J. Appl. Biol. Chem. **50**(4), 249-253 (2007)

Article

Inhibitory Activity of Edible Plant Extracts on Proliferation of Human Umbilical Vein Endothelial Cells (HUVECs)†

Myoung-Chong Song[1], Sung-Hoon Kim[2], Ho-Young Kwak[1], Hye-Joung Yang[1], Myun-Ho Bang[1], In-Sik Chung[1], Youn-Hyung Lee[3] and Nam-In Baek[1,*]

[1]*Graduate School of Biotechnology & Plant Metabolism Research Center, Kyung Hee University, Suwon 446-701, Korea*

[2]*Graduate School of East-West Medicinal Science, Kyung Hee University, Seoul 130-701, Korea*

[3]*Department of Horticultural Biotechnology, Kyung Hee University, Suwon 446-701, Korea*

Received September 6, 2007; Accepted October 24, 2007

Thirteen edible plants previously reported to show inhibitory activities on farnesyl protein transferase (FPTase) and phosphatase of the regenerating liver-3 (PRL-3) were evaluated for inhibitory activity on the proliferation of human umbilical vein endothelial cells (HUVECs). Four plant extracts, *Oenothera erythrosepala*, *Perilla frutescens*, *Panicum miliaceum*, and *Quercus acutissima*, significantly inhibited the proliferation of HUVECs induced by the basic fibroblast growth factor (bFGF) without cytotoxicity at 100 μg/mL. *Myristica fragrans*, *Rosmarinus officinalis*, and *Syringa patula* also showed inhibitory activity on the proliferation with only mild cytotoxicity.

Key words: *angiogenesis, cell proliferation, cytotoxicity, edible plant, human umbilical vein endothelial cells*

There is a Korean maxim known as 'Sig-Bo', which means consumption of food invigorates the human body by providing nutrition as well as medicinal effects [Heo, 2005]. Many vegetables growing in the fields and mountains have been used as folk medicines and clinical drugs. Thus, it is very important to investigate the available pharmaceutical sources from edible plants due to their proven safety and abundance.

In recent years, cancer has become the second leading cause of death in most countries [Kochanek *et al.*, 2004]; hence, there is a need to investigate novel and effective treatments [Weiss, 2000]. Farnesyl protein transferase (FPTase) and phosphatase from the regenerating liver-3 (PRL-3) are the key enzymes involved in the development of cancer cells. Therefore, finding the compounds with inhibitory activities on the enzymes is an effective method for the investigation of anti-cancer and tumor drugs. Focus has been placed on angiogenesis as another means of reducing the occurances of cancer and tumor. Angiogenesis is a process by which new blood vessels are formed from the pre-existing vessels, for example the normal physiological function of processing and wound healing by menstruation. [Risau, 1997; Carmeliet, 2000; Carmeliet and Jain, 2000]. The induction process of angiogenesis is characterized by the degradation of the vascular basement membrane, endothelial cell migration, proliferation, and tube formation. Angiogenesis is associated with diseases such as solid tumors, diabetes, rheumatoid arthritis, and atherosclerosis. In particular, the growths of solid tumors and metastasis were found to depend on the tumor angiogenesis [Hanaha and Folkman, 1996].

In a previous experiment, the authors evaluated 163 edible plant extracts for the inhibitory activity against FPTase and PRL-3, and found 13 methanol extracts exhibiting high inhibitory activities. Should any of these selected active extracts show anti-angiogenesis activities, they could be used as potential sources for the development of anticancer drugs. Angiogenesis is a process involving, among others, blood vessel formation, cell proliferation, cell metastasis, cell migration, and tube formation. In this study, the inhibitory effect of the identified extracts on the cell proliferation was carried out using HUVECs. Finding some active extracts will lead to further works to evaluate other anti-angiogenesis activities through cell migration, cell metastasis, tube formation, and chick chorioallantoic membrane (CAM) assays.

