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Luteolin inhibits proliferation, triggers apoptosis and modulates Akt/mTOR and MAP kinase pathways in HeLa cells

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Abstract. Flavonoids, a subclass of polyphenols, have been shown to be effective against several types of cancer, by decreasing proliferation and inducing apoptosis. Therefore, the aim of the present study was to assess the anti-carcinogenic potential of luteolin on HeLa human cervical cancer cells, through the use of a cell viability assay, DNA fragmentation assay, mitochondrial membrane potential assay, cell cycle analysis using Annexin/PI staining and flow cytometry, gene expression analysis and a protein profiling array. Luteolin treatment exhibited cytotoxicity towards HeLa cells in a dose- and time-dependent manner, and its anti-proliferative properties were confirmed by accumulation of luteolin-treated cells in sub-G₁ phases. Cytotoxicity induced by luteolin treatment resulted in apoptosis, which was mediated through depolarization of the mitochondrial membrane potential and DNA fragmentation. Furthermore, luteolin treatment increased the expression of various proapoptotic genes, including *APAF1*, *BAX*, *BAD*, *BID*, *BOK*, *BAK1*, *TRADD*, *FADD*, *FAS*, and *Caspases 3* and *9*, whereas the expression of anti-apoptotic genes, including *NAIP*, *MCL-1* and *BCL-2*, was decreased. Cell cycle regulatory genes, including *CCND1*, *2* and *3*, *CCNE2*, *CDKN1A*, *CDKN2B*, *CDK4* and *CDK2*, were decreased following

treatment. Expression of *TRAILR2/DR5*, *TRAILR1/DR4*, *Fas/TNFRSF6/CD95* and *TNFR1/TNFRSF1A*, as well as pro-apoptotic proteins, including *BAD*, *BAX* and *Cytochrome C* were consistently increased, and the expression of anti-apoptotic proteins, *HIF1 α* , *BCL-X*, *MCL1* and *BCL2*, were found to be decreased following treatment. Expression of *AKT1* and *2*, *ELK1*, *PIK3C2A*, *PIK3C2B*, *MAPK14*, *MAP3K5*, *MAPK3* and *MAPK1* was significantly decreased at the transcriptional level. Expression of *GSK3b* (p-ser9), *PRAS 40* (p-Thr246), *BAD* (p-ser112), *PTEN* (p-ser380), *AKT* (p-ser473), *ERK2* (p-Y185/Y187), *RISK2* (p-ser386), *P70S6k* (p-Thr421/ser424), *PDK1* (p-ser241), *ERK1* (p-T202/Y204) and *MTOR* (p-ser2448) was downregulated and expression of *P53* (p-ser241) and *P27* (p-Thr198) was upregulated by luteolin in a dose-dependent manner, indicating its anti-proliferative and apoptosis enabling properties, and this may have been mediated via inhibition of the *AKT* and the *MAPK* pathways.

Introduction

A major challenge with conventional cancer treatments, including chemotherapy, is the genotypic and phenotypic heterogeneity of cancer cells, and non-selective cytotoxicity of these treatment regimens, and this limits their use, increasing the risk of cancer reoccurrence (1,2). Therefore, experimental evidences need to be accumulated on plant-derived chemopreventative strategies, which may be used as adjuvants for improved cancer management. Chemopreventative agents (synthetic and natural) consist of nonsteroidal anti-inflammatory drugs (sulindac, aspirin and celecoxib) (3) and FDA-approved drugs, including Tamoxifen, Raloxifene and HPV vaccines (Cervarix and Gardasil), which all show potential anticancer effects. However, these adjuvants have also demonstrated adverse side effects in patients (4-6). Considering the constraints of these agents, studies are focused on identifying alternatives based on plant-derived compounds, which exhibit decreased toxicity with higher efficacy for the treatment of cancer (7-9).

Notably, an epidemiological study proposed that 30% of cancer types can be avoided by a healthy diet (2), and the consumption of certain fruits and vegetables may decrease

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the risk of cancer onset (5,10,11). Several flavonoids extracted from various plants have been shown to exert anticancer properties (5,7), exhibiting regulatory effects on several molecular mechanisms, including their ability to increase the activity of free radical scavenging enzymes, and increase anti-proliferative and anti-inflammatory responses (7,12,13). Furthermore, flavones, a subclass of flavonoids have been demonstrated to possess anticancer effects through decreasing cancer cell proliferation by downregulation of various pro-apoptotic proteins, including nuclear factor- κ B along with modulation of aberrant PI3K/AKT and MAPK/P38 signaling (14-18). Apoptosis is the desired outcome when treated with these agents, and if the means by which apoptosis is induced is determined, the efficacy of novel agents targeting these mechanisms may be assessed. Apoptosis is primarily mediated by the death receptor pathway and mitochondrial pathway. The death receptor family, which is comprised of tumor necrosis factor receptors, including TRAIL-R2/DR5, TRAIL-R1/DR4, TNFR1/TNFRSF1A, Fas/TNFRSF6/CD95, Fas and DR6 may initiate the extrinsic pathway, and *BCL2* family members, including Bad, Bak, Bax, Bid, Bik (pro-apoptotic molecules), and Bcl-XL and Bcl-2 (anti-apoptotic members) are involved in the mitochondrial pathway (19-21).

Luteolin (3,4,5,7-tetrahydroxy flavone) is a flavone found in vegetables and fruits, including parsley, carrots, artichoke, celery and several spices (including thyme and oregano) (14,16,17,22-25). Several studies have demonstrated the anti-inflammatory, anti-microbial, anti-diabetic and anti-carcinogenic properties of luteolin (23,26-28). Luteolin has been demonstrated to exhibit anticancer effects against several types of cancer cell lines, including liver (HepG2), colon (HT29), lung (LNM35) and breast cancer (MDA-MB-231) cells, and is a potent HDAC inhibitor (16,19). Luteolin induces apoptosis and cell cycle arrest by increasing expression of Bax, Caspase 3 and Caspase 9, as well as the MEK-ERK pathway, whilst concomitantly decreasing Bcl-2 expression in A549 lung cancer cells (18). Luteolin also possesses anti-proliferative effects against A549 lung cancer cells by arresting the cell cycle at the G2 phase and initiating programmed cell death via the mitochondrial pathway, which is mediated through activation of JNK and inhibition of NF- κ B (22,29). Luteolin suppresses the MAPK/AKT/PI3K pathway, and NF- κ B and STAT3 signalling in several types of cancer cell lines, and also decreases the expression of matrix metalloproteins and β 3 integrin in B16F10 and A431 melanoma cell lines, thereby inhibiting EMT (17,20,22,24,30,31).

Notably, flavonoids including luteolin have been reported to inhibit tumor cell proliferation and apoptosis (14-19) in various cancer cell lines e.g., HPV-18-associated cells and lung cancer cell (17,18). However, there is lack of scientific evidence to review luteolin as a probable anticancer agent. Therefore, the present study aimed to investigate the extensive molecular mechanism through which luteolin induces anticancer effects on HeLa cell as an apoptosis inducer by targeting various molecular targets; tumor inhibitors and promoters: *DAPK1*, *TP53*, *TP73*, *PTPRRR* and *TERT*; cell cycle regulatory genes: *CDK2*, *CCNE2*, *CCND2* and *CCND*; pro and anti-apoptotic genes: *BAD*, *BID*, *BOK*, *BAX*, *Bcl2*, *Caspases 3*, *7*, *8* and *9*; receptors and pathways genes: *FASLG*, *FAS*, *TNFRS10-B*, *MTOR*, *MAPK*, *PIK3CA*, *AKT1*, *ELK1*, *FOXO1* and *FOXO3*,

and to further associate it with the modulation of phosphorylated proteins in luteolin-treated HeLa cells to enhance the acceptance of luteolin as a potential anticancer agent.

Material and methods

Cell culture and drug preparation. Information of every important process that ensues in human cells whether normal or abnormal has depended to a large extent on using HeLa cells and they have also proved to be the best model for cervical cancer (32); therefore, HeLa cells were used for this study. Human cervical cancer HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA), supplemented with 10% FBS (Sigma-Aldrich; Merck KGaA), 100 μ g/ml amphotericin (Sigma-Aldrich; Merck KGaA) and 100 μ g/ml streptomycin (Sigma-Aldrich; Merck KGaA), and cells were cultured at 37°C in a humidified incubator with 5% CO₂.

Luteolin (molecular weight, 286.24 g/mol) was purchased from Sigma-Aldrich (Merck KGaA) and diluted to a concentration of 69.87 mM using dimethyl sulfoxide (DMSO; stock solution), which was then further diluted using DMEM (without FBS) to a concentration of 1 mM, which was used to make the working concentrations of 1-40 μ M.

