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Optimization of the Extraction of Antioxidative Constituents of Six Barley Cultivars and Their Antioxidant Properties

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Response surface methodology (RSM) was used to predict the optimum conditions of extraction of barley samples (organic solvent percent in the extraction medium, temperature, and time). Antioxidant capacity in the barley meals was highest under optimum extraction conditions of 80.2% methanol and 60.5 °C for 38.36 min as predicted by RSM. Phenolic antioxidative compounds of six barley cultivars, namely, Falcon, AC Metcalfe, Tercel, Tyto, Phoenix, and Peregrine, were extracted under the conditions obtained by RSM after defatting with hexane, and subsequently the extracts were assessed for their antioxidant and antiradical activities and metal chelation efficacy. The potential of barley extracts in inhibiting peroxyl and hydroxyl radical induced supercoiled DNA double-strand scission was also studied. Total phenolic content as measured according to Folin−Ciocalteu's method ranged from 13.58 to 22.93 mg of ferulic acid equiv/g of defatted material, with the highest content in Peregrine. Total antioxidant activity as measured by Trolox equivalent antioxidant capacity ranged from 3.74 to 6.82 μmol/g of defatted material. Metal chelation capacity of the extracts as measured by 2,2′-bipyridyl competition assay varied from 1.1 to 2.1 μmol of ethylenediaminetetraacetic acid equiv/g of defatted material. IC_{50} values for 1,1-diphenyl-2-picrylhydrazyl radical as measured by electron paramagnetic resonance ranged from 1.51 to 3.33 mg/mL, whereas the corresponding values for hydroxyl radical ranged between 2.20 and 9.65 mg/mL. Inhibition of peroxyl radical induced supercoiled DNA scission ranged from 78.2 to 92.1% at the concentration of 4 mg/mL of extracts, whereas the corresponding values for hydroxyl radical induced DNA scission ranged from 53.1 to 65.3%.

KEYWORDS: Antioxidants; barley; DNA; DPPH; EPR; optimization

INTRODUCTION

Barley is consumed around the world for pasta and other baked products, brewing, and malted products. Over the past few years, it has gained increasing attention due to its positive dietary role. A number of bioactives such as β-glucan, tocols, and phenolic compounds have been identified in barley. Phenolic antioxidative compounds such as benzoic and cinnamic acid derivatives, proanthocyanidins, quinines, flavanols, and flavons are reported to be present in barley (*1*, *2*). A great body of evidence has been accumulated that plant polyphenols are an important class of defense antioxidants.

Recent research has shown that the complex mixture of phytochemicals in food provides better protective health benefits than single substances through a combination of additive and/or synergistic effects (*3*). Grains contain unique phytochemicals that complement those in fruits and vegetables when consumed together. A wide array of phenolic compounds in grains is present, and these include derivatives of benzoic and cinnamic acids, anthocyanidins, quinines, flavanols, chalcones, flavones, flavanones, and aminophenolic compounds, among others (*4*). Flavonoids play different roles in the ecology of plants: defense against insects, catalysis of the light phase of photosynthesis (*5*), regulation of iron channels involved in phosphorylation (*6*), and protection against stress by scavenging reactive oxygen species produced by photosynthesis (*7*). The protective effects of flavonoids have been evidenced by in vitro, ex vivo, and animal studies (*8*).

Natural antioxidants in cereal grains may act as free radical scavengers, reducing agents, and potential complexers of metal ions (*9*). The outer layers of cereal grains (pericarp, testa, and aleurone layer) contain the highest concentration of phenolics, whereas the starchy endosperm contains a considerably lesser amount. Some of the phytochemicals, such as ferulic acid and diferulates, are predominantly found in grains, but are not present in significant quantities in fruits and vegetables (*4*).

As with any chemical process, utilization of oxygen by humans is not 100% efficient; thus, this inefficiency results in the formation of toxic free radicals. Although free radicals play a role in signal transduction and phagocytosis, overproduction leads to oxidative stress, which may result in damage to biomolecules such as DNA, RNA, lipid, and protein. Especially with DNA, over time DNA damage becomes cumulative and is believed to account for the drastic increase in cancer rate

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seen with aging in humans and animals (*10*). The phytochemicals in fruits, vegetables, and grains are known to combat oxidative stress through reducing the risk of the said disease conditions.

Response surface methodology (RSM) is a widely used tool to analyze responses, which are affected by multiple variables and their interactions (*11*). RSM is capable of examining a number of variables at a time, using special experimental designs minimizing the number of required determinations (*12*). One of the major advantages of RSM is its ability to take into account the interactions among different variables as opposed to traditional one variable at a time analyses. An appropriate experimental design can reduce the number of experiments or observations, and a mathematical model can be fitted to data acquired from a selected number of variable combinations (*13*).

The objectives of this study were to first optimize the antioxidant extraction parameters using RSM and explore the antioxidant, antiradical, and DNA-protecting properties of six barley cultivars grown in Canadian prairies.

MATERIALS AND METHODS

Materials. Six barley cultivars from the 2002 crop year, namely, Falcon, AC Metcalfe, Tyto, Tercel, Phoenix, and Peregrine (grown under the same conditions), were obtained from the Field Crop Development Center, Lacombe, AB, Canada. Samples were prepared as described in a later section.

Sodium carbonate, sodium chloride, hexane (ACS grade), methanol, and ethanol (HPLC grade) were purchased from Fisher Scientific Co. (Nepean, ON, Canada). Compounds 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), tetrazolium, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), ferulic acid, ferric chloride, DNA (pBR Plasmid *Escherichia coli* strain RRI), Trisma base, ethidium bromide, xylene cyanol, glycerine, bromophenol blue, agarose, Folin−Ciocalteu's reagent, mono- and dibasic sodium and potassium phosphates, ethylenediaminetetraacetic acid (EDTA), Tris-HCl buffer (pH 7.4), 2,2′-bipyridyl, hydroxylamine hydrochloride, Trolox, and diethylenetriaminepentaacetic acid were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

Methods. *Preparation of Barley Samples.* Barley samples were manually hulled and ground to obtain a fine powder using a laboratory mill with a 60 mesh sieve (Tecator 3420, Tecator Inc., Boulder, CO). The so-obtained barley meals were defatted by blending with hexanes (1:5 w/v, 5 min) in a Waring blender (model 33BL73, Waring Products Division, Dynamics Co. of America, New Hartford, CT) at ambient temperature. Defatted samples were vacuum packaged in polythene pouches and stored in a freezer at −20 °C until used for analysis.

