Kim *et al. Applied Biological Chemistry* (2024) 67:19
https://doi.org/10.1186/s13765-024-00872-z

KSABC
The Korean Society for Applied Biological Chemistry

ARTICLE

Open Access

Antioxidant and anti-inflammatory activities of the methanol extract from the bran of the colored wheat, 'Ariheuk'

Seung-Gyeom Kim[1†], So-Hyeon Park[1†] and Joong-Hyuck Auh[1*]

Abstract

In vitro antioxidant and anti-inflammatory activities were investigated using a 70% acidic methanol extract of the colored wheat bran, 'Ariheuk.' Active metabolites were identified via metabolomic analysis using multivariate statistical comparisons. The 'Ariheuk' bran extract (ABE) contained a higher total anthocyanin content (0.19 mg C3G/g) than the general wheat bran extract (GBE) (0.01 mg C3G/g). ABE exhibited stronger antioxidant and anti-inflammatory activities than GBE. The mechanism underlying the anti-inflammatory effects of ABE was explored by assessing the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) in RAW 264.7 cells stimulated with lipopolysaccharide (LPS). The crude ABE extract was also partially fractionated into three subfractions (ABE-F1, ABE-F2, and ABE-F3) using preparative liquid chromatography (Prep-LC) to identify the active metabolites. The total anthocyanin content was highest in ABE-F3 (1.91 ± 0.06 mg C3G/g). Among the subfractions, ABE-F2 exhibited the highest antioxidant and anti-inflammatory activities. Several distinct metabolites contributing to the activities of ABE-F2 were identified, including various cyanidin and peonidin derivatives and apigenin derivatives, such as corymboside and schaftoside.

Keywords Antioxidant, Anti-inflammatory, Colored wheat, Ariheuk bran

Introduction

Wheat (*Triticum aestivum* L.) is one of the three principal cereal grains in the world, along with rice and corn. Approximately 775 million tons of wheat is produced worldwide annually [1]. Wheat consumption is expected to increase by 12% by2030 compared to the base period (2018–2020) [2]. Moreover, different types of wheat flour, in which wheat bran (WB) is removed, serve as common raw materials for producing food products, such as noodles, biscuits, and bread [3]. WB is a byproduct of conventionally milled wheat. Previously, WB was primarily used as livestock feed due to its affordability, high amino acid content, and high protein ratio [3]. However, a growing interest exists in the potential health benefits of incorporating whole wheat (WW) foods containing WB. WW foods contain ingredients derived from physiological activities, such as dietary fiber and phytochemicals [4]. Additionally, according to recent studies, WB provides health benefits independent of other components when adjusting wheat intake and its constituents, including bran, germ, and endosperm [5]. Accumulating studies also focused on the physiological activities of WB, including its antidiabetic [6], anti-inflammatory [7], and antioxidant [8] properties.

In Korea, wheat is not the most consumed cereal grain. In fact, the annual consumption of wheat per capita is 32.4 kg, the second highest after rice [9]. Although wheat consumption is increasing due to changes in eating habits, such as a preference for diversified food choices and

†Seung-Gyeom Kim and So-Hyeon Park contribute to this work equally.

*Correspondence:
Joong-Hyuck Auh
jhauh@cau.ac.kr
[1] Department of Food Science and Biotechnology, Chung-Ang University, Anseong 17546, Republic of Korea

westernization, the self-sufficiency rate of wheat is only 1.7%. Consequently, most wheat production relies on imports [9]. As wheat production is markedly influenced by the international cereal grain market, which includes unpredictable weather phenomena and sudden increases in the demand for feed grains, the stability of the supply and demand of cereal grains must be addressed by enhancing food security and increasing self-sufficiency.

The Rural Development Administration (RDA) of Korea has successfully developed a purple wheat cultivar named 'Ariheuk' through artificial crossbreeding between native black wheat and 'Shinmichal.' This development was undertaken to enhance the quality competitiveness of domestic wheat [10]. Compared to traditional wheat varieties, 'Ariheuk' contains a higher amount of anthocyanins and is rich in minerals, such as vitamins, calcium, iron, and zinc [10]. The primary anthocyanins found in 'Ariheuk' are cyanidin-3-O-glucoside (C3G) and peonidin-3-O-glucoside (P3G) [10].

The physiological benefits of colored wheat, which is rich in bioactive compounds, such as anthocyanins, carotenoids, and other phytochemicals, have garnered global attention [11]. The qualitative and quantitative abundances of anthocyanins in colored wheat play a critical role in determining the functionality and health benefits of food [12]. Purple wheat, which contains significantly higher levels of anthocyanins than other colored wheat varieties, exhibits remarkable antioxidant activity [13]. Additionally, the anti-inflammatory activity of purple wheat has been demonstrated [11]. Colored wheat provides numerous health benefits and has been demonstrated to assist in the prevention of various chronic diseases, including cardiovascular diseases, diabetes, and inflammation [11, 13]. Therefore, 'Ariheuk' is presumed to have both physiological and functional activation effects. However, studies on 'Ariheuk' are currently limited and insufficient to draw definitive conclusions.

In this study, methanol extraction was carried out using wheat bran from general wheat (WBG), a commonly cultivated wheat variety in Korea, and 'Ariheuk' (WBA), a newly developed variety. The bran extracts were then fractionated into 'Ariheuk' bran extract (ABE) and general bran extract (GBE) using preparative liquid chromatography (Prep-LC), and their physiological activities, including antioxidant and anti-inflammatory properties, were compared. The key metabolites contributing to the physiological activity of ABE were screened and identified using metabolomic analyses.

Results

Extraction yields and total anthocyanin content (TAC)

ABE and GBE were separated into three subfractions based on their UV chromatograms. Subfraction 1 (F1) was collected within the first 8 min, whereas subfraction 2 (F2) was collected within 16 min and subfraction 3 (F3) was collected after 25 min of elution. The hydrophilic compounds were collected in the first subfractions (ABE-F1 and GBE-F1) and were colorless. The second subfraction was bright brown (ABE-F2) and yellow (GBE-F2). The third subfraction of each extract (ABE-F3 and GBE-F3) had a darker color than the corresponding second fractions. The extraction yields are described as percentages (%) of dry weight. As a result, the extraction yield of ABE was 12.4 ± 0.23%, whereas those of its subfractions were 62.75 ± 3.68% (ABE-F1), 2.79 ± 0.50% (ABE-F2), and 1.36 ± 0.13% (ABE-F3). Similarly, the extraction yield of GBE was 11.4 ± 0.06%, whereas those of its subfractions were 56.06 ± 1.71% (GBE-F1), 7.27 ± 0.64% (GBE-F2), and 3.16 ± 0.27% (GBE-F3), respectively.

The TACs of ABE, GBE, and all subfractions are summarized in Table 1 and are expressed as C3G equivalents. The TAC of ABE (0.19 mg C3G/g) was significantly higher than that of GBE (0.01 mg C3G/g). All subfractions of ABE had a higher TAC than GBE, with ABE-F3 exhibiting the highest value, followed by ABE-F2 and ABE-F1.

