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Targeting apoptosis via inactivation of PI3K/Akt/mTOR signaling pathway involving NF-κB by geraniin in HT-29 human colorectal adenocarcinoma cells

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Abstract : Dietary phytochemicals possess a variety of biological activities which can be widely discovered in fruits, vegetables and herbs. Geraniin, an ellagitannin is commonly found in fruits and herbs which possesses multitude of health benefits including anti-diabetic, hepatoprotective, anti-inflammatory and anticancer. However, the effects of geraniin on human colorectal adenocarcinoma cells have yet to be evaluated. This study aimed to investigate the apoptotic effects of geraniin against selected human cancer cell lines and elucidate its underlying mechanisms. Geraniin exhibited the strongest cytotoxic effect on HT-29 cells as determined by MTT assay. Events of apoptosis induced by geraniin in HT-29 cells was portrayed by apoptotic morphological changes, externalization of phosphatidylserine, DNA fragmentation and dissipation of mitochondrial membrane potential. Western blot analysis was then used to investigate the mechanisms underlying observed growth inhibition. Geraniin was found to initiate p53 activation further resulting in elevation of Bak/Bcl-xL ratio and caspase-3 activation. This eventually led to HT-29 cell death via apoptosis. Additionally, exposure of geraniin on HT-29 cells found to suppress the PI3K/Akt/mTOR pathway and suppression of NF-kB. Cumulative evidences in this study suggests that geraniin inhibited colorectal adenocarcinoma cell proliferation via apoptosis induction and suppression of PI3K/Akt/mTOR pathway. Thus, these findings provide novel mechanistic insight for the therapeutic potential of geraniin in the treatment of colorectal adenocarcinoma.

Keywords: Geraniin; apoptosis; colon cancer; PI3K/Akt/mTOR; NF-kB

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Introduction

Carcinogenesis is maneuvered by the loss of the balance between cell death and survival which is often associated with the mutation of oncogenes or tumor suppressor genes. The perturbation in the equilibrium of the cell death process is closely related to tumorigenesis and chemotherapy resistance[1]. Copious findings have shown that natural products or natural-derived agents targeting the programmed cell death which has no effect on normal cells are a potential anticancer drug. There are several programmed cell death modalities, for instance apoptosis, necrosis and autophagy[2,3]. Accumulating evidence has reported that chemotherapy kills cancer cells primarily by induction of growth arrest and apoptosis[4]. Apoptosis which can be typified by nuclear shrinkage, DNA fragmentation, chromatin condensation, phosphatidylserine externalization and dissipation of mitochondrial transmembrane potential is executed either through activation of death receptors or mitochondria-dependent signaling[2]. It can be governed by a myriad of apoptotic proteins including Bcl-2 family proteins, p53 and NF-kB[5,6].

Dysregulation of phosphoinositide-3-kinase (PI3K)/Akt/mTOR signaling machinery which regulates hordes of cellular processes including cell survival, growth and proliferation, migration, and angiogenesis has been associated with 60-70% of colon tumorigenesis[7,8]. Therefore, targeting the PI3K/Akt/mTOR signaling pathway has become an attractive therapeutic strategy against cancer. PI3K activation results in recruitment of Akt and mediated by mTOR which in turn activates phosphorylation of Akt and eventually promotes cell survival[9,10]. Activated Akt constitutively activates other downstream mediators such as NF-κB, p53 and Bcl-2 family members. Activation of NF-κB is frequently involved in the development of various cancers including colon cancer[5,11]. It is well documented that NF-κB, a pro-inflammatory transcription factor, promotes cell proliferation, inhibits apoptosis and facilitates metastasis[12,13]. Activated Akt phosphorylates IκB, thus releasing NF-κB dimers for translocation to the nucleus[5]. P53 tumor suppressor protein regulates cell cycle arrest and apoptosis in response of DNA damage[14]. P53 and Bcl-2 family proteins can be crucial downstream mediators of PI3K/Akt signaling. AKT directly phosphorylates MDM2, enhances MDM2-mediated ubiquitination and degradation of p53 which abrogates apoptosis[15]. Hence, inactivation of this pathway is censorious in targeting cancer. Additionally, p53 can directly promote mitochondrial outer membrane permeabilisation to trigger apoptosis by modulating the Bcl-2 family protein expression[16].

Increasing evidence has demonstrated that flavonoids exert anti-cancer[17], anti-inflammation[18], antiviral and anti-oxidant[19] activities. Geraniin ($C_{41}H_{28}O_{27}$), a member of the ellagitaniin subclass of flavonoids, is found in fruit sources, traditional medicinal plants, nuts and vegetables, including*, P. niruri, P. amarus, N. lappaceum* and *G. thunbergii*[20-23]. Recently, geraniin has been reported in abundance in *P. urinaria Linn and N. lappaceum.* In the past, *P. urinaria* is traditionally used as anti-inflammatory, hepatoprotective and anti-diarrheal medicines in Asian countries. In Thailand, this plant is practiced as an adjuvant or alternative medicine for cancer patients, including liver cancer. Numerous scientific evidences have demonstrated and further verified the ethnomedicinal properties of geraniin including anti-HIV activity, hepatoprotective[24], anti-HSV-1 and HSV-2 activities[25], and anti-tumor effect[26]. Despite the numerous beneficial roles of geraniin, the effect of geraniin on human colorectal adenocarcinoma cells so far has not yet been reported and its mechanisms remain an enigma. Therefore the present study is to investigate the anti-proliferative effect of geraniin in human colorectal cancer cells, and elucidate the underlying molecular mechanisms.

Materials and Methods

Materials

The reagents for cell culture were from Sigma (St. Louis, MO, USA) and PAA lab (Pasching, Austria). Geraniin, (>98% purity) was purchased from Chengdu Biopurify Phytochemicals Ltd. MTT (3-(4,5-dimetylthiazol-2-yl)-2,5-dipheny-tetrazoliumbromide), RPMI-1640 medium, Hoechst 33342 and TUNEL assay kit were obtained from Sigma (St. Louis, MO, USA). Antibodies of Akt, p-Akt (Ser473), mTOR, p-mTOR, p-p65, lamin B, GAPDH and β-actin were purchased from Pierce Thermo Scientific (Rockford, IL, USA) whereas pro-caspase-3, Rictor, Bak and Bcl-xL were from Cell Signaling (Beverly, MA, USA).

Methods

Cell Culture

HT-29, HCT116, Ca Ski, A549 and Jurkat cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in RPMI-1640 medium supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS), 100 µg/mL penicillin streptomycin and 50 µg/mL amphotericin B. The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. The viable cell count was done by using tryphan blue exclusion assay with haemocytometer.

