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Relative antioxidant activity of soybean isoflavones and their glycosides

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Abstract

The present study compared the antioxidant potency of genistein and daidzein with their corresponding glycosides isolated from soybean seeds. The antioxidant activity was assessed using three methods, namely human low-density lipoprotein (LDL) oxidation, the ferric reducing-antioxidant power (FRAP) and the anti-DPPH free radical assays. It was found that soybean was rich in genistein and daidzein but they were present mainly in form of glycosides. Both the FRAP and the anti-DPPH assays demonstrated that these glycosides, as well as genistin, daidzein, glycitin, malonyl glycitin and malonyl genistein possessed similar antioxidant activities to their corresponding aglycones, genistein and daidzein. In contrast, the antioxidant potency of these glycosides was much weaker than their corresponding aglycones, genistein and daidzein, in the LDL oxidation assay. The present results demonstrate that genistein and daidzein were less effective, as antioxiants in the three assays, than two well-defined antioxidants, green tea epicatechin and α-tocopherol. It is concluded that soybean isoflavones and their glycosides possess antioxidant activity but they are ineffective antioxidants compared with tea epicatechins and α-tocopherol.

Keywords: Antioxidant; Daidzein; Daidzin; Genistein; Genistin; Glycitin; Glycosides; Isoflavone; Malonyl genistin; Malonyl glycitin; Soybean

1. Introduction

The health benefits of soybean products have been well documented. Growing evidence has shown that consumption of soybean may prevent certain cancers (Alekel, Hasler, Juma, Drum, & Kukreja, 1998; Anthony, Clarkson, Hughes, Morgan, & Burke, 1996), reduce the risk of osteoporosis (Arjmandi et al., 1998; Barnes, Kirk, & Coward, 1994), have a beneficial role in chronic renal disease (Fico, Braca, Bilia, Tome, & Morelli, 2000; Ranich, Bhathena, & Velasquez, 2001), lower plasma cholesterol (Franke, Custer, Cerna, & Narala, 1995; Ho et al., 2000), exhibit an antiatherosclerotic activity (Hillis & Isoi, 1965; Huff, Roberts, & Carroll, 1982) and decrease the risk of coronary heart disease (Lucas, Khalil, Daggy, & Arjmandi, 2001). The active components of the soy products responsible for these effects have yet to be defined. But it is believed that isoflavones, genistein and daidzein, are responsible for the observed benefits (Markham, Ternai, Stanley, Geiger, & Mabry, 1978).

Genistein and daidzein possess antioxidant activity both in vivo and in vitro. The intake of genistein and daidzein has been shown to provide protection again oxidative modification of low-density lipoprotein (LDL) particles in human volunteers (Tikkanen, Wahala, Ojala, Vihma, & Adlercreutz, 1998). When incubated in human plasma, some genistein and daidzein were incorporated into LDL particles (Kerry & Abbey, 1998) and incorporation of esterified genistein and daidzein increased resistance of LDL to copper-mediated oxidation in vitro (Meng, Lewis, Wahala, Adlercreutz, & Tikkanen, 1999). Consumption of soybean

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isoflavones was also found to be protective against DNA oxidation in human lymphocytes (Mitchell & Collins, 1999). In addition, genistein and daidzein also exhibited, in vitro, strong antioxidant potency in liposomes challenged with UV exposure, peroxyl and hydroxyl free radicals (Record, Dreosti, & McInerney, 1995).

Genistein and daidzein, in soybean, are mainly present in the form of their glycosides (Fig. 1). Previous research found that malonyl genistin was the most abundant in soybean, followed by malonyl glycitin, genistin, daidzin, daidzein, genistein and glycitin in decreasing order (Ho et al., 2002). It is generally believed that isoflavone glycosides are hydrolyzed to their corresponding aglycones prior to gastrointestinal absorption (Piskula, Yamakoshi, & Iwai, 1999). However, recent investigations have shown that flavonoid glycosides, including genistin, phloretin and quercetin, can be partially absorbed without previous hydrolysis of glucose moieties (Andlauer, Kolb, & Furst, 2000; Paganga & Rice-Evans, 1997). In addition, most of the genistein and daidzein is not present in form of aglycone but instead in the form of glucuronide and sulfate conjugates in blood (Hendrich, 2002). Unfortunately, most studies have focussed on genistein and daidzein but no study to date has examined biological activity of individual soybean isoflavone glycosides on their metabolites. The present study is the first to examine relative antioxidant potency of individual soybean isoflavone and their glycosides compared with epicatechin, a well known antioxidant present in green tea.

