New Bioactive Polyphenols from *Theobroma grandiflorum* ("Cupuaçu")

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Activity-guided fractionation of *Theobroma grandiflorum* ("cupuaçu") seeds resulted in the identification of two new sulfated flavonoid glycosides, theograndins I (**1**) and II (**2**). In addition, nine known flavonoid antioxidants, (+)-catechin, (−)-epicatechin, isoscutellarein 8-*O*-*β*-D-glucuronide, hypolaetin 8-*O*-*β*-D-glucuronide, quercetin 3-*O*-*β*-D-glucuronide, quercetin 3-*O*-*β*-D-glucuronide 6″-methyl ester, quercetin, kaempferol, and isoscutellarein 8-*O*-*β*-D-glucuronide 6″-methyl ester, were identified. Theograndin II (**2**) displayed antioxidant activity (IC_{50} = 120.2 *μ*M) in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical assay, as well as weak cytotoxicity in the HCT-116 and SW-480 human colon cancer cell lines with IC_{50} values of 143 and 125 *μ*M, respectively. While **1** was less active as an antioxidant than **2**, the known compounds were more potent in the DPPH assay (IC_{50} range 39.7−89.7 *μ*M).

Theobroma (Sterculiaceae) is a tropical American genus of 22 species of understory trees that grow in evergreen rainforests between latitudes 18° N and 15° S.[1] The genus is noteworthy because it includes the economically important "cacao" or chocolate tree (*Theobroma cacao* L.). The uses and cultivation of *T. cacao* were developed by the Mayans in Central America before the arrival of Europeans.[2] Within this genus, *Theobroma grandiflorum* (Willd. ex Spreng.) K. Schum., known commonly as "cupuaçu", is second to cacao in terms of economic importance. *T. grandiflorum* is a medium-sized tree, usually 6−10 m and up to 18 m tall. Its natural distribution is in the southern half of the State of Pará, Brazil, and the adjacent Amazonian Maranhão, where it grows at low densities in the rain forest.[1]

"Cupuaçu" produces the largest pods among all *Theobroma* species. In the past 20 years, "cupuaçu" has been developed into a commercial crop in Amazonia.[3] It is frequently cultivated in Brazil as well as in the warm lowlands of other tropical American countries such as Ecuador, Costa Rica, Venezuela, Colombia, Guyana, Surinam, and French Guiana. Although *T. grandiflorum* is morphologically distinct from *T. cacao*, the two species can be hybridized and the hybrids produce a high number of fruits. For that reason *T. grandiflorum* is considered to be one of the closest relatives of *T. cacao*.[4] Recent phytochemical studies have demonstrated that *T. cacao* seeds contain potent polyphenolic antioxidants including (−)-epicatechin and (+)-catechin.[5,6] The effects of chocolate on human health are being studied with considerable interest.[7−9] Our current research on the antioxidant constituents of "cupuaçu" is an outgrowth of this extensive research on chocolate.

The "cupuaçu" fruit is appreciated for its acidic and strongly aromatic pulp that surrounds the seed. Because of its strong flavor, the fruit pulp is not typically consumed alone, but rather is used as an ingredient in various food products. This pulp is used to prepare drinks (e.g., "vinho do cupuaçu" and "suco de cupuaçu"), ice cream, liquors, jellies, preserves, and candy. In the United States, "cupuaçu" has been used in certain popular mixed fruit juice drinks. Moreover, the seeds of *T. grandiflorum* have received attention because of their potential for being used as a chocolate substitute.[10] The composition of *T. grandiflorum* seed oil has been studied, and various lipids and alkaloids have been identified, such as the xanthine alkaloids, caffeine, and theobromine.[11,12]

As part of our continuing study of polyphenols from lesser-used tropical fruits, we have examined the antioxidant and cytotoxic activities of *T. grandiflorum*. An alcohol extract of *T. grandiflorum* seeds was subjected to activity-guided fractionation using the DPPH method, and two new polyphenols, theograndins I (**1**) and II (**2**), were identified.

The seeds of *T. grandiflorum* were extracted exhaustively with MeOH and partitioned sequentially with hexane, EtOAc, and BuOH. The EtOAc and BuOH fractions were subjected to activity-guided fractionation using the DPPH method, with an initial separation by Sephadex LH-20 column chromatography (CC). Further separation of active fractions over reversed-phase (RP-18) CC and Sephadex LH-20 CC yielded two new sulfated flavonoid glycosides, **1** and **2**.

