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# Wheat seedling extract and its constituents attenuate RANKL-induced differentiation and fusion of osteoclasts and bone resorption

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## Abstract

The occurrence of osteoporosis gradually increases within the aging population. As the side effects of therapeutic agents currently used for osteoporosis are increasing, the development of preventive and therapeutic agents derived from natural products without any long-term side effects is important. Here, we investigated the effect of wheat seedling extract (WSE) on the RANKL-mediated differentiation, fusion, and function of osteoclasts. WSE inhibited the differentiation of RANKL-induced bone marrow macrophages and phosphorylation of AKT and ERK. Moreover, the protein and mRNA expression levels of c-Fos and NFATc1 as well as RANKL-induced transcription of *TRAP* and *OSCAR* were suppressed by WSE treatment. DC-STAMP and cathepsin K, which are essential for cell fusion and bone degradation, were also inhibited by WSE. Furthermore, eight components constituting WSE were confirmed to decrease the osteoclast TRAP activity. Taken together, WSE may have potential implications as a useful therapeutic or preventive agent for inhibition of bone loss.

**Keywords** Bone, Bone resorption, RANKL, Osteoclasts, Osteoclast fusion, Wheat seedling

## Introduction

Bone structurally supports the body, stores various minerals, and forms bone marrow structures necessary for the development and maturation of various immune cells through the process of remodeling. Bone homeostasis is maintained by the continuous formation and resorption of bone tissue. However, an imbalance in this process because of excessive bone resorption can lead to bone loss and osteoporosis [1]. The prevalence of osteoporosis is 7.3% in males and 38.0% in females above 50 years in Republic of Korea [2], which is very high and it is expected to accelerate further with the aging of the population. Currently, bisphosphonates account for more than 85% of osteoporosis treatment, but it is known to cause adverse effects such as fever, headache, pain in muscles or joints, and nephrotoxicity [3, 4]. Therefore, it is necessary to develop a preventive and therapeutic agent for osteoporosis derived from natural products that has no side effects and can be administered for a long time.

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Osteoclasts differentiate from the myeloid cells of the monocyte/macrophage lineage and are unique cells involved in the process of bone remodeling and bone resorption in aged individuals [5]. Osteoclastogenesis is a series of processes including differentiation, fusion, and activation of osteoclasts and is tightly regulated by many cytokines; both macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL) are necessary for osteoclast differentiation [6]. RANKL binds to a receptor called RANK on the surface of osteoclast precursors and activates AKT and mitogen-activated protein kinases (MAPKs), including the c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 pathways, which, in turn, continuously activate different transcription factors such as c-Fos and nuclear factor-activated T cells c1 (NFATc1) [7].

NFATc1 is a master transcription factor that drives terminal differentiation of osteoclasts by regulating specific genes such as *TRAP* (tartrate-resistant acid phosphatase), *cathepsin K*, *DC-STAMP* (dendritic cell-specific transmembrane protein) and *OSCAR* (osteoclast-associated receptor) [7, 8]. In addition, bone tissue damage resulting from bone resorption is related to the activation of osteoclast function via the activation of inflammation-related factors [7, 9]. Thus, many studies have been conducted on anti-resorptive activity and bone strengthening by regulating osteoclast differentiation and functional inhibition.

Plant foods, including vegetables, fruits, rice, barley, and wheat, are consumed in many countries for over thousands of years. They contain naturally occurring bioactive components that may promote human health. Wheat is one of the most important crops grown worldwide and is a major staple food crop for more than 40% of the world population [10]. Recently, wheat-based food have attracted extensive attention in the consumer market. Wheat seedlings are used as health functional supplement as they are rich in nutrients, such as amino acids, minerals, vitamins, and chlorophyll. It also produces gallic acid, epigallocathechin, epicatechin, and ρ-coumaric acid, which are undetected in wheat seed [11, 12]. Therefore, various studies have been conducted to examine the functionality, anti-mutagenicity [13], anticancer [14], and anti-oxidant effects of the compounds from wheat seedlings [15]. Furthermore, in our previous report, we identified and profiled various compounds in wheat seedling and confirmed the antioxidant effects of the extracts [16]. Additionally, the compounds we discovered in wheat seedlings have numerous known physiological effects. Isoorientin has been reported to suppress asthma and inflammation [17, 18], Isoschaftoside to alleviate fatty liver [19], and Isovitexin to have anti-inflammatory and anti-diabetic effects [20]. Particularly, Isoorientin and Isocalinoside have been reported to show effects on osteoporosis and chronic osteomyelitis in rats [21, 22]. We focused on the anti-osteoporosis effects of some wheat seedling compounds and confirmed that the impact of wheat seedling extracts(WSE) on osteoclast formation is not known. In this study, we investigated the effect of WSE and its bioactive compounds on RANKL-mediated osteoclast differentiation, fusion, and bone resorption and the potential mechanism underlying its anti-osteoclastogenic activity.

## Materials and methods

### Preparation of WSE and its compounds

The Korean wheat cultivar Keumkang was used in this study and the growth and extraction method are as follows. This cultivar was planted in 2019 using artificial soil in a growth chamber. The growth conditions were as follows: temperature, 18–20 °C; humidity, 60–70%; illumination intensity, 5500 lx; and light, 12 h → dark, 12 h (repeated alternatively). The wheat seedlings were harvested after 11 d. The harvested seedlings were air-dried at 50 °C for 2 d to remove moisture and were freeze-dried subsequently at −78 °C. The dried wheat seedlings (0.5 kg) were extracted using aqueous fermented ethanol (40%) for 12 h at 60℃. The extracts were filtered, evaporated in vacuum, and freeze-dried to obtain 56.4 g of WSE as dried powder. WSE was dissolved in 10% Dimethyl sulfoxide(DMSO), 45% MeOH, and 45% distilled water for quantitative analysis, and dissolved in 100% DMSO for biological activity experiments, and there were no insoluble components. We have previously reported on phenolic phytochemicals in WSE and method for isolation has been described in detail [16]. The list of eight compounds used in this experiment is shown in Table 1.

The structure, identification, quantitative analysis, and validation methods for the phenolic metabolites of WSE are shown in Additional file 1.

**Table 1** List of eight components constituting wheat seedlings

| Label | Identification | MW[a] |
|---|---|---|
| 1 | 3-*O*-Feruloylquinic acid | 368.3 |
| 2 | Luteolin-6-*C*-arabinoside-8-*C*-glucosdie (Isocarlinoside) | 580.5 |
| 3 | Luteolin-6-C-β-glucoside (Isoorientin) | 448.4 |
| 4 | Apigenin-6-*C*-arabinoside-8-*C*-β-glucoside (Isoschaftoside) | 564.5 |
| 5 | Isovitexin-2′′-*O*-glucoside (Meloside A) | 594.5 |
| 6 | Apigenin-6-*C*-glucoside (Isovitexin) | 432.4 |
| 7 | Isoscoparin-2′′-*O*-glucoside | 624.5 |
| 8 | Chrysoeriol-6-C-β-glucoside (Isoscoparin) | 462.4 |

[a] *MW* molecular weight

### Reagents and antibodies

Mouse soluble RANKL and M-CSF were purchased from R&D Systems (Minneapolis, MN, USA). Penicillin, streptomycin, alpha-minimum essential medium (α-MEM), and fetal bovine serum (FBS) were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Antibodies against the following proteins were purchased from the companies as indicated: actin, c-Fos, NFATc1, and secondary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA); p-AKT, AKT, and MAPKs (ERK, JNK, and p38) including the phosphorylated form (Cell Signaling Technology, Beverly, MA, USA).

