5-Geranyloxy-7-Methoxycoumarin Inhibits Colon Cancer (SW480) Cells Growth by Inducing Apoptosis

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Abstract

For the first time, three coumarins were isolated from the hexane extract of limes (*Citrus aurantifolia*) and purified by flash chromatography. The structures were identified by NMR (1D, 2D) and mass spectral analyses as 5-geranyloxy-7-methoxycoumarin, limettin, and isopimpinellin. These compounds inhibited human colon cancer (SW-480) cell proliferation, with 5-geranyloxy-7-methoxycoumarin showing the highest inhibition activity (67%) at 25 µM. Suppression of SW480 cell proliferation by 5-geranyloxy-7-methoxycoumarin was associated with induction of apoptosis, as evidenced by annexin V staining and DNA fragmentation. In addition, 5-geranyloxy-7-methoxycoumarin arrested cells at the G0/G1 phase, and induction of apoptosis was demonstrated through the activation of tumour suppressor gene p53, caspase8/3, regulation of Bcl2, and inhibition of p38 MAPK phosphorylation. These findings suggest that 5-geranyloxy-7-methoxycoumarin has potential as a cancer preventive agent.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction

Citrus aurantifolia L. Osbeck (Rutaceae) is commonly known as Mexican lime, key lime, or Kagzi lime. The fruits of this plant are rich in flavonoids, limonoids, vitamin C, coumarins, monoterpenes, and sterols [1–3]. Coumarins are phytoalexins, which are found in the Fabiaceae, Umbellifera, and Rutaceae families [4]. Coumarins are known for several biological properties such as anti-platelet-aggregating, anti-microbial, anti-mutagenic, and anti-tumor activities [5]. In addition, coumarins can inhibit oxygen radical generation in leukocytes [6]. Research from our laboratory and elsewhere has shown a potent inhibition activity against CYP3A4 and interference with the transport activity of membrane transporters [7, 8]. Recently, we have reported isolation and characterization of bergamottin, 6′,7′-dihydroxybergamottin and paradisin A, bergaptol and geranylcoumarin from grapefruit juice [7, 8]. The compound paradisin A was found to be the most potent CYP3A4 inhibitor with an IC_{50} of 1.2 µM followed by DHB and bergamottin [8]. The other coumarins isolated from grapefruit juice are epoxybergamottin and paradisin C [9].

Bioactive compounds from dietary sources are gaining recognition as important factors in reducing the risk of certain types of cancer and other chronic diseases [10]. Colon cancer is prevalent, especially in western countries, and is increasing worldwide due to rapid changes in dietary patterns and preferences. Continuing research seeks to identify novel sources, including dietary sources, of bioactive compounds that can prevent colon carcinoma [11, 12]. As one potential source of health-promoting bioactives, citrus fruits can provide multiple health benefits deriving from vitamin C and many other bioactive compounds. A number of studies have demonstrated that bioactive compounds from citrus fruits can induce cytotoxicity in different cancer cell lines by an apoptotic mechanism, as observed in human gastric cancer cells [13]. An animal study from our laboratory showed that freeze-dried grapefruit juice powder (13.7 g/kg) and its bioactive compound components naringin and limonin (200 mg/kg) are capable of inhibiting aberrant crypts in azoxymethane-challenged animals through the suppression of cyclooxygenase-2 (Cox-2) and inducible nitric oxide synthase (iNOS) [14].

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Citrus bioactives have also shown inhibition of proliferation of colon, breast [15–17], neuroblastoma [18], pancreatic [19], and prostate cancer cells [20]. In addition, citrus phytochemicals were shown to inhibit colon cancer cell proliferation in both cell culture and animal studies [21–23]. Coumarins from citrus also activated the detoxifying enzyme Nrf2, inhibited p-glycoprotein in cancer cells [24], showed anti-corpulence and tumour inhibition activities [25] and reduced chemically induced hepatotoxicity [26]. However, there is no comprehensive study on purification of putative bioactive compounds and examination of their mode of cytotoxicity on colon cancer cells.

For the first time, three coumarins were isolated from limes using flash chromatography, and their structures were confirmed by extensive NMR and mass spectral analysis. These compounds were tested for their effects on human colon cancer (SW480) cells, and biochemical studies were conducted to understand their possible mechanism of cytotoxicity.

