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Antioxidant Activity of Commercial Soft and Hard Wheat (*Triticum aestivum* L.) as Affected by Gastric pH Conditions

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Phenolic compounds from soft and hard wheat and their milling fractions were extracted into distilled deionized water, and their in vitro antioxidant activities were evaluated. Wheat samples were used as such (nontreated) or subjected to pH adjustment (treated) in order to simulate gastrointestinal pH conditions. The total phenolic content (TPC) was determined using Folin−Ciocalteu's procedure. The total antioxidant activity (TAA) was determined using Trolox equivalent antioxidant capacity assay and expressed as Trolox equivalents. The antioxidant activity of wheat extracts was also evaluated using the β-carotene bleaching assay, scavenging of 2,2-diphenyl-1-picrylhydrazyl radical, and inhibition of oxidation of human low density lipoprotein cholesterol. The TPC, TAA, and antioxidant potential, evaluated using different methods of wheat samples, were significantly increased following gastrointestinal tract-simulated pH changes. Thus, digestion taking place in the gastrointestinal tract in vivo may also enhance the antioxidant properties of the extracts.

KEYWORDS: Wheat milling fractions; antioxidant activity; free radical scavenging; inhibition of LDL oxidation; phenolic content; simulated digestion conditions

INTRODUCTION

Regular consumption of fruits, vegetables, and whole grains is known to reduce the risk of a number of chronic diseases. Hence, dietary modification by increasing the daily intake of plant foods may have a significant impact on chronic disease prevention (*1*). Whole grains, in particular, provide a wide range of nutrients and biologically active constituents, which may reduce the incidence of various diseases (*2*). In this regard, wheat (*Triticum aestivum* L.) is a staple food for a majority of the world's population and would serve as a source of potentially health-enhancing components such as dietary fiber, phenolics, tocopherols, and carotenoids (*3*) if consumed as whole grains. Many constituents in plant foods may contribute to the protective properties acting either independently or synergistically as anticancer or cardioprotective agents by a variety of mechanisms (*4*). One such protective mechanism attributed to dietary bioactive components is antioxidant activity, which is a fundamental property important to life (*4*). It has been demonstrated that many of the biological functions, including antimutagenicity, anticarcinogenicity, and antiaging arise from this property (*5*). Furthermore, many natural antioxidants exhibit a wide range of biological effects such as antibacterial, antiviral, antiinflammatory, antiallergic, antithrombic, and vasodilatory (*5*). In general, plant foods such as cereals, fruits, vegetables, nuts, and spices form the primary source of naturally occurring antioxidants in the human diet (*6*). Antioxidants are believed to play a significant role in the body's defense system against reactive oxygen species (ROS). The ROS are harmful byproducts generated during normal cellular metabolism. Consequently, there has been much interest in the antioxidant activity of phytochemicals present in the diet (*7*).

According to Vinson et al. (*8, 9*), most phytochemicals in fruits and vegetables are in the free or soluble conjugate forms of glucosides. In contrast, phenolic compounds in grains and oilseeds exist mostly in the insoluble bound form associated with cell wall polysaccharides (*10*−*12*). Among phenolic compounds, cinnamic and benzoic acid derivatives are among the antioxidant constituents universally present in plant foods (*13*). Ferulic acid is the major bound hydroxycinnamic acid derivative present in cereals (*14*). The commonly existing *trans*-ferulic acid (4-hydroxy-3-methoxy-cinnamic acid) and *trans*-*p*-coumaric acid (4-hydroxycinnamic acid) are predominantly esterified to hemicellulose via covalent links to arabinofuranose in the heteroxylans (*15*). In general, digestion of the cell wall material by endogenous enzymes in the small intestine is difficult; hence, such compounds mostly survive gastrointestinal digestion to reach the colon. However, it has been shown that colonic fermentation of such material may lead to the release of some of the bound phenolics and hence exert their unique health benefits in the colon after absorption. Therefore, bound phenolic constituents may have a significant effect on human health following digestion (*16*).

In the present study, the in vitro antioxidant activity of crude phenolic extracts of soft and hard wheat was investigated. The

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aims of the study were to (i) determine the total phenolic content (TPC), phenolic acid composition, and total antioxidant activity (TAA) of wheat; (ii) measure the antioxidant potential of wheat using various assays such as inhibition of β-carotene bleaching and scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical; and (iii) determine the ability of wheat phenolics to inhibit copper-induced oxidation of human low density lipoprotein (hLDL) cholesterol of aqueous extracts of samples of wheat as such (nontreated) and after subjecting them to simulated gastric pH conditions (treated).

MATERIALS AND METHODS

Materials. Whole grain, flour, germ, and bran of commercial soft (70% Canadian Eastern soft red spring and 30% Canadian Eastern soft white winter) and hard (90% Canadian western hard red spring and 10% Canadian Eastern hard red winter) wheat mixtures were obtained from milling suppliers of Robin Hood Multifoods Inc. (Markham, ON) in Saskatchewan. The compounds DPPH, 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfon-ate) ($ABTS^{\bullet-}$), 2,2′-azobis(2-amidinopropionamidine)-dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), β-carotene, ferulic acid, Folin−Ciocalteu phenol reagent, hLDL cholesterol, α-tocopherol, and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). All other chemicals and solvents were purchased from Fisher Scientific (Nepean, ON) and were of ACS grade or better quality.

