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Antioxidant and QR Inductive Activities of Novel Functional Soybean 'Agakong3'

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Abstract In order to evaluate the bioactivity of 'Agakong3', which was newly bred, quinone reductase (QR) inductive activity and antioxidant activity were both assessed. The methanol extract of 'Agakong3' showed a significantly stronger QR inductive activity than other soybeans. The methanol extract of 'Agakong3' also showed a significantly stronger cytotoxicity on hepa1c1c7 than other soybeans. 'Agakong3' exhibited the most potent antioxidant activity in the Trolox equivalent antioxidant capacity (TEAC) assay whereas it showed significantly weak antioxidant in the DPPH assay. In total phenol and flavonoid contents, 'Agakong3' showed the highest contents regarding phenol and flavonoid compounds. Major isoflavones such as daidzein and genistein were quantitified by high performance liquid chromatography. 'Agakong3' also showed the highest total isoflavone contents. Results of correlation analysis showed that there were high correlation coefficients between the contents of isoflavone and TEAC and the contents of isoflavone and QR inductive activity, respectively. These results suggest that 'Agakong3' will be a promising and functional food material.

Keywords: soybean, 'Agakong', quinone reductase, antioxidant effect

Introduction

Soy has been consumed as a traditional food in Asia for a long time. There are many kinds of soy food such as soymilk, *tofu*, *miso*, *tempeh*, *doenjang* (soy bean paste), and *cheonggukjang*. In the past, in Asia these soy foods were very important sources of protein, instead of meat. Many previous studies and data have shown that the consumption of soybean products was inversely correlated to human cancer (1-3), such as breast cancer (4,5), intestinal cancer (6), prostate cancer (7), and stomach cancer (8). Other studies reported that the consumption of soy products prevented osteoporosis (9) and cardiovascular diseases (10,11).

Many investigations have focused on isoflavones in soybean products because they were believed to contribute to positive biological effects such as cancer chemoprevention (12-19), osteoporosis, menopausal symptoms (20,21), and phytoestrogenic activity (22).

Wild soybeans have good agronomic characteristics such as a high isoflavone contents and resistance to disease (23). Thus, wild soybeans were considered to be good breeding materials in order to introduce valuable agronomic characteristics into cultivated soybeans. 'Agakong' was bred by means of interspecific cross breeding between 'Eunhakong' (*Glycine max*) and 'KLG10084' (*Glycine soja*) that has a green seed-coat color (24). Small seed-sized soybeans generally have good seed vigor, high hypocotyl elongation, and a high sprout yield (25,26). The One-hundred seeds weight of 'Agakong' amounts to 4.9 g and is very light when compared to other soybeans weighing 8.3-30.3 g. This characteristic indicates that 'Agakong' is a suitable soybean cultivar in regards to soybean sprouts. Furthermore, recent research has reported that the total isoflavone content of 'Agakong' was 3 times the proportion of 'Poongsannamulkong' (27). 'Agakong3' was enhanced from 'Agakong'. Thus, the total isoflavone contents of 'Agakong3' are expected to be much higher when compared to 'Agakong'.

When considering its genetic background, it is hypothesized that 'Agakong3' probably contains unique bioactivity properties. This study evaluated the bioactivity of 'Agakong3' and compared it to typical cultivated cultivars. Bioactivity of 'Agakong3' was assessed by means of quinone reductase (QR) assay that is considered to be a biomarker for chemoprevention (28) and various antioxidant assays including 1,1-diphenyl-2-picrylhydrazyl (DPPH), Trolox equivalent antioxidant capacity (TEAC), and ferric reducing ability of plasma (FRAP) assay. Total phenol, flavonoid, and isoflavone contents were also determined to understand correlation between these compounds and bioactivity.

