JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Inhibition of Lipid Peroxidation and Structure−Activity-Related Studies of the Dietary Constituents Anthocyanins, Anthocyanidins, and Catechins

NAVINDRA P. SEERAM AND MURALEEDHARAN G. NAIR*

Bioactive Natural Products and Phytoceuticals, Department of Horticulture and National Food Safety and Toxicology Center, Michigan State University, East Lansing, Michigan 48824

The antioxidant activities of a series of commonly consumed and biogenetically related plant phenolics, namely, anthocyanidins, anthocyanins, and catechins, in a liposomal model system have been investigated. The antioxidant efficacies of the compounds were evaluated on their abilities to inhibit the fluorescence intensity decay of an extrinsic probe, 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]phenylpropionic acid, caused by free radicals generated during metal ion-induced peroxidation. Distinct structure−activity relationships were revealed for the antioxidant abilities of these structurally related compounds. Whereas antioxidant activity increased with an increasing number of hydroxyl substituents present on the B-ring for anthocyanidins, the converse was observed for catechins. However, substitution by methoxyl groups diminished the antioxidant activity of the anthocyanidins. Substitution at position 3 of ring C played a major role in determining the antioxidant activity of these classes of compounds. The anthocyanidins, which possess a hydroxyl group at position 3, demonstrated potent antioxidant activities. For the cyanidins, an increasing number of glycosyl units at position 3 resulted in decreased antioxidant activity. Similarly, the substitution of a galloyl group at position 3 of the flavonoid moiety resulted in significantly decreased antioxidant activity for the catechins. Among catechins, cis−trans isomerism, epimerization, and racemization did not play a role in overall antioxidant activity. The antioxidant activities of test compounds (at 40 μM concentrations) were compared to the commercial antioxidants *tert*-butylhydroquinone, butylated hydroxytoluene, butylated hydroxyanisole, and vitamin E (all at 10 μM concentrations).

KEYWORDS: Fluorescence spectroscopy; lipid peroxidation; antioxidants; catechins; anthocyanidins; anthocyanins; SAR

INTRODUCTION

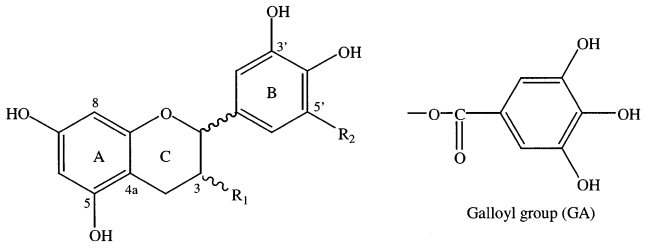
Flavonoids are a group of >4000 polyphenolics implicated with beneficial health effects due to their antioxidant properties and inhibitory role in the processes of carcinogenesis (*1*). They are categorized into major groups consisting of flavones, isoflavones, flavanones, flavanonols, flavonols, flavanols or catechins, and anthocyanidins and their glycosides. These natural antioxidant polyphenols are ubiquitously present in foods of plant origin, and it is estimated that the intake of total flavonoids by humans consuming fruit and vegetable diets can reach up to 1 g per day (*2*, *3*). There has been an increased public awareness of health-protective characteristics attributable to the flavonoid content of leaves, fruits, and vegetables.

Catechins and anthocyanins, the glycosides of anthocyanidins, contribute to a sizable proportion of total flavonoid consumption by humans. Catechins, although widely distributed in plants, are of highest yield in tea leaves, constituting up to 30% dry weight (*2*). Tea flavonoids have been reported to exhibit anticarcinogenic, antimutagenic, and cardioprotective effects that are generally associated with their antioxidant (free radical scavenging and metal chelation) properties (*4*). Tea is one of the most popular and highly consumed beverages in the world, and its health benefits have been attributed to the high flavonoid content of its leaves and extracts (*2*).

