JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antioxidant Activities of Pomegranate Fruit Extract and Its Anthocyanidins: Delphinidin, Cyanidin, and Pelargonidin

YASUKO NODA,*,† TAKAO KANEYUKI,‡ AKITANE MORI,†,§ AND LESTER PACKER†,||

Department of Molecular and Cell Biology, University of California at Berkeley, Berkeley, California 94720, and Department of Nutritional Science, Okayama Prefectural University, Okayama, Japan

Antioxidant activities of freeze-dried preparations of a 70% acetone extract of pomegranate (*Punica granatum* L.) and its three major anthocyanidins (delphinidin, cyanidin, and pelargonidin) were evaluated. Free radical scavenging activities were examined using an ESR technique with spin trapping; DMPO for hydroxyl (·OH) and superoxide ($O_2^{\cdot -}$) radicals; and [$(MGD)_2Fe^{2+}$] for nitric oxide (NO). Inhibitory effects on lipid peroxidation were estimated by the levels of malonaldehyde and 4-hydroxyalkenals in rat brain homogenates. Pomegranate extract exhibited scavenging activity against ·OH and $O_2^{\cdot -}$. Anthocyanidins inhibited a Fenton reagent ·OH generating system possibly by chelating with ferrous ion. Anthocyanidins scavenged $O_2^{\cdot -}$ in a dose-dependent manner. The ID_{50} values of delphinidin, cyanidin, and pelargonidin were 2.4, 22, and 456 μM, respectively. In contrast, anthocyanidins did not effectively scavenge NO. Anthocyanidins inhibited H_2O_2-induced lipid peroxidation in the rat brain homogenates. The ID_{50} values of delphinidin, cyanidin, and pelargonidin for them were 0.7, 3.5, and 85 μM, respectively. These findings suggest that the above anthocyanidins contribute to the antioxidant activity of pomegranate fruits.

KEYWORDS: Anthocyanidin; delphinidin; cyanidin; pelargonidin; superoxide anion radical scavenger; ferrous ion chelating; lipid peroxidation; antioxidant

INTRODUCTION

Pomegranate (*Punica granatum* L.) is a rich source of anthocyanidins. Delphinidin-3,5-diglucoside is a major anthocyanin in pomegranate juice (*1*). The seed coat of this fruit contains delphinidin-3-glucoside, delphinidin-3,5-diglucoside, cyanidin-3-glucoside, cyanidin-3,5-diglucoside, pelargonidin-3-glucoside, and pelargonidin-3,5-diglucoside (*2*). In general, epidemiological studies suggest that intake of flavonoids, a group of polyphenolic compounds found in vegetables and fruits, is beneficial for prevention of cardiovascular (*3*), inflammatory, and other diseases (*4*, *5*). It has been suggested that free radical scavenging and antioxidant activities play an important role in prevention of free radical-related diseases, including aging (*6*−*9*). Meanwhile, anthocyanidins, which differ structurally from other flavonoids except flavan-3-ol, and which do not have a carboxyl group in the C-ring, prevented lipid peroxidation of cell or liposome membranes (*10*, *11*). Pomegranate juice showed potent anti-atherogenic effects in vivo (*12*). Therefore, it is of interest to know the specific free radical scavenging and antioxidant activity of anthocyanidins in pomegranate juice. In addition, our previous study on delphinidin-3-(*p*-coumaroyl-rutinoside)-5-glucose (nasunin) strongly suggested that delphinidin in the chemical structure of nasunin may contribute to its free radical scavenging and protective effect on H_2O_2-induced lipid peroxidation (*13*). To clarify the relationship between anthocyanidin structures and their antioxidant activities we aimed to examine delphinidin and its analogues. In this study, specific free radical scavenging activities of a freeze-dried 70% acetone extract of pomegranate, and of delphinidin, cyanidin, and pelargonidin, were examined. Furthermore, malonaldehyde and 4-hydroxyalkenals levels in rat brain homogenates treated with pomegranate extract, delphinidin, cyanidin, and pelargonidin were estimated as an index of lipid peroxidation.

MATERIALS AND METHODS

Preparation of Pomegranate Extract. Fresh fruit of pomegranate, products of California, was peeled, and its edible portion (seed coats and juice) was squeezed in 70% acetone−30% distilled water (1:20, by w/v). The red extract was filtered through filter paper (Whatman, No. 1). The filtrate was condensed and freeze-dried. The freeze-dried extract was kept at 4 °C until analysis.

Chemicals. L-Ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2H-1-benzopyran-6-yl-hydrogen phosphate]

* Corresponding author. Present address: Life and Environmental Engineering, Department of Environmental Engineering, Kochi University of Technology, 185 Miyanokuchi,Tosayamada, Kami-gun, Kochi 782-8502, Japan. Phone: +011-81-887-57-2519. Fax: +011-81-887-57-5220 (or 2061). E-mail: noda.yasuko@kochi-tech.ac.jp.

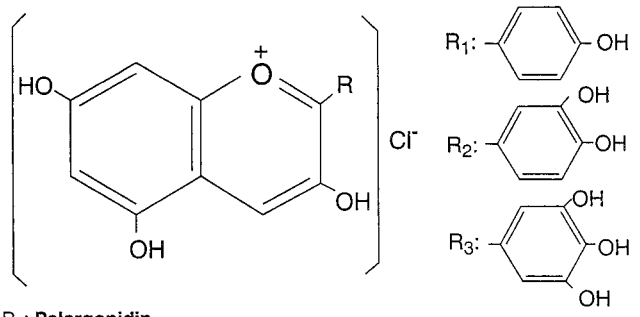
† University of California at Berkeley.

‡ Okayama Prefectural University.

