11548 J. Agric. Food Chem. 2010, 58, 11548–11552
DOI:10.1021/jf102372t

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Improved *in Vitro* Assays of Superoxide Anion and 1,1-Diphenyl- 2-picrylhydrazyl (DPPH) Radical-Scavenging Activity of Isoflavones and Isoflavone Metabolites

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Free radical-scavenging activity of isoflavones and some isoflavone metabolites have been described previously, but the results are inconsistent. The objective of the present study was to find out the pivotal factors that influence an accurate detection of both superoxide anion and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity. We here showed for the first time that organic solvents, including methanol, ethanol and acetone, were of strong superoxide radical-scavenging activity at concentrations down to 0.1% (v/v), however, no such activity was observed with acetonitrile at concentrations up to 2.0% (v/v). In DPPH assay, we found that the DPPH radical-scavenging ratio increased together with the extended reaction time. Based on our findings, improved *in vitro* assays for the detection of radical-scavenging activity of both isoflavones (daidzein and genistein) and isoflavone metabolites, including dihydrodaidzein (DHD), dihydrogenistein (DHG), and *O*-desmethylangolensin (*O*-Dma), were established.

KEYWORDS: Improved *in vitro* assay; isoflavones; isoflavone metabolites; superoxide anion; DPPH

INTRODUCTION

Isoflavones mainly composed of genistein and daidzein are a class of phenolic compounds produced in high concentration in leguminous plants, such as soybeans (*1*, *2*). After ingestion of these dietary isoflavones, they are subjected to degradation by gut microflora to diverse metabolites, including dihydrodaidzein (DHD), dihydrogenistein (DHG), equol, *O*-desmethylangolensin (*O*-Dma) and so on (*3*−*7*). Isoflavones and their metabolites are currently receiving much attention due to various health benefits. It is generally believed that many of the beneficial effects of isoflavones are at least partially related to the antioxidant activity (*8*, *9*). Oxidative stress might play a pivotal role in the pathogenesis of chronic inflammatory diseases. Many human diseases, including cancer and heart disease, have been linked to the generation of oxidation stress. Excess in reactive oxygen species, such as superoxide anion radical or hydrogen peroxide, has harmful effects on DNA leading to the cells' death. Therefore, accurate detection or comparison of antioxidant activity of isoflavones and their metabolites may provide an important guide for both SAR (structure−activity relationship) study and drug research and development. Although antioxidant activity of isoflavones daidzein, genistein and several synthesized isoflavone metabolites has been reported in several studies, the results are inconsistent. According to Guo et al. (*10*), isoflavonoids did not show any radical-scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide anion radical at concentrations up to 1.0 mmol/L by using ESR measurement. Rimbach et al. (*11*) also reported that isoflavones (daidzein and genistein) and two metabolites of daidzein, including *O*-Dma and equol, exhibited no superoxide anion radical-scavenging activity at concentrations up to 1 mmol/L by ESR. Kampkötter et al. (*12*) found that none of the isoflavones (daidzein, genistein, daidzin, genistin and glycitin) showed any DPPH radical-scavenging effect at the concentration of 50 μmol/L after 2 min reaction. Goto et al. (*13*) reported that daidzein, genistein, equol and *O*-Dma showed some DPPH radical-scavenging activity. However the ED_{50} values of the chemicals were more than 1 mmol/L, which is much larger than ours.

The aim of this study was to find out the pivotal factors which are of significant influence on the detection of radical-scavenging activity but easily neglected before. Based on our findings, improved *in vitro* assays for the detection of both superoxide anion and DPPH radical-scavenging activity were put forward, by which the radical-scavenging activity of both isoflavones and isoflavone metabolites, including DHD, DHG, *O*-Dma and equol, were measured, respectively.

MATERIALS AND METHODS

Materials. Chemicals daidzein and genistein were purchased from Indofine (Somerville, NJ). *O*-Desmethylangolensin (*O*-Dma), dihydrodaidzein (DHD), dihydrogenistein (DHG) and equol were prepared by using our previous microbial biotransformation methods (*5*, *6*). The purity of each biosynthesized compound is more than 99%. Luminol was purchased from Fluka; DPPH from Sigma-Aldrich; brain heart infusion (BHI) from Becton Dickinson. All other reagents were of analytical grade.

