Methanolic extract of *Kigelia africana* exhibits antiatherosclerotic effects in endothelial cells by downregulating RAGE and adhesion molecules

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Abstract. The Kigelia plant is used in African countries for its medicinal properties. *Kigelia africana* is an interesting example of a medicinal plant due to its pharmacological activities, including its anti-inflammatory effect. Atherosclerosis, the primary cause of cardiovascular disease, is related to lipoprotein oxidation, inflammation and immune responses involving the vascular endothelium and immune cells. Therefore, in this study we investigated the effects of *Kigelia africana* (Lam.) extract, focusing particularly on antiatherosclerotic effects in endothelial cells (ECs). The methanolic extract of *Kigelia africana* (MKA) showed no cytotoxicity on ECs at doses of 10~200 µg/ml. MKA reduced RAGE expression on oxLDL- or TNF-α-stimulated ECs in a dose dependent manner, showing significant inhibition at a concentration of 50 µg/ml. In addition, MKA significantly inhibited the oxLDL- or TNF-α-induced expression of vascular cell adhesion molecule-1 (VCAM-1) in ECs in a dose-dependent manner but did not affect intracellular adhesion molecule-1 (ICAM-1), resulting in downregulation of the migration and adhesion of THP-1 monocytes to ECs. These results suggest that MKA could be used for the treatment of atherosclerosis without cytotoxicity.

INTRODUCTION

Cardiovascular diseases including athero-sclerosis account for the major cause of morbidity and mortality over the world. It is well known that lipoprotein oxidation, inflammation and immune responses contribute to the pathogenesis of athero-sclerosis (Ross, 1999; Libby, 2002; Charo *et al.*, 2011). Atherosclerosis is initiated by lipid deposition in the subendothelial layer of the arterial wall. These lipids are converted into oxidized low-density lipoprotein (oxLDL) (Navab *et al.*, 1996). Oxidized LDL is the primary stimulus that leads to endothelial activation and injury resulting in an inflammatory response between monocytes and endothelial cells. Oxidized LDL mediates recruitment, activation and migration of monocytes to endothelial cells through upregulation of the expression of adhesion molecules (AMs) such as vascular cell adhesion molecule-1 (VCAM-1) and inter-cellular adhesion molecule-1 (ICAM-1) (Khan *et al.*, 1995; Takei *et al.*, 2001) and eventually results in endothelial dysfunction.

Proinflammatory cytokines also play a major role in the level of risk of cardio-vascular events. TNF-α is a pleiotropic proinflammatory cytokine implicated in cardiovascular disease and endothelial dysfunction (McKellar *et al.*, 2009). TNF-α has been reported to induce endothelial cell activation through the upregulation of cell adhesion molecule expression and promotion

of monocyte-endothelium interactions (Burke-Gaffney and Hellewell, 1996).

A number of researchers, including our team, have reported that the receptor for advanced glycation endproducts (RAGE) is widely implicated as a mediator of both acute and chronic vascular inflammation in conditions such as atherosclerosis (Schmidt *et al.*, 1994; Hudson *et al.*, 2003; Ramasamy *et al.*, 2005; Basta, 2008). RAGE is a 35-KDa polypeptide and is part of the immunoglobulin superfamily (Neeper *et al.*, 1992). Endothelial expression of RAGE is typically diffuse and variable but is enhanced by risk factors for various vascular diseases. RAGE has also been found in early and end stage atherosclerotic lesions in the endothelium of the human coronary artery (Ritthaler *et al.*, 1995). RAGE is able to bind a variety of structurally diverse ligands, which include advanced glycation end products (AGEs), S100/calgranulin proteins, high mobility box group protein 1 and oxLDL (Hofmann *et al.*, 1999; Basta *et al.*, 2002; Harja *et al.*, 2008; Sun *et al.*, 2009), to mediate reactive oxygen species (ROS) production as well as cell-cell adhesion in atherosclerosis (Farmer and Kennedy, 2009).

