

# Kaempferol induces ATM/p53-mediated death receptor and mitochondrial apoptosis in human umbilical vein endothelial cells

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**Abstract.** Kaempferol is a member of the flavonoid compounds found in vegetables and fruits. It is shown to exhibit biological impact and anticancer activity, but no report exists on the angiogenic effect of kaempferol and induction of cell apoptosis *in vitro*. In this study, we investigated the role of kaempferol on anti-angiogenic property and the apoptotic mechanism of human umbilical vein endothelial cells (HUVECs). Our results demonstrated that kaempferol decreased HUVEC viability in a time- and concentration-dependent manner. Kaempferol also induced morphological changes and sub-G1 phase cell population (apoptotic cells). Kaempferol triggered apoptosis of HUVECs as detecting by DNA fragmentation, comet assay and immunofluorescent staining for activated caspase-3. The caspase signals, including caspase-8, -9 and -3, were time-dependently activated in HUVECs after kaempferol exposure. Furthermore, pre-treatment with a specific inhibitor of caspase-8 (Z-IETD-FMK) significantly reduced the activity of caspase-8, -9 and -3, indicating that extrinsic pathway is a major signaling pathway in kaempferol-treated HUVECs. Importantly, kaempferol promoted reactive oxygen species (ROS) evaluated using flow cytometric assay in HUVECs. We further investigated the upstream extrinsic pathway and showed that kaempferol stimulated death receptor signals [Fas/CD95, death receptor 4 (DR4) and DR5] through increasing the levels of phosphorylated p53

and phosphorylated ATM pathways in HUVECs, which can be individually confirmed by N-acetylcysteine (NAC), ATM specific inhibitor (caffeine) and p53 siRNA. Based on these results, kaempferol-induced HUVEC apoptosis was involved in an ROS-mediated p53/ATM/death receptor signaling. Kaempferol might possess therapeutic effects on cancer treatment in anti-vascular targeting.

## Introduction

Angiogenesis is an important physiological process during promoting tumor growth or in metastatic tumors (1). Suppression of endothelial cell proliferation or induction of cell apoptosis is a good strategy for blocking tumor angiogenesis (2). The human umbilical vein endothelial cells (HUVECs) are the most widely used endothelial cell model, which can be examined through many processes for anti-angiogenic actions (3). Moreover, the induction of endothelial cell apoptosis is one of the central antiangiogenic mechanisms (3,4). Two major important pathways contribute to the apoptotic processes, including the intrinsic mitochondria-mediated pathway and the extrinsic death receptor signaling (5). Mitochondrial permeability can be regulated to release various apoptotic factors such as cytochrome *c*, Apaf-1 and pro-caspase-9 to cytosol to form apoptosome and to activate the downstream of caspase-9 (6,7). The membrane death receptors (extrinsic apoptotic pathway) located in the membrane include Fas/CD95, death receptor 4 (DR4) and DR5 that can influence the distal executioner caspases (6). In addition, reactive oxygen species (ROS) production and DNA damage caused by anticancer drugs lead to an increase of phosphorylation of ataxia-telangiectasia-mutated kinase (ATM) and p53 to trigger human cancer cell apoptosis (8). p53 phosphorylation on the residue of Ser15 has been linked to apoptosis and shown to be a transcription factor to modulate apoptotic target genes such as Fas and DR5 (9). p53 gene expression has been shown to upregulate both the extrinsic and the intrinsic apoptotic signaling pathways (6,10).

Kaempferol is a dietary flavonoid and is found in fruits and vegetables and in traditional Chinese medicines (11,12).

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The pharmacological activities of kaempferol were reported to exhibit anti-inflammatory, antioxidant, cardio-protective and antitumor activities (12). Our previous study demonstrated that kaempferol-induced apoptosis in human osteosarcoma cells is mediated through endoplasmic reticulum stress and mitochondria-dependent signaling (13). Kaempferol also induces autophagy by AMPK and AKT signaling and causes G2/M phase arrest via downregulation of CDK1/cyclin B in human hepatocarcinoma cells (14). However, there is no report addressing the possible anti-angiogenetic mechanism of kaempferol. The objective of the current study was to explore apoptotic evidence and its underlying molecular mechanism induced by kaempferol in HUVECs. Kaempferol might induce both the extrinsic and the intrinsic apoptotic pathways in HUVEC cells through ROS-mediated p53/ATM/death receptor signaling.

## Materials and methods

**Chemicals and reagents.** Caffeine, 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), kaempferol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N-acetylcysteine (NAC) and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Medium 200, Low Serum Growth Supplement (LSGS), Trypsin-EDTA, 2,7-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) and Fluo-4/AM were purchased from Thermo Fisher Scientific (Carlsbad, CA, USA). Caspase-3, Caspase-8 and Caspase-9 Colorimetric Assay kits and caspase-8 inhibitor Z-IETD-FMK were bought from R&D Systems Inc. (Minneapolis, MN, USA). Primary antibodies [Fas/CD95, DR4, DR5, p-ATM (Ser1981), ATM and  $\beta$ -actin], horseradish peroxidase (HRP)-conjugated secondary antibodies against rabbit or mouse immunoglobulin and p53 siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-caspase-3, -8 and -9 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against p53 and p-p53 (Ser15) were obtained from Abcam (Cambridge, Cambridgeshire, UK).

**Cell culture.** Human umbilical vein endothelial cells (HUVECs) were purchased from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan) and cultured in Medium 200 plus LSGS at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. The cells were used between the second to fifth passages.

