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Antioxidant Polyphenols in Almond and Its Coproducts

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Antioxidant efficacy of defatted almond whole seed, brown skin, and green shell cover extracts was evaluated by monitoring inhibition of human low-density lipoprotein (LDL) oxidation, inhibition of DNA scission, and metal ion chelation activities. The total phenolic contents of ethanolic extracts of brown skin and green shell cover of almond were 10 and 9 times higher than that of the whole seed, respectively. Brown skin extract at 50 ppm effectively inhibited copper-induced oxidation of human LDL cholesterol compared to whole seed and green shell cover extracts, which reached the same level of efficacy at 200 ppm. 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. All three almond extracts exhibited excellent metal ion chelation efficacies. High-performance liquid chromatographic (HPLC) analysis revealed the presence of quercetin, isorhamnetin, quercitrin, kaempferol 3-*O*-rutinoside, isorhamnetin 3-*O*-glucoside, and morin as the major flavonoids in all extracts.

KEYWORDS: Almond extracts; human LDL oxidation; DNA scission; metal chelating activity; phenolic antioxidants; flavonols; flavonol glycosides

INTRODUCTION

Nuts are known as a source of nutritious food with high lipid content. Replacing half of the daily fat intake with nuts lowered total and LDL cholesterol levels significantly in humans (*1*). The observed blood cholesterol lowering effects of nuts were far better than what was predicted according to their dietary fatty acid profiles (*2*, *3*). Research also shows a connection between regular nut consumption and decreased incidence of coronary heart disease (*4*). These beneficial physiological effects suggest that bioactive compounds of nuts may possess lipid-altering activities due to additive/synergistic effects and/or interactions with each other. Dietary antioxidants provide protection against oxidative attack by decreasing oxygen concentration, intercepting singlet oxygen, preventing first-chain initiation by scavenging initial radicals, binding of metal ion catalysts, decomposing primary products of oxidation to non-radical compounds, and chain breaking to prevent continuous hydrogen removal from substrates (*5*).

Almond, scientifically known as *Prunus dulcis*, belongs to the family Rosaceae and is also related to stone fruits such as peaches, plums, and cherries. It is the number one tree nut produced on a global basis, and the United States, specifically California, is the major producer (*6*). Almond, with or without the brown skin, is consumed as the whole nut or used in various confectioneries and chocolates; its discarded components are used as livestock feed (*7*). Apart from its nutritional value, almond is reported to have beneficial effects on blood cholesterol level and lipoprotein profile in humans (*8*). Diets containing almond meal or oil have been shown to reduce colon cancer risk in rats (*9*) and caused a significant reduction in plasma triacylglycerols and total and LDL cholesterol with increased levels of HDL cholesterol in humans (*10*). In addition, almonds, when used as snacks and in diets of hyperlipidemic subjects, significantly reduced coronary heart disease factors (*11*). A long-term supplementation of almond showed spontaneous nutrient modification of an individual's habitual diet that closely matched the recommendations to prevent cardiovascular and other chronic diseases (*12*). Extracts of whole almond seed, brown skin, and green shell cover possess potent free radical scavenging capacities (*13*). These activities may be related to the presence of flavonoids and other phenolic compounds in nuts. Almond hulls have been shown to serve as a rich source of triterpenoids, betulinic, urosolic, and oleanolic acids (*7*), as well as flavonol glycosides and phenolic acids (*14*) In addition, Sang and co-workers (*15*) isolated catechin, protocatechuic acid, vanillic acid, *p*-hydroxybenzoic acid, and naringenin glucoside, as well as galactoside, glucoside, and rhamnoglucoside of 3′-*O*-methylquercetin and rhamnoglucoside of kaempferol. A quantitative assessment (*16*) of the flavonol glucoside composition in blanched almond skins using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS)

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confirmed the presence of glucoside and rutinoside of kaempferol and isorhamnetin.