Antioxidant activity of the extracts

To assess the antioxidant activity, the DPPH free radical scavenging activity and ORAC values were compared (Table 1). ABE exhibited significantly higher DPPH free radical scavenging activity than GBE, with lower IC50 and higher ORAC values. The antioxidant activities of the subfractions were also evaluated. Although the anthocyanin content of ABE-F2 and ABE-F3 was significantly higher than that of the corresponding GBE subfractions, no significant differences in the IC50 values were found among the four subfractions (ABE-F2, ABE-F3, GBE-F2,

Table 1 Total anthocyanin content and antioxidant activity (DPPH and ORAC) of ABE, GBE and all subfractions

Samples	Total anthocyanin content (mg C3G/g)	Antioxidant activity	
		DPPH IC50 (mg/mL)	ORAC (μM TE/g)
ABE	0.19 ± 0.01^{c}	0.97 ± 0.12$^{c'}$	1038.45 ± 89.53$^{a''}$
GBE	0.01 ± 0.01^{e}	1.31 ± 0.07$^{bc'}$	821.70 ± 58.99$^{b''}$
ABE-F1	0.07 ± 0.01^{d}	1.46 ± 0.09$^{b'}$	5206.86 ± 168.74$^{d''}$
ABE-F2	1.48 ± 0.01^{b}	0.12 ± 0.00$^{d'}$	33021.61 ± 482.67$^{h''}$
ABE-F3	1.91 ± 0.06^{a}	0.13 ± 0.00$^{d'}$	27017.94 ± 757.70$^{g''}$
GBE-F1	0.01 ± 0.02^{e}	4.24 ± 0.59$^{a'}$	4656.57 ± 189.93$^{c''}$
GBE-F2	0.03 ± 0.03^{de}	0.44 ± 0.09$^{d'}$	30250.85 ± 591.07$^{e''}$
GBE-F3	Not Detected	0.21 ± 0.03$^{d'}$	24391.09 ± 743.31$^{f''}$

All results are expressed as mean ± SD. Different letters indicate a statistically significant difference ($p < 0.05$)

and GBE-F3). Overall, ABE-F2 exhibited the highest antioxidant activity, followed by GBE-F2. ABE-F3, which had the highest TAC, had a lower ORAC value than ABE-F2 and GBE-F2. Based on these results, antioxidant substances common to ABE and GBE might be present in F2.

Cell cytotoxicity of the extracts on RAW 264.7 cells

The MTT assay was performed to assess the cytotoxicity of the extracts on RAW 264.7 cells at various concentrations. Cell viability above 80% was considered non-cytotoxic. The appropriate concentrations of ABE, GBE, and all subfractions were determined. When 100, 200, 400, and 600 μg/mL of ABE and GBE were employed, the cell viabilities exceeded 95%, indicating no significant cytotoxicity at any concentration. Similarly, when each subfraction of ABE and GBE was screened at concentrations of 25, 50, and 100 μg/mL, cell viability ranged from 79.7 ± 2.95% to 100.9 ± 7.18%, indicating no significant cytotoxicity at the tested concentrations. Subsequent experiments were conducted with 200, 400, and 600 μg/mL of ABE and GBE, and 25, 50, and 100 μg/mL of the subfractions.

Effect of the extracts on intracellular reactive oxygen species (ROS) levels

Intracellular ROS levels in LPS-induced RAW 264.7 cells were quantified using CM-H2DCFDA as an indicator. As summarized in Fig. 1, most extracts (ABE, ABE-F2, ABE-F3, GBE, GBE-F2, and GBE-F3) significantly suppressed ROS generation in a concentration-dependent manner compared with LPS-stimulated cells. When 600 μg/mL was administered, ABE reduced ROS levels

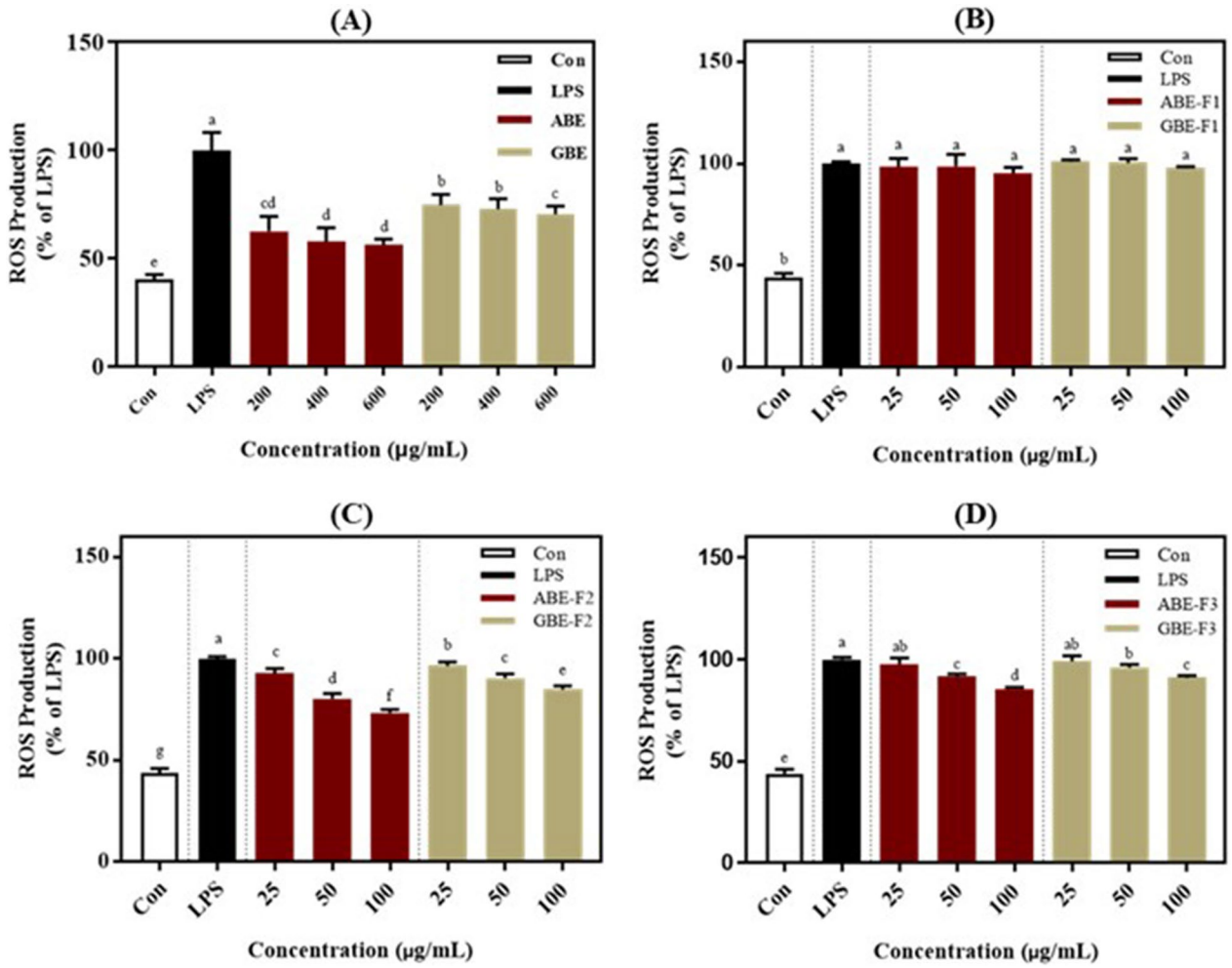

Fig. 1 Effect of ABE, GBE, and all subfractions on LPS-induced ROS production in RAW 264.7 cells. **A** ABE and GBE, **B** ABE-F1 and GBE-F1, **C** ABE-F2 and GBE-F2, and **D** ABE-F3 and GBE-F3. Intercellular ROS levels are expressed as a percentage of LPS-stimulated cells. Different letters indicate significant difference ($p < 0.05$)

to 56.3 ± 2.68% whereas GBE decreased these levels to 70.4 ± 3.73%. Among the subfractions, 100 μg/mL of ABE-F2 and GBE-F2 substantially suppressed ROS generation to 26.61 ± 0.84% and 15.20 ± 0.94%, respectively. ABE and its subfractions (ABE-F2 and ABE-F3) exhibited a stronger suppression of ROS generation than GBE and its subfractions (GBE-F2 and GBE-F3) at all concentrations. Notably, the F2 subfraction demonstrated the most potent suppression of ROS generation among all subfractions.

