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The Inhibitory Effects of *Forsythia Koreana* Extracts on the Metastatic Ability of Breast Cancer Cells and Bone Resorption by Osteoclasts

ORIGINAL ARTICLE

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Background: Breast cancer is the most common malignant disease in women. The patients with advanced breast cancer develop metastasis to bone. Bone metastasis and skeletal-related events by breast cancer are frequently associated with the invasiveness of breast cancer cells and osteoclasts-mediated bone resorption. *Forsythia koreana* is used in oriental traditional medicine to treat asthma, atopy, and allergic diseases. The aim of this study was to evaluate the inhibitory effects of *F. koreana* extracts on the invasion of breast cancer cells and bone resorption by osteoclasts.
Methods: Cell viability was measured by an MTT assay and the migration and invasion of MDA-MB-231 cells were detected by a Boyden chamber assay. The formation of osteoclasts and pit was detected using tartrate-resistant acid phosphatase staining and calcium phosphate-coated plates, respectively. The activities of matrix metalloproteinases (MMPs) and cathepsin K were evaluated by gelatin zymography and a cathepsin K detection kit.
Results: The fruit and leaf extracts of *F. koreana* significantly inhibited the invasion of MDA-MB-231 cells at noncytotoxic concentrations. The fruit extract of *F. koreana* reduced the transforming growth factor β1-induced migration, invasion and MMPs activities of MDA-MB-231 cells. In addition, the fruit, branch, and leaf extracts of *F. koreana* also inhibited the receptor activator of nuclear factor kappa-B ligand-induced osteoclast formation and osteoclast-mediated bone-resorbing activity by reducing the activities of MMPs and cathepsin K.
Conclusions: The extracts of *F. koreana* may possess the potential to inhibit the breast cancer-induced bone destruction through blocking invasion of breast cancer cells, osteoclastogenesis, and the activity of mature osteoclasts.
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Key Words: *Forsythia koreana*, Breast neoplasm, Bone metastasis, Osteoclast, Bone resorption

INTRODUCTION

Bone metastasis frequently occurs in patients with metastatic breast cancer.[1] Bone microenvironment is suitable for breast cancer metastasis because of various growth factors in bone matrix such as insulin-like factors, TGF-β, fibroblast growth factors, platelet-derived growth factors, and bone morphogenetic proteins.[2,3] In normal bone, there is balance between osteoclasts which resorb bone and osteoblasts which form bone. Metastatic breast cancer cells secrete osteolytic factors, including parathyroid hormone-related protein, interleukin (IL)-6, IL-8, and IL-11.[4] These factors stimulate osteoblasts or stromal cells to produce receptor activator of nuclear factor kappa-B ligand (RANKL) and inhibit decoy receptor osteoprotegerin. RANKL binds to RANK of osteoclasts precursors and induces osteoclast differentiation, causing bone resorption.[5,6] The growth factors are released from bone matrix by bone resorption and promote breast cancer cell proliferation and secretion of osteolytic factors.[7,8] Therefore, osteoclasts, as well as breast cancer cells, can be a strategic therapeutic target for breast cancer patients with bone metastasis.[9,10]

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Traditional medicine plants are being considered as promising research materials in drug discovery.[11] *Forsythia koreana*, a flowering plant in the family Oleaceae, is widely cultivated in South Korea and used to treat inflammatory diseases in Chinese medicine.[12] *F. koreana* extract has various pharmacological properties including antioxidant, anti-bacterial, and anti-allergic activities.[13-15] In particular, *F. koreana* fruit extract inhibits COX-2-mediated prostaglandin E_2 production and nitric oxide synthase, indicating anti-inflammatory effects.[16,17] Recent studies reported that the bioactive compounds from *F. koreana* fruit extract have cytotoxicity against Michigan Cancer Foundation (MCF)-7 human non-metastatic breast cancer cells via activation of caspase-8 and induction of the DR4/5 death receptors.[18] However, the effect of *F. koreana* extracts on metastatic breast cancer cells and osteoclasts, which are target cells for blocking cancer-induced bone destruction, remains unproved.