Compound **1**, a yellow powder, gave a molecular ion at *m*/*z* 541.0297 corresponding to $[M - H]^-$ in the negative HRESIMS, which indicated a molecular formula of $C_{21}H_{18}O_{15}S$. Compound **1** displayed four main peaks in the negative ESIMS at *m*/*z* 541 $[M - H]^-$, 461 $[M - SO_3 - H]^-$, 286 $[M - SO_3 - GlcA - H]^-$, and 255 $[M - 286 - H]^-$ and UV absorbance maxima at 271 and 335 nm, which suggested that **1** is a monosulfated flavonoid glycoside.[13,14] Acid hydrolysis of **1** confirmed the presence of glucuronic acid by comparison with a commercial sugar standard.[15] The sulfate ion was verified after acid hydrolysis by precipitating the aqueous layer with $BaCl_2$.[13] The ^1H and ^{13}C NMR data (Table 1) of **1** were similar to those of isoscutellarein 8-*O*-*β*-D-glucuronopyranoside,[16] except for the downfield chemical shifts observed for H-3″ (+1.00 ppm) and C-3″ (+8.3 ppm), showing that the sulfate group is linked to the C-3″ hydroxyl of the glucuronic acid

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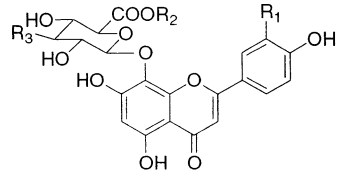
Table 1. NMR Data of Theograndins I (**1**) and II (**2**)

	1				**2**			
C/H	δ_C	δ_H (int., mult., *J* in Hz)	$^1H-^1H$ COSY (^1H No.)	HMBC (^{13}C No.)	δ_C	δ_H (int., mult., *J* in Hz)	$^1H-^1H$COSY (^1H No.)	HMBC (^{13}C No.)
2	166.8 s				167.2 s			
3	105.2 d	6.62 (1H, s)		1′, 10	103.8 d	6.62 (1H, s)		1′, 10
4	183.9 s				183.7 s			
5	159.3 s				158.9 s			
6	100.5 d	6.26 (1H, s)		8, 10	100.7 d	6.30 (1H, s)		8, 10
7	159.1 s				158.9 s			
8	126.9 s				127.3 s			
9	151.2 s				151.2 s			
10	103.2 s				104.8 s			
1′	122.8 s				123.5 s			
2′	130.3 d	8.10 (1H, d, 8.8)	3′	2, 4′, 6′	114.5 d	7.71 (1H, d, 2.3)		2, 4′, 6′
3′	117.2 d	6.96 (1H, d, 8.8)	2′	1′, 5′	146.9 s			
4′	162.9 s				150.7 s			
5′	117.2 d	6.96 (1H, d, 8.8)	6′	1′, 3′	116.8 d	6.92 (1H, d, 8.0)	6′	1′, 3′
6′	130.3 d	8.10 (1H, d, 8.8)	5′	2, 2′, 4′	120.9 d	7.43 (1H, dd, 8.0, 2.3)	5′	2, 2′, 4′
1″	107.8 d	4.86 (1H, d, 8.0)	2″	3″, 5″, 8	108.3 d	4.80 (1H, d, 7.6)	2″	3″, 5″, 8
2″	74.1 d	3.89 (1H, m)	1″, 3″	4″	74.1 d	3.90 (1H, m)	1″, 3″	4″
3″	84.4 d	4.38 (1H, t, 9.2)	2″, 4″	1″, 5″	84.2 d	4.39 (1H, t, 9.0)	2″, 4″	1″, 5″
4″	72.1 d	3.89 (1H, m)	3″, 5″	2″, 6″	71.8 d	3.90 (1H, m)	3″, 5″	2″, 6″
5″	78.9 d	3.80 (1H, d, 9.6)	4″	1″, 3″	79.4 d	3.82 (1H, d, 9.6)	4″	1″, 3″
6″	174.8 s				176.0 s			