Materials and Methods

▼

Chemicals

All solvents/chemicals were analytical grade and obtained from Fisher Scientific. Silica gel (200–400 mesh), and S-(+)-camptothecin (> 90% purity, Catalogue # C9911) were purchased from Sigma-Aldrich. TLC plates, silica gel 60 F-254, thicknesses 0.20 mm (20 × 20 cm), were obtained from Alltech Associates, Inc. Chemicals and media for cell culture were obtained from Hyclone. Anti-caspase3, caspase8, Bcl2, p53, pp53, ERK, pERK, p38, pp38, and *β*-actin were purchased from Santa Cruz Biotechnology. Anti-PARP was obtained from Cell Signaling Biotechnology. Annexin-FITC kit was obtained from BioVision Research Products, and fluorescent probes were from Molecular Probes (Invitrogen).

Plant materials

Mature limes (*Citrus aurantifolia* L. Osbeck) were harvested from the Citrus Centre, Texas A&M University-Kingsville (Weslaco, Texas, USA). The species was identified by Dr. Dale A. Kruse, and a voucher specimen (TAES Accession #255970) was deposited at the S.M. Tracy Herbarium, Texas A&M University (College Station, TX, USA). Peels were separated manually, dried under shade and powdered to obtain 40–60 mesh.

Extraction

Lime peel powder (500 g) was extracted using a Soxhlet extractor with hexane for 8 h. The extract was filtered, concentrated under vacuum to remove the solvent, freeze-dried and stored at − 20 °C until further use.

Purification of coumarins by flash chromatography

Lime peel hexane extract (25 g) was dissolved in 10 mL of chloroform, impregnated with 20 g of silica gel and air dried. The impregnated sample (45 g) was transferred to a loading cartridge. The cartridge was connected to a silica gel flash column (220 g, particle size 35–60 µm). The separation of coumarins was carried out on an automated Combiflash® RF flash chromatography system (Teledyne ISCO). First the silica column was equilibrated with hexane for 5 min. Coumarins were eluted with a binary gradient of hexane (A) and acetone (B), at a flow rate of 50 mL/min. The column was eluted as follows, 0–2 min (100% A), 2–4.5 min (100–68% A), isocratic for 4 min, and the polarity was increased to 100% B in 8 min and held as isocratic elution for 5 min. A total of 50 fractions (35 mL each) were collected. These fractions were monitored by eluting the analytes at 254 nm and 340 nm. Compounds **1, 2**, and **3** were obtained from fractions 33–34, 43–44, and 47–48, respectively. All column fractions were analysed by HPLC, and fractions containing same peaks were pooled and crystallized.

TLC analysis

Purified compounds were dissolved in chloroform and spotted on TLC plates, and hexane: ethyl acetate (4 : 1) was used as the mobile phase. The compounds were visualized as yellow-brown spots when sprayed with 10% sulphuric acid in methanol, followed by heating at 110 °C for 10 min.

HPLC analysis

High performance liquid chromatography analysis was carried out using an Agilent HPLC 1200 Series system. The compounds were eluted using a Zorbax-C18 column (250 × 4.6 mm I. D.) and detected at 240 nm with a flow rate of 0.7 mL/min. The column temperature was set at 27 °C. The elution was carried out with a gradient mobile phase with (A) 3 mM phosphoric acid and (B) acetonitrile, starting from 45% A to 10% A for 0–20 min, 10–25% A for 5 min, and 100% A for 5 min. All column fractions and purified compounds (**1**–**3**) were filtered through 0.45 micron filters and injected into the HPLC.

Identification by NMR and mass spectroscopy

The structures of the purified compounds (**1**–**3**) were identified by ^{1}H, ^{13}C NMR, and DEPT studies using a JEOL ECS-400 NMR spectrometer (JEOL USA, Inc.) at 298 K using a 5 mm broadband probe equipped with shielded z- gradient and Delta software version 4.3.6 with TMS as an internal reference. One-dimensional ^{1}H and ^{13}C spectra were obtained using one pulse sequence. One-dimensional ^{13}C spectra of distortionless enhancement of polarization transfer (DEPT-135) using a 135 degree decoupler pulse were also performed to determine the structures' identity. Homonuclear and heteronuclear two-dimensional (2D) NMR experiments, such as sensitivity-enhanced and multiplicity-edited ^{1}H-^{13}C heteronuclear multiple quantum correlation (HMQC) and ^{1}H-^{13}C gradient-enhanced heteronuclear multiple bond correlation (HMBC) experiments were also performed. For ^{1}H-^{13}C multiplicity-edited HMQC and HMBC experiments, spectral widths of 4800 Hz and 24 000 Hz were used in ^{1}H and ^{13}C dimensions, respectively. Mass spectral analyses were performed using ThermoFinnigan LCQ-DECA instrument (Thermo).