§ Presently located at Life and Environmental Engineering, Department of Environmental Engineering, Kochi University of Technology, 185 Miyanokuchi, Tosayamada, Kami-gun, Kochi 782-8502, Japan.

|| Presently located at Department of Molecular Pharmacology and Toxicology, School of Pharmacy, University of Southern California, Los Angeles, CA 90089-9121.

10.1021/jf0108765 CCC: $22.00
Published on Web 12/04/2001



R1: **Pelargonidin**
3,5,7-Trihydroxy-2-(**4-hydroxy**phenyl)-1-benzopyrylium chloride

R2: **Cyanidin**
3,5,7-Trihydroxy-2-(**3,4-dihydroxy**phenyl)-1-benzopyrylium chloride

R3: **Delphinidin**
3,5,7-Trihydroxy-2-(**3,4,5-trihydroxy**phenyl)-1-benzopyrylium chloride

Figure 1. Molecular structures of pelargonidin, cyanidin, and delphinidin.

potassium salt (EPC−K_1), standard material for hydroxyl radical scavenging activity, was a kind gift of Dr. Kazumi Ogata, Senju Pharmaceutical Co., Ltd. (Osaka, Japan). Bovine erythrocyte superoxide dismutase (SOD) standard kit was from Labotec Co., Ltd. (Osaka, Japan). The spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and *N*-methyl-D-glucaminedithiocarbamate (MGD) were from Labotec Co. (Tokyo, Japan). Xanthine oxidase was from Boehringer Mannheim Corp. (Indianapolis, IN). 1-Hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7) was from Alexis Corp. (San Diego, CA). Delphinidin-, cyanidin-, and pelargonidin-chlorides (Figure 1) were from Extrasynthese Co. (Genay Cedex, France). For the measurements of malonaldehyde plus 4-hydroxyalkenals as an indicator of lipid peroxidation, an LPO-586 kit (OXIS International, Inc., Portland, OR) was used. All other chemicals were from Sigma Chemical Co. (St. Louis, MO) and were the highest grade available.

Sample Solutions. The freeze-dried sample was dissolved in 0.1 M potassium phosphate buffer (pH 7.4) just before analysis. Delphinidin-, cyanidin-, or pelargonidin-chlorides was dissolved in deionized, distilled water (working stock solution) and diluted with 0.2 M phosphate buffer (Chelex resin-treated) to obtain appropriate concentrations in 0.1 M phosphate buffer just before use. Immediately after the dilution, ESR measurements were performed.

ESR Spectrometer. A computerized ESR spectrometer (Free Radical Monitor, JES-FR30, JEOL, Tokyo) was used. This spectrometer has the function of normalizing all spectra for accurate calculation using manganese oxide (MnO) as an internal standard.

ESR Settings. The following ESR settings were used: magnetic field, 335.6 ± 5 mT; power, 4 mT; modulation frequency, 9.41 GHz; modulation amplitude, 1 × 0.1 mT; response time, 0.1 s; amplitude, 1 × 200 or 250; sweep width, 5.000 (for DMPO−OH and DMPO−OOH) or 15.000 [for $(MGD)_2Fe^{2+}$−NO•] mT; and the sweep time, 0.5 or 2.0 min. All measurements were performed at 23 °C.

Superoxide Radical Scavenging Activity. Superoxide radicals were generated by reaction of the hypoxanthine−xanthine oxidase system. For the extract sample, an aliquot was passed through a centrifuge-type filter (capacity, 400 μL; Ultrafree-MC filters, 10,000 NMWL regenerated-cellulose membrane and 100,000 NMWL polysulfone membrane, Millipore, Bedford, MA) by centrifugation (5,000*g*, 4 °C) to obtain materials of smaller molecular weights: less than 10,000 or 100,000 (*14*). Sample solution (50 μL), 4 mM hypoxanthine (50 μL), dimethyl sulfoxide (DMSO) (30 μL), 4.5 (or 0.45) M DMPO (20 μL) and xanthine oxidase (0.4 units/mL) (50 μL) were mixed in a test tube, and the mixture was quickly transferred to a quartz flat cell (capacity 200 μL). The ESR spectra of the DMPO−OOH spin adducts were recorded (*14*).

Hydroxyl Radical Scavenging Activity. Sample solution (50 μL), 0.18 (or 0.018) M DMPO (50 μL), 2 mM H_2O_2 (50 μL), and 0.2 mM $FeSO_4$ (50 μL) were mixed in a test tube, and the mixture was quickly transferred to a flat cell. Exactly 30 s after the addition of $FeSO_4$, the ESR spectra of the DMPO−OH spin adducts were recorded (*14*). As a standard, EPC−K_1, a conjugate of ascorbate and vitamin E joined by a phosphate linkage (*15*), was used. Hydroxyl radicals were generated by an ultraviolet (UV)-H_2O_2 system. Sample solution (50 μL), 0.18 M DMPO (50 μL), 0.1 M buffer (50 μL), and 2 mM H_2O_2 (50 μL) were mixed, and the mixture was quickly transferred to a flat cell. The mixture was irradiated with UV light (254 nm) (UV lamp, Longlife filter, model ENF-240C, 0.2 amps, Spectronics Corp., Westbury, NY) for 30 s, and the ESR spectra were recorded exactly 10 s after the UV exposure (*13*).

