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Bioactivities and Antiradical Properties of Millet Grains and Hulls

Anoma Chandrasekara and Fereidoon Shahidi*

Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3X9

ABSTRACT: Antioxidant activities of phenolic extracts of kodo and pearl millet whole grains, dehulled grains, and hulls were examined by monitoring inhibition of radical-induced DNA scission, human low-density lipoprotein (LDL) cholesterol, and phospholipid liposome oxidation. Total phenolic content (TPC), hydroxyl and peroxyl radical inhibition, and antiproliferative activities against HT-29 cells were also determined. Major hydroxycinnamic acids in dehulled grains and hulls were identified and quantified using HPLC. Phenolic extract of kodo millet exhibited higher inhibition activities against oxidation of LDL cholesterol and liposome than that of pearl millet. All phenolic extracts exhibited a dose-dependent inhibition of DNA scission. The TPC of hulls of kodo and pearl millets were 3 times higher than those of their corresponding whole grains. At the end of 96 h of incubation, kodo millet extracts inhibited cell proliferation in the range of 75−100%. Antioxidant activities of phenolic extracts were in the order hull > whole grain > dehulled grain. Dehulling reduced the antioxidant potential of whole millet grains. Ferulic and *p*-coumaric acids were the major hydroxycinnamic acids, and their contents ranged from 17.8 to 1685 μg/g defatted meal and from 3.5 to 680 μg/g defatted meal, respectively. Dehulled grains, as well as the hull fraction, may serve as potential sources of nutraceutical and functional food ingredients in health promotion.

KEYWORDS: antiproliferation, bound, DNA scission, liposome, LDL cholesterol, ORAC, dehulled grains,

INTRODUCTION

The consumption of whole grain has been encouraged due to numerous health benefits arising from their bioactive constituents. The protective effect of whole grains could be due to the presence of a number of phytochemicals, including phenolic compounds that may play a major role in disease risk reduction. Several in vitro studies have shown that polyphenolic compounds from a number of cereal grains and their products are effective antioxidants.[1−11]

Cereals serve as a major staple food for many populations around the globe, and millets are placed sixth among other cereals, accounting for about 1.3% of total cereal production in 2009.[12] Pearl millet is the major millet type, cultivated mainly in Asia and Africa, and constitutes about 50% of global millet production. Kodo millet, which is considered to be a minor millet, is predominantly grown on the Indian subcontinent. Previous studies have shown that kodo millet grain extracts possess a high in vitro antioxidant capacity, among other activities.[10,11,13] In addition, an antidiabetic effect of kodo whole millet grain in alloxan-induced diabetic rats has been reported.[14]

Phenolic acids are the major phenolics identified in kodo and pearl millet grains, with flavonoids being present in small amounts.[15] Phenolics in cereal grains exist as free, soluble conjugated and insoluble bound compounds, and it has been shown that the latter fraction in cereal grains is predominant.[4,10,16,17] However, enzymatic hydrolysis under acidic and alkaline conditions in the gastrointestinal tract may release phenolic compounds and hence could affect the antioxidant activity in the intestine, locally as well as systemically upon absorption.[16] In addition, insoluble bound phenolics in cereal grains are released during fermentation in the colon.[18] Thus, determination of antioxidant and other bioactivities of bound phenolics of grains is important.

Free radical attacks on biological molecules such as lipids, protein, and DNA may be considered to be an initiating stage for several chronic diseases. Free radical induced or metal ion dependent oxidation of low-density lipoprotein (LDL) cholesterol is an important step in developing atherosclerotic lesions that lead to coronary heart diseases.[19] It is well established that elevated levels of LDL cholesterol are associated with increased risk of atherosclerosis. Thus, dietary antioxidants that inhibit LDL oxidation may help in reducing the occurrence of coronary heart disease. A number of early studies have indicated that phenolics in cereal grain are effective inhibitors of human LDL cholesterol oxidation.[20,21]

In addition, several studies have shown that phenolic compounds are effective against liposome oxidation, but no such information is available for millet grains. Cellular membrane damage and consequent peroxidation of phospholipids leads to interruption of the membrane assembly and to changes in fluidity and permeability as well as alterations of ion transport and inhibition of metabolic processes and several pathological conditions.[22] In a previous study, we showed that soluble millet phenolic extracts from whole grains were effective against DNA scission and HT-29 cell proliferation.[23] However, as millets are dehulled before consumption, it is necessary to examine the potential bioactivities of the edible part of the grain as well as hulls, as a processing byproduct, which can be used in pharmaceutical and nutraceutical applications. Therefore, the objectives of this study were (a) to evaluate the antioxidant and antiradical activities of soluble phenolic extracts of whole grains, dehulled grains, hulls, and bound fraction extracts of kodo and pearl millets and (b) to determine the bioactivities, namely, liposome oxidation inhibition, DNA oxidation inhibition, antiproliferation

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against adenocarcinoma cells, and human LDL cholesterol oxidation inhibition, of whole grains, dehulled grains, hulls, and bound fraction extracts of kodo and pearl millets.

MATERIALS

Kodo (*Paspalum scrobiculatum*) and pearl millets (*Pennisetum glaucum*) harvested in 2007 were used in this study. Kodo millet samples were obtained from the Field Crop Research and Development Center, Mahailluppallama, Sri Lanka. Pearl millet (dark green cultivar), grown in India, was kindly supplied by Dr. Uttam Chavan, Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra, India.

Folin−Ciocalteu's phenol reagent, ferulic acid, *p*-coumaric acid, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), trolox, ferrous sulfate, ethylenediaminetetraacetic acid trisodium salt (Na_3EDTA), mono- and dibasic potassium phosphates, hydrogen peroxide, L-α-phosphatidylcholine type XVI-E, pBR 322 plasmid DNA, agarose, tris acetate, bromophenol blue, xylene cyanol, glycerol, [5,5-dimethyl-1-1-pyrroline-N-oxide (DMPO)], and human LDL cholesterol were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). SYBR safe gel stain was purchased from Probes Invitrogen (Eugene, OR). Biological grade dimethyl sulphoxide (DMSO) was purchased from Sigma-Aldrich (St. Louis, MO). McCoy's 5A medium was purchased from Invitrogen Co. (Carlsbad, CA), and HT-29 cells were purchased from American Type Culture Collection (Rockville, MD). Diethyl ether, ethyl acetate, hexane, acetone, methanol, chloroform, acetonitile, formic acid, hydrochloric acid, sodium hydroxide, and sodium carbonate were purchased from Fisher Scientific Ltd. (Ottawa, ON, Canada).

