Research Article

Protective effects of *Angelica keiskei* extract against TNF-α-induced oxidative stress and vascular inflammation in human umbilical vein endothelial cells

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Abstract *Angelica keiskei*, a perennial herb from *Apiaceae* family, has been reported to improve diabetes, inhibit thrombosis, alleviate dyslipidemia, and prevent type 2 diabetes, obesity, and atherosclerosis. In this study, the protective effects of *A. keiskei* extract (AKE) against tumor necrosis factor-alpha (TNF-α)-induced oxidative stress and vascular inflammation in human umbilical vein endothelial cells (HUVECs) were investigated through cell viability analysis, antioxidant enzyme analysis, western blotting, and immunofluorescence staining. The results demonstrated that pretreatment of *Angelica keiskei* with AKE significantly inhibited the expression of key adhesion molecules such as E-selectin, ICAM-1 and VCAM-1 induced by TNF-α. AKE also showed a substantial reduction in intracellular reactive oxygen species levels and an increase in antioxidant enzyme activity, indicating potential antioxidant capabilities. This study further explained that AKE interfered with the nuclear factor-kappa B (NF-κB) pathway by inhibiting phosphorylation of IκBα and NF-κB, thereby preventing nuclear translocation. Additionally, AKE selectively inhibited the activation of c-Jun N-terminal kinase (JNK) within the mitogen-activated protein kinase (MAPK) pathway, revealing a specific action mechanism. These findings collectively suggest that AKE possesses multi-faceted protective properties, making it a potential therapeutic agent for inflammatory conditions and early atherosclerosis.

Keywords *Angelica keiskei*, atherosclerosis, oxidative stress, vascular inflammation, NF-κB

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1. Introduction

Atherosclerosis involves the build-up of lipids within the endothelium, leading to the formation of atheromatous plaques (Falk, 2006). This condition serves as the primary factor behind various cardiovascular diseases, including coronary artery disease, heart failure, stroke, ischemic gangrene, and abdominal aortic aneurysm (Hansson et al., 2006). With an increasing prevalence each year, it has become a significant global health issue, as 32% of worldwide deaths are related to cardiovascular diseases (WHO, 2017). Atherosclerosis develops through interactions of lipid-proteins, monocyte-derived macrophages, T cells, cytokines, and arterial wall cells (Glass and Witztum, 2001; Ross, 1999). The initial crucial event in atherosclerosis is the recruitment of monocytes to the arterial wall, facilitated by interactions between adhesion molecules present on the endothelium and their corresponding ligands on monocytes (Zhu et al., 2013). Pro-inflammatory cytokines and oxidized low-density lipoprotein (LDL) can increase the expression of intercellular adhesion molecule-1

(ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin in endothelial cells (Libby et al., 2010). The expression of vascular adhesion molecules can enhance adhesion of circulating monocytes to the endothelium and promote their transmigration into the subendothelial space (Rao et al., 2007). Tumor necrosis factor-alpha (TNF-α), a highly potent pro-inflammatory cytokine, can induce apoptosis in endothelial cells. It is also associated with the development and progression of atherosclerotic lesions (Dimmeler et al., 1999). The transcription factor nuclear factor-kappa B (NF-κB) plays an important role in regulating the expression of most inflammation-related genes (Beinke and Ley, 2004). It is particularly considered absolutely crucial for TNF-α-induced expression of VCAM and ICAM in endothelial cells (Zhou et al., 2007).

Reactive oxygen species (ROS) refers collectively to free radicals, oxygen ions, and peroxides. They can induce oxidative stress in vascular cells. They are among causes of atherosclerosis (Forstermann, 2008). Indeed, factors such as hypertension, hypercholesterolemia, diabetes, and smoking can increase ROS production in the vascular wall (Forstermann, 2008). Excessive ROS in the vasculature can enhance existing oxidative stress by inhibiting the activity of endothelial nitric oxide synthase (eNOS), leading to the generation of superoxide (Galan et al., 2014). Moreover, ROS can stimulate inflammatory activities of macrophages by inducing the expression of plaque, IL-6, and TNF-α through the NF-κB pathway (Li et al., 2014).

Angelica keiskei, a perennial herbaceous plant in the *Apiaceae* family, hails from the coastal areas along Japan's Pacific coast (Amalia et al., 2021). The plant is called 'Myeong-Il Yeob' in Korea and 'Ashitaba' in Japan, both literally meaning 'Tomorrow's Leaf' (Baba et al., 1998). Another common name for *A. keiskei* is 'Shin-Sun Cho', meaning 'a precious herb used by God' (Park, 2013). Inside the stem, there is a yellowish sap with main components including chalcone, triterpenoid, xanthoangelol, isobavachalcone, luteolin, cynaroside, and others (Kil et al., 2017). *A. keiskei* has been reported to possess anti-inflammatory and antioxidative activities. It has been used to treat various conditions such as diabetes, hypotension, and tumors (Jafari et al., 2023). Furthermore, *A. keiskei* has been reported to exhibit antithrombotic effects (Ohkura et al., 2018), improve dyslipidemia (Ogawa et al., 2005) and demonstrate preventive effects on type 2 diabetes, obesity, and atherosclerosis (Oh et al., 2019).

