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ScienceDirect
Food Chemistry 104 (2007) 955–961
Food Chemistry
www.elsevier.com/locate/foodchem

Anthocyanin characterization and bioactivity assessment of a dark blue grained wheat (*Triticum aestivum* L. cv. Hedong Wumai) extract

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Received 28 September 2006; received in revised form 8 December 2006; accepted 20 December 2006

Abstract

Defatted bran of dark blue grained wheat (*Triticum aestivum* L., *cv.* Hedong Wumai) was extracted using ethanol and chemically characterized by HPLC and ESI-MS. Cyanidin-3-glucoside was the predominant anthocyanin in the pigmented wheat which also contained cyanidin-3-galactoside, pelargornidin-3-glucoside, and peonidin-3-glucoside. Ferulic acid was identified as the major simple phenolic acid, with lesser amounts of *p*-hydroxybenzoic acid, caffeic acid, syringic acid and *p*-coumaric acid also present. Anthocyanins were separated from phenolic acids using solid phase extraction, and respective fractions were determined for DPPH radical, ABTS radical scavenging tests as well as oxygen radical absorption assay. Results showed that 69% of the overall free radical scavenging capacity of dark blue grained wheat was attributed to the anthocyanin content, compared to 19% for the extractable phenolic acids. In cell based models, the pigmented wheat extract significantly suppressed both hydrogen peroxide-induced intracellular oxidation ($p < 0.01$) and bacterial lipopolysaccharide-induced nitric oxide ($p < 0.05$) production. These data report for the first time the major phytochemical composition of pigmented grained wheat and associated bioactivity towards free radical scavenging and suppression of ROS and RNS activity.

Keywords: Dark blue wheat; Anthocyanin; Phenolic acid; Reactive oxygen species; Reactive nitrogen species

1. Introduction

Antioxidant properties of cereals have been reported in pigmented rice (Hu, Zawistowski, Ling, & Kitts, 2003), wheat (Li, Shan, Sun, Corke, & Beta, 2005; Zhou, Laux, & Yu, 2004) and purple corn (de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2002). Free radical scavenging capacity of black sorghum has been attributed to the presence of 3-deoxyanthocyanin (Awika, Rooney, & Waniska, 2004). Other workers have reported that ferulic acid (Li et al., 2005; Zhou, Yin, & Yu, 2005) along with tocopherol and carotenoids (Zhou et al., 2005) were responsible for the antioxidant properties of hard red winter wheat bran. A steryl ferulate extract collected from wheat bran has also been shown to protect methyl linoleate from thermal autoxidation (Nystrom, Makinen, Lampi, & Piironen, 2005). Moreover, recent data has indicated the possibility of underestimating the total antioxidant capacity of cereals due to the existence of unextractable phenolic complexes (Pearez-Jimeanez & Saura-Calixto, 2005). Analysis of the phytochemical profiles of different wheat varieties indicates little variation in total phenolic acid and total antioxidant activity, but significant differences are present in total ferulic acid and carotenoid content (Adom, Sorrells, & Liu, 2003). However, the anthocyanin composition of pigmented wheat is not well characterized (Escribano-Bailon, Santos-Buelga, & Rivas-Gonzalo, 2004).

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0308-8146/$ - see front matter
doi:10.1016/j.foodchem.2006.12.064

Many antioxidant measures on wheat have emphasized the activity derived from the phenolic acid-rich fractions (Adom & Liu, 2002); however, less is known about the antioxidant potential of pigmented wheat constituents. One former study with different wheat variety showed considerably high DPPH radical scavenging capacity of pigmented wheat than non-pigmented wheat material (Li et al., 2005). In this study, the phenolic content of a Chinese black-grained wheat was reported. One pigmented variety (Dongjian #1) was derived from a distant hybridization between different species from four genera (*Triticum*, *Agropyron*, *Haynaldia* and *Secale*) (Sun et al., 1996). The anthocyanin constituents of this wheat variety and associated antioxidant capacity are not known presently.