Anti-inflammatory activity of the extracts

To evaluate the anti-inflammatory activity of the extracts, nitric oxide (NO) generation and the expression of key biomarkers (iNOS and COX-2) were compared with LPS-induced RAW 264.7 cells; the results are summarized in Figs. 2 and 3. ABE, GBE, ABE-F2, ABE-F3, GBE-F2, and GBE-F3 significantly suppressed NO production in a concentration-dependent manner. In fact, 600 μg/mL of ABE inhibited NO production by 54.6% whereas the same concentration of GBE inhibited NO production by 40.5%. ABE-F2 (100 μg/mL) and GBE-F2 (100 μg/mL) inhibited NO production by 14.5% and 9.0%, respectively, compared to that of the LPS-stimulated group. The inhibition of NO production by F2 was stronger than that by F1 and F3 in both extract groups, which aligned with the antioxidant activity results. iNOS and COX-2 are key factors in the inflammatory pathway owing to their involvement in the induction and elevation of pro-inflammatory mediators, including NO and prostaglandin E2 (PGE2). The effects of ABE and GBE on iNOS and COX-2 expression were investigated in LPS-induced RAW 264.7 cells (Fig. 3). ABE and GBE significantly reduced iNOS and COX-2 expression in a concentration-dependent manner.

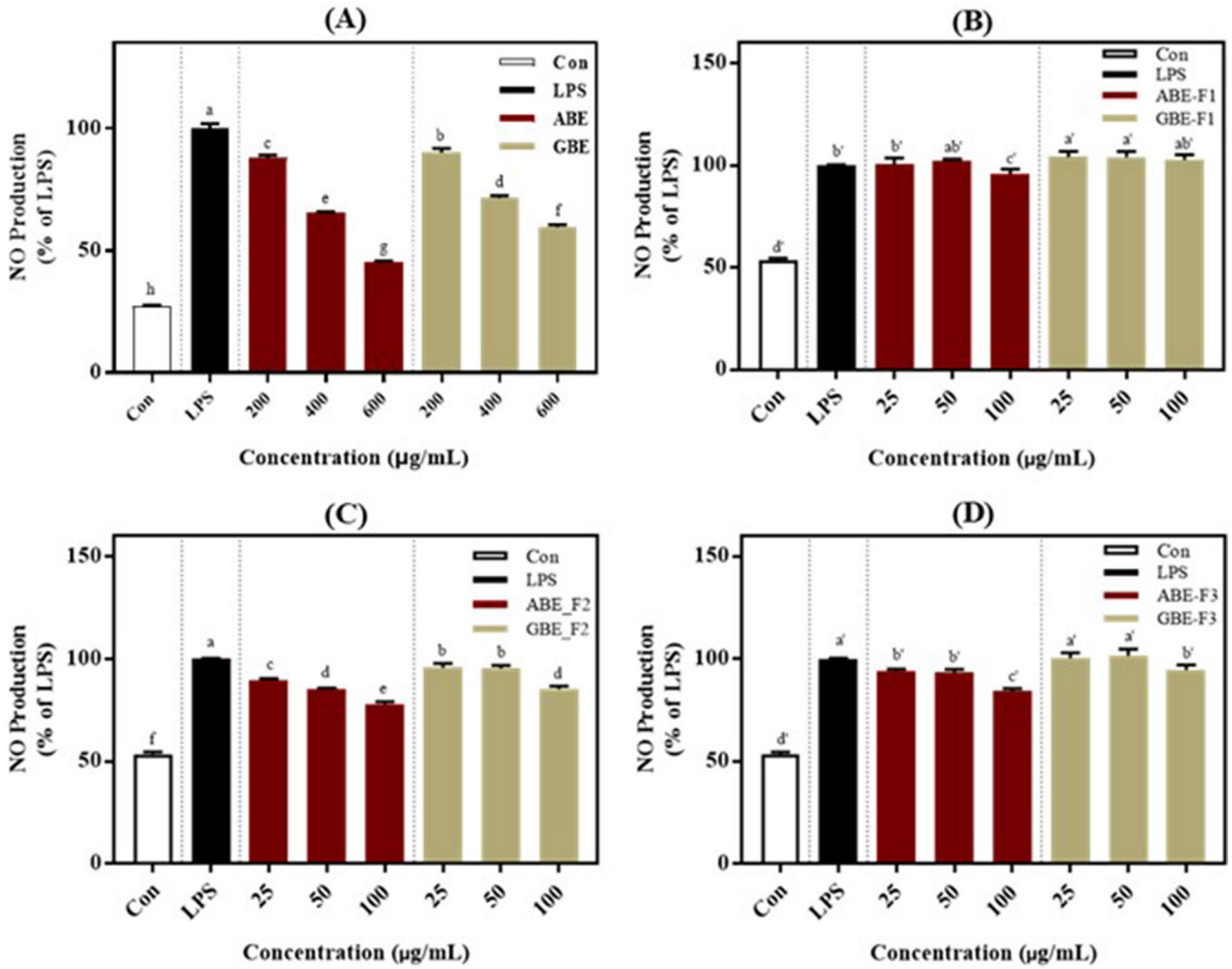

Fig. 2 Effect of ABE, GBE, and all subfractions on LPS-induced NO production in RAW 264.7 cells. **A** ABE and GBE, **B** ABE-F1 and GBE-F1, **C** ABE-F2 and GBE-F2, and **D** ABE-F3 and GBE-F3. All results are expressed as a percentage of LPS-stimulated cells. Different letters indicate significant difference ($p < 0.05$)

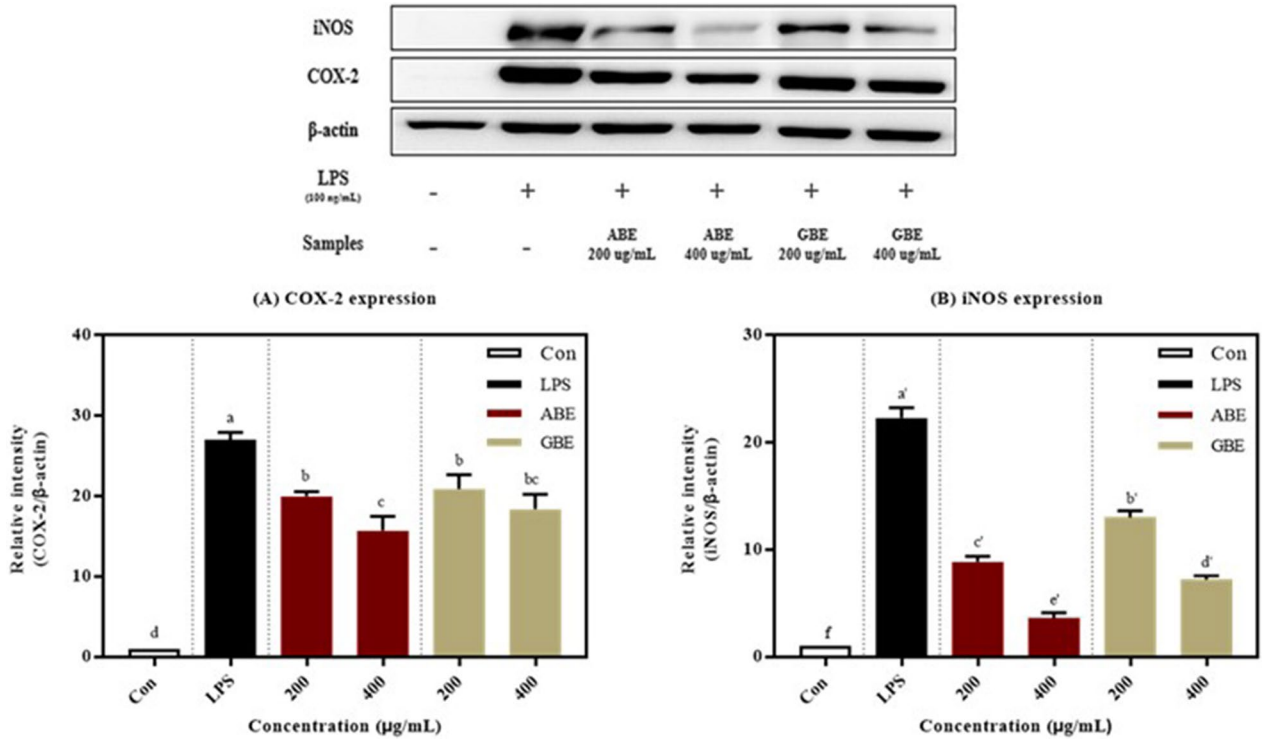

Fig. 3 Effect of ABE, GBE, and all subfractions on LPS-induced **A** COX-2 and **B** iNOS protein expression in RAW 264.7 cells. Different letters indicate significant difference ($p < 0.05$)

