ISSN (Print) 1225-9918
ISSN (Online) 2287-3406
Journal of Life Science 2017 Vol. 27. No. 9. 1059~1063 DOI : https://doi.org/10.5352/JLS.2017.27.9.1059

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Extract of Balloon-flower Inhibited *In Vitro* Angiogenesis in Human Umbilical Vein Endothelial Cells

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Received April 13, 2017 / Revised May 9, 2017 / Accepted May 10, 2017

Angiogenesis is an essential step in tumoral growth and metastasis and is regulated by a balance between stimulators and inhibitors. Recently, antiangiogenic target therapy has shown promise as a new type of chemotherapy. Natural products have attracted widespread attention worldwide as a useful source of novel therapeutic compounds. The balloon-flower has long been used as a traditional medicinal material and food in Asia. In this study, we investigated whether extract of balloon-flower would inhibit *in vitro* angiogenesis and vascular-like network formation in human umbilical vein endothelial cells (HUVECs). The extract of Balloon-flower did not affect the viability of HUVECs. However, treatment with the Balloon-flower extract suppressed tube formation of HUVECs. In addition, after treatment with the Balloon-flower extract, cell migration decreased about 80%, and cell invasion was almost completely inhibited. Taken together, these results suggest that extract of Balloon-flower may have potential as an angiogenic inhibitor and that it could be developed as an anticancer agent.

Key words : Angiogenesis, balloon-flower, chemotherapy, natural products, tumor

Introduction

Angiogenesis is a process of modification such as sprouting and growth of blood vessels [6]. It includes various cellular actions such as degradation of extracellular matrix (ECM), proliferation and migration of endothelial cells, and morphological differentiation of endothelial cells [2, 20]. These phenomena are very important process for tumor growth and metastasis [5].

Natural products have historically been used for an invaluable source of various therapeutic agents, especially for anticancer drug discovery [9, 16]. Many kinds of the medicinal natural products from plants have been identified in Korea, China, Japan, and other Asian countries for many centuries [4, 13]. It has been showed antitumor effects through many cases and have been seen as candidate for novel cancer treatment [14, 18].

Balloon-flower known as the *Platycodon grandiflorum*, is widely distributed in Northeast Asia [17]. It has long been used as a food material and also has been used as a traditional medicine for treating bronchitis, asthma, hyperlipidemia and inflammation in Asia [11, 19, 23]. Especially in Korea, it is also well known to have beneficial activities on diabetes and obesity.

In this study, we tested the anti-angiogenic activity of the extract of Balloon-flower and demonstrate a potential candidate of a therapeutic anti-cancer drug.

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Materials and Methods

Materials and reagents

Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from inopharmascreen (Chungnam, Korea). Basic fibroblast growth factor (bFGF) and heparin were obtained from Pepprotech (Gaithersbeg, USA). M199, Fetal Bovine Serum (FBS), penicillin and streptomycin were purchased from JBI (Daegu, Korea). Matrigel was purchased from Collaborative Biomedical Products (Bedford, MA. USA) and used for the tube formation assay. Trans-well filter chambers (8-μm pores) were purchased from Corning-Costar (Cambridge, USA).

Extract of Balloon-flower

Grain-alcoholic extracts derived from biomass, dried root and leaf of Balloon-flower, has been prepared upon cold son-

ication process at temperature of 20℃. Biomass was purchased from Kyung-dong medicinal herb market (Seoul, Korea). Biomass has been powdered then, mixed with 70% alcoholic solution in pure water before processing. Weight per volume percent, biomass basis, was 100 mg/ml. Cold extract was separated by centrifuge after the process.

Cell culture

HUVECs were grown in M199, supplemented with heat-inactivated 20% FBS (JBI, Daegu, Korea), 20 ng/ml of bFGF, 100 units/ml of penicillin and 100 μg/ml of streptomycin in a 37℃ incubator with a humidified atmosphere containing 5% CO_2.

MTT assay for cell viability

HUVECs were grown in M199 with 20% FBS at a density of $2x10^4$ cells on 24-well culture plates. After one night, the media was re-placed with M199 containing 1% FBS, and crude extract of Balloon-flower and the cells were then incubated for 24 hr at 37℃ under a humidified atmosphere that was comprised of 5% CO_2. Cells were treated with various concentrations of Balloon-flower extract. Next, MTT solution (5 mg/ml in H_2O) was added to the well followed by the addition of 0.3 ml of demetylsulfoxide (DMSO) to dissolve the MTT- formazan and then determined by measuring the absorbance at 540 nm. Each sample was assayed in triplicate, and the experiment was repeated three times.