METHODS

Sample Preparation. Whole millet grains, cleaned from debris and soil particles, were used in the experiments. Whole grains were directly ground using a coffee bean grinder (model CBG5 series, Black & Decker, Canada Inc., Brockville, ON, Canada) to obtain a fine powder, which passed through mesh 16 (sieve opening 1 mm, Tyler test sieve, Mentor, OH). Another portion of whole millet grains was dehulled using a Seedburo hand grinder (Seedburo Equipment Co., Chicago, IL). The hulls were separated by air classification on a 757 South Dakota seed blower equipped with a column that utilizes an air flow generated by a blower motor combination to separate seed fractions by size and density (Seedburo Equipment Co.). Portions of dehulled grains and procured hulls were ground separately to obtain fine powders in a similar manner. All samples were defatted by blending with hexane (1:5, w/v, 5 min three times) in a Waring blender (model 33BL73, Waring Products Division Dynamics Co. of America, New Hartford, CT) at ambient temperature. Defatted samples were vacuum packed in polyethylene pouches and stored at −20 °C until used within 1 week for extraction of phenolics.

Extraction of Soluble Phenolic Compounds. An ultrasound-assisted extraction procedure was used for soluble phenolic compounds. Defatted meal (5 g) was mixed with 100 mL of 70% (v/v) acetone and then placed in an ultrasonic bath (300 Ultrasonik, Whittemore Enterprises, Inc., Rancho Cucamonga, CA) and sonicated at the maximum power for 25 min under reflux conditions.[10] After centrifugation of the resulting slurry for 5 min at 4000*g* (IEC Centra MP4, International Equipment Co., Needham Heights, MA), the supernatant was collected and extraction was repeated two more times. Combined supernatants were evaporated in vacuo at 40 °C (Buchi, Flawil, Switzerland) and lyophilized for 72 h at −46 °C and 34×10^{-3} mbar (Freezone, model 77530, Labconco Co., Kansas City, MO). The residues of whole grain samples were air-dried for 12 h and stored at −20 °C until used for extraction of bound phenolic compounds within a week. During all stages, extracts were protected from light by covering them with aluminum foil. Lyophilized crude phenolic extracts were stored at −20 °C until used for further analysis.

Determination of Total Phenolic Content (TPC). The TPC of each extract was determined using the method described by Singleton and Rossi[24] with slight modifications. Briefly, the crude extracts of soluble phenolic compounds were dissolved in methanol to obtain a concentration of 1 mg/mL. Folin−Ciocalteu's phenol reagent (0.5 mL) was added to centrifuge tubes containing 0.5 mL of extract, and the contents were mixed thoroughly by vortexing. The reaction was neutralized by adding 1 mL of saturated sodium carbonate to each tube, followed by the addition of distilled water (8 mL) and thorough mixing. Tubes were allowed to stand at room temperature in the dark for 35 min followed by centrifugation at 4000*g* for 10 min. The absorbance of the resulting blue color supernatant was measured at 725 nm (model HP 8452A diode array spectrophotometer, Agilent Technologies, Palo Alto, CA) using appropriate blanks for background subtraction. The content of total phenolics in each extract was determined using a standard curve prepared for ferulic acid and expressed as micromoles ferulic acid equivalents (FAE) per gram of defatted meal.

Separation of Free and Esterified Phenolic Compounds. Free phenolic acids and those liberated from soluble esters of whole grains, dehulled grains, and hulls were separated from the lyophilized crude phenolic extract on the basis of a previously explained method.[10] In brief, the pH of the aqueous suspension of extract (250 mg in 10 mL) was adjusted to 2 with 6 M HCl; free phenolics were then extracted five times into a mixture (1:1, v/v) of diethyl ether and ethyl acetate. The free phenolic extract was evaporated to dryness under vacuum at room temperature. The water phase was neutralized to pH 7 with 2 M NaOH and then lyophilized. The resulting residue was dissolved in 10 mL of 2 M NaOH and hydrolyzed for 4 h at room temperature under a nitrogen atmosphere. After acidification to pH 2 with 6 M HCl, phenolic acids released from soluble esters were extracted from the hydrolysates five times with a mixture (1:1, v/v) of diethyl ether and ethyl acetate and evaporated to dryness under vacuum.

Extraction of Bound Phenolic Compounds. The residue of the grain samples obtained after extraction of soluble phenolics was hydrolyzed with 2 M NaOH at room temperature for 4 h with stirring under nitrogen. The resulting slurry was acidified to pH 2 with 6 M HCl and extracted five times with hexane to remove fatty acids released during alkaline hydrolysis. Bound phenolic compounds were extracted five times with diethyl ether and ethyl acetate (1:1, v/v) and subsequently desolventized to dryness at room temperature in a rotary evaporator. Phenolic compounds were reconstituted in 5 mL of HPLC grade methanol and stored at −20 °C until used.

Determination of Ferulic and *p*-Coumaric Acids Contents by HPLC and HPLC-MS. Identification and quantification of phenolic compounds in whole millet grains showed that ferulic and *p*-coumaric acids were predominant phenolic acids, among others, in soluble and bound phenolic fractions of the grains.[15] Therefore, ferulic and *p*-coumaric acids contents of free, esterifed, and bound phenolic extracts of dehulled grains and hulls were determined by HPLC analysis.[10] The RP-HPLC analysis was carried out using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA). Separations were conducted using a Supelcosil LC-18 column (4.6 × 250 mm, 5 μm; Merck, Darmstadt, Germany). The mobile phase consisted of 1% formic acid (eluent A) and methanol/acetonitrile/formic acid (94:5:1; v/v/v) (eluent B), and gradient elution was used. The phenolic acid contents of free and esterified phenolic fractions are given as the total phenolics in the soluble fraction. Ferulic and *p*-coumaric acids were identified by comparing their relative retention times and UV and ESI-MS spectra with those of authentic standards. An external standard method with ferulic and *p*-coumaric acids was used for quantification purposes.

Oxygen Radical Absorbance Capacity (ORAC). ORAC measures antioxidant scavenging activity of test compounds against peroxyl radical generated by AAPH. The ORAC assay was based on the method explained by Madhujith and Shahidi[20] as reported elsewhere.[11]

All reaction mixtures were prepared in duplicate, and three independent runs were performed for each sample. ORAC values of extracts were expressed as micromoles of trolox equivalents (TE) per gram of defatted meal using the standard curve calculated for each experiment.

Hydroxyl Radical Scavenging Activity. The hydroxyl radical scavenging capacity was determined according to the method explained by Madhujith and Shahidi[5] with slight modifications and as described by Chandrasekara and Shahidi,[11] using electron paramagnetic resonance (EPR) spectrometry. The hydroxyl radical scavenging capacity of the extracts was calculated using the following equation: hydroxyl radical scavenging capacity (%) = [(EPR signal intensity for the control − EPR signal intensity for the sample)/EPR signal intensity for the control] × 100. The hydroxyl radical scavenging activity of the extracts was expressed as micromoles of FAE per gram of defatted meal.