Sample Preparation. Whole grain wheat and its milling fractions, namely, bran and germ, were ground in a coffee bean grinder (model CBG5 series, Black and Decker Canada Inc., Brockville, ON) and passed through a sieve with mesh size 16 (Tylor Test sieve, Mentor, OH). Wheat flour was used as such for the extraction of crude phenolics. All samples tested were defatted by blending the ground material with hexane (1:5, w/v, 5 min, ×3) in a Waring blender (model 33BL73, Waring Products Division, Dynamics Corp. of America, New Hartford, CT) at ambient temperature. Defatted wheat samples were air-dried for 12 h and stored in vacuum-packaged polyethylene pouches at −20 °C until used for further analysis.

Extraction of Crude Phenolics of Wheat. Crude phenolics were extracted into aqueous media according to a procedure described by Baublis et al. (*17*). Wheat samples (10 g) were extracted with distilled deionized water (150 mL) for 30 min while continuously stirring the slurry. The resulting slurries were centrifuged at 7500*g* for 15 min at ambient temperature. To determine simulated gastrointestinal pH conditions on antioxidant activity, wheat samples were first incubated at ambient temperature for 30 min (pH 6.5−7.0). The pH of the samples was decreased to 2 using 6 N HCl and incubated at 37 °C for 30 min. Then, the pH was raised to 6 using 4 N NaOH and incubated for another 30 min at 37 °C. Finally, the pH-adjusted wheat samples were centrifuged under the same conditions (7500*g*, 15 min). The resulting supernatants from both steps were collected separately and lyophilized to obtain the crude phenolic extract. The crude phenolic extracts were stored at −20 °C in vacuum-sealed pouches until used for further analysis.

Determination of TPC. The content of total phenolics was determined according to a modified version of the procedure described by Singleton and Rossi (*18*). Extracts were dissolved in methanol to obtain a 5 mg/mL concentration solution. Folin−Ciocalteu's reagent (0.5 mL) was added to a centrifuge tube (50 mL) containing 0.5 mL of the extract. Contents were mixed, and 1 mL of saturated sodium carbonate solution was added to each tube, followed by adjusting the volume to 10 mL with distilled water. The contents in the tubes were thoroughly mixed by vortexing. Tubes were allowed to stand at ambient temperature for 45 min until the characteristic blue color developed; centrifugation was then carried out at 4000*g* for 5 min (ICE Centra M5, International Equipment Co., Needham Heights, MA). Absorbance of the clear supernatants was measured at 725 nm using a diode array spectrophotometer (model 8452A, Agilent Technologies Canada Inc., Mississauga, ON). The content of total phenolics in each extract was determined from a standard curve prepared using ferulic acid and expressed as μg ferulic acid equivalents (FAE) per gram of defatted material.

Measurement of TAA. The TAA was determined according to the Trolox equivalent antioxidant capacity (TEAC) assay described by van den Berg et al. (*19*) with slight modifications. The extracts and reagents were prepared in a 0.1 M phosphate buffer (pH 7.4) containing 0.15 M sodium chloride (PBS). A solution of $ABTS^{\bullet-}$ was prepared by mixing 2.5 mM AAPH with 2.0 mM $ABTS^{\bullet-}$ at a 1:1 (v/v) ratio and heating at 60 °C for 12 min. The absorbance of the freshly prepared radical solution at 734 nm was about 0.4. The radical solution protected from light was stored at room temperature. A standard curve was prepared using different concentrations of Trolox. Thus, the reduction in the absorbance (ΔA) of the $ABTS^{\bullet-}$ solution (1960 μL) at different concentrations of Trolox (40 μL) over a period of 6 min was measured and plotted. The TEAC values of wheat extracts (5 mg/mL) were determined in the same manner and expressed as Trolox equivalents (TE). A blank measurement was recorded for each measurement that corresponded to a decrease in absorbance without any compound added. The TEAC of an unknown compound represents the concentration of a Trolox solution that has the same antioxidant capacity as the compound.

TEAC values were determined as follows:

$$\Delta A_{\text{Trolox}} = (A_{t=0\text{minTrolox}} - A_{t=6\text{minTrolox}}) - \Delta A_{\text{radical}(0-6\text{min})}$$

$$\Delta A_{\text{Trolox}} = m \times [\text{Trolox}]$$

$$\text{TEAC}_{\text{extract}} = (\Delta A_{\text{extract}}/m) \times d$$

where ΔA = reduction of absorbance, A = absorbance at a given time, m = slope of the standard curve, [Trolox] = concentration of Trolox, and d = dilution factor.