Cell culture and maintenance

Human colon adenocarcinoma (SW480) cells were obtained from ATCC and cultured in DMEM containing 10% FBS and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin). Cells attaining 70% confluence with normal morphological features were used for the experiments. Cells were maintained in a sterile biological incubator, maintained at 37 °C with 5% carbon dioxide and 85 ± 5% relative humidity. All the experiments were performed as three biological replicates with a minimum of three independent experiments for each treatment, concentration, and time point.

Assessment of cell viability using MTT assays

Cell proliferation was measured by MTT (Sigma) reduction via mitochondrial oxidation of live cells. The SW480 cells (1×10^{4}/well) were seeded into 96-well plates and incubated for 24 h.

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The attached cells were treated with either DMSO (vehicle control) or various concentration (12.5, 25, 50, 100, and 200 µM) of 5-geranyloxy-7-methoxycoumarin (geranyloxy-7-MOC), limettin, and isopimpinellin, as well as camptothecin (3.1, 6.3, 12.5, and 25 µM), and incubated for 24, 48, and 72 h. The concentration of DMSO in the samples was maintained below 0.02% to minimize the influence of the solvent on cell viability. Camptothecin was used as positive control. After incubation, cells with samples were treated with 10 µL of MTT reagent and incubated for 2 h. Then, DMSO was added to dissolve the formazan, and the intensity was measured at 570 nm using a microplate reader (Bio-Tek). Results were expressed as % inhibition of cell viability compared to respective concentration of DMSO treatment control.

Apoptosis detection by annexin V assay

The SW480 (1×10^5) cells were grown for 24 h, and the attached cells were treated with geranyloxy-7-MOC (50 µM), camptothecin (25 µM), or DMSO for 24 h. Annexin-V reagent (BioVision) was added to the cells, and the slides were incubated at 37 °C for 10 min according to the manufacturer's instructions. Supernatant medium along with excess dye was removed, and the cells were washed three times with DMEM without phenol red. Fluorescence images of these stained cells were captured using a Stallion digital imaging workstation under FITC filter using 20× objectives.

Cell cycle analysis by flow-cytometry

SW480 cells (1×10^6) were treated with 50 µM of geranyloxy-7-MOC for 24 and 48 h. The cells were harvested, fixed with 70% ethanol at − 20 °C for 24 h and then stained with 50 µg/mL of propidium iodide (PI). After staining, the cells were analysed by flow-cytometry (Becton & Dickinson). The percentage of cells in different phases of the cell cycle was evaluated using the ModFidLT V3.2 program.

DNA fragmentation

The SW480 cells (1×10^6) were incubated with either DMSO or geranyloxy-7-MOC at 50 µM for 24, 48, and 72 h. The cells were harvested by centrifugation, and DNA was isolated as in our recent publication [27]. The DNA was dissolved in TE buffer and loaded onto a 1.5% agarose gel for electrophoresis, then stained with ethidium bromide and visualized under UV light (LAS 4000; FUJI Film).

Western blot analysis

The SW480 cells (1×10^6) were seeded in a 100 mm plate with complete media overnight. The cells were treated with 50 µM of geranyloxy-7-MOC for 48 and 72 h, and cell lysates were subjected to Western blot as described previously [27]. Briefly, the protein concentration in cell lysates was measured using BCA assay (Pierce) with BSA (bovine serum albumin) as a standard. Approximately 30 µg protein-equivalent cell lysates was separated on 10% SDS-PAGE gel and blotted onto PVDF membrane (Bio-Rad). The membrane was then incubated with primary antibodies overnight at 4 °C. The blots were then incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 h. The antibody binding was visualized by HRP-chemiluminescent detection reagents (Amersham Biosciences) using LAS 4000 (FUJI Film). To reprobe blots, the membranes were stripped with Restore Western Blot Stripping buffer (Pierce) for 15 min, and the standard procedure for Western blotting was repeated.

Fluorescence microscopy

The SW480 cells (1×10^5) were incubated in chamber slides and treated with either DMSO or 50 µM of geranyloxy-7-MOC for 48 h. The cells were stained with 5 µM acridine orange (AO) and 5 µM PI for 10 min at 37 °C. Fluorescent images were visualized using a Zeiss Axiovert 200 M microscope.

Statistical analysis

All values are expressed as means ± SE. The data was analysed by ANOVA followed by Turkey's multiple comparisons using PASW 18.0 software. The data were considered significant at $p < 0.05$.

Supporting information

Original spectra for compounds **1**–**3** are available as Supporting Information.