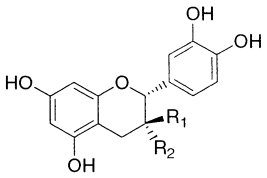
moiety.[16,17] Furthermore, negative ESIMS displayed a fragment at *m*/*z* 255 $[M - 286 - H]^-$, indicative of the loss of the isoscutellarein aglycon. The location of the glycosyl linkage was determined to be at C-8 since the anomeric proton signal at δ 4.86 showed a HMBC correlation with an oxygenated quaternary carbon at δ 126.9, assignable to C-8 on the basis of the correlation with H-6 (δ 6.26). In the ^1H NMR spectrum, the coupling constant of H-1″ (J = 8.0 Hz) confirmed a β-glycosyl linkage.[16] The "D" configuration of glucuronic acid was determined after acid hydrolysis of **1** by isolating the sugar using preparative HPLC and measuring the purified sugar's optical rotation. From these results, theograndin I (**1**) was determined to be isoscutellarein 8-*O*-β-D-glucuronopyranoside 3″-*O*-sulfate.

Compound **2** was obtained as a yellow powder, and HRESIMS of **2** in the negative mode gave *m*/*z* 557.0231 $[M - H]^-$, indicating a molecular formula of $C_{21}H_{18}O_{16}S$. The molecular formula of **2** differs from **1** by one additional oxygen atom. The ^1H NMR spectrum of **2** was similar to that of **1**, except that the spectrum of **2** displayed an AMX spin system comprised of signals at δ 7.71 (1H, d, J = 2.3 Hz), 7.43 (1H, dd, J = 8.0, 2.3 Hz), and 6.92 (1H, d, J = 8.0 Hz). The ^1H and ^{13}C NMR data (Table 1) indicated that the flavonoid skeleton for **2** is hypolaetin.[16] In a manner similar to **1**, acid hydrolysis of **2** confirmed the presence of a sulfate group and D-glucuronic acid. Comparison of the ^1H and ^{13}C data of the glucuronic acid moiety of **2** with those of **1** and hypolaetin 8-*O*-β-D-glucuronopyranoside[16] demonstrated that the sulfate group of **2** is connected to C-3″, and the HMBC experiment confirmed the D-glucuronic acid to be at the C-8 position. Therefore, theograndin II (**2**) was elucidated as hypolaetin 8-*O*-β-D-glucuronopyranoside 3″-*O*-sulfate.

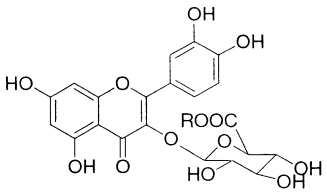
In addition to the two new sulfated flavonoid glycosides, four known flavonoids, (+)-catechin (**3**), (−)-epicatechin (**4**), quercetin (**9**), and kaempferol (**10**), were identified from *T. grandiflorum* seeds by comparing them with authentic compounds by RP-18 TLC and ESIMS. Using MS and NMR data from the literature, five known flavonoids, isoscutellarein 8-*O*-β-D-glucuronide (**5**),[16] hypolaetin 8-*O*-β-D-glucuronide (**6**),[16] quercetin 3-*O*-β-D-glucuronide (**7**),[18] quercetin 3-*O*-β-D-glucuronide 6″-methyl ester (**8**),[18] and isoscutellarein 8-*O*-β-D-glucuronide 6″-methyl ester (**11**),[19] were identified from *T. grandiflorum* seeds.



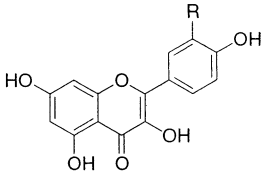
1 R_1 = H R_2 = H R_3 = OSO_3H
2 R_1 = OH R_2 = H R_3 = OSO_3H
5 R_1 = H R_2 = H R_3 = OH
6 R_1 = OH R_2 = H R_3 = OH
11 R_1 = H R_2 = Me R_3 = OH