DPPH Radical Scavenging Assay. The method described by Kitts et al. (*20*) was used with slight modifications in order to assess the DPPH radical scavenging capacity of wheat extracts. A 0.135 mM DPPH solution in ethanol (1.0 mL) was mixed with various amounts (final concentration was as 100 ppm FAE) of wheat extract (1.0 mL; 3.8−27.0 and 6.6−18.7 mg/mL of treated soft and hard wheat, respectively, and 10−37 and 9−43.5 mg/mL of soft and hard wheat as such, respectively) and vortexed thoroughly. The absorbance of the mixtures at ambient temperature was recorded for 60 min at 10 min intervals. Ferulic acid, BHT, and tocopherol were used as reference antioxidants. The absorbance of the remaining DPPH radicals was measured at 519 nm using a diode array spectrophotometer (Agilent Co.). The scavenging of DPPH was calculated according to the following equation.

$$\%\ \text{scavenging} = \{(\text{Abs}_{\text{contol}} - \text{Abs}_{\text{sample}})/\text{Abs}_{\text{control}}\} \times 100$$

where $\text{Abs}_{\text{control}}$ = absorbance of DPPH radical + methanol; and $\text{Abs}_{\text{sample}}$ = absorbance of DPPH radical + wheat extract/standard.

***β*-Carotene−Linoleate Model System.** The antioxidant activity of the wheat samples/standards was evaluated using the β-carotene−linoleate model system (*21*). A solution of β-carotene was prepared by dissolving 25 mg of β-carotene in 5 mL of chloroform. Three milliliters of β-carotene solution was pipetted into a 100 mL round-bottomed flask, and chloroform was removed under vacuum using a rotary evaporator at 40 °C. Forty milligrams of linoleic acid, 400 mg of Tween 40 emulsifier, and 100 mL of aerated distilled water were added to the flask. The contents were mixed thoroughly with vigorous shaking. Aliquots (3.0 mL) of the emulsion were transferred into a series of tubes containing 2.0 mL of the wheat extracts (final concentration was as 100 ppm FAE) in methanol (12.4−46.3 and 11.3−54.3 mg/mL of soft and hard wheat as such, respectively, and 4.7−33.8 and 8.3−23.4 mg/mL of treated soft and hard wheat, respectively). Ferulic acid, BHT, and tocopherol were used as reference antioxidants. Absorbance values were recorded over a 2 h period at 20 min intervals while keeping the samples in a water bath at 50 °C. Blank samples devoid of β-carotene were prepared for background subtraction. The

antioxidant index (AI) was calculated using the following equation.

$$AI = (\beta\text{-carotene content after 2 h of assay/initial } \beta\text{-carotene content}) \times 100$$

Measurement of Iron(II) Chelating Capacity. The ferrous ion chelating activity of wheat extracts in an aqueous medium was measured as reported by Carter (*22*). The wheat extracts (2.0 mL; final concentration was as 100 ppm FAE; 6.5−24.1 and 5.9−28.3 mg/mL of soft and hard wheat as such, respectively, and 2.4−17.6 and 4.3−12.1 mg/mL of treated soft and hard wheat, respectively) were mixed with 2 mM $FeCl_2$ (0.2 mL) and 5 mM ferrozine (0.4 mL) and followed by thorough shaking. The mixtures were left at room temperature for 10 min. The absorbance of the resulting solution was measured at 562 nm. The chelating capacities of soft and hard wheat samples were expressed as μg ethylenediaminetetraacetic acid (EDTA) equivalents/g defatted material. The blank was devoid of ferrozine.

Determination of the Effects of Hydrolysis on Oxidation of hLDL Cholesterol. The procedure described by Hu and Kitts (*23*) was employed in this study. LDL was dialyzed in 10 mM PBS (pH 7.4) at 4 °C in the dark for 24 h. LDL (0.2 mg LDL/mL) was mixed with different amounts of wheat extracts (1.0 mL; final concentration was as 100 ppm FAE; 10.0−37 and 9.0−43.5 mg/mL of soft and hard wheat, respectively, and 3.8−27.0 and 6.6−18.7 mg/mL pH treated soft and hard wheat, respectively) dissolved in 10 mM PBS. Ferulic acid was used as the reference antioxidant compound. The reaction was initiated by adding a solution of cuprous sulfate (10 μM), and samples were incubated for 22 h at 37 °C. The formation of conjugated dienes was measured at 234 nm using a spectrophotometer (Agilent Technologies Canada Inc.). The inhibitory effect of wheat extracts on the formation of conjugated dienes (% inhibition_{CD}) was calculated using the following equation. A separate blank was used for each extract that contained all of the reagents except LDL.

$$\%\ \text{inhibition}_{CD} = (\text{Abs}_{\text{oxidative}} - \text{Abs}_{\text{sample}}/\text{Abs}_{\text{oxidative}} - \text{Abs}_{\text{native}}) \times 100$$

where $\text{Abs}_{\text{sample}}$ = absorbance of LDL + $CuSO_4$ + wheat extract/standard; and $\text{Abs}_{\text{native}}$ = absorbance of LDL + PBS, and $\text{Abs}_{\text{oxidative}}$ = absorbance of LDL + $CuSO_4$ + PBS. Using percentage values, the amount of LDL (μg) that can be protected against copper-mediated oxidation by 1 g wheat sample was obtained.

Statistical Analysis. All analyses were performed in triplicate, and data were reported as means ± standard deviation unless otherwise stated. Analyses of variance were performed using General Linear Model of Minitab Release 14 Xtra for Windows (Minitab Inc., State College, PA). Significant differences ($p < 0.05$) among means were determined using the Tukey's multiple range test at a fixed level of $\alpha = 0.05$. The realtionships between TPC and other variables within the observed data range were determined as Pearson correlation coefficients in bivariate correlations.

