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Phenolic compounds and antioxidant activity of adzuki bean cultivars

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ABSTRACT

The phenolic compounds and radical scavenging activity of ethanolic extracts of five adzuki beans were evaluate according to cultivar. The predominant phenolic acids in five cultivars of adzuki bean were (+)-catechin and gallic acid. Antioxidant capacities were determined using 2,2-diphenyl-1-picrylhydrazyl and 2,2-azinobis (3-ethylbenothiazoline-6-sulphonic acid) diammonium salt, and reducing power was positively enhanced according to the total polyphenolic content. In a cell-based assay, adzuki beans showed cytoprotective effects against oxidative stress induced by tert-butyl hydroperoxide and inhibitory effects on the production of reactive oxygen species, except for the cultivar *Vigna angularis* var. nipponensis cv. *Whinnarae*. In conclusion, (+)-catechin was the predominant phenolic compound found in adzuki beans, but there were differences according to the cultivar. Overall, the adzuki bean cultivars showed different antioxidant activities and cytoprotective effects according to the concentration and composition of phenolic compounds.

Key words: Adzuki bean (*Vigna angularis* var. nipponensis), HepG2 cells, Phenolic component, Radical scavenging activity.

INTRODUCTION

Free radicals are generated by chemical reactions, radiation, and several redox reactions of various compounds associated with damage to a wide range of molecular species, including lipids, proteins, and nucleic acids (Halliwell, 1996). This oxidative stress may be linked to many disorders, including atherosclerosis, cancer, liver cirrhosis, and diabetes (Muramatsu *et al*., 1995). Oxidative stress is caused by an imbalance between free radical generation and antioxidant defenses; thus, the appropriate intake of antioxidant chemicals is important to prevent related diseases. Previous epidemiological studies have suggested that an increased intake of whole fruits, vegetables, and whole grains is associated with a reduced risk of chronic disease (Hu, 2002). This association may be attributed to natural antioxidants in plant foods such as polyphenolics, carotenoids, tocopherol, vitamin C, and flavonoids, which all prevent oxidative damage (Choi *et al*., 2007).

Grains contain diverse phenolic compounds, including benzoic acid, cinnamic acid, anthocyanidins, quinones, flavonoids, and amino phenolic compounds (Tsao, 2010). Some phenolic compounds such as ferulic acid and diferulates are commonly found in grains but not in fruits or vegetables (Adom and Liu, 2002). The composition of phenolic compounds in grains varies depending on the grain type and cultivar. The different compositions and structures of phenolic acids in grains provide different bioactive functions. For instance, anthocyanins are water-soluble flavonoids that can be red, purple, or blue depending on pH. Colored rice (e.g., red, black, and purple) contains anthocyanins as a pigment, and this may contribute to its strong antioxidant activity compared to white rice (Goufo and Trindade, 2014). However, grain studies have been limited to major grains such as rice, wheat, barley, and corn. Because other grains have not been sufficiently considered, compositional analyses of these grains are required.

Adzuki beans (*Vigna angularis*) are frequently used as ingredient in confections in East Asia. Adzuki beans are a rich source of carbohydrates, proteins, minerals, vitamins, and fiber (Yoshida *et al*., 2009); however, the composition of phenolic compounds in different adzuki bean cultivars has not been studied. Therefore, to know the presence of bioactive compounds in adzuki beans (*Vigna angularis*), phenolic compounds and antioxidant activity along with its cytotoxicity and cytoprotective effects against oxidative stress in five cultivars of adzuki beans were studied and compared their antioxidant abilities and cytotoxic effects.

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MATERIALS AND METHODS

Sample preparation and extraction: The adzuki bean cultivars (*Vigna angularis* var. nipponensis cv. Geomguseul [GGS], cv. Arari [ARR], cv. Yeonduchae [YDC], cv. Whinguseul [WGS], and cv. Whinnarae [WNR]) were grown at the National Institute of Crop Science, Rural Development Administration, Miryang, South Korea, during the 2015 growing season and stored at –20°C. The beans were pulverized using a vibrating sample mill (CMT Co. Ltd., Tokyo, Japan). The pulverized samples were extracted with a shaker (SK-71 Shaker; JEIO Tech, Kimpo, South Korea) using 80% ethanol at room temperature. The extracts were filtered through Adventec No. 2 paper to remove debris. The filtrates were evaporated by rotary evaporation (N-1000; Eyela, Tokyo, Japan) and freeze-dried (FDT-8612; Operon, Kimpo, South Korea). Each extract was dissolved in 80% ethanol and samples were used for analysis.

