Antiangiogenic Activity of Nasunin, an Antioxidant Anthocyanin, in Eggplant Peels

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Nasunin, delphinidin-3-(*p*-coumaroylrutinoside)-5-glucoside, an antioxidant anthocyanin isolated from eggplant peels, was demonstrated as an angiogenesis inhibitor. Nasunin at higher 10 μM suppressed microvessel outgrowth in an ex vivo angiogenesis assay using a rat aortic ring. The effect of nasunin was examined in various in vitro angiogenesis models using human umbilical vein endothelial cells (HUVECs). Nasunin suppressed HUVEC proliferation in a dose-dependent manner (50–200 μM); however, it had no significant effect on HUVEC chemotaxis in a Boyden chamber assay and HUVEC tube formation on a reconstituted basement membrane. These results imply that nasunin with both antioxidant and antiangiogenic activities might be useful to prevent angiogenesis-related diseases.

KEYWORDS: Angiogenesis; aortic ring; endothelial cells; nasunin

INTRODUCTION

Food-derived polyphenolic compounds have been believed to be beneficial for human health due to their antioxidant and anticarcinogenic activities, and as the mechanisms, it has been demonstrated that some of them modulate oncogenes, tumor suppression genes, cell cycle, apoptosis, angiogenesis, and signal transduction pathways (*1*). The antiangiogenic activity of food-derived polyphenols has received an increased attention as potent inhibitors of tumor growth (*2*). Angiogenesis is a formation of new blood vessels required for tumor growth and metastasis and is involved in other diseases, e.g., atherosclerosis and diabetic retinopathy. Therefore, numerous efforts have been conducted on antiangiogenic agents (*3*).

We have revealed that natural products, vitamin B$_6$ and algal polysaccharide, have antiangiogenic activities (*4*, *5*). Furthermore, we have found that petasiphenol and quercetin derivatives suppress angiogenesis (*6*, *7*). Antiangiogenic polyphenols are mainly isoflavones, flavonoids, flavones, flavanones, and catechins (*2*). The number of reports on antiangiogenic activity of anthocyanins is limited (*8*, *9*). It has been suggested that the antioxidative effect of polyphenols is linked to their antiangiogenic activity (*2*). This led us to investigate the antiangiogenic activity of antioxidative polyphenols. Nasunin is the major component of the anthocyanin pigment of eggplant and has antioxidant activity (*10*, *11*); however, it remained unknown
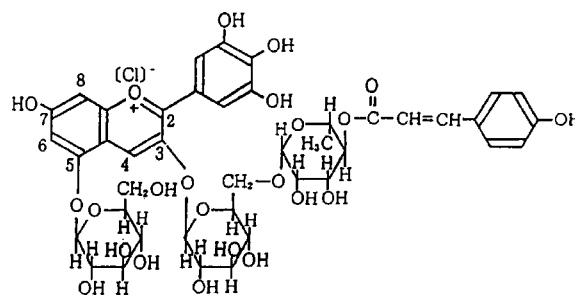
Figure 1. Chemical structure of nasunin, delphinidin-3-(*p*-coumaroylruti-noside)-5-glucoside.

whether it affected angiogenesis. This study was carried out to clarify the effect of nasunin on angiogenesis.

MATERIALS AND METHODS

Materials. Nasunin, delphinidin-3-(*p*-coumaroylrutinoside)-5-glucoside, was isolated as described previously (*12*). The chemical structure is shown in **Figure 1**. Human recombinant vascular endothelial growth factor (VEGF) was obtained from R&D systems (MN). WST-1 reagent was from Dojindo Laboratories (Kumamoto, Japan). Other reagents were special grade as commercially available.

Ex Vivo Angiogenesis Assay. Male Wistar rats (6 weeks old, Clea Japan, Inc., Tokyo, Japan) were housed two to a metal cage in a room with controlled temperature (24 ± 1°C) and a 12 h light:dark cycle (lights on, 08:00–20:00 h). They had free access to diets and deionized water. The rats were maintained according to the Guide for the Care and Use of Laboratory Animals established by Okayama Prefectural University. The ex vivo angiogenesis assay was performed according to slightly modified methods as described before (*13*, *14*). Briefly, a male Wistar rat (body weight ∼ 200 g) was sacrificed by bleeding