Production and Purification of Isoflavone Metabolites. For preparation of different isoflavone metabolites in large quantities, isoflavone biotransforming bacteria were grown in a 500 mL flask containing 200 mL of BHI liquid medium under anaerobic conditions for 18−24 h. Daidzein

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 Published on Web 10/25/2010

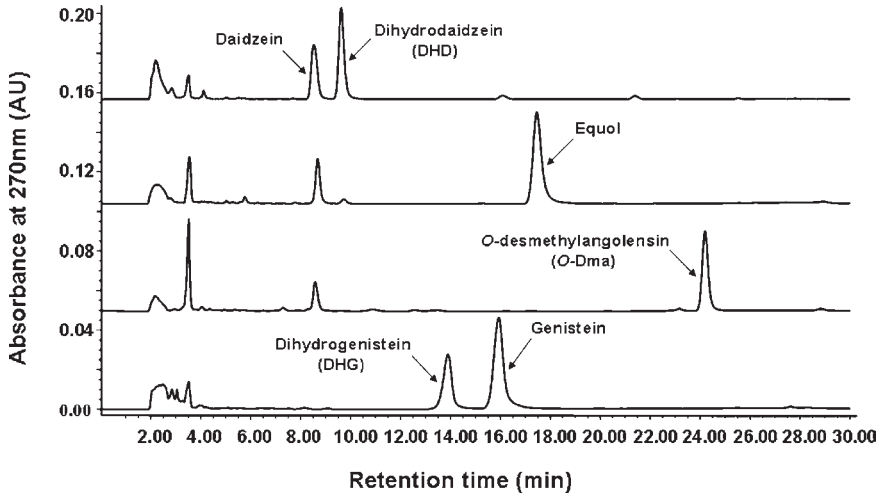

Figure 1. HPLC elution profiles of isoflavone daidzein or genistein after 3 days incubation with different isoflavone biotransforming bacteria.

and genistein stock solution (40 mmol/L in *N*,*N*-dimethylformamide (DMF) and methanol) were added to culture medium at a final concentration of 0.6 mmol/L. The culture was then continuously incubated under anaerobic conditions for 3−5 days. The culture broth was extracted with an equal volume of ethyl acetate three times, followed by evaporation to dryness with a rotary evaporator (Yarong Co. China) and a vacuum concentrator (Dai K_i Science Co., LTD, South Korea). The dried metabolites were redissolved in 100% methanol and purified by semipreparative high performance liquid chromatography (HPLC) (2487 dual λ absorbance detector, 1525 binary HPLC pump, Waters, USA).

Preparation of Stock Solutions of Isoflavone Metabolites. For superoxide anion radical-scavenging reaction assay, both isoflavones and their metabolites were dissolved in DMF, followed by an addition of 100% acetonitrile in a ratio of 1/4 at a concentration of 20 mmol/L. The stock solution was diluted to a different concentration by 100% acetonitrile before using. The same method was used for the preparation of the stock solution for DPPH scavenging reaction assay. However, 100% ethanol was used instead of 100% acetonitrile, and the concentration of the stock solution was 10 mmol/L.

Preparation of the Pyrogallic Acid 3-Aminophthalhydrazide (Luminol)−Na_2CO_3/$NaHCO_3$ Buffer (pH 10.2) System. Pyrogallic acid (0.126 g) and EDTA (7.74 mg) were dissolved in 100 mL of 1 mmol/L of HCl. The solution was stocked at 4 °C and diluted 10-fold with distilled water before being used. Two kinds of salts, including 0.1 mol/L $NaHCO_3$ (210 mL) and 0.1 mol/L Na_2CO_3 (90 mL), were mixed along with 111.6 mg of EDTA and adjusted to pH 10.2. Luminol (17.7 g) was dissolved by 0.1 mol/L Na_2CO_3 to 100 mL and stocked at 4 °C and mixed with 2-fold Na_2CO_3/$NaHCO_3$ buffer (pH 10.2) before being used.

Generation and Scavenging of Superoxide Anion Free Radicals. Generation and scavenging of superoxide anion radicals were performed as described by Guo et al. (*14*, *15*) with minor modifications. Operation steps: 20 μL of sample solution (make solvents as contrast) and 10 μL of pyrogallic acid were mixed with 970 μL of luminol−Na_2CO_3/$NaHCO_3$ buffer and put in a luminescence cup. The luminous intensity (CL) of 300 s was recorded. We can make a judgment of the scavenge capacity from the luminous intensity. The scavenging ratio can be calculated from the following formula: scavenging ratio = $(CL_{control} - CL_{sample}/CL_{control}) \times 100\%$, where $CL_{control}$ is the relative luminescent intensity of the control and CL_{sample} is the relative luminescent intensity of the experimental group.