Kigelia africana (Lam.) Benth (syn *Kigelia pinnata* DC., Bignoniaceae) is a tropical tree used in African folk medicine for its medicinal properties. Traditional remedies prepared from crushed dried fruits are used for emollient, antieczema, antipsoriasis, and skin-firming treatments and as a dressing for ulcers and wounds. Along with skin-related diseases, *Kigelia africana* is also used in Africa for its anti-inflammatory, antimicrobial, and antiaging effects. In preliminary experiments, we screened several plant extracts from natural resources which have been known to have anti-inflammatory properties. Of the many candidates, the methanolic extract of *Kigelia africana* leaves showed an effective anti-inflammatory property at nontoxic dose ranges in ECs. Thus, in this study, we examined whether the methanolic extract of *Kigelia africana* could exhibit antiatherosclerotic effect through downregulation of oxLDL- or TNF-α-mediated responses in ECs.

MATERIALS ANS METHODS

Materials
The methanolic extract of leaves of *K. africana* (Lam.) Benth (Bignoniaceae) was supplied by the International Biological Material Research Center (IBMRC, sample no. FBM025-059). The plant was collected in the Kano province of Nigeria in 2007 and authenticated by the Institute of Advanced Medical Research and Training, University of Ibadan, Oyekanmi Nash. Briefly, the dried and refined leaves of *K. africana* (50 g) were extracted with 500 ml of 95% methanol for 2 h, twice. The extract was percolated with filter paper (3 mm; Whatman PLC, Kent, UK), condensed using a rotary evaporator (Buchi, Switzerland), and lyophilized using a freeze dryer (Christ, Germany). The powder (2.44 g; yield ca. 4.88%) was dissolved in distilled water for a stock solution (200 mg/ml) and diluted with culture medium (for the *in vitro* assay) before use in experiments. Low density lipoprotein (LDL) was purchased from Calbiochem (La Jolla, CA, USA). VCAM-1 and ICAM-1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against RAGE was purchased from Cell Signaling Technology (Beverly, MA, USA). Recombinant human tumor necrosis factor-α (TNF-α) was provided by R&D Systems (Minneapolis, MN, USA). An enhanced chemiluminescence (ECL) western blotting detection reagent was obtained from Amersham (Buckinghamshire, UK). All other chemicals, including β-actin, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture
EA.hy926 cells (human umbilical vascular endothelial cell line) and THP-1 cells (human acute monocytic leukemia cell line) were originally purchased from the American Type Culture Collection. The EA.hy926 ECs and the THP-1 were cultured in Dulbecco's modified Eagle's medium and RPMI medium, respectively, containing 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin and 10 μg/ml streptomycin. All cells were incubated in a humidified 5% CO_2 incubator.

Preparation of oxLDL

Oxidized LDL was prepared as previously described (Eun *et al.*, 2014). Briefly, human LDL was oxidized with 5 µM $CuSO_4$ for 16 h at 37°C and then 1 mM EDTA was added to stop the reaction. After dialysis overnight at 4°C the solution was filtered for sterilization. The extent of LDL oxidation was assessed by the formation of thiobarbituric acid-reactive substances.

Cell viability assay

Cell viability was analyzed by MTT assay. Cells were seeded at 10^4 cells/well in 24-well plates. Cells were treated with methanolic extract of *Kigelia africana* leaf (MKA) at the indicated doses for 24 h. After treatment, MTT solution (50 µl of 5 mg/ml) was added to each well and incubated for 4 h. After aspirating the supernatants, the formazan crystals were dissolved with 200 µl of 4 N HCl-isopropanol. The optical density of the colored product was measured at 570 nm using an Infinite 200 microplate reader (Tecan Austria Gmbh, Grödig, Austria).

Measurement of ROS

ROS production was measured using H_2DCF-DA (5 µM) as described in Eun *et al.* (2014). The fluorescence intensity was measured at an emission wavelength of 535 nm and an excitation wavelength of 485 nm using a microplate fluorescence reader (Tecan Austria GmbH, Grödig, Austria).

Western blot analysis

Cells were lysed using PRO-PREP protein extraction solution (iNtRON Biotechnology, Seoul, Korea). Aliquots of 40 µg of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis for 2 h at 100 V. Separated proteins from the SDS-polyacrylamide gel were transferred onto Hybond-P+ polyvinylidene difluoride membranes (Amersham). The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.05% Tween 20 for 2 h at room temperature and then incubated with the RAGE, ICAM-1 and VCAM-1 primary antibodies. The bound antibodies were detected with horseradish peroxidase-conjugated secondary antibodies and an ECL western blotting detection reagent (Bionote, Gyeonggi-do, Korea). β-Actin was used as a loading control.