It is important to assess protective effects of antioxidants against DNA damage independently because compounds that protect lipids against oxidation may not necessarily protect other biomolecules, and some may even indirectly enhance damage to DNA (*17*). Model systems to determine damage to DNA include both site-specific and non-site-specific DNA damage by hydroxyl radical (*18*) or those induced by peroxyl radical, hydrogen peroxide, and metal ions (*19*−*21*). The objectives of this study were to investigate the inhibition of hydroxyl and peroxyl radical induced DNA scission, inhibition of copper-induced human LDL oxidation, and metal scavenging capacities of almond whole seed, brown skin, and outer green shell cover extracts and to identify the active polyphenols present in them.

MATERIALS AND METHODS

Almond seeds, skins, and shell covers were obtained from the Almond Board of California (Modesto, CA). Sodium carbonate, hexane, ethanol, methanol, hydrochloric acid, and butanol were purchased from Fisher Scientific Co. (Nepean, ON, Canada). Quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin−Ciocalteu reagent, human LDL, ethylenediaminentetraacetic acid (EDTA), ascorbic acid, tetramethylmurexide, mono- and dibasic sodium phosphate, and DNA of pBR322 (*Escherichia coli* strains PRI) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Standards of pentanal, hexanal, heptanal, and 2-heptanone were obtained from Aldrich Chemical Co. (St. Louis, MO). Hexamethylenetetramine was purchased from J. T. Baker Inc. (Phillipsburg, NJ).

Preparation of Crude Phenolic Extracts. Almond whole seed, brown skin, and green shell covers were ground in a coffee grinder (Black and Decker Canada Inc., Brockville, ON, Canada) for 10 min and then defatted by blending with hexane (1:5, w/v; 5 min × 3) in a Waring blender (model 33BL73, Waring Products Division, Dynamics Corp. of America, New Hartford, CT) at ambient temperature. Phenolic compounds present in defatted samples were extracted using 80% ethanol (6 g of sample/100 mL of solvent) under reflux condition in a thermostated water bath at 80 °C for 30 min. The resulting slurries were centrifuged at 4000*g* (ICE Centra MS, International Equipment Co., Needham Heights, MA) for 5 min and the supernatants collected. The residue was re-extracted under the same condition, and the supernatants were combined. The solvent was then removed under vacuum at 40 °C and the resulting slurry lyophilized for 72 h at −48 °C and 46 × 10^{-3} mbar (Freezone 6, model 77530, Labanco Co., Kansas City, MO).

Determination of Total Phenolics Content. Extracts were dissolved in methanol to obtain a concentration of 1 mg/mL of whole seed extract and 0.5 mg/mL of brown skin and green shell cover extracts. The content of total phenolics was determined according to a modified version of the procedure described by Singleton and Rossi (*22*). Folin−Ciocalteu 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. The volume was adjusted to 10 mL with distilled water, and the contents were thoroughly mixed. Tubes were allowed to stand at ambient temperature for 45 min and then centrifuged at 4000*g* for 5 min. Absorbance of the supernatants was read at 725 nm. A blank sample for each extract was used for background subtraction. The content of total phenolics in each extract was determined using a standard curve prepared for quercetin. Total extracted phenolics were expressed as milligrams of quercetin equivalents per gram of extract.

Cupric Ion Induced Human LDL Oxidation. Human LDL in PBS (pH 7.4, 0.01% EDTA) was dialyzed against a 10 mM solution of PBS (pH 7.4, 0.15 M NaCl) at 4 °C for 24 h. The resultant EDTA-free human LDL was further diluted to obtain a concentration of 0.2 mg of protein/mL and mixed with quercetin or almond extracts at 10, 50, 100, and 200 ppm (as quercetin equivalents). Oxidation was initiated by adding 10 μM copper sulfate and incubating at 37 °C for 20 h (*19*). The conjugated dienes from oxidation of human LDL were measured by reading the absorbance at 234 nm. The inhibitory effects of the extracts on the formation of conjugated dienes were calculated according to the equation

$$\%\ \text{inhibition of conjugated dienes formation} = \frac{\text{Abs}_{\text{oxidative}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{oxidative}} - \text{Abs}_{\text{native}}} \times 100$$

where $\text{Abs}_{\text{oxidative}}$ = absorbance of human LDL with copper sulfate, $\text{Abs}_{\text{sample}}$ = absorbance of human LDL with copper sulfate and extracts, and $\text{Abs}_{\text{native}}$ = absorbance of human LDL without copper sulfate.

