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Antioxidant and Quinone Reductase-Inducing Constituents of Black Chokeberry (*Aronia melanocarpa*) Fruits

Jie Li,[†] Ye Deng,[†] Chunhua Yuan,[‡] Li Pan,[†] Heebyung Chai,[†] William J. Keller,[§] and A. Douglas Kinghorn*,[†]

[†]Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, 500 West 12th Avenue, Columbus, Ohio 43210, United States

[‡]Nuclear Magnetic Resonance Facility, Campus Chemical Instrument Center, The Ohio State University, Columbus, Ohio 43210, United States

[§]Nature's Sunshine Products, Inc., 1655 North Main Street, Spanish Fork, Utah 84660, United States

Supporting Information

ABSTRACT: Using in vitro hydroxyl radical-scavenging and quinone reductase-inducing assays, bioactivity-guided fractionation of an ethyl acetate-soluble extract of the fruits of the botanical dietary supplement, black chokeberry (*Aronia melanocarpa*), led to the isolation of 27 compounds, including a new depside, ethyl 2-[(3,4-dihydroxybenzoyloxy)-4,6-dihydroxyphenyl] acetate (**1**), along with 26 known compounds (**2**−**27**). The structures of the isolated compounds were identified by analysis of their physical and spectroscopic data ($[\alpha]_D$, NMR, IR, UV, and MS). Altogether, 17 compounds (**1**−**4**, **9**, **15**−**17**, and **19**−**27**) showed significant antioxidant activity in the hydroxyl radical-scavenging assay, with hyperin (**24**, ED_{50} = 0.17 μM) being the most potent. The new compound (**1**, ED_{50} = 0.44 μM) also exhibited potent antioxidant activity in this assay. Three constituents of black chokeberry fruits doubled quinone reductase activity at concentrations <20 μM, namely, protocatechuic acid [**9**, concentration required to double quinone reductase activity (CD) = 4.3 μM], neochlorogenic acid methyl ester (**22**, CD = 6.7 μM), and quercetin (**23**, CD = 3.1 μM).

KEYWORDS: *Aronia melanocarpa, black chokeberry, Rosaceae, ethyl 2-[(3,4-dihydroxybenzoyloxy)-4,6-dihydroxyphenyl] acetate, antioxidant constituents, hydroxyl radical-scavenging activity, quinone reductase-inducing activity*

INTRODUCTION

Black chokeberry, *Aronia melanocarpa* (Michx.) Elliott (Rosaceae), also known as "Aronia noir" and "black apple berry", is a deciduous shrub originating from the eastern parts of North America, which has been introduced to Europe.[1] In the 1940s, black chokeberry was first included in North American plant catalogs for its ornamental value.[2] In recent years, extracts of black chokeberry fruits have become available in the United States as a botanical dietary supplement.

The most abundant secondary metabolite constituents of black chokeberry are phenolic compounds, including proanthocyanidins, anthocyanins, and flavonoids.[2,3] The content of phenolic constituents in black chokeberry is higher than that of most other berries and fruits investigated to date,[4,5] consistent with its antioxidant potency in laboratory testing.[5−7] The antioxidant effects of black chokeberry extracts have been evaluated using different in vitro assays, for example, the inhibition of methyl linoleate oxidation,[4] oxygen radical absorbance capacity (ORAC),[7] trolox-equivalent antioxidant capacity (TEAC),[8] and DPPH radical-scavenging activity.[6] Besides antioxidant effects, black chokeberry extracts have also exhibited anti-inflammatory,[9] antimutagenic,[10] hepatoprotective,[11] cardioprotective,[12] and antidiabetes[13] effects in previous biological investigations.

Cancer chemoprevention refers to a strategy of cancer control by the administration of nontoxic synthetic or natural compounds to reverse, suppress, or prevent the process of carcinogenesis.[14] Scavenging reactive oxygen species by antioxidants and enhancing carcinogen detoxification via phase II enzymes such as quinone reductase (QR) are two important cancer prevention strategies.[15,16] In vitro screening assays for antioxidants and QR inducers, for example, hydroxyl radical-scavenging and QR induction in murine hepa1c1c7 cells, have been developed as convenient and rapid assays for the discovery of novel natural product anticarcinogens.[17,18]

Despite the various biological evaluations performed on black chokeberry extracts, there are very limited studies on the isolation and biological evaluation of their bioactive constituents as purified compounds. As part of our continuing evaluation of the biologically active constituents of popular herbal remedies,[18−20] the ethyl acetate-soluble extract of black chokeberry fruits showed significant in vitro hydroxyl radical-scavenging and QR-inducing activities and thus was fractionated via bioactivity-guided isolation using these two assays. One new depside, ethyl 2-[(3,4-dihydroxybenzoyloxy)-4,6-dihydroxyphenyl] acetate (**1**), and 26 known compounds (**2**−**27**) were isolated and structurally characterized. This paper describes the isolation of the purified compounds and the structure elucidation of compound **1**, as well as the biological evaluation