Migration assay

THP-1 cells (4×10^5 cells/well) were plated in the upper chamber of the Transwell (Costar, polycarbonate filters with an 8.0 mm pore size), and the bottom compartment was filled with confluent ECs. The migration chambers were incubated overnight at 37°C. The cells that remained on the upper surface of the inserted membranes were removed by scrubbing. The cells on the lower insert membranes were stained with 4',6-diamidino-2-phenylindole (DAPI), and these migrated cells were counted under a light microscope. Each sample was measured in triplicate, and each experiment was repeated three times.

Adhesion assay

ECs and THP-1 cells were pretreated with MKA at the indicated concentrations for 1 h and then stimulated with TNF-α (10 ng/ml) or oxLDL (100 µg/ml) for 6 h. THP-1 cells (2.5×10^5 cells/ml) were then added to the ECs. After 30 min at 37°C, cell suspensions were withdrawn and the ECs were gently washed with PBS three times. The cells were then counted under a light microscope and images were taken using an Olympus microscope (CKX41) equipped with a camera (DS-U3; Nikon).

Statistical analysis

The density of the results from western blotting was analyzed using an Image Master VDS system (Pharmacia Biotech Inc., San Francisco, CA, USA). The treatment groups were compared using one-way analysis of variance (ANOVA) and the Bonferroni *post hoc* test. $P < 0.05$ was considered statistically significant. All data were checked for normality and homogeneity of variance and are expressed as the mean ± SEM.

MKA had no cytotoxicity and inhibited oxLDL- or TNF-α-induced ROS production and RAGE expression in a dose-dependent manner

First, we examined the effect of MKA on the cell viability of ECs. When ECs were treated with a range of doses of MKA (10, 50, 100, 200 µg/ml) for 24 h, MKA had no cytotoxic effect on the ECs (Figure 1A) and reduces ROS production in oxLDL or TNF-α-stimulated in ECs (Figure 1B) in a dose-dependent manner. Then, we determined whether MKA inhibits RAGE expression induced by oxLDL or TNF-α in ECs. In Figure 2A, ECs treated with oxLDL (100 µg/ml) for 6 h exhibited significantly induced RAGE expression, which was inhibited by MKA in a dose dependent manner with significant

inhibition at 50 µg/ml. Interestingly, 200 µg/ml of MKA reduced the RAGE expression level below that of the nontreated control. MKA also showed an inhibitory effect on TNF-α (10 ng/ml)-induced RAGE expression in ECs at 50 µg/ml (Figure 2B).

MKA more specifically inhibited VCAM-1 expression than ICAM-1 expression in oxLDL- or TNF-α-stimulated ECs

It is well known that upregulated adhesion molecules (AMs) such as VCAM-1 and ICAM-1 are involved in the binding of ECs and leukocytes, including monocytes and macrophages, resulting in endothelial dysfunction. Thus, we clarified the effect of MKA on the expression of AMs in oxLDL- or TNF-α-stimulated ECs. Oxidized LDL, and in particular TNF-α, dramatically induced both ICAM-1 and VCAM-1 expression, but
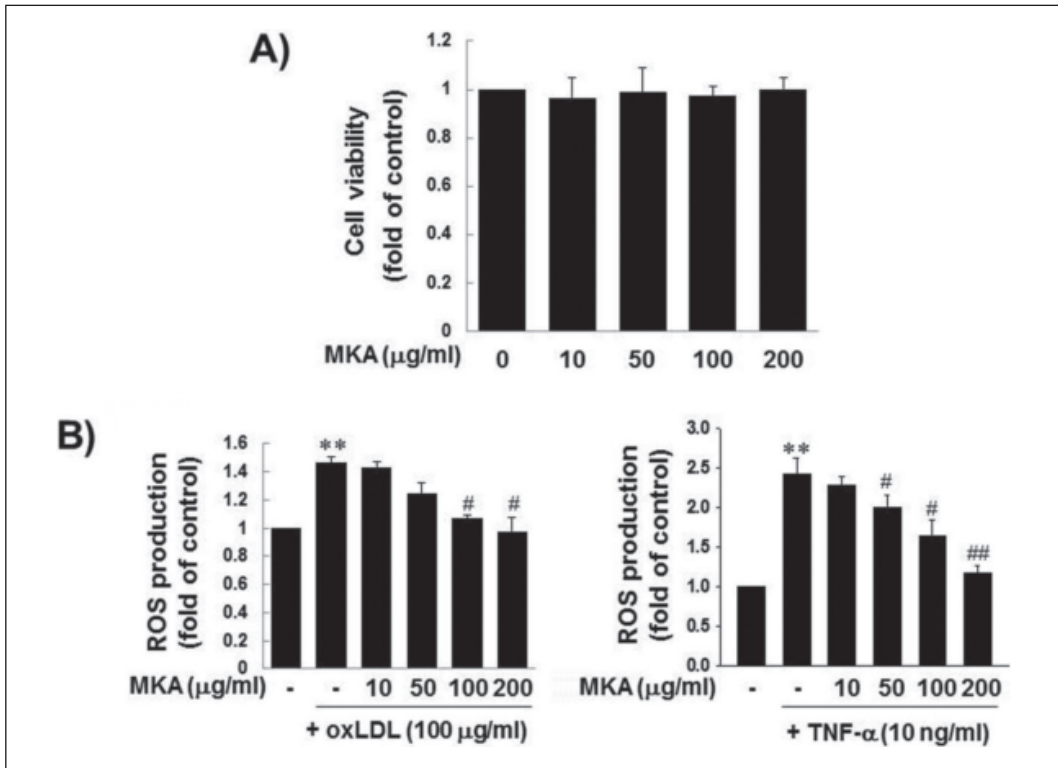

