Effects of phenolic compounds of colored wheats on colorectal cancer cell lines

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Abstract

In this study, the different colored wheat brans were analyzed and compared for phenolic content (PC), phenolic compositions, and the total antioxidant capacity (TEAC) with methods based on the ability to eliminate radicals of 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH), and anthocyanin compositions. This study also aims to characterize the bioactive components of wheat grain genotypes as well as to test the protective and rescuer effects of their extracts on colorectal cancer (CRC) cell lines. PCs in the bound insoluble fraction of red wheat bran, blue wheat bran, and purple wheat bran were determined as 369.60, 446.95, and 486.79 mg gallic acid equivalents (GAE)/100 g wheat bran, respectively, while strong relationships were detected between PC and antioxidant activity (DPPH and ABTS) results. HPLC analysis of phenolic extracts demonstrated that ferulic acid was determined as the dominant phenolic acid in the bound fractions of red, purple, and blue wheats. In the free fractions, p-coumaric acid (11.55 µg/100 g wheat bran) was the dominant phenolic acid for red wheat bran, whereas ellagic acid (14.72 and 11.55 µg/100 g wheat bran) was the highest phenolic acid for purple and blue wheat brans, respectively. In bound fractions, ferulic acid was the highest phenolic acid for red (988.39 µg/100 g wheat bran), purple (1948.76 µg/100 g wheat bran), and blue (2263.96 µg/100 g wheat bran) wheat brans. On the other hand, Cyanidin-3-O-glucoside chloride was the predominant anthocyanin in free extracts of purple and blue wheat brans. In line with the antioxidant activities and phenolic acid concentrations, the blue wheat bran extracts increased CRC cell viability nonsignificantly in HT-29 and HCT-116 cell lines, whereas purple wheat bran extract had a significantly higher ($P = 0.0361$) rescuer effect compared to vehicle control under 50 µM H_2O_2 concentration. In conclusion, the *in vitro* data here show that blue and purple wheat brans are posing a novel means to increase the defense of cells against oxidative stress and cell death.

Keywords: blue wheat bran; colorectal cancer cells; free and bound phenolic; HCT-116; HT-29; purple wheat bran; red wheat bran

Introduction

Secondary metabolites are natural chemicals produced by higher plants that serve as defensive compounds against herbivores and microbial diseases. The majority of the secondary metabolites of plants pose significant therapies for human diseases including obesity, cancer, cardiovascular disease, diabetes, and inflammation (Wink, 2008). Polyphenols are secondary metabolites with potentially beneficial properties due to their strong antioxidant activities, which include the scavenging of free radicals and reactive oxygen species. A dynamic equilibrium of antioxidants and reactive oxygen species is required for a healthy metabolic system (Rice-Evans *et al*., 1996; Sharma *et al*., 2019).

Wheat is a significant element of human diet globally. The benefits of wheat's bioactive components for promoting health and preventing disease have recently drawn more attention from academics and food producers. Wheat contains health-promoting phytochemicals such as phenolic compounds and the measurement of phenolic compounds in wheat grains is essential in determining its contribution to the antioxidant activity of wheat. The amount of phenolic content (PC) in wheat depends on the type, variety, and structural part of the wheat grain (Žilić *et al*., 2011). The PC of the bran and whole wheat flour from these cultivars is correlated with this antioxidant activity (Liu *et al*., 2010; Ma *et al*., 2016). The free radical scavenging activities of purple and blue wheat grains are significantly stronger than those of white wheat grains. Colored wheat, which is high in anthocyanin molecules, has been shown to offer several health advantages, the majority of which are related to its antioxidant characteristics (Lachman *et al*., 2017). These chemicals are helpful whether taken whole or in extract form. Colored wheat offers multiple health benefits and has been shown to help prevent chronic diseases (Garg *et al*., 2016).

PC lessens lipid peroxidation, shields DNA from single-strand breaks, and repairs harm to cellular membranes. Numerous PCs are known to cause apoptosis, which is characterized by a decrease in mitochondrial transmembrane potential, the release of cytochrome C into the cytoplasm in a dose-dependent manner, and the activation of caspase-3 (Cione *et al*., 2008; Hudson *et al*., 2000). Specific PCs inhibit colorectal cancer (CRC) cell proliferation through epidermal growth factor receptor (EGFR) downregulation (Roy *et al*., 2016). PC suppresses invasion and colony formation in prostate cancer cell lines, as well as interferes with the cell cycle and apoptotic pathways (Eroğlu *et al*., 2015). Some PCs inhibit endothelial cell proliferation through NO downregulating the ERK1/2 pathway (Hou *et al*., 2004). Also, a detailed summary of each PC and the pathways they affect *in vitro* is given by Mazewski and Gonzalez de Mejia (2018).

