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Cultivars of Apple Fruits That Are Not Marketed with Potential for Anthocyanin Production

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The red coloration of apple skin is mainly due to anthocyanins that are reported to possess health benefits. The aim of the present study was to determine the anthocyanin content in three underutilized *Malus pumila* Mill cultivars, Cranberry, Kerr, and Niedzwetzkyana, and confirm their anti-inflammatory and antioxidant activities. Our analysis revealed that the three cultivars studied contained primarily cyanidin-3-*O*-glucosyl rutinoside (**1**) at >99%. The anthocyanin was purified by C-18 medium pressure liquid chromatography and characterized by NMR spectral methods. The quantification of anthocyanins in *M. pumila* cultivars revealed that Cranberry, Kerr, and Niedzwetzkyana contained 1.12, 0.55, and 0.36 mg/g of fresh weight of **1**, respectively. The lipid peroxidation (LPO) and cyclooxygenase enzyme (COX) inhibitory activities of **1** in water were compared with the activities of cyanidin-3-*O*-rutinoside (**2**) and cyanidin-3-*O*-glucoside (**3**) found in cherries and berries. There is a significant increase in LPO and COX enzyme-inhibitory activities of anthocyanin when tested in water compared to using dimethylsulfoxide as the carrier. The LPO inhibition of anthocyanins **1**, **2**, and **3** were 53.3, 68.3, and 87.9, respectively, at a 0.25 μM concentration. They inhibited the COX-1 enzyme by 42.7, 45.2, and 50.4 and COX-2 by 52.7, 61.5, and 68.5, respectively, at 5 μM. The LPO inhibitory values for commercial standards, BHA, BHT, and TBHQ, were 85, 89, and 94%, respectively at 1 μM. Similarly, positive controls aspirin, celecoxib, and robecoxib inhibited COX-1 and -2 enzymes by 68.6, 40.7, and 0% and 26.6, 72.2, and 92.4%, respectively, at 60, 26, and 32 nM.

KEYWORDS: Apple cultivars; anthocyanin; anti-inflammatory; antioxidant; cyanidin glycoside

INTRODUCTION

A diet containing apples is considered to be beneficial to maintain good health. In addition, most apple varieties have a relatively high content of polyphenolic compounds. The genus *Malus* is comprised of about 30–35 species of small deciduous trees or shrubs in the family Rosaceae. The domesticated orchard or table apple, *Malus x. domestica* Borkh., is considered to be a complex interspecific hybrid. The main ancestor is thought to be *M. sieversii* (*1*, *2*) along with other ancestors, those being *M. sylvestris*, *M. pumila*, and *M. dasyphylla* (*3*). Most of the *Malus* species are generally known as "wild apples", "crab apples", "crab apples", or "crabs", names derived from their typically small and tart fruit (*4*, *5*). Crabapples are widely used in jams and jellies and have also been utilized in the preparation of beverages (*6*, *7*). The genus *Malus* is native to the temperate zones of the northern hemisphere, Europe, Asia, and North America.

The *Malus pumila* Mill. cv. Niedzwetzkyana produces relatively large (>400 g) fruits that show red coloration in both the skin and flesh. It is relatively nonjuicy and oxidizes easily (*8*). Niedzwetzkyana has been widely used as a parent for the development of red-fleshed novelty apples, including the popular ornamental cultivars Redflesh and Redfield. *Malus pumila* x. cv. Cranberry, developed in North Dakota from a Redflesh × Dolgo cross, a crabapple-type fruit with dark red skin and flesh, is excellent for making jelly. Another variety, *Malus pumila* x. cv. Kerr, also known as Morden 352, was developed in Alberta, Canada, by crossing Dolgo with Haralson. The fruits produced by this variety accumulate extremely high levels of anthocyanin in the skin and are purplish to nearly black when ripe. The flesh of this cultivar is yellow flecked with dark red, very juicy, sweet, and resistant to browning (*9*).