Determination of total phenolics and flvanoids : The total polyphenolic contents of the adzuki beans were measured using the Folin-Ciocalteu method (Sharma *et al*., 2017). Briefly, standards or extracts (50 µl) were mixed with 1,000 µl of a sodium carbonate solution (2%, *w/v*, Sigma-Aldrich, St. Louis, MO, USA) and 50 µl of Folin-Ciocalteu reagent (50%, *v/v*, Sigma-Aldrich). The mixtures were incubated for 30 min at room temperature and the contents were measured at 750 nm. The data were expressed as mg of gallic acid equivalents per g of extract. For total flavonoid contents, standards or extracts (250 µl) were mixed with 1,000 µl of water and 75 µl of $NaNO_2$ (5%, *w/v*, Sigma-Aldrich). After 5 min, 150 µl of $AlCl_3 \cdot 6H_2O$ (10%, *w/v*, Sigma-Aldrich) was added and the solutions were incubated for another 6 min. The reaction was terminated by the addition of 1 M NaOH (500 µl) and the absorbance was measured at 510 nm (Dewanto *et al*., 2002). The data were converted to mg (+)-catechin equivalents per g of extract.

Determination of the phenolic acid composition: The phenolic acid compositions of the adzuki beans were analyzed using HPLC (Kim *et al*., 2016). An ODS column (5 µm, 4.6 mm × 250 mm; Agilent Technologies, Santa Clara, CA, USA) was used for phenolic acid analysis. The mobile phase consisted of water containing 0.1% (*v/v*) acetic acid (solvent A) and acetonitrile (J.T. Baker, Phillipsburg, NJ, USA) containing 0.1% (*v/v*) acetic acid (solvent B). The gradient program was as follows: 0–2 min, 92% to 90% A in B (gradient); 2–27 min, 90% to 70% A in B (gradient); 27–50 min, 70% to 10% A in B (gradient); 50–51 min, 10% to 0% A in B (gradient); 51–60 min, 0% A in B (isocratic); and 60–70 min, 0% to 92% A in B (gradient). The flow rate of the mobile phase was 1 ml/min and the injection volume was 20 µl. The UV detector was set at 280 nm to identify phenolic acids, which included gallic acid, homogentisic acid, protocatechuic acid, gentisic acid, chlorogenic acid, (+)-catechin, caffeic acid, phloretic acid, ρ–coumaric acid, ferulic acid, veratric acid, naringin, hesperidin, salicylic acid, quercetin, transcinnamic acid, naringenin, hesperitin, and biochanin (Sigma-Aldrich). Peaks were verified by adding the standard phenolic acids to the samples, and each peak area was calculated in relation to a standard peak area. The total phenolic acid content was calculated by adding up the different phenolic acid component amounts.

Measurement of DPPH and ABTS radical scavenging activities: DPPH scavenging activity was determined using the spectrophotometric method (Luo *et al*., 2014; Sinha *et al*., 2013). Each extract (0.2 ml) was mixed with an aliquot (0.8 ml) of 0.2 mM DPPH (Sigma-Aldrich). The mixtures were incubated for 30 min under dark conditions and the absorbance was recorded at 515 nm. The scavenging activity for the ABTS cation radical was measured as described previously (Choi *et al*., 2006). The ABTS solution (7 mM, Sigma-Aldrich) was mixed with potassium persulfate (2.45 mM) and incubated overnight in a dark room. The ABTS mixture was diluted with methanol (J.T. Baker) to obtain an absorbance of 1.4–1.5 at 735 nm. The diluted ABTS solution (1 ml) was added to the ethanolic extracts, a standard solution, or distilled water. After 30 min, the absorbance was measured at 735 nm using a spectrophotometer (model DU-650; Beckman, Fullerton, CA, USA). The DPPH radical and ABTS cation radical scavenging activities were expressed as the trolox equivalent antioxidant capacity.