Supercoiled Plasmid DNA Strand Scission Inhibition. Inhibition activity of millet phenolics against supercoiled strand DNA scission induced by peroxyl and hydroxyl radicals was evaluated according to the methods of Hiramoto et al.[25] and Liyanapathirana and Shahidi,[21] with slight modifications. Supercoiled plasmid DNA (pBR 322 from *Escherichia coli* RRI) was dissolved at a concentration of 50 μg/mL in 0.5 M, pH 7.4, phosphate buffer solution (PBS). Different concentrations of soluble millet phenolic extracts (0.0625−0.5 mg/mL) were prepared in PBS. In an Eeppendorf tube (500 μL), 2 μL each of a solution of supercoiled plasmid DNA, PBS, phenolic extract, H_2O_2 (1 mM), and $FeSO_4$ (0.5 mM) were added in the order stated to determine the inhibitory activity of millet extracts against hydroxyl radical induced DNA strand scission. The mixture was incubated at 37 °C for 1 h in the dark.[25] The loading dye (2 μL), consisting of 0.25% bromophenol blue, 0.25% xylene cyanol, and 50% glycerol in distilled water, was added to the reaction mixture at the end of the incubation period.

In another experiment the inhibitory effect of millet extracts against peroxyl radical induced DNA scission was investigated. In this, AAPH was dissolved in PBS to attain a final concentration of 9 mM, which was then mixed with DNA and the extracts to a final volume of 10 μL. The incubation was done for 1 h at 37 °C. A control with DNA alone and a blank devoid of phenolic extracts were prepared with each set of phenolic extracts tested.

The samples were electrophoresed using a 0.7% (w/v) agarose gel prepared in Tris−acetic acid−EDTA (TAE) buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.5). SYBR safe was added at a concentration of 100 μL/L of TAE buffer as a gel stain. Submarine gel electrophoresis was run at 60 V for 5 h using a model B1A horizontal mini gel electrophoresis system (Owl Separation Systems Inc., Portsmonth, NH) and a model 300 V power supply (WMR International Inc., West Chester, PA) at room temperature in TAE buffer. The bands were visualized under transillumination of UV light using an AlphaImager gel documentation system (Cell Biosciences, Santa Clara, CA). The images were analyzed using Chemilmager 4400 software (Cell Biosciences, Santa Clara, CA) to quantify DNA scission. The protective effect of millet phenolic extracts was calculated using the retention percentage of the normalized supercoiled DNA as given: DNA retention % = (intensity of supercoiled DNA with the oxidative radical and extract/intensity of supercoiled DNA in control) × 100. The concentration of extracts that retain 50% (EC_{50}) of supercoiled DNA was then calculated.

Inhibition of Cupric Ion-Induced Human LDL Peroxidation. The method described by Andreasen et al.[26] was adopted to determine the human LDL cholesterol oxidation inhibitory activities of grain extracts. Human LDL cholesterol (in PBS, pH 7.4, with 0.01% EDTA) was dialyzed against 10 mM PBS (pH 7.4, 0.15 M NaCl) for 12 h under nitrogen at 4 °C, and EDTA-free LDL was subsequently diluted to obtain a standard protein concentration of 0.1 mg/mL with PBS. The diluted LDL cholesterol solution (0.8 mL) was mixed with 100 μL of extract (0.125 and 0.5 mg/mL) in an Eppendorf tube. Oxidation of LDL cholesterol was initiated by adding 0.1 mL of 100 μM $CuSO_4$ solution in distilled water. The mixture was incubated at 37 °C for 20 h. The initial absorbance (t = 0) was read at 234 nm immediately after mixing, and conjugated diene (CD) hydroperoxides formed at the end of 20 h were measured. The corrected absorbance at 20 h against 0 h was employed to calculate the percentage inhibition of CD formation using the following equation: % inhibition of CD formation = ($Abs_{oxidative} - Abs_{sample}$)/($Abs_{oxidative} - Abs_{native}$) × 100, where $Abs_{oxidative}$ = absorbance of LDL mixture and distilled water with $CuSO_4$ only, Abs_{sample} = absorbance of LDL with extract and $CuSO_4$, and Abs_{native} = absorbance of LDL with distilled water.

Inhibition of Liposome Oxidation. The liposome suspension was prepared according to a previously described method.[27] Briefly, α-phosphatidylcholine (PC, 20 mg) was dissolved in choloroform in a round-bottom flask, and the solvent was evaporated in a rotary evaporator in vacuo at room temperature. After traces of solvent had been removed by nitrogen flush, 10 mL of 10 mM phosphate-buffered saline (PBS) (pH 7.4, 150 mM NaCl) was added. The mixture was vortexed and sonicated for 1 and 3 min, respectively, before the PC mixture was made to a final concentration of 0.5 mg/mL in PBS. To evaluate the inhibition of liposome oxidation induced by peroxyl radicals by the extracts, 0.8 mL of PC and 0.1 mL of extracts (0.5 mg/mL for whole grain, dehulled grain, and hull extract; 50 μL/mL for bound extract) were mixed in an Eppendorf tube (1.5 mL), and the mixtures were incubated for 5 min at 37 °C before the addition of 0.1 mL of AAPH in PBS (10 mM). CD hydroperoxides formed were measured on a 100 μL sample diluted to 1 mL with methanol at 234 nm. The samples were drawn at regular intervals at 0, 6, 9, and 12 h. The control was prepared with PC mixture and PBS, and blanks were prepared with samples and PBS.

In another experiment, the inhibitory activity of grain extracts against liposome peroxidation induced by hydroxyl radicals was determined. In this, 0.8 mL of PC and 0.1 mL of extract were preincubated for 5 min at 37 °C, and 50 μL each of hydrogen peroxide (1 mM) and $FeSO_4$ (0.5 mM) were added followed by incubation; CD was measured as explained above. The results were expressed as percentage inhibition of liposome oxidation.