Materials and Methods

Preparation of extracts The 'Agakong', 'Eunhakong', 'Taekwangkong', 'Hwangkeumkong', and 'Poongsan-namulkong' soy materials were generously provided from the Plant Genetics Laboratory at Kyungpook National University. Each sample was extracted with 100% methanol 3 times. Methanol extracts evaporated under reduced pressure at temperatures below 45°C. Sample extracts were dissolved in dimethyl sulfoxide (DMSO) for purpose of the quinone reductase (QR) inductive and antioxidant assay. In regards to the determination of total

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phenol and flavonoid contents, soybean samples were ground up and filtered though a 100-mesh screen. A 0.5 g sample was weighed in a 50-mL screw cap glass tube and boiled with 50%(v/v) methanol at 95°C for 2 hr.

Chemicals and cell cultures Naringin, gallic acid, Folin Ciocalteu's phenol reagent, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), ethylendiaminetetraacetic acid (EDTA), sulforodamine B (SRB), trichloroacetic acid (TCA), potassium persulfate, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, menadione, digitonin, glucose-6-phosphate, β-nicotinamide adenine dinucleotide phosphate (β-NADP), flavin adenine dinucleotide (FAD), glucose 6-phosphate dehydrogenase, sodium dodesyl sulfate, and bovine serum albumin were all obtained from the Sigma-Aldrich (St. Louis, MO, USA). α-Minimum essential medium (α-MEM), fetal bovine serum (FBS), and various supplements were purchased from Gibco BRL (Gaitherburg, MD, USA). Hepa1c1c7 murine hepatoma cells were cultured in an α-MEM media with 10%(v/v) FBS, 100 units/mL of penicillin G and 100 μg/mL of streptomycin sulfate (37°C, 5% CO_2).

QR assay QR activity was measured with hepa1c1c7 murine hepatoma cells as described previously by Prochaska and Santamaria (29). The cells were plated at 1.2×10^4 cells/mL and cultured for 24 hr. Test samples were added and the cells were cultured for 48 hr. Then, the medium was decanted and the cells were lysed with a solution containing 0.8%(w/v) digitonin and 2 mM EDTA with a pH of 7.8. The plates were agitated on a plate shaker for an additional 10 min after the reaction mixture was added to each well. After 10 min, the plates were scanned at 595 nm with a spectrophotometer (Power Wave XS; Bio-Tek Instrument, Winooski, VT, USA). The protein contents of each well were determined by means of the crystal violet protein staining methods (30), and the specific activity was defined as nmol of MTT blue formazan formed/mg protein/min. Induction of QR activity was calculated by comparing the QR specific activity of sample-treated cells with that of solvent-treated cells.

Cytotoxicity assay Sulforhodamine B (SRB) assay was carried out as described previously by Skehan *et al.* (31). The cells were plated at 1.2×10^4 cells/mL in a 96-well plate and cultured for 24 hr. Test samples were added and the cells were cultured for 48 hr. Then, the medium was decanted. Cultures fixed with trichloroacetic acid were stained for 30 min with 0.4%(w/v) SRB dissolved in 1%(v/v) acetic acid. Unbound dye was removed by means of 4 washes with 1%(v/v) acetic acid, and protein-bound dye was solubilized with 10 mM Tris base for determination of optical density at 515 nm.

DPPH assay DPPH assay was measured as described previously by Dietz *et al.* (30). Reaction mixtures containing test samples and a 100 μM DPPH solution were incubated at room temperature for 30 min. The absorbance of the DPPH solution was measured at 515 nm and the percent inhibition was determined by comparison with DMSO-treated control groups. All samples were assessed at a final concentration of 1 mg/mL. Trolox was used as a positive control at a final concentration of 0.5 mM.

TEAC assay The TEAC assay was measured as described by Re *et al.* (32). An amount of 7 mM of ABTS ammonium was dissolved in water and treated with 2.45 mM of potassium persulfate. The reaction mixture was then allowed to stand at room temperature for 12-16 hr in order to allow it to turn into a dark blue solution. This solution was diluted with PBS (pH 7.4) until the absorbance reached 1.0. Then the reaction solution was mixed with the sample and the absorbance was recorded at 734 nm. The TEAC values were calculated based on a standard curve of Trolox standard solutions. Results were expressed in Trolox equivalents based on μM/mg of MeOH extract.

