# Citrus Limonin and Its Glucoside Inhibit Colon Adenocarcinoma Cell Proliferation through Apoptosis

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Ⓢ *Supporting Information*

**ABSTRACT:** The current study was an attempt to elucidate the mechanism of human colon cancer cell proliferation inhibition by limonin and limonin glucoside (LG) isolated from seeds of *Citrus reticulata*. The structures of purified compounds were confirmed by NMR and quantified using HPLC. These compounds of more than 95% purity were subjected to proliferation inhibition assay using human colon adenocarcinoma (SW480) cells. The $IC_{50}$ value of 54.74 and 37.39 $\mu$M was observed for limonin and LG, respectively at 72 h. Following confirmation of proliferation inhibition, pattern of DNA fragmentation and activation of caspase-3 of the cells treated with limonoids suggest involvement of apoptosis. Furthermore, reduction in the transcription ratio of *bcl2/bax* and induction of cytochrome *c* release from mitochondria to cytosol with treatment of limonoids confirm the activation of intrinsic apoptosis pathway. The activity of Bax and Bcl2 was confirmed through analysis of mitochondrial membrane potential and intracellular calcium in the cells treated with limonin and LG; the net content of caspase-8 was not affected by limonoids. Results of the current study provide compelling evidence on the induction of mitochondria mediated intrinsic apoptosis by both limonin and LG in cultured SW480 cells for the first time.

**KEYWORDS:** limonoids, colon cancer, SW480, apoptosis, intracellular calcium

## ■ INTRODUCTION

Colorectal cancer is one of the leading causes of death in the US and the Western world. Statistics from the American Cancer Society indicate 49,960 deaths due to colon cancer in 2008 with 148,810 estimated new cases in the US. The highest prevalence of colorectal cancer is found in North America, Europe, Australia and New Zealand, while it is least in South America, Africa, India and other Asian subcontinents.[1] A growing body of evidence suggests that diet and dietary components seem to help in the prevention of colorectal cancer.[2] A study conducted in Europe suggests that a diet rich in fiber can reduce the risk of colon cancer.[3] Out of 156 dietary studies, 128 have demonstrated a direct relation between low consumption of fruits and vegetables with increased risk of lung, colon, breast, cervix, esophagus, oral cavity, stomach, bladder, pancreas and ovarian cancers.[4] Results from meta analysis have prompted researchers to identify bioactive compounds present in fruits and vegetables to understand their mechanisms in cancer chemoprevention.[4]

Among fruits, citrus is widely consumed throughout the world for both nutrition and health promoting properties.[5] Bioactive molecules in citrus include furocoumarins, folate, carotenoids, pectin, limonoids, sterols and flavonoids.[6−9] Limonin is most abundant among limonoids and known for inhibition of malaria parasite,[10] radical scavenging activity[11] and induction of phase II enzymes.[12] Reports explaining the benefits of citrus limonoids in cancer prevention are available from early 1990's. Based on the *in vitro* (cell culture) and *in vivo* models, there are a number of studies explaining the ability of citrus bioactive compounds to inhibit cancer from our laboratory and elsewhere.[7,13−16] The cell culture based *in vitro* reports include differential proliferation inhibition of leukemia, ovary, cervix, stomach,

liver and breast carcinoma cells by citrus limonoids.[15] Inhibition of neuroblastoma cells by citrus limonoids through activation of caspase 3/7 was also reported.[16]

Most of the *in vivo* studies on limonoids explain induction of phase II enzymes (associated with initiation of most types of cancers) as the major mode of activity. Phase II enzymes are strongly associated with cancers of the colon, liver, lung and small bowel.[17,18] Thus, a compound capable of inducing phase II enzymes seems to have special value in colorectal cancer chemoprevention. The first *in vivo* study on limonoids explains inhibition of tumorogenesis by limonoids in benzo[*a*]pyrene induced colon cancer animals through induction of phase II enzymes.[19] Activation of phase II enzyme by limonin and nomilin was also reported in the carcinogen induced buccal pouch epidermoid carcinoma model.[20] Obacunone is another limonoid induced phase II enzyme in azoxymethane (AOM) treated animals.[21] More recently, our laboratory has demonstrated a positive influence on induction of phase II enzymes by methoxy limonin, a modified limonoid.[22] Feeding of orange juice resulted in reduced incidences of colon cancer in AOM challenged rats, and the activity seems to be due to limonin glucoside and flavonoids.[23] Another mode of colon cancer prevention by citrus limonoids as demonstrated by *in vivo* model is activation of anti-inflammatory pathway. Research from our laboratory has demonstrated ability of freeze-dried grapefruit juice powder, naringin and limonin to inhibit aberrant crypts through the

suppression of inflammatory mediators cyclo-oxygenase-2 (Cox-2) and inducible nitric oxide synthase (iNOS) in AOM induced colon carcinoma in experimental animals.[9] Limonoids isolated from fruits of *Melia toosendan* Sieb. et Zucc., namely, isotoosendanin and 1-O-tigloyl-1-O-debenzoylohchinal, have demonstrated anti-inflammatory and analgesic activity in experimental animals,[24] suggesting ability of citrus and other limonoids to act on the inflammatory pathway.

Based on the existing information regarding the role of citrus limonoids in cancer prevention from *in vitro* and *in vivo* studies, very little information is available about the influence of limonoids on the apoptosis pathway. Therefore, the current study was conducted to understand the effect of limonin and LG isolated from seeds of *Citrus reticulata* on the apoptosis pathway using the human adeno-carcinoma (SW480) cell model. The mode of action of limonoid was elucidated by measuring the activity of enzymes, expression of genes and protein related apoptosis in SW480 cells.

## ■ MATERIALS AND METHODS

**Chemicals and Reagents.** Dowex-50, silica gel and solvents were purchased from Sigma-Aldrich (St. Louis, MO). The SP-70 adsorbent resin was purchased from Supelco (Bellefonte, PA). The *Citrus reticulata* fruits were collected from Texas A&M University—Kingsville (Citrus Center, Weslaco, TX, USA). Seeds were separated manually and finely powdered. Fluorescent probes were purchased from Invitrogen molecular probes (Carlsbad, CA). Media and chemicals for cell culture were obtained from Hyclone cell culture and bioprocessing (Logan, UT). The primers used in the experiment were procured from Sigma genosys (St. Louis, MO). Both primary and secondary antibodies used in the study were procured from Santa Cruz Biotechnology (Santa Cruz, CA).

**Isolation and Quantification of Limonin and LG.** Both limonin and LG were isolated from the defatted seed powder of *Citrus reticulata* Blanco using a Soxhlet type of extractor with ethyl acetate and methanol for 8 h each successively. Ethyl acetate extract was fractionated on silica gel column chromatography to obtain limonin.[25,26] Similarly methanol extract was fractionated on SP-70 column chroma-tography as per published protocol to obtain LG.[7,27]

**Identification.** The purity of the isolated compounds was confirmed by reverse phase HPLC using a gradient elution of 3 mM phosphoric acid and acetonitrile, on a $C_{18}$ column (250 × 4.6 mm i. d., 5 $\mu$m) according to our previous publication[28] with slight modification. The flow rate was maintained at 0.8 mL/min, and compounds were detected at 210 nm. The $^1H$ and attached proton test (APT) spectra were recorded at 400 and 100 MHz respectively (JEOL USA, Inc. Peabody, MA). Tetramethylsilane (TMS) was used as an internal standard, and spectra were recorded in DMSO-$d_6$.

