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In Vitro Activity of Almond Skin Polyphenols for Scavenging Free Radicals and Inducing Quinone Reductase

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Observational studies and clinical trials suggest nut intake, including almonds, is associated with an enhancement in antioxidant defense and a reduction in the risk of cancer and cardiovascular disease. Almond skins are rich in polyphenols (ASP) that may contribute to these putative benefits. To assess their potential mechanisms of action, we tested the in vitro effect of ASP extracted with methanol (M) or a gastrointestinal juice mimic (GI) alone or in combination with vitamins C (VC) or E (VE) (1−10 μmol/L) on scavenging free radicals and inducing quinone reductase (QR). Flavonoid profiles from ASP−M and −GI extracts were different from one another. ASP−GI was more potent in scavenging HOCl and $ONOO^-$ radicals than ASP−M. In contrast, ASP−M increased and ASP−GI decreased QR activity in Hepa1c1c7 cells. Adding VC or VE to ASP produced a combination- and dose-dependent action on radical scavenging and QR induction. In comparison to their independent actions, ASP−M plus VC were less potent in scavenging DPPH, HOCl, $ONOO^-$, and $O_2^{-\bullet}$. However, the interaction between ASP−GI plus VC promoted their radical scavenging activity. Combining ASP−M plus VC resulted in a synergistic interaction, inducing QR activity, but ASP−GI plus VC had an antagonistic effect. On the basis of their total phenolic content, the measures of total antioxidant activity of ASP−M and −GI were comparable. Thus, in vitro, ASP act as antioxidants and induce QR activity, but these actions are dependent upon their dose, method of extraction, and interaction with antioxidant vitamins.

KEYWORDS: Almond skins; polyphenols; antioxidant; quinone reductase

INTRODUCTION

A generous consumption of plant foods is inversely related to the risk of many chronic diseases, a relationship often attributed to the associated intake of essential nutrients and/or fiber. However, non-nutrient phytochemicals, including alkaloids, carotenoids, organosulfur compounds, and polyphenols, may also contribute to this benefit via one or more of their putative mechanisms, including anti-oxidation, anti-inflammation, anti-proliferation, detoxification, and blood cholesterol reduction (*1–4*). Among the thousands of phytochemicals identified to date, polyphenols, especially the flavonoids, have been extensively characterized because of their wide spectrum of bioactivity, particularly their potent antioxidant activity in vitro. Reactive oxygen and nitrogen species, produced via cellular metabolism and derived from exposure to environmental pro-oxidants, appear to contribute to the pathogenesis of chronic disease via free-radical damage to lipids, nucleic acids, and proteins (*5*). Many polyphenols have been shown to modulate phase I and II detoxification pathways, mechanisms implicated in their putative chemopreventive actions (*6*). For example, flavonoids, including genistein, kaempferol, morin, quercetin, and genistein, have been reported to induce quinone reductase (QR), an enzyme often used as a biomarker of phase II metabolic activity and carcinogen elimination (*7*).

Almonds (*Prunus dulcis*) are among the richest natural food sources of vitamin E (*RRR*-α-tocopherol) (*8*). In 2003, almonds and several other nuts were qualified by the U.S. Food and Drug Administration for a B-level health claim that consuming 42 g daily "as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease" (*9*). In addition to their content of mono- and polyunsaturated fats and vitamin E, the polyphenols in almonds may also contribute to this health benefit. Recently, we and others have reported that almonds contain a variety of polyphenols, localized principally in their skin, including flavonols (kaempferol, isorhamnetin, and quercetin), flavanols (catechin and epicatechin), flavanones (naringenin), anthocyanins (cyanidin and delphinidin), and procyanidins (B2 and B3), as well as simple phenolic acids (caffeic acid, ferulic acid, *p*-coumaric acid, protocatechuic acid, and vanillic acid) (*10–14*). Further, we have demonstrated that almond skin

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flavonols and flavanols are bioavailable in hamsters and confer protection against the oxidation of low-density lipoproteins (*15*). Using in vitro radical scavenging assays, Siriwardhana and Shahidi (*16*) demonstrated almond polyphenols possess antioxidant activity against hydroxyl (OH^-), peroxyl ($ROO^\bullet$), and superoxide ($O_2^{-\bullet}$) radicals. This may be relevant to health because some radical specificity appears in the pathogenesis of different diseases, e.g., of $O_2^{-\bullet}$ in neurodegenerative conditions, peroxynitrite ($ONOO^-$) in cardiovascular disease, and reactive halide species, such as hypochlorite (HOCl), in rheumatoid arthritis.

Because each dietary antioxidant has a specific radical-quenching profile (*17*), the overall in vitro antioxidant activity of any food will be a reflection of its combination of individual ingredients as obtained following extraction and their interactions with one another. Typically, in vitro screening of plant foods for antioxidant activity is conducted after extraction with organic solvents, such as acetone, methanol, *n*-butanol, and hexane. However, the extraction of phytochemicals with gastrointestinal juice mimics (GI) has been suggested to provide a more physiologically relevant approach for this type of screening (*18, 19*). Thus, we have characterized the antioxidant activity of almond skin polyphenols (ASPs) by comparing the efficacy of acidified methanol (M) and GI extractions to (a) quench HOCl, $ONOO^-$, $O_2^{-\bullet}$, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals; (b) inhibit 2,2′-azobis(2-amidinopropane) dihydrochloride (AARP)- and Fe^{3+}-induced reduction reactions in the oxygen radical absorbance capacity (ORAC) assay of electron-donating potential and the ferric reducing antioxidant power (FRAP) assay of reducing power, respectively; and (c) induce QR in mouse hepatocytes in culture. Further, because only limited information is available regarding the relationship between polyphenols and the antioxidant vitamins C (VC) and E (VE), we have also examined their interactions with ASP. While the limitations of extrapolating in vitro characterizations of flavonoids and other polyphenols has been recognized (*20*), this approach can help substantially to generate new hypotheses regarding their mechanisms of action and inform the design of in vivo studies.

