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Manassantin A and B Isolated from *Saururus chinensis* Inhibit TNF-α-Induced Cell Adhesion Molecule Expression of Human Umbilical Vein Endothelial Cells

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Leukocyte adhesion to the vascular endothelium is a critical initiating step in inflammation and atherosclerosis. We have herein studied the effect of manassantin A (**1**) and B (**2**), dineolignans, on interaction of THP-1 monocytic cells and human umbilical vein endothelial cells (HUVEC) and expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin in HUVEC. When HUVEC were pretreated with **1** and **2** followed by stimulation with TNF-α, adhesion of THP-1 cells to HUVEC decreased in dose-dependent manner with IC_{50} values of 5 ng/mL and 7 ng/mL, respectively, without cytotoxicity. Also, **1** and **2** inhibited TNF-α-induced up-regulation of ICAM-1, VCAM-1 and E-selectin. The present findings suggest that **1** and **2** prevent monocyte adhesion to HUVEC through the inhibition of ICAM-1, VCAM-1 and E-selectin expression stimulated by TNF-α, and may imply their usefulness for the prevention of atherosclerosis relevant to endothelial activation.

Key words: Manassantin A/B, Cell adhesion molecules, TNF-α, Monocyte, Human umbilical vein endothelial cells, Atherosclerosis

INTRODUCTION

Accumulating evidence suggests that inflammation plays a major role in the development of atherosclerosis. It is recognized that inflammation stimulates cell adhesion molecule (CAM) expression at the site of atherosclerosis (Ross, 1999). Atherosclerotic plaques develop from complex multicellular processes in which the recruitment of circulating monocytes to focal areas of the arterial subendothelium is an early event. Initially, monocytes adhere to activated endothelium displaying inducible adhesive glycoproteins or CAMs, before migrating across the endothelial layer to the intima where they differentiate into macrophages and sequester cholesterol to form characteristic foam cells. Localized endothelial cell adhesion molecule up-regulation, a pre-requisite for monocyte migration, is a dynamic process which is sensitive to inflammatory cytokines, shear stress and oxidative insults (Imhof and Dunon, 1995). Levels of certain CAMs are elevated in human atherosclerotic tissue with vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin and P-selectin, in particular, being indicators of inflammation and early atherosclerosis (Van der Wal *et al.*, 1992).

Tumor necrosis factor-α (TNF-α) is one of the major inflammatory cytokines that mediates systemic inflammation and immune responses (Pfeffer *et al.*, 1993). A major site of action of TNF-α for these effects is the vascular endothelium (Pober and Cotran, 1990), where it induces inflammatory responses by enhancing adhesion molecule expression and secretion of inflammatory mediators (Springer, 1994; Modur *et al.*, 1996).

Manassantin A (**1**) and B (**2**), dineolignans, were previously isolated from *Saururus cernuus* (Rao and Alvarez, 1983) and are known to have a variety of biological activities, such as murine neuroleptic (Rao *et al.*, 1987), anti-plasmodial (Kraft *et al.*, 2002), anti-inflammatory (Hwang *et al.*, 2003), and human ACAT inhibitory activities (Lee *et al.*, 2004). We previously

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reported that **1** and **2** inhibited phorbol 12-myristate 13-acetate (PMA)-induced ICAM-1 expression of HL-60 cells (Rho *et al.*, 2003). In this study, therefore, we investigated that whether **1** and **2** affect to the interaction of monocyte and human umbilical vein endothelial cells (HUVEC) and TNF-α-induced expression of ICAM-1, VCAM-1, and E-selectin in HUVEC.

MATERIALS AND METHODS

Materials

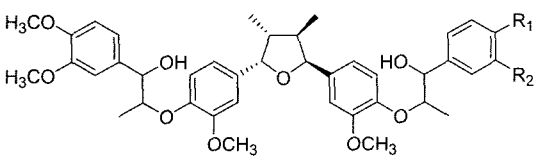
Compounds **1** and **2** were isolated from MeOH extract of *Saururus chinensis* Baill. (Saururaceae) root as reported previously (Rho *et al.*, 2003) (Fig. 1). 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM), 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), and fluorescein isothiocyanate (FITC)-conjugated antibodies were obtained from Sigma Chemical Co. (St. Louis, MO, USA). CAM monoclonal antibodies and recombinant human TNF-α were provided from R&D system (Minneapolis, MN, USA). Endothelial cell basal medium-2 (EBM-2) Bullet kit was purchased from Clonetics (San Diago, CA, USA) and all other tissue culture reagents were obtained from GIBCO-BRL (Gaithersburg, MD, USA).

