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**A B S T R A C T**

The biological activities of the ethanol extract from *Cirsium japonicum* var. *maackii* (ICF-1) and its major component, polyphenol cirsimaritin, were investigated as part of the search for possible alternative drugs for breast cancer. Three in vitro cell-based assays were used: the cell proliferation assay, tube-formation assay, and Western blot analysis. Both the ICF-1 extract and cirsimaritin inhibited the viability of HUVECs in a dose-dependent manner. The inhibition achieved was 36.89% at a level of 200 μg/ml by the ICF-1 extract and 62.04% at a level of 100 μM by cirsimaritin. The ICF-1 extract and cirsimaritin reduced tube formation by 12.69% at level of 25 μg/ml and 32.18% at the levels of 6.25 μM, respectively. Cirsimaritin inhibited angiogenesis by downregulation of VEGF, p-Akt and p-ERK in MDA-MB-231 cells, suggesting that cirsimaritin is potentially useful as an anti-metastatic agent. The present study demonstrated that *Cirsium japonicum* extract and its active component cirsimaritin is an excellent candidate as an alternative anti-breast cancer drug.

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Breast cancer is the most diagnosed cancer and the third leading cause of cancer mortality in women. Lifetime risk for breast cancer is one in eight women in developed countries.¹,² More importantly, triple-negative breast cancers (TNBCs), which lack the expressions of estrogen/progesterone receptors (ER/PR) and human epidermal growth factor receptor 2 (HER2), are associated with poor prognoses.³ To date, clinically effective drugs for the treatment of TNBC have not been reported.⁴ Metastasis of tumors, including breast cancer, involves complex processes, such as tumor cell dissociation, intravasation, extravasation, adhesion, and angiogenesis.⁵ Angiogenesis, which is the process of forming new capillaries, involves extensive interplay among cells, soluble factors, and extra cellular matrices.⁶,⁷ It also plays an important role in tumor growth.⁷,⁸ The vascular endothelial growth factor (VEGF) in tumor progression is an absolute regulator of angiogenesis.⁹ VEGF expression has been reported in a number of cancer cell lines and in several clinical specimens derived from cancer tumors. Therefore, the hostility of VEGF can effectively prevent tumor growth via incomplete blood vessel formation.¹⁰ *Cirsium japonicum*, a member of the Compositae family, is a wild perennial herb found in Korea, China and Japan. It is listed in the Korean and Chinese pharmacopoeias and has been used as a traditional antihemorrhagic, antihypertensive, antihepatitis, and uretic medicine in Korea.¹¹ *C. japonicum* contains many medicinal components including cirsimaritin, pectolinarins, pectolinarigenin, aca-cetins, thamnoglucosides, ciryneols A–E, heptadecenes, and so on.¹² To date, many studies have been conducted to explore the effects of *C. japonicum* on various diseases, including cancer. However, there have been virtually no reports on the effects on angiogenesis and its action mechanism.¹³,¹⁴
In the present study, in vitro experiments were conducted to investigate the effects of standardized *C. japonicum* extract (ICF-1) and its components, cirsimaritin (Fig. 1A) on the angiogenesis process in HUVECs and MDA-MB-231 cells. ICF-1 was prepared from the dried aerial part of *Cirsium japonicum* harvested on May and November (3:2), supplied from Imsil Herbal Medicine and extracted using 30% EtOH for 3 h (7 L × 3) under reflux at 65–75°C. The cirsimaritin content analyzed by HPLC was 7.815 ± 0.072 mg/g *C. japonicum* 30% EtOH extract (Fig. 1B). Based on HPLC results, 0.6–4.9 μM of cirsimaritin are contained in 25–200 μg/ml ICF-1 extract, respectively.

The antiangiogenic potential of the extract was assessed using HUVECs. HUVECs are cells isolated from the endothelium of veins from the umbilical cord and are widely used to study endothelial physiology and pathology. Endothelial cell proliferation is one of the complex multistep processes involved in angiogenesis. In this assay, cytotoxic substances may affect changes in morphology and cell growth and may result in cell death. Therefore, the ability of the extract to inhibit the proliferation of endothelial cells was examined using an MTT assay.

Fig. 2A shows the effect of the ICF-1 extract on percent viability of HUVECs. The ICF-1 extract inhibited the viability of HUVECs in a dose-dependent manner. The extract at concentrations of less than 25 μg/ml did not show significant effects on HUVECs viability, whereas at 50–200 μg/ml, cell viability decreased significantly. The ICF-1 extract inhibited the viability of HUVECs by 36.89% at a level of 200 μg/ml. The cell viability at this level was 63.11 ± 2.97% of the control. Fig. 2B shows photographs of tube formation of HUVECs at the level of 0 (control) and 25 μg/ml of the ICF-1 extract. When the effect of the ICF-1 extracts on the ability of HUVEC cells to form tubes was examined, the extract had reduced the tube formation by 12.69% at a level of 25 μg/ml (Fig. 2B).

