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Citrus limonoids and curcumin additively inhibit human colon cancer cells†

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In the current study, we examined the ability of limonoids, including limonin, limonin glucoside (LG) and curcumin, to inhibit proliferation of human colon cancer (SW480) cells. Additionally, we studied the effect of combining these two classes of natural compounds on inhibition of proliferation and the possible mode of cytotoxicity. The SW480 cells were treated with compounds individually and in combination to understand the effect on cell death, DNA fragmentation, caspase-3 activity and the expression of Bax, Bcl-2 and caspase-3 proteins. Results of cell proliferation assays suggest that combinations of limonoids with curcumin at three different ratios (1 : 3, 1 : 1 and 3 : 1) to a final concentration of 50 ppm demonstrated up to 96% inhibition of cell proliferation. The MTT assay results were also confirmed by counting viable cells. Further, incubation of cells with combinations of limonoids and curcumin resulted in elevation of total cellular caspase-3 activity by 3.5–4.0 fold along with a 2- to 4-fold increase in the Bax/Bcl-2 ratio. The expression of pro-caspase-3 and its cleaved products in cells treated with curcumin (individually or combination) indicates higher potency of the combination to induce apoptosis. For the first time, this study provides compelling evidence of the pharmacodynamic additive effect of limonoids and curcumin in inhibiting human colon cancer cells. The above results were also confirmed by fluorescence microscopy of SW480 cells treated with limonoids, curcumin and combination, after tagging with fluorescent probes. These results suggest that consumption of curcumin and limonoids together may offer greater protection against colon cancer.

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Introduction

Despite substantial advances in the medical sciences, finding suitable methods for prevention, treatment or management of cancer still remains a challenge. The major approaches currently practiced to treat cancer include chemotherapy, radiation therapy, surgery, hormonal replacement, complementary and alternative medicines (mostly used as a part of supportive or palliative care). In chemotherapy, one of the common strategies is treatment with combinations of drugs, which consist of either synthetic, semi-synthetic, or natural compounds that act on multiple targets. Apart from controlling the division of cancer cells, the combination therapy may help to reduce secondary pathological conditions such as inflammation and neuritis. Hence, combinations of multiple drugs are used in effective management of cancer. Some of the successful combinations, which have demonstrated synergistic activity in cell culture studies, include doxorubicin with *Ganoderma* triterpenes to induce cytotoxicity in HeLa cells,[1] cisplatin with gemcitabine to inhibit ovarian and lung cancer cell proliferation,[2] and paclitaxel, 5-fluorouracil, and cisplatin for gastric cancer.[3] Several naturally derived molecules, including dietary components, have also demonstrated benefits in prevention of progression of different forms of cancer. Phytochemicals found in fruits, vegetables and spices include polyphenols, triterpenoids and coumarins, which have demonstrated activity in inhibition of various cancers.[4,5] Among the citrus limonoids, obacunone with vincristine synergistically inhibited proliferation of mouse lymphocytic leukemia (L1210) cells by up to 10-fold.[6] Some of these combinations were employed in preclinical studies and found to be effective against different tumors. Thus, screening of potential drugs in combination with bioactive compounds for better understanding of activity is essential before testing their potency *in vivo*.

The synergistic inhibition of proliferation by combinations of synthetic and natural compounds has been demonstrated on multiple pathways in breast, lung, colon, prostate and ovarian cancer cells.[7] Thearubigin, a polyphenol found in black tea has shown synergistic inhibition of human prostate cancer cells when combined with genistein. These compounds showed inhibition of PC-3 cell proliferation and G2/M phase arrest in a dose-dependent manner. Curcumin also showed synergistic

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inhibition of breast cancer cells when combined with genistein.[7,8] These results on synergistic effects of natural compounds led us to explore the possible synergism between curcumin and limonoids.

Limonoids are colorless tetracyclic triterpenoids, which are found in citrus fruits with relatively higher concentrations in the seed compared to the juice. Limonin, the most abundant limonoid from citrus is an oxygenated compound known to possess various biological activities including larvicidal activity against the mosquito *Culex quinquefasciatus*,[9] and suppression of coronary heart disease markers.[10] Our group has characterized and explored several biological activities of citrus limonoids[11,12] and other phytochemicals with emphasis on cancer chemoprevention.[13,14] This research in our laboratory has demonstrated inhibition of colon cancer cell proliferation by limonin and other limonoids.[15,16] In a recent study, we also demonstrated a possible mechanism of apoptosis induction by both limonin and LG in SW480 cells.[15] One of our previous studies demonstrated the ability of citrus limonoids to inhibit leukemia, ovarian, cervical, stomach, liver and breast cancer cells.[17] An animal study has also shown that freeze-dried grapefruit powder, naringin and limonin were capable of inhibiting aberrant crypts through suppression of *cyclo*-oxygenase-2 and inducible nitric oxide synthase (iNOS) in an azoxymethane-induced colon cancer model.[18] All these studies suggest the ability of limonin to induce apoptosis in colon and other cancer cells.