The PC, antioxidant activities, phenolic profiles, and anthocyanin compositions of different colored wheat grains have been investigated by various groups (Abdel-Aal and Hucl, 2003; Abdel-Aal *et al*., 2006; Liu *et al*., 2010). However, the studies to associate PC, antioxidant activities, phenolic profiles, and anthocyanin composition with their effects on CRC cell lines are limited (Mazewski *et al*., 2017, 2018). In these studies, the effect of blue wheat bran extract was compared with other anthocyanin-rich plant extracts. The main novelty of the present study is the comparison of different colored wheat brans in terms of their effects on CRC proliferation. The aim of this study was to characterize PC, antioxidant activities (2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl [DPPH] and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid [ABTS]), individual phenolic and anthocyanin compositions of Red (cv. Element 22), Purple (Line Element 22 -PurplePF (2–7)), and Blue (Line 11 Element_22 Blue4Th) wheat bran extracts, and the effect of these extracts on cell viability of HCT-116 and HT-29 CRC cells were determined.

Materials and Methods

Material

Raw material

A description of the study materials is given in Table 1 and Figure 1. Red-grained cultivar Element 22, developed at Omsk SAU, served as a recurrent parent for the generation of the lines with purple and blue grain color in this study. Near-isogenic line i:S29Pp-1DPp3PF (abbreviation – i:S29PF) was used as a provider of the anthocyanin grain pigmentation. This line inherited purple pericarp color from the donor breeding lines Purple Feed (k-49426, Canada) and the line was generated in the genetic background of red-grained spring bread wheat cultivar Saratovskaya 29 (S29) having uncolored pericarp.

Chemical material

The following chemicals were purchased from Sigma-Aldrich: hexane, acetone, diethyl ether, ethyl acetate, Folin–Ciocalteu reagent, and 1,1-diphenyl-2-picrylhydrazil (DPPH) (Bornem, Belgium). ICN Biomedicals, Inc. supplied the gallic acid (Aurora, OH). Merck supplied analytical grade methanol, copper chloride, absolute ethyl alcohol, ammonium acetate, and glacial acetic acid (Darmstadt, Germany).

Cell lines

The cells of HCT-116 (CCL-247, American Type Culture Collection (ATCC)) ATCC) and HT-29 (HTB-38, ATCC) CRC cell lines were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 1% (2 mM) L-Gln, and 1% penicillin/streptomycin (which will be referred as a complete medium from this point on). All cell culture solutions were provided by Thermo Fisher Scientific (MA). All aseptic applications were performed in a laminar flow cabinet (Biovanguard, Telstar) and the cell culture vessels were incubated at 37°C in a humid incubator with 5% CO_2 pressure (MCO-230AIC-PE, Panasonic) (Mazewski *et al*., 2018).

Grain sample preparation

All wheat bran samples (Table 1) were tempered to a moisture content of 16% and left for tempering overnight. Wheat milling was then carried out by using a laboratory roller mill (Bastak 4500, Bastak Technologies, Ankara, Turkiye). Tempered wheats were passed through the corrugated break rolls section, then middlings obtained in the break section passed through the smooth reduction rolls section for obtaining flour, bran, and shorts.

Total bran yield is calculated as the ratio of the total amount of bran (coarse bran + shorts) to the total amount of all products and given as percent (%) yield. The total bran yields of the samples were in the range of 35–38%. The bran (coarse bran and shorts) was combined and milled using a Perten 3100 Laboratory Mill equipped with a 500 μm sieve and used in the extraction of phenolic compounds and various other analyses.

Extraction

The ground bran samples were defatted prior to analysis. Hexane was added to the samples at a ratio of 1 g:5 mL w/v and mixed using a vortex. The samples were shaken at 200 rpm for 10 min by using a shaker (MK200D, Yamato Scientific Co. Ltd.) and then centrifuged (Heraeus, Multifuge X3 FR, Thermo Scientific, Germany) for 5 min at 2500 xg. The defatting procedure was repeated three times and the samples were left for drying (around 12 h) in a fume hood.

Extraction of free phenolics

The extraction of free phenolics from wheat bran samples was performed as described previously (Shamanin *et al*., 2022). The defatted sample was mixed with a vortex after adding a solution of acetone and water (1:1). The extraction process was repeated three times. The supernatants were collected in a tube wrapped with aluminum foil and kept at +4°C. The dissolved samples were placed into amber-colored vials and stored at –18°C.

