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Novel triterpenoid from *Citrus aurantium* L. possesses chemopreventive properties against human colon cancer cells

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Abstract—Potential cancer preventive constituents of sour orange (*Citrus aurantium* L.) were isolated and identified from EtOAc extract of sour orange. Crude EtOAc extract was purified using silica gel column chromatography to isolate two putative bioactive compounds. The purity of the isolated compounds was analyzed by TLC and HPLC. The structures of the two compounds were identified by one-dimensional (^{1}H, ^{13}C) and two-dimensional (^{1}H–H and ^{1}H–^{13}C) NMR experiments as isolimonic acid and a novel compound named as ichanexic acid. Stereochemical assignment of the protons for both the compounds was made using one-dimensional nuclear Overhauser enhancement (nOe) experiments. The identified compounds were tested for the inhibition of human colon cancer cells (HT-29) proliferation, apoptosis, and on non-cancerous (COS-1 fibroblast) cells. Cell proliferation, arrest of cell growth, and induction of apoptosis were determined by MTT assay, flow cytometry, and nuclear staining methods, respectively. The MTT assay indicated that both the compounds exhibited differential inhibition at various concentrations. Significant arrest of cell growth by isolimonoic acid was noticed within 24 h of treatment on the HT-29 colon cancer cells at a concentration as low as 5.0 μM ($P = 0.005$) and by ichanexic acid at 10.0 μM ($P = 0.011$). None of the compounds exerted any apparent cytostatic effects on the non-cancerous COS-1 fibroblast cells. Both the compounds exerted nearly 4- to 5-fold increase in the counts of G2/M stage cells at 5 μ M indicating a potential role in the cell cycle arrest as well as possible lead structures for the development of cancer chemopreventive and therapeutic agents. To the best of our knowledge, this is the first report on isolation, identification of isolimonic acid in its native form, and compound **2** was found to a novel and identified as ichanexic acid.
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1. Introduction

Citrus belongs to family Rutaceae and several commercial citrus varieties such as sweet orange, grapefruit, lime and lemon have been very popular since ages as savory fruits and were considered to possess natural compounds with several health benefits. Our recent investigations into the chemical constituents of citrus health benefits were attributed due to several potential bioactive compounds in addition to vitamin C.[1–5] Epidemiological studies have provided strong evidence that a diet containing fruits and vegetables provide potential cancer risk reduction in humans.[6] A large number of studies on laboratory animals have also demonstrated that a wide range of non-nutritive dietary bioactive compounds derived from fruit and vegetables inhibit chemical carcinogenesis caused by electrophiles and reactive oxygen species arising from endogenous and exogenous sources. One of the possible mechanisms responsible for the protective role of fruit and vegetable consumption was the induction of phase II xenobiotic metabolizing enzymes, which include glutathione *S*-transferase, quinone reductase, and UDP-glucuronosyltransferase.[7,8]

Abbreviations: EtOAc, ethyl acetate; HPLC, high performance liquid chromatography; COS-1, normal cells; HT-29, human colonic adenocarcinoma cells; DCM, dichloromethane; QCD, quaternary carbon detection; DQF-COSY, double quantum filtered correlation-correlated spectra; HSQC, heteronuclear single quantum correlation; HMBC, heteronuclear multiple bond correlation; nOe, nuclear overhauser enhancement; BAD, background aggregation and debris; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Keywords: *Citrus aurantium* L.; Isolimonic acid; Ichanexic acid; HT-29; MTT assay; Flow cytometer.

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Colon cancer is the third most malignant neoplasm in the world and second leading cause of cancer deaths in US. It is well known that dietary factors can modulate the development of certain types of human cancer, including colon cancer. Epidemiological data suggest that ingestion of some bioactive compounds from fruits and vegetables may contribute to reduction of cancer incidence in humans. Our recent studies[1,4,9,10] and many others[11,12] have demonstrated that citrus limonoids possess numerous health benefits including but not limited to countering the effects of HIV infections, antimalarial and anti-inflammatory activity, as well as acting as cytotoxic and cytostatic agents in animals and human cell cultures. The antiproliferative effects of limonoids have been shown in various cancers including but not limited to breast cancer,[9] colon cancer,[1,12] stomach cancer,[13] and neuroblastoma cancer.[10] Miller et al. [14] proved that limonin, limonin glucoside, nomilin, and obacunone inhibited the activity of average tumor burden in DMBA-induced hamster buccal pouch carcinogenesis. Moreover, limonin and nomilin were also found to inhibit HIV-1 replication in human peripheral blood mono nuclear cell culture.[11] We were able to demonstrate that certain limonoids have antiproliferative[9] and caspase mediated apoptosis inducing effects on human cancer cells *in vitro*.[10] Consequently, the results have indicated the ability of certain glucosidic forms of limonoids, to arrest S and G2/M stages of the cell cycle.

Limonoids are tetranor-triterpenes (C_{26}) characteristically present in Rutaceae and Meliaceae families.[15] Earlier, citrus limonoids were considered a major problem for the citrus juice industry, as they cause delayed bitterness of the juices at room temperature thus lowering the quality and value of the commercial juice.[16] However, in recent years those bitter compounds have gained significant importance in the inhibition of several chronic diseases. Until now, 23 neutral and 15 acidic limonoid aglycones have been reported from citrus and its hybrids. Our recent study demonstrated that, five solvent extracts from sour orange were reported to possess good antioxidant activity *in vitro* models.[17] To further understand characteristics of bioactive compounds from sour orange, we have isolated, purified, and characterized two limonoids by extensive NMR studies for the first time and one compound was found to be novel and named as ichanexic acid. We also report the antiproliferative effects of these putative compounds on colon cancer cells (HT-29), in comparison with non-cancerous mammalian kidney fibroblast cells (COS-1).

2. Results and discussion

The present study was conducted to isolate and evaluate the cell proliferation of putative bioactive compounds present in sour orange seeds. Defatted seed powder was extracted in Soxhlet extractor with ethyl acetate and the extract was concentrated under vacuum. In order to explore potential bioactive components responsible for the antiproliferative activity, the EtOAc fractions were analyzed by TLC and HPLC for composition and were subjected to repeated silica gel column chromatography. The column was eluted with DCM and acetone to obtain two limonoids. Compounds **1** and **2** were eluted with DCM/acetone (8:2) and (1:1), respectively. The purity of the isolated compounds was analyzed by TLC and HPLC. TLC analysis of compounds **1** and **2** gave mobilities 0.47 and 0.64, respectively. No additional spots were visualized on both plates with either Ehrlich reagent or methanolic sulfuric acid followed by heating at 100 °C for 10 min, thus confirming the purity of the compounds. Furthermore, the purity of the isolated compounds was confirmed by HPLC. Figure 1 shows the HPLC chromatograms of compounds **1** and **2** and no other peaks were observed.