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from the right femoral artery under anesthesia with diethyl ether. A thoracic aorta was removed and washed with RPMI 1640 medium to avoid contamination with blood. It was then turned inside out and cut into short segments about 1–1.5 mm. Collagen gel (gel matrix solution) was made with 8 volumes of porcine tendon collagen solution (3 mg/mL) (Cellmatrix Ia, Nitta Gelatin Co., Osaka, Japan), 1 volume of 10 × Eagle's MEM (Gibco, New York), and 1 volume of reconstitution buffer (80 mM NaOH and 200 mM HEPES). These solutions were mixed gently at 4 °C. Each aortic segment was placed in the center of a well on a six well culture plate and covered with 0.5 mL of gel matrix solution reconstituted as described. The solution was allowed to gel at 37 °C for 20 min and then overlaid with 2 mL of RPMI 1640 medium (Gibco) containing 1% of ITS+ (Becton Dickinson Labware, MA). Then, the sample solution or vehicle was added. Incubation was carried out for 10 days in a fully humidified system of 5% CO_2 in the air at 37 °C. The medium was changed on day 7 of the culture. An estimation of the length of the capillary was performed under phase contrast microscopy by measuring the distance from the cut end of the aortic segment to the approximate mean point of capillary. Microscopic fields were photographed with a digital camera (Nikon, COOLPICKS 950). The length of the capillary was measured using Adobe Photoshop software. Each reported value represents the average of three culture samples.

Endothelial Cells. Human umbilical vein endothelial cells (HU-VECs) were purchased from Kurabo Industries (Osaka, Japan). Cells were grown in the medium, HuMedia EG 2 (Kurabo Industries), which was modified MCDB 131 medium containing 2% fetal bovine serum, 10 ng/mL recombinant human epidermal growth factor, 1 μg/mL hydrocortisone, 50 μg/mL gentamicin, 50 ng/mL amphotericin B, 5 ng/mL recombinant human basic fibroblast growth factor, and 10 μg/mL heparin, at 37 °C in a humidified 5% CO_2. Subcultures were obtained by treating the HUVEC cultures with 0.025% trypsin–0.01% EDTA solution. HUVECs at passages of 3–5 were used in this experiment.

HUVEC Proliferation Assay. For the HUVEC proliferation assay, HUVECs were dispersed with trypsin and suspended in HuMedia EG2 medium. A cell suspension (15000 cells/mL) was plated onto 96 well culture plates (100 μL/well) and incubated at 37 °C in a humidified 5% CO_2 for 24 h. The medium was replaced with fresh HuMedia EG2 containing nasunin (0–200 μM). After 72 h, 10 μL of WST-1 reagent was added into each well of a 96 well plate and incubated for 4 h at 37 °C. The absorbance at 450 nm was measured using a microplate spectrophotometer.

HUVEC Tube Formation Assay. The tube formation assay was performed using an In Vitro Angiogenesis Assay Kit (Chemicon International, Inc., Temecula, CA). Briefly, solid gels were prepared according to the manufacturer's manual on a 96 well tissue culture plate. HUVECs (1×10^5 cells/mL) in HuMedia EG-2 medium containing 0–200 μM nasunin were seeded 100 μL per well onto the surface of the solid gel, ECMatrix. The cells were incubated for 12 h at 37 °C in a CO_2 incubator. Tube formation was observed under an inverted light microscope at 40× magnification. Microscopic fields were photographed with a digital camera (Nikon, COOLPICKS 950). The total length of tube structures in each photograph was measured using Adobe Photoshop software. Each reported value represents the average of three samples.