Cell culture: Human hepatocyte HepG2 cells were obtained from the Korean Collection for Type Cultures (Daejeon, Korea) and cultured in Dulbecco's modified Eagle's medium high glucose with 10% heat-inactivated fetal bovine serum (FBS, Caisson, North Logan, UT, USA) and 1% penicillin streptomycin (Caisson,). The cells were maintained in a humidified incubator with 5% CO_2 at 37°C.

Cytotoxicity and cytoprotective effects: Cytotoxicity was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Tavakkol-Afshari *et al*., 2008; Kim *et al*., 2013) assay. HepG2 cells were seeded in 96-well plates at a density of 1.5×10^4 cells $well^{-1}$ and incubated for 24 h. Different adzuki bean cultivar extracts were diluted in FBS-free medium and replaced to the test plate. After 12 h of incubation, 0.5 mg ml^{-1} of MTT reagent was added and the samples were incubated for another 4 h. The medium was removed and dimethyl sulfoxide was added to dissolve the intracellular formazan. Cytotoxicity was determined by detecting the absorbance at 550 nm using a spectrophotometer (Beckman, Kim *et al*., 2013). To investigate cytoprotective effects, HepG2 cells in 96-well plates were stimulated with adzuki bean extract in FBS-free medium. After 12 h, the culture medium was discarded and the cells were treated for 3 h with tert-butyl hydroperoxide (TBHP; 200 µM) to induce oxidative stress. Cytoprotective effects were examined using an MTT assay.

Measurement of intracellular reactive oxygen species (ROS) and thiobarbituric acid reactive substances (TBARS): Intracellular ROS levels were quantified with a 2',7'-dichlorofluorescin diacetate (DCFH-DA) fluorescent probe as described previously (Wang and Joseph, 1999). HepG2 cells were seeded in a black 96-well plate and treated with extracts of five adzuki bean cultivars for 12 h. DCFH-DA (25 μM) in medium was added to the wells for 1 h and then treated with 1 mM TBHP. The fluorescence intensity was measured with a fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT, USA) for 2 h at 485/530 nm. Lipid peroxidation in HepG2 cells was measured with a TBARS assay kit (Cayman, Ann Arbor, MI, USA) according to the manufacturer's instructions.

Statistical analysis: All data were expressed as mean ± standard deviation (SD) values. The significance of differences among treatment means was determined by a one-way analysis of variance and Duncan's multiple range tests using SAS version 9.2 (SAS Institute, Cary, NC, USA) with a significance level of 0.05. We also investigated the correlations from a regression analysis between the parameters.

RESULTS AND DISCUSSION

Composition of phenolic compounds in adzuki bean extracts: The phenolic compounds in five different cultivars of adzuki bean were analyzed using HPLC. The total polyphenol content was highest in GGS (20.53 ± 0.11 mg of

Table 1: Total polyphenols, flavonoid contents, DPPH, and ABTS radical scavenging activities, and reducing power of ethanolic extracts of adzuki beans according to cultivar.

Cultivar[1)]	Total polyphenol contents[2)]	Total flavonoid contents[3)]	Radical scavenging activity[4)]		Reducing power (A700)
			DPPH radical	ABTS radical	
GGS	20.53±0.11[5) b6)]	2.87±0.22 [c]	16.47±0.15 [d]	43.65±0.46 [a]	0.768±0.010[c]
ARR	22.23±0.32 [a]	3.98±0.28 [b]	17.89±0.25 [b]	43.35±0.14 [a]	0.799±0.011[b]
YDC	21.33±1.04 [ab]	4.00±0.09 [b]	17.16±0.41 [c]	42.51±1.08 [a]	0.733±0.012[d]
WGS	21.13±0.86 [ab]	7.79±0.18 [a]	21.03±0.30 [a]	39.95±0.56 [b]	0.898±0.029[a]
WNR	12.05±0.41 [c]	0.41±0.03 [e]	1.48±0.80 [e]	32.18±1.00 [c]	0.161±0.003[e]

1) GGS: *Vigna angularis* var. nipponensis cv. Geomguseul, ARR: cv. Arari, YDC: cv. Yeonduchae, WGS: cv. Whinguseul, WNR: cv. Whinnarae.
2) mg of gallic acid equivalents per g of extract residue
3) mg of catechin equivalents per g of extract residue
4) mg of Trolox equivalents per g of extract residue
5) Each value is the mean ± SD (n = 3).
6) Values with different superscripts are significantly different at $p < 0.05$ according to Duncan's multiple range tests.