In our current study, the anthocyanin and phenolic acid compositions of a dark blue wheat grain bran extract derived from a pigmented wheat (i.e., *Triticum aestivum* L. cv Hedong Wumai) collected in China, were characterized. The antioxidant activity of the wheat extract was also assessed for its affinity to reduce both reactive oxygen radicals (ROS) and reactive nitrogen species (RNS) using both chemical and cell based models.

2. Materials and methods

2.1. Materials and chemicals

Pigmented wheat (*T. aestivum* L. cv. Hedong Wumai) grain and bran were provided by Shanxi Academy of Agricultural Sciences, China. Anthocyanin standards (e.g., cyanidin-3-glucoside, peonidin-3-glucoside, pelargonidin-3-glucoside, cyanidin-3-galactoside, and malvidin-3-glucoside), were obtained from Extrasynthèse (Genay, France). Mouse macrophage cell RAW264.7 (TIB-71) was purchased from American Type Culture Collection (Manassas, VA). 2,2′-Azobis (2-amidino-propane) dihydrochloride (AAPH) was purchased from Wako Chemical USA, Inc (Richmond, VA). 2,2′-(-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and phenolic acid standards, e.g., ferulic acid, *p*-coumaric acid, syringic acid, vanillic acid and *p*-hydroxybenzoic acid were purchased from Sigma–Aldrich Chem Co. (St. Louis, MO). All reagents used in cell culture including the cell culture media were also obtained from Sigma–Aldrich Chem Co. (St. Louis, MO).

2.2. Extraction, separation and purification of dark blue wheat grain bran

The dark blue wheat grain bran (DBWGB) was soaked in petroleum ether overnight. A ratio of wheat bran and solvent of 1:15 (w/v) was used to effectively defat the wheat bran and to improve the efficiency of anthocyanin extraction. The defatted wheat bran was re-extracted with 65% ethanol containing 0.1% HCl (~pH 3.0) in a shaker (200 rpm) at room temperature (25 °C) overnight. The crude bran extract was filtered and centrifuged (10,000 × *g*). The supernatant was concentrated under vacuum to dryness (<45 °C) and stored at 4 °C until further analysis and purification was conducted.

Primary separation of anthocyanins in the crude bran extract was performed on a Sephadex LH-20 column (25–100 μ, 50 × 3.0 cm i.d.) and eluted with 0.1% HCl–methanol. The red-colored pigment fraction was concentrated under vacuum. The final purification of the concentrated anthocyanin fraction was achieved by preparative HPLC using preparative chromatographic conditions described below. Four major anthocyanin (peak # 6, 8, 10 and 11) fractions were collected and freeze-dried (Heto FD3 freeze dryer, Heto-Holten A/S, Denmark) for later MS analysis.

A solid phase extraction (SPE) procedure was also employed in this study to separate phenolic acids from the anthocyanin fraction (Skrede, Wrolstad, & Durst, 2000). Briefly, the dark blue grain bran extract (5 mg) was dissolved in water and loaded onto a SPE cartridge (AccuBond II ODS-C18, 500 mg, Agilent) that had been pre-conditioned with 0.01% HCl–methanol, ethyl acetate and 0.01% HCl water, respectively. Sugars and other water-soluble constituents were eluted with 5 ml of 0.01% HCl–water, followed by drying cartridge under a stream of nitrogen. Ethyl acetate (5 mL) was used to elute the phenolic acids, followed by an equal volume of 0.01% HCl–methanol to recover the anthocyanin fraction. Both the phenolic acid and the anthocyanin fractions were concentrated to dryness by rotary evaporator (Büchi Rotavapor R-114, Büchi Labortechnik AG, Flawil, Switzerland) under vacuum at 35 °C and stored at −4 °C.