2.1. Structure elucidation from ^{1}H and ^{13}C NMR

Comprehensive analyses of ^{1}H and ^{13}C 1D and 2D NMR experiments were made to establish the structures of the compounds **1** and **2**. Proton spectra were quite complex with many peak patterns similar for both the compounds specifically in the region between 1 and 3 ppm. Four single peaks with characteristic chemical shifts and intensity between 1 and 1.5 ppm have been assigned tentatively to isolated methyl groups. However, initially, their positions in the molecular structure were not known. ^{1}H NMR spectra obtained in the presence of deuterium oxide provided clue to the presence of exchangeable protons since these proton signals disappeared when the spectra were recorded after the addition of a drop of D_2O. Other signals arising from CH and CH_2 groups with several overlaps could not be identified using one-dimensional spectrum. Hence, we extensively utilized two-dimensional experiments. Identification of the sequentially connected groups of protons (CH and CH_2) was greatly aided by the analysis of DQF-COSY spectra. DQF-COSY spectra provided cross peaks due to the coupling between the geminal protons (2 bond couplings between the methylene protons) as well as due to the coupling between vicinal (3 bond couplings between CH and CH or CH and CH_2) protons. Depending on the magnitude of J coupling, the intensities of the cross peaks varied with stronger cross peaks for large couplings and vice versa. Figure 2 shows a typical DQF-COSY spectrum for compound **2** showing cross peaks between protons connected through 2 bonds and 3 bonds. From the DQF-COSY spectra alone, the distinction between CH and CH_2 protons could not be made unambiguously. Therefore, we first made tentative assignment of the entire sequentially coupled proton network by tracing the cross peak positions along both the frequency dimensions. These data were then combined with hetero-nuclear (^{1}H–^{13}C) two-dimensional experiments to make additional assignments and to confirm the tentative assignments made using 1D and DQF-COSY experiments. Edited HSQC, a heteronuclear experiment, utilizes one bond ^{13}C–^{1}H coupling and hence provides information on all protons directly attached carbons along with the multiplicity information of the carbons. Thus, as shown in Figure 3 for the compound **2**, edited HSQC spectrum showed negative cross peaks for CH and CH_3 groups and positive cross peaks for CH_2 groups. The negative sign and the characteristic carbon chemical shifts of four methyl

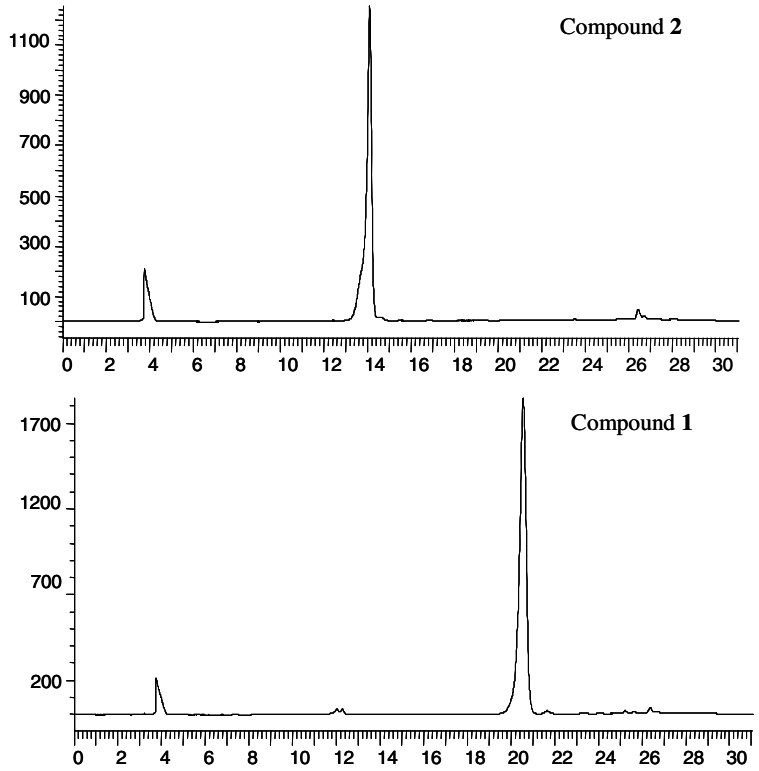

Figure 1. HPLC chromatograms of isolated compounds **1** and **2** from sour orange.

groups (between 15 and 35 ppm) are in accordance with the tentative assignment made from one-dimensional proton spectra.

The multiplicity information provided by edited HSQC was specifically more useful for distinguishing between CH and CH_2 protons. The signs of cross peaks between CH and CH_2 are opposite with their cross peaks phases shifted by 180° with respect to each other. Thus, the cross peaks arising from CH groups (negative peaks) and CH_2 (positive peaks) groups were distinguished based on the sign of the cross peaks. Further, for each CH_2 group, two cross peaks were seen along ^1H dimension due to the pair of methylene protons, attached to the same carbon, with different chemical shifts. In Figure 3, these cross peaks are shown by joining each pair by horizontal dashed lines. The methylene group, 11 CH_2 of compound **2** showed single cross peak due to the chemical shift degeneracy of these protons.

Edited HSQC spectra identified additional CH groups that were not detected in DQF-COSY spectra. In the proton spectra, such CH protons show single peaks due to their isolation in the molecular structure or due to the weak proton–proton couplings and hence the cross peaks in DQF-COSY for such peaks are either missing or too weak to be clearly observed. Thus the protons numbered 15 and 17 in compound **2** (Fig. 3) and 15, 17, and 21 in compound **1** (Fig. 5) that showed single peaks in proton spectra were not detectable in DQF-COSY spectra. On the other hand, these groups were clearly detected in the edited HSQC spectra (Fig. 3). Similarly, the protons at 22 and 23 positions are weakly coupled between them showing nearly undetectably weak cross peaks in DQF-COSY which spectra could be distinctly identified from the edited HSQC spectra (Fig. 3).

After identifying protons and their attached carbons, non-protonated carbons in the compounds were identified by the analysis of the spectra from the QCD experiments.[18] This experiment suppresses CH, CH_2, and CH_3 types of carbons and detects only non-protonated carbons such as quaternary and carbonyl carbons. Thus the carbons 3, 4, 7, 8, 10, 13, 14, 16, 20, and 21 in compound **2**, and carbons 3, 4, 7, 8, 10, 13, 14, 16, and 20 in compound **1** were distinctly detected from this experiment.

The structures of the two compounds were then built by utilizing proton and carbon chemical shifts, the multiplicity of each carbon, and combining the short and long range connectivity information provided by HMBC experiments. HMBC spectra provide proton-carbon connectivity information for up to three bonds. All the short and long range proton–carbon cross peaks in the HMBC spectra were identified by the extensive analysis. The assignment of various proton–carbon cross peaks in the HMBC spectrum of compound **2** is shown in Figure 4 and its structure shown in Figure 2. The structure of compound **1** and its proton and carbon spectra are shown in Figure 5 along with the complete assignment of proton and carbon signals.

