Antioxidant and Cytotoxic Isoprenylated Coumarins from *Mammea americana*

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Original Paper

Abstract

Antioxidant-guided fractionation of *Mammea americana* L. seeds resulted in the identification of three new isoprenylated coumarins, mammea B/BA hydroxycyclo F (**1**), mammea E/BC (**2**), and mammea E/BD (**3**). In addition, twelve known isoprenylated coumarins, mammea A/AA (**4**), mammea A/AA cyclo D (**5**), mammea A/AA cyclo F (**6**), mammea A/AC cyclo D (**7**), mammea A/AD cyclo D (**8**), mammea B/BA (**9**), mammea B/BA cyclo F (**10**), mammea B/BB (**11**), mammea B/BC (**12**), mammea B/BD (**13**), mammea E/BA (**14**), and mammea E/BB (**15**), as well as two known flavanols, (+)-catechin (**16**) and (-)-epicatechin (**17**) were identified. The fifteen isoprenylated coumarins were screened for their cytotoxicity in the SW-480, HT-29, and HCT-116 human colon cancer cell lines and antioxidant capacities in the DPPH (1,1-diphenyl-2-picrylhydrazyl) free-radical assay. Compounds **1** – **15** exhibited significant cytotoxic activities in the SW-480, HT-29, and HCT-116 human colon cancer cell lines (IC_{50} ranges 13.9 – 88.1, 11.2 – 85.3, and 10.7 – 76.7 μM, in the three cell lines, respectively) at concentrations comparable to 5-fluorouracil (IC_{50} = 53.0, 46.1, and 45.1 μM), a drug frequently used for human colon cancer treatment. Compounds **2** – **4**, **9**, and **11** – **15** displayed high antioxidant activity in the DPPH assay (IC_{50} range 86 – 135 μM), compounds **1**, **5** – **8**, and **10**, however, had no antioxidant activity (IC_{50} > 200 μg/mL) in the DPPH assay. The results of these assays were used to study the structure-activity relationships for this class of compounds. In the SW-480 cell line, the three new coumarins, **1** – **3**, also exhibited dose-dependent increases in sub-diploid cells by flow cytometry, indicating that they induce apoptosis.

Key words
Clusiaceae · *Mammea americana* · mamey · isoprenylated coumarin · cytotoxicity · antioxidant activity · SAR studies

Introduction

Mammea americana L. (Clusiaceae) is a tree native to the West Indies and northern South America. Known commonly as mamey or mammee apple, the round fruit of the plant is 4 – 8 cm in diameter, with a light-brown skin. There are 1 – 4 ovoid seeds per fruit. The fruit is generally considered edible, and is enjoyed raw, cooked, or processed into a wine. However, there are concerns in some Caribbean cultures that the fruits and seeds are poisonous [1]. For example, in Puerto Rico the fruits are widely consumed, but people are generally aware not to eat much due to gastric distress [2]. The seeds of *M. americana* are known to have insecticidal activity [1].

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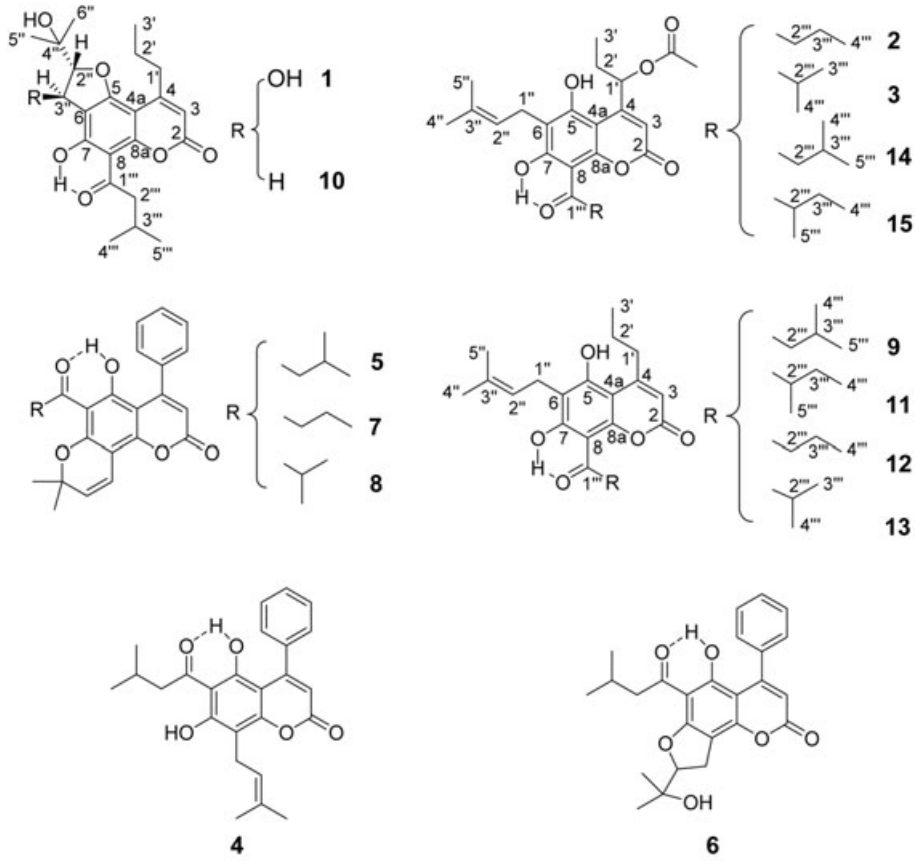

Fig. **1** Chemical structures of compounds **1** – **15**.

A number of coumarins have been identified previously from the seeds of *M. americana*[3], [4], [5], [6], [7], [8]. Some of these coumarins have been reported to be uncouplers of oxidative phosphorylation, and thus potent insecticides [8]. Several insecticidal coumarins from *M. americana* have been synthesized [9].

As part of our continuing research on polyphenolic anticancer agents and antioxidants from edible plants [10], [11], we found that extracts of *M. americana* seeds have antioxidant and cytotoxicity activities. Our work on *M. americana* seeds has resulted in the identification of three new isoprenylated coumarins, mammea B/BA hydroxycyclo F (**1**), mammea E/BC (**2**), and mammea E/BD (**3**), and twelve known isoprenylated coumarins, mammea A/AA (**4**), mammea A/AA cyclo D (**5**), mammea A/AA cyclo F (**6**), mammea A/AC cyclo D (**7**), mammea A/AD cyclo D (**8**), mammea B/BA (**9**), mammea B/BA cyclo F (**10**), mammea B/BB (**11**), mammea B/BC (**12**), mammea B/BD (**13**), mammea E/BA (**14**), and mammea E/BB (**15**) (Fig. **1**), as well as two known flavanols, (+)-catechin (**16**) and (–)-epicatechin (**17**). We now describe the chemical characterization and biological evaluation of the fifteen isolated isoprenylated coumarins from *M. americana*.