2.3. Analytical and preparative HPLC

HPLC analysis was performed using an Agilent 1100 HPLC module equipped with an Agilent 1100 diode array detector (DAD) and binary pump. Data was collected and analyzed by ChemStation Software (Agilent). Analytical HPLC was conducted on a Nucleosil 100-5 C18 column (250 × 4 mm i.d., 5 μm) with a Nucleosil 5 C18 guard column (4 × 4 mm i.d., 5 μm) (Agilent Technologies, Palo Alto, CA, USA). The mobile phase consisted of 5% aqueous formic acid (A) and HPLC grade methanol (B). Linear gradient runs were within 55 min and constituted the following: 0–1 min, 0–10% B; 1–15 min, 10–25% B; 15–55 min, 25–40% B. The injection volume of crude bran extract applied to column was 20 μL and the mobile phase flow rate was 0.8 mL/min. Detection wavelengths were set at 280 nm, 320 nm, 370 nm and 520 nm.

Preparative HPLC was conducted using a Zorbax SB-C18 column (250 × 9.4 mm i.d., 5 μm) (Agilent Technologies, Palo Alto, CA). The mobile phase consisted of 5% (v/v) aqueous formic acid (A) and 100% HPLC grade methanol (B). The solvents were delivered according to a gradient flow that was programmed at: 0 min, 90% A; 15 min, 70% A; 80 min, 0% A (100%, B). The injected sam-

ple was a concentrated anthocyanin fraction recovered from the Sephadex LH 20–100 column. The injection volume was 100 μL and a mobile phase flow rate of 3.5 mL/min was used. Detection wavelengths were 280 nm and 520 nm.

2.4. Mass spectrometry

Electrospray ionization mass spectrometry (ESI-MS) was performed using a quadrupole ion-trap mass spectrometer (Finnigan MAT LCQ) (Finnigan, San Jose, CA) equipped with electrospray ionization source. Anthocyanin samples were analyzed by ESI-MS at a flow rate of 5 μL/min under ion spray voltage of 4.5 kV; capillary temperature set at 200 °C; capillary voltage 3–8 V, tube lens offset 20–40 V and collision energy 25–40 eV.

2.5. Free radical scavenging capacity

DPPH and ABTS radical scavenging capacities of the dark blue wheat grain bran extract were conducted using procedures previously reported from our laboratory (Hu & Kitts, 2000), with modifications for use in a microtitreplate format (Multiskan Spectrometer, Thermolabsystem, Chantilly, VA). Briefly, pigmented wheat bran extracts (0–0.1 mg/ml) were mixed with 0.1 mM DPPH and absorption measurements were taken at 519 nm. For the ABTS radical, absorption measurements were recorded at 734 nm. In both assays, Trolox equivalents were determined using a Trolox standard free radical scavenger calibration curve at concentration range of 0–100 μm. The percentage inhibition of radical activity derived from each individual chemical assay method was plotted against the concentration of the pigmented wheat grain bran extract, or the standard Trolox concentration. The slope of linear regression curve of sample was divided by the slope of linear regression curve of a Trolox to obtain the Trolox equivalent, expressed as mmol Trolox/g sample.

2.6. Oxygen radical absorption capacity (ORAC) assay

The dried samples of both the crude pigmented wheat bran extract and the related purified anthocyanin and phenolic acid fractions were dissolved in 50% ethanol with distilled water, followed by appropriate dilution with phosphate buffer to conduct the ORAC test. A modified ORAC method formatted for a fluorescence microplate reader was used in this study (Kitts & Hu, 2005). The test samples and antioxidant standard (Trolox) were dissolved in phosphate buffer (50 mM, pH 7.0), followed by the addition of 60 nM fluoresecein in 96-well plate (Nunc, Fluorescent microplate). Plates were incubated at 37 °C for 15 min prior to adding the peroxyl radical initiator AAPH, and fluorescence (excitement wavelength = 485 nm, emission wavelength = 527 nm) was continuously recorded for 60 min (Fluoroskan Ascent FL, Labsystems). Data transformation and interpretation was performed according to the method of Davalos, Gomez-Cordoves, & Bartolome (2004). The ORAC value was expressed as mmol Trolox/g sample.

2.7. Cell culture conditions

Mouse macrophage RAW264.7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 unit/ml penicillin and 100 μg/ml streptomycin), under a fully humidified atmosphere with 5% CO_2 at 37 °C. Cells were maintained in culture medium that was changed every 2–3 days. Cell number was counted under microscope using a hemocytometer. All experiments were conducted in 96-well plates.

