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Antioxidant Phytochemicals in Hazelnut Kernel (*Corylus avellana* L.) and Hazelnut Byproducts

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Antioxidant efficacies of ethanol extracts of defatted raw hazelnut kernel and hazelnut byproducts (skin, hard shell, green leafy cover, and tree leaf) were evaluated by monitoring total antioxidant activity (TAA) and free-radical scavenging activity tests [hydrogen peroxide, superoxide radical, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical], together with antioxidant activity in a β-carotene−linoleate model system, inhibition of oxidation of human low-density lipoprotein (LDL) cholesterol, and inhibition of strand breaking of supercoiled deoxyribonucleic acid (DNA). In addition, yield, content of phenolics, and phenolic acid profiles (free and esterified fractions) were also examined. Generally, extracts of hazelnut byproducts (skin, hard shell, green leafy cover, and tree leaf) exhibited stronger activities than hazelnut kernel at all concentrations tested. Hazelnut extracts examined showed different antioxidative efficacies, expected to be related to the presence of phenolic compounds. Among samples, extracts of hazelnut skin, in general, showed superior antioxidative efficacy and higher phenolic content as compared to other extracts. Five phenolic acids (gallic acid, caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid) were tentatively identified and quantified (both free and esterified forms). Extracts contained different levels of phenolic acids. These results suggest that hazelnut byproducts could potentially be considered as an excellent and readily available source of natural antioxidants.

KEYWORDS: Hazelnut extracts; phenolics; phenolic acids; antioxidant activity; free radical scavenging activities; inhibition of human LDL oxidation, β-carotene−linoleate model system; inhibition of DNA scission

INTRODUCTION

Nuts are known as a source of nutritious food with a high content of healthful lipids. Recent recognition of nuts as "heart-healthy" foods by the U.S. FDA has provided a major boost to the image of nuts. Hazelnut (*Corylus avellana* L.), which belongs to the family Betulaceae, is one of the most popular tree nuts on a worldwide basis and ranks second in tree nut production after almond. Turkey, specifically the Black Sea region, is the major hazelnut-producing area, which contributes >75% to the total global production (*1*). Hazelnut is typically consumed as the whole nut (raw or roasted) or used as an ingredient in a variety of processed foods, especially in bakery and confectionery products.

Figure 1 shows the ready-to-harvest hazelnut fruit and its byproducts. The hazelnut green leafy covers, occasionally together with hazelnut tree leaves, are mechanically removed from hazelnut hard shells soon after harvesting. The hazelnut hard shell, containing a kernel, is the nut of commerce. After the hazelnut hard shell has been cracked, the hazelnut kernel may be consumed raw (with skin) or preferably roasted (without skin). In brief, hazelnut skin, hazelnut hard shell, and hazelnut green leafy cover as well as hazelnut tree leaf are byproducts of roasting, cracking, shelling/hulling, and harvesting processes, respectively. Among these, none has any commercial value except the hazelnut hard shell, which is currently used as a heating source upon burning. However, hazelnut green leafy covers and tree leaves are rarely used as organic fertilizers for the hazelnut trees and vegetables upon composting. The use of hazelnut byproducts as potential sources of natural antioxidants and functional food ingredients is of great interest to the hazelnut industry.

Plant-derived products contain a wide range of phytochemicals and phenolic compounds that possess substantial antioxidant and antiradical activities (*2*), anticarcinogenic and antimutagenic effects (*3*), and antiproliferative potential (*4*). These phytochemicals and phenolics provide protection against harmful effects of free radicals and are known to reduce the risk of

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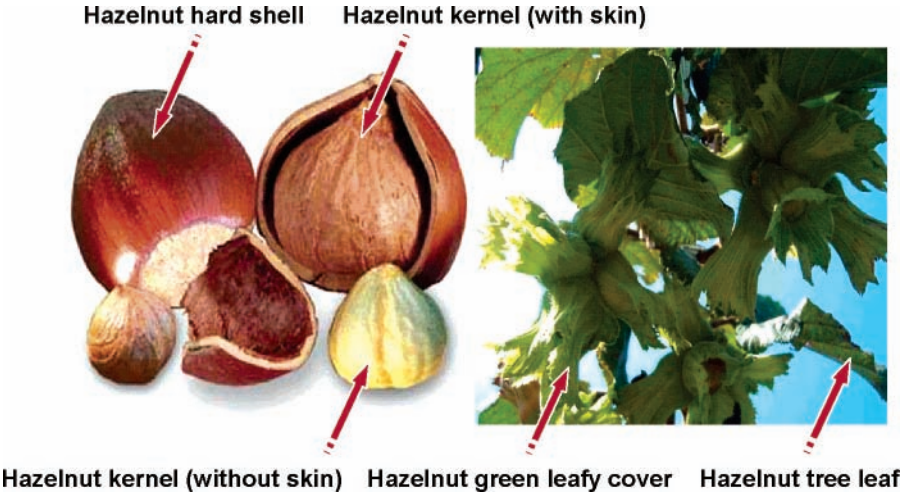

Figure 1. Hazelnut kernel and hazelnut byproducts.

certain types of cancer, CHD, CVD, stroke, atherosclerosis, osteoporosis, inflammation, and other neurodegenerative diseases associated with oxidative stress (*2*, *3*, *5*−*9*). Evaluation of antioxidant efficacy in plant products cannot be carried out accurately by any single universal method or extraction solvent due to the complex nature of the phytochemicals present (*2*, *10*, *11*). Numerous methods (*2*, *11*−*16*) have been used to evaluate and estimate the antioxidant efficacy of foods and dietary supplements, and these relate to measurement of free radical scavenging activity, reducing power, and chelation of pro-oxidative metal ions. Depending on the chemical structure-(s) of the active components, one or a combination of the above mechanisms may be operative.

The antioxidant activity of nuts (*17*) and their byproducts has been studied (*14*, *18*−*24*). These studies have acknowledged that nut byproducts are rich sources of natural antioxidants and phenolic compounds which may render beneficial biological activities and health effects.