Cell culture

Human acute monocytic leukemia THP-1 cells were obtained from American Type Culture Collection (ATCC; Rockville, MD, USA), cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), and maintained at a cellular density of 2×10^5 to 10^6 cells/mL, as described previously (Tsuchiya *et al.*, 1980). HUVEC were purchased from Clonetics (San Diego, CA, USA) and were grown on gelatin-coated culture dishes or 24-well plates in EBM-2, and used for the experiments within the first 3 to 4 passages. The cells were supplemented at 37°C in a humidified atmosphere of 5% CO_2.

Fluorescent labeling of THP-1 cells

THP-1 cells were fluorescently labeled with BCECF-AM for quantitative adhesion assay (Vaporciyan *et al.*, 1993). In brief, the fluorescence labeling of THP-1 cells was done by including cells (2×10^6 cells/mL) with 5 μM of BCECF-AM in RPMI-1640 medium for 30 min at 37°C and 5% CO_2. After loading of BCECF-AM, cells were washed three times with 1% FBS in phosphate buffered saline (PBS) to remove excess dye. Cells were then resuspended in EBM-2 at a density of 5×10^5 cells/mL.



1 $R_1 = R_2 = OCH_3$
2 $R_1 = R_2 = -OCH_2O-$

Fig. 1. The chemical structures of manassantin A (**1**) and B (**2**) isolated from roots of *Saururus chinensis* Baill. (Saururaceae).

Adhesion assay

HUVEC (5×10^5 cells/well) grown to confluence in a 24-well plate were pretreated with various concentrations of **1** and **2** at 37°C for 24 h and stimulated with 10 ng/mL of TNF-α for 8 h prior to the adhesion assay. BCECF-AM-labeled THP-1 cells (2.5×10^5 cells/well) were co-incubated with HUVEC for 60 min at 37°C. After incubation, non-adherent cells were removed by washing each well three times with 1% FBS in PBS. The attached cells were dissolved in 50 mM of Tris-HCl (pH 7.6) containing 0.1% sodium dodecyl sulfate (SDS). The fluorescence intensity of each well was measured using a fluorescence multi-well plate reader (Wallac 1420, Germany) at excitation and emission wave lengths of 485 and 530 nm, respectively.

Flow cytometry analysis

HUVEC (1×10^6 cells) were cultured to confluence in a culture dishes and treated with test samples at 37°C for 12 h. After incubation, 10 ng/mL of TNF-α was added and incubated for 4 h (E-selectin), 6 h (VCAM-1) and 12 h (ICAM-1), respectively. Following steps were performed at cold condition. The cells were harvested with a 1x trypsin/EDTA and wash once with PBS. After washing, cells were incubated with a blocking solution (2% FBS in PBS) for 1 hour. The cells were harvested and stained with 0.5 μg/mL anti-human ICAM-1, VCAM-1, E-selectin mAbs for 1 hour. After washing twice with PBS, primary antibody binding was detected with FITC-conjugated anti-mouse IgG (1:50 dilution in blocking solution) incubated for 1 hour in the dark condition. After washing three times with PBS, the cells were fixed (1% *p*-formaldehyde in PBS) for 30 minutes as a single-cell suspension (confirmed by phase-contrast microscopy). The fluorescence and light scattering properties (forward scatter and side scatter) of the cells were determined by using a FACScan flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA, USA). Cells with FITC-conjugated antibodies were excited with a 488 nm argon ion laser, and emission was recorded at 525 nm. In each sample, at least 20,000 gated viable cells were examined.

Measurement of cell viability

Cell viability was assessed by morphology and by

reduction of the tetrazolium salt MTT by mitochondrial dehydrogenase activity as described (Mosmann, 1983). The absorbance read at 540 nm by using a VERSAmax microplate reader (Molecular Devices, Palo Alto, CA, USA) with reference absorbance at 650 nm.