Extraction of bound phenolics

The bound phenolics of wheat bran samples were extracted as described previously (Shamanin *et al*., 2022). The residual pellet was hydrolyzed with 2N NaOH (20 mL) for 4 h. The pH was set arranged to 2.0 by adding 6M HCl. The extraction was performed five times to remove residual-free fatty acids. In each extraction, 10 mL of hexane was added, vortexed, shaken for 10 min at 200 rpm, and centrifuged (Heraeus, Multifuge X3 FR, Thermo Scientific, Germany) for 10 min at 4000 xg. The supernatant (hexane + free fatty acids) was removed. Then the bound phenolics were extracted with 10 mL of diethyl

Table 1. The cultivar, seed color, pedigree, and origin of wheat samples used in this study.

Cultivar	Seed coat color	Pedigree	Origin
cv. Element 22	Red	Granit / Saratovskaya29 /3/ Erythrospermum 59 // Tselinnaya 20 / Tertsiya	Omsk State Agrarian University, Russia
Line Element 22 -Purple^{PF} (2–7)	Purple	Element 22 *2 / i:S29^{PF}	Institute of Cytology and Genetics SB RAS, Russia
Line 11 Element_22 Blue^{4Th}	Blue	s:S29*4Th(4D)* / Element 22	Institute of Cytology and Genetics SB RAS, Russia

Figure 1. Grain of the recurrent parent cv. Element 22, purple-grained Line Element 22-Purple^{PF} (2–7), and blue-grained Line 11 Element_22 Blue^{4Th}.

ether-ethyl acetate (1:1, v/v). The dissolved samples were placed into amber-colored vials and stored at −18°C.

Determination of free, bound, and total PCs

The Folin–Ciocalteu method was modified to detect the concentrations of free and bound phenolic compounds (Singleton and Rossi, 1965). The optical density was determined at 760 nm by a spectrophotometer (Shimadzu 150 UV-1800, Kyoto, Japan). The PCs were reported as GAEs. The total PC was estimated from the sum of free and bound phenolic compounds.

Total antioxidant activity (TAC)

DPPH radical scavenging activity

The antioxidant activity test was performed as described by Singh *et al*. (2002) with the DPPH radical scavenging activity method. The absorbance value of the solution was measured at 515 nm by a Shimadzu 150 UV-1800 spectrophotometer following the incubation at 30°C for 60 min (Kyoto, Japan). The results are reported as mg TE/100 g ground wheat bran sample.

ABTS scavenging activity

The ABTS radical-cation scavenging activity test of the wheat bran extracts was performed with some modifications to the method described by Arnao *et al*. (2001). The absorbance was measured at 734 nm by a spectrophotometer (Shimadzu 150 UV-1800, Kyoto, Japan). The results were expressed as mg TE/100 g wheat bran.

Measurement of individual phenolic acid and anthocyanin profiles

The analysis of phenolic acid profiles of the extracts was performed with some modifications as described previously (Shamanin *et al*., 2022). The extracts were filtered using a filter (0.22 µm). An Agilent 1200 HPLC system composed of a photodiode array detector (HLPC-DAD), quaternary pump, autosampler, column oven (Shimadzu Corp., Kyoto, Japan), and Waters Atlantis column (C18, 250 mm 4.6 mm, 5 µm) was used for the analyses. A linear gradient elution procedure with solvents A and B (acetic acid: water and acetonitrile in the ratios of 0.1:99.9, respectively) was used to separate the phenolic acids. The following was the schedule for the solvent gradient: linear gradient elution from 10% B to 60% B, 0–15 min; isocratic elution of 60% B, 15–20 min; linear gradient elution from 60% B to 10% B, 20–25 min; and linear gradient elution of 10% B, 25–30 min. The chromatograms were obtained at three different wavelengths (278, 320, and 360 nm).

Anthocyanins were separated on a Waters Atlantis column (C18, 250 × 4.6 mm, 5 µm) using a linear gradient elution program with a mobile phase containing solvent A (formic acid/ acetonitrile, 7.5:92.59, v/v) and solvent B (formic acid/H_2O, 7.5:92.5, v/v) at a flow rate of 1 mL/min. The chromatograms were recorded at 524 nm. Six anthocyanins (delphinidin-3-O-galactoside, cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, petunidin-3-O-glucoside, pelargonidin-3-O-glucoside, and peonidin-3-O-glucoside) were used as standards for their separation and quantification (Demirci *et al*., 2021). The amounts of individual phenolic acids and anthocyanins were expressed as mg/100 g.