Materials and Methods

General experimental procedures

Optical rotations were measured on an Autopol III Automatic Polarimeter (Rudolph Research Analytical, Flanders, NJ, USA). UV spectra were measured on a Lambda 2 UV/Vis spectrophotometer (Perkin-Elmer, Boston, MA, USA). ^{1}H-NMR and 2D-NMR experiments were run on a Bruker Avance DMX-500 NMR spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C). The 2D experiments ^{1}H-^{1}H COSY, NOESY, HMBC, and edited-HSQC were acquired using standard Bruker software. ^{13}C NMR spectra were recorded using a Bruker Avance AV-300, operating at 75 MHz. All compounds were measured in $CDCl_3$. ESI-MS was performed with a ThermoFinnigan LCQ instrument (San Jose, CA, USA) 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. Nitrogen was used as both sheath and auxiliary gas, and their flow rates were 80 and 30 (arbitrary units), respectively. HR-ESI-MS was performed on a TofSpec mass spectrometer (Micromass). Samples were dissolved in MeOH. HPLC analyses were carried out on a Waters 2695 Separations Module (Milford, MA, USA) equipped with a Waters 996 photodiode array detector and Waters Empower software using a 250 × 4.6 mm i. d., 5 μm, Nucleosil C_{18} column (Phenomenex, Torrance, CA, USA) and an isocratic solvent system of MeCN/H_2O (75 : 25), a flow rate of 1 mL/min, column at room temperature, 30 min run time for analysis of subfractions and pure compounds. Preparative HPLC was carried out using a Waters 600 controller with a Waters 486 tunable absorbance detector and Waters Empower software with a 250 × 21.1 mm i. d., 10 μm, Nucleosil C_{18} column (Phenomenex, Torrance, CA, USA) and an isocratic solvent system of MeCN/H_2O (75 : 25), a flow rate of 5 mL/min, the column at room temperature, and a 50 min run time. TLC analyses were performed on si-

lica gel 60 F_{254} (EM Science, Darmstadt, Germany) and RP-18 F_{254} plates (EM Science, Darmstadt, Germany), with compounds visualized by spraying with a vanillin solution (1.0 g vanillin in 10 mL conc. H_2SO_4 and 90 mL EtOH) and heating at ca. 50.0 °C. Silica gel (230 – 400 mesh, EM Science, Darmstadt, Germany), silica gel 60 (particle size 0.015 – 0.040 mm, EM Science, Darmstadt, Germany), Sephadex LH-20 (25 – 100 μm; Pharmacia Fine Chemicals, Piscataway, NJ, USA), and C_{18} reversed-phase silica gel (40 μm; J. T. Baker, Phillipsburg, NJ, USA) were used for column chromatography.

Plant material

Mammea americana fruits were collected in Fruit and Spice Park, Homestead, Florida, USA (October, 2001) and identified by Chris Rollins. A voucher specimen (Kennelly, 169, NYBG) was deposited at The Steere Herbarium of The New York Botanical Garden (Bronx, NY).

Extraction and isolation of compounds

The fresh seed nuclei (3.97 kg) were obtained from the frozen fruits (17.5 kg) of *M. americana* by removing the skin, flesh, and seed coat. The seed nuclei were ground to a powder, and extracted with MeOH (4.0 L) three times at room temperature. The combined MeOH extract was concentrated under reduced pressure, and the resulting brown extract (226.3 g) was suspended in H_2O and partitioned sequentially with EtOAc and BuOH. The EtOAc (Fr_{EA}, 83.1 g), and BuOH (Fr_B, 18.4 g) partitions were tested in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, and their IC_{50} values were calculated as 39 and 16 μg/mL, respectively.

Fr_{EA} (80.0 g) was subjected to column chromatography (CC) over Sephadex LH-20 (200.0 g, 4.5 × 80.0 cm) and eluted with an isocratic system using MeOH, and 36 fractions (100 mL) were collected. Fractions were combined based upon HPLC analysis to fifteen subfractions ($Fr_{EA1to15}$). All subfractions ($Fr_{EA1to15}$) were tested in the DPPH assay, and the subfractions Fr_{EA3} and Fr_{EA15} displayed high antioxidant activity (IC_{50} = 45 and 17 μg/mL, respectively).

Fr_{EA3} (42.3 g) was separated by silica gel (600.0 g, 230 – 400 mesh, 4.5 × 100.0 cm) CC eluting with a gradient system of 1 : 0 to 0 : 1 hexane/EtOAc (increased by 10% EtOAc per step), and 51 fractions (100 mL) were collected. Fractions were combined based upon HPLC analyses to fourteen subfractions ($Fr_{EA3-1to14}$). Subfraction Fr_{EA3-4} (4.4 g, DPPH assay IC_{50} = 51 μg/mL) was purified by silica gel 60 (80.0 g, 2.0 × 45.0 cm) CC eluting with an isocratic system of $CHCl_3$/MeOH (100 : 1) to obtain eight subfractions ($Fr_{EA3-4a\,to\,h}$). Fr_{EA3-5} (7.9 g, DPPH assay IC_{50} = 37 μg/mL) and Fr_{EA3-8} (5.1 g, DPPH assay IC_{50} = 48 μg/mL) were separated and combined in a similar fashion, and eight subfractions $Fr_{EA3-5a\,to\,h}$ and nine subfractions ($Fr_{EA3-8a\,to\,i}$) were obtained, respectively.

Fr_{EA3-5a} (504.5 mg, DPPH assay IC_{50} = 34 μg/mL) was further separated and purified by preparative reversed-phase HPLC with an isocratic system of MeCN/H_2O (75 : 25) to obtain mammea B/BA hydroxycyclo F (**1**) (3.1 mg, t_R = 6.2 min), mammea B/BA (**9**) (18.0 mg, t_R = 39.3 min), mammea B/BA cyclo F (**10**) (3.7 mg, t_R = 8.9 min), mammea B/BB (**11**) (12.2 mg, t_R = 37.5 min), mammea B/BC (**12**) (1.5 mg, t_R = 15.1 min), and mammea B/BD (**13**) (4.8 mg, t_R = 13.7min).