![Fig. 1. Content of cirsimaritin in standardized *C. japonicum* 30% ethanolic extract (ICF-1). (A) The chemical structure of cirsimaritin. (B) The HPLCs of cirsimaritin in the ICF-1 extract and the standard.](image-url)
Fig. 3A shows photographs of tube formation of HUVECs at the levels of 0 (control), 3.125 and 6.25 μM of the cirsimaritin. The inhibitory effects of cirsimaritin on tube formation in HUVECs were 18.09% and 32.18% at the levels of 3.125 μM and 6.25 μM, respectively (Fig. 3B). Fig. 3C shows the effect of cirsimaritin on percent viability of HUVECs. Cirsimaritin also inhibited the viability of HUVECs in a dose-dependent manner. The cell viability (% of control) ranged from 89.94 ± 3.79% (3.12 μM) to 37.96 ± 1.27% (100 μM). Cirsimaritin inhibited the viability of HUVECs by 62.04% at the level of 100 μM. Treatments with up to 6.25 μM of cirsimaritin had no significant effect on the HUVECs, whereas treatment with 12.50–100.00 μM decreased cell viability significantly. Fig. 3D shows the effect of doxorubicin on percent viability of HUVECs. Doxorubicin is a broad spectrum antibiotic used in the treatment of breast cancers. The cell viability (% of control) ranged from 14.20 ± 1.31% (3.125 μM) to 11.66 ± 1.73% (100 μM) (Fig. 3D). Cytotoxic effect of doxorubicin in HUVECs was very stronger than cirsimaritin. Maximum doses of the ICF-1 extract and cirsimaritin showing no toxic effects in the present study were selected for further tube formation assays.

One recent report demonstrated that cirsimaritin obtained from Cirsium japonicum Fisch ex DC inhibited breast cancer stem cell-like properties and reduced the chemoresistance of the cancer cells to chemotherapy in MCF-7 breast cancer cells. In addition, cirsimaritin exhibited cytotoxic activity against human colonic carcinoma Caco-2, lung carcinoma A549 and melanoma A375 cells. However, neither its mechanism of action nor its anti-angiogenic potential in HUVECs had been elucidated until the present study.

Fig. 4A and B shows the effect of cirsimaritin on percent viability of MDA-MB-231 cell and MCF-7 human breast adenocarcinoma cells, respectively. MDA-MB-231 cells, which are invasive TNBC
cells, are extremely aggressive and resistant to several anticancer agents. Therefore, MDA-MB-231 cells are an ideal in vitro model with which to investigate the effects of antimetastatic agents. The effects obtained in the present study are shown in Fig. 4A. Cirsimaritin did not show a significant effect on MDA-MB-231 cell viability at the level of 3.125 μM (88.19 ± 4.30% of control). It reduced cell viability slightly at levels from 6.25 μM (83.19 ± 4.26% of control) to 50 μM (78.74 ± 4.01% of control). On the other hand, cirsimaritin significantly inhibited cell viability by 38.56% and 52.73% at the levels of 100.00 and 200.00 μM. However, cirsimaritin reduced MCF-7 cell viability slightly at levels from 3.125 μM (81.80 ± 0.61% of control) to 100 μM (53.35 ± 0.36% of control) (Fig. 4B). Because the results from levels less than 6.25 did not significantly inhibit the MDA-MB-231 cell proliferation, the 3.125 and 6.25 levels were selected for further mechanism studies.

It is well known that MMPs plays a major role in promoting tumor growth and metastasis and aberrant overexpression of MMP-9 has been shown to be closely associated with the progression and invasion of human breast cancer cells. RT-PCR analysis was used to evaluate the expression of MMP-9 in MDA-MB-231 cells. As shown in Fig. 4C and D, the levels of the MMP-9 mRNA were decreased, compared with control untreated cells, after 24 h of incubation with cirsimaritin (3.125 and 6.25 μM).

Vascular endothelial growth factor (VEGF), which is a pro-angiogenic factor, has been known to play an important role in tumor angiogenesis. Therefore, it is a promising candidate for therapeutic intervention. Proliferation, migration, and tube-like structure formation of cultured endothelial cells provide typical characteristics of in vitro angiogenesis through the assay. These cellular events are usually stimulated by intracellular signal cascades, such as the MEK/ERK pathway for endothelial cell proliferation and the Akt/eNOS axis for cell survival. Moreover, these signals contribute to endothelial differentiation and tube-like structure formation. Therefore, endothelial cell activation is an initial step of the angiogenic process.