Currently, research specifically addressing the relationship between atherosclerosis and *A. keiskei* is lacking. This study aimed to bridge this gap by investigating effects of *A. keiskei* ethanolic extract on oxidative stress and vascular cell adhesion molecules in TNF-α-treated human umbilical vein endothelial cells. By elucidating the potential protective properties of *A. keiskei* in the context of atherosclerosis, this research seeks to contribute valuable insights to the existing body of knowledge.

2. Materials and methods

2.1. Reagents and materials

Quanti-MAX™ WST-8 kit and WestGlow™ FEMTO ECL Chemiluminescent Substrate were purchased from BIOMAX (Guri, Korea). Murine TNF-α was obtained from PeproTech (Cranbury, NJ, USA). 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Superoxide dismutase (SOD, cat. no. 706002), glutathione (GSH, cat. no. 703002), and catalase (cat. no.707002) assay kits were obtained from Cayman Chemical (Ann Arbor, MI, USA). Antibodies against ICAM-1, VCAM-1, E-selectin, NF-κB, p-NF-κB, IκB, p-IκB, ERK, p-ERK, p38, p-p38, JNK, pJNK, β-actin, and goat anti-mouse IgG-HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-mouse IgG (H+L) Alexa Fluor plus 488 (A32723), radio-immunoprecipitation assay buffer (RIPA buffer), carboxy-H2DCFDA (general oxidative stress indicator), and eBioscience™ Flow Cytometry Staining Buffer were obtained from Invitrogen (Carlsbad, CA, USA). Diamidino-2-phenylindole (DAPI) was came from Cell Signaling Technology (Beverly, MA, USA). Bradford's assay reagent was came from Bio-Rad Laboratories (Hercules, CA, USA). SDS-PAGE loading buffer was purchased from Biosesang (Seongnam, Korea).

2.2. A. keiskei extract preparation

The *A. keiskei* used in this study was provided by 239 BIO Corporation (Iksan, Korea). The plant was identified and certified by Professor Hong Jun Kim at the College of Oriental Medicine, Woosuk University (Wanju, Korea). A reference specimen (#2023-003) has been stored in our laboratory, Department of Health Management, College of Medical Science, Jeonju University. *A. keiskei* was washed three times with distilled water and then cut into 1 cm sized pieces before being air-dried. The dried sample (10 g) was

soaked in 200 mL of 50% ethanol and extracted at room temperature with agitation at 300 RPM for 48 h. Subsequently, the extract was filtered twice using a 0.45 μm filter paper. Ethanol was then removed using a rotary evaporator (Eyela/ N-1100S-WD, Tokyo, Japan). The remaining solution was freeze-dried to obtain a powdered form of the extract. This was named *A. keiskei* extract (AKE) and stored at -80℃ for later use in experiments. To determine the optimal concentration of the extraction solvent, AKE was prepared using various concentrations of ethanol, and its DPPH, ABTS, and NO scavenging activities were tested. The results indicated that using 50% ethanol as the extraction solvent exhibited the highest antioxidant and anti-inflammatory effects. Therefore, in this study, AKE prepared with 50% ethanol was used.

2.3. Cell culture

Human umbilical vein endothelial cells (HUVECs) and Human Large Vessel Endothelial Cell Basal Medium were came from Thermo Fisher Scientific (Waltham, MA, USA). Cells were cultured at 37℃ under 5% CO_2 conditions.

2.4. Cell viability

HUVECs (2×10^5 cells/mL) were cultured in 96-well cell culture plate for 24 h. Subsequently, AKE (0-200 μg/mL) or TNF-α (0-60 ng/mL) was added to each well. After 20 h, Quanti-Max^{TM} reagent (cat. no. QM1000) (10 μL) was added to each well. Following an incubation period of 4 h, the absorbance was measured at a wavelength of 450 nm.

2.5. DPPH radical scavenging assay

DPPH was dissolved in ethanol at a concentration of 0.3 mM. Subsequently, it was mixed with AKE (0-1,000 μg/mL) at a 1:1 ratio. The mixture was protected from light and allowed to react at room temperature for 20 min. Afterward, 200 μL of the solution was aliquoted into a 96-well plate, and the absorbance was measured at 540 nm using a spectrophotometer (Tecan Group Ltd., Mannedorf, Switzerland). The DPPH radical scavenging activity was calculated as a percentage using the following formula:

DPPH radical scavenging (%)
= [1 - (absorbance of the sample / additional group)
/ absorbance of the control group] × 100   (1)

2.6. ABTS radical scavenging assay

ABTS was dissolved in deionized water at a concentration of 7.4 mM. Potassium persulphate at 2.6 mM was then added to the solution. Radical was then generated by incubating the mixture at 4℃ in the dark for 24 h. Subsequently, the solution was diluted with distilled water to achieve an absorbance of ±0.04 at 734 nm. AKE (0-1,000 μg/mL) was mixed with 50 μL of the ABTS solution. The mixture was protected from light and allowed to react at room temperature for 20 min. Following the reaction, 200 μL of the solution was aliquoted into a 96-well plate, and the absorbance was measured at 734 nm using a spectrophotometer. The ABTS radical scavenging activity was calculated as a percentage using the following formula:

ABTS radical scavenging (%)
= [1 - (absorbance of the sample / additional group)
/ absorbance of the control group] × 100   (2)

2.7. Measurement of antioxidant enzymes

HUVECs (2×10^5 cells/mL) were cultured in 60-mm cell culture dishes for 24 h. Subsequently, these cells were treated with AKE at concentrations of 50 and 100 μg/mL. After 1 h, TNF-α (20 ng/mL) was added. After an additional 24 h, protein extraction was performed using RIPA buffer, followed by quantification. Measurements of antioxidant enzymes were performed without modification according to the manufacturer's protocol using the respective kits: SOD kit (cat. no. 706002), GSH kit (cat. no. 703002), and catalase kit (cat. no.707002).

2.8. ROS measurement

HUVECs (2×10^5 cells/mL) were cultured in 6-well cell culture plate for 24 h. Subsequently, these cells were treated with AKE at concentrations of 50 and 100 μg/mL. After 1 h, TNF-α (20 ng/mL) was added. After 24 h, cells were collected, suspended in flow cytometry staining buffer, and incubated with Carboxy-H2DCFDA (1 μM) for 30 min. The cells were subsequently washed and examined via flow cytometry (Beckman Coulter, Brea, CA, USA) for non-fluorescent and fluorescent signals, allowing the quantification of ROS levels.

2.9. Protein extraction and western blot analysis

HUVECs (2×10^5 cells/mL) were cultured in 60-mm cell

culture dishes for 24 h. Subsequently, these cells were treated with AKE at concentrations of 50 and 100 μg/mL. After 1 h, TNF-α (20 ng/mL) was added. Following protein extraction using RIPA buffer after either 30 min or 24 h, quantification was performed. Subsequently, an equal quantity of protein was separated by SDS-PAGE to segregate based on size, and then transferred onto a PVDF membrane. The membrane underwent a 1 h blocking step with 5% skim milk, followed by overnight incubation with primary antibodies VCAM-1 (1:500 dilution; cat. no. sc-13160), ICAM-1 (1:500 dilution; cat. no. sc-8439), E-selectin (1:500 dilution; cat no. sc-137054), p-NF-κB (1:500 dilution; cat no. sc-271908), NF-κB (1:500 dilution; cat. no. sc-8414), p-IκB (1:500 dilution; cat. no. sc-52943), IκB (1:500 dilution; cat. no. sc-1643), p-ERK (1:500 dilution; cat. no. sc-81492), ERK (1:500 dilution; cat no. sc-514302), p-p38 (1:500 dilution; cat. no. sc-166182), p38 (1:500 dilution; cat. no. sc-1972), p-JNK (1:500 dilution; cat no. sc-293136), JNK (1:500 dilution; cat. no. sc-7345), and β-actin (1:1,000 dilution; cat no. sc-8432) at 4℃. Following three 10 min washes with TBST buffer, the membrane was incubated with a secondary antibody (1:5,000; sc-516102) for 2 h at room temperature. Protein expression was visualized using the WestGlow[TM] FEMTO Chemiluminescent substrate. ImageJ gel analysis software (National Institutes of Health, Bethesda, MD, USA) was used to analyze band density.

2.10. Immunofluorescence staining

HUVECs (2×10^5 cells/mL) were cultured in cell culture slide chamber for 24 h. Subsequently, these cells were treated with AKE at concentrations of 50 and 100 μg/mL. After 1 h, TNF-α (20 ng/mL) was added. After 30 min, the culture medium was removed and cells were fixed in 4% formaldehyde. Subsequent steps included a 10 min incubation with 0.1% Triton X-100, a 1 h blocking step with 1% BSA, overnight incubation with the primary antibody (1:50 dilution; cat no. sc-271908) at 4℃, washing, and a 2 h incubation with the secondary antibody (1:5,000; cat. no. A32723) at room temperature. After completion, slides were observed using a fluorescence microscope (ZEISS, Oberkochen, Germany).

2.11. Statistical analysis

Experimental values are presented as mean±standard deviation (mean±SD). Statistical analyses were conducted using IBM SPSS Statistics 22 (IBM, New York, NY, USA). Inter-group comparisons were made using one-way analysis of variance (ANOVA), with Duncan's multiple range test applied for post hoc analysis to identify significant differences among the groups. A significance level of $p < 0.05$ was used for all statistical tests.

3. Results and discussion

3.1. DPPH and ABTS radical scavenging activities of AKE

Table 1 presents the DPPH and ABTS radical scavenging activities of AKE. DPPH and ABTS radical scavenging activities are reported as IC_{50} values, which represent the concentration needed for the sample to inhibit 50% of the radical activity compared to the control group, based on absorbance change curves relative to sample concentration. The DPPH radical scavenging activity IC_{50} value of AKE was 375.24±8.56 μg/mL, and that of ascorbic acid, a reference drug, was 23.16±3.21 μg/mL. For ABTS, AKE was measured at 568.12±9.23 μg/mL, and ascorbic acid was measured at 35.26±5.57 μg/mL. These results indicate that the ability of AKE to scavenge DPPH and ABTS radicals is less effective than that of ascorbic acid. However, AKE has potential as an antioxidant because it effectively scavenges radicals at relatively low concentrations.

