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Antioxidant and Anti-inflammatory Activities of *Allium victorialis* subsp. *platyphyllum* Extracts

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Abstract This study was conducted to investigate antioxidant activity and anti-immunological inflammatory effect of *Allium victorialis* subsp. *platyphyllum* extracts (AVPEs). Antioxidant activities of AVPEs were determined by free radical scavenging assay and reducing power test. Leaf-part extract had comparatively better antioxidant activity than other-part extracts. Antioxidant activity of extracts had protective effect for human umbilical vein endothelial cells (HUVECs) against superoxide anions secreted from activated neutrophils. Also, we observed AVPEs had inhibitory effects on the adherence of monocytic THP-1 to HUVEC monolayer to the basal level. Inhibitory effect on cell adhesion was caused by suppression of tumor necrosis factor-α (TNF-α)-upregulated expression of vascular cellular adhesion molecule-1 (VCAM-1) and E-selectin in HUVECs. From these results, we expect to support the evidence of anti-immunological inflammatory effects of *Allium victorialis* subsp. *platyphyllum* (AVP) as a Korean traditional pharmaceutical.

Keywords: *Allium victorialis* subsp. *platyphyllum*, antioxidant, anti-inflammation, HUVEC, neutrophils, monocyte

Introduction

In inflammatory responses, the antioxidants have important roles for protective effects against the oxidative stresses. Formylmethionyl-leucyl-phenylalanine (fMLP), a pathogenic bacterial derived peptide, and phorbol 12-myristate 13-acetate (PMA), a chemoattractant, activate neutrophils and stimulate to produce superoxide anions outside neutrophils (1). Superoxides are harmful to neutrophil itself and to human umbilical vein endothelial cells (HUVECs) in vascular walls. Damaged neutrophils by harmful oxidants may destroy faster than resting neutrophils. This phenomenon may be one of the reasons that cause less healing in hurts and make worse the inflammation. Also, the damaged HUVECs increase transmigration of neutrophils to joints and cause to make rheumatoid arthritis symptom worse. In healthy blood, several enzymes and chemical compounds, such as glutaredoxin (GRX), glutathione reductase (GRD), ascorbic acid, and glutathione, play as antioxidants and reducing enzyme system for scavenging harmful superoxides and free radical compounds (2). Also, the adherence of monocytes and neutrophils to HUVEC monolayer occurs in early stage of immunological inflammation. Cellular adhesion molecules (CAMs) are stimulated by immunological inflammatory mediators, such as tumor necrosis factor-α (TNF-α) and interleukins. Upregulated CAMs increase the adherence of monocytes and neutrophils to HUVEC monolayer, and causes rheumatoid arthritis and athero-sclerosis.

Allium victorialis subsp. *platyphyllum* (AVP) is an edible plant resource, and is used for relief of a common cold, antimicrobial activity, diuresis, digestion, the strengthening a stomach, and the anti-clotting of blood in Korean traditional pharmaceuticals (3). Recently it has been reported that hexane-, acetone-, and methanol-fractions of *Allium victorialis L.* have antioxidant and anti-tumor activities (4, 5). Also, sulfur compounds with potential anti-thrombotic activity from *Allium* species were reported (6). Activity for the anti-platelet aggregation and the positive correlation with organosulfuric substances in *Allium* sp. were reported (3, 7, 8).

The aim of this study was to evaluate *Allium victorialis* subsp. *platyphyllum* extracts (AVPEs) as an antioxidant, reducing, and protective agent for HUVECs against superoxide anions from activated neutrophils. Also, through the cell adhesion assay, we examined the potential anti-immunological inflammatory activity of Korean domestic AVP.

