversion of isoflavones glycoside and conjugated forms to isoflavone aglycones created a large amount of hydroxyl groups, this is the major cause that increased the antioxidant activity in crude isoflavone aglycones extract and purified isoflavone aglycones extract [13].

*Table 3. Quality of extracts and quality of isoflavones.*

|                       | Total phytoestrogens extract | Crude isoflavone aglycones extract | Purified isoflavone aglycones extract |
|-----------------------|-----------------------------|------------------------------------|--------------------------------------|
| Quantity of extracts  | 72.20 ± 2.48                | 14.50 ± 0.36                       | 9.40 ± 0.46                          |
| (mg/ml)               |                             |                                    |                                      |
| Daidzein (mg/100ml)   | 9.20 ± 0.31                 | 40.95 ± 0.28                       | 253.20 ± 1.38                       |
| Glycitein (mg/100ml)  | 14.56 ± 0.52                | 36.31 ± 0.31                       | 193.01 ± 1.30                       |
| Genistein (mg/100ml)  | 8.85 ± 0.10                 | 32.13 ± 0.33                       | 210.42 ± 1.03                       |
| Total isoflavone aglycones (mg/100ml) | 32.62 ± 0.72            | 109.39 ± 0.38                      | 656.63 ± 2.37                       |
| Total isoflavones (mg/100ml) | 386.55 ± 0.64       | 148.06 ± 0.57                      | 753.94 ± 2.05                       |

The result of this work showed that antioxidant activity of purified aglycone phytoestrogens extract was the highest, followed by the antioxidant activity of crude aglycone phytoestrogens extract and the lowest was the total phytoestrogens extract.

4. CONCLUSIONS

Soy germ provides an interesting combination of several potential antioxidant substances. With three extracts from soy germ, our findings indicated that phytoestrogens in soy germ have antioxidant activity evaluated by DPPH radical scavenging method and reducing power assay. The ranking order of the antioxidant activity was purified isoflavone aglycone extract > crude isoflavone aglycone extract > total phytoestrogens extract.

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