3.2. Evaluation of cytotoxicity of AKE and TNF-α in HUVECs

A WST assay was conducted to investigate the cytotoxicity of AKE and TNF-α in HUVECs. Results showed no toxicity for AKE up to 200 μg/mL (Fig. 1A). TNF-α showed no toxicity up to 80 ng/mL (Fig. 1B). Therefore, in subsequent

Table 1. DPPH and ABTS radical scavenger activity of *Angelica keiskei* extract (AKE)

Samples	DPPH[1] radical scavenging activity (IC_{50}: μg/mL)	ABTS[2] radical scavenging activity (IC_{50}: μg/mL)
AKE	375.24±8.56[3]	568.12±9.23
Ascorbic acid	23.16±3.21	35.26±5.57

[1]DPPH, 2,2-diphenyl-1-picrylhydrazyl.
[2]ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid).
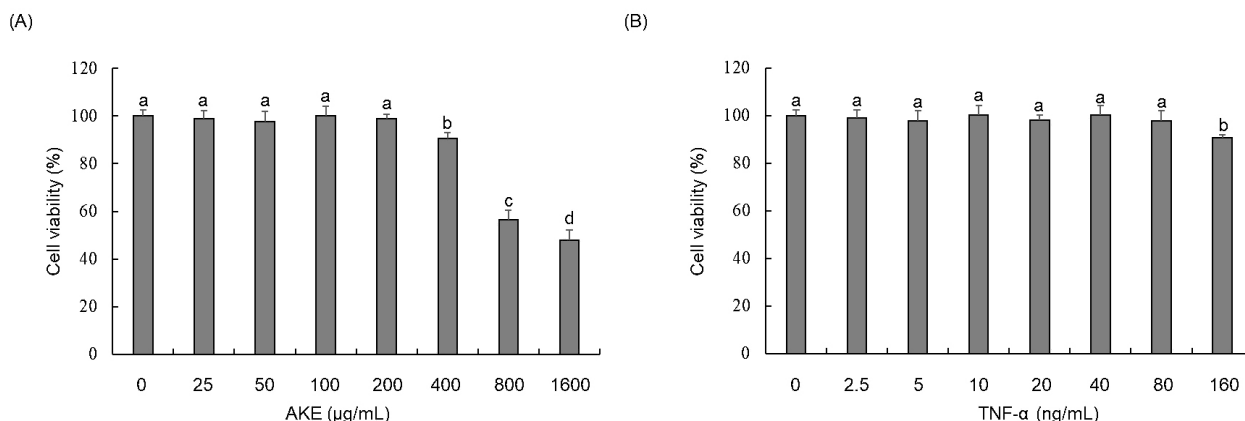[3]All values are mean±SD (n=3).

Fig. 1. Effect of AKE and TNF-α on cell viability in human umbilical vein endothelial cells (HUVECs). HUVECs were pretreated with AKE (A) or TNF-α (B) at the indicated concentrations for 24 h. The cell viability was measured using WST reagent. All values are mean±SD (n=3). Different superscript letters (a-d) on the bars indicate significant differences (p<0.05).

experiments, AKE was administered to cells at concentrations equal to or below 200 μg/mL. TNF-α was applied at concentrations equal to or below 80 ng/mL. TNF-α did not exhibit toxicity to HUVEC at concentrations up to 80 ng/mL, but excessive amounts of TNF-α can induce non-physiological responses or saturate cell receptors, potentially distorting the results. Therefore, we decided to use a treatment concentration of 20 ng/mL, which is commonly used in studies of endothelial cell damage induced by TNF-α (Ismail et al., 2018; Zhu et al., 2013).

3.3. Effects of AKE on intracellular ROS in TNF−α−treated HUVECs

In order to investigate the impact of AKE on intracellular reactive oxygen species (ROS), carboxy-H2DCFDA was administered to TNF-α-treated HUVECs followed by flow cytometric analysis. Results demonstrated that TNF-α treatment led to a significant increase in intracellular ROS levels. In contrast, treatment with AKE dose-dependently reduced previously elevated ROS levels (Fig. 2). Additionally, treatment with AKE up to the non-toxic maximum concentration of 200 μg/mL showed no significant difference compared to 100 μg/mL (data not shown). In pharmacology, it is preferred to use the lowest effective dose of a substance to minimize side effects and toxicity. Natural products have relatively fewer side effects than drugs, but to minimize the risk of potential side effects and toxicity, AKE was tested up to 100 μg/mL, the minimum dose that shows the best effect in cells. Previous

studies have documented that TNF-α stimulation can induce the generation of ROS in endothelial cells (Cominacini et al., 2002; Kim et al., 2008). Consistent with these previous findings, our study also observed a significant increase in intracellular ROS in HUVECs upon treatment with TNF-α. Under normal conditions, cells maintain a balance between ROS production and removal. However, chronic inflammation can sustainably generate high levels of ROS, leading to oxidative stress (Pan et al., 2016). AKE effectively scavenged DPPH and ABTS radicals and also reduced intracellular ROS induced by TNF-α. Therefore, we demonstrate the potential of AKE as a beneficial agent in managing conditions associated with excessive ROS production and chronic inflammation. Follow-up studies, including animal and clinical trials, are needed to determine whether the *in vitro* antioxidant effect of AKE can be replicated *in vivo* and to confirm its efficacy and safety in clinical applications.

