Comparison of Phytochemical Contents and Cytoprotective Effects of Different Rice Bran Extracts from *Indica* and *Japonica* Rice Cultivars

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ABSTRACT: The present study was aimed to evaluate the phytochemical contents and hepatocyte protective effects of functional extracts from rice bran of *indica* and *japonica* rice cultivars, Dasan 1 and Ilpum, respectively. The highest vitamin E (23.51 mg/g) and phytosterol (390.25 mg/g) content was observed in the unsaponifiable matter (USM) of Dasan 1 cultivar. However, USM of Ilpum showed the highest content of total policosanol and squalene (232.73 mg/g and 99.31 mg/g, respectively). The methanolic extract from the defatted rice bran (MEDR) of Dasan 1 showed the highest total polyphenol content, reducing power, and radical scavenging capacity, while USM of Dasan 1 showed the highest cell viability (81.3%) against oxidative stress in HepG2 cells. USM significantly increased glutathione levels and suppressed the production of reactive oxygen species in hepatocytes compared with methanolic extracts of the rice bran oils and/or MEDR. These results provide useful information on the functional extracts of rice bran from *indica* and *japonica* rice cultivars, including their antioxidant properties and cytoprotection in HepG2 cells.

Keywords: cytoprotective effect, phytochemicals, rice bran, rice cultivar, unsaponifiable matter

INTRODUCTION

In the past decade, the consumption of fresh vegetables and whole grains has increased as a result of growing health concerns. Free radicals, which cause cellular damage to DNA, and oxidation of lipid and protein are believed to be related to various diseases, including cardiovascular disease, hypertension, neurodegenerative disease, and cancer (Halliwell and Gutteridge, 1984; Steinberg et al., 1989; Muramatsu et al., 1995). A high intake of antioxidant-rich food is involved in reducing the risk of degenerative diseases (Joseph et al., 1999). This relationship may be caused by the natural antioxidants including ascorbic acid, tocopherols, polyphenols, and flavonoids, which scavenge free radicals (Xu et al., 2017).

Rice bran is a by-product of white rice production during milling. Generally, rice bran accounts for 12~20% of the total rice kernel weight. The bran is a rich source of various bioactive phytochemicals namely, phytosterols, tocopherols, and γ-oryzanol, which possess beneficial effects on human health due to their antioxidant activity, including anti-cancer, anti-coronary heart disease, and hy-

pocholesterolemic properties (Okarter and Liu, 2010; Ashraf et al., 2019). The unsaponifiable matter (USM) is defined as substances that are dissolved in lipids that are insoluble in aqueous solution but soluble in organic solvent after saponification. The USM from rice bran contains significant amounts of phytosterols, policosanols, fat-soluble vitamins, and squalenes (Afinisha Deepam and Arumughan, 2012). Previous studies have showed that the USM is beneficial in preventing the development of hyperlipidemia and skin photoaging (Sharma and Rukmini, 1987; Lee et al., 2019). As a result of an increased interest in healthy food products, interest in rice cultivars has increased due to their phytochemical content and antioxidant activity. In a previous study performed in our laboratory, the antioxidant activity and phytochemicals content of various *japonica* and *indica* cultivars—the two major subspecies of Asian rice cultivars (*Oryza sativa* L.) were investigated (Ham et al., 2013). In addition, the same study found that the *indica* cultivar Dasan 1 and the *japonica* cultivar Ilpum contained the highest tocopherol and tocotrienol content (Ham et al., 2013). Therefore, the present study was aimed to evalu-

ate the phytochemical content of functional extracts from *indica* and *japonica* rice cultivars, Dasan 1 and Ilpum. The antioxidant and cytoprotective actions of the functional extracts from rice brans against oxidative stress were also investigated.