MATERIALS AND METHODS

Chemicals and Reagents. Hepa1c1c7 cells and Dubelco's modified Eagle medium (DMEM) were obtained from American Type Culture Collection (ATCC) (Manassas, VA); fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT); $ONOO^-$ in 0.3 N NaOH was obtained from Cayman (Ann Arbor, MI); AAPH was obtained from Wako Chemicals USA (Richmond, VA); and NaOH, methanol, and HCl were obtained from Fisher Scientific (Pittsburgh, PA). All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Almond Skin Extraction. Whole raw Carmel almond kernels harvested in California during 2004 and 2005 were kindly provided by the Almond Board of California. The almonds were collected from across a wide variety of orchards throughout the state and pooled, such that no individual orchard was disproportionately represented. The almonds were stored in the dark at 4 °C until skin removal by a brief hot-water blanching (*12*). Almond skins were then lyophilized for 7 days and pulverized under liquid N_2 with a mortar and pestle. The resulting fine powder was used for M or GI extraction.

Extraction by M was achieved using a Dionex Accelerated Solvent Extraction System 200 (Sunnyvale, CA). Because of the wide range of hydrophilicity of polyphenols, 3 g of almond skin powder was sequentially extracted with 90, 60, and 30% aqueous methanol (acidified with 5% acetic acid) for 3 cycles of 15 min each, respectively. According to results from tests of *Cimicifuga racemosa* (black cohosh), *Silybum marianum* (milk thistle), and *Hypericum perforatum* (St. John's wort), all extractable phenolic constituents were obtained after 6 extraction cycles (data not shown). The solvent extraction conditions were flush volume, 50%; N_2 gas purge, 180 s; pressure, 1500 psi, static extraction cycle, 5 min; temperature, 100 °C (Dionex application note 335). Combined extracts were volumetrically measured and centrifuged for 5 min at 3000*g*. Aliquots were dried under purified N_2 gas and stored at −20 °C. On the basis of total phenolic content, intra- and interday coefficients of variation (CV) for the M extraction of St. John's wort (as a reference botanical) was 2.1 and 4.4%, respectively. The interday CV of methanol extraction for almond skins was 3.1%.

The GI extraction, which may extract more water-soluble phenolic constituents from almond skin and simulate the impact of digestive enzymes on polyphenols, was performed according to Miller et al. (*18*) and Liu et al. (*19*). Briefly, 1 g of almond skin powder was mixed with 18 mL of saline containing 150 μmol/L butylated hydroxytoulene (BHT), acidified to pH 2 with HCl, and treated for 1 h of simulated gastric digestion using pepsin at 37 °C. The pH was then increased to pH 6.9 with $NaHCO_3$ and treated for 2 h of simulated intestinal digestion at 37 °C using a pancreatin−bile solution containing 0.03 g of bile extract and 0.005 g of pancreatin in 2.5 mL of 0.1 mol/L $NaHCO_3$. All incubation steps were performed at 140 rpm in a shaking water bath (Precision Reciprocal, Chicago, IL). After centrifugation at 3000*g* for 5 min, an aliquot of the extract was mixed with an equal volume of methanol to remove protein (which interferes with radical scavenging assays), spun at 10000*g* for 10 min, dried under N_2 gas, and stored at −20 °C. On the basis of total phenolic content, the intra- and interday CV for GI extraction of ASP was 2.1 and 5.5%, respectively. Because of the addition of BHT in the GI extraction, a GI blank undergoing all of the steps was generated and used as a blank control for all assays described below.

Total Phenolic Content. After reconstitution of the dry residues of ASP−M and −GI with phosphate saline buffer (PBS, pH 7.4), total phenolic content was determined by the Folin−Ciocalteu reaction according to Singleton et al. (*21*). All results are expressed as μmol/L gallic acid equivalents (GAEs) because the phenolic compounds in almond skin represent its principal bioactive constituents. The limit of quantitation for total phenolic determination was 47 μmol/L (8.0625 μg/mL). VC and VE at 1 μmol/L were equal to 0.61 ± 0.02 and 0.48 ± 0.01 μmol/L GAE, respectively. Because 72% of the total identified almond skin flavonoids are isorhamnetin and closely related flavonoids (*12*), pure isorhamnetin (ISOR) was employed as a reference compound; ISOR at 1 μmol/L was equal to 1.24 ± 0.00 μmol/L GAE.

GAE of ASP−M, VC, VE, and ISOR at 0.1, 1, 10, and 100 μmol/L were selected for all assays to reflect the range of concentrations potentially present in the gastrointestinal tract, plasma, and cells, respectively, following ingestion. However, only 0.1, 1, and 10 μmol/L GAE were tested for ASP−GI because 100 μmol/L concentrations could not readily be achieved. When the IC_{50} (concentration of antioxidants required to decrease absorbance by 50%) of radical-scavenging assays described below could not be acquired from concentrations between 0.1 and 10 μmol/L GAE, the highest concentration of ASP−GI tested in the scavenging assays was 25 μmol/L GAE.

Radical-Scavenging Activity. DPPH-scavenging activity was performed according to Brand-Williams et al. (*22*). Briefly, 900 μL of 100 μmol/L DPPH in ethanol was mixed with 100 μL of different concentrations of the antioxidant(s), and the absorbance at 520 nm was measured after 30 min of incubation at room temperature in the dark. Intra- and interday CV was 1.4 and 7.6%, respectively.

