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Polyphenols from *Artemisia annua* L Inhibit Adhesion and EMT of Highly Metastatic Breast Cancer Cells MDA-MB-231

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Recent evidence suggests that polyphenolic compounds from plants have anti-invasion and anti-metastasis capabilities. The Korean annual weed, *Artemisia annua* L., has been used as a folk medicine for treatment of various diseases. Here, we isolated and characterized polyphenols from Korean *A. annua* L (pKAL). We investigated anti-metastatic effects of pKAL on the highly metastatic MDA-MB-231 breast cancer cells especially focusing on cancer cell adhesion to the endothelial cell and epithelial-mesenchymal transition (EMT). Firstly, pKAL inhibited cell viability of MDA-MB-231 cells in a dose-dependent manner, but not that of human umbilical vein endothelial cells (ECs). Polyphenols from Korean *A. annua* L inhibited the adhesion of MDA-MB-231 cells to ECs through reducing vascular cell adhesion molecule-1 expression of MDA-MB-231 and ECs, but not intracellular adhesion molecule-1 at the concentrations where pKAL did not influence the cell viability of either MDA-MB-231 cells nor EC. Further, pKAL inhibited tumor necrosis factor-activated MDA-MB-231 breast cancer cell invasion through inhibition of matrix metalloproteinase-2 and matrix metalloproteinase-9 and EMT. Moreover, pKAL inhibited phosphorylation of Akt, but not that of protein kinase C. These results suggest that pKAL may serve as a therapeutic agent against cancer metastasis at least in part by inhibiting the cancer cell adhesion to ECs through suppression of vascular cell adhesion molecule-1 and invasion through suppression of EMT. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: breast cancer cell; endothelial cell; metastasis; *Artemisia annua* L; VCAM-1; EMT.

Abbreviations: ECM, extracellular matrix; ECs, human umbilical vein endothelial cells; EMT, epithelial-mesenchymal transition; ICAM, intracellular adhesion molecule; KAL, Korean *Artemisia annua* L; PKC, protein kinase C; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule

INTRODUCTION

With advances in medical science, the elderly cancer patients are expected to increase due to the elderly population explosion (Jung *et al.,* 2014). This population often encounters serious side effects from conventional chemotherapeutic agents or treatment-related mortality due to short of organ reservoir. In addition, the modern chemotherapy strategy is emphasizing the quality of life of the cancer patients treated by chemotherapy. Therefore, much interest has been drawn to phytochemicals because the dietary agents can safely enhance anticancer effects without significant toxicities (Liu *et al.,* 2007; Kundu and Chun, 2014; Lu *et al.,* 2014).

Artemisia annua L is an annual weed that grows to an average height of 2 m and produces bright yellow flowers with camphor-like fragrance in September. It has been used as Korean folk medicine for many diseases such as malaria, fever, convulsion, and cancers. Recent evidence suggests that polyphenolic compounds possess anticancer properties (Sliva, 2008), so we extracted polyphenols and characterized its profile of Korean *A. annua* L (KAL). It contained quercetin and isorhamnetin derivatives and kaempferol derivatives, which have anticancer effects (Han *et al.,* 2013).

Breast cancer is the most common cancer diagnosed in Western European and North American women. In Korea, its incidence has been steadily increasing, showing the highest growth of breast cancer (Jung *et al.,* 2014). Breast cancer is considered as a systemic disease because the pattern of relapse after surgical resection is systemic dissemination rather than locoregional relapse. Thus, most of relapsed breast cancer

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patients die of metastasis like most cancer patients do. The process of cancer metastasis involves several steps: the invasion of cancer cells from the primary tumor into the vasculature, shedding into blood stream, moving to distant organs, adhesion to endothelial cells lining the blood vessels of distant organs, and new tumor mass formation at distant sites (Ali and Lazennec, 2007).

Cell adhesion molecules are involved in various processes of cancer metastasis (Okegawa *et al.,* 2004). Among them, vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) are well-known endothelial surface adhesion molecules involved in cancer metastasis (Orr *et al.,* 2000). Epithelial-mesenchymal transition (EMT) is a hypothesized process involved in cancer metastasis (Thiery, 2002; Rhim *et al.,* 2012). During EMT, cancer cells convert an epithelial phenotype to a mesenchymal phenotype by promoting the loss of cell to cell adhesion, leading to the cell's release from the surrounding tissue, and finally acquiring the migratory capability to invade. Therefore, EMT can be regarded as an initial process of metastasis and a major mechanism that is responsible for mediating the invasion and metastasis of cancer cells (Thiery, 2002; Liang, 2011). Therefore, inhibition of gene expressions involved in the metastatic cascade like adhesion, invasion, and EMT by less toxic agents such as natural compounds is one of best strategies in controlling cancer metastasis of the elderly patients with improving their quality of life. In this study, we investigated the anticancer activity of polyphenols from KAL (pKAL) on highly metastatic breast cancer cells MDA-MB-231 regarding cancer cell adhesion to the endothelial cell and EMT with elucidating its mechanism.