HUVEC Chemotaxis Assay. This was carried out by a modified Boyden chamber assay (*15*). The microporous membrane (8 μm) of 24 well cell culture inserts (BD Biosciences, MA) was coated with 0.1% gelatin. HUVECs were detached with cell dissociation buffer (Invitrogen Corp., Carlsbad, CA), collected by centrifugation, resuspended in Medium 199 (Invitrogen) with 0.1% bovine serum albumin (BSA), and seeded in triplicate in the chamber (1.0×10^5 cells/400 μL). The well was filled with 400 μL of Medium 199 containing 0.1% BSA and 10 ng/mL of VEGF with or without nasunin. The assembled chamber was incubated for 6 h at 37 °C in a humidified 5% CO_2. Nonmigrated cells on the upper surface of the membrane were removed by scrubbing with a cotton swab. The cells on the lower surface of the membrane were fixed with methanol and stained with Diff-Quik stain. Migrated cells were counted in five fields of each membrane under
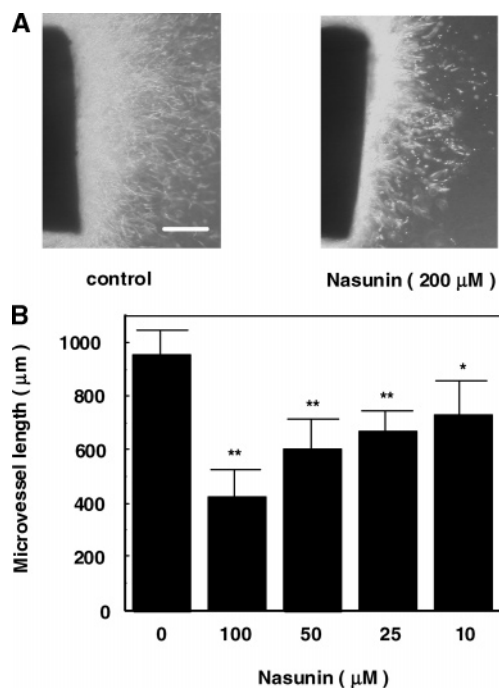
Figure 2. (**A**) Representative result of the inhibitory effect of nasunin (200 μM) on ex vivo angiogenesis using a rat aortic ring. The bar equals 500 μm. (**B**) The microvessel length was measured on day 7 of culture. Values are means ± SD ($n = 3$). Nasunin at 10 μM or more significantly inhibited microvessel growth (*$p < 0.05$ and **$p < 0.01$).

the microscope at 200× magnification, and the average number of a field was calculated. The experiments were performed in triplicate.

Statistical Analysis. Values are presented as means ± SD. Data were analyzed by one-way analysis of variance. Differences with $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Ex Vivo Angiogenesis Assay. The effect of nasunin on angiogenesis was examined in a rat aortic ring angiogenesis model. This method is widely used as a useful one to evaluate antiangiogenic agents in a complex system in which endothelial cells, fibroblasts, pericytes, and smooth muscle cells interact (*16–18*). Fibroblastic fusiform cells migrated from the ends of the aortic rings after 2–3 days, and then, they spread in the collagen gel. Microvessels appeared from the ends of aortic rings after 5–6 days and elongated (**Figure 2A**). Nasunin strongly suppressed the outgrowth of microvessels at 200 μM, and a significant inhibitory effect was observed higher at 10 μM (**Figure 2B**).

Effect of Nasunin on HUVEC Functions. The effect of nasunin on HUVEC tube formation was examined. HUVEC tube formation assay is an in vitro angiogenesis model. HUVECs on reconstituted basement membrane migrated, attached each other, and formed tube structures (**Figure 3**). Nasunin had no effect on HUVEC tube formation even at 200 μM, at which it suppressed microvessel outgrowth in a rat aortic ring assay. This result suggests that nasunin does not affect HUVEC migration and the signal transdaction pathways relating tube formation.

Next, we examined the effect of nasunin on HUVEC migration on gelatin-coated Boyden chambers (**Figure 4**). VEGF strongly stimulated HUVEC migration as shown in **Figure 4**. In the presence of 200 μM nasunin, HUVEC migration was moderately suppressed, but the effect was not statistically significant. This result was consistent with the observation on HUVEC tube formation assay.