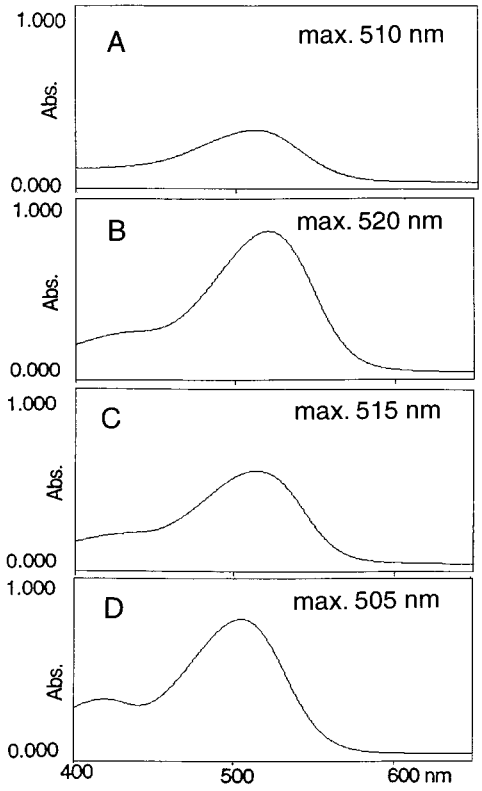


Figure 2. Spectrophotometric assay of pomegranate extract and anthocyanidins. A, Pomegranate extract; B, 0.02 mM delphinidin; C, 0.02 mM cyanidin; D, 0.05 mM pelargonidin. The extinction coefficients were 4.1, 2.7, and 1.6×10^4 M^{-1} cm^{-1} for delphinidin, cyanidin, and pelargonidin, respectively.

Nitric Oxide Scavenging Activity. NO was generated from NOC-7. All reagents except NOC-7 were dissolved in the buffer. Sample solution (100 μL), 0.1 M buffer (40 μL), 10 mM $[(MGD)_2Fe^{2+}]$ (40 μL), and 10 mM NOC-7 in 0.1 N NaOH (20 μL) were mixed, and the mixture was quickly transferred to a flat cell. NO, which reacts with $[(MGD)_2Fe^{2+}]$, was monitored by the first peak of the reaction product $[(MGD)_2Fe^{2+}$−NO•] in ESR spectra after the addition of the NOC-7 (*16*).

Spectrophotometric Measurements. Pomegranate extract, delphinidin, cyanidin, or pelargonidin was dissolved in 0.01 N HCl to get appropriate concentrations for the measurements. Immediately after the preparation, the absorption spectra were recorded using a spectrophotometer (Hitachi U-2000A, Tokyo).

H_2O_2-Induced Lipid Peroxidation in Rat Brain Homogenate. Sprague−Dawley rats (male, 350 g body weight) were anesthetized with ether and perfused through the heart with 0.9% NaCl solution (4 °C). Rat cereberal cortex was removed, and homogenized in ice-cold 20 mM Tris−HCl buffer, pH 7.4 (1:10, w/v). The homogenate was incubated with or without 5 mM H_2O_2 for 60 min at 37 °C. Anthocyanidins were used in combination with H_2O_2 (5 mM) to test for its antioxidative effect in vitro.

After incubation, the reaction was stopped by placing the homogenates into ice-cold water for 10 min, and then the homogenates were centrifuged at 15,000*g* for 10 min at 4 °C. The level of malonaldehyde and 4-hydroxyalkenals in the supernatant was measured at the wavelength of 586 nm.

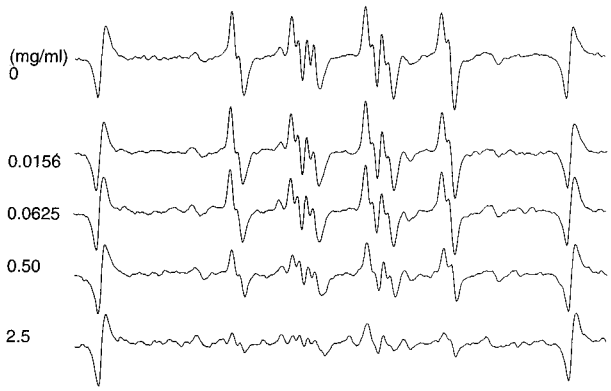


Figure 3. Superoxide anion radical scavenging activity of pomegranate.

Determination of Protein Concentration. Protein concentrations were determined by Lowry's method (*17*) using bovine serum albumin as a standard.

All data are expressed as means ± SEM.

RESULTS

Samples. The yield of freeze-dried pomegranate juice extract was 11% of edible parts (dry weight of freeze-dried sample/wet weight of fresh seed coats and juice), or 3.9% of whole fruit (dry weight of freeze-dried sample/wet weight of fresh whole fruit).

Spectrophotometric Assay of Pomegranate Extract and Anthocyanidins. Anthocyanidin content in fresh pomegranate juice was estimated using the absorbance at its maximum wavelength of 520 nm, and was calculated as 2.0 mM delphinidin equivalent if it could be assumed that delphinidin was mainly contributing to the maximal absorbance in the juice (Figure 2).

Superoxide Radical Scavenging Activity (SOD-Like Activity). Pomegranate extract showed potent activity in a dose-dependent manner (Figure 3). The SOD-like activity of pomegranate extract was 17 ± 1 SOD-equivalent units/mg of freeze-dried sample, $n = 6$. The sample solution of pomegranate extract that passed through the filter with 100,000 or 10,000 molecular weight cutoff membrane was 16 ± 2 ($n = 6$) or 15 ± 1 ($n = 5$) SOD-equivalent units/mg of freeze-dried sample (no significant difference compared to nontreated sample).

