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Free Radical Scavenging Properties of Wheat Extracts

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Three hard winter wheat varieties (Akron, Trego, and Platte) were examined and compared for their free radical scavenging properties and total phenolic contents (TPC). Free radical scavenging properties of wheat grain extracts were evaluated by spectrophotometric and electron spin resonance (ESR) spectrometry methods against stable 2,2-diphenyl-1-picryhydrazyl radical (DPPH•) and radical cation ABTS$^{•+}$ (2,2′-azino-di[3-ethylbenzthiazoline sulfonate]). The results showed that the three wheat extracts differed in their capacities to quench or inhibit DPPH• and ABTS$^{•+}$. Akron showed the greatest activity to quench DPPH radicals, while Platte had the highest capacity against ABTS$^{•+}$. The ED_{50} values of wheat extracts against DPPH radicals were 0.60 mg/mL for Akron, 7.1 mg/mL for Trego, and 0.95 mg/mL for Platte under the experimental conditions. The trolox equivalents against ABTS$^{•+}$ were 1.31 ± 0.44, 1.08 ± 0.05, and 1.91 ± 0.06 μmol/g of grain for Akron, Trego, and Platte wheat, respectively. ESR results confirmed that wheat extracts directly reacted with and quenched free radicals. The TPC were 487.9−927.8 μg gallic acid equivalents/g of grain. No correlation was observed between TPC and radical scavenging capacities for DPPH• and ABTS$^{•+}$ ($p = 0.15$ and $p > 0.5$, respectively).

KEYWORDS: Wheat; radical scavenging; antioxidant; phenolic; ESR; ABTS$^{•+}$; DPPH

INTRODUCTION

Free radical chain reaction is a chemical mechanism shared by lipid oxidation in food products and pathological processes of several aging-related health problems including cancer and heart diseases. Lipid oxidation is a primary mechanism of quality deterioration in food products, especially in high fat foods (*1*−*3*). Carbonyl and other volatile compounds produced from lipid oxidation contribute to the off-flavor of foods. Antioxidants are added in food during processing to improve food quality and stability. The demand for natural antioxidants recently has increased because of questions about the long-term safety and negative consumer perception of synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisol (BHA).

In addition to their long-term safety and capacity to improve food quality and stability, these natural antioxidants may also act as nutraceuticals to terminate free radical chain reactions in biological systems and therefore may provide additional health benefits to consumers. Severe oxidative stress, a result of an imbalance between the antioxidative defense systems and the formation of reactive oxygen species including free radicals, can damage DNA, proteins, lipids, and carbohydrates and may alter intracellular signaling processes (*4*, *5*). The damage could contribute to cell injury and death, accelerate the aging process, and promote many diseases, such as cancer, cardiovascular diseases, and Parkinson's disease (*6*). These diseases cause considerable disability and suffering, significant medical care costs, and enormous social expenses. These diseases may be prevented or improved by antioxidant treatments (*7*−*9*).

Epidemiological studies have indicated that the consumption of grains, fruits, and vegetables might reduce the risk of aging-related diseases (*10*). Several investigations were conducted to study antioxidant properties of wheat and wheat-based cereal extracts. Reported antioxidant activities of wheat (winter cultivar Almari and spring cultivar Henika) extracts included suppressing radical-induced liposome lipid peroxidation and radical cation scavenging activity (*11*). In another study, extracts from macaroni wheat (*Triticum durum*) inhibited oil oxidation using the active oxygen method (*12*). Antioxidant properties of wheat-based cereal products have also been investigated by several research groups (*10*, *13*, *14*) and reviewed by Baublis et al. (*15*). Whole grain and high bran cereal products contained higher antioxidant activity (*13*). Aqueous extracts from cereal homogenates inhibited iron/ascorbic acid-induced phosphatidylcholine liposome peroxidation (*13*).