### Ethics statement

This study was conducted in strict accordance with the recommendations of the Standard Protocol for Animal Study of Gangnam Severance Hospital Biomedical Center (Permit No. 2016 − 0238). The experimental protocol (ID No. 0238) was approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine. Every effort in this study was made to minimize the number of animals and their suffering, stress, and discomfort.

### Preparation of osteoclast precursor cells

All experiments were performed as described previously [23] with some modifications. Five-week-old male imprinting control region (ICR) mice (Damul Science Co. Deajeon, Korea) were maintained in a room illuminated daily from 07:00 to 19:00 (12-h light:12-h dark cycle), with controlled temperature (23 ± 1 °C) and ventilation (10–12 times per hour); humidity was maintained at 55 ± 5%, and the animals had free access to a standard animal diet and tap water. Bone marrow cells were obtained from the 5-week-old male ICR mice by flushing their femurs and tibias with α-MEM-containing antibiotics (100 units/ml penicillin, 100 μg/mL streptomycin). The bone marrow cells were cultured on culture dishes for 1 d in α-MEM supplemented with 10% FBS and M-CSF (10 ng/mL). The non-adherent bone marrow cells were plated on Petri dishes and cultured for 3 d in the presence of M-CSF (30 ng/mL). After washing out the non-adherent cells, the adherent cells were used as bone marrow-derived macrophages (BMMs).

### Osteoclast differentiation

The BMMs were maintained in α-MEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/mL streptomycin. The medium was changed every 3 d and BMMs were incubated a humidified atmosphere and 5% $CO_2$ at 37 °C. The BMMs ($1 \times 10^4$ cells/well in a 96-well plate or $3 \times 10^5$ cells/well in a 6-well plate) were cultured with M-CSF (30 ng/mL) and RANKL (10 ng/mL) for 4 d and multinucleated osteoclasts differentiated from BMMs were observed by microscopy.

### TRAP staining and activity assay

The mature osteoclasts were visualized by staining for TRAP, a biomarker of osteoclast differentiation. Briefly, the multinucleated osteoclasts were fixed with 10% formalin for 10 min, permeabilized with 0.1% Triton X-100 for 10 min, and then stained with TRAP solution (Sigma-Aldrich, Saint Louis, MO, USA). The TRAP-positive multinucleated osteoclasts (MNC; nuclei ≥ 3 or nuclei ≥ 10) were counted. To measure TRAP activity, the multinucleated osteoclasts were fixed in 10% formalin for 5 min, permeabilized with 0.1% Triton X-100 for 10 min, and then treated with TRAP buffer (100 mM sodium citrate, pH 5.0, 50 mM sodium tartrate) containing 3 mM *p*-nitrophenyl phosphate (Sigma-Aldrich) at 37 °C for 5 min. The reaction mixtures in the wells were transferred to new plates containing an equal volume of 0.1 N NaOH and the optical density values were measured at 405 nm on a SpectraMax M5 fluorescence spectrophotometer (Molecular Devices, San Jose, CA, USA).

### Cell proliferation assay

The BMMs were plated in 96-well plates in triplicates. After treatment with WSE and its constituents, the cells were incubated for 3 d and the cell viability was measured using the Cell Counting Kit 8 (Dojindo Molecular Technologies, Rockville, MD, USA).

### Western blotting

The BMMs were washed with ice-cold PBS and lysed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM sodium fluoride, 1 mM sodium vanadate, and 1% deoxycholate) supplemented with protease inhibitors (Roche, Basel, Switzerland). After centrifugation at 15,000 × *g* for 15 min, the protein in the supernatant was quantified using the detergent compatible (DC) protein assay kit (Bio-Rad, Hercules, CA, USA). The quantified proteins were denatured, separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 4–12% gradient gels and transferred onto a polyvinylidene difluoride membrane using the iBlot 2 Dry Blotting System (Thermo Fisher Scientific). Blots were incubated with primary antibodies in 1% BSA overnight at 4 °C and then incubated with secondary antibodies in 5% skim milk at room temperature for 2 h. The membranes were developed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and visualized on a LAS-4000 luminescent image analyzer (GE Healthcare Life Sciences, Little Chalfont, UK). Actin was used as the loading control.

### RNA isolation and real-time polymerase chain reaction (PCR) analysis

Primers were designed using the Primer3 online tool. Primer sets used in this study are shown in Table 2.

Total RNA was extracted from BMMs using Trizol reagent (Invitrogen). First-stand cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Real-Time PCR was performed using Applied Biosystems Power-Up SYBR green PCR master mix (Thermo Fisher Scientific) and quantified using Quantstudio®5 Real-Time PCR (Thermo Fisher Scientific). The gene encoding glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal standard. All reactions were performed in triplicates and data were analyzed using the $2^{-\Delta\Delta Ct}$ method [24].

### 10. F-actin ring staining

Preosteoclasts derived from BMMs in cultures with M-CSF (30 ng/mL) and RANKL (10 ng/mL) for 3 d on black clear glass plates were treated with WSE or vehicle for 1 d. Then, the cells were fixed with 10% formalin for 5 min, permeabilized with 0.1% Triton X-100 for 10 min, and then stained with phalloidin-FITC (Sigma-Aldrich) for 10 min. The actin rings of mature osteoclasts were photographed under a fluorescence microscope (10× magnification).

### Bone-pit formation analysis

Mature osteoclasts were obtained by isolating osteoblasts from the calvariae of new born mice using serial digestion with 0.1% collagenase (Gibco, Paisley, UK) as previously described [25]. The bone marrow cells were isolated as described above. Osteoblasts ($3.5\times10^5$ cells/well) and BMMs ($1\times10^6$ cells/well) were co-cultured on a collagen-coated 90-mm dish in the presence of 1α, 25-dihydroxyvitamin $D_3$ ($VitD_3$) and prostaglandin $E_2$ ($PGE_2$) for 6 d. The complete α-MEM with $VitD_3$ and $PGE_2$ was changed every 3 d. The co-cultured cells were detached from the collagen-coated dishes using 0.1% collagenase and then re-plated on a bone biomimetic synthetic surface (Corning, NY, USA) of a 24-well plate. After 1 h, the cells were treated with RANKL (10 ng/mL) and WSE for 24 h. The cells were stained for TRAP and photographed under a light microscope at 10× magnification. To observe the resorption pits, the slides were washed with PBS and treated with 5% sodium hypochlorite for 5 min. The plate was washed with PBS, dried, and photographed under a light microscope. The resorbed areas were quantified using the ImageJ software.