Materials and Methods

Reagents Dimethyl sulfoxide (DMSO), flavonoids, calcein *o,o'*-diacetate tetrakis (acetoxymethyl) ester (calcein-AM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *n*-formyl-methionyl-leucyl-phenylalanine (fMLP), rat tail collagen-I, NaCl, heparin, and histopaque-183 were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Cell culture medium and reagents, such as F-12K, RPMI-1640, fetal bovine serum (FBS), penicillin/streptomycin, endothelial cell growth supplement (ECGS), trypsin-EDTA, and Hank's balanced salt solution (HBSS) were obtained from Gibco (Invitrogen Inc., Grand Island, NY, USA). TNF-α was purchased from BD Science (San Jose, CA, USA). Dextran T-500 was obtained from Amersham Biosciences Inc. (Uppsala, Sweden). Ethanol and other reagents were used as first grade.

Preparation of AVPEs Whole *Allium victorialis* subsp. *platyphyllum* (AVP) was separated to root-, seed-, leaf-,

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flower stalk-, and stem-part, and then were washed, dried by freeze drier, and crushed. Crushed plants (30 g) were extracted with 100 mL of 70% ethanol (in water) for 24 hr at room temperature. Extracts were filtered through Whatman No.1 filter paper, and were concentrated by evaporator under reduced pressure. AVPEs were redissolved in DMSO to 100 mg/mL of concentration, and used as a stock.

Radical scavenging activity Scavenging effect of AVPEs on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was monitored according to the method described by Lee *et al.* (9). A 0.2 mL of methanolic solution containing extracts was mixed with 4 mL of methanol, and a methanolic solution of DPPH (1 mmol/L, 0.5 mL) was added. The mixture was vortexed for 15 sec and incubated at room temperature for 30 min. The absorbance was read at 517 nm.

Reducing power The reducing power of AVPEs was determined by Fe^{3+} reduction (10-12). AVPEs (10-1,000 mg/mL) in distilled water were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% $K_3Fe(CN)_6$. The mixture was incubated at 50°C for 20 min. After that, 2.5 mL of 10% trichloroacetic acid was added and centrifuged at 2,090×g for 10 min. A 2.5 mL of supernatant layer was added to 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. The absorbance of the mixture was measured at 700 nm using UV-spectrophotometry (Agilent Technologies Inc., Santa Clara, CA, USA).

Cell culture Monocytic cell line, THP-1, was obtained from Korean Cell Line Bank (KCLB, Seoul, Korea). Monocytes were cultured in RPMI-1640 medium containing 10% FBS and 100 units/mL of penicillin/streptomycin at 37°C in a 5% CO_2 incubator under controlled moisture. THP-1, the floating cell line, was subcultured after being collected by centrifugation at 2,090×g for 2 min, and were used for cell adhesion assay at passage numbers 60–70. Human umbilical vein endothelial cells (HUVECs, CRL-2480; ATCC, Manassas, VA, USA) were cultured with F-12K nutrient mixture (Kaighn's modification, Gibco/Invitrogen Inc.) containing 10% FBS, 100 units/mL of penicillin/streptomycin, 0.1 mg/mL of heparin, and 0.03 mg/mL of ECGS. HUVECs were cultured in a 5% CO_2 humidified incubator at 37°C. For subculture, HUVEC monolayer was rinsed twice with PBS (pH 7.4) to remove all traces of serum which contains trypsin inhibitor and was subdivided using 0.05% trypsin with 0.53 mM EDTA. HUVECs were used at passage numbers 20–30 for this study. WB-F344 cells, rat liver epitherial cell line, were the kind gift of Dr. J. Trosko at Michigan State University (East Lansing, MI, USA). WB-F344 cells were cultured in DMEM medium (Gibco/Invitrogen Inc.) containing 10% FBS and 100 units/mL of penicillin/streptomycin at 37°C in a 5% CO_2 incubator under controlled moisture, and continuously passaged at subconfluency with 0.05% trypsin.