Effects of wheat bran extracts on CRC cell viability

Red, blue, and purple wheat bran extracts were dissolved in 10 mL of dimethyl sulfoxide (DMSO, Merck, Damstadt, Germany) DMSO each. Subsequently, exponential dilutions of each extract were prepared in a cell culture medium with 0.1% DMSO being the highest concentration due to its toxicity *in vitro* at higher doses. The obtained extracts were dissolved in DMSO and passed through a 0.22 µm filter, and then used in the following applications. The antioxidant properties and rescuer effects of the extracts on cell viability were evaluated by application after triggering oxidative stress via 50 and 500 µM H_2O_2 for 24 h (Ransy *et al*., 2020).

In order to determine the effect of the extracts on cell viability in HCT-116 and HT-29 CRC cells, the sulforhodamine B (SRB) test was performed (Vichai and Kirtikara, 2006). To perform this test, cells were seeded in 100 µL medium at 5×10^3 cells/well in 96-well plates. After overnight incubation, extracts in 100 µL medium were applied to the cells in the test wells. In the antioxidant effect evaluation experimental setting, H_2O_2 solutions were applied after overnight incubation following seeding, and extracts were applied 24 h later. The same volume of the complete medium as the extract solution was added to the cells in the negative control (untreated) wells. After 48 h of incubation following administration of the extract solutions, 50 µL of 50% trichloroacetic acid (TCA) solution was added to the wells and fixed at 4°C for 1 h. Then the wells were washed sequentially with SRB solution (50 µL). The SRB solution was added to each well and incubated for 30 min at room temperature in the dark. The wells were washed to remove the unbound dye and the plate was dried. The protein-bound dye was dissolved with 150 µL/well of 10 mM tris base on a shaker at approximately 150 rpm for 10 min.

Then, spectrophotometric reading was performed at 564 nm/690 nm (Spectrostar, BMG Labtech). Subsequent to the subtraction of absorbance values at 690 nm from at 564 nm, the resulting signals were compared between wells equivalent to cell viability using the GraphPad Prism V9 program and visualized as dose–response curves.

Statistical analysis

The analytical results were presented as the mean of at least duplicate independent extractions. The significance of mean differences was assessed following the application of ANOVA using Tukey's post hoc test SPSS version 9.0 (SPSS Inc. Chicago, IL). The results of the *in vitro* experiments were statistically analyzed using GraphPad Prism v9. Significance levels between groups were determined by Mann–Whitney U and Kruskal–Wallis nonparametric tests. $P < 0.05$ 95% CI was accepted as significant.

Results and Discussion

Phenolic contents

Free and bound PCs of the red, blue, and purple wheat bran samples are presented in Table 2. As it can be seen in the table, the bound PCs of red, blue, and purple wheat brans were determined as 369.60, 446.95, and 486.79 mg GAE/100 g wheat bran, respectively, while their free PCs were found to be 183.11, 213.62, and 219.98 mg GAE/100 g wheat bran, respectively. The PCs of the samples significantly changed according to the wheat bran type both in free and bound fractions ($P < 0.05$).

Table 2. The phenolic content (PC) and the antioxidant activities (DPPH and ABTS) of wheat bran samples.

Samples	PC mg GAE/ 100 g bran	DPPH mg TE/ 100 g bran	ABTS mg TE/ 100 g bran
Bound			
Red	369.60 ± 2.97^{c}	100.10 ± 2.50^{c}	1069.72 ± 2.25^{c}
Purple	446.95 ± 2.33^{b}	207.49 ± 5.00^{b}	1622.80 ± 2.25^{b}
Blue	486.79 ± 1.48^{a}	239.96 ± 2.50^{a}	1784.67 ± 11.24^{a}
Free			
Red	183.11 ± 0.42^{c}	18.21 ± 0.62^{b}	52.70 ± 2.47^{b}
Purple	213.62 ± 1.27^{b}	25.07 ± 3.75^{ab}	76.87 ± 1.91^{a}
Blue	219.98 ± 1.30^{a}	36.44 ± 0.12^{a}	87.77 ± 2.25^{a}

PC (mg GAE/100 g wheat bran): phenolic content; DPPH (mg TE/100 g wheat bran): radical scavenging activity; ABTS (mg TE/100 g wheat bran): radical-cation scavenging capacity. $^{a\text{-}c}$ Means with different letters within each column are significantly different ($p < 0.05$).

The blue wheat bran showed higher bound and free PCs than those of other samples. The PCs of the bound fractions were significantly higher than those of free fractions for all wheat bran samples ($P < 0.05$). The PCs of red wheat bran lagged behind the other wheat bran samples. Significant differences in the PCs were detected between the wheat brans of different colors, indicating that these wheat grains may exhibit different levels of antioxidant activity (Liu *et al*., 2010). Liu *et al*. (2010) reported that purple wheat (Charcoal and Konini) showed the highest PC followed by red (Red Fife) and yellow (Luteus) wheat. Siebenhandl *et al*. (2007) determined the total phenolics, antioxidant capacities, anthocyanins, carotenoids, and phenolic acids of purple and blue wheat and barley samples. Their results showed that total PCs were between 64.65 and 123.14 mg/100 g for their flours.