Although some papers have been published regarding the antioxidant activity and phenolic constituents of hazelnut kernel (*17*, *25*−*27*) and some of its byproducts (*28*, *29*), little is known about the antioxidant efficacy, phenolic acids, free radical scavenging activities, inhibition of oxidation of human LDL, and inhibition of hydroxyl radical induced DNA scission of hazelnut kernel and its byproducts. Extracts of natural antioxidants from processing byproducts of hazelnut could potentially be used as nutraceuticals, dietary supplements, and pharmaceuticals or cosmetic ingredients, among others. The objectives of this study were to investigate the phenolic constituents in the extracts of hazelnut kernel and hazelnut byproducts (skin, hard shell, green leafy cover, and tree leaf) and to evaluate their antioxidant and free radical scavenging activities by a number of indicators. Inhibition of oxidation of human LDL and DNA scission was also examined.

MATERIALS AND METHODS

Samples. The premium class raw Turkish Tombul hazelnut kernels (*Corylus avellana* L.) and their byproducts (hazelnut skins, hazelnut hard shells, hazelnut green leafy covers, and hazelnut tree leaves) were obtained from the Giresun province of Turkey at the beginning of the harvest season of 2004 (**Figure 1**). Briefly, hazelnut green leafy covers were removed by hand from hazelnuts soon after harvesting. Unshelled hazelnuts, hazelnut green leafy covers, and hazelnut tree leaves were separately, but at the same time, sun-dried for 3 days at ∼20−25 °C. The sun-dried hazelnut green leafy covers and tree leaves were green/brownish in color. The skins were obtained from the Hazelnut Processing and Exporting Plant (Başkan Gida, Giresun, Turkey). For this, the sun-dried unshelled Tombul hazelnuts were cracked and then roasted at 165 °C for 25 min with an air velocity of 1 m/s to obtain skins. All samples were dispatched by DHL World Wide Express to the Department of Biochemistry, Memorial University of Newfoundland, and then kept in a dark room at 5 °C until they were analyzed. The unshelled hazelnuts were cracked prior to analysis to remove hazelnut hard shells. The remaining hazelnut kernels (with skins) were used for the analysis.

Chemicals. The compounds AAPH, ABTS, DPPH, BHT, DNA of pBR322 (*Escherichia coli* strains PRI), EDTA, Folin−Ciocalteu phenol reagent, human LDL, Trolox, Tween 40, and β-carotene were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). All other chemicals and solvents were purchased either from Sigma-Aldrich Canada Ltd. or Fisher Scientific Co. (Nepean, ON, Canada), unless otherwise stated.

Preparation of Defatted Samples. All samples that were tested were ground separately in a coffee grinder (model CBG5 series, Black and Decker Canada Inc., Brockville, ON) for 3 min and then defatted by blending with hexane (1:5, w/v, 3 × 5 min) in a Waring blender (model 33BL73, Waring Products Division, Dynamics Corp. of America, New Hartford, CT) at ambient temperature. Defatted samples were subsequently air-dried for 12 h and stored in vacuum-packaged polyethylene pouches at −20 °C until they were used for further analysis.

Extraction of Crude Phenolics. Phenolic compounds present in defatted samples were extracted using 80:20 (v/v) ethanol/water mixture (6 g of sample/100 mL of solvent) under reflux conditions in a thermostated water bath at 80 °C for 30 min (*14*). The resulting slurries were centrifuged at 4000*g* (model ICE Centra M5, International Equipment Co., Needham Heights, MA) for 5 min, and the supernatant was collected. The residue was re-extracted twice under the same conditions, and supernatants were combined. Then, the solvent was removed from the combined supernatants under vacuum at 40 °C (model Büchi Rotavapor R-114, Büchi Labortechnik AG, Flawil, Switzerland), and the remaining water in the concentrated extract was removed by lyophilization for 72 h at −48 °C and 0.046 mbar (Freezone 6, model 77530, Labconco Co., Kansas City, MO). Finally, the prepared crude phenolic extracts were stored at −20 °C in vacuum-sealed pouches (in the dark) until they were used for further analysis.

Determination of Phenolic Content. Hazelnut crude phenolic extracts were dissolved in methanol to obtain a concentration of 1 mg/mL for hazelnut kernel extract and 0.5 mg/mL for hazelnut byproducts (skin, hard shell, green leafy cover, and tree leaf) extracts. The content of phenolics in extracts was determined according to the procedure described by Singleton and Rossi (*30*) using the Folin−Ciocalteu phenol reagent as detailed by Siriwardhana and Shahidi (*14*). Folin−Ciocalteu phenol reagent (0.5 mL) was added to centrifuge tubes containing 0.5 mL of the extracts. The contents were mixed, and 1 mL of a saturated sodium carbonate solution was added to each tube, followed by adjusting the volume to 10 mL with distilled water. The contents in the tubes were thoroughly mixed by vortexing. Tubes were allowed to

stand at ambient temperature for 45 min (until the characteristic blue color developed) and then centrifuged at 4000*g* for 5 min (International Equipment Co.). Absorbance of the clear supernatant was measured at 725 nm using a diode array spectrophotometer (model 8452A, Agilent Technologies Canada Inc., Mississauga, ON). A blank devoid of any extract was used for background subtraction. The content of total phenolics in each extract was determined from a standard curve using catechin as a standard and expressed as milligrams of CE per gram of extract.

Determination of TAA. The TAA in extracts was determined according to the TEAC assay described by van den Berg et al. (*31*) and modified by Siriwardhana and Shahidi (*14*). The extracts and reagents were prepared in a 0.1 M PBS containing 0.15 M sodium chloride. A solution of $ABTS^{\bullet-}$ was prepared by mixing 2.5 mM AAPH with 2.0 mM $ABTS^{\bullet-}$ at a 1:1 (v/v) ratio. The radical solution was heated at 60 °C for 12 min, protected from light, and subsequently stored at room temperature. The absorbance of the freshly prepared radical solution at 734 nm was about 0.4. To measure the antioxidant activity, hazelnut extracts were dissolved in PBS at a concentration of 2 mg/mL and diluted to fit in the range of the assay values. A standard curve was prepared by measuring the reduction in the absorbance (ΔA) of the $ABTS^{\bullet-}$ solution at different concentrations of Trolox over a period of 6 min, as the change in absorbance after 6 min of assay was marginal. The absorbance values were corrected for the solvent. The TEAC values for the hazelnut extracts were determined in the same manner; 40 μL of extract solution was mixed with 1960 μL of $ABTS^{\bullet-}$ solution, and the absorbance was monitored over a 6-min period. A blank measurement was recorded for each case that corresponded to a decrease in absorbance without any compound added. The TEAC of an extract represents the concentration of a Trolox solution that has the same antioxidant activity as the extract. TEAC values were determined as follows:

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

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

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

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

Extraction, Hydrolysis, Identification, and Quantification of Phenolic Acids. Phenolic acids in extracts were assessed according to the HPLC method of Amarowicz and Weidner (*32*) as detailed by Alasalvar et al. (*28*). An aqueous suspension of the extract (100 mg in 10 mL) was adjusted to pH 2 (using 6 M HCl), and free phenolic acids were extracted five times, each into 10 mL of diethyl ether, using a separatory funnel. The combined extracts were then evaporated to dryness under vacuum at room temperature. The aqueous solution was neutralized and then lyophilized. The residue was dissolved in 10 mL of a 2 M NaOH and hydrolyzed for 4 h at room temperature under a stream of nitrogen. After acidification to pH 2 (using 6 M HCl), phenolic acids liberated from soluble esters were extracted five times, each into 15 mL of diethyl ether, using a separatory funnel. Ether from the combined extracts was then evaporated to dryness. The dry residues of free and esterified phenolic acids were dissolved separately in 2 mL of methanol and finally filtered through a Gelman Acrodisc LC13 PVDV 0.45-μm pore size syringe filter (PALL Life Sciences, Ann Arbor, MI) for HPLC analysis.

Phenolic acids in each fraction were analyzed using a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) consisting of an LC-10AD pump, an SCTL 10A system controller, and an SPD-M 10A photo-DAD. Twenty microliters of the sample extracts was automatically injected into a prepacked LiChrospher 100 RP-18 column (250 mm × 4 mm inner diameter, 5-μm particles, Merck, Darmstadt, Germany) at room temperature. Isocratic elution (filtered through a 0.45-μm Millipore filter prior to use) was employed with a mobile phase consisting of HPLC grade water/acetonitrile/acetic acid (88:10:2, v/v/v) at a flow rate of 1 mL/min. The wavelengths of the DAD were set at 280 and 320 nm for monitoring phenolic acids. Tentatively identified phenolic acids were quantified on the basis of their peak areas and comparison with a calibration curve obtained with the corresponding standards (gallic acid, caffeic acid, *p*-coumaric acid, *o*-coumaric acid, *m*-coumaric acid, ferulic acid, sinapic acid, vanillic acid, protocatechuic acid, syringic acid, gentisic acid, and salicylic acid). The results from free and esterified hydrolysates were calculated to represent total phenolic acids. Phenolic acids are expressed as micrograms of phenolic per gram of extract.

Determination of Hydrogen Peroxide Scavenging Activity. The method described by Ruch et al. (*33*) as modified by Siriwardhana and Shahidi (*14*) was used to determine the hydrogen peroxide scavenging activity of hazelnut extracts. Extracts were dissolved in 3.4 mL of a 0.1 M PBS and mixed with 0.6 mL of a 43 mM solution of hydrogen peroxide (prepared in the same buffer). Catechin was used as the reference compound. Final concentrations of extracts and standards were 100 or 200 ppm as catechin equivalents. (A 200 ppm solution of extracts of hazelnut kernel, hazelnut skin, hazelnut hard shell, hazelnut green leafy cover, and hazelnut tree leaf was prepared by dissolving 17.2, 0.4, 1.1, 1.86, and 1.74 mg of extracts, respectively, in 1 mL of 0.1 M PBS.) The absorbance (at 234 nm) of the reaction mixture was recorded for 40 min at 10 min intervals. For each extract concentration, a separate blank sample devoid of hydrogen peroxide was also used for background subtraction. Reduction of absorbance in a hydrogen peroxide solution alone due to its degradation was recorded, and values were corrected accordingly. The concentration of hydrogen peroxide in the assay medium was determined using a standard curve, and hydrogen peroxide scavenging activity of the extracts was calculated using the following equation.

$$H_2O_2\ \text{scavenging activity}\ (\%) = 100 - \left(\frac{H_2O_2\ \text{concentration of medium containing the additive}}{H_2O_2\ \text{concentration of the control medium}}\right) \times 100$$

Determination of Superoxide Radical Scavenging Activity. Superoxide radical was generated with an enzymatic reaction according to the procedure described by Nishikimi et al. (*34*) and detailed by Siriwardhana and Shahidi (*14*). The reaction mixture contained 1 mL of 3 mM hypoxanthine, 1 mL of xanthine oxidase (100 mIU), 1 mL of 12 mM diethylenetriaminepentaacetic acid, 1 mL of 178 mM nitro blue tetrazolium, and 1 mL of the extracts. Final concentration of the starting materials in the reaction mixture was 100 or 200 ppm. [A 200 ppm solution (as catechin equivalents) of extracts of hazelnut kernel, hazelnut skin, hazelnut hard shell, hazelnut green leafy cover, and hazelnut tree leaf was prepared by dissolving 73, 1.74, 4.68, 7.86, and 7.42 mg of extracts, respectively, in 1 mL of 0.1 M PBS.] Catechin was used as the reference antioxidant. All solutions were prepared in 0.1 M PBS. The absorbance (at 560 nm) of the medium was recorded for 60 min at 10-min intervals. The absorbance values were corrected by subtracting 0-min readings from those obtained subsequently. Superoxide radical scavenging activity (after 10 min of assay) of the additives was calculated using the following equation.

$$\text{superoxide radical scavenging activity}\ (\%) = 100 - \left(\frac{\text{absorbance of medium containing the additive}}{\text{absorbance of the control medium}}\right) \times 100$$

Determination of DPPH Radical Scavenging Activity. The method described by Kitts et al. (*35*) was used with slight modifications (*36*) to assess the DPPH radical scavenging activity of hazelnut extracts. A 0.135 mM DPPH solution in ethanol (1.0 mL) was mixed with various amounts of hazelnut extracts (a 100 ppm solution of extracts of hazelnut kernel, hazelnut skin, hazelnut hard shell, hazelnut green leafy cover, and hazelnut tree leaf was prepared by dissolving 14.6, 0.35, 0.93, 1.57, and 1.48 mg of extracts, respectively, in 1 mL of ethanol) and vortexed thoroughly. The absorbance of the mixtures at ambient temperature was recorded for 60 min at 10-min intervals. Catechin was used as the reference antioxidant. The absorbance of the remaining DPPH radicals was read at 519 nm using a diode array spectrophotometer (Agilent

