Bioactive Novel Polyphenols from the Fruit of *Manilkara zapota* (Sapodilla)

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Activity-guided fractionation of a methanol extract from the fruit of *Manilkara zapota* cv. Tikal resulted in the isolation of two new antioxidants, methyl 4-*O*-galloylchlorogenate (**1**) and 4-*O*-galloylchlorogenic acid (**2**), along with eight known polyphenolic antioxidants, namely, methyl chlorogenate (**3**), dihydromyricetin (**4**), quercitrin (**5**), myricitrin (**6**), (+)-catechin (**7**), (−)-epicatechin (**8**), (+)-gallocatechin (**9**), and gallic acid (**10**). Of the 10 polyphenols, **1** showed the highest antioxidant activity (IC_{50} = 12.9 μM) in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical assay and displayed cytotoxicity in the HCT-116 and SW-480 human colon cancer cell lines with IC_{50} values of 190 and 160 μM, respectively. Compound **2** showed high antioxidant activity (IC_{50} = 23.5 μM) in the DPPH free-radical assay and displayed cytotoxicity in the HCT-116 and SW-480 human colon cancer cell lines with IC_{50} values of 154 and 134 μM, respectively.

In living systems, free radicals are formed constantly and can cause oxidative damage to DNA, lipids, and proteins, and may lead to certain diseases such as coronary heart disease and cancer.[1] Antioxidants, which scavenge free radicals, can prevent the oxidation of biomolecules, such as low-density lipoprotein and DNA, and may thereby break the reaction chains of pathogenesis of coronary heart disease and cancer.[2] High fruit consumption has been associated with lower incidence and mortality rates of coronary heart disease and cancer,[3] and dietary antioxidants have been shown to be beneficial in reducing the risk of cognitive dysfunction in aged humans.[4] This phenomenon has been attributed to the various antioxidants contained in fruit, which are a rich source of natural antioxidants.[3] In addition to the classical nutrient antioxidants (e.g., vitamins C and E and β-carotene), fruits contain polyphenolic antioxidants, which may play an important role in the overall antioxidant activity of fruits.[5]

As well as antioxidant activity, polyphenols have other mechanisms of action that may be important in disease prevention. Epigallocatechin gallate (EGCG), for example, inhibits cell growth and the activation of the epidermal growth factor receptor (EGFR),[6] causes G1 arrest of the cell cycle, and induces apoptosis in human prostate, lung, colon, gastric carcinoma, leukemia, squamous cell carcinoma, and head and neck cancer cell lines.[7] EGCG inhibits activation of growth factor receptors and extracellular signal-regulated protein kinase (ERK) proteins and also inhibits activator protein-1 (AP-1) activity and c-*fos* promoter activity.[7] We are interested in identifying cytotoxic polyphenols from edible plants that may be important in cancer prevention by inhibiting the activation of the EGFR.

The tropical fruit tree *Manilkara zapota* (L.) P. Royen (Sapotaceae), known commonly as sapodilla or naseberry, is native to Mexico (Yucatan Peninsula) and Central America. The sapodilla is a slow-growing, long-lived tree and can reach a height of 12−18 m.[8] The ripe fruit range from nearly round to spindle-shaped, 5−10 cm in diameter, edible though grainy, and has a sweet pleasant flavor resembling that of a pear.[9] Cultivated varieties, such as *M. zapota* cv. Tikal, have been developed for enhanced flavor and edible skin.[9] Sapodilla fruit is often eaten fresh, but the pulp is also incorporated into sherbets, milkshakes, and ice cream.[8] The nutrient value of sapodilla fruit (100 g) includes 0.4 g of protein, 1.1 g of fat, 20 g of carbohydrate, 5.3 g of total dietary fiber, 210 mg of calcium, 0.8 mg of iron, 12.0 mg of magnesium, 12.0 mg of phosphorus, 193.0 mg of potassium, 12.0 mg of sodium, and 14.7 mg of vitamin C, and a total calorie count of 83 kcal.[8] The gummy latex of sapodilla, called chicle, is used to make chewing gums, and the fruit is used to treat diarrhea and pulmonary diseases.[9,10] The leaves have been used to treat cough, cold, and diarrhea, and the bark is used to treat diarrhea and dysentery.[9]