The expression of iNOS in cells treated with 400 μg/mL of ABE and GBE was inhibited by 83.9% and 67.7%, respectively. COX-2 expression was inhibited by 41.8% and 22.6%, respectively, following treatment with 400 μg/mL of ABE and GBE. Based on the assessed biomarkers, the relative anti-inflammatory activity of ABE was higher than that of GBE, indicating a significant contribution of the metabolites in each extract.

Metabolomic analysis using multivariate statistical methods for the identification of the active metabolites in the subfractions

To further identify the active metabolites with anti-inflammatory activity in subfractions (ABE and GBE), LC-mass spectrometry (MS) analysis was performed. The distinctive metabolites in each subfraction were separated using multivariate statistical analysis, and the key compounds included in the active subfraction F2 were screened and identified (Figs. 4 and 5). The principal component analysis (PCA) visually enabled the comparison of metabolomic variations between ABEs and GBEs, as depicted in Fig. 4. Irrespective of the sources of wheat brans, the fractions with the same hydrophobicity from prep-LC were consistently grouped into similar categories. PCA also revealed a metabolomic variation in ABEs, which can explain 92.6% of the variance in principal components 1 and 2 (Fig. 4). ABE-F2, the active fraction, was distinguished from ABE-F1 and ABE-F3 by principal components 1 (PC1, 73.0%) and principal component 2 (PC2, 19.6%). Most of the metabolomic variation in GBE (94.3%) was clearly explained by principal components 1 and 2 (Fig. 4). The metabolomic features of GBE-F2 relative to the other GBE subfractions were characterized by principal component 1 (PC1, 79.8%) and principal component 2 (PC2, 14.5%). The S-plot derived from the orthogonal partial least squared-discriminant analysis (OPLS-DA) revealed the key metabolites contributing to the anti-inflammatory effects of ABE-F2 and GBE-F2 (Fig. 5). The distinct metabolites in each fraction (Variable importance for the projection (VIP) value ≥ 1.5, $p < 0.05$) were successfully screened and identified. In the ABE-F2 fraction, ten metabolites were selected as significant contributors and are listed in Table 2. Corymboside (*m/z* 565), schaftoside (*m/z* 565), peonidin-3-*O*-malonylglucoside (*m/z* 549), and seven other compounds were identified as the key metabolites of ABE-F2. Similarly, the significant metabolites in GBE-F2 included DL-tryptophan (*m/z* 205), L-phenylalanine (*m/z* 166), corymboside (*m/z* 565), and 14 other compounds (Table 2). The corymbosides and schaftosides found in all F2 samples were apigenin glycosides commonly found in wheat. Apigenin glycosides exhibit anticancer, antioxidant, and

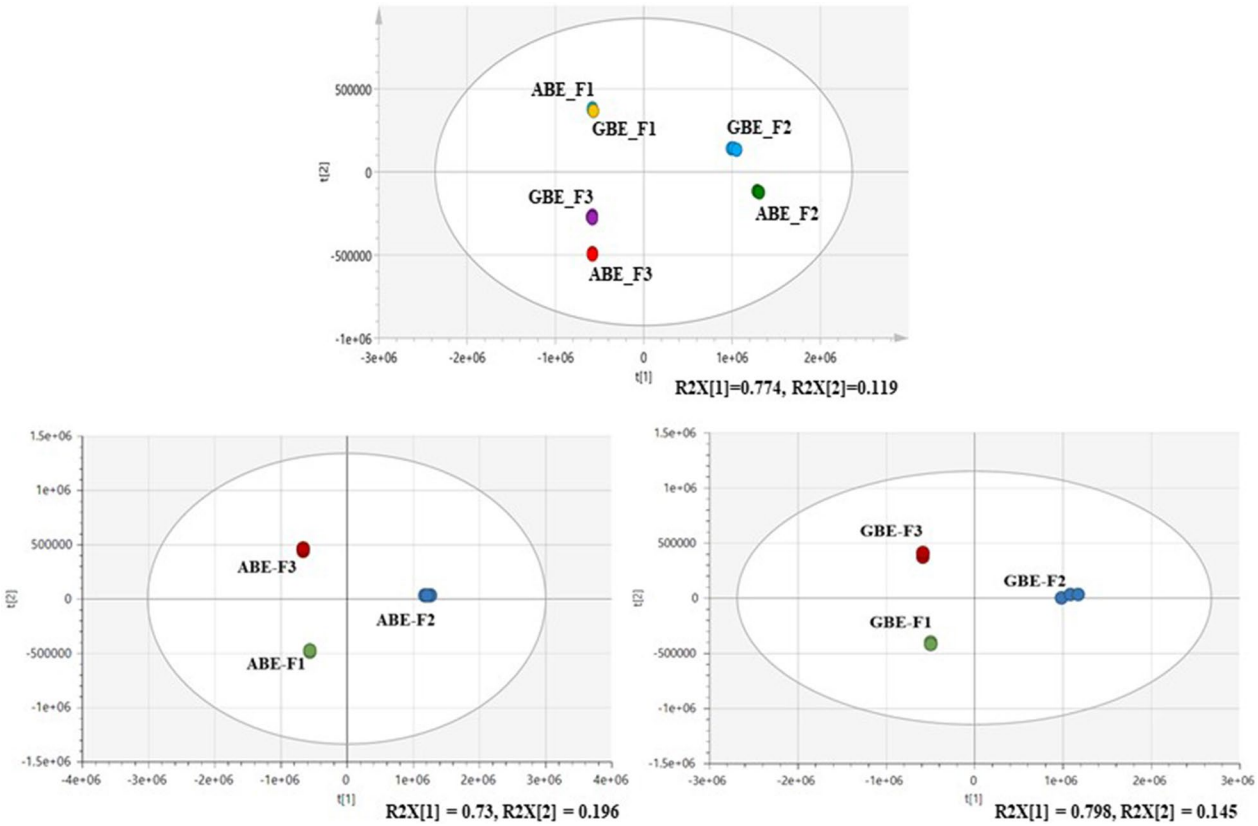

Fig. 4 Principal component analysis (PCA) score plots of the metabolomes in the ABE and GBE subfractions