Curcumin, 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is a yellow-colored phenolic pigment of turmeric (*Curcuma longa*). Curcumin is a potent anti-inflammatory molecule, which can act through inhibition of NFκB and other molecules.[19] Accumulating evidence related to the association of cancer and inflammation has led researchers to consider curcumin as a potential candidate for prevention of different types of cancers.[19] Curcumin is also known to interfere with multiple pathways to inhibit cancer cells of different organs.[20,21] In colon cancer cells, curcumin is known to modulate the cell cycle, induce apoptosis and inhibit markers of inflammation.[22]

Based on the existing literature on the ability of both limonoids and curcumin to inhibit proliferation of different cancer cells by acting through different pathways, we hypothesize that these two compounds may act synergistically. In order to test the hypothesis, experiments were conducted on colon cancer (SW480) cells to understand the influence of combinations of these two classes of natural compounds on inhibition of proliferation and induction of caspase mediated apoptosis.

Materials and methods

Chemicals, reagents and antibodies

Curcumin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). DMEM medium and chemicals used for cell cultures were purchased from Hyclone (Logan, UT, USA). All the solvents and reagents used were of analytical grade and obtained from Fisher Scientific (Pittsburg, PA, USA). A caspase-3 assay kit was procured from BD Pharmingen (Erembodegem, Belgium). All primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and HRP-conjugated goat anti-mouse and anti-rabbit secondary antisera were from Pierce Biotechnology, Inc. (Rockford, IL, USA). Fluorescent probes (acridine orange and propidium iodide) were obtained from Invitrogen (Carlsbad, CA, USA). Unless otherwise mentioned, control cells/treatment refers to SW480 cells treated with equal amount of DMSO under conditions similar to sample treatment.

Cell culture and maintenance

SW480 and 112CoN cells were obtained from ATCC (Manassas, VA, USA). The SW480 cells were cultured in DMEM containing 10% FBS (Fetal Bovine Serum) and antibiotics and maintained in a CO_2 incubator at 37 °C and 85 ± 5% RH. The 112CoN cells were grown in EMEM (ATCC) containing 10% FBS.

Isolation and characterization of limonoids

Limonin and LG were isolated and purified from citrus seeds according to our previous publications.[23,24] The purity of isolated compounds was analyzed using reversed phase HPLC[25] and structures were confirmed by NMR spectra.[15] Identified compounds were dissolved in dimethyl sulphoxide (DMSO) at known concentrations and used for assays of biological activities (maximum concentration of DMSO in culture was maintained at <0.2%).

Cell viability assay

The SW480 cells (3×10^3 cells per well) were treated with different concentrations of limonoids (12.5, 25.0 and 50.0 ppm), curcumin (25 ppm) and camptothecin (50 ppm). Three concentrations (1 : 3, 1 : 1and 3 : 1) of limonoids and curcumin were used. The cell viability was measured by the MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) assay.[26] Camptothecin, a naturally derived molecule, was used as a positive control for comparison. The cell viability was calculated in comparison to cells treated with DMSO at the same concentrations and results are expressed as % inhibition of cells with respect to DMSO at each time point.

The MTT assay was also conducted using colon normal fibroblast (112CoN) cells to know the effect on normal cells and the results are expressed as %growth with respect to corresponding DMSO concentrations.

Proliferation assay by counting viable cells

The SW480 cells (20×10^3 cells per well) were treated individually with 25, 50 ppm of limonin, LG, curcumin (25 ppm), combinations of limonin and LG with curcumin with three concentrations. Camptothecin was used as a standard for comparison. The viable cells assay was performed using a Beckman Coulter counter as described previously.[27]

DNA fragmentation assay

The SW480 cells were treated with limonoids (50 ppm), curcumin (25 ppm) and combination (1 : 1). After 24 h, genomic DNA was extracted from cells using the phenol–chloroform partition

method.[28] The amount of DNA extracted was quantified using a nanodrop spectrophotometer (ND1000, Thermofisher, Waltham, MA, USA). Equal amounts of DNA (25 μg) were separated by 1.5% agarose gel electrophoresis at 55 mV constant current. The gel was stained with EtBr and an image was captured using a LAS 4000 mini imaging system (Fuji Life Sciences, Stamford, CT, USA).