Measurement of the DPPH Radical-Scavenging Activity. Both methanol (*16*−*18*) and ethanol (*19*, *20*) were used as the solvents for the measurement of the DPPH radical-scavenging activity. Here, we used ethanol for the solvent in our study. DPPH shows a strong absorption band at 517 nm (in ethanol). An absolute ethanol solution of a sample (1 mL) was mixed with a 0.4 mmol/L DPPH absolute ethanol solution (0.5 mL). The width of the spectrocell we used is 0.5 cm. The solution was shaken and incubated at 37 °C for 72 h in the dark. The decrease in absorbance of DPPH was measured at 517 nm. The DPPH radical concentration in the reaction system with and without agent was measured by decrease in absorbance at different incubation times (30 min, 2 h, 6 h, 12 h, 24 h, 36 h, 48 h, 72 h, 96 h, 120 h, 144 h). The inhibition ratio was calculated by comparing the absorbance values of the control and those of the samples.

Statistical Analyses. All experiments were performed at least in triplicate. Data are presented as mean ± standard error of the mean (SEM) value. Statistical analyses were carried out by one-way ANOVA of variance (with LSD post hoc analysis) using SPSS version 13 for Windows. Differences were considered significant if $p < 0.05$.

RESULTS

Preparation of Isoflavone Metabolites. After being absorbed, soy isoflavones are converted into different metabolites by gastrointestinal microflora. Here, in this study all four isoflavone metabolites, including equol, DHD, DHG and *O*-Dma, were obtained by incubating isoflavone daidzein or genistein with specific isoflavone biotransforming bacterium isolated and stored by our lab in BHI medium. After incubation, different metabolites were extracted from culture broth and purified by HPLC methods described by us previously (*5*, *6*) (**Figure 1**).

Effect of Different Organic Solvents on Superoxide Anion Radical-Scavenging Activity. Different organic solvents, including methanol, ethanol, acetonitrile and acetone, were detected by their superoxide radical-scavenging activity at different concentrations. The results showed that, except acetonitrile, all three other solvents, including methanol, ethanol and acetone, were effective scavengers of superoxide anion radicals. Methanol and ethanol showed almost the same superoxide radical-scavenging capacity, which was much higher than that of acetone at all of the four tested concentrations. However, acetonitrile did not show any superoxide radical-scavenging ability, even at a concentration of 2% (v/v) in the reaction system (**Figure 2**). In order to know the exact superoxide radical-scavenging activity of isoflavones daidzein and genistein dissolved in different solvents, the radical-scavenging ratio of each compound was calculated by way of removing the part caused by the solvents themselves based on the exact volume used in the reaction system. We found that the radical-scavenging ratio of a certain amount of daidzein was significantly higher when being dissolved in acetonitrile than in any of the other three solvents (**Figure 3**). Exactly the same result was observed by using genistein instead of daidzein in our test (data not shown). Therefore, acetonitrile was chosen as the solvent for the further comparison of superoxide anion scavenging capacity of isoflavones and their metabolites.

Effect on Superoxide Anion Radical-Scavenging Activity. Both isoflavones and their metabolites were dissolved in 100% acetonitrile to detect their superoxide radical-scavenging capacity at

four different concentrations. The results showed that equol was the most effective scavenger of superoxide radical among all the tested compounds at all concentrations. The superoxide radical-scavenging activity of isoflavones daidzein and genistein was higher than that of all tested isoflavone metabolites except equol. In addition, DHG, one metabolite of genistein, was observed to be the weakest scavenger of superoxide radical among all of the tested compounds (**Figure 4**).