Antioxidant activities of free and bound fractions

Antioxidants are thought to improve health in a variety of ways, including squelching free radicals, chelating transition metals, decreasing peroxides, and promoting the body's own antioxidant defenses (Rice-Evans *et al*., 1996). The antioxidant activities evaluated as radical scavenging activities with DPPH and ABTS reagents are also included in Table 2. DPPH radical scavenging activities of the red wheat brans, blue wheat brans, and purple wheat brans in the bound fraction were 100.10, 207.49, and 239.96 mg TE/100 g, respectively, while their antioxidant activities determined using ABTS assay were 1069.72, 1622.80, and 1784.67 mg TE/100 g wheat bran, respectively. Moreover, the antioxidant activities were lower in the free fractions in line with the PCs reported above. A strong relationship was observed between PC and antioxidant activity (DPPH and ABTS) results. The sample with high PC also has high DPPH and ABTS values in both free and bound extracts. The results of the antioxidant activities of this study are in accordance with the results obtained by Subba Rao and Muralikrishna [22]. All the wheat bran samples had significantly higher antioxidant activity in the bound fractions as compared to those in the free fractions. The bound phenolics contribute a lot to the antioxidant activity, thus contributing to DPPH and ABTS radical scavenging activities. Liyana-Pathirana and Shahidi (2006) also reported that the bound phenolic fraction in both hard and soft whole wheats showed a much better antioxidant potential than the free phenolics. Significant differences might be observed in the DPPH and ABTS radical scavenging activities of different wheat genotypes. Sharma *et al*. (2020) reported that the TAC of various wheat flours varied from 6.61 to 95.04 mg/kg and followed the sequence black > blue > purple > white. Saini *et al*. (2021) also reported that the highest TAC value was observed in black (128–198 mg/kg C3G eq), blue (68–211 mg/kg C3G eq), purple (12.8–172 mg/kg

C3G eq), and red wheat (5.1–157 mg/kg C3G eq), respectively.

Individual phenolic acid profiles

Wheat grains are primarily high in ferulic and p-coumaric acids (Žilić, 2016). Within the scope of this study, Ferulic acid was determined as the dominant phenolic acid in the bound fractions of red, purple, and blue wheat bran samples (Table 3). On the other hand, different phenolic acids, including isoferulic, o-coumaric, caffeic, sinapic, vanillic, p-OH-benzoic, protocatechuic, and chlorogenic acids, were detected in various wheats in different studies (Li *et al*., 2005; Liyana-Pathirana and Shahidi, 2007; Žilić *et al*., 2012, 2014). In the free fractions, p-coumaric acid (11.55 µg/100 g wheat bran) was the dominant phenolic acid for red wheat bran, whereas ellagic acid (14.72 and 11.55 µg/100 g wheat bran) was the highest phenolic acid for purple and blue wheat brans, respectively.

In the bound fractions, ferulic acid was the highest phenolic acid for red, purple, and blue wheat brans. Ferulic acid was determined as 988.39 µg/100 g wheat bran, 1948.76 µg/100 g wheat bran, and 2263.96 µg/100 g wheat bran in the bound fractions of the red, purple, and blue wheat brans, respectively (Table 3).

Serpen *et al*. (2008), Liyana-Pathirana and Shahidi (2007), and Žilić *et al*. (2014) reported that total ferulic acid varies from 75 to 93% of total phenolic acid in whole wheat grains and 64 to 84% of total phenolic acid in wheat bran. According to the results obtained by Žilić (2016), the bound ferulic acid content provided more than 97% of the overall ferulic acid content in all varieties. Blue and purple wheat brans had higher radical scavenging activities (Table 2) as expected from their higher ferulic acid contents.

Anthocyanin profiles

The pericarp layer, which gives wheat grain its purple, blue, or red color, includes delphinidins, glycosylated cyanidins, malvidins, pelargonidins, peonidins, and petunidins. Ordinary white hexaploid wheat has no inherent grain color. The blue color came from blue-colored diploid wild einkorn wheats, while the purple color came from tetraploid emmer (*Triticum dicoccum*) (Žilić, 2016). Individual free and bound anthocyanin contents of the red, blue, and purple wheat bran samples are presented in Table 4. It was not possible to detect any free and bound anthocyanins in the respective extracts of the red wheat bran samples. Besides these, no bound phenolic compounds were detected in the purple and blue wheat bran samples. As can be seen in Table 4, cyanidin-3-O-glucoside chloride is the predominant anthocyanin in the free extract of blue and purple wheat brans. Hosseinian *et al*. (2008) and Chen *et al*. (2013) also found that cyanidin-3-O-glucoside was the most common anthocyanin in the free extract of purple wheat. The radical scavenging capacity and molecular competitive ability of cyanidin-3-O-glucoside chloride may help prevent certain inflammatory processes, cardiovascular disease, aging, and cancer (Cheynier *et al*., 2012).