Results

A hexane extract of lime peel was fractionated by silica gel flash chromatography to obtain three compounds. These compounds were observed as bluish-white fluorescent spots under UV light. Furthermore, the three compounds showed UV absorption maxima at 321 and 231 nm under UV light (366 nm), indicating the presence of a coumarin moiety. Purity of the isolated compounds (**1**–**3**) was found to be > 95% by HPLC, and chromatograms are presented in **Fig. 1S** (Supporting Information). The relative retention times of compounds **1, 2**, and **3** were found to be 19.2, 13.2, and 13.1 min, respectively. Furthermore, the yields of compounds **1, 2**, and **3** from lime peel were 92.6, 102, and 109 mg, respectively.

After confirming the purity of the isolated compounds by HPLC, NMR and EI-MS data were used to identify these compounds. Detailed NMR analysis, including ^{13}C (broadband decoupling and DEPT) and heteronuclear multiple quantum coherence (HMQC), allowed the assignments of all hydrogenated carbon atoms; HMBC (heteronuclear multiple bond correlation involving two $^2J_{CH}$ and three $^3J_{CH}$ bonds), and ^1H-^1H double quantum filtered correlation (DQF-COSY) were used for unambiguous assignments.

Proton NMR spectra of compounds **1, 2**, and **3** were complex with many overlapping peaks. Intense characteristic methyl signals were first tentatively assigned using 1D spectrum. Sensitivity-enhanced and multiplicity-edited HMQC spectra were used for the identification of methylene (CH_2) and methine (CH) protons. Assignment of all the carbons and attached protons was then made from the combination of DQF-COSY and HMBC experiments. Quaternary carbons were detected by comparing ^{13}C NMR and DEPT 135 spectra. All ^1H and ^{13}C chemical shifts were assigned completely to the three compounds, and spectra are presented in **Figs. 2S–7S**. NMR spectral assignments were unambiguously confirmed with 2D NMR, HMQC, and HMBC spectra. 1D and 2D NMR experiments allowed identification of compounds **1, 2**, and **3** as geranyloxy-7-MOC, limettin, and isopimpinellin, respectively (▶ **Fig. 1 A**). The isolated compounds were also analyzed for molecular weights, and compounds **1, 2**, and **3** showed [M + 1] ions at m/z 328.4, 206.08, and 246.07, respectively (**Fig. 8S–10S**).

To examine the anti-proliferative effects of isolated coumarins on SW480 cells, MTT assays were performed with different concentrations of geranyloxy-7-MOC, limettin, and isopimpinellin. Percent inhibition of colon cancer cell proliferation by treatment with coumarins indicated significant differences among the com-

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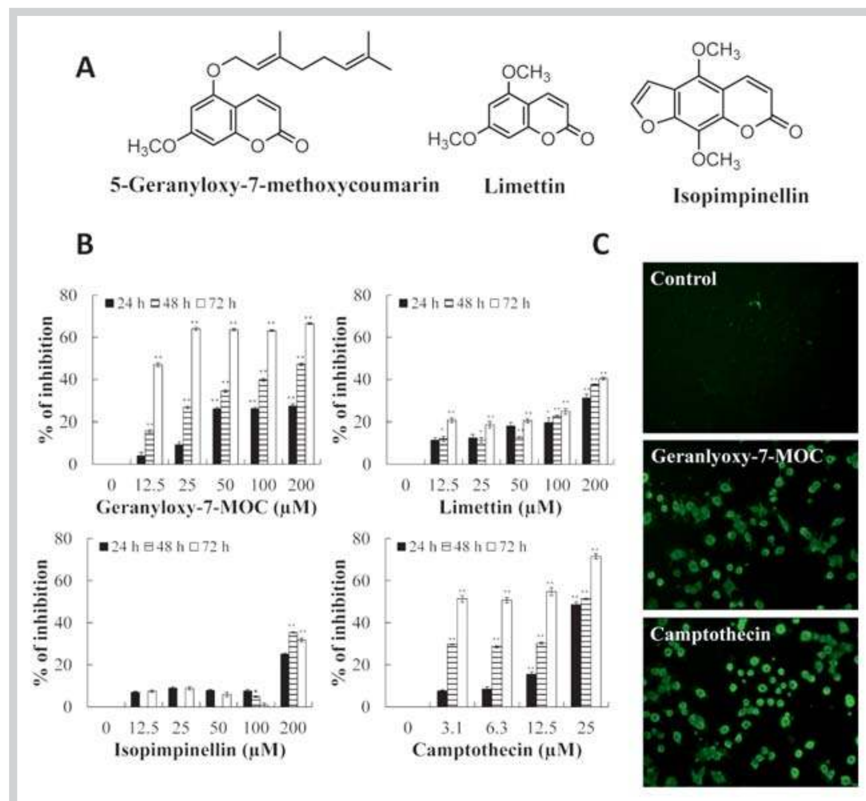