FRAP assay The FRAP assay was measured based on Benzie and Strain (33) with a slight modification. The FRAP reagent contained 2.5 mL of 10 mM TPTZ solution in 40 mM HCl and 2.5 mL of 20 mM $FeCl_3\cdot6H_2O$ and 25 mL of 0.3 M acetate buffer (pH 3.6). The FRAP reagent and soybean extracts were mixed. After 4 min, the absorbance was recorded at 593 nm. The FRAP values were calculated from a standard curve of Trolox standard solutions. Results were expressed in Trolox equivalents based on μM/mg of MeOH extract.

Determination of total phenol contents Total phenol contents were analyzed by means of the Folin-Denis method (34), using gallic acid as the standard. The assay conditions were as follows: a sample was added to 0.2 N Folin-Ciocalteu's phenol reagent. After 3 min, saturated sodium carbonate solution was added to the mixture and then, incubated at room temperature for 2 hr. The absorbance of resulting mixture was measured at 760 nm. Total phenol contents were calculated based on a standard curve with gallic acid. Results were expressed in μg of gallic acid equivalents (GAE)/g of dry weight.

Determination of total flavonoid contents Total flavonoid contents were analyzed by means of the Abeysinghe *et al.* (35) method with minor modifications in using naringin as the standard. Briefly, samples were mixed with 90%(v/v) diethylene glycol and shaken for 3 min with a microplate agitater (Titamax 101; Heidolph, Schwabach, Germany). Then 2 N NaOH solution was added. After 1 hr, the absorbance was measured at 427 nm. The total flavonoid contents were calculated based on a standard curve with naringin. Results were expressed in μg of naringin equivalents (NE)/g of dry weight.

High performance liquid chromatography (HPLC) quantification of isoflavone Soybean samples were ground up in order to be filtered though a 100-mesh screen. A 0.5 g sample was weighed in a 50 mL screw cap glass tube. The hydrolysis of isoflavone was carried out by using 50%(v/v) methanol containing 1 N HCl for 3 hr at 95°C (36). The extracts were filtered through a 0.45-μm membrane filter and analyzed with HPLC. The HPLC analysis was carried out using a Jassco system (Jasco PU2080, UV2075; Jasco Inc., Tokyo, Japan) with ZORBAX Eclipse XDB-

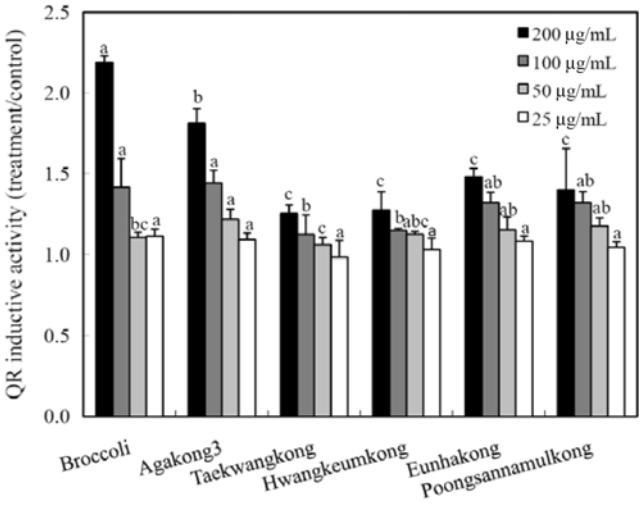

Fig. 1. QR inductive effects of methanol extracts of different soybean cultivars and broccoli. The values are expressed as the mean±SD of 3 replicates. Bars with the different lowercase letter indicate a significant difference between treatments at $p<0.05$ as determined by the Duncan's multiple-range test.