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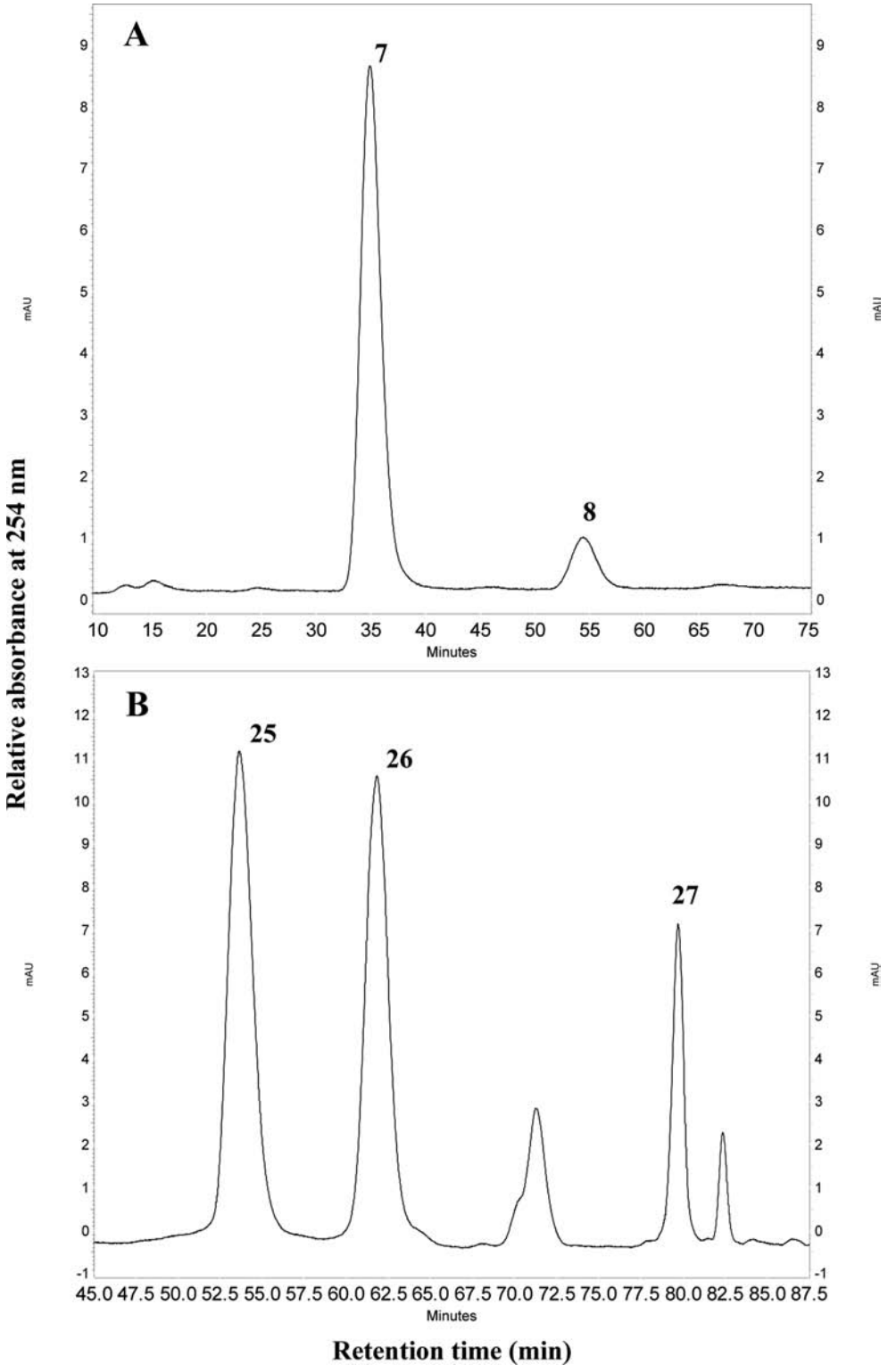

Figure 1. Representative HPLC chromatograms showing the separation of constituents of the fruits of black chokeberry (*A. melanocarpa*): (A) HPLC chromatogram of the separation of two isomers **7** and **8**; (B) HPLC chromatogram of the separation of three quercetin diglycoside analogues **25**–**27**.

of all 27 compounds obtained using the hydroxyl radical-scavenging and QR induction assays.

MATERIALS AND METHODS

Instrumentation. Optical rotations were measured on a Perkin-Elmer 343 automatic polarimeter (Perkin-Elmer, Waltham, MA, USA). UV spectra were run on a Hitachi U-2910 spectrophotometer (Hitachi, Tokyo, Japan). Circular dichroism (CD) spectra were recorded on a JASCO J-810 spectropolarimeter (JASCO Inc., Easton, MD, USA). IR spectra were obtained on a Nicolet 6700 FT-IR spectrometer (Thermo Scientific, Waltham, MA, USA). NMR spectroscopic data were recorded at room temperature on Bruker Avance DRX-400 and 600 MHz spectrometers (Bruker, Billerica, MA,

USA) using standard Bruker pulse sequences. High-resolution electrospray ionization mass spectra (HRESIMS) were obtained on a Micromass Q-Tof II (Micromass, Wythenshawe, UK) mass spectrometer operated in the positive-ion mode, with sodium iodide being used for mass calibration. Column chromatography was performed with Sephadex LH-20 (Supelco, Bellefonte, PA, USA), 65 × 250 or 230 × 400 mesh silica gel (Sorbent Technologies, Atlanta, GA, USA), and 40−63 μm C_{18}-RP silica gel (Acros Organics, Geel, Belgium). Analytical thin-layer chromatography (TLC) was conducted on precoated 250 μm thickness Partisil Si gel $60F_{254}$ glass plates, whereas preparative TLC was conducted on precoated 500 or 1000 μm thickness Partisil Si gel $60F_{254}$ glass plates (Whatman, Clifton, NJ, USA). An XBridge PrepC_{18} column (5 μm, 150 mm × 19 mm i.d., Waters, Milford, MA, USA) with a guard column (5 μm, 10 mm × 19 mm i.d., Waters) and a Sunfire PrepC_{18} column (5 μm, 150 mm × 10 mm i.d., Waters) with a guard column (5 μm, 10 mm × 10 mm i.d., Waters) were used for semipreparative HPLC, along with a Hitachi system composed of an L-2130 prep pump, an L-2200 autosampler, and an L-2450 diode array detector (Hitachi).