**Table 2** The primer sequences used in this study

| Target gene | Forward primer (5′–3′) | Reverse primer (5′–3′) |
|---|---|---|
| *c-Fos* | CCAGTCAAGAGCATCAGCAA | AAGTAGTGCAGCCCGGAGTA |
| *NFATc1* | GGGTCAGTGTGACCGAAGAT | GGAAGTCAGAAGTGGGTGGA |
| *TRAP* | GATGACTTTGCCAGTCAGCA | ACATAGCCCACACCGTTCTC |
| *OSCAR* | AGGGAAACCTCATCCGTTTG | GAGCCGGAAATAAGGCACAG |
| *DC-STAMP* | CCAAGGAGTCGTCCATGATT | GGCTGCTTTGATCGTTTCTC |
| *Cathepsin K* | GGCCAACTCAAGAAGAAAAC | GTGCTTGCTTCCCTTCTGG |
| *GAPDH* | ACCACAGTCCATGCCATCAC | TCCACCACCCTGTTGCTGTA |

### Statistical analyses

All quantitative values are presented as mean ± standard deviation. Each experiment was performed in triplicates and repeated three to five times. Figures are representative images of each experiment. Statistical differences were analyzed using the Student's *t*-test, and a value of $p<0.05$ was considered significant.

## Results

### WSE attenuated RANKL-induced osteoclast differentiation in BMMs

To investigate the effect of WSE on osteoclastogenesis, we used BMMs derived from mouse bone marrow cells. WSE strongly inhibited the RANKL-induced TRAP-positive multinucleated cells in a dose-dependent manner (Fig. 1A). Consistent with this result, WSE also significantly attenuated the number of TRAP- positive MNCs (nuclei ≥ 3 or ≥ 10) in a dose-dependent manner (Fig. 1B). The inhibitory effect of WSE on osteoclastogenesis was verified by evaluating TRAP activity (Fig. 1C). To exclude the possibility that WSE attenuated osteoclastogenesis because of its cytotoxicity, BMMs were incubated with WSE in the presence of M-CSF for 3 d, and the cell survival was evaluated. WSE did not affect the survival of BMMs, which indicated that its anti-osteoclastogenic activity was not because of cytotoxicity (Fig. 1D). Collectively, these results indicated that WSE significantly inhibited RANKL-induced osteoclast differentiation without any apparent cytotoxicity.

### WSE inhibited the RANKL-induced expression levels of c-Fos/NFATc1 by downregulating the AKT/ERK signaling pathway

To better understand the mechanisms of WSE inhibition on osteoclastogenesis, we evaluated the osteoclast differentiation-related molecules, including RANKL-induced transcriptional factors and the RANK–RANKL signaling pathways. As shown in Fig. 2A, RANKL stimulates the

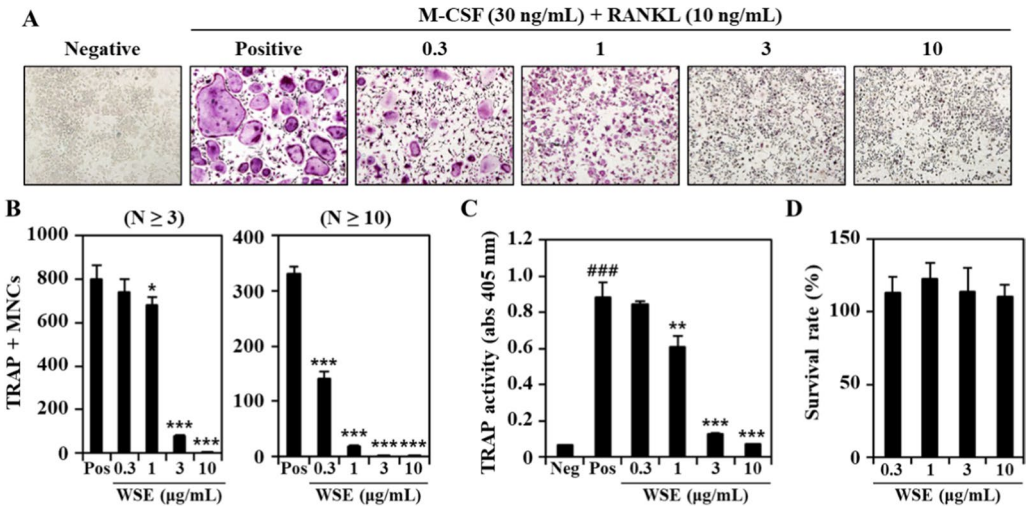


**Fig. 1** WSE inhibits RANKL-induced osteoclastogenesis. The BMMs were cultured for 4 d in the presence of RANKL (10 ng/mL) and M-CSF (30 ng/mL) in the presence of the vehicle (prethanol) or the indicated concentrations of WSE. **A** The cells were stained with TRAP solution. **B** TRAP-positive MNCs were counted as osteoclasts, and **C** TRAP activity was measured. **D** The cytotoxicity of WSE on BMMs was evaluated using the CCK-8 assay. Data are expressed as the mean ± SD and are representative of at least three experiments. $^{*}$ < 0.05; $^{***}$ < 0.001 versus the RANKL-treated group; $^{\#\#\#}$ < 0.001 versus the control

activation of RAC-alpha serine/threonine-protein kinase (AKT) and MAPKs, including ERK, c-JNK, and p38; WSE treatment blocks the RANKL-induced phosphorylation of AKT and ERK.

Next, we investigated whether WSE could regulate the osteoclastogenesis-specific transcription factors required for the RANKL-induced AKT and MAPK signaling pathways. As shown in Fig. 2B, the mRNA expression levels of *c-Fos* and *NFATc1* were elevated when treated with RANKL along with the vehicle. However, its expression was significantly attenuated by WSE treatment. In addition, WSE also significantly inhibited the expression levels of c-Fos/NFATc1-dependent genes, such as TRAP and OSCAR. Immunoblot analysis further showed that the RANKL-mediated induction of transcription factors was significantly reduced by WSE treatment (Fig. 2C). Collectively, these results demonstrated that the inhibitory effect of WSE on osteoclast differentiation could be because of the attenuation of c-Fos/NFATc1 levels via the AKT and MAPK signaling pathways.

### WSE suppressed RANKL-induced osteoclast formation at all stages of osteoclast differentiation

Because osteoclast differentiation was inhibited by WSE, we evaluated the anti-osteoclastogenic activity of WSE during the differentiation stages of osteoclasts. Treatment with WSE for 24 h at four time points (Fig. 3A) suppressed the RANKL-induced osteoclast formation (Fig. 3B). Additionally, TRAP activity was also inhibited during the indicated periods (Fig. 3C). In particular, the number of TRAP-positive multinucleated giant osteoclasts with > 10 nuclei were significantly reduced by WSE (3 μg/ml) treatment between 3 and 4 d after RANKL stimulation (Fig. 3D). These results indicated that the anti-osteoclastogenic activity of WSE could be involved at all stages of osteoclast differentiation.

### WSE inhibited the RANKL-induced osteoclasts fusion

To examine the mode of anti-osteoclastogenic action, we investigated the formation of actin ring, which is a defining characteristic of MNCs and osteoclasts fusion-mediated molecule induction. As shown in Fig. 4A, incubation of preosteoclasts with 3 μg/mL WSE for 1 d almost completely attenuates the formation of giant osteoclasts as seen with TRAP staining (Fig. 4A; upper panel) and fluorescence-conjugated phalloidin (Fig. 4A; lower panel) as compared with vehicle treatment. As described above, the presence of 3 μg/ml WSE significantly reduced the number of fused osteoclasts with > 10 nuclei (Fig. 4B). To investigate the anti-fusion action of osteoclasts by WSE treatment, we evaluated the transcriptional expression of DC-STAMP, a critical factor necessary for osteoclast fusion. As shown in Fig. 4C, the addition of WSE significantly reduces the RANKL-mediated transcriptional expression of DC-STAMP. Thus, our results showed that WSE also inhibited cell fusion in the processes of osteoclast differentiation.