Caspase-3 activity

Colon carcinoma cells were seeded at a density of 1×10^6 cells per mL in 6 well plates. After 24 h of growth and formation of monolayers, these cells were treated with limonoids (50 ppm) and curcumin (25 ppm), a combination (1 : 1) and camptothecin (50 ppm). The spectrofluorimetric assay was performed to detect the total activity of caspase-3 using the protocol described in our previous report.[15]

Immunoblotting

The SW480 cells were cultured overnight in DME medium in 100 mm cell culture plates. These plates were replenished with fresh medium containing limonin, LG (50 ppm each) and 25 ppm of curcumin and combination of limonin/LG with curcumin 1 : 1 for 24 h. The expression levels of proteins were measured after separating equal amount of protein in 12% SDS PAGE, as described in our previous publication.[15]

Fluorescence imaging of probed cells

SW480 cells were treated with compounds for 24 h and stained with acridine orange (AO) and propidium iodide (PI), then analyzed under a fluorescence microscope. Staining and image analysis were conducted as described in our recent publication.[29]

Statistical analysis

All the treatments were done in triplicate, independently and the results are expressed as mean ± SD. Evaluation of significance was performed using ANOVA and the data were compared using Tukey's post-test analysis in GraphPad Prism software version-5.00.288.

Results

Inhibition of SW480 cells using MTT assay

Results of the MTT assays clearly demonstrated inhibitory efficacy of individual test compounds, with activity ranging from 6.4–84% (Table 1). Incubation of cells with limonin and LG inhibited proliferation by 62.8 and 71.9%, respectively at 50 ppm after 72 h of incubation. The inhibition of cell proliferation using combination concentration (1 : 1) was significant compared to the control and camptothecin at 25 ppm ($P < 0.01$) at 24 and 48 h. Curcumin inhibited cell proliferation by 79.96% at 25 ppm after 48 h. The combination of curcumin with limonin demonstrated 70, 80 and 87% ($P < 0.01$) inhibition at all three concentrations, respectively, after 72 h of incubation. Similar results were also observed with combinations of curcumin with LG. These results suggest that the combination of curcumin either with limonin or LG will offer better cytotoxicity effects on SW480 cells.

The three compounds used in the study did not significantly affect the proliferation of colon normal fibroblast (112CoN) cells. For example, curcumin treatment resulted in inhibition of 3–4% of cells, after 72 h of incubation at 25 ppm and the extent of inhibition was not significant. These results suggest the possibility of lower toxicity and adverse reactions of these compounds in normal/non-cancerous colon cells (ESI Fig. S1†).

Inhibition of SW480 cells proliferation confirmed by viable cell count assay

Results of the viable cell count study further confirmed the proliferation inhibition ability of individual compounds with a maximum of 46.1 and 46.4% inhibition with treatment of limonin and LG, respectively at 50 ppm for 144 h (Fig. 1). Curcumin at 25 ppm demonstrated inhibition of up to 80% at 144 h. Combination of limonin with curcumin showed more than 95% inhibition in all three combinations after 144 h, indicating synergistic inhibition. Similar results were observed with combinations of LG with curcumin showing more than 97% inhibition at tested concentrations.

Limonoids and curcumin alter the DNA integrity in SW480 cells

The cells treated with limonin and LG individually did not show ladder formation, an indication of DNA fragmentation, at 50 ppm (Fig. 2). By contrast, the cells treated with curcumin independently and in combination with limonin and LG demonstrated clear fragmentation patterns. This provides convincing evidence that apoptosis is involved in inhibition of cell proliferation by combinations of these compounds.

Influence of combination on net caspase-3 activity

The total activity of caspase-3 was elevated by 54, 90 and 59% in the cells treated with limonin (50 ppm), LG (50 ppm) and curcumin (25 ppm), respectively, compared to control cells (Fig. 3). This suggests that limonoids and curcumin can induce apoptosis through activation of caspase-3 in SW480 cells. Furthermore, 50% reduction in the dose of both limonoids (25 ppm) resulted in the elevation of caspase-3 activity by 154%, which was significantly higher ($P < 0.01$) than the control. Similarly, the combination of LG at the same dose resulted in significant ($P < 0.01$) elevation of caspase-3 activity by 218% compared to the control. These results suggest that limonoids and curcumin can synergistically enhance caspase-3 activity in SW480 cells. Camptothecin was used as a positive control and resulted in elevation of caspase-3 activity by 90% ($P < 0.001$ compared to the control) when treated at 50 ppm.