MATERIALS AND METHODS

Chemicals
γ-Oryzanol was purchased from Oryza Oil & Fat Chemical Co., Ltd. (Ichinomiya, Japan). Tocopherol and tocotrienol were obtained from Merck KGaA (Darmstadt, Germany). The individual polycosanol standards, 5α-cholestane, β-sitosterol, campesterol, stigmasterol, diammonium salt of 1,1-diphenyl-2-picrylhydrazyl (DPPH), diammonium salt of 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ethylene diamine tetra acetic acid (EDTA), dimethyl sulfoxide (DMSO), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), 2',7'-dichlorofluorescein diacetate (DCFH-DA), glutathione reductase (GR), reduced glutathione (GSH), *tert*-butyl hydroperoxide (TBHP), potassium ferricyanide, potassium persulfate, and trichloroacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

HepG2 cells were obtained from the Korean Collection for Type Cultures (Daejeon, Korea). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were purchased from Gibco BRL (Gaithersburg, MD, USA). Rice bran provided by the Rural Development Administration of Korea (Wanju, Korea) was heated at $100^{\circ}C$ for 30 min.

Sample preparation
USM: Rice bran powder (2.0 g) was blended with 10 mL of 6% ethanolic pyrogallol in a capped flask. After sonication for 5 min, the mixture was added to 8 mL of 60% ethanolic KOH. Then, the rice bran was saponified in a water bath for 50 min at $70^{\circ}C$. The solution was shaken every 10 min to ensure a well-mixed extraction. After cooling, 30 mL of 2% NaCl was added and mixed. The resulting solution was extracted with *n*-hexane three times. After filtering the solution, the solvent was evaporated with nitrogen gas at $40^{\circ}C$. The resulting USM was collected and redissolved in DMSO and kept at $-20^{\circ}C$ until further analysis.
Methanolic extracts of the rice bran oil (MEO): The methanolic extract from the oil was prepared according to a previously described method with slight modifications (Choi and Lee, 2009). Rice bran oil was extracted using *n*-hexane (at a 7-fold volume of sample), and then filtered using filter paper (Advantec no. 2, Advantec Toyo Kaisha,

Ltd., Tokyo, Japan). The hexane-soluble extracts were then concentrated in a vacuum evaporator. The resulting oily residue was extracted using methanol (at a 7-fold volume of sample) by stirring for 24 h. The resulting methanol layer was filtered through filter paper. Lastly, the MEO were obtained by evaporating under vacuum.
Methanolic extracts of the defatted meal of rice bran (MEDR): The defatted meal of rice bran was obtained after oil solvent extraction (hexane), extracted using methanol (at a 7-fold volume of sample), and filtered using no. 2 filter paper (Advantec Toyo Kaisha, Ltd.). Lastly, the MEDR were evaporated under vacuum.

Determination of vitamin E content
Analysis of vitamin E was performed with high performance liquid chromatography (M930; Young Lin Instrument Co., Ltd., Anyang, Korea) and fluorometric detection (LC305; Thermo Separation Products, Hayward, CA, USA). Tocopherol and tocotrienol standards were obtained from Merck KGaA. A LiChrosphere® Diol column (250×4 mm i.d., 5 μm; Merck KGaA) was used with a mobile phase of 1.2% isopropanol in *n*-hexane at a flow rate of 1.0 mL/min. The wavelengths were 290 nm for excitation and 320 nm for emission (Choi et al., 2010).

Determination of policosanols, phytosterols, and squalene content
The content of phytosterols, policosanols, and squalene was measured by gas chromatography using a SACTM-5 capillary column (30 m×0.32 mm i.d.; Supelco Inc., Bellefonte, PA, USA) and a flame ionization detector. The column was initially maintained at $280^{\circ}C$ for 1 min, increased to $300^{\circ}C$ at $2^{\circ}C/min$, and then held at $300^{\circ}C$ for 20 min. The flame ionized detector and injector were set at $310^{\circ}C$ and $320^{\circ}C$, respectively, with a split ratio of 50:1 under helium atmosphere at a flow rate of 1.0 mL/min.