**Cell Culture and Maintenance.** Colon cancer (SW480) and fibroblast (112CoN) cells were obtained from ATCC (VA, USA). The SW480 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) using 10% fetal bovine serum (FBS) with 100 units of penicillin and streptomycin each. The SW480 maintained as multiple stocks were used for the experiments between passages 4−12. The 112CoN were cultured in Eagle's modified essential medium using 10% FBS. Cells were cultured and maintained during experiments in sterile, 75 cm$^2$ falcon flasks at 37 °C under 5% carbon dioxide and 85 ± 5% relative humidity, the cells attained 70% confluence were used for experiments.

**Cell Proliferation Assay.** *MTT Assay.* Viability of SW480 cells after treatment with limonoids was measured by the MTT (3-(4,5-dimethylthia-zole-2-yl)-2,5-diphenyltetrazolium bromide) method. Approximately 10 × 10$^3$ cells/well were plated into a 96-well. After 24 h of growth, media was replaced along with compounds at concentrations ranging from 6.25 to 100 $\mu$M; camptothecin was used as positive control at 25 $\mu$M for comparison purposes (compounds were dissolved in DMSO, maintaining the maximum concentration of DMSO at 0.1%). After 24, 48, and 72 h,

MTT solution (10 $\mu$L from stock of 5.0 mg/mL in sterile phosphate buffered saline) was added to each well and incubated at 37 °C for 2 h. After completion of reaction, the medium was replaced with 200 $\mu$L of DMSO and incubated for compete solubility of formazan. Optical density was measured at 550 nm with corrections at 650 nm using a micro plate reader (Bio-Tek, Instruments Inc., Vermont, USA). The viability was calculated based on the intensity of formazan, which is directly proportional to the number of viable cells. Results of the cytotoxicity were expressed as IC$_{50}$ value in $\mu$M for each incubation time point.

*Lactate Dehydrogenase (LDH) Assay.* Cells were treated with different concentrations (6.25, 12.5,25,50 and 100 $\mu$M) of limonin, LG, and camptothecin after incubation for 24, 48, and 72 h; 50 $\mu$L of supernatant medium was removed without disturbing the cells, mixed with an equal volume of LDH reagent (Roche Diagnostics, Basel, Switzerland), and incubated for 30 min in the dark at ambient temperature. Absorbance was read at 500 nm using an ELISA reader. The LDH extracted using 10% triton X-100 from one of the untreated sets was used as positive control, and an equal volume of DMEM without cells served as negative control. The results were expressed as % LDH leakage with respect to DMSO treated cells.

*Viable Cell Count Assay.* Cells were plated (20 × 10$^3$ per well) in 12 well plates and incubated for 24 h for attachment. After incubation, medium was replaced with 1.0 mL of fresh medium with different concentra-tions of limonin, LG (25, 50, 75, and 100 $\mu$M) and camptothecin (25 $\mu$M). Viable cells were counted using a Z$_1$ Coulter particle counter (Beckman Coulter) after 2, 4, and 6 days. Test compounds were redosed along with the medium once in 2 days. Each treatment was performed in triplicate, and the results were expressed as mean ± SD of viable cells.

**DNA Fragmentation Assay.** The cells grown for 24 h in a 100 mm Petri dish were treated with 100 $\mu$M of limonin, LG and 50 $\mu$M of camptothecin. Genomic DNA was extracted from these cells using the phenol:chloroform extraction method with slight modification.[29] DNA was quantified using Nanodrop (ND1000) spectrophotometer, an equal amount was separated by electrophoresis at 55 V of constant current on 1.5% agarose gel, and an image of the DNA gel was captured using LAS-4000 mini imaging system (Fuji Life sciences, CA, USA) after staining with GelRed staining reagent (Biotirum, CA, USA).

**Cellular Apoptotic Enzymes Analysis.** *Caspase-8.* The caspase 8 assay was performed to lysate obtained from the cells exposed to limonin and LG (100 $\mu$M) for 24 and 48 h, using a sensitive colorimetric assay (Chemicon international, TX, USA). Cells exposed to limonin, LG and camptothecin (50 $\mu$M) for 24 and 48 h were washed with phosphate buffered saline, centrifuged and suspended in a lysis buffer (TE buffer). Different lysate concentrations were subjected for caspase-8 assay as per manufacturer's instructions. Caspase-8 activity was measured by reading the optical density at 405 nm. Simultaneously, buffer and substrate blanks were also included in the assay. Activity was calculated a using standard curve of pNA and expressed as % caspase-8 activity.

*Caspase-3.* After treatment of SW480 cells with 100 $\mu$M of limonin, LG and 50 $\mu$M camptothecin for 24 and 48 h, cells were resuspended in lysis buffer (BD Pharmingen, San Diego, CA, USA) and kept on ice for 30 min. The lysate was subjected to caspase-3 assay as per manufac-turer's instructions. The caspase-3 activity was measured spectrofluor-imetrically using an excitation wavelength of 360 nm and an emission wavelength of 460 nm after incubating for 1 h at 37 °C.

*Cytochrome c Content.* The SW480 cells were seeded in 2 mL of DMEM at a density of 1× 10$^6$ cells/mL and incubated for 24 and 48 h with or without test compounds at 100 $\mu$M limonoids and 50 $\mu$M of camptothe-cin. After incubation, cells were washed twice with PBS and suspended in lysis buffer. The lysate was incubated at room temperature for 1 h with gentle mixing. The cytosolic fraction was obtained after centrifugation for 15 min at 1000$g$ and was diluted with 1× RD5P (Calibration diluents, R&D Systems, Inc., Minneapolis, MN, USA) and treated with antibody. Following the reaction of cytochrome *c* with antibody substrate, concentrations were

calculated by measuring absorbance at 450 nm (with correction at 540 nm) and quantification was performed using standard curve prepared from human cytochrome $c$ standard.

