Bioactive Depsides and Anthocyanins from Jaboticaba (*Myrciaria cauliflora*)

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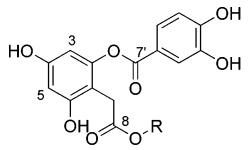
A new depside, jaboticabin (**1**), together with 17 known compounds were isolated from the fruit of jaboticaba (*Myrciaria cauliflora*). The structure of **1** was elucidated by spectroscopic data interpretation. Known compounds were identified by comparison of their spectroscopic data with literature values or by comparison to authentic standards. Compound **1** and the related depside 2-*O*-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxyphenylacetic acid (**2**) significantly inhibited chemokine interleukin (IL)-8 production before and after cigarette smoke treatment of cells. Compound **1** was cytotoxic in the HT29 colon cancer cell line (IC_{50} = 65 μM), and **2** was active against HCT116 colon cancer cells (IC_{50} = 30 μM). Compounds **1** and **2** also exhibited antiradical activity in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (IC_{50} = 51.4 and 61.8 μM, respectively). Two anthocyanins, cyanidin 3-glucoside (**3**) and delphinidin 3-glucoside (**4**), also showed good activity in these assays.

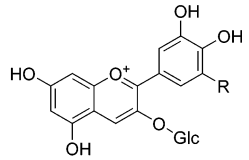
The jaboticaba (*Myrciaria cauliflora* (Mart.) O.Berg. [Myrtaceae]) is a small tree native to the Minas Gerais region near Rio de Janeiro in southern Brazil, grown for the purple, grape-like fruits it produces. Traditionally, an astringent decoction of the sun-dried skins has been used as a treatment for hemoptysis, asthma, and diarrhea and gargled for chronic inflammation of the tonsils.[1] The fruit is 3−4 cm in diameter with one to four large seeds, borne directly on the main trunks and branches of the plant, lending a distinctive appearance to the fruiting tree. It has a thick, purple, astringent skin that covers a sweet, white, gelatinous flesh. Common in Brazilian markets, jaboticabas are largely eaten fresh; their popularity has been likened to that of grapes in the United States.[2] Fresh fruit may begin to ferment 3 to 4 days after harvest, so they are often used to make jams, tarts, strong wines, and liqueurs.

In Brazil the fruit of several species, namely, *M. jaboticaba* (Vell.) O.Berg, *M. tenella* (DC.) O.Berg, and *M.* trunciflora O.Berg, share the same common name.[1−3] The phytochemistry of these fruits has not been extensively reported in the literature. The jaboticaba (no species distinguished) has been reported to contain tannins,[1] and we previously reported the presence of cyanidin 3-glucoside (**3**) in *M. cauliflora*.[4] *M. jaboticaba* reportedly contains peonidin 3-glucoside and its aglycone,[5] and the related camu-camu berry (*M. dubia*), an edible fruit known for its high levels of ascorbic acid, contains **3** and delphinidin 3-glucoside (**4**) as the main pigments.[6]

As part of our ongoing study of antioxidants and cancer chemopreventative compounds from tropical fruits,[7−9] the jaboticaba was investigated, and crude methanolic extracts were shown to have strong antiradical activity in the DPPH assay (IC_{50} = 35 μg/mL). Fruit extracts were subsequently subjected to bioactivity-guided fractionation using the DPPH assay, resulting in the isolation of a new depside, jaboticabin (**1**). In addition, the related depside 2-*O*-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxyphenylacetic acid (**2**), **4**, pyranocyanin B, quercetin, isoquercitrin, quercimeritrin, quercitrin, rutin, myricitrin, cinnamic acid, *O*-coumaric acid, gallic acid, protocatechuic acid, methyl protocatechuate, and ellagic acid were identified from this species for the first time.