**Cell viability.** HUVECs were plated onto 96-well microplates at a density of 5x10<sup>3</sup> cells/100  $\mu$ l per well and then incubated with kaempferol at the concentrations of 0, 50, 100, 150 and 200  $\mu$ M for 24, 48 and 72-h treatment. Cell viability was determined by MTT assay as previously described (15), and the optical density ratio of the treatment to the control (% of control) was calculated.

**Cell morphological detection and DNA content analysis by flow cytometry.** HUVECs were treated with 100  $\mu$ M kaempferol for 24 and 48 h. The cells were examined and photographed using a phase-contrast microscope. Then the cells were collected and fixed in 75% ethanol overnight at -20°C before being stained with 0.1 M phosphate/citric acid buffer (0.2 M NaHPO<sub>4</sub> and 0.1 M citric acid, pH 7.8) and 40  $\mu$ g/ml

PI for 30 min at room temperature in the dark. The cells were determined with BD FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA) as previously described (6).

**DAPI staining and comet assay.** HUVECs were treated with 100  $\mu$ M kaempferol for 48 h. The cells were fixed and incubated with 1  $\mu$ g/ml DAPI following a previously reported method (6,16). After being harvested, cells were combined with molten low-melting agarose (Sigma-Aldrich) at a density of 1x10<sup>5</sup> cells/ml. The agarose-cell mixture (50  $\mu$ l) was immediately pipetted onto comet slides. The slides were then immersed in pre-chilled lysis solution for 30 min at 4°C as previously described (10). After lysis, horizontal electrophoresis was performed for 30 min at 300 mA. The slides were fixed by 70% ethanol for 5 min before being stained with 50  $\mu$ l nuclear counterstain DAPI solution (final concentration: 1  $\mu$ g/ml) and viewed under a fluorescence microscope.

**Immunofluorescence staining.** HUVECs (5x10<sup>4</sup> cells/well) on 4-well chamber slides were treated with 100  $\mu$ M kaempferol for 24 h. Cells were fixed in 3% formaldehyde (Sigma-Aldrich) for 15 min, permeabilized with 0.1% Triton-X 100 in PBS for 1 h with blocking of non-specific binding sites using 2% bovine serum albumin (BSA) as previously described (6,17). These fixed cells were stained with cleaved caspase-3 antibody (1:100 dilution, Cell Signaling Technology) overnight before being detected using a goat anti-mouse IgG secondary antibody conjugated fluorescein isothiocyanate (FITC) (1:500 dilution, green fluorescence) (Merck Millipore, Billerica, MA, USA), followed by nuclei counterstaining using and PI (red fluorescence). Images were collected with a Leica TCS SP2 Confocal Spectral Microscope (Leica Microsystems, Heidelberg, Mannheim, Germany)

**Determination of caspase-3/-8/-9 activities and effects of their specific inhibitors.** HUVECs (5x10<sup>6</sup> cells) were pretreated with or without 10  $\mu$ M Z-IETD-FMK (a specific caspase-8 inhibitor) for 1 h and incubated in 75-T flasks and treated with kaempferol for 24 and 48 h. After treatment, cells were harvested and lysed, and cell lysates (50  $\mu$ g proteins) were incubated to check relative caspase activity using Caspase-3, Caspase-8 and Caspase-9 Colorimetric Assay Kits (R&D Systems Inc.) following the manufacturer's instructions.

**Measurements of intracellular Ca<sup>2+</sup> levels and mitochondrial membrane potential ( $\Delta\Psi$ m).** HUVECs were treated with 100  $\mu$ M kaempferol for 6, 12 and 24 h. Cells were then harvested and labeled with 2  $\mu$ M Fluo-4/AM (a specific intracellular Ca<sup>2+</sup> fluorescence probe) and 500 nM DiOC<sub>6</sub>(3), respectively, at 37°C for 30 min. Consequently, intracellular Ca<sup>2+</sup> and  $\Delta\Psi$ m were individually analyzed for fluorescence intensity by flow cytometry as previously described (17).

**Western blot analysis.** HUVECs (5x10<sup>6</sup> cells) were incubated in 100  $\mu$ M kaempferol for 0, 12 or 24 h. After being harvested and lysed, the 10% SDS-polyacrylamide electrophoresis (SDS-PAGE) gels were used to separate equal amount of protein extract from cell lysate as detailed by Yang *et al* (18). The appropriate the primary antibodies were hybridized to observe the specific protein signals. Then the HRP-conjugated

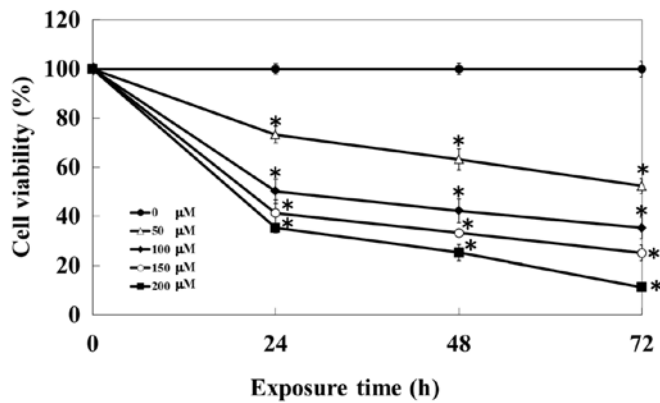


Figure 1. Kaempferol reduces cell viability in HUVECs. Cells were exposed to various concentrations of kaempferol (0, 50, 100, 150 or 200  $\mu$ M) for 24, 48 and 72 h and determined and analyzed the viability using the MTT assay. The values are presented as the means  $\pm$  SD (n=3). \*P<0.05 versus untreated control.

secondary antibodies were applied before using Immobilon Western HRP substrate kit (Merck Millipore). The densitometric quantification of each band was performed utilizing NIH ImageJ 1.47 software.