Table 2: The phenolic acid contents in ethanolic extracts of adzuki beans according to cultivar.

Phenolic acid	Contents of phenolic acids (μg per g extract residue)				
	GGS[1)]	ARR	YDC	WGS	WNR
Gallic acid	16.56±1.48[2)]	16.3±0.71	43.57±2.19	32.32±2.88	15.99±1.79
Homogentisic acid	ND[3)]	ND	ND	ND	ND
Protocatechuic acid	2.42±0.16	ND	2.65±0.49	ND	ND
Gentisic acid	42.86±8.66	ND	46.27±3.69	ND	ND
Chlorogenic acid	0.48±0.37	ND	ND	14.26±3.17	ND
(+)-Catechin	467.89±20.87	584.08±33.88	302.47±4.47	644.17±13.79	133.55±5.73
Caffeic acid	0.16±0.13	ND	ND	ND	ND
Phloretic acid	107.71±5.53	ND	ND	35.08±3.34	ND
ñ-Coumaric acid	46.81±4.51	ND	19.32±0.24	6.75±0.87	ND
Ferulic acid	2.79±0.12	2.31±0.36	9.88±1.34	2.27±0.27	ND
Veratric acid	1.96±0.4	ND	6.2±0.53	ND	ND
Naringin	ND	ND	ND	ND	ND
Hesperidin	2.72±0.45	5.25±0.24	3.56±0.41	ND	ND
Salicylic acid	ND	ND	ND	ND	ND
Cinnamic acid	ND	ND	ND	ND	ND
Naringenin	ND	ND	ND	ND	ND
Hesperitin	ND	ND	ND	ND	ND
Biochanin	1.99±0.10	ND	ND	ND	ND
Total	651.33±29.56	607.94±33.89	387.65±5.62	734.85±13.46	149.54±6.54

1) GGS: *Vigna angularis* var. nipponensis cv. Geomguseul, ARR: cv. Arari, YDC: cv. Yeonduchae, WGS: cv. Whinguseul, WNR: cv. Whinnarae.
2) Each value is the mean ± SD (n = 3).
3) ND: Not detected

gallic acid g^{-1} of extract), and the flavonoid content was highest in WGS (7.79 ± 0.18 mg of catechin g^{-1} of extract; Table 1). The predominant phenolic compound in the adzuki beans was (+)-catechin; it accounted for 71.84, 96.08, 78.03, 87.66, and 89.31% of the content in GGS, ARR, YDC, WGS, and WNR, respectively (Table 2). Gallic acid was also ubiquitously found in the adzuki beans; it comprised 2.54, 2.68, 11.24, 4.40, and 10.69% of the total phenolic acids in GGS, ARR, YDC, WGS, and WNR, respectively. The other phenolic acids differed according to the cultivar. GGS contained the most varied phenolic acids, including caffeic acid and biochanin, which were only found in this cultivar. In ARR and WNR, phenolic compounds were not detected or minor amounts were detected, except for gallic acid and (+)-catechin. In YDC and WGS, gentisic acid and phloretic acid were the second most substantial compounds, respectively.

Adzuki beans can have several seed coat colors, including red, black, speckled purple, brown, green, and white (Hori *et al*., 2006). The cultivars that were analyzed in this study, GGS, ARR, and YDC, have black, red, and green seed coats, respectively. WGS and WNR have yellow-white seed coats. Interestingly, GGS (with a black seed coat) contained the most varied types of phenolic acids (Gallic acid, protocatechuic acid, gentisic acid, chlorogenic acid, (+)-catechin, caffeic acid, phloretic acid, ρ-coumaric acid, ferulic acid, veratric acid, hesperidin, and biochanin) while WNR (with a yellow-white seed coat) showed limited phenolic acids composition (Gallic acid and (+)-catechin) However, another yellow-white cultivar, WGS, contained the highest flavonoid and phenolic acid contents compared to the other cultivars. Therefore, there was no relationship between seed coat color and phenolic compounds content.