After identifying the structures based on proton and carbon connectivity, one-dimensional nOe experiments

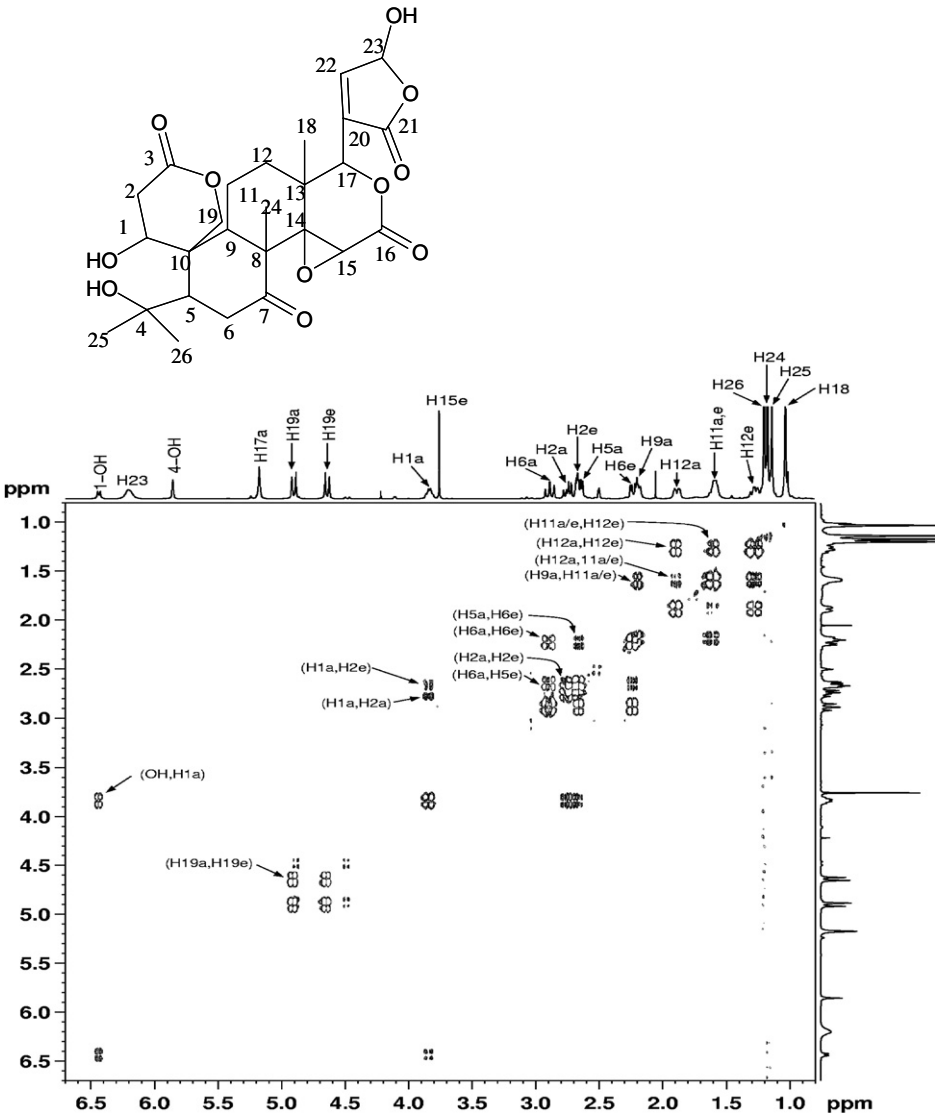

Figure 2. ^{1}H–^{1}H DQF-COSY spectrum of compound **2** (ichanexic acid) obtained on a Bruker Biospin Avance 400 MHz spectrometer, along with the stereochemical assignments of the cross peaks. The proton labels with 'a' refer to axial protons and with 'e' refer to equatorial protons. The structure of compound **2** along with the numbering of various $^{1}H/^{13}C$ nuclei is shown at the top.

were performed to stereochemically assign various protons in both the compounds. Each signal in the proton spectra was selectively irradiated and the nOe buildup to the neighboring protons arising from through space interaction was determined. Stereochemical assignment of the protons was then made based on the relative nOe cross peak intensity in the nOe difference spectra. The percentage nOe for the proximal protons was higher when the two protons lie on the same side of the ring, whereas, the enhancement was either absent or less when the protons were on the opposite side of the ring. We used the H18 methyl group that is axial in such steroid class of compounds as the starting point for these assignments. For example, irradiation of the H18 proton in compound **2** resulted in nOe enhancement of the peaks at 1.88 and 5.17 ppm and therefore they were assigned to protons 12a and 17a, respectively. Chemical shifts of compounds **1** and **2** for all the protons and carbons along the stereochemical assignments for the protons are presented in Table 1. Moreover, the isolated compounds have been analyzed for mass. Compound **1** showed [M−1] ion at *m/z* 487.1 and compound **2** showed [M+1] ion at 521.1 (100%). In addition, compound **2** showed fragments at 503.1 (45%), 485.1 (25%), and 381.1 (7%). On the basis of above results, the structures of compounds **1** and 2, were elucidated as isolimonic acid ichanexic acid. Earlier, isolimonic acid has been isolated and identified as the methyl ester.[19] Isolation of isolimonic acid (**1**) is the first report in its native form and its chemical shifts are compared with reported methyl ester of isolimonate[18] in $CDCl_3$. Even after considering the solvent effect the proton and carbon chemical shifts of our compound (**1**) are comparably matched with its reported methyl ester. All the proton and carbon chemical shifts of ester[18] have not been assigned completely. Although the authors have proposed the structure, some of the protons (H21 and H23) and carbon chemical shifts (C21, C23, C2 and

