2010 년 2 월
박사학위논문

# Effects of kahweol on inflammation and metastasis

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Kahweol 의 염증 및 암 전이 조절 연구

2009 년 2 월 25 일

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이 논문을 이학 박사학위 논문으로 제출함.

2009 년 10 월

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# List of Abbreviations

| | |
|---|---|
| **Akt** | Protein kinase B |
| **BCECF** | 2',7'-bis-(carboxyethyl)-5,6-carboxyfluorescein |
| **BSA** | Bovine serum albumin |
| **COX-2** | Cyclooxygenase-2 |
| **DAPI** | 4′,6-diamidino-2-phenylindole |
| **DMEM** | Dulbecco's modified Eagle's Medium |
| **DMSO** | Dimethylsulfoxide |
| **ERK1/2** | Extracellular signal-related kinase1/2 |
| **FBS** | Fetal bovine serum |
| **GFP** | Green fluorescent protein |
| **ICAM** | Intercellular cell adhesion molecule |
| **IKK1** | IκB kinase 1 |
| **IκBα** | Inhibitor κBα |
| **iNOS** | inducible nitric oxide synthase |
| **JAK2** | Janus kinase 2 |
| **JNK1/2** | c-Jun N-terminal kinase1/2 |
| **KA** | Kahweol |
| **MAPK** | Mitogen-activated protein kinase |
| **MMP** | Matrix metalloproteinase |

**MTT** 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

**NF-κB** Nuclear factor κB

**PARP** Poly ADP-ribose polymerase

**PBS** Phosphate-buffered saline

**PD** Parkinson's disease

**PI3K** Phosphatidylinositol 3-kinase

**PKC** Protein kinase C

**PMA** Phorbolmyristate acetate

**ROS** Reactive oxygen species

**RT-PCR** Reverse transcriptase polymerase chain reaction

**SDS-PAGE** Sodium dodecyl sulfate-polyacryamide gel electrophoresis

**STAT3** Signal transducer and activator of transcription 3

**TFA** Trifluoroacetic acid

**TNF-α** Tumor necrosis factor-α

**TUNEL** Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling

**VCAM** Vascular cell adhesion molecule

**VEGF** Vascular endothelial growth factor

# ABSTRACT

## Effects of kahweol on inflammation and metastasis

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**The coffee diterpene kahweol inhibits tumor necrosis factor-α-induced expression of cell adhesion molecules in human endothelial cells**

Endothelial cells produce adhesion molecules after being stimulated with various inflammatory cytokines. These adhesion molecules play an important role in the development of atherogenesis. Recent studies have highlighted the chemoprotective and anti-inflammatory effects of kahweol, a coffee-specific diterpene. This study examined the effects of kahweol on the cytokine-induced monocyte/human endothelial cell interaction, which is a crucial early event in atherogenesis. Kahweol inhibited the adhesion of TNFα-induced monocytes to endothelial cells and suppressed the TNFα-induced protein and mRNA expression of the cell adhesion molecules, VCAM-1 and ICAM-1. Furthermore, kahweol inhibited the TNFα-

induced JAK2-PI3K/Akt-NF-κB activation pathway in these cells. Overall, kahweol has anti-inflammatory and anti-atherosclerotic activities, which occurs partly by down-regulating the pathway that affects the expression and interaction of the cell adhesion molecules on endothelial cells.

**Kahweol blocks STAT3 phosphorylation and induces apoptosis in human lung adenocarcinoma A549 cells**

Kahweol, the coffee-specific diterpene, has been reported to have anti-carcinogenic properties. Animal data support such a chemopreventive effect of coffee. However, the precise underlying protective mechanisms are poorly understood. In this study, the apoptotic effect of kahweol in human lung adenocarcinoma A549 cells was investigated. In cell viability assays and cell proliferation assays, kahweol exhibited anti-proliferative and pro-apoptotic effects on A549 cells in a time- and dose-dependent manner. Kahweol considerably inhibited the expression of Bcl-2 but increased that of Bax; it also stimulated the cleavage of caspase-3 and PARP (poly ADP-ribose polymerase). In addition, kahweol-induced apoptosis was confirmed by TUNEL assays. Furthermore, kahweol inhibited dose-dependent phosphorylation of signal transducer and activator of transcription 3 (STAT3). An overexpression in STAT3 led to resistance to kahweol-induced apoptosis, suggesting that STAT3 was a critical target of kahweol. These findings suggest that kahweol inhibited A549 cell growth and induced apoptosis via down-regulation of STAT3 signaling pathway.

**Kahweol inhibit angiogenesis and metastasis through suppression of STAT3 activation**

Kahweol, a coffee-specific diterpene, is reported to have anti-cancer properties, although its precise chemopreventive mechanism remains unclear. In this study, the antiangiogenic and anti-metastatic activities of kahweol was investigated. Kahweol effectively inhibited interleukin-6-induced production of vascular endothelial growth factor (VEGF), as well as the migration and tube formation of human umbilical vein endothelial cells. Kahweol also inhibited cell migration, VEGF production, and matrix metalloproteinase (MMP)-2 and -9 activities in the MDA-MB231 breast cancer cell line. Moreover, kahweol inhibited the phosphorylation of signal transducer and activator of transcription 3 (STAT3), suggesting that STAT3–responsive regions in the VEGF and MMP promoters may underlie the inhibitory effects of kahweol. The transient expression of constitutively active STAT3 significantly reduced the inhibitory effects of kahweol on cell migration, invasion, and VEGF expression. Collectively, these data suggest that kahweol inhibits metastasis and angiogenesis, at least in part, through the disruption of STAT3-mediated transcription of the MMP and VEGF genes.

# I. Introduction

**The coffee diterpene kahweol inhibits tumor necrosis factor-α-induced expression of cell adhesion molecules in human endothelial cells**

The activation of the vascular endothelium, the increased adhesion of circulating monocytes to the injured endothelial layer, and their subsequent infiltration into the vessel wall and differentiation into macrophages are critical early events in the development of atherosclerosis (Price and Loscalzo, 1999; Ross, 1999; Iiyama et al., 1999; Glass and Witztum, 2001). Endothelial cells recruit monocytes by selectively expressing various cell surface adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1) (Iiyama et al., 1999). Proinflammatory cytokines such as tumor necrosis factor-alpha (TNFα), which is commonly found in atherosclerotic lesions, can induce chemotactic factors, other cytokines, and cell adhesion molecules, all of which can contribute to the inflammatory process (DiDonato et al., 1997; Ross, 1999). TNFα can activate nuclear factor-κB (NF-κB) (Sen and Paker, 1996; DiDonato et al., 1997; Tak and Firestein, 2001) and NF-κB plays a key role in the development of the inflammatory response by upregulating the expression of many inflammatory mediators (Tak and Firestein, 2001; Chen et al., 2002). In addition, the transcriptional activation of NF-κB is an important process for the expression of the

proinflammatory cell adhesion molecules (Ledebur and Parks, 1995; Ross, 1999; Griendling et al., 2000; Harrison et al., 2003). Janus kinases (JAKs) are cytoplasmic protein tyrosine kinases that mediate cytokine signaling by phosphorylating different intracellular targets (Rane and Reddy, 2000). It has been reported that TNFα activates JAKs through TNFR1 (Guo et al., 1998). JAK-triggered receptor phosphorylation potentially functions via activation of the STAT signaling pathway, Ras-mitogen-activated protein kinase pathway, and the phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B) pathway after the interaction of cytokine receptors with their ligands (Rane and Reddy, 2000).

The ligands (integrins) for ICAM-1 and VCAM-1 are expressed on the monocytes. The PI3K/Akt and NF-κB signaling molecules regulate the interactions between the endothelial cell adhesion molecules and their ligands. It has been reported that PI3K is essential for the chemokine-triggered conversion of leukocyte rolling to firm adhesion, and is sufficient to enhance the arrest of monocyte under physiological flow conditions (Gerszten et al., 2001). The activation of PI3K activates serine-threonine kinase Akt by phosphorylation (Coffer et al., 1998). NF-κB is composed of p65 and p50 subunits, and inactive NF-κB dimers are sequestered in the cytosol in association with various inhibitory molecules of the IκB family. The stimulation of cells with TNFα causes the phosphorylation of the inhibitor, kappa B alpha (IκBα), which leads to its polyubiquitination and proteasome-mediated degradation

(Zandi and Karin, 1999; Tak and Firestein, 2001; Chen et al., 2002). The release from IκB unmasks the nuclear localization signal of NF-κB, which mediates its translocation to the nucleus, and regulates the expression of the cell adhesion molecules and pro-inflammatory mediators (DiDonato et al., 1997; Tak and Firestein, 2001).

Many foods contain non-nutritional constituents that may have beneficial health effects, such as anti-inflammatory and anti-carcinogenic properties (Bellisle et al., 1998). Kahweol and its dehydro derivative, cafestol, are two diterpenes found in considerable quantities in coffee beans and in the final, unfiltered beverage, e.g. Turkish or Scandinavian style coffees (Gross et al., 1997). They have been shown to have both adverse and chemoprotective properties (De Roos et al., 1999; Cavin et al., 2002). Both kahweol and cafestol increase the blood cholesterol level in both humans and animals (De Roos et al., 1999). However, animal studies have shown that kahweol and cafestol can protect against the action of well-known carcinogens (Huber et al., 1997; Cavin et al., 2001). Moreover, there is epidemiological evidence suggesting that in humans, the consumption of coffee with a high level of kahweol and cafestol is associated with a lower rate of colon cancer, which one of the most frequent cancers in the western world (Giovannucci, 1998). Thus far, studies on the chemoprotective effects of kahweol and cafestol have shown an

association between these compounds and the beneficial modifications of the xenobiotic metabolism (Huber et al., 1997; Cavin et al., 2001, 2002).

There is a paucity of reports on the effects of kahweol on the expression of cell adhesion molecules as well as on the adhesion of monocytes to endothelial cells. Recently, we reported the anti-inflammatory properties of kahweol and cafestol, which inhibit cyclooxygenase-2 (COX-2) and the inducible nitric oxide synthase (iNOS) gene expression through NF-κB inhibition by targeting the IKK complex in macrophages. Kahweol was found to be much more effective in inhibiting the expression of the inflammatory gene than cafestol (Kim et al., 2004a, 2004b, 2006). These findings suggest that kahweol attenuates the transmigration of mononuclear cells, possibly by down-regulating the endothelial-leukocyte interaction in endothelial cells. This study examined the effects of kahweol on the JAK2-PI3-kinase-Akt-NF-κB pathway in terms of its ability to regulate the expression of the cell adhesion molecules on activated endothelial cells as well as their interaction with monocytes.

**Kahweol blocks STAT3 phosphorylation and induces apoptosis in human lung adenocarcinoma A549 cells**

It is increasingly being acknowledged that foods contain non-nutritional constituents that may possess biological activities with beneficial health effects, such as anti-inflammatory and anti-carcinogenic properties (Karihtala and Soini, 2007). Coffee is one of the most widely consumed pharmacologically active beverages in the world. Considerable quantities of the diterpenes, kahweol and its dehydro derivative, cafestol, are found in coffee beans and in the final unfiltered beverage, *e.g.*, Turkish or Scandinavian style coffee (Gross et al., 1997). These diterpenes exhibit both adverse and chemoprotective properties (de Roos et al., 1999; Cavin et al., 2002). Animal studies have shown that kahweol affords protection against well-known carcinogens such as 7,12-dimethylbenz[*a*]anthracene, aflatoxin $B_1$, and 2-amino-l-methyl-6-phenylimidazo[4,5-*b*]-pyridine (Cavin et al., 2001; Huber et al., 1997). Moreover, there is epidemiological evidence suggesting that, in humans, the consumption of coffee with a high level of kahweol is associated with a lower rate of colon cancer, which is one of the most frequent cancers in the western world and world-wide (Giovannucci, 1998).

Apoptosis is a highly regulated process that involves activation of a series of molecular events leading to cell death that is characterized by cellular morphological changes, chromatin condensation, and more (Reed, 2000). The Bcl-2

gene product protects cells against apoptosis in a variety of experimental systems. Suppression of Bcl-2 has been shown to promote apoptosis in response to a number of stimuli, including anticancer drugs (Fisher et al., 1993). Bcl-2 and Bcl-$X_L$ exert their anti-apoptotic effects, at least in part, by binding to Bax and related pro-apoptotic proteins. They also prevent Bax and pro-apoptotic proteins from inducing the release of cytochrome C. Signal transducer and activator of transcription 3 (STAT3) is a key signaling molecule for many cytokines and growth factor receptors (Mitsuyama et al., 2007). In addition, STAT3 is constitutively activated in a number of human tumors and possesses oncogenic potential and anti-apoptotic activity (Kim et al., 2007). Recently, the oncogenic transcription factor STAT3 has attracted much attention as a pharmacologic target, although *in vivo* evidence suggesting that inhibiting STAT3 could counteract cancer remains incomplete (Aggarwal et al., 2006).

The results indicated that kahweol inhibited A549 cell growth and induced apoptosis. Furthermore, a role for signal transducer and activator of transcription 3 (STAT3) in kahweol-induced apoptosis was ascertained.

**Kahweol inhibit angiogenesis and metastasis through suppression of STAT3 activation**

Tumor angiogenesis is a critical event for solid tumor growth and metastasis (Jain, 2002). Angiogenesis is a complex process consisting of several steps, including secretion of angiogenic factors by tumor and host cells, activation of proteolytic enzymes, endothelial cell migration and invasion, and capillary formation (Karamysheva, 2008). These events are regulated by several antiangiogenic and proangiogenic factors, of which vascular endothelial growth factor (VEGF) is the most potent. VEGF is commonly overexpressed in several types of human cancers, including breast cancer, and plays a crucial role in angiogenesis and tumor progression (Ferrara and Davis-Smyth, 1997; Hsieh et al., 2005). The inhibition of VEGF has produced promising results as an antiangiogenesis therapy for tumors in animal models and cancer patients (Ferrara, 2005). Metastasis is also a complex, multi-step process and involves the separation of cells from the primary tumor, intravasation, extravasation, and the establishment of tumor cells at a secondary site (Geiger and Peeper, 2009). The invasive ability of tumor cells is important in all of these steps. Invasion requires degradation of the basement membrane to permit the egress and eventual ingress of malignant cells from the primary tumor. Several invasion-related proteases, including matrix metalloproteinases (MMPs), cysteine proteinases, and serine proteases, are responsible for this process (Sreenath et al.,

1992; Denhardt et al., 1987; Achbarou et al., 1994). In particular, MMP-2 and MMP-9, the two predominately expressed MMPs in endothelial cells capable of degrading type IV collagen, are thought to be directly involved in endothelial cell migration and vascular remodeling during angiogenesis, as well as in tumor cell growth and progression during metastasis (Sluijter et al., 2006; Deryugina and Quigley, 2006). Increased MMP activity is found in human breast, prostate, lung , and ovarian tumors (Kohrmann et al., 2009; Roy et al., 2009).

The signal transducer and activator of transcription (STAT) proteins are transcription factors with known oncogenic potential (Darnell, 2002). STAT is activated by phosphorylation of a critical tyrosine residue via Janus-activated kinases (JAK) or Src family kinases. This leads to the formation of homo- and heterodimers and translocation to the nucleus, followed by binding to specific DNA response elements in the promoter regions of target genes. Among the STAT proteins, STAT3 may be the one most intimately linked to tumorigenesis (Aggarwal et al., 2006). STAT3 regulates the expression of gene products involved in cell survival, proliferation, angiogenesis, and tumor cell invasion, and constitutive activation of STAT3 is sufficient to induce tumor formation (Haura et al., 2005; Niu et al., 2002a). Constitutive activation of STAT3 is frequently detected in breast cancer specimens from patients with advanced disease and in breast carcinoma cell lines, but not in normal breast epithelial cells (Hsieh et al.,

2005). Furthermore, there is evidence that STAT3 protein regulates genes involved in angiogenesis and metastasis (Aggarwal et al., 2006).

Several attempts have been made to inhibit tumor formation, tumor invasion, and metastasis using components of beverages (Shukla, 2007). Coffee is among the most widely consumed, pharmacologically active beverages in the world. Most studies on the beneficial properties of coffee in humans are observational (Cadden et al., 2007). Considerable quantities of the diterpene kahweol and its derivative, cafestol, are present in coffee beans and in the final unfiltered beverage, *e.g.*, Turkish or Scandinavian style coffee (Gross et al., 1997). Kahweol exhibits a wide variety of activities, including anticarcinogenic, antitumor, and anti-inflammatory properties (Cavin et al., 2002; Tao et al., 2008; Kim et al., 2004). Moreover, epidemiological evidence suggests that the consumption of coffee containing a high level of kahweol is associated with a lower rate of colon cancer, one of the most common cancers worldwide (Giovannucci, 1998). Recently, attention has focused on the biological effects of these diterpenes; however, the mechanism responsible for the chemopreventive effects of kahweol is not fully understood. The results indicated that the inhibition of activated STAT3 by kahweol not only significantly suppressed VEGF and MMPs but also reduced the invasiveness of human breast cancer cells with high metastatic potential. Collectively, these data provide evidence that the activation of the STAT3 signaling pathway may be critical for the

invasive and metastatic properties of breast cancer cells. The inhibition of this pathway may offer a novel strategy for breast cancer intervention.

# II. Materials & Methods

### 1. Chemicals and materials

All the chemicals and cell culture materials were obtained from the following sources: Kahweol acetate from Sigma Co.; 2',7'-bis-(carboxyethyl)-5,6-carboxyfluorescein (BCECF), Alexa-488-coupled goat anti-rabbit IgG and DAPI (4′,6-diamidino-2-phenylindole) from Molecular Probe; LY294002, Wortmannin and AG490, Cell Proliferation ELISA BrdU Kit and MTT-based colorimetric assay kit from Roche; LipofectAMINE Plus, Dulbecco's modified Eagle's medium (DMEM), DMEM/nutrient mixture F-12 (DMEM/F12), RPMI 1640, fetal bovine serum (FBS), and penicillin-streptomycin solution from Gibco-BRL-Life Technologies; pGL3-4κB-Luc, DeadEnd$^{TM}$ Fluorometric TUNEL System and the luciferase assay system and the luciferase assay system from Promega; pCMV-β-gal from Clonetech; STAT-3 reporter constructs from Panomics (Redwood City, CA); NF-κB–GFP plasmid from System Biosciences; recombinant human TNFα, anti-β-actin and mouse anti-human VCAM-1 and ICAM-1 antibodies from BD Pharmingen; $^{125}$I-TNF□ from PerkinElmer; anti-Bcl-2, anti-Bax, anti-cleaved caspase-3, anti-cleaved poly(ADP) ribose polymerase (PARP), VEGF, p-STAT3, STAT3, anti-Akt and phospho-Akt (Ser) antibodies from Cell Signaling Technology, Inc.; anti-NF-κB p65 and anti-JAK2 antibodies from Santa Cruz Biotechnology,

Inc.; phospho-specific anti-JAK2 antibody from BIOSOURCE International; Western blotting detection reagents (ECL) from Amersham Pharmacia Biotech.; the other chemicals were of the highest commercial grade available.

**2. Cell culture and treatment**

ECV304 cells, which are a spontaneously transformed immortal endothelial cell line established from the vein of an apparently normal human umbilical cord, and U937 cells, which are a type of human monocytic leukemia cell, and A549 cells, which are a type of human lung cancer cell, were obtained from the American Type Culture Collection (Bethesda, MD). Human endothelial cell line ECV304, A549 and U937 cells were grown in DMEM and RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% $CO_2$ humidified incubator. The human breast cancer cell line MDA-MB231 and the normal breast epithelial cell line MCF-10A (American Type Culture Collection, Manassas, VA) were grown in DMEM (MDA-MB231) or DMEM/F12 (MCF-10A) supplemented with 2 mM glutamine and 10% FBS, at 37°C in an atmosphere containing 5% $CO_2$. Human umbilical vein endothelial cells (HUVECs) were cultured in EGM-MV medium (Clonetics Corp., San Diego, CA). Kahweol acetate was dissolved in dimethylsulfoxide and added directly to the culture medium. The control cells were treated with solvent only. The final concentration of these

solvents never exceeded 0.1%, and did not have any noticeable effect on the assay systems.

**3. Cell viability and proliferation**

A549 cells were plated at 1 x $10^4$ cells per well in 48-well plates and allowed to grow in growth medium for 24 h. Cells were treated with or without kahweol for 24 or 48 h. Cell proliferation was determined daily for 2 days using the Cell Proliferation ELISA BrdU Kit as described by the manufacturer. Cell viability was determined using the MTT assay as described (Choi et al., 2001).