The polyphenols catechin, epicatechin, leucocyanidin, leucodelphinidin, leucopelargonidin, chlorogenic acid, and gallic acid have been reported previously from unripe sapodilla fruit.[11−13] From ripe fruit, the level of 5-caffeoyl quinic acid was measured recently in the peel (94.6 ± 9.50 mg/kg) and the pulp (32.1 ± 18.7 mg/kg).[14] The antioxidant activity of sapodilla fruit has been reported to be very high in the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay (AEAC = 3396 ± 387.9 mg/kg).[15] This paper describes the isolation and structural elucidation of two new chlorogenic acid derivatives from *M. zapota* with antioxidant and cytotoxicity activities.

The fresh frozen *M. zapota* cv. Tikal fruit was extracted with MeOH and partitioned with hexane and EtOAc sequentially. The EtOAc-soluble fraction displayed high antioxidant activity in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and cytotoxicity against the HCT-116 and SW-480 human colon cancer cell lines. Activity-guided fractionation of the EtOAc-soluble fraction was performed to isolate the antioxidant constituents. The EtOAc-soluble fraction was separated by Sephadex LH-20 column chromatography (CC). Fractions were combined according to

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Table 1. ^{1}H and ^{13}C NMR Data for **1** and **2** (100 and 400 MHz, CD_3OD)

	1			**2**		
C/H	δ_C	δ_H (int., mult., *J* in Hz)	HMBC (^{13}C No.)	δ_C	δ_H (int., mult., *J* in Hz)	HMBC (^{13}C No.)
1	74.5			76.1		
2	37.2	2.12−2.23 (2H, m)	4, 5, 7	37.6	2.05−2.25 (2H, m)	4
3	67.2	4.41 (1H, m)		69.2	4.39 (1H, m)	4
4	73.8	5.18 (1H, dd, 8.1, 2.9)	2, 3, 6, 7″	76.1	5.14 (1H, dd, 9.9, 2.9)	3, 7″
5	67.8	5.59 (1H, m)		68.2	5.75 (1H, m)	4, 9′
6	37.2	2.12−2.23 (2H, m)	4, 7	39.3	2.05−2.25 (2H, m)	3, 4
7	174.0			179.1		
1′	126.3			126.4		
2′	113.9	7.01 (1H, d, 2.2)	4′, 6′, 7′	113.8	6.98 (1H, d, 2.2)	4′, 6′, 7′
3′	145.2			145.1		
4′	148.4			148.2		
5′	115.3	6.77 (1H, d, 8.2)	1′, 3′, 4′, 6′	115.2	6.74 (1H, d, 8.2)	1′, 3′, 4′
6′	121.9	6.91 (1H, dd, 8.2, 2.2)	2′, 4′, 5′, 7′	121.8	6.88 (1H, dd, 8.2, 2.2)	2′, 4′, 7′
7′	146.4	7.50 (1H, d, 16.0)	1′, 2′, 6′, 9′	146.0	7.49 (1H, d, 15.8)	2′, 6′, 9′
8′	113.3	6.16 (1H, d, 16.0)	1′, 9′	113.6	6.17 (1H, d, 15.8)	1′, 9′
9′	166.7			167.3		
1″	119.9			120.1		
2″	109.2	7.12 (1H, s)	1″, 3″, 4″, 7″	109.1	7.10 (1H, s)	1″, 3″, 4″, 7″
3″	145.5			145.4		
4″	138.8			138.6		
5″	145.5			145.4		
6″	109.2	7.12 (1H, s)	1″, 4″, 5″, 7″	109.1	7.10 (1H, s)	1″, 4″, 5″, 7″
7″	166.5			166.7		
OCH_3	51.9	3.72 (3H, s)	7			

their reversed-phase C18 (RP18) thin-layer chromatography (TLC) profiles to give 11 combined fractions in total (A−K). All fractions were tested in the DPPH assay. Active fractions A, B, and K were further separated by RP18 CC. Two new antioxidants, **1** and **2**, were isolated from fraction B.