Apples contain several health-beneficial constituents including dietary fiber, sugars, vitamins, and phenolic compounds (*5*). The red-skin apple is reported to contain anthocyanins such as cyanidin-3-*O*-galactoside, cyanidin-3-*O*-arabinoside, cyanidin-

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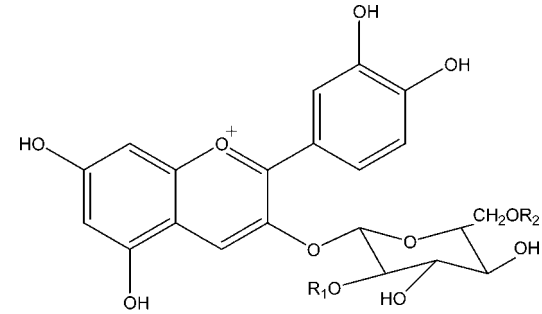
7-*O*-arabinoside, cyanidin-3-*O*-rutinoside, cyanidin-3-*O*-xyloside, and cyanidin-3-*O*-glucose (*10–15*). The intensity of anthocyanins in apples is very important since it impacts the market value. Bioactive phytochemicals, especially anthocyanins, in fruits and vegetables have been shown to be of great interest to consumers and researchers alike due to their wide array of health benefits (*5*). The consumption of fruits and vegetables containing anthocyanins has been shown to be effective in the prevention of chronic diseases (*14*). Food enriched with anthocyanins is being perceived as a desirable functional food. The dietary anthocyanin pigments may have preventive and therapeutic roles in a number of human diseases. Research reports suggest that anthocyanins are not only nontoxic and nonmutagenic but possess antioxidant (*16, 17*), anti-inflammatory (*17–19*), anticarcinogenic, and antidiabetic (*20–22*) properties.

In our ongoing efforts to produce bioactive anthocyanins, we have studied fruits from the not-so-popular *M. pumila* Mill. cultivars Cranberry, Kerr, and Niedzwetzkyana. Apples from these varieties are not marketed for consumption. The red flesh seen in Niedzwezkyana and Cranberry suggests that these varieties may represent an unusually rich source of anthocyanins. Therefore, understanding the concentration, nature, and bioactivity of the anthocyanins present in these apple cultivars is critical in order to develop them for the production of anthocyanins. The present work describes the isolation, characterization, and quantification of anthocyanin in these cultivars of apples for the first time. In addition, we report the lipid peroxidation (LPO) and cyclooxygenase (COX) enzyme-inhibitory activities for aqueous solutions of three cyanidin glycosides, which include cyanidin-3-*O*-glucosyl rutinoside, the only anthocyanin present in these varieties.

MATERIALS AND METHODS

General Experimental. The NMR (^{1}H and ^{13}C) experiments were recorded on Varian INOVA (300 MHz) and VRX (500 MHz) instruments. The chemical shifts were measured in a CD_3OD/DCl solution and are expressed in δ (parts per million). Samples were homogenized using a Kinematica CH-6010 (Roxdale, ON, Canada) homogenizer and centrifuged (model RC5C, Sorvall Instruments, Hoffman Estates, IL) at 10 000*g* for 20 min at 4 °C. The fractionation of anthocyanin was carried out on an XAD-2 column (500 g, amberlite resin, mesh size 20–50; Sigma Chemical Co., St. Louis, MO) and purified on a C-18 MPLC column (350 × 40 mm). The quantification of anthocyanin was carried out on a Waters 2010 high-performance liquid chromatography (HPLC) system (Waters Corp.) equipped with Empower Software, a Shodex degasser, an auto sampler (Waters 717), a photodiode array detector (Waters 996), and a Capcell Pak (Dichrome, Santa Clara, CA) C-18 column (150 × 4.6 mm i.d.; 5 μm particle size). ACS-grade solvents were used for isolation and purification. *tert*-Butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), aspirin, and 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide were purchased from Sigma-Aldrich Chemical Company Co. (St. Louis, MO). Aspirin, celecoxib (Celeberex), and robecoxib (Vioxx) were used as positive controls in a COX enzyme-inhibitory assay. Celebrex and Vioxx were provided by Dr. Subash Gupta, Sparrow Hospital, MI. The COX-1 enzyme was prepared from ram seminal vesicles purchased from Oxford Biomedical Research, Inc. (Oxford, MI). The COX-2 enzyme was prepared from prostaglandin endoperoxide H synthase-2 cloned insect lysate.