**4. Monocyte-endothelial cell adhesion assay**

The endothelial cells were grown to confluence in 48-well plates and pretreated with kahweol for 1 h followed by stimulated with TNFα for 6 h. The cells were then washed twice with PBS. The U937 cells were labeled for 1 h at 37°C with 10 μM BCECF and washed twice with growth medium. 2.5 x $10^5$ of the labeled cells were then added to the endothelial cells and incubated in a $CO_2$ incubator for 1 h. The non-adherent cells were removed from the plate by washing them with PBS. The U937 cells bound to the endothelial cells were lysed with 50 mM Tris-HCI, pH 8.0, containing 0.1% SDS, and their fluorescent intensity was measured using a spectrofluorometer (Varioskan, Thermo Electron Co., Vantaa, Finland) at an

emission and excitation wavelength of 485 nm and 535 nm, respectively. The adhesion data are represented in terms of the fold change compared with the control values.

**5. Cell ELISA**

ELISA was used to determine the ICAM-1 and VCAM-1 expression levels on the cell surface, as previously described with minor modifications (Noguchi et al., 2003). Briefly, the endothelial cells in 96-well plates were pretreated with or without kahweol for 1 h, which was followed by stimulation with TNFα for 6 h at 37°C. After the treatments, the cells were fixed by 1% glutaraldehyde and exposed to the mouse anti-human VCAM-1 or ICAM-1 antibodies at a 1:1000 dilution in PBS containing 1% skim milk for 2 h at room temperature. The cells were washed and incubated with the horseradish peroxidase-conjugated secondary antibody. The VCAM-1 or ICAM-1 expression levels were quantified by adding a peroxidase substrate solution and the absorbance of each well was measured at 490 nm using a microplate reader.

**6. RNA isolation and reverse transcriptase polymerase chain reaction**

The endothelial cells were pretreated with kahweol for 1 h and stimulated with TNFα for 3 h. This was followed by then washing them twice with PBS. The total

RNA was isolated from the endothelial cells using the Trizol reagent. cDNA synthesis, semiquantitative RT-PCR for VCAM-1, ICAM-1, TNFR1 and GAPDH mRNA, and the analysis of the results were all performed using the methods reported elsewhere (Scholzen et al., 2003; Smolnikar et al., 2000). The cycle number was determined from a linear amplification curve as being within the linear amplification range. The PCR reactions were electrophoresed through a 1.5% agarose gel and visualized by ethidium bromide staining and UV irradiation.

**7. Transient transfection and luciferase and β-galactosidase assays**

The endothelial cells were grown to 60-80% confluence and transfected with a total of 1 μg of the constitutive active Akt plasmid DNA (pCMV5-HA-Akt) using the LipofectAMINE Plus Reagent according to the manufacturer's protocol. Twenty-four h after transfection, the cells were treated with kahweol for 1 h, which was followed by stimulation with TNFα for 6 h for the adhesion assay. The NF-κB luciferase activity was examined by co-transfecting the endothelial cells with the pGL3-4κB-Luc reporter plasmid (0.5 μg) and pCMV-β-gal (0.2 μg) in the presence or absence of co-transfected pCMV5-HA-Akt (active form). Twenty-four h after transfection, the cells were treated with kahweol for 1 h. They were then stimulated with TNFα for 12 h, and then lysed. A549 cells were plated on 24-well plates for 12 h and then transiently co-transfected with pGL3-STAT3-Luc (0.5 μg) and pCMV-β-

gal (0.2 μg) plasmids using LipofectAMINE Plus according to the manufacturer's protocol. After 18 h, the cells were treated with kahweol for 24 h and lysed. The luciferase and β-galactosidase activity were determined as described elsewhere (Kim et al., 2004a; Choi et al., 2001). The luciferase activity was normalized with respect to the β-galactosidase activity and was expressed relative to the activity of the TNFα group. The GFP (green fluorescent protein) was detected in the NF-κB-GFP-transfected cells by transfecting the endothelial cells with NF-κB-GFP plasmid according to the above mentioned transfection protocol. The cells were stimulated with TNFα for 12 h, washed with cold PBS and viewed under a Fluorescent microscope (Carl Zeiss).

**8. Nuclear staning with hoechst 33258**

After the cells were treated with kahweol for 48 h, cells were harvested and fixed with 4% formaldehyde in PBS for 10 min, stained with Hoechst 33258, and then subjected to fluorescence microscopy.

**9. Western immunoblot analysis**

Cells were centrifuged, washed with PBS, and lysed at 4°C for 30 min in lysis buffer [20 mM Hepes (pH 7.4), 2 mM EGTA, 50 mM □-glycerol phosphate, 1% Triton X-100, 10% glycerol, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 10

μg/ml aprotinin, 1 mM $Na_3VO_4$, and 5 mM NaF]. Protein concentration was determined using the Bio-Rad dye-binding microassay, and 20 μg of protein per lane was subjected to electrophoresis on 10% SDS-polyacrylamide gels. Proteins were blotted onto Hybond ECL membranes (Amersham Biosciences) and the membranes were immunoblotted with the indicated primary antibodies as recommended by the manufacturers. Proteins were detected using the enhanced chemiluminescence detection system (Amersham Biosciences).

**10. Immunofluorescence assay**

Endothelial cells were seeded on sterile slide coverslips in 6-well plates overnight and pretreated with kahweol for 1 h before stimulated with TNFα for 30 min. Cells were fixed with 3.5% (w/v) paraformaldehyde for 15 min at room temperature and permeabilized with 0.2% (v/v) Triton X-100 in PBS for 1 min. Cells were proved with rabbit anti-NF-κB p65 as the primary antibody for 1 h at room temperature. After washing steps, Alexa-488-coupled goat anti-rabbit IgG as the secondary antibody was added. Chromosomal DNA was visualized by 4′,6-diamidino-2-phenylindole (DAPI), and stained cells were mounted on glass slides and examined using a Carl Zeiss Axiovert 200 M microscope.

**11. TNFR binding assay**

Binding assay to TNFR was performed using $^{125}$I-TNFα as described previously (Higuchi and Aggarwal, 1992).

**12. Determination of caspase-3 activity**

A modified method described by Wang et al. was used to examine caspase-3 activation (Datta et al., 1997). A549 cells were treated for 48 h with kahweol in the absence or presence of the caspase-3 inhibitor z-DEVD-FMK and then lysed in hypotonic buffer [20 mM Tris-HCl (pH7.5), 1 mM EDTA, 100 μM PMSF, 2 μg/ml each of aprotinin, pepstatin, and leupeptin]. The supernatants were collected and incubated with 100 μM of the substrate DEVD-pNA at 37°C. The change in absorbance at 405 nm was measured using a plate reader.

**13. TUNEL assay**

Apoptosis was detected by the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay using the DeadEnd$^{TM}$ Fluorometric TUNEL System (Promega) according to the manufacturer's instructions. A549 cells were seeded on sterile slide coverslips in 12-well plates overnight and stimulated with kahweol for 48 h. Cells were then washed twice with PBS, fixed with 4% methanol-free paraformaldehyde for 10 min, washed twice with PBS, and permeablized with 0.2% Triton X-100 for 5 min. After two more washes, each glass

slide was covered with equilibration buffer for 10 min. The buffer was then aspirated, and the glass slides were incubated with TdT buffer at 37°C for 1 h. Chromosomal DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI), and stained cells were mounted on glass slides and examined using a Carl Zeiss Axiovert 200 M microscope.

**14. Real-time PCR**

Total RNA was isolated from kahweol-treated cells using an RNA isolation kit (Takara Shuzo, Kyoto, Japan) according to the manufacturer's protocol. The quality of the RNA was confirmed by a ratio of >1.8 for the optical densities at 260 and 280 nm. PCR product formation was continuously monitored during PCR using Sequence Detection System software, version 1.7 (Applied Biosystems, Foster City, CA). Accumulated PCR products were detected directly by monitoring the increase in the reporter dye (SYBR) signal. The primers used in this study were: 5′-GGCAGAGTACGCAAACACTT-3′ and 5′-GGCTGTAGCTCCCCGTTAG-3′ for VEGF; 5′-AGTCTGAAGAGCGTGAAG-3′ and 5′-CCAGGTAGGAGTGAGAATG-3′ for MMP-2; 5′-TGACAGCGACAAGAAGTG-3′ and 5′-CAGTGAAGCGGTACATAGG-3′ for MMP-9; and 5′-GATGATATCGCCGCGCTCGTCGTCGAC-3′ and 5′-AGCCAGGTCCAGACGCAGGATGGCATG-3′ for β-actin. The quantity of each

transcript was calculated as described in the instrument manual and was normalized to the amount of β-actin, a housekeeping gene.

**15. Tube formation assay**

The wells of a 24-well culture plate were coated with 50 μl of low-growth-factor synthetic matrix (Matrigel; BD Biosciences, San Jose, CA) and incubated for 30 min at 37°C. HUVECs were pretreated with kahweol for 1 h and then incubated with or without interleukin (IL)-6 (10 ng/ml) for 24 h at 37°C. The HUVECs were seeded on the synthetic matrix at a density of $1 \times 10^4$ cells per well and incubated for 17 h at 37°C. Tube formation was determined under an inverted light microscope and photographed using a Carl Zeiss Axiovert 200 M microscope. Images of tube formation were selected randomly from three fields at ×100 magnification.

**16. Cell migration and invasion assay**

Cell migration was assessed with a Transwell insert system (Millipore Corp., Bedford, MA) using polycarbonate membranes of 8-μm pore size. Invasion assays were performed using Transwell inserts pre-coated with Matrigel. HUVECs and MDA-MB231 cells were cultured in serum-free, supplement-free EGM-MV and DMEM, respectively, for at least 6 h. Each cell suspension, in the presence or

absence of the indicated treatments, was added to the upper chamber of a Transwell and incubated overnight at 37°C. For all cells, DMEM containing 10% FBS was used as the chemoattractant in the lower chamber. Migrating cells were stained with crystal violet, imaged, eluted, and transferred to a 96-well plate for absorbance readings at 595 nm.

**17. Gelatin zymography**

The activities of the gelatinases MMP-2 and MMP-9 in the conditioned medium were measured using gelatin-zymogram protease assays, as described previously (Wang et al., 2006). Cells were seeded on 6-well plates in DMEM supplemented with 10% FBS and then treated with the indicated compounds for 48 h in serum-free medium. Briefly, conditioned medium was diluted with 4× sample buffer (8% SDS, w/v, 0.04% bromophenol blue, w/v, 0.25 M Tris) and incubated at 37°C for 30 min. Ten micrograms of total protein were electrophoresed in a 10% polyacrylamide gel containing 0.5 mg/ml gelatin (Difco Laboratories, Detroit, MI). After electrophoresis, the gel was washed with 50 mM Tris-HCl, pH 7.5, containing 2.5% Triton X-100, followed by incubation in calcium assay buffer (50 mM Tris, 10 mM $CaCl_2$, 1 mM $ZnCl_2$, 1% Triton X-100, pH 7.5) for 24 h at 37°C. The gel was stained with Coomassie Brilliant Blue R250 in 50% methanol and

10% acetic acid and destained in 10% acetic acid. Areas of gelatin degradation appeared as clear bands.

**18. Statistical analysis**

All experiments were repeated at least three times. For quantitation analysis, the sum of the density of bands corresponding to protein blotting with the anti-body under study was calculated, and the amount of β-actin normalized. One-way analysis of variance (ANOVA) was used to determine the significance of differences between treatment groups. The Newman–Keuls test was used for multi-group comparisons. Statistical significance was accepted for $p$ values of $<0.01$.

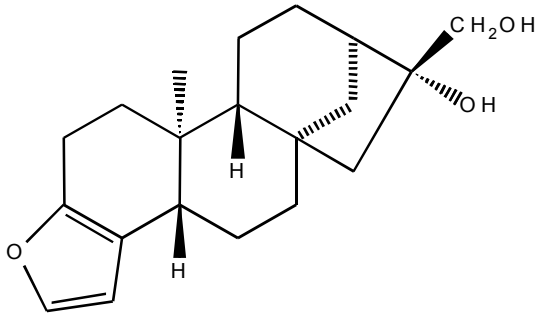
# III. Results

**The coffee diterpene kahweol inhibits tumor necrosis factor-α-induced expression of cell adhesion molecules in human endothelial cells**

**1. Kahweol inhibits monocyte adhesion to TNFα-activated endothelial cells**

The increased expression of cell adhesion molecules on endothelial cells promotes the adhesion of monocytes, which is regarded as the molecular basis for the inflammatory response observed in various diseases (Price and Loscalzo, 1999; Ross, 1999; Iiyama et al., 1999; Glass and Witztum, 2001). This study examined the effect of kahweol (Fig. 1) on the adhesion of monocytes to endothelial cells. The endothelial cells were pretreated with kahweol for 1 h before the TNFα treatment for 6 h, which was followed by an adhesion process and quantification of the adhered fluorescent monocytes. As shown in Fig. 2, TNFα significantly increased the adhesion of monocytes to the endothelial cells compared with the untreated (control) cells. However, kahweol significantly inhibited this adhesion in a dose-dependent manner. An examination of the cytotoxicity of kahweol in the endothelial cells using a MTT assay indicated that kahweol had no adverse effect on the cell viability (> 90% cell viability, Fig. 2). Therefore, the inhibition of the

adhesion of monocytes on endothelial cells by kahweol was not the result of any cytotoxicity.




『 kahweol 』

Fig. 1. Chemical structure of kahweol.

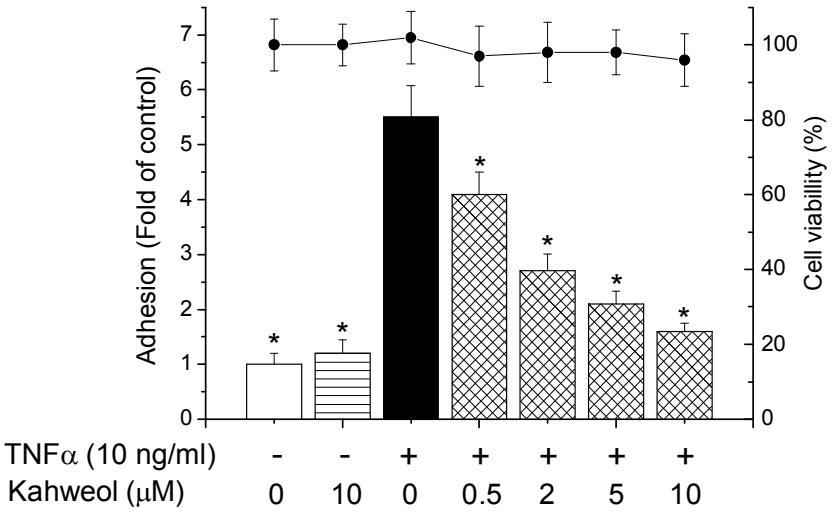



Fig. 2. Effects of kahweol on the monocyte adhesion to endothelial cells.

The endothelial cells were pretreated with kahweol for 1h, and then stimulated with TNFα for 6 h. Cell adhesion assay was performed. The values are expressed as a mean ± SD of three individual experiments, performed in triplicate. *, $P < 0.01$ compared with the TNFα alone.

**2. Kahweol inhibits cell-surface expression of cell adhesion molecules**

The effects of kahweol on the TNFα-induced VCAM-1 and ICAM-1 expression on the endothelial cell surface were determined using cell-ELISA (Fig 3). Exposure of the cells to TNFα for 6 h induced the strong up-regulation of the cell surface expression of VCAM-1 and ICAM-1. Pretreatment with kahweol significantly inhibited the TNFα-induced cell surface expression of VCAM-1 and ICAM-1.

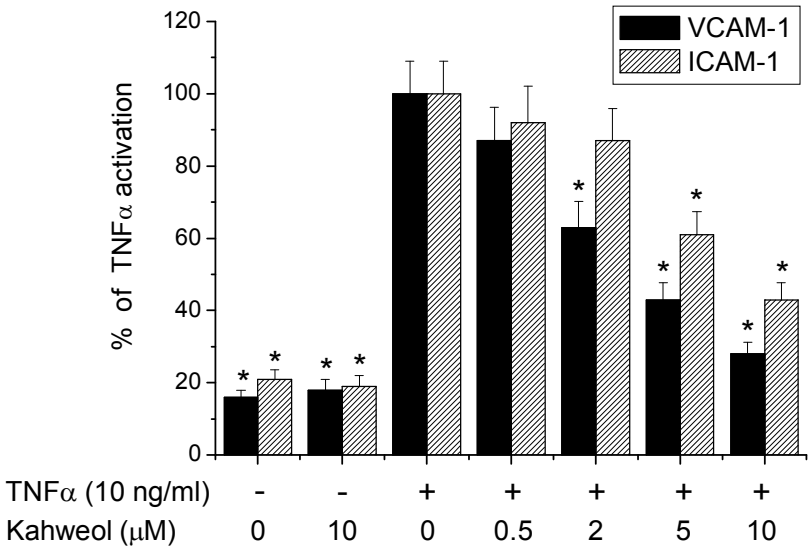



Fig. 3. Effects of kahweol on the endothelial cell surface expression of VCAM-1 and ICAM-1.

The endothelial cells were pretreated with kahweol for 1 h and stimulated with TNFα for 6 h. Cell surface expressions of VCAM-1 and ICAM-1 were analyzed using cell ELISA. The values are expressed as a mean percentage of TNFα-induced adhesion molecule expression ± SD of three individual experiments, performed in triplicate. *, $P < 0.01$ compared with the TNFα alone.

**3. Kahweol inhibits ICAM-1 and VCAM-1 protein and mRNA expression**

Western blotting of the cell lysate protein was carried out in order to confirm the effects of kahweol on the expression of the cell adhesion molecules in endothelial cells. Pretreating the cells with kahweol significantly inhibited the TNFα-induced VCAM-1 and ICAM-1 expression (Fig. 4A). The observed changes in the levels of the cell adhesion molecules might indicate a change in protein synthesis or degradation. The mRNA levels if the cell adhesion molecules were measured by RT-PCR in order to further clarify the mechanism responsible for the changes in the level of the cell adhesion molecules. Pretreating the cells with kahweol markedly decreased the VCAM-1 and ICAM-1 mRNA levels (Fig. 4B). This suggests that kahweol suppresses VCAM-1 and ICAM-1 expression at the transcriptional level, which lowers the production of their protein and reduces the level of monocytes adhesion.

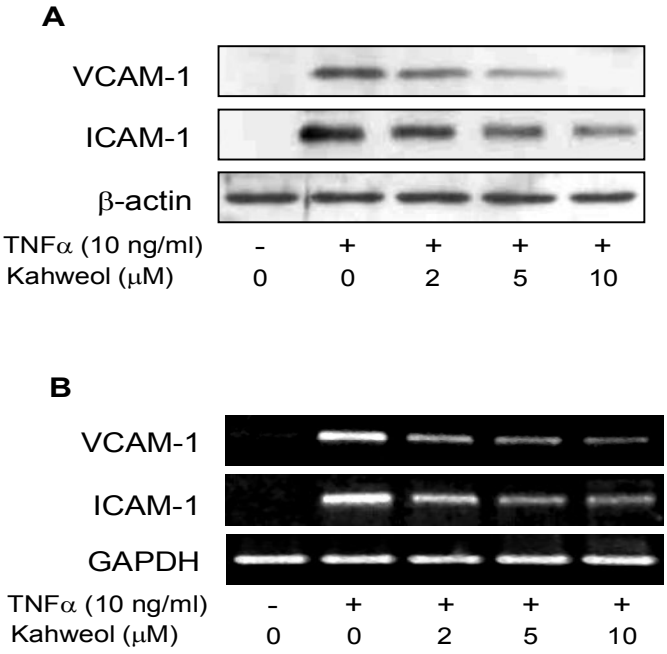



Fig. 4. Effects of kahweol on TNFα-induced expression of VCAM-1 and ICAM-1 protein and mRNA.

The endothelial cells were pretreated with kahweol for 1h and stimulated with TNFα. (A) Western blot analysis. After 6 h of incubation, the cell lysates were blotted with the anti-ICAM-1, VCAM-1 or β-actin antibody. (B) RT-PCR analysis. After 3 h of incubation, the total RNA was prepared and RT-PCR was performed. These blots (A, B) are a representative of three independent experiments. One of three representative experiments is shown.

**4. Kahweol inhibits TNFα-induced PI3K/Akt activation**

The role of PI3K, an important the process of monocyte adhesion (Gerszten et al., 2001), was examined in order to further understand the mechanism of action of kahweol on the monocyte-endothelial cell interaction. Akt is a downstream target of PI3K, which has been implicated in monocyte chemotaxis in response to chemokines (Fresno Vara et al., 2004; Hanada et al., 2004). When the endothelial cells were pretreated with an inhibitor of PI3K (LY294002) or Akt (Wortmannin) for 30 min, the adhesion of monocytes to the endothelial cells was significantly inhibited, suggesting a role in the adhesion process (Fig. 5). Therefore, this study examined the effect of kahweol on the TNFα-induced Akt activation. Endothelial cells were treated with kahweol for 1 h, which was followed by a TNFα treatment for 30 min. TNFα induced significant Akt phosphorylation, which was markedly reduced in the kahweol-pretreated endothelial cells (Fig. 6). To support this observation, the cells were transiently transfected with the constitutively active form of Akt and the level of monocyte adhesion to the endothelial cell was examined. As shown in Fig. 7, the constitutively active form of Akt reversed the inhibitory effect of kahweol on monocyte adhesion to the endothelial cell. Therefore, kahweol inhibits the adhesion of monocytes to endothelial cells by down-regulating the PI3K/Akt signaling pathway.

