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Cyclooxygenase Inhibitory and Antioxidant Compounds from Crabapple Fruits

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Crabapple trees belong to the *Malus* genus (Rosaceae) and bear fruits that are sparingly consumed and used in the preparation of fruit beverages. Cyclooxygenase (COX) enzyme inhibitory and antioxidant bioassay-guided fractionation of the aqueous and methanol extracts of *Malus* × *kornicensis* and *Malus* × *Indian Summer* yielded (+)-catechin (**1**), (−)-epicatechin (**2**), cyanidin-3-*O*-β-galactopyranoside (**3**), and amygdalin (**4**). Pure compounds **1**−**4** were obtained by HPLC, identified by LC-ES/MS, CD, and NMR spectroscopic methods and evaluated for their COX enzyme inhibitory and antioxidant activities. In COX-1 and -2 enzyme inhibitory assays, compounds **1**−**3** (all at 80 μM) showed activities of 20.4, 46.3%; 57.6, 47.9%; and 8.2, 13.7%, respectively, compared to naproxen (54.3, 41.3%; 10 μM), ibuprofen (47.5, 39.8%; 10 μM), Celebrex (46.2, 66.3%; 1.67 ppm), and Vioxx (23.8, 88.1%, 1.67 ppm). In the antioxidant assay, the catechins (**1**−**2**) and anthocyanin (**3**) (all at 40 μM) showed activities of 61.3, 62.5, and 60.1%, respectively. The synthetic antioxidants, *tert*-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and vitamin E (all tested at 10 μM), gave 75.2, 80.1, 70.0, and 10.2% activities, respectively. The cyanogenic glycoside, amygdalin (**4**), and its hydrolysis products, mandelonitrile (**5**) and benzaldehyde (**6**), were not active in the antioxidant or COX enzyme inhibitory assays at 80 μM concentrations.

KEYWORDS: ***Malus*; Rosaceae; crabapple; catechin; anthocyanin; cyanogenic; antioxidant; cyclooxygenase**

INTRODUCTION

There has been an increase in demand for the utilization of plant-derived compounds, rather than synthetic compounds, by consumers around the world due to negative perceptions of the safety of synthetic food additives (*1*). Phytoceuticals and nutraceuticals have grown in popularity especially for their reputed prevention of crippling inflammatory conditions such as arthritis and gout and for their antioxidant properties. This has resulted in the phytoceutical and botanical supplement industries investigating new and previously unexplored sources for purposes of formulating new commercial products. Fruits are well-known to be some of the most important candidates that can supply health beneficial compounds in substantial yields.

In our ongoing phytoceutical investigation of under-utilized fruits for the improvement of health and the production of value-added food (*2*, *3*), we have turned our attention to crabapples, *Malus* × *kornicensis* and *Malus* × *Indian summer* (Rosaceae). Although the phytochemistry of fruits of *Malus* genus, such as apples, has been widely investigated (*4*, *5*), a survey of the literature has revealed only one previous report on the chemical constituents of crabapples (*6*). Fruits of *Malus baccata* L. were reported to contain long chain alcohols, ursolic acid, and β-sitosterol, and campesterol and their D-glucosides (*6*). Crabapple trees, e.g., *M. baccata*, are grown widely in the USA for landscape and ornamental purposes, and the fruits are sparingly consumed and utilized in the preparation of fruit beverages (*6*).

In this study, we report the isolation of compounds from crabapples guided by cyclooxygenase (COX) enzyme inhibitory and antioxidant bioassays. We have reported the antiinflammatory activities of extracts and pure compounds of several plant species by determining their ability to inhibit the two isoforms of the COX enzyme, COX-1 and COX-2 (*2*, *3*). These enzymes catalyze the synthesis of prostaglandins, inflammation-causing hormones, thereby mediating the physiological effect of pain (*7*, *8*). The COX-1 enzyme is the constitutive form, expressed in most cells and performs protective functions, whereas COX-2 is an inducible form and expressed in response to inflammatory and other physiological stimuli (*7*, *8*). In the present study, we assessed the antioxidant activity by evaluating the ability to inhibit lipid peroxidation induced by Fe (II) ions in a liposomal model. Natural antioxidants may function as reducing agents

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or donors of hydrogen atoms and act as free radical scavengers and chain breakers, complexers of pro-oxidant metals, and quenchers of the formation of singlet oxygen (*9*).