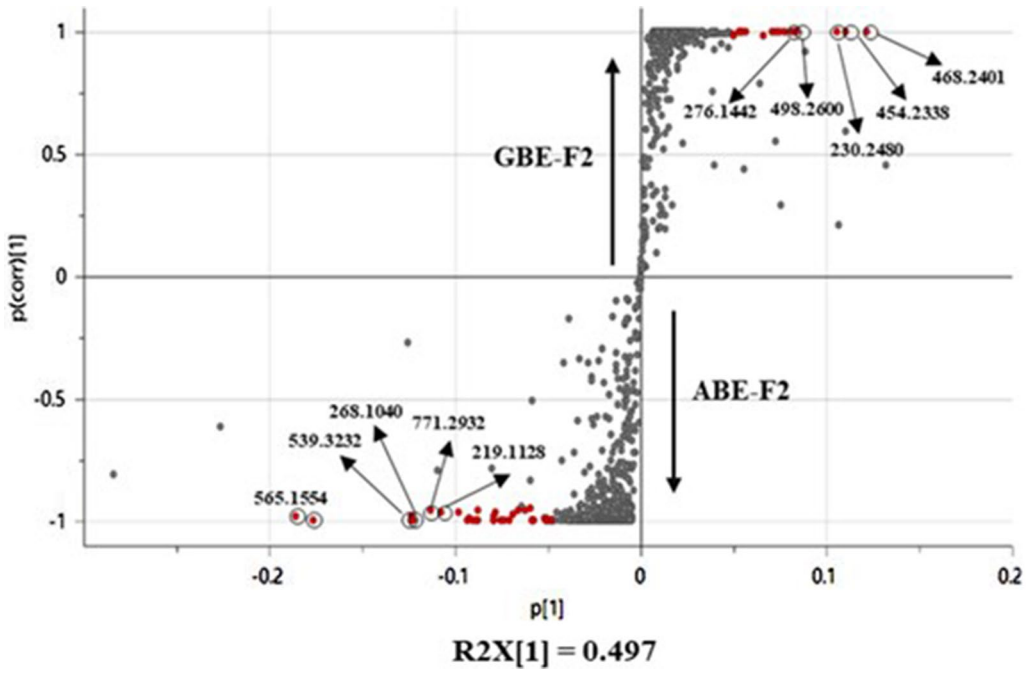

Fig. 5 The S-plot derived from OPLS-DA for the metabolomes in ABE-F2 and GBE-F2

Table 2 Tentative identification of distinctive metabolites in active subfractions of ABE-F2 and GBE-F2

m/z	RT (min)	Subfractions	Tentative identification	MS/MS fragments	Error (ppm)
565.1554	14.06	ABE-F2	Corymboside	391.0809, 295.0598, 325.0705	0.39
565.1557	13.46		Schaftoside	379.0811, 325.0703, 295.0597	0.92
595.1662	14.69		Apigenin-6,8-di-C-glucoside	409.0914, 355.0811, 325.0700	0.76
268.1042	0.86		Adenosine	136.0619, 137.0657, 268.1038	0.63
144.0809	6.56		6-Methylquinoline	144.0808, 145.0842, 143.0731	0.86
160.0757	11.81		6-Methoxyquinoline	160.0757, 145.0522, 118.0653	0.06
206.0812	11.77		Indole-3-lactic acid	118.0653, 130.0652, 146.0600	0.15
136.062	0.86		Adenine	136.0618, 91.0546, 137.0	1.68
229.1548	0.86		Prolylleucine	229.1545, 142.0862, 70.0657	0.57
549.1245	15.35		Peonidin-3-O-malonylglucoside	301.0705, 286.0471, 302.0738	1.09
463.1238	18.75		Peonidin-derivatives	301.0704, 286.0470, 302.0739	-1.70
188.0708	2.61	GBE-F2	trans-3-Indoleacrylic acid	146.0600, 118.0653, 144.0808	1.04
205.0972	2.61		DL-Tryptophan	146.0601, 188.0707, 118.0654	0.22
146.0601	2.61		Indole-4-carbaldehyde	118.0653, 146.0600, 144.0808	0.41
166.0864	1.72		L-Phenylalanine	120.0809, 121.0844, 103.0545	0.87
144.0809	2.61		6-Methylquinoline	144.0808, 145.0842, 117.0701	0.87
118.0654	2.60		Indole	118.0654, 91.0547, 119.0686	2.32
217.0973	6.78		2,3,4,9-Tetrahydro-1H-β-carboline-3-carboxylic acid	144.0808, 145.0842, 74.0242	0.67
229.1548	0.87		Prolylleucine	229.1546, 142.0863, 70.0657	0.57
268.1041	0.87		Adenosine	136.0617, 137.0665, 268.1042	0.26
565.1556	13.50		Corymboside	379.0807, 325.0702, 349.0703	0.74
132.081	2.96		6-Methylindole	132.0809, 117.0575, 133.0844	1.70
146.0602	4.51		8-Hydroxyquinoline	146.0600, 118.0653, 147.06355	1.10
182.0814	1.16		L-Tyrosine	136.0757, 123.0442, 119.0493	1.26
160.0759	11.83		6-Methoxyquinoline	160.0757, 145.0524, 132.0808	1.31
152.0568	1.17		Guanine	152.0567, 153.0407, 135.0301	0.75
136.062	0.87		Adenine	136.0618, 137.0458, 91.0547	1.68

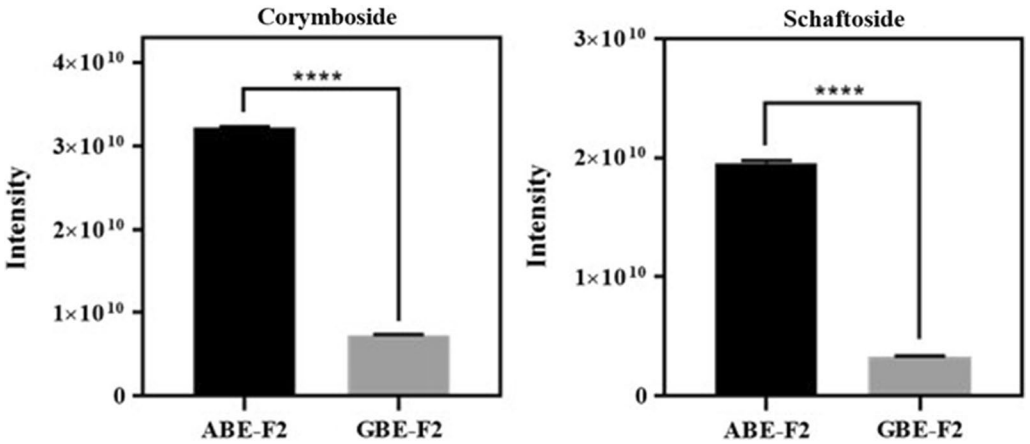

Fig. 6 Relative abundance of corymboside and schaftoside in ABE-F2 and GBE-F2. Significant differences between the second subfraction (F2) of ABE and GBE are depicted by **** ($p < 0.0001$)

anti-inflammatory properties. In this study, ABE contained higher levels of corymboside and schaftoside than GBE (Fig. 6).

Discussion

Colored wheat is a significant ingredient for gaining enhanced nutritional properties relative to normal wheat [14]. Liu et al. [13] compared the antioxidant activities of six different colored wheat varieties and correlated these activities with the profiles of phenolic compounds. They found that anthocyanins were the primary contributors to the antioxidant activity among the various classes of phenolic compounds, particularly in purple wheat varieties. Abdel-Aal et al. [15] also reported that the compositional and antioxidant properties of purple wheat bran, in which the anthocyanins were highly localized, are closely related to antioxidant activity. They also identified cyanidin-3-glucoside as the predominant anthocyanin in the purple wheat variety. The anthocyanin pigments in wheat are primarily situated in the outer layers of the kernel, specifically in the aleurone or pericarp layers. Therefore, it is both economically and technologically feasible to concentrate anthocyanins in the bran fraction as a source of antioxidants.