RESULTS AND DISCUSSION

Effects of Simulated Gastric pH Changes on TPC of Wheat. The yields of extracts and TPC of whole wheat and its different milling fractions, namely, flour, germ, and bran, as such (nontreated) and after subjecting to gastric pH conditions (treated) are shown in **Table 1**. Both the yield and the TPCs increased significantly ($p < 0.05$) following simulated gastric pH treatment. The increase in the yield of crude phenolic extracts of soft and hard wheat was 1.25−2.5- and 1.7−2.3-fold, respectively. The amount of total phenolics in different milling fractions as such varied from 22 ± 1 to 356 ± 8 and 23 ± 1 to 251 ± 7 μg FAE/g for defatted soft and hard wheat, respectively. The corresponding values following simulated gastric pH conditions were 74 ± 6 to 959 ± 43 and 118 ± 9 to 699 ± 23 μg FAE/g defatted material. In the soft wheat samples, the TPC was increased by 3.5−5.3-fold while this was 2.4−5.1-fold for hard wheat samples. The extractability of free and esterified phenolic compounds may be enhanced due to acidification of wheat samples. According to Baublis et al. (*17*), gastrointestinal pH conditions caused a dramatic increase in antioxidant activity of aqueous extracts of wheat-based ready-to-eat (RTE) breakfast cereals. The authors reported that acid conditions resulted in the alteration of activity, composition, and/or concentration of water soluble low molecular weight antioxidants. Moreover, gastric conditions may influence phenolic compositions since phenolics are commonly esterified to sugars or acids (*17*). The authors further proposed the release of low molecular weight compounds such as phytataes and/or a decrease in the activity of prooxidants in the cereal extracts. It can also be suggested that pH treatment resulted in some hydrolysis leading to the release of some of the bound phenolics present in the wheat samples. However, this has not been verified. The high-performance liquid chromatography (HPLC) analysis of crude phenolic extracts prepared from wheat samples as such and after subjecting to gastric pH treatment revealed a significant increase in the phenolic acid content (**Table 2**). The extraction of hydrocolloidal materials (*24*) such as hemicellulose, proteins, amino acids, peptides, and soluble sugars, among others, in water has also been suggested. The bound phenolic acids in the wheat samples as such are not available for extraction but may be released under simulated digestion conditions. Kroon et al. (*16*) reported that acid conditions in the gastrointestinal tract may

Table 1. Crude Yield (%) and TPC (μg of FAE/g Defatted Material) of Soft and Hard Wheat as Such and after Subjected to Simulated Gastric Conditions[a]

	yield of extract		TPC	
milling fraction	nontreated[b]	treated[c]	nontreated	treated
		soft wheat		
whole grain	0.6 ± 0.04[a]	1.2 ± 0.02[b]	62 ± 4[a]	294 ± 17[b]
flour	0.4 ± 0.02[a]	1.0 ± 0.03[b]	22 ± 1[a]	74 ± 6[b]
germ	2.0 ± 0.1[a]	2.5 ± 0.02[b]	356 ± 8[a]	850 ± 28[b]
bran	0.9 ± 0.03[a]	1.8 ± 0.04[b]	181 ± 12[a]	959 ± 43[b]
		hard wheat		
whole grain	0.6 ± 0.03[a]	1.4 ± 0.06[b]	81 ± 4.2[a]	252 ± 11[b]
flour	0.5 ± 0.01[a]	1.1 ± 0.04[b]	23 ± 0.9[a]	118 ± 9[b]
germ	1.7 ± 0.01[a]	2.9 ± 0.02[b]	251 ± 7[a]	699 ± 23[b]
bran	1.1 ± 0.02[a]	1.9 ± 0.02[b]	243 ± 2[a]	574 ± 17[b]

[a] Results are means of three determinations ± standard deviation. Values in each row having the same superscript are not significantly different ($p > 0.05$); means of nontreated and treated samples were compared for significance; data for soft and hard wheat were treated separately. [b] Aqueous extracts of whole grains and their milling fractions. [c] Samples were subjected to simulated gastric pH treatment.

Table 2. Content (μg/g) of Vanillic and Ferulic Acid in Soft and Hard Wheat Samples as Such and after Treatment under Simulated Gastric pH[a]

	vanillic		ferulic	
milling fraction	nontreated	treated	nontreated	treated
		soft wheat		
whole grain	trace	16	trace	33
flour	ND	trace	ND	trace
germ	30	155	189	227
bran	42	134	102	110
		hard wheat		
whole grain	trace	25	trace	67
flour	ND	trace	ND	trace
germ	28	37	143	145
bran	59	61	54	84

[a] Results are based on a single HPLC determination; ND, not detected.