The aim of this study is to evaluate the protective potential of *F. koreana* extracts on breast cancer cell-induced bone destruction. We investigated the effects of *F. koreana* extracts on the migration and invasion of breast cancer cells and the formation and activation of osteoclasts.

MATERIALS AND METHODS

1. Materials

F. koreana Nakai branches and leaves were extracted with methanol and concentrated. Fruit extract was provided by Korea National Research Resource Bank. The extracts were dissolved with dimethyl sulfoxide (DMSO). Dulbecco's modified Eagle medium (DMEM), FBS, Dulbecco's PBS, α-minimum essential medium (α-MEM), and antibiotics were purchased from Gibco BRL (Grand Island, NY, USA). Recombinant mouse soluble RANKL (sRANKL) and macrophage-colony stimulating factor (M-CSF) were obtained from R&D Systems (Minneapolis, MN, USA). Histopaque-1083, MTT, DMSO were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2. Animal

Four-week old male Institute of Cancer Research (ICR) mice were purchased from the Nara Biotech (Pyeongtaek, Korea). The mice were provided free access to a commercial rodent chow and tap water *ad libritum*, and housed under specific pathogen-free conditions with a 12 hours light/dark cycle at 22°C ± 2°C. All animal experimental procedures were conducted in compliance with the guidelines and regulations for the use and care of animals established by Yonsei University College of Dentistry (Seoul, Korea).

3. Cell cultures

Human mammary carcinoma cell line MDA-MB-231 was obtained from the Korea Cell Line Bank (Seoul, Korea) and the cells were maintained in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic mixture at 37°C. Mouse bone marrow-derived macrophages (BMMs) were isolated from tibiae of 4-week-old male ICR mice and cultured in a α-MEM containing 10% FBS, 30 ng/mL M-CSF, and 1% antibiotic-antimycotic mixture at 37°C. All of the cells were incubated in a humidified atmosphere of 5% CO_2.

4. Cell viability

MDA-MB-231 cells (1×10^3 cells/well) were seeded into a 96-well plate with DMEM containing 10% FBS and the attached cells were incubated in serum-free media with the various concentrations of *F. koreana* fruit, branch or leaf extract for 24 hours. BMM cells (5×10^4 cells) were treated with the indicated concentration of *F. koreana* fruit, branch or leaf extract in α-MEM containing 10% FBS and 30 ng/mL M-CSF for 5 days with replacement to fresh medium every second day. The cell viability was measured using an MTT assay.[19]

5. Cell migration and invasion

Migration assay was performed in 24-well transwell chamber (8 mm pore size; Corning Costar, Cambridge, MA, USA) coated with 10% (w/v) gelatin. Invasion assay was performed using 24-well transwell chamber coated with 10% (w/v) gelatin and 1 mg/mL Matrigel (BD Biosciences, Palo Alto, CA, USA). MDA-MB-231 cells (5×10^4 cells/200 μL) were incubated in serum-free DMEM containing *F. koreana* fruit extract at the indicated concentrations and/or TGF-β1 (10 ng/mL) in the upper part of a transwell chamber for 6 hours or 24 hours. The lower part of chamber were added with 600 μL DMEM containing 5% FBS and *F. koreana* fruit extract at the indicated concentrations. The migrated or invaded cells to the lower surface of the membrane were fixed with 70% methanol, stained with hematoxylin, and then counted using a Zeiss Axio imager microscope (Zeiss, Oberkochen, Germany).

6. Osteoclast formation

BMMs (5×10^4 cells/well) were seeded in 96-well plate and cultured with α-MEM containing 10% FBS, M-CSF (30 ng/mL), sRANKL (100 ng/mL), and the various concentrations of *F. koreana* extracts for 5 days. The medium was replaced with fresh medium every 2 days. The cells were fixed with 3.7% (v/v)

formaldehyde and stained using the Acid Phosphatase Leukocyte kit (Sigma-Aldrich). The multinucleated tartrate-resistant acid phosphatase-positive cells (≥ 3 nuclei) were counted (× 100, magnification).