This study aimed to confirm whether WBA, a colored wheat cultivar developed in Korea, can be used as a nutraceutical source with antioxidant and anti-inflammatory properties. To identify the active components, ABE and GBE were analyzed by dividing each extract into three subfractions. ABE displayed higher TAC than GBE and the subfractions of the normal wheat cultivar. Anthocyanins, which are primarily found in the bran of cereal grains, are water-soluble pigments responsible for red, purple, and blue colors in nature and possess antioxidant and anti-inflammatory activities [14, 16]. Therefore, ABE could be expected to have higher antioxidant and anti-inflammatory activities than GBE. According to a previous study, approximately 69% of the free radical scavenging capacity of blue wheat is attributed to its anthocyanin content, with 19% attributed to phenolic acids [14]. Thus, the ABE subfractions exhibited low IC50 and high ORAC values, significantly influencing their antioxidant activity due to their high anthocyanin content. Oxidative stress and inflammatory reactions are closely linked. Oxidative agents, such as ROS and reactive nitrogen species, are produced in large quantities during every inflammatory response [17]. Therefore, compounds with high antioxidant activity can inhibit inflammation [18]. This result is supported by those of our study, which revealed that ABE and its subfractions exhibited anti-inflammatory activity by inhibiting the production of ROS and NO. During the inflammatory process, significant quantities of pro-inflammatory mediators, such as NO and PGE2, are generated by iNOS and COX-2 [19]. Our study also showed that the anti-inflammatory mechanism of ABE is related to the regulation of iNOS and COX-2 protein expression, which produce substances that mediate inflammatory responses.

The TAC of ABE was 0.19 mg C3G/g, whereas that of GBE was negligible. Nevertheless, both extracts exhibited antioxidant and anti-inflammatory activities, inhibiting LPS-induced NO and ROS levels in RAW 264.7 cells without causing cytotoxicity. The inhibitory effects of ABE and GBE on pro-inflammatory mediators (NO and ROS) were likely associated with modulation of the expression of the inflammatory biomarkers, iNOS and COX-2. ABE and GBE were divided into three subfractions, with ABE-F3 showing the highest TAC (1.96 mg C3G/g), followed by ABE-F2 (1.48 mg C3G/g). Through LC–MS/MS analysis and

multivariate statistical analysis, several anthocyanins were identified in ABE: peonidin-(6-coumaroyl)-3-O-glucoside, peonidin-3-O-glucoside, peonidin-3-O-malonylglucoside, cyanidin-3-O-rutinoside, delphinidin-3-O-rutinoside, cyanidin-3-O-(6″-malonyl-glucoside), and peonidin-3-O-glucoside. Effective inhibition of ROS and NO production was observed with F2 of ABE and GBE, respectively. Additionally, corymboside, an apigenin glycoside, was identified in the F2 subfractions of ABE and GBE. The observed antioxidant and anti-inflammatory effects were assumed to be the result of a synergistic interaction between anthocyanins and various polyphenolic compounds, such as corymboside and schaftoside. Corymboside and schaftoside, known to be found in wheat, are apigenin glycosides with antioxidant and anti-inflammatory activity [20, 21]. Schaftoside is known to effectively suppress mitochondrial fission through a mechanism associated with the TLR4/Myd88 signaling pathway [20]. In this study, ABE contained significantly higher levels of corymboside and schaftoside than GBE (Fig. 6), and the presence of anthocyanins in ABE may enhance its physiological activity. Thus, the physiological activity of ABE may be due to the synergistic effects of anthocyanins and apigenin glycosides.

Methods

Chemicals and reagents

2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Alfa Aesar (Ward Hill, MA, USA). 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Dulbecco's modified Eagle's medium (DMEM) with high-glucose and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Biowest (Rue de la Caille, France) for cell culture. Fetal bovine serum (FBS), N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES), and penicillin–streptomycin (P/S) were obtained from Gibco (N.Y., USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CM-H_2DCFDA was purchased from Invitrogen (Carlsbad, CA, USA). Primary antibodies for β-actin and COX-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and inducible nitric oxide synthase (iNOS) was purchased from Invitrogen (Carlsbad, CA, USA). Secondary antibodies, including anti-mouse IgG-HRP and anti-rabbit IgG-HRP), were purchased from Cell Signaling Technology (Beverly, MA, USA), and anti-goat IgG-HRP was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Guaranteed reagent (GR)-grade methanol for Prep-LC and Extra pure (EP)-grade hexane were purchased from Duksan (Ansan, Korea). Mass spectrometry- (MS)-grade water, acetonitrile, and formic acid were purchased from Thermo Fisher Scientific (Waltham, MA, USA) for LC–MS/MS analysis.

Sample preparation

WBA was kindly donated by the RDA of Korea, and WBG was purchased from a local market in Korea. Each type of WB was milled (UNB-A9001; Ningbo, China) to facilitate efficient defatting, which was achieved by stirring in hexane for 2 h. In particular, 750 mL of hexane was added to 50 g of each sample (at a ratio of 1:25). The defatted samples were then dried in a fume hood. For the extraction process, 5 g of the dried sample was extracted with 100 mL of 70% acidic methanol (0.1% HCl) via bath sonication (B3510-DTH, Branson, MO, USA) for 30 min. The extracts were centrifuged at 15,000×g for 30 min, and the supernatant was purified through Whatman filter papers with a diameter of 150 mm (G.E. Healthcare, Little Chalfont, UK). The filtrate of the extracts was concentrated using a rotary vacuum evaporator (Eyela, Tokyo, Japan) at 36 ℃ and lyophilized to a dried powder. All procedures were performed in the dark. The dried powder was stored at -80 ℃ until use.

Fractionation of ABE and general wheat bran extract (GBE)

ABE and GBE were fractionated using a preparative HPLC system (LC-Forte/R, YMC, Koyto, Japan) equipped with a diode array detector (DAD). The DAD was monitored at 210, 254, and 285 nm. The separation was performed using YMC Triart C18 column (250×15 mm, 20 μm). The flow rate was 10 mL/min and the injection volume was 4 mL. The following mobile phase and gradient were employed: water (A) and methanol (B); 0–3 min 0% B, 3–5 min 0% B, 5–11 min 0–20% B, 11–12 min 20% B, 12–19 min 20–35% B, 19–21 min 35% B, 21–25 min 35–100% B, 25 min 100% B.

Total anthocyanin content (TAC)

TAC was determined using the pH differential method [22]. The extracts (20 mg/mL) were diluted with 25 mM potassium chloride (pH 1.0) buffer and 0.4 M sodium acetate (pH 4.5) buffer. These dilutions were left to equilibrate for 30 min before measurement of the absorbance at 520 and 700 nm.

The absorbance of the diluted extracts was calculated as follows:

$$A = (A_{520} - A_{700})_{pH1.0} - (A_{520} - A_{700})_{pH4.5}$$

The TAC in each extract was determined as follows:

$$\text{TAC (mg C3G/g)} = (sA \times MW \times DF \times V \times 10^3) \Big/ \varepsilon \times W$$

where *M.W.* is the molecular weight (449.2 g/mol) for C3G, *DF* is the dilution factor, *V* is the volume of the extracting solution, *W* is the weight of the powder in grams, and ε is the extraction coefficient (26,900 $L \times cm^{-1} \times mol^{-1}$) for C3G.

Evaluation of antioxidant activity

The antioxidant activity was determined using a DPPH free radical scavenging assay, with slight modifications [23]. A methanolic (≥99.9%) 0.2 mM DPPH solution was prepared. Ascorbic acid (as a positive control) and each extract were dissolved using 70% methanol. Thereafter, the dissolved solutions were diluted using methanol (≥99.9%) and 200 µL of the resulting solutions was incubated with methanolic 0.2 mM DPPH solution (100 µL) at 37 °C for 30 min in the dark. Additionally, the sample blank was measured based on the sample color. The absorbance of the diluted solution was measured at 517 nm using a plate reader. The DPPH free radical scavenging activity of each extract was calculated as follows:

$$\text{DPPH free radical scavenging activity}(\%) = 100 - \left(\frac{Sample\,Abs}{Blank\,Abs} \times 100 \right)$$

All results are expressed as half-maximal inhibitory concentration values (IC50).