Supercoiled DNA Strand Scission by Peroxyl and Hydroxyl Radicals. Plasmid DNA (pBR322) was dissolved in 10 mM PBS (pH 7.4, 0.15 mM sodium chloride). DNA (150 ng) was mixed with quercetin and almond extracts dissolved in the same buffer to attain final concentrations of 2, 5, 10, 50, and 100 ppm (as quercetin equivalents). Peroxyl radical was generated using AAPH at a final concentration of 9 mM, and reactants (total volume = 12 μL) were incubated at 37 °C for 2 h in the dark (*19*). The hydroxyl radical was generated using 100 μM EDTA, 100 μM ferric chloride, 100 μM ascorbic acid, and 1 mM hydrogen peroxide for non-site-specific hydroxyl radical generation (*23*). For site-specific hydroxyl radical generation EDTA was replaced with PBS (*18*). Ferric chloride and ascorbic acid were dissolved in deionized water immediately before use. Reaction mixtures were incubated at 37 °C for 1 h. After incubation, 2 μL of the loading dye (consisting of 0.25% bromophenol blue, 0.25% xylene cyanol, and 40% sucrose in distilled water) was added to the sample and loaded to an 0.8% (w/v) agarose gel prepared in 40 mM Tris−acetate/2 mM EDTA buffer (pH 8.5). Gel electrophoresis was performed in the same buffer using a horizontal gel electrophoresis apparatus at 60 V for 3 h. The gels were stained with ethidium bromide (0.5 μg/mL) and visualized under ultraviolet light. Images were analyzed using AlphaEase Stand Alone software (Alpha Innotech Corp., San Leandro, CA). The protective effect of the additives/extracts was measured using the retention percentage of supercoiled DNA as given below:

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

Evaluation of Fe^{2+} Chelating Activity of Almond Extracts. Ferrous sulfate (400 μM), almond extracts, quercetin, and 1 mM tetramethylmurexide were respectively dissolved in a hexamine−HCl buffer (10 mM, pH 5.0) containing 10 mM potassium chloride. Solutions of 1 mL ferrous sulfate and 1 mL extracts were mixed followed by the addition of 0.1 mL of a 1 mM tetramethylmurexide solution. The final concentration of quercetin and almond extracts was 100 or 200 ppm (as quercetin equivalents). Absorbance at 460 nm ($A_{460\text{nm}}$) and 530 nm ($A_{530\text{nm}}$) was read and the ratio of $A_{460\text{nm}}/A_{530\text{nm}}$ calculated. A standard curve was used to determine the amount of free Fe^{2+} left after chelation by the extracts (*24*). Iron chelation capacity of additives was calculated using the following equation:

$$\text{iron } (Fe^{2+}) \text{ chelation capacity, \%} = 100 - (\text{absorbance ratio for medium containing the additive of concern/ absorbance ratio for the control}) \times 100$$

Fractionation of Almond Extracts. Almond extracts (1 g) were adsorbed onto 50 g of Sephadex LH-20 preconditioned by soaking in methanol for 24 h and packed in a 7 × 120 cm i.d., glass column. Elution was carried out at a slow rate using 200 mL of methanol followed by 200 mL aliquots of increasing concentrations of water. To isolate pure compounds, the fractions were concentrated under vacuum and chromatographed one-dimensionally on Whatman no. 1 chromatography paper (PC) using either BAW (*n*-butanol/acetic acid/water, 4:1:5, v/v/v, upper phase) or acetic acid/water (15:85, v/v). Further fractionation was carried out on a Sephadex LH-20 column using methanol as a solvent, and fractions were tested for purity by high-performance liquid chromatography (HPLC).