2. Materials and methods

2.1. Solvents and reagents

All solvents used in chromatographic separation and analysis were of AR grade and purchased from BDH (Poole, England). EDTA, thiobarbituric acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), α-tocopherol, and (−)-epicatechin were purchased from Sigma Chemical Company (St. Louis, MO, USA). NaN_3 was obtained from Acros Organics (New Jersey, USA) while $CuSO_4$ was purchased from Riedel-Dehaen (Seelze, Germany).

2.2. Purification and isolation of soybean isoflavones and their glycosides

Dried soybeans were ground into powder (3 kg) and extracted with 18 l of 70% ethanol three times at 60 °C. Ethanol was evaporated under vacuum in a rotary evaporator to yield 1550 g of ethanol extract, which was then dissolved in 2 l of distilled water. The extract was thrice partitioned with chloroform in a ratio of 1:1. The ethanol–water fraction was thrice further partitioned with butanol in a ratio of 1:1. The butanol

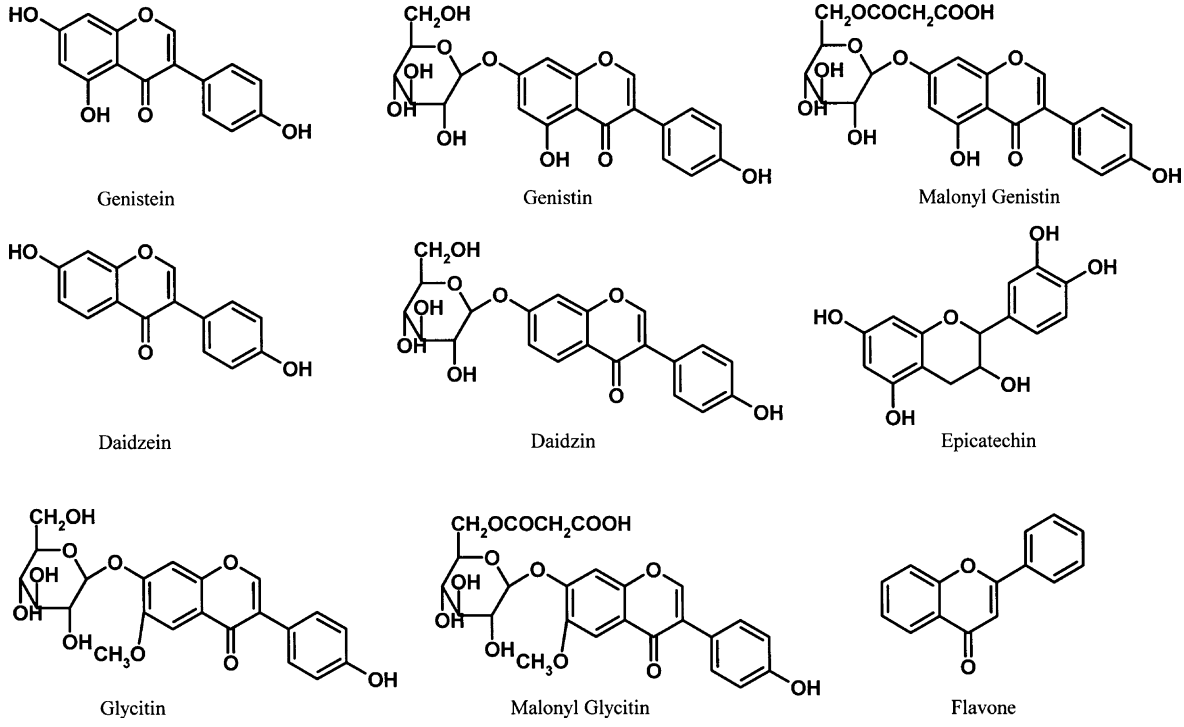

Fig. 1. Chemical structures of daidzein, genistein and their glycosides.

fraction was dried under vacuum to yield 78 g of extract.