Scavenging activity against $ONOO^-$ was measured by monitoring the increase in fluorescence from the oxidation of dihydrorhodamine 123 (DHR 123) according to a slightly modified method of Choi et al. (*23*). The concentration of $ONOO^-$ stock solution was determined by a spectrophotometric method after alkalization using a cold 0.3 mol/L NaOH solution at a ratio of 1:40, and aliquots were stored at −80 °C. Immediately before use, $ONOO^-$ was diluted to a final concentration of 100 μmol/L. Fluorescence at 485 nm excitation and 530 nm emission generated from DHR123 oxidation 5 min after the addition of $ONOO^-$ was recorded using a FLUOstar Optima multifunctional plate reader (BMG LABTECH, Inc., Durham, NC). Intra- and interday CV was 4.7 and 3.6%, respectively.

Scavenging activity against HOCl was assessed via the oxidation of ferrocyanide $[Fe^{II}CN)_6]$ in a phosphate buffer as a reference reaction

to investigate the stoichiometry of the reaction according to the modification of the methods described by Zhu et al. (*24*) and Prutz et al. (*25*). The concentration of HOCl stock solution obtained from Sigma was determined according to Hussain et al. (*26*). Briefly, the test antioxidant(s) were incubated with HOCl for 5 min at room temperature before the addition of $Fe^{II}(CN)_6$, and then, absorbance was monitored at 420 nm using a Shimadzu UV1601 spectrophotometer (Japan). Intra- and interday CV was 0.9 and 2.9%, respectively.

Scavenging activity against $O_2^{-\bullet}$ was measured in a xanthine/xanthine oxidase system with spectrophotometric determination of the reduction product of nitroblue tetrazolium (NBT) according to a slight modification of the method described by Chun et al. (*27*). Briefly, after 10 min of incubation of the antioxidant(s) at room temperature with a reaction mixture of 50 μmol/L NBT, 50 μmol/L xanthine, and 0.05 unit/mL xanthine oxidase (final concentrations), the change in absorbance of NBT was measured at 560 nm using a Shimadzu UV1601 spectrophotometer. Intra- and interday CV was 1.9 and 7.7%. Inhibition in xanthine oxidase activity by the antioxidant(s) was monitored by the spectrophotometric determination of uric acid production (*26*).

Results of radical-scavenging activity were expressed as a percentage of the appropriate control (no test antioxidants present), and the IC_{50} (concentration of antioxidants required to decrease absorbance by 50%), in μmol/L GAE, was calculated using a spline function.

Antioxidant Activity Assay. The "total antioxidant activity" was assessed by the ORAC and FRAP assays. The ORAC assay was conducted according to Ou et al. (*28*). Briefly, the ORAC assay employs the area under the curve (AUC) of the magnitude and time to the oxidation of fluorescein because of peroxyl radicals generated by the addition of AAPH. The assay was carried out on a FLUOstar OPTIMA plate reader using fluorescence filters with 485 nm excitation and 520 nm emission. ORAC values of unknowns were calculated on the basis of standard curves established using Trolox at 5−50 μmol/L. All data are expressed as μmol of trolox equivalents (TEs)/μmol of GAE. Intra- and interday CV was 3.0 and 7.3%, respectively.

The FRAP assay determines the capability of antioxidants as reductants in a redox-linked colorimetric reaction of the reduction of Fe^{3+}−2,4,6-tripyridyl-*S*-triazine to a blue-colored Fe^{2+} complex at low pH, which is measured spectrophotometrically at 593 nm (*29*). The antioxidant(s) were incubated at room temperature with the FRAP reagent, and the absorbance was recorded after 1 h. FRAP values of unknowns were calculated on the basis of standard curves established using trolox at 31.25−500 μmol/L. The reducing power was expressed as μmol of TE/μmol of GAE. Intra- and interday CV was 0.7 and 4.2%, respectively.

Quinone Reductase Activity. The modulation of QR activity in murine hepatoma Hepa1c1c7 cells has been widely employed as a tool to examine the potential chemopreventive activity of phytochemicals and other compounds (*30*). Hepa1c1c7 cells were cultured until confluent in minimum essential medium eagle α modified supplemented with 10% heat inactivated, charcoal-treated FBS, 1% penicillin/streptomycin, and 1% L-glutamine in a Napco incubator with 5% CO_2 at 37 °C. After confluence, cells were plated at a concentration of 2×10^4 cells per well in 96-well clear plates and allowed to settle for 24 h. After the medium was aspirated, cells were treated with ASP−M, VC, VE, and ISOR at 0.1 to 100 μmol/L GAE and with ASP−GI at 0.1−10 μmol/L GAE in medium for 48 h. QR activity was measured by an NADPH-generating system, coupling the oxidation of menadione to the reduction of the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) according to Kang and Pezzuto (*31*). The resulting blue−brown color was measured at 570 nm using a FLUOstar Optima plate reader. The protein content of cells in each well was determined by a BCA protein kit (Pierce, Rockford, IL). After adjustment of the protein content, QR activity was expressed as nmol (mg of protein)$^{-1}$ min^{-1}. β-Napthoflavone at a concentration of 1 μmol/L was employed as a positive control and increased QR activity by 2.4 ± 0.5-fold of the negative control (absent antioxidants).

Statistics. All results are reported as mean ± standard error (SE). After significant differences were obtained by one-way analysis of variation (ANOVA), the Tukey−Kramer honestly significant difference (HSD) test was used for all assays. A Student's *t* test was performed to determine the significance of the antagonism or synergism between ASP or ISOR and VC or VE in all assays by comparing the detected activities of combinations with the expected values calculated from the individual antioxidants alone based on the formula: (actual value − expected value)/expected value × 100%. The expected values of combined antioxidants for radical-scavenging assays were obtained using the spline function of additive values of ASP, ISOR, VC, or VE alone at selected concentrations. Further, the expected values for antioxidant activity and QR assays were derived from the arithmetic mean of values for the individual antioxidants. For radical-scavenging activity assays, positive percentages from the formula above indicate an antagonistic relationship. For the ORAC, FRAP, and QR assays, positive percentages indicate a synergistic relationship. Pearson correlation tests were performed to reveal any associations between outcome values. Differences with $p < 0.05$ were considered significant. The JMP IN 4 statistical software package (SAS Institute, Inc., Cary, NC) was used to perform all statistical analyses.