Anthocyanins are pigments primarily responsible for the attractive colors in fruits, fruit juices, wines, flowers, and vegetables. Anthocyanins have been implicated with beneficial activities as food ingredients and as promoters of human health, and their antioxidant activities have been well established (*3*, *5*, *6*). Previous studies in our laboratory have shown that the anthocyanins isolated from tart cherries and their major anthocyanidin, cyanidin, exhibit in vitro antioxidant and anti-inflammatory activities, comparable to those of commercial products (*5*, *6*). The "French paradox", the low incidence of cardiovascular diseases coupled with a high-fat diet and red wine consumption of inhabitants of some regions of France, is a subject of constant discussion. A recent study has suggested

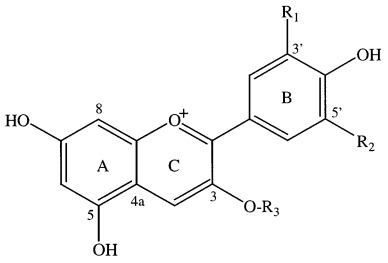
* Author to whom correspondence should be addressed [telephone (517) 353-2915; fax (517) 432-2310; e-mail nairm@msu.edu].

10.1021/jf025671q CCC: $22.00
Published on Web 08/09/2002



Catechins	Symbol	Configuration	R_1	R_2
(+)- catechin	(+)-C	2R, 3S	OH	H
(+)- epicatechin	(+)-EC	2S, 3R	OH	H
(-)-catechin	(-)-C	2S, 3R	OH	H
(-)-epicatechin	(-)-EC	2R, 3R	OH	H
(±)-catechin	(±)-C	2R, 3S; 2S, 3R	OH	H
(-)-epigallocatechin	(-)-EGC	2R, 3R	OH	OH
(-)-gallocatechin	(-)-GC	2S, 3R	OH	OH
(-)-epicatechin gallate	(-)-ECG	2R, 3R	GA	H
(-)-catechin gallate	(-)-CG	2S, 3R	GA	H
(-)-epigallocatechin gallate	(-)-EGCG	2R, 3R	GA	OH
(-)-gallocatechin gallate	(-)-GCG	2S, 3R	GA	OH

Figure 1. Structures of catechins that were studied for antioxidant activity.



Anthocyanidins & Anthocyanins	R_1	R_2	R_3
Pelargonidin	H	H	H
Cyanidin	OH	H	H
Delphinidin	OH	OH	H
Peonidin	OMe	OH	H
Malvidin	OMe	OMe	H
Pelargonidin-3-galactoside	H	H	galactose
Cyanidin-3-galactoside	OH	H	galactose
Cyanidin-3-rutinoside	OH	OH	rutinose
Cyanidin-3-glucosylrutinoside	OH	OH	glucose-rutinose
Delphinidin-3-galactoside	OH	OH	galactose

Figure 2. Structures of anthocyanidins and anthocyanins that were studied for antioxidant activity.

that these health benefits may be attributed to phenolic antioxidants, which are abundant in red wines and are not related to alcohol intake (*3*). Red wines contain substantial amounts of flavonoids, mostly anthocyanins, reaching concentrations as high as 3200 mg/L (*3*).

Natural antioxidants may function as reducing agents or donors of hydrogen atoms. Hence, they act as free radical scavengers and chain breakers, complexers of pro-oxidant metals, and quenchers of the formation of singlet oxygen (*7*). The mechanism of the protective action of flavonoids is a subject of considerable debate, but metal chelation may play a larger role in determining the antioxidant activities of these compounds than previously reported (*8*). In the present study, we assessed the ability of a series of flavonoids, which are commonly consumed by humans, to inhibit lipid peroxidation induced by Fe(II) ions in a liposomal model. The antioxidant activities of the compounds were compared to the commercial standards *tert*-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and vitamin E. The fluorescent assay described by Arora and Strasburg was used to evaluate the antioxidant efficacy of the compounds (*9*). In this assay, the peroxidative degradation of the fluorescent probe 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]phenylpropionic acid (DPH-PA), indicated by a decay in its fluorescence, is used to monitor the sensitivity of the phospholipid membrane toward oxidative stress. Our study represents the first time that catechins (**Figure 1**) are being evaluated for antioxidant activity using analysis of model liposome oxidation by fluorescence spectroscopy. This is also the first comparison of the antioxidant activities of anthocyanidins (**Figure 2**) in a liposomal model system. In addition, this is the first comparative evaluation of the structure−activity relationships (SAR) of these two major classes of flavonoids as related to their antioxidant activities using this model system. Because anthocyanidins enter the diet as glycosides, several anthocyanins (**Figure 2**) were also included in the study as the naturally occurring forms of their aglycons.