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) for Apoptosis Related Genes.** Total RNA was extracted from cultured SW480 cells after exposure of limonoids ($100 \mu$M) for 24 and 48 h. RNA was isolated according to the instruction from manual (SV total RNA isolation kit, Promega, WI, USA). Isolated RNA was subjected to DNase (Invitrogen Corporation, CA, USA) treatment. Reverse-transcription reaction was carried out using Taqman reverse transcription reagents kit (Applied Biosystems, CA, USA) with 400 ng of total RNA and oligo (dT) primers in a 10 $\mu$L volume. The resulting cDNA was subjected to PCR amplification using primer 5′-CCCTCAAGATTGTCAGCAATGC-3′ forward and 5′-GTCCTCAGTGTAGCCCAGGAT-3′ reverse for glyceraldehyde-3-phosphate dehydrogenase (*gapdh*; control), 5′-TAGCAAACTGGT GCTCAA-GG-3′ forward and 5′-CGAAGTAGGAGAGGAGGCC-3′ reverse for *bax* and 5′-GGATGCCTTTGTGGAACTGT-3′ forward and 5′-AGCCTG-CAGCTTTGTTTCAT-3′ reverse for *bcl$_2$*. The primers for *gapdh* and *bax* were designed using Primer III program[30]and *bcl2* was used from the reference Yeung et al.[31] The PCR conditions were as follows: 94 °C for 5 min; 30 cycles of 94 °C for 45 s, annealing for 45 s, and extension at 72 °C for 60 s; and a final extension at 72 °C for 10 min. Annealing temperatures used were 58, 55, and 60 °C for *gapdh, bax* and *bcl2*, respectively. These PCR products were electrophoresed on 1.6% agarose gel and visualized following ethidium bromide staining on a transilluminator (FBTIV-816, Fisher biotech, USA). The images were captured with a CCD camera (Alpha innotech corporation, CA, USA), and band intensities were quantified with alpha images 5.5 software.

**Immunoblotting for Expression of Apoptosis Related Proteins.** Approximately, $2 \times 10^6$ cells were cultured in a 100 mm Petri dish overnight prior to treatment of test compounds. These cells were treated with $100 \mu$M of limonin or LG for 24 and 48 h. The treated cells were lysed in RIPA buffer (containing 130 mM NaCl, 1 mM dithiothreitol, 2 $\mu$g/mL leupeptin, 10 mM NaF, 1 mM PMSF and 20 mM Tris, pH 7.4), and lysates were centrifuged at 3000$g$ for 15 min (4 °C) and the supernatant was used for experiment. Protein content of these lysates was estimated using BCA assay with BSA as standard (Pierce biotechnology, Inc., IL, USA). Each sample equivalent to 50 $\mu$g of protein were resolved on 12% SDS−PAGE, using Mini-PROTEAN tetra electrophoresis system (Bio-Rad laboratories, CA, USA). Separated proteins were transferred to nitrocellulose membrane (0.45 $\mu$m, Trans-Blot, transfer medium, Bio-Rad laboratories, CA, USA) using a semidry transfer system (TRANS-BLOT SD, Bio-Rad, CA, USA) at 10 V for 40 min. These membranes were blocked for 30 min in tris-buffered saline-Tween-20 (TBST; consisting of 150 mM NaCl, 10 mM Tris pH 7.4, 0.05% Tween-20) containing 6% dried fat-free skimmed milk powder. Membranes were dried and probed with mouse monoclonal anti-AR antibodies Bax, Bcl$_2$, caspase-3 (34 kDa considered as procaspase) and $\beta$-actin (1:2500 dilution in TBST containing 3% dried skimmed milk) (Santa Cruz Biotechnology Inc., CA, USA) overnight at 4 °C. These membranes containing proteins were washed four times (10 min each time) in TBST, followed by incubation with HRP-conjugated goat anti-mouse secondary antisera (1:25000 dilution in TBST containing 3% dried skimmed milk) (Pierce protein research products, IL, USA) for 1 h at room temperature. Protein bands were visualized using supersignal west femto maximum sensitivity substrate as described by the manufacturer (Pierce Biotechnology, Inc., IL, USA). The images were captured using a LAS-4000 mini imaging system (Fuji Life Sciences, CA, USA) and band intensities were quantified with alpha images 5.5 software.

**Measurement of Intracellular Calcium.** The SW480 cells were cultured up to 80% confluence on a two-chambered borosilicate sterile cover glass (Lab-Tek brand, Nalge nunc International, NY) and treated with $100 \mu$M limonin and LG. After 12, 24, and 48 h of treatment with compounds, cells were incubated for 45 min with 1 $\mu$M of fluo-4 AM dye (Molecular Probes, Eugene, OR) in DMEM without phenol red. Images of these cells were captured on a "zeiss" digital imaging

workstation using excitation filter of 485 nm and emission filters of 525−585 nm, with a 20× objective. Thirty images were captured from each well, and samples were treated in four independent wells as replications. Amount of Ca$^{2+}$ was quantified using slide book software (Intelligent Imaging Innovations, Inc., CO, USA) using mean of 30 selected images and is expressed as mean fluorescence intensity.

**Measurement of Change in Mitochondrial Membrane Potential ($\Delta\Psi$m).** Mitochondrial membrane potential was monitored by measuring uptake of mitochondria specific dye Rhodamine-123. The SW480 cells were treated with limonin, LG ($100 \mu$M) and camptothecin ($50 \mu$M) for 24 and 48 h, followed by incubation of cells with 2.0 $\mu$M Rhodamine-123 for 30 min at 37 °C. The treated cells were washed twice with PBS and resuspended in 100 $\mu$L of sterile PBS. The fluorescence intensity of the cell suspensions was measured fluorometrically using an excitation wavelength of 480 nm and an emission wavelength 530 nm using an ELISA reader.[32]

**Morphology of Mitochondrial Membrane.** The SW480 cells were treated with either limonin or LG ($100 \mu$M) for 24 and 48 h, and the treated cells were incubated with 2.0 $\mu$M Rhodamine-123 for 25 min. The images of mitochondria of treated cells were captured using a Zeiss digital imaging workstation with 63× (water immersion) objective with excitation at 505 nm and emission of 533 nm.

**Microscopic Study of Limonoids Treated Cells.** Approximately $1 \times 10^5$ cells well$^{-1}$ were grown for 24 h on borosilicate sterile two-chambered cover glass slides, which were preincubated with fetal bovine serum for four hours, and the cells were incubated with $100 \mu$M of limonin, LG or 50 $\mu$M camptothecin for 24 h. The cells were incubated with 5 $\mu$M of acridine orange (AO) and propidium iodide (PI) for 10 min at 37 °C. The supernatant medium and excess dye were removed after 10 min and washed twice with DMEM (without phenol red), and fluorescence images of stained cells were captured using a Zeiss digital imaging workstation.

**Statistical Analysis.** All the experiments were conducted in triplicate, and analyses were performed in duplicate for each sample. Results are expressed as mean ± standard error. ANOVA was performed for all the values of activity, and data was analyzed by Tukey's post-test analysis using GraphPad prism software version 5.00.288.

## ■ RESULTS

**Isolation and Characterization of Limonin and LG.** Both limonin and LG were purified from the seeds of *C. reticulata*. The purity of limonoids was analyzed by our previously established reverse phase HPLC method.[28] The retention time for limonin and LG was 20.38 and 4.53 min respectively (Figure 1A). Furthermore, structures of the isolated compounds were confirmed by NMR spectra. APT spectra of limonin and LG are presented in Figure 1B. In the APT spectrum methyl (CH$_3$) and methane (CH) signals are on the negative side and methylene (CH$_2$) and quaternary carbon signals are on the positive side. These chemical shifts are consistent with reported values.[25] The purity of limonin and LG was found to be more than 96%, and they were dissolved in DMSO for further cell culture experiments.