In vitro MTT Cytotoxicity Assay

To investigate the cell viability, viable cells were seeded to 96-well plates and incubated overnight prior to the treatment with different concentrations (1.56-100.00 µM) of geraniin. Untreated cells (negative control) were treated with vehicle dimethyl sulfoxide (DMSO). After 72 h incubation, MTT (5 mg/mL) was then added and incubated for 4 h at 37°C. After 4 h incubation, the medium was gently discarded and replaced by 150 µL of DMSO to dissolve the formazan crystals. The amount of formazon product was read at the absorbance of 570nm and 650nm as a background by microplate reader (Asys UVM340, Eugendorf, Austria). The percentage of cell viability was calculated according to the following formula: Percentage of cell viability (%) = (Absorbance of treated cells/absorbance of untreated cells) × 100%.

Nuclear Morphology Detection using Hoechst 33342/PI

HT-29 cells (1×10^6 cells) were plated in 60 mm^2 culture dishes. After 24 h incubation, the cells were treated with different concentrations (18, 36 and 72 µM) of geraniin while negative control was treated with vehicle DMSO for 24 h. After 24 h, the cells were harvested and washed with PBS followed by Hoechst 33342 (40 µg/mL) and propidium iodide (10 µg/mL) staining at room temperature in the dark for 30 min. Subsequently, the cells were observed under fluorescence microscope (Leica DM1600B, Wetzlar, Germany).

Detection of Externalization of Phosphatidylserine by Annexin V and PI Staining

HT-29 cells (1×10^6 cells) were treated with different concentrations (18-72 µM) of geraniin while negative control was treated with vehicle DMSO. Doxorubicin (2 µg/mL) was used as the positive control. Thereafter, cells were harvested, washed twice with PBS and proceed to detect the externalization of phosphatidylserine. Annexin V (BD) and propidium iodide (PI) (50 µg/mL) were added and incubated for 15 min in dark at room temperature. Subsequently, 1x binding buffer (BD) was added into

each tube and analyzed by Accuri C6 flow cytometry using quadrant statistics for apoptotic and necrotic cell populations. The fluorescence intensity was detected in FL1-A (x-axis) and FL2-A channel (y-axis).

Assessment of Mitochondrial Membrane Potential

The alteration in mitochondrial membrane potential (ΔΨm) was assessed by using the fluorescent cationic dye, JC-1. After 24 h treatment with geraniin, the cells were harvested, washed and stained with medium containing JC-1 for 15 min at 37°C. Subsequently, the cell suspension was centrifuged, washed and re-suspended in the medium. The cells were subjected to flow cytometry analysis by detecting the green and red fluorescence signals in FL1-A and FL2-A channels respectively.

Terminal Deoxynucleotidyl Transferase UTP Nick End Labeling (TUNEL) Assay

For detection of DNA fragmentation, a TUNEL assay was carried out following the protocol provided by the manufacturer (Sigma). Geraniin-treated cells were harvested, and fixed with 1% (w/v) paraformaldehyde in PBS on ice for 15 min. The cells were washed and incubated in DNA labeling solution [containing terminal deoxynucleotidyl transferase enzyme, bromodeoxyuridine (BrdU), and TdT reaction buffer] for 1 h at 37 °C. The cells subsequently incubated with FITC-conjugated anti-BrdU antibody for 30 min at room temperature in the dark following by the addition of the PI/RNase A solution to the cell for 30 min incubation in the dark. The cells were then analyzed by using flow cytometry and the fluorescence intensity in X-axis and Y-axis were detected in FL1-A and FL2-A channel respectively.

Determination of p53 Tumor Suppressor Protein Expression Level

HT-29 cells (1×10^6 cells) were treated with 36 µM of geraniin for 6, 12, 18 and 24 h. Negative control was treated with vehicle DMSO. The cells were then harvested, washed twice with cold PBS and resuspended in fixation/permeabilisation solution. After 20 min incubation at 4 °C, the cells were centrifuged and washed twice with 1 mL 1x Perm/Wash buffer. To detect p53, the fixed and permeabilized cells were incubated in Perm/Wash buffer containing FITC Mouse Anti-Human p53 antibody (BD Pharmingen) or FITC Mouse IgG2b isotype control (anti-dansyl) at 4 °C for 30 min. The cells were then washed with Perm/Wash buffer and resuspended in PBS. The cells were subjected to the flow cytometry analysis and detected in FL1-A channel.

*NF-*κB *translocation assay*

NF-κb translocation was determined by using NF-κB activation HCS kit which includes Hoescht 33342 and Alexa Fluor 488 conjugated anti-NF-κB dyes. HT-29 cells were seeded in 96-well plate and treated with different concentration of geraniin range from 18-72 µM for 1 h followed by stimulation of 10 ng/mL of TNF-α for 30 min at 37°C. Culture medium was removed and pre-warmed fixation solution was added into each well for 10 min incubation. Then, plate was washed by 1X wash buffer twice. Permeabilisation buffer was added into each well and incubated for 10 min following by washing with 1X wash buffer twice. 50 µL of primary antibody solution containing NF-kB antibody was added into each well and incubated for 1 h. After incubation, wash buffer II was added for 15 min and washed by 1X wash buffer twice. Cells were then incubated with the staining solution containing Hoescht 33342 dye and DyLight 488 goat anti-rabbit secondary antibody for 1 hr protected from light and followed by washing steps using wash buffer. The cells were then subjected to analysis using ImageXpress Micro XLS (Molecular devices, USA).

Western blot analysis

HT-29 cells (1×10^6 cells) were treated with 36 µM of geraniin for 6 and 12 h. The cells were harvested, washed with cold PBS and resuspended in cold RIPA buffer containing protease and phosphatase inhibitors. The cells were then kept on ice for 5 min and centrifuged at 14,000 × g for 15 min at 4 °C. The total protein content was determined by using Bradford assay. 25 µg of the total protein of each lysate was separated by electrophoresis on the 12 % SDS-PAGE gel and transferred onto a nitrocellulose membrane followed by blocking using either skim milk or 5% BSA for 1 h prior to incubation with various primary antibodies at 4°C overnight. Subsequently, the membrane was washed with TBST (0.05 % Tween-20 in TBS) and incubated with corresponding anti-mouse/rabbit immunoglobulin G-horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing, the membrane was incubated using enhanced chemiluminescence (ECL) detection kit and visualized using gel documentation system. Protein bands were analyzed quantitatively and qualitatively with Vilber Lourmart.

Statistical analysis

In all the experiments, data were expressed as means ± standard error. A significant difference from the respective control for each experiment was assessed using one way ANOVA followed by Dunnett's test or Student's *t*-test, with p values < 0.05 being regarded as statistically significant.