Table 3. TAA (μmol TE/g Defatted Material) of Soft and Hard Wheat as Such and after Subjected to Simulated Gastric Conditions

type of extract	nontreated[b]	treated[c]
	soft wheat	
whole grain	0.6 ± 0.01[a]	1.5 ± 0.09[b]
flour	0.2 ± 0.002[a]	1.0 ± 0.02[b]
germ	4.4 ± 0.11[a]	6.3 ± 0.08[b]
bran	1.2 ± 0.03[a]	2.5 ± 0.08[b]
	hard wheat	
whole grain	0.5 ± 0.02[a]	2.0 ± 0.02[b]
flour	0.3 ± 0.01[a]	1.1 ± 0.02[b]
germ	1.1 ± 0.01[a]	6.4 ± 0.14[b]
bran	1.4 ± 0.01[a]	3.7 ± 0.06[b]

[a] The final concentration of extracts was as 100 ppm FAE. Results are means of three determinations ± standard deviation. Values in each row having the same superscript are not significantly different ($p > 0.05$); data for soft and hard wheat were treated separately. [b] Aqueous extracts of whole grains and their milling fractions. [c] Samples are subjected to simulated gastric pH treatment.

release the phenolics esterified to carbohydrates from wheat bran. Most antioxidants/phenolics in cereals are in the bran (*25*−*28*). In this study, both bran and germ samples of soft and hard wheat possessed greater antioxidative components as compared to endosperm, which constitutes the flour fraction.

Effects of Hydrolysis on TAA of Wheat. The antioxidant activity of wheat extracts was examined using the TEAC assay. The TEAC assay measures the relative ability of antioxidant substances to scavenge the $ABTS^{\bullet-}$ in comparison with a standard amount of Trolox, a water soluble analogue of α-tocopherol (*4*). The total antioxidant activities of defatted wheat samples expressed as μmol of TE/g are shown in **Table 3**. The simulated gastric conditions had a dramatic effect on TAA of wheat, reflecting a significant ($p < 0.05$) increase in TAA. For soft wheat, the TAA varied from 0.4 ± 0.01 to 4.4 ± 0.1 μmol TE and 1.0 ± 0.02 to 6.3 ± 0.08 μmol TE/g of deaftted wheat samples as such or after simulated gastric pH treatment, respectively. The corresponding values for hard wheat varied from 0.3 ± 0.01 to 1.4 ± 0.3 μmol TE and 1.1 ± 0.02 to 6.4 ± 0.1 μmol TE/g defatted material. The germ fraction, except that of hard wheat as such, produced the highest TAA while the flour fractions had the lowest under both conditions examined. In hard wheat as such, bran produced the highest TAA. Thus, the antioxidant activities of phenolics were different among various milling fractions. In oat, the aleurone layer or the outermost layer of the endosperm contained the highest concentration of antioxidants. Because the aleurone layer is often removed with the bran during milling, the bran fraction possessed the greatest antioxidant capacity among different oat fractions (*29*). This has mainly been attributed to the release of a portion of bound phenolics upon hydrolysis resulting from the extreme pH changes. The TAA of wheat samples as such represents the antioxidant activity of free and soluble conjugates of phenolic acids while the TAA of wheat samples subjected to simulated gastric pH conditions may represent the antioxidant activity of free phenolics and soluble phenolic esters mainly while bound phenolics might also have some contribution if hydrolysis had occurred as anticipated. The TPC and TAA of wheat samples of both soft and hard wheat as such showed quite a strong correlation ($R^2 = 0.96$, $p < 0.05$). Hard wheat samples after subjecting to pH adjustment also reflected a strong correlation ($R^2 = 0.95$, $p < 0.05$) between the two parameters. However, the correlation of TPC and TAA of treated soft wheat samples was weak ($R^2 = 0.68$). Hence, there was no consistency in the correlation between TPC and TAA. Thus, the present study lends further support to the findings of Zeilinski and Kozlowska (*30*), who failed to observe any relationship between the TAA of aqueous wheat extracts and their contents of phenolics. However, many authors have reported that the antioxidant activity of plant materials correlates with their phenol content (*28*, *31*). The method of extraction, type of compounds, and procedures employed for evaluation of antioxidant activity may vary greatly in different studies, thus making it difficult to compare the results. The inconsistent relationships between TAA and TPC of wheat extracts may also be attributed to the poor specificity of Folin−Ciocalteu's procedure for phenolics. In this method, the reagent detects all phenolic groups and nonphenols in the samples. Thus, the TPC measured by the Folin−Ciocalteu's procedure does not give a full picture of the quantity or quality of the phenolic constituents in the extracts (*32*). For instance, extractable proteins may be included in the measurement (*33*); hence, the TPC could be overestimated. There may also be some interference from sugars and ascorbic acid in these determinations (*33*). Phenolics do not exert the same antioxidant activity; some may demonstrate strong antioxidative properties while others may demonstrate poor activity. They may also display antagonistic or synergistic effects among themselves or with other constituents in the extracts (*4*). Thus, the antioxidant activity of the extracts may originate from the combined action of phenolic constituents and other compounds such as extractable proteins, among others (*30*). Cereal proteins have been known to exert strong antioxidant properties (*34*) and hence some water soluble proteins as well as phenolics other than phenolic acids that might be present in the extracts which could have made a contribution to the antioxidant activity observed in the flour samples despite lack of any phenolic acids. However, according to the Folin−Ciocalteu procedure, the soft and hard wheat samples as such contributed 22 ± 1 and 23 ± 1 μg FAE/g, respectively, to the TPC. This may be due to the presence of other components that reacted with Folin−Ciocalteu's reagent. Because the antioxidant activity does not necessarily correlate with high amounts of phenolics, it is important to consider both phenolic content and antioxidant activity when evaluating the antioxidant potential of an extract (*32*).