**Cell Proliferation Assay.** *MTT Assay.* Both limonin and LG were tested for their effect on viability of SW480 cells at 6.25 to $100 \mu$M by MTT assay for incubation period of 24, 48, and 72 h. The inhibition results were expressed in terms of IC$_{50}$ value in $\mu$M, and camptothecin was used as positive control. The IC$_{50}$ values for limonin were 137.23, 72.5, and 54.74 $\mu$M, respectively at 24, 48, and 72 h of treatment and for LG values were of 52.5, 51.6, and 37.4 $\mu$M at 24, 48, and 72 h (Table S1 in the Supporting Information), respectively. Furthermore, neither limonin nor LG was toxic to normal colon fibroblast (112CoN) cells (data not shown).

*LDH Assay.* Maximum release of LDH into the medium was observed after 48 h of incubation with limonoids. The leakage of
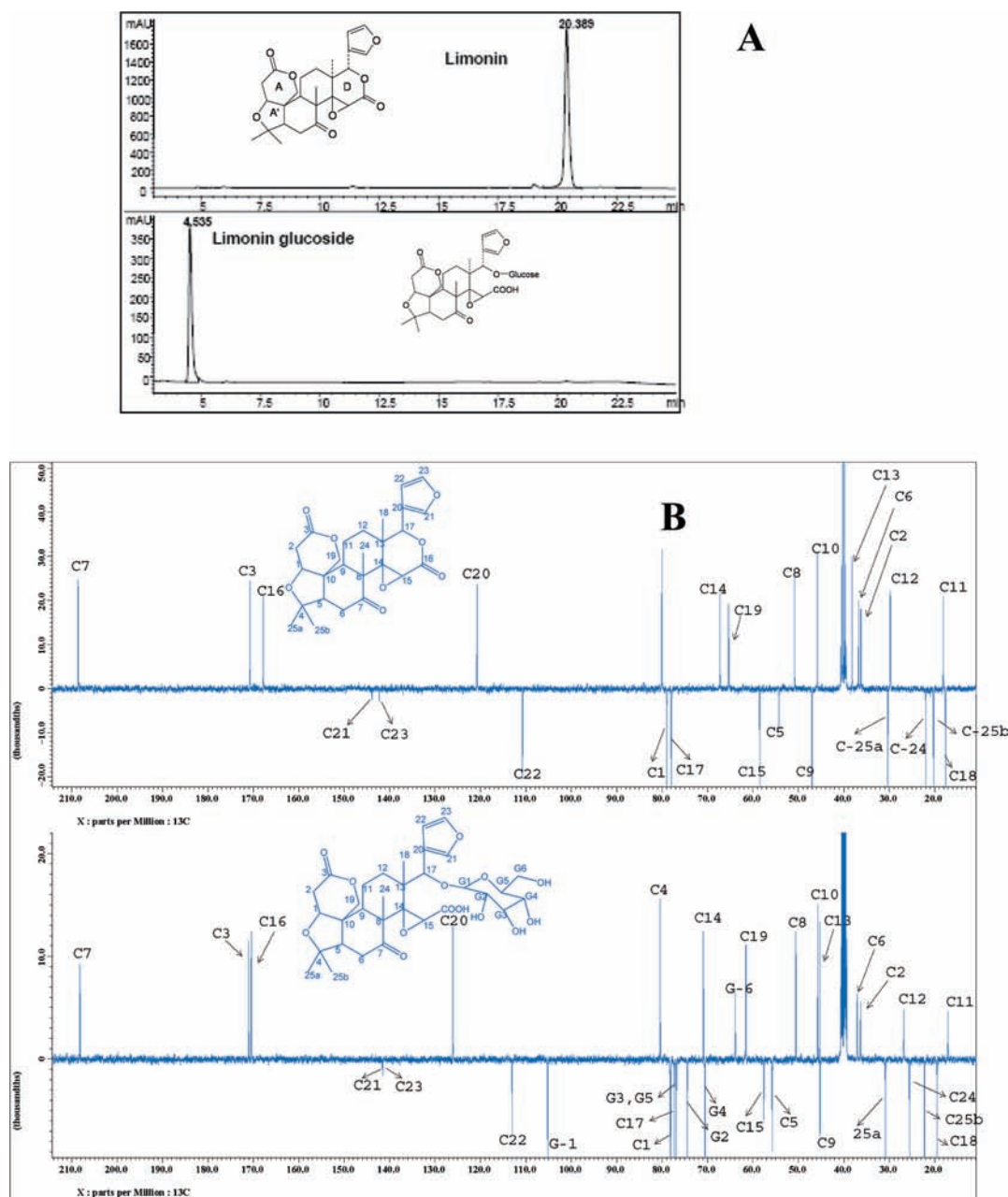
**Figure 1.** Quantification and identification of limonin and limonin glucoside. (A) HPLC chromatograms of purified limonin and LG from seeds of *C. reticulata* and their structures. Limonoids were eluted with gradient mobile using a $C_{18}$ column ($250 \times 4.6$ mm i.d., $5\,\mu$m particle size) with a flow rate of 0.8 mL/min, and detection was set at 210 nm. (B) $^{13}$C NMR spectra of limonin and limonin glucoside recorded in DMSO-$d_6$ on a JEOL ECS spectrometer at 100 MHz. Assignments of all the carbons are made on the respective spectrum. In the APT spectrum, the $CH_3$ and CH carbons are negative while the $CH_2$ and quaternary carbons are positive in intensity.

LDH was significant in cells incubated with 25, 50, and 100 $\mu$M of LG compared to camptothecin at 25 $\mu$M ($P < 0.001$). Interestingly, LDH leakage was reduced in cells incubated for 72 h with both limonoids in comparison with DMSO treatment (Figure S1 in the Supporting Information).

*Viable Cell Count Assay.* Based on the results from MTT assay, concentrations of 25, 50, 75, and 100 $\mu$M were used to reconfirm the proliferation inhibition ability of limonoids through viable cell count assay. Results of this assay were in agreement with MTT assay, and both limonoids demonstrated a dose and time dependent inhibition of SW480 cell proliferation. Limonin treatment inhibited cell proliferation by 89% after incubation for 4 days

and 75% after 6 days of incubation at 100 $\mu$M. Incubation of LG at 100 $\mu$M has resulted in inhibition up to 95 and 69%, respectively after 4 and 6 days (Figure S2 in the Supporting Information). Both limonin and LG exhibited optimum cytotoxic effect at 100 $\mu$M, as measured by MTT, LDH and viable cell count assay, and this led to use of this concentration for further studies.