Table 1. Percent Inhibition of Conjugated Diene Formation by Almond Extracts and Quercetin (10–200 ppm) in Copper-Induced LDL Oxidation[a]

extract	10 ppm	50 ppm	100 ppm	200 ppm
whole seed	54 ± 3c	88 ± 1c	94 ± 1c	99 ± 1b
brown skin	72 ± 2d	90 ± 1c	99 ± 1d	99 ± 1b
green shell cover	36 ± 2b	65 ± 3b	90 ± 1b	99 ± 1b
quercetin	29 ± 4a	53 ± 4a	84 ± 2a	95 ± 1a

[a] Results are mean values of three determinations ± standard deviation. Means in a column sharing the same letter are not significantly ($p > 0.05$) different from one another.

Analytical and Semipreparative HPLC Analysis. A Waters Delta Prep 4000 LC system equipped with a 996 photodiode array detector was used for HPLC analysis. For analytical work, dilute solutions of freeze-dried crude extracts (10 mg/mL; both with and without spiking with standards) were passed through Acrodisc filters (0.45 μm), and 20 μL aliquots were injected onto a Waters reverse-phase analytical column of 150 mm × 3.9 mm (i.d.) packed with Nova-Pak C18 (4 μm, 60 Å) material. A gradient profile using two solvents was applied at room temperature (*25*), with solvent A = 5% aqueous formic acid and solvent B = acetonitrile/methanol (5:95, v/v) and a flow rate of 0.9 mL/min. Compounds of interest were detected on the basis of characteristic UV−vis spectra (spectral range of 220−400 nm) and retention times. Semipreparative HPLC was carried out using two Waters preparative cartridges in series, each 100 mm × 25 mm (i.d.) and packed with Nova-Pak HR C18 (6 μm, 60 Å) material. A gradient profile, using two solvents, was applied at room temperature with solvent A = water, solvent B = methanol, and flow rate = 3 mL/min. This procedure was applied to those fractions identified from analytical HPLC as containing flavonol compounds.

Identification of Isolated Compounds. UV spectra were recorded on a UV−vis Beckman DU 7 spectrophotometer. Structures of purified compounds were determined according to the standard methods (*26*−*28*), acid hydrolysis in 2 or 0.1 M HCl at 100 °C for 60 min, and UV spectroscopy, and by comparison with authentic samples.

Statistical Analysis. All experiments were carried out in triplicates. The significance of differences among mean values was determined at $p \leq 0.05$ using analysis of variance (ANOVA) followed by Tukey's multiple-range test (*29*).

RESULTS AND DISCUSSION

Antioxidant Efficacy of Almond Extracts. Preliminary studies and close scrutiny of literature data indicated that extraction conditions were best when carried out in 80% ethanol at 80 °C for 30 min to obtain the highest amount of phenolic extracts from almond. The extract yields after lyophilization of almond whole seed, brown skin, and green shell cover were 19 ± 2, 8 ± 1, and 41 ± 3 g/100 g of defatted sample, respectively. Preliminary experiments revealed the presence of quercetin or a structurally related compound. The total phenolic contents of almond whole seed, brown skin, and green shell cover extracts were 8 ± 1, 88 ± 2, and 71 ± 2 mg of quercetin equiv/g of ethanolic extract, respectively.

Human LDL Oxidation. 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 (*30*). Breakdown products of hydroperoxides may modify proteins and nucleic acids via Schiff base reactions (*31*). Cupric ion has been shown to be a strong oxidizing agent for EDTA-free human LDL and, hence, has been used extensively to evaluate inhibitory effects on oxidation of various compounds in in vitro models (*32*). The inhibitory effect of extracts was significantly higher than that observed for quercetin (**Table 1**). When a series of 3,5,7,3′,4′-pentahydroxy flavonoids with identical arrangements around their A and B rings were examined for their effect against LDL oxidation, it was revealed that the presence of a double bond in the C ring of quercetin and the 4-oxo group contributed to a lower antioxidant activity (*33*).