Isolation of neutrophils (PMNs) Neutrophils were isolated from heparinized whole blood collected from healthy male volunteers (13). Neutrophils were separated from other cellular components by histopaque-1083 density centrifugation, followed by dextran sedimentation and osmotic lysis of residual erythrocytes with hypotonic saline. Neutrophils were suspended in HBSS without calcium, magnesium, and phenol red (pH 7.4) to a concentration of 1×10^7 cells/mL using particle count and size analyzer (ZM; Coulter Corp., Middletown, NY, USA).

Calcein-AM labeling of cells Prior to adhesion assay, monocytes were fluorescent-labeled by the incubation with 5 mM of calcein-AM in PBS (pH 7.4) for 30 min at 37°C. After loading calcein-AM, cells were washed 3 times with PBS to remove excess calcein-AM. Then the monocytes were resuspended in RPMI-1640 medium for adhesion assay.

Cytotoxicity test Cytotoxicity was examined using the MTT assay. HUVECs and WB-F344 cells were seeded at 1×10^4 cells/well in 96 well culture plate (Corning Inc., Corning, NY, USA), and were incubated for 24 hr at 37°C in a humidified 5% CO_2 incubator. AVPEs were added to the plate and incubated for 24 hr. MTT was added to 0.5 mg/mL final concentration in cell culture plate and incubated for 4 hr at 37°C. After discarding all medium from the plates, 100 μL of DMSO was added to all wells. The plates were placed for 5 min at room temperature with shaking, so that complete dissolution of formazan was achieved. The absorbance of the MTT formazan was determined at 540 nm by UV-spectrophotometric plate reader (Emax; Molecular Devices Inc., Sunnyvale, CA, USA). Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells.

Cell adhesion assay HUVECs were seeded at 1×10^5 cells/well in 96 well tissue culture plate (Corning 3603; Corning Inc.). After 24 hr incubation at 37°C, HUVEC monolayer was treated with AVPEs for 24 hr, and then was stimulated with 5 ng/mL of TNF-α for 24 hr. The monolayer was washed 3 times with PBS before cell adhesion assay (14). Calcein-AM labeled monocytes, THP-1, were cocultured at a density of 5×10^5 cells/well with monolayer for 1 hr in a 5% CO_2 humidified incubator at 37°C. Non-adherent monocytes were removed by 4 times-washing with PBS. Adherence of calcein-AM labeled monocytes was determined by fluorescent intensity, measured using a fluorescent plate reader (FL600; Bio-Tek Instruments Inc., Winooski, VT, USA). The excitation and emission wavelengths for the calcein-AM molecule were 485 and 530 nm, respectively.

For photographs, HUVECs were seeded on 24-well culture plates. Calcein-AM-labeled THP-1 cells attached to HUVEC monolayer were photographed at a magnification of 100× or 200× using an inverted fluorescence microscope (IX 71; Olympus Inc., Tokyo, Japan) connected to an Olympus DP50 camera with Imaging software (ViewfinderLite, Ver. 1.0.134, Pixera Corporation, Los Gatos, CA, USA and OLYSIA BioAutoCell Ver. 3.2, Soft Imaging System, Tokyo, Japan).

RT-PCR analysis for CAMs transcription Total RNA was isolated from HUVECs using the RNeasy kit (Qiagen