MTT assay. MTT assay (Sigma-Aldrich; Merck KGaA) was used to study the cytotoxic effects of luteolin on HeLa cells. In brief, 1×10^4 cells/well were plated onto a 96-well plate and treated as follows: Blank control, DMSO control (0.11%), positive control (cisplatin 5 μ M) and luteolin-treated (1-40 μ M); and treatment was performed for 24 or 48 h, after which images were captured. Subsequently, plates were decanted, and the cells were incubated with MTT at final concentration of 5 mg/ml (dissolved in PBS) at 37°C for 2-4 h, the MTT was discarded, 100 μ l DMSO was added to the wells and plates were incubated at 37°C for 30 min in the dark. Viable cells convert the tetrazolium salt into insoluble formazan violet crystals that is quantified using a colorimeter at 570 nm. A graph was plotted by dividing the absorbance of luteolin-treated cells with that of DMSO controls. Data are presented as the mean \pm standard deviation of three independent repeats.

Nuclear staining of HeLa cells with propidium iodide (PI) following luteolin treatment. PI staining of cells treated with luteolin for different concentrations was performed to assess morphological variations in the nucleus in cells undergoing apoptosis. In total, $\sim 2.5 \times 10^5$ cells were seeded onto glass coverslips and treated with luteolin for 24 or 48 h. The cells were then washed with 1x PBS (pH 7.4) twice and a mixture of ice cold methanol:acetone (1:1) was used to fix the cells at -20°C for 10 min followed by staining with PI (10 mg/ml in PBS) for 30 sec in the dark at room temperature, and the cover slips were subsequently mounted on glass slides. The images were captured at 515 nm using a fluorescent microscope (Olympus Corporation) at x40 magnification.

DNA fragmentation assay. Inter-nucleosomal DNA disintegration is one of the primary features of apoptosis. During apoptosis, nucleases are activated causing fragmentation of nuclear chromatin into 50-300 kbp fragments (33). The

ApoTarget™ Quick Apoptotic DNA Ladder Detection kit (cat. no. #KHO1021; Invitrogen; Thermo Fisher Scientific, Inc.) was used for rapid extraction of chromosomal DNA of untreated cells, and cells treated with 5, 10 and 20 μ M luteolin for 48 h, according to the manufacturer's protocol. The fragmented DNA was extracted from cells and run on a horizontal electrophoresis gel containing ethidium bromide and the DNA was resolved on a 1.2% agarose gel.

Tetramethyl rhodamine, ethyl ester (TMRE) mitochondrial membrane potential assay. A TMRE Mitochondrial Membrane Potential assay kit (cat. no. ab113852; Abcam) was used to assess the mitochondrial membrane potential. In total, $\sim 5 \times 10^3$ HeLa cells were plated onto clear bottom 96-well plates and treated with 5, 10 and 20 μ M luteolin for 48 h at room temperature. TMRE at a final concentration of 400 nM was used and the cells were incubated in the dark for 30 min at room temperature. Following washing several times with the wash buffer provided with the aforementioned kit, the plate was read on a fluorescence microplate spectrophotometer (Ex/Em=549/575 nm; Synergy H1 Biotech Plate Reader) and images were captured using a fluorescent microscope (Progress Fluorescent Microscope; Olympus Corporation) at x40 magnification.

Apoptosis detection using FITC-conjugated Annexin V/PI. Quantitation of the apoptosis was performed using a FITC-conjugated Annexin V/PI assay kit (cat. no. ab 14085; Abcam) and analyzed using a flow cytometer. In brief, 2×10^5 cells were plated onto six-well plates, and cells were treated with 5, 10 and 20 μ M luteolin for 48 h at 37°C. Treated and untreated cells were collected and washed with PBS and resuspended in 500 μ l binding buffer, followed by staining with PI (50 μ g/ml) and FITC-conjugated Annexin V (10 mg/ml) for 15 min at room temperature in the dark, and the cells were analyzed using a BD FACS-Aria™ III Flow cytometer (BD Biosciences). Flow cytometry data were analyzed using the FlowJo software (FlowJo LLC; Version 10.1).

Cell cycle analysis by flow cytometry. HeLa cells were treated with 0, 5, 10 and 20 μ M luteolin for 48 h, and all the cells (floating and attached) were collected. This was followed by washing with 1x PBS and fixed with absolute ethanol at -20°C overnight. The following day, cells were washed with 1x PBS and staining with PI (1 mg/ml), containing 0.1% citrate buffer, Triton X-100 and 0.5% RNase for 30 min at room temperature. BD FACS-Aria™ III Flow cytometer (BD Biosciences) was used to analyze the cells for DNA content, and FlowJo software (FlowJo LLC; version 10.1) was used for data analysis.

Gene expression analysis using TaqMan arrays. RNA extraction was performed on luteolin-treated HeLa cells (10 and 20 μ M for 48 h at 37°C) and untreated cells according to the manufacturer's protocol using a GenElute Mammalian Genomic Total RNA kit (Sigma-Aldrich; Merck KGaA). Extracted total RNA was quantified using Nanodrop (Thermo Fisher Scientific, Inc.). RNA was then subjected to first strand synthesis, according to the manufacturer's protocol, using an Applied Biosystems™ High-Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.).

TaqMan® Gene Expression assays are a comprehensive collection of pre-designed, pre-formulated primer and probe sets to perform quantitative gene expression studies (Apoptosis Array and oncogene array (cat. nos. 4414072 and 4391524). TaqMan® Gene Expression assays that were manufactured and stocked in advance were used to detect the expression of genes associated with cell cycle signaling pathways and apoptosis in the treated and untreated cells. A total of 10 μ l cDNA with a concentration of 100 ng per well and 10 μ l master mix provided in the kit was used. Expression analysis was performed according to the manufacturer's protocol. The qPCR array was run on a thermocycler (QuantiStudio3, Applied Biosystems, USA) with the following reaction conditions: Enzyme activation at 95°C for 10 min and 40 cycles, denaturation at 95°C for 15 sec, annealing at 60°C for 1 min. Results were analyzed by the $2^{-\Delta\Delta C_q}$ method using DataAssist™ software version 3.01 (Thermo Fisher Scientific, Inc.) as previously described (34).

Caspase 3 activity. Caspases are the final executioners of apoptosis and Caspase-3 serves a role in the extrinsic and intrinsic pathways of apoptosis (35). A Caspase-3 Activity kit (cat. no. CASP 3C; Sigma-Aldrich; Merck KGaA) was used. A total of 1×10^6 cells were plated and treated (5, 10 and 20 μ M luteolin at 37°C for 48 h) with various concentrations of luteolin. Cell pellets were taken and resuspended in the lysis buffer (1X) at a concentration of 1×10^7 cell/100 μ l and incubated for 30 min, prior to being centrifuged at 12,000 x g for 20 min at 4°C. The lysates of the DMSO control and treated (5, 10 and 20 μ M luteolin) HeLa cells and the assay were set up according to the manufacturers' protocol. The plate was incubated overnight at room temperature and read at 405 nm, and fold-changes were calculated by comparing the treated sample reading with the DMSO control. The experiment was repeated 3 times and all results are expressed as mean \pm standard deviation of three independent experiments.

Determination of protein expression using a proteome profiler array. To determine the expression of genes associated with apoptosis and cell cycle progression at the protein level, a protein profiler array was purchased from R&D Systems, Inc. (cat. no. ARY009). The cells were treated with 10 and 20 μ M luteolin for 48 h at 37°C and the lysis was performed with lysis buffer. The assay was performed according to the manufacturer's protocol to assess the expression of all the proteins associated with apoptosis. Protein quantitation of lysates was performed using a Pierce™ BCA Protein assay kit (Thermo Fisher Scientific, Inc; cat. no. 23225). A further 400 μ g protein of the diluted cell lysate was used for each membrane, the membranes were incubated with the lysates on a rocking platform overnight at 2-8°C. The signal was developed using Streptavidin-HRP and chemiluminescent detection reagents. Signal intensity was measured using a chemiluminescent detector Gel Doc system (Bio-Rad Laboratories, Inc.), and analyzed using Image Lab software (version 6.1; Bio-Rad Laboratories, Inc.). The experiment was repeated three times and the results are expressed as the mean \pm standard deviation from three independent experiments (*P<0.005).

