Bioorganic & Medicinal Chemistry Letters 28 (2018) 3150–3154

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Eupatilin inhibits angiogenesis-mediated human hepatocellular metastasis by reducing MMP-2 and VEGF signaling

Jun Yeon Park[a,1], Do Hwi Park[b,1], Youngsic Jeon[c], Young-Joo Kim[d], Jaemin Lee[b], Myoung-Sook Shin[b], Ki Sung Kang[b], Gwi Seo Hwang[b], Hyun Young Kim[e,*], Noriko Yamabe[a,*]

[a] Department of Food Science and Biotechnology, Kyonggi University, Suwon 16227, Republic of Korea
[b] College of Korean Medicine, Gachon University, Seongnam 13120, Republic of Korea
[c] Department of Pathology, Brain Korea 21 PLUS Project for Medical Science, Yonsei University College of Medicine, Seoul, Republic of Korea
[d] Natural Products Research Center, Korea Institute of Science and Technology, Gangneung, Gangwon-do, Republic of Korea
[e] Department of Food Science, Gyeongnam National University of Science and Technology, Jinju 660-758, Republic of Korea

ARTICLE INFO

Keywords:
Eupatilin
Metastasis
HUVEC
MMPs
VEGF

ABSTRACT

Metastasis is responsible for the great majority of deaths in cancer patients. Matrix metalloproteinases (MMPs) have critical functions in cancer metastasis. Especially, MMP-2 and MMP-9 play a major role in tumor-cell migration and invasion. Therefore, to first find out the inhibitory effect of eupatilin on expression of MMPs in SNU182 cells, we used quantitative real-rime PCR to measure MMP-2 and MMP-9 mRNA levels. Eupatilin suppressed transcription of MMP-2 in SNU182 cells more than did the corresponding controls. Also, eupatilin significantly blocked tube formation when treated with a concentration of 3.125 or 6.25 μg/mL on human umbilical vein vascular endothelial cells (HUVECs). Eupatilin induced significant anti-angiogenic potential associated with down-regulation of hypoxia-inducible factor 1-alpha (HIF-1α), vascular endothelial growth factor (VEGF), and phosphorylated Akt expression. Thus, tube-formation inhibition and MMP-2-mediated migration are likely to be important therapeutic targets of eupatilin in hepatocellular carcinoma metastasis.

Hepatocellular carcinoma (HCC) is one of the most malignant cancers and the third leading cause of cancer-related deaths in the world.[1,2] The primary cause of HCC death is metastasis, and it is urgent to understand the mechanism of HCC metastasis and to improve the effectiveness of current treatment medications. Despite the development of treatments such as transplantation, surgery, and carotid embolization, the prognosis of advanced HCC is still very poor, and the management of HCC remains a significant challenge for physicians.[3]

Invasion of tumor cells is necessary for tumor establishment and migration. Invasiveness is an active process in which cancer cells pass through the anatomical boundary between tissue components, and in this process, degrading the extracellular matrix (ECM) is required.[4] Matrix metalloproteinases (MMPs) degrade ECM components as a zinc-dependent endopeptidases family. Also, the activity of the MMPs family is an important factor associated with HCC metastasis. Collagenases MMP-2 and MMP-9, also known as type IV collagenases, are the major enzymes for collagen degradation and removal of extracellular matrix molecules in the extracellular matrix and basement membrane.[5] Abnormal expression of MMP-2 and MMP-9 is associated with multiple stages of tumor growth, vascular invasion, tumor progression, and metastasis.[6]

Angiogenesis is a cancer feature that is essential for supplying oxygen and nutrients to all proliferating tumor cells.[7] Tumor cells secrete a variety of growth factors that form blood vessels at the tumor site because of tissue stimulation, which is mainly induced by vascular endothelial growth factor (VEGF).[8]

Eupatilin (5,7-dihydroxy-3,4,6-trimethoxyflavone, Fig. 1(A)) is an active ingredient of the oriental herb *Artemisia asiatica* Nakai and is widely used for the treatment of gastritis and peptic ulcers. Eupatilin has shown various pharmacological activities of anti-oxidation, anti-inflammation, and cyto-protection.[9,10] Actually, eupatilin has been reported to ameliorate gastric mucosal injury, cerulein-induced pancreatitis, and dextran sulfate sodium-induced colitis and reflux esophagitis.[11] In animal models of liver disease, eupatilin has protected against hepatic fibrosis induced by dimethylnitrosamine and liver damage by acetaminophen or carbon tetrachloride.[12] The therapeutic effect of eupatilin can be involved in anti-apoptotic effects in hepatocytes, unlike apoptosis in other cells.[13] However, little is known about

