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Inhibition of colon cancer cell growth and antioxidant activity of bioactive compounds from *Poncirus trifoliata* (L.) Raf.

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Abstract—Recently several plant derived natural compounds have been screened for their anticancer activity in order to identify putative compounds with novel structures or mechanism of action. In the present study, fruits of *Poncirus trifoliata* were extracted with acetone and loaded onto silica gel column chromatography. The column was eluted with different solvents to obtain two bioactive compounds. The purity of compounds was analyzed by HPLC and their structures were identified by ^{1}H and ^{13}C NMR experiments as β-sitosterol and 2-hydroxy-1,2,3-propanetricarboxylic acid 2-methyl ester (HPCME). β-Sitosterol, HPCME, and trolox were tested for their antioxidant capacity by oxygen radical absorbance capacity (ORAC) measurement. Further, these compounds were tested for their inhibition of cancer cell proliferation and apoptosis using human colon cancer cell line (HT-29). These results were compared with the corresponding activity on non-cancerous (COS-1 fibroblast) cells. Cell proliferation and induction of apoptosis were determined by MTT assay and nuclear staining. The MTT assay indicated that both the compounds exhibited differential inhibition at various concentrations. Significant arrest of cell growth was observed with β-sitosterol even at low concentration such as 0.63 μM in 48 h and none of the compounds exerted any apparent cytostatic effects on the non-cancerous COS-1 fibroblast cells. Growth inhibition assay suggested the potential use of bioactive compounds as cancer chemopreventive and therapeutic agents. This is the first report on HPCME isolation and identification from Rutaceae family and β-sitosterol from *P. trifoliata*.

1. Introduction

Investigations into the health maintaining properties of fruits and spices have resulted in the identification of a wide array of bioactive compounds in plants that include flavonoids, phenolics, limonoids, carotenoids, coumarins, phytosterols, etc.[1–5] Consumption of fruits and vegetables is associated with the protection or prevention from several ailments such as chronic heart diseases, cancer, diabetes, and osteoporosis, etc.[6–11] The plant family Rutaceae, consisting of several genera such as *Citrus, Poncirus, Fortunella, Microcitrus, Eremocitrus* and *Clymenia*, is regarded as an excellent source for the bioactive compounds. Commercial genus of *Citrus* alone contains at least 170 bioactive compounds including limonoids, flavonoids, vitamin C, folic acid, potassium, and pectin that are documented to possess health promoting characteristics.[2,4,9,12–14]

Based on the recent research, it is clear that several compounds from fruits and vegetables were found to posses anticarcinogenic and antioxidant activities. Furthermore, flavonoids and carotenoids have also been shown to inhibit cancer cell proliferation in vitro.[15,16] Antioxidant activity by scavenging of reactive oxygen species is important in preventing potential damage to cellular components such as DNA, proteins, and lipids. Such an oxidative damage can cause major events such as carcinogenesis. Vast majority of epidemiological studies have demonstrated protective effects of vitamin C,[17] while plasma carotenoids have also been found to inversely correlate with indices of lipid peroxidation and oxidative DNA damage.[18]

Traditionally, trifoliata oranges (*Poncirus trifoliata*) have been widely used in folk medicine as a remedy

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for gastritis, dysentery, inflammation, digestive ulcers, etc. A scientific investigation into the health maintaining properties of trifoliata orange fruit has revealed its anti-inflammatory, anti-bacterial, and anti-anaphylactic activities.[19,20] Recent in vitro studies conducted by Yi et al.[21] suggested *Poncirus* fruit to be a potent anti-leukemic agent by promoting apoptosis of cancer cells. Several compounds such as poncirin, coumarins, auraptine, hesperidin, and naringin have been identified from poncirus fruits.[22,23]

Phytosterols or plant sterols are the complement of cholesterol in animals. They exist in the diet in many forms: the two most abundant ones among these being β-sitosterol and campesterol. Several in vitro and animal studies suggest that plant sterols offer protection from the most common cancers in the developed countries including colon, prostate, and breast.[24,25] It has been shown that dietary consumption of plant sterols is lower in developed countries (80 mg/day) as compared to the consumption in developing countries (400 mg/day) which seems to be the possible reason for lower incidence of cancer or cancer related deaths in developing countries.[26] Talapatra et al.[27] have first reported the isolation of 2-hydroxy-1,2,3-propanetricarboxylic acid 2-methyl ester (HPCME) from the fruits of *Rhus parviflora*. However, until now there has been no report on the isolation and identification of HPCME from Rutaceae. In this study, we have focused on the isolation and identification of bioactive compounds from *P. trifoliata*. The isolated compounds were assayed for their biological activities on colon cancer cells (HT-29). Potential of these isolated compounds on cell proliferation and induction of apoptosis were tested along with their oxygen radical absorbance capacity.