3.4. Effects of AKE on antioxidant enzyme activity in TNF−α−treated HUVECs

To investigate the influence of AKE on activities of antioxidant enzymes (SOD, GSH, and catalase), protein samples from TNF-α-treated HUVECs were utilized to measure the activity of each enzyme. Results revealed that TNF-α treatment significantly inhibited activities of SOD, GSH, and catalase enzymes. In contrast, activities of SOD and GSH showed concentration-dependent increases in AKE-treated (Figs. 3A and 3B). For catalase, a significant increase
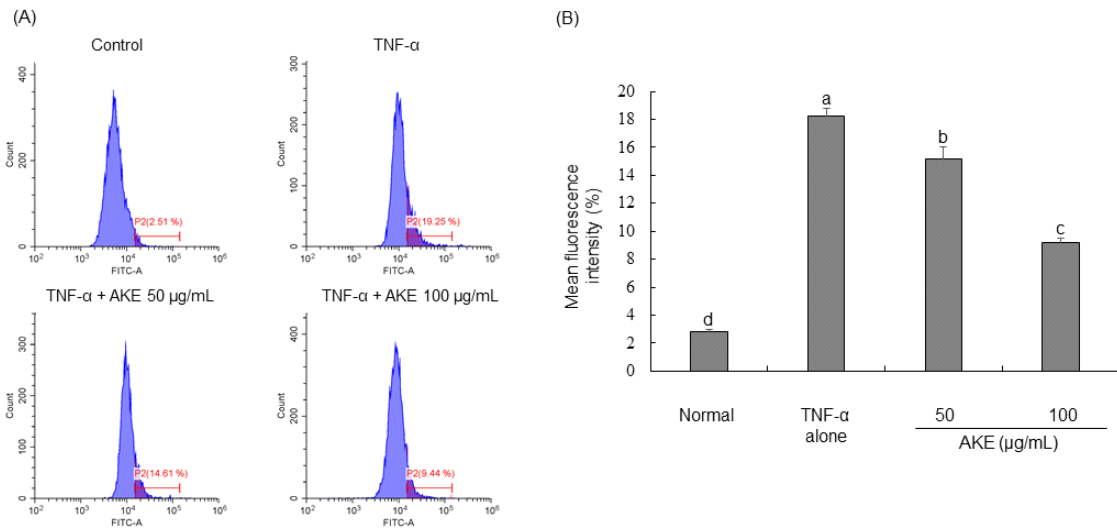
Fig. 2. Effect of AKE on intracellular ROS level in TNF-α stimulated HUVECs. HUVECs were pre-treated with or without AKE (50 or 100 µg/mL) for 1 h prior to incubation with or without TNF-α (20 ng/mL) for 24 h. Intracellular ROS level was measured by flow cytometer. All values are mean±SD (n=3). Different superscript letters (a-d) on the bars indicate significant differences (p<0.05).
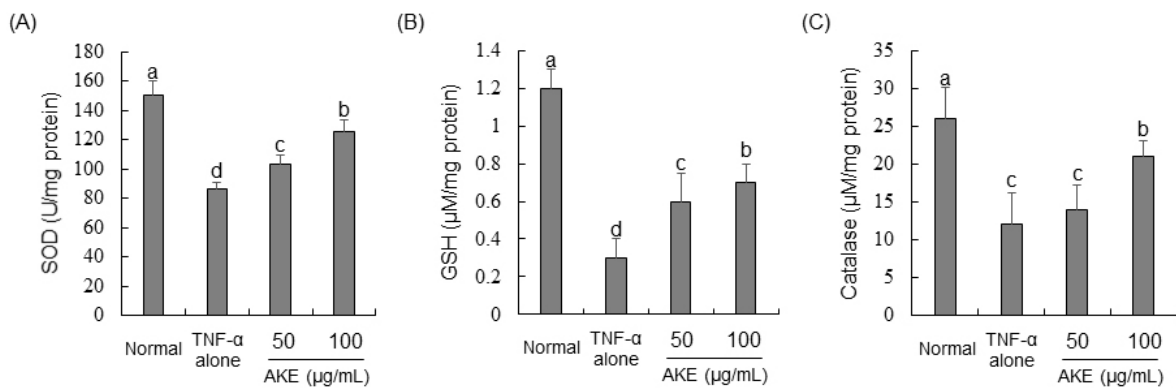

Fig. 3. Effect of AKE on SOD, GSH and catalase level in TNF-α stimulated HUVECs. HUVECs were pre-treated with or without AKE (50 or 100 µg/mL) for 1 h prior to incubation with or without TNF-α for 24 h. After extracting the protein, each enzyme was measured. All values are mean±SD (n=3). Different superscript letters (a-d) on the bars indicate significant differences (p<0.05).