To the best of our knowledge, this study represents the first report of these compounds from crabapples. Compounds were isolated by HPLC and characterized by LC-ES/MS, CD, and NMR spectroscopic methods. We also report the cyclooxygenase inhibitory and antioxidant activities of the pure compounds from crabapple fruits in these assays.

MATERIALS AND METHODS

General Experimental Procedures. NMR spectra (^{1}H and ^{13}C) were recorded on a Varian VXR 500 MHz spectrometer. ^{13}C NMR spectra were recorded at 126 MHz. Chemical shifts for compounds **1**, **2**, and **4** were recorded in DMSO-d_6 and are in δ (ppm) relative to DMSO at 2.45 for ^{1}H NMR and at 39.51 ppm for ^{13}C NMR. Chemical shifts for compound **3** was acquired in CD_3OD/DCl and the values are in δ (ppm) relative to CD_3OD at 3.31 for ^{1}H NMR and at 49.15 ppm for ^{13}C NMR. CD analyses for compounds **1** and **2** were performed on a JASCO J710 CD-ORD spectropolarimeter. Nitrogen was generated by a nitrogen generator model NG-150 at a rate of 25 L min^{-1}. Samples were dissolved in methanol separately and the CD was determined at 200−400 nm. For preparative HPLC purification, (LC-20, Japan Analytical Industry Co., Tokyo), a JAIGEL-ODS, A-343-10, 250 × 20 mm i.d., 10 μm (Dychrom, Santa Clara, CA) column was used. Peaks were detected at 210 nm, unless otherwise stated, using a UV detector equipped with a model D-2500 chromatointegrator (Hitachi, Tokyo). Positive controls used in the antioxidant (TBHQ, BHA, BHT, and α-tocopherol) and COX enzyme inhibitory (Ibuprofen and Naproxen) bioassays, benzaldehyde, and mandelonitrile 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, Sparrow Hospital, Lansing, MI. All solvents were ACS reagent grade and were purchased from Spectrum Chemical Co.

HPLC Conditions for the Analysis of Anthocyanin (3). Samples (20 μL injection volume) were analyzed on an Xterra (Waters Corp.) RP-18 column, 250 × 4.6 mm i.d, 5 μm, as reported previously (*3*). The mobile phase, 4% aqueous H_3PO_4/CH_3CN (9:1 v/v), was used under isocratic conditions at a flow rate of 0.75 mL/min; column temperature 35 °C. Anthocyanins were detected at 520 nm using a PDA detector (Waters Corp., Milford, MA).

LC-ES/MS Analysis. HPLC-ES/MS analyses were carried out as previously reported (*3*). The mobile phase was solvent (A) 0.1% TFA/H_2O (v/v), (B) 50.4% H_2O/48.5% ACN/1.0% CH_3COOH/0.1% TFA (v/v/v/v); gradient % B: initial: 20%, 26 min: 60%, 30 min: 20%, 35 min: 20%; run time 35 min; flow rate 0.80 mL/min; column temperature 30 °C. Peak identities were obtained by matching their molecular ions (M^+) obtained by ES/MS with the expected theoretical molecular weight from literature data (*10*).

Fruits. Fruits (2.5 g average weight, brownish in color) of *Malus* × *Indian summer* were collected in mid-October from trees in Okemos, MI. *Malus* × *kornicensis*, crabapples of similar size but red in color, were also collected in mid-October from the campus of Michigan State University (MSU), East Lansing, MI. The locations of the trees are recorded in the Michigan State University Herbarium Plant database. The fruits were stored in plastic ziplock bags at −20 °C prior to extraction. Analytical HPLC profiles of the fruit extracts from the two locations were similar except for the presence of anthocyanins (detected at 520 nm) in the red fruits collected from the campus of MSU. Also, the extracts of both fruits showed similar COX-1 and -2 inhibitory and antioxidant activities.