The brown skin of almond exerted the highest preventive effect against LDL oxidation, whereas the green shell cover extract had the lowest antioxidant activity up to 100 ppm; the effects at 200 ppm were similar ($p > 0.05$) (**Table 1**). Factors such as differences in the solubility and partitioning between aqueous and lipid phases in the LDL system might be responsible for the observed differences. It is well-known that lipophilic antioxidants such as α-tocopherol provide a greater protection against LDL oxidation than hydrophilic antioxidants (*34*). This is because lipophilic antioxidants enter LDL particles, whereas hydrophilic ones act only on the surfaces of the LDL particles and, hence, are less effective (*35*). It has been reported that copper-mediated oxidation of tryptophan residues in the LDL−apolipoprotein B is responsible for lipid oxidation in the LDL particles (*36*). Both free radical scavenging and copper chelation activity of antioxidants are found to be responsible for inhibition of LDL oxidation (*30*).

Supercoiled DNA Strand by Peroxyl and Hydroxyl Radicals. Oxidative damage of DNA results in strand breakage and sister chromatid exchange, DNA−DNA and DNA−protein cross linking, and base modification (*37*, *38*). All three almond extracts tested showed a reduction (**Table 2**) in the three types of free radical induced strand scissions in a concentration-dependent manner. Green shell cover extract showed the highest activity at 2, 5, 10, 50, and 100 ppm levels against peroxyl-induced strand scission, which was superior even to that of quercetin. Total DNA retention was achieved by green shell cover extract at the 50 ppm level, whereas other extracts showed the same activity at 100 ppm. On the other hand, for hydroxyl radical induced DNA strand scission, all three almond extracts exerted a total protection at 50 ppm against both non-site-specific and site-specific strand scissions. However, the activity of whole seed extract against strand scission by hydroxyl radical was significantly ($p \leq 0.05$) higher than both brown skin and green shell cover extracts at 2, 5, and 10 ppm. It has been observed that compounds such as mannitol, glucose, and thiourea exhibit strong hydroxyl radical scavenging activity (*39*), and this might explain the effective antioxidative properties of whole seed extract despite its low phenolic content. Due to the low concentration of phenolics in whole seed extract, a higher weight of it had to be used to obtain the required phenolic levels. This inadvertently caused extraction of nonphenolic compounds such as free amino acids and soluble carbohydrates into ethanol, which may possess antioxidant properties. If proteins capable of iron binding were present in whole seed extract, in addition to phenolic compounds, they could "wrap around" the iron ion and intercept a high percentage of hydroxyl radical than the interception by an iron−EDTA complex due to the structural differences (*40*). The concept of site-specific toxicity of hydroxyl radicals was first described by Gutteridge (*39*). He observed that in the absence of EDTA iron ions bind to deoxyribose molecules and bring about a site-specific reaction on the molecule. However, in the presence of EDTA, which competes with deoxyribose for transition metal ions, iron is removed form the binding site to form a Fe^{2+}−EDTA complex and produce hydroxyl radical that could be readily removed by hydroxyl radical scavengers. It was further explained that most of the scavengers of hydroxyl radical show poor inhibitory capacities for site-specific hydroxyl reaction. This was observed with