Analysis of expression of phosphorylated AKT pathway proteins. To determine the effect of luteolin treatment on

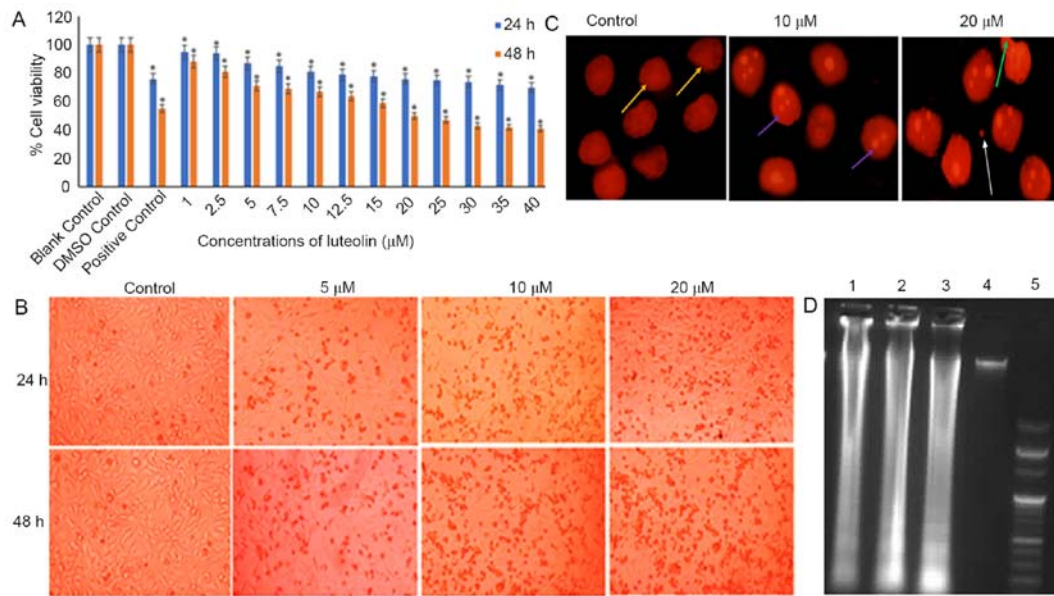


Figure 1. Anti-proliferative effect of luteolin on HeLa cells. (A) Induction of cytotoxicity by luteolin at various concentrations and time points. The graph represents the dose- and time-dependent decrease in cell viability of HeLa cells treated with 1-40 μM luteolin for 24 and 48 h. For comparison, 0.11% DMSO was used as the loading control and 5 μM cisplatin was used as the positive control. The 24 and 48 h treated samples were compared with 24 and 48 h DMSO controls, respectively. The data are expressed as the mean \pm standard deviation of three independent experiments. * $P < 0.05$. The IC_{50} of luteolin was found to be 20 μM at 48 h. (B) Microscopic images of HeLa cells treated with 10, 15 and 20 μM luteolin for 24 and 48 h. Cells exhibited a characteristic rounding off of the cells, indicating apoptosis. Magnification, $\times 10$. (C) Changes in nuclear morphology of treated HeLa cells (10 and 20 μM luteolin) compared with the untreated controls. These nuclear changes were observed following staining with propidium iodide under a fluorescence microscope (magnification, $\times 40$). A dose-dependent increase in the apoptotic index, characterized by features such as nuclear condensation, nuclear blebbing, nuclear fragmentation and apoptotic bodies, was observed. Yellow, large and prominent nuclei; purple, nuclear fragmentation; green, blebbing; white, apoptotic bodies. (D) Luteolin induces DNA fragmentation in a dose-dependent manner in HeLa cells. Lane 5 shows the 100 bp ladders; lanes 1, 2, 3 and 4 show fragmentation of DNA in cells treated with 5, 10 and 20 μM luteolin and the control, respectively. The experiment was performed three times. DMSO, dimethyl sulfoxide.

proteins involved in the AKT pathway, a phosphorylated AKT pathway array was obtained from Ray-Biotech, Co., Ltd. (cat. no. AAH-AKT-1-8). The lysates of treated and untreated cells were quantitated using a Pierce BCA assay (Thermo Fisher Scientific, Inc.) and $\sim 500 \mu\text{g}$ lysate was used on each nitrocellulose membrane. To begin with, the membranes were blocked with blocking buffer from the aforementioned kit, followed by incubation with the cell lysate for 24 h, and the assay was performed according to the manufacturer's protocol. The chemiluminescent gel doc system (BioRad Laboratories, Inc.) was used to capture the image of the blot and analysis was performed using Image Lab software (version 6.1).

Statistical analysis. Statistical analysis was performed using SPSS software (version 21; IBM Corp.). The data was analyzed using one-way analysis of variance followed by Tukey's HSD *post hoc* test. All experiments were performed in triplicate. Results are expressed as the mean \pm standard deviation of three separate experiments * $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Luteolin inhibits the growth and proliferation of HeLa cells. HeLa cells treated with varying concentrations of luteolin (1-40 μM for 24 or 48 h) exhibited a dose and time-dependent decrease in the viability of HeLa cells when compared with the untreated DMSO control. When cells were treated with 20 μM luteolin for 48 h, $\sim 50\%$ cell death was observed (Fig. 1A).

Luteolin induced substantial morphological changes in HeLa cells, including rounding off of the cells and subsequent detachment of the cells from the surface, and the changes became more significant as the dose of luteolin was increased (Fig. 1B).

Luteolin induces nuclear morphological abnormalities and causes DNA fragmentation in HeLa cells. HeLa cells treated with 10 and 20 μM luteolin exhibited condensation and disintegration of nuclear material, blebbing and formation of apoptotic bodies and nuclear debris, which increased with concentration, while the untreated HeLa cells possessed a distinct and prominent nucleus (Fig. 1C). Furthermore, the observation that luteolin induced apoptosis was supported by the results of a DNA laddering assay in HeLa cells. Treatment of HeLa cells with 5, 10 and 20 μM luteolin for 48 h resulted in fragmentation of DNA. A typical DNA ladder-like pattern was observed in all the treated samples when compared with the controls upon gel electrophoresis (Fig. 1D).

Luteolin treatment arrests cell cycle progression of HeLa cells at sub-G1 phases. Inhibition of proliferation and growth may be a result of cell cycle arrest. Analysis of cell cycle progression was performed on luteolin-treated cells using PI staining and flow cytometry. Luteolin induced accumulation of cells at the G2/M phase when treated with 5 μM , and when treated with 10 and 20 μM , accumulation of cells was observed at sub-G1 phases, and $\sim 50\%$ of cells were arrested when treated with 20 μM luteolin for 48 h, with a decrease in the number of

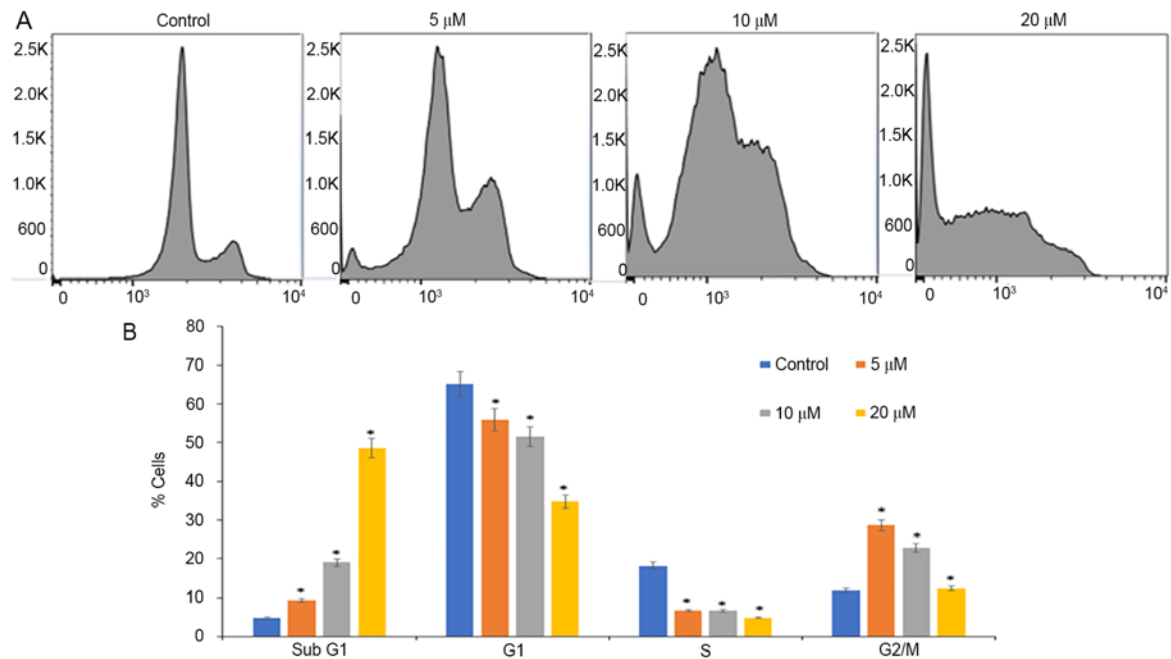


Figure 2. Luteolin induces cell cycle arrest in HeLa cells in sub-G1 phases. (A) DNA contents were analyzed by flow cytometry following treatment with different concentrations of luteolin (0, 5, 10 and 20 μM) for 48 h. (B) Percentage of cells in the G0/G1, S and G2/M phases of the cell cycle are compared between the 5, 10, 20 μM treated HeLa cells and dimethyl sulfoxide control cells. The results are presented as the mean \pm standard deviation of three independent experiments *P<0.05.

cells in the S and G2/M phases when compared with controls wherein a normal distribution of cells was observed (Fig. 2). In the present study, luteolin treatment increased the sub-G1 peak, which is an indicator of endonuclease activation and apoptosis induction due to DNA fragmentation, which appears to be the crucial anti-proliferative mechanism of luteolin treatment (36).

