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ARTICLE

Bioactive Compounds from Sour Orange Inhibit Colon Cancer Cell Proliferation and Induce Cell Cycle Arrest

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Epidemiological studies suggest that dietary limonoids and phytosterols offer protection from certain types of cancers. Potential cancer preventive constituents of sour orange (*Citrus aurantium* L.) were isolated and identified from ethyl acetate extract. The structures of the compounds were identified by one-dimensional (^{1}H or ^{13}C) and two-dimensional (^{1}H−H and ^{1}H−^{13}C) nuclear magnetic resonance experiments as limonexic acid and β-sitosterol glucoside (SG). The identified compounds were tested for the potential inhibition of human colon cancer cell (HT-29) proliferation, apoptosis, and also noncancerous cells (COS-1). Cell proliferation, arrest of the cell cycle, and induction of apoptosis were assessed by MTT assay, flow cytometry, and nuclear staining methods, respectively. The MTT assay indicated that both compounds exhibited significant inhibition at various concentrations. These compounds did not show any toxic effects on noncancerous cells. These compounds caused 4−5-fold increases in the counts of G2/M stage cells at 50 μM, indicating a potential role in cell cycle arrest. These findings support the hypothesis that limonoids and phytosterols are effective apoptosis-promoting agents and incorporation of enriched fractions of these compounds in the diet may serve to prevent colon cancer. To the best of our knowledge, this is the first report of the isolation, identification, and cell proliferation assay for limonexic acid and SG from sour orange.

KEYWORDS: *Citrus aurantium* L.; limonexic acid; β-sitosterol glucoside; HT-29; MTT assay; flow cytometer

1. INTRODUCTION

Epidemiological data suggest that ingestion of some bioactive compounds from fruits and vegetables may contribute to cancer reduction in humans. Since 1970, there has been tremendous interest in *Citrus* fruits because of the presence therein of large amounts of putative bioactive compounds and their health benefits. Among the human health-maintaining phytonutrients in citrus juices are limonoids, flavonoids, sterols, furocoumarins, and vitamins (*1*−*3*). Our recent investigations of citrus found that several potential bioactive compounds have been identified in addition to vitamin C (*2*−*6*).

Phytosterols (PS) are the counterparts of cholesterol in animals. In vitro and animal studies suggest that PS offer protection from the most common cancers in developed countries, including colon, prostate, and breast (*7*). However, the exact mechanism by which dietary phytosterols offer this protection is not fully understood. Dietary consumption of PS is lower in developed countries (80 mg/day) as compared to developing countries (400 mg/day). It seems that the incidence and/or the death rate from these cancers that is minimal in developing countries may be due to the consumption of PS (*8*).

Colorectal carcinoma is the second leading cancer in the United States (*9*). It is well-known that dietary factors can modulate the development of certain types of human cancer, including colon cancer. The antiproliferative effects of limonoids have been shown in various cancers, including but not limited to breast cancer (*10*), colon cancer (*11*, *12*), stomach cancer (*13*, *14*), and neuroblastoma cancer (*15*). Our recent studies (*15*, *16*) and many others (*17*−*19*) have demonstrated that citrus limonoids have numerous health benefits, including but not limited to countering the effects of antimalarial and anti-inflammatory activity and acting as cytotoxic and cytostatic agents in animals and human cell cultures.

Citrus limonoids inhibit average tumor burden in DMBA-induced hamster buccal pouch carcinogenesis (*19*). In addition, limonin and nomilin were found to inhibit HIV-1 replication in human peripheral blood mononuclear cell cultures (*20*). Our group has demonstrated that certain citrus bioactive compounds exhibit differential levels of inhibition of colon cancer (*16*) and caspase-mediated apoptosis inducing effects on human nuroblastoma cancer cells in culture models (*15*, *21*). The results of the study described above demonstrate that certain limonoid glucosides have arrested significantly S and G2/M stages of the

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cell cycle (*21*). Our recent study demonstrated that different solvent extracts from sour orange were reported to possess significant antioxidant activity in in vitro models (*22*). To further characterize the bioactive compounds from sour orange, we have purified and characterized two bioactive compounds by nuclear magnetic resonance (NMR) studies for the first time. Moreover, identified compounds have been tested for the inhibition of colon cancer cells (HT-29) for the first time, and these compounds were found to be nontoxic to normal cells.

2. MATERIALS AND METHODS

2.1. Materials. All solvents used in this study for extraction and high-performance liquid chromatography (HPLC) were obtained from Sigma-Aldrich (St. Louis, MO). TLC plates [silica gel 60 F-254, thickness of 0.20 mm (20 cm × 20 cm)] were obtained Fisher Chemicals. *p*-(*N*,*N*-Dimethylamino)benzaldehyde (Ehrlich's reagent) and silica gel (200−400 mesh) were obtained from Sigma Chemical Co. (St. Louis, MO).

2.2. Plant Material. Sour oranges (*Citrus aurantium* L.) were harvested from the orchard of Texas A&M University-Kingsville Citrus Center (Weslaco, TX) in October and November 2006. The seeds were separated, dried, and powdered to pass through a 40−60 mesh sieve using a mixer grinder (VitaMix Corp., Cleveland, OH).

2.3. Extraction. Ground citrus seed powder (4800 g) was defatted using a Soxhlet apparatus with hexane (15 L) for 24 h. The defatted powder was extracted for 8 h with ethyl acetate (15 L) at 60−70 °C. The extracts were filtered and concentrated under vacuum (Buchi) to yield a viscous concentrate.

2.4. Purification. The viscous liquid described above was impregnated with silica gel and loaded onto a silica gel (250 g) column. The column was eluted thoroughly with 5 L of dichloromethane (DCM), mixtures of DCM and acetone (95:5 to 25:75), and acetone. Fractions (500 mL each) were collected and concentrated under vacuum. The fractions containing the same HPLC peaks were combined and further purified as mentioned above using silica gel column chromatography. Compounds **1** (56 mg) and **2** (49 mg) were eluted using 7:3 and 4:6 DCM/acetone mixtures, respectively.