Limonoids and curcumin alter the expression of intrinsic apoptosis-related proteins in SW480 cells

The expression of major proteins related to apoptosis, namely Bax, Bcl-2 and caspase-3, was measured to confirm the

Table 1 Colon cancer cells were treated with limonoids, curcumin, and their different combinations and camptothecin and the viability of SW480 cells was measured by the MTT assay[a]

		%Inhibition of proliferation		
Treatment	Conc. (ppm/ratio)	24 h	48 h	72 h
Limonin	12.5	6.42 ± 0.23	15.45 ± 0.94	18.98 ± 1.21
	25	11.38 ± 1.72	25.07 ± 0.11	31.77 ± 0.40
	50	25.58 ± 0.85	51.89 ± 2.01	62.78 ± 2.05
LG	12.5	9.18 ± 1.68	16.60 ± 2.45	20.43 ± 3.40
	25	12.02 ± 0.48	19.25 ± 2.32	21.35 ± 1.75
	50	28.18 ± 0.28	67.84 ± 2.56*	71.85 ± 1.86**
Curcumin	25	36.90 ± 3.03	79.96 ± 0.72*	84.28 ± 0.22**
Limonin and curcumin	1 : 3	35.94 ± 2.86	61.78 ± 2.58*	70.25 ± 1.15**
	1 : 1	40.56 ± 2.14	65.92 ± 0.11*	80.69 ± 0.98**
	3 : 1	62.01 ± 2.01*	78.99 ± 0.03**	87.17 ± 0.16**
LG and curcumin	1 : 3	34.49 ± 2.21	38.90 ± 1.74	60.46 ± 1.52*
	1 : 1	40.57 ± 2.10	52.99 ± 0.74	72.09 ± 0.42**
	3 : 1	68.73 ± 2.20*	77.64 ± 3.14**	87.37 ± 0.69**
Camptothecin	12.5	22.14 ± 2.97	23.53 ± 2.76	55.80 ± 0.73
	25	26.60 ± 4.94	29.15 ± 2.47	59.18 ± 1.93

[a] SW480 cells were treated with the specified concentrations of compounds for 24, 48 and 72 h and percentage viable cells were spectrophotometrically measured using MTT reagent. Results were expressed as %inhibition in viability of cells compared to the vehicle (DMSO) control. [*$P < 0.05$ and **$P < 0.01$ compared to control treatment (DMSO)].

induction of programmed cell death by these compounds (Fig. 4). β-Actin was used as a loading control and the expression of proteins was normalized to actin. The levels of pro-apoptotic Bax were higher (compared to control) in cells treated with individual compounds. By contrast, the anti-apoptotic protein Bcl-2 was reduced in the cells treated with curcumin and the combination of curcumin with limonoids, which clearly suggests the involvement of programmed cell death. Due to homo- and heterodimerization of Bcl-2 family proteins, the ratio of Bax to Bcl-2 is considered to be a more precise indicator for measuring apoptosis. The Bax/Bcl-2 ratio was elevated by 1.6- and 2.7-fold, compared to the control, in the cells treated with limonin and LG at 50 ppm. Furthermore, a 5.13-fold increase in the ratio was observed in cells treated with limonin and curcumin (1 : 1) in comparison with the control. The expression ratio was enhanced by 3.6-fold after treatment with LG and curcumin (1 : 1) compared to the control. Treatment of curcumin alone increased the ratio by 8.8-fold (Fig. 4). The elevated activity of caspase-3 was also supported by an observed increase in the expression of caspase-3. Cells treated with both limonin and LG did not show a marked difference in the expression of pro-caspase-3 (34 kDa) compared to the control. The SW480 cells treated with curcumin alone and the combination of curcumin and limonoids demonstrated the down-regulated expression of pro-caspase-3. This clearly indicates the extent of caspase-3 mediated apoptosis induction.