Compound **1** was obtained as a yellow amorphous powder. In the positive ion HRFABMS, a peak corresponding to $[M + H]^+$ was observed at *m*/*z* 521.1294, showing the molecular formula of **1** to be $C_{24}H_{24}O_{13}$. The ^{1}H NMR spectrum of **1** displayed an AMX spin system consisting of δ 7.01 (1H, d, J = 2.2 Hz), 6.77 (1H, d, J = 8.2 Hz), and 6.91 (1H, dd, J = 8.2, 2.2 Hz), thereby indicating a 1′,3′,4′-trisubstituted benzene ring.[16] The presence of a *trans* double bond was noted in the ^{1}H NMR spectrum of **1**, δ 6.16 (1H, d, J = 16.0 Hz) and 7.50 (1H, d, J = 16.0 Hz), suggesting the presence of a *trans* caffeoyl moiety in **1**. A gallic acid moiety in **1** was determined by ^{1}H NMR δ 7.12 (2H, s, H-2″ and 6″) and ^{13}C NMR, δ 166.5 (C-7″), 145.5 (C-3″ and 5″), 138.8 (C-4″), 119.9 (C-1″), 109.2 (C-2″). The ^{1}H and ^{13}C NMR data of **1** are consistent with previously published data for methyl chlorogenate and gallic acid.[17−19] HMBC correlations (Table 1) clearly revealed that H-4 (δ 5.18) in the quinic acid moiety of chlorogenic acid is long-range coupled to the C-7″ carbonyl group (δ 166.5) of gallic acid. Therefore, the gallic acid moiety is attached at the 4 position of the quinic acid moiety. In addition, the *O*-methyl protons (δ 3.72) are long-range coupled to the C-7 carbonyl group (δ 174.0) in the quinic acid moiety of chlorogenic acid. This indicates the methyl group is attached at the 7 position of the quinic acid moiety.

Analysis of ^{1}H NMR experiments was used to determine the stereochemistry of **1**. The ^{1}H NMR spectrum of **1** displayed H-4 as a dd at δ 5.18 with J values of 2.9 and 8.1 Hz, indicating *axial*−*axial* (J = 8.1 Hz) and *axial*−*equatorial* (J = 2.9 Hz) couplings. Homodecoupling experiments were carried out on **1** by irradiating H-3 (δ 4.41) and H-5 (δ 5.59). The large coupling of H-4 and H-5 (J = 8.1 Hz) demonstrates *axial*−*axial* coupling, and the small coupling of H-3 and H-4 (J = 2.9 Hz) shows *axial*−*equatorial* coupling. These results confirm **1** has the same stereochemistry in its quinic acid moiety as chlorogenic acid.[20] Therefore, **1** was identified as methyl 4-*O*-galloylchlorogenate. The configuration of chlorogenic acid derivatives is of biological significance, as in the protection against lipid peroxidation[21] and as inhibitors of hepatic glucose-6-phosphate translocase,[22] but there is confusion in the literature on the nomenclature of these compounds.[23] Previous studies have found the quinic acid moiety of chlorogenic acid to be in the chair conformation,[23] and this conclusion is further supported by the NMR data of **1**.

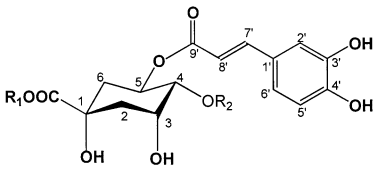
Compound **2**, a yellow amorphous powder, displayed a peak at *m*/*z* 507.1135 in the positive ion HRFABMS, corresponding to $[M + H]^+$, indicating a molecular formula of $C_{23}H_{22}O_{13}$. In a manner similar to **1**, homodecoupling experiments were carried out on **2**. The results showed that **2** has the same stereochemistry in the quinic acid moiety as chlorogenic acid.[20] Compound **2** has 1D and 2D NMR data similar to those of **1**, except **2** does not display chemical shifts for an *O*-methyl group (Table 1). Therefore, **2** was identified as 4-*O*-galloylchlorogenic acid.