Extraction and Isolation of Compounds 1, 2, and 4. Crabapples (1 kg), *Malus* × *Indian summer*, were blended with H_2O (500 mL), and the puree was filtered through cheesecloth to afford an aqueous extract (945 mL). The residual fruit pulp was sequentially and exhaustively extracted to afford MeOH (54.6 g) and $CHCl_3$ (8.3 g) extracts after removal of the solvents in vacuo. COX enzyme inhibitory and antioxidant bioassays showed that the aqueous extract had the best activity; therefore, it was adsorbed on an Amberlite XAD-16 resin column, washed exhaustively with H_2O (3 L) to remove sugars and acids, then eluted with MeOH (500 mL) to afford a final XAD-MeOH extract (3.55 g). Further COX enzyme inhibitory bioassays showed that the activity was confined to this XAD-MeOH extract. The XAD-methanol extract (2 g) was fractionated by medium pressure liquid chromatography (MPLC) on a C-18 column, 350 × 40 mm i.d., and eluted with MeOH/H_2O solvent system, under gradient conditions, starting with 30% MeOH to 100% MeOH. Five fractions, I: 425 mL, II: 100 mL, III: 125 mL, IV: 300 mL and V: 200 mL, were collected. The highly COX enzyme inhibitory and antioxidant bioactive fraction III was further purified by high performance liquid chromatography (HPLC). The purification of fraction III (464 mg) by preparative HPLC utilizing an isocratic mobile phase of CH_3CN/H_2O (2:8; v/v) and a flow of 3 mL/min, gave (+)-catechin (**1**) (25.7 mg, t_R 39.9 min), (−)-epicatechin, **2** (27.1 mg, t_R 46.6 min), and amygdalin (**4**) (18.3 mg, t_R 51.5 min).

*Compounds **1** and **2***. ^{1}H and ^{13}C NMR and CD data of compounds **1**, (+)-catechin, and **2**, (−)-epicatechin, were consistent with literature data (*11*−*13*). For CD spectral analyses, compounds **1** and **2** were dissolved in MeOH at 1.4 and 1.3 mg/mL, respectively, and carried out at 25 °C. The CD spectra of **1** showed a positive cotton effect at 280 nm with 12.25 mdeg. The CD spectra of **2** showed a negative cotton effect at 283 nm with 13.17 mdeg.

*Compound **4***: ^{1}H and ^{13}C NMR of compound **4**, amygdalin, were consistent with literature data (*14*, *15*).

Extraction and Isolation of Anthocyanin (3). Analytical HPLC profiles and bioassays of the extracts showed that *Malus* × *Indian summer* and *Malus* × *kornicensis* were similar except for the presence of anthocyanins in the latter. Crabapples, *Malus* × *kornicensis*, were extracted for anthocyanins according to the previously published method (*2*). Analytical HPLC profiles showed the presence of one major anthocyanin that was isolated by fractionation by MPLC on a C-18 column, 350 × 40 mm i.d. The MeOH extract (4 g) was eluted with MeOH (0.01% TFA)/H_2O solvent system, under gradient conditions, starting with 30% MeOH to 100% MeOH (0.01% TFA). Three fractions, I: 250 mL, II: 100 mL, and III: 275 mL, were collected. HPLC analyses of these fractions revealed that fraction II (20 mg) (scarlet band) contained anthocyanin **3**. Two minor anthocyanin peaks were also detected at 520 nm but were not isolated due to their low yields.