Results

Geraniin abrogated the growth of different cancer cell lines in a dose-dependent manner

To determine the inhibitory growth effect of geraniin on five different human cancer cell lines (HT-29, HCT116, Ca Ski, A549 and Jurkat), MTT assay was utilized to determine the cell viability at different concentrations of geraniin ranging from 0.78 to 100 µM for 72 h. The results showed that geraniin significantly reduced the cell viability of the five different cancer cells in a dose-dependent manner (Figure 1B). Geraniin exhibited the most potent inhibition on HT-29 cells with the IC_{50} value of 18.13 ± 0.53 µM, which was the lowest among all the cancer cell lines. In addition, the HT-29 cells was significantly inhibited by geraniin in a time-dependent manner (Figure 1C). The IC_{50} values of geraniin for HT-29 cells were 73.71 ± 0.86 and 39.94 ± 0.43 µM at 24

and 48 h, respectively. However, geraniin did not show significant reduction in the viability of normal colon cells CCD841 CoN with the IC_{50} value of greater than 100 µM.

Induction of nuclear morphological changes by geraniin

To determine if the inhibition of cell viability by geraniin was due to the induction of apoptosis, the alteration of nuclear morphology when stained with Hoescht 33342/PI was observed by using fluorescence microscopy. In the control untreated cells, the nuclei appeared to be round in shape with intact cell membrane and chromatin which was stained homogenously with dim blue fluorescence (Figure 1D). However, treatment with varying concentrations of geraniin (18-72 µM), typical nuclear morphological changes including nuclear shrinkage (Figure 1E, Arrow 1), chromatin condensation (Figure 1F, Arrow 2), DNA fragmentation (Figure 1G, Arrow 3) and late apoptosis (Figure 1G, Arrow 4) were observed. In increasing concentrations of geraniin, the nuclear changes were more apparent in HT-29 cells.

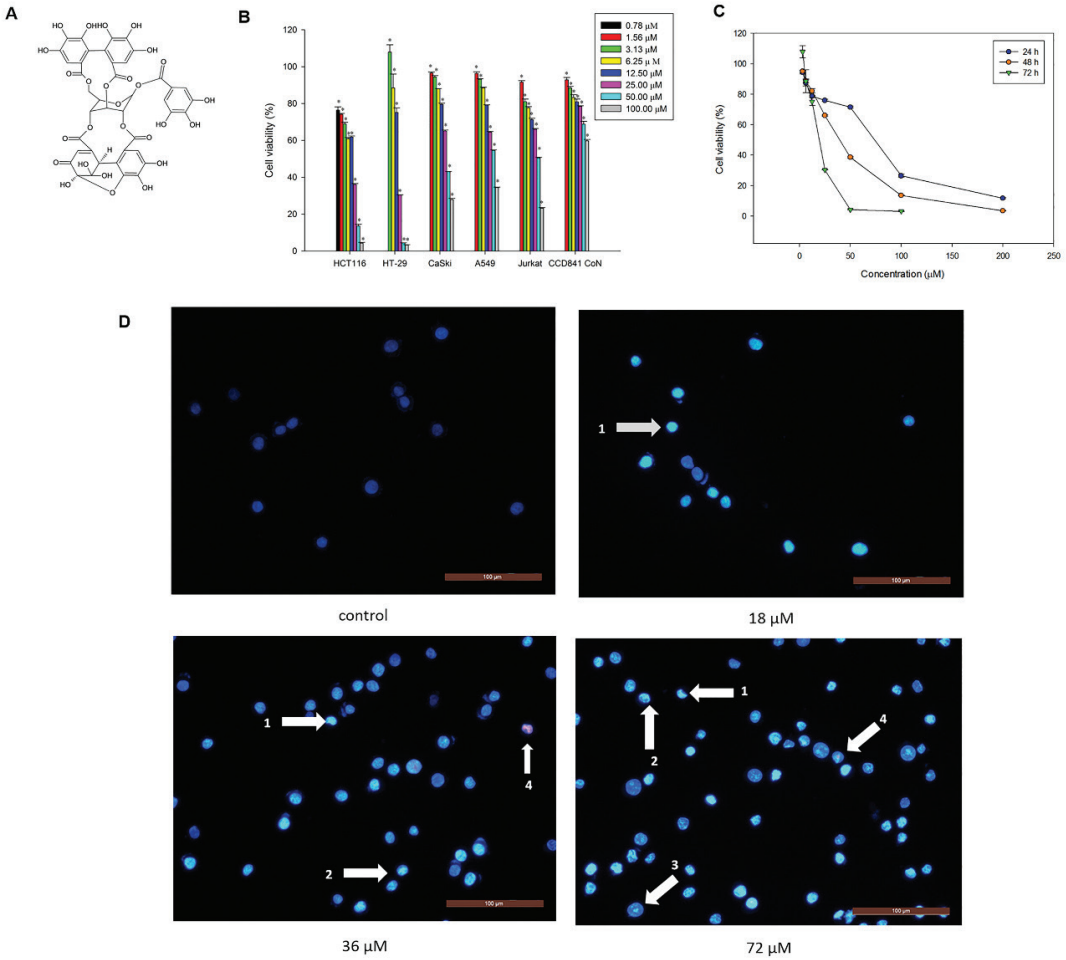

Figure 1. Geraniin reduced the viability of various human cancer cells and induced nuclear morphological changes in HT-29 cells. (A) Chemical structure of geraniin. (B) It displayed the cytotoxicity of geraniin against HT-29, HCT 116, Ca Ski, Jurkat, A549 cancer cell lines and CCD841 CoN normal colon cells which determined by MTT assay. (C) Geraniin inhibited cell proliferation of HT-29 cells after treated at different time incubation (24, 48 and 72 h). (D) Untreated control cells. After exposure to 18 µM (E), 36 µM (F) and 72 µM (G) of geraniin for 24 h, cells were stained with Hoechst 33342 and PI. The viable cells were stained uniformly blue. The early apoptotic cells were stained bright blue and late apoptotic cells were stained with purple. The data expressed as mean ± S.E. of three independent experiments (n=9). Asterisks indicate significantly different value from control (*$p < 0.05$). Magnification: 200×. Arrow 1 indicates nuclear shrinkage, 2 Chromatin condensation, 3 DNA fragmentation, 4 Late apoptosis

Externalization of phosphatidylserine induced by geraniin

To further investigate whether geraniin induced apoptosis in HT-29 cells, Geraniin-treated HT-29 cells were subjected to annexin V/PI staining assay. As shown in dual parametric dot plots, the four quadrants correspond to viable at lower left ($Annexin^-/PI^-$), early apoptosis at lower right ($Annexin^+/PI^-$), late apoptosis at upper right ($Annexin^+/PI^+$) and necrosis at upper left ($Annexin^-/PI^+$). Treatment with varying concentrations of geraniin (18-72 µM) on the HT-29 cells resulted in a progression from viable to early and late apoptosis in the dose-dependent manner (Figure 2A) revealing translocation of phosphatidylserine to outer membrane. Thus, exposure to different concentrations of geraniin on HT-29 cells was found to elicit apoptosis as evidenced by the percentages of annexin V positive cells (Figure 2A). The total annexin V positive cells were dose- dependently increased from 7.02 ± 0.70 to 10.34 ± 0.48, 15.10 ± 0.63 and 21.82 ± 1.42 %.