The possibility that **1** is a MeOH extraction artifact of **2** was studied by extracting *M. zapota* cv. Tikal fruit with 95% EtOH and partitioning with hexane and EtOAc sequentially. The EtOAc-soluble fraction was separated by Sephadex LH-20 CC with 95% EtOH. By TLC, fraction I from the Sephadex separation contained a compound with the same R_f value as **1**. Fraction I was further analyzed by liquid chromatography−electrospray ionization mass spectrometry (LC-ESIMS) in the negative mode, and a peak with retention time 11.7 min displayed an ion with *m*/*z* 519 $[M - H]^-$. Under the same LC-MS conditions, standard compound **1** also had a retention time 11.7 min and displayed an ion with *m*/*z* 519 $[M - H]^-$. These results confirmed the presence of **1** in the EtOH extract of sapodilla fruit, and therefore we conclude that **1** is not a MeOH extraction artifact of **2**.

In the DPPH assay, **1** and **2** showed high antioxidant activity, IC_{50} = 12.9 and 23.5 μM, respectively. The IC_{50} value of commercially available chlorogenic acid is 39.5 μM. Thus, the addition of a gallic acid moiety to chlorogenic acid improves the latter's antioxidant activity. In the microtetrazolium (MTT) assay, **1** displayed cytotoxicity against the HCT-116 and SW-480 human colon cancer cell

lines with IC_{50} values of 190 and 160 μM, respectively. Compound **2** displayed cytotoxicity in the HCT-116 and SW-480 human colon cancer cell lines with IC_{50} values of 154 and 134 μM, respectively. EGCG, known to inhibit the activation of the EGFR,[7] displayed IC_{50} values of 161 and 195 μM in the HCT-116 and SW-480 human colon cancer cell lines, respectively. Chlorogenic acid displayed cytotoxicity against the HCT-116 and SW-480 human colon cancer cell lines with IC_{50} values of 367 and 353 μM, respectively. Thus the addition of a gallic acid moiety to chlorogenic acid results in a more cytotoxic compound.

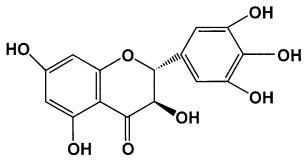
Eight additional known antioxidants, namely, methyl chlorogenate (**3**), dihydromyricetin (**4**), quercitrin (**5**), myricitrin (**6**), (+)-catechin (**7**), (−)-epicatechin (**8**), (+)-gallocatechin (**9**), and gallic acid (**10**), were isolated from fractions A, B, and K and identified by spectroscopic methods.



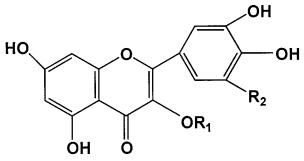
1 $R_1 = CH_3$ $R_2 = R$ methyl 4-*O*-galloylchlorogenate