Figure 1. MKA reduces ROS production in oxLDL- or TNF-α-stimulated ECs at nontoxic doses. (A) ECs were treated with MKA at 10, 50, 100 and 200 µg/ml. After 24 h, cell viability was determined by MTT assay. (B) ECs were pretreated with MKA at 10, 50, 100 and 200 µg/ml for 1 h and then stimulated with oxLDL (100 µg/ml) (B) or TNF-α (10 ng/ml) for additional 1 h. ROS production was measured using H_2DCF-DA (5 µM) as described in the Methods section. The results are the means ± SEM from three independent determinations. **$P<0.01$ compared with control group; #$P<0.05$, ##$P<0.01$ compared with oxLDL- or TNF-α-treated group.
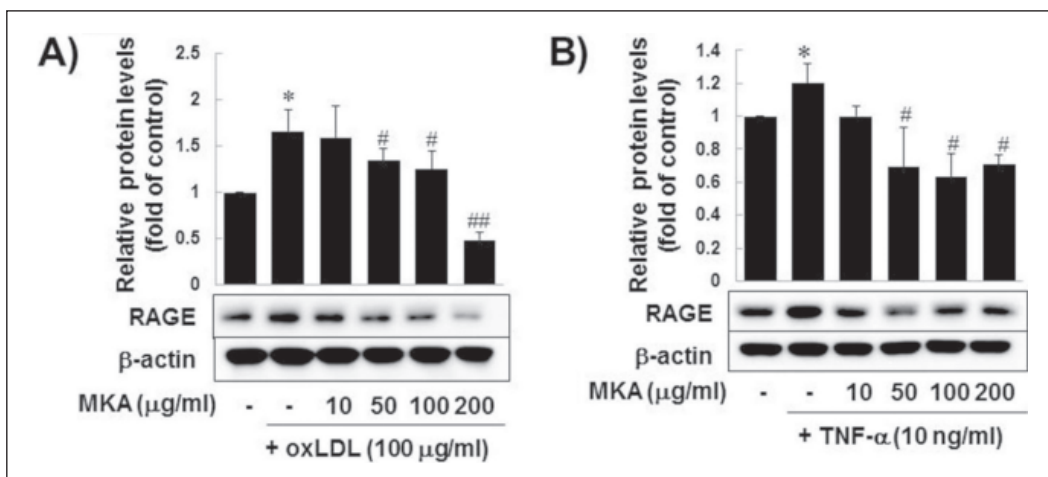
Figure 2. MKA inhibits oxLDL- or TNF-α-induced expression of RAGE in ECs. ECs were pretreated with MKA at the indicated concentrations for 1 h and then stimulated with oxLDL (100 µg/ml) (A) or TNF-α (10 ng/ml) (B) for 6 h. After treatments, RAGE and β-actin protein levels were determined by western blot analysis and quantified. Values are expressed as the mean ± SEM from three independent determinations. *P<0.05 compared with control group; #P<0.05, ##P<0.01 compared with oxLDL- or TNF-α-treated group.
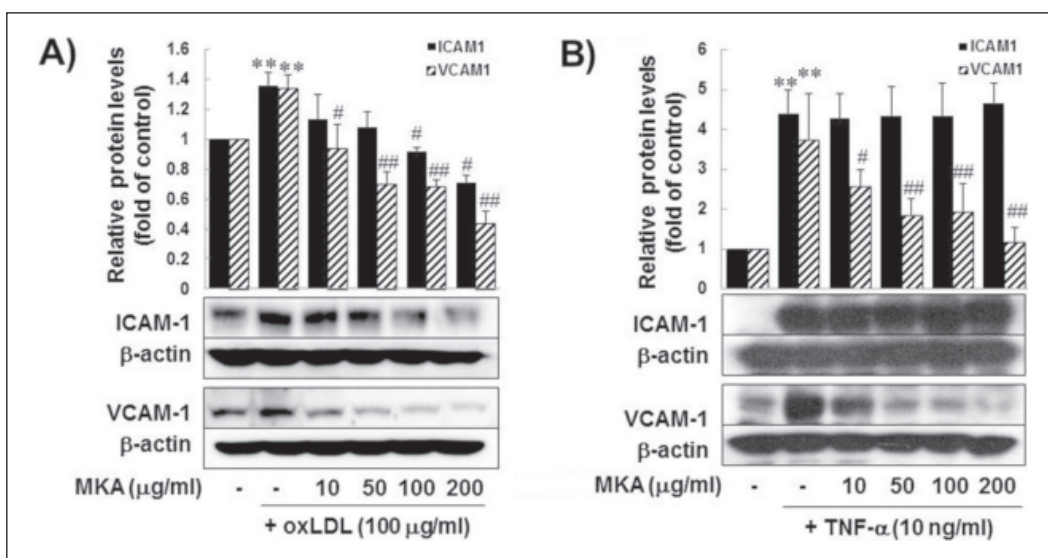