2. Results and discussion

The present study was conducted to isolate and evaluate the activity of putative bioactive compounds present in *Poncirus* fruits. Freeze-dried fruits of *Poncirus* were extracted with acetone and fractionated on column chromatography. The column fractions were pooled according to their TLC and HPLC profiles. The combined fractions were concentrated under vacuum and allowed to crystallize two compounds. The purity of compounds was confirmed by HPLC (Fig. 1). One and two-dimensional NMR spectroscopy was employed to elucidate the structures of the isolated compounds. Typical ^{1}H and ^{13}C NMR spectra of both the compounds with complete assignments of various signals are shown in Figures 2 and 3. ^{1}H and ^{13}C chemical shift assignments of both the compounds are given in Tables 1 and 2. On the basis of NMR results, the structures of compounds **1** and **2** were identified as β-sitosterol and 2-hydroxy-1,2,3-propanetricarboxylic acid 2-methyl ester (HPCME), respectively (Fig. 4). The structures and the NMR data obtained independently in this study are in close conformity with reported literature.[27,28] This is the first report on isolation and identification of HPCME from Rutaceae family and β-sitosterol from *Poncirus*. Further, this is the second report on isolation of HPCME from natural source so far.

The antioxidant capacity of β-sitosterol, HPCME, and trolox was determined by the ORAC-FL method. Figure 5 depicts the decay curves for purified compounds, trolox, and control at 10 μM concentration. In the case of control, equivalent amount of buffer was used in place of sample/trolox. The linearity between the net area under the curve (AUC) and the concentration was checked for both the compounds. Trolox equiva-

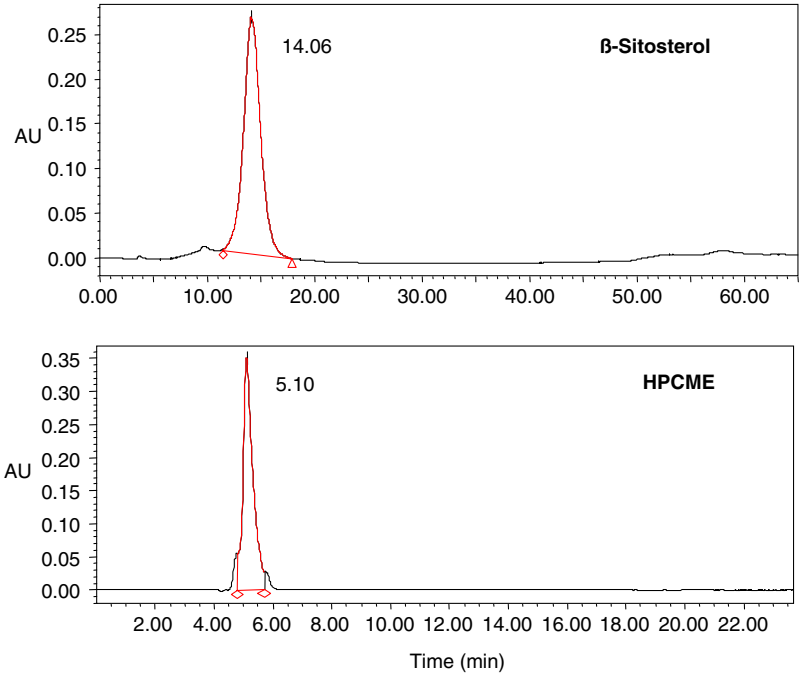


Figure 1. HPLC chromatograms of two isolated compounds from *Poncirus*.