The butanol extract was fractionated in a column packed with Sephadex LH-20 (100 M, Pharmacia Fine Chemical Co., Ltd., Germany) and eluted with 5 l of 70% ethanol (250 ml each time) to produce 20 fractions (F1–F20). Fractions F1–F6 and F17–F20 contained mainly sugars, organic acids and some other components that had no UV absorption, while F7–F16 contained mainly a mixture of isoflavone aglycones and their glycosides. Each fraction of F7–F16 was loaded on a Sephadex LH-20 column. Pure ethanol was used to elute each isoflavone and its glycoside. The eluates were monitored using a high performance liquid chromatograph (HPLC) equipped with a UV detector. The purity of each flavone and its glycoside was ca. 60–80%. These isoflavones or glycosides were further purified on a C18 HPLC preparative column (Hypersil ODS, 250×22 mm, 10 m, Alltech, Deerfield, IL, USA) and eluted with 10% acetonitrile in 1% acetic acid solution, leading to purification of seven isoflavones or glycosides, namely daidzin, daidzein, glycitin, malonyl glycitin, genistin, malonyl genistin and genistein. Each fraction was evaporated to dryness, followed by dissolving in ethanol. These isoflavones and their glycosides were identified using their ultraviolet (UV) and mass spectrophotometry (MS) as previously described (Barnes et al., 1994; Ho et al., 2002). The purity of these isoflavone/glycosides was greater than 98%, based on HPLC analysis.

2.3. HPLC analysis

Soybean isoflavones and their glycosides were analyzed in a Hewlett Packard series 1100 HPLC (Hewlett Packard, palo alto, CA, USA) equipped with a binary pump delivery system (G1322A) and a diode array detector (G1315A). In brief, 10 μl of the sample was loaded onto a Waters C18 column (Hypersil ODS, 4.6×250 mm, 5 μM, Wexford, Ireland) through an autosampler (G1313A). The diode array detector was set from 200 to 400 nm, and the eluting components were monitored at 260 nm. The mobile phase consisted of 1% acetic acid in water (v/v) (solvent A) and acetonitrile (solvent B). After injection of the sample, solvent B was increased from 10% to 20% in 40 min and then increased from 20% to 100% in the next 30 min. The flow rate was maintained at 0.8 ml/min. A typical HPLC chromatogram of soybean extract is shown in Fig. 2.