2 $R_1 = H$ $R_2 = R$ 4-*O*-galloylchlorogenic acid

3 $R_1 = CH_3$ $R_2 = H$ methyl chlorogenate

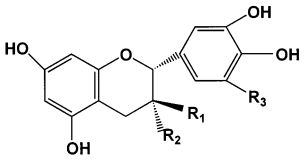


4 Dihydromyricetin



5 R_1 = Rha $R_2 = H$ quercitrin

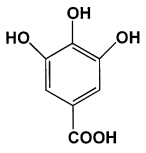
6 R_1 = Rha $R_2 = OH$ myricitrin



7 R_1= OH $R_2 = H$ $R_3 = H$ (+)-catechin

8 R_1 =H $R_2 = OH$ $R_3 = H$ (-)-epicatechin

9 $R_1 = OH$ $R_2 = H$ $R_3 = OH$ (+)-gallocatechin



10 Gallic acid

Experimental Section

General Experimental Procedures. Melting points were determined on a Mel-Temp II melting point apparatus (Laboratory Devices) and are uncorrected. Optical rotations were measured on an Autopol II automatic polarimeter (Rudolph Research). ^{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 Unity Plus 500 MHz. All compounds were measured in CD_3OD.[20] ESIMS was performed on a Finnigan LCQ Deca instrument from ThermoQuest Finnigan (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 was offset at 0 V. The capillary temperature was 230 °C. HRFABMS was performed on a 70-SE-4F mass spectrometer (Micromass). Samples were dissolved in MeOH. HPLC analysis was carried out on a Waters 2690 separations module equipped with a Waters 996 photodiode array detector and Millenium^{32} software using a Phenomenex Nucleosil C18 column (4.6 × 250 mm, 5 μm) and a solvent system of 1:19 to 1:1 MeCN/H_2O linear gradient, and a flow rate of 1 mL/min, 20 min running time. TLC analysis was performed on RP-18 F_{254} plates (Merck, Darmstadt, Germany), with compounds visualized by spraying with vanillin in 10% (v/v) H_2SO_4 in EtOH. Sephadex LH-20 (25−100 μm; Pharmacia Fine Chemicals, Piscataway, NJ) and C18 reversed-phase silica gel (40 μm; J. T. Baker, Phillipsburg, NJ) were used for column chromatography.

Plant Material. Fruit of *M. zapota* cv. Tikal was collected from the Fruit and Spice Park (Homestead, FL) and Dr. Richard J. Campbell's tropical fruit collection at the Montgomery Botanical Center (Coral Gables, FL). Frozen fruit was shipped to New York by overnight courier and stored at −20 °C until extracted. A voucher specimen of *M. zapota* was prepared, identified, and deposited at the herbarium of The New York Botanical Garden (Bronx, NY).

Extraction and Isolation. The fresh frozen fruit (20.2 kg) of *M. zapota* was twice extracted with MeOH at room temperature for 1 h per extraction. After the MeOH was removed in vacuo, the resulting aqueous extract was partitioned with hexane and EtOAc, respectively. The EtOAc fraction (IC_{50} = 10.7 μg/mL in the DPPH assay, IC_{50} = 95 μg/mL for HCT-116, and IC_{50} = 100 μg/mL for SW-480) was concentrated in vacuo to give 14.6 g of a residue, of which 13.8 g was separated by Sephadex LH-20 CC (from 100% H_2O to 100% MeOH in 10% steps). The resulting fractions were combined according to their RP18 TLC profiles to give 11 combined fractions in total (A−K). All fractions were tested in the DPPH assay, and fractions A (2.2 g), B (1.1 g), and K (0.6 g) showed high activity, 5.2, 7.1, and 19.2 μg/mL, respectively.

Fraction A was separated over RP18 CC (from 1:9 to 3:2 MeOH/H_2O). Subfraction A-1 was purified by RP18 CC eluting with a gradient of 100% H_2O to 1:9 MeOH/H_2O to yield gallocatechin (**9**) (114.6 mg) and gallic acid (**10**) (95.6 mg). Subfraction A-2 was purified by RP18 CC eluting with a gradient of 1:9−4:21 MeOH/H_2O to obtain catechin (**7**) (14.9 mg). Subfraction A-3 was purified by RP18 CC eluting with a gradient of 1:4−3:7 MeOH/H_2O to obtain epicatechin (**8**) (30.9 mg).