Human Colon Adenocarcinoma Cell Proliferation Inhibition. Cell proliferation inhibition was studied using the HT-29 colorectal cancer (CRC) cell line, according to the method of Wang et al.[28] HT-29 cells were propagated in T-150T flasks in McCoy's 5A medium supplemented with 10% fetal bovine serum albumin (FBS) and 1% antibiotic/antimycotic. Cells were grown in a humidified atmosphere containing 5% CO_2 at 37 °C.[29,30] The cells were plated at 2500 cells per well in a 96-well microplate and incubated for 24 h at 37 °C. Soluble millet phenolic extracts dissolved in DMSO were introduced into the wells containing cell culture media to obtain final concentrations of 0.1 and 0.5 mg/mL. The concentrations of bound extracts dissolved in ethanol were 0.015 and 0.003 μL/mL for high and low doses, respectively. The control consisted of cell culture media and DMSO. Both treatment levels and the control contained a final concentration of 0.99% DMSO. The live cells on each of the wells were studied using an ATP-Lite 1 step kit (Perkin-Elmer, Shelton, CT), which produces luminescence proportionately to the amount of ATP present in viable cells. Luminescence readings were taken using a Victor multiwell plate reader (Perkin-Elmer) immediately prior to the treatment and 4, 24, 48, 72, and 96 h afterward. The treatment and control media were replaced every 24 h up to 96 h during the incubation period. The antiproliferative effects of millet phenolics against HT-29 cells were expressed as percent inhibition calculated against the control.

Statistical Analysis. All experiments were carried out in triplicates, and data were reported as the mean ± standard deviation. The differences of mean values among millet grain fractions were determined using one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Differences (HSD) multiple-rank test at $p < 0.05$ significance level. All statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL).

Table 1. Phenolic Contents and Antioxidant Activities of Whole Millet Grains, Dehulled Grains, and Hulls[a]

millet	whole grain (soluble)	dehulled grain (soluble)	hulls (soluble)	whole grain (bound)
Total Phenolic Content (ferulic acid equiv μmol/g defatted meal)				
kodo	32.4 ± 0.93*c[1]	6.86 ± 0.19d[1]	112 ± 1.37a[1]	81.6 ± 0.15*b[1]
pearl	8.63 ± 0.38*b[2]	8.50 ± 0.03b[2]	34.3 ± 1.69a[2]	9.14 ± 0.17*b[2]
Oxygen Radical Absorbance Capacity (trolox equiv μmol/g defatted meal)				
kodo	95.7 ± 2.37c[1]	41.0 ± 1.39d[1]	216 ± 1.54b[1]	793 ± 7.76a[1]
pearl	60.3 ± 8.38d[2]	95.9 ± 10.1c[2]	214 ± 0.35a[1]	176 ± 18.2b[2]
Hydroxyl Radical Scavenging Activity (ferulic acid equiv μmol/g defatted meal)				
kodo	56.4 ± 6.76c[1]	16.4 ± 1.13d[1]	133 ± 23.0b[1]	499 ± 34.0a[1]
pearl	59.6 ± 1.01c[1]	2.13 ± 0.89d[2]	196 ± 8.26a[2]	80.9 ± 0.49b[2]

[a] Values in each row having the same letter are not significantly different ($p > 0.05$). For individual assays, values in each column having the same superscript number (1 or 2) are not significantly different ($p > 0.05$). Values with an asterisk (*) are adapted from ref 10.

Table 2. Ferulic and *p*-Coumaric Acid Contents of Whole Millet Grains, Dehulled Grains, and Hulls[a]

	ferulic acid (μg/g defatted meal)		*p*-coumaric acid (μg/g defatted meal)	
millet	kodo	pearl	kodo	pearl
whole grain (soluble)	202 ± 5.70*c[1]	82.5 ± 1.40*c[2]	19.2 ± 0.46*c[1]	20.8 ± 0.18*b[1]
dehulled grain (soluble)	17.8 ± 0.50d[1]	50.0 ± 0.39d[2]	3.46 ± 0.16d[1]	9.90 ± 0.62c[2]
hulls (soluble)	617 ± 0.14b[1]	222 ± 0.89b[2]	25.7 ± 1.69b[1]	73.6 ± 0.49a[2]
whole grain (bound)	1685 ± 1.00*a[1]	639 ± 2.86*a[2]	680 ± 6.33*a[1]	20.7 ± 0.04*b[2]

[a] Values in each column having the same letter are not significantly different ($p > 0.05$). For individual phenolic acids, values in each row having the same superscript number (1 or 2) are not significantly different ($p > 0.05$). Values with an asterisk (*) are adapted from ref 10.

RESULTS AND DISCUSSION

This study reported for the first time in vitro human LDL cholesterol oxidation inhibition, antiproliferation of adenocarcinoma cells, DNA scission inhibition, and liposome oxidation inhibitory activities of phenolics extracted from dehulled grains of kodo and pearl millets and their corresponding hulls. These two millets were selected in this study on the basis of the importance of pearl millet as the major type in global production and the reported high antioxidant activity of kodo millet. The information generated in this study demonstrated the potential of using dehulled millet grains as therapeutic dietary agents and hulls, the processing byproducts, as potential nutraceuticals for reduction of diseases in which oxidative stress plays a role in their initiation and progression. As can be seen, the efficacy of phenolics involved may be exerted by different pathways and mechanisms, both antioxidative and otherwise.

Total Phenolic Content (TPC) and Antioxidant Activities. Table 1 presents the TPC and free radical scavenging activities of whole grain, dehulled grain, hull soluble phenolic extracts, and whole grain insoluble bound phenolic extracts from kodo and pearl millet grains. The TPC ranged from 6.9 to 112 μmol FAE/g defatted meal and from 8.5 to 34.3 μmol FAE/g defatted meal for kodo and pearl millets, respectively. As expected, hulls of both millet types had 4−16 times higher ($p \leq 0.05$) TPC compared to their dehulled grain counterparts. Our work lends further support to the earlier studies reporting that dehulling significantly reduced the polyphenolic content of the two pearl millet cultivars Standard and Ugandi.[31] In addition, hulls of barley, oat, and buckwheat grains showed higher TPC than the corresponding dehulled grains.[32] Soluble extracts of kodo whole grain millet showed 3.8 times higher TPC than pearl millet.

Upon dehulling pearl millet grains showed more TPC in the edible part than did kodo millet, indicating that the distribution of phenolic compounds in different grain fractions is dependent upon the variety tested. On the other hand, kodo millet hulls had 3 times more TPC than pearl millet hulls, suggesting their potential use as a source of natural antioxidant.

ORAC showed that millet grain phenolics were effective peroxyl radical scavengers. In biological systems peroxyl radicals are formed through autoxidation of fats. In addition, compared to other oxygen-centered radical species, peroxyl radicals are stable and thus have the ability to diffuse to distant cellular locations.[33] Bound extracts of kodo millet grains showed the highest ORAC value, expressed as μmol trolox equiv/g defatted meal followed by soluble extracts of hulls, whole grains, and dehulled grains (Table 1). It is noteworthy that despite the high TPC observed in the hull extracts of kodo millet, bound extracts showed the highest ORAC value, suggesting that the content and composition of phenolics contribute to the antioxidant activity. In addition, the ORAC value of kodo hulls was 5-fold higher than that of the corresponding dehulled grains. Similarly, it was noted that pearl millet grain hulls had the highest ORAC value, which was 2.2 times higher than that of its dehulled grain counterpart. The edible fraction, pearl dehulled grains, had a 2-fold higher ORAC value compared to that of kodo.