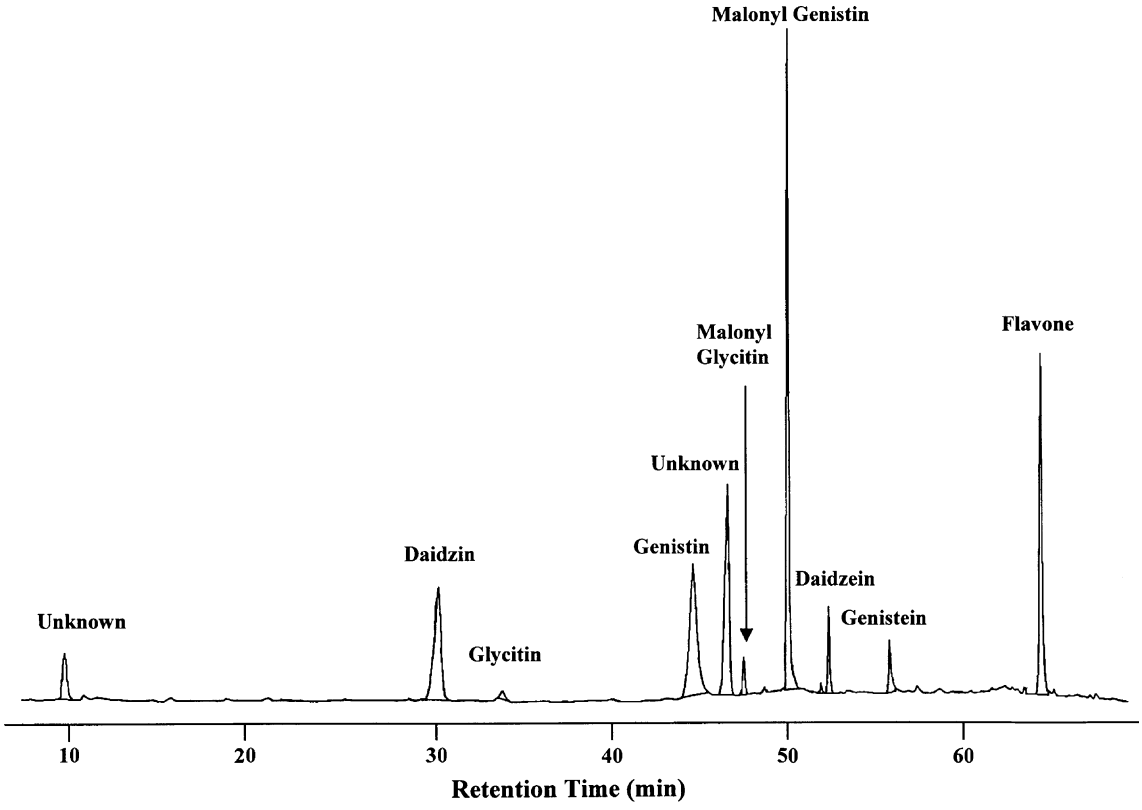

Fig. 2. HPLC profile of soybean butanol fraction at 280 nm. Soybean powder (3 kg) was thrice extracted with 18 l of 70% ethanol at 60C. Ethanol was evaporated under vacuum in a rotary evaporator and the extract was then dissolved in 2 l of distilled water followed by partition with chloroform in a ratio of 1:1, three times. The water fraction was saved and further partitioned with butanol in a ratio of 1:1, three times. The butanol fraction was subjected to HPLC analysis and dried under vacuum to yield 78 g extract. See text for HPLC conditions and peak identification.

2.4. LDL isolation

Fresh blood was collected at the Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong. To protect the lipoprotein from oxidative modification, EDTA (2.7 mM) and NaN_3 (7.7 mM) solutions were immediately added before LDL was isolated from the serum according to the method previously described (Zhang, Chan, Luk, Ho, & Chen, 1997). The protein content of isolated LDL was determined using Lowry's method (Lowry, Rosebrough, Farr, & Randall, 1951). The protocol was approved by the Committee of Human Ethics, The Chinese University of Hong Kong.

2.5. LDL-oxidation

Oxidation of LDL was conducted as previously described (Zhang et al., 1997). In brief, the stock LDL fraction (5 g protein/l) was dialyzed against 100 volumes of the degassed dialysis solution (pH = 7.4) containing 0.01 M sodium phosphate, 9 g/l NaCI, 10 μM EDTA and 7.7 mM NaN_3 in the dark for 24 h. The dialysis solution was changed at least four times. Then, the dialyzed LDL was diluted to 250 mg protein/l with 0.01 M sodium phosphate buffer (pH = 7.4). For the control incubation tubes, 0.4 ml LDL (250 mg/l) was mixed with 50 μl of 50 μM $CuSO_4$ solution and 50 μl of 0.01 M sodium phosphate buffer (pH = 7.4), and incubated at 37 °C for up to 20 h. For the experimental tubes, 0.4 ml LDL (250 mg protein/l) was pre-incubated with 12 μM individual isoflavone and glycoside for 5 min. Then, 50 μl of 50 μM CuSO4 solution were added to initiate the oxidation, followed by incubation at 37 °C for up to 20 h. The oxidation was then stopped by addition of 25 μl of 27 mM EDTA and cooled at 4 °C. The degree of LDL-oxidation was monitored by measuring the production of thiobarbituric acid-reactive substances (TBARS) as previously described (Zhang et al., 1997). The LDL-incubated tubes were immediately treated with 2 ml of 0.67% thiobarbituric acid and 15% trichloroacetic acid in 0.1 N HCI solution. The incubation mixture was then heated at 95 °C for 1 h, cooled on ice, and centrifuged at 1000*g* for 20 min. TBARS were then determined by measuring the absorbance at 532 nm. The calibration was done using a malondialdehyde (MDA) standard solution prepared from tetramethoxylpropane. The value of TBARS was expressed as nmol MDA/mg LDL protein.