RESULTS AND DISCUSSION

Exposure of endothelial cells to inflammatory cytokines, oxidized low density lipoprotein (ox-LDL), lipopolysaccharides (LPS), and oxidative stress induces leukocyte adhesion by increasing the surface expression of the various CAMs and contributes to the formation of fatty streaks and the development of atherosclerosis (Cybulsky and Gimbrone, 1991; Bevilacqua, 1993). In our previous report, **1** and **2** inhibited PMA-induced homotypic aggregation of the HL-60 cells and PMA-induced ICAM-1 expression in HL-60 cells (Rho *et al.*, 2003). In this study, therefore, we examined whether **1** and **2** could influence adhesion of monocyte to endothelial cells stimulated by TNF-α.

Incubation of confluent HUVEC with 10 ng/mL of TNF-α for 8 h caused an almost 10-fold increase in adhesion of THP-1 monocytic cells compared with non-stimulated HUVEC. This increase in HUVEC adhesiveness was dose-dependently reduced by **1** and **2** (1 ng/mL - 1μg/mL) with IC_{50} values of 5 ng/mL and 7 ng/mL, respectively (Fig. 2). This result suggests that possibility of **1** and **2** inhibit the CAMs expression on HUVEC stimulated by TNF-α.

To determine whether the compounds modulate TNF-α-induced CAM expression, we performed that effect of these compounds on TNF-α-induced surface expression of the ICAM-1, VCAM-1 and E-selectin by flow cytometric analysis. HUVEC have a cobblestone appearance when confluent and display endothelial antigens and CAMs, some of which were up-regulated in response to cytokines. In the basal state, HUVEC expressed CD34, thrombomodulin, PECAM-1 and ICAM-1. HUVEC were mostly negative for VCAM-1 and E-selectin in the resting state, but both membrane and cytoplasmic expression increased on exposure to cytokines. ICAM-1 was constitutively expressed, but was super-induced by cytokine treatment (Stannard *et*

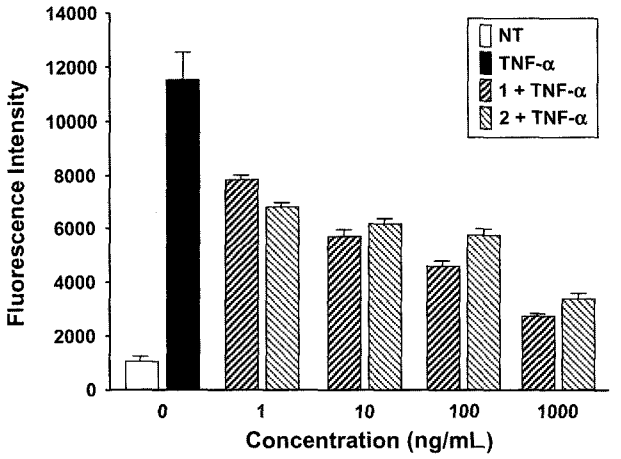


Fig. 2. Effects of **1** and **2** on the adhesion of THP-1 cells to TNF-α-stimulated human umbilical vein endothelial cells (HUVEC). Confluent HUVEC were pre-incubated with **1** and **2** for 24 h at indicated concentrations, and then stimulated with 10 ng/mL of TNF-α for 8 h. Thereafter BCECF-AM-labeled THP-1 cells were co-incubated with HUVEC for 60 min at 37°C. Fluorescence intensity was then determined as described in Materials and Methods. Values are mean ± SE (n=3).