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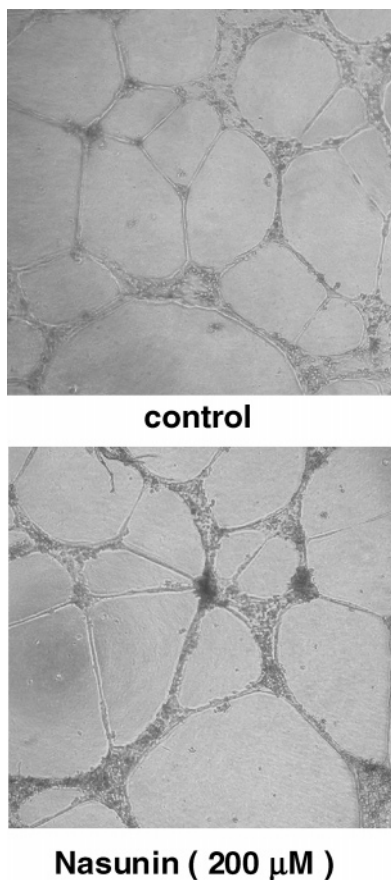
Figure 3. Effect of nasunin on HUVEC tube formation on reconstituted basement membrane gel. Cells were plated on reconstituted gel and observed 12 h later. The representative result of nasunin was shown. Nasunin had no effect on HUVEC tube formation.
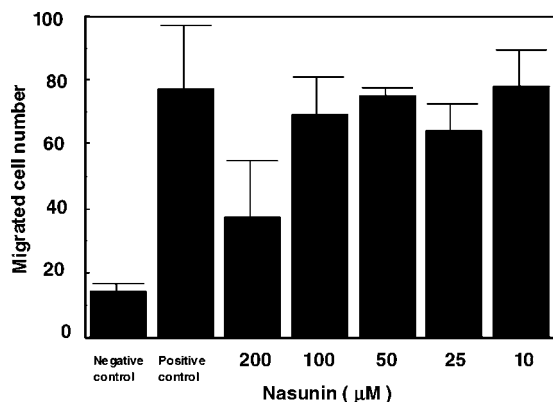
Figure 4. Effect of nasunin on HUVEC chemotaxis. HUVECs that migrated after 6 h of incubation to lower surface of the filter were counted in five 200× fields of a filter. Means of a field of three filters ± SD ($n = 3$) are shown. Negative control, medium without VEGF and nasunin. Positive control, VEGF containing medium without nasunin. No significant inhibitory effect on HUVEC chemotaxis was observed.

Many angiogenesis inhibitors suppress endothelial cell proliferation in vitro. Nasunin was applied to HUVEC proliferation assay. HUVECs were treated for 72 h with various concentrations of nasunin, and the proliferation rate was measured by a colorimetric method. Nasunin inhibited HUVEC proliferation in a dose-dependent manner, and significant differences ($p <$ 0.01) are found between control and nasunin-treated HUVECs (50−200 μM) (**Figure 5**). Taken together, nasunin, an antioxi-
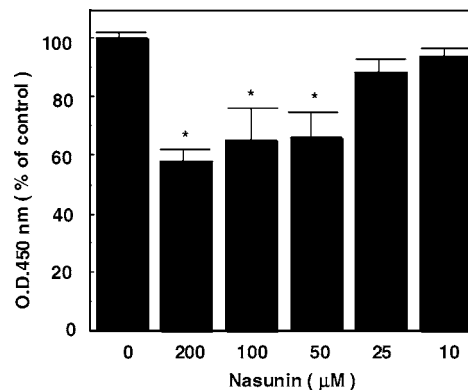
Figure 5. Effect of nasunin on HUVEC proliferation. Means ($n = 3$) with an asterisk (*) are significantly different ($p < 0.01$) from control.

dant anthocyanin, exerted the antiangiogenic activity through inhibiting endothelial cell proliferation.

In the present study, we revealed the antiangiogenic activity of nasunin, an antioxidant anthocyanin in eggplant. Nasunin inhibited microvessel outgrowth in a rat aortic ring assay. We demonstrated that nasunin exerted the antiangiogenic activity through suppressing endothelial cell proliferation; however, it did not affect HUVEC tube formation and migration significantly. Many antiangiogenic plant polyphenols suppress endothelial cell proliferation (*2*). The mechanisms by which antiangiogenic polyphenols suppress endothelial cell proliferation are very complex and not fully understood yet. Antiangiogenic polyphenols affect receptor phosphorylation, protein kinase C, tyrosine kinase, and phosphoinositide 3-kinase (*19*, *20*). Cell cycle and matrix metalloproteinases coupled with angiogenesis are also their target (*21*, *22*). The antioxidant activity of plant polyphenols would be involved in the antiangiogenic effect, since reactive oxygen species stimulate angiogenesis (*23*, *24*). Nasunin is a derivative of delphinidin, which has an antioxidant activity (*10−12*). The antiangiogenic activity of delphinidin has been demonstrated recently (*25*). Delphinidin suppresses endothelial cell migration and proliferation at more than 5 μg/mL (about 16 μM). Thus, the antiangiogenic effect of delphinidin is stronger than nasunin. The inhibitory effect of delphinidin on H_2O_2-induced lipid peroxidation is about 2-fold stronger than nasunin (*10*, *26*). These observations suggest that the difference of antioxidant activity would be related to antiangiogenic activity. However, it remains unknown that the different effects of nasunin and delphinidin on endothelial cells are due to structural differences or antioxidant activities. The molecular mechanisms and the structure−activity relationship should be addressed in the future study.