In vitro tube formation assay

0.3 ml matrigel was transferred to 24-well plate and incubated for 30 min. HUVECs (2×10^4 cells) were plated on a layer of previously polymerized matrigel and treated with or without extract of Balloon-flower. Matrigel culture was incubated at 37℃ during 24 hr. Cell morphological changes were captured through a phase contrast microscope and photographed at 40x magnification. Each sample was assayed in duplicate, and independent experiments were repeated three times.

In vitro wounding migration assay

HUVECs were seeded onto 24-well culture plate until confluence and left overnight. Media was aspirated the next day, and cells were scratched with a 200 μl pipette tip along the diameter of the well. Cells were washed twice with PBS and incubated at 37℃ and 5% CO_2. After wounding, the cells were incubated in M199 with 1% serum, 1 mM thymidine, and/or extract of Balloon-flower. These culture conditions minimized proliferation of HUVECs. Wound diameters were photographed at 24 hr. Wound closure was determined by measurement with optical microscopy at 40x magnification. Each sample was assayed in duplicate, and independent experiments were repeated three times.

In vitro invasion assay

Invasion assay was performed *in vitro* using a trans-well chambers system (COSTAR, USA) with 8.0 μm pore polycarbonate filter inserts. The upper side of trans-well was coated with 10 μl of matrigel (0.5 mg/ml) at room temperature for 1 hr. Complete media was plated in the lower parts of the trans-well chamber filters, and HUVECs ($2x10^4$ cells) and extracts of Balloon-flower in serum-free media were placed in the upper part. Cells were incubated at 37℃ for 24 hr, trans-wells were fixed with methanol, and then stained with hematoxyline/eosin. Cells on the upper surface of the filter membrane were removed by wiping with a cotton swab. Invaded cells were determined with optical microscopy at 40x magnification. Each sample was assayed in duplicate, and independent experiments were repeated three times.

Data analysis and statistics

Statistical comparison was performed using Student's t-test. $p<0.001$ was considered statistically significant. Data are presented as means ± standard deviation (SD). All experiments were performed in triplicate.

Results and Discussion

Angiogenesis is the developmental process of new vessels by sprouting from pre-existing vasculature [10, 21], and is needed significant component of various physiological and pathological conditions [8]. J. Folkman was firstly established in 1971 that angiogenesis is an essential process in tumor growth [7]. Since then, angiogenesis has been recognized as important target for cancer therapy [1, 15]. Tumor initiation and progression are also closely related to angiogenesis. Therefore, controlling of angiogenic actions in tumor could be strategies for development of anti-cancer therapeutic drugs [5].

The investigations for novel anticancer drugs from natural products have been continued through the research of scientists around the world [1]. Many numbers of natural prod-

ucts show anticancer effects *in vitro* and *in vivo* [3, 9]. Balloon-flower is commonly used for food materials and is also one of the traditional medicinal products [12]. In particular, roots of Balloon-flower show excellent anti-cancer effects against various cancer cell lines mainly by inhibiting cell proliferation, promoting cell cycle arrest and apoptosis [22].

The cytotoxic effect of Balloon-flower extract on the HUVECs was examined by treatment of various concentrations (from 50 to 500 μg) using the MTT assay. Treatment of Balloon- flower extract for 24 hr did not affect the cell viability of HUVECs (Fig. 1A). This result indicates that the treatment of extract of Balloon-flower did not show any cytotoxic effect on the HUVECs at the tested concentrations.