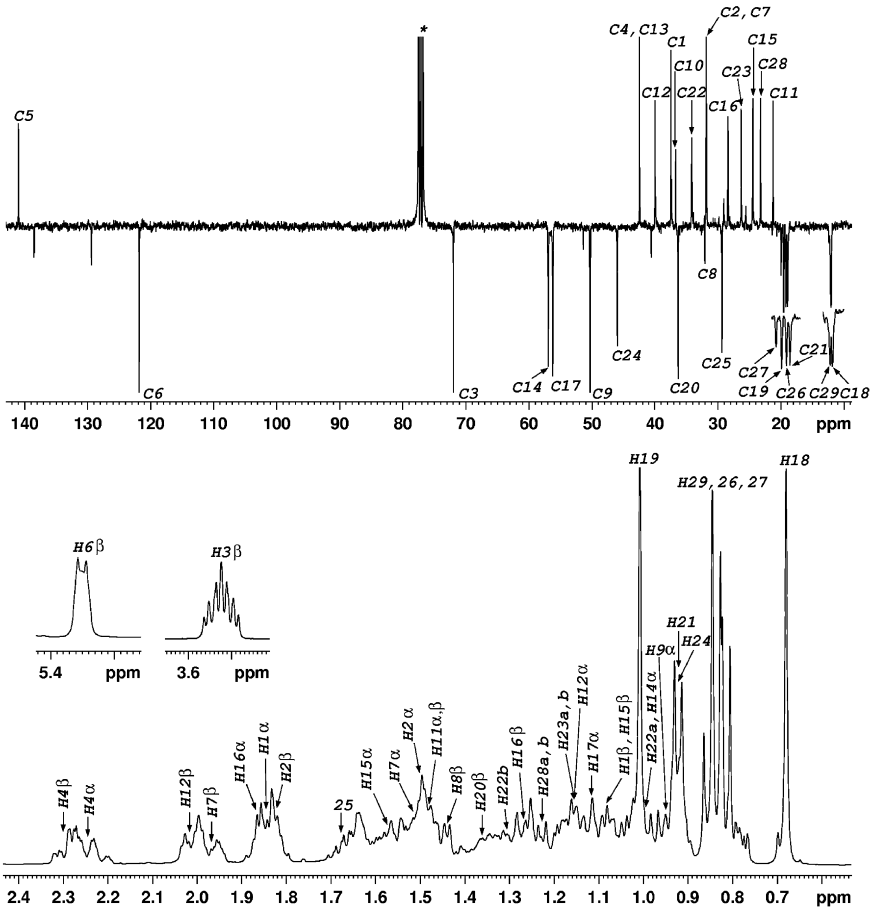


Figure 2. ^{1}H (bottom) and ^{13}C (top) NMR spectra of compound **1** (β-sitosterol) with the complete assignments of individual proton and carbon signals.

lents for the β-sitosterol and HPCME obtained from the ORAC-fluorescein assay have been presented in Figure 6. β-Sitosterol showed significantly more activity than HPCME. The ORAC assay depends on the free radical damage to a fluorescent probe through the change in its fluorescence intensity. The change of fluorescence intensity is an index of the degree of free radical damage. The inhibition of free radical damage by an antioxidant is reflected in the protection against the fluorescence change in the ORAC assay which is a measure of its antioxidant capacity against the free radical (Fig. 5).

The antiproliferative effects of both the compounds were measured in terms of the percent survivability of the HT-29 cancer cells using MTT reduction assay. The results indicated a considerable potential of both compounds inhibiting cancer cell growth even at very low concentrations. β-Sitosterol and HPCME showed noticeable inhibition at a concentration as low as 0.63 μM and significant inhibition was observed at 5 μM levels within 24 h ($P < 0.05$) (Fig. 7). These results clearly indicated a linear increase in the inhibitory effect over the period of 48 h and relative effects were found to be much stronger for β-sitosterol as compared to HPCME (Fig. 8). Figures 9 and 10 show clearly that β-sitosterol and HPCME had no inhibitory or toxic effects against the non-cancerous cells (COS-1) at four concentrations up to 48 h.

β-Sitosterol is structurally similar to cholesterol; its absorption has been reported to be 800–1000 times less than cholesterol. β-Sitosterol, found ubiquitously in plants, has been shown to have several beneficial bioactivities including anticarcinogenic properties demonstrated in human and animal studies.[29] In a skin carcinogenesis study using female ICR mice treated with DMBA and tumor promoter (TPA) to induce tumors, application of 0.005 mmol β-sitosterol topically resulted in a reduced incidence of tumor-bearing mice by 20% as compared to untreated mice.[30] Furthermore, studies in rats showed that inclusion of β-sitosterol in their diet decreased the occurrence of chemically induced cancers in the colon.[31] Moreover, β-sitosterol was shown to be effective for the treatment of benign prostatic hyperplasia.[32] The present study establishes the potential role of β-sitosterol and HPCME in the health maintaining properties of the *Poncirus* fruit. It is clear that *Poncirus* is a novel source for β-sitosterol and HPCME, in addition to several other bioactive compounds previously identified. The results augment the evidence of β-sitosterol as a potent anticarcinogenic agent. For the first time, the antiproliferative and apoptotic characteristics of the HPCME suggest that, this compound can be used