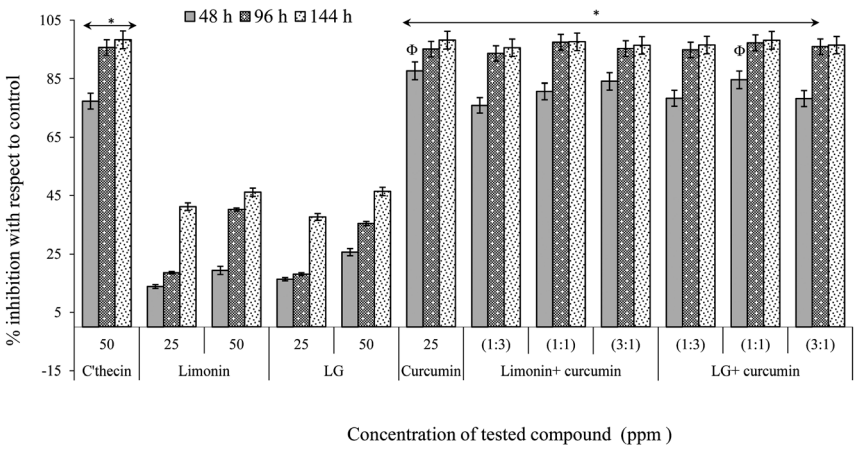

Fig. 1 Proliferation inhibition of SW480 cells by limonoids, curcumin, camptothecin and combination treatments by the cell count assay. Cells were treated with the specified concentrations of compounds and incubated for 48, 96 and 144 h and the number of viable cells was counted using a Beckmann Z_1 particle counter. Results of the inhibition are expressed as %dead cells compared to the vehicle (DMSO) control (Φ – significant compared to camptothecin at the same incubation period $P < 0.05$; *significant compared to the control at $P < 0.01$, note: C'thecin – camptothecin; LG – limonin glucoside).

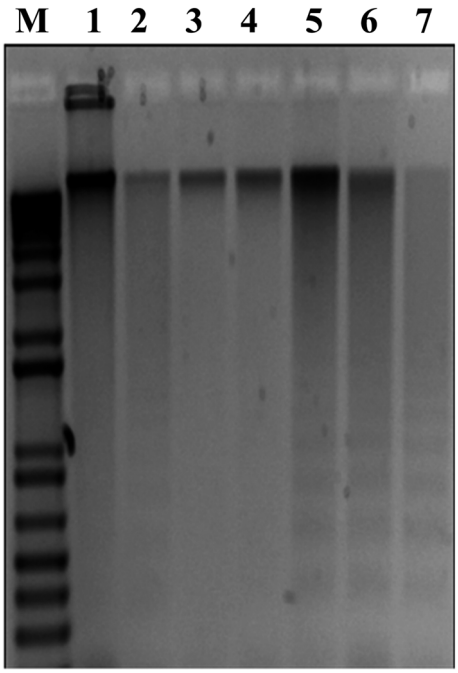

Fig. 2 Effect of limonoids, curcumin, camptothecin and combination treatments on integrity of DNA of SW480 cells. The cells were treated with specified concentrations of various compounds for 24 h and DNA was extracted from the treated cells using the phenol–chloroform partition method. The purified DNA was electrophoresed on 1.5% agarose gel electrophoresis at 55 mV constant current. The gel was stained with EtBr and the image was captured using a LAS 4000 mini imaging system. Note: M – marker, 1 – control, 2 – camptothecin (50 ppm), 3 – limonin (50 ppm), 4 – LG (50 ppm), 5 – curcumin (25 ppm), 6 – limonin + curcumin (1 : 1), 7 – LG + curcumin (1 : 1).