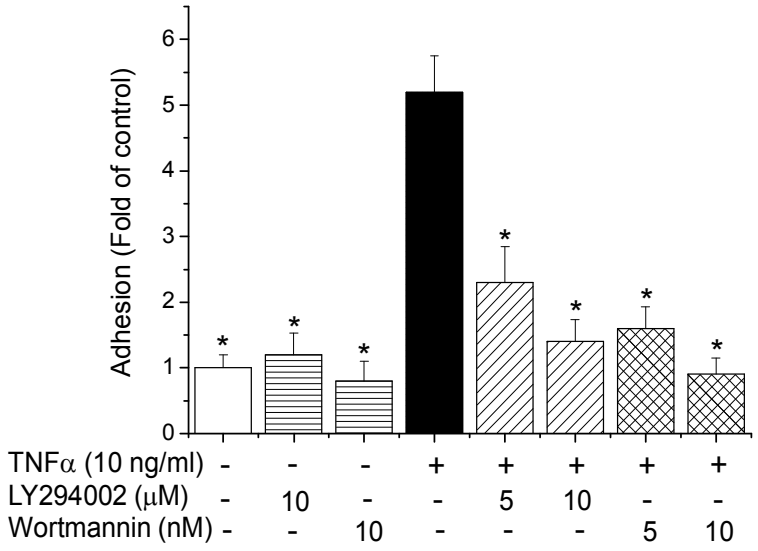



Fig. 5. Effects of Akt on kahweol-mediated inhibition of monocytes adhesion: Akt inhibitor.

The endothelial cells were pretreated with LY294002 or Wortmannin for 30 min and then stimulated with TNFα for 6 h. Cell adhesion assay was performed. The values are expressed as a mean ± SD of three individual experiments, performed in triplicate. *, $P < 0.01$ compared with the TNFα alone.

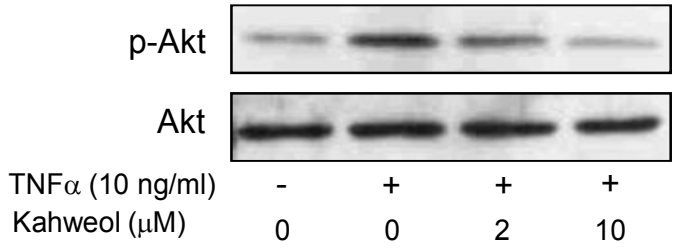



Fig. 6. Effects of kahweol on TNFα-induced Akt phosphorylation.

The endothelial cells were pretreated with kahweol for 1 h and then stimulated with TNFα for 30 min. Western blot analysis of Akt phosphorylation was performed. The blots are a representative of three independent experiments.

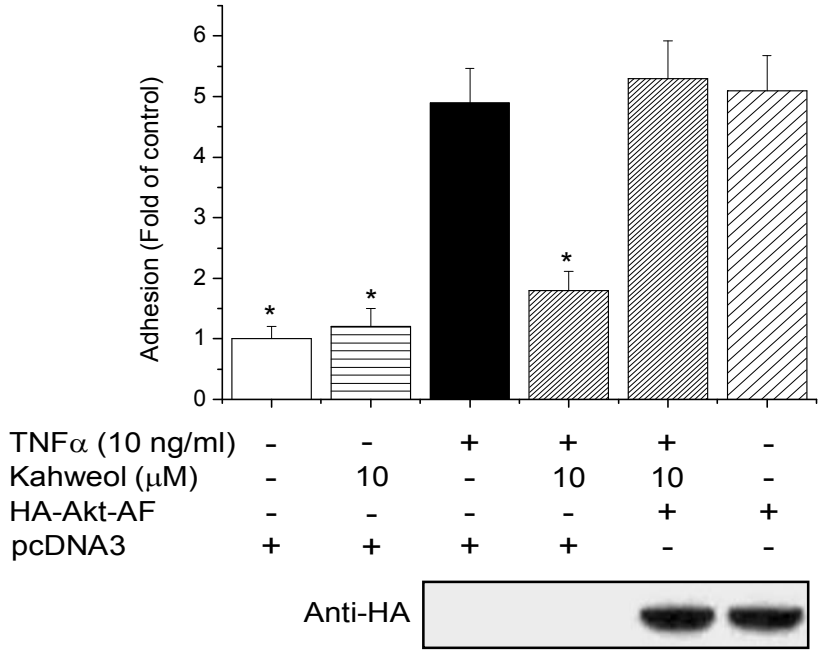



Fig. 7. Effects of Akt on kahweol-mediated inhibition of monocytes adhesion: Akt expression.

The endothelial cells were transfected with the constitutively active form of Akt (HA-Akt-AF). After 24 h of transfection, the cells were pretreated with kahweol for 1 h, and then stimulated TNFα for 6 h. Cell adhesion assay was performed. After 6 h of incubation, the cell lysates (30 μg protein) were separated by SDS-PAGE, transferred to a nitrocellulose membrane and blotted with the anti-HA (active Akt). The values are expressed as a mean ± SD of three individual experiments, performed in triplicate. *, $P < 0.01$ compared with the TNFα alone.

**5. Kahweol inhibits TNFα-induced JAK2 activation**

It has been reported that TNFα activates and tyrosine-phosphorylates JAK2, a member of Janus kinases family, through TNFR1 (Guo et al., 1998). JAK2 has been suggested to be upstream of the PI3K-Akt cascades (Yamauchi et al., 1998; Al-Shami et al., 1999; Rane and Reddy, 2000), and our data showed that inhibitors of PI3K/Akt and kahweol block TNFα-induced the adhesion of monocytes to the endothelial cells (Fig. 5). Therefore, this data speculated that JAK2 is located upstream from PI3K/Akt activation in the TNFα-induced signaling pathway of endothelial cells. To determine whether JAK2 is tyrosine phosphorylated after TNFα stimulation, endothelial cells were stimulated with TNFα. Phosphorylated JAK2 was measured in Western blots using tyrosine phospho-JAK2 antibody. As previously shown (Guo et al., 1998), these findings that TNFα induced tyrosine phosphorylation of JAK2 and pretreatment with AG490 (a JAK2 tyrosine kinase inhibitor) or kahweol inhibited JAK2 phosphorylation by TNFα (Fig. 8). To examine whether JAK2 and PI3K/Akt were two independent pathways for TNFα signaling or whether they were contingent on one another, the present study was performed Western blots to define these two pathways. The present study detected phospho-Akt after TNFα stimulation (Fig. 9). The present study examined whether phospho-Akt induced by TNFα were JAK2-related. Cells were incubated for 30 min in the presence or absence of AG490 and then stimulated with TNFα for 30

min. AG490 markedly inhibited TNFα-induced activation of Akt (Fig. 9). Next, it was studied to examine whether JAK2 is also involved in cell adhesion, because it have already shown that JAK2 acts upstream of PI3K/Akt in TNFα-induced signaling (Fig. 9). Cells were pretreated in the presence or absence of AG490 for 30 min before TNFα stimulation, and cell adhesion were assayed. Pretreatment with AG490 significantly inhibited the adhesion of monocytes to endothelia cells (Fig. 10). These results indicate that a JAK2 act upstream of PI3K/Akt cascades in TNFα-induced signaling pathways and that kahweol inhibits JAK2 and down-regulates the PI3K/Akt signaling pathway in TNFα-induced the adhesion of monocytes to endothelial cells.

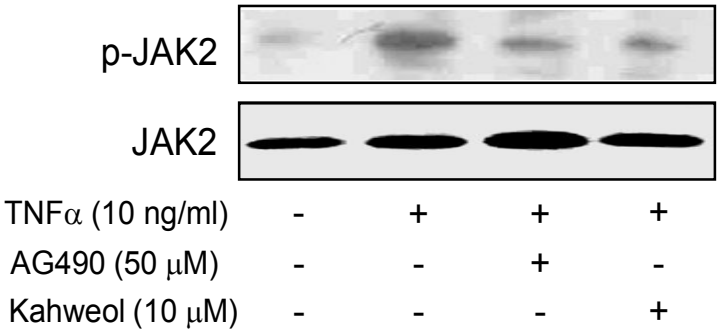



Fig. 8. Effects of JAK2 on kahweol-mediated inhibition of monocytes adhesion: JAK2 phosphorylation.

The endothelial cells were pretreated with AG490 for 30 min or kahweol for 1 h and then stimulated with TNFα for 30 min. Western blot analysis of JAK2 phosphorylation was performed. The blots are a representative of three independent experiments.

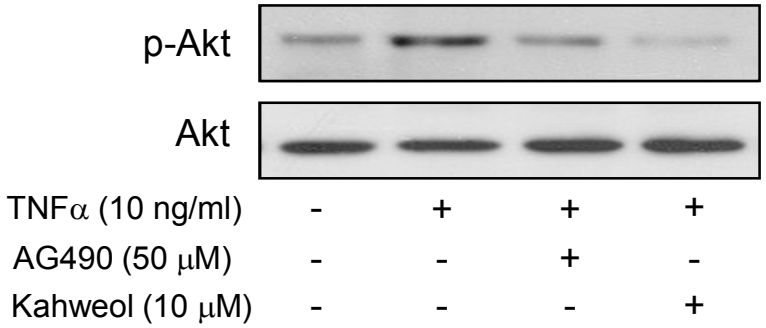



Fig. 9. Effects of JAK2 on kahweol-mediated inhibition of monocytes adhesion: Akt phosphorylation.

The endothelial cells were pretreated with AG490 for 30 min or kahweol for 1 h and then stimulated with TNFα for 30 min. Western blot analysis of Akt phosphorylation was performed. The blots are a representative of three independent experiments.

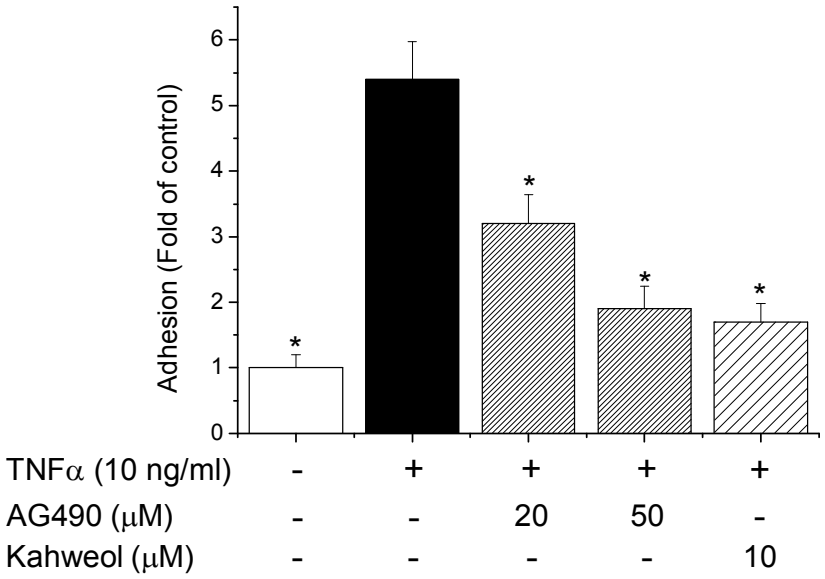



Fig. 10. Effects of JAK2 on kahweol-mediated inhibition of monocytes adhesion: adhesion.

The endothelial cells were pretreated with AG490 for 30 min or kahweol for 1 h and then stimulated with TNFα for 6 h. Cell adhesion assay was performed. The values are expressed as a mean ± SD of three individual experiments, performed in triplicate. *, $P < 0.01$ compared with the TNFα alone.

**6. Kahweol inhibits TNFα-induced NF-κB activation**

NF-κB is a well-known transcription factor of various proinflammatory genes including the cell adhesion molecules (Glass and Witztum, 2001). The effect of kahweol on the NF-κB reporter activity was examined because kahweol inhibited both the adhesion of monocytes to the endothelial cells and expression of the cell adhesion molecules on the endothelial cells. Endothelial cells were transiently transfected with pGL3-4κB-Luc. Kahweol significantly inhibited the TNFα-induced NF-κB luciferase activity in a dose-dependent manner compared with the untreated cells (Fig. 11). This effect was also evident from the NF-κB-GFP expression pattern in the endothelial cells. As shown in Fig. 12, TNFα-stimulation significantly increased the GFP expression level, which is regulated by NF-κB activation. The control cells did not express GFP. However, the kahweol pretreatment reduced the GFP expression level. TNFα can activate NF-κB and induces translocation of p65 to the nucleus. Next, it was studied to the effect of kahweol on TNFα-induced nuclear translocation of p65 by immunofluorescence assay. As shown in Fig. 13, TNF*α* induced the nuclear translocation of p65 and kahweol suppressed TNF*α*-induced p65 nuclear translocation. The inhibitory effects of kahweol on the TNFα-induced NF-κB luciferase reporter activity and nuclear translocation of p65 in endothelial cells may be due to the down-regulation of JAK2-PI3K/Akt activities. The endothelial cells were co-transfected with the

constitutively active form of Akt along with the NF-κB luciferase reporter. The cells were then pretreated with kahweol for 1 h or AG490 for 30min and stimulated with TNFα for 12 h. The kahweol-mediated inhibition of NF-κB luciferase activity was reversed by co-transfecting the cells with the constitutively active form of Akt (Fig. 14). Furthermore, pretreatment with AG490 significantly inhibited TNFα-induced NF-κB luciferase activity (Fig. 14). These highlight the involvement of JAK2 and Akt in the kahweol-mediated mechanism of the JAK2-PI3K/Akt-NF-κB pathway in endothelial and monocyte cells adhesion.

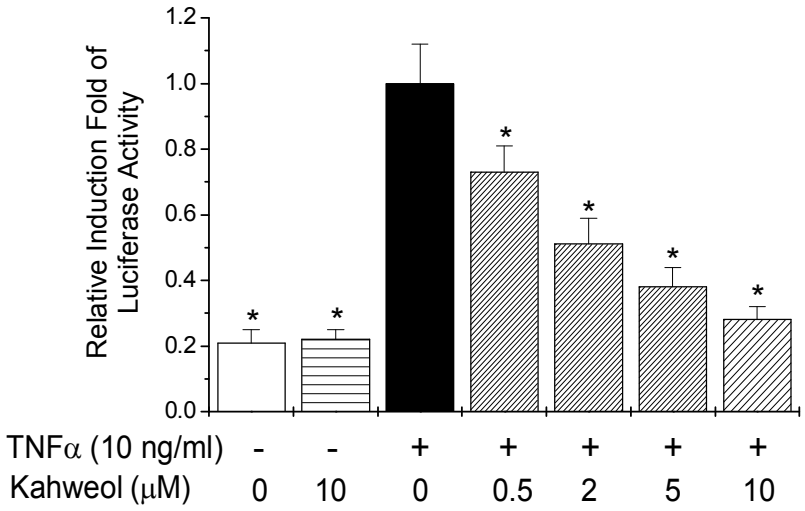



Fig. 11. Effects of kahweol on TNFα-induced NF-κB transcriptional activity.

The endothelial cells were transiently transfected with pGL3-4κB-Luc and pCMV-β-gal. After 18 h, the cells were pretreated with kahweol for 1 h, and then stimulated with TNFα for 12 h. Luciferase and β-galactosidase activities were determined. The luciferase activities are expressed relative to the TNFα. The values are expressed a mean ± SD of three individual experiments, performed in triplicate. *, $P < 0.01$ compared with the TNFα alone.

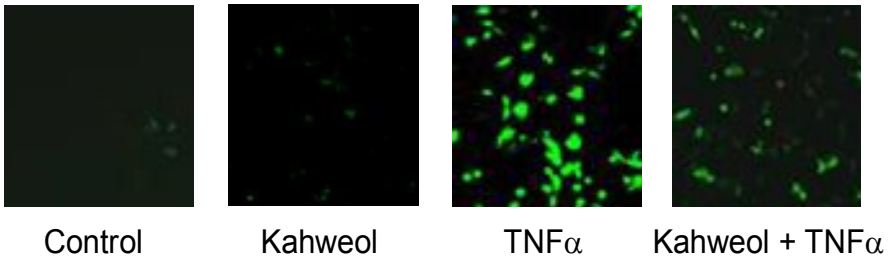



Fig. 12. Effects of kahweol on TNFα-induced NF-κB activation.

The endothelial cells were transiently transfected with NF-κB-GFP. After 18 h, the cells were pretreated with kahweol (10 μM) for 1h and then stimulated with TNFα (10 ng/ml) for 12 h. GFP expression in the cells was observed.

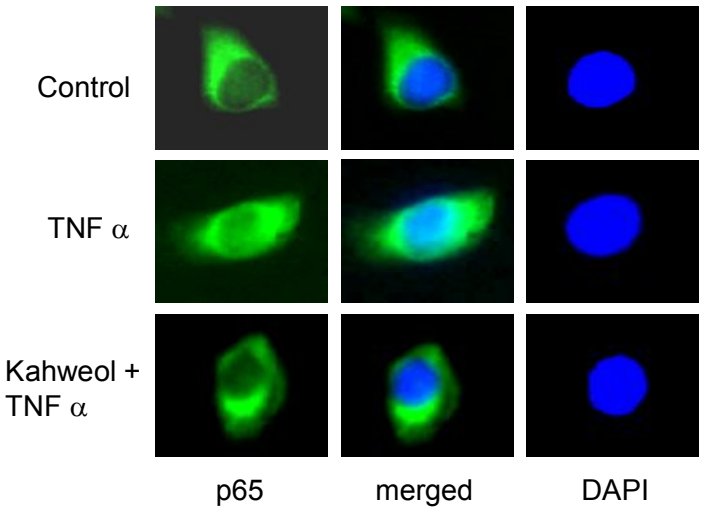



Fig. 13. Kahweol inhibited TNFα-induced nuclear translocation of NF-κB.

Intracellular localization of NF-κB p65 was investigated by indirect immunofluorescence using NF-κB p65 antibody. The endothelial cells were pretreated with kahweol (10 μM) for 1h and then stimulated with TNFα (10 ng/ml) for 30 min. NF-κB p65 (green) localization is shown. Nuclear DNA was visualized with DAPI.

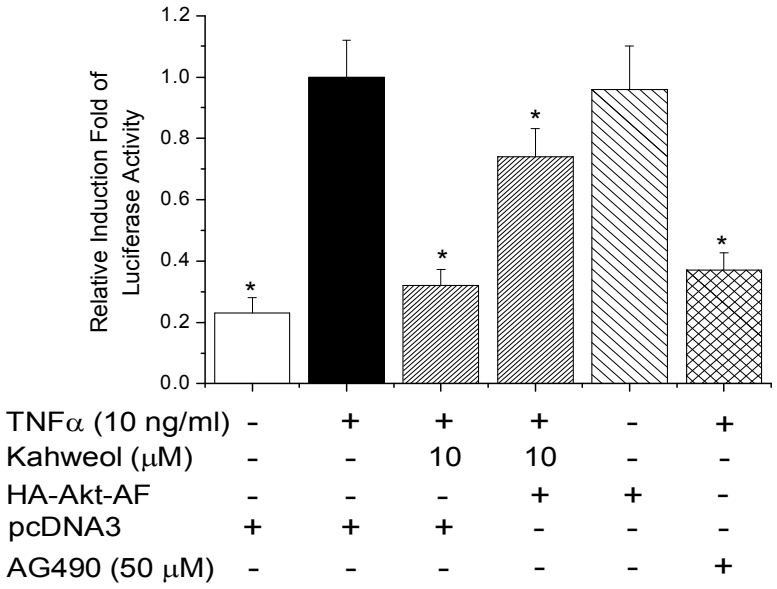



Fig. 14. Effects of kahweol on PI3K/Akt-NF-κB pathway.

The endothelial cells were transiently co-transfected with pGL3-4κB-Luc, along with the constitutively active form of Akt (HA-Akt-AF) or pcDNA3 and pCMV-β-gal. After 24 h of transfection, the cells were pretreated with kahweol for 1 h or AG490 for 30 min and then stimulated TNFα for 12 h. Luciferase and β-galactosidase activities were determined. The luciferase activities are expressed relative to the TNFα. The values are expressed a mean ± SD of three individual experiments, performed in triplicate. *, $P < 0.01$ compared with the TNFα alone.

**7. Kahweol has no effect on TNFR**

Because kahweol blocked signals activated by TNFα, it is possible that kahweol down-regulated TNFR. Therefore, this study examined the effects of kahweol on TNFR1 expression and binding activity to TNFR by RT-PCR and receptor-binding assays, respectively. Kahweol did not affect level of TNFR1 mRNA in endothelial cells (Fig. 15). When the endothelia cells were treated with $^{125}$I-TNFα, it bound to the cells. However, this binding was unaffected by pretreatment of the cells with kahweol (Fig. 16). These results suggesting that the effects of kahweol were not due to down-regulation of TNFR1 expression and TNFα binding activity.