Fr_{EA3-8a} (684.6 mg, DPPH assay IC_{50} = 36 μg/mL) was further separated and purified by preparative reversed-phase HPLC with an isocratic system of MeCN/H_2O (75 : 25) to yield mammea E/BC (**2**) (2.2 mg, t_R = 34.1 min), mammea E/BD (**3**) (8.8 mg, t_R = 32.8 min), mammea E/BA (**14**) (15.8 mg, t_R = 48.3 min), and mammea E/BB (**15**) (6.7 mg, t_R = 46.5 min).

The compounds mammea A/AA (**4**) (3.8 mg, t_R = 21.0 min), mammea A/AA cyclo D (**5**) (1.4 mg, t_R = 36.2 min), mammea A/AA cyclo F (**6**) (7.5 mg, t_R = 12.6 min), mammea A/AC cyclo D (**7**) (1.5 mg, t_R = 30.7 min), and mammea A/AD cyclo D (**8**) (1.4 mg, t_R = 29.3 min) were obtained from Fr_{EA3-4e} (266.5 mg, DPPH assay IC_{50} = 40 μg/mL) by preparative reversed-phase HPLC with a isocratic system of MeCN/H_2O (75 : 25).

In addition, subfraction Fr_{EA15} (2.9 g) was subjected to CC over silica gel 60 (60.0 g, 2.0 × 32.0 cm) and eluted with an isocratic system of $CHCl_3$/MeOH (9 : 1) to afford nine subfractions ($Fr_{EA15a\,to\,i}$). Further separation of $Fr_{EA15\,g}$ (373.9 mg, DPPH assay IC_{50} = 14 μg/mL) was achieved by RP-18 (50.0 g, 2.0 × 30.0 cm) CC using a gradient system of 1 : 9 to 1 : 0 MeOH/H_2O (increased by 10% MeOH per step) to give (+)-catechin (**16**) (61.8 mg) and (–)-epicatechin (**17**) (152.2 mg).

Mammea B/BA hydroxycyclo F {2,3-dihydro-3,4-dihydroxy-2-(1-hydroxy-1-methylethyl)-5-(3-methly-1-oxobutyl)-9-propyl-7*H*-furo[2,3-*f*]-1-benzopyran-2-one} (**1**): yellow semisolid, $[\alpha]_D^{25}$: + 15° (*c* 0.00065, MeOH); UV (MeOH): λ_{max} (log ε) = 222 (3.40), 233 (3.29), 298 (3.43) nm; ^{1}H- and ^{13}C-NMR, see Table **1**; ESI-MS (negative ion): *m/z* = 403 $[M-H]^-$; HR-ESI-MS (positive ion): *m/z* = 405.1927 $[M+H]^+$ (calcd. for $C_{22}H_{29}O_7$: 405.1913).

Mammea E/BC {5,7-dihydroxy-4-[1-(acetoxy)propyl]-6-(3-methyl-2-butenyl)-8-(1-oxobutyl)-2*H*-1-benzopyran-2-one} (**2**): yellow semisolid, $[\alpha]_D^{25}$: –28° (*c* 0.00092, MeOH); UV (MeOH): λ_{max} (log ε) = 221 (3.1), 294 (3.2), 326 (3.2) nm; ^{1}H- and ^{13}C-NMR, see Table **1**; ESI-MS (negative ion): *m/z* = 415 $[M-H]^-$, 355 $[M-AcOH-H]^-$; HR-ESI-MS (positive ion): *m/z* = 417.1920 $[M+H]^+$ (calcd. for $C_{23}H_{29}O_7$: 417.1913).

Mammea E/BD {5,7-dihydroxy-4-[1-(acetoxy)propyl]-6-(3-methyl-2-butenyl)-8-(2-methyl-1-oxopropyl)-2*H*-1-benzopyran-2-one} (**3**): yellow semisolid, $[\alpha]_D^{25}$: –46° (*c* 0.0043, MeOH); UV (MeOH): λ_{max} (log ε) = 221 (2.4), 294 (2.5), 326 (2.5) nm; ^{1}H- and ^{13}C-NMR, see Table **1**; ESI-MS (negative ion): *m/z* = 415 $[M-H]^-$, 355 $[M-AcOH-H]^-$; HR-ESI-MS (positive ion): *m/z* = 417.1924 $[M+H]^+$ (calcd. for $C_{23}H_{29}O_7$: 417.1913).

Mammea B/BA (**9**): ^{13}C-NMR ($CDCl_3$, 75.0 MHz): δ = 13.9 (C-3′), 18.1 (C-5″), 21.6 (C-1″), 22.6 (C-4‴ and 5‴), 22.7 (C-2′), 25.6 (C-3‴), 25.8 (C-4″), 38.6 (C-1′), 53.6 (C-2‴), 102.3 (C-4a), 104.5 (C-8), 109.7 (C-3), 110.2 (C-6), 120.2 (C-2″), 138.4 (C-3″), 156.4 (C-8a), 158.8 (C-4), 159.2 (C-5), 159.3 (C-2), 165.4 (C-7), and 206.3 (C-1‴).

Mammea B/BA cyclo F (**10**): $[\alpha]_D^{25}$: + 5.2° (*c* 0.0019, MeOH); ^{13}C-NMR ($CDCl_3$, 75.0 MHz): δ = 13.9 (C-3′), 22.6 (C-4‴ and 5‴), 22.7 (C-2′), 24.7 (C-6″), 25.6 (C-3‴), 26.1 (C-5″), 26.6 (C-3″), 37.3 (C-1′), 53.4 (C-2‴), 71.6 (C-4″), 92.8 (C-2″), 99.4 (C-4a), 105.1 (C-8), 109.5 (C-3), 109.9 (C-6), 157.1 (C-4), 157.5 (C-8a), 159.4 (C-2), 162.1 (C-5), 163.1 (C-7), and 206.1 (C-1‴).

Mammea B/BB (**11**): $[\alpha]_D^{25}$: -1.3° (*c* 0.0061, MeOH); ^{13}C-NMR ($CDCl_3$, 75.0 MHz): δ = 11.9 (C-4‴), 14.0 (C-3′), 16.9 (C-5‴), 18.3 (C-5″), 21.9 (C-1″), 23.0 (C-2′), 26.1 (C-4″), 27.7 (C-3‴), 38.9 (C-1′), 47.1 (C-2‴), 102.5 (C-4a), 104.6 (C-8), 109.9 (C-3), 110.0 (C-6), 120.4 (C-2″), 138.4 (C-3″), 156.2 (C-8a), 158.8 (C-4), 159.1 (C-5), 159.7 (C-2), 165.4 (C-7), and 210.9 (C-1‴).