* Corresponding authors.
E-mail addresses: hykim@gntech.ac.kr (H.Y. Kim), norikoy@gachon.ac.kr (N. Yamabe).
[1] These two authors contributed equally to the work described in this study.

https://doi.org/10.1016/j.bmcl.2018.08.034
Received 17 July 2018; Received in revised form 23 August 2018; Accepted 27 August 2018
Available online 28 August 2018
0960-894X/

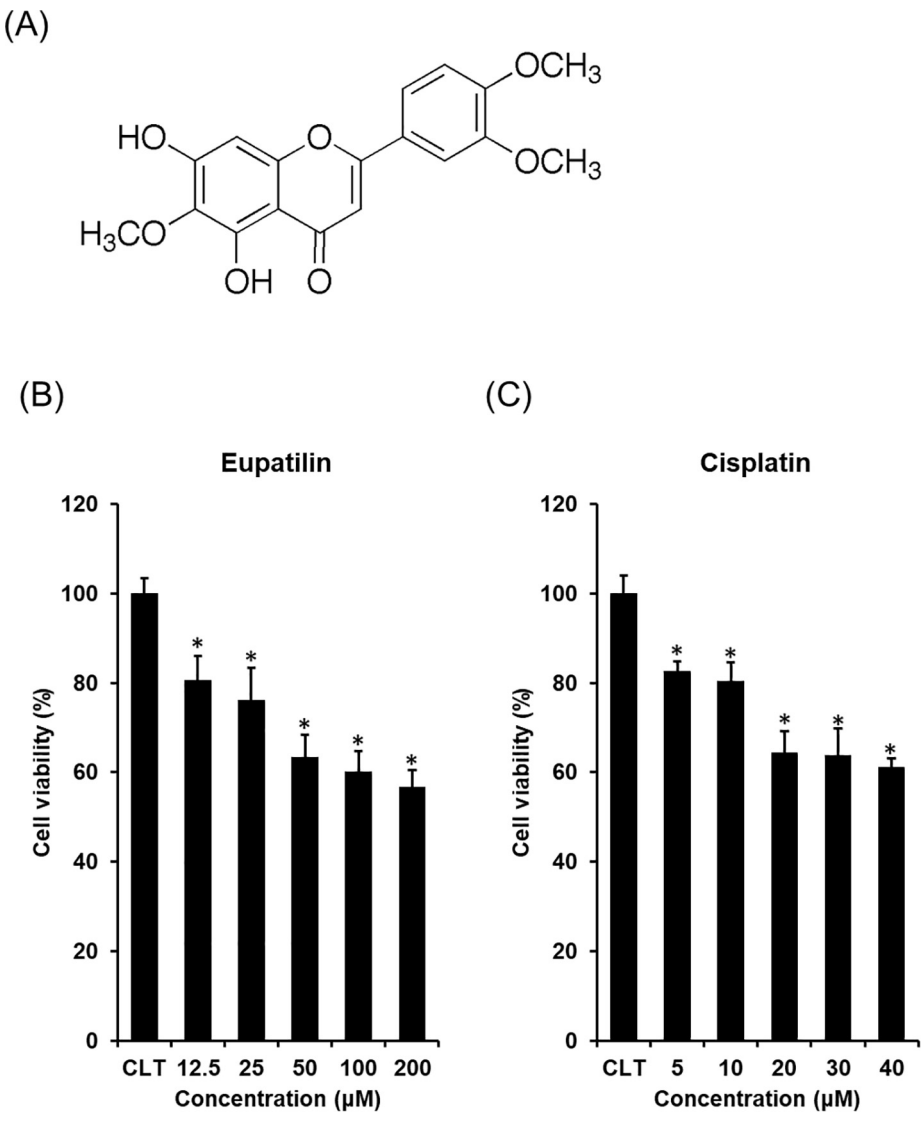

Fig. 1. The cytotoxic effect of eupatilin in HepG2 hepatocarcinoma cell line. (A) Chemical structure of eupatilin. (B) Cytotoxic effect of eupatilin in HepG2 cells, which were treated with indicated concentrations of eupatilin for 24 h. Cell viability was measured by using an MTT assay and is represented by the percentage of control. (C) Cytotoxic effect of cisplatin in HepG2 cells, which were also exposed to the indicated concentrations of cisplatin, as a positive control, for 24 h. The graph shows the percentage of cell viability compared with that of the control. Data are expressed as means ± s.e.m. Similar results were obtained in three independent experiments; $^{*}p < 0.05$ compared to the control value.

the effects of eupatilin on metastasis.