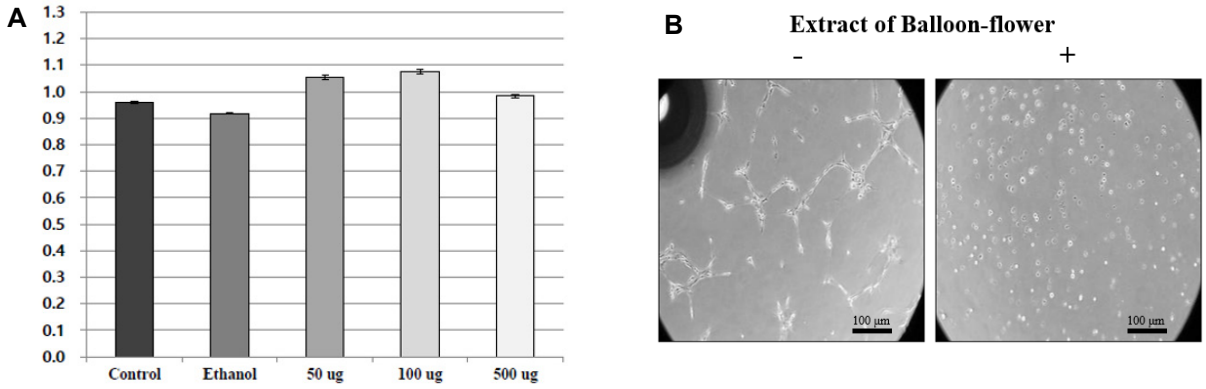

Fig. 1. Extracts of Balloon-flower inhibits the vascular network formation. (A) The cytotoxic effect of Balloon-flower extract on the HUVECs was determined using the MTT assay. The treatment for 24 hr did not affect the cell viability of HUVEC cells in the tested concentrations. (B) Balloon-flower extract (100 μg/ml) was added and incubated for 24 hr. The changes of cell morphology were captured through a phase contrast microscope (x40) and photographed. Representative photographs of the tube formation of endothelial cells cultured on polymerized matrigel layers reveal the inhibitory effect of extracts of Balloon-flower on the formation of capillary-like structure. This independent experiment was repeated three times.

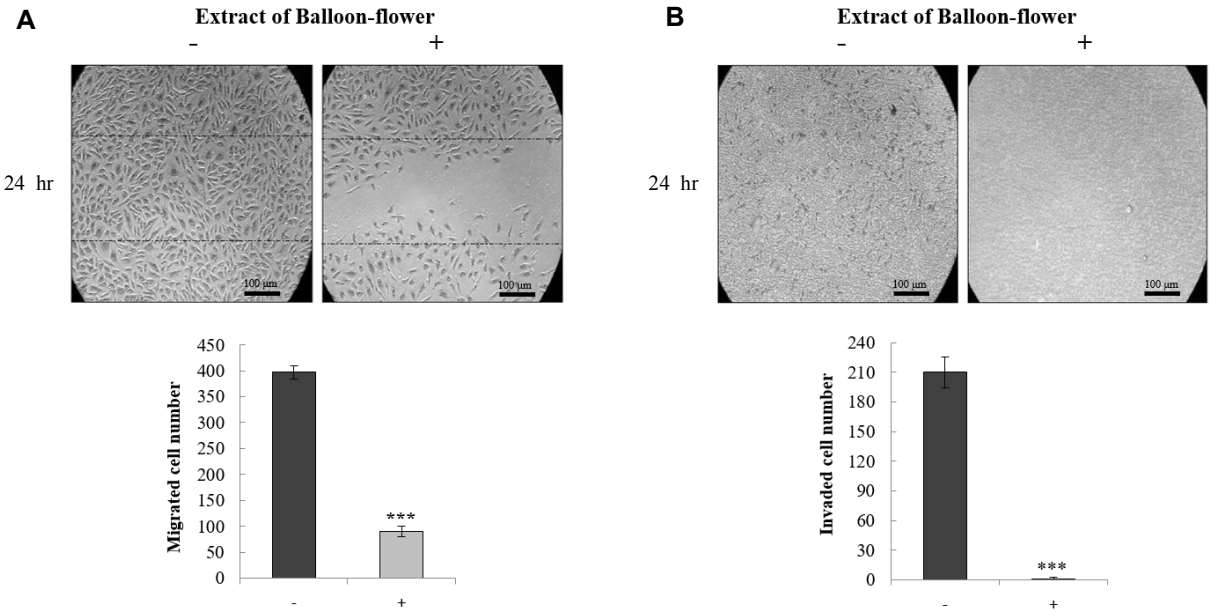

Fig. 2. Extracts of Balloon-flower suppress migration and invasion of HUVECs. (A) Extracts of Balloon-flower (100 μg/ml) inhibits migration of HUVECs. Migration ability of HUVECs was measured by wounding migration assay. This independent experiment was repeated three times. Bars correspond to mean ± s.d. ***p<0.001 as compared with control. (B) Extracts of Balloon-flower (100 μg/ml) inhibits invasion of HUVECs. Invasion capacity was examined using a trans-well system coated with matrigel. This independent experiment was repeated three times. Bars correspond to mean±s.d. ***p<0.001 as compared with control.