The oxygen radical absorbance capacity (ORAC) was determined based on a slight modification [24]. All substrates were dissolved in 75 mM potassium phosphate buffer (pH 7.0). A 50-µL volume of Trolox (as a positive control) and each extract were mixed with 50 µL of 3 µM fluorescein sodium salt solution. The mixture was incubated at 37 °C for 15 min. After the addition of 25 µL of 221 mM AAPH, the fluorescence was measured using an excitation wavelength of 485 nm and emission wavelength of 535 nm. All results are expressed as micromole of Trolox equivalents (TE), derivative of water-soluble vitamin E, per gram of each extract (µM TE/g sample).

Cell culture

RAW 264.7, a mouse macrophage cell line, was obtained from the American Type Culture Collection (ATCC, Rockville, USA). RAW 264.7 cells were cultured in DMEM with high-glucose supplemented with 10% FBS, 1% penicillin–streptomycin, and 1% HEPES, in an atmosphere of 5% CO_2 and 95% humidity at 37 ℃. When RAW 264.7 cells reached 80% confluence, they were rinsed with DPBS and isolated for subculture. RAW 264.7 cells were seeded at a density of 2.5×10^6 and used before passage 30. For the experiments, RAW 264.7 cells were seeded at 4×10^4 cells/well for 18 h, unless otherwise stated.

Cell cytotoxicity

The cytotoxicity of the extracts on RAW 264.7 cells was determined using the MTT assay, as previously described [25]. RAW 264.7 cells were incubated in a 96-well plate and treated with 100, 200, 400, and 600 µg/mL of ABE and GBE for 20 h. After the addition of MTT (20 µL) reagent, which was dissolved at 1 mg/mL in DPBS, and incubation for 4 h, the media in each well were removed, and DMSO (200 µL) was added to dissolve the insoluble formazan crystals. The absorbance of the dissolved solutions was measured at 570 nm using a microplate reader (Molecular Devices, CA, USA). All results are expressed as cell viability compared to the control.

Intracellular reactive oxygen species (ROS) levels

Intracellular ROS levels were detected by using a previously reported method, with a slight modification, and CM-H_2DCFDA as the fluorescent probe [26]. RAW 264.7 cells were cultured in black 96-well plates, treated with the extract, and stimulated with 100 ng/mL LPS for 24 h. Thereafter, the cells were incubated with serum-free culture medium containing 20 µM CM-H_2DCFDA for 30 min [27]. Following washing and resuspension in DPBS, CM-H_2DCFDA fluorescence was measured using an excitation wavelength of 492 nm and an emission wavelength of 522 nm. All results are expressed as the percentage of LPS-stimulated cells.

Evaluation of anti-inflammatory activity

LPS-induced Nitric Oxide production in RAW 264.7 cells

Nitrite, the end product of nitric oxide (NO) production by activated RAW 264.7, was quantified using a colorimetric assay [28]. RAW 264.7 cells in 96-well plates were treated with LPS (100 ng/mL) and the extracts. After 24 h of incubation, 50 µL of sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) was supplemented to the cell culture media (50 µL) for 10 min. Thereafter, 50 µL of 0.1% NED solution was added and the mixture was incubated for 10 min. The absorbance was measured at 540 nm using a microplate reader. All results are expressed as the percentage of LPS-stimulated cells.

Expression level of inflammatory biomarkers (iNOS and COX-2)

Western blot analysis was performed according to an optimized method [29] to confirm the expression level of inflammatory biomarkers (iNOS and COX-2). RAW 264.7 cells (1×10^6) cultured in 6 cm culture dishes were stimulated with 100 ng/mL LPS and exposed to the extracts for 24 h. The cells were washed with DPBS and harvested with a 0.1% protease inhibitor in PBS. The supernatant was removed via centrifugation at 13,000 × g for 5 min. The cell pellets were resuspended in lysis buffer

(1% protease inhibitor and 1% phosphatase inhibitor in RIPA buffer) and incubated at 4 ℃ for 10 min. Cell debris was removed via centrifugation at 13,000 × g for 15 min. The protein (30 μg) was separated via 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (PVDF). Blocking was carried out with a solution of 5% BSA in TBST (0.075% Tween 20) for 1 h at room temperature. The membranes were incubated overnight with primary antibodies (β-actin, COX-2, iNOS) at 4 ℃. Thereafter, the membranes were incubated with secondary antibodies (anti-mouse IgG-HRP, anti-rabbit IgG-HRP, anti-goat IgG-HRP) for 1 h at room temperature. The expression levels of the proteins were normalized using EZ capture MG (ATTO, Tokyo, Japan) and quantified using CS analyzer ver. 3.0 (ATTO, Tokyo, Japan).

LC–MS/MS analysis

The analyses were performed using a Vanquish HPLC system with a DAD/multiple-wavelength detector (VF-D11-A; Thermo Fisher Scientific, Waltham, MA, USA) and a Q Exactive Orbitrap mass spectrometer fitted with a heat electrospray ionization (HESI) interface. A Thermo Hypersil Gold AQ C18 column (1.9 μm internal 100 × 2.1 mm) was used at 36 ℃ for the chromatographic separation. The following gradient elution program was employed using 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B): 0–5 min 5% B, 5–30 min 5–40% B, 30–31 min 40–100% B, 31–31.5 min 100% B, 31.5–32 min 100–5% B, 32–35 min 5% B. The extract was dissolved in 70% methanol and then 5 μL was injected into the system at a flow rate of 0.3 mL/min. The UV spectra were measured at 254, 280, 320, and 520 nm. A full MS scan was performed for each target component in positive ionization mode, and the scan range was 100–1500 m/z at a spray voltage of 3.80 kV.

Data processing and statistical analysis

LC–MS/MS data were processed using the Compound Discoverer 3.1 software (Thermo Fisher Scientific, Waltham, USA). Several steps were implemented, including raw data import, spectral filtering, peak detection, isotope classification, chromatographic alignment, gap filling, and data export. SIMCA software (version 17.0; Umetrics, Umeå, Sweden) was used for the multivariate statistical analysis. Active metabolites were identified based on their MS/MS spectra and compared with those of mzCloud (Thermo Fisher Scientific) and related studies to ensure high accuracy.

The results are expressed as mean ± standard deviation of triplicate measurements. One-way analysis of variance (ANOVA) and Duncan's test ($p < 0.05$) were performed using SPSS software (Statistical Package for the Social Sciences, version 26; USA). To identify differences or similarities among the experimental groups, principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) were performed using SIMCA software (Umetrics). The variable importance in the projection (VIP) value and p-value were used to compare differences between the two groups.

Abbreviations

ABE	Extract of 'Ariheuk' bran
ABE-F1	First eluant of the fractionated 'Ariheuk' bran extract
ABE-F2	Second eluant of the fractionated 'Ariheuk' bran extract
ABE-F3	Third eluant of the fractionated 'Ariheuk' bran extract
COX-2	Cyclooxygenase 2
C3G	Cyanidin-3-O-glucoside
P3G	Peonidin-3-O-glucoside
GBE	Extract of 'Geumgang' bran
GBE-F1	First eluant of the fractionated 'Geumgang' bran extract
GBE-F2	Second eluant of the fractionated 'Geumgang' bran extract
GBE-F3	Third eluant of the fractionated 'Geumgang' bran extract
HESI	Heated electrospray ionization
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MS	Mass spectrometry
NO	Nitric oxide
OPLS-DA	Orthogonal partial least squared-discriminant analysis
ORAC	Oxygen radical absorbance capacity
PCA	Principal component analysis
PC1	Principal component 1
PC2	Principal component 2
Prep-LC	Preparative liquid chromatography
ROS	Reactive oxygen species
TAC	Total anthocyanin content
TE	Trolox equivalents
VIP	Variable importance for the projection

Acknowledgements

We thank the BT research facility center, Chung-Ang University.