3 R_1 = OH R_2 = H
4 R_1 = H R_2 = OH



7 R = H
8 R = Me



9 R = OH
10 R = H

Theograndin I (**1**) showed low antioxidant activity (IC_{50} = 341.1 μM) in the DPPH free-radical assay and displayed weak cytotoxicity in the HCT-116 and SW-480 human colon cancer cell lines with IC_{50} values of 205 and 164 μM, respectively. Theograndin II (**2**) displayed higher antioxidant activity (IC_{50} = 120.2 μM) in the DPPH free-radical assay and cytotoxicity in the HCT-116 and SW-480 human colon cancer cell lines with IC_{50} values of 143 and 125 μM, respectively. The nine known compounds, **3**−**11**, were also screened for their antioxidant capacities in the DPPH assay, and all displayed high antioxidant activity, with IC_{50} values of 49.0, 49.0, 68.1, 58.2, 44.4, 41.6, 39.7, 89.7, and 69.2 μM, respectively. Therefore, the order of antioxidant potency, as defined by IC_{50} values in the DPPH assay, was quercetin (**9**) > quercetin 3-*O*-β-D-glucuronide (**7**) ≈ quercetin 3-*O*-β-D-glucuronide 6″-methyl ester (**8**) > (+)-catechin (**3**) = (−)-epicatechin (**4**) > hypolaetin 8-*O*-β-D-glucuronide (**6**) > isoscutellarein 8-*O*-β-D-glucuronide (**5**) ≈ isoscutellarein 8-*O*-β-D-glucuronide 6″-methyl ester (**11**) > kaempferol (**10**) > theograndin II (**2**) > theograndin I (**1**). These results are in agreement with previous reports of structure−activity relationship of antioxidant polyphenols. For example, increasing the number of hydroxyl groups in a flavonoid often increases antioxidant activity.[20] The *O*-dihydroxy structure in the B ring of a flavonoid confers higher stability to the radical form and participates in electron delocalization,[21] and thus the dihydroxylation in the 3′, 4′ positions of the B ring plays an important role in antioxidant activity, as in both (+)-catechin (**3**) and (−)-epicatechin (**4**). The glycosylation of flavonoids may reduce their antioxidant activity when compared to that of the corresponding aglycons, such as quercetin (**9**) and its glycoside quercetin 3-*O*-β-D-glucuronide (**7**).[22] The unsaturation in the C ring as in quercetin (**9**) and kaempferol (**10**) allows electron delocalization across the molecule for stabilization of aryloxy radicals due to existing conjugation.[21] The C-3 and C-5 hydroxyl groups with a 4-oxo function in the A and C rings allow for maximum radical-scavenging potential, such as in quercetin (**9**).[21]

The antioxidant activities of sulfate flavonoid glycosides **1** and **2** are significantly less than those of their corresponding flavonoid glycosides **5** and **6**. The decreased antioxidant activity may be due to the inter- and intramolecular hydrogen bond formed between the oxygen of the sulfated group and the hydrogen of the hydroxyl group connected to a flavonoid skeleton. This hydrogen bond may prevent the ionization of hydrogen from the hydroxyl group and reduce the available hydrogen donors and therefore decrease the antioxidant activity of sulfated flavonoid glycosides. The low water solubility of many flavonoids limits their therapeutic application.[15] Sulfated flavonoid glycosides are highly soluble in water, although the antioxidant activities of sulfated flavonoid glycosides are less than their corresponding flavonoids and flavonoid glycosides. The sulfated flavonoid glycosides might improve bioavailability because they are both easily dissolved in water and hydrolyzed to their corresponding flavonoid glycosides and flavonoids; however, in vivo and clinical studies are needed to understand the significance of the sulfated flavonoid glycosides to human health.

Experimental Section

General Experimental Procedures. Melting points were determined on a Mel-Temp II melting point apparatus (Laboratory Devices Inc., Holliston, MA) and are uncorrected. Optical rotations were measured on an Autopol III automatic polarimeter (Rudolph Research Analytical, Flanders, NJ). UV spectra were measured on a Lambda 2 UV/vis spectrophotometer (Perkin-Elmer, Boston, MA). ^{1}H NMR and ^{13}C NMR spectra were recorded using a JEOL GX-400 MHz, operating at 400 and 100 MHz, respectively. 2D NMR experiments were run on a Varian Inova 400 MHz. All compounds were measured in CD_3OD. ESIMS was performed with a ThermoFinnigan LCQ instrument (San Jose, CA) equipped with Xcalibur software. Samples were dissolved in MeOH and introduced by direct injection. The capillary voltage was 10 V, the spray voltage was 4.5 kV, and the tube lens offset was 0 V. The capillary temperature was 230 °C. HRESIMS was performed on a 70-SE-4F mass spectrometer (Micromass). Samples were dissolved in MeOH. HPLC analyses were carried out on a Waters 2690 separations module equipped with a Waters 996 photodiode array detector and Waters Millenium^{32} software using a Phenomenex Aqua C_{18} column (4.6 × 250 mm, 5 μm) and a solvent system of 5:95 to 50:50 MeCN/H_2O linear gradient, a flow rate of 1 mL/min, column at room temperature, 20 min run time for analysis of subfractions, and an isocratic solvent system of MeCN/H_2O (70:30), flow rate of 1 mL/min, column at room temperature, 10 min run time for sugar identification. Preparative HPLC was carried out using a Waters 600 controller with a Waters 486 tunable absorbance detector and Waters Empower software with a Phenomenex Nucleosil 10 C_{18} column (21.1 × 250 mm, 10 μm) and an isocratic solvent system of MeCN/H_2O (70:30), a flow rate of 5 mL/min, column at room temperature, and 30 min run time. TLC analyses were performed on RP-18 F_{254} plates (Merck, Darmstadt, Germany), with compounds visualized by spraying with 1.0 g of vanillin in 10 mL of H_2SO_4 (concentrated) and 90 mL of EtOH. Sephadex LH-20 (25−100 μm; Pharmacia Fine Chemicals, Piscataway, NJ) and C_{18} reversed-phase silica gel (40 μm; J. T. Baker, Phillipsburg, NJ) were used for column chromatography.