Falcon cultivar that yielded an average Trolox equivalent antioxidant activity (TEAC) in a series of preliminary experiments was used as the representative barley sample for optimization of the extraction conditions using RSM. It is important to know the range of each variable (solvent percent, temperature, and time course) for which the maximum response (antioxidant activity) may be obtained. Thus, before RSM was employed, approximate conditions for extraction, namely, the solvent percentage, temperature, and time, were determined by varying one factor at a time while the other factors were kept constant.

Determination of Three Levels of Independent Variables (X_1, X_2, and X_3). Three levels for each independent variable were selected using the procedure outlined below. Phenolic components present in the barley meals were extracted using different solvents (ethanol, methanol, and acetone) at various temperatures over a series of time courses. Barley meals (6 g) were extracted twice into different solvent systems (100 mL, 30−100%, v/v, ethanol, methanol, or acetone) at different temperatures (30−80 °C) over 20−80 min in a thermostated water bath with continuous mixing. Defatted Falcon sample (6 g) was extracted with 100 mL of organic solvent (ethanol, methanol, and acetone, each 30−100%, v/v) under reflux conditions at 60 °C for 30 min. The resulting slurries were centrifuged at 4000*g* for 5 min (ICE Centra M5, International Equipment Co., Needham Heights, MA) and the supernatants collected. The pooled supernatants were evaporated in vacuo at 35 °C, and the resulting concentrated solution was lyophilized for 72 h at −47 °C and 30×10^{-3} mbar (Freezone model 77530, Labconco Co., Kansas City, MO). The lyophilized powder was placed in screw-capped glass vials until used for analysis. The total antioxidant capacity (TAC) of the extracts was determined by TEAC assay as explained in a later section. On the basis of the TEAC values, the best solvent type and the suitable solvent percentages were selected.

Three levels of temperature variable (X_2) were determined by carrying out a series of extractions at different temperatures (40−80 °C) with 80% methanol for 30 min. The extraction was repeated once more, and the slurries were combined, evaporated, and lyophilized as explained earlier. In another series of experiments, the samples were extracted with 80% methanol at 60 °C for different time periods (20−80 min) to determine the appropriate three levels (X_3); extraction was repeated once more, and the slurries were treated as mentioned earlier.

Use of RSM To Establish the Optimum Extraction Parameters. Three-factor, three-level face-centered cube design with 17 different design points was adapted for this purpose (*12*, *14*). Three independent factors studied were organic solvent content (%, v/v, X_1), extraction temperature (°C, X_2), and extraction time (min, X_3) (**Table 1**). Response (Y) was the antioxidative index (TEAC values) calculated for the extracts. The extractions were carried out as detailed in **Table 2**, and the TEAC value of each extraction was determined. The following was used in the RSM.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{I<j=I}^{3} \Sigma \beta_{ij} X_i X_j$$

In this equation β_0, β_i, β_{ii}, and β_{ij} are regression coefficients for intercept, linear, quadratic, and interaction terms, respectively. X_i and X_j are independent variables. Antioxidant activity (TEAC values) was analyzed

Table 1. Variable (X_1, X_2, and X_3) Levels Used for Response Surface Methodology

		coded variable levels		
variable	symbol	(−1)	(0)	(+1)
solvent (methanol) (% v/v)	X_1	70	80	90
extraction temperature (°C)	X_2	40	60	80
extraction time (min)	X_3	20	40	60

Table 2. Face-Centered Cube Design and Observed Responses[a]

	independent variables[b]			
design point	X_1[c]	X_2[d]	X_3[e]	response (Y)[f]
1	−1	−1	−1	63.1
2	−1	−1	+1	64.3
3	−1	+1	−1	65.4
4	−1	+1	+1	66.2
5	+1	−1	−1	68.6
6	+1	−1	+1	67.2
7	+1	+1	−1	66.9
8	+1	+1	+1	65.9
9	−1	0	0	74.9
10	+1	0	0	75.8
11	0	−1	0	87.6
12	0	+1	0	90.6
13	0	0	−1	92.7
14	0	0	+1	96.7
15	0	0	0	99.2
16	0	0	0	102
17	0	0	0	101

[a] Nonrandomized. [b] See **Table 1** for actual values. [c] X_1, solvent (methanol) content (% v/v) in the extraction media. [d] X_2, extraction temperature (°C). [e] X_3, extraction time (min). [f] Y, TEAC value (μmol of Trolox equivalents/g of extract). Mean of duplicate determinations except for points 15–17, for which trials were run in triplicate.

using a general linear model (GLM), and response surface regression coefficients were substituted in the quadratic polynomial equation. Response surfaces and contour plots were obtained using the fitted model. Verification experiments were carried out using combinations of variables predicted by the model to determine the adequacy of the model fitted. The extraction parameters (X_1, X_2, and X_3) established by RSM were used to extract the phenolic components from barley extracts.

Determination of Total Phenolic Content. Extracts were dissolved in methanol to obtain a concentration of 3 mg of extract/mL of solution. The total content of phenolics was determined according to the procedure explained by Singleton and Rossi (*15*) with some modifications. Total phenolic content was expressed as milligrams of ferulic acid equivalents per gram of defatted material.

TAC. TAC was determined by TEAC assay as reported by van den Berg et al. (*16*) with some minor modifications. TEAC values were expressed as micromoles of Trolox equivalents per gram of defatted material.

Determination of Metal Chelation Activity. Fe(II) chelation activity of the barley extracts (6 mg/mL) was measured using a 2,2′-bipyridyl competition assay as explained by Yu et al. (*17*) and Yamaguchi et al. (*18*).

Determination of DPPH Radical Scavenging Capacity Using Electron Paramagnetic Resonance (EPR). The DPPH radical scavenging assay was carried out using the method explained by Diaz et al. (*19*) with slight modifications. Two milliliters of a 0.18 mM solution of DPPH in methanol was added to 400 μL of various concentrations (0.67−3.33 mg/mL, final concentration) of extracts dissolved in methanol. Contents were mixed well, and after 1 min, the mixture was passed through the capillary tubing, which guides the sample through the sample cavity of a Bruker e-scan EPR spectrometer (Bruker E-scan, Bruker Biospin Co., Billercia, MA). The spectrum was recorded on a Bruker E-scan food analyzer (Bruker Biospin Co.). The parameters were set as follows: 5.02×10^2 receiver gain, 1.86 G modulation amplitude, 2.621 s sweep time, 8 scans, 100.000 G sweep width, 3495.258 G center field, 5.12 ms time constant, 9.795 GHz microwave frequency, 86.00 kHz modulation frequency, 1.86 G modulation amplitude. For quantitative measurements of radical concentration remaining after reaction with the extracts, the method of comparative determination based on the corresponding signal intensity of first-order derivative of absorption curve was used. DPPH radical scavenging capacities of the extracts were calculated using the following equation:

DPPH radical scavenging capacity, % =
100 − (EPR signal intensity for the medium containing the additive/ EPR signal intensity for the control medium) × 100

Determination of Hydroxyl Radical Scavenging Capacity Using EPR. The hydroxyl radical was generated via Fe(II)-catalyzed Fenton reaction and spin trapped with DMPO. The resultant DMPO-OH adduct was detected using a Bruker E-scan EPR (Bruker Biospin Co.). Barley extracts were dissolved in deionized water and diluted to obtain various concentrations (4−20 mg/mL). Extracts (100 μL) were mixed with 100 μL of 10 mM H_2O_2, 200 μL of 17.6 mM DMPO, and100 μL of 1 mM $FeSO_4$. All solutions were prepared in deionized water except $FeSO_4$, which was dissolved in deoxygenated distilled water to maintain reduced status until mixed with the other reagents. After 1 min, the mixtures were passed through the plastic capillary tubing, which guides the sample through the sample cavity of the magnet unit of EPR spectrometer. The EPR spectrum was recorded at 5.02×10^2 receiver gain, 1.86 G modulation amplitude, 2.621 s sweep time, 8 scans, 100.000 G sweep width, 3495.258 G center field, 5.12 ms time constant, 9.795 GHz microwave frequency, 86.00 kHz modulation frequency, and 1.86 G modulation amplitude. Hydroxyl radical scavenging capacities of the extracts were calculated using the following equation:

hydroxyl radical scavenging capacity, % =
100 − (EPR signal intensity for the medium containing the additive/ EPR signal intensity for the control medium) × 100

Inhibition of Peroxyl Radical and Hydroxyl Radical Induced Supercoiled DNA Double-Strand Scission. Supercoiled plasmid DNA scission was carried out in 10 mM saline phosphate buffer solution (pH 7.4). The reaction mixture contained 4 μL of 25 ng/μL supercoiled plasmid DNA (pBR 322 strain RRI, 43 kbp), 4 μL of barley extracts of different concentrations (1.33−6.67 mg/mL, final concentration), and 4 μL of 1 mM (final concentration) APPH. The reaction was carried out in microcentrifuge tubes with a final volume of 12 μL. The reaction mixture was incubated at 37 °C for 2 h in an incubator in the dark. After incubation, 3 μL of loading dye (0.25% bromophenol blue, 0.25% of xylene cyanol, and 50% glycerine) was added, and the mixture was loaded onto a 0.7% agarose (w/v) gel prepared in Tris−acetic acid−EDTA buffer (40 mM Tris acetate and 2 mM EDTA, pH 8.5). Agarose gel was subjected to horizontal submarine gel electrophoresis for 5 h at 60 V using the same buffer. The gel was unloaded, stained with 0.5 μg/mL ethidium bromide staining solution, and destained with excess amounts of deionized water for 30 min. The bands were visualized under UV light, and the images were photographed by a GelDoc apparatus equipped with a Sony camera. The images were analyzed using AlphaEase stand-alone software (Alpha Innotech Co., San Leandro, CA). The level of inhibition was determined by comparing the intensity of the band of initial supercoiled with the intensity of the supercoiled bands treated with antioxidative extracts. A control devoid of AAPH and extracts was used.

In another experiment, the effect of extracts on inhibiting hydroxyl radical induced supercoiled DNA scission was investigated. AAPH was replaced with a mixture of 2 μL each of 100 μM H_2O_2, 10 μM $FeCl_3$, 100 μM EDTA, and 10 μM ascorbic acid, which generate hydroxyl radical (*20*).

Statistical Analysis. One-way analysis of variance (ANOVA) and Tukey's studentized range test (*14*) were carried out at the $p < 0.05$ significance level.

RESULTS AND DISCUSSION

Selection of Factor Levels for Solvent Concentration (X_1). Panels **a**, **b**, and **c** of **Figure 1** show the effect of extraction with aqueous organic solvents on the antioxidant activity of the resultant extracts. The response (TEAC value) followed a second-order function of the solvent composition (X_1). The TEAC value increased with increasing organic percentage of the solvent for all solvents, reached a maximum, and then started decreasing. Maximum antioxidant activity was obtained when the solvent content in the extraction medium was 80% for methanol and ethanol, whereas 70% acetone yielded the highest antioxidant activity. Existing differences in the simple phenolics versus condensed tannins might be responsible for this observation. Although the former compounds are more soluble in methanol, along with other impurities the latter group is known to be stronger antioxidants (*21*). Among the solvents used, methanol yielded the highest antioxidant activity. Thus, aqueous methanol was selected as the medium of extraction. The solvent concentrations of 70, 80, and 90% were selected as the lower, middle, and upper design points, respectively. No attempt was made to release insoluble bound phenolics in this study.

Selection of Factor Levels for Temperature (X_2) and Time (X_3). The effect of extraction temperature and time on antioxidant activity also followed a polynomial function (**Figure 1d**). Extraction at a low temperature yielded lower antioxidant activity as measured by TEAC value, which gradually increased up to about 60 °C and then exhibited a downward trend. Therefore, 40, 60, and 80 °C were selected as the lower, middle, and upper points, respectively. Extraction of antioxidative compounds was not efficient at low temperatures, whereas high temperatures might lead to destruction of antioxidative components, thus leading to low TEAC values. Times of <30 min did not yield high antioxidant activity due to insufficient time duration available for the extraction process, whereas prolonged extraction time might lead to destruction of antioxidative compounds (**Figure 1e**).