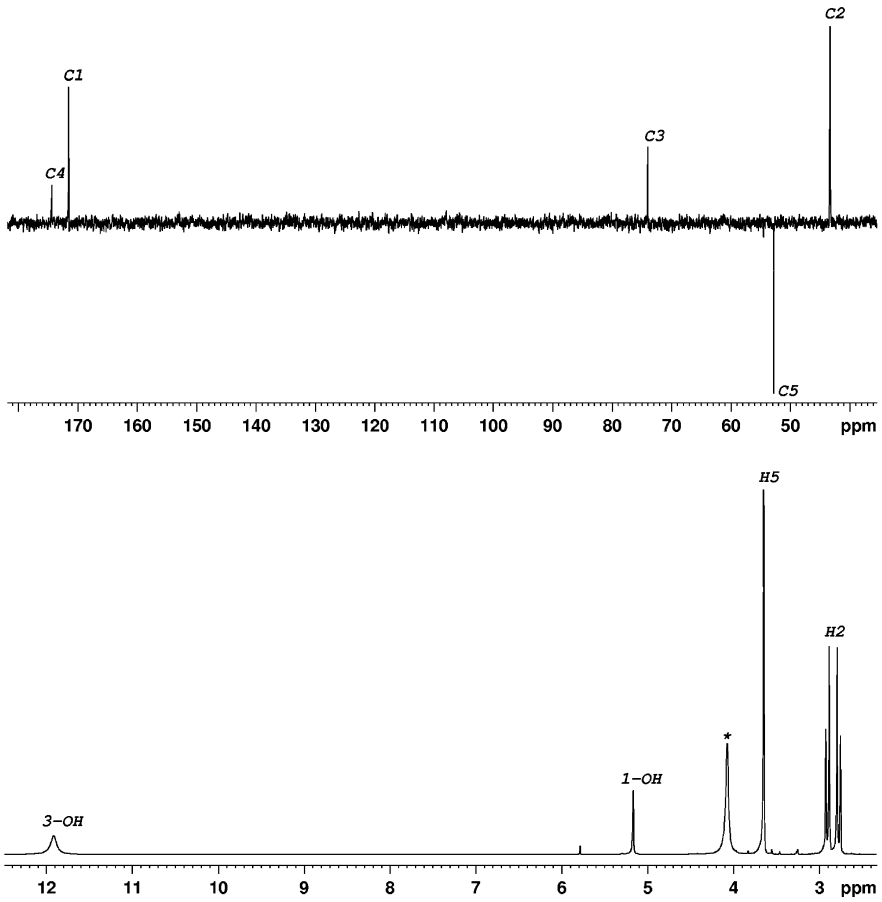


Figure 3. ^{1}H (bottom) and ^{13}C (top) NMR spectra of compound **2** (2-hydroxy-1,2,3-propanetricarboxylic acid 2-methyl ester) with the complete assignments of individual proton and carbon signals.

as potential strong chemopreventive agent after confirmation of similar results in animal and human clinical trials. The addition of HPCME to the list of several health maintaining compounds present in Rutaceae reinforces the importance of this group of fruits. Further, studies using biochemical approaches to elucidate the pathway/mode of action of these bioactive compounds using cancer cells are essential for their use in preventing cancer and related diseases.

Recently, the importance of botanicals as alternative remedies for disease prevention and therapy is being recognized due to several adverse repercussions of the use of conventional medicines such as side effects and therapeutic limitations besides high costs. At least 40% of American population is estimated to consider herbal medicines as alternate to conventional medicine.[33] *Poncirus* fruit plays an important role in traditional oriental medicine among the oriental countries including China, Korea, and Taiwan. A recent study conducted in South Korea by Yi et al.[21] showed an induction of apoptosis with *Poncirus* fruit extracts in human leukemia cells (HL-60) by triggering the caspase-3 enzyme activity. *Poncirus* fruit extracts are shown to exhibit anti-hepatitis-C virus activity and inhibit interleukin 4 (IL-4) dependent immunoglobulin E (IgE) production, resulting in anti-allergic activity.[19] The aforementioned beneficial effects of *Poncirus* fruits can be attributed to its bioactive compounds such as hesperidin, neohesperidin, limonene, naringin, poncirin, umbelliferone, linalool, auraptene, synephrine, and imperatorin,[34–36] Bennet and Hasegawa[37] reported the presence of a unique group of limonoids, 7α-oxygenated limonoids, in the seeds of *Poncirus*.

Further, in a pursuit of understanding the biological activities of the β-sitosterol and HPCME from *Poncirus*, we have measured apoptotic index, based on the ethidium bromide and acridine orange staining technique (Fig. 11). This figure depicts the effect of β-sitosterol, HPCME, and camptothecin on apoptosis of human colon cancer cells which were viewed under a fluorescence microscope. The nuclei of control cells showed round and homogeneous nuclei, whereas apoptotic cells from treated group showed condensed and fragmented nuclei. As predicted, the results showed a significant decrease in the number of viable cells that were treated with β-sitosterol and HPCME. Interestingly, the data revealed that both the compounds exerted more than 60% of the effect shown by camptothecin, a known potent anticarcinogenic drug, when monitored under identical conditions of concentration and treatment time. These results clearly supported that both the compounds are highly effective in inhibiting the cancer cell proliferation. We also believe that the apoptotic bodies visualized in our results are a specific effect of activation of the cell death

Table 1. ^{1}H and ^{13}C NMR chemical shifts of β-sitosterol (compound **1**) ($CDCl_3$)

No.	Carbon type	^{1}H	^{13}C
1	CH_2	1.84; 1.08	37.42
2	CH_2	1.50; 1.82	31.82
3	CH	3.52	71.97
4	CH_2	2.23; 2.27	42.48
5	C	—	140.93
6	CH	5.35	121.86
7	CH_2	1.514; 1.97	31.87
8	CH	1.43	32.07
9	CH	0.93	50.31
10	C	—	36.67
11	CH_2	1.49; 1.49	21.25
12	CH_2	1.16; 2.00	39.94
13	C	—	42.48
14	CH	0.99	56.93
15	CH	1.57; 1.07	24.46
16	CH_2	1.86; 1.27	28.39
17	CH	1.107	56.23
18	CH_3	0.68	12.02
19	CH_3	1.01	19.55
20	CH	1.36; 1.36	36.30
21	CH_3	0.92	18.93
22	CH_2	1.32; 1.00	34.12
23	CH_2	1.16; 1.16	26.28
24	CH	0.928	46.02
25	CH	1.66	29.34
26	CH_3	0.82	19.19
27	CH_3	0.84	19.90
28	CH_2	1.24; 1.24	23.24
29	CH_3	0.85	12.13