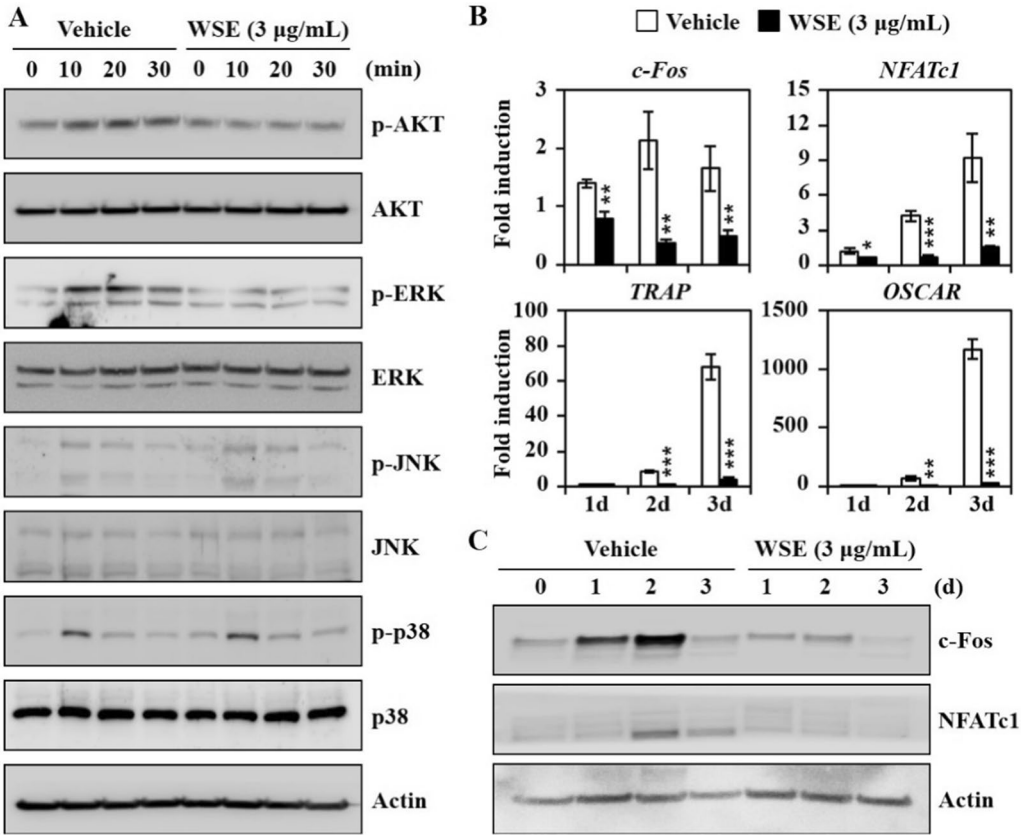


**Fig. 2** WSE inhibits c-Fos/NFATc1 transcription factors by attenuating AKT/ERK phosphorylation. **A** In the condition of serum starvation for 1 h, the BMMs were pretreated with or without WSE (3 μg/mL) for 1 h prior to RANKL stimulation (10 ng/mL) at the indicated time periods. Then, the protein expression levels were evaluated using western blot analysis, and one representative result obtained from three independent experiments yielding similar results is shown. **B** The BMMs were stimulated with RANKL (10 ng/mL) and M-CSF (30 ng/mL) in the presence or absence of WSE (3 μg/mL) for the indicated times. Total RNA was isolated using TRIzol reagent and the mRNA expression levels were evaluated using RT-qPCR. Relative levels of *c-Fos, NFATc1, TRAP*, and *OSCAR* were normalized to *GAPDH*. $^{**}$ < 0.01; $^{***}$ < 0.0010 versus the vehicle control. **C** The effect of WSE on the protein expression level of RANKL-induced transcription factors was evaluated using western blot analysis. Actin was used as the internal control. Data are representative of at least three experiments

### WSE attenuated the bone-resorbing activity of mature osteoclasts

Because osteoclast differentiation and fusion were inhibited by WSE, we speculated that WSE could subsequently repress the osteoclast function or the bone-resorbing activity of mature osteoclasts. When purified mature osteoclasts from the co-culture were re-plated on a bone biomimetic synthetic surface and cultured in the presence or absence of WSE for 1 d, WSE treatment did not affect the formation and number of TRAP-positive multinucleated cells as compared to vehicle treatment, as seen by TRAP staining (Fig. 5A; upper panel). However, the addition of WSE significantly reduced the bone resorption areas (Fig. 5A; lower panel). Furthermore, the resorbed areas quantified using bone-pit measurement were remarkably reduced in WSE-treated osteoclasts (Fig. 5B). Hence, we examined the expression of molecules related to bone resorption; WSE significantly attenuated the levels of RANKL-induced transcription of cathepsin K, which plays a vital role in bone resorption by osteoclasts (Fig. 5C). These results indicated that the addition of WSE could inhibit the RANKL-induced bone-resorptive function of mature osteoclasts.

### Constituents of WSE suppressed the RANKL-induced osteoclastogenic activity

In order to identify the anti-osteoclastogenic potential of compounds constituting WSE during the differentiation stages of osteoclasts, we isolated and identification of eight major constituents from WSE (Fig. 6A). Their identified chemical structures are shown in Additional file 1. Next, we studied their inhibitory effects on osteoclastogenicity by TRAP staining. Constituents of WSE inhibited the RANKL-induced TRAP-positive multinucleated cells (Fig. 6B). The inhibitory effect on osteoclastogenesis for each compound was verified by evaluating