**Measurements of ROS production after N-acetylcysteine and caffeine pre-treatment for cell viability.** HUVECs were treated with 100  $\mu$ M kaempferol for 6, 12 and 48 h. Cells were then harvested and labeled with 20  $\mu$ M H<sub>2</sub>DCFDA (a specific ROS fluorescent probe) at 37°C for 30 min. Consequently, ROS was analyzed for fluorescence intensity by flow cytometry as previously described (19). Cells were incubated with 100  $\mu$ M kaempferol for 48 h before individual pretreatment with or without the 10 mM N-acetylcysteine (NAC, an antioxidant) or 1 mM caffeine (an ATM kinase inhibitor) for 1 h. After that, cells were determined for cell viability by MTT assay as described above.

**Small interference RNA transfection.** HUVEC cells were grown to 70% confluence in 6-well culture plates, and control siRNA (100 nM) or p53 siRNA (100 nM) was transfected using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. After transfection, cells were seeded and thereafter exposed to 100  $\mu$ M kaempferol for 48 h before analyses using western blot and MTT assay, respectively.

**Statistical analysis.** The data represent the mean  $\pm$  standard deviation (SD) from at least three separate experiments. Statistical analysis was carried out using Student's t-test, and P<0.05 was considered statistically significant.

## Results

**Kaempferol induces growth inhibition in HUVECs.** At first, our study focused on the growth inhibition effects of kaempferol on HUVECs. Cells were treated with 0, 50, 100, 150 and 200  $\mu$ M of kaempferol, and cell number was counted at 24, 48 and 72 h. Our results showed that kaempferol decreased viable HUVECs in a concentration- and time-dependent manner

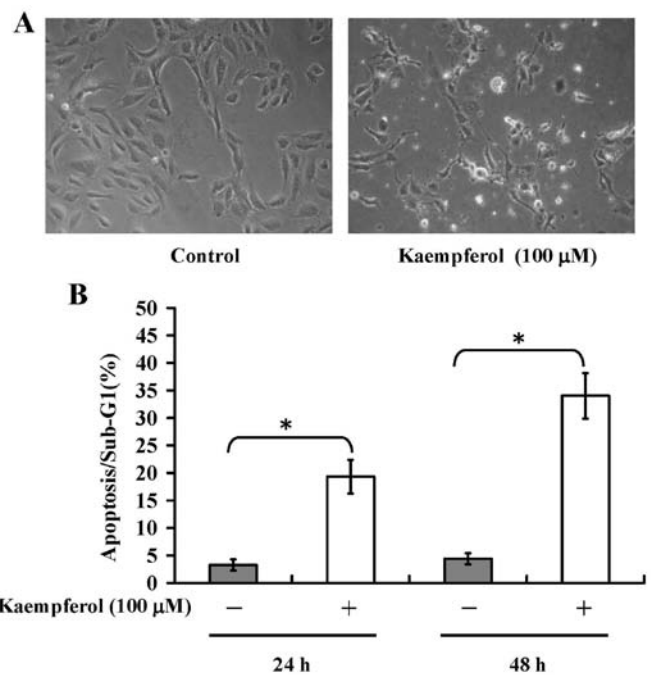


Figure 2. Kaempferol induces morphological changes and hypodiploid DNA contents in HUVECs. (A) Cells in response to 100  $\mu$ M kaempferol for a 24-h exposure were photographed at x20 magnification and showed apoptotic morphological changes. (B) Cells were treated with 100  $\mu$ M of kaempferol for 24 and 48 h. Cells with hypodiploid DNA contents (%) represent the fractions undergoing apoptotic DNA degradation by flow cytometry and the Modfit program. The values are presented as the means  $\pm$  SD (n=3). \*P<0.05 versus untreated control.

(Fig. 1). The IC<sub>50</sub> of kaempferol was 103.25 $\pm$ 4.15  $\mu$ M after 24-h treatment.

**Kaempferol triggers morphological changes and apoptosis in HUVECs.** To understand whether apoptotic mechanisms are involved in kaempferol-treated HUVECs, the morphological changes and DNA content using flow cytometric analysis were investigated. Our results showed that HUVECs were detached from the surface of the plate and showed shrinkage in kaempferol-treated cells (Fig. 2A, right), and the control group showed normal morphology (Fig. 2A, left). The results from the DNA content demonstrated that kaempferol induced an increase of hypodiploid apoptotic cell population (sub-G1 phase) at 24 and 48 h treatments (Fig. 2B). These effects are time-dependent. Our results indicated that kaempferol provoked apoptotic cell death in HUVECs.

