NMR structural characterization and inhibition of colon cancer cells by components of Citrus aurantium L.

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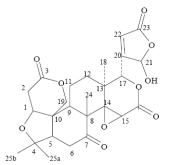
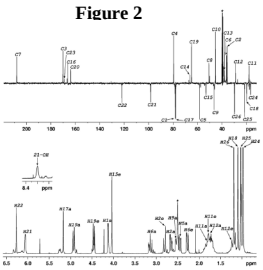
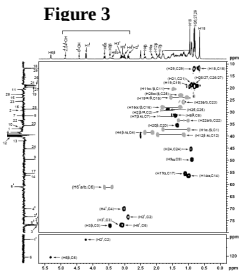
Introduction: Epidemiological studies suggest that dietary limonoids and phytosterols offer protection from some types of cancers (1). In the present study, cancer preventive components from sour orange (*Citrus aurantium* L.) were isolated and identified for the first time. Isolated compounds were tested for the inhibition of human colon cancer cells (HT-29) proliferation, apoptosis.

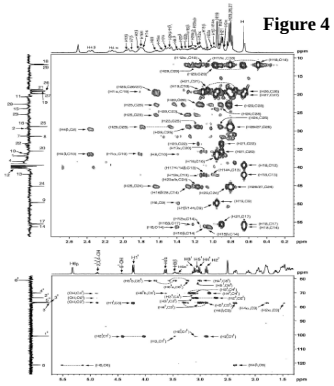
Methods: Sour oranges (*Citrus aurantium* L.) seed powder (4800 g) was extracted using a Soxhlet apparatus with hexane (15 L) for 24 h for the removal of fatty matter. The defatted powder was extracted for 8 h with ethyl acetate (15 L) at 60-70°C. Purification was carried out by column chromatography using dichloromethane and acetone in ratio 7:3 to obtain limonexic acid and sitosterol glucoside at 4:6. Both the compounds were subjected to HPLC analysis using gradient mobile phase (A) 3mM phosphoric acid and (B) acetonitrile. Cell viability, cell cycle analysis and apoptosis assessment was performed using MTT assay, flow cytometry measurements. and acridine orange and ethidium bromide staining under Olympus FV1000 confocal microscope. Two isolated compounds were characterized by extensive analysis of ^{1}H and ^{13}C NMR experiments 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. Two-dimensional NMR experiments such as ^{1}H-^{1}H DQF-COSY, edited ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC were performed to solve the structure of isolated compounds.

Results: Purity of the isolated compounds was analyzed by TLC and HPLC. Comprehensive analysis of ^{1}H and ^{13}C 2D NMR experiments of isolated compounds facilitated us to identify and assign the compounds as **limonexic acid and sitosterol glucoside** (**Fig. 1**). Typical ^{1}H and ^{13}C NMR spectra of limonexic acid with assignments is displayed in **Fig. 2**. Proton spectra of the compounds were complex with many overlapped peaks in the region 0.98 to 2 ppm (sitosterol glucoside) and 1.2 to 2.8 ppm (limonexic acid). In order to resolve the chemical shift values of CH and CH_2 protons from the overlapped region, isolated compounds were subjected to edited HSQC and SEFT experiments. Edited ^{1}H-^{13}C HSQC spectrum of the Sitosterol glucoside is shown in **Fig.3**. From the well resolved HSQC spectrum the cross peaks indicative of proton and respective carbon chemical shifts of CH groups (H3β C3), (H6β C6), (H8β C8), (H9α C9), (H14α C14), (H17α C17), (H20β C20), (H24 C24), (H25 C25) and CH_2 groups (H1α/β C1), (H2β/α C2), (H4β/α C4), (H7α/β C7), (H11α/β C11), (H12β/α C12), (H15α/β C15), (H16α/β C16), (H22 C22), (H23 C23), (H28α/β C28) are assigned to Sitosterol moiety of the Sitosterol glucoside. In the Glucoside moiety the chemical shifts (H1' C1'), (H2' C2'), (H3' C3'), (H4' C4'), (H5' C5') and CH_2 (H6'a/b C6') are clearly assigned to their positions in the structure. From HSQC spectrum of limonexic acid (data not shown) the proton and carbon chemical shifts from CH groups (H1 C1), (H5 C5), (H9 C9), (H15 C15), (H17 C17), (H23 C23) (H22 C22) and CH_2 groups (H2 C2), (H6 C6), (H11 C11), (H12 C12), (H19 C19) are assigned in the structure of limonexic acid. With the prior knowledge of individual proton and carbon chemical shifts from edited HSQC spectra, subsequently identification of sequentially connected protons of CH and CH_2 groups of both the compounds were achieved by extensive analysis of ^{1}H-^{1}H DQF-COSY spectrum. In sitosterol glucoside, the OH groups (2'-OH, 3'-OH, 4'-OH and 6'-OH) can not be identify in the edited HSQC spectrum are clearly showed correlation to their respective vicinal protons in DQF-COSY spectrum. ^{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, however their positions in the structure were not know. In order to trace the chemical shift of non-protonated carbon atoms, both compounds were subjected to quaternary carbon detection (QCD) experiments(2). Quaternary carbons C5, C10, C13 are assigned in sitosterol moiety and C5' is assigned in Glucoside moiety of sitosterol glucoside. The carbons C3, C4, C7, C8, C13, C14, C16, C20 and C21 are assigned in limonexic acid. Eventually the heteronuclear molecular connectivity (up to 3 bonds) of both the isolated compounds was achieved by ^{1}H-^{13}C HMBC experiments. All possible proton to carbon cross peaks are identified and assigned in HMBC spectrum of sitosterol glucoside (**Fig. 4**). In sitosterol glucoside the key correlation which connects the Sitostirol and Glucoside moiety is H3 to C1 (across oxygen) is identified and assigned in HMBC spectrum. The proton to carbon key correlations such as H17 to C20-C21, H19 to C3 (across oxygen), H17 to C16 (across oxygen) which skeletons the structure are clearly assigned in HMBC spectrum of limonexic acid. With the rigorous analysis of all the observed correlations from HSQC, DQF-COSY and HBMC data the structure of the two isolated compounds were framed unambiguously as sitosterol glucoside and limonexic acid.