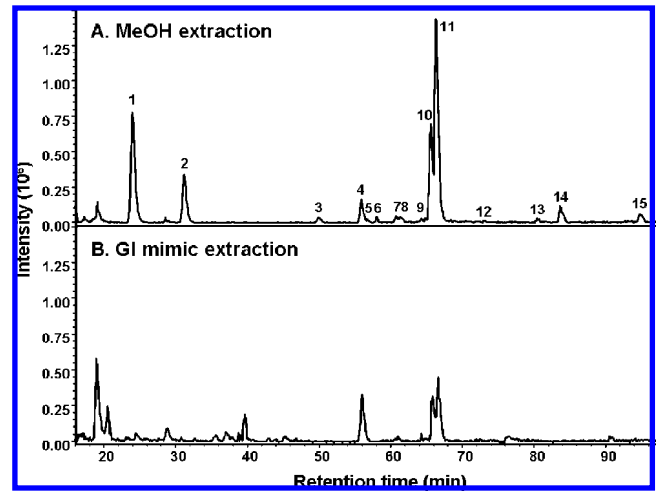

Figure 1. Typical LC−MS/MS extracted ion chromatograms of almond skin polyphenolics from (A) acidified methanol extraction and (B) gastrointestinal juice mimic extraction. Peak numbers correspond to compounds established by Milbury et al. (*12*) as (1) catechin, (2) epicatechin, (3) quercetin-3-*O*-galactoside, (4) naringein-7-*O*-glucoside, (5) rutin, (6) quercetin-3-*O*-glucoside, (7) dihydroxylkaempferol, (8) kaempfer-3-*O*-galactoside, (9) kaempferol-3-*O*-glucoside, (10) kaempferol-3-*O*-rutinoside, (11) isorhamnetin-3-*O*-runtinoside, (12) eriodictyol, (13) quercetin, (14) naringenin, and (15) isorhamnetin.

RESULTS

After ASP−M and −GI extractions, the total phenolic content of the almond skins was significantly different by 4.8-fold (1829 ± 13.8 versus 313 ± 4.1 μmol/L GAE, $p \leq 0.0001$). By extrapolation, 1 g almond skins produced 91.2 ± 0.7 μmol of GAE (15.5 mg) and 14.9 ± 0.2 μmol of GAE (2.5 mg) by M and GI extraction, respectively. The liquid chromatography−mass spectrometry (LC−MS) chromatograms of ASP−M and −GI at the same GAE concentration showed qualitatively different profiles of flavonoids, largely because of the absence in ASP−GI of catechin, epicatechin, kamperfol-3-*O*-glucoside, kaemperfol-3-*O*-galactoside, dihydroxy-kampferol, quercetin-3-*O*-glucoside, quercetin-3-*O*-galacoside, rutin, isorhamnetin, kaempferol, naringenin, quercetin, and eriodictyol (**Figure 1**) (*12*).

VE at concentrations ≤100 μmol/L GAE was only effective in scavenging the DPPH radical (**Table 1**). Of all antioxidants tested, VE was the most potent DPPH scavenger, followed by VC. ISOR was 49% more effective scavenging DPPH than ASP−M ($p \leq 0.05$), while ASP−GI ≤ 10 μmol/L GAE was ineffective. In scavenging HOCl, ISOR proved to be the most potent of the antioxidants tested ($IC_{50} = 1.6$ μmol/L). In contrast, ASP−M, ASP−GI, and VC required 12.3-, 1.8-, and 18.4-fold

Table 1. Radical Scavenging and Antioxidant Activity of Vitamins C and E, Isorhamnetin, and Almond Skin Polyphenols[a]

assay	VC	VE	ISOR	ASP−M	ASP−GI
Radical Scavenging			IC_{50} (μmol/L GAE)		
DPPH	13.8 ± 0.1 a	9.1 ± 0.3 b	29.0 ± 0.3 c	43.2 ± 0.5 d	[b]
HOCl	31.0 ± 1.0 a	[b]	1.6 ± 0.0 b	21.3 ± 0.1 c	4.4 ± 0.0 d
$ONOO^-$	9.5 ± 0.2 a	[b]	5.8 ± 0.8 bc	6.1 ± 0.0 b	4.2 ± 0.5 c
$O_2^{-\bullet}$	29.0 ± 1.8 a	[b]	[b]	57.5 ± 1.5 b	[b]
Antioxidant Activity			(μmol of TE/μmol of GAE)		
FRAP	0.7 ± 0.0 b	0.5 ± 0.0 cd	1.6 ± 0.1 a	0.6 ± 0.0 bc	0.4 ± 0.0 d
ORAC	7.1 ± 0.1 b	0.0 ± 0.0 d	13.1 ± 0.1 a	3.0 ± 0.1 c	2.7 ± 0.1 c

[a] Means in the same assay without the same letter differ, determined by Tukey's HSD test, $p \leq 0.05$. [b] Antioxidants at selected concentrations ranging from 0.1 to 25 μmol/L GAE for ASP−GI and from 0.1 to 100 μmol/L GAE for others were unable to scavenge 50% of radicals.