Table 2. ^{1}H and ^{13}C NMR chemical shifts of 2-hydroxy-1,2,3-propanetricarboxylic acid 2-methyl ester (compound **2**) (DMSO-d_6)

No.	Carbon type	^{1}H	^{13}C
1	C	—	171.5
2	CH_2	2.95; 2.81	43.3
3	C	—	74.1
4	C	—	174.4
5	CH	3.69	52.8

process in the cancerous cells by the treatment with either of the compounds and not just a mere overall toxic effect of the chemicals. Earlier reports on β-sitosterol indicated an antiproliferative activity in human cancer cells, exerted through the inhibition of DNA synthesis on the Ras dependent signaling pathway.[38] In human colonic adenocarcinomas, the β-sitosterol has been shown to reduce about 50% the membrane sphingomyelin at a concentration as low as 16 mM along with altered fatty acid composition of minor phospholipids and increase in the unsaturated index of the membranes.[39] These results are in agreement with our observations that these compounds exerted a profound effect on the membrane integrity, leading to the collapse of membrane and eventual death of the cancerous cells. In vitro studies have suggested that β-sitosterol will increase the production of ceramide which in turn activates protein phosphotase 2A (PP2A), ultimately resulting in the inhibition of cell proliferation and induction of apoptosis.[40]

Numerous studies were performed in determining the beneficial effects of several bioactive compounds, however the mode of action of these potent compounds including β-sitosterol remain elusive. Recent research insights into this area showed that β-sitosterol stimulates antioxidant enzymes through an estrogen receptor/PI3-kinase-dependent pathway.[41] While other classes of compounds present in *Poncirus* and related citrus species have been proven to possess anticancer properties, our findings provide considerable evidence for a further investigation into the importance of β-sitosterol and HPCME. Information obtained from such investigations on the mode of action of these compounds will further help to understand their anticancer properties and lead us to evaluate strategies in their eventual development as cancer preventive compounds.

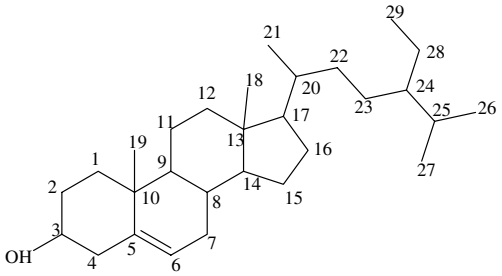
3. Materials and methods

3.1. Plant material

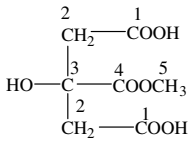
Poncirus trifoliata (L.) Raf. fruits were harvested in October 2004 from the orchard of Texas A&M University-Kingsville, Citrus Center, Weslaco, Texas, USA. Whole fruits were cut into sections and freeze-dried. The dried fruit material was then finely pulverized using a hand blender.

3.2. Chemicals

All solvents/chemicals used were of analytical grade and obtained from EM Science (NJ, USA). Silica gel (200–



ß-Sitosterol



2-Hydroxy-1,2,3-propanetricarboxylic acid 2-methyl ester

Figure 4. Structures of isolated compounds from *Poncirus*.

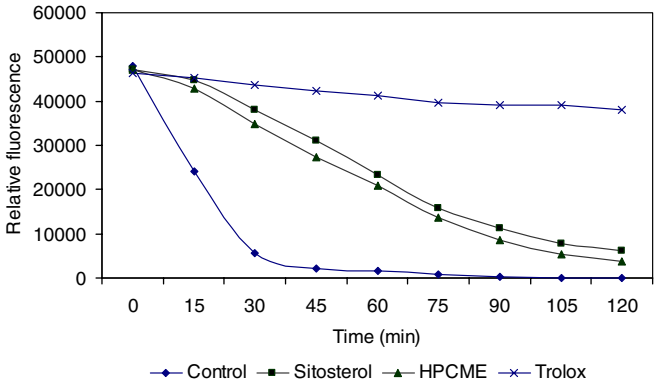


Figure 5. Time course of the reaction of fluorescein with AAPH in the β-sitosterol, HPCME, and trolox at 10 μM concentration.