Baublis et al. (*17*) reported the antioxidative capacity of extracts from high bran, whole grain, and refined RTE breakfast cereals in a phosphatidylcholine liposome model system. They observed that high bran and whole grain RTE cereals had a higher antioxidant activity than refined RTE cereals. Furthermore, when aqueous extracts were subjected to simulated gastrointestinal pH treatment, the antioxidative capacities of treated extracts were greater than those of their untreated counterparts. The results of this study confirm the finding of Baublis et al. (*17*) for RTE breakfast cereals.

Effects of Hydrolysis on Free Radical Scavenging of Wheat. The antioxidant potential of wheat extracts was evaluated using the stable DPPH radical. The DPPH scavenging capacity of soft and hard wheat samples as such and after simulated gastric pH treatment is shown in **Table 4**. The DPPH scavenging capacity of reference compounds such as ferulic acid, α-tocopherol, and BHT at 100 ppm is also shown in **Table 4**. Ferulic acid, α-tocopherol, and BHT scavenged DPPH radical efficiently at a 100 ppm concentration. The defatted soft and hard wheat samples as such, respectively, scavenged 1.8 ± 0.01 to 63.6 ± 1.1 μmol/g and 1.9 ± 0.03 to 40.1 ± 0.06 μmol/g DPPH radical while the corresponding values increased to 7.8 ± 0.1 to 168.3 ± 1.0 μmol/g and 12.3 ± 0.1 to 126.4 ± 2 μmol/g when wheat samples were subjected to simulated gastric pH conditions (**Table 4**). Thus, the highest DPPH radical scavenging activity was rendered by samples of both soft and

Table 4. DPPH Scavenging Activity (μmol/g Defatted Material) of Soft and Hard Wheat as Such and after Subjected to Simulated Gastric Conditions[a]

type of extract	nontreated[b]	treated[c]
	soft wheat	
whole grain	6.5 ± 0.03[a]	32.4 ± 0.1[b]
flour	1.8 ± 0.01[a]	7.8 ± 0.08[b]
germ	63.6 ± 1.1[a]	168.3 ± 1.0[b]
bran	24.0 ± 0.06[a]	164.8 ± 0.8[b]
	hard wheat	
whole grain	8.9 ± 0.1[a]	31.0 ± 0.4[b]
flour	1.85 ± 0.03[a]	12.3 ± 0.1[b]
germ	40.1 ± 0.06[a]	126.4 ± 2.0[b]
bran	30.3 ± 0.03[a]	78.1 ± 1.1[b]
	reference antioxidants[d]	
ferulic acid	46.2 ± 0.3	
α-tocopherol	48.3 ± 0.3	
BHT	40.9 ± 0.4	

[a] The final concentration of extracts was as 100 ppm FAE. Results are means of three determinations ± standard deviation. Values in each row having the same superscript are not significantly different ($p > 0.05$); data for soft and hard wheat were treated separately. [b] Aqueous extracts of whole grains and their milling fractions. [c] Samples were subjected to simulated gastric pH treatment. [d] The antioxidant activity of reference compounds was expressed at 100 ppm.

Table 5. Retention of β-Carotene (nmol/g Defatted Material) by Soft and Hard Wheat Samples as Such and after Subjected to Simulated Gastric Conditions[a]

type of extract	nontreated[b]	treated[c]
	soft wheat	
whole grain	52 ± 1[b]	292 ± 10[b]
flour	12 ± 0.3[b]	51 ± 2[b]
germ	396 ± 4[b]	1034 ± 6[b]
bran	189 ± 3[b]	1207 ± 14[b]
	hard wheat	
whole grain	91 ± 2[b]	228 ± 4[b]
flour	13 ± 1[b]	76 ± 2[b]
germ	278 ± 3[b]	832 ± 15[b]
bran	295 ± 6[b]	672 ± 11[b]
	reference antioxidants[d]	
ferulic acid	92.6 ± 1.9	
α-tocopherol	96.7 ± 2.7	
BHT	89.0 ± 1.2	

[a] The final concentration of extracts was as 100 ppm FAE. Results are means of three determinations ± standard deviation. Values in each row having the same superscript are not significantly different ($p > 0.05$); data for soft and hard wheat were treated separately. [b] Aqueous extracts of whole grains and their milling fractions. [c] Samples were subjected to simulated gastric pH treatment. [d] The antioxidant activity of reference compounds was expressed as a percentage at 100 ppm.

hard wheat, when they were treated to mimic gastric conditions. In our study, the correlation between the TPC and the DPPH scavenging ability was strong ($R^2 = 0.98$ or 0.99, $p < 0.05$).