Table 2. Scavenging and Antioxidant Activity of Combined Almond Skin Polyphenols and Vitamin C with Equal Concentrations[a]

assay	ASP−M plus VC	ASP−GI plus VC	ISOR plus VC
Radical Scavenging		IC_{50} (μmol/L GAE)	
DPPH	21.6 ± 0.3 (6%)[b]	22.5 ± 0.2 (−19%)[c]	18.1 ± 0.2 (−5%)[d]
HOCl	45.8 ± 1.3 (84%)[c]	6.5 ± 0.5 (−38%)[e]	3.3 ± 0.0 (−59%)[c]
$ONOO^-$	7.8 ± 0.3 (8%)	7.5 ± 0.3 (−9%)	4.1 ± 0.0 (−46%)[c]
$O_2^{-\bullet}$	45.5 ± 0.5 (35%)[e]	[f]	26.7 ± 0.2 (−54%)[e]
Antioxidant Activity		(μmol of TE/μmol of GAE)	
FRAP	1.2 ± 0.0 (78%)[c]	1.1 ± 0.0 (86%)[c]	1.5 ± 0.0 (31%)[d]
ORAC	2.1 ± 0.1 (−58%)[c]	2.6 ± 0.0 (−48%)[c]	12.4 ± 0.0 (23%)[e]

[a] Numbers in the parentheses show the percent difference between actual and expected values (actual value − expected value)/expected value × 100%), with positive percentages indicating antagonism for radical-scavenging assays and synergism for antioxidant activity assays, respectively. [b] $p \leq 0.05$, tested by a Student's t test. [c] $p \leq 0.0001$, tested by a Student's t test. [d] $p < 0.01$, tested by a Student's t test. [e] $p \leq 0.001$, tested by a Student's t test. [f] ASP−GI and VC at combined concentrations up to 25 μmol/L GAE were unable to scavenge 50% of superoxides.

larger concentrations, respectively ($p \leq 0.05$), to quench HOCl. The scavenging activity of ASP−M, ASP−GI, and ISOR for $ONOO^-$ was comparable, albeit significantly different between ASP−M and −GI, while VC was 1-fold less potent than ASP−GI ($p \leq 0.05$). Only VC and ASP−M were effective in reducing $O_2^{-\bullet}$, with IC_{50} values at 29.0 and 57.5 μmol/L GAE, respectively ($p \leq 0.05$).

Total antioxidant activity values determined by the FRAP and ORAC assays are presented in **Table 1**. By extrapolation, in 1 g of Carmel almond skins, FRAP values were 54.7 and 6 μmol of TE and ORAC values were 273.6 and 40.2 μmol of TE by M and GI extraction, respectively. Using the FRAP assay, the reducing power of ISOR was >1-fold larger than the other antioxidants ($p \leq 0.05$), while the value of ASP−GI was 33% lower than ASP−M. Similarly, using the ORAC assay, ISOR had the highest antioxidant value compared to the other compounds ($p \leq 0.05$). The ORAC assay was not sensitive to the hydrophobic antioxidant VE. In contrast to the differences detected between ASP−M and −GI with the FRAP and radical-scavenging assays, screening with the ORAC assay revealed no differences between these two extracts. Among radical-scavenging and total antioxidant activity assays employed to assess antioxidant potency of ASP, VC and VE, only FRAP values were significantly correlated with ORAC values ($r = 0.83$, $p = 0.0017$).

Interactions between ASP and VC and VE were explored using equal GAE concentrations of each (**Table 2**). ASP−GI plus VC and ISOR plus VC resulted in a 19 and 5% greater efficacy, respectively ($p \leq 0.0001$ and 0.01), in scavenging DPPH radicals than their calculated additive values, suggesting a synergistic interaction. However, ASP−M plus VC provided an IC_{50} of 21.6 μmol/L GAE, suggesting an antagonistic interaction of 6% ($p \leq 0.05$). A synergism between ASP−GI plus VC and ISOR plus VC of 38 and 59% ($p \leq 0.001$ and 0.0001) was noted in the ability of these combinations to inhibit HOCl oxidation. However, ASP−M plus VC had an antagonistic interaction ($p \leq 0.0001$) with regard to scavenging HOCl. ASP−M and −GI had an additive effect with VC on $ONOO^-$-scavenging activity. In contrast, combining ISOR produced a 47% synergistic interaction ($p \leq 0.0001$). At ≤100 μmol/L GAE, ISOR did not prevent $O_2^{-\bullet}$-induced reactions, but ISOR plus VC yielded a 54% greater inhibition than their calculated sum ($p \leq 0.001$). ASP−GI plus VC at ≤10 μmol/L GAE was inadequate to inhibit $O_2^{-\bullet}$ oxidation by 50%. ASP−M plus VC produced a 35% smaller effect in quenching $O_2^{-\bullet}$ than their calculated sum ($p \leq 0.001$). Importantly, the $O_2^{-\bullet}$ scavenging activity of these antioxidants cannot be ascribed to an inhibition of $O_2^{-\bullet}$ generation from the xanthine/xanthine oxidase reaction because uric acid production was not altered. ISOR plus VC and ASP plus VC were also found to produce synergistic interactions of 31−86% ($p \leq 0.01$) when assessed by the FRAP assay. While ISOR plus VC had a 23% larger ORAC value than expected from their calculated additive effect ($p \leq 0.001$), ASP−M plus VC and ASP−GI plus VC produced a 58 and 48% antagonism in the ORAC assay ($p \leq 0.0001$).

When ASP−M and −GI were combined with VE, a synergistic 9 and 17% enhancement in scavenging the DPPH radical was noted ($p \leq 0.001$ and 0.0001, respectively), while a simple additive interaction was observed with ISOR plus VE (**Table 3**). VE alone at selected concentrations was ineffective in scavenging HOCl, but ISOR plus VE produced an 81% synergistic interaction ($p \leq 0.0001$). ASP−M and −GI were effective scavengers of HOCl; however, ASP plus VE was inactive in this assay. In the $ONOO^-$-scavenging assay, ISOR plus VE had a 34% synergistic interaction ($p \leq 0.01$). In contrast, ASP−M plus VE showed an additive relationship and ASP−GI plus VE resulted in a 122% antagonism ($p \leq 0.0001$). Although ASP−M effectively scavenged $O_2^{-\bullet}$, its combination with VE was without effect. ASP−GI plus VE and ISOR plus VE produced 28−74% synergistic increases in FRAP and ORAC values ($p \leq 0.001$), and ASP−M plus VE showed a simple additive effect in these assays.