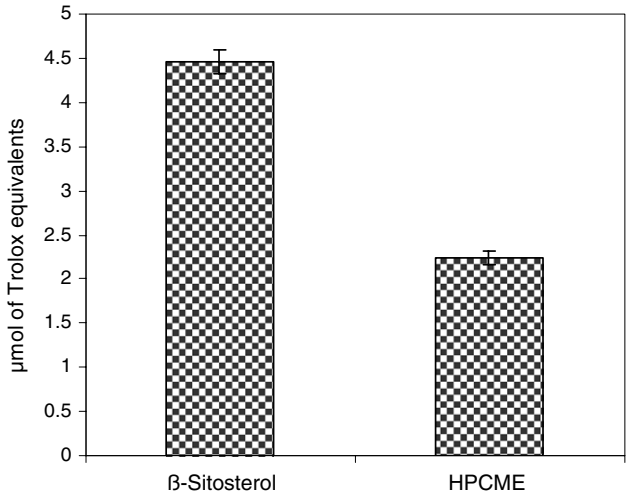


Figure 6. Trolox equivalents as measured by the ORAC-fluorescein assay.

400 mesh) was purchased from Aldrich (MO, USA). Fluorescein and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were obtained from Sigma (St. Louis, USA). TLC plate's Silica gel 60 F-254 (20 × 20 cm) was obtained from Alltech Associates, Inc. Trypsin EDTA, tetrazolium salt were obtained from Roche (Annapolis, MD, USA).

3.3. Extraction

Lyophilized fruit powder (700 g) was extracted with 3 L of acetone in a Soxhlet type extraction apparatus for 8 h at 60–70 °C. The extract was concentrated under vacuum (Buchi, Switzerland) to obtain a viscous liquid (60 g), which was stored at 4 °C until further use.

3.4. Purification

The concentrated extract (60 g) was impregnated with 50 g of silica gel and chromatographed on silica column (100 cm × 35 mm). The column was washed with 2 L of dichloromethane (DCM), mixtures of DCM and acetone with increasing polarity. Fractions (500 ml) were collected and analyzed by TLC. Fractions containing similar spots were pooled and further concentrated under vacuum. The yields of the two compounds (compound **1** and compound **2**) eluted with DCM (100%) and DCM: acetone (85:15) were 11.0 mg and 276.0 mg, respectively.

3.5. HPLC analysis

The purity of the compounds **1** and **2** was analyzed by HPLC using Spectra System Model P-4000 (Thermo Separation Products, USA) equipped with a quaternary HPLC pump. A Waters Prevail C18 analytical column (15 cm × 4.6 mm id, 5 μm particle size; Alltech, IL, USA) was used. The auto-injection system (Spectra System AS 3000) consisted of a 50 μl sample loop. An isocratic mobile phase of 40% acetonitrile in water for 30 min, with a flow rate of 1.0 ml/min, was employed for elution. A UV 6000 LP wavelength detector at 210 nm was used to detect the compounds. Both the compounds were quantified using ChromQuest software.

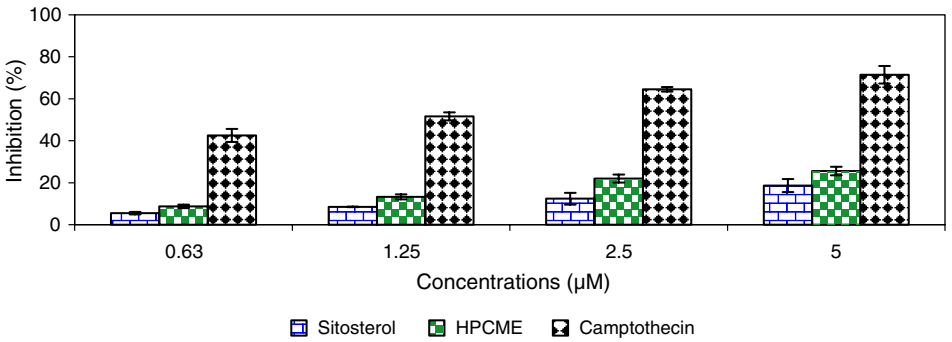


Figure 7. Percentage inhibition of HT-29 cancer cells by β-sitosterol, HPCME, and camptothecin after 24 h.

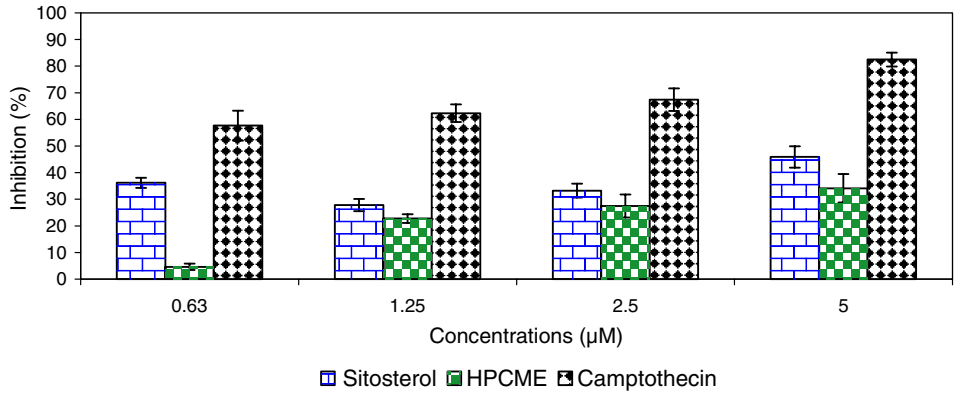


Figure 8. Percentage inhibition of HT-29 cancer cells by β-sitosterol, HPCME, and camptothecin after 48 h.

