JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. 2009, 57, 1239–1246 1239

Anthocyanin Content, Lipid Peroxidation and Cyclooxygenase Enzyme Inhibitory Activities of Sweet and Sour Cherries

VANISREE MULABAGAL,[†] GREGORY A. LANG,[‡] DAVID L. DEWITT,[§] SANJEEV S. DALAVOY,[†] AND MURALEEDHARAN G. NAIR*[,†]

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

Cherries contain bioactive anthocyanins that are reported to possess antioxidant, anti-inflammatory, anticancer, antidiabetic and antiobese properties. The present study revealed that red sweet cherries contained cyanidin-3-*O*-rutinoside as major anthocyanin (>95%). The sweet cherry cultivar “Kordia” (aka “Attika”) showed the highest cyanidin-3-*O*-rutinoside content, 185 mg/100 g fresh weight. The red sweet cherries “Regina” and “Skeena” were similar to “Kordia”, yielding cyanidin-3-*O*-rutinoside at 159 and 134 mg/100 g fresh weight, respectively. The yields of cyanidin-3-*O*-glucosylrutinoside and cyanidin-3-*O*-rutinoside were 57 and 19 mg/100 g fresh weight in “Balaton” and 21 and 6.2 mg/100 g fresh weight in “Montmorency”, respectively, in addition to minor quantities of cyanidin-3-*O*-glucoside. The water extracts of “Kordia”, “Regina”, “Glacier” and “Skeena” sweet cherries gave 89, 80, 80 and 70% of lipid peroxidation (LPO) inhibition, whereas extracts of “Balaton” and “Montmorency” were in the range of 38 to 58% at 250 μg/mL. Methanol and ethyl acetate extracts of the yellow sweet cherry “Rainier” containing β-carotene, ursolic, coumaric, ferulic and cafeic acids inhibited LPO by 78 and 79%, respectively, at 250 μg/mL. In the cyclooxygenase (COX) enzyme inhibitory assay, the red sweet cherry water extracts inhibited the enzymes by 80 to 95% at 250 μg/mL. However, the methanol and ethyl acetate extracts of “Rainier” and “Gold” were the most active against COX-1 and -2 enzymes. Water extracts of “Balaton” and “Montmorency” inhibited COX-1 and -2 enzymes by 84, and 91 and 77, and 87%, respectively, at 250 μg/mL.

KEYWORDS: ***Prunus avium*; *Prunus cerasus*; antioxidant; anti-inflammatory**

INTRODUCTION

Cherries and berries are rich sources of dietary phenolics that are associated with a wide spectrum of health benefits (*1−3*). Both sweet (*Prunus avium* L.) and sour (*Prunus cerasus* L.) cherries are popular fruit crops in the United States. They are also economically and nutritionally important crops worldwide and are grown in more than 45 countries (*4*). The worldwide production of sweet cherries (1.86 million metric tons) is about 50% higher than that of sour cherries. Similarly, the crop value of sweet cherry ($1.44 billion) is about 135% higher than that for sour cherries (*5*). In the USA, Michigan ranks first and fourth, respectively, in sour and sweet cherry production.

Sour cherry, grown primarily for processing (frozen, brined, canned, dried, etc.), in the U.S. is dominated by the red-skinned, clear juice variety “Montmorency”, although there has been recent interest in the red-juiced variety “Balaton”. Sweet cherries, cultivars of which can be red- or yellow-fleshed, are grown for both processed and fresh markets. Most red-fleshed sweet cherries have a dark-red flesh, juice, and skin. Yellow-fleshed sweet cherry varieties may have a totally yellow flesh and skin with clear juice (e.g., “Gold” and “Nugent”) or a yellow flesh, clear juice, and yellow skin that becomes blushed with varying levels of red pigment, depending upon exposure to light (e.g., “Rainier”, “Emperor Francis”, “Napoleon”).

A number of sweet cherry cultivars are reported to contain cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, cyanidin-3-*O*-sophoroside, pelargonidin-3-*O*-glucoside, pelargonidin-3-*O*-rutinoside, peonidin-3-*O*-glucoside and peonidin-3-*O*-rutinoside (*6−8*). Similarly, sour cherries are reported to contain cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, cyanidin-3-*O*-glucosylrutinoside, cyanidin-3-*O*-sophoroside, pelargonidin-3-*O*-glucoside, peonidin-3-*O*-rutinoside and cyanidin-3-*O*-arabinosy lrutinoside (*7−10*). The anecdotal health benefits of anthocyanins continue to prompt producers to add more value to fruits

* Author to whom correspondence should be addressed. E-mail: nairm@msu.edu. Fax: (517) 432 2310.

[†] Bioactive Natural Products and Phytoceuticals, Department of Horticulture and National Food Safety and Toxicology Center.

[‡] Department of Horticulture.

[§] Department of Biochemistry and Molecular Biology.

10.1021/jf8032039 CCC: $40.75 © 2009 American Chemical Society
Published on Web 01/30/2009

Table 1. Sweet and Sour Cherry Fruits Used for Anthocyanin Content, LPO and COX Enzyme Inhibitory Studies

species	fruit type	cultivar
Prunus avium	red sweet cherry	Benton
		BlackGold
		Glacier
		Hedelfingen
		Kiona
		Kordia
		Kristin
		Regina
		Selah
		Skeena
	yellow sweet cherry	Gold
		Rainier[a]
Prunus cerasus	red sour cherry	Balaton
		Montmorency

[a] Cherries grown under a plastic-covered tunnel and without any tunnel structure.

that have high content of anthocyanins. Therefore, in this manuscript we report comparative findings on anthocyanin content in sweet and sour cherry cultivars and their health-beneficial properties based on in vitro LPO and COX enzyme inhibitory activities.