in its activity was not observed in cells treated with AKE 50 µg/mL. However, after treatment with AKE at 100 µg/mL, there was a noticeable enhancement in catalase activity (Fig. 3C). GSH, catalase, and SOD are vital antioxidant enzymes crucial for shielding cells against oxidative damage. They function by eliminating reactive oxygen species, breaking down hydrogen peroxide into harmless water and oxygen, and transforming superoxide radicals into oxygen and hydrogen peroxide, respectively (Ma et al., 2017). Considering this, the ROS-reducing effect of AKE shown in Fig. 2 appears to be due to increased activity of antioxidant enzymes

or direct radical scavenging ability, as shown in Table 1. This suggests that AKE could restore the balance of the redox state in endothelial cells impaired by TNF-α, highlighting its potential therapeutic value in oxidative stress-related vascular diseases.

3.5. Effects of AKE on adhesion molecules in TNF-α-treated HUVECs

To investigate the influence of AKE on the expression of vascular adhesion molecules, Western blot analysis was conducted for TNF-α-treated HUVECs. Results demonstrated

a marked rise in the expression of E-selectin, ICAM-1, and VCAM-1 in the TNF-α-treated group (Fig. 4). In the case of E-selectin, there was no change after treatment with AKE at 50 μg/mL. However, a significant decrease was observed at AKE 100 μg/mL. For ICAM-1 and VCAM-1, there was a concentration-dependent decrease in expression at AKE 50 and 100 μg/mL concentrations. TNF-α, a crucial cytokine widely utilized in the study of molecular mechanisms associated with vascular adhesion molecules in HUVECs, plays a key role in the recruitment and activation of inflammatory cells (Wu et al., 2015; Zhang et al., 2002). E-selectin, ICAM-1, and VCAM-1 play important roles in the initiation of early atherosclerosis. They are expressed during inflammatory conditions. They can mediate leukocyte movement across the endothelial barrier (Wu et al., 2015). Additionally, as a key mediator in signaling pathways, ROS can regulate the expression of vascular adhesion molecules (Cominacini et al., 2002; Kim et al., 2008; Park et al., 2010). Thus, the proven inhibitory effect of AKE on the expression of vascular adhesion molecules in this study is also presumed to be a result of ROS elimination. These findings imply that AKE may have therapeutic potential in preventing or treating inflammatory vascular diseases by reducing the endothelial expression of adhesion molecules and thereby limiting leukocyte recruitment and adhesion.

3.6. Effects of AKE on MAPKs activation in TNF-α-treated HUVECs

We evaluated the effect of AKE on the phosphorylation of MAPK by Western blot analysis to investigate its mechanism of action, as AKE effectively inhibited the expression of adhesion molecules in TNF-α-stimulated HUVECs. Results showed that TNF-α treatment significantly increased the phosphorylation of ERK, JNK, and p38 (Fig. 5). In contrast, AKE treatment concentration-dependently decreased the phosphorylation of JNK. ERK and p38 showed no significant changes in phosphorylation after AKE treatment. Mitogen-activated protein kinases (MAPKs), such as p38, extracellular signal-regulated kinases 1 and 2 (ERK1/2), and Jun N-terminal kinases (JNKs), are involved in a variety of signaling pathways within cells. They are known to regulate gene transcription, protein synthesis, and cell cycle control (Widmann et al., 1999). In endothelial cells, p38 and JNK are activated by TNF-α stimulation, inducing the expression of pro-inflammatory molecules (Hoefen and Berk, 2002). ERK, including ERK1/2 and BMK1, plays a crucial role in promoting cell growth and survival (Hoefen and Berk, 2002).
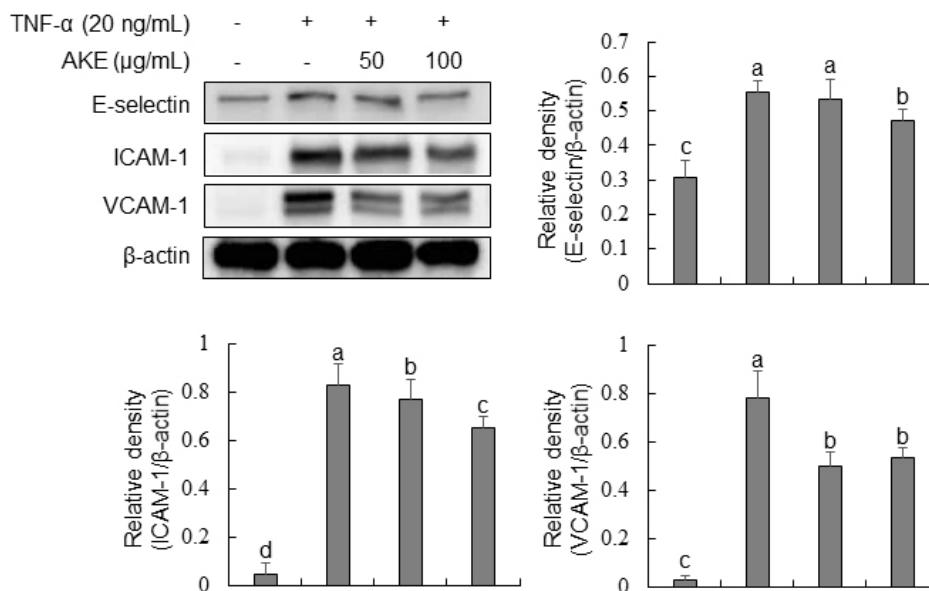

Fig. 4. Effect of AKE on protein expression level of E-selectin, ICAM-1 and VCAM-1 in TNF-α stimulated HUVECs. HUVECs were pre-treated with or without AKE (50 or 100 μg/mL) for 1 h prior to incubation with or without TNF-α for 24 h. Protein expression levels were measured by western blot analysis and analyzed using ImageJ software. All values are mean±SD (n=3). Different superscript letters (a-d) on the bars indicate significant differences (p<0.05).