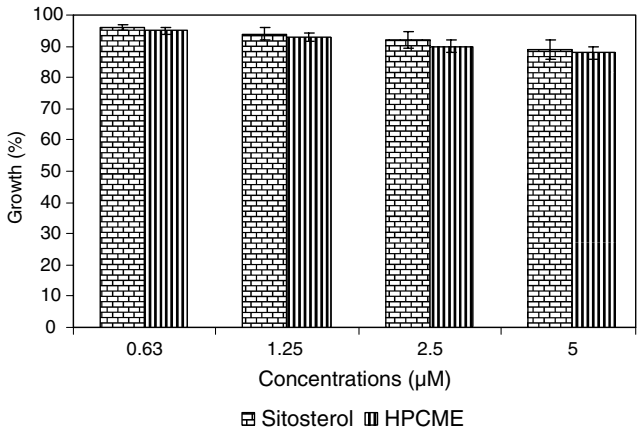


Figure 9. Percentage growth of normal cells (COS-1) by β-sitosterol and HPCME after 24 h.

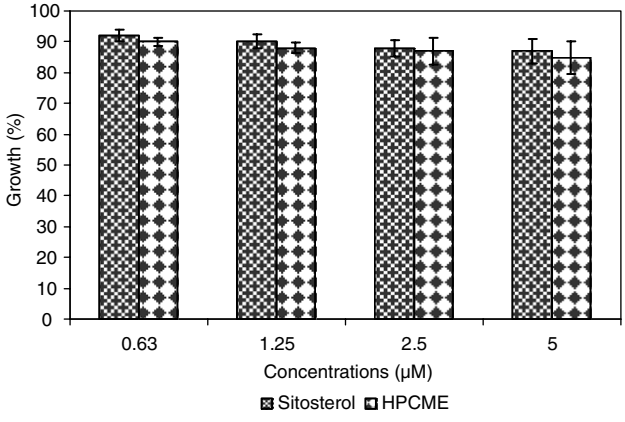


Figure 10. Percentage growth of normal cells (COS-1) by β-sitosterol and HPCME after 48 h.

3.6. NMR equipment and experiments

The structures of the compounds **1** and **2** were identified and confirmed by ^{1}H and ^{13}C NMR experiments using 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. One-dimensional ^{13}C spectra using Spin Echo Fourier Transform (SEFT) and Quaternary Carbon Detection (QCD)[42] sequences were also performed to aid the structure identification. Homonuclear and heteronuclear two-dimensional (2D) NMR experiments such as ^{1}H–^{1}H double quantum filtered correlation (DQF-COSY), sensitivity enhanced, and multiplicity edited ^{1}H–^{13}C heteronuclear single quantum correlation (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 free induction decays (FIDs) were obtained with t_1 increments, each of 2048 complex data points. Number of transients and the relaxation delay used were 32 and 2.5 s. 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. Five hundred and twelve 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.

3.7. ORAC-fluorescein assay

Antioxidant capacity was assessed by the ORAC-fluorescein assay with slight modification of the method presented by Ou et al.[43] Trolox standards (10–100 μM), fluorescein (7.0 μM), and AAPH (63 mM) solutions were prepared prior to use in phosphate buffer (75 mM, pH 7.4). Ten milllimolar solution of β-sitosterol and HPCME was prepared in chloroform, acetone, and trolox in phosphate buffer. Blanks solutions in triplicate were also prepared using corresponding solvents to serve as controls. Different volumes of β-sitosterol, HPCME, and blanks