Mammea B/BC (**12**): ^{13}C-NMR ($CDCl_3$, 75.0 MHz): δ = 13.5 (C-4‴), 13.7 (C-3′), 18.2 (C-3‴), 18.3 (C-5″), 21.6 (C-1″), 22.7 (C-2′), 25.7 (C-4″), 38.7 (C-1′), 47.0 (C-2‴), 102.2 (C-4a), 104.3 (C-8), 109.8 (C-3), 110.0 (C-6), 120.1 (C-2″), 138.2 (C-3″), 156.3 (C-8a), 158.8 (C-4), 159.2 (C-5), 159.5 (C-2), 165.2 (C-7), and 206.5 (C-1‴).

Mammea B/BD (**13**): ^{13}C-NMR ($CDCl_3$, 75.0 MHz): δ = 13.9 (C-3′), 18.1 (C-5″), 19.1 (C-4‴), 19.3 (C-3‴), 21.8 (C-1″), 22.9 (C-2′), 25.9 (C-4″), 38.9 (C-1′), 40.8 (C-2‴), 102.3 (C-4a), 104.5 (C-8), 109.7 (C-3), 110.1 (C-6), 120.4 (C-2″), 138.2 (C-3″), 156.2 (C-8a), 158.6 (C-4), 159.1 (C-5), 159.4 (C-2), 165.4 (C-7), and 210.7 (C-1‴).

Mammea E/BA (**14**): $[\alpha]_D^{25}$: –37° (*c* 0.0064, MeOH); ^{13}C-NMR ($CDCl_3$, 75.0 MHz): δ = 10.1 (C-3′), 18.1 (C-5″), 21.0 ($OCOCH_3$), 21.7 (C-1″), 22.6 (C-4‴ and 5‴), 25.6 (C-3‴), 25.9 (C-4″), 28.8 (C-2′), 53.2 (C-2‴), 73.7 (C-1′), 100.4 (C-4a), 104.3 (C-8), 106.5 (C-3), 110.2 (C-6), 120.0 (C-2″), 138.8 (C-3″), 156.1 (C-8a), 157.2 (C-5), 158.1 (C-4), 159.3 (C-2), 165.8 (C-7), 170.4 ($OCOCH_3$), and 206.3 (C-1‴).

Mammea E/BB (**15**): $[\alpha]_D^{25}$: –62° (*c* 0.0034, MeOH); ^{13}C-NMR ($CDCl_3$, 75.0 MHz): δ = 10.1 (C-3′), 11.7 (C-4‴), 16.7 (C-5‴), 18.9 (C-5″), 21.0 ($OCOCH_3$), 21.7 (C-1″), 25.9 (C-4″), 27.1 (C-3‴), 28.8 (C-2′), 47.0 (C-2‴), 73.7 (C-1′), 100.4 (C-4a), 104.3 (C-8), 106.5 (C-3), 110.1 (C-6), 120.0 (C-2″), 138.7 (C-3″), 156.1 (C-8a), 157.2 (C-5), 158.1 (C-4), 159.4 (C-2), 165.8 (C-7), 170.4 ($OCOCH_3$), and 210.8 (C-1‴).

The specific rotation of compound **6** was also measured, and the $[\alpha]_D^{25}$ value is + 4.6° (*c* 0.0024, MeOH).

DPPH free-radical scavenging assay

The DPPH assay was performed on fractions and purified isolates as described previously [12]. 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 – 50 µg/mL for extracts and fractions, and from 2 – 140 μM for pure compounds. 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

SW-480, HT-29, and HCT-116 human colon cancer cell lines were purchased from the American Type Culture Collection. All three cell lines were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY, USA) with 10% fetal bovine serum (Gibco) at 5% CO_2 and 37 °C. All cultures were passaged weekly, and the medium was changed three times a week. No antibiotics were added at any time during the experiments. Prior to treatment with coumarins, the cells were grown to 30% confluence for the MTT assay and to 40% confluence for flow cytometry experiments. In all experiments, coumarins were dissolved in DMSO and added to the medium at the start of the incubation. The incubation time was 72 h for the MTT assay, and 24 h for the flow cytometry experiments.

Microtetrazolium (MTT) assay

The MTT assay (Boeringher-Mannheim, Indianapolis, IN, USA) was carried out according to the manufacturer's instructions. In brief, about 30,000 cells per well were plated in 96-well flat-bottom plates in 100 μL of medium. When the cells reached 30% confluence, the medium was changed and cells were exposed to various concentrations of coumarins. After 72 h of incubation, the 96-well plates were gently washed three times with PBS and 100 μL of fresh medium was added. Subsequently, 10 μL of a 5 mg/mL MTT solution in PBS was added to each well, and the plates were incubated at 37 °C for 4 h. Finally, 100 μL of solubilization solution was added to each well and the plates were incubated at 37 °C overnight to dissolve the formazan crystals that formed in the presence of viable cells. The absorbance at 570 nm was determined using a Biokinetics plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Octuplicate wells were assayed for each condition, and means and standard deviations were determined. The IC_{50} values (concentration of compound that caused 50% inhibition) were determined by linear regression analysis.

Flow cytometry

Apoptosis and cell cycle distribution were quantified on a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA). The flow cytometry procedure to quantify apoptotic cells was previously described in detail [13]. Briefly, the culture media containing the floating SW-480 cells was harvested. The remaining adherent cells were trypsinized and harvested as well, and pooled with the floating cells. The cells were then washed twice with PBS, fixed with 70% ethanol chilled at –20 °C, and stored in 70% ethanol overnight at –20 °C. Before analysis the cells were washed again with PBS, resuspended, and then incubated for 30 min in a staining solution containing 0.05 mg/mL propidium iodide (Sigma Chemicals Co.) and 1 mg/mL RNAse A in PBS (Sigma Chemical Co.). At least 20,000 gated cells were analyzed by flow cytometry. Data were plotted on FL2-A histograms and the sub-diploid population of cells in the area corresponding to apoptotic nuclei was considered apoptotic. Cell cycle analysis and sub-diploid cell analysis were performed using the FlowJo software version 6.0. Apoptosis is expressed as percentage of sub-diploid cells in the total population of the analyzed cells. Cell cycle distribution is expressed as percentage of analyzed cells, after subtraction of the subG1 population, in G1, S, or G2/M phase of the cell cycle.