2.6. Free radical-scavenging assay

Anti-free radical activity of each isoflavone or glycoside was also examined, as previously described (Blois, 1958). In brief, 0.5 ml of methanol containing individual isoflavone on glycoside was mixed in a test tube with 2.5 ml methanol containing 75 μM DPPH, which is a stable free radical and has a typical absorbance at 517 nm. The reaction mixture was maintained in dark at room temperature for 90 min and the absorbance at 517 nm was then recorded. The free radical-scavenging activity was calculated by the following equation:

$$\text{Scavenging activity } (\%) = [A_a - (A_b - A_c)]/A_a \times 100,$$

Where A_a is the absorbance of the incubation DPPH solution without addition of the tested isoflavone or glycoside; A_b is the absorbance of the incubation mixture containing both the tested isoflavone or glycoside and DPPH and A_c is the absorbance of the blank solution without DPPH.

2.7. Ferric reducing-antioxidant power

The ferric reducing-antioxidant power (FRAP) assay was conducted according to Benzie and Strain (1999). The principle of the assay is based on the reduction of the Fe^{3+}-TPTZ complex to the ferrous form (Fe^{2+}). The antioxidant activity of individual isoflavone or glycoside was measured by monitoring the change in absorption at 593 nm. Acetate buffer (0.3 M, pH 3.6) was prepared by dissolving 3.1 g C2H3O2Na · 3H2O and 16 ml of acetic acid in 1 l of double-distilled water. TPTZ solution was prepared by dissolving 10 mol of TPTZ in 1 l of 40 mM HCl solution. Ferric solution (20 mM) was prepared using FeCl3 · 6H2O. The final working FRAP reagent was prepared freshly by mixing acetate buffer, TPTZ and ferric solutions at a ratio of 10:1:1. In brief, 500 μl FRAP working reagent was mixed with 480 μl double distilled water and was warmed to 37 °C in a water bath. The reagent blank reading was recorded at 593 nm, followed by adding 20 μl of pure isoflavone or glycoside solution. The absorbance was taken at 10 min when the reading was constant. The difference in absorbance between the tested sample and the blank reading was calculated and the data were expressed as mmol/l of ferric reduced to ferrous form.

3. Results and discussion

Soybean isoflavones and their glycosides were analysed using HPLC at 260 nm and quantified according to the amount of internal standard flavone added. The reason for choosing flavone was that it had a UV absorption similar to that of isoflavones at 260 nm and showed no overlap with native soybean isoflavones and glycosides during the HPLC analysis. The results showed that soybean was rich in genistein and daidzein but they were present mainly in the form of glycosides (Fig. 2). The HPLC analysis found that malonyl genistin was the most abundant, accounting for 1.38 mg/g soy-

bean, followed by malonyl daidzin (1.01 mg/g), genistin (0.32 mg/g) and daidzin (0.25 mg/g). In contrast, genistein and daidzein were quantitatively minor, accounting for less than 0.01 mg/g soybean (Fig. 2). The present result is in agreement with that of Lori, Michelle, Marion, and Stephen (1998), who showed that soybean contains predominately 6″-O-malonylglucoside conjugates. It was further demonstrated that the thermal processing of tofu could significantly change profile and degrade malonylglucoside conjugates of genistein and daidzein (Grun et al., 2001). Most interesting was that the genistein and daidzein were absorbed faster and in greater amounts than their glucosides in humans, implying that isoflavone aglycone-rich products may be more effective than glucoside-rich products in preventing chronic diseases such as coronary heart disease and cancer (Izumi et al., 2000).