2.5. High-Performance Liquid Chromatography Analysis. HPLC analysis was conducted using an Agilent (Foster City, CA) HPLC 1200 Series system consisting of a degasser, a quaternary pump, an auto sampler, a column oven, and a diode array detector. Compound **1** and column fractions were eluted using a Zorbax SB-C18 column [250 mm × 4.6 mm (inside diameter)] and detected at 210 nm with a flow rate of 0.8 mL/min. The elution was conducted with a gradient of mobile phase A (3 mM phosphoric acid) and mobile phase B (acetonitrile): from 100 to 50% A for 0−26 min, from 50 to 10% A for 4 min, and 100% A for 5 min. Compound **2** was analyzed at 207 nm with a flow rate of 0.5 mL/min with a linear gradient from 100 to 0% A in 26 min. The column fractions were filtered through 0.45 μm filters and subjected to HPLC.

2.6. NMR Experiments. Two isolated compounds, **1** and **2**, were characterized by NMR studies. ^{1}H and ^{13}C NMR experiments were performed on a Bruker Biospin Avance 400 NMR spectrometer (^{1}H frequency of 400.13 MHz, ^{13}C frequency of 100.62 MHz) at 298 K using a 5 mm broadband inverse probehead equipped with a shielded z-gradient and XWIN-NMR version 3.5 using TMS as an internal reference. One-dimensional ^{1}H and ^{13}C spectra were recorded using a one-pulse sequence. ^{1}H spectra were also recorded after the addition of a drop of deuterium oxide to identify exchangeable protons. One-dimensional ^{13}C spectra using spin-echo Fourier transform (SEFT) and quaternary carbon detection (*23*) (QCD) sequences were also recorded to aid in structure identification. Homonuclear and heteronuclear two-dimensional (2D) NMR experiments such as ^{1}H−^{1}H double-quantum-filtered correlation (DQF-COSY), sensitivity-enhanced and multiplicity-edited ^{1}H−^{13}C heteronuclear single-quantum correlation (*24*) (edited HSQC), and ^{1}H−^{13}C gradient-enhanced heteronuclear multiple-bond correlation (HMBC) experiments were performed. For the DQF-COSY experiment, a spectral width of 4800 Hz was used in both the dimensions and 512 FIDs were obtained with t_1 increments, each with 2048 complex data points. The number of transients and the relaxation delay used were 32 and 5 s, respectively. Phase sensitive data were obtained using the TPPI method. For ^{1}H−^{13}C multiplicity-edited HSQC and HMBC experiments, spectral widths of 4800 and 24000 Hz were used in the ^{1}H and ^{13}C dimensions, respectively; 512 FIDs were collected with t_1 increments each of 2048 data points, 32 transients, and a 2 s recycle delay. Phase sensitive data for the edited HSQC experiment were obtained using the echo−anti-echo mode, whereas for HMBC, magnitude mode data were obtained. The resulting 2D data were zero filled to 1024 points in the t_1 dimension and double Fourier transformed after being multiplied by a squared sine-bell window function shifted by $\pi/2$ along both dimensions. For the stereochemical assignments of the protons, one-dimensional nuclear Overhauser enhancement (NOE) experiments were performed on both compounds. The NOE buildup on the proximal protons was monitored from the difference spectrum of on- and off-resonance irradiation of individual proton signals.

2.7. Cell Culture. Human HT-29 colorectal adenocarcinoma cells and noncancerous COS-1 monkey kidney fibroblast cells (CRL-1650) were obtained from American Type Culture Collection (Bethesda, MD). Both types of cells were grown at 37 °C with 5% CO_2 and 90% relative humidity. HT-29 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 1.5 mM L-glutamine, 2.2 g/L sodium bicarbonate, 100 μg/mL penicillin G, streptomycin, and 1 μg/mL amphotericin B. The COS-1 cells were grown in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, with 10% fetal bovine serum, 100 μg/mL penicillin G, streptomycin, and 1 μg/mL amphotericin B.

2.8. Cell Viability Assay. Colon cancer cells (HT-29) and normal cells (COS-1) cells were cultured in 96-well plates in triplicate separately and in three different trials, at a density of 10^3 cells/well in 200 μL of medium. The cells were grown to ∼70% confluence, and treatments were initiated by supplementing the cells with 5, 10, 20, and 40 μM limonexic acid, SG, and camptothecin in DMSO (final concentrations in medium). The plates were incubated for 24, 48, and 72 h. The final concentration of DMSO in culture medium was maintained at $<0.005\%$. Camptothecin was used as a positive control. The viability of the cells was assayed on the basis of the ability of the live cells to reduce MTT as previously described (*21*). The color product formazon was measured spectrophotometrically at 550 nm using a KC-4 microplate reader (BioTek Instruments, Winooski, VT). After the incubation (24, 48, and 72 h) with the compounds, 20 μL of the MTT solution (5 mg/mL in PBS) was added to each well and the wells were incubated for 2 h at 37 °C in a humidified chamber with 5% CO_2. The colored product (formazon), formed by the reduction of MTT by the active mitochondria of the live cells, was released into the solution using cell lysis buffer and solubilized via addition of 50 μL of DMSO. The absorbance of formazon was measured at 550 nm using a microplate reader. Three different replicate trials were conducted for each compound, and the results are presented as means ± the standard deviation (SD).

2.9. Cell Cycle Analysis by Flow Cytometry. HT-29 cells were grown in 25 mL Falcon culture flasks at 37 °C under 5% CO_2. At 70−80% confluence, the cells were detached using Pucks EDTA and distributed to 24-well plates. After the cells reached nearly 90% confluence in the plates, the medium (1.0 mL) was changed and limonexic acid, SG, and camptothecin were used to reach concentrations of 25 and 50 μM. The plates were incubated at 37 °C for 48 h. After the incubation, the cells were harvested using Pucks EDTA. The cells were washed three times using PBS (0.01 M, pH 7.4). The supernatant was removed carefully, and the cells were washed with 1.0 mL of PBS and centrifuged. Finally, the supernatant was removed slowly without disturbing the cells; 200 μL of 70% ethanol and 200 μL of PBS were added, and the solutions were gently mixed and stored at −80 °C until further use. Before the sample preparation for the flow cytometer was begun, the cells were centrifuged and the supernatant was removed and washed with PBS buffer twice. The cell pellet was suspended in 1 mL of staining reagent (1.25 mL of propidium iodide, 5 mg of RNase, 2.5 mL of sodium citrate, 0.250 mL of Triton X, and 18.5 mL of water). After a 15 min incubation at 37 °C, the suspension was passed through a nylon filter and collected in tubes. DNA fluorescence readings were taken using a Beckton Dickenson FACS Caliber flow cytometer with an excitation blue light set at 488 nm and a detector emission of red fluorescence through a 585 nm filter. Pulse width area signals were used to discriminate between G_2 cells and cell doublets. The data were analyzed using ModFitLT version 3.1 (PMac). The relative distribution of 10000 events for each sample was analyzed for background aggregates and debris (BAD), an indicator of apoptosis as well as G_1, S, G_2, and M phases of the cell cycle.