Figure 3. MKA preferentially inhibits VCAM-1 expression in oxLDL- or TNF-α-stimulated ECs. ECs were pretreated with MKA (10, 50, 100 and 200 µg/ml) for 1 h and then stimulated with oxLDL (100 µg/ml) (A) or TNF-α (10 ng/ml) (B). Six hours later, ICAM-1 and VCAM-1 protein levels were determined from the cell lysates. Values are expressed as the mean ± SEM from three independent determinations. **P<0.01 compared with control group; #P<0.05, ##P<0.01 compared with oxLDL- or TNF-α-treated group.

interestingly, MKA differentially regulated oxLDL- or TNF-α-mediated VCAM-1 and ICAM-1 expression. MKA significantly downregulated VCAM-1 expression by both oxLDL and TNF-α from 10 µg/ml (Figure 3A and B). MKA significantly downregulated VCAM-1 expression by both oxLDL and TNF-α from 10 µg/ml (Figure 3A and B). MKA, however, did not show any inhibitory effect on TNF-α-induced VCAM-1 expression but did have an inhibitory effect on oxLDL-induced VCAM-1 at 100 and 200 µg/ml (Figure 3A and B).

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MKA inhibited oxLDL- or TNF-α-induced migration of THP-1 monocytes and adhesion of THP-1 monocytes to ECs