Radical scavenging activity and reducing power of adzuki beans: Cell-free antioxidant activities were compared within five cultivars of adzuki bean (Table 1). The DPPH radical scavenging activities were highest in WGS (by 21.03 mg of Trolox g^{-1} of extract) followed by ARR, YDC, GGS, and WNR. The ABTS radical scavenging properties were highest in GGS (by 43.65 mg of Trolox g^{-1} of extract) followed by ARR, YDC, WGS, and WNR. Reducing power is the capacity to donate hydrogen and electrons in reduction reactions. WGS showed the highest reducing power (A_{700} = 0.898) followed by ARR, GGS, YDC, and WNR.

WGS was the most effective cultivar against DPPH radical scavenging and with regard to reducing power. This may be related to its high phenolic content, including (+)-catechin. A strong correlation (r2 value 0.966) was observed between phenolics with antioxidant capacity in legumes (Marathe *et al.*, 2011) Thus, presence of phenolic and flavonoid contents positively contributes to antioxidant activity (Mahatma *et al.*, 2016). Catechin is a polyphenolic compound that is widely found in plants. We found that (+)-catechin was the major phenolic acid in adzuki beans. Catechin is a major contributor to antioxidant properties in biological systems (Lu *et al*., 2011). Catechin effectively scavenges reactive oxygen and nitrogen species, including singlet oxygen, superoxide, peroxynitrite, peroxyl radicals, and hypochlorous acid (Frei and Higdon, 2003). This antioxidant capacity increases the resistance of plasma low-density lipoprotein oxidation, and thus may prevent coronary heart disease (Hayek *et al*., 1997).

Cytotoxicity and cytoprotective effects of adzuki beans against oxidative stress: We investigated cytotoxicity using HepG2 cells after stimulation with adzuki bean extracts (Fig. 1). Cell viability was significantly reduced in 50 µg ml^{-1} of YDC and 20 µg ml^{-1} of WNR; however, the toxicity was not considerable (cell viability > 90%). Next, we stimulated HepG2 cells using TBHP, which is an organic peroxide that induces oxidative stress. Cell viability was dramatically reduced by TBHP and recovered by GGS, ARR, YDC, or WGS extract (50 µg ml^{-1}). WNR was less effective at cytoprotection after oxidative damage (Fig. 2).

Previous studies have reported that polyphenol-rich plant extracts protect against TBHP-mediated damage via antioxidant activities. In a fermented tea study, tea polyphenols suppressed membrane leakage and the disruption of intracellular antioxidant machinery caused by TBHP (Bhattacharya *et al*., 2011). In our study, the total polyphenol contents were positively correlated with cytoprotective effects against TBHP. In contrast, polyphenol contents of less than 20 mg of gallic acid g^{-1} of extract (i.e., WNR) were less likely to have a protective effect after TBHP stimulation.

Antioxidant activities of adzuki beans in HepG2 cells: ROS production in HepG2 cells was induced by TBHP treatment. However, ROS production was markedly ameliorated after treatment with 50 µg mL^{-1} of GGS, ARR, YDC, and WGS, respectively. WNR had no effect on ROS production (Fig. 3).

In addition, inhibitory effects of lipid peroxidation were established in HepG2 cells after adzuki bean stimulation (Fig. 4). Every cultivar of adzuki bean significantly reduced lipid peroxidation at 0 to 5 h of incubation. WGS showed the strongest inhibitory effects on cellular lipid peroxidation compared to the other derivatives.