MATERIALS AND METHODS

Preparation of polyphenols from Korean *Artemisia annua* L. Polyphenols from Korean *A. annua* L (pKAL) were extracted and characterized by Professor Shin (Gyeongsang national university, Jinju, Korea). Briefly, the lyophilized KAL tissues including roots, stems, leaves, and flowers (10 g) were ground into powder and extracted in ethyl acetate (300 mL) at 80°C for 20 h, and eluted using a mixture of methanol:dichloromethane (1:5, 25 mL). The isolated polyphenol mixtures were identified by HPLC-MS/MS according to the previous method (Yun *et al.,* 2010). The thirty-two polyphenols in the KAL were as follows: caffeic acid (**1**), syringic aldehyde (**2**), dicaffeoylquinic acid isomer (**3**, **4**, **11**), Quercetin 3-O-galactoside (**5**), dicaffeoylquinic acid isomer (**6**), mearnsetin 3-O-hexoside isomer (**7**, **10**), kaempferol 3-O-glucoside (**8**), quercetin 3-O-glucoside (**9**), ferulic acid (**12**), caffeoylferuloylquinic acid isomer (**13**, **17–19**), isorhamnetin 3-O-glucoside (**14**), diosmetin 7-O-glucoside (**15**), luteolin 7-O-glucoside (**16**), diferuloylquinic acid (**21**), quercetin (**22**), dicaffeoylferuloylquinic acid isomer (**23**), 3-O-methylquercetagetin (**24**), dicaffeoylferuloylquinic acid isomer (**25**), luteolin (**26**), 8-methoxykaempferol (**27**), 3,5-dimethoxyquercetagetin (**28**), caffeoyldiferuloyl quinic acid (**29**), kaempferol (**30**), 3,5-dihydroxy-6,7,4'-trimethoxyflavone (**31**), and 3,5-dihydroxy-6,7,3',4'-tetramethoxyflavone (**32**). Quercetin and kaempferol derivatives formed 84.8% of the total polyphenols (Song *et al.,* 2014). Twelve hydroxycinnamic acids (**1**, **3**, **4**, **6**, **11**, **13**, **17–19**, **21**, **23**, and **25**) and ten flavonoids (**7**, **9**, **10**, **14**, **16**, **22**, **26**, and **30–32**) were previously reported in Chinese, Brazilian, and Italian *A. annua* (Carbonara *et al.,* 2012). The rest of the hydroxycinnamic acids (**2**, **12**, **20**, and **29**) and flavonoids (**5**, **8**, **15**, **24**, **27**, and **28**) were detected for the first time in *A. annua* tissues in this study.

Cell culture and chemicals. Human breast cancer cell line, MDA-MB-231, was obtained from Korea Cell Line Bank (Seoul, Korea) and cultured in RPMI 1640 (Invitrogen Corp, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (GIBCO BRL, Grand Island, NY, USA), 2 mM L-glutamine, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 25 mM $NaHCO_3$, 100 IU/mL penicillin, and 10 μg/mL streptomycin. Human umbilical vein endothelial cells (ECs, EA.hy 926 cells) were obtained from American Type Culture Collection (ATCC) and cultured in medium 199 (GIBCO BRL, Grand Island, NY, USA) supplemented with 20% fetal bovine serum, 2 mM L-glutamine, 5 U/mL heparin, 100 IU/mL penicillin, 10 μg/mL streptomycin and 50 μg/mL EC growth supplements. Cells were cultured in 100 mm dishes at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Antibodies against VCAM-1, ICAM-1, Snail, N-cadherin, E-cadherin, Akt, protein kinase C (PKC), and β-catenin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against phospho-Akt, and phospho-PKC was purchased from

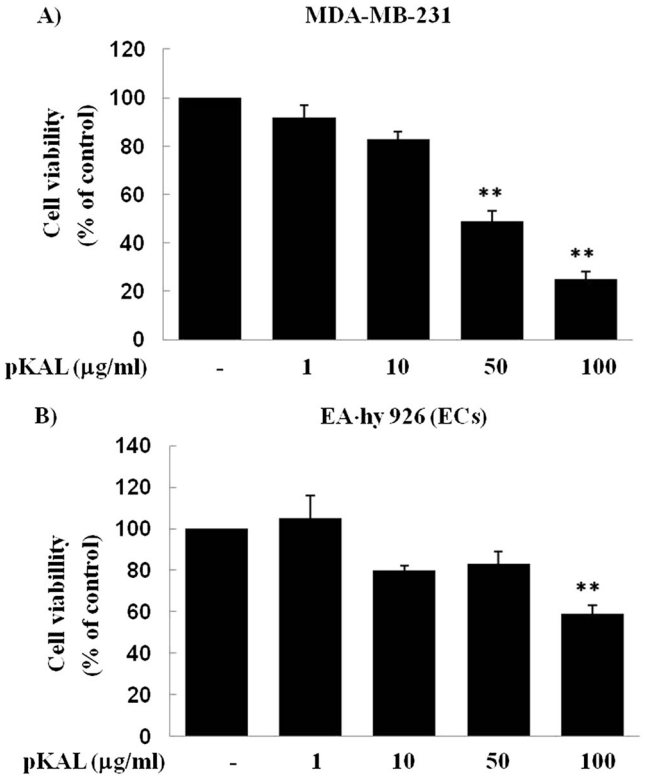

Figure 1. The anticancer effect of polyphenols from Korean *Artemisia annua* L (pKAL) on cell viability of breast cancer cells MDA-MB-231 or ECs. (A) MDA-MB-231 cells and (B) human umbilical vein endothelial cells (ECs) were starved for 16 h and then treated with pKAL of the indicated concentrations. After 24 h, cell viability was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. The data are expressed as mean ± standard error of three replicates. $^{**}P < 0.01$ compared with vehicle-treated group.