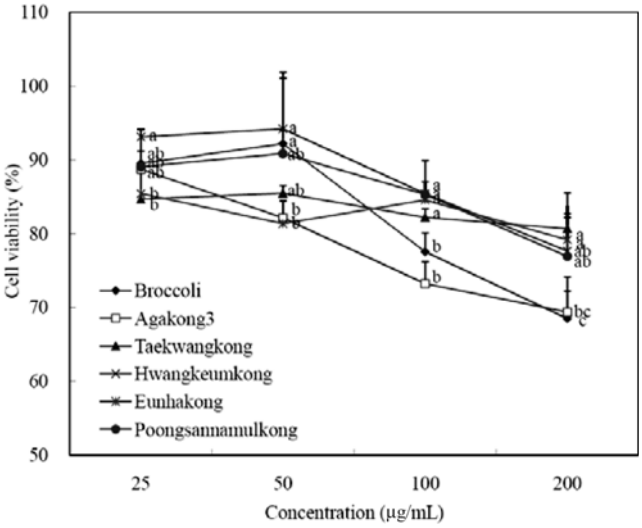

Fig. 2. Cytotoxicity of methanol extracts of different soybean cultivars and broccoli by SRB assay. The values are expressed as the mean±SD of 3 replicates. Bars with the different lowercase letter indicate a significant difference between treatments at $p<0.05$ as determined by the Duncan's multiple-range test.

C18 column (4.6×250 mm, 5 μm, Agilent Inc., Santa Clara, CA, USA). The UV detector (Jasco Inc.) was used at 254 nm, and the solvent flow rate was 1.0 mL/min. Eluent solvent contained 50%(v/v) methanol as part of the isocratic system and the injection volume was recorded at 20 μL.

Data analysis The Duncan's multiple-range tests and correlation analysis were conducted by using SAS 9.1.3 (SAS Inc., Cary, NC, USA). In all cases, a p value of <0.05 was considered to indicate significance. Experimental values are expressed as mean±standard deviation (SD).

Results and Discussion

QR activity of 'Agakong3' The effect of each sample on the QR activity using *in vitro* bioassay with hepa1c1c7 cells was assayed. As shown in Fig. 1, the methanol extract of 'Agakong3' significantly induced QR activity in a dose dependent manner. However, the methanol extract of 'Taekwangkong', 'Hwangkeumkong', 'Eunhakong', and 'Poongsannamulkong' respectively did not exhibit strong activity when compared to the methanol extract of 'Agakong3'.

Broccoli was used as a positive control because broccoli showed strong QR inductive activity in the previous study (37) and sulforaphane which is anticarcinogenic agent was isolated from broccoli (38). While the methanol extract of broccoli induced stronger QR activity than that of 'Agakong3' at 200 μg/mL, the methanol extract of 'Agakong3' induced stronger QR activity when compared to the methanol extract of broccoli at the concentration of 50 μg/mL. Considering the strong activity of the methanol extract 'Agakong3' at the low concentration compare to the methanol extract of the broccoli, 'Agakong3' can be a good chemopreventive material.

Cytotoxicity of soybean extracts The cytotoxicity of each sample using hepa1c1c7 cells was measured. As shown in Fig. 2, the methanol extracts of 'Agakong3' showed significantly stronger cytotoxicity than other cultivated soybeans. Broccoli, one of the most frequently consumed vegetables as chemoprevention food, was used as a

Table 1. Total phenol, flavonoid, and isoflavone contents

Cultivar	μg/g Dry weight				
	Total phenol contents[1)]	Total flavonoid contents[2)]	Isoflavone contents		
			Genistein	Daidzein	Total[3)]
'Akakong3'	5,925±316a	1,103±6a	1,442±30a	3,906±39a	5,348±70a
'Taekwangkong'	3,886±164d	686±42b	188±5d	447±10d	635±15e
'Hwangkeumkong'	3,204±99 c	414±41c	441±21c	432±34d	873±54d
'Eunhakong'	3,779±130c	625±98b	413±8c	935±24c	1,348±24c
'Poongsannamulkong'	4,595±174b	639±32b	576±8b	1,335±36b	1,911±44b

[1)]Represents as gallic acid equivalent (GAE) concentration.
[2)]Represents as naringin equivalent (NE) concentration.
[3)]Contents of genistein and dadizein. The values are expressed as the mean±SD of 3 or 4 replicates. Different letters in the column are significantly different by Duncan's multiple-range test ($p<0.05$).