Technologies Canada Inc., Mississagua, ON). The scavenging of DPPH radical of the extracts were calculated according to the equation

$$\text{DPPH radical scavenging activity (\%)} = \left(\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right) \times 100$$

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

***β*-Carotene−Linoleate Model System.** The antioxidative activity of the extracts was evaluated using a β-carotene−linoleate model system (*36*, *37*). A solution of β-carotene was prepared by dissolving 25 mg of β-carotene in 5 mL of chloroform. β-Carotene solution (3 mL) was pipetted into a 100-mL round-bottom flask, and chloroform was removed under vacuum using a rotary evaporator at 40 °C (Büchi Labortechnik AG). Forty milligrams of linoleic acid, 400 mg of Tween 40 emulsifier, and 100 mL of distilled water were added to the flask. The contents were mixed thoroughly with vigorous shaking. Aliquots (3.0 mL) of the emulsion were transferred into a series of tubes containing 2.0 mL of the hazelnut extracts in methanol. [A 100 ppm solution (as catechin equivalents) of extracts of hazelnut kernel, hazelnut skin, hazelnut hard shell, hazelnut green leafy cover, and hazelnut tree leaf was prepared by dissolving 18.25, 0.43, 1.17, 1.96, and 1.86 mg of extracts, respectively, in 1 mL of methanol.] Catechin was used as the reference antioxidant. Absorbance values were recorded over a 120-min period at 20-min intervals while the samples were kept in a water bath at 50 °C. Blank samples devoid of β-carotene were prepared for background subtraction. The AI was calculated using the following equation.

$$\text{AI} = \left(\frac{\beta\text{-carotene content after 120 min of assay}}{\text{initial } \beta\text{-carotene content}}\right) \times 100$$

Determination of the Effects of Hydrolysis on Oxidation of Human LDL Cholesterol. The procedure originally described by Hu and Kitts (*38*) and slightly modified by Liyana-Pathirana and Shahidi (*36*) was employed in this study. Human LDL from the same source was dialyzed in 10 mM PBS at 4 °C in the dark for 24 h. Human LDL (0.2 mg of human LDL/mL) was mixed with various amounts of hazelnut extracts dissolved in 10 mM PBS. [A 100 ppm solution (as catechin equivalents) of extracts of hazelnut kernel, hazelnut skin, hazelnut hard shell, hazelnut green leafy cover, and hazelnut tree leaf was prepared by dissolving 14.6, 0.35, 0.93, 1.57, and 1.48 mg of extracts, respectively, in 1 mL of 10 mM PBS.] Catechin was used as the reference antioxidant compound. The oxidation was initiated by adding a 10 μM solution of $CuSO_4$, and subsequently samples were incubated at 37 °C for 22 h. The formation of conjugated dienes from the oxidation of human LDL was recorded at 234 nm using a diode array spectrophotometer (Agilent Technologies Canada Inc.). The inhibitory effect of hazelnut extracts on the formation of conjugated dienes (% $\text{inhibition}_{\text{CD}}$) was then calculated according to the equation

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

where $\text{Abs}_{\text{sample}}$ = absorbance of LDL + $CuSO_4$ + hazelnut extract/standard, $\text{Abs}_{\text{native}}$ = absorbance of LDL + PBS, and $\text{Abs}_{\text{oxidative}}$ = absorbance of LDL + $CuSO_4$ + PBS. A separate blank that contained all of the reagents except human LDL was used for each extract. The amount of LDL (μg) that can be protected against copper-mediated oxidation by different amounts of hazelnut extract was calculated from percentage values.

Inhibition of Strand Breaking of Supercoiled DNA. DNA strand-breaking studies by hydroxyl radical were performed according to the method described by Johnson and Grossman (*39*) and Hiramoto et al. (*40*) with slight modifications. The reaction was carried out in 1 M PBS. The reaction mixture contained 2 μL of PBS, 4 μL of a solution of extract at the indicated final concentration (5−50 ppm as catechin equivalents), 2 μL of a solution of supercoiled plasmid pBR 322 DNA (4300 base pairs) at 100 μg/mL, 6 μL of 0.33 mM H_2O_2, and 6 μL of 0.33 mM $FeSO_4$ added in the order stated. The reaction was carried out in an Eppendorf tube (1 mL) and incubated at 37 °C for 1 h. Simultaneously, the plasmid DNA was also incubated with the restriction endonuclease *Hin*dIII. [*Hin*dIII has one restriction site on the pBR 322 plasmid DNA, thus producing one fragment having the original number of base pairs. The reaction mixture contained 8 μL of DNA (100 μg/mL), 2 μL of *Hin*dIII restriction enzyme, 2 μL of restriction buffer (× 10) and 8 μL of distilled water.] For identification, the base pair ladder DRIgest III was run along with the extracts. After incubation, 2 μL of the loading dye (0.25% bromophenol blue/0.25% xylene cyanol/50% glycerol) was added, and the whole mixture was loaded onto an 0.8% (w/v) agarose gel prepared in TBE electrophoresis buffer (pH 8.3). Agarose gel electrophoresis was performed using TBE electrophoresis buffer at 116 V for 75 min. The gel was stained with 0.5 μg/mL ethidium bromide, and bands were visualized under ultraviolet light. The images were analyzed using AlphaEase Stand Alone software (Alpha Innotech Corp., San Leandro, CA). The protective effects of the crude extracts were measured using the retention percentage of supercoiled DNA.

$$\text{DNA retention (\%)} = \left(\frac{\text{DNA content with the oxidative radical and extract}}{\text{DNA content without the oxidative radical}}\right) \times 100$$

Statistical Analysis. Results were expressed as means ± SD (n = 3) for each extract. The statistical significance (t test: two-sample equal variance, using two-tailed distribution) was determined using Microsoft Excel statistical software (Microsoft Office Excel 2003, Microsoft Corp., Redmond, WA). Differences at $p < 0.05$ were considered to be significant.