Dissipation of mitochondrial membrane potential

To evaluate the loss of mitochondrial membrane potential, JC-1 probe was used. The mitochondrial depolarization which precedes the onset of apoptosis converts JC-1 from red to green fluorescence. As shown in Figure 2B, the upper quadrant represents the JC-1 aggregates whereas the lower quadrant corresponds to the green JC-1 monomers. In the untreated cells, most of the red JC-1 aggregates fluorescence emerged in the up-

per quadrant indicating healthy mitochondria. In contrast, geraniin-treated cells showed progressive loss of red aggregate fluorescence (upper quadrant) in mitochondria and enhanced green monomer fluorescence (lower quadrant) in cytoplasm (Figure 2B). Upon exposure to increasing concentrations of geraniin for 24 h, the cell populations shifted progressively from the upper quadrant to the lower quadrant. The percentage of red/green ratio fluorescence of HT-29 cells was reduced when treated with different concentration of geraniin, indicating a substantial dose-dependent loss of mitochondria membrane potential and mitochondrial dysfunction.

Induction of DNA fragmentation

To further verify the induction of apoptosis by geraniin, the TUNEL assay which detects DNA breakage during late stage of apoptosis was performed. The results revealed that TUNEL-positive cells, shown in P4 region were detected in geraniin-treated HT-29 cells, evidenced by the shift of TUNEL-negative cells from the P3 region (Figure 2C). There was a dose-dependent augmentation of TUNEL positivity following treatment with increasing concentrations of geraniin (18-72 µM). The percentage of apoptotic cells (apoptotic index) was distinctly increased to 18.55 ± 1.24 % compared to the control group. Additionally, the percentage of TUNEL-positive cells was increased in time-dependent manner (Figure 2C). The apoptotic index was markedly increased from 3.52 ± 0.59 % to 14.62 ± 0.91, 23.50 ± 0.92 and 26.76 ± 0.71 % when treated with geraniin for 24, 48 and 72 h, respectively. These results were in parallel with the morphological changes by the Hoechst 33342/PI dual staining.

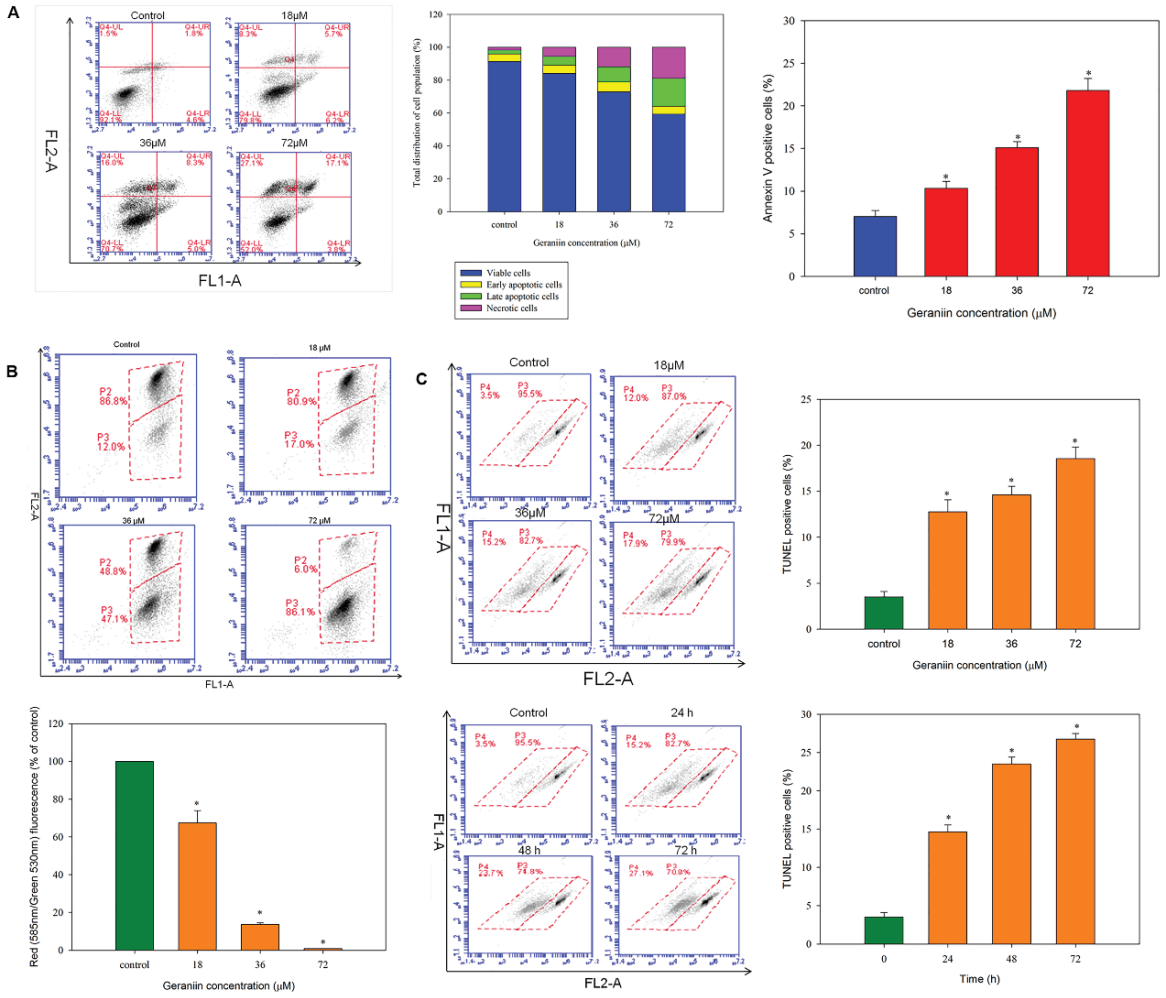

Figure 2. Induction of apoptosis by geraniin in HT-29 cells. (A) Phosphatidylserine externalization induced by different concentrations of geraniin (18-72 µM) in HT-29 cells at 24 h. Cells were stained by using Annexin V/FITC and PI and analyzed by flow cytometry. (B) Dose-dependent disruption of mitochondrial membrane potential observed in geraniin-treated HT-29 cells detected by JC-1 staining. (C) Dose- and time-dependent induction of DNA fragmentation after exposure of geraniin in HT-29 cells by TUNEL assay. The data expressed as mean ± S.E. from three individual experiments. Asterisks indicate significantly different value from control (*$p < 0.001$).