Plant Material. *Theobroma grandiflorum* fruits were collected in Cayenne, French Guiana (May 1999), and identified by Dr. Scott Mori, Nathaniel Lord Britton Curator of Botany at The New York Botanical Garden.

Extraction and Isolation. The dried-powder seeds (962.3 g) of *T. grandiflorum* were extracted with MeOH three times at room temperature. The MeOH was removed in vacuo, and the resulting brown extract (60.8 g) was suspended in H_2O and partitioned sequentially with hexane, EtOAc, and BuOH. The hexane (Fr_H, 12.8 g), EtOAc (Fr_{EA}, 5.5 g), and BuOH (Fr_B, 9.8 g) extracts were tested in the DPPH assay, and their IC_{50} values were calculated as 1204.8, 40.9, and 137.0 μg/mL, respectively.

Fraction Fr_{EA} was subjected to column chromatography over Sephadex LH-20 (100.0 g) and eluted with an isocratic system using MeOH, and 25 fractions (50 mL) were collected. Fractions were combined based upon HPLC analysis to 11 subfractions (Fr_{EA1-11}). Fraction Fr_B was separated and combined in a similar fashion, and nine subfractions (Fr_{B1-9}) were obtained.

Fraction Fr_{B7} (1.2 g) was separated by RP-18 (50.0 g) CC eluting with a gradient system of 1:9 to 1:0 MeOH/H_2O to yield six subfractions (Fr_{B7a-f}), and two of these subfractions, Fr_{B7b} (100.2 mg) and Fr_{B7e} (163.5 mg), were purified by Sephadex LH-20 (10.0 g) CC eluting with an isocratic system of MeOH to obtain theograndin I (**1**) (5.0 mg) and isoscutellarein 8-*O*-β-D-glucuronide 6″-methyl ester (**11**) (7.0 mg), respectively.

Fraction Fr_{B9} (1.6 g) was separated by RP-18 (50.0 g) CC eluting with a gradient system of 1:9 to 1:0 MeOH/H_2O to yield seven subfractions (Fr_{B9a-h}), and subfraction Fr_{B9d} (230.0 mg) was purified by Sephadex LH-20 (10.0 g) CC eluting with an isocratic system of MeOH to obtain theograndin II (**2**) (7.2 mg).

The fractions Fr_{EA3} (0.9 g), Fr_{EA4} (0.6 g), Fr_{EA8} (2.1 g), and Fr_{B1} (0.5 g) were separated by RP-18 (50.0 g) CC and Sephadex LH-20 (10.0 g) CC, using a gradient system of 1:9 to 1:0 MeOH/H_2O for RP-18 CC and an isocratic system of MeOH for Sephadex LH-20 CC to yield (+)-catechin (**3**) (8.6 mg) and (−)-epicatechin (**4**) (6.2 mg) from Fr_{EA3}, to afford isoscutellarein 8-*O*-β-D-glucuronide (**5**) (3.4 mg) from Fr_{EA4}, to obtain hypolaetin 8-*O*-β-D-glucuronide (**6**) (277.6 mg), quercetin 3-*O*-β-D-

glucuronide (**7**) (5.8 mg), quercetin 3-*O*-*β*-D-glucuronide 6″-methyl ester (**8**) (3.6 mg), and quercetin (**9**) (12.2 mg) from Fr_{EA8}, and to give kaempferol (**10**) (4.2 mg) from Fr_{B1}.