Apple Fruits. Ripe fruits of *Malus pumila* Mill. cv. Cranberry (Accession PI589180), *Malus pumila* Mill. cv. Kerr (Accession PI588866), and *M. pumila* Mill. cv. Niedzwetzkyana (Accession PI589857) were collected from the United States Department of Agriculture Plant Genetic Resources *Malus* germplasm collection in Geneva, New York.



	R_1	R_2
1	Glu	Rha
2	H	Rha
3	H	H

Figure 1. Structures of anthocyanins **1**, **2**, and **3**.

Preparation of Samples for HPLC Analysis. *Malus* hybrid cv. Cranberry (15.4 g), *Malus* hybrid cv. Kerr (48.6 g), and *Malus* hybrid cv. Niedzwetzkyana (86.3 g) fruits were weighed and homogenized separately with methanol (1% HCl, g/2 mL) for 10 min and centrifuged (model RC5C, Sorvall Instruments, Hoffman Estates, IL) at 10 000*g* for 20 min at 4 °C. The supernatants were filtered through a 0.2 μm filter disc and analyzed by HPLC on the same day.

Isolation and Characterization of Anthocyanin. The apple variety used for the extraction of anthocyanin was *M. pumila* Mill. cv. Niedzwetzkyana. The fresh fruits (300 g) were blended with acidic methanol (1% HCl, 3 × 500 mL) for 2 min at room temperature and centrifuged for 20 min at 4 °C. The residue was extracted further with acidic methanol (500 mL). The combined supernatants were concentrated at reduced pressure (50 mbar) and at 35 °C. The resulting concentrate was then partitioned with ethyl acetate (3 × 200 mL). The aqueous methanol fraction was concentrated to dryness (25.4 g). A part of this crude anthocyanin extract (20 g) was dissolved in 100 mL of water and fractionated on an XAD-2 column (35 × 6 cm, Amberlite resin, 875 mL, mesh size 20–50; Sigma Chemical Co., St. Louis, MO) as per published work from our laboratory (*23*). The column was eluted with water (2 × 1 L), and the water fraction was discarded. The adsorbed anthocyanin was then eluted with methanol (2 × 500 mL) and concentrated at reduced pressure to yield a dark red residue (1.4 g; **Figure 1**). It was purified further with a C-18 MPLC column (350 × 40 mm) using water/methanol (1% HCl) as the mobile phase under gradient conditions, starting with 80% H_2O. Fraction I (500 mL) was discarded since it did not contain anthocyanin, as confirmed by HPLC. Fractions II (500 mL, 9.8 mg) and III (500 mL, 940 mg) were collected when the H_2O/CH_3OH gradient was at 75:25 and 65:35 (v/v). Both of these fractions contained anthocyanin, as confirmed by HPLC. Also, the HPLC and NMR analyses confirmed that fraction II contained pure cyanidin-3-*O*-glucosyl rutinoside (**1**) (*24*). Fraction III was stored at –20 °C as a source of cyanidin-3-*O*-glucosyl rutinoside.