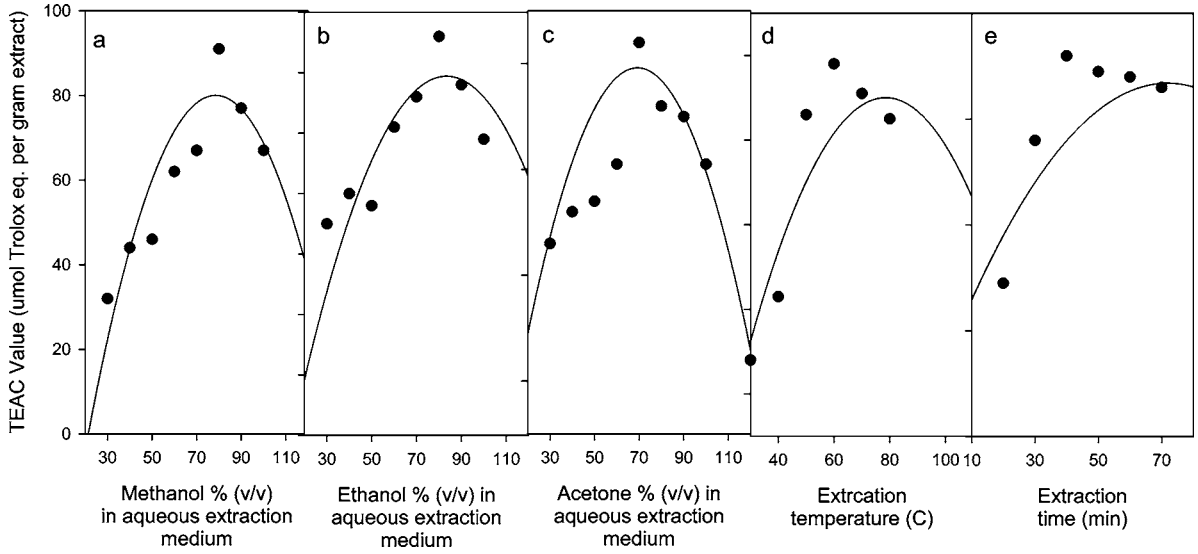

Figure 1. Effect of different extraction media (**a**, methanol; **b**, ethanol; **c**, acetone), extraction temperature (**d**), time (**e**), and duration of extraction on antioxidant capacity of Falcon barley extract.

Response Surface Analysis. RSM is an optimization technique that determines optimum process conditions by combining special experimental designs with modeling by a first- or second-order polynomial equation in a sequential testing procedure. Three independent variables (X_1, X_2, and X_3) were assigned a code (**Table 1**). The center point of each independent variable level was given a code of 0, whereas the lowest and highest levels of interest for each variable were coded −1 and +1, respectively.

In this particular case, the face-centered cube design was used as the experimental design in analyzing the data. The response (TEAC value) obtained for 17 different combinations (**Table 2**) was analyzed using the face-centered cube design model. Regression coefficients of intercept, linear, quadratic, and interaction terms of the experimental model were calculated and their levels of significance determined using the *t* test. Multiple regression coefficients obtained by employing a least-squares procedure to predict the quadratic polynomial model for the antioxidant capacity are summarized in **Table 3**. Examination of these parameters with the *t* test indicated that linear and quadratic terms of solvent percent were highly significant ($p = 0.0001$).

The polynomial model fitted to experimental data was highly significant ($p < 0.05$). The coefficient of determination (r^2) was 0.98; thus, the model explained most of the observed variation. The coefficient of variation (CV) of 2.90% indicated that the model was reproducible. The predicated second-order polynomial model was

$$Y = 98.906 - 2.7693w^2_1 - 8.3585w^2_2 - 22.1418w^2_3$$

where Y is response (TEAC) and w_1, w_2, and w_3 are the axes of response surfaces.

Response surface analysis revealed that the methanol content (X_1) in the extraction medium had the greatest effect on the antioxidant activity of extract followed by linear effect of temperature (X_2) and time (X_3).

Figure 2 illustrates the nature of the response surfaces. The stationary point was determined by performing canonical analysis of the response surface. Contour plots were generated using data obtained from canonical analysis. All eigenvalues were negative, indicating a maximum or minimum stationary point. On the basis of the response surface mesh plots, the stationary point was a maximum. Canonical analysis revealed the critical values as $X_1 = 80.24\%$, $X_2 = 60.47$ °C, and $X_3 = 38.36$ min. Extractions carried out using the parameters predicted by the model indicated that the observed responses were in agreement with predicted values.

Table 3. Estimated Regression Coefficients of the Quadratic Polynomial Models for Extraction of Antioxidative Components from Barley

parameter[a]	estimated coefficient	standard error
intercept	–1461.254****[b]	149.227
β_0		
linear	36.2222****	3.8645
β_1	2.6576	0.9196
β_2	1.1681	0.7637
β_3		
quadratic	–0.2256****	0.0239
β_{11}	–0.0283	0.0059
β_{22}	–0.0068	0.0059
β_{33}		
interaction	–0.0077	0.0069
β_{12}	–0.0091	0.0069
β_{13}		
β_{123}		
R^2[c]	0.9895	
F value	73.41	
P value	<0.0001	
CV[d] (%)	2.9034	

[a] Parameters refer to the general linear model where β_0, β_i, β_{ii}, β_{ij}, and β_{ijk} are regression coefficients for intercept, linear, quadratic, and interaction terms, respectively. [b] ****, significant at 0.0001 level. [c] R^2, regression coefficient. [d] CV, coefficient of variation.

Total Phenolic Content. The percentage yield obtained under optimum extraction conditions established by RSM varied between 4.86 and 5.47%. These values lie in the range reported by Zielinski et al. (*22*). Xing and White (*23*) reported that methanol was one of the best solvents for extracting phenolics and other polar materials from cereals. The total phenolic content of the barley cultivars tested ranged from 0.81 to 1.38 mg of ferulic acid equiv/g of defatted material. The values ranged from