Table 2. Retention (Percent) of Supercoiled DNA by Almond Extracts and Quercetin (at 2–100 ppm) in Free Radical Induced Strand Scission[a]

radical	extract	2 ppm	5 ppm	10 ppm	50 ppm	100 ppm
peroxyl	whole seed	67 ± 3b	74 ± 2a,b	87 ± 2b	94 ± 1b	96 ± 1a
	brown skin	55 ± 2a	68 ± 4a	78 ± 3a	87 ± 1a	95 ± 1a
	green shell cover	74 ± 3b	88 ± 2c	95 ± 1c	99 ± 1c	99 ± 1b
	quercetin	69 ± 2b	79 ± 2b	93 ± 1c	97 ± 1c	99 ± 1b
site-specific hydroxyl	whole seed	36 ± 3c	85 ± 2d	91 ± 1d	99 ± 1b	99 ± 1b
	brown skin	5 ± 2b	14 ± 5b	38 ± 4b	99 ± 1b	99 ± 1b
	green shell cover	9 ± 2b	37 ± 5c	63 ± 4c	99 ± 1b	99 ± 1b
	quercetin	0a	0a	0a	0a	0a
non-site-specific hydroxyl	whole seed	70 ± 5c	90 ± 1c	93± 1d	99 ± 1b	99 ± 1b
	brown skin	0a	28 ± 3a	79 ± 2a	99 ± 1b	99 ± 1b
	green shell cover	5 ± 1b	86 ± 1c	89 ± 1c	99 ± 1b	99 ± 1b
	quercetin	8 ± 2b	80 ± 2b	84 ± 1b	85 ± 3a	86 ± 2a

[a] Results are mean values of three determinations ± standard deviation. Means in a column sharing the same letter for a particular radical are not significantly ($p > 0.05$) different from one another.

almond extracts and quercetin. In general, almond extracts performed less effectively against site-specific hydroxyl radical reaction, whereas quercetin did not exert any protection toward site-specific hydroxyl radical scission, perhaps due to a pro-oxidant activity in the absence of EDTA. In contrast, the presence of EDTA, which is a strong metal chelator, reversed the activity of quercetin to an antioxidant, which inhibited oxidative activity of hydroxyl radical. DNA damage was not observed when DNA was incubated with quercetin in the presence of diethylenetriaminepenataacetic acid (*20*), whereas in the presence of metal ions, quercetin at higher concentrations was capable of exerting pro-oxidant effects and bringing about DNA damage in rat liver nuclei (*41*). Although plant-derived phenolic compounds could act as pro-oxidants and damage biomolecules (*42*), the three almond extracts tested showed protective effects even up to a level of 100 ppm.

Iron Chelating Activities. In iron chelating experiments, tetramethylmurexide (absorption maximum of 530 nm) was used to quantitatively determine the ferrous ion (Fe^{2+}) chelating capacity of almond extracts. However, shifting of the absorption maximum to 460 nm occurs when a metal ion chelates with tetramethylmurexide. The ratio of absorbance at 460 nm to that of 560 nm is linearly correlated with the metal ion concentration (*43*). When a known concentration of Fe^{2+} is added to a buffered solution of additives, some of the Fe^{2+} present chelates with additives, whereas some are left as free Fe^{2+} in the solution. When tetramethylmurexide is added to the solution, it chelates the remaining free Fe^{2+} and the absorption maximum shifts from 530 to 460 nm. This free Fe^{2+} can then be determined using a calibration line and the concentration of the chelated Fe^{2+} by additives calculated by subtracting free Fe^{2+} concentration from that initially present.

Metal-catalyzed oxidation can take place by acceleration of hydroperoxide decomposition, direct reaction with unoxidized substrates, and activation of molecular oxygen to give rise to singlet oxygen and peroxyl radical *(44)*. Iron is one of the most important biocatalysts, and free iron found in cells originates from ferritin, which is the major iron storage protein in cells (*45*). This iron together with that present in myoglobin, hemoglobin, and transferrin may cause lipid oxidation in muscle tissues (*46*). Ferrous ion is found to stimulate lipid peroxidation by generating hydroxyl radicals and by breaking down lipid peroxides to form alkoxyl radicals (*45*). All transition metal ions (iron, cobalt, manganese, copper, and nickel) having two or more valency states are potent pro-oxidants even at low concentrations such as 0.1 ppm concentration (*44*).