Results and Discussion

The seeds of *M. americana* were extracted exhaustively with MeOH, and partitioned sequentially with EtOAc and BuOH. The EtOAc fraction was subjected to antioxidant-guided fractionation, with an initial separation by Sephadex LH-20 column chromatography (CC). Extensive separation of active fractions by sili-

Table **1** ^{1}H-NMR ($CDCl_3$, 500 MHz, *J* in Hz) and ^{13}C-NMR ($CDCl_3$, 75 MHz) spectral data of compounds **1** – **3**

Position	*1*		*2*		*3*	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
2	159.1 s		159.4 s		159.5 s	
3	109.7 d	6.06 (brt, 0.7)	106.4 d	6.29 (brd, 0.7)	106.3 d	6.29 (brd, 0.7)
4	157.1 s		157.1 s		157.3 s	
4a	99.6 s		100.4 s		100.5 s	
5	162.4 s		158.2 s		158.4 s	
OH-5				7.11 (s)		7.11 (s)
6	112.3 s		110.1 s		110.4 s	
7	164.2 s		165.6 s		165.9 s	
OH-7		14.51 (s)		14.67 (s)		14.59 (s)
8	105.1 s		104.6 s		103.7 s	
8a	157.4 s		156.4 s		156.0 s	
1′	37.3 t	2.89 (brt, 7.2)	73.7 d	6.49 (dd, 8.0, 2.5)	73.7 d	6.50 (dd, 8.0, 2.5)
$OCOCH_3$			170.4 s		170.4 s	
$OCOCH_3$			21.1 q	2.17 (s)	21.0 q	2.17 (s)
2′	22.7 t		28.7 t		28.8 t	
2′a		1.71 (m)		1.96 (ddq, 14.5, 7.5, 3.0)		1.96 (ddq, 14.5, 7.5, 3.0)
2′b		1.71 (m)		1.69 (ddq, 14.5, 7.5, 7.5)		1.69 (ddq, 14.5, 7.5, 6.0)
3′	13.9 q	1.07 (t, 7.5)	10.1 q	1.01 (t, 7.5)	10.1 q	1.01 (t, 7.5)
chromene moiety						
1″			21.7 t		21.7 t	
1″a				3.55 (dd, 17.0, 7.0)		3.55 (dd, 17.0, 7.0)
1″b				3.44 (dd, 17.0, 7.0)		3.44 dd (17.0, 7.0)
2″	99.0 d	4.59 (d, 6.3)	119.9 d	5.24 (t, 7.0)	120.1 d	5.24 (t, 7.0)
3″	70.4 d	5.66 (d, 6.3)	138.8 s		138.5 s	
4″	71.4 s		25.9 q	1.83 (s)	25.9 q	1.83 (s)
5″	26.0 q	1.42 (s)	18.1 q	1.87 (s)	18.8 q	1.88 (s)
6″	25.1 q	1.40 (s)				
8-acyl moiety						
1‴	206.4 s		206.5 s		210.9 s	
2‴	53.5 t	3.17 (d, 6.9)	46.7 t	3.29 (t, 7.0)	40.4 d	4.06 (septet, 6.5)
3‴	25.6 d	2.29 (m)	18.1 t	1.79 (sextet, 7.0)	19.3 q	1.26 (d, 6.5)
4‴	22.6 q	1.06 (d, 6.6)	13.8 q	1.04 (t, 7.0)	19.2 q	1.27 (d, 6.5)
5‴	22.6 q	1.06 (d, 6.6)				

ca gel CC, reversed-phase (RP-18) CC, and preparative RP-18 HPLC yielded three new isoprenylated coumarins, **1** – **3**.

The molecular formula of compound **1** ($C_{22}H_{28}O_7$) was established by positive HR-ESI-MS analysis of its molecule ion $[M + H]^+$ at *m/z* = 405.1927. Compound **1** is optically active ($[\alpha]_D^{25}$: + 15°, *c* 0.00065, MeOH). The UV spectrum of **1** (MeOH) supported an 8-acyl-5,7-dihydroxycoumarin type with maximum absorptions at 222, 233, and 298 nm [6]. The ^{1}H-NMR spectra of **1** (Table **1**) showed a broad triplet at δ = 6.06 (*J* = 0.7 Hz, H-3) characteristic of a 4-substituted coumarin. This signal showed long-range allylic coupling with the triplet at δ = 2.89 (2H, brt, *J* = 7.2 Hz, H-1′) in the COSY experiment, which was coupled to the signal at δ = 1.71 (2H, m, H-2′), and in turn with the signal at δ = 1.07 (3H, t, *J* = 7.5 Hz, H-3′), confirming the presence of an *n*-propyl group attached to C-4. This connection was also supported by the long-range couplings observed in the HMBC spectrum of **1** (Fig. **2**). The combined analysis of the 1D ^{1}H, ^{13}C, and 2D HSQC NMR spectra of **1** (Table **1**) revealed proton signals at δ = 3.17 (2H, d, *J* = 6.9 Hz, H-2‴), 2.29 (1H, m, H-3‴), 1.06 (6H, d, *J* = 6.6 Hz, H-4‴ and H-5‴), and carbon signals at δ = 206.4 (C-1‴), 53.5 (C-2‴), 25.6 (C-3‴), and 22.6 (C-4‴ and C-5‴), which are due to the presence of a 3-methylbutyryl substituent. As expected, the relevant cross-correlation peaks for the 3-methylbutyryl were observed in the COSY and HMBC spectra of **1** (Fig. **2**). Additionally, through ^{1}H-NMR experiments, the phenolic hydroxy proton signal (δ = 14.51, 1H, s, OH-7) was found to be exchangeable with D_2O, and its high chemical shift suggested that it is involved in intermolecular hydrogen bond with the carbonyl function of the acyl group at C-8. This downfield proton signal showed cross-peaks with the carbon signals of C-6 (δ = 112.3), C-7 (δ = 164.2), C-8 (δ = 105.1), and C-1‴ (δ = 206.4) in the HMBC spectrum of **1** (Fig. **2**). These results indicated that this phenolic hydroxy is attached to C-7, thereby locating the 3-methylbutyryl chain at C-8. Thus, **1** is a 4-propyl-8-acyl-5,7-dihydroxycoumarin derivative. The absence of any other phenolic OH function signals in the ^{1}H-NMR spectrum and the presence of a quaternary carbon at δ = 162.4 (C-5) then suggested the presence of an oxygenated ring system in the 5,6-position in **1**. Examination of the COSY and HMBC spectra of **1** (Fig. **2**) con-