1 R = Me
2 R = H



3 R = H
4 R = OH

Depsides are phenolic compounds composed of two or more monocyclic aromatic units linked by an ester bond. They are most often found in lichens, but have also been isolated from higher plants, including species of the Ericaceae, Lamiaceae, and Papaveraceae.[10,11] They have not been previously reported in the Myrtaceae. Depsides have antibiotic, anti-HIV, and antiproliferative activity.[12,13] As inhibitors of prostaglandin biosynthesis and leukotriene B_4 biosynthesis, depsides are potent nonsteroidal anti-inflammatories.[14]

Compound **1** was isolated as a reddish, amorphous powder. The negative ESI mass spectrum showed a $[M - H]^-$ molecular ion of m/z = 333. Positive HRESIMS gave a $[M + Na]^+$ molecular ion of m/z = 357.0581, corresponding to a molecular formula of $C_{16}H_{14}O_8$. The UV spectrum exhibited a peak at 267 nm with a shoulder at 298 nm, typical of a phenolic acid ester. The ^1H and ^{13}C NMR experiments were similar to literature values for **2**, with an additional methoxy signal at δ 3.57 (3H, s, OCH_3-8) and 50.9 (OCH_3-8).[11] The position of the methoxy group was established through HMBC correlations between the proton signal at δ 3.57 (OCH_3-8) and δ 172.9 (C-8). The C-1 attachment for the methyl ethanoate group followed from HMBC correlations; the methylene proton signal at δ 3.50 (H-7) showed HMBC correlations with C-1, C-2, C-6, and the carbonyl C-8. A detailed analysis of 1-D and

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2-D NMR spectra and comparison with **2** confirmed the structure of jaboticabin (**1**) as methyl 2-[(3,4-dihydroxybenzoyloxy)-4,6-dihydroxyphenyl]acetate.

An ethanolic extract of jaboticaba fruits was analyzed by LC-MS in selected ion monitoring (SIM) mode to address concerns that **1** could be a methyl ester artifact from the initial MeOH extraction. A $[M - H]^-$ molecular ion $m/z = 333$ with the same retention time as **1** was detected in ethanolic extracts, indicating that **1** is produced by the plant itself.

Compounds **2**−**4**, pyranocyanin B, protocatechuic acid, methyl protocatechuate, ellagic acid, quercimeritrin, and quercitrin were isolated and identified by comparison of spectroscopic measurements to published literature values.[11,15] Quercetin, isoquercitrin, rutin, myricitrin, cinnamic acid, *O*-coumaric acid, and gallic acid were identified by comparison of retention time, UV, and MS data to authentic standards.

Depsides **1** and **2** exhibited antiradical activity in the DPPH assay ($IC_{50} = 51.4$ and 61.8 μM, respectively) and colon cancer cell cytotoxicity and significantly inhibited chemokine interleukin (IL)-8 production in human small airway epithelial (SAE) cells before and after treatment with cigarette smoke extract (CSE). Compound **1** decreased IL-8 production in untreated SAE cells by 81.3% and decreased production in SAE cells treated with 5% CSE by 47.3%. Compound **2** inhibited IL-8 production by 74.9% in untreated SAE cells and 70.3% in treated SAE cells. The anthocyanins **3** and **4**, major constituents of jaboticaba fruits, also displayed significant activity against IL-8 production in SAE cells. IL-8 was not detected in SAE cells treated with compound **4**, which caused a 96% reduction of IL-8 production in SAE cells treated with CSE; **3** inhibited IL-8 production by 65.3% and 36.4%, respectively. Compounds **1**−**4** were more effective at blocking IL-8 production in untreated SAE cells than catechin, and **2** and **4** were more effective than catechin at blocking cigarette smoke-induced inflammation.

IL-8 is a cytokine implicated in some cancers and a wide range of chronic inflammatory conditions, including rheumatoid arthritis and heart and lung diseases.[12,16,17] The ability of depsides **1** and **2** to reduce IL-8 production suggests an important anti-inflammatory action of these compounds. Chronic obstructive pulmonary disease (COPD) is a complex lung disease characterized by irreversible airflow obstruction due to chronic inflammation. COPD includes chronic obstructive bronchiolitis (fibrosis and obstruction of small airways) and emphysema (permanent enlargement of the airspaces distal to the terminal bronchioles accompanied by destruction of lung parenchyma). COPD is considered steroid-resistant, and it has been noted that nonsteroidal anti-inflammatories that target chemokine pathways are needed as new therapies.[16,18] The demonstration that jaboticaba depsides and anthocyanins can reduce inflammation secondary to smoke exposure could provide a novel therapeutic role for these compounds in COPD.