Table 1. Yield, Content of Phenolics, and TAA in Extracts of Hazelnut Kernel and Hazelnut Byproducts[a]

extract	yield[b]	phenolics[c]	TAA[d]
hazelnut kernel (with skin)	2.26 ± 1.11 e	13.7 ± 0.5 e	29.0 ± 3.5 e
hazelnut skin	10.28 ± 1.02 f	577.7 ± 1.1 f	132.0 ± 4.0 f
hazelnut hard shell	2.53 ± 0.33 e	214.1 ± 0.3 g	120.0 ± 3.0 g
hazelnut green leafy cover	3.59 ± 0.85 e	127.3 ± 0.7 h	117.0 ± 2.5 g
hazelnut tree leaf	1.64 ± 1.87 e	134.7 ± 1.0 i	148.0 ± 2.1 h

[a] Data are expressed as means ± SD (n = 3) on an extract. Means ± SD followed by the same letter, within a column, are not significantly different ($p > 0.05$). [b] Expressed as grams per 100 g of defatted samples. [c] Expressed as milligrams of CE per gram of extract. [d] Expressed as micromoles of TE per gram of extract.

RESULTS AND DISCUSSION

Yield. The extract yields after lyophilization of hazelnut kernel, hazelnut skin, hazelnut hard shell, hazelnut green leafy cover, and hazelnut tree leaf were 2.26, 10.28, 2.53, 3.59, and 1.64 g/100 g of defatted samples, respectively (**Table 1**). These values (except for hazelnut skin) were significantly different ($p < 0.05$) from each other.

Content of Phenolics. The phenolic contents, as milligrams of CE per gram of ethanol extract, were 13.7, 577.7, 214.1, 127.3, and 134.7 for hazelnut kernel, hazelnut skin, hazelnut hard shell, hazelnut green leafy cover, and hazelnut tree leaf, respectively (**Table 1**). Significant differences ($p < 0.05$) in the phenolic contents existed among hazelnut extracts. Siriwardhana and Shahidi (*14*) evaluated the antiradical activity of extracts of whole almond seed (kernel) and its byproducts (brown skin and green shell cover) and found that the 80% (v/v) ethanol extracts of whole almond seed, brown skin, and green shell (leafy) cover had a phenolic content of 8.1, 87.8, and 71.1 mg of CE/g of extract, respectively. A higher concentration of total phenolics in almond seed using 80% (v/v) acetone extract (16.1 mg of CE/g of extract) was obtained by Amarowicz et al. (*41*). Alasalvar et al. (*28*) found that extracts obtained from 80% (v/v) ethanol were characterized as having significantly lower

Table 2. Contents of Phenolic Acids (Free and Esterified) in Extracts of Hazelnut Kernel and Hazelnut Byproducts[a]

extract	gallic	caffeic	*p*-coumaric	ferulic	sinapic
hazelnut kernel (with skin)	127 ± 5 b	81 ± 2 b	208 ± 15 b	105 ± 5 b	93 ± 5 b
hazelnut skin	387 ± 9 c	trace c	231 ± 17 b	124 ± 8 c	124 ± 4 c
hazelnut hard shell	3261 ± 79 d	212 ± 13 d	757 ± 31 c	333 ± 25 d	235 ± 17 d
hazelnut green leafy cover	892 ± 43 e	158 ± 6 e	1662 ± 43 d	327 ± 15 d	64 ± 3 e
hazelnut tree leaf	157 ± 8 f	362 ± 10 f	884 ± 19 e	237 ± 12 e	241 ± 11 d

[a] Data are expressed as means ± SD ($n = 3$) on an extract. Phenolic acids, expressed as micrograms per gram of extract. Means ± SD followed by the same letter, within a column, are not significantly different ($p > 0.05$).

($p < 0.05$) content of phenolics compared to those of extracts obtained from 80% (v/v) acetone. Yu et al. (*22*) compared three different extraction solvent systems [water, 80% (v/v) ethanol, and methanol] and peeling methods (direct, blanching, and roasting) using peanut skin phenolics and found that a combination of roasting and ethanol extraction was the most efficient recovery method. The content of phenolics resulting from ethanol extract of roasting peeling was 125 mg of GAE/g of non-defatted dry peanut skin. By comparison with almond and peanut skins, skin obtained from hazelnut had much higher levels of phenolics.

TAA. The total antioxidant activities of hazelnut extracts ranged from 29 to 148 μmol of TE/g of ethanol extract, being lowest in hazelnut kernel and highest in hazelnut tree leaf (**Table 1**). TAA values of hazelnut byproduct extracts were approximately 4−5-fold greater than that of hazelnut kernel at the same extract concentration. In other words, at a given concentration, hazelnut byproduct extracts would be more effective antioxidants than hazelnut kernel extract. Similar results were obtained by Siriwardhana and Shahidi (*14*), who evaluated the TEAC of almond and its byproduct extracts and found that the 80% (v/v) ethanol extracts at the same extract concentration were in the order of brown skin > green shell cover > whole seed. TEAC values of brown skin and green shell cover extracts were 12.6- and 9.8-fold higher than that of whole seed extract, respectively. Alasalvar et al. (*28*) observed that extracts obtained from 80% (v/v) ethanol were characterized as having significantly lower ($p < 0.05$) TAA compared to those of extracts obtained from 80% (v/v) acetone.

Wu et al. (*17*) measured the lipophilic and hydrophilic antioxidant capacities of common foods in U.S. markets and found that hazelnut had the third highest value (96.45 μmol of TE/g on an as-is basis) of total antioxidant capacity (by combining L-$ORAC_{FL}$ and H-$ORAC_{FL}$) among 10 nut samples that were examined, with pecan and walnut having the highest. Consideration of defatted hazelnut (on an extract basis) and hazelnut (as-is weight basis) makes the quantitative comparison between the two studies impossible.

Phenolic Acids. The contents of total soluble phenolic acids (free and esterified) in ethanol extracts of hazelnut kernel and hazelnut byproducts are listed in **Table 2**. A total of five phenolic acids were tentatively identified, one of which was a hydroxylated derivative of benzoic acid (gallic acid) and four of which were cinnamic acid derivatives (caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid). In addition, there were several unknown compounds in both free and esterified phenolic acids. The same number, but different concentrations, of phenolic acids were also identified earlier in hazelnut kernel and hazelnut green leafy cover (*28*). The order of total phenolic acid concentration was as follows: hazelnut hard shell > hazelnut green leafy cover > hazelnut tree leaf > hazelnut skin > hazelnut kernel. However, the dominance of each acid in the products depended upon its location in the samples examined. Among the identified phenolic acids, *p*-coumaric acid was most abundant in hazelnut kernel, hazelnut green leafy cover, and hazelnut tree leaf, whereas gallic acid was most abundant in hazelnut skin and hazelnut hard shell, possibly implying the presence and perhaps the dominance of tannins in the latter samples.