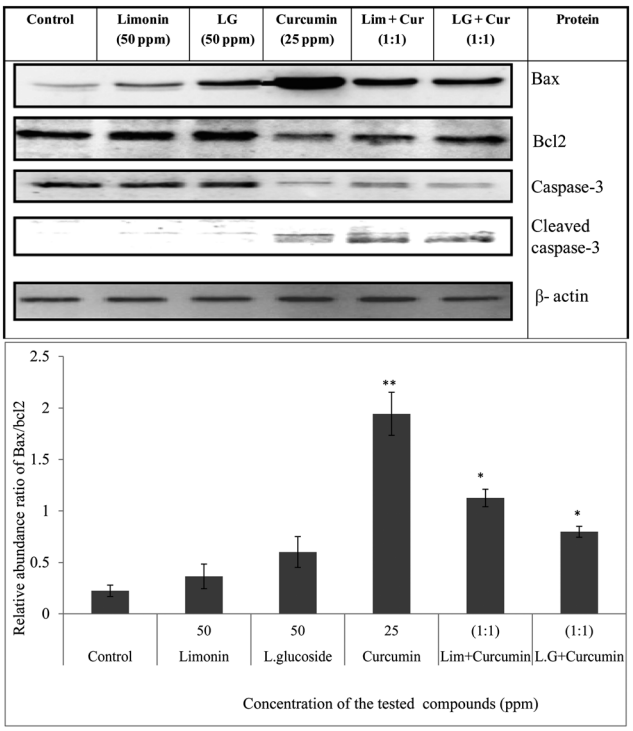

Fig. 4 Effect of limonoids curcumin and combinations on expression levels of apoptosis-related proteins of SW480 cells. Cells were treated with the specified concentrations of compounds for 24 h and the total protein was separated by 12% SDS-PAGE. The separated protein band was incubated with specific primary antibodies overnight at 4 °C and tagged with the secondary HRP-conjugated anti-mouse antibody for 2 h. The binding was detected using the 'super signal west femto-maximum sensitivity substrate' and the chemiluminescence image was captured using a LAS 4000 image analyzer. The β-actin was used as a loading reference (top). Results of the relative abundance ratio of Bax/Bcl-2 was measured using densitogram (bottom). $^{**}P < 0.01$ and $^{*}P < 0.05$ compared to control (DMSO) treated cells [note: Lim – limonin; LG – limonin glucoside; Cur – curcumin].

Fluorescence microscopy evidence for induction of apoptosis by compounds

The induction of apoptosis by combination treatments was further confirmed by double staining with acridine orange (AO) and propidium iodide (PI). Control cells treated with DMSO showed normal cell morphology with intact cell organelles; these cells were stained with AO and impermeable to PI, indicating normal morphological features and intact DNA. Treatment with limonin (50 ppm) resulted in a decreased number of

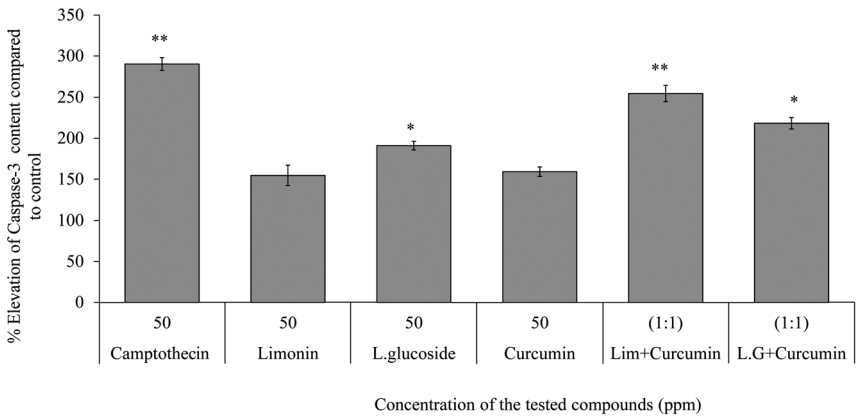

Fig. 3 Elevation of caspase-3 activity by limonoids, curcumin, camptothecin and combination treatments in SW480 cells. Cells were treated with the specified concentrations of compounds for 24 h and the total content of caspase-3 was measured spectrofluorimetrically using the substrate Ac-DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin). The AMC liberated as a result of caspase-3 activity was measured at an excitation wavelength of 360 nm and an emission wavelength of 460 nm [**highly significant ($P < 0.001$) and *significant ($P < 0.01$) compared to the control, Lim – limonin; LG – limonin glucoside].

live cells and those present were of irregular shape. The cell number was decreased by treatment of LG and several cells were permeable to PI, suggesting the loss in membrane integrity. Images of cells treated with curcumin showed clear features of cell death, which was evident from staining of most cells with PI. Treatment with curcumin and limonoids resulted in permeability of PI in all the cells, indicating the loss in both cellular and nuclear membrane integrity. Additionally, very few cells stained green (live cells) and most of them had lost membrane permeability and had condensed nuclei. There were no cells with normal features in these two sets of samples, suggesting the maximum apoptosis inducing potency of the combination.

Discussion

Synergy between natural compounds and synthetic molecules has proven to be a successful approach in prevention and treatment of chronic diseases.[30] Benefits from multiple compounds are achieved either due to pharmacokinetic or pharmacodynamic interactions resulting in synergistic or additive biological benefits. In the current study, two limonoids isolated from citrus were investigated in combination with curcumin for potential benefits in inhibiting human colon cancer cells. Both limonoids and curcumin are only part of the regular diet in Asian subpopulations; therefore we hypothesize that the positive results of this study will provide great benefits worldwide. Based on the literature, it is evident that curcumin is capable of inducing apoptosis at less than 50 μM in colon and other cancer cells,[31] which is accounting the concentration of curcumin 18.419 ppm. Here, we demonstrated the inhibition of cell proliferation of limonin and LG at 50 μM accounting for 23.52 ppm.[15] Based on these results, we used 25 ppm of each with a ratio of 1 : 1. Furthermore, we used different ratios of compounds to understand the influence on colon cancer cells. Three combinations were selected to examine the influence of limonoids on the activity of curcumin and its bioavailability. The ratio of 1 : 1 was used to understand the additive or synergistic activity and the ratio of 1 : 3 was to test whether limonoids can influence the bioavailability of curcumin and *vice versa* (3 : 1) to understand contribution of each compound. Experiments were performed to elucidate the effects on the extent of proliferation inhibition, DNA fragmentation, content of caspase-3 enzyme and expression levels of apoptosis related proteins.