7. Pit formation

BMMs (5×10^4 cells/well) were seeded in an Osteo assay plate (Corning, Cambridge, MA, USA) and cultured in α-MEM containing 10% FBS, M-CSF (30 ng/mL), and sRANKL (100 ng/mL) for 5 days to induce osteoclastogenesis. The cells were then treated with the various concentrations of *F. koreana* extracts for additional 2 days. The media were collected to measure the activities of MMPs and cathepsin K and the adherent cells were removed using 5% sodium hypochlorite solution. The resorbed pits were observed under light microscope (× 100, magnification).

8. Activities of matrix metalloproteinases and cathepsin K

MDA-MB-231 cells (5×10^5 cells/dish) were plated in 60 mm culture dishes and incubated with indicated concentrations of *F. koreana* fruit extract in DMEM containing 1% FBS and TGF-β1 (10 ng/mL) for 24 hours. The culture media of MDA-MB-231 cells and RANKL-induced osteolcasts were collected by centrifugation at 200 ×*g* for 5 minutes. The activities of MMP-2 and MMP-9 were detected by gelatin zymography and cathepsin K activity was measured with commercially available kit as described previously.[20]

9. Statistics analysis

Data were expressed as mean ± SE of three independent experiments. Statistical analysis was performed with a one-way ANOVA and Student's *t*-test to express the difference between the two groups. Results with values of $P < 0.05$ were considered statistically significant. Statistical analysis was performed with SPSS statistical software ver. 21 (IBM Co., Endicott, NY, USA).

RESULTS

1. *Forsythia koreana* extracts inhibited the invasion of MDA-MB-231 human breast cancer cells

We first investigated the effects of *F. koreana* fruit, branch, and leaf extracts on the viability of MDA-MB-231 metastatic human breast cancer cells. The fruit extract inhibited the cell viability by 15% at 40 μg/mL and by 33% at 100 μg/mL (Fig. 1A) but the branch (Fig. 1B) and leaf (Fig. 1C) extracts did not affect. Next, we found