Luteolin induces early and late apoptosis. Annexin V-FITC and PI were used for the detection of early and late apoptosis. Annexin V-stained cells were considered early apoptotic cells, and cells stained with PI and annexin V and were considered late apoptotic cells. The percentage of viable cells decreased from 90.5% in the control cells to 65.4 and 47.9% when treated with 10 and 20 μM luteolin, respectively, for 48 h. Additionally, there was an increase in early apoptotic cells from 2.89% in control samples to 9.75 and 12.4% when treated with 10 and 20 μM luteolin. A notable increase in the proportion of late apoptotic cells was detected, increasing from 1.89% in the untreated samples to 7.85 and 14% when treated with 10 and 20 μM luteolin 48 h, respectively (Fig. 3).

Luteolin decreases the mitochondrial membrane potential of HeLa cells. Mitochondrial membrane potential is a reliable measure of cell stress and apoptosis, as it is compromised early during the stress response and a good indicator of ongoing cell death. Several dyes may be used to detect mitochondrial membrane potential, which allows flexibility in wavelengths and use in combination with other fluorescence markers. In the present study, TMRE was used. The percentage of TMRE fluorescence was calculated compared with the control; 84, 64 and 56% of cells fluoresced with TMRE when HeLa cells were

treated with 5, 10 and 20 μM luteolin for 48 h, respectively (Fig. 4).

Luteolin inhibits cell cycle progression by modulation of cell cycle regulatory genes. Following treatment of HeLa cells with 5, 10 and 20 μM luteolin for 48 h, the untreated and treated cells were subjected to cell cycle progression analysis. The results demonstrated that 5 μM luteolin induced G2/M phase arrest in HeLa cells, and when treated with 20 μM luteolin, there was a substantial accumulation of cells at sub G1 phases. This was further verified by assessing the expression of cell cycle regulatory genes (oncogene array, cat. no. 4369514); down-regulation of cell cycle regulatory genes, including *CCND1*, *CCND2*, *CCND3*, *CCNE2*, *CDKN1A*, *CDKN2B*, *CDK4* and *CDK2*, was observed. All these genes serve an important role in the G1 phase transition. Additionally, telomerase reverse transcriptase (TERT) expression was decreased and PTPRR expression was significantly increased when treated with 10 and 20 μM luteolin. TERT adds repeat sequences of DNA (TTAGGG) to the end of chromosomes, thereby increasing the replicative properties, and its inhibition controls proliferation, while PTPRR inhibits ERK and MAPK pathways. FOXO1 and FOXO3 expression was also upregulated, and they serve an important role in apoptosis [Fig. 5A; RQ (relative quantitation)] values are presented in Table I).

Luteolin increases apoptosis in HeLa cells. HeLa cells treated with 10 and 20 μM luteolin for 48 h along with untreated cells were used to assess gene expression of apoptotic genes using a TaqMan Array (apoptosis array; cat. no. 4414072). Gene expression levels of *APAF1*, *BID*, *BAK1*, *BOK*, *BAD*, *BAX*, *NOD1*, *NOD2*, *BCL10* and *BCL2L14*, as well as *Caspase-3*, *Caspase-7* and *Caspase-9* increased (10 μM , RQ \geq 1.4; 20 μM ,

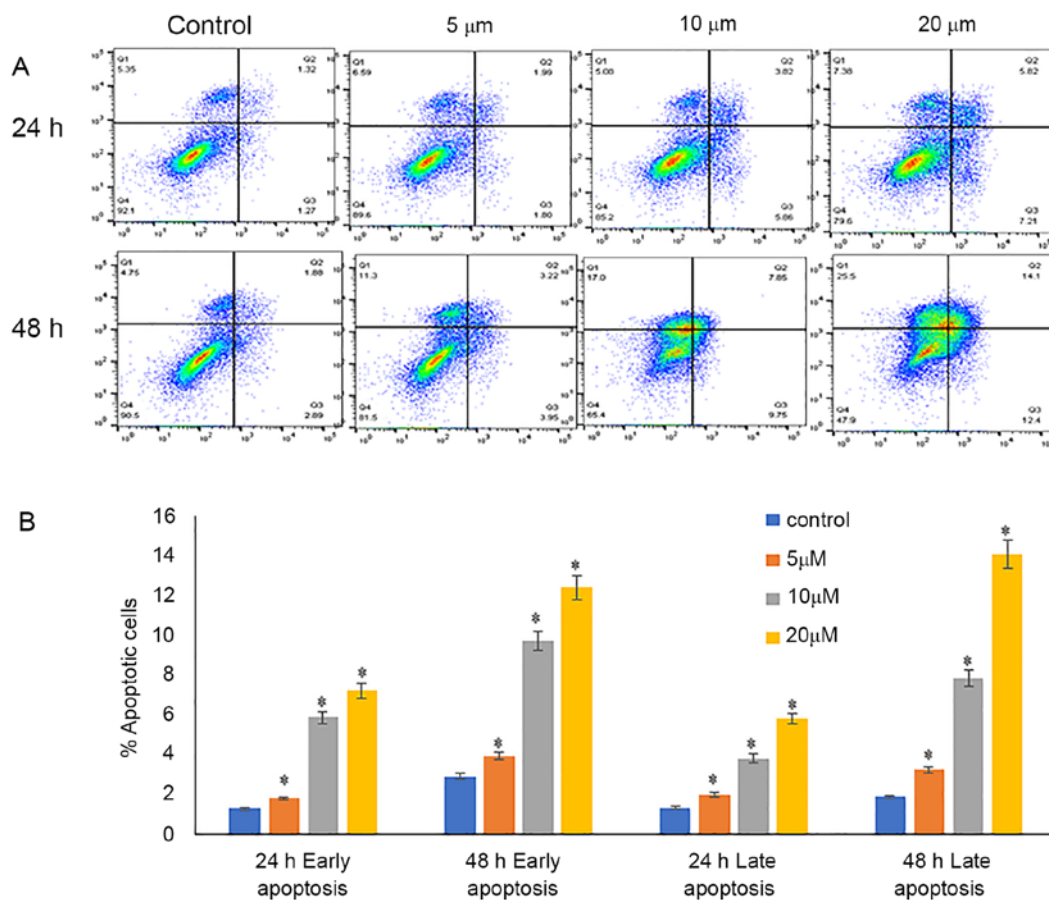


Figure 3. Evaluation of apoptosis using Annexin V-PI double staining following treatment of HeLa cells with 5, 10 and 20 μ M luteolin for 24 and 48 h and DMSO control cells. (A) Representative flow cytometry dot plots of apoptosis. In all four plots, viable cells are seen in the left lower quadrant (FITC-/PI-), early apoptotic cells in the right lower quadrant (FITC+/PI-), and late apoptotic cells in the right upper quadrant (FITC+/PI+). (B) Quantification of the proportion of cells in different stages of apoptosis, as determined by flow cytometry. The proportion of early and late apoptotic cells increased in a dose-dependent manner, compared with DMSO control cells. Data are presented as the mean \pm standard deviation of three independent experiments * P <0.05. DMSO, dimethyl sulfoxide.