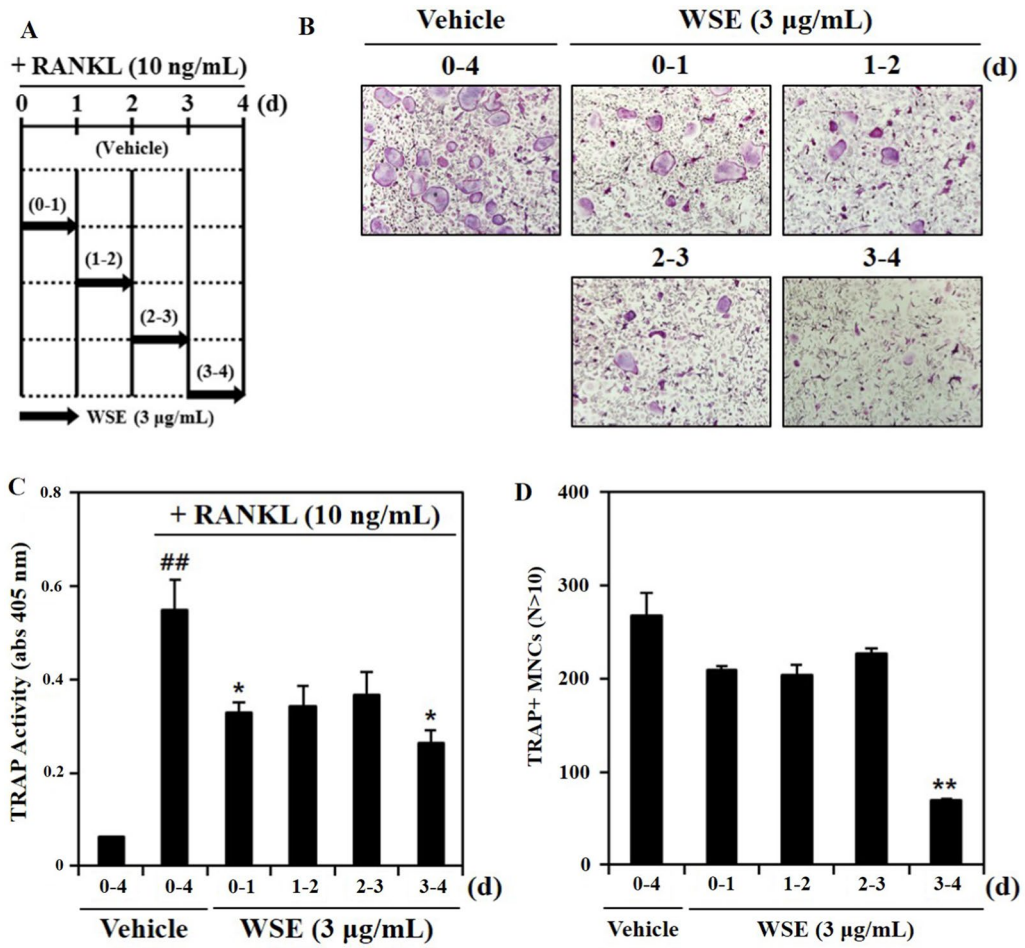


**Fig. 3** WSE impairs the fusion of preosteoclasts. **A** Based on the exposure schedule, the BMMs were cultured with WSE (3 μg/mL) for various times periods in the presence of M-CSF (30 ng/mL) and RANKL (10 ng/mL). **B** The cells were stained with TRAP solution. **C** TRAP activity was measured, and **D** TRAP-positive MNCs were counted as osteoclasts. Each exposure period of WSE was indicated as "0–4" for the vehicle. "0–1" for 0–1 d, "1–2" for 1–2 d, "2–3" for 2–3 d, and "3–4" for 3–4 d. $^{\#\#\#}$ < 0.001 versus the control; $^{*}$ < 0.05, $^{**}$ < 0.01 versus the RANKL-treated group

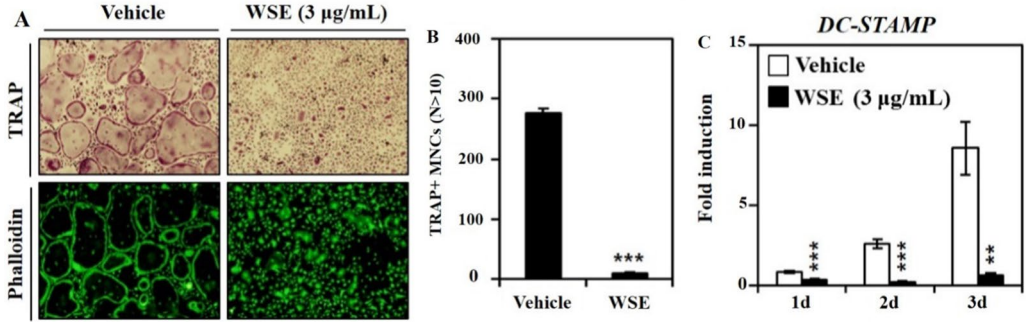


**Fig. 4** WSE attenuates RANKL-induced actin ring formation. **A** Preosteoclasts incubated with or without 3 μg/mL WSE for 1 d. The formation of giant osteoclasts was observed using TRAP staining and fluorescence-conjugated phalloidin, and **B** TRAP-positive MNCs were counted as osteoclasts. **C** The effect of WSE on the mRNA expression of *DC-STAMP* was analyzed using RT-qPCR, and the relative-fold change of the mRNA expression level was normalized to *GAPDH*. $^{**}$ < 0.01; $^{***}$ < 0.001 versus the vehicle control. Data are representative of at least three experiments

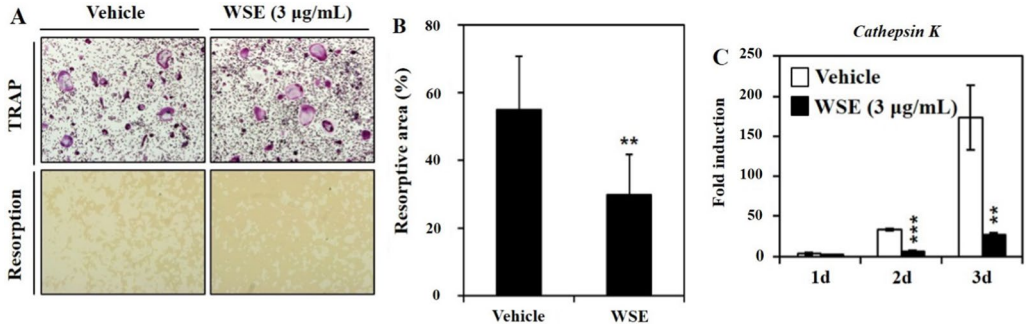


**Fig. 5** WSE inhibits the function of mature osteoclasts. **A** The mature osteoclasts were plated on bone biomimetic synthetic surface and treated for 24 h with WSE (3 μg/ml). The cells stained with TRAP and TRAP + MNC formation was visualized under a light microscope (top images). The resorption areas formed by the cells were photographed under a light microscope (bottom images). **B** The resorptive areas (%) were quantified using the ImageJ software. ** < 0.01 versus the vehicle control. One representative result achieved from three independent experiments yielding similar results is shown. **C** The mRNA expression of *Cathepsin K* was evaluated during osteoclast differentiation in the absence or presence of WSE (3 μg/ml) using RT-qPCR and presented in comparison with the control. ** < 0.01; *** < 0.001 versus the vehicle control

TRAP activity (Fig. 6C). At a concentration of 100 μM, luteolin-6-*C*-arabinoside-8-*C*-glucosdie (isocarlinoside, compound 2), luteolin-6-*C*-β-glucoside (isoorientin, compound 3), and chrysoeriol-6-*C*-β-glucoside (isoscoparin, compound 8) reduced the TRAP activity compared to the positive control (vehicle only) by 70, 81, and 73%, respectively. Furthermore, all compounds significantly reduced TRAP activity at 300 μM, which is consistent with the staining results. In particular, luteolin-6-*C*-arabinoside-8-*C*-glucosdie (isocarlinoside, compound 2) and luteolin-6-*C*-β-glucoside (isoorientin, compound 3) were reduced by 22% and 25%, respectively. Cell viability test was performed to verify whether the decrease in TRAP activity was a result of cytotoxicity in BMM cells (Fig. 6D). Although the cell viability was slightly increased by chrysoeriol-6-*C*-β-glucoside (isoscoparin, compound 8), the results showed that all compounds had no significant effect on the cytotoxicity of BMM cells. Collectively, these results indicated that all constituents of WSE contributed to inhibit RANKL-induced osteoclast differentiation without cytotoxicity.