MATERIALS AND METHODS

General Experimental. NMR spectra (^{1}H and ^{13}C) were recorded on Varian INOVA (300 MHz) and VRX (500 MHz) spectrometers. The chemical shifts were measured in $CDCl_3$, DMSO-d_6 and CD_3OD/DCl solution and are expressed in δ (ppm). Solvents used for purification and HPLC analysis are ACS grade and were purchased from Sigma-Aldrich Chemical Co., Inc. (St. Louis, MO). Cherry fruits were homogenized using a Kinematica CH-6010 (Roxdale, ON, Canada) homogenizer. Samples were centrifuged using a model RC5C centrifuge (Sorvall Instruments, Hoffman Estates, IL) at 10000*g* for 20 min at 4 °C. Quantification of anthocyanin was carried out on a Waters 2010 HPLC system (Waters Corp.) equipped with Empower Software, Shodex Degasser, Auto sampler (Waters 717), Photodiode Array Detector (Waters 996) and Capcell Pak (DyChrom, Santa Clara, CA) C-18 column (150 × 4.6 mm i.d.; 5 μm particle size). Fractionation and purification of anthocyanin were carried out on an XAD-2 column (500 g, Amberlite resin, mesh size 20−50; Sigma Chemical Co., St. Louis, MO) and C-18 MPLC column (350 × 40 mm). Merck silica gel (60-mesh size, 35−70 μm) and C-18 columns were used for medium pressure liquid chromatography (MPLC). For preparative TLC separation, 250 and 500 μ (20 × 20 cm) silica gel plates (Analtech Inc., Newark, DE) were used. TLC plates were viewed under UV-light at 254 and 366 nm or sprayed with 10% sulfuric acid solution in methanol. 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 insect cells cloned with human prostaglandin H_2 synthase (PGHS-2) enzyme. The liposome was prepared by mixing the phospholipid 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (SLPC) and a fluorescence probe [3-[*p*-(6-phenyl)-1,3,5-hexatrienyl] phenylpropionic acid (DPH-PA)]. Positive controls *t*-butyl hydroquinone (TBHQ), butylated hydroxyanisol (BHA) and butylated hydroxytoluene (BHT) used in the lipid peroxidation assay and aspirin in the COX inhibitory assay were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Celebrex capsules and Vioxx tablets were physician's professional samples provided by Dr. Subash Gupta, Sparrow Pain Center, MI.

Cherry Fruits. Ripe fruits from sweet (*P. avium* L.) and sour cherry (*P. cerasus* L.) cultivars (**Table 1**) were harvested during summer 2006 from 5-to-7-year-old trees at Michigan State University's Clarksville Horticultural Experiment Station (Clarksville, MI). Two harvest samples of the blushed yellow variety "Rainier" were collected, one from trees grown under a plastic-covered protective "high tunnel" structure (Haygrove Tunnels, Ledbury, U.K.) and the other from adjacent, unprotected trees. The plastic film (Luminance THB polyethylene, BPI-Agri, Stockton-on-Rees, U.K.) used to cover the tunnel has the following light transmissive properties when new, according to the manufacturer's specifications (*11*). The plastic used in this study was in its second season (4 to 5 months of total use by the time of fruit harvest) with 87% transmission of photosynthetically active light (400−700 nm, consisted of 90% diffused, 43% of infrared and mixture of short- and long-wave ultraviolet light).

Preparation of Samples for HPLC Analysis. Fresh and pitted cherry fruits were homogenized separately in acidic methanol (1% HCl) for 5 min in a Kinematica CH-6010 (Roxdale, ON, Canada) homogenizer and centrifuged (model RC5C, Sorvall Instruments, Hoffman Estates, IL) at 10000*g* for 20 min at 4 °C. The extraction procedure was repeated twice, and the combined supernatant was quantitatively made up to 25 mL with methanol (1% HCl), filtered through 0.2 μm filter disk and analyzed by HPLC on the same day.

HPLC Analysis of Anthocyanin in Cherries. The anthocyanin analysis was carried out by using HPLC according to the published procedure from our laboratory (*12*). Quantification of anthocyanins was performed on a Capcell Pak (DyChrom, Santa Clara, CA) C-18 column (150 × 4.6 mm i.d.; 5 μm particle size) maintained at 25 °C. The gradient solvent system used was solvents A [water−trifluoroacetic acid (TFA) 99.9:0.1 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 5 min. The flow rate was 0.75 mL/min. The injection volume for all samples was 25 μL, and detection of the anthocyanins was performed at 520 nm. Anthocyanins cyanidin-3-*O*-glucosyl rutinoside (**1**) and cyanidin-3-*O*-glucoside (**2**) were obtained from sour cherry "Balaton". Pure cyanidin-3-*O*-rutinoside (**3**) was isolated from the sweet cherry "Regina". The stock solutions were diluted with acidic methanol to yield 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.01525 and 0.007625 mg/mL concentrations, respectively, and analyzed in duplicate. Calibration curves were obtained by plotting the average of the mean peak areas of triplicate injections against concentrations.

To quantify anthocyanins, ten cherries per cultivar were extracted separately and standardized to yield equal volume of extract per/g fresh weight (FW) of fruit. Each extract was then analyzed in duplicate. The mean peak areas from the duplicate analyses were used to read the concentration of anthocyanin from the standard curve. The data collected for ten such replicates were then averaged to determine the quantity of cyanidin-3-*O*-rutinoside and cyanidin-3-*O*-glucosyl rutinoside in each variety.

Purification of Cyanidin-3-*O*-rutinoside and Cyanidin-3-*O*-glucosyl Rutinoside from Cherry Fruits. "Regina" fruits were used for the extraction of cyanidin-3-*O*-rutinoside. Freshly harvested fruits (1.16 kg) were blended with acidic water (1% HCl, 2 × 500 mL) for 2 min at room temperature and centrifuged (Sorvall Instruments, Hoffman Estates, IL) for 20 min at 4 °C to separate water insoluble materials, and the supernatants were lyophilized. The crude anthocyanin extract (14.5 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 (*10*). The column was eluted with water (3 × 2 L) until the colorless washings gave a pH of about 7. The adsorbed anthocyanin was then eluted with methanol (3 × 500 mL), concentrated under reduced pressure and lyophilized to yield a dark red powder (1.4 g). A part of the lyophilized extract (0.5 g) was purified further with a C-18 MPLC column (100 × 10 mm) using water:methanol (1% HCl) as the mobile phase under gradient conditions, starting with 80% H_2O. Fraction I (50 mL) was discarded since it did not contain anthocyanin, as confirmed by HPLC. Fractions II (150 mL, 20 mg) and III (200 mL, 9.8 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 analysis confirmed that fraction III contained pure cyanidin-3-*O*-rutinoside. The structures of compounds **1**, **2** and **3** were confirmed by NMR and mass spectral data (ref *13*, **Figure 2**)

Isolation of Chemical Constituents from "Rainier" Grown under Tunnel. Fresh fruits of the yellow sweet cherry "Rainier", grown under tunnel (1 kg), were extracted with water (1 L × 3) and centrifuged.