Quantification of Cyanidin-3-*O*-glucosyl Rutinoside. Anthocyanin in *M. pumila* Mill. cultivars Cranberry, Kerr, and Niedzwetzkyana was quantified by HPLC according to the method published from our laboratory (*25*). The quantification was performed on a Capcell Pak (Dichrom, Santa Clara, CA) C-18 column (150 × 4.6 mm i.d.; 5 μm particle size) maintained at 25 °C. A gradient solvent system was used consisting of solvents A [water–trifluoroacetic acid (TFA) 99.99:0.1 v/v] and B (water–acetonitrile–acetic acid–TFA, 50.4, 48.5, 1, and 0.1%, respectively). The linear gradient began at 80% A and 20% B, reached 40% A and 60% B in 26 min, and then returned to the initial conditions of 80% A and 20% B in 30 min, where it remained for 10 min. Under these gradient conditions, all anthocyanins elute before reaching a 30 min interval. In between injections, the column was equilibrated for 10 min. The flow rate was 1 mL/min. The injection volume for all samples was 25 μL, and detection of the anthocyanins was performed at 520 nm. Pure cyanidin-3-*O*-glucosyl rutinoside (**1**), isolated from the *Malus* cultivar Niedzwetzkyana, was weighed (0.8 mg) and dissolved in acidic methanol. The stock solutions were diluted with acidic methanol to yield 0.40, 0.20, 0.10, 0.05, 0.025, and 0.0125 mg/mL concentrations, respectively, and analyzed in triplicate. The

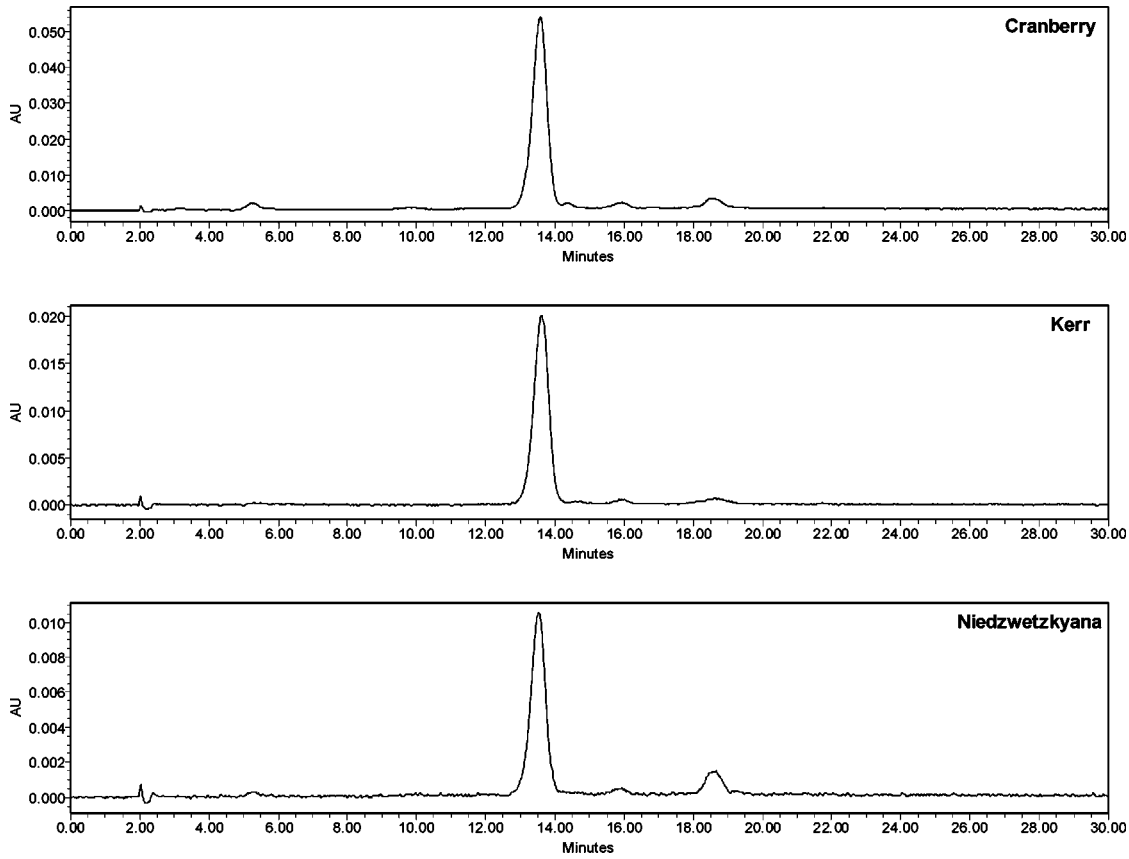


Figure 2. HPLC profile of anthocyanin in three cultivars of apples *Malus* hybrid cv. Cranberry, *Malus* hybrid cv. Kerr, and *Malus pumila* Mill. cv. Niedzwetzkyana.

experiment was replicated. Calibration curves were obtained by plotting the average of the 10 mean peak areas of triplicate injections of each standard against concentrations. The extracts from cultivars were also analyzed in triplicate at two different concentrations, and mean peak areas were used for the quantification of cyanidin-3-*O*-glucosyl rutinoside in each extract.