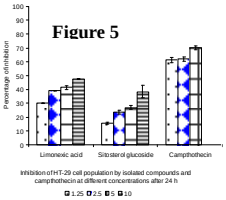


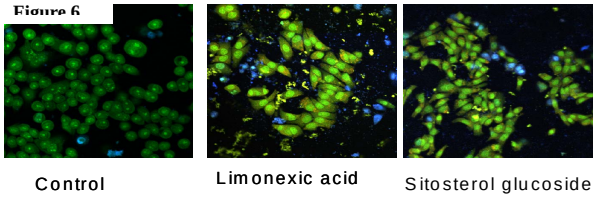






Anti proliferative effect of isolated compounds was studied on HT-29 colon cancer cell line for 24 h (**Fig. 5**), 48 and 72 h of incubation. Limonexic acid at 24 h of post treatment showed significant cytotoxic effect at low concentration as compared to untreated cells. However sitosterol glucoside showed significant cytotoxic effect only at 10.0 µM ($p<0.05$) concentration. The supplementation of both the compounds over a period of 72 h increased the cell death by another 20% at concentrations over 5.0 µM. Both the compounds exhibited significant effect on proliferation of HT-29 cells, mediated through the ability of the compounds to arrest certain stages of the cell cycle. Cell cycle analysis from Flow cytometry measurements unambiguously indicates, both the compounds have the ability to arrest DNA synthesis and G2/M phases of cell the cycle. Evidence of three to seven fold increase in the background aggregates and debris (% BAD: 34.8% and 42.9%) and G2/M: 6.1% and 8.7%), is a profound indicator of apoptosis. These results were further confirmed on confocal fluorescent microscopy (**Fig 6)**, where the DAPI nuclear staining indicated a loss of membrane integrity over the period of time of HT 29 cells.





Conclusion: Isolated compounds were unambiguously identified and assigned as limonexic acid and sitosterol glucoside by 1D and 2D HSQC, DQF-COSY and HBMC experiments. Both the compounds were found to be potent inhibitors of colon cancer cell line at the lowest concentration of 1.25 µM. Ability to arrest DNA synthesis and G2/M phases of cell the cycle is unambiguously confirmed by flow cytometry analysis. Based on MTT reduction results it is observed that both the compounds arrest the cell cycle significantly at 5µM concentration. **Reference:** (1) Jayaprakasha, et. al. Bio. & Med. Chem. (2007) 15, 4923-4932.(2) Jayaprakasha, et. al. Bio. & Med. Chem (2008) 16:5939-5951. (3) Nagana Gowda, G. A. Magn.Reson.Chem. (2001) 39, 581–585. This project was supported by the USDA-CSREES # 2007-34402-17121 "Designing Foods for Health" through the Vegetable & Fruit Improvement Center.