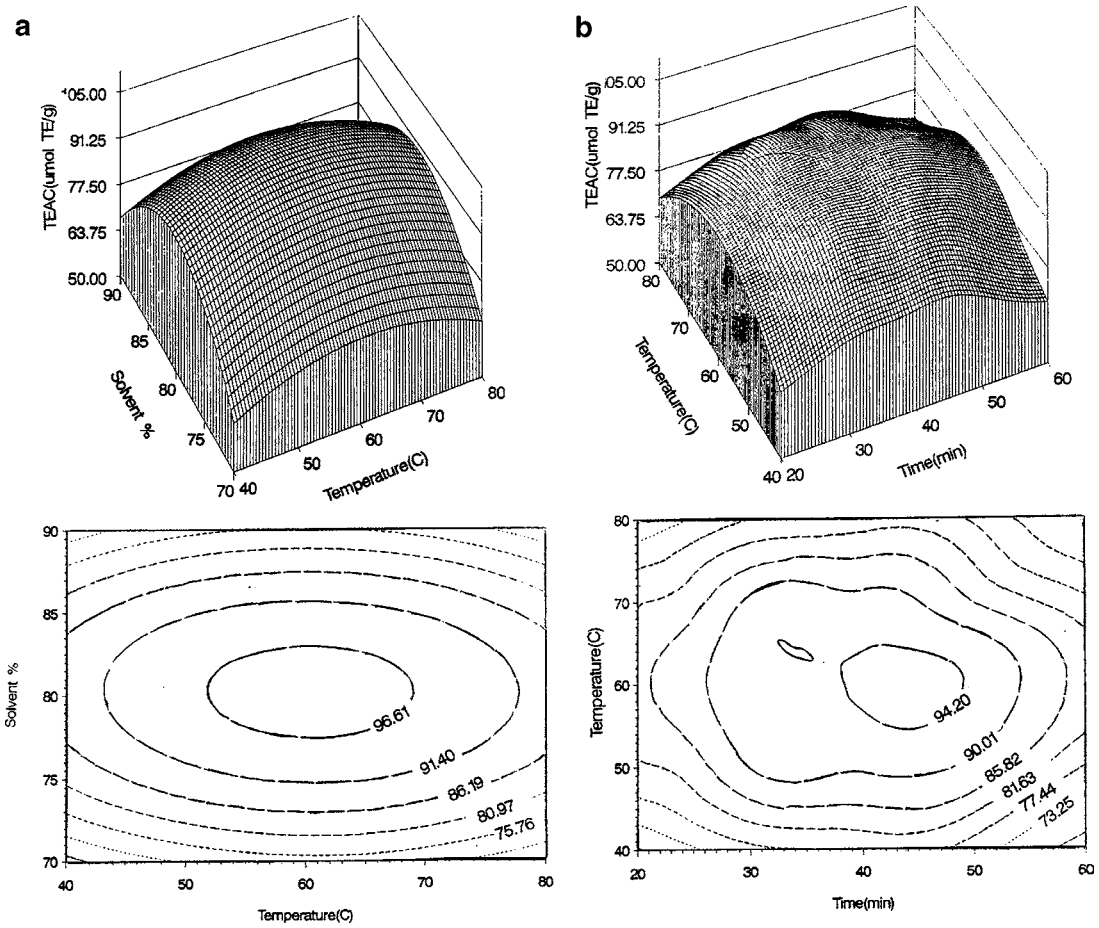

Figure 2. Dependence of the antioxidant activity of Falcon barley extract on (**a**) methanol content in the aqueous medium and extraction temperature and (**b**) extraction temperature and time.

Table 4. Yield, Total Phenolic Content (TPC), and Total Antioxidant Capacity (TAC) As Measured by Trolox Equivalent Antioxidant Capacity (TEAC) of Barley Extracts[a]

barley cultivar	extract yield (%)	TPC (mg of ferulic acid/g of defatted material)	TEAC (μmol/g of defatted material)
Falcon	5.1 ± 0.3	16.31 ± 0.22d	5.27 ± 0.03b
AC Metcalfe	5.7 ± 0.9	19.99 ± 0.15e	5.33 ± 0.20c
Tyto	4.7 ± 0.7	15.53 ± 0.23c	4.92 ± 0.04a
Tercel	5.2 ± 0.6	13.58 ± 0.09a	3.74 ± 0.05a
Phoenix	4.6 ± 0.6	14.87 ± 0.10b	4.23 ± 0.24a
Peregrine	5.2 ± 0.9	22.93 ± 0.07f	6.82 ± 0.26d

[a] Results are means of three determinations ± standard deviation. Values in each row (within a single cultivar category) having the same letter are not significantly different ($p > 0.05$).

13.58 to 22.93 mg of ferulic acid equiv/g on the basis of lyophilysate weight. The total phenolic contents of the six barley cultivars on a defatted weight basis were Peregrine > AC Metcalfe > Falcon > Tyto > Phoenix > Tercel (**Table 4**). Zielniski and coauthors (*22*) reported that barley cultivars Mobek and Gregor contained 26.9 and 24.3 mg of catechin equiv/g of lyophilysate extracted using 80% methanol. Barley cultivars tested in our study contained more total phenolic content than wheat, rye, and oat reported by Zielinski et al. (*22*).

The Folin−Ciocalteu method used to measure TPC in this study is not a specific test for phenolic compounds but a general test that determines readily oxidizable groups found in extracts. The method involves two components in the reaction mixture, antioxidant and oxidant (also serves as the probe), which follows the following electron transfer reaction:

$$\text{probe (oxidant)} + \text{e (from antioxidant)} \rightarrow \text{reduced probe} + \text{oxidized antioxidant}$$

The probe is an oxidant that accepts an electron from the antioxidant, causing color change of the probe (*24*). The heteropoly phosphotungstic−molybdic complex (Folin−Ciocalteu reagent) used in the assay is oxidized by reducing agents present in the extract, mainly phenolic groups. Thus, this method is not specific to phenolic compounds as the complex can also be reduced by nonphenolic compounds. However, the relationship between TPC and TAC was strong, with a regression coefficient of 0.97. On the other hand, the efficacy of inhibiting DNA scission and DPPH and hydroxyl radical scavenging capacities did not strongly correlate with TPC, indicating that factors other than TPC may play a role in the antioxidant activity of the extracts. Moreover, all phenolics do not bear the same level of antioxidant efficacy. Phenolic compounds may have antagonistic or synergistic effects with themselves or with other constituents of the extracts (*25*). Other than phenolic compounds, protein in barley that was extracted in aqueous methanol may contribute to antioxidant activity. Iwama et al. (*26*) reported that cereal proteins exert a strong antioxidant activity.

Goupy et al. (*27*) reported that flavan-3-ols constitute the major class of phenolics in barley. They included catechin and epicatechin, whereas the most abundant dimers were prodel-

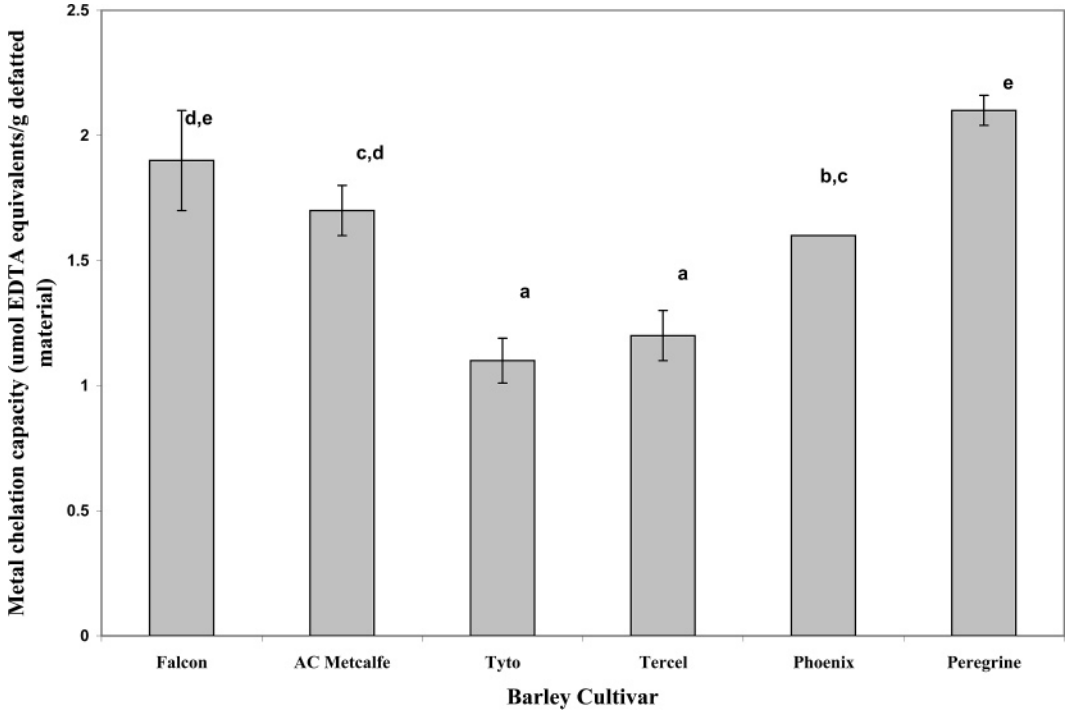

Figure 3. Fe(II) chelation capacity of barley extracts as determined by 2,2′-bipyridyl competition assay.