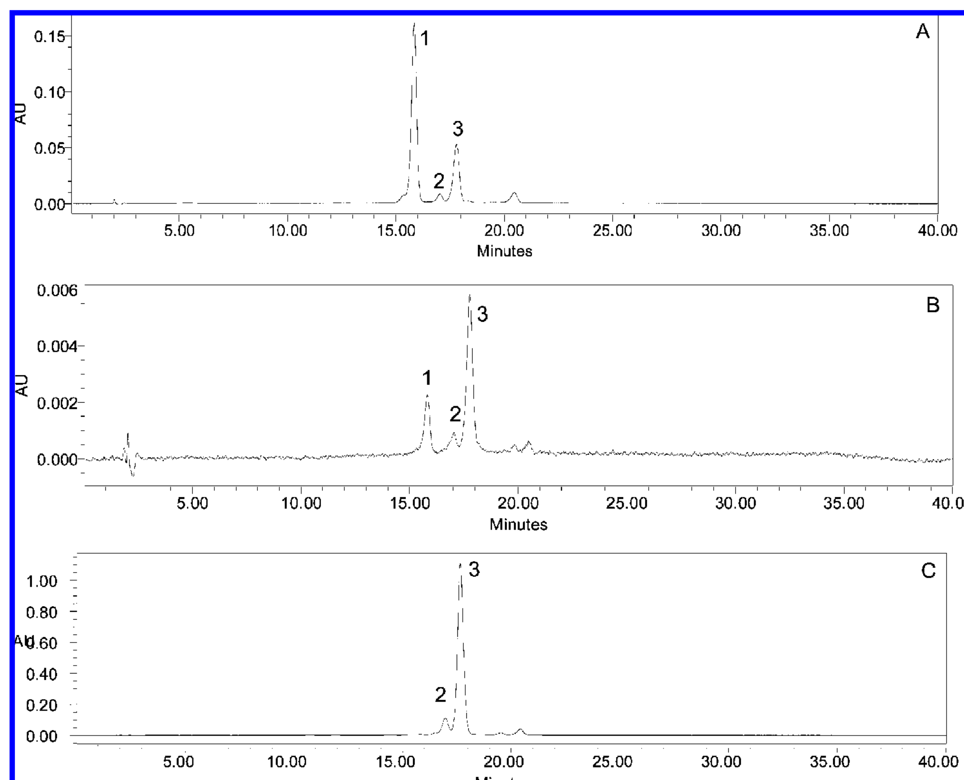

Figure 1. Anthocyanin profiles of the sour cherry "Balaton" (**A**), yellow sweet cherry "Gold" (**B**), and red sweet cherry "Kordia" (**C**). Peak 1: cyanidin-3-*O*-glucosyl rutinoside. Peak 2: cyanidin-3-*O*-glucoside. Peak 3: Cyanidin-3-*O*-rutinoside.

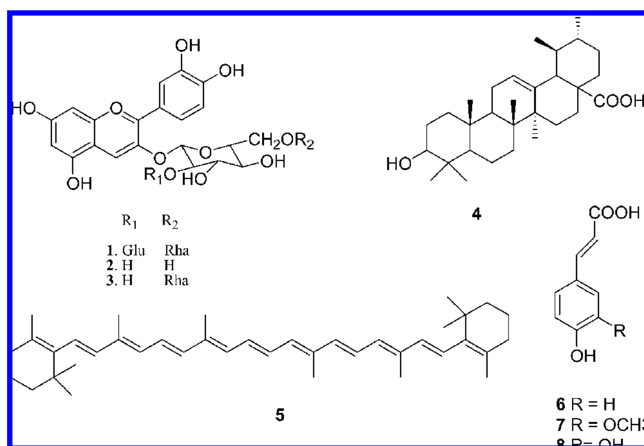

Figure 2. Structures of cyanidin-3-*O*-glucosyl rutinoside (**1**), cyanidin-3-*O*-glucoside (**2**), cyanidin-3-*O*-rutinoside (**3**), ursolic acid (**4**), β-carotene (**5**), coumaric (**6**), ferulic (**7**) and caffeic acid (**8**).

The supernatants were combined and lyophilized (10.8 g). The residue obtained after aqueous extraction was sequentially extracted further with methanol (500 mL × 3) and ethyl acetate (500 mL × 3) and evaporated under reduced pressure to afford 2.3 and 1.7 g, respectively, of methanolic and ethyl acetate extracts. The methanolic extract was dissolved in water (50 mL) and partitioned with *n*-butanol (50 mL) followed by ethyl acetate (50 mL). The resulting fractions were concentrated and monitored by TLC. The *n*-butanol extract (0.2 g) was further purified by silica gel MPLC with $CHCl_3$:MeOH (7:3) as the mobile phase to yield a colorless solid and identified as ursolic acid (**4**, 23 mg) (*14*). The combined ethyl acetate extract (2 g) was purified by using silica gel MPLC starting with hexane: acetone (4:1, 3:1, 2:1), acetone:$CHCl_3$ (1:1) and $CHCl_3$:MeOH (8:2, 7:3, 1:1) followed by methanol (100%) as eluting solvents. Fractions were collected in 50 mL aliquots, monitored by TLC and pooled to obtain fractions I (0.06 g), II (0.35 g), III (0.65 g), and IV (0.72 g). Fraction I gave a single yellow spot, identified as β-carotene based on NMR spectral data (**5** (*15*)). Fraction II gave a UV active spot (silica TLC, hexane:ethyl acetate, 7:3) and was further purified by preparative TLC using hexane: ethyl acetate (7:3) as the mobile phase. The resulting solid (7.8 mg) was identified as coumaric acid (**6** (*16*)). Similarly, purification of fraction III by silica gel MPLC using $CHCl_3$:MeOH mixtures (8:2, 7:3, 1:1) as mobile phases yielded two compounds which were identified as ferulic (**7**, 11.6 mg (*18*)) and caffeic acids (**8**, 9.5 mg (*17*)). Purification of fraction IV gave another batch of ursolic acid (18.3 mg) (*14*). The structures of compounds **4**−**8** (**Figure 2**) were analyzed by NMR spectral data.