Sigma-Aldrich Co. (St. Louis, MO, USA). Recombinant human tumor necrosis factor (TNF) was purchased from R&D system (Minneapolis, MN, USA). Matrigel™ basement membrane matrix is supplied by BD Biosciences (San Diego, CA, USA). Enhanced chemiluminescence western blotting detection reagent was purchased from Amersham (Buckinghamshire, UK). All other chemicals, including β-actin, were purchased from Sigma-Aldrich (St, Louis, MO, USA).

Cell viability assay. Cells were seeded at 10^4 cells per well in 24-well plates and treated with pKAL at the indicated doses for 24 h. After treatments, 50 μL of 5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide solution was added to each well and incubated for 4 h. The supernatants were aspirated, and the formazan crystals were dissolved with 200 μL of 4 N HCl-isopropanol in each well. The absorbance was measured at 570 nm by an Infinite 200 microplate reader (TECAN Austria GmbH, Grödig, Austria).

Adhesion assay. Human umbilical vein endothelial cells (ECs) and MDA-MB-231 cells were treated with pKAL for 24 h and subsequently stimulated with TNF for 6 h. Then, MDA-MB-231 cells (7.5×10^5 cells/mL) were

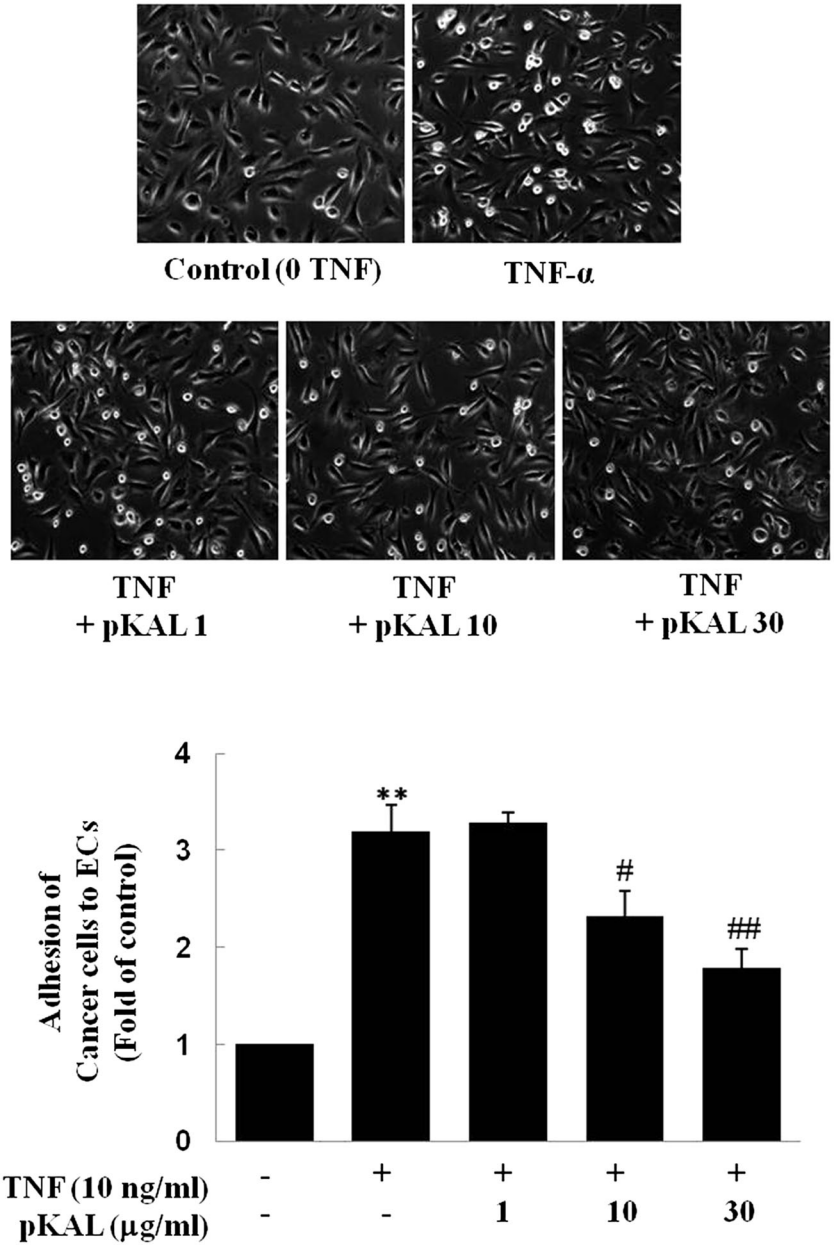

Figure 2. The inhibitory effect of polyphenols from Korean *Artemisia annua* L (pKAL) on adhesion of MDA-MB-231 cells to human umbilical vein endothelial cells (ECs). Human umbilical vein endothelial cells were pretreated with pKAL of the indicated concentrations for 1 h, and then, cells were stimulated with tumor necrosis factor (TNF) (10 ng/mL) for 6 h. Human umbilical vein endothelial cells were washed with serum-free medium, and MDA-MB-231 cells were added onto ECs and incubated for 30 min at 37°C. The cells were gently washed, and adhered MDA-MB-231 cells to ECs were counted under light microscope (×400). The image depicts that the TNF-induced cancer cell adhesion to ECs was significantly inhibited by pKAL. The data are expressed as mean ± standard error of three replicates. $**P < 0.01$ compared with control group; $\#P < 0.05$, $\#\#P < 0.01$ compared with TNF-treated group.

added to the ECs at 37°C. After 30 min, cell suspensions were withdrawn, and the ECs were washed with phosphate-buffered saline three times. The MDA-MB-231 cells were counted under a light microscope, and images were taken using an Olympus microscope (CKX41) equipped with a camera (Nikon, DS-U3).