MATERIALS AND METHODS

Materials. The lipid substrate 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (SLPC) was obtained from Avanti Polar Lipids (Alabaster, AL). The fluorescent probe DPH-PA was obtained from Molecular Probes (Eugene, OR). Positive controls, TBHQ, BHA, BHT, and vitamin E (α-tocopherol), were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Malvidin and peonidin were obtained from Chromadex (Laguana Hills, CA). Cyanidin 3-rutinoside and cyanidin 3-glucosylrutinoside were isolated from Balaton tart cherries in our laboratory, and their aglycon, cyanidin, was prepared by acid hydrolysis as previously reported (*6*). The 3-galactosides of pelargonidin, cyanidin, and delphinidin were isolated from fruits of dogwood, *Cornus mas*, commonly known as Cornellian cherries, as previously reported (*10*). Pelargonidin and delphinidin aglycons were prepared from their respective galactosides similar to the preparation of cyanidin (*6*). (+)-Catechin [(+)-C], (+)-epicatechin [(+)-EC], (−)-catechin [(−)-C], (−)-epicatechin [(−)-EC], (±)-catechin [(±)-C], (−)-epigallocatechin [(−)-EGC], (−)-gallocatechin [(−)-GC], (−)-epicatechin gallate [(−)-ECG], (−)-catechin gallate [(−)-CG], (−)-epigallocatechin gallate [(−)-EGCG], and (−)-gallocatechin gallate [(−)-GCG] were purchased from Sigma-Aldrich Chemical Co.

Antioxidant Assay. Antioxidant assays were conducted by analysis of model liposome oxidation using fluorescence spectroscopy (*9*). The lipid, SLPC, and fluorescent probe, DPH-PA, were combined in DMF and dried at room temperature under vacuum. Large unilamellar liposomes (LUVs) were produced by resuspension of the lipid−probe mixture (0.15 M NaCl, 0.1 mM EDTA, and 0.01 M MOPS maintained over chelating resin Chelex 100) followed by 10 freeze−thaw cycles in a dry ice/EtOH bath and extrusion (29 times) through a 100 nm pore size membrane (Avestin Inc., Ottawa, ON, Canada). The final assay volume was 2 mL, consisting of 100 μL of HEPES buffer (50 mM HEPES and 50 mM Tris, pH 7.0), 200 μL of 1 M NaCl, 1.64 mL of N_2-sparged water, 20 μL of test sample or DMSO (control), and a 20 μL aliquot of liposome suspension. Peroxidation was initiated by addition of 20 μL of $FeCl_2 \cdot 4H_2O$ (0.5 mM) for positive controls and test samples. Fluorescence was measured at 384 nm and monitored at 0, 1, 3 min and every 3 min thereafter up to 21 min using a Turner model 450 digital fluorometer (Barnstead Thermolyne, Dubuque, IA). The decrease of relative fluorescence intensity with time indicated the