Hard winter wheat (*Triticum aestivum*), which has not been investigated for potential antioxidant properties, is an important component of both dryland and irrigated production systems in the Great Plains of the United States. Between 1983 and 1999, harvested winter wheat acreage in Colorado alone has averaged

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6.2 million hectares (2.5 million acres) with average yields from dryland and irrigated production of 2200 kg/ha (33 bushels/acre) and 3977 kg/ha (59 bushels/acre), respectively. The annual farm gate value of this production (5 year average) is $311 million. Roughly 80% of the wheat produced in Colorado is sold in export markets. Largely because of recent depressions in export markets and high production levels in other wheat growing areas of the world, domestic market prices have fallen to record lows and the farm economy has suffered tremendously. To overcome some of the inherent difficulties in these volatile commodity markets, wheat producers are searching for value-added opportunities for marketing wheat grain. Determination of antioxidant properties of current hard winter wheat varieties may lead to the production of value-added wheat varieties rich in antioxidants for preparing functional foods to benefit human health, as well as novel wheat antioxidants for improving food quality and stability in place of the synthetic antioxidants.

Free radical scavengers are a group of potential antioxidants to be used as food additives and nutraceuticals. Free radical scavenging activity can be evaluated by spectrophotometric and electron spin resonance (ESR) spectrometry methods (*16*, *17*). ESR is considered to be the least ambiguous method for the detection of free radicals (*16*−*18*). ESR has successfully been used to study the free radical scavenging activities of antioxidants. Spectrophotometric methods are also widely used to study free radical scavenging capacities of antioxidants using stable radicals and trapping agents (*19*−*21*).

The objective of this investigation was to characterize the free radical scavenging properties of three hard winter wheat varieties. Results from this study may promote the development of wheat varieties with enhanced antioxidant properties and demonstrate the potential of developing value-added foods or antioxidant preparations from wheat.

MATERIALS AND METHODS

Materials. Grain samples of three winter wheat varieties (Akron, Trego, and Platte) adapted for production in Colorado were used for this study. The variety Akron is a hard red winter wheat, while Trego and Platte are both hard white winter wheat varieties. Samples were obtained at harvest from breeding trials conducted at a single dryland testing location in eastern Colorado. This field location was considered to be representative of typical wheat production conditions in eastern Colorado. Grain samples were cleaned using seed cleaners to remove all nongrain debris present following harvest. 2,2-Diphenyl-1-picryhydrazyl radical (DPPH•) was purchased from Sigma-Aldrich (St. Louis, MO). A total antioxidant status kit was purchased from Randox Laboratories Ltd. (San Francisco, CA). All other chemicals and solvents were the highest commercial grade and were used without further purification.

Extraction and Testing Sample Preparation. Wheat grain of each variety was ground and extracted for 3 h with absolute ethanol under nitrogen, using a Soxhlet extractor. The ethanol extracts were concentrated to a final volume of 250 mL using a rotary evaporator and kept in the dark under nitrogen until further analysis. To prepare dimethyl sulfoxide (DMSO) solution, ethanol was removed under vacuum from a known volume of the ethanol extract, and the solid residue was quantitatively redissolved in DMSO. The resulting DMSO solution was kept under nitrogen in the dark until further analysis. For the benzene solution preparation, the residue was quantitatively redissolved in benzene.

ESR Analysis. ESR analysis of radicals was conducted at room temperature using stable free DPPH radical and a Bruker EMX ESR spectrometer (Bruker Instruments, Inc., Billerica, MA) in the Department of Chemistry at Colorado State University. The benzene solutions of wheat grain extracts and DPPH• were mixed and kept at 20−25 °C until ESR analysis. ESR measurements were conducted at 10 and 60 min following the start of the reactions, with a modulation frequency of 100 kHz and a sweep width of 100.00 G (*17*, *22*). The reaction mixture contained 2.0 mM DPPH and 2.4 mg/mL wheat extracts.