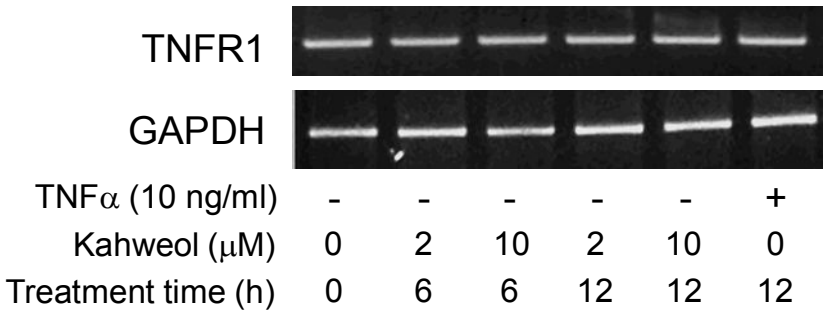



Fig. 15. Effects of kahweol on TNFR1 expression.

RT-PCR analysis of TNFR1. The endothelial cells were treated with kahweol for 6 h or 12 h and the total RNA was prepared and RT-PCR was performed. This blot is a representative of three independent experiments. One of three representative experiments is shown.

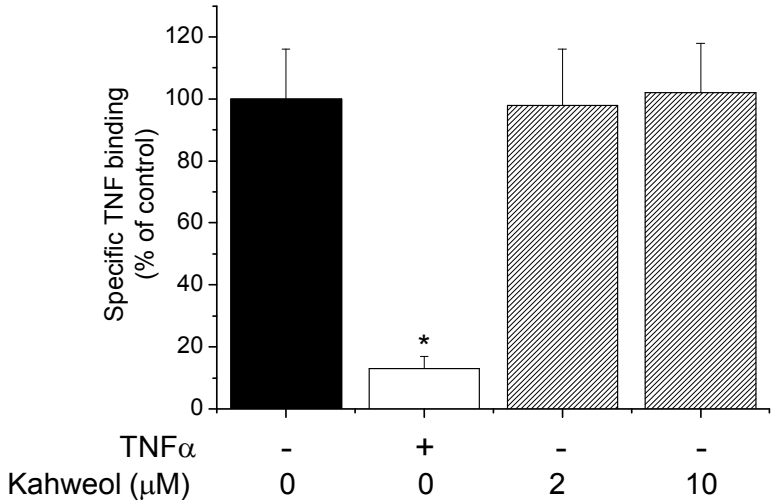



Fig. 16. Effects of kahweol on TNFR-binding activity.

The endothelial cells were incubated with labeled $^{125}$I-TNFα (0.1 mCi) in the presence or the absence of 100 nM excess cold TNFα or kahweol (mM) for 1 h at 4°C. Thereafter, cells were washed and radioactivities were counted. The values are expressed as a mean ± SD of three individual experiments, performed in triplicate. *, $P < 0.01$ compared with the control.

**Kahweol blocks STAT3 phosphorylation and induces apoptosis in human lung adenocarcinoma A549 cells**

## 8. Kahweol inhibits cell growth and viability in human lung adenocarcinoma A549

First, it was studied to determine whether kahweol treatment inhibited the growth of A549 cells. For time-course and dose-response experiments, human lung adenocarcinoma A549 cells were treated with 10, 20, 30, and 40 μM kahweol for 24 and 48 h, and then cell viability and cell growth were assessed by the MTT assay and BrdU incorporation, respectively. Control cells were treated with 0.1% DMSO. As seen in Fig. 17 and 18, kahweol caused a time- and dose-dependent reduction in cell viability and DNA synthesis. Significant inhibition of BrdU incorporation was observed as early as 24 h post-treatment. Cell viability was reduced by 50% at a dose of 20 μM kahweol after 48-h incubation (Fig. 17). Under the phase contrast microscope kahweol-treated cells exhibited a rounded and granulated morphology, and eventually detached from culture plates after 48 h of treatment (Fig. 19). Moreover, Hoechst staining demonstrated that kahweol induced a change in nuclear morphology. Compared to the typical round nuclei of the control, kahweol-treated cells displayed condensed and fragmented nuclei (Fig. 20). To determine if kahweol reduced cell number by inducing apoptosis, TUNEL assays were performed. Fig. 21 shows that DNA fragmentation was detected in kahweol-treated cells. Kahweol treatment increased the extent of DNA fragmentation in A549 cells in a dose-

dependent manner (Fig. 21). These data indicate that kahweol inhibits cell proliferation and induces apoptotic death in human lung adenocarcinoma A549 cells.

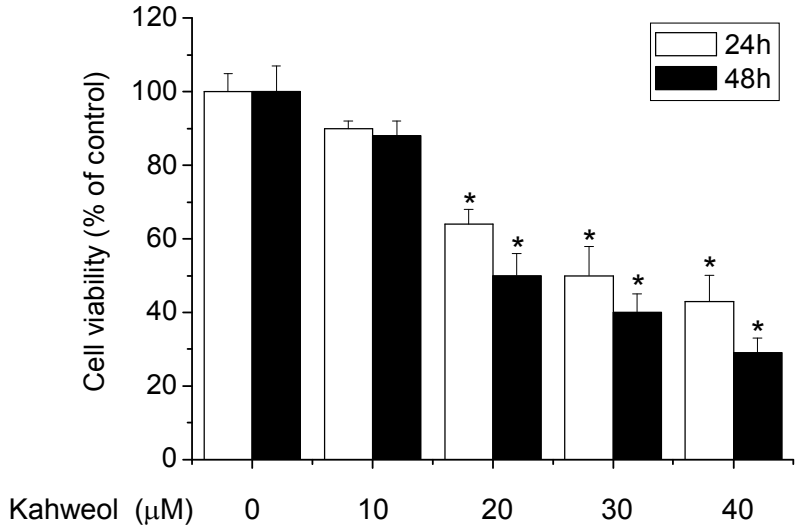



Fig. 17. Effects of kahweol on proliferation and apoptosis in A549 cells:

cell viability.

Cells were treated with serum-free medium containing either 0.1% DMSO or indicated doses of kahweol for 24 and 48 h as described under Materials and Methods. Cell viability were determined by MTT assays, respectively. Each bar shows the mean ± SD of three independent experiments performed in triplicate. *$P < 0.01$; significantly different from the control.

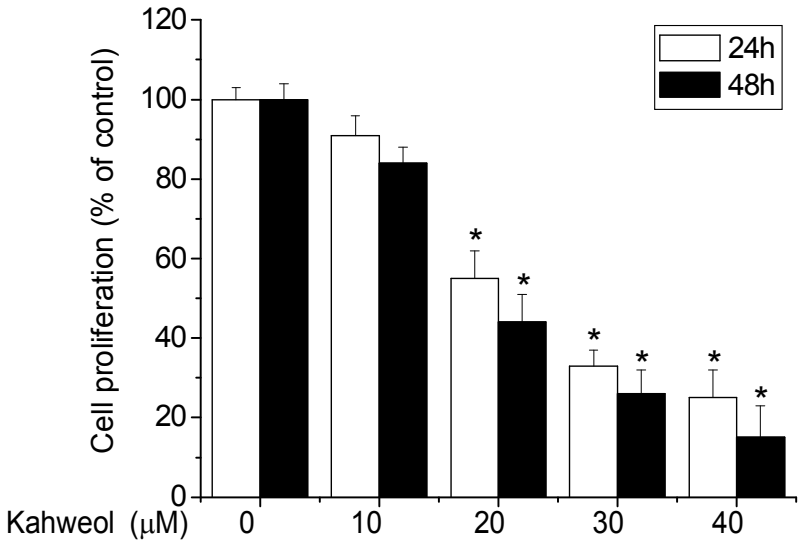



Fig. 18. Effects of kahweol on proliferation and apoptosis in A549 cells:

cell proliferation.

Cells were treated with serum-free medium containing either 0.1% DMSO or indicated doses of kahweol for 24 and 48 h as described under Materials and Methods. Cell proliferation were determined by bromo-uridine incorporation, respectively. Each bar shows the mean ± SD of three independent experiments performed in triplicate. *$P < 0.01$; significantly different from the control.

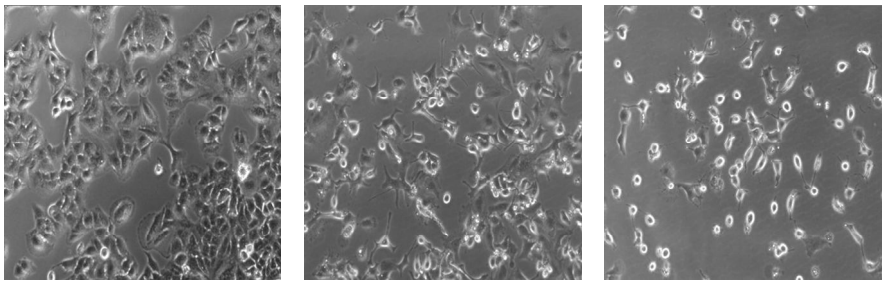



Fig. 19. Effects of kahweol on A549 cells morphology.

Morphological changes were measured using light microscopy; phase contrast. Cells were treated with 0.1% DMSO or 20 μM or 40 μM kahweol for 48 h.

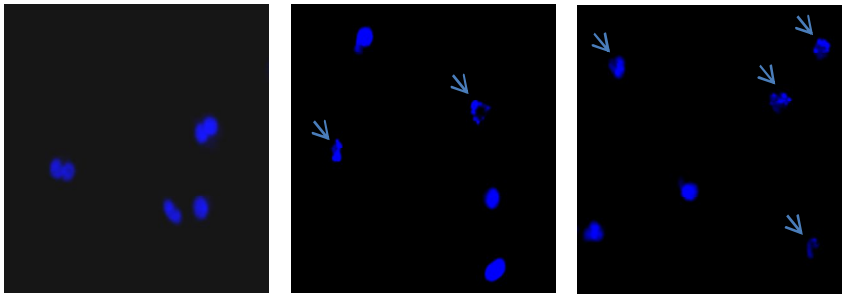



Fig. 20. Effects of kahweol on apoptosis in A549 cells.

Cells were treated with serum-free medium containing either 0.1% DMSO or indicated doses of kahweol 48 h as described under Materials and Methods. Morphology of apoptotic cells stained with Hoechst 33258 in cells. Cells were treated as indicated.

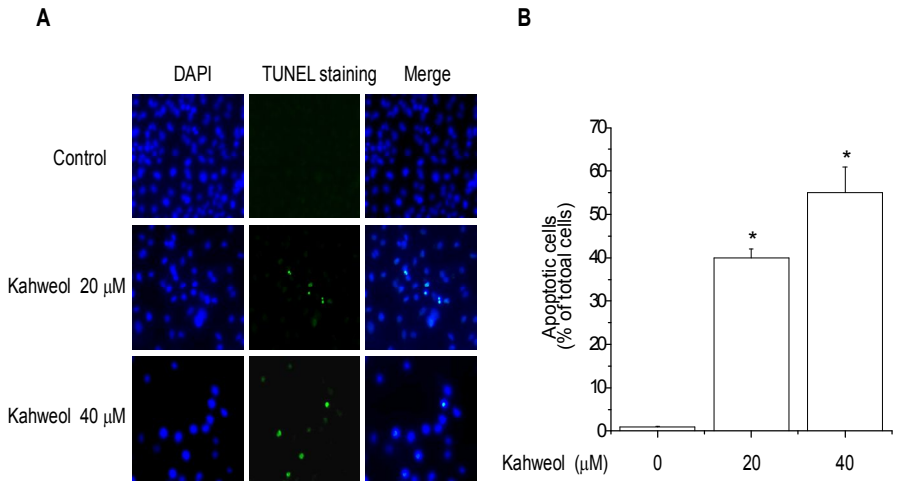



Fig. 21. Induction of apoptosis by kahweol in A549 cells.

TUNEL assays were performed according to the manufacturer's instructions. Apoptotic cells were visualized under a fluorescent microscope (A). The rate of apoptosis was expressed as percentage of total cells counted and is shown in (B). Each bar shows the mean ± SD of three independent experiments, performed in triplicate. *$P < 0.01$; significantly different from the control.

**9. Kahweol induces apoptosis through activation of the mitochondrial pathway**

The Bcl-2 family proteins are important regulators in both the inhibition and the promotion of apoptosis (Thomadaki and Scorilas, 2008). To elucidate whether Bcl-2 family molecules were involved in kahweol-induced apoptosis, effects of kahweol on the constitutive protein levels of Bcl-2, Bcl-$X_L$ and Bax were investigated in A549 cells. As shown in Fig. 22, kahweol induced a significant elevation in the expression of pro-apoptotic Bax. Treatment of A549 cells with 20 and 40 μM of kahweol led to 4.7 and 5.6-fold increase in Bax. In contrast, anti-apoptotic Bcl-2 expression was decreased by 0.5 and 0.2-fold on treatment with 20 and 40 μM of kahweol, respectively (Fig. 22)**.** Similar the protein expression of Bcl-$X_L$ was significantly decreased by kahweol treatment at 20 and 40 μM. A significant dose-dependent shift in the ratio of Bax to Bcl-2 was observed after kahweol treatment, indicating induction of the apoptotic process.

Caspase-3 has been shown to play a pivotal role in the terminal execution phase of apoptosis induced by diverse stimuli (Zhao-Yang et al., 2008). As PARP cleavage is a hallmark of caspase activation, it was studied to determine whether apoptotic machinery was activated by kahweol treatment, using an anti-specific-cleaved PARP antibody that detects only cleaved products of PARP. As shown in Fig. 23, the 89-kDa cleaved PARP fragment was detected in kahweol-treated samples. As caspase-3 plays a central role in PARP cleavage, activation of caspase-3 was determined by using antibodies capable of detecting cleaved caspase-3. Cell extracts prepared from kahweol-treated cells showed dose-dependent activation of

caspase-3 and caspase-9 (Fig. 24, 25). To monitor the enzymatic activity of caspase-3 during kahweol-induced apoptosis used caspase-3 specific colorimetric peptide substrate DEVD-pNA. As shown in Fig. 24, kahweol dramatically increased DEVD-specific caspase-3 activity in A549 cells. Furthermore, after cells treated with a caspase-3 inhibitor (z-DEVD-fmk), the activity of caspase-3 was completely blocked. These data demonstrated an apoptotic effect of kahweol in A549 cells.

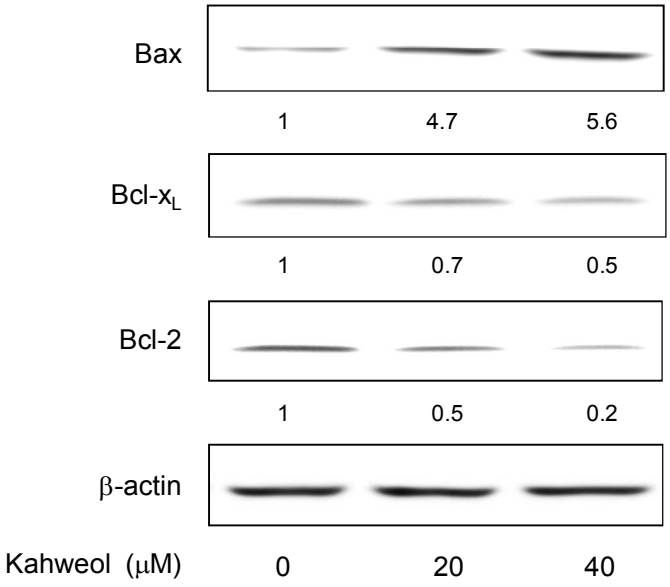



Fig. 22. Effects of Kahweol on the levels of Bcl-2 family in A549 cells.

Western blotting analysis of Bcl-2 family proteins in A549 cells following 20 or 40 μM kahweol treatment for 48 h. Actin was used as a loading control. The densitometry data presented below the bands are "fold change" compared with control after normalization with respective loading control value.

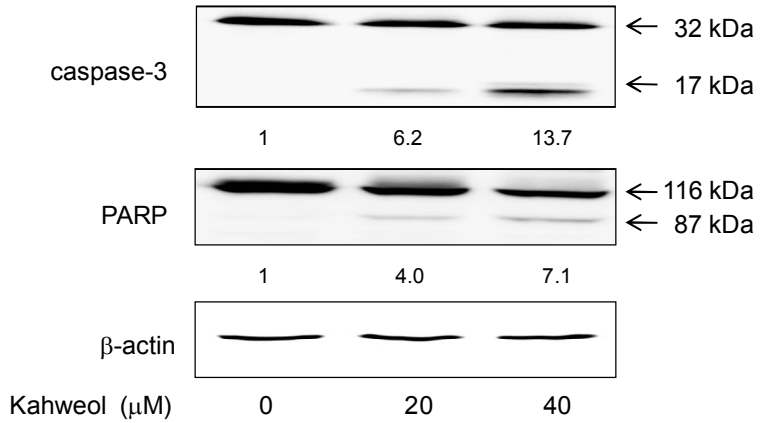



Fig. 23. Effects of kahweol on cleavage of caspase-3 and PARP in A549 cells. Western blotting of caspase-3 activation and PARP proteolytic fragment in A549 cells treated with different concentrations of kahweol for 48 h. Actin was used as a loading control. The densitometry data presented below the bands are "fold change" compared with control after normalization with respective loading control value.

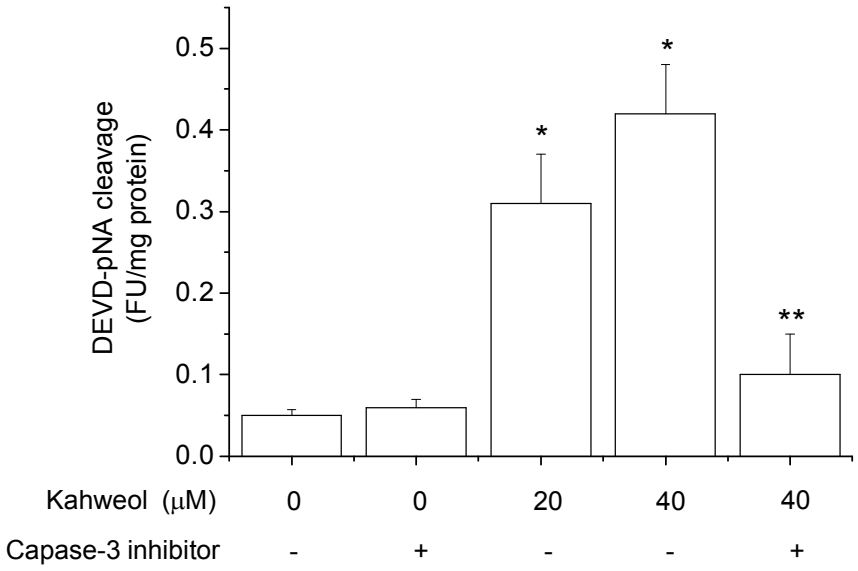



Fig. 24. Activation of caspase-3 by kahweol in A549 cells.

A549 cells were pretreated with z-DEVD-fmk for 1 h, and then exposed to kahweol for additional 48 h. The catalytic activities of caspase-3 in cell lysates were assayed using the specific substrate DEVD-pNA. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. *$P < 0.01$; significantly different from the control. **$P < 0.01$; significantly different from kahweol treatment alone.

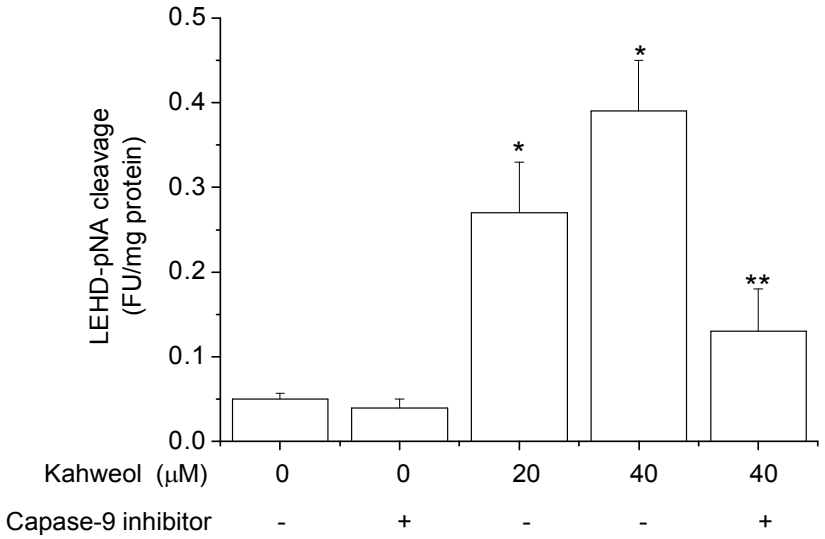



Fig. 25. Activation of caspase-9 by kahweol in A549 cells.