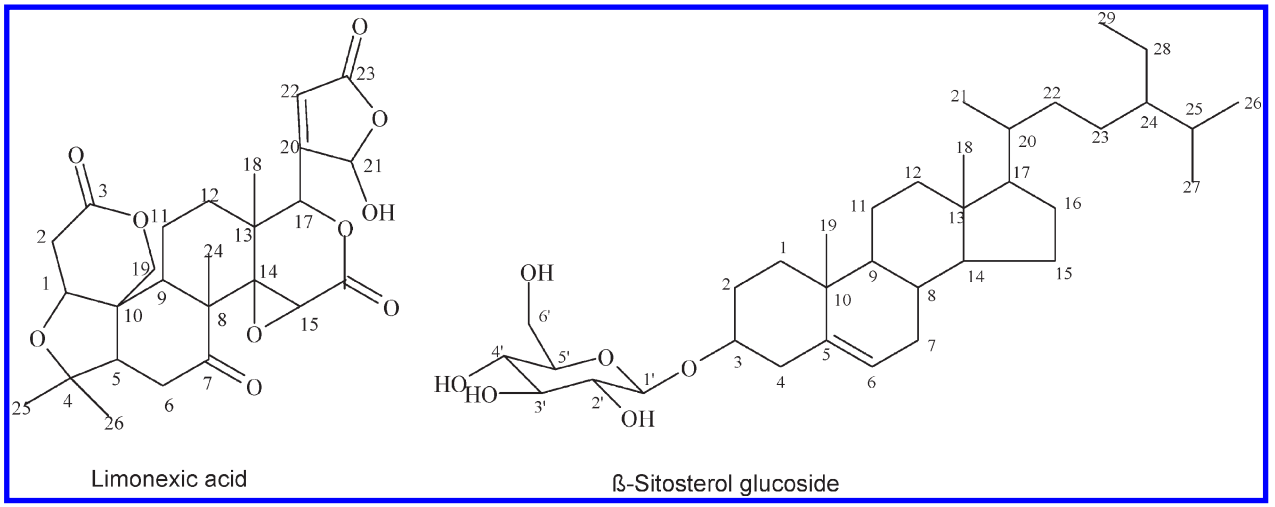

Figure 1. Structures of isolated compounds from sour orange: limonexic acid (compound **1**) and β-sitosterol glucoside (compound **2**).

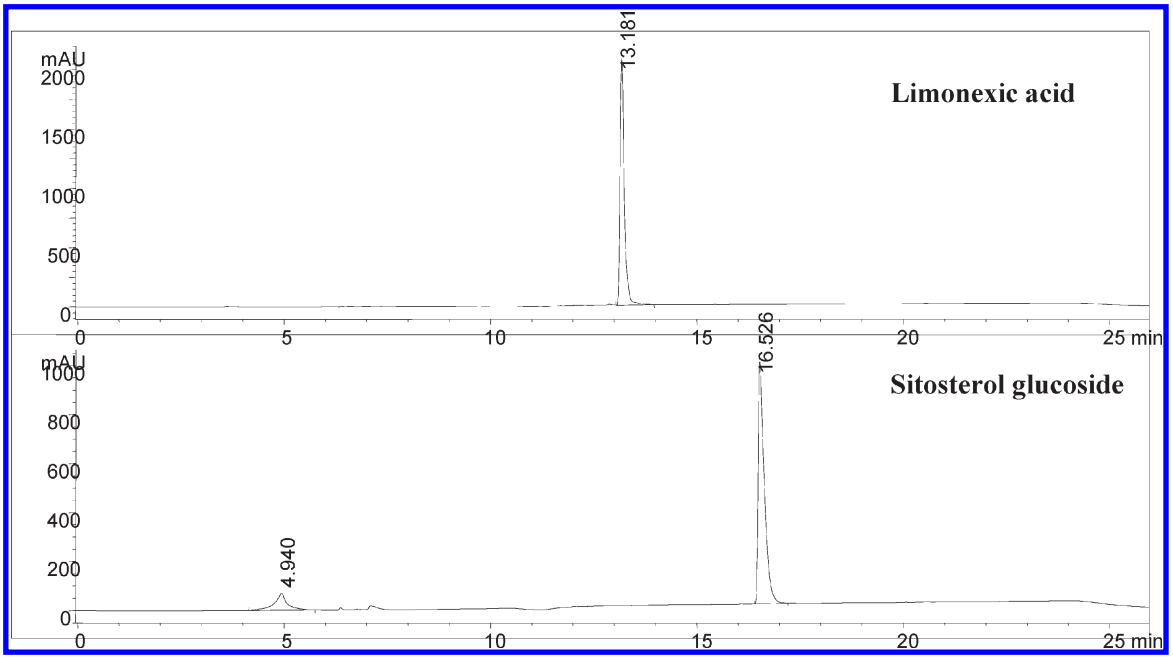

Figure 2. HPLC chromatograms of isolated compounds from sour orange.