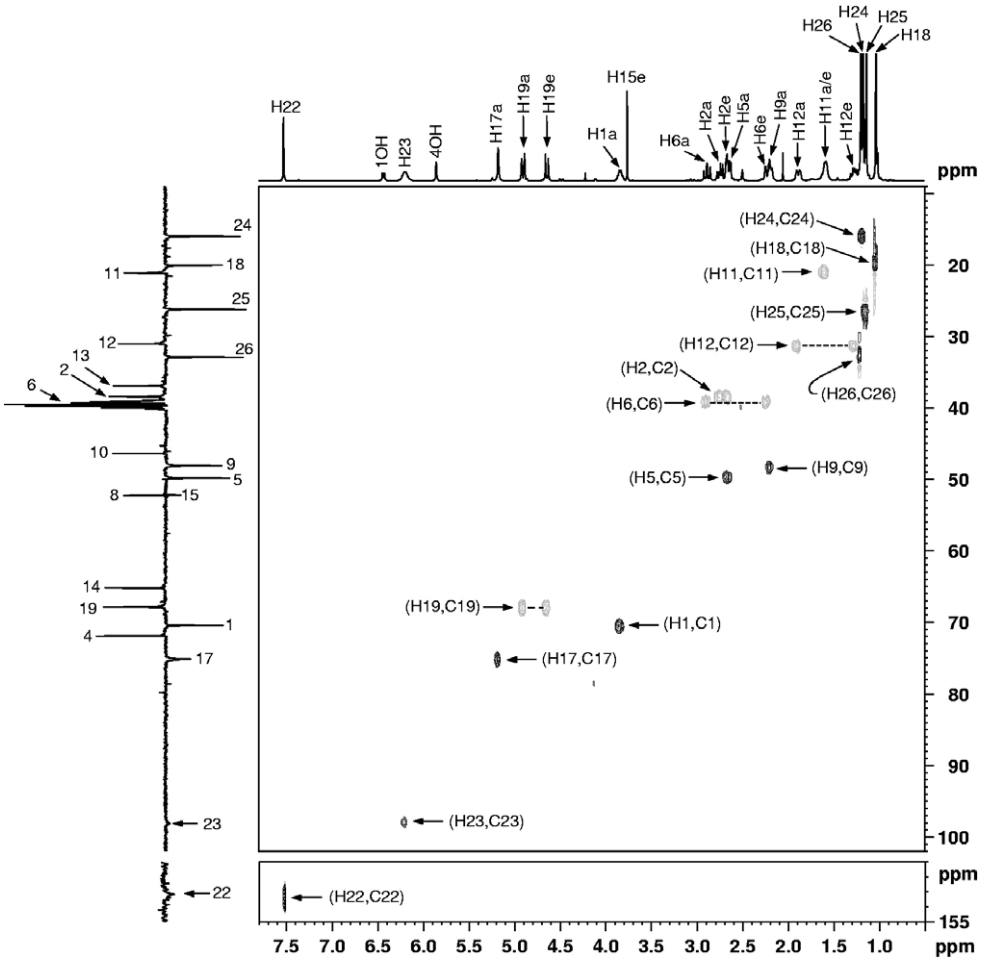

Figure 3. Sensitivity enhanced, multiplicity edited ^{1}H–^{13}C HSQC spectrum of compound **2** (ichanexic acid) obtained on a Bruker Biospin Avance 400 MHz spectrometer, along with the assignments of all the protons and proton attached carbons. For clarity, CH and CH_3 cross peaks are shown as dark continuous contours whereas CH_2 cross peaks are shown in light discontinuous contours. Wherever methylene protons are chemically different (non-degenerate), two distinct cross peaks were observed corresponding to axial and equatorial protons (each pair of cross peaks is joined by dashed lines). Traces of one-dimensional ^{1}H spectrum and ^{13}C SEFT spectrum (CH and CH_3 negative and C and CH_2 positive) are also shown. The proton labels with 'a' refer to axial protons and with 'e' refer to equatorial protons. Refer to the structure of compound **2** shown in Figure 2 for the numbering of various $^{1}H/^{13}C$ nuclei.

C6) are not precisely assigned, though the C2 and C6 are in a different chemical environment. The quaternary carbons C8 and C10 chemical shifts are reversed. All methyl (18, 24, 25 and 26) proton and carbon chemical shifts are not assigned to their respective positions (numbers). All these above exceptions of the reported methyl ester have been rectified in compound **1** and all the proton and carbon chemical shifts are precisely and unambiguously assigned and are confirmed by performing 2D HSQC and HMBC experiments (data not shown). However, this is the first report of the isolation and identification of isolimonic acid as such and the isolation of a novel compound, that is, ichanexic acid.

2.2. Cell viability

Significant cytotoxic effects of isolimonic acid were noticed by 24 h of treatment on the HT-29 colon cancer cells when compared to that of untreated cells at a concentration of 5.0 μM ($P = 0.005$) and 10.0 μM ($P = 0.005$) (Fig. 6). However, the significant difference in cytotoxic effects for ichanexic acid were observed only at 10.0 μM ($P = 0.011$). The amount of formazon formed because of MTT reduction by the live cells sharply decreased by 48 h, a result of fewer number of live cells, due to cessation of cell growth and cell death (Fig. 7). By 48 h the cytotoxic effects of isolimonic acid were highly significant even at concentrations as low as 2.5 μM ($P = 0.023$). Although the initial effects of ichanexic acid were mild, by 48 h this novel compound had as much effect as that of isolimonic acid with a concentration of 2.5 μM significantly affecting the cell growth and survival ($P < 0.05$). An intriguing result of this experiment was an observation of initial surge in the cytotoxic effect with isolimonic acid (24 h) and a gradual reduction in the effects over the next 48 h that was maintained at a constant level. The multiple replicates of this experiment yielded similar results, indicating the possibility of an initial saturation phase for the compounds or a higher turnover rate or susceptibility of a specific population within the cell type. The supplementation of both the compounds over a period of 72 h increased the cell death

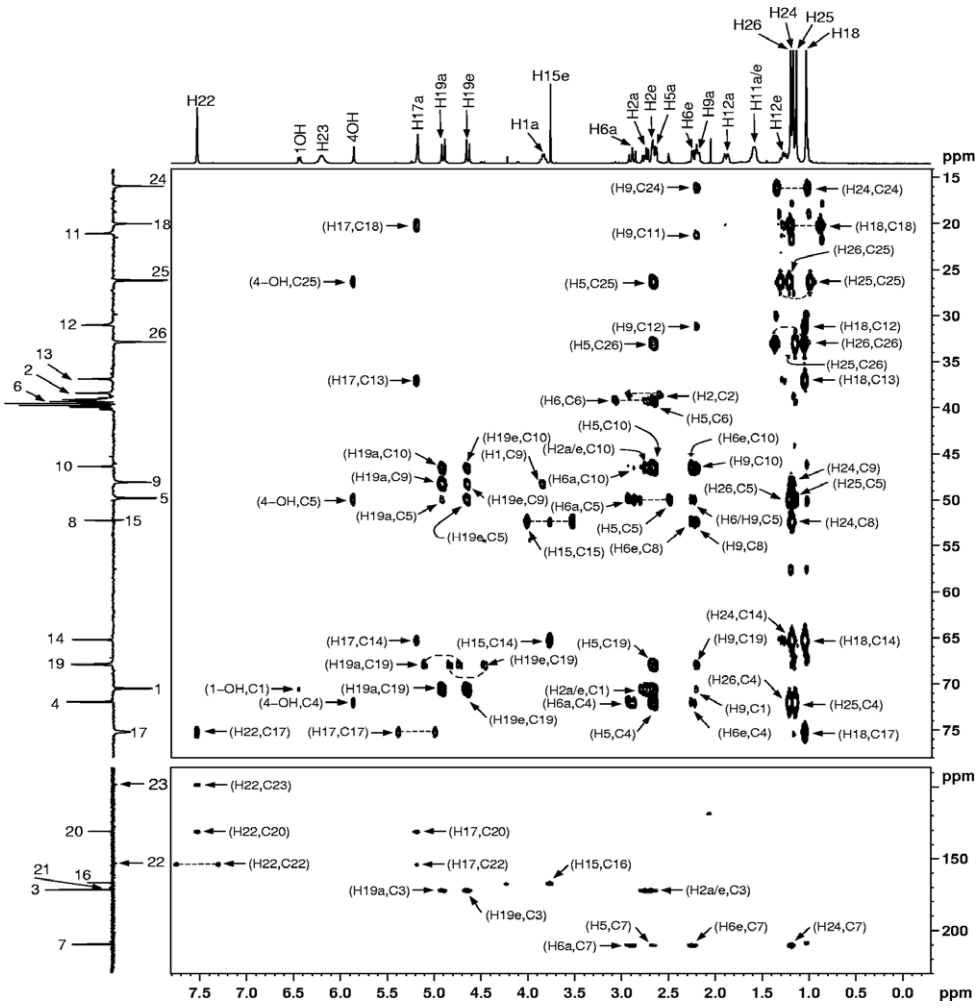

Figure 4. ^{1}H–^{13}C HMBC spectrum of compound **2** (ichanexic acid) obtained on a Bruker Biospin Avance 400 MHz spectrometer. Assignments of the entire cross peaks in the 2D spectrum are made. One-dimensional ^{1}H and ^{13}C SEFT spectra are also shown on the top and left of the 2D spectrum along with the assignments of various proton and carbons resonances. In the ^{13}C SEFT spectra, quaternary and CH_2 carbons are positive and, CH and CH_3 carbons are negative in intensity. The proton labels with 'a' refer to axial protons and with 'e' refer to equatorial protons. Refer to the structure of compound **2** shown in Figure 2 for the numbering of various $^{1}H/^{13}C$ nuclei.

by another 20% at concentrations over 5.0 μM (Fig. 8). However, the compounds had no significant effect on the growth of non-cancerous mammalian COS-1 kidney fibroblast cells indicating a cell type specific effect of these compounds (Figs. 9 and 10).