Fig. 5A shows a photograph of the electrophoresis of the Western blot analysis obtained in the present study. Fig. 5B shows the levels of VEGF, phosphorylated-Akt (p-Akt) and phosphorylated-extracellular-signal-regulated kinases (p-ERK). As shown in Fig. 5B, the Western blot analysis showed that the expressions of VEGF (0.78 ± 0.02 and 0.78 ± 0.01-fold at 3.12 and 6.25 μM, respectively), p-Akt (0.68 ± 0.01 and 0.66 ± 0.02-fold at 3.12 and 6.25 μM, respectively), and p-ERK (0.80 ± 0.02 and 0.57 ± 0.01-fold at 3.125 and 6.25 μM, respectively) were lower in the cells treated with cirsimaritin than in the control. In particular, cirsimaritin reduced the p-ERK activation value by 43.00% at the level of 6.23 μM. The present study thus demonstrates that cirsimaritin
could inhibit angiogenesis by downregulation of VEGF, p-Akt and p-ERK in MDA-MB-231 cells, suggesting that cirsimaritin is potentially useful as an anti-metastatic agent.

Some results in the present study are consistent with the results found in sesame seeds (Sesamum indicum) and green tea studies. Sesamin, found in sesame seeds, effectively inhibited the macrophage-induced VEGF and proangiogenic activity in breast cancer cells. Akt and p38 activities were also strongly inhibited by epigallocatechin-3-gallate (EGCG), which also inhibited VEGF production by inhibiting both the constitutive activation of Stat3 and NF-kappa B. However, it did not inhibit the ERK or Akt, in MDA-MB-231 cells.

Cirsium japonicum extract and its active component cirsimaritin inhibited tube formation in HUVECs. Cirsimaritin reduced the cell viability of MDA-MB-231 cells in a dose dependent manner. The protein expressions of VEGF, p-Akt and p-ERK, markers for angiogenesis, were also inhibited by cirsimaritin in MDA-MB-231 cells. These findings suggest that cirsimaritin is potentially useful as an anti-metastatic agent in breast cancer. Also, HPLC analysis showed that the amount of cirsimaritin in the extract was 7.82 ± 0.07 mg/g (0.78%) in the present study, suggesting that cirsimaritin plays important role in the beneficial effects of Cirsium japonicum extract. Cirsium japonicum extract and its active component cirsimaritin can be an excellent candidate for an alternative drug to fight breast cancer.

**Conflict of interest**

The authors declare no competing financial interest.

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**Fig. 4.** Antimetastatic effectiveness of cirsimaritin on MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with cirsimaritin at a series of concentrations (3.125–200 μM) or the DMSO vehicle (control) for 24 h, and then cell viability was evaluated by the MTT assay. (B) MCF-7 cells were treated with cirsimaritin at a series of concentrations (3.125–200 μM) or the DMSO vehicle (control) for 24 h, and then cell viability was evaluated by the MTT assay. (C) Expression of the MMP-9 mRNA in cirsimaritin-treated MDA-MB-231 cells. MDA-MB-231 cells (2.0 × 10^6 cells/6-cm dish) were treated with cirsimaritin at the indicated concentrations (0, 3.125, and 6.25 μM) for 24 h. Total RNA was extracted from cirsimaritin-treated MDA-MB-231 cells, and MMP-9 expression was analyzed by reverse transcription-PCR (RT-PCR). The size of the product is 272 bp. β-Actin was used as an internal control. (D) Bar graphs showing quantification of the expression level of MMP-9 compared with the expression of β-actin.
Fig. 5. Effects of cirsimaritin on angiogenic protein expressions in MDA-MB-231 Cells. (A) Results of the Western blot show the levels of VEGF, phosphorylated-Akt, Akt, phosphorylated-extracellular-signal-regulated kinases (ERK) and ERK in MDA-MB-231 cells treated with cirsimaritin at different concentrations for 24 h. Whole cell lysates (20 μg) 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 cirsimaritin on the angiogenic protein expressions in MDA-MB-231 cells. Results of the Western blot show the levels of VEGF, phosphorylated-Akt, Akt, phosphorylated-extracellular-signal-regulated kinases (ERK) and ERK in MDA-MB-231 cells treated with cirsimaritin at indicated concentrations for 24 h.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.07.070.

References

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