Chemicals. All solvents used for chromatographic separations were purchased from Fisher Scientific (Fair Lawn, NJ, USA). 2′,7′-Dichlorodihydrofluorescin diacetate (H_2DCF-DA), esterase, ferrous sulfate ($FeSO_4$), hydrogen peroxide (H_2O_2), quercetin, dimethyl sulfoxide (DMSO), digitonin, EDTA, Trizma base, Tween 20, flavin adenine dinucleotide phosphate (FAD), glucose-6-phosphate (G-6-P), nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate dehydrogenase (G-6-P-D), menadione, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), bovine serum albumin (BSA), L-sulforaphane, and deuterated NMR solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture media and supplements were obtained from Life Technologies, Inc. (Grand Island, NY, USA).

Plant Material. Spray-dried black chokeberry (*A. melanocarpa*) fruit juice (lot 0164144) used in this study was obtained from Nature's Sunshine Products, Inc. (Spanish Fork, UT, USA). A representative sample (OSUADK-CCP0022) was deposited in the Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University.

Extraction and Isolation. The spray-dried, powdered black chokeberry fruit juice sample (1.95 kg) was suspended in H_2O (2 L) to produce an aqueous solution and then partitioned in turn with *n*-hexane (3 × 2 L), $CHCl_3$ (3 × 2 L), EtOAc (3 × 2 L), and *n*-BuOH (3 × 2 L) to afford dried hexanes (0.04 g), $CHCl_3$ (0.31 g), EtOAc (7.7 g), *n*-BuOH (60.8 g), and H_2O-soluble (ca. 1850 g) extracts. The EtOAc-soluble extract (D3), the most potent among these extracts in the in vitro hydroxyl radical-scavenging and QR induction assays, was subjected to open column chromatography over coarse silica gel, eluted with a step gradient of $CHCl_3$/MeOH mixtures (40:1, 30:1, 20:1, 15:1, 8:1, 6:1, 4:1, 2:1, 1:1, and 1:2), and then pure MeOH, and collected in 250 mL aliquots. The aliquots were combined on the basis of TLC analysis to afford nine fractions: F01 (eluted with $CHCl_3$/MeOH, 40:1), F02 (eluted with $CHCl_3$/MeOH, 30:1), F03 (eluted with $CHCl_3$/MeOH, 20:1), F04 (eluted with $CHCl_3$/MeOH, 15:1), F05 (eluted with $CHCl_3$/MeOH, 15:1), F06 (eluted with $CHCl_3$/MeOH, 8:1), F07 (eluted with $CHCl_3$/MeOH, 4:1), F08 (eluted with $CHCl_3$/MeOH, 2:1), and F09 (eluted with $CHCl_3$/MeOH, 1:1 and 1:2). Fractions showing activity in either the hydroxyl radical-scavenging assay or the QR induction assay were chosen for further purification.

Fraction F02 (699 mg) was chromatographed over a silica gel column with a $CHCl_3$/MeOH solvent system (15:1, 10:1, 7:1, 3:1, and 1:1) to afford eight subfractions (F0201−F0208). F0203 (eluted with $CHCl_3$/MeOH, 10:1) was further separated by preparative TLC (20 × 20 cm, 500 μm), developed by $CHCl_3$/MeOH/AcOH (7:1:0.1), to yield compounds **4** (R_f = 0.27; 11.6 mg) and **5** (R_f = 0.62; 4.8 mg). F0204 (eluted with $CHCl_3$/MeOH, 7:1) was further purified by Sephadex LH-20 column chromatography, with elution by MeOH/H_2O (1:1), to afford compound **6** (1.0 mg) and a further subfraction, F020404. This subfraction was then purified by HPLC (see Figure 1), using a Sunfire PrepC_{18} column (5 μm, 150 mm × 10 mm i.d.) with MeOH/H_2O (5:95) at a flow rate of 4.0 mL/min, to afford two isomers, **7** (t_R = 35.1 min; 1.5 mg) and **8** (t_R = 54.8 min; 0.8 mg).

Fraction F03 (200 mg) was chromatographed over a silica gel column with a $CHCl_3$/MeOH solvent system (15:1, 8:1, 4:1, and 1:1). The third subfraction, F0303 (eluted with $CHCl_3$/MeOH, 8:1), was further purified by preparative TLC (20 × 20 cm, 500 μm), developed by $CHCl_3$/MeOH/AcOH (7:1:0.1), to yield compound **9** (R_f = 0.20; 43.8 mg).

Fraction F04 (445 mg) was subjected to Sephadex LH-20 column chromatography, with elution by MeOH/H_2O (25:75, 50:50, 75:25, and 100:0), to afford five subfractions (F0401−F0405). F0402 (eluted with MeOH/H_2O, 50:50) was then chromatographed over a silica gel column, with a $CHCl_3$/MeOH/AcOH solvent elution system (7:1:0.1), to yield compounds **10** (2.1 mg) and **11** (3.0 mg).

Fraction F05 (699 mg) was chromatographed over a reversed-phase C_{18} open column, using MeOH/H_2O gradient mixtures (0:100, 20:80, 40:60, 60:40, 80:20, and 100:0) as eluting solvents, to afford 12 subfractions (F0501−F0512). F0502 was then purified by HPLC, with a Sunfire PrepC_{18} column (5 μm, 150 mm × 10 mm i.d.), using a MeOH/H_2O gradient (6−12% MeOH from 0 to 70 min) at a flow rate of 4.0 mL/min, to yield compound **12** (t_R = 27.1 min; 4.2 mg). F0503 was purified using the same HPLC column, with a MeOH/H_2O gradient (flow rate of 4.0 mL/min, 6−20% MeOH from 0 to 30 min, 20−22% MeOH from 30 to 45 min, then 100% MeOH from 45 to 55 min), to yield compounds **13** (t_R = 18.9 min; 18.6 mg) and **19** (t_R = 36.2 min; 2.4 mg). F0504 was also chromatographed using this same HPLC column, with a MeOH/H_2O gradient (flow rate of 4.0 mL/min, 10−40% MeOH from 0 to 40 min), to yield a broad peak (t_R from 30.0 to 35.1 min), which consisted of a mixture of compounds **1**−**3**. This mixture was then purified by preparative TLC (20 × 20 cm, 500 μm), developed twice by $CHCl_3$/MeOH/AcOH (6:1:0.1), to yield compounds **1** (R_f = 0.30; 2.2 mg), **2** (R_f = 0.27; 3.8 mg), and **3** (R_f = 0.08; 14.2 mg). F0507 was also chromatographed using the same HPLC column, with a MeOH/H_2O gradient (25−38% MeOH from 0 to 60 min, then 38% MeOH from 60 to 70 min) at a flow rate of 4.0 mL/min, to yield compound **23** (t_R = 57.8 min; 3.6 mg).