Lipid Peroxidation Inhibitory Assay. The anthocyanins were tested in vitro for their ability to inhibit the oxidation of large unilamellar vesicles (LUVs). The assay was conducted in a buffer consisting of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (100 μL), NaCl (200 μL), N_2-sparged water (1.64 mL), a test sample (20 μL) in water, and a LUV (20 μL) suspension. The peroxidation was initiated by the addition of an $FeCl_2$ (20 μL, 0.5 mM) solution and was monitored by observing the fluorescence at 0, 1, 3, 6, 9, 12, 15, 18, and 21 min using a Turner model 450 digital fluorometer (Barnestead Thermolyne, Dubuque, IA) at 384 nm. The decrease in relative fluorescence intensity with time indicated the rate of lipid peroxidation. All compounds were tested at 0.25 μM. The antioxidant standards BHA, BHT, and TBHQ were tested at 10 μM. Inhibition of lipid peroxidation by anthocyanins was reported earlier from our laboratory in dimethylsulfoxide (DMSO) solutions (*26*). In the present study, we have performed the assay of anthocyanin in aqueous solutions.

Cyclooxygenase Inhibitory Assay. COX activities of anthocyanins were assessed by monitoring the initial rate of O_2 uptake using a micro-oxygen chamber and electrode (Instech Laboratories, Plymouth Meetings, PA) attached to a YSI model 5300 biological oxygen monitor (Yellow Springs Instrument, Inc., Yellow Springs, OH) at 37 °C. The assay was conducted according to the previously reported procedure (*26*). The test samples and controls were dissolved in water. Each assay mixture contained a tris(hydroxymethyl)aminomethan buffer (0.6 mL, 0.1 M, pH 7), phenol (1 mM), hemoglobin (85 μg), and water or test samples (10 μL). The COX-1 or COX-2 enzyme (10 μL) was added to the chamber and incubated for 3 min. The reaction was initiated by the addition of arachidonic acid (10 μL of a 1 mg/mL solution). A duplicate analysis was performed for each sample, and the standard deviation was calculated for $n = 2$. The data were recorded using the QuickLog for Windows data acquisition and control software (Strawberry Tree, Inc., Sunnyvale, CA). Commercial anti-inflammatory drugs aspirin, celecoxib, and robecoxib were tested at 60, 26, and 32 nM concentrations, respectively. Compounds were tested at a 5 μM concentration. COX enzyme-inhibitory activities of anthocyanins for DMSO solutions were reported earlier from our laboratory (*26*).

RESULTS AND DISCUSSION

Prior to the isolation of anthocyanin for characterization, apple cultivars Cranberry, Kerr, and Niedzwetzkyana were analyzed by HPLC under identical conditions. The data revealed that the cultivars studied showed identical anthocyanin profiles (**Figure 2**) and indicated that they contained only one predominant anthocyanin, which was at >99%. In order to quantify the anthocyanin content in these apple varieties, a standard solution of pure cyanidin-3-*O*-glucosyl rutinoside was prepared and serially diluted. The solutions were analyzed by HPLC in triplicate. A calibration curve was then prepared by plotting the mean peak area of anthocyanin against the concentration. The analysis yielded a linear curve with an R^2 value of 0.99. The quantification data suggested that the cyanidin-3-*O*-glucosyl rutinoside content in the cultivars studied varied significantly. For example, *Malus* cv. Cranberry showed the highest amount of cyanidin-3-*O*-glucosyl rutinoside (1.12 mg/g fresh wt), whereas Kerr and Niedzwetzkyana contained 0.55 and 0.36 mg/g of fresh weight of anthocyanin, respectively (**Figure 3**).