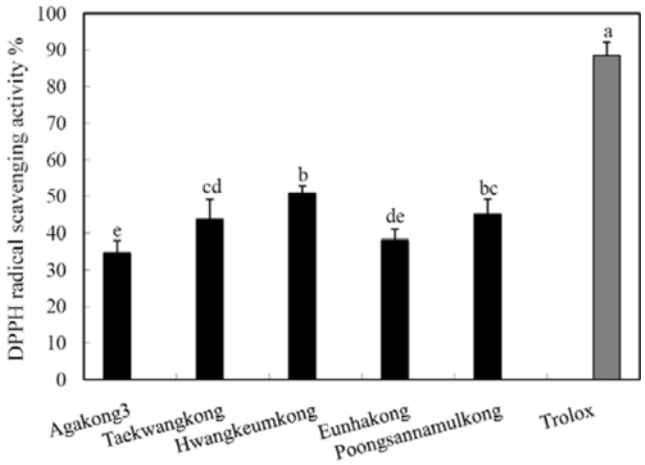

Fig. 3. DPPH radical scavenging activity of different soybean cultivars. The values are expressed as the mean±SD of 3 replicates. Bars with the different lowercase letter indicate a significant difference between treatments at $p<0.05$ as determined by the Duncan's multiple-range test.

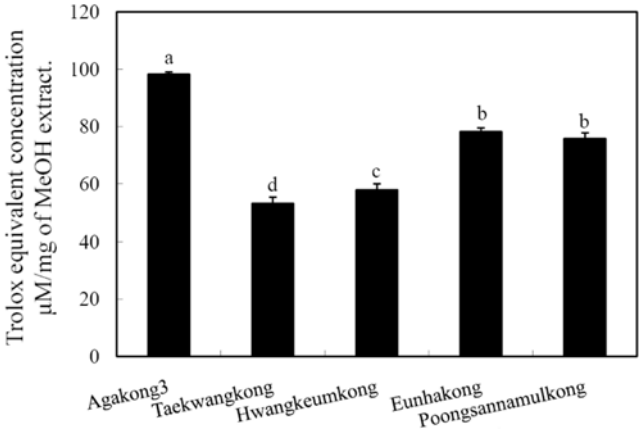

Fig. 4. ABTS radical scavenging activity of different soybean cultivars in TEAC assay. The values are expressed as the mean±SD of 3 replicates. Bars with the different lowercase letter indicate a significant difference between treatments at $p<0.05$ as determined by the Duncan's multiple-range test.

positive control (37,38). As shown in Table 1, it is speculated that the high amount of genistein contained in 'Agakong3' induced strong cytotoxicity (39).