Radical Cation $ABTS^{•+}$ (2,2′-Azino-di[3-ethylbenzthiazoline sulfonate]) Scavenging Activity. The radical cation $ABTS^{•+}$ scavenging activity was determined using a commercial kit from Randox Laboratories Ltd. (San Francisco, CA). Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) was used as an antioxidant standard. DMSO was used to prepare the solutions of trolox and wheat extracts and to determine the reagent blank. The trolox equivalent was calculated and used to compare the radical cation scavenging activity of each antioxidant (*19*). The tests were conducted in triplicate for each wheat extract.

Comparison of Radical DPPH• Scavenging Capacity. The total free radical scavenging capacity of each wheat extract was estimated and compared to vitamin E, vitamin C, and BHT according to the previously reported procedure using the stable DPPH• (*23*). Briefly, freshly made DPPH• solution was added into ethanol solutions of individual wheat extracts, vitamin E, vitamin C, and BHT to start the reaction. The final concentration was 100 μM for DPPH• and 50 mM for vitamin E, vitamin C, and BHT. The concentrations of wheat extract were 0.9 and 2.0 mg/mL for Akron extract, 3.0 and 12.0 mg/mL for Trego extract, and 1.3 and 3.4 mg/mL for Platte extract. A control containing no antioxidant was included. The absorbance at 517 nm was measured against a blank of pure ethanol after the reaction was carried out at ambient temperature for 60 min. Radical DPPH• scavenging capacity was estimated from the difference in absorbance with or without antioxidants and expressed as percent DPPH• remaining. All tests were conducted in triplicate.

Dose and Time Effects of Wheat Extract to Quench DPPH Radicals. A kinetic study was also conducted using a spectrophotometric method to evaluate the free radical scavenging properties of wheat extracts using stable DPPH• according to a previously reported procedure (*7*, *23*, *24*). Briefly, 1000 μL of testing antioxidant solution was mixed and reacted with 1000 μL of 200 μM DPPH• at ambient temperature. The absorbance at 517 nm was measured at certain time points and used to calculate the remaining radical levels according to a standard curve. Seven levels of each extract were used to show dose and time effects. The ED_{50} of each wheat extract was obtained by plotting the percent DPPH• remaining at the steady state (60 min) of the reaction against the corresponding antioxidant level. The ED_{50} is the concentration of antioxidant to quench 50% DPPH• under the experimental conditions. Triplicate reactions were carried out for each level of antioxidant from each wheat variety.

Total Phenolic Contents (TPC). The TPC of wheat extracts were determined using Folin−Ciocalteu reagent (*25*, *26*). The reaction mixture contained 100 μL of wheat extracts, 500 μL of the Folin−Ciocalteu reagent, and 1.5 mL of 20% sodium carbonate. The final volume was made up to 10 mL with pure water. After 2 h of reaction, the absorbance at 765 nm was measured and used to calculate the phenolic contents using gallic acid and catechin as standards. Triplicate reactions were conducted.

Statistic Analysis. Data were reported as mean ± standard deviation. The analysis of variance and least significant difference tests were conducted to identify differences among means, while a Pearson correlation test was conducted to determine the correlations among means. Statistical significance was declared at $p < 0.05$.

RESULTS

ESR Analysis. ESR spectra showed that extracts from all three wheat varieties directly reacted and quenched free DPPH radicals at the tested concentration. The ESR data for Akron wheat are illustrated in **Figure 1A**−**D**. No change of absorbance was observed in the control sample at 10 and 60 min (**Figure 1A**,**C**), while Akron extracts resulted in the disappearance of radical absorbance at both 10 and 60 min (**Figure 1B**,**D**). The 60 min reaction time resulted in a greater reduction in free radicals than the 10 min reaction time (**Figure 1B**,**D**). Similar ESR spectra were obtained for Trego and Platte (data not shown).