Results of MTT based proliferation assays clearly suggested synergy between limonoids and curcumin in inhibition of proliferation (Table 1). The inhibition potency of the individual limonoids was lower compared to curcumin. However, combining these limonoids with curcumin showed a net increase in the activity compared to individual compounds. The results from MTT assays were further confirmed by viable cell count assays (Fig. 1).

To understand the possible causes for cell death, DNA integrity was measured. Fragmentation of DNA is a classical hallmark of apoptosis, and helps differentiate apoptotic cell death from necrosis. Fragmentation of oligonucleosomes of 180–200 bp is considered to be biochemical hallmark for programmed cell death induction. Appearance of large fragments (50–300 kb) is also reported in some types of cells, such as rat thymocytes and lymphocytes. These fractions are known to serve as precursors for smaller DNA fragments.[32] Results of the current study suggest that the combination of curcumin with limonin and LG may have an additive effect in inducing apoptosis as evident from fragmentation of DNA in cells treated with combinations, compared to individual compounds (Fig. 2).

Upon confirming the induction of apoptosis, the next step was to analyze the activity of major caspase proteins involved in apoptosis. Caspase-3 (also referred as executor caspase) is one of the major enzymes involved in the induction of apoptosis.[33] Approximately, 42–58 known caspase substrates are specifically cleaved by caspase-3 and related proteases and can act on procaspases 2, 6, 7 and 9 to induce cell death. In addition, caspase-3 is also known to induce biochemical events such as chromatin condensation, upstream cytochrome-c release from mitochondria through cleavage of Bcl-2, converting Bcl-2 an anti-apoptotic protein into pro-apoptotic.[34] Activation of caspase-3 is one of the confirmed targets of apoptosis induction. Curcumin is known to activate caspase-3 through various pathways including production of ROS, Ca^{2+} ions[35] and activation of caspase-8.[4] Considering the fact that limonoids are not active against caspase-8,[15] the synergy may be due to activation of intrinsic apoptosis and/or production of ROS. To further confirm the activity, expression levels of major intrinsic apoptosis inducing proteins were studied.

The Bcl-2 family proteins are key regulators of apoptosis. Upon activation, loss of mitochondrial membrane potential leading to a release of cytochrome-c was observed, which is known to activate caspase-3 to induce apoptosis. Bcl-2 is an upstream effector molecule in the apoptotic pathway and is identified as anti-apoptotic. The Bcl-2 family proteins are known to form a heterodimer complex with Bax, which is a pro-apoptotic member. This complex neutralizes the pro-apoptotic effect of Bax to prevent cell death.[36] Therefore, the ratio of Bax/Bcl-2 is considered as one of the major markers of apoptosis. Both curcumin and limonoids are known to activate Bcl-2 family proteins leading to apoptosis in different cancer cells.[15,37] Contradictory to these results, treatments of curcumin in SW480 cells showed no net effect on the ratio of Bax/Bcl-2.[38] Activation of the Bcl-2 family finally resulted in activation of death inducing caspase-3 to cause apoptosis. Therefore, in the current study, the expression of caspase-3 in the cells treated with combinations of compounds confirmed the additive effect of these compounds in the induction of caspase-3 mediated apoptosis.

Detection of cleaved caspase-3 (17–20 kDa) bands indicates the complete activation of caspase-3 by cleavage of pro-caspase-3.[39] Activated caspase-3 initiates apoptotic DNA fragmentation by inactivation of DNA fragmentation factor-45, an inhibitor of caspase-activated DNase.[40] Hence, activation of caspase-3 is considered to be a confirmatory marker for apoptosis.[41] In the current study, the presence of cleaved caspase-3 in the cells treated with curcumin indicates the ability of curcumin to induce cleaved caspase-3. Curcumin is known to activate caspase-3 through down-regulation of anti-apoptotic Bcl-2, Bcl-X_L and IAP (inhibitor of apoptosis) proteins in renal cancer cells.[42]