2.8. Cell viability assay

Cell viability measurements was performed using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, according to the method of (Hu et al., 2003). Briefly, cells were seeded in a 96-well plate overnight, prior to the addition of test samples added to each well in serial concentrations. Cells were incubated with test samples for 24 h and culture medium was replaced with fresh medium containing 0.5 mg/ml MTT and kept under cell culture condition for 4 h. SDS (10% w/v) was added to each well to dissolve the MTT crystal and absorption measurements were taken at 570 nm.

2.9. Intracellular oxidation assay

Intracellular oxidation measurement were conducted in cultured RAW264.7 cells exposed to 0.1 mM hydrogen peroxide in 96-well plates (Hu, Kwok, & Kitts, 2005). Briefly, a range of pigmented wheat bran extract, e.g., 0–200 μg/ml was incorporated into the cell culture medium, followed by the addition of 5 μM 2′,7′-dichlorofluroscin diacetate (DCF-DA), and 0.1 mM H_2O_2. Fluorescence (excitement wavelength = 485 nm, emission wavelength = 527 nm) was measured every 10 min over a 2 h period using a fluorescence microplate reader (Fluoroskan Assent FL, Labsystems). Individual fluorescence readings were corrected for the fluorescence reading occurring at time zero. Intracellular antioxidant activity was expressed as corrected fluorescence units.

2.10. Nitric oxide assay

Mouse macrophage RAW264.7 cells were seeded in 96-well plates (4×10^4 cell/well) and cultured with a range of pigmented wheat samples, e.g., 0–1.25 mg/ml and 1 μg/ml bacterial lipopolysaccharide (LPS). Nitrite concentration in culture medium was measured 24 h later using the Greiss reagent. Background readings were made on the same microplate. Concentrations of nitrite were calculated according to a calibration curve obtained using sodium

nitrite standard and expressed as percentage of inhibition (Hu et al., 2003).

3. Statistical analysis

All data are expressed as mean ± SD ($n = 6$). Triplicate wells were used for the different treatment conditions. Results were analyzed by one way ANOVA, with level of significance set as *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

4. Results and discussion

Four anthocyanin constituents (peaks 6, 8, 10 and 11) present in the crude bran extract were identified to be cyanidin-3-galactoside (Rt = 27.5 min), cyanidin-3-glucoside (Rt = 31.0 min), pelargonidin-3-glucoside (Rt = 36.0 min) and peonidin-3-glucoside (Rt = 40.4 min) (Table 1, Fig. 1a). Based on UV–Vis spectra (λ_{max}), peaks 1 and 3 could also be anthocyanins; a theory that is substantiated by the relative retention times that indicate delphinidin glycosides (Escribano-Bailon et al., 2004). Cyanidin-3-glucoside has been identified as the principal anthocyanin in purple wheat (*T. aestivum* L. cv. Konini), and the second major anthocyanin in blue wheat (*T. aestivum* L. cv. Purendo 38). Peonidin-3-glucoside was also shown to be a minor anthocyanin constituent in both purple and blue wheat; while other anthocyanins have not been identified (Abdel-Aal & Hucl, 2003). We confirm that cyanidin-3-glucoside is a principle anthocyanin found in dark blue wheat grain, and add that two other principal anthocyanins (pelargonidin-3-glucoside and cyanidin-3-galactoside) are also present. Other sources of pigmented cereals contain cyanidin-3-glucoside (Escribano-Bailon et al., 2004; Hu et al., 2003). Pelargonidin-3-glucoside and cyanidin-3-glucoside have also been identified in pigmented corn (Escribano-Bailon et al., 2004).