Because the migration of monocytes and the interaction between monocytes and ECs are in regulated in part by specific AMs, we investigated whether MKA downregulates migration of monocytes and binding to ECs after stimulation with oxLDL or TNF-α. Figure 4 shows that incubation of THP-1 monocytes with MKA significantly attenuated the oxLDL- or TNF-α-induced migration of THP-1 (Figure 4A and B). In addition, adhesion of THP-1 monocytes to
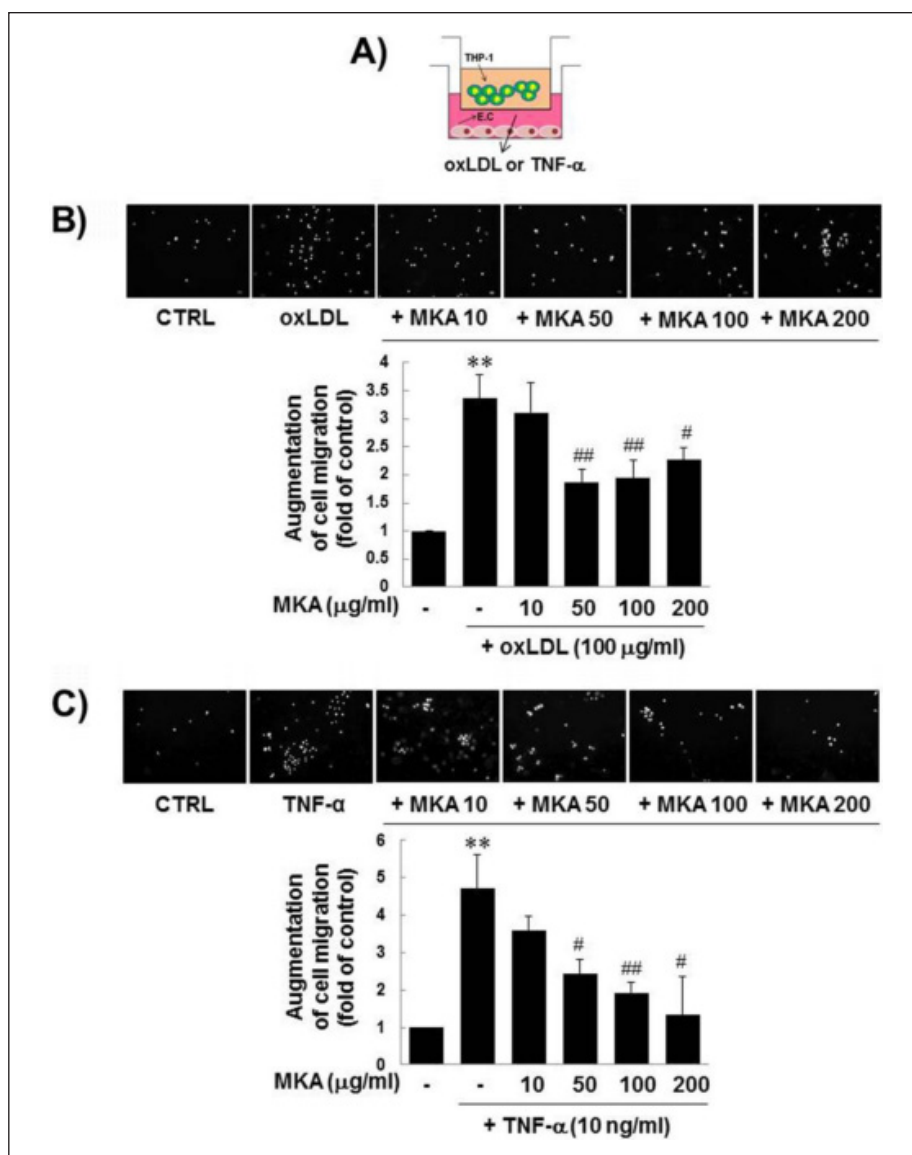

Figure 4. MKA inhibited oxLDL- or TNF-α-induced migration of THP-1 monocytes. (A) ECs seeded in the bottom chamber were pretreated with MKA for 1 h and stimulated with oxLDL (B) or oxLDL (C) for 6 h. Transwell inserts were then placed into the wells and THP-1 cells (4×10^5 cells/well) were seeded in the upper chamber of the Transwell insert. After incubation overnight at $37°$C, the cells which migrated across the Transwell membrane were stained with DAPI and counted under a light microscope. The results are the means ± SEM from three independent determinations (**P<0.01, compared with untreated cells; #P<0.05, ##P < 0.01, compared with oxLDL or TNF-α).

ECs increased 3.4-fold or 2-fold after stimulation for 6 h with oxLDL (100 µg/ml) or TNF-α (10 ng/ml), respectively. When ECs were pretreated with MKA for 1 h, there was a significant reduction in adherent cells to ECs in a dose-dependent manner (Figure 5A and B).