Anthocyanidins are unstable to light and are rapidly destroyed by alkali (*1*). In this study, anthocyanidin chloride was dissolved in distilled water as working stock solution, and then diluted with buffer to get appropriate concentrations just before measurements. The IC_{50} values obtained under this experimental condition are summarized in Table 1. By using the ID_{50} values and constant value ($k_{scavenger} = k_{DMPO} \times [DMPO]/ID_{50}$, where [DMPO] = 0.45 M and $k_{DMPO} = 16.9$ M/s) (*18*), the second-order rate constants were calculated as 3.2×10^6, 3.5×10^5, and 1.7×10^4 (M/s) for delphinidin, cyanidin, and pelargonidin, respectively. When the concentration of DMPO was changed to 10-fold (from 450 to 45 mM, final concentrations), the ID_{50} was shifted to a 10-fold lower concentration (Figure 4), i.e., delphinidin, cyanidin, or pelargonidin directly scavenged superoxide radicals. The scavenging activity was 1360 ± 214 ($n = 4$), 240 ± 19 ($n = 5$), and 52 ± 13 ($n = 4$) SOD-equivalent units/mg for delphinidin, cyanidin, and pelargonidin, respectively.

Table 1. Superoxide Anion Radical Scavenging Activity[a]

	IC_{50}	
	μg/mL	μM
pelargonidin	130	420
cyanidin	7.0	22
delphinidin	0.8	2.4

[a] Pelargonidin chloride (MW 306.7); cyanidin chloride (MW 322.7); delphinidin chloride (MW 339.7).

Hydroxyl Radical Scavenging Activity. Pomegranate extract showed hydroxyl radical scavenging activity (Figure 5). Pomegranate extract showed scavenging activity (0.35 ± 0.04 EPC−K_1-equivalent μmol/mg of freeze-dried sample, $n = 13$). Delphinidin, cyanidin, and pelargonidin apparently showed scavenging activity in a dose-dependent manner (data not shown). The results were 0.93 ± 0.11 ($n = 12$), 0.40 ± 0.10 ($n = 15$), 0.37 ± 0.10 ($n = 15$) EPC−K_1-equivalent μmol/mg or 0.31 ± 0.04, 0.13 ± 0.02, 0.11 ± 0.02 EPC−K_1-equivalent μmol/μmol for delphinidin, cyanidin, and pelargonidin, respectively. From the dose-dependent curves obtained by using 0.18 M DMPO (45 mM, final) or 10-fold lower concentration of DMPO (4.5 mM, final), the ID_{50} did not shift to 10-fold lower concentration in all these anthocyanidins (Figure 6) indicating that the observed activity is not due to the direct scavenging.

By the UV−H_2O_2 system, typical ESR quartet signals of the DMPO−OH spin adducts were obtained, and they were identified by the hyperfine coupling constants ($\alpha_H^\beta = \alpha_N = 1.49$). Delphinidin, cyanidin, and pelargonidin, when alone, produced ESR signals of DMPO spin adducts upon UV irradiation, in the absence of H_2O_2. Figure 7 shows typical ESR signals observed in delphinidin: the second signal from left in quartet signal (a) of DMPO−OH (1:2:2:1), $g = 2.0062 \pm 0.0002$; the fourth signal (b) of DMPO−H, $g = 2.0066 \pm 0.0002$) (9 signals, triplets of 1:2:1). Only the second signal intensity (a) of DMPO−OH did not overlap with DMPO−H signals. This second signal intensity in delphinidin (cyanidin or pelargonidin)/

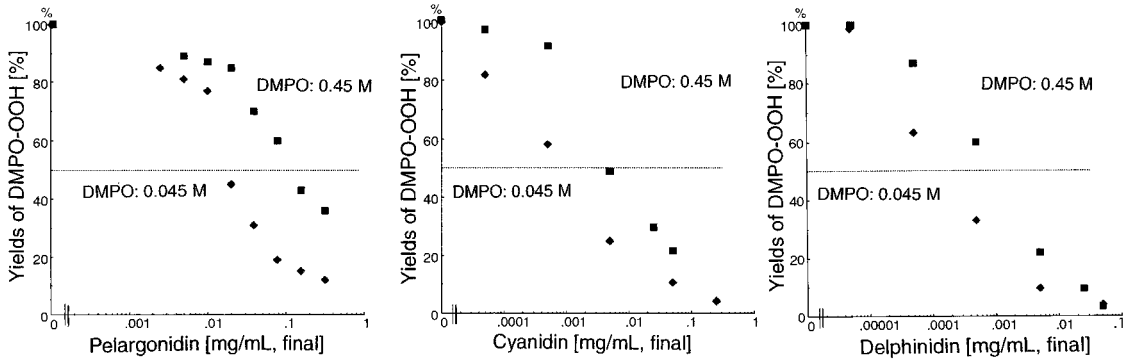


Figure 4. Relationship between the signal intensity of the DMPO–OOH spin adducts and concentration of pelargonidin, cyanidin, or delphinidin (concentration of DMPO, 0.45 M or 0.045 M).

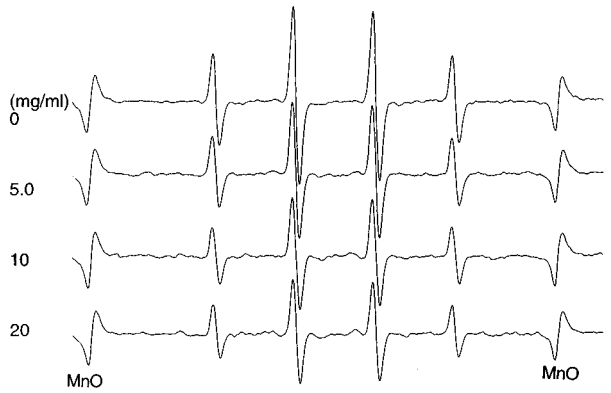


Figure 5. Hydroxyl radical scavenging activity of pomegranate extract.