Determination of γ-oryzanol content
The concentration of γ-oryzanol was measured using a spectrophotometer (JASCO UV-900, JASCO, Tokyo, Japan). γ-Oryzanol standards were obtained from Oryza Oil & Fat Chemical Co., Ltd.. The absorbance of solution was read at 315 nm, and then calculated using the extinction coefficient (358.9) for γ-oryzanol (Miller and Engel, 2006).

Determination of polyphenolic content
The phenolic content was measured using the Folin-Ciocalteu method (Dewanto et al., 2002). An aliquot (200 μL) of standard solution (gallic acid) or extract was added to 2 mL of 2% sodium carbonate and incubated for 3 min. Then, 100 μL of a 50% Folin-Ciocalteu reagent was added, thoroughly mixed, and incubated for 30 min. The absorbance of solution was measured at 750 nm.

Antioxidant property

Antioxidant activity was determined using DPPH assay (Pellegrini et al., 2000). One-mL DPPH radical solution (0.2 mM) was added to 20 μL of extract or Trolox$^{®}$ solution. The solution was vortexed for 10 s and incubated for 10 min. The absorbance was measured at 520 nm.

The ABTS radical scavenging activity of the extracts was evaluated using a previously reported method (Re et al., 1999). The ABTS radical cation solution was prepared by reaction of ABTS (7 mM) and potassium persulfate (2.45 mM). The mixture was placed in the dark overnight. Then, an aliquot (50 μL) of sample was added and vortexed to 1 mL of ABTS radical cation solution for 30 min. Then, the absorbance was read at 734 nm.

The ferric reducing power assay was measured using a previously reported method (Benzie and Strain, 1996). An aliquot (250 μL) of sample was added and mixed to 250 μL of sodium phosphate buffer (20 mM, pH 6.6) and 250 μL of 1% potassium ferricyanide, and then placed at 50°C. After 20 min, 10% trichloroacetic acid (250 μL) was added to the solution and centrifuged at 1,000 g for 3 min. The resulting supernatant (500 μL) was added to 1% ferric chloride (100 μL) and distilled water (500 μL) and allowed to react. The absorbance was read at 700 nm.

Cell culture and determination of cytotoxicity

HepG2 cells were grown in DMEM supplemented with 10% FBS, 100 μg/mL streptomycin, and 100 U/mL penicillin in a humidified 5% CO_2 atmosphere at 37°C. The cell viability was measured using the lactate dehydrogenase (LDH) assay kit (Sigma Chemical Co.). HepG2 cells were seeded in 96-well plates (1×10^4 cells/well) and incubated for 24 h. The residue samples (100 μg/mL) were added to cells. After 6 h, the LDH activity in the medium was analyzed. The released LDH activity was calculated according to the manufacturer's instructions.

Examination of cytoprotective effects

HepG2 cells were seeded at a density of 10,000 cells/well in 96-well plates. After 24 h, the medium was replaced with serum-free medium containing the corresponding concentration of the residue samples (100 μg/mL). The cytoprotective effects of samples were determined according to a previous study (Lee et al., 2014). Cells were treated with the samples for 6 h. Medium was removed and cells were incubated with TBHP (500 μM) for 3 h. Then, cell viability was assessed by measuring the amount of released LDH.

Reactive oxygen species (ROS)

The intracellular ROS levels were measured according to the previously described method (Wang and Joseph, 1999). HepG2 cells were seeded in 96-well black plates (5×10^4 cells/well). After 24 h, 100 μg/mL of samples were added to the cells. The medium containing samples was withdrawn after 6 h, and serum-free medium containing 25 μM of DCFH-DA was added and incubated for 1 h at 37°C. HepG2 cells were exposed to 1.5 mM of TBHP for 2 h. The ROS production was measured at 485 nm for excitation and 530 nm for emission by using a fluorescence spectrophotometer (PerkinElmer, Inc., Norwalk, CT, USA).