As shown in **Table 3**, almond extracts exhibit strong metal chelating capacities. Almond extracts at 100 and 200 ppm (as quercetin equivalents) chelated 95−98 and 97−100% of Fe^{2+}, respectively. As a reference antioxidant, quercetin chelated 96% of Fe^{2+} at both 100 and 200 ppm levels. The Fe^{2+} chelating capacities of extracts may be attributed to metal chelating agents, mainly phenolics, present in the extracts. Flavonoids are capable of chelating metal ions depending on their structural features (*47*, *48*). The stability of the metal−antioxidant complex is higher in six-membered ring than five-membered ring complexes (*49*). The iron chelating capacities of the additives and quercetin could arise from their formation of six-membered complexes with iron ions. In addition to iron chelation activities, almond also has shown strong hydrogen peroxide neutralizing and hydroxyl, superoxide, and DPPH radical scavenging capacities, which confirms their efficient hydrogen-donating capabilities (*13*).

Table 3. Concentration and Proportion of Chelated Ferrous Ions by Almond Extracts at 100 and 200 ppm[a]

	100 ppm		200 ppm	
additive	concn (μM)	proportion (%)	concn (μM)	proportion (%)
no additive	0 ± 0a	0	0 ± 0a	0
whole seed extract	380 ± 3b	95	387 ± 3b	97
brown skin extract	385 ± 3bc	96	390 ± 2b	98
green shell cover extract	390 ± 2c	98	399 ± 1c	100
quercetin	383 ± 3b	96	385 ± 3b	96

[a] Results are mean values of three determinations ± standard deviation. Means in a column sharing the same letter are not significantly ($p > 0.05$) different from one another. The initial ferrous ion concentration was 400 μM.

Table 4. Spectral Peaks and Retention Times of the Phenolic Compounds Identified by HPLC Analysis of Almond Extracts

	compound	spectral peak(s) (nm)	retention time (min)
1	protocatechuic acid	275	2.1
2	quercetin 3-*O*-rhamnoside	257, 350	29.4
3	kaempferol 3-*O*-glucoside	266, 356	30.4
4	morin	253, 354	30.9
5	kaempferol 3-*O*-rutinoside	266, 348	31.5
6	isorhamnetin 3-*O*-glucoside	255, 354	32.1
7	quercetin	365	33.4
8	isorhamnetin	365	42.7

Phenolic Compounds in Almond Extracts. The free radical scavenging and metal chelating activities of almond extracts could be attributed to the presence of phenolic compounds. **Table 4** shows the spectral peaks and retention times of each of the eight compounds identified in the extracts (**Figure 1**).