**Kaempferol induces DNA condensation, DNA damage and caspase-3 protein expression in HUVECs.** To confirm the apoptotic evidence in kaempferol-treated HUVECs, DAPI stain for DNA condensation and comet assay for DNA damage were monitored. Our results showed that kaempferol induced DNA condensation (Fig. 3A) and DNA damage (Fig. 3B) in HUVECs cells. It is well known that caspase-3 is a key mediator of cell apoptosis (13,14). Next, we used caspase-3 immunofluorescence staining and confocal laser scanning microscopy to observe the caspase-3 protein expression. The caspase-3 protein expression (green color) was observed in the cytosol of kaempferol-treated HUVECs (Fig. 3C). Our results

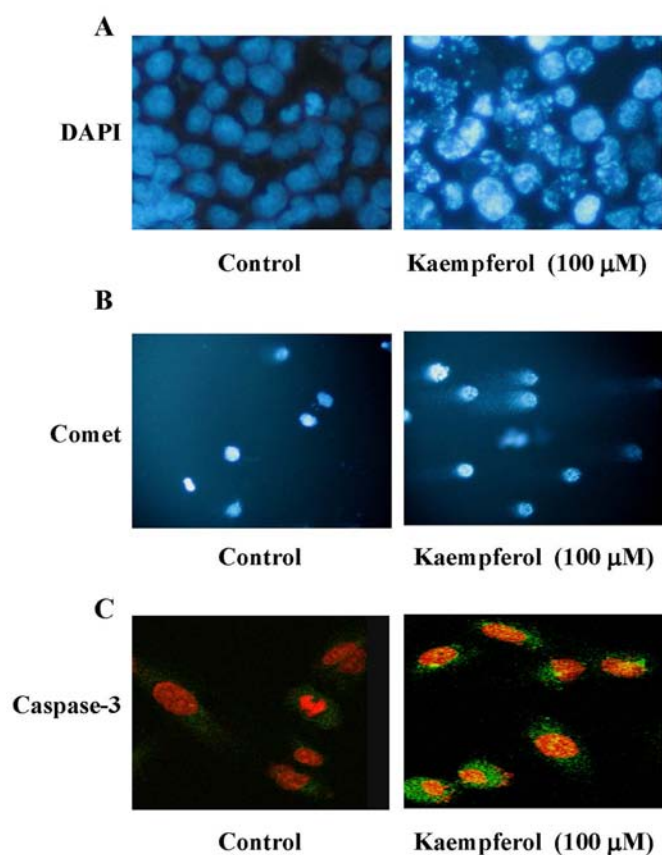


Figure 3. Kaempferol induces DNA condensation, DNA damage and caspase-3 protein expression in HUVECs. Cells were treated with 100  $\mu$ M kaempferol for 48 h. (A) DAPI staining to analyze chromatin condensation (a cataphoretic of apoptosis). (B) Comet assay to analyze DNA damage. The images were observed and captured using a fluorescent microscope. (C) Kaempferol stimulated the translocation of caspase-3 trafficking to nuclei in HUVECs by confocal laser scanning microscope as described in Materials and methods.

demonstrated that kaempferol provoked apoptotic cell death through DNA damage and caspase-3 activation in HUVECs.

*Kaempferol stimulates intracellular  $Ca^{2+}$  levels and loss of  $\Delta\Psi_m$  in HUVECs.* To determine the roles of intracellular  $Ca^{2+}$  levels and  $\Delta\Psi_m$  levels of apoptotic death induced by kaempferol, we detected the intracellular  $Ca^{2+}$  by Fluo-4/AM dye and  $\Delta\Psi_m$  by DiOC<sub>6</sub>(3) dye at 6, 12 and 24 h, respectively. Kaempferol increased intracellular  $Ca^{2+}$  levels (Fig. 4A) and depletion of  $\Delta\Psi_m$  (Fig. 4B) in HUVECs. The data indicated that kaempferol-provoked apoptotic death in HUVECs might be mediated through  $Ca^{2+}$  signal and mitochondrial pathway.

*Kaempferol induces the activity of caspase-8, -9 and -3 in HUVECs.* To determine the major caspase pathway of apoptotic death induced by kaempferol, we further detected the activity of caspase-8, -9 and -3 at 24 and 48 h, respectively. Our data indicated that the activities of caspase-8, -9 and -3 were significantly increased in kaempferol-treated HUVECs in a time-dependent manner (Fig. 5A). These results suggested that both the intrinsic mitochondria-mediated pathway and the extrinsic death receptor signaling are involved in kaempferol-induced apoptosis in HUVECs. To confirm our suggestion, we used western blotting to detect the cleaved form of caspase-8,

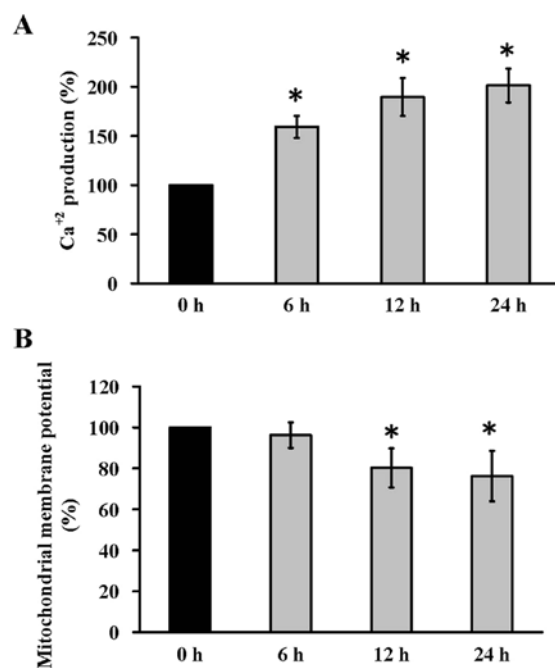


Figure 4. Kaempferol stimulates intracellular  $Ca^{2+}$  levels and loss of  $\Delta\Psi_m$  in HUVECs. (A) Intracellular  $Ca^{2+}$  levels of HUVECs treated with 100  $\mu$ M kaempferol as determined by Fluo-4/AM fluorescent dye using flow cytometry analysis. (B) The levels of HUVECs after kaempferol treatment as determined by DiOC<sub>6</sub>(3) fluorescent dye using flow cytometry analysis. The values are presented as the means  $\pm$  SD (n=3). \*P<0.05 versus untreated control.