Fraction F06 (533 mg) was subjected to passage over a reversed-phase C_{18} open column, using MeOH/H_2O mixtures (0:100, 20:80, 40:60, 60:40, 80:20, and 100:0) as eluting solvents, to afford 10 subfractions (F0601−F0610). F0603 was chromatographed over a column containing Sephadex LH-20, with elution by MeOH/H_2O (3:2), to afford compound **15** (2.2 mg). F0605 was also purified by Sephadex LH-20 column chromatography, eluting with MeOH/H_2O (4:1), to give a subfraction, F060503, which was then purified using a HPLC Sunfire PrepC_{18} column (5 μm, 150 mm × 10 mm i.d.) with isocratic elution (5% MeOH−95% H_2O), at a flow rate of 4.0 mL/min, to yield compound **14** (t_R = 46.2 min; 4.2 mg). F0606 was subjected to passage over a Sephadex LH-20 column, eluted by MeOH/H_2O (40:60, 60:40, 80:20, and 100:0), to furnish six subfractions (F060601−F060606). Of these subfractions, F060602 was purified by HPLC [Sunfire PrepC_{18} column (5 μm, 150 mm × 10 mm i.d.) with isocratic elution (18% MeOH/82% H_2O), at a flow rate of 4.0 mL/min], to yield compound **18** (t_R = 82.6 min; 3.6 mg). In addition, F060605 was also purified using this same HPLC column, with isocratic elution (25% MeOH−75% H_2O), at a flow rate of 4.0 mL/min, to yield compound **16** (t_R = 94.8 min; 2.0 mg).

Fraction F07 (1.79 g) was chromatographed initially over a Sephadex LH-20 column, with elution by MeOH/H_2O gradient mixtures (0:100, 25:75, 50:50, 75:25, and 100:0), to afford five subfractions (F0701−F0705). F0702 was then purified by HPLC on a Sunfire PrepC_{18} column (5 μm, 150 mm × 10 mm i.d.), with a MeOH/H_2O gradient (flow rate of 4.0 mL/min, 30−60% MeOH from 0 to 30 min), to yield compounds **17** (t_R = 12.3 min; 1.9 mg) and **24** (t_R = 18.8 min; 38.3 mg). F0703 was subjected to separation on a reversed-phase C_{18} open column, using MeOH/H_2O mixtures of decreasing polarity (0:100, 20:80, 40:60, 60:40, 80:20, and 100:0) as eluting solvents, to afford eight subfractions (F070301−F070308). F070302 was further purified by preparative TLC (20 × 20 cm, 1000 μm), developed by $CHCl_3$/MeOH/AcOH (4:1:0.2), to yield compounds **20** (R_f = 0.67; 20.6 mg), **21** (R_f = 0.20; 12.2 mg), and

Table 1. ^{1}H [δ in ppm, Multiplicity (J in Hz)] and ^{13}C NMR (δ in ppm) Spectroscopic Data of Compounds 1−3[a]

	1		2		3	
position	δ_H[b]	δ_C[c]	δ_H[b]	δ_C[c]	δ_H[b]	δ_C[c]
1		107.2		107.1		107.6
2		152.4		152.4		152.3
3	6.15, d (2.3)	102.1	6.14, d (2.3)	102.1	6.15, d (2.3)	102.0
4		158.6		158.6		158.5
5	6.26, d (2.3)	101.0	6.25, d (2.3)	101.0	6.26, d (2.3)	101.1
6		158.4		158.4		158.4
7	3.48, s	30.1	3.48, s	29.9	3.45, s	30.2
8		173.9		174.3		176.1
1′		121.5		121.6		121.7
2′	7.52, d (2.1)	117.7	7.52, d (2.1)	117.8	7.55, d (2.1)	117.9
3′		146.5		146.5		146.4
4′		152.8		152.5		152.4
5′	6.87, d (8.0)	116.1	6.86, d (8.0)	116.1	6.87, d (8.0)	116.1
6′	7.54, dd (8.0, 2.1)	124.4	7.54, dd (8.0, 2.1)	124.3	7.57, dd (8.0, 2.1)	124.4
7′		166.3		166.3		166.4
OCH_2-8	4.00, q (7.1)	61.8				
CH_3	1.10, t (7.1)	14.3	3.55, s	52.3		

[a]Obtained in CD_3OD. Assignments are based on ^{1}H−^{1}H COSY, HSQC, and HMBC spectroscopic data. [b]Measured at 400 MHz for ^{1}H NMR with residual signals of CD_3OD at δ 3.31 used as reference. [c]Measured at 100 MHz for ^{13}C NMR with residual signals of CD_3OD at δ 49.0 used as reference.

22 (R_f = 0.52; 3.6 mg). F070305 was subjected to Sephadex LH-20 column chromatography, eluted by MeOH/H_2O (25:75, 50:50, 75:25, and pure MeOH). Subfraction F07030502, eluted by MeOH/H_2O (50:50) from the Sephadex LH-20 column, was further purified by HPLC on an XBridge PrepC_{18} column (5 μm, 150 mm × 19 mm i.d.), using a CH_3CN−H_2O (0.05% TFA) gradient (flow rate of 8.0 mL/min, 11% CH_3CN from 0 to 55 min, 11−19% CH_3CN from 55 to 85 min), to yield compounds **25** (t_R = 53.8 min; 7.8 mg), **26** (t_R = 61.7 min; 5.6 mg), and **27** (t_R = 79.5 min; 2.7 mg).