The concentration of different anthocyanins in blue wheat free extract was found to be in the range of 3.48 µg/100 g to 148.44 µg/100 g. The main anthocyanins

Table 3. Individual phenolic acid profiles (µg/100 g wheat bran).

	Red		Purple		Blue	
Phenolic acids	**Free**	**Bound**	**Free**	**Bound**	**Free**	**Bound**
Ferulic acid	9.15	988.39	10.75	1948.76	9.28	2263.96
Gallic acid	10.85	223.33	9.49	114.39	10.14	302.51
Protocatechuic acid	9.70	11.42	9.77	19.06	6.51	10.75
Catechin	3.02	6.72	4.28	20.29	n.d.	12.34
Syringic acid	3.74	6.43	4.37	12.66	4.49	9.38
Ellagic acid	10.12	38.86	14.72	47.94	11.89	47.88
m-coumaric acid	n.d.	0.27	n.d.	3.10	n.d.	2.26
o-coumaric acid	n.d.	n.d.	n.d.	n.d.	n.d.	0.19
Chrysin	3.60	4.76	3.02	16.02	3.36	8.33
Caffeic	5.49	6.28	5.16	6.26	4.93	6.17
p-coumaric acid	11.55	11.78	11.83	15.91	2.04	13.94
Kaempferol	7.86	8.77	7.85	27.16	6.63	29.30

n.d.: no peak is detected.

Table 4. Anthocyanin compositions of red, purple and blue wheat bran samples (µg/100 g).

	Red		Purple		Blue	
Anthocyanin	Free	Bound	Free	Bound	Free	Bound
Cyanidin-3,5-O-diglucoside	n.d.	n.d.	n.d.	n.d.	6.34	n.d.
Cyanidin-3-O- glucoside chloride	n.d.	n.d.	59.80	n.d.	148.44	n.d.
Cyanidin-3-O- rutinoside chloride	n.d.	n.d.	n.d.	n.d.	3.48	n.d.
Delphinidin 3-O- β-D- glucoside chloride	n.d.	n.d.	n.d.	n.d.	13.97	n.d.
Malvidin-3-O- glucoside chloride	n.d.	n.d.	33.18	n.d.	49.44	n.d.
Pelargonidin 3-O- glucoside chloride	n.d.	n.d.	4.32	n.d.	12.97	n.d.
Peonidine-3-O- glucoside chloride	n.d.	n.d.	10.77	n.d.	31.43	n.d.

*n.d.: not detected.

in the blue wheat bran extract were cyanindin-3-O-glucoside chloride, delphinidin-3-O-galactoside chloride, malvidin-3-O-glucoside chloride, and petunidin-3-O-glucoside chloride. Many studies have found cyanidin 3-glucoside, cyanidin 3-rutinoside, delphinidin 3-glucoside, delphinidin 3-rutinoside, malvidin 3-glucoside petunidin-3-glucoside, and petunidin-3-rutinoside as the major anthocyanins in blue wheat (Abdel-Aal *et al*., 2006, 2008; Chen *et al*., 2013; Tyl and Bunzel, 2012). Cyanidin-3,5-O-diglucoside, cyanidin-3-O-glucoside chloride, cyanidin-3-O-rutinoside chloride, delphinidin-3-O-β-D-glucoside chloride, malvidin-3-O- glucoside chloride, pelargonidin 3-O- glucoside chloride, and peonidine-3-O-glucoside chloride are also found in the blue wheat bran used in the present study. Anthocyanin content is greatest in black-grained wheat and progressively decreases in the sequence of blue–purple–red–amber colored wheat (Sharma *et al*., 2018). The anthocyanin values of the colored wheats obtained in the study by Sharma *et al.* (2018) are similar to the anthocyanin content of colored wheat in our study. Abdel-Aal *et al*. (2006) investigated the anthocyanin compounds of purple wheat extracts and cyanidin-3-glucoside and peonidin malonyl glucoside were determined as the main ones.