Determination of glutathione content

HepG2 cells were seeded at a density of 1×10^6 cells/well in 6-well plates. After 24 h, the medium was replaced with serum-free medium containing the corresponding concentration of the residue samples (100 μg/mL) and incubated for 6 h. The cells were treated with 1.5 mM of TBHP for 3 h. Then, the cells were harvested and sonicated (Sonics VCX 750 Vibra Cell^{TM}, Sonics & Materials Inc., Newtown, CT, USA). After sonication, the suspension was centrifuged at 10,000 g for 10 min at 4°C and the supernatants were transferred to new tubes. The GSH assay was carried out as reported earlier (Baker et al., 1990).

Statistical analysis

The results were expressed as the mean±standard error (SE). The significant difference was determined by one-way analysis of variance (ANOVA) and Duncan's multiple range test ($P<0.05$) using SAS version 9.2 (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Phytochemical content

The extraction yield of the functional extracts is shown in Table 1. Each yield was calculated based on the weight of rice bran (USM), the oil (MEO), or defatted meal of rice bran (MEDR). The vitamin E, policosanol, phytosterols, squalene, and γ-oryzanol content of the functional extracts was analyzed. The phytochemical components are listed in Table 2. Generally, it has been reported that the *japonica* rice contained higher contents of vitamin E and γ-oryzanol than the *indica* rice (Heinemann et al.,

Table 1. Extraction yields of functional rice bran extracts from two different rice cultivars　　　　　　　　　　　(unit: %)

	Dasan 1			Ilpum		
	USM	MEO	MEDR	USM	MEO	MEDR
Yield	1.00	7.30	5.80	1.14	7.35	7.10

USM, unsaponifiable matter of rice bran; MEO, methanolic extract of rice bran oil; MEDR, methanolic extract of defatted rice bran.

Table 2. Phytochemical content of functional rice bran extracts from two different rice cultivars · (unit: mg/g of residue)

Components	Dasan 1			Ilpum		
	USM	MEO	MEDR	USM	MEO	MEDR
Vitamin E						
α-T	3.05	0.68	0.03	5.16	1.55	0.10
α-T3	0.99	0.32	0.01	5.13	1.97	0.09
β-T	0.20	0.03	0.00	0.27	0.08	0.01
β-T3	−	−	−	−	−	−
γ-T	2.81	0.77	0.04	0.30	0.08	0.01
γ-T3	15.49	5.47	0.22	3.53	1.41	0.08
δ-T	0.14	−	−	−	−	−
δ-T3	0.82	0.35	0.02	0.36	0.20	0.02
Total	23.51	7.61	0.33	14.75	5.30	0.31
Phytosterols						
Campesterol	52.57	9.50	2.28	65.92	5.23	1.67
Stigmasterol	147.42	18.40	5.34	120.15	14.10	6.30
β-Sitosterol	190.27	28.33	5.59	148.20	20.75	6.39
Total	390.25	56.24	13.22	334.27	40.08	14.36
Polycosanols						
C23	17.90	0.72	0.33	33.16	0.44	0.28
C24	−	0.52	−	−	0.36	0.27
C26	22.24	0.72	−	38.36	0.60	−
C27	2.58	0.50	−	4.69	0.44	−
C28	29.13	3.65	−	59.84	1.53	−
C30	21.12	0.31	−	96.68	0.31	−
Total	92.97	6.43	0.33	232.73	3.68	0.54
Squalene	11.57	0.5	0.45	99.31	4.02	0.7
γ-Oryzanol	11.67	51.04	13.1	10.77	34.95	10.8

USM, unsaponifiable matter of rice bran; MEO, methanolic extract of rice bran oil; MEDR, methanolic extract of defatted rice bran. T, tocopherols; T3, tocotrienols.
Values are the mean of duplicate determinations. −: not determined.