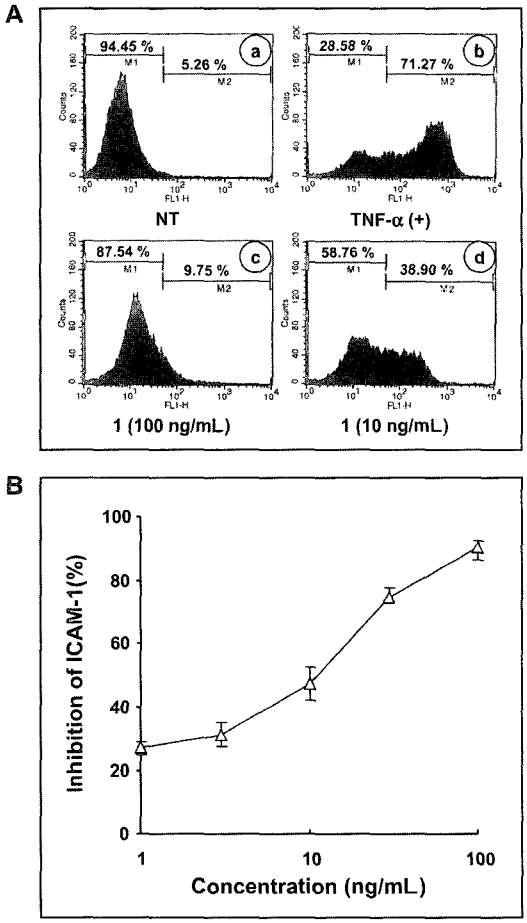


Fig. 3. Flow cytometric analysis of **1** on TNF-α-induced cell surface expression of ICAM-1 in HUVEC. (A) Data were given as histograms of cell number versus log-FITC fluorescence intensity and the histograms shown are from one representative experiment but were reproduced in other independent experiments. Values were presented to % gated (M1; marker of basal level of expression, M2; marker of TNF-α-induced expression). (B) The inhibitory effect of **1** on each CAM expression was down-regulated in a dose dependent manner. The % inhibition of ICAM-1 expression was presented by more or less of intensity on the M2 values and calculated as follows: [1 - M2 value for TNF-α plus compound treated cells M2 value for unstimulated cells - M2 value for TNF-α treated cells M2 value for unstimulated cells)] x 100 %. Values are mean ± SE (n=3).

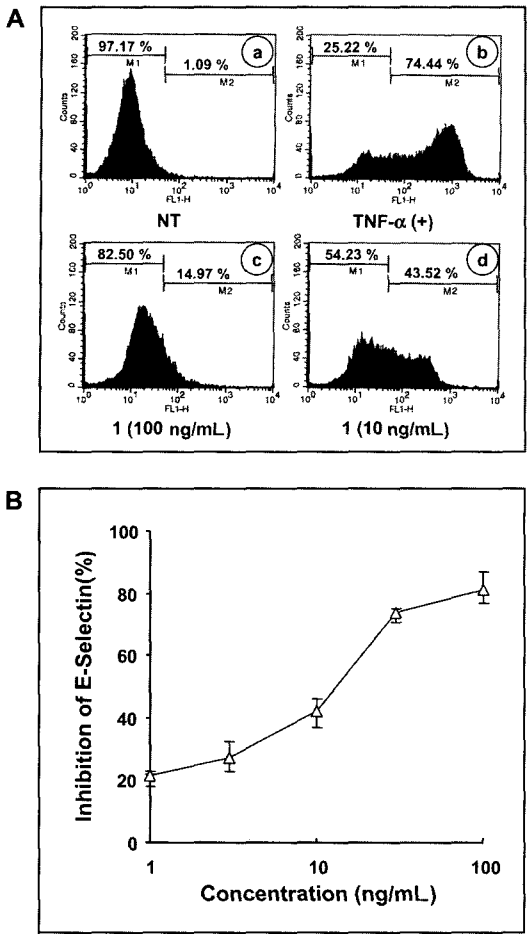


Fig. 4. Flow cytometric analysis of **1** on TNF-α-induced E-selectin expression in HUVEC. Detailed methods of flow cytometic analysis were performed as described in Fig. 3 and Materials and Methods.