ASP−M increased QR activity by 25% ($p \leq 0.05$) only at a dose of 100 μmol/L GAE. In contrast, ASP−GI at 10 μmol/L GAE inhibited QR by 16% ($p \leq 0.05$). ISOR at 10 and 100 μmol/L GAE upregulated QR activity by 45 and 70% ($p \leq 0.05$). VC at 100 μmol/L GAE produced a 56% increase in QR ($p \leq 0.05$). VE at 1 μmol/L GAE inhibited QR activity by 13% ($p \leq 0.05$), but larger concentrations did not lead to a further decrease (**Table 4**).

Table 3. Scavenging and Antioxidant Activity of Combined Almond Skin Polyphenols and Vitamin E with Equal Concentrations[a]

assay	ASP−M plus VE	ASP−GI plus VE	ISOR plus VE
Radical Scavenging	IC_{50} (μmol/L GAE)		
DPPH	14.4 ± 0.1 (−9%)[b]	16.7 ± 0.1 (−17%)[c]	14.9 ± 0.1 (−1%)
HOCl	[d]	[d]	4.1 ± 0.3 (−81%)[c]
$ONOO^-$	30.1 ± 0.2 (−1%)	40.1 ± 0.3(122%)[c]	9.5 ± 0.3 (−34%)[e]
$O_2^{-\bullet}$	[d]	[d]	[d]
Antioxidant Activity	(μmol of TE/μmol of GAE)		
FRAP	0.5 ± 0.0 (−2%)	0.6 ± 0.0 (28%)[b]	1.7 ± 0.1 (64%)[b]
ORAC	1.5 ± 0.1 (2%)	2.3 ± 0.1 (70%)[b]	11.4 ± 0.2 (74%)[c]

[a] Numbers in the parentheses show percent difference between actual and expected values ((actual value − expected value)/expected value × 100%), with positive percentages indicating antagonism for radical-scavenging assays and synergism for antioxidant activity assays, respectively. [b] $p \leq 0.001$, tested by a Student's t test. [c] $p \leq 0.0001$, tested by a Student's t test. [d] Antioxidants at combined concentrations ranging from 0.1 to 25 μmol/L GAE for ASP−GI and from 0.1 to 100 μmol/L GAE for others were unable to scavenge 50% of radicals. [e] $p < 0.01$, tested by a Student's t test.

ASP−M plus VC at 1 and 10 μmol/L GAE synergistically increased QR activity by 18 and 13%, respectively ($p \leq 0.001$ and 0.01), even though neither antioxidant at these doses affected QR activity. ASP−M plus VC at 100 μmol/L GAE led to a 10% antagonism ($p \leq 0.01$). ASP−GI plus VC at 0.1 μmol/L GAE increased QR activity by 17% in a synergic manner ($p \leq 0.05$) but, at higher concentrations, led to an antagonistic interaction. In particular, ASP−GI plus VC at 10 μmol/L GAE reversed the inducing effect of VC at the same concentration. ISOR and VC alone at 10 and 100 μmol/L GAE enhanced QR activity, but their combination at these concentrations gave rise to a 17% antagonism and a 41% synergy, respectively ($p \leq 0.001$ and 0.0001). In contrast to the inhibitory action of VE at >1 μmol/L GAE on QR, ASP−M plus VE at the same concentration resulted in a synergistic upregulation of QR ($p \leq 0.001$). ASP−GI plus VE at 1 and 10 μmol/L GAE produced additive outcomes on QR activity but, at 0.1 μmol/L GAE, inhibited QR activity in an antagonistic manner. ISOR plus VE at 0.1−10 μmol/L GAE had additive interactions, but their combination at 100 μmol/L GAE led to a 26% synergy ($p \leq 0.05$) (**Table 5**).

DISCUSSION

Almonds are a rich source of VE, providing 7 mg/28 g serving and a high value tree nut. In contrast, almond skins, although rich in bioavailable flavonoids and other antioxidant polyphenolic compounds, when removed via blanching, are generally considered as a waste byproduct with little economic value (*12, 15, 32*). We have previously found that ASP works in a synergistic manner with VE and VC in vitro to protect LDL lipid and protein phases from oxidation and stabilizes its conformation when challenged with Cu^{2+}, a pro-oxidant stress (*33*). Feeding almonds to habitual smokers, we have demonstrated the antioxidant actions of the whole food increases antioxidant defenses and reduces biomarkers of oxidative stress in vivo (*34*). Here, we further examine the antioxidant and detoxification activity of ASP extracted with M and GI solvents and their respective interactions with VC and VE. These results may provide useful information about in vivo interactions between VE and ASP on antioxidant reactions and detoxification pathways when whole almonds are consumed and between VC and ASP when almonds are eaten with VC-rich foods, such as citrus fruit.