Cells were treated as indicated. The catalytic activities of caspase-9 in cell lysates were assayed using a specific substrate LEHD-pNA. Experiments were done in triplicates and the means and standard deviations are shown. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. *$P < 0.01$; significantly different from the control. **$P < 0.01$; significantly different from kahweol treatment alone.

**10. Kahweol inhibits constitutive STAT3 phosphorylation**

**in human lung adenocarcinoma A549 cells**

Human lung cancer A549 cells express constitutively active STAT3 (Zhu et al., 2007). Under resting conditions and in the non-phosphorylated state, STAT3 is retained in the cytoplasm. It translocates to the nucleus when phosphorylated (Snyder et al., 2008). Posphorylation induces STAT3 dimerization, thus permitting its translocation into the nucleus. The effect of kahweol on STAT3 activation in A549 cells was examined. Western blot analyses with specific antibodies for phosphorylated STAT3 indicated that kahweol inhibited constitutively active STAT3 in A549 cells in a dose- and time-dependent manner (Fig. 26). This inhibition appeared as early as 15 min, with complete abrogation at 45 min after 40 μM kahweol treatment. Kahweol treatment did not alter the overall expression of STAT3 protein. Activation of STAT3 can be determined by assessing STAT3 tyrosine phosphorylation or its ability to increase transcription of reporter genes containing multiple copies of STAT3 binding sites in the promoter region (STAT3-luc). The effect of kahweol on STAT3 transcriptional activation in A549 cells was assessed in cells transfected with a plasmid containing the STAT3-binding DNA consensus site linked to a luciferase reporter gene. IL-6 was used as a positive control. As shown in Fig. 27, kahweol decreased the transcriptional activity of STAT3 in a dose-dependent manner.

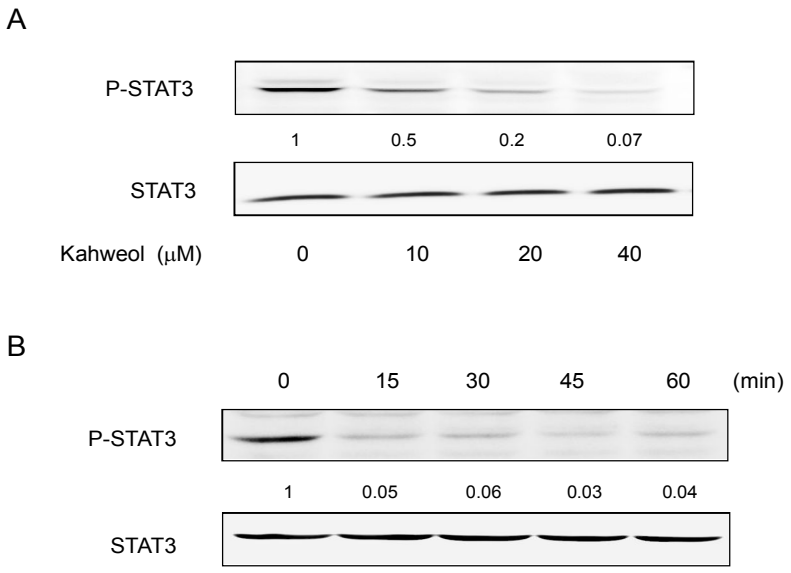



Fig. 26. Kahweol inhibits constitutively active STAT3 in A549 cells.

A and B, cells were treated with 40 μM kahweol for the indicated times. Cells lysates were prepared, and Western blotting was performed using antibodies against phosphorylated (p)-STAT3 and STAT3 (A). A549 cells were treated with the indicated concentrations of kahweol for 60 min, after which western blotting was performed as described for panel (A). The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. The densitometry data presented below the bands are "fold change" compared with control after normalization with respective loading control value.

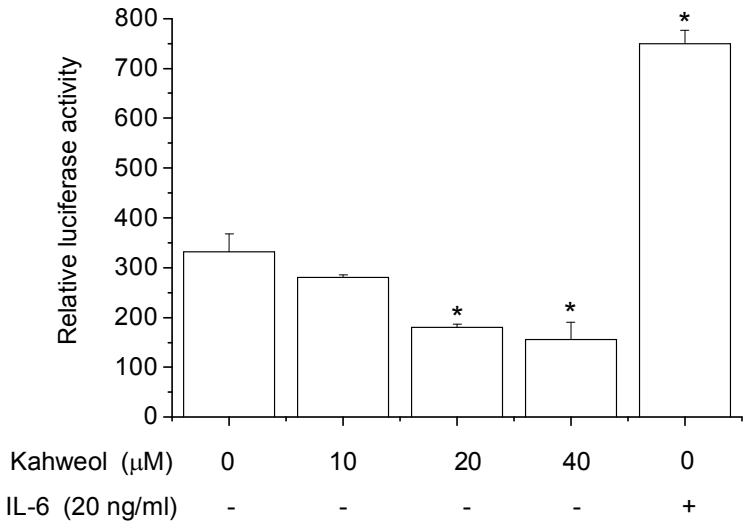



Fig. 27. Kahweol decreases the transcriptional activity of STAT3 in A549 cells in a dose-dependent manner.

A549 cells were transiently transfected with a STAT3-responsive luciferase reporter (STAT3-Luc). Cells were then treated with indicated amounts (μM) of kahweol and IL-6 for 24 h and assayed for luciferase activities. Experiments were performed in triplicate and normalized to b-galactosidase expression, as determined by b-galactosidase assays. Each bar shows the mean ± SD of three independent experiments performed in triplicate. *$P < 0.01$; significantly different from the control.

**11. STAT3 overexpression leads to resistance to kahweol-induced apoptosis**

If STAT3 is a critical target of kahweol, profound expression of STAT3 should attenuate kahweol-induced apoptosis. STAT3 activation has been shown to regulate the expression of various gene products involved in cell survival and proliferation (Klampfer, 2008). To determine whether the disruption of STAT3 signaling is required for the observed apoptotic effects of kahweol, the effect of kahweol on apoptotic events in cells transiently expressing STAT3 were examined. The wild-type STAT3-overexpressing A549 cells showed increased expression of total STAT3 and Bcl-2 (Fig. 28). Despite kahweol treatment, the STAT3-overexpressing cells had a clearly detectable level in the expression of phosphorylated STAT3, which was fragile against kahweol in this system. The kahweol-treated STAT3-overexpressing cells showed no enhanced cleavages in caspase-3, in comparison with the controls. The kahweol-treated STAT3-overexpressing cells decreased expression of Bcl-2, in comparison with the STAT3-overexpressing cells, suggesting that kahweol might target, at least in part, STAT3 in the A549 cells. Also, the effects of wild-type STAT3 on the kahweol-induced apoptosis were measured using MTT assay and TUNEL assay. Wild-type STAT3 completely blocked the apoptotic effect of kahweol (Fig. 28, 29, 30). Furthermore, inhibitors of the JAK/STAT pathway, namely AG490, also induced apoptosis. These results suggest that kahweol-induced A549 cell apoptosis involves inhibition of STAT3 activation.

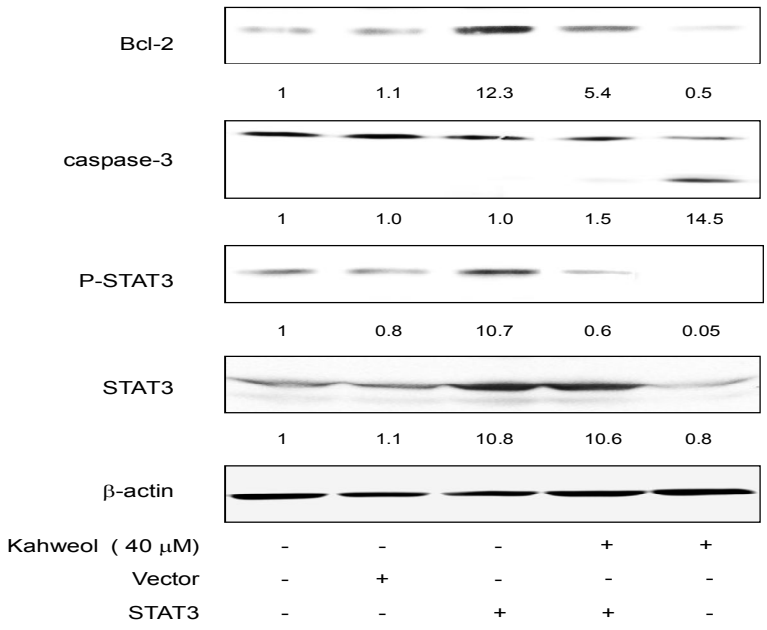



Fig. 28. Effects of Kahweol on the levels of Bcl-2, caspase, STAT3 in STAT3-overexpressing A549 cells.

The STAT3 gene was transiently transfected into A549 cells by the lipofection method. At 24 h after transfection of the STAT3 gene, kahweol were added into the culture medium of the cells. Total cell lysate was prepared for western blot analysis as described under Materials and methods. Western blotting was performed using antibodies against actin, Bcl-2, caspase-3, phosphorylated (P)-STAT3 and STAT3. Actin was used as a loading control. The densitometry data presented below the bands are "fold change" compared with control after normalization with respective loading control value.

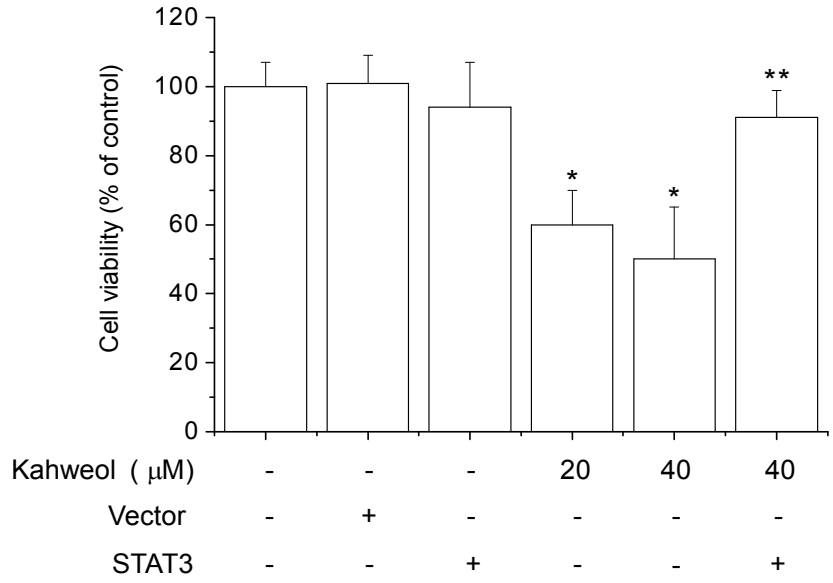
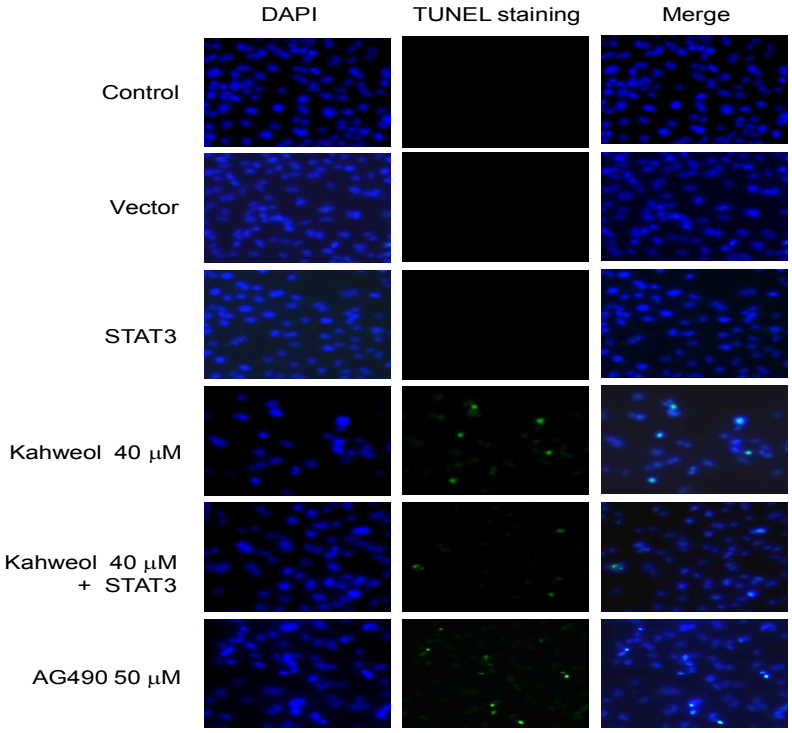



Fig. 29. Effects of Kahweol on cell viability in STAT3-overexpressing A549 cells. The STAT3 gene was transiently transfected into A549 cells by the lipofection method. At 24 h after transfection of the STAT3 gene, kahweol were added into the culture medium of the cells. Cells were treated with 20 and 40 μM kahweol for 48 h. Cell viability was determined by MTT assay. Each bar shows the mean ± SD of three independent experiments performed in triplicate. *$P < 0.01$; significantly different from the control. **$P < 0.01$; significantly different from kahweol treatment alone.

A


DAPI
TUNEL staining
Merge
Control
Vector
STAT3
Kahweol 40 μM
Kahweol 40 μM
+ STAT3
AG490 50 μM

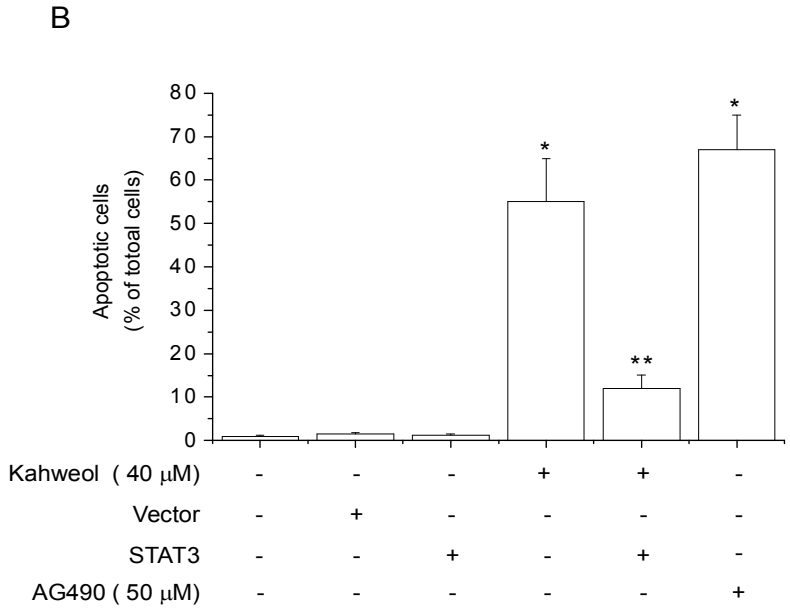



Fig. 30. Resistance to kahweol-induced apoptosis in STAT3-overexpressing A549 cells.

TUNEL assays were performed according to the manufacturer's instruction. Apoptotic cells were visualized under a fluorescent microscope (A). The rate of apoptosis was expressed as percentage of total cells counted and is shown in (B). Each bar shows the mean ± SD of three independent experiments, performed in triplicate. *$P$ < 0.01; significantly different from the control. **$P$ < 0.01; significantly different from kahweol treatment alone.

**Kahweol inhibit angiogenesis and metastasis through suppression of STAT3 activation**

**12. Cytotoxicity of kahweol in HUVECs**

To determine the optimal concentrations for use in our studies, kahweol was tested for potential cytotoxicity in HUVECs. Fig. 31 shows that kahweol at concentrations of 1, 5, 10 μM did not affect cell viability. However, at 20 and 40 μM, the highest concentrations tested, kahweol caused 20% and 40% decreases, respectively, in cell viability. Accordingly, this study employed kahweol at non-cytotoxic concentrations (i.e., <10 μM) and focused on the effect of kahweol on IL-6-induced angiogenesis in subsequent experiments.

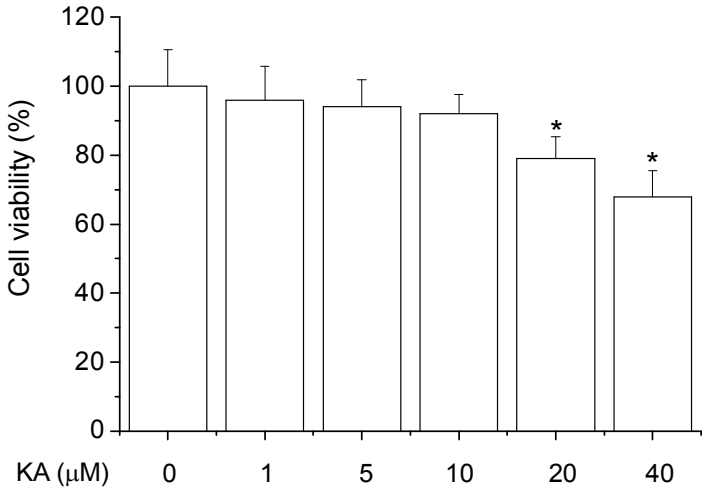



Fig. 31. Effects of kahweol on cell viability.

To test the cytotoxicity of kahweol, cell viability of HUVECs 24 h after treatment with kahweol (0, 1, 5, 10, 20, and 40 μM) was determined by MTT assay. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. *$P$ < 0.01; significantly different from the control.

**13. Kahweol inhibits angiogenesis by HUVECs**

Angiogenesis is initiated by the release of angiogenic factors, and VEGF is the most potent angiogenic factor associated with tumor angiogenesis. IL-6 is also important for both physiological and pathological angiogenesis (Kim et al., 2009; Fan et al., 2008). To investigate whether kahweol inhibits IL-6-induced angiogenesis, the effect of kahweol on IL-6-induced VEGF expression, cellular migration, and tube formation were measured. HUVECs were pretreated with various doses of kahweol for 1 h and then co-treated with IL-6 for 24 h. Treatment with kahweol resulted in a dose-dependent inhibition of IL-6-induced VEGF mRNA and protein expression in HUVECs (Fig. 32 and 33). To further test the effect of kahweol on VEGF production, HUVECs were incubated with various concentrations of kahweol for 24 h, and the VEGF levels in the culture supernatants were measured by ELISA. As shown in Fig. 34, IL-6-induced VEGF production was significantly and dose-dependently decreased by kahweol. To examine further the effect of kahweol on VEGF promoter activity in cells transfected with pVEGF-Luc. Kahweol suppressed IL-6-induced VEGF-luciferase activity in a dose-dependent manner (Fig. 35).

The migration of endothelial cells through the basement membrane is a crucial step for the establishment of new blood vessels (Geiger and Peeper, 2009). To assess the effect of kahweol on IL-6-induced HUVEC migration, HUVECs were treated with

kahweol at concentrations of 1, 5, and 10 μM and then placed in an 8-□m Transwell system. As shown in Fig. 36, kahweol significantly inhibited the IL-6-induced migration of HUVECs, in a dose-dependent manner. The angiogenic effects of kahweol were also determined by examining the effects on morphologic differentiation of HUVECs into capillary-like structure using the Matrigel tube formation assay. The treatment of cells with various concentrations of kahweol resulted in significant inhibition of IL-6-stimulated tube formation (Fig. 37). Our results demonstrate that kahweol is effective in reducing IL-6-stimulated angiogenesis by endothelial cells via the regulation of VEGF gene expression.

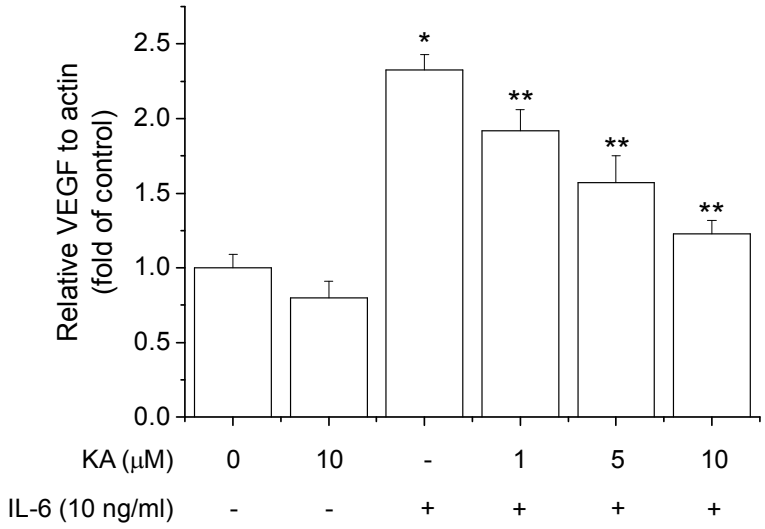



Fig. 32. Effects of kahweol on VEGF mRNA expression in HUVECs.

Endothelial cells were pretreated with kahweol for 1 h and incubated with or without IL-6 (10 ng/ml) for 24 h. Total RNA was prepared, and VEGF mRNA expression was analyzed by real-time PCR analysis. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. *$P < 0.01$; significantly different from the control. **$P < 0.01$; significantly different from IL-6 treatment.