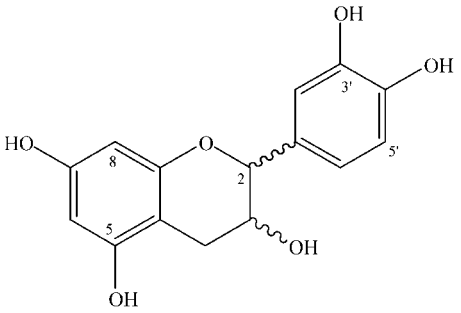
*Compound **3***. ^{1}H and ^{13}C NMR of compound **3**, cyanidin-3-*O*-β-galactopyranoside, were consistent with literature data (*3*). The LC-ES/MS gave the following major peaks at *m*/*z* (% intensity) for **3**, 449 (M^+, 100), 312 (10), 287 (25, aglycone). In HPLC analysis, the t_R of compound **3** was identical with that of an authentic sample of cyanidin-3-*O*-β-galactopyranoside (*3*).

Cyclooxygenase Inhibitory Assay. The COX-1 enzyme inhibitory assay was conducted with an enzyme preparation from ram seminal vesicles. COX-2 activity was determined using a preparation from insect cell lysate cloned with human HPGHS2. COX assays were measured at 37 °C by observing the initial rate of O_2 consumption as previously reported (*2*, *3*).

Antioxidant Assay. Bioassays were conducted by analysis of model liposome oxidation using fluorescence spectroscopy as reported previously (*2*, *3*). Peroxidation was initiated by addition of 20 μL of $FeCl_2$·$4H_2O$ (0.5 mM) for positive controls (BHA, BHT, TBHQ, and α-tocopherol/vitamin E, all 10 μM) and test samples. Fluorescence was measured at 384 nm and the decrease of relative fluorescence intensity with time indicated the 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).

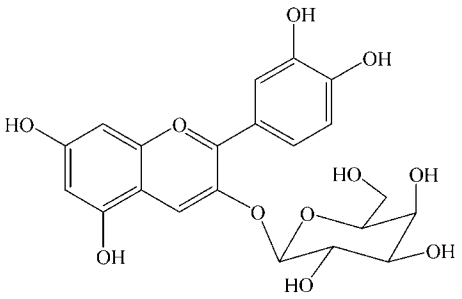
RESULTS AND DISCUSSION

Fruits of *Malus* × *Indian summer* were harvested, blended with water, and filtered to yield an aqueous extract. The residue was sequentially extracted with methanol/chloroform. The aqueous extract showed activity in the cyclooxygenase enzyme inhibitory and antioxidant assays and was further fractionated by separation over Amberlite XAD-16 resin to yield fractions I−II, eluted with water and methanol, respectively. Fraction II was found to be active in the cyclooxygenase inhibitory and



Catechins	Configuration
(+)- catechin (**1**)	2R, 3S
(-)- epicatechin (**2**)	2R, 3R

Figure 1. Catechins (**1**–**2**) isolated from crabapples.



(**3**) cyanidin-3-galactosidc

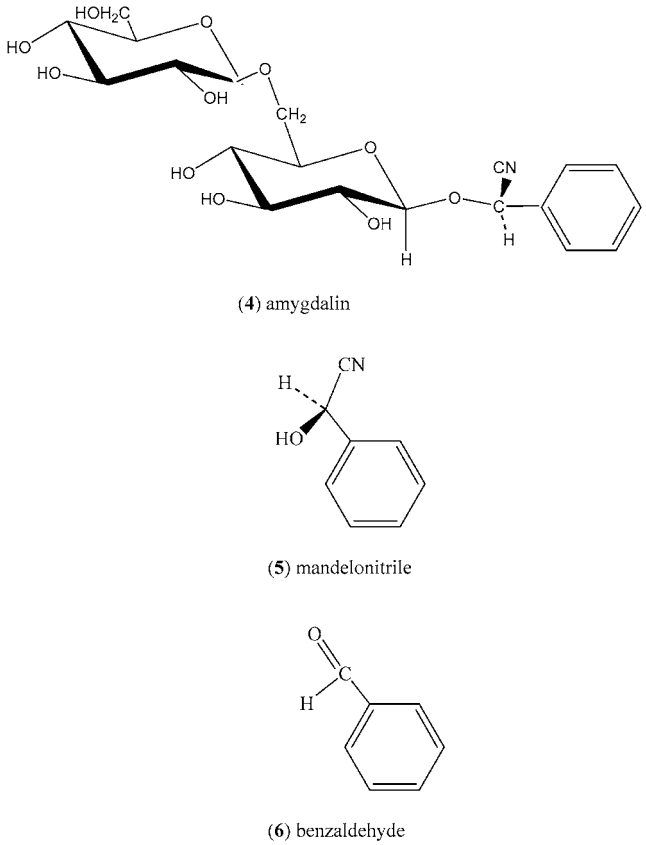
Figure 2. Anthocyanin (**3**) isolated from crabapples.