Cyclooxygenase Inhibitory Assay. Cherry extracts were evaluated for COX-1 and -2 enzyme inhibitory activities according to the published procedure (*18*). The assay mixture contained 0.6 mL of 0.1 M Tris buffer (pH = 7), 1 mM phenol, 85 μg of hemoglobin and 27 μM arachidonic acid. DMSO and aqueous solutions of test samples (10 μL) were added to the assay chamber, and the reaction mixture was incubated for 2 min at 37 °C. The reaction was initiated by the addition of arachidonic acid (10 μL of 1.64 μM solution). The rate of O_2 consumption was determined by Instech oxygen micro chamber with an oxygen electrode attached to a YSI 5300 biological oxygen monitor. The data were recorded using QuickLog for Windows data acquisition and control software (Strawberry Tree, Inc., Sunnyvale, CA) and each sample was assayed in duplicate. Water extracts of cherries were dissolved in water to prepare test solutions, whereas methanol and ethyl acetate extracts were dissolved in DMSO. All extracts were tested at 250 μg/mL concentration. Each sample was assayed in duplicate and the percent inhibition was calculated with respect to water or DMSO control. Standard anti-inflammatory drugs, aspirin (60 μM), Celebrex (26 nM) and Vioxx (32 nM), were used as positive controls. COX inhibitory activities of pure cyanidin-3-*O*-rutinoside and cyanidin-3-*O*-glucosyl rutinoside were reported previously from our laboratory (*19*).

Lipid Peroxidation Inhibitory Assay. Cherry fruit extracts were also assayed for LPO inhibitory activities by using fluorescence spectroscopy according to the reported procedure (*20*). The peroxidation was initiated by the addition of 20 μL of $FeCl_2 \cdot 4H_2O$ (0.5 mM) to the assay mixture [HEPES (100 μL), 1 M NaCl (200 μL), N_2-sparged water (1.64 mL), test sample or DMSO (20 μL)] and 20 μL of liposome suspension. The fluorescence was monitored at 0, 1, 3 and every 3

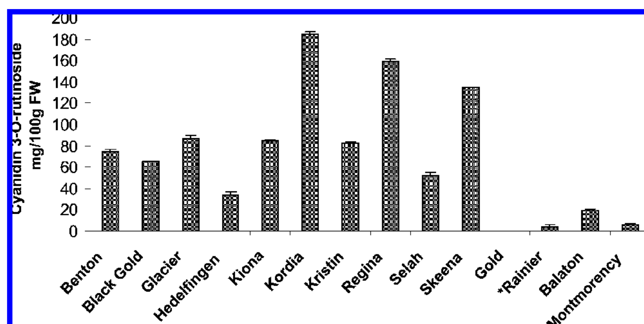

Figure 3. Relative amounts of cyanidin 3-*O*-rutinoside in fresh pitted cherry fruits. *Rainier fruits grown under no tunnel structure.

min thereafter up to 21 min using a Turner model 450 digital fluorometer. The decrease in fluorescence intensity over time (21 min) indicated the rate of peroxidation ($n = 2$). Water extracts were assayed for aqueous solutions, whereas methanol and ethyl acetate extracts were assayed for DMSO solutions at 250 μg/mL concentration. Each sample was assayed in duplicate, and the percent inhibition was calculated with respect to water or DMSO control. Commercial antioxidants, BHA, BHT and TBHQ, were tested as positive controls at 1 μg/mL. The LPO inhibitory activity of pure cyanidin-3-*O*-rutinoside and cyanidin-3-*O*-glucosyl rutinoside were reported previously from our laboratory (*19*).

RESULTS AND DISCUSSION

The anthocyanin content varied among the sweet and sour cherry genotypes (**Table 1**) analyzed in the present study. The HPLC profile of red sweet cherries contained primarily cyanidin-3-*O*-rutinoside (**Figure 1C**). However, the sour cherries "Montmorency" and "Balaton" showed the presence of cyanidin-3-*O*-glucosylrutinoside and cyanidin-3-*O*-rutinoside at a ratio of 3:1 (**Figure 1A**). yellow sweet cherry "Gold" showed a similar profile to that of sour cherries, but the ratio of cyanidin-3-*O*-glucosylrutinoside and cyanidin-3-*O*-rutinoside was 1:3 (**Figure 1B**).

Among the cherries analyzed, the sweet cherry "Kordia" (aka "Attika") showed the highest amount of anthocyanin, cyanidin-3-*O*-rutinoside, at 185 mg/100 g fresh weight (**Figure 3**). Two other red sweet cherries, "Regina" and "Skeena", were next to "Kordia" in anthocyanin yield, having cyanidin-3-*O*-rutinoside at 159 and 134 mg/100 g fresh weight, respectively (**Figure 3**). Published reports suggest that cyanidin-3-*O*-rutinoside is always the predominant anthocyanin in sweet cherries, although the total anthocyanin content in the dark red and yellow sweet cherry genotypes varied significantly (*8*). These studies have also shown that the anthocyanin content in dark red sweet cherry cultivars ranged from 82 to 297 mg/100 g and from 2 to 41 mg/100 g for the yellow cherries (*8*). Color attenuation in red sweet cherries is an indication of maturity and also is used to predict processing grades (*21*).

The yields of cyanidin-3-*O*-glucosylrutinoside and cyanidin-3-*O*-rutinoside were 57 and 19 mg/100 g fresh weight in "Balaton" and 21 and 6 mg/100 g FW in "Montmorency", respectively. Both of these sour cherries also gave minor quantities of cyanidin-3-*O*-glucoside (5 and 2 mg/100 g FW). The anthocyanin contents in other red sweet cherries, "Hedelfingen", "Kristin", "Glacier", "Kiona", "BlackGold" and "Benton", were 34, 82, 87, 85, 65, and 74 mg of cyanidin-3-*O*-rutinoside in 100 g FW (**Figure 3**). The yellow sweet cherry "Gold" contained the lowest amount of cyanidin-3-*O*-glucosylrutinoside and cyanidin-3-*O*-rutinoside (<1 mg/100 g FW). The yellow sweet cherry "Rainier" did not produce any anthocyanin when grown under the tunnel. However, "Rainier" grown without tunnel protection (exposed to light) attained a slightly red color and yielded cyanidin-3-*O*-rutinoside at 4 mg/100 g fresh weight. The impact of light on anthocyanin production in "Rainier" is in agreement with our previous report on the impact of light on anthocyanin production in *Pennisetum setaceum* cv Rubrum (*22*).