We performed this study to investigate the anti-metastasis activity of eupatilin and to elucidate its underlying mechanisms. We investigated the effects of various concentrations of eupatilin and cisplatin on the proliferation of HepG2 cells by using an MTT assay. Eupatilin (≥98% purity, Fig. 1(A)) was prepared from an ethanolic extract of *A. asiatica* as reported previously.[10] As shown in Fig. 1(B), eupatilin inhibited the proliferation of HepG2 cells in a dose-dependent manner. Eupatilin inhibited the viability of HepG2 cells by 43.24% at a concentration of 200 μM. Cisplatin inhibited the viability of HepG2 cells by 38.84% at a concentration of 40 μM (Fig. 1(C)). However, the anticancer effect was so weak we could not measure the value of IC_{50} when the high concentrations of the two compounds were treated, because HepG2 cells are resistant to toxicity.

Next, we used SNU hepatocellular carcinoma-cell lines to confirm the effect on metastasis. SNU hepatocellular carcinoma-cell lines (such as SNU 182 cells) have proven valuable in carcinogenicity studies on the development of hepatocellular carcinoma.[14] Prior to investigating the anti-metastatic effect of eupatilin, we examined the cytotoxicity of eupatilin in SNU182 cells. As shown in Fig. 2(A and B), eupatilin did not affect cell viability for 48 h at concentrations up to 20 μM. Therefore, 20 μM eupatilin was used for the following experiments. MMPs have critical functions in cancer metastasis. Especially, MMP-2 and MMP-9 play a major role in tumor cell migration and invasion.[15,16] Therefore, to measure the inhibitory effect of eupatilin on expression of MMPs in SNU182 cells, we did quantitative real-rime PCR to measure MMP-2 and MMP-9 mRNA levels. As shown in Fig. 2B, the levels of the MMP-2 mRNA were decreased more than in untreated control cells, after incubation with eupatilin in SNU182 cells. In contrast, there were no changes in MMP-9 mRNA expression following the treatment with eupatilin.

MMP-2 and -9 have similarity as type IV collagenase, however there are differences; MMP-2 is expressed constantly in most normal cells including fibroblasts, keratinocytes, vascular endothelial cells, chondrocytes, monocytes, and osteoblasts in vitro, and it is also normally present in serum. However, MMP-9 is expressed constantly in polymorphonuclear leukocytes, and expression by TPA (12-O-tetradecanoylphorbol-13-acetate), growth factors, cytokines in normal keratinocytes.[17,18] According to the previous report, MMP-2 is strongly positive in gastric cancer, but not over-expressing in precancerous lesions. Conversely, MMP-9 is overexpressed not only in gastric cancer but also pre-cancerous polyp and it may be involved as an early contributor to cancer.[19] Therefore, further study is needed to confirm that eupatilin does not participate in the inhibition of MMP-9 expression.

In the following study, we conducted an experiment on angiogenesis using HUVECs to confirm the effect of the blood vessels on metastasis. The angiogenesis process is completed by the proliferation, migration, tube formation and differentiation into vascular forms of vascular