To determine the anti-angiogenic effect of extract of Balloon-flower, we carried out *in vitro* tube formation assay. It is important step of angiogenesis which promote morphological differentiation into capillary-like structure. As shown in Fig. 1B, HUVECs on matrigel formed blood vessel network in the absence of extract of Balloon-flower. However, after treatment of Balloon-flower extract for 24 hr, we could not observe the tube networks. This result demonstrates that extract of Balloon-flower suppressed the tube formation of HUVECs *in vitro*.

Endothelial cell migration and invasion are one of the critical steps in the formation of new blood vessels [7]. The effect of Balloon-flower extract on the migration was examined by wounding migration assay. The result shown in Fig. 2A shows that Balloon-flower extract clearly inhibited migration of HUVECs. The migration of HUVECs decreased about 80%. In addition, *in vitro* invasion assay was performed using a trans-well chambers system to examine the effect of Balloon-flower extract on the invasion of HUVECs. Treatment with Balloon-flower extract for 24 hr markedly almost completely inhibited invasion of HUVECs compared with that of control (Fig. 2B).

In conclusion, we showed that the extract of Balloon-flower inhibited *in vitro* angiogenesis steps in HUVECs including tube formation, migration and invasion. Therefore, Balloon-flower extract might be a strong angiogenic inhibitor and may have a potential to be developed as a therapeutic anti-cancer drug.

Acknowledgments

This work was supported by a 2-Years Research Grant of Pusan National University (2015-2016). We thank Dr. Han KS for his help in supplying the ingredients (Balloon flower Extracts).

References

1. Adriana, B. R., Rafael, M. L. and Gilberto, S. 2001. Natural products in anticancer therapy. *Curr. Opi. Phamacol*. **1**, 364-369.
2. Carmeliet, P. and Jain, R. K. 2000. Angiogenesis in cancer and other diseases. *Nature* **407**, 249-257.
3. Chen, X., Bao, J., Guo, J., Ding, Q., Lu, J., Huang, M. and Wang, Y. 2012. Biological activities and potential molecular targets of cucurbitacins: a focus on cancer. *Anticancer Drugs* **23**, 777-787.
4. Efferth, T., Li, P. C., Konkimalla, V. S. and Kaina, B. 2007. From traditional Chinese medicine to rational cancer therapy. *Trends Mol. Med*. **13**, 353-361.
5. Ferrara, N. and Kerbel, R. S. 2005. Angiogenesis as therapeutic target. *Nature* **438**, 967-974.
6. Folkman, J. 1971. Tumor angiogenesis; therapeutic implications. *N. Engl. J. Med*. **285**, 1182-1186.
7. Folkman, J. and Shing, Y. 1992. Angiogenesis. *J. Biol. Chem*. **267**, 10931-10934.
8. Grivennikov, S. I. and Karin, M. 2010. Inflammation and oncogenesis: a vicious connection. *Curr. Opin. Genet. Dev*. **20**, 1-12.
9. Huang, M., Lu, J. J., Huang, M. Q., Bao, J. L., Chen, X. P. and Wang, Y. T. 2012. Terpenoids: natural products for cancer therapy. *Expert. Opin. Investig. Drugs* **21**, 1801-1818.
10. Karin, M. and Greteo, F. R. 2005. NF-kB: Linking inflammation and immunity to cancer development and progression. *Nat. Rev. Immunol*. **5**, 749-759.
11. Lee, J. S., Choi, M. S., Seo, K. I., Lee, H. I., Lee, J. H., Kim, M. J. and Lee, M. K. 2014. Platycodi radix saponin inhibits glucosidase *in vitro* and modulates hepatic glucose-regulating enzyme activities in C57BL/KsJ -db/db mice. *Arch. Pharm. Res*. **37**, 773-782.
12. Lee, W. H., Gam, C. O., Ku, S. K. and Choi, S. H. 2011. Single oral dose toxicity test of platycodin d, a saponin from platycodin radix in mice. *Toxicol. Res*. **27**, 217-224.
13. Meng, Z., Garrett, C. R., Shen, Y., Liu, L., Yang, P., Huo, Y., Zhao, Q., Spelman, A. R., Ng, C. S., Chang, D. Z. and Cohen, L. 2012. Prospective randomized evaluation of traditional Chinese medicine combined with chemotherapy: a randomised phase II study of wild toad extract plus gemcitabine in patients with advanced pancreatic adenocarcinomas. *Br. J. Cancer* **107**, 411-416.
14. Morishita, S., Shoji, M., Oguni, Y., Ito, C., Higuchi, M. and Sakanashi, M. 1992. Pharmacological actions of "kyushin," a drug containing toad venom: cardio-tonic and arrhythmogenic effects, and excitatory effect on respiration. *Am. J. Chin. Med*. **20**, 245-256.
15. Naldini, A. and Carraro, F. 2005. Role of inflammatory mediators in angiogenesis. *Curr. Drug Targets Inflam. Allergy* **4**, 3-8.
16. Neychev, V. K., Nikolova, E., Zhelev, N. and Mitev, V. I. 2007. Saponins from Tribulusterrestris L. are less toxic for normal human fibroblasts than for many cancer lines: Influence on apoptosis and proliferation. *Exp. Biol. Med*. **232**, 126-133.
17. Park, D. I., Lee, J. H., Moon, S. K., Kim, C. H., Lee, Y. T., Cheong, J., Choi, B. T. and Choi, Y. H. 2005. Induction of apoptosis and inhibition of telomerase activity by aqueous extract from *Platycodon grandiflorum* in human lung carcinoma cells. *Pharmacol. Res*. **51**, 437-443.
18. Qi, F., Li, A., Zhao, L., Xu, H., Inagaki, Y., Wang, D., Cui, X., Gao, B., Kokudo, N., Nakata, M. and Tang, W. 2010. Cinobufacini, an aqueous extract from Bufo bufo gargarizans Cantor, induces apoptosis through a mitochondria-mediated pathway in human hepatocellular carcinoma cells. *J. Ethnopharmacol*. **128**, 654-661.