Fig. 1 Inhibition of SW480 cell growth by purified coumarins from lime. **A** Structures of identified compounds. **B** Different concentrations of coumarins and camptothecin (positive control) were incubated with SW480 (1×10^4) cells for 24, 48, and 72 h. The number of inhibited cells was determined by MTT assay. **C** Geranyloxy-7-MOC induces early apoptosis in SW480 cells as assessed by fluorescence-conjugated annexin V. The cells (1×10^4) were treated with DMSO (control), 50 µM of geranyloxy-7-MOC, and 25 µM of camptothecin (positive control) for 24 h. Apoptotic cells were detected by Zeiss Axiovert 200 M microscope.
(Color figures available online only.)

pounds at different concentrations. As shown in ◉ **Fig. 1 B**, all coumarins produced a significant inhibition of cell growth in a time-dependent manner. At 200 µM concentration, geranyloxy-7-MOC, limettin, and isopimpinellin inhibited cell growth by 67%, 41%, and 32%, respectively at 72 h. Particularly, 5-geranyloxy-7-MOC also produced a 64% decrease in cell survival at 25 µM after 72 h, but increasing the concentration to 200 µM had only a minor increase in its activity. Based on these results, geranyloxy-7-MOC was selected for further study.

To examine the mechanism of cytotoxicity, cells were treated with geranyloxy-7-MOC and then stained with annexin V to examine the translocation of membrane phosphatidylserine (PS) from the cytosol to the surface of the cells, which occurs soon after initiating apoptosis. The control (DMSO-treated) cells did not show annexin V fluorescence. However, geranyloxy-7-MOC-treated cells exhibited strong fluorescence around the cell surface (◉ **Fig. 1 C**). Camptothecin was used as positive control for induction of apoptosis.

To determine whether geranyloxy-7-MOC inhibits cell cycle progression of SW480 cells, the treated cells were stained with PI, and the distribution of cells in different phases of the cell cycle was analysed by flow cytometry. It was observed that the geranyloxy-7-MOC at 50 µM for 24 and 48 h altered the cell cycle distribution. In particular, the percentage of cells in the sub G1/G0 phase increased from 5.3% (in control cells) to 18% in geranyloxy-7-MOC treated cells, after 48 h incubation (◉ **Fig. 2**). This result indicated that geranyloxy-7-MOC is also capable of inducing apoptosis through cell cycle arrest.

Apoptotic progression of cells leads to distinct morphological characteristics, such as membrane blebbing and chromatin condensation [28]. To determine whether the inhibition of SW480 cell proliferation was due to apoptosis, geranyloxy-7-MOC-treated cells were stained with AO and PI and observed by fluorescence microscopy. The PI staining of cells treated with geranyloxy-7-MOC shows that the dye was able to penetrate the cells, which indicates a loss of cell membrane integrity (◉ **Fig. 3 A**). In addition, SW480 cells treated with 50 µM of geranyloxy-7-MOC showed fragmented DNA suggesting the induction of apoptosis (◉ **Fig. 3 B**). To determine the major genes involved in induction