Induction of p53 protein expression by geraniin

The p53 tumor suppressor protein is a DNA-binding transcription factor that activates the transcription of genes regulating cellular responses such as apoptosis or cell cycle arrest in response to DNA damage[14]. To elucidate the crucial roles of p53, the protein level of p53 in geraniin-treated HT-29 cells was investigated by flow cytometry. Cells treated with 36 µM of geraniin exhibited an increase in fluorescence intensity when stained with p53 antibody compared to the control. The elevation of p53 protein expression level shown in Figure 3B is evidenced by the shift of the histogram from left to right (Figure 3A). These results suggested that p53 protein expression was time-dependently up-regulated to a maximum of 1.52 ± 0.03 fold at 24 h postreatment with geraniin (Figure 3B).

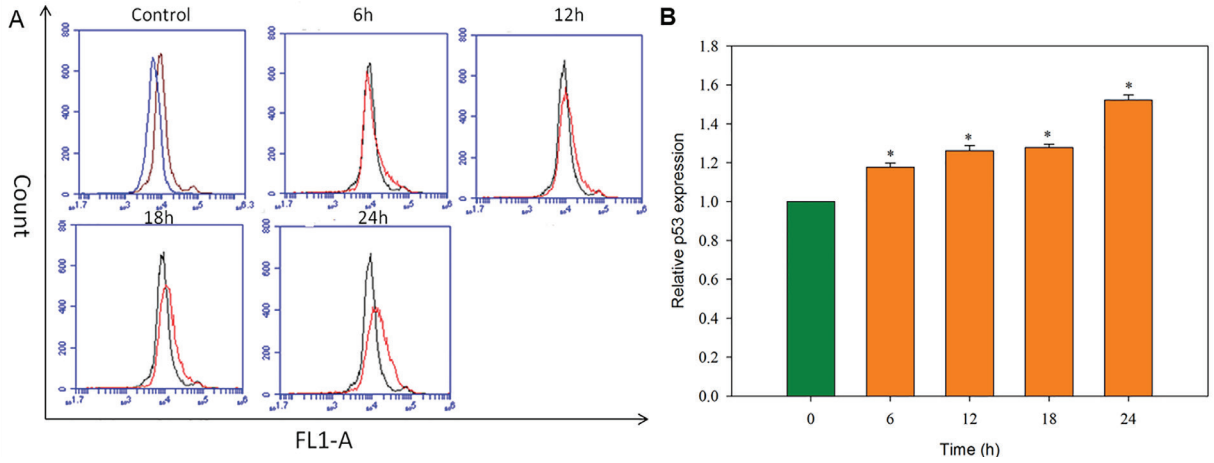

Figure 3. Effect of geraniin on p53 protein expression level in HT-29 cells. Cells were exposed with 36 μM of geraniin for different incubation periods. (A) The histogram showed the p53 expression in HT-29 cells at increasing incubation periods. (B) Bar chart showed the relative p53 expression level. The data expressed as mean ± S.E. from three individual experiments. Asterisks indicate significantly different value from control (*p < 0.001).

Perturbation of Anti-apoptotic/Pro-apoptotic Balance by Geraniin

Perturbation of the balance between proliferation and apoptosis in these aberrant cancer cells is the key mechanism in preventing colon cancer. The results demonstrated that the expression of anti-apoptotic protein Bcl-xL was suppressed and the expression of pro-apoptotic protein, Bak was stimulated (Figure 4A). There was an up-regulation of Bak expression in a time-dependent manner upon exposure to 36 μM of geraniin which significantly increased to 1.87 ± 0.27 fold at 12 h. In contrast there was a significant reduction of Bcl-xL protein expression that resulted in a decrease of 0.45± 0.07 and 0.34 ± 0.08 fold at 6 and 12 h, respectively. The elevated Bak protein expression has increased the Bak/Bcl-xL ratio to 6.02 ± 1.72 fold at 12 h (Figure 4B).

Activation of caspase-3 mediates apoptosis

To gain further understanding into the mechanism by which geraniin induces cell death, apoptosis related proteins were investigated. In our study, we found that geraniin induced apoptosis via caspase-3 activation evident by the time-dependent down-regulation of procaspase-3 on HT-29 cells. Relative procaspase-3 expression significantly decreased to 0.22 ± 0.01 at 12 h (Figure 4C).

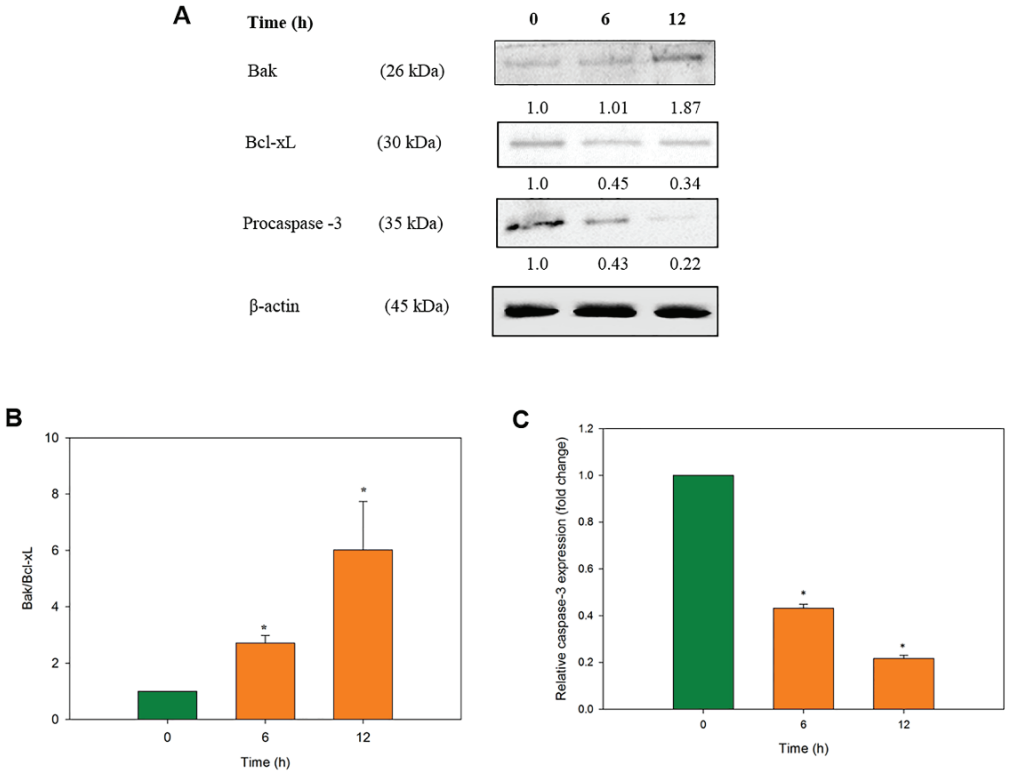

Figure 4. Effect of geraniin on apoptosis related-protein expression level including Bak, Bcl-xL and procaspases-3. (A) Bak, Bcl-xL and procaspases-3 expression level with their respective western blot band intensity image. (B) The ratio of Bak/Bcl-xL (C) Bar chart showed the relative procaspases-3 protein expression level. β-actin was used as internal control. The data expressed as mean ± S.E. from three individuals experiments. Asterisks indicate significant different value from control (*p<0.05).