2008). In the same study, the most abundant tocols present in the *japonica* rice were α-tocopherol, whereas γ-tocopherol was predominant in the *indica* rice. In the results, the Dasan 1 cultivar showed comparatively higher levels of γ-tocotrienol than the Ilpum cultivar. The highest total vitamin E and phytosterol content were observed in the USM of the Dasan 1 cultivar (23.51 mg/g and 390.25 mg/g, respectively). However, the highest content of total policosanol and squalene were found in the USM of Ilpum (232.73 mg/g and 99.31 mg/g, respectively). The highest content of γ-oryzanol was 51.04 mg/g in the MEO of Dasan 1. γ-Oryzanol is mainly composed of ferulic acid esters of triterpene alcohols and sterols in rice bran oil (Lerma-García et al., 2009). It has been previously reported that the γ-oryzanol content was reduced by alkaline hydrolysis (Truong et al., 2017). Therefore, it appears that γ-oryzanol in USM is broken down by alkaline treatment during lipid saponification. Except for γ-oryzanol, USM contained higher levels of phytochemicals compared to MEO and/or MEDR irrespective of the cultivar. In previous studies, USM obtained by saponification were found to possess more phytochemicals, including tocols, phytosterols, and polycosanol, than oil (Choi et al., 2010; Afinisha Deepam and Arumughan,

2012). These phytochemicals are associated with various properties that are beneficial to health, including antioxidant activity and hypocholesterolemic effects (Wilson et al., 2007; Okarter and Liu, 2010; Zhu and Sang, 2017). These data indicate that USM of *japonica* rice (Ilpum) but also *indica* rice (Dasan 1) contains high amounts of bioactive substances, such as tocols, phytosterol, squalene, and polycosanol, which could contribute to the development of phytochemical-enriched functional foods.

Antioxidant activity of functional extracts
The polyphenolic content is shown in Fig. 1A. In the results, the MEDR of Dasan 1 was found to contain the highest total polyphenol content. The antioxidant capacity was measured based on the DPPH and ABTS radical scavenging activities, and reducing power (Fig. 1B~1D). As expected, MEDR had a higher ABTS and DPPH radical scavenging activity than MEO and USM, as well as a significantly higher reducing power than the other extracts, regardless of the cultivars. Taken together, MEDR showed the highest radical scavenging activity, whereas MEO had the lowest. Phenolic compounds are more soluble in organic solvents that are less polar than water. It has been reported that absolute methanol is the best sol-
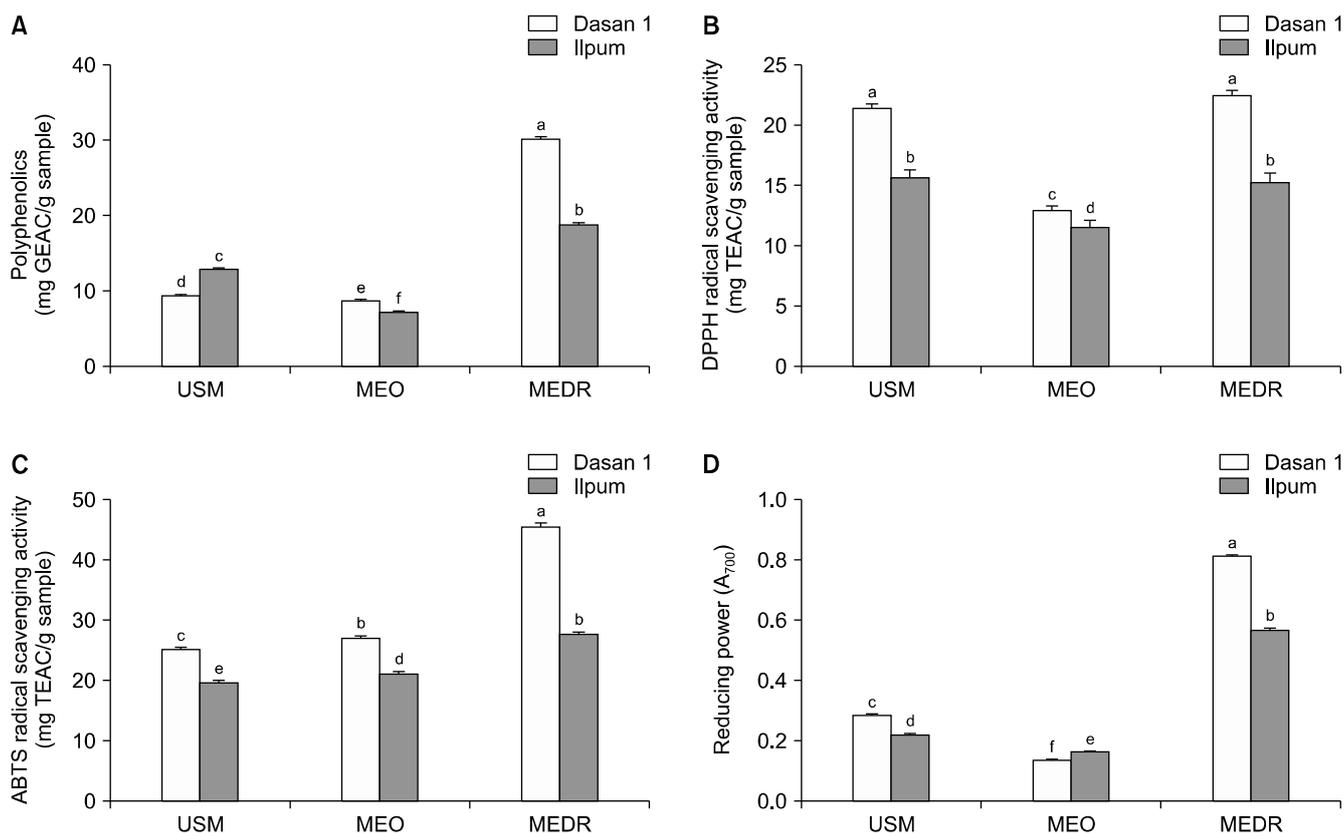
Fig. 1. Polyphenolic content (A), DPPH radical scavenging activity (B), ABTS radical scavenging activity (C), and reducing power (D) in functional extracts from two different rice cultivars. The vertical bars represent the mean±SE (n=3). Different letters (a-f) denote significant differences ($P<0.05$). GEAC, gallic acid equivalent antioxidant capacity; TEAC, Trolox equivalent antioxidant capacity. USM, unsaponifiable matter of the rice bran; MEO, methanolic extract of the rice bran oil; MEDR, methanolic extract of the defatted rice bran.