Endogenous ROS production occurs in mitochondria, the plasma membrane, endoplasmic reticulum, and peroxisomes through enzymatic reactions or auto-oxidation of several compounds, including catecholamines and hydroquinone (Ayala *et al*., 2014). ROS formation is suppressed by the inhibition of enzymes or chelating trace elements. This could be inhibited by flavonoids through efficiently chelated irons and stabilized ROS in cells (Heim

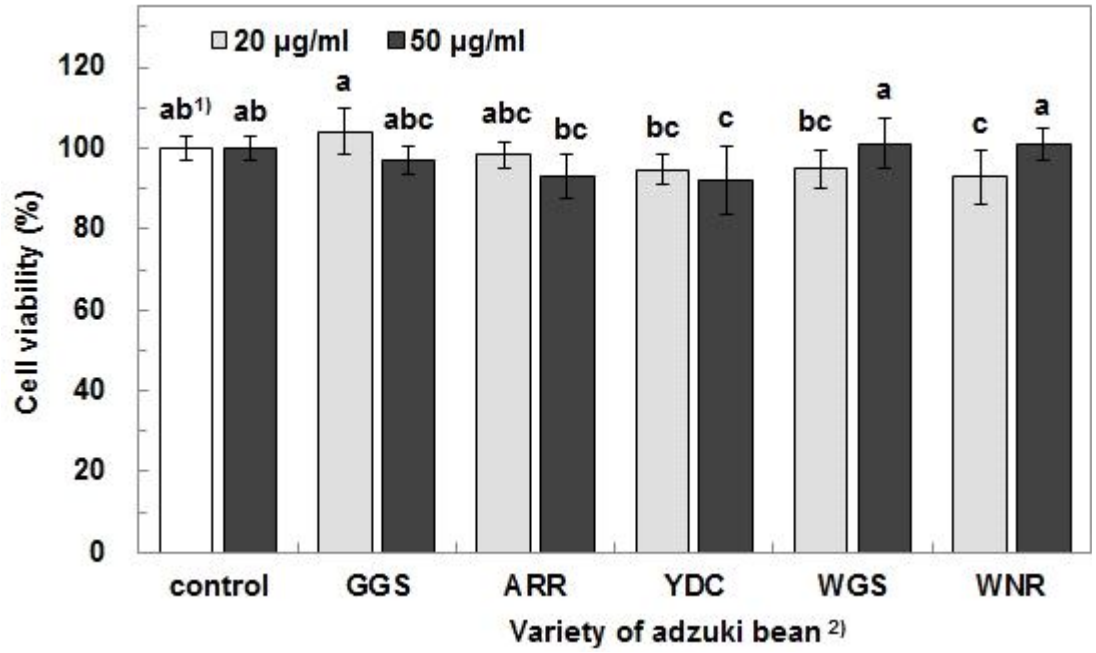

Fig 1: Cytotoxicity in HepG2 cells treated with ethanolic extracts of adzuki beans according to variety.
1). Values with different superscripts are significantly different at $p < 0.05$ according to Duncan's multiple range tests. 2) GGS: *Vigna angularis* var. nipponensis cv. Geomguseul, ARR: cv. Arari, YDC: cv. Yeonduchae, WGS: cv. Whinguseul, WNR: cv. Whinnarae

