Antioxidant and Antiinflammatory Activities of Anthocyanins and Their Aglycon, Cyanidin, from Tart Cherries

Haibo Wang,[†] Muraleedharan G. Nair,*[,†] Gale M. Strasburg,[‡] Yu-Chen Chang,[‡] Alden M. Booren,[‡] J. Ian Gray,[‡] and David L. DeWitt[§]

Bioactive Natural Products Laboratory, Department of Horticulture and National Food Safety and Toxicology Center, Food Science and Human Nutrition, and Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824

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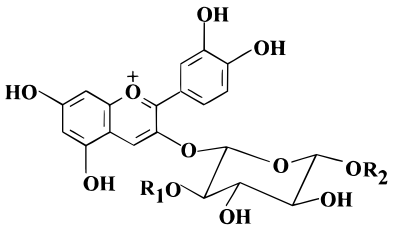
The anthocyanins (**1**−**3**) and cyanidin isolated from tart cherries exhibited in vitro antioxidant and antiinflammatory activities comparable to commercial products. The inhibition of lipid peroxidation of anthocyanins **1**−**3** and their aglycon, cyanidin, were 39, 70, 75, and 57%, respectively, at 2-mM concentrations. The antioxidant activities of **1-3** and cyanidin were comparable to the antioxidant activities of *tert*-butylhydroquinone and butylated hydroxytoluene and superior to vitamin E at 2-mM concentrations. In the antiinflammatory assay, cyanidin gave IC_{50} values of 90 and 60 mM, respectively, for prostaglandin H endoperoxide synthase-1 and prostaglandin H endoperoxide synthase-2 enzymes.

Public interest in phytoceuticals to inhibit chronic diseases and aging is gathering momentum. Reactive oxygen species such as hydroxyl (OH•) and peroxyl radicals (ROO•) and the superoxide anion (O_2•) are constantly produced as a result of metabolic reactions in living systems.[1] Living systems are protected from oxidative damage by these reactive species by enzymes such as superoxide dismutase and glutathione peroxidase and by antioxidant compounds such as ascorbic acid, tocopherols, and carotenoids.[2] However, when free-radical production exceeds the antioxidant capacity of the organism, these radical species attack lipids, proteins, and DNA, thus damaging structural integrity and function of cell membranes, enzymes, and genetic material.[3] A growing body of evidence indicates that various pathological conditions, including cardiovascular disease, arthritis, various cancers, and Alzheimer's disease, are associated, at least in part, with the damaging effects of uncontrolled free-radical production.[3]



1 R_1 = glucose, R_2 = rhamnose

2 R_1 = H, R_2 = rhamnose

3 R_1 = H, R_2 = H

Many foods contain nonnutritive components such as flavonoids and other phenolic compounds that may provide protection against chronic diseases through multiple effects, which are as yet poorly understood.[4] These compounds may act as antioxidants by reacting with free radicals and thus interrupting the propagation of new free radical species, or by chelating metal ions such as Fe^{2+}, which catalyze lipid oxidation to alter their redox potentials. In addition, it has been shown that antioxidant supplements can significantly improve certain immune responses.[5]

Consumption of cherries was reported to alleviate arthritic pain and gout,[6] although there is no evidence for its active components or mode of action. These beneficial effects may be partially associated with the abundance of anthocyanins, the glycosides of cyanidin. Anthocyanins have been investigated as antioxidant substances.[7] Several anthocyanins isolated from the seed coat of red beans inhibited lipid peroxidation.[8−10] Also, some of these anthocyanins were investigated for their antioxidant activity on human low-density lipoprotein.[11]

Tart cherries are now incorporated into meat products for improved nutritional qualities. Liu et al. reported that cooked low-fat ground beef with approximately 12% tart cherries showed less rancidity development.[12] Also, the addition of cherry tissue to ground beef before frying significantly inhibited the formation of heterocyclic aromatic amines (HAAs).[13] HAAs are dietary compounds that are formed naturally during the cooking of muscle foods and are thought to arise from reactions involving creatine or creatinine, sugars, and amino acids.[14] To evaluate the reported antioxidant activity and anecdotal health claims associated with tart cherries, we have studied the antioxidant[15] and antiinflammatory[16] efficacies of anthocyanin and its aglycon, cyanindin, isolated from tart cherries.