The cytotoxicity of **1**, **2**, and **4** is comparable to IC_{50} values for 5-fluorouracil (5-FU), a drug used for colon cancer treatment, epigallocatechin gallate (EGCG), and Polyphenon E (Poly E), a standardized decaffeinated green tea extract.[8,19] Compound **1** is cytotoxic against HT29 colon cancer cells ($IC_{50} = 65$ μM), and **2** is cytotoxic against HCT116 colon cancer cells ($IC_{50} = 30$ μM). Consistent with published literature, **4** was more cytotoxic than **3**.[20] Compound **4** showed good activity against both the HCT116 and SW480 cell lines ($IC_{50} = 12$ and 20 μM, respectively), while **3** inhibited 50% cell growth only at the 100 μM range. Compounds **1**−**4** also exhibit good antiradical activity in the DPPH assay.

The anthocyanins are a group of well-studied phenolic compounds with antioxidant, anti-inflammatory, antimutagenic, and cancer chemopreventative activities.[21] In one study, it was shown that UVB-exposed HaCaT keratinocytes pretreated with **3** were protected from UVB-induced inflammation, inhibiting NF-kB and AP-1 activation and IL-8 mRNA expression.[22] Here we show that jaboticaba anthocyanins and depsides exhibit good antiradical activity and cytotoxicity and inhibit IL-8 production in both untreated SAE cells and those treated with proinflammatory CSE. Depsides from foods and botanicals are less well-studied than the anthocyanins, possibly as a result of their limited distribution in higher plants, and this is the first report of their ability to inhibit IL-8 production and cytotoxcity against colon cancer cells. The jaboticaba is rich in anthocyanins, phenolic acids, and flavonoids and contains depsides with antiradical, anti-inflammatory, and cytotoxic activity, and therefore we believe it has potential to be developed as a functional food.

Experimental Section

General Experimental Procedures. UV spectra were measured on a Perkin-Elmer Lambda 35 UV/vis spectrometer. NMR experiments were collected on a Bruker Avance AV300 NMR spectrometer operating at 300.13 MHz for ^{1}H and 75.48 MHz for ^{13}C using standard Bruker software. Mass spectra were obtained on a ThermoFinnigan LCQ utilizing both ESI and APCI in the positive and negative modes. HRESIMS was performed on a Micromass Q-TOF Ultima mass spectrometer. HPLC was done on a Waters 2695 using a Phenomenex Aqua column (250 × 4.6 mm, 5 μm) and monitored using a Waters 996 PDA scanning from 240 to 600 nm. Column chromatography was accomplished using Sephadex LH-20 (Pharmacia, 25−100 μm), reversed-phase C18 silica gel (J. T. Baker, 40 μm), and Diaion HP-20 (Mitsubishi, Japan). Separations were monitored using silica gel 60 F254 and RP18 F254 TLC plates (1 mm thickness, EM Science, Germany). Quercetin, rutin, cinnamic acid, *O*-coumaric acid, gallic acid, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma (St. Louis, MO). Isoquercitrin and myricitrin were previously isolated in our laboratory.[23]

Plant Material. Fruits of *M. cauliflora* were collected at the Fruit and Spice Park in Homestead, FL, immediately frozen, and shipped by overnight courier on dry ice to the laboratory, where they were kept in cold (−20 °C), dark storage until processed. A voucher specimen (Reynertson 39) was prepared, identified, and deposited at the Steere Herbarium of The New York Botanical Garden (Bronx, NY).

Extraction and Isolation Procedures. Deseeded fresh fruits (6.2 kg) were homogenized in a blender with MeOH, extracted exhaustively, and concentrated in vacuo at temperatures not exceeding 40 °C to give a thick syrup, which was diluted with water. The aqueous solution was separated over Diaion HP-20 and eluted using H_2O, MeOH, and acetone. The MeOH fraction was concentrated to give a residue (52 g). A portion (44 g) of that residue was subjected to Sephadex LH-20 column chromatography (∼125 g) in amounts of 8, 11, 12, and 13 g and eluted with formic acid−water−MeOH (1:9:10). Fractions from all four columns were recombined to give eight fractions (A−H). Fractions D (3.74 g) and E (111 mg) were chromatographed over Sephadex LH-20 and eluted using MeOH−formic acid (9:1). The recombined fraction A1 was then separated in a smaller Sephadex LH-20 column (12 g) using an isocratic system of acetonitrile−water. Fractions $A1_{35-59}$ were recombined as fraction A2 and subjected to reversed-phase C18 column chromatography (6 g) using 10% formic acid−acetonitrile (95:5 to 50:50; 5% gradient; 20 mL each eluant, fractions of 3 mL). Fractions $A2_{12-27}$ were recombined and subjected to final purification using Sephadex LH-20 and water−acetonitrile to give 16 mg of **1**. Compound **2** was isolated according to a similar scheme to give 10 mg.