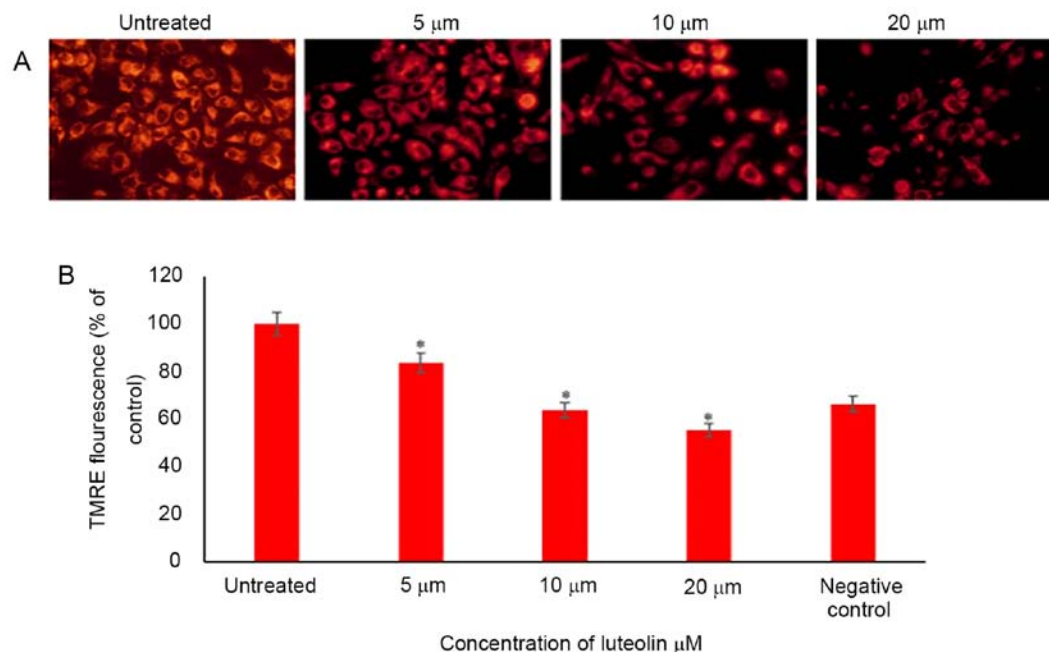


Figure 4. Luteolin decreases the membrane potential of HeLa cells in a concentration-dependent manner. (A) HeLa cells were treated with 5, 10 and 20 μ M luteolin for 48 h, and the mitochondrial membrane potential was determined using TMRE staining and a fluorescent microscope (magnification, $\times 40$). (B) TMRE fluorescence of treated cells was compared with the untreated control cells and the results are expressed as the percentage of fluorescence. Data are presented as the mean \pm standard deviation of three independent experiments * P <0.05.

Table I. Relative quantitation values of cell cycle regulatory genes and pathway genes in HeLa cells following 10 and 20 μ M luteolin treatment for 48 h.

Genes	Gene ensemble no.	Gene description	Luteolin 10 μ M	Luteolin 20 μ M
CDK4	Hs00175935_m1	Cyclin dependent kinase inhibitor 1A	0.06	0.04
ESR1	Hs01046816_m1	Estrogen receptor 1	0.12	0.04
MTOR	Hs00234522_m1	Mechanistic target of rapamycin	0.23	0.18
PIK3C2B	Hs00898499_m1	Phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 beta	0.37	0.10
CCNE2	Hs00372959_m1	Cyclin E2	0.39	0.28
CCND3	Hs00236949_m	Cyclin D3	0.45	0.24
CDK2	Hs01548894_m1	Cyclin-dependent kinase 2	0.45	0.42
MAPK3	Hs00385075_m1	Mitogen-activated protein kinase 3	0.45	0.19
AKT2	Hs01086102_m1	AKT serine/threonine kinase 2	0.45	0.30
ELK1	Hs00428286_g1	ELK1C	0.48	0.25
CCND1	Hs00765553_m1	Cyclin D1	0.50	0.31
MAPK1	Hs01046830_m1	Mitogen-activated protein kinase 1	0.50	0.18
AKT1	Hs00178289_m1	AKT serine/threonine kinase 1	0.52	0.31
TERT	Hs00972650_m1	Telomerase reverse transcriptase	0.53	0.40
PIK3CA	Hs00907957_m1	Phosphatidylinositol-4C	0.53	0.35
CCND2	Hs00153380_m1	Cyclin D2	0.60	0.25
MAPK14	Hs00176247_m1	Mitogen-activated protein kinase 14	0.60	0.32
MAP3K5	Hs00178726_m1	Mitogen-activated protein kinase kinase kinase 5	0.69	0.40
PIK3CD	Hs00192399_m1	Phosphatidylinositol-4	0.71	0.23
TP53	Hs01034249_m1	Tumor protein p53	1.20	2.23
FOXO3	Hs00818121_m1	Forkhead box O3	1.60	4.10
FOXO1	Hs00231106_m1	Forkhead box O1	1.90	4.70
TP73	Hs01056231_m1	Tumor protein p73	2.80	5.40
PTPRR	Hs00373136_m	Protein tyrosine phosphatase	9.50	10.00

RQ \geq 2), while expression of *BCL2* and *MCL-1* expression was decreased in a dose-dependent manner (20 μ M, RQ \leq 0.5), and NAIP demonstrated a marginal decrease in expression. Notably, the receptors and ligands responsible for stimuli-based apoptosis, including *FAS*, *FASL*, *FADD*, *TNF*, *TNFRSF10B*, *TNFRSF1A*, *Caspase-8* and *Caspase-8AP2* (RQ \geq 2) were found to be increased in a dose-dependent manner following luteolin treatment as shown in Fig. 5B (RQ values in Table II).

Luteolin increases Caspase-3 activity in HeLa cells. Caspases are vital proteins that modulate the apoptotic response. Caspase 3 is the primary effector molecule, with the crucial role of induction of the extrinsic and intrinsic pathway of apoptosis (35). Luteolin treatment upregulated Caspase-3 activity in HeLa cells, and the activity increased in a dose-dependent manner. Luteolin-treated HeLa cells exhibited increased expression of Caspase-3 activity by 2-, 4- and 8-fold when treated with 5, 10 and 20 μ M luteolin for 48 h, respectively. The increase in Caspase activity following luteolin treatment, in comparison to the control, is shown in Fig. 5C.

Luteolin alters protein expression of apoptotic genes. The fold-changes of 35 proteins that are involved in apoptosis, were investigated using an R&D proteome profiler. Protein concentration was quantified using a Pierce BCA assay

(Thermo Fisher Scientific, Inc.; cat. no. 23225). Luteolin significantly upregulated the levels of the pro-apoptotic proteins involved in mitochondrial apoptosis, including HSP60, BAD, p-p53 (S15), BAX, SMAC/Diablo, pro-Caspase-3, Cytochrome c and HTRA2/Omi, as well as increasing the expression of receptors and ligands responsible for receptor based apoptosis, including TNF RI/TNFRSF1A, TRAIL R2/DR5, Fas/TNFRSF6/CD95, FADD, p-p53 (S392), p-RAD-17 (S635), HSP27, TRAIL R1/DR4, Catalase, p-Rad17 (S635) and cleaved-Caspase3 (all exhibiting a fold-change \geq 1.5-fold with 20 μ M luteolin treatment), while the levels of the anti-apoptotic proteins, including Pon2, BCL-X, cIAP-2, Clusterin, p27/Kip1, HIF-1 α , BCL2, cIAP-1, Claspin and XIAP were all down-regulated (all exhibiting a fold-change \leq 0.5 with 20 μ M luteolin treatment); only HSP70 expression was marginally decreased (Fig. 6; fold-changes are presented in Table III).

Luteolin modulates the Ras-Raf/MAPK and AKT/MTOR/PIK3 pathways. The MAPK and the PI3K/AKT/MTOR pathways serve a crucial role in the regulation of cell growth, differentiation and cell viability. Cancer development is associated with aberrant activation of genes in these pathways, and this may lead to increased cell proliferation and survival. Treatment of HeLa cells with 10 and 20 μ M luteolin for 48 h caused