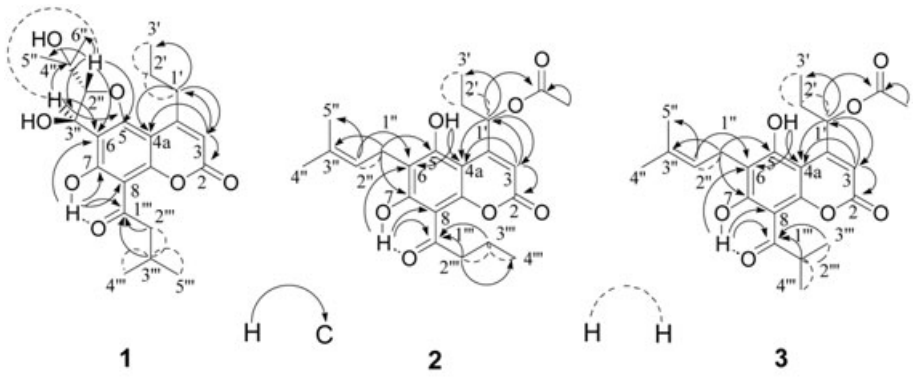

Fig. **2** Key correlations observed in COSY and HMBC spectra of compounds **1** – **3**.

firmed the presence of a 2,3-dihydro-3-hydroxy-2-(1-hydroxy-1-methylethyl)-furan ring system. The relative stereochemistry of C-2″ and C-3″ was determined by nOe cross-correlation peaks in the NOESY spectrum between H-3″ and the methyl protons H-5″ and H-6″. These signals are associated with a relative stereochemistry of this furan ring as shown in Fig. **1**. Therefore, **1** was identified as 2,3-dihydro-3,4-dihydroxy-2-(1-hydroxy-1-methylethyl)-5-(3-methyl-1-oxobutyl)-9-propyl-7*H*-furo[2,3-*f*]-1-benzopyran-2-one or, according to the established nomenclature of Crombie, et al. [6], as mammea B/BA hydroxycyclo F.

Compound **2** was isolated as a yellow, optically active ($[\alpha]_D^{25}$: –28°, *c* 0.00092, MeOH) semisolid, and revealed a molecular formula of $C_{23}H_{28}O_7$ by positive HR-ESI-MS (*m/z* = 417.1920 $[M + H]^+$). The UV spectrum (MeOH) (λ_{max} = 221, 294, 326 nm) of **2** indicated a 4-alkyl-5,7-dihydroxycoumarin with an 8-acyl substituent [8], [14]. The negative ion ESI-MS showed the molecular ion at *m/z* = 415 $[M – H]^-$ and a fragment ion at *m/z* = 355 $[M – AcOH – H]^-$, and the NMR spectrum (Table **1**) showed signals at δ = 6.49 (1H, dd, *J* = 8.0, 2.5 Hz, H-1′), 1.96 (1H, ddq, *J* = 14.5, 7.5, 3.0 Hz, H-2′a), 1.69 (1H, ddq, *J* = 14.5, 7.5, 7.5 Hz, H-2′b), 1.01 (3H, t, *J* = 7.5 Hz, H-3′), and 2.17 (3H, s, $OCOCH_3$), attributed to the presence of a 1-acetoxypropyl group. The signal at δ = 14.67 (1H, s, OH-7) was ascribed to a phenolic group hydrogen bonded to an acyl group, and a signal at δ = 7.11 (1H, s, OH-5) was assigned to a free phenolic group. Two singlets of three hydrogens each at δ = 1.87 (H-5″) and 1.83 (H-4″), and two doublets of doublets of one hydrogen each at δ = 3.55 (*J* = 17.0, 7.0 Hz, H-1″a) and 3.44 (*J* = 17.0, 7.0 Hz, H-1″b), and a triplet of one hydrogen at δ = 5.24 (*J* = 7.0 Hz, H-2″) established the presence of a 3-methylbut-2-enyl [3], [15]. The broad doublet at δ = 6.29 (*J* = 0.7 Hz, H-3) showed long-range allylic coupling with H-1′ in the COSY spectrum, indicating the substitution at C-4 by the 1-acetoxypropyl moiety. The nature of the substituent at C-8 was deduced to be a butyryl chain from proton signals appeared at 3.29 (2H, t, *J* = 7.0 Hz, H-2‴), 1.79 (2H, sextet, *J* = 7.0 Hz, H-3‴), and 1.04 (3H, t, *J* = 7.0 Hz, H-4‴) and carbon signals at δ = 206.5 (C-1‴), 46.7 (C-2‴), 18.1 (C-3‴), and 13.8 (C-4‴) [15]. The presence of the three substituents described above was also confirmed by the 2D NMR experiments COSY and HMBC of **2** (Fig. **2**). From the proton-decoupled ^{13}C NMR spectrum of **2** (Table **1**), 23 signals were observed. Examination of both the ^{13}C NMR and HSQC spectra of **2** determined that the compound contains: five methyl carbon atoms at δ = 10.1 (C-3′), 25.9 (C-4″), 18.1 (C-5″), 13.8 (C-4‴), and 21.1 (methyl carbon atom of acetoxyl group); four methylene carbon atoms at δ = 28.7 (C-2′), 21.7 (C-1″), 46.7 (C-2‴), and 18.1 (C-3‴); two olefinic methine carbon atoms at δ = 106.4 (C-3) and 119.9 (C-2″); one methine carbon atom at δ = 73.7 (C-1′); and eleven quaternary carbon atoms at δ = 159.4 (C-2), 157.1 (C-4), 100.4 (C-4a), 158.2 (C-5), 110.1 (C-6), 165.6 (C-7), 104.6 (C-8), 156.4 (C-8a), 170.4 ($OCOCH_3$), 138.8 (C-3″), and 206.5 (C-1‴). Additionally, on the basis of the HMBC spectrum of **2** (Fig. **2**), the positions of the 3-methylbut-2-enyl, butyryl, 1-acetoxypropyl, and two phenolic groups (OH-5 and 7) were also established in a similar fashion to compound **1**. Thus, **2** was characterized as 5,7-dihydroxy-4-[1-(acetoxy)propyl]-6-(3-methyl-2-butenyl)-8-(1-oxobutyl)-2*H*-1-benzopyran-2-one, or mammea E/BC.