Hydroxyl radicals can be generated in the body and may attack all biological molecules such as DNA, proteins, and polyunsaturated fatty acids (PUFA) in membranes, among others. In the present study hydroxyl radicals were generated through the iron-catalyzed Harber−Weiss reaction, and EPR spectrometry was used to measure the presence of a stable spin adduct formed between hydroxyl radical and DMPO. The hydroxyl radical scavenging activities (HRSA) of kodo and pearl millet grain fractions were 16.4−499 and 2.1−196 μmol FAE/g defatted meal, respectively. In addition, dehulled grains of both millet

types had the least HRSA in accordance with the ORAC values and TPC. It is worthwhile to note that although dehulled pearl millet grains showed a 2.3-fold higher ORAC value than kodo, the reverse trend existed for hydroxyl radical scavenging activity, which was 8 times lower in pearl millet than in kodo millet. Thus, the use of multiple test systems in the assessment of antioxidant activities of the extracts is warranted as antioxidant activity may differ on the basis of the type of millet grain as well as the radical species used in the evaluation system employed. The present results demonstrated that millet hulls have a high potential as an attractive source of natural antioxidants. In general, the outer layers of plants such as peels, shells, and hulls contain a high amount of phenolic compounds that protect the inner material from pest attacks, microbial invasion, and protection from environmental stress.

Determination of Ferulic and *p*-Coumaric Acids Contents. Table 2 summarizes the contents of ferulic and *p*-coumaric acids of different extracts of kodo and pearl millets as analyzed by HPLC and HPLC-MS. Ferulic and *p*-coumaric acids were mainly found in the insoluble bound extracts of the grain for both kodo and pearl millet grains, and their contents ranged from 17.8 to 1685 μg/g defatted meal and from 3.5 to 680 μg/g defatted meal. In addition, the results of the present study further established the fact that phenolic acids were mainly concentrated in the hulls of the grains. Kodo and pearl millets contained 34- and 4-fold more ferulic acid, respectively, compared to their dehulled counterparts. In general, the *p*-coumaric acid content in hulls of kodo and pearl millet grains was 7 times higher when compared to their corresponding dehulled grains. In a previous study, Hernanz et al.[34] showed that the outermost fraction, which mainly consists of husk and outer layers, of barley grains had the highest concentrations of ferulic and *p*-coumaric acids as well as ferulic acid dehydrodimers that could contribute to their high antioxidant activity.

Supercoiled Plasmid DNA Strand Scission Inhibition. DNA molecules are easily attacked by free radicals and induced base modification as well as DNS strand scission that leads to mutagenesis, which could possibly progress to cancer. Table 3 presents the effective concentrations of millet extracts needed to retain 50% (EC_{50}) of the supercoiled DNA (10 mM in the assay) in the presence of peroxyl and hydroxyl radicals. Thus, the extract with the least concentration required for 50% inhibition shows superior protection against oxidative damage to DNA. Kodo millet grain extracts showed higher ($p \leq 0.05$) protection against peroxyl radical induced DNA damage compared to corresponding pearl millet extract. Figure 1 shows that decreased concentration of the whole grain extracts of kodo and pearl millets rendered a lesser protection against peroxyl radical induced DNA scission, thus demonstrating a dose-dependent protection of extracts against oxidative stress on DNA molecules. The EC_{50} values of kodo extracts ranged from 0.02 to 0.17 mg/mL (4−34 mM in the assay). The results of the present work showed that millet antioxidants protected against both hydroxyl and peroxyl radical-induced DNA scission. In agreement with this finding, Madhujith and Shahidi[6] demonstrated that barley extracts at a concentration of 4 mg/mL inhibited oxidation of DNA by peroxyl and hydroxyl radicals by 83−92 and 53−65%, respectively.

Inhibition percentages of DNA strand scission induced by peroxyl and hydroxyl radicals for different grain extracts of kodo and pearl millets at a concentration of 0.5 mg/mL (100 mM in the assay) are shown in Figure 2. All extracts tested in this study effectively protected DNA against scission, and this may be partly attributed to the free radical scavenging activity of the extracts as demonstrated by ORAC values and HRSA in this study.

Table 3. EC_{50} Values of Grain Extracts in Retention of Supercoiled DNA in the Presence of Peroxyl and Hydroxyl Radicals[a]

	peroxyl radical		hydroxyl radical	
millet	kodo	pearl	kodo	pearl
whole grain[b]	$0.06 \pm 0.00b^1$	$0.53 \pm 0.01a^2$	$0.07 \pm 0.00c^1$	$0.11 \pm 0.02b^2$
dehulled grain[b]	$0.17 \pm 0.04a^1$	$0.61 \pm 0.01a^2$	$0.23 \pm 0.04a^1$	$0.22 \pm 0.01a^2$
hulls[b]	$0.02 \pm 0.00c^1$	$0.20 \pm 0.01b^2$	$0.15 \pm 0.04b^1$	$0.10 \pm 0.01b^1$
whole grain[c]	3.00 ± 0.00^1	7.30 ± 0.40^2	3.80 ± 0.40^1	5.40 ± 1.20^2

[a] Values in each column having the same letter are not significantly different ($p > 0.05$). For individual radicals, values in each row having the same superscript number (1 or 2) are not significantly different ($p > 0.05$). [b] Soluble extracts and the concentration of extract is in mg/mL. [c] Bound extracts and the concentration of extract is in μL/mL; 1 μL contains bound phenolics extracted from 1 μg of defatted meal.