The oxidation of LDL has been implicated in the development of atherosclerosis (Steinberg, Parthasarathy, Carew, Khoo, & Witztum, 1989). The present study examined the protective effect of soybean isoflavones and their glycosides against Cu2+-mediated LDL oxidation. TBARS was used as an index of LDL oxidation. When all individual soy isoflavones and their glycosides were compared at the concentration of 12 mol/l, the antioxidant activity was in the following order: Genisteing $\geqq$ genistin > malonyl glycitin > daidzein > malonyl genisting $\geqq$ daidzin > glycitin > flavone (Fig. 3). It clearly demonstrated all tested soybean isoflavones and their glycosides could inhibit LDL oxidation to a certain extent. This result is in agreement with several previous reports by Hodgson, Croft, Puddey, Mori, and Beilin (1996), Kerry and Abbey (1998), and Tsai and Chait (1995), who only studied the antioxiant activity of genistein and daidzein. The present study is the first to demonstrate that not only genistein and daidzein but also their glycosides possessed antioxidant potency against Cu^{2+}-mediated LDL oxidation. In general, aglycones, genistein and daidzein, were more effective than their corresponding glycosides against LDL oxidation (Fig. 3). However, under the same experimental conditions, all tested soybean isoflavones and glycosides appeared to be much weaker antioxidants than green tea epicatechin (Fig. 3).

The free radical-scavenging activity of all soybean isoflavones and their glycosides was also compared at a concentration of 12 μmol/l (Fig. 4). Genistin had greater activity than other isoflavones and glycosides. In general, all tested soybean isoflavones and their glycosides were less effective in scavenging DPPH free radical than the two positive controls, epicatechin and α-tocopherol. The results are in agreement with those of Mitchell et al. (1998), who demonstrated that genistein and daidzein did not strongly scavenge DPPH or galvinoxyl free radicals. The present study suggests that anti-free radical activity associated with consumption of soybean isoflavones and glycosides is not comparable to that achieved by drinking green tea or intake of α-tocopherol on the same molar basis.

The result of FRAP assay was consistent with those of the anti-DPPH free radical assay. As shown in Fig. 5, genistin was the most effective as an antioxidant among

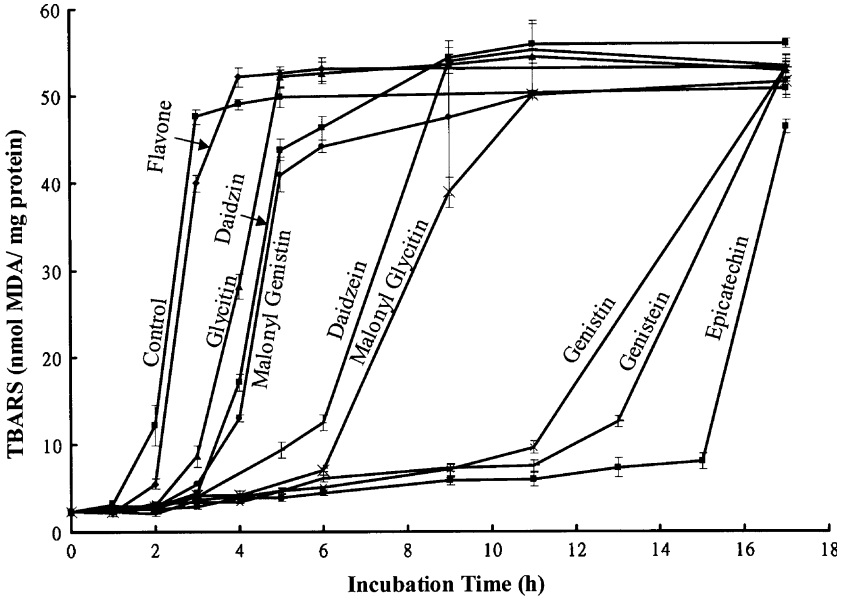

Fig. 3. Inhibitory effect of daidzein, genistein and their glycosides (12 μM) on production of thiobarbituric acid-reactive substances in Cu^{+2}-mediated oxidation of human low-density lipoprotein. (−)-Epicatechin and flavone (12 μM) were used as reference antioxidants. The LDL (100 mg protein/l) was incubated in sodium phosphate buffer (pH7.4) containing 5 μM CuSO4. The oxidation was conducted at 37 °C. Data are expressed as means ± SD of $n = 5$–7 samples.