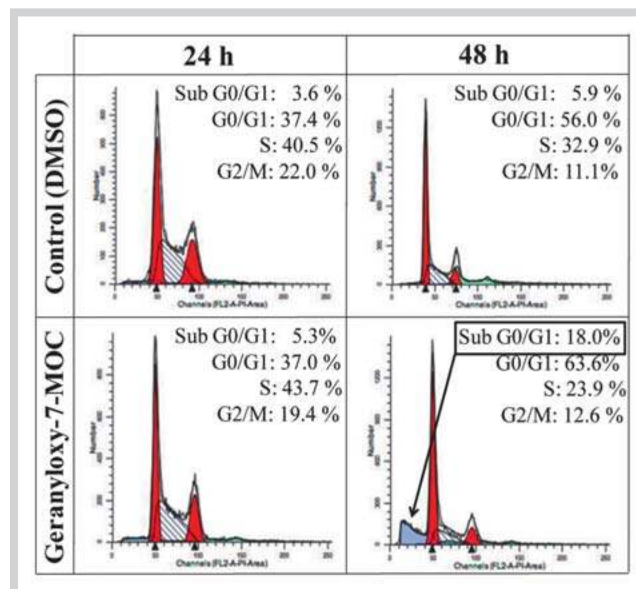

Fig. 2 Geranyloxy-7-MOC exhibits cell cycle arrest in SW480 cells. The incubated cells (1×10^6) with DMSO or compound (50 µM) were harvested, and DNA content was determined by flow-cytometry.
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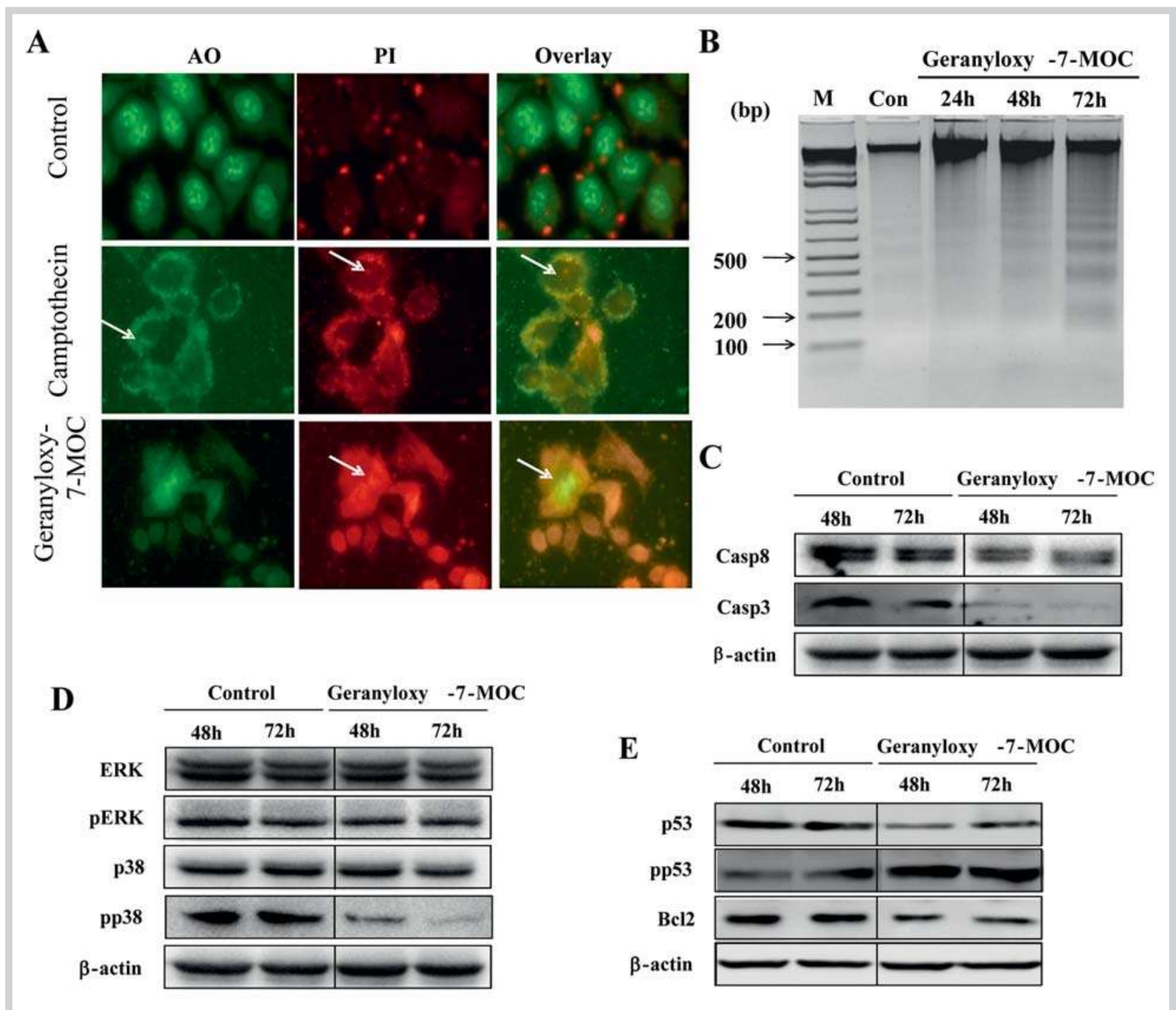

Fig. 3 Geranyloxy-7-MOC induces apoptosis in SW480 cells. **A** The cells (0.7×10^6) were incubated with DMSO (control), 50 µM of geranyloxy-7-MOC, or 50 µM of camptothecin (positive control) for 48 h, stained with acridine orange (AO) and propidium iodide (PI) and assessed by fluorescence microscopy. The fluorescence image was viewed using a Zeiss Axiovert 200 M microscope. **B** Geranyloxy-7- MOC promotes DNA fragmentation in SW480 cells. The incubated cells (1×10^6) with DMSO, geranyloxy-7-MOC (50 µM) were harvested after different incubation periods and analyzed by 1.5% gel electrophoresis. **C, D**, and **E** Inhibition of SW480 cell proliferation by geranyloxy-7-MOC was caused by apoptosis through inhibition of the p38 MAPK signaling pathway. The incubated cells (1×10^6) without or with geranyloxy-7-MOC (50 µM) were harvested after 48 h and 72 h and subjected to immunoblotting as explained in methods.
(Color figures available online only.)