phinidn B_3 and procyanidin B_3 (*28*). The major trimers were procyanidin C_2 and proanthocyanidins T_1, T_2 and T_3. Monomeric, dimeric, and trimeric flavan-3-ols accounted for 58−68% of total phenolic content (*29*).

In a separate study, we found that ferulic acid was the major phenolic acid present in barley among other phenolic acids, namely, vanillic acid, *p*-coumaric acid, and caffeic acid (*30*). Hydroxycinnamic acid and hydroxybenzoic acids were the primary phenolic antioxidants acting as free radical acceptors (*27*).

TAC Measured by TEAC. TEAC values for barley cultivars varied from 3.74 to 6.82 μmol/g of defatted material. The order of TEAC values of the six cultivars was Peregrine > AC Metcalfe = Falcon > Tyto = Phoenix > Tercel (**Table 4**). TEAC values were well correlated ($r^2 = 0.97$) with total phenolic contents of the cultivars. The TEAC value of a compound represents the concentration of Trolox (a water-soluble vitamin E analogue without the side-chain moiety) that has the same antioxidant capacity as the compound or a mixture of compounds of interest (*16*). Thus, the TEAC value may be considered as a stoichiometric number related to TEAC, for Trolox of 1.

Fe(II) Chelation Activity of Barley Extracts. The chelation activities of barley extracts were examined against Fe(II) and reported as EDTA equivalents. The Fe(II) chelation capacity of the barley cultivars varied from 1.1 to 2.1 μmol of EDTA equiv/g of defatted material. The Fe(II) chelation capacity of Peregrine cultivar was the highest of all the cultivars (**Figure 3**). Yu et al. (*17*) reported that wheat bran contained 1.55−1.85 μmol of EDTA equiv/g of defatted material. In another study, we found that the metal chelation activity of barley bran extracts was significantly higher than that of wheat (results not reported). Fe(II) is one of the important transition metal ions that contribute to initiation of lipid peroxidation in both food and biological systems. It also contributes to the generation of hydroxyl radicals via Fenton's reaction, which in turn attack biomolecules. Thus, the Fe(II) chelating ability of an antioxidant extract is one of the key features used in assessing its antioxidant efficacy.

Table 5. IC_{50} Values of Barley Extracts for DPPH and Hydroxyl Radicals As Measured by Electron Paramagetic Resonance

	IC_{50} value (mg/mL)	
barley cultivar	DPPH radical	hydroxyl radical
Falcon	2.12 ± 0.21bc	2.20 ± 0.04a
AC Metcalfe	1.65 ± 0.17ab	2.81 ± 0.12b
Tyto	2.60 ± 0.02c	2.40 ± 0.06a
Tercel	>3.33 ± 0.36d	9.65 ± 0.04e
Phoenix	3.20 ± 0.09d	5.31 ± 0.13d
Peregrine	1.51 ± 0.02a	3.14 ± 0.11c

[a] Results are means of three determinations ± standard deviation. Values in each row (within a single cultivar category) having the same letter are not significantly different ($p > 0.05$).

DPPH Radical Scavenging Capacity by EPR. The DPPH radical has been widely used in model systems to investigate the scavenging activities of antioxidative compounds. All of the barley extracts tested exhibited a strong antioxidant activity against DPPH$^\bullet$ in a concentration-dependent manner. The IC_{50} value is defined as the amount of extract (milligrams per milliliter) required to lower the initial DPPH radical concentration by 50%, and this was extrapolated from the dose-dependent curves. The IC_{50} values obtained for six barley cultivars ranged from 1.51 to 3.33 mg/mL, with smaller IC_{50} values corresponding to greater radical scavenging activity. The IC_{50} value for Tercel could not be extrapolated as it did not yield 50% reduction even at the highest concentration used. The order of DPPH radical scavenging activity of the extracts was as follows: Peregrine > AC Metcalfe > Falcon > Tyto > Phoenix > Tercel (**Table 5**). **Figure 4a** depicts the EPR spectra obtained for Falcon extracts at 1.33, 2, and 3.33 mg/mL concentrations.

Hydroxyl Radical Scavenging Capacity by EPR. Hydroxyl radical is an extremely reactive, short-lived species that can hydroxylate DNA, protein, and other biomolecules (*30*). Thus, it is important to assess the hydroxyl radical scavenging activity of antioxidative extracts. Hydroxyl radicals generated were spin-