Antioxidant activity of soybean extracts As shown in Fig. 3, in regards to the methanol extract of soybean, 'Hwangkeumkong' exhibited significantly stronger antioxidant activity than other soybeans in the DPPH assay. Even though the isoflavone contents of 'Agakong3' were the highest among soybeans (Table 1), it did not show strong antioxidant activity in the DPPH assay. This result is consistent with previous data of Mun *et al.* (40). They reported that isoflavone was underestimated in the DPPH assay system in determining soybean antioxidants. According to their data illustrated, isoflavone and glutathione showed very low or undetectable activities in the DPPH assays, while isoflavone and glutathione showed remarkable antioxidant activities in the TEAC assay. As shown in Fig. 4, the methanol extract of 'Agakong3' showed significantly stronger antioxidant activity than other samples in the TEAC assay. Especially, the antioxidant activity of 'Agakong3' was 30% higher than 'Poongsannamulkong' which was the most widely used cultivar in regards to sprouts. Considering previous finding by Mun *et al.* (40), it is supposed that the strong antioxidant activity of 'Agakong3' resulted from high contents of isoflavone in 'Agakong3' (Table 1). In the FRAP assay, 'Eunhakong' showed the strongest reducing power among soybeans. 'Agakong3' also exhibited strong reducing power compared to 'Taekwangkong', 'Hwangkeumkong', and 'Poongsannamulkong' (Fig. 5).

Total phenol contents As phenolic compounds have been proven to possess various biological activities (41), the total phenol contents of soybeans were examined. The total phenol contents of test samples were presented in Table 1. Total phenol contents were recorded in the following order: 'Agakong3'>'Poongsannamulkong'>'Eunhakong'>'Hwangkeumkong'>'Taekwangkong'. The total phenol contents of 'Agakong3' (5,925 μg of GAE/g of dry weight) were the highest among all samples tested. The total phenol contents of soy beans showed wide ranges from 3,204 to 5,925 μg of GAE/g of dry weight.

Total flavonoid contents The total flavonoid contents were assessed because some flavonoids were proven to be QR inducers (19,42). The total flavonoid contents of 'Agakong3', 'Taekwangkong', 'Hwangkeumkong', 'Eunhakong', and 'Poongsannamulkong' were 1,103, 686, 414, 625, and 576 μg of NE/g of dry weight respectively. 'Agakong3' showed the highest contents in total flavonoid as well as in total phenol.

Contents of isoflavones In this study, daidzein and genistein, both major isoflavones derived from soybeans were quantitified by means of the HPLC method. The genistein contents of 'Agakong3', 'Taekwangkong', 'Hwangkeumkong', 'Eunhakong', and Poongsannamulkong were 1,442, 188, 441, 413, and 576 μg/g of dry weight respectively. The genistein contents of soybeans ranged from 188 to 1,442 μg/g of dry weight in this study. Lee *et al.* (43) reported that the average genistein contents was 342 μg/g of dry weight derived from 46 soybean cultivars. Considering the results compiled by Lee *et al.* (43), 'Agakong3' has higher genistein contents than other cultivated soybeans. The daidzein contents of 'Agakong3', 'Taekwangkong', 'Hwangkeumkong', 'Eunhakong', and Poongsannamulkong were 3,906, 447, 432, 935, and 1,335 μg/g of dry weight, respectively. Total isoflavone (genistein plus daidzein) contents of 'Agakong3', 'Taekwangkong', 'Hwangkeumkong', 'Eunhakong', and 'Poongsannamulkong' were 5,348, 635, 873, 1,348, and 1,911 μg/g of dry weight, respectively. The isoflavone contents of 'Agakong3' to 'Taekwangkong' and 'Poongsannamulkong' were approximately 8.4- and 2.8-fold, respectively. 'Taekwangkong' and 'Hwangkeumkong', both of which are typical large-seed cultivars, have been used as materials for *cheonggukjnag* based on crop yields and economical factor. 'Eunhakong' and 'Poongsannamukong', both of which are typical small-