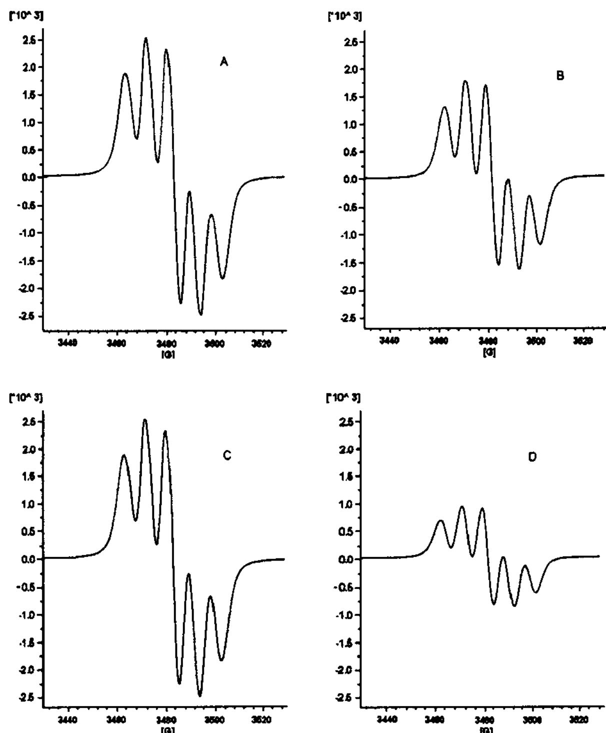

Figure 1. Free radical scavenging activity of Akron wheat extracts determined by ESR. The final concentration of DPPH was 2.0 mM in all tested samples. A = 0 Akron extract reacted for 10 min; B = 2.4 mg/mL Akron extract reacted for 10 min; C = 0 Akron extract reacted for 60 min; and D = 2.4 mg/mL of Akron extract reacted for 60 min.

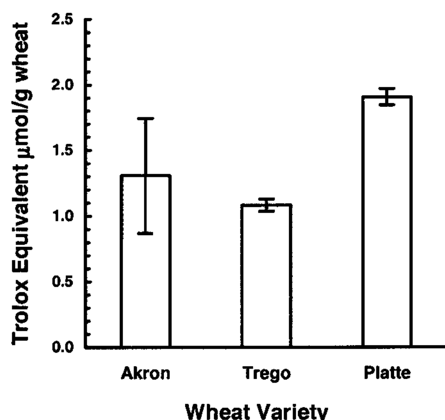

Figure 2. Radical cation scavenging capacity of wheat grain extracts. The radical cation scavenging capacity of three wheat extracts was expressed as trolox equivalent. Vertical bars represent the standard deviation of each data point.

Radical Cation Scavenging Activity. Wheat extracts were measured and compared for their free radical scavenging activities against radical cation $ABTS^{\bullet+}$. Extracts from all three wheat varieties showed $ABTS^{\bullet+}$ scavenging capacity (**Figure 2**), although some differences among varieties were noted. Platte wheat had the greatest activity to quench $ABTS^{\bullet+}$, followed by Akron and Trego. The trolox equivalents were 1.31 ± 0.44, 1.08 ± 0.05, and 1.91 ± 0.06 μmol/g grain for Akron, Trego, and Platte wheat, respectively.

Comparison of Radical DPPH Scavenging Capacity. Total DPPH• scavenging capacities of each wheat extract at two selected concentrations were measured and compared to vitamin E, vitamin C, and BHT. Significant DPPH radical scavenging capacity was detected in all tested wheat extracts at selected levels, although the tested wheat varieties differed in their activity to react and quench DPPH radicals (**Figure 3**, **Table 1**).