Ethyl 2-[(3,4-Dihydroxybenzoyloxy)-4,6-dihydroxyphenyl] Acetate (1). **1** was isolated as a colorless, amorphous solid: UV (MeOH) λ_{max} (log ε) 205.5 (4.32), 222.0 (4.15), 267.5 (3.85), 299.0 (3.64) nm; IR (film) ν_{max} 3315, 2987, 2933, 1716, 1605, 1522, 1464, 1446, 1373, 1341, 1297, 1218, 1192, 1137, 1085, 1029, 761 cm^{-1}; ^{1}H and ^{13}C NMR data shown in Table 1; HRESIMS obsd m/z 371.0733 $[M + Na]^+$ (calcd for $C_{17}H_{16}O_8Na$, 371.0743).

Evaluation of Hydroxyl Radical-Scavenging Activity. Hydroxyl radical-scavenging activity was performed according to a method described previously.[18,21,22] A 160 μL aliquot of freshly prepared mixed solution (80 μL of 1.25 mM H_2O_2 and 80 μL of 0.2 mM $FeSO_4$ in 50 mM phosphate buffer at pH 7.4) was added into each well containing 10 μL of test sample in 25% DMSO solution and then incubated at 37 °C for 5 min. Then, 80 μL of esterase (1.0 unit/mL)-treated H_2DCF-DA (2 μM) in 50 mM phosphate buffer (pH 7.4) was rapidly added and mixed well. The final assay volume was 250 μL. Changes in hydroxyl radicals were measured using an FL 800 fluorescence spectrophotometer (Bio-Tek) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm after 30 min. Quercetin was used as a positive control. Data were processed using nonlinear regression analysis (TableCurve2DV4; AISN Software Inc., Mapleton, OR, USA).

Evaluation of Quinone Reductase Induction Activity. The potential QR-inducing activity of the extracts, fractions, and pure isolates was assayed as described previously.[22,23] Briefly, the murine hepa1c1c7 cells (ATCC CRL-2026) were seeded onto 96-well plates at a density of 1.5 × 10^4 cells/mL in 190 μL of cell culture medium/well. After incubation for 24 h, the cells were then dosed with 10 μL of each test compound in 10% DMSO, or the positive control (L-sulforaphane), or the negative control (10% DMSO), and were incubated for 48 h. Two plates were used for each test sample to determine both cytotoxicity, using crystal violet staining of protein content, and QR-inducing activity, by measuring the NADPH-dependent reduction of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) mediated by menadiol. Both the cytotoxicity and QR-inducing activity were measured at 595 nm with an ELISA plate reader. QR-inducing activity was presented as the concentration required to double the specific activity of QR (CD). Cytotoxicity was expressed as the concentration inhibiting cell growth by 50% (IC_{50}). The chemoprevention index (CI, IC_{50}/CD), was also determined. Data were processed using nonlinear regression analysis (TableCurve2DV4; AISN Software Inc.).

RESULTS AND DISCUSSION

The EtOAc-soluble extract of the fruits of black chokeberry, *A. melanocarpa*, was found to be the most potent among the hexanes, $CHCl_3$, EtOAc, *n*-BuOH, and H_2O-soluble extracts in the in vitro hydroxyl radical-scavenging (ED_{50} = 0.3 μg/mL) and QR induction (CD = 8.4 μg/mL) assays; therefore, it was selected for further detailed purification. Fractions F02−F07 of the EtOAc-soluble extract showed potent activity in the hydroxyl radical-scavenging assay, with ED_{50} values of 0.3, 0.6, 2.4, 2.6, 0.6, 0.3 μg/mL, respectively. In addition, fractions F03 and F05−F07 exhibited QR-inducing activity, with CD values of 1.9, 2.6, 3.6, and 2.2 μg/mL, respectively. Accordingly, fractions F02−F07 were used for further purification. In this way, bioactivity-guided fractionation of ethyl acetate-soluble extract of the fruits of black chokeberry, using in vitro hydroxyl radical-scavenging and QR induction assays, led to the isolation of a new depside (**1**), along with 26 known compounds (**2**−**27**). On the basis of physical and spectroscopic data measurements ($[\alpha]_D$, CD, ^{1}H NMR, ^{13}C NMR, DEPT, 2D NMR, and MS) and comparison of the data obtained with published values, the structures (see Figure 2) of the known compounds **2**−**27** were identified as jaboticabin (**2**),[24−26] 2-*O*-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxyphenylacetic acid (**3**),[27] 2,4,6-trihydroxybenzaldehyde (formylphloroglucinol) (**4**),[28] benzoic acid (**5**),[29] 2-furoic acid (pyromucic acid) (**6**),[30] *p*-hydroxybenzoic aicd (**7**),[31,32] *m*-hydroxybenzoic acid (**8**),[33] protocatechuic acid (**9**),[31] L-malic acid (**10**),[34] L-malic acid methyl ester (**11**),[35] 1-β-*O*-benzoyl-D-glucopyranoside (**12**),[36] prunasin (**13**),[37,38] 4,6-dihydroxy-2-*O*-(β-D-glucopyranosyl)-