†Development of Biological Active Compounds from Edible Plant Sources - XXIII

*Corresponding author
Phone: +82-31-201-2661; Fax: +82-31-201-2157
E-mail: nibaek@khu.ac.kr

Materials and Methods

Plant materials. One hundred and sixty-three edible plants, permitted as food by the Korea Food & Drug Administration, were purchased from agricultural and marine products markets located in Suwon, Korea in 2005. The plants were taxonomically identified with respect to the morphology by Prof. Dae-Keun Kim, College of Pharmacy, Woosuk University, Jeonju, Korea. Voucher specimens (KHU0501001æKHU0501163) were deposited in the Laboratory of Natural Products Chemistry, Graduate School of Biotechnology & Plant Metabolism Research Center, Kyung Hee University, Suwon, Korea.

General chemicals and instruments. First grade methanol (MeOH) was purchased from Daejung Chemicals (Seoul, Korea). The optical density was measured using a microplate reader (Molecular Devices Co., Sunnyvale, CA, USA).

Solvent extraction of the plants. The dry ingredients (50 g) including grains, nuts, and seeds were ground and extracted with 80% MeOH (0.5 L × 2) for 24 h at room temperature. The wet ingredients (500 g) such as green algae, flesh of fruits, leaves, mushrooms, roots, and stems were cut and extracted with 100% MeOH (1 L × 2) for 24 h at room temperature. The filtrates were concentrated *in vacuo* at 40°C to render the MeOH extracts.

Cell culture of HUVECs. HUVECs were isolated from fresh human umbilical cord veins using the collagenase treatment as described previously. The cells were cultured in M199 (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS), 3 ng/mL basic fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MN), 5 units/mL heparin, and 100 units/mL antibiotic-antimycotic in 0.1% gelatin-coated flasks. The cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 [Huh *et al.*, 2005; Lee *et al.*, 2006; Lee *et al.*, 2006].

Cytotoxicity assay in the absence of growth factors. The HUVECs were seeded in 100 μL of their respective complete media on the 96-well culture plates. After preincubation for 48 h, the medium was replaced with the endothelial-serum free medium, which contained various concentrations of the 13 edible plant extracts, without the growth factors for the HUVECs. The cultures were incubated for 24 h (HUVECs), and the metabolically viable cell fractions were determined by the 2,3-bis[l2-methoxy-4-nitro-5-sulfo]-2*H*-tetrazolium-5-carboxanilide (XTT) colorimetric method [Jost *et al.*, 1992], which measures the ability of the mitochondria to metabolize the redox-sensitive dye [Roehm *et al.*, 1991].

Proliferation assay. Proliferation of the cells was examined by a cell proliferation assay using XTT as previously described [Jost *et al.*, 1992]. The HUVECs (5×10^3) were seeded into the 0.1% gelatin-coated 96-well and incubated in a humidified incubator for 24 h. The cells were starved for 6 h in M199 containing 5% heat-inactivated FBS and treated with various concentrations of MeOH extracts in M199 containing 5% heated-inactivated FBS, 5 ng/mL bFGF, and 5 unit/mL hepalin. After 48 h incubation, XTT working solution was added, and the optical density was measured using a microplate reader (Molecular Devices Co.) at 450 nm. The cell proliferation was calculated as a percentage compared with the bFGF only treated-HUVECs.

Results and Discussion

For the development of useful anti-cancer and anti-tumor agents, 163 kinds of edible plant extracts were previously examined for their inhibitory activities against FPTase and PRL-3 [Kwak *et al.*, 2007]. Results showed 13 and 23 of the 163 MeOH extracts exhibited higher than 50% inhibition on FPTase and PRL-3, respectively. The 13 plants showing inhibitory activities on both FPTase and PRL-3, *Carya illinoensis*, *Chlorella vulgaris*, *Eugenia caryophyllata*, *Gossypium indicum*, *Juglans regia*, *Myristica fragrans*, *Oenothera erythrosepala*, *Panicum miliaceum*, *Perilla frutescens*, *Pinus densiflora*, *Rosmarinus officinalis*, *Rubus coreanus*, and *Quercus acutissima*, were examined for their angiogenic effects *in vitro* using HUVECs.