Results and Discussion

We have reported that 100 g of fresh and pitted cherries (ca. 20 cherries) contain 12.5−25.0 mg of anthocyanin, depending on the variety and seasonal variations.[17] The cherry anthocyanins were assayed for antioxidant activity using the method developed by Arora and Strasburg.[15] This assay is based on the reaction between a fluorescent probe incorporated with a phospholipid and free radicals generated by a pro-oxidant such as Fe^{2+}. As the reaction proceeds, the fluorescent probe will be degraded, resulting in the decline of fluorescent intensity. Therefore, in the presence of an antioxidant, the rate of fluorescence decay will be reduced. Our experiments indicated that antioxidant activities of anthocyanins **1**−**3** and of the aglycon, cyanidin, compared favorably with the commercial anti-

* To whom correspondence should be addressed: Tel: (517) 353-2915. Fax: (517) 432-2242. E-mail: nairm@pilot.msu.edu.
[†] Bioactive Natural Products Laborary.
[‡] Food Science and Human Nutrition.
[§] Department of Biochemsitry.

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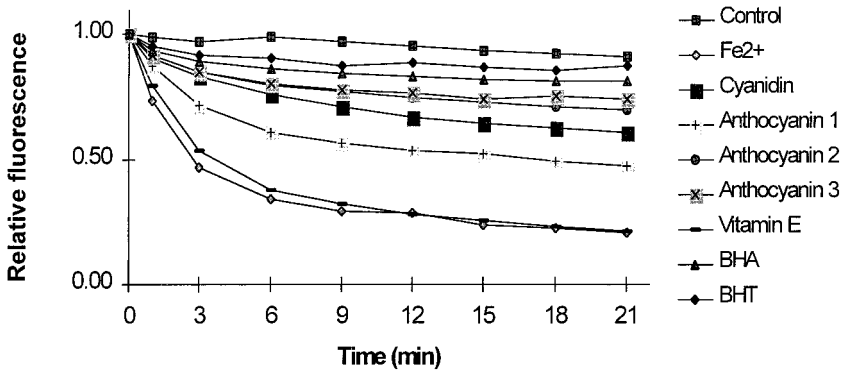

Figure 1. The antioxidant efficacy of anthocyanins and commercial antioxidants in a liposomal model system. Oxidation was initiated by the addition of ferrous ions. In the presence of test compounds, the rate of decay of fluorescence was decreased. Control samples contained no added Fe^{2+}, and Fe^{2+} contains no added test compounds. Other samples contained Fe^{2+} plus 2 μM of test compound.

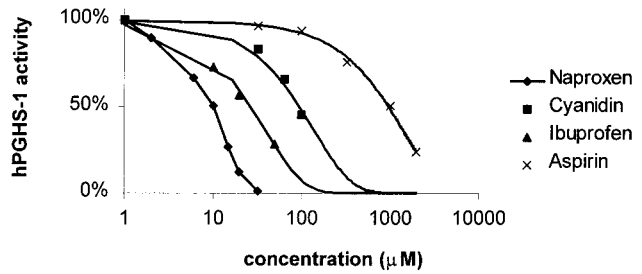

Figure 2. Dose-response curve for the inhibition of the human PGHS-1 enzyme by cyanidin. The antiinflammatory activity of cyanidin was estimated by its ability to inhibit the cyclooxygenase activity of the PGHS-1 enzyme. Cyanidin gave an IC_{50} value of 90 μM for PGHS-1 enzyme, while the NSAID aspirin, naproxen, and ibuprofen gave IC_{50} values of 1050, 11, and 25 μM, respectively.