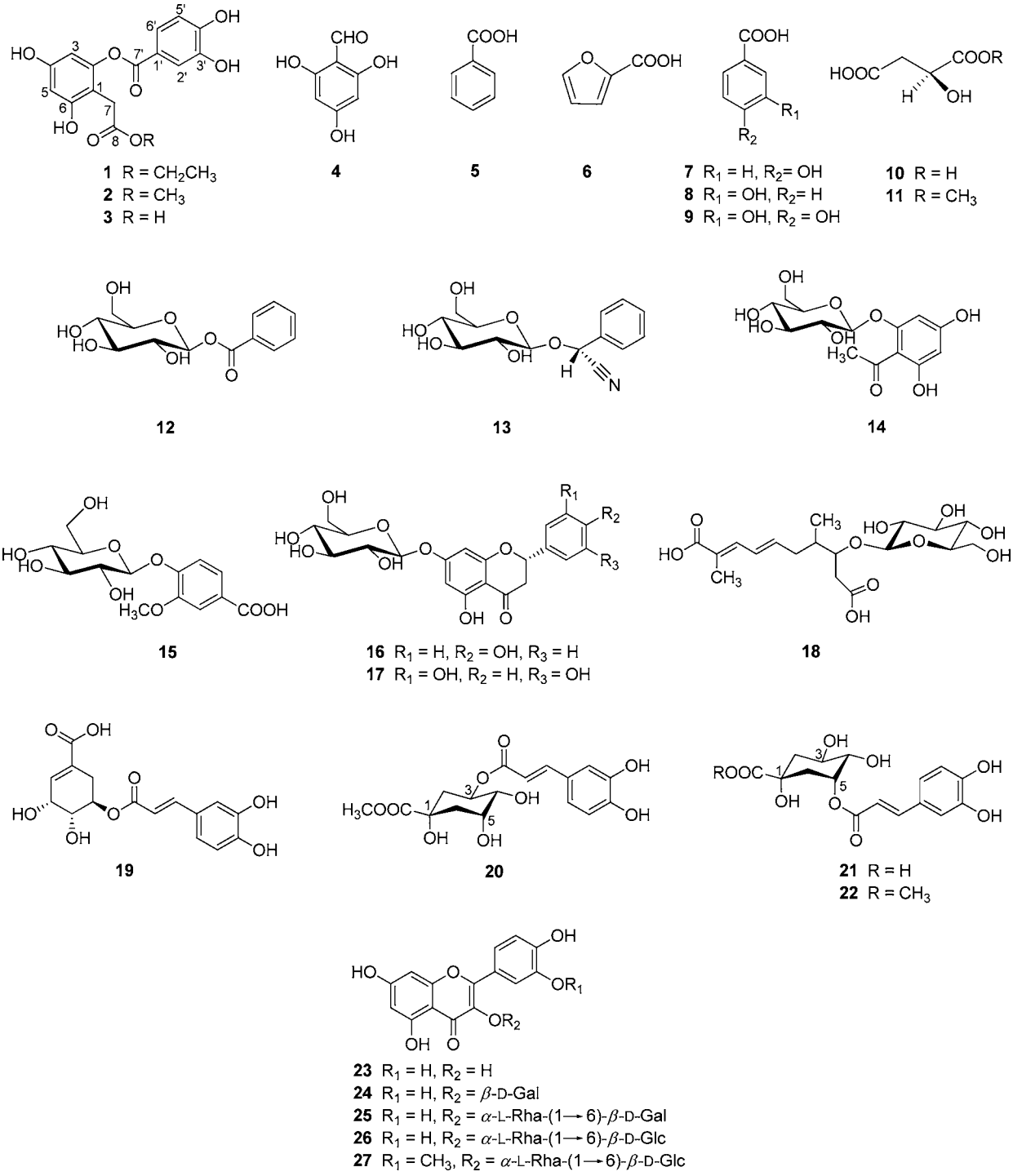

Figure 2. Structures of compounds isolated from the fruits of black chokeberry (*A. melanocarpa*).

acetophenone (**14**),[39] vanillate glucoside (**15**),[40] naringenin 7-*O*-*β*-D-glucopyranoside (prunin) (**16**),[31,41,42] (2*S*)-5,7,3′,5′-tetrahydroxyflavanone 7-*O*-*β*-D-glucopyranoside (**17**),[43] (2*E*,4*E*)-8-*O*-*β*-D-glucopyranosyloxy-2,7-dimethyl-2,4-decadiene-1,10-dioic acid (**18**),[44] 5-*O*-caffeoylshikimic acid (**19**),[45] chlorogenic acid methyl ester (**20**),[46] neochlorogenic acid (**21**),[47] neochlorogenic acid methyl ester (**22**),[46] quercetin (**23**),[48] quercetin 3-*O*-*β*-D-galactopyranoside (hyperin) (**24**),[42] quercetin 3-*O*-*β*-D-robinobioside (**25**),[42] quercetin 3-*O*-*β*-D-rutinoside (rutin) (**26**),[48] and isorhamnetin 3-*O*-*β*-D-rutinoside (**27**).[31] Compounds **1**−**3**, **5**−**9**, **12**, **14**−**20**, **22**, **23**, and **27** were isolated from *A. melanocarpa* for the first time. Compounds **3**, **9**, **13**, **20**, **21**, and **24** were found to be the major constituents isolated.