Seven plant extracts inhibited HUVECs proliferation. To define an exposure range of MeOH extracts without acute cytotoxicity to the quiescent endothelial cells, we measured HUVEC viability by XTT assay after 24 h exposure in the serum-free medium lacking the angiogenic factor supplements. The serum-free condition was used to more sensitively reveal the unwanted cytotoxic effects. *Q. acutissima* and *M. fragrans* showed the viabilities of 65 and 64%, respectively, compared to the reference treatment at the concentration of 50 μg/mL. *R. officinalis* and *S. patula* showed 48 and 68% at 200 mg/mL, respectively. However, it became evident that the exposure concentrations of ≤100 μg/mL were not cytotoxic to the HUVECs, because viability of less than 50% generally indicates non-cytotoxicity (Fig. 1).

Because the angiogenic factor-stimulated endothelial cell proliferation is a key component of the angiogenic response, we determined the effects of the MeOH extracts of the edible plants on the bFGF (3 ng/mL)-induced mitogenic response in HUVECs with the treatment duration of 48 h. Results showed that the inhibitory effects on HUVECs were not due to the cytotoxicity of

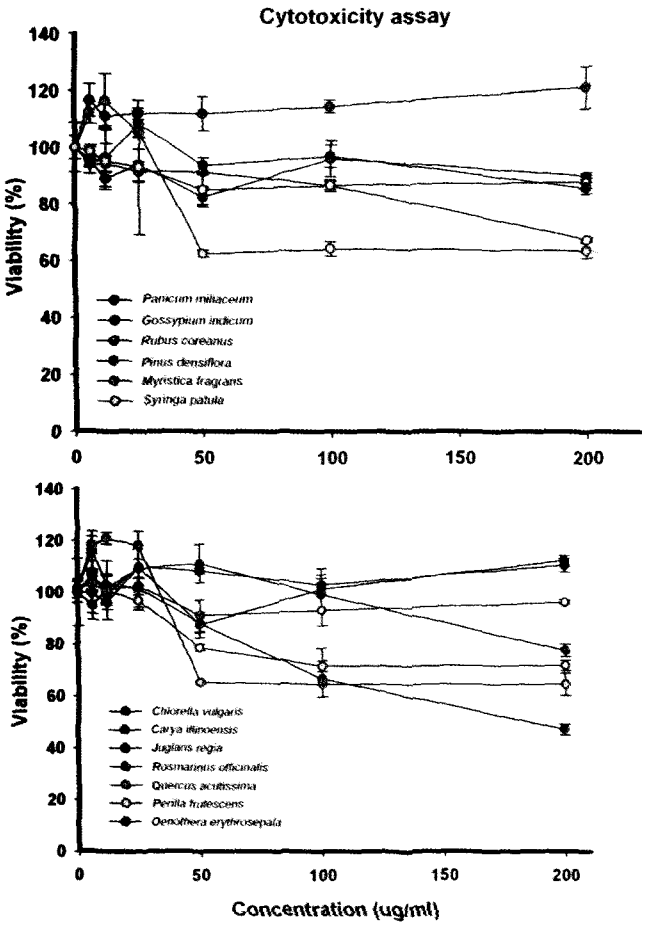

Fig. 1. Cytotoxicity of some edible plants on human umbilical vein endothelial cells (HUVECs).