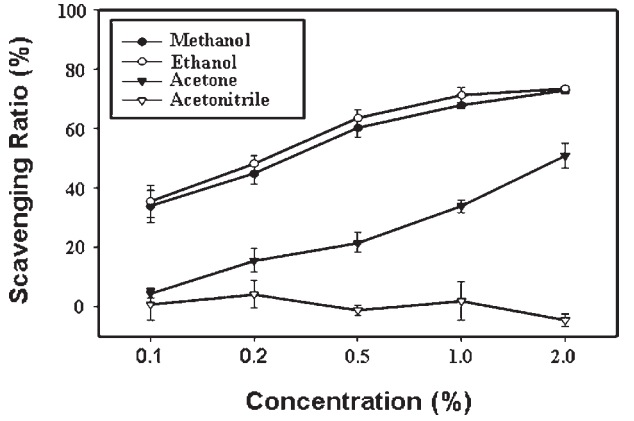

Figure 2. Superoxide anion radical-scavenging capacity of different organic solvents.

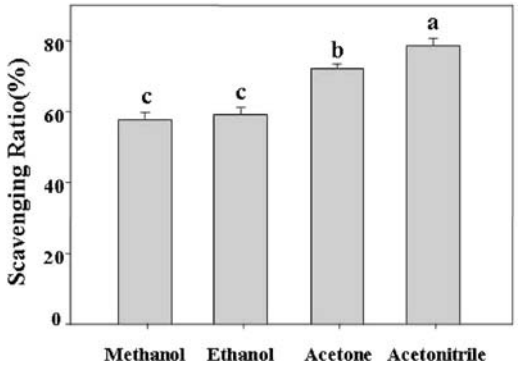

Figure 3. Superoxide anion radical-scavenging capacity of daidzein at 0.4 mmol/L in different organic solvents.

Effect on DPPH Radical-Scavenging Activity. One phenomenon observed in this study was that the DPPH scavenging activity of both isoflavones and their metabolites was increased with the extended reaction time. DPPH is a stable free radical. In order to determine the proper reaction time, the scavenging activity of both daidzein and equol was detected at different incubation times. The kinetics curves of daidzein, equol and the control are shown in **Figure 5**. Since the increasing slope of the scavenging ratio became much lower after 72 h incubation (**Figure 5**), 72 h was chosen as the reaction time for detection of the DPPH radical-scavenging activity of both isoflavones and their metabolites. Consistent with superoxide radical-scavenging assay, equol also showed the highest DPPH scavenging ability at all concentrations. Even though both isoflavones daidzein and genistein showed relatively high superoxide radical-scavenging activity, none of them was a strong scavenger of DPPH. Isoflavone daidzein was observed to have the lowest DPPH radical-scavenging activity at all four tested concentrations. Genistein and the other three isoflavone metabolites, including DHD, DHG and *O*-Dma, showed similar scavenging capacity at the concentrations of 0.33 mmol/L and 0.67 mmol/L. However, when the concentration was increased, the increasing slope of DPPH radical-scavenging capacity of different compounds was not the same. The DPPH radical-scavenging activity of all isoflavone metabolites used in this study was higher than that of isoflavones at the concentrations of 1.33 mmol/L and 3.33 mmol/L (**Figure 6**). The inhibitor concentrations leading to 50% activity loss (IC_{50}) of all the tested agents in both of the two assays were calculated. Except DHG, the assay of superoxide anion radical-scavenging activity was much more sensitive than that of the DPPH assay (**Table 1**).

DISCUSSION

In agreement with Kampköttera et al. (*12*) as well as Goto et al. (*13*), neither isoflavone daidzein, genistein nor their metabolites