Matrigel invasion assay. The matrigel invasion assays were performed on EC-coated matrigel. Human umbilical vein endothelial cells were pretreated with pKAL for 24 h and washed with phosphate-buffered saline three times. EC-coated matrigel was treated with TNF for 6 h, and MDA-MB-231 cells were added to EC-coated matrigel wells and incubated for 24 h. The non-invasive cells that remained on the upper side of the matrigel membranes were removed. The cells on the lower part of matrigel membranes were stained with 4',6-diamino-2-phenylindole and counted under a light microscope.

Gelatin zymography. The gelatinolytic activities for secreted matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) in the culture medium were assessed by electrophoresis on 8% polyacrylamide gels containing gelatin (1 mg/mL) at 4°C. Media were concentrated by Pierce protein concentrators 7 mL/9 K, MWCO devices (Thermo, Pierce, Rockford, IL, USA). Protein in the media was precipitated with 80% cold acetone. Precipitated proteins were mixed with sample buffer (0.03% bromophenol blue, 0.4 M Tris-HCl pH 7.4, 20% glycerol, 5% SDS) and separated on 8% SDS-polyacrylamide gels containing gelatin (1 mg/mL). Subsequently, gels were washed with renaturing buffer (2.5% Triton X-100) for 1 h and then incubated for 24 h at 37°C in developing buffer (50 mM Tris, 20 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij35, pH 7.5). Gels were stained with 0.05% Coomassie Brilliant Blue R-250 and destained with 50% methanol and 10% acetic acid. White lysis zones indicating gelatin degradation were revealed by staining with Coomassie brilliant blue.

Western blot analysis. The cells were lysed using PRO-PREP protein extraction solution (iNtRON Biotechnology, Seoul, Korea), and proteins in conditioned media were concentrated 20-fold with Pierce concentrator 7 mL/9 K, MWCO devices (Thermo, Pierce, Rockford, IL, USA). The concentrated proteins were quantified using the BioRad protein assay (BioRad Lab., Hercules, CA, USA). Aliquots of 50 μg of protein were subjected to 7.5–12.5% sodium dodecyl sulfate–

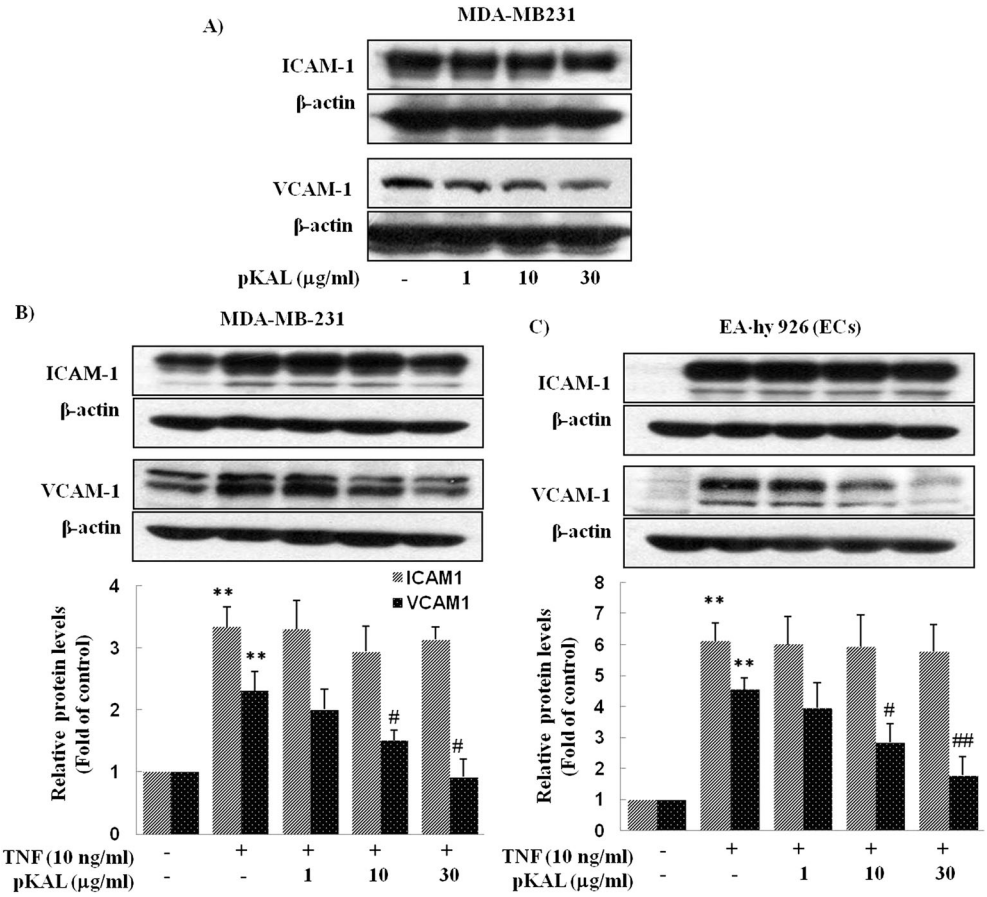

Figure 3. The effects of polyphenols from Korean *Artemisia annua* L (pKAL) on the expression of adhesion molecules [intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) in MDA-MB-231 and human umbilical vein endothelial cells (ECs). (A) MDA-MB-231 cells were treated with pKAL of the indicated concentrations for 6 h. (B) MDA-MB-231 cells or (C) ECs were pretreated with pKAL of the indicated concentrations for 1 h and then stimulated with tumor necrosis factor (TNF) (10 ng/mL) for 6 h. After treatments, proteins were extracted from the cells, and ICAM-1, VCAM-1, and β-actin protein levels were determined by western blot analysis and quantified. The data are expressed as mean ± standard error of three replicates. $^{**}P < 0.01$ compared with control group; $\#P < 0.05$, $\#\#P < 0.01$ compared with TNF-treated group.