Ferulic acid was identified as the predominant phenolic acid in the crude bran extract with other minor phenolic acids including *p*-hydroxybenzoic acid, caffeic acid, syringic acid and *p*-coumaric acid (Fig. 1b). Ferulic acid is a primary phenolic acid that exists in both free and in bound form in the outer layer of cereal grains (Abdel-Aal, Sosulski, Graf, Gillott, & Pietrzak, 2001). This composition of phenolic acids reported in this study is similar to another wheat variety found in China (Li et al., 2005). Other studies have reported the presence of vanillic acid (Zhou et al., 2004), which was not detected in our analysis of pigmented wheat. It is noted, however, that our results do not necessarily reflect the bound phenolics, which may not have been totally recovered in this study due to the limitations of our extraction method. Recently, treatment of plant materials with alkaline reagents to chemically hydrolyze bound phenolics in the cereal matrix has proven to be successful in recovering a greater phenolic fraction (Liyana-Pathirana & Shahidi, 2006). Notwithstanding this, however, is the fact that the physiological importance of specific phenolic acids that exhibit antioxidant properties will depend on its availability for absorption and subsequent interaction with target tissues. To what extent hydrolysis of bound phenolic occurs naturally under conditions of the gastrointestinal tract thus resulting in release of potentially greater amounts of antioxidant agents remains to be determined.

A concentration-dependent free radical scavenging capacity is shown for the wheat bran extract using both the DPPH stable radical and $ABTS^{\cdot+}$ radical procedures (Fig. 2). $DPPH^{\cdot}$ radical scavenging capacity has been reported for other wheat cultivars (Li et al., 2005; Zhou et al., 2004) and rice (Hu et al., 2003; Li et al., 2005; Zhou et al., 2004). The radical scavenging capacity was 0.28 and 0.94 mmol Trolox equivalent/g sample by DPPH and ABTS methods, respectively (Fig. 3, insert). The higher ($p < 0.01$) total antioxidant activity of the pigmented wheat bran extract using the ABTS method could represent an overestimation due to an interference resulting from the higher affinity of an intermediate reaction product 3′,4′,7-trihydroxyflavylium ion (anthocyanin) chalcone to neutralize the ABTS radical in aqueous phase (Arts, Haenen, Voss, & Bast, 2004), which other wise does not occur in the same extent with the less hydrophilic DPPH solvent. This explanation also further substantiates the antioxidant activity of anthocyanins (Dangles, Gargeix, & Dufour, 2000).

Table 1
Chromatographic and spectral data of phenolic acids and anthocyanins identified from dark blue wheat grain bran (DBWGB) by HPLC and MS

Peak	Rt (min)	Identity	UV–Vis λ_{max} (nm)	$[M+H]^+$ (*m*/*z*)	Fragments $[M+H]^+$ (*m*/*z*)
1[a]	15.1	Unidentified anthocyanin	286, 522	–	–
2[a]	15.7	*p*-Hydroxybenzoic acid	210 sh, 252	–	–
3[a]	17.5	Unidentified anthocyanin	286, 522	–	–
4[a]	20.6	Caffeic acid	216, 240 sh, 292 sh, 320,		–
5[a]	22.8	Syringic acid	268, 296	–	–
6	27.5	Cyanidin-3-galactoside	284, 518	449	287
7[a]	28.5	*p*-Coumaric acid	209, 230 sh, 292 sh, 310,	–	–
8	31.0	Cyanidin-3-glucoside	284, 518	449	287
9[a]	33.3	Ferulic acid	216, 239 sh, 290 sh, 324,	–	–
10	36.0	Pelargonidin-3-glucoside	282, 334, 426, 506	433	271
11	40.4	Peonidin-3-glucoside	282, 516	463	301

[a] Were not collected for MS analysis. See peak number in Fig. 1.