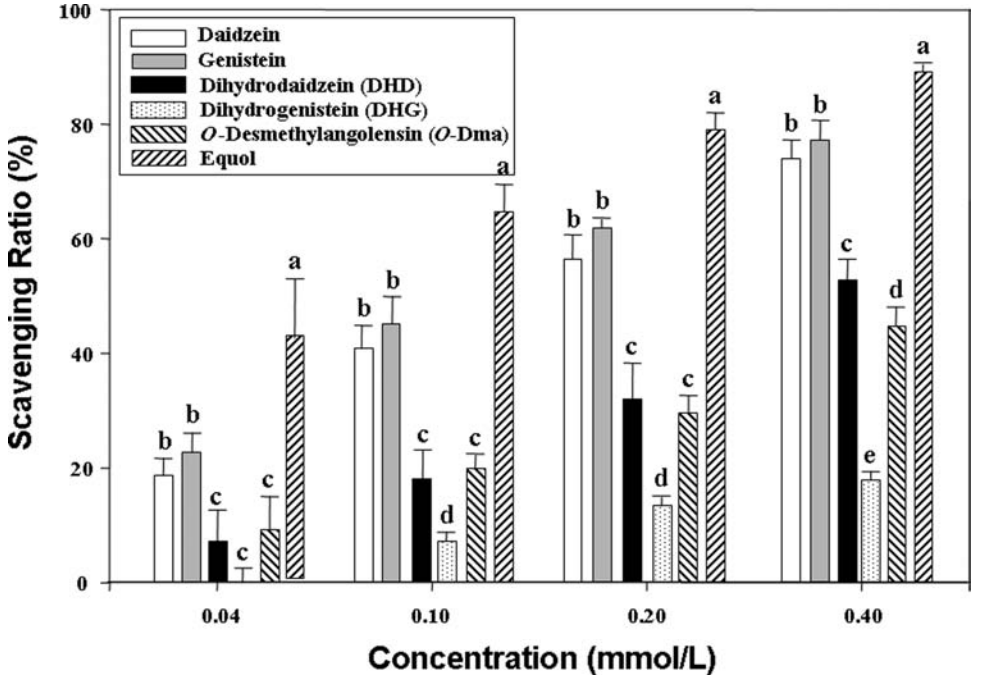

Figure 4. Superoxide anion radical-scavenging capacity of isoflavones and isoflavone metabolites.

showed any radical-scavenging effects in the DPPH assay when the reaction time was within 30 min. However, when the reaction time was extended to 72 h, all of the tested compounds were observed to be effective scavengers at a concentration of 0.33 mmol/L. The most effective scavenger of DPPH was equol, whose IC_{50} was 0.295 mmol/L (**Table 1**). However, according to Goto et al. (*13*), the IC_{50} of equol was up to 1 mmol/L when the reaction time was restricted to 30 min. DPPH radical is stable, and the DPPH test is a nonenzymatic method currently used to scavenge free radicals, so it is proper to get a higher scavenging activity by extending the reaction time. In fact, the beneficial effect of dietary isoflavone intake on scavenging free radicals in animal and human bodies is a long-time process. The most reasonable way for studying DPPH radical-scavenging capacity is to extend the reaction time until almost all of the scavenger molecules have played their role in the reaction system.

Superoxide effects are involved in many pathological processes, such as inflammation (*21*), cancer (*22*) and aging (*23*). In this study, superoxide anion produced by using pyrogallic acid in alkaline conditions was trapped by luminol and detected by chemiluminescence. This detection method is much more sensitive than that of the DPPH assay (**Table 1**). Except DHG, all three other metabolites of daidzein, i.e. DHD, *O*-Dma and equol, were of detectable superoxide radical-scavenging ability even at the concentration of 0.04 mmol/L. It is worth noticing that the superoxide scavenging ratio of equol was up to 40% at the concentration of 0.04 mmol/L. However, on the other hand, the method for measuring superoxide anion radical-scavenging activity also has some limitation. Except equol, the superoxide anion radical-scavenging activity of all three other metabolites (DHD, DHG and *O*-Dma) was obviously lower than that of their parental compounds (daidzein or genistein). This result seems abnormal since the reduction activity of DHD, DHG and *O*-Dma would be higher based on their structures (obtained by reduction reactions), which would lead to a stronger antioxidant activity than their parent compounds. Rimbach et al. also reported that isoflavone metabolites exhibited higher antioxidant activity than parent compounds in standard antioxidant (FRAP and TEAC) assays (*11*). The deficiency of the assay for measuring superoxide anion radical-scavenging activity was found in our study. The method itself has some limitation in detecting acidic or weak acidic compounds (like DHG in this study) since the pH of the reaction system is too high (10.2). Therefore, both the pH value of the reaction system and the physicochemical characteristics of the tested compound need to be under consideration before choosing a proper method for the detection of free radical-scavenging activity.