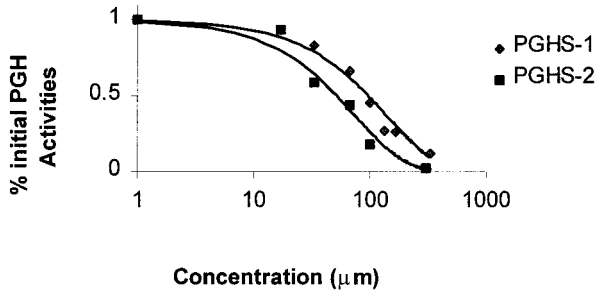

Figure 3. Dose-response curve for the inhibition of PGHS-1 and PGHS-2 enzymes by cyanidin. Cyanidin gave IC_{50} values of 90 and 60 mM for PGHS-1 and PGHS-2 enzymes, respectively.

oxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Figure 1). Also, these cherry compounds showed better antioxidant activity than α-tocopherol. At 2-mM concentrations, the extent of peroxidation of the sample containing α-tocopherol was indistinguishable from that of the Fe^{2+}-containing sample with no added antioxidant (Figure 1).

The aglycon of anthocyanins, cyanidin, has higher efficacy than its glycosides, suggesting that the antioxidant activity of anthocyanins is due to their aglycon moiety. Anthocyanins **1**−**3** contain 3, 2, and 1 sugar residues, respectively, which explains the lowest antioxidant activity observed for anthocyanin **1**. The number of sugar residues at the C_3 position seems to be very important for antioxidant activity. The smaller the number of sugar units at C_3, the higher the antioxidant activity. Also, it is reported that the stability of aryloxyl radical affected the antioxidant activities of compounds and may give rise to pro-oxidant effects.[18,19] Therefore, the antioxidant activity of cyanidin may depend on the stability of its aryloxyl radical. The *ortho*-dihydroxy substitution in the B ring of anthocyanins and cyanidin is important to stabilize the resulting free radical generated through the 3′ and 4′-OH moieties. Also, *ortho*-dihydroxy groups in anthocyanins have the potential to chelate metal ions and thus prevent iron-induced lipid peroxidation.

The antiinflammatory assays [16] were conducted using prostaglandin endoperoxide H synthase-1 and -2 isozymes (PGHS-1, and -2) and were based on their ability to convert arachidonic acid to prostaglandins (PGs). The positive controls used in this experiment were aspirin, naproxen, and ibuprofen. Aspirin gave an IC_{50} value of 1050 μM each against PGHS-1 and PGHS-2 enzymes (Figure 2). Naproxen and ibuprofen gave IC_{50} values of 11 and 25 mM against PGHS-1 enzyme, respectively (Figure 2). A preliminary experiment with the mixture containing anthocyanins **1**−**3** showed PGHS-1 and PGHS-2 activities at 33 ppm concentration. However, pure anthocyanins **1**−**3** showed little or no activity against PGHS-1 and PGHS-2 at 300-mM test concentrations. Higher concentrations of anthocyanins **1** and **2**, on the contrary, increased the activity of enzyme. This is probably due to the ability of anthocyanins **1** and **2** to act as oxygen carriers at high concentration and enhance the oxygen uptake. However, the aglycon cyanidin showed good PGHS-1 and -2 inhibitory activities, with IC_{50} values of 90 and 60 mM, respectively (Figures 2, 3). The ratio of IC_{50} values for PGHS-1 to PGSH-2 was about 0.56 (Figure 3).

For measurements of time-dependent inhibition of PGHS-2 enzyme activity by cyanidin, the enzyme was preincubated at 37 °C with 15 mM of cyanidin (one-fourth of the concentration of IC_{50}) and added to an oxygen electrode chamber with arachionic acid substrate to initiate the reaction. Our results suggest that the rate of inhibition of PGSH-2 did not change with time. Further experiments should be carried out to determine the arachidonic acid metabolites in order to understand the mechanism of action of anthocyanins and cyanidin on PGHS-1 and -2 enzymes.

The specific inhibition of the PGHS-2 enzyme will be a major advance in antiinflammatory therapy because it significantly reduces the adverse effects of nonsteriodal antiinflammatory drugs (NSAIDs).[20] It is generally believed that ulcerogenic and other adverse properties of NSAIDs result from the inhibition of PGHS-1, whereas the therapeutically desirable effects come from the inhibition of PGHS-2 enzyme.[21]

Our experiments with anthocyanins and cyanidin, isolated from tart cherries, indicate that they possess antioxidant activity comparable to commercial antioxidants. Similarly, cyanidin showed better antiinflammatory activity than aspirin in the inflammatory assays. The antioxidant and antiinflammatory properties of anthocyanins and

cyanidin suggest that consumption of cherries may have the potential to reduce cardiovascular or chronic diseases in humans.