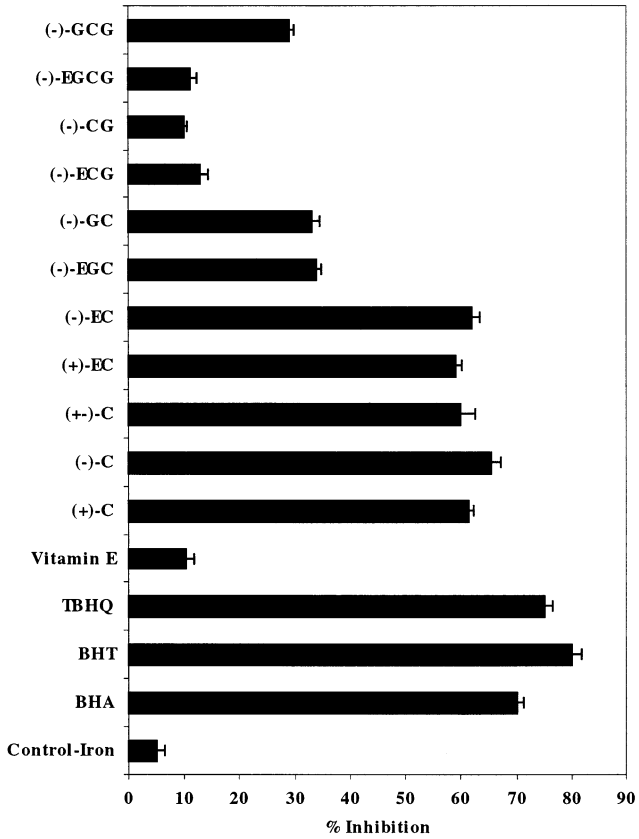


Figure 3. Effects of catechins, vitamin E, and synthetic antioxidants on rates of Fe(II)-induced peroxidation in the LUVs. The rate of peroxidation was monitored by a decrease in fluorescence intensity as a function of time. Relative fluorescence represents the fluorescence intensity at time = 21 min over the fluorescence intensity at time = 0 min. Results are expressed as the mean percent inhibition of triplicate measurements ± one standard deviation.

rate of peroxidation. Relative fluorescence (F_t/F_0) was calculated by dividing the fluorescence value at a given point (F_t) by that at $t = 0$ min (F_0). All compounds were tested in triplicate, and results are reported as the mean ± one standard deviation after 21 min of incubation (**Figures 3** and **4**).

RESULTS

The rates of Fe(II)-induced lipid peroxidation of the catechins, anthocyanidins, and anthocyanins are illustrated in **Figures 3** and **4**. The antioxidant efficacies of the test compounds were evaluated by their abilities to inhibit the fluorescent decay of the extrinsic probe DPH-PA. Antioxidant activities varied among the compounds tested, and distinct SAR were observed.

The presence of the 3′,4′,5′-trihydroxyl groups attached to the B-ring of the anthocyanidin skeleton elevated their ability to inhibit the rate of Fe(II)-induced lipid peroxidation. Delphinidin was the most active under these conditions and inhibited lipid oxidation by 70.3 ± 1.1%. The commercial synthetic antioxidants TBHQ, BHT, and BHA and vitamin E showed antioxidant activities of 75.2 ± 1.4, 80.1 ± 1.7, 70.0 ± 1.3, and 10.2 ± 1.5%, respectively. Cyanidin and pelargonidin with 3′,4′-dihydroxyl and 4′-hydroxyl groups, respectively, exhibited considerably less antioxidant activity with 60.1 ± 1.3 and 40.4 ± 0.8% inhibition, respectively. In contrast, the 3′,4′,5′-trihydroxyl groups on the B-ring of the flavan skeleton of the catechins diminished their inhibitory efficiency in comparison to those with 3′,4′-dihydroxyl groups. Hence, (−)-EGC (34.2 ± 1.2%) and (−)-GC (33.7 ± 1.6%) showed lower activities

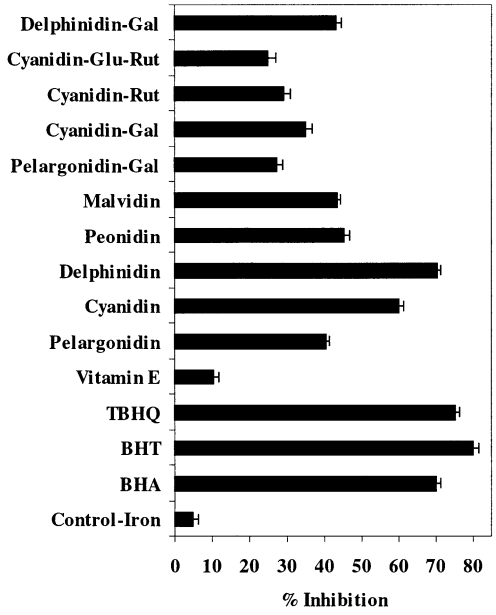


Figure 4. Effects of anthocyanidins and anthocyanins, vitamin E, and synthetic antioxidants on rates of Fe(II)-induced peroxidation in the LUVs. Results are expressed as the mean percent inhibition of triplicate measurements ± one standard deviation.