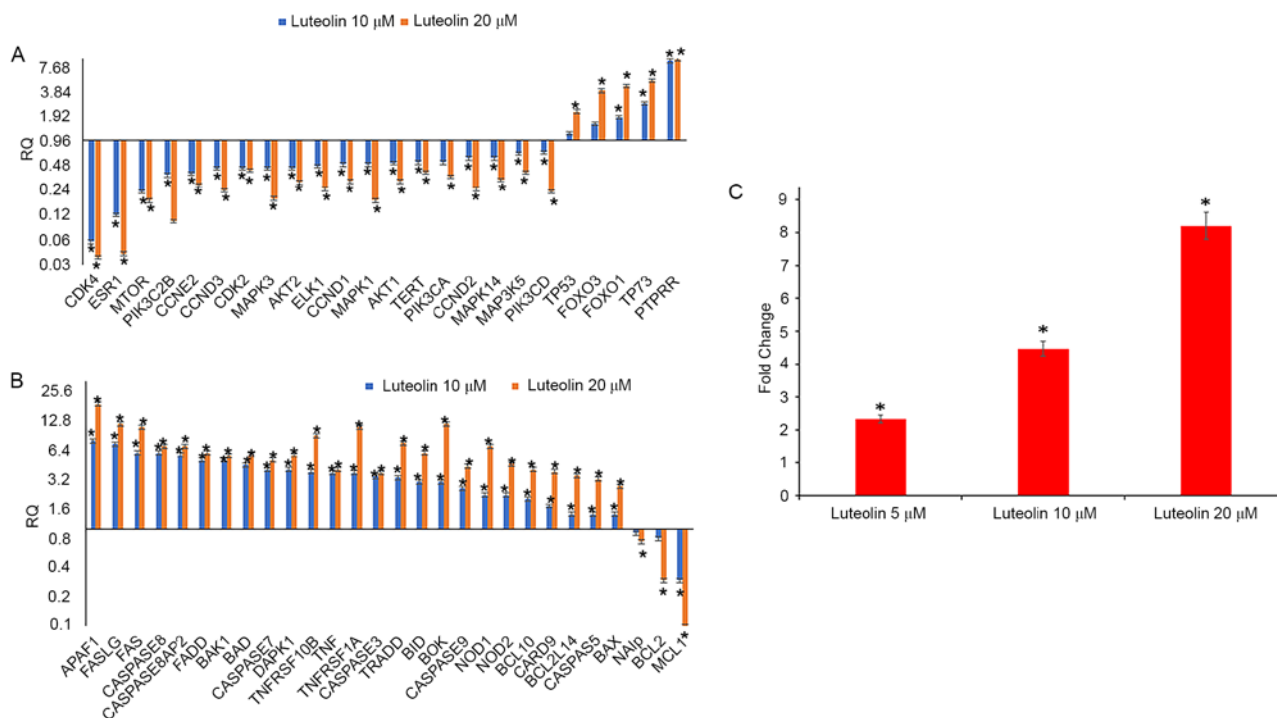


Figure 5. Treatment of HeLa cells with 10 and 20 μ M luteolin for 48 h resulted in a decrease in various cell cycle regulatory and apoptotic genes in a dose-dependent manner. (A) Decrease in cell cycle regulatory and AKT/PI3K and MAPK pathway genes. (B) Increase in the RQ values of pro-apoptotic genes and downregulation of anti-apoptotic genes following treatment with 10 and 20 μ M luteolin for 48 h. The results were normalised using the endogenous control, GAPDH, and the expression in treated samples was compared with DMSO control samples. (C) Analysis of Caspase 3 activity following treatment with 5, 10 and 20 μ M luteolin for 48 h. Luteolin treatment of HeLa cells increases the caspase activity by 2-, 4- and 8-fold following treatment with 5, 10 and 20 μ M for 48 h, respectively. Results depict the caspase activity of treated samples as compared with the DMSO controls. Data are presented as the mean \pm standard deviation of three independent experiments * P <0.05. RQ, relative quantitation.

decreased expression of MAPK pathway genes, including *MAPK1*, *MAK14*, *MAPK3* and *MAP3K5*, as well as decreased expression of AKT/MTOR/PIK3 pathway genes, such as *AKT1*, *AKT2*, *MTOR*, *PIK3CD*, *PIK3C2A* and *PIK3C2B* (all with an $RQ \leq 0.5$; Fig. 5A and Table I).

Luteolin alters the phosphorylation of proteins involved in the AKT signaling pathway. An AKT pathway phosphorylated array (cat. no. AAH-AKT-1-8) was used to assess the effects of luteolin treatment on phosphorylation of proteins involved in the AKT pathway. AKT pathway genes were modulated following treatment with 10 and 20 μ M of luteolin for 48 h. Certain proteins, including P53 (p-ser241) and P27 (p-Thr198) exhibited a marginal increase in fold-change, while others, including GSK3a (p-ser21) and GSK3b (p-ser9) were not affected by luteolin treatment. MTOR (p-ser2448), PRAS 40 (p-Ther246), BAD (p-ser112), PTEN (p-ser380), AKT (p-ser473), ERK2 (p-Y185/Y18, RISK2 (p-ser386), P70S6k (p-Thr421/ser424, PDK1 (p-ser241) and ERK1 (p-T202/Y204) were downregulated. The fold-changes with respect to the control are presented in Fig. 7 and Table IV.

Discussion

Despite advances in the early detection of cancer, and developments in the prevention and treatment of cancer, it still remains the principal cause of death worldwide (37). Cancer treatment includes a wide range of chemotherapeutic drugs administered either alone or as an adjuvant with surgery or

radiotherapy, and they are extremely effective in killing highly proliferative cells, including normal cells, and this underlies many of the associated side effects (19,29,37,38). These drawbacks highlight the requirement for more specific and less toxic cancer treatment strategies, including plant-derived chemopreventative agents. Several studies have demonstrated that plant derived bioactive compounds exert their effects at various stages of cancer, including initiation, promotion and progression, and may serve as promising preventative or therapeutic agents. There is a strong association between a high intake of fruits and vegetables and a decreased risk of cancer (5,10,11). Various dietary agents, including curcumin, resveratrol, genistein, chrysin, sulforaphane and various other flavonoids possess the ability to reverse or attenuate aberrantly active signaling pathways associated with carcinogenesis (19,28,38-42). Flavones exhibit strong anticancer effects in a range of cancer cell lines via regulation of various molecular mechanisms (14,24,25,43). In addition to their ability to restore the aberrant signaling of pathways associated with cancer, flavones may cause cell cycle arrest, induce apoptosis, and inhibit invasion, metastasis and inflammation (14,19). Cancer cells may evade apoptosis; therefore, apoptosis induction by the therapeutic regimen is required, and this is possible through administration of several different flavones (14,19,43).

The aim of the present study was to determine the molecular mechanisms regulated by luteolin in human cervical cancer cells. Luteolin is the major bioactive compound found in several different fruits and vegetables, including oregano,

Table II. Relative quantitation values of apoptotic genes in HeLa cells following treatment with 10 and 20 μ M luteolin treatment for 48 h.

Genes	Ensemble gene no.	Gene description	Luteolin 10 μ M	Luteolin 20 μ M
APAF1	Hs00559441_m1	Apoptotic peptidase activating factor 1	8.00	19.00
FASLG	-Hs00181225_m1-	Fas ligand (TNF)superfamily	7.40	12.00
FAS	Hs00163653_m1	Fas (TNF receptor superfamily)	6.00	11.00
CASPASE 8	Hs01018151_m1	Caspase 8	6.00	7.00
CASPASE 8AP2	Hs01594281_m1	CASP8 associated protein 2	5.70	7.00
FADD	Hs00538709_m1	Fas (TNFRSF6)-associated via death domain	5.00	6.00
BAK1	Hs00832876_g1	BCL2-antagonist/killer 1	5.00	5.60
BAD	Hs00188930_m1	BCL2-antagonist of cell death	4.50	5.70
CASPASE 7	-Hs00169152_m1	Caspase 7	4.00	5.00
DAPK1	Hs00234480_m1	Death-associated protein kinase 1	4.00	5.70
TNFRSF10B	Hs00366272_m1	Tumor necrosis factor receptor superfamily	3.80	9.00
TNF	Hs00174128_m1	Tumor necrosis factor (TNF superfamily)	3.70	4.00
TNFRSF1A	Hs01042313_m1	Tumor necrosis factor receptor superfamily	3.70	11.00
CASPASE 3	Hs00234387_m1	Caspase 3	3.40	3.70
TRADD	Hs00182558_m1	TNFRSF1A-associated via death domain	3.30	7.60
BID	Hs00609632_m1	BH3 interacting domain death agonist	3.00	6.00
BOK	Hs00261296_m1	BCL2-related ovarian killer	3.00	12.00
CASPASE 9	Hs00154260_m1	Caspase 9	2.60	4.30
NOD1	Hs00196075_m1	Nucleotide-binding oligomerization domain 1	2.20	7.00
NOD2	Hs00223394_m1	Nucleotide-binding oligomerization domain 2	2.20	4.60
BCL10	Hs00961847_m1	B-cell CLL/lymphoma 10	2.00	4.00
CARD9	Hs00261581_m1	caspase recruitment domain family	1.70	3.80
BCL2L14	Hs00373302_m1	BCL2-like 14 (apoptosis facilitator)	1.40	3.50
CASP 5	Hs00362072_m1	Caspase 5	1.40	3.20
BAX	Hs00751844_s1	BCL2-associated X protein	1.40	2.70
NAIp	Hs01847653_s1	NRL family	0.90	0.74
BCL2	Hs00608023_m1	B-cell CLL/lymphoma 2	0.80	0.29
MCL1	Hs00172036_m1	Myeloid cell leukemia sequence 1 (BCL2-related)	0.29	0.10