2.3. Effect on the specific stages of the cell cycle

We have previously hypothesized that the antiproliferative effects of limonoids could be in part due to their ability to affect certain stages of the cell cycle in the cancerous cells. In this study, we noticed a significant effect of the two tested compounds on the proliferation of HT-29 cells, mediated through the ability of the compounds to arrest certain stages of the cell cycle (Table 1). While the results explicitly indicate a three to 7-fold increase in the background aggregates and debris (BAD), a profound indicator of apoptosis, it was unambiguous that these compounds have the ability to arrest DNA synthesis and G2/M phases of cell cycle as well. While isolimonic acid had a profound effect on the arrest of DNA synthesis phase, ichanexic acid inhibited the G2/M phase inhibiting the separation of two daughter cells, thereby inducing apoptosis. Based on MTT reduction results, a significant effect on the cell cycle arrest by both the compounds at 5 μM concentration was observed, as indicated by a profound increase in the % BAD (34.8% and 42.9%) and G2/M (6.1% and 8.7%) cell contents (Table 2; Fig. 11). The flow cytometry data of a 3- to 4-fold increase in the cell cycle. The results were further confirmed on confocal fluorescent microscopy, where the DAPI nuclear staining indicated a loss of membrane integrity over the period of time for HT 29 cells (Fig. 12). Apparently, there was no effect of the selected compounds on the non-cancerous COS-1 cells (Figs. 9 and 10). Hence it can be discerned from our current and previous studies that purified limonoids had a specific toxic to lethal effect on a variety of human cancer cells, and sparing the non-cancerous cells.

Manthey and Guthrie[20] showed that flavonoids in compounds in citrus have the effects of antiproliferative

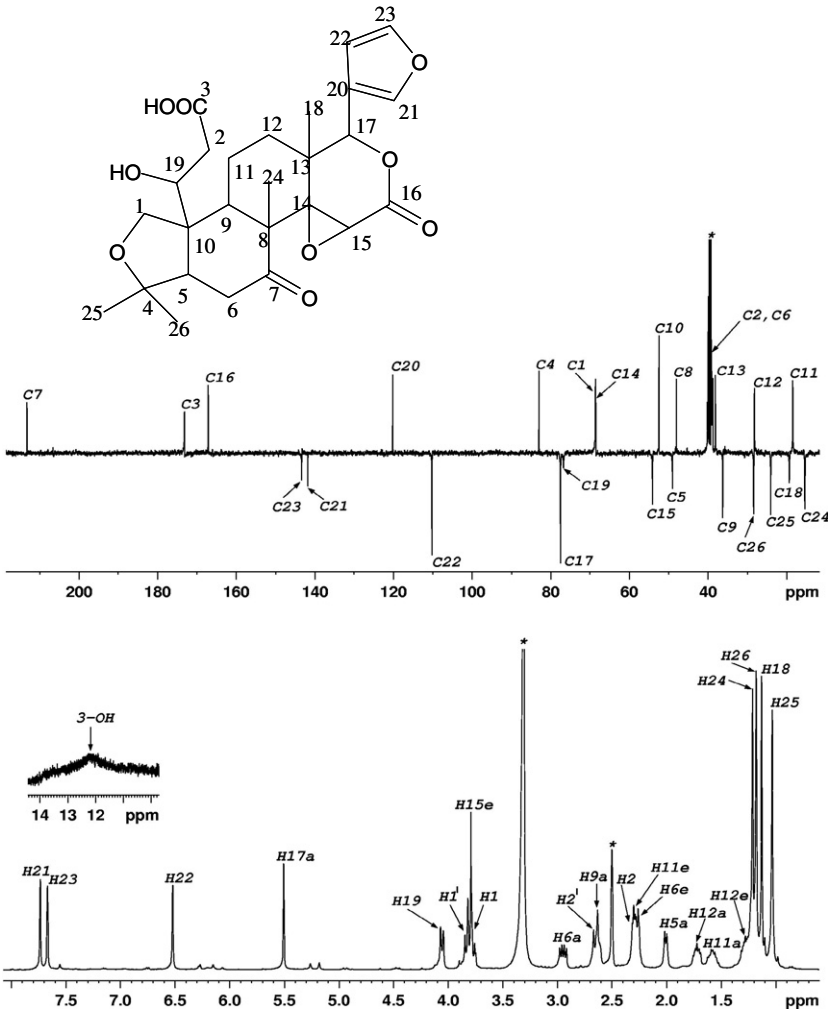

Figure 5. ^{1}H (bottom) and ^{13}C SEFT (top) spectra of compound **1** (isolimonoic acid) obtained on a Bruker Biospin Avance 400 MHz Spectrometer. Assignments of various protons and carbons are made on the respective spectrum. In the SEFT spectrum, the CH and CH_3 carbons are negative while the quaternary and CH_2 carbons are positive in intensity. The proton labels with 'a' refer to axial protons and with 'e' refer to equatorial protons. The structure of the molecules along with the numbering of various $^{1}H/^{13}C$ nuclei is shown at the top. Peaks from the DMSO solvent are marked with asterisks. ∗a = axial and e = equatorial protons.

activities on six human cancer cell lines, including human colon and breast cancer cells. Manthey et al.[21] reported high anti-inflammatory activities of citrus flavonoids in oxidative stress. Our study further confirms the apoptosis inducing effects of bioactive compounds from citrus fruits on cancerous cells which is in agreement with the previous reports.[9] The mode of action associated with the antineoplastic activity observed for citrus limonoids has not been determined. However, most of the citrus limonoids have a furan ring. Furan containing molecules from coffee bean and citrus limonoids have been found to induce phase II enzyme glutathione *S*-transferase activity *in vivo*.[8,13,22] Another study examining the effect of limonin and nomilin on phase I P450 and phase II GST activity in rats confirmed that the two limonoids induced GST in a dose-dependent manner in rat liver.[23] In a recent animal study, using eight limonoids including limonin and nomilin, we have established that nomilin increased GST activity 3 times above that of control in the small intestinal mucosa of test mice. Interestingly, limonin was not as effective.[24] The compounds in the present study are rare and novel. Inhibition of colon cancer cells is the first biological activity reported for these compounds. However, inhibition of breast cancer cells by citrus limonoids such as ichangin, methyl isolimonate, and 15 other compounds have been tested. The IC_{50} values for most of these compounds were found to be less than 25 μg/ml[25].

Plant derived triterpenoids have shown significant promise for the prevention of wide array of human cancers. Naturally, citrus juice contains significant quantities of limonoids and has the ability to inhibit colon cancer cells. 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. As accelerated cell growth or abnormal cell cycle regulation defines the progression of the cancer, compounds specifically affecting the stages of this abnormal cycle are often considered as important blue prints for drug development. For the first time isol-

Table 1. ^{1}H and ^{13}C NMR chemical shifts for isolated compounds **1** and **2** (solvent: DMSO-d_6)