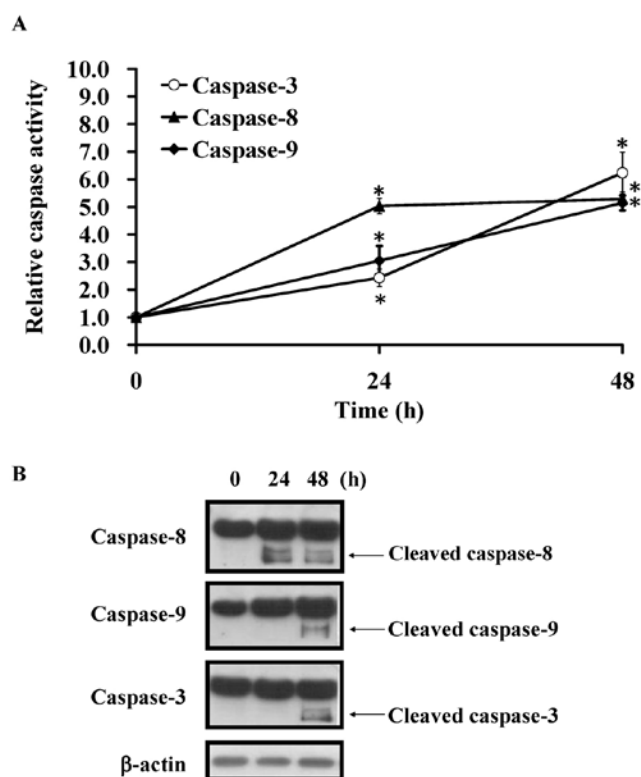


Figure 5. Kaempferol triggers caspase-8/-9/-3 activities and protein levels in HUVECs. Cells were treated with 100  $\mu$ M kaempferol for 0, 24 and 48 h. (A) Kaempferol-stimulated the activities of caspase-8, -9 and -3 in HUVECs were detected by colorimetric assay kits. The values are presented as the means  $\pm$  SD (n=3). \*P<0.05 versus untreated control. (B) The total proteins were harvested and determined the protein levels of caspase-8, -9 and -3 by western blotting.  $\beta$ -actin served as a loading control.

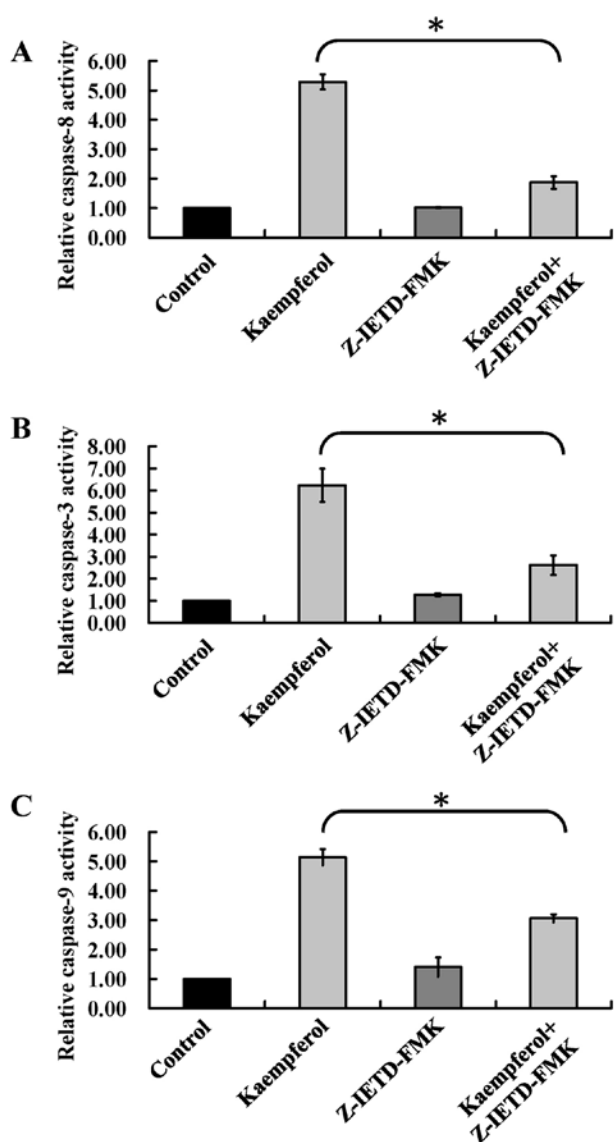


Figure 6. Caspase-8 inhibitor (Z-IETD-FMK) decreases kaempferol-induced caspase-8/-9/-3 activities in HUVECs. Cells were pretreated with 10  $\mu$ M caspase-8 inhibitor (Z-IETD-FMK) for 2 h prior to treatment with 100  $\mu$ M kaempferol for 48 h. Cells were harvested for measuring the caspase-8 (A), caspase-9 (B) and caspase-3 (C) activities as described in Materials and methods. The values are presented as the means  $\pm$  SD (n=3). \*P<0.05 versus kaempferol alone sample.