Fig. 5. Effects of AKE on MAPKs singnaling pathway in in TNF-α stimulated HUVECs. HUVECs were pre-treated with or without AKE (50 or 100 μg/mL) for 1 h prior to incubation with or without TNF-α for 30 min. Protein expression levels were measured by western blot analysis and analyzed using ImageJ software. All values are mean±SD (n=3). Different superscript letters (a-d) on the bars indicate significant differences (p<0.05).
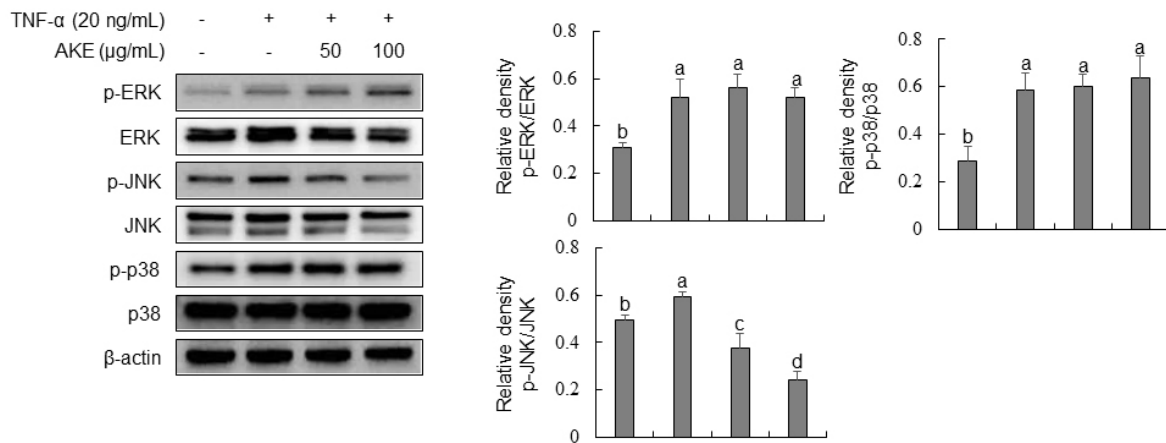
Additionally, members of MAPKs can regulate the NF-κB-dependent pathway (Schulze-Osthoff et al., 1997). In this study, we demonstrated that AKE could inhibit the phosphorylation of JNK induced by TNF-α in HUVECs, while it did not affect the phosphorylation of ERK1/2 or p38. This finding aligns with previous reports indicating selective inhibition of JNK by compounds such as Glycyrrhetinic acid in TNF-α-stimulated HUVEC and Berberine in LPS-stimulated HUVECs (Chang et al., 2010; Guo et al., 2016). These results suggest that the anti-inflammatory effects of AKE may be primarily mediated through the inhibition of JNK signaling, rather than through ERK or p38 pathways.

3.7. Effects of AKE on NF-κB Signaling pathways in TNF-α-treated HUVECs

Due to inhibition of JNK phosphorylation by AKE, we investigated the impact of AKE on the NF-κB signaling pathway, a JNK cascade member. Results revealed that TNF-α treatment for 30 min significantly increased the phosphorylation of NF-κB and its inhibitor, IκBα (Fig. 6A). In contrast, AKE treatment concentration-dependently decreased the phosphorylation of IκBα and NF-κB. Immunofluorescence staining was performed to observe NF-κB nuclear translocation. Results demonstrated TNF-α-induced nuclear translocation of NF-κB, while treatment with AKE at 50 or 100 μg/mL inhibited such nuclear translocation (Fig. 6B). The NF-κB pathway plays a crucial role in triggering the activation of HUVEC in response

to TNF-α stimulation (Monaco and Paleolog, 2004). In the absence of stimulation, NF-κB resides in the cytoplasm in association with IκB proteins. Upon inflammatory stimuli, including TNF-α, IκBα undergoes phosphorylation and degradation, allowing NF-κB to translocate to the nucleus, initiating the transcription of various genes, including E-selectin, ICAM-1, and VCAM-1 and (Collins, 1993; Lu et al., 2012). In conclusion, our study suggests that AKE's anti-inflammatory effects in TNF-α-stimulated HUVECs are primarily mediated through the selective inhibition of the JNK/NF-κB signaling pathway, offering potential therapeutic implications for vascular inflammation.