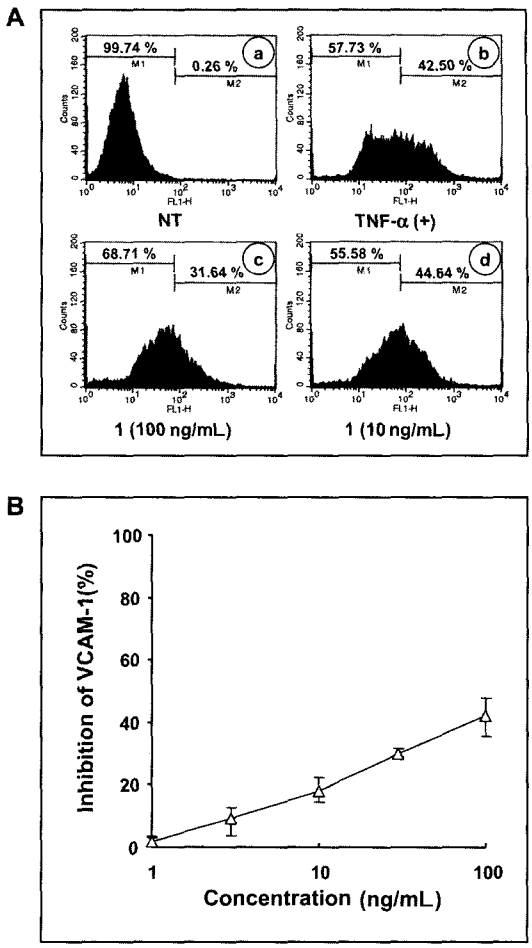


Fig. 5. Flow cytometric analysis of **1** on TNF-α-induced VCAM-1 expression in HUVEC. Detailed methods of analysis were performed as described in Fig. 3 and Materials and Methods.

al., 1998). On the vascular endothelium, TNF-α transiently increased E-selectin with a maximum level reached after 4 h of incubation. VCAM-1 was maximally induced after 6 h of incubation with TNF-α. ICAM-1 is present at low levels under normal conditions and is dramatically up-regulated by treatment with TNF-α for 5-6 h. ICAM-1 was strongly up-regulated by TNF-α with maximum levels reached after 12 h of incubation and persists for several days in cultured endothelial cells (Bevilacqua *et al.*, 1994; Zhang and Frei, 2001; Chen *et al.*, 2001). In this study, therefore, time-courses of TNF-α stimulation were decided to 4 h (E-selectin), 6 h (VCAM-1) and 12 h (ICAM-1), respectively. The FITC fluorescence profiles are shown in Fig. 3-5. Basal levels of ICAM-1 (Fig. 3A-a), E-selectin (Fig. 4A-a) and VCAM-1 (Fig. 5A-a) were negligible with the majority of cell fluorescence values under 10 units, giving a similar profile to that of isotype-treated control cells (not shown). After exposure to 10 ng/mL of TNF-α there was a clear right shift in the fluorescence peak, indicating up-regulation of cell surface CAMs (Fig. 3A-b; 4A-b; 5A-b). Pretreatment with 100 ng/mL and 10 ng/mL of **1** markedly down-regulated the fluorescence profile or mean fluorescence intensity of ICAM-1 (Fig. 3A-c, d) and E-selectin (Fig. 4A-c, d), respectively, but the inhibitions of VCAM-1 expression of **1** was less an intensity than ICAM-1 or E-selection (Fig. 5A-c, d). In addition, the inhibitory effects of **1** on each CAM expression were down-regulated in a dose dependent manner (Fig. 3B; 4B; 5B). The IC_{50} values of **1** on the ICAM-1 expression was observed at the concentration of 12 ng/mL (Fig. 3B), and the E-selectin expression were observed at 14 ng/mL (Fig. 4B). However, the effect of **1** was presented at value of $IC_{50} \geq 100$ ng/mL on VCAM-1 expression (Fig. 5B). These results suggest that **1** is more effective on ICAM-1 and E-selectin than VCAM-1 expression. In addition, **2** also presented similar activity to **1** on CAMs expression but the inhibitory effects of **2** was not effective than of **1** (data not shown).

Because cytotoxicity may have an effect on cell to cell adhesion and CAMs expression, cell viability was assessed