The antioxidant activity of wheat extracts as measured by the inhibition of bleaching of β-carotene after 2 h of assay is presented in **Table 5**. The absorbance readings at 470 nm reflected the efficacy of inhibiting oxidation of β-carotene by wheat samples/standards. Bran, germ, and whole grain of both soft and hard wheat exhibited a significantly ($p < 0.05$) higher antioxidative activity than the flour fraction. In both types of wheat examined, the antioxidant capacity was significantly enhanced when the samples were subjected to simulated gastric pH conditions. Different milling fractions exhibited various degrees of antioxidant activity. The antioxidant activity of reference compounds was determined at a 100 ppm concentration. Even at this level, reference antioxidants exhibited a stronger activity than that of samples; α-tocopherol showed the strongest activity against β-carotene bleaching. According to Singh et al. (*35*), the mechanism involved in bleaching of β-carotene is a free radical-mediated process resulting from hydroperoxides of linoleic acid oxidation. In this model system, β-carotene undergoes rapid discoloration in the absence of an antioxidant as observed in the control. Wheat extracts, especially those of bran and germ, exhibited considerable antioxidative activity by retaining β-carotene in the medium. The absorbance was decreased over the time although the decrease became insignificant toward the end. The correlation analysis between TPC and β-carotene scavenging by all wheat samples examined demonstrated an excellent association ($R^2 = 0.99$, $p < 0.01$). Germ and bran samples examined demonstrated a significantly ($p < 0.05$) higher antioxidant activity than that of whole wheat and flour. Results indicated that the increase in activity was 5.6-, 4.3-, 2.6-, and 6.3-fold for whole grain, flour, germ, and bran of soft wheat, respectively, when the samples were subjected to simulated gastric pH conditions. The corresponding values for hard wheat were 2.5, 5.8, 3.0, and 2.3.

Table 6. Iron(II) Chelation Capacity (μg EDTA Equivalents/g Defatted Material) of Soft and Hard Wheat Samples as Such and after Subjecting to Simulated Gastric Conditions[a]

type of extract	nontreated[b]	treated[c]
	soft wheat	
whole grain	19.1 ± 0.9[b]	110.0 ± 3.0[b]
flour	6.9 ± 0.6[b]	28.2 ± 1.7[b]
germ	53.7 ± 1.8[b]	239.0 ± 4.7[b]
bran	85.4 ± 2.0[b]	345.0 ± 4.0[b]
	hard wheat	
whole grain	23.4 ± 2.1[b]	87.7 ± 3.4[b]
flour	6.9 ± 1.0[b]	42.5 ± 1.5[b]
germ	58.9 ± 1.5[b]	193.2 ± 5.8[b]
bran	61.2 ± 1.1[b]	200.5 ± 4.7[b]
	reference antioxidants[d]	
EDTA	100.0 ± 0	
Trolox	0	
ferulic acid	4.7 ± 0.7	

[a] The final concentration of extracts was as 100 ppm FAE. Results are means of three determinations ± standard deviation. Values in each row having the same superscript are not significantly different ($p > 0.05$); data for soft and hard wheat were treated separately. [b] Aqueous extracts of whole grains and their milling fractions. [c] Samples were subjected to simulated gastric pH treatment. [d] The iron chelation capacity of reference compounds was expressed as a percentage at 100 ppm.

Effects of Hydrolysis on Metal Chelation. Iron(II) chelation capacity of soft and hard wheat samples as such and after simulated gastric pH treatment is given in **Table 6**. Iron(II) chelation capacity was increased when samples were subjected to simulated digestion prior to extraction. Thus, treated samples of soft and hard wheat produced a greater iron chelation capacity than that of their nontreated counterparts. The chelation capacity of different milling fractions decreased in the order of bran > germ > whole grain > flour for both soft and hard wheat irrespective of the sample treatment. Hence, bran showed the highest chelation capacity while the flour exhibited the lowest in each instance. As expected, EDTA exhibited the strongest chelation capacity and at 100 ppm there was a complete chelation of iron(II). On the contrary, Trolox did not chelate iron(II) at all while ferulic acid exhibited only 4.7% chelation capacity at 100 ppm phenolics. According to Graf (*36*), ferulic acid may not exert antioxidant activity via metal chelation. The TPC and iron(II) chelation capacity demonstrated poor correla-

Table 7. Retention of Human LDL (μg LDL/g Defatted Material) against Copper-Induced Oxidation by Soft and Hard Wheat Samples as Such and Following Simulation Gastric pH Treatment[a]

type of extract	nontreated[b]	treated[c]
	soft wheat	
whole grain	63 ± 1^{b}	334 ± 3^{b}
flour	21 ± 1^{b}	81 ± 1^{b}
germ	335 ± 7^{b}	1034 ± 7^{b}
bran	194 ± 4^{b}	1163 ± 12^{b}
	hard wheat	
whole grain	78 ± 1^{b}	280 ± 7
flour	20 ± 1^{b}	118 ± 2^{b}
germ	275 ± 3^{b}	831 ± 19^{b}
bran	257 ± 4^{b}	676 ± 10^{b}
	reference antioxidants[d]	
ferulic acid	81.7 ± 2.4	
Trolox	100 ± 0	

[a] The final concentration of extracts was as 100 ppm FAE. Results are means of three determinations ± standard deviation. Values in each row having the same superscript are not significantly different ($p > 0.05$); data for soft and hard wheat were treated separately. [b] Aqueous extracts of whole grains and their milling fractions. [c] Samples were subjected to simulated gastric pH treatment. [d] Retention human LDL against copper-induced oxidation by reference compounds at 100 ppm.