than (+)-C, (−)-C, (±)-C, (+)-EC, and (−)-EC (61.3 ± 0.98, 65.4 ± 1.8, 60.0 ± 1.3, 59.2 ± 1.7, and 62.5 ± 0.8%, respectively). Substitution of the hydroxyl groups of the B-ring with methoxyl groups also resulted in a decrease in antioxidant activity. The anthocyanidins malvidin (43.3 ± 0.9%) and peonidin (45.1 ± 1.5%), which have 3′,5′-dimethoxyl and 3′-methoxyl substituents, respectively, showed lesser antioxidant activities than their 3′,4′,5′-trihydroxylated counterpart, delphinidin (70.3 ± 1.1%).

From an SAR perspective, substitution at position 3 of ring C played a major role in the ability of catechins and anthocyanidins to inhibit the rate of Fe(II)-induced lipid peroxidation. A galloyl substituent at position 3 of the catechin moiety resulted in diminished inhibitory activities as observed for (−)-ECG, (−)-CG, and (−)-EGCG (13.0 ± 1.6, 10.3 ± 1.3, and 11.6 ± 0.75%, respectively). However, the antioxidant activity for (−)-GCG was higher at 29.1 ± 1.3%. Similarly, substitution of glycosidic moieties of the anthocyanidins at C-3 of ring C resulted in a decrease in antioxidant activity. This trend was evident in the superior activities of the anthocyanidins (aglycons) compared to their respective anthocyanins (glycosides). Also, the antioxidant activity decreased as the number of sugar residues increased. The monogalactosides of pelargonidin, cyanidin, and delphinidin showed antioxidant activities of 27.3 ± 1.4, 35.1 ± 1.6, and 43.6 ± 1.7%, respectively. Cyanidin 3-glucoserhamnose and cyanidin 3-glucoserhamnoseglucose inhibited lipid oxidation only by 29.3 ± 1.3 and 25.0 ± 0.75%, respectively.

DISCUSSION

Catechins and anthocyanidins are biogenetically derived from a common C-15 tetrahydroxychalcone precursor, naringenin, formed by a condensation reaction between 4-coumaroyl-coenzyme A and malonyl coenzyme A in the pivotal step of flavonoid biosynthesis (*11*). This is the first report of the comparative efficacy of these two major classes of flavonoids to inhibit metal ion-induced peroxidation by analysis of model liposome oxidation monitored by fluorescent decay. It is well

established that flavonoids act as antioxidants by a free radical scavenging mechanism to form less reactive flavonoid phenoxyl radicals (*12*, *13*). However, through their abilities to chelate transition metal ions, flavonoids can complex and inactivate iron ions, thus suppressing the superoxide-driven Fenton reactions that are thought to be the most important route to the formation of active oxygen species (*14*). Flavonoids are reported to be more effective at inhibiting Fe(II) and Fe(III) ion-induced peroxidations than free radical-induced peroxidations (*8*). Our aim was to measure and conduct SAR studies of the antioxidant activities of flavonoids, which are commonly consumed by humans, by a liposome model oxidation where peroxidation was initiated by the addition of Fe(II) ions. We have not investigated structural diversity in ring A of the flavonoids. It has been proposed that the A-ring has poor reactivity toward peroxyl radicals and is not a significant contributor to antioxidant activity (*13*, *15*).