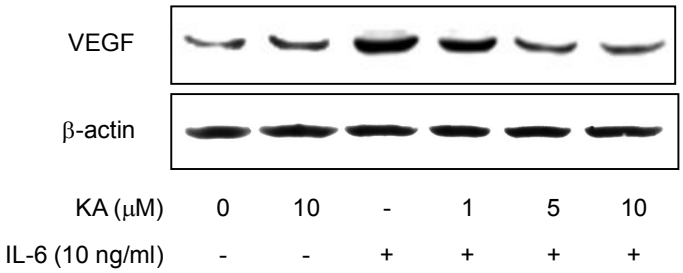



Fig. 33. Effects of kahweol on VEGF protein expression in HUVECs.

After cells were incubated for 24 h with and without kahweol, cell lysates were prepared and analyzed by Western blotting with anti-VEGF and anti-β-actin antibodies. One representative blot from three independent experiments is shown.

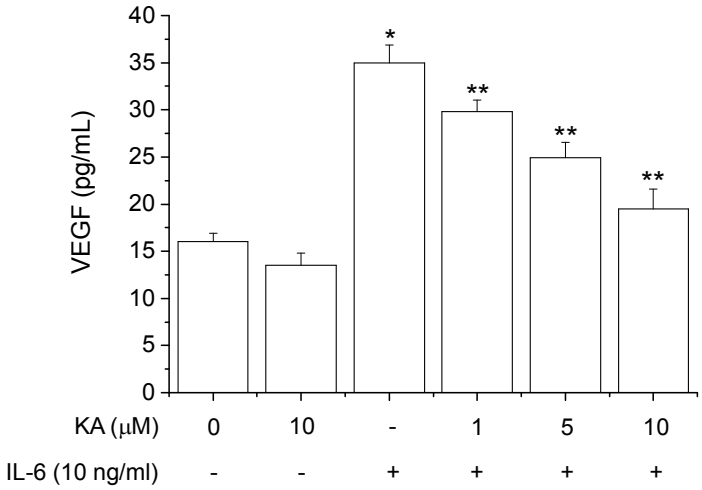



Fig. 34. Effects of kahweol on VEGF production in HUVECs.

HUVECs in fresh medium containing 1% serum were treated with increasing doses of kahweol (1, 5, and 10 μM) for 24 h, and then the cells were incubated with or without IL-6 (10 ng/ml) for 24 h. The concentration of VEGF in the culture medium was determined by quantitative ELISA. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. *$P < 0.01$; significantly different from the control. **$P < 0.01$; significantly different from IL-6 treatment.

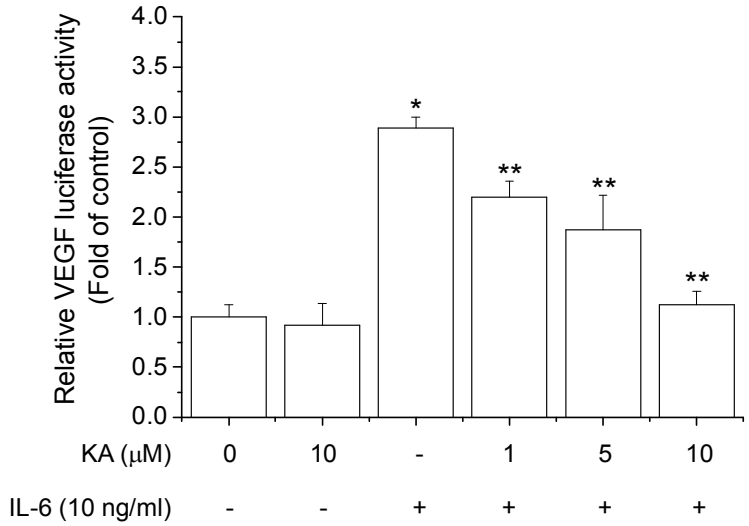



Fig. 35. Effects of kahweol on IL-6-induced VEGF transcriptional activity.

Cells were transiently transfected with pGL3-VEGF-Luc and pCMV-β-gal. After 4 h of transfection, cells were treated with kahweol for 1 h and then incubated with or without IL-6 for 24 h. Luciferase and β-galactosidase activities were determined. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. *$P$ < 0.01; significantly different from the control. **$P$ < 0.01; significantly different from IL-6 treatment.

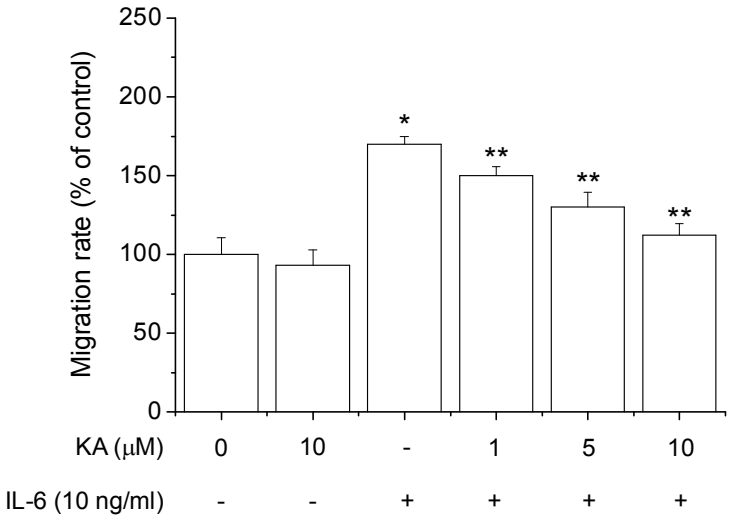



Fig. 36. Kahweol inhibited IL-6-induced migration in HUVECs.

HUVECs were cultured with various concentrations of kahweol in the upper chamber of a Transwell; the lower chamber contained IL-6 (10 ng/ml). After 24 h, cell migration was determined by counting the cells that had migrated through the membrane. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. *$P < 0.01$; significantly different from the control. **$P < 0.01$; significantly different from IL-6 treatment.

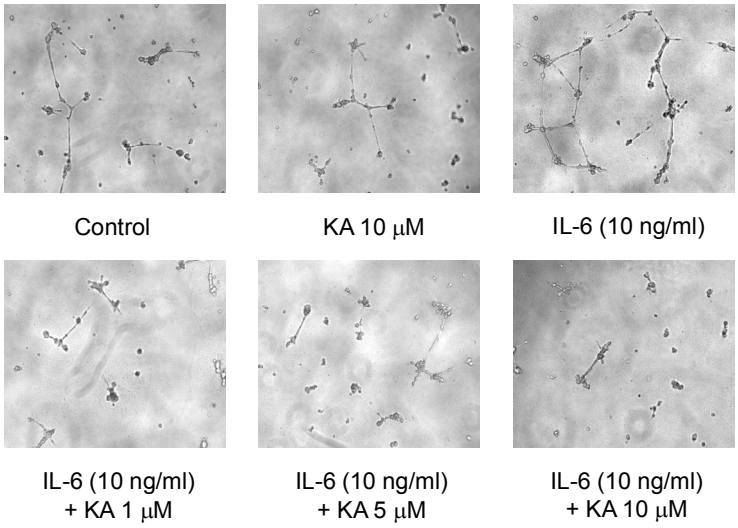



Fig. 37. Effects of kahweol on angiogenesis in HUVECs.

HUVECs were incubated on 96-well plates with IL-6 and/or kahweol. Micrographs taken under a phase contrast light microscope at 200× magnification show the degree of morphological differentiation of the HUVECs into capillary-like structures.

**14. Kahweol inhibits VEGF expression in breast cancer cells**

VEGF is commonly overexpressed in several types of human cancers, including breast cancer (Hsieh et al., 2005). Furthermore, estrogen receptor (ER)-independent breast cancers are extremely aggressive, with high potential for metastasis (Anandappa et al., 2000). To examine the antiangiogenic effects of kahweol in breast cancer, ER-independent human breast cancer cells (MDA-MB231) and normal human breast epithelial cells (MCF-10A) were cultured for 24 h in the absence or presence of kahweol. Conditioned media from the MCF-10A and MDA-MB231 cells were concentrated and assessed for VEGF by ELISA analysis. As shown in Fig. 38, kahweol inhibited VEGF secretion by up to 50% in MDA-MB231 cells, compared with controls. In contrast, kahweol had no effect in normal MCF-10A cells. To assess whether the inhibition occurred at the transcriptional level, VEGF mRNA and protein expression were measured by real-time PCR and Western blotting. Both VEGF mRNA and protein expression were significantly and dose-dependently reduced in MDA-MB231 cells after 24 h of kahweol treatment (Fig. 39 and 40), whereas kahweol had no effect on VEGF mRNA or protein expression in MCF-10A cells. To examine the effect of kahweol on VEGF transcription, MCF-10A and MDA-MB231 cells were transiently transfected with a luciferase reporter construct containing the 5' regulatory region of the human VEGF

gene. Cells were transfected with pVEGF-Luc and treated for 24 h with the indicated concentrations of kahweol. Similar to the gene expression results, kahweol inhibited VEGF promoter activity by up to 50% in MDA-MB231cells, compared with controls, but it did not affect promoter activity in MCF-10A cells (Fig. 41). Taken together, these results suggest that kahweol mediates VEGF expression at the transcriptional level in MDA-MB231 cells.

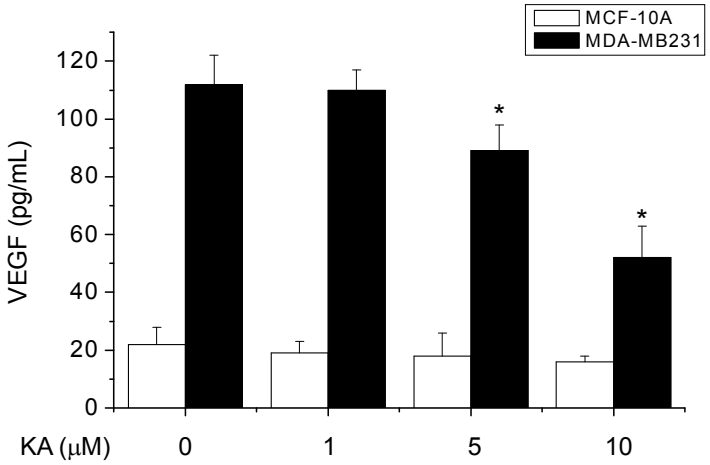



Fig. 38. Effects of kahweol on VEGF secretion in MCF-10A and MDA-MB231 cells.

VEGF release into the cell culture medium in the presence and absence of kahweol was determined by ELISA assay. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. *$P < 0.01$; significantly different from the control.

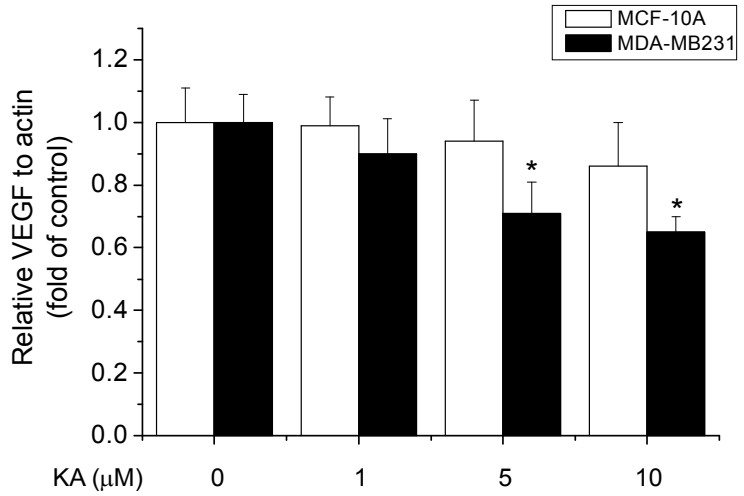



Fig. 39. Effects of kahweol on VEGF mRNA expression in MCF-10A and MDA-MB231 cells.

VEGF mRNA expression in MDA-MB231 and MCF-10A cells cultured with the indicated concentrations of kahweol was measured by real-time PCR analysis. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. *$P < 0.01$; significantly different from the control.

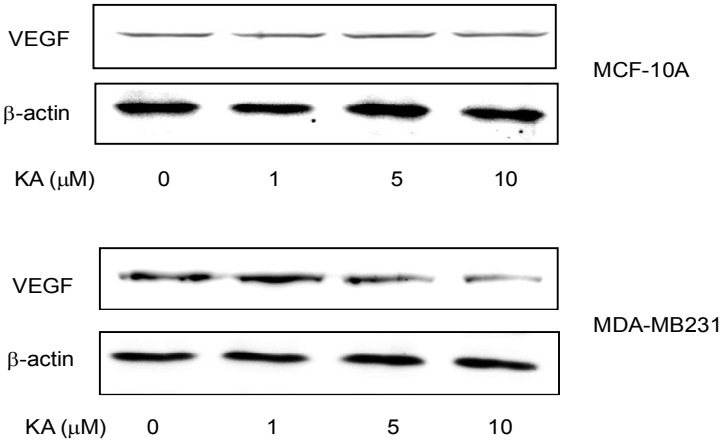



Fig. 40. Effects of kahweol on VEGF protein expression in MCF-10A and MDA-MB231 cells.

After cells were incubated for 24 h with and without kahweol, cell lysates were prepared and analyzed by Western blotting with anti-VEGF and β-actin antibodies. One representative blot from three independent experiments is shown.

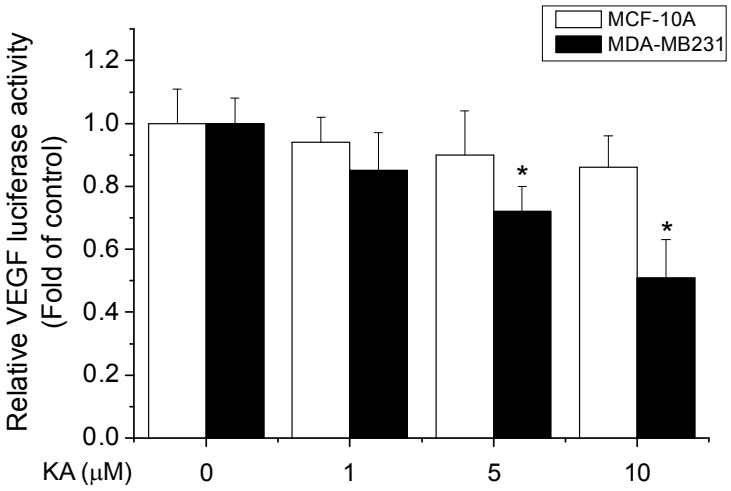



Fig. 41. Effects of kahweol on VEGF transcriptional activity in MCF-10A and MDA-MB231 cells.

MCF-10A and MDA-MB231 cells were transfected with pGL3-VEGF-Luc and pCMV-β-gal, treated with kahweol for 24 h, harvested, and assayed for luciferase and β-galactosidase activities. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. *$P < 0.01$; significantly different from the control.

**15. Kahweol modulates gene expression and activities of MMP-2 and -9**

Metalloproteinases such as MMP-2 and MMP-9 are involved in both angiogenic and tumorigenic processes (Deryugina and Quigley, 2006). Real-time PCR analysis was performed to determine the effect of kahweol on MMP-2 and -9 mRNA expression levels. Treatment of MDA-MB231 cells with kahweol decreased the mRNA expression levels of both MMPs, in a dose-dependent manner (Fig. 42A and 42B). To examine the direct effect of kahweol on MMP-2 and -9 gene transcription, cells were transfected with a reporter plasmid containing the 5'-flanking region of the MMP-2 or -9 gene. Treatment with kahweol decreased the activity of both the MMP-2 and -9 promoters in a dose-dependent manner, compared with the activity in untreated cells (Fig. 43). As MMP-2 and -9 belong to the group of MMPs that use gelatin as their substrate, the gelatinolytic activities of secreted MMP-2 and -9 were determined by zymographic analysis of MDA-MB231 cell supernatants. As shown in Fig. 44, kahweol suppressed MMP-2 activity in a dose-dependent manner, and a similar effect was observed for MMP-9 activity. These results indicate that kahweol modulates MMP-2 and -9 activities via transcriptional mechanisms in MDA-MB231 cells.

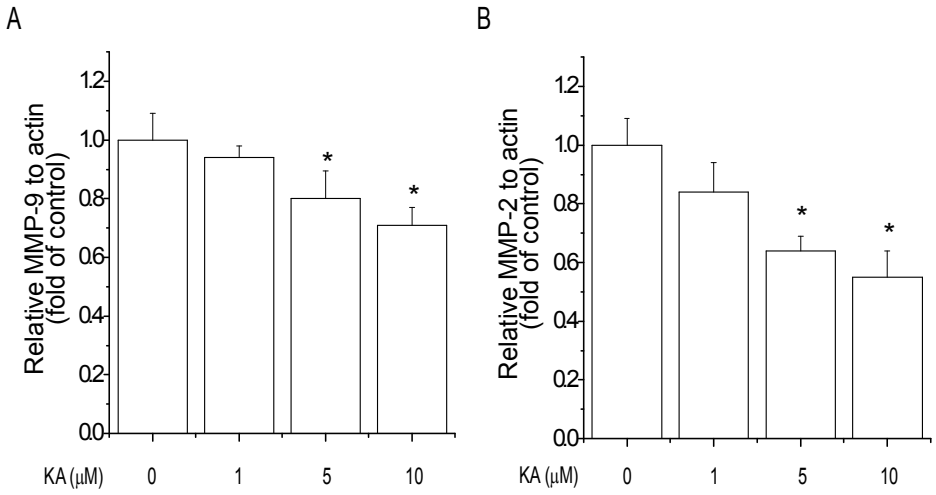



Fig. 42. Kahweol inhibits the expression of MMP-2 and -9.

MDA-MB231 cells were exposed to kahweol for 24 hr. (A, B) After MDA-MB231 cells were incubated with and without kahweol at the indicated concentrations for 24 h, total RNA was prepared and the mRNA expression levels of MMP-2 and -9 were measured by real-time PCR. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. *$P < 0.01$; significantly different from the control.

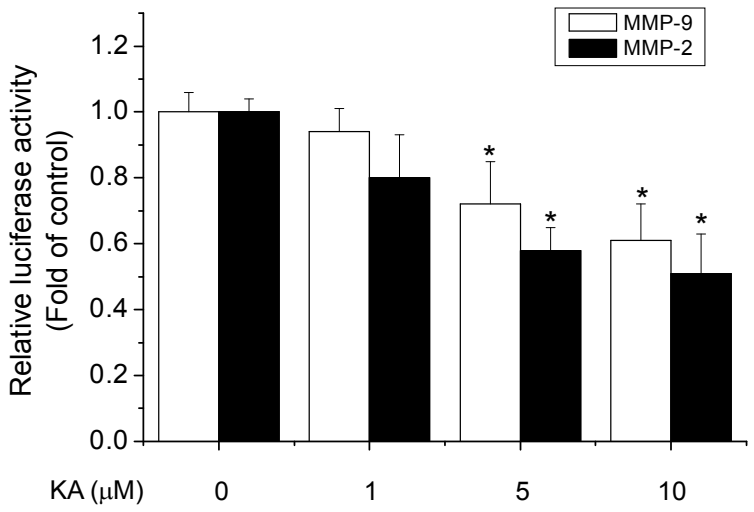



Fig. 43. Effects of kahweol on MMP-2 and -9 transcriptional activity in MDA-MB231 cells.

To test the effect of kahweol on MMP-2 and -9 promoter activities, MDA-MB231 cells transiently transfected with pCMV-β-gal and pGL3-MMP-2-Luc or pGL3-MMP-9-Luc were treated with kahweol for 24 h, harvested, and assayed for luciferase and β-galactosidase activities. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. *$P < 0.01$; significantly different from the control.

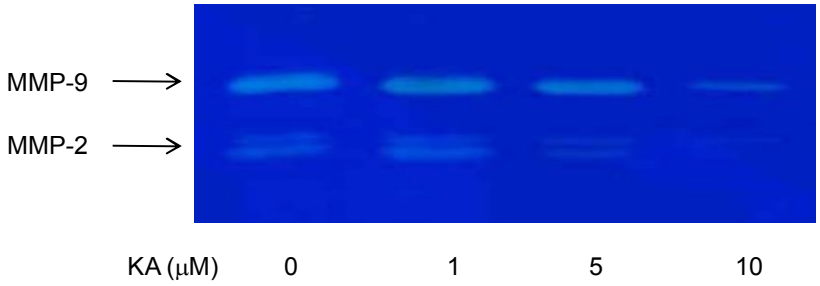



Fig. 44. Effects of Kahweol on MMP-2 and MMP-9 activities.

Gelatin zymography assays were performed to analyze the gelatinolytic activity of MMP-2 (72 kDa) and MMP-9 (92 kDa) in the presence of various concentrations of kahweol. One representative zymograph from three independent experiments is shown.