## Discussion

Osteoporosis is a skeletal disease characterized by microstructural abnormalities and decreased bone mass and is caused by the enhanced activity of osteoclasts that destroy bone as compared to the bone forming osteoblasts [26]. Therefore, for the treatment and prevention of osteoporosis, strategies for inhibition of bone resorption include inhibition of the osteoclast activity or promotion of bone formation by functional activation of osteoblasts [27].

In this study, we found that the non-cytotoxic concentration of WSE not only reduced the number of differentiated osteoclasts but also inhibited the activity of TRAP (Fig. 1). Osteoclast precursor proliferation and survival is because of ERK and AKT activation through c-Fms, a tyrosine kinase receptor belonging to the type III protein tyrosine kinase family, in the presence of M-CSF [28]. Upon differentiation of pre-osteoclast because of M-CSF stimulation, RANK is expressed on the cell membrane and TRAF6 is activated upon RANKL-RANK binding [29]. Activated TRAF6 further stimulates MAPKs, including ERK, JNK and p38, to activate c-Fos or directly activates AKT, which subsequently induces the expression of NFATc1, a major differentiation factor for osteoclasts [30]. WSE suppressed the activation of c-Fos as well as AKT and ERK (Fig. 2). In addition, we demonstrated that the mRNA and protein expression levels of NFATcl decreased during the osteoclast differentiation. NFATc1 contributes to osteoclast differentiation by producing various proteins such as OSCAR, DC-STAMP, TRAP, and cathepsin K [31]. Our results indicated that WSE inhibited the expression of NFATc1 in osteoclast formation and blocked the differentiation of osteoclasts by reducing the action of various factors responsible for osteoclast differentiation.

Cell-cell fusion is essential for multinucleated osteoclast formation and maturation, and the preferential expression of DC-STAMP in the late stage is involved in this process [32]. In DC-STAMP-deficient mice, multinucleated osteoclasts are lost and the activity of bone resorption is significantly reduced, eventually resulting in osteopetrosis [33], indicating that DC-STAMP is essential for osteoclastogenesis and bone maintenance. We found that cell fusion and mRNA expression of

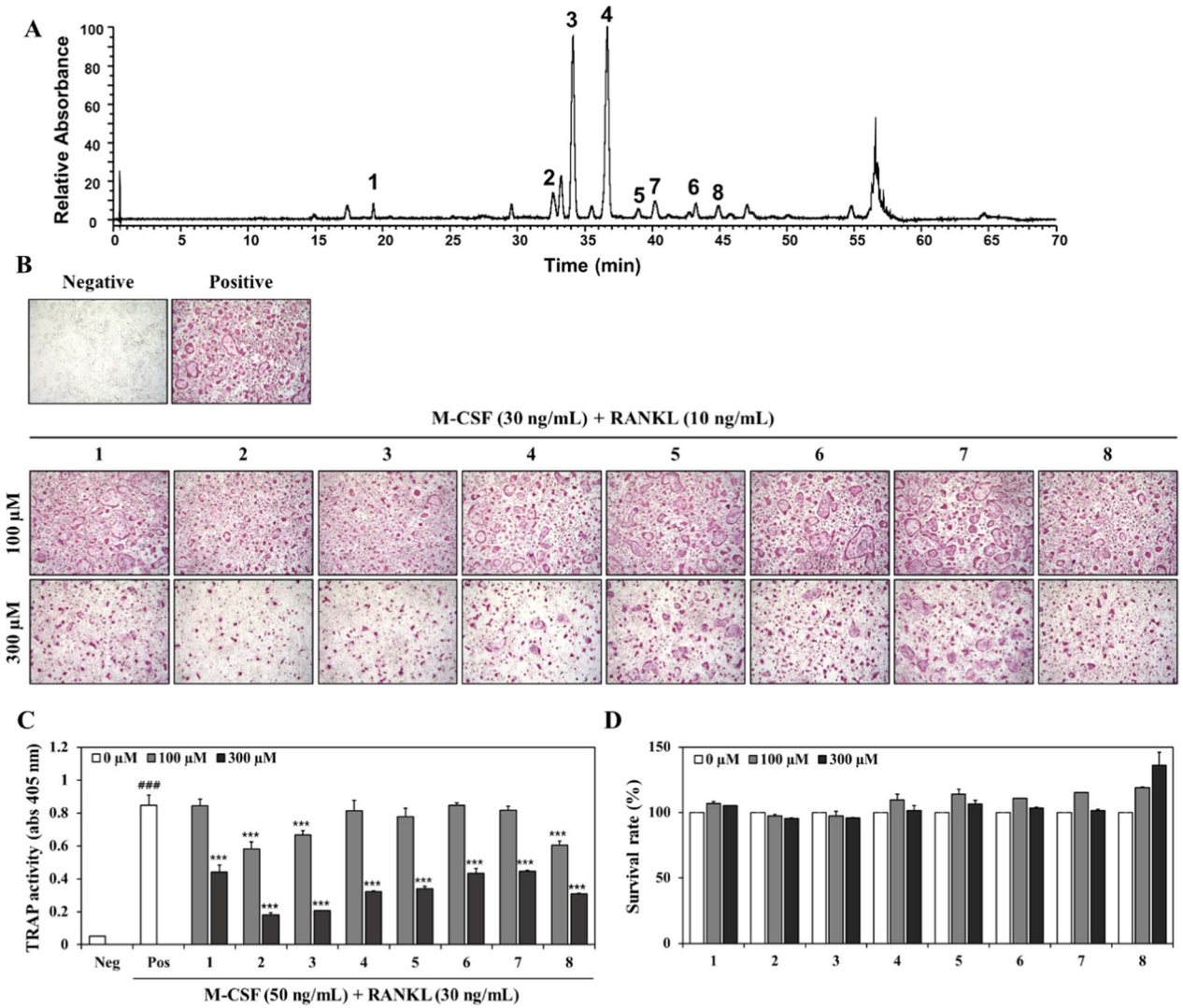


**Fig. 6** Eight key compounds of WSE inhibits RANKL-induced osteoclastogenesis. **A** Typical chromatogram detected at 235 nm of phenolic phytochemical compositions in wheat seedlings extracts. The BMMs were cultured for 4 d in the presence of RANKL (10 ng/mL) and M-CSF (30 ng/mL) in the presence of the vehicle (DMSO) or the indicated concentrations of each compound. **B** The cells were stained with TRAP solution. **C** TRAP activity was measured. **D** The cytotoxicity of compounds on BMMs was evaluated using the CCK-8 assay. Data are expressed as the mean ± SD and are representative of at least three experiments. $^{###}$ < 0.001 versus the negative control; $^{***}$ < 0.001 versus the positive control

*DC-STAMP* were inhibited by WSE during osteoclast differentiation (Figs. 3 and 4). Collectively, these results suggested that WSE with anti-osteoclastogenic activity is involved in osteoclast differentiation from the initial to the terminal phases of osteoclastogenesis.