vent to extract phenolic compounds among various solvents (Haminiuk et al., 2014). Moreover, a significant correlation between radical scavenging capacity and phenolic content has been widely reported in previous studies (Kılıçgün and Altıner, 2010; Basu and Maier, 2016). These results indicate that the high polarity of phenolics in MEDR increased its antioxidant capacity compared to USM and MEO.

Cytoprotective effects

The protective effect of the functional extracts on hepatocytes was examined in HepG2 cells using the LDH assay. None of the tested samples exhibited cytotoxicity (Fig. 2A). The cytoprotective effects of functional extracts against TBHP-induced oxidative stress were evaluated (Fig. 2B). The cell viability of TBHP-treated cells was reduced by approximately 50%. However, the pretreatment of the cells with functional extracts at a concentration of 100 µg/mL increased their viability. The treatment with USM from the Dasan 1 cultivar showed highest cell viability. Various phytochemicals have been found to provide cells with protection against oxidative damage. In a previous study, tocotrienols from grape seeds were found to increase the cell viability of HepG2 cells against oxidative damage via the modulation of antioxidant enzyme

activity (Choi et al., 2010). Moreover, procyanidins extracted from cocoa have been found to protect PC12 cells against oxidative stress (Cho et al., 2008). In particular, a previous study reported that β-sitosterol induced the cytoprotective action via activation of the Nrf2/ARE signaling pathway (Kang et al., 2008; Zhang et al., 2015). Therefore, the excellent cytoprotective properties of USM may be associated with its much higher content of phytosterols compared to other extracts. The present study showed that USM, MEO, and MEDR from two different rice cultivars had a cytoprotective effect on HepG2 cells, where USM provided the most effective protection against TBHP-induced oxidative insult, regardless of the cultivars.