Inc., Valencia, CA, USA) after treated with AVPEs. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using an One-Step RT-PCR kits (Qiagen Inc. and Bioneer Corp., Seoul, Korea) and primers at a final concentration of 1 μM. For PCR, human primers for CAMs were used as follows: vascular cellular adhesion molecule-1 (VCAM-1) forward primer: 5'-ATGCCTGGG-AAGATGGTCGTGA-3', VCAM-1 reverse primer: 5'-TG-GAGCTGGTAGACCCTCGCTG-3', intracellular adhesion molecule-1 (ICAM-1) forward primer: 5'-GGTGACGCT-GAATGGGGTTCC-3', ICAM-1 reverse primer: 5'-GTCC-TCATGGTGGGGCTATGACTC-3', E-selectin forward primer: 5'-ATCATCCTGCAACTTCACC-3', E-selectin reverse primer: 5'-ACACCTCACCAAACCCTTC-3'. Total RNA (1 μg/μL) of basal HUVECs was used as a control, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, the house-keeping gene (15), were used to determine PCR efficiency; GAPDH forward primer: 5'-A-TGACAACAGCCTCAAGATCATCAG-3', GAPDH reverse primer: 5'-CTGGTGGTCCAGGGGTCTTACTCCT-3'. Thermal cycling was performed according to modified method (16, 17). For cDNA synthesis and predenaturation, 1 cycle of 50°C for 30 min and 95°C for 15 min was performed on total RNA. For PCR amplification, 30 cycles of 95°C for a 1 min denaturation, 55°C for 2 min annealing, 72°C for a 3 min extension, and 1 cycle of 72°C for a 10 min final extension were performed using Bio-Rad thermal cycler (MJ Mini; Bio-Rad Inc., Hercules, CA, USA). And RT-PCR product was stored at 4°C until agarose gel separation.

Protein assay For standadization of cell adhesion assay data, the amount of plated monolayer was measured by BCA protein assay (Pierce Inc., Rockford, IL, USA) using bovine serum albumin as a standard, following solubilizing cells using 0.1 N NaOH and 1% of 3-(3-cholamido-propyl)dimethylammonio-1-propanesulfonate.

Statistical analysis The Student's *t*-test was used to determine the statistical significance of data value-differences. Data was expressed as means ± standard deviation (SD) of at least triplicate values.

Results and Discussion

Antioxidant activity of AVPEs Several part-extracts (1,000 μg/mL) of AVP and 500 μM of authentic apigenin, quercetin, and rutin were used for radical scavenging activity assay, as shown in Table 1. Hydrogen-donating capability (approximately 51.4%) of leaf-part extracts to DPPH radicals was superior than other part-extracts of AVP. The root- and seed-extracts showed a little DPPH-scavenging activity, approximately 5.29 and 1.69% for the control, respectively. However, stem- and flower stalk-part extracts had no radical scavenging activity. According to the HPLC and LC-MS analysis of flavonoids, quercetin, and kaempferol were determined as major flavonoid components in various part-extracts of AVP. The amount of quercetin and kaempferol in leaf-part extracts was 18.6 ±0.5 and 12.1±1.0 μg/g powder, respectively, and were superior to other part-extracts (data not shown). DPPH scavenging results were consistent with flavonoids analysis data in AVP. Quercetin and rutin showed great DPPH radical scavenging activity, but apigenin was examined to have comparatively low antioxidant activity, as reported by Nuutila *et al.* (18).

Table 1. DPPH radical scavenging activity of AVPEs

Extracts	DPPH radical scavenging activity (%)[1]
AVPEs (1,000 μg/mL)	
Seed	1.69±0.72
Leaf	51.39±4.51
Flower stalk	ND[2]
Stem	ND
Root	5.29±0.66
Flavonoid (500 μM)	
Apigenin	18.06±0.58
Quercetin	80.32±0.34
Rutin	78.67±0.63

[1]DPPH radical scavenging activity was expressed by the percentage for control.
[2]Not detected.

Reducing power of AVPEs Leaf-part extract showed the highest reducing power of Fe^{3+} (Table 2), and the reducing power was increased in concentration-dependent manner (data not shown). These results were consistent with DPPH radical scavenging assay results. Also, flower stalk- and seed-part extracts had higher reducing power than root- and stem-part extracts.