Compound 2					Compound 1				
No.	Carbon/ proton type	$^{1}H^{*}$		^{13}C	No.	Carbon/proton type	$^{1}H^{*}$		^{13}C
		a	e				a	e	
1	CH	3.83		70.45	1	CH_2	3.77	3.83	68.62
2	CH_2	2.74	2.67	38.39	2	CH_2	2.28	2.64	38.82
3	C	—		171.58	3	C	—		173.23
4	C	—		71.90	4	C	—		83.02
5	CH	2.65	—	49.77	5	CH	2.01	—	49.03
6	CH_2	2.88	2.22	39.17	6	CH_2	2.93	2.26	39.22
7	C	—		209.64	7	C	—		213.24
8	C	—		52.20	8	C	—		48.04
9	CH	2.19	—	48.07	9	CH	2.62	—	36.25
10	C	—		46.37	10	C	—		52.46
11	CH_2	1.59	1.59	21.06	11	CH_2	1.57	2.28	18.49
12	CH_2	1.88	1.26	30.99	12	CH_2	1.72	1.27	28.23
13	C	—		36.87	13	C	—		38.13
14	C	—		65.18	14	C	—		68.49
15	CH	—	3.75	52.16	15	CH	—	3.79	54.11
16	C	—		166.46	16	C	—		167.13
17	CH	5.17	—	75.16	17	CH	5.50	—	77.57
18	CH_3	1.03		20.00	18	CH_3	1.12		19.38
19	CH_2	4.90	4.64	67.83	19	CH	4.06	—	76.81
20	C	—		130.59	20	C	—		120.18
21	C	—		170.38	21	CH	7.72		141.83
22	CH	7.52		153.14	22	CH	6.51		110.24
23	CH	6.20		98.04	23	CH	7.65		143.45
24	CH_3	1.17		15.92	24	CH_3	1.21		15.41
25	CH_3	1.14		26.14	25	CH_3	1.03		24.06
26	CH_3	1.20		32.86	26	CH_3	1.18		28.38
1	OH	6.43		—	3	OH	12.17		—
4	OH	5.85		—					
23	OH	7.96		—					

* a = axial and e = equatorial protons.

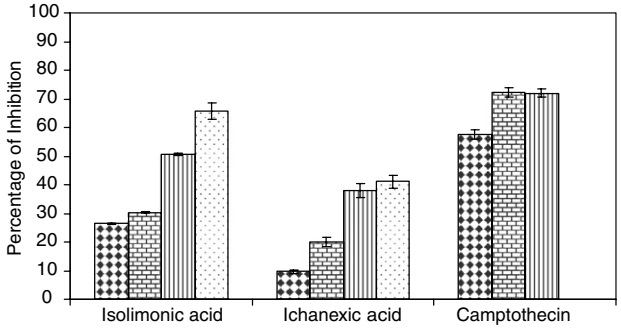

Figure 6. Inhibition of HT-29 cell population by limonoids and camptothecin at different concentrations (μM) after 24 h. ◩ 1.25, ▤ 2.5, ▥ 5, ⬚ 10.

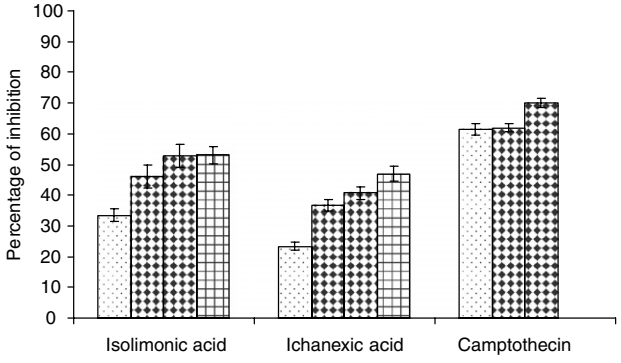

Figure 7. Inhibition of HT-29 cell population by limonoids and camptothecin at different concentrations (μM) after 48 h. ⬚ 1.25, ◪ 2.5, ◯ 5, ▣ 10.

imonoic acid and ichanexic acid 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 regulates the cell cycle complexing with other cyclin ligands and G2/M transition is governed by the active complexation of CDKs with the mitotic cyclins.[26] Our results indicate a highly promising arrest of G2/M phase of the cell cycle eventually leading to apoptosis, but the specific mechanism of these compounds on these kinase receptors and other transcription factors is yet to be determined. However, both isolimonoic acid and ichanexic acid possess potential chemopreventive properties and therefore provide significant leads to the development therapies to check the incessant progression of colonic adenocarcinomas. Further studies are required to better understand structure–activity which will provide a basis for selective chemical or biochemical methods to enhance antitumor activity. Moreover, bioavailability of citrus limonoids and mechanism of absorption are important future research objectives.

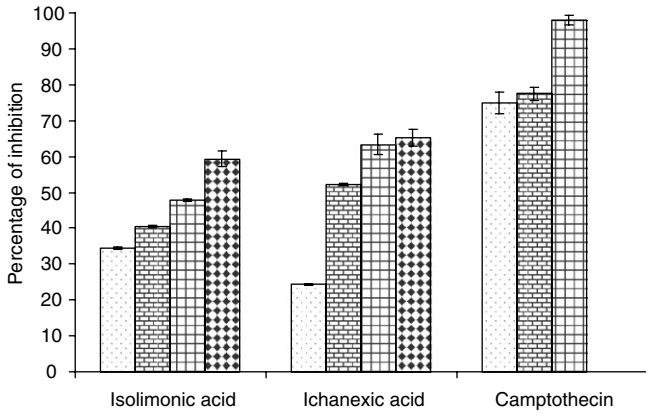

Figure 8. Inhibition of HT-29 cell population growth by limonoids and camptothecin at different concentrations after 72 h. ☐ 1.25, ▦ 2.5, ▥ 5, ▩ 10.

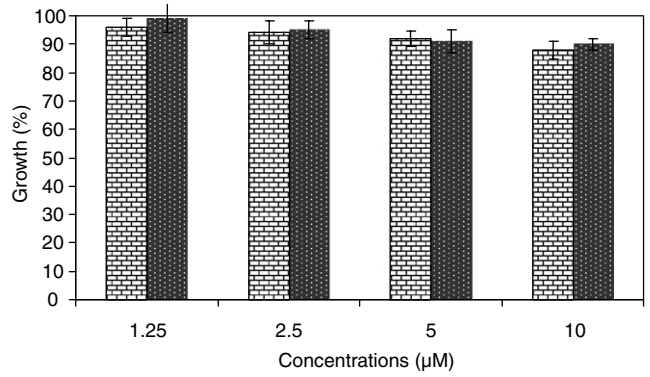

Figure 9. Percentage growth of COS-1 normal cells by isolated compounds after 24 h. ▦ isolimonic acid, ▩ ichanexic acid.

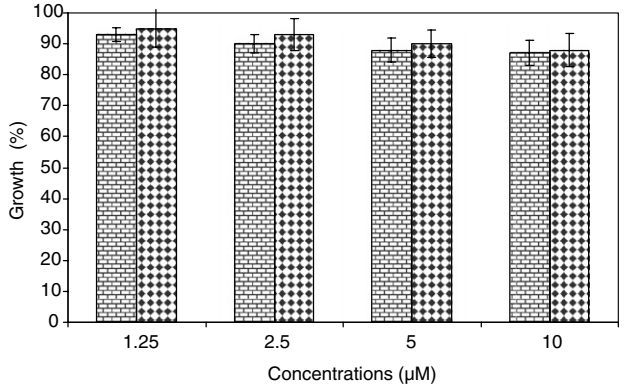

Figure 10. Percentage growth of COS-1 normal cells by isolated compounds after 48 h. ▦ isolimonic acid, ◆ ichenexic acid.

3. Experimental

3.1. Chemicals

Silica gel (200–400 mesh) was purchased from Aldrich (MO, USA). TLC plate's Silica gel 60F-254, thicknesses 0.20 mm (20 × 20 cm), were obtained from (Alltech Associates, INC.). *para*-*N*,*N*-Dimethyl amino benzaldehyde (Ehrlich reagent) was obtained from Sigma Chemical Co., (St. Louis, MO).