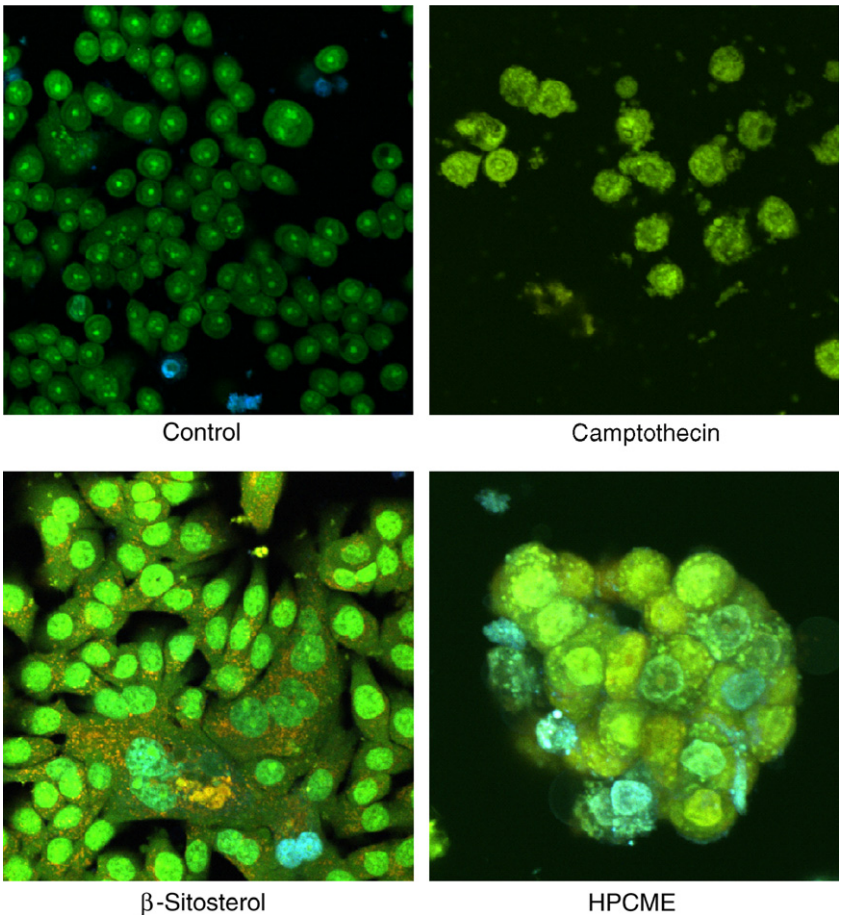


Figure 11. Effect of camptothecin, β-sitosterol, and HPCME on apoptosis of human colon cancer cells (HT-29) at 5 μM concentration. Morphological changes of nuclear chromatin were viewed under a fluorescence microscope. The nuclei of control cells showed round and homogeneous nuclei; whereas treated cells showed condensed and fragmented nuclei. Data from a typical representative of three similar experiments. Magnification ×100.

(10, 20, 30, and 40 μl) were placed in the well of a Costar 3631 assay plate (Corning Incorporated, Corning, NY) and fluorescein (200 μl) was added to each well. The total volume in each well was made up to 240 μl using buffer solution. The plate was incubated in the preheated (37 °C) microplate reader for 10 min with slow shaking and, subsequently, AAPH (20 μl) was added. The plate was top read at excitation and emission wavelengths of 485 and 535 nm, respectively, at 37 °C and at 3 min intervals for 60 min. The plate reader was controlled by KC4™ version 3.4 (revision 10) and area under the curve (AUC) was calculated using KC-4 instrument software. The standard curve was obtained by plotting trolox concentrations against the average net AUC of the three measurements for each concentration. The net AUC corresponding to a sample was calculated by subtracting the AUC of the blank. A standard curve was generated from the net AUC of the trolox standards and used to assign trolox equivalence values to the samples. Final ORAC values were calculated using the regression equation between trolox concentration and the net AUC and are expressed as micromol of trolox equivalents.

3.8. Cell culture

Human HT-29 colorectal adenocarcinoma cells (HTB-38), non-cancerous COS-1 monkey fibroblast cells (CRL-1650) were purchased from American Type Culture Collection (Bethesda, MD, USA). 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, incubated at 37 °C under 5% CO_2, and 90% relative humidity. 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 grown under similar conditions as similar to HT-29 cells.

3.9. Cell viability assay

HT-29 and COS-1 cells were cultured in 96-well plates in triplicates separately and three different trials, at a density of 10^4 cells/well. After 24 h the cells β-sitosterol, HPCME, and camptothecin with a series of concentrations (0.63,

1.25, 2.5 and 5 μM) for 24 and 48 h time intervals. Camptothecin was used as a positive control. Viability of the cells was assayed based on the ability of the live cells to reduce MTT tetrazolium salt as previously described.[8] The cleavage product formazon was measured spectrophotometrically at 551 nm using a microplate reader. Briefly, a 20 μl MTT solution (5 mg/ml in PBS) was added to each well after incubation with the compounds **1** and **2** for about 24 and 48 h. MTT was incubated for one h at 37 °C and the absorbance of the cleaved product formazon was measured at 550 nm using an ELISA plate reader. Three replications were used for each compound and cell viability tests were repeated for three times and the results were averaged.

3.10. Assessment of apoptosis

Apoptosis assessment was performed using the acridine orange and ethidium bromide staining method,[44] which is based on the differential staining of viable and apoptotic cells in a mixture of acridine orange and ethidium bromide.[45] The 10^4 cells/ml were grown (HT-29) in 8-well chamber glass culture slides. At 70% confluence the cells, 2.5 μl of 5 μM of β-sitosterol, HPCME, and camptothecin was treated and fresh media were added. The plates were incubated at 37 °C for 24 h and stained with 1–2 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 are bright green.

3.11. 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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