Table 2. Percent Yields of Cherry Extracts (mg) in 100 g of Fresh and Pitted Cherries

cherry cultivar	water extract	MeOH extract	EtOAc extract
Kordia	11.4	6.1	2.3
Regina	6	3.7	2.2
Skeena	4.7	3.6	1.6
Benton	8.9	3.9	1.9
Glacier	7.6	2.9	0.3
Kiona	7.4	3.5	1.8
Hedelfingen	9.9	3.8	1.6
Gold	6.5	4.9	1.4
BlackGold	7.4	3.4	1.2
Rainier	8.6	2.5	1.3
Selah	6.1	3.5	1.2
Balaton	7.1	5.3	1.7
Montmorency	9	4.1	1.4

Purification of anthocyanins was performed using Amberlite XAD-2 and C-18 medium pressure liquid chromatography (MPLC). The structures of the anthocyanins cyanidin-3-*O*-glucosylrutinoside (**1**), cyanidin-3-*O*-glucoside (**2**) and cyanidin-3-*O*-rutinoside (**3**) were elucidated by NMR and mass spectral data, and the data were in agreement with the data published (**Figure 2**) (*13*). Calibration curves for cyanidin-3-*O*-glucosylrutinoside and cyanidin-3-*O*-rutinoside were prepared and analyzed in triplicate. The total anthocyanin content in cherries was analyzed from their calibration curves resulting from the mean peak area of duplicate injections of each extract.

Cherries were extracted sequentially with water, methanol and ethyl acetate in order to determine the presence and biological activities of both lipid and water-soluble compounds. **Table 2** represents the quantity of extracts resulting from 100 g of fresh fruit of each cultivar. All extracts were assayed for COX enzyme inhibitory activity at 250 μg/mL. The water extracts of "Kordia", "Regina", "Skeena", "Hedelfingen", and "Glacier" sweet cherries inhibited COX-2 enzyme by 96, 94, 94, 94 and 93%, respectively (**Figure 4a**). The inhibition of COX-1 enzyme for these extracts ranged from 80 to 89%. Similarly, "Balaton" and "Montmorency" sour cherries inhibited COX-1 and -2 enzymes by 84, 76 and 91, 87% (**Figure 4a**). The water extracts of yellow sweet cherries "Gold" and "Rainier" displayed low COX-2 enzyme inhibition with values of 36 and 37%, respectively. However, they did not inhibit COX-1 enzyme, and this data is of great interest to those who seek pain relief from consumption of fruits. The water extracts of these cherries did not inhibit COX-1 enzyme, suggesting specificity in COX enzyme inhibition similar to some of the pharmaceutical COX-2 inhibitors.

The COX enzyme inhibitory activity of methanolic extracts was similar to those of the water soluble extracts for the red sweet and sour cherry cultivars. However, "Gold" and "Rainier" inhibited COX-1 and -2 enzymes by 81, 94 and 79, 85%, respectively (**Figure 4b**). Similarly, the ethyl acetate extracts of these cultivars showed good COX-1 and -2 enzyme inhibitions (**Figure 4c**). We have reported the COX enzyme inhibitory activities of pure cyanidin-3-*O*-glucosylrutinoside and cyanidin-3-*O*-rutinoside recently (*19*). The positive controls used in our assays were aspirin (60 μM), Celebrex (26 nM), and Vioxx (32 nM) (**Figure 4c**).