of apoptosis, SW480 cells were treated with 50 µM of geranyloxy-7-MOC and incubated for 48 and 72 h. As shown in **Fig. 3 C** and **E**, geranyloxy-7-MOC treatment inhibited caspase 8 and Bcl2 protein expression and activated p53. In addition, the effector caspase 3 was suppressed by geranyloxy-7-MOC (**Fig. 3 C**). Furthermore, the cells exposed to geranyloxy-7-MOC demonstrated decrease in the phosphorylated form of p38 (remarkable change) and extracellular signal regulated kinase (ERK), compared with the total p38 and ERK (**Fig. 3 D**), indicating that the p38 MAPK might be the upstream signalling pathway to regulate p53. These results clearly demonstrate that geranyloxy-7-MOC inhibits SW480 colon cancer cell growth by inducing apoptosis.

Discussion

In the present study, two coumarins (geranyloxy-7-MOC and limettin) and a furanocoumarin (isopimpinellin) were isolated from lime peel using flash chromatography for the first time. There are numerous reports on the presence of coumarins in citrus fruits and other crops [29, 30]. However, very few studies have reported the purification of these compounds using flash separation, which is essential to understand the biological role of each compound and their contributions to health benefits.

Inhibition of cancer cell proliferation by any molecule usually involves either induction of programmed cell death or cell cycle arrest. Among the phytochemicals from dietary constituents, sulphoraphen and polyphenols including flavonoids and pigments [31, 32] have shown significant activity in colon cancer cell in an-

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imal models. Coumarins are the class of phytochemicals widely found in plants and are known for numerous biological activities [33]. Coumarins found in plants have shown significant inhibition of human lymphoblastoid, lung cancer [34], and skin cancer [35] cells. The major known modes of cancer cell inhibition by coumarins are through Bcl2 proteins [34], modulation of the cell cycle [36], inhibition of hormone receptors [37], and inhibition of cytochrome enzymes [38]. These examples motivated us to screen lime coumarins for their anti-proliferative activity.

Among the three coumarins tested for anti-proliferative activity, geranyloxy-7-MOC showed the highest inhibition activity, and the inhibition was found to be time-dependent. An inhibition of more than 60% of cell proliferation was observed at 25 µM after 72 h of incubation (**Fig. 1 B**). Furthermore, the inhibitions by both limettin and isopimpinellin were less than 40% after 72 h of treatment at 200 µM. This suggests that geranyloxy-7-MOC is a potent inhibitor of SW480 cell proliferation; hence we performed further experiments to examine the mode of toxicity using 50 µM of the compound.

Before understanding the possible mechanism, we have studied the possibility that apoptosis was involved through DNA fragmentation assays and annexin V staining in geranyloxy-7-MOC treated SW480 cells. Most of the cells showed translocation of phosphatidylserine to the outer plasma membrane (**Fig. 1 C**), which is one of the early events of apoptosis and a common technique to test for the occurrence of apoptosis [39]. Formation of distinct DNA fragments of 180–200 bp is one of the biochemical features of many cancer cells undergoing apoptosis [40]. Treatment with geranyloxy-7-MOC at 50 µM induced fragmentation of DNA (~ 200 bp) after 48 and 72 h (**Fig. 3 B**), but cleaved PARP was not observed (data not shown). Since the PARP cleavage is dispensable to activate caspase 3 [41], the results clearly suggest that inhibition of proliferation by geranyloxy-7-MOC involves apoptosis.

After confirming the involvement of apoptosis, we were interested in knowing the effect of geranyloxy-7-MOC on signalling molecules involved in apoptosis, i.e., caspase, tumour suppressor protein (p53), and the major anti-apoptotic protein Bcl2. A family of proteins known as caspases (cysteinyl aspartate-specific proteases) are key regulators during apoptosis [42]. In early stage of apoptosis, initiator caspases (including caspase 8, 9, and 10) are auto-activated, and the stimulation triggers a cascade of downstream caspases (called effector caspases, including caspase 3, 6, and 7) activation [42]. Previously, coumarins induced apoptosis by activation of mainly caspase-3 [43, 44]. Indeed, geranyloxy-7-MOC treatment of SW480 cells has demonstrated inhibition of caspase 3 through activation of caspase 8 (**Fig. 3 C**).