Suppression of NF-κB activation by geraniin

The role of geraniin in the inhibition of TNF-α-induced NF-κB activation was investigated in HT-29 cells in which cell proliferation, survival and invasion are known to be regulated by NF-κB. High NF-κB fluorescence intensity was found in the nuclei when cells were stimulated by TNF-α alone as compared to the control untreated cells. However, geraniin inhibited the activation of NF-κB in a dose-dependent manner, indicating that geraniin attenuated NF-κB activation and translocation from cytoplasm to nuclei (Figure 5A). The translocation of NF-κB was further verified and quantified in western blot result (Figure 5B). The cytoplasmic phospho-p65 protein expression was up-regulated by 4.53 ± 0.14 folds at 72 μM of geraniin (Figure 5C). In contrast, nuclear phospho-p65 protein expression was down-regulated to 0.29 ± 0.01 at 72 μM of geraniin (Figure 5D).

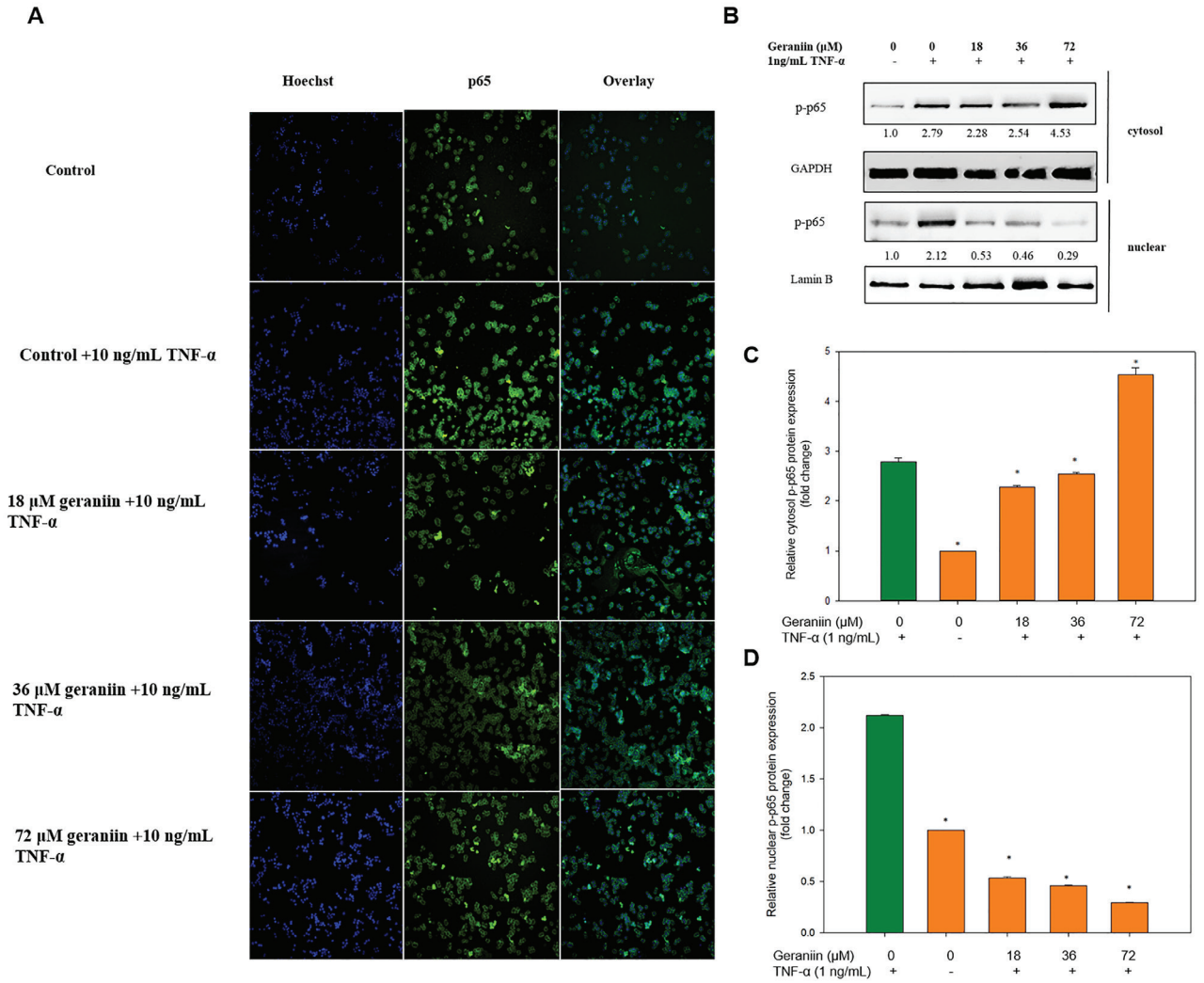

Figure 5. Attenuation effect of geraniin on TNF-α induced NF-κB nuclear translocation. HT-29 cells were pretreated with different concentrations of geraniin for 1 h followed by stimulation of 10ng/mL TNF-α for 30 min. (A) Representative image of NF-κB fluorescence are shown in HT-29 cells. (B) Cytosolic and nuclear p-p 65 expression level with their respective western blot band intensity image. Bar chart showed the relative (C) cytosol p-p65 and (D) nuclear p-p65 protein expression level. GAPDH and Lamin B were used as internal control for cytosol and nuclear protein, respectively. The data expressed as mean ± S.E. from three individual experiments. Asterisks indicate significantly different value from control (*p< 0.001).

Geraniin induced modulation of the PI3K/Akt/mTOR pathway

Next, an investigation of the involvement of PI3K/Akt/mTOR was conducted on geraniin-induced apoptosis in HT-29 cells. The Akt expression level was shown to be down-regulated compared to the control untreated cells (Figure 6A). The relative Akt expression was decreased to 0.46 ± 0.12 and 0.68 ± 0.08 fold at 6 and 12 h, respectively (Figure 6B). The results revealed that the phosphorylated Akt (p-Akt) expression was time-dependently downregulated in geraniin-treated HT-29 cells. The attenuation in p-Akt expression resulted in a significant decreased ratio of p-Akt/Akt by 0.51 ± 0.09 fold at 12 h (Figure 6C). Thus, the expression level of mTOR and Rictor were investigated in geraniin-treated cells. The relative mTOR expression was decreased to 0.94 ± 0.05 fold at 12 h as well as the relative p-mTOR expression level was markedly reduced to 0.31 ± 0.07 fold at 12 h. This decline of p-mTOR expression led to the reduction of p-mTOR/mTOR ratio by 0.83 ± 0.07 and 0.34 ± 0.05 fold at 6 and 12 h, respectively (Figure 6C). Furthermore, treatment with geraniin was shown to abrogate Rictor expression as compared to the control. The relative Rictor expression was significantly decreased to 0.38 ± 0.01 fold at 12 h (Figure 6D).