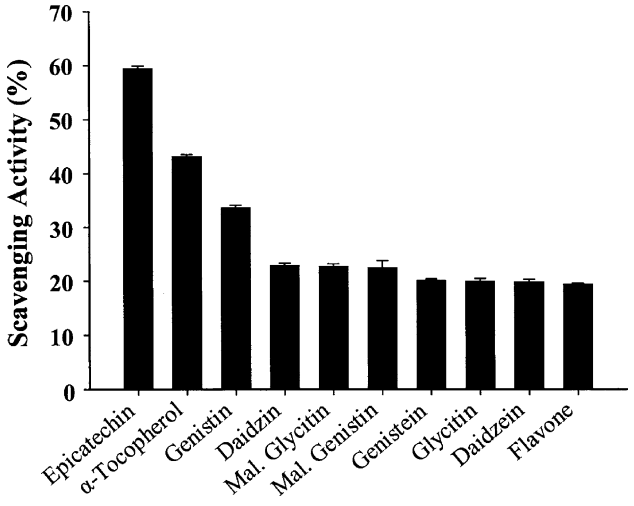

Fig. 4. Free radical-scavenging activity of daidzein, genistein and their glycosides (12 μM). DPPH (2,2-diphenyl-1-picrylhydrazyl) was used as a stable free radical. See text for experimental conditions. Data are expressed as means ± SD of $n = 5$ samples.

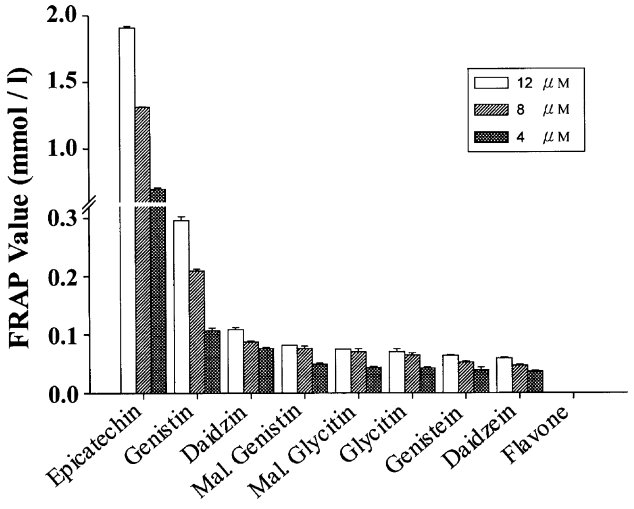

Fig. 5. Ferric reducing-antioxidant power of daidzein, genistein and their glycosides (4–12 μM). Data are expressed as means ± SD of $n = 5$ samples. See text for experimental conditions.

all tested soybean isoflavones and their glycosides. It is expected that processing alters antioxidant value of soy products because the respective malonyl conjugate would yield genistin after demalonylation during processing. The FRAP assay used only epicatechin but not α-tocopherol as a reference antioxidant because the latter was not soluble in the medium. The antioxidant potency of all tested isoflavones and their glycosides was much weaker than that of epicatechin. A dose-dependent activity was observed when all soybean isoflavones and their glycosides were tested at concentrations of 4, 8 and 12 μM (Fig. 5). The results of FRAP and anti-DPPH free radical assays were somehow different from that of the Cu^{2+}-mediated LDL oxidation. In the two former assays, genistin showed relatively higher antioxidant potency while in the latter, it showed a lower activity than other soybean isoflavones and glycosides. The mechanism responsible for this inconsistency remains unclear at the present time, but it is speculated to be due to the "steric effect" of the hydroxyl groups and sugar moieties on genistein and its glycosides that may behave differently in the three reaction media, leading to change in antioxidant efficacy.

4. Conclusion

Daidzein and genistein are present mainly in form of their glycosides and malonylglycosides in soybean. The present results showed that the soybean isoflavone glycosides possessed at least a similar antioxidant potency to the two aglycones, genistein and daidzein, when the potency was assessed using the anti-DPPH free radical and FRAP assays. In contrast, the LDL oxidation assay demonstrated that the soybean isoflavone glycosides had weaker antioxidant activities than genistein and daidzein. The present study clearly demonstrated that the soybean isoflavones and their glycosides were ineffective antioxidants compared with green tea epicatechin and α-tocopherol.

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