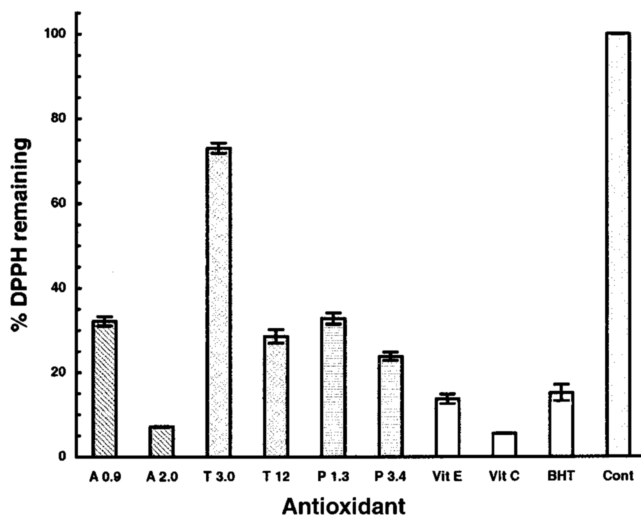

Figure 3. Comparison of radical DPPH scavenging capacity. Radical DPPH scavenging capacities of two levels of each wheat extract, vitamin E (Vit E), vitamin C (Vit C), and BHT, as compared to the control containing no antioxidant. The final concentration of DPPH radical was 100 μM, while the concentrations of Vit E, Vit C, and BHT were 50 mM. A0.9 and A2.0 represented final concentrations of Akron extracts of 0.9 and 2.0 mg/mL; T3.0 and T12 represented final concentrations of Trego extract of 3.0 and 12.0 mg/mL; and P1.3 and P3.4 represented the final concentrations of Platte extract of 1.3 and 3.4 mg/mL. Cont represented the control containing no antioxidants. Vertical bars represent the standard deviation of each data point (n = 3).

Table 1. ED_{50} of Wheat Extracts against DPPH Radicals[a]

variety	ED_{50} (mg extracts/mL)	corresponding grain weight (mg)
Akron	0.60	15.4
Trego	7.1	254.0
Platte	0.95	31.3

[a] ED_{50} is the concentration of wheat extracts to quench 50% DPPH radicals under experimental conditions.

Reaction Kinetics of Wheat Extract−DPPH•. The antioxidant extracts from these three wheat varieties had similar reaction kinetics curves against DPPH• as determined by spectrophotometric measurement (**Figure 4a−c**). Both dose and time effects were observed (**Figure 4a−c**). Higher concentrations of wheat extracts were more effective in quenching free radicals in the system. The ED_{50} of Akron wheat grain extracts to quench DPPH radical is about 0.60 mg/mL, which corresponds to 15.4 mg of Akron wheat grain (**Table 1**). ED_{50} is the concentration of wheat extracts to quench 50% DPPH radical under the experimental conditions. Therefore, Akron extract had the greatest free radical scavenging activity against DPPH radical, followed by Platte extract with an ED_{50} of 0.95 mg/mL and Trego extract with an ED_{50} of 7.10 mg/mL (**Table 1**).

TPC. The three wheat varieties differed in their TPC expressed as gallic acid equivalents per gram of grain or milligram of the extract and catechin equivalents per milligram of the extract (**Table 2**). Akron seed had the highest phenolic content, while Platte had the lowest TPC value. There was no significant correlation between TPC and radical scavenging activity for DPPH radicals ($p = 0.15$) and $ABTS^{\bullet+}$ ($p > 0.5$).