The presence of hydroxyl groups on the B-ring of the flavonoids greatly influenced antioxidant activity as observed for all of the compounds of this study. Evidence for the highly significant contribution of the 3′,4′-dihydroxyphenyl (catechol) substitution pattern on the B-ring to antioxidant activity was provided by epicatechin and catechin (cis and trans isomers, respectively) and their various epimers. Similarly, cyanidin exhibited outstanding antioxidant activity. The *o*-dihydroxy structure of the B-ring is an important determinant for antioxidative potential because it confers a higher degree of stability to the flavonoid phenoxyl radicals by participating in electron delocalization (*16*). The antioxidant activity of flavonoids have also been reported to be governed by the position and number of hydroxyl groups on the B-ring (*8*, *13*). It should be noted that the conversion of the 3′,4′-dihydroxyphenyl B-ring to a 3′,4′,5′-trihydroxyphenyl ring increased activity for the anthocyanidins but reduced activity for the catechins. Hence, delphinidin was the most effective antioxidant in this system. EGC and GC, however, showed lower activities than their 3′,4′-dihydroxyl-substituted analogues. This could possibly be attributed to contributions of the flavylium ion of anthocyanidins to the overall stability of the flavonoid phenoxyl radicals when the *o*-dihydroxy structure is affected. It is noteworthy that cis−trans isomerization, epimerization, and racemization were not significant contributors to antioxidant activity in the case of the catechins.

Substitution of the hydroxyl groups of the B-ring with methoxyl groups also resulted in a decrease in antioxidant activity. The reduction potential of phenols is reported to be influenced by the presence of electron-donating or electron-withdrawing groups (*17*). The altered redox potential could affect the ability of the flavonoids to scavenge deleterious oxy radicals by limiting the lipid peroxidation reactions in which they can participate. The anthocyanidins malvidin and peonidin, which have 3′,5′-dimethoxyl and 3′-methoxyl substituents, respectively, showed significantly lower antioxidant activities than the 3′,4′,5′-trihydroxylated analogue, delphinidin.

We have also observed that antioxidant activity was influenced by susbstitution of the hydroxyl group at the C-3 position. Derivatization of this group could probably reduce the potential of these compounds to chelate metal ions and thus prevent iron-induced peroxidation. In addition, substitution of the hydroxyl group at this position could affect the planarity of the molecule, causing ring B to lose coplanarity to rings A and C (*8*). It has been determined that the torsion angle of ring B with the rest of the flavonoid molecule can be correlated with its overall scavenging activity (*14*). Substitution of the 3-OH group with a bulkier galloyl group or sugar residues could increase the torsion angle of ring B with the rest of the molecule, causing a loss of coplanarity and decreased conjugation. Hence, the anthocyanidins are better antioxidants than their corresponding glycosidic forms, the anthocyanins. For the cyanidin glycosides, the number of sugar residues seems to play an active role in antioxidant activity by decreasing activity with an increase in the number of sugar moieties. Also, a reduction in antioxidant activity was observed when a galloyl group was conjugated at position 3 of the catechins. However, an anomaly was observed in this trend for the activity of GCG as compared to ECG, CG, and EGCG. The slight increase in the activity of GCG compared to its epimer, EGCG, could probably be attributed to the change in stereochemistry.

In summary, our SAR studies have shown that a 3′,4′-dihydroxyphenyl B-ring is a critical criterion for antioxidant activity of catechins. However, for the anthocyanidins, activity increased with the number of hydroxyl groups on the B-ring, where the flavylium ion of ring C contributed to overall stabilization of the resulting phenoxyl radical. In addition, susbstitution of the B-ring hydroxyls with methoxyl groups significanty decreased antioxidant activity. Another important determinant of antioxidant activity was the conjugation of various substituents at position 3 of ring C, which decreased the antioxidant activity for all compounds. For anthocyanidins, antioxidant activity decreased as the number of sugar residues at position 3 increased. It is important to note that cis−trans isomerization, epimerization, and racemization did not influence the antioxidant activity among catechins. Anthocyanins, cyanidin, and catechins have significant popularity in the consumer market for their powerful antioxidant activity and their ability to reduce cardiovascular diseases. On the basis of our SAR studies, delphinidin, cyanidin, EC, and C may have useful biological applications as antioxidants.