DISCUSSION

Endothelial dysfunction is a key factor in the development of vascular disorders. When ECs are activated by stimuli such as oxLDL or TNF-α, ECs increases ROS production and upregulate the expression of adhesion
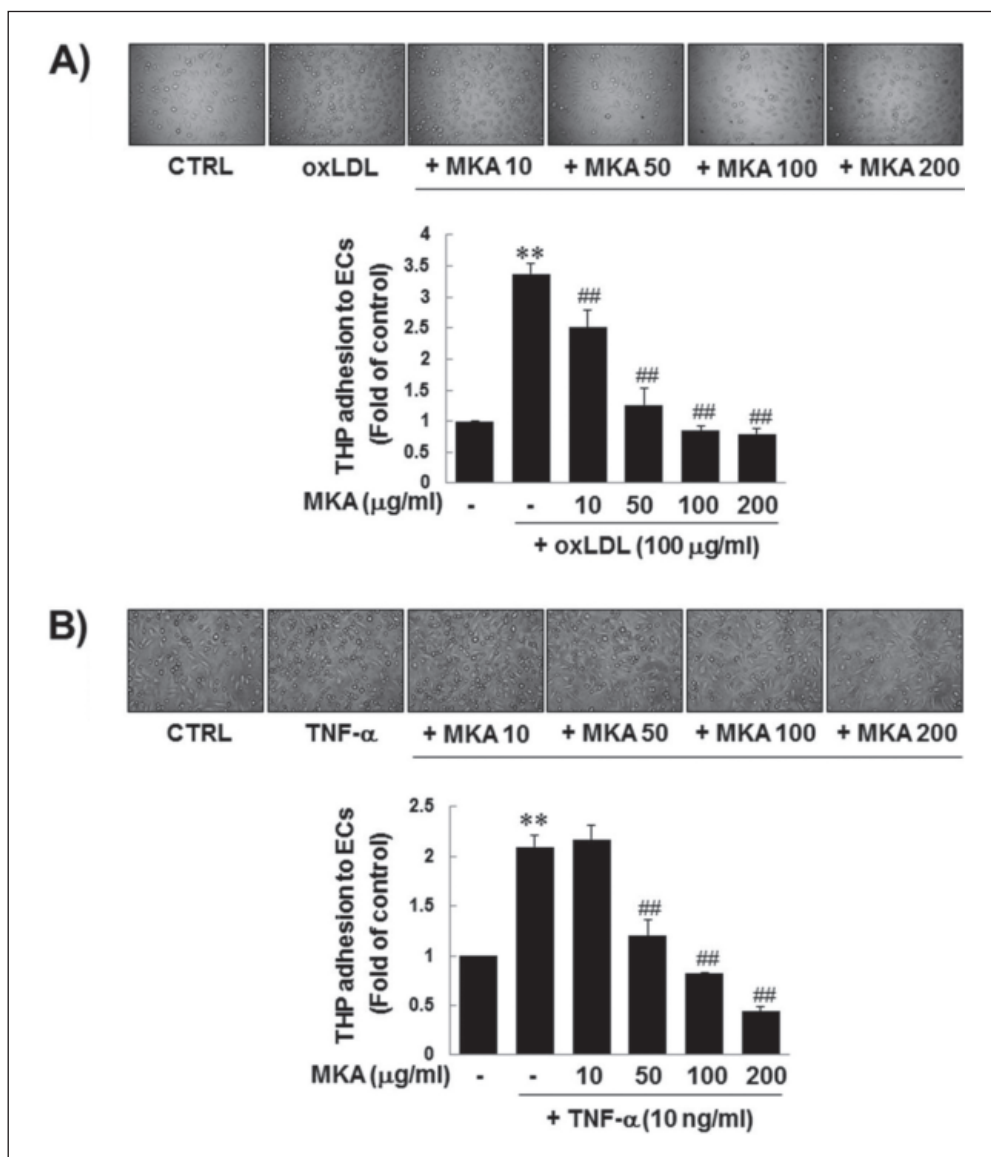

Figure 5. MKA down-regulates the binding of THP-1 monocyte to ECs. ECs and THP-1 cells were pretreated with MKA of the indicated concentrations for 1 h and then stimulated with oxLDL (100 µg/ml) (A) or TNF-α (10 ng/ml) (B) for 6 h. Then, THP-1 cells (2.5×10^5 cells/ml) were added to the ECs. After 30 min at 37°C, cell suspensions were withdrawn, and the ECs were gently washed with PBS three times. The adherent cells were then counted under a light microscope as described in the Method. Values are expressed as mean ± SEM from three independent determinations. **$P<0.01$ compared with control group; ##$P<0.01$ compared with oxLDL- or TNF-α-treated group.