**DNA Fragmentation.** Fragmentation of DNA was observed in SW480 cells treated with 100 $\mu$M limonin and LG for 24 and 48 h. The fragmentation was initiated at 12 h. This characteristic DNA cleavage between nucleosomes was visible as ladder and suggests possible involvement of apoptosis to inhibit the proliferation of SW480 cells (Figure 2A).
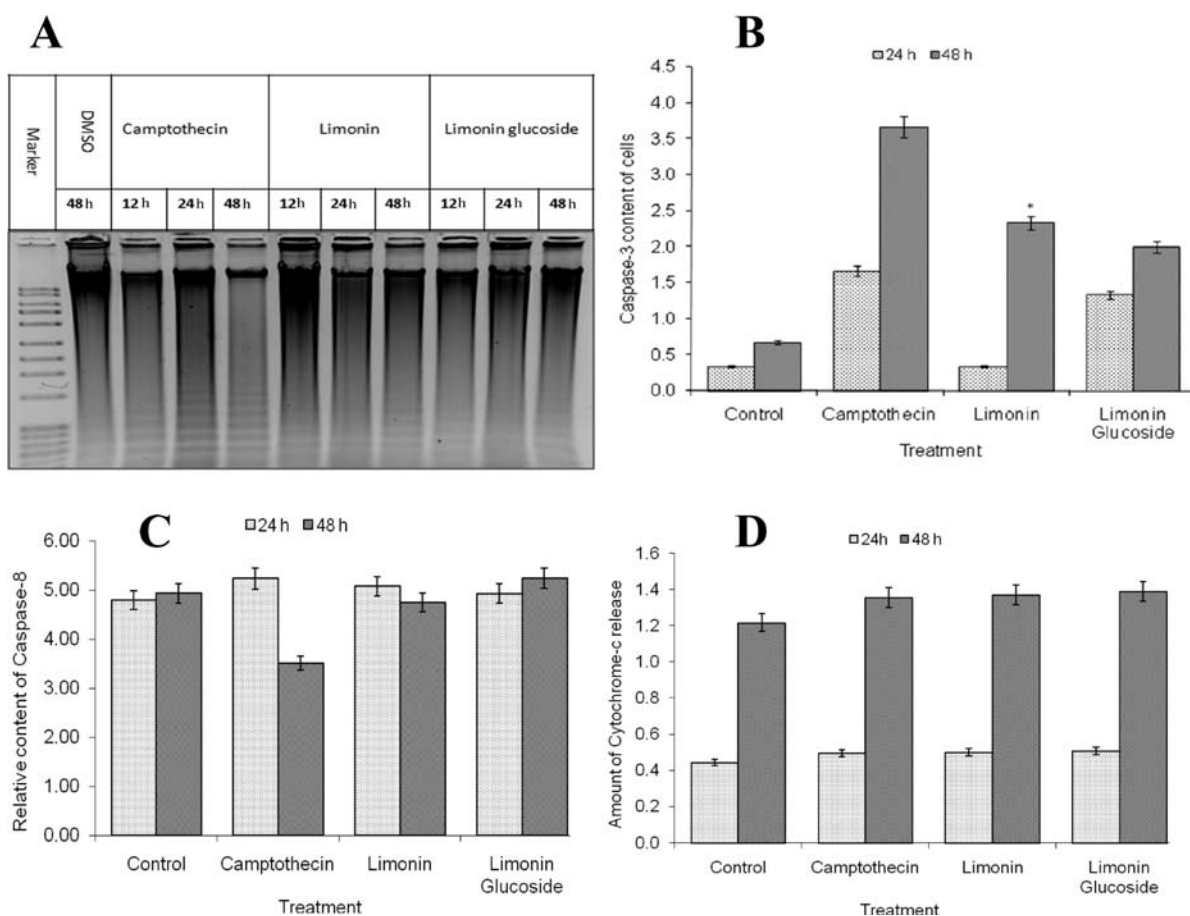
**Figure 2.** Effect of limonin and LG on apoptosis markers in SW480 cells. (A) Detection of DNA fragmentation (DNA laddering) in SW480 cells treated with camptothecin, limonin and LG for 12, 24, and 48 h. DNA was isolated and subjected to electrophoresis as explained in Materials and Methods. (B) Effect of limonin and LG treatment on caspase-3, (C) caspase-8 and (D) cytochrome $c$, which were measured spectroscopically as described in Materials and Methods. Values are mean ± SE, $n = 6$ (* significant $p < 0.05$ with respect to control cells).

**Total Cellular Apoptotic Enzymes Content Analysis.** *Caspase-3 Activity.* Caspase-3 activity was measured in the cells incubated with limonoids for 24 and 48 h. Both limonin and LG resulted in significant ($P < 0.05$) activation of caspase-3 by 3.5- and 2.5-fold higher compared to control, after 48 h of incubation (Figure 2B). These results clearly suggest the involvement of caspase mediated apoptosis to inhibit the proliferation of colon cancer cells by limonoids.

*Caspase-8 Content.* Incubation of cells with limonin and LG at 100 $\mu$M for 24 or 48 h did not show changes in the net caspase-8 content. Interestingly, camptothecin treatment resulted in depletion in caspase-8 content by 25% after 48 h (Figure 2C). This could be due to its cytotoxic effect, resulting in lower number of biochemically active viable cells.

*Cytosolic Cytochrome c Content.* In the current study, neither limonin nor LG (100 $\mu$M) exhibited a profound effect after 24 h of incubation. However, the content of cytosolic cytochrome $c$ increased significantly ($p < 0.05$) by 11.6% and 12.9% following incubation with limonin and LG for 48 h (Figure 2D), indicating the involvement of intrinsic apoptosis to induce cytotoxicity.

**RT-PCR of Bcl$_2$ Family Gene Expression in Limonoids Treated Cells.** Semiquantitative RT-PCR results have shown significantly ($p < 0.05$) higher expression of bax in cells incubated with LG for 48 h in comparison with control. The *gapdh* was used as a loading control to ensure uniformity of cDNA in various reactions (Figure 3A). Approximately, 40% higher induction of

*bax* expression was observed following the incubation of SW480 cells with LG for 48 h (Figure 3B).

Furthermore, reduced expression of antiapoptotic $bcl_2$ was observed in cells incubated with limonin and LG confirming the involvement of the apoptotic pathway. The expression of $bcl2$ was not affected at 24 h with limonoid treatment, but treatment for 48 h specifies significant ($P < 0.01$) depletion of $bcl2$ expression (2.5-fold) with LG incubation (Figure 3C). In the current study, ratio of $bcl2/bax$ in the cells incubated with LG for 48 h was significantly ($p < 0.05$) decreased (by 89%) compared to control (Figure 3D).

**Expression of Apoptosis Related Proteins in Limonoids Treated Cells.** Expression of Bax was 3.0 and 5.0-fold higher with limonin (100 $\mu$M) treatment for 24 and 48 h, respectively, compared to control (Figure 4A), and the same was 2.4 (24 h) and 5.0-fold (48 h) higher in the case of LG. On the other hand, expression of Bcl$_2$ was depleted by 30 and 40% in the case of limonin and LG treatments after 48 h, respectively. Expression of caspase-3 (34 kDa) was 50% lesser in comparison with control after 24 h of treatment with limonoids. Expression of caspase-3 was 60% lower in cells incubated with limonoids for 48 h compared to control. The ratio of Bcl2 to Bax was significantly lower (up to 9-fold), after 48 h of incubation with limonin or LG (Figure 4B).