Isolation of the pure anthocyanin was carried out first by adsorbing the methanol-soluble fraction on an Amberlite XAD-2 column according to a previously published procedure (*23*). The anthocyanin was characterized as cyanidin-3-*O*-glucosyl rutinoside by comparison of its NMR and mass spectral data to the published spectral data of cyanidin-3-*O*-glucosyl rutinoside

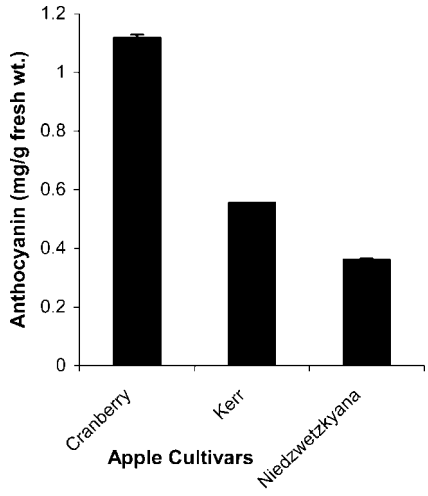


Figure 3. Relative amounts of cyanidin-3-*O*-glucosyl rutinoside (mg/g fresh wt) in three cultivars of apple fruits, *Malus* hybrid cv. Cranberry, *Malus* hybrid cv. Kerr, and *Malus pumila* Mill. cv. Niedzwetzkyana.

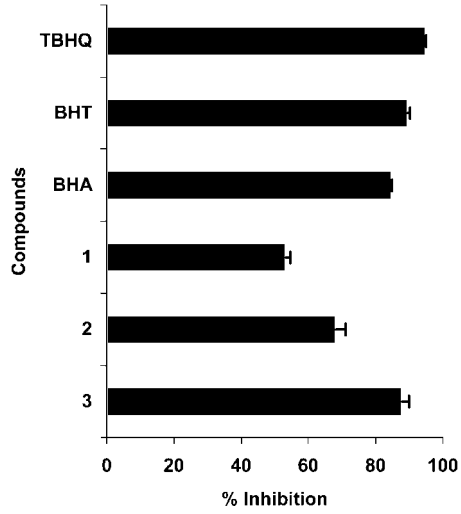


Figure 4. Antioxidant activities of anthocyanins **1**, **2**, and **3** when tested at a 0.25 μM concentration in H_2O. Commercial antioxidants BHA, BHT, and TBHQ were used as positive controls and tested at a 10 μM concentration. Positive controls inhibited lipid peroxidation by $\leq$100% at this concentration. Lipid peroxidation was initiated by Fe^{2+}, and the rate of decrease in fluorescence intensity was monitored over 21 min. The percent inhibition was calculated with respect to solvent controls (DMSO), and H_2O values represent mean $\pm$ SD ($n = 2$).

(**Figure 1**) (*24*). This is the first report of the isolation of cyanidin 3-*O*-glucosyl rutinoside from an apple variety.

We have reported LPO and COX enzyme–inhibitory activities of cyanidin-3-*O*-glucosyl rutinoside, cyanidin-3-*O*-rutinoside and cyanidin-3-*O*-glucoside, present in sweet and tart cherries, for DMSO solutions (*17*, *18*, *25*). We have observed that dissolving anthocyanins in DMSO cause a color change of anthocyanins to blue. This indicated that the anthocyanins are either forming a complex with DMSO or the solvent is affecting the stability of anthocyanins. Therefore, we have determined the LPO and COX enzyme activities in water for cyanidin-3-*O*-glucosyl rutinoside (**1**) from apple varieties along with the anthocyanins (**2** and **3**) isolated from cherries (**Figures 4** and **5**). The anthocyanins **1**–**3** were compared for their LPO activity by using an iron-catalyzed liposome model as per the published procedure from our laboratory (*22*). The cyanidin-3-*O*-glucosyl rutinoside (**1**) inhibited LPO by 53.3% at a 0.25 μM concentration, while anthocyanins **2** and **3** were inhibited by 68.3 and 87.9%,