19. Qin, H., Du, X., Zhang, Y. and Wang, R. 2014. Platycodin D, a triterpenoid saponin from Platycodon grandiflorum, induces G2/M arrest and apoptosis in human hepatoma HepG2 cells by modulating the PI3K/Akt pathway. *Tumour Biol*. **35**, 1267-1274.
20. Siddiqui, F. A., Desai, H., Siddiqui, T. F. and Francis, J. L. 2002. Hemoglobin induces the expression and secretion of vascular endothelial growth factor from human malignant cells. *Hematol. J*. **3**, 264-270.
21. Yoshimura, A. 2006. Signal transduction of inflammatory cytokines and tumor development. *Cancer Sci*. **97**, 439-447.
22. Yu, J. S. and Kim, A. K. 2010. Platycodin D induces apoptosis in MCF-7 human breast cancer cells. *J. Med. Food* **13**, 298-305.
23. Zhang, L., Wang, Y., Yang, D., Zhang, C., Zhang, N., Li, M. and Liu, Y. 2015. Platycodon grandifloras-An ethnopharmacological, phytochemical and pharmacological review. *J. Ethnopharmacol*. **164**, 147-161.

초록 : 도라지 추출물에 의한 인간 제대 정맥 내피 세포의 *in vitro* 혈관신생 억제

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혈관신생과정은 종양 형성과 이동에 필수적인 과정으로 촉진제와 저해제에 의하여 조절되며, 이러한 혈관신생 과정의 저해는 새로운 항암치료 기법으로 이용하고 있다. 최근, 한약재와 식료품으로부터 추출한 천연물을 새로운 치료 물질로 널리 이용하고 있으며, 실제 *in vitro* 뿐만 아니라 *in vivo* 상에서도 항암 효과가 나타나는 것을 확인하였다. 그 중 도라지는 아시아에서 한약재와 식료품으로 오랫동안 사용 되어왔다. 본 연구에서는 도라지 추출물이 *in vitro* 상에서 인간 제대 정맥 내피 세포의 혈관신생을 억제하는 효과에 대해 조사하였다. 도라지 추출물은 세포 독성 없이, 혈관 형성 및 이동, 침윤 현상을 모두 억제하는 효과를 보였다. 특히, 세포 이동은 80% 정도 감소 시켰으며 침윤 현상이 거의 나타나지 않는 것을 확인하였다. 이러한 결과를 토대로 도라지 추출물은 혈관신생 억제제로 이용할 수 있으며, 더 나아가 항암제로 개발될 수 있을 것이라 사료된다.