2.10. Assessment of Apoptosis. Assessment of apoptosis was performed using the acridine orange and ethidium bromide staining method, which is based on the differential staining of viable and apoptotic cells in a mixture. A total of 10^4 cells/mL were grown (HT-29) in eight-well chamber glass culture slides at 70% confluence. The cells were treated with 50 μM limonexic acid, SG, and camptothecin supplemented through the fresh medium. The plates were incubated at 37 °C for 24 h and stained with 1 μL of 1× working solution of nuclear stains consisting of ethidium bromide (500 μg), DAPI (100 μg), and acridine orange (100 μg) in 1 mL of PBS. After being incubated for 10 min, the cells were observed under an Olympus FV1000 confocal microscope with spectral imaging and photoactivation (Olympus America Inc., Center Valley, PA) with 495 nm primary and 515 nm secondary filters. Dead cells were stained a bright orange color, while viable cells were stained a bright green color.

2.11. Statistical Analysis. SPSS software was used for statistical analyses. Mean values among treatment groups were compared by the ANOVA test. P values of ≤ 0.05 were considered significant.

3. RESULTS AND DISCUSSION

A recent study demonstrated that different solvent extracts from sour oranges possess significant antioxidant activity in in vitro models (*22*). Hence, our study was focused on isolation and identification of putative bioactive compounds present in *C. aurantium*. Seed powder of sour orange was extracted with ethyl acetate (EtOAc) for 8 h in a Soxhlet type extractor, and the extract was concentrated under vacuum. The concentrate of EtOAc was freeze-dried. To explore potential bioactive components responsible for the antiproliferative activity, the EtOAc fraction was analyzed by TLC and HPLC for chemical composition. The EtOAc extract was subjected to repeated silica gel column chromatography. The column was eluted with DCM and acetone with increasing polarity to yield two compounds. Compounds **1** (56 mg) and **2** (49 mg) were eluted with 7:3 and 4:6 DCM/acetone mixtures, respectively (**Figure 1**). The purity of the isolated compounds was analyzed by TLC and HPLC. No additional spots were observed on TLC plates with either Ehrlich's reagent or methanolic sulfuric acid followed by heating at 100 °C for 10 min, thus confirming the purity of the compounds. Moreover, the purity of the isolated compounds was further confirmed by HPLC (**Figure 2**).

Table 1. ^{1}H and ^{13}C NMR Chemical Shifts for Limonexic Acid (compound **1**) (solvent, DMSO-d_6)

		^{1}H		
no.	group	a	e	^{13}C
1	CH	4.13	–	78.45
2	CH_2	2.66	2.80	35.70
3	C	–		170.25
4	C	–		79.59
5	CH	2.44	–	58.46
6	CH_2	3.14	2.27	36.23
7	C	–		207.99
8	C	–		50.80
9	CH	2.53	–	46.55
10	C	–		45.37
11	CH_2	1.81		17.76
12	CH_2	1.24	1.73	28.84
13	C	–		37.89
14	C	–		66.13
15	CH	–	4.03	53.07
16	C	–		166.19
17	CH	5.17		77.85
18	CH_3	1.09 (α)		19.80
19	CH_2	4.93	4.45	64.97
20	C	–		163.82
21	CH	6.06		98.25
22	CH	6.27		122.07
23	C	–		169.13
24	CH_3	0.97 (β)		16.51
25	CH_3	1.03 (β)		21.45
26	CH_3	1.19 (α)		29.71
21	OH	8.20		–

3.1. Elucidation of Structure from ^{1}H and ^{13}C Two-Dimensional (2D) NMR. After confirming the purity of the isolated compounds by HPLC, we employed one and two-dimensional NMR spectroscopy to elucidate the structures. Proton spectra of these compounds were complex with many overlapped peaks. Intense characteristic methyl signals were first assigned tentatively using the one-dimensional (1D) spectrum. Assignment of the resonances from the overlapped region due to methylene (CH_2) and methine (CH) protons of both compounds was not possible from the 1D spectrum alone. Hence, sensitivity-enhanced and multiplicity-edited $^{1}H-^{13}C$ heteronuclear single-quantum correlation (*23*) (edited HSQC) and spin-echo Fourier transform (SEFT) spectra were used first for the identification of these groups. The assignment of all the carbons and the attached protons was then made from the combination of $^{1}H-^{1}H$ double-quantum-filtered correlation (DQF-COSY) and $^{1}H-^{13}C$ gradient-enhanced heteronuclear multiple-bond correlation (HMBC) experiments. To trace the signals from nonprotonated carbons, a quaternary carbon detection (*24*) (QCD) experiment was used. This experiment detects only nonprotonated carbons by effectively suppressing signals from CH, CH_2, and CH_3 types of carbons. Typical 1D ^{1}H and ^{13}C NMR spectra of both compounds and 2D spectra for compound **2** are shown in Figures S1−S5 of the Supporting Information. Stereochemical assignment of the protons was subsequently made on the basis of the relative NOE peak intensity. ^{1}H and ^{13}C chemical shifts of both the compounds thus derived are listed in **Tables 1** and **2**. Analysis of ^{1}H and ^{13}C 1D and 2D NMR experiments with the compounds facilitated identification of compounds **1** and **2** as limonexic acid and β-sitosterol glucoside, respectively (**Figure 1**).

3.2. Cell Viability. The MTT assay was performed using limonexic acid, SG, and camptothecin at different concentrations with colon cancer cells. This method is based on the reduction of yellow tetrazolium salt to a purple formazan by active mitochondria which was assessed with a spectrophotometer. These absorbances were converted to the percentage of cell viability with respect to control. Both the compounds inhibited cell proliferation in a linear dose-dependent manner. We have compared the antiproliferative effect of these compounds for 24, 48, and 72 h (**Figure 3**). Interestingly, our results showed that limonexic acid (47.5%) was a more potent inhibitor of colon cancer cells than SG (38.4%) at 40 μM after 24 h. On the other hand, significant cytotoxic effects of limonexic acid (66.5%) were noticed after treatment for 72 h on the colon cancer cells when compared to that of untreated cells at a concentration of 20 μM ($P < 0.005$). However, the significant difference in the cytotoxic effects for SG was observed only at 40 μM ($P < 0.05$). In the case of positive control (campthothecin), the growth inhibition of colon cancer cell is significant at 5 μM onward in 24 h. On the other hand, these compounds had no significant toxic effect on the growth of noncancerous mammalian COS-1 kidney fibroblast cells (**Figure 3**), which indicates a specific effect of these compounds only for cancer cells. In this study, we have observed significant inhibition at higher SG concentrations. This confirms the previously published results (*25*−*27*).