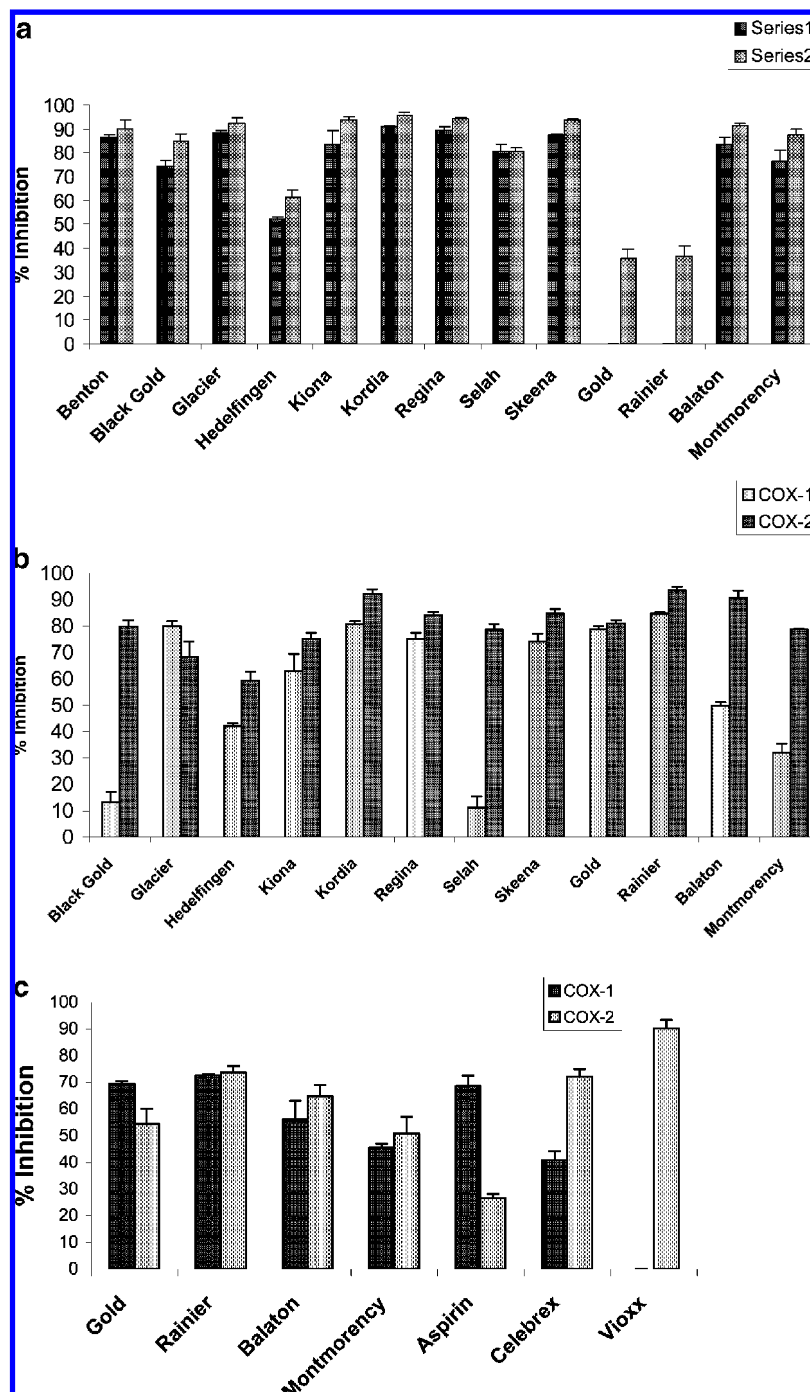

Figure 4. **a**−**c**. Inhibition of COX-1 and -2 enzymes by water (**a**), methanol (**b**) and ethyl acetate (**c**) extracts from sweet and sour cherries. Extracts were tested at 250 μg/mL. Water or DMSO was used as the solvent control. The percent inhibition was calculated with respect to solvent control and the values represent the mean ± SD. The anti-inflammatory drugs aspirin, Celebrex and Vioxx were tested at 60 μM, 26 nM, and 32 nM concentrations.

The lipid peroxidation (LPO) assay revealed that the water extract of "Kordia" sweet cherry was best with an inhibition value of 88% at 250 μg/mL (**Figure 5a**). However, water extracts of "Regina", "Skeena", "Glacier", and "Kiona" showed 82, 79, 82, and 72% of inhibition (**Figure 5a**). The yellow "Gold" and "Rainier" sweet cherries and "Balaton" and "Montmorency" sour cherries inhibited LPO by 48, 63 and 59, 49%, respectively. The methanol and ethyl acetate extracts of "Rainier" and "Gold" showed 79, 82 and 33, 37% of inhibition (**Figure 5a−c**).

Even though "Rainier" did not produce anthocyanins when grown under plastic-covered tunnels, the methanol and ethyl acetate extracts from it were the most active against LPO and COX enzymes. Purification of these extracts yielded compounds that were responsible for the biological activity. They were characterized as ursolic acid (**4**), coumaric (**6**), ferulic (**7**) and caffeic acids (**8**), along with β-carotene (**5**) by NMR spectral methods (refs *14−17*, **Figure 2**).

Several research reports indicate that sweet cherries are rich in phenolic acids. The most abundant phenolics present are hydroxycinnamic acids, such as caffeic and coumaric acids, with antioxidant and anti-inflammatory properties (*23*). Ursolic acid is known for its antioxidant (*24*), anti-inflammatory (*25*) and anticancer activities (*26*), and its consumption via cherry fruits

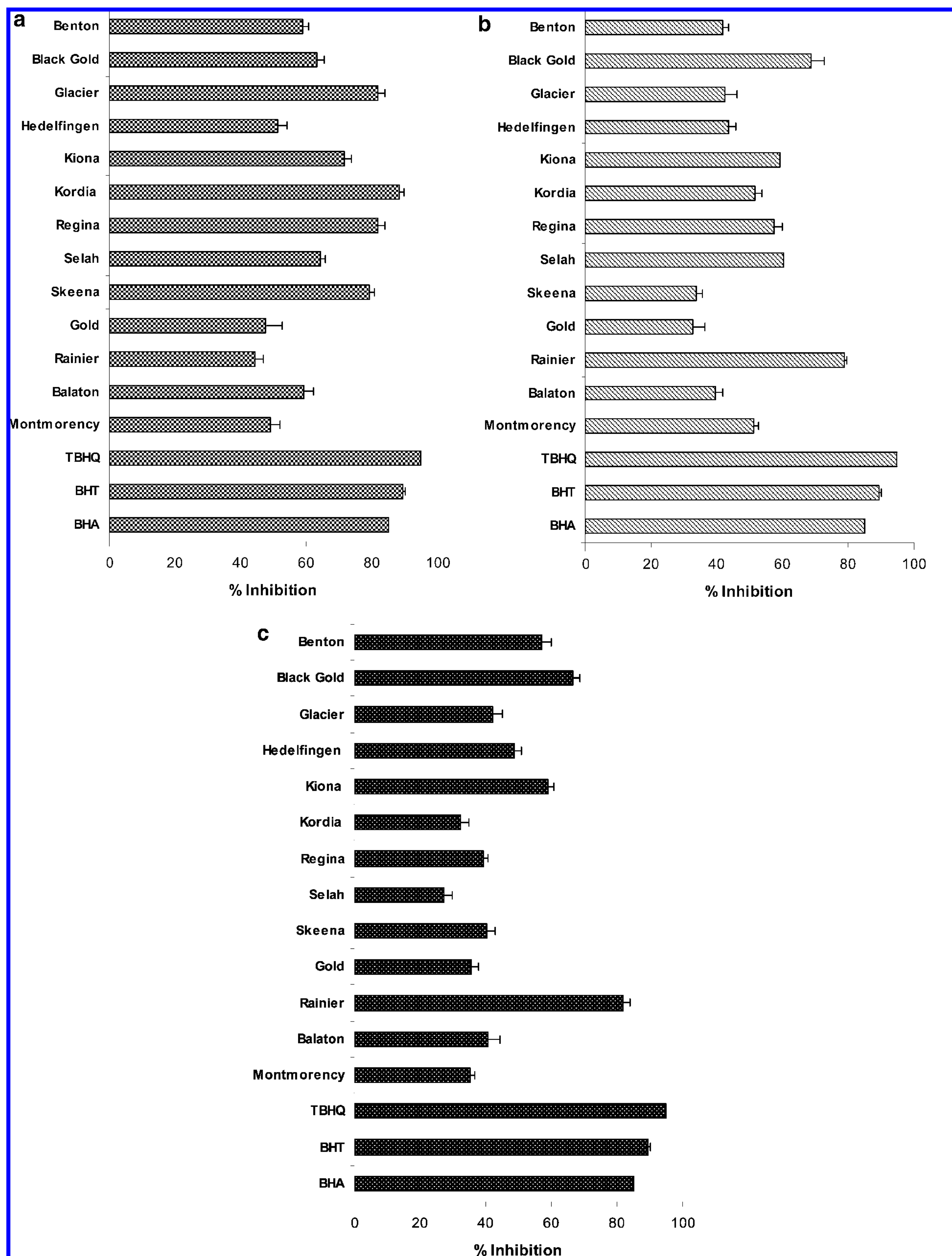

Figure 5. **a**−**c**. Lipid peroxidation inhibition by water (**a**), methanol (**b**) and ethyl acetate (**c**) extracts from sweet and sour cherries. Extracts were tested at 250 μg/mL. Water or DMSO was used as the solvent control. The percent inhibition was calculated with respect to solvent control and the values represent the mean ± SD. Positive controls BHA, BHT and TBHQ were tested at 1 μg/mL.