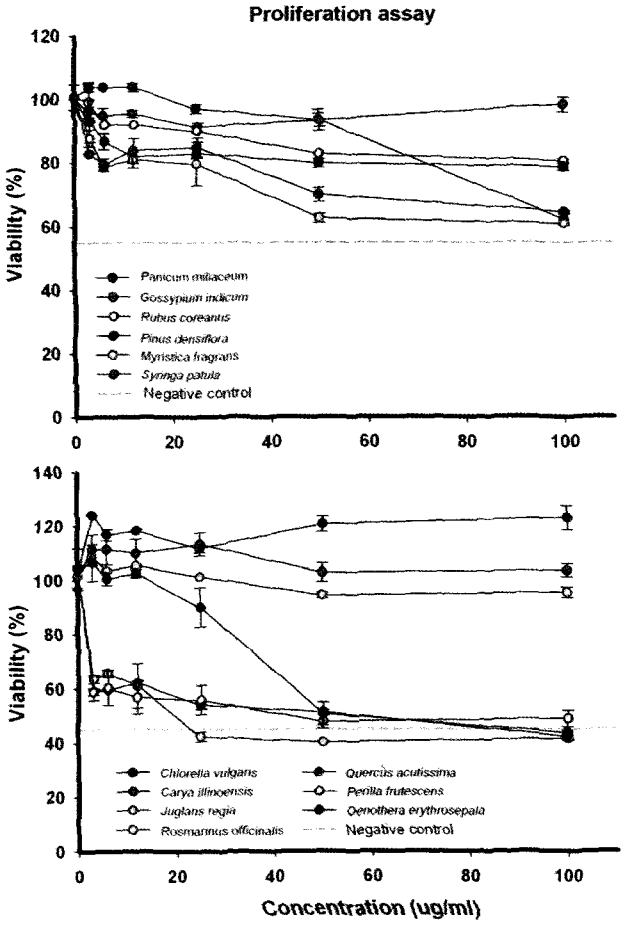

Fig. 2. Inhibitory activity on the proliferation of human umbilical vein endothelial cells (HUVECs) induced by basic fibroblast growth factor (bFGF).

the 13 edible plant extracts, because the extracts did not show any significant cytotoxic effect on the nonstimulated HUVECs at 100 μg/mL. *P. frutescens*, *Q. acutissima*, and *R. officinalis* inhibited the bFGF-induced endothelial proliferation by 42 , 43, and 36%, respectively, at 2.5 μg/mL; in particular, *R. officinalis* showed lower cell viability than that of the cell with non-treatment of growth factors at 25 μg/mL. *O. erythrosepala* and *M. fragrans* exhibited inhibition on the cell proliferation by 48 and 38%, respectively, at 50 μg/mL, whereas *P. miliaceum* and *S. patula* showed 38 and 36%, respectively, at 100 μg/mL (Fig. 2). *O. erythrosepala*, *P. frutescens*, *P. miliaceum*, and *Q. acutissima* significantly inhibited the proliferation of the endothelial cells induced by the basic fibroblast growth factor (bFGF) without cytotoxicity at 100 μg/mL. *M. fragrans*, *R. officinalis*, and *S. patula* also showed inhibitory activities on the proliferation as well as mild cytotoxicity (Fig. 3).

The plant extracts showing effective inhibition on the proliferation of HUVECs were studied for the anticancer activities reported in the literature. The methanol extract of the *M. fragrans* seeds demonstrated potent inhibitions on the proliferations of the cultured human tumor cells such as A549 (non-small cell lung), SK-OV-3 (ovary), SK-MEL-2 (melanoma), XF498 (central nerve system), and HCT-15 (colon) [Lee *et al.*, 2005]. Meso-Dihydroguaiaretic acid, naturally occurring in *M. fragrans*, exhibits a neuroprotective effect and also exerts cytotoxicity to certain cancer cells [Park *et al.*, 2005]. The antitumor effect of oenothein B, a macrocyclic ellagitannin from *O. erythrosepala*, was studied [Miyamaoto *et al.*, 1993]. The MeOH extracts of *R. officinalis* inhibited the growths of human gastric adenocarcinoma (MK-1), human uterine carcinoma (HeLa), and murine melanoma (B16F10) cells, and the bioactivity-guided fractionation resulted in the isolation of several phenolic constituents [Nagao *et al.*, 2004]. The anti-mammary cancer activities of the rosemary extract and its main antioxidative constituents were studied [Cao *et al.*, 2001]. Rosmarinic acid from *R. officinalis*, a water-soluble polyphenolic compounds with anti-oxidative and anti-inflammatory activities, inhibited