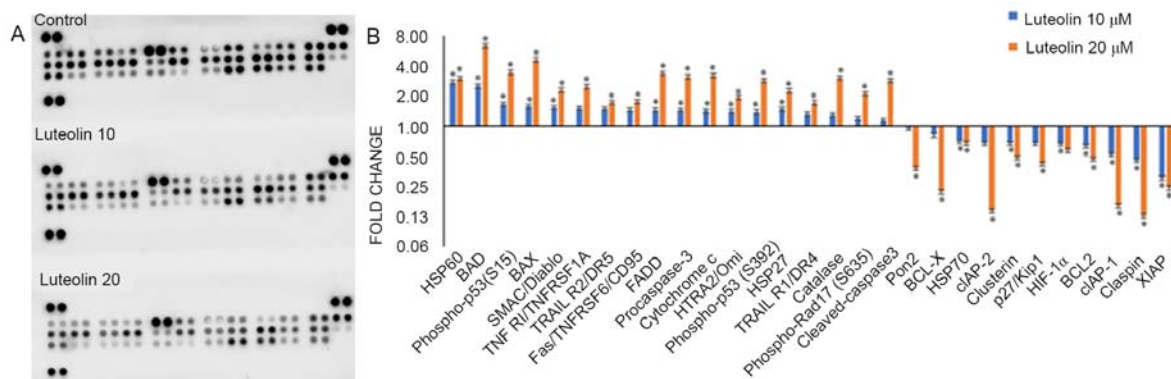


Figure 6. Change in expression of apoptotic proteins following treatment with luteolin. (A) Images of the nitrocellulose proteome profiler membrane showing differences in the expression of apoptotic and cell cycle regulatory proteins in the control and treated samples. (B) Luteolin-treated (10 and 20 μ M for 48 h) HeLa cells exhibited upregulated expression of pro-apoptotic proteins and downregulation of anti-apoptotic proteins in a concentration-dependent manner. Fold-changes of various apoptotic proteins was quantitated by comparison with the dimethyl sulfoxide controls. The data are expressed as the mean \pm standard deviation of three independent experiments. * $P < 0.05$.

parsley and artichoke, and it possesses anti-neoplastic, anti-inflammatory, anti-microbial, anti-viral and anti-diabetic

properties *in vitro* and *in vivo* (19,24,26,27,44). The present study demonstrated that luteolin increased cell death in a

Table III. Fold-change in expression of apoptotic proteins following luteolin treatment (10 and 20 μ M for 48 h).

Gene	Luteolin 10 μ M	Luteolin 20 μ M
HSP60	2.73	2.97
BAD	2.50	6.32
Phospho-p53 (S15)	1.64	3.43
BAX	1.56	4.58
SMAC/Diablo	1.53	2.28
TNF RI/TNFRSF1A	1.50	2.46
TRAIL R2/DR5	1.48	1.72
Fas/TNFRSF6/CD95	1.44	1.75
FADD	1.44	3.36
Pro-caspase-3	1.43	3.08
Cytochrome c	1.41	3.20
HTRA2/Omi	1.40	1.94
Phospho-p53 (S392)	1.38	2.85
HSP27	1.47	2.24
TRAIL R1/DR4	1.31	1.71
Catalase	1.29	3.03
Phospho-Rad17 (S635)	1.20	2.10
Cleaved Caspase3	1.15	2.85
Pon2	0.95	0.38
BCL-X	0.82	0.22
HSP70	0.70	0.67
cIAP-2	0.68	0.14
Clusterin	0.67	0.48
p27/Kip1	0.67	0.42
HIF-1 α	0.66	0.57
BCL2	0.63	0.47
cIAP-1	0.52	0.16
Claspin	0.45	0.13
XIAP	0.30	0.24

dose- and time-dependent manner, and the IC₅₀ was 20 μ M at 48 h. Treatment of HeLa cells with luteolin caused cell death that was clearly visible using a microscope, with dying cells exhibiting rounding off and detaching from the culture plates. This is in agreement with several other studies, which have demonstrated that luteolin inhibits the growth of cancer cells, with an IC₅₀ ranging between 10 and 60 μ M based on cell line (24,29,43-47). The changes to nuclear morphology of HeLa cells treated with 10 and 20 μ M for 48 h also justifies the assumption that apoptosis was induced by luteolin, as nuclear condensation, disintegration, nuclear blebbing and nuclear debris formation were visualized following luteolin treatment, while untreated cells did not show any significant changes in nuclear morphology. Several studies have reported that flavones, including luteolin, show similar nuclear morphological changes in several cancer cell lines, including HeLa, glioma, EC1 and KYSE450 cells (20,24,48).

DNA fragmentation is a characteristic feature of apoptotic cells (33), and this was evident in luteolin treated HeLa cells. Exposure of HeLa cells to 5, 10 and 20 μ M luteolin for 48 h

Table IV. Fold changes observed following treatment of HeLa cells with luteolin concentrations of 10 and 20 μ M for 48 h.

Gene	Luteolin 10 μ M	Luteolin 20 μ M
P53(P-ser241)	1.46	1.57
P27(P-Thr198)	1.48	1.57
GSK3b(P-ser9)	0.98	0.68
Mtor(P-ser2448)	0.67	0.48
PRAS 40(P-ther246)	0.67	0.57
BAD(p-ser112)	0.56	0.45
PTEN(P-ser380)	0.62	0.51
AKT(P-ser473)	0.80	0.48
ERK2(P-Y185/Y187)	0.80	0.50
RISK2(P-ser386)	0.66	0.51
P70S6k(P-Thr421/ser424)	0.70	0.48
PDK1(P-ser241)	0.70	0.48
ERK1(p-T202/Y204)	0.65	0.50

resulted in disintegration of the DNA in a ladder-like pattern, while intact DNA was observed in the untreated control. The degree of fragmentation increased in a dose-dependent manner when treated for 48 h. Flavonoids, including chrysin and quercetin, have also been demonstrated to fragment DNA in different cell lines (20,23,49,50). Cell cycle analysis revealed that luteolin induced sub-G1 arrest following treatment with 20 μ M for 48 h, in agreement with previous studies, where arrest at the G2/M or G1 phase was observed when treated with luteolin in several different cell lines (14,22,24,27,43). The determination of membrane potential using TMRE and apoptosis induction by Annexin/PI also supported the notion that apoptosis was induced by luteolin. Treatment with 5, 10 or 20 μ M luteolin for 48 h resulted in mitochondrial depolarization and Annexin/PI staining showed an increase in the proportion of cells in early and late apoptosis, and this increased in a dose-dependent manner. Several studies have reported that flavonoids induce DNA fragmentation, decrease mitochondrial potential and increase the proportion of early and late apoptotic cells in various cancer cell lines (20,24,50,51). Notably, it has also been demonstrated that luteolin decreased the expression levels of important cell cycle regulatory genes, including *CCND2*, *CCND3*, *CCND1*, *CCNE2*, *CDK2*, *CDK4* and *TERT*, and increased the expression of *FOXO1* and *FOXO3*. The cyclin D proteins, *CCNE2* along with *CDK4/CDK6* complex, promotes progression through the G1-S phase by inhibiting the retinoblastoma protein. *hTERT* is responsible for maintenance of the telomeric ends of the chromosome, and thus, its downregulation assists in the arrest of cell cycle progression, and similar results have been reported in different cell lines following treatments with polyphenols (20,52,53). *FOXO1/3* are considered tumor suppressors that increase transcription of pro-apoptotic, DNA damage repair and cell cycle regulatory genes, and their expression is decreased in various cancer cell lines (20,54). *FOXO1* is involved in tumor progression and is significantly associated with late-stage tumors, and

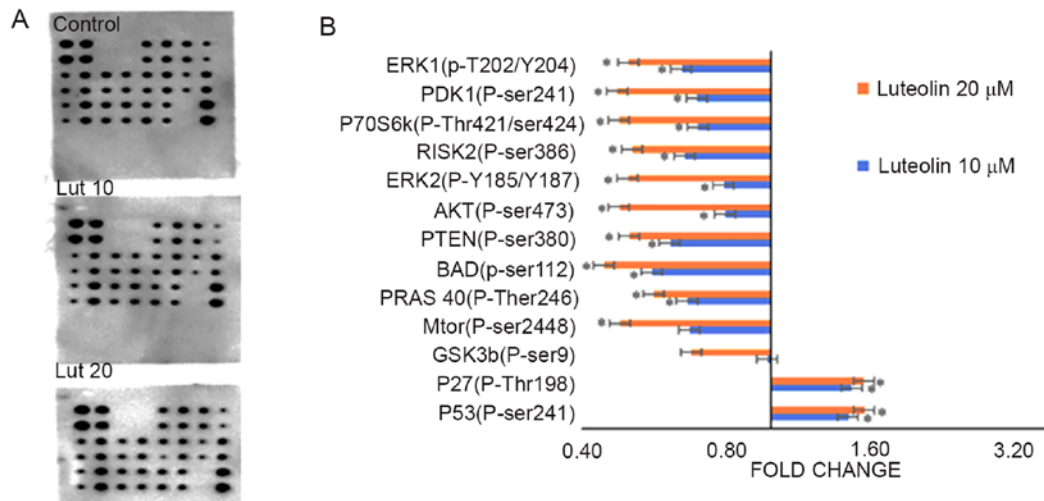


Figure 7. Differential expression of phosphorylated proteins in the AKT pathway. (A) Images of the AKT profiler membranes showing differential expression of the phosphorylated proteins involved in the AKT signaling pathway at 10 and 20 μ M of luteolin treatment for 48 h. (B) Luteolin treatment decreased expression of phosphorylated proteins involved in the AKT signaling pathway, including MTOR (p-ser2448), PRAS 40 (p-Ther246), BAD (p-ser112), PTEN (p-ser380), AKT (p-ser473), ERK2 (p-Y185/187), RISK2 (p-ser386), P70S6k (p-Thr421/ser424), PDK1 (p-ser241) and ERK1 (p-T202/Y204), while the expression of P53 (p-ser241) and P27 (p-Thr198) was increased, all in a dose-dependent manner (10 and 20 μ M). The fold-changes were calculated by comparing the treated samples with the DMSO controls. Data are presented as the mean \pm standard deviation of three independent experiments * P <0.05.

its expression is important in terms of prognosis of cervical cancer (20,55).