Experimental Section

General Experimental Procedures. Arachidonic acid and a microsomal fraction of ram seminal vesicles containing PGHS-1 enzyme suspended in 100 mM Tris pH 7.8 and 300 μM diethyldithiocarbamic acid (DDC) as a preservative were purchased from Oxford Biomedical Research (Oxford, MI). Recombinant human PGHS-2 enzyme was initially obtained from Dr. David Dewitt (Department of Biochemistry, Michigan State University) and then purchased from Oxford Biomedical Research (Oxford, MI). Naproxen, ibuprofen, and hemoglobin were purchased from Sigma Chemical Co. (St. Louis, MO). Anthocyanins **1**−**3** were purified from Balaton tart cherry by HPLC and were identified by ^{1}H and ^{13}C NMR spectral data.[17]

Cyanidin. The anthocyanin mixture containing **1**−**3** (500 mg) was stirred with 3N HCl (20 mL) at 80 °C for 10 h. The reaction mixture was purified on a XAD-4 column as in the preparation of anthocyanins.[17] The MeOH solution of cyanidin was evaporated to dryness to yield a red amorphous powder (190 mg) and stored at −30 °C until use.

Antioxidant Assay. The buffers were stored in Chelex 100 to remove metal ions. A mixture containing 5 μM of 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine (Avanti Polar Lipids, Inc., Alabaster, AL) and 15 nM of the fluorescence probe 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl] phenylpropionic acid (Molecular Probes, Inc., Eugene, OR) was dried under vacuum. The resulting film was suspended in 500 μL of buffer (NaCl, 0.15 M; EDTA 0.1 mM; MOPS 10 mM) and was then subjected to 10 freeze−thaw cycles in an EtOH−dry ice bath. The suspension was then passed 29 times through a polycarbonate membrane with a pore size of 100 nm using a LiposoFast extruder (Avestin, Inc., Ottawa, Canada). The resulting liposomes (200 nM) were then suspended in 2 mL of buffer (100 mM NaCl, 50 mM HEPES, pH 7.0). The peroxidation was initiated by the addition of 4 nM of Fe^{2+}. Anthocyanins, BHT, propyl gallate, and α-tocopherol (vitamin E) were tested at 2-μM concentration. Control samples contained no added Fe^{2+} or test compound. Fluorescent intensity of lipid suspension was monitored 21 min with or without test compounds, immediately followed by the addition of Fe^{2+}, using a SLM 4800 spectrofluorometer (SLM Instruments, Urbana, IL). The values of relative fluorescence were determined by dividing the fluorescence value at a given time point by that at $t = 0$ min.[15]

Antiinflammatory Assay. Cyclooxygenase activities were measured by using PGHS-1 enzyme vesicles (ca. 5 mg protein/mL in 0.1 M TrisHCl, pH 7.8), a homogeneous protein purified from ram seminal. Assays were performed at 37 °C by monitoring the initial rate of O_2 uptake using an O_2 electrode (Yellow Springs Instrument Inc., Yellow Springs, OH). Each assay mixture contained 3 mL of 0.1M Tris HCl, pH adjusted to 7 by the addition of 6M HCl, 1 mM phenol, 85 μg hemoglobin, and 10 μM of arachidonic acid. Reactions were initiated by the addition of 5−25 μg of microsomal protein in a volume of 15−50 μL. Instantaneous inhibition of enzyme activity was determined by measuring the cyclooxygenase activity initiated by adding aliquots of microsomal suspensions of PGHS-1 or PGHS-2 (10 μM O_2/min cyclooxygenase activity/aliquot) to assay mixtures containing 10 μM arachidonate and various concentrations of the test substances (10−1100 μM). The IC_{50} values represent the concentrations of the test compound that gave half-maximal activity under the standard assay conditions.

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