Polyphenols are ubiquitous in plants, and we consume them in food daily. Physiological roles of polyphenols in food remain unknown; however, consumption of polyphenols would be beneficial for human health (*1*). It has been believed that the antioxidant activity of polyphenols is beneficial for preventing against oxidative stress. Recent evidence shows that such food-derived polyphenols have pharmacological effects. Antiangiogenic activity is one of the attractive biological activities of polyphenols, since angiogenesis inhibitors have potency for preventing various diseases including cancer, atherosclerosis, and diabetic retinopathy (*3*). Although many antiangiogenic polyphenols have been reported (*2*), antiangiogenic anthocyanins are very few (*8*, *9*, *25*). In the present study, the antiangiogenic activity of nasunin, an antioxidant anthocyanin, was revealed. Our finding suggests that nasunin would be expected as a useful

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anthocyanin against oxidation stress and diseases accompanied with abnormal angiogenesis.

LITERATURE CITED

(1) Yang, C. S.; Landau, J. M.; Huang, M. T.; Newmark, H. L. Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annu. Rev. Nutr.* **2001**, *21*, 381−406.

(2) Cao, Y.; Cao, R.; Bråkenhielm, E. Antiangiogenic mechanisms of diet-derived polyphenols. *J. Nutr. Biochem.* **2002**, *13*, 380−390.

(3) Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.* **1995**, *1*, 27−31.

(4) Matsubara, K.; Mori, M.; Matsuura, Y.; Kato, N. Pyridoxal 5′-phosphate and pyridoxal inhibit angiogenesis in the serum-free rat aortic ring assay. *Int. J. Mol. Med.* **2001**, *8*, 505−508.

(5) Matsubara, K.; Mori, M.; Matsumoto, H.; Hori, K.; Miyazawa, K. Antiangiogenic properties of a sulfated galactan isolated from a marine alga, *Codium cylindricum. J. Appl. Phycol.* **2003**, *15*, 87−90.

(6) Matsubara, K.; Mori, M.; Mizushina, Y. Petasiphenol which inhibits DNA polymerase λ is an inhibitor of in vitro angiogenesis. *Oncol. Rep.* **2004**, *11*, 447−451.

(7) Matsubara, K.; Ishihara, K.; Mizushina, Y.; Mori, M.; Nakajima, N. Anti-angiogenic activity of quercetin and its derivatives. *Lett. Drug Des. Discovery* **2004**, *1*, 329−333.

(8) Roy, S.; Khanna, S.; Alessio, H. M.; Vider, J.; Bagchi, D.; Bagchi, M.; Sen, C. K. Anti-angiogenic property of edible berries. *Free Radical Res.* **2002**, *36*, 1023−1031.

(9) Bagchi, D.; Sen, C. K.; Bagchi, M.; Atalay, M. Anti-angiogenic, antioxidant, and anti-carcinogenic properties of a novel anthocyanin-rich berry extract formula. *Biochemistry* (*Moscow*) **2004**, *69*, 75−80.

(10) Noda, Y.; Kaneyuki, T.; Igarashi, K.; Mori, A.; Packer, L. Antioxidant activity of nasunin, an anthocyanin in eggplant. *Res. Commun. Mol. Pathol. Pharmacol.* **1998**, *102*, 175−187.

(11) Noda, Y.; Kaneyuki, T.; Igarashi, K.; Mori, A.; Packer, L. Antioxidant activity of nasunin, an anthocyanin in eggplant peels. *Toxicology* **2000**, *148*, 119−123.

(12) Igarashi, K.; Yoshida, T.; Suzuki, E. Antioxidative activity of nasunin in Chouja-nasu (Little eggplant, *Solanum melongena* L. 'Chouja'). *Nippon Shokuhin Kogyo Gakkaishi* **1993**, *40*, 138−143.