Table 2. ^{1}H and ^{13}C NMR Chemical Shifts for β-Sitosterol Glucoside (compound **2**) (solvent, DMSO-d_6)

		^{1}H		
no.	group	α	β	^{13}C
1	CH_2	1.79	0.98	36.86
2	CH_2	1.46	1.83	28.75
3	CH	–	3.46	76.97
4	CH_2	2.12	2.36	38.34
5	C	–		140.49
6	CH	–	5.32	121.23
7	CH_2	1.50	1.93	31.41
8	CH	–	1.40	31.46
9	CH	0.88	–	49.64
10	C	–		36.25
11	CH_2	1.47	1.47	20.63
12	CH_2	1.96	1.14	39.13
13	C	–	–	41.89
14	CH	0.98	–	56.20
15	CH	1.54	1.03	23.88
16	CH_2	1.80	1.25	27.83
17	CH	1.09	–	55.47
18	CH_3	0.64		11.70
19	CH_3	0.95		19.13
20	CH	–	1.34	35.51
21	CH_3	0.89		18.65
22	CH_2	1.30	1.00	33.39
23	CH_2	1.15/1.15		25.48
24	CH	0.91		45.18
25	CH	1.63		28.75
26	CH_3	0.81		18.96
27	CH_3	0.81		19.72
28	CH_2	1.25	1.19	22.64
29	CH_3	0.82		11.81
1′	CH	4.21		100.80
2′	CH	2.88		73.37
3′	CH	3.11		76.71
4′	CH	3.01		70.01
5′	CH	3.05		76.61
6′	CH_2	3.63/3.39		61.01
2′	OH	4.90		–
3′	OH	4.90		–
4′	OH	4.90		–
6′	OH	4.43		–

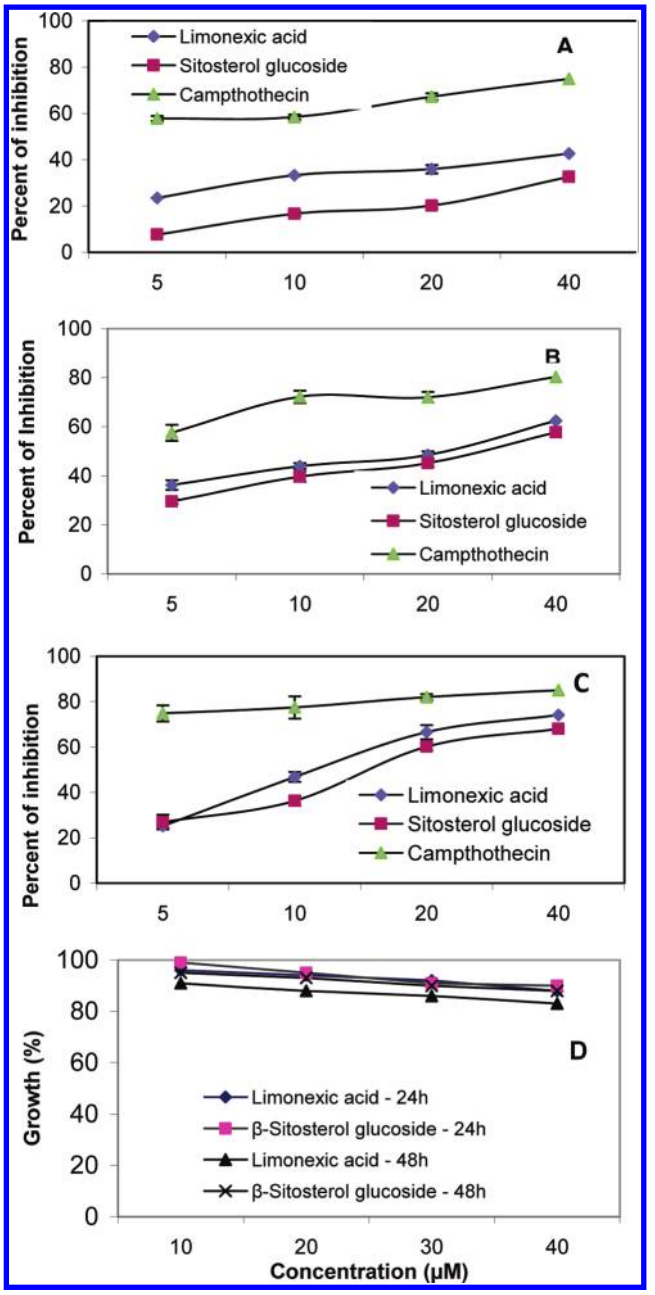

Figure 3. Determination of the percent inhibition of colon cancers (HT-29) and growth of normal cells by limonexic acid, β-sitosterol glucoside, and camptothecin at different concentrations after incubation for (**A**) 24, (**B**) 48, and (**C**) 72 h. (**D**) Growth of normal (COS-1) cells after 24 and 48 h. The results are averages of three independent experiments conducted in triplicate and expressed as means ± SD.

Phytosterols have inhibitory effects on breast cancer cells, and these effects occurred after incubation for 3−5 days at 8−16 μM. The growth inhibitory effect of phytosterols occurred at this concentration relevant to vegetarian diets similar to that observed with other tumor cell lines such as human prostate cancer (LNCaP) cells and colon cancer (HT-29) cells (*26*, *27*). These findings indicated that phytosterols inhibit the growth of several cancer cell lines representing common cancers of Western society. Phytosterols such as β-sitosterol, campesterol, and stigmasterol are the predominant sterols reported in foods. SG is a plant sterol similar in structure to cholesterol. However, SG is not a commonly found sterol in seeds and nuts. Epidemiological studies suggest that consumption of phytosterols may have protective

Table 3. Distribution (%) of HT-29 Cells at Various Stages of the Cell Cycle at Two Concentrations of the Isolated Compounds after 48 h Determined by Flow Cytometry Using Propidium Iodide Staining[a]

		% BAD	% G_0/G_1	% S	% G_2/M	% CV
control	0 μM	8.2	91.4	1.1	2.5	3.4
limonexic acid	25 μM	42.9	72.2	2	13.2	4.6
	50 μM	75.1	8.8	14.7	16.1	5.5
β-sitosterol glucoside	25 μM	56.6	72	1.9	9	4
	50 μM	72.1	89.1	17.2	10.8	6.2
camptothecin	25 μM	80.2	79.7	0	9.3	3.4
	50 μM	97.9	90.7	0	20.3	4.1

[a] Abbreviations: BAD, background aggregates and debris; CV, coefficient of variation; G_0, gap 1; G_1, gap 2; M, mitosis; S, DNA synthesis phase.