Theograndin I (Isoscutellarein 8-*O*-*β*-D-glucuronopyranoside 3″-*O*-sulfate) (1): yellow powder, mp 164.2−165.8 °C; $[\alpha]_D^{20}$ +62.35° (*c* 0.00085, MeOH); UV (MeOH) λ_{max} (log ϵ) 271 (4.1), 335 (4.2) nm; ^{1}H and ^{13}C NMR, see Table 1; negative ESIMS *m*/*z* 541 $[M - H]^-$, 461 $[M - SO_3 - H]^-$, 286 $[M - SO_3 - GlcA - H]^-$, 255 $[M - 286 - H]^-$; negative HRESIMS *m*/*z* 541.0297 $[M - H]^-$ (calcd for $C_{21}H_{17}O_{15}S$, 541.0288).

Theograndin II (Hypolaetin 8-*O*-*β*-D-glucuronopyranoside 3″-*O*-sulfate) (2): yellow powder, mp 157.0−158.6 °C; $[\alpha]_D^{20}$ +110° (*c* 0.0008, MeOH); UV (MeOH) λ_{max} (log ϵ) 270 (4.0), 355 (3.9) nm; ^{1}H and ^{13}C NMR, see Table 1; negative ESIMS *m*/*z* 557 $[M - H]^-$, 477 $[M - SO_3 - H]^-$, 301 $[M - SO_3 - GlcA - H]^-$, 255 $[M - 302 - H]^-$; negative HRESIMS *m*/*z* 557.0231 $[M - H]^-$ (calcd for $C_{21}H_{17}O_{16}S$, 557.0237).

Acid Hydrolysis. Compounds **1** (0.5 mg) and **2** (0.3 mg) were each refluxed separately in a mixture of MeOH (1 mL) and 2% HCl (1 mL) for 2 h.[15] After evaporation of MeOH, the aglycons were extracted with EtOAc; the water layer was neutralized with a saturated solution of $NaHCO_3$. The water layers of **1** and **2** were purified by preparative HPLC to yield 0.12 and 0.09 mg of sugar, respectively. The sugar constituent of both **1** and **2** was determined to be D-glucuronic acid by comparison of their HPLC retention times with that of an authentic compound (t_R: 2.3 min) and measurement of their optical rotations (positive). In addition, the presence of the sulfate ions in the water layers of **1** and **2** was confirmed by precipitating with $BaCl_2$.[13]

DPPH Free-Radical Scavenging Assay. The DPPH assay was performed on fractions and purified isolates as previously described.[23] Reaction mixtures containing test samples (dissolved in DMSO) and 300 μM DPPH ethanolic solution in 96-well microtiter plates were incubated at 37 °C for 30 min, and absorbances were measured at 515 nm. Final concentrations of test materials were typically in a range from 1 to 50 μg/mL. Percent inhibition by sample treatment was determined by comparison with a DMSO-treated control group. IC_{50} values denote the concentration of sample required to scavenge 50% DPPH free radicals.

Cell Culture. HCT-116 and SW-480 human colon cancer cells were maintained in Dulbecco's modified Eagle medium (Gibco-BRL, Grand Island, NY) with 10% fetal bovine serum (Gibco) in a normal atmosphere with 5% CO_2 at 37 °C. Cells were grown to 40% confluence prior to treatment with polyphenols. All cultures were passaged weekly, and the media was changed three times a week. No antibiotics were added at any time during the experiments. In all experiments, compounds to be studied were dissolved in DMSO and added to the medium at the start of the incubation.

Microtetrazolium (MTT) Assay. The MTT assay (Boeringher-Mannheim, Indianapolis, IN) was carried out according to the manufacturer's instructions. In brief, about 30 000 cells were plated in 96-well flat-bottom plates in 100 μL of medium. When cells reached 40% confluence, the medium was changed and cells were exposed to the plant extracts or isolates. After 72 h, cells were washed three times with PBS followed by the addition of 100 μL of Dulbecco's modified Eagle medium, and 10 μL of 5 mg/mL MTT solution in PBS was added to each well for 4 h. Finally, 100 μL of MTT solubilization solution was added to each well to dissolve the formazan crystals. The absorbance at 570 nm was determined using a Biokinetics plate reader (Bio-Tek Instruments Inc., Winooski, VT). Octuplicate wells were assayed for each condition, and mean as well as standard deviations were determined. The IC_{50} values were determined by linear regression analysis.

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