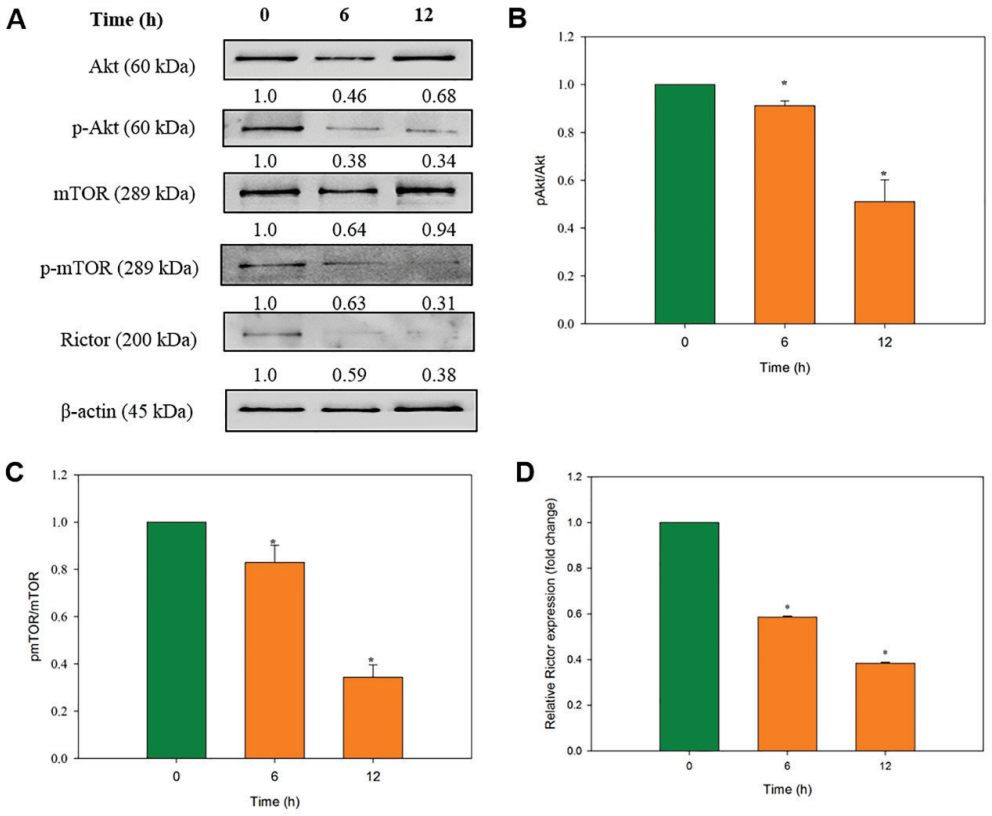

Figure 6. Effect of geraniin on PI3K/Akt/mTOR signaling pathway in HT-29 cells. Cells were treated with 36 μM of geraniin for different time incubation. (A) Akt, p-Akt, mTOR, p-mTOR and rictor expression level with their respective western blot band intensity image. (B) The ratio of p-Akt/Akt. (C) The ratio of p-mTOR/mTOR. (D) Bar chart showed the relative Rictor protein expression. β-actin was used as internal control. The data expressed as mean ± S.E. from three individual experiments. Asterisks indicate significantly different value from control (*p< 0.05).

Discussion

Despite of the advancement treatment against malignancy, there has been massive effort and renewed interest in discovering safer yet effective therapeutic candidates especially natural derived-phytochemicals[27-40]. Geraniin, a multifunctional plant-based compound, which is found in various plants and possess multitude of health benefits[41]. Thus, our current study also demonstrated that geraniin is toxic to HT-29 colorectal adenocarcinoma cells but does not have adverse effect on normal colon cells. Treatment with geraniin resulted in a significant decrease in the viability of HT-29 cells among all the cancer cell lines. Thus, HT-29 cells was selected for further investigation on the underlying cell death mechanisms. Our current data reported that geraniin treatment caused HT-29 cells to execute apoptosis, as evident by nuclear morphology alterations, an increase in the total Annexin V-positive cells and DNA fragmentation. Mechanistically, the present study revealed the PI3K/Akt/mTOR mediated-apoptosis signaling pathway.

To investigate the geraniin-induced apoptosis initiation in HT-29 cells, the levels of apoptosis related proteins of procaspase-3, Bak and Bcl-xL were assessed by western blot analysis. The expression of procaspase-3 was significantly decreased in HT-29 cells treated with geraniin in a time-dependent manner. The present findings demonstrated that geraniin significantly downregulated Bcl-xL and upregulated Bak protein expression. Consequently, the Bak/Bcl-xL ratio increased by 6.02 ± 1.72 fold to evoke apoptosis by triggering mitochondrial outer membrane permeabilisation as evident by the increase of green JC-1 monomers fluorescence, hence allowing cytochrome c released into the cytosol in HT-29 cells. Subsequently, cytochrome c binds Apaf-1 and procaspase-9 leading to cleavage of procaspase-9 and procaspase-3[42]. Mechanistically, activation of caspase-3 is followed by DNA fragmentation and eventually apoptosis[43]. Accordingly, geraniin resulted in mitochondrial dysfunction, caspase-3 activation, DNA fragmention and eventually apoptosis in HT-29 cells suggesting that HT-29 cells underwent apoptosis involving mitochondria-mediated pathway after exposure of geraniin.