may reduce the incidence of initiation of cancer and heart disease (*27, 28*). There are anecdotal reports that phenolics in sour cherries relieve pain resulting from arthritis and gout. While the research on the exact mechanisms of pain relief from cherry consumption is ongoing, many consumers are convinced that tart cherry juice and related sour cherry products can stave off pain. Processed sour cherries also are claimed to be effective in reducing the pain associated with gout.

The COX enzymes play an important role in processes such as inflammation, carcinogenesis, apoptosis, cell proliferation and angiogenesis (*29*). Cherry anthocyanins are reported to exhibit antioxidant and anti-inflammatory activities comparable to over-the-counter drugs, and results from cherry extracts in this study are consistent with those of published data (*18, 29−32*). The cherry extracts, both sweet and sour, that we have studied so far are good candidates to inhibit these enzymes, and hence their consumption is beneficial to maintaining overall health.

Studies have shown that the prevention of cancer, cardiovascular disease, obesity and hyperglycemia are beneficial health effects attributable to phenolics in fruits and vegetables (*33, 34*). Cherries contain higher amounts of anthocyanins and phenolics when compared to several other fruits. It is implied that regular consumption of cherries is beneficial in reducing risk factors for heart disease, diabetes and certain cancers (*35, 36*). The use of LPO and COX enzyme inhibitory studies with the extracts from commercial sweet and sour cherry varieties allowed us to compare the potential dietary advantages among them. It is evident that all cherry varieties studied have significant LPO and COX enzyme inhibitory activities. However, the bioactive anthocyanins and phenolics vary among varieties of both sweet and sour cherries, with bioactive anthocyanins being most important. "Kordia" sweet cherry is the most beneficial when consumed, with regard to anthocyanin as the target bioactive molecule. It also yields the highest amount of extracts containing phenolics, followed by "Benton" and "Hedelfingen" (**Table 2**). Although the sour cherries "Balaton" and "Montmorency" yielded about the same amount of total extract between them, "Benton" and "Hedelfingen" contained lower amounts of anthocyanins compared to "Kordia" and other sweet cherry varieties. Among the phenolics present in sweet and sour cherries, anthocyanin constitutes the largest percentage and hence the advantage goes to "Kordia", "Benton" and "Hedelfingen". However, the methanol and ethyl acetate extracts from "Rainier", which included ursolic, coumaric, ferulic and caffeic acids as well as β-carotene, were the most active against LPO and COX enzymes. Based on our studies, it is evident that consumption of cherries, either sweet or sour, may be advantageous to better health.

ACKNOWLEDGMENT

This is a contribution from the Michigan State University Agricultural Experiment Station. The authors thank Tara Valentino for her help in collecting cherry fruits from orchards.

LITERATURE CITED

(1) Rashidkhani, B.; Lindblad, P.; Wolk, A. Fruits, vegetables and risk of renal cell carcinoma: a perspective study of Swedish women. *Int. J. Cancer* **2005**, *113*, 451–455.

(2) Hollman, P. C. H. Evidence for health benefits of plant phenolics: local or systemic effects. *J. Sci. Food Agric.* **2001**, *81*, 842–852.

(3) Johnsen, S. P.; Overvad, K.; Stripp, C.; Tjonneland, A.; Husted, S. E.; Sorensen, H. T. Intake of fruit and vegetables and the risk of ischemic stroke in a cohort of Danish men and women. *Am. J. Clin. Nutr.* **2003**, *78*, 5764..

(4) Webster, A. D.; Looney, N. E. World distribution of sweet and sour cherry production: national statistics. In *Cherries: Crop physiology, production and uses*; Webster, A. D., Looney, N. E., Eds.; CAB International: Wallingford, U.K., 1996; pp 25−69.

(5) FAOSTAT. 2005 Food and agricultural commodities production: sour cherries. http://www.fao.org/es/ess/top/commodity.html.

(6) Mozetic, B.; Hribar, J. Determination and quantification of anthocyanins and hydroxycinanmic acids in different cultivars of sweet cherries (Orunus avium L.,) from Nova Gorica Region (Slovena). *Food Technol. Biotechnol.* **2002**, *40*, 207–212.

(7) Chaovanalikit, A.; Wrolstad, R. E. Total anthocynains and total phenolics of fresh and processed cherries and their antioxidant properties. *J. Food Sci.* **2004**, *69*, 67–72.

(8) Gao, L.; Mazza, G. Characterization, quantification, and distribution of anthocyanins and color less phenolics in sweet cherries. *J. Agric. Food Chem.* **1995**, *43*, 343–346.

(9) Chandra, A.; Nair, M. G.; Lezzoni, A. Evaluation and characterization of the anthocyanin pigments in tart cherries (*Prunus cerasus* L.). *J. Agric. Food Chem.* **1992**, *40*, 967–969.

(10) Chandra, A.; Rana, J.; Li, Y. Separation, identification, quantification, and method evaluation of anthocyanins in botanical supplement raw materials by HPLC and HPLC-MS. *J. Agric. Food Chem.* **2001**, *49*, 3515–3521.

(11) http://www.bpiagri.com/pdf/greenhouse-film-facts.pdf.

(12) Vareed, S. K.; Reddy, M. K.; Schutzki, R. E.; Nair, M. G. Anthocyanins in *Cornus alternifolia* Cornus controversa, Cornus kousa and Cornus florida fruits with health benefits. *Life Sci.* **2006**, *78*, 777–784.

(13) Wang, H.; Nair, M. G.; Iezzoni, A. F.; Strasburg, G. M.; Booren, A. M.; Gray, J. I. Quantification and characterization of anthocyanins in Balaton tart cherries. *J. Agric. Food Chem.* **1997**, *45*, 2556–2560.