polyacrylamide gel electrophoresis and transferred onto Hybond-P+ polyvinylidene difluoride membranes (Amersham Biosciences UK Ltd). The membranes were incubated with the indicated primary antibodies followed by a horseradish peroxidase-conjugated secondary antibody. Blots were developed with an enhanced chemiluminescence detection system. β-Actin was used as a loading control.

Statistical analysis. Scanning densitometry was performed using Image Master® VDS (Pharmacia Biotech Inc., San Francisco, CA, USA). The treatment groups were compared using one-way analysis of variance and the *post hoc* test Neuman–Keuls in the cases at least three treatment groups and Student's *t* test for two group comparison. All data were expressed as the mean ± standard error. $P < 0.05$ was considered statistically significant.

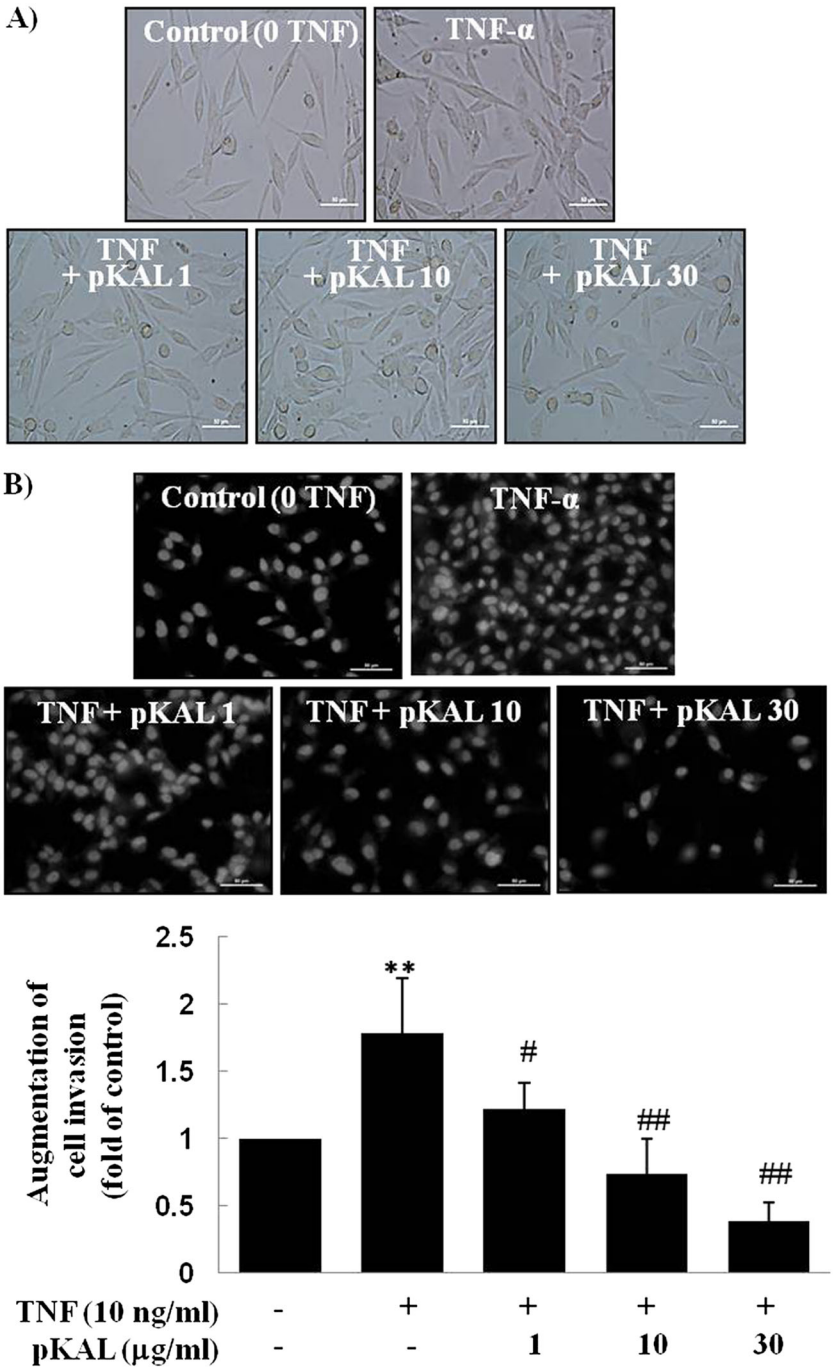

Figure 4. The inhibitory effects of polyphenols from Korean *Artemisia annua* L (pKAL) on the invasion of MDA-MB-231 cells augmented by tumor necrosis factor (TNF). MDA-MB-231 cells were pretreated with pKAL of the indicated concentrations for 1 h and then stimulated with TNF (10 ng/mL) or not for 6 h, and (A) the morphologic changes of cells were observed under the microscope (×400). Polyphenols from Korean *Artemisia annua* L induced morphologic changes of MDA-MB-231 cells from spindle (mesenchymal) form to round (epithelial) form in a dose-dependent manner. (B) After treatments, cells were collected, applied in matrigel-coated insert well, and then incubated overnight (for 16 h) at 37°C. The non-invasive cells that remained on the upper side of the matrigel insert were removed, and the cells on the lower part of insert membranes were stained with 4',6-diamino-2-phenylindole. The number of cells that invaded through the membrane was counted under a fluorescence microscope. TNF significantly increase cancer cell invasion, which was significantly inhibited by pKAL in a dose-dependent manner. The data are expressed as mean ± standard error of three replicates. $**P < 0.01$ compared with control group; $\#P < 0.05$, $\#\#P < 0.01$ compared with TNF-α treated group.