Cathepsin K is a type of lysosomal cysteine protease and plays a critical role in osteoclastic bone resorption by cleaving the organic matrix of the bone. Wilson et al. [34] report that cathepsin K is required for the initial formation of actin rings and activation of osteoclasts. Our results showed that WSE effectively suppressed the mRNA expression of RANKL-induced cathepsin K and inhibited its bone-resorbing activity (Fig. 5). Taken together, WSE could inhibit the bone resorption function of osteoclasts.

The current results suggest that the eight key compounds constituting WSE exhibit anti-osteoclastogenetic activity at high concentrations. (Fig. 6). Although individual compounds require higher concentration treatment compared to the extract, it is worth considering the synergy of various compounds within the plant extract [35, 36]. Often, plant extracts, or fractions thereof, have been observed to demonstrate greater clinical biological activity than single constituents,

providing uniqueness and novelty derived from the individual characteristics of the plant, rather than the effects of single compounds [37, 38]. The effects of isoorientin and isoscoparin, which are present in high quantities in wheat extracts, have been reported to decrease the production of nitric oxide and Tumor Necrosis Factor-alpha (TNF-α), thus showing anti-inflammatory effects [39]. TNF-α has been demonstrated to be involved in bone resorption associated with inflammatory diseases of bone [40]. In addition, it was reported that TNF-α in combination with RANKL significantly stimulated the osteoclasts differentiation and upregulated the mRNA expression of osteoclasts marker [41]. Taken together, it was hypothesized that various compounds that inhibit osteoclast differentiation and bone resorption exist in WSE, and TNF-α is involved in this process. Based on the previously reported results and the present findings, isoorientin and isoscoparin will have value in the treatment and prevention of bone-related diseases including inflammatory diseases of the bone such as osteoarthritis.

In the present study, the inhibitory effects of WSE on differentiation of osteoclasts and bone-resorbing activity were demonstrated in vitro and the plausible inhibitory mechanism was validated. Also, eight compounds constituting WSE had potent effects on TRAP activity in BMM cells. It was considered necessary to examine the effects of WSE on osteoblast differentiation and validate its efficacy using an animal model. Although the in vitro and in vivo observations may differ, the above results suggest that WSE can be an effective candidate for the treatment of bone-related diseases such as osteoporosis, rheumatoid arthritis, osteoarthritis, and periodontitis.

**Abbreviations**

| | |
|---|---|
| AKT | RAC-alpha serine/threonine-protein kinase |
| BMMs | Bone marrow macrophages |
| DC-STAMP | Dendritic cell-specific transmembrane protein |
| DMSO | Dimethyl sulfoxide |
| ERK | Extracellular signal-regulated kinase |
| FBS | Fetal bovine serum |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| ICR | Imprinting control region |
| JNK | c-Jun N-terminal kinase |
| MAPKs | Mitogen-activated protein kinases |
| M-CSF | Macrophage colony-stimulating factor |
| MNC | Multinucleated cells |
| NFATc1 | Nuclear factor-activated T cells c1 |
| OSCAR | Osteoclast-associated receptor |
| RANKL | Receptor activator of nuclear factor-κB ligand |
| TNF-α | Tumor necrosis factorα |
| TRAP | Tartrate-resistant acid phosphatase |
| WSE | Wheat seedling extract |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-023-00819-w.

**Additional file 1: Figure S1.** Chemical structures of the identified phenolic phytochemicals from wheat seedlings. **Table S1.** WSE compound content used in this study.

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Not applicable.

**Author contributions**
HGL and JYY wrote the manuscript and performed the analysis. SHK. carried out the experiment and performed analysis. JKK, CSK, and KHK contributed to the interpretation or the results. SAL verified the analytical methods. SWC and WDS conceived the idea, revised the manuscript, and supervised the findings of this work. All authors have read and agreed to the published version of the manuscript.

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**Availability of data and materials**
Any data generated or analyzed in the study is included in this published article.

## Declarations

**Competing interests**
There are no competing interest to declare.

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**References**

1. Chen X, Wang Z, Duan N, Zhu G, Schwarz EM, Xie C (2018) Osteoblast-osteoclast interactions. Connect Tissue Res 59(2):99–107. https://doi.org/10.1080/03008207.2017.1290085
2. Park EJ, Joo IW, Jang MJ, Kim YT, Oh K, Oh HJ (2014) Prevalence of osteoporosis in the korean population based on Korea National Health and Nutrition Examination Survey (KNHANES), 2008–2011. Yonsei Med J 55(4):1049–1057. https://doi.org/10.3349/ymj.2014.55.4.1049
3. Loes S (2012) Side effects of bisphosphonates. Tidsskr Nor Laegeforen 132(18):2036. https://doi.org/10.4045/tidsskr.12.0993
4. Toussaint ND, Elder GJ, Kerr PG (2009) Bisphosphonates in chronic kidney disease; balancing potential benefits and adverse effects on bone and soft tissue. Clin J Am Soc Nephrol 4(1):221–233. https://doi.org/10.2215/CJN.02550508
5. Zaidi M (2007) Skeletal remodeling in health and disease. Nat Med 13(7):791–801. https://doi.org/10.1038/nm1593
6. Balogh E, Paragh G, Jeney V (2018) Influence of iron on bone homeostasis. Pharmaceuticals (Basel). https://doi.org/10.3390/ph11040107
7. Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H et al (2002) Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. Dev Cell 3(6):889–901. https://doi.org/10.1016/s1534-5807(02)00369-6