The molecular formula $C_{23}H_{28}O_7$ of **3** was established by positive high-resolution ESI mass measurement showing a quasi-molecular ion at *m/z* = 417.1924 $[M + H]^+$. The UV and negative ion ESI-MS data for compound **3** closely resemble those for **2**. The ^1H- and ^{13}C-NMR spectral data (Table **1**) of **3** are almost identical with those of **2**. However, **3** showed ^1H- and ^{13}C-NMR spectral data that were different from those of **2** only in the signal of the 8-acyl group. Compound **3**, which has a 2-methylpropionyl group, showed proton signals at 4.06 (septet, *J* = 6.5 Hz, H-2‴), 1.26 (d, *J* = 6.5 Hz, H-3‴), and 1.27 (d, *J* = 6.5 Hz, H-4‴), and carbon signals at δ = 210.9 (C-1‴), 40.4 (C-2‴), 19.3 (C-3‴), and 19.2 (C-4‴). The COSY and HMBC spectra data (Fig. **2**) of **3** gave further evidence to support the presence of 2-methylpropionyl, and the HMBC and UV spectra indicated that the 2-methylpropionyl substituent is attached at C-8 [14]. The structure of this compound was thus deduced as 5,7-dihydroxy-4-[1-(acetoxy) propyl]-6-(3-methyl-2-butenyl)-8-(2-methyl-1-oxopropyl)-2*H*-1-benzopyran-2-one, or mammea E/BD.

Twelve known coumarins, mammea A/AA (**4**) [4], [14], [16], mammea A/AA cyclo D (**5**) [4], mammea A/AA cyclo F (**6**) [7], [17], mammea A/AC cyclo D (**7**) [14], [18], mammea A/AD cyclo D (**8**) [14], [16], mammea B/BA (**9**), mammea B/BA cyclo F (**10**) [6], mammea B/BB (**11**), mammea B/BC (**12**) [3], mammea B/BD (**13**) [9], mammea E/BA (**14**), and mammea E/BB (**15**) [8], isolated from *M. americana* were identified by NMR (including HSQC and HMBC), ESI-MS, and comparison of their UV and ^1H-NMR data with those reported in the literature. The ^{13}C-NMR data of mammea B/BA (**9**), mammea B/BA cyclo F (**10**), mammea B/BB (**11**), mammea B/BC (**12**), mammea B/BD (**13**), mammea E/BA (**14**), and mammea E/BB (**15**) have been assigned here since these data have not been reported previously. In addition, two known flavonols, (+)-catechin (**16**) and (–)-epicatechin (**17**), isolated from *M. americana* seeds were identified by comparison with commercial standards by RP-18 TLC and ESI-MS.

The new compounds **2** and **3** were suggested in Crombie's report in 1972 [8]. The authors hypothesized that either compound **2** or **3** was present in a colorless crystalline material isolated from *M. americana* seeds, because they found an ion at *m/z* = 416 among other ions in the EI-MS of the material from which they identified mammea E/BA (**14**) and mammea E/BB (**15**) [8]. Their supposition was proven to be correct by our results on the same fruit seeds. Separation of compounds **2**, **3**, **14**, and **15** from *M. americana* seeds by traditional column chromatography and preparative thin layer chromatography is difficult because of their similarity to each other. Small amounts of **2**, **3**, **14**, and **15** were obtained only by repeated reversed-phase preparative HPLC.

The HMBC experiments were run on each coumarin (**1** – **15**) isolated from *M. americana* in our investigation to determine definitively the position of the acyl moiety. The cross-peak of the downfield phenolic hydroxy proton signal with the carbon signal of the carbonyl function of an acyl group was observed clearly in the HMBC experiments in addition to other long-range correlations. For example, in compound **1** the acyl group is attached at C-8, and we observed a correlation from the phenolic hydroxy proton (δ = 14.51, 1H, s, OH-7) to C-1‴ (δ = 206.4) in the HMBC experiment. Similar correlations were observed in the HMBC experiments of compounds **2**, **3**, **9**, **10**, and **11** – **15**. On the other hand, for compound **4,** the acyl group is attached at C-6, and in the HMBC experiment we observed correlation from the phenolic hydroxyl proton (δ = 14.38, 1H, s, OH-5) to C-1″ (δ = 207.1). The HMBC experiments of compounds **5** – **8** also showed similar correlations. These long-range correlations may be observed because of the hydrogen-bond formed between the downfield phenolic hydroxy proton and the carbonyl function of the acyl group or due to ^4J allylic coupling. These HMBC correlations thus gave additional evidence to determine the position of the acyl moiety for each of the coumarins (**1** – **15**).

The fifteen isoprenylated coumarins (**1** – **15**) isolated from *M. americana* in our study were screened for their cytotoxicity in the SW-480, HT-29, and HCT-116 human colon cancer cell lines and antioxidant capacities in the DPPH assay, and their IC_{50} values are shown in Table **2**. 5-Fluorouracil (5-FU), a drug frequently used for human colon cancer treatment, was used as a positive control to compare its cytotoxicity in the SW-480, HT-29, and HCT-116 human colon cancer cell lines (IC_{50} = 53.0, 46.1, and 45.1 μM, respectively) with the fifteen isoprenylated coumarins.

Compounds **1** – **15** exhibited significant cytotoxic activities in the SW-480, HT-29, and HCT-116 human colon cancer cell lines (IC_{50} ranges 13.9 – 88.1, 11.2 – 85.3, and 10.7 – 76.7 μM, respectively). For a given coumarin (**1** – **15**), its cytotoxicity in each of these three human colon cancer cell lines showed similar potency (Table **2**). The eight 4-propylcoumarins (**2**, **3**, **9**, and **11** – **15**) with an isoprenyl side-chain showed the highest cytotoxicity in the SW-480, HT-29, and HCT-116 human colon cancer cell lines (IC_{50} ranges 13.9 – 17.7, 11.2 – 16.8, and 10.7 – 16.1 μM, respectively). The one 4-phenylcoumarin (**4**) with an isoprenyl side-chain displayed the second highest cytotoxicity in the three cell lines (IC_{50} = 25.1, 27.1, and 25.9 μM, respectively). The three 4-phenylcoumarins (**5**, **7**, and **8**) with an unsaturated chromene moiety (pyran ring) displayed the third highest cytotoxicity in the three cell lines (IC_{50} ranges 38.4 – 38.9, 35.9 – 42.1, and 30.8 – 32.2 μM, respectively). The two 4-propylcoumarins (**1** and **10**) and one 4-phenylcoumarin (**6**) without any unsaturated side-chains revealed the least cytotoxicity in the three cell lines (IC_{50} ranges 48.9 – 88.1, 61.8 – 85.3, and 46.4 – 76.7 μM, respectively). These results suggest that: (a) the presence of an unsaturated side-chain in a coumarin increases the cytotoxicity (e.g., **2** – **5**, **7** – **9**, **11** – **15**), especially the presence of an isoprenyl side-chain (e.g., **2** – **4**, **9**, **11** – **15**); and (b) a change in the 8-acyl moiety does not result in loss of cytotoxicity (e.g., **2**, **3**, **5**, **7** – **9**, **11** – **15**). These conclusions follow the same patterns of anti-HIV activity and cancer