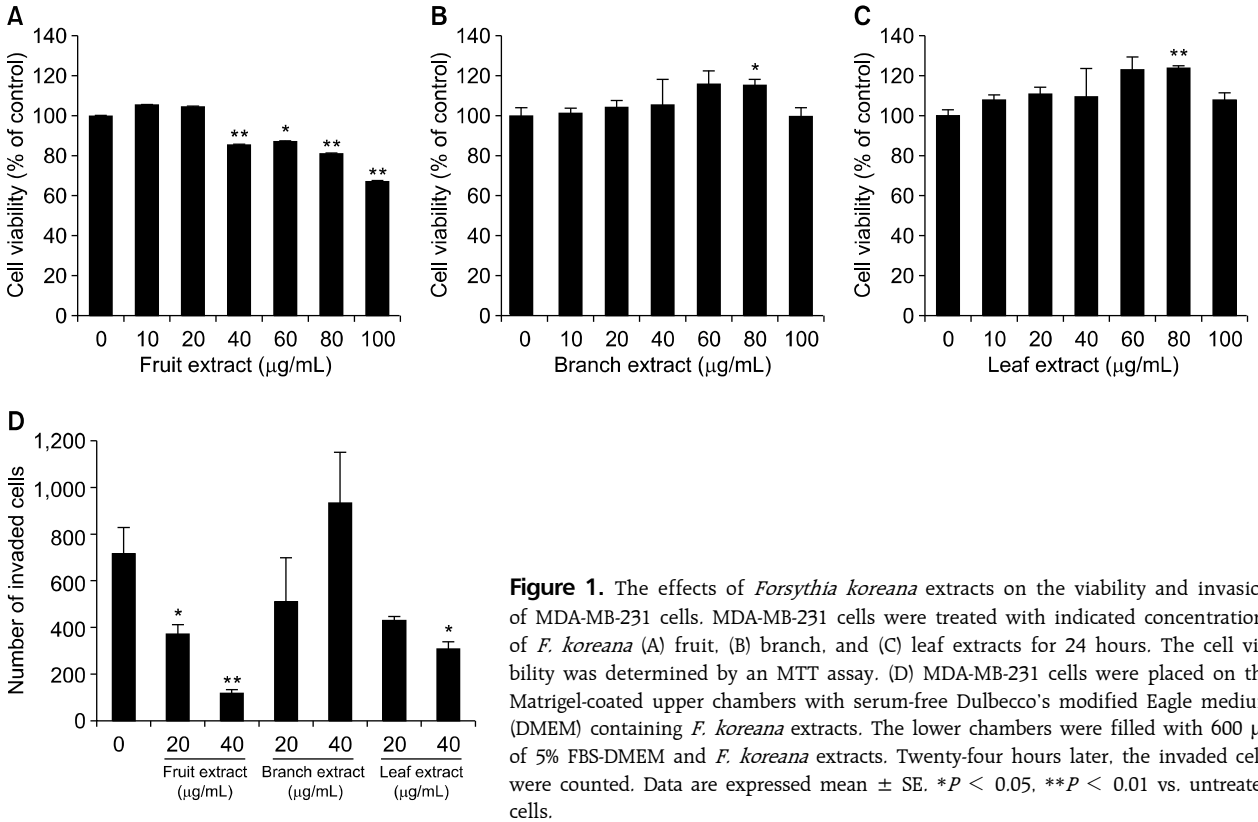

Figure 1. The effects of *Forsythia koreana* extracts on the viability and invasion of MDA-MB-231 cells. MDA-MB-231 cells were treated with indicated concentrations of *F. koreana* (A) fruit, (B) branch, and (C) leaf extracts for 24 hours. The cell viability was determined by an MTT assay. (D) MDA-MB-231 cells were placed on the Matrigel-coated upper chambers with serum-free Dulbecco's modified Eagle medium (DMEM) containing *F. koreana* extracts. The lower chambers were filled with 600 μL of 5% FBS-DMEM and *F. koreana* extracts. Twenty-four hours later, the invaded cells were counted. Data are expressed mean ± SE. $*P < 0.05$, $**P < 0.01$ vs. untreated cells.