-9 and -3. Our results showed that the cleaved caspase-8, -9 and -3 protein level were significantly increased in HUVECs prior to kaempferol challenge at 48 h (Fig. 5B). Strikingly, caspase-8 activity was significantly increased at 24-h treatment in treated HUVECs. The data indicated that extrinsic death receptor pathway is a key signal in kaempferol-induced apoptosis of HUVECs.

*Z-IETD-FMK blocks the activity of caspase-8, -9 and -3 in kaempferol-treated HUVECs.* Our hypothesis that kaempferol-provoked apoptosis is mediated mainly through extrinsic death receptor pathway. To prove our hypothesis, Z-IETD-FMK (a specific caspase-8 inhibitor) was used to block caspase-8, -3, and -9 activities. Our results demonstrated that pre-incubation with the specific inhibitor of Z-IETD-FMK strongly decreased

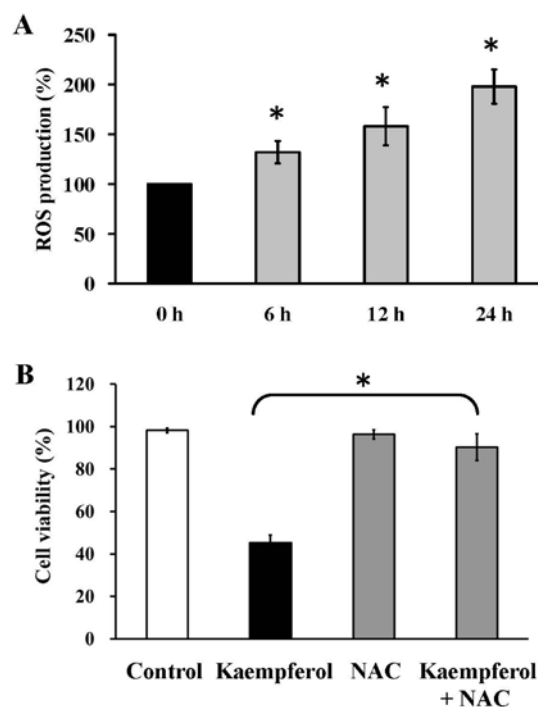


Figure 7. Kaempferol induces ROS generation and N-acetylcysteine reduces kaempferol-induced growth inhibition effect on HUVECs. (A) Treatment with 100  $\mu$ M kaempferol for 0, 6, 12 and 24 h was subjected to ROS productions by flow cytometry. (B) Pre-treatment with NAC (10 mM, a ROS scavenger) in kaempferol-treated HUVECs restored the cell viability by MTT assay. The values are presented as the means  $\pm$  SD (n=3). \*P<0.05 versus untreated control or kaempferol alone sample.

the activity of caspase-8 (Fig. 6A), caspase-3 (Fig. 6B), and caspase-9 (Fig. 6C) compared with kaempferol treatment alone. Overall, these data demonstrated that extrinsic death receptor pathway is a crucial element in kaempferol-triggered apoptosis of HUVECs.

*Kaempferol increases ROS generation and N-acetylcysteine reduces kaempferol-induced growth inhibition effect in HUVECs.* Our findings showed that kaempferol increased intracellular ROS production at 6, 12 and 24 h in HUVECs by using flow cytometry and H<sub>2</sub>DCFDA (a specific fluorescent probe) (Fig. 7A). Cells showed a significant inhibitory effect on kaempferol-induced growth inhibition after pre-treatment with NAC (Fig. 7B). These data indicated that ROS production is important in kaempferol-triggered apoptosis of HUVECs.

*ATM-p53-mediated death receptor pathway is involved in kaempferol-induced apoptosis.* It was reported that ROS can modulate death receptor pathway in cancer cells (8,10). Our hypothesis showed that extrinsic death receptor pathway is a central component in kaempferol-triggered apoptosis of HUVECs. The results revealed that kaempferol stimulated the death receptor-associated protein levels, including Fas/CD95, DR4 and DR5 in HUVECs (Fig. 8A). It is well documented that p53 gene and its phosphorylation at the Ser15 interact Fas/CD95 activation during cell apoptosis (6,20). To elucidate the possible signaling pathway in kaempferol-provoked apoptosis, the levels of associated proteins were evaluated. Kaempferol increased the protein level of ATM, p53, phosphorylation of

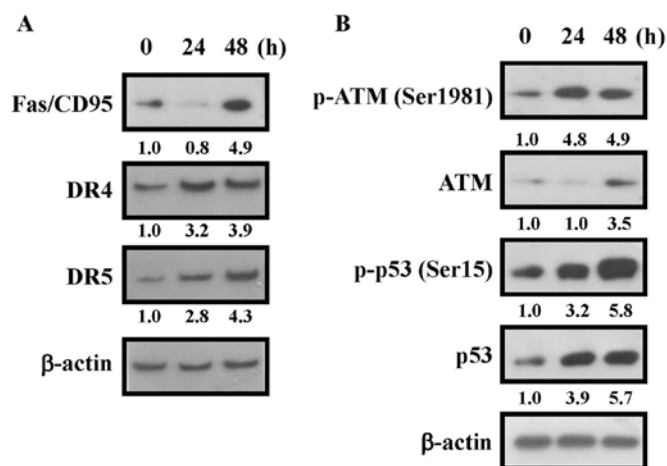


Figure 8. Kaempferol contributes to p53-correlated ATM/Fas/DR4/DR5 apoptotic signaling in HUVECs. Cells were treated with 100  $\mu$ M kaempferol for 0, 24 and 48 h, and total proteins were prepared and subjected to western blot analysis. The membranes were incubated with (A) anti-Fas/CD95, anti-DR4 and anti-DR5 antibodies; (B) p-ATM (Ser1981), ATM, p-p53 (Ser15) and p53 antibodies. The blot was probed with anti- $\beta$ -actin antibody to confirm equal loading. Each band was quantified using ImageJ software.