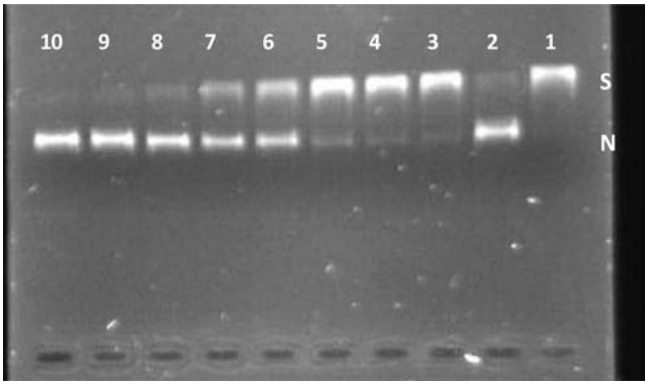

Figure 1. Agarose gel electrophoresis of DNA treated with peroxyl radical (R) in the presence of millet whole grain (WG) soluble (S) phenolic extracts at 37 °C. Concentrations of extracts are given in μg/mL of final assay. Lanes: 1, DNA, blank; 2, DNA + R, control; 3, DNA + R + kodo WG(S) 100; 4, DNA + R + kodo WG(S) 50; 5, DNA + R + kodo WG(S) 25; 6, DNA + R + kodo WG(S) 12.5; 7, DNA + R + pearl WG(S) 100; 8, DNA + R + pearl WG(S) 50; 9, DNA + R + pearl WG(S) 25; 10, DNA + R + pearl WG(S) 12.5; S, supercoiled DNA strands; N, nicked DNA strands

The percentage inhibitions of supercoiled DNA scission induced by peroxyl radicals of kodo and pearl millet extracts were 85−95 and 31−88%, respectively (Figure 2A). Despite the low content of TPC (Table 1) and phenolic acids (Table 2), dehulled grains of kodo millet had nearly 3-fold higher protection against peroxyl radical induced supercoiled DNA scission than pearl millet. This could be due to the presence of other phenolic compounds belonging to hydroxybenzoic acids and flavonoids that may possess effective antiradical activity.[15] Percentage DNA strand scission inhibitions induced by hydroxyl radicals by kodo and pearl millet extracts were 46−96 and 67−89%, respectively (Figure 2B). The hydroxyl radical is extremely reactive, although short-lived, and has been implicated as a highly damaging free radical in cells. On the other hand, peroxyl radicals have a long half-life and, thus, greater affinity to diffuse into cells, which leads to more macromolecular damage.[35] This lends further support to previous findings that wheat phenolic extracts protected supercoiled plasmid pBR 322 DNA from scission by hydroxyl radicals.[21] The outermost fractions obtained as byproducts of

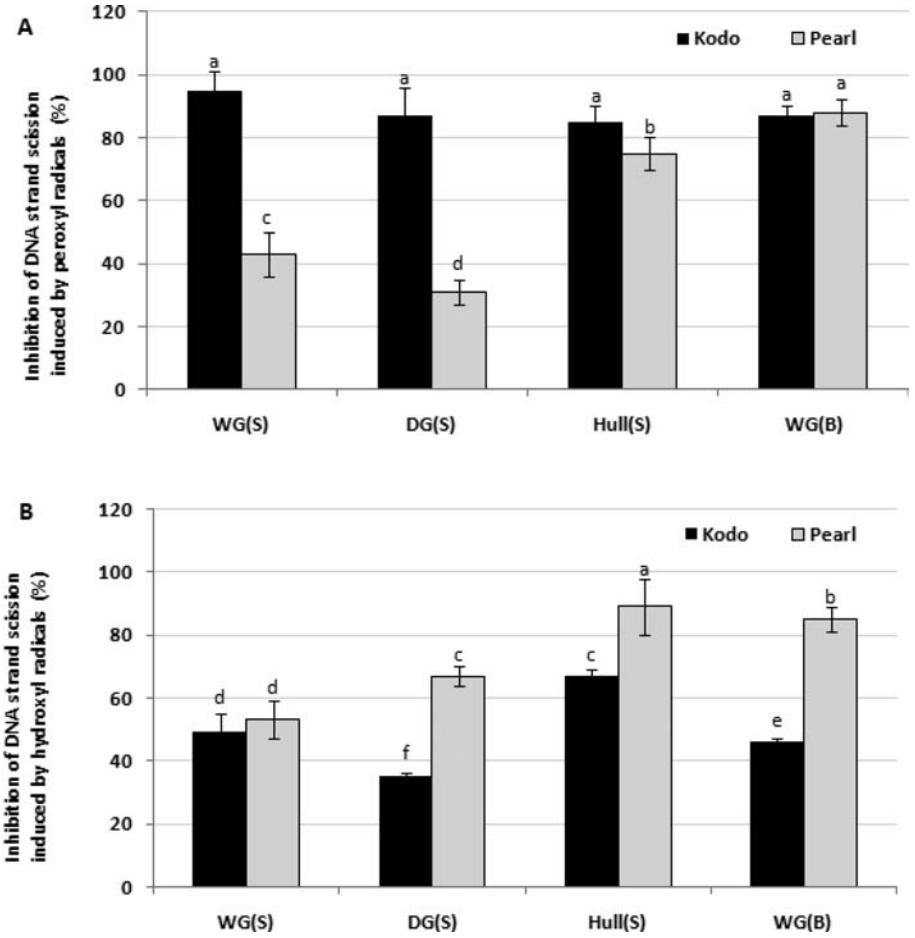

Figure 2. Retention percentage of supercoiled pBR 322 plasmid DNA in peroxyl radical-mediated (A) and hydroxyl radical-mediated (B) in vitro systems with phenolic extracts. WG(S), whole grain soluble; DG(S), dehulled grain soluble; hull(S), hulls soluble; WG(B), whole grain bound at a concentration of 100 μg/mL and 20 μL/mL in final assay. Bars with different letters are significantly ($p < 0.05$) different from one another; "a" represents the highest value.

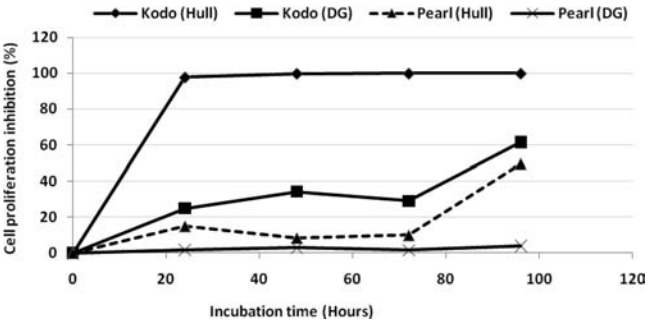

Figure 3. Percent inhibition of colon adenocarcinoma cell (HT-29) proliferation by millet soluble extracts. Dehulled grain (DG) and hulls are at a concentration of 0.5 mg/mL, from day 0 to day 4 of incubation.

pearling were found to be more effective in the inhibition of DNA strand scission. The present work indicates that natural antioxidants in kodo and pearl millets dose-dependently protected against DNA scission induced by both peroxyl and hydroxyl radicals, thus suggesting their potential use as a functional food ingredient to prevent carcinogenesis.