We have identified and quantified 21 flavonoids and phenolic acids in ASP extracted with M (*12*). However, it has been suggested that phytochemicals obtained via extraction with organic solvents may not reflect those that are actually bioaccessible and bioavailable during human digestion (*18, 19*). While there are marked limitations in extrapolating results from any in vitro simulation of the dynamic gastrointestinal milieu to in vivo conditions, it is interesting to note the 18-fold larger extraction efficiency of the M versus GI solvents and their significantly different profile of flavonoids and phenolic acids. As anticipated, the more hydrophobic flavonoid aglycones, e.g., catechin, epicatechin, isorhamnetin, kaempferol, naringenin, quercetin, dihydroxy-kampferol, and eriodictyol, were found at lower levels or absent in ASP−GI compared to ASP−M. In addition, even the relatively more water-soluble flavonoids, e.g., kamperfol-3-*O*-glucoside, kaemperfol-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-galacoside, and rutin, were also absent in the ASP−GI. However, because LC−MS/MS analysis of ASP−GI and −M revealed the same concentration of total phenolics, the GI solvent appears to be extracting compounds not obtained via the M method, suggesting that a more complete characterization of the phenolic profile in ASP−GI is warranted.

The quantification of the total phenolic content of plant foods is substantially dependent upon the extraction conditions, e.g., hydrophilicity and acidity of the solvent, temperature, and pressure. Our results show that extraction with an organic solvent and high temperature and pressure can produce a 6-fold enhancement in extraction efficiency compared to a process designed to simulate the extraction that would occur in the gastrointestinal tract. Nonetheless, the total phenolic content obtained from the GI extraction was comparable to our previously reported value of 3.09 mg/g obtained using an extraction protocol with 16 h rocking in acidified methanol (*12*). Although the total phenolic content in Carmel almond skins obtained from the M extraction in this study was similar to that obtained by Garrido et al. (*35*), such comparisons must be made with caution because these results can be affected by a wide variety of factors, including plant variety, season, weather, cultivation practices, processing, and storage.

Consistent with observations that ASP extracted with organic solvents increase the resistance of LDL to oxidation (*15, 32*), ASP−M was an effective antioxidant against all of the free radicals that we tested, albeit with different degrees of scavenging efficacy. However, even at the same GAE concentration, ASP−GI was more potent against HOCl and $ONOO^-$ than ASP−M but ineffective against DPPH and $O_2^{-\bullet}$ even at 10 μmol/L GAE. ISOR glycosides contribute to 72% of flavonoids from ASP−M (*12*). Interestingly, ISOR was more potent against DPPH, HOCl, and $ONOO^-$ radicals than ASP−M but ineffective against $O_2^{-\bullet}$. The DPPH- and $ONOO^-$-scavenging activity of ISOR detected here was ≥70% lower than that of ISOR−3-glucoside and −rutinoside reported by Hyun et al. (*36*). While the basis for this difference is not clear, our study suggests that interactions between ASP constituents serve to decrease the antioxidant contributions of ISOR glycosides. In comparison to VC, ASP−GI was a more potent scavenger of HOCl and $ONOO^-$ but less effective in quenching DPPH and $O_2^{-\bullet}$ radicals. At lower concentrations, ASP−M was effective only against $ONOO^-$. However, it is important to recognize potential limitations in accurately comparing the radical-scavenging activity of antioxidants with different molecular conformations because of the complexity of the reaction kinetics in these assays; e.g., the rates of the kinetic behavior of VC, VE, and phenolics in the DPPH were rapid, intermediate, and slow,

Table 4. Changes in Quinone Reductase Activity in Hepa1c1c7 Cells after Incubation with Antioxidants for 48 h[a]

concentration	VC	VE	ISOR	ASP−M	ASP−GI
μmol/L GAE			(nmol (mg of protein)$^{-1}$ min^{-1})		
0	23.4 ± 1.5 a	23.4 ± 1.5 a	23.4 ± 1.5 a	23.4 ± 1.5 a	23.4 ± 1.5 a
0.1	24.6 ± 0.8 a	22.6 ± 0.7 ab	21.7 ± 0.8 a	23.3 ± 0.7 a	20.2 ± 0.6 ab
1	24.5 ± 0.8 a	20.4 ± 0.4 c	24.2 ± 0.6 a	22.1 ± 0.8 a	20.8 ± 1.4 ab
10	26.1 ± 0.8 a	20.1 ± 0.4 c	34.0 ± 1.3 b	23.9 ± 0.3 a	19.7 ± 0.8 b
100	36.5 ± 1.0 b	20.9 ± 0.2 bc	39.8 ± 1.9 c	29.3 ± 0.8 b	NA[b]

[a] Means in the same column without the same letter differ, determined by Tukey's HSD test, $p \leq 0.05$. [b] ASP−GI at a concentration of 100 μmol/L GAE was unachievable.

Table 5. Changes in Quinone Reductase Activity of Hepa1c1c7 Cells after Incubation with Combined Almond Skin Polyphenols and Vitamins C and E with Equal Concentrations for 48 h[a]

μmol/L	ASP−M plus VC	ASP−GI plus VC	ISOR plus VC
	(nmol (mg protein)$^{-1}$ min^{-1})		
0.1	23.5 ± 1.7 (−2%)	26.3 ± 1.4 (17%)[b]	23.2 ± 1.0 (0%)
1	27.7 ± 0.5 (18%)[c]	20.3 ± 0.6 (−11%)[b]	23.5 ± 0.7 (−4%)
10	28.4 ± 1.1 (13%)[d]	18.3 ± 1.2 (−20%)[d]	24.8 ± 0.5 (−17%)[c]
100	29.7 ± 0.6 (−10%)[d]	NA[e]	53.7 ± 2.4 (41%)[f]

μmol/L	ASP−M plus VE	ASP−GI plus VE	ISOR plus VE
	(nmol (mg protein)$^{-1}$ min^{-1})		
0.1	20.8 ± 1.0 (−9%)[b]	18.4 ± 1.0 (−14%)[b]	24.1 ± 1.1 (8%)
1	24.4 ± 0.4 (16%)[f]	20.0 ± 0.8 (−3%)	22.5 ± 0.6 (1%)
10	27.3 ± 0.8 (24%)[f]	20.9 ± 1.4 (5%)	27.7 ± 1.5 (2%)
100	32.3 ± 1.3 (29%)[c]	NA	38.3 ± 2.0 (26%)[d]