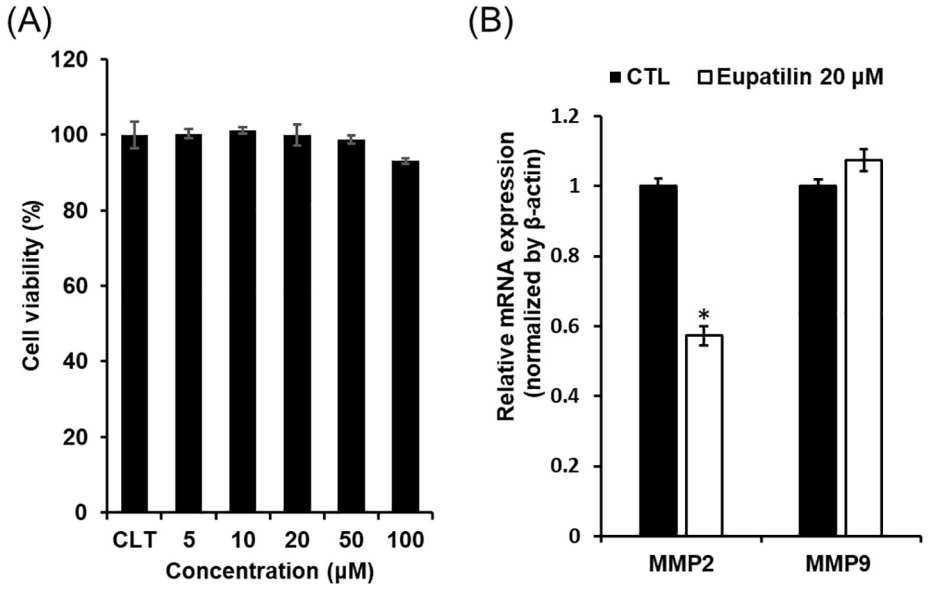

Fig. 2. The effect of eupatilin on MMP2 and MMP9 mRNA expression in SNU-182 cells. (A) Cytotoxic effect of eupatilin in SNU182 cells, which were treated with the indicated concentrations of eupatilin for 24 h. Cell viability was measured by using an MTT assay and is represented by the percentage of control. (B) SNU182 cells were treated with the indicated concentrations of eupatilin for 24 h. The expression of MMP2 and MMP9 mRNA in SNU182 was detected using RT-PCR analysis. The relative mRNA expression was quantitatively analyzed and is represented by fold-increases compared with control. Data are expressed as means ± s.e.m. Similar results were obtained in three independent experiments; $^{*}p < 0.05$ compared to the control value.