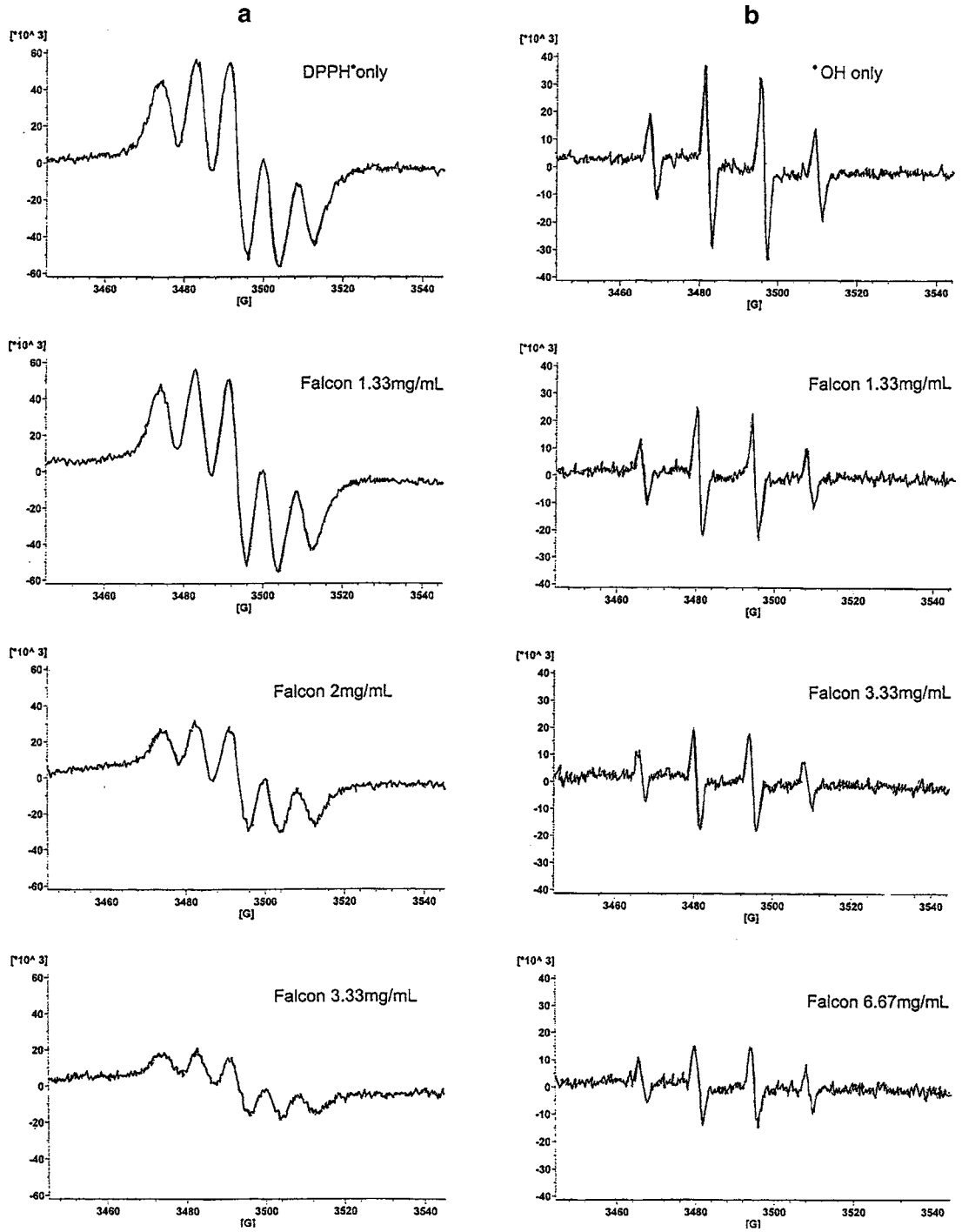

Figure 4. Electron spin resonance spectra showing the effect of different concentrations of Falcon barley extract on scavenging of DPPH• (**a**) and •OH (**b**).

trapped with DMPO due to the very short life of the radical. DMPO adduct, a relatively stable free radical, can easily be detected with EPR. The intensity of the characteristic 1:2:2:1 quartet with a hyperfine coupling constant of 14.9 G (*31*) diminished with the addition of the extract. This could be due to either scavenging of the hydroxyl radical by the extract, chelation of Fe(II) by the extract, or, most likely, the combination of both actions. The IC_{50} value is defined as the amount of extract (milligrams per milliliter) required to lower the initial hydroxyl radical concentration by 50% and was extrapolated from dose−response curves. IC_{50} values for hydroxyl radical ranged from 2.20 to 9.65 mg/mL. Lower IC_{50} values correspond to higher antioxidant potential. Hence, Falcon (IC_{50} = 2.2 mg/mL) was the most active hydroxyl radical scavenger. The hierarchy of extracts based on the hydroxyl radical scavenging activity was as follows: Falcon > Tyto > AC Metcalfe > Peregrine > Phoenix > Tercel (**Table 5**). This pattern is fairly different from the pattern observed for DPPH radical scavenging activity. This is possibly due to the existing difference in kinetics and scavenging power of the two radicals. **Figure 4b** depicts the EPR spectra obtained for Falcon extract at 1.33, 3.33, and 6.67 mg/mL concentrations.

Inhibition of Supercoiled Plasmid DNA Scission Induced by Peroxyl Radical. Peroxyl radical generated through AAPH

Table 6. Effects of Barley Extracts at 4 mg/mL on the Retention of Supercoiled Strand of PBR322 DNA under Peroxyl and Hydroxyl Radical Induced Scission[a]

sample	peroxyl radical	hydroxyl radical
Falcon	84.3 ± 2.3a	59.8 ± 2.7ab
AC Metcalfe	86.7 ± 1.7a	65.3 ± 3.1bcd
Tyto	82.6 ± 4.6a	58.6 ± 1.2ad
Tercel	87.9 ± 5.2ab	62.8 ± 3.7bc
Phoenix	78.2 ± 2.9a	53.1 ± 3.3a
Peregrine	92.1 ± 3.2bc	64.2 ± 4.2cb
ferulic acid[b]	99.2 ± 4.7c	78.2 ± 2.6e

[a] Results are means of three determinations ± standard deviation. Values in each row (within a single cultivar category) having the same letter are not significantly different ($p > 0.05$). [b] 70 μg/mL.

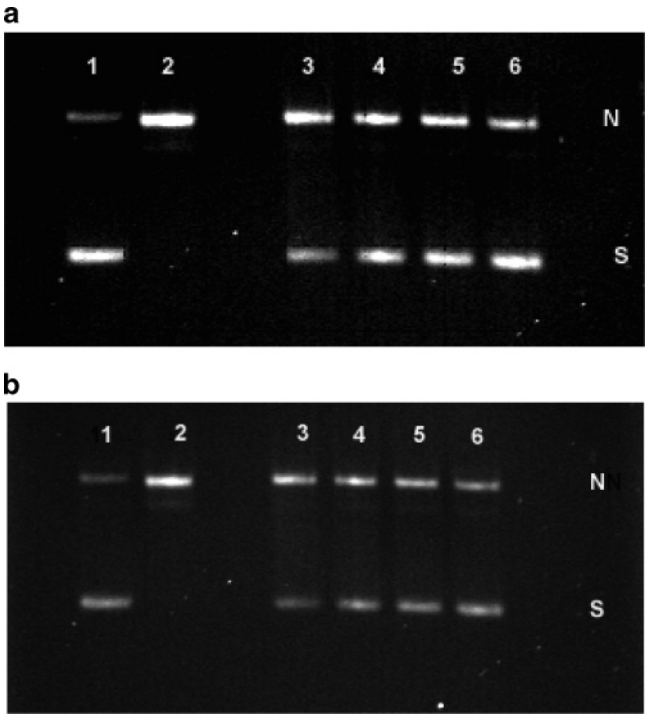

Figure 5. Effect of Falcon barley extract on preventing peroxyl radical (**a**) and hydroxyl radical (**b**) induced DNA scission: (**a**) lane 1, DNA + PBS; lane 2, DNA + 1 mM AAPH; lane 3, DNA + 1 mM AAPH + 1.33 mg/mL extract; lane 4, DNA + 1 mM AAPH + 2.67 mg/mL extract; lane 5, DNA + 1 mM AAPH + 4 mg/mL extract; lane 6, DNA +1 mM AAPH + 6.67 mg/mL extract; N, nicked DNA; S, supercoiled DNA; (**b**) lane 1, DNA + PBS; lane 2, DNA + •OH; lane 3, DNA + •OH + 1.33 mg/mL extract; lane 4, DNA + •OH + 2.67 mg/mL extract; lane 5, DNA + •OH + 4 mg/mL extract; lane 6, DNA + •OH + 6.67 mg/mL extract; N, nicked DNA; S, supercoiled DNA.