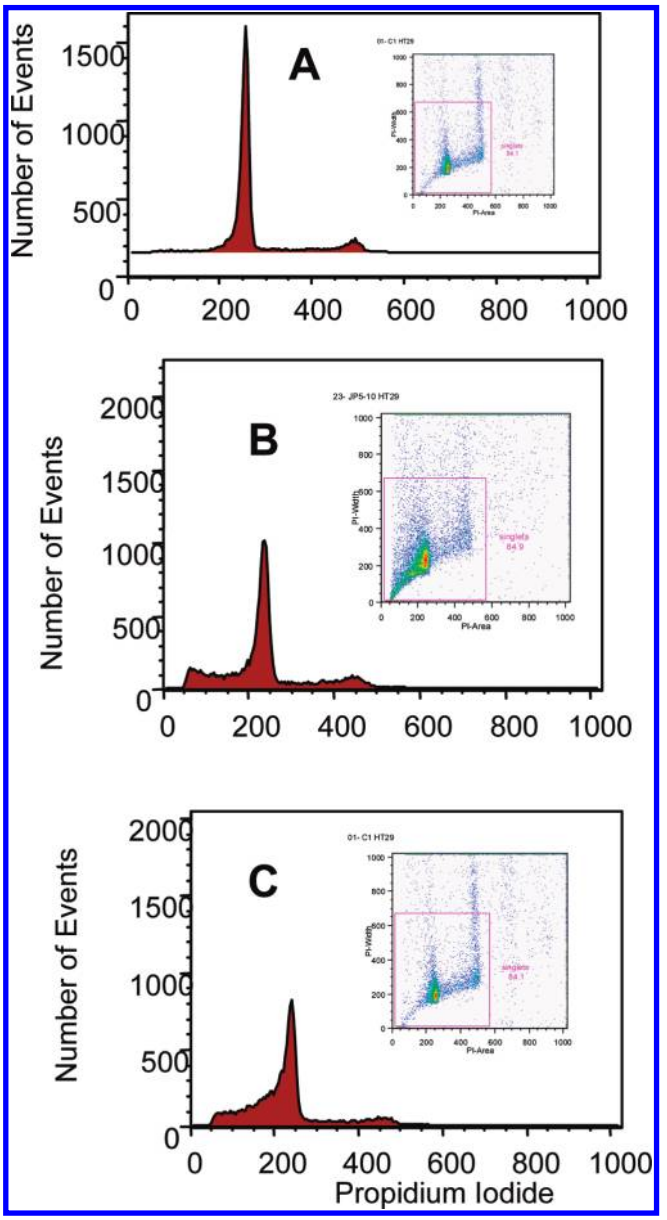

Figure 4. Inhibition of HT-29 cell proliferation and cell cycle progression by (**A**) control, (**B**) limonexic acid, and (**C**) β-sitosterol glucoside, at a concentration of 50 μM after 48 h. Horizontal and vertical axes indicate the relative nuclear DNA content and number of cells, respectively.

effects against several types of cancer, including colon (*26*), prostate (*27*), and breast carcinoma (*7*). Chang et al. (*25*) tested the inhibition of colon cancer cells by several compounds along

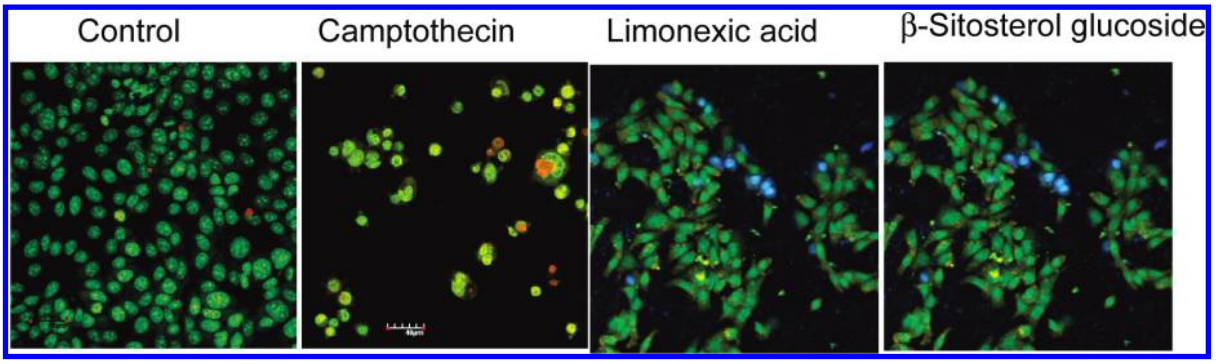

Figure 5. Effect of limonexic acid and β-sitosterol glucoside on apoptosis of colon cancer cells (HT-29) at a concentration of 50 μM after 24 h. After cells had been treated and stained as described in Materials and Methods, samples were analyzed under a fluorescence Olympus FV500 confocal microscope. The nuclei of control cells showed round and homogeneous nuclei, whereas nuclei of treated cells were condensed and fragmented. Apoptotic morphology is demonstrated by shrunken or dot-shaped nuclear fragments. Data are from a typical representative of three similar experiments. The magnification was 100×.

with SG from *Casearia memvranacea*. The IC_{50} value for SG was 26.55 ppm. In this study, we have demonstrated that SG can significantly inhibit the cell cycle and apoptosis in colon cancer cells at around same concentration after incubation for 24 h.