antioxidant assays, and further purification by preparative HPLC afforded compounds **1**, **2**, and **4**. The anthocyanin, compound **3**, was isolated from *Malus* × *kornicensis* according to the previously published method (*2*).

Compounds **1** and **2** were identified as catechins, compounds reported with beneficial health effects due to their antioxidant properties and inhibitory role in the processes of carcinogenesis (*16*). Compounds **1** and **2** were identified by their ^{1}H and ^{13}C NMR and CD spectral data to be (+)-catechin and (−)-epicatechin, respectively (**Figure 1**). The structures of **1** and **2** were unequivocally confirmed by comparison of their NMR and CD spectral data with that of literature reports (*11*−*13*).

The identity of compound **3** was established as cyanidin-3-*O*-*β*-galactopyranoside (**Figure 2**) by examination of its LC-ES/MS and NMR data, which were consistent with literature data (*3*). The LC-ES/MS gave the following major peaks at *m*/*z* (% intensity) for **3**, 449 (M^+, 100), 312 (10), 287 (30, aglycone). Two minor anthocyanins were also detected but were not identified due to low yields. Anthocyanins are pigments primarily responsible for the attractive colors in fruits, fruit juices, wines, flowers, and vegetables. Anthocyanins are plant flavonoids implicated with beneficial activities as food ingredients and as promoters of human health. Also, their cyclooxygenase inhibitory and antioxidant activities have been well established (*2*, *3*).

Compound **4** was identified as amygdalin (**Figure 3**) by examination of its ^{1}H and ^{13}C NMR data, which were identical to the literature values (*14*, *15*). The presence of the cyanogenic glycoside, amygdalin (**4**), in kernels of fruits such as cherries, apricots, peaches, and almonds are well-known (*17*). Amygdalin



(**4**) amygdalin

(**5**) mandelonitrile

(**6**) benzaldehyde

Figure 3. Amygdalin (**4**) isolated from crabapples. Commercially obtained hydrolysis products of amygdalin, mandelonitrile (**5**), and benzaldehyde (**6**) were also tested for cyclooxygenase enzyme inhibitory and antioxidant activities.

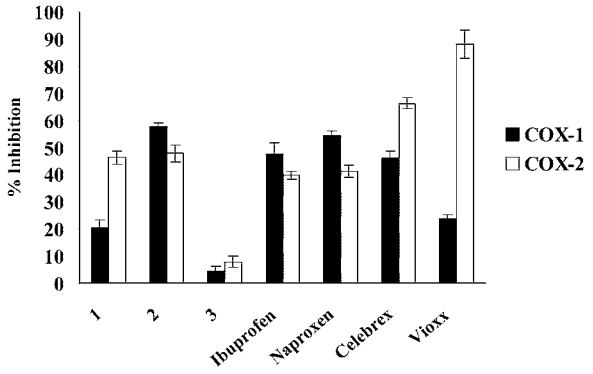


Figure 4. COX-1 and -2 enzyme inhibitory activities of catechins (**1**) and (**2**) and anthocyanin (**3**) isolated from crabapples. Samples were tested at 80 μM and at pH 7. Positive controls were naproxen (10 μM), ibuprofen (10 μM), Celebrex (1.67 ppm), and Vioxx (1.67 ppm). DMSO as solvent control did not show any inhibition. Vertical bars represent the standard deviation of each data point ($n = 2$).