Reducing power and radical scavenging activity play a critical role for quenching superoxide anions and maintaining the cellular redox homeostasis against harmful oxidants and free radicals. Reducing power is generally associated with reductones, which have been shown to exert an antioxidant effect by donating a hydrogen atom and breaking the free radical chain (19, 20). Reductones react with certain peroxide precursors, which preventing peroxide formation (21). In rheumatoid arthritis, the cellular reducing power may support to quench superoxide anions secreted from activated neutrophils in synovial membrane and to prevent the inflammation responses in joint-cells. Also, the radical quenching property and reducing power are able to protect HUVECs against

Table 2. Reducing power of AVPEs[1]

AVPEs (1,000 μg/mL)	Reducing power (Absorbance at 700 nm)
Seed	0.11±0.00
Leaf	0.42±0.01
Flower stalk	0.27±0.01
Stem	0.09±0.00
Root	0.05±0.00

[1]Five concentration test-groups (from 10 to 1,000 μg/mL) of AVPEs were used for reducing power test, but only 1,000 μg/mL data was shown in this paper.

superoxides in vascular system.

Effect of AVPEs on protection of HUVECs against superoxide secreted from neutrophils In primary inflammation responses, the activated neutrophils produce a huge amount of superoxide anions into blood vessel. The superoxide anions have the bactericidal effect against pathogenic bacteria, such as *E.coli* CP9 (22). However, the over-controlled superoxides are harmful to neutrophils itself and HUVECs (23), simultaneously. Also, the activated neutrophils, secreting the abundant superoxide anions, may damage spleen in mice with type II-collagen-induced arthritis (CIA) (24).

The antioxidant properties of AVPEs have been shown already in Table 1 and 2. In cellular system, the quenching effect of AVPEs on superoxide anions secreted from activated neutrophils, were shown in Fig. 1. The activated neutrophils produce about 4 nmol/10^6 cells of superoxide anions in 5 min incubation (25), and this amount is 6-8 times more than resting condition (13). Protective activity for HUVECs was expressed as a percentage of cell viability for the control groups (Fig. 1). Neutrophils with or without chemoattractant were used as controls. Chemoattractant causes the abundant secretion of superoxide anions from neutrophils. Seed- and leaf-part extracts (1,000 μg/mL) showed about 94 and 85% protective effect, respectively. Erythrocyte antioxidant systems protect the cultured endothelial cells against oxidant damage (23). From these results, AVPEs are expected to help erythrocyte antioxidant function for the protection of HUVECs.

Cell proliferation was expressed as percentage for the control (HUVEC monolayer with only neutrophils-group). All results are average for at least 4 samples. Significantly different at $^*p<0.5$ and $^{**}p<0.001$, as compared to the control.

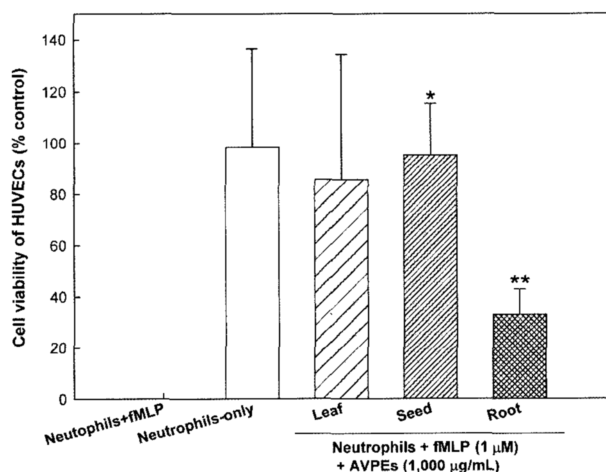

Fig. 1. Effects of AVPEs on protection of HUVECs against the activated neutrophils. Cell proliferation was expressed as percentage for the control (HUVEC monolayer with only neutrophils-group). All results are average for at least 4 samples. Significantly different at $^*p<0.5$ and $^{**}p<0.001$, as compared to the control.