3.3. Effect on the Specific Stages of the Cell Cycle and Apoptosis. It has been hypothesized that the antiproliferative effects of limonexic acid and SG could be due to their ability to affect certain stages of the cell cycle in the cancerous cells. In this study, we have noticed a significant effect of limonexic acid and SG on the proliferation of HT-29 cells, mediated through the ability of the compounds to arrest certain stages of the cell cycle (**Table 3** and **Figure 4**). Limonexic acid exhibited 4- and 9-fold more background aggregates and debris (BAD) at concentrations of 25 and 50 μM, respectively, which is a profound indicator of apoptosis. On the other hand, 6- and 9-fold increases in BAD were observed with SG. Overall, our results showed that limonexic acid and SG induced arrest of DNA synthesis, G2/M phases of the cell cycle, and apoptosis in colon cancer cells (**Figures 4** and **5**). Both the compounds exhibited approximately 15% arrest of the DNA synthesis phase at 50 μM. Interestingly, a very negligible amount of cell arrest was observed at a concentration of 25 μM (**Table 3**). The flow cytometry data indicated a 3−4-fold increase in the cell cycle. The results were further confirmed via confocal fluorescence microscopy, where the DAPI nuclear staining indicated a loss of membrane integrity (**Figure 5**). This figure depicts the effect of limonexic acid, SG, and camptothecin on apoptosis of human colon cancer cells which were viewed under a fluorescence microscope. The nuclei of control cells showed round and homogeneous nuclei, whereas apoptotic cells from treated group showed condensed and fragmented nuclei. As predicted, the results showed a significant decrease in the number of viable cells that were treated with limonexic acid and SG.

These results clearly support the idea that both compounds are effective in inhibiting cancer cell proliferation. Apparently, there was no effect on the noncancerous COS-1 cells. Hence, it can be discerned from our study that purified limonoids had a specific toxic to lethal effect on human cancer cells, while they were nontoxic to normal cells. Plant-derived triterpenoids have shown significant promise for the prevention of a wide array of human cancers. Citrus fruits and juice contain significant quantities of limonoids. The concentration of limonoids in citrus juice ranges from approximately 140 to 300 ppm, while the limonoid glucoside content of citrus peel and flesh solids is ~500 ppm. Citrus seeds contain limonoid glucosides in ~1% on a dry weight basis (*28*, *29*). These compounds may significantly inhibit colon cancer cells. The specific correlation of the chemistry or biochemistry of these compounds in relation to their mode of action will enhance their potential realization as antineoplastic therapeutics. Accelerated cell growth or abnormal cell cycle regulation defines the progression of the cancer. Bioactive compounds from citrus may specifically inhibit the different stages of cancer cells, and these types of bioactive compounds are often considered as important candidates for drug development. For the first time, limonexic acid and SG have been purified from sour oranges and tested for their ability to affect the cell cycle, inducing cytotoxicity and induction of apoptosis. Cyclin-dependent kinases regulate the formation of a complex of the cell cycle with other cyclin ligands, and the G2/M transition is governed by the active formation of a complex of CDKs with the mitotic cyclins (*30*). Our results indicate a highly promising arrest of the G2/M phase of the cell cycle eventually leading to apoptosis, but the specific mechanisms of these compounds on these kinase receptors and other transcription factors are yet to be determined. However, both limonexic acid and SG possess potential chemopreventive properties and therefore provide significant leads in development therapies for checking the incessant progression of colonic adenocarcinomas. Further studies are required to improve our understanding of structure−activity relationships, which will provide a basis for selective chemical or biochemical methods for enhancing antitumor activity. Moreover, the bioavailability of citrus limonoids and the mechanism of absorption are important future research objectives.

Supporting Information Available: Complete assigned 2D NMR spectra for the isolated compounds **1** (limonexic acid) and **2** (β-sitosterol glucoside) (Figures S1−S5). This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Manners, G. D. Citrus Limonoids: Analysis, Bioactivity, and Biomedical Prospects. *J. Agric. Food Chem.* **2007**, *55* (21), 8285–8294.

(2) Girennavar, B.; Poulose, S. M.; Jayaprakasha, G. K.; Bhat, N. G.; Patil, B. S. Furocoumarins from grapefruit juice and their effect on human CYP 3A4 and CYP 1B1 isoenzymes. *Bioorg. Med. Chem.* **2006**, *14* (8), 2606–2612.

(3) Jayaprakasha, G. K.; Singh, R. P.; Pereira, J.; Sakariah, K. K. Limonoids from *Citrus reticulata* and their moult inhibiting activity in mosquito *Culex quinquefasciatus* larvae. *Phytochemistry* **1997**, *44* (5), 843–846.

(4) Mandadi, K. K.; Jayaprakasha, G. K.; Bhat, N. G.; Patil, B. S. Red Mexican grapefruit: A novel source for bioactive limonoids and their antioxidant activity. *Z. Naturforsch., C: J. Biosci.* **2007**, *62* (3−4), 179–188.