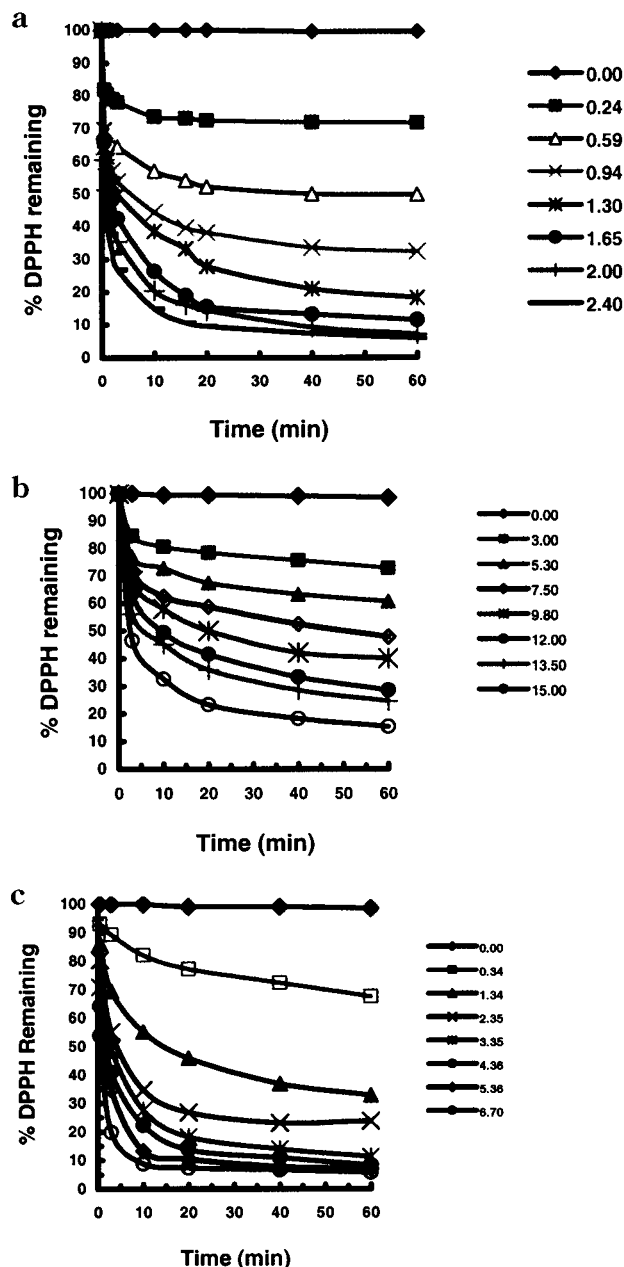

Figure 4. Reaction kinetics of wheat extracts with DPPH radical. The DPPH radical concentration was 100 μM in all reaction mixtures. All tests were conducted in triplicate, and the means are used. (**a**) Reaction kinetics of Akron extracts; 0, 0.24, 0.59, 0.94, 1.30, 1.65, 2.00, and 2.40 represent the final Akron extract concentrations of 0, 0.24, 0.59, 0.94, 1.30, 1.65, 2.00, and 2.40 mg/mL in the reaction mixtures. (**b**) Reaction kinetics of Trego extracts; 0, 3.00, 5.30, 7.50, 9.80, 12.00, 13.50, and 15.00 represent the final Trego extract concentrations of 0, 3.00, 5.30, 7.50, 9.80, 12.00, 13.50, and 15.00 mg/mL in the reaction mixtures. (**c**) Reaction kinetics of Platte extracts; 0, 0.34, 1.34, 2.35, 3.35, 4.36, 5.36, and 6.70 represent the final Platte extract concentrations of 0, 0.34, 1.34, 2.35, 3.35, 4.36, 5.36, and 6.70 mg/mL in the reaction mixtures.

DISCUSSION

The mechanisms involved in the beneficial actions of antioxidants in biological or food systems include directly quenching free radicals to terminate the radical chain reaction, chelating transition metals to suppress the initiation of radical formation, acting as reducing agents, or stimulating the antioxidative defense enzyme activities (*11*, *23*). This study was conducted to evaluate (i) free radical scavenging properties of three hard winter wheat varieties currently produced in the United States and (ii) whether the radical scavenging activities may be related to the TPC. The information obtained from this study will be used to produce improved wheat varieties high in antioxidants through a wheat breeding program for commercial production and promote the new utilizations of existing wheat varieties.