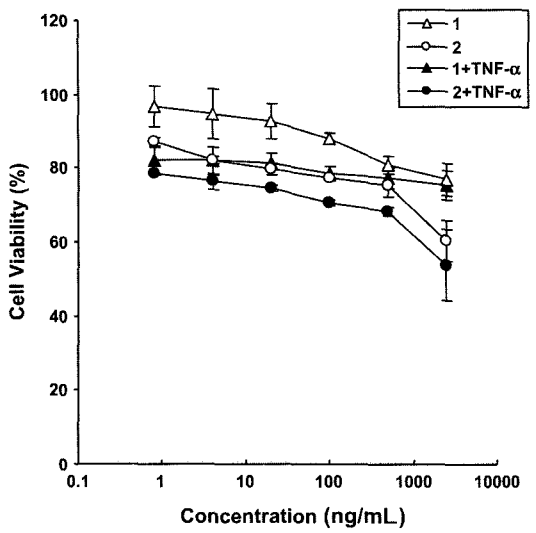


Fig. 6. Cell viability of **1** and **2** in HUVEC. Cells were incubated in the absence or presence of TNF-α with the indicated concentrations of **1** and **2** for 48 hours, and the viability was determined by the MTT-based cytotoxicity assay as described in Materials and Methods. The % viability was calculated as follows: (absorbance for compound treated cells / absorbance for untreated cells) x 100 %. Data are presented as the mean ± SE (n=3).

by the MTT assay. Treatment of HUVEC with 10 ng/mL of TNF-α for 48 h was shown to 76.7 ± 6.9% of cell viability. In the presence of 100 ng/mL of **1** or **2**, TNF-α-stimulated HUVEC viability was 78.6 ± 1.8% or 70.7 ± 0.9%, respectively. On the other hand, non-stimulated cell viability was 88.1 ± 1.7% or 77.6 ± 0.9%, respectively (Fig. 6). Therefore, the highest concentrations of **1** and **2** used in these experiments (100 ng/mL) did not affect cell viability as assessed by cell number, cellular morphology, and MTT reduction.

TNF-α has been shown to stimulate activation of a wide variety of putative second messengers. In the context of adhesion molecule expression by TNF-α, involvement of mitogen-activated protein kinases (MAPK) have been reported (Berk *et al.*, 2001). Activation of MAPK is known to lead activation of transcription factors, such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB), which may promote adhesion molecule expression and resultant atherosclerosis (Surapisitchat *et al.*, 2001). The previous report demonstrated that **1** and **2** are potent inhibitors of NF-κB activation by the suppression of transcriptional activity of RelA/p65 subunit of NF-κB, although these compounds did not prevent the DNA-binding activity of NF-κB and degradation of IκB-α (Lee *et al.*, 2003). Therefore, the present study suggest that **1** and **2** prevent TNF-α induced endothelial activation through the inhibition of ICAM-1, VCAM-1 and E-selectin expression, and may imply their usefulness for the prevention of atherosclerosis relevant to endothelial activation.

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REFERENCES

Berk, B. C., Abe, J. I., Min, W., Surapisitchat, J., and Yan, C., Endothelial atheroprotective and anti-inflammatory mechanisms. *Ann. N. Y. Acad. Sci.*, 947, 93-111 (2001).

Bevilacqua, M. P., Endothelial-leukocyte adhesion molecules. *Annu. Rev. Immunol.*, 11, 767-804 (1993).

Bevilacqua, M. P., Nelson, R. M., Mannori, G., and Cecconi, O., Endothelial-leuckocyte adhesion molecules in human disease. *Annu. Rev. Med.*, 45, 361-378 (1994).

Chen, Y. -H., Lin, S. -J., Ku, H. -H., Shiao, M. -S., Lin, F. -Y., Chen, J. -W., and Chen, Y. -L., Salvianolic acid B attenuates VCAM-1 and ICAM-1 expression in TNF-α-treated human aortic endothelial cells. *J. Cell Biochem.*, 82, 512-521 (2001).

Cybulsky, M. I., and Gimbrone, M. A. Jr., Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science*, 251, 788-791 (1991).

Hwang, B. Y., Lee, J. -H., Nam, J. B., Hong, Y. -S., and Lee, J. J., Lignans from *Saururus chinensis* inhibiting the transcription factor NF-κB. *Phytochemistry*, 64, 765-771 (2003).

Imhof, B. A. and Dunon, D., Leukocyte migration and adhesion. *Adv. Immunol.*, 58, 345-416 (1995).

Kraft, C., Jenett-Siems, K., Köhler, I., Tofern-Reblin, B., Siems, K., Bienzle, U., and Eich, E., Antiplasmodial activity of sesquilignans and sesquineolignans from *Bonamia spectabilis*. *Phytochemistry*, 60, 167-173 (2002).