led to breakage of supercoiled plasmid DNA. All barley extracts exhibited strong protection against peroxyl radical induced DNA breakage in a concentration-dependent manner. The level of protection against breakage was presented as percentage inhibition by comparing the amount of DNA remaining at the end of the incubation with the amount of DNA present in the native DNA sample (**Table 6**). **Figure 5a** depicts the effect of hydroxyl radical on supercoiled DNA incubated without any extracts. S and N represent supercoiled and nicked DNA bands, respectively. Lane 1 represents the native supercoiled DNA sample without any additives. A high-intensity S band with a low-intensity N band indicates a high concentration of supercoiled DNA and low concentrations of nicked DNA in the native DNA sample, respectively. Lane 2 shows the effect of radical attack on the native supercoiled DNA samples. The presence of a high-intensity N band and the disappearance of the S band in lane 2 indicate that supercoiled DNA was completely nicked. Wells 3−6 contained supercoiled DNA, along with the same concentration of radical together with increasing concentrations of barley extracts (1.33−6.67 mg/mL). The intensity of the S band gradually increased from lane 3 through lane 6, reflecting a higher level of retention of supercoiled DNA due to the protection offered by increasing concentrations of barley extracts. On the other hand, the intensity of the N band gradually decreased from lane 3 through lane 6, depicting lower nicked DNA concentrations with increasing level of protection offered by the extracts. To compare the DNA protection efficiencies among the barley extracts, a 4 mg/mL concentration was chosen as the reference concentration. The percentage inhibition offered by the extracts ranged from 78.2 to 92.1% at the extract concentration of 4 mg/mL. Ferulic acid standard yielded 99.2% protection at a concentration of 60 μg/mL, which is equivalent to the extracts in terms of total phenolic content. Peregrnie exhibited the highest inhibition against DNA scission, whereas Phoenix exhibited the lowest inhibition of 78.2% at 4 mg/mL. It is important to note that no pro-oxidant activity was observed at any level tested with any of the extracts. All extracts showed a similar pattern of concentration dependence in protecting supercoiled plasmid DNA. The affinity of reactive oxygen species (ROS) to initiate DNA damage has been well characterized in vitro; however, the effect of peroxyl radical on breakage of DNA has only recently been established (*20*, *32*).

Hydroxyl Radical Induced Supercoiled Plasmid DNA Scission. Whole barley extracts were effective in suppressing hydroxyl radical induced DNA damage in non-site-specific protocol in a concentration-dependent manner. It was clearly seen that the level of protection against hydroxyl radical is substantially lower compared to the level of protection against peroxyl radical. The extracts were tested at different concentrations (1.33, 2.62, 4, and 6.67 mg/mL). Concentrations lower than 4 mg/mL did not exhibit effective protection. Thus, for the purpose of comparison among the cultivars, percent inhibition at 4 mg/mL was used. The levels of protection exerted by whole barley extracts are listed in **Table 6**.

Figure 5b is a representative gel photograph depicting the effect of Falcon cultivar on supercoiled DNA at four different concentrations. S and N represent supercoiled and nicked DNA, respectively. Lane 2 of **Figure 5b** shows the presence of supercoiled DNA, radical, and PBS. The presence of a high-intensity N band and the disappearance of the S band in lane 2 indicate that supercoiled DNA was completely nicked. Wells 3−6 contained supercoiled DNA, along with the same concentration of hydroxyl radical together with increasing concentrations of barley extracts (1.33−6.67 mg/mL). The intensity of the S band gradually increased from lane 3 through lane 6, reflecting a higher level of retention of supercoiled DNA due to the protection offered by increasing concentrations of barley extracts. On the other hand, the intensity of the N band gradually decreased from lane 3 through lane 6, depicting lower nicked DNA concentrations with increasing levels of protection offered by the extracts.

The reactions of hydroxyl radical are mainly addition to the double bond of pyrimidine bases and abstraction of hydrogen from the sugar moiety, resulting in chain scission of DNA. These effects can cause cell mutagenesis and carcinogenesis (*33*). The effects of barley extracts toward protecting plasmid DNA strand

scission provide further evidence of antioxidant efficacy exhibited by the antioxidative constituents in barley.

The putative hydroxyl radical is an extremely reactive and short-lived species that can hydroxylate DNA; thus, the direct scavenging of hydroxyl radical by dietary antioxidants is unrealistic as the cellular concentration of antioxidants is negligible compared with those of DNA and other molecules (*24*). On the other hand, it is possible to prevent the formation of hydroxyl radical by deactivating free metal ions such as Fe(II) through chelation. Non-site-specific protocol distinguishes Fe(II) chelation and hydroxyl radical scavenging capabilities of test extracts by eliminating the contribution of the extracts toward the Fe(II) chelation effect. Therefore, this protocol identifies the true hydroxyl radical scavenging capacity of the extracts.

This study demonstrated that barley contained substantial amounts of phenolic antioxidants that effectively scavenge free radicals. They were very effective against peroxyl, DPPH, and hydroxyl radicals. The antioxidative constituents in barley effectively inhibited DNA double-strand scission induced by peroxyl and hydroxyl radicals. The cultivars Peregrine, Falcon, and AC Metcalfe were in general more effective than the rest of the cultivars, perhaps due to a higher phenolic content. The genetic differences are mainly attributable to the different antioxidant capacities among the cultivars.

Polyphenolics protect cell constituents against oxidation damage and, therefore, limit the risk of various degenerative diseases associated with oxidative stress (*34*). A number of animal studies have demonstrated that the consumption of phenolic compounds limits atherogenesis, which is a key factor in the development of cardiovascular diseases. Experimental studies on animals and human cultured cell lines support the role of polyphenols in the prevention of cancer, neurodegenerative diseases, diabetes, and osteoporosis, among others (*34*). Although phenolic compounds are present in minor quantities in cereals, the dietary intake of polyphenols through cereals remains high due to the considerably large amounts of cereals consumed in the daily diet. Thus, among other cereals, barley significantly contributes to the daily polyphenolic compound intake. Furthermore, barley has great potential in the development of nutraceuticals rich in antioxidants.

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