The new compound **1** was obtained as a colorless, amorphous solid. The molecular formula was determined as $C_{17}H_{16}O_8$ on the basis of the sodiated molecular ion peak at *m*/*z* 371.0733 $[M + Na]^+$ (calcd 371.0743) in the HRESIMS. The IR spectrum exhibited absorptions of hydroxy (3315 cm^{-1}), ester carbonyl (1716 cm^{-1}), and phenyl (1605, 1522, 1446, and 761 cm^{-1}) groups. The UV spectrum showed a peak at 267.5 nm with a shoulder at 299 nm that is typical of a

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phenolic acid ester. The splitting pattern in the ^{1}H NMR spectrum at lower field and the ^{1}H−^{1}H COSY correlations observed indicated that compound **1** exhibits two isolated aromatic spin systems (see the Supporting Information). One was characterized by a 1,3,4-trisubstituted ABX spin system at δ_H 7.54 (1H, dd, J = 8.0, 2.1 Hz, H-6′), 7.52 (1H, d, J = 2.1 Hz, H-2′), and 6.87 (1H, d, J = 8.0 Hz, H-5′), as well as a phenyl carboxyl group (δ_C 166.3), suggesting the presence of a protocatechuic acid moiety.[27,31] The other moiety was a 1,2,4,6-tetrasubstituted AX spin system at δ_H 6.26 (1H, d, J = 2.3 Hz, H-5) and 6.15 (1H, d, J = 2.3 Hz, H-3), along with three oxygenated aromatic carbons (δ_C 158.6, 158.4, and 152.4, respectively). In addition, a methylene group (δ_C 30.1; δ_H 3.48, 2H, s, H-7) and an ethoxy group (δ_C 61.8, δ_H 4.00, 2H, q, J = 7.1 Hz, OCH_2-8; δ_C 14.3, δ_H 1.10, 3H, t, J = 7.1 Hz, CH_3) were evident. The HMBC correlations (Figure 3) of the ethoxy

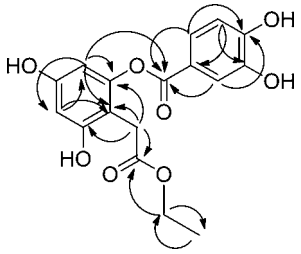

Figure 3. Selected HMBC correlations of compound **1**.

signal at δ_H 4.00 (2H, q, J = 7.1 Hz, OCH_2-8) and the methylene signal at δ_H 3.48 (2H, s, H-7) to C-8 (δ_C 173.9) revealed that they form an ethyl acetate unit. The attachment of this ethyl acetate unit to C-1 was established through HMBC correlations of H-7 to C-1 (δ_C 107.2), C-2 (δ_C 152.4), and C-6 (δ_C 158.4). Accordingly, the second moiety was characterized as a phloroglucinol unit substituted with an ethyl acetate unit. The relatively large chemical shift difference between H-3 (δ_H 6.15) and H-5 (δ_H 6.26) indicated that the ester linkage between the two moieties occurs at C-2 instead of C-4; otherwise, the symmetrical structure would lead to the virtually identical chemical shift for both H-3 and H-5. More importantly, only H-3, but not both H-3 and H-5, exhibited a four-bond HMBC correlation to C-7′ (δ_C 166.3). This four-bond HMBC correlation, also observed in compounds **2** and **3**, was facilitated using a long delay (125 ms) for evolution of long-range couplings for the detection of small multiple-bond coupling. Compared to the chemical shift of the free carboxyl carbon of protocatechuic acid (**9**), the upfield shift of 4.1 ppm of C-7′ in compound **1** provided indirect evidence that C-7′ is the site of esterification in the protocatechuic acid moiety,[25] which was confirmed by the direct evidence of the four-bond HMBC correlation of H-3 (δ_H 6.15) to C-7′ (δ_C 166.3). All of these data together led to the determination of the ester linkage at C-2 and C-7′ between the two moieties. Furthermore, the H-3 and H-5 signals were distinguishable by the four-bond HMBC correlation with C-7′ (because H-5 is too distant to show this HMBC correlation with C-7′), which helped further establish the full NMR assignments for **1**. Therefore, the structure of compound **1** was established unambiguously as ethyl 2-[(3,4-dihydroxybenzoyloxy)-4,6-dihydroxyphenyl] acetate.

Compound **1** and the known compounds **2** and **3** represent a novel class of depsides that has been reported only relatively recently. Thus, compound **3** was isolated from the petals of *Papaver rhoeas* in 2004,[27] whereas compound **2** was purified

Table 2. Hydroxyl Radical-Scavenging and Quinone Reductase-Inducing Activities of the Isolated Compounds 1−27

	hydroxyl radical scavenging		quinone reductase induction	
compound[a]	ED_{50}[b] (μM)	CD[c] (μM)	IC_{50}[d] (μM)	CI[e]
1	0.44 ± 0.04	>20	>100	NA[f]
2	0.60 ± 0.07	>20	>100	NA
3	0.31 ± 0.04	>20	>100	NA
4	2.6 ± 0.32	>20	>100	NA
9	1.9 ± 0.16	4.3 ± 0.66	>100	>23.3
15	6.4 ± 0.66	>20	>100	NA
16	2.3 ± 0.33	>20	>100	NA
17	1.3 ± 0.12	>20	>100	NA
19	0.59 ± 0.08	>20	>100	NA
20	0.27 ± 0.02	>20	>100	NA
21	0.56 ± 0.07	>20	>100	NA
22	0.81 ± 0.11	6.7 ± 1.2	>100	>14.9
23	1.1 ± 0.13	3.1 ± 0.57	>100	>32.3
24	0.17 ± 0.03	>20	>100	NA
25	0.25 ± 0.04	>20	>100	NA
26	0.20 ± 0.03	>20	>100	NA
27	2.4 ± 0.34	>20	>100	NA
quercetin[g]	1.1 ± 0.11			
L-sulforaphane[h]		0.53 ± 0.08	13.2 ± 1.8	24.9 ± 3.1

[a]Compounds **5**−**8**, **10**−**14**, and **18** did not show hydroxyl radical-scavenging (ED_{50} > 20 μM) or quinone reductase-inducing (CD > 20 μM) activity. [b]ED_{50}, concentration scavenging hydroxyl radical by 50%. Each value represents the mean ± SD (n = 3). Compounds with ED_{50} values of <20 μM are considered to be active. [c]CD, concentration required to double quinone reductase activity. Each value represents the mean ± SD (n = 3). Compounds with CD values of <20 μM are considered to be significantly active. [d]IC_{50}, concentration inhibiting cell growth by 50%. [e]CI, chemoprevention index (= IC_{50}/CD). [f]NA, not applicable. [g]Positive control for hydroxyl radical-scavenging assay. [h]Positive control for quinone reductase-induction assay.