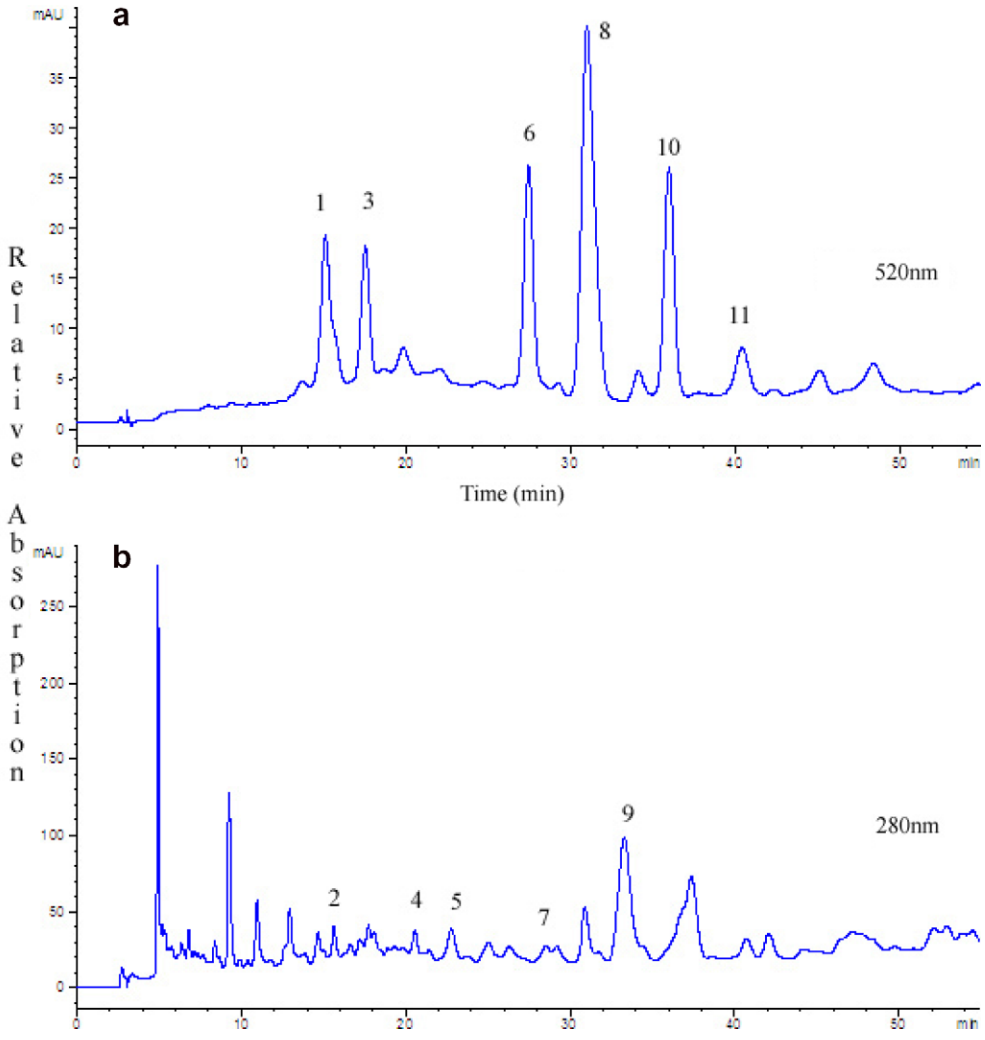

Fig. 1. HPLC-DAD chromatograms of anthocyanins (a) and phenolic acids (b) from crude extract (~20 mg/ml) of black blue wheat grain bran at 520 nm and 280 nm, respectively Refer to peak numbering in Table 1. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.).

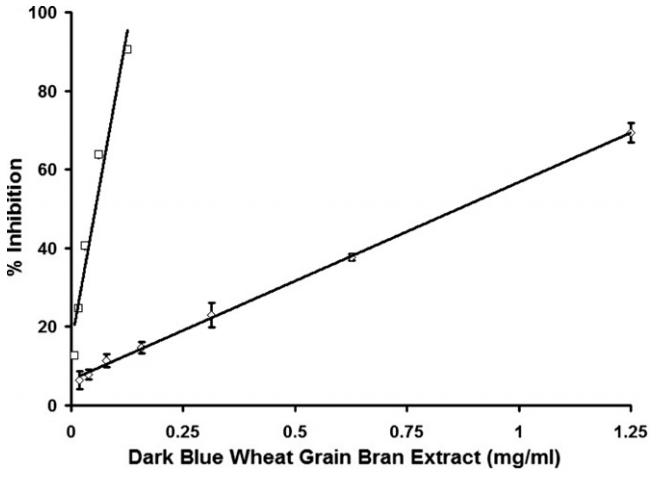

Fig. 2. Free radical scavenging activity of dark blue wheat grain bran extract on DPPH (◇) and ABTS (□) radical.