RESULTS

Polyphenols from Korean *Artemisia annua* L inhibited cell viability of MDA-MB-231 breast cancer cells in a dose-dependent manner, but not that of human umbilical vein endothelial cells

First, we examined the cell viability of MDA-MB-231 breast cancer cells and ECs at the indicated concentrations of pKAL (1, 10, 50, 100 μg/mL). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide test revealed that pKAL decreased the cell viability of MDA-MB-231 cells in a dose-dependent manner, but not that of ECs until at the doses of 50 μg/mL of pKAL (Fig. 1). This finding suggests that pKAL may have cancer-specific cytotoxicity, indicating that pKAL may serve as an anticancer agent with minimal cytotoxicity in normal cells.

Polyphenols from Korean *Artemisia annua* L inhibited the adhesion of MDA-MB-231 breast cancer cells to human umbilical vein endothelial cells

The aforementioned finding demonstrated that the cell viability of MDA-MB-231 cells and ECs was not influenced by 48-h pKAL treatment up to the concentration of 50 μg/mL. Then, we investigated the effect of pKAL on adhesion of MDA-MB-231 to ECs at the concentrations of 1–30 μg/mL where pKAL did not show antiproliferative effects. The adhesion of MDA-MB-231 cells to ECs was dramatically increased by 6-h TNF treatment (10 ng/mL) compared with untreated ECs (Fig. 2). The results revealed that pKAL significantly inhibited TNF-induced cancer cell adhesion to ECs, and it has anti-adhesive effect on cancers to human endothelial cells of blood vessel. This finding suggests that pKAL may serve as a therapeutic agent against cancer metastasis with minimal cytotoxicity in normal cells.

Polyphenols from Korean *Artemisia annua* L reduced vascular cell adhesion molecule-1 expression of MDA-MB-231 and human umbilical vein endothelial cells, but not intracellular adhesion molecule-1

Intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) have been shown to be involved in cell–cell and cell–extracellular matrix (ECM) interactions and are mechanistically important for the extravasation of cancer cells (Price and Thompson, 2002). We first examined whether pKAL inhibited VCAM-1 and ICAM-1 expressions of MDA-MB-231 cells. Polyphenols from Korean *A. annua* L significantly inhibited VCAM-1 expression, but not ICAM-1 expression of MDA-MB-231 cells (Fig. 3A). In advanced or metastatic cancers, TNF is highly expressed (Correia *et al.,* 2007) and is involved in the expression of VCAM-1 and ICAM-1 expressions. Then, we examined whether pKAL inhibited VCAM-1 and ICAM-1 expressions induced by TNF in MDA-MB-231 cells and ECs. Treatment of pKAL significantly inhibited TNF-induced VCAM-1 expression, but not TNF-induced ICAM-1 expression in both MDA-MB-231 cells and ECs (Fig. 3B and C). These findings suggest that the anti-adhesive effect of pKAL may be associated with reduction of VCAM-1 expression of both the cancer cells and ECs.

Polyphenols from Korean *Artemisia annua* L affected MDA-MB-231 breast cancer cell morphology and inhibited tumor necrosis factor-activated MDA-MB-231 breast cancer cell invasion

Next, we observed changes of MDA-MB-231 cells in morphology after pKAL treatment; pKAL induced morphologic changes of MDA-MB-231 cells from spindle (mesenchymal) form to round (epithelial) form in a dose-dependent manner (Fig. 4A). Then, we determined whether pKAL inhibit MDA-MB-231 cell invasion