Author contributions

SGK, SHP and JHA conceived and designed the experiments; SGK and SHP performed the experiments; SGK, SHP and JHA analyzed the data and wrote the paper.

Funding

This research was supported by the Chung-Ang University Graduate Research Scholarship in 2021, and by the Cooperative Research Program of the Agriculture Science and Technology Department (Project No. PJ016031) funded by the Rural Development Administration (Republic of Korea).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Competing interests

The authors declare that they have no competing interests.

Received: 6 November 2023 Accepted: 5 February 2024
Published online: 23 February 2024

References

1. USDA (2022) World agricultural production. United States Department of Agriculture, Washington, DC, p 22
2. OECD/FAO. Agricultural Outlook 2021-2030;131.
3. Sáenz JAC: Importance of wheat in animal feed and production. 2021. https://www.veterinariadigital.com/en/articulos/importance-of-wheat-in-animal-feed-and-production/.
4. Liu J, Yu LL, Wu Y (2020) Bioactive components and health beneficial properties of whole wheat foods. J Agric Food Chem 68:12904–12915. https://doi.org/10.1021/acs.jafc.0c00705
5. Stevenson L, Phillips F, O'Sullivan K, Walton J (2012) Wheat bran: its composition and benefits to health, a European perspective. Int J Food Sci Nutr 63:1001–1013. https://doi.org/10.3109/09637486.2012.687366
6. Budhwar S, Chakraborty M, Sethi K, Chatterjee A (2020) Antidiabetic properties of rice and wheat bran-A review. J Food Biochem 44:e13424. https://doi.org/10.1111/jfbc.13424
7. Moon J, Choi S, Lee S, Yim D (2015) Antioxidant activities and anti-inflammatory effects of rice bran and wheat bran extracts. Kor J Pharmacogn 46:140–147
8. López-Perea P, Guzmán-Ortiz FA, Román-Gutiérrez AD, Castro-Rosas J, Gómez-Aldapa CA, Rodríguez-Marín ML, Falfán-Cortés RN, González-Olivares LG, Torruco-Uco JG (2019) Bioactive compounds and antioxidant activity of wheat bran and barley husk in the extracts with different polarity. Int J Food Prop 22:646–658. https://doi.org/10.1080/10942912.2019.1600543
9. Jin H-Y, Jeon S-H, Kim K-H, Kang C-S, Choi H-S, Youn Y (2021) Phytochemical components and physiological activities of purple wheat bran 'Arr-iheuk' extracts. Kor J Food Preserv 28:372–383. https://doi.org/10.11002/kjfp.2021.28.3.372
10. Kim K-H, Kim K-M (2019) A new variety of wheat (KCTC18591P) and food composition for anti-oxidative activity comprising thereof. Korean Patent 10–2035666.
11. Gupta R, Meghwal M, Prabhakar PK (2021) Bioactive compounds of pigmented wheat (*Triticum aestivum*): potential benefits in human health. Trends Food Sci Technol 110:240–252. https://doi.org/10.1016/j.tifs.2021.02.003
12. Hosseinian FS, Li W, Beta T (2008) Measurement of anthocyanins and other phytochemicals in purple wheat. Food Chem 109:916–924. https://doi.org/10.1016/j.foodchem.2007.12.083
13. Liu Q, Qiu Y, Beta T (2010) Comparison of antioxidant activities of different colored wheat grains and analysis of phenolic compounds. J Agric Food Chem 58:9235–9241. https://doi.org/10.1021/jf101700s
14. Saini P, Kumar N, Kumar S, Mwaurah PW, Panghal A, Attkan AK, Singh VK, Garg MK, Singh V (2020) Bioactive compounds, nutritional benefits and food applications of colored wheat: a comprehensive review. Crit Rev Food Sci Nutr 61:3197–3210
15. Abdel-Aal E-SM, Hucl P, Rabalski I (2018) Compositional and antioxidant properties of anthocyanin-rich products prepared from purple wheat. Food Chem 254:13–19
16. Miguel MG (2011) Anthocyanins: Antioxidant and or anti-inflammatory activities. J Appl Pharm Sci 01(6):7–15
17. Virgilio F (2004) New pathways for reactive oxygen species generation in inflammation and potential novel pharmacological targets. Curr Pharm Des 10:1647–1652
18. Oomah BD, Corbe A, Balasubramanian P (2010) Antioxidant and anti-inflammatory activities of bean (*Phaseolus vulgaris* L.) hulls. J Agric Food Chem 58:8225–8230. https://doi.org/10.1021/jf1011193
19. Kim J-B, Han A-R, Park E-Y, Kim J-Y, Cho W, Lee J, Seo E-K, Lee K-T (2007) Inhibition of LPS-induced iNOS, COX-2 and cytokines expression by poncirin through the NF-κB inactivation in RAW 264.7 macrophage cells. Biol Pharm Bull 30:2345–2351
20. Zhou K, Wu J, Chen J, Zhou Y, Chen X, Wu Q, Xu Y, Tu W, Lou X, Yang G (2019) Schaftoside ameliorates oxygen glucose deprivation-induced inflammation associated with the TLR4/Myd88/Drp1-related mitochondrial fission in BV2 microglia cells. J Pharmacol Sci 139:15–22
21. Zou Y, Yang M, Zhang G, He H, Yang T (2015) Antioxidant activities and phenolic compositions of wheat germ as affected by the roasting process. JAOCS 92:1303–1312
22. Giusti MM, Wrolstad RE (2001) Characterization and measurement of anthocyanins by UV-visible spectroscopy. Curr Protocols Food Anal Chem 1:F1-2
23. Fimbres-Olivarria D, Carvajal-Millan E, Lopez-Elias JA, Martinez-Robinson KG, Miranda-Baeza A, Martinez-Cordova LR, Enriquez-Ocaña F, Valdez-Holguin JE (2018) Chemical characterization and antioxidant activity of sulfated polysaccharides from *Navicula* sp. Food Hydrocoll 75:229–236
24. Cao G, Alessio HM, Cutler RG (1993) Oxygen-radical absorbance capacity assay for antioxidants. Free Rad Biol Med 14:303–311
25. Lee J, Kim S, Namgung H, Jo YH, Bao C, Choi HK, Auh JH, Lee HJ (2014) Ellagic acid identified through metabolomic analysis is an active metabolite in strawberry ('Seolhyang') regulating lipopolysaccharide-induced inflammation. J Agric Food Chem 62:3954–3962
26. Hernandez-Ledesma B, Hsieh CC, de Lumen BO (2009) Antioxidant and anti-inflammatory properties of cancer preventive peptide lunasin in RAW 264.7 macrophages. Biochem Biophys Res Commun 390:803–808
27. Lee SG, Brownmiller CR, Lee SO, Kang HW (2020) Anti-inflammatory and antioxidant effects of anthocyanins of Trifolium pratense (Red Clover) in lipopolysaccharide-stimulated RAW-267.4 macrophages. Nutrients 12:1089
28. Choi SY, Hwang JH, Ko HC, Park JG, Kim SJ (2007) Nobiletin from citrus fruit peel inhibits the DNA-binding activity of NF-kappaB and ROS production in LPS-activated RAW 264.7 cells. J Ethnopharmacol 113:149–155
29. Kim GD, Lee JY, Auh JH (2019) Metabolomic screening of anti-inflammatory compounds from the leaves of *Actinidia arguta* (Hardy Kiwi). Foods 8:47

Publisher's Note