Mounting evidence has highlighted the paramount importance of targeting PI3K/Akt pathway involved in the malignant transformation and cell survival[9,10,44] can be an alternative therapeutic strategy for the treatment of a variety tumors including colorectal cancer[45]. In addition, it has been demonstrated that inhibition of PI3K/Akt pathway may inhibit cell growth and increase the sensitivity of solid tumors to conventional chemotherapeutic agents[46]. This signaling pathway is controlled by several main regulatory proteins including PI3K, Akt and mTOR. Akt is in turn regulated by PI3K, which recruits Akt to the cell membrane to be activated by PDK1[47]. The current findings showed that geraniin resulted in a significant time-dependent suppression of phosphorylated Akt proteins with a concomitant decreased ratio of p-Akt/Akt. This phenomenon was in accordance with the previous study showing that geraniin exhibited significant dose-depen-

dent reduction of p-Akt in both Jurkat and HepG2 cells[48]. Activated Akt leads to the survival of tumor and attenuates apoptosis via phosphorylation of multiple downstream targets including mTOR and GSK-3β. The mammalian target of rapamycin complex 2 (mTORC2), which contains the regulatory proteins Rictor and mTOR is activated via a cascade that activates PI3K and phosphatidylinositol-3, 4, 5 phosphate-mediated activation of Akt. Therefore, it is conceivable that the modulation in phosphorylation of Akt is closely related to the phosphorylation of mTORC2[47]. The current study revealed that treatment by geraniin abrogates the phosphorylation of mTOR (p-mTOR) and rictor expression along with decreased phosphorylation of Akt corroborated well with the growth suppression and induction of apoptosis in HT-29 cells. These data suggest that by targeting the PI3K/AKT/mTOR signaling pathway may lead to the development of novel therapeutic agents for cancer treatment. Cumulatively, these results highlighted the crucial role of PI3K/AKT/mTOR signaling pathway in contributing the apoptotic cell death effect mediated by geraniin. Additionally, activated Akt phosphorylates one of the downstream mediators, NF-kB which is aberrantly regulated in colon cancer[5] and conferred the resistance of colon cancer cells to chemotherapy[49]. The involvement of NF-kB signaling enhances cancer cells survival via upregulation of anti-apoptotic protein such as Bcl-xL[50]. Previously, xanthohumol was found to inhibit NF-kB nuclear translocation and downregulate Bcl-xL protein expression that eventually induces apoptosis in leukemia[51]. Similarly, our results clearly showed that geraniin inhibited the nuclear translocation of NF-kB which eventually induced apoptosis via suppression of Bcl-xL protein.

P53 is a crucial orchestrator in regulating various cellular responses including cell cycle regulation, induction of apoptosis and cellular senescence[14,35]. Constitutive activation of Akt signaling which results in activation of MDM2 and inhibition of p53, contributes to the tumor development[52,53]. Hence, suppression of Akt has been implicated in targeting various cancers. For instance, curcumin-treated chemoresistant ovarian cancer cells led to suppression of Akt concomitantly with activation of p53 which facilitating the cell death mechanism via apoptosis[54]. Additionally, DNA damage may activate p53 and subsequently inhibit cell growth via inhibition of the PI3K/Akt pathway[55]. It was also reported that p53 can provoke mitochondrial permeabilisation and apoptosis by interacting with anti-apoptotic protein Bcl-xL and releasing pro-apoptotic protein Bak[56,57]. Resveratrol, a well-known antioxidant, mediates apoptosis in human prostate carcinoma via modulation of PI3K/Akt pathway and Bcl-2 family proteins[58]. In consistent, our results suggested that geraniin inhibits cell growth by activating p53 through the inhibition of PI3K/Akt/mTOR pathway and modulation of Bak/Bcl-xL ratio in HT-29 cells. Based on the collective results, the underlying apoptotic mechanism conferred by geraniin in HT-29 cells is summarized in schematic illustration (Figure 7).

The present study revealed the apoptosis inducing effect conferred by geraniin was through inhibition of PI3K/Akt/mTOR signaling pathway. Interestingly, geraniin suppressed the Akt and mTOR phosphorylation through inhibition of PI3K/Akt/mTOR signaling pathway and further attenuates the nuclear translocation of NF-κB thus leading to the suppression of cell proliferation. Activation of p53 in response to either DNA damage or inactivation of Akt signaling eventually triggered mitochondrial permeabilisation by modulating the anti- and pro-apoptotic proteins (Bcl-xL and Bak). This was followed by the activation of caspase cascades and eventually leading to apoptosis in HT-29 cells.

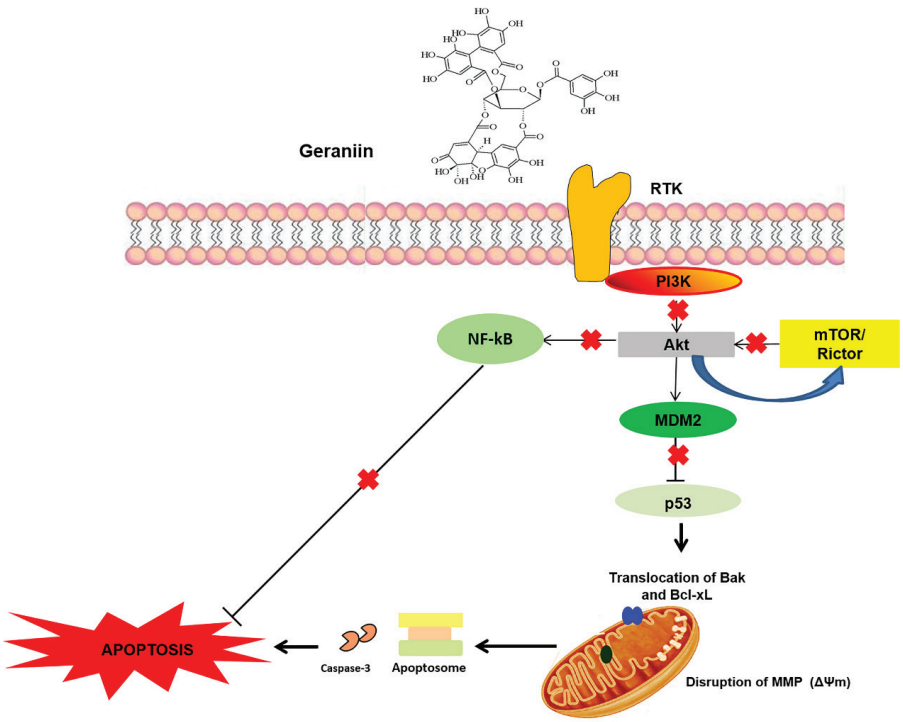

Figure 7. Schematic illustration showing the apoptosis conferred by geraniin via inhibition of PI3K/Akt/mTOR pathway and suppression of NF-kB through modulation of p53 and Bcl-2 family member proteins in colon adenocarcinoma cells.

Conclusion

In conclusion, geraniin imparts the most potent inhibition against HT-29 human colorectal adenocarcinoma cells. Geraniin induced apoptosis via inhibition of PI3K/Akt/mTOR signaling pathway and suppression of NF-κB in HT-29 cells which is preceded by the mitochondrial damage leading to a disruption in the balance between Bak and Bcl-xL proteins followed by caspase activation. Hence, our study provides novel insights in the application of geraniin as a potential therapeutic agent for the treatment of colon cancer and warrants further investigation to delineate the possible mechanisms of apoptosis in *in vivo* model.

Conflict of Interest

The authors declare that there is no conflict of interest in this work.

Acknowledgements

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