While Abdel-Aal *et al*. (2006) found that delphinidin-3-glucoside and delphinidin-3-rutinoside were the most common anthocyanins in blue wheat, Abdel-Aal and Hucl (2003) reported the frequent anthocyanins as cyanidin-3-glucoside and peonidin-3-glucoside in the blue wheat.

The anthocyanin composition of blue wheat bran differed from that of purple wheat bran. Whereas purple wheat bran free extract contained only cyanidin-3-O-glucoside chloride, pelargonidin 3-O-glucoside chloride, peonidin 3-O-glucoside chloride, and malvidin-3-O-glucoside chloride, blue wheat bran free extract contained all anthocyanins investigated.

Protective and rescuer effects of the wheat bran extracts on CRC cells

Cell viability values obtained 48 h after the extract solutions were applied to HCT-116 and HT-29 CRC cells showed that blue wheat bran extract increased CRC cell viability nonsignificantly (Figure 2).

Seventy-two hours' cell viability results vary little among extracts and the differences between them were also nonsignificant. Following the application of 50 and 500 µM H_2O_2, the extracts were applied to HT29 CRC cells and their antioxidant properties and their rescuer effects from oxidative damage were evaluated (Figure 3). Accordingly, it was determined that purple wheat bran extract had a significantly higher ($P = 0.0361$) rescuing effect compared to VC under 50 µM H_2O_2. The viability of CRC cells was observed to have increased in the blue wheat bran extracts, albeit not significantly, in response to the extracts of the three different colored wheat grains. Purple wheat bran extract, on the other hand, was discovered to have a substantial therapeutic effect on CRC cells. On the other hand, the rescuer effect of the purple wheat bran extract could be associated with the antioxidative properties of the purple wheat bran extract (Gordeeva *et al*., 2019).

A previous study on rats showed that wheat class, not state of wheat refinement, seems to influence colon cancer risk, with hard red wheat being more protective relative to soft white wheat. Thus, epidemiological associations of reduced colon cancer risk with whole grain consumption may actually reflect different wheat classes (Buescher and Gallaher, 2014). This provides us with the perspective that the genotype of colored wheat could pose a novel aspect of the therapeutic approach to cancer.

A study performed on HCT-116 and HT-29 CRC cell lines, testing a variety of anthocyanin-rich extracts *in vitro*: red and purple corn, black lentil, red and purple grapes, sorghum, black rice, black and purple beans,

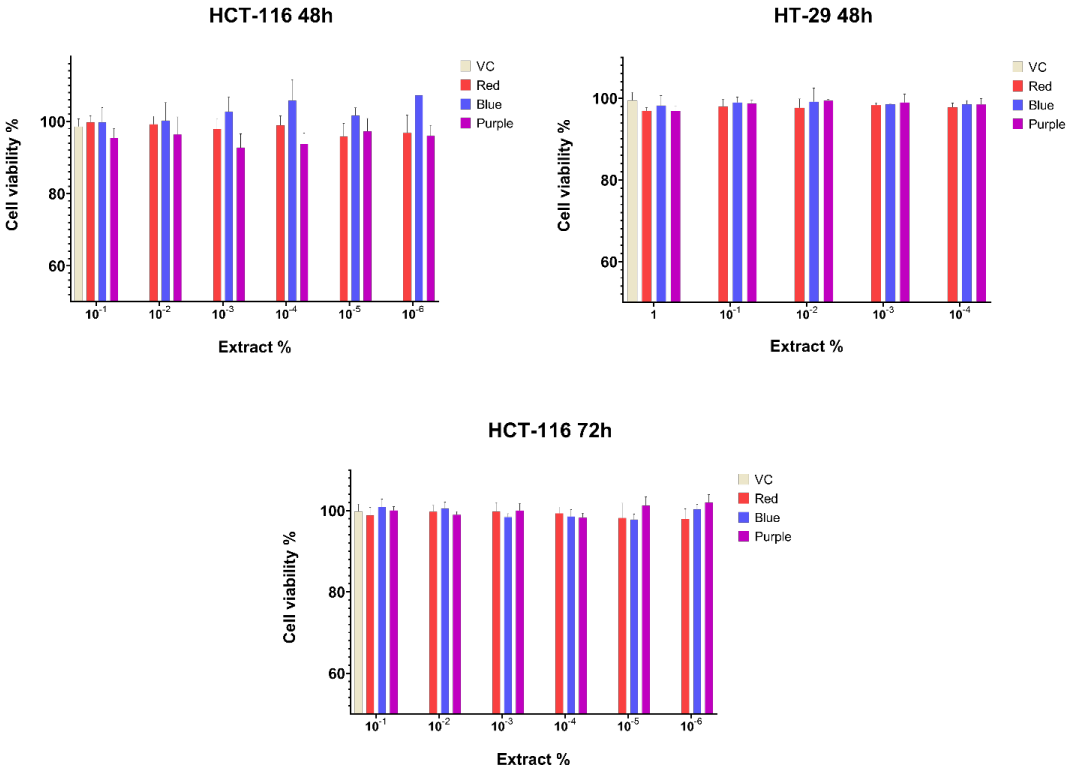

Figure 2. Cell viability assay results of HCT-116 (left, top 48 h and left, bottom 72 h) and HT-29 (right, 48 h) cells demonstrated a subtle proliferative effect of the blue wheat bran extract. (VC: Vehicle Control, Complete medium with 0.1% DMSO).