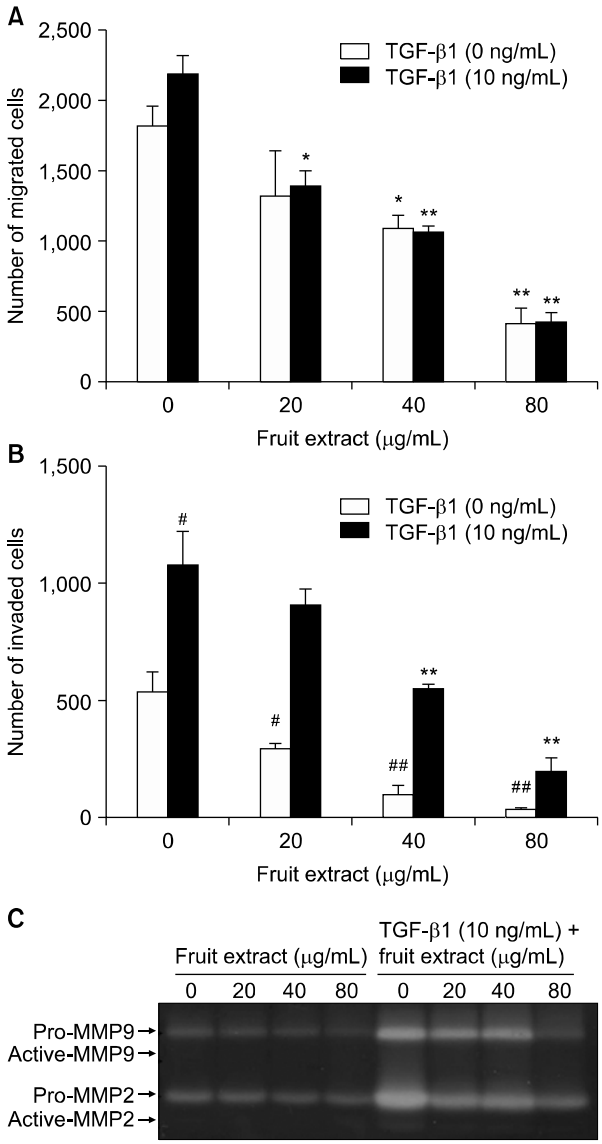

Figure 2. The effects of *Forsythia koreana* fruit extract on TGF-β1-induced migration and invasion of MDA-MB-231 cells. (A) MDA-MB-231 cells were placed on the upper chambers with serum-free Dulbecco's modified Eagle medium (DMEM) containing *F. koreana* fruit extract and TGF-β1 (10 ng/mL). The lower chambers were filled with 600 μL of 5% FBS-DMEM containing *F. koreana* fruit extract. The cells were incubated for 6 hours. (B) MDA-MB-231 cells were placed on the Matrigel-coated upper chambers with serum-free DMEM containing *F. koreana* fruit extract and TGF-β1 (10 ng/mL). The lower chambers were filled with 600 μL of 5% FBS-DMEM containing *F. koreana* fruit extracts. The cells were incubated for 24 hours. The migrated or invaded cells were fixed, stained, and counted as a described in Materials and Methods. Data are expressed mean ± SE. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. untreated cells, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. MDA-MB-231 cells with TGF-β1 alone. (C) MDA-MB-231 cells were cultured with *F. koreana* fruit extract and 1% FBS-DMEM in the absence or presence of TGF-β1 (10 ng/mL) for 24 hours. The levels of matrix metalloproteinase (MMP)-2 and MMP-9 in the collected medium were examined using gelatin zymography. The clear bands indicate the activities of MMPs.

that *F. koreana* fruit and leaf extracts significantly reduced the invasion of MDA-MB-231 cells by 48% and 41% at 20 μg/mL and 84% and 58% at 40 μg/mL, respectively (Fig. 1D).

2. *Forsythia koreana* fruit extract attenuated the TGF-β1-induced migration and invasion of MDA-MB-231 cells

TGF-β1 plays a critical role in the migration and invasion of breast cancer cells.[21,22] MMPs are closely associated with cancer invasion and metastasis.[23] The treatment with *F. koreana* fruit extract decreased the migration (IC_{50} = 50.6 μg/mL; Fig. 2A) and invasion (IC_{50} = 22.5 μg/mL; Fig. 2B) of MDA-MB-231 cells by 75% and 92% at 80 μg/mL, respectively. TGF-β1-induced migration (IC_{50} = 49.5 μg/mL; Fig. 2A) and invasion (IC_{50} = 40.9 μg/mL; Fig. 2B) were also reduced by *F. koreana* fruit extract by 79% and 82% at 80 μg/mL, respectively. In addition, the fruit extract suppressed the proteolytic activities of MMP-2 and MMP-9 in the culture media of MDA-MB-231 cells with or without TGF-β1 (Fig. 2C).

3. *Forsythia koreana* extracts blocked the receptor activator of nuclear factor kappa-B ligand-induced osteoclast formation

Bone loss induced by metastasis of breast cancer is caused by excessive osteoclasts.[5] The viability of BMMs as osteoclast precursors was not reduced by the treatment with *F. koreana* fruit (Fig. 3A), branch (Fig. 3B) or leaf (Fig. 3C) extracts for 5 days. RANKL-induced osteoclast formation was significantly decreased by 24%, 58%, and 92% at 10 μg/mL and 79%, 95%, and 100% at 20 μg/mL of the fruit, branch, and leaf extracts, respectively (Fig. 3D).

4. *Forsythia koreana* extracts suppressed the bone-resorbing activity of mature osteoclasts

In bone mimetic material-coated plates, three *F. koreana* extracts substantially inhibited the formation of osteoclast-mediated resorption pits (Fig. 4A). In gelatin zymography, *F. koreana* branch extract suppressed the activities of MMP-2 and MMP-9 derived from RANKL-induced osteoclasts but the fruit and leaf extracts did not show noticeable inhibition (Fig. 4B). In addition, the cathepsin K activities induced by RANKL stimulation were significantly decreased by the treatment with *F. koreana* fruit, branch, and leaf extracts by 67%, 80%, and 78% at 40 μg/mL, respectively (Fig. 4C).