(13) Mori, M.; Sadahira, Y.; Kawasaki, S.; Hayashi, T.; Notohara, K.; Awai, M. Capillary growth from reversed rat aortic segments cultured in collagen gel. *Acta Pathol. Jpn.* **1988**, *38*, 1503−1512.

(14) Kawasaki, S.; Mori, M.; Awai, M. Capillary growth of rat aortic segments cultured in collagen gel without serum. *Acta Pathol. Jpn.* **1989**, *39*, 712−718.

(15) Kim, K. S.; Hong, Y. K.; Joe, Y. A.; Lee, Y.; Shin, J. Y.; Park, H. E.; Lee, I. H.; Lee, S. Y.; Kang, D. K.; Chang, S. I.; Chung, S. I. Anti-angiogenic activity of the recombinant kringle domain of urokinase and its specific entry into endothelial cells. *J. Biol. Chem.* **2003**, *278*, 11449−11456.

(16) Nicosia, R. F.; Ottinetti, A. Growth of microvessels in serum-free matrix culture of rat aorta. *Lab. Invest.* **1990**, *63*, 115−122.

(17) Kruger, E. A.; Duray, P. H.; Tsokos, M. G.; Venzon, D. J.; Libutti, S. K.; Dixon, S. C.; Rudek, M. A.; Pluda, J.; Allerga, C.; Figg, W. D. Endostatin inhibits microvessel formation in the ex vivo rat aortic ring angiogenesis assay. *Biochem. Biophys. Res. Commun.* **2000**, *268*, 183−191.

(18) Zogakis, T. G.; Costouros, N. G.; Kruger, E. A.; Forbes, S.; He, M.; Qian, M.; Feldman, A. L.; Figg, W. D.; Alexander, H. R.; Liu, E. T.; Kohn, E. C.; Libutti, S. K. Microarray gene expression profiling of angiogenesis inhibitors using the rat aortic ring assay. *Biotechniques* **2002**, *33*, 664−666, 668, 670.

(19) Peterson, G. Evaluation of the biochemical targets of genistein in tumor cells. *J. Nutr.* **1995**, *125*, 784S−789S.

(20) Stewart, J. R.; Ward, N. E.; Ioannides, C. G.; O'Brian, C. A. Resveratrol preferentially inhibits protein kinase C-catalyzed phosphorylation of a cofactor-independent, arginine-rich protein substrate by a novel mechanism. *Biochemistry* **1999**, *38*, 13244−13251.

(21) Fotsis, T.; Pepper, M. S.; Aktas, E.; Breit, S.; Rasku, S.; Adlercreutz, H.; Wahala, K.; Montesano, R.; Schweigerer, L. Flavonoids, dietary-derived inhibitors of cell proliferation and in vitro angiogenesis. *Cancer Res.* **1997**, *57*, 2916−2921.

(22) Garbisa, S.; Biggin, S.; Cavallarin, N.; Sartor, L.; Benelli, R.; Albini, A. Tumor invasion: Molecular shears blunted by green tea. *Nat. Med.* **1999**, *5*, 1216.

(23) Shono, T.; Ono, M.; Izumi, H.; Jimi, S. I.; Matsushima, K.; Okamoto, T.; Kohno, K.; Kuwano, M. Involvement of the transcription factor NF-kappaB in tubular morphogenesis of human microvascular endothelial cells by oxidative stress. *Mol. Cell. Biol.* **1996**, *16*, 4231−4239.

(24) Yasuda, M.; Ohzeki, Y.; Shimizu, S.; Naito, S.; Ohtsuru, A.; Yamamoto, T.; Kuroiwa, Y. Stimulation of in vitro angiogenesis by hydrogen peroxide and the relation with ETS-1 in endothelial cells. *Life Sci.* **1999**, *64*, 249−258, 1999.

(25) Favot, L.; Martin, S.; Keravis, T.; Andriantsitohaina, R.; Lugnier, C. Involvement of cyclin-dependent pathway in the inhibitory effect of delphinidin on angiogenesis. *Cardiovasc. Res.* **2003**, *59*, 479−487.

(26) Noda, Y.; Kaneyuki, T.; Mori, A.; Packer, L. Antioxidant activities of pomegranate fruit extract and its anthocyanidins: Delphinidin, cyanidin, and pelargonidin. *J. Agric. Food Chem.* **2002**, *50*, 166−171.