Human Colon Adenocarcinoma Cell Proliferation Inhibition. Colon cancer is the second most important cancer type in North America, and its prevention and therapy are important. Consistent epidemiological evidence has indicated that a diet rich in fruits, vegetables, and whole grains reduces the risk of many types of cancer, suggesting that the dietary antioxidants could be effective in the reduction of cancer incidence.[36] Figure 3 and Table 4 show that millet extracts effectively inhibited the proliferation of HT-29 adenocarcinoma cells in vitro. These results

Table 4. Percentage Inhibition of HT-29 Cell Proliferation in the Presence of Millet Extracts[a]

	kodo		pearl	
millet	0.5 mg/mL	0.1 mg/mL	0.5 mg/mL	0.1 mg/mL
whole grain[b]	100 ± 0.01a	35.5 ± 2.27b	53.6 ± 2.83b	52.7 ± 4.80a
dehulled grain[b]	75.5 ± 7.40b	24.8 ± 4.01c	33.3 ± 0.54c	43.8 ± 3.40b
hulls[b]	100 ± 0.01a	99.6 ± 0.01a	67.8 ± 3.71a	37.9 ± 0.51b
whole grain[c]	34.7 ± 3.29c	18.4 ± 4.44d	18.1 ± 2.06d	12.9 ± 0.99c

[a] Values in each column having the same letter are not significantly different ($p > 0.05$). [b] Soluble extracts. [c] Bound extracts, the concentrations of extracts are 0.015 and 0.003 μL/mL for high and low doses, respectively, and 1 μL contains bound phenolics extracted from 1 μg of defatted meal.

further showed that cell proliferation inhibition by millet grain phenolic extracts was time and dose dependent. As shown in Figure 3, hull extracts at a concentration of 0.5 mg/mL inhibited cell proliferation nearly 100% after a 24 h incubation and throughout the entire 96 h incubation period. Table 4 presents the percentage of antiproliferative activity of millet extracts against HT-29 cells at the end of a 96 h incubation period. At a concentration of 0.5 mg/mL, kodo and pearl hull extracts displayed 100 and 68% inhibition activity, respectively. On the other hand, even at a 5-fold lower concentration (0.1 mg/mL), kodo hull extracts demonstrated 100% inhibition of cell proliferation. It was noted that hull extracts of both kodo and pearl millets exhibited antiproliferative activity superior to that of the corresponding dehulled grain extracts, suggesting their potential use as natural agents in cancer therapy. The present results show that at a 5-fold lower concentration of the extracts pearl millet dehulled grain had 1.3 times higher antiproliferative activity than at high concentration. It should be noted that at the lower concentration whole pearl millet grain and dehulled grain had higher inhibition of 52.7 and 43.8%, respectively, against HT-29 cells compared to their kodo millet counterparts. These results suggest that the varying phenolic profiles of these two millet varieties may explain those observed differences. In a previous study, we reported that soluble extracts of kodo millet grains also contain flavonoids such as vitexin, isovitexin, quercetin, luteolin, and apigenin at a higher concentration than found in pearl millet grains.[15] The potential of flavonoids as chemopreventive agents in carcinogenesis has been considered.[37]

Table 5. Percentage Inhibition of LDL Cholesterol Oxidation in the Presence of Millet Extracts[a]

	kodo		pearl	
millet	0.5 mg/mL	0.125 mg/mL	0.5 mg/mL	0.125 mg/mL
whole grain[b]	$35.7 \pm 0.67c^1$	$20.5 \pm 0.12c^2$	$5.35 \pm 1.80c^1$	$0.20 \pm 0.09d^2$
dehulled grain[b]	$27.7 \pm 1.43d^1$	$16.2 \pm 0.32d^2$	$1.42 \pm 0.29d^1$	$5.50 \pm 0.35c^2$
hulls[b]	$58.2 \pm 0.16b^1$	$24.5 \pm 0.96b^2$	$9.34 \pm 0.82b^1$	$14.5 \pm 3.02b^2$
whole grain[c]	$70.8 \pm 0.58a^1$	$34.1 \pm 1.54a^2$	$32.9 \pm 1.67a^1$	$18.6 \pm 1.10a^2$

[a] Values in each column having the same letter are not significantly different ($p > 0.05$). For individual millet types, values in each row having the same superscript number (1 or 2) are not significantly different ($p > 0.05$). [b] Soluble extracts. [c] Bound extracts, the concentrations of extract are 50 and 25 μL/mL for high and low doses, respectively, and 1 μL contains bound phenolics extracted from 1 μg of defatted meal.

Inhibition of Human LDL Cholesterol Peroxidation. It has been shown that oxidation of LDL cholesterol plays a key role in the pathogenesis of atherosclerosis.[38] Thus, prevention of LDL cholesterol oxidation reduces the disease's risk and complications. The antioxidant activity of millet grain phenolic extracts was determined by measuring the concentration of conjugated