Intracellular antioxidative effects

Next, we measured the production of intracellular ROS to investigate whether functional extracts ameliorate TBHP-induced oxidative stress in hepatocytes. Treatment with all of the USM and the MEO inhibited ROS generation compared with TBHP alone treatment (Fig. 3A). Moreover, pretreatment with USM of Dasan 1 was the most effective for the inhibition of ROS generation. Generally, excessive ROS levels are harmful and can cause various degenerative diseases such as cancer, neurodegenerative,
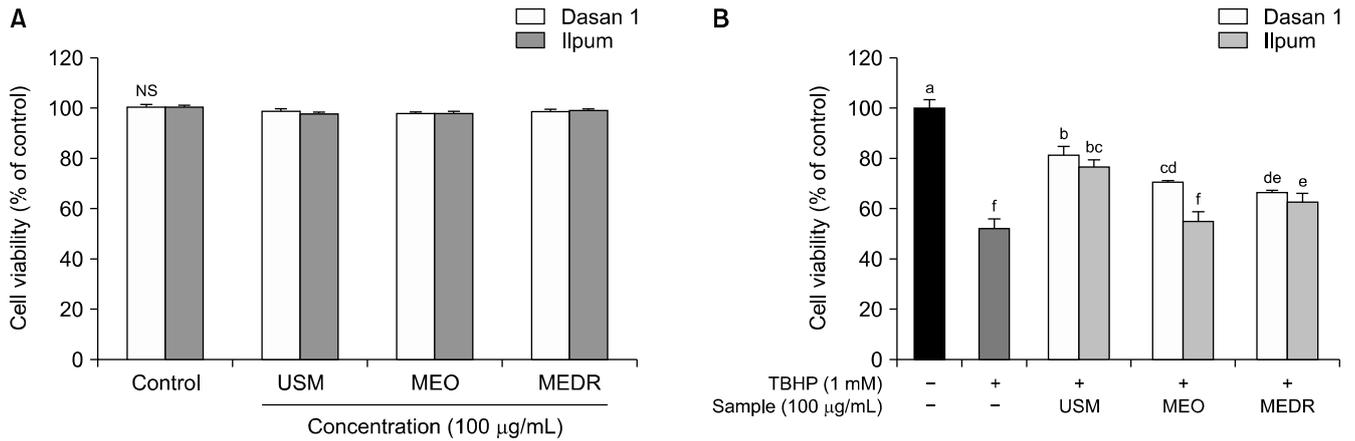
Fig. 2. Effects of functional rice bran extracts on cytotoxicity (A) and cytoprotection (B) in HepG2 cells. Oxidative stress was induced by *tert*-butyl hydroperoxide (TBHP). The vertical bars represent the mean±SE (n=3). Different letters (a-f) denote significant differences ($P<0.05$). NS, not significant. USM, unsaponifiable matter of the rice bran; MEO, methanolic extract of the rice bran oil; MEDR, methanolic extract of the defatted rice bran.
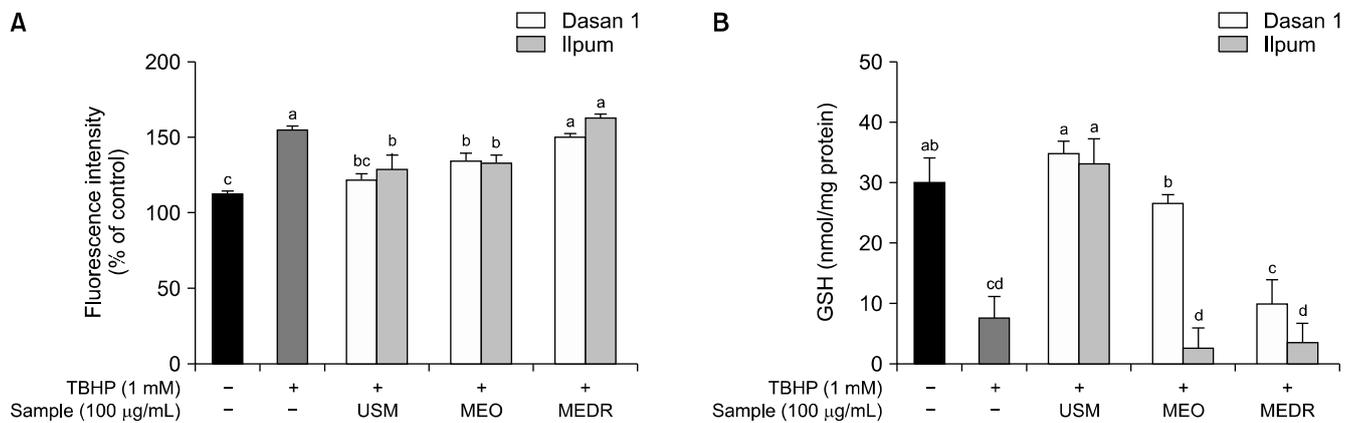