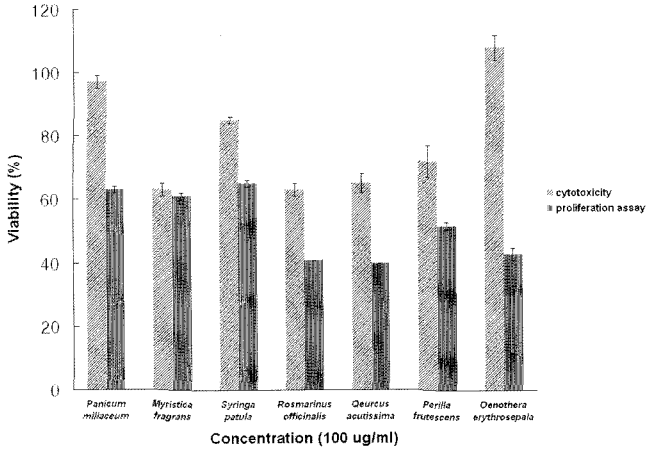

Fig. 3. Cytotoxicity on human umbilical vein endothelial cells (HUVECs) and inhibitory activity on the proliferation of HUVECs induced by bFGF at the concentration of 100 μg/mL.

several important steps of the angiogenesis including proliferation, migration, adhesion, and tube formation of HUVECs in a concentration-dependent manner [Huang and Zheng, 2006]. Bioassay-directed fractionation of the clove terpenes from the plant *E. caryophyllata* has led to the isolation of the following five active known compounds; β-caryophyllene, β-caryophyllene oxide, α-humulene, α-humulene epoxide I, and eugenol showed significant activities as the inducers of the detoxifying enzyme glutathione S-transferase in the mouse liver and small intestine [Zheng *et al.*, 1992]. A glycoprotein fraction, derived from the unicellular green alga *C. vulgaris*, exhibited a pronounced antitumor effect against both spontaneous and experimentally induced metastasis in mice [Tanaka *et al.* 1998]. *P. frutescens* extracts showed marked reductions of the tumor genesis in a murine, a two-stage skin carcinogenesis model [Osakabe *et al*, 2004]. Tormentic acid from red perilla and green perilla (*P. frutescens*) exhibited strong antitumor-promoting activity in an *in vivo* 2-stage carcinogenesis test of the mouse tumor when 7,12-dimethylbenz(a)anthracene was used as an initiator and 12-*O*-tetradecanoylphorbol-13-acetate as a promoter [Banno *et al.*, 2004]. The effects of *P. frutescens* leaf extract on the proliferation and apoptosis inducing human hepatoma HepG2 cells were evaluated through a cell proliferation assay, flow cytometry, and cDNA microarrays [Lin *et al.*, 2007].

Selected plants in this study such as *P. frutescens*, *P. miliaceum*, and *Q. acutissima* that showed inhibitory activity on the proliferation without cytotoxicity in HUVECs and *S. patula* with mild cytotoxicity in HUVECs could be useful sources in the development of drugs for the prevention and remedy of cancers.

References

Banno N, Akihisa T, Tokuda H, Yasukawa K, Higashihara H, Ukiya M, Watanabe K, Kimura Y, Hasegawa JI, and Nishino H (2004) Triterpene acids from the leaves of Perilla frutescens and their anti-inflammatory and antitumor-promoting effects. *Biosci Biotechnol Biochem* **68**, 85-90.

Cao S, Yu Y, Wen H, and Singletary KW (2001) Study on anti-mammary cancer activity of rosemary extract and its antioxidative constituent. *Yingyang Xuebao* **23**, 225-229.

Carmeliet P and Jain RK (2000) Angiogenesis in cancer and other diseases. *Nature* **407**, 249-257.

Carmeliet P (2000) Mechanisms of angiogenesis and arteriogenesis. *Nature Med* **6**, 389-395.

Hanaha D and Folkman J (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86**, 353-364.

Heo J (2005) In *Dongeui Bogam* (3rd ed.), YeoGang Press, Seoul.

Huang SS and Zheng RL (2006) Rosmarinic acid inhibits angiogenesis and its mechanism of action *in vitro*. *Cancer Lett* **239**, 271-280.

Huh JE, Lee EO, Kim MS, Kang KS, Kim CH, Cha BC, Surh YJ, and Kim SH (2005) Penta-O-galloyl-β-D-glucose suppresses tumor growth via inhibition of angiogenesis and stimulation of apoptosis: roles of cyclooxygenase-2 and mitogen-activated protein kinase pathways. *Carcinogenesis* **26**, 1436-1445.