DISCUSSION

In the present study to investigate whether *F. koreana* extracts

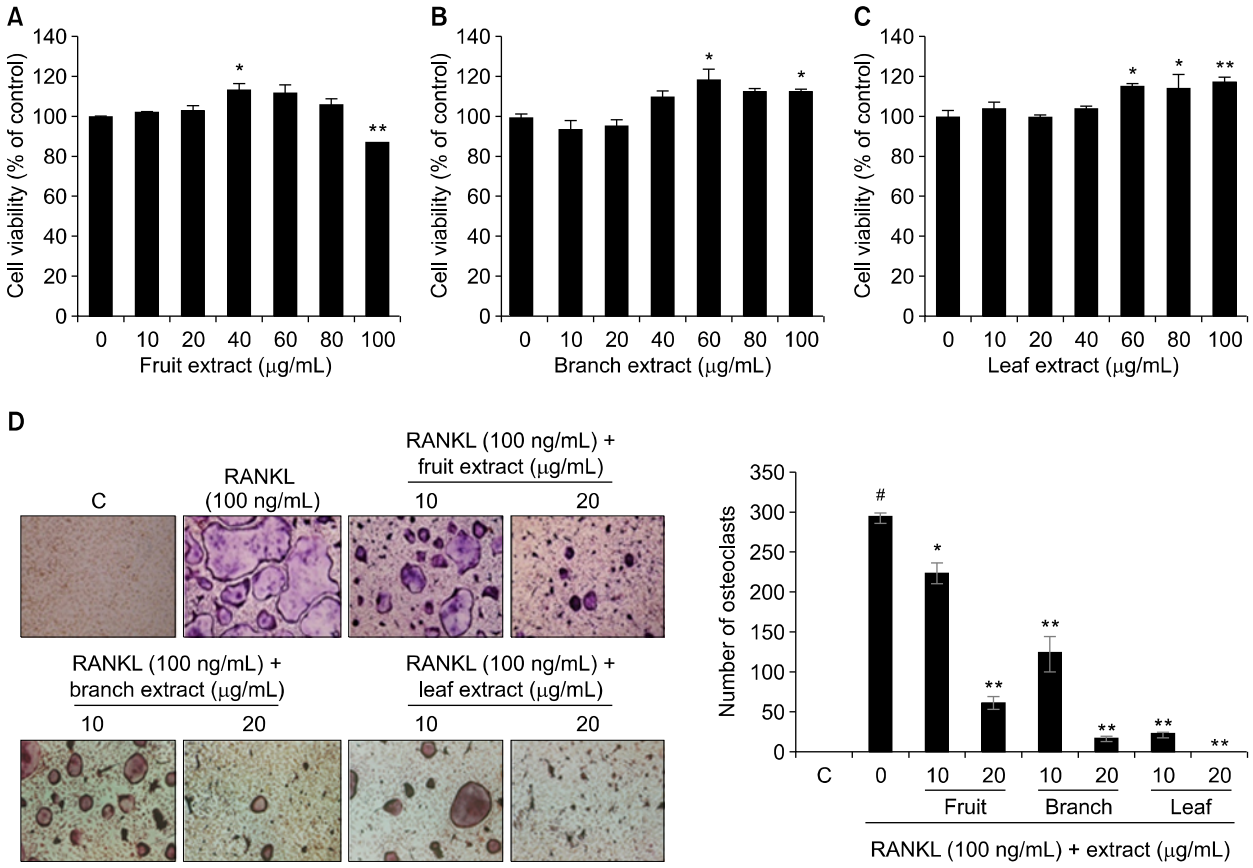

Figure 3. The effects of *Forsythia koreana* extracts on receptor activator of nuclear factor kappa-B ligand (RANKL)-induced osteoclast formation. The bone marrow-derived macrophages (BMMs) were treated with M-CSF (30 ng/mL) and indicated concentrations of *F. koreana* (A) fruit, (B) branch, or (C) leaf extracts for 5 days. The cell viability was determined by an MTT assay. Data are expressed as the mean ± SE. $*P < 0.05$, $**P < 0.001$ vs. untreated cells. (D) The BMMs were cultured in α-minimum essential medium with M-CSF (30 ng/mL), RANKL (100 ng/mL) and *F. koreana* extracts at the indicated concentrations for 5 days. The cells were detected by tartrate-resistant acid phosphatase staining and osteoclasts were counted (× 100, magnification). Data are expressed as the mean ± SE. $^{\#}P < 0.05$ vs. BMMs without RANKL (C, control). $*P < 0.05$, $**P < 0.001$ vs. BMMs with RANKL alone.