H_2O_2/UV system was compared to the DMPO−OH signals from the H_2O_2/UV system and the delphinidin (cyanidin or pelargonidin)/UV system (Figure 8). These anthocyanidins did not show direct scavenging from the H_2O_2/UV system.

Inhibitory Effect of Pomegranate Extract and Anthocyanidins on H_2O_2-Induced Lipid Peroxidation. Pomegranate extract lowered the levels of malonaldehyde plus 4-hydroxyalkenals produced by H_2O_2-induced lipid peroxidation in rat brain homogenates during the incubation (Figure 9a). The ID_{50} value of the inhibition was 10 mg/mL. Anthocyanidins inhibited lipid peroxidation in a dose-dependent manner (Figure 9b). The ID_{50} values were 0.7, 3.5, and 85 μM for delphinidin, cyanidin, and pelargonidin, respectively.

Nitric Oxide Radical Scavenging Activity. Pomegranate extract did not show NO scavenging activity up to 2.5 mg/mL. Delphinidin, cyanidin, and pelargonidin did not show NO scavenging under this experimental condition.

DISCUSSION

Pomegranate extract showed potent scavenging activity for superoxide radicals. A filtered sample from the extract only slightly decreased the activity when compared to that of the nontreated sample. These results suggest that the scavenging activity of pomegranate extract is due to its relatively smaller molecular weight components (MW < 10,000). Anthocyanidins showed potent activity in the order of delphinidin, cyanidin, and pelargonidin. Anthocyanidins, aglycons of anthocyanins, are known to exist in red fruits (*19*). Delphinidin-3,5-diglucoside is a major anthocyanin in pomegranate juice (*1*). From the spectrophotometric observation, the estimated value of delphinidin content in fresh juice was approximately 0.06% if it was calculated on the basis of delphinidin. The yield of freeze-dried sample was about 11% of the fresh juice and seed coats. Meanwhile, the activity of pomegranate extract and delphinidin were 17 ± 1 and 1360 ± 214 SOD-equivalent units/mg. Therefore, the activity would most likely be due to delphinidin which is a major component contained as delphinidin-3,5-diglucoside in pomegranate juice (*1*), and may be in part due to cyanidin which is also contained in the seed coats (*2*). In this study, a hypoxanthine−xanthine oxidase system was used for superoxide radical generation. It has been reported that flavonoids, such as flavones, flavonols, and isoflavones, inhibited xanthine oxidase (*20*). Meanwhile, it was mentioned that flavonoids inhibited various enzymes such as alkaline phos-

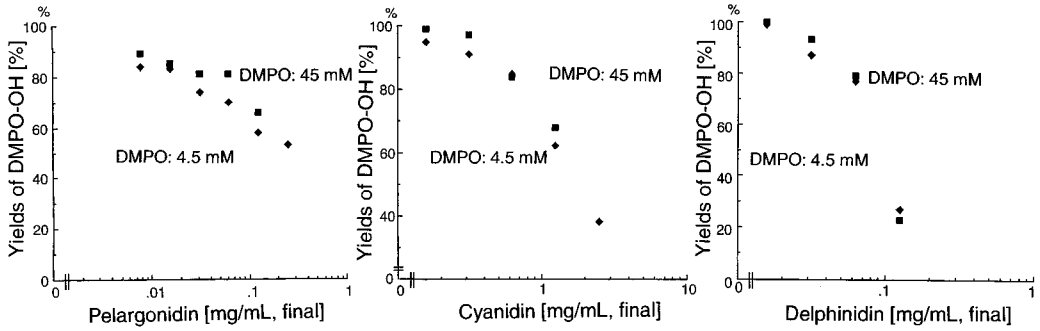


Figure 6. Relationship between the signal intensity of the DMPO-OH spin adducts and concentration of pelargonidin, cyanidin, or delphinidin (concentration of DMPO. 45 mM or 4.5 mM).

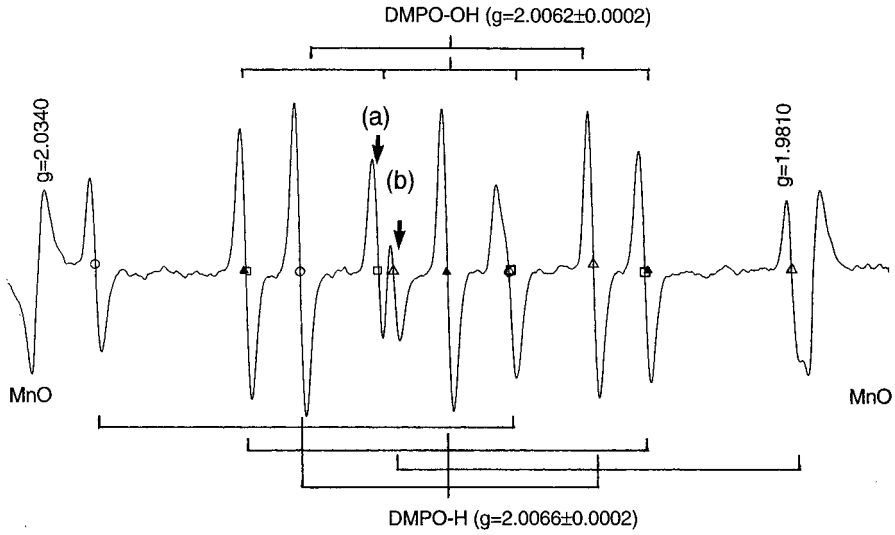


Figure 7. Delphinidin-produced ESR signal of DMPO spin adducts by UV irradiation.