ATM and p53, followed by increasing levels of Fas, DR4 and DR5 based on the exposure time (Fig. 8B). Our results indicated that kaempferol increased the protein level of Fas/CD95, DR4 and DR5 through the ATM-p53-dependent regulation of transcription levels. We also re-checked the kaempferol-caused ATM-p53-dependent signal in HUVECs, and caffeine (an ATM kinase inhibitor) and p53 siRNA were used to block ATM and p53 function. Pre-treatment with caffeine reversed the inhibition of cell viability in treated group (Fig. 9A). Moreover, p53 siRNA also had a similar effect in kaempferol-treatment group (Fig. 9B). The results from our experimental approaches indicate that kaempferol-induced apoptosis of HUVECs is mediated through ATM-p53-mediated pathway.

## Discussion

Kaempferol is a flavonol present in fruits and vegetables, including onions, kale, broccoli, apples, cherries, berries, tea and red wine (11,12). Kaempferol has many biological properties, including anti-cancer, antioxidant activity, anti-inflammatory effects (12,21). Kaempferol induces apoptosis and cell cycle arrest in various cancer cell lines, including colon cancer (22), liver cancer (23), gastric cancer (24), and bladder cancer (25) cells. Kim *et al* (26) demonstrated that kaempferol can modulate angiogenesis and immune-endothelial cell adhesion. Zhao *et al* (27) showed that kaempferol from Pu-erh tea has anti-cancer and anti-angiogenesis effects. Currently, the mechanism involved in the kaempferol anti-angiogenesis effects is unknown. In this study, we are the first to report that kaempferol induced growth inhibition (Fig. 1) and apoptosis (Figs. 2 and 3) in HUVEC cells. Our results also showed that kaempferol induced the activity of caspase-8, -9 and -3 (Fig. 4) and Fas/CD95, DR4 and DR5 at protein levels (Fig. 8) in HUVEC cells. Moreover, major cell signaling involved in kaempferol-treated HUVECs were investigated, we focused on the ROS-ATM-p53 signaling. Our results demonstrated that kaempferol induced ROS production (Fig. 7), ATM,

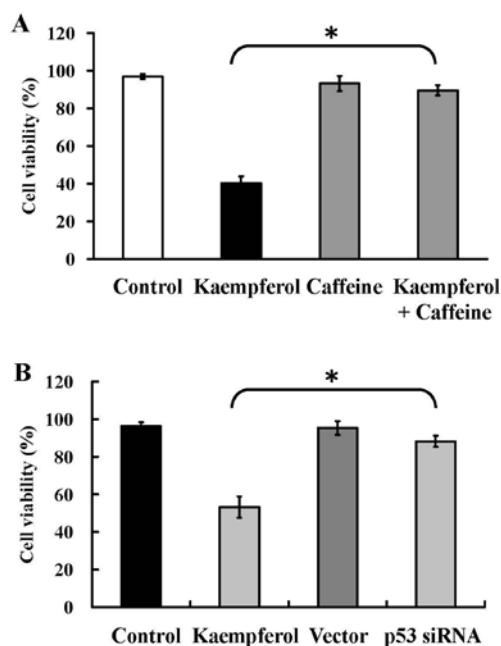


Figure 9. Caffeine and p53 siRNA reduce kaempferol-induced growth inhibition effect in HUVECs. (A) Cells were pretreated with or without 2 mM caffeine (an ATM/ATR inhibitor) for 2 h and further incubated with 100  $\mu$ M kaempferol. After a 48-h exposure, cell viability was calculated by MTT assay. (B) HUVECs after being transfected with or without control vector or p53 siRNA were exposed to 100  $\mu$ M kaempferol. Transfected HUVECs following exposure to kaempferol for 48 h was determined for cell viability by MTT assay. The values are presented as the means  $\pm$  SD (n=3). \*P<0.05 versus kaempferol alone sample.

p53, phosphorylation of ATM and phosphorylation of p53 protein levels (Fig. 9). We used the specific inhibitors that include Z-IETD-FMK (a specific caspase-8 inhibitor), N-acetylcysteine (NAC, an antioxidant), caffeine (an ATM kinase inhibitor) and p53 siRNA to confirm this pathway. We found that kaempferol triggered HUVEC apoptosis through the ROS-mediated ATM/p53 signaling.

The p53 tumor suppressor protein is an essential regulator in controlling cell growth and cell death (6,20). In response to intracellular and extracellular stress, p53 is activated and serves as a transcription factor that orchestrates various targets, which in turn modulate multitude of cellular processes such as DNA repair, cell cycle arrest and apoptosis (28,29). It is reported that p53-inducible pro-apoptotic genes trigger apoptosis through both the extrinsic and the intrinsic apoptotic molecular pathways (30). Our results showed that kaempferol significantly increased ROS production (Fig. 7A) and the protein levels of Fas/CD95, DR4, DR5, ATM, p-ATM (Ser1981), p53, and p-p53 (Ser15) in HUVECs (Fig. 8). In addition, knockdown of p53 expression by p53 siRNA significantly inhibited the cell growth inhibitory effects (Fig. 9A) after treatment with kaempferol in HUVECs. Based on our results, we suggest that p53 might be involved in kaempferol-upregulated death receptor signaling.