Lee, J. -H., Hwang, B. Y., Kim, K. -S., Nam, J. B., Hong, Y. S., and Lee, J. J., Suppression of RelA/p65 transactivation activity by a lignoid manassantin isolated from *Saururus chinensis*. *Biochem. Pharmacol.*, 66, 1925-1933 (2003).

Lee, W. S., Lee, D. -W., Baek, Y. -I., An, S. -J., Cho, K.- H., Choi, Y. -K., Kim, H. -C., Park, H. -Y., Bae, K. -H., and Jeong, T. -S., Human ACAT-1 and -2 inhibitory activities of saucerneol B, manassantin A and B isolated from *Saururus chinensis*. *Bioorg. Med. Chem. Lett.*, 14, 3109-3112 (2004).

Modur, V., Zimmerman, G. A., Prescott, S. M., and McIntyre, T. M., Endothelial cell inflammatory responses to tumor necrosis factor-α. Ceramide-dependent and -independent mitogen-activated protein kinase cascades. *J. Biol. Chem.*, 271, 13094-13102 (1996).

Mosmann, T., Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, 65, 55-63 (1983).

Pfeffer, K., Matsuyama, T., Kundig, T. M., Wakeham, A., Kishihara, K., Shahinian, A., Wiegmann, K., Ohashi, P. S.,

Kronke, M., and Mak, T. W., Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection. *Cell,* 73, 457-467 (1993).

Pober, J. S. and Cotran, R. S., Cytokines and endothelial cell biology. *Physiol. Rev.*, 70, 427-451 (1990).

Rao, K. V. and Alvarez, F. M., Manassantins A/B and saucerneol: Novel biologically active lignoids from *Saururus cernuus*. *Tetrahedron Lett.*, 24, 4947-4950 (1983).

Rao, K. V., Puri, V. N., Diwan, P. K., and Alvarez, F. M., Preliminary evaluation of manassantin A, a potential neuroleptic agent from *Saururus cernuus*. *Pharmacol. Res. Commun.*, 19, 629-638 (1987).

Rho, M. C., Kwon, O. E., Kim, K., Lee, S. W., Chung, M. Y., Kim, Y. H., Hayashi, M., Lee, H. S., and Kim, Y. K., Inhibitory effects of manassantin A and B isolated from the roots of *Saururus chinensis* on PMA-induced ICAM-1 expression. *Planta Med.*, 69, 1147-1149 (2003).

Ross, R., Atherosclerosis - An inflammatory disease. *N. Engl. J. Med.*, 340, 115-126 (1999).

Springer, T. A., Traffic signals for lymphocyte recirculation and leukocyte emigration: The multistep paradigm. *Cell*, 76, 301-314 (1994).

Stannard, A. K., Riddell, D. R., Bradley, N. J., Hassall, D. G., Graham, A., and Owen, J. S., Apolipoprotein E and regulation of cytokine-induced cell adhesion molecule expression in endothelial cells. *Atherosclerosis*, 139, 57-64 (1998).

Surapisitchat, J., Hoefen, R. J., Pi, X., Yoshizumi, M., Yan, C., and Berk, B. C., Fluid shear stress inhibits TNF-α activation of JNK but not ERK1/2 or p38 in human umbilical vein endothelial cells: inhibitory crosstalk among MAPK family members. *Proc. Natl. Acad. Sci. U.S.A.,* 98, 6476-6481 (2001).

Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., and Tada, K., Establishment and characterization of a human acute leukemia cell line (THP-1). *Int. J. Cancer*, 26, 171-176 (1980).

Van der Wal, A. C., Das, P. K., Tigges, A. J., and Becker, A. E., Adhesion molecules on the endothelium and mononuclear cells in human atherosclerotic lesions. *Am. J. Pathol.*, 141, 1427-1433 (1992).

Vaporciyan, A. A., Jones, M. L., and Ward, P. A., Rapid analysis of leukocyte-endothelial adhesion. *J. Immunol. Methods*, 159, 93-100 (1993).

Zhang, W. J. and Feri, B., α-Lipoic acid inhibits TNF-α-induced NF-κB activation and adhesion molecule expression in human aortic endothelial cells. *FASEB J.*, 15, 2423-2432 (2001).