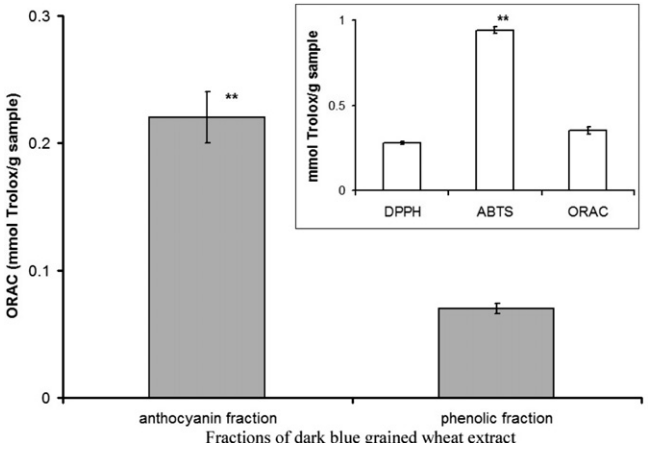

Fig. 3. Comparison of ORAC value of anthocyanin-rich fraction and phenolic-rich fraction of dark blue grain wheat extract. (*Insert*: Comparison of free radical inhibition capacity of wheat bran extract with DPPH, ABTS and ORAC methods (**, $p < 0.01$).

ORAC measurement of total antioxidant activity of a crude pigmented wheat bran extract was equivalent to 0.35 mmol Trolox/g sample (Fig. 3). A similar antioxidant activity was obtained for both ORAC (0.35 mmol Trolox/g) and DPPH (0.28 mmol Trolox/g), both of which were significantly ($p < 0.01$) lower than ABTS (0.94 mmol Trolox/g) (Fig. 3 insert). Further separation of the bran extract with C18-SPE into non-bound phenolic- and anthocyanin-rich fractions, showed that the majority of antioxidant activity in pigmented wheat bran resided with the anthocyanins, e.g. 68% or 0.22 mmol Trolox/g component compared to the free phenolic content, e.g. 19% or 0.07 mmol Trolox/g.

Hydrogen peroxide quickly initiated intracellular oxidation in RAW264.7 macrophage cells, as indicated by the rapid increase in fluorescence (Table 2). The addition of the pigmented wheat bran extract resulted in a significantly concentration-dependent suppression of H_2O_2-induced fluorescence that was particularly evident at longer incubation. A 2 h IC_{50} value calculated for the pigmented wheat extract towards intracellular oxidation was 87 μg/ml. Similar IC_{50} values for suppressing intracellular oxidation in anthocyanin rich soft fruits has been reported in Saskatoon berries (Hu et al., 2005) and black berries (Elisia, Hu, Popovich, & Kitts, 2007) and attributed to the presence of cyanidin-3-galactoside and cyanidin-3-glucoside, respectively.

Cell viability of RAW264.7 was not affected by the addition of the pigmented wheat grain extract relative to control (e.g., >89%) at concentrations up to 1.25 mg/ml. Meanwhile the production of nitric oxide in LPS-activated macrophage was significantly suppressed by the pigmented wheat extract (Fig. 4). Previous studies have reported that both anthocyanins and food extracts rich in anthocyanin have the capacity to suppress nitric oxide production in RAW264.7 cells activated by bacterial LPS, by suppressing the inducible nitric oxide synthase (iNOS) expression pathway (Hu et al., 2003; Wang & Mazza, 2002). Our previous study also showed that greater efficiency to suppress nitric oxide production occurs with cyanidin-3-glucoside compared to peonidin-3-glucoside (Hu et al., 2003). Taken together, these findings indicate that anthocyanin-rich natural products can suppress the overproduction of nitric oxide in activated macrophage cells, mediated by iNOS, thus contributing to an anti-inflammatory effect in addition to an antioxidant property. Additional bioactive properties