Senter et al. (*42*) compared phenolic acids of nine edible tree nuts produced in the United States. The extracts from the nut samples showed great diversity in the phenolic acids present. Qualitative and quantitative differences existed among nut samples in the phenolic acids present, with gallic acid being predominant except in pine nut, almond, and hazelnut (filbert). A total of eight phenolic acids were isolated and identified among nine nuts (*p*-hydroxybenzoic acid, *p*-hydroxyphenylacetic acid, vanillic acid, protocatechuic acid, syringic acid, gallic acid, caffeic acid, and ferulic acid). Protocatechuic acid has been reported to be the predominant phenolic acid in testa (skin) of hazelnut with a concentration of 0.36 μg/g. This phenolic acid was not detected in this study or in our previous study (*28*). It has been reported that caffeic acid, sinapic acid, ferulic acid, and *p*-coumaric acid are better antioxidants than syringic acid, vanillic acid, and protocatechuic acid (*43*).

Yurttas et al. (*25*) isolated and tentatively identified six phenolic aglycones in Turkish and American hazelnut extracts; these were gallic acid, *p*-hydroxybenzoic acid, epicatechin and/or caffeic acid, sinapic acid, and quercetin. However, the variety of hazelnut and extraction solvents used in this study were different from that used by Yurttas et al. (*25*). Variety and extraction exerted a great influence on the concentration and variability of phenolic acids present. Recently, Amaral et al. (*29*) identified and quantified four phenolic acids, namely, 3-caffeoylquinic acid, 5-caffeoylquinic acid, caffeoyltartaric acid, and *p*-coumaroyltartaric acid, in hazelnut leaves from 10 different cultivars grown in Portugal. Like hazelnut, some other tree nuts and their processing byproducts have been reported to contain different patterns and levels of phenolic acids (*2*).

Hydrogen Peroxide Scavenging Activity. The scavenging activity of hydrogen peroxide by hazelnut extracts and catechin as the reference antioxidant was measured spectrophotometrically at 234 nm (**Table 3**). At 200 ppm concentration, all extracts exhibited 97−99% scavenging of hydrogen peroxide with the exception of hazelnut kernel, which scavenged only 77% of hydrogen peroxide. Thus, scavenger concentration was mainly in the outer portions of the hazelnut kernel. Scavenging activity varied between 60 and 95% at the 100 ppm level. Catechin exhibited 91 and 96% hydrogen peroxide scavenging activity at 100 and 200 ppm concentrations, respectively. Compared to catechin, extracts of hazelnut byproducts showed stronger ($p < 0.05$) hydrogen peroxide scavenging activity than did catechin at 200 ppm concentration, except hazelnut green leafy cover ($p > 0.05$). Hence, hazelnut byproducts may serve as effective scavengers and thereby protect cells from oxidative damage. Hydrogen peroxide has the potential to cause damage through

Table 3. Radical Scavenging Activities in Extracts of Hazelnut Kernel and Hazelnut Byproducts[a]

	hydrogen peroxide scavenging activity (%)		superoxide radical scavenging activity (%)		DPPH radical scavenging activity (%)	
extract	100 ppm	200 ppm	100 ppm	200 ppm	50 ppm	100 ppm
hazelnut kernel (with skin)	60 ± 3 b	77 ± 2 b	82 ± 1 b	94 ± 1 b	86.1 ± 0.1 b	92.2 ± 0.1 b
hazelnut skin	95 ± 1 c	99 ± 1 c	88 ± 1 c	99 ± 1 c	93.4 ± 0.2 c	99.5 ± 0.2 c
hazelnut hard shell	94 ± 3 cd	99 ± 1 c	88 ± 2 c	99 ± 1 c	93.5 ± 0.1 c	99.4 ± 0.2 c
hazelnut green leafy cover	85 ± 2 e	97 ± 2 cd	86 ± 2 c	99 ± 1 c	97.3 ± 0.1 d	99.5 ± 0.1 c
hazelnut tree leaf	93 ± 2 cf	99 ± 1 c	87 ± 2 c	99 ± 1 c	94.8 ± 0.2 e	99.4 ± 0.2 c
catechin	91 ± 1 df	96 ± 1 d	90 ± 2 c	91 ± 1 d	100.0 ± 0.0 f	100.0 ± 0.0 d

[a] Data are expressed as means ± SD ($n = 3$) on an extract. Means ± SD followed by the same letter, within a column, are not significantly different ($p > 0.05$).

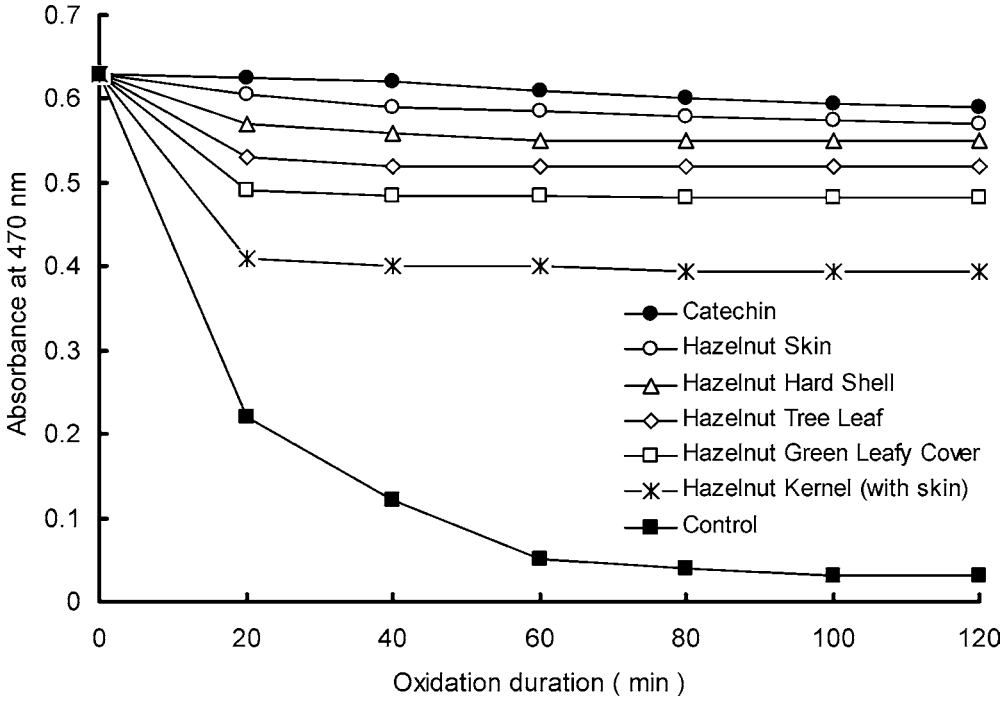

Figure 2. Antioxidant activity in extracts of hazelnut kernel and hazelnut byproducts in a β-carotene–linoleate model system.

the formation of highly reactive oxygen species, such as hydroxyl radical. Effective scavenging of hydrogen peroxide can, therefore, prevent oxidative damage to lipids.