4. Conclusions

This study aimed to evaluate the antioxidant, cytotoxic, and anti-inflammatory properties of AKE in HUVECs treated with TNF-α. DPPH and ABTS assays determined the radical scavenging activities of AKE, yielding IC_{50} values of 375.24±8.56 μg/mL and 568.12±9.23 μg/mL, respectively. WST assay revealed no cytotoxicity of AKE up to 200 μg/mL. Flow cytometric analysis showed that AKE significantly reduced intracellular ROS levels in TNF-α-treated HUVECs, with enhanced activities of antioxidant enzymes such as SOD, GSH, and catalase. Western blot analysis demonstrated that AKE inhibited the expression of vascular adhesion molecules (E-selectin, ICAM-1, and VCAM-1) and selectively reduced the phosphorylation of JNK without affecting ERK and p38
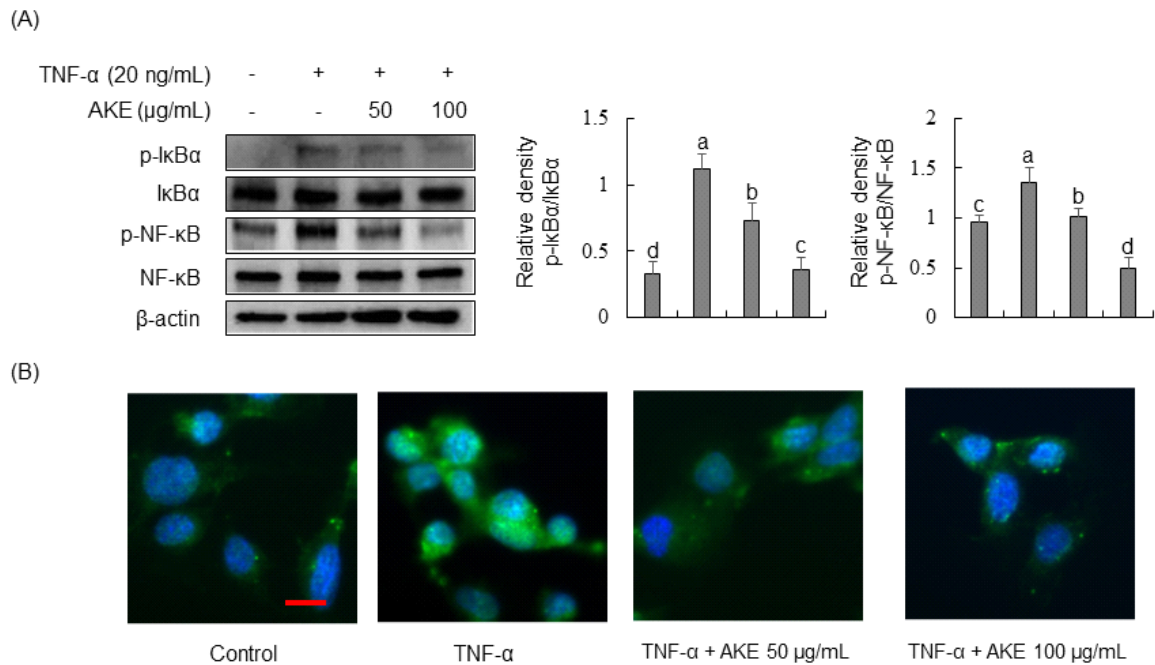
Fig. 6. Effect of AKE on NF-κB and IκB activation in TNF-α-stimulated HUVECs. HUVECs were pre-treated with or without AKE (50 or 100 μg/mL) for 1 h prior to incubation with or without TNF-α for 30 min. Phosphorylation of NF-κB and IκB (A) were measured by western blot analysis and analyzed using ImageJ software. Translocation of NF-κB p65 (B) were determined by immunofluorescence staining. Representative fluorescence microscope images of NF-κB p65 staining (green) and nucleus (blue) (scale bar, 20 μm). All values are mean±SD (n=3). Different superscript letters (a-d) on the bars indicate significant differences (p<0.05).

in the MAPK pathway. Additionally, AKE decreased the phosphorylation of IκBα and NF-κB, inhibiting NF-κB nuclear translocation in TNF-α-stimulated HUVECs. The observed vascular endothelial cells protection effect of AKE may be attributed to its flavonoid content, which includes chalcone, xanthoangelol, isobavachalcone, luteolin, and cynaroside (Kil et al., 2017). These flavonoids are known for their potent antioxidant and anti-inflammatory properties (Zhang et al., 2011) which likely contribute to the overall beneficial effects of AKE in mitigating oxidative stress and inflammation in vascular cells. Further research into these specific flavonoids could provide deeper insights into their individual and synergistic roles in the protective effects of AKE. These findings suggest that AKE possesses significant antioxidant and anti-inflammatory properties, making it a promising candidate for managing oxidative stress and inflammatory vascular conditions.

Conflict of interests
The authors declare no potential conflicts of interest.

Author contributions
Conceptualization: Shin JY, Kim YS, Jang SI. Methodology: Shin JY, Cho BO, Joo MH. Formal analysis: Park JH, Kang ES. Validation: Cho BO, Joo MH. Writing - original draft: Shin JY. Writing - review & editing: Kim YS, Jang SI.

Ethics approval
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