Table 2. TPC[a] of Wheat Grains

variety	TPC (mg gallic acid/g grain)	TPC (μg gallic acid/mg extract)	TPC (μg catechin/mg extract)
Akron	927.8 ± 9.6	24.1 ± 0.2	30.8 ± 0.2
Trego	642.2 ± 3.7	23.0 ± 0.1	29.4 ± 0.1
Platte	487.9 ± 9.6	16.2 ± 0.3	20.7 ± 0.4

[a] TPC was expressed as gallic acid and catechin equivalences; $n = 3$.

In this study, all three tested wheat varieties showed significant scavenging activities against DPPH and $ABTS^{•+}$ radicals. Trego grain had the lowest activity against both DPPH and $ABTS^{•+}$ radicals. The relative radical quenching activities of Akron, Platte, and Trego grains are 16.5:8.1:1 against DPPH•, while Platte had the stronger radical scavenging activity against $ABTS^{•+}$ than Akron, on a per weight basis. The different relative radical scavenging capacity of individual wheat extract against different testing radicals may be explained by the different mechanisms involved in the radical−antioxidant reactions. In this study, the $ABTS^{•+}$ was generated by incubating ABTS with a peroxidase and hydrogen peroxide. Chemical compounds that inhibit the peroxidase activity may reduce the production of $ABTS^{•+}$. This reduction results in a decrease of the total $ABTS^{•+}$ in the system and contributes to the total $ABTS^{•+}$ scavenging capacity. Other factors, such as stereoselectivity of the radicals or the solubility of wheat extracts in different testing systems, may also affect the capacity of individual wheat extract to react and quench different radicals. This observation is supported by the study of Wang et al. (*19*). In their study, the different relative activity of selected antioxidants against DPPH• and $ABTS^{•+}$ was also detected. In addition, Wang and Jiao (*27*) evaluated the radical scavenging capacity of berry crops using superoxide radicals, hydroxyl radicals, and other reactive oxygen species. The berry crop that had a greater scavenging activity against superoxide radicals did not necessarily have a higher activity to quench hydroxyl radicals (*27*). ESR is considered as the most reliable method for radical detection and has been successfully used for evaluating radical quenching capacity of antioxidants (*17*, *22*). ESR data further confirm that wheat antioxidants can directly react with and quench free radicals.

As compared to vitamin E, vitamin C, and BHT, the well-known synthetic antioxidants, wheat extracts showed great DPPH radical quenching capacity, although they differed to each other in their relative activities. Akron extract at a level of 2.0 mg/mL quenched 92.9% DPPH radicals, which was greater than 50 mM of vitamin E (21.5 mg/mL) or BHT (11.0 mg/mL) and was comparable to vitamin C at the level of 50 mM or 8.8 mg/mL (quenched 94.5% DPPH radicals) under the experimental conditions. About 51 mg of Akron grain is able to provide 2.0 mg of extract. These data suggest that wheat may serve as a potential source for natural antioxidants.

Zielinski and Kozlowska (*11*) reported 54 and 150 μmol trolox equivalents/g of lyophilized wheat extract prepared with 80% methanol. In the present study, the scavenging activity of wheat extracts against $ABTS^{•+}$ was lower (1.08−1.91 μmol trolox equivalents) on a per gram wheat grain basis but similar (37−62 μmol trolox equivalents) to the low range reported by