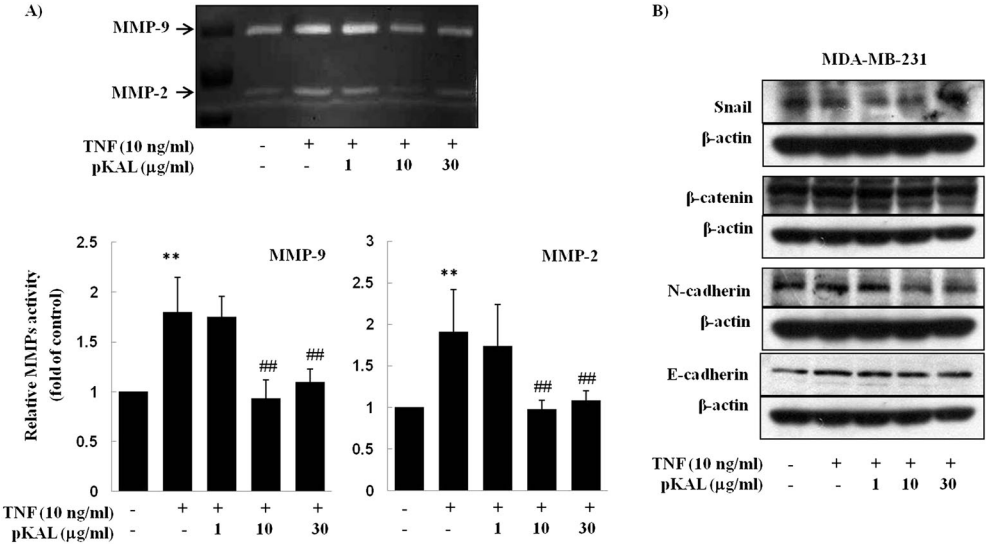

Figure 5. The inhibitory effects of polyphenols from Korean *Artemisia annua* L (pKAL) on secreted matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) and the mesenchymal markers levels of MDA-MB-231 cells. (A) Cells were seeded at 5×10^4 cells/mL and pretreated with pKAL of the indicated concentrations for 1 h. Then, cells were treated with tumor necrosis factor (TNF) (10 ng/mL) furan additional 12 h. Matrix metalloproteinase-2 and MMP-9 protein levels were measured by gelatin zymography. The gelatinolytic activity was measured by densitometry. The data are expressed as mean ± standard error of three replicates. $^{**}P < 0.01$ compared with control group; $^{\#\#}P < 0.01$ compared with TNF-α treated group. (B) The levels of Snail, β-catenin, N-cadherin, and E-cadherin from cell lysates were determined by western blot analysis. The results are representative of three replicates.

activated by TNF. TNF significantly increases cancer cell invasion, which was significantly inhibited by pKAL in a dose-dependent manner (Fig. 4B). These findings suggest that pKAL inhibit the cancer cell invasion at least in part through suppression of EMT.

Polyphenols from Korean *Artemisia annua* L inhibits secreted matrix metalloproteinases and downregulates TNF-induced mesenchymal markers of MDA-MB-231 cells

Tumor invasion is the first step for metastasis. The process includes proteolytic digestion of the ECM, in which MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) are involved (Liabakk *et al.,* 1996). Thus, we investigated the expression of MMP-2 and MMP-9. Gelatin zymography analyses revealed that pKAL suppressed MMP-2 and MMP-9 expression augmented by TNF (Fig. 5A). Next, to confirm whether pKAL inhibit the cancer cell invasion at least in part through suppression of EMT, we assessed the effects of pKAL on EMT-associated proteins (Snail, β-catenin, N-cadherin, and E-cadherin). Western blot analysis revealed that pKAL significantly reduced the mesenchymal markers Snail and N-cadherin expression, but not β-catenin and E-cadherin levels (Fig. 5B). These findings suggest that pKAL suppress EMT by downregulating the mesenchymal markers, especially, N-cadherin (Fig. 5B).

Polyphenols from Korean *Artemisia annua* L inhibited phosphorylation of Akt, but not that of protein kinase C

To investigate upstream signaling pathways, we examined the p-Akt and p-PKC expression because adhesion molecules (ICAM-1 and VCAM-1) and EMT-associated proteins (Snail, β-catenin, N-cadherin, and E-cadherin) are regulated by PI3K/Akt and PKC pathways (Li and Zhou, 2011; Moon *et al.,* 2011). Polyphenols from Korean *A. annua* L suppressed the phosphorylation of Akt in a dose-dependent manner, but increased the phosphorylation of PKC in a dose-dependent manner (Fig. 6A). Then, we tested the effects of pKAL on the p-Akt and p-PKC expression activated by TNF in both MDA-MB-231 cells and ECs. As shown in Fig. 6B, TNF significantly increased phosphorylation of Akt, but not phosphorylation of PKC in MDA-MB-231 cells. Polyphenols from Korean *A. annua* L suppress phosphorylation of Akt and increased phosphorylation of PKC in a dose-dependent manner in MDA-MB-231 cells (Fig. 6B). However, in ECs, TNF significantly increased phosphorylation of Akt and PKC, and pKAL suppressed phosphorylation of Akt, but not that of PKC. These findings suggest that the Akt pathway may be a main upstream signaling pathway involved in pKAL-induced anticancer effects (adhesion and invasion).