Fraction B was separated over RP18 CC (from 1:9 to 3:2 MeOH/H_2O) to yield methyl 4-*O*-galloylchlorogenate (**1**) (45.6 mg) and dihydromyricetin (**4**). Subfraction B-7 was purified by RP18 CC eluting with a gradient of 1:4−2:3 MeOH/H_2O to obtain B-7-2. B-7-2 was further purified by continual RP18 CC eluting with a gradient of 1:9−1:4 MeCN/H_2O to yield 4-*O*-galloyl chlorogenic acid (**2**) (15.9 mg). Subfraction B-8 was purified by RP18 CC eluting with a gradient of 3:7−1:1 MeOH/H_2O to obtain B-8-4. B-8-4 was further purified by continual RP18 CC eluting with an isocratic solvent system of 1:4 MeCN/H_2O to yield myricitrin (**6**) (14.3 mg). Subfraction B-10 was purified by RP18 CC eluting with a gradient of 3:7−2:3 MeOH/H_2O to obtain B-10-4. B-10-4 was further purified by continual RP18 CC eluting with an isocratic solvent system of 1:4 MeCN/H_2O to yield quercitrin (**5**) (4.0 mg). Fraction K was separated over RP18 CC (from 1:4 to 2:3 MeOH/H_2O) to yield methyl chlorogenate (**3**) (145.2 mg).

Methyl 4-*O*-galloylchlorogenate (1): yellow powder, mp 183−185 °C; $[\alpha]^{25}_D$ −137.4° (*c* 0.0048, MeOH); negative ESIMS *m/z* 519 $[M - H]^-$ (negative ESIMS/MS *m/z* 357 $[M - H - 162]^-$; negative ESIMS/MS *m/z* 367 $[M - H - 152]^-$); positive HRFABMS *m/z* 521.1294 $[M + H]^+$ (calcd for $C_{24}H_{25}O_{13}$, 521.1295); ^{1}H and ^{13}C NMR data, see Table 1.

4-*O*-Galloylchlorogenic acid (2): yellow powder, mp >300 °C (began decomposing at 180 °C); $[\alpha]^{25}_D$ −65.3° (*c* 0.00075, MeOH); negative ESIMS *m/z* 505 $[M - H]^-$ (negative ESIMS/MS *m/z* 343 $[M - H - 162]^-$); positive HRFABMS *m/z* 507.1135 $[M + H]^+$ (calcd for $C_{23}H_{23}O_{13}$, 507.1139); ^{1}H and ^{13}C NMR data, see Table 1.

LC-ESIMS Analyses. The fresh frozen fruit (500 g) of *M. zapota* cv. Tikal was twice extracted with 95% EtOH at room temperature for 1 h per extraction. After the EtOH was removed in vacuo, the resulting aqueous extract was partitioned with hexane and EtOAc, respectively. The EtOAc fraction was concentrated in vacuo, and the resulting residue (527 mg) was separated by Sephadex LH-20 CC using an

isocratic solvent system of 95% EtOH to yield 20 fractions, A−T. These fractions, along with **1**, were analyzed by RP18 TLC using a solvent system of 7:3 MeCN/H_2O, and fraction I contained a compound with the same R_f value as **1**. Fraction I was further analyzed by LC-ESIMS in the negative mode using a Finnigan LCQ mass spectrometer (San Jose, CA) with a Waters 2690 separations module and a Waters 2487 dual λ absorbance detector. The instrument was equipped with an electrospray ionization source and controlled by Xcalibur software. The capillary temperature was 230 °C. Nitrogen was used as the sheath gas and helium as the auxiliary gas at flow rates of 80 and 20 units, respectively. The capillary voltage was 10 V, the spray needle voltage was 4.5 kV, and the tube lens offset was 0 V. A mass range of 75−1500 was scanned in the negative mode. Compounds were also monitored using the absorbance detector at 280 nm. Compounds were separated using a Phenomenex Nucleosil C18 column (4.6 × 250 mm, 5 μm) and a solvent system of 1:19−1:1 MeCN/H_2O linear gradient, with a flow rate of 1 mL/min, 20 min running time. Compound **1** was run under the same LC-MS conditions.

DPPH Free-Radical Scavenging Assay. The DPPH assay was performed on fractions and purified isolates as previously described.[24] 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 the 1−50 μg/mL range. Percent inhibition by sample treatment was determined by comparison with a DMSO-treated control group. IC_{50} values denoted 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 medium 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 manufacturer's instructions. Briefly, about 3.0×10^4 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 included in the assay 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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