LITERATURE CITED

(1) Hollman, P. C. H.; Katan, M. B. Health effects and bioavailability of dietary flavonols. *Free Radical Res*. **1999**, *31*, S75−S80.

(2) Ho, C.-T. Antioxidant properties of plant flavonoids. *Food Factors Cancer Prev. (Int. Conf.)* **1995**, 593−597.

(3) Lapidot, T.; Harel, S.; Granit, R.; Kanner, J. Bioavailibility of red wine anthocyanins as detected in human urine. *J. Agric. Food Chem*. **1998**, *46*, 4297−4302.

(4) Khokhar, S.; Magnusdottir, S. G. M. Total phenol, catechin, and caffeine contents of teas commonly consumed in the United Kingdom. *J. Agric. Food Chem*. **2002**, *50*, 565−570.

(5) Wang, H.; Nair, M. G.; Strasburg, G. M.; Chang, Y.-C.; Booren, A. M.; Gray, J. I.; Dewitt, D. L. Antioxidant and anti-inflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries. *J. Nat. Prod*. **1999**, *62*, 294−296.

(6) Seeram, N. P.; Momin, R. A.; Nair, M. G.; Bourquin, L. D. Cyclooxygenase inhibitory and antioxidant cyanidin glycosides in cherries and berries. *Phytomedicine* **2001**, *8*, 362−369.

(7) Pratt, D. E. Natural antioxidants from plant materials. *ACS Symp. Ser*. **1992**, *No. 507*, 54−71.

(8) Arora, A.; Nair, M. G.; Strasburg, G. M. Structure−activity relationships for antioxidant activities of a series of flavonoids in a liposomal system. *Free Radical Biol. Med*. **1998**, *24*, 1355−1363.

(9) Arora, A.; Strasburg, G. M. Development and validation of fluorescence spectroscopic assays to evaluate antioxidant efficacy. Application to metal chelators. *J. Am. Oil Chem. Soc*. **1997**, *74*, 1031−1040.

(10) Seeram, N. P.; Schutzki, R.; Chandra, A.; Nair, M. G. Characterization, quantification and bioactivities of anthocyanins in *Cornus* species. *J. Agric. Food Chem*. **2002**, *50*, 2519−2523.

(11) Strack, D.; Wray, V. The anthocyanins. In *The Flavonoids: Advances in Research since 1986*; Harborne, J. B., Ed.; Chapman and Hall: London, U.K., 1993; pp 1−19.
(12) Hanasaki, Y.; Ogawa, S.; Fukui, S. The correlation between active oxygen scavenging and antioxidative effects of flavonoids. *Free Radical Biol. Med.* **1994**, *13*, 211−219.
(13) Pietta, P.-G. Flavonoids as antioxidants. *J. Nat. Prod*. **2000**, *63*, 1035−1042.
(14) Afanas'ev, I. B.; Dorozhko, A. I.; Brodskii, A. V.; Kostyuk, V. A.; Potapovitch, A. I. Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem. Pharmacol*. **1989**, *38*, 1763−1769.
(15) Van Acker, S. A. B. E.; de Groot, M. J.; van den Berg, D.-J.; Tromp, M. N. J. L.; den Kelder, G. D.-O.; van der Vijgh, W. J. F.; Bast, A. A quantum chemical explanantion of the antioxidant activity of flavonoids. *Chem. Res. Toxicol*. **1996**, *9*, 1305−1312.
(16) Bors, W.; Heller, W.; Michel, C.; Saran, M. Flavonoids as antioxidants: Determination of radical scavenging efficiencies. *Methods Enzymol*. **1990**, *186*, 343−355.
(17) Steenken, S.; Neta, P. One-electron potentials of phenols. Hydroxy- and aminophenols and related compounds of biological interest. *J. Phys. Chem*. **1982**, *86*, 3661−3667.

Received for review May 14, 2002. Revised manuscript received June 27, 2002. Accepted July 19, 2002. Partial funding for this project was provided by the Agricultural Experimental Station and the Center for Plant Products and Technologies (CPPT) at Michigan State University.

JF025671Q