(**4**), generally in the R form, is the naturally occurring cyanogen found in the Rosaceae family (*15*). However, rapid epimerization converts the R form to the unnatural S form, neo-amygdalin, leading to isoamygdalin, an equilibrium mixture of the epimers (*14*, *15*). In the ^{1}H NMR spectrum of compound **4**, we have observed a pair of singlets at δ 5.98 and 6.05 ascribable to methine protons in R and S aglycones of isoamygdalin, respectively (*15*). Since amygdalin (**4**) is reported to undergo enzymatic hydrolysis to produce benzaldehyde (**6**) and hydrogen cyanide (HCN) via mandelonitrile (**5**) (**Figure 3**), we have also tested commercially available **5** and **6** in our bioassays.

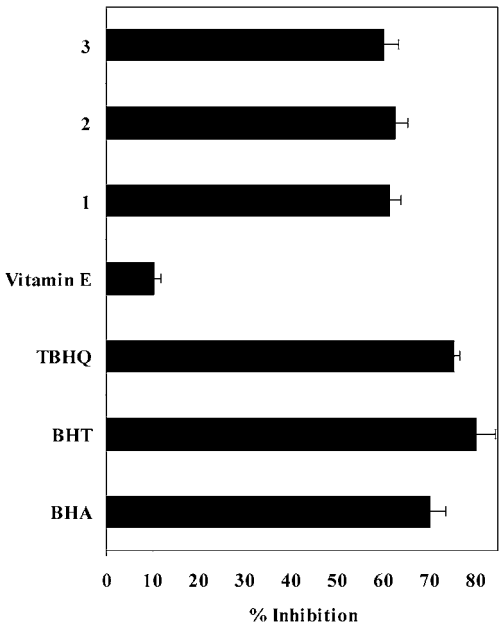


Figure 5. Antioxidant activities of catechins (**1**) and (**2**) and anthocyanin (**3**) isolated from crabapples and assayed in a liposomal model system at 40 μM. Commercial antioxidants TBHQ, BHT, BHA, and vitamin E were assayed at 10 μM. The rate of peroxidation was monitored by a decrease in fluorescence 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 compared to Fe^{2+} control and are for triplicate measurements ± one standard deviation. DMSO as solvent control did not show any inhibition.

However, at 80 μM concentrations, compounds **4**−**6** were not active in the COX enzyme inhibitory and antioxidant bioassays.

In COX enzyme inhibitory assay, catechins **1** and **2** and anthocyanin **3** were evaluated at 80 μM concentrations. Compounds **1**, **2**, and **3** displayed 20.4, 46.3%; 57.6, 47.9%; and 4.6, 7.9%, COX-1 and -2 inhibitory activities, respectively (**Figure 4**). The positive controls, ibuprofen and naproxen, showed 47.5 and 54.3% of COX-1 and 39.8 and 41.3% of COX-2 inhibitory activities, respectively, at 10 μM concentrations. Celebrex and Vioxx showed 46.2, 23.8% and 66.3, 88.1% COX-1 and COX-2 inhibition, respectively, at 1.67 ppm concentrations (**Figure 4**).

Compounds **1**−**3** were tested at 40 μM concentrations for antioxidant activity by using an iron-catalyzed liposomal model and fluorescence spectroscopy to monitor the inhibition of lipid peroxidation as described before (*2*). At a test concentration of 40 μM, the catechins **1** and **2** and anthocyanin **3** showed antioxidant activities of 61.3 ± 0.98%, 62.5 ± 0.8%, and 60.1 ± 0.75%, respectively. The commercial antioxidants, TBHQ (75.2%), BHT (80.1%), and BHA (70.0%), and vitamin E (10.2%) were tested at 10 μM concentrations (**Figure 5**).

In conclusion, we have identified catechins (**1**−**2**), an anthocyanin (**3**), and a cyanogenic glycoside, amygdalin (**4**), from cyclooxygenase enzyme inhibitory and antioxidant bioassay-guided isolation of crabapple fruits. The antioxidant and antiinflammatory activities of the bioactive compounds in crabapple fruits suggest that they could be considered as potential phytoceuticals from which value-added products could be generated.

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