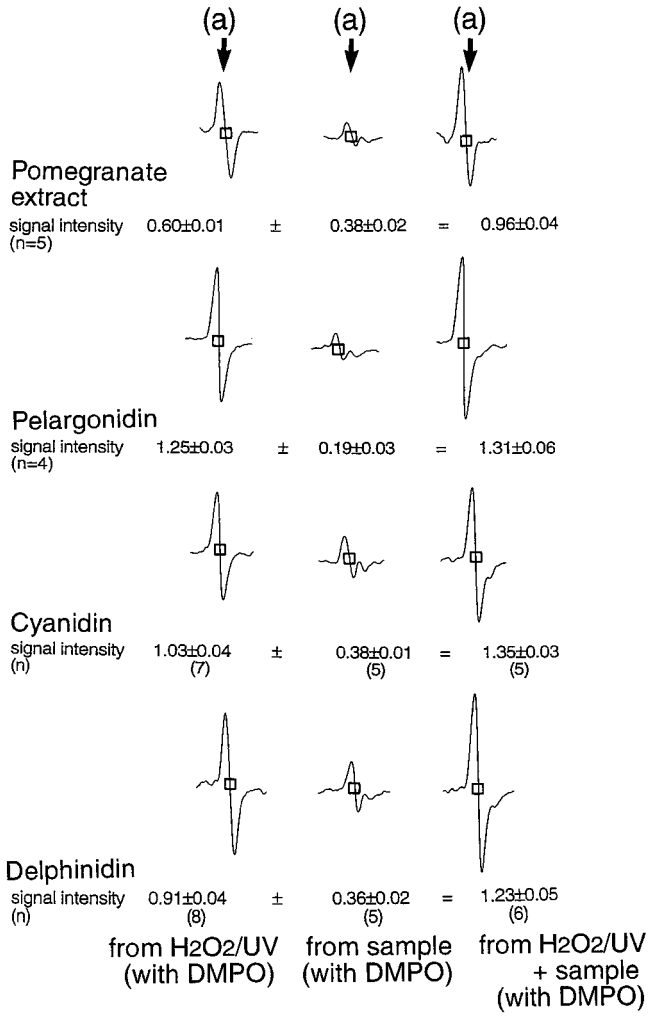


Figure 8. Comparison of second signal of DMPO–OH spin adduct. ESR recordings showing that pomegranate extract, pelargonidin, cyanidin, or delphinidin did not scavenge •OH radicals generated from H_2O_2/UV system.

phatase, arylsulfatase, DOPA decarboxylase, and so forth (*21*). To examine whether the observed activity reflected the direct radical scavenging and/or inhibition of xanthine oxidase, an ESR method was used for this analysis (*18*). As shown in Figure 4, when the concentration of DMPO was lowered 10-fold, the ID_{50} values were shifted to 10-fold lower concentrations, indicating that pomegranate delphinidin, cyanidin, and pelargonidin directly scavenged superoxide radicals when these compounds encountered the radicals. In the presence of acetoaldehyde and *tert*-butyl hydroperoxide, it was reported that the chemiluminescence intensity of anthocyanins was in the order nasunin > rubrobrassicin > delphinidin > malvin = cyanidin > malvidin; i.e., a hydroxyl group at around 4′ position in B-ring and total number at 3′, 4′, and 5′ position in B-ring, and glucosylation at C-3 and C-5 positions of the anthocyanidin ring enhances chemiluminescence of the parent compound (*22*). Dihydroxylation at the 3′ and 4′-positions in the B-ring is very important for antioxidant activity (*23*−*26*). In our study, in particular, specific scavenging activity for superoxide radicals was in the order dephinidin > cyanidin > pelargonidin, indicating that (i) hydroxyl groups at the 3′, 4′, and 5′ positions in the B-ring are contributing to their activity, and also important are (ii) adjacent hydroxylation at 3′ and 4′ positions in B-ring, and (iii) stabilization by its resonance structure. It is not apparent why the order of nasunin and delphinidin in our study is different from the chemiluminescence observation (*22*, *27*).