**16. Kahweol inhibits breast cancer cell migration and invasion**

Cancer cell tissue invasion is important during metastasis, and MDA-MB231 cells are able to migrate through a Matrigel matrix (Walter-Yohrling et al., 2003). To investigate whether kahweol inhibits breast cancer cell migration and invasion, Transwell migration and Matrigel invasion assays were performed in kahweol-treated MDA-MB231 cells. As shown in Fig. 45, serum-induced cell migration was reduced by kahweol treatment, in a concentration-dependent manner. In addition, the number of cells that invaded a Matrigel matrix was significantly decreased after a 24-h treatment with kahweol (Fig. 46A and 46B). These findings suggest that kahweol can inhibit the invasive migration of MDA-MB231 cells.

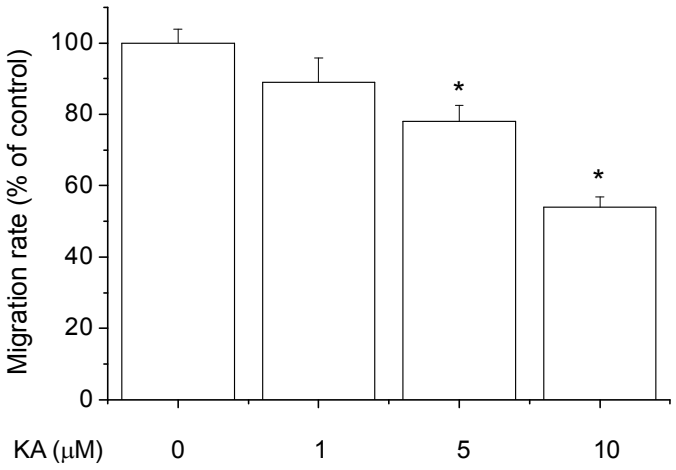



Fig. 45. Effects of kahweol on migration of MDA-MB231 cells.

MDA-MB231 cells in the upper half of a Transwell chamber were incubated in the presence or absence of kahweol at the indicated concentrations and allowed to migrate through a porous membrane toward the lower chamber, which contained medium with 10% FBS as a chemoattractant. Data from three experiments are expressed as a percentage of untreated cells. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. *$P < 0.01$; significantly different from the control.

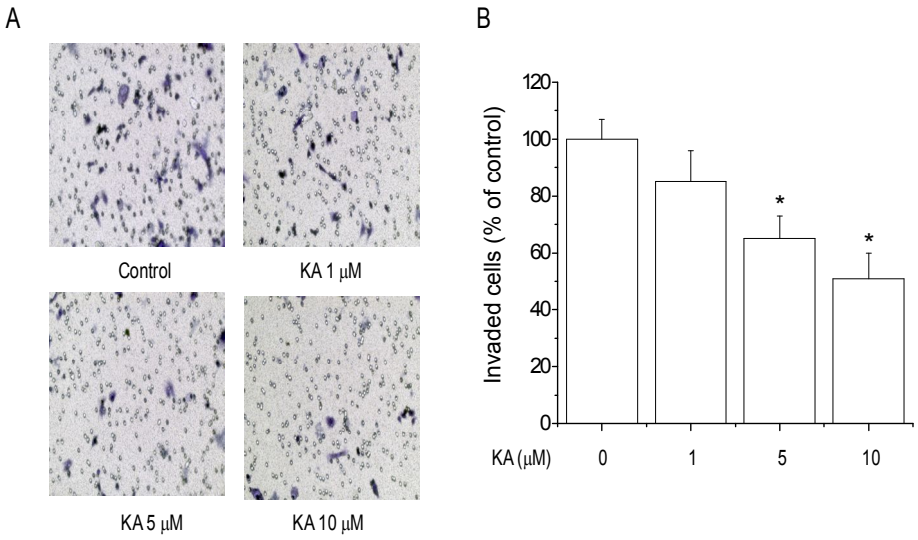



Fig. 46. Effects of kahweol on invasion of MDA-MB231 cells.

MDA-MB231 cells were treated with indicated concentrations of kahweol. (A) MDA-MB231 cells that penetrated to the lower surface of a Matrigel matrix were stained with crystal violet and photographed under a light microscope at 200×. (B) The number of cells that invaded the Matrigel is expressed as a percentage of untreated cells. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. *$P < 0.01$; significantly different from the control.

### 17. Kahweol inhibits constitutive STAT3 phosphorylation in breast cancer cells

STAT family proteins participate in tumorigenesis (Silva, 2004). STAT3 activity is commonly upregulated in breast cancer, and STAT3 regulates the expression of proangiogenic genes, including VEGF and MMP genes (Aggarwal et al., 2006; Hsieh et al., 2005). To confirm that this effect correlated with differences in STAT3 activation, the level of STAT3 tyrosine phosphorylation in MDA-MB231 and MCF-10A cells were measured by Western blotting. Compared with MCF-10A normal breast epithelial cells, MDA-MB231 breast cancer cells showed increased STAT3 expression and phosphorylation (Fig. 47). The effect of kahweol on STAT3 activation in MDA-MB231 cells was examined. As shown in Fig. 48A and 48B, kahweol inhibited constitutive STAT3 activation in a dose- and time-dependent manner, but did not affect STAT3 protein expression. Kahweol applied at 10 μM for 2 h completely inhibited constitutive STAT3 activation. The effect of kahweol on STAT3 transcriptional activation in MDA-MB231 cells was assessed in cells transfected with a plasmid containing the STAT3-binding DNA consensus site linked to a luciferase reporter gene. AG490, a specific JAK/STAT inhibitor, was used as a negative control. As shown in Fig. 49, kahweol decreased STAT3 transcriptional activity in a dose-dependent manner. AG490 also reduced STAT3 transcriptional activity. These results indicate that the level of STAT3 activation may correlate with the inhibition of angiogenesis and metastasis produced by kahweol.

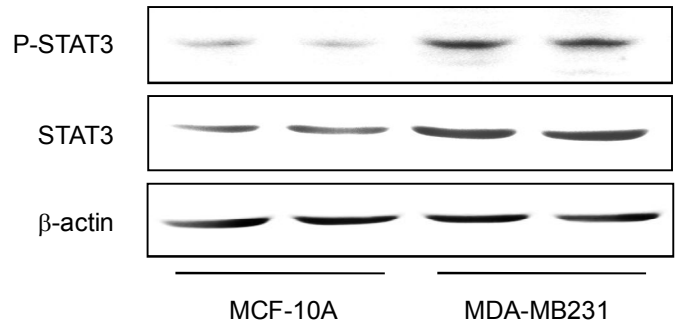



Fig. 47. Expression of Stat3 phosphorylation and STAT3 in MCF-10A and MDA-MB231 cells.

Endogenous STAT3 protein expression and phosphorylation levels in MCF-10A and MDA-MB231 cells were assessed by Western blot analysis. Actin was used as a loading control.

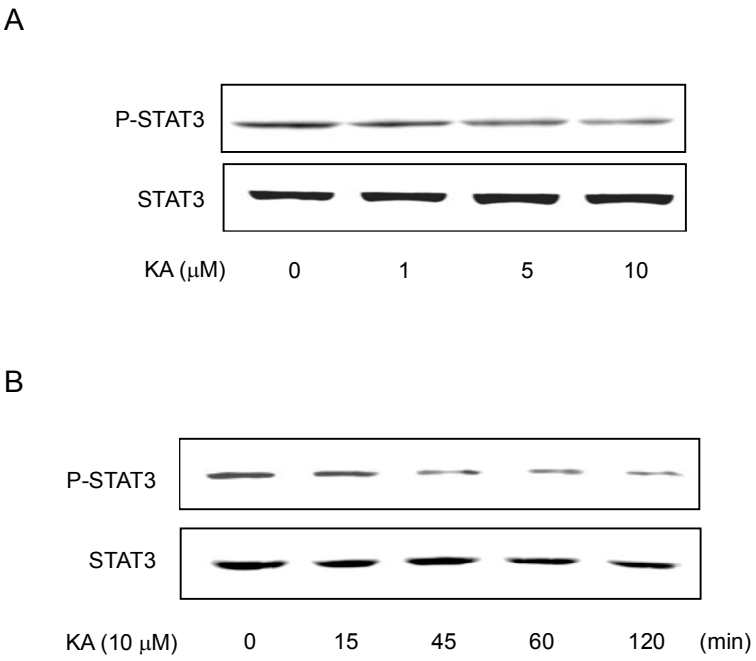



Fig. 48. Effects of Kahweol on STAT3 activation in MDA-MB231 cells.

MDA-MB231 cells were treated with the indicated concentrations of kahweol for 1 h (A) or with 10 mM kahweol for the indicated times (B). Cells lysates were prepared, and Western blotting was performed using antibodies against phosphorylated (p)-STAT3 and STAT3.

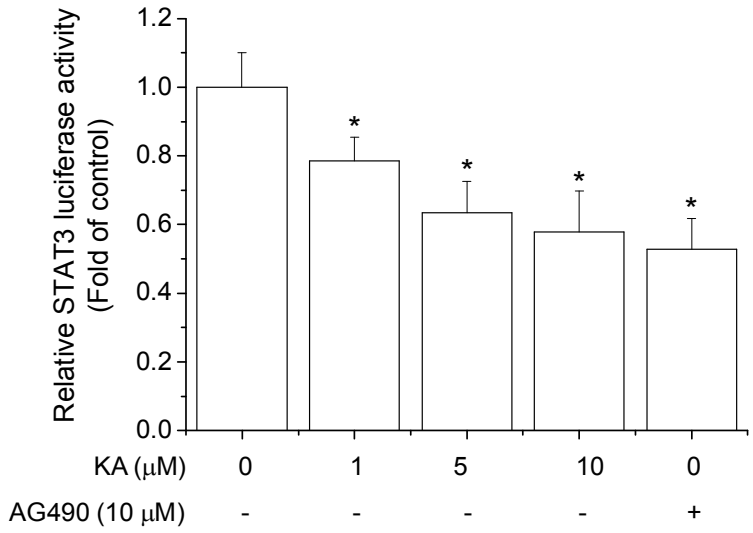



Fig. 49. Effects of kahweol on STAT3 transcriptional activity

in MDA-MB231 cells.

MDA-MB231 cells transiently transfected with a plasmid containing a STAT3-responsive luciferase reporter (STAT3-Luc) and pCMV-β-gal were treated with kahweol at the indicated concentrations and/or AG490 for 24 h. Luciferase and β-galactosidase activities were determined. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. *$P < 0.01$; significantly different from the control.

**18. STAT3 mediates the antiangiogenic effects of kahweol in breast cancer cells**

To further confirm that the inhibition of angiogenesis and metastasis by kahweol is mediated through the inhibition of STAT3 activation, this study was examined the migration and invasion by tumor cells containing a plasmid encoding a constitutively active mutant form of STAT3 (STAT3C), which can dimerize spontaneously and bind to DNA to activate transcription (Bromberg et al., 1999). STAT3C-expressing MDA-MB231 cells showed increased total STAT3 expression and elevated VEGF mRNA and protein expression (Fig. 50 and 51). Kahweol reduced the STAT3C-induced increases in the levels of both VEGF mRNA and VEGF protein (Fig. 51). However, kahweol did not downregulate the expression of STAT3C in these cells. Cell migration and invasion assays were performed to confirm the requirement of STAT3 inhibition for the anti-metastatic effects of kahweol. In cells expressing only the control vector, kahweol treatment for 24 h produced a significant inhibition of migration and invasion (Fig. 52), whereas in STAT3C-expressing cells, the anti-invasive effects of kahweol were completely blocked (Fig. 53). These results indicate that the inhibition of migration and invasion by kahweol is mediated at least in part by the inhibition of STAT3 activity in breast cancer cells.

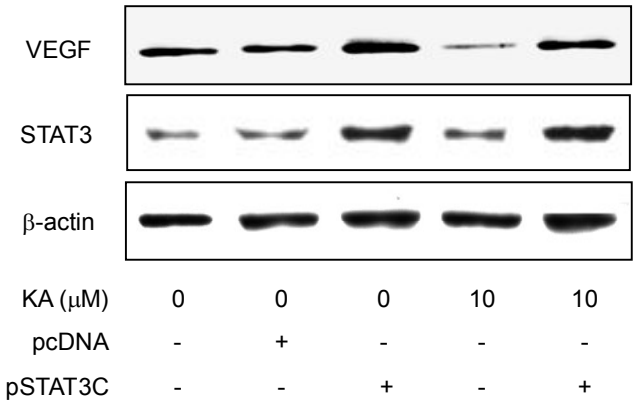



Fig. 50. Constitutively-activated STAT3 mutant protein up-regulates VEGF expression.

MDA-MB231 cells transiently transfected with pSTAT3C or control pcDNA expression vector were treated with kahweol for 24 h. Total cell lysates were prepared and analyzed by Western blotting using antibodies against actin (as a loading control), VEGF, phosphorylated (p)-STAT3, and STAT3.

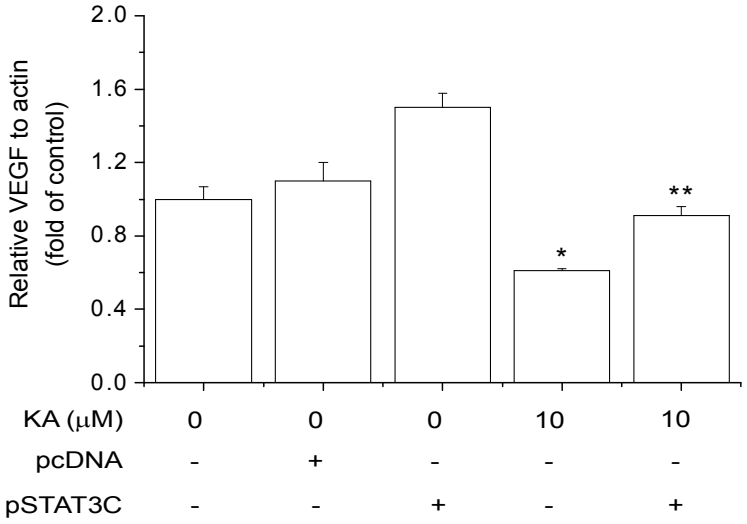



Fig. 51. Kahweol inhibits of VEGF expression effects through STAT3 dependent mechanisms.

MDA-MB231 cells transiently transfected with pSTAT3C or control pcDNA expression vector were treated with kahweol for 24 h. Total RNA was prepared and VEGF mRNA expression was measured by real-time PCR analysis. Results are expressed as fold induction relative to expression in control cells transfected with pcDNA in the absence of kahweol treatment. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. *$P < 0.01$; significantly different from the control. **$P < 0.01$; significantly different from kahweol treatment.

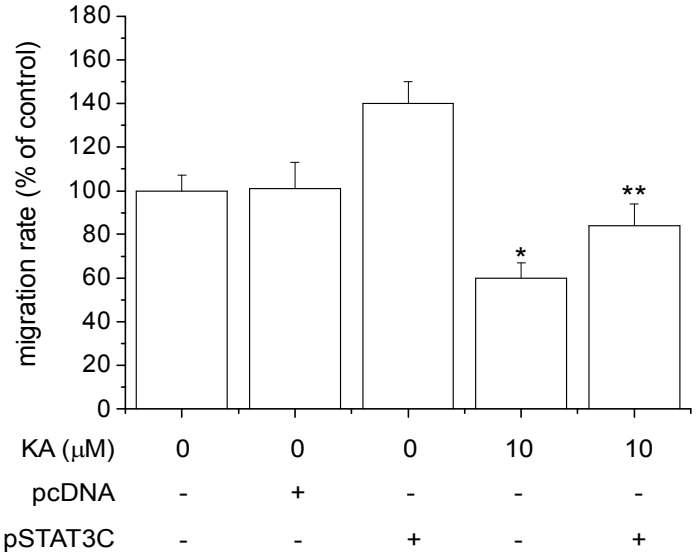
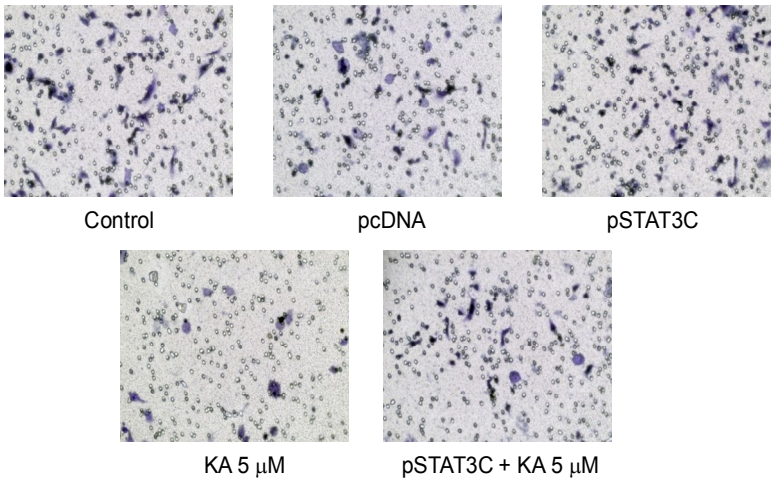



Fig. 52. Kahweol mediates anti-migration effects through STAT3 dependent mechanisms.

MDA-MB231 cells transiently transfected with pSTAT3C or control pcDNA expression vector were treated with kahweol for 24 h. MDA-MB231 cells transiently transfected with pSTAT3C or control pcDNA were assayed in a Transwell chamber, with and without kahweol treatment for 24 h. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. *$P$ < 0.01; significantly different from the control.

A


Control
pcDNA
pSTAT3C
KA 5 μM
pSTAT3C + KA 5 μM

B

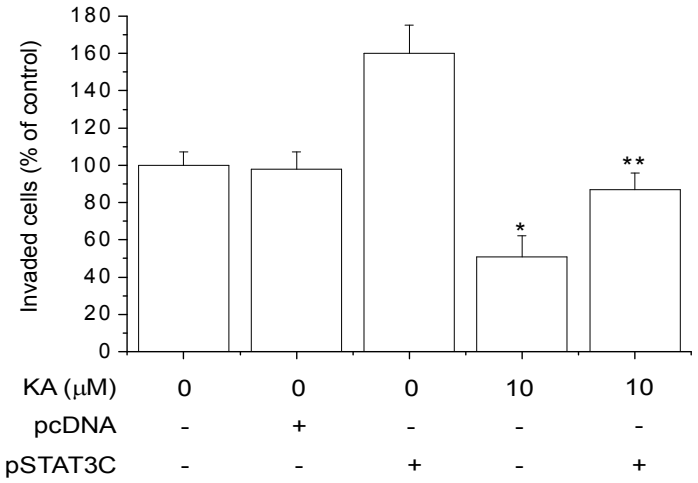

Invaded cells (% of control)
180
160
140
120
100
80
60
40
20
0
*
**
KA (μM) 0 0 0 10 10
pcDNA - + - - -
pSTAT3C - - + - +

Fig. 53. Kahweol mediates anti-invasion effects through STAT3 dependent mechanisms.

MDA-MB231 cells transiently transfected with pSTAT3C or control pcDNA expression vector were treated with kahweol for 24 h. (A) MDA-MB231 cells transiently transfected with pSTAT3C or control pcDNA were assayed in a Matrigel-coated Transwell chamber, with and without kahweol treatment for 24 h. Cells invading the Matrigel-coated membrane were stained and counted under a bright-field microscope. (B) The number of invasive cells is presented as a percentage of untreated cells. Each bar shows the mean ± SD of three independent experiments, performed in triplicate. *$P < 0.01$; significantly different from the control. **$P < 0.01$; significantly different from kahweol treatment.

# IV. Discussion

**The coffee diterpene kahweol inhibits tumor necrosis factor-α-induced expression of cell adhesion molecules in human endothelial cells**

In the previous reported the anti-inflammatory properties of kahweol, which inhibits COX-2 and iNOS gene expression through NF-κB inhibition by targeting the IKK complex in macrophages (Kim et al., 2004a, 2004b). However, it is unclear if kahweol inhibits the expression of the cytokine-induced cell adhesion molecules and reduces level of monocyte adhesion to endothelial cells. This study investigated the effects of kahweol on the expression of the endothelial cell adhesion molecules and the adhesion monocytes to human endothelial cells. The results suggest that in cultured endothelial cells, a kahweol pretreatment significantly suppressed the TNFα-induced activation of the JAK2-PI3K/Akt-IKK-NF-κB pathway, VCAM-1 and ICAM-1 expression, and the adhesiveness to monocytes, which is an *in vitro* sign of atherogenesis. This suggests that kahweol can inhibit vascular inflammation and prevent in vitro atherogenesis.