from the fruits of jaboticaba (*Myrciaria cauliflora*),[24] cranberry (*Vaccinium macrocarpon*),[25] and kiwifruit (*Actinidia chinensis*),[26] all in 2006. It should be noted that although the position of the ester linkage of compound **3** was established correctly in the original literature by indirect evidence of an upfield shift of C-7′ compared to authentic protocatechuic acid, it was difficult to distinguish H-3 and H-5 as well as the carbons associated with them (C-3/C-5, and C-2/C-6) in the absence of an HMBC correlation of H-3 to C-7′. Therefore, the NMR assignments of H-3/H-5, C-3/C-5, and C-2/C-6 of compound **3** were left as interchangeable in the published literature.[27] The structure elucidation of compound **2** in the original literature[24−26] followed that of compound **3**, and MS fragmentation was used to provide further evidence for the position of the ester linkage. However, the NMR assignments of H-3/H-5, C-3/C-5, and C-2/C-6 were not consistent in these literature values.[24−26] In the present study, the four-bond HMBC correlation of H-3 to C-7′ provided direct evidence to clearly distinguish H-3 and H-5, and thus all of the protons and carbons of compounds **2** and **3** could be assigned unambiguously (see Table 1), following the assignment of compound **1**, as discussed above. In terms of the occurrence of these compounds, most natural depsides are biosynthesized in lichens via predominately the acetyl polymalonyl pathway and to a lesser extent via the shikimic acid pathway,[49,50] and it is rare to find depsides in higher plants. Although a previous investigation has indicated that ionizing radiation can break down quercetin into various depsides and small phenolic acids,[51] the formation of depsides in higher plants still deserves further investigation.

All of the 27 compounds isolated in this investigation were tested in the in vitro hydroxyl radical-scavenging and QR induction assays. The results are shown in Table 2. Seventeen compounds (**1**−**4**, **9**, **15**−**17**, and **19**−**27**) showed significant antioxidant activity in the hydroxyl radical-scavenging assay, with hyperin (**24**, ED_{50} = 0.17 μM) being of the greatest potency. The new compound **1** also exhibited potent antioxidant activity (ED_{50} = 0.44 μM) in this assay. The high potency of these pure isolates for radical scavenging may explain, at least in part, the very high antioxidant capacity of black chokeberry extracts shown in previous studies. On the basis of an analysis of the structure−activity relationship of the active compounds in the hydroxyl radical-scavenging assay, it may be suggested that the presence of a catechol group in compounds **1**−**3**, **9**, and **19**−**26** greatly enhances their ability to scavenge hydroxyl radicals. This observation is well supported by previous studies[52,53] showing that catechol is a radical target because its *o*-dihydroxy groups have better electron-donating properties to form ketones after scavenging radicals.

The in vitro QR assay showed that three compounds, namely, protocatechuic acid (**9**, CD = 4.3 μM), neochlorogenic acid methyl ester (**22**, CD = 6.7 μM), and quercetin (**23**, CD = 3.1 μM), doubled quinone reductase activity at concentrations <20 μM. On the basis of the QR-inducing results obtained, two observations can be made. First, certain positional isomers showed very different QR-inducing activities. For example, the only difference between neochlorogenic acid methyl ester (**22**) and chlorogenic acid methyl ester (**20**) is the position at which the caffeoyl moiety is linked to quinic acid (i.e., the C-5 position for neochlorogenic acid methyl ester and the C-3 position for chlorogenic acid methyl ester). However, neochlorogenic acid methyl ester (**22**, CD = 6.7 μM) was more active than chlorogenic acid methyl ester (**20**, CD > 20 μM). The different spatial orientation of these two compounds may result in different binding affinities to their corresponding targets and, thus, lead to their different QR-inducing activities. Second, the in vitro QR-inducing activity of quercetin aglycone (**23**, CD = 3.1 μM) was much more potent than that of either of the quercetin monoglycoside **24** or the quercetin diglycosides **25**−**27** (all CD > 20 μM). However, these quercetin glycosides may have in vivo QR-inducing activity, if the present knowledge concerning their bioavailability and metabolism is considered. Thus, there is evidence that suggests quercetin glycosides, depending on the different sugar moieties, may have increased bioavailability.[54,55] Also, quercetin glycosides are converted to quercetin aglycone by primary hydrolysis when ingested and are further subjected to glucuronidation, sulfation, and methylation to a large extent. A relatively small percentage of quercetin aglycone could still be absorbed intact into the circulation to exert the QR-inducing activity.[56] Moreover, if the conjugated metabolites of quercetin aglycone retain the QR-inducing activity, the increased bioavailability of the quercetin glycosides would lead to additional amounts of the conjugated metabolites of quercetin aglycone and thus would contribute to enhanced in vivo QR-inducing activity.

Finally, because of the high percentage of bioactive constituents present in *A. melanocarpa* in the present investigation, black chokeberry is an excellent source of leads as potential cancer chemopreventive agents and as a possible functional food ingredient of natural origin. Several of the major constituents isolated could also serve as standard markers for black chokeberry preparations with regard to antioxidant activities.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H NMR, ^{13}C NMR, ^{13}C DEPT 135 NMR, ^{1}H−^{1}H COSY, HSQC, HMBC, HRESIMS, IR, and UV spectra for compound **1**; ^{1}H NMR, ^{13}C NMR, and HMBC spectra for compounds **2** and **3**; HPLC chromatograms showing the separation of constituents of black chokeberry fruits. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 1-(614) 247-8094. Fax: 1-(614) 247-8119. E-mail: kinghorn.4@osu.edu.

Notes

The authors declare no competing financial interest.

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