(5) Perez, J. L.; Jayaprakasha, G. K.; Valdivia, V.; Munoz, D.; Dandekar, D. V.; Ahmad, H.; Patil, B. S. Limonin Methoxylation Influences the Induction of Glutathione S-Transferase and Quinone Reductase. *J. Agric. Food Chem.* **2009**, *57* (12), 5279–5286.
(6) Abeysinghe, D. C.; Li, X.; Sun, C.; Zhang, W.; Zhou, C.; Chen, K. Bioactive compounds and antioxidant capacities in different edible tissues of citrus fruit of four species. *Food Chem.* **2007**, *104* (4), 1338–1344.
(7) Awad, A. B.; Down, A. C.; Fink, C. S. Inhibition of growth and stimulation of apoptosis by β-sitosterol treatment of MDA-MB-231 human breast cancer cells in culture. *Int. J. Mol. Med.* **2000**, *5*, 541–546.
(8) Messina, J.; Barnes, S. The role of soy products in reducing the risk of cancer. *J. Natl. Cancer Inst.* **1991**, *38*, 541–546.
(9) Martin, R.; Paty, P.; Fong, Y.; Grace, A.; Cohen, A.; Dematteo, R.; Jarnagin, W.; Blumgart, L. Simultaneous liver and colorectal resections are safe for synchronous colorectal liver cancer. *Am. J. Coll. Surg.* **2003**, *197*, 233–241.
(10) Gutherie, N.; Morley, K.; Hasegawa, S.; Manners, G. D.; Vandenberg, T. Inhibition of human breast cancer cells by citrus limonoids. In *Citrus limonoids: Functional chemicals in agriculture and foods*; Berhow, M., Hasegawa, S., Manners, G., Eds.; American Chemical Society: Washington, DC, 2000; pp 164−174.
(11) Tanaka, T.; Maeda, M.; Kohno, H.; Murakami, M.; Kagami, S.; Miyake, M.; Wada, K. Inhibition of azoxymethane-induced colon carcinogenesis in male F344 rats by the citrus limonoids obacunone and limonin. *Carcinogenesis* **2001**, *22* (1), 193–198.
(12) Tanaka, T.; Kohnu, H.; Kawabata, K.; Honjo, S.; Miyake, M.; Wada, K. Citrus limonoids obacunone and limonin inhibit the development of a precursor lesion, aberrant crypt foci, for colon cancer in rats. In *Citrus limonoids: Functional chemicals in agriculture and foods*; Berhow, M., Hasegawa, S., Manners, G., Eds.; American Chemical Society: Washington, DC, 2000; pp 145−163.
(13) Lam, L. K. T.; Li, Y.; Hasegawa, S. Effects of citrus limonoids on glutathione S-transferase activity in mice. *J. Agric. Food Chem.* **1989**, *37* (4), 878–880.
(14) Lam, L. K. T.; Hasegawa, S.; Bergstrom, C.; Lam, S. H.; Kenney, P. Limonin and nomilin inhibitory effects on chemical-induced tumorigenesis. In *Citrus limonoids: Functional chemicals in agriculture and foods*; Berhow, M. A., Hasegawa, S., Manners, G. D., Eds.; American Chemical Society: Washington, DC, 2000; pp 185−200.
(15) Poulose, S. M.; Harris, E. D.; Patil, B. S. Citrus limonoids induce apoptosis in human neuroblastoma cells and have radical scavenging activity. *J. Nutr.* **2005**, *135*, 870–877.
(16) Jayaprakasha, G. K.; Mandadi, K. K.; Poulose, S. M.; Jadegoud, Y.; Gowda, G. A. N.; Patil, B. S. Inhibition of colon cancer cell growth and antioxidant activity of bioactive compounds from *Poncirus trifoliata* (L.) Raf. *Bioorg. Med. Chem.* **2007**, *15* (14), 4923–4932.
(17) Miller, E. G.; Porter, J. L.; Binnie, W. H.; Guo, I. Y.; Hasegawa, S. Further Studies on the Anticancer Activity of Citrus Limonoids. *J. Agric. Food Chem.* **2004**, *52* (15), 4908–4912.
(18) Tanaka, T.; Kawabata, K.; Kakumoto, M.; Matsunaga, K.; Mori, H.; Murakami, A.; Kuki, W.; Takahashi, Y.; Yonei, H.; Satoh, K.; Hara, A.; Maeda, M.; Ota, T.; Odashima, S.; Koshimizu, K.; Ohigashi, H. Chemoprevention of 4-nitroquinoline 1-oxide-induced oral carcinogenesis by citrus auraptene in rats. *Carcinogenesis* **1998**, *19* (3), 425–431.
(19) Miller, E. G.; Fanous, R.; Rivera-Hidalgo, F.; Binnie, W. H.; Hasegawa, S.; Lam, L. K. T. The effects of citrus limonoids on hamster buccal pouch carcinogenesis. *Carcinogenesis* **1989**, *10* (8), 1535–1537.
(20) Battinelli, L.; Mengoni, F.; Lichtner, M.; Mazzanti, G.; Saija, A.; Mastroianni, C. M.; Vullo, V. Effect of limonin and nomilin on HIV-1 replication on infected human mononuclear cells. *Planta Med.* **2003**, *69*, 910–913.
(21) Poulose, S. M.; Harris, E. D.; Patil, B. S. Antiproliferative Effects of Citrus Limonoids Against Human Neuroblastoma and Colonic Adenocarcinoma Cells. *Nutr. Cancer* **2006**, *56* (1), 103–112.
(22) Jayaprakasha, G. K.; Girennavar, B.; Patil, B. S. Radical scavenging activities of Rio Red grapefruits and Sour orange fruit extracts in different in vitro model systems. *Bioresour. Technol.* **2008**, *99* (10), 4484–4494.
(23) Nagana Gowda, G. A. One-dimensional pulse technique for detection of quaternary carbons. *Magn. Reson. Chem.* **2001**, *39*, 581–585.
(24) Nagana Gowda, G. A. Improved sensitivity and gradient-enhanced multiplicity edited two-dimensional heteronuclear shift correlation technique. *Chem. Phys. Lett.* **2002**, *353* (1−2), 49–54.
(25) Chang, K.-C.; Duh, C.-Y.; Chen, I.-S.; Tsai, I.-L. A Cytotoxic Butenolide, Two New Dolabellane Diterpenoids, a Chroman and a Benzoquinol Derivative Formosan Casearia membranacea. *Planta Med.* **2003**, *69* (07), 667–672.
(26) Awad, A. B.; Chen, Y. C.; Fink, C. S.; Hennessey, T. B-Sitosterol inhibits HT-29 human colon cancer cell growth and alters membrane lipids. *Anticancer Res.* **1996**, *16*, 2797–2804.
(27) von Holtz, R. L.; Fink, C. S.; Awad, A. B. B-Sitosterol activates the sphingomyelin cycle and induces apoptosis in LNCaP human prostate cancer cells. *Nutr. Cancer* **1998**, *32*, 8–12.
(28) Fong, C. H.; Hasegawa, S.; Herman, Z.; Ou, P. Limonoid glucosides in commercial citrus juices. *J. Food Sci.* **1990**, *54*, 1505–1506.
(29) Ozaki, Y.; Ayano, S.; Inaba, N.; Miyake, M.; Berhow, M. A.; Hasegawa, S. Limonoid glucosides in fruit, juice and processing by-products of Satsuma Mandarin (*Citrus unshiu* Marcov.). *J. Food Sci.* **1995**, *60*, 186–189.
(30) Hunter, T.; Pines, J. Cyclins and cancer. *Cell* **1991**, *66*, 11071–1074.

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