Zielinski and Kozlowska when considered on a per gram of wheat extract basis, although the difference in $ABTS^{•+}$ activities and the lacking of correlation between TPC and $ABTS^{•+}$ scavenging capacity observed in the two studies could be explained by the wheat varieties, extraction solvent, and the solvent used to prepare the antioxidant solution for $ABTS^{•+}$ scavenging activity assays. Individual wheat varieties may contain different antioxidant compounds at different levels. This explains well why individual wheat varieties may differ in their radical scavenging activity regardless of extraction solvent and the testing radical systems and is supported by our observations of the three hard winter wheat varieties for their $ABTS^{•+}$ and DPPH• scavenging activities as well as by the previous observation that two spring wheat varieties also differed in their radical quenching capacity (*11*). DMSO was used to prepare wheat antioxidant solution and dilute trolox standard and used to determine the reagent blank in the present study, while Zielinski and Kozlowska dissolved 80% methanol extract lyophilizate with pure methanol for the assay (*11*). DMSO was used in our study since precipitation was observed in the reaction mixture when the ethanol solution of wheat antioxidants was used in the assay. Solvents may have interactions with other reagents in the assay system, including the enzyme, and consequently alter the testing results; however, this would be unlikely to account for the differences if the standard solution and reagent blank were prepared with the same solvent used to dissolve the antioxidant. In addition, the extracting solvent and extraction method may contribute to the different radical scavenging activities of wheat extracts. Aqueous alcohols and acetone, with different levels of water, have been widely used to extract phenolic components from botanical materials, especially herbs. The solvent system is generally selected according to the purpose of extraction, polarity of the interested components, polarity of undesirable components, overall cost, and safety. The efficiency of ethanol (80%) and methanol (80%) on wheat antioxidant extraction was compared and reported similar by Zielinski and Kozlowska (*11*). Although methanol might improve extraction efficiency as observed by Zielinski and Kozlowska, ethanol is less toxic and has a better recovery ratio from the reduced pressure distillation for reuse. Additional value would be gained for wheat producers if both the wheat antioxidant extracts and the grain fractions could be utilized (*11*). While adding a certain amount of water in ethanol might improve the extracting efficiency, this will increase the total cost of the wheat antioxidants since the solvent cannot be easily reused, and additional steps will be required to further remove water from both antioxidant preparation and grain fractions. A freeze-dry procedure may have to be employed to remove water from the aqueous antioxidant extracts after the organic solvent is removed by distillation under reduced pressure (*11*). Freeze-dry operation limits the scale-up of production and increases the total cost of the antioxidants. Therefore, absolute ethanol was used in the present study.

Recently, antioxidative phytochemicals in grains, vegetables, and fruits have obtained great attention for their potential roles in human disease prevention and food quality improvement. Phenolics were considered as a major group of compounds that contribute to the antioxidant activities of the grains. The phenolic contents of the three tested wheat varieties were 487.9−927.8 mg/kg grain, which is higher than the reported total phenolic content of 500 mg/kg for edible cereals (*11*). Furthermore, the phenolic contents of the three wheat extracts were greater than those reported for lyophilizate of 80% methanol extracts reported by Zielinski and Kozlowska (*11*), suggesting that Soxhlet extraction with absolute ethanol is an acceptable procedure to prepare wheat antioxidants. In contrast to the previous report by Zielinski and Kozlowska (*11*), no correlation between TPC and free radical scavenging activities against both DPPH• and $ABTS^{•+}$ was detected in this study. This indicates the presence of other components in wheat extracts that can directly react with radicals in addition to phenolic compounds, although this may be partially explained by the fact that the conclusion was highly influenced by the high concentration points in Zielinski and Kozlowska's report (*11*). These components may have a wide variety of chemical structures that could react with radicals by donating protons (free radical quenching), radical addition, redox reaction (electron transfer), and radical recombination. Some examples of these compounds are commonly accepted antioxidants such as vitamin C, aromatic amines, and sulfur-containing compounds. Further composition analysis of wheat extracts is necessary to clearly explain the different observations of the correlation between TPC and radical scavenging activities from this and Zielinski and Kozlowska's studies (*11*).

In conclusion, this study indicates that wheat contains significant free radical scavenging activities against stable DPPH• and radical cation $ABTS^{•+}$. Wheat varieties significantly differed in their free radical scavenging properties against stable DPPH•, radical cation $ABTS^{•+}$, and TPC, except that they shared the same reaction kinetics when reacting with DPPH radical, suggesting that it is necessary to further screen for and breed to obtain value-added wheat varieties containing high levels of antioxidants for production. More research is needed to investigate the chemical components that contribute to total antioxidant activities and the relationship between antioxidant activity and health benefits.

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