**Intracellular Calcium.** Treatment with limonin at 100 $\mu$M resulted in 2-fold elevation of intracellular calcium level, and the same was increased by 2.6-fold at 48 h ($p < 0.001$). Similarly,

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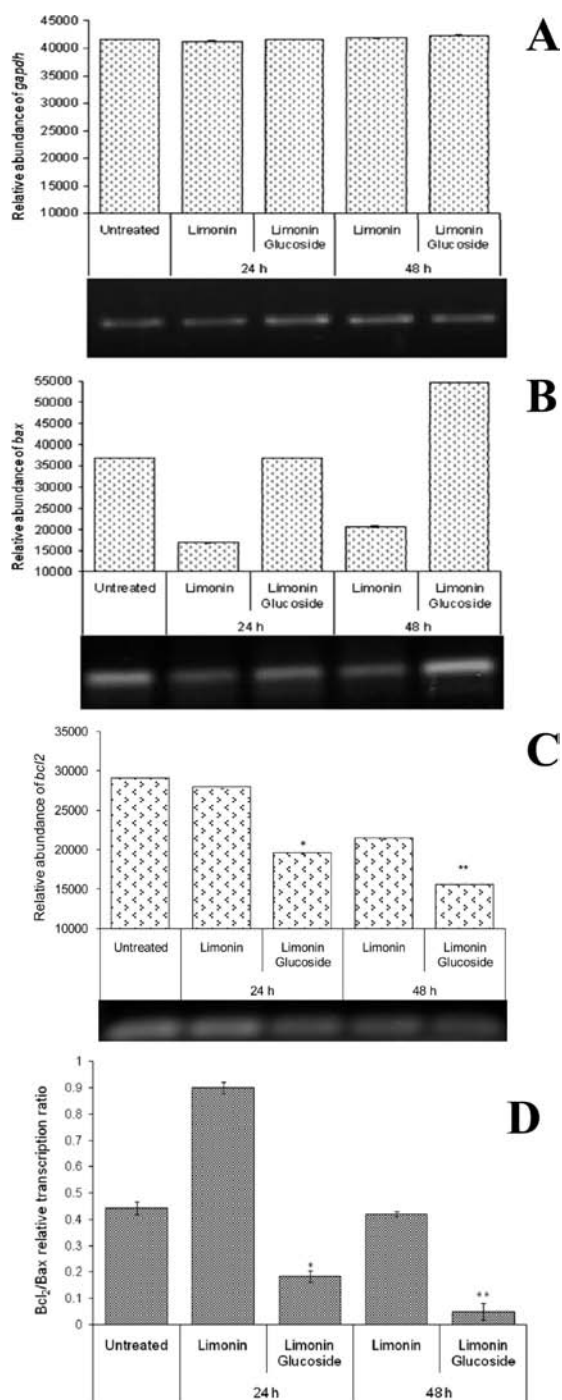
**Figure 3.** Role of limonin on LG on transcription of apoptosis related genes in SW480 cells. RT-PCR-based transcription analysis for (A) *gapdh*, (B) *bax*, and (C) *bcl₂* in SW480 cells. RNA was isolated from SW480 cells treated with limonin and LG at 100 $\mu$M for 24 and 48 h and subjected to RT-PCR as explained in methods [Gel lanes are in the following order from left to right: control cells, limonin-24 h, LG-24 h, limonin-48 h and LG-48 h]. (D) Gene expression ratio for *bcl₂/bax* in limonin and LG treated colon cancer cells for 24 and 48 h (values were calculated from the densitometry scores and expressed as mean ± SE, n = 3; * significant $p < 0.05$ and ** highly significant $p < 0.01$ compared to control group).

significant ($p < 0.01$) increases of intracellular calcium level by 1.7- and 2.5-fold compared to control were observed at 12 and 48 h with LG (Figure 5A). The content of calcium was also observed
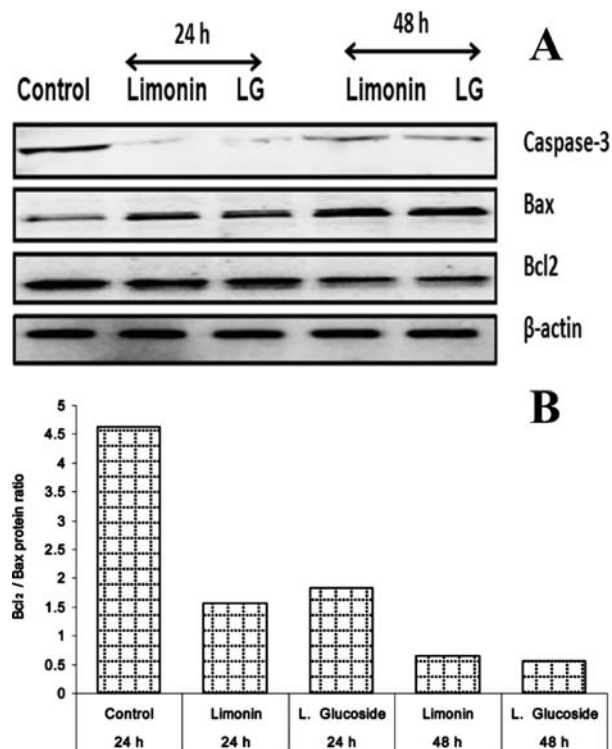


**Figure 4.** Influence of limonoids on expression of apoptosis related proteins in SW480 cells. (A) Expression of apoptosis related proteins in SW480 cells treated with limonin and LG for 24 and 48 h. Proteins from limonoid treated SW480 cells were separated on 12% SDS−PAGE, and Western blot was performed as described in Materials and Methods. (B) Expression ratio for Bcl₂/Bax proteins in limonin and LG treated colon cancer cells for 24 and 48 h. Values were calculated from the densitometry scores.

in microscopic images, which appears as green fluorescent in presence of fluo-4 AM dye (Figure 5B).

**Mitochondrial Membrane Potential and Morphology of SW480 Cells.** Higher fluorescence intensity was observed in the cells treated with limonin and LG, which is due to enhanced permeation of probe into mitochondria due to loss in membrane integrity. Treatment of camptothecin at 50 $\mu$M decreased membrane potential by 26%, while reductions of 22 and 31% were observed in cells incubated with limonin and LG, respectively, for 48 h compared to 24 h of incubation (Figure 5C). Furthermore, this was also supported by images of mitochondria of SW480 cells treated with limonin or LG. Swollen and irregular shaped mitochondria were observed in cells incubated with limonoids. Images of the cells incubated with LG for 24 and 48 h showed the presence of irregular and fragmentation, confirming induction of mitochondrial membrane damage by limonoids (Figure 5D).

**Morphology of Limonoid Treated SW480 Cells.** The SW480 cells treated with both limonin and LG were stained with PI, suggesting loss in plasma membrane integrity. Relative permeation of PI was higher in cells treated with LG compared to limonin. The cells treated with camptothecin were abnormal with disrupted cell surface, irregular shape and stained completely with PI. These results further support the data on DNA laddering and apoptosis (Figure 6A).