Inhibition of AVPEs on the adherence of monocytes to HUVEC monolayer The adhesion of the monocytes to HUVECs acts as one of the initial steps for immunological inflammatory responses. First, the cell toxicity of AVPEs on HUVECs was determined by proliferation test. Leaf-, seed-, and root-part extracts (0-1,000 μg/mL) were added to plated HUVEC monolayer. AVP showed no cell toxicity on HUVEC monolayer in tested concentration-range, as shown in Table 3. However, the cytotoxicity of AVPEs had different results dependent on the cell lines. AVPEs showed a significant cytotoxicity on rat liver epithelial cells, WB-F344. Proliferation of WB-F344 was inhibited by AVPEs (0-100 μg/mL) in a dose-dependent manner. From this result, AVPEs had no harmful effects specifically on proliferation of HUVEC monolayer, related with cell adhesion, whereas showed the cytotoxicity on WB-F344 cells. AVPEs (1,000 mg/mL) were used for cell adhesion assay without damages to HUVEC monolayer.

HUVEC monolayer was stimulated by adding TNF-α for cell adhesion assay. In response to TNF-α, we observed a significant increase of THP-1 adherence, and AVPEs reduced the adhesion of THP-1 to HUVEC monolayers (Fig. 2). Figure 2 showed the representative images of the stimulation of adhesion and the reduction of TNF-α-stimulated adhesion of THP-1 to HUVEC monolayers by pre-incubation with AVPEs. Fluorescent images and the analysis by fluorescent plate reader (Table 4) showed that seed-part extracts reduced the adhesion of calcein-AM labeled THP-1 to HUVEC monolayer to basal level (HUVEC monolayer background was not shown in fluorescent images, but was appeared in half-fluorescent images).

Table 3. Cytotoxicity of AVPEs on HUVEC monolayer and WB-F344[1)]

AVPEs (μg/mL)	Seed	Leaf	Root
		HUVEC	
0	100.4±5.7	100.4±5.7	100.4±5.7
50	95.6±4.8	98.8±11.6	105.9±4.2
100	93.8±7.5	92.3±7.9	106.5±4.7
250	100±6.8	104.8±6.9	117.4±1.3
500	107.2±2.5	126.9±4.2	139.2±6.1
1,000	122.0±5.4	181.3±9.6	132.5±8.0
		WB-F344	
0	100±2.2	100.0±5.4	100.0±5.4
3.125	85.3±2.1	86.3±3.2	80.1±8.9
6.25	80.2±5.5	80.0±5.3	79.2±3.5
12.5	78.1±3.3	82.2±4.9	70.7±6.3
25	72.5±1.1	68.2±4.2	74.8±5.4
50	60.1±3.1	74.5±4.6	70.1±3.7
100	57.2±2.2	77.6±7.8	66.5±6.0

[1)]AVPEs were added to the HUVEC monolayer and WB-F344 cells, and incubated in humidified 5% CO_2 incubator for 24 hr in 37°C. Cell viability against AVPEs was performed by MTT assay. Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells.

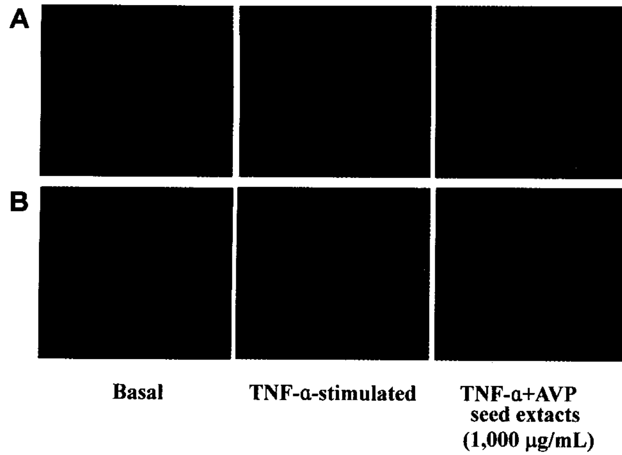

Fig. 2. Inhibitory effects of AVP seed-part extracts on the cell adhesion of THP-1 to HUVEC monolayer. A, fluorescent image (100×); B, half- fluorescent image (200×).