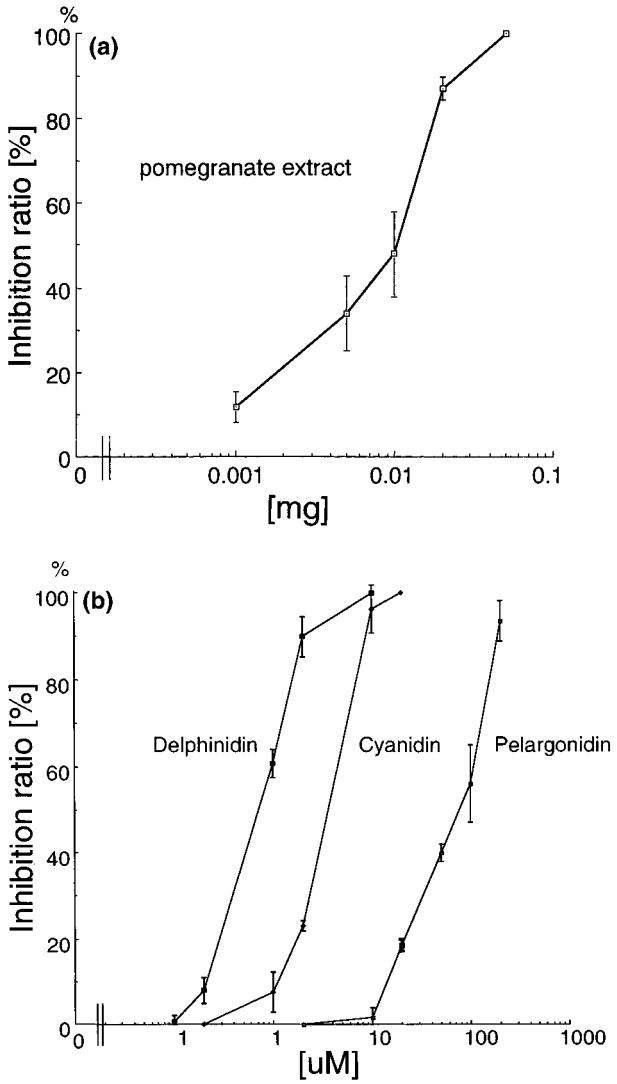


Figure 9. Inhibitory effects of pomegranate extract, pelargonidin, cyanidin, or delphinidin on H_2O_2-induced lipid peroxidation in rat brain homogenate. The control value of the levels of malonaldehye and 4-hydroxyalkenals was 5.6 ± 0.6 nmol/mg protein ($n = 5$). (a) Pomegranate extract (0–0.05 mg of pomegranate extract in 0.5 mL of the incubation mixture); (b) pelargonidin, cyanidin, or delphinidin (0–200 μM in the incubation mixture).

Pomegranate extract and anthocyanidins showed apparent hydroxyl radical scavenging activity. By changing the concentration of DMPO from 45 to 4.5 mM (final concentrations), the ID_{50} values did not shift to 10-fold lower concentrations, indicating that the apparent activity was not due to the direct radical scavenging but may be due to the chelating with ferrous ion in the hydroxyl radical generation system. From the observation using the UV/H_2O_2 system for hydroxyl radical generation, pomegranate and anthocyanidins did not show significant differences in the second peak intensity of DMPO−OH between anthocyanidin/H_2O_2/UV system and the sum of H_2O_2/UV and anthocyanidin/UV systems, indicating that these compounds did not directly scavenge hydroxyl radicals. Anthocyanidins, which possess a hydroxyl group at 3′, 4′, and 5′ (delphinidin) or 3′ and 4′ (cyanidin) positions in the B-ring, chelate with iron and certain metals such as copper, tin, and aluminum (*28*, *29*). However, pelargonidin, which has no ortho-dihydroxy substitution in the B-ring, has been reported to have no chelating capacity with metals (*30*, *31*). Therefore, the results observed with pelargonidin may be due to a different oxidation profile (*32*) or due to oxidation of a hydroxy group at 3′ position

in the B-ring at neutral pH (*33*, *34*), although the mechanism in this case needs clarification.

Delphinidin, cyanidin, and pelargonidin inhibited H_2O_2-induced lipid peroxidation in rat brain homogenates in the order of delphinidin > cyanidin > pelargonidin. It was suggested that delphinidin, the main component in pomegranate juice, is the most contributing to the inhibitory effect of pomegranate on H_2O_2-induced lipid peroxidation.

Under in vitro experimental conditions, pomegranate extract and anthocyanidins did not show nitric oxide scavenging activity.

ABBREVIATIONS USED

ESR, electron spin resonance; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; MGD, *N*-methyl-D-glucamine-dithiocarbamate; $[(MGD)_2Fe^{2+}]$, complex Fe^{2+} with *N*-methyl-D-glucamine dithiocarbamate; DMSO, dimethyl sulfoxide; SOD, superoxide dismutase; EPC−K_1, L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2H-1-benzopyran-6-yl hydrogen phosphate] potassium salt.

LITERATURE CITED

(1) Harborne, J. B. The Anthocyanin Pigments. In *Comparative Biochemistry of the Flavonoids*; Academic Press: New York, 1967; pp 1−30.

(2) Du, C. T.; Wang, P. L.; Francis, F. J. Anthocyanins of pomegranate, *Punica granatum*. *J. Food Sci.* **1975**, *40*, 417−418.

(3) Gaziano, J. M.; Manson, J. E.; Branch, L. G.; Colditz, G. A.; Willett, W. C.; Buring, J. E. A prospective study of consumption of carotenoids in fruits and vegetables and decreased cardiovascular mortality in the elderly. *Ann. Epidemiol*. **1995**, *5*, 255−260.

(4) Middleton, E.; Kandaswani; C. Effects of flavonoids on immune and inflammatory cell functions. *Biochem. Pharmacol.* **1992**, *43*, 1167−1179.

(5) Cook, N. C.; Samman, S. Flavonoids − Chemistry metabolism, cardioprotective effects, and dietary sources. *J. Nutr. Biochem*. **1996**, *7*, 66−76.

(6) Packer, L. Oxidative stress, antioxidants, aging and disease. In *Oxidative Stress and Aging*; Culter R. G., Packer, L., Bertram, J., Mori, A., Eds.; Birkhauser Verlag: Basel, Switzerland, 1995; pp 1−14.

(7) Halliwell, B. Antioxidant activity and other biological effects of flavononids. *Int. Congr. Symp. Ser.−R. Soc. Med.* **2000**, 226, 13−23.

(8) Harman, D. Aging: a theory based on free radical and radiation chemistry. *J. Gerontol.* **1956**, *11*, 298−300.

(9) Harman, D. Aging: overview. *Ann. N. Y. Acad. Sci*., **2001**, 928, 1−21.

(10) Tsuda, T.; Watanabe, M.; Ohshima, K.; Norinobu, S.; Choi. S.-W.; Kawakishi, S.; Osawa, T. Antioxidative activity of the anthocyanin pigments cyanidin 3-*O*-*β*-D-glucoside and cyanidin. *J. Agric. Food Chem.* **1994**, *42*, 2407−2410.

(11) Tsuda, T.; Shiga, K.; Ohshima, K.; Kawakishi, S.; Osawa, T. Inhibition of lipid peroxidation and the active oxygen radical scavenging effect of anthocyanin pigment isolated from *Phaseolus vulgaris* L. *Biochem. Pharmacol*. **1996**, *52*, 1033−1039.