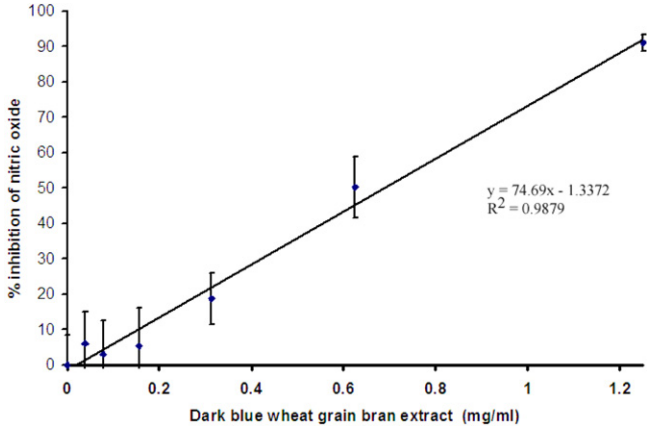

Fig. 4. Effect of dark blue wheat grain bran extract on inhibition of nitric oxide production in bacterial LPS activated mouse macrophage RAW264.7 cells. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

of anthocyanins reported by other researchers include inhibition of activator protein-1 (AP-1), nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs) signaling, induced by UVB in mouse epidermal JB6 cells (Wang et al., 2005).

In conclusion, we have characterized the anthocyanin and phenolic acid content of a pigmented wheat bran extract using HPLC and MS and have assessed the affinity of both phytochemicals in both a crude mixture as well as fractionated isolates to suppress ROS and RNS. Cyanidin-3-glucoside, followed by cyanidin-3-galactoside and pelargonidin-3-glucoside were the main anthocyanins recovered in pigmented wheat bran which contributed to the noted antioxidant activity. Furthermore, suppression of both intracellular oxidation and production of nitric oxide in cell culture suggested that the bioactive components present in the pigmented wheat extract are active in cell culture. It is important to note that metabolic studies on anthocyanin recovered from small berry fruits have demonstrated absorption of principle components after methylation, glucuronidation and/or as intact anthocyanins; the result of which corresponds to an increased serum antioxidant potential (Prior, 2004; Wu, Cao, & Prior, 2002). Thus, the presence of anthocyanins in pigmented wheat provides a considerable source of bioactive material for potential use in the functional food industry.

Table 2
Effect of dark blue wheat grain bran extract on the inhibition of 0.1 mM hydrogen peroxide-induced intracellular oxidation

	0	0	25 μg/ml sample	50 μg/ml sample	100 μg/ml sample	200 μg/ml sample
	No H_2O_2	0.1 mM H_2O_2	0.1 mM H_2O_2	0.1 mM H_2O_2	0.1 mM H_2O_2	0.1 mM H_2O_2
0	1.00	1.00	1.00	1.00	1.00	1.00
25 min	1.07 ± 0.02	12.80 ± 1.75	9.61 ± 1.28	7.23 ± 0.81*	5.41 ± 1.11**	3.34 ± 0.19***
85 min	1.39 ± 0.06	43.93 ± 8.66	42.20 ± 6.94	26.03 ± 2.57**	19.95 ± 4.26***	11.43 ± 1.18***
110 min	1.53 ± 0.06	51.78 ± 8.46	50.31 ± 5.91	31.03 ± 2.72**	23.25 ± 4.64***	13.43 ± 1.31***

* $p < 0.05$ denotes the level of significant difference between treatment protection and positive control.
** $p < 0.01$ denotes the level of significant difference between treatment protection and positive control.
*** $p < 0.001$ denotes the level of significant difference between treatment protection and positive control.

Acknowledgements

This work was supported in part by a grant from the National Centers of Excellence to D.D.K., and a grant from the University of Hong Kong (Seed Funding for Basic Research).

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