(14) Vareed, S. K.; Schutzki, R.; Nair, M. Lipid peroxidation, cyclooxygenase enzyme and tumor cell proliferation inhibitory compounds in *Cornus kousa* fruits. *Phytomedicine* **2007**, *14*, 706–709.

(15) Vaya, J.; Belinky, P. A.; Aviram, M. Antioxidant Constituents from Licorice Roots: Isolation, Structure Elucidation and Antioxidative Capacity Toward LDL Oxidation. *Free Radical Biol. Med.* **1997**, *23*, 302–313.

(16) Wei, X. L.; Yang, C. H.; Liang, J. Y. Constituents of the Barks of *Fraxinus chinensis* Roxb. *Chin. J. Nat. Med.* **2005**, *3*, 228–230.

(17) Jayaprakasam, B.; Vanisree, M.; Zhang, Y.; DeWitt, D. L.; Nair, M. G. Impact of alkyl esters of caffeic and ferulic acids on tumor cell proliferation, cyclooxygenase enzyme and lipid peroxidation. *J. Agric. Food Chem.* **2006**, *54*, 5375–5381.

(18) Seeram, N. P.; Momin, R. A.; Bourquin, L. D.; Nair, M. G. Cycloozygenase and antioxidant cyaniding glycosides in cherries and berries. *Phytomedicine* **2001**, *8*, 362–369.

(19) Mulabagal, V.; Van Nocker, S.; DeWitt, D. L.; Nair, M. G. Cultivars of apple fruits that are not marketed with potential for anthocyanin production. *J. Agric. Food Chem.* **2007**, *55*, 8165–8169.

(20) Arora, A.; Nair, M. G.; Strasburg, G. M. Antioxidant activities of isoflavones and their biological metabolites in a liposomal system. *Arch. Biochem. Biophys.* **1998**, *356*, 133–141.

(21) Drake, S. R.; Proebsting, E. L.; Spayd, S. E. Maturity index for the color grade of caned dark sweet cherries. *J. Am. Soc. Hortic. Sci.* **1982**, *107*, 180.

(22) Bechwith, A. G.; Zhang, Y.; Seeram, N. P.; Nair, M. G. Relationship of light quantity and anthocyanin production in *Pennisetum setaceum* Cvs. rubrum and red riding hood. *J. Agric. Food Chem.* **2004**, *52*, 456–461.

(23) Kang, S.-Y.; Seeram, N. P.; Bourquin, L. D.; Nair, M. G. Tart cherry anthocyanins inhibit tumor development in ApcMin mice and reduce proliferation of human colon cancer cells. *Cancer Lett.* **2003**, *194*, 13–19.

(24) Adhikari, D.; Schutzki, R. E.; DeWitt, D. L.; Nair, M. G. Effect of Amenlanchier fruit isolates on cyclooxygenase enzymes and lipid per oxidation. *Food Chem.* **2006**, *97*, 56–64.

(25) Dufour, D.; Pichette, A.; Mshvildadze, V.; Bradette-Hebert, M. E.; Lavoie, S.; Longtin, A.; et al. Antioxidant, anti-inflammatory and anticancer activities of methanolic extarcts from Ledum groenlandicum Retzius. J. *Ethno. Pharmacol.* **2007**, *111*, 22–28.

(26) Ma, C. M.; Cai, S. Q.; Cui, J. R.; Wang, R. Q.; Tu, P. F.; Hattori, M.; et al. The cytotoxicity activity of ursolic acid derivatives. *Eur. J. Med. Chem.* **2005**, *40*, 582–589.

(27) Kim, D. K.; Baek, J. H.; Kang, C. M.; Yoo, M. A.; Sung, J. W.; Chung, H.; Kim, N. D.; Choi, Y. H.; Lee, S. H.; Kim, K. W. Apoptotic activity of ursolic acid may correlate with the inhibition

of initiation of DNA replication. *Int. J. Cancer* **2000**, *87*, 629–636.

(28) Tokuda, H.; Ohigashi, H.; Koshimizu, K.; Ito, Y. Inhibitory effects of ursolic and oleanolic acid on skin tumor promotion by 12-O-tetradecanoylphorbol-13-acetate. *Cancer Lett.* **1986**, *33*, 279–285.

(29) Marnett, L. J.; Dubois, R. N. COX-2: a target for colon cancer prevention. *Annu. Rev. Pharmacol. Toxicol.* **2002**, *42*, 55–80.

(30) Wang, H.; Nair, M. G.; Strasburg, G. M.; Booren, A. M.; Gray, J. I. Novel antioxidant and anti-inflammatory compounds from tart cherries. *J. Nat. Prod.* **1999**, *62*, 86–88.

(31) Wang, H.; Nair, M. G.; Strasburg, G. M.; Change, Y.-C.; Booren, A. M.; Gray, J. I.; DeWitt, D. L. Cyclo-oxygenase active bioflavonoids from Balaton tart cherry and their structure activity relationships. *Phytomedicine* **2000**, *7*, 15–19.

(32) Tall, J. M.; Seeram, N. P.; Zhao, C.; Nair, M. G.; Meyer, R. A.; Raja, S. N. Tart cherry anthocyanins suppress inflammation-induced pain behavior in rat. *Behav. Brain Res.* **2004**, *153*, 181–188.

(33) Ness, A. R.; Fowles, J. W. Fruit and vegetables and cardiovascular disease: a review. *Int. J. Epidemiol.* **1997**, *26*, 1–13.

(34) Block, G.; Patterson, B.; Subar, A. Fruit, vegetables and cancer prevention: a review of the epidemological evidence. *Nutr. Cancer* **1992**, *18*, 1–29.

(35) Bolleddula, J.; Vareed, S. K.; Olson, L. K.; Nair, M. G. Insulin secretion by bioactive anthocyanins and anthocyanidins present in fruits. *J. Agric. Food Chem.* **2005**, *53*, 28–31.

(36) Bobe, G.; Wang, B.; Seeram, N. P.; Nair, M. G.; Bourquin, L. D. Dietary anthocyanin-rich tart cherry extract inhibits intestinal tumorigenis in APC (Min) mice fed sub optional levels of sulindac. *J. Agric. Food Chem.* **2006**, *54*, 9322–9328.

Received for review October 14, 2008. Revised manuscript received December 11, 2008. Accepted January 5, 2009.

JF8032039