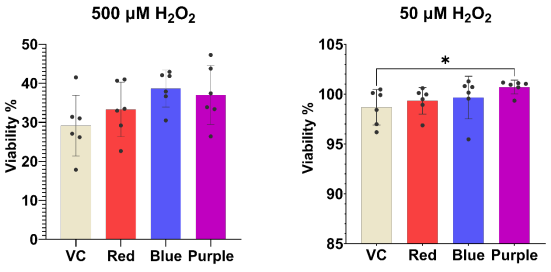

Figure 3. Rescuer potentials of wheat bran extracts due to their antioxidant properties were assessed via SRB viability assay to show a significant rescuer effect of purple wheat bran extract. (VC: Vehicle Control, Complete medium with 0.1% DMSO).

purple sweet potato, blue wheat, purple carrot, and black peanut, showed that blue wheat had a low level of viability inhibition in both cell lines. The authors indicated that these results were expected due to the very low phenolic, condensed tannin, and anthocyanin concentrations of blue wheat used in their study (Mazewski, 2019). This outcome correlates with our findings here.

Higher ferulic acid contents of purple and blue wheat bran extracts may have enhanced the rescuer effects on CRC cell lines. According to the study by Ma *et al.*, the bound ferulic acid contents in purple wheat bran varieties were significantly higher than those of white and red wheat brans as well (Ma *et al.*, 2016). Although free ferulic acid is well absorbed, the ferulic acid present in wheat flour is almost entirely in a bound form and is minimally absorbed (Adam *et al.*, 2002), thus our finding indicating a higher level of bound ferulic acid could still be useful in terms of anticancer effects. In their study on carcinogen-treated rats, Kawabata *et al.* (2000) concluded that rats fed a diet containing ferulic acid (250 ppm) had considerably fewer colonic tumors than rats given a control diet devoid of ferulic acid.

Ferulic acid was also shown to dose-dependently suppress lipid peroxidation with inhibitory ratios comparable to that obtained by α-tocopherol and superoxide dismutase (SOD) (Toda *et al.*, 1991), which could be correlated with higher rescuer effects of the extracts of the blue and purple wheat brans used in the present study that are rich in this phenolic acid. Indeed, many recent studies have focused on the anticancer effects of ferulic acid and showed that it could be beneficial to use it in CRC therapies either individually or in combination with other therapeutics (Akkoc *et al.*, 2019; Alazzouni *et al.*, 2021; El-Gogary *et al.*, 2022; Roy *et al.*, 2016; Singh Tuli *et al.*, 2022; Zheng *et al.*, 2019).

Conclusions

Among the extracts of three colored wheat brans, the blue wheat bran extract was found to have increased the viability of CRC cells, yet nonsignificantly. Meanwhile, the purple wheat bran extract was found to have a significant rescuer effect on CRC cells. There were strong relationships between total phenolics, antioxidant activities (DPPH and ABTS), individual phenolics, and anthocyanins of purple wheat bran extracts with the rescuer effect of purple wheat bran extract. Blue and purple wheat brans had higher radical scavenging activities as expected from their higher ferulic acid contents. The radical scavenging capacity and molecular competitive ability of cyanidin-3-O- glucoside chloride which is the predominant anthocyanin in the extract of blue and purple wheat brans may contribute to their health benefits. Altogether, the *in vitro* data here show that blue and purple wheat brans are posing a novel means to increase the defense of cells against oxidative stress and cell death. In order to better understand the mechanism of how this is enabled, investigation of apoptotic and autophagic pathways under treatment of the extracts would provide a valuable insight. Subsequent to further investigations and clarification of the exact mechanism of effects, utilization of these kinds of wheat bran in daily consumed food products to increase the bioavailability of protective agents in our body could be considered. In order to make a more accurate correlation between the viability-promoting and oxidative stress–rescuing properties of these extracts, the *in vitro* experiments should also be implemented on immortalized healthy colorectal epithelial cells. Future studies are very likely to contribute to these novel aspects of endemic wheat brans.

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