Fig. 3. Effect of functional rice bran extracts on the formation of intracellular reactive oxygen species (A) and glutathione production (B) induced by *tert*-butyl hydroperoxide (TBHP). The vertical bars represent the mean±SE (n=3). Different letters (a-d) denote significant differences ($P<0.05$). USM, unsaponifiable matter of the rice bran; MEO, methanolic extract of the rice bran oil; MEDR, methanolic extract of the defatted rice bran.

and cardiovascular disease (Halliwell et al., 1992; Termini, 2000). On the other hand, various phytochemicals have been reported to exert cytoprotective effects against oxidative insult by reducing ROS formation and scavenging free radicals (Moskaug et al., 2005; Choi et al., 2010). Our results suggest that the generation of intracellular ROS is reduced by the action of phytochemicals in the functional extracts of rice brans.

The intracellular levels of GSH were examined to elucidate the cytoprotective action. Compared to the control group, TBHP significantly decreased the GSH content (Fig. 3B). However, pretreatment with USM in both Dasan1 and Ilpum for 6 h increased intracellular GSH levels. Remarkably, USM recovered GSH depletion to the level of the control. GSH is synthesized from cysteine, glutamate, and glycine. GSH plays a major role in the detoxification of various chemical substances conjugated by the catalytic action of glutathione *S*-transferases (Choi et al., 2010). Moreover, GSH is an important endogenous

antioxidant against oxidative stress (Kwon et al., 2019). In a previous study, flavonoids were found to elevate the glutathione levels by the transactivation of the catalytic subunit promoter of γ-glutamyl cysteine synthetase (Myhrstad et al., 2002). In addition, rice bran USM restored the GSH depletion and ROS production back to the control level against oxidative stress (Ham et al., 2015). As such, the cytoprotective effects of the functional extracts from rice brans against oxidative stress appear to be mainly attributed to phytochemicals, which prevent the depletion of GSH and production of ROS. This suggests that the high cytoprotective effects provided by USM against oxidative stress can be attributed its high levels of phytosterols, including tocols, phytosterols, and squalene.

In conclusion, this study evaluated the phytochemical content and the antioxidant activity of functional rice bran extracts from *indica* and *japonica* rice cultivars, Dasan 1 and Ilpum, respectively. The highest vitamin E and

phytosterol content were found in the USM of the *indica* rice (Dasan 1). The highest total polyphenol content, reducing power, and radical scavenging capacity were observed in the MEDR of Dasan 1. Pretreatment with functional extracts from Dasan1 and Ilpum provided the cytoprotection against oxidative damage by decreasing the levels of ROS production and GSH depletion. In particular, intracellular ROS and GSH depletion was greatly reduced by USM, regardless of the cultivar used. Taken together, these results demonstrate that USM of *japonica* rice and *indica* rice contains considerable amounts of phytochemicals and showed the cytoprotective properties against oxidative stress. Our findings indicate that the USM from rice bran represents a potential natural source of antioxidants and a hepatic cytoprotective material.

ACKNOWLEDGEMENTS

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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