In addition to the death receptor pathway, our results suggest that kaempferol induced apoptosis through mitochondria-dependent pathway. The elevation of DiOC<sub>6</sub>(3) fluorescence indicated the loss of  $\Delta\Psi_m$  in kaempferol-treated HUVECs (Fig. 4B). The dissipation of  $\Delta\Psi_m$  is attributed to the opening of mitochondrial permeability transition (MTP) pore. Hence,

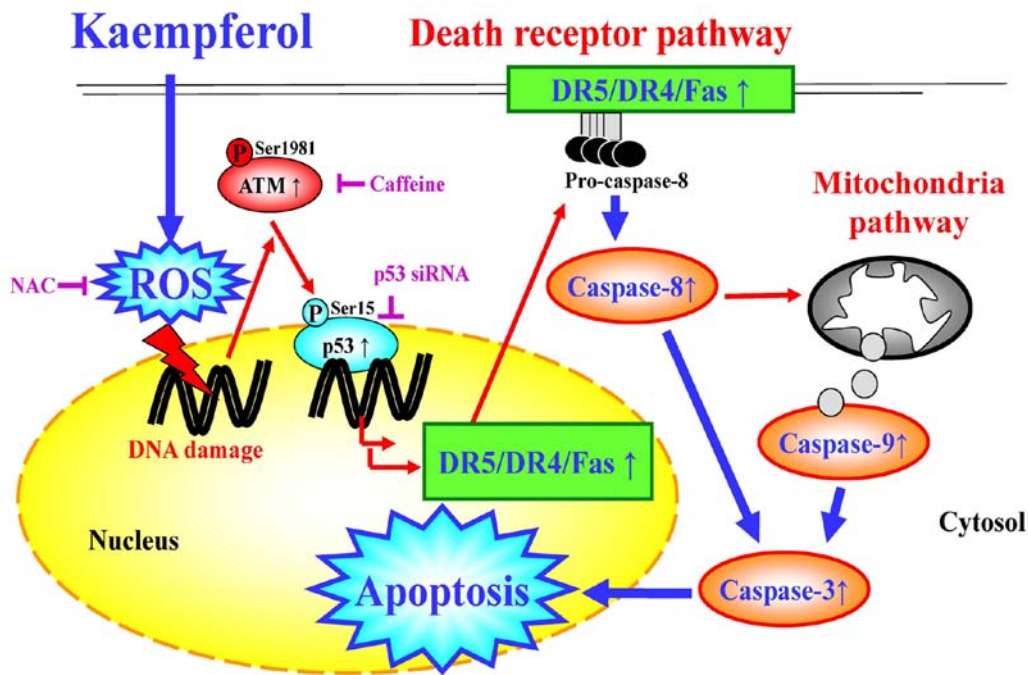


Figure 10. A proposed model for the action and possible signaling pathways of kaempferol on HUVECs. Kaempferol induces apoptosis through both extrinsic and intrinsic apoptotic pathways, resulting from p53-mediated ATM/Fas/DR4/DR5 signaling, which counteracts the induction of apoptotic death in HUVECs.

we suggest that kaempferol led to the persistent opening of the MTP pore, which resulted in mitochondrial swelling and the rupture of mitochondrial outer membrane, ultimately the release of intermembrane proteins such as cytochrome *c*, Apaf-1, pro-caspase-9, AIF and Endo G that trigger cell apoptosis (6,19).

Oxidative stress is closely related to cancer and often associated with cancer prevention and cancer therapy agents (15,31). It was report that reactive oxygen species (ROS) not only function as a regulator of subcellular events but are also able to induce cell apoptosis (8). Yang *et al* (32) demonstrated that kaempferol reduced the glutamate-induced oxidative stress in mouse-derived hippocampal neuronal HT22 cells. Ondricek *et al* (33) showed that kaempferol rescued RGC-5 cells from iodoacetic acid-induced cell death, as well as reduced caspase activation and ROS generation. However, Jeong *et al* (34) demonstrated that kaempferol caused an increase in generation of reactive oxygen species (ROS), and induced cell death in human glioma cells. Kim *et al* (35) also showed that kaempferol induced the generation of fluorescent DCF in the MCF-7 cells, and treatment with N-acetylcysteine suppressed kaempferol-induced PARP cleavage. Sharma *et al* (36) also showed kaempferol in glioblastoma cells induced apoptosis through oxidative stress. In this study, kaempferol was found to be less cytotoxic towards HUVECs after pre-treatment with N-acetylcysteine, suggesting that kaempferol induced oxidative stress in HUVECs (Fig. 7B). Based on the result from H<sub>2</sub>DCFDA assay, surprisingly kaempferol was found to stimulate the ROS formation in HUVECs.

In conclusion, the molecular signaling pathway in HUVECs caused by kaempferol is summarized in Fig. 10. Our study discovered that kaempferol reduced HUVEC viability and induced DNA damage and DNA fragmentation through activating the levels of caspase-3, -8, and -9 signaling, which

were upregulated by ROS-mediated p53/ATM molecules following stimulations of p53 downstream protein levels of Fas/CD95, DR4, and DR5. Our results suggest that kaempferol warrants further development as an anti-angiogenic agent.

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