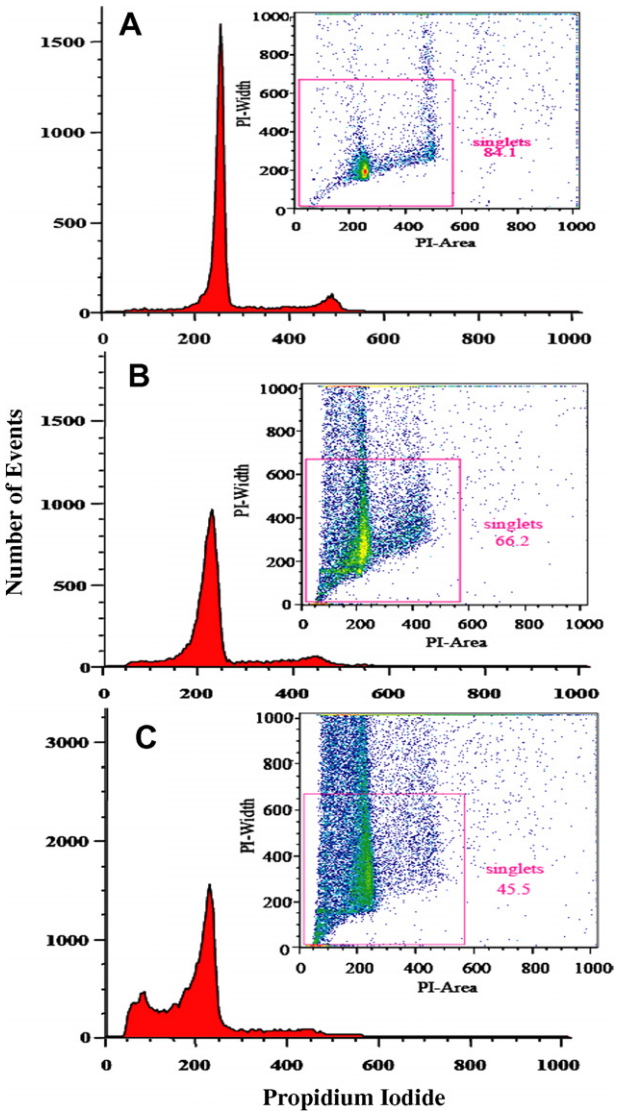

Figure 11. Results of flow cytometric cell cycle analysis of (A). Control (B). Isolimonic acid (C). Ichanexic acid at 10 μM concentration after 48 h. Histograms of the untreated cells were compared with those of untreated control cells. Horizontal and vertical axes indicate relative nuclear DNA contents and number of cells, respectively.

3.2. Plant material

Sour oranges (*Citrus aurantium* L.) were harvested from the orchard of Texas A&M University-Kingsville Citrus Center, Weslaco, Texas, in the month of October. The seeds were separated from the fruits and dried under shade at 25 °C and powdered using a blender.

3.3. Extraction

Ground citrus seed powder (7200 g) was extracted using a Soxhlet apparatus with hexane (15 L) for 24 h for the removal of fatty matter. The defatted powder was extracted for 8 h with ethyl acetate (15 L) at 60–70 °C. The extracts were filtered, concentrated under vacuum

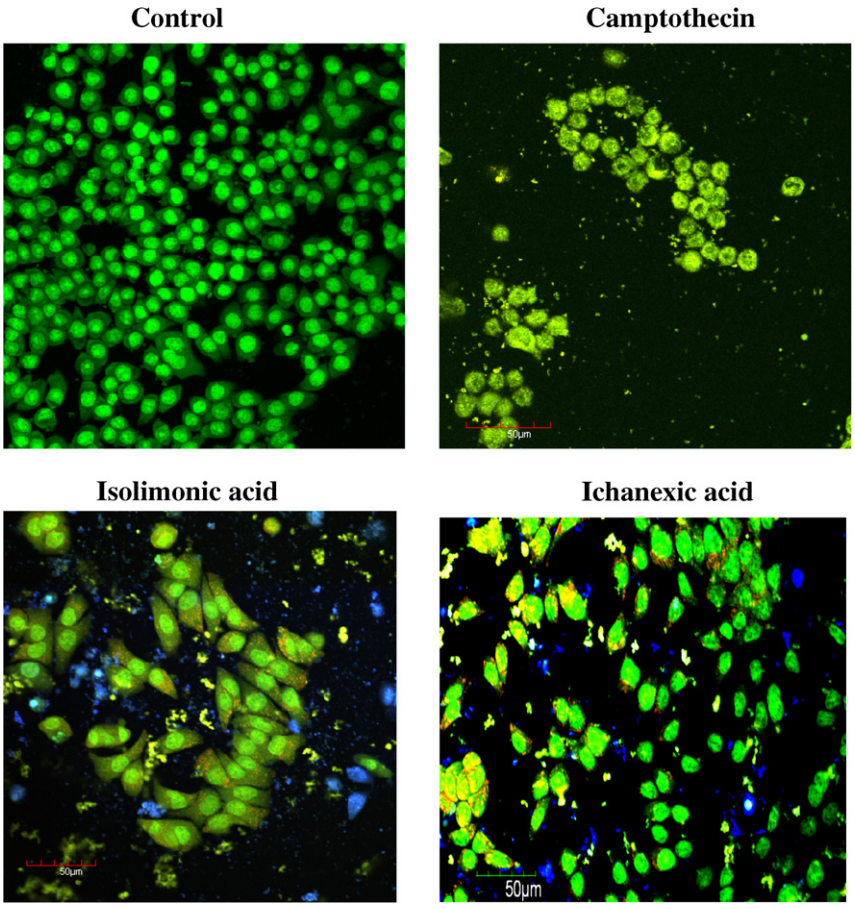

Figure 12. Effect of camptothecin and limonoids on apoptosis of HT-29 cells at 10 µM concentration. Morphological changes of nuclear chromatin were viewed under a fluorescence microscope. The nuclei of control cells show round and homogeneous nuclei; whereas treated cells show condensed and fragmented nuclei. Data from a typical representative of three similar experiments. Magnification 100×.

Table 2. 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

	µM	% BAD	% G_0/G_1	% S	% G_2/M	% CV
Control	0	9.2	92.4	3.6	1.0	3.1
Isolimonic acid	5	34.8	91.0	4.0	6.1	6.0
	10	75.5	69.9	12.7	17.4	8.0
Ichanexic acid	5	42.9	91.3	4.1	8.7	4.0
	10	75.1	70.2	6.0	23.8	8.2

(Buchi, Switzerland) to obtain a viscous concentrate and freeze dried.

3.4. Purification

The freeze dried ethyl acetate fraction was re-extracted on a Soxhlet apparatus using DCM for 1 h. The DCM extract was separated and dried under vacuum. The dried extract (62.0 g) was loaded onto silica gel (100 cm × 35 mm) column. The column was washed thoroughly with 2 L of DCM and was eluted with a linear gradient solvent of 0.5% acetone in DCM to 70% acetone in DCM. Fractions (500 ml each) were collected and concentrated under vacuum (Buchi, Switzerland). The fractions containing same HPLC peaks were combined and further purified as mentioned above using silica gel column chromatography. Compounds **1** (26 mg) and **2** (105 mg) were eluted using DCM/acetone (8:2) and (1:1), respectively.