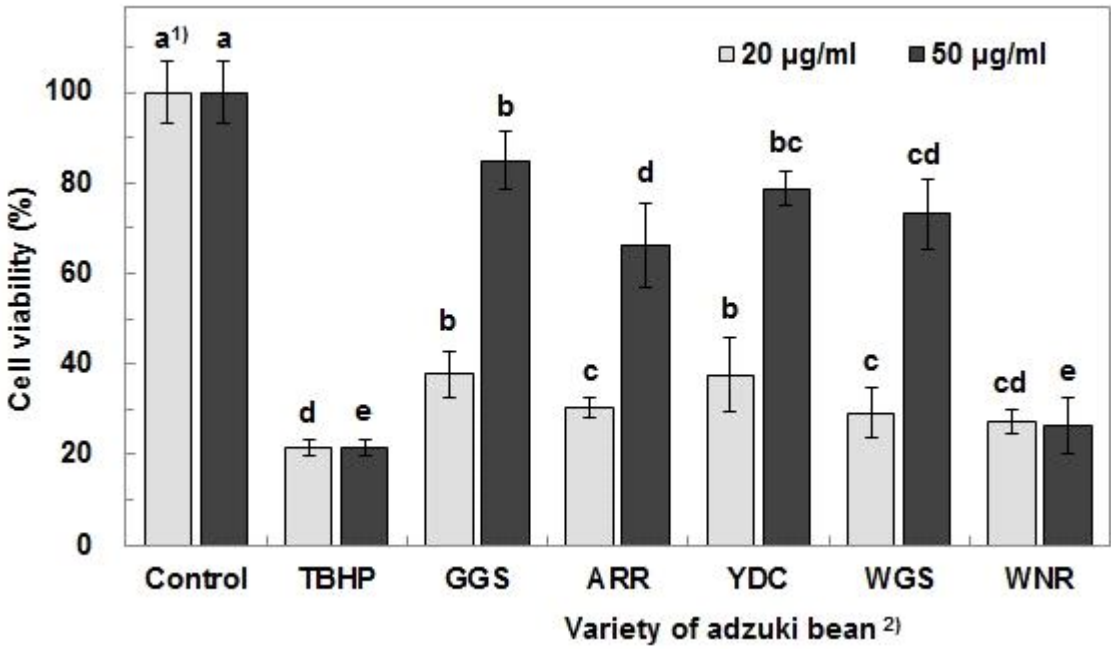

Fig 2: Protective effects of adzuki beans ethanolic extracts against oxidative stress in HepG2 cells.
1). Values with different superscripts are significantly different at $p < 0.05$ according to Duncan's multiple range tests. 2)GGS: *Vigna angularis* var. nipponensis cv. Geomguseul, ARR: cv. Arari, YDC: cv. Yeonduchae, WGS: cv. Whinguseul, WNR: cv. Whinnarae.

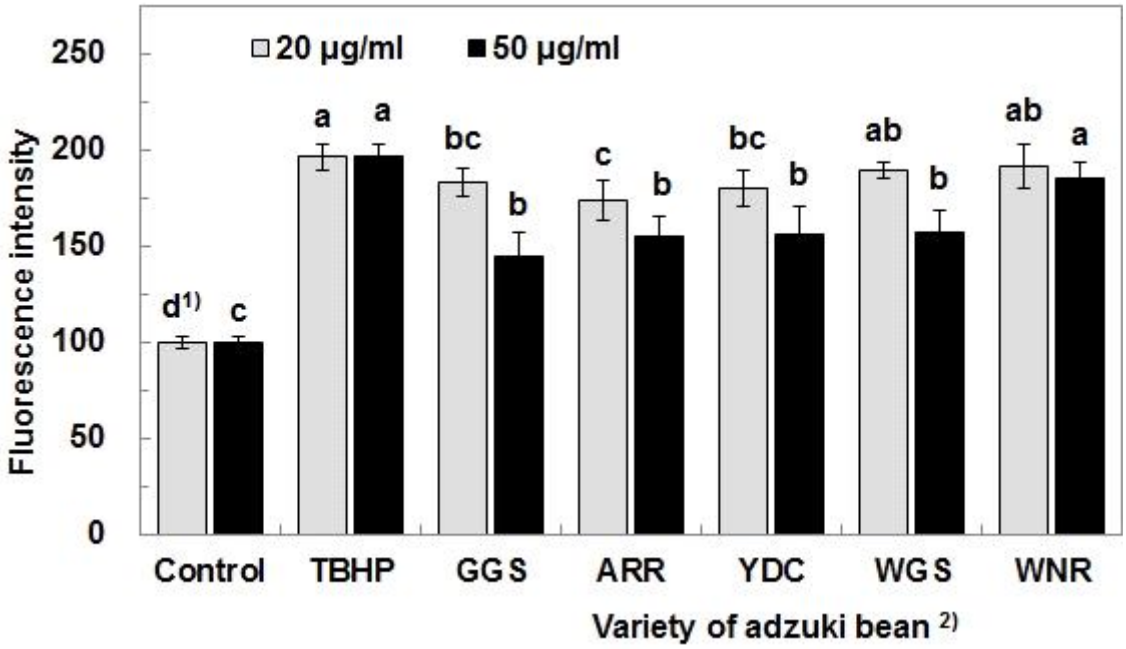

Fig 3: The generation of ROS in HepG2 cells exposed to ethanolic extracts of adzuki beans according to variety.
1). Values with different superscripts are significantly different at $p < 0.05$ according to Duncan's multiple range tests. 2) GGS: *Vigna angularis* var. nipponensis cv. Geomguseul, ARR: cv. Arari, YDC: cv. Yeonduchae, WGS: cv. Whinguseul, WNR: cv. Whinnarae.