Although some organic solvents, like methanol, ethanol and dimethyl sulfoxide (DMSO), were reported to share an ability to scavenge hydroxyl radicals (*24*−*26*), similar study has not been performed thoroughly in superoxide anion radicals. For the first time, some organic solvents used for hydrophobic pharmaceutical agents, including methanol, ethanol and acetone, were of strong superoxide radical-scavenging activity at very low concentrations (0.1%, v/v); however, acetonitrile did not show any scavenging activity. In fact, in our previous study, we have observed similar phenomenon in a hydroxyl radical-scavenging assay (*27*). Therefore, in order to obtain

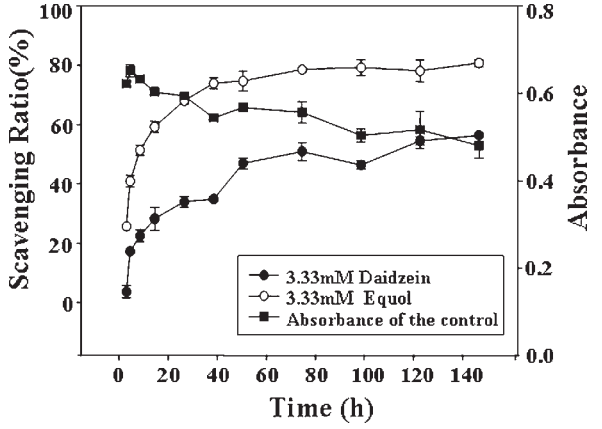

Figure 5. Kinetic curve of DPPH radical concentrations measured by the change in absorbance (the control) and scavenging ratio of daidzein and equol added to the reaction system.

Table 1. IC_{50} of Isoflavones and Their Metabolites in Both Superoxide Anion and DPPH Assays

items	daidzein	genistein	DHD	DHG	*O*-Dma	equol
superoxide anion assay	0.149	0.125	0.349	22.040	0.435	0.053
DPPH assay	2.292	1.485	1.964	0.800	0.942	0.295

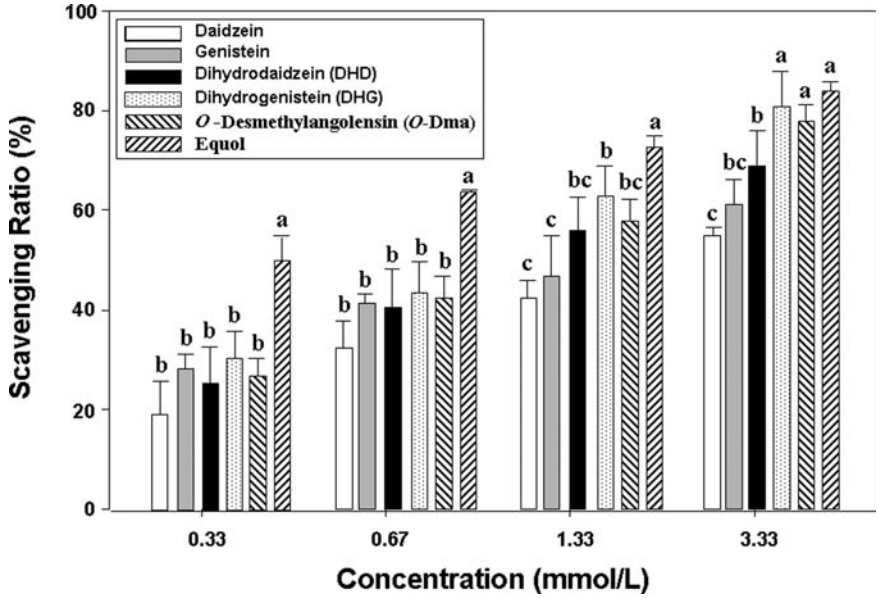

Figure 6. DPPH radical-scavenging capacity of isoflavones and isoflavone metabolites.

an accurate result, a suitable solvent should be chosen first before carrying out the experiment for detecting or comparing the scavenging ratio of the candidate compound. If there is no such proper solvent, the solvent used instead must be in an accurate amount, based on which the influence of the solvent itself can be removed. Another hint obtained from this study is that daily intake of a small amount of alcohol may help aging people to scavenge excessive free radicals being produced in their bodies.

ABBREVIATIONS USED

DHD, dihydrodaidzein; DHG, dihydrogenistein; *O*-Dma, *O*-desmethylangolensin; DPPH, 1,1-diphenyl-2-picrylhydrazyl ; SAR, structure−activity relationship; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; IC_{50}, inhibitor concentration leading to 50% activity loss.

ACKNOWLEDGMENT

The authors thank Maggie Carstarphen, a foreign teacher in Agricultural University of Hebei, for grammar check.

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Received for review June 19, 2010. Revised manuscript received September 28, 2010. Accepted October 7, 2010. This research was supported by NSFC (No. 30570035) funded by the Chinese government and CPRC (No. 027) funded by the government of Hebei Province.