have preventive potential on breast cancer-induced bone destruction, *F. koreana* fruit extract suppressed the viability of MDA-MB-231 metastatic breast cancer cells but did not show high cytotoxic effect. The fruit and leaf extracts at the non-cytotoxic concentrations considerably inhibited the invasiveness of MDA-MB-231 cells. Cancer cells interact with many growth factors and cytokines during progression and metastasis.[24,25] Among them, TGF-β1 enhances the metastatic ability of breast cancer cells through stimulation of epithelial-mesenchymal transition processes and the activation of the MMPs, which are required for cancer cells to degrade physical barriers during local expansion, intravasation, extravasation, and invasion at a distant location.[8,26] TGF-β1 modulates the homeostasis between MMPs and MMP inhibitors in highly invasive breast cancer cells.[27] In our data, *F. koreana* fruit extract, with weak cytotoxic activity but remarkable anti-invasive activity in MDA-MB-231 cells, inhibited TGF-β1-induced migration, invasion, and activities of MMP-2 and MMP-9. These results demonstrate the anti-metastatic potential of *F. koreana* fruit extract against metastatic breast cancer cells.

In breast cancer patients with bone metastasis, osteoclast formation, and activation were abnormally increased.[2] Several enzymes such as cathepsin K and MMPs were released from mature osteoclasts and these hydrolytic enzymes digest organic components of the bone matrix and promote bone resorption.[28] Thus, many researchers have paid much attention to osteoclast-targeting new agents for the treatment of patients with bone metastasis of breast cancer.[9,10] Recent studies suggest that breast cancer induced-bone destruction can be prevented by controlling RANKL-induced osteoclast formation and activities of these enzymes.[20,29-31] In our study, *F. koreana* fruit, branch, and leaf extracts did not show cytotoxicity at less than 100 μg/mL and significantly inhibited the RANKL-induced formation of osteoclasts