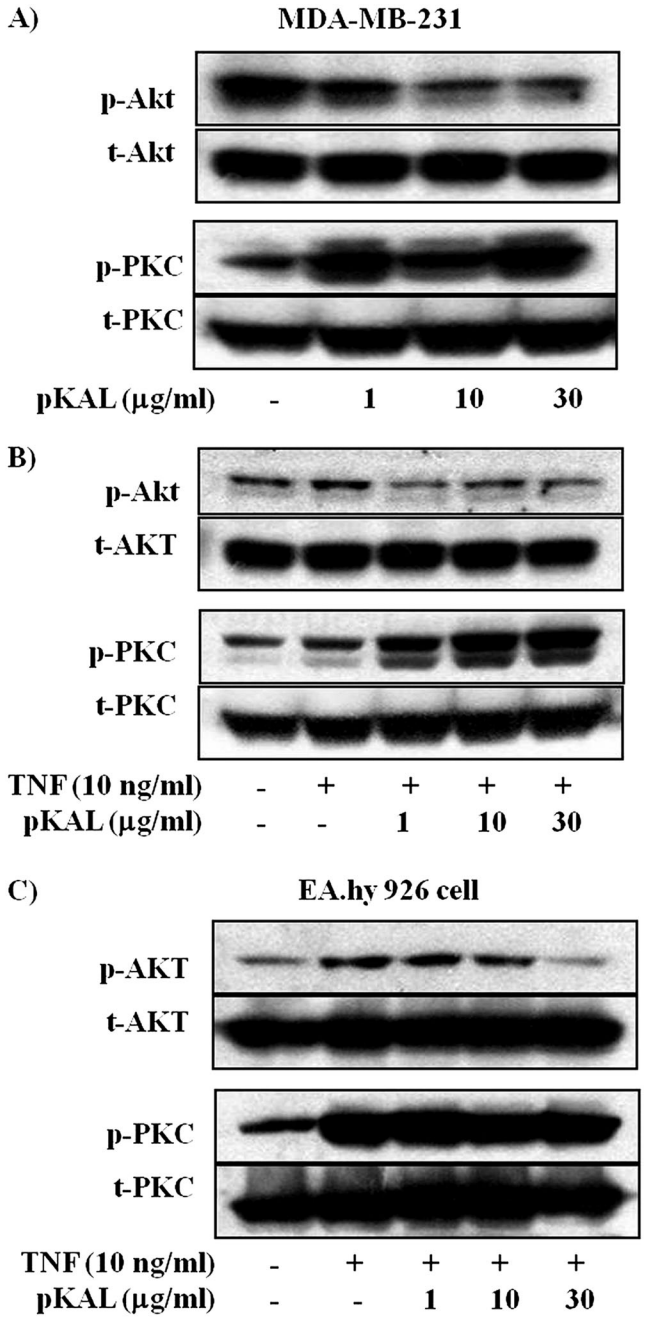

Figure 6. The effects of polyphenols from Korean *Artemisia annua* L (pKAL) on Akt and protein kinase C (PKC) in MDA-MB-231 and human umbilical vein endothelial cells. Human umbilical vein endothelial cells were pretreated with pKAL for 1 h, followed by incubation with tumor necrosis factor (TNF) or not for 6 h. Whole-cell extracts were prepared, and 30 μg of the whole-cell lysate was analyzed by western blot analysis for phospho-Akt, Akt, phospho-PKC, and PKC, in (A and B) MDA-MB-231 cells and (C) human umbilical vein endothelial cells. The results are representative of three replicates.

DISCUSSION

This study was designed to investigate the effects of pKAL on the cancer metastasis focusing on cancer cell adherence to endothelial cells of human blood vessel and EMT because adhesive interaction of cancer cells with the endothelial cells, and EMT process are crucial steps for cancer metastasis. Therefore, we investigated the effect of pKAL on adherence of cancer cells to the endothelial cells and on the expression of adhesion proteins (VCAM-1, ICAM-1) and EMT-associated proteins (Snail, β-catenin, N-cadherin, and E-cadherin). This study showed that pKAL suppressed TNF-induced cancer cell adhesion to ECs through inhibiting VCAM-1 expression of both the MDA-MB 231 cells and ECs.

Previous studies demonstrated that the regulatory pathway for VCAM-1 can be different from that for ICAM-1 (Moon *et al.,* 2011). Here, we also demonstrated that pKAL suppressed N-cadherin involved in EMT. The major components of pKAL were quercetin and kaempferol. The two flavonoids exert anti-metastatic effects on cancer cells by inhibiting EMT (Bhat *et al.,* 2014). In addition, PI3K/Akt pathway is important in controlling EMT process of cancer cells (Bhat *et al.,* 2014). Because PKC also plays an important role in cancer metastasis (Jain and Basu, 2014; Liu *et al.,* 2014) and quercetin also inhibits cancer invasion through inhibition of PKC (Lin *et al.,* 2008), we investigated the effects of pKAL on PKC activation. The results were opposite to our expectation, pKAL activated PKC. Protein kinase C has many isozymes. In cancer cells, PKC isozymes are involved in cell proliferation, survival, invasion, migration, apoptosis, angiogenesis, and anticancer drug resistance through various mechanisms. Role of PKC isozymes in cancer cells largely depend on the types of cancer (Dowling and Kiely, 2015). Therefore, the role of PKC in pKAL-induced anticancer effects needs further evaluation.

For cancer invasion, proteolytic digestion of ECM is the essential process, in which MMP-2 and MMP-9 are key molecules (Davies *et al.,* 1993). The two molecules are the target molecules to prevent cancer invasion because cancer invasion is prerequisite for cancer metastasis, which most of cancer patients eventually die of. The finding that pKAL has anti-invasive effects through suppression of MMP-2 and MMP-9 is consistent with the previous reports using quercetin or kaempferol (Lindstedt *et al.,* 2013)

In this study, we used MDA-MB 231 cells for the experiments of anti-invasive effects of pKAL, because it is aggressive in terms of invasion and metastasis to distant organs (Anandappa *et al.,* 2000). In addition, we used TNF to clearly demonstrate adherence of cancer cells to ECs and EMT. Cancer metastasis is a complex process involving release of variable growth factors and cytokines including TNF from cancer and infiltrating inflammatory cells (Hamaguchi *et al.,* 2011; Mikami *et al.,* 2015). In addition, TNF is highly expressed in advanced cancer, and TNF inhibitor can suppress cancer metastasis (Hamaguchi *et al.,* 2011). In addition, this finding is similar to the experiments we previously performed with anthocyanins of which effects was verified *in vivo* study (Yun *et al.,* 2010). Therefore, the effects of pKAL would not be limited to MDA-MB 231 cells treated by TNF. In conclusion, this study demonstrated that pKAL suppressed the cancer cell adhesion to ECs by suppression of VCAM-1 expression and invasion at least in part by inhibition of EMT. This study provides evidence that pKAL might have anti-metastatic activity against human breast cancer cells with minimal cytotoxicity on normal cells.

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Conflict of interest

We declare that we have no conflict of interest

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