(12) Aviram, M.; Dornfeld, L.; Rosenblat, M.; Volkova; N.; Kaplan, M.; Coleman, R.; Hayek, T.; Presser, D.; Fuhrman; B. Pomegranate juice consumption reduces oxidative stress, atherogenic modification to LDL, and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E-deficient mice. *Am. J. Clin. Nutr*. **2000**, *71*, 1062−1076.

(13) Noda, Y.; Kaneyuki, T.; Igarashi, K.; Mori, A.; Packer, L. Antioxidant activity of nasunin, an anthocyanin in eggplant. *Res. Commun. Mol. Pathol. Pharmacol.* **1998**, *102*, 175−187.

(14) Noda, Y.; Kohno, M.; Mori, A.; Packer, L. Automated electron spin resonance free radical detector assays for antioxidant activity in natural extracts. *Methods Enzymol.* **1999**, 299, 28−34.

(15) Mori, A.; Edamatsu, R.; Kohno, M.; Ohmori, A. A new hydroxyl radical scavenger: EPC−K_1. *Neurosciences* **1989**, *15*, 371−376.

(16) Noda, Y.; Mori, A.; Ogata, K.; Packer, L. Hydroxyl and nitric oxide radical scavenging activities of new synthetic derivatives of α-tocopherol and glutathione; 2-*S*-[2-(*N*-carbonylaminoethylsulfonic acid)-1-(α-tocopheryl-6-yloxy-carbonyl)ethyl] glutathione and 2-*S*-[2-(*N*-carbonyl-3-*β*-aminoethyl-5- hydroxyindol)-1-(α-tocopheryl-6-yloxy-carbonyl)ethyl] glutathione. *Res. Commun. Biochem. Cell & Mol. Biol.* **1999**, *3*, 131−143.

(17) Lowry, O. J.; Rosebrough, N. J.; Farr, A. L.; Randall, R. L. Protein measurement with folin-phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

(18) Kimura, K. Mechanism of active oxygen species reduction by non-steroidal anti-inflammatory drugs. *Int. J. Biochem. Cell Biol.* **1997**, *29*, 437−446.

(19) Harborne, J. B.; Grayer, R. J. The anthocyanins. In *The Flavonoids: Advances in Research Since 1980*; Harborne, J. B., Ed.; Chapman and Hill: London, 1988; pp 1−20.

(20) Hayashi, T.; Sawa, K.; Kawasaki, M.; Arisawa, M.; Shimizu, M.; Morita, N. Inhibition of cow's milk xanthine oxidase by flavonoids. *J. Nat. Prod.* **1988**, *51*, 345−348.

(21) Havsteen, B. Flavonoids, a class of natural products of high pharmacological potency. *Biochem. Pharmacol.* **1983**, *32*, 1141−1148.

(22) Yoshiki, Y.; Okubo, K.; Igarashi, K. Chemiluminescence of anthocyanins in the presence of acetaldehyde and *tert*-butyl hydroperoxide. *J. Biolumin. Chemilumin.* **1995**, *10*, 335−338.

(23) Bors, W.; Heller, W.; Michel C.; Saran, M. Flavonoids as antioxidants: Determination of radical-scavenging efficiencies. *Methods Enzymol.* **1990**, *186*, 343−355.

(24) Rice-Evans, C. A.; Miller, N. J.; Bolwell, P. G.; Bramley, P. M.; Pridham, J. B. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Rad. Res*. **1995**, *22*, 375−383.

(25) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Rad. Biol. Med.* **1996**, *20*, 933−956.

(26) Cao, G.; Sofic, E.; Prior, R. L. Antioxidant and prooxidant behavior of flavonoids: Structure−activity relationships. *Free Radical Biol. Med.* **1997,** *22*, 749−760.

(27) Noda, Y.; Kaneyuki, T.; Igarashi, K.; Mori, A.; Packer, L. Antioxidant activity of nasunin, an anthocyanin in eggplant peels. *Toxicology* **2000**, *148*, 119−123.

(28) Jurd, L.; Asen, S. The formation of metal and copigment complexes of cyanidin-3-glucoside. *Phytochemistry* **1966**, *5*, 1263−1271.

(29) van Acker, S. A.; van Balen, G. P.; van den Berg, D.-J.; Bast, A.; van de Vijgh, W. J. Influence of iron chelation on the antioxidant activity of flavonoids. *Biochem. Pharmacol.* **1988**, *56*, 935−943.

(30) Wrolstad, R. E.; Erlandson, J. A. Effect of metal ions on the color of strawberry puree. *J. Food Sci.* **1973**, *38*, 460−463.

(31) Francis, F. J. Food colorants: anthocyanins. *Crit. Rev. Food Sci. Nutr*. **1989**, *28*, 273−314.

(32) Van Acker, S. A.; van de Berg, D.-J.; Tromp, M. N.; Griffoen, D. H.; van Bennekom, W. P.; van den Vijgh, W. J.; Bast, A. Structural aspects of antioxidant activity of flavonoids. *Free Radical Biol. Med*. **1996**, *20*, 331−342.

(33) Timberlake, C. F.; Bridle, P. Anthocyanins. In *The Flavonoids;* Harborne J. B., Mabry, T. J., Mabry, H., Eds.; Academic Press: New York, 1975; pp 214−256.

(34) Hrazdina, G. Anthocyanins. In *The Flavonoids: Advances in Research*; Harborn, J. B., Mabry, T. J., Eds.; Chapman and Hill: New York, 1982; pp 135−188.

Received for review July 9, 2001. Revised manuscript received October 9, 2001. Accepted October 9, 2001.

JF0108765