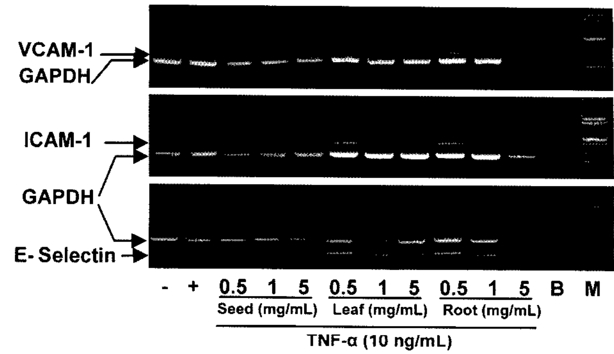

Fig. 3. Influence of AVPEs on TNF-α-stimulated mRNA transcription of CAMs. HUVECs were preincubated with AVPEs for 30 min and stimulated 10 ng/mL of TNF-α for 6 hr. Transcriptional levels of CAMs were determined by RT-PCR. B, blank; M, size marker.

When HUVECs in vascular system are exposed to the immunological inflammatory mediators, such as interleukin-1 and TNF-α, CAMs are expressed for the attachment of monocytes to HUVECs (26, 27). We expect AVPEs affect the expression of TNF-α-upregulated CAMs in HUVEC.

Effect of AVPEs on endothelial adhesion molecules transcription There was no signal of the basal mRNA transcription of CAM proteins in HUVECs, as shown in Fig. 3. However, the transcription of CAM proteins was significantly increased by stimulation of cytokine, TNF-α. We examined the suppression of mRNA transcriptional level for CAM proteins by AVPEs. Seed-part extracts reduced TNF-α-stimulated transcription of VCAM-1 and E-selectin mRNA, whereas has little effect on ICAM-1 mRNA level. VCAM-1 and E-selectin transcriptional level were reduced as the concentration of extract increased. Also, leaf- and root-part extracts reduced VCAM-1 transcription. These results were consistent with inhibitory effect data of cell adhesion in Table 4.

In immunological inflammatory responses, TNF-α and interleukin family activate neutophils and upregulate CAMs expression in HUVEC monolayer (28). Upregulated expression of CAMs protein causes the adherence of monocytes and neutrophil to blood vessel wall, especially HUVEC monolayer. These responses are closely related to rheumatoid arthritis (14) and atherosclerosis (29). VCAM-1, ICAM-1, and E-selectin are constitutive, and were expressed by immunological inflammatory agent-modulation. Also, the expression of CAMs protein is regulated by mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) pathways (31). We are planning to study the effect of AVPEs on these pathways in the future.

Through these cell adhesion and CAM proteins transcription assay, we investigated AVPEs prevented the adhesion of monocyte to HUVEC monolayer, and expect that this activity may have anti-inflammatory function. AVPEs may be used as the functional food materials and the alternative medicine to help for the relief and the prevention of rheumatoid arthritis symptoms.

Table 4. Inhibitory effects of AVPEs on the cell adhesion of THP-1 to HUVEC monolayer[1)]

AVPEs (1,000 μg/mL)	Inhibitory effect (%) of cell adhesion of THP-1 to HUVEC monolayer
Seed	105.75
Leaf	ND[2)]
Root	45.74

[1)]Prior to the cell adhesion assay, monocytes were fluorescently labeled with calcein-AM (5 ng/mL) for 30 min. HUVEC monolayer was stimulated with 10 ng/mL of TNF-α for 24 hr. Calcein-AM labeled monocytes and HUVEC monolayer were co-incubated in humidified 5% CO_2 incubator for 1 hr at 37°C. Adhesion of THP-1 to HUVEC monolayer was expressed as percent fluorescent intensity for the control at 485 and 530 nm. All value is average of at least 6 samples.
[2)]Not detected.

Acknowledgments

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