Approximately 50 g of freeze-dried fruits was extracted in EtOH and subjected to LC-MS SIM analysis to address concerns that **1** might be a methyl ester artifact of **2** following extraction in MeOH. Analysis was performed in negative ESI mode, using a gradient of 0.1% formic acid (A) and acetonitrile (B) from 95% A to 50% A over 30 min, monitoring $[M - H]^-$ m/z 332 to 334.

Jaboticabin (methyl 2-[(3,4-dihydroxybenzoyloxy)-4,6-dihydroxyphenyl]acetate, 1): reddish, amorphous solid (MeOH); UV (MeOH) λ_{max} (log ϵ) 267.5 (3.35), 298.5 (3.16) nm; ^{1}H NMR (CD_3OD, 300.13 MHz) δ 7.55 (1H, dd, $J = 2.1$, 7.8 Hz, H-6′), 7.54 (1H, d, $J = 2.1$ Hz, H-2′), 6.88 (1H, d, $J = 7.8$ Hz, H-5′), 6.27 (1H, d, $J = 2.4$ Hz, H-5), 6.16 (1H, d, $J = 2.4$ Hz, H-3), 3.57 (3H, s, OCH_3-8), 3.50 (2H, s, H-7); ^{13}C NMR (CD_3OD, 75.48 MHz) δ 172.9 (C, C-8), 164.9 (C, C-7′), 157.2 (C, C-4), 157.0 (C, C-2), 151.1 (C, C-6), 145.0 (C, C-3′),

122.9 (CH, C-6′), 120.3 (C, C-1′), 116.4 (CH, C-2′), 114.7 (CH, C-5′), 105.7 (C, C-1), 100.7 (CH, C-5), 99.6 (CH, C-3), 50.9 (OCH_3-8), 28.5 (CH_2, C-7); ESIMS *m*/*z* 333 $[M - H]^-$ ($C_{16}H_{14}O_8$), HRESIMS *m*/*z* 357.0581 $[M + Na]^+$ (calcd for $C_{16}H_{14}O_8Na$, 357.0586).

DPPH Assay. The DPPH assay was performed on extracts, fractions, and purified compounds as previously described, using 400 μM DPPH.[9] Gallic acid was used as a positive control ($IC_{50} = 30.0 \pm 2.9$ μM).

IL-8 Immunoassay. Human SAE cells were cultured according to supplier instructions (Clonetics, CA) and maintained in a controlled atmosphere of air−5% CO_2 at 37 °C. Confluent SAE cells at passages 4−8 were used for experiments.

Cigarette smoke extract (CSE) was prepared using a modified protocol.[24] Briefly, a Barnet vacuum pump operating at constant flow was used to draw the smoke of one unfiltered 2R1 reference cigarette (University of Kentucky) through 25 mL of Dulbecco's phosphate-buffered saline. This solution (100% CSE) was adjusted to pH 7.4, filtered, diluted with small airway growth medium to a final concentration of 5%, and added to the cells immediately.

Cells were treated with 5% CSE or pure compounds (100 μM) or pretreated with pure compounds 30 min prior to 5% CSE exposure. After 24 h, measurement of human IL-8 in cell culture supernates was performed by ELISA (R&D Systems Inc., MN). Statistical analyses were performed by Student's *t*-test (two-sided) using the JMP Statistics software package (SAS Institute Inc., NC) and defined at the 5% level.

Cytotoxicity Assays. Colon cancer cell lines HT29, HCT116, and SW480 (10 000 cells) were plated into 24-well plates in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). After 24 h, cells were treated with six concentrations (1, 5, 10, 30, 50, and 70 μM) of compounds and incubated for 72 h under DMEM containing 1% FBS. The plates were washed with PBS once, and the attached cells were collected by tripsinization. The numbers of cells were counted using a Coulter Counter (Beckman Coulter Co., Fullerton, CA) as previously described.[25] EGCG and Poly E were used as positive controls (HT29 $IC_{50} = 27$ and 22 μg/mL, respectively).

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