3.5. Identification

3.5.1. Thin layer chromatography (TLC) analysis. Compounds **1** and **2** were spotted on silica gel 60F-254 TLC plates and developed using $CHCl_3$/MeOH (80:20) as mobile phase. The developed plates were sprayed with Ehrlich's reagent (2% *N*,*N*- dimethyl amino benzaldehyde in EtOH) and were exposed in HCl gas chamber. Typical pink/reddish colored spots were visualized for limonoids.[3]The plates were further sprayed with 10% sulfuric acid in MeOH followed by heating at 100 °C for 10 min to detect any other impurities.

3.5.2. High performance liquid chromatography (HPLC) analysis. All the column fractions, compounds **1** and **2**, were subjected to HPLC analysis using Spectra System Model P-4000 (Thermo Separation Products, USA) equipped with a quaternary HPLC pump. A Waters Prevail C18 (Alltech, IL, USA) analytical column (15 cm × 4.6 mm I.D, 5 μ particle size) was used. The auto injection system (Spectra System AS 3000) consisted of a 100 μl sample loop. The detection was done by a UV 6000 LP wavelength detector at 210 nm. Compounds **1** and **2** were quantified using ChromQuest software. The gradient mobile phase consisted of (a) 3 mM phosphoric acid (b) acetonitrile at a flow rate of 1.0 ml/min. The elution program involved a linear gradient from 0 to 100% of solvent A to B in 0 to 30 min, 30 to 35 min, 100 to 0% B to A and isocratic run from 35 to 40 min followed by 5 min of equilibrium with 100% A.

3.6. Nuclear magnetic resonance experiments

Compounds **1** and **2** were identified by extensive analysis of ^{1}H and ^{13}C NMR experiments, which were performed on a Bruker Biospin Avance 400 NMR spectrometer (^{1}H frequency = 400.13 MHz, ^{13}C frequency = 100.62 MHz) at 298 K using 5-mm broad band inverse probe head equipped with shielded *z*-gradient and XWIN-NMR software version 3.5 using TMS as an internal reference. One-dimensional ^{1}H and ^{13}C spectra were obtained using one pulse sequence. ^{1}H spectra were also obtained 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 (QCD) sequences were also performed to aid the structure identification.[18] 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 (edited HSQC) and ^{1}H–^{13}C gradient enhanced heteronuclear multiple bond correlation (HMBC) experiments were performed. For DQF-COSY experiment, sweep width of 4800 Hz was used in both the dimensions and 512 FIDs were obtained with t_1 increments, each of 2048 complex data points. Number of transients and the relaxation delay used were 32 and 5.0 s, respectively. Phase sensitive data were obtained using TPPI method. For ^{1}H–^{13}C multiplicity edited HSQC and HMBC experiments, spectral widths of 4800 Hz and 24,000 Hz were used in ^{1}H and ^{13}C dimensions, respectively. 512 FIDs were collected with t_1 increments each of 2048 data points, 32 transients, and 2 s recycle delay. Phase-sensitive data for edited HSQC experiment were obtained using echo-anti echo mode whereas for HMBC, magnitude mode data were obtained. Resulting 2D data were zero filled to 1024 points in t_1 dimension and double Fourier transformed after multiplying by a squared sine-bell window function shifted by $\pi/2$ along both the dimensions. For stereochemical assignments of the protons, one-dimensional nuclear Overhauser enhancement (nOe) experiments were performed on both compounds. The nOe build up on the proximal protons was monitored from the difference spectrum of on- and off-resonance irradiation of individual proton signals.

3.7. Mass spectral data analysis

Mass spectrometric analyses were performed using ThermoFinnigan LCQ-DECA instrument (Thermo, San Jose, CA, USA). Conditions used were 450 °C vaporizer with 300 μl of flow of methanol with 5 μA current and 30 PSI sheath and 15 PSI aux.

3.8. Cell culture

Human HT-29 colorectal adenocarcinoma cells (HTB-38) and non-cancerous COS-1 monkey kidney fibroblast cells (CRL-1650) were purchased from American Type Culture Collection (Bethesda, MD, USA). The cells were grown at 37 °C with 5% CO_2, 90% relative humidity. HT-29 cells were grown in RPMI 1640 media 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.

3.9. Cell viability assay

HT-29 and COS-1 cells were cultured in 96-well plates in triplicates separately and in three different trials, at a density of 10^3 cells/well in 200 μl of medium. After the cells were grown to about 70% confluence, treatments were initiated by supplementing to get 1.25, 2.5, 5.0, and 10.0 micromoles of final media concentration of compounds **1**, **2**, and camptothecin in DMSO. The plates were incubated for 24 and 48 h. The final concentration of DMSO in culture medium was maintained at <0.005%. Camptothecin was used as a positive control. Viability of the cells was assayed based on the ability of the live cells to reduce MTT as previously described.[10] The cleavage product formazon was measured spectrophotometrically at 551 nm using a KC-4 microplate reader (BioTek Instruments, Winooski, VT). Briefly, after 24 and 48 h of incubation with the compounds, 20 μl MTT solution (5 mg/ml in PBS) was added to each well and incubated for 2 h at 37 °C in a humidified chamber with 5% CO_2. After the incubation period the cleaved product of MTT, namely, formazon, formed by the reduction MTT by the active mitochondria of the live cells, was released into the solution using cell lysis buffer (100 mM Tris–HCl, pH 7.4, 154 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% NP-40) and solubilized by adding 50 μl of DMSO. The absorbance of formazon was measured at 550 nm using an ELISA plate reader. Three different replicated trials were conducted for each compound and the results were averaged.

3.10. Assessment of apoptosis

Apoptosis assessment was performed using the acridine orange and ethidium bromide staining method,[27] which is based on the differential staining of viable and apoptotic cells in a mixture.[28] The 10^4cells/ml were grown (HT-29) in 8-well chamber glass culture slides and at

70% confluence. The cells were treated with 10 μM compounds **1**, **2**, and camptothecin, supplemented through the fresh media to get final concentration of 10 μmol. The plates were incubated at 37 °C for 4 h and stained with 1 μl of 1× working solution of nuclear stains consisting of ethidium bromide (500 μg) and acridine orange (100 μg) in 1 ml of PBS. After 10 min of incubation, the cells were observed under 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 bright orange color while the viable cells were bright green.

3.11. Cell cycle analysis 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 with Pucks EDTA. Based on a hemocytometer counts, 10^4 cells were subcultured into 24-well plates. After the cells reached nearly 90% confluence in the plates, the media (1.0 ml) were changed and isolimonoic acid, ichanexic acid, and camptothecin were treated to get 5 and 10 μmol concentration. 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). 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. Two hundred micro liters of 70% ethanol and 200 μl of PBS were added, gently mixed, and stored at −80 °C until further use. Before starting sample preparation for flow cytometer, the cells were centrifuged and supernatant was removed and washed with PBS buffer two times. The cell pellet was suspended in 1 ml of staining reagent (propidium iodide 1.25 ml + RNase 5 mg + sodium citrate 2.5 ml + Triton X 0.250 ml + water 18.5 ml). After 15-min incubation at 37 °C, the suspension was passed through a nylon filter and collected in tubes. DNA fluorescence readings were taken using 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 software version 3.1 (PMac). The relative distribution of 10,000 events for each sample was analyzed for background aggregates and debris (BAD), an indicator of apoptosis as well as G_1, S, G_2,M phases of the cell cycle.

3.12. Statistical analysis

SPSS computer software was used for statistical analyses. Mean values among treatment groups were compared by the ANOVA test. All tests were two sided. *P*-values $\leqslant 0.05$ were considered significant.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.04.063.

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