One of the earliest events in atherogenesis is the adhesion of monocytes to the endothelium, which is followed by their infiltration and differentiation into macrophages. This key step is mediated by an interaction between monocytes and

the molecules expressed on the surface of endothelial cells (Price and Loscalzo, 1999; Ross, 1999; Iiyama et al., 1999; Glass and Witztum, 2001). These cell adhesion molecules mediate the adhesion of the monocytes that are specifically found in atherosclerosis lesions to the vascular endothelium (Price and Loscalzo, 1999; Ross, 1999). This study found that a pretreatment with kahweol inhibited the TNFα-induced VCAM-1 and ICAM-1 expression at both the protein and mRNA level. Furthermore, the adhesion of monocytes to the endothelial cells was markedly inhibited. It was demonstrated that atherosclerosis is a chronic inflammatory disease (Diaz et al., 1997; 1999; Ross, 1999), and that NF-κB is involved in its development (Price and Loscalzo, 1999; Ross, 1999; Glass and Witztum, 2001) and the signal transduction pathways for the TNFα-induced cell adhesion molecules such as ICAM-1 and VCAM-1 (Ledebur and Parks, 1995; Collins et al., 1995). This study found that kahweol inhibited the activation of TNFα-stimulated NF-κB, suggesting that kahweol at least partially impairs the NF-κB pathways specific to the cytokine-stimulated induction of VCAM-1 and ICAM-1 expression. It was observed that kahweol inhibits the adhesion of monocytes to endothelial cells. Therefore, the effect of kahweol on the signal processes involved in adhesion was examined to further understand the inhibitory mechanism. PI3K is one of the key molecules implicated in cell adhesion and motility (Gerszten et al., 2001). Akt is the downstream target of PI3K. Kahweol was found to inhibit the

TNFα-mediated activation of Akt (phosphorylation of Akt at Ser) in endothelial cells. The partial restoration of the inhibitory effects of kahweol on the adhesion as a result of the transfection of the constitutively active form of Akt further supports this.

Recently, kahweol was reported to have anti-inflammatory effects by suppressing the production of proinflammatory mediators, such as prostaglandin $E_2$ and NO in macrophages, via the inhibition of the NF-κB-dependent transcription of inflammatory genes such as COX-2 and iNOS (Kim et al., 2004a, 2004b). The results of this study are in agreement with these findings and clearly demonstrate that kahweol inhibits NF-κB activation by suppressing IKK activity (Kim et al., 2004a, 2004b). Akt is one of the upstream kinases for IKKs (Ozes et al., 1999; Romashkova and Makarov, 1999), and the inhibition of PI3K/Akt activity by the kahweol treatment and the reversal of its inhibitory effect via the co-transfection with the constitutively active form of Akt further supports the role of IKKs regulation by Akt in the mechanism of kahweol-mediated inhibition of the adhesion process. Janus kinase-signal transducers and activators of transcription (JAK-STAT) signaling pathways have been reported to be involved in many signaling events such as the immune response-induced cytokines (Rane and Reddy, 2000). It is recognized that JAK2, a member of Janus kinases family, is activated and tyrosine-phosphorylated by TNFα (Guo et al., 1998). The major function of the JAK family

kinases is generally considered to be activation of the STAT transcription factors; however, this function is not the sole role of the Jak family. Several studies have reported the involvement of JAK2 in the activation of PI3K/Akt in several signal transductions (Al-Shami et al., 1999; Yamauchi et al., 1999; Rane and Reddy, 2000). The present study demonstrated that kahweol inhibits TNFα-induced tyrosine phosphorylation of JAK2. Furthermore, JAK2 is required for the adhesion of monocytes to endothelia cells via the activation of the PI3K/AKT pathway. These studies show that the down-regulation of the adhesion process by kahweol includes the inhibition of the JAK2-PI3K/Akt-IKK-NF-κB pathway. Although the down-regulatory ability of kahweol on the cell adhesion molecules expressions was demonstrated by the inhibition of NF-κB activation in the TNFα-stimulated endothelial cells, the precise mechanisms are unclear. The JAK family is important protein tyrosine kinases (Rane and Reddy, 2000). JAK2 is also tyrosine phosphorylated after stimulation via several other receptors. Furthermore, tyrosine kinases inhibitors suppress TNFα-induced activation of Akt (Deutsch et al., 2004) and NF-κB (Reddy et al., 1994; Natarajan et al., 1998). The molecular mechanism how kahweol inhibits JAK2 activation remains obscure. Therefore, the effects of kahweol on the protein tyrosine kinases are necessary to elucidate whether kahweol plays a pivotal role in the inhibition of TNFα-induced the adhesion of monocytes to endothelia cells. The activation of the MAPK members, such as ERK1/2, JNK1/2,

and p38 MAP kinases, is involved in stimulating the NF-κB activity and the subsequent expression of the cell adhesion molecules in TNFα-activated endothelial cells (Sethi et al., 2002). Kahweol might also inhibit the activity of these kinases leading to NF-κB activation before or during the IκB phosphorylation step, which would account for the suppression of NF-κB activation. Therefore, further studies on the effects of kahweol will be also needed to understand how kahweol regulates the expression of the cell adhesion molecules and other signal transduction pathways.

This study showed that kahweol inhibits the expression of cell adhesion molecules, such as VCAM-1 and ICAM-1, that mediate the recruitment of monocytes to the arterial wall. NO is a potent oxidant with diverse functions in the cardiovascular system. The impaired production of NO plays a key role in the development of cardiovascular diseases (Gerszten et al., 2001). NO has both atherogenic and vascular protective effects, which are dependent on the source and level of production. NO produced by endothelial NO synthase (eNOS) has a vasodilator function and a protective effect. However, iNOS in macrophages produces large amounts of NO as a response to stimuli, and the potent oxidative properties of NO produced by iNOS appear to induce atherosclerosis. A previous study, which reported the inhibitory effect of kahweol on iNOS expression in LPS-stimulated macrophages, also explained the anti-atherosclerotic activity of kahweol (Yamauchi

et al., 1998). Therefore, the properties of kahweol appear to inhibit the pathogenesis of atherosclerosis.

In conclusion, kahweol inhibits the TNFα-induced VCAM-1 and ICAM-1 expression and hinders the adhesion of monocytes to endothelial cells via a mechanism involving the inhibition of the JAK2-PI3K/Akt-NF-κB signaling pathways in response to TNFα. Monocyte adhesion to endothelial cells and their subsequent recruitment into the vascular wall is a key step in the pathogenesis of atherosclerosis. These findings should provide a new insight into the mechanism responsible for the anti-inflammatory and anti-atherosclerotic properties of kahweol.

**Kahweol blocks STAT3 phosphorylation and induces apoptosis**

**in human lung adenocarcinoma A549 cells**

Many chemopreventive agents act through the induction of apoptosis. Therefore, induction of apoptosis by chemopreventive agents may be an effective strategy in anti-tumorigenesis. Current studies provide new insights into the biological and molecular mechanisms for the anti-tumor effects of kahweol, which is a coffee-specific diterpene (Cavin et al., 2002). Epidemiological studies have revealed a protective association between coffee consumption and the risk of certain types of cancer including colon cancer (Cavin et al., 2001; Inoue et al., 1998). However, the precise mechanisms of its anti-tumorigenic or chemopreventive activities remain unclear. The present study demonstratesd that kahweol inhibited A549 cell growth and induced apoptosis via down-regulation of the STAT3 signaling pathway. The results that kahweol-treated A549 cells exhibit rounded and granulated morphology, and subsequent aggregation of nuclear chromatin. These observations are confirmed by TUNEL assays, which clearly show DNA fragmentation. The regulation of apoptosis is a complex process and involves a number of gene products including Bcl-2 protein family and cell cycle-regulatory proteins. The Bcl-2 family proteins play an important regulatory role in apoptosis, either as inhibitors or as activators. Thus, it has been suggested that the ratio between the level of pro-apoptotic Bax protein and that of the anti-apoptotic factor Bcl-2 protein determines

whether a cell responds to an apoptotic signal (Vieira et al., 2000). In the present study, there was a concentration-dependent increase of Bax protein levels in cells treated with kahweol while the levels of Bcl-2 decreased dramatically, resulting in an increase in the ratio of Bax/Bcl-2 (Fig. 22). Another hallmark of the apoptotic pathway is cleavage of caspase-3. Kahweol significantly increased cleavage of caspase-3, thus promoting PARP cleavage to its proteolyzed products, a phenomenon that is known to result from caspase-3 activation.

Furthermore, this is the first report to identify kahweol as a novel blocker of the STAT3 pathway. The present study demonstrated that kahweol suppressed constitutive STAT3 activation and transcriptional activity of STAT3 in human lung adenocarcinoma A549 cells. STAT3 activation requires phosphorylation, resulting in dimerization, nuclear translocation, DNA binding, and transcriptional activation of target genes (Groner et al., 2008). Human lung cancer A549 cells express constitutively active STAT3. Besides multiple myeloma cells, other forms of cancer, including breast and colon cancers and leukemia, also express constitutively active STAT3. The suppression of constitutively active STAT3 in multiple myeloma cells raises the possibility that this novel STAT3 inhibitor might also inhibit constitutively activated STAT3 in other types of cancer cells. These results indicated that kahweol inhibited the constitutive phosphorylation and activation of STAT3. No changes were noted in STAT3 proteins. Down-regulation of STAT3

was associated with a gradual decrease in the number of viable cells. The decrease in cell viability can be attributable to the significant increase in apoptotic cell death and the occurrence of cell cycle arrest after inhibition of STAT3 signaling. STAT3 has been reported to be activated by soluble tyrosine kinases of the Janus family (Murray, 2007). Our previous studies have shown that kahweol inhibits TNFα-induced tyrosine phosphorylation of JAK2 (Kim et al., 2006). STAT3 phosphorylation plays a critical role in the transformation and proliferation of tumor cells (Aggarwal et al., 2006). Downstream target genes of activated STAT3 include cyclin D1, Bcl-$X_L$, Bcl-2, and vascular endothelial growth factor (Bhutani et al., 2007; Xiong et al., 2008), and deregulated expression of these target genes influences cell cycle progression, apoptosis, and angiogenesis. These results indicated that kahweol-induced cell death in A549 cells was associated with decreased expression of anti-apoptotic Bcl-2 and Bcl-$X_L$ proteins and increased expression of pro-apoptotic Bax protein (Fig. 22). This is in line with previous studies showing that STAT3 can upregulate the expression of Bcl-$X_L$ and Bcl-2 (Lassmann et al., 2007). Thus, inhibition of STAT3 by kahweol may decrease the levels of the Bcl-$X_L$ and Bcl-2 proteins. In the present study, the kahweol-treated STAT3-overexpressing cells show no enhanced apoptosis compared with the kahweol-treated cells, suggesting that kahweol might target STAT3 in human lung adenocarcinoma A549 cells. Indeed, the transfected cells clearly expressed

phosphorylated STAT3 despite exposure to cytocidal concentrations of kahweol, resulting in less apoptosis. This finding strongly supports the concept that kahweol may induce apoptosis in A549 cells by targeting the oncogenic transcription factor STAT3. Consistent with our observations, dominant-negative STAT3 has also been shown to induce apoptosis in with constitutively active STAT3 (Barton et al., 2004). Furthermore, inhibitors of JAK/STAT pathway, namely AG490, also induce apoptosis (Fig. 30) in A549 cells, much like the kahweol treatment, confirming inactivation of STAT3 as one of the key pathways involved in A549 cell apoptosis. In conclusion, this study demonstrated that kahweol inhibited A549 cell growth and induced apoptosis via down-regulation of the STAT3 signaling pathway. The present findings warrant preclinical and clinical investigation on the potential use of this compound as a cancer chemopreventive or chemotherapeutic agent.

**Kahweol inhibit angiogenesis and metastasis through suppression of STAT3 activation**

Several studies have revealed an association between coffee consumption and reduced risk for certain types of cancer (Cavin et al., 2001; Inoue et al., 1998). More recent reports have provided new insights into the anticarcinogenic effects of kahweol, a coffee-specific diterpene (Cavin et al., 2002). However, the precise mechanisms of the antiangiogenic and antimetastatic activities of kahweol remain unclear. In the present study, kahweol not only eliminated the secretion of VEGF but also decreased the expression of MMP-2 and -9. Furthermore, both of these effects required the inhibition of STAT3 activation, as constitutive STAT3 activation blocked the antiangiogenic effect of kahweol.

Angiogenesis is essential for tumor growth and metastasis, and VEGF is one of the most potent stimulators of angiogenesis (Shibuya, 2008). Tumor and normal cells secrete angiogenic growth factors such as VEGF, which bind to specific receptors on endothelial cells and mediate angiogenesis through selective mitogenic activity (Ferrara, 2005). This ligand-receptor interaction leads to endothelial cell proliferation, migration, invasion, and eventually angiogenesis (Risau, 1994). In the present study, kahweol greatly inhibited VEGF protein secretion in HUVECs and MDA-MB231 cells. The inhibitory effects of kahweol on VEGF production may represent a therapeutically important approach for inhibiting angiogenesis and

tumor invasion.

Interactions between tumor cells and the basement membrane are critical for tumor invasion and the metastatic cascade (Geiger and Peeper, 2009). Several investigators have proposed a three-step process of tumor cell invasion: tumor cell attachment to the basement membrane, creation of proteolytic defects in the basement membrane, and migration of tumor cells through the defects (Geiger and Peeper, 2009). Of the MMPs, MMP-2 and -9 are the most important for degrading basement membrane type IV collagen (Deryugina and Quigley, 2006). Overexpression of MMP-2 in tumor cells has been suggested by the positive correlation between MMP activity in a primary tumor and metastasis of the tumor (Xie et al., 2004). It is well known that MMP inhibitors block endothelial cell activities that are essential for the new vessel development necessary for proliferation and invasion tumor cells (Tosetti et al., 2002). Therefore, based on their basement membrane-degrading activities, MMP-2 and -9 are thought to be potential therapeutic targets of anticancer drugs. Kahweol suppressed MMP-2 and -9 gene expression in MDA-MB231 cells, which may represent the primary mechanism by which kahweol prevents tumor cell invasion and metastasis.

STAT3 also plays a critical role in regulating tumor angiogenesis. It was demonstrated that the antiangiogenic effect of kahweol in breast cancer cells was mediated via STAT3 inhibition. To further clarify which STAT3-regulated genes

may be associated with the elevated levels of STAT3 phosphorylation in breast cancer, this examined the protein expression of potential target genes in breast cancer cell lines. VEGF expression was significantly higher in MDA-MB-231 breast cancer cells than in MCF-10A normal human breast epithelial cells. MCF-10A cells lack constitutive STAT3 activity and show low levels of STAT3 phosphorylation. In contrast, MDA-MB-231 cells express a constitutively active form of STAT3 and show elevated levels of STAT3 phosphorylation. Kahweol inhibited the phosphorylation and activation of STAT3 in MDA-MB231 cells, and kahweol-treated STAT3C-overexpressing cells showed decreased expression of VEGF and MMPs, compared with control cells. The observed downregulation of STAT3 activity was essential for the inhibition of VEGF and MMP gene expression and the invasive activity of MDA-MB231 cells. Therefore, as STAT3 is frequently activated in breast cancer and can contribute to cancer progression, it will be important to identify other downstream targets of STAT3 that are involved in oncogenesis of breast carcinoma. Previous studies have demonstrated that STAT3 activation upregulated VEGF expression and correlated with increased MMP-9 activity, whereas the expression of dominant-negative STAT3 led to reduced angiogenesis and suppression of cell growth, including breast cancer cell growth (Qiu et al., 2007; Devarajan and Huang, 2009; Niu et al., 2002b). As the expression of constitutively active STAT3 nearly completely eliminated the effects of kahweol,

this study suggest that STAT3 inhibition is the primary mechanism by which kahweol exerts an antiangiogenic effect in MDA-MB231 cells.

Elevated STAT3 activity has been associated with breast cancer progression and is inversely correlated with a response to chemotherapy (Aggarwal et al., 2006; Devarajan and Huang, 2009). Thus, STAT3 inhibitors such as kahweol, when combined with chemotherapy, may improve the therapeutic response in breast cancer patients. STAT3 lies at the point of convergence of a number of oncogenic signaling pathways, and the suppression of STAT3 activity further highlights the potent antitumor effects of kahweol.

# V. References

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# [국문 초록]

## Kahweol의 염증 및 암 전이 조절 연구

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지도교수: 강건욱

본 학위 논문에서는 커피 원두 함유물질인 카와웰 (kahweol:KA)의 염증, 세포사멸, 신생 혈관 및 암전이 억제에 대한 영향과 그 작용 기전을 규명하였다. 동맥경화증은 만성 염증 과정에서 생성되는 산화 스트레스의 증가에 의해서 발생 된다. 정상 혈관내피세포들은 염증세포와의 상호작용이 일어나지 않으나 염증이 일어나는 부위의 혈관이나 혈관 벽 내의 산화 스트레스 및 염증이 발생하였을 경우 혈관 벽의 내피세포에서 adhesion molecule 이라고 지칭되는 VCAM-1, ICAM-1 등이 발현하게 되고 이들이 염증세포와 상호작용을 하게 된다. 따라서, 혈관내피세포에서 카와웰 (kahweol:KA)의 adhesion molecule 에 대한 영향 및 작용 기작을 조사하였다. Proinflammatory cytokines 인

TNFα 에 의해서 증가된 VCAM-1, ICAM-1 의 발현이 KA 에 의해서 억제됨을 확인하였다. 또한, KA 의 adhesion molecule 에 대한 조절 기전은 adhesion molecule 의 주요 전사조절인자인 NF-kB 및 PI3K/AKT 의 활성 억제를 통하여 조절되었다. 이는 KA 의 동맥경화 및 염증 반응을 억제하는 기능을 가지고 있음을 규명하였다.

세포사멸은 외부로부터 세포사멸를 유도하는 신호를 받거나, 세포가 내부적 손상 즉 DNA 의 손상 등으로 인하여, 세포내부에서 세포사멸에 관계된 protein kinase 와 cell death protein 이 활성화되고 세포사멸에 관계된 유전자가 발현되어 세포사멸을 유도한다. 세포의 사멸과 관련된 세포 내 신호 전달 체계는 세포의 종류 및 죽음을 초래하게 하는 외부 신호에 따라 각기 다르다. 따라서, KA 에 의한 암세포 사멸에 대한 영향 및 그 작용기전을 조사하였다. KA 에 의해서 암세포 증식 억제 및 세포 사멸이 증가 되었다. 또한, 세포 사멸 유전자 (Bax, caspase, PARP)의 활성 및 발현이 억제되었으나 세포 사멸 억제 유전자 (Bcl-2, BCl-XL)의 발현은 증가하였다. KA 의 세포사멸에 대한 영향은 유전자의 발현 및 세포사멸에 중요한 역할을 하는 전사조절인자인 STAT3 의 활성 억제를 통하여 조절되었다. 이는 KA 의 암세포 내에서 STAT3 의 신호전달 체계 억제를 통하여 항암효과를 규명한 것으로 향후 암세포 억제방법 및 치료제 개발에 기초 자료로 활용될 수 있을 것으로 사료된다.

암의 발생은 일반적으로 유발단계 (initiation), 촉진단계 (promotion), 진행단계 (progression) 및 암전이 단계(metastasis)의 매우 복잡한 다단계의 발암과정을 수반하게 된다. 암전이 과정은 전이성을 가진 악성 암세포가 최초의 암 발생 장소에서 다른 새로운 조직으로 침투하여 혈관신생이 동반되는 이차종양을 형성하는 것으로, 부착 (adhision), 침윤 (invasion), 혈관신생 (angiogenesis)의 세가지 주요 단계로 구성되어 있다. 따라서, Human unbilical vein endothelial cell (HUVEC)을 이용한 *in vitro* 혈관 형성 (Metrigel assay)에 대한 KA 의 억제 효과와 암세포 주에서 KA 의 종양혈관 생성억제 유전자들 (VEGF, MMPs)의 발현 측정 및 혈관신생 억제 기전을 조사하였다. KA 을 처리한 결과 암세포 및 혈관 내피세포에서 migration, invasion 및 *in vitro* 혈관 형성이 억제되었다. 또한, KA 에 의해서 MMP-2, 9 의 활성이 억제되었으며 KA 의 농도 의존적으로 신생혈관 촉진 유전자로 알려진 Vascular endothelial growth factor (VEGF)의 생성량이 감소되었다. KA 의 암전이 및 신생혈관에 대한 기전은 암전이 관련 유전자의 발현 및 혈관 신생에 중요한 역할을 하는 전사조절인자인 STAT3 의 활성 억제를 통하여 조절되었다. 이와 같은 결과로 미루어 KA 는 암세포의 성장을 저해 하고, 혈관 내피세포의 혈관 신생 및 암세포의 전이를 억제됨을 확인하였다.

위 연구 결과들은 커피 원두 함유물질인 카와웰 (kahweol:KA)의 염증 및 암 전이 억제에 대한 영향을 규명한 것으로 동맥경화 및 암과 관련한 질병의 예방 및 치료를 위한 기초 자료로 활용할 수 있으며 생체방어증강 활성을 지닌 신소재 개발에 활용될 수 있을 것으로 사료된다.

# 저작물 이용 허락서

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| | 영문 : Effects of kahweol on inflammation and metastasis | | | | |

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함.
2. 위의 목적을 위하여 필요한 범위 내에서의 편집과 형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물 이용의 허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음.
7. 소속 대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의 여부 : 동의 ( ○ ) 반대 ( )

2010년 2 월 일

저작자: 김 형 균 (인)

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