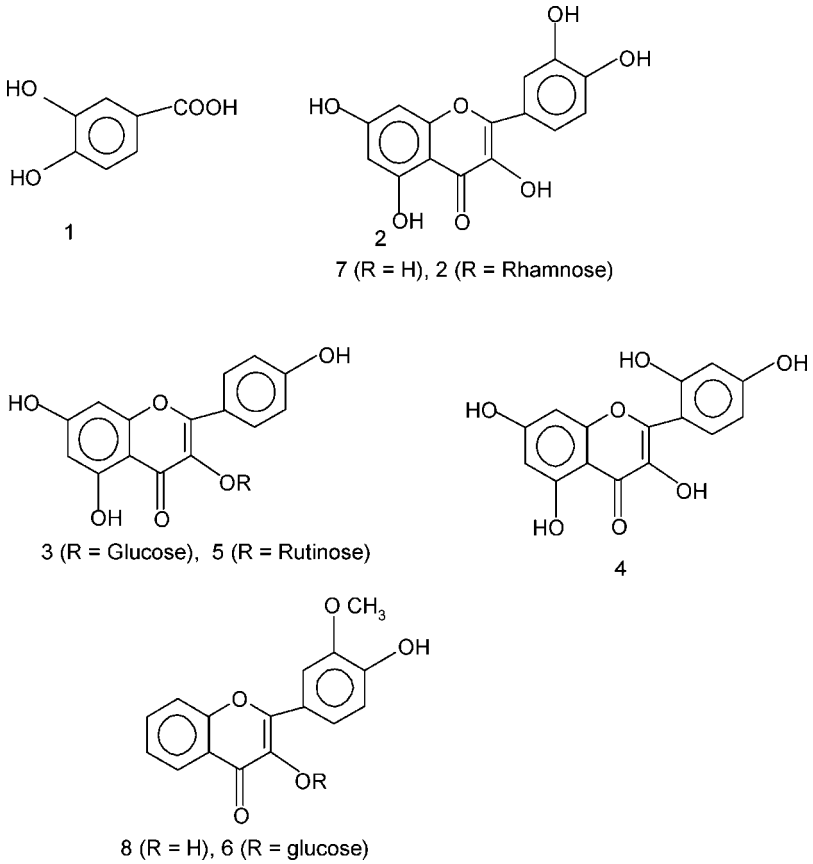

Figure 1. Chemical structures of phenolics of almond and its byproducts.

Upon hydrolysis, compound **2** yielded quercetin, and compounds **3** and **5** gave kaempferol, whereas compound **6** afforded isorhamnetin. Compounds **3** and **6** gave glucose, compound **2** yielded rhamnose, and compound **5** released rutinose as sugar moieties. These compounds were identified as flavonols, namely, quercetin, isorhamnetin, and morin; four flavonol glycosides, namely, quercetin 3-*O*-rhamnoside (quercitrin), kaempferol 3-*O*-glucoside (astragalin), kaempferol 3-*O*-rutinoside, and isorhamnetin 3-*O*-glucoside. The structures of the compounds were determined by spectral analysis as well as cochromatography with authentic samples using PC and HPLC.

Quercetin is the aglycone of a number of other flavonoids such as rutin, quercitrin, isoquercetin, and hyperoside. Methylation of oxygen at the 3′-position of quercetin gave rise to isorhamnetin (*50*). Studies on the inhibitory effects of quercetin and isorhamnetin on copper-induced lipid peroxidation in human LDL revealed that isorhamnetin was less effective than quercetin, which implies that introduction of a conjugated group to the position of the dihydroxyl group in the B ring markedly decreases the inhibition of LDL oxidation (*51*). The sugar molecule, when replacing one of the hydroxyl groups on the C ring, changes the activity of the molecule (*52*). Ioku et al. (*53*) reported that flavonoid glucosides possess a lower peroxyl radical scavenging activity than their corresponding aglycones. The antioxidant activity of flavonols such as quercetin and isorhamnetin is mainly based on the hydroxyl group at C-3 and the double bond between C-2 and C-3 atoms, whereas the metal chelating capacity is centered around the C-3′ and C-4′ atoms (*52*). Chelation occurs at the 3-hydroxy, 4-keto groups and/or at the 5-hydroxy, 4-keto groups of the A ring, which is hydroxylated in the 5-position (*54*). The 5,7-hydroxylation on the A ring has little effect on the antioxidant activity (*55*). These results show that the majority of the flavonols are present in their glycosylated forms, which would account for the hydrophilic compounds. Quercetin is rather a lipophilic antioxidant compared with its glycosides or conjugates and seems to interact with the polar head of phospholipid bilayers by locating near the surface of the membranes (*56*). This location may be favorable for trapping of peroxyl radicals originating from the aqueous phase (*57*). It has been revealed that quercetin is more powerful than morin in preventing oxidation of lipids, protein, and DNA (*58*). Moreover, quercetin has shown a higher iron chelating (*59*) and hydroxyl radical scavenging activity than morin (*60*). The difference in activity has been attributed to the *o*-diphenol structure of the B ring in quercetin.

The free radical scavenging and metal chelating activities of almond may be, in part, due to the presence of flavonols and the flavonol glycosides. The possible cytotoxic, genotoxic, and allergenic potencies of these compounds remain to be studied. Therefore, it is imperative to conduct further research not only on the chemistry of the almond constituents but also on their absorption, metabolism, excretion, and behavior in experimental models and humans.

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