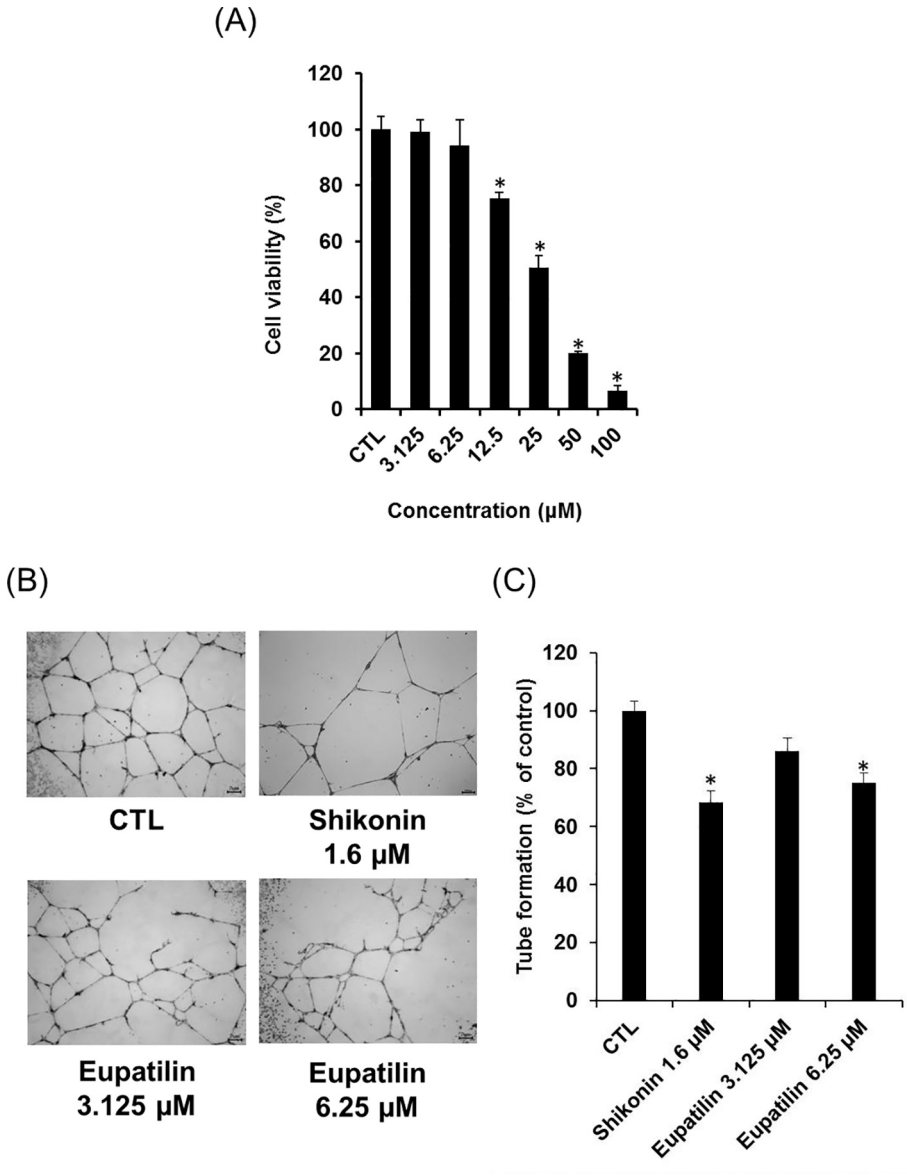

Fig. 3. The effect of eupatilin on a tubule formation in HUVEC cells. (A) HUVEC cells were treated with the indicated concentrations of eupatilin for 24 h, and cell viability was measured using an MTT assay. The graph indicates the percentage of viable cells. (B) Representative images for tubule formation were photographed after the exposure to the indicated concentrations of eupatalin and 1.6 μM shikonin (positive control). (C) The length of tubes was measured using the Image J soft and is represented by the percentage of tubule formation compared with control. Data are expressed as means ± s.e.m. Similar results were obtained in three independent experiments; $^{*}p < 0.05$ compared to the control value.