Jost LM, Kirkwood JM, and Whiteside TL (1992) Improved short- and long-term XTT-based colorimetric cellular cytotoxicity assay for melanoma and other tumor cells. *J Immunol Methods* **147**, 153-165.

Kochanek KD, Murphy SL, Anderson RN, and Scott C (2004) Deaths: Final data for 2002. *National Vital Statistics Reports* **53**, 1-116.

Kwak HY, Kwon BM, Song MC, Lee JH, Yang HJ, Kim DK, Ahn EM, and Baek NI (2007) Development of biologically active compounds from edible plant sources-XXIV. Anti-cancer activity of alcohol extracts from edible plants. *Kor J Pharmacogn*, in press.

Lee EO, Lee HJ, Hwang HS, Ahn KS, Chae C, Kang KS, Lu J, and Kim SH (2006) Potent inhibition of Lewis lung cancer growth by heyneanol A from the roots of Vitis amurensis through apoptotic and anti-angiogenic activities. *Carcinogenesis* **27**, 2059-2069.

Lee HJ, Lee EO, Rhee YH, Ahn KS, Li GX, Jiang C, Lu J, and Kim SH (2006) An oriental herbal cocktail, ka-mi-kae-kyuk-tang, exerts anti-cancer activities by targeting angiogenesis, apoptosis and metastasis. *Carcinogenesis* **27**, 2455-2463.

Lee JW, Lee SO, Seo JH, Kwon JW, Choi SU, Lee KR, Kwon DY, Kim YK, Kim YS, and Ryu SY (2005) Inhibitory effects of the seed extract of Myristica fragrans on the proliferation of human tumor cell lines. *Kor J Pharmcogn* **36**, 240-244.

Lin CS, Kuo CL, Wang JP, Cheng JS, Huang ZW, and

Chen CF (2007) Growth inhibitory and apoptosis inducing effect of Perilla frutescens extract on human hepatoma HepG2 cells. *J Ethnopharmacol* **112**, 557-567.

Miyamaoto K, Nomura M, Sasakura M, Matsui E, Koshiura R, Murayama T, Furukawa T, Hatano T, Yoshida T, and Okuda T (1993) Antitumor activity of oenothein B, a unique macrocyclic ellagitannin. *Jpn J Canc Res* **84**, 99-103.

Nagao T, Kinjo J, and Okabe H (2004) Anti-proliferative phenolic constituents in plants and structure-activity relationships. *Foods Food Ingredients J Jpn* **209**, 3-12.

Osakabe N, Yasuda A, Natsume M, and Yoshikawa T (2004) Rosmarinic acid inhibits epidermal inflammatory responses: anticarcinogenic effect of Perilla frutescens extract in the murine two-stage skin model. *Carcinogenesis* **25**, 549-557.

Park EY, Shin SM, Ma CJ, Kim YC, and Kim SG (2005) Meso-dihydroguaiaretic acid from machilus thunbergii down-regulates TGF-1 gene expression in activated hepatic stellate cells via inhibition of AP-1 activity. *Planta Med* **71**, 393-398.

Risau W (1997) Mechanisms of angiogenesis. *Nature* **386**, 671-674.

Roehm NW, Rodgers GH, Hatfield SM, and Glasebrook AL (1991) An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. *J Immunol Methods* **142**, 257-265.

Tanaka K, Yamada A, Noda K, Hasegawa T, Okuda M, Shoyama Y, and Nomoto K (1998) A novel glycoprotein obtained from *Chlorella vulgaris* strain CK22 shows antimetastatic immunopotentiation. *Cancer Immunol Immunother* **45**, 313-320.

Weiss L (2000) Metastasis of cancer: a conceptual history from antiquity to the 1990s. *Cancer Metastasis Rev* **19**, 193-383.

Zheng GQ, Kenney PM, and Lam LKT (1992) Sesquiterpenes from clove(*Eugenia caryophyllata*) as potential anticarcinogenic agents. *J Nat Prod* **55**, 999-1003.