Dysfunction or alteration of the p53 activity is associated with most types of cancer. Normally, this is connected with activation of oncogenes, induction of DNA damage, hypoxia, etc. Since geranyloxy-7-MOC demonstrated induction of DNA fragmentation in colon cancer cells, the measurement of p53 expression and its activation (phosphorylated product, i.e., pp53) would also provide additional insight of the efficacy of the coumarins. Phosphorylation of p53 serves as one of the confirmatory components of DNA fragmentation. Based on these results of DNA fragmentation, cells treated with geranyloxy-7-MOC for 48 and 72 h were subjected to immunoblotting to know the expression of proteins. Results demonstrated the clear activation of pp53 after 47 and 72 h of treatment with geranyloxy-7-MOC compared to control (**Fig. 3 E**). This was evidenced from expression of p53 as well as its phosphorylated product in comparison with control cells. The p53 protein is one of the key tumour suppressors that induce cell death by coordinating cellular responses to various stress conditions [45]. A number of molecules capable of activating p53 have emerged as chemotherapeutic drugs [46, 47]. Therefore, the results of this study suggest the potential of geranyloxy-7-MOC as a cancer preventive. Although the abnormal functions have been studied *in vitro* and *in vivo* due to p53 mutation in SW480 cells, the transcriptional function is still unclear. Therefore, we could not rule out the possible p53 involvement as well as p53 independent signalling pathway in apoptosis by geranyloxy-7-MOC.

A number of studies on apoptosis demonstrated that kinases such as mitogen-activated protein (MAP) kinases (p38, ERK, JNKs) and phosphoinositide 3-kinase (PI3K)/Akt regulate p53-dependent apoptosis [48]. Geranyloxy-7-MOC induced significant inhibition of phosphorylated p38 MAP kinase at 50 µM concentration after 48 h incubation (**Fig. 3 D**). Phosphorylated ERK were also slightly activated by geranyloxy-7-MOC, but the effect was negligible compared to p38 (**Fig. 3 D**). The data clearly support that p53-dependent apoptosis by geranyloxy-7-MOC is mediated by inhibition of phosphorylated p38 MAP kinase.

The proteins of the Bcl2 family act as both pro-and anti-apoptotic factors, and the balance of these proteins is therefore critical in cancer prevention [49]. Among the anti-apoptotic proteins, Bcl2 is one of the key proteins in regulating apoptosis, and its expression is found to be higher in breast [50], colon, and most other organ-specific cancers [51]. Hence, down-regulation of Bcl2 is also one of the more successful targets of cancer chemoprevention. Results from the current study show down-regulation of Bcl2 after 48 and 72 h of geranyloxy-7-MOC treatment, suggesting the involvement of Bcl2 in apoptosis induction in response to this coumarin (**Fig. 3 E**). Several studies showing the inverse relationship between p53 and Bcl2 in cancer cells/tumours suggest that these two markers may target common death pathways [52]. This was also observed in non-small lung cancer [53].

In terms of structure–activity relationship, the presence of a geranyloxy side chain coupled to a sterically hindered group such as OCH_3, at position 3 of the aromatic ring seems to represent the best combination for COX inhibitory activities [54]. When such a group is not present in position 3, shortening the O-side chain to isopentenyl provided a better activity compared to compounds having a geranyloxy moiety in the para position. The presence of a medium sterical hindrance group like methyl or chlorine abolished the activity either in the presence of a C-10 or C-5 O-side chain [54]. Hori et al. [55] tested synthesized imidazoles with geranyl moiety and demonstrated high fungicidal activity in the *in vivo* efficacy test with experimental dermatophytosis. The inhibitory effect was reduced when the geranyl group of imidazole was replaced by a longer farnesyl or a shorter prenyl group. Saturation of two double bonds of the geranyl moiety reduced the inhibitory effect. On the other hand, hydroxy-7-methoxycoumarin derivatives were reported to be cytotoxic to the tumor cell line HSC-2 [56]. In the present study, the presence of a side chain with geranyloxy and methoxy group favors the attainment of maximum activity as compared to limettin and isopimpinellin. Considering the structural features, it may be possible that the geranyloxy group at the 5th position is critical for the proliferation inhibition activity of coumarins (**Fig. 1 A**). However, further studies with derivatives are essential to understand the structure-function relation.

For the first time, three coumarins were purified and characterized from lime peel using flash chromatography. The purity of these compounds was confirmed by HPLC, and the structures

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were confirmed by 1D, 2D NMR and mass spectral analysis. Among the three compounds, geranyloxy-7-MOC showed significant inhibition of SW480 cell proliferation. Furthermore, activation of p53 and caspase 8/3 and negative regulation of Bcl2 and p38 MAPK phosphorylation were associated with induction of apoptosis in SW480 colon cancer cells. To the best of our knowledge, this is the first report to explain the role of geranyloxy-7-MOC in inducing apoptosis in human colon cancer cells.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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