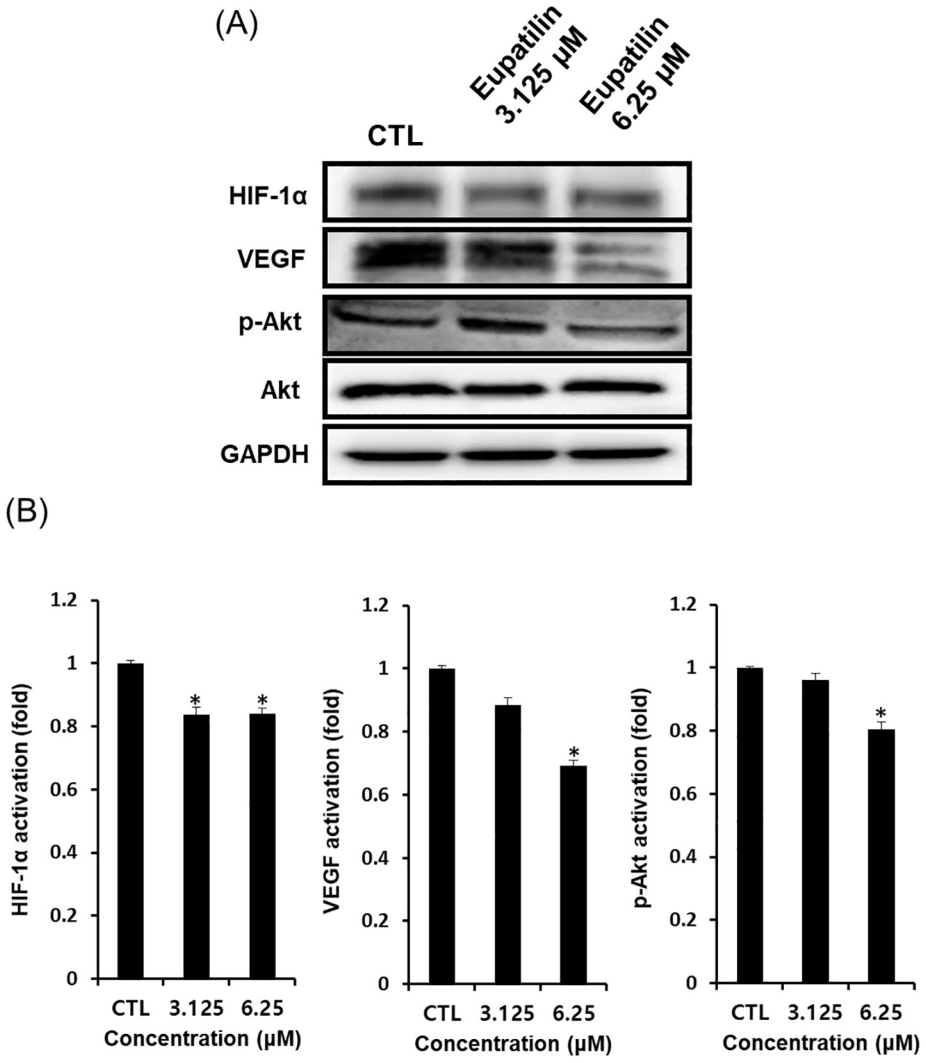

Fig. 4. Effects of eupatilin on angiogenic protein expressions in HUVECs. (A) Results of the Western blot show the levels of HIF-1α (124 kDa), VEGF (21 kDa), phosphorylated-Akt (60 kDa), and Akt (60 kDa) in HUVECs treated with eupatilin at different concentrations for 24 h. Whole cell lysates (20 mg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene fluoride (PVDF) transfer membranes, and probed with the indicated antibodies. Proteins were visualized using an ECL detection system. (B) Quantified graphs for the effects of eupatilin on the angiogenic protein expressions in HUVECs. Results of the Western blot show the levels of HIF-1α, VEGF, phosphorylated-Akt, and Akt in HUVECs treated with eupatalin at indicated concentrations for 24 h. Data are expressed as means ± s.e.m. Similar results were obtained in three independent experiments; $^{*}p < 0.05$ compared to the control value.

endothelial cells.[20] A method of verifying the inhibition of angiogenesis in the development of new blood vessels is vascularization of vascular endothelial cells on Matrigel containing various growth factors.[21] Tube formation was examined as reported previously with some modification.[22,23] As shown in Fig. 3A, eupatilin inhibited HUVECs proliferation in a dose-dependent manner. Treatments with up to 6.25 μM of eupatilin had no effect on the HUVECs, whereas treatment with 12.5–100 μM decreased cell viability. The effect of a nontoxic dose (3.125 and 6.25 μM) of eupatilin and 1.6 μM shikonin (positive control) on HUVEC tube formation is shown in Fig. 3(B and C). Representative tube images are shown (Fig. 3B); eupatilin at a concentration of 3.125 or 6.25 μM significantly blocked tube formation (Fig. 3C). Compared with the control, 14.08% inhibition of tube formation was achieved with 3.125 μM eupatilin, 24.77% inhibition with 6.25 μM eupatilin, and 31.77% inhibition with 1.6 μM shikonin, respectively.

HIF-1α is a constitutive protein of hypoxic-specific transcription factor, which induces mass induction in a hypoxic state, and expresses genes related to red blood-cell production, angiogenesis, and glucose metabolism in order to maintain the oxygen homeostasis in the cells.[24,25] Also, the process of angigenesis begins when endothelial cells are stimulated by angiogenic factors.[26] The expression of Akt is known to be an important protein involved in the mechanism of angiogenesis. These roles are involved in such angiogenic processes as endothelial-cell proliferation, migration, and tube formation, and are known to regulate angiogenic factors, such as VEGF and bFGF.[27,28] Previously, experiments related to angiogenesis have demonstrated that eupatilin inhibits angiogenesis, but the question of what mechanism causes it is not yet resolved. Western blot, preparation of cell lysates, supernatant collection and protein quantification were performed as previous studies with minor modification.[29] Fig. 4A is a photograph of the electrophoresis of the Western blot analysis we obtained. Fig. 4B shows the levels of HIF-1α, VEGF, phosphorylated-Akt (p-Akt), and Akt. As shown in Fig. 4B, the Western blot analysis showed that the expressions of HIF-1α (0.83 ± 0.02 and 0.84 ± 0.02-fold at 3.125 and 6.25 μM, respectively), VEGF (0.88 ± 0.01 and 0.69 ± 0.01-fold at 3.125 and 6.25 μM, respectively), and p-Akt (0.82 ± 0.02 and 0.80 ± 0.02-fold at 3.125 and 6.25 μM, respectively) were lower in the cells treated with eupatilin than in the control. In particular, eupatilin reduced the VEGF activation value by 31% at the level of 6.25 μM.

In conclusion, we confirmed the toxic effect of eupatilin in liver-cancer cells and confirmed the decrease in the level of MMP-2 mRNA after incubation with eupatilin in SNU182 cells. Eupatilin also inhibited tube formation through down-regulation of HIF-1α, VEGF, and Akt in HUVECs. Thus, tube-formation inhibition and MMP-2 mediated migration are likely to be important therapeutic targets of eupatilin in HCC metastasis.