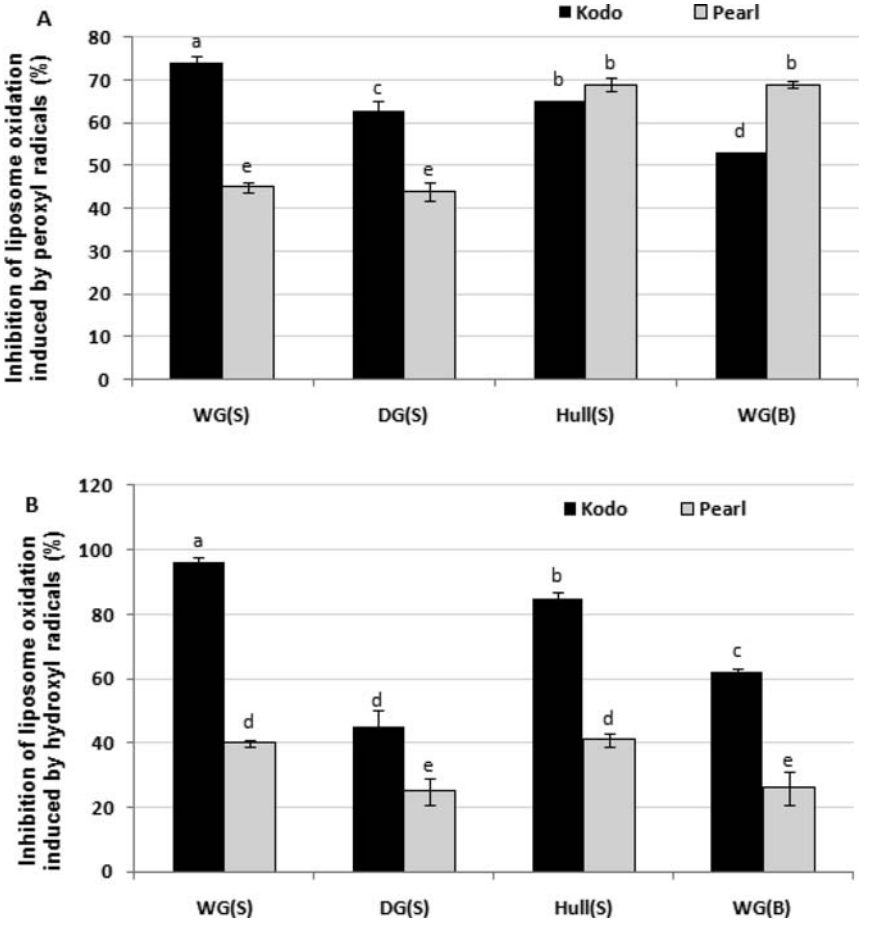

Figure 4. Inhibition of liposome oxidation induced by peroxyl radicals (A) and hydroxyl radicals (B) in the presence of phenolic extracts. WG(S), whole grain soluble; DG(S), dehulled grain soluble; hull(S), hulls soluble; WG(B), whole grain bound. Bars with different letters are significantly ($p < 0.05$) different from one another; "a" represents the highest value.

dienes formed during copper-catalyzed human LDL cholesterol oxidation in vitro, and the results were expressed as percentage inhibition of oxidation based on the CD value after 20 h of incubation (Table 5). Bound phenolic extracts of kodo and pearl whole grain millets showed 71 and 31% inhibition, respectively, at a concentration of 50 μL/mL. At each concentration of millet grain extract of 0.5 and 0.125 mg/mL, hulls displayed a higher inhibition against LDL cholesterol than that of dehulled grains. This may be attributable to the higher phenolic content of hulls as shown in the present work (Tables 1 and 2). Copper catalyzes the oxidation of unsaturated fatty acid moieties in the cholesterol molecule. The inhibition of LDL cholesterol oxidation by phenolics could account for chelation of cupric ions as well as scavenging of peroxyl radicals formed.[39] Kodo millet extracts at 0.5 mg/mL had a higher inhibition percentage compared to that of the extracts at a lower concentration. On the other hand, pearl millet hulls and dehulled grain extracts at a low concentration (0.125 mg/mL) showed 1.6 and 3.9 times higher inhibition, respectively, than that at high concentration. Although the exact reason for this observation is not clear, there is a possibility that phenolic compounds at high concentrations may complex with protein moieties of the LDL cholesterol molecules, which makes them unavailable to inhibit oxidation of cholesterol. Several earlier studies have shown that phenolic compounds can inhibit protein oxidation by virtue of binding to the proteins and forming complexes with protein molecules.[40−42] In addition, pearl millet contained proanthocyanidins at 5 μmol catechin equiv/g defatted meal, which may contribute to these observed effects.[10]

Pure phenolic acids and flavonoids are known to inhibit copper-induced LDL cholesterol oxidation.[43,44] Among different hydroxycinnamic acids, ferulic and *p*-coumaric acids showed weak inhibition of LDL cholesterol, whereas caffeic acid displayed a high activity.[45] The caffeic acid contents of kodo and pearl millet grains were 48 and 30 μg/g defatted whole grain, respectively, suggesting their contribution to the observed effects.[15] Furthermore, it has also been shown that mixtures of phenolic compounds are more effective in the inhibition of LDL cholesterol oxidation.[45] The synergistic activity of different phenolic compounds in a mixture could result in high antioxidant activity as demonstrated in the present work. In a previous study, Madhujith and Shahidi[20] showed that inhibition of LDL cholesterol oxidation of whole barley extracts ranged from 19 to 34% after 100 min of incubation. Furthermore, Liyana-Pathirana and Shahidi[21] showed that bound phenolic extracts of hard and soft wheats had a higher inhibition capacity of LDL oxidation than the soluble extract as observed in the present work.

Inhibition of Liposome Oxidation. Figure 4 shows that millet grain phenolics are effective against inhibition of liposome peroxidation induced by peroxyl (panel A) and hydroxyl (panel B) radicals. The presence of kodo millet grain extracts caused a 53−74% inhibition of liposome oxidation induced by peroxyl radical, generated by AAPH after incubation for 6 h at 37 °C. Pearl millet extracts rendered 44−69% inhibition. Similarly, hull extracts of both millet types demonstrated a significantly ($p \leq 0.05$) higher liposome inhibition compared to that of dehulled grains, essentially due to their high phenolic content. Zielinski and Kozlowska[32] earlier demonstrated that hull extracts of buckwheat, oat, and barley exhibited antioxidant activity in AAPH-induced lipid peroxidation in a PC−liposome system, which was higher than that of dehulled grains. The protection of kodo and pearl millet extracts against hydroxyl radical induced liposome oxidation was 45−96 and 25−41%, respectively (Figure 4B). Furthermore, hulls of kodo and pearl millet extracts had 1.9- and 1.6-fold higher inhibition percentage, respectively, compared to the dehulled grains. In addition, kodo millet grain extracts demonstrated higher protection against hydroxyl radical than that of pearl millet. Heinonen et al.[46] showed that ferulic acids had the highest antioxidant activity in a lecithin−liposome system and suppressed hydroperoxide formation. Furthermore, ferulic acid esters also showed potent inhibitory activity in PC liposome oxidation.[47] The present results clearly show that millet grain phenolics are capable of protecting membrane lipids from oxidation caused by free radical reactions, thus saving cell membrane integrity and function. This protective effect could be due to the free radical scavenging and metal ion chelation activities of millet phenolics. Furthermore, phenolic compounds may interact with membrane phospholipids by hydrogen bonding to the polar head groups of phospholipids and may accumulate at the surface of the membrane, thus preventing access of radicals to the lipid region. Verstraeten et al.[48] demonstrated that flavan-3-ols and procyanidins can potentially reduce liposome oxidation by limiting the access of oxidants to the bilayer.

The results of the present work demonstrated that dehulled grains of millet and hulls inhibited DNA scission, LDL cholesterol, liposome oxidation,and proliferation of HT-29 adenocarcinoma cells. Bound phenolic extracts showed considerable bioactivity and release of these compounds in the colon upon microbial fermentation and, hence, may impart health benefits locally. Hydroxycinnamic acids, mainly ferulic and *p*-coumaric acids, may contribute to the observed action of millet phenolics in addition to hydroxybenzoic acids and flavonoids. The results of the present study suggest that millet grains could serve as a promising cereal grain for therapeutic diets. However, the bioavailability and bioefficacy of millet phenolics upon absorption are yet to be unraveled.

AUTHOR INFORMATION

Corresponding Author

*Phone: (709) 864-8552. Fax: (709) 864-4000. E-mail: fshahidi@mun.ca.

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