Table **2** Cytotoxic and antioxidant activities of compounds **1** – **15** and 5-FU

Compound	*IC_{50} (μM)* *DPPH (± S.D.)[a]*	*SW-480 (95% CI)*	*HT-29 (95% CI)*	*HCT-116 (95% CI)*
1	NA[b]	88.1 (76.7 – 103.9)	80.9 (74.3 – 86.6)	76.7 (70.5 – 81.7)
2	88 ± 1.3	14.2 (13.2 – 15.1)	12.3 (10.6 – 13.9)	11.5 (10.6 – 14.4)
3	88 ± 1.4	14.7 (13.9 – 16.3)	12.5 (11.3 – 13.5)	12.0 (11.5 – 14.2)
4	135 ± 2.1	25.1 (23.6 – 26.1)	27.1 (23.4 – 29.6)	25.9 (21.7 – 29.1)
5	NA	38.4 (32.2 – 44.6)	42.1 (37.1 – 52.0)	32.2 (28.0 – 37.1)
6	NA	80.6 (71.1 – 80.6)	85.3 (73.5 – 94.8)	75.8 (59.2 – 97.2)
7	NA	38.9 (34.4 – 43.5)	39.7 (34.6 – 44.8)	32.0 (26.9 – 37.1)
8	NA	38.5 (35.6 – 41.2)	35.9 (30.8 – 38.4)	30.8 (25.9 – 35.9)
9	90 ± 0.7	17.5 (16.1 – 18.5)	13.7 (13.4 – 15.0)	16.1 (14.8 – 18.3)
10	NA	48.9 (45.3 – 53.6)	61.8 (51.5 – 69.6)	46.4 (38.7 – 63.4)
11	91 ± 1.1	17.7 (16.1 – 19.1)	13.2 (11.3 – 15.0)	12.9 (11.0 – 15.6)
12	90 ± 0.8	15.7 (14.5 – 16.8)	15.3 (12.8 – 17.3)	14.5 (12.8 – 17.6)
13	92 ± 0.9	15.4 (14.2 – 16.2)	16.8 (14.0 – 19.8)	15.9 (14.8 – 20.1)
14	86 ± 1.4	13.9 (13.5 – 15.3)	11.2 (8.1 – 13.0)	10.7 (9.3 – 12.8)
15	88 ± 1.3	15.4 (14.9 – 16.3)	11.6 (10.9 – 13.2)	13.9 (11.6 – 16.5)
5-FU		53.0 (47.7 – 60.7)	46.1 (43.0 – 50.7)	45.4 (42.3 – 53.0)

[a] Mean ± S.D. (n = 3).
[b] NA: no activity

chemopreventive effect observed previously by other groups with isoprenylated coumarins [19], [20], [21], [22].

The coumarins **1** – **3** were studied by DNA flow cytometry with SW-480 colon cancer cells. In the assay the sub-diploid population of cells represents cells undergoing apoptosis. These three compounds were chosen since they are new coumarins, and they range from relatively weak (compound **1**) to strong (compounds **2** and **3**) activity with respect to cytotoxicity (Table **2**). Compound **1** exhibited a dose-dependent increase in sub-diploid cells from 2.5% for untreated controls to 4.9% at 24.7 μM, 6.2% at 74.3 μM and 9.2% at 148.5 μM at 24 h. Both **2** and **3** exhibited more potent dose-dependent increases in sub-diploid cells than compound **1**, at both 24 and 48 h. At 24 h, compound **2** exhibited an increase in sub-diploid cells from 2.5% in untreated controls to 5.1% at 7.2 μM and 35% at 12.0 μM. Compound **3** also exhibited an increase in sub-diploid cells from 2.5% in controls to 5.5% at 7.2 μM and 26.1% at 12.0 μM at 24 h. Both **2** and **3** induced 90% sub-diploid cells at 12.0 μM at 48 h compared to 12% in the untreated controls. There were no significant changes in the distribution of cells in the G0/G1, S, or G2/M phases of the cell cycle after treatment with compounds **1**, **2**, or **3**. Thus, compounds **2** and **3** are active with respect to both cytotoxicity and induction of apoptosis.

Compounds **2** – **4**, **9**, and **11** – **15** displayed antioxidant activity in the DPPH assay (IC_{50} range 86 – 135 μM), but compounds **1**, **5** – **8**, and **10** had no antioxidant activity (IC_{50} > 200 μg/mL). These results are in agreement with previous reports of structure-activity relationship of antioxidant polyphenols [23]. The hydrogen donor plays an important role in the antioxidant activity of polyphenols. The more hydrogen donors, the stronger is the antioxidant activity. Fifteen isoprenylated coumarins isolated from *M. americana* in this study are derivatives of 5,7-dihydroxycoumarin. These derivatives should display antioxidant activity if one or both of these hydroxy groups are free, since they are known to be good hydrogen donors [23]. Thus compounds **2** – **4**, **9**, and **11** – **15** displayed relatively high antioxidant activity since 5-OH in these coumarins is free although 7-OH forms a hydrogen bond with the carbonyl function of an acyl group. Compounds **1**, **5** – **8**, and **10**, however, had no available hydrogen donors because the two hydroxy groups in these coumarins are not free: one forms a hydrogen bond with the carbonyl function of an acyl group and the other is cyclized to a dihydrofuran ring (e. g., compounds **1**, **6**, and **10**) or pyran ring (e. g., compounds **5**, **7**, and **8**). Therefore, compounds **1**, **5** – **8**, and **10** were inactive in the DPPH assay**.** Thus, there is no correlation between the cytotoxic and antioxidant activities in this series of compounds (Table **2**).

On the basis of the present findings, the above-mentioned coumarins, especially those with the isoprenyl side-chain, may be valuable as potential anticancer agents. However, further *in vitro*, *in vivo*, and clinical studies are needed to understand the significance of these coumarins to human health. We are now in the process of examining the molecular mechanism by which compounds **1** – **3** inhibit growth and induce apoptosis in human colon cancer cells.

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