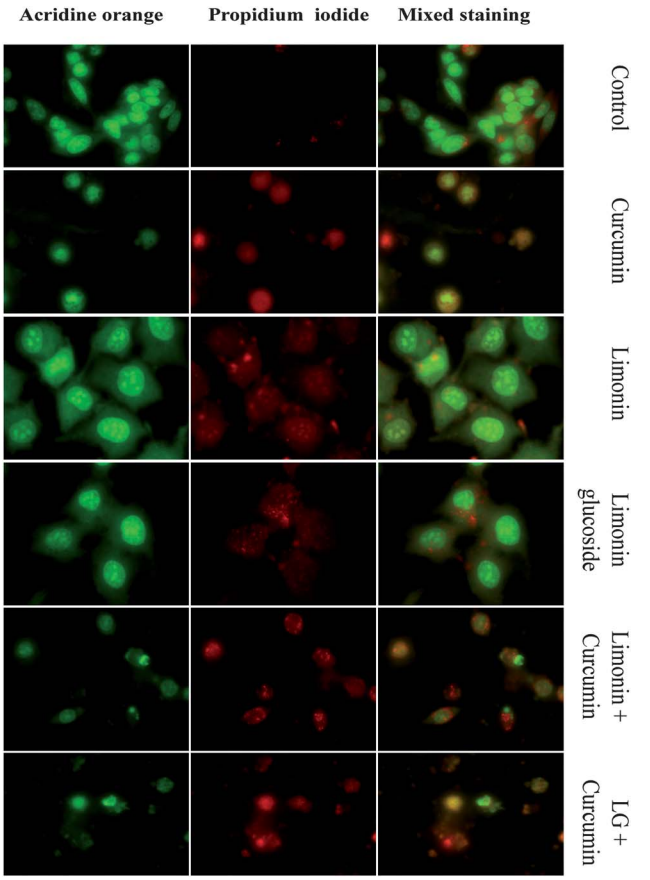

Fig. 5 Fluorescence microscopy images of SW480 cells treated with DMSO (control), limonoids (50 ppm each), curcumin (25 ppm), and combination (1 : 1). Following 24 h of treatment, cells were stained with acridine orange (5 μM) and propidium iodide (5 μM) for detection of apoptosis related characters. Images were captured using a fluorescent lamp at 500 (excitation) and 530 (emission) for AO and 535 (excitation) and 617 (emission) for PI using a Stallion digital imaging workstation.

Additionally, caspase-3 is also activated *via* caspase-8, which is known to be activated as a result of suppression of tumor necrosis factor (TNF).[43] Since curcumin is a well-known suppressor of TNF-α, activation of caspase-3 may be due to activity on both Bcl-2 family proteins and TNF. Although the proliferation results suggest synergy between the compounds, results of other biochemical analyses indicate that activity is more of an additive effect. At this point, it is also important to consider the ability of curcumin and limonoids to induce apoptosis through other pathways for correlation of proliferation inhibition results with expression levels of the proteins measured in the current study.

Finally, cell death was also confirmed through fluorescence microscopy of treated SW480 cells stained with AO and PI. Acridine orange is a cationic dye specific to nucleic acids and known for interaction with both DNA and RNA. Cells stain with green fluorescence when AO binds to double stranded nucleic acids, indicating live cells; cells stain red when AO binds to single stranded nucleic acids, indicating dead cells. AO also enters the acidic compartment of cell organelles.[44] PI is a DNA-specific dye known to enter only late apoptotic and necrotic cells. It can intercalate into nucleic acids every 4–5 base pairs without preference.[45] The extent of permeability of treated cells to PI suggests the extent of cytotoxicity (Fig. 5); this was also confirmed by the reduced number of viable cells (stained with AO). The characteristic features of cells with different treatments clearly demonstrate the ability of these compounds to induce apoptosis, as well as their additive effect.

Conclusions

The results of the current study demonstrated the inhibition of human colon cancer cell proliferation through programmed cell death by treatment with a combination of limonoids and curcumin, for the first time. The combinations of limonoids and curcumin were effective in inducing apoptosis in SW480 cells. Furthermore, limonoids and curcumin exhibited synergistic inhibition of proliferation of colon cancer cells. These results were also supported by total caspase-3 activity in the cells treated with combinations of limonoids and curcumin. However, experiments on expression levels of proteins suggest that the activity is more of an additive effect between these two classes (triterpenoids and phenolic) of compounds, with respect to the Bcl-2 pathway. These results indicate the possibility of influencing other pathways by these compounds to induce synergism and provide a new dimension to explore the benefits using *in vivo* models for effective prevention of colon and other cancers.

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