Acknowledgements

This work was supported by a Gyeongnam National University of Science and Technology Grant in 2017. This research was also supported by the Basic Science Research Program of the National Research

Foundation of Korea (NRF), funded by the Ministry of Science, ICT & Future Planning (2017R1C1B5015841).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2018.08.034.

References

1. Farazi PA, DePinho RA. *Nat Rev Cancer.* 2006;6:674.
2. El-Serag HB, Rudolph KL. *Gastroenterology.* 2007;132:2557.
3. Yamamoto J, Kosuge T, Takayama T, et al. *Br J Surg.* 1996;83:1219.
4. He X, Lee B, Jiang Y. *Adv Exp Med Biol.* 2016;936:73.
5. Westermarck J, Kahari VM. *FASEB J.* 1999;13:781.
6. Deryugina EI, Quigley JP. *Cancer Metastasis Rev.* 2006;25:9.
7. Hanahan D, Weinberg RA. *Cell.* 2011;144:646.
8. Finley SD, Popel AS. *J Natl Cancer Inst.* 2013;105:802.
9. Seol SY, Kim MH, Rew JS, Choi MG. *Korean J Gastrointest Endosc.* 2004;28:230.
10. (a) Oh TY, Lee JS, Ahn BO, et al. *Gut.* 2001;49:364;

    (b) Seo HJ, Park KK, Han SS, et al. *Int J Cancer.* 2002;100:456.
11. Ryoo SB, Oh HK, Yu SA, et al. *Physiol Pharmacol.* 2014;18:383.
12. Ryu BK, Ahn BO, Oh TY, Kim SH, Kim WB, Lee EB. *Arch Pharm Res.* 1988;21:508.
13. Park JY, Lee D, Jang HJ, et al. *Evid Based Complement Alternat Med.* 2015;2015:483980.
14. Park JG, Lee SK, Hong IG, et al. *J Natl Cancer Inst.* 1994;86:700.
15. Gialeli C, Theocharis AD, Karamanos NK. *FEBS J.* 2011;278:16.
16. Yamaguchi H, Condeelis J. *Biochim Biophys Acta, Mol Cell Biol Lipids.* 2007;1773:642.
17. Bergmann U, Michaelis J, Oberhoff R, Knäuper V, Beckmann R, Tschesche H. *Clin Chem Lab Med.* 1989;27:351.
18. Salo T, Lyons JG, Rahemtulla F, Birkedal-Hansen H, Larjava H. *J Biol Chem.* 1991;266:11436.
19. Grigioni WF, D'errico A, Fortunato C, et al. *Modern pathol.* 1994;7:220.
20. Jung S, Kim HW, Lee JH, et al. *Cancer Res Clin Oncol.* 2002;128:469.
21. Park EH, Park JY, Yoo HS, Yoo JE, Lee HL. *Bioorg Med Chem Lett.* 2016;26:3291.
22. Park JY, Lee DS, Kim CE, et al. *J Ginseng Res.* 2017;41:1.
23. Hwang SH, Lee BH, Choi SH, et al. *J Ginseng Res.* 2016;40:325.
24. Iyer NV, Kotch LE, Agani F, et al. *Genes Dev.* 1998;12:149.
25. Semenza GL, Agani F, Iyer N, et al. *Ann N Y Acad Sci.* 1999;874:262.
26. Markowska AI, Liu FT, Panjwani N. *J Exp Med.* 1981;2010:207.
27. Milanini J, Vinals F, Pouyssegur J, Pages G. *J Biol Chem.* 1998;273:18165.
28. Yamaoka-Tojo M, Ushio-Fukai M, Hilenski L, et al. *Circ Res.* 2004;95:276.
29. (a) Kim NY, Koh HJ, Li H, Lee HJ, Ryu JH. *Nat Prod Sci.* 2017;23:92;

    (b) Taher M, Aminuddin A, Susanti D, et al. *Nat Prod Sci.* 2016;22:122;

    (c) Yoon DH, Han C, Fang Y, et al. *Nat Prod Sci.* 2017;23:162;

    (d) Guon TE, Chung HS. *Nat Prod Sci.* 2017;22:209;

    (e) Kim NH, Yang MH, Heo JD, Sung SH, Jeong EJ. *Nat Prod Sci.* 2016;22:53.