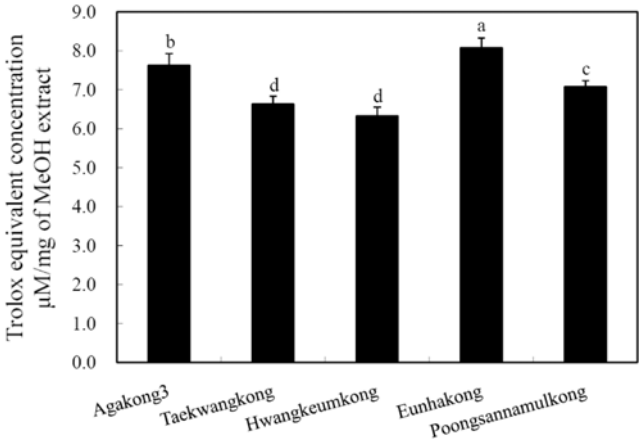

Fig. 5. Reducing power of different soybean cultivars in FRAP assay. The values are expressed as the mean±SD of 3 replicates. Bars with the different lowercase letter indicate a significant difference between treatments at $p<0.05$ as determined by the Duncan's multiple-range test.

seed cultivars, have been used for sprouts based on germination yields. Considering the high isoflavone contents and one hundred seeds weight regarding 'Agakong3', this will be good material, not only regarding soybean sprouts, but also in processing functional foods such as *cheonggukjang* and *doenjang*.

Correlation coefficients between bioactivities and functional constituents In order to determine the principles of QR and antioxidant activities in soybeans, the correlation coefficients between bioactivities (QR and antioxidant activities) and functional constituents (total phenol, flavonoid, and isoflavone contents) were analyzed by means of SAS (SAS Inc.) There is a positive correlation between functional compounds and QR inductive activity. The correlation coefficient between total phenol contents and QR inductive activity was recorded at 0.7938 (r). The correlation coefficient between total flavonoid contents and QR inductive activity was recorded at 0.7654 (r). The correlation coefficient between total isoflavone contents (daidzein and genistein) and QR inductive activity was recorded at 0.8392 (r). The analysis of the relationship between daidzein contents and QR inductive activity especially showed a high positive correlation coefficient (r=0.8416). Although Kim *et al*. (44) reported that genistein was a stronger QR inducer than daidzein in regards to soybeans, an analysis of the relationship between QR inductive activity and daidzein contents determined that a higher correlation was observed, compared to the correlation between QR activity and genistein levels. It was speculated that daidzein contents is generally 48% higher in comparison to the genistein contents in soybeans (43). There was a higher negative correlation between functional compounds and SRB result. The correlation between total flavonoid contents and SRB results particularly showed the most significant correlation among functional compounds. Although the relationship between the FRAP and DPPH results and functional compounds showed a negative or relatively low correlation coefficients, the relationship between TEAC results and all functional constituents showed high positive correlation coefficients. Interestingly, the relationship between QR inductive activity and TEAC results showed a high positive correlation coefficient (r=0.8478, $p<0.001$) because isoflavones such as genistein and daidzein were sensitive to both assays (40,44).

Acknowledgments

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Table 2. Correlation coefficients between bioactivities and functional constituents

	QR	SRB	TEAC	DPPH	FRAP	Total phenol	Total flavonoid	Daidzein	Genistein	Daidzein+ Genistein
QR	1									
SRB	-0.6453**	1								
TAEC	0.8478***	-0.5987*	1							
DPPH	-0.5496*	0.3331^{ns}	-0.6987***	1						
FRAP	0.5743*	-0.2140^{ns}	0.7254***	-0.6415**	1					
Total phenol	0.7938***	-0.7027**	0.8707***	-0.3858^{ns}	0.3600^{ns}	1				
Total flavonoid	0.7654***	-0.7814***	0.8264***	-0.4568*	0.3662^{ns}	0.9173***	1			
Daizein	0.8416***	-0.7229**	0.9047***	-0.6379*	0.4561^{ns}	0.9353***	0.8998***	1		
Genistein	0.8194***	-0.7530**	0.8769***	-0.5367*	0.3774^{ns}	0.9586***	0.9568***	0.9802***	1	
Daidzein+ Genistein	0.8392***	-0.7332**	0.9011***	-0.5367*	0.4380^{ns}	0.9447***	0.9175***	0.9988***	0.9889***	1

***$p<0.001$, **$p<0.01$, *$p<0.05$, ^{ns}=non significant.

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