molecules (AMs) such as ICAM-1 and VCAM-1. Cell adhesion molecules (CAMs) play a key role in several pathologies, including inflammatory diseases. AMs mediate the interactions between ECs and immune cells, and these interactions are some of the key factors in the pathogenesis of atherosclerosis. According to Cybulsky *et al.* (2001), VCAM-1 plays a more major role than ICAM-1 in the initiation of atherosclerosis, although expression of both VCAM-1 and ICAM-1 is regulated in atherosclerotic lesions. Therefore, it has been suggested that there is a need to discover therapeutic agents that have specific suppression effects on adhesion molecules such as VCAM-1. Our previous study reported that some natural products, including anthocyanins from black soybeans, have no cytotoxicity in normal cells and preferentially inhibit VCAM-1 rather than ICAM-1 (Nizamutdinova *et al.*, 2008).

Medicinal plants which have relatively no toxicity in normal cells are becoming more popular in the treatment of various diseases because current therapies using synthetic chemicals have various adverse effects. In this study, we found that the methanolic extract of *Kigelia africana* (MKA) had no cytotoxicity in ECs, and more importantly, MKA dose-dependently reduced RAGE expression in oxLDL- or TNF-α-stimulated ECs. In addition, MKA significantly inhibited the oxLDL- or TNF-α-induced VCAM-1 expression but not ICAM-1 expression, in ECs in a dose-dependent manner, resulting in a decrease in the migration and adhesion of THP-1 monocytes to ECs. Our results suggest that MKA could be used for the treatment of atherosclerosis without cytotoxicity. Actually, high doses of MKA (100 and 200 μg/ml) slightly reduced ICAM-1 expression induced by oxLDL, whereas any dose of MKA did not reduced TNF-α-mediated ICAM-1 induction. We think there could be two possibilities for the discrepancy of responses of MKA on ICAM inductions by oxLDL and TNF-α. Firstly, as mentioned before, TNF-α more dramatically induces ICAM-1 and VCAM-1 expression compared to oxLDL. The inhibitory effect of MKA on TNF-α-induced ICAM-1 could be hidden if the inhibitory effect of MKA on ICAM-1 is weak compared to the inhibitory effect on VCAM-1. Secondly, the mechanisms which are involved in the oxLDL-induced ICAM-1 and TNF-α-induced ICAM-1 might be different each other, even though there are common pathways such as ROS-NF-κB-mediated pathway. MKA might inhibit the pathway which is involved in the oxLDL-induced ICAM-1 but not TNF-α-induced ICAM-1, even though we can't suggest the pathway in this study. Further study regarding to the differential mechanisms between oxLDL-induced ICAM-1 and TNF-α-induced ICAM-1 is needed.

The active ingredients from medicinal plants play a significant part in the prevention and treatment of inflammatory diseases. Bello *et al.* (2016) reported that *Kigelia africana* has anti-inflammatory, analgesic, antioxidant and anticancer activities and that the bioactive constituents responsible for these activities are found to be present in all parts of the plant. So far, approximately 150 compounds have been characterized from different parts of the plant. Iridoids, naphthoquinones, flavonoids, terpenes and phenyl ethanoglycosides are the major classes of compounds that have been isolated from the plant. Novel compounds with potent antioxidant, antimicrobial and anticancer effects such as verbascoside, verminoside and pinnatal, among others, have already been identified. The polar extract of *K. africana* fruit contains verminoside as the major constituent and a series of other phenols (verbascoside, caffeic acid, *p*-coumaric acid and caffeic acid methyl ester) (Picerno *et al.*, 2005). In particular, regarding the anti-inflammatory activity of the extract, it has been reported that verminoside, an iridoid, and verbascoside, one of the poly-phenols, may have contributed to this activity (Moncada, 1999; Picerno *et al.*, 2005).

CONCLUSION

Taking the results of this paper and several additional reports together, *K. africana* could be considered an alternative medicine with no cytotoxicity that is effective for a wide range of diseases,

especially for cardiovascular diseases such as atherosclerosis.

Conflict of interest
Authors declare no conflicts of interest.

Contributions of authors
We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. YSK carried out the experiments and analyzed the data. ON and SC supplied material. HJK designed the study and wrote the manuscript. All authors read and approved the final manuscript.

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