tion ($R^2 = 0.644$) for soft wheat samples as such. However, hard wheat samples showed excellent correlation ($R^2 > 0.97$, $p < 0.05$) between TPC and metal chelation capacity. In addition to phenolic compounds, phytic acid may have a role in the iron chelation assay. Phytic acid is found in high concentrations in the seeds of grains, pulses, and oleaginous products. In cereals, approximately 1−2% of the seed weight is phytic acid. This amount may even reach 3−6% in cereals (*37*). In wheat, a greater proportion of phytic acid is distributed in outer layers in the pericarp and in the aleurone layer (*38*). It has been shown that phytate is a potent chelator of free iron. According to Jayarajah et al. (*39*), phytate was released when samples of wheat bran were incubated with water acidified to pH 5.1 with HCl, at room temperature. Thus, under the conditions that we employed, there is a possible release of phytates that may have contributed to iron chelation.

Effects of Hydrolysis on LDL Oxidation. Oxidative modification of LDL plays a major role in the pathogenesis of coronary heart disease (*40*). Kinsella et al. (*41*) reported the importance of dietary antioxidants in the inhibition of LDL cholesterol oxidation thereby reducing the risk of atherogenesis and coronary heart disease. Numerous studies have reported the antioxidant activities of various crude plant extracts in in vitro LDL models (*23*, *28*, *35*, *42*). The inhibition of copper-induced LDL oxidation by wheat phenolics is summerized in **Table 6**. The inhibition was increased significantly ($p < 0.05$) following treatment of samples of both soft and hard wheat to simulate gastric digestion. The increase was 5.3, 3.9, 3.1, and 6.0-fold for whole grain, flour, germ, and bran, respectively, for soft wheat. The corresponding values for hard wheat were 3.6, 5.9, 3.0, and 2.6. A strong correlation has been observed between the content of phenolic acids in the extracts of rye and the antioxidant activity against copper-mediated LDL oxidation (*25*). Results of the current study indicated that samples of germ and bran possessed a higher TPC than that of whole grain and flour and demonstrated the highest inhibition against LDL oxidation despite the sample treatment. The flour fraction exhibited the lowest inhibition against copper-induced LDL oxidation. The correlation coefficient between TPC and inhibition of LDL was strong ($R^2 \geq 0.99$, $p < 0.01$) for all wheat samples examined.

Direct binding of copper to LDL has been reported to be crucial for the reactivity of copper with LDL (*43*). Thus, the ability of an antioxidant to inhibit copper-mediated LDL oxidation may be attributed to the efficient removal of copper from the surface of LDL (*44*). According to Giessauf (*45*), copper-mediated oxidation of tryptophan residues in LDL-apolipoprotein B may be a major cause in initiating lipid oxidation in LDL molecule. A unique antioxidant mechanism that involves the blockage of copper binding sites on apolipoprotein B tryptophan has been proposed (*46*). This may be achieved by the binding of antioxidants to apolipoprotein B on the LDL molecule. Hence, structural features that confer differences in protein binding may affect the antioxidant activity of phenolics in inhibiting oxidation of LDL (*46*). It has been suggested that hydroxycinnamates act mainly as peroxyl radical scavengers and hence increase the resistance of LDL to oxidation (*47*). Ferulic acid does not chelate metal ions effectively (**Table 6**) and hence may not exhibit an antioxidant activity against copper-mediated LDL oxidation by chelating copper (*36*). Under certain circumstances, a phenolic compound may not exhibit any metal chelation ability but still could show high lipid peroxidation inhibition activity. This could be explained by their lipophilicity that renders better antioxidant activity (*48*). Moreover, hydroxycinnamates such as ferulic acid and *p*-coumaric acid bind to apolipoprotein B of LDL and then block copper access to LDL particle (*46*). Natella et al. (*49*) reported that inhibition of copper-catalyzed oxidation represents the association of both chelation of metal ions and scavenging of free radical species in the LDL system. The main phenolic compound in rye bran was ferulic acid, and this inhibited LDL oxidation by approximately 34% at 40 μM (*25*). The authors further reported that rye bran extracts exhibited a much higher antioxidant activity than what can be explained from concentration of individual phenolic acids, thus reflecting their synergistic action in the assay medium. Therefore, compounds other than ferulic acid must have contributed to antioxidant activity (*25*). Ferulic acid exhibited 81.7% inhibition against copper-induced LDL oxidation at 100 ppm while this was 100% for Trolox.

In conclusion, aqueous extracts of wheat contained phenolic compounds that contributed to TAA. The antioxidative activity was significantly enhanced when wheat samples were subjected to simulated gastrointestinal pH treatment prior to extraction. The low pH could have improved extractability of the phenolic compounds from wheat. The simulated gastrointestinal conditions may also solubilize some phenolics bound to cell wall polymers. Moreover, release of some phytates could also be possible that may contribute to the antioxidant activity of wheat. However, these phenomena have not been verified in this study. Among different fractions of wheat, bran, and germ exhibited the highest antioxidative capacities while the endosperm showed the lowest. Thus, consumption of wheat as whole grains or use of wheat bran as a dietary supplement may render the maximum health benefits associated with wheat.

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