## ■ DISCUSSION

Consumption of citrus fruit and juice is associated with reduced risk of chronic diseases due to the presence of bioactive
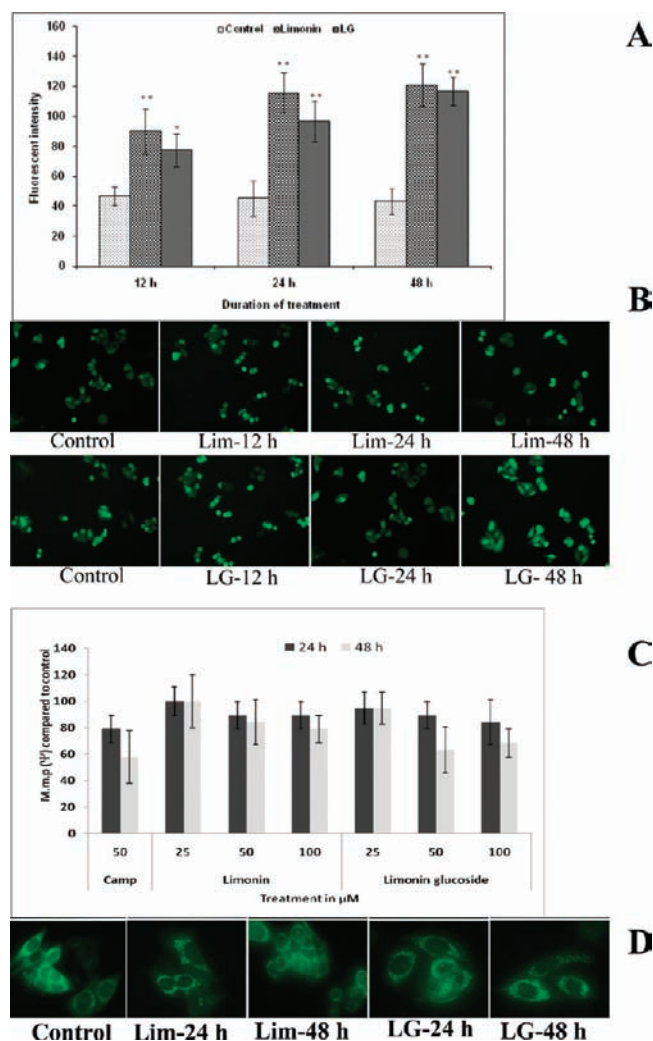
**Figure 5.** Role of limonin on LG on intracellular calcium and mitochondrial membrane potential ($\Delta\Psi$m) of SW480 cells. (A) Intracellular calcium levels in SW480 treated with limonin and LG for 12, 24, and 48 h as measured by fluorescence intensity. (Values are mean ± SE, $n = 3$, images are typical representative of three identical experiments (* significant $p < 0.01$ and ** highly significant $P < 0.001$ compared to control). (B) Fluorescence images of SW480 cells incubated with or without limonoids. Calcium specific Fluo-4 AM was used for both these experiments to measure calcium content. (C) Effect of limonin and LG treatment on mitochondrial membrane potential ($\Delta\Psi$m) measured by the intensity of Rhodamone-123. (D) Images of mitochondria of SW480 cells treated with limonin and LG for 24 and 48 h. (Values are mean ± SE, $n = 9$; images are typical representative of three similar experiments.)
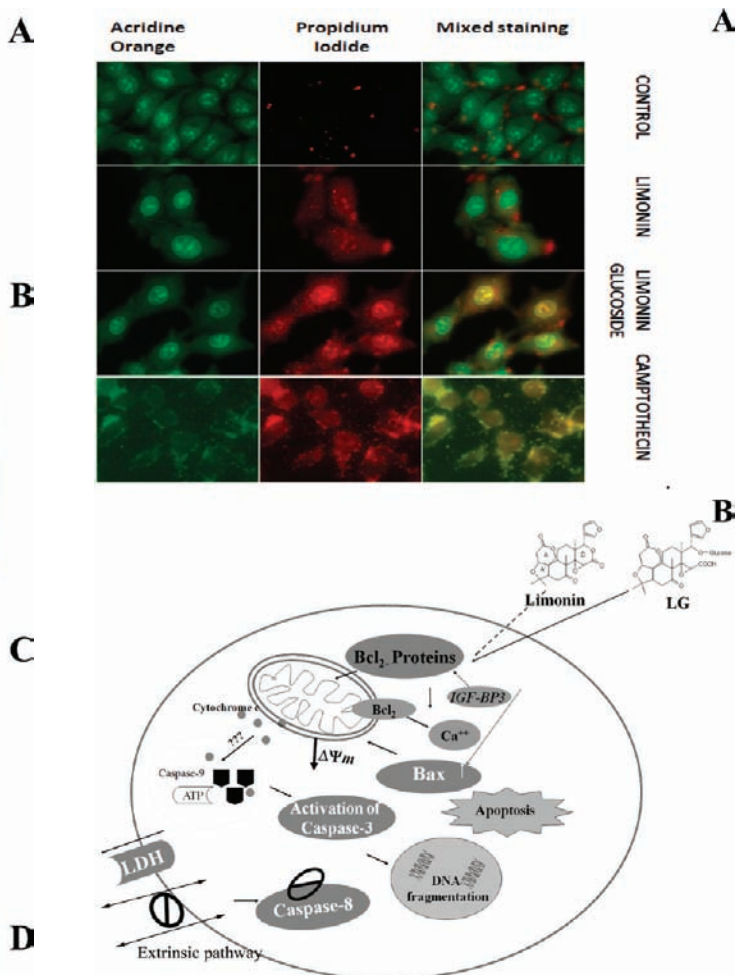


**Figure 6.** Fluorescent image of SW480 cells treated with limonoids and possible mode of apoptosis induction. (A) Fluorescent images of SW480 cells treated with limonin and LG at 100 $\mu$M and camptothecin at 50 $\mu$M for 24 h. Both treated and control cells were stained, and image was captured as explained in Materials and Methods. Each treatment was done in three independent slides, and representative images of three such identical experiments were presented. (B) Schematic drawing depicting a plausible mechanism of apoptosis induction by limonin and LG in colon cancer (SW480) cells. Briefly, both limonin and LG induce apoptosis through mitochondrial pathway, via upregulation of *bax* and downregulation of *bcl₂*, which results in the reduction of mitochondrial membrane potential leading to release of cytochrome *c* into the cytosol and elevation of intracellular Ca$^{2+}$ content. Released cytochrome *c* will combine with caspase-9 and ATP to form apoptosome, which further activates caspase-3. The activated caspase-3 ultimately executes apoptosis by DNA fragmentation. LDH leakage from cell membrane indicating membrane disintegration is also indicated (Ø indicates no effect observed).

compounds such as flavonoids, terpenoids, coumarins and volatile principles. Recent research suggests the role of oxygenated triterpenoids in the prevention of cancer and other chronic diseases.[33,34] However, very little information is available on the mode of inhibition. Therefore, the current investigation is an attempt to understand the possible mode of apoptosis induction by citrus limonoids. The SW480 cells treated with limonoids were analyzed for influence on proliferation, followed indicators of apoptosis, such as DNA fragmentation, caspase activity and expression of Bcl2 family genes. Effect of limonoids on caspase-8 was analyzed to understand the influence of limonoids on receptor mediated apoptosis. Intracellular Ca$^{2+}$ and mitochondrial membrane potential were measured in the

cells treated with limonoids to understand the activity of Bcl2 and Bax. Finally, the nature and extent of cytotoxicity are confirmed through fluorescent images of limonoids treated SW480 cells tagged with fluorescent probes.