Siriwardhana and Shahidi (*14*) reported that hydrogen peroxide scavenging activities at 100 ppm concentration were 59, 63, and 66% and at 200 ppm were 86, 91, and 91% for whole almond seed, brown skin, and green shell (leafy) cover extracts, respectively. The results obtained from both hazelnut and almond clearly show that extracts from byproducts scavenged organic free radicals more effectively than kernel or seed extracts.

Superoxide Radical Scavenging Activity. The superoxide radical is a powerful oxidizing agent that can react with biological membranes and induce tissue damage (*44*). It may also decompose to singlet oxygen, hydroxyl radical, or hydrogen peroxide (*45*). Data on the efficacy of hazelnut extracts and catechin to scavenge superoxide radical are presented in **Table 3**. The decrease of absorbance at 560 nm in the presence of antioxidants indicates the consumption of superoxide anions in the reaction mixture. The activity of both hazelnut kernel and hazelnut byproducts increased with increasing concentration. Extracts from byproducts demonstrated superior activity (99%) compared to hazelnut kernel (94%) and catechin (91%) at 200 ppm. A closer scrutiny of the results assembled indicates that all extracts are more effective ($p < 0.05$) than catechin at 200 ppm. Therefore, superoxide radical scavenging activity of hazelnut extracts, especially that of byproducts, would be one of the major mechanisms contributing to their antioxidant activities.

DPPH Radical Scavenging Activity. The use of the DPPH radical scavenging assay is advantageous in evaluating antioxidant effectiveness because the DPPH radical is more stable than hydroxyl and superoxide radicals (*14*). The antioxidant potential of hazelnut extracts was evaluated using the stable DPPH radical. This method has been used extensively to predict antioxidant activity because of the relatively short time required for analysis (*46*). The DPPH scavenging activities of all extracts together with that of catechin at 50 and 100 ppm concentrations are shown in **Table 3**. All hazelnut extracts exhibited fairly effective DPPH radical scavenging activity at both concentrations tested. On the other hand, catechin scavenged the DPPH radical nearly completely at both concentrations. Thus, the phenolic compounds present may have acted as free radical scavengers by virtue of their hydrogen-donating ability (*47*).

Antioxidant Activity in the β-Carotene−Linoleate Model System. The antioxidant activity of hazelnut extracts, as measured by the β-carotene−linoleate model system, is presented in **Figure 2**. As oxidation progressed, the absorbance of β-carotene at 470 nm decreased and its yellow color faded. The reference compound, catechin, exhibited a more powerful antioxidant activity than all other extracts that were examined. The antioxidant activity in this model system was in the

Table 4. Retention of β-Carotene and Inhibition of Oxidation of Human LDL Cholesterol in Extracts of Hazelnut Kernel and Hazelnut Byproducts[a]

	retention of β-carotene–linoleate model system (%)		inhibition of oxidation of human LDL cholesterol (%)	
extract	50 ppm	100 ppm	50 ppm	100 ppm
hazelnut kernel (with skin)	62.5 ± 1.1 b	63.5 ± 1.5 b	42 ± 2 b	99 ± 1 b
hazelnut skin	83.3 ± 1.2 c	93.3 ± 2.0 c	99 ± 1 c	99 ± 1 b
hazelnut hard shell	83.1 ± 0.9 c	89.1 ± 1.1 d	56 ± 3 df	99 ± 1 b
hazelnut green leafy cover	76.4 ± 1.9 de	76.5 ± 1.8 e	93 ± 1 e	99 ± 1 b
hazelnut tree leaf	78.5 ± 1.6 e	83.3 ± 1.0 f	61 ± 2 d	99 ± 1 b
catechin	83.6 ± 1.1 c	83.6 ± 1.2 f	53 ± 3 f	99 ± 1 b
control	3.6 ± 0.3 f	3.6 ± 0.3 g		

[a] Data are expressed as means ± SD ($n = 3$) on an extract. Means ± SD followed by the same letter, within a column, are not significantly different ($p > 0.05$).

Table 5. Retention (Percent) of Supercoiled DNA in Extracts of Hazelnut Kernel and Hazelnut Byproducts in Free Radical Induced Strand Scission[a]

extract	5 ppm	10 ppm	25 ppm	50 ppm
hazelnut kernel (with skin)	33.3 ± 1.9 b	39.6 ± 1.2 b	53.4 ± 1.7 bf	59.2 ± 2.1 b
hazelnut skin	64.7 ± 2.7 c	73.2 ± 3.3 c	90.7 ± 0.9 c	95.4 ± 2.5 c
hazelnut hard shell	48.1 ± 2.2 d	68.7 ± 3.7 c	86.3 ± 2.4 d	94.7 ± 1.9 cd
hazelnut green leafy cover	44.2 ± 1.9 de	54.4 ± 1.4 d	83.0 ± 2.5 de	89.9 ± 1.3 e
hazelnut tree leaf	38.9 ± 1.3 f	45.2 ± 0.9 ef	56.1 ± 1.4 f	65.7 ± 2.4 f
catechin	33.1 ± 2.0 b	44.2 ± 2.1 f	51.8 ± 1.2 b	60.3 ± 1.7 b

[a] Data are expressed as means ± SD ($n = 3$) on an extract. Means ± SD followed by the same letter, within a column, are not significantly different ($p > 0.05$).

following order: catechin > hazelnut skin > hazelnut hard shell > hazelnut tree leaf > hazelnut green leafy cover > hazelnut kernel > control. Although the same order was obtained at 50 ppm concentration, at 100 ppm hazelnut skin extract exhibited the highest antioxidative activity by retaining β-carotene in the medium, followed by hazelnut hard shell, catechin, hazelnut tree leaf, hazelnut green leafy cover, hazelnut kernel, and control (**Table 4**). The differences in the activity order between the two sets of experiments at 100 and 200 ppm concentrations (50 and 100 ppm) could be explained.