Pro-apoptotic and anti-apoptotic genes are the primary proteins involved in regulation of apoptosis, and they form part of the intrinsic and extrinsic apoptotic pathways. Aberrant expression of these genes contributes toward the process of carcinogenesis; therefore, these genes may be targeted for the prevention and treatment of cancer (20,24,51). Luteolin treatment of HeLa cells resulted in upregulation of pro-apoptotic genes involved in the mitochondrial apoptosis pathway, including *APAF1*, *BAX*, *BAK1*, *BAD*, *BID*, *BOK*, *Caspase7*, *Caspase9*, *Caspase 3*, *NOD1*, *NOD2*, *BCL10*, *CARD9*, *BCL2L14* and *Caspase 5*, as well as extrinsic pathway genes, including *FASLG*, *FAS*, *Caspase 8*, *Caspase 2*, *FADD*, *TNFRSF10B*, *TNF*, *TNFRSF1A* and *TRADD*, at the transcriptional level. By contrast, anti-apoptotic genes, including *BCL2*, *Mcl1* and *NAIP* were found to be down-regulated in luteolin-treated HeLa cells. Caspase-3 is the primary effector caspase and serves a significant role in the induction of apoptosis via regulation of the extrinsic and intrinsic pathways. Treatment with 5, 10 and 20 μ M luteolin for 48 h enhanced the biochemical activity of Caspase-3 by 2, 4 and 8-fold, respectively. The association of caspase 3 activation with decreased cell survival could be hypothesized by treating the cells with caspase inhibitor (Z-VAD-FMK), but that was beyond the scope of the present study. However, Islam *et al* (35) has reported role of Caspase-3 in EGCG mediated apoptosis of Human Chondrosarcoma Cells (HTB-94). Numerous studies have demonstrated that flavonoids induce apoptosis by either the intrinsic or extrinsic pathways (14,19,20,22,48). Additionally, protein expression of various genes was in agreement with the changes observed in their gene expression levels. Treatment of HeLa cells with luteolin resulted in the upregulation of pro-apoptotic genes at the protein level, including upregulation of HSP60, BAD, p-p53(S15), BAX, SMAC/Diablo, TNF, RI/TNFRSF1A,

TRAILR2/DR5, Fas/TNFRSF6/CD95, FADD, pro-caspase-3, Cytochrome c, HTRA2/Omi, p-p53(S392), HSP27, TRAILR1/DR4, Catalase, p-Rad17 (S635), cleaved Caspase3 and p53, which is consistent with earlier reports on HeLa and leukemia cells treated with the flavonoids quercetin and luteolin (17,20,23). Downregulation of antiapoptotic proteins at the mRNA/protein level, including Pon2, BCL-X, HSP70, cIAP-2, clusterin, p27/Kip1, HIF-1 α , p21/CIP1/CDKN1A and BCL2, as well as upregulation of BAX, p-p53, release of Cytochrome C, SMAC/DIABLO and cleavage of Caspase 3, Caspase 9 and PARP1 suggests the involvement of intrinsic apoptosis. Furthermore, upregulation of receptors and ligands involved in the extrinsic pathway at the mRNA/protein level, including TNF, TNFR, RI/TNFRSF1A, TRAIL R1/DR4, TRAIL R2/DR5, Fas/TNFRSF6/CD95 and Caspase-8/Caspase-8Ap2 highlights the involvement of the extrinsic pathway. Similar results have been reported when treated with several different flavonoids and various cell lines (19,20,56,57). Based on the aforementioned results, it may be stated that luteolin activates the extrinsic and the intrinsic pathway of apoptosis, in agreement with certain previous studies; however, other studies have reported that luteolin induces either the extrinsic or intrinsic pathway exclusively (46,47,49). In cancer cells, alterations of p27 protein-protein interaction alters p27 from a CDK inhibitor to an oncogene.

Activation of the AKT/RAS-RAF pathway by phosphorylation averts apoptosis; however, dephosphorylation likely induces apoptosis (57,58). BAD is phosphorylated at Ser-136 and Ser-112 by AKT and RAS/RAF, respectively (58). Phosphorylation of BAD leads its association with 14-3-3 protein, whereas dephosphorylation of BAD results in its dimerization with BCL2 family proteins, and accumulation in the mitochondrial membrane, in turn increasing apoptosis (59). AKT may further activate other pathways and molecules, including the MTORC pathway (60), and is

initiated by various growth factors and cytokines through receptor tyrosine kinases, including HER, and promotes cell survival by inactivating pro-apoptotic proteins and the forkhead (FoxO1/3a) transcription factors (61). Luteolin treatment of HeLa cells decreased the expression levels of important members of AKT/PI3K signaling; specifically, AKT1, AKT2, MTOR, PIK3CD, PIK3C2A and PIK3C2B were downregulated. Luteolin also modulated the phosphorylation of members of the AKT signaling pathway, including P53 (p-ser241) and P27 (p-Thr198), which were significantly upregulated, while GSK3b (p-ser9) was slightly downregulated, MTOR (p-ser2448), PRAS 40 (p-Thr246), BAD (p-ser112), PTEN (p-ser380) and AKT (p-ser473) were more notably downregulated, and ERK2 (p-Y185/Y187, RISK2 (p-ser386), P70S6k (p-Thr421/ser424), PDK1 (p-ser241) and ERK1 (p-T202/Y204) were significantly downregulated. Several studies have reported similar results; however, with fewer phosphorylated proteins assessed. Therefore, the present study is the first to extensively demonstrate the molecular effects of luteolin-mediated modification of phosphorylated proteins involved in the AKT pathway, to the best of our knowledge (14,18,21). The MAPK pathway serves a potential role in cell proliferation and differentiation; luteolin treatment of HeLa cells showed downregulation of various members of the MAPK signaling pathway, including *MAPK1*, *MAK14*, *MAPK3* and *MAP3K5*, a substantial decrease in gene expression was observed in *ELK1* and *ESR1* together with an increase in *PTPRR*, which is an inhibitor of the MAPK pathway. Notably, flavonoids have been demonstrated to inhibit the AKT/MTOR/MAPK signaling pathways through modulation of various molecules involved in these pathways (20,22,48,49,61).

Plant-derived agents, such as flavonoids, including luteolin, have proven to be potentially effective options for the treatment of cancer through modulation of apoptotic pathways. However, the effect of these agents vary notably when comparing their effects *in vitro* and *in vivo*, and this may be due to their low bioavailability *in vivo*. Several studies have attempted to improve the bioavailability of various agents, with success in certain cases; quercetin treatment using a nano delivery system improved its effectiveness in *in-vivo* studies (62,63). As the majority of the results cited regarding luteolin in the present study are based on *in vitro* and *in vivo* studies, and these do not necessarily always accurately represent the outcomes observed in humans, additional studies on the different pharmacokinetic parameters are required, as well as human clinical trials, before it can be considered a suitable treatment for cancer. Further study on other cancer cells, including blood tumor cells, will support the use of luteolin in a broad spectrum.

In conclusion, the various effects of luteolin highlight its efficacy on the inhibition of cell proliferation, depolarization of the mitochondrial membrane potential, induction of cell cycle arrest and impeding the RAS-RAF/MAPK/AKT/PI3K signaling pathway. Therefore, induction of apoptosis in HeLa cells may qualify it as a potential chemo-preventive agent. However, it is first necessary to perform additional *in vivo* studies and clinical trials to standardize the dosage and assess the safety of luteolin, before it can be recommended as a treatment for cervical cancer treatment.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

RR and SP performed the experiments, collected the data and wrote the manuscript. NR and SH revised the manuscript and validated the data. JS and KB analyzed and validated the data. MH and AH conceived and designed the study, validated the data and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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