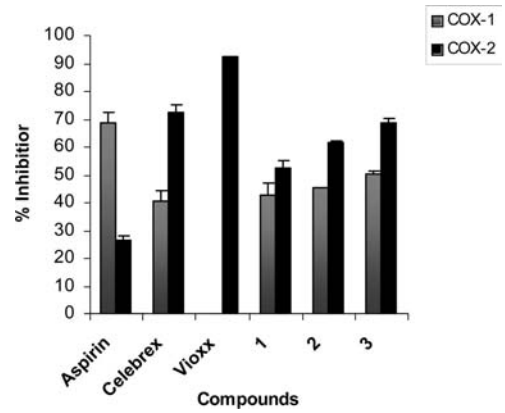


Figure 5. COX-1 and -2 enzyme-inhibitory activities of anthocyanins **1**, **2**, and **3**, at a concentration of 5 μM. Commercial standards aspirin, Celebrex, and Vioxx were used as positive controls and tested at 60, 26, and 32 nM concentrations, respectively. These drugs inhibit the COX enzymes at different levels at an identical concentration. This explains the different concentrations of the drugs tested in the assay and was done to obtain the inhibition at $\geq$50%. DMSO and H_2O were used as solvent controls. Percent inhibition was calculated with respect to DMSO and H_2O controls. Vertical bars represent the average of two experiments $\pm$ SD.

respectively (**Figure 4**). In earlier reports, these anthocyanins were tested for LPO inhibitory activity in DMSO (*28*). Interestingly, the activity was increased 4-fold for a water solution when compared with the activity observed for DMSO solutions. Also, the results indicated that the activity increased with a decreasing number of sugar units.

The COX enzyme-inhibitory activity of apple anthocyanin was carried out by using COX-1 and -2 enzymes (*29*, *30*). The assay is based on the ability of the enzymes to covert arachidonic acid to prostaglandin, which evokes the physiological response of the inflammation. Anthocyanins **1**–**3** were assayed in water for their COX inhibitory activity as in the case of the LPO assay. Anthocyanins **1**–**3** inhibited the COX-1 enzyme by 42.7, 45.2, and 50.4%, and COX-2 did so by 52.7, 61.5, and 68.5%, respectively, at 5 μM (**Figure 5**). The nonsteroidal anti-inflammatory drugs aspirin, Celeberex, and Vioxx were used as positive controls, at 60, 26, and 32 nM concentrations, respectively. The activity profile was similar to the LPO inhibitory activity by showing the highest activity for cyanidin-3-*O*-glucoside. However, the result indicated that there was a significant increase in the COX enzyme-inhibitory activity for anthocyanins dissolved in water (**Figure 5**) compared to the activity for DMSO solutions reported earlier from our laboratory (*25*).

Consumption of fruits and vegetables has been implicated in the prevention of chronic diseases. These benefits are often attributed mainly to their high antioxidant content. Apples are one of the primary sources of polyphenolic compounds in European and North American diets (*15*). Anthocyanin accumulation in apples is an important determinant of fruit quality related to the presence of bioactive anthocyanins. The pigments provide apple cultivars an added value since consumers are interested in the health attributes of the anthocyanins in the fruit they consume (*27*). Our in vitro bioassay results illustrated that *Malus* cv. Cranberry was the best variety among the three studied for biologically active anthocyanin, cyanidin-3-*O*-glucosyl rutinoside. On the basis of our earlier reports on anthocyanin content in cherries and berries, the apple cultivar Cranberry provided a higher anthocyanin content per gram of

fresh weight of the fruit when compared to per gram fresh weight of cherries (*16–18*, *24*).

Our results also indicated that the apple cultivar *M. pumila* Mill. cv. Cranberry is a significant source for anthocyanin production. Although this cultivar is not marketed along with other cultivars of apples, it has the potential to be commercially grown for its high anthocyanin value. It is highly recommended that these fruits should be marketed since they contain high amounts of health-beneficial anthocyanins along with other commercial apples in the market.

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