8. Kodama J, Kaito T (2020) Osteoclast multinucleation: review of current literature. Int J Mol Sci. https://doi.org/10.3390/ijms21165685
9. Sapkota M, Li L, Kim SW, Soh Y (2018) Thymol inhibits RANKL-induced osteoclastogenesis in RAW264.7 and BMM cells and LPS-induced bone loss in mice. Food Chem Toxicol 120:418–429. https://doi.org/10.1016/j.fct.2018.07.032
10. Shewry PR (2009) Wheat. J Exp Bot 60(6):1537–1553. https://doi.org/10.1093/jxb/erp058
11. Nagaoka H (2005) Treatment of germinated wheat to increase levels of GABA and IP6 catalyzed by endogenous enzymes. Biotechnol Prog 21(2):405–410. https://doi.org/10.1021/bp0496777
12. Donkor ON, Stojanovska L, Ginn P, Ashton J, Vasiljevic T (2012) Germinated grains–sources of bioactive compounds. Food Chem 135(3):950–959. https://doi.org/10.1016/j.foodchem.2012.05.058
13. Peryt B, Szymczyk T, Lesca P (1992) Mechanism of antimutagenicity of wheat sprout extracts. Mutat Res 269(2):201–215. https://doi.org/10.1016/0027-5107(92)90201-c
14. Bonfili L, Amici M, Cecarini V, Cuccioloni M, Tacconi R, Angeletti M et al (2009) Wheat sprout extract-induced apoptosis in human cancer cells by proteasomes modulation. Biochimie 91(9):1131–1144. https://doi.org/10.1016/j.biochi.2009.06.001
15. Calzuola I, Marsili V, Gianfranceschi GL (2004) Synthesis of antioxidants in wheat sprouts. J Agric Food Chem 52(16):5201–5206. https://doi.org/10.1021/jf0307752
16. Lee H, Yeong Yang J, Eun Ra J, Ahn H-J, Ja Lee M, Young Kim H et al (2023) Elucidation of phenolic metabolites in wheat seedlings (Triticum aestivum L.) by NMR and HPLC-Q-Orbitrap-MS/MS: changes in isolated phenolics and antioxidant effects through diverse growth times. Food Chemistry: X 17:100557. https://doi.org/10.1016/j.fochx.2022.100557
17. Li Y, Zhao Y, Tan X, Liu J, Zhi Y, Yi L et al (2020) Isoorientin inhibits inflammation in Macrophages and Endotoxemia mice by regulating glycogen synthase kinase 3beta. Mediators Inflamm 2020:8704146. https://doi.org/10.1155/2020/8704146
18. Liang S, Zhao Y, Chen G, Wang C (2022) Isoorientin ameliorates OVA-induced asthma in a murine model of asthma. Exp Biol Med (Maywood) 247(16):1479–1488. https://doi.org/10.1177/15353702221094505
19. Su Y, Kang Y, Yi J, Lin Q, Zhang C, Lin Z et al (2022) Isoschaftoside reverses nonalcoholic fatty liver Disease via activating autophagy in vivo and in Vitro. Evid Based Complement Alternat Med 2022:2122563. https://doi.org/10.1155/2022/2122563
20. He M, Min JW, Kong WL, He XH, Li JX, Peng BW (2016) A review on the pharmacological effects of vitexin and isovitexin. Fitoterapia 115:74–85. https://doi.org/10.1016/j.fitote.2016.09.011
21. Cao Z, Liu W, Bi B, Wu H, Cheng G, Zhao Z (2022) Isoorientin ameliorates osteoporosis and oxidative stress in postmenopausal rats. Pharm Biol 60(1):2219–2228. https://doi.org/10.1080/13880209.2022.2142614
22. Ge S, Ren H, Guo Q, Wang X, Liu Y, Lin B et al (2023) Wuweixiaoduyin regulates TAZ-mediated immunoregulatory properties of Treg/TH17 cells in chronic osteomyelitis. Biotechnol Genet Eng Rev. https://doi.org/10.1080/02648725.2023.2166706
23. Choi SW, Kim SH, Lee KS, Kang HJ, Lee MJ, Park KI et al (2017) Barley seedling extracts inhibit RANKL-Induced differentiation, Fusion, and maturation of osteoclasts in the early-to-late stages of Osteoclastogenesis. Evid Based Complement Alternat Med 2017:6072573. https://doi.org/10.1155/2017/6072573
24. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2 − ΔΔCT method. Methods 25(4):402–408. https://doi.org/10.1006/meth.2001.1262
25. Choi SW, Son YJ, Yun JM, Kim SH (2012) Fisetin inhibits osteoclast differentiation via downregulation of p38 and c-Fos-NFATc1 Signaling Pathways. Evid Based Complement Alternat Med 2012:810563. https://doi.org/10.1155/2012/810563
26. Bi H, Chen X, Gao S, Yu X, Xiao J, Zhang B et al (2017) Key triggers of osteoclast-related Diseases and available strategies for targeted therapies: a review. Front Med (Lausanne) 4:234. https://doi.org/10.3389/fmed.2017.00234
27. Feng X, McDonald JM (2011) Disorders of bone remodeling. Annu Rev Pathol 6:121–145. https://doi.org/10.1146/annurev-pathol-011110-130203
28. Feng X, Teitelbaum SL, Osteoclasts (2013) New insights. Bone Res 1(1):11–26. https://doi.org/10.4248/BR201301003
29. Liu W, Zhang X (2015) Receptor activator of nuclear factor-kappab ligand (RANKL)/RANK/osteoprotegerin system in bone and other tissues (review). Mol Med Rep 11(5):3212–3218. https://doi.org/10.3892/mmr.2015.3152
30. Wei ZF, Tong B, Xia YF, Lu Q, Chou GX, Wang ZT et al (2013) Norisoboldine suppresses osteoclast differentiation through preventing the accumulation of TRAF6-TAK1 complexes and activation of MAPKs/NF-kappaB/c-Fos/NFATc1 Pathways. PLoS ONE 8(3):e59171. https://doi.org/10.1371/journal.pone.0059171
31. Hwang SY, Putney JW (2012) Orai1-mediated calcium entry plays a critical role in osteoclast differentiation and function by regulating activation of the transcription factor NFATc1. FASEB J 26(4):1484–1492. https://doi.org/10.1096/fj.11-194399
32. Zhang C, Dou CE, Xu J, Dong S (2014) DC-STAMP, the key fusion-mediating molecule in osteoclastogenesis. J Cell Physiol 229(10):1330–1335. https://doi.org/10.1002/jcp.24553
33. Yagi M, Miyamoto T, Sawatani Y, Iwamoto K, Hosogane N, Fujita N et al (2005) DC-STAMP is essential for cell-cell fusion in osteoclasts and foreign body giant cells. J Exp Med 202(3):345–351. https://doi.org/10.1084/jem.20050645
34. Wilson SR, Peters C, Saftig P, Bromme D (2009) Cathepsin K activity-dependent regulation of osteoclast actin ring formation and bone resorption. J Biol Chem 284(4):2584–2592. https://doi.org/10.1074/jbc.M805280200
35. Williamson EM (2001) Synergy and other interactions in phytomedicines. Phytomedicine 8(5):401–409. https://doi.org/10.1078/0944-7113-00060
36. Wagner H, Ulrich-Merzenich G (2009) Synergy research: approaching a new generation of phytopharmaceuticals. Phytomedicine 16(2):97–110. https://doi.org/10.1016/j.phymed.2008.12.018
37. Ma XH, Zheng CJ, Han LY, Xie B, Jia J, Cao ZW et al (2009) Synergistic therapeutic actions of herbal ingredients and their mechanisms from molecular interaction and network perspectives. Drug Discovery Today 14(11):579–588. https://doi.org/10.1016/j.drudis.2009.03.012
38. Rasoanaivo P, Wright CW, Willcox ML, Gilbert B (2011) Whole plant extracts versus single compounds for the treatment of malaria: synergy and positive interactions. Malar J 10(Suppl 1):S4. https://doi.org/10.1186/1475-2875-10-s1-s4
39. Luyen BT, Tai BH, Thao NP, Cha JY, Lee YM, Kim YH (2014) A new phenolic component from Triticum aestivum sprouts and its effects on LPS-stimulated production of nitric oxide and TNF-alpha in RAW 264.7 cells. Phytother Res 28(7):1064–1070. https://doi.org/10.1002/ptr.5097
40. Dong J, Cui X, Jiang Z, Sun J (2013) MicroRNA-23a modulates tumor necrosis factor-alpha-induced osteoblasts apoptosis by directly targeting Fas. J Cell Biochem 114(12):2738–2745. https://doi.org/10.1002/jcb.24622
41. Luo G, Li F, Li X, Wang ZG, Zhang B (2018) TNFalpha and RANKL promote osteoclastogenesis by upregulating RANK via the NFkappaB pathway. Mol Med Rep 17(5):6605–6611. https://doi.org/10.3892/mmr.2018.8698


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