The effect of limonin and LG on the proliferation was measured by both MTT and viable cell count assay. Results of MTT assay indicate that both the limonoids can induce apoptosis at less than 100 $\mu$M, which corresponds to ∼47 and 68 ppm. Similar results were observed in cell count assay with more than 50% cell inhibition at 50 $\mu$M and higher concentration. These results were further supported by LDH content in the medium of

the cells treated with limonoids, which is an indicator of loss in cell membrane integrity. Loss in membrane integrity occurs due to both necrosis and apoptosis death events.[35] The content of LDH was 30 and 40% higher in cells treated with more than 25 $\mu$M of limonin and LG, respectively, for 48 h compared to control. Induction of apoptosis was evident from fragmentation of DNA in the SW480 cells pretreated with limonoids. The initiation of fragmentation was observed at 12 h, however the clear indication was observed after 24 and 48 h of incubation (Figure 2A).

The signals induced by external agents such as chemotherapeutic/bioactive molecules follow either an intrinsic or extrinsic/receptor mediated pathway to initiate programmed cell death. In both of these pathways, caspase-3 is the common enzyme, which is also known as executor of apoptosis.[36] In the current study, activation of casaspe-3 was observed with treatment of limonoids for 24 and 48 h (Figure 4A), suggesting involvement of either an intrinsic or extrinsic pathway to induce apoptosis. This result is concurrent with the reported effect of other natural terpenoids on leukemia cells.[37]

Caspase-8 is one of the key indicators of the extrinsic apoptosis, which is known as initiator caspase. In the current study, pretreatment of SW480 cells with limonin and LG did not alter the net content of caspase-8, suggesting less possibility of activating the extrinsic apoptosis pathway by limonoids.

The content of cytochrome $c$ released into cytosol was measured to understand the influence of compounds on intrinsic pathway. Treatment of limonoids enhances the content of cytosolic cytochrome $c$ in SW480 cells (Figure 2D), suggesting that limonoids may trigger cytochrome $c$ mediated intrinsic apoptosis. Transcription levels of $bax$ (pro-apoptotic) and $bcl_2$ (antiapoptotic) genes offer further information regarding intrinsic apoptosis. Treatment of cells with limonoids significantly reduced transcription ratio of $bcl_2/bax$, which is considered as one of the major biochemical markers of cancer chemoprevention.[38] Limonoids which can alter the transcription ratio of $bcl_2/bax$ are known to be effective in prevention of different types of cancer.[39] The expression ratio of Bcl$_2$/Bax protein was in concurrence with gene transcription results, further confirming the activity of limonoids. This result clearly suggests the efficacy of limonoids to inhibit proliferation of SW480 cells by activating the intrinsic apoptosis pathway.

Calcium is known for induction of apoptosis, by direct as well as indirect mechanisms, as evidenced from increase in the intracellular Ca$^{2+}$ of cancer cells undergoing apoptosis.[40,41] In colon cancer, decrease in the cell proliferation as a result of consuming calcium rich diets further confirms involvement of calcium in cell proliferation.[42] Bcl$_2$ is known to regulate intracellular calcium. Hence, in the current study the effect of limonin and LG on intracellular calcium was measured as an indicator of Bcl2 activity and also to understand the role in apoptosis. Incubation of cells with limonoids resulted in increase in the intracellular calcium content by more than 2-fold (Figure 5A), confirming induction of Bcl$_2$ mediated apoptosis.

Results of cytochrome $c$ suggest the possibility of loss in mitochondrial membrane potential, which is also associated with increased content of calcium due to opening of megachannel (also known as PT pores).[43] Mitochondrial membrane potential of SW480 cell incubated with limonin and LG was measured using the fluorescent probe Rhodamine-123. The result clearly demonstrates loss in the membrane integrity by 20–30%, upon incubation of cells with 100 $\mu$M limonin and LG (Figure 5C). This was also evidenced through microscopic imaging of mitochondria of cells incubated with limonoids (Figure 5D). Finally,

the results were also supported by fluorescent tagged images. The SW480 cells treated with limonin and LG were stained with PI indicating loss in permeability of plasma membrane, a characteristic feature of cells undergoing apoptosis.

These results demonstrate for the first time that both limonin and LG can target Bcl-2 mediated intrinsic apoptosis to inhibit colon cancer cell proliferation. Furthermore, results also indicate that limonoids are capable of depleting mitochondrial membrane potential by altering intracellular calcium content, releasing of cytochrome $c$ to cytosol and activate caspase-3 to induce apoptosis. The effect of limonoids on caspase-8 suggests that the putative compounds may not trigger the extrinsic apoptosis pathway. These results provide an impetus for further investigations toward utilization of limonin and LG for colon cancer prevention.

In summary, limonin and LG were successfully isolated from seeds of *Citrus reticulata* Blanco and characterized. Both limonoids inhibit proliferation of SW480 cells in a dose and time dependent manner. The cytotoxicity was by means of intrinsic apoptosis pathway, which is mediated through Bcl2 family protein and cytochrome. Limonoids were also found to facilitate the apoptosis process by increasing intracellular Ca$^{2+}$ content. Results of the study provide comprehensive evidence on the possible mechanisms of colon cancer cell proliferation inhibition by citrus limonin and its 17-$\beta$ glucoside. Based on the results, a cartoon depicting a possible mode of inhibition of colon cancer cell proliferation by limonoids is presented in Figure 6B.

## ■ ASSOCIATED CONTENT

**ⓢ Supporting Information.** IC$_{50}$ values of limonoids as measured by MTT assay was presented in Table S1. Cytotoxicity by LHD leakage and cell viability assay by cell count assay were depicted in Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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## ■ ACKNOWLEDGMENT

## ■ ABBREVIATIONS USED

Bax, Bcl2-associated X protein; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GST glutathione S-transferase; HPLC, high performance liquid chromatography; LDH, lactate dehydrogenase; LG, limonin glucoside; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; QR, quinone reductase; RT-PCR, reverse transcriptase-polymerase chain reaction; TE buffer,

2321

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TIRS-EDTA buffer; TMS, tetramethylsilane; $\Delta\Psi m$, change in mitochondrial membrane potential; $Ca^{2+}$, calcium ion concentration.

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