[a] Quinone reductase activity in Hepa1c1c7 cells incubated without antioxidants was 23.4 ± 1.5 nmol (mg protein)$^{-1}$ min^{-1}. Numbers in the parentheses show percent difference between actual and expected values ((actual value − expected value)/expected value × 100%), with positive percentages indicating synergism and vise versa. [b] $p \leq 0.05$, tested by a Student's *t* test. [c] $p \leq 0.001$, tested by a Student's *t* test. [d] $p < 0.01$, tested by a Student's *t* test. [e] ASP−GI at a concentration of 100 μmol/L GAE was unachievable. [f] $p \leq 0.0001$, tested by a Student's *t* test.

respectively (*22*). Nevertheless, our in vitro results suggest that ASP would offer little protection against $O_2^{-\bullet}$ attack in vivo, although they might protect the gastrointestinal tract, where they could reach mmol/L concentrations (*37*).

Recently, ORAC and FRAP values for numerous foods have been generated with whole almonds reported as 44.5 μmol TE/g and 41.3 μmol Fe^{2+}/g, respectively (*38, 39*). The ORAC value of almond skins has been reported to range from 331 to 1080 μmol TE/g, which was comparable to the 273.6 μmol TE/g found in Carmel almond skins by M extraction (*35*). Interestingly, in contrast to the markedly different radical-quenching potencies of the tested antioxidants, their ORAC and FRAP values were found to have a similar rank order of ISOR > VC >ASP−M > ASP−GI (except for VE, which is ineffective in the ORAC assay), in a consistent manner with reports that flavonoid aglycones are more potent than their corresponding glycones (*15, 40*).

We have previously found that ASP−M as well as oat polyphenols interact with VC and VE to increase the resistance of LDL to oxidation in a dose-dependent, synergistic fashion (*15, 33, 41*). In contrast to these results, combining ASP with VC or VE produced additive, synergistic, or antagonistic responses to free-radical scavenging dependent upon the specific antioxidant combination and assay. ASP−M plus VC consistently produced lower antioxidant potency in radical scavenging compared to their calculated additive effect, while ASP−GI plus VC produced an antioxidant synergy in these assays. ASP plus VC were antagonistic in the ORAC assay but synergistic in the FRAP assay. In contrast, combining ISOR with VC or VE in these assays always produced a synergistic result.

Flavonoids and other polyphenols can modulate phase II metabolism, in part via an impact on signal transduction pathways that affect the antioxidant-response element (*8, 42*). Assessing QR activity as a biomarker of phase II metabolism has commonly been employed to screen the potential chemopreventive activity of phytochemicals (*43*). Although ASP provides antioxidant protection, its effect on QR activity in mouse hepatocytes was dependent upon the extraction solvent, with QR induced by ASP−M and inhibited by ASP−GI, a result that further illustrates the different constituents of these two extracts. ISOR had a greater impact on QR induction than ASP−M at the same GAE concentration. The underlying mechanisms for these relationships remains to be elucidated, although the greater antioxidant activity of ISOR and/or the presence in ASP−M of less potent QR inducers, such as catechins and flavanones, may contribute to the observed effects (*44, 45*). Wang and Higuchi (*46*) reported that VC and VE at 0.01−10 μmol/L enhanced QR activity of Colo205 colon cancer cells in a dose-dependent manner. We found VC induced and VE inhibited QR in Hepa1c1c7 cells in a dose-dependent manner. Combinations of ASP with VC or VE produced additive, synergistic, or antagonist responses dependent upon the specific combination and dose. At the intermediate 1 and 10 μmol/L GAE doses, ASP−M plus VC or VE induced QR in a synergistic manner. At the 0.1 μmol/L GAE, ASP−GI plus VC induced QR, while neither component alone at the same dose had an effect.

In conclusion, ASP possesses a range of potencies in quenching free radicals, increasing measures of antioxidant activity, and modulating phase II metabolism, dependent upon their dose, radical species, and method by which they are extracted. ASP−GI, potentially a more physiologically relevant extract for estimating the activity of bioavailable almond skin constituents, was particularly effective against HOCl and $ONOO^-$. Although flavonoid profiles from ASP−M and −GI were different, on the basis of their total phenolic content, their total antioxidant activity as determined with the FRAP and ORAC assays was unexpectedly comparable. Interactions between ASP and VC or VE were dependent upon a specific combination, dose, and radical species. At concentrations achievable in vivo, QR activity was inhibited by ASP−GI yet unaltered by ASP−M. Interactions between ASP and VC or VE produced additive, synergistic, or antagonistic actions on QR, again dependent upon the extract, combination, and dose. In part, the complexity of these relationships reflects the dynamic interactions within the antioxidant defense network. Undoubtedly, this complexity is further increased in vivo following almond consumption by factors such as bioaccessibility, bioavailability, metabolism, and distribution of their constituent phytochemicals and other nutrients. However, even with the controls and relative precision available for in vitro studies, the very different antioxidant and QR responses of ASP obtained in these experiments illustrate the limitations of trying to assess their antioxidant action via a single assay (*47*). Simple ap-

proaches to defining the complex nature of these phytochemicals and the foods that contain them will often provide incomplete or misleading information. Thus, while further in vivo studies on ASP are warranted to determine their impact on physiological functions, additional in vitro experiments directed at understanding their molecular mechanisms of action would help generate new hypotheses and better inform study designs with animal models and clinical trials.

ABBREVIATIONS USED

ASP, almond skin polyphenol; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalent; GI, gastrointestinal juice mimic extraction solvent; IC_{50}, 50% inhibition constant; ISOR, isorhamnetin; M, acidified methanol extraction solvent; ORAC, oxygen radical absorbance capacity; QR, quinone reductase; TE, trolox equivalent.

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