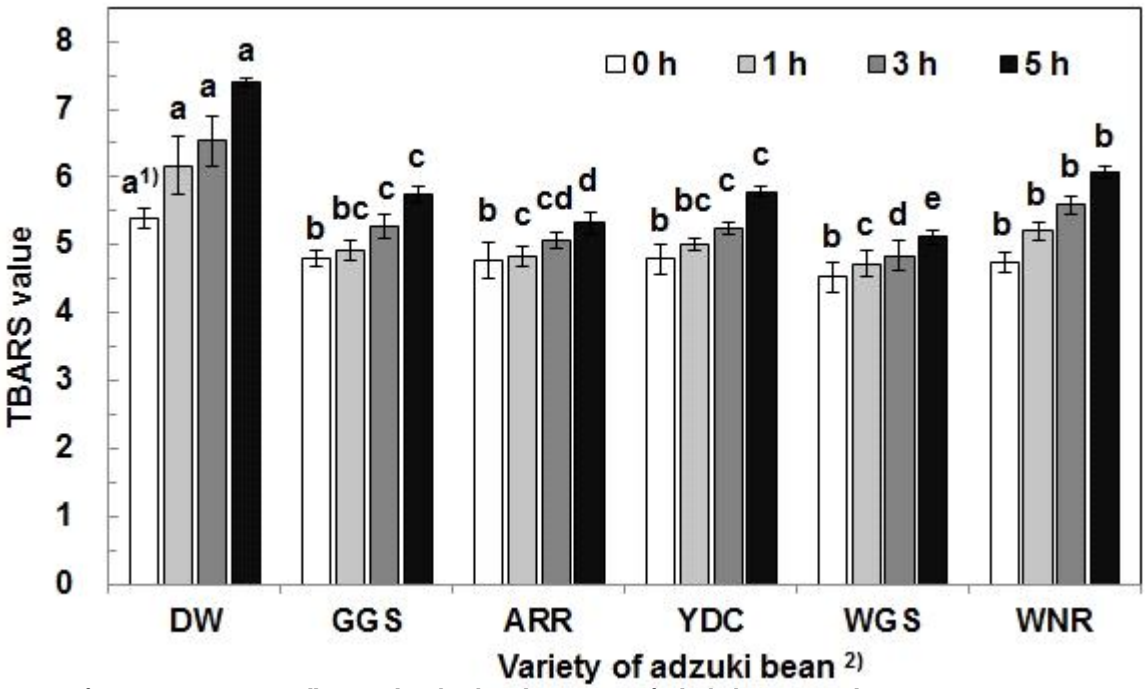

Fig 4: TBARS formation in HepG2 cells treated with ethanolic extracts of adzuki beans according to variety.
1). Values with different superscripts are significantly different at $p < 0.05$ according to Duncan's multiple range tests. 2) GGS: *Vigna angularis* var. nipponensis cv. Geomguseul, ARR: cv. Arari, YDC: cv. Yeonduchae, WGS: cv. Whinguseul, WNR: cv. Whinnarae.

et al., 2002). As a result, the flavonoid-rich extracts of GGS, ARR, YDC, and WGS reduced ROS generation, which may protect against DNA, protein, and lipid damage in cells.

In summary, adzuki beans exhibited a diverse composition of phenolic compounds depending on the cultivar. The effect of free radical scavengers and cell protection from oxidative damage were positively correlated with the content of phenolic compounds in adzuki beans. The differences were minor in GGS, ARR, YDC, and WGS, while WNR had a markedly lower polyphenol content and weak antioxidant activity. Adzuki beans with high polyphenol concentrations may have therapeutic effects on oxidative stress-related disorders.

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