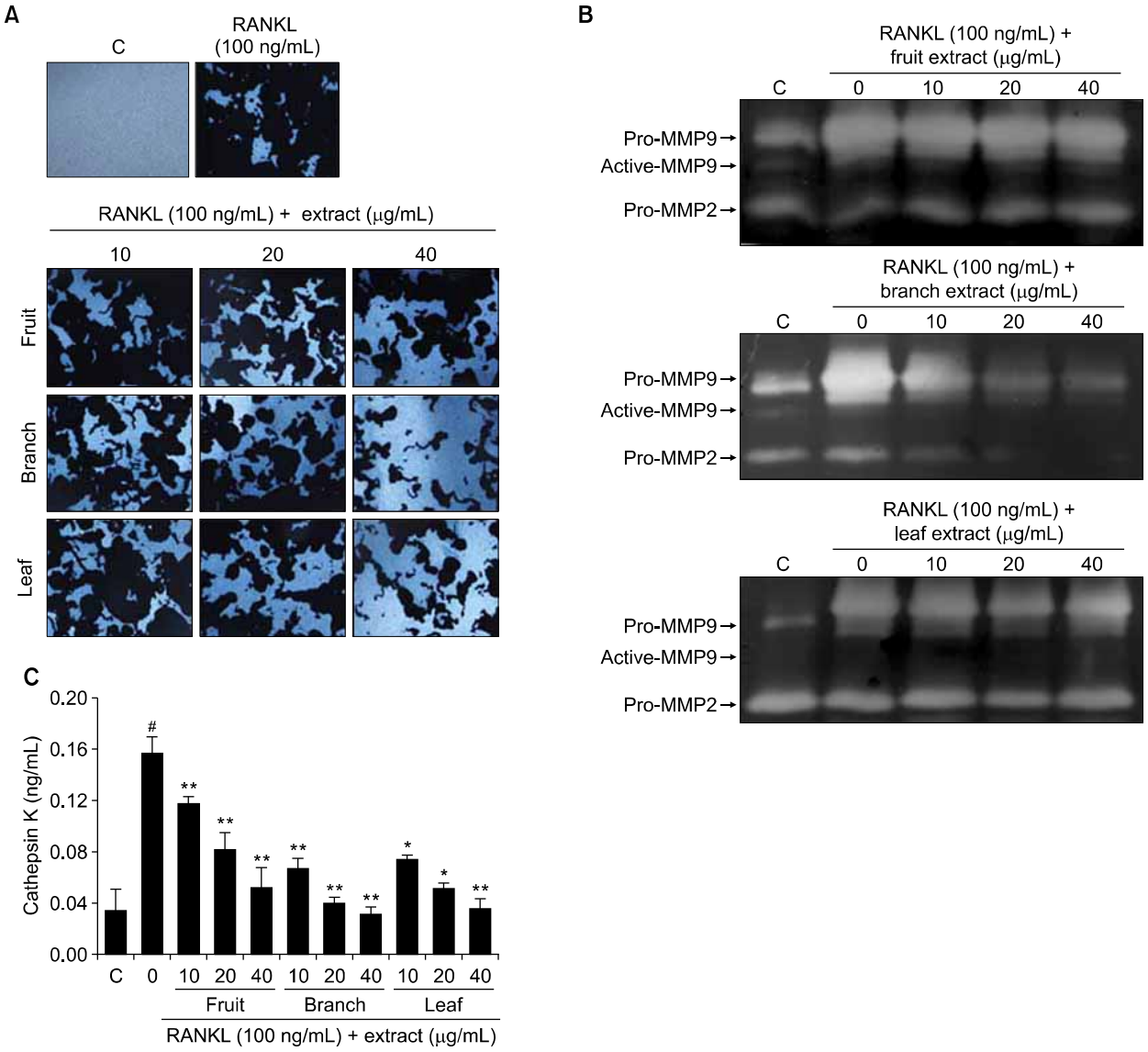

Figure 4. The effects of *Forsythia koreana* extracts on bone resorbing-activity of osteoclasts. Bone marrow-derived macrophages (BMMs) were seeded in Osteo assay plate and treated with M-CSF (30 ng/mL) and receptor activator of nuclear factor kappa-B ligand (RANKL) (100 ng/mL) for 5 days. After induction of osteoclasts, the cells were treated with *F. koreana* extracts for an additional 2 days. (A) The resorption pits were observed under a light microscopy (× 100, magnification). (B) The activities of matrix metalloproteinase (MMP)-2 and MMP-9 in cultured media of osteoclasts were detected by gelatin zymography as described in Materials and Methods. (C) Cathepsin K levels in cultured media of osteoclasts were measured using the Sensizyme Cathepsin K activity kit. Data are expressed as the mean ± SE. $^{\#}P < 0.05$ vs. BMMs without RANKL (C, control). $^{*}P < 0.05$, $^{**}P < 0.001$ vs. BMMs with RANKL alone.

and resorption pits at 20 μg/mL. Moreover, *F. koreana* branch extract, rather than the fruit and leaf extracts, substantially inhibited the secreted levels of MMP-2 and MMP-9. Three extracts decreased the release of osteoclast-derived cathepsin K into the culture media. These results indicate that *F. koreana* extracts may prevent breast cancer-related bone resorption by attenuating RANKL-induced osteoclast formation and activation.

Taken together, *F. koreana* fruit and leaf extracts have anti-metastatic potential on breast cancer cells and *F. koreana* extracts have anti-osteoclastogenic and anti-bone-resorbing activities. Therefore, *F. koreana* extracts can target both breast cancer cells and osteoclasts, thereby they may be promising agents for prevention of cancer-mediated bone loss and the related skeletal diseases.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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