Alasalvar et al. (*28*) found a similar order of antioxidant efficacy when using two solvent systems (80%, v/v, ethanol and 80%, v/v, acetone) for comparing defatted hazelnut kernel and hazelnut green leafy cover; BHT was used as a reference compound. In that study, acetone was found to be a more effective solvent for the extraction process compared to the corresponding ethanol. Antioxidant activity in the acetone (80%, v/v) extract of defatted almond and its two fractions in the same model system showed a weaker antioxidant activity for defatted almond extract (*41*) compared to that of ethanol extract of hazelnut kernel presented in this study.

Inhibition of Oxidation of Human LDL Cholesterol. The inhibition of copper-induced human LDL oxidation by hazelnut extracts is summarized in **Table 4**. Hazelnut skin and hazelnut green leafy cover extracts at 50 ppm concentration effectively inhibited copper-induced oxidation of human LDL cholesterol (99 and 93%, respectively) compared to hazelnut kernel (42%), hazelnut hard shell (56%), and hazelnut tree leaf (61%) extracts, which reached the same level of efficacy (99%) at 100 ppm. It is worth nothing that at the 50 ppm level, all hazelnut extracts, except hazelnut kernel, were far more effective in inhibiting human LDL oxidation than catechin (53%) used as a standard. At 100 ppm, catechin exhibited 99% inhibition, the same as that shown by all hazelnut extracts. Factors such as differences in the solubility and partitioning between aqueous and lipid phases in the LDL system are among factors responsible for the observed trends. Similar to this study, Wijeratne et al. (*24*) found that the brown skin of almond exerted the highest preventive effect against LDL oxidation at 10, 50, and 100 ppm levels, compared to those of whole almond and its green shell (leafy) cover. At 200 ppm, all extracts exerted the same effects.

Oxidative modification of LDL plays a major role in the pathogenesis of CHD (*48*). Kinsella et al. (*49*) reported the importance of dietary antioxidants in the inhibition of LDL cholesterol oxidation, thereby reducing risk of atherosclerosis and CHD. Dietary antioxidants, including those from hazelnut extracts, may therefore moderate risk factors involved in CHD. It has been reported that oxidation of human LDL by free radicals arising from lipid oxidation products may be involved in the pathogenesis of atherosclerosis, and transition metal ions could promote oxidative modification through interaction with hydroperoxides (*50*). Both free radical scavenging and copper chelation activity of antioxidants were found to be responsible for the inhibition of LDL oxidation. Moreover, Natella et al. (*51*) reported that inhibition of copper-catalyzed oxidation represents mainly the scavenging of free radical species in the LDL system.

Supercoiled DNA Strand by Hydroxyl Radical. Hydroxyl radical induced DNA single-strand breaks may be a better index in the evaluation of the effects of phenolic compounds against hydroxyl radical (*52*). Oxidative damage of DNA results in strand breakage and sister chromatid exchange, DNA−DNA and DNA−protein cross linking, and base modification (*53*). The effect of hazelnut extracts on DNA single-strand breaks, induced by Fenton reagent, was examined, and the results are summarized in **Table 5**. Hazelnut skin extract showed the highest inhibition, whereas hazelnut kernel extract exhibited the lowest effect at all four concentrations tested (5, 10, 25, and 50 ppm). Extracts from byproducts (skin, hard shell, green leafy cover, and tree leaf) exhibited stronger inhibition ($p < 0.05$) than hazelnut kernel extract (except between tree leaf and hazelnut kernel extracts at the 25 ppm level, $p > 0.05$), which was either better than or similar to catechin in activity against the hydroxyl radical. Although plant-derived phenolic compounds could act as pro-oxidants and damage biomolecules (*54*), all hazelnut extracts tested showed protective effects even up to a level of 50 ppm. The inhibitory effects of hazelnut extracts may be attributed to their ability to scavenge hydroxyl radical. Hence,

hazelnut products may also participate in cancer prevention. Wijeratne et al. (*24*) investigated the inhibition of peroxyl and hydroxyl radical induced DNA scission of almond whole seed, brown skin, and green shell (leafy) cover extracts between 2 and 100 ppm levels. Green shell cover extract at 50 ppm level completely arrested peroxyl radical induced DNA scission, whereas 100 ppm of brown skin and whole seed extracts was required for similar efficiencies. On the other hand, for hydroxyl radical induced DNA strand scission, all three almond extracts exerted a total protection at 50 ppm against both site-specific and non-site-specific strand scissions (*24*).

In summary, different assays used for examining antioxidant efficacies of hazelnut extracts revealed that hazelnut byproducts exhibited superior antioxidant activities compared to that of hazelnut kernel and could potentially be considered as inexpensive sources of natural antioxidants. All hazelnut extracts performed differently. The overall activity of hazelnut extracts was different and may depend on the type of individual phenolic compounds present in each extract, their relative activities, and possible synergistic and antagonistic effects brought about by different interactions among the compounds involved. Therefore, it is imperative to conduct further research not only on the chemistry of the hazelnut constituents but also on their absorption, metabolism, excretion, and behavior in experimental models and humans. Further research is also required to identify and quantify the composition of polyphenolic compounds, especially flavonoids and other phenolic acid fractions (glycoside and ester-bound) in hazelnut kernel and hazelnut byproducts.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-methylpropionamidine) dihydrochloride; ABST, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); $ABTS^{\bullet-}$, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical anion; AI, antioxidant index; BHT, butylated hydroxytoluene; CE, catechin equivalents; CHD, coronary heart disease; CVD, cardiovascular disease; DAD, diode array detector; DNA, deoxyribonucleic acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EDTA, ethylenediaminetetraacetic acid; FDA, U.S. Food and Drug Administration; GAE, gallic acid equivalents; $H\text{-}ORAC_{FL}$, hydrophilic oxygen radical absorbance capacity with fluorescein; HPLC, high-performance liquid chromatography; LDL, low-density lipoprotein; $L\text{-}ORAF_{FL}$, lipophilic oxygen radical absorbance capacity with fluorescein; PBS, phosphate buffer (pH 7.4) saline; ROS, reactive oxygen species; SD, standard deviation; TAA, total antioxidant activity; TBE, Tris/borate/EDTA; TE, Trolox equivalents; TEAC, Trolox equivalent antioxidant capacity; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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